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Photohighlighting approaches to access membrane dynamics of the Golgi apparatus

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Abstract

By providing quantitative, visual data of live cells, fluorescent protein-based microscopy techniques are furnishing novel insights into the complexities of membrane trafficking pathways and organelle dynamics. In this chapter, we describe experimental protocols employing fluorescent protein-based photo-highlighting techniques to quantify protein movement into and out of the Golgi apparatus, an organelle that serves as the central sorting and processing station of the secretory pathway. The methods allow kinetic characteristics of Golgi-associated protein trafficking to be deciphered, which can help clarify how the Golgi maintains itself as a steady-state structure despite a continuous flux of secretory cargo passing into and out of this organelle. The guidelines presented in this chapter also can be applied to examine the dynamics of other intracellular organelle systems, elucidating mechanisms for how proteins are maintained in specific organelles and/or circulated to other destinations within the cell.

Keywords

Photoactivation; photobleaching; Golgi; organelle dynamics; protein trafficking; secretory pathway

I. Introduction

The Golgi apparatus functions as a carbohydrate factory at the crossroads of the secretory pathway, receiving proteins and lipids derived from the endoplasmic reticulum (ER) and sorting them into different plasma membrane (PM) intersecting pathways. During this process, proteins destined for the PM, endosomes and lysosomes are separated from those to be retained in the Golgi and/or recycled back to the ER. How the Golgi is able to perform this selective filtration, including retaining its own specific proteins and lipids in the face of continuous outward trafficking of molecules, has been the subject of intense study and speculation (Altan-Bonnet et al., 2004; Emr et al., 2009; Jackson, 2009; Lippincott-Schwartz, 2011; Nakano and Luini, 2010; Pelham and Rothman, 2000).

The development of fluorescent protein (FP)-based highlighting methodologies to directly visualize movement of proteins and organelles in live cells have provided important tools for

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addressing the dynamics of the Golgi apparatus (De Matteis and Luini, 2008; Lippincott-Schwartz and Patterson, 2003; Lippincott-Schwartz et al., 2000; Lippincott-Schwartz et al., 2001; Presley, 2005; Storrie et al., 2008). In these approaches, protein populations tagged with FPs in the Golgi are made visible either by photobleaching molecules outside the Golgi region-of-interest or through photoactivation of molecules inside it. Alternatively, FP-tagged molecules outside the Golgi are highlighted following photobleaching of the Golgi pool of fluorescent molecules. Thereafter, the highlighted proteins are tracked as they move into and out of the region-of-interest in real time (Presley et al., 2002; Ward et al., 2001; Zaal et al., 1999). The spatial and temporal resolution achieved therein has enabled quantification of protein export rates from and import rates into the Golgi (Hirschberg and Lippincott-Schwartz, 1999; Patterson et al., 2008), as well as protein residence times within the Golgi (Zaal et al., 1998; Storrie et al., 2008; Ward et al., 2001; Altan-Bonnet 2004). What has emerged from such measurements is a clearer picture of how membrane trafficking and sorting occurs within the Golgi.

In this chapter, we describe these fluorescent highlighting protocols for precisely measuring export rates of proteins into and out from the Golgi. Use of the protocols can provide quantitative insights into the kinetics of Golgi membrane trafficking, either retrograde back to the ER or anterograde toward the PM. The imaging schemes can furthermore be used to measure dynamic associations of protein and lipids in other intracellular contexts in live cells (e.g., transport in/out of the nucleus). As such, they provide a universal toolkit for probing intracellular trafficking of molecules.

II. Objectives and Rationale

Proteins that traffic to the Golgi apparatus are primarily of two types: cargo molecules that transit through the secretory pathway en route to different final destinations, and resident molecules such as Golgi enzymes that are retained in the Golgi for extended periods. Optical highlighting techniques using live cell imaging together with fluorescence recovery after photobleaching (FRAP) approaches or photoactivation have been valuable in teasing out the Golgi trafficking dynamics of these two classes of proteins, including the time frame of their association with the Golgi apparatus and their export and import kinetics from this organelle. This, in turn, has shed light on the mechanisms of protein sorting in the Golgi and the governing principles of Golgi maintenance.

Here, we discuss the operational principles of these optical highlighting techniques in the context of understanding the dynamics and steady-state organization of the Golgi apparatus. Using FRAP, a subset of fluorescent molecules is photobleached in a defined region inside a cell, either to measure the rate of movement of fluorescent molecules into the bleached, dark region, or to highlight a fluorescent region of the cell against a dark background followed by monitoring the movement of fluorescent molecules out of the highlighted region. Using photoactivation, a defined region inside a cell is made bright by switching on the fluorescent molecules in this region followed by the tracking of the molecules out of the photactivated region. These two approaches (FRAP and photoactivation) can yield quantitative complementary data characterizing the parameters controlling cargo efflux out from and

Golgi enzyme retention within the Golgi apparatus. In so doing, the methods can provide important new insights into the dynamic organization of the Golgi apparatus.

III. Materials and Instruments

A. Reagents

Chinese Hamster Ovary (CHO) cells

COS-7 cells

HeLa cells

Plamid constructs encoding Galactosyl Transferase-EGFP, Mannosidase II-PAmCh, Sialyl Transferase-EGFP, VSVG-GFP, GFP-prcollagen, and ss-YFP.

Dulbecco minimum essential medium (DMEM)

Fetal bovine Serum

Glutamine Penicillin

Streptomycin

Fibronectin

Cycloheximide

HEPES buffer

B. Instrument

Zeiss LSM 510 confocal microscope (or equivalent microscope)

IV. Methods

A. Cell plating and transfection

CHO and COS-7 cells are maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a 5%-CO₂ incubator. Ideally, the cells should be grown in phenol red free DMEM to minimize the background fluorescence during quantitative fluorescence imaging experiments. Cells with flat morphology should be used for quantitative fluorescence microscopy experiments, so that the total fluorescence signal from the entire depth of the cell can be collected during image acquisition. Cells are plated on fibronectin coated Labtek or Mattek chambers at a density such that they are 30-40% confluent after 24 hours. Cells are transfected with plasmid constructs tagged with EGFP 24 hours after plating.

B. Cell imaging

Prior to imaging, cells are incubated with fresh media containing cycloheximide (100-150 µg/mL) for 2-3 hours at 37°C for experiments requiring block of protein synthesis.

Cycloheximide is an inhibitor of mammalian protein synthesis and prevents the synthesis of new copies of the FP-tagged protein-of-interest. This ensures that quantification of the time-lapse images is not complicated by the presence of newly synthesized proteins. The cells are then transferred to imaging medium, which is regular growth medium (with no phenol red) with HEPES buffer (20mM, pH7.4). In addition, the imaging media is supplemented with cycloheximide (100-150 µg/mL) for experiments where imaging needs to be done in absence of new protein synthesis. The imaging chamber should be kept humidified by passage of humid air during image acquisition.

C. Image Protocols and Acquisition

The imaging protocols for the experimental strategies discussed in this paper involve two distinct steps: (1) An initial photobleaching scheme when a user defined region-of-interest (ROI) of a cell is selectively photobleached (Strategies 1, 2; Figures 1 and 2) or photoactivated (Strategy 3; Figure 3), (2) collection of time-lapse images to monitor either the loss (Strategy 1, Figure 1) or gain (Strategies 2 and 3, Figures 2 and 3) of fluorescence within a specific area of the cell.

Imaging conditions are chosen upon careful calibration of laser power and exposure time such that there is minimal loss of fluorescence signal due to photobleaching during the acquisition of the time series. For quantitative imaging, the total amount of fluorescence signal collected from the cell should stay constant, and should not vary due to redistribution of fluorescent proteins between various organelles within the cell. In order to achieve this, the images acquired during the time-lapse should contain the fluorescence signal from the entire volume of the cell. In order to achieve this, images are acquired with a low magnification objective (40× or 63 ×) and an open pinhole, so that the collecting volume matches the z-dimensional thickness of the cell and the fluorescence signal from the entire depth of the cell is collected.

In time-lapse imaging, definite focusing feature of the microscope can be used to compensate for any movement of the object being imaged in the plane-of-focus due to internal vibrations of the microscope. The definite focusing operation uses the coverslip surface as a fixed reference plane, and ensures that the images are captured at a user-defined distance from this reference plane.

Extra care should be taken to ensure that none of the pixels are saturated during collection of images. The laser power, exposure, contrast and black levels are adjusted so that the image pixel intensities stay within the measurable range during the course of the experiment (0-4,095 gray levels for 12-bit image, 0-65,535 for 16 bit images), while sampling the maximum possible dynamic range for the camera.

D. Experimental Strategies

Strategy 1. Measurement of cargo export out of the Golgi

Background and Objective: Different models of intra-Golgi trafficking make different predictions regarding the export kinetics of cargo out of the Golgi during secretory transport. For example, in the classic cisternal maturation model for Golgi transport, cargo is proposed

to traverse the Golgi stack of cisternae while associated with a single cisterna, without leaving it (Bonfanti et al., 1998; Glick and Malhotra, 1998; Glick and Nakano, 2009). This predicts that cargo should leave the Golgi at a constant, linear rate, correlating with the pace that an individual cisterna progresses through the Golgi cisternal stack to its exit face, where the cisterna disintegrates into transport vesicles. In models of Golgi transport invoking rapid mixing of cargo prior to Golgi export (Lippincott-Schwartz and Phair, 2010; Patterson et al., 2008), by contrast, cargo export from the Golgi is predicted to be an exponential process (akin to radioactive decay), with the export kinetics dependent on cargo becoming concentrated in an export domain present in potentially every cisterna. Fluorescent highlighting approaches can provide valuable information for distinguishing between these two models of intra-Golgi transport due to their ability to precisely quantify cargo export rates out of the Golgi during secretory transport

One way to measure cargo export out of the Golgi using fluorescent highlighting is by photobleaching the entire pool of fluorescent cargo molecules outside a defined Golgi region-of-interest (ROI) followed by recording of the loss of fluorescence from the ROI (Figure 1A). The selective photobleaching of fluorescence from the entire cell outside the Golgi-region-of-interest highlights the Golgi pool of FP-tagged cargo molecules, which because they have not been photobleached, are bright in relation to the rest of the photobleached molecules in the cell. Subsequent time-lapse imaging permits visualization and quantification of the fluorescent cargo molecules as they move out from the Golgi apparatus. From the collected data, one can determine whether cargo proteins leave the Golgi at a constant rate (predicted by the classic cisternal progression model) or whether the rate is constantly changing over time as a function of the amount of cargo in the Golgi (predicted by the rapid mixing model).

Here, we describe Golgi export kinetics for three different classes of cargo that have been obtained using this experimental approach. The three classes of cargo include: a transmembrane protein (temperature sensitive ts045 VSVG protein, VSVG-GFP); a large soluble cargo (GFP-procollagen); and a small soluble cargo (YFP with a cleavable signal sequence, ss-YFP). VSVG-GFP is retained as a misfolded protein in the ER at 40°C before being released into the secretory pathway by temperature shift to 32°C, which induces the protein to properly fold and leave the ER (Hirschberg et al., 1998). Cells are transfected with one of each of the cargo molecules. Once expressed, the cargo protein is allowed to achieve a Golgi distribution. The Golgi pool is then pulse-labeled by selective photobleaching and cargo export from the Golgi is measured over time (Figure 1B).

Flow of experiment: 1. Transfect COS-7 cells plated in three different Mattek dishes (or Labtek chambers) with VSVG-GFP, GFP-Procollagen and ss-YFP, respectively. Perform the following steps with each of the dishes.

2. Mount the dish (or chamber) for imaging 20-24 hours post transfection.

3. Choose a field of view with a spread out cell expressing the FP-labeled cargo.

4. Select an ROI (*ROI_1*) that includes the entire area of the cell, excluding the perinuclear Golgi network. The pool of the FP-tagged cargo molecule outside the Golgi is present within this ROI.
5. Select a complementary ROI (*ROI_2*) that encompasses the Golgi network.
6. Selectively bleach the FP-cargo fluorescence inside *ROI_1* using few short pulses of high intensity laser. Use 488-nm laser for EGFP tagged cargo molecules and 514-nm laser for YFP tagged cargo molecules.
7. Next, acquire time-lapse images with low intensity laser at 1 min intervals for 1 hour to record the export of cargo molecules out from the Golgi.
8. Measure the background subtracted fluorescence intensity of the *ROI_2* at each successive time point of the time series.
9. Plot the fluorescence intensities of *ROI_2* against time to obtain a graph of the loss of fluorescence from the Golgi over time due to exit of the FP-cargo molecules.
10. Examine whether the data is best fit to a linear or exponential plot. The data will favor the classic cisternal progression model if the data fits a linear curve, and favors the rapid mixing model if it fits an exponential curve. For an exponential curve, the rate constant for cargo export, λ , can be calculated from $t_{1/2}$ ($\lambda = \ln(2)/t_{1/2}$) of the curve, whereas $1/\lambda$ gives an estimate of the residency time of the cargo in the Golgi.

Considerations: The fluorescence intensity in the Golgi region decreases during the time course of the experiment due to the exit of cargo from the Golgi. The imaging conditions at the start of the time lapse imaging (immediately after the photobleaching step) should be set such that the intensity at the Golgi region is as close to the saturation value as possible (maximum recordable value by the camera), while ensuring minimal photobleaching. This enables utilization of a larger portion of the dynamic range of the camera, enabling higher detection sensitivity for changes in fluorescence intensity.

Results: The integration of background subtracted fluorescence intensity in *ROI_2* measures the fluorescence of the FP-tagged cargo molecule within the Golgi, and is directly proportional to the number of FP-cargo molecules present in this region. The fluorescence intensity of the *ROI_2* is measured at each successive time-point and plotted against time to obtain a profile of the exit of the FP-cargo molecules from the Golgi.

In the experiment shown in Figure 1, all of the cargo varieties exhibited exponential exit kinetics, with each cargo type having a distinct Golgi export rate (i.e., VSVG-GFP=0.039 min⁻¹; GFP-procollagen= 0.065 min⁻¹; and ss-YFP= 0.07 min⁻¹) (Figure 1C). Therefore, the observed export kinetics supports a rapid mixing model for trafficking of these cargo proteins through the Golgi. For more discussion for how rapid mixing of cargo molecules within the Golgi can lead to selective export out of the Golgi, see Patterson et al., 2008, which posits an additional membrane partitioning step within the Golgi for export of transmembrane cargo proteins.

Strategy 2. Exchange of Golgi between Golgi and ER

Background and Objective: Even though the majority of Golgi enzymes reside within the Golgi at any particular moment within the cell, various lines of research have suggested these enzymes undergo constitutive recycling back to the ER (Miles et al., 2001; Storrie et al., 1998; Ward et al., 2001; Zaal et al., 1999). By photo-highlighting fluorescently tagged Golgi enzymes in the Golgi and following their fate over time, it is possible to measure the length of time the enzymes remain within the Golgi prior to cycling back into the ER (Figure 2A). Moreover, by photobleaching the entire pool of FP-tagged Golgi enzymes and watching recovery of fluorescence into the Golgi from the non-bleached pool of molecules outside the Golgi in the presence of cycloheximide, one can estimate how quickly Golgi enzymes outside the Golgi (including those in the ER and transport intermediates) are retrieved back into the Golgi (Figure 2B).

To perform these experiments, the entire population of fluorescently tagged Golgi proteins localized within (Figure 2B) or outside the Golgi (Figure 2A) is photobleached with a short, high intensity laser pulse. Subsequently, fluorescence recovery (into or out of the Golgi) is measured by time-lapse imaging. This approach interrogates whether a Golgi protein cycles between the Golgi and ER during its normal trafficking itinerary, and can provide rates for exchange of the Golgi enzyme between the Golgi and the ER.

Flow of experiment

Stage #1. Dynamics of ER-pool of GalT-EGFP: 1. Transfect CHO cells with a FP-tagged Golgi enzyme, Galactosyl Transferase-EGFP (GalT-EGFP), and allow the protein to equilibrate throughout the secretory pathway by maintaining the transfected cells at 37°C for 20-24 hours. At steady state, GalT-EGFP is primarily localized in the Golgi with a minor fraction in the ER.

2. Choose a field of view (Window 1) having two cells transfected with GalT-EGFP. One of the cells is subjected to the photobleaching protocol, while the second cell is used as a control to assess the amount of bleaching during the collection of time-lapse images.
3. Choose a region of interest (*ROI_1*) in the first field of view (Window 1) that includes the entire cell, but excluding the perinuclear Golgi network. The fluorescence in this ROI represents the GalT-EGFP present in the ER
4. Record a few images (5-10) of the initial GalT-EGFP fluorescence distribution. Make sure that the pinhole is open.
5. Selectively bleach the entire ER pool of GalT-EGFP by illuminating *ROI_1* with a few short pulses of high intensity 488nm-laser.
6. Monitor the recovery of fluorescence in the ER by performing time-lapse images at 5 minutes intervals using low laser power. Collect the time-series for 1.5-2 hours, or until you see recovery of fluorescence in the ER reach pre-bleach levels.
7. Choose a second field of view with one or two GalT-EGFP expressing cells (Window 2).

8. Choose an ROI that includes an entire cell excluding the Golgi apparatus (*ROI_2*)
9. Photobleach the complete pool of GalT-EGFP in the entire cell by illuminating the whole cell with short pulses of high intensity 488nm-laser.
10. Collect time-lapse images with low intensity laser for 1 hour to check if there is any recovery of fluorescence in *ROI_2*.
11. Calculate the total amount of fluorescence in the ER in each image of the time series by integrating the background-subtracted fluorescence over all the pixels inside *ROI_1* in the corresponding image.

$$I_{ER}^i = \sum ROI_1 I^i$$

where I_{ER}^i represents ER fluorescence in image frame #*i* of the time series, and ROI_1^i is the *ROI_1* in image frame #*i*, $\Sigma()$ represents the integration of fluorescence over all pixels in the defined region.

12. Calculate the fluorescence intensity of Golgi pool of FP-labeled enzyme in each frame of the time-lapse images by subtracting the ER-pool of fluorescence (I_{ER}^i) from the total cell fluorescence in the corresponding image.

$$I_{Golgi}^i = \sum Cell^i - I_{ER}^i$$

Where I_{Golgi}^i and I_{ER}^i represent the Golgi-pool and ER-pool of fluorescence in image frame #*i* of the timeseries, respectively, and $Cell^i$ is the total cell fluorescence in image frame #*i*.

13. Plot I_{ER}^i against time to obtain a graph of recovery of fluorescence in ER with time.

Stage #2. Dynamics of Golgi-pool of GalT-EGFP: 14. Next, choose a new region with two transfected cells in the field of view.

15. Choose a region of interest (*ROI_3*) that encompasses the perinuclear Golgi network. The Golgi pool of GalT-EGFP resides inside this ROI.
16. Acquire a few images to record the initial distribution of the Golgi enzyme
17. Bleach the fluorescence from the Golgi pool of GalT-EGFP by selectively illuminating *ROI_3* with few pulses of high intensity 488nm-laser.
18. Record time-lapse images at 5 min intervals for 2 hours to monitor the recovery of fluorescence in the Golgi network.

19. Measure the Golgi-pool of fluorescence in each image by integrating the background subtracted fluorescence intensity inside *ROI_3* in each image of the time series.

$$I_{Golgi}^i = \sum ROI_3^i$$

Where I_{Golgi}^i represents ER fluorescence in image frame $\#i$ of the timeseries, and ROI_3^i is the *ROI_3* in image frame $\#i$

20. Calculate the fluorescence in the ER in each image frame by subtracting the Golgi-pool of fluorescence from the total cell fluorescence in the corresponding image.

$$I_{ER}^i = \sum Cell^i - I_{Golgi}^i$$

Where I_{Golgi}^i and I_{ER}^i represent the Golgi-pool and ER-pool of fluorescence in image frame $\#i$ of the timeseries, respectively, and $Cell^i$ is the total cell fluorescence in image frame $\#i$.

21. Plot I_{ER}^i against time to obtain a graph of recovery of fluorescence in the Golgi with time.

22. Fit the measured values of I_{Golgi} and I_{ER} at each acquisition timepoint for the two stages of the experiments to a two-compartment model to obtain rate constants for flow of GalT-EGFP from Golgi to ER (retrograde) and from ER to Golgi (anterograde).

Considerations: Cells should be treated with a protein synthesis inhibitor like cycloheximide (100-150 μ g/mL) for 2 hours before starting the image acquisition. They also should be maintained in media containing cycloheximide during the entire course of time-lapse imaging. This ensures that the changes in fluorescence intensities do not arise from synthesis of new FP-tagged proteins but reflect exchange of FP-tagged protein molecules between Golgi and ER. For situations where protein synthesis inhibition can affect the results of the experiments (by affecting relevant physiological process), however this strategy should not be used.

Golgi enzymes typically have a half-life of over 20 h or more; thus, the fluorescence intensities should be minimally affected by degradation of the FP-tagged Golgi enzymes. However, if the protein-of-interest has a shorter lifetime, then protein degradation can change the protein amounts significantly. This has to be taken into account for quantitative interpretation of the time lapse-images.

For the second set of experiments (involving photobleaching the Golgi pool of FP labeled protein), the movement of proteins from Golgi to ER will lead to an overall higher average fluorescence intensity in the Golgi region since the proteins are concentrated within a smaller volume at the Golgi compared to the source organelle ER. Thus, the imaging conditions should be chosen such that the maximum fluorescence intensity at the ER

immediately after photobleaching of Golgi is significantly below the maximum pixel value that can be recorded by the camera. This will ensure that the increase in fluorescence intensity at the Golgi region stays within the linear range and does not saturate the pixels during timelapse imaging.

Results: At steady state, the localization of GalT-EGFP is primarily restricted to two organelles, the Golgi and the ER. By measuring the recovery of GalT-EGFP fluorescence in Golgi and ER independently following depletion of the fluorescent GalT-EGFP in either of these compartments, this experimental protocol can provide information about the exchange of the enzyme between the two organelles.

Following selective bleaching of the GalT-EGFP present within *ROI_1*, most of the fluorescent signal in ER is recovered within 1 hour (Figure 2A). Following the photobleaching protocol, the only fluorescent pool of GalT-EGFP is present in the Golgi. In the absence of synthesis of new GalT-EGFP, recovery of fluorescence in the ER can only occur from recycling of GalT-EGFP from the Golgi to the ER. Furthermore, there is no fluorescence recovery in the ER when the entire cell is photobleached (*ROI_2* in Window 2), indicating the fluorescence recovery in *ROI_1* is indeed due to movement of GalT-EGFP back to ER.

In the second stage of the experiment, the fluorescence in *ROI_3* (Golgi pool of GalT-EGFP) is recovered within 2 h and reaches the same percentage of total cellular fluorescence as recorded prior to the bleaching step (Figure 2B). This recovery can only happen as a result of movement of GalT-EGFP from the only available source, the ER, and indicates that the ER pool of GalT-EGFP is also in constant exchange with the Golgi apparatus.

The mean rate constant for retrograde and anterograde movement of GalT-EGFP obtained from this analysis is 1.8% per minute and 3.6% per minute, respectively (Figure 2C). Taken together, these results indicate that the steady state distribution of Golgi enzymes like GalT arise by continuous cycling of these proteins between these compartments, rather than by stable retention of the proteins in the lipid bilayer of the compartments.

Strategy 3. Use of photoactivation to evaluate exchange of Golgi enzymes between Golgi and ER

Background and Objective: A different strategy for measuring flux of Golgi enzymes in and out of the Golgi apparatus is using photactivable fluorescent proteins (PA-FP) to label a protein-of-interest and record its trafficking itinerary. Upon irradiation with light of specific wavelength, photoactivable proteins exhibit dramatic changes in their spectral properties, manifested either by conversion from a dark state to a fluorescent state or emission in a different spectral window (Lippincott-Schwartz and Patterson, 2008). As a consequence, it is possible to selectively illuminate only a fraction of the total proteins within a defined region or an organelle inside the cell, and then track the spatial and temporal evolution of the fluorescent signal against a dark background (Figure 3A). Since only the PA-FPs that are “switched on” during the initial photoactivation step are recorded during the imaging, experiments using PA-FPs are not affected by newly synthesized, dark PA-FP tagged proteins. This permits quantitative imaging experiments without the need for protein

synthesis inhibitor drugs. Consequently, experiments can be performed under more physiological conditions.

We utilized PA-FPs to evaluate whether Golgi enzymes concentrated in the perinuclear Golgi apparatus recycle back to the ER in HeLa cells. Mannosidase II (ManII), an enzyme involved in N-linked glycan processing within the Golgi, is labeled with photoactivable mCherry1 (ManII-PAmCh). Prior to photoactivation, ManII-PAmCh exists in a non-fluorescent dark state. However, upon irradiation with 405 nm light, ManII-PAmCh is converted to a fluorescent state, which absorbs maximally at 561 nm and emits between 590-650 nm. We selectively photoactivated ManII-PAmCh present in the Golgi, and then performed time-lapse imaging to interrogate whether ManII-PAmCh moves out of Golgi into the dark ER network (Figure 3B). Because it is challenging to identify the Golgi apparatus for selective photoactivation, we coexpressed a different Golgi enzyme, Sialyl Transferase-EGFP (SiT-EGFP) to identify the Golgi region. It is this region that we then irradiate with 405 nm light to switch on the ManII-PAmCh molecules there.

Flow of experiment: 1. Co-transfect cells with ManII-PAmCh and SiT-EGFP.

2. Use the excitation 488nm-laser and the green collection channel to find an expressing cell to image. This cell will only show the SiT-EGFP signal when imaged with 488 nm light. The image from the red channel (565nm-excitation) will have no fluorescence signal since ManII-PAmCh will be in the inactive, dark state.

3. Using the green channel image, select a region of interest (*ROI_1*) that circumscribes the entire Golgi network highlighted by the green fluorescence from SiT-EGFP.

4. Record a few images (~five) of the initial SiT-EGFP fluorescence distribution.

5. Selectively photoactivate the Golgi pool of ManII-PAmCh by illuminating *ROI_1* with a few short pulses of 405nm-laser.

6. Acquire a couple of images of the activated ManII-PAmCh in the red collection channel using 561 nm-laser line at low laser intensity.

7. Now collect time-lapse images at 10 min interval using the same laser power and imaging parameters as in Step5 to capture the loss of fluorescence in the Golgi and the concomitant increase in ER fluorescence due to exchange of fluorescent ManII-PAmCh molecules with dark ones. Continue collecting the time-series for 1.5-2 hours, or until the ManII-PAmCh fluorescence signal has equilibrated between the Golgi apparatus and ER network.

8. Calculate the Golgi and ER-pool of ManII-PAmCh in each of the image frame of the timeseries as described above for **Strategy 2, Stage #1**.

9. Plot the measured Golgi- and ER-pool of fluorescence in successive frames against time.

Considerations: The photoactivable proteins that can be used in these experiments are many. However for optimal performance a careful consideration should be made of their photophysical properties like brightness, photostability and contrast ratio (i.e., measure of

the brightness of the activated or photoswitched form relative to the initial form) (Lippincott-Schwartz and Patterson, 2009; Sengupta and Lippincott-Schwartz, 2012). PAGFP, for example, has appreciable fluorescence in the inactive form, leading to a low contrast ratio. Thus, when possible, it is preferable to replace PAGFP with a different photoactivable probe. We recommend PAmCh or mEos3, which have significantly higher contrast ratio than PAGFP.

We and other labs have noticed that mEos2, a widely used photoswitchable fluorescent protein, often causes mislocalization of the protein-of-interest due to its tendency to form aggregates at physiological concentrations. Thus, PA-FP labeled protein labeled proteins should be tested for proper localization and function. The recently developed mEos3 (Zhang et al., 2012) is a brighter and more monomeric version of mEos2, and is recommended as a replacement to mEos2.

In addition to carefully choosing the photoactivable label, design the protein constructs so that the photoactivable protein is present in an environment where it can be efficiently activated and imaged. For example, certain photoactivable proteins like PAGFP cannot be efficiently activated and imaged when they are present in the oxidizing environment of the ER lumen. Thus, for applications involving visualizing protein populations in the ER lumen, PAmCh or mEos2 should be used instead of PAGFP.

Results: Following photoactivation of ManII-PAmCh at the Golgi, fluorescence signal in the ER is detected, which progressively increases over the course of 2 hours (Figure 3B). Concomitantly, the ManII-PAmCherry fluorescence signal at the Golgi decreases by an equivalent amount. The photoactivation strategy ensured that the Golgi is the only source of fluorescent ManII-PAmCh, since the newly synthesized ManII-PAmCh molecules, which have not been photoactivated, are in dark, non-fluorescent state. The build up of fluorescent ManII-PAmCh in the ER is due to the retrograde movement of photoactivated ManII-PAmCh from Golgi to the ER. Thus, these Golgi enzymes constitutively recycle, from the Golgi back to the ER in interphase cells.

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Figure 1. Workflow and results for Strategy 1

(A) Workflow for Strategy 1. (B) The fluorescent pool of three different cargo (left: GFP-procollagen, center: ss-YFP, right: VSVG-GFP) outside the Golgi apparatus is selectively photobleached, and the movement of cargo out of Golgi is monitored by time-lapse imaging. (C) The plot of loss of fluorescence of cargo from Golgi (black circles) shows an exponential decay for all the three cargo molecules. Red line represents the fit of the decay curve to a model involving rapid partitioning of cargo molecules between two different lipid environments in the Golgi and exit of cargo from the export domain according to first order kinetics. Blue line represents the expected decay for cisternal maturation model of cargo trafficking through the Golgi. Image adapted from Patterson et al., 2008.

Figure 2. Workflow and results for Strategy 2

(A) The ER-pool of GalT-EGFP is selectively photobleached by illuminating a defined ROI comprising of the entire cell except the perinuclear Golgi apparatus. Next, time-lapse images are collected to record the exchange of fluorescent GalT-EGFP between the Golgi and the dark ER-network. (B) The GalT-EGFP present in the Golgi is photobleached using an ROI that circumscribe the Golgi apparatus, leaving the ER-pool of GalT-EGFP as the only source of fluorescent GalT-EGFP. Timelapse images are acquired to evaluate the replenishment of fluorescent GalT-EGFP at the Golgi apparatus by movement of GalT-EGFP from ER to Golgi. (C) Plot of fluorescence intensity in ER network (black squares) and Golgi apparatus (white circles) against time for time-lapse images following selective photobleaching of ER-pool and Golgi-pool of GalT-EGFP, respectively. (D) Rate constants for exchange of GalT-EGFP between ER and Golgi obtained by fitting the data from the selective photobleaching experiments. Image adapted from Zaal et al., 1999.

Figure 3. Workflow and results for Strategy 3

(A) Workflow for Strategy 3. (B) ManII-PAmCh is selectively photoactivated within the Golgi apparatus by illuminating an ROI encompassing the Golgi with 405nm laser. Fluorescence signal from SiT-EGFP highlights the Golgi in the green channel, and is used to select the ROI. The exchange of photoactivated ManII-PAmCh between Golgi and ER against a dark background is recorded by time-lapse images.