

Imaging Zinc: Old and New tools

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Dark Ages

As recently as 20 years ago, the very idea of “imaging zinc” was heresy among the cognoscenti. This was because dogma held that all zinc in biological systems should be tightly bound to the proteins where it was functional (or to zinc-chaperone molecules) and would therefore be impossible to image. The very idea of “free” zinc that could be imaged was, as late as the 1980’s, simply not acceptable.

Free Zn^{2+} is a Signal

Times have changed.

It is now completely clear that at least a dozen types of mammalian cells sequester rather startling amounts of “free” zinc in their secretory granules and secrete that zinc in regulated fashion, under the precise control of action potentials (in neurons) and secretagogues (in non-neural cells^{1,2}). Furthermore, it is becoming more and more clear that the secreted zinc is not just some epiphenomenon that accompanies secretion of the “real” messengers from these cells. Indeed, to paraphrase McLuhan, it appears that “the cationic medium is the message.” Specifically, the brief “puffs” of ionic (rapidly-exchangeable) Zn^{2+} that cells deliver into their immediate microenvironment are now recognized as pivotal and essential modulators and mediators of cell-to-cell signaling^{3,4}. Even more intriguing, cases are emerging in which the Zn^{2+} released by one cell travels through specific, gated, zinc-permeable channels in adjacent cells to enter the latter. This makes the Zn^{2+} ion an orthograde, transcellular, transmembrane signal, a completely novel signal type^{5,6}.

The zinc imaging tools were the agents that freed zinc from proteins. Thus, as early as 1955, Maske (review in reference⁷) published the odd result that the colorimetric zinc-binding reagent, dithizone, when injected intravitaly, produced a rather stunning band of bright-red, apparent zinc:dithizonate staining in the

hippocampal formation of the brain (Fig 1). Similar vivid staining filled the islets of Langerhans in the pancreas and (unbeknownst to Maske) also stained odd places like the secretory cells of the submandibular salivary gland², the prostatic fluid ducts⁸, and other scattered types of secretory cells, all with apparent zinc:dithizonate. This result implied that loosely-bound (“free”) zinc was richly abundant in select cell types and cell organelles in brain and other tissues.

The definitive identification of Maske’s bright red band as a pool of rapidly-exchangeable zinc that was selectively localized in neuronal presynaptic vesicles took another 25 years. Two new, complementary methods of imaging zinc were required. First, Timm and his followers developed the beautiful methods of silver-enhancing weakly-bound metals in tissue. In the hands of Finn-Mogen Haug⁹ and Gorm Danscher^{10,11}, these silver-enhancement methods were refined and fine-tuned until (In the current Danscher variants¹⁰) they now quite selectively label only transition metals (Cu, Zn, Fe, etc) that are weakly bound in situ in their normal, physiological milieu. When used this way, the Timm-Danscher method stains essentially nothing in the mammalian cerebrum except presynaptic vesicles of certain (now called “zinc-containing”) neurons (Fig 1). The confirmation that the labeled metal in the vesicles is zinc was provided by the second method, the TSQ fluorescent method, which produces bright fluorescence when complexed with zinc, but not when complexed with other Timm-positive biologically-relevant transition metals (Fe, Cu, etc.)¹². Moreover, since prior Timm’s reagents (S⁻) blocks all TSQ staining, there is essentially no doubt that (i) Timm-Danscher and TSQ stain the same pool of metal (ii) which is zinc, and (iii) which is located exclusively in the vesicles of neurons in the cerebrum.

This combined approach of silver staining and inspection at the electron-microscopic level and fluorescent labeling (to verify the cationic species) remains the definitive method for establishing the nature and fine-structural location of metal deposits in tissue (e.g., Fig 1,2).

Three Separate Zn²⁺ Pools (and counting?)

In the brain, we now have three separate “pools” of “free” zinc that can be (indeed *must* be) imaged separately.

i. Secretory Granule (Vesicular) Zinc

First is the Zn²⁺ in the presynaptic vesicles. This Zn²⁺ can be imaged by the Danscher-type silver staining (Fig 1) in post-mortem histologic sections. For imaging intravitaly (or in brain slices or slice cultures), one must use a probe that can penetrate both the plasma membrane and the vesicular membrane. The original TSQ and its congeners (Zinquin, TFL-Zn) will penetrate into the vesicles, but they have unstable partition coefficients, and bind Zn²⁺ very tightly (Kd~ 10⁻¹²) and so are of limited use for in vitro zinc studies¹³.

A better choice for imaging the vesicular pool of zinc is the new probe ZP1^{14,15}, which is stable in physiological conditions, remains in vesicles (probably in the membranes), and gives a sufficiently vigorous change in emission intensity upon binding zinc that individual synaptic boutons can be resolved in acute brain slices with routine confocal microscopy (Fig 1). Similar images can be obtained with the new zinc-sensing probe of Kikuchi¹⁶. The probe ZP1 has low-nanomolar affinity for the zinc in vesicles, but the concentration of Zn²⁺ in the vesicles is believed to be as much as 1 million-fold higher than that, around 1 mM or so. Thus, the ideal probe for work on the biophysics of zinc release, reuptake, and general “life-cycle” in neuronal vesicles would be a small, lipophilic molecule binding zinc in the low mM to high μ M range, such as the one recently reported by Burdette and Lippard¹⁷.

Concerning the Zn²⁺ in other secretory cell granules, the situation seems to be about the same as in neuronal secretory vesicles. Thus, the silver methods show the exquisitely-precise labeling of zinc-filled secretory vesicles in the pancreas¹⁸, for example (Fig 2), and the pancreas has been imaged often using the vesicle-permeating TSQ and zinquin stains¹⁹.

ii. Released Zn²⁺ Signals

The second pool of “free” zinc of both clinical and basic science interest is the Zn²⁺ that is released from secretory vesicles into the extracellular milieu. In the brain, this synaptically-released zinc has been successfully imaged with a number of methods, all involving the use of membrane-impermeable fluorescent zinc probes. The first visualization of synaptic zinc release was done using the protein biosensor derived from apocarbonic anhydrase (CA) coupled to a fluorescent reporter that changes emission when the CA binds zinc²⁰. The ratiometric, quantitative nature of this apoCA system has recently been harnessed to “image” released zinc collected from microdialysis probes placed in the living brain. Optical fiber probes for Zn²⁺ (and Cu⁺⁺) are now in testing phase (Thompson & Frederickson, unpublished), and these should allow real-time monitoring of Zn²⁺ release in vivo.

Another method of monitoring released Zn²⁺ optically has now evolved using single-reagent, membrane impermeable dyes, such as Newport Green^{5,6} and newer compounds (e.g., FluoZin) from Molecular Probes (Eugene OR.)²¹. While not ratiometric, these methods can be used for semi-quantitative studies of the timing and magnitude of zinc translocation within neurons (Fig 2) and zinc release from other sources, such as the pancreatic beta cells (Fig 2).

iii. Transmembrane Intracellular Zinc Signals

Beyond the zinc sequestered in secretory vesicles and the just-secreted zinc in the interstices, there is a third “pool” of rapidly-exchangeable zinc that is transiently present in some cells under some conditions. This is the zinc that is

found in the perikaryal and dendritic cytoplasm of neurons that have been through situations that allow zinc currents to flow across the cell membrane (Fig 1). This will occur in neurons in the brain whenever (i) there is a release of Zn^{2+} into the synaptic space around the target neuron, and simultaneously (ii) the postsynaptic neuron has zinc-permeable channels opened by a ligand or by depolarization²². This situation is illustrated beautifully by the image of Weiss and Sensi (Fig 2), which shows the virtual absence of any zinc staining in a normal cultured neuron at rest (inset) and the vivid zinc signal throughout several dendrites after exposing the neuron briefly to a channel-opening ligand (kainate) and elevated extracellular zinc (Fig. 2). Importantly, the zinc-permeable channels operating in this case (the Ca-A/K channels) are known to be preferentially located along the more distal dendrites, where the zinc influx has occurred²³.

These transitory intracellular (and transmembrane) Zn^{2+} fluxes are reminiscent of similar intracellular (and transmembrane) Ca^{2+} fluxes. We recently adduced evidence that this flux of synaptically-released Zn^{2+} into neuronal dendrites is necessary and (with glutamate) sufficient for the induction of a lasting potentiation of synaptic strength at one type of zinc-modulated synapse^{5,6}.

Whereas the zinc fluxes across the plasma membrane and the resulting intracellular Zn^{2+} currents are likely involved in normal, physiological cell signaling, they are fairly certainly involved in the injury of neurons in so-called excitotoxicity. For in excitotoxicity caused by ischemia, seizure, or head trauma, neurons that are injured (eosinophilic, acidophilic) routinely show vivid zinc staining in the perikaryal cytoplasm, proximal dendrites, and (sometimes) in the nucleus as well (Fig 1). This latter, injury-associated accumulation of free zinc in the cytosol can be imaged in living tissue by the membrane trappable dyes such as Newport Green and compounds from Molecular Probes. In post-mortem harvested tissue sections, the best method for imaging this pathological zinc is to use a membrane-impermeable probe so that the perikaryal staining will not be overshadowed by the background staining of the neuronal presynaptic vesicles. Thus, the new probe ZP4¹⁴, which is membrane impermeable will hardly stain vesicles in frozen sections at all, but will easily stain the cut-open somata of 20-30 μm diameter neurons in 10-15 μm histologic sections (Fig. 1).

On the Horizon

In Vitro and in Vivo Imaging:

Biosensors are coming in increasingly sophisticated varieties. For example, Wolfgang Maret and colleagues have developed a metallothionein-based sensor that capitalizes on the fast and selective binding of Zn^{2+} by metallothionein²⁴. By attaching two halves of a FRET pair to the two ends of the MT molecule, he has produced a sensor that will give a proportional report to Zn^{2+} signals occurring in the cytoplasm.

Another biosensor is under development by the Thompson-Fierke team, who have developed several exquisitely-sensitive zinc and copper sensors²⁵⁻²⁷. In this ingenious model, one naturally fluorescent protein is attached to the carbonic anhydrase molecule, and another is added which will coordinate with the holo but not the apoprotein. Because the two proteins form a FRET pair, the detection of Zn^{2+} can be done using excitation wavelength ratiometry, a powerful quantitative method.

Even further out on the horizon are possibilities for non-invasive imaging of Zn^{2+} deep in tissue. We have for example preliminary data that T1 and T2 weighted imaging of Zn^{2+} by in vivo MRI may be possible, and others^{28*} have proposed other MRI imaging methods for Zn^{2+} .

The Wish List

For intravital imaging studies of Zn^{2+} in living systems, one needs a ratiometric probe that has a strong fluorescent signal and has: (i) the right affinity (K_D roughly in the middle of the range of expected signals), (ii) the right on- and off-rates (fast enough to see the expected signals), (iii) the right permeability (able to permeate and be trapped in the cellular compartment under study), and (iv) lack of toxicity to the cells or tissues. In the case of Zn^{2+} , probe development is still in the bootstrapping phase, because, for example, neither the basal extracellular level of Zn^{2+} nor the basal level of intracellular Zn^{2+} is known for mammalian cells or tissues. Various indirect evidence indicates that both of these are in the range of 10^{-13} to 10^{-9} in normal healthy tissue²⁹, but direct quantitative determinations are still absent. Transient zinc signals, in contrast, have been measured in the 10^{-7} to 10^{-5} range within the micro-domains of signaling^{5,6}. Like the absolute concentrations, the absolute signal speeds are not fully understood, but it is clear that signals in the single-digit millisecond regime must be detectable.

From its modest beginnings buried within proteins, the zinc ion has now emerged as an intracellular and intercellular signal ion that rivals calcium in the ubiquity and essentiality of its functions. As the toolkit for zinc research grows, biomedical science will surely be galvanized.

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Figure Legends

Figure 1

Top row: Zinc release from beta cells imaged in green with membrane-impermeable dye from Molecular Probes (FluoZin-30), the sequential time-lapse pictures cover 66 msec, from left to right. (Courtesy Kyle Gee and Bob Kennedy), Second row: the diagram at right shows in false-color the release of zinc (Newport Green - impermeant) into the extracellular fluid of a brain slice during electrical stimulation of the zinc-rich axons of the dentate granule neurons (circles), in 33 msec (4 stimulus pulses) the released zinc could be reliably detected, and the peak release was about 20 μ M in the fluid (Courtesy Yang Li and John Sarvey). Bottom row: This false-color image a cortical neuron resting in culture has virtually no fluorescence for zinc (faint blue cell body in lower left inset) but a brief stimulation with kainate opens zinc-permeable Ca-A/K channels, causing middle and distal dendrites to rapidly fill with zinc (white color) (Courtesy Stefano Sensi & John Weiss; probe from Molecular Probes). Bottom row, right: the Timm-Danscher method of silver staining was used to label the zinc in the pancreatic beta cell secretory granules. Degranulation of these granules causes the release of zinc shown in the top row (Courtesy Gorm Danscher).

Figure 2

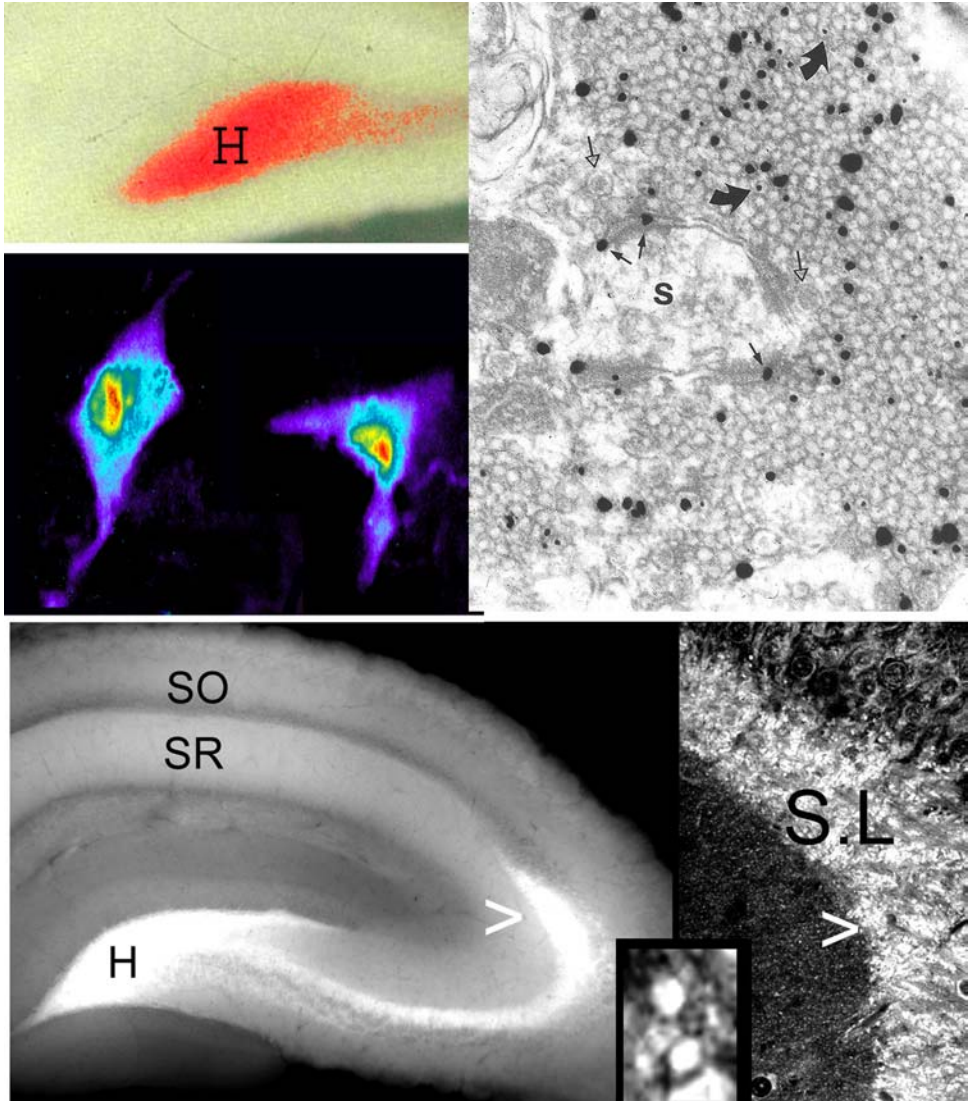
Top Left: The hilus (H) of the dentate gyrus is shown stained for zinc with dithizone, as discovered by Maske. Below are two individual neurons from the hilus of the dentate gyrus stained for zinc with ZP4 and imaged in by confocal microscopy. Pseudo-color image shows that after excitotoxic injury (seizures, in this case) neurons fill with zinc that is rich in the nucleus as well as the perinuclear cytosol (Courtesy Cathy Frederickson, NeuroBioTex, Inc.) Right panel, the Timm-Danscher method shows individual silver labeling of zinc in the vesicles of a single, giant mossy bouton of the hippocampus (courtesy Jeus Perez-Clausell). Two such giant boutons are shown labeled with a fluorescent zinc probe (ZP1) in the living brain slice in the bottom row. Bottom row: ZP1 (Steve Lippard; NeuroBioTex) stains all of the presynaptic zinc in the various zinc-positive regions of hippocampal neuropil of a live slice; left side shows confocal image with individual axon terminals (bright spheres in inset) visible in the living slice (Courtesy of Stefano Sensi and John Weiss).

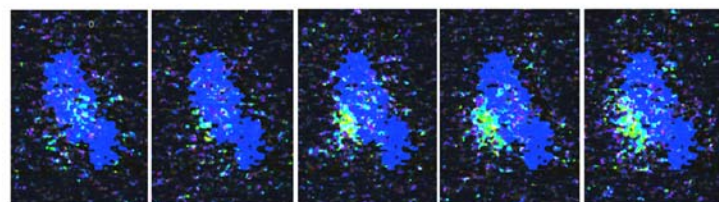
References

1. Y. Morita, M. Sawada, H. Seno, S. Takaishi, H. Fukuzawa, N. Miyake, H. Hiai, T. Chiba. Identification of xanthine dehydrogenase/xanthine oxidase as a rat Paneth cell zinc-binding protein. *Biochim.Biophys.Acta* 1540, 43-49 (2001).
2. C. J. Frederickson, J. Perez-Clausell, G. Danscher, Zinc-containing 7S-NGF complex. Evidence from zinc histochemistry for localization in salivary secretory granules. *J.Histochem.Cytochem.* 35, 579-583 (1987).
3. X. Xie and T. G. Smart, Modulation of long-term potentiation in rat hippocampal pyramidal neurons by zinc. *Pflugers Arch.* 427, 481-486 (1994).
4. M. Hershinkel, A. Moran, N. Grossman, I. Sekler, A zinc-sensing receptor triggers the release of intracellular Ca²⁺ and regulates ion transport. *Proc.Natl.Acad.Sci.U.S.A* 98, 11749-11754 (2001).
5. Y. Li, C. J. Hough, S. W. Suh, J. M. Sarvey, C. J. Frederickson, Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. *J.Neurophysiol.* 86, 2597-2604 (2001).
6. Y. Li, C. J. Hough, C. J. Frederickson, J. M. Sarvey, Induction of mossy fiber --> Ca³ long-term potentiation requires translocation of synaptically released Zn²⁺. *J.Neurosci.* 21, 8015-8025 (2001).
7. C. J. Frederickson, Neurobiology of zinc and zinc-containing neurons. *Int.Rev.Neurobiol.* 31, 145-238 (1989).
8. M. B. Sorensen, M. Stoltenberg, S. Juhl, G. Danscher, E. Ernst, Ultrastructural localization of zinc ions in the rat prostate: an autometallographic study. *Prostate* 31, 125-130 (1997).
9. F. M. Haug, Electron microscopical localization of the zinc in hippocampal mossy fibre synapses by a modified sulfide silver procedure. *Histochemie.* 8, 355-368 (1967).
10. G. Danscher, S. Juhl, M. Stoltenberg, B. Krunderup, H.D. Schroder, A. Andreasen. Autometallographic silver enhancement of zinc sulfide crystals created in cryostat sections from human brain biopsies: a new technique that makes it feasible to demonstrate zinc ions in tissue sections from biopsies and early autopsy material. *J.Histochem.Cytochem.* 45, 1503-1510 (1997).

11. G. Danscher, M. Stoltenberg, S. Juhl, How to detect gold, silver and mercury in human brain and other tissues by autometallographic silver amplification. *Neuropathol.Appl.Neurobiol.* 20, 454-467 (1994).
12. C. J. Frederickson, E. J. Kasarskis, D. Ringo, R. E. Frederickson, A quinoline fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. *J.Neurosci.Methods* 20, 91-103 (1987).
13. V. Snitsarev, T. Budde, T. P. Stricker, J. M. Cox, D. J. Krupa, L. Geng, A.R. Kay. Fluorescent detection of Zn(2+)-rich vesicles with Zinquin: mechanism of action in lipid environments. *Biophys.J.* 80, 1538-1546 (2001).
14. S. C. Burdette, C. J. Frederickson, W. Bu, S. J. Lippard. ZP4, an improved neuronal Zn²⁺ sensor of the Zinpyr family. *J.Am.Chem.Soc.* 125, 1778-1787 (2003).
15. S. C. Burdette, G. K. Walkup, B. Spingler, R. Y. Tsien, S. J. Lippard. Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. *J Am Chem Soc.* 123(32):7831-41 (2001).
16. S. Maruyama, K. Kikuchi, T. Hirano, Y. Urano, T. Nagano. A novel, cell-permeable, fluorescent probe for ratiometric imaging of zinc ion. *J Am Chem Soc.*, 124(36):10650-1 (2002).
17. S. C. Burdette and S. J. Lippard, The rhodafluor family. An initial study of potential ratiometric fluorescent sensors for Zn²⁺. *Inorg.Chem.* 41, 6816-6823 (2002).
18. L. H. Kristiansen, J. Rungby, L. G. Sondergaard, M. Stoltenberg, G. Danscher, Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas. *Histochem.Cell Biol.* 115, 125-129 (2001).
19. A. T. Maghasi, K. T. Schlueter, H. B. Halsall, W. R. Heineman, H. L. Rodriguez Rilo, Development and characterization of simulant pancreatic islets. *Anal.Biochem.* 314, 38-45 (2003).
20. R. B. Thompson, W. O. Whetsell, Jr., B. P. Maliwal, C. A. Fierke, C. J. Frederickson, Fluorescence microscopy of stimulated Zn(II) release from organotypic cultures of mammalian hippocampus using a carbonic anhydrase-based biosensor system. *J.Neurosci.Methods* 96, 35-45 (2000).
21. K. R. Gee, Z. L. Zhou, W. J. Qian, R. Kennedy. Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. *J Am Chem Soc.* 2002 Feb 6;124(5):776-8.

22. C. J. Frederickson and A. I. Bush, Synaptically released zinc: physiological functions and pathological effects. *Biometals* 14, 353-366 (2001).
23. K. R. Gee, Z. L. Zhou, D. Ton-That, S. L. Sensi, J. H. Weiss, Measuring zinc in living cells. A new generation of sensitive and selective fluorescent probes. *Cell Calcium* 31, 245-251 (2002).
24. S. H. Hong and W. Maret, A fluorescence resonance energy transfer sensor for the beta -domain of metallothionein. *Proc.Natl.Acad.Sci.U.S.A* 100, 2255-2260 (2003).
25. C. A. Fierke and R. B. Thompson, Fluorescence-based biosensing of zinc using carbonic anhydrase. *Biometals* 14, 205-222 (2001).
26. R. B. Thompson, B. P. Maliwal, V. L. Feliccia, C. A. Fierke, K. McCall, Determination of picomolar concentrations of metal ions using fluorescence anisotropy: biosensing with a "reagentless" enzyme transducer. *Anal.Chem.* 70, 4717-4723 (1998).
27. R. B. Thompson, D. Peterson, W. Mahoney, M. Cramer, B. P. Maliwal, S. W. Suh, C. J. Frederickson, C. Fierke, P. Herman. Fluorescent zinc indicators for neurobiology. *J.Neurosci.Methods* 118, 63-75 (2002).
28. K. Hanaoka, K. Kikuchi, Y. Urano, M. Narazaki, T. Yokawa, S. Sakamoto, K. Yamaguchi, T. Nagano. Design and synthesis of a novel magnetic resonance imaging contrast agent for selective sensing of zinc ion. *Chem.Biol.* 9, 1027-1032 (2002).
29. C. E. Outten and T. V. O'Halloran, Femtomolar Sensitivity of Metalloregulatory Proteins Controlling Zinc Homeostasis. *Science* 292, 2488-2492 (2001).





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