

A hexahistidine-Zn²⁺-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 Zn²⁺ ions binds tightly but reversibly to hexahistidine (His₆) 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 Ca²⁺ from the endoplasmic reticulum. Whereas N-terminal fluorescent protein fusions interfere with surface exposure of STIM1, short His₆ 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/Ni²⁺-nitrilotriacetate (His₆-Ni²⁺-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 antidotes are required to minimize arsenical toxicity. Ni²⁺ 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 Ni²⁺ ions by Zn²⁺, which is ubiquitous in biological systems, nutritionally and physiologically essential, diamagnetic, and redox-inert. Because Zn²⁺ 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 Zn²⁺ binding. The Zn²⁺-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 Zn²⁺ ions while leaving enough vacancy in the metal coordination shells for further binding to multiple imidazole side chains of the His₆ motif

(Fig. 1A). Placement of the two pyridylsulfonamides at the 2',7'-positions insures that they bind separate Zn²⁺ ions, whereas 4',5'-substituents can jointly coordinate a single Zn²⁺ 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 Ca²⁺ signaling mechanisms.

Recently, STIM1 (stromal interaction molecule 1) has been recognized as a key mediator by which depletion of intracellular Ca²⁺ stores, particularly the endoplasmic reticulum (ER), triggers compensatory Ca²⁺ influx across the plasma membrane (PM) (13–18). When the ER is replete with Ca²⁺, 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 Ca²⁺ is sensed by a Ca²⁺-binding domain (“EF hand”) on STIM1, somehow triggering translocation of STIM1 to the PM, where it plays a crucial role in activating channels for Ca²⁺ 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 Ca²⁺ store depletion, based on immunoelectron microscopy of permeabilized cells and Western blotting for STIM1 after neutravidin collection of surface biotinylated complexes (13). Also, pretreatment of intact cells with antibodies against the N terminus of STIM1 reduced subsequent Ca²⁺ 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; GPI, glycosylphosphatidylinositols.

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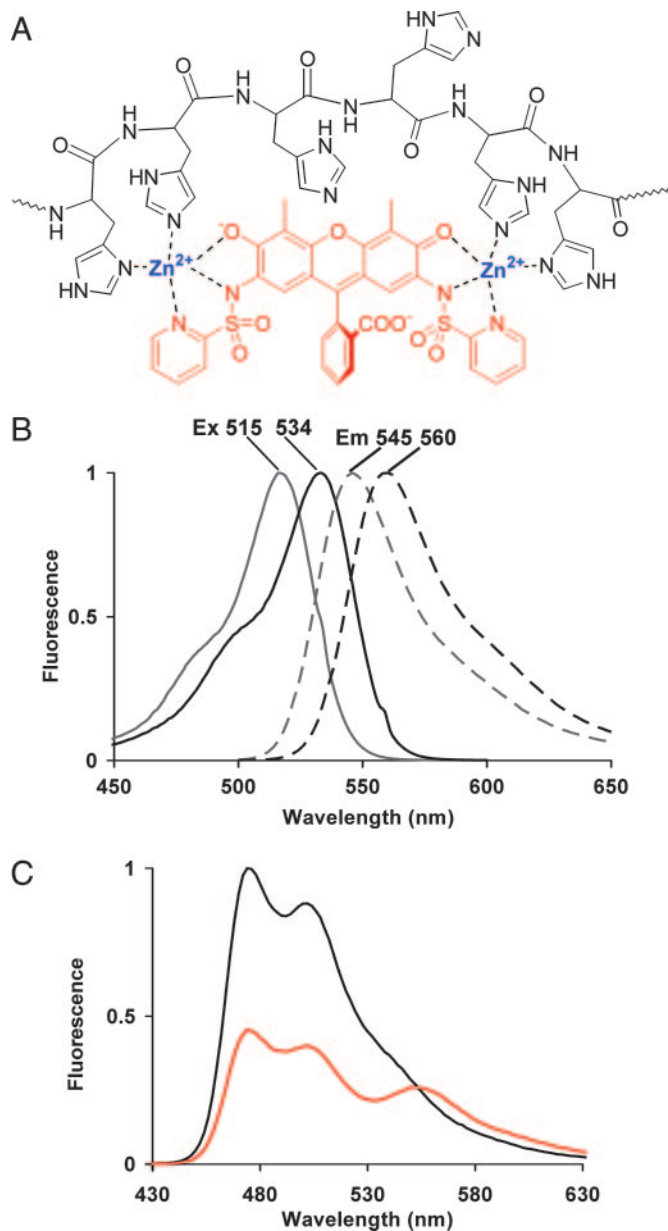


Fig. 1. Structure and spectra of HisZiFiT. (A) Structure of HisZiFiT (red) and proposed mode of Zn^{2+} -mediated binding to a His₆ sequence (black), in flattened projection. (B) Normalized fluorescence excitation (solid curves) and emission (dashed) spectra of 1 μ M HisZiFiT without Zn^{2+} (gray curves) and with saturating (10 μ M) free Zn^{2+} (black). (C) FRET response of His₆-CFP to HisZiFiT binding (ex420). Emission spectrum of 1 μ M His₆-CFP with 10 μ M buffered free Zn^{2+} before (black curve) and after (red curve) addition of 1 μ M HisZiFiT.

HisZiFiT rapidly, directly, and nondestructively labels very short surface-exposed His₆ motifs, it is well suited to resolve this controversy.

Results

The synthesis of HisZiFiT [supporting information (SI) Fig. 5] began with nitration of 4',5'-dimethylfluorescein, where the methyl substituents confined nitration to the 2',7' positions. Reduction of the nitro groups yielded 4',5'-dimethyl-2',7'-diaminofluorescein, which was reacted with 2 equivalents of 2-pyridylsulfonyl chloride (23) to give the desired fluorescent chelator. Formation of the Zn-dye complex shifts fluorescence excitation/emission maxima from 515/545 nm to 534/560 nm (Fig. 1B) and slightly increases the

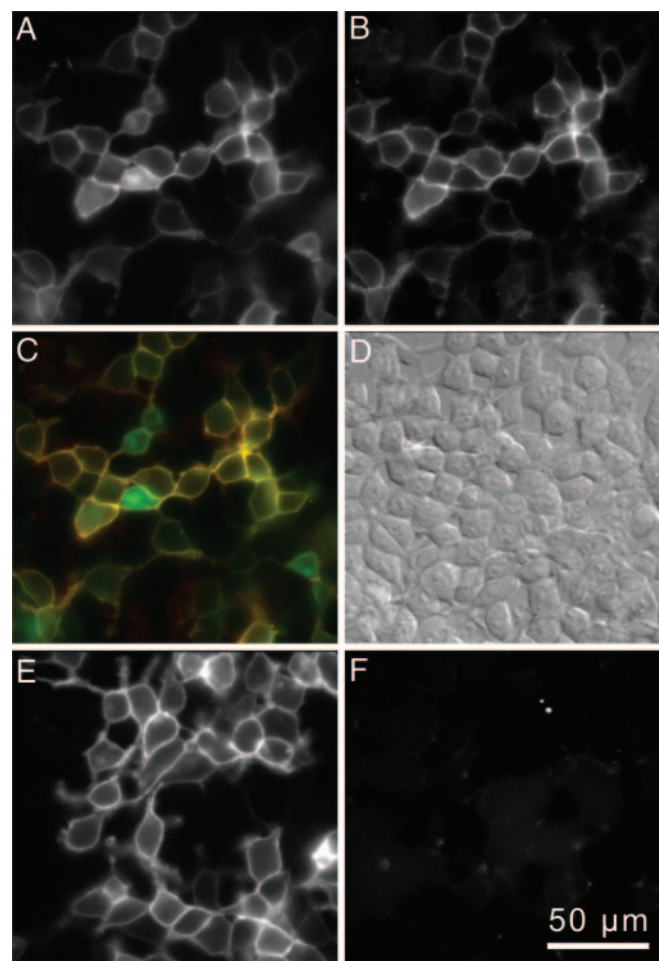


Fig. 2. HisZiFiT labeling is specific for cell-surface His₆-tagged proteins. (A–D) HEK293T cells expressing His₆-CFP-GPI, labeled with 100 nM HisZiFiT in 1 μ M buffered free Zn^{2+} . (A) CFP channel, ex420/em475, shows both surface and internal His₆-CFP-GPI. (B) HisZiFiT channel, ex540/em595, of the same cells shows only plasma membrane outlines. (C) Merge of A (displayed in green) and B (red). (D) Differential interference contrast image of the same cells. (E and F) Control HEK293T cells expressing nontagged CFP (CFP-GPI), identically exposed to HisZiFiT. (E) CFP channel. (F) HisZiFiT channel shows negligible staining of CFP-GPI protein.

quantum yield from 0.3 to 0.4. These spectral shifts were nearly complete at 1 μ M free Zn^{2+} , so buffers with 1 or 10 μ M free Zn^{2+} 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 His₆-CFP to HisZiFiT (Fig. 1C). Addition of 1 μ M HisZiFiT to stoichiometric amounts of His₆-CFP protein in saturating 10 μ M Zn^{2+} buffer quenched the CFP by \approx 50%. In the absence of either Zn^{2+} or His₆-fusion, binding to the FP was not observed (SI Fig. 6). The proposed binding mode of the HisZiFiT- Zn^{2+} complex to the oligohistidine peptide is schematized in Fig. 1A. The binding affinity of the HisZiFiT- Zn^{2+} complex to a His₆ 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 K_D of HisZiFiT- Zn^{2+} to His₆ of \approx 40 nM (SI Fig. 7), comparable to K_D values for His₆ binding to bis- Ni^{2+} -NTA ligands (68 nM), but much lower than mono- Ni^{2+} -NTA ligands (1–20 μ M K_D values) (6, 7).

The first biological tests of HisZiFiT were on live HEK293T cells expressing His₆-tagged CFP anchored via glycosylphosphatidyli-

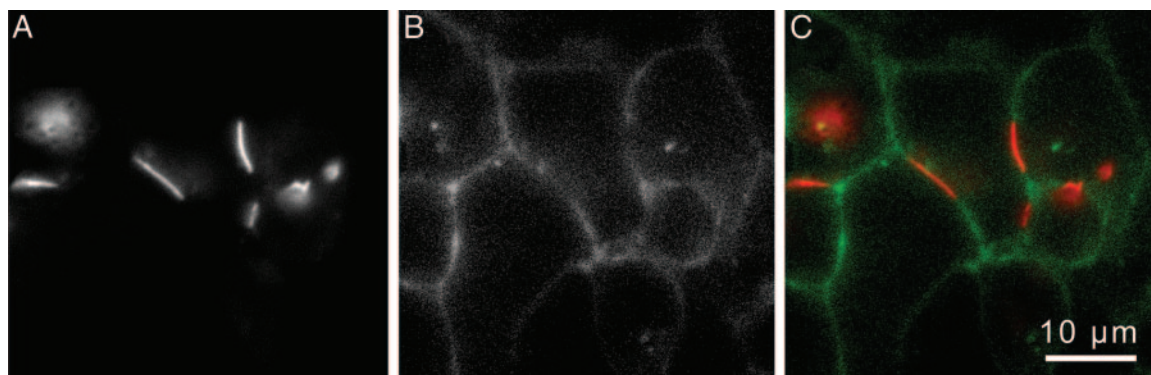


Fig. 3. Orthogonal staining of HEK293T cells expressing two proteins on the PM. (A) Connexin43–4C with an intracellular tetracysteine motif, stained with ReAsH (ex568/55, em653/95). (B) His₆ on extracellular PM surface stained and visualized with HisZiFiT (ex495/10, em535/45). (C) Merge of images A and B representing HisZiFiT staining of His₆-CFP-GPI (green) and ReAsH staining of Connexin43–4C (red).

nositols (GPIs) to the surface of the PM membrane (His₆-CFP-GPI) (24) although some His₆-CFP-GPI also remains in intracellular organelles (Fig. 2*A*). HisZiFiT (100–200 nM) was administered with free Zn²⁺ buffered to 1 μM with histidine. Surface staining by the impermeant HisZiFiT-Zn²⁺ complex was complete in <1 min. After two washes with dye-free HBSS, transfected cells clearly exhibited membrane specific fluorescent staining (Fig. 2*B*). When images in Fig. 2*A* and *B* were overlaid with the CFP channel in green and the HisZiFiT channel in red (Fig. 2*C*), the PM contained both signals and appeared yellow, whereas His₆-CFP-GPI that had not yet reached the cell surface remained green. Control cells expressing the CFP-GPI protein without a His₆-tag (Fig. 2*E*) showed no specific staining (Fig. 2*F*). HisZiFiT attached to His₆-CFP-GPI could be rapidly removed by washing with a strong Zn²⁺ chelator such as EDTA (SI Fig. 8).

To demonstrate that His₆/HisZiFiT-Zn²⁺ and the tetracysteine/biarsenical systems do not interfere with each other chemically or spectrally, we coexpressed His₆-CFP-GPI and connexin43 C-terminally tagged with a tetracysteine motif (Cx43–4C) (25) in HEK293T cells. ReAsH staining of the gap junctions formed by Cx43–4C for 30 min (Fig. 3*A*), followed by 1 min of HisZiFiT staining of the surface His₆-CFP (Fig. 3*B*), permitted site-specific labeling of two different proteins in the same PM (merge of images Fig. 3*A* and *B* in Fig. 3*C*). Interestingly, the His₆-CFP-GPI appears partially excluded from the gap junctions, which consist of a paracrystalline array of connexins occupying most of the surface area within the plaque (26).

We then applied HisZiFiT to a currently controversial question in cell biology, the surface exposure of the stromal interaction protein STIM1 after depletion of Ca²⁺ stores. Because endogenous STIM1 is reported to become incorporated into the PM and therefore surface exposed (13, 17, 21, 22) whereas YFP, GFP, an HA tag, or horseradish peroxidase fused to the N terminus of STIM1 remain unexposed (15, 19, 20), we wondered whether bulky tags could prevent membrane insertion of the STIM1 fusion. We therefore created two His₆-tagged STIM1 fusion proteins, SP(signal peptide)-His₆-CFP-STIM1 and SP-His₆-STIM1-CFP (Fig. 4; complete sequences in SI Fig. 9), differing only in the placement of the CFP initially within the lumen of the ER or in the cytosol respectively. Imaging the CFP channel 24–48 h after transient transfection of the genes into HEK293T cells revealed that both fusions showed the expected ER expression before stimulation (data not shown) as well as translocation to the PM after thapsigargin-induced depletion of stored Ca²⁺ (Fig. 4*B*, *E*, *H*, and *K*). Cells expressing the His₆-STIM1-CFP protein (Fig. 4*I*), but not those expressing His₆-CFP-STIM1 (Fig. 4*C*), showed HisZiFiT labeling of cell-surface N-terminal His₆ tags. This difference between the two constructs was confirmed by staining of live unper-

meabilized cells with a monoclonal antibody against His₅ 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. 4*L*), but not when CFP was additionally inserted at the N terminus of STIM1 (Fig. 4*F*). 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. 4*L*, 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 *K_D*. Dyes with two Ni²⁺-NTA units have tighter affinities as expected, 0.06–1 μM, but have not been demonstrated on cells (4, 7). The *K_D* of Zn²⁺-loaded HisZiFiT for His₆ (40 nM) is better yet, perhaps because the fluorescein 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⁺ quenches nearby fluorophores (5, 6). Nonfluorescent Ni²⁺-dye 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 (*K_D* 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

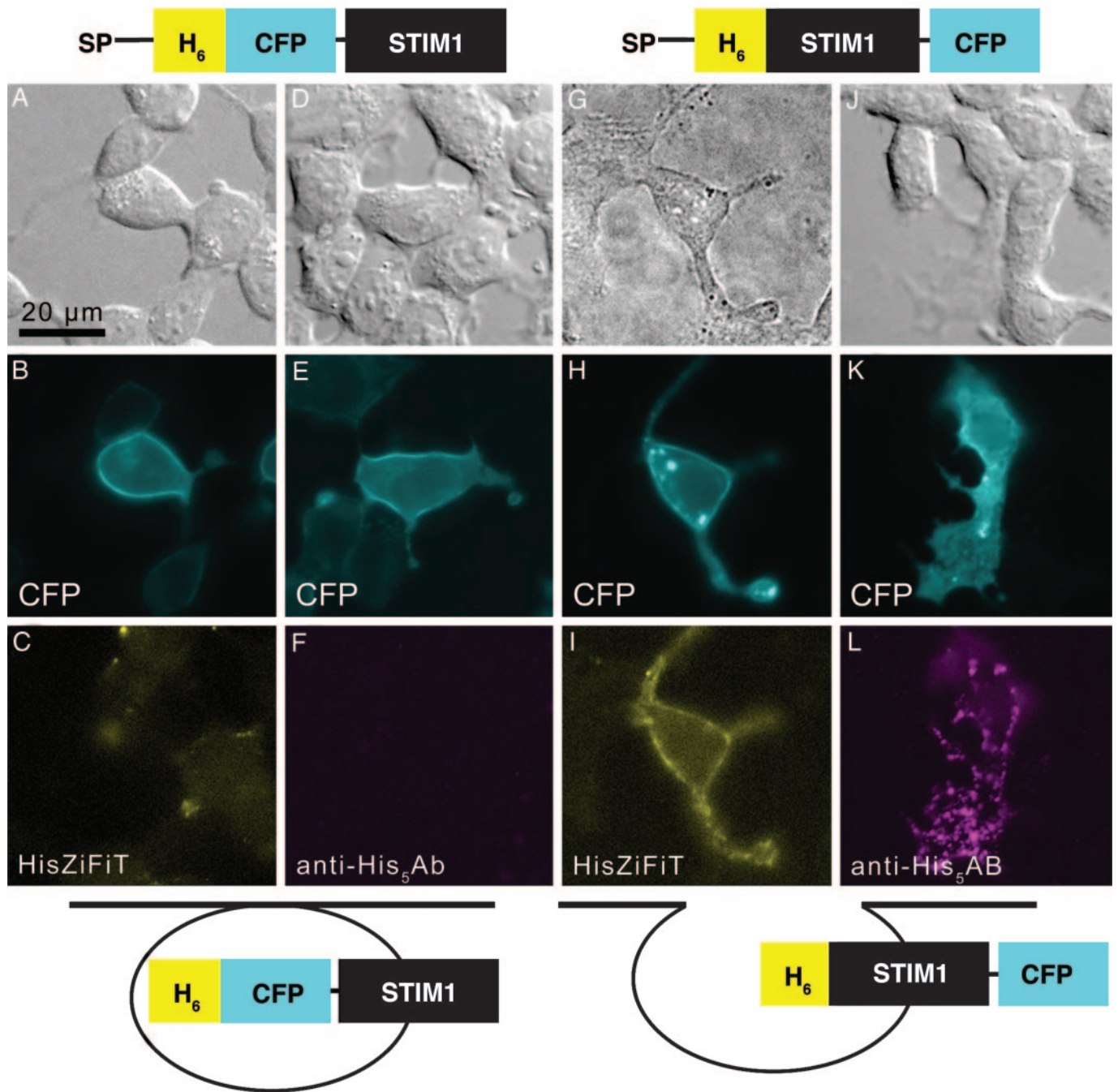


Fig. 4. N-terminal tagging with His₆ but not CFP allows cell surface exposure of STIM1. (A–L) Images of cells transiently transfected with His₆-CFP-STIM1 (A–F) or His₆-STIM1-CFP protein (G–L). Cartoons above A–L schematize the expressed polypeptide domains, where SP denotes signal peptide and H₆ stands for His₆. (A, D, G, and J) Transmitted light images; images in A, D, and J show differential interference contrast. (B, E, H, K) CFP fluorescence (cyan) of the same fields shows that all of the STIM1 fusions are recruited to the plasma membrane after depletion of Ca²⁺ stores with 2 μM thapsigargin. (C) Lack of specific staining by HisZiFiT (yellow) indicates absence of extracellularly exposed His₆-tags on the same cells as in B. (F) Lack of antibody staining for His₅ (magenta) confirms absence of extracellularly exposed His₆ tags on the same live cell as in E. (I) Surface exposed His₆-tags of the same cell as in H are labeled by HisZiFiT (yellow). (L) Live cell His₅-Ab staining (detected with Alexa Fluor 568-labeled secondary Ab, magenta) confirms the exposure of N-terminal His₆-tags on the same cells as in K. Exposure times with a 5% neutral density filter on excitation were 200, 1,000, and 50 ms for CFP, HisZiFiT, and Alexa Fluor 568, respectively. Cartoons show schematically how His₆ remains luminal in His₆-CFP-STIM1 (B, C, E, and F) but becomes extracellularly accessible in His₆-STIM1-CFP (H, I, K, and L).

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 Zn²⁺ can be separately delivered with ionophores (12). Another conceivable application would be to

provide a prolonged mark at sites of endogenous Zn²⁺ release as in glutamatergic synapses or insulin-secreting beta cells.

The results with STIM1 (Fig. 4) exemplify the two major advantages of the His₆-Zn²⁺-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

