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From the Bench

Quantitative analysis of endocytic recycling of membrane proteins by monoclonal antibody-based recycling assays†

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Running head: Quantification of endocytic recycling

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Abstract

In this report, we present an analysis of several recycling protocols based on labeling of membrane proteins with specific monoclonal antibodies (mAbs). We analyzed recycling of membrane proteins that are internalized by clathrin-dependent endocytosis, represented by the transferrin receptor, and by clathrin-independent endocytosis, represented by the Major Histocompatibility Class I molecules. Cell surface membrane proteins were labeled with mAbs and recycling of mAb:protein complexes was determined by several approaches. Our study demonstrates that direct and indirect detection of recycled mAb:protein complexes at the cell surface underestimate the recycling pool, especially for clathrin-dependent membrane proteins that are rapidly reinternalized after recycling. Recycling protocols based on the capture of recycled mAb:protein complexes require the use of the Alexa Fluor 488 conjugated secondary antibodies or FITC-conjugated secondary antibodies in combination with inhibitors of endosomal acidification and degradation. Finally, protocols based on the capture of recycled proteins that are labeled with Alexa Fluor 488 conjugated primary antibodies and quenching of fluorescence by the anti-Alexa Fluor 488 displayed the same quantitative assessment of recycling as the antibody-capture protocols.

Abbreviations list

1^{st}Ab, primary antibody; 2^{nd}Ab, secondary antibody; AF, Alexa Fluor; TCCF, total corrected cell fluorescence; EE, early endosome; fMHC-I, fully conformed MHC-I proteins; LE, late endosome; MFI, mean fluorescence intensity; MHC-I, major histocompatibility class I; mAb, monoclonal antibody; PICT, Protease inhibitor cocktail tablets; PM, plasma membrane; RF, recycling fraction; Tf, transferrin; TfR, transferrin receptor.

Introduction

Membrane proteins and their ligands are continuously internalized by endocytosis into the endosomal system and distributed into the recycling or degradation circuits of endosomal organelles (Huotari and Helenius, 2011). Endocytic recycling is a highly regulated, dynamic and complex cellular process which in coordination with endocytic uptake and lysosomal degradation is essential for the structuring of the plasma membrane and membranous organelle composition (reviewed by Maxfield and McGraw, 2004; Grant and Donaldson, 2009). Consequently, endocytic recycling has irreplaceable role in cellular physiology processes, such as nutrient uptake, cell adhesion, proliferation, migration, cytokinesis, antigen presentation, morphogenesis, immune response, learning or memory and important role in pathophysiological processes such as neurodegeneration, toxin delivery, infective diseases, metastasis and cancer, atherosclerosis and metabolic disorders (Muhkejee et al., 1997; Maxfield and McGraw, 2004; Grant and Donaldson, 2009). Despite that, the physiology of endosomal recycling routes and mechanisms are still at early stage of integration into the cellular physiology, and especially into the higher-order physiology (Grant and Donaldson, 2009). With the emergence of molecules whose recycling mechanisms have been studied, it was becoming clear about the need for integration of endosomal recycling into physiology and pathophysiology of many processes, but also about diversity of endosomal routes and complexity of the regulatory mechanism.

Studies of endosomal trafficking suggest that membrane proteins use various recycling pathways. However, much knowledge about recycling route and the regulatory mechanism was generated for transferrin receptor (TfR), a clathrin-dependent cargo protein (reviewed Grant and Donaldson, 2009). After fast endocytic uptake, TfR is transported into early endosomes (EEs) and recycled back to the plasma membrane either by the rapid (Hao and Maxfield, 2000) and fast recycling route from EEs or collected in the juxtanuclear tubular recycling compartment (JRC) which slowly generates recycling carriers (Mukherjee et al., 1997; van Dam et al, 2002; Maxfield and McGraw, 2004; Grant and Donaldson, 2009). Recycling route, recycling rate, and recycling efficiency are well established for TfR due to the availability of an exquisite tool appropriate for radioactive, chemical or fluorescent labeling (Ciechanover et al., 1983; Hao and Maxfield, 2000; Van Dam and Stoorvogel, 2002; Mukherjee et al., 1997; Majeed, 2014). In contrast to clathrin-dependent cargo proteins, for many proteins that are endocytosed by the clathrin-independent mechanism, the rate of endocytic uptake was not established and remains largely unknown. The best characterized is the recycling route of Major Histocompatibility Class I (MHC-I) proteins (Weigert at al., 2004; Jovanovic et al., 2006; Donaldson and Williams, 2009; Grant and Donaldson, 2009), although recycling of several clathrin-independent proteins was described in the last decade (Vargas et al., 2004; Weigert at al., 2004; Chen et al., 2005; Klein et al., 2006; Barral et al., 2008; Zuo et al., 2011; Maldonado-Báez et al., 2013; Grant and Donaldson, 2009; Majeed et al., 2014; Hsu et al., 2015; Reineke et al., 2015; Li et al., 2015).

For detection and quantification of recycling, several assays were used. The main obstacles in studies of endosomal recycling routes and mechanisms are the lack of appropriate experimental settings and reliable ligands, especially for membrane proteins that do not bind soluble ligands. Thus, specific antibodies arose as a valuable tool for labeling and detection of recycled proteins and, thereby, trace the recycling pathway (Ghosh et al., 1998; Tanowitz and Zastrow, 2003; Park et al., 2004; Lin et al., 2004; Scott et al., 2004; Vargas and Von Zastrow, 2004; Weber et al., 2004; Weigert et al., 2004; Chen et al., 2005; Jovanovic et al., 2006; Barral et al., 2008; Millman et al., 2008; Henriques et al., 2010; Ilić Tomaš et al., 2010; Mahmutefendić et al., 2011; Zuo et al., 2011; Zagorac et al., 2012; Finetti et al., 2014; Hsu et al.,

2015; Reineke et al., 2015). Although antibodies were used in tracing the recycling routes more than a decade, still there were many inconsistencies in using different experimental approaches.

In this report, we performed a systematic study of various protocols in which antibodies are used as tools to study endosomal recycling. We used TfR recycling, as a paradigm of rapidly endocytosed clathrin-dependent cargo molecule (McMahon and Boucrot, 2011), and fully conformed MHC-I proteins as a paradigm for constitutively endocytosed clathrin-independent cargo molecule (Donaldson and Williams, 2009). Our study demonstrates that some antibody-based assays, with proposed upgrades aligned with the cellular physiology of endosomal trafficking and in combination with kinetic modeling, may be used for quantitative assessment of endosomal recycling of both clathrin-dependent and clathrin-independent cargo proteins. Thus, our study contributes to the development of experimental settings needed for further expansion of endocytic recycling research.

Materials and Methods

Cell lines

HeLa cells were obtained from American Type Culture Collection (ATCC). J26-Cw6 cells are murine Ltk-cells expressing human beta-2-microglobulin (ß2m) that were transfected with HLA-Cw6 heavy chain (HC) genes (Ferrier et al., 1985). Cells were grown in DMEM, supplemented with 10% (v/v) of fetal bovine serum (FBS), 2mM L-glutamine, 100 mg/ml of streptomycin, and 100 U/ml penicillin. Also, J26 transfectants were grown with 1% pyruvate. Cell culture medium and supplements were from Gibco (Invitrogen, Eugene, OR).

Antibodies and reagents

We used the following monoclonal antibodies (mAbs) produced by hybridoma cells: W6/32 (mouse IgG_{2a}, ATCC HB-95) that reacts with HCs of fully conformed human MHC-I molecules (Dangoria et al., 2002), HC-10 (mouse IgG_{2a}, obtained from Dr Hidde Ploegh, Massachusetts Institute of Technology, Cambridge, MA) that recognizes peptide-empty ß2m-unassociated HLA class I HCs (Stam et al., 1986), and R17 217.1.3 (rat IgG_{2a}, ATCC TIB 219) that recognize murine transferrin receptor (TfR). MAb to human TfR (236-15375), AF^{488}- and AF^{555}-conjugated transferrin (Tf), anti-Alexa Fluor 488, and AF^{488}- and AF^{555}-conjugated secondary antibody reagents (2^{nd}Ab) to mouse IgG_{2a} and rat Ig were from Molecular Probes (Eugene, OR). FITC-anti-mouse Ig was from Becton Dickinson (New Jersey, USA), and AF^{488}-conjugated W6/32 antibody was from BioLegend (San Diego, CA). Propidium iodide and Concanamycin A were from Sigma-Aldrich Chemie GmbH (Germany), saponin was from Roth (Karlsruhe, Germany), protease inhibitor cocktail tablets (PICT) from Roche (Mannheim, Germany), and Cytofix/Cytoperm from Becton Dickinson (New Jersey, USA).

Quantification of cell surface protein expression by flow cytometry

Cells were collected by short trypsin treatment, washed in culture medium and incubated at 4^{o}C for 30-60 min with 5 µg/ml of primary mAbs in PBS containing 10mM EDTA, HEPES pH=7.2, 0.1% NaN_3 and 2% FCS (PBS-A). Unbound antibodies were removed by three washes with cold PBS-A, and the cells were incubated 30 min at 4^{o}C with 5 µg/ml of FITC-conjugated secondary antibody reagents (FITC-2^{nd}Ab) in PBS-A. After three washes with PBS-A, cells were analyzed by flow cytometry using FACSCalibur flow cytometer (Becton Dickinson & Co, San Jose, CA). Dead cells were excluded by propidium iodide (1 µg/ml) and a total of 5,000 cells were acquired. Fluorescence signal was determined as mean fluorescence intensity (MFI) after subtraction of the background fluorescence (ΔMFI) determined in the same cells by incubation with nonreactive mAbs of the same isotype and appropriate FITC-2^{nd}Ab.

To determine the relative cell surface expression, cells were fixed and permeabilized with Cytofix/Cytoperm (12 min at 4°C) either before (total cellular expression) or after (cell surface expression) incubation with primary mAbs at 4^{o}C in PBS-A. After three washes with 0.1% saponin, cells were incubated with FITC-2^{nd}Abs for 30 min at 4^{o}C, and analyzed by flow cytometry as described above. The percentage of cell surface expressed molecules was calculated as

$$(\Delta MFI_{cell\ surface})/(\Delta MFI_{total})x100 \quad (1)$$

Internalization and plasma membrane dynamics of cell surface proteins labeled with mAbs

Cells were washed with PBS and incubated with TfR or MHC-I-specific mAb (2µg/ml) at 4^{o}C for 60 min. Unbound mAbs were removed by the three washes with the cell culture medium and internalization was initiated by the addition of

pre-warmed cell culture medium in pre-warmed tubes. After incubation at 37°C for different times (t=x min), the level of cell surface bound mAbs was determined using FITC-2ndAbs (5μg/ml) and flow cytometry. The ΔMFI was calculated for each time point ($\Delta MFI_{t=x}$), representing proteins that remained at the cell surface. The ΔMFI of the control cells, which were kept on ice, accounts for the total cell surface expression before internalization was initiated ($\Delta MFI_{t=0}$). The percentage of proteins that remained on the cell surface at each time point was calculated as

$$(\Delta MFI_{t=x})/(\Delta MFI_{t=0})x100. \quad (2)$$

Immunofluorescence and confocal analysis of internalized proteins

To visualize internalized proteins inside cells, the internalization was performed on adherent cells grown on coverslips. Cells were incubated with mAb reagents (2μg/ml) at 37°C for 60 min, washed with PBS (3x), and uninternalized mAbs were acid stripped (1 min, pH 2.2) from the cell surface. Internalized mAbs bound to intracellular proteins were visualized on fixed (20 min at r.t. with 4% formaldehyde) and permeabilized (20 min at 37°C with 0.5% Tween) cells using AF^{488}- or AF^{555}-2ndAbs (60 min at 4°C). Unbound reagents were washed with PBS and cells were embedded in Mowiol (Fluka Chemicals, Selzee, Germany)-DABCO (Sigma Chemical Co, Steinheim, Germany) in PBS containing 50% glycerol and analyzed by confocal microscopy. In some experiments, the TfR was labeled with fluorochrome-conjugated Tf (20 μg/ml) at 37°C for 60 min.

Images were obtained using Olympus Fluoview FV300 confocal microscope (Olympus Optical Co., Tokyo, Japan) with 60 x PlanApo objectives and either 4x or 8x zoom. Z-axis was 0.5 μm, if not indicated otherwise. Images of single cells were acquired at the same magnification, exported in a TIFF format, and processed by Fluoview, Version 4.3 FV 300 (Olympus Optical Co., Tokyo, Japan). Images were quantified by Image J software according to the published protocols (McCloy et al., 2014). Briefly, cells of interest were selected by using freeform selection tool, and area, integrated density and mean gray value was measured for each cell that was selected. For background correction, we used region next to selected cells that had no fluorescence. Total corrected cell fluorescence (TCCF) for each cell of interest was calculated by using the formula: integrated density – (area of selected cell x mean fluorescence of background readings).

Kinetic modeling of TfR and MHC-I trafficking

Kinetic modeling of endosomal trafficking of TfR and MHC-I was performed by the modified multi-compartment model with first order kinetics integrated into the in-house developed software (Mahmutefendić et al., 2016). A detailed description of the kinetic modeling is presented in the supplemental material (Supplemental Materials and Methods).

Recycling assays

Flow cytometric quantification of recycling by the release of the recycled fluorescent ligand

Recycling of TfR was quantified by detection of fluorochrome-conjugated Tf loss from cells after pulse internalization (Rahbek-Clemmensen et al., 2014; Jovic et al., 2014). Cells were incubated with AF^{488}-Tf (20 μg/ml) at 37°C for 60 minutes to load intracellular compartments, washed three times in medium with unlabeled Tf, and acid washed (1 min, pH 2.0) to remove uninternalized cell surface-bound AF^{488}-Tf. The amount of internalized AF^{488}-Tf was quantified by flow cytometry ($\Delta MFI_{int,\ t=60}$) and the loss of fluorescence by recycling determined after incubation at 37°C for

different periods of time ($\Delta MFI_{rec,\ t=x}$) in the medium containing 200 μg/ml of unlabeled Tf. The percentage of the recycled was calculated as:

$$(1-\Delta MFI_{rec,\ t=x}/\Delta MFI_{int,t=60})x100. \quad (3)$$

Flow cytometric quantification of recycling of cell surface proteins labeled with mAbs

Cell surface and endosomal TfR and MHC-I proteins were labeled by incubation with mAbs (2 μg/ml) at 37°C for 60 min. For labeling, we used either unconjugated (protocols A, B, and C) or AF^{488}-conjugated (protocol D) mAbs. Unbound mAbs were washed with cell culture medium, and mAbs bound to cell surface proteins were acid stripped (1 min, pH 2.0) leaving only intracellular pool of mAb-bound complexes (mAb:protein). The surface fluorescence signal after acid stripping, determined by flow cytometry using FITC- or AF^{488}-2^{nd}Abs (30 min at 4°C) for protocols A, B, and C, or anti- AF^{488} mAbs (30 min at 4°C) for protocol D was used as a background ($\Delta MFI_{non\ rec}$). The efficiency of the acid stripping was ~95% according to the ratio of the fluorescent signal before and after the acid stripping. After labeling of the intracellular pool, the recycling was initiated by incubation at 37°C (0-60 minutes). The amount of mAb:proteins that recycled was quantified using the following approaches:

Protocol A - direct detection of recycled mAb-proteins at the cell surface. The recycling was stopped by chilling cells on ice and fluorescence signal of recycled mAb-proteins at the cell surface was determined by flow cytometry for each time point of recycling ($\Delta MFI_{rec,\ t=x}$) by 30 min incubation at 4°C with FITC-2^{nd}Abs. The intracellular pool of mAb:proteins ($\Delta MFI_{int,\ t=60}$) was determined after 60 min of internalization and the acid stripping on Cytofix/Cytoperm fixed and permeabilized cells using FITC-2^{nd}Abs (30 min at 4°C). The percentage of internalized mAb:proteins that recycled to the cell surface was calculated as:

$$(\Delta MFI_{rec,\ t=x} - \Delta MFI_{non\ rec})/(\Delta MFI_{int,\ t=60})x100 \quad (4)$$

Cells incubated with non-binding antibodies, and FITC-2^{nd}Abs served as negative control.

Protocol B - indirect detection of recycled proteins by measurement of the intracellular pool that remains after acid stripping of recycled mAb:proteins from the cell surface. After labeling of the intracellular pool, cells were incubated at 37°C (recycling) for various periods of time and mAb:proteins that recycled to the cell surface were again removed by acid stripping. Cells were fixed and permeabilized using Cytofix/Cytoperm, and intracellular mAb:proteins quantified by FITC-2^{nd}Abs (30 min at 4°C) and flow cytometry ($\Delta MFI_{rec,\ t=x}$). The decrease of fluorescent signal by acid stripping of the cell surface represents the number of recycled mAb:proteins as:

$$100 - (\Delta MFI_{rec,\ t=x} - \Delta MFI_{non\ rec})/(\Delta MFI_{rec,\ t=0})x100 \quad (5)$$

Protocol B' - Since the intracellular pool of mAb:proteins may also be reduced by degradation and, thus, degraded mAb:proteins can be incorrectly interpreted as recycled, we modified this protocol as follows. After labeling of the intracellular pool and recycling, cells were chilled and divided into two groups. In the first group, the surface mAb:proteins were removed by acid stripping and remaining intracellular mAb:proteins determined by FITC-2^{nd}Abs on Cytofix/Cytoperm treated cells ($\Delta MFI_{deg+rec,\ t=x}$). Using the calculation

$$100 - (\Delta MFI_{deg+rec,\ t=x}/\Delta MFI_{deg+rec,\ t=0})x100 \quad (6)$$

we determined the amount of mAb-proteins that was degraded or recycled at indicated period. In the second group, mAb:proteins were determined on fixed and permeabilized non-acid stripped cells by FITC-2ndAbs, giving mAb-proteins that were degraded at indicated time ($\Delta MFI_{deg,\ t=x}$) using the following calculation:

$$100 - (\Delta MFI_{deg,\ t=x}/\Delta MFI_{deg,\ t=0})x100 \quad (7)$$

The proportion of recycled mAb:proteins was the difference in the percentage of both recycled and degraded and the percentage of degraded molecules.

Protocol C - the direct capture of recycled mAb:proteins that reach the cell surface by the fluorochrome-conjugated secondary antibody. During recycling, cells were incubated at 37°C for a various period (0-60 minutes) in the presence of either FITC- or AF^{488}-2ndAbs (2 µg/ml) to capture mAb:proteins complexes that recycled to the cell surface and fluorescence signal quantified by flow cytometry ($\Delta MFI_{rec,\ t=x}$). The intracellular pool of mAb:proteins before recycling ($\Delta MFI_{int,\ t=60}$) was determined as described above, and the percentage of internalized mAb:proteins that reached the cell surface by recycling calculated using the Eq. 4. Cells incubated with non-binding antibodies and fluorochrome conjugated-2ndAbs served as negative control.

Protocol D – the immunofluorescence quenching of recycled proteins labeled with AF^{488}-conjugated primary antibodies. After 60 min labeling with AF^{488}-conjugated primary Abs (AF^{488}-1stAbs) at 37°C, cells were acid stripped and divided into two groups: one to follow degradation and the other to follow degradation and recycling. To calculate degradation, we incubated cells at 37°C for various periods of time (0-60 minutes) and determined cell-associated fluorescence by flow cytometry. The loss of fluorescence represents the level of mAb:protein degradation at the indicated time ($\Delta MFI_{deg,\ t=x}$) as described in Eq. 7. To determine both degradation and recycling, the other part of cells was incubated in the presence of anti-AF^{488} Abs, which quench with the AF^{488} emission with ~92% efficiency (Lin *et al*., 2004, our data). Thus, the presence of anti-AF^{488} will quench the fluorescence of AF^{488}-1stAbs labeled protein complexes that reappeared at the cell surface by recycling. The loss of fluorescent signal, thus, represents the level of mAb-proteins that were either degraded or recycled at the indicated time periods ($\Delta MFI_{deg+rec,\ t=x}$) and the percentage was calculated using Eq. 6. The percentage of recycled mAb:proteins was calculated as the difference between the percentage of recycled plus degraded and proportion of degraded.

Kinetic analysis of recycling

The integrated recycling rate constant (k_r) was determined the slope of the first-order rate single exponential decay between time intervals in the experimental quantification of recycling and by the best fit to the experimental data throughout the period of 60 min of recycling.

The recycling fraction (RF), the fraction of internalized ligand-labeled proteins that undergo recycling, was determined as a fraction that gave plateau of recycling after the best fit to the experimental data using calculated recycling rate constant.

The amount of recycled proteins $R(t_n)$ was calculated as:

$$R(t_n) = 100RF - 100RF(1-k_r\Delta t) \quad (8)$$

For antibody-based recycling assays the amount of recycled 1stAb-bound complexes was corrected for the number of 2ndAb captured 1stAb-bound complexes that degraded (RD) within the time period as:

$$R(t_n) = 100RF - 100RF(1-k_r\Delta t) - \Sigma_{i=1}^{n} RD(t_i) \quad (9)$$

Since experimental data demonstrated that the degradation of cell surface 2^{nd}Ab:1^{st}Ab:protein complexes starts after 15 min with the degradation rate constant (k_d) that is determined experimentally and adjusted by the best fit, RD was calculated as:

$$RD(t_n) = R(t_n\text{-}15) - R(t_n\text{-}15)(1-k_d\Delta t) \quad (10)$$

Kinetic modeling of recycling

Kinetic modeling of endosomal distribution 1^{st}Ab-bound TfRs and MHC-I proteins after cell surface labeling at 4°C and after 60 min labeling at 37°C and kinetic modeling of recycling by the 2^{nd}-Ab capture of 1^{st}Ab-bound TfRs and MHC-I proteins is described in Supplementary material.

Quantification of MHC-I and TfR recycling by immunofluorescence image analysis

Simultaneous recycling of MHC-I proteins and TfR was determined by immunofluorescence using two protocols that differ in the reagent for quantification of TfR recycling: either AF^{488}-Tf or mAbs that react to TfR.

Recycling of TfR after labeling of with AF^{488}-Tf. Cells grown on coverslips were 60 min incubated with mAbs to MHC-I proteins and AF^{488}-Tf (20 μg/ml) at 37°C (*internalization*). To display internalized mAb-MHC-I and AF^{488}-TfR uninternalized ligand were acid stripped (1 min, pH 2.0) from the cell surface, cells were fixed and permeabilized and mAb-MHC-I stained with AF^{555}-2^{nd}Abs (5 μg/ml). To allow recycling cells were incubated at 37°C with AF^{555}-2^{nd}Abs (1 μg/ml) for 60 min and processed for confocal analysis without permeabilization. Recycling of TfR was quantified as a loss of green fluorescence and recycling of MHC-I proteins as the accumulation of red fluorescence within the same cell. Approximately 20 cells per coverslip were randomly selected and imaged with Olympus Fluoview FV300 confocal microscope (Olympus Optical Co., Tokyo, Japan) with 60 x PlanApo objectives. All images were taken with identical settings that were optimized for the signals to be in the dynamic range. Images were quantified by Image J software, as described previously. TCCF was calculated for each selected cell and the percentage of recycled MHC-I molecules was calculated as:

$$TCCF_{rec}/TCCF_{int}*100 \quad (11)$$

The percentage of recycled TfR was calculated as:

$$100 - (TCCF_{rec}/TCCF_{int}*100) \quad (12)$$

Recycling of TfR after labeling with anti-TfR mAb. TfRs and MHC-I proteins were labeled by the 60 min incubation at 37°C with mAbs, and recycled complexes were captured by AF^{488}- and AF^{555}-conjugated secondary reagents. Recycling of both TfR and MHC-I was quantified on confocal images as the accumulation of green and red fluorescence within the same cell. Percentage of recycled was calculated using Eq. 11.

Statistics and data fitting

Data are presented as mean ± S.D. The significance of difference was tested using Student's *t*-test (p <0.05 was considered significant).

To quantify goodness of any of the models obtained by the described fitting processes we used the coefficient of determination (R^2)

$$R^2 = 1 - \frac{SS_E}{SS_T} \tag{13}$$

Here

$$SS_E = \sum_{l=1}^{m} \left(Q_1^{(l)} - q_1\left(t^{(l)}, \lambda_1, \dots, \lambda_N\right)\right)^2 \tag{14}$$

is the residual sum of squares,

$$SS_T = \sum_{l=1}^{m} \left(Q_1^{(l)} - \overline{Q_1}\right)^2 \tag{15}$$

is the total sum of squares, and $\overline{Q_1} = \frac{1}{m}\sum_{l=1}^{m} Q_1^{(l)}$ is the mean of the measured concentrations. Value of the coefficient of determination is equal to the part of the variability of the data that is covered by the model. The fitting was based on the adjustment of kinetic parameters until the R^2 value was larger than 0.99.

Results

Kinetic parameters of intracellular trafficking of TfR and MHC-I

In this study, we used monoclonal antibodies (mAbs) as a tool for analysis of recycling of transferrin receptor (TfR) and fully conformed MHC-I (fMHC-I) proteins in human HeLa cells and murine fibroblastic cell line J26-Cw6 stably transfected with human HLA-Cw6. MAb W6/32 detected fMHC-I at a relatively high level at the cell surface of both cell lines (Fig. 1A), whereas anti-TfR mAbs detected low level of TfR at the cell surface of HeLa cells and moderate level of TfR at the surface of J26-Cw6 cells, despite the high intracellular level (Fig. 1A). Thus, for analysis of TfR recycling using mAbs as a reagent, we used J26-Cw6 cells. We also followed recycling of open MHC-I conformers (peptide-empty MHC-I proteins, eMHC-I) as a control, because they do not recycle from early endosomal recycling circuit by the fast and slow recycling route (Mahmutefendić et al., 2010; Zagorac et al., 2011).

After surface labeling with mAbs at 4^{o}C and various periods of incubation at 37^{o}C (chase), the amount of mAb-bound proteins (mAb:protein) at the cell surface of each time-point reflects integrated rate of the plasma membrane and intracellular cycling, including endocytic rate, recycling rates, and inter endosomal trafficking rates. Thus, we determined the kinetics of cell surface expression of mAb-labeled TfRs and MHC-I proteins by flow cytometric quantification after different periods of the chase (Fig. 1C). We used these data and parameters from existing literature (Mukherjee et al., 1997; Hao and Maxfield, 2000) to model their intracellular trafficking and to determine kinetic parameters of their recycling. For modeling, we used in-house developed software, which integrates multicompartment trafficking based on the first-order rate reactions (Fig. 1C). In our model, we divided the early endosomal (EE) recycling circuit into three compartments: pre-EEs, early stages of EEs as a source of rapid recycling; EEs, tubular or vesicular EEs as a source of fast recycling; and the JRC as a source of slow recycling (Hao and Maxfield, 2000, Grant and Donaldson, 2009; Hsu et al., 2012). To model trafficking within the EE recycling circuit, we set up six kinetic rate constants (k_1-k_6) and rate constant of transit from EEs towards late endosomes (k_7) (Fig. 1C). Fitting these parameters to the experimental data (cell surface expression of mAb-proteins) demonstrated that both TfR and MHC-I must use all three routes of recycling (rapid, fast and slow) to justify cell surface levels established by intracellular cycling after a chase at 37^{o}C (Fig. 1C). Optimized parameters (k_1-k_7) and the onset of transit between compartments is presented in tables within the Fig. 1C. These parameters suggest that TfR and MHC-I use distinct endosomal domains or even subsets for recycling within the EE recycling circuit. Additionally, modeling of inter endosomal trafficking demonstrates also relative distribution within plasma membrane and endosomal compartments with time (Fig. 1C). All these parameters were used for further modeling of recycling.

Recycling protocols based on antibody reagents as a tool for membrane protein labeling

To determine endocytic recycling, we tested four recycling protocols based on mAbs as reagents for detection of cell surface and an endosomal fraction of proteins. As a reference, we used the well-established protocol of TfR recycling by detection of the fluorescent ligand loss (Tf).

Cells were incubated with unconjugated (Fig. 2, protocols A-C) or AF^{488}-conjugated (Fig. 2, protocol D) primary mAbs at 37^{o}C over 60 min to label all proteins that are present at the cell surface and that appear at the cell surface (recycled from the endosomal system, from the secretory pathway) during this time and to establish steady-state distribution within endosomal compartments as a consequence of endosomal cycling. Residual cell surface bound mAbs were then

acid stripped and cells were incubated at 37°C for various time periods to allow the recycling. The intracellular (endosomal) fraction that reappears at the cell surface (recycle) was determined by the following approaches (Fig. 2, protocols A-D).

In protocol A, after recycling at 37°C, mAb-bound proteins that reappeared at the cell surface (recycled) were determined directly by staining cells at 4°C with fluorochrome-conjugated 2ndAbs (Fig. 2, protocol A). This approach has been used in several studies (Tanowitz and von Zastrow, 2003; Park et al., 2004; Vargas et al., 2004; Weber et al., 2004; Weigert et al., 2004; Barral et al., 2008; Millman et al., 2008; Henriques et al., 2010; Zuo et al., 2011; Finetti et al., 2014; Hsu et al., 2015; Reineke et al., 2015).

In protocol B, recycled mAb-bound proteins were detected indirectly, by quantification of non-recycled mAb:proteins that remained in the cell interior after acid-stripping of those that reached the cell surface. The intracellular mAb-proteins were determined by fluorochrome-conjugated 2ndAbs and the loss of fluorescence represented recycled mAb-proteins that were removed by the acid stripping. This protocol has been used for quantification of recycling of MHC-I proteins (Jovanovic et al., 2006). Given that the loss of fluorescence may also be a result of degradation of internalized mAb-proteins, we upgraded this protocol (protocol B') for quantification of total fluorescence (cell surface and intracellular) by permeabilization and staining of non-acid stripped cells (Fig. 2, protocol B). Using this approach, the loss of fluorescence in acid-stripped cells represents recycling plus degradation, the loss of fluorescence in non-acid stripped cells represents degradation and the recycling was calculated as the difference.

In protocol C, during recycling at the 37°C, fluorochrome-conjugated secondary Abs were present in incubation media and thus recycled mAb-proteins were captured by the fluorochrome-conjugated 2ndAbs while reappearing at the cell surface at 37°C (Fig. 2, protocol C), irrespective whether they remained at the cell surface or were re-internalized after recycling. This approach was used in several studies (Weber et al., 2004; Scott et al., 2004; Mahmutefendić et al., 2010; Zagorac et al., 2011; Ilić Tomaš et al. 2010).

In protocol D we used AF^{488}-conjugated 1stAbs for labeling of cell surface proteins. After internalization at 37°C (60 min) and acid-stripping, cells were divided into two samples. In one sample, anti-AF^{488} Abs were present in incubation media during recycling at 37°C, which quenches AF^{488} ~ 90% efficiency (Lin et al., 2004; our unpublished data). AF^{488}-mAb-bound proteins were captured with anti-AF^{488} Abs when reappeared at the cell surface (recycled), and the fluorescence was quenched, irrespective whether they remained at the cell surface or were re-internalized. To include the degradation of mAb-proteins into the calculation, the loss of fluorescence was determined in cells that were chased in the absence of anti-AF^{488} Abs (the second sample). Therefore, the loss of fluorescence in this group was used as a measure of degradation (Fig. 2, protocol D). Recycling is calculated as the difference between the first and the second sample. The protocol D was used in several studies (Ghosh et al., 1998; Lin et al., 2004; Chen et al., 2005), but without the second sample modification that also includes degradation.

Quantification of recycling of clathrin-dependent membrane protein by antibody reagent-based recycling protocols

To validate the recycling protocols, we first compared quantification of TfR recycling using a standard protocol based on the release of fluorescent ligand (AF^{488}-Tf) with antibody reagent-based protocols A-C (Fig. 2). The recycling rate of the TfR was first determined as the loss of immunofluorescence after loading of endosomal compartments with the fluorescent ligand (Tf-AF^{488})-labeled receptors and subsequent incubation in the absence of fluorescent ligand and the

excess of the free ligand, as described in many studies (Matsui and Fukuda, 2014). In both, J26-Cw6 and HeLa cells the integrated recycling rate constant (k_r) was ~0.09 min^{-1} (Fig. 3A) and recycling fraction (RF) of 0.91 and 0.85, respectively. This is consistent with the well-established rates in other cell lines (Ciechanover et al., 1983; Hao and Maxfield, 2000; Mukherjee et al., 1997; Van Dam and Stoorvogel, 2002) and suggests that in both HeLa and J26-Cw6 cells the TfR recycling system operates on similar principles.

Direct detection of mAb-labeled TfRs that reappeared at the cell surface (Protocol A) revealed only 34% receptors (RF 0.34) that recycle with the integrated rate constant (k_r) of 0.115 min^{-1} (Fig. 3B). This result indicates that mAb-labeled receptors recycle as efficiently as unlabeled receptors, but due to the low retention time at the cell surface only a fraction of receptors can be detected by recycling protocol A. This fraction corresponds to the cell surface fraction of TfRs that can be determined at steady state (Fig. 1B). Quantification of recycling receptors by the indirect detection (Protocol B) also discovered 35% of recycled receptors (RF 0.35) and suggested higher integrated recycling rate (0.142 min^{-1}, Fig. 3B). Thus, these two protocols are not appropriate for quantification of recycling of cellular proteins that are constitutively internalized by clathrin-dependent endocytosis and thereby have short retention time at the cell surface.

The capture of recycled mAb-TfRs by FITC-2^{nd}Ab (Protocol C) revealed 87% of recycling receptors that reappeared at the cell surface (RF 0.87) with the integrated recycling rate constant (k_r) of ~0.105 min^{-1} (Fig. 3C), which is close to the recycling efficiency and the recycling rate determined by the loss of fluorescent ligand (Fig. 3A). However, by this protocol, the plateau could not be reached, and a decline of the fluorescent signal was observed after incubation longer than 30 min (Fig. 3C). This decrease may be associated with the rapid reinternalization and degradation of 2^{nd}Ab captured mAb:TfR complexes. Kinetic simulation of recycling with the integration of the degradation demonstrated that the best-fit can be achieved by the integration of degradation after 20 min of recycling with the rate constant (k_d) of ~0.012 min^{-1} (Fig. 3C, full line). Thus, if experimental data were supplemented with the kinetic simulation which includes degradation, the antibody-capture protocol will give higher recycling efficiency (~0.99) than the ligand release protocol (Fig. 3C, dashed line; compare with the data in Fig. 3A). Thus, the inclusion of the kinetic simulation into the 2^{nd}Ab capture protocol may overcome technical problems that often arise with the use of a fluorescent ligand (i.e. fluid-phase uptake).

To simulate recycling, we used parameters mAb:TfRs cycling presented in Fig. 1C and extended it to the conditions established by the recycling protocol C (Fig. 3C). After 60 min of cycling the cell surface level was set up to 0 (acid strip), the overall cycling continued with the same kinetic parameters (Fig. 1C) and the recycled receptors redirected into the fast degradation route with the constant rate of ~0.012 min^{-1}. Using these parameters, the recycling kinetic (Fig. 3D, light blue line) fully aligns with the calculated first-order rate (Fig. 3D, dashed line). However, to align kinetic of recycled receptors captured by the 2^{nd}Ab (Fig. 3D, red line) we introduced efficiency of the 2^{nd}Ab catch of 0.55 min^{-1}. Thus, if the 2^{nd}Abs recognize mAb:TfRs with the 55% efficiency in a minute and redirect complexes into the fast degradation track, we modeled similar result (Fig. 3D) as determined by the recycling protocol C (Fig. 3C). Also, the modeling displays relative contribution of EE compartments in the recycling of TfRs (Fig. 3D).

Altogether, the antibody capture-based recycling protocol C supplemented with the kinetic modeling may accurately display recycling kinetic of rapidly endocytosed clathrin-dependent membrane proteins.

Quantification of recycling of clathrin-independent membrane protein by antibody-based protocols

The antibody-based protocols were further tested on the recycling of fMHC-I proteins. Protocol A detected ~22% of recycled fMHC-I proteins (RF 0.22) with the integrated recycling rate constant (k_r) of ~0.145 min^{-1}, and detected recycling of ~3% of eMHC (Fig. 4). Similar to TfR (Fig. 3B), the protocol A may underestimate the recycling fraction because a fraction of recycled MHC-I proteins can also be re-internalized.

In contrast to protocol A, protocol B detected 75% of recycled fMHC-I and 45% of recycled eMHC (Fig. 4). The protocol B detects mAb-bound proteins that are retained intracellularly as non-recycled molecules. Thus, molecules that leave EEs towards the LEs and enter the degradative route are recognized as recycled, which gives false high recycling fraction. When this protocol was corrected for degradation of mAb-bound proteins during the period of recycling, the recycling of eMHC-I was less than ~2%, but the overall recycling fraction of fMHC was ~20% (data not shown). Thus, the values obtained by the recycling protocol B, in fact, represented recycled and degraded MHC-I proteins. With the modification which includes degradation (protocol B'), the recycling protocol B measured similar recycling efficiency as the protocol A.

The protocol C detected little recycling of eMHC-I and detected ~38% of recycled fMHC-I (RF 0.38) with integrated recycling rate constant (k_r) of ~0.172 min^{-1} (Fig. 4). In this protocol the gain of fluorescence is used as a basis for calculation of recycling. As with the TfR (Fig. 3C), however, the fluorescence signal decreased after 30 min with the predictable constant rate (k_d) of ~0.011 min^{-1} (Fig. 4).

In the protocol D, which is based on labeling of fMHC-I proteins with AF^{488}-conjugated primary Abs and capture of reappeared mAb:MHC-I complexes by the anti-AF^{488} mAb that quenches the fluorescent signal, the loss of fluorescence was used for calculation of recycling. The protocol D detected ~35% of recycled fMHC-I (RF 0.35) and displayed the integrated recycling rate constant (k_r) of ~0.165 min^{-1} without reduction (k_d=0) after prolonged incubation (Fig. 4).

Clarification of the protocol C

In the 2^{nd}Ab-capture assay (protocol C), we used FITC-2^{nd}Ab reagents, most frequently used and economically suitable reagents for flow cytometric studies. FITC-conjugated reagents may reduce emission in the acidic environment similar to that of LEs/lysosomes (Lanz et al., 1997), thus, the loss of signal in the protocol C may be associated with the decreased fluorescence emission after rapid internalization of 2^{nd}Ab:1^{st}Ab:fMHC-I complexes into acidic endosomes, or by their degradation, or both. To clarify these options we performed the following experiments.

First, we determined the onset of the loss of fluorescence signal after internalization of 2^{nd}Ab:1^{st}Ab:fMHC-I complexes. The complexes were established at the cell surface by incubations at 4^oC followed by incubation at 37^oC and quantification of the fluorescence signal by flow cytometry. Under the same experiment, the recycling assay was performed. As shown in Fig. 5A, the loss of fluorescence was detectable after 20 min and continued at the constant rate (~0.009 min^{-1}). The loss of fluorescence of 2^{nd}Ab:1^{st}Ab:fMHC-I complexes established at 4^oC coincided with the loss of signal in the recycling assay, suggesting that the 2^{nd}Ab:1^{st}Ab:fMHC-I complexes established by the Ab-capture in the protocol C were rapidly internalized and reached acidic endosomes after 15 min.

Second, to distinguish contribution of the entry of 2^{nd}Ab:1^{st}Ab:MHC-I complexes into acidic endosomes and degradation, we performed the recycling assay in the presence of Concanamycin A (ConA), an inhibitor of endosomal acidification and degradation (Dröse and Altendorf, 1997), or protease inhibitory cocktail (PICT), inhibitor of lysosomal degradation (Durrington et al., 2010). As shown in Fig. 5B, the addition of ConA or PICT during the 2^{nd}Ab-capture prevented loss of the fluorescence, suggesting that lysosomal degradation is one of the main causes of the loss of fluorescence. However, the fraction of recycled mAb:fMHC-I that was detected in Con A-treated cells was higher than in PICT-treated cells (0.45 v.s. 0.4), suggesting that endosomal acidification also contributes to the loss of fluorescence.

Third, we performed 2^{nd}Ab capture using AF^{488}-conjugated secondary reagent, since it is known that AF^{488} emission is insensitive to pH changes (Panchuk–Voloshina et al., 1999). As shown in Fig. 5C, the loss of fluorescence was not present when the 1^{nd}Ab capture was performed with AF^{488}-anti mouse IgG. Under identical experimental conditions, anti-mouse IgG-FITC displayed 38% whereas anti-mouse IgG-AF^{488} displayed 44% of intracellular mAb:fMHC-I as recycled. Therefore, although economically less favorable, AF^{488}-2^{nd}Ab reagents are more feasible for Ab-capture assay and flow cytometric quantification. However, AF^{488} did not undergo degradation (Fig. 5D) and, thereby, cannot be appropriate for the assays which consider lysosomal degradation.

Finally, as with the TfR (Fig. 3D), we performed a simulation of recycling of fMHC-I (Fig. 5E), using parameters of kinetic MHC-I cycling (Fig. 1C) and recycling parameters obtained by protocol C (Fig. 4). To simulate conditions of the recycling assay, which is based on the continuous presence of 1^{st}Ab during 60 min of labeling, the cell surface level of MHC-I was set to 100%. Thus, in addition to preexisting MHC-I proteins at the cell surface, we also included in 1^{st}Ab labeling those that reach the cell surface within 60 min either from the secretory pathway or endosomal system. After setting up the cell surface to 0 (simulation of the acid strip), mAb:fMHC-I continued traveling through endosomes (Fig. 5E) and recycled were displayed as caught with the 2^{nd}Ab (Fig. 5E, light blue line) with an efficiency of 0.55 min^{-1}. The simulation of recycled was well aligned with the 1^{st} order rate kinetic (Fig. 5E dashed black line) determined experimentally (0.165 min^{-1}) and demonstrated that under these trafficking conditions (k_1-k_8) a fraction of ~42% of mAb:fMHC-I can be detected. A major reason for that was the relocation of mAb:fMHC-I into late endosomes (Fig. 5E). The introduction of degradation of reinternalized 2^{nd}Ab-1^{st}Ab-MHC-I complexes with the rate of 0.011 min^{-1} gave similar recycling curve (Fig. 5E, red line), as determined experimentally (Figs. 4 and 5A).

Altogether, our analysis indicates that both protocol C and protocol D may be used for flow cytometric quantification of recycling of Ab labeled clathrin-independent proteins. The protocol C is less experimentally demanding, but requires the use of AF^{488}-conjugated secondary reagents or requires upgrades with an inhibitor of endosomal acidification and degradation (i.e. ConA) when FITC-conjugated secondary reagents are used. When performed simultaneously under identical experimental conditions, the protocol D gave the identical results as ConA-upgraded protocol C (Fig. 6). If experimental conditions do not support the use an inhibitor of endosomal acidification, either AF^{488}-2^{nd}Ab reagents are recommended or FITC-2^{nd}Ab reagents in combination with the kinetic modeling that integrates degradation of the reinternalized 2^{nd}Ab captured complexes.

mAb-bound membrane proteins stably recirculate throughout the endosomal system

Modeling of intracellular distribution, kinetic parameters, and modeling of recycling was based on the assumption that mAb-bound proteins stably recirculate in the endosomal system. To test this, we designed window experiments (Fig. 7A) in which we first incubated cells with mAbs to fMHC-I (1^{st}Abs) for 60 min (internalization), washed excess of unbound Abs and performed antibody capture protocol (protocol C) for 30 min, starting at various periods after internalization and incubation at 37°C. Before antibody capture with AF^{488}-2^{nd}Ab, the cell surface bound 1^{st}Abs were removed by the short acid wash and the intracellular amount of 1^{st}Abs was quantified on permeabilized cells by flow cytometry. The same approach was also tested by immunofluorescence. After 60 min of internalization, mAb:fMHC-I was found in punctate and tubular endosomal carriers and the juxtanuclear compartment, and the same pattern was observed after 150 min of the chase in mAb-free medium (data not shown). Flow cytometric quantification of intracellular mAb:fMHC-I (Fig. 7B) and TfR (data not shown) demonstrated similar amounts in comparison with the intracellular pool after 150 min of the chase as immediately after labeling. Furthermore, the overall amount of mAb:fMHC-I (Fig. 7C) and TfR (data not shown) in the EE recycling domain(s) was similar and recycling fraction determined by the antibody-capture protocol after 30 mins were indistinguishable. These data indicate that mAb-bound fMHC-I proteins and TfR stably recirculate in the early endosomal system, and that antibody binding does not elicit their degradation.

Quantification of recycling of clathrin-dependent and clathrin-independent cargo molecules within the same cell

Fluorescent-secondary antibody capture of recycled mAb-labeled proteins also enables the establishment of the assay based on simultaneous quantification of recycling together with clathrin-dependent cargo protein. We simultaneously labeled TfRs with fluorescent transferrin (Tf-AF^{488}) and fMHC-I proteins with mAbs by incubation at 37°C for 60 min (Fig 8A). This approach labels all proteins that reside at the plasma membrane and that load early endosomal system, as described above. Essentially, the same could be achieved by incubation at 4°C and internalization at 37°C, but this approach resulted in lower TfR signal inside the cells. After 60 min of internalization, the un-internalized reagents were acid stripped from the cell surface and the cells were again incubated at 37°C in the presence of AF^{555}-2^{nd}Abs (recycling). During incubation, recycled TfRs released AF^{488}-Tf from the cell, resulting in the loss of green fluorescence, and recycled mAb:fMHC-I complexes were caught with AF^{555}-2^{nd}Abs, leading to the increase of red fluorescence at the cell surface and inside the cell (Fig 8A). Overlaid sequence of images of cells was quantified for the intensity of fluorescence. By this quantification, we measured that 90% of TfRs and 45% were recycled within 60 min, which is similar to data obtained by flow cytometric assays (Fig 8B). Similar results have been achieved under conditions when both TfRs and fMHC-I recycling was detected by fluorescent secondary antibodies (Fig 8C and D).

Discussion

To understand the cellular physiology of a membrane protein, including feedback mechanisms that determine their intracellular distribution and consequently function, it is essential to construct their intracellular itinerary, which includes endocytic uptake, inter endosomal trafficking, and endosomal recycling. Quantitative analysis of endosomal recycling is, therefore, required for integration of cellular physiology of endocytic trafficking of membrane proteins. For the vast majority of membrane proteins, there is no appropriate ligand that would enable quantitative analysis of endocytic recycling. Thus, in this study, we performed analysis of protocols for quantitative assessment of endocytic recycling based on antibodies as a tool for convenient labeling of membrane proteins and monitoring their intra-endosomal itinerary. Antibodies have been used for monitoring of endosomal recycling in a number of studies using various approaches and with diverse depth and fidelity of quantitative assessment (Ghosh et al., 1998; Tanowitz and Zastrow, 2003; Park et al., 2004; Lin et al., 2004; Scott et al., 2004; Vargas and Von Zastrow, 2004; Weber et al., 2004; Weigert et al., 2004; Chen et al., 2005; Jovanovic et al., 2006; Barral et al., 2008; Millman et al., 2008; Henriques et al., 2010; Ilić Tomaš et al., 2010; Mahmutefendić et al., 2011; Zuo et al., 2011; Zagorac et al., 2012; Finetti et al., 2014; Hsu et al., 2015; Reineke et al., 2015). In this report, we demonstrate that antibody-based assays may be used for quantitative assessment of both clathrin-dependent and clathrin-independent membrane proteins, with the introduction of several upgrades that were required to fit the cellular physiology of endocytic trafficking and the inclusion of kinetic modeling. Thus, our study contributes to the development of experimental settings needed for further expansion of endocytic recycling research.

First, the antibody-based protocols for monitoring of endocytic recycling should consider the nature of the endocytic uptake of a membrane protein. It has been accepted that the constitutive endocytic uptake occurs by clathrin-coated pits and by a clathrin-independent mechanism (reviewed by Mayor and Pagano, 2007; Maldonado-Báez et al., 2013). Although several membrane proteins were taken into studies, still there is no clear distinction between clathrin-dependent and clathrin-independent membrane cargo proteins (Maldonado-Báez et al., 2013). Recent evidence indicates that at least 95% of cellular endocytic uptake is based on clathrin-coated pits (Bitsikas et al., 2014) and that different cell surface proteins can be sorted into distinct clathrin-coated pits (Mundell et al., 2006; Puthenveedu et al., 2006; Mettlenet al., 2010). Thus, it appears that membrane proteins cannot be put into two categories but rather classified into wider spectrum regarding engagement of endocytic machinery and thereby kinetics of the endocytic uptake. Therefore, intracellular itinerary after endocytic uptake, irrespective of the composition of the pits, will determine the cell surface presence and intracellular routing of a membrane protein. For the purpose of this study, we analyzed two proteins that span opposite strands of this spectrum, the TfR as a paradigm for clathrin-dependent protein and MHC-I proteins as a paradigm for clathrin-independent protein.

The constitutive uptake of clathrin-dependent membrane proteins is rather fast and thereby they do not spend much time at the cell surface (Mousavi et al., 2004; Hao and Maxfield, 2000; McMahon and Boucrot, 2011). The best example of such protein is the TfR, which is a basic molecule used by now to construct the knowledge about recycling pathways. To study recycling of such proteins, it is essential to have a tool that can catch those that reappear at the cell surface. We demonstrated that antibody-based protocols could be used for quantification of recycling of clathrin-dependent cargo proteins. Hover, protocols based on direct (protocol A) and indirect (protocol B) detection of recycled proteins at the cell surface are not suitable for clathrin-dependent proteins since recycled proteins are

rapidly reinternalized which do not allow quantitative assessment of recycling. On the contrary, protocol C, which is based on capturing of mAb-labeled recycled clathrin-dependent proteins while reappearing at the cell surface at 37°C, irrespective whether they will remain at the cell surface or will be re-internalized after one round of recycling, enable assessment of the recycling rate and dissection of recycling. This protocol can directly measure the recycling fraction when AF^{488}-conjugated secondary reagents are used since AF^{488} have stable emission in the acidic environment (Kreft et al., 2007) and does not undergo degradation. However, when FITC-conjugated secondary reagents are used, the protocol should be upgraded either with the introduction of inhibitors of endosomal acidification and degradation or by kinetic modeling because recycled 2^{nd}Ab:1^{st}Ab:protein complexes are rapidly reinternalized into acidic endosomes (LE/lysosomes), which reduces FITC emission (Lanz et al., 1997), and degraded.

In this study, we did not test protocol D on TfR model. However, based on the data with fMHC-I proteins we can conclude that this protocol can also be suitable for quantitative assessment of recycling of clathrin-dependent proteins that do not have useful ligand tool as TfRs. In contrast to many published papers with TfR labeling with the ligand (van Dam et al, 2002; Van Dam and Stoorvogel, 2002; Jovic et al., 2014; Rahbek-Clemmensen et al., 2014; HSU et al., 2015) which has many limitations in studying the route of recycling receptors, recently SS-biotin approach was used to label TfR and to quantify recycled receptors (Majeed et al., 2014; Margadant et al., 2012). However, antibodies were rarely used (Finetti et al., 2014).

Although clathrin-independent proteins spend much more time at the cell surface than clathrin-dependent proteins, our study demonstrates that direct (protocol A) or indirect (protocol B) identification of mAb-bound recycled receptors at the cell surface are less suitable for these proteins and underestimate the recycling fraction, although used in several studies (Barral et al., 2008, Zuo et al., 2011). An explanation for this may be reinternalization of recycled clathrin-independent proteins and their redistribution inside the cell, especially in the rapidly recycling pre-EE circuit. Additionally, the indirect quantification (protocol B) overestimates recycling and discovers recycling of non-recycling proteins (eMHC-I) when degradation is not considered. Although this protocol was used for characterization of the earliest studies of postendocytic transport of MHC-I proteins (Weigert et al., 2004; Jovanovic et al., 2006) they should be used with the modification that includes degradation fraction of proteins. On the contrary, the antibody-capture protocols (protocols C and D), based either on the 2^{nd}Ab capture of 1^{st}Ab-labeled recycling protein (protocols C) or anti-AF^{488} capture of AF^{488}-1^{st}Ab-labeled recycling proteins and fluorescence quenching (protocols D), enable quantitative detection of recycling rate and fraction of clathrin-independent proteins. The protocol C has been used for estimation of recycling of MHC-I proteins (Ilić-Tomaš et al., 2010; Mahmutefendić et al., 2011; Zagorac et al., 2012), chemokine receptor D6 (Weber et al., 2004), and NMDA receptors (Scott et al., 2004).

In addition to the endocytic uptake, the antibody-capture based protocols should be sufficiently sensitive to reveal recycling rate and recycling fraction. Our study demonstrates that antibody-capture protocols of endosomal recycling, which detect the gain (protocol C) or loss (protocol D) of fluorescence determine the recycling rate. However, regarding the recycling fraction, the both protocols should consider the degradation of captured antibody complexes. Our analysis indicates that free mAb-proteins and 2^{nd}Ab-captured complexes do not follow the same route. FITC-2^{nd}Ab-captured complexes were rapidly reinternalized into endosomes distinct from that loaded with mAb-proteins (data not shown) and reach LEs/lysosomes within 15 min (Fig. 5A) and undergo degradation. This route is not well characterized, but may be used for molecular targeting of bioactive molecules to lysosomes and navigating therapeutic application (Moody et al., 2015). We have demonstrated that the problem of degradation of the FITC-

2^{nd}Ab complexes can be overcome by kinetic modeling or by the introduction of inhibitors of lysosomal degradation into recycling protocol.

Our study suggests that the recycling assay based on quenching of the fluorescence (protocol D) may be as good as a 2^{nd}Ab-capture protocol for analysis of recycling routes of both clathrin-dependent and clathrin-independent proteins. However, this protocol requires high-quality primary antibody reagents conjugated with AF^{488}. A major advantage of the recycling assay based on the capture with fluorescent 2^{nd}Ab reagents and the gain of fluorescence (protocol C) is the simplicity (not too many steps that may affect results) and opportunity for quantification using flow cytometry or immunofluorescence without permeabilization.

A concern for the Ab-capture protocols may be the time required for binding of fluorescent 2^{nd}Ab to recycled mAb-protein complexes and some recycled complex that re-internalize rapidly that 2^{nd}Ab does not have enough time to bind may escape detection. Although this option cannot be excluded, it is not very likely to contribute significantly since in our hands and with the combination of primary and secondary antibodies used in our studies, approx. 80-90% was bound within 1 min at 37°C.

Our study also confirms the previous observation that TfR and MHC-I proteins are sorted in distinct endosomal domains or even subsets. Almost all endocytosed TfRs load the recycling early endosomal domain and are returned to the cell surface, whereas a significant fraction of MHC-I proteins is directed into late endosomes. Thus, it appears that there is a mechanism or biophysical property of early endosomes that allows only a fraction of internalized MHC-I proteins to entry into the recycling domain, from which they are rapidly returned to the plasma membrane. Growing evidence challenge the traditional concept of early endosomes as stable sorting station (Gruenberg, 2001; Mellman et al., 1986; Zerial and McBride, 2001) and indicate that early endosomes are functionally heterogeneous and composed of distinct membrane sub-domains within an individual organelle (Hayakawa et al., 2006; Lakadamyali et al., 2006; Miaczynska et al., 2004; Sonnichsen et al., 2000). Therefore, to study mechanisms of recycling it is also important to analyze conditions within the same cells. Immunofluorescence-based protocols, which demonstrate two proteins within the same cell, thus, may be a choice.

Altogether, our study demonstrate that recycling protocols based on labeling of membrane proteins with specific antibodies and capture with antibodies in the second step (protocol C and protocol D) may be used for quantification of the recycling rate and for studies that are focused on the analysis of recycling mechanisms based on the analysis of the recycling rate.

Authors' contributions

G.B.Z. established variants of recycling protocols, carried out internalization and recycling assays (flow cytometric and confocal analysis) on HeLa and J26 cells, and participated in the drafting of the manuscript. H.M. established internalization assays and antibody-capture recycling assays by flow cytometric and confocal analysis. LJ.K. carried out recycling assays. SM performed mathematical modeling and performed statistical analysis. P.L., G.B.Z., and H.M. conceived the study and participated in its design and coordination. P.L. carried image analysis, conceived figure presentation, and drafted the manuscript. All authors read and approved the final manuscript.

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Literature Cited

Barral DC, Cavallari M, McCormick PJ, Garg S, Magee AI, Bonifacino JS De Libero G, Brenner MB. 2008. CD1a and MHC class I follow a similar endocytic recycling pathway. Traffic 9:1446-1457.

Bitsikas V, Corrêa IR, Nichols BJ. 2014. Clathrin-independent pathways do not contribute significantly to endocytic flux. eLife 3:e03970.

Bolte S, Cordeliéres FP. 2006. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224: 213–232.

Ciechanover A, Schwartz AL, Dautry-Varsat A, Lodish HF. 1983. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. J Biol Chem 258:9681–9689.

Chen ZY, Ieraci A, Tanowitz M, Lee FS. 2005. A novel endocytic recycling signal distinguishes biological responses of Trk neurotrophin receptors. Mol Biol Cell 16: 5761-5772.

Dangoria NS, DeLay ML, Kingsbury DJ, Mear JP, Uchanska-Ziegler U, Ziegler A, Colbert RA. 2002. HLA-B27 misfolding is associated with aberrant intermolecular disulfide bond formation (dimerization) in the endoplasmic reticulum. J Biol Chem 277:23459–23468.

Donaldson JG, Williams DB. 2009. Intracellular assembly and trafficking of MHC class I molecules. Traffic 10: 1745–1752.

Dröse S, Altendorf K. 1997. Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J Exp Biol* 200:1-8.

Durrington HJ, Upton PD, Hoer S, Boname J, Dunmore BJ, Yang J, Crilley TK, Butler LM, Blackbourn DJ, Nash GB, Lehner PJ, Morrell NW. 2010. Identification of a Lysosomal Pathway Regulating Degradation of the Bone Morphogenetic Protein Receptor Type II. J Biol Chem 285:37641-37649.

Ferrier P, Layet C, Caillol DH, Jordan BR, Lemonnier FA. 1985. The association between murine beta 2-microglobulin and HLAclass I heavy chains results in serologically detectable conformational changes of both chains. J Immunol 135: 1281–1287.

Finetti F, Patrussi L, Masi G, Onnis A, Galgano D, Lucherini OM, Pazour GJ, Baldari CT. 2014. Specific recycling receptors are targeted to the immune synapse by the intraflagellar transport system. J Cell Sci 127:1924-1937.

Ghosh RN, Mallet WG, Soe TT, McGraw TE, Maxfield FR. 1998. An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. J Cell Biol 142:923-936.

Grant BD, Donaldson JG. 2009. Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10:597-608.

Hao M, Maxfield FR. 2000. Characterization of rapid membrane internalization and recycling. J Biol Chem 275:15279–15286.

Henriques CM, Rino J, Nibbs RJ, Graham GJ, Barata JT. 2010. IL-7 induces rapid clathrin-mediated internalization and JAK3-dependent degradation of IL-7Ralpha in T cells. Blood 115:3269-3277.

Hsu VW, Bai M, Li J. 2012. Getting active: protein sorting in endocytic recycling. Nat Rev Mol Cell Biol 13:323-328.

Hsu F, Hu F, Mao Y. 2015. Spatiotemporal control of phosphatidylinositol 4-phosphate by Sac2 regulates endocytic recycling. J Cell Biol 209:97-110.

Huotari J, Helenius A. 2011. Endosome maturation. EMBO J 30:3481–3500.

Ilić Tomaš M, Kučić N, Mahmutefendić H, Blagojević G, Lučin P. 2010. Murine cytomegalovirus perturbs endosomal trafficking of MHC class I molecules in the early phase of infection. J Virol 84:11101-11112.

Jovanovic OA, Brown FD, Donaldson JG. 2006. An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. Mol Biol Cell 17:327-335.

Jovic M, Kean MJ, Dubankova A, Boura E, Gingras AC, Brill JA, Balla T. 2014. Endosomal sorting of VAMP3 is regulated by PI4K2A. J Cell Sci 127:3745-56.

Klein S, Franco M, Chardin P, Luton F. 2006. Role of the Arf6 GDP/GTP cycle and Arf6 GTPase-activating proteins in actin remodeling and intracellular transport. J Biol Chem 281:12352-12361.

Kreft O, Javier AM, Sukhorukov GB, Parak WJ. 2007. Polymer microcapsules as mobile local pH-sensors. J Mater Chem 42:4471-4476.

Lakadamyali M, Rust MJ, Zhuang X. 2006. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124:997–1009.

Lanz E, Gregor M, Slavik J, Kotyk A. 1997. Use of FITC as a fluorescent probe for intracellular pH measurement. J Fluorescence 7: 317-319.

Li X, Lavigne P, Lavoie C. 2015. GGA3 mediates TrkA endocytic recycling to promote sustained Akt phosphorylation and cell survival. Mol Biol Cell 26:4412–4426.

Lin SX, Mallet WG, Huang AY, Maxfield FR. 2004. Endocytosed cation-independent mannose 6-phosphate receptor traffics via the endocytic recycling compartment en route to the trans-Golgi network and a subpopulation of late endosomes. Mol Biol Cell 15:721-733.

Mahmutefendić H, Blagojević G, Ilić Tomaš M, Kučić N, Lučin P. 2011. Segregation of open Major Histocompatibility class I conformers at the plasma membrane and during endosomal trafficking reveals conformation-based sorting in the endosomal system. Int J Biochem Cell Biol 43:504-515.

Mahmutefendić H, Blagojević G, Grabušić K, Karleuša Lj, Maćešić S, Momburg F, Lučin P. 2016. Late endosomal recycling of open MHC-I conformers. J Cell Physiol, in press

Majeed SR, Vasudevan L, Chen CY, Luo Y, Torres JA, Evans TM, Sharkey A, Foraker AB, Wong NM, Esk C, Freeman TA, Moffett A, Keen JH, Brodsky FM. 2014. Clathrin light chains are required for the gyrating-clathrin recycling pathway and thereby promote cell migration. Nat Commun 5:3891.

Maldonado-Báez L, Williamson C, Donaldson JG. 2013. Clathrin-independent endocytosis: A cargo-centric view. Exp Cell Res 319:2759–2769.

Margadant C, Kreft M, de Groot DJ, Norman JC, Sonnenberg A. 2012. Distinct Roles of Talin and Kindlin in Regulating Integrin α5β1 Function and Trafficking. Cur Biol 22:1554-1563

Maxfield FR, McGraw TE. 2004. Endocytic recycling. Nat Rev Mol Cell Biol 5:121-132.

Mayor S, Pagano RE. 2007. Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol 8:603–612.

Matsui T, Fukuda M. 2014. Methods of analysis of the membrane trafficking pathway from recycling endosomes to lysosomes. Methods Enzymol 534:195-206.

McCloy RA, Rogers S, Caldon CE, Lorca T, Castro A, Burgess A. 2014. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. Cell Cycle 13: 1400-1412.

McMahon HT, Boucrot E. 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12:517-533.

Mettlen M, Loerke D, Yarar D, Danuser G, Schmid SL. 2010. Cargo- and adaptor-specific mechanisms regulate clathrin-mediated endocytosis. J Cell Biol 188:919-933.

Millman EE, Zhang H, Zhang H, Godines V, Bean AJ, Knoll BJ, Moore RH. 2008. Rapid recycling of beta-adrenergic receptors is dependent on the actin cytoskeleton and myosin Vb. Traffic 9:1958-1971.

Moody PR, Sayers EJ, Magnusson JP, Alexander C, Borri P, Watson P, Jones AT. 2015. Receptor crosslinking: a general method to trigger internalization and lysosomal targeting of therapeutic receptor:ligand complexes. Mol Ther 23:1888-1898.

Mundell SJ, Luo J, Benovic JL, Conley PB, Poole AW. 2006. Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. Traffic 7:1420-1431.

Mukherjee S, Ghosh RN, Maxfield FR. 1997. Endocytosis. Physiol Rev 77:759-803.

Panchuk–Voloshina N, Haugland RP, Bishop–Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung WJ, Haugland RP. 1999. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. J Histochem Cytochem 47:1179-1188.

Park M, Penick EC, Edwards JG, Kauer JA, Ehlers MD. 2004. Recycling endosomes supply AMPA receptors for LTP. Science 305:1972-1975.

Puthenveedu MA, von Zastrow M. 2005. Cargo regulates clathrin-coated pit dynamics. Cell 127:113-124.

Rahbek-Clemmensen T, Bay T, Eriksen J, Gether U, Jørgensen TN. 2014. The serotonin transporter undergoes constitutive internalization and is primarily sorted to late endosomes and lysosomal degradation. J Biol Chem 289:23004-23019.

Reineke JB, Xie S, Naslavsky N, Caplan S. 2015. Qualitative and quantitative analysis of endocytic recycling. Methods Cell Biol 130:139-155.

Sandvig K, Pust S, Skotland T, van Deurs B. 2011. Clathrin-independent endocytosis: mechanisms and function. Curr Opin Cell Biol 23:413–420.

Scott DB, Michailidis I, Mu Y, Logothetis D, Ehlers MD. 2004. Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. J Neurosci 24:7096-7109.

Stam NJ, Spits H, Ploegh HL. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. J Immunol 137:2299–2306.

Tanowitz M, von Zastrow M. 2003. A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. J Biol Chem 278:45978-45986.

van Dam EM, Stoorvogel W. 2002. Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. Mol Biol Cell 13:169-182.

Vargas GA, Von Zastrow M. 2004. Identification of a novel endocytic recycling signal in the D1 dopamine receptor. J Biol Chem 279:37461-37469.

Weber M, Blair E, Simpson CV, O'Hara M, Blackburn PE, Rot A, Graham GJ, Nibbs RJ. 2004. The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. Mol Cell Biol 15:2492–2508.

Weigert R, Yeung AC, Li J, Donaldson JG. 2004. Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. Mol Biol Cell 15:3758–3770.

Zagorac GB, Mahmutefendić H, Ilić Tomaš M, Kučić N, Le Bouteiller P, Lučin P. 2012. Early endosomal rerouting of Major Histocompatibility Class I conformers. J Cell Physiol 227:2953–2964.

Zuo J, Quinn LL, Tamblyn J, Thomas WA, Feederle R, Delecluse HJ, Hislop AD, Rowe M. 2011. The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways. J Virol 85:1604-1614.

Figure Legends

Figure 1. **Kinetic parameters of intracellular trafficking of MHC-I and TfR.**

(**A**) Flow cytometric profiles of cell surface expression of transferrin receptor (TfR) and fully conformed MHC-I proteins (fMHC-I) in untreated HeLa and J26-Cw6 cells. ΔMFI represents the difference between mean fluorescence intensities (MFI) of specific mAb-stained (blue histograms) and unspecific mAb-stained cells (white histograms). (**B**) Cellular distribution of TfRs and fMHC-I in untreated HeLa and J26Cw6 cells (**C**) Kinetic modeling of cell surface and inter endosomal distribution of internalized TfR and fMHC-I proteins in J26-Cw6 and HeLa cells, respectively. Multicompartment model is shown in the left panel and the kinetic modeling on the right. Screenshot of the modeling software shows relative distribution of mAb-labeled TfR and fMHC-1 after internalization. The modeling was based on experimental data. The data (◊) represent the mean values ± S.D. of cell surface expression of mAb-bound TfR and fMHC-I after internalization obtained in 11 and 9 independent experiments, respectively. Kinetic parameters (rate constants and the onset of transition between compartments) of distribution shown in the modeling software screenshots are presented in tables.

Figure 2. **mAb-labeling based recycling protocols.**

Cell surface proteins were labeled with specific unconjugated (protocol A-C) or AF^{488}-conjugated (protocol D) mAbs by continuous exposure to mAbs at 37°C for 60 min. Un-internalized mAbs were acid stripped (pH 2.0 for 1 min), and cells were incubated at 37°C up to 60 min for recycling. Recycled mAb-labeled proteins were detected by flow cytometry either directly, by detection of recycled complexes at the cell surface using AF^{488}-2^{nd}Abs (protocol A), or indirectly, by detection of recycled proteins by measurement of the intracellular pool that remain after acid stripping of recycled mAb-bound complexes from the cell surface (protocol B), or by capture of all recycled mAb-bound complexes at 37°C that reach the cell surface by AF^{488}-2^{nd}Abs (protocol C). Recycled AF^{488}-primary mAs-labeled proteins were captured by anti-AF^{488} mAbs at 37°C and the loss of fluorescence by quenching was quantified by flow cytometry (protocol D). For details see *Material and Methods*.

Figure 3. **Quantification of TfR recycling by antibody reagent-based protocols.**

(A) Kinetics of TfR recycling determined by release of the fluorescent ligand (AF^{488}-Tf). J26-Cw6 and HeLa cells were incubated 60 min with AF^{488}-Tf at 37°C (internalization) and the loss of the intracellular fluorescence (recycling) determined by flow cytometry. Data represent the mean values ± S.D. from independent experiments (n=6 for J26-Cw6 cell and n=9 for HeLa). The curve integrates first-order kinetics based on the integrated recycling rate constant (k_r) and recycling fraction (RF). **(B)** Kinetics of TfR recycling determined by the mAb-based protocol of recycling. J26-Cw6 cells were incubated 60 min with R17 mAbs (internalization), un-internalized mAbs were acid stripped, and cells were incubated at 37°C up to 60 min (recycling). Percentage of recycled receptors was determined by direct (protocol A), and indirect (protocol B) flow cytometric detection of recycled mAb:TfR using AF^{488}-anti-rat-IgG. Data represent the mean value ± S.D. from 4 independent experiments. (**C**) Kinetics of TfR recycling determined by the antibody-capture protocol C. TfRs were labeled with R17 mAbs as described above and mAb-bound recycling receptors captured by the FITC-anti-rat IgG at 37°C. The curve integrates the first-order kinetics based on integrated recycling

rate constant (k_r) and recycling fraction (RF) with (full line) and without (dashed line) the degradation rate constant of reinternalized recycled complexes (k_d). Data represent the mean value ± S.D. from 5 independent experiments. (**D**) Kinetic modeling of TfR recycling under the conditions of protocol C, using the data obtained in the Protocol C and kinetic parameters of TfR cycling presented in Fig. 1C. The light blue line represents calculated recycling kinetic, the dashed line first-order rate reaction and the red line kinetics of detection of the 2^{nd}-Ab captured receptors with the efficiency of 0.55 min^{-1} and degradation of internalized 2^{nd}Ab-captured complexes after 20 min with the rate of 0.012 min^{-1}. Shown is the relative contribution of EE compartments in recycling.

Figure 4. **Quantification of recycling of MHC-I proteins using antibody-based recycling protocols.**

The four protocols described in Fig. 2 were tested on HeLa cells. The recycling of fully conformed MHC-I (fMHC-I) and open MHC-I conformers (eMHC-I) was quantified using flow cytometry. The curve integrate the first-order kinetics based on integrated recycling rate constant (k_r), recycling fraction (RF) and either endocytic rate constant (k_e) in protocol A or degradation rate constant of reinternalized recycled complexes (k_d) in protocols C and D. Data represent the mean value ± S.D. from at least 4 independent experiments.

Figure 5. **Analysis of antibody-based recycling protocol C**

(**A**) Loss of fluorescence after internalization of FITC-2^{nd}-Ab captured complexes. Recycling of mAb:fMHC-I complexes was determined by the capture with FITC-2^{nd}Ab (Protocol C) and under the same experimental conditions determined the onset and the rate of degradation of FITC-2^{nd}Ab:1^{st}Ab:MHC-I complexes formed at the cell surface at 4^{o}C. The data represent the mean ± S.D. from three independent experiments. (**B**) Effect of inhibition of lysosomal degradation on the outcome of recycling protocol C. Recycling of mAb:fMHC-I was determined by the FITC-2^{nd}Ab capture (Protocol C) in the presence of the PICT, lysosomal protease inhibitors cocktail, or 100 nM of Concanamycin A (ConA,). Data represent integral representative experiment (n=3). (**C**) Comparison of AF488- and FITC-2^{nd}Abs in the antibody capture. The protocol C was performed under identical conditions using AF^{488}- or FITC-anti-mouse IgG. Data represent integral representative experiment (n=3). (**D**) AF^{488} does not degrade in lysosomes. Cells were incubated with AF^{488}-EGF (2.5 µg/ml) for 50 min and degradation was quantified by flow cytometry on saponin (0.05 %) permeabilized and non-permeabilized (untreated) cells. Data represent integral representative experiment (n=3). (**E**) Kinetic modeling of MHC-I recycling under the conditions of protocol C, using the data obtained in the Protocol C and kinetic parameters of MHC-I cycling presented in Fig. 1C. The cell surface level was set up to 0 (simulation of the acid wash) and cycling throughout the endosomal system continued with the same kinetic parameters (k_1-k_7) and the introduction of k_8 which represents the rate constant of entry from LEs into lysosomes (degradation). The light blue line represents calculated recycling kinetic, the dashed line first-order rate reaction and the red line kinetics of detection of the 2^{nd}Ab captured complexes with the efficiency of 0.55 min^{-1} and degradation of reinternalized 2^{nd}Ab-captured complexes after 20 min with the rate of 0.011 min^{-1}. Also, shown is the simulation of the relative distribution of mAb:fMHC-I in endosomal compartments over 60 min.

Figure 6. **Comparison of the recycling protocol C and D**

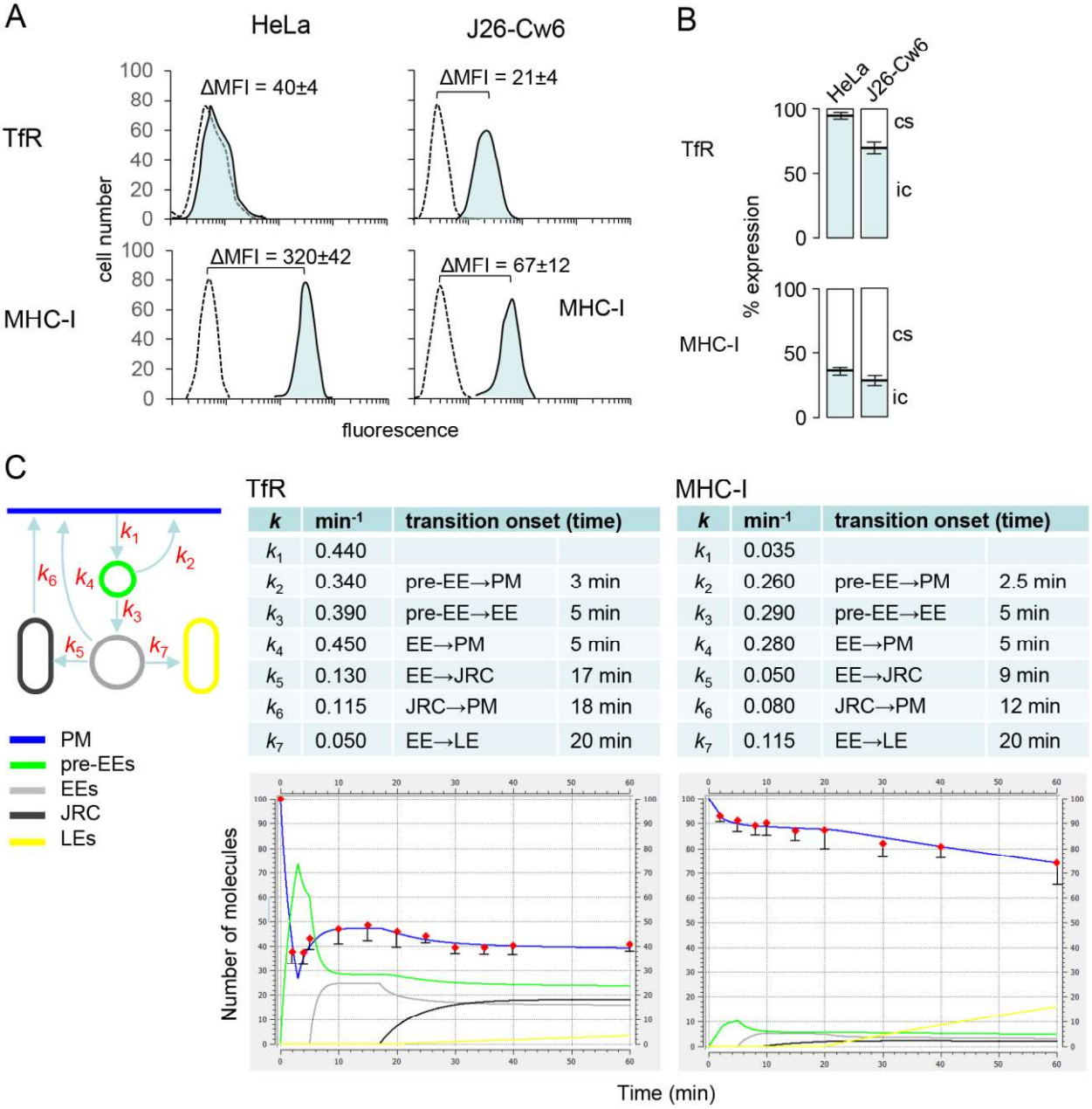
Comparison of recycling kinetic obtained by the protocol C under the presence of ConA, using FITC-2ndAbs, and protocol D. Data represent integral representative experiment (n=3).

Figure 7. **mAb-bound MHC-I proteins stably cycle in the early endosomal system**.

(A) Experimental design. HeLa cells were incubated for 60 min at 37°C with mAbs W6/32 (5ug/ml) to label cell surface and an early endosomal pool of MHC-I. After 60 min unbound mAbs were washed and cells incubated in mAb-free medium (chase). After various periods of the chase, the mAbs exposed at the cell surface were removed by the acid wash and cells incubated for 30 min in medium containing AF^{488}-anti-mouse IgG to capture recycled mAb:fMHC-I. (**B**) Flow cytometric quantification of intracellular mAb:fMHC-I on permeabilized cells. Data represent the mean ΔMFI ± S.D. of three independent experiments. (**C**) Quantification of 30-min recycling efficiency, determined by the antibody capture protocol (protocol C), after various periods of the chase. Data represent the mean ΔMFI ± S.D. of three independent experiments.

Figure 8. **Quantification of recycling of Clathrin-dependent and clathrin-independent cargo proteins by immunofluorescence assay within the same cell.**

(A) Simultaneous recycling of mAb-labeled MHC-I and AF^{488}-Tf labeled TfR. HeLa cells were 60 min incubated with W6/32 mAbs and AF^{488}-Tf at 37^{o}C (*internalization*). After brief acid wash, cells were incubated with AF^{555}-anti-mouse IgG$_{2a}$ for 60 min (*recycling*) and processed for confocal analysis without permeabilization. Recycling of TfR was quantified as loss of green fluorescence and recycling of MHC-I proteins as the accumulation of red fluorescence within the same cell. Stacked confocal images were quantified by Image J software. **(B)** *Quantification of recycling based on the immunofluorescence imaging*. Data represent the mean value ± S.D. Kinetics of recycling of TfR and fMHC-I obtained by the immunofluorescence assay described above. **(C)** Simultaneous recycling of mAb-labeled MHC-I and TfR on J26-Cw6 cells. Internalized TfRs and fMHC-I were labeled with specific mAbs for 60 min at 37°C, un-internalized mAbs acid stripped and cells incubated with AF^{488}-anti-rat IgG and AF^{555}-anti-mouse IgG$_{2a}$ for 60 min at 37°C. (**D**) Quantification of recycling of mAb-labeled TfRs and fMHC-I proteins by immunofluorescence and image analysis. Both TfR and MHC-I recycling was quantified on confocal images as the accumulation of green (TfR) and red (MHC-I) fluorescence within the same cell. Stacked confocal images were quantified by Image J software. TCCF, total corrected cell fluorescence.



TfR

k	min^{-1}	transition onset (time)	
k_1	0.440		
k_2	0.340	pre-EE→PM	3 min
k_3	0.390	pre-EE→EE	5 min
k_4	0.450	EE→PM	5 min
k_5	0.130	EE→JRC	17 min
k_6	0.115	JRC→PM	18 min
k_7	0.050	EE→LE	20 min

MHC-I

k	min^{-1}	transition onset (time)	
k_1	0.035		
k_2	0.260	pre-EE→PM	2.5 min
k_3	0.290	pre-EE→EE	5 min
k_4	0.280	EE→PM	5 min
k_5	0.050	EE→JRC	9 min
k_6	0.080	JRC→PM	12 min
k_7	0.115	EE→LE	20 min

Figure 1

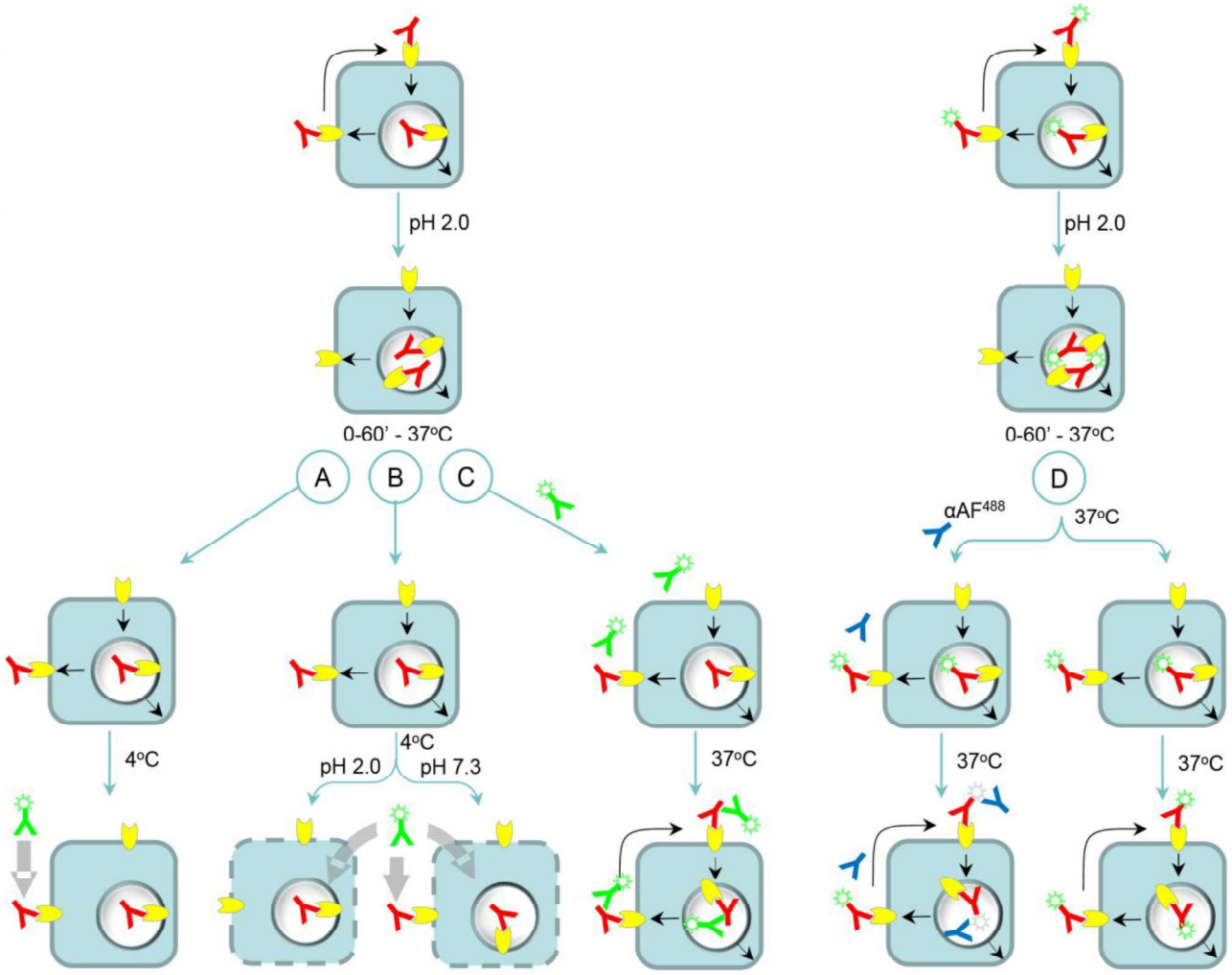


Figure 2

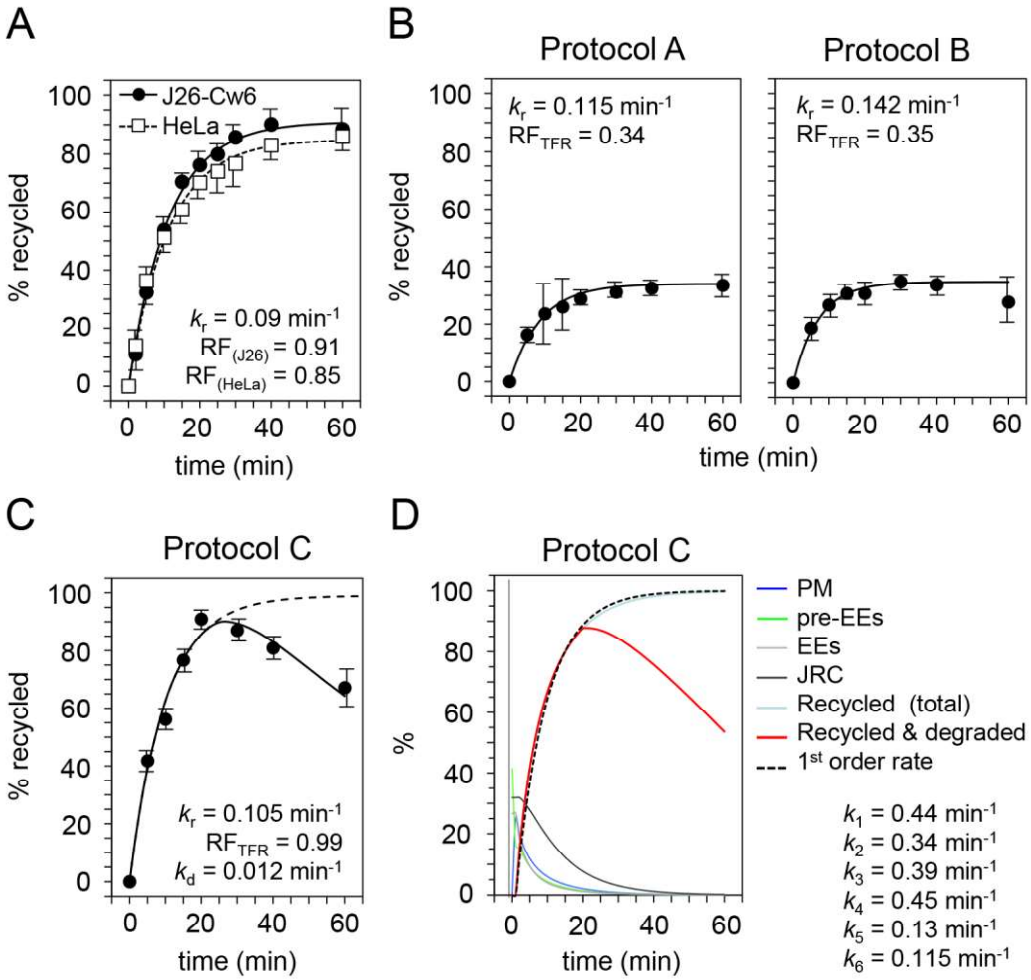


Figure 3

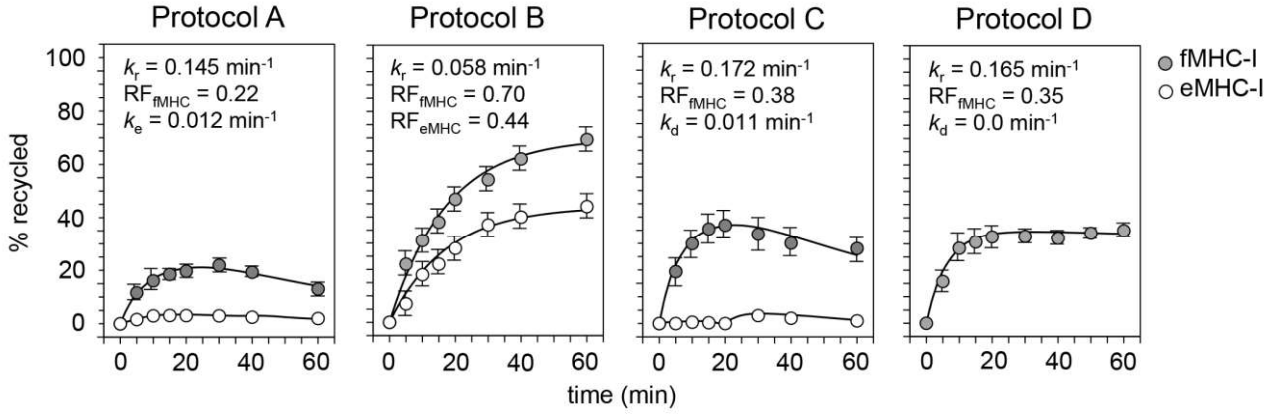


Figure 4

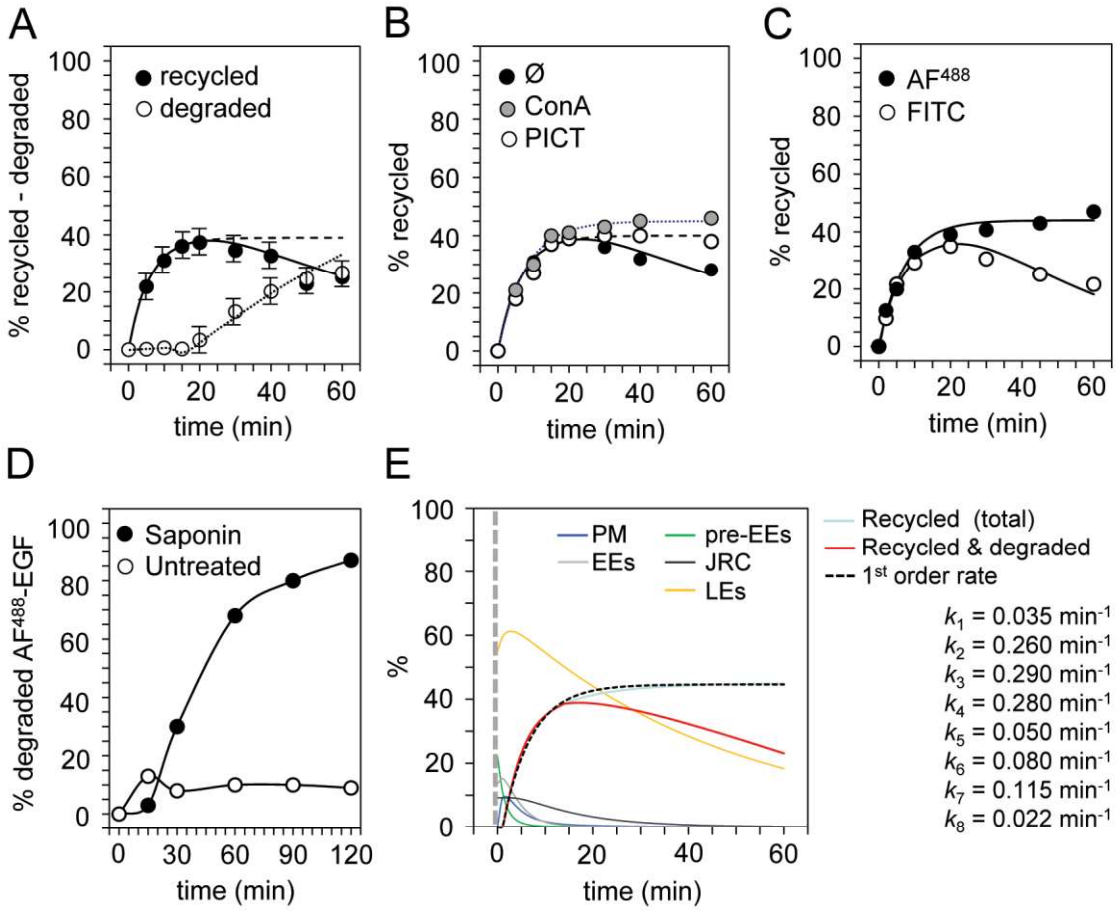


Figure 5

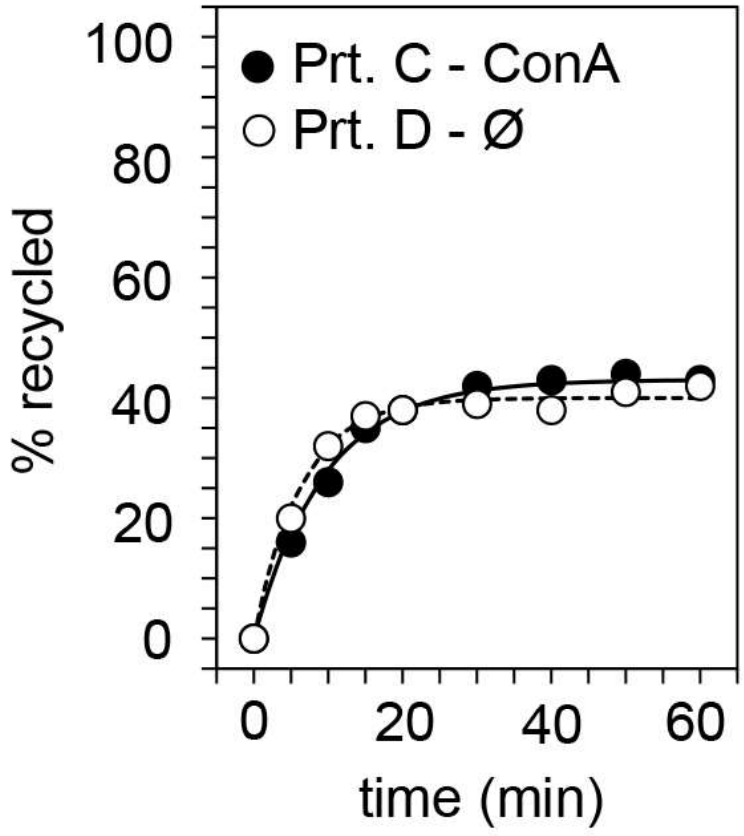


Figure 6

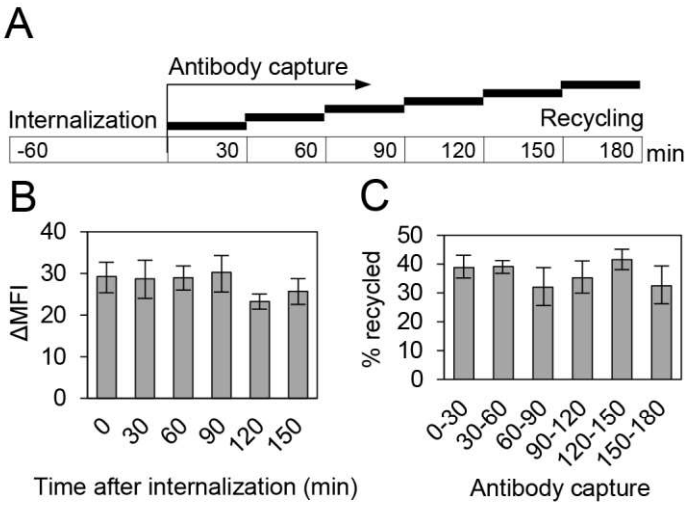


Figure 7

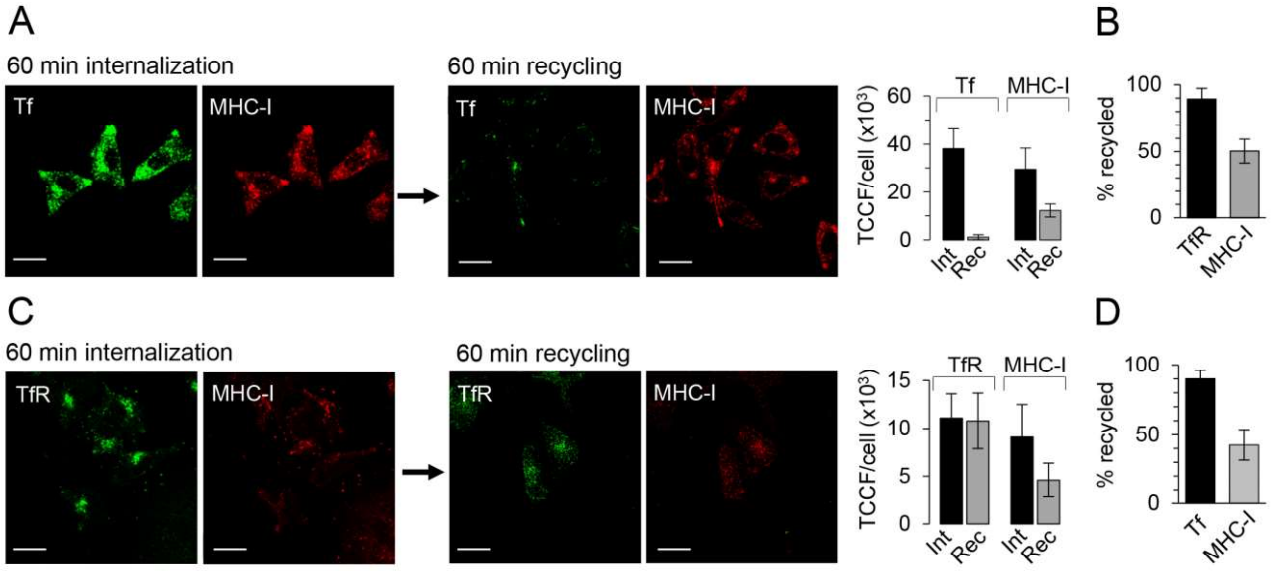


Figure 8