

# Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks

Hieng Chiong Tie, Divyanshu Mahajan, and Lei Lu

*Corresponding Author(s): Lei Lu, Nanyang Technological University*

---

## Review Timeline:

Submission Date:	2021-09-24
Editorial Decision:	2021-10-25
Revision Received:	2021-12-03
Editorial Decision:	2021-12-16
Revision Received:	2022-03-23
Editorial Decision:	2022-03-24
Revision Received:	2022-04-04

---

*Monitoring Editor: Sean Munro*

*Scientific Editor: Andrea Marat*

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

**DOI:** <https://doi.org/10.1083/jcb.202109114>

October 25, 2021

Re: JCB manuscript #202109114

Dr. Lei Lu  
Nanyang Technological University  
60 Nanyang Drive  
Singapore, 637551

Dear Dr. Lu,

Thank you for submitting your manuscript entitled "Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are all positive about the work, but raise quite a lot of issues that need to be clarified or discussed further in the text. Reviewer #2 mentions immuno-EM localisation of some of the markers. I do not think that this is necessary (unless you are keen to do it), but you should discuss the markers that you localise and that have also been localised by immuno-EM so as to compare the results. If there are suitable reagents available it may be worth applying your method to a couple proteins for which there is good quality immuno-EM data in HeLa cells, but which you have not tested yet. The reviewers also comment on the possibility that the mini stacks in nocadazole-treated cells differ from the Golgi in untreated cells. This needs to be discussed, and although beyond the scope of this study, in the future you could try looking at cell lines where the Golgi is naturally scattered into smaller stacks.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

#### GENERAL GUIDELINES:

**Text limits:** Character count for Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

**Figures:** Toolss may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <https://jcb.rupress.org/site/misc/fora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

**\*\*\*IMPORTANT:** It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision. \*\*\*

**Supplemental information:** There are strict limits on the allowable amount of supplemental data. Toolss may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. If your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: <https://rupress.org/jcb/pages/submission-guidelines#revised>

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Sean Munro, PhD  
Monitoring Editor

Andrea L. Marat, PhD  
Senior Scientific Editor

Journal of Cell Biology

-----  
Reviewer #1 (Comments to the Authors (Required)):

The Lu lab has been developing microscopy methods that use sophisticated averaging algorithms to resolve protein localizations within mammalian Golgi stacks. Individual "ministacks" are generated using nocodazole. Here, they describe a side averaging strategy that uses super-resolution side views of ministacks to localize fluorescent resident proteins or secretory cargo proteins. This approach extends and complements earlier methods described by the same authors. The relative distributions of Golgi proteins are reported with unprecedented resolution. This work supports the authors' provocative prior conclusion that a constitutive secretory cargo exits from the trans compartment of the stack rather than from the furthest trans-Golgi network compartment as is generally assumed.

This study is compelling. The method is clearly described and the logic makes sense. Overall this work is an important contribution because reliable, high-resolution localization data are crucial for understanding the Golgi. Models for Golgi function need to fit with what is seen by microscopy, and the approach described here sets a new standard in this regard.

My one major concern is that the authors devote considerable attention to interpreting the results obtained with secretory cargos but provide little commentary about the results for endogenous Golgi proteins. To demonstrate that this method is informative regarding the Golgi traffic machinery, they should illustrate with a couple of examples. I was particularly struck by the COPI data. COPI-coated vesicles have been seen by EM to bud throughout the Golgi stack, yet the results here place COPI in a fairly tight distribution at a cis/medial location. Moreover, GOLPH3 is thought to be a COPI adaptor, yet GOLPH3 is found much further along the Golgi axis than COPI. How can these various findings be reconciled?

Minor points:

1. Emphasize early in the text that GM130 resides in the interior of the stack whereas giantin is at the rims.
2. I don't quite understand Figure 1D, where giantin puncta are plotted against giantin. This experiment seems to be a control, but the rationale should be better explained. What is the significance of the lateral widths of the giantin puncta?
3. Polishchuk et al. (JCB 2000 148: 45-58) previously showed by EM that VSVG is not exclusively in the TGN at 20{degree sign}C and is visible earlier in the stack.
4. The data reveal a "prolonged pause" of secretory cargos in the trans-Golgi. My impression is that this pause is much longer for some proteins (e.g., CD59) than for others (e.g., VSVG). Perhaps the authors could comment on this point.

Reviewer #2 (Comments to the Authors (Required)):

This paper builds upon previous work from the same lab describing the localization of Golgi resident proteins and secretory

cargo that is transiting across the Golgi apparatus. High resolution light microscopy is used to image Golgi ministacks that are formed after depolymerization of microtubules, which makes imaging of the Golgi much simpler, albeit with the caveat that the Golgi ribbon structure has been lost. Sample averaging and quantitation are used to build a picture of lateral and axial position of proteins, resulting in a molecular model of protein localization Golgi stack organization. The results suggest that secretory cargo exits the Golgi at the trans-cisternae as opposed to the TGN, which is traditionally thought of as the Golgi exit station.

The study appears to be well done and is likely to be of interest to the community. I do however have a number of points that the authors should address.

- 1.) I understand why nocodazole-induced mini-stacks were imaged, but I also worry that the disruption of the Golgi ribbon could lead to changes in protein localization or compartment organization from the situation in a 'native' Golgi e.g. the organization of the TGN is likely to be significantly disrupted, and inter-cisternal connections will be lost. It also seems odd that COPII would align with the Golgi rims- is this an artefact of looking at mini-stacks? Such caveats should be discussed.
- 2.) The lateral and en face views of the Golgi look clear, but it wasn't clear to me how Golgi stacks with an oblique orientation were accounted for or excluded from the analysis. If a Golgi stack is at an angle, this will affect distances, and hence any measurements made. How were these Golgi stacks identified and excluded?
- 3.) Giantin was used to measure lateral distances across the cisternae. However, this protein may extend over extremely long distances itself, which could lead to an over-estimate in the distance measured, or should the conformation of giantin vary, it may in turn account for the quite high variability in lateral distances seen with this marker.
- 4.) Quite a few of the markers were over-expressed, which could in principle affect their distribution. Can the authors exclude the possibility of over-expression affecting localization?
- 5.) The protein distributions were obtained from averaging of multiple images. I worry that this method, by reinforcing particular enrichment at a certain place, will lead to a loss of the subtlety in any pattern of localization. For example, COPI has been shown in cryo-EM studies to reside in a gradient across the Golgi stack, with enrichment at the cis-side. But the imaging here would suggest it has a quite restricted localization in the stack. Could the same be true for the other proteins e.g. many Golgi enzymes are present in several cisternae e.g. GALNT2, which EM indicates is present across the stack. Is this a major issue or limitation of the imaging method described here? It would have been good to see additional EM to provide validation for some of the described localizations.
- 6.) The model in Fig 3B shows 10 cisternae, which is derived from calculations of distances seen in the light microscope images combined with previous EM data on physical dimensions of the Golgi cisternae. I am puzzled by this, since HeLa cells would normally have 3-5 cisternae. It suggests there is a mistake in the calculations somewhere.
- 7.) Regarding cargo export from the Golgi, can the authors exclude the possibility that the cargo might still exit via the TGN, but that its transit time in this compartment is very short. So that as it enters it leaves almost straight away, which could explain why no enrichment is seen there in the RUSH experiments?

#### Reviewer #3 (Comments to the Authors (Required)):

In this manuscript Tie and Lu provide a novel and updated method to pin-point the intra-Golgi localization of Golgi residents and cargoes. The method builds from previous quantification methods from the Lu lab 1) Golgi localization by imaging the center of mass (GLIM) (Tie et al., 2016,2017) and a method to identify Nocodazole-induced ministacks that have an orientation favorable to clearly separate Golgi cisternae from each other and rim vs the center of the cisternae. In this paper the authors suggest a new method based on side-averaging of various mini-stacks with the same orientation in respect to the imaging plane. With this method they were able to obtain an average map of 30 Golgi localized proteins. Additionally, they looked at trafficking of various cargoes and were able to show that while VSVG and CD59 exit the Golgi at the level of the trans-Golgi, CD8 exits from the TGN as a CD8 variant where the sorting motif in the cytosolic tail is mutated behaves like the VSVG and CD59 cargo and exits the Golgi at the trans cisterna.

It is extremely hard to quantify the localization of Golgi proteins in mammalian cells because of the non-symmetrical geometry and the complexity of the Golgi ribbon that twists and is very tridimensional so that no single Golgi look like the other. Much more progress has been made to quantitatively map components in symmetrical structures like clathrin coated pit and nuclear pore complexes which are substantially easier targets due to their symmetrical geometry. Work like this is very important if we really want to understand how the Golgi functions and I strongly recommend the paper for publications after minor revisions.

Line 49: I do not agree with the statement that super-resolution cannot reliably resolve intra-Golgi localization. Many super-resolution methods have now been used to look at Golgi proteins (Hao et al., Nature Methods 2021, Zhang et al., Nature methods 2020, Bottanelli et al., MBoC 2017). But I agree that super-resolution microscopes are cost prohibitive and technically challenging to use and maintain and the method proposed is based on widely available confocal microscopy.

Line 55: The authors should at least discuss that breaking down the ribbon with nocodazole may affect trafficking of some cargoes. In particular, nocodazole has been shown to affect the trafficking of large cargoes like collagen (Lavie et al., MBoC 2014).

Line 73: Airyscan is not a super-resolution microscopy method. The authors should call it enhanced confocal microscopy or

simply airyscan microscopy.

Line 80: the authors use an antibody against Giantin which is a very extended protein. Have the authors previously discussed which part of the protein the antibody bind? How do they interpret their measurement considering that? Our own experience is that different giantin antibodies binding different parts of the protein give completely different localization patterns...

Line 85 and Supp Fig 1B. GM130 is not written in the figure.

Line 109: The authors use Giantin to identify ministacks with a side-view. Wouldn't the authors also need a trans or TGN marker to distinguish perfectly flat ministacks from ones in a tilted position? Could you comment please?

Line 113-117: How were 80 pixels, 8-fold numbers for expanding the image chosen? Could the author better explain their manipulations? Line 116: 701 is missing a unit? Why are the authors normalizing the intensities?

Line 121: The authors should mention this is a Fiji macro. Could the authors provide instructions on how to use the macro to make it accessible and easy to use?

Line 140-141: Could the authors translate what the parameters mean in terms of Golgi localization to make the text more accessible?

Line 163-165: What does the "dramatically" different size average mean in terms of Golgi localization? Post-Golgi carriers further away from the TGN?

Line 175: I would disagree that sample size is a limiting factor: there can be 100s of ministacks in one cell and if only 1/3 has the correct orientation that seems more than enough. Could the authors comment?

Line 184: By providing the average of the axial localization one does not convey that some proteins may be more broadly distributed along the Golgi stack. Is there a way to express how broad is protein distribution? Very interestingly it seems like COPI (Figure 2B) is pretty much distributed around the cis cisternae and not much around the trans-Golgi? I understand that this may be a controversial topic but I find it useful information that maybe should be highlighted in the text?

Line 193: I believe the authors mean GOLPH3?

Line 209: could the author explain what they mean for "unimodal" in terms of Golgi localization to make the text more understandable?

Line 226 and Figure 4G: could the authors add an extra color line for the TGN to show that CD8 furin exits where one would expect a TGN marker?

Line 283: I find it very interesting that cargoes stay in the inner part of the cisternae and do not enrich at the rims where vesicular carriers markers are. I understand that this may be a controversial topic but very cool info that the authors could highlight in their discussion?

Point-by-point response letter

Manuscript number: JCB manuscript #202109114

Manuscript title: Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks

Reviewer #1 (Comments to the Authors (Required)):

The Lu lab has been developing microscopy methods that use sophisticated averaging algorithms to resolve protein localizations within mammalian Golgi stacks. Individual "ministacks" are generated using nocodazole. Here, they describe a side averaging strategy that uses super-resolution side views of ministacks to localize fluorescent resident proteins or secretory cargo proteins. This approach extends and complements earlier methods described by the same authors. The relative distributions of Golgi proteins are reported with unprecedented resolution. This work supports the authors' provocative prior conclusion that a constitutive secretory cargo exits from the trans compartment of the stack rather than from the furthest trans-Golgi network compartment as is generally assumed.

This study is compelling. The method is clearly described and the logic makes sense. Overall this work is an important contribution because reliable, high-resolution localization data are crucial for understanding the Golgi. Models for Golgi function need to fit with what is seen by microscopy, and the approach described here sets a new standard in this regard.

My one major concern is that the authors devote considerable attention to interpreting the results obtained with secretory cargos but provide little commentary about the results for endogenous Golgi proteins. To demonstrate that this method is informative regarding the Golgi traffic machinery, they should illustrate with a couple of examples. I was particularly struck by the COPI data. COPI-coated vesicles have been seen by EM to bud throughout the Golgi stack, yet the results here place COPI in a fairly tight distribution at a cis/medial location. Moreover, GOLPH3 is thought to be a COPI adaptor, yet GOLPH3 is found much further along the Golgi axis than COPI. How can these various findings be reconciled?

Reply:

We want to thank this reviewer for his/her constructive and insightful comments and suggestions.

The steady-state localization of Golgi resident proteins was discussed in our previous publication using en face-averaging (Tie et al., *elife*, 2018; PMID: 30499774). The main discovery was that glycosylation enzymes are at the interior of the Golgi stack while components of trafficking machinery are at the periphery of the Golgi stack.

As this reviewer correctly noticed, Figs. 3 A and S3 show that the signal density of COPI mainly localizes to the *cis* and medial Golgi cisternae. There are two types of  $\gamma$ COPs —  $\gamma$ 1 and  $\gamma$ 2COP (Moelleken *et al.*, *PNAS*, 2007, PMID: 17360540). COPI coats with  $\gamma$ 1 and  $\gamma$ 2COP have significantly different localizations within the Golgi. Those with  $\gamma$ 1COP mainly localize to the *cis*-region, while those with  $\gamma$ 2COP prefer the *trans*-region. We realized that our antibody (Santa Cruz #SC-393977) is specific to  $\gamma$ 1COP. Therefore, the *cis* and medial-cisternae localizations are consistent with what we know of  $\gamma$ 1COP. The *trans*-Golgi localization of COPI might be revealed by using other COPI antibodies.

We have modified the corresponding text at line 208 as shown below (newly added text is underlined).

It also highlights the striking distributions of protein coats, including COPI (labeled by  $\gamma$ 1COP), COPII (sec31a-GFP), and clathrin coats (GFP-GGA1). GGA1-labeled clathrin coat appears to spread across a large area, capping the ministack like a crown at its *trans*-side, consistent with our knowledge that it decorates vesicles, buds, and tips of tubules at the TGN (De Matteis and Luini, 2008; Guo et al., 2014). Both COPI ( $\gamma$ 1COP) and COPII distributions appear as a ring at the periphery of a ministack in en face-views (Figs. 2, A and B). In the axial direction, COPI ( $\gamma$ 1COP) extends from the *cis* to medial-cisternae, while the localization of COPII is distal to the *cis*-cisternae and away from COPI ( $\gamma$ 1COP). Our observation is consistent with previous reports on the tightly adjacent localization of COPII and COPI at the ER-proximal and distal region, respectively, near the ERES (Scales et al., 1997; Stephens et al., 2000; Weigel et al., 2021), and the *cis*-Golgi localization of COPI with  $\gamma$ 1COP (Moelleken et al., 2007).

Minor points:

1. Emphasize early in the text that GM130 resides in the interior of the stack whereas giantin is at the rims.

Reply:

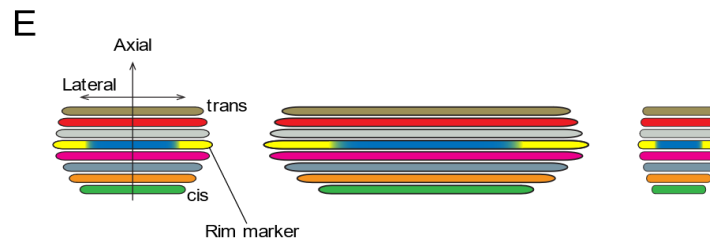
The rim-localization of giantin has been mentioned at the beginning of the Results. As suggested by this reviewer, we have directly indicated the interior localization of GM130 when it first appears in the Results at line 90. Below is the sentence with the modified part underlined.

Similarly, the lateral line intensity profile of GM130, which localizes to the interior of the *cis*-cisternae (Tie et al., 2018), was fitted to a Super-Gaussian function (Fig. S1 B).

2. I don't quite understand Figure 1D, where giantin puncta are plotted against giantin. This experiment seems to be a control, but the rationale should be better explained. What is the significance of the lateral widths of the giantin puncta?

Reply:

The aim of Fig. 1 D is to show that ministacks vary proportionally in the lateral direction. For example, the lateral width of giantin puncta is expected to increase by a factor of 2 if the lateral size increases by a factor of 2. We have modified Fig. 1 E to give a better visual illustration of this point. The figure and its legend are shown below, with the modified part underlined.



**(E)** A schematic diagram illustrating the morphometric changes of Golgi ministacks — the axial sizes are conserved while the lateral ones vary proportionally. The two yellow segments schematically represent the distribution of a rim marker.

3. Polishchuk et al. (JCB 2000 148: 45-58) previously showed by EM that VSVG is not exclusively in the TGN at 20°C and is visible earlier in the stack.

Reply:

We believe that this reviewer referred to the below description of the EM localization of VSVG-GFP from Polishchuk *et al.*: "Fig. 6 a confirms that, as expected, cargo protein was retained in the Golgi at 20°C. Interestingly, it was present not only in the TGN but also in medial and trans-cisternae. The cis-Golgi, ER, and PM were unlabeled". Both Polishchuk *et al.* and our results agree on the *medial* and *trans*-localization of VSVG. However, we did not observe the TGN localization of VSVG. Although Polishchuk *et al.* claimed the TGN localization of VSVG in their Fig. 6a, they did not molecularly identify the TGN in their EM; instead, they used the morphological appearance to determine the TGN. It is a common issue for similar EM studies, in which authors used the morphological appearance instead of molecular labeling to identify the TGN. However, we know that the *trans*-cisternae can also generate tubules and vesicles (Ladinsky *et al.*, *J. Cell Biol.*, 1999, PMID: 10087259; March *et al.*, *PNAS*, 2001, PMID: 11226251; Mogelsvang *et al.*, *Traffic*, PMID: 15086783). Hence, it is unreliable to use morphological appearance to distinguish the TGN from the *trans*-cisternae under the conventional EM. On the other hand, in our side-averaging and GLIM, the TGN is defined by the distribution of TGN markers such as furin, M6PRs, AP1, GGAs, and clathrin.

4. The data reveal a "prolonged pause" of secretory cargos in the trans-Golgi. My impression is that this pause is much longer for some proteins (e.g., CD59) than for others (e.g., VSVG). Perhaps the authors could comment on this point.

Reply:

We have studied the pause of secretory cargos and reported our findings in our recent publications (Sun *et al.*, *J. Cell Sci.*, 2021; Sun *et al.*, *J. Biol. Chem.*, 2020, PMID: 32826314). The Golgi residence time of GPI-anchored mCherry (a protein similar to CD59 as both are GPI-anchored) is 17 min. In comparison, the Golgi residence time of VSVG is 12 min, indicating that GPI-anchored mCherry resides in the Golgi longer than VSVG, supporting this reviewer's impression. However, in side-averaging, the image intensity is normalized to a fixed value,  $5 \times 10^7$ . Therefore, the intensity cannot be used to compare the Golgi residence of secretory cargos.

As suggested by this reviewer, we have added the following text at line 244 in the Results. The length of time that VSVG pauses at the *trans*-Golgi is indicated by its Golgi residence time, which can be measured by the half-life of the Golgi fluorescence decay after the release of 20 °C block (Sun *et al.*, 2021; Sun *et al.*, 2020). We have recently measured the Golgi residence time of VSVG to be 12 min. Furthermore, we found that different constitutive transmembrane secretory cargos have distinct Golgi residence times, which are determined by their Golgi export signals, such as the linked glycan, the transmembrane domain, and the cytosolic tail (Sun *et al.*, 2021; Sun *et al.*, 2020).

Reviewer #2 (Comments to the Authors (Required)):

This paper builds upon previous work from the same lab describing the localization of Golgi resident proteins and secretory cargo that is transiting across the Golgi apparatus. High resolution light microscopy is used to image Golgi ministacks that are formed after depolymerization of microtubules, which makes imaging of the Golgi much simpler, albeit with the caveat that the Golgi ribbon structure has been lost. Sample averaging and quantitation are used to build a picture of lateral and axial position of proteins, resulting in a molecular model of protein localization Golgi stack organization. The results suggest that secretory cargo exits the Golgi at the trans-cisternae as opposed to the TGN, which is traditionally thought of as the Golgi exit station.

The study appears to be well done and is likely to be of interest to the community. I do however have a number of points that the authors should address.

1.) I understand why nocodazole-induced mini-stacks were imaged, but I also worry that the disruption of the Golgi ribbon could lead to changes in protein localization or compartment organization from the situation in a 'native' Golgi e.g. the organization of the TGN is likely to be significantly disrupted, and inter-cisternal connections will be lost. It also seems odd that COPII would align with the Golgi rims- is this an artefact of looking at mini-stacks? Such caveats should be discussed.

Reply:

We want to thank this reviewer for his/her constructive and insightful comments and suggestions.

The native Golgi stack and nocodazole-induced ministack are known to share similar organizational features (see below revamped text for references). Therefore, the nocodazole-induced ministack should be an excellent model to study the more complicated native Golgi. However, differences exist, as commented by this reviewer. Reviewer #3 (Line 55) has pointed out that nocodazole treatment might affect the trafficking of large cargoes such as collagen (Lavieu *et al.*, *MBoC*, 2014, PMID: 25103235). Therefore, we have modified the corresponding text at line 57. Below is the revamped text with newly added text underlined.

Ministacks are known valid representations of the native Golgi stack (Cole *et al.*, 1996; Rogalski *et al.*, 1984; Tie *et al.*, 2018; Trucco *et al.*, 2004; Van De Moortele *et al.*, 1993), although the intra-Golgi transport of large secretory cargos such as protein aggregates and collagen I might be compromised in ministacks (Lavieu *et al.*, 2014).

As noted by this reviewer, COPII subunits indeed appear as a ring surrounding the ministack in the en face-average (Figs. 2 A and S2 A). Consistent with our finding, Trucco *et al.* previously reported that, under EM tomography, the ERGIC, which is tightly adjacent to the COPII-positive ERES (ER exit site), surrounds the Golgi ministack under the nocodazole treatment (Trucco *et al.*, *Nat. Cell Biol.*, 2004, PMID: 15502824). However, the ring localization of COPII has only been observed in the nocodazole-induced Golgi ministacks, where ministacks assemble immediately near the ERES. The relative localization of COPII in the context of native Golgi stacks is poorly studied at the super-resolution or EM level. Hence, it is premature to conclude that the ring-localization of COPII is an artifact. Interestingly, it has been known that COPII staining surrounds the Golgi cisternae in the native Golgi complex under light microscopy (an example can be seen in Tang *et al.*, *J. Biol. Chem.*, 2000, PMID: 10788476). We have added the below text at line 208 to describe the distribution of COPI, COPII, and clathrin coats.

It also highlights the striking distributions of protein coats, including COPI (labeled by  $\gamma$ 1COP), COPII (sec31a-GFP), and clathrin coats (GFP-GGA1). GGA1-labeled clathrin coat appears to spread across a large area, capping the ministack like a crown at its *trans*-side, consistent with our knowledge that it decorates vesicles, buds, and tips of tubules at the TGN (De Matteis and Luini, 2008; Guo *et al.*, 2014). Both COPI ( $\gamma$ 1COP) and COPII distributions appear as a ring at the periphery of a ministack in en face-views (Figs. 2, A and B). In the axial direction, COPI ( $\gamma$ 1COP) extends from the *cis* to medial-cisternae, while the localization of COPII is distal to the *cis*-cisternae and away from COPI ( $\gamma$ 1COP). Our observation is consistent with previous reports on the tightly adjacent localization of COPII and COPI at the ER-proximal and distal region, respectively, near the ERES (Scales *et al.*, 1997; Stephens *et al.*, 2000; Weigel *et al.*, 2021), and the *cis*-Golgi localization of COPI with  $\gamma$ 1COP (Moelleken *et al.*, 2007).

2.) The lateral and en face views of the Golgi look clear, but it wasn't clear to me how Golgi stacks with an oblique orientation were accounted for or excluded from the analysis. If a Golgi stack is at an angle, this will affect distances, and hence any measurements made. How were these Golgi stacks identified and excluded?

Reply:

The lateral view is termed as the side-view in our study. The orientation of a Golgi ministack and the selection criteria for side (when the axial angle is  $\sim 0^\circ$ ) and en face-view (when the axial angle is  $\sim 90^\circ$ ) ministacks have been discussed in detail in our previous publication (Tie *et al.*, *elife*, 2018, PMID: 30499774). Briefly, we used giantin to identify side and en face-view ministacks. The giantin staining should appear double-punctum and circle in the side and en face-view, respectively. Its appearance is oval in the oblique view (when the axial angle significantly differs from 0 and  $90^\circ$ ). Ministacks with oblique views are excluded from our analysis. The visual inspection involved in this method is subjective, but the method seems to work well based on our side and en face-averaging data.

3.) giantin was used to measure lateral distances across the cisternae. However, this protein may extend over extremely long distances itself, which could lead to an over-estimate in the distance measured, or should the conformation of giantin vary, it may in turn account for the quite high variability in lateral distances seen with this marker.

Reply:

The epitope of our anti-giantin antibody lies at the N-terminus of giantin, while giantin anchors onto the Golgi membrane by its C-terminal transmembrane domain. Therefore, if completely extended, the filamentous structure of giantin could overestimate the diameter of the cisternal membrane, as this reviewer pointed out.

However, in our previous study, we have investigated the possible extension of giantin and other golgins (Tie *et al.*, *elife*, 2018, PMID: 30499774). We found that their N and C-termini are closely adjacent to each other in space. See Figure 1 and Figure 1-figure supplement 2 from Tie *et al.*, *elife*, 2018 (PMID: 30499774). Figure 1-figure supplement 2 is copied below. We have stained the endogenous giantin using antibodies against its N and C-termini. Our quantification indicated that the diameter of the giantin-ring stained by the C-terminus antibody is only 95% or 50 nm less than that of the N-terminus antibody, assuming that the diameter of the giantin-ring is 1000 nm (Figure 1-figure supplement 2A). Similar observations were made for GM130 and GCC185 by selectively labeling their N and C-termini using antibodies or fluorescence proteins (Figure 1-figure supplement 2 B and C). Thus, our data indicate that golgins are primarily in a collapsed instead of extended configuration. Therefore, the ring pattern of giantin staining should closely represent the cisternal membrane rim.

To make it more clear, we added the following text at line 80 in the Results.

Previously, we demonstrated that the N-termini of golgins such as giantin, GCC185, and GM130 are closely adjacent to their C-terminal membrane anchors (Tie *et al.*, 2018). Therefore, the giantin-ring stained by anti-N-terminus antibody should closely represent the membrane at the cisternal rim.

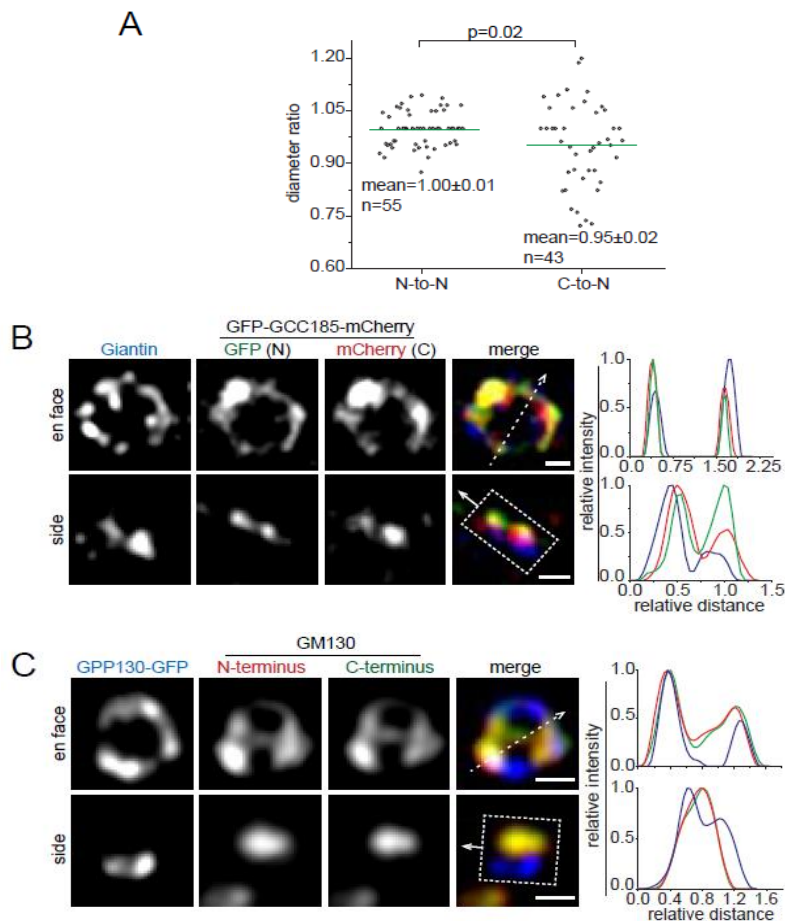


Figure 1-supplement figure 2 from Tie *et al.*, *elife*, 2018 (PMID: 30499774).

4.) Quite a few of the markers were over-expressed, which could in principle affect their distribution. Can the authors exclude the possibility of over-expression affecting localization?

Reply:

This is a common concern for almost all studies involving tagged proteins. However, it is challenging to address it. Here, we only examined if the expression level affects a Golgi protein's *LQ*, since *LQ* is a quantitative axial localization indicator. We plotted *LQs* and relative expression levels of a GFP-tagged Golgi protein at individual ministacks. The relative expression level was calculated as the integrated intensity of the tagged protein divided by that of endogenous GM130 at each ministack. The new results in Fig. S1 C (see below) demonstrate that the expression level does not substantially affect the *LQ*. We have added the following text at line 140 with newly added or modified text underlined. We applied the side-averaging method to 36 Golgi proteins – 8 endogenous and 28 fluorescence protein or Myc-tagged ones. Their steady-state localization (indicated by their *LQs*) ranged from the ERES, ERGIC, *cis*, medial, *trans*-Golgi to TGN. We selected 10 GFP-tagged Golgi proteins to assess the effect of overexpression on localization. We plotted the *LQ* against the corresponding relative expression level of a protein for each ministack (Fig. S1 C). The relative expression level was calculated as the ratio of the integrated intensity of the protein to that of endogenous GM130. Our plots demonstrated that overexpression

seems not to grossly affect the LQ, a quantitative metric for the axial localization. However, we could not rule out the effect of overexpression on the lateral localization.

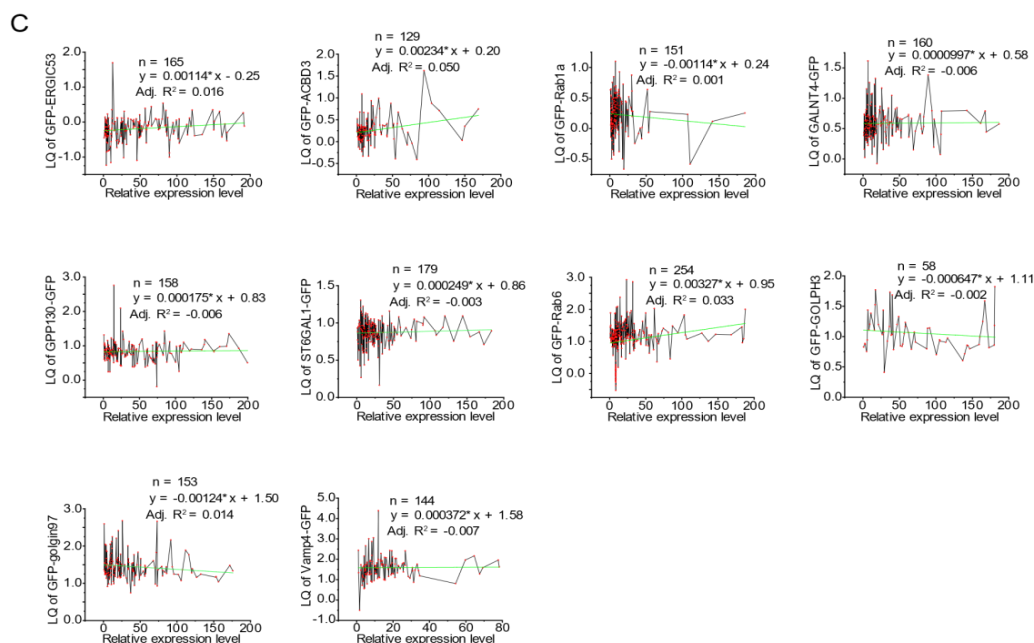


Fig. S1 C

5.) The protein distributions were obtained from averaging of multiple images. I worry that this method, by reinforcing particular enrichment at a certain place, will lead to a loss of the subtlety in any pattern of localization. For example, COPI has been shown in cryo-EM studies to reside in a gradient across the Golgi stack, with enrichment at the cis-side. But the imaging here would suggest it has a quite restricted localization in the stack. Could the same be true for the other proteins e.g. many Golgi enzymes are present in several cisternae e.g. GALNT2, which EM indicates is present across the stack. Is this a major issue or limitation of the imaging method described here? It would have been good to see additional EM to provide validation for some of the described localizations.

Reply:

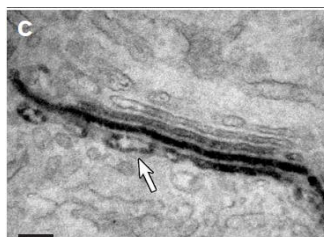
In theory, when properly conducted, averaging could enhance the common structural features, including subtle ones. Averaging should not broaden or narrow the axial distribution of a Golgi protein.

Regarding the localization of COPI, we have discussed it in our reply to Point 1 of Reviewer #2's comments. We employed  $\gamma$ COP antibody to localize COPI. There are two types of  $\gamma$ COPs —  $\gamma$ 1 and  $\gamma$ 2COP (Moelleken *et al.*, *PNAS*, 2007, PMID: 17360540). COPI coats with  $\gamma$ 1 and  $\gamma$ 2COP have significantly different localizations within the Golgi. Those with  $\gamma$ 1COP mainly localize to the *cis*-region, while those with  $\gamma$ 2COP prefer the *trans*-region. We realized that our antibody (Santa Cruz #SC-393977) was specific to  $\gamma$ 1COP based on the epitope used to raise this antibody. Therefore, the *cis* and medial-localization from our side-averaging is consistent with the EM localization of  $\gamma$ 1COP. We have added the corresponding text at line 212, as shown below.

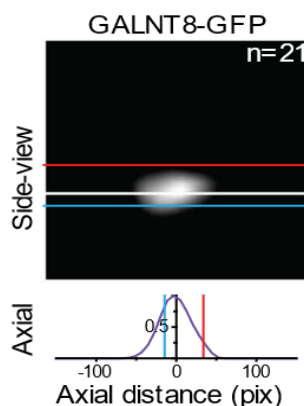
Both COPI ( $\gamma$ 1COP) and COPII distributions appear as a ring at the periphery of a ministack in en face-views (Figs. 2, A and B). In the axial direction, COPI ( $\gamma$ 1COP) extends from the *cis* to medial-cisternae, while the localization of COPII is distal to the *cis*-cisternae and away

from COPI ( $\gamma$ 1COP). Our observation is consistent with previous reports on the tightly adjacent localization of COPII and COPI at the ER-proximal and distal region, respectively, near the ERES (Scales et al., 1997; Stephens et al., 2000; Weigel et al., 2021), and the *cis*-Golgi localization of COPI with  $\gamma$ 1COP (Moelleken et al., 2007).

Regarding the axial distribution of Golgi enzymes, it seems that Golgi enzymes do not homogenously distribute from the *cis* to *trans*-cisternae; instead, their gradient distributions peak at 1-2 cisternae. As an example, the EM localization of GalNAc-T2-GFP by photooxidation of DAB is shown below (Figure 1c of Grabenbauer *et al.*, *Nat. Methods*, 2005, PMID: 16278657).



We suggest the usage of the axial line intensity profile for quantitative assessment of the axial distribution (Figs. 2 and S2). Below is an example of GALNT8 (Fig. S2 C), which could be similar to GALNT2. As shown in the axial line intensity profile (copied below), the axial intensity of GALNT8 spreads continuously from the *cis* (blue line) to *trans*-cisternae (red line) but peaks at the medial cisternae ( $LQ=0.34$ ). In summary, our side-averaging demonstrates that Golgi enzymes distribute across the ministack but peak at different axial positions. Therefore, there seems no fundamental discrepancy between our data and the EM result cited by this reviewer.



Regarding EM validation, we have compared our side-averaging data of the following Golgi proteins with corresponding EM results from the literature review: sec31a, GS27, ERGIC53, GM130,  $\gamma$ 1COP, MGAT2, giantin, GPP130, GalT-mCherry, Rab6, GOLPH3, CI-M6PR, Vamp4, clathrin light chain B, GGA2, and  $\gamma$ -adaptin. The comparison is summarized in the newly made Table S2, which demonstrates that side-averaging is consistent with available EM data. We have added the following text at line 189 in the revamped manuscript.

We compared our side-average data and corresponding published EM localization results in Table S2, which includes sec31a (Tang et al., 2000), GS27 (Hay et al., 1998), ERGIC53 (Klumperman et al., 1998), GM130 (Trucco et al., 2004),  $\gamma$ 1COP (Moelleken et al., 2007), MGAT2 (Tie et al., 2018), giantin (Tie et al., 2018; Trucco et al., 2004), GPP130 (Tie et al., 2018), GalT-mCherry (Trucco et al., 2004), Rab6 (Antony et al., 1992), GOLPH3 (Bell et al.,

2001), GGA1 (Puertollano et al., 2003), CI-M6PR (Doray et al., 2002), Vamp4 (Steegmaier et al., 1999), furin (Bosshart et al., 1994), CLCB (Staehelin and Kang, 2008), GGA2 (Doray et al., 2002), and  $\gamma$ -adaptin (Doray et al., 2002). Our comparison demonstrates that side-averaging is generally consistent with EM in localizing proteins with sub-Golgi accuracy.

We added the EM results that support our side-average findings on the spindle shape of the ministack and the distribution of VSVG at 15 °C temperature block at lines 179 and 285, respectively (newly added text is underlined).

(line 179)

Therefore, consistent with our composite side-average images (Fig. 2F), our morphometric analysis demonstrated the spindle shape of the ministack, which was previously observed by our en face-averaging (Tie et al., 2018) and noticeable in the EM tomography of plant Golgi ministacks (Staehelin and Kang, 2008).

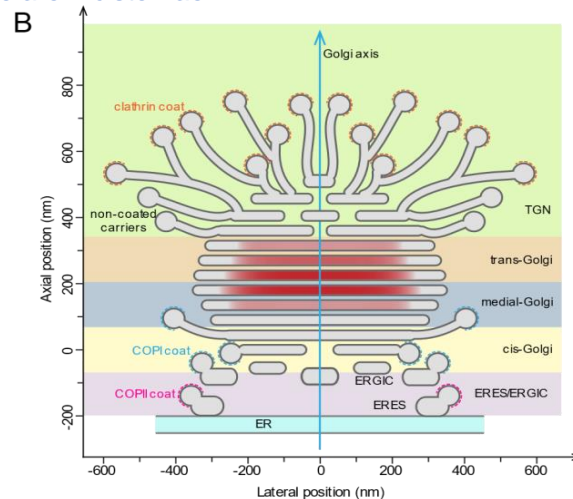
(line 285)

When VSVG RUSH reporter was released from the ER at 15 °C for 2 hours, side-averaging revealed that its traffic wave stopped at a pre-Golgi position in between sec23a-mCherry ( $LQ^{\text{side}} = -0.30$ ) and GS27 ( $LQ^{\text{side}} = -0.06$ ) with the  $LQ^{\text{side}} = -0.23$  (Fig. 5 A), consistent with the EM result in NRK ministacks (Trucco et al., 2004).

6.) The model in Fig 3B shows 10 cisternae, which is derived from calculations of distances seen in the light microscope images combined with previous EM data on physical dimensions of the Golgi cisternae. I am puzzled by this, since HeLa cells would normally have 3-5 cisternae. It suggests there is a mistake in the calculations somewhere.

Reply:

The 3D tomography of the NRK Golgi demonstrates 7 cisternae (Ladinsky et al., *JCB*, 1999, PMID: 10087259). The Golgi stack in HeLa cells probably has a similar number of cisternae. 3-5 could be an underestimation for the number of cisternae in HeLa cells, as there lacks 3D EM tomography of the HeLa Golgi stack, and conventional thin-section EM could underestimate the number of cisternae. Our study did not directly reveal the average number of cisternae in a ministack in HeLa cells. Hence, Fig. 3B is only a schematic model. We have modified it so that there are 7 cisternae.



7.) Regarding cargo export from the Golgi, can the authors exclude the possibility that the cargo might still exit via the TGN, but that its transit time in this compartment is very short. So that as it enters it leaves almost straight away, which could explain why no enrichment is

seen there in the RUSH experiments?

Reply:

We cannot rule out the scenario suggested by this reviewer. We have added our argument and discussion at line 313 in the Discussion.

Our data do not agree with the prevailing model, which posits the TGN as the Golgi exit site of the constitutive secretory cargos (De Matteis and Luini, 2008; Di Martino et al., 2019). Our data agree with several EM tomography studies using cryofixation (Ladinsky et al., 1999; Marsh et al., 2001; Mogelsvang et al., 2004). The 3D tomographic organization of the Golgi demonstrates that the *trans*-most cisternae generate a clathrin-positive tubular and vesicular membrane network characterizing the TGN. Furthermore, it uncovers that the two adjacent *trans*-cisternae assemble clathrin-negative vesicles and tubules, characterizing the constitutive secretory carriers, therefore supporting our data that constitutive secretory cargos can exit at the *trans*-Golgi. We can not rule out the possible scenario where cargos might transit through the TGN so rapidly that their presence in the TGN is undetectable under our imaging condition. If the scenario is true, we argue that it would have been impossible for EM to detect cargo's presence in the TGN either. Further high-speed and high-sensitivity live-cell imaging is necessary to resolve these two different views. However, the most straightforward explanation to our data is that constitutive secretory cargos exit at the *trans*-Golgi instead of the TGN.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript Tie and Lu provide a novel and updated method to pin-point the intra-Golgi localization of Golgi residents and cargoes. The method builds from previous quantification methods from the Lu lab 1) Golgi localization by imaging the center of mass (GLIM) (Tie et al., 2016,2017) and a method to identify Nocodazole-induced ministacks that have an orientation favorable to clearly separate Golgi cisternae from each other and rim vs the center of the cisternae. In this paper the authors suggest a new method based on side-averaging of various mini-stacks with the same orientation in respect to the imaging plane. With this method they were able to obtain an average map of 30 Golgi localized proteins. Additionally, they looked at trafficking of various cargoes and were able to show that while VSVG and CD59 exit the Golgi at the level of the trans-Golgi, CD8 exits from the TGN as a CD8 variant where the sorting motif in the cytosolic tail is mutated behaves like the VSVG and CD59 cargo and exits the Golgi at the trans cisterna.

It is extremely hard to quantify the localization of Golgi proteins in mammalian cells because of the non-symmetrical geometry and the complexity of the Golgi ribbon that twists and is very tridimensional so that no single Golgi look like the other. Much more progress has been made to quantitatively map components in symmetrical structures like clathrin coated pit and nuclear pore complexes which are substantially easier targets due to their symmetrical geometry. Work like this is very important if we really want to understand how the Golgi functions and I strongly recommend the paper for publications after minor revisions.

Line 49: I do not agree with the statement that super-resolution cannot reliably resolve intra-Golgi localization. Many super-resolution methods have now been used to look at Golgi proteins (Hao et al., Nature Methods 2021, Zhang et al., Nature methods 2020, Bottanelli et al., MBoC 2017). But I agree that super-resolution microscopes are cost prohibitive and technically challenging to use and maintain and the method proposed is based on widely available confocal microscopy.

Reply:

We want to thank this reviewer for his/her constructive and insightful comments and suggestions.

We agree with this reviewer that super-resolution light microscopy, even the conventional one, can resolve the intra-Golgi localization to various extents. The original text was hence modified as below at line 46 (new text is underlined).

Although conventional and especially super-resolution light microscopy can resolve the intra-Golgi localization to various extents, the results are mainly qualitative and only reveal the relative cisternal distributions of 2-3 proteins in individual Golgi stacks (Bottanelli et al., 2016; Deigaard et al., 2007; Hao et al., 2021; Tie et al., 2016; Zhang et al., 2020). In contrast, the complete understanding of the molecular and cellular organization of the Golgi demands quantitative and systematic imaging methods to localize the Golgi proteome, which comprises ~ 1,000 proteins (Chen et al., 2010).

Line 55: The authors should at least discuss that breaking down the ribbon with nocodazole may affect trafficking of some cargoes. In particular, nocodazole has been shown to affect the trafficking of large cargoes like collagen (Lavieu et al., MBoC 2014).

Reply:

As suggested by this reviewer, we have added the reference. Please also see our reply to Point 1 of Reviewer #2's comments. We have modified the corresponding text at line 57, as shown below.

Ministacks are known valid representations of the native Golgi stack (Cole et al., 1996; Rogalski et al., 1984; Tie et al., 2018; Trucco et al., 2004; Van De Moortele et al., 1993), although the intra-Golgi transport of large secretory cargoes such as protein aggregates and collagen I might be compromised in ministacks (Lavieu et al., 2014).

Line 73: Airyscan is not a super-resolution microscopy method. The authors should call it enhanced confocal microscopy or simply airyscan microscopy.

Reply:

This might be trivial and controversial. Since super-resolution microscopy is defined as "techniques that manage to surpass the 'classical' diffraction limit of optical resolution of about half the wavelength of the emitted light" (review by Schermelleth *et al.*, *Nat. Cell Biol.*, 2019, PMID: 30602772), Airyscan microscopy can be regarded as a super-resolution microscopy. "Airyscan super-resolution microscopy" also frequently appears in the literature.

However, Airyscan is a diffraction-limited super-resolution microscopy, which is different from the diffraction-unlimited super-resolution microscopy techniques such as STED and STORM (Schermelleth *et al.*, *Nat. Cell Biol.*, 2019, PMID: 30602772). Thus, in this aspect, we agree with this reviewer to use "Airyscan microscopy" instead of "Airyscan super-resolution microscopy". Accordingly, we have modified our manuscript.

Line 80: the authors use an antibody against giantin which is a very extended protein. Have the authors previously discussed which part of the protein the antibody bind? How do they interpret their measurement considering that? Our own experience is that different giantin antibodies binding different parts of the protein give completely different localization patterns...

Reply:

Please see our reply to Point 3 of Reviewer #2's comments. Briefly, we ruled out this possibility using experiments in our previous study (Figure 1 and Figure 1-figure supplement 2 from Tie *et al.*, *elife*, 2018, PMID: 30499774).

Line 85 and Supp Fig 1B. GM130 is not written in the figure.

Reply:

As suggested by this reviewer, we have labeled GM130 in Figure S1 B.

Line 109: The authors use giantin to identify ministacks with a side-view. Wouldn't the authors also need a trans or TGN marker to distinguish perfectly flat ministacks from ones in a tilted position? Could you comment please?

Reply:

Please see our reply to Point 2 of Reviewer #2's comments. We have discussed this issue in our previous study (Tie *et al.*, *elife*, 2018, PMID: 30499774). Briefly, giantin should be sufficient to identify side and en face-views.

Line 113-117: How were 80 pixels, 8-fold numbers for expanding the image chosen? Could the author better explain their manipulations? Line 116: 701 is missing a unit? Why are the authors normalizing the intensities?

Reply:

80 pixels and 8-fold are numbers that we think should be sufficiently large for direct visualization. Eventually, x and y-coordinates are normalized so that the pixel unit is isotropic.

Regarding line 123 (previously line 116), we have added "pixel" as the unit for  $701 \times 701$ .

Regarding the intensity normalization, we have added the following sentence at line 124 to explain it.

The intensity normalization is necessary so that each image has the same weightage in subsequent averaging.

Line 121: The authors should mention this is a Fiji macro. Could the authors provide instructions on how to use the macro to make it accessible and easy to use?

Reply:

As suggested by this reviewer, we have modified the corresponding text to indicate Fiji macros at line 129.

We have developed Fiji macros (Note S1 and S2) to perform these procedures semi-automatically.

More details on the usage of Fiji macros developed in this work can be found at the beginning of each macro as comments. Below is an example of the comments of macro "P1-Rotate\_Resize\_Normalize".

The code for the Fiji macro "P1-Rotate\_Resize\_Normalize".

```
//P1-Rotate_Resize_Normalize

//Part I of side-averaging and analysis (three parts in total)

//Function:           To rotate, resize and normalize side-view images for the subsequent
side-averaging. It prepares images for averaging, but it does not average the images. To
average images, you need to use ImageJ-> image ->Stacks->Z-project.

//Input:              Side-view multi-color image stack (one channel must be the giantin
double-punctum image) that meets the below requirements: (1) the background is subtracted to
zero; (2) the image is a square; (3) the trans-side of the Golgi is up; and (4) two giantin
puncta are manually marked as two ROIs and selected in the ROI Manager.
```

```
//Usage:          Open the multi-color image stack. Select the giantin channel as the
                  active window. Execute this macro. A window is popped up to prompt the selection of the folder
                  directory where you'd like to save your processed images.
```

```
//              Each channel image is saved individually with "C" and the
                  channel number appended at the beginning of its file name. Relevant information, file names,
                  and folder paths of processed images are displayed in the "log" window.
```

Line 140-141: Could the authors translate what the parameters mean in terms of Golgi localization to make the text more accessible?

Reply:

As suggested by this reviewer, we have added the below sentence at line 156.

Both  $LQ$  and  $LQ^{side}$  are metrics of the axial localization of a Golgi protein, but  $LQ^{side}$  is calculated from the side-average.

Line 163-165: What does the "dramatically" different size average mean in terms of Golgi localization? Post-Golgi carriers further away from the TGN?

Reply:

We have modified the corresponding text to make it more clear (line 181).

TGN proteins have two types of morphologies in side-average (Figs. 2 G and S2 E). Some TGN proteins, such as golgin-97, Cab45, Vamp4, SMS1, and furin, appear as a compact lump along the Golgi axis (Fig. S2 E), similar to Golgi stack proteins (Figs. 2, B-D and S2, B-D). However, other TGN proteins appear to have dramatically different side and en face-averages from Golgi stack proteins (Figs. 2 G and S2 E), consistent with our previous report (Tie et al., 2018). For example, clathrin coat machinery and its transmembrane cargos, such as clathrin light chain B (CLCB), GGA2,  $\gamma$ -adaptin, and Cl-M6PR, scatter away from the stacked region and compact lumps of golgin-97, Cab45, Vamp4, SMS1, and furin as puncta (Figs. 2 G and S2 E).

Line 175: I would disagree that sample size is a limiting factor: there can be 100s of ministacks in one cell and if only 1/3 has the correct orientation that seems more than enough. Could the authors comment?

Reply:

As advised by this reviewer, we have removed the sentence at line 199 (previously line 175).

Line 184: By providing the average of the axial localization one does not convey that some proteins may be more broadly distributed along the Golgi stack. Is there a way to express how broad is protein distribution? Very interestingly it seems like COPI (Figure 2B) is pretty much distributed around the cis cisternae and not much around the trans-Golgi? I understand that this may be a controversial topic but I find it useful information that maybe should be highlighted in the text?

Reply:

The broadness of the axial distribution might be quantified by the FWHM of the axial line intensity profile. We measured that FWHMs of most Golgi proteins are in the range of 200 – 250 nm. However, the FWHM is convoluted by the PSF (point spread function) of Airyscan, which has a size of ~ 140 - 170 nm. Hence, we do not think that the FWHM is a good indicator of the axial distribution. We are currently exploring a better way to quantify it.

Regarding the *cis*-localization of COPI, see our reply to Point 5 of Reviewer #2's comments. Briefly, our  $\gamma$ COP antibody is specific to  $\gamma$ 1COP. COPI with  $\gamma$ 1COP mainly localizes to the *cis*-Golgi under immuno-gold EM (Moelleken *et al.*, *PNAS*, 2007, PMID: 17360540).

Therefore, the *cis* and medial localization from our side-averaging is consistent with the EM localization of  $\gamma$ 1COP. We have modified the corresponding text.

Line 193: I believe the authors mean GOLPH3?

Reply:  
Yes. We have corrected the typo.

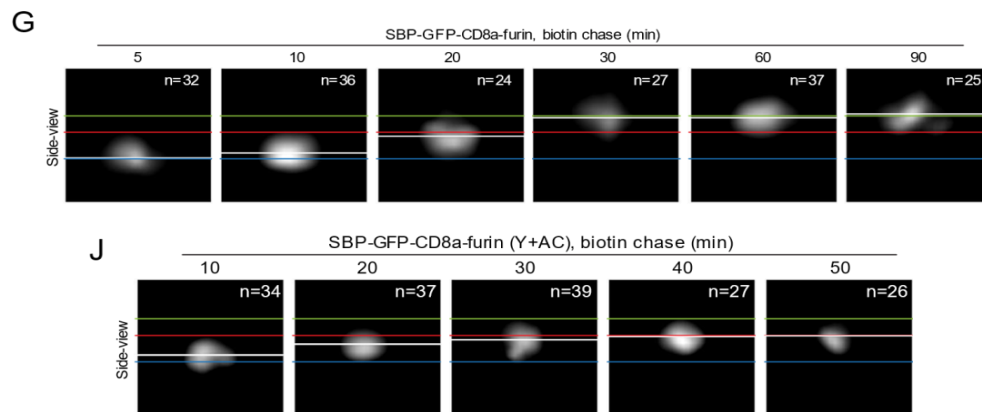
Line 209: could the author explain what they mean for "unimodal" in terms of Golgi localization to make the text more understandable?

Reply:  
Unimodal means that there is a clear single-peak. When first used in the text at line 151, it is explained in the following sentence "All Golgi proteins examined in this study display a single-peak or unimodal axial distribution, ....".

"Unimodal" at line 209 (now line 253) has been further explained by "...suggesting that it should not homogeneously distribute across the ministack".

Line 226 and Figure 4G: could the authors add an extra color line for the TGN to show that CD8 furin exits where one would expect a TGN marker?

Reply:  
We have modified Figure 4 G and J by adding a green line representing the axial position of clathrin light chain B (see below).



Line 283: I find it very interesting that cargoes stay in the inner part of the cisternae and do not enrich at the rims where vesicular carriers markers are. I understand that this may be a controversial topic but very cool info that the authors could highlight in their discussion?

Reply:  
We have discussed this interesting observation in our previous study (Tie *et al.*, *elife*, 2018, PMID: 30499774). We hypothesized that Golgi enzymes densely localize to the cisternal interior to organize as the "enzyme matrix". Conventional secretory cargos reside in the interior enzyme matrix so that glycosylation enzymes can process them. Their weak rim localization is probably due to their rapid transition through the rim. It could be due to the retrieval and retention of cargos by the enzyme matrix. Alternatively, cargos can be rapidly packed into post-Golgi exocytic carriers to leave the Golgi.

Supporting the latter explanation, CD59, a conventional secretory cargo, can occasionally be observed at the rim and rim-connected exocytic carriers and tubules when it reaches the *trans*-Golgi 60 min after chase (arrows in Fig. 5A below; images are not en face averaged).

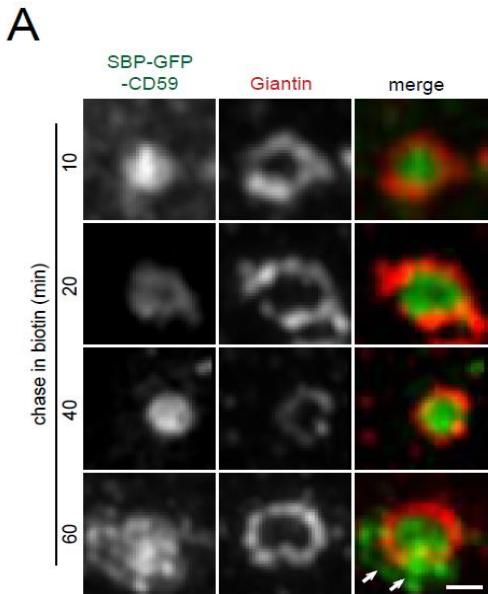


Figure 5A from Tie *et al.*, *elife*, 2018, PMID: 30499774

December 16, 2021

Re: JCB manuscript #202109114R

Dr. Lei Lu  
Nanyang Technological University  
60 Nanyang Drive  
Singapore, I am not located in the U.S. or Canada 637551  
Singapore

Dear Dr. Lu,

Thank you for submitting your revised manuscript entitled "Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks" that has been modified in response to the comments of the three reviewers.

To expedite the process I assessed the revisions myself rather than return the paper to the three reviewers. Overall you have done a good job of addressing their various concerns, and your point-by-point response was extremely clear. All three of the reviewers asked question about the distribution of COPI, and you have noted in replying to all three that the antibody that you used recognises only one of the two isoforms of gamma-COP. COPI is clearly very important for Golgi organisation, as reflected in all the reviewers being interested in this question. Thus I feel that your paper would have been considerably strengthened by instead using a different antibody to examine the distribution of all of COPI - ie an antibody against one of the subunits that has only one isoform.

As such I would in principle be happy to accept the paper, but would like you to first add this experiment using another COPI antibody. There were also comments about the distribution of COPII, which you discussed by referring to previous EM studies of Golgi mini-stacks. Thus although it is less essential, it would add to the paper if you could add an analysis of a different ER exit site marker such as Sec16. Your method is powerful, and because your lab is clearly expert at applying it, then your data will provide a very valuable map of the Golgi for the field. This data would also add further novelty to the paper as this is the third publication on the general method. I should add that acceptance is no contingent on the distribution of COPI matching the reviewers' expectations, and indeed I would not necessarily be surprised if the entire population of COPI was similar to that of gamma1-COPI, but rather it is the inclusion of this data that I would like to see before publication.

I hope that you can understand the reason for this request, and that it is straightforward to address, especially as there are commercially available antibodies against COPI and Sec16. In addition to this, you should cite John Presley's paper that reported a basic version of this assay (PubMed ID 17341478), as well as a couple more of the papers on EM of Golgi mini-stacks in HeLa cells in the context of the location of COPII and the number of cisternae in the mini-stacks (PubMed IDs 23555793 and 9852147). Finally, you should discuss the work from Gaelle Boncompain that reports that some mini-stacks in nocodazole-treated HeLa cells take several hours to become functional (27411366). Your use of giantin as a the key marker will mean that you focused on the functional stacks, but it seems important to note this for other reproducing the method.

If you can add this additional data and the literature discussion, I will then be in a position to approve publication.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Sean Munro, PhD  
Monitoring Editor

Andrea L. Marat, PhD  
Senior Scientific Editor

Journal of Cell Biology

-----

Point-by-point response letter

Manuscript number: JCB manuscript #202109114R

Manuscript title: Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks

December 16, 2021

Re: JCB manuscript #202109114R

Dr. Lei Lu  
Nanyang Technological University  
60 Nanyang Drive  
Singapore, I am not located in the U.S. or Canada 637551  
Singapore

Dear Dr. Lu,

Thank you for submitting your revised manuscript entitled "Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks" that has been modified in response to the comments of the three reviewers.

To expedite the process I assessed the revisions myself rather than return the paper to the three reviewers. Overall you have done a good job of addressing their various concerns, and your point-by-point response was extremely clear. All three of the reviewers asked question about the distribution of COPI, and you have noted in replying to all three that the antibody that you used recognises only one of the two isoforms of gamma-COP. COPI is clearly very important for Golgi organisation, as reflected in all the reviewers being interested in this question. Thus I feel that your paper would have been considerably strengthened by instead using a different antibody to examine the distribution of all of COPI - ie an antibody against one of the subunits that has only one isoform.

As such I would in principle be happy to accept the paper, but would like you to first add this experiment using another COPI antibody. There were also comments about the distribution of COPII, which you discussed by referring to previous EM studies of Golgi mini-stacks. Thus although it is less essential, it would add to the paper if you could add an analysis of a different ER exit site marker such as Sec16. Your method is powerful, and because your lab is clearly expert at applying it, then your data will provide a very valuable map of the Golgi for the field. This data would also add further novelty to the paper as this is the third publication on the general method. I should add that acceptance is no contingent on the distribution of COPI matching the reviewers' expectations, and indeed I would not necessarily be surprised if the entire population of COPI was similar to that of gamma1-COPI, but rather it is the inclusion of this data that I would like to see before publication.

I hope that you can understand the reason for this request, and that it is straightforward to address, especially as there are commercially available antibodies against COPI and Sec16. In addition to this, you should cite John Presley's paper that reported a basic version of this assay (PubMed ID 17341478), as well as a discuss a couple more of the papers on EM of Golgi mini-stacks in HeLa cells in the context of the location of COPII and the number of cisternae in the mini-stacks (PubMed IDs 23555793 and 9852147). Finally, you should

discuss the work from Gaelle Boncompain that reports that some mini-stacks in nocodazole-treated HeLa cells take several hours to become functional (27411366). Your use of giantin as a the key marker will mean that you focused on the functional stacks, but it seems important to note this for other reproducing the method.

If you can add this additional data and the literature discussion, I will then be in a position to approve publication.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Sean Munro, PhD  
Monitoring Editor

Andrea L. Marat, PhD  
Senior Scientific Editor

Journal of Cell Biology

Point-by-point response letter

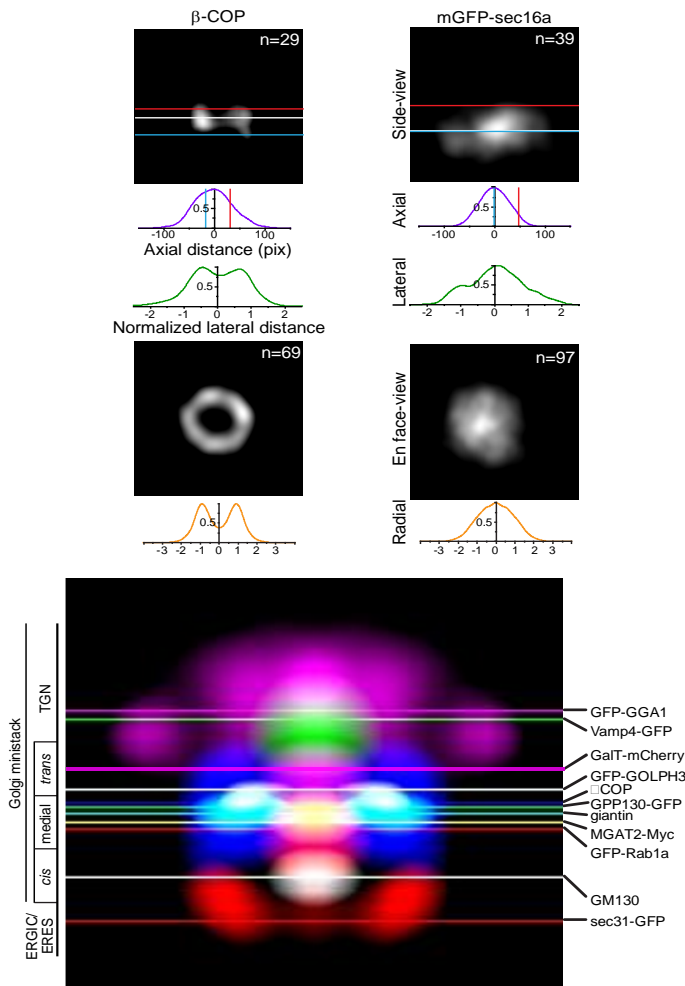
Manuscript number: JCB manuscript #202109114R

Manuscript title: Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks

1 The side and en face-averaging of two more proteins, sec16a and  $\beta$ COP.

Reply:

We have purchased mGFP-sec16a DNA plasmid from Addgene (Glick lab's deposit) and a new antibody against  $\beta$ COP, and performed the requested imaging (see below). The model in Fig. 3A has been modified accordingly (see below). As expected, COPI labeled by  $\beta$ COP has a similar appearance to  $\gamma$ 1COP — a ring. Interestingly,  $\beta$ COP signal is more *trans* than  $\gamma$ 1COP, as reported previously (Moelleken *et al.*, 2007, PMID: 17360540). However, different from our expectation, sec16a appears as a filled disk instead of a ring, the significance of which needs to be further investigated.



We have included the following text in the Results section to describe sec16a's localization in side-average.

Sec16a seems to be the only one with the gross discrepancy in Table S2. Our side-averaging localizes it to the *cis*-Golgi ( $LQ^{\text{side}} = 0.04$ ) (Fig. S2 A), which does not agree with its well-documented ERES localization (Bhattacharyya and Glick, 2007; Hughes et al., 2009), although our GLIM supports its ERES localization ( $LQ = -0.81$ ). The very asymmetrical and off-axial distribution of sec16a puncta could contribute to such discrepancy.

We have modified the text in the Results section accordingly for COPI (changed text underlined).

Our observation is consistent with previous reports on the tightly adjacent localization of COPII and COPI at the ER-proximal and distal region, respectively, near the ERES (Scales et al., 1997; Stephens et al., 2000; Weigel et al., 2021), and the cisternal rim distribution of COPI from the *cis* to *trans*-Golgi (Orci et al., 1997). Interestingly, we found that, in contrast to  $\beta$ COP,  $\gamma$ 1COP, one of the two paralogs of COPI's  $\gamma$ -subunit (Moelleken et al., 2007), localizes primarily from the pre-Golgi to the medial-Golgi in side-average (Fig. S2 B), as previously discovered under EM (Moelleken et al., 2007).

- 2 The citation of John Presley's paper.

Reply:

We cited Presley lab's paper—Dejgaard et al. in the introduction section as shown below.

Dejgaard et al. previously proposed quantitatively localizing a Golgi protein by intensity line scan or peak distances for nocodazole-induced Golgi ministacks (hereafter referred to as ministacks) (Dejgaard et al., 2007).

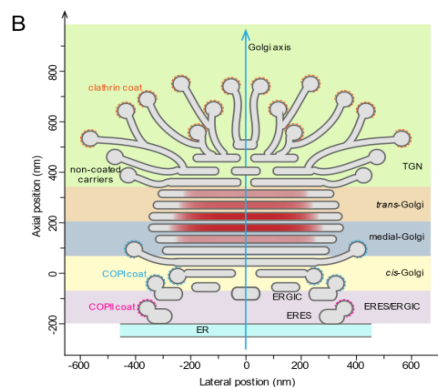
- 3 The discussion of Koreishi *et al.*, 2013 (PMID: 23555793).

Reply:

It is now cited as an EM localization evidence to support our side-averaging of giantin.

- 4 The discussion of Storrie *et al.*, 1998 (PMID: 9852147) for the number of cisternae in ministacks.

Reply:



Here is how we estimated the number of cisternae.

The membrane thickness (5 nm), the distance of the intra- (30 nm) and inter-cisternal (15 nm) membrane, and the diameter of a vesicle/bud (60 nm) are roughly drawn in

scale according to published EM data (Ladinsky et al., 1999; Trucco et al., 2004). Hence, the distance from the *cis* to the *trans*-side of the ministack (*LQ* from -0.25 to 1.25) has roughly seven cisternae by the below calculation.

$$\frac{(1.25 + 0.25) \times 274 \text{ nm}}{(5 + 30 + 5 + 15)} \approx 7$$

Our number of cisternae per ministack, seven, is roughly consistent with the 3D EM tomography of Golgi in NRK cells (Ladinsky et al., 1999). The number of observed Golgi cisternae per stack varies in cell lines and depends on EM techniques. 2D EM thin sections are the most commonly used techniques. However, this approach could grossly underestimate the number of cisternae as the sectioning plane is randomly oriented and cannot be controlled to align with the Golgi axis. Therefore, we think that the number of cisternae per ministack in HeLa cells reported by Storrie et al. (PMID: 9852147), five, is an underestimation. Hence, we have decided not to include this reference.

We have included the following sentences in the Results section.

Hence, the distance from the *cis* to the *trans*-side of a ministack (*LQ* from -0.25 to 1.25) has roughly seven cisternae, calculated by the above morphometric parameters. The number of cisternae per ministack, seven, is consistent with the 3D EM tomography data acquired in NRK cells (Ladinsky et al., 1999).

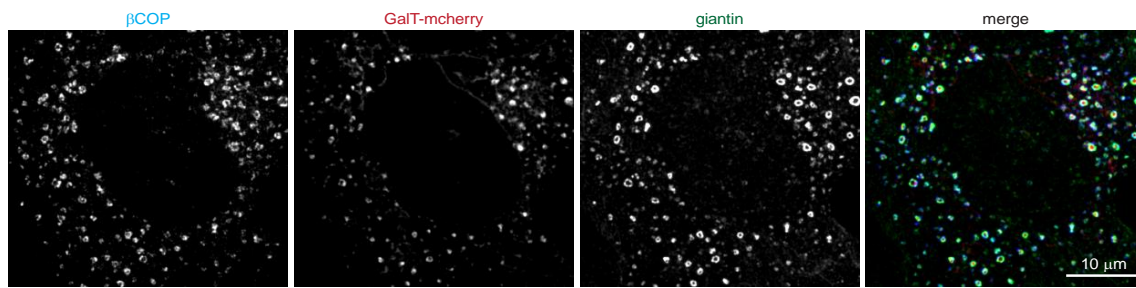
- 5 The discussion of Boncompain lab's paper—Fourriere et al., 2016 (PMID: 27411366)  
Reply:

We have included the following sentence in the Introduction section.

Extensive studies have demonstrated that different from acute treatment (Fourriere et al., 2016), ministacks under prolonged nocodazole treatment ( $\geq 3$  hours), the condition under which GLIM employs, are valid representations of the native Golgi stack (Cole et al., 1996; Rogalski et al., 1984; Tie et al., 2018; Trucco et al., 2004; Van De Moortele et al., 1993), although the intra-Golgi transport of large secretory cargos might be compromised in ministacks (Lavieu et al., 2014).

We found that almost all peripheral ministacks (newly generated ones) are positive for giantin after 3 hours of nocodazole treatment (Fig. S1 A). Therefore, we also included the following sentences at the beginning of the Results section.

Fourriere et al. reported that, under the acute nocodazole treatment, newly assembled ministacks initially lack certain Golgi proteins such as giantin, which are gradually acquired by ministacks after more extended treatment (Fourriere et al., 2016). Under our nocodazole treatment ( $\geq 3$  hours), we found that almost all ministacks are positive for giantin (Fig. S1 A), suggesting that ministacks might be homogenous in the organization and functionally matured.



- Bhattacharyya, D., and B.S. Glick. 2007. Two mammalian Sec16 homologues have nonredundant functions in endoplasmic reticulum (ER) export and transitional ER organization. *Mol Biol Cell*. 18:839-849.
- Cole, N.B., N. Sciaky, A. Marotta, J. Song, and J. Lippincott-Schwartz. 1996. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol Biol Cell*. 7:631-650.
- Dejgaard, S.Y., A. Murshid, K.M. Dee, and J.F. Presley. 2007. Confocal microscopy-based linescan methodologies for intra-Golgi localization of proteins. *J Histochem Cytochem*. 55:709-719.
- Fourriere, L., S. Divoux, M. Roceri, F. Perez, and G. Boncompain. 2016. Microtubule-independent secretion requires functional maturation of Golgi elements. *J Cell Sci*. 129:3238-3250.
- Hughes, H., A. Budnik, K. Schmidt, K.J. Palmer, J. Mantell, C. Noakes, A. Johnson, D.A. Carter, P. Verkade, P. Watson, and D.J. Stephens. 2009. Organisation of human ER-exit sites: requirements for the localisation of Sec16 to transitional ER. *J Cell Sci*. 122:2924-2934.
- Ladinsky, M.S., D.N. Mastronarde, J.R. McIntosh, K.E. Howell, and L.A. Staehelin. 1999. Golgi structure in three dimensions: functional insights from the normal rat kidney cell. *J Cell Biol*. 144:1135-1149.
- Lavieu, G., M.H. Dunlop, A. Lerich, H. Zheng, F. Bottanelli, and J.E. Rothman. 2014. The Golgi ribbon structure facilitates anterograde transport of large cargoes. *Mol Biol Cell*. 25:3028-3036.
- Moelleken, J., J. Malsam, M.J. Betts, A. Movafeghi, I. Reckmann, I. Meissner, A. Hellwig, R.B. Russell, T. Sollner, B. Brugger, and F.T. Wieland. 2007. Differential localization of coatamer complex isoforms within the Golgi apparatus. *Proc Natl Acad Sci U S A*. 104:4425-4430.
- Orci, L., M. Starnes, M. Ravazzola, M. Amherdt, A. Perrelet, T.H. Sollner, and J.E. Rothman. 1997. Bidirectional transport by distinct populations of COPI-coated vesicles. *Cell*. 90:335-349.
- Rogalski, A.A., J.E. Bergmann, and S.J. Singer. 1984. Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. *J Cell Biol*. 99:1101-1109.
- Scales, S.J., R. Pepperkok, and T.E. Kreis. 1997. Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell*. 90:1137-1148.
- Stephens, D.J., N. Lin-Marq, A. Pagano, R. Pepperkok, and J.P. Paccaud. 2000. COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J Cell Sci*. 113 ( Pt 12):2177-2185.
- Tie, H.C., A. Ludwig, S. Sandin, and L. Lu. 2018. The spatial separation of processing and transport functions to the interior and periphery of the Golgi stack. *Elife*. 7.
- Trucco, A., R.S. Polishchuk, O. Martella, A. Di Pentima, A. Fusella, D. Di Giandomenico, E. San Pietro, G.V. Beznoussenko, E.V. Polishchuk, M. Baldassarre, R. Buccione, W.J. Geerts, A.J. Koster, K.N. Burger, A.A. Mironov, and A. Luini. 2004. Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. *Nat Cell Biol*. 6:1071-1081.

- Van De Moortele, S., R. Picart, A. Tixier-Vidal, and C. Tougard. 1993. Nocodazole and taxol affect subcellular compartments but not secretory activity of GH3B6 prolactin cells. *Eur J Cell Biol.* 60:217-227.
- Weigel, A.V., C.L. Chang, G. Shtengel, C.S. Xu, D.P. Hoffman, M. Freeman, N. Iyer, J. Aaron, S. Khuon, J. Bogovic, W. Qiu, H.F. Hess, and J. Lippincott-Schwartz. 2021. ER-to-Golgi protein delivery through an interwoven, tubular network extending from ER. *Cell.* 184:2412-2429 e2416.

March 24, 2022

RE: JCB Manuscript #202109114RR

Dr. Lei Lu  
Nanyang Technological University  
60 Nanyang Drive  
Singapore, 637551  
Singapore

Dear Dr. Lu:

Thank you for submitting your revised manuscript entitled "Visualizing intra-Golgi localization and transport by side-averaging Golgi ministacks". We find that you have done an excellent job in addressing the remaining issues and would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

#### A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, <https://jcb.rupress.org/submission-guidelines#revised>.

**\*\*Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.\*\***

- 1) Text limits: Character count for Tools is < 40,000, not including spaces. Count includes abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.
- 2) Figures limits: Tools may have up to 10 main text figures.
- 3) \* Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.
- 4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."
- 5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.
- 6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. \* Please provide full descriptions in the text for readers who may not have access to referenced manuscripts, for example the immunofluorescence labeling and details of the RUSH reporter previously published must be described here. \*
- 7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.
- 8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
  - a. Make and model of microscope
  - b. Type, magnification, and numerical aperture of the objective lenses
  - c. Temperature
  - d. Imaging medium
  - e. Fluorochromes
  - f. Camera make and model

g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed. Please note that supplemental references are not permitted.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: <https://rupress.org/jcb/pages/submission-guidelines#revised>

## B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander ([lhollander@rockefeller.edu](mailto:lhollander@rockefeller.edu)).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, <https://jcb.rupress.org/fig-vid-guidelines>.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**\*\*It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.\*\***

**\*\*The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before**

choosing the appropriate license.\*\*

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit <https://rupress.org/jcb/pages/submission-guidelines#videoSummaries>.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Sean Munro, PhD  
Monitoring Editor

Andrea L. Marat, PhD  
Senior Scientific Editor

Journal of Cell Biology

---