Visualizing endocytotic pathways at transmission electron microscopy via diaminobenzidine photo-oxidation by a fluorescent cell-membrane dye

S. Grecchi, M. Malatesta
Department of Neurological and Movement Sciences, Anatomy and Histology Section, University of Verona, Italy

Abstract

The endocytotic pathway involves a complex, dynamic and interacting system of intracellular compartments. PKH26 is a fluorescent dye specific for long-lasting cell membrane labelling which has been successfully used for investigating cell internalization processes, at either flow cytometry or fluorescence microscopy. In the present work, diaminobenzidine photo-oxidation was tested as a procedure to detect PKH26 dye at transmission electron microscopy. Our results demonstrated that DAB photo-oxidation is a suitable technique to specifically visualise this fluorescent dye at the ultrastructural level: the distribution of the granular dark reaction product perfectly matches the pattern of the fluorescence staining, and the electron density of the fine precipitates makes the signal evident and precisely detectable on the different subcellular compartments involved in the plasma membrane internalization routes.

Materials and Methods

Cell culture and treatments

Human HeLa cells (ATCC, Rockville, MD, USA) were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 U of penicillin and streptomycin (Cellbio S.r.l., Milan, Italy), in a humidified air atmosphere containing 5% CO2. Cells were seeded onto glass coverslips in six-multiwell plates (1×10^6 per well) 48 h before being incubated with PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich, Catalog Nos. MINI26 and PKH26GL) to stain the plasma membrane. After staining according to manufacturer’s recommendations, the medium containing PKH26 dye was removed and replaced with fresh medium. The cells were observed as fresh preparations by fluorescence microscopy or processed for transmission electron microscopy (see below) either immediately after staining or after 30 min, 1 h and 3 h.

Fluorescence microscopy

Confocal laser scanning microscopy was performed with a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope using a HeNe laser at 543 for excitation. Spaced (0.5 µm) optical sections were recorded using a Leica oil-immersion objective (63X; NA 3.2). Images were collected in the 1024 x 1024 pixels format, stored on a magnetic mass memory and processed by the Leica Confocal Software.

Transmission electron microscopy

PKH26 stained HeLa cells on coverslips were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 1 h, washed and incubated with 3.3’ diaminobenzidine (DAB) (20 mg/10 mL in 0.05 M Tris HCl, pH 7.6) under simultaneous irradiation with two 8W Osram Blacklite 350 lamps for 2 h at room temperature. These lamps have two emission peaks of high intensity at 550 and 580 nm, thus being compatible with the excitation maximum (551 nm) of PKH26 dye. The cells were then post-fixed with 1% OsO4 at room temperature for 1 h, dehydrated with acetone and embedded in Epon. As controls, some PKH26 stained cells were processed as described above but omitting either DAB incubation or exposure to light; in addition, unstained cells were exposed to DAB incubation and/or exposure to light.

Thin sections were weakly stained with 2.5% aqueous solution of uranyl acetate for 1-2 min or 2.5% to 5% aqueous solution of gadolinium triacetate for 10 min (gadolinium triacetate is a good substitute for uranyl acetate).
Results and Discussion

At confocal microscopy, the HeLa cells stained in suspension with the PKH26 dye showed a marked fluorescent labelling at the cell periphery, where numerous fluorescent membrane protrusions were clearly visible (Figure 1 a,b). No signal was detected inside the cells, probably due to the reduced endocytotic activity of detached HeLa cells in suspension as much as it occurs in mitotic cells.\(^{31}\) When PKH26 dye was applied to HeLa cells adhering to the glass coverslips, a marked labelling was visible both at the periphery and inside the cells: the cell membrane was evident as a continuous fluorescent rim, while many fluorescent spots of different size and intensity were present throughout the cytoplasm (Figure 1 c,d). No fluorescent signal was ever observed inside the cell nucleus. The membrane internalization was so rapid and massive that the amount of cytoplasmic spots was apparently similar between 30 min and 3 h.

DAB photo-oxidation allowed to detect the fluorescent signal of PKH26 dye at transmission electron microscopy as fine granular electron-dense precipitates, and to specifically identify the structural components involved in the intracellular pathways of cell membrane internalization. At short times after PKH26 dye treatment most cells showed the fine dark reaction product diffusely distributed along their plasma membranes, including the finger-like protrusions observed at fluorescence microscopy (Figure 2a). Many small invaginations containing the photo-oxidation precipitates were observed along the plasma membranes, and several labelled small vesicles were found just beneath the cell surface (Figure 2 b,c). These findings clearly depict successive steps of the plasma membrane internalization process and early endosome formation. At longer times after PKH26 staining, the DAB photo-oxidation product was mostly found inside multivesicular bodies and multilamellar bodies ubiquitously distributed in the cytoplasm, even very close to the nuclear envelope (Figure 2 d-f).

Multivesicular bodies are morphologically distinctive late endosomes filled with small luminal vesicles originating from invaginations of the endosomal membrane. They play a key role in the transport dynamics between the plasma membrane, the secretory pathway and the lysosomes; in particular, they incorporate transmembrane proteins and lipids as well as cytoplasmic material in the luminal vesicles that may be then either delivered to lysosomes for degradation, or released by exocytosis or stored in the cytoplasm.\(^{32}\) Thus, the central position of multivesicular bodies in the endocytotic pathway accounts for the presence of the photo-oxidated DAB in their intraluminal vesicles.

Multilamellar bodies represent lipid storage/secretory organelles that have been described in a plethora of physiological and pathological conditions characterized by defective lipid metabolism and/or autophagic activities.\(^{33}\) In our experimental model it is likely that PKH26 dye, which is stably incorporated with its long aliphatic tails into the lipid regions of the cell membranes, may interfere with the metabolism of phospholipids that accumulate in lysosomes by the endocytotic process. Similarly, many amphipathic drugs induce abnormal accumulation of multilamellar bodies either by forming indigestible drug-lipid complexes or by interfering directly with the lysosomal enzymes.\(^{34}\) No DAB precipitates were ever found free in the cytoplasm or inside the cell nuclei, thus confirming the durable linking of PKH26 dye to the plasma membrane (as reported in the manufacturer’s technical datasheet) and to cell-membrane-derived organelles. Control samples never showed DAB precipitates (not shown). In order to facilitate the visualization of the DAB photo-oxidation product, the ultrathin sections were observed by omitting any additional staining, the mere osmication providing sufficient contrast to clearly distinguish all cellular organelles. In fact, even a weak staining with uranyl acetate provided the finest granular deposits (as reported in the manufacturer’s technical datasheet) and to cell-membrane-derived organelles. Control samples never showed DAB precipitates (not shown). In order to facilitate the visualization of the DAB photo-oxidation product, the ultrathin sections were observed by omitting any additional staining, the mere osmication providing sufficient contrast to clearly distinguish all cellular organelles. In fact, even a weak staining with uranyl acetate masked the finest granular deposits (not shown), while staining with gadolinium triacetate did not improve DAB precipitate visualization (Supplementary Figure 1). On the other hand, lead citrate staining was unsuitable due to its tingly granular appearance,\(^{35}\) which may mimic the photo-oxidation product.
DAB-photo-oxidation thus proved to be a suitable technique to specifically visualize at ultrastructural level the PKH26 red fluorescent dye for cell membrane labelling; the distribution of the granular dark reaction product precisely matches that of the fluorescing staining and the electron density of the fine precipitates makes the signal evident and precisely detectable in the different subcellular compartments. Future application of this technique may be foreseen for investigating, at the high resolution of electron microscopy, endosome or exosome dynamics under physiological or experimental conditions.

References