Electronic Supplementary Material (ESI) for Metallomics.

Supplementary Information for:

High-resolution complementary chemical imaging of bio-elements in *Caenorhabditis elegans*

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Experimental Methods

Caenorhibditis elegans strains:
Wild type (strain N2) was obtained from the Caenorhabditis Genetics Center. The ferritin::GFP reporter was generated using a previously reported plasmid construct[1] microinjected into wild-type worms. Strain GMC02 was recovered with an extra chromosomal transgenic array *gmcEx02* [*Pftn-1*::*ftn-1*::*gfp*]. This strain was backcrossed four times to wild type prior to further use. All experiments were performed on 5-day old (post egg lay) adult hermaphrodites cultured at 20 °C and on nematode growth media (NGM) using standard methods.[2]

Sample preparation for X-ray fluorescence microscopy
The sample preparation method was adapted from those previously described.[3-4] Developmentally synchronous 5-day old adults were removed from NGM and washed four times in excess S-basal (0.1 M NaCl; 0.01 M K_2HPO_4; 0.05 KH_2PO_4; 5 mg L^{-1} cholesterol) to remove any excess *Escherichia coli* feed. Specimens were then anaesthetized in ice-cold sodium azide (NaN_3; 0.2% w/v), which inhibits mitochondrial function.[5] Once immobilized, the specimens were washed in ice-cold ammonium acetate (CH_3COONH_4; 1.5% w/v) and then deposited via transfer pipette onto a 0.5 μm-thick silicon nitride (Si_3N_4) window (Silson). Excess buffer from transfer was removed using fine tapered paper wicks (MicroWicks, MiToGen). The window was then immediately frozen in liquid nitrogen-chilled propane in a KF-80 plunge freezer (Leica). The samples were then lyophilized *in situ* overnight at -40 °C. Prepared window was then kept under vacuum until analysis.

X-ray fluorescence microscopy:
X-ray fluorescence microscopy (XFM) was performed at the Australian Synchrotron using the Maia Revision C large solid angle energy dispersive detector (see http://www.synchrotron.org.au/aussyncbeamlines/x-ray-fluorescence-microscopy/technical-information for technical specification of Rev C) adapted from described methods for previous revisions of the Maia detector.[3] Principles of the detector design can be found in Ryan *et al*.[7] Two beam energies were used; 12.9 keV, and 18.5 keV (for strontium imaging, shown in Figure 1b and 2b) in consecutive experiments using the same specimen. The beam was focused to a spot of approximately 2 μm FWHM using a Kirkpatrick-Baez (KB) mirror pair with a flux of approximately 1.9×10^{10} photons s^{-1}. The sample on the Si_3N_4 window was mounted on a Perspex sample holder using double-sided tape on the top and bottom of the window frame, and then placed in a nitrogen environment. The sample was scanned continuously through the incident beam in a raster pattern, with data binned at intervals equivalent to a 400 nm step size in the *x*-axis, with an effective dwell time of 22 ms. A vertical transition step of 400 nm was used to produce an effective pixel size of approximately 400 nm. On average, images contained approximately 1.1 megapixels, and took approximately 6 hours to acquire. Quantitation of was performed elements using dynamic analysis[8] with two single-element manganese and platinum foil calibrants (Micromatter, Canada). Spectral fits and quantification was performed using GeoPIXE.[9] All images report pixel values in areal density (*i.e.* μ or ng cm^{-2}).

Sample preparation for Perls staining:
Perls staining was performed using the method previously described.[6] 5-day old adults were washed in S-basal and fixed overnight in neutral buffered formalin (10% v/v) and then embedded in agar (2% w/v in phosphate buffered saline; PBS). Samples were fixed overnight in neutral buffered formalin again and then dehydrated in increasing ethanol concentrations to xylene and paraffin-infiltrated in an 8-hour cycle. The paraffin blocks were then sectioned at 5 µm on a microtome for Perls staining.

Perls staining:
The 5-µm thick sections mounted on microscope slides were dewaxed in three changes of xylene and then decreasing ethanol concentration, then extensively rinsed in running water. Hydrated sections were incubated at 37 °C for 1 hour in potassium ferrocyanide ($K_4[Fe(CN)_6]\cdot 3H_2O$; 7% w/v) in HCl (3% v/v) and then enhanced using a solution of 3.5 µM 3,3'-diaminobenzidine (DAB) in H_2O_2 (0.015% v/v) for 5-10 minutes, or until a clear reaction had occurred. After quenching the reaction by immersing in running water, samples were counterstained with hemotoxylin for 2 minutes and washed in water before dehydration in increasing ethanol concentration, xylene and coverslipping. Micrographs were recorded using a Leica DM2500 optical microscope with a 20×/0.50 NA lens and Leica DFC310FX digital camera.

Sample preparation for confocal microscopy:
Adults (5-day old post egg lay) bearing the ferritin::GFP reporter were anesthetized as outlined above, immobilized on a thin agar (1% w/v) with sodium azide (NaN_3; 0.5% w/v) pad mounted on a microscope slide, coverslipped and immediately imaged by confocal microscopy.

Confocal imaging:
Images were acquired by using a Zeiss LSM 780 microscope equipped with 10×/0.45 NA and 40×/1.4 NA lenses. High-resolution image stacks were acquired within Nyquist sampling parameters and were deconvolved using Huygens software (Scientific Volume Imaging). 3D reconstruction was performed using Amira (Version 6.0.0; FEI Visualization Sciences Group). Image contrast and brightness were adjusted for presentation purposes. Supplementary Movie S1 was generated using Amira and colored in ImageJ, then exported as an .AVI file.

XFM image analysis:
XFM images were exported from GeoPIXE as .TIF files and imported into ImageJ. Contrast was adjusted in a linear fashion and standard look-up tables applied. All images underwent the 'Remove Outliers' function, with a pixel radius of 2.0 and threshold of 50. Backgrounds were subtracted by selecting a minimum 100 x 30 pixel region of interest (ROI) and application of the 'BG Subtraction from ROI' plugin function with a scaling factor of 3.

Intensity correlation analysis:
Intensity colocalization analysis (ICA) was performed according to the methods described by Li *et al.*[10] and subsequent ImageJ plugins released, which can be accessed at http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm. Background-subtracted images were downsampled from 32-bit to 8-bit in ImageJ prior to application of the 'Intensity Correlation Analysis' plugin. Product of difference from the mean (PDM) images were exported, showing local distribution of

the ICA quotient (Q) values, as well as total image Pearson's ρ, Mander's R and ICA Q values for the whole organism (shown in Figure 2). PDM is defined as:

$$(A_i - a)(B_i - b)$$

Where A_i/B_i are the pixel intensity (in this case, areal concentration) of variable A or B (K and Cl in Figure 2a) and a/b is the mean values of the variables. Frequency distribution and PDM plots were generated in Prism 5.0d (GraphPad) after exporting from ImageJ.

Figure construction:

Final image panels were exported as .PNG files for the generation of figures. Figures were constructed using Adobe Photoshop and Illustrator CS 5.1. Image contrast and brightness were adjusted for presentation purposes *after* analysis was completed.

Animal Experimentation:

The use of *Caenorhabditis elegans* for research in this study is exempt from the *Australian code for the care and use of animals for scientific purposes* 8th edition 2013 (National Health and Medical Research Council, Australia).

***Author contributions*:**

G.M. conceived and designed the experiments; M.W.M.J., V.C.W. and G.M. performed experiments; D.J.H., M.W.M.J., V.C.W. and G.M. analyzed the data; M.D.J. and A.I.B. provided material support, D.J.H., M.W.M.J., N.L.J. and G.M. prepared the manuscript following discussion and contribution from all authors.

Supplementary Results

Figure S1-2: Full resolution XFM maps of *C. elegans* adults (Samples 1 and 2, at 2876 x 376 and 2951 x 376 pixels per map, respectively) for elements P to Sr presented as a single 8-bit .PNG file and converted to .PDF files. Scale bar = 50 μm.

Movie S1: Animation of GFP fluorescence measured by confocal fluorescence microscopy of ferritin distribution in strain GMC02 in three dimensions, showing punctate deposits similar in size and appearance to Perls images of non-heme Fe in Figure 3.

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