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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.

1. 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 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

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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.

2. 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 Fig. 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 DNAzyme, which can be activated with light and cleaves in the presence of Zn^{2+} separating a fluorophore, fluorescein, from a quencher, dabcyl. 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].

3. 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. Fig. 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

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