**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.**

**Editorial comments:**  
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. <b>Please download the .docx file and use this updated version for any future revisions</b>. The updated manuscript is also attached.

JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript (text and figures). All commercial products should be sufficiently referenced in the table of materials/reagents. Please replace all commercial sounding language in your manuscript with generic names that are not company-specific. Examples of commercial sounding language in your manuscript are: Opti-MEM, Lipofectamine 2000, Mowiol, Teflon, Excel, OriginPro, etc.

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.

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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.

JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

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