LETTER TO THE EDITOR

**USE OF FLUORESCEIN-PHALLOIDIN AND DAPI AS A
COUNTERSTAIN FOR IMMUNOFLUORESCENCE
MICROSCOPIC STUDIES WITH SEMITHIN
FROZEN SECTIONS**

Although the immunofluorescence method is a versatile procedure, it is sometimes difficult to specify the positive sites against the dark background. In the immunoperoxidase method, counterstaining of the nuclei with methylgreen or hematoxylin greatly helps the identification of cells and tissues. When semithin frozen sections are employed in immunofluorescence studies, a discrete staining pattern is obtained against the low background (5, 7, 8). Confocal imaging also provides clear fluorescence images with very low background (11). However, identification of the positive structure becomes very difficult in these cases. For correction of this problem, it is often useful to record the bright field images with phase-contrast or Nomarski differential interference contrast optics along with the fluorescent images and to compare them side by side. One of the advantages of the fluorescence microscopy is that multi-parameter fluorescence imaging can be easily achieved by the use of proper probes and instruments (2). We show here a simple and useful counterstaining procedure for use in the immunofluorescence technique.

We use the combination of fluorescein-phalloidin (1, 13) and DAPI (4', 6-diamidino-2-phenylindole) (12) as counterstain in rhodamine (Texas Red)-labeled semithin frozen sections. Jejunum was removed from a male ddY mouse, washed with phosphate-buffered saline (PBS), opened flat on a Millipore filter (type HA), and fixed in 3% formaldehyde-PBS for 2 hr at room temperature. After being infused in 2.3 M sucrose-0.1 M phosphate buffer, pH 7.4, semithin frozen sections of 1-1.5 μm in thickness were cut with a Sorvall MT-2B ultramicrotome equipped with a LTC-2 cryoattachment (5, 7-9). Sections were mounted on glass slides precleaned with 1% HCl ethanol and deionized water. The specimens were first covered with 1% bovine serum albumin (BSA) for 10 min, and then with biotinylated *Ricinus communis* agglutinin I (RCA, Vector Laboratories, USA) in 0.1% BSA-PBS for 1 hr. After being washed with PBS, the sections were incubated with Texas Red-streptavidin (1 : 100 dilution, Amersham, UK) in 0.1% BSA-PBS for 40 min. After a brief rinse with PBS, the sections were incubated with a mixture of fluorescein-phalloidin (1 : 50 dilution, Molecular Probes, USA) and DAPI (2 ng/ml, Boehringer-Mannheim, West Ger-

FIGS. 1a-d. A portion of jejunum epithelium stained with biotinylated RCA/streptavidin-Texas Red (red), fluorescein-phalloidin (green), and DAPI (blue). a. A single exposure image for RCA/Texas Red showing the localization of RCA binding sites. b. A double-exposure image for RCA/Texas Red and DAPI. c. A double-exposure image for fluorescein-phalloidin and DAPI. d. The corresponding Nomarski differential interference image. The arrows show nuclei, and the arrowheads point to the supranuclear staining for RCA. M: microvilli, G: goblet cells. $\times 1,300$

FIG. 2. Ultrastructural localization of RCA binding sites in the spranuclear region of the epithelial cells of jejunum. The Golgi apparatus with lamellae and associated vesicles (G), the lateral plasma membrane (arrowheads), and the cytoplasmic vesicles (arrow) are positively labeled for RCA. $\times 27,000$

Inset shows a cross section of the apical microvilli. Colloidal gold particles are seen preferentially on the outer surface of the plasma membrane. $\times 40,000$

many) in PBS for 30 min. After a wash with PBS, they were mounted in 1 mg/ml *p*-phenylenediamine-90% glycerol-0.1 M Tris-HCl buffer, pH 8.5 (3). Specimens were examined with a Nikon Microphot FX microscope equipped with epifluorescence (G, B1 with Olympus G520 barrier filter, and UV excitation) and Nomarski differential interference optics. Single- and double-exposure images were photographed. In addition to semithin frozen sections, ultrathin frozen section were also cut, and labeled with RCA-colloidal gold (1:10 dilution, 15 nm in diameter, E.-Y.

Laboratories, USA). These sections were osmicated and ultrathin-embedded in LR White (London Resin, UK) according to Keller *et al.* (4).

RCA binding sites were seen in the epithelial cells, goblet cells, and cells of the lamina propria (Fig. 1a). DAPI is a specific probe for DNA (12), and double-exposure images for biotinylated RCA/Texas Red-streptavidin and DAPI clearly showed RCA binding sites and nuclei (Fig. 1b). Since F-actin is, in general, localized in the cellular cortex in tissue cells (6, 10), fluorescein-phalloidin preferentially delineated the plasma membranes (Fig. 1c). Microvilli, which are also rich in F-actin, were intensely stained. A combination of DAPI and fluorescein-phalloidin, therefore, clearly showed two of the basic features of cells, namely, plasma membranes and nuclei, thereby serving as an ideal counterstaining procedure for Texas Red- or rhodamine-labeled specimens. Location of nuclei and cell shape were also observed by Nomarski differential interference contrast images (Fig. 1d).

Comparison of images of biotinylated RCA/Texas Red-streptavidin/DAPI with those of fluorescein-phalloidin/DAPI clearly showed that: 1) the entire plasma membrane of epithelial cells was positive for RCA; 2) cytoplasmic granular staining was scattered in the apical cytoplasm, and 3) cytoplasmic worm-like staining was located at the apical side of nuclei, suggesting it to indicate the Golgi apparatus (14). Electron microscopic examination of the ultrathin frozen sections confirmed that the Golgi apparatus and cytoplasmic vesicles, as well as the plasma membrane were positive for RCA (Fig. 2).

In conclusion, when F-actin staining with fluorescein-phalloidin is combined with nuclear staining with DAPI, the combination serves as a useful counterstaining procedure in immunofluorescence microscopy.

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Kuniaki Takata and Hiroshi Hirano
Department of Anatomy, Kyorin University
School of Medicine, Shinkawa, Mitaka,
Tokyo 181