**φXANES: In vivo imaging of metal-protein coordination environments**

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We have developed an X-ray absorption near edge structure spectroscopy method using fluorescence detection for visualizing in vivo coordination environments of metals in biological specimens. This approach, which we term fluorescence imaging XANES (φXANES), allows us to spatially depict metal-protein associations in a native, hydrated state whilst avoiding intrinsic chemical damage from radiation. This method was validated using iron-challenged Caenorhabditis elegans to observe marked alterations in redox environment.

Metal cofactors represent a fundamental component of biochemistry via their ability to facilitate electron transport and stabilize biomolecules. An important requirement for understanding the role of transition metals in normal biochemistry and disease processes is the determination of their coordination environment1,2. The synergy of synchrotron-based X-ray fluorescence microscopy (XFM) and X-ray absorption near edge structure (XANES) spectroscopy represents a powerful analytical approach for studying metal biochemistry at the micro-scale. This permits both quantitative mapping of metal distribution and profiling of the native coordination environment without the need for exogenous molecular probes3. We combined these two measurement strategies using the same synchrotron beamline to develop an imaging approach we have called fluorescence imaging (‘fi’, or φ for the Greek ‘phi’) φXANES.

XANES has traditionally been used in biology to profile coordination environments in fixed locations (‘point’ XANES)4, rather than functioning as a fine resolution imaging technique. In addition to lack of spatial information, the problem of extended exposure to the ionizing X-rays (>10 keV) that can damage the sample by disrupting chemical bonds. Photoreduction of redox metals can occur at doses around 107 Gy5,6. Exposure of XANES samples can be increased to 1010 Gy using cryogenic conditions (~100 °C)2, though samples are still susceptible to morphological damage8 and the requirement to maintain the specimen at low temperatures during preparation and measurement increases logistical complexity. Ideally, analysis of samples that remain hydrated and at physiological temperature is preferable. Here, we demonstrate the development of non-destructive φXANES imaging at standard laboratory conditions, validated in a Caenorhabditis elegans model of disrupted metal metabolism.

To determine the optimal conditions for φXANES (see Supplementary Note), we tested two experimental scenarios to establish the appropriate dose of radiation to which hydrated and anesthetized C. elegans could be exposed without inducing morphological changes and to avoid photoreduction of endogenous iron. These were: i) ‘high dose’ φXANES, where elemental maps of high statistical precision were obtained using long dwell and spatial oversampling in two directions with a symmetrical beam profile; and ii) ‘low dose’ φXANES, where a shorter dwell time was used along with a vertically-elongated beam (reducing the X-ray flux density), while undersampling in the vertical direction (for details of the focused beam see Supplementary Figure 1). Together, these measures reduced sampling time and localized beam exposure by a factor of 100. A representative whole-body XFM elemental map of calcium and iron distribution in a separate cryofixed and lyophilized specimen is presented for anatomical reference (Fig. 1a). For high dose φXANES, four hydrated adults were mapped using standard XFM parameters (Fig. 1b), with an anterior region containing iron-rich intestinal cells in one specimen selected for φXANES. Here, the region underwent XANES analysis spanning the iron K-edge incident energies (7100 to 7220 eV), exposing this region to an estimated 5 × 108 Gy, within the radiation dose range previously reported to stimulate photoreduction of iron6.

These four individuals were mapped again using standard XFM following φXANES, approximately 5 hours after the initial XFM scan. We performed intensity correlation analysis (ICA)9 on a region of interest representative

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Figure 1. Optimization of ϕXANES parameters to minimize sample perturbation. (a) Reference X-ray fluorescence micrograph showing Compton scatter (greyscale) and quantitation of calcium (yellow) and iron (rainbow color scale) in a dried adult *C. elegans* as a reference of general anatomy. (b) A map of iron distribution (rainbow color scale) was recorded at 7282 eV and used to select two sub-regions for reanalysis: the dashed white box was selected for high dose ϕXANES (114 scans) followed by a final map at 7282 eV, while the ‘control’ area (solid white box) was mapped at 7282 eV twice only; prior to and post completion of ϕXANES on the
first region. With the exception of incident energy, all scan parameters were held constant for this series of measurements and the dose associated with recording each map was ~5 MGy. Comparing the first (blue) and last (yellow) maps (over a 5 hour period, shown in overlay where white represents colocalization) from each sub-region showed that high dose \( \varphi \)-XANES induced significant redistribution (ICA quotient \( Q = 0.006 \)) of iron compared to the region mapped only twice (\( Q = 0.41 \)). (c) The distribution of iron was also mapped using low dose \( \varphi \)-XANES. Total dose for these maps was 0.05 MGy. ICA comparing the first and last maps revealed strong agreement of iron signal (\( Q = 0.37 \)), consistent with a minimally disturbed system. Scale bar for all images = 100 \( \mu \)m.

of the pre and post-\( \varphi \)-XANES scanned area and found that distribution of pixel intensities significantly differed between the two maps (ICA quotient \( Q = 0.006; 0 = \) no correlation), demonstrating clear sample damage. In parallel, we selected an anatomically equivalent adjacent specimen that received ~10\(^8\) Gy from the two XFM maps alone which, in contrast, maintained a consistent iron distribution (\( Q = 0.37; 0.5 = \) perfect correlation).

Low dose \( \varphi \)-XANES and XFM of a matching sample group were then examined (Fig. 1c), encompassing an additional four adults. These samples were exposed to 4 \( \times \) 10\(^6\) Gy, approximately 100-fold less than the high dose method. Iron spatial distribution pre- and post \( \varphi \)-XANES was maintained (\( Q = 0.41 \)). Although low dose \( \varphi \)-XANES does sacrifice some spatial detail (5.6 \( \mu \)m \( \times \) versus 0.64 \( \mu \)m \( \times \) sampling area), the reduced radiation dose (< 2 \( \times \) 10\(^6\) Gy) ensures photoreduction of iron\(^6\) and other metals\(^10\) is minimized while endogenous spatial distribution is maintained.

To demonstrate the potential of \( \varphi \)-XANES to profile bioinorganic chemistry \textit{in vivo}, we examined a combined genetic and exogenously challenged model of severe iron dyshomeostasis. \textit{C. elegans} lacking the iron-storage protein ferritin (both genes \textit{ftn-1} and \textit{ftn-2} are ablated via mutation; hereafter referred to as ferritin nulls) have increased oxidative load from elevated ferrous iron\(^11\), and have a shortened lifespan compared to wild type (Supplementary Figure 2). We designed two experimental paradigms, exposing both wild type and ferritin nulls to either basal iron levels \( \varphi \) normal cultivating conditions, or high iron through supplementation of their growth media. Adults were anesthetized and quantitatively mapped by XFM (Fig. 2a). Exposure to high iron increased levels in wild type animals compared to equivalent animals raised under basal conditions (one-way ANOVA with Tukey’s \textit{post hoc} test; \( p < 0.001 \); Fig. 2b), whilst ferritin nulls raised on basal iron exhibited a decrease in total levels compared to wild type (\( p < 0.001 \)). As ferritin is not involved in iron uptake, the reduced load is consistent with an inability to store iron\(^12\); ferritin nulls on high iron still displayed increased total body burden (\( p < 0.001 \)).

Each experimental group was mapped \( \varphi \)-XANES using our optimized low dose parameters, scanning the iron K-edge (Fig. 2c). This range encompasses the characteristic pre-edge (\( \sim 7115\) eV), shoulder (\( \sim 7124\) eV), and crest (\( \sim 7130\) eV) features, arising from 1s \( \rightarrow \) 3d, 1s \( \rightarrow \) 4s and 1s \( \rightarrow \) 4p electronic transitions, respectively. The precise energy of the pre-edge reflects the relative abundance of ferrous [Fe(II)] and ferric [Fe(III)] iron, and shifts to lower energies in the presence of increased Fe(II)\(^13\). When comparing the pooled XANES spectra (\( i.e. \) the mean for all pixels) for each measured individual, we observed that the centroid energy for the pre-edge transition in wild type \( 7114\) eV, Fig. 2d) cultured on high iron was unchanged but the reduced intensity was indicative of an increase in the number of octahedral Fe(III) centres\(^14\), consistent with increased buffering of iron within ferritin, where it is arranged in such coordination geometry\(^15\). However, ferritin nulls, regardless of iron load, demonstrated a shift to lower centroid energies away from \( 7114\) eV, indicating increased Fe(II). Comparing the relative intensity of the shoulder and crest features of the iron K-edge in each group further confirmed a disruption in the iron coordination environment. First-derivative iron XANES spectra exhibited a significant alteration in the cumulative Fe(III):total iron ratio between wild type and ferritin null groups (one-way ANOVA with Tukey’s \textit{post hoc} test; \( p < 0.001 \); Supplementary Fig. 3a,b). This effect was independent of iron loading. In addition, we observed increased variability between ferritin nulls compared to wild type (Bartlett’s test for homogeneity of variances \( \chi^2 = 16.54; p < 0.001 \); Supplementary Fig. 3c), consistent with a homeostatic system in distress.

Potentially hundreds of individual iron-binding proteins contribute to the proteome (the ferroproteome), although even in microbes the precise number remains unclear\(^16\). These include proteins containing heme moieties, iron-sulfur clusters, ferrihydrite-like crystalline structures (as in ferritin), and multi-dentate ligands arising from specific amino acid conformations\(^17\). When examining the cumulative \( \varphi \)-XANES spectra, we are assessing the aggregate distribution of iron-protein coordination complexes in a whole organism. Spatial mapping by \( \varphi \)-XANES allows for individual tissue or cell types to be objectively assessed for changes to iron coordination in response to specific challenges at the \( \mu \)m scale. We applied principal component analysis (PCA) and \( k \)-means clustering (CA) as implemented in the Multivariate AAnalysis Tool for Spectromicroscopy (MANTiS)\(^18\) package after tiling \( \varphi \)-XANES maps to directly compare spatial coordination states in wild type and ferritin nulls raised on high iron. Pixels with similar XANES spectra were assigned to six distinct regions of interest (ROIs), color coded in Fig. 2e as descending Fe(III):total iron. Of these six regions, ROIs 1 and 5 differed in proportions of total iron compared to the region mapped only twice (\( Q = 0.41 \)). All ROIs further support the higher levels of Fe(II) in the ferritin null animals, consistent with the well characterized role of ferritin in buffering reactive ferrous iron as a redox-silenced mineralized Fe(III) species. For comparison,
Figure 2. Loss of ferritin skews iron load and Fe(III):total iron ratio. (a) XFM of wild type and ferritin nulls ± high iron. Ferritin nulls exhibited reduced total iron, but, as expected, retained capacity to uptake iron via a mechanism independent of ferritin. White outline demarcates the boundary of each animal, color table defines iron areal density (μg cm⁻²) and the scale bar = 100 μm. (b) Median iron areal density for each specimen, showing elevated iron load following exposure to high iron (n = number of specimens per group; data presented as the mean of the medians ± 1 SD). (c) Iron XANES spectra (across all pixels) extracted from low dose XANES for the groups shown in (a). The starting position of iron K-edge (7112 eV) is marked with a vertical line and for clarity the integrated XANES spectra from each group has been offset vertically. (d) Expanding the pre-edge region (grey box in (c)), following subtraction of the rising edge (dashed line), highlights changes in both the energy and intensity of the 1s → 3d pre-edge feature between groups. The extracted data (colored circles) and fitted Gaussian (solid black lines; 95% confidence interval in grey) are superimposed to determine the centroid values (~7114 eV for wild type; marked for reference). Loss of ferritin changed the pre-edge feature to exhibit two centroid energies (7113 eV and 7117 eV), whereas high iron exposure retained a single centroid energy of 7114 eV. (e) Areas of similar iron XANES spectra identified via principal component analysis and k-means clustering marked as distinct regions of interest (ROIs, six per specimen). The XANES spectra for each cluster were highly structured and allowed the Fe(III):total iron ratio to be calculated for each ROI. The spatial extent of each region as a proportion of the area scanned is shown and highlights that, with the exception of portions of the intestine, the majority of wild type tissues possess relatively low Fe(II) levels despite a higher iron load. In particular, two regions differed significantly in Fe(III):total iron ratio (ROIs 1 and 5; both localized along the intestinal tract) between wild type and ferritin nulls. Scale bar = 100 μm. (f) XANES from a purified horse spleen ferritin standard was compared to the
cumulative XANES spectra from ROIs 1 and 5. (g) The difference (ΔXANES) between these two ROIs and the ferritin standard spectra showed that ROI1 had stark similarities with the ferritin profile, whilst ROI5, which was practically absent in wild types demonstrated significant variation from the ferritin XANES spectra, further supporting complete ablation of ferritin from these animals and an altered coordination environment. (h) Features characteristic of electronic transitions used to differentiate between iron oxidation states also revealed that ROI5 had a greater level of abundant Fe(II) compared to ROI1 and the ferritin standard, where the majority of iron is stabilized in a mineralized Fe(III) form.

XANES spectra of additional iron-protein ligands (oxidized and reduced heme-containing cytochrome c) are shown in Supplementary Figure 4.

In summary, we have demonstrated that ϕXANES is a powerful method for mapping coordination environments in vivo, with no displacement of target elements and measurement dose well below previous studies of biological iron redox status inline with bulk XAS measurements, and without the need for cryogenic sample environment. ϕXANES in conjunction with PCA-CA is ideal for assessing changing coordination environments in tissue sections, small model organisms (including C. elegans and Drosophila melanogaster, which has previously been used for point XANES) and cell culture. Although we validated this method using iron coordination, ϕXANES can be applied to any element to which XFM is sensitive, drugs that elicit a change in cellular redox environment, and longitudinal studies that require real-time assessment of changing coordination conditions in a biological system.

Methods

Methods and any associated references are available in the online version of this paper.

References

1. Song, W. J., Sontz, P. A., Ambroggio, X. I. & Tezcan, F. A. Metals in protein-protein interfaces. *Annu. Rev. Biophys.* 43, 409–431 (2014).
2. Chang, C. J. Searching for harmony in transition-metal signaling. *Nat. Chem. Biol.* 11, 744–747 (2015).
3. Hare, D. J., New, E. I., de Jonge, M. D. & McColl, G. Imaging metals in biology: balancing sensitivity, selectivity and spatial resolution. *Chem. Soc. Rev.* 44, 5941–5958 (2015).
4. Lombi, E. & Susini, J. Synchrotron-based techniques for plant and soil science: opportunities, challenges and future perspectives. *Plant Soil* 320, 1–35 (2009).
5. Yano, J. et al. X-ray damage to the Mn₇Ca complex in single crystals of photosystem II: a case study for metalloprotein crystallography. *Proc. Natl. Acad. Sci. USA* 102, 12047–12052 (2005).
6. George, S. J., Fu, J., Guo, Y., Druzy, O. B. & Friedrich, S. X-ray photochemistry in iron complexes from Fe (0) to Fe (IV)—Can a bug become a feature? *Inorg. Chim. Acta* 361, 1157–1165 (2008).
7. Bacquart, T. et al. Subcellular speciation analysis of trace element oxidation states using synchrotron radiation micro-X-ray absorption near-edge structure. *Anal. Chem.* 79, 7353–7359 (2007).
8. Deng, J. et al. Simultaneous cryo X-ray ptychography and fluorescence microscopy of green algae. *Proc. Natl. Acad. Sci. USA* 112, 2314–2319 (2015).
9. Li, Q. et al. A syntaxin 1, Galphao(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolor localization. *J. Neurosci.* 24, 4070–4081 (2004).
10. Ascone, I., Meyer-Klaucke, W. & Murphy, L. Experimental aspects of biological X-ray absorption spectroscopy. *J. Synchrotron Radiat.* 10, 16–22 (2003).
11. James, S. A. et al. Direct in vivo imaging of ferrous iron dyshomeostasis in ageing Caenorhabditis elegans. *Chem. Sci.* 6, 2952–2962 (2015).
12. Honarmand Ebrahimi, K., Hagedoorn, P. L. & Hagen, W. R. Unity in the Biochemistry of the Iron-Storage Proteins Ferritin and Bacterioferritin. *Chem. Rev.* 115, 295–326 (2015).
13. Berry, A. J., O’Neill, H. S. C. & Jayasuriya, K. D. XANES calibrations for the oxidation state of iron in a silicate glass. *Am. Mineral.* (2003).
14. Westre, T. et al. A multiplet analysis of Fe K-edge 1s → 3d pre-edge features of iron complexes. *J. Am. Chem. Soc.* 119, 6297–6314 (1997).
15. Brown, D. A., Herlihy, K. M. & O’Shea, S. K. Kinetics of Iron(III) Chelation from Polynuclear Oxo-Hydroxy Aggregates by Hydroxamic Acids: Understanding Ferritin Iron(III) Sequestration. *Inorg. Chem.* 38, 5198–5202 (1999).
16. Cvetkovic, A. et al. Microbial metalloproteomes are largely uncharacterized. *Nature* 466, 779–782 (2010).
17. Philpott, C. C. Coming into view: eukaryotic iron chaperones and intracellular iron delivery. *J. Biol. Chem.* 287, 13518–13523 (2012).
18. Loretic, M., Mak, R., Wirick, S., Meier, F. & Jacobsen, C. MANTiS: a program for the analysis of X-ray spectromicroscopy data. *J. Synchrotron Radiat.* 21, 1206–1212 (2014).
19. Kondapalli, K. C., Kok, N. M., Dancis, A. & Stemmler, T. L. Drosophila Frataxin: An Iron Chaperone during Cellular Fe—S Cluster Bioassembly. *Biochemistry* 47, 6917–6927 (2008).

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Author Contributions

S.A.J., M.D.dJ. and G.M. devised the study. S.A.J., D.J.H. and G.M. performed the experiments. S.A.J., D.J.H. and G.M. analyzed the data. All authors contributed to the scientific interpretation of the data and writing the manuscript.
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Erratum: ϕXANES: *In vivo* imaging of metal-protein coordination environments

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In this Article, the online methods were omitted. The online methods should read:

**Strains.** Wild type *C. elegans* (strain N2) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota), and *ftn-2(ok404)* and the ferritin null *ftn-2(ok404); ftn-1(ok3625)* (strain GMC005) have been previously described⁵. All strains were cultured at 20°C on standard nematode growth media (NGM) with *E. coli* (strain OP50)². Populations were developmentally synchronized via a 3 hour egg lay and then developed at 20°C for three days. Cohorts were then transferred to either standard NGM (basal media) or NGM supplemented with 5 mg ml⁻¹ ammonium ferric (III) citrate (FAC, C₆H₈O₇·xFe³⁺·yNH₃, Sigma Aldrich; high iron media) for 48 h at 25°C. The concentration of iron was 246 ng g⁻¹ in the basal media and 1.17 mg g⁻¹ for high iron media for our batch of reagents¹.

**Lifespan analysis.** The effects of *ftn-2(ok404)* and *ftn-2(ok404); ftn-1(ok3625)* null mutants on lifespan at 25°C were compared to wild type using protocols previously published¹.

**Elemental mapping: X-ray fluorescence microscopy (XFM).** Specimens for analysis were repeatedly washed with s-basal, anesthetized, and mounted on Si₃N₄ windows (window area 16 mm², 2 μm thick, Silson, United Kingdom) for analysis at the XFM beamline at the Australian Synchrotron as described previously¹. In brief, the specimens were cushioned on an agarose pad supported by the Si₃N₄ window and overlaid with Ultralene film (4 μm thick, Volga Instruments) to prevent dehydration. Element localization for iron and lighter elements was mapped using an incident beam of 7282 eV X-rays in order to induce K-shell ionisation and to clearly separate the Elastic and Inelastic peaks from the elemental peaks. Dynamic Analysis and deconvolution of fluorescence via GeoPIXE 7.1 (CSIRO) was used to produce quantitative elemental maps³. Two single-element thin metal foils of known areal density (manganese 18.9 μg cm⁻² and iron 52.2 μg cm⁻², Micromatter, Canada) were used to establish elemental quantification.

X-ray fluorescence (XRF) was corrected for an assumed specimen composition and thickness⁴ using the known composition and thickness of the Si₃N₄ window and Ultralene film and composition and density of the agarose. Small deviations from these assumptions are not significant for results presented in this study as the effects of beam attenuation and self-absorption on calcium and iron XRF are negligible for a specimen of this type and size. The sample preparation and elemental mapping of the freeze-dried five-day old (post egg lay) wild type were performed as previously described⁴.

**Fluorescence imaging X-ray absorption near edge structure (ϕXANES) spectroscopy.** Iron K-edge ϕXANES was measured as a series of XRF maps (as described above) at a range of incident energies spanning the iron K-edge (7112 eV). Incident energy was calibrated by defining the first derivative peak of the iron foil standard to be 7112.0 eV and the spectrum from this foil was recorded at the beginning and end of the experiment to monitor energy stability. XRF was normalized to the incident beam flux monitored by an ionization chamber with a 27 cm path length placed upstream of the focusing optics and filled with 100% N₂. The XANES spectra were extracted from pixels within the selected areas and background- and baseline-corrected using methods implemented in ATHENA, an interface to IFEFFIT⁵. The relative intensity of the 1s → 3d, 1s → 4s and 1s → 4p electronic transitions were determined as previously described⁴.
High dose \( \varphi \)XANES. Specimens were continuously scanned with the resultant XRF binned at 0.8 \( \mu \)m horizontal intervals (Supplementary Fig. 1a). Upon completion of each row the sample was translated 0.8 \( \mu \)m vertically before continuing the raster scan. This process produced elemental maps containing up to 882,096 0.64 \( \mu \)m\(^2\) pixels with a pixel transit time of 15.6 msec. The full width at half max (FWHM) of a Gaussian distribution fitted to the beam profile defined the spatial extent of illumination as 2.1 \( \mu \)m in the horizontal and 2.8 \( \mu \)m vertical. High dose \( \varphi \)XANES imaging was then collected across 114 energies spanning the Fe K-edge (7112 eV) using the following incident energy increments: 7042 eV to 7102 eV in 10 eV steps; 7102 eV to 7110 eV in 1 eV steps; 7110 eV to 7115 eV in 0.5 eV steps; 7115 eV to 7152 eV in 1 eV steps; and 7152 eV to 7256 eV in 2 eV steps. This series of maps delivered a total absorbed dose estimated at 500 MGy. The distribution of iron was mapped at 7282 eV, using identical scan parameters, immediately prior and post high dose \( \varphi \)XANES.

Low dose \( \varphi \)XANES. During data collection specimens were continuously scanned through X-ray focus with the resultant XRF binned at 0.8 \( \mu \)m horizontal intervals. Upon completion of each row the sample was translated 7 \( \mu \)m vertically before continuing the raster scan (Supplementary Fig. 1b). This process produced elemental maps ranging up to 102,000 pixels and containing 5.6 \( \mu \)m\(^2\) pixels with a pixel transit time of 1.9 msec. As above the beam profile defined the spatial extent of illumination as 1.5 \( \mu \)m in the horizontal and 3.8 \( \mu \)m vertical. Low dose \( \varphi \)XANES imaging was collected at 82 energies spanning the Fe K-edge using the following incident energy increments: 7100 eV to 7162 eV in 1 eV steps; 7162 eV to 7192 eV in 2 eV steps; and 7192 eV to 7217 eV in 5 eV steps. This series of maps delivered a total estimated measurement dose of 4 MGy.

Image analysis. Analysis of elemental XRF maps, including colocalization calculations of ICA quotients\(^6\), was performed using a combination of tools native to GeoPIXE and ImageJ, a java- based image-processing program developed by the National Institutes of Health (USA)\(^6,7\). Principal component analysis followed by cluster analysis (PCA-CA) of the \( \varphi \)XANES stack was achieved by grouping pixels based on spectral similarity using MANTiS v2.0\(^8\).

Statistics. Lifespan data were compared via a Kaplan–Maier survival curve and non-parametric log rank test. Median iron per individual derived from the iron maps was compared between experimental groups via 1-way ANOVA with Tukey’s post hoc tests. Variability of individual Fe(III):total iron ratios derived from \( \varphi \)XANES of the various treatment groups was assessed via Bartlett’s test for homogeneity of variances. Non-normally distributed data for each spectra (D’Agostino’s K\(^2\) test; \( p < 0.05 \); shown in Fig. 2f–h) required comparing the group medians to an expected value of zero (i.e. \( \Delta \)XANES for ferritin = 0). All tests were performed using Prism v5.0d (GraphPad).

References
1. James, S. A. et al. Direct in vivo imaging of ferrous iron dyshomeostasis in ageing Caenorhabditis elegans. *Chem. Sci.* **6**, 2952–2962 (2015).
2. Brenner, S. The genetics of Caenorhabditis elegans. *Genetics* **77**, 71–94 (1974).
3. Ryan, C. G. et al. Large detector array and real-time processing and elemental image projection of X-ray and proton microprobe fluorescence data. *Nucl Instrum Meth B* **260**, 1–7 (2007).
4. James, S. A. et al. Direct in vivo imaging of essential bioinorganics in Caenorhabditis elegans. *Metallomics* **5**, 627–635 (2013).
5. Ravel, B. & Newville, M. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. *J. Synchrotron Radiat.* **12**, 537–541 (2005).
6. Li, Q. et al. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J. Neurosci.* **24**, 4070–4081 (2004).
7. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Meth.* **9**, 671–675 (2012).
8. Lerotic, M., Mak, R., Wirick, S., Meirer, F. & Jacobsen, C. MANTiS: a program for the analysis of X-ray spectromicroscopy data. *J. Synchrotron Radiat.* **21**, 1206–1212 (2014).

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