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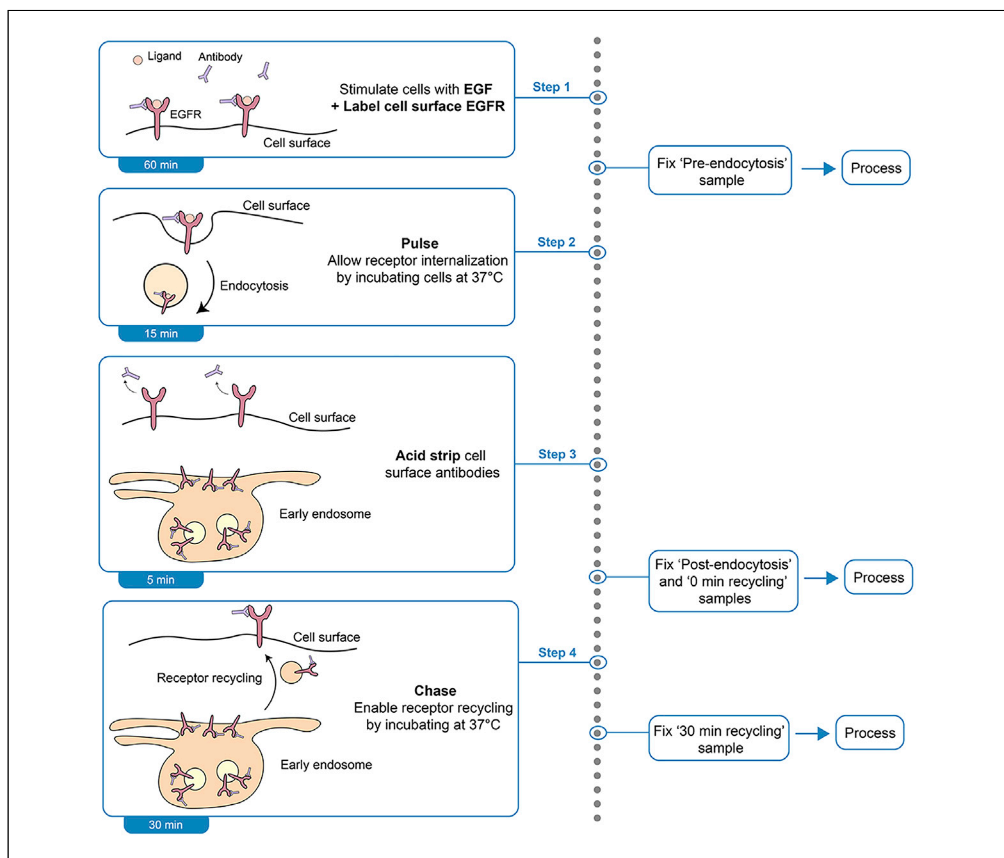
Protocol

Quantifying EGFR endosomal recycling via immunofluorescence in breast cancer cells



Previously published protocols for quantification of endosomal recycling are limited by the use of radioactive reagents, washing of cells in reducing buffers, or the requirement for large numbers of cells. Here, we describe a protocol for quantification of endosomal recycling using immunofluorescence that is optimized for EGFR in BT-549 breast cancer cells but could be applied to other RTKs and cell lines. Our protocol enables quick assessment of recycling and uses a relatively small number of cells.

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Highlights

An optimized protocol for analysis of EGFR endosomal recycling via immunofluorescence

Used to analyze endosomal recycling in triple negative breast cancer cell line BT-549

Can be used to analyze recycling of other receptor tyrosine kinases

Allows assessment of alterations in endosomal recycling with small numbers of cells

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Quantifying EGFR endosomal recycling via immunofluorescence in breast cancer cells

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SUMMARY

Previously published protocols for quantification of endosomal recycling are limited by the use of radioactive reagents, washing of cells in reducing buffers, or the requirement for large numbers of cells. Here, we describe a protocol for quantification of endosomal recycling using immunofluorescence that is optimized for EGFR in BT-549 breast cancer cells but could be applied to other RTKs and cell lines. Our protocol enables quick assessment of recycling and uses a relatively small number of cells.

For complete details on the use and execution of this protocol, please refer to Lonic et al. (2021).

BEFORE YOU BEGIN

The protocol is based on tagging of cell surface receptors with antibody followed by induction of endocytosis, after which the tags on receptors that remained on the surface are removed prior to initiating recycling of internalized receptors back to the cell surface. Receptors with bound antibody tag that have returned to the cell surface are then quantified using a fluorescently labeled secondary antibody.

The protocol below describes the procedure optimized for detection of endogenous epidermal growth factor receptor (EGFR) recycling in BT-549 breast cancer cells.

For the protocol described, an antibody (Ab3) recognizing the extracellular domain of EGFR was used, which was verified not to induce ligand-independent internalization of antibody-bound EGFR (Figure 1). If other antibodies are to be used in this protocol, **they must be validated not to induce ligand-independent endocytosis of the receptor of interest, as well as not interfere with ligand binding to the receptor or the ligand-induced endocytosis of the receptor**. As shown in Figure 1, following up to 30 min endocytosis, fluorescent signal was not detected with Ab3 alone indicating that binding of antibody alone did not induce internalization. Also, the internalized fluorescent signal in the EGF alone and EGF/Ab3 treatments are comparable indicating antibody binding does not interfere with ligand induced internalization (Figure 1).

1. Acid stripping of antibody bound to receptors present on the cell surface, is a crucial step in the procedure and needs to be optimized prior to attempting the recycling protocol. The optimal acid strip procedure - selected from options a), b) or c) indicated below - will be determined in

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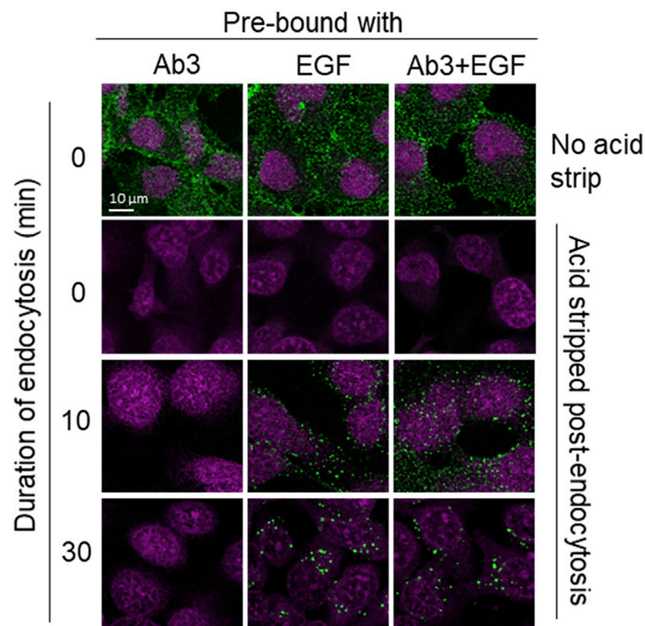

Figure 1. The EGFR-ECD antibody (Ab3) alone does not induce endocytosis
Representative immunofluorescence micrographs showing cells prebound (at 4°C) with the Ab3 antibody alone and EGF (20 ng/mL) alone or with the Ab3 antibody in the presence of EGF. Cells were either fixed without inducing endocytosis (0 min, no acid strip) or induced to undergo endocytosis for various times, as indicated, by incubation at 37°C, followed by an acid wash to remove Ab3 antibody and EGF remaining on the cell surface (acid strip) after endocytosis. Cells were then permeabilized and incubated with secondary Ab (cells prebound with Ab3, Ab3 + EGF) or with the Ab3 antibody followed by secondary antibody (cells prebound with EGF only) to detect endocytosed EGFR. Scale bar 10 µm. Figure reprinted with permission from Lonic et al. (2021).

part by antibody and cell type used. The optimized acid strip will remove all antibody bound resulting in no detectable fluorescent signal and not alter the morphology or viability of the cells (as determined by visual inspection of cells). To provide additional evidence on cell viability following acid strip, a duplicate well can be trypsinized and analyzed by trypan blue staining.

Acid strip solution optimization:

a. 0.2 M acetic acid/0.5 M NaCl/ 5 min incubation.
b. 0.5 M acetic acid /0.2 M NaCl/ 2 min incubation.
c. 25 mM glycine/3%BSA/PBS pH 2.7/ 10 min incubation.

2. A pulse of endocytosis is performed to accumulate receptor inside the cell, so prior to performing the endocytosis pulse, a time-course of endocytosis is required to optimize the time for maximal intracellular accumulation. Following the endocytosis pulse, there needs to be a large enough internalized receptor population to ensure the amount of receptor returned to the cell surface through recycling is quantifiable. Time points up to 15 min (5, 10, 15 min) would be optimal (as the effects of degradation would still be minimal) but up to 30 min may be required in some circumstances. At longer time points, loss of internalized receptors may occur due to degradation. If longer time points are utilized, the contribution of degradation to the loss of internalized receptors should be assessed by assaying the rate of degradation (in the presence of the protein synthesis inhibitor cycloheximide).
3. Prior to starting, ensure there are enough buffers/solutions at the correct temperature. All required buffers/solutions and their correct temperature are listed in Table 1.
4. Prepare fixation solution just prior to use.
5. Install Fiji/ImageJ and Bioformats plugin.

Table 1. Recycling assay solutions

Solution	Components	Required (on ice)	Required pre warmed (37°C)	Required RT (21°C–25°C)
Cell surface labeling solution	RPMI medium 0.1% FBS EGF 20 ng/mL Ab3 0.4 μg/mL	yes	no	no
Trafficking solution	RPMI medium 0.1% FBS	yes	yes	no
Acid strip solution	0.2 M acetic acid 0.5 M NaCl pH 2.5	yes	no	no
Fixation solution	RPMI medium 0.1% FBS 3.7% formaldehyde	no	no	yes
Wash buffer (non-perm)	TBS (1×)	no	no	yes
Wash buffer (perm)	TBS (1×) Triton × 0.1%	no	no	yes
Block buffer (non-perm)	TBS (1×) 2% BSA	no	no	yes
Block buffer (perm)	TBS (1×) Triton × 0.1% 2% BSA	no	no	yes
PBS	PBS (1×)	no	no	yes

https://imagej.net/software/fiji/.

(Schindelin et al., 2012).

https://docs.openmicroscopy.org/bio-formats/6.7.0/.

(Linkert et al., 2010).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Epidermal Growth Factor Receptor extracellular domain (EGFR-ECD) Ab-3 (Clone EGFR.1)	Thermo Scientific	Cat#MS-311-P
Anti-human-IgG Alexa Fluor 488+	Life Technologies	Cat#A32766
Chemicals, peptides, and recombinant proteins		
Recombinant Human EGF Protein	R&D Systems	Cat#236-EG
Formaldehyde solution	Sigma-Aldrich	Cat# F8775
Acetic acid (Glacial)	Merck	Cat#695092
RPMI 1640 1×	Gibco	Cat#11875-093
Sodium Chloride	CSA Scientific	Cat#SA046
Potassium Chloride	Sigma-Aldrich	Cat#P9541
Sodium phosphate dibasic	Sigma-Aldrich	Cat#S5136
Potassium phosphate monobasic	Sigma-Aldrich	Cat#P0662
UltraPure Tris Buffer (powder form)	Thermo Fisher Scientific	Cat#15504-020
Triton X-100	Sigma-Aldrich	Cat#T8787
Bovine Serum Albumin	Sigma-Aldrich	Cat#A9418
Fibronectin	Roche	Cat#10838039001
Fetal Bovine Serum	HyClone	Cat#SH30084

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
BT-549	ATCC	Cat#HTB-122
Software and algorithms		
Fiji	Open source	https://imagej.net/software/fiji/downloads
Bioformats	Open Microscopy Environment	https://www.openmicroscopy.org/bio-formats/downloads/
Other		
Lab-tek II chamber slide	Thermo Fisher Scientific	Cat#177402
ProLong Gold antifade Mountant with DAPI	Invitrogen	Cat#P36935

MATERIALS AND EQUIPMENT

Cell surface labeling solution

Reagent	Final concentration	Amount
RPMI 1640 1×		9.78 mL
FBS	0.1%	100 µL
EGF (2 µg/mL)	20 ng/mL	100 µL
Ab3 (200 µg/mL)	0.4 µg/mL	20 µL
Total	n/a	**10 mL**

Solution needs to be prepared immediately prior to use and stored on ice until added to cells.

Tris-buffered saline (TBS) (1×)

Reagent	Final concentration	Amount
Tris	50 mM	6.05 g
NaCl	150 mM	8.76 g
ddH_2O	n/a	1,000 mL
Total	n/a	**1,000 mL**

Dissolve Tris and NaCl in 800 mL of H_2O. Adjust pH to 7.6 with 1 M HCl and make volume up to 1 l with H_2O. TBS is stable at room temperature (21°C–25°C) for 3 months-2 years.

⚠ CRITICAL: HCl is toxic if inhaled and causes severe skin burns and eye damage. Tris and NaCl may cause eye, respiratory or skin irritation. Handle with appropriate PPE.

Phosphate buffered saline (PBS) (1×)

Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
ddH_2O	n/a	1,000 mL
Total	n/a	**1,000 mL**

Dissolve NaCl, KCl, Na_2HPO_4, KH_2PO_4 in 800 mL of H_2O. Adjust pH to 7.4 with 1 M HCl and make volume up to 1 l with H_2O. Store PBS at room temperature (21°C–25°C).

⚠ CRITICAL: HCl is toxic if inhaled and causes severe skin burns and eye damage. NaCl may cause eye, respiratory or skin irritation. Na_2HPO_4 causes skin and serious eye irritation and

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may cause respiratory irritation. KH_2PO_4 causes eye irritation. Handle with appropriate PPE.

Block buffer (with permeabilization)		
Reagent	Final concentration	Amount
TBS	1×	9.99 mL
Triton X-100	0.1%	10 µL
BSA	2%	0.2 g
Total	n/a	**10 mL**

Solution needs to be prepared on the day of use and stored at room temperature (21°C–25°C).

⚠ CRITICAL: Triton X-100 causes skin irritation and serious eye damage. Handle with appropriate PPE.

Fixation solution		
Reagent	Final concentration	Amount
RPMI 1640 1×		8.99 mL
FBS	0.1%	10 µL
Formaldehyde solution (37%)	3.7%	1 mL
Total	n/a	**10 mL**

Solution needs to be prepared immediately prior to use and stored room temperature (21°C–25°C) until added to cells.

⚠ CRITICAL: Formaldehyde solution is toxic and causes severe skin burns and eye damage. Handle formaldehyde solution in the fume hood, with appropriate PPE and discard appropriately.

STEP-BY-STEP METHOD DETAILS

Seeding cells

Timing: 30 min–1 h

1. For culture of adherent cells precoat Lab-tek chamber slides with fibronectin (0.1 mg/mL) by adding 200 µL per well and then incubate for minimum 15 min at 37°C.
2. Aspirate fibronectin and seed cells at 20,000 cells per well into RPMI supplemented with 10% FBS and 0.023 IU/mL insulin.
3. After 48 h, replace medium with low serum medium (RPMI/0.1% FBS) to starve cells for 20 h, this will maximize cell surface presentation of EGFR.

Note: Washing cells ×2 with RPMI prior to addition of starve medium may be needed with other cell lines/receptors.

⚠ CRITICAL: Cells need to be evenly seeded onto the coated chamber slide (clumping in the middle or on the edges needs to be avoided as this will obstruct the imaging of individual cells). Visually inspect the health and morphology of the cells before and after the low serum starve to ensure cells are healthy and ready to proceed to the next stage. To maximize cell surface presentation of EGFR a 20 h starve works well for BT-549 cells but should be optimized for different cell lines or when analyzing other receptors. Starve times of 2 h–24 h may be appropriate with certain cell lines or receptors.

Table 2. Cell surface labeling solution

Reagent	Volume (μL)	Final concentration
RPMI/0.1% FBS	10,000	
EGF (2 μg/mL)	100	20 ng/mL
Ab3 (200 μg/mL)	20	0.4 μg/mL

Recycling assay

Timing: 2–2.5 h

4. On ice prepare master mix for labeling cell surface presented EGFR, volumes below are enough for 6 chamber slides using 8 well/chamber slide:

Note: Cell surface labeling solution master mix needs to be cold when added to cells.

5. Place cells on ice, aspirate medium and replace with 200 μL of cell surface labeling solution (Table 2) per well.
   a. For all aspiration steps, gentle manual aspiration using an Eppendorf P1000, or similar, is preferable to prevent detaching of cells from the slide.
   b. Concentration of EGF will affect the proportion of EGFR that undergoes recycling vs degradation, with higher EGF concentrations favoring degradation. To determine an optimal concentration for the recycling assay a range of EGF concentrations 0.1–50 ng/mL can be tested in different cell lines.
   c. Concentration of antibody to be used in the labeling solution can be determined by performing immunofluorescence staining with the antibody of interest in unstimulated cells. For most antibodies, concentration test range of 0.2 μg/mL – 1 μg/mL would be appropriate.
6. Label baseline cell surface receptors by incubating chamber slides with cell surface labeling solution at 4°C for 60 min with gentle rocking on rocking platform mixer.
7. Following surface labeling, place all chamber slides on ice.
   a. Fix the baseline (pre-endocytosis) sample with fixation solution for 5 min on ice followed by 10 min at room temperature (21°C–25°C).
   b. Following fixation wash ×2 with PBS (1×) then store at 4°C covered with PBS (1×) until processing.
8. For the remaining chamber slides, while still on ice, aspirate cell surface labeling solution and replace with pre-warmed trafficking solution.
9. Perform an endocytosis pulse by incubating cells at 37°C for 15 min to allow EGF dependent internalization of antibody labeled EGFR.
10. Following the pulse, place all chamber slides on ice.
11. On ice, aspirate trafficking solution (to remove unbound EGF and antibody) and add cold acid strip solution (to strip antibody from remaining surface receptors) and incubate with gentle rocking for 5 min.
12. Aspirate acid strip solution and wash gently ×4 with cold trafficking solution.

Note: It is very important that all strip solution is aspirated prior to adding the warm trafficking solution. RPMI with Phenol Red can be used as a check to confirm effective washing (presence of residual acid will result in yellow color).

   a. Fix the post-endocytosis pulse sample and 0 min recycling sample with fixation solution for 5 min on ice followed by 10 min at room temperature (21°C–25°C).
   b. Following fixation wash wells ×2 with PBS (1×) then store at 4°C covered with PBS until processing.

13. For the remaining chamber slide, while still on ice, aspirate cold trafficking solution and replace with prewarmed trafficking solution to allow recycling.
14. Perform receptor recycling by incubating cells at 37°C for 30 min to allow antibody labeled EGFR back to the cell surface.

Note: Time course (5–60 min) of recycling should be initially performed to determine the best time point to use for assessing recycling.

15. After the 30 min recycling time, fix cells with fixation solution for 5 min on ice followed by 10 min at room temperature (21°C–25°C).
16. Following fixation wash ×2 with PBS (1×) then store at 4°C covered with PBS (1×) until processing.

Pause point: Once cells are fixed, they can be stored at 4°C covered with PBS (1×) for up to 7 days prior to processing.

Slide processing

Timing: 1.5 h

17. Aspirate PBS and wash cells ×3 in wash buffer (non-perm) or **×3 in wash buffer (perm) [for the post-endocytosis pulse only]** (Table 1).
18. Add 200 μL block solution (non-perm) or **block solution (perm) [for the post-endocytosis pulse only]** per well and incubate at room temperature (21°C–25°C) for 60 min.
19. Aspirate block solution and add 200 μL of secondary antibody solution (anti-mouse-Alexa488^{+} 2 μg/mL) diluted in appropriate block solution) and incubate in the dark at room temperature (21°C–25°C) for 60 min with gentle rocking.
20. Wash cells ×3 in wash buffer (non-perm) or **×3 in wash buffer (perm) [for the post-endocytosis pulse only]** then mount using ProLong Gold Antifade Mountant with DAPI.

Imaging

Timing: 3–4 h

In this protocol fluorescent images were captured at ambient temperature using Leica TCS SP8 with HC PL APO CS2 40×/NA 1.1 water immersion objective. Images were acquired using LAS X software (version 3.5.7) (saved in lif format) on the Leica SP8. Image processing was performed using Fiji/ImageJ.

For quantification, z-slices (0.4 μm steps spanning 2.8 μm) were taken per field of view (FOV = 193 × 193 μm) with a minimum of 6 fields of view per condition (total of 80–100 cells per condition).

Images for comparison were acquired with identical acquisition settings and post-acquisition processing.

EXPECTED OUTCOMES

We assessed EGFR recycling in BT-549 cells expressing wt-PKCδ or Y374F-PKCδ and demonstrated reduced recycling in the Y374F-PKCδ expressing cells. Figure 2.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. To quantify EGFR fluorescent signal, drag Leica lif file onto the Fiji floating task bar and open using the Bioformats Import plugin.

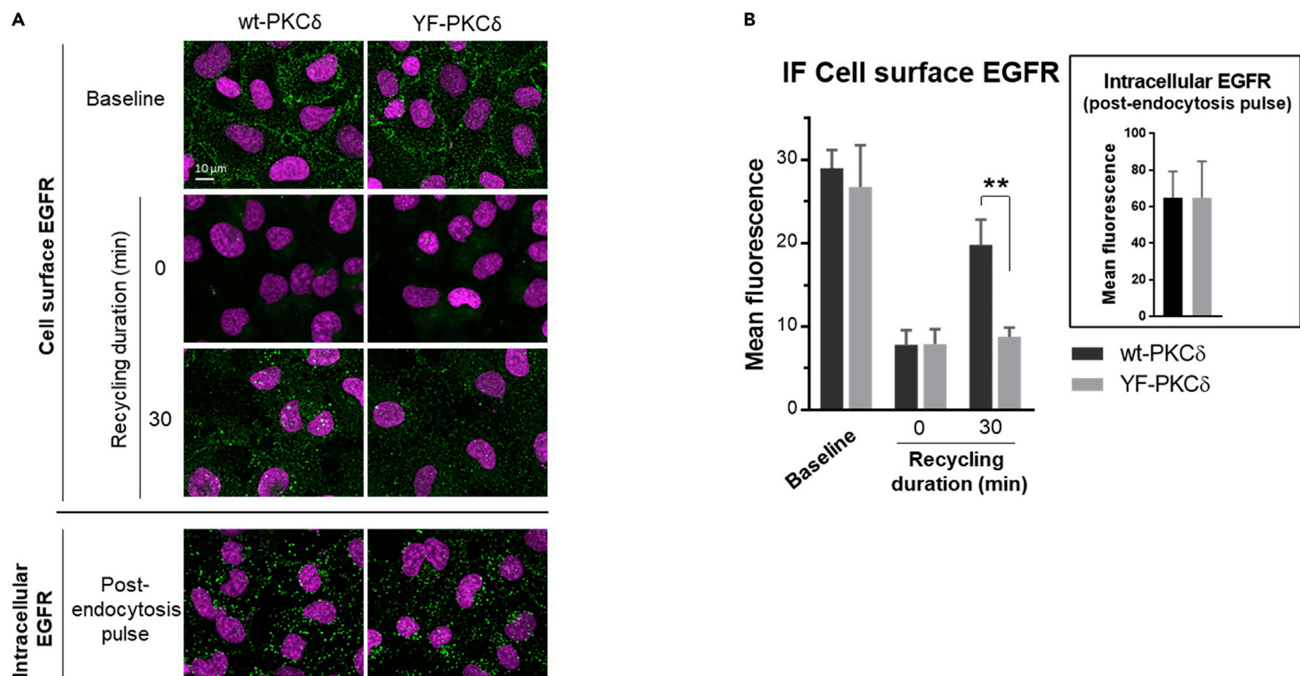

Figure 2. Phosphorylation on Y374 on PKCδ is required to promote EGFR recycling

(A and B) BT-549 cells overexpressing either WT (wt) or Y374F-PKCδ (YF-PKCδ), where the tyrosine (Y) on position 374 has been mutated to a non-phosphorylatable phenylalanine (F), were prelabeled with the mouse Ab3 antibody in the presence of 20 ng/mL EGF (4°C), and endocytosis was initiated by incubation at 37°C. After stripping any Ab3 antibody and EGF remaining on the cell surface with an acid wash at 4°C after the pulse of endocytosis, EGFR recycling was initiated by incubation at 37°C and allowed to continue for 30 min. Representative immunofluorescence micrographs (A) show either cell surface EGFR in nonpermeabilized cells or intracellular EGFR in permeabilized cells as indicated and (B) quantified data from n = 2 independent experiments (mean ± SD; **, $p < 0.01$ using multiple t tests; Prism). Scale bar 10 μm. Figure reprinted with permission from Lonic et al. (2021).

2. In the Bioformats Import window select "View using Hyperstack" and "Split channel" options.
3. Individually save the DAPI channel hyperstack (this will be used for performing cell counts for normalization of EGFR signal) and EGFR channel hyperstack (this will be used for quantifying the amount of recycling).
4. For background subtraction:
   a. Create a duplicate EGFR stack through Image>Duplicate selecting duplicate entire stack.
   b. For the duplicate stack proceed to Process>Filters>Gaussian blur with sigma radius 10 this will create the background image.
   c. Under Process>Image calculator select your original image as "Image 1" and background image as "Image 2" operation "Subtract" and ensure "create new window" is selected.
   d. The result image is your background subtracted EGFR signal which will be used for quantification of EGFR signal.
5. Apply threshold to background subtracted image using Image>Adjust>Threshold, select Otsu threshold.
6. Under Analyze>Set measurements ensure "Integrated density", "Mean gray value" and "Limit to threshold" are selected.
7. Select Analyze>Analyze particles.
8. Both Mean gray value or Integrated density can be used as a measure of EGFR signal.

Note: The EGFR signal can be normalized to the number of cells per field of view or if per cell quantification is required, each cell can be defined as a region of interest (ROI) then EGFR fluorescent signal quantified within each ROI. To generate a cell outline ROI following the recycling protocol, cells need to be post-stained with a marker that will stain the cell membrane

(any plasma membrane protein could be used). The cell membrane stain can be used to generate an ROI of individual cells and thus allow quantification of EGFR fluorescent signal per individual cell.

9. To quantify cell number per field of view (binary DAPI mask counts), open DAPI channel stack.
10. Perform background subtraction as previously for EGFR channel.
11. Using background subtracted DAPI image apply threshold using Image>Adjust>Threshold, select Otsu threshold.
12. Select Analyze>Analyze particles and in the size box in μm^2 select "50-infinity" (the size selection will depend on the size of the nuclei) this will select only nuclei and select "Show – Masks" (compare the masks generated to the original DAPI image to confirm all the cells are being counted correctly) and press ok.
13. In the result table the "counts" column will be the number of nuclei/cells.
14. Calculate the EGFR signal/ number of cells in field of view.

LIMITATIONS

The recycling assay can be complicated by effects of receptor degradation. For example, if there is evident receptor degradation, during the time frame of the receptor recycling assay, then loss of fluorescence signal may be due to reduced recycling or loss of protein due to degradation. A potential solution may be to incubate the cells in the presence of lysosomal inhibitors for the duration of the recycling time course but, if possible, would be preferable to work with a recycling period where degradation is minimal.

While this recycling protocol is able to provide quantitative data on relative receptor recycling occurring under different conditions it would not be appropriate for determining recycling rates as done by using radiolabeled EGF (Sorkin and Duex 2010).

TROUBLESHOOTING

Problem 1

Fluorescent signal is detected in the 0 min recycling sample.

Potential cause:

- Unsuccessful stripping of antibody from cell surface bound EGFR (step 11).
- Fixation induced partial permeabilization of the cells resulting in intracellular antibody bound EGFR being detected after secondary antibody staining (step 12 a).

Potential solution

- Include control wells to test effectiveness of acid strip step (two wells where cells are surface stained one of which is acid stripped). Compare fluorescence signal between stained and stained/stripped samples. If fluorescence is detected in stained/stripped samples increase time for acid strip with current strip buffer or increase concentration of acetic acid.
- Reduce fixation time to 10 min total or perform fixation on ice for the entire 15 min. Alternatively use 16% Paraformaldehyde Aqueous Solution (EM grade, methanol free) to prepare the fixation solution.

Problem 2

No receptor detected on the surface of the cell following the recycling step.

Potential cause:

- Insufficient receptor internalization during the endocytosis pulse (step 9).

- Insufficient receptor recycling to the cell surface (step 14).

Potential solution

- Refer to the post endocytosis pulse control (permeabilized) to determine if there is sufficient fluorescent signal following endocytosis step. If insufficient receptor is internalized, increase the amount of time for the endocytosis pulse to accumulate more intracellular receptors.
- If sufficient receptors are detected after endocytosis, it may be necessary to increase amount of time for recycling to occur.

Problem 3

Weak signal detected in the baseline (pre-endocytosis) sample.

Potential cause:

- Antibody concentration used in the cell surface labeling solution was too dilute (step 5).
- Cell surface presentation of receptor was not successfully maximized (step 3).

Potential solution

- Optimize the concentration of antibody used in the cell surface labeling solution. Concentration test range of 0.2 μg/mL – 1 μg/mL would be appropriate.
- Optimize the cell starvation period to obtain maximal cell surface presentation of the receptor. Starve times of 2 h–24 h may be appropriate with certain cell lines or receptors.

Problem 4

Cell patches remaining on the slide after recycling assay.

Potential cause:

Non-manual aspiration used during wash steps or cell wash solutions added to wells with too much force.

Potential solution

Perform gentle manual aspiration using an Eppendorf P1000, or similar, to prevent detaching of cells from the slide. Always add wash solutions to wells gently by pipetting along the edge of the well.

Problem 5

Difficulty capturing images in focus particularly in center or on the edges of the well.

Potential cause:

- Cells have become too confluent which is causing clumping.
- Cells were not evenly seeded which has caused clumping in the middle or on the edges of the well (step 2).

Potential solution

- Seed a lower number of cells per well or culture cells for a shorter period (24 h) prior to performing recycling assay.
- Check that cells are evenly seeded by inspecting under a microscope after seeding. If uneven seeding is evident gently tap slide under each well to redistribute cells within the well.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yeesim Khew-Goodall (yeesim.khew-goodall@unisa.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.L. and Y.K-G. conceived the project and analyzed the data. A.L. developed the recycling protocol and performed the experiments. W.O. prepared the graphics.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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