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COMPARISON OF CRYOPREPARATION TECHNIQUES FOR ELECTRON PROBE MICROANALYSIS OF CELLS AS EXEMPLIFIED BY HUMAN ERYTHROCYTES

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Abstract

Erythrocytes in human blood were used to evaluate the reliability of cryopreparation techniques for electron probe X-ray microanalysis of biological cells and tissues. The elemental content determined by X-ray microanalysis of ultrathin freeze-dried cryosections was found to be consistent with data known from the literature. Considerable redistribution of the intracellular elemental composition was found after freeze-substitution as well as after freeze-drying followed by resin embedding. Two conclusions are drawn from this study: 1. Erythrocytes in human blood are a suitable reference specimen for evaluation of specimen preparation techniques for microanalysis. 2. At present, freeze-dried cryosections are the most reliable specimen type for quantitative electron probe microanalysis of cells.

Key Words: Cryopreparation, cryosection, erythrocyte, freeze-drying, freeze-substitution, resin embedding, intracellular ions, X-ray microanalysis.

Introduction

The quantitative localization of elements in cells by electron probe microanalytical methods requires a preparation technique which preserves the elemental distribution of the functional state of interest up to the analysis in the electron microscope. The proper evaluation of different preparation techniques used for this purpose turns out to be difficult due to two reasons: 1. A reference specimen with known elemental content including known concentration gradients representing the compartmentation of biological cells and tissues is hardly found. 2. Mobile elements in cells can change their location very rapidly depending on the particular experimental conditions. Therefore, most reports in the literature evaluate specimen preparation techniques for quantitative biological microanalysis rather qualitatively or by plausibility considerations (Morgan 1980, 1985; Chandler, 1985; Roos and Barnard, 1985; Wroblewski and Wroblewski, 1986; Edelmann 1986, 1991; Zierold and Steinbrecht, 1987; Zierold and Schäfer, 1988; Condron and Marshall, 1990).

In this paper, human erythrocytes in fresh blood are presented as an easily available sensitive reference specimen with known elemental content and gradients across the cell membrane for the evaluation of preparation techniques for biological microanalysis. Since non-cryo methods including chemical fixation, immersion into dehydration liquids or air drying are well known to redistribute intracellular elements (Morgan, 1980, 1985; Zierold and Schäfer, 1988), this study concentrates on the comparison of different cryopreparation methods.

Materials and Methods

Cryopreparation methods studied in this report started by cryofixation of fresh human blood droplets approximately 10-30 seconds after puncturing a finger tip with a needle. A droplet of about 1 mm in size was mounted onto a gold Balzers freeze etch planchet by means of a syringe and plunged into liquid propane cooled by liquid nitrogen by means of an air pressure

Table 1. Elemental contents of human erythrocytes and blood serum measured by EDX of freeze-dried cryosections ± standard deviation in comparison to literature data taken or derived from Geigy (1968).

	erythrocytes			serum	
	EDX mmol/kg drymass	EDX mmol/kg water	literature mmol/kg water	EDX mmol/kg water	literature mmol/kg water
n*	24			8	
d**	0.33±0.03		0.33±0.01	0.23±0.03	
Na	21±21	10±11	11±3	134±22	138±6
Mg	10±8	5±4	5±1	4±3	1.6±0.5
P	48±10	24±5	23±3	13±3	4±1
S	123±12	62±6	59	71±5	24
Cl	79±27	40±14	68±9	103±19	103±4
K	200±29	100±14	88±12	7±3	4±1
Ca	3±2	1±1	0.1	5±2	5±1
Fe	41±5	21±3	28±3	---	

*n = number of measurements; **d = dry mass portion.

driven device with an entry velocity of 2 m/s. Then, the specimen was stored in liquid nitrogen for further processing. In total ten different cryopreparation procedures were used, which can be divided into three essentially different techniques:

1. **Freeze-substitution**: Three different substitution liquids were used for this purpose: methanol, acetone or diethylether. Before use, these liquids were kept dry by molecular sieve. Freeze-substitution was done in the pure liquids without the addition of fixatives or staining substances as used for morphological studies. Freeze-substitution was done in the Balzers FSU 010 freeze substitution apparatus. Frozen specimens were incubated in 1.5 ml substitution liquid in plastic vials at 183 K. Freeze-substitution in methanol or acetone was performed successively for 8 hours at 183 K, 8 hours at 213 K and 8 hours at 243 K. In addition, other frozen blood droplets were freeze-substituted in acetone or diethylether successively for 3 days at 183 K, 3 days at 213 K and 3 days at 243 K. Then the specimens were allowed to warm up to room temperature overnight and transferred into a desiccator for drying in nitrogen gas atmosphere. The specimens were coated by carbon and studied by X-ray microanalysis in the scanning electron microscope (SEM).

2. **Freeze-drying followed by resin embedding**: The blood droplets were freeze-dried in the Leybold GT1 freeze-drying apparatus at 190 K for 24 hours. Some droplets were warmed above room temperature, quickly transferred to an evaporation unit, and coated by carbon for X-ray microanalysis in SEM. Some blood droplets were warmed up above room temperature and embedded in Epon 812 or LR white after short transfer through the air. Other droplets were transferred at a temperature below 240 K into a refrigerator, embedded in Lowicryl K4M or HM20 and polymerized by irradiation of UV-light at 243 K for at least 4 days. After polymerization, ultrathin sections were cut dry from all embedded specimens by a glass knife and analyzed in the scanning transmission electron microscope (STEM).

3. **Freeze-dried cryosections**: After cryofixation as described above, approximately 100 nm thick cryosections were cut dry at a temperature below 170 K by use of a glass knife in a Reichert FC4 Ultracut cryoultramicrotome. The sections were placed onto Pioloform coated and carbon evaporated grids by means of an eyelash probe, cryotransferred to the STEM under cold nitrogen gas, freeze-dried in the cryotransfer chamber and studied by X-ray microanalysis as described previously (Zierold, 1986a).

Energy dispersive X-ray microanalysis (EDX) in SEM was performed using the Link 860 system attached to an Autoscan SEM operating at an accelerating voltage of 12.5 kV. The obtained X-ray spectra were evaluated quantitatively by the ZAF-PB program provided by Link Systems. The resin embedded sections and cryosections were analyzed by an energy dispersive SiLi detector (Nuclear Semiconductors) combined with a Link AN10000 system, attached to a Siemens ST 100F STEM operating at an accelerating voltage of 100 kV. For quantitative data evaluation, the Quantem FLS program provided by Link Systems was used. As standards for quantitation, droplets consisting of 20% dextran and 80% salt solution of known concentration were cryofixed and freeze-dried for EDX in SEM or cryosectioned, cryotransferred and freeze-dried for EDX in STEM.

Table 2: Elemental ratios measured by X-ray microanalysis of human erythrocytes after different cryopreparation techniques.

		Na/P	S/P	Cl/P	K/P	Fe/P	K/Na
Freeze-substitution	methanol, 1 day	0.26	10.7	0.1	1.47	1.5	(5.6)
	acetone, 1 day	4.49	1.75	5.95	1.35	1.5	0.30
	acetone, 9 days	5.58	6.1	5.4	1.75	1.6	0.31
	diethylether, 9 days	9.5	5.1	17.3	2.67	2.9	0.29
Freeze-drying followed by resin embedding	no resin	4.37	2.2	8.1	3.18	0.78	0.67
	Epon 812	3.56	3.96	31	2.1	0.6	0.59
	LR white	2.67	2.5	6.5	1.8	0.83	0.67
	Lowicryl K4M	2.95	3.28	5.95	3.38	0.86	1.14
	Lowicryl HM20	3.77	2.83	6.16	1.9	0.67	0.5
Freeze-dried cryosection		0.44	2.56	1.65	4.17	0.85	9.5

The dry mass portion of cryosections was estimated by the relative dark field intensity method as described previously (Zierold, 1986b).

Results

Fig. 1 shows a freeze-dried cryosection of human blood with qualitative elemental maps recorded according to the energy window method as described by Zierold *et al.* (1991a). The maps show the homogeneous distribution of K in the cells and of Na and Cl mainly in the extracellular serum. The low intensity of the Na map is due to absorption of low energy X-rays by the Be-window in front of the detector. Elemental maps in Fig. 1 as well as measurements of single spectra give no indication for elemental shifts across cell membranes. Microanalytical data on the element content measured in freeze-dried cryosections from erythrocytes and the surrounding serum are compiled in Table 1. Literature data on the element content of erythrocytes and blood serum are included in Table 1 in order to compare the conformity with the data obtained by X-ray microanalysis of cryosections.

For comparison of data obtained from resin embedded and non-embedded specimens, the element contents found after different preparation paths are related to phosphorus, a mainly bound element in the cells which does not occur in the embedding resins used. In addition, the K/Na ratio in the erythrocytes is indicated as a particularly sensitive criterion for cell viability. All ratioed data derived from mean values of X-ray microanalytical measurements are compiled in Table 2. X-ray microanalytical data are based on measurements of at least 10 cells.

The data show clearly that in particular elemental gradients of mobile electrolyte ions such as Na, Cl and K across the erythrocyte cell membrane are lost or greatly reduced after freeze-substitution or resin embedding. Electrolyte gradients across cell membranes were found to be preserved only in freeze-dried cryosections.

Discussion

The data on the elemental content in erythrocytes and serum of human blood determined by EDX of cryosections agree fairly well with the literature, as can be seen in Table 1. In particular, the EDX results of mobile electrolyte ions such as Na, Cl and K coincide with few exceptions with known data. Intracellular Cl and Fe contents determined by EDX were 30% lower than those reported in the literature and extracellular S and P were found to be higher than expected. Despite these discrepancies, similar element contents in erythrocytes and serum were found by X-ray microanalysis of cryosections from five preparations of blood droplets originating from three different persons. Surprisingly, there are only few electron microprobe studies on erythrocytes. Kirk *et al.* (1974) found a similar K and Na content in erythrocytes placed on Be-discs as measured in cryosections and compiled in Table 1. Loss of intracellular elements was prevented by dibutyl phthalate. Lechène *et al.* (1977) found rather broad distributions of Na, S, K and Fe in erythrocytes by wavelength dispersive X-ray microanalysis. Tormey (1978) and Tormey and Platz (1979) used nominally 500 nm thick cryosections of erythrocytes to validate electron probe X-ray microanalysis of diffusible elements in biological samples. After appropriate correction of data they found similar values of intracellular Na and K as reported here.

Most studies on quantitative X-ray microanalysis of cells are based on chemically prepared standards, for review, see Roomans (1988) and Warley (1990). The

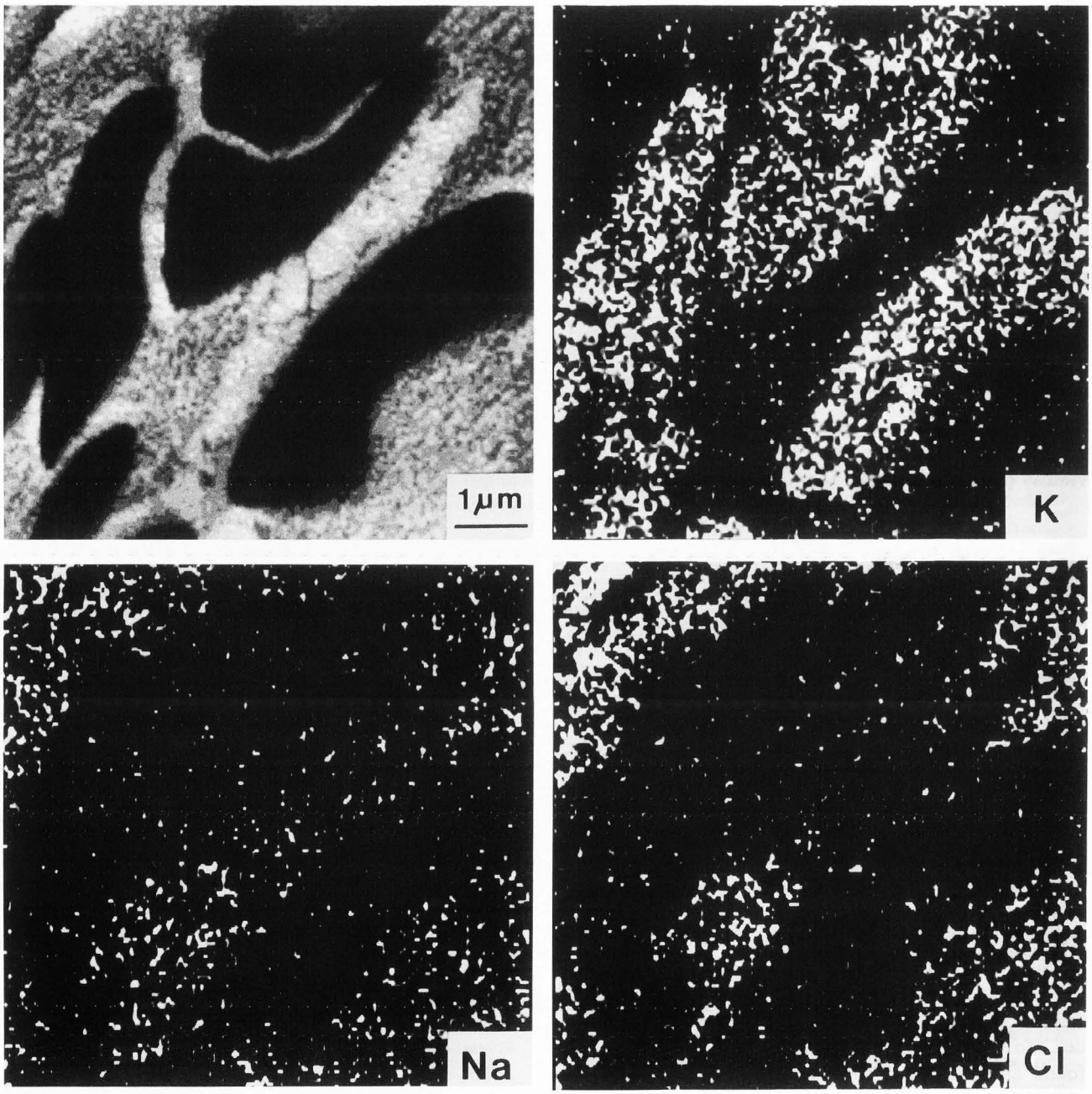

Figure 1. Elemental maps of cryosections of erythrocytes in human blood. The bright field/dark field image (upper left) and the corresponding element distribution maps for K, Na and Cl were recorded by scanning the electron beam in a frame of 128 x 128 pixels with a dwell time of 10 ms per pixel. Ten frames were superimposed in order to improve the intensity. The elemental maps were corrected for specimen thickness variations by normalization to the continuum map in the energy range from 4.5 to 6 keV.

advantage of human blood as reference specimen to test the effects of specimen preparation techniques on the intracellular element content is the easy availability and the known elemental composition of erythrocytes and serum. The use of other cells, for example, cells from tissues such as liver, kidney, muscle etc., induces more severe problems with respect to the preservation of the elemental composition as tissue pieces have to be dissected from an intact organ of usually unknown physiological state (Zierold and Schäfer, 1988; Zierold 1993). Le Furgey *et al.* (1988) have compiled quantitative electron probe microanalysis data from cryosections of a variety of cells and tissues. Most of the cited data look reasonable, and probably they can serve as a starting point for further investigations, but due to the problem to reproduce the experimental conditions before cryofixation, the compiled data can hardly be taken as biological standards. This problem was encountered recently by comparing X-ray microanalytical data from cryosections of pancreas, where large variations were found among different tissue blocks, from animal to animal and among the data from different research groups (Tobler *et al.*, 1992).

Table 2 shows the effects of the preparation techniques used on the element content in erythrocytes in detail. The K/Na-ratio is the most sensitive parameter for preparation induced changes of the element composition as high K and low Na in the cells borders on low K and high Na beyond the cell membrane in the extracellular serum. Obviously, none of the studied preparation techniques represented in Table 2 can compare with cryosectioning with respect to the preservation of the intracellular K/Na-ratio.

Redistribution of ions in biological specimens by use of non-cryo-techniques, for example chemical fixation, was studied previously (Morgan *et al.*, 1978; Morgan, 1980; Zierold and Schäfer, 1988) and will not be further discussed here.

Immobilization of intracellular elements can be improved remarkably by cryofixation. Recently, a comparison of different cryofixation techniques (plunge freezing, impact freezing and high pressure cryofixation) has shown similar results of the elemental content of human erythrocytes (Zierold *et al.*, 1991b; Zierold, 1993). Therefore, all cryopreparation techniques considered in this article start with plunge freezing.

The freeze-substitution protocols used in the presented study have obviously failed to immobilize diffusible elements. As described in a previous paper, solubility of electrolyte ions in the substitution medium increases with increasing polarity of the substitution liquid (Zierold and Schäfer, 1988). In particular, by use of the highly polar methanol (dielectric constant 33.5) diffusible elements are dissolved and spread into the substitution medium. The high K/Na ratio of 5.6 is caused by chance due to small values resulting from X-ray microanalytical evaluation. Theoretically an apolar substitution medium such as diethylether (dielectric constant 4.3) should preserve the elemental distribution better. Indeed, freeze-substitution with diethylether for 9 days enhanced potassium retention as compared to more polar substitution media. Due to the low polarity of diethylether, extracellular ions such as sodium and chloride are not dissolved but attracted to polar "binding sites" in the cells, thus increasing the intracellular content of sodium and chloride. In the older literature, high portions of ion retention after freeze-substitution are reported. For compilation of data see Morgan *et al.* (1978) and Morgan (1980). Harvey (1982) reports high retention of ions in botanic specimens after freeze-substitution with diethylether. The apparent discrepancy to the results obtained after freeze-substitution of erythrocytes shown in Table 2 could be explained by the rigid cell walls surrounding plant cells. Probably, these cell walls hinder the loss of ions from the cells and the influx of extracellular ions into the cells. Wroblewski and Wroblewski (1986) report similar elemental ratios in muscle after freeze-drying or freeze-substitution with ether and a remarkable loss of K as compared to cryosections. Roos and Barnard (1986) show considerable elemental loss in pancreas tissue freeze-substituted with acetone and embedded in Spurr's resin. Edelmann (1991) concludes from experiments on freeze-substitution of skeletal muscle with acetone and embedding in Lowicryl resins that in the best case 67% of the intracellular K was retained. The increased Fe/P ratios after freeze-substitution in comparison to the data from cryosections indicate that substitution media not only redistribute electrolyte ions but also phosphorus or organic molecules containing phosphorus.

Dehydration by freeze-drying followed by resin embedding is a widely used preparation technique for biological microanalysis (e.g., Ingram and Ingram, 1984; Chandler, 1985; Wroblewski and Wroblewski, 1986; Edelmann, 1986; Fritz, 1989; Condron and Marshall, 1990; Elder *et al.*, 1992). The evaluation of this method with respect to the preservation of elemental distribution as described in the literature cited above is hampered by the fact that the peculiar effects of dehydration and embedding were not discerned clearly. The present study shows the mere effect of the embedding resin on the intracellular elemental content by comparison with data obtained from freeze-dried blood droplets and cryosections of erythrocytes. The mainly bound elements P, S and Fe seem to be well preserved in LR white and in the Lowicryl resins, whereas Epon exhibits a slight loss of these elements. The high Cl/P ratio in Epon is due to the chlorine content of Epon.

The best retention of K in the cells after freeze-drying and embedding was found after using Lowicryl K4M at low temperature. Embedding with K4M at room temperature led to a slight intracellular decrease of K and increase of Cl whereas the more apolar Lowicryl HM20 caused the opposite effect: Embedding at room temperature increased the intracellular K and decreased the Cl slightly (data not shown). This observation corresponds to the dielectric properties of the two embedding resins. In the polar resin K4M the solubility of ions increases with increasing temperature, in the apolar resin HM20 the solubility of ions decreases with increasing temperature.

All embedding resins used cause an increase of intracellular Na and Cl accompanied by some loss of K. The low K/Na ratios found after embedding are rather due to the uptake of extracellular Na than due to the loss of intracellular K. Thus, embedding resins are unsuitable for quantitative microanalysis of diffusible elements. This result agrees with previous comparative studies on rat pancreas (Roos and Barnard, 1985).

Of course, the frozen-hydrated cryosection would be the most attractive specimen type with respect to the preservation of the original distribution of elements. This method was developed successfully for X-ray microanalysis of approximately 1 μm thick cryosections (Gupta and Hall, 1979; Hall and Gupta, 1983). Unfortunately, this technique is unsuitable for the analysis of approximately 100 nm thick sections necessary for higher spatial resolution because of radiation damage during electron irradiation (Zierold 1986a, 1988). Freeze-dried cryosections usually are stable in the electron beam, however, in compartments with a dry mass portion of less than 10%, shrinkage of structures and redistribution of elements caused by freeze-drying have to be taken into account. These problems are discussed in detail elsewhere (Zierold, 1988).

Conclusion

Erythrocytes in human blood are a suitable reference specimen for evaluation of specimen preparation techniques for electron probe X-ray microanalysis. Freeze-substitution as well as resin embedding affect the original intracellular element composition. Reliable quantitative data are obtained from freeze-dried cryosections.

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Discussion with Reviewers

H.Y. Elder: Our group has repeatedly shown that the resin-embedding route is capable of delivering very reproducible results and can consistently detect physiologically induced relative changes in the concentrations of intracellular elements such as Na, K, Cl (for review, see e.g., Elder *et al.*, 1992). Your data (Table 1) from the freeze-dried cryosections, with quoted standard deviations, usefully give a measure of the spread of the values obtained. Can you give an indication of the spread of the data obtained by the other methods which you used, the resin-embedding route in particular?

Author: In principle, there is no contradiction between reproducibility of experimental results and artifacts. This is particularly true for systematic artifacts which are inherent, more or less, in every preparation technique. Thus, physiologically meaningful data can also be obtained by experimental methods causing artifacts. The presented microanalytical data from erythrocytes merely show that intra- and extracellular element contents, measured in freeze-dried cryosections, fit better to literature data obtained by other chemical methods than data determined after use of freeze-substitution or resin embedding. The standard deviation of microanalytical data obtained from freeze-dried and embedded erythrocytes were in the same range as that given for data from cryosections in Table 1.

H.Y. Elder: It is agreed that the K/Na ratios are a sensitive criterion of elemental retention and the greater than ten fold difference (Table 2) between the freeze-dried cryosections and the other preparative routes is striking. You report (Table 1) a K/Na ratio of ~10 by EDX of the freeze-dried cryosections of erythrocytes. In Elder *et al.* (1992) cited above K/Na ratios from sweat gland cells were usually in the range of 2.5 to 3.5, which is some five fold higher than the ratios found by you in red blood cells. Please comment.

G.M. Roomans: The changes in ion content found after freeze-drying/embedding do not suffer from artifacts

and form a valid criticism against this method. It is, of course, too early to say whether the method could not be improved by minor changes. A very important point to discuss is also that blood is a very sensitive test specimen since it has a high ratio of extracellular space to intracellular space. If redistribution occurs, it will show up in blood cells first, because there is such a large amount of Na and Cl to redistribute. Therefore, the changes in, e.g., liver may be much smaller than those given here for blood.

Author: I agree that the K/Na ratio in erythrocytes probably is a very sensitive criterion for ion redistributions by preparation artifacts. The elemental composition of other cells can be different and less sensitive for preparative manipulations, in particular, dehydration processes. For example, isolated rat liver cells were processed parallel to the erythrocytes. The K/Na ratio was similarly low after freeze-substitution and freeze-drying followed by embedding in comparison to the data from the cryosections. However, some experimental protocols provided K/P-ratios very similar to those determined in cryosections, e.g., freeze-substitution with diethylether and embedding in Lowicryl K4M or Epon after freeze-drying. This can be seen from the data compiled in Table 3. Despite some remarkably good agreement with data from cryosections, I would not recommend any specific freeze-substitution or embedding protocol for quantitative microanalysis because of remarkable variation of the intracellular ion content depending on the particular cell suspension. For example, potassium may vary up to 50% in different rat liver cell suspensions. In comparison to rat liver cells, where intracellular compartmentation of ions has to be taken into account, human healthy erythrocytes exhibit a remarkably stable and homogeneous elemental composition.

T. von Zglinicki: A comparison of freeze-dried, embedded and cryosectioned specimens will show the sole influence of the embedding procedure only if the drying schedules were completely the same. As I understand it, in your case, cryosections were never warmed above 190 K and might still contain some (structural) water, while embedded specimens probably lost all of it before or during embedding at 243 K. Could this difference contribute to ion shifts?

Author: This seems unlikely to me. Freeze-dried cryosections warmed up after X-ray microanalysis in the electron microscope to room temperature overnight showed only slight, if any, differences to the data measured before.

Reviewer 4: The drying of cells freeze-substituted with solvents is not an equivalent process to freeze-substitution followed by embedding. Furthermore, translocations may occur during the drying process as well as collapse of cell structures. After drying, were the cells kept in anhydrous conditions? Absorption of water vapour from the atmosphere could occur very readily in such specimens and can lead to ion movements.

Author: I agree. In my experience, dehydrated specimens are very sensitive for contamination by humidity of the air. Therefore, freeze-dried as well as freeze-substituted specimens were warmed slightly above room temperature before they were transferred through the air to the desiccator filled with nitrogen gas or to the evaporation unit for carbon coating. Although this transfer was done rapidly within a few seconds, artifacts by humidity of the air cannot be excluded completely. However, this effect should not be overemphasized. Comparative studies on freeze-dried cryosections have shown that humidity affects recognizability of cellular structures much more than the distribution of elements [von Zglinicki T, Zierold K, (1989). Elemental concentrations in air-exposed and vacuum-stored cryosections of rat liver cells. J. Microsc. **154**, 227-235).

H.Y. Elder: In the light of the information from differential thermal analysis which MacKenzie (1981) [In: Microprobe Analysis in Biological Systems. Hutchinson TE, Somlyo AP (eds.), Academic Press, New York, pp. 397-421] presents about the discrete temperatures at which significant quantities of water vapour are released during warm-up, do you not think that the "room temperature" steps which you employed during freeze-substitution were too great?

Author: The temperature course used during freeze-substitution was the same as that used for preparation of cells for morphological studies by transmission electron microscopy and SEM with good success. Since a step from lower to higher temperature during freeze-substitution simply means that the cold aluminum block surrounding the substitution liquid is not cooled further and allowed to warm up, I do not think that the temperature steps are too great. In comparison to the "rapid freezing" rate used by MacKenzie of approximately 1000 K/min, blood droplets were frozen with a rate of at least 5000 K/s as determined previously by thermocouple measurements. Thus, the cellular matrix is expected to be immobilized in a state far away from the eutectic equilibrium state, and reorder of solvents and solutes measured as "antemelting" in differential thermal analysis is expected to take place at low temperature where mobility of ions is also relatively low. In order to avoid rapid reordering, prolongation of substitution times seems to me sufficient.

Reviewer 4: The use of ZAF-PB for analysis of thin dried cells of this type may lead to erroneous results.

The depth from which X-rays can be emitted in samples of this type at 12.5 kV will almost certainly be much greater than the thickness of the blood cells. This means that background would be generated by and detected from the coverslips which would invalidate the quantitation.

Author: The dehydrated blood droplets analyzed in SEM were several cell layers thick. By forming ratios of the microanalytical data, the influence of the support is largely eliminated.

Reviewer 4: Presumably you cut *dry* ultrathin sections of freeze-dried embedded blood cells. Did you use any special method to obtain ultrathin sections in this way?

Author: Dry sections were obtained by glass knives in an ultramicrotome with a little bit more patience than necessary for usual ultramicrotomy. Sometimes sections were cut with a cold knife in order to reduce curling.

H.Y. Elder: I invite you to expand your comments about the inverse trends in solubility of ions with temperature between the Lowicryls of higher (K4M) and lower dielectric properties (HM20), especially in the context of the suggestion of Wroblewski *et al.* (1990) [A low temperature embedding procedure for X-ray microanalysis of biological specimens at subcellular level. Scanning Microsc. **4**, 787-793], who show that low temperature embedding in the polar Lowicryls can be useful in microanalysis.

Author: Unfortunately, Wroblewski and coworkers do not provide quantitative microanalytical data in the paper you mention in your question. Based on my studies presented in this paper, I cannot recommend Lowicryl-resins for microanalysis. In addition, Lowicryl-resins suffer from high mass loss during electron irradiation as also described by Wroblewski *et al.* However, my skepticism should not discourage other investigators from a search for a more suitable embedding medium.

Table 3. Elemental ratios measured by X-ray microanalysis of cryofixed rat liver cells.

Freeze-substitution	**Na/P**	**Cl/P**	**K/P**	**K/Na**
methanol, 1 day	0.27	0.07	0.36	1.34
acetone, 1 day	0.86	1.68	0.38	0.44
acetone, 9 days	1.44	1.14	0.32	0.22
diethylether, 9 days	1.98	4.06	0.55	0.28
Freeze-drying resin-embedding	**Na/P**	**Cl/P**	**K/P**	**K/Na**
Epon 812	0.46	6.25	0.58	1.27
LR white	0.52	1.23	0.32	0.61
Lowicryl K4M	0.35	0.69	0.67	1.92
Lowicryl HM20	1.16	1.49	0.46	0.39
freeze-dried cryosection	0.10	0.33	0.65	6.4