Discussion

This novel approach to the measurement of the optical density of linear transects of stained tissue sections has several advantages. It is relatively fast (particularly with batching of the samples for analysis), requires no additional capital expense, is easy to perform, and is not especially labour intensive. Nevertheless, an estimation of the thickness of the sections[2] at intervals along the chosen plane of the transects is a recommended adjunct to the procedure.

We believe that this method could be useful in other instances when both quantitative and qualitative assessments of optical densities in other anisotropic pathological tissues are required.[2] The magnification of the tissue is independent of the method. Suitable enlargements of ultrastructural micrographs can be analysed in the same way as images obtained by low power light microscopy. The use of filters in the initial stage of photomicrography may enhance the selectivity of the method, depending on the light absorption characteristics of the dyes used. When studying nuclei stained by haematoxylin, as in our investigation, with a reasonably modest background of eosin this is not necessary when using the red in sensitive film.

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Simple technique for fluorescence staining of blood cells with acridine orange

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Fluorescence techniques, which were introduced at the beginning of the century,[1 2] are now widely used as a research tool in cytology.

Blood has been studied by several fluorochromes, including auramine and acridine. Acridine orange is the most popular fluorochrome for studies on whole blood,[2–6] reticulocyte counting,[1 7 8] and identification of nucleic acids.[9–11] It has also been used in the study of vaginal and cervical cancer,[12] cell culture,[13] blood parasites,[14 15] and certain automated techniques.[16 17] The initial object of this study was to develop a more sensitive method for reticulocyte counting. As the study progressed it became clear that the method was also suitable for the

detection of malarial parasites. In addition, it is possible to identify white cells and to assess red cell morphology in terms of size, shape, and staining intensity.

Material and methods

Different concentrations of acridine orange solution (Sigma Chemical Company) were tested in a pH range 5·6—8·2 using a large number of slides with different staining and incubation times. The blood samples were taken from normal subjects and from patients with a variety of blood disorders. The following solution of acridine orange gave optimal cellular staining in the minimum time: acridine orange 0·5 g and sodium chloride 8·5 g in 1 litre of distilled water.

The procedure for staining is as follows. Fresh blood (50 μl) is delivered into a small clean test tube, and an equal volume of the dye solution is added. The tube is then mixed gently for two minutes, after which thin smears are made in the usual manner. Rapid drying—for example, using an electric fan—improves the colour development in the cells. The slides are then examined under an oil immersion lens (x40 or x100 as appropriate) using incident ultraviolet light. In our experiments this light was produced by a

Watan HBO 50W super pressure mercury lamp, using a Leitz dialux 20 EB visible light and fluorescence microscope (E Leitz Instruments Ltd).

To avoid dilution of a blood sample the stain may be made in higher concentration (1g/l), dispensed in 25 µl aliquots into several tubes, and incubated at 37°C to dry. These tubes can then be stored for at least a year. To perform the test, 50 µl of blood is added to a prepared tube and mixed for two minutes. This procedure is recommended for some tests where the blood needs to be undiluted—for example, when identifying malarial parasites.

Although the slides are often sufficiently stained after 30 seconds, a longer period of up to two minutes is recommended to ensure adequate staining of all cells.

Fixatives such as methanol, ethanol, formalin, or Carnoy's solution contain destaining materials. Preparations stained with acridine orange cannot therefore be fixed since the smear would be decolourised and the cells would not then fluoresce. The cells stained by this solution of acridine orange do not undergo autolysis; moreover, under ultraviolet light not only is cellular detail preserved, but the cell inclusions also have different refractive indices which are easily distinguished. Counterstaining is not recommended as the dye could easily mask the fluorescence. Thus an acridine orange stained slide should be examined without further preparation, fixation, counterstaining, or mounting.

Results

Studies of many types of blood and bone marrow cells are possible using this method. The cells are easy to differentiate and well illuminated. Features such as shape, size, and intracellular material are readily seen.

Red cells are clearly visible, being surrounded by acridine orange green luminescence. The part of the red blood cell containing haemoglobin remains dark, whereas the centre of the cell which contains little haemoglobin, fluoresces. Therefore, the lower the haemoglobin concentration the brighter the illumination, and the higher the haemoglobin concentration the darker the illumination (figs 1–3). The red cells may fade within minutes of exposure to ultraviolet light, but this is not a problem because the fading occurs only in the field of vision and many fields are available for analysis. The assessment of anisocytosis, poikilocytosis, and so on is not affected by fading. Other cells do not fade, and, in fact, become more prominent as the red cells fade (figs 4 and 5).

The reticular material of reticulocytes emits a fiery red or bright orange-red fluorescence by this method. This red fluorescence is sometimes reticular in shape and may appear stippled. Reticulocytes can thus be readily distinguished since the red fluorescence of the reticulum stands out against the green stroma of the reticulocyte (figs 1 and 6).

Howell-Jolly bodies in the red cells appear as bright, yellow, small, spherical inclusions which are easily distinguishable from the reticular network of the reticulocytes.

The technique is also useful for detecting malarial parasites. The parasites are seen clearly in the red cells as ring forms or as irregularly shaped inclusions. They are green or yellowish green and have a large, dark, centrally located vacuole (fig 6).

Neutrophils exhibit bright green or yellowish green nuclei with a distinct chromatin network and small, bright orange cytoplasmic granules. Sometimes a few granules are dispersed over the nucleus giving an orange tinge. Occasionally, one or two brown vacuoles are present in the cytoplasm, especially in old samples (fig 4).

Eosinophils have bright deep green nuclei which are distinguishable from the nuclei of neutrophils; they have a chromatin network similar to that of neutrophils. The cytoplasmic granules are regular, spherical, and larger; they develop a bright orange colour (fig 5).

Basophils are smaller than eosinophils and their nuclei emit a deep dull green colour; they are not as bright as the nuclei of the neutrophils. Their granules are larger and fluoresce with a bright red rather than orange colour; occasionally, they cover part of the nucleus (fig 2).

Lymphocytes are distinguishable by their very bright yellowish green nuclei, the brightest elements in an acridine orange preparation. There is a small amount of cytoplasm around the nucleus emitting an orange hue because of a paucity of orange granules. The chromatin pattern of the nucleus usually gives a lobulated or divided shape, and is partly covered with orange granules. Usually, one or two large clearly visible orange nucleoli are open in the nucleus. (figs 3 and 4).

Monocytes exhibit dull green nuclei, which often have a convoluted or irregular shape. The nucleus has a pale chromatin pattern and an indistinct margin, while the cytoplasm is full of orange granules; a few brown vacuoles are present in the cytoplasm located either near the cell margin or near the nucleus (fig 3).
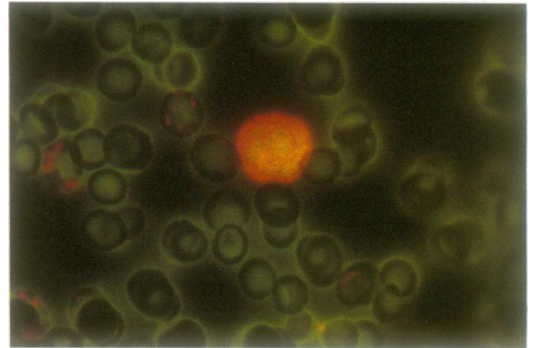
Platelets appear as bright greenish yellow bodies with a compact and almost centrally located inclusion, visible as a pale orange granule. Platelets do not usually have a distinct sharp margin (fig 6).
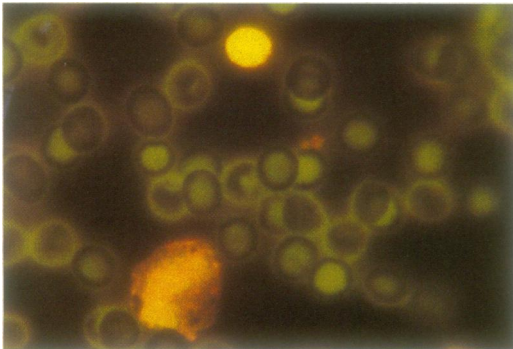
Discussion

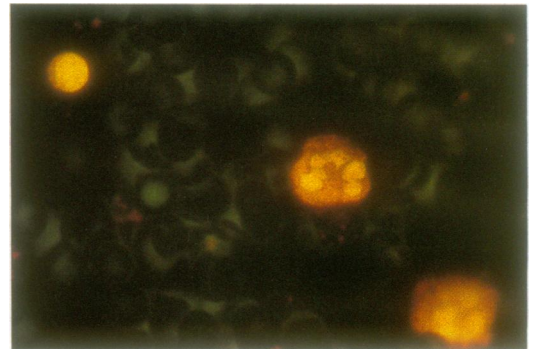Fluorescence techniques fall broadly into two groups:
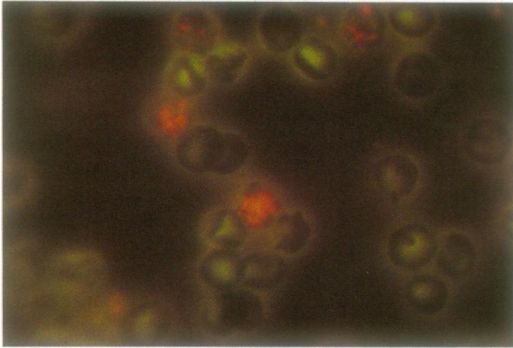
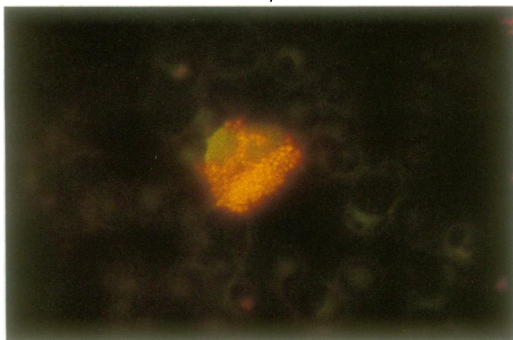Fig 1   *Four reticulocytes in a field of red cells; morphology of the red cells is easy to assess.*

Fig 2   *A basophil and four reticulocytes.*
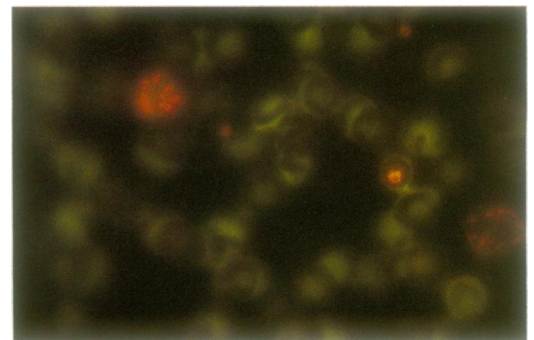

Fig 3   *A lymphocyte (upper field) and a monocyte (lower field).*

Fig 4   *A lymphocyte (top left) and two neutrophils (centre and below)*

Fig 5   *An eosinophil.*

Fig 6   *A malarial parasite (bright element in the middle left), two platelets, and two reticulocytes (left and below).*

wet and dry. Wet preparations[1 3 6 8] are generally less satisfactory; they are time consuming and the cells may require more than 30 minutes to settle into a single layer. The slides need careful handling otherwise the cells resuspend and require further time to settle. This type of preparation is unsuitable for storage, with a maximum life of about two hours.

Previously described dry techniques require the preparation of a series of solutions, which takes a relatively long time.[4 5 7] The staining takes at least 30 minutes, and the slides require staining in 10 different solutions.[4] Despite the complexity of the technique the slides do not usually give sufficient detail under ultraviolet light. This is often due to suboptimal concentration of dye and pH adjustment, among other factors. As a result, these methods are not always reproducible. The method of Schiffer[4] and other similar dry techniques usually result in a slide which fades quickly when exposed to ultraviolet light,[18] so that there may be insufficient time to evaluate the cells. The storage of such preparations is unsatisfactory because the slides fade after one or two days.

Clinical laboratories require simple, reliable, and reproducible methods. These requirements have not been met by any previously described fluorescence method, but are satisfied by the method described here. Preparation of the staining solution is simple, and there is no need to make stock and working solutions consecutively. A simple solution is required for staining any smear whether made from whole blood, buffy coat, bone marrow, or other biological fluid. The solution is stable for up to a year and does not need to be filtered before use or have the pH adjusted. Stained slides may be kept for at least a year without deterioration provided that light is excluded.

Another potential application of this technique is the study of mature red blood cells. Previous attempts to study mature red blood cells by fluorescence microscopy have proved unsatisfactory.[3 4 6 8 14] In this new method, although the haemoglobin in the red cells does not fluoresce by acridine orange the study of red cells is possible, and morphological features of red cells such as anisocytosis, poikilocytosis, and the degree of hypochromia can be assessed (figs 1–3). In addition, it is a simple matter to identify the normal physiological classes of white cell by this technique.

Our studies began with an evaluation of various methods of staining reticulocytes. We found that the technique described in this paper is greatly superior to the standard methods. It is easy to perform, reliable, and much more sensitive. It is also a simple and sensitive method for the detection of malarial parasites; even a relatively inexperienced observer can distinguish between parasites and reticular material in red cells.

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