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TECHNIQUES

MICROPHOTOMETRY IN IMMUNOFLUORESCENCE

R. C. NAIRN, F. HERZOG, H. A. WARD AND W. G. R. M. DE BOER

Department of Pathology, Monash University, Melbourne

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SUMMARY

A specially sensitive version of the Reichert microphotometer provides a means of obtaining accurate measurements of the emission of immunofluorescent microscopical preparations. The fluorescence of single particles down to 0·8 μ in diameter may be measured with all background excluded. The photometer has permitted determinations of titre of antisera, assessment of the validity of conventional visual estimation of fluorescence, and comparative studies of fluorescence intensity and fading under a variety of conditions. Preliminary studies by double immunofluorescent staining of two distinct antigenic constituents of cells which might be present either separately or together, suggest future value in investigations of cell differentiation and dedifferentiation.

INTRODUCTION

Conventional photomicroscopy exposure meters and the majority of photomultiplier microphotometers available commercially are insufficiently sensitive to measure the fluorescence emission of the smallest microscopical particles stained with fluorescent antibody. The problem has been overcome by some individual workers with specially constructed equipment (e.g. Mansberg & Kusnetz, 1966; Goldman, 1967) and a few suitable instruments are now marketed by the largest of the microscope manufacturers. One such instrument (Gabler *et al.*, 1960), designed for use with the Reichert Zetopan Binolux microscope, has been specially modified for low intensity fluorescence work and successfully applied by us to a number of immunofluorescence situations demanding exact measurements.

This communication reports our experience with the instrument in studies of immunofluorescent preparations: (a) correlating fluorimetric values with antibody titre and subjective estimates of fluorescence intensity made on visual inspection; (b) assessing the general level of fluorescence and of fading under various preparative situations; and (c) measuring two distinct antigenic constituents of cells which might contain them either separately or

Correspondence: Professor R. C. Nairn, Department of Pathology, Monash University Medical School, Commercial Road, Prahran, Victoria, Australia, 3181.

together. The results obtained confirm the value of the fluorimeter in yielding objective measurements which have important implications for the future practice of immunofluorescence.

METHODS

Microscope and photometer

The important features of the microphotometer are illustrated diagrammatically in Fig. 1. The beam splitter (4) and the viewing telescope (5) are so adjusted that the image

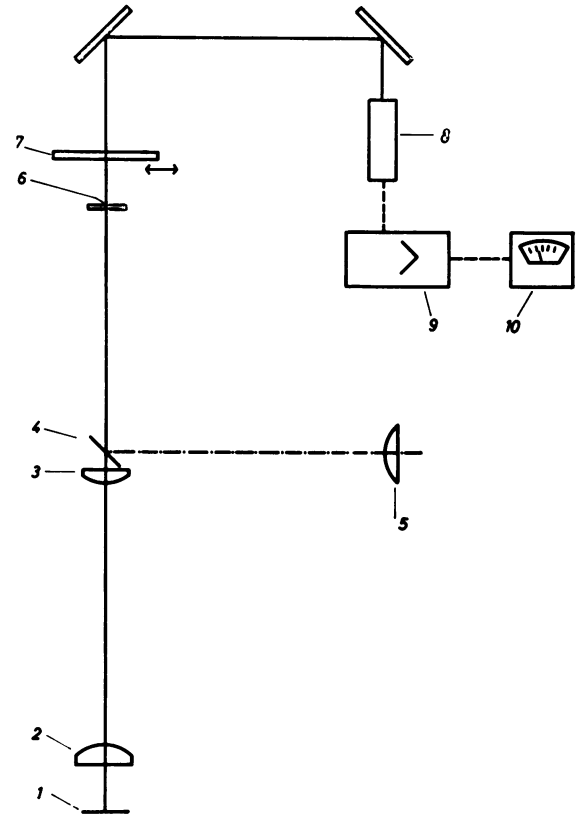

FIG. 1. Simplified diagram of microphotometer. 1, Specimen; 2, objective; 3, ocular; 4, beam splitter; 5, observation telescope; 6, measuring diaphragms; 7, interference wedge filter, with arrow indicating direction of movement; 8, photomultiplier tube; 9, amplifier and supply unit; 10, micro-ammeter. (Figure from Nairn, 1969.)

of the specimen (1) is in the same plane as the image of the appropriate diaphragm (6). A series of eight diaphragms with apertures varying in diameter from 0·5 to 10 mm is provided in a rotating turret, and one is selected to cover exactly the area to be measured and exclude completely any background fluorescence or transmitted light. With a 6·3× ocular (3) and a 95× glycerol immersion objective (2), the smallest diaphragm permits the selective inspection of a microscopic field only 0·8 μ in diameter.

The specimen is illuminated by an HBO 200 high pressure mercury lamp (Wotan, German Osram) through a heat filter (KG2/2 mm) and an ultraviolet-passing filter (UG 1/1·5 mm) and a darkground condenser of high numerical aperture (Reichert, 1·18/1·40). As a first barrier filter above the specimen we have usually employed the virtually colourless Wratten 2A film sandwiched between 1+3 mm thick glass slips (GG385). When making measurements this is supplemented by an interference wedge filter (7) which permits the selection of a narrow waveband corresponding to the emission peaks of the fluorochromes

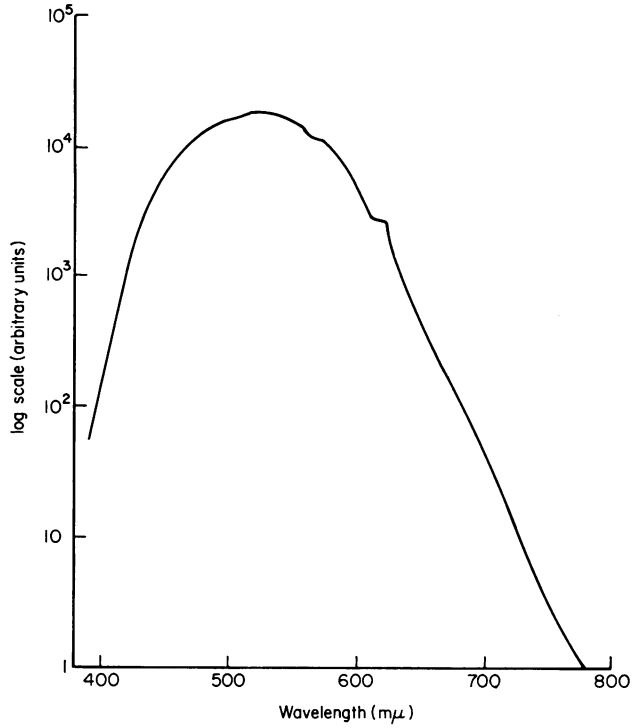

FIG. 2. Spectral sensitivity of phototube (Philips 150 AVP.S11 cathode).

used as antibody labels. The wavelengths selected were 520 mμ for fluorescein isothiocyanate (FITC) labelling, and 595 mμ for lissamine rhodamine B (RB 200). For comparison with ultraviolet–blue illumination, BG12/3 mm was employed as primary filter and a combination of GG9/1 mm with GG14/3 mm as first barrier filter. Apart from the Wratten film all filter glasses were manufactured by Schott and Genossen.

The emission is received by (8) a phototube (Philips 150 AVP.S11 cathode) of appropriate spectral sensitivity (Fig. 2), connected to an amplifier (9) and ammeter (10) of specially high sensitivity. This could be coupled to a pen recorder as desired.

The power both for the microscope lamp and the microphotometer was supplied through a voltage stabilizer with a measured output of 215 V±1%.

Microscopical preparations

All immunofluorescence staining procedures were carried out by routine standardized methods fully described elsewhere (Nairn, 1968, 1969). Fresh frozen sections of human

stomach, rat stomach and rat liver were employed for the majority of experiments, although a few confirmatory studies of nuclear staining were carried out on human blood films and mouse lymphocyte cytocentrifuge deposits. Immunofluorescent staining was accomplished either with specific rabbit antisera to gastrointestinal antigens (Nairn *et al.*, 1962; Lynraven & de Boer, 1969) labelled with FITC or RB 200, or by sandwich staining with unlabelled gastrointestinal antisera or human sera containing antibodies against gastric parietal cells or containing antinuclear factors, followed by the appropriate labelled antiglobulin.

For some purposes preparations were examined dry and unmounted, but for the majority of experiments they were mounted in buffered glycerol consisting of 9 parts glycerol (analytical reagent grade) and 1 part phosphate saline; for routine studies this consisted of 0·145 M-NaCl, 0·01 M-phosphate (pH 7·1). The glycerol phosphate saline had a pH value of 6·8 as measured by a glass electrode; higher values were obtained by varying the proportions of mono-, di- and tri-sodium phosphates. Dry unmounted preparations could be stored overnight at 2°C; once they had been mounted, microphotometry had to be completed within 1 hr, i.e. before there was any significant diffusion of fluorochrome into the mountant. In order to minimize the influence of fading of fluorescence, great care was taken to examine microscopic fields with the least possible exposure to ultraviolet radiation before taking measurements. For the most critical studies, focusing and aligning of the field to be studied in the appropriate diaphragm was accomplished by darkground illumination with visible light from the low voltage tungsten lamp provided by the Binolux equipment: photometer readings could then be taken from the first moment the selected object was exposed to the ultraviolet radiation.

Microphotometry procedure

Individual cells in the microscopical preparation were selected for microphotometry by a strictly standard procedure. The specimen was first inspected by visible illumination from the low voltage tungsten lamp and, after choosing a suitable field, ultraviolet illumination was employed to permit optimum focusing of the condenser to give maximum fluorescence. At this condenser setting individual cells were brought into the field, aligned with the appropriate diaphragm and measured, the preliminary steps being normally carried out by tungsten lamp illumination. The emission of not less than twenty cells of one type was measured for each experiment and the arithmetical means of the values obtained were used for comparisons between experiments. The variation of values in any given experimental situation was not great and the raw figures in each set of observations mainly fell within $\pm 25\%$ of their mean: for example, the standard deviation was 4·8 at photometer readings in the region of 20. A sensitivity range of the recording device was selected to give an adequate galvanometer reading and this range was adhered to for any one series of experiments.

As a fluorescent standard, a 0·17 mm thick uranium glass (GG17) was employed: it was mounted on a glass slide and accurate focusing on the fluorescent back surface was facilitated by marking with a small black dot. Neutral filters were required to reduce the fluorescence to the same order of intensity as given by the immunofluorescence preparations.

The sensitivity of the microphotometer was remarkably constant under stable laboratory conditions, i.e. in a darkened room and at times when substantial electrical apparatus in the building was not being switched on and off. Continuous recordings for periods up to

24 hr revealed variation in readings of no more than $\pm 1\%$; this is the same as the value given for the voltage variation, which might be largely responsible, therefore, for such observed fluctuations in photometer sensitivity. All experimental measurements were made under such stable conditions.

Specific procedures adopted for the different experiments will be dealt with in the appropriate part of the following section.

RESULTS

Correlation of fluorimetric readings with antibody titre and visual estimates

Immunofluorescent staining of rat gastric parietal cells by the sandwich method with human pernicious anaemia serum and FITC-conjugated goat anti-human-globulin was employed for this study. The pernicious anaemia serum and the conjugate were first diluted to give optimal staining, whereafter there was a linear relationship between the fluorimetric

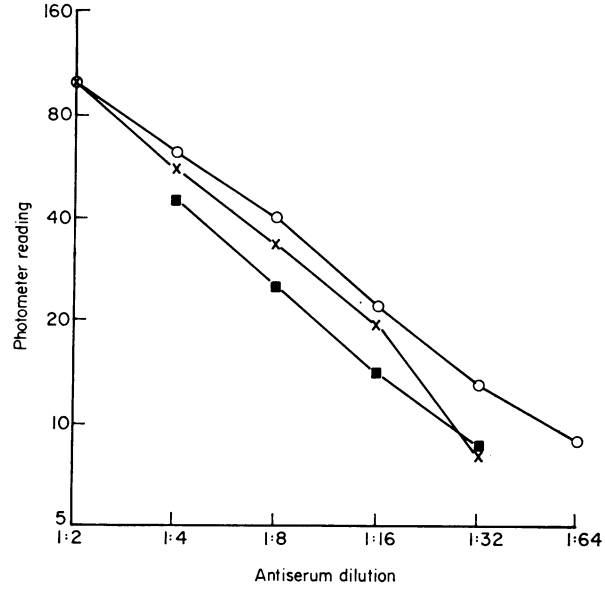

FIG. 3. Linear relationship between photometric reading and antiserum dilution. Rat gastric parietal cells stained with pernicious anaemia serum and FITC-conjugated anti-human-globulin. Logarithmic scales on both axes.

reading and further dilution of the pernicious anaemia serum. The results of three experiments are summarized in Fig. 3. This variation of fluorescence emission with antibody titre is also demonstrated in Table 1, in which the results of a further two experiments are recorded to show in addition that routine subjective visual assessments of fluorescence intensity roughly conform to the photometric readings.

In contrast with the results of gastric parietal cell staining, nuclear staining by human sera containing antinuclear factors showed no consistent correlation between fluorimetric values and serum dilution. Two or more fluorimetric peaks were observed in any series of dilutions, and irregular variations occurred when experiments were repeated. Attempts to

obtain consistent results by modifications of procedure or use of different sera containing antinuclear factors were unsuccessful.

Level of fluorescence and degree of fading under different conditions

The effect of pH of the mountant on intensity of fluorescence and on its fading when exposed to ultraviolet or ultraviolet–blue light was measured by rat gastric parietal cell staining as for the previous experiments. The results of a typical experiment with FITC

TABLE 1. Comparison in two separate experiments between photometric readings and visual assessments of immunofluorescent staining of rat gastric parietal cells by two pernicious anaemia sera at different dilutions

Serum dilution	Photometric reading	Visual assessment	Photometric reading	Visual assessment
1:2	100	+++++	100	+++++
1:4	84	++++	94	++++
1:8	74	+++	51	+++
1:16	39	++	36	++
1:32	24	+	26	+
1:64	18	(+)	10	(+)
1:128	12	—	8	—

(+) = Between + and −.

TABLE 2. Variation of fluorescence emission and of fading with mounting conditions and type of illumination (rat gastric parietal cells stained with pernicious anaemia serum and FITC-conjugated anti-human-globulin)

	Ultraviolet				Ultraviolet–blue			
		Buffered glycerol				Buffered glycerol		
	Dry	pH			Dry	pH		
		7·4	8·6	9·3		7·4	8·6	9·3
Fluorescence intensity in arbitrary units (highest reading recorded as 100)	70	74	100	77	91	57	71	100
Time to fade to half initial value (sec)	13	23	35	28	7	10	14	17

labelling are summarized in Table 2, which gives the readings obtained with unmounted preparations and with three different pH values of the mountant, viz. 7·4, 8·6 and 9·3. It is clear that fading, expressed as the time taken for the fluorescence to reach half the initial value, was slowest in the mounted preparation at pH 8·6 exposed to ultraviolet radiation only, and that this preparation also gave the most intense emission of all the ultraviolet-irradiated preparations. The emission was as brilliant in the preparation at pH 9·3 illuminated by ultraviolet–blue radiation but here fading was twice as rapid.

Comparative data for FITC- and RB 200-labelled antisera at equivalent titre are given in Fig. 4, which shows the decrease of fluorescence under different conditions after exposure to ultraviolet irradiation for 3 min. In contrast to the results with FITC, the highest initial intensity with RB 200 conjugates was given by the unmounted preparation and fading was not materially affected by the mounting. Moreover, although the initial fluorescence of the RB 200 preparations was up to six times lower than for FITC, the rate of fading of the RB 200 conjugate was so much slower that within 2 or 3 min the emission of either conjugate was of much the same order of intensity.

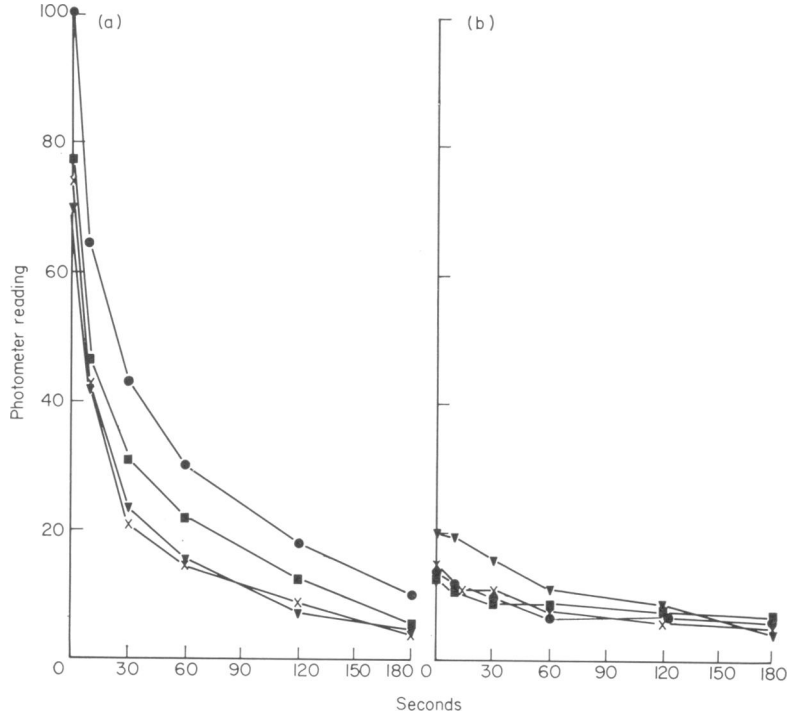

FIG. 4. Fluorescence intensity and fading time. Ultraviolet irradiation and different mounting conditions. (a) FITC conjugate. (b) RB 200 conjugate. ▼, Unmounted; ×, pH = 7·4; ●, pH = 8·6; ■, pH = 9·3.

The photochemical degradation of the conjugates is accompanied by a change of fluorescence colour as well as reduction in brilliance. In the case of RB 200 there is loss of the red component of the emission (Nairn, 1969).

Measurement of two antigens in different or the same cells

Sections of human stomach showing intestinal metaplasia were stained with FITC-conjugated anti-gastric mucous epithelium serum and RB 200-conjugated anti-intestinal serum. Visual inspection of the mucosa by ultraviolet fluorescence microscopy disclosed the following appearances in the superficial gastric mucous epithelium: (a) normal cells with bright green staining, apparently containing only stomach-specific mucin; (b) metaplastic cells with bright orange staining, apparently containing only intestinal-specific

mucin; and (c) intermediate cells with yellowish staining containing both antigens in varying proportions. Photometric readings at the appropriate emission maximum for each of the two fluorochromes confirmed these observations and permitted measurement of minor proportions of one or other antigen in cells not certainly classifiable as intermediate by visual inspection alone. No intermediate cells contained more than about half of the maximum amount of stainable antigen present in any single antigenic cell type, but it was not possible to determine any meaningful relationship between the relative amounts of the two antigens in these cells. Accurate figures for both antigens in individual cells could not be ascertained because of the inevitable fading of the second antigen to be measured during microphotofluorimetry of the first antigen.

DISCUSSION

Microphotometry in immunofluorescence provides for some systems, such as gastric parietal cell staining by pernicious anaemia serum, an accurate means of measuring serum antibody titre over a limited range of values, but strictly standardized conditions are needed. Essential requirements are stabilization of voltage supply, uniform microscopical equipment, in particular a high performance darkground condenser, and the use of an unvarying standard fluorescence source for initial photometer calibration in each series of experiments. For actual serum titrations, comparison must be made with a standard antiserum of known potency. We were unable to obtain consistent results for nuclear staining by antinuclear factors, possibly because of multiplicity of antibody–antigen reactions involved and variable diffusion of soluble nuclear antigens during processing. Our data are insufficient to confirm that the method gives an accurate absolute measure of antigenic content of individual cells but it does permit more certain recognition of cells containing very small amounts of an antigen than is possible by ordinary visual inspection.

The most useful application of microphotometry at the present stage of development of immunofluorescence is in permitting objective comparisons between the effects of variations in technical procedure. Such application has already been demonstrated by Pittman *et al.* (1967) in studies of non-specific staining, and we have reported here preliminary investigation of the effect of pH of mountant on fluorescent emission and fading of specimens. It seems that mountant at pH 8·6 is of definite advantage for tracing with FITC-labelled conjugates, but more extensive trial with a wide range of staining reactions should be made before replacing well-proven methods already in use. The greater initial intensity of fluorescence of FITC conjugates as compared with RB 200 conjugates has confirmed the advantages of the former for most routine work. However, the much slower fading of RB 200 conjugates on ultraviolet irradiation suggests their being especially applicable for fluorimetry which requires more prolonged exposure of microscopic specimens. The relative independence of RB 200 conjugate-stained specimens, of mounting or variations in pH within the range used is also an interesting observation. The very rapid initial decrease in fluorescence of FITC conjugates was an unexpected observation: it could only be measured by avoiding exposure of specimens to ultraviolet radiation until ready for immediate photometry after making all preliminary adjustments by tungsten lamp. Unless this be done, the first intense brilliance of FITC conjugate fluorescence and its fast fading will not be appreciated.

Measurement of two antigens in the same cell by double immunofluorescent staining is

difficult because of fading of one during photometry of the other. Because FITC conjugates fade more rapidly, their emission should normally be measured first. Ideally, double staining photometry should be checked on parallel microscopical preparations stained with pairs of antisera for which the fluorochrome labelling has been reversed. This is to say that four conjugates of two antisera should be available for any experiment (FITC serum 1, RB 200 serum 1, FITC serum 2, RB 200 serum 2) and each of two microscopical preparations should be stained respectively by the two different antisera in contrasting colours. Even with such precautions large numbers of individual cells from all representative areas of a tissue must be examined to obtain meaningful results and it may well be that this kind of application will not yield the cytogenetic data of which it is capable until we have available automatic scanning and subject the results therefrom to digital computer analysis.

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