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Supplementary Information for:

VIPER is a genetically-encoded peptide tag for fluorescence and electron microscopy

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Materials and Methods

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, ThermoFisher Scientific, or Lumiprobe and used as received. Anhydrous ethanol (Decon Labs; Cat.# 2716) and hexamethyl-disilazane (HMDS; Electron Microscopy Sciences; Cat.# 16700) were used to dehydrate samples for SEM.

Summary of genetic constructs

A summary of all genetic constructs used in the current work, including peptide and protein sequences, is provided in **Table S5**. Bacterial strains and plasmids are summarized in **Table S6**. Oligonucleotides were purchased from Integrated DNA Technologies (IDT; **Table S7**).

Mammalian cell culture and maintenance

Chinese Hamster Ovary (CHO) TRVb cells were generously provided by Prof. Timothy E. McGraw (Cornell University, New York). These cells do not express functional transferrin receptor 1 (TfR1) or transferrin receptor 2 (TfR2)(1). CHO TRVb cells were maintained in Ham's F12 media (Gibco) with 5% fetal bovine serum (FBS) in 10 cm polystyrene dishes. Cells were grown at 37 ºC in a humidified incubator with 5% CO_2. Cells were passaged when they reached 80-90% confluency. Cells were detached with 0.25% trypsin/1 mM EDTA (TRED) and seeded at a 1:10 dilution (2 x10^6 cells/dish).

U-2 OS cells were purchased from ATCC (Cat. #HTB-96). Cells were maintained in Dulbecco's Modified Eagle Media (DMEM; Gibco) with 10% FBS in 10 cm polystyrene dishes. Cells were grown at 37 ºC in a humidified incubator with 5% CO_2. Cells were passaged when they reached 80-90% confluency. They were detached with TRED and seeded at a 1:10 dilution (10^6 cells/dish).

Transfection of plasmid DNA in CHO TRVb or U-2 OS cells

All imaging experiments were conducted in transiently transfected cell lines. For transfections, 5 x 10^4 cells were seeded into each well of an 8-well chambered slide (Cellvis) and grown overnight to 70-90% confluency. Transfection was performed using Lipofectamine 2000 (ThermoFisher Scientific) following the manufacturer's instructions. For each well, the transfection mixture contained 500 ng plasmid DNA and 1 µg Lipofectamine 2000 in 400 µL Opti-MEM. After 2 h, the transfection media was removed and replaced with fresh media with serum for recovery. Cells were imaged approximately 24 h after transfection.

Genetic construction of pET28b(+)_CoilR and pET28b(+)_CoilE

Genes were synthesized using gene assembly PCR, as described previously(2). Oligonucleotides were designed using DNAworks(2)(http://helixweb.nih.gov/dnaworks/). Restriction enzyme cut sites *Nco*I and *Hind*III were included in the primers to allow for compatible insertion of *coilR* or *coilE* into the pET28b(+) vector backbone. The *coilE* gene was assembled using primers CoilE-1, CoilE-2, CoilE-3, CoilE-4, CoilE-5 and CoilE-6 (see **Table S7** for oligonucleotide sequences). The *coilR* gene was derived from the c*oilR-Lys56* gene, which was assembled using primers CoilR-1, CoilR-2, CoilR-3, CoilR-4, CoilR-5, and CoilR-6.

The amplified *coilR-Lys56* PCR gene product and purified pET28b(+) plasmid (Novagen) were doubly-digested with *Nco*I (NEB) and *Hind*III (NEB), ligated into the digested pET28b(+) plasmid using T4 DNA ligase (NEB), and transformed into chemically-competent *E. coli* cells (DH5α; ThermoFisher Scientific). Transformed *E. coli* cells were grown and propagated on LB agar plates supplemented with kanamycin (50 µg/mL). Recombinant colonies were screened with colony PCR and plasmids from positive hits were submitted for sequencing analysis. The pET28b(+)_CoilR-Lys56 plasmid was altered by site-directed mutagenesis to introduce a cysteine at position 56 (Lys56Cys). This generated the pET28b(+)_CoilR plasmid, which was used to express thiol-containing CoilR probe peptides, as described below.

An analogous approach was used to insert the *coilE* gene into pET28b(+) to generate pET28b(+)_CoilE.

Genetic construction of mEmerald constructs

Three mEmerald constructs were obtained from Addgene (Michael Davidson's Collection): mEmerald-Actin-C18 (Addgene #53978), Mito-7-mEmerald (Addgene #54160), and H2B-6-mEmerald (Addgene #54111). The parent plasmid for these constructs is mEmerald-C1 (Addgene #53975), which has multiple cloning sites and includes a CMV promoter and neomycin/kanamycin resistance. mEmerald-C1 is adapted from the discontinued Clontech vector pEGFP-C1, with mutations introduced to convert EGFP into mEmerald

(mEmerald = EGFP + L64L [silent C192T transition], S72A, N149K, M153T, I167T, A206K). The sequence information for these vectors is on the Addgene website (www.addgene.org).

The mEmerald-Actin-C18 vector was digested with *Bgl*II (NEB). Vectors Mito-7-mEmerald and H2B-6-mEmerald were both digested with *Age*I (NEB). Digested vectors were then purified from a 0.8% low-melting point agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo). The *coilE* gene was introduced intragenically, between the mEmerald and the target protein using a restriction site (*Bgl*I or *Age*I) located in the middle of the linker (Gly-Gly-Gly-Gly-Pro-Val-Ala-Thr) designed by the Davidson lab (**Table S5**). In-Fusion primers were designed following the protocol provided in the In-Fusion HD Cloning Kit user manual (Takara Clontech). Primer sequences for the In-Fusion cloning are provided in **Table S7**. For insertion into mEmerald-Actin-C18, the *coilE* gene was amplified with the CoilE *Bgl*I Actin F and CoilE *Bgl*I Actin R primers. For insertion into Mito-7-mEmerald, *coilE* was amplified with the CoilE *Age*I Mito F and CoilE *Age*I Mito R primers. For insertion into H2B-6-mEmerald, *coilE* was amplified with the CoilE *Age*I H2B F and CoilE *Age*I H2B R primers. After PCR, the products were analyzed on a 2% agarose gel in TAE. One unit of *Dpn*I (NEB) was added to each PCR reaction to digest pET28b(+)_CoilE and then PCR products were purified via spin column (Takara Clontech).

In-Fusion HD reactions were set up with ~100 ng of linearized vector and 200 ng InFusion PCR product following manufacturer's protocol (In-Fusion HD, Takara Clontech). The reactions were incubated at 50 ºC for 15 min followed by transformation into *E. coli* (Stellar) competent cells (Takara Clontech). Cells were grown and propagated on LB agar plates supplemented with kanamycin (50 µg/mL). Recombinant colonies were screened and the sequences from positive hits were confirmed via Sanger sequencing.

Genetic construction of pcDNA3.1_TfR1-CoilE from pcDNA3.1_TfR1

The pcDNA3.1_TfR1 vector, which encodes murine TfR1, was previously described(3). CoilE-tagged TfR1 was constructed by Gibson assembly. To make pcDNA3.1_TfR1-CoilE, the *coilE* gene was inserted at the C-terminal end of the *tfr1* gene. The *tfr1* stop codon was moved to the end of the *coilE* gene and a sequence encoding a Gly-Ser-Gly-Ser-Gly-Ser-Thr-Gly linker was added between the two genes. Novel *Age*I cut sites were inserted flanking the *coilE* gene on both ends.

Primers for generating Gibson assembly fragments were designed using the NEBuilder® Assembly tool (http://nebuilder.neb.com/) (**Table S7**). Gibson assembly fragment 1 was generated by PCR using primers TfR1-CoilE 1 F and TfR1-CoilE 1 R with the vector pcDNA3.1_TfR1 as the template. Gibson assembly fragment 2 was PCR generated using primers TfR1-CoilE 2 F and TfR1-CoilE 2 R and pET28B(+)_CoilE as the template. Gibson assembly fragment 3 was generated using PCR using primers TfR1-CoilE 3 F and TfR1-CoilE 3 R and pcDNA3.1_TfR1 as the template. All PCRs yielded a single product and were purified via PCR and Gel Clean Up Kit (Takara Clontech). The pcDNA3.1_TfR1 vector was digested with *Sma*I and *BstE*II (NEB) and the vector backbone was gel-purified using a 0.6% agarose gel. The vector backbone and all inserts were combined (molar ratio of 1:5 vector-to-insert) and ligated using the Gibson Assembly Mastermix (NEB). The reaction was incubated at 50 ºC for 1 h, transformed to TOP10 *E. coli*, and plated on LB agar plates supplemented with ampicillin (100 µg/mL). Vector-transformed colonies were identified using colony PCR and positive clones were confirmed by Sanger sequencing.

Expression and purification of CoilR

We used the pET28b(+)_CoilR plasmid transformed into *E. coli* BL21(DE3) cells (ThermoFisher) to express the CoilR probe peptide. A starter culture was used to inoculate a 1 L flask of LB media with kanamycin (50 µg/mL). When the OD_{600} reached 0.8, we induced expression of CoilR by adding 0.5 mM IPTG for 1 h at 37 ºC. Cells were pelleted by centrifugation and frozen at -20 ºC until purification.

The CoilR peptide was purified under denaturing conditions. Cells were thawed on ice and re-suspended in denaturing lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 8 M urea, 1 mM DTT, pH 7.5). Re-suspended cells were sonicated on ice with a 0.5-inch horn for 8 min with 30 sec on-off intervals (Branson A-450; duty cycle 80% and output control: 8). The lysate was clarified by centrifugation and then incubated with 1 mL of Ni-NTA resin (QIAGEN; resin pre-equilibrated with denaturing lysis buffer) for 1 h at 4 ºC. The resin was then loaded into an Econo-Pac® Chromatography Column (Bio-Rad) and washed with 10 mL denaturing lysis buffer containing 20 mM imidazole and 10 mL lysis buffer containing 50 mM imidazole. The His_6-tagged CoilR was eluted from the resin with 20 mL lysis buffer containing 500 mM imidazole. Fractions containing CoilR were combined, concentrated, and buffer exchanged into a low imidazole buffer (20 mM sodium

phosphate, 500 mM NaCl, 8 M urea, 10 mM imidazole, 1 mM DTT, pH 7.5) using Amicon Ultra centrifugal filters (3kDa MWCO, Millipore).

The peptides were further purified on a HisTrap FF 1 mL column (GE Healthcare) via FPLC (GE Äkta Purifier). The peptides were eluted with a linear gradient of 20 mM to 500 mM imidazole in lysis buffer over 20 column volumes. Fractions (1 mL) were collected and analyzed by SDS-PAGE. Fractions containing purified peptides were combined, concentrated, and buffer exchanged into 1x TBS/Urea (50 mM Tris pH 8, 150 mM NaCl, 6 M urea) using 3000 kDa Amicon Ultra centrifugal filters. Peptide purity was monitored by SDS-PAGE. Purified peptide solutions were quantified using the Pierce bicinchoninic acid (BCA) assay kit (ThermoFisher) following the manufacturer's instructions. Peptides were stored frozen (-20 °C) in 10% glycerol.

SDS-PAGE analysis

Protein samples were analyzed by SDS-PAGE using Criterion XT gels (BioRad). Samples were combined with SDS-PAGE loading dye (50 mM Tris pH 6.9, 100 mM TCEP, 2% SDS, 0.1% Ponceau Red, 10% glycerol) and boiled (5-10 min). Samples were centrifuged briefly before loading and then resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and de-stained before imaging on a flat-bed scanner (Canon LiDE220).

Generation of CoilR-BODIPY and CoilR-Cy5 dye-labeled peptides

Purified CoilR peptide, containing a single reactive cysteine residue, was buffer-exchanged into Maleimide Labeling Buffer (MLB: 20 mM Tris pH 7.2, 150 mM NaCl, 8 M urea) using Amicon Ultra centrifugal filters (3kDa MWCO, Millipore). MLB was de-gassed under vacuum with stirring immediately before use. CoilR peptide (30 nmol, from 2 mg/mL stock) was reduced in TCEP (30-fold molar excess) in de-gassed MLB (200 µL) for 30 min at 37 ºC.

Reactive maleimide dyes were purchased from Lumiprobe, and stock solutions were prepared in anhydrous DMSO (Sigma-Aldrich). The maleimide dye (40 µL of 100 mM BODIPY or 50 mM Cy5) was added to the reduced CoilR peptide in MLB and incubated on a rotisserie inverter at 4 ºC overnight, protected from light. To make CoilR-BODIPY, the peptide was reacted with a 133-fold molar excess of BODIPY-maleimide (Lumiprobe, #21480). Some of the BODIPY-maleimide precipitated upon addition to the peptide in MLB. To make CoilR-Cy5, the peptide was reacted with a 67-fold molar excess of Sulfo-Cy5-maleimide (Lumiprobe, #23380).

After the reaction, excess fluorophore was removed by centrifugation (Amicon Ultra 3 kDa MWCO) and exchanged into Ni-NTA binding buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris, 10 mM imidazole, pH = 8.0). Labeled peptide was bound to Ni-NTA resin (Qiagen), washed, and eluted with pH 4.5 elution buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris, 10 mM imidazole, pH = 4.5). Fractions containing CoilR were combined, concentrated (Amicon Ultra 3kDa MWCO), and buffer-exchanged into MLB, yielding a clear-orange (CoilR-BODIPY) or clear-dark-blue (CoilR-Cy5) solution. Final peptide concentration was determined using the Pierce BCA assay kit (ThermoFisher Scientific) following the manufacturer's instructions. Dye labeling efficiency was estimated using Lumiprobe's suggested protocol. Briefly, we measured absorbance of the labeled peptide at 280 nm and at the excitation maximum of the dye (503 for BODIPY or 646 nm for Sulfo-Cy5) in MLB in a 1-cm quartz cuvette. Labeling efficiency was calculated using the equation:

$$\frac{mol\ dye}{mol\ protein} = \frac{A_{dye}}{\varepsilon_{dye} \times \frac{A_{280} - \left(A_{dye} \times CF\right)}{\varepsilon_{peptide}}}$$

In this equation, A_{dye} is the absorbance of the labeled peptide at 503 nm (BODIPY) or 646 nm (Sulfo-Cy5), ε_{dye} is the molar extinction coefficient of the dye, A_{280} is the absorbance of the labeled peptide at 280 nm, *CF* is the dye absorbance correction factor at 280 nm, and $\varepsilon_{peptide}$ is the molar extinction coefficient of the peptide at 280 nm (2980 $L \cdot mol^{-1} \cdot cm^{-1}$; calculated using ExPasy, https://web.expasy.org/protparam). See **Table S8** for fluorophore values provided by Lumiprobe. Using this equation, we estimated that the CoilR-BODIPY was 30% labeled and the CoilR-Cy5 peptide was 90% labeled. The peptides were diluted to 100 µM in MLB with 10% glycerol and stored at -20 ºC, protected from light.

Generation of biotinylated probe peptide: CoilR-biotin

Purified CoilR peptide was buffer-exchanged into de-gassed MLB using Amicon Ultra centrifugal filters (3kDa MWCO, Millipore). CoilR peptide (100 nmol) was reduced with TCEP (1 µmol, 10-fold excess) for 30 min at 37 ºC. The reduced peptide solution was combined with 2 mg (35-fold excess) of EZ-Link-PEG2-biotin (ThermoFisher #21901BID). The reaction mixture (1 mL total volume) was incubated on a rotisserie inverter at 4 ºC overnight. After the reaction, excess biotin was removed by centrifugation (Amicon Ultra, 3 kDa MWCO) by washing exhaustively. The biotinylated peptide was purified by affinity column chromatography. CoilR-biotin was exchanged into 10 mL TBS (20 mM Tris, 150 mM NaCl pH 8.0) before purification of CoilR-biotin on Pierce monomeric avidin agarose resin (ThermoFisher Scientific; Pierce #20228) following the manufacturer's protocol. Fractions were analyzed by SDS-PAGE and anti-biotin Western blot. Fractions containing CoilR-biotin were combined and concentrated by centrifugation (Amicon Ultra 3 kDa MWCO). Final protein concentration was determined by the Pierce BCA assay kit (ThermoFisher Scientific). CoilR-biotin stocks were stored with 10% glycerol at -20 °C.

Preparation of samples for FM:

A. Organelle imaging of VIPER-tagged proteins

U-2 OS cells expressing mEmerald-actin, mEmerald-CoilE-actin, H2B-mEmerald, H2B-CoilE-mEmerald, Mito-mEmerald, or Mito-CoilE-mEmerald were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 15 min at room temperature. Cells were subsequently washed twice with PBS and permeabilized with 0.1% Triton X-100 (10 min). Cells were washed twice to remove detergent. Cells were blocked with 10% FBS, 5% sucrose, 2% BSA (Fraction V) in PBS ("Blocking Solution") for 30 min. Cells were then treated with 100 nM CoilR-Cy5 in Blocking Solution (15 min, room temperature). Cells were washed and then imaged using a Zeiss LSM880 Airyscan line-scanning confocal microscope. This experiment was repeated multiple times, and **Figure 2** shows representative images.

B. Competition binding assay with unlabeled CoilR peptide

U-2 OS cells expressing mEmerald-actin, mEmerald-CoilE-actin, H2B-mEmerald, H2B-CoilE-mEmerald, Mito-mEmerald, or Mito-CoilE-mEmerald were washed twice with PBS and fixed with 4% PFA in PBS (pH 7.4) for 15 min at room temperature. Cells were subsequently washed twice with PBS and permeabilized with 0.1% Triton X-100 (10 min). Cells were washed twice to remove detergent. Cells were blocked with Blocking Solution for 30 min at room temperature.

Fixed cells expressing CoilE-tagged proteins were treated with increasing concentrations of unlabeled CoilR peptide (0, 100, 1000, 10,000, and 100,000 nM) in Blocking Solution (30 min, room temperature). Cells expressing untagged protein were treated with 0 nM or 100,000 nM unlabeled CoilR peptide. Cells were washed twice and then treated with 100 nM CoilR-Cy5 (30 min, room temperature). Cells were washed three times. Nuclei were stained with 10 µg/mL Hoechst 33342 (10 min) and then washed twice. Cells were imaged using a Zeiss Yokogawa spinning disk confocal microscope. The brightness and contrast (B/C) were optimized for all samples using the images for 0 nM CoilR treated samples during image processing. This experiment was repeated twice, and **Figure S1** shows representative images.

C. Live cell imaging comparison of TfR1-CoilE and TfR1-mCherry

CHO TRVb cells expressing TfR1-CoilE or TfR1-mCherry were incubated with 10 µg/mL Hoechst 33342 in F12 media supplemented with 10% FBS and 6% BSA for 30 min at 37 ºC. Cells were cooled on ice to pause endocytosis and then incubated with Tf-AF488 (50 µg/mL) in ice-cold F12 with 5% FBS. TfR1-CoilE cells were additionally treated with CoilR-Cy5 (100 nM). After 30 min, cells were washed three times with ice-cold PBS. Cold PBS with 20 mM HEPES was added to cells prior to fluorescence imaging. The Zeiss Yokogawa spinning disk confocal microscope was housed in an incubation chamber (37 ºC) and cells were imaged at 0 and 30 min. Time point captures were focused and acquired manually without the use of microscope automation. This experiment was performed multiple times and **Figure 3** shows representative images.

D. Time-lapse imaging of TfR1-CoilE labeled with CoilR-Cy5 and Tf-AF488

CHO TRVb cells expressing TfR1 or TfR1-CoilE were incubated with 10 µg/mL Hoechst 33342 in F12 media supplemented with 10% FBS and 6% BSA for 30 min at 37 ºC. Cells were cooled on ice to pause

endocytosis and then incubated with CoilR-Cy5 (500 nM) and Tf-AF488 (50 µg/mL) in ice-cold F12 with 5% FBS (no BSA). After 30 min, cells were washed three times with ice-cold PBS. Cold PBS with 20 mM HEPES was added to cells prior to fluorescence imaging. The Zeiss LSM880 Airyscan line-scanning confocal microscope was housed in an incubation chamber and cells were imaged at 37 ºC over the course of 1 h. Time point captures were focused and acquired manually without the use of microscope automation due to drift in image focus over time. Images of AF488 and Cy5 were acquired every 2-4 min for 25 min and a final capture was taken at 60 min. The nuclear stain, Hoechst 33342, was imaged at the 0, 25, and 60 min. This experiment was performed three times and **Figure S3** shows representative images.

We found that VIPER labeling was highly specific, with Cy5 signal only observed for cells expressing TfR1-CoilE and not for untagged TfR1. For TfR1-CoilE, both the receptor and ligand were found on the cell surface at 0 min and localized in endosomes within 5 min. We observed colocalization of the Tf-AF488 (green) and VIPER (magenta) signal. We saw changes in intracellular distribution of TfR1-CoilE between 5 and 25 min, with more Cy5 signal observed in vesicles within the cytoplasm over time. For both TfR1 and TfR1-CoilE, the Tf ligand trafficked into the cell quickly, with few vesicles observed near the cell surface at 5 min, and a greater number of vesicles in the cytoplasm by 15 min.

E. Pulse-chase labeling of distinct populations of TfR1-CoilE using CoilR probe peptides

CHO TRVb cells expressing TfR1 or TfR1-CoilE were incubated with 10 µg/mL Hoechst 33342 in F12 media supplemented with 10% FBS and 6% BSA for 30 min at 37 ºC. Cells were cooled on ice to pause endocytosis and then "pulse" labeled with CoilR-Cy5 (500 nM, 15 min) in ice-cold F12 supplemented with 5% FBS. Cells were washed, returned to media, and incubated at 37 ºC for 5, 30, or 120 min. Cells were returned to 4 °C for the "chase" labeling with CoilR-BODIPY (500 nM, 15 min). Cells were washed, fixed with 4% PFA, and imaged on a Zeiss Yokogawa spinning disk confocal microscope. This experiment was performed twice and **Figures 4** and **S4** show representative images.

Line-scanning confocal imaging

Micrographs for **Figures 2** and **S3** were acquired on a Zeiss LSM 880 confocal microscope (OHSU Advanced Light Microscopy Core). We used a 63X/1.4 NA oil immersion objective lens and the Zeiss Airy detector. Images were acquired with 2X zoom scanning, resulting in 128X total magnification. Hoechst 33342 was imaged using 405 nm excitation and a 450/50 nm emission filter. AF488 was imaged using 488 nm excitation and a 525/50 nm emission filter. Cy5 was imaged using 633 nm excitation and a 670/30 emission filter. In each experiment, images were acquired as single confocal slices (450 nm depth) with identical acquisition settings optimized for each channel. The Airyscan detector is a 32-channel GaAsp array that uses the additional channels to collect out of focus light for each capture. This additional data was then used to deconvolve the image using the ZEN 2.0 software package (Zeiss).

Spinning disk confocal imaging

Micrographs for **Figures 3**, **4, S1, S2** and **S4** were acquired on a Zeiss Yokogawa CSU-X1 spinning disk confocal microscope (OHSU Advanced Light Microscopy Core). We used a 63X/1.4 NA oil immersion objective lens. Hoechst 33342 was imaged using 405 nm excitation and a 450/50 nm emission filter. AF488 or mEmerald were imaged using 488 nm excitation and a 525/50 nm emission filter. TfR1-mCherry was imaged using 534 nm excitation and a 562/45 nm emission filter. Cy5 was imaged using 633 nm excitation and a 670/30 emission filter. In each experiment, the images were captured as single confocal slices (450 nm depth) with identical acquisition settings optimized for each channel.

Image processing: fluorescence micrographs

Image processing and analysis was carried out using Fiji Software (Version 2.0.0-rc-46). The brightness and contrast (B/C) were optimized and the same settings were applied for each channel across all samples within an experiment. For example, all images of Tf-AF488's green fluorescence in **Figure S3** were set to the same B/C settings (150 to 3,000). Images were sometimes manually cropped to enlarge or highlight a particular feature. Images were false-colored using standard lookup tables: mEmerald (green); AF488 (green); BODIPY (green); mCherry (magenta); Cy5 (magenta); Hoechst 33342 (blue). Colocalization analysis and channel intensity plots for **Figure S2** were generated using the Coloc 2 plugin in Fiji (Analyze > Colocalization > Coloc 2). Pearson's correlation values were calculated without thresholding or use of a region of interest.

Preparation and imaging of samples for multi-scale microscopy:

A. Plating and transfection of CHO TRVb for CLEM

CHO TRVb cells were plated (10^6 cells/well) and grown to 90% confluence on indium tin oxide (ITO)-coated coverslips (2SPI Cat#06486-AB) in 6-well dishes. Cells were transfected with 2 µg of pcDNA3.1_TfR1 or pcDNA3.1_TfR1-CoilE and 4 µg of Lipofectamine 2000 in 3 mL Opti-MEM. After 2 h, cells were returned to serum-containing media. After 24 h, cells were labeled and processed for CLEM imaging.

B. Imaging TfR1 and TfR1-CoilE by CLEM

CHO TRVb cells were blocked with 10% FBS with 6% BSA in F12 (30 min, 37 ºC). Cells were labeled cold with CoilR-biotin (100 nM) and Tf-A488 (50 µg/mL) in F12 with 5% FBS (30 min, 4 ºC). Cells were washed with cold PBS and fixed in cold 4% PFA (20 min). Cells were washed and then blocked with 10% FBS with 6% BSA in PBS (1 h, room temperature). Cells were subsequently labeled with 10 nM streptavidin-Qdot655 (ThermoFisher Scientific #Q10121MP) in PBS with 6% BSA (1 h, room temperature). Cells were washed with PBS before fluorescence imaging. Cells were mapped using the FEI Corrsight MAPS software.

This experiment was performed three times and **Figure 5** shows representative images. Additional images are provided in **Figures S5** and **S6**.

C. Comparison of VIPER with immunolabeling for CLEM

We evaluated four anti-TfR1 antibodies: 8D3 (Novus Biologicals), Ab1086 (Abcam), Ab216665 (Abcam), and H68.4 (ThermoFisher Scientific). We evaluated one anti-Tf antibody: Ab82411 (Abcam). A summary of the primary and secondary antibodies used for this study are summarized in **Table S9**.

All antibodies were evaluated first by FM. We applied each primary antibody to transfected cells live or post-fixation. Secondary Qdot655 conjugates were applied to cells after fixation and samples were evaluated for Qdot fluorescence. Primary and secondary antibodies were used at the dilution recommended by the manufacturer for immunofluorescence. Antibodies were tested against both TfR1 and TfR1-CoilE; no differences in immunolabeling were observed. We found that Ab1086 and Ab216665 were unable to label TfR1 or TfR1-CoilE (**Figure S7**). We verified that each secondary antibody was specific for the corresponding primary antibody (e.g., with a no primary control).

For CLEM imaging, transfected CHO TRVb cells were treated with 100 nM Tf-AF488 (30 min at 4 °C). Cells were then washed and fixed with 4% PFA (15 min, 4 °C). For H68.4 immunolabeling, cells were permeabilized with 0.1% Triton-X-100 (10 min, room temperature) and then washed with PBS to remove detergent. Samples were blocked in 1% BSA/PBS (30 min, room temperature). For immunolabeling, primary antibodies were diluted 1:100 (10 µg/mL) in 1% BSA/PBS and applied to cells (1h, room temperature). Cells were washed and treated with the appropriate Qdot655-conjugated secondary antibody diluted 1:200 (5 nM) for 1 h at room temperature and then washed. For VIPER labeling, cells were treated with 100 nM CoilR-biotin (1 h, room temperature), washed, and then treated with 10 nM streptavidin-Qdot655 (1 h, room temperature), and washed. Cells were imaged by FM to detect Tf-AF488 and Qdot655 fluorescence.

This CLEM experiment was performed three times and **Figures 6**, **S7**, **S10**, and **S11** show representative images.

D. Qdot detection of VIPER compared to Qdot detection of Tf ligand

We used CLEM to directly compare streptavidin-Qdot655 detection of biotinylated TfR1 versus biotinylated Tf. CHO TRVb cells transfected with TfR1-CoilE were blocked with 10% FBS with 6% BSA in F12 (30 min, 37 ºC). Cells were treated with either CoilR-biotin (100 nM) or 100 nM Tf-biotin (ThermoFisher Scientific #T23363, Lot# 1853655). This lot of Tf-biotin was reported to have an average of 5 biotins/ligand, with a range of 2-5. Both biotinylated ligands were added in F12 media for 30 min at 4 °C. Cells treated with CoilR-biotin were also treated with Tf-AF488 (100 nM) as a counterstain for TfR1. Cells were then washed, fixed with 4% PFA (15 min, 4 °C), and blocked with 6% BSA, 10% serum in PBS (1 h, room temperature). Cells were then treated with 10 nM streptavidin-Qdot655 (ThermoFisher Scientific #Q10121MP; Lot# 1843526) in 6% BSA in PBS (1 h, room temperature). Cells were washed with PBS and imaged by FM to detect Tf-AF488 and/or Qdot655 fluorescence.

This experiment was performed three times and **Figures S8** and **S12** show representative images.

E. FEI Corrsight FM

ITO coverslips were imaged on an FEI Corrsight spinning disk confocal fluorescence microscope. Fiducial markers were added to each ITO-coverslip using a diamond scribe. Then coverslips were mounted on a custom-machined aluminum slide, which prevented disruption of the cells during wet imaging and allowed coverslips to be removed from the metal slide after acquisition. First, the slide was imaged using a 5X/0.25 N.A. objective lens with transmitted light to capture fiducial markers. Then fluorescence micrographs were acquired using a 63X/1.4 N.A. objective lens. Individual cells were imaged for green fluorescence (Tf-AF488) and Qdot655 fluorescence. Transfected cells within each sample were selected for imaging based on Tf-AF488 fluorescence. We attempted to select cells that exhibited similar levels of AF488 fluorescence in order to normalize for cell to cell variations in TfR1 or TfR1-CoilE expression. When Tf-AF488 fluorescence could not be compared (such as loss of fluorescence due to detergent treatment), cells were selected based on average Qdot655 signal on the coverslip. AF488 signal was detected using 488 nm excitation and a 525/50 nm filter to collect the emitted light. Then, Qdot655 signal was detected using 405 nm excitation and a 690/650 filter to collect the emitted light. Fluorescent cells were mapped using FEI's MAPS software for subsequent imaging via SEM.

F. CLEM processing of ITO coverslips

The FEI's MAPS software enables the same cell to be imaged by FM and SEM. After FM imaging, coverslips were returned to PBS and prepared for SEM. Coverslips were rinsed with deionized water and then dehydrated using 5 min washes with an ethanol gradient: 25%, 50%, 75%, 90%, and 100% (twice). Slides for **Figures 5, S5, and S6** were then chemically dehydrated using 5 min washes of 50%, 75% and 100% (twice) hexamethyl-disilazane (HMDS) in ethanol and then left to fully dry in a fume hood. In contrast, samples for **Figures 6**, **S7**, **S8**, and **S10-S12** were first dehydrated with an ethanol gradient and then critical point dried using a Leica EM CPD300 critical point dryer. Coverslips were glued to SEM mount pins using conductive silver paint (Pelco Cat#16035). Samples were dried overnight inside a desiccator and flash-coated with 10 nm of carbon using a Leica ACE600 sputter/coater machine.

G. FEI Helios Nanolab 660 FIB SEM

SEM images were acquired on a FEI Helios Nanolab 660 DualBeam. The instrument was set to acquire at 3 kV accelerating voltage and beam current of 0.2 nA. Back scattered electron (BSE) images were acquired via a dedicated BSE detector. MAPS software allowed previously-selected cells to be re-located. Briefly, a three point alignment on the engraved fiduciary pattern was used to globally align the SEM image to the previously captured light image of the ITO slide. Cells could be located using MAPS after global alignment. Cells were imaged at 65,000X magnification (**Figures 5, S5-S6**) or 100,000X magnification (**Figure 6**, **S7**, **S8, and S10-S12**). We imaged 2 non-overlapping fields-of-view per cell and six cells (**Figures 5, S5, and S6**) or three cells (**Figure 6**, **S7**, **S8, and S10-S12)** per condition.

The SEM micrographs display the surface of cells. At high magnification, the cell surface appears dark gray and textured, with cell features such as protrusions or ruffles appearing lighter gray or white due to their closer proximity to the BSE detector. For an example of a cell with many raised features, see **Figure S6A**: Cell 3 Image 1 (*lower left panel*). In contrast, recessed areas of the cell surface appear darker gray. The ITO coverslip appears white in BSE mode, and areas where the cell is thin often appear lighter gray due to signal from the coverslip penetrating through the cell. For an example of a thin cell feature, see **Figure S10B:** Cell 3 Image 2 (*lowest right panel*).

Quantitative image analysis of Qdot labeling in SEM images

For computer-assisted counting of SEM images, images were acquired at high magnification (65,000X or 100,000X) for optimal particle detection and segmentation. To detect and count particles, segmentation was implemented in MATLAB with a two-step procedure. First, we detected bright objects of interest in a dark background using morphological top-hat filtering(4). This method computes the morphological opening of the image and then subtracts the result from the original image to enhance the original image. Second, simple intensity thresholding (i.e., Otsu's method(5)) was applied to segment the objects followed by applying a Gaussian blur(6) to the improved image. Finally, segmentation was validated by visual assessment to refine parameters and exclude objects falsely annotated. False-annotations were rare, but typically resulted from unspecific intensity background, intensity variations, background artifacts, or errors in segmentation overlooked

by the automated procedure described above. A representative Qdot655 image, with segmentation applied, is provided in **Figure S5B.**

To separate multiply-clustered objects within SEM images, we differentiated the object's foreground and background. However, to successfully segment the locally clustered or overlapping particles, we performed marker-controlled watershed segmentation(7). We computed the watershed transform(5) of the distance transform of good foreground markers from the segmented mask and looked for the watershed ridge lines of the result. Then, we counted the segmented single particles with results provided in **Tables S1-S4**.

Statistical analysis of Qdot655 particle counts

Scatter plots of counted Qdots are provided in **Figures S5** and **S9**. Statistical analysis was done in Graphpad Prism (Version 6.02). Raw counts for each labeling method were selected for Welch's t-test analysis. For determining statistical significance, labeling methods (*i.e.* Tf-biotin, 8D3, etc.) were compared against VIPER using an unpaired, two-tail t-test. We assumed a Gaussian distribution and unequal variances. Significance values were reported in the figure captions. VIPER (live-labeling protocol) was compared with Tf-biotin. VIPER (fixed-labeling protocol) was compared to 8D3, Ab82411, H68.4, Ab1086, and Ab216665.

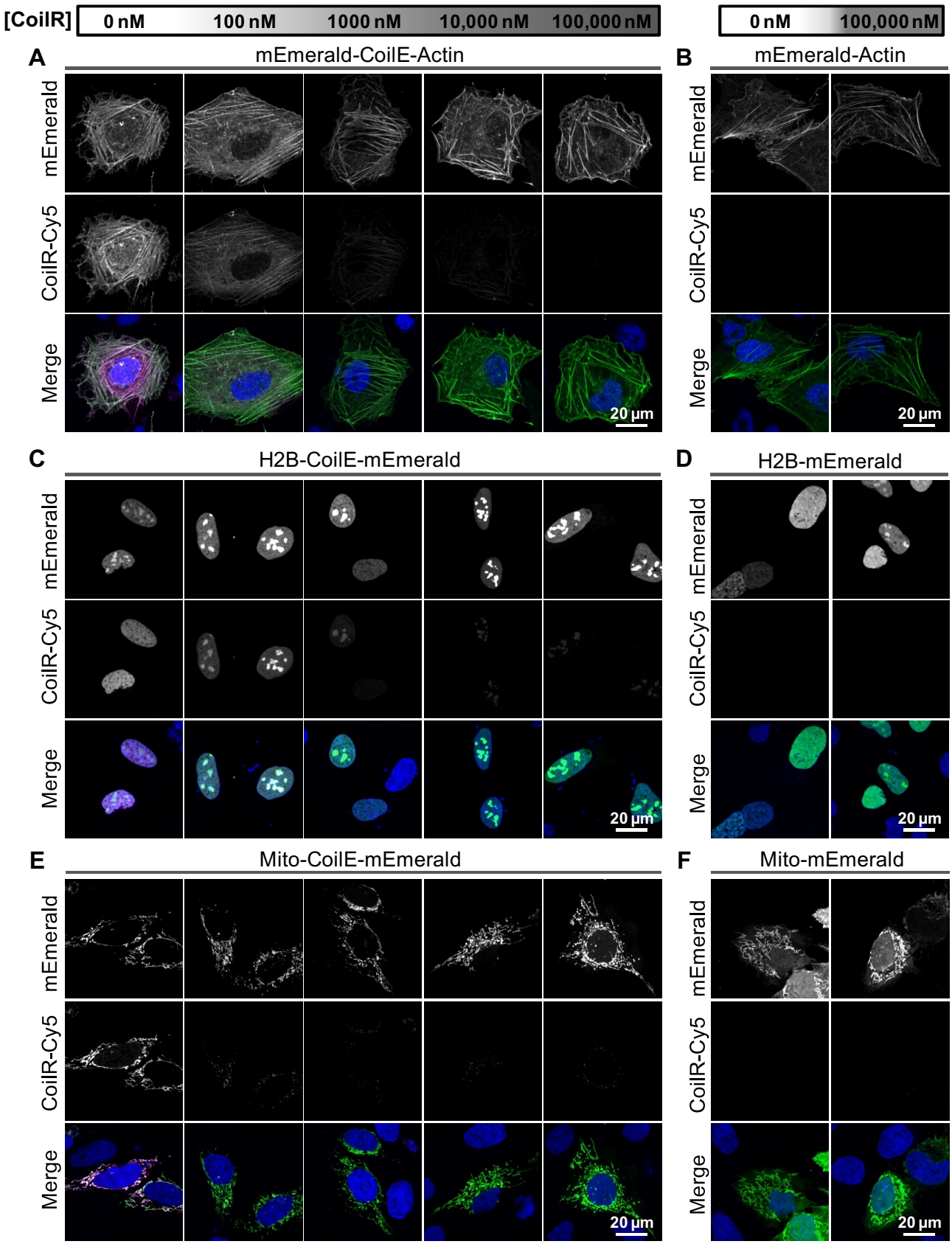


Figure S1. Reduction of VIPER labeling by pre-treatment of fixed cells with unlabeled CoilR peptide. U-2 OS cells were transfected to express CoilE-tagged proteins (**A**: mEmerald-CoilE-Actin; **C**: H2B-CoilE-mEmerald; **E**: Mito-CoilE-mEmerald). Cells were fixed, permeabilized, and treated with increasing concentrations of unlabeled CoilR peptide (0, 100, 1000, 10,000, or 100,000 nM). Next, cells were washed,

treated with 100 nM CoilR-Cy5, and imaged by confocal FM. Without pre-treatment with unlabeled CoilR, Cy5 fluorescence was observed and co-localized with mEmerald signal (*Column 1*; **A**, **C**, and **E**). Cy5 labeling was reduced for samples pre-treated with 100 nM unlabeled CoilR (*Column 2*). For cells expressing mEmerald-CoilE-Actin or Mito-CoilE-mEmerald, Cy5 fluorescence became nearly undetectable after pre-treatment with 1000 nm CoilR. Cy5 signal localized to nucleoli was detected for cells pre-treated with ≥1000 nM CoilR, but the signal was reduced and was increasingly difficult to detect. Cells expressing mEmerald-Actin (**B**), H2B-mEmerald (**D**), or Mito-mEmerald (**F**) were treated with 0 or 100,000 nM unlabeled CoilR before treatment with 100 nM CoilR-Cy5. Cy5 fluorescence was not detected for cells expressing these proteins. In merged images, mEmerald is false-colored green, Cy5 is false-colored magenta and Hoechst 33342 (nuclei) is false-colored blue.

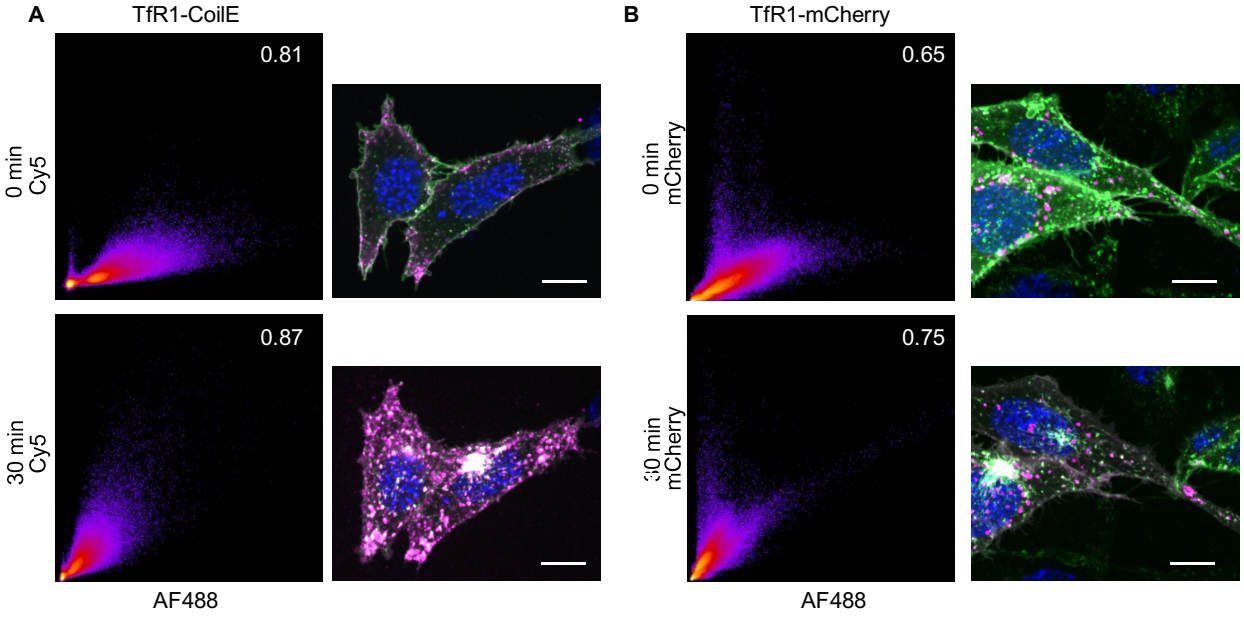


Figure S2. Colocalization analysis of Tf with TfR1. We generated pixel intensity plots to analyze the colocalization of Tf-AF488 fluorescence with red fluorescence from Cy5-labeling of TfR1-CoilE (**A**) or from TfR1-mCherry (**B**). We analyzed micrographs from **Figure 3** for the 0 min (*top*) and 30 min (*bottom*) time points. Plots were generated using Fiji software (Coloc 2 analysis) with Tf-AF488 signal intensity on the horizontal axis and either Cy5 signal intensity (**A**) or mCherry signal intensity (**B**) on the vertical axis. Pearson's correlation values are reported in the upper-right corner of the intensity plot. Micrographs from **Figure 3** are provided next to each plot and the scale bars represent 10 μm.

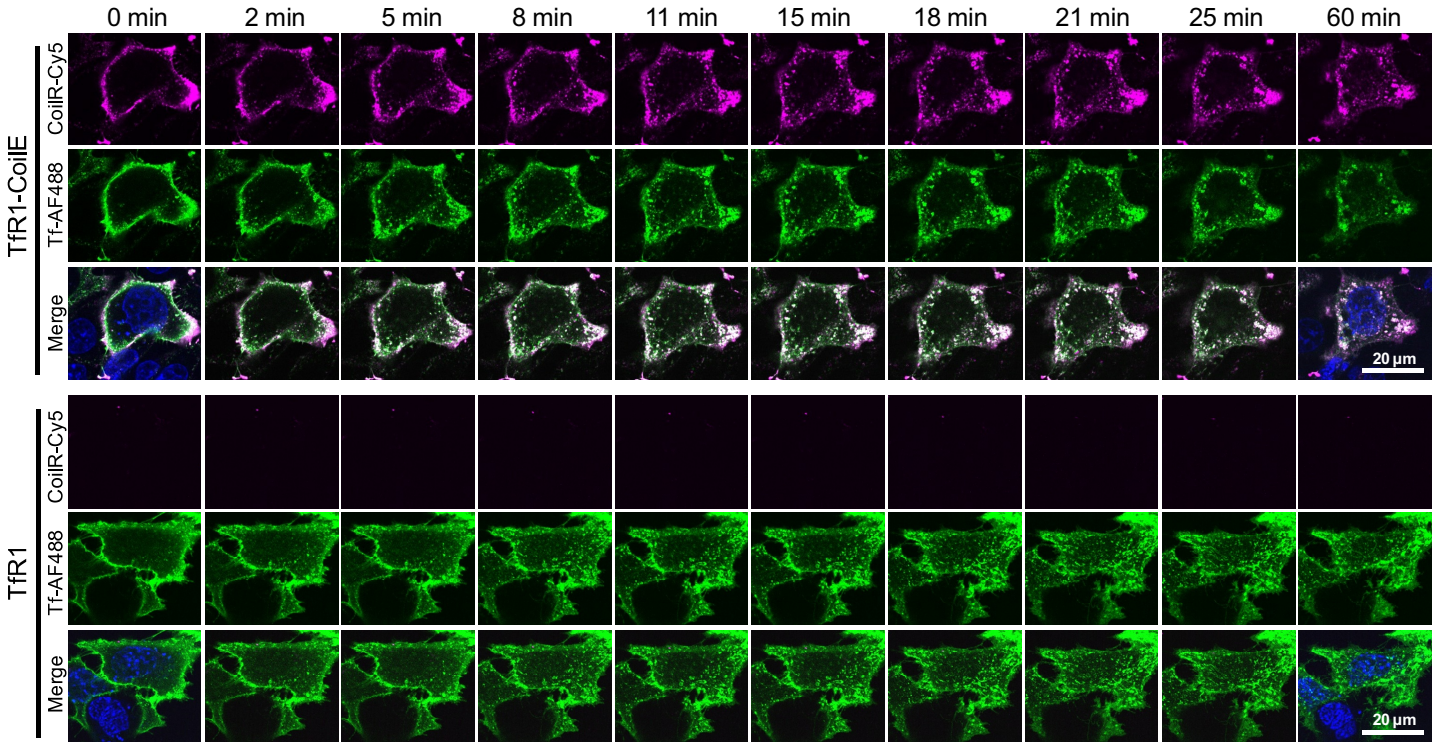


Figure S3. Time-lapse imaging of TfR1 following Tf-AF488 and CoilR-Cy5 treatment. Live CHO TRVb cells expressing TfR1-CoilE (*top*) or untagged TfR1 (*bottom*) were treated with CoilR-Cy5 and Tf-AF488 and then imaged by confocal fluorescence microscopy. Cells were imaged every 2-3 min for the first 25 min to capture endocytosis of TfR1 and TfR1-CoilE. A final image was taken after 60 min at 37° C. Nuclear stain (Hoechst 33342; blue) was imaged during the first and last capture only to minimize UV-light exposure. Areas where AF488 (green) and Cy5 (magenta) overlap are white in the merge. Images are single confocal slices (450 nm depth) acquired at 63X magnification (1.4 NA). Fluorescence signal was normalized in each channel.

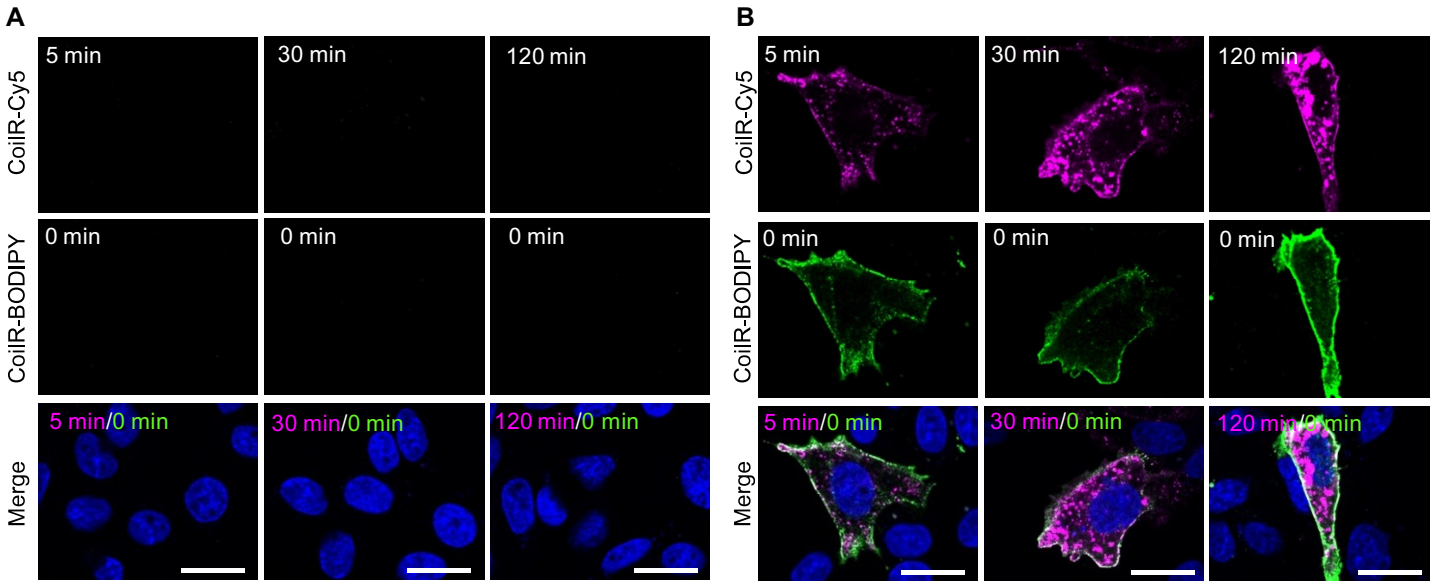


Figure S4. Two-color pulse-chase labeling of untagged TfR1 compared to TfR1-CoilE. Cells expressing TfR1 (**A**) or TfR1-CoilE (**B**) were pulse-labeled with CoilR-Cy5 (500 nM, 15 min), washed, and returned to 37 °C for 5, 30, or 120 min. Cells were chase-labeled by treatment with CoilR-BODIPY (500 nM, 15 min), fixed, and imaged to detect both Cy5 (magenta) and BODIPY (green) signal. Nuclear stain (Hoechst 33342) is blue and scale bars represent 20 μm. Images are single confocal slices (450 nm depth) acquired at 63X magnification (1.4 NA) with fluorescence signal normalized in each channel. There was no Cy5 or BODIPY fluorescence observed for untagged TfR1-expressing cells treated with CoilR-Cy5 and CoilR-BODIPY. The micrographs in **B** are reproduced from **Figure 4B**.

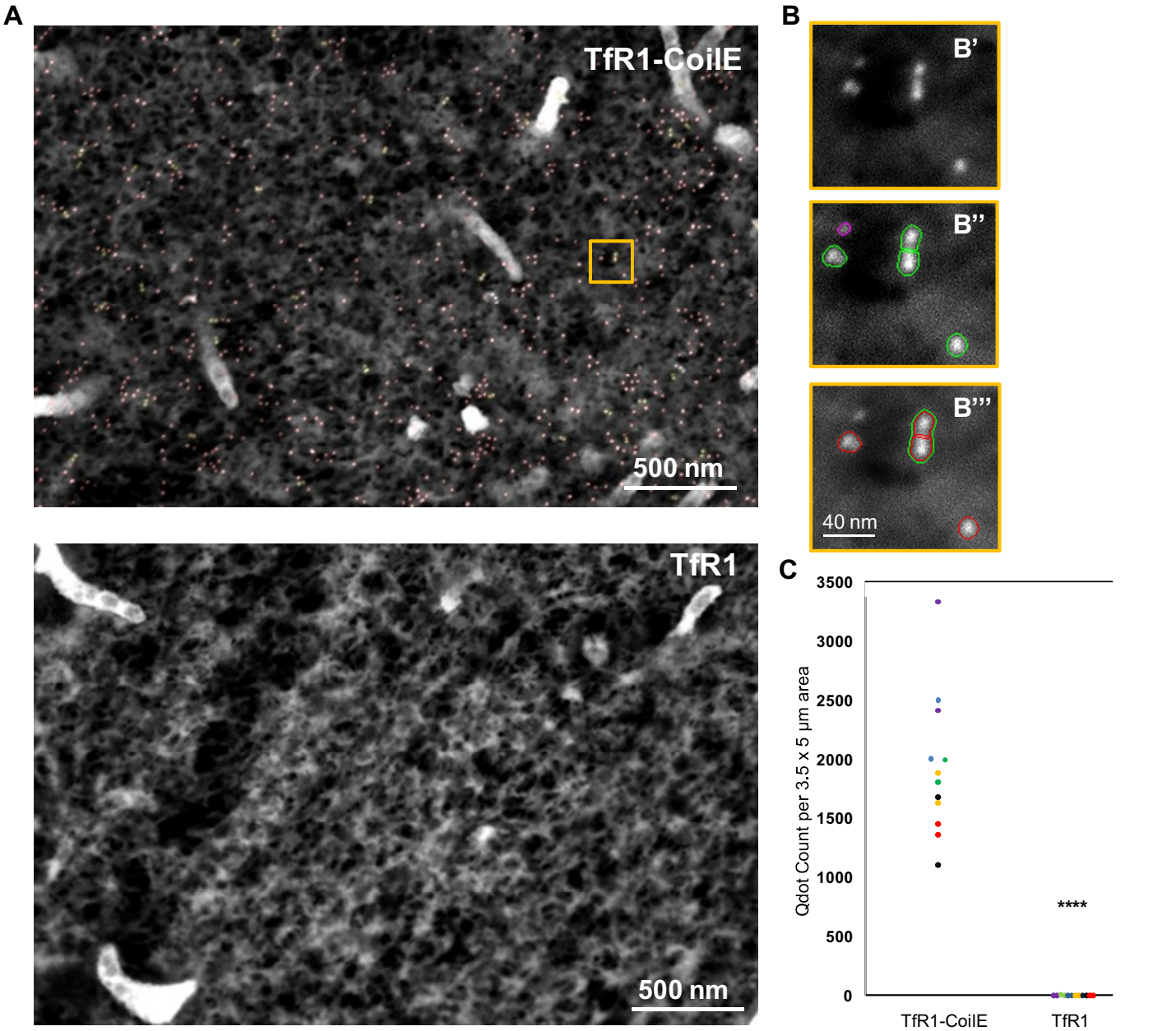
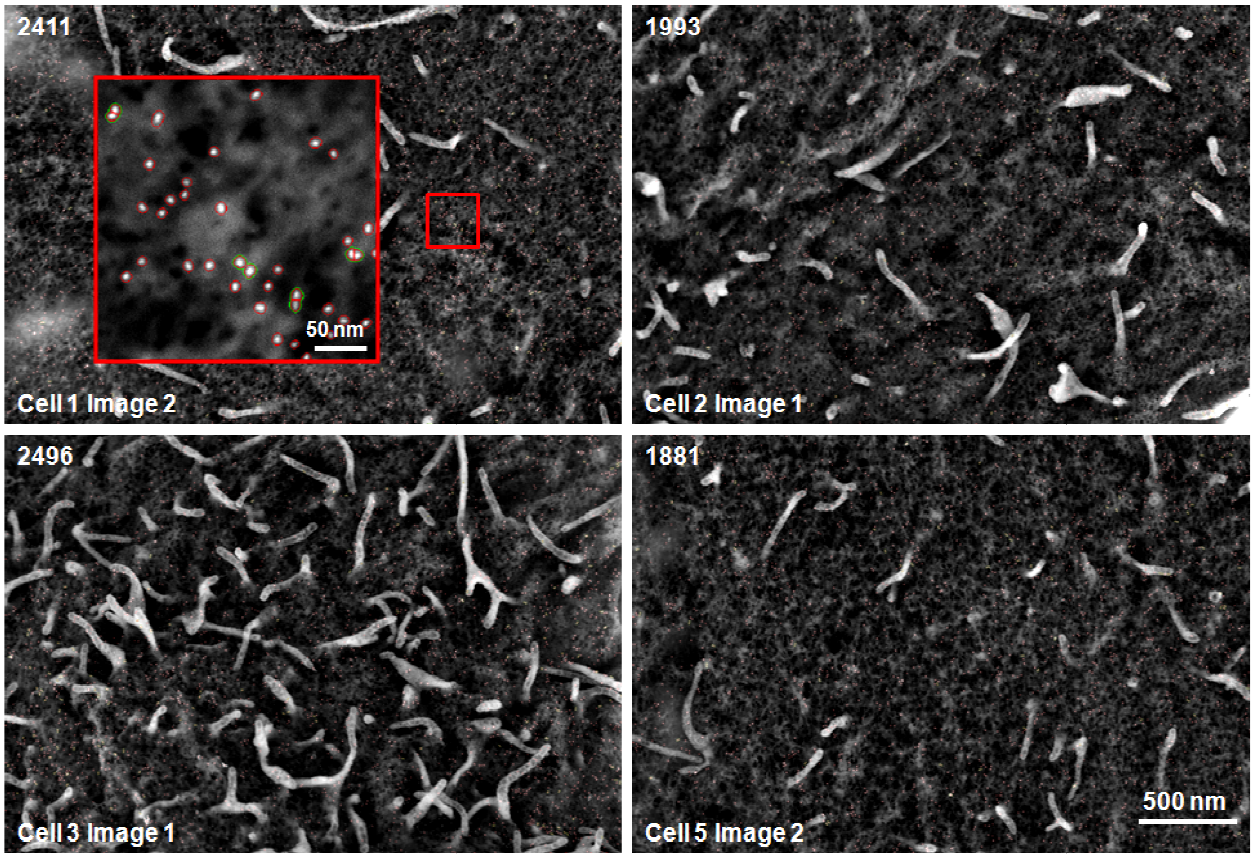


Figure S5. Computer-assisted counting of Qdot655-labeled TfR1 in SEM micrographs. (**A**) Micrographs were acquired at 65,000X magnification with back scatter electron capture of the cell surface. The field of view shown is 1.75 x 2.5 µm. CHO TRVb cells were transfected with TfR1-CoilE (*top*) or untagged TfR1 (*bottom*) and treated with CoilR-biotin and streptavidin-Qdot655. The counting mask overlay appears as a red outline, with clusters additionally outlined in green. The yellow box defines the inset shown in **B**. (**B**) Magnified view and segmentation analysis of the region designated in **A**. **B'** is the magnified view of the unprocessed image. **B''** shows the top-hat, initial particle detection (green outline) and Watershed separation of contiguous particles. Non-Qdot particles were filtered out (magenta outline) based on size. **B'''** shows the final mask with counted particles outlined in red and clusters (≥2 particles) outlined in green. **C.** Scatter plot of total counted Qdot655 particles per field of view (3.5 x 5 µm) for TfR1 and TfR1-CoilE. We analyzed 2 non-overlapping images per cell and a total of 6 cells per conditions (i.e., TfR1-CoilE and untagged TfR1). See **Table S1** for a summary of the data obtained from SEM image analysis. Same-colored data points in **C** and **Table S1** correspond to data obtained from the same cell. The difference in Qdot counts for TfR1 versus TfR1-CoilE was statistically significant (**** = $p < 0.0001$).

A TfR1-CoilE



B TfR1

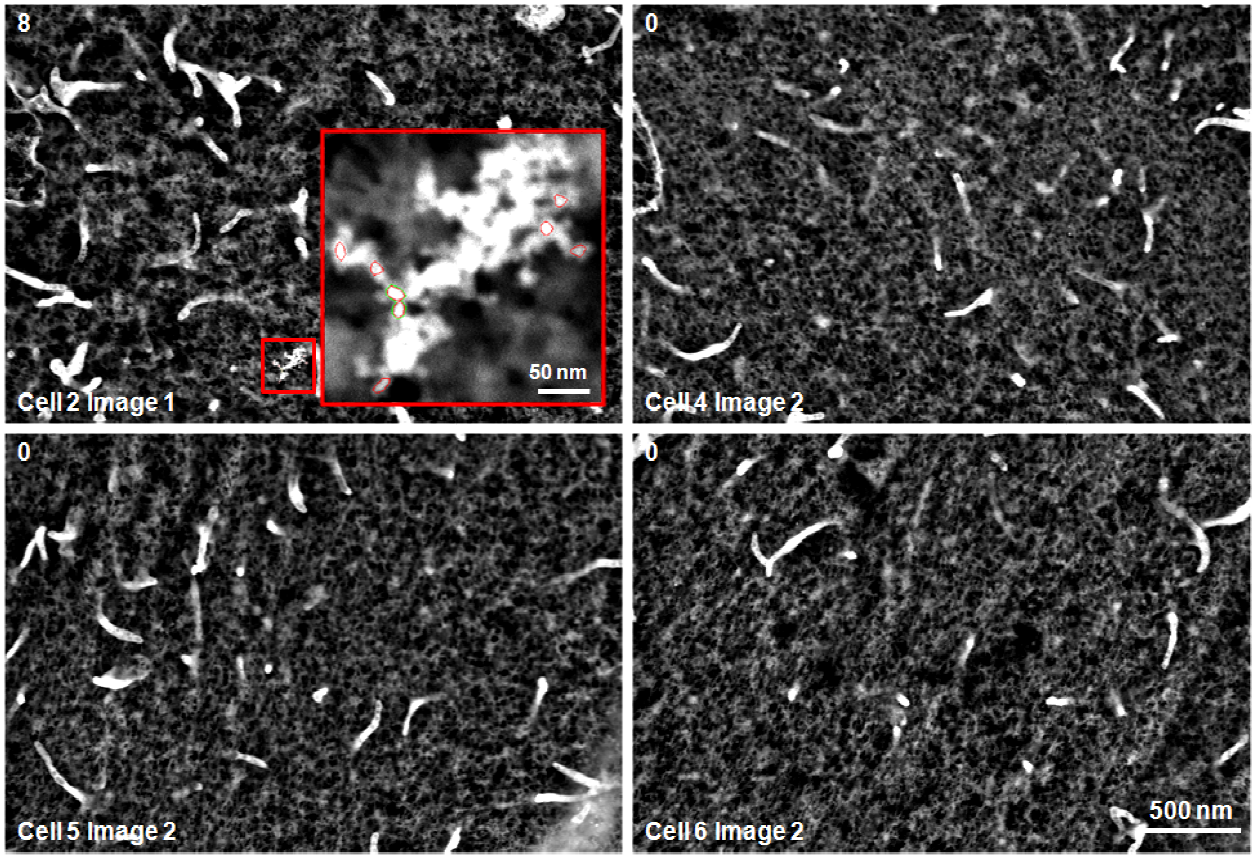


Figure S6. Representative SEM micrographs of CHO TRVb cells expressing TfR1-CoilE (A) or untagged TfR1 (B). Cells were treated with CoilR-biotin and streptavidin-Qdot655, as described in the SI Methods. The counting mask overlay appears as a red outline, with clusters additionally outlined in green. Images shown are the full field of view from a 65,000X magnification capture (3.5 x 5 μm). Micrograph names correspond to names provided in **Table S1** (e.g., Cell X Image Y) and the total Qdot count for each image is reported in the upper left corner. A magnified inset (red box) is provided with the upper left micrograph of **A** and **B** to highlight an area of the cell with particles segmented as Qdots.

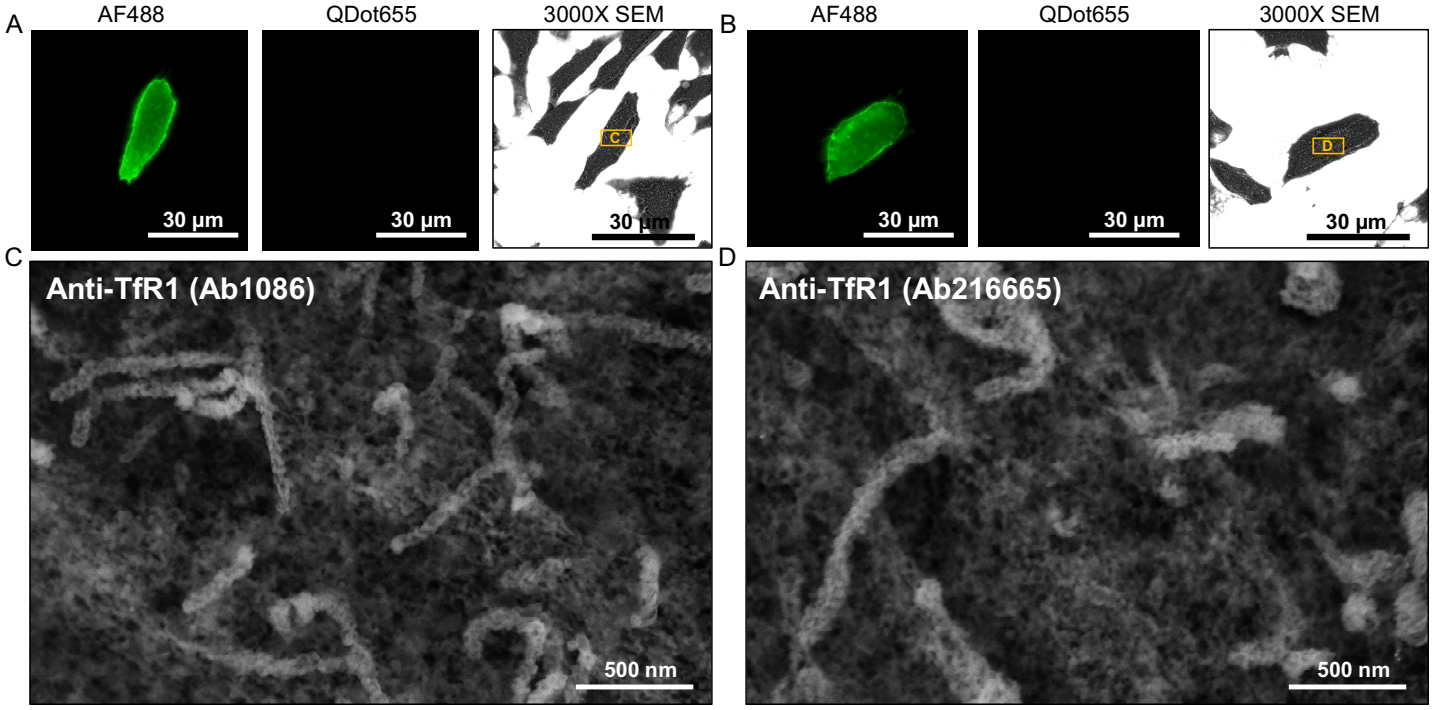


Figure S7. Target labeling and CLEM imaging of TfR1-Coil by anti-TfR1 antibodies Ab1086 and Ab216665. CHO TRVb cells were transfected with TfR1-CoilE and treated live with 100 nM Tf-AF488**.** After fixation, cells were treated with a primary antibody against TfR1: Ab1086 (**A, C**) or Ab216665 (**B, D**). Cells were treated with the appropriate secondary antibody conjugated to Qdot655. Fluorescence micrographs of cells treated with Ab1086 (**A**) and Ab2166665 (**B**) were acquired, mapped, and imaged by high-resolution SEM. Transfected cells were identified by Tf-AF488 fluorescence, and Qdot fluorescence was not detected for either antibody. We selected region C (in **A**) and region D (in **B**) for SEM imaging at 100,000X magnification. Less than 20 particles were detected on cell surfaces treated with Ab1086 or Ab216665. See **Figure S9** and **Table S4** for particle counting data.

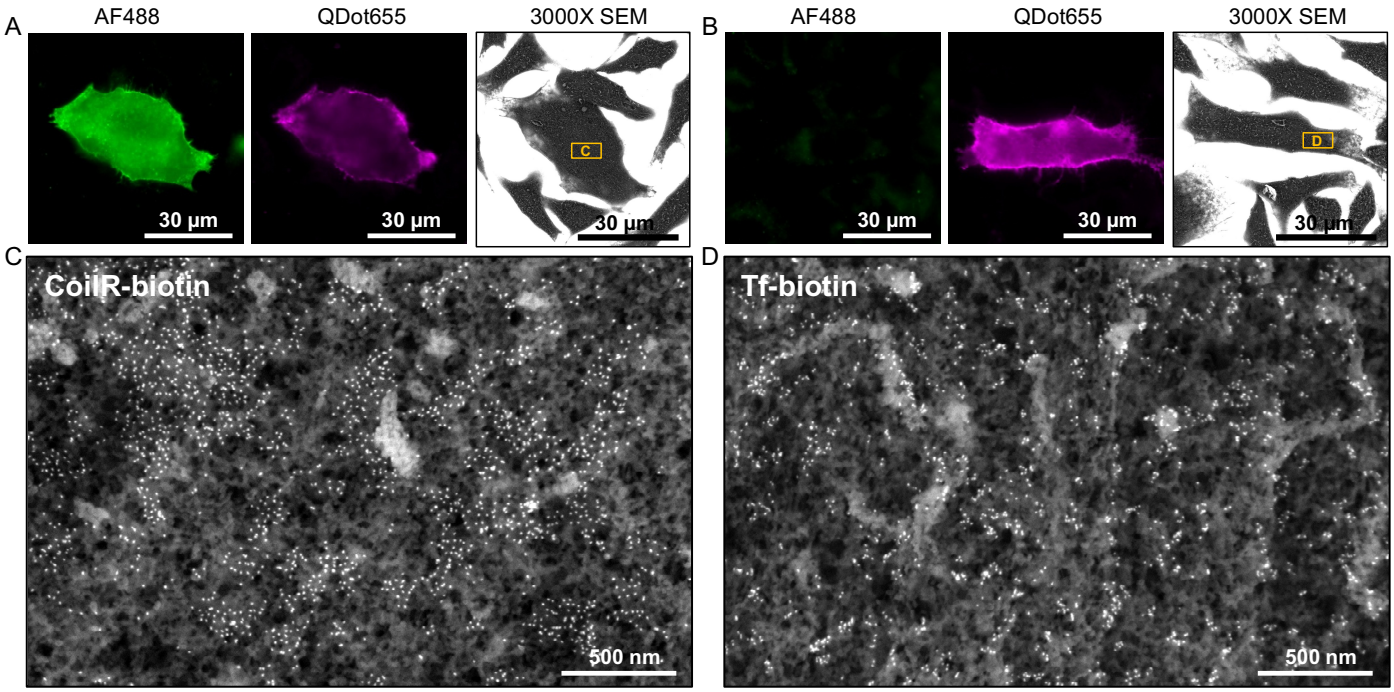


Figure S8. Qdot detection of VIPER compared to Qdot detection of Tf ligand. CHO TRVb cells were transfected with TfR1-CoilE and treated live with 100 nM CoilR-biotin and 100 nM Tf-AF488 (**A**) or with 100 nM Tf-biotin only (**B**). After fixation, cells were treated with streptavidin-Qdot655 to detect biotinylated TfR1 receptors (**A**, **C**) or biotinylated Tf (**B**, **D**). Cells expressing TfR1-Coil were identified by Tf-AF488 (green) and Qdot655 (magenta) labeling (**A**) or by Qdot655 labeling (**B**). MAPS software was used to select cells for high-resolution SEM. We selected *Region C* (in **A**) and *Region D* (in **B**) for SEM imaging. Samples were processed by chemical dehydration, carbon coated, and imaged at 100,000X magnification. High-resolution SEM micrographs of cells expressing TfR1-CoilE (**C**) showed selective Qdot labeling of the TfR1 receptor. SEM micrographs of cells expressing TfR1-CoilE treated with biotinylated Tf (**D**) showed selective Qdot labeling of the ligand. See **Figure S9A** and **Table S2** for particle counting data.

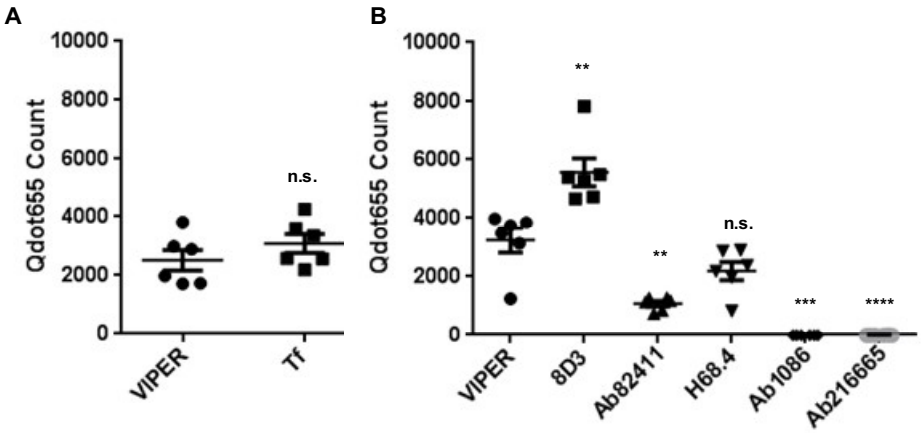


Figure S9. Scatter plot of total Qdot particles counted per field-of-view (4.15 µm x 2.75 µm) for SEM micrographs of cells. A. Total Qdot particles counted for cells treated with CoilR-biotin versus cells treated with Tf-biotin. Both biotinylated ligands were detected by streptavidin-Qdot655. Data were analyzed using a Welch's t-test and were not statistically significant (n.s.; $p > 0.05$). **B.** Total Qdot particles counted for cells treated with VIPER or immunolabeled. Data were analyzed using a Welch's t-test comparing counts for VIPER versus counts for each antibody. In **B**, n.s. = not significant ($p > 0.05$); ** = $p < 0.01$; *** = $p < 0.001$; and **** = $p < 0.0001$). Raw images with counting masks are provided in **Figures S10-S12**. Raw data counts are provided in **Tables S2-S4**.

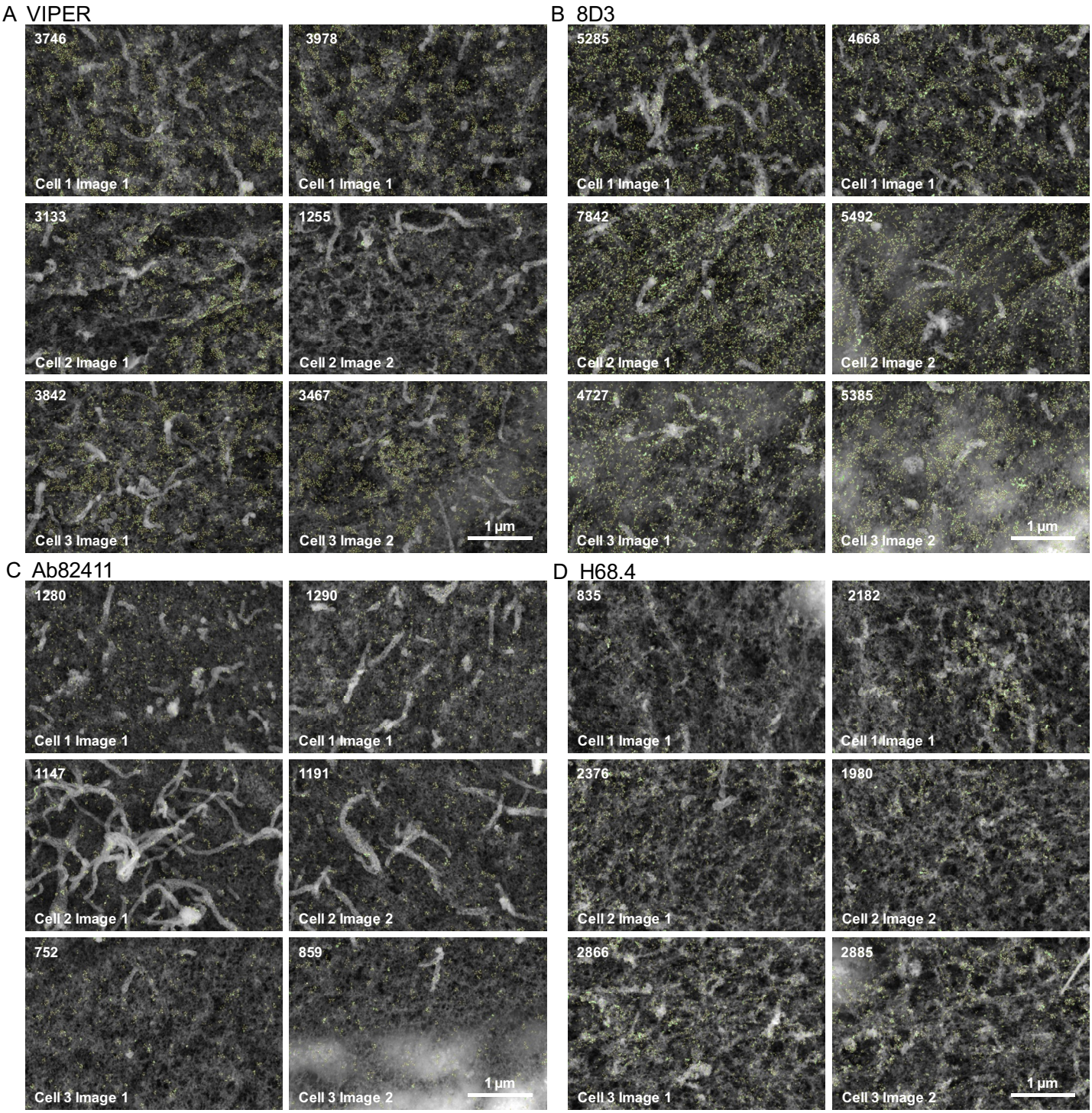


Figure S10. SEM micrographs of CHO TRVb cells expressing TfR1-CoilE labeled with VIPER (A), 8D3 (B), Ab82411 (C), or H68.4 (D). A. Fixed cells were treated with CoilR-biotin and streptavidin-Qdot655. **B-D.** Fixed cells were treated with the indicated primary antibody and a secondary antibody conjugated to Qdot655. In the micrographs, the counting mask overlay appears as a red outline, with clusters additionally outlined in green. Images shown are the full field of view from a 100,000X magnification capture (4.15 x 2.75 µm). Micrograph names correspond to names provided in **Table S3** (e.g., Cell X Image Y) and the total Qdot count for each image is reported in the upper left corner of each micrograph.

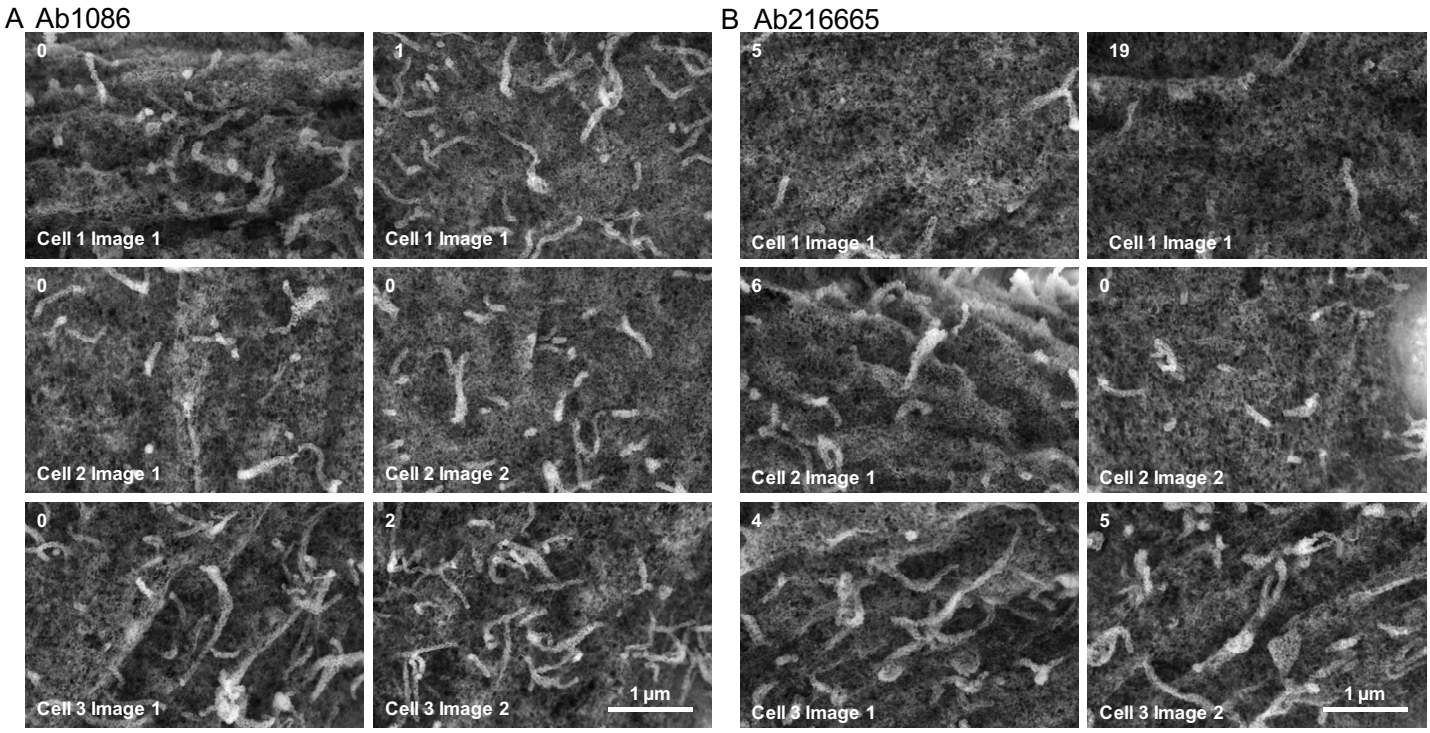


Figure S11. SEM micrographs of CHO TRVb cells expressing TfR1-CoilE labeled with Ab1086 (A) or Ab216665 (B). Fixed cells were treated with an anti-TfR1 antibody, either Ab1086 (A) or Ab216665 (B), and a secondary antibody conjugated to Qdot655. Neither of these primary antibodies labeled TfR1. In the micrographs, the counting mask overlay appears as a red outline, with clusters additionally outlined in green. Images shown are the full field of view from a 100,000X magnification capture (4.15 x 2.75 µm). Micrograph names correspond to names provided in **Table S4** (e.g., Cell X Image Y) and the total Qdot count for each image is reported in the upper left corner of each micrograph.

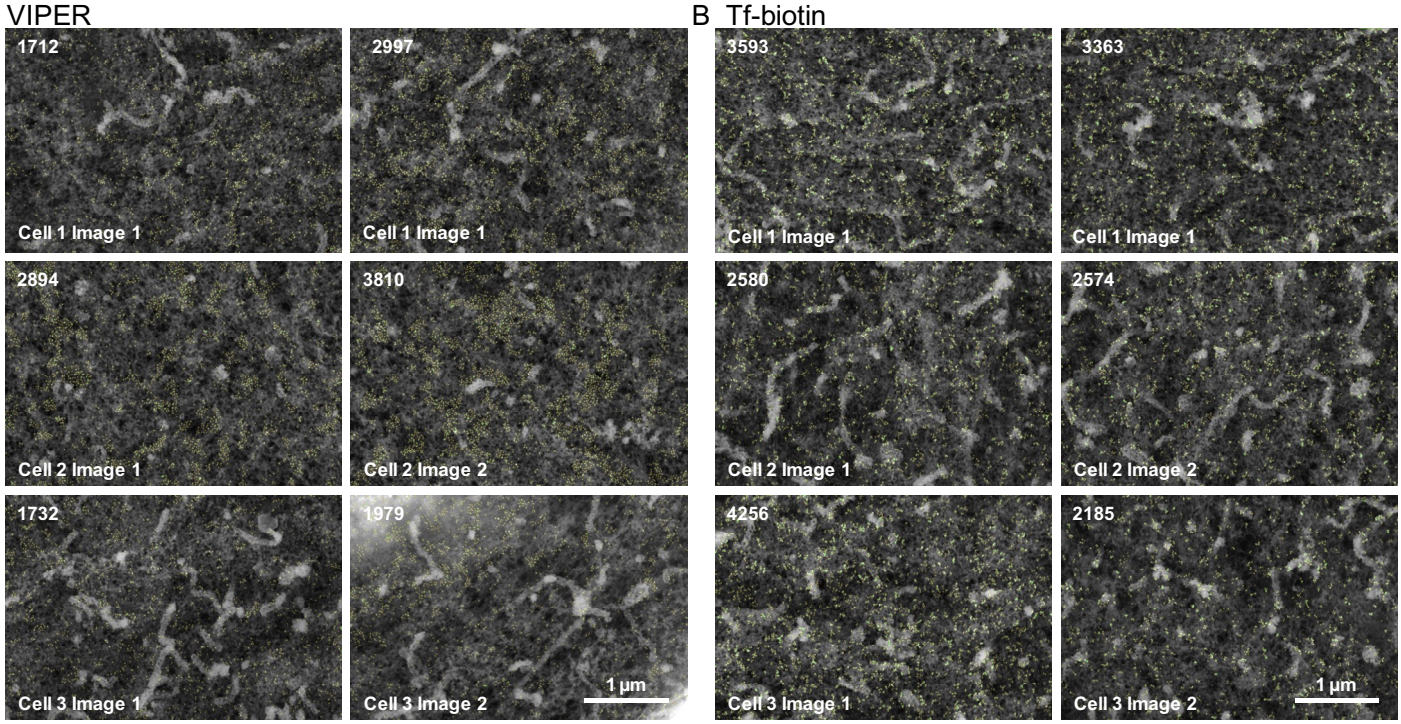


Figure S12. SEM micrographs of CHO TRVb cells expressing TfR1-CoilE labeled with VIPER (A) or Tf-biotin (B). Live cells were treated with CoilR-biotin (**A**) or Tf-biotin (**B**), fixed, and then treated with streptavidin-Qdot655. In the micrographs, the counting mask overlay appears as a red outline, with clusters additionally outlined in green. Images shown are the full field of view from a 100,000X magnification capture (4.15 x 2.75 µm). Micrograph names correspond to names provided **Table S2** (e.g., Cell X Image Y) and the total Qdot count for each image is reported in the upper left corner.

Table S1: Quantification of Qdots in SEM images comparing TfR1-CoilE and TfR1[‡]

	Micrograph	count/μm^2	total count[§]	monomer	dimer	≥ 3
TfR1-CoilE	Cell 1 Image 1	190.3	3331	2841	187	37
	Cell 1 Image 2	137.8	2411	2196	92	10
	Cell 2 Image 1	113.9	1993	1827	72	6
	Cell 2 Image 2	103	1803	1627	82	4
	Cell 3 Image 1	142.6	2496	2181	133	14
	Cell 3 Image 2	114.3	2000	1747	106	13
	Cell 4 Image 1	63.1	1104	1032	30	4
	Cell 4 Image 2	95.9	1679	1607	30	4
	Cell 5 Image 1	93.1	1629	1487	62	6
	Cell 5 Image 2	107.5	1881	1735	70	2
	Cell 6 Image 1	77.6	1358	1228	55	6
	Cell 6 Image 2	82.8	1449	1300	64	7
	AVERAGE	110±34	1928±598	1734±493	82±44	9±9
TfR1	Cell 1 Image 1	0	0	0	0	0
	Cell 1 Image 2	0	0	0	0	0
	Cell 2 Image 1	0.5	8	6	1	0
	Cell 2 Image 2	0.1	1	1	0	0
	Cell 3 Image 1	0	0	0	0	0
	Cell 3 Image 2	0	0	0	0	0
	Cell 4 Image 1	0.1	1	1	0	0
	Cell 4 Image 2	0	0	0	0	0
	Cell 5 Image 1	0	0	0	0	0
	Cell 5 Image 2	0	0	0	0	0
	Cell 6 Image 1	0	0	0	0	0
	Cell 6 Image 2	0	0	0	0	0
	AVERAGE	0±0	1±2	1±2	0±0	0±0

[‡]*Entries are color-coded to match points shown in* ***Figure S5C****.*

[§] *Welch's t-test = 11.16, p value <0.0001*

Table S2: Quantification of Qdots in SEM images comparing VIPER-biotin and Tf-biotin

	Micrograph	count/μm^2	total count	monomer	dimer	≥ 3
VIPER-biotin	**Cell 1 Image 1**	142.7	1712	1337	146	25
	Cell 1 Image 2	249.8	2997	2169	297	70
	Cell 2 Image 1	241.2	2894	2000	304	86
	Cell 2 Image 2	317.5	3810	2469	468	118
	Cell 3 Image 1	144.3	1732	1393	128	26
	Cell 3 Image 2	164.9	1979	1435	188	49
	AVERAGE	**210 ± 71**	**2521 ± 848**	**1800 ± 477**	**255 ± 128**	**62 ± 36**
Tf-Biotin	**Cell 1 Image 1**	299.4	3593	1238	447	359
	Cell 1 Image 2	280.2	3362	1249	395	342
	Cell 2 Image 1	215.0	2580	1177	317	216
	Cell 2 Image 2	214.5	2574	1140	313	216
	Cell 3 Image 1	354.7	4256	1672	505	409
	Cell 3 Image 2	182.1	2185	892	244	214
	AVERAGE	**258 ± 65**	**3092 ± 779**	**1228 ± 253**	**370 ± 97**	**293 ± 88**

Table S3: Quantification of Qdots in SEM images: VIPER versus immunolabeling

	Micrograph	count/μm^2	total count	monomer	dimer	≥ 3
VIPER	**Cell 1 Image 1**	312.2	3746	2148	429	200
	Cell 1 Image 2	331.5	3978	2331	462	192
	Cell 2 Image 1	261.1	3133	1587	398	196
	Cell 2 Image 2	104.6	1255	840	130	40
	Cell 3 Image 1	320.2	3842	2363	452	165
	Cell 3 Image 2	288.9	3467	2056	430	156
	AVERAGE	**270 ± 85**	**3237 ± 1016**	**1888 ± 584**	**384 ± 126**	**158 ± 61**
Anti-TfR1 (8D3)	**Cell 1 Image 1**	440.4	5285	2294	595	457
	Cell 1 Image 2	389.0	4668	1861	504	452
	Cell 2 Image 1	653.5	7842	2734	833	802
	Cell 2 Image 2	457.8	5493	2357	633	462
	Cell 3 Image 1	393.9	4727	1782	540	453
	Cell 3 Image 2	448.8	5385	1952	646	515
	AVERAGE	**464 ± 97**	**5567 ± 1167**	**2163 ± 364**	**625 ± 115**	**524 ± 139**
Anti- Tf (Ab82411)	**Cell 1 Image 1**	106.7	1280	857	148	36
	Cell 1 Image 2	107.5	1290	810	156	47
	Cell 2 Image 1	95.6	1147	607	138	73
	Cell 2 Image 2	99.3	1191	669	153	59
	Cell 3 Image 1	62.7	752	467	89	32
	Cell 3 Image 2	71.6	859	492	102	42
	AVERAGE	**91 ± 19**	**1087 ± 227**	**650 ± 161**	**131 ± 28**	**48 ± 15**
Anti-TfR1 (H68.4)	**Cell 1 Image 1**	69.6	835	530	86	34
	Cell 1 Image 2	181.8	2182	1149	232	146
	Cell 2 Image 1	198.0	2376	1392	265	120
	Cell 2 Image 2	165.0	1980	1164	213	106
	Cell 3 Image 1	238.8	2866	1484	301	204
	Cell 3 Image 2	240.4	2885	1492	324	196
	AVERAGE	**182 ± 63**	**2187 ± 756**	**1202 ± 362**	**237 ± 85**	**134 ± 63**

Table S4: Quantification of Qdots in SEM images: Immunolabeling with Ab1086 and Ab216665

	Micrograph	count/μm^2	total count	monomer	dimer	≥ 3
Ab1086	**Cell 1 Image 1**	0.0	0	0	0	0
	Cell 1 Image 2	0.1	1	1	0	0
	Cell 2 Image 1	0.0	0	0	0	0
	Cell 2 Image 2	0.0	0	0	0	0
	Cell 3 Image 1	0.0	0	0	0	0
	Cell 3 Image 2	0.2	2	2	0	0
	AVERAGE	**0±0**	**1±1**	**1±1**	**0±0**	**0±0**
Ab216665	**Cell 1 Image 1**	0.4	5	5	0	0
	Cell 1 Image 2	1.6	19	15	2	0
	Cell 2 Image 1	0.5	6	6	0	0.5
	Cell 2 Image 2	0.0	0	0	0	0.0
	Cell 3 Image 1	0.3	4	4	0	0
	Cell 3 Image 2	0.4	5	5	0	0
	AVERAGE	**1±1**	**7±6**	**6±5**	**0±1**	**0±0**

Table S5. Summary of genetic constructs

Protein Name	**Sequence** (1-letter amino acid code) Sequence annotation key: **Coil tag**; linker; mEmerald; mCherry	**Molecular Weight (Daltons)**‡	**pI**‡	**Vector name**
CoilR	MGGS**LEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPL**GGGAAAL GCLAAALEHHHHHH	7,502.35	6.00	pET28b(+)_CoilR
CoilR-Lys56	MGGS**LEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPL**GGGAAAL GKLAAALEHHHHHH	7,527.38	6.27	pET28b(+)_CoilR-Lys56
CoilE	MGGS**LEIEAAFLERENTALETRVAELRQRVQRLRNRVSQYRTRYGPL**GGGCLEH HHHH	6,737.56	9.29	pET28b(+)_CoilE
Transferrin receptor 1 (TfR1)	MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENADNNMKA SVRKPKRFNGRLCFAAIALVIFFLIGFMSGYLGYCKRVEQKEECVKLAETEETD KSETMETEDVPTSSRLYWADLKTLLSEKLNSIEFADTIKQLSQNTYTPREAGSQ KDESLAYYIENQFHEFKFSKVWRDEHYVKIQVKSSIGQNMVTIVQSNGNLDPVE SPEGYVAFSKPTEVSGKLVHANFGTKKDFEELSYSVNGSLVIVRAGEITFAEKV ANAQSFNAIGVLIYMDKNKFPVVEADLALFGHAHLGTGDPYTPGFPSFNHTQFP PSQSSGLPNIPVQTISRAAAEKLFGKMEGSCPARWNIDSSCKLELSQNQNVKLI VKNVLKERRILNIFGVIKGYEEPDRYVVVGAQRDALGAGVAAKSSVGTGLLLKL AQVFSDMISKDGFRPSRSIIFASWTAGDFGAVGATEWLEGYLSSLHLKAFTYIN LDKVVLGTSNFKVSASPLLYTLMGKIMQDVKHPVDGKSLYRDSNWISKVEKLSF DNAAYPFLAYSGIPAVSFCFCEDADYPYLGTRLDTYEALTQKVPQLNQMVRTAA EVAGQLIIKLTHDVELNLDYEMYNSKLLSFMKDLNQFKTDIRDMGLSLQWLYSA RGDYFRATSRLTTDFHNAEKTNRFVMREINDRIMKVEYHFLSPYVSPRESPFRH IFWGSGSHTLSALVENLKLRQKNITAFNETLFRNQLALATWTIQGVANALSGDI WNIDNEF	85,731.40	6.13	pcDNA3.1_TfR1
TfR1-CoilE	MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENADNNMKA SVRKPKRFNGRLCFAAIALVIFFLIGFMSGYLGYCKRVEQKEECVKLAETEETD KSETMETEDVPTSSRLYWADLKTLLSEKLNSIEFADTIKQLSQNTYTPREAGSQ KDESLAYYIENQFHEFKFSKVWRDEHYVKIQVKSSIGQNMVTIVQSNGNLDPVE SPEGYVAFSKPTEVSGKLVHANFGTKKDFEELSYSVNGSLVIVRAGEITFAEKV ANAQSFNAIGVLIYMDKNKFPVVEADLALFGHAHLGTGDPYTPGFPSFNHTQFP PSQSSGLPNIPVQTISRAAAEKLFGKMEGSCPARWNIDSSCKLELSQNQNVKLI VKNVLKERRILNIFGVIKGYEEPDRYVVVGAQRDALGAGVAAKSSVGTGLLLKL AQVFSDMISKDGFRPSRSIIFASWTAGDFGAVGATEWLEGYLSSLHLKAFTYIN LDKVVLGTSNFKVSASPLLYTLMGKIMQDVKHPVDGKSLYRDSNWISKVEKLSF DNAAYPFLAYSGIPAVSFCFCEDADYPYLGTRLDTYEALTQKVPQLNQMVRTAA EVAGQLIIKLTHDVELNLDYEMYNSKLLSFMKDLNQFKTDIRDMGLSLQWLYSA RGDYFRATSRLTTDFHNAEKTNRFVMREINDRIMKVEYHFLSPYVSPRESPFRH IFWGSGSHTLSALVENLKLRQKNITAFNETLFRNQLALATWTIQGVANALSGDI WNIDNEFGSGSGSTG**MLEIEAAFLERENTALETRVAELRQRVQRLRNRVSQYRT RYGPL**GGGCLETG	92,312.77	6.34	pcDNA3.1_TfR1-CoilE
TfR1-mCherry	MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAVDEEENADNNTK ANVTKPKRCSGSICYGTIAVIVFFLIGFMIGYLGYCKGVEPKTECERLAGTES PVREEPGEDFPAARRLYWDDLKRKLSEKLDSTDFTSTIKLLNENSYVPREAGS QKDENLALYVENQFREFKLSKVWRDQHFVKIQVKDSAQNSVIIVDKNGRLVYL VENPGGYVAYSKAATVTGKLVHANFGTKKDFEDLYTPVNGSIVIVRAGKITFA EKVANAESLNAIGVLIYMDQTKFPIVNAELSFFGHAHLGTGDPYTPGFPSFNH TQFPPSRSSGLPNIPVQTISRAAAEKLFGNMEGDCPSDWKTDSTCRMVTSESK NVKLTVSNVLKEIKILNIFGVIKGFVEPDHYVVVGAQRDAWGPGAAKSGVGTA LLLKLAQMFSDMVLKDGFQPSRSIIFASWSAGDFGSVGATEWLEGYLSSLHLK AFTYINLDKAVLGTSNFKVSASPLLYTLIEKTMQNVKHPVTGQFLYQDSNWAS KVEKLTLDNAAFPFLAYSGIPAVSFCFCEDTDYPYLGTTMDTYKELIERIPEL NKVARAAAEVAGQFVIKLTHDVELNLDYERYNSQLLSFVRDLNQYRADIKEMG LSLQWLYSARGDFFRATSRLTTDFGNAEKTDRFVMKKLNDRVMRVEYHFLSPY VSPKESPFRHVFWGSGSHTLPALLENLKLRKQNNGAFNETLFRNQLALATWTI QGAANALSGDVWDIDNEFSEFGSTGSTGSTGADPPVATMVSKGEEDNMAIIKE FMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSP QFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQD GEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLK DGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRH STGGMDELYK	113,413.42	5.87	mCherry-TFR-20 Addgene: 55144
mEmerald-actin	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGK LPVPWPTLVTTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGN YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHKVYITADKQK NGIKVNFKTRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDP	69,948.42	5.58	mEmerald-actin-C18 Addgene: 53978

	NEKRDHMVLLEFVTAAGITLGMDELYKSGLRSGSGGGGSASGGSGSDDDIAALV VDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKR GILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKAN REKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEG YALPHAILRLDLAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVAL DFEQEMATAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGI HETTFNSIMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKI KIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF			
mEmerald-CoilE-actin	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGK LPVPWPTLVTTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGN YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHKVYITADKQK NGIKVNFKTRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDP NEKRDHMVLLEFVTAAGITLGMDELYKSGLRSM**LEIEAAFLERENTALETRVA ELRQRVQRLRNRVSQYRTRYGPL**GGGRSGSGGGSASGGSGSDDDIAALVVDNG SGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILT LKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALP HAILRLDLAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQ EMATAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETT FNSIMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIA PPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF	75,678.93	5.83	mEmerald-CoilE-actin-C18
H2B-mEmerald	MPEPAKSAPAPKKGSKKAVTKAQKKGGKKRKRSRKESYSIYVYKVLKQVHPDT GISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRLLLPGE LAKHAVSEGTKAITKYTSAKDPPVATMVSKGEELFTGVVPILVELDGDVNGHK FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFARYPDHMK QHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYNSHKVYITADKQKNGIKVNFKTRHNIEDGSVQLADHYQQN TPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK	41,320.19	9.26	H2B-6-mEmerald Addgene: 54111
H2B-CoilE-mEmerald	MPEPAKSAPAPKKGSKKAVTKAQKKGGKKRKRSRKESYSIYVYKVLKQVHPDT GISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRLLLPGE LAKHAVSEGTKAITKYTSAKDPPVM**LEIEAAFLERENTALETRVAELRQRVQR LRNRVSQYRTRYGPL**GGGGPVATMVSKGEELFTGVVPILVELDGDVNGHKFSV SGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFARYPDHMKQHD FFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGN ILGHKLEYNYNSHKVYITADKQKNGIKVNFKTRHNIEDGSVQLADHYQQNTPI GDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK	47,060.74	9.39	H2B-6-CoilE-mEmerald
Mito-mEmerald	MSVLTPLLLRGLTGSARRLPVPRAKIHSLGDPPVATMVSKGEELFTGVVPILV ELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQ CFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR IELKGIDFKEDGNILGHKLEYNYNSHKVYITADKQKNGIKVNFKTRHNIEDGS VQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAG ITLGMDELYK	30,728.05	6.56	Mito-7-mEmerald Addgene: 54160
Mito-CoilE-mEmerald	MSVLTPLLLRGLTGSARRLPVPRAKIHSLGDPPVM**LEIEAAFLERENTALETR VAELRQRVQRLRNRVSQYRTRYGPL**GGGGPVATMVSKGEELFTGVVPILVELD GDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFA RYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL KGIDFKEDGNILGHKLEYNYNSHKVYITADKQKNGIKVNFKTRHNIEDGSVQL ADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITL GMDELYK	36,468.60	7.77	Mito-7-CoilE-mEmerald

‡*Calculated by ExPasy Protparam, https://web.expasy.org/protparam*

Table S6. Bacterial strains and plasmids

	Characteristics	Source
E. coli strains		
TOP10	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG	ThermoFisher Scientific
BL21(DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	ThermoFisher Scientific
Plasmids		
pET28b(+)	T7 promoter, His-tag coding sequence, MCS, *lacI* coding sequence, (KanR)	Novagen
pcDNA3.1	CMV promoter, MCS, BGH polyadenylation signal, SV40 origin, (AmpR), (NeoR)	Invitrogen
Mito-7-mEmerald	CMV promoter, COX8A, mEmerald (C terminal on backbone) (KanR, NeoR)	Addgene: 54160
H2B-6-mEmerald	CMV promoter, HIST1H2BJ, mEmerald (C terminal on backbone) (KanR, NeoR)	Addgene: 54111
mEmerald-Actin-C18	CMV promoter, ACTB, mEmerald (C terminal on backbone) (KanR, NeoR)	Addgene: 53978

Table S7. Oligonucleotide sequences

Primer Name	**Sequence** (restriction sites underlined)
CoilR-1 (*Nco*I)	GATATACCATGGGCGGCAGCCTGGAAATTGAAGCGGCGTTT
CoilR-2	TCCAGCGCGGTATTTTCACGTTCCAGAAACGCCGCTTCAATTTCC
CoilR-3	GTGAAAATACCGCGCTGGAAACCCGTGTGGCGGAACTGCGTCAGC
CoilR-4	GCTCACACGATTACGCAGACGCTGCACACGCTGACGCAGTTCCGC
CoilR-5	TCTGCGTAATCGTGTGAGCCAGTATCGTACCCGTTATGGCCCGTT
CoilR-6 (*Hind*III)	GCAAGCTTGCCCAGCGCAGCAGCCCCTCCGCCTAACGGGCCATAACGGGT
CoilE-1 (*Nco*I)	GATATACCATGGGCGGCAGCCTGGAAATTGAAGCGGCGTTT
CoilE-2	TCCAGCGCGGTATTTTCACGTTCCAGAAACGCCGCTTCAATTTCC
CoilE-3	GTGAAAATACCGCGCTGGAAACCCGTGTGGCGGAACTGCGTCAGC
CoilE-4	GCTCACACGATTACGCAGACGCTGCACACGCTGACGCAGTTCCGC
CoilE-5	TCTGCGTAATCGTGTGAGCCAGTATCGTACCCGTTATGGCCCGTT
CoilE-6 (*Hind*III)	GCAAGCTTGCCCAGCGCAGCAGCCCCTCCGCCTAACGGGCCATAACGGGT
TfR1-CoilE 1 F	AAAGCAGCATTGGTCAAAACATGGTGACCATAGTGCAGTCAAATGGTAAC
TfR1-CoilE 1 R	TACCAAACTCATTGTCAATATTCCAAATGTC
TfR1-CoilE 2 F	TATTGACAATGAGTTTGGTAGCGGCAGC
TfR1-CoilE 2 R	CATGTTACATTTAACCGGTCTCGAGACAG
TfR1-CoilE 3 F	GACCGGTTAAATGTAACATGCATAATTAAATAAGAG
TfR1-CoilE 3 R	AAATGGATATACAAGCTCCCGGGAGCTTTTTGCAAAAGCCTAG
CoilE *Bgl*I Actin F	GTCCGGACTCAGATCTATGCTGGAAATTGAAGCGGCGT
CoilE *Bgl*I Actin R	CACCGCTGCCAGATCTGCCGCCACCCAGCGGGCCATAA
CoilE *Age*I H2B F	CTAAGGATCCACCGGTAATGCTGGAAATTGAAGCGGCG
CoilE *Age*I H2B R	CATGGTGGCGACCGGTCCGCCGCCACCCAGCGGGCC
CoilE *Age*I Mito F	TGGGGGATCCACCGGTAATGCTGGAAATTGAAGCGGCG
CoilE *Age*I Mito R	CATGGTGGCGACCGGTCCGCCGCCACCCAGCGGGCC

Table S8. Properties of Sulfo-Cyanine5 maleimide and BODIPY-FL maleimide‡

Fluorophore	Vendor	Excitation maximum	Emission maximum	Quantum Yield	ε_{dye} ($L \cdot mol^{-1} \cdot cm^{-1}$)	CF_{280}
Sulfo-Cyanine5 Maleimide	Lumiprobe	646 nm	662 nm	0.28	271,000	0.04
BODIPY-FL Maleimide	Lumiprobe	503 nm	509 nm	0.97	80,000	0.027

‡*Values provided on the Lumiprobe website: www.lumiprobe.com.*

Table S9: Summary of primary and secondary antibodies used for immunolabeling.

Primary Antibody (1:100 dilution)	Protein Target	Commercial Source (Catalog #)	Lot #	Secondary Antibody (1:200 dilution)	Commercial Source (Catalog.#)	Lot #
8D3	TfR1 (extracellular)	Novus Biologicals (#NB100-64979)	1607	F(ab')2-Goat anti-Rat IgG (H+L) Secondary Antibody, Qdot 655	Thermofisher Scientific (#Q-11621MP)	1863945
H68.4	TfR1 (cytoplasmic)	ThermoFisher Scientific (#13-6800)	RB232679	F(ab')2-Goat anti-Mouse IgG (H+L) Secondary Antibody, Qdot 655	Thermofisher Scientific (#Q-11021MP)	1863429
Ab82411	Tf	Abcam (#ab82411)	GR3207592-3	F(ab')2-Goat anti-Rabbit IgG (H+L) Secondary Antibody, Qdot 655	Thermofisher Scientific (#Q-11421MP)	1996360
Ab1086	TfR1 (extracellular)	Abcam (#ab1086)	GR3211582-6	F(ab')2-Goat anti-Mouse IgG (H+L) Secondary Antibody, Qdot 655	Thermofisher Scientific (#Q-11021MP)	1863429
Ab216665	TfR1 (extracellular)	Abcam (#ab216665)	GR3192662-5	F(ab')2-Goat anti-Rabbit IgG (H+L) Secondary Antibody, Qdot 655	Thermofisher Scientific (#Q-11421MP)	1996360

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