Technical methods

Modification of an incident light microscope for fluorescence

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A recent ACP Broadsheet (Taylor, Tomlinson, and Heimer, [1973]) described the simple and efficient apparatus required for the fluorescence microscopy of fluorescein-labelled antibody. If other fluorescent tracers, such as tetracycline and quinacrine, are also of interest an illumination system using the Ploem incident light technique may be preferred. It is the most effective method of exciting fluorescence (Kaufman, Nester, and Wasserman, 1971) and the small, high intensity lamps it employs allow narrow band filters to be used for the selective excitation of the fluorescent tracer in the presence of autofluorescent tissue. An unrestricted range of objectives can be employed for examining both transparent and opaque objects.

Using for this technique a Leitz Ortholux microscope with the standard HBO 50 mercury lamp we encountered a number of difficulties. Appropriate modifications, which resulted in a significantly improved microscope performance, are detailed below.

Mirror Fluorescence

The autofluorescence of the dichroic mirrors fitted to the Leitz/Ploem illuminator, noted by Kaufman *et al* (1971), we found intolerable, even for visual work. The introduction of a primary filter (Schott GG 420/4 mm), which removed all the light below 420 nm emitted by the HBO 50 lamp, reduced mirror fluorescence considerably. With tracers which require excitation below 420 nm, such as tetracycline, fluorescence of the appropriate mirror (no. 2) may be a handicap.

Many objectives require an adaptor (37 mm to 45 mm) which contains a lens. Removal of this lens did not degrade the performance of our 40 × fluorite objective noticeably but reduced mirror autofluorescence caused by light reflected from the lens surface.

Primary Filters

Following normal practice, coloured glass primary filters (Schott BG3 etc) were used for the blue and violet light illumination. Koch (1972) recommended an interference filter system (two Leitz KP 490 and K 480) for the blue light excitation of fluorescein at 480 to 490 nm with a mercury lamp. This system gives negligible background tissue (and mirror) fluorescence, but is expensive and sacrifices most of the useful light output of the mercury lamp, which is below 440 nm. For the excitation of fluorochromes in the band 440 to 540 nm xenon lamps like the XBO 75 are far more efficient. Stabilized and unstabilized power supplies for this lamp are available from Chandos Intercontinental, New Mills, Stockport.

Lamp Characteristics

The hot electrodes of a mercury lamp emit an excessive amount of red light. The electrodes should, therefore, not be visible when an image of the mercury arc is brought into focus on a microscope slide, or extremely uneven illumination of the field of view will result. We found it necessary to enlarge this image by fitting an extension tube on the front of the lamphouse to increase the light path by about 10 cm.

Barrier Filters

In an incident light microscope the barrier filter may be strongly illuminated by the mercury source and barrier filter combinations which fluoresce only weakly are essential. In this respect the Leitz gelatine filters were satisfactory. Barrier filters must also have sharp cut-off characteristics (Rygaard and Olsen, 1971). All-glass filters have cut-off characteristics which are superior to those of gelatine filters. Selected Schott glass filters were therefore used in place of the upper Leitz barrier filters, which are carried in the filter slide, with satisfactory results. A selected Schott OG 515, obtained from Polaron Ltd, Watford, replaced the Leitz K510 for work with fluorescein conjugates.

Slide Fluorescence

With a dark ground illumination system the yellow

fluorescence of ordinary glass slides is not readily visible. With incident light illumination the fluorescent area of the slide lies immediately below the specimen and may obscure specific fluorescence. The primary filter GG 420/4 mm reduced this interference. Non-fluorescent multi-specimen slides are made by Hendley & Co, Buckhurst Hill, Essex.

References:

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Improved mountant for immunofluorescence preparations

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Fluorescence emission of fluorescein isothiocyanate (FITC) from labelled antibody in microscopical preparations may be influenced by the characteristics of the mounting medium, in particular, its pH, its ionic strength, its viscosity, and the presence of quenching agents (Cherry, 1970). Fluorescence emission is greater at alkaline than at acid pH (Hiramoto, Bernecky, Jurand, and Hamlin, 1964). Pital and Janowitz (1963) claimed maximum fluorescence with glycerol buffered at pH 9.0 but Nairn (1969) recommended a slightly lower pH (8.6) so as to avoid impairment of immunological reactivity and fine tissue morphology. In a quantitative study of emission from FITC bound to cells mounted in buffered glycerol, Jongasma, Hijmans, and Ploem (1971) observed maximum fluorescence at pH 8.5. Small variations around this pH did not significantly influence fluorescence emission.

In spite of this well documented information it is still common practice to use a mounting medium which consists of glycerol and phosphate-buffered saline at pH 7.1. Furthermore, such a mountant does not give firm adhesion between the cover glass and the microscope slide.

A semi-permanent medium containing polyvinyl alcohol grade 51-05 Elvanol (PVA)¹ and glycerol was described by Rodriguez and Deinhardt (1960). This alternative to buffered glycerol solidifies on drying without trapping air bubbles provides an adhesive film between the cover glass and the microscope slide, and was used by Taylor, Heimer, Lea, and Tomlinson (1964) to mount immunofluorescence preparations of *Shigella sonnei*. Several semi-permanent mountants containing PVA were later evaluated in immunofluorescence studies by Thomason and Cowart (1967). The method of preparing the mountant described by Rodriguez and Deinhardt includes mechanical stirring for 32 hours so as to dissolve the PVA and centrifugation at 12 000 rpm to clear the solution. The final pH of this

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