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MAPPING METAL DISTRIBUTIONS IN THIN CRYOSECTIONS WITHOUT SCANNING TRANSMISSION ELECTRON MICROSCOPE WITH THE PHILIPS ELECTRON BEAM AND IMAGE DEFLECTION (EBID) UNIT

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Abstract

The purpose of the present paper was to describe the use of a commercially-available, and relatively inexpensive, beam and image deflection unit that can facilitate digital X-ray (element) mapping in a standard transmission electron microscope not furnished with a STEM attachment. The test specimen was a thin freeze--dried section of the metal-sequestering chloragogenous tissue from the earthworm, *Lumbricus rubellus*, inhabiting a soil naturally contaminated with Pb, Zn and Cd. Qualitative maps obtained from this material confirmed the efficacy of the deflection unit, and revealed the presence of three compositionally distinct metal-accumulating compartments within the chloragocytes: (i) ovoid, electron-dense, phosphate-bearing and Ca-, Pb- and Zn-containing chloragosomes; (ii) relatively electron lucent, morphologically featureless, Cd- and S-rich "cadmosomes"; an extremely electron-dense compartment lying close to individual chloragosomes, containing high concentrations of Ca, Pb and Zn but deficient in P. Azo dye histochemistry indicated that the chloragogenous tissue did not contain either diffuse or focal acid phosphatase activity. The possible relationships of the three metal-sequestering compartments to each other and to the lysosomal system was discussed.

Key Words: Digital X-ray mapping, cryosections, metal compartmentation.

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Introduction

Essential elements impair biological function if their concentrations become either deficient or excessive (Hopkin, 1990). Many bio-essential elements exert their metabolic influences through their high intrinsic reactivity. Such bio-inorganic events must often be transient, highly regulated and specifically directed, otherwise severe cytotoxic damage will ensue. Cellular Ca^{2+} regulation illustrates these principles very well. Calcium overload can cause irreversible cell injury (Buja *et al.*, 1988). Low (50-200 nM) cytoplasmic free Ca^{2+} concentrations are maintained in healthy cells through a combination of active calcium transporters located at the plasma membrane extruding Ca^{2+} against its steep electrochemical gradient (Borle, 1988), binding to high affinity intracellular proteins (Upton and Moor, 1992), and storage by organelles (Somlyo *et al.*, 1988a). There is a large body of evidence indicating that in non-contractile cells the endoplasmic reticulum plays a major role in the physiological storage and regulation of Ca^{+}, and that many cellular functions are modulated by a cascade of biochemical reactions, initiated by cell-surface receptors, and resulting in the episodic release of Ca^{2+} into the cytosol from endoplasmic reticulum stores (Somlyo *et al.*, 1988b; Baumann *et al.*, 1991).

Some invertebrates can be described as macroconcentrators of certain non-essential toxic metals (Dallinger, 1993). As a rule the body burdens of these metals are not homogeneously distributed amongst the constituent tissues (Hopkin and Martin, 1984; Morgan and Morgan, 1990). Indeed, the metals are preferentially accumulated in a limited number of cell types in particular tissues (Morgan, 1984; Morgan and Winters, 1987; Nott, 1991, 1993). Within these metal-accumulating cells the metals tend to be confined within discrete compartments according to their ligand-seeking properties (Morgan and Winters, 1987; Morgan *et al.*, 1993), and are immobilized so that the risk of their intrusion into sensitive biochemical pathways is alleviated.

From the above resume it is apparent that the

distributions of essential and non-essential metals are often focalized at the cellular and subcellular levels of organisation. In order to better understand the contributions of specific elements to specified physiological or pathological processes techniques must be available that make it possible to measure subcellular compartments *in situ*. The electron microprobe X-ray analyser (EPXMA) yields information about subcellular chemical heterogeneity (for an historical overview: Gupta, 1991). EPXMA is normally employed in the static "spot" or "restricted raster" modes, where the probe is allowed to dwell on the subcellular compartments of interest selected on a morphological basis. Data collected from a number of individual compartments are then combined and statistically compared with data either from different compartments or similar compartments in altered physiological states. This approach, especially when applied to thin cryosections, has yielded a wealth of useful information. However, static probe EPXMA can be subject to two significant drawbacks. The first relates to sampling strategy. The selection of compartments for analysis by applying morphological criteria introduces a form of analytical bias, because only prominent structures will be included; structurally less-imposing, but possibly functionally significant, compartments could be overlooked and eliminated from consideration. Second, it fails to provide a direct visual correlation between element distribution heterogeneities and cellular structure, and also of the relative spatial distributions of two or more elements.

Digital X-ray mapping of element distributions has been introduced relatively recently to overcome some of the problems alluded to above. Despite the fact that digital mapping offers certain specific advantages over static probe X-ray microanalysis (LeFurgey *et al.*, 1992), the former approach has not been used in a large number of microprobe laboratories. Doubtless, a major reason for this is that the transmission electron microscope must normally be furnished with scanning transmission unit (STEM). In this conventional configuration, the energy-dispersive analyser is interfaced with the scanning coils via an external analog interface board, which drives and sequentially positions a fine probe within a regular frame on the specimen. Complete X-ray spectra are collected and displayed, if necessary with full quantitative spectral processing, for each pixel separately (Somlyo *et al.*, 1989). The present paper briefly describes a system that permits digital X-ray mapping without the need for a STEM unit. An ecotoxicological application of the novel mapping facility is also presented.

Materials and Methods

Mapping hardware

A Philips CM12 transmission electron microscope was interfaced with an EDAX PV 9900 X-ray analyser. However, the scan generator of the analyser was connected to the pre-specimen deflection coils of the microscope via a special drive unit, the Philips Electron Optics External Beam and Image Deflection (EBID; PW6439) unit (Fig. 1). [EBID can be interfaced with the scan generator of any X-ray analyser equipped for X-ray mapping]. The microscope was set up in the normal analytical mode (i.e., "nanoprobe" setting on the CM12). Spot size was selected. The EBID unit modifies the incoming wave and frame signals from the scan generator to change the magnification of the X-ray map. The area to be scanned was visualized on the fluorescent microscope screen by setting the mapping software to perform rapid continuous scans. Accurate alignment of the scanned area was achieved by using the microscope beam shifts. Activating the mapping software then initiated the mapping sequence: the stepwise movement of the probe could be observed, and the X-ray maps were displayed and processed in a qualitative manner.

Biological sample: source and preparation

Clitellate earthworms (*Lumbricus rubellus*) were collected from the contaminated metalliferous soil in the vicinity of Roman Mine, Draethen, South Wales (Ordnance Survey Map Reference: ST 214876). Previous atomic absorption spectrophotometric analyses (Morgan and Morris, 1982) have shown that this calcareous soil is heavily contaminated with Pb, Zn and Cd; these metals are accumulated in the posterior alimentary fraction of the earthworms, which includes the intestine and multifunctional chloragogenous tissue that overlies it (Morgan *et al.*, 1993). Qualitative X-ray fluorescence confirmed the metalliferous nature of the soil at the sampling site (Fig. 2).

Small pieces (~ 1 mm) of fresh posterior alimentary fraction were mounted on Ag or Al pins and plunged into liquid propane. Ultrathin sections of the frozen specimens were cut on glass knives at -125 C in a Reichert Ultracut E with FC4 cryo attachment. The frozen-hydrated sections, mounted on Ti grids, were transferred to a vacuum evaporation unit in a lidded, liquid N -cooled, container where they were freeze-dried overnight and then carbon coated. Dried sections were stored over silica gel.

Mapping conditions

The Philips CM12 was operated at 80 kV with a tungsten filament, using a 50 m "top-hat" C2 aperture,

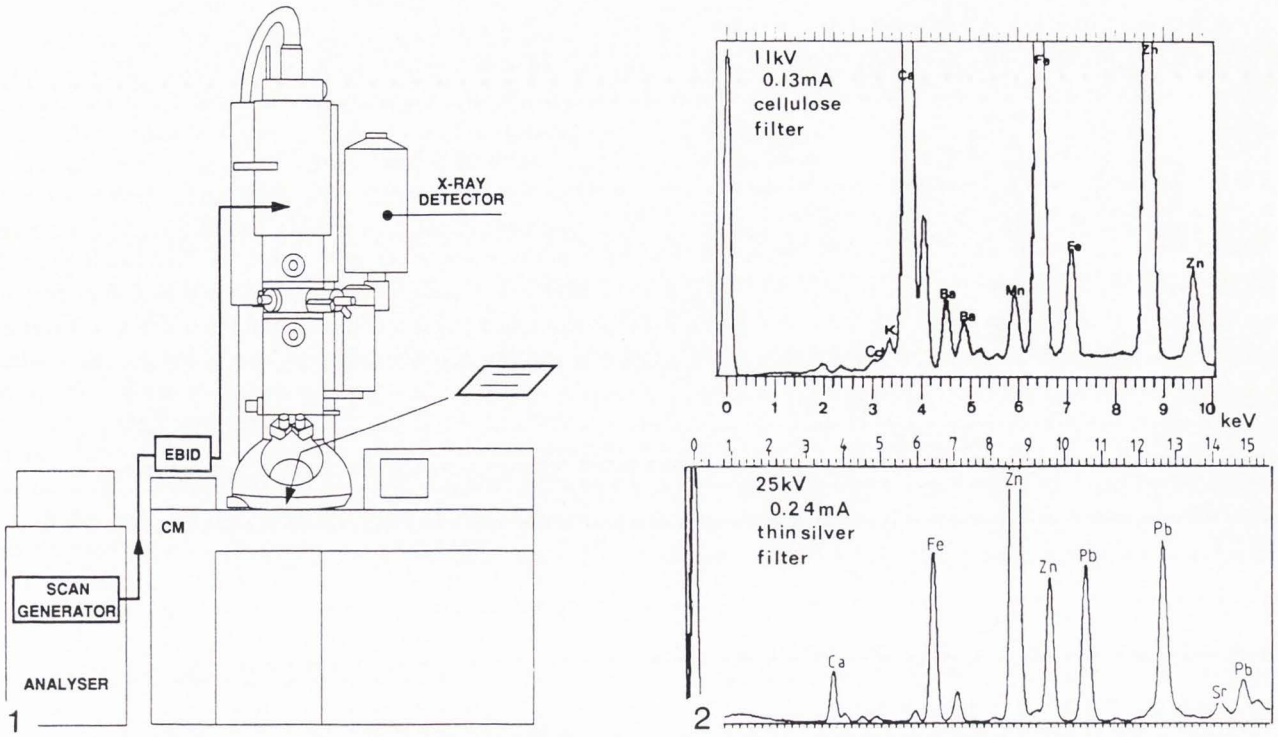

Figure 1: Schematic diagram of the EBID interface between the energy-dispersive X-ray (EDX) analyser and the deflection coils of the transmission electron microscope.

Figure 2: X-ray fluorescence spectra of a ground sample of Draethen soil to confirm that it is contaminated with Cd, Pb and Zn. Top = 0 - 10 keV; Bottom = 0 - 15 keV.

and with a probe of 12 nm diameter. Screen resolution was 256 200 pixels; dwell time per pixel = 1 s, yielding a total map acquisition time of 14 hours 14 mins. Digital intensity (peak-background) maps were plotted and superimposed in selected element pairs. Typical maximal counts in individual spectra/pixels were: P (53), S (85), Cd L (34), K (27), Ca (60), Zn (39), PB L (27). The contribution of the Pb M line to the convoluted S/Pb M signal was estimated from the measured Pb L intensity after establishing that the Pb M: Pb L ratio in a thin aminoplastic standard (Morgan and Winters, 1988; Morgan *et al.*, 1989) under the given analytical conditions was 1.45. No attempt was made to convert the X-ray intensities into element-concentration distribution maps.

Acid phosphatase histochemistry

Acid phosphatase was localized in the posterior alimentary fraction of *L. rubellus* from Draethen using the Burstone (1962) Azo Dye simultaneous coupling method. Fresh tissue was fixed in a 1:9 (v/v) mixture of acetone and 10% neutral buffered formalin, dehydrated in acetone, and embedded in methacrylate resin (Lewis and Bowen, 1985). Sections (2 m thick) were incubated for 2.5 hours in a medium consisting of the substrate, naphthol-AS-phosphate, and the diazonium capturing agent, Red-Violet LB, in 0.2 M acetate buffer, pH 5.2. After counterstaining in haematoxylin and eosin, the sections were mounted in Gurr's Xam medium and examined in an Olympus BH-2 photomicroscope.

Results

A selected area in the cryosectioned chloragogenous tissue of *L. rubellus* (Fig. 3) yielded the X-ray distribution maps shown in Fig. 4. Cd appeared to be confined to a S-containing chloragocytic compartment in which Ca, Zn, Pb and P were non-detectable. No appreciable specimen drift or shrinkage was observed during the map acquisition period.

Apart from identifying the previously well-characterized Ca-, Pb- and Zn-accumulating, phosphate--bearing, chloragosome granules, the X-ray maps revealed a third metal sequestering compartment that is exceptionally rich in Ca, Pb and Zn but deficient in P (Fig. 4). This compartment was usually tightly opposed

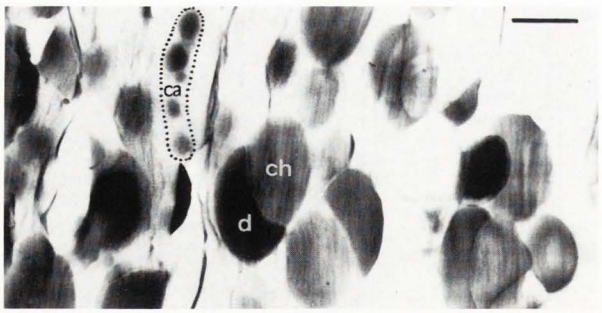

Figure 3: Thin freeze-dried cryosection of the chloragogenous tissue of an earthworm, *L. rubellus*, sampled from the metalliferous Draethen site. Note the presence of the relatively electron dense chloragosome (ch) granules with the very dense, crescent-shaped, bodies (d) tightly opposed to them in some cells. The "cadmosomes" (ca) were not apparent until after X-ray mapping had been completed. [Note that the correspondence between the photographed region of the section and the mapped field, Fig. 5, is not perfect: the maps represent a larger area of the section than the micrograph].

to individual chloragosome granules, and may be structurally continuous with and functionally derived from them.

Azo dye histochemistry for acid phosphatase indicated that this lysosomal hydrolase was not detectable either as diffuse or focal reaction products in earthworm chloragocytes (Fig. 5); acid phosphatase activity was intense in localized regions of the adjacent intestinal epithelium (Fig. 5).

Discussion

The rationale and practicalities of the technique of digital X-ray (element) mapping have been described by several authors (Fiori *et al.*, 1988; Somlyo *et al.*, 1989; LeFurgey *et al.*, 1992; Wood, 1993). The potential advantages of the technique are summarized in Table 1, and a selected and deliberately diverse list of biological applications is presented in Table 2 to provide a biological context for the technically unconventional mapping procedure described in the present paper.

Before discussing the primary observations made during our study, it is germane to comment on some of the claims made in Table 1, notably those relating to sampling and productivity. Whilst X-ray mapping certainly provides unbiased sampling across a selected area of the specimen, this demands a judgement of the image where it is possible to misidentify a structure or compartment (Roomans, personal communication). But many cellular compartments should not, indeed cannot, be identified solely by morphological criteria. Elements of the endocytotic pathway and Golgi often cannot be defined with certainty without fine-structure immunolocalization of specific marker molecules (Griffiths *et al.*, 1993); likewise, Ca^{2+}-storage compartments in non-contractile cells (Burgoyne and Cheek, 1991). By analogy, metal-sequestering subcellular compartments can be partially characterized, or at least located, by their elemental compositions, even though they may not otherwise possess distinguishing morphological features as revealed in cryosections. The chloragocytic "cadmosome" is an instructive example (see below). We do not presently know the origin or relationships of this compartment, but it is indisputably true that even finding such a structurally bland feature by static probe analyses of cryosections would be improbable. The problem of characterization is compounded because the compartment cannot be located in conventionally-fixed thin sections due to Cd leaching. Clearly there is considerable scope in the field of metal compartmentation studies to develop correlative electron microscopic strategies, permitting immunogold "mapping" of key antigenic markers and elemental mapping in adjacent sections. Freeze-substituted thin sections may offer the best prospects of achieving such unifying approaches.

Roomans (personal communication) has raised two further important comments relating to Table 1: (i) if the question or hypothesis under investigation concerns a known and easily identifiable compartment, then the productivity of X-ray microanalysis may be higher with static probes rather than with digital mapping; (ii) the protracted map acquisition times often employed to derive detailed quantitative information across a region of a single cell could militate the statistically desirable objective of analysing more cells, in more sections, from more individual organisms. Both are valid points. However, our study shows that functionally significant data can be obtained from qualitative maps. Such maps can be produced under favourable circumstances within a few minutes (Morgan *et al.*, 1994). "Speed-mapping" can be useful, particularly for locating structurally intransigent metal-sequestering compartments that can then be quantitatively probed with static beams. The purpose of the present paper was, however, to demonstrate that meaningful qualitative maps (and, potentially, given appropriate processing software, quantitative maps) can be generated without STEM in a transmission electron microscope furnished with a relatively inexpensive beam and image deflector.

Several features of the subcellular distributions of essential (Ca, Zn) and non-essential (Cd, Pb) metals, as revealed in element maps derived from cryosectioned

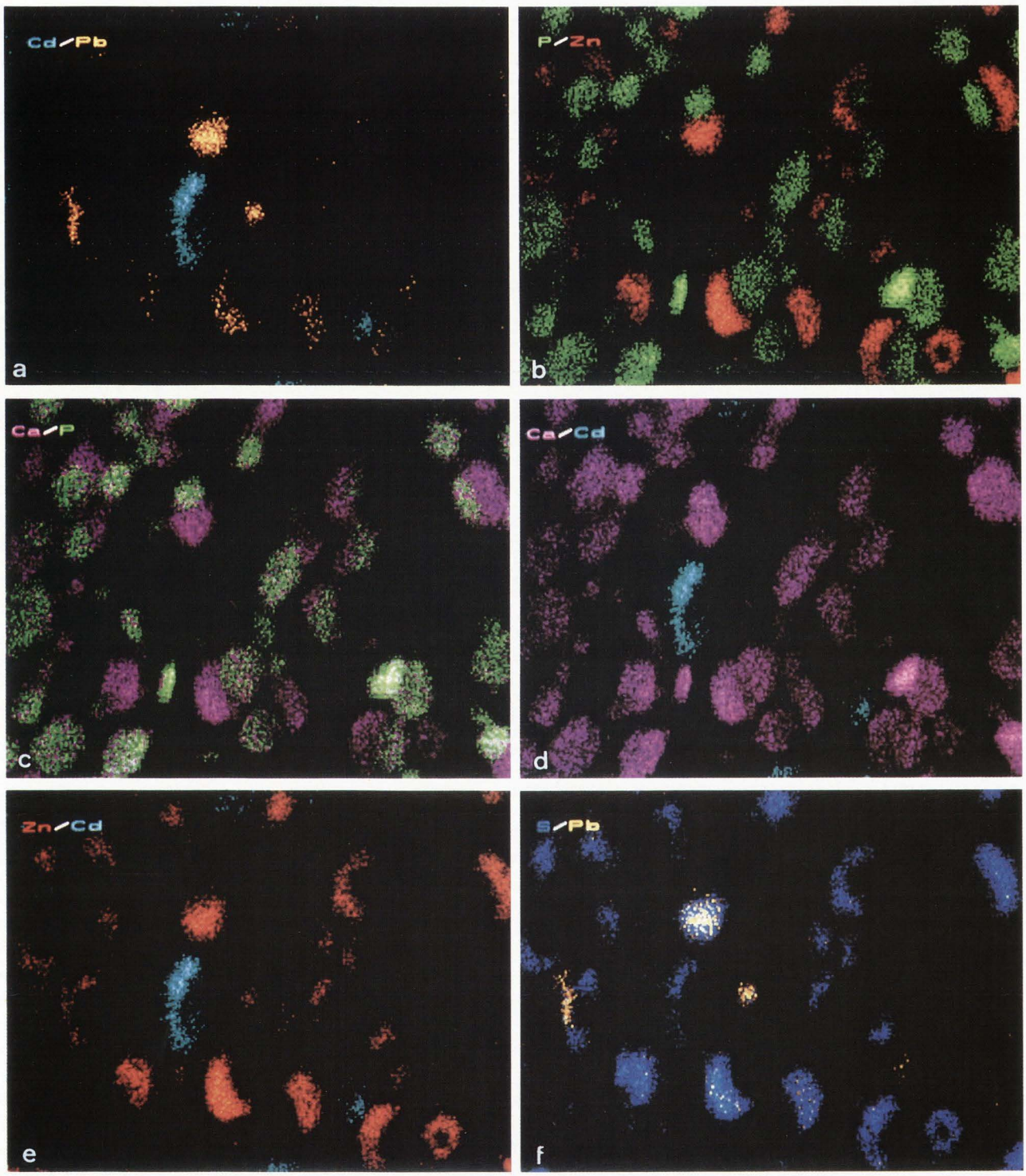

Figure 4: Superimposed pairs of digital X-ray maps of an area slightly larger than that depicted in Fig. 3 (a) Cd/Pb: Cd, light blue; Pb, yellow. (b) P/Zn; P, green; Zn, orange. (c) Ca/P; Ca, magenta; P, green. (d) Ca/Cd; Ca, magenta; Cd, light blue. (e) Zn/Cd; Zn, orange; Cd, light blue. (f) S/Pb; S, dark blue; Pb, yellow.

Table 1: Key attributes of the technique of digital X-ray (element) mapping in biology*

Attribute	*Comments*
1. Unbiased sampling	Does not require operator judgement on where to place the (static) probe in unstained cryopreparations where the morphology may or may not be good, and where the beam-sensitive specimen cannot be exposed to a detailed pre-analysis examination.
2. Identification of element compartments	- Element concentrations: morphological features relationships determined and interpreted.
	- Provides a good impression of element-elements co-distributions in two-dimensional morphological space.
	- It can register elements at unpredicted, perhaps morphologically indistinct, sites.
3. Quantitative considerations	- X-ray count statistics good for high "local" element concentrations (e.g. 12 nmol/kg dry wt. Ca), but poor for low concentrations.
	- Overall productivity is high, even though the acquisition time for quantitative maps may be high (typically 10 - 30 hours). However the plethora of data extant in a single map is derived from multiple organelles often in part of a single cell. [An appreciation of variability at higher levels or organization is time-demanding].
	- Spatial resolution is restricted in thin specimens by probe diameter or pixel size.
	- Element maps can be morphometrically analysed, and element correlations statistically analysed on a pixel-to-pixel basis.

* Information compiled from: Saubermann and Heyman (1987); Le Furgey *et al.* (1992); de Bruijn *et al.* (1993).

earthworm chloragocytes, deserve comment.

Biochemical characterisation of extracted proteins has shown that the major proportion of the Cd burden of the posterior alimentary fractions of two earthworm species (*L. rubellus* and *Dendrodrilus rubidus*) inhabiting the Cd-contaminated Draethen soil is bound by low molecular weight, cysteine-rich proteins (Morgan *et al.*, 1989). Static probe X-ray microanalyses (Morgan and Morris, 1982; Morgan *et al.*, 1989, 1993) has identified a S-rich, Cd-accumulating organelle in the chloragocytes of these worms. The present X-ray maps confirmed the existence of these so-called "cadmosomes". Until suitable antibodies become available that will facilitate a definitive immunocytochemical localization it will remain a matter of conjecture whether these cadmosomes contain the Cd-binding protein isolated from the whole tissue. The fact that there is a mis-match between the biochemically determined Cd:S ratios in the protein and the rather preliminary Cd:S ratios determined by EPXMA in the cadmosomes (Morgan, Morgan and Winters, 1989) does not preclude this possibility. It is very likely, for example, that the contents of the cadmosome consist of a mixture of the "primary" Cd-binding metalloprotein and degradative products, conceivably including the released but compartment-confined metal (see: George, 1983a, b).

The Cd-binding proteins isolated by Morgan *et al.* (1989) from *L. rubellus* exposed to Cd in its natural environment, and the proteins isolated by others (Suzuki *et al.*, 1980; Yamamura *et al.*, 1981) from a related earthworm species exposed to Cd in the laboratory, can be considered to be metallothioneins (Roesijadi and Fowler, 1992). Metallothionein gene expression is generally considered to be induced by Zn, and it has been considered that the protein donates the metal to zinc metalloenzymes, DNA-binding transcription factors and for membrane stabilization (Schroeder and Cousins, 1992). Despite the fact that the affinity of Cd for binding sites on metallothionein exceeds that of Zn (Vasak, 1992), the unequivocal absence of Zn accumulations within the cadmosome (Fig. 5) is surprising. In the earthworm chloragocyte the "borderline" protein-binding status of Zn (Christianson, 1991) is "weighted" towards a distinct preference to accumulate in the oxygen-dona-

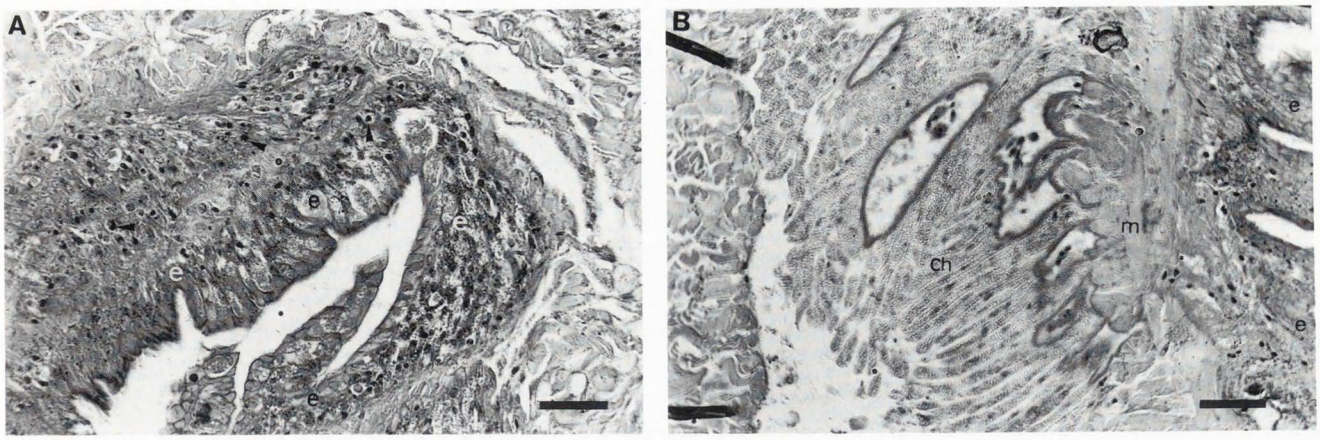

Figure 5: Light micrographs of the posterior alimentary tissues of *L. rubellus* from Draethen exposed to the Burstone Azo Dye histochemical test for acid phosphatase activity. A = Part of the intestinal epithelium (e). Note the dense regions indicative of focal acid hydrolase activity (arrows), found nearer the basal rather than the apical region of the absorptive tissue. [The hydrolase-positive regions are stained an intense scarlet in the actual sections]. B = A region of chloragog tissue (ch) separated from the intestinal epithelium (e) by a narrow muscle layer (m). The chloragog tissue did not show any acid phosphatase activity. Bar = 10 μm.

ting, phosphate-rich chloragosomal compartment. One can only speculate about the possible functional significance of the Zn versus Cd discrimination in this cell type with prodigious metal sequestering capacity. A possible consequence of the discrimination is that the essential metal (Zn) can continue to participate in various cellular activities, whilst the non-essential toxic metal (Cd), with which it shares binding tendencies in many biological systems, is entrapped in a discrete organelle. If there is any basis to this speculation, then at least two important questions arise. First, is Zn capable of inducing the metallothionein gene in earthworm chloragocytes? Second, how is the Cd-metallothionein complex specifically incorporated into the cadmosome? Unlike vertebrate cells, Cd has been found to accumulate with S within membrane-limited vesicles in a number of invertebrate groups (George and Pirie, 1979; Lauverjat *et al.*, 1989; Klerks and Bartholomew, 1991) (Fig. 4). It is premature to suggest that this represents a fundamental phylogenetic difference, but it does suggest that these Cd-sequestering compartments, including the earthworm cadmosome, possess a real detoxification function.

It is possible that the metal-enriched dense structures we observed lying contiguous with some chloragosome granules correspond to the rather empty-looking "debris vesicles" described by Ireland and Richards (1977) in conventionally fixed sections of chloragogenous tissue from earthworms inhabiting a Pb-contaminated soil. By implication these debris vesicles lost a large proportion of their metallic contents during aqueous processing for electron microscopy, and they may be homologous with "residual bodies" and "tertiary lysosomes". Although one isolated report (Varute and More, 1972) suggested that the chloragosome granules themselves have lysosomal characteristics, there is no supportive evidence for this. However, it is possible to hypothesize that the metal-loaded granules "converge" with the chloragocytic lysosomal system, and that the combination of low intralysosomal pH and the action of hydrolytic, notably phosphatase, enzymes autolyses the chloragosome matrix. Phosphate could then be re-cycled to participate in cellular metabolism and/or the formation of new chloragosomes. In this way the debris vesicle/residual body/tertiary lysosome would become a P-poor, metal enriched organelle. This hypothesis has been tested in a preliminary manner by attempting to detect acid phosphatase in the chloragogenous tissue by azo dye histochemistry. Our findings (Fig. 5) do not support the hypothesis. Acid phosphatase activity was detected in localized parts of the intestinal epithelium, thus acting as an "internal" positive control. These histochemical observations on metal-exposed chloragogenous tissues were consistent with the biochemical assays of acid phosphatase activity and distribution in "control" *L. terrestris* (Prentø, 1986, 1987). We are currently engaged in studies involving the localization of different hydrolytic enzymes at the electron microscope level in *L. rubellus* from Draethen, together with subcellular pH measurements in the same tissue, to obtain a better

Table 2: Some selected biological applications of digital X-ray mapping

Applications: Major Findings	*Technical Remarks*	*Author(s)*
Vertebrates		
1. Ca, P, S, Cl in the atrial-specific granules of rat heart. High Ca (75 mmol/kg dry weight) and Cl and low P in the granules; no Ca or Cl compartimentalization in nucleus or mitochondria. [Incubation in NaBr showed that atrial granules can accumulate Br].	Thin freeze-dried cryosections of atrial muscle. Quantitative X-ray maps, dwell-time per pixel= 5 sec; 10 nm diameter; 10 nA probe from field-emission gun; specimen drift compensation.	Somlyo *et al.*, 1988b, 1989.
2. Na, Mg, P, K, Cl, Ca and mass thickness in skeletal muscle. Ca is highly localized in the junctional sarcoplasmic reticulum (JSR); P is localized not only in the JSR of the I band, but also in the M rete of the free SR.	Thin freeze-dried cryosection. Quantitative X-ray maps. Typical conditions: X-ray images at 64x64 and 128x128 pixel resolution at mags. of x15,000 - x30,000 (pixel side = about 0.065-0.13 μm); 10 nA current and about 20 nm probe diameter; dwell time: 1 to several seconds per pixel. [128x128 at 4 sec/pixel map requires about 26 hours mapping time]. Maps obtained at ambient temperature.	LeFurgey *et al.*, 1992
3. Na, Mg, P, S, K, Cl, and Ca in kidney proximal tubules and glomerular mesangial cells.	(Idem)	LeFurgey *et al.*, 1992
4. P, Cl and Ca in human nasal epithelium. Selective localization of Ca in the mitochondria of a single cell in a small population of mapped cells.	(Idem)	LeFurgey *et al.*, 1992
Prokaryotes and Invertebrates		
1. Mg, Ca, P, in *Escherichia coli* B cells. Ca uniformly distributed around periphery of cells; Mg and P distributed equally and uniformly throughout cell.	Cryosections (thin, freeze-dried) of water-washed and unwashed centrifuged pellets. Slow-scan quantitative X-ray maps. [see Somlyo *et al.*, 1988b, 1989 for details].	Chang *et al.*, 1986

Applications: Major Findings	*Technical Remarks*	*Author(s)*
2. Na, Cl, K, P, H_2O in leech (*Macrobdella decora*) ganglia.	0.5 μm thick cryosections of fresh tissues frozen-hydrated then freeze-dried under microscope vacuum. Quantitative maps: 64x64, 128x128, or 256x256 pixels resolution; 1.4 nA probe current; dwell time= 4 sec/pixel.	Saubermann and Heyman, 1987
3. Fe, Cu, Ca, Cd, Pb, Zn, P, S, in the B and S cells of terrestrial isopod hepatopancreas (*Oniscus asellus, Porcellio scaber*) and in the digestive cells of the spider *Dysdera crocata*. Cd and Cu bound permanently in S cells; Fe excreted by breakdown of B cells; excretion of Pb and Zn depends on relative distribution between B and S cells.	Glutaraldehyde (2.5%, 0.1 M cacodylate buffer) fixed, Spurr-resin-embedded, unstained sections. Qualitative maps: 256x200 or 512x400 pixel screen resolution; spot diameter 50nm; dwell time=50 or 100 msec/pixel.	Hopkin *et al.*, 1989
4. Ca, P, Zn, Fe in type A calcium phosphate granules in digestive cells of *D. crocata*. Zn and Pb, but not Fe are accumulated by these granules.	Idem, except: 256x200 pixels screen resolution; dwell time=50 ms/pixel; probe diameter=20nm.	Hopkin, 1990
5. Ca, P, S and mass (continuum) in photoreceptors of honey bee (*Apis mellifera*) drones. The Ca concentration in the endoplasmic reticulum of dark-adapted photoreceptors was high ($\approx$48 mmol/kg dry weight). [Spot analyses showed that light stimulation caused a release of Ca from, and an entry of Mg into, the endoplasmic reticulum].	Freeze-dried thin cryosections. Quantitative maps; dwell time=15 sec/pixel, using field emission source. Specimen drift compensation applied.	Baumann *et al.*, 1991
6. S, K, Sr, Ca in the mucocytes of oral or free gastroderms in the coral, *Galaxea fascicularis*. Mucous granules contained high concentrations of S, K, Ca and Sr; intergranule compositional variability was noted.	Thin, freeze-substituted (ether/acrolein) sections of undecalcified polyps embedded in Spurr's resin. Semi-Quantitative (peak-to-continuum, background corrected) maps.	Marshall and Wright, 1991

Applications: Major Findings	*Technical Remarks*	*Author(s)*
7. Mg, P, S, K, Ca in the nematocytes of the cnidaria, *Hydra vulgaris* and *Actinia equina*. In *A. equina* two types of cyst were identified: one with high Mg and S concentrations, the other with high Ca concentrations.	Freeze-dried thin cryosections. Qualitative (X-ray intensity mapping: 128x128 pixels screen resolution; dwell time=10 ms recording time of about 3 mins per frame; 5-10 frames superimposed to produce a map).	Zierold *et al.*, 1991
8. Zn and Cl in the mandible of the locust, *Schistocerca gregaria*. Zn and Cl co-distributed in the outer shell of left, and inner shell of right, mandibles; also in the toothed incisor edges. These elements harden these foodcutting cuticular surfaces.	Whole air-dried mandibles cast in epoxy resin, ground and polished. Qualitative maps: 128x128 pixel screen resolution; dwell time=1 s/pixel.	Smith *et al.*, 1992
9. P, Ca, Cd, Pb, Zn in earthworm, *Lumbricus rubellus* chloragocytes. The heterogenous chloragosome granule population are P-rich, and accumulate Ca, Zn, Pb. Cadmium and S were co-distributed in a separate compartment in the same cells.	Thin, freeze-dried cryosections. Quantitative maps: 128x128 pixels screen resolution; dwell time=3 s/pixel (total aquisition time=17 hours); drift correction.	Morgan *et al.*, 1992 (see also: Morgan *et al.*, 1993)

understanding of the relationship between chloragosomes and debris vesicles that the X-ray mapping appears to pinpoint.

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Discussion with Reviewers

K. Zierold: The acquisition time of more than 14 hours

seems to me to be too long for collecting qualitative maps as shown in Fig. 4. Were these maps further evaluated to get quantitative data? Have you observed specimen drift or shrinkage during X-ray mapping.
Authors: Certainly, the total acquisition time was well in excess of that needed to produce qualitative maps. We collected our data under conditions that were probably acceptable for quantitative mapping, but were unable to display such maps because the analytical system to which we had access was not furnished with the appropriate software. Whilst this was regrettable, it does not detract from the fact that the study met its primary objective of showing that digital maps can be obtained with a deflector unit on a microscope without STEM. The long acquisition time did serve a very practical purpose, however, because it showed that the hardware system employed did not cause any significant specimen drift or shrinkage.

K. Zierold: How do the present data compare with the element distribution in similar cells of this earthworm from uncontaminated soil?
Authors: Our observations using static probe and mapping show that the chloragosome granule containing high concentrations of P, Ca and Zn is present in "control" earthworms, but that the "cadmosome" is absent. It is not surprising that the latter does not occur because if the matrix of the granule consists of metallothionein, or a product of metallothionein degradation, then it is worth noting that this metalloprotein is not constitutive but is induced by Cd and other S-seeking metals. We have not detected the third metal-rich compartment ("residual bodies"/"debris vesicles") in control tissue. If this is merely a chloragosome-derived compartment then it may be present in "controls", albeit at low frequency if the turnover of the chloragosomes is low when metal burdens are physiological. On the other hand, the debris-vesicles may only be formed when the metal-sequestering capacity of chloragosomes is overloaded.