The electron optical analog of immunofluorescence microscopy utilizes emitted electrons instead of emitted light. The excitation source is still an UV lamp but the optical microscope is replaced by an electron emission-type microscope (photoelectron microscope). The resolution is determined not by the excitation source but by the wavelength of the emitted particle—in this case, a low-energy electron. A comparison of immunofluorescence microscopy and the electron optical analog is shown in Fig. 1. In immunofluorescence, light incident on the biological specimen stimulates fluorescence emission from dye molecules (e.g., fluorescein or rhodamine) that are covalently linked to antibody molecules (Fig. 1 Left). In immunophotoelectron microscopy, light of somewhat shorter wavelength stimulates the emission of electrons from suitable markers also linked to antibody molecules (Fig. 1 Right). The theoretical resolution of the photoelectron microscope using conventional electron optics is about 5 nm so that the image contains information on the order of single protein resolution (and using corrected electron optics the resolution could approach the diffraction limit of 1 nm for a 1-eV electron). The electrons are then accelerated to high velocities (30-50 kV) and imaged by an electron optical system that gathers information from all emitting points on the specimen simultaneously to form the image. Immunophotoelectron microscopy was first proposed in 1972 (4). Three developments were needed to bring this idea to fruition: the development of a high-resolution ultra-high vacuum photoelectron microscope with image intensification (5), theory and experiments on the photoelectric behavior of biological macromolecules (6-9), and a search for suitable photoemissive markers (10-12). While this work was progressing two important developments, the production of monoclonal antibodies (13) and the introduction of colloidal gold as a marker in electron microscopy (14), have extended the capabilities of immunological mapping approaches, including immunophotoelectron microscopy. In this paper we present a comparison of immunophotoelectron microscopy with immunofluorescence on the same specimens, using, as an example, microtubules in CV-1 epithelial cells.

MATERIALS AND METHODS

Cell Line and Antibodies. CV-1 (African green monkey) kidney epithelial cells were grown on sterile tin oxide-coated 5-mm glass coverslips (Belloco Glass) prepared as described (15). The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO), 25 mM Hepes, and 2 mM L-glutamine in a 10% CO2/90% air incubator at 37°C. The mouse monoclonal IgM antibody against microtubules were a gift from L. R. Chen. Rhodamine-conjugated goat anti-mouse IgM (GAM) and the rabbit anti-goat IgG (RAG) were obtained from Cap- pel Laboratories (Cooper City, PA) and E Y Laboratories (San Mateo, CA), respectively.

Abbreviations: DTSP, dithiobis(succinimidyl propionate); RAG, rabbit anti-goat IgG; GAM, goat anti-mouse IgM.
In both cases markers have been coupled to antibodies directed against a cell surface antigen. In immunofluorescence, the emitted photons mark the distribution of antigen and in immunophotolabeling methods provide similar information but at much higher spatial resolution. The diagram is not drawn to scale (the markers and cell surface components are much smaller than drawn here).

**Fig. 1.** Schematic diagram of an immunofluorescence experiment (Left) and the corresponding immunophotolabeling technique (Right).

<table>
<thead>
<tr>
<th>Visible Light</th>
<th>UV Light</th>
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<td>Immunofluorescence Microscopy</td>
<td>Immunophotolabeling Microscopy</td>
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**Colloidal Gold and Immunogold Complexes.** Colloidal gold particles, 20 nm and 30 nm in diameter, were prepared by the method of Frens (16). Antibody–colloidal gold complexes were made by the procedures of De Mey et al. (17) and were stored at 4°C in 20 mM Tris/0.15 M NaCl/20 mM NaN3/1% bovine serum albumin, pH 8.2.

**Detergent Treatment and Immunolabeling.** The cells were treated with dithiobis(succinimidyl propionate) (DTSP, from Pierce) for 5 min at 37°C (18). DTSP was freshly diluted from 100 mM HEPES/1 mM EGTA/0.15 M NaCl, pH 7.4. Following the DTSP treatment, the cells were treated with 1% bovine serum albumin in HEN buffer for 3 min at 37°C to block any unreacted DTSP. The DTSP-treated cells were then rinsed with microtubule stabilization buffer (MTS buffer) as described by Osborn and Weber (19) but without GTP (100 mM HEPES/1 mM EGTA/4% polyethylene glycol (6000 molecular weight), pH 7.0) and incubated for 5 min at 37°C in MTS buffer containing 2.5% Triton X-100. Following a rinse with MTS buffer, the extracted cells were fixed for 10 min at 37°C, then treated with normal rabbit serum for 20 min at 37°C to reduce nonspecific binding. Antibodies were applied as follows. The first antibody was a murine monoclonal IgM recognizing myelin-associated antigen, supplied as unlabeled conditioned medium from hybridoma lines H3-45 or H2-1B2. The second antibody was rhodamine-conjugated GAM or rhodamine-GAM bound to colloidal gold. On this basis alone the fibers seen in the fluorescence image (15) and the microtubules in the same cell. The first, second, and third antibodies were monoclonal IgG recognizing microtubules, rhodamine-conjugated GAM, and RAG bound to 20-nm colloidal gold, respectively. The photolabeled microtubules are clearly visible in Fig. 2A, and immunophotolabeling (Fig. 2B) microscopy of microtubules in the same cell. The first, second, and third antibodies were monoclonal IgM recognizing microtubules, rhodamine-conjugated GAM, and RAG bound to 20-nm colloidal gold, respectively. The photolabeling technique is both a fluorescent marker (rhodamine) and a photolabeled marker (20-nm colloidal gold). Arrows identify some of the many microtubules that can be seen in both the fluorescence and photolabeling micrographs. The diagonal dashed lines in A indicate the positions of the microtubules after photolabeling so that the cell has the same horizontal orientation in A, B, and C, n= nuclei. (Bars are 30 and 5 μm for A and C, respectively.)

**RESULTS**

Fig. 2A is a typical immunofluorescence image of microtubule networks in a CV-1 cell. The cell has been extracted with Triton X-100 in a MTS buffer, followed by a three-step antibody application to allow both immunofluorescence (Fig. 2A) and immunophotolabeling (Fig. 2B) microscopy of microtubules in the same cell. The first antibody rather than a third antibody was bound to the gold label and not the rhodamine fluorochrome gave similar images. How ever, the immunophotolabeling micrographs of Fig. 3A and B clearly illustrate the distribution of microtubules in this cell, with minimal nonspecific binding. Other two-step labeling experiments in which the second antibody carried only the gold label and not the rhodamine fluorochrome gave sim-
We selected colloidal gold as an initial marker for immunopho
toelectron microscopy because of its photoemissive properties (22). Although materials that are more photoemis
tive, colloidal gold is enjoying wide use as an electron
dense marker for transmission electron microscopy and
scanning electron microscopy and can be seen in some cases by optical microscopy (14, 17). There have even been a few reports of double-labeling for fluorescence and transmission
electron microscopy using fluorescent protein conjugates
coupled to colloidal gold (23, 24), although not the specific
combination used here. Thus, at present colloidal gold
comes closest to being a universal marker and makes possi
ble a wide range of comparative experiments.

The double-labeling experiment of Fig. 2 provides a direct
comparison of immunofluorescence microscopy and immu
nophotoneto electron microscopy on the same specimen. The
graphical contrast has been used alone to visualize cytoskel
etal structures on the specimen surface (9). This sensitivity to topo
graphical detail is very useful for imaging fine details but can
extend the useful range in cases of larger-scale structures,
such as some nuclear regions and rounded-up cells. Topo
graphical contrast has been used alone to visualize cytoskel
etal structures by photoneto electron imaging in a previous study (12). In that case, the cytoskeletal elements were indirectly
identified by comparison with immunofluorescence images of
the same cells. The present study represents a significant
advance in that photoneto electron microscopy bound to site-specific antibodies antibodies are used to directly identify specific cytoskeletal elements.

To summarize, the purpose of the present study is to dem
strate the use of photoneto electron imaging in conjunction with antibody techniques as the electron optical analog of immuno
detection by fluorescence microscopy. Immunophotoneto electron microscopy employs both photoemissive markers and topo
graphical information to provide an image of the distribution
of specific antigenic sites with respect to cellular structures. Immunophotoneto electron microscopy retains the essential basis of
immunofluorescence— that is, the emission of signals from
coupled to colloidal gold. Moreover, the topographical features of sibling fibers appearing in the electron microscope images, generally require the specimen to be either dehy
drated or frozen. Photoelectron microscopy has been com
pared with transmission electron microscopy to be either dehy
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