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Techniques for measuring cellular zinc

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Abstract

The development and improvement of fluorescent Zn^{2+} sensors and Zn^{2+} imaging techniques have increased our insight into this biologically important ion. Application of these tools has identified an intracellular labile Zn^{2+} pool and cultivated further interest in defining the distribution and dynamics of labile Zn^{2+} . The study of Zn^{2+} in live cells in real time using sensors is a powerful way to answer complex biological questions. In this review, we highlight newly engineered Zn^{2+} sensors, methods to test whether the sensors are accessing labile Zn^{2+} , and recent studies that point to the challenges of using such sensors. Elemental mapping techniques can complement and strengthen data collected with sensors. Both mass spectrometry-based and X-ray fluorescence-based techniques yield highly specific, sensitive, and spatially resolved snapshots of metal distribution in cells. The study of Zn^{2+} has already led to new insight into all phases of life from fertilization of the egg to life-threatening cancers. In order to continue building new knowledge about Zn^{2+} biology it remains important to critically assess the available toolset for this endeavor.

Introduction

Zinc is an essential metal, and the proper balance of zinc is critical to the health of organisms [1]. At the molecular level, the coordination of zinc ions (Zn^{2+}) to individual proteins and enzymes either to stabilize protein structure or to create a catalytic center has been well characterized [2]. Further, bioinformatics studies predict that 10% of human proteins require Zn^{2+} for their structure and function [3], indicating that Zn^{2+} is necessary for the proper function of thousands of proteins. Work at the cellular level strives to connect our molecular understanding of Zn^{2+} biology to observations correlating organismal Zn^{2+} status and health. Mammalian cells maintain a total concentration of Zn^{2+} in the hundreds of micromolar [4]. While most of this Zn^{2+} is bound to proteins and inaccessible, a pool of labile Zn^{2+} , which is non-protein bound and complexed to a variety of small molecule ligands [5], has been detected in the cytosol with a concentration in the hundreds of picomolar. Furthermore, there is growing evidence of a labile Zn^{2+} pool in organelles [4, 6]. The concentration of Zn^{2+} in cells and across organelles is maintained by a complex set of 24 Zn^{2+} transporters [7, 8]. This pool of labile Zn^{2+} is available to bind to newly synthesized

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proteins, but the importance of Zn^{2+} to organismal and proteomic stability and the cellular energy allocated to the transportation of Zn^{2+} has led to the hypothesis that Zn^{2+} may also serve as a signal [9].

In order to gain insight into the biology of Zn^{2+} at a fundamental level it is important to understand both the distribution and dynamics of accessible Zn^{2+} in cells. Research into the distribution of Zn^{2+} generally strives to generate a detailed quantitative map of where Zn^{2+} is located in order to define possible sources and sinks of labile Zn^{2+} and identify whether there is a heterogeneous distribution of total Zn^{2+} . To rigorously assign Zn^{2+} to a specific organelle, experimental approaches must be high enough resolution to unambiguously distinguish organelle structures (such as electron microscopy techniques) or the probe being used to measure Zn^{2+} must be restricted to a specific organelle. Fluorescent probes and elemental mapping techniques (see below) applied can both be used to develop such a Zn^{2+} map. As detailed in this review, the two approaches provide complementary information on different types of samples: live cells in the case of probes, and fixed samples or fixed time points in the case of elemental mapping techniques. On the other hand, research into the dynamics of Zn^{2+} must be carried out in live cells using fluorescent sensors in order to obtain temporal information about Zn^{2+} fluxes. Ideally, such tools will be sensitive to small changes in Zn^{2+} concentrations. For measuring both distribution and dynamics, tools must respond specifically and selectively to labile Zn^{2+} .

A growing number of fluorescent small molecule probes and protein-based sensors are being developed to measure both the dynamics and subcellular distribution of labile Zn^{2+} in live cells. A wide range of sensors has been developed with diverse characteristics including: targeting to different organelles, signal detection at various wavelengths, and binding to Zn^{2+} with altered affinities. The application of probes and sensors in live cell imaging experiments requires many controls to evaluate whether the sensor is selectively measuring labile Zn^{2+} , targeted only to the cellular area of interest, perturbs Zn^{2+} homeostasis and regulation, and validate that the signal changes are sensitive only to fluctuations in Zn^{2+} [6]. Thus, it is both wise and prudent to employ complementary imaging methods on fixed samples or fixed time points to corroborate and further define the cellular distribution of zinc, without adding probes to cells. These data can strengthen and confirm interesting observations gathered through the development and use of Zn^{2+} sensors and probes.

In the past several years, several elemental mapping methods including mass-spectrometry-based and X-ray fluorescence-based techniques have greatly improved in sensitivity and spatial resolution, allowing for the study of trace metal distribution at the cellular and subcellular levels [10–13]. By analyzing the significance of heterogeneous metal distributions at high resolution and quantifying biologically relevant changes in these distributions, researchers have gained insight into physiology, pharmacology, toxicology, pathology, and other disciplines. Besides providing a snapshot of total elemental distribution, mass-spectrometry approaches are capable of resolving individual isotopes of Zn^{2+} and other metals, and X-ray techniques can be used to determine chemical speciation. The information obtained from these approaches can complement studies using tools for measuring labile Zn^{2+} pools in live samples.

Probes and Sensors

Several types of systems have been developed to study Zn^{2+} in cells by fluorescence microscopy. We refer the reader to several recent reviews that provide comprehensive coverage of these probes and sensors [4, 6, 14]. In this review, we will briefly summarize the types of sensors available for Zn^{2+} detection, and move on to a discussion of the experimental challenges associated with using probes and important controls that should be carried out to minimize misinterpretation of data. Many of the available tools fall under two general classes of probes: small molecule probes and protein sensors based on Förster Resonance Energy Transfer (FRET) [6, 14, 15]. The strengths and weaknesses of these two classes of probes, as well as summary of commonly used probes are highlighted in Figure 1. Small molecule probes usually increase in fluorescence upon chelation of Zn^{2+} . The strengths of small molecule probes are that they can be cell permeable and therefore are easy to apply to cells, they are bright and yield a high fluorescence signal over background autofluorescence of cells, and can be made to fluoresce at various wavelengths. Small molecule probes have also been adapted to give ratiometric signals that allow for normalization for changes in fluorescence that are not due to chelation of Zn^{2+} [16, 17]. FRET-based protein sensors have also been developed to measure Zn^{2+} in cells. These sensors consist of two fluorescent proteins (FPs) and a Zn^{2+} coordinating site that is designed to change the relative orientation and distance of the FPs upon binding leading to a change in FRET signal from the donor FP to the acceptor FP. The ratiometric nature of these sensors allows for correction for protein concentration, sample thickness, and movement. The sensors are genetically encoded, can be targeted to organelles, and, through mutation, can be tuned to bind Zn^{2+} with a variety of affinities. Different colored FRET based sensors have been derived from the many available FPs, increasing flexibility in experimental protocols.

Beyond these two general classes of probes, other platforms have been developed that rely on different strategies to detect and report Zn^{2+} . Hybrid sensors combine a synthetic portion with a genetically encoded portion. The Lippard lab has used SNAP-tag to genetically target the small molecule sensor ZinPyr-1 [18]. In a similar vein, the Fierke group created sensors that combine the Zn^{2+} binding enzyme, carbonic anhydrase, with a small molecular fluorophore and a FP [19]. These systems aim to combine the advantages of both small molecule and protein-based systems: the modularity and brightness of the small molecules with the targetability and ratiometric signals of protein based systems. A new DNA based probe for Zn^{2+} has recently been developed [20]. This system relies on a photocaged DNzyme, which can be activated with light and cleaves in the presence of Zn^{2+} separating a fluorophore, fluorescein, from a quencher, dabcyI. This process creates a molecular beacon for Zn^{2+} . Further development of this platform might allow for a new class of Zn^{2+} sensing molecules. It is useful to note the diversity of strategies for sensor design, as different sensors have different strengths and application of complementary sensor platforms can strengthen cell-based studies. Below we turn to recent work that points to the need for careful controls in the application of these molecules and the importance of understanding of the underlying chemistry that allows these molecules to detect Zn^{2+} . We also refer the reader

to an excellent recent review that discusses the complex solution chemistry and speciation of zinc ions in biological environments [5]

Progress and challenges in applying FRET-based protein sensors to measure Zn^{2+}

FRET-based sensors have been applied to measure the concentration of Zn^{2+} in the cytosol and organelles in a variety of cell lines. Figure 2 outlines the *in situ* calibration that is carried out in order to use ratiometric sensors to convert the FRET ratio to a normalized parameter for comparing relative levels of labile Zn^{2+} in different cells or under different conditions. Briefly, once cells are expressing the sensor, the ‘resting’ FRET ratio or the signal of the sensor before manipulation of Zn^{2+} , is measured. Subsequently, the FRET ratios of the apo-sensor and the fully saturated sensor must be measured in order to determine the fractional saturation of the sensor at rest in each individual cell. To measure the FRET ratio of the apo sensor, the Zn^{2+} concentration is lowered to its extreme by adding excess cell permeable Zn^{2+} chelators, N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) or Tris(2-pyridylmethyl)amine (TPA). To measure the FRET ratio of the Zn^{2+} -saturated sensor, the Zn^{2+} concentration is elevated by adding excess concentrations of Zn^{2+} combined with either a cell permeabilizing agent (digitonin or saponin) or an ionophore (pyrithione) [21]. The ratio of the maximum signal of the sensor over the minimum signal of the sensor is called the dynamic range (DR).

The *in situ* calibration is typically used to calculate the fractional saturation of the sensor in individual cells, and can be used along with the K_D value to estimate the concentration of Zn^{2+} in a particular location. The fractional saturation of the sensor provides a relative comparison of Zn^{2+} in different cells or under different conditions, where higher saturation suggests higher levels of labile Zn^{2+} and lower saturation suggests lower levels. Estimation of the Zn^{2+} concentration requires further assumptions and data processing, including accurate measurement of the sensor K_D . The dissociation constants of these sensors are usually determined through *in vitro* titration of the sensors with known amounts of Zn^{2+} , and fitting of the resulting data using a ratio-based approach [22]. This approach assumes that the donor fluorophore signal decreases at the same rate that the acceptor fluorophore signal increases during FRET. Recently, Pomorski et al. have shown that many ratiometric sensors do not conform to this assumption and have developed a more rigorous intensity-calibrated approach for fitting the titration data that should be applied to determining the apparent binding constants of ratiometric sensors [23]. It is worth noting that the reported K_D values of many FRET sensors will likely need to be adjusted using the approach of Pomorski et al. In this review, we report the currently published K_D values for consistency. It has also been suggested that the isosbestic wavelength of FRET sensors can be used as an internal standard to determine the binding affinities of sensors; this approach decreases the dynamic range by using the intensity change at a single wavelength [24]. Finally, some attempts have been made to measure *in situ* K_D s, but this is challenging due to the complexity of the cellular environment.

Four different FRET-based protein sensor platforms, Zif, Zap, eCALWY, and eZinCh, have been applied using the above procedure to measure the concentration of Zn^{2+} in the cytosol of a variety of cell types. Interestingly, these sensors have very different modes of Zn^{2+} sensing. The ZifCY and ZapCY sensors, developed by the Palmer Lab, connect two FPs, truncated ECFP and Citrine, through zinc finger zinc-binding domains (ZBDs) that are unstructured in the unbound state with a sensor architecture of: donor FP – ZBD – acceptor FP [25–27]. Coordination of Zn^{2+} to the zinc-binding domain structures the linker between the two FPs leading to an increase in FRET signal. Mutation of the zinc binding domain of the ZapCY proteins led to the development two sensors with different apparent zinc disassociation constants at pH 7.4: ZapCY1, $K_D' = 2.5$ pM, and ZapCY2, $K_D' = 811$ pM [26]. The FPs have also been exchanged in the ZapCY platform to give a range of colors of sensors [28]. The Merckx Lab has developed two sensor platforms, the eCALWY and eZinCh sensors. In the eCALWY sensors, the FPs were engineered by making two mutations in the FPs (S208F and V224L) creating intermolecular contacts between the two FPs [29]. Two copper binding proteins, Atox1 and WD4 were modified to decrease binding of copper and linked between the two FPs, yielding a Zn^{2+} -specific sensor with the following sensor architecture: donor FP – Atox1 – linker – WD4 – acceptor FP. Coordination of Zn^{2+} by the two copper binding domains breaks the contacts between the FPs lowering the FRET signal. In the final platform, eZinCh, the FPs have been engineered to create a Zn^{2+} binding site between the two FPs using two mutations (A206H and S208C) in each FP. These mutated FP are linked by an unstructured, flexible linker with the overall architecture: donor FP – linker – acceptor FP [30]. When Zn^{2+} is coordinated to the sensor there is an increase in the FRET ratio. Members of both the eCALWY and eZinCh platforms have been developed to bind zinc with a variety of binding constants and have a variety of colors of FPs [14, 30–32]. Although these platforms have very different modes of sensing, they converge on a labile Zn^{2+} concentration in the hundreds of picomolar in the cytosol of many cell types.

There are a few concerns that should be addressed in order to ensure the sensors are functioning well: the sensor should be localized to the area of the cell of interest, the concentration of the sensor should be such that it does not perturb the labile Zn^{2+} pool, the apparent disassociation constant of the sensor for Zn^{2+} should be close to the concentration of labile Zn^{2+} in the cellular compartment of interest, and the binding modality should be such that the sensor doesn't complex Zn^{2+} bound to proteins [6, 33]. A few key experiments suggest that genetically encoded FRET-based protein sensors are measuring the native labile Zn^{2+} pool in the cytosol. The lessons learned from measuring Zn^{2+} concentrations in the cytosol may help to inform protocols for measuring Zn^{2+} in other organelles.

Qin and coworkers critically compared FluoZin-3 AM and ZapCY2 to demonstrate that ZapCY2 was measuring the labile Zn^{2+} pool [34]. First, the localization of the sensors was compared. By incorporating a nuclear exclusion signaling sequence, ZapCY2 can be localized only to the cytosol and not to other organelles. In comparison, the small molecule probe, FluoZin-3 AM, co-localizes with a Golgi marker as well as localizing in the cytosol. To ensure that the concentration of ZapCY2 was not perturbing the Zn^{2+} pool, the concentration of Zn^{2+} measured by both ZapCY2 and FluoZin-3 AM was measured as a function of sensor concentration. While raising the extracellular concentration of FluoZin-3 AM from 0–10 μM appeared to deplete the Zn^{2+} pool, the fractional saturation of ZapCY2

remained constant across a range of intracellular sensor concentrations from ~0–80 μM demonstrating that ZapCY2 does not detectably deplete the Zn^{2+} pool. Depletion of the Zn^{2+} pool by FluoZin-3 AM may be caused by the high concentration of the probe that accumulates inside the cell as hydrolysis of AM-ester probes traps the probes intracellularly. By this process, the intracellular concentration of these probes reaches hundreds of micromolar which may allow the probes to interfere with the homeostasis of the hundreds of picomolar concentrations of labile Zn^{2+} [35]. In contrast to the dye that is added acutely to cells and measured a few hours later, the long-term expression of the genetically-encoded sensors over the course of days at a relatively low concentration (~10 μM) may allow these sensors to become part of the cellular Zn^{2+} buffer and therefore non-perturbing to the labile Zn^{2+} pool.

Another important concern to address when using sensors to quantify Zn^{2+} is whether the sensor is truly accessing the labile pool. The Zn^{2+} coordination environment is flexible in both geometry and number of ligands [5]. Consequently, probes have the potential to form ternary complexes by binding to Zn^{2+} that is already coordinated to a protein. The propensity of ternary complex formation is likely much lower for FRET-based sensors than small molecular probes, due to the steric bulk of FPs and protein-based zinc binding domains compared to small chelates, however this has yet to be tested. Sensors with low K_D values or present at high concentrations could also shift the native equilibrium of Zn^{2+} thereby perturbing the labile pool. In either scenario the sensor would access Zn^{2+} from outside of the labile pool. If this were the case then sensors of various affinities would be able to access different pools of Zn^{2+} and measurement of Zn^{2+} with sensors possessing different affinities for Zn^{2+} would give rise to different results for the amount of Zn^{2+} . Vinkenborg and co-workers tested this hypothesis by applying a panel of eCALWY variants to the cytosol of INS-1 cells with a range of apparent Zn^{2+} disassociation constants (K_D') from 2 pM to 2.9 nM at pH 7.4 [29]. A plot of the fractional saturation of each variant against the disassociation constants of the variants gives a sigmoidal shape and a consistent cytosolic concentration of ~ 400 pM. Similar experiments were carried out by Qin et al in HeLa cells, yielding a cytosolic concentration of ~ 180 pM [34]. These experiments provide evidence that both sensor platforms are accessing the same pool of labile Zn^{2+} regardless of the affinity of the sensor.

These kinds of experiments strengthen the measurements collected through the application of sensors. As more sensors are developed and more measurements are made in a variety of cell types and in many organelles it is important to think critically about the various aspects of the sensors that could be affecting the measurements. Next we will examine measurements of Zn^{2+} concentrations using FRET-based sensors in the endoplasmic reticulum (ER), Golgi apparatus, and mitochondria where measurement of Zn^{2+} with different classes of probes has been less consistent. The variability in these measurements points to a need for critical examination of the fundamental chemistry of these tools, and to an interesting area of zinc biology as the distribution and dynamics of Zn^{2+} in the organelles continues to be studied.

There are a number of challenges associated with measuring Zn^{2+} in organelles, including ensuring that the sensor is properly localized, measuring Zn^{2+} in a small crowded

environment with an uncertain Zn^{2+} buffering capacity, and measuring Zn^{2+} in different chemical environments (pH, redox state, etc). Table 1 summarizes the estimates of organelle Zn^{2+} obtained using three different sensor platforms and reveals the variability in estimates of Zn^{2+} in organelles. ZapCY1 and ZifCY1 were the first FRET-based genetically encoded sensors to be targeted to the ER and the Golgi in HeLa cells. Colocalization with canonical markers of the ER and Golgi was used to demonstrate localization to the targeted organelle [26]. Treatment of the cells with TPEN resulted in a small decrease in the high affinity sensor, ZapCY1 ($K_D' = 2.5$ pM, pH 7.4), and no response in the low affinity sensor, ZifCY1 ($K_D' = 1$ μ M, pH 7.4), suggesting that Zn^{2+} concentration in the organelles was lower than the detection limit of ZifCY1. In situ calibrations suggested that ZapCY1 (dynamic range in the ER = ~ 2) was 26% saturated in the ER and 18% saturated in the Golgi. Using these data and the *in vitro* K_D' , the concentrations of labile Zn^{2+} in the ER and the Golgi were estimated to be 0.9 ± 0.1 pM and 0.6 ± 0.1 pM, respectively. When ZapCY1 and ZifCY1 were targeted to the mitochondria each sensor reported a very different concentration of labile Zn^{2+} in the organelle [27]. Because of the very low dynamic range of ZifCY1 in the mitochondria, this sensor was re-engineered by replacing the YFP with circularly-permuted Venus to increase the dynamic range. The circular permutation of Venus changes the orientation of the fluorophore with respect to the CFP, altering the FRET efficiency between the two FPs. This resulted in the development of a high dynamic range (DR=2.5), but lower affinity sensor, mito-ZifCV1.173. Importantly, Zif sensors that incorporated other versions of circularly-permuted Venus had very low dynamic ranges and appear more saturated with Zn^{2+} in comparison to the high dynamic range sensors ($\sim 40\%$ vs $\sim 10\%$) although all the sensors had the same Zif1 binding domain and probably similar affinities for Zn^{2+} . This observation highlights the need for high dynamic range sensors (above ~ 1.2 based on these data) for accurate measurement of Zn^{2+} concentration. Because the environment in the mitochondrial matrix is crowded and has a variable pH, the K_D' of ZapCY1 was determined *in situ* to be 1.6 pM. This value matched the *in vitro* K_D measured at the same pH. ZapCY1 was saturated $16 \pm 10\%$ in the mitochondria (dynamic range = ~ 2), and, using these data and the *in situ* K_D' for the sensor, the concentration of Zn^{2+} in the mitochondria of HeLa cells was estimated to be about 0.14 pM. Although the range of sensor concentration was limited in the organelles, perturbation the labile Zn^{2+} concentration at increasing levels of sensor expression was not detected [26, 27].

The Merckx Lab has also applied their sensors to the mitochondria and ER. Three different sensors were targeted to the ER of a variety of cell lines (eCALWY-4, $K_D' = 630$ pM; eCALWY-6, $K_D' = 2.9$ nM; eZinCh-2, $K_D' = 1.0 \pm 0.1$ nM) [30, 36]. The dynamic range of the sensors in the ER varied from 1.1 to 1.4, depending on the sensor and the cell type, yielding a range of Zn^{2+} concentrations from 800 pM to 7.2 nM. eZinCh-2 and eCALWY-4 were both used to measure the labile Zn^{2+} concentrations in the mitochondria of HeLa cells where the dynamic range in mitochondria ranged from 1.2 to 1.9 and estimations of Zn^{2+} , determined using the *in vitro* K_D' values, ranged from 3.3 ± 1.2 pM applying eZinCh-2 to 180 ± 300 pM applying e-CALWY-4 [30].

In summary, while it is easy to point to the variability in Zn^{2+} estimates obtained using genetically encoded sensors and find fault with the FRET-based protein sensors [4], a better

understanding the details of why the variability occurs will lead to the development of more robust tools that can be targeted to organelles to uncover the details of Zn^{2+} biology.

Re-examination of data collected using small molecule probes

Over the last 30 years small molecule probes have been engineered for and applied in a variety of contexts to study Zn^{2+} biology in cells and tissues. Over this period, progress has been made in designing probes to gain enhanced photophysical properties, to tune the affinities of probes for Zn^{2+} , to be ratiometric, to be reaction-based, and to target different organelles. These improvements have led to the application of probes to complex biological questions and led to important new knowledge about Zn^{2+} homeostasis and signaling [6]. However, recent work has led to re-examination of data collected using small molecule probes that emphasizes the need to better understand the chemistry of these probes and to develop clear controls for verifying the reactivity and properties of probes in cells. Studies carried out by the Petering Lab have pointed out the subtleties of the interaction of small molecule probes, Newport Green, TSQ, and Zinquin with Zn^{2+} in the cell [37, 38]. Briefly, it was always assumed that these sensors substituted for weakly bound ligands in cells, coordinating labile Zn^{2+} , and increasing in fluorescence intensity. However, examination of the dyes under a variety of conditions shows that these probes have multiple interactions with both labile and bound Zn^{2+} that cause detectable fluorescence increases. Although papers have claimed to use Newport Green to image native Zn^{2+} dynamics, the dye has a micromolar affinity for Zn^{2+} , and it is therefore difficult to find an application where it detects changes in the labile Zn^{2+} pool, given that estimates of cytosolic Zn^{2+} are in the hundreds of picomolar range. TSQ and Zinquin were both found to form fluorescent ternary sensor-Zn-protein adducts, meaning that the probes access and report Zn^{2+} that is tightly bound to proteins in addition to labile Zn^{2+} . This observation is interesting in light of recent work done by the Merckx Lab that re-examined data that was collected in fixed cells using Zinquin with live cells and genetically encoded FRET sensors. Work with Zinquin suggested that Zn^{2+} was mobilized from ER stores by activation of the Zn^{2+} transporter Zip7 in the presence of EGF and ionomycin in Tamoxifen-resistant MCF-7 cells. Through application of eCALWY-4 and eZinCh-2 in the cytosol and ER, no such dynamics of Zn^{2+} were seen [31]. The application of Zn^{2+} sensors allows for the start of interrogation of the biology of Zn^{2+} , but studies like these are excellent reminders that other imaging techniques can lend increased rigor to the data obtained with sensors.

Total elemental imaging and mapping

Numerous analytical techniques are available for visual mapping of elemental distribution in biological samples. Here we will discuss several approaches that can be divided into two classes: mass spectrometry-based and X-ray fluorescence-based imaging techniques. A schematic of the differences between these techniques is outlined in Figure 3. Due to space limitations, this review will cover only a subset of the various methods in these categories, focusing on the most widely used techniques reported in the literature. We will describe the basic properties of each imaging technique and provide a few examples of how they have been applied to the field of Zn^{2+} biology. An important distinguishing feature of these techniques is that they provide a measure of the total amount of a metal of interest, as

opposed to just the labile or accessible metal pool, and most techniques permit measurement of multiple elements at once, allowing for correlation between metals and other abundant biological elements, such as phosphorus. These techniques are traditionally applied to fixed samples of cells, or biological specimens such as plants or seeds at a fixed time point. While these techniques cannot be applied to live cell imaging, they can offer complementary elemental snapshots at multiple time points.

Mass-spectrometry techniques

Mass-spectrometry based techniques involve identification of specific chemicals within a sample by separation of ions according to their mass-to-charge ratio. Early mass-spectrometry applications did not provide spatial resolution, but newer approaches often contain mapping capabilities, thus enabling researchers to define the spatial distribution of metal ions. Mass-spectrometry-based spatial imaging allows for a point by point mass-spectrum distribution map generated by ablation of a sample at thousands of spots [13]. The different types of massspectrometry techniques can be distinguished by their method of ionization. Here we will discuss laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and secondary ion mass spectrometry (SIMS), the major two mass-spectrometry imaging techniques that are used to study the localization of metals in biological samples.

LA-ICP-MS

LA-ICP-MS has been widely applied in the life sciences for analyzing biological samples for metal localization in medical studies, such as tumor growth analysis, and biochemistry of animal model diseases [13, 39]. This method uses high-energy focused lasers to ablate particles from a solid sample; the particles are then carried by gas (Ar or He) into an ICP-MS platform where ionization occurs and charged ions are detected by their mass-to-charge ratio. Although LA-ICP-MS has a relatively low spatial resolution compared to other imaging techniques (1 μm limit), it offers very high sensitivity (ng/g) and multi-elemental imaging capability [11, 40, 41]. Particularly unique to both LA-ICP-MS and SIMS (discussed below) is the ability to detect multiple isotopes of individual elements, allowing for snapshots of dynamic trace element transport and distribution [40].

One notable application of this technology was the use of laser ablation coupled to multi-collector-ICP-MS (LA-MC-ICP-MS) to measure Zn^{2+} isotope incorporation in rat brain thin sections in an effort to identify the rate of uptake into different regions of the brain [42]. This is particularly important because the mechanisms of Zn^{2+} acquisition and distribution at the tissue and organismal level are not well understood. Further, alterations in brain Zn^{2+} homeostasis have been linked to the development of neurological diseases and thus it is critical to understand Zn^{2+} metabolism in the brain [43]. In this study, animals were injected with solutions enriched in two stable isotopes of Zn^{2+} , ^{67}Zn and ^{70}Zn , at two time points compared to control animals injected with either saline only or a Zn^{2+} solution of natural isotopic composition [44]. Isotope ratio maps revealed a heterogeneous distribution of Zn^{2+} isotope ratios in different physiological features of brain tissue (hippocampus, amygdala, cortex, and hypothalamus) suggesting different turnover kinetics in specific regions of the

brain. In another study using LA-ICP-MS, researchers analyzed brain tissue sections and found that the $^{31}\text{P}/^{66}\text{Zn}$ ratio decreased beyond tumor boundaries, demonstrating that changes in this ratio can be used to identify healthy vs. cancerous tissue [45]. Similarly, a study of liver sections demonstrated that metals can be used as disease biomarkers; Fe and Cu levels are higher in diseased liver tissue, while Zn^{2+} levels are decreased, and these alterations can be used to predict changes from healthy to diseased liver status [46]. Further examples demonstrating the application of LA-ICP-MS include cerebral imaging metals including Zn^{2+} in the pathophysiology in the mouse models of Parkinson's disease and Alzheimer's disease [47–49].

SIMS

Another analytical mass-spectrometry technique used for imaging elements and their isotopes is SIMS. SIMS uses a focused primary ion beam to disperse particles from a sample surface. The ions that are ejected after this surface bombardment are called secondary ions, which are subsequently analyzed in a mass spectrometer. The primary strength of SIMS is that it can offer excellent spatial resolution, particularly for NanoSIMS, which has a spatial resolution of 50 nm. In general, the width and energy of the beam dictates the achievable resolution; a wide, high intensity beam allows for depth profiling, while a narrower, low intensity beam may only erode the surface, making quantification difficult [11]. Further limitations include: the technique does not provide information about chemical state [11] and SIMS analysis requires samples to be in a vacuum, and hence samples must either be cryo-fixed or must be fixed at low temperatures, dehydrated, and resin-embedded [10]. For such preparations, care must be taken to ensure elemental distribution is not altered as a result of sample preparation. Although SIMS can be used to detect most elements in the periodic table and their different isotopes, a few elements such as Zn^{2+} , Cd, and Mn have poor secondary ion yield and are therefore difficult to detect at standard concentrations (Zhao, 2014, Moore 2012). SIMS has, however been used to successfully determine Zn^{2+} localization in Poplar leaves from plants grown on Zn^{2+} -contaminated soil [50], suggesting that although SIMS may not be ideal for detecting low levels of Zn^{2+} , it may be appropriate to study biological samples with elevated Zn^{2+} levels. Further, in studies where another approach is applied to study Zn^{2+} localization, SIMS may offer the ability to study localization of other lighter metals of interest such as P and Si that cannot be examined as effectively by other approaches, such as X-ray fluorescence (discussed in detail in the next section of this review) [51]. In cases where X-ray fluorescence and SIMS are used in combination, adjacent thin sections can be analyzed.

X-ray fluorescence techniques

In addition to mass-spectrometry techniques, the use of X-ray fluorescence techniques provide a complementary approach for mapping metal distribution in biological specimens, and offer the additional advantage of obtaining information on multiple elements and chemical speciation of elements of interest. X-ray fluorescence methods rely on the photoelectric effect; when an atom is exposed to high-energy radiation, electrons are ejected from inner shells, leading to core vacancies, which are filled by outer-shell electrons. As outer shell electrons decay, energy is released in the form of fluorescence. Because the

energy of the emitted photons is characteristic for each element, and the intensity is proportional to concentration, X-ray fluorescence provides information on the identity and amount of a given element in a sample. Samples can be raster scanned line by line through a focused incident X-ray beam to yield a high resolution, high sensitivity elemental map [10, 11]. X-ray fluorescence techniques are categorized according to the type of incident radiation. Here we will discuss synchrotron X-ray fluorescence (SXRF), which uses X-rays, and electron-probe energy-dispersive spectroscopy (EDS), which uses electrons and is often combined with a scanning electron microscope (SEM). Other techniques beyond the scope of this review include micro-particle-induced X-ray emission (μ PIXE), which uses charged atoms such as protons.

Synchrotron X-ray fluorescence

SXRF possesses a number of advantages for mapping metals and other elements in biological samples. Synchrotron facilities have high-intensity photon sources that are more than ten times brighter than those of conventional X-ray tubes [10], thus giving rise to high sensitivity and submicron spatial resolution [13, 52]. As opposed to mass spectrometry-based techniques, SXRF is generally non-destructive and does not require vacuum. Further, the sensitivity of SXRF increases with increasing atomic number, making it well-suited to study trace elements and heavy metals/metalloids, including Zn^{2+} .

A recent application of SXRF fluorescence demonstrates the strengths of this approach for mapping metals and other elements with sub-cellular resolution, where the location and redistribution of metals was monitored during mitosis of mammalian cells. Briefly, washed and dried NIH 3T3 cells attached to Si-nitride windows were analyzed by SXRF and researchers found that Zn^{2+} , Cu, and S remained colocalized throughout mitotic states and that Zn^{2+} increased during mitosis compared to interphase, suggesting a role for Zn^{2+} in the cell cycle [53]. Because SXRF imaging has the potential to study metal distribution at the subcellular level in studies such as these, important questions are raised about the significance of variable sub-cellular metal accumulation and speciation. To confirm subcellular localization, SXRF may be performed in combination with organelle localization using fluorescent markers. For a recent review on the application of this concept, see Roudeau *et al.*, 2014 [54].

X-ray absorption spectroscopy

In addition to mapping elemental localization by SXRF, X-ray absorption spectroscopy (XAS) is another synchrotron-based technique that can be used to analyze the chemical speciation of individual elements. This capability is unique to synchrotron-based approaches and is not offered by the previously discussed mass-spectrometry techniques. An XAS analysis involves progressively increasing the incident X-ray beam energy at a specific point in a sample and collecting X-ray fluorescence at each individual energy. The XAS spectrum includes the X-ray absorption near-edge structure (XANES) and the extended X-ray absorption fine structure (EXAFS). XANES covers the energy range from about -50 to $+200$ eV of the absorption edge and is sensitive to the oxidation states of elements, while EXAFS covers energies from the absorption edge to approximately $+800$ eV and is well-suited to elucidate coordination chemistry, including the identity and number of coordinating

atoms and their interatomic distance [10]. Depending on the researcher's specific needs, these techniques can be applied to several regions of interests in samples using a micro-focused beam or alternatively applied to bulk tissues using a larger mm beam. It is important to note that in order to properly identify speciation within a sample, an XAS spectrum must be compared to known standards and thus researchers may be limited by the availability of appropriate standards.

Synchrotron beam-lines generally allow for collection of both SXRF mapping and XANES or EXAFS allowing researchers to map metal distribution and perform more detailed analysis of the chemical speciation in specific regions of the sample. For example, SXRF analysis of frozen murine macrophage cells identified Zn^{2+} hotspots, or putative Zn^{2+} vesicles, called "zincosomes" [55]. These spots were then analyzed by XAS to elucidate chemical speciation information of the zincosomal Zn^{2+} . XANES and EXAFS revealed that Zn^{2+} was bound to one sulfur atom at a distance of 2.28 Å and two and a half histidine atoms and one oxygen atom at 1.97 Å. For a few recent reviews on the application of X-ray absorption spectroscopy for metal speciation, refer to Gräfe *et al.* 2014, West *et al.*, 2015, and Zhao, *et al.* 2014 [10, 56, 57].

EDS

Although Synchrotron-based X-ray fluorescence techniques are powerful, access to Synchrotrons can limit the widespread availability of these techniques. An additional approach for elemental mapping by X-ray fluorescence is electron-probe energy-dispersive spectroscopy (EDS), also called electron probe microanalysis (EPMA), which uses an electron beam to bombard a sample. As with Synchrotron-induced fluorescence, the X-rays emitted following bombardment of a sample with an electron-beam, are characteristic for individual elements and the intensity is proportional to the amount of the element in the sample. EDS is usually used in combination with a scanning electron microscope (SEM), a transmission electron microscope (TEM), or a scanning transmission electron microscope (STEM) to allow for analysis of additional morphological features of the sample. Simultaneous high resolution imaging of subcellular structures in combination with elemental analysis within these structures is particularly useful for studying composition of whole cell samples that are a few micrometers thick [13]. Recently, this technique has been applied by fitting a cryo-compatible STEM with an EDS detector. Researchers demonstrated the ability to spatially map K, Fe, and Zn^{2+} in red blood cells with STEM-EDS [58]. They found an even distribution of these metals throughout RBCs and because of the combination with STEM, they were also able to study these metal distributions in the context of the flexible RBC morphology. We will discuss an additional example of STEM-EDS in the section entitled "combinatorial imaging approach".

Perspectives

Each technique highlighted above offers a different degree of sensitivity, specificity, and spatial resolution; the advantages and limitations of each should be carefully considered for individual experimental design. Besides the fundamental pros and cons of each approach, access to equipment may present additional limitations for researchers. For example, a

STEM-EDS system may not be available at a researcher's home university. Further, many of the X-ray fluorescence techniques, such as SXRF, require access to specialized synchrotron facilities. Access to these facilities usually necessitates a prior arrangement of beam time through award of successful written proposal submitted well in advance. Even with awarded time, researchers are allotted a limited period of time and each scan may take upwards of several hours. Researchers may have to compromise resolution of their elemental maps to decrease scanning time in order to study more samples. Because of issues such as these, studies using these techniques are often limited in sample size/biological replicates. Thus, these approaches are better suited for targeted hypothesis-driven experiments, rather than discovery-based screens or high-throughput studies.

Quantitative analysis of zinc distribution using complementary approaches

As emphasized above, different tools permit measurement of different metal pools; small molecule probes and sensors can be used to quantify the subcellular distribution of labile Zn^{2+} , whereas mass-spectrometry and X-ray fluorescence-based techniques can be used to map the distribution of total Zn^{2+} . Each analytical tool offers a unique piece of information such that combination of multiple techniques provides a powerful means to generate a comprehensive picture of Zn^{2+} distribution.

An elegant example of this combinatorial approach is illustrated by the recent study of Zn^{2+} fluxes involved in the fertilization mammalian egg. Previous studies found that labile Zn^{2+} is released from eggs during a meiotic checkpoint in what is referred to as a " Zn^{2+} spark" [59]. To elucidate the molecular mechanism of these Zn^{2+} sparks, researchers used a combination of four approaches to resolve zinc distribution and measure Zn^{2+} concentrations in single cells before and after sparks. Using a synthetic fluorescent Zn^{2+} probe and an extracellular zinc dye for live-cell imaging at different stages of egg development, researchers that Zn^{2+} -rich compartments are the source of Zn^{2+} that is exocytosed during Zn^{2+} sparks. Further, elemental composition of meiosis II (MII) eggs was analyzed at an ultrastructural level with a STEM microscope equipped with EDS detectors, allowing for anatomical and elemental imaging. Thin sections of fixed, resin-embedded MII eggs were analyzed with STEM-EDS. Bright, vesicularlike structures near the ooplasmic membrane were found in Z-contrast STEM images, indicating elements of higher molecular weight. The EDS spectra measured in these regions identified by STEM compared to nearby cytoplasmic regions revealed an increased Zn^{2+} intensity in the vesicular-like bodies. Authors point out that STEM-EDS is not very quantitative, so they further turned to high-resolution SRXF to confirm their results by mapping thin sections of the MII eggs. In these experiments, they also saw Zn^{2+} -enriched compartments, consistent with both the live-cell imaging and the STEM-EDS analysis. Finally, quantification by highly sensitive SRXF allowed for determination of total Zn^{2+} concentration in the vesicular stores. Application of four complementary technique allowed the authors to conclude that Zn^{2+} -containing vesicles near the membrane are the source of Zn^{2+} -sparks, and the amount of Zn^{2+} within these vesicles is consistent with the amount of Zn^{2+} released.

Conclusions

We have discussed the methods and considerations for imaging labile and total Zn^{2+} using various tools and approaches. As others have pointed out, there is room for improved communication between the chemists who develop tools such as FRET sensors, experts of particular imaging techniques (i.e. synchrotron beamline scientists), and the biologists who apply them [11, 60]. With the extensive and ongoing development of a vast array of chemical and analytical tools available to study labile and total Zn^{2+} pools, the scientific community is now well-poised to answer fundamental questions about Zn^{2+} biology. Understanding the dynamics and distribution of metals is essential for deciphering how particular distribution patterns are set up, which genes control these processes, how they differ between cell-types, and how they are altered in healthy vs. diseased states. To aid in answering these questions, Zn^{2+} pools can be depleted or elevated, manipulated by pharmacology, or altered by genetics (i.e. knockdowns or overexpression of particular Zn^{2+} transport/homeostatic genes). By combining these biologically relevant perturbations with the chemical and analytical analysis methods, we will continue to elucidate fundamental aspects of Zn^{2+} biology.

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A diverse library of zinc sensors have been developed to image zinc in live cells. Studies presented that illustrate need for care in the proper application of sensors. Mass-spectrometry and X-ray fluorescence methods map cellular distribution of Zn^{2+} . Elemental mapping can complement data gathered through Zn^{2+} sensors and probes.

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Figure 1.

Panel A) Comparison of properties of small molecule and FRET based protein sensors.

Panel B) Small molecule probes typically contain a fluorophore attached to a Zn^{2+} chelating moiety (FluoZin-3 is shown as an example). FRET-based protein probes typically contain two fluorescent proteins separated by a Zn^{2+} -binding domain. Small molecule probes tend to increase in fluorescence intensity upon binding Zn^{2+} , whereas FRET-based probes are characterized by an increase in intensity at one wavelength and decrease at another (Sample fluorescence emission spectra are depicted with arrows indicating the change upon Zn^{2+} addition.) Panel C) Most commonly used probes and sensors. Companies and labs that provide these sensors for general use are included in parentheses.

Figure 2.

Diagrams of Zn^{2+} calibrations: Panel A. depicts a sensor that increases in FRET ratio when Zn^{2+} binds. Sensors with this mechanism include the Zif, Zap, and eZinCh families. Panel B. depicts a sensor that decreases in FRET ratio when Zn^{2+} binds. The eCALWY family reports Zn^{2+} abundance by this mechanism. The equations used to determine the resting concentration of zinc are given where n equals the hill coefficient and K_D is the dissociation constant determined by titration of the sensor *in vitro* [25–31]. These equations assume that the probes form a 1:1 complex, that the probes are at a low enough concentration in cells for the fluorescence intensity to be linearly proportional to the concentration, and that the probes behave similarly in cells and *in vitro* [22].

Figure 3.

Schematic of mass-spectrometry and X-ray fluorescence-based imaging techniques. A) Mass-spectrometry-based imaging techniques rely on point-by-point sample ablation using a laser or primary ion beam to disperse charged particles or secondary ions that are subsequently analyzed by mass-spectrometry. Ions are separated by their mass-to-charge ratio. B) X-ray fluorescence techniques rely on the photoelectric effect; when an incident beam (X-ray or e^-) bombards a sample, electrons are ejected from inner shells and vacancies are filled by outer-shell electrons. In this process, X-rays are released and detected at characteristic energies for each element.

Table 1

Summary of measurements of labile Zn^{2+} made in organelles with protein based sensors.

Sensor	Dynamic Range in Organelle	Percent Saturation	<i>in vitro</i> K_D (pM)	pH of K_D measurement	Estimated Zn^{2+} concentration (pM)
ER-CALWY-4 [29, 36]	1.25 – 1.4 *	> 80% *	630	7.1	1600 – >5000
ER-CALWY-4 [29, 30]	1.4 *	33%	630	7.1	390 ± 170
ER-CALWY-6 [29, 36]	1.1 *	0 – 50 %	2900	7.1	5600 – 7200
ER-ZinCh-2 [30]	1.4 *	~ 55 %	1000 ± 100	7.1	800 ± 600
ER-ZapCY1 [26]	2.16 ± 0.06	26%	2.5	7.4	0.9 ± 0.1
Golgi-ZapCY1 [26]	2.09 ± 0.07	18%	2.5	7.4	0.6 ± 0.1
mito-CALWY-4 [36]	1.2 – 1.55 *	~ 80 %	60	7.8	180 – 300
mito-CALWY-4 [30, 36]	1.6 *	~ 66 %	60	7.8	42 ± 28
mito-ZinCh-2 [30]	1.9 *	23 ± 6 %	10	7.8	3.3 ± 1.2
mito-ZapCY1 [27]	3.22 ± 0.57	8.7 ± 5.8 %	1.6	8 (<i>in situ</i>)	0.14
mito-CA based sensor [19]	1.66 *		0.15 ± 0.05	<i>in isolated mitochondria</i>	0.15

Starred (*) values were estimated by the authors from published data. Note the K_D values are those reported in the relevant references.