

# Site-specific protein labeling by Sfp phosphopantetheinyl transferase

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**Sfp phosphopantetheinyl transferase covalently attaches small-molecule probes including biotin and various organic fluorophores to a specific serine residue in the peptidyl carrier protein (PCP) or a short 11-residue peptide tag ybbR through a phosphopantetheinyl linker. We describe here a protocol for site-specific protein labeling by Sfp-catalyzed protein post-translational modification that includes (i) expression and purification of Sfp, (ii) synthesis of small-molecule probe–CoA conjugates, (iii) construction of target protein fusions with PCP or the ybbR tag, (iv) labeling PCP- or ybbR-tagged target protein fusions in cell lysates and on live cell surfaces and (v) imaging fluorophore-labeled cell surface receptors by fluorescence microscopy. To follow this protocol, we advise that you allow 3 d for the expression and purification of Sfp phosphopantetheinyl transferase, 1 d for the synthesis and purification of the small-molecule probe–CoA conjugates as the substrates of Sfp, 3 d for the cloning of target protein genes as fusions to the PCP or the ybbR tag in the appropriate plasmids and another 3 d for transfecting cell lines with the plasmids and the expression of PCP- or ybbR-tagged proteins. Labeling of the PCP- or the ybbR-tagged proteins in cell lysates or on cell surfaces should require only 15–30 min.**

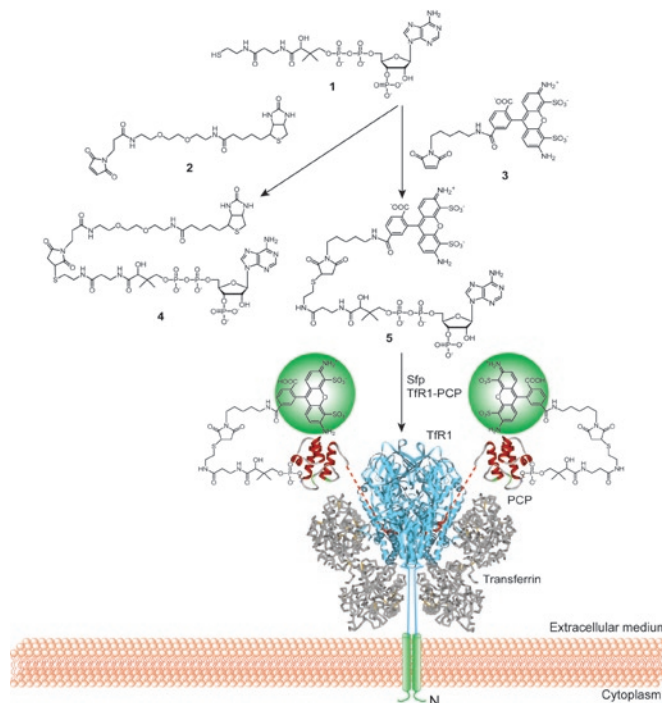
## INTRODUCTION

Sfp phosphopantetheinyl transferase covalently transfers 4'-phosphopantetheinyl (Ppant) groups from CoA to conserved serine residues on PCP and acyl carrier protein (ACP) domains in non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) in *Bacillus subtilis*<sup>1</sup>. The post-translational modification of PCP and ACP domains by Ppant is crucial for the activation of NRPS and PKS, because the flexible 20-Å Ppant group functions as a swinging arm providing the anchoring point for the attachment of the peptide or polyketide chain as it elongates along the biosynthetic assembly line<sup>2</sup>.

Although the substrate for Sfp must contain CoA, the enzyme is otherwise quite tolerant of a range of small molecules attached to CoA. In fact, Sfp transfers small molecules of diverse structures from CoA to the conserved serine on the PCP or ACP domain<sup>3–6</sup>. Sfp-catalyzed post-translational modification of PCP or ACP takes advantage of the substrate promiscuity of Sfp and the small size of PCP and ACP domains (~80 residues) to provide an attractive method for one-step site-specific protein labeling with small molecules. The *Escherichia coli* phosphopantetheinyl transferase AcpS is functionally similar to Sfp, although AcpS preferably modifies ACP domains<sup>7–9</sup>.

Sfp and AcpS have been used for site-specific protein labeling in cell lysates or on live cell surfaces. In these applications the target protein is expressed as a fusion to PCP or ACP at either the N or the C terminus, and Sfp is used to enzymatically attach a small-molecule probe–Ppant conjugate to the expressed PCP or ACP tag. This method allows the site-specific labeling of target proteins by small molecules of diverse structures and functionalities, such as biotin, fluorophores, porphyrin, sugars and peptides (Fig. 1)<sup>10–12</sup>. The labeling reaction proceeds with high efficiency and can be carried out in cell lysates or in culture media. A sufficient number of cell surface protein molecules can be labeled for imaging purposes within 15 min at submicromolar concentrations of

the enzyme and CoA-conjugated small-molecule probes. Thus far, protein labeling catalyzed by Sfp and AcpS has been used for high-throughput printing of protein microarrays<sup>11</sup>, site-specific



**Figure 1** | Synthesis of biotin-CoA (4) and Alexa Fluor 488-CoA (5) using CoA (1) and maleimide-linked biotin (2) and Alexa Fluor 488 (3), respectively. In a subsequent step, Alexa Fluor 488-CoA (5) is used to label the Tfr1-PCP fusion by Sfp-catalyzed site-specific modification of a conserved serine residue on PCP.

attachment of small molecules to phage particles<sup>12</sup> and live cell imaging of cell surface proteins (Fig. 1)<sup>10, 13, 14</sup>. Most recently, the ybbR tag, a short (11-residue) peptide, was found to be an efficient substrate for Sfp-catalyzed protein labeling, thereby replacing the full-length PCP or ACP domain for the construction of smaller fusions of the target protein. The ybbR tag can be fused to the N or C terminus of the target protein, or as an alternative, be inserted into a flexible loop of a protein; all of these fusions can then be efficiently labeled with small-molecule probes by Sfp, further improving the versatility of Sfp-catalyzed protein labeling<sup>15</sup>.

Because CoA-conjugated small molecules are not membrane permeable, presumably as a result of the many negative charges on the ATP moiety of CoA, Sfp- or AcpS-catalyzed protein labeling is currently limited to labeling proteins in cell lysates or on the cell surface. One advantage of the inability of the CoA-fluorescent dye conjugate to penetrate the membrane is that the intracellular background fluorescence is very low after extracellular labeling of the cell with the conjugate. This feature allows high-contrast imaging of the intracellular trafficking of internalized cell surface receptors<sup>10, 13, 14</sup>.

### Experimental design

In this protocol, small-molecule probe–CoA conjugates are synthesized by one-step Michael condensation<sup>16</sup> of the maleimide-functionalized small-molecule probe with the free thiol group of CoA at the end of the Ppant arm, resulting in a thioether linkage

between the small-molecule probe and CoA. Maleimide-linked biotin and fluorophores are commercially available from Pierce and Molecular Probes. Besides the thioether linkage, small molecules can also be conjugated to CoA through the formation of disulfide or thioester bonds for their use as the substrates of Sfp for protein labeling<sup>5, 12</sup>.

We have been using the PCP domain from the NRPS enzyme GrsA as a tag for Sfp-catalyzed protein labeling<sup>17</sup>. The gene encoding GrsA-PCP was cloned between the *NcoI* and *BglII* restriction sites in the pQE60 vector (Qiagen) to give the plasmid pQE60-GrsA-PCP, which carries an ampicillin-resistance gene and expresses GrsA-PCP with a C-terminal His<sub>6</sub> tag. The DNA sequence of pQE60 can be downloaded from the vendor's website at <http://www1.qiagen.com/> (for the DNA sequence of the GrsA-PCP see REAGENTS list). PCP can be cloned as a fusion to either the N or C terminus of the target protein. If a cell surface protein is to be labeled, the membrane topology of the protein must be verified first, and a terminus that is exposed on the cell surface should be fused to PCP. A linker between PCP and the target protein is not necessary. The 11-residue ybbR tag with the peptide sequence DSLEFIASKLA can also be fused to the N or C terminus of the target protein or inserted within a flexible loop of the protein with the underlined serine as the site of covalent attachment by the small-molecule–Ppant group<sup>15</sup> (for the DNA sequence of the ybbR tag see REAGENTS list).

## MATERIALS

### REAGENTS (TIMING ~2–3 h)

- Fluorophores (Pierce or Molecular Probes)
- pET29-Sfp (contact C.T.W. for the plasmid)
- pQE60-GrsA-PCP (contact C.T.W. for the plasmid)
- PCP sequence: GCGGAACCTGATTTAACTTTCGGGATGAGGGTAGACTATGAAGCGCCGCGAA ATGAAATCGAGGAAACGCTTGTACTATCTGGCAGGATGATATTAGGTATTGAGAAAATCGGTATTAAGATAATTTCTATGCATTA GGTGGAGATTCTATTAAAGCAATACAGGTTGCTGCTCGCCTGCATTCCTA CCAATTAAGCTAGAAACAAAGATTATTAAAGTATCCAACAATCGATCA GCTCGTTCAATATATAAAGATAGT
- ybbR tag sequence: GATTCTCTGAATTTATTGCTAGTAAGCTTGCG
- *E. coli* BL21 DE3 (Invitrogen)
- Kanamycin (Sigma)
- LB medium and LB agar (Difco)
- Isopropyl-1-thio-β-D-galactoside (IPTG) (Sigma)
- DNase I (Sigma)
- Ni-NTA agarose (Qiagen)
- CoA trilithium salt (Sigma)
- EZ-Link PEO-maleimide activated biotin (Pierce; cat. no. 21901)
- Alexa Fluor 488 C5 maleimide (Invitrogen; cat. no. A10254)
- 4–15% SDS-PAGE precast ready gel (Bio-Rad)
- Immun-Blot PVDF membrane (Bio-Rad)
- ImmunoPure Streptavidin–horseradish peroxidase (HRP) conjugate (Pierce)
- ECL plus western blotting detection kit (GE Healthcare)
- FuGENE 6 transfection reagent (Roche Diagnostics; cat. no. 1814443)
- DMEM–F-12 medium (1×) (Invitrogen; cat. no. 21041-025)
- Penicillin–Streptomycin (Invitrogen; cat. no. 15140-122)
- Alexa Fluor 568-conjugated transferrin (Molecular Probes)
- Dulbecco's phosphate-buffered saline (1× D-PBS) (Invitrogen; cat. no. 14190-250)
- SlowFade Antifade Kit (Invitrogen)

- Tris-buffered saline (TBS), Tris-HCl 50 mM, pH 7.5, NaCl 0.15 M
- Formaldehyde ! **CAUTION** Respiratory irritant and potential cancer hazard. May cause eye damage.
- Glycerol (10%)
- Sodium phosphate (100 mM, pH 7.0)
- Acetonitrile
- Trifluoroacetic acid (TFA), 0.1%
- MgCl<sub>2</sub> (10 mM)
- HEPES (50 mM, pH 7.5)
- Streptavidin-coated agarose beads (Promega)

### EQUIPMENT

- Innova 4230 floor incubator/shaker (New Brunswick Scientific)
- RC-5C plus centrifuge (Sorvall)
- French pressure cell press (Thermo Spectronic)
- Centriprep YM-10 concentrator (Millipore)
- HPLC system (Beckman)
- Protein peptide preparative HPLC column (Vydac)
- Voyager matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) system (Applied Biosystems)
- Mini-PROTEAN 3 gel electrophoresis system (Bio-Rad)
- Mini Trans-blot cell (Bio-Rad)
- PowerPac power supply (Bio-Rad)
- Lyophilizer (Savant)
- Nikon TE2000U inverted microscope (Nikon)
- Ultraview spinning disk confocal system (PerkinElmer)
- Orca ER Cooled Charge-Coupled Device (CCD) camera (Hamamatsu)
- MetaMorph Software (Universal Imaging)

## PROTOCOL

### PROCEDURE

#### Expression and purification of Sfp phosphopantetheinyl transferase ● TIMING 3 d

**1** | Clone Sfp as a C-terminal His<sub>6</sub>-tagged protein in the pET29 vector carrying a kanamycin resistance gene to give the plasmid pET29-Sfp. (For detailed information on cloning and protein expression with pET vector, please refer to the vendor's manual, which can be downloaded at the Novagen website at <http://www.emdbiosciences.com/home.asp>.) Transform the Sfp expression plasmid pET29-Sfp into BL21 DE3 *E. coli* cells following the vendor's protocol. Plate the cells on a LB agar plate containing 50 µg ml<sup>-1</sup> kanamycin. Incubate the plate at 37 °C overnight. The next day, inoculate a 10-ml LB starting culture containing 50 µg ml<sup>-1</sup> kanamycin and shake at 200 r.p.m. on a New Brunswick Innova 4230 floor incubator/shaker at 37 °C overnight.

**2** | Use the starting culture to inoculate 1 liter of LB medium containing 50 µg ml<sup>-1</sup> kanamycin. Shake at 200 r.p.m. at 37 °C on a New Brunswick Innova 4230 floor incubator/shaker until the OD<sub>600</sub> reaches 0.6. Add IPTG to the LB culture to a final concentration of 1 mM. Continue to shake at 200 r.p.m. at room temperature (25 °C) for 6 h, and then harvest the cells by centrifugation in two 500-ml centrifuge bottles at 5,000 r.p.m. for 10 min, and finally resuspend the cells in 20 ml of a solution of 5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9, containing 2 units ml<sup>-1</sup> DNase I.

**3** | Lyse the cells by passing the cell suspension twice through a French pressure cell press (18,000 p.s.i.). Spin down the cellular debris from the lysate by centrifugation (30 min at 17,000g) with a Sorvall RC-5C plus centrifuge and a SLA-3000 rotor at 4 °C.

**4** | Purify the Sfp protein by nickel column chromatography using 2 ml Ni-NTA agarose in a 15-mm diameter column according to the manufacturer's instructions.

**5** | Analyze the fractions of the eluant from the Ni-NTA column by SDS-PAGE, and pool the fractions that contain Sfp (26 kDa) and are >90% pure.

**6** | Dialyze the protein twice against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 10% glycerol.

**7** | After dialysis, concentrate the protein to 100–200 µM with a Centriprep YM-10 concentrator. Aliquot the concentrated protein solution into Eppendorf tubes, flash-freeze the tubes in liquid nitrogen and store the tubes at –80 °C. The purification procedure typically yields ~25 mg of pure (>90%) Sfp per liter of culture.

■ **PAUSE POINT** Sfp is very stable and stores well at the above conditions for years without significant loss of activity.

#### Synthesis and purification of small molecule–CoA conjugates ● TIMING 1 d

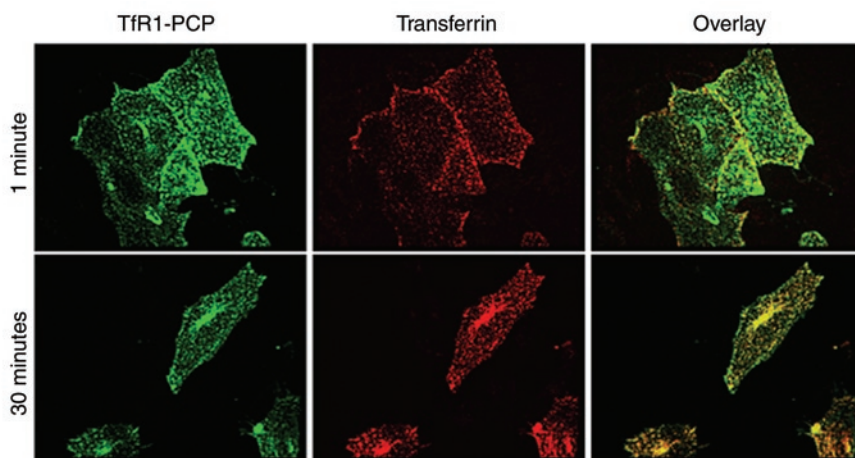
**8** | We use the synthesis of Alexa 488–CoA as an example (Fig. 1, compound 5). To a solution of Alexa Fluor 488 C<sub>5</sub> maleimide (1.0 mg, 1.4 µmol) in 0.25 ml DMSO, add CoA trilithium salt (1.6 mg, 2.1 µmol) in 0.75 ml sodium phosphate 100 mM, pH 7.0, and stir the reaction mixture at room temperature for 1 h in the dark.

**9** | Directly purify the reaction mixture by preparative HPLC using a reverse-phase protein peptide C18 column with a gradient of 0–50% (vol/vol) acetonitrile in 0.1% (vol/vol) TFA/water over 30 min.

**10** | Lyophilize the purified compound and confirm its identity by MALDI-TOF (negative mode): calculated for C<sub>51</sub>H<sub>62</sub>N<sub>11</sub>O<sub>28</sub>P<sub>3</sub>S<sub>3</sub>: 1465.21, found 1465.28.

#### Construction of PCP- or ybBR-tagged fusion proteins ● TIMING 3 d

**11** | Design primers to amplify the PCP gene from pQE60-GrsA-PCP with appropriate restriction sites at the ends of the PCR fragment, and clone the PCP gene in frame as a fusion to the target protein gene with those restriction sites.



**Figure 2** | Laser confocal images of TRVb cells transiently transfected with Tfr1-PCP, labeled with CoA-conjugated Alexa Fluor 488 (green) in the presence of Sfp, and incubated with Tf-Alexa 568 (red) for 1 min (top panels) or 30 min (bottom panels). The right column shows the overlay of the green and red channels; yellow indicates the overlap of green and red pixels, suggesting colocalization of ligand and receptor.

One can also fuse the PCP gene to the target protein gene by DNA overlap extension<sup>18</sup>. If the *ybbR* tag is to be fused to the target protein, design a PCR primer to incorporate the DNA sequence of the *ybbR* tag and appropriate restriction sites so as to insert the *ybbR* tag as N- or C- terminal fusion to the target protein or within a flexible loop of the target protein.

**12** | There are two ways of conducting the Sfp-catalyzed protein labeling: (A) with purified protein or proteins in cell lysates or (B) on the cell surface.

**(A) Sfp-catalyzed protein labeling with purified protein or proteins in cell lysates** ● **TIMING 15–30 min**

- (i) In a total volume of 100  $\mu\text{l}$  containing 10 mM  $\text{MgCl}_2$  and 50 mM HEPES pH 7.5, add 0.1  $\mu\text{M}$  Sfp, 5  $\mu\text{M}$  biotin–CoA (**Fig. 1**, compound **4**) and 5  $\mu\text{M}$  PCP- or *ybbR*-tagged protein (all final concentrations). Incubate the reaction mixture at room temperature for 30 min. PCP- or *ybbR*-tagged proteins in cell lysates can be directly labeled by Sfp following the same protocol.

▲ **CRITICAL STEP**

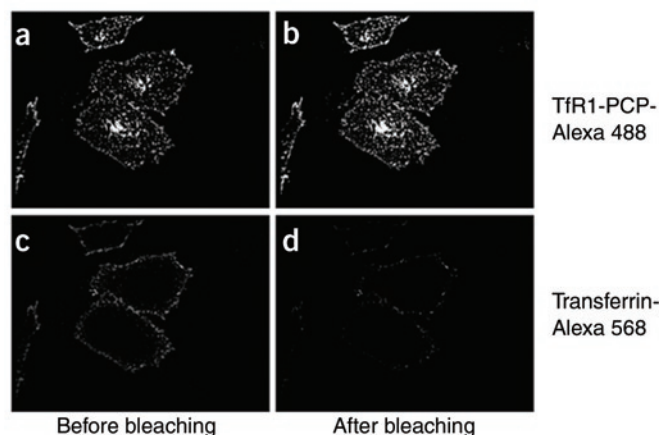
- (ii) Western blotting probed with streptavidin–HRP conjugates allows verification of the formation of biotinylated protein. To detect biotin labeling, load the labeling reaction mixture containing 0.5  $\mu\text{g}$  protein on a 4–15% SDS-PAGE gel and run the gel at 200 V with a Mini-PROTEAN 3 gel electrophoresis system.
- (iii) After electrophoresis, electroblot the protein bands onto a PVDF membrane with a Mini Trans-blot cell.
- (iv) Block the membrane with 3% (wt/vol) BSA in TBS for 2 h.
- (v) Incubate with 0.1  $\mu\text{g ml}^{-1}$  streptavidin–HRP conjugate in 1% (wt/vol) BSA for 1 h.
- (vi) Wash the membrane five times with 0.05% (vol/vol) Tween 20 and 0.05% (vol/vol) Triton X-100 in TBS and then five washes in TBS alone.
- (vii) Detect streptavidin binding using an ECL luminescence detection kit following the manufacturer’s instructions.

**(B) Sfp-catalyzed protein labeling on cell surface** ● **TIMING 3d (transfection) and 15–30 min (labeling)**

- (i) Live-cell labeling of transferrin (Tf) receptor 1 (TfR1) serves as an example (**Fig. 1**). Fuse PCP to TfR1 at the C terminus of the receptor, which is exposed on the cell surface. The construction of TfR1–PCP fusion in the pcDNA3.1(+) plasmid has been reported<sup>14</sup>.
- (ii) Grow TRVb cells<sup>19</sup> on sterilized coverslips to 50–60% confluency in cell culture medium containing DMEM–F-12 with 10% FBS and 1% penicillin–streptomycin.
- (iii) For each 35-mm well in a six-well plate, prepare one vial of transfection medium containing 3  $\mu\text{l}$  of FuGENE 6 transfection reagent and 1  $\mu\text{g}$  of the pcDNA3.1(+)TfR1–PCP plasmid diluted in 100  $\mu\text{l}$  of DMEM–F-12 (1 $\times$ ).
- (iv) Mix the transfection medium well and allow it to come to equilibrium for 20 min at room temperature.
- (v) Incubate the cells with transfection medium for 5 h, and then incubate the cells in DMEM–F-12 medium (1 $\times$ ) with penicillin and streptomycin for 24 h to allow time for protein expression.
- (vi) After 24 h of incubation in serum-containing medium, incubate the transfected cells in serum-free medium (DMEM–F-12 alone) for 2 h before labeling.
- (vii) To label TfR1–PCP, incubate the cells with 0.5  $\mu\text{M}$  Sfp and 1  $\mu\text{M}$  Alexa 488–CoA (**5**) in serum-free medium for 20 min at 37  $^\circ\text{C}$  (**Fig. 1**).

▲ **CRITICAL STEP**

- (viii) Wash the labeled cells three times with D-PBS. Fix the cells with a 3.7% (vol/vol) formaldehyde solution in D-PBS for 15 min.
- (ix) Mount the slide with SlowFade Antifade Kit for optical microscopy studies.
- (x) Perform confocal microscopy analysis of samples using a Nikon TE2000U inverted microscope in conjunction with a PerkinElmer Ultraview spinning disk confocal system equipped with a Hamamatsu Orca ER Cooled CCD camera. Acquire images using a 60 $\times$  differential interference contrast oil immersion objective lens, and analyze them using MetaMorph software.
- (xi) Where applicable, process images by MetaMorph (Universal Imaging) using 2D no-neighbors deconvolution before 3D reconstruction. For fluorescence resonance energy transfer (FRET) measurements<sup>14</sup>, acquire two images in each field



**Figure 3** | FRET analysis of Tf–TfR1 interactions in TRVb cells transfected with TfR1–PCP. TfR1–PCP is fluorescently labeled with CoA–Alexa Fluor 488, and cells are then incubated with Tf–Alexa 568 for 2 min. Shown are images of TfR1–PCP/CoA–Alexa Fluor 488 (top panels) and Tf–Alexa 568 (bottom panels) before (**a**, **c**) and after (**b**, **d**) photobleaching of the FRET acceptor Alexa Fluor 568. Fluorescence intensity is indicated by gray scale in each image.

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of view in the Alexa Fluor 488 (donor) and Alexa Fluor 568 (acceptor) channels using laser confocal microscopy. Then photobleach the field at 568 nm by high-intensity light from a mercury arc lamp for 60 s, and acquire a second set of images.

(xii) Process the four images, and calculate relative FRET intensities according to protocols described elsewhere<sup>14</sup>.

### ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

PROBLEM	SOLUTION
Expression yield of Sfp is low and <1 mg per liter of culture.	After isopropyl-1-thio- $\beta$ -D-galactoside induction, shake the culture at 15 °C overnight before harvesting the cells.
Yield of CoA–small-molecule coupling by thiol maleimide condensation is low.	Check the buffer pH and make sure it is neutral or slightly basic (pH >7).
No labeling of the cell surface receptor–PCP <sup>a</sup> or –ybbR fusion proteins.	Make sure the terminus of the cell surface protein to which PCP or ybbR is fused is exposed on the surfaces of the cells. Check the expression level of the cell surface protein fused to PCP or ybbR by western blot with antibodies that bind to the cell surface protein. First label the PCP- or ybbR-tagged cell surface proteins with biotin-CoA ( <b>4</b> ), and try to detect the biotinylation of the target protein by streptavidin-HRP on a western blot so as to evaluate the expression level of PCP- or ybbR-tagged protein, and test whether the PCP or the ybbR tag can be accessed and labeled by Sfp on the cell surface.

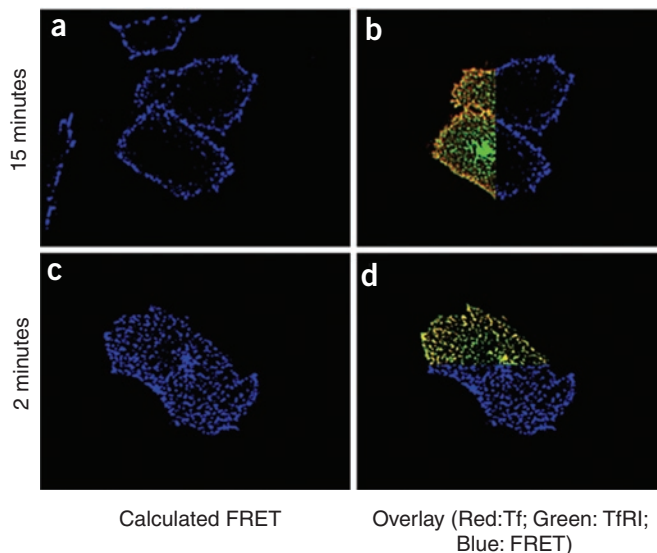
<sup>a</sup>PCP, Peptidyl carrier protein.

### ANTICIPATED RESULTS

To quantify the yield of the protein labeling reaction, EGFP proteins fused to the ybbR tag at the N or C terminus or with the ybbR tag inserted inside the protein were labeled with biotin Ppant after incubation with Sfp and biotin-CoA for 30 min. Streptavidin-coated agarose beads were then added, and >80% of the ybbR-tagged EGFP could be immobilized on the streptavidin beads, suggesting the high efficiency of the labeling reaction<sup>15</sup>.

The Tf–TfR1 system illustrates the use of Sfp-catalyzed protein labeling for live-cell fluorescence imaging and single-cell FRET measurements. TfR1-PCP is transiently transfected into TRVb cells<sup>19</sup>, a Chinese hamster ovary cell line that lacks endogenous TfR1. The expressed fusion receptors are labeled with CoA-conjugated Alexa Fluor 488 in the presence of Sfp (**Fig. 1**). Cells are then incubated with Tf-conjugated Alexa Fluor 568 and fixed at different time points for observation under the confocal microscope. **Figure 2** shows projections of stacks of optical slices taken at 0.25  $\mu$ m per slice; green represents TfR1-PCP and red represents Tf. At 1 min after the addition of Tf–Alexa 568, the ligand is observed mainly in the vicinity of the plasma membrane (**Fig. 2**, top). Longer incubation with Tf–Alexa 568 results in trafficking of Tf and TfR1-PCP into the cell and aggregation and colocalization of ligand and receptor (**Fig. 2**, bottom). These observations agree with current models for TfR1-mediated Tf uptake, in which the ligand is internalized through receptor-mediated endocytosis and collected in recycling endosomes before leaving the cell by exocytosis.

We further probe the dynamic interactions between TfR1-PCP and Tf by applying single-cell FRET imaging and analysis techniques. We use a donor dequenching method to quantify FRET, in which the acceptor is photobleached and the subsequent recovery of donor fluorescence is measured; a partial photobleaching protocol is also used to prevent photodamage<sup>14</sup>. For each field of view, a set of four confocal images is taken and processed as described earlier to obtain a calculated FRET image<sup>14</sup>. **Figures 3a** and **b** show the distribution of TfR1-PCP labeled with Alexa 488 before and after photobleaching of Alexa 568, respectively, whereas **Figure 3c** show the distribution of Tf–Alexa 568 before and



**Figure 4** | FRET analysis of Tf–TfR1 interactions in TRVb cells transfected with TfR1-PCP. The top panels show the results at 2 min after the addition of Tf–Alexa 568. (a) The calculated relative intensity of FRET between donor and acceptor fluorophores. (b) Overlay of TfR1-PCP (green), Tf–Alexa 568 (red) and calculated FRET (blue). The bottom panels show the calculated FRET (c) and overlay (d) images at 15 min after the addition of Tf–Alexa 568.

after photobleaching of Alexa 568, respectively. All of the images in **Figure 3** were acquired 2 min after the addition of Tf–Alexa 568. The integrated fluorescence intensities of the FRET acceptor Tf–Alexa 568 before and after photobleaching yield the factor for partial photobleaching, which is then used to calculate pixel-by-pixel relative FRET intensities. The calculated FRET intensities are reconstructed into an image (**Fig. 4a**) that reveals the subcellular distribution of ligand–receptor interactions. **Figure 4b** shows the overlay of Alexa 488–labeled TfR1–PCP (green) after photobleaching of the acceptor, Tf–Alexa 568 (red) before photobleaching of the acceptor, and calculated FRET (blue). Similar analysis of images acquired 15 min after the addition of Tf–Alexa 568 results in calculated FRET (**Fig. 4c**) and overlay (**Fig. 4d**) images for this time point. **Figure 4** reveals that ligand–receptor interactions are initially detected at or near the plasma membrane; by 15 min of incubation large aggregates of Tf/TfR1–PCP complexes are observed, indicating the collection of these complexes in the recycling endosomes. This example shows that Sfp-catalyzed site-specific protein labeling combined with single-cell FRET imaging methods allow tracking of the interaction and trafficking of ligand and receptor molecules in live cells.

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**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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