NOTES

Technique for Preparing High-Quality Microphotographs by Fluorescence Microscopy

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Simple techniques resulting in high-quality microphotographs by fluorescence microscopy are described for the preparation of Salmonella cultures and slides.

Simple techniques were developed for preparing high-quality color microphotography of flagellated cells of salmonellae detected by immunofluorescent techniques. For the preparation of high-contrast slides, it was necessary to optimize and integrate the optical system of the microscope, the preparation of slides, and the photographic procedures used for film development and printing.

Optical system. In this study microphotographs were made with a Leitz Artholux ultraviolet research microscope equipped with a Zeiss Ikon camera attachment capable of automatic exposures. The highest-power oil immersion objective lens (×95) equipped with a variable diaphragm was used, and a numerical aperture of 1.11 was selected for all experimentation. This apochromat-type lens was best suited for color photomicrography because of its extensive color correction, both spherically and chromatically. A high degree of correction was found necessary for fine resolution and high image quality. A compensating-type eyepiece located in the camera attachment was used in conjunction with the achromatic objective to compensate for the residual color errors present in the apochromatic objective. For proper color and contrast with ultraviolet light, it was necessary to use a dark-field condenser since the mercury vapor lamps contained only line spectra. With a dark-field condenser, care was taken to select slides of proper thickness; the correct range is usually specified on the condenser mount. If the slide was too thin, the oil seal collapsed when the condenser was racked up very close, whereas if the slide was too thick, it was impossible to ensure the best focus of the condenser. The slides used in this study had a thickness of approximately 1.2 mm.

Introduction of air bubbles in the immersion oil caused considerable difficulty due to flare and loss of image contrast. Therefore, care was taken to avoid the introduction of air bubbles in any of the oil seals. Best results were obtained with cover glasses 0.16 to 0.19 mm thick, with number 1-1/2 cover glasses serving very well. The thickness of cover glasses may be more critical with objectives of higher numerical aperture. The immersion oil used must not exhibit fluorescence. Cargille immersion oil, type A, was quite satisfactory.

A medium-blue exciter filter was incorporated in the optical system of the microscope to transmit the ultraviolet and blue light and absorb the remaining visible light. A deep-yellow barrier filter was used to absorb the ultraviolet light transmitted through the slide and to transmit the visible fluorescence.

Proper alignment of the mercury vapor lamp and the optical system was found to be very critical for optimal results. In addition, the lenses must be ultra-clean.

Preparation of slides. The flagellated cells were prepared by transferring an 8-h culture of Salmonella carrau (2933-54) (obtained from the Center for Disease Control, Atlanta, Ga.) into a test tube of motility test medium (Difco) and allowing it to grow throughout the medium overnight. This procedure significantly increased the number of heavily flagellated cells since it selected against those with few flagella. The culture was then transferred from the opposite end of the tube into H broth (2) to which 25 μg of vancomycin-hydrochloride per ml and 1.5% tryptic soy broth (Difco) had been added. After incubation for 6 h at 37 C, cells were centrifuged and washed three times with buffered saline (pH 7.8).
The cell suspension was then placed on acid-cleaned slides and allowed to dry. Smears were fixed by treating for 2 min in ethanol-chloroform-formalin (6:3:1, vol/vol). After drying, a drop of fluorescein isothiocyanate-labeled H antibody fraction was added to each smear and allowed to incubate for 30 min in a petri dish containing moistened filter paper. The conjugate was removed and slides were washed in saline, phosphate-buffered (0.066 M) saline (pH 7.8), and distilled water, and allowed to dry. A drop of phosphate-buffered (0.066 M) glycerine (0.1 M, pH 9.0) was used under the cover slip. Phosphate-buffered glycerine was found to be far superior to glycerine since it significantly enhanced fluorescence.

**Photographic procedures.** When making photomicrographs, all sources of vibration must be

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**Fig. 1.** Ultraviolet microphotographs of S. carrau grown at 37 C (A) and 45 C (B). Note the presence of flagella on cells incubated at 37 C and their absence in cells incubated at 45 C.
eliminated. In addition to the normal vibration sources (air conditioner, centrifuge, fraction collector, etc.), it was found that the power supply for the mercury vapor lamp caused enough vibration to blur the image. Focal plane shutters should be avoided completely. The Zeiss camera system had a magnetically damped leaf-type shutter, which was best suited for making photomicrographs.

Illumination should be maintained at a high level without losing contrast, and the system should be adjusted to achieve maximal brightness of cells in order to decrease exposure time, which in turn reduces the possibility of blur due to fluorescent fading of cells. It is highly desirable that a camera system be used that diverts at least 80% of the light from the subject to the film. The Zeiss automatic exposure system used 20% of the available light from the occular. The system could be further adjusted to permit 100% of the light available from the occular to reach the film plane.

Due to fluorescent fading, exposure times were kept to a minimum, necessitating the use of a very high-speed film. Unfortunately, to increase film speed, one must compromise for lower resolution. Several films were evaluated. Choice of a fast film over a slow, high-resolution film has the advantage of achieving greater contrast and brightness with less exposure time. The higher-speed film does not have as large a degree of reciprocity failure in each of the color-sensitive emulsions, thus producing a truer color and more overall contrast on the transparency. Anscochrome 64 (ASA 64), Kodak High Speed Ektachrome Daylight (ASA 160), and Anscochrome 500 (ASA 500) films were evaluated for optimal results. Photomicrographs made with the Anscochrome 64 required a 480-s exposure and were developed with an Anscochrome 75F developing kit to an ASA rating of 125. As expected, poor results were obtained even though the exposure time yielded a bright reproduction. The images were blurred, and poor color reproduction was obtained due to reciprocity failure. Kodak High Speed Ektachrome Daylight film was used for exposure intervals up to 360 s and was then processed by an authorized Kodak processor. Poor results were also obtained due to insufficient exposure. It is possible that satisfactory results could be obtained by force developing this film to ASA ratings of 400 or 640. Kodak will commercially force develop this film to an ASA rating of 400, and Kodak publishes information for developing film to an ASA rating of 640 with the use of an E-4 processing kit.

Use of Anscochrome 500 proved to be much more satisfactory. Exposure times were cut to 90 s by force developing this film to approximately 750 ASA with the 75F processing kit by increasing the processing time in the first developing solution by approximately 30%. This combination proved to be the best compromise for faithful reproduction of color, high contrast, brightness, and sharpness of details.

Microphotographs of S. carrau grown at 37 C (A) and 45 C (B) were prepared as described above and are shown in Fig. 1. Note the clarity and detail obtained with these procedures. These microphotographs were reported by Elliot et al. (1).

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LITERATURE CITED

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