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Quantitative localization of a Golgi protein by imaging its center of fluorescence mass

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Abstract:	The Golgi complex consists of serially stacked membrane cisternae which can be further categorized into sub-Golgi regions, including the cis-Golgi, medial-Golgi, trans-Golgi and trans-Golgi network. Cellular functions of the Golgi are determined by the characteristic distribution of its resident proteins. The spatial resolution of conventional light microscopy is too low to resolve sub-Golgi structure or cisternae. Thus, the immuno-gold electron microscopy is a method of choice to localize a protein at the sub-Golgi level. However, the technique and instrument are beyond the capability of most cell biology labs. We describe here our recently developed super-resolution method called Golgi protein localization by imaging centers of mass (GLIM) to systematically and quantitatively localize a Golgi protein. GLIM is based on standard fluorescence labeling protocols and conventional wide-field or confocal microscopes. It involves the calibration of chromatic-shift aberration of microscopic system, image acquisition and post-acquisition analysis. The sub-Golgi localization of a test protein is quantitatively expressed as the localization quotient. There are four main advantages of GLIM. 1) It is rapid and 2) based on conventional methods and tools; 3) The localization result is quantitative; 4) And it affords ~ 30 nm practical resolution along the Golgi axis. Here we describe the detailed protocol of GLIM to localize a testing Golgi protein.
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Mala Mani, Ph.D.
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Dear Mala,

Thanks for reviewing our manuscript titled “Quantitative localization of a Golgi protein by imaging its center of fluorescence mass” (JoVE55996). We have addressed all concerns raised from the editor and reviewers. We believe that our manuscript is ready for publishing in JoVE.

Feel free to contact with me if you have any further questions.

Regards

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TITLE:
Quantitative Localization of a Golgi Protein by Imaging Its Center of Fluorescence Mass

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Golgi, Golgi mini-stack, protein localization, quantitative localization, fluorescence microscopy, super-resolution imaging.

SHORT ABSTRACT:
The precise localization of Golgi residents is essential for understanding the cellular functions of the Golgi. However, conventional optical microscopy is unable to resolve the sub-Golgi structure. Here we describe the protocol for a conventional microscopy based super-resolution method to quantitatively determine the sub-Golgi localization of a protein.

LONG ABSTRACT:
The Golgi complex consists of serially stacked membrane cisternae which can be further categorized into sub-Golgi regions, including the *cis*-Golgi, medial-Golgi, *trans*-Golgi and *trans*-Golgi network. Cellular functions of the Golgi are determined by the characteristic distribution of its resident proteins. The spatial resolution of conventional light microscopy is too low to resolve sub-Golgi structure or cisternae. Thus, the immuno-gold electron microscopy is a method of choice to localize a protein at the sub-

Golgi level. However, the technique and instrument are beyond the capability of most cell biology labs. We describe here our recently developed super-resolution method called Golgi protein localization by imaging centers of mass (GLIM) to systematically and quantitatively localize a Golgi protein. GLIM is based on standard fluorescence labeling protocols and conventional wide-field or confocal microscopes. It involves the calibration of chromatic-shift aberration of the microscopic system, the image acquisition and the post-acquisition analysis. The sub-Golgi localization of a test protein is quantitatively expressed as the localization quotient. There are four main advantages of GLIM; it is rapid, based on conventional methods and tools, the localization result is quantitative, and it affords ~ 30 nm practical resolution along the Golgi axis. Here we describe the detailed protocol of GLIM to localize a test Golgi protein.

INTRODUCTION:

The Golgi complex plays essential roles in secretory/endocytic trafficking of proteins and lipids (hereafter cargos) in mammalian cells[1-3]. At the Golgi, cargos are not only sorted to various sub-cellular compartments but also modified by diverse types of glycosylation. The mammalian Golgi complex comprises numerous laterally connected Golgi stacks, which typically consists of 4–11 tightly adjacent and flat membrane sacs called cisternae. The serially stacked Golgi cisternae are further categorized, from one end to the other, as *cis*, medial and *trans*-cisternae. At the *trans*-side of a Golgi stack, the *trans*-most membrane sac develops into a tubular and reticulum membrane network called the *trans*-Golgi network (TGN)[4]. In the secretory pathway, cargos derived from the endoplasmic reticulum (ER) enter a Golgi stack at its *cis*-side and then sequentially pass through medial and *trans*-cisternae. Cargos eventually exit the Golgi at the *trans*-Golgi or TGN destining to the plasma membrane, endosomes or secretory granules.

The molecular and cellular mechanisms of how cargos transit a Golgi stack and how the Golgi maintains its cisternal organization remain mysterious and are currently still under a heated debate[1]. One of difficulties in this field is that Golgi cisternae can only be resolved under the electron microscopy (EM) since the resolution of an optical microscope (~ 200 nm) is insufficient to resolve individual Golgi cisternae (< 100 nm in both cisternal thickness and distance). Therefore, the sub-Golgi localization of resident proteins and transiting cargos are conventionally determined by the immuno-gold EM. However, the immuno-gold EM is very technically demanding and it is beyond the capability of most cell biology labs. Although the resolution of the EM can be sub-nanometer, the resolution afforded by the immuno-gold EM is greatly hampered by the size of the antibody complex (primary plus the secondary antibody) and the gold particle, and it can be worse than 20 nm. Furthermore, EM images are obtained from 2D thin-sections instead of a 3D global view of the Golgi, which can result in erroneous conclusions depending on the relative position and orientation of the 2D section[5]. For example, studying an EM single-section is unable to reliably differentiate a vesicle from the orthogonal view of a tubule since both can display identical round membrane profiles. The recent advent of super-resolution microscopy techniques, such as 3D-structured illumination microscopy (3D-SIM), stimulated emission depletion (STED), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), makes it possible to resolve sub-Golgi structures under light

microscopes[6]. However, there are at least four drawbacks that can significantly limit their uses in the cell biological study of the Golgi. 1) Current super-resolution techniques require expensive and special hardware configuration which is beyond most cell biology labs. 2) Special fluorescence labeling protocols are needed for some super-resolution techniques. 3) Although, under the best condition, these techniques claim 20–110 nm in spatial resolution, the practical resolution obtained in real samples can be much worse. 4) In comparison to conventional microscopy, these super-resolution techniques still have difficulties in conducting multicolor, 3D or live cell imaging, either singly or in combination. Probably most importantly, both immuno-gold EM and the super-resolution microscopy techniques yield qualitative instead of quantitative localization data.

Attempting to partially solve problems mentioned above, we have recently developed a conventional light microscopy based method, which is named Golgi protein localization by imaging centers of mass (GLIM), to systematically and quantitatively localize a Golgi protein at a resolution equivalent to that of the immuno-gold EM[7]. In this method, the Golgi in cultured mammalian cells is dispersed as Golgi mini-stacks by the treatment of nocodazole, a microtubule depolymerizing drug. Extensive studies have demonstrated that nocodazole-induced Golgi mini-stacks (hereafter Golgi mini-stacks) closely resemble native Golgi stacks in both organization and cellular functions[8-11]. The localization quotient (LQ) of a test protein can be acquired through GLIM and it denotes the quantitative sub-Golgi localization. The numerical values of LQs can be compared and a LQ database of more than 25 Golgi markers has been available.

In GLIM, Golgi mini-stacks are triple-labeled by endogenous or exogenously expressed GM130, GalT-mCherry and the test protein (x). GM130 and GalT-mCherry, *cis*- and *trans*-Golgi markers respectively[12,13], provide reference points. The triple fluorescence, red (R), green (G) and far-red (B), are artificially displayed as red, green and blue, respectively. Center of fluorescence mass (hereafter center) is adopted to achieve sub-pixel resolution. The Golgi axis is defined as the vector from the center of GM130 to that of GalT-mCherry. The Golgi mini-stack is modeled as a cylindrical structure with infinite rotational symmetry around the Golgi axis. Therefore, a Golgi mini-stack can be further modeled as an one-dimensional structure along the Golgi axis. The LQ of the test protein x is defined as d_x/d_1, in which d_x is the distance from the center of x to that of GM130, while d_1 is the distance from the center of GalT-mCherry to that of GM130. If the center of x is off-axis, its projection axial distance is used for the calculation. The variables, including Golgi axis, axial angle, d_x, d_1, angle α and angle β, for GLIM are schematically illustrated in **Figure 1**. LQ is independent of the Golgi axial angle though Golgi mini-stacks orient randomly in a cell.

Golgi mini-stacks appear inhomogeneous in images. We developed three criteria to select analyzable Golgi mini-stacks for GLIM. 1) The signal-to-noise ratio criterion, in which the ratio of the total intensity of a Golgi mini-stack to the standard deviation (SD) of the background is ≥ 30 in each channel. This criterion is to ensure the positioning accuracy of the center of mass, which depends on the signal-to-noise ratios of Golgi mini-stacks. 2) The axial angle or distance criterion, which requires $d_1 \geq 70$ nm. d_1 decreases with the increase of the Golgi axial angle. When the axial angle is

approaching 90° or vertical, the mini-stack becomes non-resolvable as d_1 is approaching 0. $d_1 \geq 70$ nm can effectively exclude near vertical Golgi mini-stacks. 3) The co-linearity criterion, in which either $|\tan \alpha|$ or $|\tan \beta|$ is ≤ 0.3. This criterion ensures that the three centers of a mini-stack are sufficiently co-linear for our one-dimensional model of the Golgi mini-stack. All light microscopes suffer from chromatic aberration which can seriously distort the relative positions of red, green and far-red fluorescence centers. Chromatic aberration of microscope systems is experimentally calibrated by imaging 110 nm beads, which are triple-labeled by red, green and far-red fluorescence. For each bead image, the center of red is defined as the true position of the bead and chromatic-shifts of green and far-red centers are fitted by first-order polynomial functions. Centers of Golgi mini-stacks are subjected to the polynomial functions to correct the chromatic-shifts in green and far-red channels.

Through GLIM we can achieve a resolution of ~ 30 nm along the Golgi axis under standard conditions. Importantly, it provides a systematical method to quantitatively map any Golgi protein. GLIM can be performed by conventional microscopes, such as wide-field or confocal microscopes, using common fluorescence labeling protocols. The imaging and data processing can take as short as an hour. Through GLIM, we have directly demonstrated the progressive transition of the secretory cargo from the *cis*- to *trans*-side of the Golgi[7].

PROTOCOL:

Note: Below is a step-by-step protocol of GLIM for determining the LQ of EGFP-tagged tyrosylprotein sulfotransferase 1 (TPST1), a Golgi resident enzyme, in HeLa cells.

1 Preparation of Fluorescence-labeled Golgi Mini-stacks

1.1 Prepare glass coverslips

1.1.1 Aliquot 0.3 mL sterile Dulbecco Modified Eagle's Medium (DMEM) to a well of a 24-well plate in a tissue culture hood.

1.1.2 Wipe a piece of Φ 12 mm No.1.5 glass coverslip using soft tissue paper dabbed with 70% ethanol to remove debris on the surface of the glass coverslip. Use a pair of sharp tweezers to briefly soak the coverslip in 70% ethanol, transfer it into the 24-well plate and sink it to the bottom of the well containing sterile DMEM.

Note: Glass coverslips are conventionally sterilized by flaming. However, coverslips under such treatment easily crack during subsequently handling. 70% ethanol soaking is effective in sterilizing glass coverslips without damaging them.

1.1.3 With the lid covered, leave the 24-well plate in the 37 °C CO_2 incubator until use.

1.2 Seed cells

1.2.1 Culture HeLa cells (hereafter cells) in a T-25 flask in DMEM supplemented with 10% Fetal Bovine Serum (FBS) (hereafter complete medium) in a 37 °C CO_2 incubator supplied with 5% CO_2. Antibiotics are not necessary here.

1.2.2 When cells reach ~ 80% confluency, aspirate the culture medium. Add 1 mL of 0.25% Trypsin-EDTA into the flask and incubate the cells in a 37 °C CO_2 incubator for 2 min. Add 1 mL complete medium into the flask and gently flush the cells off the flask wall by pipetting.

1.2.3 Transfer detached cells to a sterile centrifugation tube and spin at 500 x g for 2 min to pellet the cells. Aspirate the supernatant and suspend the cell pellet in 1 mL complete medium.

1.2.4 Aspirate the medium in the well of 24-well plate containing the sterilized glass coverslip and seed ~ 1 x 10^5 cells in the well. Top up the volume of the well with complete medium to 0.5 mL. Incubate in a 37 °C CO_2 incubator supplied with 5% CO_2. Culture the cells to ~ 80% confluency.

1.3 Transfect cells

Note: Well-spread cells are advantageous for imaging dispersed Golgi mini-stacks.

1.3.1 Transfect ~ 80% confluent cells with 80 ng GalT-mCherry[7] and 320 ng TPST1-EGFP (see **Table of Materials**) DNA plasmids using a transfection reagent according to the protocol provided by the manufacturer. Incubate in a 37 °C CO_2 incubator. Change the medium after 4–6 h incubation. The cells are ready ~ 12 h later.

1.4 Nocodazole treatment to generate Golgi mini-stacks

1.4.1 Prepare a nocodazole stock solution (33 mM) by dissolving 10 mg nocodazole powder in 1 mL dimethyl sulfoxide. Aliquot the stock solution and store at -20 °C for long term storage.

1.4.2 Dilute 1 µL of nocodazole stock solution (33 mM) in 1 mL 37 °C complete medium (final concentration 33 µM). Centrifuge at top speed to remove particulate matter.

1.4.3 Aspirate the medium in the well and add 0.5 mL of 33 µM nocodazole containing complete medium. Incubate the cells in a 37 °C CO_2 incubator for 3 h. Proceed to immunofluorescence labeling (section 1.5).

1.5 Immunofluorescence labeling

Note: Keep cells in the dark to avoid photobleaching of GalT-mCherry and TPST1-EGFP.

1.5.1. Prepare reagents for immunofluorescence labeling

1.5.1.1. To prepare 4% paraformaldehyde solution, dissolve 4% (w/v) paraformaldehyde powder in hot 1X phosphate buffered saline (PBS) and filter the solution through 0.45 µm filter. The solution can be stored at -20 °C.

CAUTION: Paraformaldehyde is toxic and carcinogenic and it can cause skin irritation. Wear the appropriate personal protective equipment (PPE).

1.5.1.2. To prepare fluorescence dilution buffer (FDB), mix 5% (v/v) FBS, and 2% (w/v) bovine serum albumin (BSA) in 1X PBS. Filter the solution through 0.45 µm filter and store the solution at -20 °C.

1.5.1.3. Dissolve 5.35 g NH_4Cl powder in 1 L water to prepare 100 mM NH_4Cl and store the solution at room temperature.

1.5.1.4. Dissolve 10% (w/v) saponin powder in water to prepare 10% saponin, and aliquot and store the solution at -20 °C.

1.5.2. Fixation

1.5.2.1. Rinse the well once with 0.5 mL 1X PBS solution and add 0.5 mL 4% paraformaldehyde solution. Incubate for 20 min at room temperature. Rinse the well twice with 0.5 mL 1X PBS and twice with 0.5 mL 100 mM NH_4Cl. Rinse the well twice with 0.5 mL 1X PBS. Cells can be kept in 1X PBS at 4 °C overnight in the dark.

1.5.3. Fluorescence labeling

1.5.3.1 Dilute 1 µL mouse anti-GM130 primary antibody in 500 µL FDB containing 0.1% saponin. Reverse the lid of the 24-well plate and apply 10 µL primary antibody mixture onto the lid.

1.5.3.2 Use a pair of sharp tweezers to extract and transfer the glass coverslip onto the drop of antibody mixture ensuring that the cell side is in contact with the mixture. Incubate the cells with the primary antibody mixture for 1 h at room temperature.

Note: The lid with the coverslip can be placed in a humidified plastic bag in the dark.

1.5.3.3 Use a pair of sharp tweezers to extract and transfer the coverslip to the well with the cell side up and rinse it in 0.5 mL 1X PBS for three times in ≥ 30 min. Shaking is unnecessary.

1.5.3.4 Dilute 1 µL far-red fluorophore conjugated goat anti-mouse IgG (secondary antibody) in 500 µL FDB containing 0.1% saponin.

1.5.3.5 Wash and clean the reverse surface of the lid of the 24-well plate. Apply 10 µL far-red secondary antibody mixture onto the lid. Repeat steps 1.5.3.2–1.5.3.3.

1.5.4. Mounting

1.5.4.1. Prepare the mounting medium by mixing 1 g glycerol, 2.2 g poly(vinyl alcohol) (MW ~ 31,000 Da), 1.2 mL 1 M Tris (pH 8) and 8.8 mL H_2O. Dissolve the mixture in a 60 °C water bath with occasionally vortexing. Store the solution at -20 °C.

1.5.4.2. Thaw the mounting medium and transfer 10 µL onto a glass slide. Overlay the coverslip (cell side down) onto the drop of mounting medium. Incubate the glass slide at 37 °C for 30 min or room temperature for 1 h to harden the mounting medium. Seal the coverslip with colorless-nail polish and store the glass slide at -20 °C.

2. Preparation of Fluorescent Beads for Chromatic-shift Correction

2.1 Coverslip cleaning

2.1.1 Carefully place a piece of Φ 25 mm No.1.5 glass coverslip onto a plastic rack.

2.1.2 Transfer the rack with the coverslip to a 400 mL beaker filled with 300 mL 1 M NaOH and sonicate in a bath sonicator (35 Watts) for 15 min. Briefly rinse the rack in a 400 mL beaker filled with 300 mL deionized water. Transfer the rack to a 400 mL beaker filled with 300 mL 99% ethanol and sonicate in the bath sonicator for 15 min. Briefly rinse the rack in a 400 mL beaker filled with 300 mL deionized water.

2.1.3 Repeat step 2.1.2 two more times.

2.1.4 Rinse the rack with the coverslip in 300 mL deionized water in a 400 mL beaker for 10 min. Repeat the step two more times. Transfer the rack to a 60 °C oven and dry the coverslip for 1 h. Store the dried coverslip in a dust-free Petri dish.

2.2 Immobilization of fluorescent beads on the glass coverslip

2.2.1. Dilute 110 nm multi-color fluorescent beads 80-fold in 1X PBS containing 0.1 µg/µL BSA. Briefly vortex the tube to disperse bead aggregates. Spread 60 µL diluted beads onto the cleaned Φ 25 mm No.1.5 glass coverslip (see section 2.1) using a pipette tip. Dry the coverslip in a desiccator connected to a vacuum pump in the dark and mount the coverslip onto a glass slide in 50 µL mounting medium (see step 1.5.4.2).

3. Image Acquisition

Note: GLIM requires images of high signal-to-noise ratio (SNR) for high precision center of mass calculation. The image can be acquired by conventional microscopes such as the laser scanning confocal, spinning disk confocal or wide-field microscope. A wide-field microscope equipped with plan-apochromatic objective lenses and a low noise

image sensor, such as a charge-coupled device (CCD) and scientific complementary metal-oxide semiconductor (sCMOS) can be used. Parameters for the image sensor are adjusted to ensure a low read-noise and high dynamic range. The microscope must be equipped with the optimal configuration of fluorescence filters for green, mCherry and far-red fluorophore, and it must have negligible fluorescence cross-talk. Ideally, the imaging system achieves a Nyquist sampling rate in the x, y and z axis, which typically requires the x, y and z size of a voxel to be less than 100, 100 and 200 nm, respectively. The x and y size of the voxel are always equal and are referred to as pixel_size. The pixel_size can be calculated by dividing the camera sensor size by the system magnification.

3.1. Image multi-color beads

3.1.1. Image multi-color beads in green, red and far-red channels at the beginning and at the end of every imaging session.

Note: Here, images were acquired through a conventional wide-field epi-fluorescence microscope (inverted) equipped with 100X NA 1.4 plan-apochromatic objective, motorized stage, 200 Watt metal-halide light source and 16-bit sCMOS camera. The wavelengths for excitation filter (band pass), dichroic mirror (long pass) and emission filter (band pass) of the green channel filter cube are 465–495, 505 and 515–555 nm; those for the red channel filter cube are 528–553, 565 and 578–633 nm; and those for the far-red channel filter cube are 590–650, 660 and 663–738 nm. During acquisition, the size of a voxel along the x, y and z axis is 64, 64 and 200 nm, respectively.

3.1.2. Find a field of view with good bead density.

3.1.3. Acquire 3D image stacks in green, red and far-red channels ($channel_G$, $channel_R$, $channel_B$, respectively). Take 3 sections above and below the best focal plane (7 sections per stack). Save the three stacks as three TIFF files.

Note: The exposure time for each channel is empirically determined to maximize the dynamic range, avoid pixel saturation and minimize photobleaching. Other parameters, such as filters and the x, y and z size of the voxel, are chosen as discussed above.

3.2. Image Golgi mini-stacks

3.2.1. Use TPST1-EGFP and GalT-mCherry transfected and fluorescently labeled (section 1.5) slides to find cells that express TPST1-EGFP and a low or medium level of GalT-mCherry. Acquire 3D image stacks as described in step 3.1.3.

4. Image Analysis

4.1. Acquire centers of fluorescent beads

4.1.1. In ImageJ, open a set of bead images consisting of three TIFF files (File>Image>Open).

4.1.2. Average 3 consecutive sections around the best focused section in channel$_R$ by Image>Stacks>Z Project. Input the section number of “Start slice” and “Stop slice”, and among options of “projection type”, select “Average Intensity”.

4.1.3. Draw regions of interest (ROIs) that contain no beads in the image using “Polygon selections”. In “Analyze>Set Measurements”, check only “Mean gray value” and “Standard deviation”. Then execute “Analyze>Measure” to obtain the mean and SD of the ROI.

4.1.4. Calculate the background intensity as “mean+6×SD”. Subtract the image with the corresponding background intensity values by “Process>Math>Subtract”, input the background intensity value corresponding to this channel.

4.1.5. Repeat steps 4.1.2 to 4.1.4 for channel$_G$ and channel$_B$ image stacks by using the same start and stop slice and the same background ROI. Note that the ROI used in step 4.1.3 can be copied to channel$_G$ and channel$_B$ images.

4.1.6. Merge the three background-subtracted images (Image>Color>Merge Channels) by selecting channel$_R$ as red, channel$_G$ as green and channel$_B$ as blue. A single composite image consisting of three channels will be obtained.

4.1.7. Launch ROI manager (Analyze>Tools>ROI Manager). Draw a square ROI around single beads ensuring that there is only one bead within each ROI. Add the ROI to ROI manager by pressing “t” on the keyboard. Repeat this process and add as many ROIs as possible.

4.1.8. In “Analyze>Set Measurements”, check only “Center of mass”.

4.1.9. Select channel$_R$, in ROI Manager, click “Measure” to obtain centers; two columns corresponding to the x and y coordinate of centers of ROIs will be displayed in the “Result” window. Copy and paste the two columns into a spreadsheet.

4.1.10. Repeat step 4.1.9 for channel$_G$ and channel$_B$.

4.1.11. Arrange coordinates of centers in a single spreadsheet in the following order: x_R, y_R, x_G, y_G, x_B, and y_B. Save the spreadsheet in “.csv” format as “*beads.csv*”. Leave no space in the file name.

4.2. Select and measure Golgi mini-stacks

4.2.1. Install two macros (attached in **Supplemental Materials**) in ImageJ, “Macro-Golgi ROI inspection” and “Macro-Output 3 channels data” by Plugins>Macros>Install”. Clear ROI manager and Result window.

4.2.2. Open a set of Golgi mini-stack images consisting of three TIFF files (File>Image>Open).

4.2.3. Repeat steps 4.1.2–4.1.5 and save the three background-subtracted images for later use. Record the background SDs for $channel_R$, $channel_G$ and $channel_B$ as SD_R, SD_G and SD_B, respectively, for later use.

4.2.4. Duplicate the three background-subtracted images generated from step 4.2.3 (Ctrl+Shift+D).

4.2.5. In "Process>Image Calculator", first add the background-subtracted $channel_G$ to $channel_R$ images. Add the resulting image to the background-subtracted $channel_B$ image. To the resulting image, in "Image>Adjust>Threshold", select "set" to input "1" as "Lowest Threshold Level". After pressing "Apply", a black and white binary image is resulted.

4.2.6. In "Analyze>Analyze particles", input the size range for "Size (pixel^2)". Input "50-infinity". Check "Excludes on edges" and "Add to Manager". ROIs containing Golgi mini-stacks are now added to the ROI manager.

Note: The size range must be empirically determined to exclude noises which are generally small.

4.2.7. Merge the three background-subtracted images (Image>Color>Merge Channels) from step 4.2.3 by selecting $channel_R$ as red, $channel_G$ as green and $channel_B$ as blue. A single composite image consisting of three channels is generated.

4.2.8. Run the macro "Macro-Golgi ROI inspection" by selecting "Plugins> Macro-Golgi ROI inspection". In the interactive dialog box, visually inspect each ROI to keep or reject it. Select ROIs that contain a single object in all three channels.

Note: After running this tool, rejected ROIs are eliminated from the ROI manger.

4.2.9. Check only "Area", "Mean gray value" and "Center of mass" in "Analyze>Set Measurements". Acquire data by launching the macro tool "Macro-Output 3 channels data" (Plugins> Macro-Output 3 channels data).

Note: Areas, Mean intensities, and centers (x and y) of ROIs in $channel_R$, $channel_G$ and $channel_B$ are displayed in the "Result" window.

4.2.10. Copy x and y coordinates of centers into a spreadsheet and arrange them in the following order: x_R, y_R, x_G, y_G, x_B, and y_B. Save the spreadsheet as a ".csv" file (*ministacks*.csv). Leave no space in the file name. Proceed to chromatic-shift correction of the centers (section 4.3) and LQ calculation (section 4.4).

4.3. Chromatic-shift correction of centers

4.3.1. Install Matlab Compiler Runtime (MCR).

4.3.2. Install the following files in a dedicated working folder: my_train.exe and my_test.exe. Copy and paste "*beads.csv*" and "*ministacks*.csv" files in the same folder.

4.3.3. Launch "Command Prompt" of Windows and go to the working folder by typing the following command "**cd** *path_of_working_folder"*.

4.3.4. Generate a chromatic-shift calibration file by using centers of beads. Type the following command "**my_train.exe** *beads*.csv *calibration*.mat **1**". A file named "*calibration*.mat" is created in the working folder. Ignore other files that are generated.

4.3.5. Correct the chromatic-shift of centers of Golgi mini-stacks by typing the following command "**my_test.exe** *ministacks*.csv *corrected_ministacks*.csv *calibration*.mat **1**".

Note: A file named "*corrected_ministacks*.csv" is created in the working folder. It contains chromatic-shift corrected coordinates of centers, which are arranged in the order of x_G, y_G, x_B and y_B. Ignore other files that are created. Take note that the red channel is defined as free of chromatic aberration and therefore x_R and y_R are the same as raw data.

4.4. Calculation of LQs

4.4.1. Launch the data analysis software and copy and paste, in the below sequence, mean gray values, areas, background SDs obtained from step 4.2.3 (place them at row 1) and chromatic-shift corrected x and y coordinates of Golgi mini-stacks in $channel_R$ into a worksheet as columns A to E. Similarly, transfer corresponding data for $channel_G$ and $channel_B$ to columns F to J and K to O, respectively.

4.4.2. Add eight new columns P to W, named "integrated intensity of channel R", "integrated intensity of channel G", "integrated intensity of channel B", d1, dx, ABS(tan a), ABS(tan b) and LQ.

4.4.2.1. Right click the top of the respective column and select "Set Column Values" to calculate values of each column. For column P, input "Col(A)∗Col(B)"; for column Q, input "Col(F)∗Col(G)"; for column R, input "Col(K)∗Col(L)"; for column S, input "pixel_size*Distance(Col(D), Col(E), Col(N), Col(O))" where "pixel_size" is the size of the pixel in nm; for column T, input "pixel_size*((Distance(Col(I), Col(J), Col(N), Col(O))^2+Distance(Col(D), Col(E), Col(N), Col(O))^2-Distance(Col(D), Col(E), Col(I), Col(J))^2)/(2∗Distance(Col(D), Col(E), Col(N), Col(O)))"; for column U, input "Abs(Tan(Acos((Distance(Col(I), Col(J), Col(N), Col(O))^2+Distance(Col(D), Col(E), Col(N), Col(O))^2-Distance(Col(D), Col(E), Col(I), Col(J))^2)/(2∗ Distance(Col(I), Col(J), Col(N), Col(O))∗Distance(Col(D), Col(E), Col(N), Col(O))))))"; for column V, input "Abs(Tan(Acos((Distance(Col(D), Col(E), Col(I), Col(J))^2+Distance(Col(D), Col(E),

Col(N), Col(O))^2-Distance(Col(I), Col(J), Col(N), Col(O))^2)/(2∗ Distance(Col(D), Col(E), Col(I), Col(J))∗Distance(Col(D), Col(E), Col(N), Col(O))))))"; for column W, input "Col(T)/Col(S)".

4.4.3. Filter the Golgi mini-stacks by the three criteria described in the **Introduction**.

4.4.3.1. In the data analysis software, go to "Worksheet>Worksheet Query" and select column variables for "If" test. Assign the following aliases I1, I2, I3, d1, A and B for columns "integrated intensity of channel R", "integrated intensity of channel G", "integrated intensity of channel B", d1, ABS(tan a) and ABS(tan b), respectively. In the "If condition" box, input "I1>=30∗cell(1,3) AND I2>=30∗cell(1,8) AND I3>=30∗cell(1,13) AND d1>=70 AND (A<=0.3 or B<=0.3)".

4.4.3.2. Select "Extract to New Worksheet", click "Apply". Columns A–W of analyzable Golgi mini-stacks are extracted to a new worksheet.

4.4.4. In the new worksheet, right click the top of column W, select "Statistics on Column>Open Dialog". Check "N total", "Mean", "SE of mean", "Histograms". The statistical analysis of LQs is then displayed.

REPRESENTATIVE RESULTS:

The modern research grade light microscope equipped with a plan apochromatic lens, such as the one used in our lab, shows minimal chromatic aberration (**Figure 2A**). However, a careful examination of the multi-color fluorescent bead image can reveal the shift of different color images of the same bead (**Figure 2B**). We define that the red channel is free of chromatic aberration and therefore centers of red fluorescence are the true positions of beads. The relative chromatic-shifts of green and far-red fluorescence can be represented by green and blue vectors originating from the center of red fluorescence (**Figure 2C**). The chromatic-shift within the imaging-plane is empirically illustrated by the green and blue vector for each bead (**Figure 2D**). For our microscope, the chromatic-shifts are not uniform as both magnitudes and directions of shifts change according to x and y positions. The chromatic-shift can significantly affect the accuracy of GLIM as the shift of far-red centers can be as much as 50 nm. Therefore chromatic-shift must be corrected. Once centers of beads are acquired, we employ first-order polynomial fitting to calibrate and subsequently correct the chromatic-shifts of centers. The chromatic-shift aberration is greatly improved by this approach (**Figure2 D–G**).

In mammalian cells, the Golgi complex is aggregated at the perinuclear area which is usually not resolvable under a conventional optical microscope (**Figure 3A**). After nocodazole treatment for 3 hours, the perinuclear Golgi complex disappears and dozens of Golgi mini-stacks assemble at the endoplasmic reticulum exit sites throughout the cytoplasm (**Figure 3B**). Large chunks of Golgi membrane and aggregated Golgi mini-stacks are present and they are not selected for analysis. An example image illustrating the selection of Golgi mini-stacks is shown in **Figure 3C**. Using the "Macro-Golgi ROI inspection" tool, 40 selected Golgi mini-stacks are listed in **Figure 3D**. After applying the three criteria, 21 Golgi mini-stacks were analyzable and

chosen for the calculation of LQs of TPST1-EGFP (**Figure 3D**; labeled as "L"), with their statistics shown in **Figure 3E**. In total, 111 analyzable Golgi mini-stacks were selected from 12 cells and the LQ of TPST1-EGFP was measured to be 0.76 ± 0.04 (mean ± SEM; n = 111) (**Figure 3F**). The LQ of TPST1-EGFP positions it between giantin (LQ = 0.59) and EGFP-Rab6 (LQ = 1.04)[7]. It is near GS15 (LQ = 0.83) and ST6GalT1-AcGFP1 (LQ = 0.82). We have defined the sub-Golgi regions according to LQs by considering qualitative localization data from literature: the ERES/ERGIC, < -0.25; *cis*, 0.00 ± 0.25; medial, 0.50 ± 0.25; *trans*-Golgi, 1.00 ± 0.25 and TGN, 1.25–2.00. The LQ of TPST1-EGFP therefore indicates its *trans*-Golgi localization, which is in agreement with a previous study[14].

FIGURE LEGENDS:

Figure 1. Schematic illustrations showing variables used in the calculation of GLIM. (**A**) xz view of a Golgi mini-stack that has co-axial centers of GM130, GalT-mCherry and protein x fluorescence showing Golgi axis, axial angle, d_x and d_1. The image of the Golgi mini-stack is its projection onto the image plane. (**B**) xy view of a Golgi mini-stack with off-axis center of protein x showing angle α, angle β and projection axial distance d_x. **A** and **B** are adapted from **Figure 1E** and **1F** of our previous publication[7], respectively.

Figure 2. Correction of the chromatic-shift. (**A**) The three-color merged image of 110 nm multi-color beads acquired by a wide-field microscope. Each bead emits green, red, and far-red (artificially colored as blue) fluorescence. Scale bar, 10 μm. (**B**) Enlarged view of a merged single-bead image with a noticeable chromatic-shift. Scale bar, 200 nm. (**C**) A schematic diagram showing that shifts of green and far-red bead images can be represented by vectors from the center of the red bead image (0,0) to centers of green (green vector) and far-red (blue vector) bead images, respectively. (**D**, **E**) The effect of the chromatic-shift correction. Within the same field of image, green and blue vectors are plotted before and after shift correction. To visualize the tiny shift, the magnitude of each vector is amplified 200-fold. (**F**, **G**) Scatter plots showing centers of green (green dots) and far-red (blue dots) beads before and after shift correction.

Figure 3. A typical example of GLIM, here acquiring the LQ of TPST1-EGFP. A HeLa cell expressing TPST1-EGFP (green) and GalT-mCherry (red) were stained for endogenous GM130 (blue). (**A**) A typical cell. (**B, C, D, E**) A cell treated with nocodazole was imaged (**B**). From the image, analyzable Golgi mini-stacks were selected as shown in **C**. Selected Golgi mini-stacks are labeled by identification numbers. Images of these masked Golgi mini-stacks are displayed below in **D** with their identification numbers. "L" denotes that the Golgi mini-stack is valid for the calculation of the LQ (analyzable Golgi mini-stacks). Histogram of LQs of 21 mini-stacks from the cell is shown in **E**. (**F**) Histogram of LQs of 111 mini-stacks from 12 cells. The value shown in the histogram is mean ± SEM.

SUPPLEMENTAL MATERIALS: Macro-Golgi ROI inspection.ijm, Macro-Output 3 channels data.ijm, Matlab codes, My_train.exe, My_test.exe, and Spreadsheet template.opj.

DISCUSSION:

Previously, the localization of a Golgi protein under the light microscopy was mainly quantified by the degree of correlation or overlapping of the image of the protein with the image of a Golgi marker of known localization[15-17]. The resulting correlation or overlapping coefficient reflects how close the testing protein is to the Golgi marker spatially. There are at least three caveats for this approach. First, the correlation or overlapping coefficient is nonlinear and it does not directly indicate spatial distance. Second, the degree of correlation is critically dependent on the resolution of the microscopic system. Therefore, the coefficient between two Golgi proteins is not a system-independent constant. Third, two Golgi proteins with the same axial localization but different lateral distribution along cisternae can have distinct coefficients. Hence, the correlation or overlapping coefficient does not indicate the axial localization of a Golgi protein. In an alternative method proposed by Dejgaard *et al*., *cis*, *trans*-Golgi markers and the protein of interest are triple-labeled in nocodazole treated Golgi mini-stacks, similar to GLIM. Within each Golgi mini-stack, positions of the three maximum intensity pixels are acquired and the relative position of the test protein is calculated as a distance ratio[18]. However, their method is unable to achieve sub-pixel resolution, which greatly limits its application. Compared to previous quantitative methods, GLIM, which was independently developed but bears a similar concept to that of Dejgaard *et al*., is able to quantify the axial localization of a Golgi protein with unprecedented accuracy and consistency.

We presented the protocol of GLIM to acquire the LQ of exogenously expressed GFP-tagged protein — TPST1-EGFP. The LQ of endogenous protein can be determined if its antibody is available. Depending on the species of the primary antibody, mouse or rabbit, then a rabbit or mouse anti-GM130 antibody, respectively, can be used in the triple-labeling protocol. Both rabbit and mouse anti-GM130 antibodies for immunofluorescence labeling are commercially available. We have previously demonstrated that rabbit and mouse anti-GM130 antibodies give the same results in GLIM[7]. If the test protein is intrinsically red in fluorescence emission, such as mCherry fusion, a GFP-tagged Golgi marker protein with known LQ in combination with GM130 antibody labeling can be used to triple-label cells and indirectly deduce the LQ of the test protein. Assuming the marker protein's LQ is LQ_m and the distance from the center of the marker protein to that of GM130 is d_m, the LQ of the test protein x can be calculated as $(d_x/d_m)\times LQ_m$. One of the greatest advantages of GLIM in comparison to super-resolution microscopy is that it can easily be applied to the live cell imaging in conventional microscopic setups. We have tried a combination of three fluorescence proteins, GFP-Golgin84, mCherry-GM130 and GalT-iRFP670, for dynamically monitoring the LQ of GFP-Golgin84 in single Golgi mini-stacks[7].

In GLIM, the most time-consuming step is the post-acquisition analysis, especially on the selection of analyzable Golgi mini-stacks. As Golgi mini-stacks are heterogeneous in both size and molecular composition[19], it is unclear if the distribution of LQs (**Figure 3F**) represents the heterogeneous architecture of Golgi mini-stacks or the uncertainty of our calculation of the LQ. Regardless of the causes, we found that it is important to

have a large number of analyzable Golgi mini-stacks (n) to ensure the accuracy of the LQ, which is compromised when n is small. Typically, data with n ≥ 100 from multiple cells yields reliable LQs. Therefore, GLIM gives ensemble-averaged localization data, obscuring the individuality of Golgi mini-stacks. Another limitation of GLIM is that it assumes that all Golgi proteins have a narrow distribution around a single center. Some Golgi proteins, such as COPI subunits, ARF1 and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs)[20,21], have been reported to distribute broadly from the *cis* to the *trans*-Golgi and the TGN. It is probably inappropriate to study their Golgi localization by the LQ. As GLIM currently involves manual selection of the Golgi mini-stacks, which is both laborious and subjective, the future development of GLIM will be to implement a software tool to automatically select Golgi mini-stacks with minimal human interference.

What is the spatial resolution of GLIM? Since the LQ is a ratio, which is unitless, it does not indicate a spatial distance. Here, we attempt to give a very rough estimation. The spatial resolution of GLIM can be defined as the smallest axial distance between two resolvable Golgi proteins. Examining LQs of various Golgi proteins[7], we found that SEMs of datasets with n ≥ 100 range around 0.03. Assuming two Golgi proteins have the same n = 100 and SEM = 0.03, the two Golgi proteins have significant different localization by *t-test* ($p < 0.05$), *i.e.*, resolvable, if the difference between their LQs is ≥ 3 × SEM = 0.09. From the EM data, we can estimate that the axial length of the Golgi mini-stack, which is also the distance from GM130 to GalT-mCherry in GLIM, is ~ 300 nm[8]. Hence, the resolution of GLIM is estimated to be 0.09 × 300 ≈ 30 nm along the Golgi axis. In GLIM, a larger n generally yields smaller SEM, which in turn results in higher resolution.

It is probably inappropriate to directly compare the resolution of GLIM to that of the immuno-gold EM since the latter technique does not directly yield quantitative localization data. Similar to GLIM, the resolution of the immuno-gold EM along the Golgi axial can be defined as the smallest distance between two resolvable Golgi markers. To measure its resolution requires the study of dual-immuno-gold labeling of two Golgi proteins that are closely adjacent to each other along the Golgi axis. Such systemic study is probably unavailable according to our knowledge. As discussed in the introduction, one of the limiting factors in the spatial resolution of the immuno-gold EM is the large size of the antibody complex, which makes the resolution to be worse than ~ 20 nm. Another important limiting factor of the image resolution is the labeling density of antigen molecules. According to the Nyquist sampling theory, there must be at least two gold particles per resolution unit. In most immuno-gold EM studies, the distances between neighbor gold particles are not < 15 nm due to the very low labeling efficiency; this makes it difficult for this technique to achieve an image resolution less than 30 nm. From this point of view, we estimate that the resolution of GLIM is at least comparable to that of the immuno-gold EM.

GLIM is a robust method that gives highly consistent results. It is independent of the type of microscope used. We have tested that wide-field and spinning disk confocal microscopes yielded the same results. LQs of the same Golgi proteins acquired at

different batches of experiments were also in good agreement with each other. A LQ database consisting of a diverse range of Golgi resident proteins has been generated for comparison and interpretation. We expect that more usage of GLIM in the research community will significantly expand the database. Consequently, a more complete database quantitatively describing the localization of a large number of Golgi proteins will greatly help in understanding the organization and function of the Golgi complex.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure 1

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Fig. 1

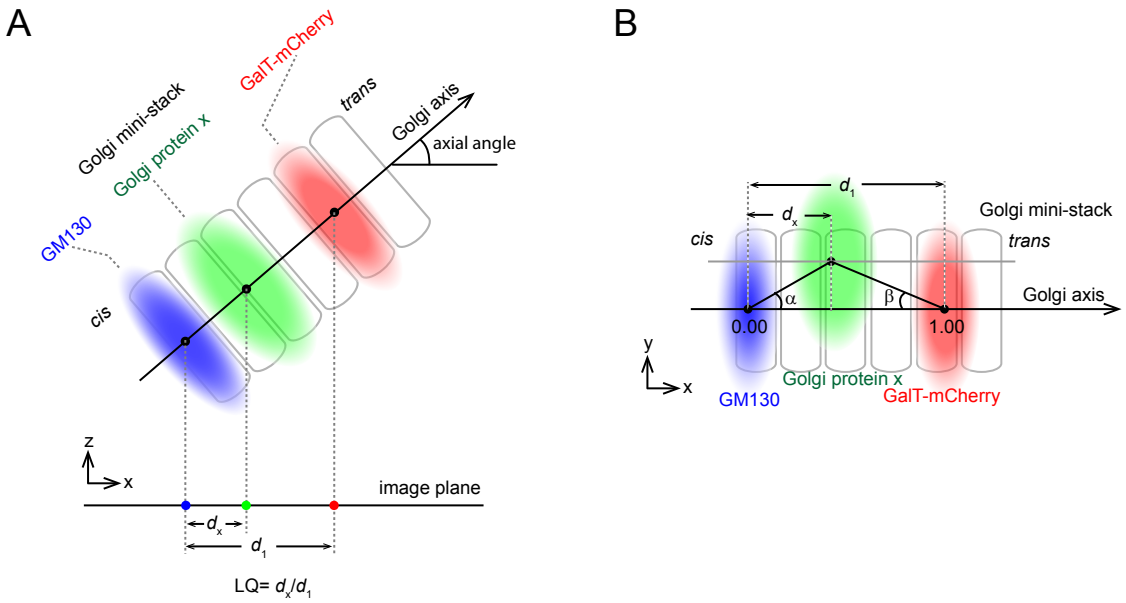

Figure 2

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Fig. 2

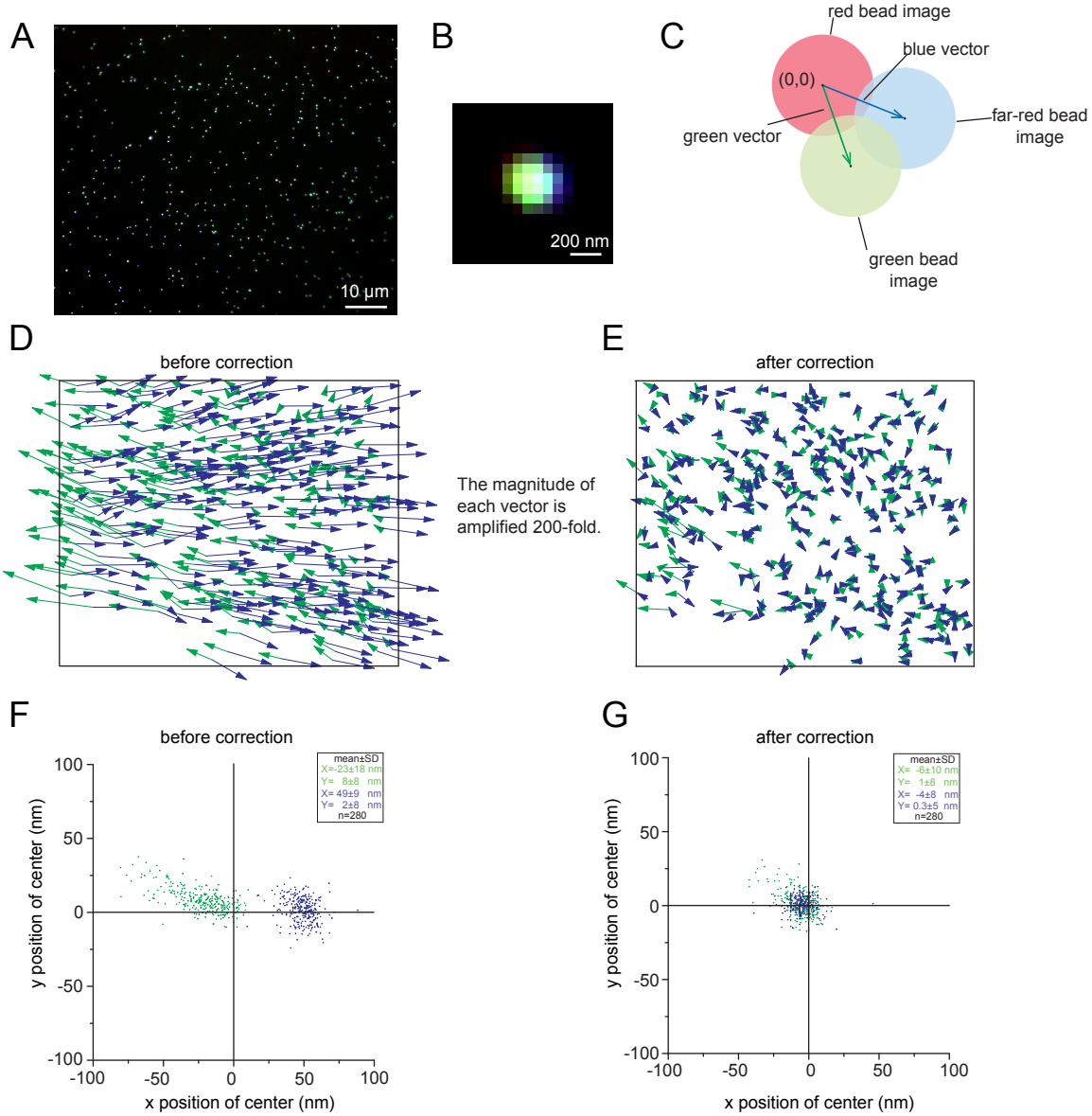

Figure 3

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Fig. 3

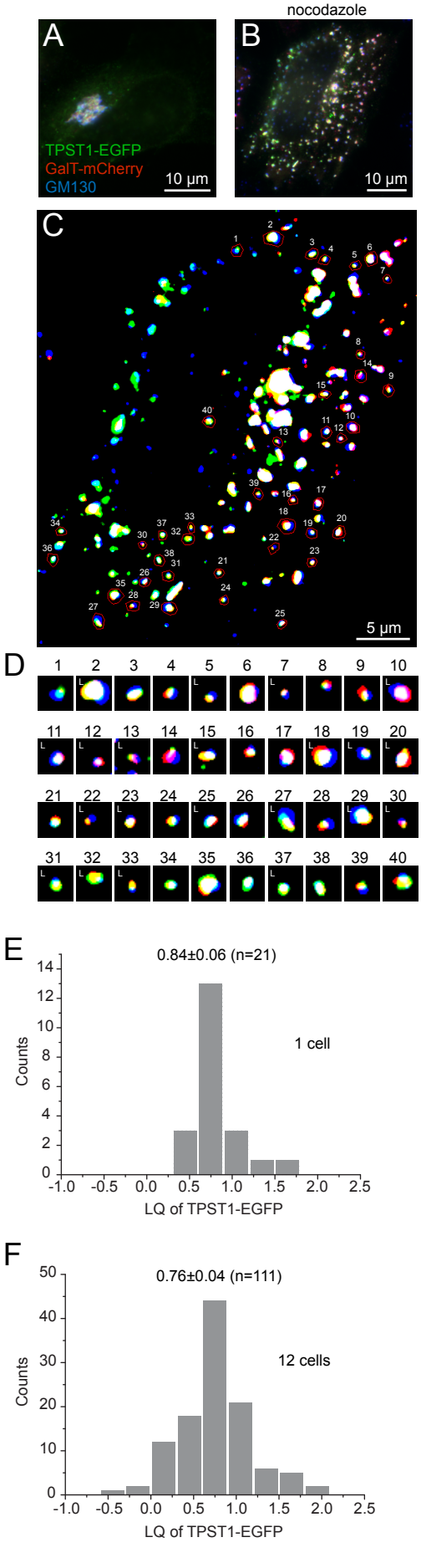

Table of Materials/Equipment

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Name of Material	Company	Commercial Name	Catalog Number	Comments/Description
fluorescence beads	Invitrogen	TetraSpeck beads	T7279	As multi-color beads to calibrate chromatic-shift of the microscope.
Glass coverslip Φ12 mm (No. 1.5)	Menzel		CB00120RAC	
Glass coverslip Φ25 mm (No. 1.5)	Menzel			
DMEM	Capricon		DMEM-HPA-P50	
Trypsin-EDTA				
FBS	GE Hyclone		SV30160.03	
Nocodazole	Merck		487928	
transfection reagent	Invitrogen	Lipofectamine 2000	11668-019	
transfection medium	Invitrogen	OptiMEM	31985070	
TPST1-EGFP	Addgene		66617	A gift from D. Stephens (University of Bristol, Bristol, United Kingdom)
GalT-mCherry				Made in our lab.
paraformaldehyde	Merck		1.04005.1000	
saponin	Sigma-Aldrich		47036	
poly(vinyl alcohol) (Mw ~31,000)	CALBIOCHEM	Mowiol-488	475904	
BSA	Sigma-Aldrich		A9647	
Mouse anti-GM130	BD Biosciences		610823	Primary antibody for human GM130

far-red fluorescence conjugated goat anti-mouse IgG	Invitrogen	Alexa Fluor 647 conjugated goat anti-mouse IgG	A-21235	Far red fluorescence conjugated secondary antibody

Name of Equipment	Company	Catalog Number
bath sonicator	Elma	
microscope	Olympus	IX83
objective	Olympus	Plan apochromatic, 100×, NA 1.40
motorized stage	Prior Scientific	
camera	Andor	Neo
excitation light	Prior Scientific	Lumen Pro 200
filter set for green channel	Chroma	31001
filter set for red channel	Chroma	31002
filter set for far-red channel	Chroma	41008
Metamorph software	Molecular Devices	
ImageJ	freeware	
Microsoft Office Excel 2007	Microsoft	
OriginPro8.5	OriginLab	
Matlab Compiler Runtime	Math works	

Comments/Description
Power output: 35 watts
scMOS. Parameters used: central quadrant, rolling shutter, 200 MHz digitizer and 16-bit (low noise, high well capacity).
200 Watt metal-halide
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CORRESPONDING AUTHOR:

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Article Title:	Quantitative localization of a Golgi protein by imaging its center of fluorescence mass
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We would like to thank the editor and three reviewers for their very constructive comments and feedbacks, which have benefited our manuscript tremendously. We believe that our manuscript has been further improved and is ready for publishing in JoVE. Please see our replies to the editor and reviewers' comments or suggestions below.

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Reply:
We have modified the manuscript accordingly.

Please ensure that all items mentioned have been included in the Materials/Equipment list, and are accompanied by a catalog number. For e.g. Trypsin-EDTA, etc.

Reply:
We have modified the manuscript accordingly.

Please define all abbreviations before use. For e.g., 3D-SIM, STED, PALM, STORM, DMEM, etc.

Reply:
We have modified the manuscript accordingly.

Please use h for hour(s), min for minute(s) and s for seconds throughout the manuscript (including figures and tables).

Reply:
We have modified the manuscript accordingly.

Please include spaces between all numbers and units.

Reply:
We have modified the manuscript accordingly.

Please include at least six keywords.

Reply:
6 keywords have been added accordingly.

Please ensure that **all** text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. For e.g. please re-write steps 1.1.4, 1.2.1, 2.2.3, 3.1.1, etc. in the imperative tense.

Reply:
We have modified the manuscript accordingly.

1.1.4: Approximately how many hours can the slide be incubated?

Reply:
We have modified the text.

1.2.1: Are antibiotics added to the medium?

Reply:
They are not necessary. We have modified the text.

1.3.2: Please provide references/citations for the plasmids.

Reply:
We have modified the manuscript accordingly.

1.5.1.1: Please provide a Caution statement for PFA.

Reply:
A caution statement has been added for PFA.

1.5.3.5: transfer how?

Reply:
Modified by adding "use a pair of sharp tweezers to extract and transfer.."

Section 3 (image acquisition): Please mention the type of microscope, setting and parameters (filters, wavelength, apertures, etc.) used in your studies. If image acquisition is to be filmed, please provide stepwise detail on how to acquire image stacks. For steps that involve software, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item.

Reply:
We have modified the manuscript accordingly.

3.1.3: Please provide a reference for acquisition.

Reply:
We have added a note.

3.2.1: Please provide the microscope, setting and parameters for imaging.

Reply:
It is the same as 3.1.3.

The Note at the beginning of the Image analysis section (section 4) should be moved to section 3 (image acquisition).

Reply:
We have modified the text accordingly.

After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Reply:
OK. We have prepared a 3-page video script which is included as a supplementary file.

Visualization and highlighting: Please note that scripting and calculations (e.g. section 4.3, 4.4) cannot be filmed.

Reply:
OK.

If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reply:

We have supplied the permission document as a supplementary file. Please also see below.

Please expand your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Reply:
We have expanded our discussion to cover these topics.

References: Please abbreviate all journal titles.

Reply:
We have used JoVE style to format our references in Endnote.

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Reply:
We have used JoVE style to format our references in Endnote.

Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors.

Reply:
We have tried our best to proofread our manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors show a novel technology to gain high resolution images of the Golgi apparatus. The Golgi consists of several subdomains for instance required for cargo import and export. However, visualization and quantification of these domains have been difficult because of the resolution of conventional light microscopes. The manuscript shows a novel super-resolution imaging technique that allows to systematically and quantitatively localize Golgi proteins called GLIM (Golgi protein localization by imaging centers of mass). The method can be applied with classical Golgi markers and state of the art light microscopy.

Major Concerns:

N/A

Minor Concerns:
I would discuss the limitation of GLIM in the article discussion not in the abstract.
The Figures are not labeled as (Figure1, Figure2 etc)

Reply:
We would like to thank this reviewer for his/her comments. We have removed the limitation from the abstract as it is discussed in Discussion. Figures are now labeled.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
It is appreciated that the authors developed a novel method to quantitatively localize proteins to Golgi cisternaes. The autors provided the detailed protocols for the method, which should be useful for other scientists who work on Golgi associated proteins. The manuscript was well written up.

Major Concerns:
N/A

Minor Concerns:
1. Are there any other quantitative analysis investigations before? if yes, the author should discuss.

Reply:
We would like to thanks this reviewer for his/her comments. As suggested, a paragraph has been added in "Discussion".

2.In protocol section: for the labeling procedure, the author skipped the permeablization step,instead of incubation with antibody in 0.1% saponin, I wonder if this is cell type dependent protocol.

Reply:
We didn't skip the permeabilization step. 0.1% saponin serves as a mild detergent to permeabilize cells.

3.Have the authors used other cell types to verify this protocol? there may have variances in different cell types, the authors may give discuss.

Reply:
We have reported the findings on other cell types in our MBoC paper (Tie *et al*., 2016). In conclusion, Golgi proteins have very similar LQs in various mammalian cells types.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The Golgi stack plays a central role in the secretory pathway, serving both as a major sorting station but also as the site of extensive post-translational processing of glycoproteins and lipids. Thus there are many Golgi resident proteins and these typically are found in only a subset of the cisternae that make the stack. Determining the precise localization of proteins in the stack is challenging as the gap between cisternae is less that the wavelength of light, and immuno-localization of proteins by electron microscopy is technically very difficult and tends to work best with abundant proteins.
The authors of this paper have recently reported the use of a light microscopy based method in which the distribution of a particular protein within the stack is determined relative to two reference proteins, and the data from many stacks averaged so that the centre of the distribution of localization by light microscopy gives a position within the stack relative to the ends that has a better resolution that a single micrograph would allow. This is potentially a very useful method, but it also quite involved as it requires specialized image analysis methods. Thus this manuscript gives an introduction to the method and the provides a detailed description of how the authors have performed the imaging and the image analysis, along with providing some plug-ins for ImageJ that help with the analysis.
Overall the manuscript is clearly written, and seems likely to be of considerable help to any lab interested in trying this method. As such it is potentially suitable for publication, although there are a few relatively minor issues that would need to be addressed before it is ready to be published.

Major Concerns:
N/A

Minor Concerns:
a) The figures in the paper are similar to the first three figures in the authors' recent paper that reported the development and application of the method (Tie et al (2016) MBoC 27, 848). Figures 2 and 3 are different, even if they illustrate the same points, but Figure 1 is identical to Figure 1 in Tie et al which might raise issues.

Reply:

We would like to thank this reviewer for very constructive comments and questions, which prompt us to think more about our method.
Please also see above in this rebuttal letter. Yes, Figure 1 is identical to our published articles in MBoC. We can do so since, for MBoC articles, the authors retain their copy right. We have supplied the permission as a supplementary file.

b) The abstract should state the actual resolution that the method can achieve.

Reply:
OK. We have stated it in the abstract and it is explained in the newly added paragraph in discussion.

c) The introduction refers to Golgi cisternae having a thickness of <100 nm. However, for resolution, the key parameter is the distance between the cisternae, and so the authors should state this value.

Reply:
OK. We have modified the corresponding text to "<100 nm in both cisternal thickness and distance".

d) Does the method really produce a resolution comparable to immuno-EM as the authors state? This is hard to assess as the actual resolution is not stated.

Reply:
Yes, we think so. We have discussed the comparison between the two techniques in resolving Golgi proteins in the newly modified Discussion.

e) The authors claim that nocodazole-treated Golgi stacks "closely resemble" the normal Golgi. However, the authors should mention and cite a recent paper from the Perez lab that indicates that some of the mini-stacks are not fully functional for some hours after nocodazole addition (Fourriere et al (2016) J. Cell Sci. 129, 3238-50).

Reply:
OK. We have discussed this point accordingly in the Discussion.

f) In part 1.5.3.3 the authors should describe how they remove the coverslip from the well for transfer.

Reply:
OK. We have modified the text accordingly.

g) The section at the start of section 4, "Image Analysis" describes what sort of microscopes should be used and this would be better at the start of Section 3 on Image Acquisition.

Reply:
OK. We have modified the manuscript accordingly.

h) The authors should discuss the issue of over-exposure of images. Surely it is critical to ensure that no part of the Golgi has a maximum intensity - ie that the image does not become saturated. Indeed it is a bit disturbing that many of the mini-stacks in Figure 3C and 3D appear white suggesting that they have been over-exposed.

Reply:

As for other quantitative image analysis, intensity-saturated images cannot be used in GLIM. The intensities in Figure 3C and D are artificially scaled up so that individual Golgi mini-stacks are visible to readers, but they are not saturated. We also added “avoid pixel saturation” in the beginning of the Section 3 “Image acquisition”.

j) In section 4.4.2 the authors described a lot of editing that is required in Excel. Would it be possible to also provide a template file that already has this editing inserted and which could then be downloaded?

Reply:
OK. In Section 4.4.2, OriginPro but not Excel is used. The OriginPro template is provided as suggested.

Additional Comments to Authors:
N/A

Spreadsheet template

ImageJ macro file

ImageJ macro file

Supplemental code file (if applicable)

executable file

executable file