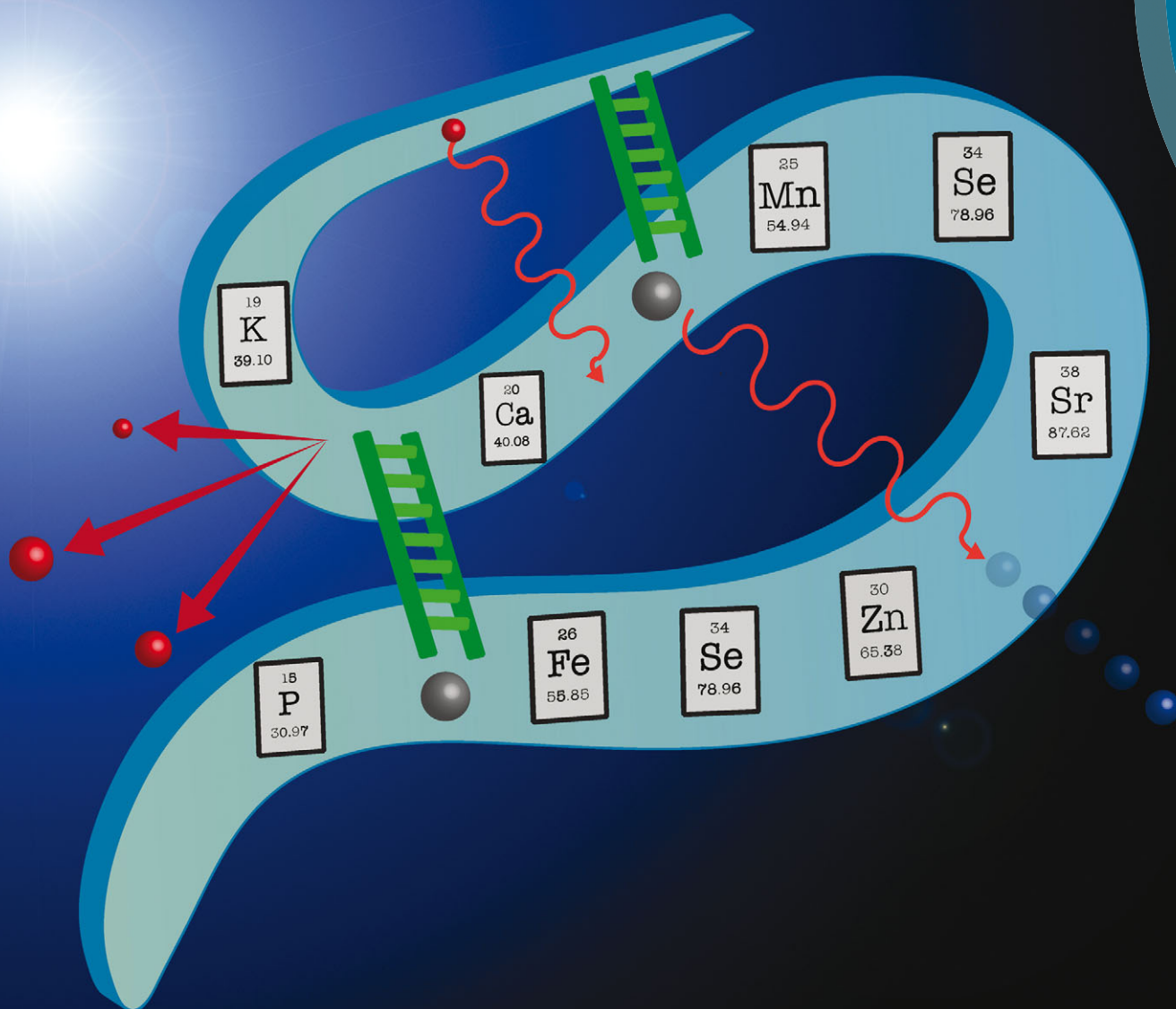


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## COMMUNICATION

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## High-resolution complementary chemical imaging of bio-elements in *Caenorhabditis elegans*<sup>†</sup>

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**Here, we present a sub- $\mu\text{m}$  multimodal approach to image essential elements in *Caenorhabditis elegans*. A combination of chemical imaging technologies reveals total metal concentration, chemical state and the protein to which an element is associated. This application of distinct yet complementary chemical imaging techniques provided unique insight into essential and trace elements at the subcellular level.**

*Caenorhabditis elegans* is a model system that displays highly compartmentalised elemental distribution ranging from high abundance species to ultra-trace elements. When imaging these elements, abundance within the sample does not necessarily equate to high sensitivity or the capacity for high spatial resolution.<sup>1</sup> Application of synchrotron-based X-ray fluorescence microscopy (XFM) is no exception; a number of factors determine whether an analyte can be detected and spatially mapped at subcellular resolution. These factors relate to both technical limitations and the nature of the sample itself. Considerations for imaging elements in *C. elegans* include: the energy of the incident beam, duration of exposure, the atomic mass of the element, the energy of the fluorescence, the characteristics of the detector used, the composition and thickness of the sample, and the environmental conditions in which the analysis takes place.<sup>2</sup>

Fluorescence profiles of specific elements must be discerned from elastic (Rayleigh) and inelastic (Compton) scattering;<sup>3</sup> *i.e.* element-specific fluorescence must not be obscured by scatter peak tails. The incident X-ray energy determines which excitation events can occur, but also positions the scattering

peaks within the X-ray spectrum. Lower mass (*Z*) elements have reduced cross-sections, fluorescent yields and are easily absorbed by the sample matrix, all limiting sensitivity. While self-absorption may be negligible for heavier elements in thinner specimens, it places real limitations on low-*Z* elements, even within single cells.<sup>4,5</sup> In addition, for specimens measured in air, argon (Ar) fluorescence causes a major interfering peak in the collected spectra (with associated tail) that can overwhelm the signal derived from lighter bio-elements, making their detection impossible.<sup>6</sup> Previously, within whole animals biologically important mid-*Z* elements, such as calcium (Ca), zinc (Zn) and redox-active metals have often been analysed without corresponding data on low-*Z* elements (*e.g.* phosphorus (P) and sulfur (S)).

*C. elegans* have been a successful test bed for pushing the boundaries of microscopy,<sup>7,8</sup> and are particularly well suited for whole-organism imaging of fundamental biochemistry. Examples span from Raman vibrational spectroscopy for imaging lipid metabolism<sup>9</sup> to scanning electron microscopy for profiling the *C. elegans* connectome.<sup>10,11</sup> We have used this nematode to extensively study metal metabolism *via* XFM, conducting population studies,<sup>12</sup> tomography<sup>13</sup> and X-ray absorption near-edge structure (XANES) spectroscopy<sup>14</sup> to appraise the complex biochemistry of metals *in vivo*. *C. elegans* are highly resistant to ionising radiation,<sup>15</sup> which permits analysis of hydrated and anaesthetised samples for mid-*Z* elements using hard X-rays (> 10 keV).

While hydrated imaging of anaesthetised samples is preferred in principle, the water content increases the absorption of low energy fluorescence, and therefore dehydration aids detection of elements with atomic masses below potassium (K; *Z* < 19). Preserving subcellular distribution of elements is challenging, particularly following chemical fixation. Even brief (< 30 s) formalin fixation of thin tissue sections can cause redistribution and leaching of transition metals and electrolytes.<sup>16</sup> However, we previously demonstrated that cryofixation of *C. elegans* in liquid N<sub>2</sub>-cooled propane followed by lyophilisation does not cause significant variation in elemental content or subcellular distribution.<sup>12</sup>

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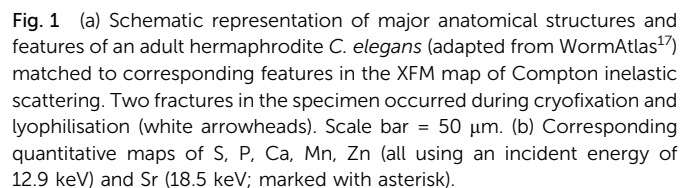
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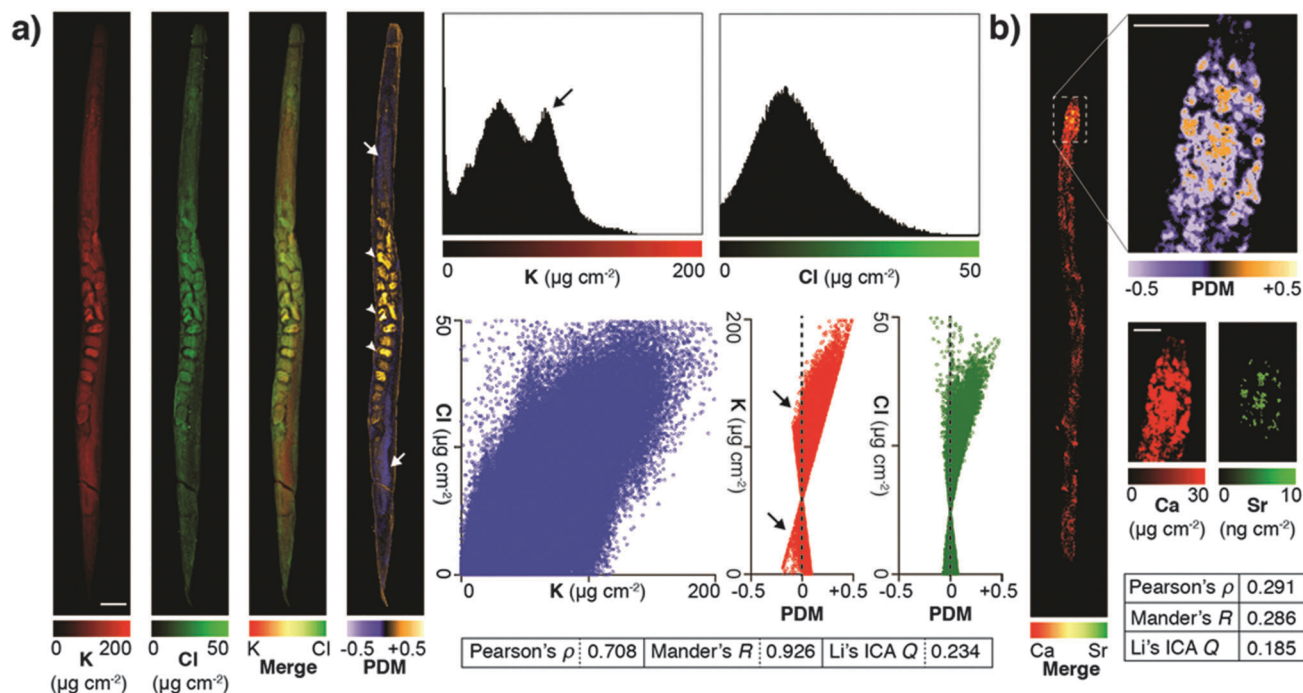
<sup>‡</sup> These authors contributed equally.



As we have previously shown, Ca and manganese (Mn) were highly compartmentalised along the intestinal lumen,<sup>13</sup> as was Zn, in addition to being rich within the gonad and embryos, consistent with Zn finger transcription factors necessary for early development. Using the higher incident energy of 18.5 keV, we also found subcellular concentrations of Sr in the most anterior intestine. Strontium commonly substitutes for Ca in biological systems at a greatly reduced concentration;<sup>23</sup> higher



Li's ICA Q measure is particularly useful for visualising the degree of spatial co-localisation; to demonstrate this we assessed the correlation between Ca and Sr, which within the whole organism was less distinct due to low Sr concentration (ICA Q = 0.185). In the anterior intestinal cells where Sr was detectable, we observed high correlation between Ca and Sr in the resulting PDM image (Fig. 2b), consistent with intestinal co-localisation observed in other taxa.<sup>26</sup> These results illustrate



**Fig. 2** (a) High abundance and low-Z elements K and Cl were analyzed for co-localisation in an individual *C. elegans* specimen. Images were merged and underwent whole organism correlation analysis using Pearson's, Mander's and Li's intensity correlation analysis (ICA; inset table).<sup>25</sup> Visualisation of the product of difference from the mean (PDM; presented on a black background) at high resolution improved interpretation by presenting the ICA quotient ( $Q$ ) on a pixel-by-pixel basis. Using this method, both elements showed marked correlation in embryos ( $0 > Q > +0.5$ ; white arrowheads), as well as distinct potassium-rich regions within the distal and proximal gonad ( $-0.5 < Q < 0$ ; white arrows). Scale bar = 50  $\mu\text{m}$ . Histograms of pixel values revealed a bimodal distribution for K, consistent with K enrichment in the gonad (black arrow). (b) The advantages of visualising correlation are clear when comparing co-localisation of high abundance Ca with low abundance Sr, which shares similar biochemistry but is close to the XFM limit of detection. Co-localisation is less robust (lower Pearson's  $\rho$ , Mander's  $R$  and ICA  $Q$ ) across the whole organism (inset table); though in the anterior intestine where both Ca and Sr are most concentrated shows high spatial correlation ( $0 < Q < +0.5$ ).

that PDM imaging allows both spatial correlation at a sub  $\mu\text{m}$  level of detail, as well as the within the whole organism.

The XFM methods used here significantly improved spatial resolution for *in vivo* mapping. Previously we have shown a distribution of iron (Fe) about the intestine at approximately 2  $\mu\text{m}$  resolution.<sup>12</sup> Our sub- $\mu\text{m}$  imaging approach permitted assessment of Fe revealing a level of detail comparable to histological staining and light microscopy. We found that punctate Fe deposits (Fig. 3a) resembled Fe distributions in formalin-fixed, paraffin-embedded sections stained using the Perls method for non-heme Fe (Fig. 3b).<sup>27</sup> In addition to localised Fe deposits, XFM mapping also showed a more generalised Fe distribution not seen in Perls staining, indicative of heme. The correspondence between these two diverse imaging modalities suggests that both approaches accurately report on *in vivo* Fe. Although Perls staining is not quantitative, these results differ from those reported by Hackett *et al.*,<sup>16</sup> who suggested formalin fixation alone alters Fe distribution in biological tissue. We suggest that neither radiation damage from XFM, nor extensive chemical processing for histological staining necessarily disturbs the distribution of non-heme Fe in *C. elegans*.

To further explore consistency between complementary imaging methods, we examined the *in vivo* localisation of the dominant Fe storage protein ferritin.<sup>28</sup> Using a green fluorescent protein

(GFP) fusion to ferritin we compared high-resolution confocal fluorescence *in vivo* microscopy to XFM mapping and Perls staining (Fig. 3c and d). Distribution of GFP fluorescence, and thereby ferritin localisation, was again remarkably similar to the Fe puncta previously imaged (see Movie, ESI<sup>†</sup>). Ferritin accounts for almost half of the Fe content of *C. elegans* and therefore represents a good proxy for non-heme Fe. The multiple imaging methods used serve as validation of each respective technique, providing the first consistent representation of subcellular Fe within a whole organism. Future directions of this complementary imaging approach could employ *C. elegans* with mutated genes that affect Fe metabolism, as well as ageing studies (such as those described in James *et al.*<sup>14</sup>) to exploit the higher resolution mapping protocol described here. Here we focused on Fe to demonstrate complementary imaging of metal distribution; similar studies could employ the genetically encoded fluorescent calcium sensor GCaMP,<sup>29</sup> which has been used in the *C. elegans* model system,<sup>30</sup> to compare total body Ca concentration with cell-specific  $\text{Ca}^{2+}$  content.

In summary, we have demonstrated sub- $\mu\text{m}$  XFM mapping of bio-elements, both rare and ubiquitous, ranging from low-Z to highly abundant transition metals in a model organism ideally suited for studying metal metabolism. Mapping low-Z elements with confidence will facilitate new experimental paradigms.







**Fig. 3** (a) Iron distribution in a single *C. elegans* sample was overlaid on Compton map showed high concentration along the intestinal lumen, as well as distinct punctate deposits varying from 1  $\mu\text{m}$  in diameter. Scale bars = 50  $\mu\text{m}$ . (b) These features were reflective of non-heme Fe (regions i–iii), confirmed by staining of 5  $\mu\text{m}$  thick sections using 3,3'-diaminobenzidine (DAB)-enhanced Perls staining of an additional specimen. Iron (stained brown) was notably absent in the head (region iv) using both methods. Scale bars = 50  $\mu\text{m}$ . (c) 10× brightfield micrograph overlaid with GFP fluorescence and three-dimensional GFP fluorescence-only image of ferritin::GFP distribution in adult *C. elegans*. (d) 40× high-resolution confocal micrographs revealed ferritin distribution similar to the punctate deposits of non-heme Fe observed in Perls stained micrographs and XFM mapping. Scale bar = 25  $\mu\text{m}$ .

With image resolution approaching that of light microscopy, implementation of high-level correlative image analysis methods previously reserved for standard fluorescence microscopy are now within reach of contemporary XFM. We also demonstrated how multiple modality imaging provides a greater appreciation of subcellular metal distribution. Using both well-established histochemical analysis and confocal fluorescence imaging provides a more comprehensive picture of Fe distribution within *C. elegans*, an essential element for development and health. A unified approach to imaging using multiple methods accessible to chemists and biologists alike will permit further advances in understanding metal biochemistry.

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