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RUSH-synchronized secretion to measure local exocytosis

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RUSH-synchronized secretion to measure local exocytosis

Abstract

Proteins destined to be exposed to the extracellular space enter the secretory pathway at the level of the endoplasmic reticulum. Proteins are then transported to the Golgi apparatus and addressed to their destination compartment, such as the plasma membrane for exocytic cargos. Exocytosis constitutes the last step of the anterograde transport of secretory cargos. Exocytic vesicles fuse with the plasma membrane releasing soluble proteins to the extracellular milieu and transmembrane proteins to the plasma membrane. In order to monitor local exocytosis of cargos, we describe in this chapter how to perform synchronization of the anterograde transport of an exocytic cargo of interest using the Retention Using Selective Hooks (RUSH) assay in combination with Selective Protein Immobilization (SPI). SPI is based on the coating of coverslips with anti-GFP antibodies, which capture the GFP-tagged RUSH cargos once exposed to the cell surface after its release by the addition of biotin.

Keywords (5-10):

exocytosis, secretory transport, RUSH, antibody coating, protein immobilization, real-time imaging

1. Introduction

Exocytosis is the last step of the anterograde transport of proteins destined to the cell surface through the secretory pathway. It represents an essential step to ensure cell homeostasis such as the release of growth factors, the deposition of extracellular matrix components or the expression of surface receptors. The exocytosed cargos can be proteins either soluble, transmembrane or glycosylphosphatidylinositol (GPI)-anchored. At steady state, exocytic events observed at the cell surface originate either from anterograde transport or from recycling pathways. To analyze events of exocytosis linked to the anterograde transport of the cargo of interest and distinguish them from the recycling pathway, the best method is to synchronize cargo transport along the secretory pathway. Several years ago, we developed the Retention Using Selective Hooks (RUSH) assay to synchronize the anterograde transport of virtually any cargo of interest in physiological conditions for cells (Boncompain et al., 2012). The RUSH assay is a two-state secretory assay based on the retention of the cargo of interest (reporter) in a donor compartment and its release to the acceptor compartment. The donor compartment is usually the endoplasmic reticulum (ER) when using the RUSH assay while various final compartments can be targeted such as various domains of the plasma membrane, the endosomes or the lysosomes for example. Here, we will focus on the study of transport to the cell surface and exocytosis (Figure 1A). Retention of the reporter is mediated by the interaction between streptavidin fused to an ER-resident hook and a streptavidin-binding peptide (SBP) fused to the reporter. Interaction is then released by addition of biotin. Biotin has a higher affinity for streptavidin compared to SBP (Barrette-Ng et al., 2013; Wilson et al., 2001)

competing out the SBP. The reporter is then free to traffic to its destination compartment in a synchronous wave. To facilitate the observation of the reporter, a fluorescent protein such as enhanced green fluorescent protein (EGFP) is usually fused to the SBP-bearing reporter.

The transport kinetics varies depending on the cargo of interest. Most of the cargos reach the Golgi apparatus after 15 min of incubation with biotin and the plasma membrane starting from 30 min of treatment with biotin (see RUSH-synchronized transport of gp135/podocalyxin as an example, Figure 1B).

Once exocytic vesicles delivered their content at the cell surface, soluble proteins will be released to the extracellular medium. Integral or membrane-attached proteins will diffuse in the plasma membrane, be internalized or recycled with different kinetics based on their intrinsic diffusion properties and on their interacting partners. To be able to analyze local delivery of these diverse classes of cargos, we developed Selective Protein Immobilization (SPI) to be used in combination with the RUSH assay (Fourriere et al., 2019). SPI is based on the extracellular capture of RUSH-synchronized proteins after their exocytosis thanks to the presence on the coverslip of specific antibodies. The antibodies coated on the coverslip prior to cell seeding, in contact with the ventral surface of cells, will immobilize the RUSH cargo once exposed to the extracellular space after exocytosis. Because a lot of RUSH cargos available are fused to EGFP and because a large variety of anti-GFP antibodies is accessible, we developed and describe here SPI capturing GFP (see Notes 3 and 4 for tips to set up SPI using antibodies directed to other proteins) (Figure 2).

This chapter describes how to perform SPI by coating coverslips with anti-GFP antibodies, how to express and induce the trafficking of the RUSH-synchronized

cargo. Methods to detect local exocytosis are also described using surface immunolabeling or real-time imaging in living cells (using total internal reflection microscopy or spinning disk microscopy).

2. Materials

2.1. Coating of coverslips

1. Carbonate buffer 0.1 M, pH 9.5. Weigh 8.40 g of NaHCO₃ and 3.56 g of Na₂CO₃. Add water to a volume of 900 mL. Mix and adjust pH to 9.5. Make up to 1 L with water. Sterilize by filtration through a filter with pores of 0.2 µm of diameter. Store at 4°C.

2. Poly-L-lysine solution: dilute poly-L-lysine in sterile water to a final concentration of 0.01%

3. Anti-GFP antibody. A large source of anti-GFP antibodies is available from various providers. We set-up the SPI assay using a homemade rabbit polyclonal antibody from the Recombinant antibody platform of the Institut Curie. This anti-GFP antibody was purified and concentrated at 5 mg/mL. As an indication, homogenous and efficient coating was obtained using 0.1 mg/mL as final concentration of the anti-GFP antibody diluted in carbonate buffer. (See Notes 7 and 9 for tips to check the homogeneity and the efficiency of the coating)

2.2. Cell seeding, transfection and release of the RUSH-synchronized cargo

1. Glass coverslips

2. Cell culture Petri dishes or multi-well plates

3. Culture medium adapted to the cells of interest
4. EGFP-tagged RUSH cargo of interest (RUSH plasmids from the Perez lab are available from Addgene: https://www.addgene.org/Franck_Perez/).
5. Transfection reagent adapted to the cell line used (see Note 2)
6. 100X biotin stock solution (at 4 mM): weigh 48 mg of D-biotin (Sigma, ref B4501), dissolve into 50 mL of culture medium. After complete dissolution, sterilize by filtration through a filter with pores of 0.2 μm of diameter. Store at 4°C for 3 months.

2.3. Fixation and detection of the released cargo by surface immunolabeling

1. Ice-cold PBS.
2. 1 % paraformaldehyde solution prepared in PBS.
3. Primary antibody: either anti-GFP or anti-SBP antibodies. Fluorochrome-conjugated secondary antibodies adapted to the primary antibody used
4. Mounting medium: weigh 6 g of glycerol and 2.4g of Mowiol 4-88. Add 6 mL of distilled water. Incubate for 2 h at room temperature under agitation. Add 12 mL of 0.2 M Tris-HCl pH 8.5. Agitate for 2 h at 50°C until it completely dissolved. Centrifuge for 15 min at 4,000 *g*. Store aliquots at -20°C for several months.

2.4. Real-time imaging of local exocytosis by total internal reflection fluorescence (TIRF) or spinning disk microscopy

1. Imaging medium such as Leibovitz's medium. This medium does not need a high CO₂ environment to image the cells during several hours. Better to select Leibovitz without phenol red to reduce background.
2. Chambers adapted to glass coverslips or ChamSlide magnetic chamber

3. Inverted fluorescence microscope equipped with a thermostated incubation chamber either spinning disk or TIRF microscope
4. Immersion oil

3. Methods

3.1. Coating of coverslips with anti-GFP antibodies

1. Immerse glass coverslips in carbonate buffer 0.1M pH9.5. Incubate for 1 h at 37°C.
2. Transfer coverslips in poly-L-lysine 0.01% solution. Coverslips will float on the solution. Keep in mind that the poly-L-lysine coated surface is the one facing the solution. Incubate for 1 h at 37°C.
3. Wash coverslips three times in PBS. Let the coverslips dry before proceeding to the next step.
4. Prepare 40 µL drops for 12mm glass coverslips or 100 µL drops for 25mm coverslips of anti-GFP solution diluted in carbonate buffer on a parafilm foil.
5. Flip coverslips upside down with the face coated with poly-L-lysine immersed in the antibody solution.
6. Add a humidified filter or tissue to create a humid environment and prevent dehydration.
7. Incubate for at least 3 h at 37°C (incubation might be prolonged overnight if more convenient).
8. Wash coverslips twice with PBS.

9. Transfer the coated coverslips in a multi-well plate or a dish containing complete culture medium adapted for the cells of interest.

10. Seed the cells at the appropriate confluence for the transfection method of choice. Allow cell seeding for several hours or overnight prior to proceed to transfection of the plasmid coding for the RUSH cargo.

3.2. Expression and release of the RUSH synchronized cargo

1. Transfect the cells to introduce the plasmid coding for the EGFP-tagged RUSH cargo. (See Note 2 for comments about transfection methods compatible with the RUSH assay).

2. The day after transfection, replace medium containing transfection mix by pre-warmed complete medium containing biotin at 40 μ M final.

3. Incubate for the desired time at 37°C. Trafficking kinetics is variable depending on the cargo. Consequently, it is critical to assess the trafficking kinetics of the cargo of interest prior using the SPI assay. Proceed to the detection of local exocytosis either by surface immunolabeling or real-time imaging in living cells. If real-time imaging is used, please read the section 3.4. since the release of the cargo should be performed in chamber or dish adapted to the microscope to be used. Instead of using glass coverslips, some imaging dishes might be used meaning that these dishes have to be coated with the anti-GFP antibodies.

3.3. Analysis of local exocytosis by surface immunolabeling

To visualize the exocytosed cargo only, an immunolabeling without permeabilization of the cells may be performed (Figure 3). Antibodies directed to EGFP or to SBP might be used. Please note that using antibodies from an animal species different

from the one used for the coating of coverslips is critical. It also makes sense to use an anti-EGFP antibody with a different epitope than the one used for coating to avoid any competition for binding to the exocytosed EGFP-tagged RUSH cargo. To perform surface immunolabeling, not fixing the cells is preferable to avoid the entry of the antibodies through small holes induced by fixation.

1. Following the steps described in the previous section, immediately wash coverslips with ice-cold PBS. Keeping the cells at 4°C is essential to stop trafficking of the cargo preventing both further exocytosis of the cargo and endocytosis, which would lead to internalization of the antibody used for immunostaining.

2. Dilute the primary antibody (e.g. anti-EGFP or anti-SBP) in ice-cold PBS at the recommended concentration for immunofluorescence.

3. Prepare drops of the diluted primary antibody on a parafilm foil (or another type of holder) placed on ice. 25 – 50 µL and 100 - 150 µL drops will be sufficient for immunolabeling of cells seeded on 12 mm or 25 mm glass coverslips respectively.

4. Incubate coverslips upside down on the drops for 45 min on ice. The cells are immersed in the antibody solution.

5. Wash three times with ice-cold PBS.

6. Fix with paraformaldehyde 1 % for 15 min at room temperature

7. Wash three times with PBS. The cells are fixed at this stage and the rest of the protocol might be performed at room temperature.

8. Dilute the fluorochrome-conjugated secondary antibody in PBS at the recommended concentration for immunofluorescence.

9. Prepare drops of the diluted secondary antibody on a parafilm foil (or another type of holder).
10. Incubate coverslips upside down on the drops for 30 min at room temperature.
11. Wash three times with PBS.
12. Mount the coverslip in a drop of Mowiol deposited on a glass slide. The cells are immersed in the Mowiol between the coverslip and the slide. The mounting solution might also contain a nuclear dye such as Hoechst or DAPI to counterstain nuclei.
13. Allow the mounting medium to polymerize for several hours prior proceeding to observation with a fluorescence microscope.

3.4. Analysis of local exocytosis using real-time imaging in living cells

Real-time fluorescence microscopy in living cells allows monitoring dynamically the first events of exocytosis of the RUSH-synchronized cargo. This can be achieved using spinning disk microscopy (Figure 4A) or using total internal reflection fluorescence (TIRF) microscopy (Figure 4B). TIRF microscopy enables observation of the events occurring only at the ventral surface of cells and thus events occurring at the plasma membrane (Axelrod, 1981). The protocol for imaging local exocytosis in real-time is the same using either a spinning disk microscope or a TIRF microscope.

1. Follow the protocols described in section 3.1 and 3.2 until transfection.
2. After transfection, transfer the coverslips in a chamber adapted to the coverslips and the microscope stage.
3. Fill with medium adapted to video-microscopy such as Leibovitz's medium without phenol red

4. Set up acquisition parameters (e.g. focal plane, wavelengths, exposure time, interval time between acquisitions...).
5. Acquire three or more pictures. These pictures correspond to the retention state (absence of biotin) when no exocytic events are expected to happen.
6. Replace medium by pre-warmed imaging medium containing 40 μM biotin.
Alternatively, imaging chamber might be filled directly with medium containing 40 μM biotin if the time taken to set-up acquisition parameters is shorter than the time of appearance of the first exocytic events. In any case, writing down the time of addition of biotin is essential to define time zero.
7. Resume acquisition.
8. Continue time-lapse acquisition as long as necessary to visualize exocytic events.

4. Notes

1. The efficacy of SPI depends on the relative speed of immobilization (linked antibody-antigen affinity, concentration of the coated antibody) vs. the relative speed of diffusion. For example, it is particularly efficient to analyze the exocytosis of the poorly diffusive Collagen X while the signal is less contrasted when monitoring secretory soluble GFP (see Fourriere et al., 2019).
2. One critical parameter for the use of the RUSH assay to synchronize the transport of cargos is the absence or the very low amount of biotin contained in the culture medium (and/or serum). The presence of biotin prevents the efficient retention of the reporter. DMEM GlutaMax from Thermo that we routinely use does not contain biotin. We noticed that OptiMEM, DMEM-F12

and RPMI-1640 from Thermo do contain biotin and prevent synchronization of the transport of the RUSH cargo. To overcome this problem, low amount of avidin (or streptavidin) might be added in the medium to trap biotin contained in the medium and enable efficient retention. For example, we use 10^{-7} M (7 $\mu\text{g/mL}$) of avidin in OptiMEM and $0.4 \cdot 10^{-8}$ M (0.28 $\mu\text{g/mL}$) in DMEM-F12 (both supplemented with 10 % serum). In this condition, the use of biotin at 40 μM as final concentration is still efficient to induce the release of the RUSH cargo.

3. We routinely use calcium phosphate method to transfect HeLa cells (Jordan et al., 1996). Some transfection reagents such as Lipofectamine induce a very strong expression per cell, which often prevents protein trafficking to occur. The expression level of the cargo will also impact on its transport kinetics. Consequently testing several transfection methods (calcium phosphate, JetPEI, JetPRIME, electroporation...) might be useful to use the RUSH assay efficiently. Please note that lentiviral transduction is also usable. This is adapted in particular to establish cell lines stably expressing RUSH cargos and choosing medium-expressing population (see (Boncompain and Perez, 2013) for a detailed protocol to establish a RUSH stable cell line).
4. While using combination of the RUSH and SPI assays, make sure that the antibody chosen for the SPI assay is directed to the extracellular part of the protein of interest. Alternatively, if using an antibody directed against SBP or the fluorescent protein, make sure that these tags are fused to the luminal part of the protein of interest.
5. As mentioned above, we set-up the SPI assay using a homemade rabbit polyclonal anti-GFP antibody. However, this assay can be set-up using other antibodies either other anti-EGFP antibodies or antibodies directed to other

targets. In a recent study (Fourriere et al., 2019), we also used anti-mCherry and anti-VSVG antibodies for immobilization of mCherry-tagged RUSH cargo and RUSH-VSVG respectively. If the antibody chosen is directed against your protein of interest, which is also endogenously expressed, the endogenous expression of the protein of interest might compete with the RUSH-synchronized exogenously expressed protein for capture. For this reason, using an antibody directed to the tag is an advantage. This also avoids performing multiple set up experiments while changing antibodies.

6. The combination of the RUSH and SPI assays is also usable for multiple color imaging. In this case, two or more RUSH cargos tagged with different fluorescent proteins will be used. In consequence, the SPI assay mixing two or more specific antibodies has to be set up.
7. Carbonate buffer is quite easy to prepare. However, commercial ELISA coating buffer can be used to treat coverslips and dilute the antibodies for SPI.
8. Homogeneity of the coating can be assessed by incubating the anti-GFP coated coverslips with a fluorochrome-conjugated appropriate secondary antibody. Then using an epifluorescence microscopy, observe coverslips. Focal plane might be difficult to set up. Fluorescence is expected to be homogeneously distributed. If some zones are not fluorescent, bubbles were probably present while incubating the coverslips during the coating procedure.
9. Be careful to not scratch the coverslips while handling them with tweezers during the coating procedure and during their transfer to dishes or plates prior to cell seeding.
10. To check efficacy and saturation of the coating of coverslips with anti-GFP antibodies, coated coverslips can be incubated with serial dilutions of

recombinant EGFP. Measure fluorescence intensity of the coverslip surface using an epifluorescence microscope.

11. The co-expression of a fluorescently tagged protein stably localized at the plasma membrane (e.g. Myr-Palm-mCherry or paxillin-mCherry) may be useful to set up the TIRF angle given that before the first exocytic events occurred, the RUSH-tagged cargo is absent from the plasma membrane.

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Figure legends

Figure 1: Principle of the Retention Using Selective Hooks (RUSH) assay

A. The RUSH system is two-state secretory assay allowing synchronization of the trafficking of a cargo of interest (reporter). Retention of the reporter in the donor compartment is mediated by interaction between the Streptavidin binding peptide (SBP) fused to it and streptavidin fused to a protein stably resident in the donor compartment. Synchronous release of the reporter is induced by addition of biotin, which competes out the SBP. The reporter is then free to traffic to its target compartment. To facilitate the observation of the transport of the reporter an EGFP (or another fluorescent protein) is fused to the reporter.

B. HeLa cells expressing RUSH synchronized gp135/podocalyxin (Str-KDEL_SBP-EGFP-gp135) were seeded on non-coated glass coverslips. Cells were fixed after the indicated time of incubation with biotin. Pictures were acquired using an epifluorescence. Scale bar: 10 μm .

Figure 2: Principle of Selective Protein Immobilization (SPI) combined to the RUSH assay

A, B. Glass coverslips are coated with anti-GFP antibodies prior to cell seeding and transfection. Cells are then transfected to express a GFP-tagged RUSH cargo. After addition of biotin, the GFP-tagged RUSH cargo is released and exocytosed at the cell surface where it is immobilized by the anti-GFP antibodies present on the surface of the coverslip.

Figure 3: Local exocytosis of RUSH-synchronized CollagenX analyzed using surface immunolabeling.

HeLa cells seeded on glass coverslips coated with rabbit polyclonal anti-GFP antibodies were transfected to express Str-KDEL_SBP-EGFP-ColX (first row and green on merge). Biotin was added for 35 min and then cells were processed for surface immunolabeling using mouse monoclonal anti-GFP antibodies (second row (inverted colors) and red on merge). Scale bar: 10 μ m.

Figure 4: Local exocytosis of RUSH-synchronized CollagenX monitored using real-time imaging in living cells.

HeLa cells transiently co-expressing RUSH-synchronized CollagenX (Str-KDEL_SBP-EGFP-ColX) and Paxillin-mCherry were seeded on coverslips coated with rabbit polyclonal anti-GFP antibodies. Biotin was added at time zero. Transport of SBP-EGFP-ColX was analyzed using real-time imaging with a spinning disk microscope (A) or a TIRF microscope (B). Scale bars: 10 μ m. Figure 4A : ©2019 Fourriere et al. Originally published in The Journal of Cell Biology. <https://doi.org/10.1083/jcb.201805002>

Figure 1
A

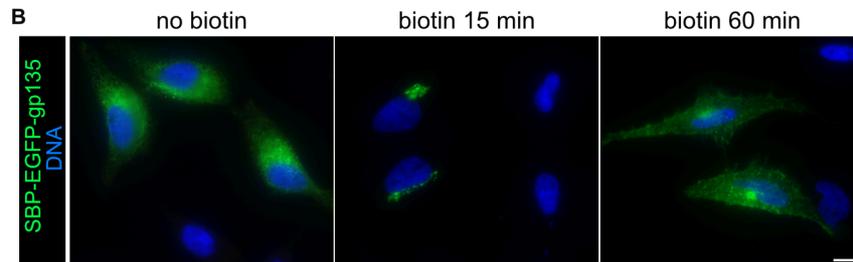
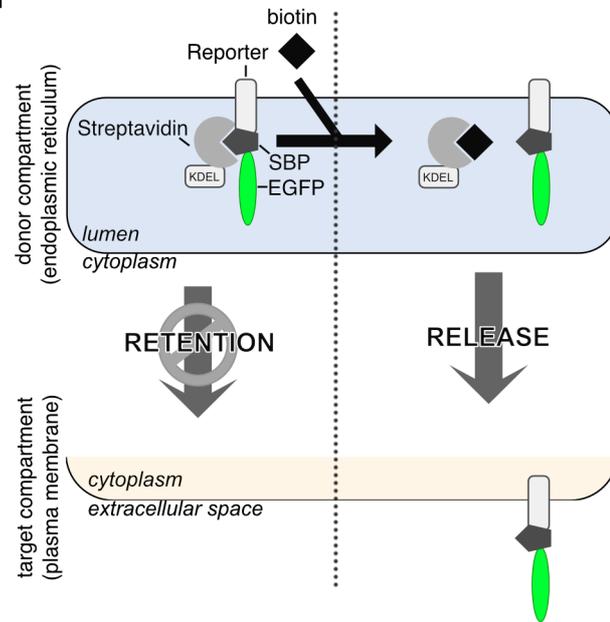


Figure 2

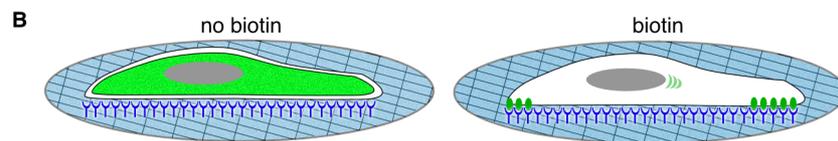
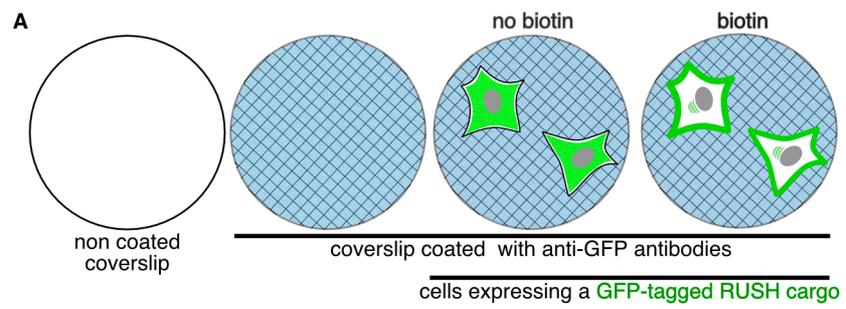


Figure 3

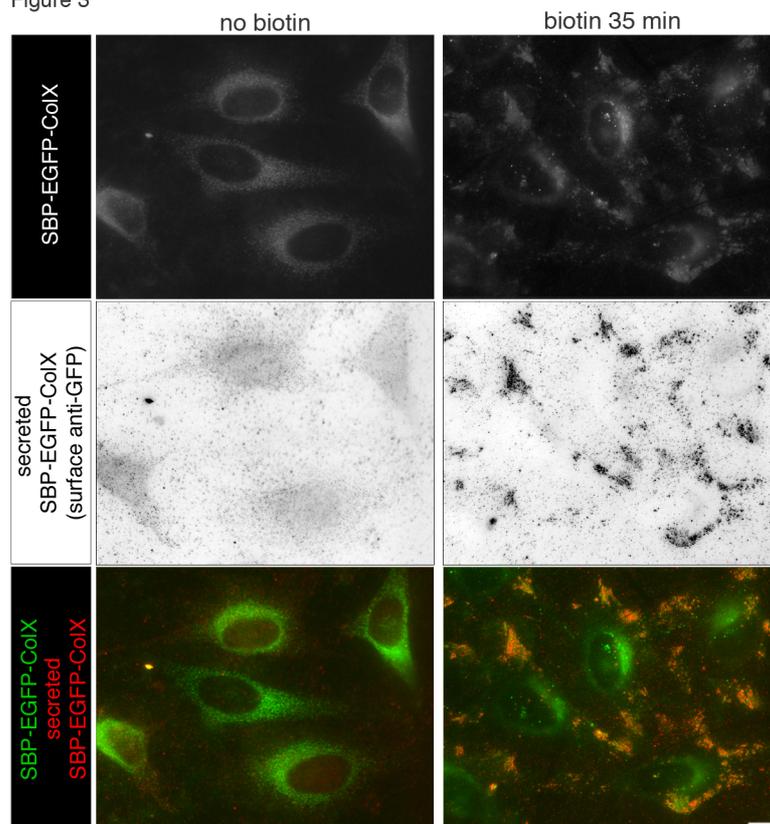
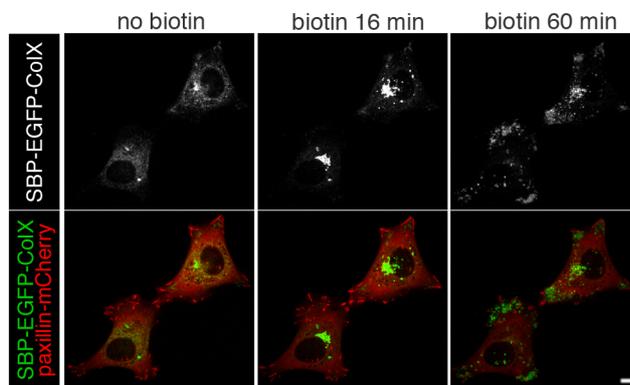


Figure 4

A *spinning disk microscopy*



B *TIRF microscopy*

