Digital Image Mapping of Water and Dry Mass Content Using Ultrathin Freeze-Dried Cryosections

S. V. Buravkov, K. Zierold, and V. A. Shakhlamov

UDC 612.014.461.3.08

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny* , Vol. 116, № 9, pp. 325-328, September, 1993
Original article submitted March 31, 1993

Key Words: *x-ray microanalysis; ultrathin freeze-dried cryosections; water digital image mapping*

The water content of various cell organelles and the cytoplasm is an important parameter related to the life cycle and functional activity of the cell. The need to obtain quantitative data on the local content of tissue dry mass and water content is dictated by two important circumstances: first, dry mass and water content may change significantly in some pathological processes, this being manifested in edema and shrinking of organelles, the cells themselves, and the tissue as a whole [1,2], and second, data on the ionic content of the cell cytoplasm and various organelles, assessed by x-ray local microanalysis, are usually expressed in mmoles per kg dry mass [3]. To convert these units of concentration into the mmoles per liter of intracellular water which are used in cytophysiology and which are more easily interpreted, we need to know the local concentration of water or dry mass.

To preserve intracellular water and ion distributions when making preparations, quick-freezing methods should be used, followed by the preparation of ultracryosections, their transfer in the frozen state to the scanning electron microscope, and freeze drying [9]. Such a technique of preparation allows for satisfactory preservation of the ultrastructure and distribution of dry matter and ions in the cells and their organelles.

Use of digital image analysis methods not only helps map the dry mass and water distribution in the sections but also permits their detailed study at any site of the resultant image.

In the present study the possibility was investigated of using a dark-field electron-microscopic image of 100-nm ultrathin freeze-dried sections for mapping dry mass and water in these sections.

MATERIALS AND METHODS

Experiments were carried out with isolated rat liver cells. The methods of suspension preparation were described previously [10]. A drop of suspension in

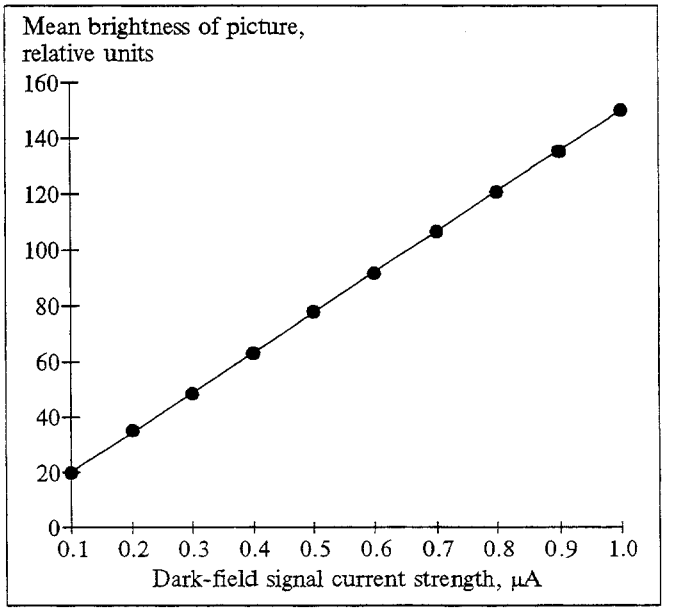

Fig. 1. Calibration curve reflecting relationship between digitalized picture mean brightness and dark–field signal intensity in an electron microscope.

Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow; Max Planck Institute of Molecular Physiology, Dortmund, Germany. (Presented by N. K. Permyakov, Member of the Russian Academy of Medical Sciences)

0007-4888/93/0009-1174$12.50

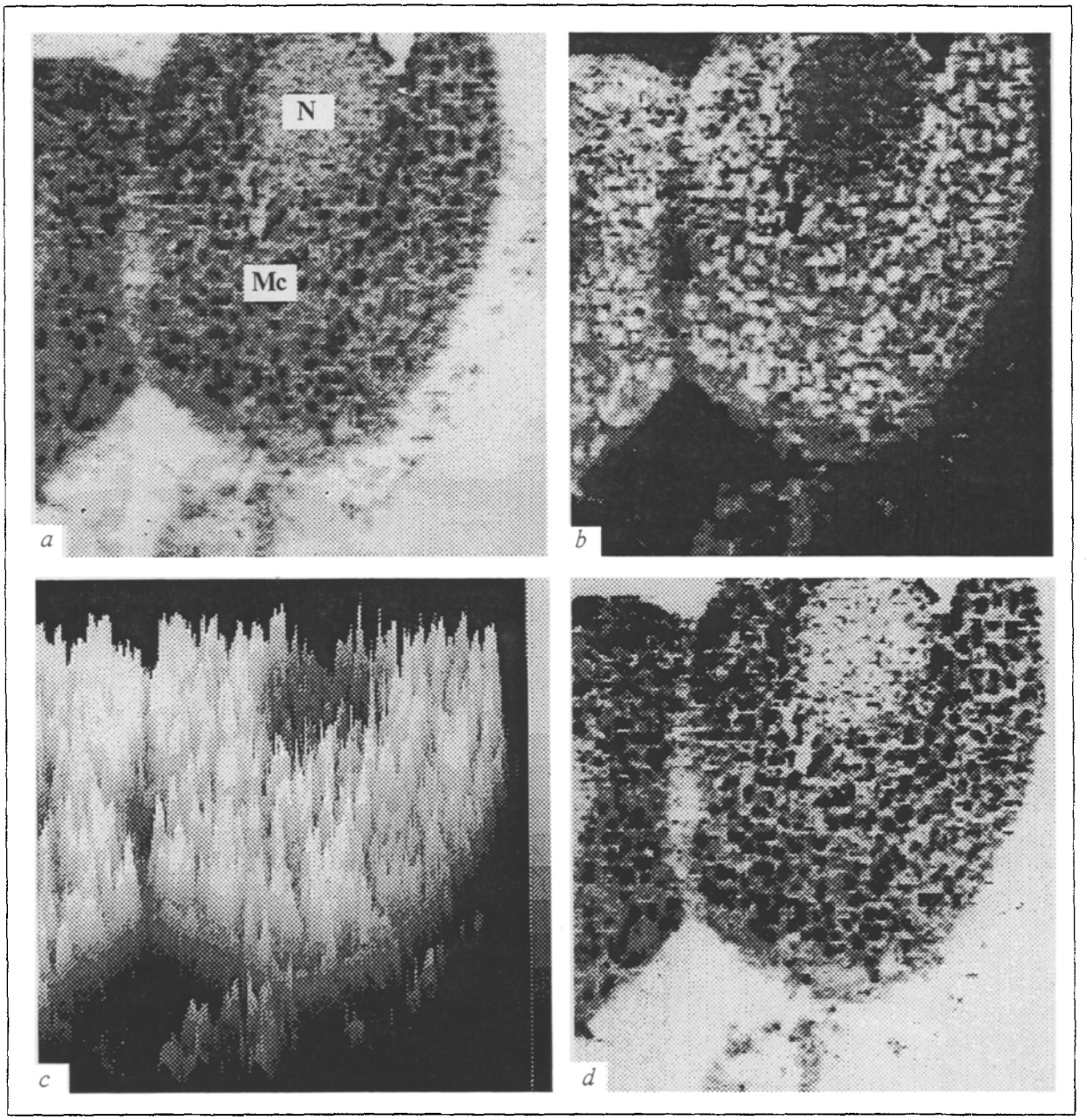

Fig. 2. Mapping of local content of water and dry mass. *a*) digital image of 100 nm ultracryosection of rat hepatocyte suspension in secondary electrons (N – cell nucleus, Mc – mitochondria); *b*) dry mass distribution map in ultracryosection of rat hepatocyte suspension after conversion of dark–field signal current strength values. Right: scale representing dry mass content as 16 discrete values from 0% (black) to 50% (white); *c*) heterogeneity of dry mass distribution in the same section after recalculation with an inclination angle of 25°. White peaks correspond to the highest dry mass values (50%) in rat hepatocyte section; *d*) water distribution map in 50–100% range. ×5000.

normal saline was placed onto a special holder and immediately frozen in liquid propane cooled to -160°C with liquid nitrogen. The technique for preparing ultrathin frozen sections 100 mn thick has also been described [9]. Frozen sections were dried at 138 K for 2-30 min after they had been transferred to a Siemens Elmiskop ST 100F scanning transmission electron microscope. X-ray spectra were analyzed by a SiLi detector hooked up to a Link AN10000 x-ray analyzer (Great Britain).

Local dry mass in a section was measured with an anglular dark-field detector situated under an objective diaphragm 30 μ in diameter and electron beam flux of 1.3 nA, the signal collection half-angle being less than 10 mrad. For calibration of the relationship between the dark-field signal and dry mass content, 5 to 50% dextran solutions were used, from which sections were prepared in a similar manner. The methodological particulars and theoretical rationale for the method

TABLE 1. Dry Mass Content in Various Compartments of Isolated Rat Liver Cells

Hepatocyte organelles	Dry mass content (%, $M\pm m$)	Number of masurements
Nucleus	13.24±4.221	5
Mitochondria	38.094±6.509	25
Cytoplasm	19.631±4.471	25

used are described in detail in an earlier paper [10].

A Link AN10000 x-ray analyzer analog digital converter was used to collect digital section images in the dark field. The image-processing package in this analyzer permitted point-by-point arithmetic operations on the digitalized image and allowed us to obtain, after the appropriate transformations, values of local content of dry mass or water.

RESULTS

Before carying out any transformations on digital images, one should make sure that there is a linear relationship between the dark-field signal current and the digital image brightness. For this purpose a calibration signal was fed to the analog-digital converter input over the entire range of values which were to be expected from the real object (0.1×10^{-7} - 1.0×10^{-7} nm). After complete image digitalization (128×128 dots, 8 bit per dot) the mean brightness level per frame and the standard deviation were estimated. The resultant calibration curve is represented in Fig. 1), in which a strict linear relationship between the digitalized signal brightness and the input signal level is determined. For further estimation a linear regression equation was calculated: $L=3.74+145.7\times I$, where L is the digitalized signal brightness level, and I is the amplifier output current (in μA).

Figure 2, *a* shows a scanning transmission image of ultrathin freeze-dried sections of isolated rat liver cells dried in the microscope column. The nucleus and mitochondria are clearly seen. Dry mass distribution in the same section is shown in Fig. 2, *b*, with a scale on the right reflecting dry mass content. The sixteen gradations on this scale (from black to white) make it possible to assess the local content of dry mass at any site of this section within a range from 0 to 50%. The dry mass distribution varied widely in different hepatocyte compartments, being low in the nucleus and much higher in the mitochondria. A more accurate analysis was carried out during an investigation of five different cells. The results are summarized in Table 1. The dry mass content of the nucleus measured by averaging all points constituting the nuclear area) was 13.24±4.22%, whereas the dry mass content of the mitochondria and cytoplasm was 38.09±6.51 and 19.63±4.47%, respectively.

For a more graphic representation of the distribution it is possible to convert the image into a three-dimensional isomeric form (Fig. 2, *c*), where white peak height determines dry mass content in a given section.

The local water content may be easily estimated from the ratio presented previously [10]: $M_{dry}+M_{wat}=1$, where M_{dry} is the dry mass content and M_{wat} the water content at a given cell site. The resultant water distribution map is depicted in Fig. 2, *d*. A scale with a water content range from 50 to 100% is shown on the right.

The maps of water and dry mass distribution in isolated hepatocytes demonstrate a clear-cut heterogeneity and considerable variability. Values of dry mass content similar to our results were obtained in experiments with isolated fibroblast suspension [10]. The local redistribution of water and dry mass is known to be involved in pathological reactions of the body, such as choleragenic intoxication [1,3], and in common hydroreceptor adaptation reactions, for example, the adaptation of *Bombyx mori* to changes in environmental humidity [8]. Knowledge of local water redistribution in many cases might help us better understand the mechanisms of physiological and pathological processes and their genesis.

One more factor which prompted us to develop the dry mass and water mapping method was the fact that a study of the quantitative distribution of ions in ultrathin freeze-dried sections of cells and tissues calls for the preparation of final ionic charts in units used in physiology, that is, mmoles per liter of intracellular water. A variety of methods for estimating dry mass using different electron microscopic signals are known [4-7]. The scope of this paper does not allow us to discuss all their advantages and short-comings. However, we believe mapping making use of digital measurement of current strength in a dark field under an electron microscope operating in the scanning transmission mode to be most effective, simplest, and at the same time sufficiently accurate method. Still, we have to mention in conclusion that this method may be used only with ultrathin sections of a reproducible thickness up to 500 nm.

REFERENCES

1. A. P. Avtsyn and V. A. Shakhlamov, *Ultrastructural Foundations of Cell Pathology* [in Russian], Moscow (1979).

2. N. N. Bogolepov, *Ultrastructure of the Brain in Hypoxia* [in Russian], Moscow (1975).
3. V. A. Shakhlamov and S. V. Buravkov, *Arkh. Anat*, № 4, 95-107 (1983).
4. M. J. Ingram and F. D. Ingram, *Scan. Electron. Microsc.*, 3, 1249-1254 (1983).
5. R. D. Leapman, C. E. Fiori, and C. R. Swyt, *J. Microsc.*, **133**, 239-253 (1984).
6. P. W. J. Linders and P. Hagemann, *Ultramicroscopy*, **11**, 13-20 (1983).
7. P. W. J. Linders, R. A. Van der Vorstenbosch, and A. M. Stadhouders, *J. Histochem. Cytochem.*, **33**, № 3, 185-190 (1985).
8. R. A. Steinbrecht and B. Muller, *Cell Tiss. Res.*, **266**, 441-456 (1991).
9. K. Zierold, *Ultramicroscopy*, **14**, 201-209 (1984).
10. K. Zierold, *Scan. Electron Microsc.*, **11**, 713-724 (1986).

METHODS

Improving the Results of Statistical Comparison of the Means by Allocation of Animals to Experimental Groups

S. G. Kornilov

UDC 616-092.9-07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 116, № 9, pp. 328-329, September, 1993
Original article submitted March 31, 1993

Key Words: *statistical analysis of observations; comparison of the means; planning of comparisons*

Prior to any investigation, planning has to be carried out. We will call the planning primary if it concerns a study still to be begun, and secondary if we are dealing with a continuation of a study which is currently being performed. The methods of planning are different for different cases.

The present publication is devoted to primary planning.

Since, in contrast to the situation with secondary planning, information on the phenomena of interest is generally not available for primary planning, no recommendations can be offered with respect to the total number of objects (animals, for example) to be involved in the study. This number (N) is to be chosen by the researcher himself or herself, based on intuition and the resources available. The goal of primary planning is to allocate this collection of objects to groups, taking into account that statistical comparison of the means will be the main method of mathematical processing. Primary planning ensures that actually existing differences of the mean will be found with the highest probability.

In the simplest investigation only two groups of objects are involved: control and experimental. But more often than not, there will be a greater number of groups. Let K denote the number of groups. Assume that just one of the characteristics of the objects under study (for example, the weight of the liver) will be analyzed. Let H denote the total number of comparisons to be performed with respect to this parameter (second-order and optional comparisons are not to be included in this number). Let us designate the groups of objects by

Saratov Medical Institute. (Presented by M. Ya. Studenikin, Member of the Russian Academy of Medical Sciences)

0007-4888/93/0009-1177$12.50