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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.

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Highlights

A novel method to quantify lysosomal degradation using a GFP-RFP-fused POI

Purification of GFP-RFP fused proteins from conditioned medium of mammalian cells

Flow cytometry enables cell biological analysis of the internalization of POI

Immunoblotting enables biochemical analysis of the lysosomal degradation of POI

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

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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% CO_2.
   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 (FBS)	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
DAPI	Nacalai Tesque	Cat # 11034-56
Newborn calf serum	Hyclone	Cat # SH30401.01
Benzylsulfinyl Fluoride (PMSF)	FUJIFILM	Cat # 022-15371
L-Alanyl-L-glutamine Solution	Nacalai Tesque	Cat # 04260-64
Bafilomycin A_1	LC Laboratories	Cat # B-1080
Recombinant luciferase	Promega	Cat # E1702
Signal Enhancer HIKARI for Western Blotting and ELISA	Nacalai Tesque	Cat # 02270-81
Experimental models: Cell lines		
Human: Flp-in T-Rex HEK293 cell line	Thermo Fisher Scientific	Cat # R78007
Human: 293FT cells	Thermo Fisher Scientific	Cat # R70007
Recombinant DNA		
Plasmid: pcDNA5/FRT/TO Vector Kit	Thermo Fisher Scientific	Cat # V652020
Plasmid: pOG44 Flp-Recombinase Expression Vector	Thermo Fisher Scientific	Cat # V600520
Plasmid: pHR-scFv-GCN4-sfGFP-GB1-NLS-dWPRE	Addgene	Cat # 60906
Plasmid: mCherry-ER-3	Addgene	Cat # 55041
Software and algorithms		
CytExpert 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 # MCP030C46
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 × PBS	1 ×	20 mL
ddH_2O	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 × PBS	1 ×	20 mL
ddH_2O	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 × PBS	1 ×	20 mL
ddH_2O	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
ddH_2O	n/a	8 mL
Total	**n/a**	**100 mL**
Store at 20°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
ddH_2O	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 Na_2HPO_4, 1.47 mM KH_2PO_4 Store at 20°C
1M Imidazole	1 M Imidazole in ddH_2O, 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% CO_2 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 Micro-sep 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.

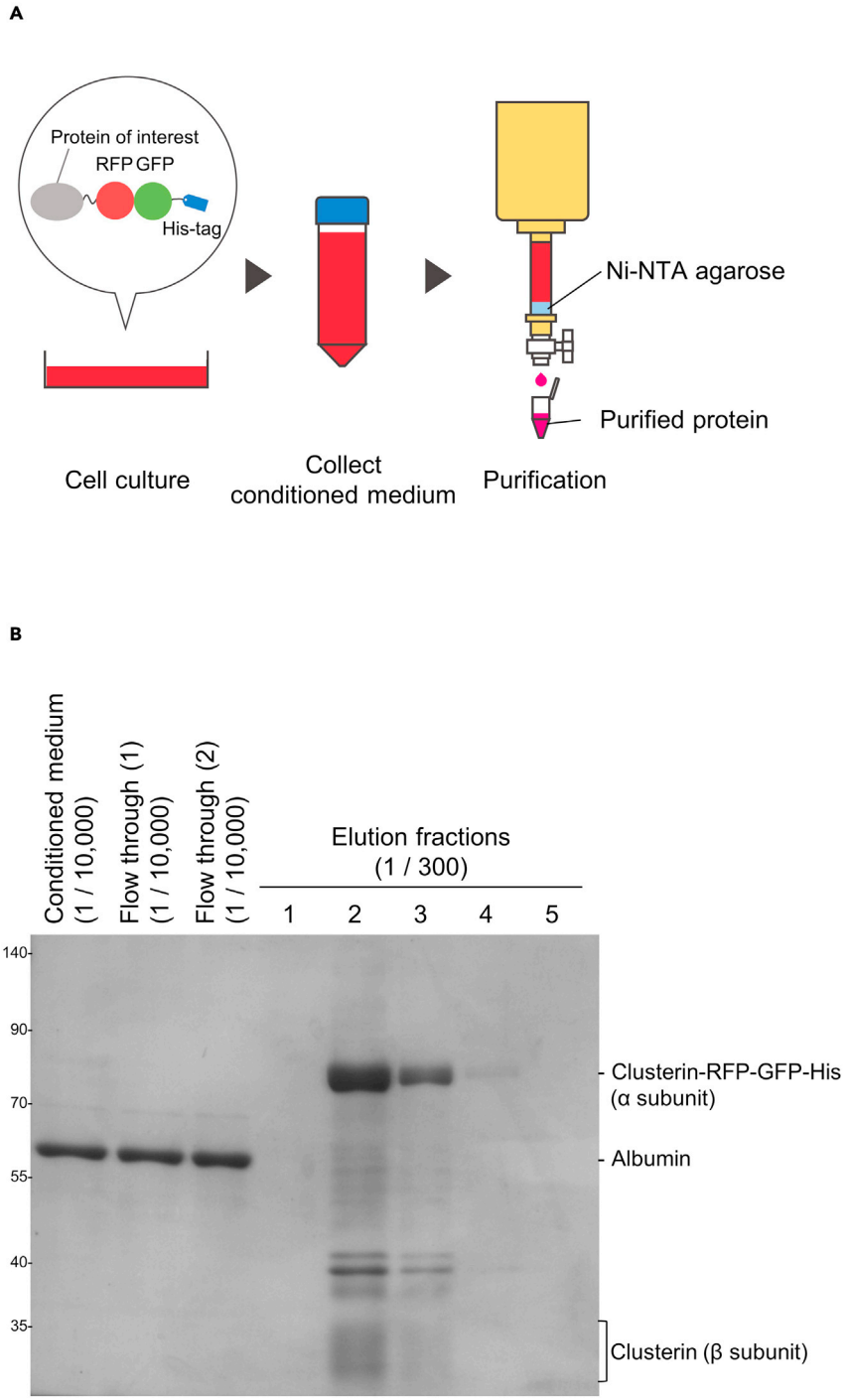


Figure 1. Purification of clusterin-RFP-GFP-His protein from conditioned medium of mammalian cells

(A) Schematic of the purification of secreted protein from conditioned medium.

(B) Coomassie brilliant blue (CBB) staining of clusterin-RFP-GFP-His. Samples were subjected to SDS-PAGE and CBB staining.

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% CO_2. 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.

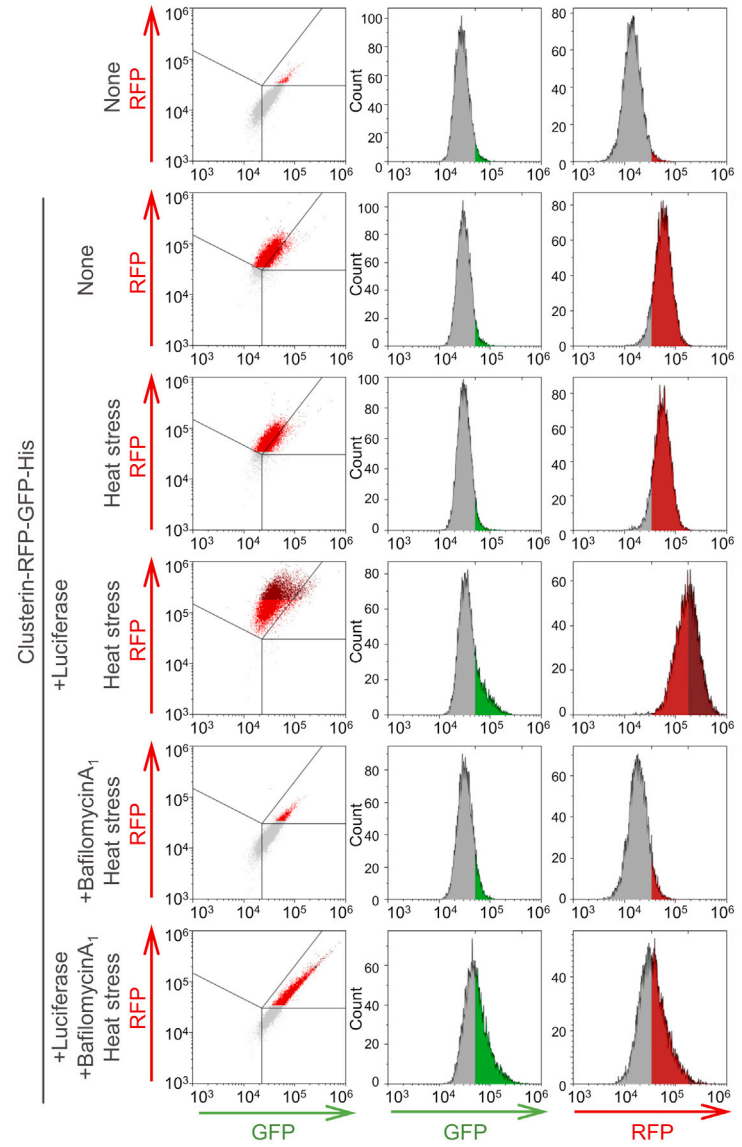
10. On day 1, passage HEK293 cells (or the desired cell line for internalization of the POI) and seed at 20–40% confluence in a 12-well plate with complete DMEM.

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% CO_2.

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.


Clusterin-RFP-GFP-His
+Luciferase
+Luciferase +BafilomycinA1 Heat stress
+BafilomycinA1 Heat stress
Heat stress
None
RFP
Count
GFP
GFP
RFP

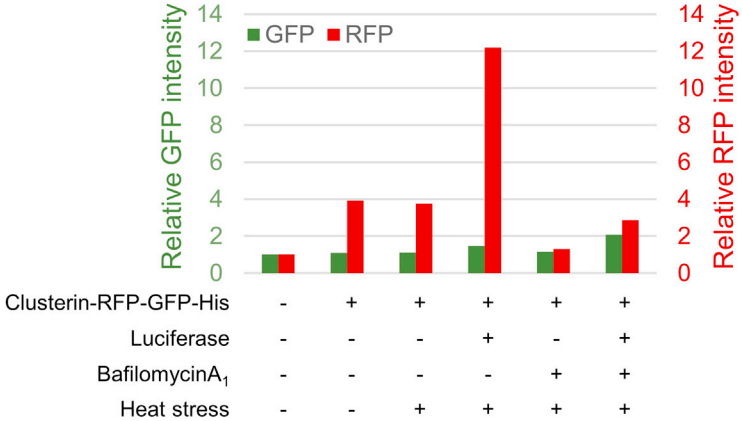

Relative GFP intensity
Relative RFP intensity
GFP
RFP
Clusterin-RFP-GFP-His - + + + + +
Luciferase - - - + - +
BafilomycinA1 - - - - + +
Heat stress - - + + + +

Figure 2. Analysis of the internalization of clusterin-RFP-GFP-His by flow cytometry

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 A_1 for 18 h at 37°C and analyzed by flow cytometry.

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 standard method).
    ii. Mix 40 μL of the supernatant with 10 μL of 6× sample buffer and boil for 5 min at 95°C.
i. Load 10–20 μg of protein per lane and perform SDS-PAGE and immunoblotting using standard 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 A_1, 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 A_1 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.

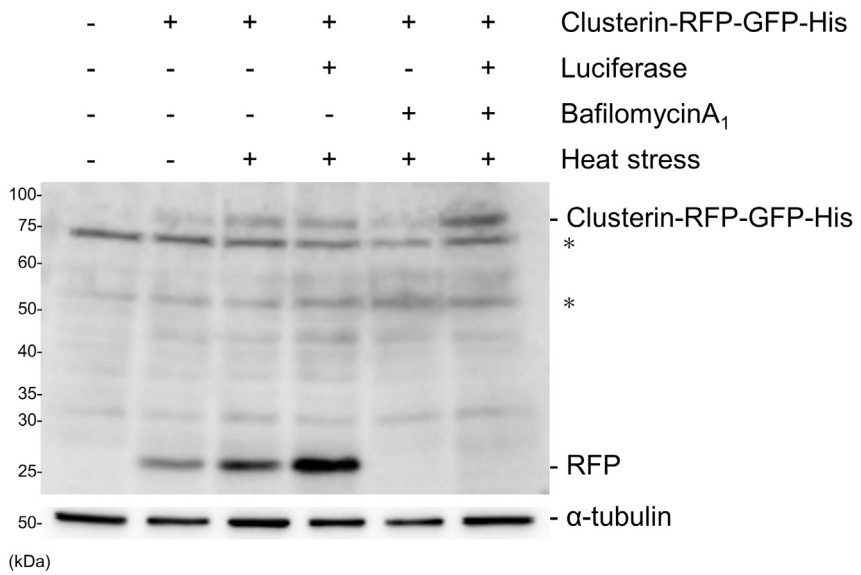


Figure 3. Analysis of the internalization of clusterin-RFP-GFP by immunoblotting.

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 A_1 for 18 h at 37°C and analyzed by immunoblotting. Asterisks indicate non-specific bands.

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).

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.

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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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