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QUANTITATIVE ELECTRON MICROPROBE ANALYSIS OF CRYOSECTIONS

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Abstract

There are numerous approaches to preparation and analysis of biological specimens which have been developed in many laboratories involving the use of cryosections. Because there is no clear consensus on how such samples should be produce or analyzed, it can be a formidable problem to choose or even review possible options. Therefore, it is the purpose of this tutorial to consider, in an instructional manner the problems of specimen preparation and analysis in terms of the solutions and procedures used by the author. The discussion, consequently, focuses on the problems of specimen preparation and analysis as seen by the author. It is hoped that the reader will appreciate the inherent bias introduced by this strategy, yet be able to use the information presented as a framework for approaching the literature in this field with sufficient understanding to make an informed decision about the diverse techniques which have been used for x-ray analysis of cryosections. In this tutorial, the author has considered the problems and limitations in the critical freezing step in contrast to what are widely held assumptions. Lastly, popular analytical algorithms have been reviewed including the use of x-ray analysis for forming quantitative elemental images.

Key Words: EPMA, cryosectioning, biological x-ray analysis, frozen-hydrated cryosections, specimen preparation

Introduction

Quantitative x-ray microprobe analysis of biological specimens using cryosections is an approach to determining elemental and water content of cellular structures which has offered much promise over the past 20 years. Still there appears to be little which one can consider as "standard" methodology. This tutorial will present our approach to quantitative x-ray analysis of cryosection along with rationale, compromises, and results. In the process, the author will attempt to compare, and to consider the advantages and disadvantages of other approaches currently used in other laboratories. It is hoped that the reader will understand the difficulties of these methods while appreciating the principles behind them.

Preparation of Cryosections

Freezing

While considerable information is available about freezing ice, the relationship to this process in biological specimens is far less clear. Much effort has been expended to achieve what is called vitreous freezing or vitreous ice or the glass state. In this state, ice has an amorphous structure and each element or ion within the ice matrix should be located in its original position as if stopped in time and space. Such ice is really a plastic material in that it can be considered a highly viscous liquid. The mechanical properties would make the achievement of this kind of freezing highly desirable for purposes of analysis and cryosectioning. In a practical sense, achieving this state of freezing in a biological specimen is essentially improbable except for very specific situations [13] where thin films of aqueous suspensions could be frozen with sufficient rapidity to result in ice with vitreous ice properties. However, achieving this state imposes a number of technical problems in so far as it is necessary to maintain sufficiently low temperatures to prevent warming and

recrystallization [13]. This concept of preventing recrystallization has greatly influenced cryosectioning methods despite the unlikelihood of achieving vitreous ice states in a piece of biological tissue sufficiently large to be cryosectioned.

For most biological specimens, one removes a piece of material and then freezes it. While excellent methods are available to freeze *in situ* using either Freon popsicles or highly polished metal surfaces [11], these methods all have in common the need to remove heat by contact. Heat removal rate is limited by the biological tissue (and its state) through which the heat is removed. Ice is a good insulator and slows heat removal sufficiently to prevent formation of vitreous states. At best, it has repeatedly been shown that freezing damage and ice crystal size is a function of distance from the freezing surface. This depth, in practical terms, is in the range of 10-20 μm. What is usually achieved using rapid freezing methods is frozen biological material where ice crystal size ranges from the imperceptible and hence insignificant, to areas where ice crystal size is in the range of 1 μm and typical of that seen in specimens frozen by slower methods (such as freezing in liquid nitrogen). Thus, the degree and size of ice crystals within a biological specimen will depend upon severe factors including: 1) the volume of tissue frozen; 2) the shape of tissue frozen; 3) the composition or how much water there is in the tissue; and 4) the rate of heat removal at the surface.

Interestingly there are several important assumptions about freezing which have strongly influenced how frozen tissue samples are subsequently handled and cryosectioned. These assumptions are: 1) that the better the tissue is frozen (i.e., the smaller the ice crystals and the closer to the vitreous state one achieves - best of all achieving the vitreous state) the easier it will be to cryosection and the better quality of the cryosections; and 2) that it is essential to keep frozen specimens below the theoretical recrystallization temperature of ice (vitreous to crystalline if possible) so that less ice crystal damage and less elemental movement and displacement will occur.

These assumptions have resulted in the widely held principle of the desirability of cutting frozen material at the lowest possible temperature. Interestingly, very little attention has been paid to the actual process of cryosectioning and the effects of temperature on material properties of frozen tissue. In essence, it is important to confront these assumptions noted above and consider the first of many compromises necessary to do quantitative x-ray analysis of cryosectioned biological material.

Cryosectioning

Cryosectioning is clearly a critical step for specimen preparation. It is intuitively obvious that to cut something requires some information about the object one cuts. For example if someone was asked to cut an object they would clearly want to know something about the object before selecting the tool to use in cutting, since the object to be cut might be a brick or a piece of paper. It is a basic idea that one needs some idea of the material properties of an object in order to choose the tool for cutting. Suppose one picked another object whose material properties varied as a function of temperature such as butter. At very cold temperatures butter is difficult to cut and can even be broken or fractured, while at warmer temperatures it is ductile and cuts easily. Biological material has properties which are temperature dependent [19]. It is not accurate, or even helpful, to consider biological material as simply a frozen ice cube. This raises the issue of the first assumption about good freezing, namely that vitreous or microcrystalline ice is easier to cut. Vitreous ice, assuming the tissue sample's water is frozen in the vitreous phase and that the material properties of the frozen tissue are determined by the material properties of the ice, has many of the material properties of glass. One could expect the frozen specimen to be hard and brittle, and since the ice is highly viscous, its flow characteristics, up to a point, are dependent upon temperature. Thus, in theory, it is not an ideal material to cut. The other half of the assumption about whether the ice controls the material properties relates to the variable composition and heterogenicity of biological materials. When tested, liver has material properties which are very dependent upon temperature [19]. At about -60 °C, frozen liver undergoes a rather abrupt transition from a ductile to a brittle state depending upon direction of temperature change. This change in ductility is not a function of the ice but rather a function of the other materials present in the tissue such as protein and lipids. What our findings have shown, is that biological material when it is too cold becomes so brittle that it cannot be cut any more than any other highly brittle material. The implications of this fact are quite interesting. Firstly, it suggests that while rapid freezing, to minimize ice crystal damage and ice crystal size, is important for morphological factors, rapid freezing is of less importance (but nonetheless of some importance) for cryosectioning. This idea is true up to a point and some qualification is important. If ice crystal damage is extremely large, it could result in sufficient damage to the morphological structure of the lipid and protein to alter the specimen's material properties. However, if one attempted to keep the frozen material below a

recrystallization point for vitreous ice, it would require using other compensations so as to make the brittle biological material ductile enough to cryosection. That is, it becomes necessary to raise the temperature at some point during the cryosectioning process. This can occur without consciously raising specimen temperature. There are many reports of cryosectioning at -80 to -100 °C. How is this possible, if biological material becomes brittle at -50 to -60 °C? The answer to this can be found in the well known principles of metal machining. In fact, most who cryosection at such low temperatures probably are not even aware that they have to be raising the temperature sufficiently to alter their specimen's material properties for cryosections to be formed.

To cut something requires work. The work of cutting is in part a function of the material properties. Soft and ductile material requires less work to cut than hard and less ductile material. Much of the work of cutting occurs in what is called the shear zone or shear plane. This is a zone located at some angle incident to the cutting tool. It is in this zone that cutting forces are exerted. When these forces are exerted, the material either fails and fractures along the shear plane or the material deforms by plastic deformation in the shear zone. A substantial quantity of the work of cutting can be deposited in the shear zone and chip along with the tool. In fact, heat from friction is a major cause of tool wear and often the reason for use of lubricants (actually coolants) in metal machining. The amount of heat rise in the shear zone is a function of heat input and heat removal. With a simple understanding of these concepts, it is easy to appreciate some of the common variables in cryosectioning which are considered part of the "art" of cryosectioning but which are in reality the science.

Karp et al. [12] performed an interesting experiment which illustrates this point, although the purpose of their experiment and their interpretation failed to convey the significance of their data. In this experiment, they cut a section of frozen toluene several degrees below its melting point and did not observe melting. They interpreted this finding as demonstrating that there was no rise in temperature during cryosectioning. Acutually what they demonstrated was completely predictable by metal machining theory and our previous studies in the work of cryosectioning. Toluene is a homogeneous material, and like butter, becomes softer and more ductile as its temperature rises toward its melting point. As the toluene becomes softer, less work (just like warm butter) is required to cut it. Because the amount of heat transferred to the shear zone is related to the work of cutting (which is related to the material properties of the material being cut), lower work means there will be less heat transferred. We pointed this phenomenon out in our previous studies. Thus as heat from the work of cutting is transferred to the shear zone, the material properties are altered so as to make the specimen more ductile, less work is necessary to cut and heat transfer is reduced. This heat transfer process is a function of heat input versus heat loss and the rate constants will be a function of the material (thermal) properties of knife, specimen, lubricant (air, etc) and power (rate of work). It is therefore quite wrong to consider Karp et al. [12] as having disproved well known physical laws of nature, instead it is clear that their results are completely supported by well known and well proven concepts of machining theory.

Consider the indisputable physical fact that heat must be generated in the cutting process and that cryosectioning is analogous to metal machining except that we are interested in preserving and using the chip (section) and throwing away the work piece (block). It is a well established truism that the thinner the cryosection, the lower the cutting temperature. This is true because of the smaller mass in the shear zone of a thin cryosection. Heat gain rate for a given power input (work/time) would be greater for a fixed heat removal rate. That is, the momentary rise in temperature would be greater for a thinner section than a thicker section. It is during that short, but important, period of heat input and rise in temperature of the shear zone, that the material properties of the shear zone become sufficiently ductile for plastic deformation of the lipid and protein in the specimen to occur. The ice crystals are probably minimally changed but are likely to move within the matrix. The ice crystals are in fact very hard material in a soft matrix and can act as an abrasive. Much of the fine surface structure seen in frozen hydrated sections probably is the result of ice crystals being dragged across the surface like a piece of sand paper.

The thermal conductivity of the knife also can play a role in thermal control over the shear zone and hence ductility. Glass knives or diamond knives have lower thermal conductivity and greater heat capacity than metal knives. Using glass or diamond knives probably results in higher shear zone temperatures than with metal knives. Another factor which is important is knife angle, since this parameter determines the amount of change in shape which the cryosection must undergo. Taking into consideration all of these variables, it is easy to see that shear zone temperature and its ductility can be easily affected during the cutting process. It is also of importance to control ambient temperature, since any differential temperatures between knife and specimen, or time varying temperatures within the cryochamber, will lead to changes in shape and

hence alterations in section thickness with the obvious consequences as noted above.

It has been our experience that an awareness of the importance of temperatures, the shear zone properties, and the factors which affect that temperature lead to reasonably predictable cryosectioning. The basic principle being that one chooses the ambient temperature, most likely to give a ductile specimen for the thickness of section desired. However, to go for the lowest chamber temperature because of theoretical reasons related to specimen freezing considerations and then be forced to increase cutting speed (heat input rate) and decrease section thickness (reduce shear zone mass thereby increasing heat/mass ratio) and think that one is cutting at an ideal low temperature, fails to appreciate what is happening to the cryosection in a physical sense. The actual thermal effects from cutting are essentially unavoidable but controllable. It should be noted that there are reports on cryosectioning very small samples of biological material which has been frozen in what appear to be the vitreous state [13]. Those cryosections also exhibit x-ray diffraction patterns suggesting that the amorphous state is retained. This phenomenon is as yet unexplained given the material properties of vitreous ice and heat input from cryosectioning. It is possible that the thermal effects of cryosectioning result in this special case, to a decrease in viscosity and hence alter flow characteristics of the vitreous sample. If this is true, and it eventually becomes possible to freeze relatively large pieces of tissue samples in a vitreous state, then these studies hold much promise.

Cryosection Handling

After a cryosection is cut, there appears to be a number of ways in which the section be handled. Frozen hydrated cryosections can be placed directly into the scanning electron microscope or electron microprobe on to a cold stage for direct analysis, or freeze drying, and subsequent analysis [10, 18]. Several investigators have freeze dried cryosections prior to transferring into the electron probe [1, 6, 15, 23]. This process has led to some controversy which is not yet resolved. Frozen dried biological material is hygroscopic and transfer through air with water vapor present has been implicated in elemental translocation [8]. Data available suggests that this phenomenon may be related to the specimen since the effects are not entirely predictable nor uniform between tissues and laboratories. The question of whether translocation occurs during air transfers of frozen dried cryosections and to what degree remains unclear. It is probably reasonable for anyone using these methods to be cognizant of these effects and take appropriate care. Minimal controls are probably valuable, if not essential.

Frozen hydrated sections present several important problems for analysis. In the first place, morphology is usually very poorly defined [5, 18]. Secondly, the specimen is sensitive to radiation induced mass loss [2, 24]. Thirdly, any significant loss or gain of water could lead to analytical errors.

Most of the published methods for x-ray analysis of cryosections have section handling systems which are carefully integrated into the analytical algorithm. Specimen holders are designed as thermal interfaces as well as optimized for their analytical properties. For example, the question of correction for background radiation can be approached from two major directions: 1) eliminate sources of extraneous radiation and 2) correct for sources of extraneous radiation [14]. A number of laboratories use copper grids, measure the Cu K_α and calculate the L_α lines and continuum to be able to strip those extraneous contributions to the analytic spectrum. As the ratio of background to specimen increases, it is clear that the errors inherent in the counting statistics become more significant in reducing accuracy and precision. Several laboratories have used sample sandwiching techniques for both frozen hydrated and dried cryosections [8, 15]. The disadvantage to this method is that it requires additional correction for the added film. If the combined film thickness is such that it becomes thicker than the specimen, then the extraneous signal becomes a more important source of error. We have used highly specialized holders and supports to minimize extraneous background while giving maximal thermal support [16, 18]. By optimizing cryosectioning to produce flat sections we have found that sandwiching the top film layer is not necessary. For our support system we use beryllium holders with Be grids covered with a carbon coated nylon support film. By using an area of support film adjacent to the section we have been able to correct for background. Since the specimen is transferred in the frozen hydrated state and freeze dried in the microscope chamber, analytical integrity is maintained without concern for questions of translocation effects when freeze dried cryosections are transferred through air.

Analytical Algorithms

There are two major analytical approaches to the analysis of cryosectioned biological material. One method uses the continuum normalization technique developed by Hall et al. [9]. The other method uses a peripheral standard and intensity ratio to determine elemental mass fractions [15]. It is important to understand that x-ray analysis measures elemental concentrations not ion concentration.

Under many circumstances those may be the same, but not always. It is important to keep in mind that elemental relationships may be unique in a given cell type and may change. This will be discussed in more detail later.

Rick et al. developed a method which uses a peripheral standard [15]. In this method the specimen is dipped into a standard (known) solution usually made of an albumin solution doped with known concentrations of salts in biologically relevant concentrations. The specimen, and its adherent albumin standard solution, is then frozen and cryosectioned so that each cryosection has a piece of standard adherent to it. The cryosection is then freeze dried and analyzed in the dried state. Characterstic x-rays from the specimen and from the standard are then collected under identical conditions and their calculated ratio obtained in order to determine the absolute concentration of that element. Water, or dry weight fraction, is determined the same way:

$$C_x = (I_x/I_{sx})C_{sx} \quad (1)$$

Where I_x is the characteristic x-ray intensity for element x after background correction and peak overlay stripping, I_{sx} is the characteristic x-ray intensities of element x in the standard(s), and C is the concentration. There are several important assumptions which are inherent in this method. Firstly, it is assumed that section and standard have the same thickness. Secondly, it is assumed that section and standard shrink uniformly. Both of these assumptions are generally true and have not proven to be a problem with this method. However, there are two other inherent assumptions which appear to have caused major analytical problems. These assumptions are that the standard is not altered by the specimen and that the specimen is not altered by the standard. Unless these two conditions can be proven to have been met, this method is likely to be unreliable and unpredictable. For a detailed description of the experimental evidence for this problem the reader is referred to Saubermann et al. [17, 22]. The Hall method can also be used with a peripheral standard [7]. While the first two assumptions, as noted above, are probably reasonable and correct, the last two assumptions are still a problem requiring separate controls and standards.

The continuum normalization method (Hall method) uses the continuum generation rate as a measure of specimen mass [9]. Characteristic x-ray intensities can be normalized to the continuum, or mass, to give a ratio which is proportionate to the mass fraction. By using appropriate standards, such as an albumin solution doped with biologically relevant elemental concentration, it is possible to compare the unknown ratio to a known mass fraction ratio to obtain absolute standardization. This method is widely used and has proven quite reliable [17, 22, 23]. The mathematics of this approach can be summarized as follows:

$$R_x = (P_x - b_x)/(W_T - W_E) \quad (2)$$

$$C_x = R_x S_x \quad (3)$$

$$C_{H2O} = (1 - W_{Dry}/W_{Hyd}) \quad (4)$$

$$C_{x(wet\ wt)} = C_x(1 - C_{H2O}) \quad (5)$$

where R is the relative mass fraction of element x, P is the integrate total x-ray counts, b is the background counts for the energy range of P, W_T is continuum counts for the total counts over the energy range used, W_E is the extraneous continuum counts, and C is the absolute mass fraction standardized against standard value S.

To apply this method we analyze the frozen hydrated cryosection in the hydrated state first, then the dried state [21]. The reason for this is that it is very difficult in the frozen hydrated state to determine morphological criteria for identification of the location at which analysis will be done. Another reason is that in the hydrated state the elemental mass fraction is less than in the dried state, consequently the peak to background ratio of an element is less favorable for accurate detection and measurement than in the dried state. Furthermore, the hydrated specimen is considerably more sensitive to radiation damage than the dried specimen. In essence we use the hydrated cryosection to determine the continuum generation rate of the hydrated section for later comparison with the dried specimen. To accomplish this requires that we measure the hydrated continuum in a blinded manner. This measurement is made by arbitrarily dividing the frozen hydrated cryosection into a pattern of squares, each having a label denoting its location and the section from which it came. The continuum is then measured over that area using a relatively low probe current (0.4 nA) and acceleration voltage (20 keV). The continuum measurement is then corrected for extraneous background by subtracting a measured continuum generation rate from adjacent support film. We use an area of continuum taken from the spectral energy range of 4.00-6.00 keV. This region is chosen because it normally has no distinguishable characteristic x-rays and the region is sufficiently wide so that there are enough counts/sec to make the sample reasonably accurate. Others have suggested using continuum at the low end of the spectrum after correcting for detector window absorption and extraneous peaks [23]. Both approaches seem to give good results and it

probably doesn't matter where the continuum sample is taken from as long as it is sufficiently large to have good counting statistics. With our methods, the final value obtained for that particular area of section is stored and labeled for later retrieval. After all the usable regions are analyzed in that manner, the section is freeze dried by slowly raising the temperature of the cold stage to -60 °C for 30 min. After the section is fully dried, the cold stage is recooled to -185 °C; areas which are of interest can usually be readily discerned for subsequent analysis. Values of elemental mass fraction in the dried state are then measured and their dry weight mass calculated. To calculate the hydrate concentration (wet weight) the water content is determined from a comparison of the dried continuum generation rate and the hydrated continuum generation rate. Important assumptions, inherent in our method, are that the original hydrated area, arbitrarily chosen, was uniformly thick and had a homogeneous average atomic mass thickness. The former assumption can be experimentally determined and proven, the later assumption is generally true provided there are little heavy elements present (i.e., calcified bone, Os, etc.).

Digital Imaging

The algorithm just described (Figs. 1 & 2) can be applied to digital x-ray imaging [4]. In this technique the electron beam used to excite characteristic and continuum x-rays is placed over the specimen in a matrix pattern. The spectra so collected are then processed and the data used to form maps or images. There are several considerations of analysis of cryosections which pertain to imaging.

Since the image resolution is influenced by the specimen sample, it is appropriate to begin this discussion with consideration of spatial resolution limits imposed by hardware and instrumentation. Beam diameter is a relative limitation to spatial resolution while ice crystal damage is an absolute limit to spatial resolution. Beam diameter, in analysis of a cryosection, defines the analytical volume in a practical sense assuming the cryosection is thin with respect to the beam. That is, that the electron beam has sufficient energy for the beam to penetrate completely. Under these circumstances, the information obtained for a particular pixel represents the location and extent (area) defined by the electron beam. Resolution under these circumstances can be defined as the ability to separate the beam edge from adjacent beam edges. Once beam diameters begin to overlap significantly, the analytical volumes overlap and pixel values begin to reflect the elemental content of adjacent pixels. In a sense, overlapping beam placements tend to average elemental mass fractions from adjacent areas. The converse to

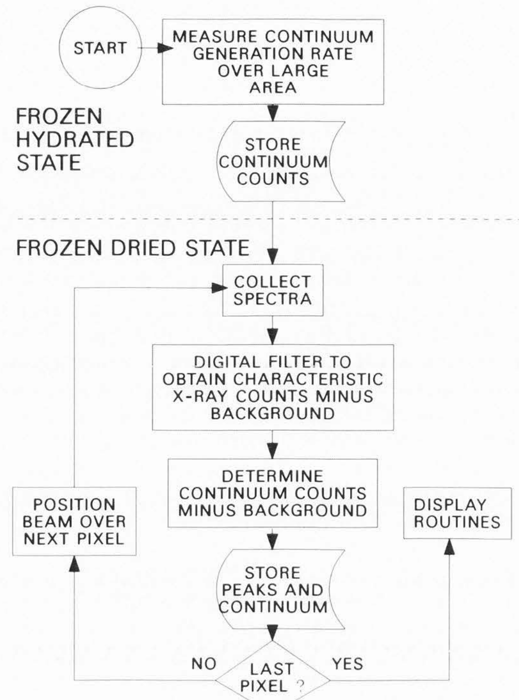


Figure 1. Imaging algorithm used to acquire data to produce quantitative elemental images.

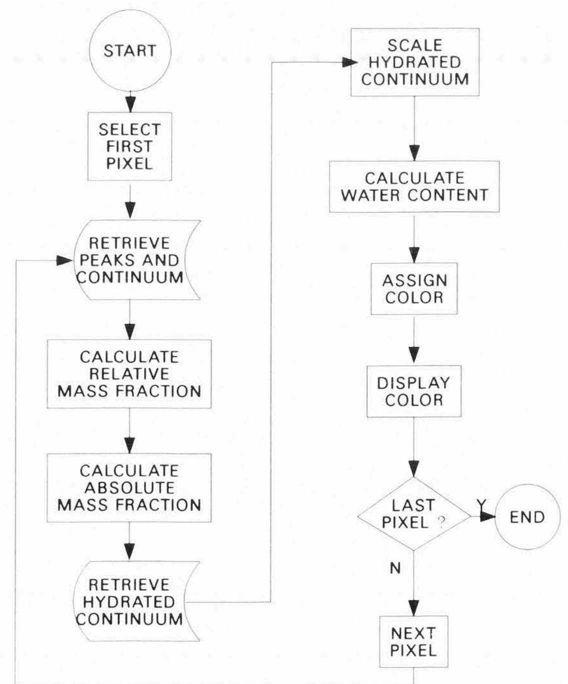


Figure 2. Display algorithm used to produce quantitative elemental images. More specific displays are applied for data retrieval.

this problem is that beam placement at intervals which are too far from each other distorts the apparent spatial location of elements since pixel values, when displayed, are usually displayed as being contiguously located. Figure 3 shows the minimal distance between beam diameter edges, which can be used as a working definition of spatial resolution. The important point is to be aware of the possibility of using too high a pixel density for a structure resulting in overlap of analytical volume.

Another serious problem is the spatial resolution which is limited by the size and structure of ice crystal damage. Since the analytical volume is defined by the beam diameter, ice crystals which exceed the beam diameter degrade spatial resolution by directly disrupting elemental position and location. This is a consideration for any type of biological microanalytical method.

Consideration must also be given to section thickness, since beam dwell time generally has to be short for practical reasons. This means that sufficient material must be present to increase the probability of characteristic x-ray generation at a rate sufficient to obtain a statistically reasonable count rate. For analysis at a static location, it is possible to use 300 s or longer dwell times to obtain a reasonable characteristic x-ray signal to background. However, such long counting times are impractical for mapping. We have found that cryosections cut at nominal thickness of 100-500 nm are generally acceptable for mapping. Such sections are technically obtainable using conventional cryo-microtomy methods.

Obviously, a major problem is to obtain sufficient x-ray generation rates to obtain acceptable counting statistics for quantitation. Direct analysis of frozen hydrated cryosections is a particular problem since this type of cryosection is sensitive to beam damage [20]. While radiation damage to biological material is dependent upon multiple factors, many poorly understood, it is clear that current density is important in causing loss of water from biological frozen hydrated material. This fact alone may preclude direct analysis of frozen hydrated cryosections, since for mapping purposes, it is difficult to obtain sufficient x-ray generation rates without relatively high current densities.

Another major limitation on probe current is dead time. As current is increased, dead time can be a problem since compensation for dead time increases beam dwell time [20]. This increases the likelihood of beam damage and makes the total time required for obtaining a map quite long. Like other biological x-ray microanalysis algorithms, there are a number of tradeoffs which must be made.

Quantitative imaging, that is where each pixel contains values which have been standardized and quantitated, requires sufficient

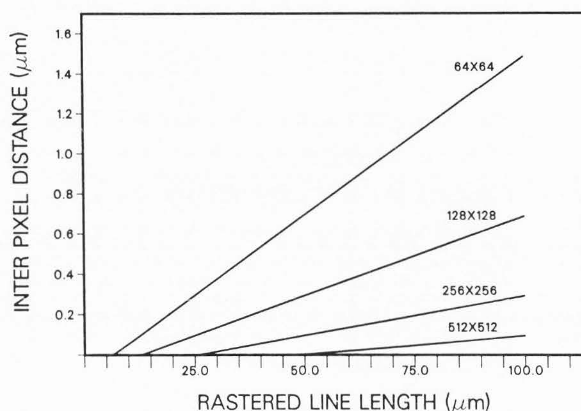
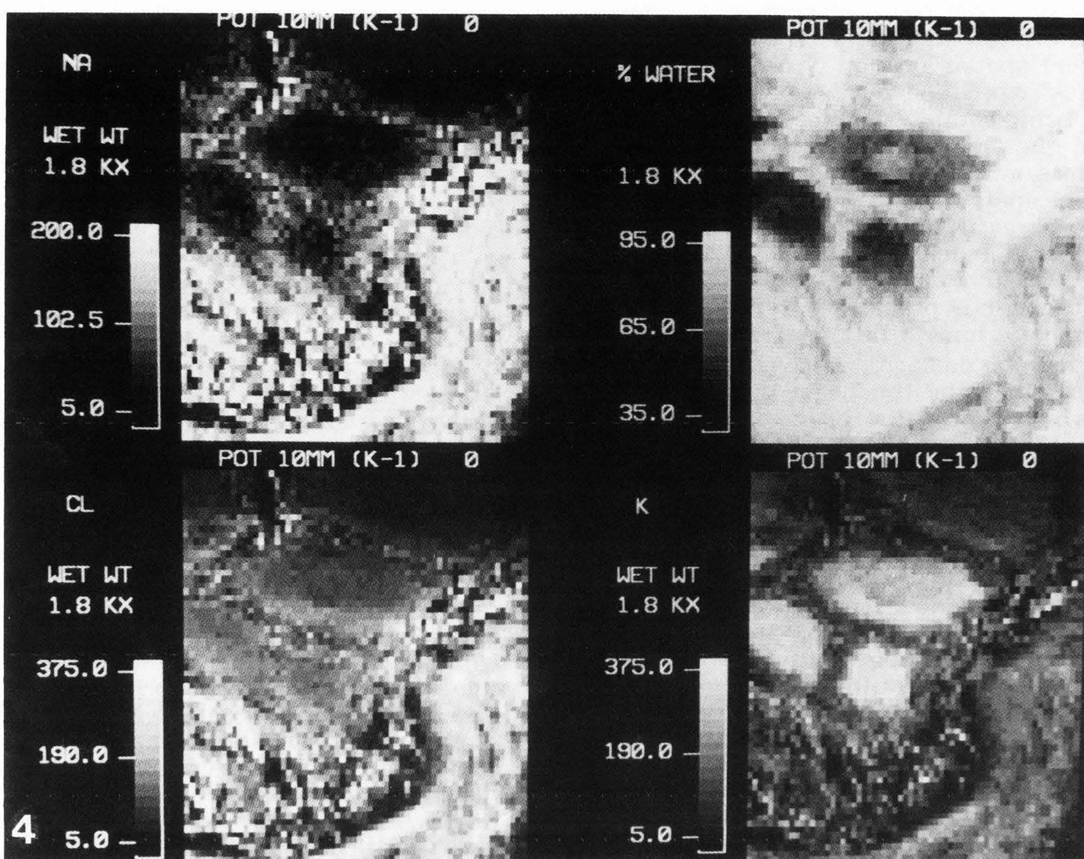


Figure 3. Pixel resolution is plotted as a function of specimen sizes (assumes beam diameter is 100 nm).

information be obtained to have a statistically acceptable sample of the analytical volume. There appears to be two major approaches to this problem having differing advantages and disadvantages. One approach is to minimize dwell time by minimizing the number of x-ray pulses collected per pixel and use statistical methods to obtain maximum information from each pixel [3, 4]. In this approach, counts are dead time corrected by scaling each count rate. Thus integer count rates corrected for dead time become highly precise real numbers. This approach clearly provides high quality images in reasonable times with good precision and accuracy. A second approach which we use increases the amount of x-ray pulses collected per pixel by increasing dwell time [20]. This approach uses a hardware correction for dead time as opposed to a mathematical approach. The advantage to our approach is that each pixel contains a relatively large sample which is statistically accurate prior to signal processing. The obvious disadvantage is the increased dwell time results in a long acquisition time where instrumental drift can be a problem. However, the major tradeoff is that each pixel is a reasonably accurate quantitative analysis equivalent in precision and accuracy to conventional operator selected analytic methods [20].

In the methods described, we use frozen hydrated cryosections to obtain a measure of the mass thickness using continuum generation rate. This measurement can be obtained using low probe currents and larger counting times with the beam rastered over an area. This has the effect of providing minimal dwell times at low current density so that there is negligible beam damage.


POT 10MM (K-1) 0
NA
WET WT
1.8 KX
200.0
102.5
5.0
POT 10MM (K-1) 0
% WATER
1.8 KX
95.0
65.0
35.0
POT 10MM (K-1) 0
CL
WET WT
1.8 KX
375.0
190.0
5.0
POT 10MM (K-1) 0
K
WET WT
1.8 KX
375.0
190.0
5.0
4

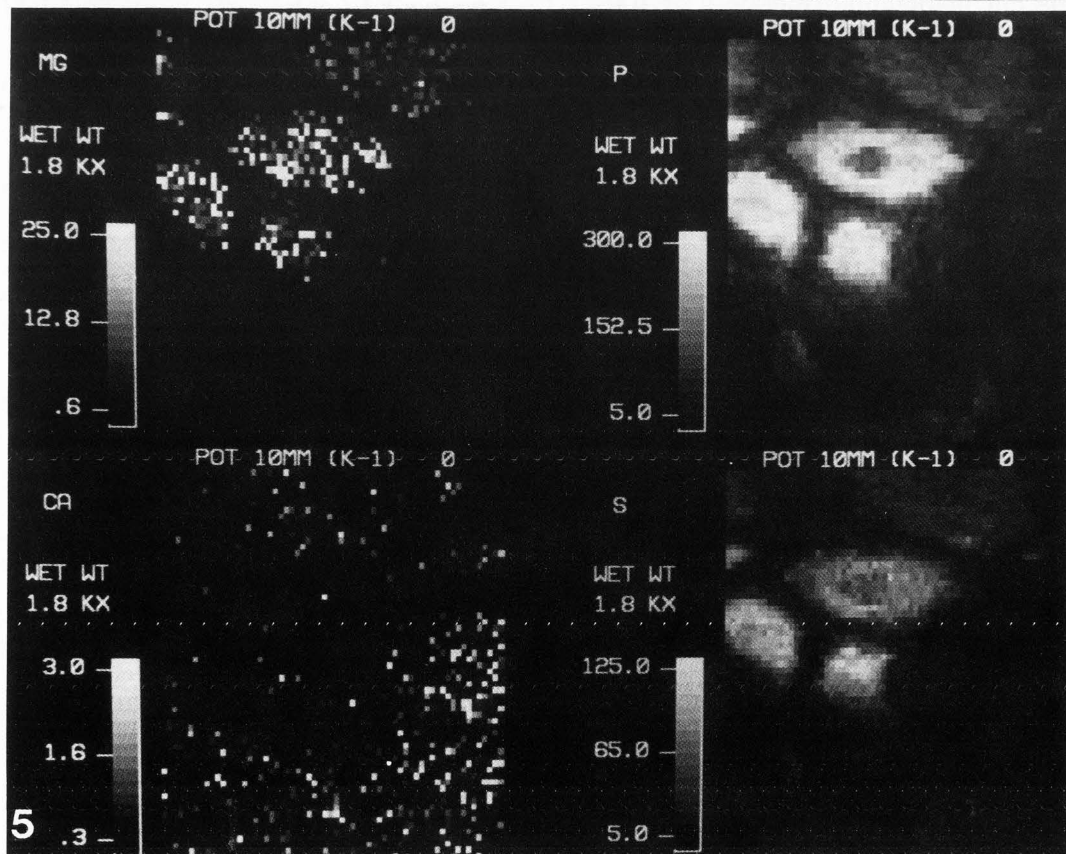
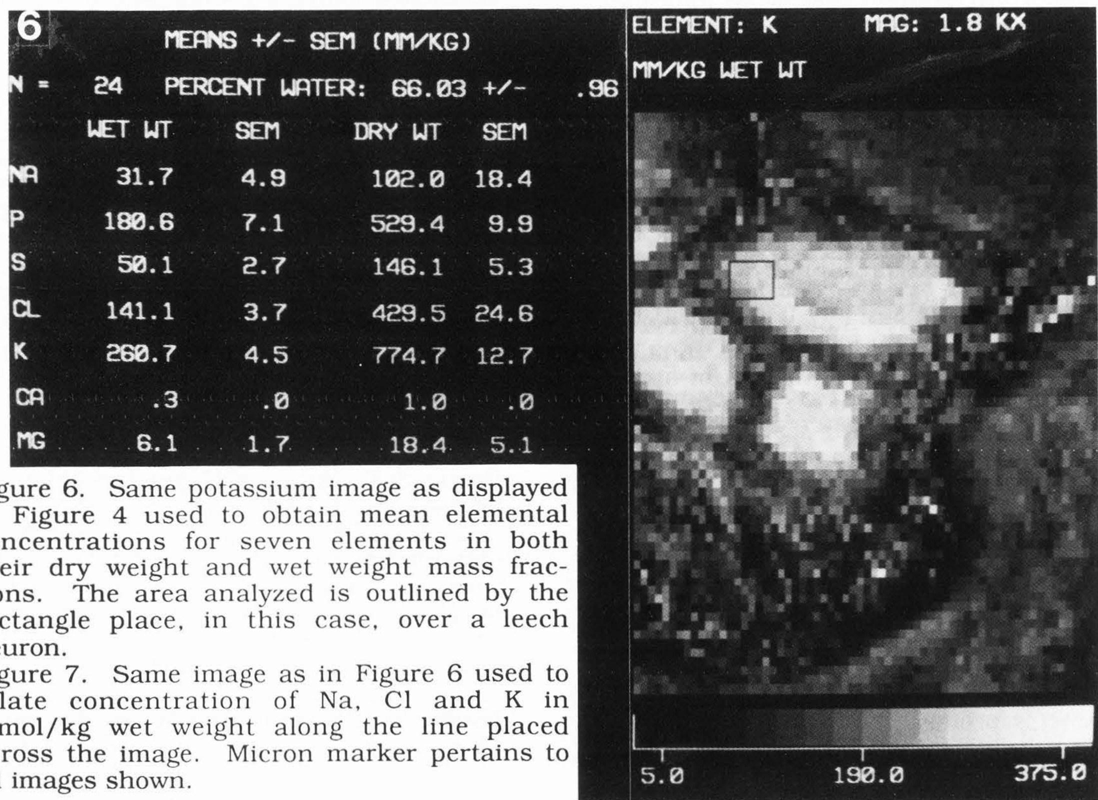

POT 10MM (K-1) 0
MG
WET WT
1.8 KX
25.0
12.8
.6
POT 10MM (K-1) 0
P
WET WT
1.8 KX
300.0
152.5
5.0
POT 10MM (K-1) 0
CA
WET WT
1.8 KX
3.0
1.6
.3
POT 10MM (K-1) 0
S
WET WT
1.8 KX
125.0
65.0
5.0
5

Figure 4. Quantitative digital x-ray images of leech ganglia. Maps (64 X 64 pixels) are displayed with 16 levels of grey for Na, Cl, K and % water. Figure 5. Same specimen as Figure 4 displaying Mg, Ca, P and S.


6

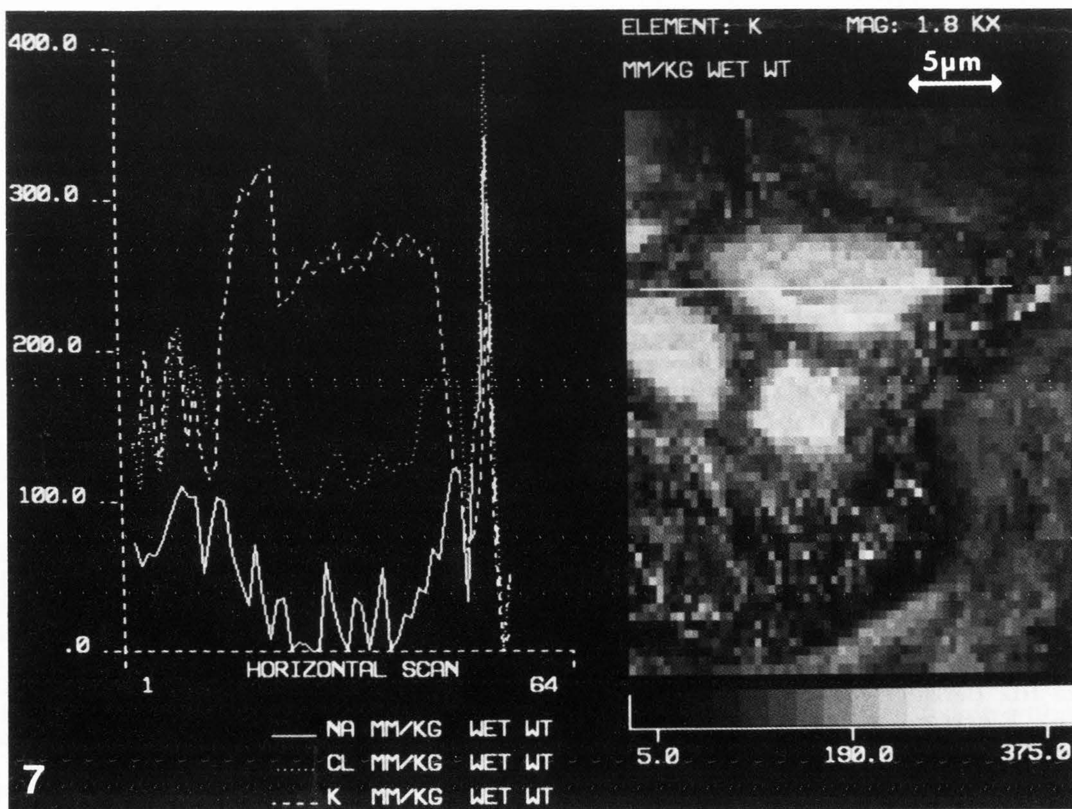
MEANS +/- SEM (MM/KG)

N = 24 PERCENT WATER: 66.03 +/- .96

	WET WT	SEM	DRY WT	SEM
NA	31.7	4.9	102.0	18.4
P	180.6	7.1	529.4	9.9
S	50.1	2.7	146.1	5.3
CL	141.1	3.7	429.5	24.6
K	260.7	4.5	774.7	12.7
CA	.3	.0	1.0	.0
MG	6.1	1.7	18.4	5.1

Figure 6. Same potassium image as displayed in Figure 4 used to obtain mean elemental concentrations for seven elements in both their dry weight and wet weight mass fractions. The area analyzed is outlined by the rectangle place, in this case, over a leech neuron.

Figure 7. Same image as in Figure 6 used to relate concentration of Na, Cl and K in mmol/kg wet weight along the line placed across the image. Micron marker pertains to all images shown.



Interpretation of Data

It is important to appreciate what information is provided through x-ray analysis of biological cryosections. X-ray analysis combines electron microscopy (morphology) with quantitative analytical information (amount) (Figures 4-7). The quantitative analysis provides a measurement of mass fraction of a particular element. The information, thus obtained, combines both where an element is located, or positional information, with how much is present. X-ray imaging provides a determination of location of element by using elemental intensity or amount to provide morphological information. One can think of these maps as being chemical images of a cell analogous to say the carbon, oxygen, nitrogen density images one sees with scanning transmission electron imaging. Like conventional microscopy, where one studies effects of various perturbations or structure presented by structural elements, one can also apply various perturbations to study elemental morphology which is more labile and less structural. To translate this into physiologically meaningful information requires knowledge of both elemental activity and factors affecting that location.

X-ray maps promise to provide a great deal of information which can be used to appreciate changes in distribution of elements in relationship to carefully defined conditions imposed on known systems.

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Discussion with Reviewers

G.M. Roomans: The method for determination of water content by using the continuum intensity only has been criticized (e.g., by Gupta and Hall, Tissue and Cell,13:623-643,1981). The author should acknowledge this criticism and present his defence. It may well be that Dr. Saubermann's method is adequate in practice, but this needs further arguments.

Author: Dr. Roomans refers to an unreviewed, higly personal and specific evaluation (written by Drs Gupta and Hall) of our papers which were published in the J. Cell Biol. (text ref 18). What Dr. Roomans may not be aware of is that the same criticisms were sent to the J. Cell Biol. in the form of a letter to the editor by Drs Gupta and Hall which was withdrawn by them for reasons not stated. Their critique of the use of the continuum intensity as a means of determining water content was based on theory. We have, in our original publications in J. Cell Biol. and in other high impact and extensively peer reviewed journals (e.g. text refs. 17, 20, and 21) shown that accurate and precise measurements of water content and wet weight elemental content can by made using the continuum intensity method. Given the proven success of this method, and the theory that says it can't work, perhaps it is time to reconsider the theory.

G.M. Roomans: In his discussion on the choice of pixel distance, the author states that the analytical volume is defined by the beam diameter. This totally neglects the effect of electron scattering in the sample, which certainly at 500 nm is not negligible!

Author: Dr. Roomans is quite correct that electron scattering would effect the analytical volume. Our imaging is actually done in the dried state so that the actual thickness of the now dried cryosection is significantly less than the original 500 nm. Consequently, scattering effects are probably less than in the hydrated state. Beam diameter used for analysis of a freeze-dried 500 nm thick cryosection is probably, however, the major determinant of analytical volume and hence the limitation to pixel size.

G.M. Roomans: The experiment on liver reflects the 'bulk' characteristics of the tissue, i.e., those of the poorly frozen inner part. How relevant are these data for the well-frozen outer layer, which is what one hopes to section and analyze?

Author: The answer to this question is not known. To rephrase the question, one might ask what effects does the structure and size of the ice crystals in the frozen biological specimen have on its material properties? This question remains to be answered by careful experimentation rather than widely held and popular assumptions.

T. von Zglinicki: There are two additional assumptions inherent in your method of water fraction estimation. The first is the assumption of constant mean atomic mass both in hydrated and dried sections. Is the bias induced due to that small enough to be neglected even in cells with high hydration (10% dry mass,say)? Secondly, it is assumed that the sections do not shrink during drying. However, a shrinkage of at least 10% (linearly) is known to occur, though the dry mass fraction measured will be biased by at least 20% in the direction of an overestimation. Did you perform any control experiments to estimate the influence of shrinkage during freeze-drying experimentally?

Author: Experimentally, we have determined that presumed differences in hydrated average atomic number (Z^2/A) and dried specimen can be neglected as being negligible even when the relative dry weight fraction is 10% or less. This may be due to a number of factors such as the relative contribution of inorganic elements, expansion during freezing, or specimen elemental composition just to name a few possibilities. Likewise effects of section shrinkage on quantitation are also negligible. For experimental evidence we refer Dr. von Zglinicki to our published papers (see for example text refs. 16 and 22). It is possible that a number of factors are cancelling out any effects of these two assumptions. However, since we have repeatedly shown that accurate and precise measurements of elemental content in wet and dry weight mass fractions can be made using our methods, it would appear illogical to continue to deny the obvious because theory says we cannot do what we have proven can be done.

K. Zierold: Well cryofixed tissue with small ice crystal damage can be cut at considerably lower temperature down to -160 °C. Specimens with large ice crystal segregation

(larger than 0.5 μm) can be cut smoothly only at elevated temperature, preferably above -80 °C, whereas they are brittle at lower temperature. Why do you claim that frozen liver becomes brittle below -60 °C?
Author: We have measured the stress/strain material properties of frozen liver at various temperatures and determined that a transition from the ductile to the brittle state occurs at around -55 °C. In addition, we have measured the actual work of cryosectioning and the effects of temperature on that work. We refer Dr. Zierold to this work (text ref 19, Saubermann AJ, Heyman RV, Mechanical properties of frozen biological tissue. Abstr. Texas Section of the Amer. Physical Soc., p. 24, 1985, and Saubermann AJ, Riley WD, Beeuwkes R, Cutting work in thick section cryomicrotomy. J Microsc. 111:39-49, 1977).

K. Zierold: You claim that cryosectioning requires elevation of the temperature until the ductile state of the specimen is achieved. How can you prove that? In my opinion cryosectioning is a cleavage process and has nothing to do with temperature input to the cleavage plane. Please comment!
Author: We have applied well known metal machining principles to the process of cryosectioning. So far there is absolutely no evidence to contradict this approach (but much "opinion") and very good experimental evidence (see above) to consider machining principles as applicable (also see Frederik PM, Busing WM, Persson A, Concerning the nature of the cryosectioning process. J. Microsc. 125:167-175, 1982). On the other hand, there is no evidence supporting Dr. Zierold's opinion that cryosectioning is a cleavage process. Furthermore, if Dr. Zierold believes that cryosectioning is a cleavage process, then clearly a microtome is an irrational device to use for cryo-cleavage.

K. Zierold: How have you overcome the problem of radiation damage during x-ray microanalysis of frozen-hydrated cryosections? Please comment on irradiation conditions (electron dose, irradiation area and time, mass loss).
Author: We use a low current density to determine the continuum generation rate in our hydrated specimen in order to minimize radiation damage. Actual elemental analysis occurs in the dried state which is more radiation resistant than the hydrated specimen. Under these conditions relatively higher current densities, than those used in the hydrated state, can be applied without quantitatively significant mass loss. For a more detailed discussion and information on irradiation conditions we refer Dr. Zierold to our previous publications (see for example text ref 20).

K. Zierold: Freeze-drying causes shrinkge of cryosections. How do you correlate the x-ray counts obtained in pixels of the frozen-hydrated state with pixels of the same area in the freeze-dried state?
Author: The continuum generation rate, measured in the frozen hydrated state, comes from relatively large areas (100 μm x 100 μm) which are marked on a micrograph of a secondary image of the frozen hydrated cryosection. After freeze drying in the microscope, those areas are readily apparent in the freeze dried state (for details please see text ref 20).