Diaminobenzidine photoconversion allows detection of fluorescently-labelled nanoparticles at transmission electron microscopy after embedding in epoxy and acrylic resins

B. Cisterna,[1]* M. Costanzo,[1,2]* M. Giagnacovo,[3] V. Galimberti,[3] M. Malatesta,[1] C. Zancanaro[1]

[1]*Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona, Italy*
[2]*Consorzio Interuniversitario Nazionale per la Scienza e la Tecnologia dei Materiali (INSTM)*
[3]*Department of Biology and Biotechnology, Laboratory of Cell Biology, University of Pavia, Italy*

**These authors contributed equally to the work*

Corresponding author: Dr. Barbara Cisterna
Dipartimento di Scienze Neurologiche, Neuropsicologiche, Morfologiche e Motorie, Università di Verona, Strada Le Grazie, 8
37134 Verona, Italy.
Tel. +39.045.8027157 - Fax: +39.045.8027163
E-mail: bacys79@gmail.com

Summary
Photoconversion is a correlative technique used to combine the capabilities of conventional light microscopy with the high spatial resolution and fine specific localization provided by transmission electron microscopy. In fact, photoconversion allows to translate fluorescence signals into electron-dense diaminobenzidine (DAB) deposits which are detectable in ultrastructural analysis. In this view, photoconversion may have promising application as a method for detecting fluorescently-labelled nanoparticles (NPs); in fact, the intracellular localization of NPs may elucidate the dynamic process of cell internalization, as well as their organelle targeting and final fate. Our previous study showed that photoconversion can be used to track the intracellular location of fluorescently-labeled chitosan NPs in Epon embedded samples. In this work we verify whether photoconverted samples can be suitably processed to transmission electron microscopy not only for morphological analysis but also for immunocytochemistry. Cells loaded with fluorescent NPs in vitro were fixed with aldehydes and photoconverted, and embedded in either epoxy or acrylic resins. Results demonstrate that specimens embedded in acrylic resin were of sufficiently high quality in terms of organelle preservation and definition while having much higher potential for cytochemical analyses than the samples embedded in epoxy resins for pure morphology.

Key words: nanoparticles, photoconversion, immunohistochemistry, transmission electron microscopy.

Introduction

Photoconversion is a correlative technique used in order to combine the capabilities of light microscopical method with the unique power of resolution of Transmission Electron Microscopy (TEM).

Plugging the gap between these two microscopy techniques (Garini *et al.*, 2005; Heintzmann and Ficz, 2006; Sartori *et al.*, 2007), photoconversion allows to translate fluorescence signals into electron-dense diaminobenzidine (DAB) deposits which are detectable with ultrastructural analysis. In fact, illumination with high energetic light oxidizes the fluorescent dye with the production of a stable DAB reaction product (Sosinsky *et al.*, 2007), which appears as a fine granular precipitate at the sites where the fluorescent marker was located. Thus, the information that can be obtained with conventional light microscopy is completed by the high spatial resolution and fine

specific localization provided by TEM.

Maranto (1982) first realized the photoconversion of a non-stable fluorescent marker molecule into an insoluble stable product. Originally developed for neuronal mapping through the injection of lucifer-yellow in neurons, this method has been broadly extended to a wide spectrum of fluorescent probes (Sandell and Masland, 1988; Von Bartheld *et al.*, 1990; Lubke, 1993) including fluorescent proteins, quantum dots and enzyme- or particle-base probes, in addition to classical fluorochromes (Giepmans *et al.*, 2006; Sosinsky *et al.*, 2007).

Therefore, the fundamental evidence of the immunofluorescence localization of molecules, as well as of their transport pathways and dynamics in living cells (Giepmans *et al.*, 2006; Lippincott-Schwartz and Patterson, 2003; Yuste, 2005; Jyoti *et al.*, 2004) can be integrated by TEM through a detailed characterization of the subcellular structures where the molecules of interest move, interact and carry out their specific function.

In this view, photoconversion may have promising application as a method for detecting fluorescently-labelled nanoparticles (NPs): the intracellular localization of NPs may, in fact, helps elucidating the dynamic process of cell internalization, as well as their organelle targets and final fate. Taking into account the growing importance of NPs for their potential application in drug delivery, medical imaging and tissue engineering, the possibility of visualizing NPs in an ultrastructural snapshot opens amazing perspectives to the investigator; the simultaneous application of fluorescently-labeled nanoparticles photoconversion and immunocytochemistry at TEM would significantly increase the potential of this *in situ* approach, allowing to analyze the molecular factors involved in different cellular mechanisms of NPs handling.

The aim of this investigation was to verify whether cultured cells containing photoconverted NPs can be processed to allow morphological analysis and ultrastructural immunocytochemistry at a time. To do this, cell samples loaded with fluorescent NPs were fixed with aldehydes and photoconverted, and embedded in acrylic resin: sections from these specimens proved to be of sufficiently high quality, in term of organelle preservation and definition, while having much higher potential for cytochemical analyses than conventional epoxy resin-embedded samples.

Materials and Methods

Cell cultures

Rat neuronal B50 cells were cultured in Dulbecco Modified Eagles Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 100 U of penicillin and 100 μg/mL streptomycin (Celbio, Milan, Italy), at 37°C in a 5% CO_2 humidified atmosphere. Cells were trypsinized when subconfluent and seeded on glass coverslips in 12 multiwell dishes (5×10^3 cells per well) for the following fluorescence and TEM preparations. Two days after seeding, the initial medium was replaced with 450 μL of fresh medium plus 50 μL of a suspension of FITC-labelled chitosan NPs (Colonna *et al.*, 2008). The incubation time with NPs varied from 10 min to 24 h.

Diaminobenzidine (DAB)-photoconversion

B50 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, and then DAB-photoconversion was performed as follows: cells were washed and incubated with 3,3' diaminobenzidine (20 mg/10 mL in Tris HCl 0.05 M, pH 7.6) under simultaneous irradiation with two 8W Osram Blacklite 350 lamps for 2 h at room temperature (these lamps emits with high intensity in the spectral range between 430 and 470 nm, thus being suitable for FITC excitation).

Transmission electron microscopy (TEM)

Some DAB-photoconverted cells were washed with phosphate buffered saline (PBS) and then post-fixed with 1% OsO_4 at room temperature for 1 h. The cells were then dehydrated with acetone and embedded in Epon 812. As controls, some samples were processed as described above but omitting both DAB incubation and exposure to the excitation light.

Some other DAB-photoconverted cell samples, after washing in PBS, were dehydrated with ethanol and embedded in LRWhite resin.

Ultrathin sections (80-100 nm) were cut with an Ultracut E Ultramicrotome (Reichert, Wien, Austria), transferred to copper grids, weakly stained with 2.5% aqueous solution of uranyl acetate for 2 min and observed in a Philips Morgagni transmission electron microscope (FEI

Company Italia Srl, Milan, Italy) operating at 80kV. The microscope was equipped with a Megaview II camera for digital image acquisition.

Results

Photoconverted B50 cells embedded in resins especially suitable for either ultrastructural morphology (Epon) or immunohistochemistry (LRWhite) were observed.

After incubation with DAB and irradiation with high energetic lamps before embedding, NPs were easily recognized in the cytoplasm by the electron-dense product of DAB photoconversion. This typical granular precipitate was specifically and exclusively located on NPs, which appeared as roundish electron-dense structures occurring in the cytoplasm of cells embedded in epoxy (Figure 1a-b) as well as acrylic (Figure 1c-d) resin. No reaction product was ever observed over organelles or free in the cytosol.

In epoxy resin-embedded specimens (Figure 1a), NPs were unequivocally recognized in a well-preserved cytoplasm, showing that photoconver-

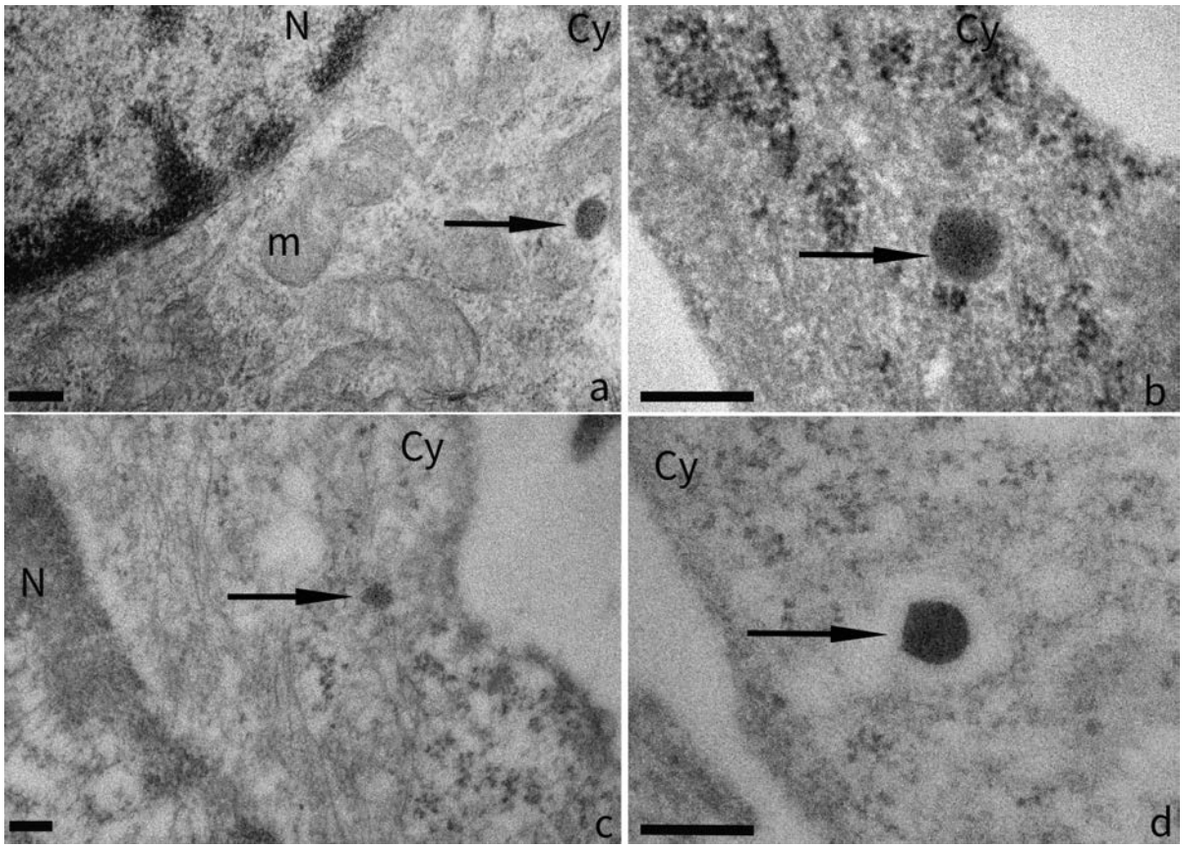

Figure 1. Transmission electron micrographs of B50 cells fixed with aldehydes and post-fixed in osmium tetroxide, and embedded in Epon (a, b) or fixed with aldehydes and embedded in LRWhite (c, d). The NPs (*arrows*), located in the cell cytoplasm (Cy), are detected as electron-dense roundish structures stained by the granular reaction product of DAB photoconversion in both Epon and LRWhite samples. N: nucleus; m: mitochondria. Scale bars: 200 nm.

sion did not damage cell ultrastructure. The nuclear envelope and the inner and outer mitochondria membranes were well preserved due to the post-fixation with osmium tetroxide. Figure 1b shows at a higher magnification the fine granular reaction deposits on the NPs.

In the LRWhite embedded-specimens NPs were clearly detectable thanks to the electron-dense DAB precipitates (Figure 1c-d), and the overall cell morphology was good: despite the lack of osmium tetroxide, the nucleus and the other membrane-bounded cytoplasmic organelles can be easily recognized after uranyl staining (Figure 1c).

In the control samples where DAB incubation and/or light exposure were omitted we never found DAB reaction product (*not shown*).

Discussion

Photoconversion is a unique technique to plug the gap between fluorescence microscopy and electron microscopy. In fact, the former allows to study the localization and function of molecular components, even providing dynamic data on the cell mechanisms in living cells, but it is unable to reveal the ultrastructural context. On the contrary, TEM reveals fundamental data on the cell complexity and the relationships between the molecules of interest and subcellular structures in a correlative image.

Upon illumination, most fluorophores generate oxidizing chemical species such as free oxygen radicals that can locally photo-oxidize DAB (Giepsman *et al.*, 2006), yielding a stable electron-dense precipitate. The half-life of these oxidizing chemical species is very short (from 1 ns to 1 μs) with a mobility between 1 to 30 nm (Karuppanapandian *et al.*, 2011): thus DAB deposits do localize in close proximity of the real place where photoactive molecules elicited the production of reactive oxygen species upon light irradiation. Precise spatial localization of DAB deposits is allowed by transmission electron microscopy.

Aldehyde fixation preserves a good morphology and signal specificity, and stabilizes the tissue thus reducing the risk of diffusion of the photoconversion reaction products (Deerinck *et al.*, 1994). Moreover, it may be hypothesized that the structure of chitosan NPs itself contributes retaining the DAB precipitates, improving detectability. In addition, using our fixation and embedding procedures we did never observe any interference from the potentially disturbing endogenous oxidative activity of organelles such as peroxisomes or mitochondria (Dantuma *et al.*, 1998; Tabas *et al.*, 1990).

As Malatesta *et al.* (2012) already reported, photoconversion can be used to track the intracellular location of fluorescently-labeled chitosan NPs in Epon embedded samples. Our results confirm and extend this observation: the exposure to osmium tetroxide after photoconversion enhances the electron density of the DAB deposits on the NPs, at the same time making the plasmalemma and all the intracellular membrane systems easily recognizable.

As a fully original finding, we also observed that the photoconversion products on NPs can unambiguously be detected in LRWhite embedded samples: this is extremely interesting since these samples may suitably be used for immunolabelling with specific antibodies thus paving the way to refined molecular investigations. The concomitant application of photoconversion and immunohistochemical techniques will represent an especially penetrating tool for studying the internalization pathways of NPs, their time of permanence and the dynamics of degradation within the cell and, even more important, their interaction with specific organelles which could become the targets for mechanistically oriented treatments.

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