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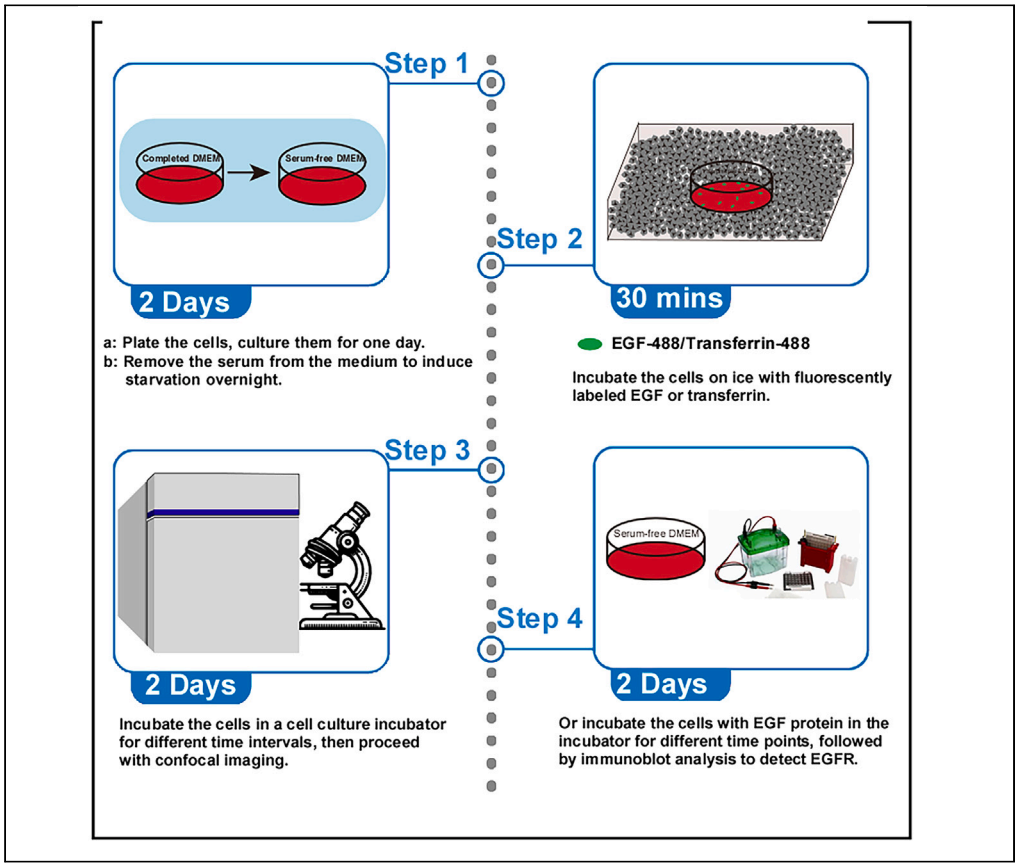
Protocol

Protocol for monitoring the endosomal trafficking of membrane proteins in mammalian cells



Here, we present a protocol to study epidermal growth factor (EGF) receptor (EGFR) or transferrin trafficking in mammalian cells. We describe steps for using fluorescent ligands or antibodies, confocal imaging, and quantitative analysis to track their movement. We detail procedures for cell culture preparation, labeling membrane proteins, optimizing imaging, and isolating cell lysates for the biochemical analysis of EGFR degradation after EGF treatment. This protocol is adaptable to various cell types and for assessing genetic or pharmacological impacts on endosomal trafficking.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Instructions for labeling EGFR or transferrin receptor on the cell surface of live cells

Steps for assessing the endosomal trafficking of membrane proteins

Guidance on assessing the degradation of EGFR after EGF treatment

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Protocol for monitoring the endosomal trafficking of membrane proteins in mammalian cells

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SUMMARY

Here, we present a protocol to study epidermal growth factor (EGF) receptor (EGFR) or transferrin trafficking in mammalian cells. We describe steps for using fluorescent ligands or antibodies, confocal imaging, and quantitative analysis to track their movement. We detail procedures for cell culture preparation, labeling membrane proteins, optimizing imaging, and isolating cell lysates for the biochemical analysis of EGFR degradation after EGF treatment. This protocol is adaptable to various cell types and for assessing genetic or pharmacological impacts on endosomal trafficking.

For complete details on the use and execution of this protocol, please refer to Ye et al.,[1] Ye et al.,[2] and Wang et al.[3]

BEFORE YOU BEGIN

Endosomal trafficking is a critical cellular process that regulates the sorting, distribution, and degradation of various biomolecules, including receptors, lipids, and proteins.[4,5] It is vital in maintaining cellular homeostasis and mediating responses to external stimuli. Given its involvement in a wide range of physiological functions and its dysregulation in numerous diseases, such as neurodegenerative disorders, cancer, and infectious diseases,[6–8] a detailed protocol for studying endosomal trafficking is critical. This protocol provides researchers with the tools and methodologies to accurately monitor and analyze the dynamics of endosomal trafficking of plasma membrane protein(s), e.g., EGFR or transferrin receptor,[9,10] facilitating a deeper understanding of cellular function and the development of therapeutic strategies targeting endosomal pathways.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
EGFR mouse mAb (1:2,000)	Proteintech	CAT#66455-1-1g
EEA1 rabbit mAb (1:2,000)	CST	CAT#C45B10
GAPDH mouse mAb (1:2,000)	Proteintech	CAT#60004-1-1g
Donkey anti-rabbit IgG(H + L) 647(1:1,000)	Invitrogen	CAT#A32795
Goat anti-mouse IgG(H + L)-HRP (1:4,000)	Jackson ImmunoResearch	CAT#115-035-003

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
EGF-biotinylated-488 streptavidin	Invitrogen	CAT#E13345
Transferrin from human serum Alexa Fluor 488 conjugated	Invitrogen	CAT#T13342
Human EGF recombinant protein	Thermo Fisher Scientific	CAT# PHG0313
DMEM	Gibco	CAT#12100-061
BSA	Maclin	CAT#B824162-100g
$NaHCO_3$	Aladdin	CAT#S118600-500g
HEPES	Maclin	CAT#H917470-2.5kg
0.25% trypsin-EDTA	Gibco	CAT#25200-056
PBS	Beyotime	CAT#ST448-1L
NP40	Maclin	CAT#N823211-25g
Tris-HCl	BBI	CAT#A600194-0005
4% PFA	BBI	CAT#E672002-5000
Prolong Gold Antifade with DAPI	CST	CAT#8961S
SDS PAGE	GenScript	CAT#M00663
Protein marker	SMOBIO	CAT#PM2510
Tris-MOPS-SDS buffer	GenScript	CAT#M00138
Transfer buffer	Beyotime	CAT#P0572-2L
PVDF	Millipore	CAT#IPVH00010
Pen strep	Gibco	CAT#15140-122
TBS	Beyotime	CAT#ST667-1L
Tween 20	Aladdin	CAT#T434506-100mL
Protease inhibitor	MCE	CAT#HY-K0010
Software and algorithms		
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij
GraphPad Prism	GraphPad Software	https://www.graphpad.com/

MATERIALS AND EQUIPMENT

Cell culture medium: DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin.

Note: Repeated freezing and thawing are prohibited for FBS and Penicillin/Streptomycin. Pre-warm the media to 37°C before use.

EGFR degradation solution buffer		
Reagent	Final concentration	Amount
BSA	0.2%	0.1 g
HEPES	20 mM	0.238 g
DMEM		Bring to 50 mL

Note: The EGFR degradation solution buffer should be stored at 4°C. Flocculent precipitates indicative of protein denaturation or bacterial contamination suggest solution expiration.

EGF uptake solution buffer		
Reagent	Final concentration	Amount
BSA	2%	1 g
HEPES	20 mM	0.238 g
DMEM		Bring to 50 mL

Note: The EGF uptake solution buffer should be stored at 4°C. Repeated freezing and thawing of EGF is prohibited, and EGF is diluted with buffer on the day of the experiment and used promptly to prevent protein degradation.

Cell lysis buffer (Image)		
Reagent	Final concentration	Amount
NP40	0.1%	0.5 g
PBS		Bring to 500 mL

Note: The cell lysis buffer (Image) should be stored at 4°C. The occurrence of crystal precipitation within the solution could serve as an indication of its degradation beyond the designated shelf life

Cell lysis buffer (western blot)		
Reagent	Final concentration	Amount
NP40	1%	5 g
NaCl	200 mM	5.85 g
Tris-HCl (8.0)	50 mM	3 g
ddH_2O		Bring to 500 mL

Note: The cell lysis buffer should be stored at 4°C and used with protease inhibitors for western blot analysis. The occurrence of crystal precipitation within the solution could serve as an indication of its degradation beyond the designated shelf life.

Blocking buffer (Image)		
Reagent	Final concentration	Amount
BSA	2%	1 g
PBS		Bring to 50 mL

Note: Blocking buffer (Image) should not be stored. Use a freshly prepared.

Blocking buffer (western blot)		
Reagent	Final concentration	Amount
NP40	5%	2.5 g
TBST	Tween-20 (0.1%)	Bring to 50 mL

Note: The blocking buffer (western blot) should not be stored. Use a freshly prepared.

STEP-BY-STEP METHOD DETAILS

EGFR degradation (western blot analysis)

Timing: 4 days

1. Plate healthy HeLa cells into four wells of a six-well plate at a density of 1×10^6 cells per well. (Healthy cells are those that exhibit normal morphology, maintain high viability, proliferate at typical rates, and are free from any contamination.)
   a. Add 2 mL of DMEM supplemented with 10% FBS and 1% PS as the culture medium.
   b. Incubate the cells in a cell culture incubator at 5% CO_2 and 37°C for 24 h.

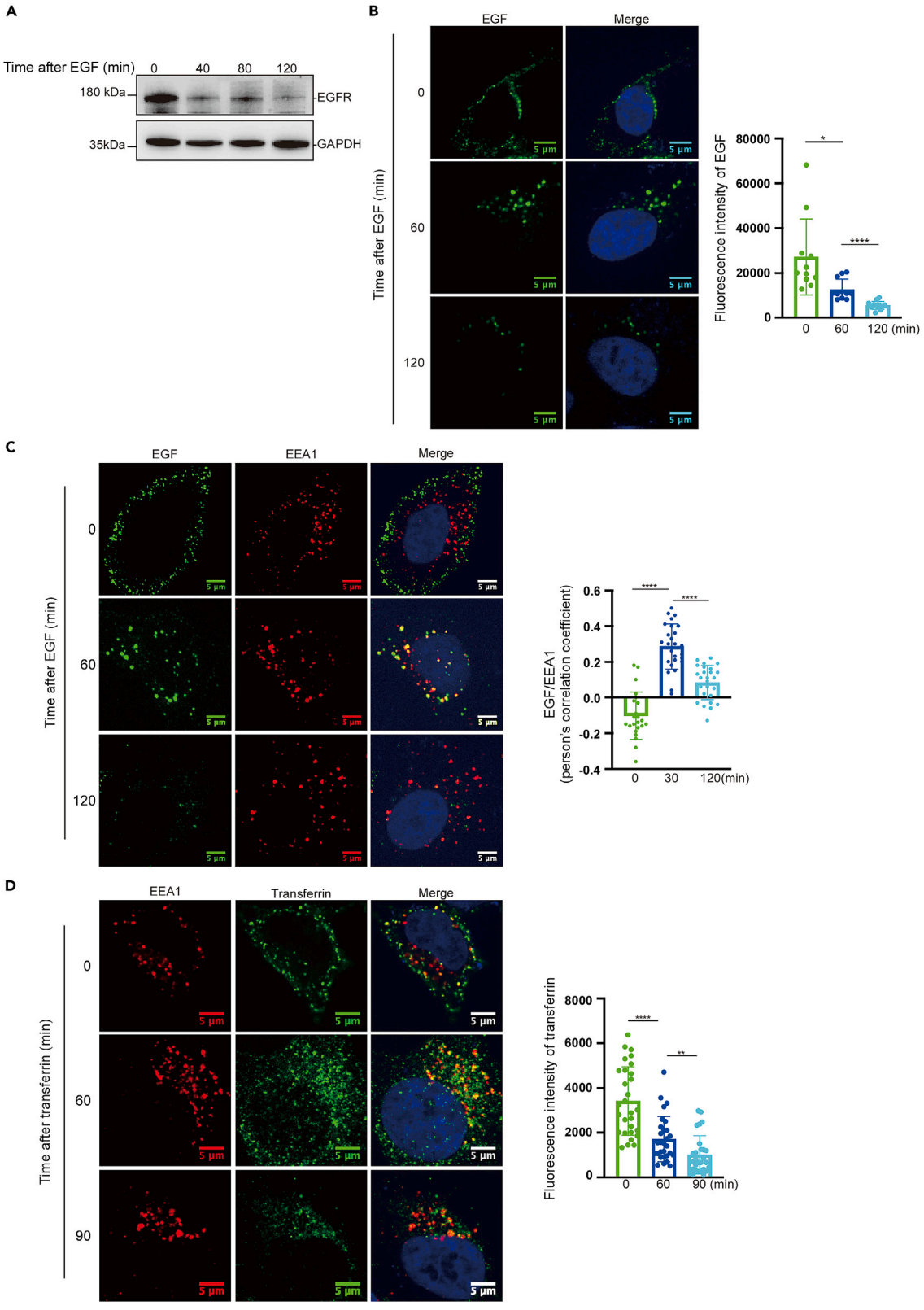
A
Time after EGF (min) 0 40 80 120
180 kDa
EGFR
35kDa
GAPDH
B
EGF
Merge
Time after EGF (min)
0
60
120
5 μm
Fluorescence intensity of EGF
80000
60000
40000
20000
0
*
****
0 60 120 (min)
C
EGF
EEA1
Merge
Time after EGF (min)
0
60
120
5 μm
EGF/EEA1
(person's correlation coefficient)
0.6
0.4
0.2
0.0
-0.2
-0.4
****
****
0 30 120(min)
D
EEA1
Transferrin
Merge
Time after transferrin (min)
0
60
90
5 μm
Fluorescence intensity of transferrin
8000
6000
4000
2000
0
****
**
0 60 90 (min)

Figure 1. Endosomal trafficking of EGFR or transferrin

(A) HeLa cells were treated with EGF for 0 h, 40 min, 80 min, or 120 min. The cell lysates were subjected to EGFR immunoblot analysis.

(B) HeLa cells were treated with EGF-488 on ice for 1 h, then incubated at 37°C for the indicated times, followed by confocal imaging. The fluorescence intensity of EGF-488 was subsequently quantified.

(C) HeLa cells were treated with EGF-488 on ice for 1 h, then incubated at 37°C for the indicated times, fixed, and immunostained with anti-EEA1 antibody, followed by confocal imaging. Pearson correlation coefficient analysis was performed to assess colocalization between EGF-488 and EEA1.

(D) HeLa cells were incubated with transferrin-594 on ice for 1 h. The cells were then incubated at 37°C for the indicated times, fixed, and immunostained with anti-EEA1 antibody, followed by confocal imaging. The fluorescence intensity of transferrin-594 was quantified.

2. Replace the existing medium with DMEM without FBS and PS, and incubate the cells in the cell culture incubator at 5% CO_2 and 37°C overnight to induce serum starvation.
3. EGF treatment of cells (critical steps):
   a. Prepare an EGF-containing medium by dissolving EGF in DMEM supplemented with 20 mM HEPES and 0.2% BSA, achieving a final EGF concentration of 200 ng/mL.
   b. Discard the medium from the serum-starved cells.
      i. Wash the cells three times with ice-cold PBS.
      ii. Add 2 mL of the prepared EGF-containing medium to each well.
      iii. Incubate the cells at 5% CO_2 and 37°C for 0, 40, 80, and 120 min, respectively.
   c. At each designated time point, discard the supernatant and detach the cells with trypsin. Centrifuge the cell suspension at 800g for 3 min, then collect the cell pellets. Wash the pellets three times with PBS, and after the final wash, collect the final cell pellets for further analysis.

   Note: EGF protein should be aliquoted and stored at −80°C at a concentration of 2 μg/mL to avoid repeated freeze-thaw cycles. Additionally, each cell sample should be lysed immediately after collection at the designated time points rather than waiting until all samples are collected.

4. Perform cell Lysis:
   a. To harvest the cells
      i. Aspirate the DMEM culture medium and wash the cells once with PBS to remove residual serum and medium components.
      ii. Add sufficient trypsin to cover the cell surface and incubate at 37°C for 2 min to promote cell detachment. Stop the digestion by adding DMEM containing serum to neutralize the trypsin.
      iii. Centrifuge the cell suspension at 600g for 3 min to collect the cell pellet.
      iv. Resuspend the cell pellet and wash it once with PBS to remove any remaining trypsin or medium components.
   b. Lyse the cells on ice for 30 min using 300 μL of lysis buffer containing 1% NP-40, 200 mM NaCl, and 50 mM Tris-HCl (pH 8.0).
   c. Centrifuge the lysates at 12,000g for 10 min at 4°C and collect the supernatant.
   d. Quantify the protein concentration using a protein assay kit (Epizyme). Adjust all samples to equal protein concentrations, then add 5× protein loading buffer. Heat the samples at 95°C for 10 min before proceeding with western blot analysis.
5. Perform SDS-PAGE followed by western blot analysis:
   a. Assemble an 8% polyacrylamide gel in the electrophoresis chamber. Load 20 μL of protein sample with a total protein amount of 10–20 μg and perform SDS-PAGE at a constant voltage of 150 V for approximately 40–45 min.
   b. After electrophoresis, immerse the gel in transfer buffer and activate the PVDF membrane by soaking it in methanol for at least 15 s.
   c. Transfer the proteins to the PVDF membrane at a constant current of 200 mA for 180 min. Carry out the entire transfer process on ice, using a pre-cooled transfer buffer stored at 4°C.
   d. After the transfer, block the PVDF membrane in the blocking solution at room temperature for 1 h. Then, dilute the primary antibodies (EGFR and GAPDH, Proteintech) in blocking solution at a 1:2000 ratio and incubate the membrane overnight at 4°C.

Note: Since EGFR is a large protein, an 8% polyacrylamide gel is optimal, and extending the transfer time to 180 min helps enhance the protein signal.

e. Wash the PVDF membrane three times with TBST, each for 5 min, to remove any excess primary antibody.
f. Incubate the membrane with the secondary antibody diluted in blocking solution at room temperature for 1 h.
g. Wash the membrane three times with TBST, each for 5 min, to remove any excess secondary antibody.
h. Finally, use a chemiluminescence imaging system to detect the signal of EGFR and GAPDH (Figure 1A).

EGFR trafficking (confocal image analysis)

Timing: 3 days

6. Place 14 mm diameter round coverslips in 75% ethanol for 30 min. Using tweezers, transfer three coverslips into a six-well plate.
7. Rinse each coverslip three times with PBS to thoroughly remove any ethanol residue, then allow them to air dry.
8. Once the coverslips are dry, add 2 mL of DMEM supplemented with 10% FBS and 1% PS to each well. The cell culture conditions are the same as steps one and two from the previous experimental method.
9. EGF Treatment of cells:
   a. Discard the supernatant from the serum-starved cells and wash the cells three times with ice-cold PBS. Keep the cells in the six-well plate on ice during the process.
   b. Dilute Alexa Fluor 488–EGF (Invitrogen) in DMEM supplemented with 20 mM HEPES and 2% BSA to a final concentration of 1 μg/mL.
   c. Add 2 mL of the prepared solution to each well and incubate on ice for 30 min. After incubation, wash the wells three times with ice-cold PBS to remove excess EGF dye.
   d. Remove one coverslip to serve as the 0-min time point sample. For the remaining two coverslips, add 2 mL of complete medium to each well and incubate them at 5% CO_2 and 37°C for 60 and 120 min, respectively.
10. Fixation and Staining:
   a. At each designated time point, transfer the coverslips to a 24-well plate and wash them three times with 500 μL of PBS.
   b. Fix the cells with 500 μL of 4% PFA at room temperature for 15 min. Wash away excess PFA by rinsing the cells three times with PBS.
   c. Permeabilize the cells by adding 500 μL of 0.1% NP-40 in PBS for 10 min at room temperature. After permeabilization, wash the cells thoroughly with PBS three times to remove any excess permeabilization buffer.
   d. Block the cells by adding 500 μL of 3% BSA in PBS and incubate for 1 h at room temperature.
   e. Dilute the primary antibody EEA1 at a ratio of 1:100 in blocking solution, and incubate the cells with the antibody overnight at 4°C.
   f. Wash the coverslips three times with PBS to remove excess primary antibody. Then, dilute the fluorescent secondary antibody at a ratio of 1:1000 in blocking solution and incubate at room temperature for 2 h. Next, dilute DAPI at a ratio of 1:1000 in PBS and incubate at room temperature for 15 min. Finally, wash the coverslips three times with PBS to remove excess dye.
   g. Mount the coverslips with 10 μL of antifade mounting medium (CST) and allow them to incubate for 30 min. Seal the edges of the coverslips using nail polish, and upon complete drying of the nail polish, perform imaging using a confocal microscope (Figures 1B and 1D).

Transferrin recycling assay (confocal imaging)

Timing: 4 days

11. Submerge 14 mm diameter round coverslips in 75% ethanol for 30 min to sterilize them.
12. Using tweezers, transfer four coverslips to a six-well plate.
13. Rinse the coverslips three times with PBS to thoroughly remove ethanol.
14. Allow the coverslips to air dry.
15. Add 2 mL of DMEM containing 10% FBS and 1% PS to each well.
16. Seed each well with 0.5×10^6 cells.
17. Incubate the six-well plate in a cell culture incubator at 5% CO_2 and 37°C for 24 h.
18. Replace the complete medium with DMEM lacking FBS and PS.
19. Incubate the plate overnight in the cell culture incubator at 5% CO_2 and 37°C to induce serum starvation.
20. Transferrin treatment of cells:
    a. Discard the supernatant from the serum-starved cells and wash the cells three times with ice-cold PBS.
    b. Keep the cells in a six-well plate on ice.
    c. Dilute transferrin-Alexa Fluor 488 (Invitrogen) in DMEM containing 20 mM HEPES and 2% BSA to a final concentration of 25 μg/mL.
    d. Add 2 mL of the prepared solution to each well and incubate on ice for 1 h.
    e. Wash the wells three times with ice-cold PBS to remove excess transferrin dye.
    f. Remove one coverslip to serve as the 0-min time point sample.
    g. For the remaining coverslips, add 2 mL of complete medium and incubate at 37°C for 30, 60, and 90 min, respectively.
21. Fixation and Staining:
    a. At each designated time point, transfer the coverslips to a 24-well plate and wash three times with 500 μL PBS.
    b. Fix the cells with 500 μL of 4% PFA at room temperature for 15 min.
    c. Wash off the excess PFA with PBS.
    d. Permeabilize the cells using 500 μL of 0.1% NP-40 in PBS for 10 min at room temperature.
    e. After permeabilization, wash the cells with PBS three times to remove excess permeabilization buffer.
    f. Block the cells with 500 μL of 3% BSA in PBS for 1 h at room temperature.
    g. Dilute the EEA1 antibody (CST, Rabbit) 1:200 in blocking solution and incubate the cells overnight at 4°C.
22. Wash the cells three times with PBS.
23. Dilute the secondary antibody 1:1000 in blocking solution and incubate at room temperature for 2 h.
24. Wash the cells three times with PBS to remove excess secondary antibodies.
25. Mount the coverslips using 10 μL of antifade mounting medium and allow them to incubate for 30 min.
26. Secure the coverslips with nail polish.
27. After the nail polish layer has fully polymerized and hardened, commence with the acquisition of images via confocal microscopy (Figure 1C).
28. Conduct imaging using an Olympus spinning disk confocal microscope at 60× magnification.

Note: The fluorescence exposure times are set to 200 ms for transferrin or EGF (green fluorescence), 100 ms for EEA1 (red fluorescence), and 20 ms for DAPI (blue fluorescence). The imaging parameters are optimized according to the specifications and settings of different microscopes.

Quantification of EGF fluorescence intensity in confocal images using ImageJ

29. Open ImageJ: Start by launching the ImageJ software.
30. Import the Image: Load the confocal image into ImageJ via *File > Open.*
31. Convert to 8-bit: Select *Image > Type > 8-bit* to convert the image to grayscale, as intensity measurements are easier in this format.
32. Thresholding: Adjust the threshold by selecting *Image > Adjust > Threshold*. Set an appropriate threshold value that works for all images, ensuring consistency. Tick the *Dark background* option to enhance signal differentiation, and then click *Apply*.
    a. Define Regions of Interest (ROI): Use the selection tool to draw around individual cells (or areas of interest). Open *Analyze > Tools > ROI Manager* and click *Add* to store each selection.
    b. Measure Intensity: Once the regions are defined, select *Analyze > Measure*. The software will record the intensity values such as *IntDen* (integrated density) for each selected area.
    c. Export Data: The recorded intensity values can then be exported for statistical analysis.
    d. Graph Analysis in Prism: Import the intensity data into GraphPad Prism to generate bar graphs or other plots to compare signal intensity across different conditions.

Note: The method for quantifying transferrin fluorescence intensity in immunofluorescence images follows the same approach as the EGF quantification process.

Colocalization analysis of EGF and EEA1 fluorescence signals using ImageJ

33. Import Images: Open ImageJ and import the confocal images corresponding to the EGF (green fluorescence channel) and EEA1 (red fluorescence channel).
34. Convert to 8-bit: Select *Image > Type > 8-bit* for both images to convert them into grayscale, ensuring compatibility with the subsequent analysis steps.
35. Merge Channels: Go to *Image > Color > Merge Channels*, select the green channel for EGF and the red channel for EEA1, and merge them into a combined image.
36. Cell Selection: Use the selection tool to draw a region of interest (ROI) around a single, complete cell.
37. Image Enlargement: Use *Image > Scale* to enlarge the selected cell region for better visualization and precise analysis.
38. Split Channels: Go to *Image > Color > Split Channels* to separate the merged image back into its original two channels.
39. Colocalization Analysis:
    a. Select *Analyze > Colocalization > Coloc2* from the menu
    b. Choose the two corresponding channels (EGF and EEA1) to analyze the degree of colocalization.
    c. Coloc2 will generate several statistical parameters, including Pearson's R value and Mander's coefficient.
40. Data Collection: When the fluorescence signal-to-noise ratio is high, use the Pearson's R value (above threshold) as the main statistical metric for colocalization analysis.
41. Data Visualization: Export the Pearson's R values and use GraphPad Prism to generate bar graphs for statistical analysis and visualization.

EXPECTED OUTCOMES

By adhering to the specified experimental protocols, we observed a time-dependent decrease in total cellular EGFR protein levels via western blot analysis, indicative of EGFR degradation (Figure 1A). This degradation is attributed to the endolysosomal breakdown of the internalized EGFR-EGF complex, a process that terminates EGFR signaling. Immunofluorescence assays further monitored the intracellular trafficking of EGF or transferrin at various time points. At 0 min, EGF or

transferrin signals were predominantly localized at the plasma membrane. Over time, these signals translocated into the cytoplasm, reflecting their endocytic uptake.

The EGF signal diminished due to the degradation of the EGFR-EGF complex within endolysosomal compartments, a key step in regulating EGFR signaling and ensuring proper signal termination (Figure 1B).[9] The fluorescent signal for transferrin, conjugated to an iron-binding fluorescent dye, reflects its interaction with the transferrin receptor (TfR), which triggers the internalization of the iron-transferrin-TfR complex via clathrin-mediated endocytosis. Within acidic endosomes, iron dissociates from transferrin and is released into the cytoplasm. Subsequently, the transferrin-TfR complex is recycled back to the plasma membrane, where transferrin is released from its receptor into the extracellular space. This recycling process leads to a progressive decrease in the transferrin fluorescence signal (Figure 1C).[10] These findings confirm that our experimental approach effectively captures the dynamics of endocytosis and facilitates quantitative analysis of these processes.

LIMITATIONS

Endosomal trafficking is a highly dynamic process; however, experimental methods such as western blot and imaging techniques measure protein levels or localization at specific time points. This limitation prevents real-time monitoring of protein trafficking in live cells. Additionally, these approaches are confined to *in vitro* studies using cultured cell lines, making them unsuitable for investigating endocytosis or endosomal trafficking in live animal models.

TROUBLESHOOTING

Problem 1

In the western blot analysis, the EGFR signal appeared diffuse, with no distinct protein band observed, indicating potential issues with protein separation or transfer efficiency (see step 5).

Potential solution

First, during serum starvation treatment on the second day, it is critical to thoroughly wash the cells with complete medium followed by PBS prior to replacing it with serum-free medium to ensure removal of residual serum and maintain experimental consistency.

Second, given that EGFR is a large protein, the use of an 8% polyacrylamide gel is recommended for optimal separation. Additionally, extending the transfer time to 180 min improves the detection of the protein signal by enhancing its transfer efficiency.

Problem 2

In the EGF trafficking experiment, at 0 min, the signal was not localized on the cell membrane (see step 2).

Potential solution

Initially, the dilution ratio of the EGF dye was excessively high, resulting in suboptimal experimental conditions. This was subsequently corrected by adjusting the dye to an appropriate dilution ratio. Furthermore, a 30-min incubation of cells on ice was determined to be optimal, as extended incubation times increased the likelihood of dye internalization into the cells.

Problem 3

For various cell lines, a clear reduction in protein signal during EGF or transferrin transport was not consistently detectable (see step 3).

Potential solution

In cell biology research, variations in protein trafficking efficiency across different cell lines necessitate careful optimization of experimental conditions. Preliminary experiments are often required to determine the optimal time points for analysis, tailored to the unique characteristics of each cell line.

For quantitative confocal microscopy analysis, the application of standardized image processing protocols is essential to ensure data reliability. This includes maintaining consistent threshold values across all images to effectively exclude background signals. Such standardization enhances the accuracy and comparability of results, ultimately strengthening the validity and credibility of the conclusions drawn.

RESOURCE AVAILABILITY

Lead contact

Further information and reasonable requests for reagents may be directed to and will be fulfilled by the lead contact, Jianbo Yue (jianbo.yue@dukekunshan.edu.cn).

Technical contact

Questions about the technical specifics of this protocol should be directed to the technical contact, Peng Xu (peng.xu@dukekunshan.edu.cn).

Materials availability

All unique reagents generated in this study are available upon reasonable request from the technical contact.

Data and code availability

This study did not generate any codes.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.X. performed the experiments. P.X. and J.Y. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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