Electron Probe Microanalysis of Cytoplasmic Concentrations of Elements in a Single Cell in Culture and Suspension

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Electron probe microanalysis is a method for evaluation of concentrations of elements at the subcellular level, effective in studies of an individual cell in culture or suspension. Intracellular concentrations of cytoplasmic ions (K^+, Na^+, Cl^-) most rapidly and markedly reacting to changes is an adequate criterion for evaluating the traumatism of manipulations used in cell technologies. Using electron probe microanalysis it will be possible to develop special procedures (for example, therapeutic cloning) for reprogramming or cryopreservation, when intactness of intracellular balance of element serves as the criterion of cell status.

Key Words: *isolated cell; culture; suspension; early mammalian embryo; electron probe microanalysis; cytoplasmic element balance*

According to the classical theory of biological electron probe microanalysis (EPMA) [38], comprehensive understanding of the role of ions in the regulation of cell function requires quantitative data characterizing the cell status (concentration of the analyzed element in the cell and its organelles; distribution of the element in the cell; element composition of the space directly adjacent to the cytoplasmic membrane from the outside and forming the cell microenvironment; content of water and dry residue weight in the studied cell compartment; concentrations/activities of free ions).

EPMA provides information on the element concentration and distribution in the cell, element composition of the space, composition of cell compartment for all elements of the periodical table, starting from beryllium (Z=4) [36]. This electron microscopic method developed with consideration for the technological characteristics of an electron microscope suggests *in situ* cell studies by adequate methods for preparation of the object of investigation fixing the cell status in time. It is impossible to register changes in the element concentration in the online mode. This limitation involves the preparation of a series of samples, each characterizing the cell in a certain physiological status.

The method is local and universal, permitting analysis of a wide spectrum of elements at the same imaged site of the section with subcellular resolution. Activities of ions directly in the cell can be evaluated by EPMA, for example, by ion-selective electrodes, but this excludes *in situ* measurement at the cell level. The method of ion-sensitive dues, *e.g.* Fura-2 for Ca^{2+}, can be used for only one element. We may ask here the traditional question about the specificity of the stain and the implication of the staining protocol for cell function. Measurements of ion concentrations in organelles and/or fragments of the cell isolated by biochemical method are strategically not justified, because the object of the study (nuclei, mitochondria, pigment granules, vesicles with secretory vesicles, *etc.*) uncontrollably change their element composition irrespective of the method of isolation, after which it is not correct to extrapolate the data to an *in vivo* situation.

In biology the theoretical and experimental basis of EPMA was investigated in many fundamental

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studies [33,34,39,41,47,51]. "Biological" microanalysis was also intensely practiced by Russian scientists [16,22,37]; its development in our country has been reviewed [54]. The main limitation of electron microscopy is impossibility of direct *in vivo* study of the cell, because of high water content (about 80%) of the cytoplasm. High content of water in "soft" biological tissues necessitates the development of special approaches.

Cryofixation. Traditional methods of electron and light microscopy based on chemical fixation and dehydration of the cell result in an uncontrolled redistribution of cell composition [28,60]. Cytochemical methods of precipitation of easily diffused elements in the form of insoluble precipitate were developed for some of these elements [42,45]. However, the specificity of cytochemical reaction rules out the possibility of simultaneous precipitation of the entire element composition in the cell. For this reason, new approaches were needed, not violating the intact distribution of cytoplasmic ions.

At present, several methods for preparation of tissue sections for EPMA are known; all of them are based on cryofixation as the initial stage of the sample preparation [31]. A method of low-temperature fixation by application of a sample onto metal surface cooled to the temperature of liquid nitrogen is developed [29]. This method provides rapid cryofixation only at sites of contact with the metal. Freezing by plunging the whole sample in liquid cryoagent is preferred. Several cryofixatives are suggested for this purpose [27]. The freezing velocity is the highest in liquid propane, re-cooled in liquid nitrogen to 85°K.

Due to cryofixation, a "glacial" sample can be obtained, which can be processed by different methods [31]. Depending on the tasks of the experiment, a massive object or a section is prepared. In the latter case cryotomy, cryotomy with subsequent dehydration of the cryosection, low-temperature dehydration of frozen object with its subsequent embedding in epoxy medium and microtomy is used (Fig. 1).

The scheme of manipulation is presented in Fig. 1; using these manipulations, several methods for the preparation of objects for EPMA were developed. Due to succession of events, no aqueous phase forms and no solvents are needed in any of the variants. Cryomethods serve not only as instruments for preservation of a life-time distribution of element composition, but promote optimal sensitivity and spatial resolution of EPMA [12,53,55,57].

It is difficult to obtain an amorphous ice structure reaching to a sufficient thickness of the sample during cryofixation of biological objects [21]. High pressure freezing provides a zone of vitrified ice up to 500 μ deep [50], but this method requires special equipment and is very expensive. Usually the depth of the area free from crystals is no more than 30 μ from the surface of the sample [30]. It was shown in experiments with tissue samples that the size of cell destruction caused by crystallization/recrystallization of the ice did not surpass 0.1 μ [28]. This value characterizes the main artifact of cryofixation caused by local redistribution of the element in the ice crystal. Possible ion displacement in the cell by several tenths of a micron do not lead to apparent disorders in its distribution in analysis of a cytoplasm fragment and even an organelle of several microns in size.

EPMA of an isolated cultured cell in a monolayer. Tissue sections were analyzed for evaluating sodium distribution in the frog skin epithelium [37], potassium and calcium content in the insect complex eye cell [22], ion compartmentalization in the frog oocyte [23], element concentrations in rat thymocytes [66], and changes in the cytoplasmic concentration of potassium, sodium, and chlorine in myocytes of ischemic rat heart [9-11,55].

The interest to an isolated cell of primary or surviving culture as the main object of cell technologies is explained by the fact that manipulations with extracellular environment are easier in studies of an isolated cell, in comparison with tissue. Specialized function of only one isolated cell type is studied, which can be directly observed over the course of the entire experiment [21,46]. The prospects of EPMA use in studies of element composition of cultured isolated cell were discussed not once [66,72]. A specific feature of the culture (in comparison with tissue) is small volume of material, which makes impossible to form a preparation convenient for mechanical manipulations at various stages of the study. In manipulations with the culture the underlayer to which cells adhere can serve as the visual check-point.

TABLE 1. Cytoplasmic Concentration (mM) of Potassium and Sulfur in a Chondrocyte after Washout of the Culture with Different Media ($M \pm m$)*

Washing medium	Potassium**	Sulfur
Distilled water	46±2	82±2
0.3 M sucrose	84±7	78±5
0.15 M ammonium acetate	12±1	82±7

Note. Results from a previous study [74] are presented. *Results are conversed from mmol/kg dry weight [74] into mM (dry residue of an animal cell after its lyophilization being taken for 80%). **The data are presented for a group of 10 cells.

TABLE 2. Intracellular Concentrations of K^+ and Na^+ (mM) in a Cardiomyocyte, Determined by EPMA on Semithin (2 μ) Section of a Sample Embedded in Epon 812 (Epoxy Resin-Based Medium) (*M*±*m*)

Studied sample	Potassium	Sodium
Rabbit rectangular cardiomyocytes*, culture (*n*=39)	128	40
Rat heart papillary muscles (*n*=62)	120±10	35±7
Rat rectangular cardiomyocytes (type I), culture (*n*=21)	116±13	30±11
Rat rectangular cardiomyocytes (type II), culture (*n*=33)	76±8	50±21
Rat round cardiomyocytes, culture (*n*=41)	50±7	97±29

Note. *Results of EPMA for rabbit cardiomyocyte on a lyophilized cryosection of suspension presented by A. Warley [68]. *n*: number of analyzed cells in a group.

EPMA method is developed for evaluating the *on mass* element concentration in the entire cell (Fig. 1). A cell layer was applied onto a solid underlayer [15,26,75] or a net covered with a film [43,73] and analyzed after lyophilization. The method of preparation in this case included washout from culture medium before low-temperature dehydration, otherwise salts from the medium deposited on the preparation surface as a result of lyophilization can distort the results of the analysis.

The data on the effect of even a short-term exposure of a cell in a washing solution on its status are contradictory. Comparative experiments with fibroblasts and chondrocytes showed that Na^+/K^+ ratio in cell culture and tissue is virtually the same [26, 73]. However, significant changes in this parameter after washout no longer than 2 sec were observed for vascular smooth muscle myocyte [43].

Further studies showed that not only the time, but also the washout medium are critical factors [74]. The authors of this study analyzed the concentrations of six elements (Na, P, S, Cl, K, Ca) in a chondrocyte after using several washing solutions. The levels of all elements changed depending on the solution; only the content of sulfur remained constant; it seems that it belongs to cell composition components (*e.g.*, proteins) which are difficult to wash out. The data for sulfur and the main intracellular ion K^+, whose content is particularly sensitive to changes in the cell status, are presented (Table 1) [74].

TABLE 3. Potassium and Sodium Concentrations (mM) in Mouse Two-Cell Embryo Blastomer Cytoplasm (*M*±*m*)*

Group	Potassium	Sodium
Control (*n*=7)	130±6	120±6
Experiment (equilibration/ ethylene glycol washing; *n*=9)	47±3	60±5

Note. *Results presented by D. V. Gol'dstein *et al.* [1]. *n*: number of analyzed embryos in a group.

Analyzing a whole cell, it is impossible to measure the concentrations of elements in subcellular structures; that is, analysis by this method gives just intermediate values between the concentration of a certain element in the cytoplasm and in subcellular structure. These limitations can be overcome, if EPMA is carried out on a cell section [46, 73,75]. In this case we make a transverse cryosection of an underlayer together with the culture growing on it, expecting (with good grounds) that the section plane will include fragments of the monolayer cells [62,63]. As a rule, after cryotomy the section is lyophilized before EPMA (Fig. 1).

Human prostatic carcinoma PC-3 cells are used as a model of *in vitro* dividing cells [20]. Monovalent cations are regarded as one of the main factors regulating normal proliferation and transformation [25,48]. Many EPMA studies confirmed the hypothesis that the Na^+/K^+ ratio increases during cell transformation and is characteristic of malignant neoplasms [24,76].

EPMA of an isolated cell in suspension. A variant of the method was developed for suspensions. Cells for this variant are presedimented by centrifugation forming a compact precipitate, which can be used for subsequent freezing and cryotomy. This method was used for the analysis of HeLa S3 [17,70], thymocytes [44,67], and PC3 cells derived from human adenocarcinoma [62]. Unfortunately, the method suggested for suspensions cannot be used for monolayer cultures, because the cytoplasmic membrane is damaged during removal of the cells from the underlayer and formation of precipitate. The fundamentals of the method and the data of EPMA for suspension of isolated cells are described in detail as exemplified by primary culture of rabbit heart myocytes [68]. Interesting results of this study were obtained on lyophilized cryosections of precipitate consisting of cardiomyocytes.

A known fact, the presence of two types of cells, differing by shape, in rabbit cardiomyocyte suspension, was confirmed experimentally [68]. High concentration of sodium, low concentration of K^+, and very high content of Ca^{2+} (250 mmol/kg dry weight) was recorded for round cells. The presence of a group of round cells in the suspension is attributed to the negative effect of disintegration procedure during isolation of cardiomyocytes. Damaged cells are characterized by high Na^+/K^+ ratio and accumulation of calcium in the cytoplasm [49,71].

In contrast to round cells, rectangular (normal) cardiomyocytes visually demonstrate the features characteristic of intact tissue [68]. In this study [68] for conversion into mM units the level of intracellular water was taken as 76% [64]. With this amendment, the cytoplasmic concentrations of elements in normal isolated myocytes is 128 mM for K, 40 mM for Na, and 11 mM for Mg. Special attention in discussion of EPMA results is paid to high concentration of sodium (40 mM) in normal myocytes in suspension in comparison with the value obtained for tissue (about 20 mM). This disagreement is caused by great variations in the results of the element concentration measurements in a group of isolated cardiomyocytes. It seems that high level of intracellular sodium is due to damage to the plasma membrane. It is noteworthy that the increase in sodium concentration after tissue disaggregation recorded for other types of isolated cells as well (for example, rat thymocytes) is leveled during subsequent incubation of primary culture [65].

EPMA of isolated cultured cell embedded in epon. In parallel with EPMA methods based on low-temperature dehydration of frozen cell or its cryosections, sections can be prepared from lyophilized tissue embedded in epon [6]. This protocol was used for the analysis of intracellular potassium and phosphorus in a single-cell *Acanthamoeba Castellanii* [58,59]. It was shown that phosphorus and potassium were unevenly distributed in the cell [59].

Embedding of a lyophilized cell in epon at ambient temperature permits making sections of needed thickness using ultramicrotomes. Due to design of these devices and stability of physical efforts, the cutting step (critical parameter for estimating element concentrations in EPMA) is highly accurate [5,53,55,57]. In order to rule out the impact of the cryosection thickness variations, a special method was developed, when the results of each measurement were standardized with consideration for the intensity of inhibitory X-ray radiation proportional to the local bulky thickness of the section [40,41].

The adequacy of the method of "embedded" sections for EPMA was verified on tissues [55,57] and in comparative experiments on preparations of primary cardiomyocyte culture [8,56]. The method was used with lyophilized cryosections of primary culture of cardiomyocytes; data for isolated myocyte were reported [68]. The results are summed up in Table 2.

Cardiomyocytes were analyzed in two states: in papillary muscle tissue and in suspension of rat heart primary culture [8]. The samples were prepared by cryofixation of with subsequent lyophilization and embedding in epon. Analysis of semithin (2-μ) sections showed that primary cardiomyocyte culture contained three cell types differing by shape, size, and potassium concentration. Potassium content in rectangular cells, which were classified as "normal" according to electrophysiological measurements, was close to its intracellular concentration in tissue. Presumably, differences in potassium concentration in different types of myocytes can be caused by disruption of the cell membrane during tissue disintegration for primary culture isolation. It seems that membrane repair during subsequent culturing is paralleled by continuing dissociation of cell ensembles.

Single cell EPMA. In this case the volume of experimental material is confined to the size of one cell, which extremely impedes manipulations during section preparation. The embryo at the stage of cleavage divisions is an example of a single cell. The detection of the object is easy for an early embryo (fish egg) of, *e. g.*, fish or amphibians, due to their large size and specific features of their development [23].

The requirements to preparation of sections of a mammalian early embryo are quite different. All preparative manipulations are carried out with a single microobject, whose optical characteristics virtually do not differ from the characteristics of saline or epoxy resin. For this reason the main methodological problem was to provide, after multi-staged preparation of the sample, the presence of the embryo in the small volume of embedded block, with coordinates identified in space.

Using experimental approaches for preparation of a whole isolated cell (Fig. 1), a method intended for murine early embryo was created [18]. All the flaws of whole cell analysis were present: uncontrolled washing of the preparation from physiological medium and *on mass* analysis of the cell without subcellular resolution. Further development of mammalian embryo EPMA was arrested for a long period because of the absence of technology for making semithin sections. We solved this problem

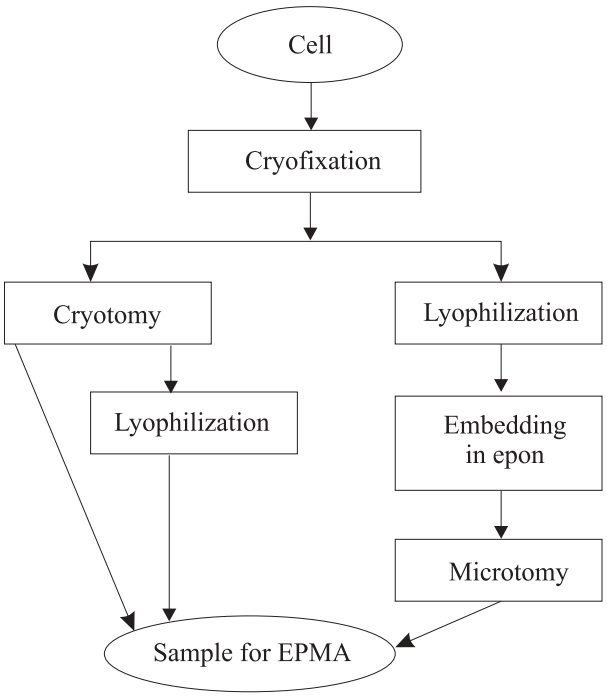

Fig. 1. Scheme of manipulations used for making cell preparations for electron probe microanalysis (EPMA) of easily diffused elements (potassium, sodium, chlorine, *etc.*).

[1,14]. The sequence of manipulations in our EPMA technology is presented in Fig. 2.

The use of our method for making semithin (2-μ) sections of mouse embryo gave unique results for such promising cell technologies as cryopreservation of an early embryo [4,13] or therapeutic cloning [2,7].

Analysis of the results (Table 3) suggests that cytoplasmic concentration of potassium in mature two-cell mouse embryos is close to the value recorded in specialized cells. Sodium level in a blastomer significantly surpasses the corresponding parameter for differentiated cell. It is hardly possible that the element is present in ionized form. It seems that sodium is mainly deposited in cytoplasmic structures of embryonic cell.

As a result of cryopreservation, the content of intracellular potassium decreased from 130±6 to 47±3 mM. It could be expected, as ethylene glycol, penetrating into the blastomer, impairs the membrane integrity and/or inhibits active potassium transport. The equilibration/washing protocol modified the status of embryonic cell. Cytoplasmic concentration of potassium in the embryo (in the G_1/S phase) was established at the level corresponding to mitosis (that is, equilibration/washing of the cryoprotector (ethylene glycol) imbalanced the K^+/Na^+ ratio in the two-cell embryo blastomer). Presumably, potassium imbalance induced by the cryoprotector at the stage of equilibration can disorder embryo development.

A two-fold decrease in the cytoplasmic concentration of sodium is intriguing (Table 3). The level of this element drops opposite to the gradient, which is possible in the presence of active transport. This effect can be due to activation of Na^+/K^+-ATPase directed to compensation for potassium deficiency formed in the blastomer. The presence of Na^+-ATPase (active Na^+ transport) without Na^+/K^+-ATPase cannot be ruled out either. The presence of Na^+-ATPase is probable in a mammalian early embryonic cell with its unique physiology. One more example of EPMA use in cell technologies for studies of an isolated cell status can be reprogramming of the zygote by introduction of genetic material.

Enucleation causes an appreciable decrease in potassium content in the zygote (Table 4). It seems that this disorder is caused by the procedure of pronucleus selection, as the registered changes virtually do not depend on the method of nuclear material introduction. As a result of microsurgical manipulations, potassium level in the zygote cytoplasm decreased to a level characteristic of the embryo at the beginning of mitosis. We know now that modification of the concentration of K^+ (the main intracellular ion) is essential for the molecular genetic mechanisms, critical for cell function: DNA

TABLE 4. Potassium Concentration (mM) in the Cytoplasm of Mouse One-Cell Embryo (Zygote) in the G_1/S Phase, Determined by EPMA* (*M*±*m*)

Experiment protocol	*n*	K concentration, mM
Intact zygote in the G_1/S phase	20	119±6
Introduction of one's own nuclear material back into zygote in the G_1/S phase	5	29±7
Introduction of somatic cell nucleus in zygote in the G_1/S phase	13	21±3
Intact zygote in mitosis prophase	9	35±3

Note. *Presented by D. V. Gol'dstein *et al.* [2]. *n*: number of analyzed embryos in a group.

recombination and degradation [52], gene expression [61], polymerization/depolymerization of actin (structural element of the cytoskeleton) [35]. For this reason a shift of potassium balance can significantly modify further development of chimerical embryo.

EPMA approaches developed for early mouse embryo can be used in other studies, when the size of the studied object are comparable with those of a single embryo. An example of this effective application is analysis of human clonogenic neurospheres obtained by neural stem cell culturing [3].

Great variety of the data reflects the variability of physiological status in the clonogenic group of human neurospheres: according to EPMA, the mean concentrations of Na and K in a group of 6 clonogenic neurospheres were 100±10 and 130±30 mM. Even with consideration for the impact of extracellular space, high concentration of sodium, not characteristic of a differentiated cells, attracts special interest. It was shown that potassium was unevenly distributed in a neurosphere [3]. This observation does not contradict the heterogeneous structure of the neurosphere demonstrated at an ultrastructural and morphological levels [19]. Morphological signs of all phases of cell cycle, mitosis and apoptosis, are detected in the clone [19]. Presumably, local alteration of potassium concentration in a neurosphere corresponds to cells at different stages of differentiation.

The main cytoplasmic ion (K^+) most rapidly and intensely reacts to changes in the situation, and hence, intracellular concentration of this element is an important criterion for evaluating the traumatism of manipulations used in cell technologies. Using this approach it is possible to purposefully develop procedures for, *e. g.*, therapeutic cloning, when intracellular balance of elements should be retained.

Among the fundamental and methodological problems to be solved, we should like to name analysis of element balance in synchronized cell culture in different mitosis phases, the velocity of Na^+/K^+ balance recovery during early mammalian embryogenesis after manipulations in enucleation or cryopreservation, K^+-induced polymerization of actin as a component of the mechanism of pronucleuses approximation in mammalian zygote, role of periviteline space as buffer volume in the regulation of Na^+/K^+ ratio in the embryonic cytoplasm, when EPMA advantages are obvious.

In this connection the problem of correspondence of cellular *in vitro* and *in vivo* status acquires special importance, because under culturing conditions the extracellular environment modulates cell homeostasis and hence, cell function. This problem is essential for cells with different *in vitro* and *in vivo* status: hepatocyte [69], cardiomyocyte [8,68], and neuron [57]. In this respect it seems interesting to compare the element balance in a mammalian embryonic totipotent cell at the morula or blastocyst stage as analog of cell clone and in a clonogenic structure grown from a stem cell, *e. g.*, human neurosphere. Among the environmental parameters which can modify cell development in culture are, in addition to the main factors, experimental microadditives (vital stains, probes, antibodies, physiological substitutes of substrates and chemical elements, isotopes, *etc.*). It is noteworthy that EPMA is an effective method for the study of a single cell in culture.

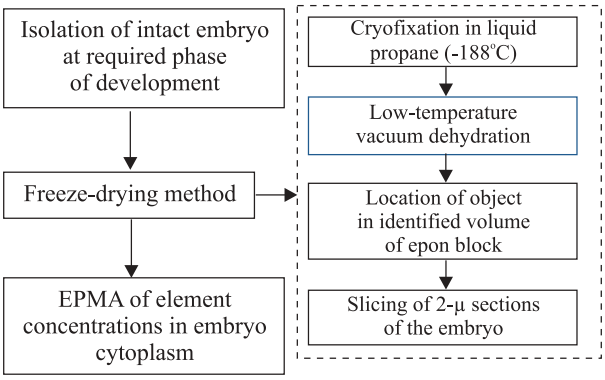

Fig. 2. Technology of preparation of semithin sections for EPMA of cytoplasmic concentration of elements in early mouse embryo.

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