

Detection of Bacteriochlorophyll-containing Micro-organisms by Infrared Fluorescence Photomicrography

By BEVERLY K. PIERSON AND H. M. HOWARD

*Department of Biology, University of Oregon,
Eugene, Oregon, 97403, U.S.A.*

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SUMMARY

Fluorescence in the near infrared of individual photosynthetic bacteria was photographed through the microscope with infrared sensitive film. The fluorescence was also observed directly with an infrared sensitive image converter.

INTRODUCTION

The principles of fluorescence microscopy have been applied previously to reveal the presence of chlorophyll *a* in micro-organisms (Brock, 1968). When algae are illuminated with blue exciting light, they are easily seen by their bright red fluorescence. This property has been used to confirm the identity of chlorophyll *a*-containing micro-organisms in mixed field collections of blue-green algae and heterotrophic bacteria from hot springs (Brock, 1968). In such collections it would also be desirable to identify the photosynthetic bacteria which may appear morphologically identical to blue-green algae and chemotrophic bacteria. Since photosynthetic bacteria contain bacteriochlorophylls that fluoresce in the near infrared, it is impossible to detect visually the fluorescence with standard light microscopy. This report describes the use of two techniques that permit detection of infrared bacteriochlorophyll fluorescence with microscopy. The first technique uses infrared-sensitive film; the second uses an infrared image converter.

METHODS

Film technique. Purple photosynthetic bacteria containing bacteriochlorophyll *a* show a fluorescence maximum *in vivo* at approximately 900 nm (Olson & Stanton, 1966). Eastman spectroscopic safety film type 1-M* (Eastman Kodak Co., Rochester, New York, U.S.A.) is sensitive in this region (Fig. 1). To increase sensitivity, the film was hypersensitized immediately before use at 6 °C in 1% ammonia for 3 min followed by immersion in 95% ethanol for 2.5 min. The film was dried in a Kinderman rapid drier (Ehrenreich Photo-Optical Industries, New York, U.S.A.) and stored in metal cannisters before loading the camera. This treatment gave the film an exposure index of ASA (American Standards Association) 400, which is equivalent to the European Din 27, for bright field photomicrography. The film is so sensitive to visible and infrared radiation that it must be handled minimally and only in total darkness, stored below 0 °C, and cannot be used in film loaders or stored in plastic cassettes without a high degree of fogging which results from infrared exposure.

* The film is available in 35 mm 100 ft rolls and standard cut film sizes on estar base and as standard glass plates.

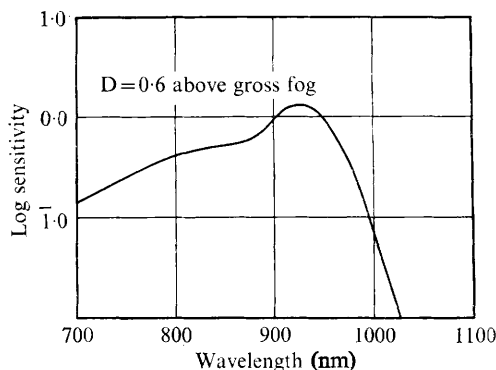
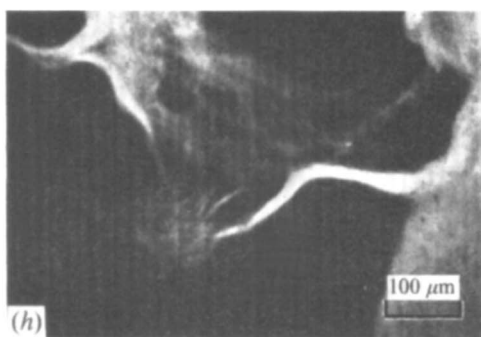
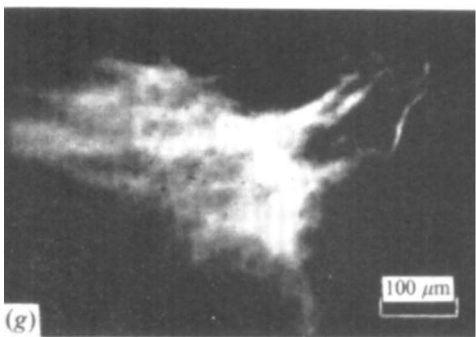
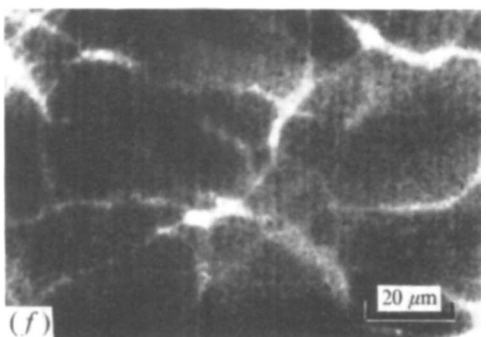
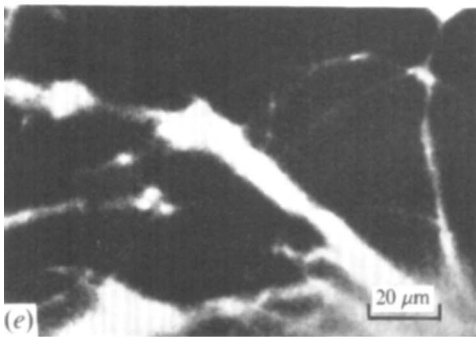
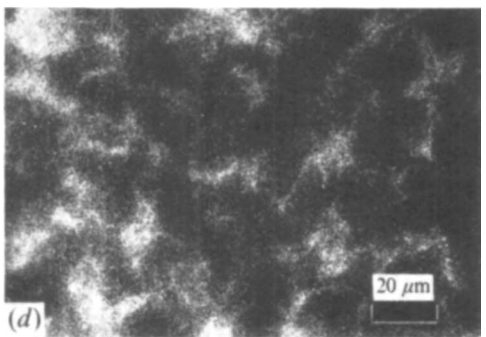
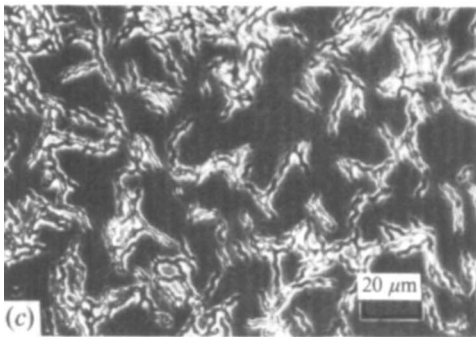
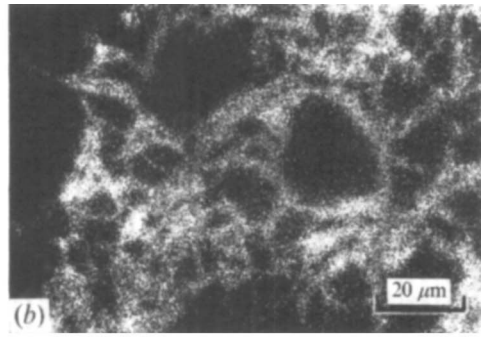
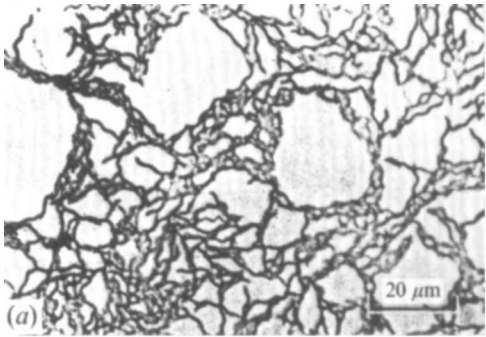


Fig. 1. Spectrum of sensitivity of hypersensitized Eastman spectroscopic safety film type 1-M. From *Kodak Plates and Films for Science and Industry*, p. 14d (Kodak Publication no. P-9, Eastman Kodak Co., Rochester, New York).

To record infrared fluorescence using this film, the bacteria to be examined were suspended in distilled water and dried on a microscope slide. The mount was placed on a Zeiss Universal microscope (Zeiss, Oberkochen, W. Germany) and was illuminated with a mercury arc lamp (HBO 200 W/4) (Osram, Berlin, W. Germany) with emission peaks at 334, 365 to 366, 405, 435, 546 and 577 to 579 nm. The exciting beam was passed through a standard Zeiss heat-absorbing filter, a Zeiss BG 38 red suppressor filter, 4 mm of Corning 1-59 heat-absorbing filters (Corning Glass Works, Corning, New York, U.S.A.) and 8 mm of Corning 4-76 infrared-absorbing filters, in that order. A 13% copper sulphate solution in water may be substituted for the Corning 4-76 filters (Eastman Kodak Co., 1968). With the condenser focused for Köhler illumination and the condenser diaphragm open completely, the exciting beam had an intensity of 7.3×10^5 ergs. $\text{cm}^{-2} \cdot \text{s}^{-1}$ at the bacterial layer, measured on a YSI-Kettering Model 65 radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The exciting-filter system was used to remove the little infrared output from the mercury arc. The emission peaks of the mercury arc lamp fall within the absorption peaks of bacteriochlorophyll *a* in purple bacteria (Olson & Stanton, 1966). Absorption of the 546 nm light by carotenoids in the bacteria may also contribute to fluorescence from bacteriochlorophyll *a*.

For high apertures, 10, 25 and 40 power plan-apochromatic objectives were used. The barrier-filter system consisted of a Kodak Wratten no. 87C filter (Eastman Kodak Co.). This is a cut-off filter transmitting no radiation below 800 nm and having a transmittance of 70 to 80% in the range of 880 to 920 nm. The filter was cut to fit the aperture in

Fig. 2. (a) Bright-field photomicrograph of dried cell masses of *Rhodospirillum rubrum*. (b) Infrared fluorescence photomicrograph of the same field at the same magnification as in (a). Exposure time 15 min. (c) Phase-contrast photomicrograph of dried cell masses and individual cells of *R. rubrum*. (d) Infrared fluorescence photomicrograph of the same field at the same magnification as in (c). Exposure time 15 min. (e) Infrared fluorescence photomicrograph of isolate OH-64-fl Clone 5 filaments containing bacteriochlorophylls *a* and *c*. Dried preparation. Exposure time 15 min. (f) Infrared fluorescence photomicrograph of isolate OK-70-fl filaments containing bacteriochlorophylls *a* and *c*. Dried preparation. Exposure time 15 min. (g) Photomicrograph of visible image produced by Wild M500 infrared converter unit from infrared fluorescence of the same organism as in (e). Dried preparation. Exposure time 4 min. (h) Photomicrograph of visible image produced by Wild M500 infrared converter unit from infrared fluorescence of bacteriochlorophyll *a* containing filaments embedded in agar. Exposure time 7 min.



a Zeiss slider which fits into the microscope tube above the objectives. It was held in place in the slider with a snap ring to prevent edge diffraction. The film was loaded in a Nikon camera (Nikon, New York, U.S.A.) and exposures of 10 to 15 min were used to record the fluorescence image. When desired, a bright-field or phase-contrast picture of the same field could be taken on the same film by switching to a tungsten lamp and using an automatic camera box with an ASA setting of 400. In this way it was possible to photograph a mixed sample of organisms and to follow it with a fluorescence image of the same field to identify the cells producing infrared fluorescence. After exposure the film was developed in total darkness in D19 developer (Eastman Kodak Co.) for 6 min.

Image-converter technique. For this technique the Zeiss Universal microscope was used with a Wild M500 infrared image-converter unit (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). The lamp, exciting-filter system, objectives and barrier filter were identical with those used in the film technique. The barrier filters provided with the Wild unit were not satisfactory for this work. The fluorescence image is converted by the Wild unit to a visible image seen through a special eyepiece focused on a fluorescent viewing screen. The same image was photographed in this case with Polaroid pack film (ASA 3000) (Polaroid Corporation, Cambridge, Massachusetts, U.S.A.) with exposure times of 2 to 7 min.

RESULTS AND DISCUSSION

Representative results from the infrared-sensitive film technique can be seen in Fig. 2*a-f*. Fig. 2(*a*)–(*d*) compares the fluorescence images from masses of *Rhodospirillum rubrum* with bright-field and phase-contrast photomicrographs of the same fields. Fig. 2(*e*) and (*f*) are fluorescence images from two different isolates of the filamentous bacteria containing bacteriochlorophylls *a* and *c* described by Pierson & Castenholz (1971). These Figures illustrate two of the problems of the infrared film technique – the large grain size which reduces clarity and the difficulty of achieving good focus when photographing infrared fluorescence images for long exposure periods. When the bacteriochlorophylls were extracted from all of the above organisms with methanol, no fluorescence images were detectable.

Fig. 2(*g*) and (*h*) are photomicrographs of the visible images produced by the Wild infrared image-converter unit from the fluorescence of two different filamentous bacteria. The masses in Fig. 2(*g*) are the same organism illustrated in 2(*e*) with the film technique. The filament masses in 2(*h*) were living cells embedded in agar and not dried as in the other preparations. They are bacteriochlorophyll *a*-containing filaments described previously (Pierson & Castenholz, 1971).

By using these techniques in association with visible fluorescence microscopy, morphologically similar organisms may be distinguished from each other. For example, the following taxonomically distinct organisms may be found together in a collection from a hot spring algal-bacterial mat: the blue-green alga *Phormidium* sp. (Brock, 1968), the heterotrophic flexibacterium *Herpetosiphon geysericola* (Lewin, 1970), and the bacteriochlorophyll-containing filaments described by Pierson & Castenholz (1971). All are filamentous and range from 0.5 to 1.5 μm in diameter. All may appear orange in colour when collected *en masse*. Conventional visible fluorescence microscopy reveals characteristic red fluorescence only from the *Phormidium* containing chlorophyll *a*. Infrared fluorescence microscopy reveals fluorescence from the *Phormidium* and the bacteriochlorophyll-containing filaments. It is assumed that the infrared fluorescence detected beyond 800 nm from blue-green algae is due to a tailing fluorescence emission in this region. Published emission spectra that have been determined as far as 750 or 770 nm

for some blue-green algae still show considerable emission at these wavelengths (Bergeron & Olson, 1967; Brown, 1969). No fluorescence was revealed under any conditions from *Herpetosiphon*.

The advantage of the infrared film method is that it is relatively inexpensive and can be done on any microscope. The film is difficult to handle however, focusing is difficult, exposure times are long, and there is considerable delay between examining the cells and obtaining the results. The infrared image converter seems ideal for this work since the image can be focused, and the field examined quickly, producing immediate results.

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