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Rapid Detection of *Plastmodium* by a New 'Thick Smear' Method using Transmission Fluorescence Microscopy : Direct Staining with Acridine Orange

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ABSTRACT

A new staining technique of 'thick smears' was developed tor diagnosis of malaria using transmission fluorescence microscopy. Plasmodium falciparum cultured in vitro was used as a model. The infected blood was mixed 1 : 1 -1 :2 with acridine orange (AO) solution (final concentrations of 50-lOOu.g/ml in 0.01 M Tris-HCI buffer or in PBS, pH 7.0-7.5).
Immediately or several minutes later, haemolyzed or non-haemolyzed 'thick smears' were observed directly by fluorescence microscopy using an interference filter specially designed for excitation of AO or commercially available, three (interference-, glass- and triacetyl cellulose film-) types of B-excitation filters with halogen- or daylight-illuminated microscopes. All of these filters were capable of detecting the parasites rapidly in these wet mounts at a magnification of x200 using standard lenses. Among them, the interferencetype filters having higher transmission efficiencies gave the best results. Film filter system, which was the cheapest, could also be used, especially in combination with daylightilluminated microscopes, although it was necessary to be exposed accurately to the direct rays of the sun. These results strongly suggest that transmission fluorescence microscopy using any B-excitation filter in light microscopes may be a useful economic system for rapid diagnosis of malaria.

INTRODUCTION

Fluorescence microscopy combined with fluorochrome stainings has become a valuable means for rapid examination of cell components. Staining with acridine orange (AO) has been reported to be more sensitive than the Romanowsky technique for the detection of malaria parasites (Richards et al. 1969; Sodeman 1970; Shute and Sodeman 1973): AO binds DNA and RNA, and emits two fluorescences of green (530 nm) and red (650 nm) when excited at 430 nm and 492~495 nm, respectively. Therefore, AO staining permits differential colouration of green (nuclei) and red (cytoplasm) in stained parasites; the outlines of the parasites stained by these dyes are well preserved and the general morphology is comparable to specimens stained by Giemsa, but pigment granules and some organelles such as flagella are not stained (Kawamoto and Kumada 1987). This stainingtechnique has been also applied successfully to the 'QBC' tube system for rapid diagnosis of malaria and other parasitic infection (Spielman et al. 1988; Rickman et al. 1988; Long et al. 1990). This system was reported to be about 8 times more sensitive than the Giemsa-stained thick smears for detecting malaria parasites (Spielman et al. 1988; Rickman et al. 1989), although some investigators (Moody et al. 1990; Wongsrichanalai et al. 1991; Chiodini et al. 1991) stated that this system was not able to speciate malaria parasites nor to count

parasitaemias as effectively as conventional microscopy. Recently, a new staining technique for diagnosis of malaria using a fluorescence dye, benzothiocarboxypurine (BCP method) has been developed (Makler et al. 1990; Makler 1991).

Fluorochrome staining techniques are much easier for inexperienced workers and less time-consuming for detecting parasites in the dark field than the Giemsa stain. A great disadvantage of fluorochrome staining methods is their reliance upon an expensive fluorescence microscope. In the 'QBC' tube and BCP methods, cheaper fluorescence microscopic systems have been developed, both of which include epi-illuminated, special objectives and battery-powered halogen lights. However, these systems still seem to be expensive for most of tropical countries endemic for malaria.

Application of interference filter to standard light microscope is another possibility for making a cheaper system, 'transmission' fluorescence microscopy. This system can be applied to fluorochromes whose excitation wavelengths are over 400 nm, and is easily capable of detecting the emitted fluorescence. In previous papers (Kawamoto 1991a, b), the author presented a preliminary description of a new diagnostic technique for malaria by transmission fluorescence microscopy using a light microscope and a new interference filter specially designed for excitation of AO; application of the AO-interference filter system succeeded in observing two fluorescence colours from the stained parasites with standard light microscopes. In this paper, 1 report a new staining technique for rapid diagnosis of malaria in 'thick smears' by direct haemolyzing and simultaneous staining with AO. Furthermore, I also report the details of the comparison of the AO-interference filter with several B-excitation filters which are commercially available.

MATERIALS AND METHODS

Parasite and AO: *Plasmodium falciparum* (FCR-3 strain) was obtained from Prof. M. Suzuki, Gunma Univ., Japan, and cultured in vitro by the method described by Trager and Jensen (1976). The infected blood was taken from culture bottles, centrifuged and resuspended with human serum to be a 50% haematocrit. AO-hydrochloride was obtained from Sigma Chemicals Co. (Cat. No. A 4921), and stock solutions (1mg/ml) in sterilized, 0.01 M Tris-HCI buffer or PBS (pH 7.0-7.5) were kept in the dark.

A new 'thick smear' method: The infected blood (5μl)was mixed 1:1~1:2 with the AO-staining solution, 100~300μg/ml in the above buffers. A coverslip was placed on the specimens, and sealed with nail vanish, if necessary. Immediately (for non- or partially-haemolyzed samples) or several minutes later (for haemolyzed samples), these wet mounts were observed by transmission fluorescence microscopy using various B-excitation filters. Stainings of semi-dried or dried thin smears were also examined without fixation of methanol; the AO solution was put on a coverslip (ca. 50μl per a 24 x 50mm coverslip). Then, the coverslip was inverted and placed on smears. This procedure permitted an equal staining in the whole of smears.

Transmission fluorescence microscopy using B-excitation filters and light microscopes:
In this study, three types of B-excitation filters were used; (1) interference-type filters such as a new AO-interference filter (32 and 45mm ; Kawamoto 199la, b), Nikon B-3A (20mm , see Fig. 1A-a), Olympus BP-49C (20mm 0) and Olympus FITC-interference filter (45mm 0); (2) glass-type filters of Nikon FL-B, Zeiss BG-12 (see Fig. 1A-b) and Toshiba Glass V-40 (all 32mm 0); (3) film (triacetyl cellulose)-type filters of Fuji Film BPB-45 (Fig. IB-a) and SP-9, both of which were cut from each sheet (7.5x7.5 cm) into 32 and 45 mm 0 by scissors. FITC and film filters have red diffraction beams between about 640 and 700 nm (known as 'red light' for contrasting dark field) which disturb observation of the red fluorescence emitted from AO-stained specimens (Kawamoto 1991a). To suppress these beams, a suppression filter set (Olympus) composed of BG 14-3, 38-2 and 38-4 (Fig. 1C), or a suppression film

filter (Fuji PG-C100, Fig. 1B-b) cut from a sheet (10x12.5 cm) were used in combinations with the FITC filter or film filters, respectively. In all systems, a barrier filter (20 mm 0), each of Hoya Y-520 (glass-type), or Fuji SC-54 (film-type) which had been cut from a sheet (7.5x7.5 cm), was inserted into the body of the microscopes. There was no significant difference between two types of barrier filters.

Observations were made with two types of microscopes, i.e., halogen-illuminated microscopes (Nikon Optiphot-2 and Olympus BH-2; 100W) or simple binocular micro-scopes (Nikon type S and SE-Mirror) using the daylight illumination system. To fit small excitation filters to daylight (32mm 0)- or halogen (45mm 0)-illuminated microscopes, ring-shaped adaptors made by cutting a hard paper (32mm 0 with a hole of 18mm 0, and likewise, 45-18mm 0 or 45-30mm 0, Fig. 1A) were used. The condensers of all these microscopes were fully open, and ND suppression filters and frosted glasses in halogen-illuminated microscopes were removed to obtain stronger light. All of the objective lenses were standard (x10~40, without immersion oil). Optical halogen light sources (Nikon and Toyo Kogaku, 100~150W) were also examined, instead of the sunlight, with a combination of the simple microscopes.

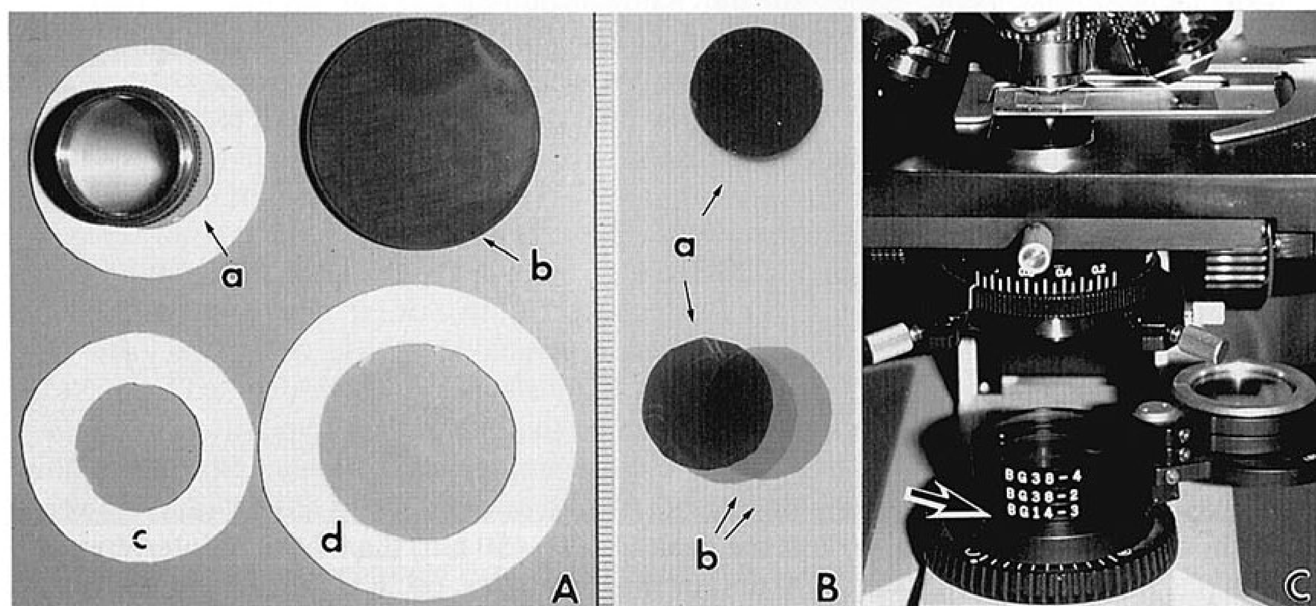

Fig. 1. Commercially available, B-excitation filters and ring-shaped adaptors for setting them to daylight-and halogen-illuminated microscopes.
A, an interference filter (a, B-3A) and a glass filter (b, BG-12) with their ring-shaped adaptors for daylight-(c, 32-18mm 0) or halogen- (d, 45-30mm 0) illuminated microscope. B, film filters (32mm 0) cut by scissors for excitation (a, BPB-45) and suppression (b, PG-C100). C, application of an FITC-interference filter with three suppression filters. The FITC filter (arrow) is hidden in a filler adaptor.

RESULTS

Application of the AO-interference filter to halogen-illuminated microscopes: Fig. 2A shows a fluorescence micrograph of 'thick smears' of P. *falciparum* haemolyzed and simultaneously stained with AO solution in Tris-HCI buffer. The nuclei of the parasites emitted yellowish green fluorescence, whereas the cytoplasm strongly fluoresced bright red. The morphology of the stained parasites was similar to that seen in standard thick smear stained with Giemsa, and this new 'thick smear' enabled rapid detection of

the parasites at low magnifications of x200 or less. It was also easy to detect the parasites in ‘thick smears’ partially-haemolyzed with the AO solution by mixing 1:1 (Fig. 2B), but thicker mounts of blood sometimes blocked physically the fluorescence emitted from stained parasites. Non-haemolyzed smears stained with AO in PBS gave results similar to those using partial haemolysis (see Fig. 2C). Similarly, semi-dried or dried thin smears stained with AO solution in Tris-HCI or PBS were also capable of detecting the parasites rapidly (see Figs. 2D-F). All the developmental stages of the parasites were easily detected from both these smears. The marked differences in size, shape and staining properties between the parasites and leucocytes enabled easy discrimination. Ability to detect the parasites in ‘thick smears’ was improved in comparison with the use of thin smears, and the new staining technique was superior in terms of simplicity and rapidity to the standard thick smears stained by the Giemsa. However, accurate identification of four species in human malaria parasites on the basis of new ‘thick smears’ alone, particularly in the haemolyzed method, may be as difficult as reported in the standard thick smear method. In this respect, non-haemolyzed ‘thick smears’ or semi-dried or dried thin smears stained with the AO solution in PBS seemed to be superior to the haemolyzed method. The best result was obtained in the AO staining at final concentrations of 50-100 μg/ml with halogen-illuminated microscopes.

<u>Application of other B-excitation filters to halogen-illuminated microscopes:</u> Two interference-type filters (B-3A and BP-49C) were capable of detecting fluorescing parasites as clearly as the full-sized AO filter did, although brightness of microscopic fields was little darker, probably because of narrow light-path. Application of the FITC-interference filter to halogen-illuminated microscopes also gave similar results (Fig. 2C) to those by the AO-interference filter, only when three suppression filters (BG 14-3, 38-2 and 38-4) were additionally used. However, these suppression filters overlaid on the FITC filter caused a darker microscopic field and a little weaker fluorescence than that by the AO filter alone. In cases of film filters (BPB-45 and SP-9), two or three suppression filters of PG-C100 overlaid on each excitation filter were also needed to observe fluorescence emitted from stained specimens (see Fig. 2E). Fluorescence strengths excited by these film filters were almost same, but much weaker than by the interference-type filters. On the other hand, glass filters (FL-B, BG-12 and V-40) which transmit wavelengths only below 500 nm could be used without any modification. All of these filters having much lower transmission efficiencies (ca. 40% at the peak of 420-450 nm) than those of interference types (ca. 80% between 430 and 490 nm) or film filters (ca. 60 % at the peak of 450 nm) gave darker field and weaker fluorescence, but they could also be usable for detecting the parasites (Fig. 2D).

<u>Application of various B-excitation filters to simple microscopes using the daylight illumination:</u> Simple microscopes using the daylight illumination system with interference-type filters such as AO (Fig. 2F), B-3A and BP-49C could detect two fluorescences emitted from stained parasites more clearly than halogen-illuminated microscopes. However, it was necessary to be exposed accurately to the direct rays of the sun, and only flat mirrors, but not concave ones, could be applicable to this system (Fig. 3A). The brightness of microscopic fields was uneven, strong only in the central zone and weak in the peripheral zone (see Figs. 2D-E), but it was no problem to detect the parasites. In addition, black paper placed around the revolver and the mechanical stage was required to suppress excess light (Fig. 3A).

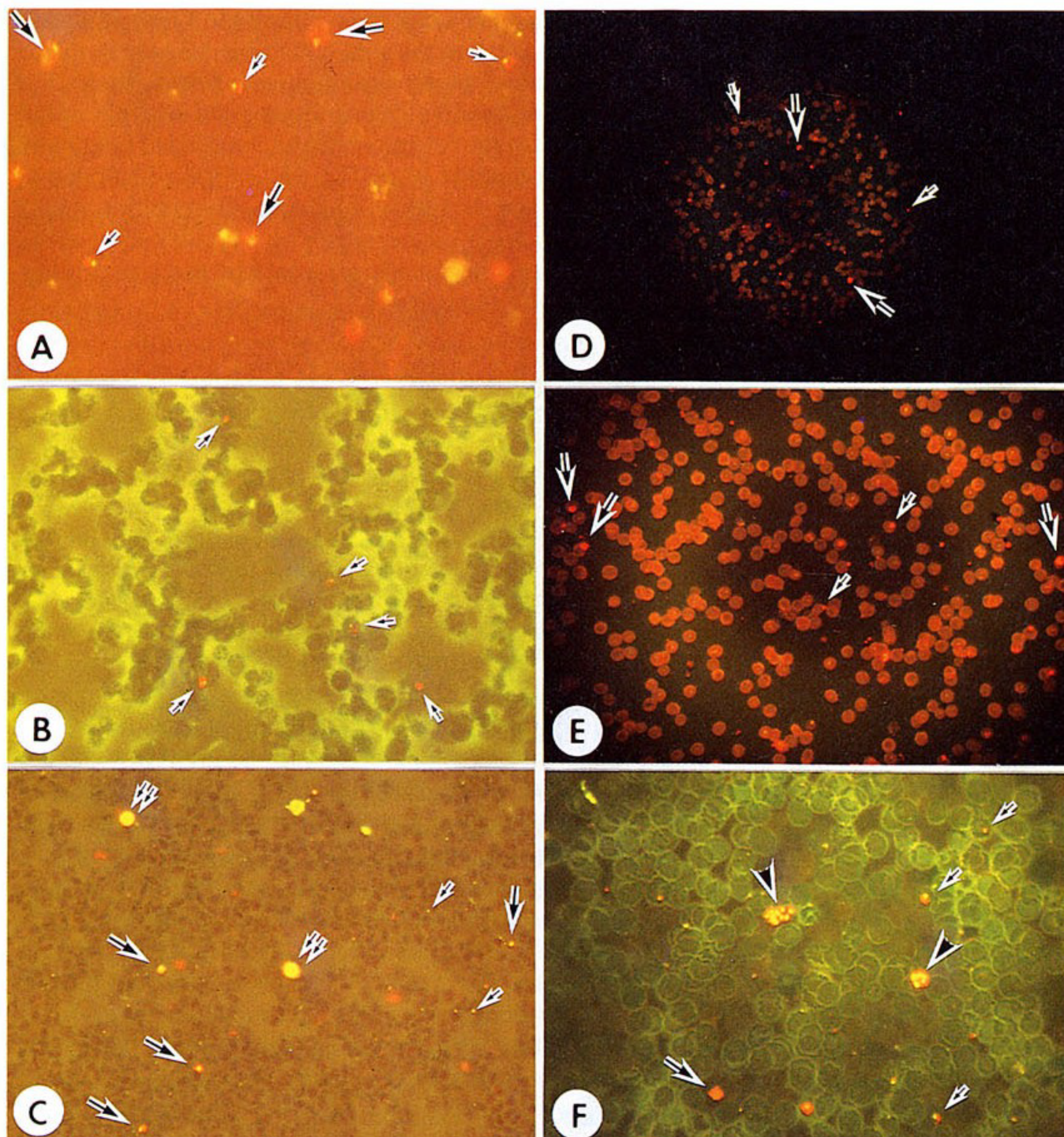

Fig. 2. Fluorescence microphotographs of *P. falciparum* in new 'thick smears' (A-C) and thin smears (D-F) using a halogen- and a daylight-illuminated microscope, respectively. Thick smears are stained with two volumes (A) or equal volume (B-C) of the AO solution at a final concentration of 100μg/ml (A-B, Tris-HCl buffer; C, PBS). Small arrows show early trophozoites, and large ones indicate later trophozoites or young gametocytes.
A-B, haemolyzed blood (3% parasitaemia) and partially-haemolyzed blood (1 % parasitaemia) observed by the AO filter system, respectively. A, x540; B, x360. **C**, non-haemolyzed blood (3% parasitaemia) observed by the F1TC filter system. Double arrows indicate lymphocytes, x170. **D-F**, air-dried thin smears (5% parasitaemia) observed by glass (BG-12, D), film (BPB-45, E) and AO (F) filler systems in a daylight-illuminated microscope with x10, x20 and x40 objectives, respectively. Note that brightened areas in D-E are limited in the central zones. Arrowheads in F indicate mature and maturing schizonts. D, x95; E, x190; F, x540.

When the microscope was directed at the sun, other objectives may make shadow on the mechanical stage, whereby no black paper was required. Setting up a separate, stand-type mirror near the window or outside for introducing the sunlight only into the flat mirrors (and not to the mechanical stage) of microscopes succeeded in allowing observation of fluorescence without any other modification (Fig. 3B). Optical halogen light sources gave darker microscopic fields than daylight- or halogen-illumination, but they were applicable to this system at night or cloudy, especially with a combination of the interference-type filter (Fig. 3C). In this case, concave mirrors could be used, providing brighter field and stronger fluorescence than by flat mirrors.

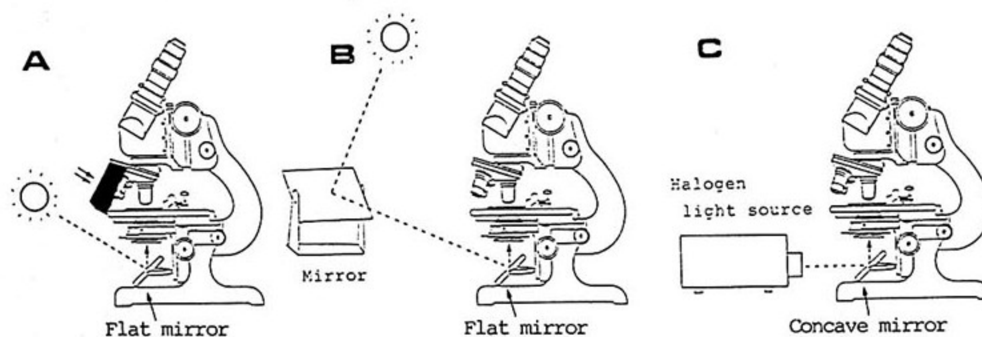

Fig. 3. Schematic drawings of practical applications of B-excitation filter system to daylight-illuminated microscopes. Double arrows in A show a black paper for blockade of the excess light. See text.

Similarly, other B-excitation filters, both of glass and film types, were capable of detecting the parasites more clearly than halogen-illuminated microscopes (Figs. 2D-E), although the microscopic fields were darker than by the interference types. In all applications, a darkroom was not needed to observe the emitted fluorescence. The AO staining at a final concentration of 100μg/ml was recommended in the use of daylight-illuminated microscopes.

DISCUSSION

The interference filter system designed for AO has been demonstrated as useful for the laboratory diagnosis of haematoparasites including *Plasmodium* (Kawamoto 1991a), *Trypanosoma* and microfilariae of the genus *Brugia* (unpublished). The morphology of fluorescing parasites in the dark field was easily identified at low magnifications. The author has routinely been using this system, instead of the Giemsa stain, to count the numbers of parasites in the laboratory work on *P. falciparum* cultivated in vitro.
Fluorescence strength emitted by the AO-interference filter system was weaker in the comparison with that by an epi-illuminated, mercury vapor fluorescence microscope (Kawamoto 1991a, b). However, fluorescence emitted by the filter was sufficient to observe all the parasites tested. In the present study, the author has shown that all of commercially available B-excitation filters such as interference-, film- or glass-type filters can be substituted with the AO filter, although some of them require additional suppression filters. Furthermore, transmission fluorescence microscopy using all of these filters reported here was capable of detecting a green fluorescence emitted form the benzothiocarboxypurine-stained *P. falciparum* (unpublished). In addition, these filters can transmit the wavelength for excitation of FITC (490 nm), indicating that they can also be used as an FITC filter for immunological investigations (Kawamoto 199la).

AO is one of the vital staining dyes, and its characteristics have been applied successfully to the 'QBC' tube method. The new staining technique for 'thick smears' reported in this paper was also based on these characteristics. AO staining has been reported to be unreliable because the green colour is not easily revealed (Kawamoto and Kumada 1987); careful adjustment of pH (Sodeman 1970) and cleaning with $CaCl_2$ solution, or rinsing with buffer solutions or water (Richards et al. 1969; Sodeman 1970; Shute and Sodeman 1973) have been recommended for colour differentiation. Actually, final concentrations of the AO solution higher than 100μg/ml were inadequate since nuclei of stained parasites appeared red or orange (Kawamoto and Kumada 1987; Kawamoto 199la). In the AO staining method reported previously (Kawamoto I991a, b) and in the present study, it was clear that cleaning with $CaCl_2$ or any rinse was not required as in the 'QBC' tube method. However, commercially available AOs have different characteristic staining properties (personal communication from Prof. S. Takada). When other AOs (e.g., hemi-[zincchloride] salt, A 6021, Sigma Chemicals) are used, concentration to give best results should be tested for each AO dye. The new 'thick smears' method was also applicable to detect *Trypanosoma* and microfilariae in blood samples, whereby the AO staining in PBS (non-haemolyzed method) was recommended (unpublished).

These above findings suggest strongly that any B-excitation filter including the AO-interference filter may become a useful and economic piece of equipment in tropical countries endemic for malaria. Particularly, film filter system, despite its lower transmission efficiency, might be promising in these areas since an estimated cost for a set of excitation and barrier filters is much cheaper than other filters, e.g., about one fifteenth of the AO-interference filter or less than one fiftieth of other interference filters. It should be emphasized that combination of these filters with simple microscopes using daylight illumination are capable of detecting the parasites, indicating a possibility that this method may be used as a practical diagnostic technique in these endemic countries, since the sunlight must be stronger than that in other areas. In tropical areas, the author is rather concerned about quenching of AO by strong UV-light of the sun. For preventing this, it is recommended to add 2-mercaptoethanol (0.5~1.0%, v/v) or 2-mercaptoethylamine (10mM) in the AO solution (Kawamoto et al. 1987; Kawamoto and Kumada 1987).

In the B-excitation filter system reported here, all of the AO-stained parasites emit strong red fluorescence. Therefore, the author recommends that detection of the parasites in new 'thick smears' or thin smears should be started at a low magnification of x200, and small red spots emitted from the specimens should be searched first. Subsequent observations of shapes and staining properties at a higher magnification of x400 may then permit reliable detection of the parasites. Identification of the four species of human malaria parasites should be performed from thin smears after the observations of 'thick smears'. Observed thick smears might be air-dried after removed coverslips, and directly stained with Giemsa for storage.

Unfortunately, tungsten-illuminated microscopes were inapplicable in the filter system (unpublished) because of its low excitation energy than halogen light or the sunlight. Improvement of the film filters to obtain more intense fluorescences, and development of a new system for tungsten-illuminated microscopes, are now in progress.

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