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A New Method to Enhance Contrast of Electron Micrograph of Rat Tissues Sections

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***Abstract*—**This report presents an alternative technique of application of contrast agent *in vivo*, i.e. before sampling. By this new method the electron micrograph of tissue sections have an acceptable contrast compared to other methods and present no artifact of precipitation on sections. Another advantage is that a small amount of contrast is needed to get a good result given that most of them are expensive and extremely toxic.

***Keywords*—**Image quality, Microscopy research, Staining technique, Ultrathin section.

I. Introduction

SINCE the beginning of the microscopy, improving the image quality has always been a concern of its users. Especially for transmission electron microscopy (TEM), the problem is even more important due to the complexity of the sample preparation technique and the many variables that can affect the conservation of structures, proper operation of the equipment used and then the quality of the images obtained [1]-[3]. Animal tissues being transparent it is necessary to apply a contrast agent in order to identify the elements of their ultrastructural morphology. Several methods of contrastation of tissues for TEM imaging have already been developed. The most used are the "en block" and "*in situ*" contrastation i.e pre- and post- embedding contrastation [4]. Studies in cell and tissue morphology were always dependents to the use of a microscope which is a remarkable tool for observation of biological phenomena. A lot of types of microscopes with more or less important performances have been developed over the last century to meet the demands of increasingly sophisticated current research. All of these devices use the image as a means of analysis and storage of the information they provide. The acquisition of a good quality image is still dependent on the aspect of the material to be observed and in particular its been contrast. Transmission electron microscopy (TEM) is not an exception to this condition, and a battery of techniques has developed for the preparation of biological samples to observe them [1]-[2]. As for light microscope, the main steps of samples preparation for conventional TEM are: fixation, inclusion, microtomy, and contrastation [3]-[7]. Fixation that keeps the tissue as it was when he was in the living organism (in a life-like state) can be performed before sample collection by perfusion-fixation method when the fixative is injected directly to the animal intravenously under anesthesia, or after the collection of the samples that are immediately immerged in the fixative solution. This step may or may not be followed by a post-fixation step with osmium tetroxide to strengthen the fixation and which also acts as a contrast agent. It is followed by a dehydration step with a series of alcohol solutions to permit infiltration of hydrophobic resins into tissues that contain a large amount of water. Inclusion is the step that hardens the samples for carrying out the next step, the microtomy. This step consists of cutting tissues in ultrathin sections of 50 to 100 nm thick which is formed using an ultramicrotom because higher resolution imaging requires thinner samples that let pass more easily electrons. As biological tissues are completely transparent, the contrastation is an important step that allows differentiating cellular elements during the ultrastructural analysis by TEM. It can be performed before or after the inclusion. When contrastation is applied before inclusion (pre-embedding), fixed specimens are immersed in the contrast agent as is done with osmium tetroxide. When the constrastation is performed after inclusion therefore after microtomy (post-embedding), the contrast solution is applied directly on the ultrathin sections placed on grids. Thus, in most cases, the contrastation step is always the last sample preparation step for TEM observation. The contrast of details of a sample is enhanced by applying heavy metals on sections, such as osmium, lead and/or uranium whose atomic mass is respectively 190, 207, 238. They are more or less fixed by the samples according to the chemical composition of the biological structures. They interact with the electrons of the incident beam and can deflect, absorb or let them pass [8]. The electrons passing through the sample form an image on the fluorescent screen of the electron microscope. For certain tissues, in order to have a satisfactory image contrast, it is necessary to increase the concentration of the contrast agent or add other heavy metals, which may reveal some artifacts like the presence of electron dense deposits on tissue sections. Sometimes electron micrographs obtained may lack contrast if the contrast agent, applied by the conventional method, cannot penetrate well into the resin used for inclusion. For some experimental studies, the contrast agent should not be used in order to do not interfere in the observation of a particular

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phenomenon as for detection of particular studies on metal deposit or precipitation in biological structures. Without using a method of contrastation of samples, electron micrographs may lack contrast, making difficult identification of the structures as well as the interpretation of possible lesions, alterations or damages that were objectives of the studies. Despite their radioactivity, compounds containing uranium are the most used for the processing of samples. They are the best contrasts agents for biological tissues due to the high atomic number of uranium, thus electrons strongly react with it and give a satisfactory contrast of structures that fixed this element [9]-[14].

This new method consists in the application of the contrast solution at the beginning of the protocol of sample preparation, before samples collection. This is a process of contrastation of tissues *in vivo* that can be achieved by intravenous injection (single or by perfusion), intraperitoneal injection, or by ingestion. The protocol chosen for our study of the contrastation of intestinal mucosa sections is the ingestion route to improve the contrast of the electron micrographs without evidence of artifacts or tissue damages due to uranium radioactivity.

II. Materials and Method

A. Protocol and Samples Processing

Four groups of three male rats were used in this study respectively for *in situ, in vivo* contrastation and controls (without contrastation). Two groups will be reserved for the *in vivo* method of the samples that were analyzed 3 hours and 6 hours after ingestion of the contrast agent. The rats in the first group underwent the protocol generally used for transmission electron microscopy [4]-[8], i.e. after their anesthesia by intraperitoneal injection of ketamine and xylazine (80-120 mg/kg:10-16 mg/kg), their intestine was removed and samples of 5 mm side were fixed by 3% glutaraldehyde in sodium cacodylate buffer (0.1 M; pH=7.4) at 4°C and were post-fixed in 1% osmium tetroxide in the same buffer at room temperature. After dehydration of the samples in successive baths of alcohol solutions of 50, 75, 90 and 100°, they were included in a resin Epon (EMBed812/Electron Microscopy Sciences). The formed blocks were cut into ultrathin sections of 60 nm thick using an ultramicrotom (Ultracut/Leica), which were then deposited on copper grids of 5 mm in diameter and 200 Mesh. The contrast agent used is a saturated solution of uranyl acetate ($UO_2(CH_3CO_2)_2 \cdot 2H_2O$), with a molecular weight of 424.15. It is applied by floating the grids face down on the surface of the uranyl acetate solution and followed by a lead salt solution. *In situ* contrasted sections were analyzed by TEM (FEI Morgagni 820) at 80 kV. For assessment of *in vivo* contrastation, rats of the second or third group, fasted for 3 h, received 1 ml of an aqueous solution of uranyl acetate (30mG/mL) to the stomach using a gavage needle 3 or 6 hours prior to samples collection. Thus almost 0.2 µG of depleted uranium was administrated to the rats. This dose was determined from a preliminary study that allowed us to know that 3 hours after ingestion, there were no lesions or electron-dense deposits of uranium in the duodenal mucosa [15]-[22]. But a part of uranyl acetate can remain in soluble form, we first test the time required after ingestion of the contrast agent to determine if later the soluble forms of uranyl acetate can concentrate and precipitate or not, depending on the time of contact with the tissue and then if there is occurrence of radiation-induced damage to samples by uranium. Under anesthesia, intestine of all animals was dissected and then cut into small fragments which were then prepared by the same steps, reagents and equipment of the previous method. After being deposited on copper grids, the cuts obtained were then analyzed by TEM since they were not contrasted with uranyl acetate or lead salts. Control samples were taken from the rats of the fourth group were prepared by the same method but did not receive any type of contrastation either *in vivo* or *in situ*.

B. Images Acquisition

In order to compare the different types of contrastation procedure, all tissue sections were observed at the same time, using a digital transmission electron microscope (Morgani^{TM} 268D - Philips/FEI). The microscope was control through the Morgani user interface and the images taken with its digital camera through the Analysis software. All micrographs were taken at the same magnification and the same conditions of the instrument setting (voltage, filament emission, targets openings, sample illumination intensity, opening objective, bias and camera). The same cellular structures of the different cuts which present inherently different contrast due to their structure, thickness and composition, were photographed such as nucleus, cytoplasm, organelles and membrane specialties. For all TEM section observations ten micrographs were taken by method. Because the low wavelength of the electrons, the micrographs are shown in black and white with shades of gray. The intensity of black, gray and white depends on the interaction of incident electrons with atoms of the biological structures of the sample [7], [8]. The presence of the contrast agent (heavy metal with high atomic number such as uranium and lead) accentuates this phenomenon. In brief, because of their affinity difference for the heavy metals, some biological structures are stained in black (high affinity), white (low affinity) or grayscale (mean activity). If the electrons are not absorbed or reflected by the biological structures which is then called "non-electron dense" structures, electrons will cross through and interact with the fluorescent screen of the TEM or with the detector of the digital camera and form a white or gray point on micrograph according to the intensity of absorption of the electron by the sample. Because electrons are transmitted through the specimen, it is the origin of the name of this type of microscope. Conversely, a structure is called "electron-dense" when it absorbs or reflects the incident electrons, thereby preventing to cross through. Thus, these electrons will not interact with the underlying fluorescent screen of the TEM or the digital camera and a black dot will appear at this place on the micrograph. The intensity of staining by heavy metals and the shades of gray of the micrograph also depends on the thickness of the sample. Thinner it is (up to 100 nm), more it will let pass the electrons and areas will be clearer on the micrograph. Conversely,

thicker it is fewer electrons will be transmitted and the area of the micrograph will be darker [9]-[14].

C. Assessment of Micrographs Contrast

The contrast of our digital micrographs was assessed using public domain software, ImageJ, developed by Wayne and available at the NIH site [23]. Histograms were used as a measuring tool in order to appreciate the contrast level of the digital image, contrast deficiencies such as low or high contrast [24]. Histogram represents the number of pixels (vertical y-axis) in an image at each different intensity value or each tone (horizontal x-axis) found in the image. In an 8-bit grayscale image, there are 256 possible intensity values or tones. The tonal range is spread from the minimum and maximum values of 0 and 255 respectively. On the left side of the histogram are represented the black (0-25) and shadow (26-63) tones, the middle (64-178) is the area of exposition, and on the right region are the light grays (179-229) and writes (229-255). The TEM digital images of the sections were burned to a digital media, transferred to another computer and imported into *ImageJ* for analysis. TEM settings were not changed during analysis of the tissues sections and acquisition of their images. Digital micrographs were not artificially manipulated during their acquisition by the digital camera of the TEM or with any image processing software. The *ImageJ* software was only used to evaluate the intensity profile of brightness of the micrograph.

III. Results

It was observed that the micrographs of tissue sections contrasted by the *in vivo* method have sufficient contrast to identify the biological structures. Indeed it is possible to distinguish different elements normally present in the duodenal mucosa such as cells and their organelles. Fig. 1 shows the tissue sections with various structures that are found in the duodenal mucosa, such as enterocytes, goblet cells, plasmocytes, connective fibers near fibroblasts and blood cells in the capillaries or between the enterocytes. It can be seen very clearly the structures of the cytoplasm of these cells such as nucleus, nucleolus, the two types of chromatin, endomembranes (nuclear membrane with pores, rough and smooth reticulum and Golgi), ribosomes, secretory vesicles, crystals within the granules of eosinophyles, mitochondria with their inner membrane, cristae and matrix, and lysosomes. Three hours after administration of uranyl acetate, the duodenal mucosa appears without any radiation-induced ultrastructural alteration, and no significant sign of injury cell death, apoptosis or necrosis. At this post-ingestion time, careful analysis of the sections failed to highlight electron-dense deposits of uranium in tissues analyzed. But after 6 hours post-ingestion, it was detected small round black deposits in lysosomes (Fig. 2) and sometimes large precipitates of irregular shape scattered in duodenum lumen. For the highest post-ingestion time, it was also observed cellular alterations such as the presence of dilated mitochondria whose inner membrane and the cristae are altered which are signs of cellular radiotoxicity as one of early steps of radiation-induced apoptosis. By comparing the *in vivo* contrasting method with conventional one that we can consider as a control method, it was detected in the cytoplasm of intestinal cells no injured mitochondria or damaged endomembrane system. The microvilli of enterocytes show no structural difference. There has been found no traces of phagocytosis such as the presence of macrophages with phagolysosomes with cell debris.

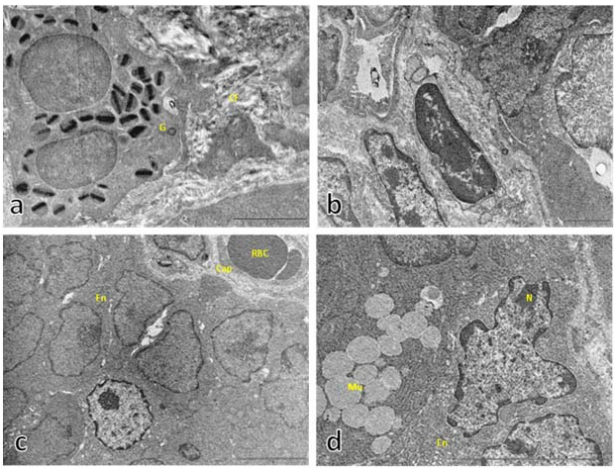

Fig. 1 Electron micrographs showing representative sections of rat duodenal mucosa 3 hours after in vivo staining by oral administration of uranyl acetate (see text). *Enterocyte (En), red blood cell (RBC) into capillary lumen (Cap), mucus grains of goblet cells (Mu), nucleus (N), electron-dense granules of an eosinophil (Gr), and connective fibers (CF)*

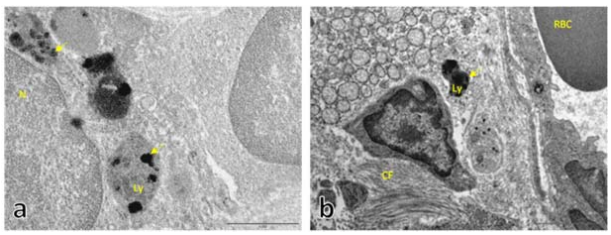

Fig. 2 Electron micrographs showing representative sections of rat duodenal mucosa 6 hours after in vivo staining by oral administration of uranyl acetate (see text). Note presence of cytoplasmic black deposits inside lysosomes (arrow). *Lysosome (Ly), Nucleus (N), red blood cell (RBC), and connective fibers (CF)*

Fig. 3 shows some representative digital micrographs of transversal sections of rat duodenal mucosa without contrastation (a), after post-embedding (b), or *in-vivo* contrastation (c) and their respective histogram above each of them. Micrographs of unstained tissues sections with any of the three available contrast reagents (osmium, uranyl or lead) are too light to distinguish the ultrastructure of the duodenal mucosa (Fig. 3 (a)) and it is not possible to identify the elements that compound the tissue. The contrast histogram associated with this type of image is a single narrow peak located in the highest brightness area. For these micrographs, the peak extends from grayscale (value of 165) to the brightest

shade (value of 255) corresponding to the most intense white. It is centered on the average value of 219.480, which means that a great number of pixels contains this very clear shade of gray. As seen on Fig. 3 (a), the histogram indicates that there is no pixel containing dark or black shades (left graph area). Such micrograph corresponds to an overexposed photograph which then has a bad contrast.

Figs. 3 (b) and (c) both allow distinguishing all the cellular elements such as epithelial cells of the intestinal mucosa (enterocyte and goblet cells), granulocyte in the connective tissue and endothelial cell of capillaries which contains erythrocytes. These images contain the key elements that are found in biological tissues we study and also it can be seen different cellular structures like cell membrane, cytoplasm, eu- and heterochromatin, crystals, proteins and fibers of collagen whose composition have a different affinity for the contrast agents we used. There are areas of the sample that are more or less electron-dense due to their thickness or their mode of absorption of the contrast agent thereby forming a gray scale. After contrastation with the conventional method, the micrographs present a very good contrast, which is the objective of this method allow distinguish all the cellular elements present normally in the duodenal mucosa (Fig. 3 (b)). Their histogram reveals a uniform distribution of pixels of the graph from the most intense black shade (value of 0) to the brightest white shade (value 255). As for all *in vivo* contrasted micrographs, the histogram of Fig. 3 (b) of shows a bell-shaped distribution of pixels as a Gaussian curve with a maximum of pixels containing gray tones (central region of the graph with a mean value 0f 149.930) which is the best exhibition area. There are more black pixels than white ones. A small peak, visible in the left half of the histogram, represents a very dark part of the micrograph which is due to the presence of a red blood cell in this cut and it is very electron-dense. Such micrograph is equivalent to a picture with good exposure that presents an excellent contrast. The micrographs of the *in vivo* contrasted samples herein histogram similar to that of the conventional method only it is smaller and less spread (Fig. 3 (c)).

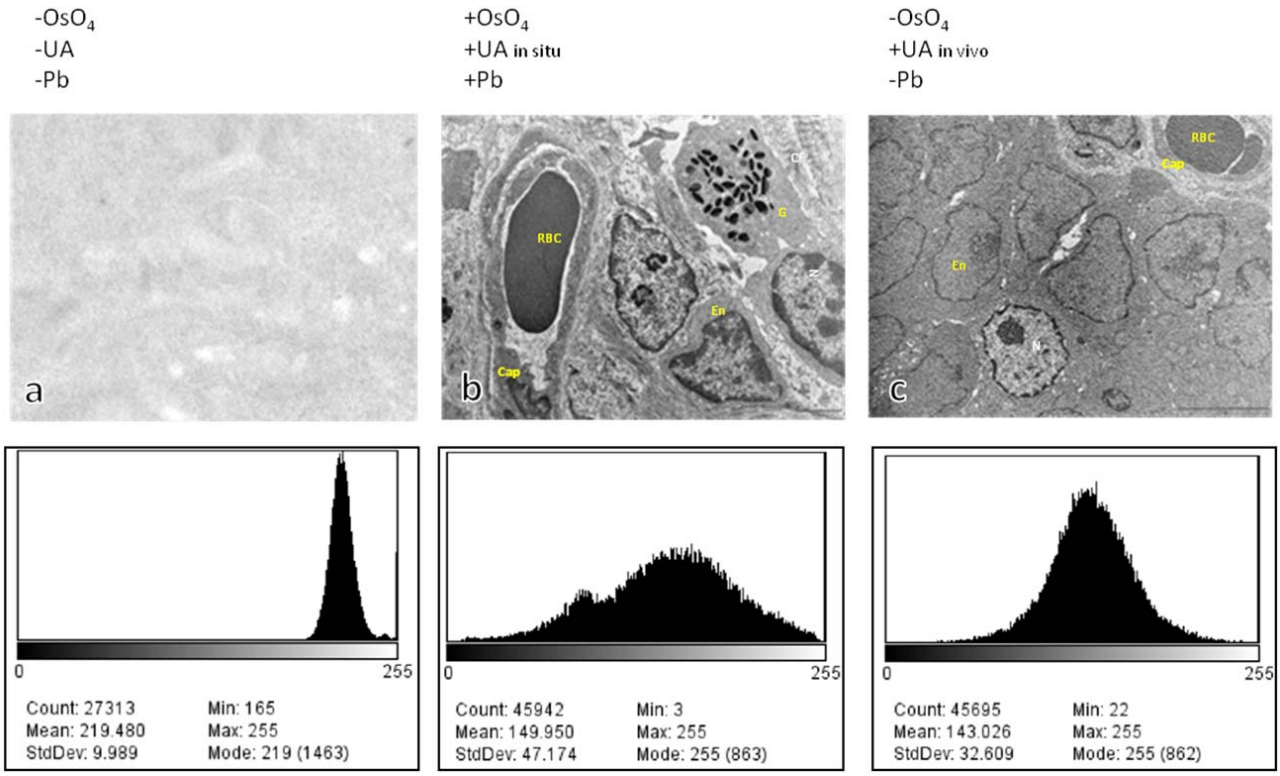

Fig. 3 Assessment of images contrast using the picture processing program *ImageJ*. There are representative electron micrographs of ultrathin sections of rat small intestine without contrastation (a), after post-embedding conventional contrastation (b) or 3 hours after in vivo contrastation with uranyl acetate (c) and above them their respective histogram. It is not possible to distinguish the ultrastructure of the sample in the cuts without contrast (Fig. 3 (a)). By cons, many cellular structures and organelles can be observed in the contrasting cuts by the conventional method and by the in vivo method such as (RBC=red blood cell; Cap=capillary; En=enterocyte; N=nucleus; G=granules of an eosinophil; CF=connective fibers; OsO4=osmium tetroxide, UA=uranium acetate; Pb=lead salt)

IV. Discussion

Biological structures have between them and with their environment, very small differences in refractive index. Then under bright-field illumination it cannot or difficult to discern them unless they have a significant thickness. The contrast is linked to a notion of opposition as in photography whose beginnings were mainly black and white; pictures were extremely contrasted and were to have black areas very pronounced, very strong white areas and a reduced number of gray areas. Then the grayscale was preferred and the photos have acquired a contrast much more suave. Several solutions were found for light microscopy to enhance contrast of structures of living cells as applying a small phase difference of the light passing through the sample using the phase contrast method invented by Zernike in 1930 [25]. In the case of fixed tissues, specific dyes which have a different affinity for each biological structure can be applied on samples and thus the staining creates a good contrast of the sample image [11]-[14]. For electron microscopy, illumination of the sample is not made with photons but with electrons. Therefore, it is not a difference in refractive index which allows contrast between the structures but a difference of interaction and absorption of these electrons by the sample molecules. As they almost have the same atomic composition there is very little difference of electron interactions between the structures that absorb or let pass the electrons. Thus the image formed on the fluorescent screen of the microscope has very little darkness or brightness difference and then a poor contrast to distinguish the different structures of the sample. Ou result have shown that uranyl acetate applied *in vivo* the samples micrographs have a sufficient contrast to identify cellular ultrastructures. If the administration of this contrast agent is carried out at a short time after the sample collection there will be no occurrence of tissue damage due to the radioactivity of uranium. We observe certain radiotoxicity from 6 hours after ingestion but no injury or electron dense deposits were detected in shorter times.

V. Conclusion

This work was conceived after observing sections of duodenum of rats that received a solution of uranyl acetate orally in order to highlight the sites of concentration of uranium in the lining of the digestive tract. The cuts had a significant contrast as they were not subjected to any contrasting treatment with osmium tetroxide or uranyl acetate. This in vivo contrastation method can also be applied to other biological material such as bacteria, fungi, protozoa or plants after determining the dose of uranyl acetate to use and the exposure time in order to obtain satisfactory contrast without the occurrence of radiation-induced damage.

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