High pressure freezing of *in vivo*-DAB stained organelles

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Questions as to the structural and functional organization of complex and dynamic membranous cell compartments, such as those of the biosynthetic and endocytic systems, require research methods that allow both, high temporal together with high spatial resolution. Studying the endocytic pathway one is forced to continuously pose questions how to discriminate anterograde and retrograde transport systems and to recognize distinct endosomal intermediates. Novel methods combine morphological, kinetic and molecular criteria to distinguish these compartments (1).

Here, we describe a hybrid technique – a combination of cytochemical staining of endosomal compartments in living cells with high pressure cryo-immobilization and subsequent preparation for electron tomography and 3D-analysis. The method, adapted from procedures used earlier for cell fractionation and immunolabeling (2), was further developed for combination with high pressure freezing (HPF) and 3D-analysis. Basically, labelled ligands after endocytic uptake and cytochemical visualization leave their marks along the endocytic pathway and define these compartments. The method consists of the following main steps:

1. Endocytic uptake of HRP-labelled molecules,
2. *in vivo* generation of peroxidase – diaminobenzidine (DAB) precipitates,
3. Vitrification of the samples by means of HPF,
4. Freeze-substitution, and embedding in resin
5. Generation of 3-D profiles by three dimensional analysis by means of electron microscopic tomography.

First, endocytic compartments in human hepatoma cells ($HepG_2$) are labelled with internalized HRP-conjugated ligands; here wheat germ agglutinin (WGA) is used. The method makes use of the cross-linking process that occurs during peroxidase-catalyzed oxidation and polymerization of DAB (Fig. 1).

In order to gain sufficient membrane and DAB-contrast for the 3D-analysis, osmification during freeze-substitution and low temperature embedding had to be adapted. Carbon-coated sapphire discs are the standard supports for cell monolayers: two modes, either sandwiching in the cavity of the supporting aluminium moulds (100µm) and covering free spaces with hexadecane and flat lids, or using 2 sapphire discs with the cell layers face to face in tandem, separated by a 50µm spacer without any further supports, were used. Manipulation from the take off of the sapphire discs out of the culture medium to the moment of freezing was standardized to a maximum of 30 seconds (average 20). Substitution with OsO_4 / UA as fixatives was done according to a short time protocol (-90°C, -60°C, -30°C 8hrs each) altogether lasting approximately 24hrs; at least for the cell cultures, results of the short version was comparable to prolonged substitution protocols.

Contrasting had to take care for both, membrane and DAB-precipitate mapping. The intensity of membrane staining was controlled by the duration of osmification at the end of the substitution protocol (approximately 1hr between 4°C → 20°C). Considerable amounts of internalized WGA are taken up into subcompartments of the Golgi apparatus; labelled Golgi

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regions are encircled by yellow lines (Fig. 2). Because of the electron dense precipitates, the method is well suitable for 3D-electron microscopy of endocytic compartments and organelles. The 3D-model in figure 3 shows the partial reconstruction of a Golgi region; endocytic parts at the trans side of the stack are indicated by coloured luminal contents.

1. M. Mari et al. Traffic **9** (2008) p380
2. W. Stoorvogel et al. J Cell Biol **132** (1996) p21
3. The valuable assistance of Mr. Peter Auinger, Mr. Ulrich Kaindl, Ms. Beatrix Mallinger, and Mrs. Barbara Kornprat is gratefully acknowledged.

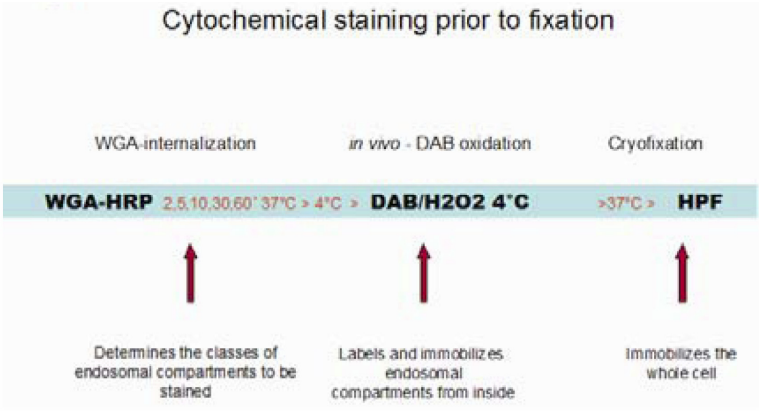

Figure 1.

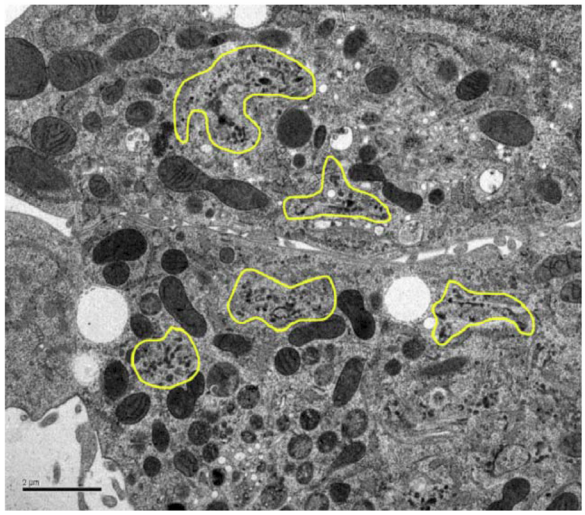

Figure 2.

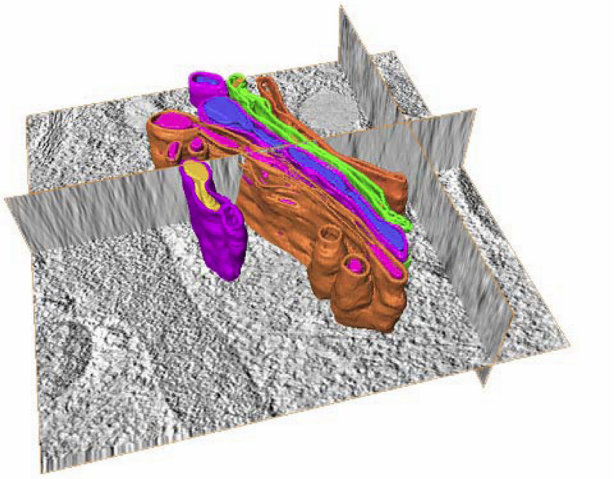

Figure 3.

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