Live CHO-K1 cells transiently transfected with pACP-GPI. Cells were labeled with CoA 547 (red) in the presence of ACP Synthase for 60 minutes.

Introduction
CoA 547 is a photostable fluorescent substrate that can be used to label ACP-tag and MCP-tag fusion proteins exposed on the surface of living cells. This cell-impermeable substrate is based on the Dymocks dye DY-547, and is suitable for standard TAMRA and Cy3 filter sets. It has an excitation maximum at 554 nm and an emission maximum at 568 nm. This package contains 50 nmol of CoA 547 substrate, sufficient to make 10 ml of a 5 µM ACP-tag or MCP-tag fusion protein labeling solution.

The ACP-tag and MCP-tag are small polypeptide tags (8 kDa) based on the acyl carrier protein. MCP-tag contains two mutations (D36T and D39K). Both allow the specific, covalent attachment of virtually any molecule to a protein of interest. Substrates are derivatives of coenzyme A (CoA).

In the labeling reaction, the substituted phosphopantetheine group of CoA is covalently attached to the ACP-tag or MCP-tag by a phosphopantetheinyl transferase (SFP Synthase or ACP Synthase).

Materials Required but not Supplied
ACP Synthase (NEB #P9301) for labeling ACP-tag
SFP Synthase (NEB #P9302) for labeling ACP-tag or MCP-tag
Cells expressing ACP-tag or MCP-tag fusion proteins
Tissue culture materials and media
Transfection reagents
Fluorescence microscope with suitable filter set
DMSO

Storage
CoA 547 should be stored at −20°C (long term) or at 4°C in the dark (short term, less than 4 weeks). Protect the substrate from light and moisture. With proper storage at −20°C the substrate should be stable for at least 2 years dry or 3 months dissolved in DMSO.

Quality Controls
Purity and Characterization: Purity of CoA 547 was determined to be 97% by HPLC analysis. Molecular weight [M+H]+ was determined by MS to be 1506.3 (1506.4 expected).

In vitro Protein Labeling: Reaction of CoA 547 (10 µM) with purified ACP-MBP (Maltose Binding Protein, 5 µM) and SFP Synthase (1 µM) in vitro, followed by mass spec analysis, indicated an efficiency of labeling of ≥95%.

Cellular Protein Labeling: Cells transfected with pACP-GPI expressing ACP-GPI (cell surface) were labeled with 5 µM CoA 547 using SFP Synthase for 60 minutes and visualized by confocal microscopy. Surface target was efficiently labeled.

Instructions for Labeling on the Surface of Cells
ACP-tag and MCP-tag fusion proteins are expressed by transient transfection. For expression of fusion proteins with the ACP-tag and MCP-tag, refer to instructions supplied with the pACP-tag and pMCP-tag plasmids. For cell culture and transfection methods, refer to established protocols.

Dissolve one vial of CoA substrate (50 nmol) in 50 µl of DMSO to give a labeling stock solution of 1 mM CoA substrate. Mix by vortexing for 10 minutes until all the substrate is dissolved. Store this stock solution at 4°C in the dark (short term, less than 4 weeks). Proper storage at −20°C the substrate should be stable for at least 2 years dry or 3 months dissolved in DMSO.

Optimizing Labeling
Optimal substrate concentrations and reaction times range from 1–10 µM and 30–60 minutes, respectively, depending on experimental conditions and expression levels of the ACP-tag and MCP-tag fusion protein. Best results are usually obtained at concentrations between 1 and 5 µM substrate and 60 minutes reaction time. Increasing substrate concentration and reaction time usually results in a higher background and does not necessarily increase the signal to background ratio.

Usage Notes
Five We recommend routinely labeling one well of non-transfected or mock-transfected cells as a negative control.

3 Wash the cells three times with tissue culture medium with serum.

4 Image the cells using an appropriate filter set. ACP-tag and MCP-tag fusion proteins labeled with CoA 547 should have an excitation maximum at 554 nm and an emission maximum at 568 nm and can be imaged with standard TAMRA and Cy3 filter sets.

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Protocol for Labeling Reaction
1. Dilute the substrate stock solution 1:200 in medium to a final concentration of 5 µM. Mix dye with medium thoroughly by pipetting up and down 10 times (necessary for reducing backgrounds). For best performance, add the CoA substrate to complete medium, including serum (0.5% BSA can be used for experiments carried out in serum-free medium). Add MgCl2 to a final concentration of 10 mM. Finally, add ACP Synthase or SFP Synthase to a final concentration of 1 µM, a dilution of 1:40. Do not prepare more medium with substrate, MgCl2, and synthase than will be consumed within one hour.

2 Replace the medium on the cells expressing an ACP-tag or MCP-tag fusion protein with the labeling medium and incubate at 37°C, 5% CO2, for 60 minutes.

3 Wash the cells three times with tissue culture medium with serum.

4 Image the cells using an appropriate filter set. ACP-tag and MCP-tag fusion proteins labeled with CoA 547 should have an excitation maximum at 554 nm and an emission maximum at 568 nm and can be imaged with standard TAMRA and Cy3 filter sets.

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Stability of Labeling
The turnover and internalization rates of the ACP-tag and MCP-tag fusion protein under investigation may vary widely depending on the fusion partner. Where protein turnover is rapid, we recommend analyzing the cells under the microscope immediately after the labeling reaction or fixing the cells directly after labeling.
Fixation of Cells
After labeling the ACP-tag or MCP-tag fusion proteins, the cells can be fixed with standard fixation methods such as para-formaldehyde, ethanol, methanol, methanol/acetone etc., without loss of signal. We are not aware of any incompatibility of the CoA label with any fixation method.

Counterstaining
Cells can be counterstained with any live-cell dye that is compatible with the fluorescent properties of the substrate for simultaneous microscopic detection. We routinely add 5 µM Hoechst 33342 to the labeling medium as a DNA counterstain for nuclear visualization.

Troubleshooting for Cellular Labeling

No Labeling
If no labeling is seen, the most likely explanation is that the fusion protein is not expressed. Verify the transfection method to confirm that the cells contain the fusion gene of interest. If this is confirmed, check for expression of the ACP- or MCP-tag fusion protein via Western blot.

Weak Labeling
Weak labeling may be caused by insufficient exposure of the fusion protein to the substrate. Try increasing the concentration of CoA substrate and/or the incubation time, following the guidelines described above. Alternatively, the protein may be poorly expressed and/or turn over rapidly. If the protein has limited stability in the cell, it may help to analyze the samples immediately after labeling.

High Background
Background fluorescence may be controlled by reducing the concentration of CoA substrate used and by shortening the incubation time. The presence of fetal calf serum or BSA during the labeling incubation should reduce non-specific binding of substrate to surfaces.

Signal Strongly Reduced After Short Time
If the fluorescence signal decreases rapidly, it may be due to instability of the fusion protein. The signal may be stabilized by fixing the cells. Alternatively, try switching the ACP- or MCP-tag from the N-terminus to the C-terminus or vice versa. Photobleaching is generally not a problem as the CoA 547 substrate is very photostable. However, if problems with photobleaching are experienced, addition of a commercially available anti-fade reagent may be helpful.

Instructions for Labeling of Proteins in vitro:

1. Dissolve the vial of CoA 547 substrate (50 nmol) in 50 µl of DMSO to yield a stock solution of 1 mM CoA substrate. Mix by vortexing for 10 minutes until all the CoA substrate is dissolved. Dilute this 1 mM stock solution 1:4 in fresh DMSO to yield a 250 µM stock for labeling proteins in vitro.

2. Set up the reactions, in order, as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>28.25 µl</td>
<td></td>
</tr>
<tr>
<td>1 M HEPES</td>
<td>2.5 µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>50 mM DTT</td>
<td>1 µl</td>
<td>1 mM</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>10 µl</td>
<td>10 mM</td>
</tr>
<tr>
<td>50 µM ACP-tag</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>Purified Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µM ACP</td>
<td>1.25 µl</td>
<td>1 µM</td>
</tr>
<tr>
<td>Synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 µM CoA</td>
<td>2 µl</td>
<td>10 µM</td>
</tr>
<tr>
<td>Substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>50 µl</td>
<td></td>
</tr>
</tbody>
</table>

3. Incubate in the dark for 60 minutes at 37°C.

4. Run sample on an SDS-PAGE gel and detect using a fluorescent gel scanner or store samples at −20°C or −80°C in the dark.

Removal of Unreacted Substrate (optional)
After the labeling reaction, the unreacted substrate can be separated from the labeled CoA fusion protein by gel filtration or dialysis. Please refer to the vendor’s instructions for the separation tools used.

Notes for Labeling in vitro
We recommend the routine addition of 1 mM DTT to all buffers used for handling, labeling and storage of the ACP- or MCP-tag. The stability of the ACP- or MCP-tag is improved in the presence of reducing agents; however it can also be labeled in their absence, if handling at temperatures above 4°C is minimized.

ACP- or MCP-tag fusion proteins can be purified before labeling, but the labeling reaction also works in non-purified protein solutions (including cell lysates).

Troubleshooting for Labeling in vitro

Solubility
If solubility problems occur with the ACP- or MCP-tag fusion protein, we recommend testing a range of pH (pH 5.0–pH 10.0) and ionic strengths. The salt concentration may also need to be optimized for the particular fusion protein (50–250 mM).

Loss of Protein Due to Aggregation or Sticking to Tube
If stickiness of the fusion protein is a problem, we recommend adding Tween 20 at a final concentration of 0.05% to 0.1%. The ACP-tag/MCP-tag activity is not affected by this concentration of Tween 20.

Incomplete Labeling
If exhaustive labeling of a protein sample is not achieved using the recommended conditions, try the following protocol modifications: Increase the incubation time to two hours total at 25°C or to 24 hours at 4°C; or halve the volume of protein solution labeled. Both approaches may be combined.

If the ACP- or MCP-tag fusion has been stored in the absence of DTT or other reducing agent, or has been stored at 4°C for a prolonged period, its activity may be compromised. Include 1 mM DTT in all solutions of the ACP- or MCP-tag fusion protein, and store the fusion protein at −20°C.

Using less than the recommended amount of substrate stock solution can significantly slow down the reaction rate.

Loss of Activity of Protein of Interest
If the fusion protein is particularly sensitive to degradation or to loss of activity, try reducing the labeling time or decreasing the labeling temperature. We recommend overnight incubation when labeling at 4°C.

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