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SHORT COMMUNICATION

Direct Visualization of Vesicle Maturation and Plasma Membrane Protein Trafficking

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Abstract Internalization and intracellular trafficking of membrane proteins are now recognized as essential mechanisms that contribute to a number of cellular processes. Current methods lack the ability to specifically label the plasma membrane of a live cell, follow internalization of labeled membrane molecules, and conclusively differentiate newly formed membrane-derived vesicles from pre-existing endocytic or secretory structures in the cytoplasm. Here, we detail a visualization method for surface biotinylation of plasma membrane-derived vesicles that allows us to follow their progress from membrane to cytosol at specific time points. Using the transmembrane receptor RET as a model, we demonstrate how this method can be applied to identify plasma membrane-derived vesicle maturation, determine RET's presence within these structures, and monitor RET's recycling to the cell surface. This method improves on static and less discriminatory methods, providing a tool for analysis of real-time vesicle trafficking that is applicable to many systems.

Keywords Membrane protein · Internalization · Recycling · Biotinylation · RET

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Introduction

The demand for sensitive methods to study endocytosis has been one of the driving forces behind many recent advances in cell- and tissue-based imaging technology. Internalization of membrane proteins is no longer viewed as simply an 'off' switch that targets active molecules to the lysosome, but is recognized as an intrinsic element of cellular signalling and other processes (reviewed in [1]). The canonical mechanism of Receptor Mediated Endocytosis (RME), whereby activated transmembrane receptors are internalized through clathrin coated pits, sorted to early, late, and recycling endosomes, and eventually delivered to the lysosome or back to the cell membrane (reviewed in [2]), occurs within the cell in four dimensions and is ideally investigated by direct microscopic visualization.

Traditionally, plasma membrane protein internalization and trafficking has been visualized by colocalization of the protein of interest with known markers of specific endosomal structures. Colocalization is determined either by multi-colour immunofluorescent staining of fixed samples, or using fluorescent fusion proteins in live cells. Although these methods yield indirect evidence of a protein's subcellular localization, they are unable to visualize newly formed membrane vesicles as they are internalized from the membrane and trafficked to their final destination within the cell.

Biotinylated cell surface molecules have been shown to accumulate over time in cytoplasmic vesicles that have been inferred, but not proven, to be components of the endocytic pathway [3]. As well, poor resolution during the imaging of this process requires the user to allow internalization to occur over a relatively long period of time, ensuring the accumulation of a large number of

internalized vesicles. This, in turn, prevents an appreciation of the rapidity with which the internalization process takes place and a lack of continuity between experiments as the chosen time points can vary widely. Here, we utilize biotinylation of cell surface molecules as a label for visualization of plasma membrane-derived vesicles as they traffic from the membrane to the cytoplasm. Triple fluorophore staining (biotinylated vesicle, protein of interest, and endosomal marker) allows the internalization and maturation of plasma membrane-derived vesicles to be studied by determining both the nature of the vesicle and the presence of a protein of interest within it. Further, this method eliminates the inclusion of secretory vesicles, or vesicles formed during fusion events between the secretory and endocytic pathways, from the final analyses, as these do not contain a biotin label.

Methods and results

Trafficking of the transmembrane receptor tyrosine kinase RET post-activation was used as a model to demonstrate the effectiveness of this method. We have previously shown RET to be internalized by RME after activation by its ligand and co-receptor GDNF and GFRα1, respectively [4, 5]. HELA cells, plated on glass coverslips coated with 0.2% gelatin, were transiently transfected with expression constructs for RET and GFRα1 [6]. Twenty-four hours after transfection, cells were serum starved for 2 h and incubated with 100 μg/mL of the translation inhibitor cyclohexamide (CHX) for 30 min. Cells were biotinylated at 4°C (to inhibit endocytosis), as previously described [7], using 1 mM EZ Link NHS-SS-Biotin (Fisher, Nepean, ON, Canada). Serum-free growth medium, supplemented with 100 μg/mL CHX and 100 ng/mL GDNF (Peprotech, Rocky Hill, NJ, USA), was added, and cells were returned to a 37°C incubator. Cells were incubated for the indicated amounts of time, fixed, permeabilized, and blocked, as previously described [8]. Cells were incubated with Alexa 594 conjugated Streptavidin to detect biotin-labelled molecules (1:200 dilution in 3% BSA; Invitrogen, Burlington, ON, Canada) and Hoechst 33342 nuclear stain (1:1,000 dilution; Invitrogen) for 45 min. Coverslips were mounted in MOWIOL (EMD, Gibbstown, NJ, USA) and images captured using a Leica TCS-SP2 inverted confocal microscope. Figure 1a displays data captured at 0, 5, 15, 30, and 60 min after biotinylation, showing an accumulation of intracellular vesicles of increasing intensity over time.

Sub-diffraction microscopy has suggested that traditional co-localization studies may over-estimate the spatial proximity of two fluorophores due to the optical diffraction introduced by widefield and confocal microscopes [9]. Therefore, to reduce error introduced by optical diffraction, we used a small pinhole size of 1 airy disc (equivalent to 91 μm), a high numerical aperture (NA=1.4) 100× Plan-Apo objective that produces minimal spherical aberration, and performed deconvolution on raw data images using the 'Iterative Deconvolve 3D' plug-in (Dr. Robert Dougherty, OptiNav, Redmond, WA, USA) for the freeware imaging program, Image J [10]. Theoretical Point Spread Functions (PSFs) were determined using the 'Diffraction PSF 3D' plug-in for Image J (Dr. Robert Dougherty, OptiNav) and the settings outlined in Supplemental Table 1. Deconvolved images are shown next to the raw images in Fig. 1a. For all remaining figures, only deconvolved images are shown.

As strong membrane staining has the potential to interfere with the identification of vesicles near the cell surface and small vesicles containing limited fluorescence, a modified procedure was used to increase internalization and improve resolution of biotinylated vesicles. First, to increase the number of vesicles, 10% fetal bovine serum (Sigma, Oakville, ON, Canada) was added to cultures during the internalization step. Second, because EZ Link NHS-SS-Biotin contains a thio-cleavable disulphide bond, residual surface biotin can be removed by washing cells twice for 15 min at 4°C with MeSNa buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.6, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 50 mM MeSNa (Sigma)) after internalization and prior to fixation. Figure 1b shows that addition of 10% fetal bovine serum induced more rapid internalization, as vesicles were visible after only five minutes, and removal of surface biotin made the vesicles present easier to resolve. The addition of FBS during the internalization step appears to be beneficial if non-specific membrane internalization is the goal. However, in our system, serum-free medium was essential to ensure internalization was specifically due to GDNF, not any other soluble factors present in FBS.

In Fig. 2a, immunofluorescent staining was used to identify colocalization of RET, and two markers of endocytic structures with which RET has previously been shown to colocalize, EEA1 (early endosomes, [4]) and LAMP2 (lysosomes, [5]). After permeabilization and blocking, cells were incubated with primary antibodies to RET (Santa Cruz Biotechnologies, Santa-Cruz, CA, USA), and either EEA1 (BD Biosciences, Mississauga, ON, Canada) or LAMP2 (Santa-Cruz) diluted 1:100 in 3% BSA, for 45 min. Cells were washed 3× with PBS and further incubated with anti-goat Alexa 488 (RET) and anti-mouse Alexa 546 (EEA1 or LAMP2) conjugated antibodies (1:200 dilution in 3% BSA; Invitrogen) along with Alexa 594 streptavidin, as indicated above. After deconvolution, co-localization of RET and EEA1 or LAMP2 was clearly visible in some, but not all, biotin positive vesicles as confirmed by pixel intensity plots (Fig. 2a).

The addition of CHX to the medium before and during internalization inhibits de novo synthesis of membrane

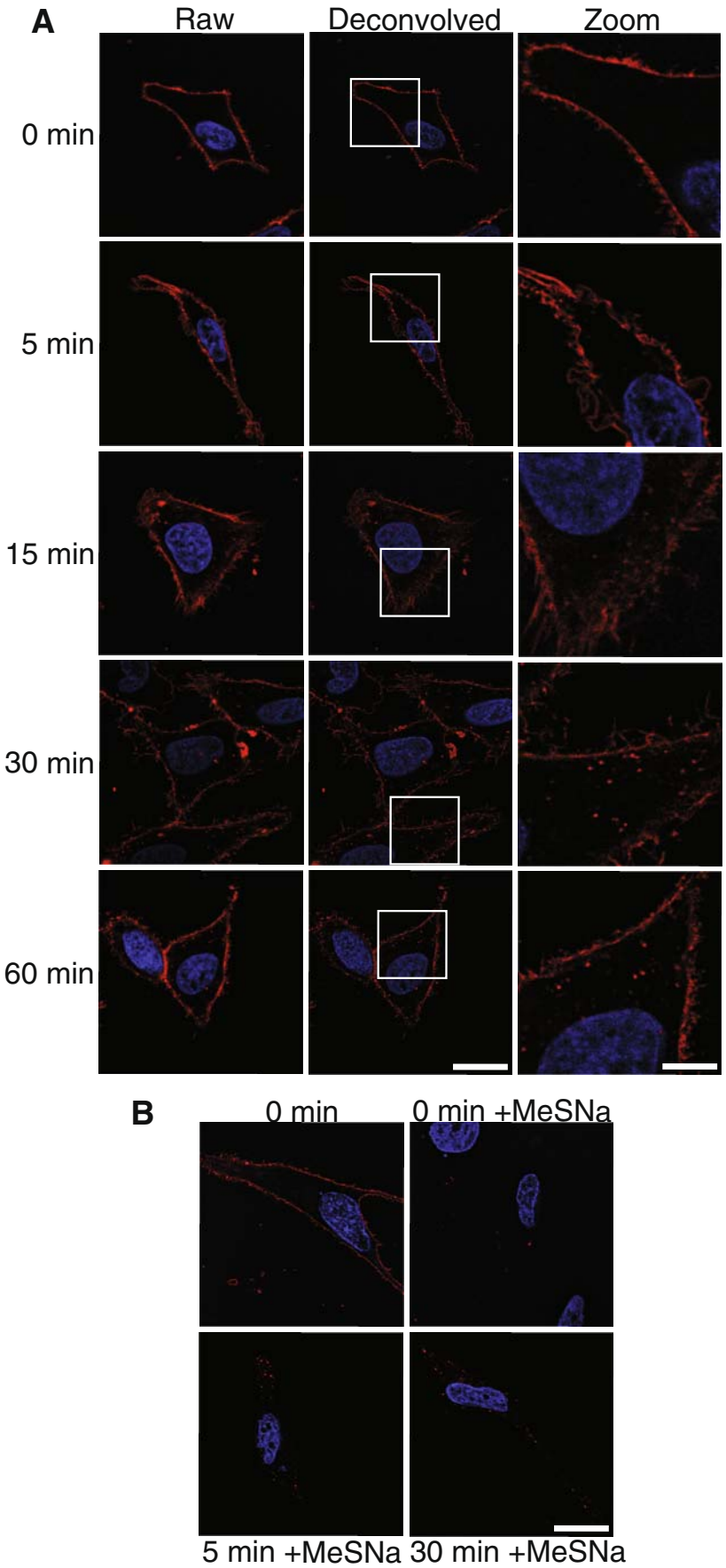

Fig. 1 Internalization of cell surface proteins. **a** HELA cells, transiently transfected with RET and GFRα1 constructs were serum starved for 2 h, incubated with CHX for 30 min, surface biotinylated, then incubated at 37°C for 0, 5, 15, 30 or 60 min. After fixation, cells were stained with Alexa 594 labeled streptavidin to detect biotin-labeled molecules (*red*) and Hoechst nuclear counterstain (*blue*). Images on the left are raw data captured by confocal microscopy. Images in the middle column have been deconvolved. Images in the Zoom column are enlargements of the white boxes in the corresponding Deconvolved image. **b** HELA cells were processed as in **a**. Where indicated (+MeSNa), remaining surface biotin was stripped using the MeSNa reducing agent after internalization, before fixation. All images in **b** were deconvolved. Scale bars, 7 μm in Zoom images; 20 μm in all other panels

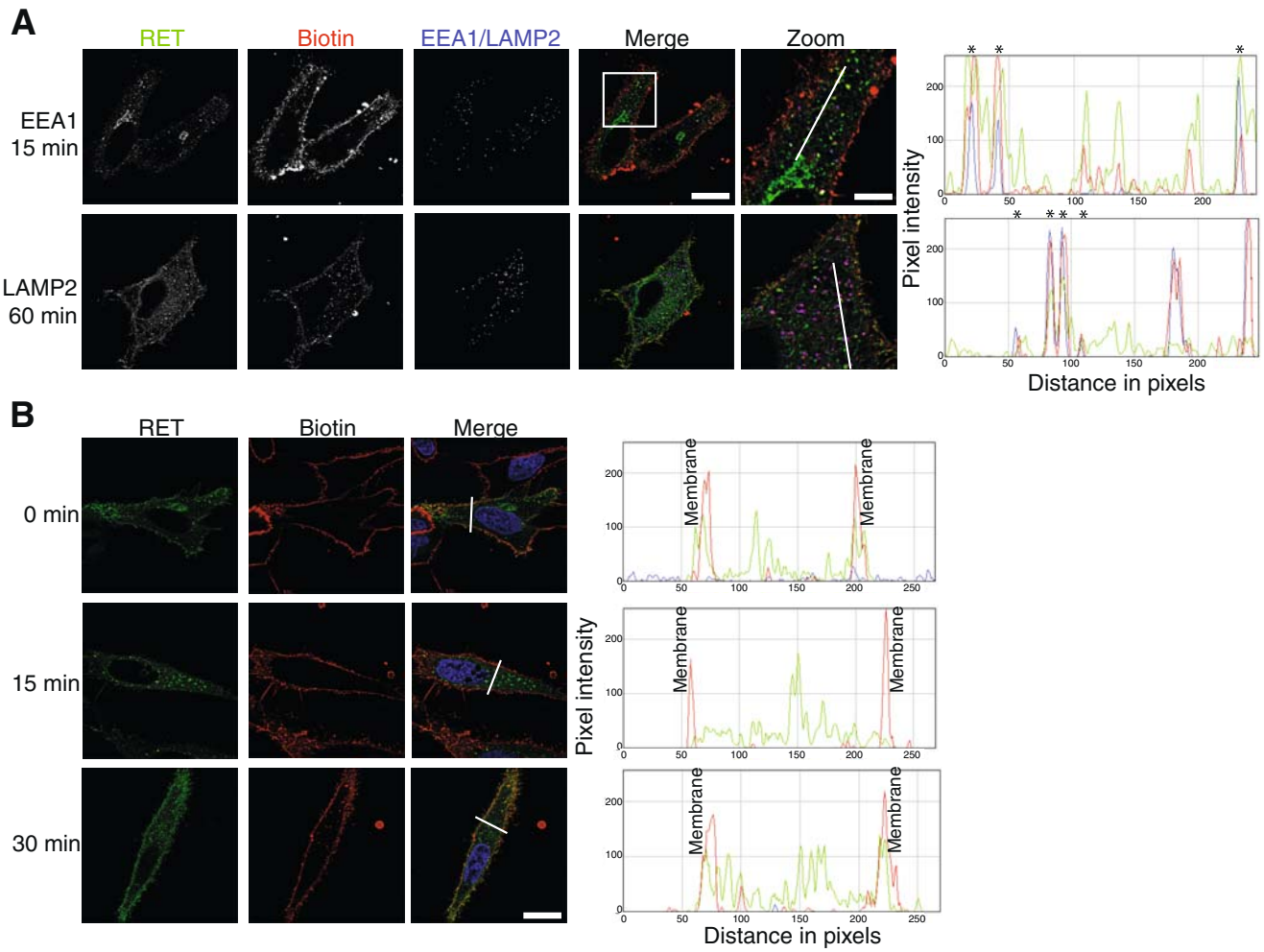

Fig. 2 Assessing colocalization of RET with biotin positive vesicles and markers of endocytic vesicles. **a** HELA cells were processed as in Fig. 1a. Along with fluorescent streptavidin staining to detect biotin-labeled molecules (*red*), cells were immunostained for RET (*green*) and either the early endosome marker EEA1 or the lysosomal marker LAMP2 (*blue*). Merge represents a composite image of all three channels, the Zoom column displays an enlargement of the boxed area in Merge. Pixel intensity plots for all three channels along the lines in the Merge image are displayed. *, indicates vesicles in which all three fluorophores colocalize. **b** HELA cells were processed as in Fig. 1a and stained for biotin (*red*), RET (*green*), and counterstained with Hoechst (*blue*). Pixel intensity plots for all three channels along the lines in the Merge image are displayed. Fluorescent signal originating from the plasma membrane is indicated. Scale bars, 7 μm in Zoom images, 20 μm in all other panels

proteins, which allows easy assessment of membrane protein recycling via this methodology. In Fig. 2b, cells were biotinylated and surface proteins allowed to internalize, as described above. Surface biotin was not stripped and fixed cells were stained for RET and biotin and counterstained with Hoechst. Strong colocalization of RET with membrane bound biotin can be seen in the 0 and 30 min time points, but is absent at 15 min (Fig. 2b). This is the first observation suggesting RET is quickly internalized, but is partially recycled back to the cell surface as has been shown with other receptor tyrosine kinases [11–13]. The presence of CHX in culture ensures the return of RET to the cell surface is not due to de novo synthesis of RET molecules. This method represents a quick and easy procedure to measure receptor recycling in comparison to traditional, more complicated recycling assays requiring multiple stripping steps in conjunction with western blotting [14].

Summary and applications

Together, the methods presented here allow the user to acquire an accurate representation of the RME and recycling processes by removing confounding contributions from the complex and promiscuous network of interacting intracellular vesicles. These methods are of potential use for studying all forms of internalization, not just RME, as the

high-resolution images attained here will assist in the study of synaptic release and uptake, viral fusion and budding, and mechanisms of non-clathrin mediated endocytosis; processes that occur on a scale of hundreds of nanometers. Intracellular trafficking occurs in four dimensions and the ability to visualize internalization in a live cell-based system using this methodology and the applicable 3D and 4D reconstruction software is exciting. The use of streptavidin conjugated non-protein photoswitchable fluorophores to track the internalization of sub-sets of the membrane, or certain vesicles in particular relative to others, in real-time is another exciting potential application. Ultimately, studies using these methods and variations and improvements thereof, will provide an unmatched level of visualization, and future insights into intracellular trafficking.

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