**Promises and Pitfalls of Metal Imaging in Biology**

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**A picture may speak a thousand words, but if those words fail to form a coherent sentence there is little to be learned. As cutting-edge imaging technology now provides us the tools to decipher the multitude of roles played by metals and metalloids in molecular, cellular and developmental biology, as well as health and disease, it is time to reflect on the advances made in imaging, the limitations discovered, and the future of a burgeoning field. In this Perspective, the current state-of-the-art is discussed from a self-imposed contrarian position, as we not only highlight the major advances made of the years but use them as teachable moments to zoom in on challenges that remain to be overcome. We also describe the steps being taken towards being able to paint a completely undisturbed picture of cellular metal metabolism, which is, metaphorically speaking, the Holy Grail of the discipline.**

## Introduction

Although all scientists strive for accuracy, and we now have tools at our disposal that were once the stuff of fantasy, there is still no such thing as a perfect measurement. This is true for all scientific disciplines, including those focused on observing biological systems where inherent complexity and 'noise' of biological processes make accurate observations often very challenging. Every scientific measurement comes with two numbers, the measurement and the error of that measurement, and two terms that are loosely related to them, accuracy and precision. Accuracy is used to describe how close a measurement is to the reality, and precision describes how well the measurement has been performed. For a scientific measurement to be of maximal value, it should be both accurate and precise. But, that is not to say that a measurement that lacks either accuracy or precision is useless. Provided we understand, and can quantify, the extent of inaccuracy or imprecision we can report the observation and discuss its implications and limitations.

The ideal scenario for an analytical biochemist involves a stable analyte that does not undergo changes during the sample preparation and handling, or the duration of measurement due to either internal or external effects. The types of sample analytical biochemists handle are different types of biomolecules, such as proteins, nucleic acids, lipids or saccharides. Each one of these categories comes with its own properties that must be taken into account when conducting measurements *in vitro* and *in vivo*. Additionally, within each one of these large categories of biomolecules, there is a huge amount of variation and variability that requires fine-tuning and optimization of every single step along the measurement trajectory, from selecting the method and sample to best fit the scientific question one is asking, to making an observation and interpreting the results.

But, an analytical biochemist may also have interests that don't focus on biomolecules but on small molecules or even ions that surround and oftentimes bind biomolecules with specific structural and functional consequences. Here, we offer a viewpoint of analytical biochemists with a specific interest in metal ions and their biological roles.

Why metal ions? All life has evolved to harness the unique chemistry of metals in order to carry out a multitude of functions. It has been estimated that at least a third of all proteins interact with a metal (Holm et al., 1996) and half of all enzymes require metals to function (Waldron et al., 2009), in addition to the key roles played by free metal ions in regulating resting and action potentials, neurotransmission, osmotic equilibrium and pH. However, their study has been somewhat restricted by limitations that make interpreting multiple roles of a single metal species challenging. Further, *in vitro* evidence of metal-protein interaction does not necessarily reflect a primary biological role (Andreini et al., 2004), while conversely many proteins have metal-binding capacities that are yet to be experimentally characterised (Cvetkovic et al., 2010). With the advent of protein and genomic databases comes new bioinformatics approaches for predicting metal binding sequences (Valasatava et al., 2015), though applying these to visualisations of biological systems in multi-dimensional space remains an analytical and computational challenge. Interactions are often transient, and many regulators of metal homeostasis have indirect effects. For instance, the hormone hepcidin sits atop the hierarchy of iron regulatory proteins, though has no chemical interaction with an iron atom (Hare, 2017). Adverse effects may also be the result of effects not as obvious as hypertension in response to excessive circulating sodium levels: a redistribution of from safe storage in specific protein complexes to indiscriminate reactivity as labile metals can confer toxicity while not altering total metal levels (Valko et al., 2016). The development of analytical tools to accurately visualise *and* speciate metals in biological systems is therefore paramount (New, 2013). The chemical reactivity that makes many metals biologically desirable is also responsible for a wide range of detrimental effects, and careful regulation of metal metabolism ensures that unwanted bioinorganic processes are minimal.

In this Perspective, we discuss how some of these have been overcome and the future directions of imaging metals in biological systems, identifying the prevailing challenges in gaining a clear picture of true metal physiology.

**Techniques for imaging metals in biological systems**

Novel biological roles of metal ions are steadily being discovered, and the well-established one are constantly evolving and being refined. The untangling the intricacies of metal

metabolism is an important scientific pursuit with major implications for understanding not only basic biology but informing and improving clinical practice and biotechnological solutions to challenges like climate change and environmental remediation. For instance, metal levels and chemical state within ice cores can be related to epochal weather patterns spanning millions of years (Dansgaard et al., 1993) or serve as temporal measures of anthropogenic activity in the comparatively short post-industrial revolution era (Barbante et al., 2004).

As mentioned, a powerful way to understand what metal ions are doing in the context of biological systems is to image them directly, preferably *in vivo*. Techniques for imaging metal ions in biological systems are vast and varied, with the resolutions varying from several hundred μm to close to ten nm (Figure 1), and have been reviewed extensively elsewhere (Ackerman et al., 2017; Hare et al., 2015; McRae et al., 2009). These highly sophisticated methods are all based on traditional analytical tools, from fluorescence microscopy with commercially-available or bespoke sensors (Carter et al., 2014) to mass spectrometry and emission spectroscopy techniques (Pozebon et al., 2017; Pushie et al., 2014) Some techniques that have been in use for decades within the broader inorganic chemistry disciplines are now starting to find their way into biology, and include laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), nano-secondary ion mass spectrometry (nanoSIMS), micro-particle induced X-ray emission (μPIXE) spectroscopy and synchrotron-based X-ray fluorescence microscopy (XFM). With these newfound biological applications comes a need for extensive reassessment of the analytical capabilities of each respective technique.

Improving sensitivity, specificity and spatial resolution allows observation of cellular and organ-specific metal metabolism with ever-increasing detail (Hare et al., 2015), yet also introduces new sources of error and potential misinterpretation. Each technique, with all its complexity, requires a comprehensive understanding of the technology to extract a true measure of spatial metal distribution within biological systems. This challenge is further compounded by the critical, yet often-overlooked fourth dimension of imaging—time, which reflects the dynamic nature of metal metabolism and biological activity.

**An incomplete picture: perturbations induced by the imaging technique**

For most biologically relevant metal ions, such as calcium, zinc, and iron, contemporary techniques have adequate sensitivity to detect physiological concentrations, and produce distribution maps. However, native metal ion metabolism is highly susceptible to extrinsic factors that can perturb how the total metal complement of a biological system is distributed. Metal homeostasis is dynamic, and capturing an unadulterated snapshot is the biggest analytical challenge facing the discipline. The primary question is whether images are true reproductions of physiological metal ion metabolism, or merely a depiction of the effects external perturbations introduced by the measuring technique have (summarised in Figure 2).

**Sample preparation.** For centuries, the ability of metal ions to form dyes visible to the naked eye has been used to profile their spatial distribution (e.g. Perls Prussian blue for non-haem iron (II, III); Timm's stain for zinc (II) ions) (McRae et al., 2009). Effective as they are in a clinical setting (Kim Suvarna et al., 2013), they have limited utility as research tools for assessing true quantitative distribution and spatial coordination of metal ions in their native state. Chemical processing required for staining exerts some influence on metal ion levels. Specimens are typically immersed in formaldehyde, dehydrated with alcohol and xylene, infiltrated with paraffin, sectioned, rehydrated, exposed to numerous chemicals during staining, dehydrated again, and coverslipped in a glycerol medium. Not only does each step offer the opportunity for labile metal ions to be lost or contamination introduced, but the very nature of the stain may chemically alter metal ions within the sample.

It is now well accepted that preparative treatments and immunolabelling of cell structures can have a profound impact on both total metal ions' levels and spatial distribution at the micro-scale (Roudeau et al., 2014). Fourier-transform infrared (FTIR) microspectroscopy of brain tissue before and after formalin fixation revealed marked effects on the organic composition of the sample, including leaching of oxidised proteins and lipids, indicating disruption of cell membranes, as well as redistribution and influx of metal ions (Hackett et al., 2011). Post-processing of tissue is often a necessity to prevent motion artefacts as a sample dries during scanning and enable assessment of relationships between spatial distributions of metal ions and their regulatory proteins or organelles. Quantitative assessment of several fixatives showed that concentrations and distributions, as measured by XFM and μPIXE, vary

depending on the chemical used (James et al., 2011; Perrin et al., 2015). The effect of fixatives, or indeed any solvent, on metal redistribution is likely to vary greatly with metal species. What this means for the protein-bound metal ions is that the stronger their metal-protein bond the less susceptible they are to redistribution upon fixation. In general, the strength of metal-protein bond follows the Irving-Williams series (Irving and Williams, 1948), with divalent metals ranked from lowest to highest as follows: $Mg^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+}, Zn^{2+}$ (Foster et al., 2014), with the typical $K_d$ for $Mg^{2+}$ in the mM range (Grabarek, 2011) while $Zn^{2+}$ ions form strong complexes with $K_d$ values at the pM level (Krężel and Maret, 2016), depending on the ligand(s), protein conformation and pH. On the other hand, sparingly soluble monovalent (*e.g.* $Cu^{+}$) and trivalent (*e.g.* $Fe^{3+}$) metal ions are typically found sequestered in strong metal-protein complexes, due to their detrimental effect on cellular physiology. Although reported $K_d$ values for $Cu^{+}$ are broad, spanning $10^{-5}$ to $10^{-18}$ M for the $Cu^{+}$-binding Atox1 (Xiao et al., 2011), this range is still indicative metal-protein complexes being the favourable species. For $Fe^{3+}$ the association is even stronger; transferrin binds ferric iron with a $K_d$ of $10^{-23}$ M (Ponka, 1999). Overall, knowing how tightly the metal ion of interest is bound to its protein partner can help make appropriate precautionary measures to maintain the integrity of the sample and the measurement.

We will use recent updates to XFM protocols to illustrate some ways in which external perturbations can be minimized. Having said that, we would like to point out that the best way to minimize artifacts in any analytical methodology is to have deep understanding of both the method used, the sample used, and the specific analyte in the context of that sample. In this case, XFM is a method that takes advantage of the fact that each metal ion (actually each atom) has unique spectral signature and upon exposure to an X-ray beam emits fluorescent X-rays with specific, metal ion dependent wavelengths. This technique has been successfully used in material science to analyse samples such as ceramics or glass. What limits application of XFM to biological samples is the strength of X-ray beams needed, which causes radiation-induced damage, and leads to perturbed biochemical activity, altered metal speciation and redistribution (Paunesku et al., 2006). One major way to protect biological samples from radiation-induced damage is the use of cryogenic preservation. Upgrades to XFM beamlines, including those at the three 'third generation' synchrotrons, the Advanced Photon Source in the USA (Chen et al., 2014b; Deng et al., 2017), the European Synchrotron Radiation Facility

in France (Cotte et al., 2017) and Spring8 in Japan (Matsuyama et al., 2010), combine both high-energy capabilities for sub-micron resolution imaging with cryo-cooling (<110 K) in a vacuum chamber for sample preservation to image metal ions in cells that exist in a state of 'suspended animation'. The XFM beamline at the Australian Synchrotron (Paterson et al., 2011) recently installed a 'cryostream', where a continuous flow of liquid $N_2$ is directed onto the sample at atmospheric pressure (Figure 3a).

Cryopreservation can be used to provide the closest biological 'standard' of an undisturbed cellular system, and is currently being used to examine the effects of other perturbations such as chemical processing. For example, plunge-freezing and subsequent lyophilisation of murine fibroblasts was compared to fixation by glutaraldehyde or paraformaldehyde (Jin et al., 2017). As expected, fixation had different effects on each analyte. For example, calcium was most prone to contamination, leading to two to four-fold increase. Additionally, efflux of chloride and potassium ions was observed relative to lyophilised samples (72-84% loss of $Cl^-$; 99.3-99.7% loss of $K^+$), while the first-row transition metal ions were somewhat stable. Compared to cryopreserved (100 K) cells, fixation and lyophilisation appeared to have little influence on compartmentalisation at 200-250 nm resolution, although there was some evidence of incongruity in nuclear zinc ion distribution beyond this scale.

For some intact multicellular organisms, such as *Caenorhabditis elegans*, XFM can be performed on fully hydrated samples at room temperature, with minimal disruption to both metal levels (including mobile $Ca^{2+}$) and spatial distribution at the micron level compared to lyophilised samples (James et al., 2013a). While *C. elegans* are remarkably resistant to ionising radiation, room temperature analysis is not a viable option for more sensitive samples. Hard X-rays induce photoreduction of metals, precluding spatial measurement of oxidation state *via* X-ray absorption near-edge structure (XANES) spectroscopy, where beam dwell time must be increased, thereby potentially mobilising the static metal pool. High doses of radiation (50 MGy) results in total loss of iron compartmentalisation in *C. elegans* (James et al., 2016b); and the suggested limit for hydrated blood cells is ~1 MGy (Fayard et al., 2009), based on morphological damage to the cell; metal redistribution likely occurs prior to physical disruption of the cell membrane. Photoreduction, like metal-protein complex stability, is species-specific, affecting lower mass elements more markedly. X-ray absorption near-edge

structure experiments using soft X-rays recommend a limit of 0.1 MGy to prevent photoreduction of copper (II) in organic complexes (Yang et al., 2011), and XANES tomography delivering a total radiation dose of approximately 2.2 MGy to intact lyophilised *Drosophila melanogaster* larvae has been used to spatially differentiate regions of predominant cupric and cuprous ligands (James et al., 2016a).

**Challenges of using cultured cells.** The ideal medium for observing native metal distribution is in a living organism, making the work done on imaging fully hydrated samples of *Caenorhabditis elegans* or intact lyophilised *Drosophila melanogaster* larvae that much more valuable and significant. However, most work done in imaging metal ions in biological systems today is done in cell cultures under physiological conditions. Using the cell cultures in this context is not without shortcomings as metal ion levels present in the growth media can be substantially different from the in vivo metal ion levels. For example, cultured neurons and astrocytes can subsist on iron supplies that are orders of magnitude lower than that within the brain (Hare et al., 2013). This does not necessarily preclude information on cellular metal ion metabolism being extracted from cell culture work, though it does suggest that these factors should be taken into account when interpreting the results and their implications.

Maintaining the *in vivo* redox environment is another common challenge of imaging fixed and live cells alike. The redox activity in cells and biofluids maintained in normal physiological conditions continues *ex vivo* (Lam et al., 2016), and exposure of samples to air can induce autoxidation. For example, metallothionein is extremely sensitive to changes in redox state and a shift to oxidising conditions disrupts the thiolate cluster binding zinc and copper, releasing it into the cytoplasm where it either remains ionic or is incorporated into other proteins (Kang, 2006).

**Sensor interference.** A subset of techniques currently employed to image metal ions in biological system requires the use of imaging agents, which themselves can affect metal distribution. For example, fluorescent metal sensors are often used to report on the presence of a specific metal ion and its intracellular distribution and dynamics (Carter et al., 2014). Their most common sensor design includes a fluorophore that generates a measurable signal, or a measurable change in the signal, upon metal ion binding to a metal binding part of the sensor.

Given that metal binding is a required step for the sensor to work, this means that the sensor itself can perturb the distribution and concentration of its intended target. This is especially problematic when investigating metal ions with the labile pool, where probe binding can trigger unintended transfer of protein-bound metal to the sensor, or movement of a metal ion from one intracellular location to another upon probe binding. Such perturbations can be minimised by the design of probes that are sufficiently bright and sensitive to be used at concentrations far below that of the labile pool by tuning the binding affinities to be well below those of metalloproteins (Carter et al., 2014), or using reaction-based probes that do not directly bind metal ions (Ackerman et al., 2017).

Since it is not possible to completely eliminate the potential sensor interference, it is important to determine appropriate protocols to control for any such effects. For example, measurement of metal levels with varying sensor concentration can enable determination of anomalous effects. This has been successfully applied to observe a genetically-encoded $Zn^{2+}$ sensor present in the cells at micromolar concentrations had minimal effects on cellular $Zn^{2+}$ pools, while higher concentrations of an exogenous $Zn^{2+}$ sensor altered cellular $Zn^{2+}$ buffering (Qin et al., 2013). At the same time, measurement of other parameters such as cellular viability, mitochondrial function and levels of reactive oxygen species enable confirmation that the probe does not have antecedent effects. In the future, the simultaneous application of other methods to assess metal ion levels, such as expression levels of metal-sensing proteins, will have significant impact on the interpretation of data from fluorescent sensors.

***In vivo* metal ion imaging.** *In vivo* imaging methods may provide a window into the unperturbed system, as long as both phototoxicity and fluorescent metal sensor interference are well controlled for. Combination of virally transfected genetically encoded indicators with well-established imaging modalities like confocal or multiphoton microscopy have enabled subcellular observation, for example, of calcium dynamics in intact brains and multicellular model organisms (Helmchen and Denk, 2005; Logan et al., 2014; Tian et al., 2009); however, the trade-offs include high light exposure and slow frame rates, which lead to cell stress and reduced temporal resolution of dynamic intracellular processes. Swept confocally-aligned planar excitation (SCAPE) microscopy, a recently developed method that permits high speed, translationless volume imaging of a variety of samples including behaving animals through a

single objective lens dramatically reduces light exposure and increases imaging speed, while maintaining cells in their natural environment (Bouchard et al., 2015).

In summary, the factors that can influence metal distribution and chemical speciation are diverse and often application-specific, and therefore experiments should be approached on a case-by-case basis, factoring every possible source of exogenous interference into the analysis and controlling them where possible. These factors by no means immediately invalidate a picture created, though not appreciating the potential effects can leave the analyst prone to misinterpretation.

**All that glitters is not gold: artefacts in source of the signal**

The potential perturbation of homeostasis by the imaging process itself is a significant source of error in understanding metal-based biological systems. However, even in cases where any perturbation is eliminated or controlled for, it is possible that the imaging technique generates artefacts that can wrongly be interpreted as positive signals.

For example, LA-ICP-MS is a powerful method to analyse elemental composition of solid materials. It uses a focused micrometre-range diameter laser beam to discretely vapourise the sample surface, with the ejected particles swept to an argon plasma where they are atomised, ionised and transferred to a mass spectrometer for separation on the basis of the mass-to-charge ratio. First proposed in 1985 as a means to circumvent the need for dissolution of minerals and other difficult solids in concentrated acids (Gray, 1985), the technique is now commonplace in the earth and environmental sciences (Durrant and Ward, 2005). Quadrupole mass analysers in typical LA-ICP-MS systems are extremely sensitive, but are unable to differentiate between isobaric (*i.e.* two elements with the same isotopic mass) and polyatomic (*i.e.* comprised of two or more constituent elements with equal atomic mass to the analyte, usually oxygen or argon adducts) interferences. Solution nebulisation ICP-MS uses collision gases to remove polyatomic species, though the significantly smaller sample volume and resultant signal intensity in LA-ICP-MS precludes its use for metals at low abundance. Reaction gases like $H_2$ have been optimised for specific analytes (*e.g.* removal of $^{40}Ar^{16}O^+$ on $^{56}Fe^+$ (Lear et al., 2012b)). Both types of interferences can be avoided where

alternative isotopes are available, though at the potential cost of sensitivity when natural isotopic abundance of the alternate isotope is lower. In extremely rare cases, monoisotopic elements may be subject to polyatomic interference (*e.g.* $^{59}Co^{16}O^{+}$ on $^{75}As^{+}$ in a cobalt-rich matrix). The recent application of mass-shifting to LA-ICP-MS imaging (Bishop et al., 2016), where gases with favourable reaction kinetics are used to preferentially form charged adducts that are detected as proxies (*e.g.* $^{80}Se^{+} + O_2 \longrightarrow {}^{80}Se^{16}O^{+}$ to avoid isobaric interference from $^{40}Ar_2^{+}$), can be used in such a scenario.

XFM suffers from similar matrix-based interference *via* emission energy overlap (*e.g.* As $K_{\alpha 12}$ edge emission obscures Pb $L_{\alpha 12}$ emission). Increasing the monochromator energy to excite alternate fluorescent emission lines can overcome this potential artefact in most biologically-relevant situations (Pushie et al., 2014)

For application of fluorescent sensors, artefacts can be introduced by the application of intensity-based probes, for which changes at a single wavelength signal metal concentration (New, 2016). For systems in which the metal-free probe emits residual signal at this wavelength, the accumulation of metal-free probe is indistinguishable from a lower concentration of metal-bound probe, and regions of high probe accumulation can therefore be mistaken for the presence of metal ion. For the subset of probes that turn off as a response to metal ion binding, exemplified by the widely-used calcein AM iron sensor, image analysis is further complicated by the fact that the absence of probe gives the same output as the presence of both probe and metal (Hirayama and Nagasawa, 2017). A further interference that can confound the use of fluorescent sensors is the presence of multiple metal ions that bear structural similarities (Foster et al., 2014). Sensors must therefore exhibit exquisite selectivity for the metal of interest, particularly for metal ions that exist in much lower buffered intracellular concentrations, such as copper (I) (New, 2013).

The data obtained from bioanalytical experiments therefore must not be interpreted blindly without consideration of potential artefacts. Most importantly, a comprehensive set of control experiments should accompany all data collection, and the scientific community must call for rigour in this regard.

**Location, location, location! Imaging sub-cellular localisation**

The term *metallomics*, coined in 2004 by Hiroki Haraguchi, was intended to encompass all aspects of the cell involved in metal metabolism, not just the metal ion itself (Haraguchi, 2004, 2017). In accordance with this definition, correlating spatial metal ion distribution with cell type, organelles and regulatory proteins remains of paramount importance. Thus, new approaches for imaging organellar metal ion distribution in the wider context of cellular regulatory pathways and biological function are essential research priorities (Chang, 2015).

Studying how metal ions associate with cellular compartments bears some of the same restrictions as we discussed above, although there are some additional issues to keep in mind. For example, when trying to understand whether a metal ion and a protein co-localise and/or interact, simple visual colocalisation of the signal that originates from the protein and the metal ion species can't be taken as the proof for the interaction. Thus, much work within the microscopy community has been aimed at developing robust quantitative measures of real correlation between pairs of biological molecules (Dunn et al., 2011), and this is also true for imaging in metallomics. The two most commonly used are spatial measurements of Pearson's correlation (Barlow et al., 2010) and Mander's overlap coefficient, with Pearson's correlation suggested to be superior, due partly to its simplicity (Adler and Parmryd, 2010). Li's intensity correlation analysis and quotient, which measures the sum of deviation from the mean pixel intensity in entire images (Li et al., 2004) is a powerful alternative for correlation analysis of metal ion images (Hare et al., 2016a) and assessing redistribution as a result of extended exposure to ionising radiation (James et al., 2016b). Open source image analysis software, such as Fiji, offers simple modular add-ons for this type of analyses (Schindelin et al., 2012). Specialised software for specific imaging modalities, such as LA-ICP-MS, have also been developed, including the Biolite add-on (Paul et al., 2015) for *iolite* (Paton et al., 2011), which integrates chemometric capabilities, LA-iMageS for advanced data analysis (López-Fernández et al., 2016), scripts for fast data reduction (Sforna and Lugli, 2017), and *R*-based tools with the ability to examine colocalisation of metal ions using Mander's overlap with photomicrographs of immunolabelled sections within a web-based interface (Niedzwiecki et al., 2016). In the latter example, where immunolabelling and LA-ICP-MS imaging were

performed on alternate sections, the robustness of correlation analysis is somewhat constrained by changes in analyte distribution moving through the depth of the sample.

For LA-ICP-MS imaging, correlation analysis should always be approached with caution, even when imaging multiple metal ions in a single tissue section. Over 90% of systems use sequential quadrupole-based mass analysers (Potter, 2008), which can result in a 'spectral skew' where abrupt changes in sample composition may not be reflected in each measurement cycle of transient signal (Sylvester and Jackson, 2016). This can be mitigated by decreasing integration times for each measured mass, reducing the number of analytes, decreasing the laser scan speed (Lear et al., 2012a) or using faster quadrupole analysers (Van Malderen et al., 2016). Recently developed time-of-flight ICP-(TOF)-MS systems with simultaneous detection capabilities will overcome this limitation (Gundlach-Graham et al., 2015), though achieving adequate sensitivity for high-resolution imaging of bio-elements remains a technical challenge (Gundlach-Graham and Günther, 2016). Coupling TOF-based ICP-MS systems with fast-washout ablation cell designs (Wang et al., 2013) and high-repetition rate lasers (Diwakar et al., 2014) will be a significant technological leap, reducing analysis time by at least a factor of five and allowing simultaneous detection of nearly every element on the periodic table. Although not an example of native metals, imaging using LA coupled to a CyTOF 'mass cytometer' (an ICP-TOF-MS design variant) for multiplexed imaging of 32 lanthanide-isotope tagged antibodies in a single experiment at 1 µm resolution (Giesen et al., 2014) cannot be overlooked, nor should a similar application using nanoSIMS for imaging 10 lanthanides at 200-300 nm resolution (Angelo et al., 2014). Although the CyTOF is unable to detect < 80 atomic mass units, where most biologically-relevant elements fall, it demonstrates the future capabilities of 'true' resolution LA-ICP-MS imaging using simultaneous detectors.

Fluorescent metal sensors can be readily tailored to probe organelle-specific metal pools. A number of organelle-targeting groups have been reported, both those based on short peptide sequences (*e.g.* nuclear-localisation sequences (Lange et al., 2007)) and small molecules (*e.g.* lipophilic cations such as triphenylphosphonium for mitochondrial targeting (Murphy, 2008)). While organelle targeting is straightforward for genetically-encoded sensors, there are some organelles such as the endoplasmic reticulum and Golgi apparatus for which targeting of small

molecule probes cannot be reliably achieved. Organelle-targeted sensors generally requires verification of localisation with more common organelle markers (*e.g.* DAPI staining of DNA in the nucleus).

Examples of complementary imaging techniques being used to construct a more complete picture of metal ion metabolism are emerging as technology becomes more accessible. For instance, Que et al. used a combination of a chemical sensor (ZincBY-1) with dynamic live-cell fluorescence microscopy, scanning transmission electron microscopy, XFM and three-dimensional tomography to temporally characterise the formation of zinc-loaded vesicles that precede extracellular 'zinc sparks' essential for egg-to-embryo maturation post-fertilisation (Que et al., 2014).

Where we previously proposed a more unified approach to metal imaging that assesses concentration, spatial distribution and chemical speciation (Hare et al., 2015), we now emphasise the importance of employing complementary imaging protocols that enable visualisation of metal-associated biomolecules and direct association with organelles and cell types. For instance, Compton inelastic scatter emitted during XFM can be used for discerning micron-scale neuroanatomy, as the degree of scatter is proportional to density. This approach was used to examine apparent accumulation of iron, copper and zinc within β-amyloid plaques in the APP/PS1 model of Alzheimer's disease with no sample pre-treatment (James et al., 2017). Plaque density was used both as a fiducial marker for appraising metal ion content and to normalise metal ion concentrations to total protein levels. In agreement with other studies that employed off-line FTIR measurement of protein density in plaques to normalise metal concentration (Leskovjan et al., 2011; Leskovjan et al., 2009), only zinc showed significant elevation within aggregates, with iron actually decreased relative to surrounding tissue.

No biological process functions in isolation, and similarly interactions between metals and biomolecules, or metals and other metals, can have a domino effect on the entire biochemical environmental of a living system. These bioinorganic 'ripples' are both spatial, as the signalling effects or a chemical change spreads from the initial site of a chemical reaction, and also temporal, as a cell or system responds to the stimulus of a sudden change. Determining

if a response is a cause, effect or entirely separate response begins with pinpointing a precise location, though no two chemical species are immediately 'guilty by association'. Complete interpretation of the specific role of a metal-associated biomolecule cannot be drawn from one picture alone; rather, multiple outputs, as well as observation within the context of biochemical mechanisms that have been previously elucidated combine to give a greater overall picture, both literally and metaphorically.

**Big trouble in little images: the challenges of increasing resolution**

With the development of technologies capable of probing cell and metal ion biochemistry at the nanometre scale comes new technical challenges. Examining colocalisation at the nanoscale is still applicable to images where multiple analytes were measured simultaneously, like in XFM mapping of copper and zinc ions within neuron dendrites and spines (Perrin et al., 2017). However, limitations raised above are amplified at sub-micron resolution, particularly when imaging uses independent measurements that require separate techniques. For example, some measurements require off-line live cell imaging of organelles marked with fluorescent dyes prior to metal ion mapping. In subsequent steps, the organelle images are aligned with metal ion images to create a complete picture, and this alignment of sequential imaging techniques has added uncertainty as image resolution increases. Thus, data should be viewed with some caution, as corresponding images presented side-by-side are only indicative of colocalisation between metal ions and cell ultrastructure, even when the same sample is analysed (see (Dučić et al., 2017; Grubman et al., 2014; Jiang et al., 2014; Kashiv et al., 2016; Matsuyama et al., 2009) for examples). High precision microscopy stages can be adapted for XFM configurations, as demonstrated by McRae et al., who applied Li's correlation analysis to profile changes in transition metal distribution through stages of cell division (McRae et al., 2012). In future, there is hope and an expectation that XFM beamlines will adopt online fluorescence microscopy to allow true correlative imaging, as has been demonstrated on soft X-ray nanoprobes (Hagen et al., 2012).

Information on cellular structure should be obtained either simultaneous to metal ion imaging, or using complementary online methods that do not require sample repositioning. Micro-PIXE is an attractive option, in that both Rutherford backscattering and ion

transmission can be collected for physically characterising the physical properties of the sample (in this case density and thickness, respectively) concurrent to detection of elemental X-ray emission (Hare et al., 2015).

In some cases, the unique properties of specific cellular features can be used as a proxy for determining their spatial location when simultaneously measuring metal ion levels. Typically, this is limited to dense proteinaceous inclusions, or biological structures that natively have high affinity to metal ions compared to the surrounding space, such as neuromelanin (Bohic et al., 2008). Neuromelanin indiscriminately binds most biological elements due to the high areal density of available ligands (Hong and Simon, 2007) and this feature is a useful biochemical marker for assessing the changing concentration of metals within pigmented dopaminergic neurons in Parkinson's disease (Davies et al., 2014), as this biopolymer is the end-stage product of dopamine metabolism and is depleted in the degenerating brain (Hare and Double, 2016).

Nanoparticles are of great interest for drug-delivery (Chen et al., 2013) and as potential cytotoxins (James et al., 2013b), and may also be useful markers due to their unique elemental composition. Using nanoparticles as proxies for specific cell types or fiducial markers is typically secondary to a biological purpose, and thus any impact they may have on native metal ion homeostasis is not well understood. Gold nanoparticles tagged to antibodies have been used in electron microscopy for several decades to visualise specific biomolecules (Faulk and Taylor, 1971) and have been used in LA-ICP-MS imaging to examine associations between metals and immunolabelled proteins (Hare et al., 2014).

XFM is probably the most versatile imaging technique for simultaneously obtaining structural and metal ion composition information. The interactions between the sample matrix and hard X-ray beam generates not only fluorescent photon emission from metal ions but also diffraction images and phase distribution maps. From this data, an approach like ptychography can extract information on electron density, and thus cellular structure (Giewekemeyer et al., 2010). Overlapping illumination as the samples traverses the X-ray beam permits construction of images at low nanometre resolution. A limitation of ptychography is the wide dynamic range of diffraction patterns, requiring long dwell times or

repeated measurements to collect sufficient signal to reconstruct an image. Soft X-rays (typically < 1 keV) are preferable for ptychography (Maiden et al., 2013), though the fluorescent yield is sub-optimal for XFM at sensitivities necessary for most biological specimens. Fluorescence can be deconvoluted from ptychographic data— ptychographic algorithms used to deconvolute images can actually enhance XFM resolution (Deng et al., 2015)—though self-absorption limits application to thin samples such as single cells (Vine et al., 2012). Additionally, at such high resolution, artefacts in ptychographic images resulting from sample preparation become particularly troublesome as structural damage from plunge freezing and lyophilisation becomes highly apparent at the nanoscale. Cryogenically-preserved samples are preferable, though optimised pytchography with 0.52 keV soft X-rays and XFM using a 10.1 keV hard X-ray incident beam performed sequentially has been demonstrated for room temperature imaging of hydrated fibroblast cultures (Jones et al., 2016).

At smaller scales, self-absorption effects within the sample also become problematic in XFM. Transmission of fluorescence is dependent on the sample matrix, the incident X-ray beam energy and thickness of the sample (Figure 4; (Davies et al., 2015)). According to the inverse square law, decreasing incident beam diameter to the sub-micron level reduces the flux reaching the detector, compounded by fewer emitted photons and self-absorption events.

For scanning techniques such as XFM and mass spectrometry, the precision of sample stage mechanics must exceed the resolution of the resulting image. Vibration of stage components, such as coarse-scanning stepper motors or piezo-flexure stages for nanoscale imaging can produce motion artefact when scanning at high resolution. Stage movement is also complicated when using cryogenic conditions; thermal drift can be as high as 0.4 nm $s^{-1}$ at 110 K (Maser et al., 2000). Cryogenic conditions intended to prevent ice crystal formation and maintain a stable temperature (Figure 3b) can also be unpredictable; an example study that performed simultaneous ptychography and XFM of a single *Chlamydomonas reinhardtii* cell highlighted artefact ptychographic images, with a build-up of ice due to a 33 K increase in sample temperature over the course of a 6.5 hour scan, as well as localised damage from radiation exposure when stage motion was abruptly halted for an undisclosed amount of time (Deng et al., 2015). As these technicalities are overcome (Deng et al., 2017), nanoimaging of

both structure and metal ion content will peer deeper into the inner workings of the cell. One noteworthy example is the recently-described metal ion distribution within a single chromosome (Yan et al., 2016).

Super-resolution microscopy techniques achieve spatial resolution that breaks through the diffraction-limited 200 nm resolution barrier of a conventional light microscope and allow the observation of many biological structures not resolvable in conventional fluorescence microscopy (Fernández-Suárez and Ting, 2008). Super-resolution methods can be broadly classified into two groups: i) illumination-based techniques that employ spatially or temporally modulated excitation light to extract super-resolution information from multiple fluorophores; and ii) single molecule localisation microscopy (SMLM) which accurately determines the position of individual fluorescent molecules. In SMLM only a fraction of the fluorescent molecules present in the sample are fluorescent at any point in time, and this stochastic switching can be achieved *via* photo-activatable or photo-switchable proteins, or by inducing reversible blinking in photochromic dyes (Fernández-Suárez and Ting, 2008). While the latter approach is often more readily adaptable in the laboratory, its use in metal ion imaging is hampered by the fact that reversible blinking is induced by addition of chemicals that generate an anoxic and acidic chemical environment, and substitution with simple media (Keller et al., 2013) would still be expected to affect the natural equilibrium of cellular metals.

In practical terms, this means that the application of SMLM in metallomics is limited to the use of fluorescent protein based sensors. Developing improved sensors will require a closer collaboration between inorganic chemists, who are developing the sensors, and microscopists, who are applying them (Hare and New, 2016), as the next generation probes require higher quantum yields, long fluorescent lifetimes and, ideally, improved ‘on-off’ states (New, 2016). Additionally, differentiating between regions of interest and background becomes more challenging at the low-nanometre scale (Carter et al., 2014), and often this necessitates the use of a total internal reflection fluorescence (TIRF) microscope, thereby essentially limiting the imaging to the cell membrane.

While SMLM methods yield the most dramatic improvement in resolution, their inherently slow speed—a consequence of needing to collect large numbers of frames to assemble one super-resolved image—limits our ability to image fast dynamic processes in living cells as well as image in three dimensions. Furthermore, high speed and limited photo-toxicity are advantageous in avoiding changes of metal ion homeostasis caused by the imaging process itself.

Generally speaking, lightsheet microscopy offers this desired increase in imaging speed compared to point-scanning techniques. In lightsheet microscopy only a thin slice of the sample is illuminated perpendicularly to the direction of observation, thereby reducing photodamage and increasing acquisition rates more than 1,000 times compared to point-scanning methods. The limiting factor with respect to the axial resolution is the thickness of the lightsheet, but development of ultrathin lattice light sheets in combination with structured illumination microscopy (SIM) (Chen et al., 2014a) now allows super-resolution 3D imaging of live cells and tissues at <4s intervals, at bleaching rates that are an order of magnitude lower than with confocal microscopy. Vast improvements in maintaining cell health and continued development of probes for redox-active metals that meet the necessary criteria for super resolution microscopy will see this field blossom in coming years and move beyond traditional targets, such as $Ca^{2+}$ (Oheim et al., 2014). It is encouraging that metal sensors, such as a reversible bipyridine derivative on a DNA scaffold for $Cu^{2+}$ sensing, are being developed with both specific biological questions (*i.e.* temporal changes in cellular $Cu^{2+}$) and the capabilities of nanoscale microscopy in mind (Schwering et al., 2011).

The application of the latest technology for ultra-high-resolution imaging of metals in biology has depended on the technique used. Although fluorescent sensors for standard-resolution confocal microscopy are widespread there are few examples of applications employing super resolution microscopy, while nearly all synchrotron microprobes being brought online have sub-micron imaging capabilities. New applications will undoubtedly be driven by need.

## Conclusion

Imaging the metal complement of the living cell or intact organism, in real time with no external influences could be described as the proverbial Holy Grail of metallomics, and like its mythical counterpart, will likely remain out of reach. It is impossible to have complete certainty that sample preparation, cellular chemistry of a fluorescent sensor, or even the technique itself does not perturb biochemical conditions to even the slightest degree. Unlike traditional approaches to analytical method validation, getting a standard reference for native biological states is, and will remain, unobtainable. In truth, few of these approaches have undergone the rigours of extensive analytical method validation typical for more routine applications (Green, 1996).

Throughout this perspective we highlighted many of the pitfalls of techniques commonly used to image metals in biology. Overall, the points where errors can be introduced and propagated revolve around the sample preparation, image interpretation and the application of new analytical methods where limitations are not yet fully understood.

Does this mean that bioimaging of metal ion metabolism to date is invalid? Not at all. As mentioned in the Introduction, provided the methods are used appropriately, with a full acknowledgement of their limitations and the results interpreted conscientiously, these studies can offer illuminating insights into metal ion metabolism and physiology. This does not mean that the work of analytical chemists is done, as we will continue to refine methods to ensure error in measurement is as small as practicable, and the range of questions that can be asked is a broad as possible. Well-designed imaging experiments that control every addressable variable, apply multiple imaging modalities for comparative purposes and, importantly, acknowledge the potential limitations and sources of error will continue to provide new insight into metal metabolism in health and disease. Imaging metals has provided invaluable insight into the biology of life, development, ageing and disease; has aided in the development of new metal-containing drugs and therapies that target dysfunctional metal metabolism; and will continue to provide a window into the fundamental biochemical processes that make biological systems function, or not function properly. The onus will be equally shared between the scientists developing the tools and those asking

pertinent biological questions to ensure that the latest technical developments are applied and interpreted appropriately.

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**Main figure titles and legends**

**Figure 1: Metal-specific techniques for macro-to-nanoscale imaging.** Choice of imaging approach is dependent on desired spatial resolution, sample type and species-specific capabilities. LA-ICP-MS (red) has a wide spatial range, and is well suited for quantitative imaging of human tissue sections (left; frontal cortex, reproduced from (Hare et al., 2016b) with permission from Elsevier, Copyright 2016) and whole organs in model systems (centre; coronal section of murine brain, reproduced from (Hare et al., 2012) with permission from the American Chemical Society, Copyright 2012). Custom-designed ablation cells coupled to simultaneous TOF-based MS detection have allowed subcellular imaging of 32 lanthanide isotope-tagged antibodies (right; three-colour image of human breast cancer section, reproduced from (Giesen et al., 2014) with permission from Nature Publishing Group, Copyright 2014). XFM (orange) is capable of ~100 nm resolution for single cells (left, P, S, Ca and K distribution in cryopreserved *Chlamydomonas reinhardtii*) with simultaneous ptychographic reproduction of structure at <20 nm (reproduced from (Deng et al., 2017) Creative Commons CC-BY 4.0 License); and micron resolution for whole organisms (right bottom panel, Fe in *Caenorhabditis elegans*). XFM can also be used to perform X-ray absorption near-edge structure (XANES) imaging; here principal component analysis and *K*-means clustering is used to spatially assign predominant Fe XANES spectra to spatial regions of interest (left bottom panel, both reproduced from (James et al., 2016b), Creative Commons CC-BY 4.0 License). X-ray emission from µPIXE (gold) has a similar spatial resolution to XFM (Ca, Fe and Zn in ammonium acetate washed, plunge-frozen and lyophilised PC12 cells, reproduced from (Perrin et al., 2015) with permission from the Royal Society of Chemistry, Copyright 2015). Confocal fluorescence microscopy (light blue) resolution is diffraction-limited, though ~500 nm resolution is sufficient to visualise fluorescence emitted by metal sensors at the subcellular level (top, ZincBY-1 fluorescence in green showing $Zn^{2+}$ at the extent of a mammalian oocyte with DNA marked using a Hoechst 33342 probe in blue, reproduced from (Que et al., 2014) with permission from Nature Publishing Group, Copyright 2014); bottom, InCCu1 ratiometric sensor emitting flue fluorescence in the presence of mitochondrial copper (I), reproduced from (Shen et al., 2016) Electronic Supplementary Material with permission from the Royal Society of Chemistry, 2016). NanoSIMS (green) uses a focused ion beam to emit secondary ions detected by mass spectrometry to achieve ~200

nm spatial resolution images (composite image of lanthanide-isotope labelled antibodies in human breast tumour, reproduced from (Angelo et al., 2014) with permission from Nature Publishing Group, Copyright 2014). To date, the highest resolution metal-specific super resolution microscopy (dark blue) approach produced 30 nm resolution images of a $Cu^{2+}$ reversible bipyridine derivative-sensor localised within microtubules (reproduced from (Schwering et al., 2011) with permission from John Wiley and Sons, Copyright 2011).

**Figure 2: Mechanisms of metal loss and cellular redistribution.** Subcellular metal imaging modalities, such as XFM and fluorescent sensing can perturb cellular distribution; as can sample preparation steps. These include, but are not limited to: membrane permeabilisation *via* chemical fixation; photoreduction and subsequent mobilisation of metals; motion artefact as the sample is scanned; organelle redistribution, non-physiological conditions or *ex vivo* enzymatic activity; freeze-fracturing; and altered equilibria between labile and static metal pools.

**Figure 3: Cyrostream configuration at the Australian Synchrotron XFM beamline.** a) The standard X-ray fluorescence configuration at the Australian Synchrotron includes the use of a multi-channel array detector positioned in the backscatter geometry, with an additional silicon drift detector positioned at 90° to the sample. A vertically-mounted cryostream of liquid $N_2$ is used to maintain sample temperature at approximately 100 K. b) Ice build-up on the sample mount is present after extended cryostream use (red box in (a)).

**Figure 4: Sample thickness and transmission efficiency for XFM.** Predicted transmission efficiency of iron, copper and zinc K-edge X-ray fluorescence emission using an approximated empirical formula based on literature values for the elemental composition of ovine, porcine and bovine neurological tissue (reproduced from (Davies et al., 2015) with permission from the American Chemical Society, Copyright 2015).