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Research Article

Inverted Ferritin Images: Electron Microscopy of a Natural Enhanced Biomarker

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Keywords

- Iron storage
- Iron-rich ferritin
- Ferritin visualization
- Hemosiderin
- Siderosomes
- Internalization
- Transcytosis
- *Spalax*

Abstract

Ferritin is a naturally occurring iron storage protein present in mineral, vegetal and living organisms, endowed with the possibility of both storage and release of iron compounds, according to demand. Ferritins have been studied extensively including their metabolism in experimental animals and humans. The mineral core of the ferritin molecule (hydroxyl apatite), when loaded with iron, enables visualization by transmission electron microscopy, especially on unstained ultrathin sections. Numerous studies revealed the localization and quantitation of ferritin, and its value in portraying physiological and pathological processes at the cellular and subcellular level. Here we are showing a technical-photographic variation of ferritin identification, localization and quantification, obtained by inverting the standard ultrastructural image (bright field) and producing an image similar to that of dark field or negative staining. The advantages of this procedure are illustrated and application on additional cells and tissues exemplified.

ABBREVIATIONS

TEM: Transmission Electron Microscopy; EDS: Energy Dispersive Analysis

In memory of Professor Iancu

After the acceptance of the article and right before final publication, the main author, Professor Theodore C. Iancu, has passed away. He was author of more than 150 publications and scientific books, many of which are related to the study of ultrastructural aspects of iron storage, transport and metabolism. Professor Iancu was a noble physician, scientist and family man. May he rest in peace'

INTRODUCTION

Ferritin is classically defined as an oligomeric protein of 24 identical or similar subunits forming a hollow protein shell ("cage") of about 12.5nm in its external diameter and an internal mineral core of about 8nm [1,2]. The crystalline iron oxide forming the core in vertebrate ferritin has the structure of ferrihydrite, which is a natural mineral [3]. Mineral cores of ferrihydrite are growing crystal(s) with a heterogeneous composition (according to species) and variable content of atomic iron: frequently quoted are contents of 4500 atoms of iron in each ferritin molecule [3]; however, according to Massover [3], the actual maximum iron content per molecule of horse spleen ferritin is 2250 atoms. When using electron microscopy, iron-loaded ferritin particles become visible because of the electron density of the core. While most particles are apparently larger when "fully loaded" with iron, other factors determine the relationship between ferritin molecule iron content and apparent size, e.g. sectioning or beam angle, focusing hydration of the molecule and crystal contrast [3,4].

In parallel to ferritin cores, other, smaller (10-30 nm) electron-dense particles become visible in thin sections of iron-containing samples. They represent hemosiderin, an iron storage compound present in lysosomes (siderosomes) and form aggregates. Iron-loaded ferritin should not be confused with iron-poor serum ferritin, an acute phase reactant, which provides information on status of iron reserves of the organism.

During the study of iron metabolism and storage, electron-microscopy emerged as a powerful tool, following ferritin molecule assembly, storage, overload or deficiency and also involvement in numerous physiologic and pathologic conditions [5-7]. In recent years, numerous ferritin studies revealed the importance of its nano-molecules in various domains. After the seminal review by Massover [3], researchers focused on additional ferritin-related aspects, including those of biological/medical areas: in 2017, Wang and collaborators published a review on "Functional ferritin nanoparticles for biomedical applications"[8]. An additional study describes the use of ferritin-

based nanocores as a contrast medium [9].The value of ferritin as a tracer was already known and used in biological and clinical electron microscopy. More recently, while studying newly born of the Middle Eastern subterranean blind mole rat *Spalax* (previous named *Spalax ehrenbergi*) we detected an increased amount of electron-dense iron storage compounds in liver and heart samples [10]. It is usual for newborn mammals, including humans, to show during the early post-natal period, raised levels of hemoglobin, hematocrit, and red-blood-cells. In *Spalax*, the newborn rodents showed these features apparently even more, following their long hypoxic prenatal period. Three weeks later, their post-partum hematological data returned to normal and repeated tissue examination showed no storage of iron excess. Ferritin here was key to expanding our understanding of the physiology of macromolecular internalization and transcytosis.

MATERIALS AND METHODS

Animals

The *Spalax* rodents (newborn and adults) were kept in the Animal Facility of the Institute of Evolution, University of Haifa, Israel. The study was approved by the Animal Care and Use Committee of the University of Haifa.

Electron microscopy

We describe here the procedure used for the handling of the *Spalax* rodents. Routine preparation of samples included: immersion in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4, for 2hours, post-fixed in 2% osmium tetroxide, dehydrated in alcohol series and then embedded in epoxy resin. Semi-thin sections (1µm) were stained with toluidine blue and were used for orientation, and cell identification. Ultrathin sections (60 nm) were either left unstained or stained with 1% lead citrate for 2 minutes. Sections placed on 300-mesh copper grids were examined using a transmission electron microscope (Tecnai T12, Eindhoven, Holland), operated at 120 kV. To document the presence of iron in siderosomes, an unstained section a typical siderosome was examined by Energy dispersive X-ray spectroscopy (EDS) which confirmed the iron in the spectrum [10].

Inverted images

To obtain inverted micrographs, images selected by bright field for their electron-dense content (of ferritin or hemosiderin), were inverted using the Irfan View picture viewer (© Irfan Skiljan, Graduate of Vienna University of Technology). Ferritin and hemosiderin nanoparticles were visible at magnifications higher than X 10,000, but the details of individual molecular structures, location and amount, were better resolved at X 30,000. Magnification higher than 50,000 are useless in routine TEM and generate distorted images in both bright field or inverted images. Although the micrographs here presented illustrate our recent findings in *Spalax*, we could observe advantageous inverted images in other TEM investigations, such as organelles, lipid droplets, crystals, collagen fibers, nuclear structure and intercellular components.

RESULTS AND DISCUSSION

Iron is a vital component of living organisms, vegetal and animal. Storage iron, in the form of ferritin, is a delicate buffer capable of responding to the iron needs or excess, on demand.

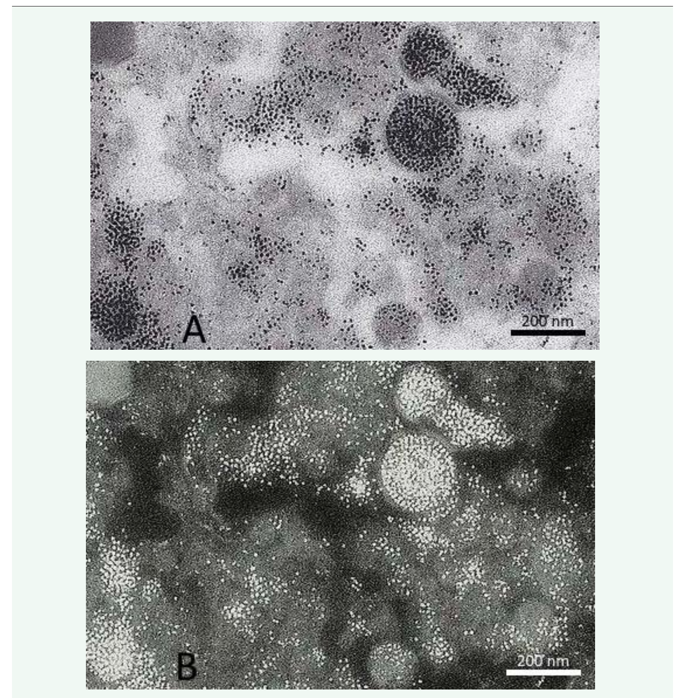

Figure 1 Electrospun nanofibers membrane of poly-ε-caprolactone visualization after 21 days of human Osteoblasts culture (Cells visualization in blue (nucleus /DAPI) and PLL^{FITC} labelled nanofibers in green): colonization and proliferation of osteoblasts into the nanofibers membrane.

Figure 1 A and B present a few inverted images of ferritin, retrieved from newborn blind rodents, mole rat *Spalax* living in subterranean burrows, in upper Galilee, Israel [10]. Lysosomes-siderosomes are frequently seen as circular, multilayered structures on which ferritin particles occasionally form symmetrical arrays consistent with the association between ferritin and phospholipids [11]. Hemosiderin is usually stored in lysosomes-siderosomes and its iron is less accessible upon request. Throughout our study, we also investigated inverted images, similar but not identical, to dark field and negative staining micrographs of the storage proteins. At a later stage, we extended our observations to include inverted images of TEM of other cells and conditions (e.g. neonatal hemochromatosis, β-thalassemia major, experimental iron overload, fatty liver, glycogen and other storage diseases, cholestasis, Wilson disease) (not shown). These inverted electron-micrographs require additional study to establish their informative value when compared to regular bright field TEM.

In **Figures 2-6** Electron micrographs from *Spalax* liver and heart (bright field) provided information on the presence, localization and relative amount of the iron storage proteins ferritin and hemosiderin. Moreover, we were able to follow the process of internalization, from the cell surface, endocytosis, through transcytosis, up to the deposition of the storage iron in lysosomes (i.e. siderosomes). In parallel with the images obtained by bright field TEM, we also used the procedure of inverting the images, resulting in photomicrographs similar, but not identical,

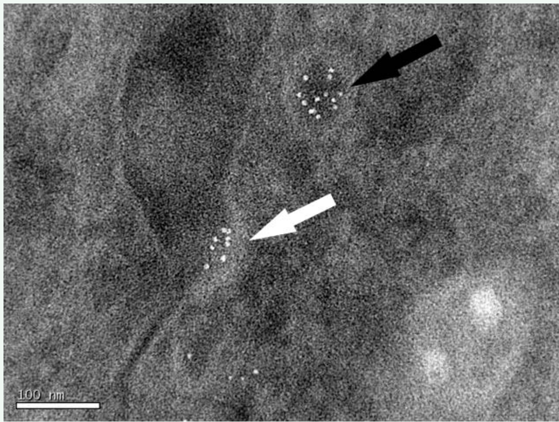

Figure 2 Ferritin particles in the initial phase of internalization-endocytosis, are seen within a pit (white arrow) and in a vesicle (black arrow), within an endothelial cell of Spalax heart. Unstained, inverted image.

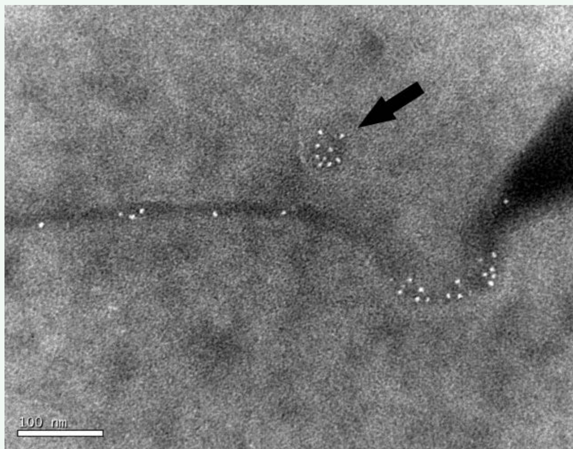

Figure 3 Early stages of internalization: ferritin particles are seen between cells and in a vesicle; (arrow), Unstained, Spalax liver, endothelial cell, inverted image.

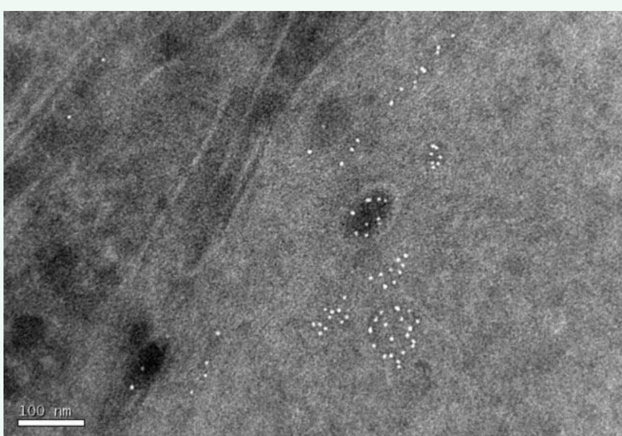

Figure 4 During endocytosis-internalization, ferritin particles are seen within vesicles or tubular structures. Unstained, Spalax heart, inverted image.

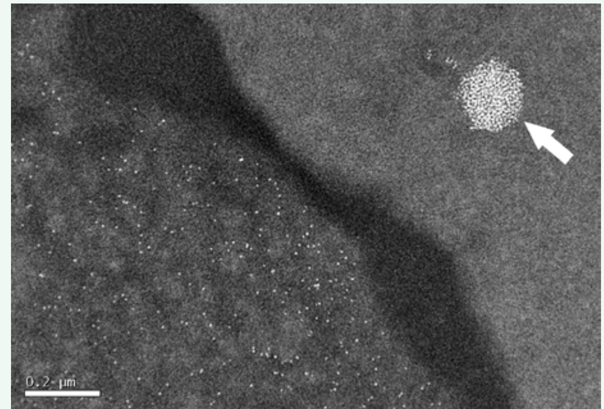

Figure 5 Completed endocytosis: ferritin particles are seen scattered in the cytosol and assembled in a heart muscle nuclear cluster (arrow). Unstained, Spalaxheart, inverted image.

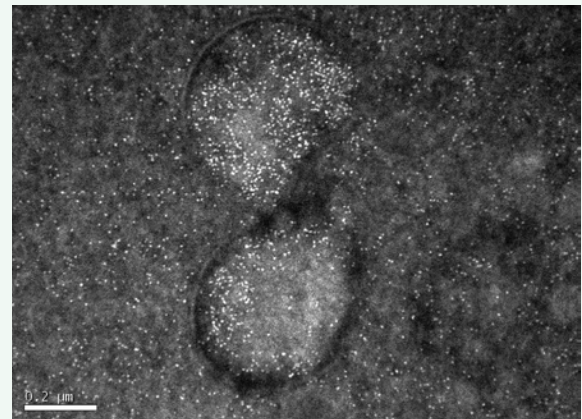

Figure 6 Advanced stage of ferritin segregation in siderosomes: numerous particles are encircled in a siderosome; note their increased frequency as well as relative thicker siderosome limit membrane. Unstained, liver endothelial cell, Spalax, inverted image.

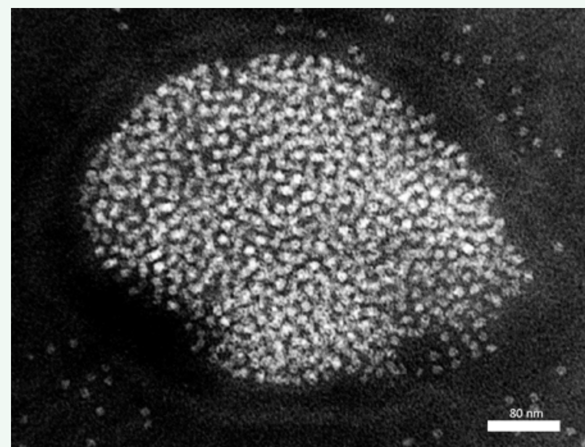

Figure 7 Higher magnification of a siderosome. The limit membrane is visible and densely packed ferritin particles which can still be individually resolved. Spalax, myocardiocyte, unstained, inverted image.

to the dark field images or negative staining. The sequences of internalization, also confirmed by the inverted image photo-microscopy, were:

In **Figure 2,** Apposition of ferritin molecules along external cell membranes, especially in pits or bottle-shaped caveolae as well as other forms of invaginations.

In **Figure 3,** Identification of ferritin molecules within juxta-luminal vesicles and/or tubular structures seen at different distance from the plasmalemma.

In **Figures 6-9,** Assembly of ferritin within single-membrane organelles-lysosomes (siderosomes) Propensity of ferritin molecules to form circular structures in association with phospholipid membranes (demonstrated *in vitro* by including cationized ferritin in phospholipid vesicles [11].

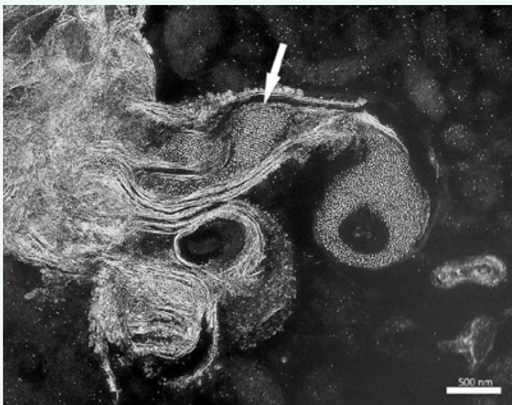

Figure 8 Ferritin particles intermingled with phospholipid-collagen fibrils. Note the tendency to form hexagonal crystals (arrow). The particles in the surrounding cytosol appear smaller, without implying that they are less iron loaded. Unstained, neonatal hemochromatosis. Liver, space of Disse, inverted image.

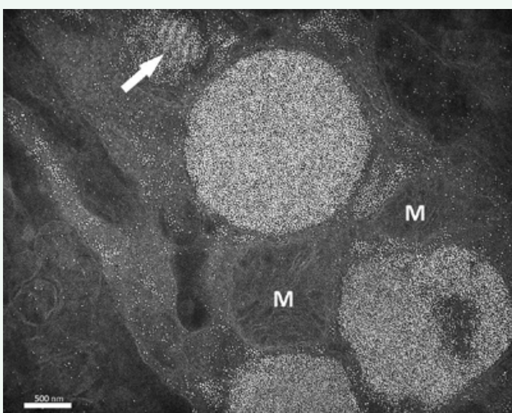

Figure 9 Extreme iron load: particles fill membrane-limited organelles; as these particles cannot be individually resolved, the compound is now named hemosiderin [12]. Outside particles, which can be resolved, represent ferritin. Note absence of particles from the mitochondria (M) and formation of crystal in the siderosome (arrow). Unstained, liver biopsy - thalassemia major, space of Disse. Inverted image.

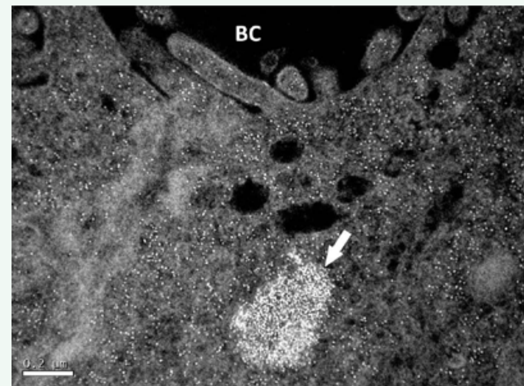

Figure 10 Reversed exocytosis. Ferritin particles present around a bile canaliculus (BC) opening are expelled with bile into the small intestine. Arrow points to a siderosome. Iron compounds have been found in biliary secretion in iron overload and normal subjects. Unstained, transfusional siderosis, liver biopsy, inverted image.

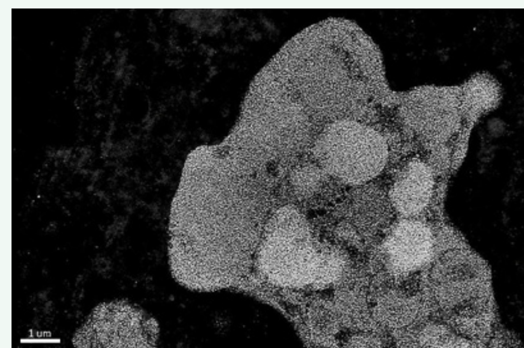

Figure 11 Hemosiderin 'clump' – an aggregate of electron dense particles which cannot be individually resolved and therefore corresponds to Fishbach's definition of hemosiderin [12]. In histopathology they appear as Perls' stain positive granules, i.e. iron containing bodies; liver,transfusional siderosis, unstained, inverted image.

1. Additional ferritin-related observations, not necessarily related to the process of internalization itself, were: Assessment of randomly dispersed ferritin in the cytosol (enabling morphometry) [5]
2. **Figure 3**: Presence of inter-cellular ferritin.
3. **Figure 5:** Ferritin within the nucleus.
4. **Figures 9 & 11**: Hemosiderin, TEM definition and images.

We here present a number of inverted image photomicrographs and list advantages in the parallel use of this method.

Advantages

- The inverted image provides increased "sharpness" and contrast: on the dark background, the particles have enhanced visibility and cannot be confused with other particles.
- Clear, striking presence of randomly dispersed ferritin particles in cytosol and smaller particles (spicules,

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aggregates, 'clumps' of hemosiderin?) in cytosol and siderosomes.

- Features of the inverted image - The core has a typical shape, previously considered octahedral (now polygonal and only quasi-spherical); the iron sequestered in the cage central cavity is formed by one or more crystallites of ferrihydrite ($9Fe_2O_3 \cdot 5H_2O$) [3].The inverted image affords easier apparent size measurement of the core and surrounding apoferritin coat, facilitating morphometry. Inter-core distance: suspected hemosiderin formation. According to Fishbach et al. [12], ferritin molecules must be separated by minimum 5.0 nm, representing twice the 2.5 nm apoferritin coat, in order to be considered un-degraded ferritin molecules (Figure 7).
- Inverted images are similar to negative stain and dark field images but without the need for the processing and the relatively expensive equipment.
- Such images, like those exemplified here, can help in unraveling the cellular molecular transport of nano-molecules, like ferritin and contribute in our understanding of conditions associated with overload and the putative results of chelating therapy.
- For images other than those of iron-storing compounds, details are projected from an additional angle, providing different structure information: e.g. membranes, collagen fibers, organelles, storage constituents, lipid droplets in cytosol, nuclei and intercellular spaces (Figures. 12 A, B and Figures 13 A, B) [13]. Additional liver biopsies which can be studied by inverting images can use micrographs published in "Electron microscopy of Liver Biopsies" [14].

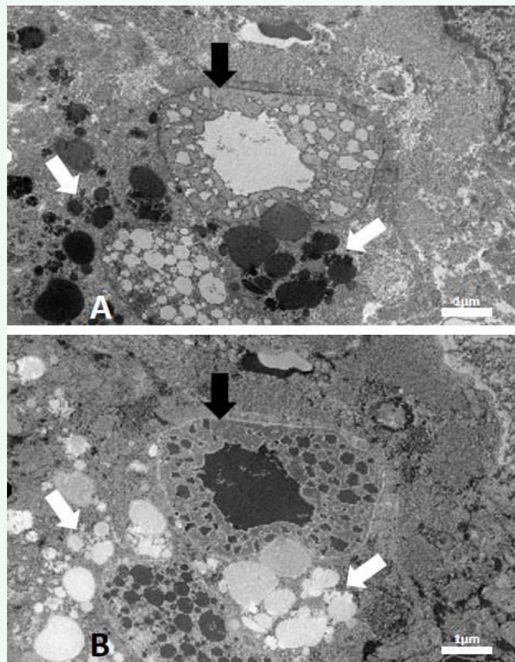

Figure 12 Regular (12A) and inverted (12B) micrographs showing lipolysosomes (black arrow)adjacent to lipofuscin droplets (white arrows). Fatty liver, uranyl acetate, lead citrate stain.

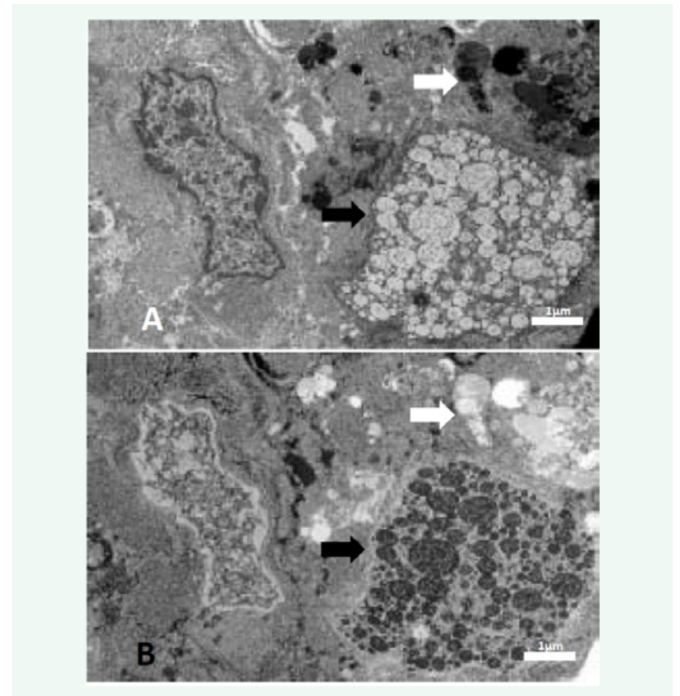

Figure 13 Regular (13A) and inverted (13B) micrographs showing lipolysosomes (black arrow) adjacent to lipofuscin droplets (white arrows). Note the unusual content of lipolysosome droplets. Fatty liver, uranyl acetate, lead citrate stain.

Disadvantages

The inverted images result from a photographic-optic manipulation based on the original bright field image. As such, it can contain only elements existing in the original micrograph. In contrast, dark field illumination circumvents the central light beam and records also particles located at the periphery of the central beam. Thus, the dark field "sees" a more widespread surface and is therefore more informative.

Of note, dark field illumination and negative staining are procedures that require expertise, experience and availability of advanced, expensive equipment. These conditions are fulfilled today only in rare research institutions. In contrast, many medical centers still use standard electron microscopes which can yield quality TEM micrographs and their inverted images.

CONCLUSIONS

Ferritin and hemosiderin play a major role in iron metabolism in general and in tissues in particular. These nanoparticles can be viewed, localized and quantitated, on unstained ultrathin sections, by TEM. We presently report on a technique of optical inversion of the iron-containing particles present in tissues and revealed by electron microscopy. Even though the information gained is less comprehensive when compared with dark field or negative staining, it nonetheless provides enhanced viewing of individual ferritin and hemosiderin particles, their location at unusual sites and the possibility of following their formation and/or disposal from the cell or intercellular structures. As an archeoprotein and as a natural biomarker, ferritin provides confirmation of the cellular metabolism pathways. Moreover, when extended to other TEM images, the inverted viewing

provides a 'new dimension', of details not recognized before, during bright field electron microscopy.

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