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Protocol for quantification of the lysosomal degradation of extracellular proteins into mammalian cells

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https://doi.org/10.1016/j.xpro.2021.100975

SUMMARY
Endocytic internalization of extracellular proteins plays roles in signaling, nutrient uptake, immunity, and extracellular protein quality control. However, there are few protocols for analyzing the lysosomal degradation of extracellular protein. Here, we purified secreted proteins fused with pH-sensitive GFP and acid- and protease-resistant RFP from mammalian cells and describe an internalization assay for mammalian cells. This protocol enables quantification of cellular uptake and lysosomal degradation of protein-of-interest (POI) via cell biological and biochemical analyses.
For full details on the use and execution of this protocol, please refer to Itakura et al. (2020).

BEFORE YOU BEGIN
To detect lysosomal degradation of extracellular proteins, we developed a protocol for an internalization assay using His-GFP-RFP-POI. GFP (superfolder GFP (sfGFP)) is pH-sensitive, whereas RFP (mCherry) is acid-resistant and protease-resistant. Because the lysosome accumulates RFP, but not GFP, an increase in the RFP/GFP ratio is indicative of lysosomal degradation of the His-GFP-RFP-POI. In this protocol, we describe an internalization assay of the extracellular chaperone, clusterin, which interacts with misfolded protein and is internalized for lysosomal degradation. Clusterin-RFP-GFP must be purified from mammalian cells, because clusterin is a secreted protein modified by glycosylation, disulfide bonds, and heterodimerization of two subunits. If POI is cytosolic or does not have modifications, GFP-RFP-POI can be purified from bacteria by the standard method using tag (e.g., His-Tag purification).

Preparation buffer

© Timing: 1 h

1. Prepare buffers and culture medium (see "Materials and equipment").

Generation of Flp-In T-REx 293 cells stably expressing POI (Clusterin)-RFP-GFP-(6x) His

© Timing: 1 month

Note: In this protocol, the Flp-in system, which introduces a Flp recombination target site in the pcDNA5 FRT TO vector into the genome of Flp-in 293 cells, is used to generate a stable
cell line (Itakura et al., 2017). This system ensures stable high-level expression of even large proteins. As well as the Flp-in system, virus vector and other expression systems including transient transfection also be suitable, as long as the POI is highly overexpressed.

**Note:** The T-REx system (Tet-on inducible system) is not required for the expression of POI-GFP-RFP, but is useful if overexpression of POI is cytotoxic.

2. Subclone POI-RFP-GFP-6xHis or SS (signal sequence)-6xHis-RFP-GFP-POI into the pcDNA5 FRT TO vector. In this protocol, we use Clusterin-RFP-GFP-6xHis (Clusterin-RG-His).

△ **CRITICAL:** If the POI is a secreted protein, the signal sequence for the POI should be replaced with one for the N-terminus region of 6xHis-RFP-GFP-POI.

**Note:** If there is no information on the structure or epitope tagging of the POI, examining both POI-RFP-GFP and RFP-GFP-POI is recommended.

**Note:** mCherry, an acid- and protease-resistant fluorescent protein, is used as an RFP (Katayama et al., 2008). Because sfGFP is pH-sensitive, monomeric, resistant to disulfide bond formation, and lacks N-linked glycosylation sites (Costantini and Snapp, 2013), we recommend mCherry and sfGFP for fusion to secreted proteins.

3. Seed Flp-In T-REx 293 cells into a 12-well plate at ~2 × 10^5 cells per well. Culture cells for 1 day to 30–50% confluence, and cotransfect cells with the pcDNA5 FRT TO Clusterin-RG-His and pOG44 vectors.
   a. Mix the reagents as follows and incubate for more than 5 min at 20°C.

   | PEI | 5 μL |
   | Opti-MEM | 120 μL |
   | Total | 125 μL |

   b. Mix the reagents as follows and combine the DNA solution with the PEI solution.

   | pcDNA5 FRT TO Clusterin-RG-His | 1 μg |
   | pOG44 | 1 μg |
   | Opti-MEM | 125 μL |
   | Total | 125 μL |

c. Incubate for 30 min at 20°C.
d. Remove the culture medium and replace with fresh medium without penicillin-streptomycin.
e. Add 250 μL of the mixture to well containing cells and culture the cells at 37°C in 5% CO2.
f. Replace with fresh medium after 4–12 h.

4. Culture the cells for 3–5 days.
5. Replace with fresh medium containing 100 μg/μL hygromycin and culture for more than 2 weeks to remove non-stable cells.

**Note:** After antibiotic selection, polyclonal cells efficiently express the POI without single cell cloning. For detailed information on the Flp-In T-Rex cell line, refer to the manufacturer’s manual (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/flpintrexcells_man.pdf).
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-RFP | MBL    | Cat # M204-3 |
| Mouse monoclonal anti-α tubulin | FUJIFILM | Cat # 071-25031 |
| Horse Anti-mouse IgG, HRP-linked Antibody | Cell Signaling Technology | Cat # 7076S |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Blasticidin         | Wako   | Cat # 029-18701 |
| Hygromycin B        | Nacalai Tesque | Cat # 07296-11 |
| Polyethylenimine (PEI) | Polysciences | Cat # 24765-2 |
| Opti-MEM            | Gibco  | Cat # 31985070 |
| DMEM                | Nacalai Tesque | Cat # 08459-35 |
| Advanced DMEM/F-12  | Gibco  | Cat # 12634010 |
| Fetal bovine serum  | MPbio  | Cat # 2917354H |
| Penicillin-streptomycin | Nacalai Tesque | Cat # 09367-34 |
| Doxycycline         | Clontech | Cat # Z1311N |
| Imidazole           | FUJIFILM | Cat # 099-00013 |
| Ni-NTA Agarose HP   | FUJIFILM | Cat # 141-09683 |
| 2.5 g/L Trypsin, 1 mmol/L EDTA Solution | Nacalai Tesque | Cat # 32777-15 |
| **Experimental models: Cell lines** |        |            |
| Human: Flp-in T-Rex | Thermo Fisher Scientific | Cat # R78007 |
| HEK293 cell line    | Thermo Fisher Scientific | Cat # R70007 |
| Human: 293FT cells  | Thermo Fisher Scientific | Cat # R70007 |
| **Recombinant DNA** |        |            |
| Plasmid: pcDNAs/PRT/TO Vector Kit | Thermo Fisher Scientific | Cat # V652020 |
| Plasmid: pOG44 Flp-Recombinase Expression Vector | Thermo Fisher Scientific | Cat # V600520 |
| Plasmid: pHK-scFv-GCN4-sGFP-GB1-NLS-dWPRE | Addgene | Cat # 60906 |
| Plasmid: mCherry-ER-3 | Addgene | Cat # 55041 |
| **Software and algorithms** |        |            |
| CytoExpert 2.4      | BECKMAN COULTER | https://www.beckman.jp/flow-cytometry/instruments/cytoflex/software |
| **Other**           |        |            |
| Gravity flow column | Bio-Rad Laboratories | Cat # 7370712B02 |
| Microsep Advance Centrifugal Devices with Omega Membrane 30 K | PALL | Cat # MCP030CA46 |
| 5 mL round-bottom tube | Falcon | Cat # 352058 |
| Cell strainer 70 μm | Greiner | Cat # 542070 |
| Flow cytometer (CytoFLEX S) equipped with 405, 488, 561 nm lasers. | BECKMAN COULTER | N/A |
## MATERIALS AND EQUIPMENT

### Column wash buffer 1

| Reagent               | Final concentration | Amount  |
|-----------------------|---------------------|---------|
| 5M NaCl               | 300 mM              | 30 mL   |
| 1M Imidazole          | 10 mM               | 5 mL    |
| 25 x PBS              | 1 x                 | 20 mL   |
| ddH₂O                 | n/a                 | 445 mL  |
| **Total**             | n/a                 | 500 mL  |

Store at 4°C.

### Column wash buffer 2

| Reagent               | Final concentration | Amount  |
|-----------------------|---------------------|---------|
| 5M NaCl               | 500 mM              | 50 mL   |
| 1M Imidazole          | 10 mM               | 5 mL    |
| 25 x PBS              | 1 x                 | 20 mL   |
| ddH₂O                 | n/a                 | 425 mL  |
| **Total**             | n/a                 | 500 mL  |

Store at 4°C.

### Column elution buffer

| Reagent               | Final concentration | Amount  |
|-----------------------|---------------------|---------|
| 5M NaCl               | 150 mM              | 15 mL   |
| 1M Imidazole          | 200 mM              | 100 mL  |
| 25 x PBS              | 1 x                 | 20 mL   |
| ddH₂O                 | n/a                 | 365 mL  |
| **Total**             | n/a                 | 500 mL  |

Store at 4°C.

### DMEM complete medium

| Reagent               | Final concentration | Amount  |
|-----------------------|---------------------|---------|
| DMEM                  | n/a                 | 500 mL  |
| FBS                   | 10%                 | 56 mL   |
| penicillin-streptomycin | 1%                 | 5.6 mL  |
| **Total**             | n/a                 | 561.6 mL|

Store at 4°C.

### Advanced DMEM complete medium

| Reagent               | Final concentration | Amount  |
|-----------------------|---------------------|---------|
| Advanced DMEM         | n/a                 | 500 mL  |
| 200mmol/L L-Alanyl-L-glutamine Solution | 2 mmol/L. | 5 mL |
| **Total**             | n/a                 | 50 mL   |

Store at 4°C.
### Trypsin solution

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| 2.5 g/L Trypsin, 1 mmol/L EDTA Solution | 0.5 g/L Trypsin, 0.2 mmol/L EDTA | 5 mL   |
| PBS                            | n/a                 | 20 mL  |
| **Total**                      | **n/a**             | **25 mL** |

Store at 4°C.

### FACS buffer

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| Newborn serum (or fetal bovine serum) | 5%                  | 250 μL |
| 1 mg/mL DAPI                     | 1 μg/mL             | 5 μL   |
| 1 × PBS                          | n/a                 | 4.745 mL |
| **Total**                        | **n/a**             | **5 mL** |

On ice. Prepare immediately before use.

### 10 × Lysis Buffer

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| 1M Tris-HCl pH 7.5             | 500 mM              | 50 mL  |
| 5M NaCl                         | 1500 mM             | 30 mL  |
| 0.5M EDTA pH 8.0                | 10 mM               | 2 mL   |
| Triton X-100                    | 10%                 | 10 mL  |
| ddH₂O                           | n/a                 | 8 mL   |
| **Total**                       | **n/a**             | **100 mL** |

Store at 4°C.

### Lysis Buffer

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| 10 × Lysis Buffer              | 1 ×                 | 100 μL |
| 100 × protease inhibitor cocktail | 1 ×              | 10 μL  |
| 0.1M PMSF                      | 1 mM                | 10 μL  |
| ddH₂O                           | n/a                 | 880 μL |
| **Total**                      | **n/a**             | **1000 μL** |

On ice. Prepare immediately before use.

### Other solutions

| Name                           | Reagents                                                                 |
|--------------------------------|--------------------------------------------------------------------------|
| PBS                            | 137 mM NaCl, 2.7 mM KCl, 7.7 mM Na₂HPO₄, 1.47 mM KH₂PO₄                  |
|                                | **Store at 20°C**                                                        |
| 1M Imidazole                   | 1 M Imidazole in ddH₂O, pH adjusted to 8.0 using HCl                     |
|                                | **Store at 20°C**                                                        |
| 6 × Sample buffer              | 0.28 M Tris-HCl pH 6.8, 10% SDS, 30% Glycerol, 0.03% bromophenol blue, 0.93% Dithiothreitol |
|                                | **Store at ~20°C**                                                      |
| 2 × Sample buffer              | 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 0.01% bromophenol blue, 10% 2-Mercaptoethanol |
|                                | **On ice. Prepare immediately before use.**                             |
STEP-BY-STEP METHOD DETAILS

Collection of conditioned medium of mammalian cells containing secreted POI (clusterin)-RFP-GFP-His protein

🎯 Timing: 6 days

Collect the secreted POI-RFP-GFP-His protein from conditioned medium. Two hundred milliliters of conditioned medium (from 20 100-mm culture dishes) yields about 1 mg of purified protein.

1. Culture Flp-in T-REx HEK293 cells stably expressing clusterin-RFP-GFP-His (~1.5 × 10^6 cells per dish) in 20 100-mm culture dishes with complete DMEM containing 0.1 mg/mL doxycycline at 37°C in 5% CO2 for 2 days.

2. When the cells reach 70–80% confluence (~6 × 10^6 cells per dish), exchange the medium for advanced DMEM medium with 0.1 mg/mL doxycycline.

⚠️ CRITICAL: Do not add FBS to advanced DMEM, because this decreases the purity of the purified protein.

a. Remove the culture medium and wash the cells with 3 mL of PBS per 100-mm dish.
b. Add 10 mL of advanced DMEM medium with doxycycline to each 100-mm dish and culture for 4 days.

3. Collect the conditioned medium

a. Transfer the conditioned medium into 50 mL conical tubes.
b. Centrifuge the 50 mL tubes at 780 × g for 20 min at 4°C to remove dead cells and debris.
c. Transfer supernatant to a 200 mL bottle (any freezable bottle or tube) and store at −80°C until purification.

⚠️ CRITICAL: To prevent photobleaching, samples should be shielded from light in subsequent experiments.

⚠️ Pause point: The collected medium can be stored at −80°C for a few years.

Purification of secreted POI (clusterin)-RFP-GFP-His protein from conditioned medium of mammalian cells

🎯 Timing: 10 h

Purify the fusion protein secreted by the stable cell line from the conditioned medium by Ni-NTA affinity chromatography.

4. Purify secreted clusterin-RFP-GFP-His protein by Ni-NTA affinity chromatography.

a. Transfer 1 mL (bed volume) of Ni-NTA agarose resin into a gravity-flow column.
b. Equilibrate the Ni-NTA agarose resin with 10 mL of column wash buffer 1.
c. Add 10 mM imidazole to the supernatant (containing POI) to reduce non-specific binding of protein to resin.

Optional: Retain 20 μL of the supernatant for the analysis by SDS-PAGE.

d. Load the supernatant onto the column.

Optional: Retain 20 μL of the flowthrough for the analysis by SDS-PAGE.
e. Wash the resin four times with 10 mL of column wash buffer 2.

f. Elute the clusterin-RFP-GFP-His protein with column elution buffer and collect each elution fraction into a 1.5 mL tube.
   i. Apply 0.5 mL of elution buffer and collect the eluate into a fresh 1.5 mL tube (Fr.1).
   ii. Apply 1 mL of elution buffer and collect the eluate into a fresh 1.5 mL tube (Fr.2).
   iii. Apply 1 mL of elution buffer and collect the eluate into a fresh 1.5 mL tube (Fr.3).
   iv. Apply 0.5 mL of elution buffer and collect the eluate into a fresh 1.5 mL tube (Fr.4).
   v. Apply 0.5 mL of elution buffer and collect the eluate into a fresh 1.5 mL tube (Fr.5)

Optional: Retain 20 μL of each elution fraction for CBB staining.

Note: Detect the fractions containing protein by measuring the absorbance at 280 nm and pool (fractions 2 and 3 usually contain eluted protein).

5. Remove imidazole from the purified protein by ultrafiltration.

△ CRITICAL: More than 2 mM imidazole in culture medium interferes with lysosomal activity (Liu et al., 2015). Therefore, it is necessary to remove imidazole from purified proteins for the internalization assay.

Optional: Dialysis is also suitable instead of ultrafiltration.

a. Concentrate the purified protein and bring the buffer to a 0.2-mL final volume using the Microsep advance centrifugal (ultrafiltration) device (30 kDa MWCO) at 2,380 × g for 30 min at 4°C.
   b. Discard the flow-through and add 2 mL of cold PBS to the centrifugal device.
   c. Repeat steps a and b at least three times.
   d. Transfer the concentrate to a fresh 1.5 mL tube.
   e. Measure the concentration by determining the OD$_{280}$ and store at −80°C.

Note: 10% glycerol in storage buffer may prevent the aggregation of purified protein during freeze/thaw.

6. Analyze the purification by SDS-PAGE with CBB staining (Figure 1).
   a. Add an equal volume of 2× sample buffer to each fraction and boil at 95°C for 5 min.
   b. Separate the fractions by SDS-PAGE and stain with CBB-R250.

Flow-cytometric analysis of the internalization of GFP-RFP-labeled protein

© Timing: 3 days

This step describes measurement of lysosomal degradation of extracellular clusterin-RFP-GFP-His with misfolded proteins in mammalian cells based on the green and red fluorescence intensities.

7. On day 1, passage HEK293 cells (or the desired cell line for internalization of the POI) and seed at ~5 × 10^4 cells per well in a 24-well plate with complete DMEM.

8. On day 2, cells should be 40%–60% confluent (~1 × 10^5 cells per well). Culture cells with or without clusterin-RFP-GFP-His and unfolded protein substrate.
   a. Add 0.06 μM Clusterin-RFP-GFP-His protein with or without 2.2 μM luciferase to 250 μL advanced DMEM in a 1.5 mL tube.

Note: For the internalization of other POIs, it may be necessary to optimize the concentration of POI and composition of culture medium.
b. To denature the substrate (luciferase), incubate advanced DMEM with or without luciferase and clusterin-RFP-GFP-His protein at 42°C for 20 min.

c. Wash HEK293 cells with serum-free DMEM and exchange the medium for advanced DMEM treated as in b, and culture for 14 h in a humidified atmosphere with 5% CO2. If HEK293 cells
are aggregated after medium exchange, dissociate the cells by pipetting so as not to interfere with internalization efficiency.

**Note:** Contamination by FBS might affect the internalization of clusterin.

**Note:** Culture time is dependent on internalization efficiency.

9. On day 3, collect the cells and analyze by flow cytometry.
   a. Wash the cells with PBS.
   b. Discard the PBS, add 100 µL of trypsin solution, and incubate at 37°C for 5 min.
   c. Add 200 µL of FACS buffer and suspend the cells by pipetting.

**Note:** Bovine serum in FACS buffer is used for trypsin inhibition.

d. Pass the resuspended cells through a cell strainer and transfer the cells to a 5 mL round-bottom tube.
e. Set up a flow cytometer. Place the 5 mL tube on the sample collector and run the samples.
   i. Select a cell population and exclude debris by setting the cell size and granularity parameters (forward-scatter area [FSC-A] vs. side-scatter area [SSC-A]).
   ii. Exclude duplet cells (side-scatter height [SSC-H] vs. side-scatter width [SSC-W]).
   iii. Exclude dead cells (forward-scatter area [FSC-A] vs. DAPI area [DAPI-A]). DAPI is detected at 405 nm excitation with a 450/45-nm bandpass filter.
   iv. sfGFP is detected at 488 nm excitation with a 525/40-nm bandpass filter. mCherry is detected at 561 nm excitation with a 585/42-nm bandpass filter.

**Immunoblot analysis of the internalization of GFP-RFP labeled protein**

© Timing: 4–5 days

This step describes measurement of the lysosomal degradation of extracellular Clusterin-RFP-GFP-His with misfolded protein in mammalian culture cells by immunoblot of RFP, which is acid- and protease-resistant.

10. On day 1, passage HEK293 cells (or the desired cell line for internalization of the POI) and seed at 20–40% confluence in a 12-well plate with complete DMEM.

11. On day 2, cells should be 40–60% confluent. Culture cells with or without clusterin-RFP-GFP-His and unfolded protein substrate.

**Note:** Treat as in step 8.

a. Add 0.06 µM clusterin-RFP-GFP-His protein with or without 2.2 µM luciferase to 500 µL of advanced DMEM in a 1.5 mL tube.
b. To denature the substrate (luciferase), incubate advanced DMEM with or without luciferase and clusterin-RFP-GFP-His protein at 42°C for 20 min.
c. Wash HEK293 cells with serum-free DMEM and exchange the medium for advanced DMEM treated as in b, and culture for 14 h in a humidified atmosphere with 5% CO₂.

12. On day 3, lyse the cells and perform immunoblotting.
   a. Wash the cells with cold PBS.
   b. Discard the PBS and add 500 µL of cold PBS.
   c. Scrape and transfer the cells to a fresh 1.5 mL tube.
   d. Centrifuge at 2,380 × g for 2 min at 4°C.
e. Discard the supernatant and lyse the cells with 50 μL of lysis buffer.

f. Incubate the lysate on ice for 15 min.

g. Centrifuge the lysate at 20,620 × g for 5 min at 4°C.

h. Transfer the supernatant to a fresh 1.5 mL tube, and prepare for immunoblotting
   i. Measure the protein concentration of the supernatant by Bradford assay (or another stan-
   dard method).
   ii. Mix 40 μL of the supernatant with 10 μL of 6× sample buffer and boil for 5 min at 95°C.
   iii. Load 10–20 μg of protein per lane and perform SDS-PAGE and immunoblotting using stan-
   dard procedures with a monoclonal anti-RFP antibody (1:1,000 dilution) and monoclonal
   anti-α-tubulin antibody (1:1,000 dilution) together with an anti-mouse IgG (1:2,000 dilution).

Optional: If needed, lysosomal degradation of POI can be examined using anti-POI antibody.

EXPECTED OUTCOMES

Analysis of the internalization of clusterin-RFP-GFP by flow cytometry
Mammalian lysosomes contain a variety of acid hydrolases and degrade proteins, including GFP. RFPs (mCherry) derived from sea anemone are resistant to acidic pH and lysosomal proteases (Katayama et al., 2008). Consequently, transport of POI-RFP-GFP into lysosomes leads to the accumulation of RFP, but GFP is degraded or quenched, in cells. Therefore, increased RFP indicates increased lysosomal internalization of POI. Increased RFP and GFP indicates cell-surface or endosomal POI accumulation, which are associated with lysosomal inhibition. Flow cytometry enables measurement of the fluorescence intensities of RFP and GFP, and quantification of the amount of internalized POI.

The protocol describes the treatment of cells for assay of the internalization of clusterin-RFP-GFP-His. Flow cytometry showed that the RFP, but not GFP, signal in a cell treated with clusterin-RFP-GFP-His and heat-denatured luciferase was markedly elevated compared to a cell treated with clusterin-RFP-
GFP-His alone (Figure 2). Note that treatment with Clusterin-RFP-GFP-His alone increased the RFP signal, indicating that extracellular proteins are constitutively internalized by fluid-phase endocytosis. The elevated RFP signal was suppressed by bafilomycin A₁, which disrupts the lysosome and endocytosis.

Analysis of the internalization of clusterin-RFP-GFP-His by immunoblotting
By exploiting the protease-resistant properties of RFP, internalization assay of POI-RFP-GFP can be applied to biochemical analysis using immunoblot. In lysosomes, lysosomal proteases digest clusterin and GFP, but not RFP. Consequently, increased uptake of clusterin-RFP-GFP leads to an accumulation of cleaved RFP, which can be detected by immunoblotting. Flow cytometry enables quantification of the RFP signal, whereas an increased RFP signal does not always indicate lysosomal accumulation of RFP. Detection of cleaved RFP by immunoblotting ensures that POI-RFP-GFP was internalized by lysosomes.

Treatment of Clusterin-RFP-GFP-His increased the cleaved RFP level in the cells (Figure 3), which was further increased by cotreatment with luciferase. By contrast, lysosomal inhibition by bafilomycin A₁ completely suppressed cleaved RFP, and increased full-length clusterin-RFP-GFP-His. These results suggested that clusterin-RFP-GFP-His is internalized in lysosomes.

LIMITATIONS
Under conditions of lysosomal inhibition, flow-cytometric analysis cannot determine whether POI-RFP-GFP is localized on the cell membrane or has been taken up into the cell. Fluorescent microscopy is needed to determine the localization of POI-RFP-GFP on the cell surface or in endosomes.
Random conjugations of fluorescent molecules to amino acid residues of the POI might interfere with its functions. In addition, detection of fluorescence does not distinguish between the cell surface and endosomes, and fluorescent molecules are costly. In this protocol, we performed N- or C-terminal fusion of tandem fluorescent proteins, which did not affect the structure of a majority of proteins, to detect an acidic environment. However, tandem fluorescent proteins are large, which may affect structure and/or function. If there is no information on the structure or epitope tagging of the POI, trying both POI-RFP-GFP and RFP-GFP-POI is recommended.

**TROUBLESHOOTING**

**Problem 1**
The fluorescent protein-fused POI is not detected in conditioned medium (step 1–3).

**Potential solution 1**
First, check that the signal sequence is correctly introduced into the plasmid. Second, check that the fluorescent protein-fused POI is expressed by immunoblotting or fluorescence microscopy. If the POI is a tissue-specific gene, a tissue-specific cell line is needed for its expression.

**Problem 2**
In the step of collection of conditioned medium containing POI, cells expressing the POI died during 4 days of culture in advanced DMEM (step 2).

**Potential solution 2**
Collect conditioned medium within 3 days after culture in advanced DMEM.

**Problem 3**
Incorporation of POI is less efficient (step 4).

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_Figure 3. Analysis of the internalization of clusterin-RFP-GFP by immunoblotting._
Purified clusterin-RFP-GFP-His was added with or without recombinant luciferase to advanced DMEM and preincubated at 4°C or 42°C (heat stress) for 20 min. HEK293 cells were cultured in medium with or without bafilomycin A₁ for 18 h at 37°C and analyzed by immunoblotting. Asterisks indicate non-specific bands.
Potential solution 3
Reconsider the incubation duration and POI concentration in cell culture medium. Cell number during internalization is also affected by uptake efficiency. Because the uptake of each protein might differ among cell lines, use other cell lines that can take up the POI.

Problem 4
Lysosomal function is inhibited during culture with purified fusion protein (step 8–9, 11–12).

Potential solution 4
More than 2 mM imidazole in culture medium inhibits lysosomes (Liu et al., 2015). Remove imidazole from purified protein solution by ultrafiltration or dialysis.

Problem 5
For analyzing fluorescent protein fusion proteins in the presence of a compound, the fluorescence intensity changed without addition of the purified fluorescent protein-fused POI (step 9).

Potential solution 5
Some compounds exhibit autofluorescence. Include a negative control (cells treated with compound only) for flow cytometry.

Problem 6
Cleaved RFP is not detected by immunoblotting (step 12).

Potential solution 6
Add more POI-RFP-GFP. Some monoclonal anti-RFP antibodies do not recognize cleaved RFP. The monoclonal anti-RFP antibody (MBL, Cat #M204-3) used in this protocol or polyclonal anti-RFP antibodies for immunoblotting are recommended.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Eisuke Itakura (eitakura@chiba-u.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any new datasets or code.

ACKNOWLEDGMENTS
This work was supported by JSPS KAKENHI (grant numbers 19K22413, 20H03249, and 20H05312 to E.I.), JST FOREST (grant number 20351166 to E.I.), the Takeda Science Foundation (to E.I.), the Terumo Life Science Foundation (to E.I.), and the Hamaguchi Foundation for the Advancement of Biochemistry (to E.I.).

AUTHOR CONTRIBUTIONS
All authors contributed to the conceptualization and editing of the manuscript. M.C. performed the experiment and generated the figures. A.T. and E.I. contributed to the writing and generation of tables.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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