

Modification of the Fluorescent Staining Method for Mycobacterial Cells^{*)}

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ABSTRACT

The fluorescein diacetate-ethidium bromide (FDA/EB) staining method for determining the viability of mycobacterial cells reported by Kvach and Veras was modified for applying the method to *Mycobacterium leprae* and *Mycobacterium lepraemurium*. Our method clearly differentiated green-stained cells from red-orange ones with either mycobacterial species and permitted their original fluorescence for over 4 min to be maintained.

Fluorescein diacetate (FDA), a non-polar and non-fluorescent fatty acid ester, passes easily into intact cells, where it is hydrolyzed by esterases of the cells to yield polar fluorescent fluorescein. When observed by a fluorescent microscope, living cells are stained green⁷⁾. In contrast, ethidium bromide (EB) is excluded by viable cells but enters cells with damaged membrane. Inside these cells, EB combines with double-stranded nucleic acid and produces red-orange fluorescence²⁾. Both FDA and EB have been used for measuring the viability of granulocytes^{1, 6)} and the semiquantitative assessment of mycobacterial viability³⁾. Recently, Kvach and Veras⁴⁾ reported the feasibility of using FDA/EB staining method for evaluating the viability of mycobacterial cells. However, they encountered the difficulty in observing *Mycobacterium leprae* cells microscopically, chiefly because of quicker loss in fluorescence of the green-stained cells, and could take no color photographs. The present paper describes preliminary results of modified FDA/EB method for staining *Mycobacterium lepraemurium* and *M. leprae*.

M. lepraemurium, the Hawaiian strain, sus-

pensions. Cells of *in vitro* grown *M. lepraemurium* were harvested 6 weeks after incubation at 37°C on Ogawa egg yolk medium⁵⁾. The cells were washed twice with and suspended in Dubos albumin-liquid medium (DLM) (Eiken, Tokyo, Japan) instead of Hanks' balanced salt solution containing 0.05% Tween 80, and homogenized gently in a Teflon homogenizer on crashed ice. The homogenate was centrifuged at 500 × *g* for 5 min. The supernatant was used as the single cell suspension.

Cells of *in vivo* grown *M. lepraemurium* were obtained from the liver of a mouse 6 months previously infected intravenously with the organism. The liver was minced, trypsinized and suspended in a tissue culture medium supplemented with 10% fetal calf serum. The tissue-cell suspension was centrifuged at 200 × *g* for 10 min. The supernatant was centrifuged at 17,000 × *g* for 30 min in the cold. The pellet was suspended in DLM and the single cell suspension was prepared in the same way as mentioned above.

Needle biopsy of the liver infected with *M. lepraemurium*. Needle biopsies were taken from the liver of a mouse previously mentioned.

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The tissue was touched on microscopic slides and air-dried at room temperature.

M. leprae suspension. Cell suspension of *M. leprae*, the Kurume-Naha strain, harvested from lepromatous lesion of a nude mouse was supplied by K. Kohsaka, Research Institute for Microbial Diseases, Osaka University, Japan. For the FDA/EB staining, the suspension was treated with DLM in the same way as that of the *M. lepraemurium* suspension.

FDA/EB staining solution. A stock solution of FDA (Sigma, St. Louis, Missouri, USA) was prepared to give a concentration of 5 mg per ml in acetone, and that of EB (Sigma, St. Louis, Missouri, USA) was to give a concentration of 2 mg per ml in 1/75 M phosphate buffered saline (PBS), pH 6.5, containing 0.05% Tween 80. One-ml volume of each stock solution was distributed to small screw-capped tubes and stored at -20°C in the dark.

A fresh working solution of FDA/EB was prepared immediately before operation. The formulation by Kvach and Veras was considerably modified. The FDA stock solution was diluted 1:5 in acetone and a 0.5-ml volume was added to a 4.5-ml volume of PBS-Tween 80. The EB stock solution was diluted 1:250 in PBS-Tween 80 containing the FDA. A final concentration of FDA and EB in the working solution was per ml 100 μg and 8 μg , respectively.

Staining of bacterial cell with FDA/EB. For the bacterial cell suspensions, a 0.5-ml

volume of the sample was mixed with a 0.25-ml volume of FDA/EB working solution and incubated at 37°C for 60 min. One drop of the mixture was placed on a microscopic slide and a wet mount was prepared. The coverslip was pressed tightly and sealed with colorless nail polish. For the smear, FDA/EB working solution was placed directly over the smear which was incubated under the same condition as the above, preventing evaporation. A wet mount was prepared and the coverslip was sealed.

Fluorescent microscopy. Stained preparations were observed with an incident fluorescent microscope, Olympus BH2-RFL, equipped with a UG1 exciting filter, DM400 beam splitting mirror and L420-L435 suppression filter, at a magnification of $500\times$. For taking color photographs, 4-min exposure was made with Kodak Ektachrome ASA 400 and a ND-25 filter.

Results. Our staining method enabled the cells of either *M. leprae* or *M. lepraemurium* to be stained clearly. The green-stained mycobacterial cells maintained their original fluorescence for over 4 min and permitted percent green-stainability of the cells to be determined as well as color photographs to be taken easily (Figs. 1 and 2). A green fluorescence in background generated by host-tissue derived esterases was minimum and did not disturb the observation. Subsequently, the single cell suspension of *M. leprae* or *M. lepraemurium* was exposed for various lengths of time to a tem-

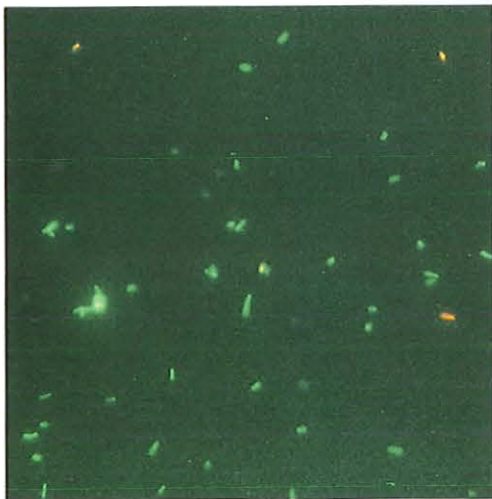


Fig. 1. *In vitro* grown *M. lepraemurium*, Hawaiian strain. Treated with modified FDA/EB staining method for 60 min at 37°C .

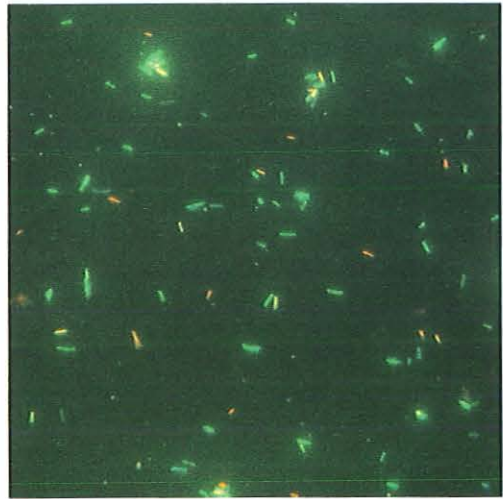


Fig. 2. *M. leprae*, Kurume-Naha strain. See legend in Fig. 1.

perature of 60°C and percent green-stained cells was determined. The reduction in the percentage was proportional to the exposure time to heating in either case (the data will be published later).

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