DAB photo-oxidation as a tool for detecting low amounts of free and membrane-bounded fluorescent molecules at transmission electron microscopy

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Summary

DAB photo-oxidation is a well-established cytochemical technique, originally proposed in light microscopy to transform the fluorescence signals into stable reaction products. The electron-density of oxidized DAB made it possible to detect the precise location of the fluorescent probes at the high resolution of electron microscopy. Especially in the last decade, this technique has been extensively used for correlative light and electron microscopy to investigate the molecular composition of subcellular structures as well as dynamic cell processes. In the present article, we summarize the results we obtained by DAB photo-oxidation experiments performed using different fluorescent molecules under variable experimental conditions. In our experience, DAB photo-oxidation proved to be a very sensitive technique, and allowed to locate even small amounts of fluorophores irrespective of their localization either within membrane-bounded organelles or vesicles, or free in the cytosol.

Key words: calcium, nanoparticles, PKH26 dye, photosensitizing molecules.

Photo-oxidation: theory and applications

Diaminobenzidine (DAB) photo-oxidation was first applied by Maranto (1982) to convert the fluorescent dye Lucifer Yellow injected in neurons into a stable signal for electron microscopy. Subsequently, many fluorochromes with specificity for different substances were photo-oxidated into reaction products visible at both light and electron microscopy (Sandell and Masland, 1988; Lubke, 1993; Singleton and Casagrande, 1996).

Photo-oxidation is based on simple physico-chemical principles: when a fluorophore is exposed to light of an appropriate wavelength, the orbital electrons are first excited from the ground state to a higher energy level and then reverts to the native state; the relaxation process generates highly reactive singlet oxygen, which in turn induces the oxidation of DAB into a granular electron-dense precipitate whose contrast may be enhanced by osmium treatment (Maranto, 1982; Sandell and Masland, 1988; Lubke, 1993; Singleton and Casagrande, 1996). Since the half-life of oxidizing chemical species such as singlet oxygen, hydroxyl or superoxide radicals is very short (1-1000 ns), their mobility is limited to 1 to 30 nm (Karuppanapandian *et al.*, 2011), thus producing DAB precipitates very close to the site where the fluorochromes produced reactive oxygen species upon light irradiation.

Following Maranto's pioneer study, photo-oxidation initially became the technique of choice for detecting fluorescent molecules to investigate the nervous tissue, thus allowing tracing neuronal networks (Balercia *et al.*, 1992; Buhl, 1993; Gan *et al.*, 1999; Hanani *et al.*, 1999) and analyzing synaptic vesicle turnover (Harata *et al.*, 2001; LoGiudice *et al.*, 2009; Meunier *et al.*, 2010; Welzel *et al.*, 2011;

Hoopmann *et al.*, 2012). In recent years, photo-oxidation has been widely used to correlate fluorescence and transmission electron microscopy with the aim to elucidate cellular dynamic processes: it has been applied to study the three-dimensional relationships of the endoplasmic reticulum and the Golgi apparatus (Pagano *et al.*, 1989; Meisslitzer-Ruppitsch *et al.*, 2008; Röhrl *et al*, 2012), to describe the fine features of microtubule ends (Kukulski *et al.*, 2011), and to investigate endocytosis and exocytosis (Fomina *et al.*, 2003; Kishimoto *et al.*, 2005; Liu *et al.*, 2005; Lichtenstein *et al.*, 2009; Kukulski *et al.*, 2011; Schikorski, 2010; Röhrl *et al.*, 2012).

Photo-oxidation to reveal low amounts of fluorescent molecules

Due to its high sensitivity, DAB photo-oxidation represents a valuable method to precisely detect both free and membrane-bounded fluorescent molecules. In a series of papers, we have been able to validate the reliability of this technique in different experimental issues, even when the fluorophores occur in very low amounts.

Photosensitizing molecules

Photodynamic therapy is an emerging approach for treating tumors of the head and neck and, more generally, those which can be reached endoscopically (Wolfsen, 2005; Agostinis *et al.*, 2011), as well as for the therapy of non-tumoral diseases especially in dermatology (Lee and Baron, 2011; Darlenski and Fluhr, 2013) and ophthalmology (Ziemssen and Heimann, 2012). Photosensitizing molecules have a cytotoxic action after being excited by an appropriate light wavelength: they are able to dissipate the absorbed energy through photochemical processes rather than by fluorescence emission, thus producing oxidizing chemical species that damage the cell molecular structures, with possible induction of cell death (Garg *et al.*, 2010; Santin *et al.*, 2013).

Although photodynamic therapy is currently used in the clinical practice, the mechanisms leading to the penetration and action of most of the photosensitizing molecules at the tissue, cellular, and subcellular level are still incompletely understood. In particular, information about the intracellular accumulation and subcellular localization of the photoactive molecules is essential to obtain an effective cytotoxic effect (Oleinick *et al.*, 2002; Piette *et al.*, 2003; Agostinis *et al.*, 2004).

In our investigations, we used the fluorogenic substrates Rose Bengal-Acetate and Hypocrellin B-Acetate: these acetate derivatives of Rose Bengal and Hypocrellin B exhibit a much more efficient cellular accumulation and greater cytotoxic effects than their native forms (Bottiroli *et al.*, 1997; Croce *et al.*, 2002, 2011). After entering the cells, the acetate groups are cleaved by the cellular esterases, and fluorescence microscopy showed that the restored photoactive molecules occurred in the cytoplasm (Bottiroli *et al.*, 1997; Soldani *et al.*, 2007; Croce *et al.*, 2011): a few minutes post-incubation, fluorescing spots occur near the plasma membrane, then they aggregate in clusters close to the nucleus and, after long incubation times, a diffuse fluorescence also appears suggesting a cytosolic diffusion of the photoactive molecules (Figure 1a). By DAB photo-oxidation (Pellicciari *et al.*, 2013; Malatesta *et al.*, 2014b) we were able to detect the Rose Bengal or Hypocrellin B molecules at the plasma membrane surface, inside endocytic vesicles, in secondary lysosomes and multivesicular bodies, thus demonstrating that the internalized molecules follow the endosome-lysosome route (Figure 1b). In addition, we found DAB precipitates free in the cytoplasm (Figure 1b), suggesting that lysosome-derived vesicles may undergo spontaneous breakage (Ono *et al.*, 2003), thus releasing the photosensitizing molecules and accounting for the diffuse cell damage induced by the irradiation in the course of photodynamic therapy (Soldani *et al.*, 2007; Santin *et al.*, 2013).

Fluorescent nanoparticles

Nanoparticles (NPs) are receiving great attention in the diagnostic and therapeutic fields as biocompatible carriers for tracing molecules or drugs. They are especially investigated as efficient drug delivery systems, able to cross biological barriers (such as the blood-brain barrier), to easily enter the cells, to allow drug accumulation at the targeted sites, and to prolong drug activity by stabilizing the encapsulated drugs and modulating their release (Béduneau *et al.*, 2007; Jallouli *et al.*, 2007; Mundargi *et al.*, 2008). NPs have also been considered for theranostics, the integrated diagnostics and therapy for personalized medicine (Kim *et al.*, 2013).

However, designing a drug delivery strategy

requires preliminary studies on target cells to elucidate the NP uptake mechanisms and timing, and their intracellular fate and relationships with cell organelles. NPs frequently enter the cells by endocytosis, and their intracellular degradation pathway is crucial for estimating their efficacy as drug carriers: in fact, after being endocytosed the endosome-entrapped NPs generally fuse with the acidic lysosomes, resulting in sequestration and degradation of the loaded molecules by the lysosomal enzymes (Panyam *et al.*, 2002).

Our groups studied chitosan NPs (Kumar *et al.*, 2004, Freier *et al.*, 2005; Rinaudo, 2006) as promising drug delivery systems for targeting molecules of potential biomedical interest to the central nervous system (Malatesta *et al.*, 2007, 2014a). Unfortunately, the elucidation of the exact intracellular trafficking pathway of these NPs is difficult to perform at transmission electron microscopy, due to their homogeneous and moderate electron density, which makes them hardly distinguishable from the cytosol. DAB photo-oxidation applied to FITC-labeled chitosan NPs represented a successful solution of this technical problem (Figure 2). We tested fluorescent chitosan NPs in different cell lines *in vitro* (Malatesta *et al.*, 2012, 2014b, 2015) and we could easily recognize them at transmission electron microscopy thanks to the finely granular electron dense DAB precipitates. It is worth noting that the photo-oxidation procedure allowed not only the unambiguous visualization of NPs inside endosomes or free in the cytosol after endosomal escape, but also the detection of NP remnants inside late lysosomes and residual bodies (Figure 2b), where they occur intermingled with heterogeneous material and are morphologically unrecognizable due to the action of lytic enzymes.

The fluorescent membrane dye, PKH26

PKH26 (Sigma-Aldrich) is a red fluorescent dye specific for cell membrane labelling (Figure 3a,b) that has been successfully used for investigating, at either flow cytometry or fluorescence microscopy, macrophage phagocytosis (Bratosin *et al.*, 1997; Pricorp *et al.*, 1997; Williams *et al.*, 2005; Swan *et al.*, 2007; Healey *et al.*, 2007), virus absorption (Balogh *et al.*, 2011), and NP internalization (Malatesta *et al.*, 2012). This dye is stably incorporated with its long aliphatic tails into the phospholipid leaflets of the cell membranes and is therefore suitable for long-lasting labelling.

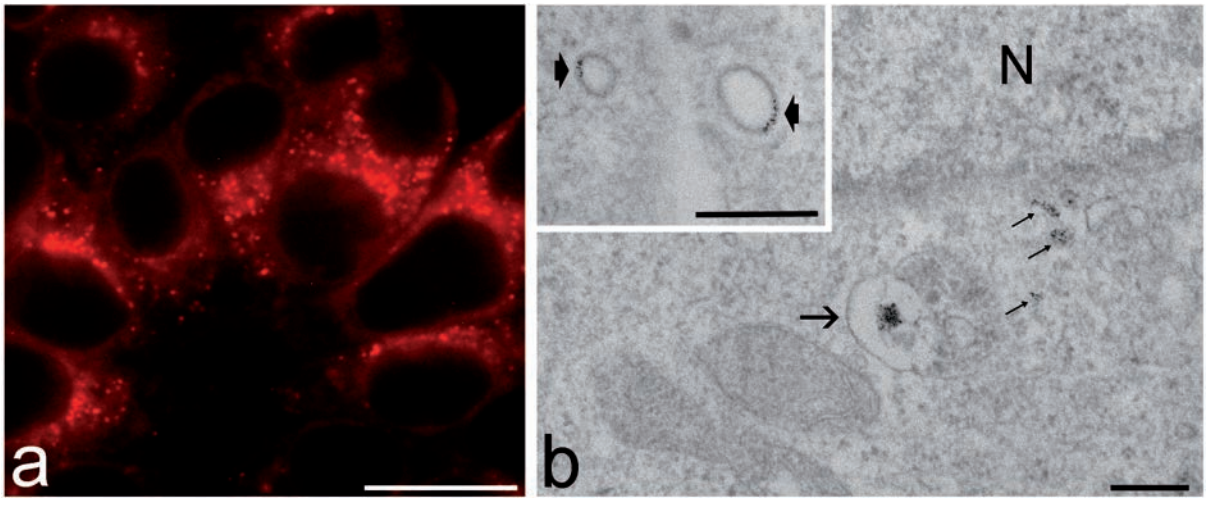

Figure 1. HeLa cells incubated with Hypocrellin B-Acetate. a) Conventional fluorescence micrograph. The fluorescing red signal appears as bright spots as well as diffused in the cytoplasm. Bar, 10 µm. b) Transmission electron micrographs. The finely granular electron dense DAB precipitates are visible in endocytic vesicles (arrowheads), in secondary lysosomes (arrow) and free in the cytoplasm (small arrows). N, cell nucleus. Bars, 250 nm.

Based on these characteristics, we used PKH26 dye for DAB photo-oxidation experiments with the aim of specifically identifying at transmission electron microscopy the structural components involved in the intracellular pathways of cell membrane internalization (Grecchi and Malatesta, 2014). DAB-photo-oxidation proved to be a suitable technique; in fact, the distribution of the fine precipitates precisely matched the signal observed at fluorescence microscopy. The electron density of the reaction product was made detectable in the different subcellular compartments, even when it was quite scarce, by omitting any additional staining, osmication providing sufficient contrast to clearly distinguish the cell components. We could therefore track PKH26 dye from the plasma membrane, where it occurred as weak finely granular precipitates distributed along finger-like protrusions and in small invaginations (Figure 3b), to the resulting small vesicles (early endosomes) just beneath the cell surface, and finally in the multivesicular bodies (playing a central role in the endocytotic pathway; Hanson *et al.*, 2012) and multilamellar bodies (Figure 3d) (lipid storage/secretory organelles related to defective lipid metabolism and/or autophagic activities; Schmitz and Muller, 1991).

Calcium ions

The ultrastructural localization of non electron-dense ions is generally difficult, in particular when they are highly diffusible. Precipitation techniques have been widely used, as in the case of calcium ions, which represent a challenge even when protein-bound. Tandler and coworkers (1970) used pyroantimonate to precipitate Ca^{2+}, but the technique was shown to suffer from a cross-reaction with other divalent cations. In this view, the possibility to make use of a selective fluorescent molecule to be utilized for photo-oxidation becomes an interesting tool for localizing calcium at high resolution. Several Ca^{2+}-sensitive fluorochromes, such as Fura-2, Indo-1 and Fluo-4 (Tsien *et al.* 1982) have been used to image changes in intracellular Ca^{2+} concentration (Bootman *et al.*, 2013). We have recently shown that Mag-Fura 2 can be effi-

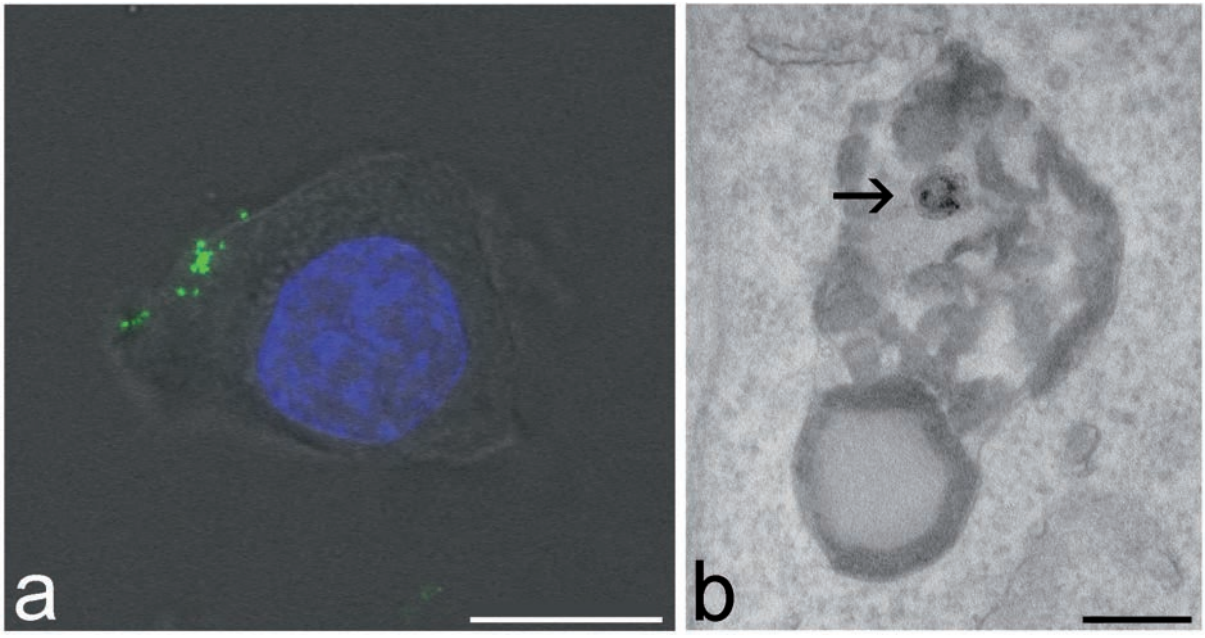

Figure 2. B50 cells incubated with chitosan nanoparticles. a) Confocal fluorescence micrograph. Some FITC-labelled nanoparticles (green) are visible in the cytoplasm; DNA is stained with Hoechst 33258 (blue). Bar, 10 µm. b) Transmission electron micrograph. The dark reaction product of DAB photo-oxidation identifies a nanoparticle remnant (arrow) inside a residual body containing heterogeneous material. Bar, 250 nm.

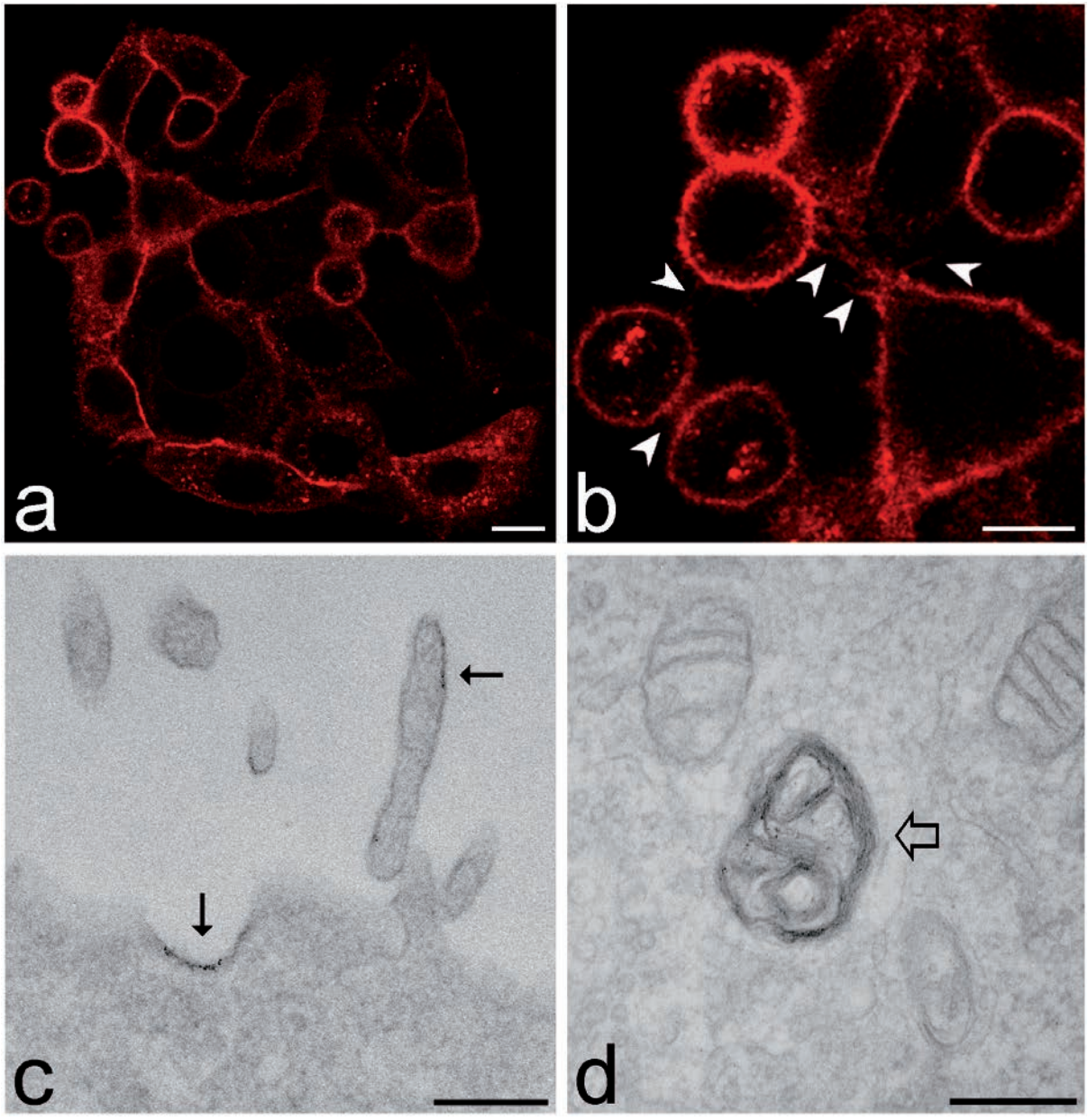

Figure 3. HeLa cells incubated with the membrane red fluorescent dye PKH26. a,b) Confocal fluorescence micrographs. The fluorescent signal occurs along the cell surface as well as inside the cytoplasm as discrete spots. Note the labelled plasma membrane protrusions (arrowheads in b). Bars, 10 µm. c,d) Transmission electron micrographs. The fine granular reaction product of DAB photo-oxidation is visible along the cell surface, especially in finger-like protrusions and in plasma membrane invagination (arrows in c). DAB precipitates are present inside a multilamellar body (open arrow in d). Bars, 250 nm.

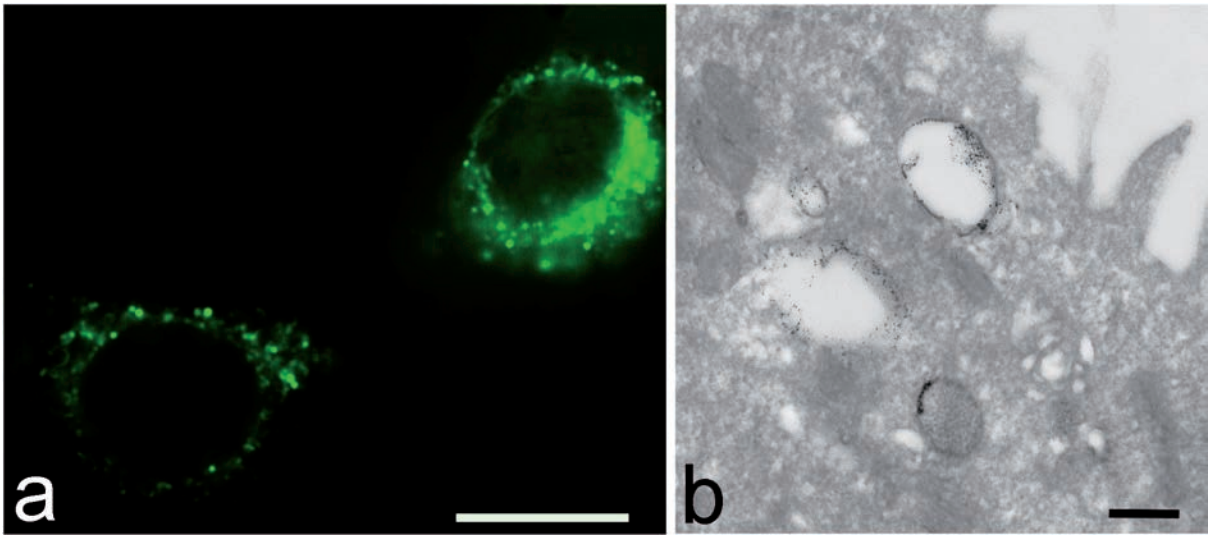

Figure 4. HeLa cells stained with Mag-FURA 2 and observed in conventional fluorescence microscopy (a). Bar, 10 µm. b) After photo-oxidation, the finely granular end-product is visible at transmission electron microscopy inside the vesicles, in particular near the membrane. Bar, 400 nm.

ciently photo-oxidized (Poletto *et al.*, 2015) for localizing Ca^{2+} ions at transmission electron microscopy (Figure 4). With this technique, it is not only possible to visualize Ca^{2+} ions but also to stabilize their intracellular presence, thus limiting the loss of ions. Interestingly enough, the final yield in terms of cells which can then be processed for electron microscopy is rather large, since the direct irradiation of the fluorochrome-stained samples is obtained via a germicide lamp.

The end product is sufficiently electron dense to be clearly detected when present in sufficient amount within vesicles or membrane-limited tubules of the smooth endoplasmic reticulum.

It must be noted that the technique is very sensitive: in comparison with the whole-cell images obtained in fluorescence, the satisfactory final yield at electron microscopy is due to the very low amount of end product which is present in a thin section of 60-80 nm.

Concluding remarks

DAB photo-oxidation is a well-established technique which has originally been proposed in light microscopy to transform the (intrinsically unstable) fluorescence signals into stable reaction products. The DAB electron-density, especially after osmium intensification, paved the way to the refined application of photo-oxidation experiments to detect the fluorescent probes at the high resolution of electron microscopy.

Especially in the last decade, this techniques has been extensively used for correlative light and electron microscopy (Kukulski *et al.*, 2011; Meisslitzer-Ruppitsch *et al*, 2008), also in the attempt to investigate dynamic cell processes: with this aim, the green fluorescent protein proved to be especially appropriate (Grabenbauer, 2012; Horstmann *et al*. 2013).

It is apparent from the cited literature, that the photo-oxidation products are easily visualized when the fluorescent molecules are present in a relatively high quantity and are located inside membrane-bounded organelles or vesicles. In our experience, DAB photo-oxidation is extremely sensitive and allows to detect even small amounts of both membrane-bounded and free fluorophores, provided that the appropriate fluorescent substrates are used under the proper excitation conditions and fixation/embedding/staining procedures.

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