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A hexahistidine-Zn\textsuperscript{2+}-dye label reveals STIM1 surface exposure

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Site-specific fluorescent labeling of proteins in vivo remains one of the most powerful techniques for imaging complex processes in live cells. Although fluorescent proteins in many colors are useful tools for tracking expression and localization of fusion proteins in cells, these relatively large tags (>220 aa) can perturb protein folding, trafficking, and function. Much smaller genetically encodable domains (<15 aa) offer complementary advantages. We introduce a small fluorescent chelator whose membrane-impermeant complex with nontoxic Zn\textsuperscript{2+} ions binds tightly but reversibly to hexahistidine (His\textsubscript{6}) motifs on surface-exposed proteins. This live-cell label helps to resolve a current controversy concerning externalization of the stromal interaction molecule STIM1 upon depletion of Ca\textsuperscript{2+} from the endoplasmic reticulum. Whereas N-terminal fluorescent protein fusions interfere with surface exposure of STIM1, short His\textsubscript{6} tags are accessible to the dye or antibodies, demonstrating externalization.

fluorescent protein | HisZiFiT | live cell labeling | zinc

Nondestructive optical imaging of protein localization, fate and function in live cells is presently most commonly achieved by expressing the protein as a fusion to a fluorescent protein (FP) (1). The exquisite selectivity of genetic targeting allows high sensitivity of detection with no background except cellular autofluorescence. However, FPs are full-sized proteins of at least 220 aa, so they can severely perturb folding, trafficking, and function of proteins to which they are fused. FPs require up to several hours to mature and develop fluorescence, which is too slow for some applications. Genetically encoded tags for most spectroscopic readouts other than fluorescence are unknown. Therefore, several groups have devised hybrid genetic/organic alternatives in which a short (<20 aa), genetically encoded peptide motif binds a small-molecule spectroscopic reporter with sufficient specificity and affinity to be useful in or on living cells (2). Two of the best-known examples are the tetracysteine/biarsenical (1, 3) and the hexahistidine/Ni\textsuperscript{2+}-nitrilotriacetate (His\textsubscript{6}-Ni\textsuperscript{2+}-NTA systems (4–8), which have their own limitations. Tetracysteines function only when fully reduced and are therefore most applicable to the cytosolic and nuclear compartments. Smelly 1,2-dithiol antibiotics are required to minimize arsenical toxicity. Ni\textsuperscript{2+} is also a toxic heavy metal and a listed human carcinogen (9–11). Its paramagnetism tends to quench any nearby fluorophores.

Within the genetically encoded amino acids, histidine has the greatest ability to bind a variety of transition metals in an oxidizing environment. We have replaced the bridging Ni\textsuperscript{2+} ions by Zn\textsuperscript{2+}, which is ubiquitous in biological systems, nutritionally and physiologically essential, diamagnetic, and redox-inert. Because Zn\textsuperscript{2+} is not a fluorescence quencher, we designed the fluorophore to participate directly in metal chelation, resulting in a more compact structure as well as excitation and emission wavelength shifts upon Zn\textsuperscript{2+} binding. The Zn\textsuperscript{2+}-chelating molecule, 2,7-bis(pyridyl-2-sulfonamido)-4,5-dimethylfluorescein (histidine-zinc fluorescent in vivo tag, HisZiFiT), consists of a fluorescein derivatized with a pair of 2-pyridylsulfonamido functionalities to bind two Zn\textsuperscript{2+} ions while leaving enough vacancy in the metal coordination shells for further binding to multiple imidazole side chains of the His\textsubscript{6} motif (Fig. 1A). Placement of the two pyridylsulfonamides at the 2′,7′-positions insures that they bind separate Zn\textsuperscript{2+} ions, whereas 4′,5′-substituents can jointly coordinate a single Zn\textsuperscript{2+} ion (12), saturating its coordination capacity and leaving no detectable affinity for oligo-histidine binding. After describing the synthesis, spectra, and binding properties of HisZiFiT and its complexes, we show that it is a useful label for surface-exposed hexahistidine-tagged proteins. In particular, it resolves a current controversy in Ca\textsuperscript{2+} signaling mechanisms.

Recently, STIM1 (stromal interaction molecule 1) has been recognized as a key mediator by which depletion of intracellular Ca\textsuperscript{2+} stores, particularly the endoplasmic reticulum (ER), triggers compensatory Ca\textsuperscript{2+} influx across the plasma membrane (PM) (13–18). When the ER is replete with Ca\textsuperscript{2+}, STIM1 is largely resident in the ER membrane as an integral membrane protein with its N terminus in the lumen of the ER and its C terminus in the cytosol. Depletion of ER Ca\textsuperscript{2+} is sensed by a Ca\textsuperscript{2+}-binding domain (“EF hand”) on STIM1, somehow triggering translocation of STIM1 to the PM, where it plays a crucial role in activating channels for Ca\textsuperscript{2+} entry. The controversy is whether STIM1 docks to the cytosolic face of the PM without surface exposure (15, 19, 20), or instead becomes inserted into the PM (13, 17, 21, 22), presumably via some sort of exocytosis in which the formerly luminal N terminus becomes extracellular (17). A fusion of yellow fluorescent protein (YFP) to the N terminus of the STIM1 (YFP-STIM1) translocated rapidly from the ER to punctate aggregates near the PM, but antibodies against the FP did not detect surface-exposed YFP, leading to the conclusion that STIM1 does not incorporate into the PM (15, 19). An analogous GFP-STIM1 fusion was not quenched by extracellular acidification, again showing that the pH-sensitive GFP was not surface-exposed (20). A triple-HA tag (41 aa including linkers) or horseradish peroxidase (>300 aa) fused to the N terminus of STIM1 likewise remained intracellular (20). By contrast, external exposure of STIM1 was reported to increase after Ca\textsuperscript{2+} store depletion, based on immunoelectron microscopy of permeabilized cells and Western blotting for STIM1 after neutrophil collection of surface biotinylated complexes (13). Also, pretreatment of intact cells with antibodies against the N terminus of STIM1 reduced subsequent Ca\textsuperscript{2+} entry (17). However, the surface biotinylation results (see also refs. 21 and 22) have been criticized as possibly reflecting either dead cells or complexes between buried STIM1 and another surface-exposed protein (19, 20). The immunoelectron microscopy (13) was criticized as having insufficient spatial resolution to distinguish surface exposure from close apposition to the cytosolic face of the plasma membrane (20). Because

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Abbreviations: FP, fluorescent protein; PM, plasma membrane; ER, endoplasmic reticulum; HisZiFiT, histidine-zinc fluorescent in vivo tag; GFP, glycosylphosphatidylinositol.

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HisZiFiT rapidly, directly, and nondestructively labels very short surface-exposed His<sub>6</sub> motifs, it is well suited to resolve this controversy.

**Results**

The synthesis of HisZiFiT [supporting information (SI) Fig. 5] began with nitration of 4<sup>-</sup>,5<sup>-</sup>-dimethylfluorescein, where the methyl substituents confined nitration to the 2<sup>-</sup>,7<sup>-</sup> positions. Reduction of the nitro groups yielded 4<sup>-</sup>,5<sup>-</sup>-dimethyl-2<sup>-</sup>,7<sup>-</sup>-diaminofluorescein, which was reacted with 2 equivalents of 2-pyridylsulfonyl chloride (23) to give the desired fluorescent chelator. Formation of the Zn<sup>-</sup>-dye complex shifts fluorescence excitation/emission maxima from 515/545 nm to 534/560 nm (Fig. 1B) and slightly increases the quantum yield from 0.3 to 0.4. These spectral shifts were nearly complete at 1 μM free Zn<sup>2+</sup>, so buffers with 1 or 10 μM free Zn<sup>2+</sup> were used for binding to hexahistidine motifs. Although such binding caused no further spectral shifts, it could be monitored by fluorescence resonance energy transfer (FRET) from His<sub>6</sub>-CFP to HisZiFiT (Fig. 1C). Addition of 1 μM HisZiFiT to stoichiometric amounts of His<sub>6</sub>-CFP protein in saturating 10 M Zn<sup>2+</sup> buffer quenched the CFP by ~50%. In the absence of either Zn<sup>2+</sup> or His<sub>6</sub>-fusion, binding to the FP was not observed (SI Fig. 6). The proposed binding mode of the HisZiFiT-Zn<sup>2+</sup> complex to the oligohistidine peptide is schematized in Fig. 1A. The binding affinity of the HisZiFiT-Zn<sup>2+</sup> complex to a His<sub>6</sub> peptide was determined by surface plasmon resonance (Biacore, Piscataway, NJ). Approximately 0.2 pmol of the N-terminally biotinylated peptide was coupled to streptavidin-coated sensor chips. Refractive index changes due to HisZiFiT binding were fitted to a KD of HisZiFiT-Zn<sup>2+</sup> to His<sub>6</sub> of ~40 nM (SI Fig. 7), comparable to KD values for His<sub>6</sub> binding to bis-Ni<sup>2+</sup>-NTA ligands (68 nM), but much lower than mono-Ni<sup>2+</sup>-NTA ligands (1–20 M KD values) (6, 7).

The first biological tests of HisZiFiT were on live HEK293T cells expressing His<sub>6</sub>-tagged CFP anchored via glycosylphosphatidylinositol.
transfected cells clearly exhibited membrane specific fluorescent staining of the surface His6-CFP (Fig. 3). Cells expressing the His6-STIM1-CFP protein (Fig. 4), therefore created two His6-tagged STIM1 fusion proteins, SP(sig- nal peptide)-His6-CFP-STIM1 and SP-His6-STIM1-CFP (Fig. 4; L), but not those expressing His6-CFP-STIM1 (Fig. 4C), showed HisZiFiT labeling of cell-surface N-terminal His6 tags. This difference between the two constructs was confirmed by staining of live unpermeabilized cells with a monoclonal antibody against His6 and visualization with a secondary Alexa Fluor 568-conjugated antibody (Fig. 4 F and L). Again, surface exposure was detectable when His6 was the only N-terminal tag (Fig. 4L), but not when CFP was additionally inserted at the N terminus of STIM1 (Fig. 4F). However, HisZiFiT labeling appeared laterally more uniform, whereas antibodies gave a more punctate or beaded staining pattern. This difference may arise because HisZiFiT binds a single His6 motif, whereas primary antibodies are bivalent and are further cross-linkable by secondary antibodies, thus promoting lateral aggregation or capping. Antibody staining of His6-STIM1 without the CFP at the C terminus gave images (SI Fig. 10) similar to Fig. 4L, showing that STIM1 location is not perturbed by a C-terminal fluorescent protein fusion.

Discussion

HisZiFiT is the first small-molecule chelator that binds to His6 sequences using nontoxic Zn2+ in place of Ni2+. Ni2+ is an acute blocker of Ca2+ channels and a chronic toxin, allergen, mutagen, and human carcinogen (9–11). Although these long-term pathologies need not affect short-term experiments on cultured cells, we see no advantages for Ni2+. Previous His6-binding dyes used on cells (5, 6) have contained single Ni2+-NTA units, so their affinities for His6 were quite modest, 2–12 μM, compared to some His6-NTA units with tighter affinities as expected, 0.06–1 μM, but have not been demonstrated on cells (4, 7). The Kd of Zn2+-loaded HisZiFiT for His6 (40 nM) is better yet, perhaps because the fluorescence scaffold rigidly holds the two Zn2+-binding sites in fairly close proximity, whereas the long floppy arms that connect NTA units in previous designs may reduce intramolecular cooperativity between the two Ni2+ ions. The diamagnetism of Zn2+ allows HisZiFiT to remain robustly fluorescent when Zn2+-bound, whereas paramagnetic Ni2+-dyes complexes are invisible on their own and have only been detected by their ability to quench neighboring FPs by FRET (5). HisZiFiT is chemically orthogonal and functionally complementary to tetracysteine/biarsenical labeling (Fig. 3). HisZiFiT is naturally redox-independent and specific for cell surface tags, whereas tetracysteines are best suited to the reducing environment of the cytosol or nucleus. Restriction of labeling to surface-expressed tetracysteines is possible, but requires temporary reduction of disulfides with a membrane-impermeant reducing agent plus a sulfonated biarsenical (27), which is not commercially available. Tetracysteine/biarsenical labeling displays much higher affinity (Kd values in the low pM range or lower) but much slower on and off rates. Just as tetracysteine sequences and biarsenical dyes have benefited greatly from multiple rounds of improvement (26, 27), the His6 sequence and HisZiFiT should be similarly optimized to
provide tighter binding, increased photostability, decreased background staining, and additional wavelengths. Eventually it may become possible to label intracellular histidine-rich sequences, because it should be possible to synthesize membrane-permeant versions of HisZiFiT and Zn$^{2+}$/H11001 can be separately delivered with ionophores (12). Another conceivable application would be to provide a prolonged mark at sites of endogenous Zn$^{2+}$ release as in glutamatergic synapses or insulin-secreting beta cells.

The results with STIM1 (Fig. 4) exemplify the two major advantages of the His$_6$-Zn$^{2+}$/H11001-HisZiFiT system over previous fusions, the much smaller size of the peptide tag and the ability to distinguish surface-exposed proteins from those docked to the
cytosolic face of the PM. The controversy over surface exposure of STIM1 is partially resolved by the findings that externalization of the N terminus of STIM1 is prevented by N-terminal fusions of HA tags, FPs, and horseradish peroxidase (41, 238, and >300 residues, respectively) but tolerates N-terminal His6 tags and C-terminal FP fusion. Anti-His6 antibody staining confirmed these HisZiFiT results. Although the antibody gave lower background signal, immunostaining is a slower, more complex, and less reversible procedure than HisZiFIT staining. Furthermore, antibodies promote lateral aggregation, add far more mass, and are known to perturb STIM1 function, possibly by dominantly modifying multimerization (17). Further studies will be required to quantify how much and how quickly surface exposure may increase upon stores depletion. We do not know the precise mechanism by which the N terminus of STIM1 moves from the ER lumen to the extracellular space. A prime candidate would be exocytotic fusion of vesicles budded off from the ER, consistent with observations that Ca2+ influx due to stores depletion can be inhibited by interference with proteins involved in exocytosis (28, 29). However, such exocytosis would differ from classical regulated secretion with respect to source organelle (ER vs. acidic secretory granules), mode of triggering by Ca2+ (luminal depletion vs. cytosolic elevation), and tolerance for luminal FP fusions (nonpermissive vs. permissive; ref. 30). Exocytosis of the N terminus of STIM1 is probably not essential for triggering Ca2+ elevation of the N terminus should expose the EF hand of STIM1 to the cytosolic domain of Orai1/CRACM1 (16, 18, 19). Externalization of the cytosolic C terminus of STIM1 to the PM, in particular the N terminus of STIM1 moves from the ER lumen to the extracellular space. A prime candidate would be exocytotic fusion of vesicles budded off from the ER, consistent with observations that Ca2+ influx due to stores depletion can be inhibited by interference with proteins involved in exocytosis (28, 29). However, such exocytosis would differ from classical regulated secretion with respect to source organelle (ER vs. acidic secretory granules), mode of triggering by Ca2+ (luminal depletion vs. cytosolic elevation), and tolerance for luminal FP fusions (nonpermissive vs. permissive; ref. 30). Exocytosis of the N terminus of STIM1 is probably not essential for triggering Ca2+ influx, because overexpression of the nonexocytosis-yielding GFP-STIM1 fusion is sufficient to increase influx (15, 19). Presumably the activating interaction is the docking of the cytosolic C terminus of STIM1 to the PM, in particular the cytoplasmic domain of Orai1/CRACM1 (16, 18, 19). Externalization of the N terminus should expose the EF hand of STIM1 to the cytosolic domain of Orai1/CRACM1 (16, 18, 19). Externalization of the N terminus should expose the EF hand of STIM1 to the cytosolic domain of Orai1/CRACM1 (16, 18, 19). Externalization of the N terminus should expose the EF hand of STIM1 to the cytosolic domain of Orai1/CRACM1 (16, 18, 19). Externalization of the N terminus should expose the EF hand of STIM1 to the cytosolic domain of Orai1/CRACM1 (16, 18, 19).

Materials and Methods

The synthesis of the HisZiFIT dye, surface plasma resonance experiments, and detailed cloning information with the final peptide sequences of the STIM1-containing constructs are described in SI Text.

HEK293T cells were plated onto sterilized glass coverslips on 2-cm imaging dishes and grown to 50–80% confluency in DMEM ( Gibco-BRL) supplemented with 10% FBS and 5% penicillin/streptomycin at 37°C in 5% CO2. Cells were transfected with FUGENE-6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After a 24- to 48-h incubation at 37°C in culture medium, the cells were washed twice with HBSS supplemented with 20 mM Hepes (pH 7.4) and 1 g/liter D-glucose before staining. His6-CFP-GPI-expressing cells were stained by adding ~100 nM HisZiFIT (aliquots of 1 mM DMSO stock, g = 72,000 mol−1 cm−1) in labeling buffer with 1 μM free Zn2+ (Zn2+ buffered by 8.099 mM L-histidine, 2.603 mM ZnO in 10 mM Hepes, 5 mM KCl, 140 mM NaCl, pH 7.4). See SI Text for details on Zn2+ buffers. After 1 min, the cells were washed twice with HBSS and imaged. Orthogonal staining of Connexin43–4C and His6-CFP-GPI-expressing cells was achieved by staining with 1 μM ReAsH10/μM ethanediol (EDT) in HBSS for 30 min. Staining was followed by two 10-min washes with 0.1 mM 2,3-dimercaptopropanol in HBSS and 1 min of HisZiFIT staining as described above.

Cells expressing STIM1 fusions were stained and imaged 48 h after transfection. The culture medium was removed, and the cells were rinsed once with HBSS and a second time with Ca2+-free saline (25 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM d-glucose, 1 mM EDTA, pH 7.4). Before staining, Ca2+ store depletion was triggered by addition of 2 μM thapsigargin in Ca2+-free medium. The associated STIM1 plasma membrane translocation of the CFP-fusion proteins was monitored with CFP fluorescence. After ~15 min, no further translocation was observed. The cells were washed twice with 2 ml of HBSS and stained by treating either with ~100 nM HisZiFIT/1 μM Zn2+ staining solution as described above or with penta-His antibody (mouse IgG1, Qiagen). An aliquot of the penta-His antibody (His5-Ab) stock solution (0.2 mg/ml) was diluted 1:40 into DMEM with 10% FBS and added to the cells for 15 min. For visualization of the His5-Ab, the cells were washed twice with HBSS and stained for 15 min with 1:300 dilution of secondary Ab stock solution (Alexa Fluor-568 goat anti-mouse IgG3, 2 mg/ml; Invitrogen) into DMEM/10% FBS. After secondary Ab staining, cells were washed two times with HBSS and imaged. Ab staining was highly specific to transfected cells, and control dishes stained with secondary Ab alone did not exhibit staining.