nature research

Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Super-resolved 3D Tracking of Cargo Transport Through Nuclear Pore Complexes
Corresponding author name(s): Siegfried Musser

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Dr Musser,

Your manuscript, "Super-resolved 3D Tracking of Cargo Transport Through Nuclear Pore Complexes", has now been seen by 3 referees, who are experts in single-molecule tracking (referee 1); single-molecule and super-resolution tools; NPC/nuclear transport (referee 2); and NPC (referee 3). As you will see from their comments (attached below) they find this work of potential interest but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

As you may know, Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, the reviewers - and we agree - shared strong interest in the description of the direct visualization of cargo transport trajectories at high spatial and temporal resolution in cells. To strengthen the main conclusions, however, we feel that revision efforts should be dedicated to the following points:

A) The work focuses on the new method to delineate nucleocytoplasmic transport in live cells at high

resolution whilst also obtaining well-resolved NPC structures, and this is a significant technical achievement for the field, as echoed by the reviewers. We therefore feel that the paper would best fit the NCB "Technical Report" format. At resubmission and during revision, please convert the format to a Technical Report. Essentially the format itself would not alter the paper as it allows up to 8 main figures and 10 ED figures; the main change at this stage is the name of the format, and please let me know if you'd like to discuss this further.

B) As suggested by Rev#2 and Rev#3, testing another NLS-cargo would provide a stronger test of your model in terms of cargo translocation dynamics and/or enrich our understanding of nucleo-cytoplasmic transport mechanisms for different types of cargo, and provide a stronger demonstration of the value of the method. We feel that adding these data would further establish the importance of the method for the field and therefore feel additions along these lines are essential.

C) Please address all the reviewers' comments and questions about the method, including better describing the method (E.g., Rev#2 last major comment). Thoroughly addressing the reviewers' questions about the approach and their technical remarks is especially essential for a Technical Report.

D) All other referee concerns pertaining to strengthening existing data, providing controls, clarifications and textual changes should also be addressed. The reviewers raise interesting discussion points that should be incorporated into the manuscript.

E) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime. Our typical revision period is 6 months; please do let me know if you would like to discuss this timeline further or anticipate any pandemic-related challenges; we are happy to discuss further as

needed.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Editorial Policy Checklist (found here https://www.nature.com/authors/policies/Policy.pdf), and Reporting Summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf). This is essential for reconsideration of the manuscript and these documents will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope that you will find our referees' comments and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Melina

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Senior Editor, Nature Cell Biology
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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
A. Summary of the key results
Chowdhury et al. visualized interactions of single M9-βGal-8C cargo complexes with highly resolved NPCs in 3D on a millisecond timescale. Visualization of NPCs was achieved through chromosomally expressed NUP96-mEGFP labeled with anti-GFP nanobodies tagged with the spontaneous blinking dye HMSiR. By using a home-built adaptive optics (AO) system, astigmatism was introduced into the imaging system, by which positional 3D information of single particle images could be obtained. Using dSTORM combined with a smart computational approach the authors derived a high resolution 3D image of the eightfold symmetrical structure of the NPC including the nucleoplasmic and the cytoplasmic rings. For a duration of 11 min the authors showed that the nuclear envelope with the incorporated NPCs can be considered as a rigid structure. By combining the highly resolved NPC structures with individual 3D particle trajectories of M9-βGal-8C cargo complexes, the authors could demonstrat that translocation through the NPC occurred through an hourglass-shaped conduit with the particle distribution narrowest at the center of the pore and wider and more diffuse at the openings of the pore. Finally, their data also suggested that the cargo molecules distributed in distances up to 100 – 150 nm from the NPC openings in both the cytoplasm and nucleoplasm suggesting that the FG-Nups protrude into this space probably impeding the translational mobility in this region.

B. Originality and significance:
This is the first time that the pathway of individual trajectories of cargo complexes through NPCs could be visualized in living cells in true three dimensions on a millisecond timescale. This approach opens a fascinating view on the translocation of molecules passing the NPC and specifically their interactions with the FG-Nups in permeabilised cells.

C. Data & methodology:
Single-molecule fluorescence microscopy has been pushed to its current limits in this study, which certainly will become a reference publication. The experimental approach, the resulting data, the analysis and the conclusions were presented in a very clear and comprehensive manner. The approach is technically extremely complex, but was realized and characterized in convincing manner. The presentation is clear and easy to follow. The data quality is simply excellent. The derived NPC structures have a remarkably high resolution. The quality of the data was excellent due to the application of several filtering steps as well as the double-circle fit to determine the NPC centroid and the angular global fit to align the eight segments of each ring. Also, the removal of all M9-βGal-8C particles present for less than 9ms improved the outcome of the single-molecule data strongly. All these measures were well explained and are comprehensible. In summary, certainly, the raw data were strongly filtered, fitted and averaged – but for good reasons and in a well justified manner. Excellent.

D. Appropriate use of statistics and treatment of uncertainties
The number of M9-βGal-8C trajectories (N = 168) is significant, considering that these data resulted after filtering out the short time events. The randomness of those short-time events was demonstrated via simulation (Supplementary Figure 7). All uncertainties occurring by the used methodology have been considered, presented and discussed in an exemplary manner. The comparison between experimental vs simulated data reinforced the conclusions drawn from the experimental data.

E. Conclusions: robustness, validity, reliability
As said the data were evaluated appropriately so that the resulting findings can be considered as very reliable.

F. Suggested improvements: experiments, data for possible revision

The overall quality of presentation of this paper is extraordinary. It would be valuable to know for the reader how many NPCs were measured to yield the 168 trajectories of M9-βGal-8C? Could it be possible that the high number of localizations outside the central pore (>90%) has an alternative reason besides FG-Nups extending deep into the cytoplasm/nucleoplasm (e.g. due to a kind of traffic jam)?

The authors determined a high rigidity of the nuclear envelope (NE). Two steps in the sample preparation pose questions with respect to the measured rigidity. Firstly, by cell permeabilization all proteins, cofactors, and nutrients were washed out of the cytoplasm. Could this have affect the fluidity of the NE or NPC? Secondly, the cells were attached to the coverslip surface by poly-L-lysine. Could this influence the cytoskeleton in a way that the fluidity of the NE and respectively the mobility of the NPC was altered?

Typo in Supplementary Information page 8, line 4: moment – movement

G. References: appropriate credit to previous work?
Yes.

H. Clarity and context:
lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions.

The abstract gives a good introduction to the topic and summarizes the important findings in a clear manner. Introduction and discussion are concise but complete and were clearly written.

Ulrich Kubitscheck

Reviewer #2:
Remarks to the Author:
In this work, the authors aim to "develop an approach that would allow the characterization of the real time dynamics of cargo translocation in the context of a well-resolved NPC scaffold". For obtaining a well-resolved NPC scaffold image, they use Nup96-EGFP tagged with nanobody conjugated with HMSiR. They validate that this scaffold is sufficiently positionally stable in unfixed permeabilized cells in transport buffer for 11 minutes. Their data on Nup96 localization is in good agreement with previously reported values. Then, for the characterization of cargo translocation dynamics, they use a ~500 kDa cargo tagged with Atto532 and track the localizations which persist for 3 or more frames (<9 ms) in permeabilized cells within 2 minutes. To obtain the context of NPC scaffold for cargo transport, they then image the NPC for 8 minutes. In this way, their approach allows the real-time imaging of cargo translocation through individual, well-resolved NPCs. With this method, they are able to differentiate between abortive or completed import and export. The cargo localization data suggests three interesting findings. First, they observe particle localization close to the NPC in both cytoplasmic and nucleoplasmic sides, suggesting that NPC structural elements such as FG-Nups may extend 100-150 nm from the double-ring structure and interact with the cargo. Second, surprisingly, they observe that cargoes which passed the NPC mid-plane almost invariably were transported to the other side, while abortive transport events almost never crossed this plane. Third, their data suggests that cargo passes close to the surface of the NPC scaffold. I believe the third point is the major new point, finally shown in a proper convincing way. Repeating the measurement for another condition or cargo that might go a different path or be very different in size would have helped to increase the impact, but I understand that the data is hard to acquire. However, the text more emphasizes how easy and well suitable the method is, and if that is indeed the case, showing another meaningful biological condition would help the story to gain more biological significance.
This work aims to track cargo passing through individual, well-resolved NPCs in a single-molecule level using super-resolution. This could provide valuable in-situ experimental evidence for or against the various available models regarding the NPC inner channel and cargo transport.
The approach of the authors is straightforward to explain, but these experiments are very hard to perform: first, they validate the NPC localization precision and positional stability throughout their planned measurements. Then, in their experiments, they localize the cargo first, then the NPC which the cargo went through. Finally, they analyze the localizations of the cargo and the NPC to observe their interaction. Throughout this procedure, the various sources of instrumentation and biological error are measured or simulated and reported. In the end, the authors are able to reach some conclusions on the cargo interaction with the NPC and its transport through it. The initial measurements of the NPC give results which agree with previous reports. The cargo localization data set is rather small and not validated by previous or further current results. However the data hint at interesting results which could be explored further using the outlined method.

In summary the paper is rich in technological rigor, and an impressive and useful combination of SRM and tracking applied to a highly challenging biological problem that was certainly very hard work to achieve. Experimental data wise, it is less rich and at many places thus has to remain rather descriptive, and in that sense it might be a little less then what you expect from a Nature Cell Biology paper in that respect.

Major comments:
- The benefit of non-fixation is overemphasized, as they work with permeabilized cells, and many cell biologists would also see the digitation treatment already critical. The authors also just mention cytoskeletal elements which hold the NPC stable. Again, with respect to the cytoskeleton one should take into account that the digitonin treatment is quite invasive. Please do not get me wrong, the permeabilized treatment is very suitable to study the functional transport machinery, and none of their conclusions are wrong. Just overall the impression emerges, that this is a life cell study, and that is not appropriate. I recommend downtowning those statement.
- Abstract can be re-phrased to better fit the main body. SPEED microscopy is not mentioned in the main text. While it is refreshing to see aa technical nearly flawless work compared to much of the SPEED literature, I suggest they authors move the SPEED aspect to the discussion. Also, the rigid cytoskeletal framework which is suggested to mediate NPC stability in unfixed permeabilized cells is mentioned in the abstract, but only passingly in the text, only indicating that there must be such a framework. No evidence or further comment is given on this.
- From the text, the conditions under which the cells were measured first (data for Figure 1) are not clear. Were the transport proteins and GTP present? Was cargo present? If no, then do the findings of the data for Figure 1 (positional stability and localization errors) still hold? Howe were cells identified, carton in Figure 1 with imaging desiply.
- Precision should come form tracking labelled cargo in fixed cells. Using bead measurement only gives technical precision, which is not a useful parameter for the precision of the actual measurement.
- The results are mainly discussed in relevance to a kap centric model. Taking the complexity of the NPC barrier into account, I do not think that the model itself (that NTRs and an integral part of the barrier) make to strong statement about actual transport routes. So I found the discussion here a bit too focused, and biased. I do not think the data excludes a kap centric model. The data is about transport routes, and does not exclude or include any of the most commonly discussed models (which are mode fundamental in nature). The data on transport routes is significant though.
- Evidence for interaction with NUP extending to 150 nm is indirect, soley based on tarcking data. The conclusion thus needs to be toned down, as this is very descriptive, and thei ri sno drect evidence of interaction with Nup. This could also be a result of the boxing, or traking routine, or other interactions at the nuclear envelope. One can simply not say when looking only at cargo trajectories. Speculation and result need to be more clearly expressed.
- I would suggest to show more movies than 1 for the crago movement within the cannel, so that one

can see how reproducible this is. The movie is impressive.
- Authors make comments on export (ex.: “Notably, the analysis in Fig. 2l suggests that both import and export occur through the same (or similar) channels since trajectories from both processes were included.”). However, the export does not happen under well-controlled conditions, as they also outline in the text. Therefore, it seems rather hard to reach a meaningful conclusions on export.
- It is not clear from the text what were the conditions of the cells for the initial measurements of the positional stability and localization precision of the NPC (Figure 1). In the methods section, only the tracking experiments are described. In the main text, the transport mix is only mentioned after the results of initial NPC localizations are discussed. During transport tracking experiments, the NPCs are measured and localized in the presence of transport proteins, energy and large cargo. The possible differences between the conditions may affect the NPC localization precision or positional stability.

Minor comments
- A cartoon of how cells are measured and the NPC oriented with respect to the measurement plane should go into figure 1.
- No data on various controls is shown such as binding of nanobody-HMSiR to control cells, leakage of fluorescent signal to other channels, etc.
- E.g. the authors claim that nanobody-HMSiR does not bind to U-2 OS control cells but do not show the data.
- For Figure 1, the text does not explain the different parts of the figure in alphabetic order.
- The authors can give a more specific description on the scope, validity and limitations of their proposed method.

Reviewer #3:
Remarks to the Author:
The authors tackle a difficult problem that has proven quite controversial in recent years, namely, measuring the path and kinetics taken by individual nuclear transport cargoes as they cross the NPC. One particular issue has been the use of certain types of rotational symmetrization in some of the microscopic methods used, with lively debate ensuing (e.g. “Deconstructing transport-distribution reconstruction in the nuclear-pore complex” comment and reply, PMID 30518848). Here, the authors avoid such issues by directly resolving the eight-fold ring structure of each NPC directly and – taking advantage of their demonstration of NE stability over the time course of the experiment - using that as the reference for transport events going through these NPCs in a permeabilized cell nuclear transport assay.

The methodology described appears extremely elegant, is thoroughly and carefully controlled and

documented, and represents a significant technical achievement in the field – potentially opening the way to far superior high-resolution time-resolved studies of nuclear transport pathways in situ.

One notable finding is the considerable extent to which the FG repeats seem to emanate from the NPC's scaffold; this is consistent with numerous recent data on the extent of these domains – contrasting more condensed states of the FG repeats ('gels') seen in some studies with more extended and fluid states (such as observed 'polymer brushes' and 'liquid phases') - as well as several models of the nucleocytoplasmic transport mechanism, only some of which are only touched on in this manuscript. The hourglass-shaped translocation conduit reported here is also a significant finding, and one that seems to fit well with both 'classic' (Akey and Rademacher, 1993) and recent (e.g. Kim et al., 2018) ultrastructural observations supporting an hourglass-shaped central 'transporter' density in the NPC, postulated to consist of transport factors and cargo in transit across FG repeat domains. Perhaps a more robust discussion of these issues is warranted, i.e., how do the data in this work advance our understanding of the selective transport mechanism at the NPC in the light of the various major models proposed and more recent published data?

A potential drawback of this work is the use of only one nuclear transport reporter, even though as the authors point out, a tremendous potential of this method – untapped in this manuscript – is to untangle the possible alternative pathways that different pathways may utilize. Would it be possible and within the scope of this study to test this by using a different NLS and import factor, such as an IBB sequence and importin / karyopherin beta-1? Or a ribosomal NLS and importin / karyopherin beta-4?

Minor comments:

Do the authors assume circularity of the NPC for their generation of the octagonal reference rings? How would deviations from circularity (as have been observed) in the NPC affect the generation of these references?

Is it possible to determine if there are any radial preferences for the import / export pathways (i.e., relative to the radial position of the GFP/dye)?

One possibility has been that the preference for RNA export through the center of the channel is due to a greater crowding effect pushing these large cargoes towards the center, leaving the periphery for the smaller protein cargoes by default. However, presumably there is no such RNA export here, so that would tend to indicate instead that the peripheral localization of the transport paths seen here is a true preference?

In yeast, a stronger concentration of GLFG type FG repeats is predicted for the channel periphery, while FXFG repeats are predicted to be found more towards the central axis (Kim et al., 2018). If this is

conserved in mammalian cells, would this make any sense with known importin preferences vs RNA export factors such as NXF/NXT?

Recently, an analog of nuclear transport was reconstituted in vitro showing that different types of FG repeat were not needed to be differentially localized in a pore to support transport events (Fragasso et al., 2021 PMID 33790297). Do the authors believe that these events would also be seen to be peripheral to their pores, or that differential FG repeat support the strong path preferences through the central channel shown here?

“distinct locals” should likely read “distinct locales”

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the

text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail,

identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement at the end of the Methods section. For Springer Nature policies on data availability see http://www.nature.com/authors/policies/availability.html; for more information on this particular policy see http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf. The Data availability statement should include:

• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

• Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details http://www.nature.com/sdata/data-policies/repositories).

• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

• If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs $600 for the first, and $300 for each subsequent colour figure.

All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in ‘type layers’ and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects’ - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – To improve the quality of methods and statistics reporting in our papers we have recently revised the reporting checklist we introduced in 2013. We are now asking all life sciences authors to complete two items: an Editorial Policy Checklist (found here https://www.nature.com/authors/policies/Policy.pdf) that verifies compliance with all required editorial policies and a reporting summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf) that collects information on experimental design and reagents. These documents are available to referees to aid the evaluation of the manuscript. Please note that these forms are dynamic 'smart pdfs' and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical

significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Author Rebuttal to Initial comments

REVIEWER 1:

1. Chowdhury et al. visualized interactions of single M9-βGal-8C cargo complexes with highly resolved NPCs in 3D on a millisecond timescale. Visualization of NPCs was achieved through chromosomally expressed NUP96-mEGFP labeled with anti-GFP nanobodies tagged with the spontaneous blinking dye HMSiR. By using a home-built adaptive optics (AO) system, astigmatism was introduced into the imaging system, by which positional 3D information of single particle images could be obtained. Using dSTORM combined with a smart computational approach the authors derived a high resolution 3D image of the eightfold symmetrical structure of the NPC including the nucleoplasmic and the cytoplasmic rings. For a duration of 11 min the authors showed that the nuclear envelope with the incorporated NPCs can be considered as a rigid structure. By combining

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the highly resolved NPC structures with individual 3D particle trajectories of M9-βGal-8C cargo complexes, the authors could demonstrate that translocation through the NPC occurred through an hourglass-shaped conduit with the particle distribution narrowest at the center of the pore and wider and more diffuse at the openings of the pore. Finally, their data also suggested that the cargo molecules distributed in distances up to 100 – 150 nm from the NPC openings in both the cytoplasm and nucleoplasm suggesting that the FG-Nups protrude into this space probably impeding the translational mobility in this region.

We thank the Reviewer for concisely highlighting the key results of this paper.

2. This is the first time that the pathway of individual trajectories of cargo complexes through NPCs could be visualized in living cells in true three dimensions on a millisecond timescale. This approach opens a fascinating view on the translocation of molecules passing the NPC and specifically their interactions with the FG-Nups in permeabilised cells.

We appreciate the Reviewer's comments. However, while likely unintentionally stated, we clarify that all of our experiments were performed in permeabilized cells, not living cells.

3. Single-molecule fluorescence microscopy has been pushed to its current limits in this study, which certainly will become a reference publication. The experimental approach, the resulting data, the analysis and the conclusions were presented in a very clear and comprehensive manner. The approach is technically extremely complex, but was realized and characterized in convincing manner. The presentation is clear and easy to follow. The data quality is simply excellent. The derived NPC structures have a remarkably high resolution. The quality of the data was excellent due to the application of several filtering steps as well as the double-circle fit to determine the NPC centroid and the angular global fit to align the eight segments of each ring. Also, the removal of all M9-βGal-8C particles present for less than 9ms improved the outcome of the single-molecule data strongly. All these measures were well explained and are comprehensible. In summary, certainly, the raw data were strongly filtered, fitted and averaged – but for good reasons and in a well justified manner. Excellent.

We thank the Reviewer for his kind comments regarding our analysis methods.

4. The number of M9-βGal-8C trajectories (N = 168) is significant, considering that these data resulted after filtering out the short time events. The randomness of those short-time events was demonstrated via simulation (Supplementary Figure 7). All uncertainties occurring by the used methodology have been considered, presented and discussed in an exemplary manner. The comparison between experimental vs simulated data reinforced the conclusions drawn from the experimental data.

We appreciate the Reviewer's recognition of the value of our simulations and statistical analysis.

5. As said the data were evaluated appropriately so that the resulting findings can be considered as very reliable.

We appreciate this comment on data robustness.

6. The overall quality of presentation of this paper is extraordinary. It would be valuable to know for the reader how many NPCs were measured to yield the 168 trajectories of M9-βGal-8C? Could it be possible that the high number of localizations outside the central pore (>90%) has an alternative reason besides FG-Nups extending deep into the cytoplasm/nucleoplasm (e.g. due to a kind of traffic jam)?

The 168 trajectories previously indicated were the total displayed as representative trajectories. The Figure 4 caption now indicates the total number of trajectories for which the initial and destination compartments are known (*N* = 239), as well as the number of NPCs (142) and nuclei (10) from which these were obtained (Fig. 4e-h).

The reason for the large number of localizations outside of the central pore is a debatable point that will only be resolved with future data. We consider an extended FG-Nup network the most likely possibility as it would readily provide the numerous binding sites needed to retain complexes within the observed volume. While the traffic jam concept is a reasonable explanation for trajectories that cross through the pore to the other side, over 40% of 3+ point trajectories never penetrate the pore, suggesting a cluster of binding sites rather than a reflection of necessary waystations on the way to/from the pore. This uncertainty is now more completely discussed (lines 158-167, 287-296).

7. The authors determined a high rigidity of the nuclear envelope (NE). Two steps in the sample preparation pose questions with respect to the measured rigidity. Firstly, by cell permeabilization all proteins, cofactors, and nutrients were washed out of the cytoplasm. Could this have affected the fluidity of the NE or NPC? Secondly, the cells were attached to the coverslip surface by poly-L-lysine. Could this influence the cytoskeleton in a way that the fluidity of the NE and respectively the mobility of the NPC was altered?

We do indeed expect that the rigidity of the system has increased upon cell permeabilization, and that this increased stability is essential for the success of the experimental approach. The precise explanation for the increased stability will require further study, but the absence of the dynamics inherent in living systems due to competing and opposing pathways almost certainly contributes. With these caveats, we nonetheless emphasize that the stability of the system is truly remarkable, as an entirely rigid framework must exist from the coverslip, through the poly-L-lysine layer, through the fragmented plasma membrane, most likely through a stably polymerized cytoskeletal structure to the nuclear envelope, which must itself prevent both lateral and axial motion of the individual NPCs. We now provide a clearer discussion in the revised manuscript (lines 108-116, 244-252).

8. Typo in Supplementary Information page 8, line 4: moment – movement

Corrected. This figure has been moved to Fig. 3 within the main manuscript to provide support for the high structural stability. Some of text in the figure caption has been moved to the computational methods section.

9. The abstract gives a good introduction to the topic and summarizes the important findings in a clear manner. Introduction and discussion are concise but complete and were clearly written.

We thank the Reviewer for his kind comments, and hope that the revised manuscript in which these sections were significantly modified retain the same clarity.

REVIEWER 2:

1. In this work, the authors aim to "develop an approach that would allow the characterization of the real time dynamics of cargo translocation in the context of a well-resolved NPC scaffold". For obtaining a well-resolved NPC scaffold image, they use Nup96-EGFP tagged with a nanobody conjugated with HMSiR. They validate that this scaffold is sufficiently positionally stable in unfixed permeabilized cells in transport buffer for 11 minutes. Their data on Nup96 localization is in good agreement with previously reported values. Then, for the characterization of cargo translocation dynamics, they use a ~500 kDa cargo tagged with Atto532 and track the localizations which persist for 3 or more frames (≥9 ms) in permeabilized cells within 2 minutes. To obtain the context of NPC scaffold for cargo transport, they then image the NPC for 8 minutes. In this way, their approach allows the real-time imaging of cargo translocation through individual, well-resolved NPCs. With this method, they are able to differentiate between abortive or completed import and export. The cargo localization data suggests three interesting findings. First, they observe particle localization close to the NPC in both cytoplasmic and nucleoplasmic sides, suggesting that NPC structural elements such as FG-Nups may extend 100-150 nm from the double-ring structure and interact with the cargo. Second, surprisingly, they observe that cargoes which passed the NPC mid-plane almost invariably were transported to the other side, while abortive transport events almost never crossed this plane. Third, their data suggests that cargo passes close to the surface of the NPC scaffold. I believe the third point is the major new point, finally shown in a proper convincing way. Repeating the measurement for another condition or cargo that might go a different path or be very different in size would have helped to increase the impact, but I understand that the data is hard to acquire. However, the text more emphasizes how easy and well suitable the method is, and if that is indeed the case, showing another meaningful biological condition would help the story to gain more biological significance.
This work aims to track cargo passing through individual, well-resolved NPCs in a single-molecule level using super-resolution. This could provide valuable in-situ experimental evidence for or against the various available models regarding the NPC inner channel and cargo transport.
The approach of the authors is straightforward to explain, but these experiments are very hard to perform: first, they validate the NPC localization precision and positional stability throughout their planned measurements. Then, in their experiments, they localize the cargo first, then the NPC which the cargo went through. Finally, they analyze the localizations of the cargo and the NPC to observe their interaction. Throughout this procedure, the various sources of instrumentation and biological error are measured or simulated and reported. In the end, the authors are able to reach some conclusions on the cargo interaction with the NPC and its transport through it. The initial measurements of the NPC give results which agree with previous reports. The cargo localization data set is rather small and not validated by previous or further current results. However the data hint at interesting results which could be explored further using the outlined method.
In summary the paper is rich in technological rigor, and an impressive and useful combination of SRM and tracking applied to a highly challenging biological problem that was certainly very hard work to achieve. Experimental data wise, it is less rich and at many places thus has to remain rather descriptive, and in that sense it might be a little less than what you expect from a Nature Cell Biology paper in that respect.

We thank the Reviewer for his/her careful and balanced assessment of our work, and for the recognition of the many challenges faced and surmounted. We agree that expanding the manuscript to include a second cargo would increase the impact of the work, and have accordingly added data for a substantially smaller cargo (NLS-2xBFP) that utilizes the Imp α/Imp β import pathway. Also, considering that the weight of the manuscript reports a technically challenging method and on the advice of the Editor, we have converted the format of the manuscript to a "Technical Report". The revised manuscript demonstrates that the well-resolved NPC scaffold is reproducible, and that the two cargos follow similar import paths. The additional dataset and new manuscript format enable a more extensive technical discussion and description of the cell biological impact of our findings.

Major Comments

2. The benefit of non-fixation is overemphasized, as they work with permeabilized cells, and many cell biologists would also see the digitation treatment already critical. The authors also just mention cytoskeletal elements which hold the NPC stable. Again, with respect to the cytoskeleton one should take into account that the digitonin treatment is quite invasive. Please do not get me wrong, the permeabilized treatment is very suitable to study the functional transport machinery, and none of their conclusions are wrong. Just overall the impression emerges, that this is a life cell study, and that is not appropriate. I recommend toning down these statements.

We understand the Reviewer's concerns. It was never our intention to give the impression that this was a live cell study, as it clearly is not. However, we contend that it remains nonetheless important to emphasize that our samples were not fixed cells, which are used in most super-resolution studies of the NPC scaffold and which are substantially different from permeabilized cells. A more expanded description of the model system is now provided (lines 19-21, 67-71; see also our response to point 7 of Reviewer #1).

3. Abstract can be re-phrased to better fit the main body. SPEED microscopy is not mentioned in the main text. While it is refreshing to see a technically nearly flawless work compared to much of the SPEED literature, I suggest the authors move the SPEED aspect to the discussion. Also, the rigid cytoskeletal framework which is suggested to mediate NPC stability in unfixed permeabilized cells is mentioned in the abstract, but only passingly in the text, only indicating that there must be such a framework. No evidence or further comment is given on this.

We agree that the results from SPEED microscopy should be better integrated into the text, and have done so in our revision (lines 33-39, 48-50, 232-234, 254-256). We have also elaborated on the stability of the permeabilized cell system (lines 244-252; see also our response to point 7 of Reviewer #1).

4. From the text, the conditions under which the cells were measured first (data for Figure 1) are not clear. Were the transport proteins and GTP present? Was cargo present? If no, then do the findings of the data for Figure 1 (positional stability and localization errors) still hold? How were cells identified, carton in Figure 1 with imaging desiply (sic).

We apologize for not being clearer – the data in Figures 1 and 2 of the earlier version of the manuscript were collected at the same time with all reagents present for both. The only things

that changed between the different color datasets were the illumination laser and a spherical aberration correction with the adaptive optics to ensure that the two colors had similar focal planes. A new Figure 1 was added to clarify the experimental design and the imaging protocol is now more clearly described (lines 61-71, 119-139, 151-153).

5. Precision should come from tracking labelled cargo in fixed cells. Using bead measurement only gives technical precision, which is not a useful parameter for the precision of the actual measurement.

We were confused by this comment. The Editor provided the following clarification from the Reviewer:

The authors use bead tracking to determine the resolution. This is technical resolution that basically just captures the precision of the instrument. Beads are typically much brighter than the actual fluorescent signal they track, so technical resolution is usually an overestimate of the precision relevant to their biological tracking experiment. More representative is to track a static object that is labelled the way they label their cargo. This can be achieved by fixing the cell after applying the cargo. This will arrest movement of the cargo, and whatever mobility is left in the sample is thus due to imprecision, and this value should be given to the reader.

For precision measurements, the illumination intensities were adjusted and varied so that the fluorescence recovered from beads (1000-4000 photons/spot) was in the range recovered from the NPC and particle tracking measurements. Precision estimates were obtained from repeated position measurements (100 frames; see Supplementary Fig. 2c). It is simply not possible to obtain sufficient measurements with the requisite photons for our dye labeled proteins, whereas one can readily obtain hundreds of such measurements from beads with minimal photobleaching. We specifically measured the precision at different photon levels to determine how the *x*, *y*, and *z* precisions for the 3D measurements varied with the number of photons recovered. These precisions scaled well according to $1/(\text{photons})^{1/2}$ for all three coordinates (Supplementary Fig. 2c). Consequently, we used this correction within our simulation model, which was used to estimate the average precision in the experiments, based on experimental parameters. A more detailed description of the 3D microscope and the precision analysis will follow in a subsequent paper (in preparation).

6. The results are mainly discussed in relevance to a kap centric model. Taking the complexity of the NPC barrier into account, I do not think that the model itself (that NTRs and an integral part of the barrier) make to strong statement about actual transport routes. So I found the discussion here a bit too focused, and biased. I do not think the data excludes a kap centric model. The data is about transport routes, and does not exclude or include any of the most commonly discussed models (which are more fundamental in nature). The data on transport routes is significant though.

We agree with the Reviewer that the focus on the Kap centric model was inappropriate and biased. We have instead re-focused the discussion on the impact of the findings regarding transport routes (lines 259-286), which is significantly strengthened by the addition of the new data on the Imp α/Imp β import pathway. Included in this discussion are predictions inherent in models describing the characteristic of the permeability barrier (lines 270-274).

7. Evidence for interaction with NUPs extending to 150 nm is indirect, solely based on tracking data. The conclusion thus needs to be toned down, as this is very descriptive, and there is no direct evidence of interaction with Nups. This could also be a result of the boxing, or tracking routine, or other interactions at the nuclear envelope. One can simply not say when looking only at cargo trajectories. Speculation and result need to be more clearly expressed.

We thank the Reviewer for raising these important issues. Similar concerns regarding interactions with the FG-polypeptides far from the pore were addressed earlier in our response to Reviewer #1 (point 6). Regarding the boxing/tracking routine, we note that i) assembled import complexes that do not transport are found within a large region over ~100 nm from the entrance to the pore (Figs. 4f and 5f), and ii) no multi-frame localizations were obtained for Imp α/Imp β complexes in the absence of NLS-2xBFP (Table 1). These findings indicate that binding (multi-frame localization) in this region requires fully assembled transport complexes, arguing against the boxing or tracking routine as explanations for the clustered observations. This issues are now more clearly described (lines 158-167, 287-296)

8. I would suggest to show more than 1 movie for the cargo movement within the channel, so that one can see how reproducible this is. The movie is impressive.

We are glad that the Reviewer liked the movie. A total of 9 movies are included with the resubmitted manuscript to more fully illustrate the method and the reproducibility of the measurements. Three pairs of movies (illustrating movement in different planes) show the super-resolved tracks of cargo complexes.

9. Authors make comments on export (ex.: "Notably, the analysis in Fig. 2l suggests that both import and export occur through the same (or similar) channels since trajectories from both processes were included."). However, the export does not happen under well-controlled conditions, as they also outline in the text. Therefore, it seems rather hard to reach a meaningful conclusions on export.

We agree with the Reviewer that the export conditions were not well-controlled. We have refocused the discussion on import. The data in Fig. 6 illustrating the two transport pathways examined only includes data from import and abortive import trajectories.

10. It is not clear from the text what were the conditions of the cells for the initial measurements of the positional stability and localization precision of the NPC (Figure 1). In the methods section, only the tracking experiments are described. In the main text, the transport mix is only mentioned after the results of initial NPC localizations are discussed. During transport tracking experiments, the NPCs are measured and localized in the presence of transport proteins, energy and large cargo. The possible differences between the conditions may affect the NPC localization precision or positional stability.

This comment is related to point 4 of this Reviewer. We apologize that the sequential process to acquire the measurements were not clear. For the reported results, all NPC scaffold and cargo tracking measurements were made sequentially on the same samples without any reagent changes. This is critical for the stability of the sample. These issues are now clarified (Figure 1; and lines 61-71, 119-139, 151-153).

Minor Comments

11. A cartoon of how cells are measured and the NPC orientation with respect to the measurement plane should go into figure 1.

This is an excellent suggestion. As indicated in our response to point 4 for this Reviewer, a new Figure 1 was added to clarify the experimental design and the imaging protocol.

12. No data on various controls is shown such as binding of nanobody-HMSiR to control cells, leakage of fluorescent signal to other channels, etc. E.g. the authors claim that nanobody-HMSiR does not bind to U-2 OS control cells but do not show the data.

We thank the Reviewer for pointing out the absence of these important controls. Since the HMSiR blinking dye is fluorescent only ~1% of the time, the specificity of nanobody-HMSiR is difficult to visualize in a single image. Instead, the nanobody's specificity was verified by addition of nanobody-Alexa568 to wild type and NUP96-mEGFP U-2 OS cells (Supplementary Fig. S1a). Movie 1 illustrates the blinking of the HMSiR dye from a nanobody-decorated nuclear envelope.

The Atto542 and HMSiR dyes have well-separated excitation spectra. Atto542 has essentially no excitation by the 647 nm laser. While the HMSiR dye has minimal excitation by the 532 nm laser, this blinking dye is in the off-state ~99% of the time, effectively eliminating any background from this dye. However, substantial background from GFP fluorescence arises from 532 nm excitation. Thus, the GFP fluorescence of the NUP96-mEGFP labeled NPCs was photobleached before imaging the Atto542-labeled cargo complexes (Supplementary Fig. 1b).

13. For Figure 1, the text does not explain the different parts of the figure in alphabetic order.

This has been fixed in the revised manuscript (the original Figure 1 is now Figure 2).

14. The authors can give a more specific description on the scope, validity and limitations of their proposed method.

Based on the Reviewers' critiques and specific concerns, and the reconfiguring into a Technical Report format, which allows for a more expanded description and discussion, we hope that the revised manuscript addresses these concerns.

REVIEWER 3:

Major Comments

1. The authors tackle a difficult problem that has proven quite controversial in recent years, namely, measuring the path and kinetics taken by individual nuclear transport cargoes as they cross the NPC. One particular issue has been the use of certain types of rotational symmetrization in some of the microscopic methods used, with lively debate ensuing (e.g. "Deconstructing transport-distribution reconstruction in the nuclear-pore complex" comment and reply, PMID 30518848). Here, the authors

avoid such issues by directly resolving the eight-fold ring structure of each NPC directly and – taking advantage of their demonstration of NE stability over the time course of the experiment - using that as the reference for transport events going through these NPCs in a permeabilized cell nuclear transport assay.

The methodology described appears extremely elegant, is thoroughly and carefully controlled and documented, and represents a significant technical achievement in the field – potentially opening the way to far superior high-resolution time-resolved studies of nuclear transport pathways in situ.

One notable finding is the considerable extent to which the FG repeats seem to emanate from the NPC's scaffold; this is consistent with numerous recent data on the extent of these domains – contrasting more condensed states of the FG repeats ('gels') seen in some studies with more extended and fluid states (such as observed 'polymer brushes' and 'liquid phases') - as well as several models of the nucleocytoplasmic transport mechanism, only some of which are only touched on in this manuscript. The hourglass-shaped translocation conduit reported here is also a significant finding, and one that seems to fit well with both 'classic' (Akey and Rademacher, 1993) and recent (e.g. Kim et al., 2018) ultrastructural observations supporting an hourglass-shaped central 'transporter' density in the NPC, postulated to consist of transport factors and cargo in transit across FG repeat domains. Perhaps a more robust discussion of these issues is warranted, i.e., how do the data in this work advance our understanding of the selective transport mechanism at the NPC in the light of the various major models proposed and more recent published data?

We thank the Reviewer for highlighting the key points of this manuscript and the laudatory comments. The hourglass-shaped translocation conduit is indeed a significant finding, and it is reproduceable, as a similar translocation conduit was identified for the newly added NLS-2xBFP cargo complex. We have included a more expanded discussion of the impact of our findings in the context of previous work and proposed models (lines 231-241, 259-274).

2. A potential drawback of this work is the use of only one nuclear transport reporter, even though as the authors point out, a tremendous potential of this method – untapped in this manuscript – is to untangle the possible alternative pathways that different pathways may utilize. Would it be possible and within the scope of this study to test this by using a different NLS and import factor, such as an IBB sequence and importin / karyopherin beta-1? Or a ribosomal NLS and importin / karyopherin beta-4?

This was also a question/concern of Reviewer #2 (point 1), and, as we indicated in our earlier response, we have now added data for a second cargo (NLS-2xBFP) utilizing the Imp α/Imp β import pathway (Figs. 5 & 6).

<u>*Minor Comments*</u>

3. Do the authors assume circularity of the NPC for their generation of the octagonal reference rings? How would deviations from circularity (as have been observed) in the NPC affect the generation of these references?

The 3D position and orientation of individual NPCs obtained from our HMSiR localizations does indeed rely on an assumption of circularity – clusters of localizations were fit to a double-circle model (Figure 2f,g; and lines 800-809). The well-resolved octagonal reference

rings suggests that any non-circularity is moderate or present in only a low percentage of NPCs. Non-circularity would reduce the precision of the NPC centroid estimates and increase the width of the radial distribution of the localizations. These are inherent errors that are not expected to be significant relative to the current localization error. The very low tilt and jiggle values that produced simulated distributions comparable to the experimental results (Fig. 3) support this assumption.

4. Is it possible to determine if there are any radial preferences for the import / export pathways (i.e., relative to the radial position of the GFP/dye)?

The radial preference is already established by the identified translocation paths at the periphery of the pore (Fig. 6). However, we presume that the Reviewer is interested in whether there is any *angular* preference, i.e., relative to the octagonal scaffold. Such an angular preference would be expected if there are distinct peripheral channels rather than a single annular channel. This is a key question motivating our ongoing work. We are currently limited by the low number of localizations within the pore, and potentially by resolution (temporal and spatial). This issue and limitations of our current data are discussed (lines 275-286).

5. One possibility has been that the preference for RNA export through the center of the channel is due to a greater crowding effect pushing these large cargoes towards the center, leaving the periphery for the smaller protein cargoes by default. However, presumably there is no such RNA export here, so that would tend to indicate instead that the peripheral localization of the transport paths seen here is a true preference?

Yes, the data do indeed suggest that the peripheral localization is a true preference. This preference is now more thoroughly discussed (lines 222-241, 259-274).

6. In yeast, a stronger concentration of GLFG type FG repeats is predicted for the channel periphery, while FXFG repeats are predicted to be found more towards the central axis (Kim et al., 2018). If this is conserved in mammalian cells, would this make any sense with known importin preferences vs RNA export factors such as NXF/NXT?

While the local concentration of different types of FG repeats could indeed help determine the preferential translocation path, there are other potential biasing mechanisms (lines 262-269). A central mRNA export conduit and peripheral protein import channels would seemingly increase the efficiency of a bi-directional transporter, and is consistent with the current data.

7. Recently, an analog of nuclear transport was reconstituted in vitro showing that different types of FG repeat were not needed to be differentially localized in a pore to support transport events (Fragasso et al., 2021 PMID 33790297). Do the authors believe that these events would also be seen to be peripheral to their pores, or that differential FG repeat support the strong path preferences through the central channel shown here?

If the model nanopores of Fragasso et al. are indeed accurate mimics (key assumption) of the NPC, we would argue that translocation near the periphery of their model pores must occur,

as we have seen here for authentic NPCs. However, there are notable differences in the simplified model system used in the Fragasso et al. paper: cargo complexes vs. empty transport receptors; an entire complement of FG-Nups of variable length vs. a single consensus, short, model Nup; and the potential for physiological factors not washed away in the authentic system. As we have indicated (lines 262-269), there are other factors that can bias translocation path that may not be appropriately reproduced within the Fragasso model system. Note, however, that molecular dynamics simulations predict that the Kap95 density in the Fragasso et al pores (Fig. 5a of their paper) follow an annular distribution. This may indeed predict a peripheral translocation path.

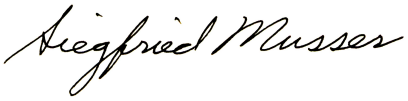
8. "distinct locals" should likely read "distinct locales"

Fixed.

Overall, the manuscript has been improved significantly by directly addressing the questions and comments of the Reviewers. We are grateful for the thoughtfulness and time expended by the Reviewers in critiquing this manuscript. Lastly, we thank you for your efforts in handling this manuscript. We hope that the Reviewers and you find the current version of this manuscript suitable for publication in *Nature Cell Biology*.

Please contact me if you have any questions, or need additional information.

Sincerely,



Siegfried M. Musser

Decision Letter, first revision:

Our ref: NCB-M45733A

30th September 2021

Dear Dr. Musser,

Thank you for submitting your revised manuscript "Super-resolved 3D Tracking of Cargo Transport Through Nuclear Pore Complexes" (NCB-M45733A). It has now been seen by two of the original referees and their comments are below. As Reviewer #1 was not available to re-review, we asked Reviewer #2 to please comment on your responses to Rev#1. Overall I am pleased to share that the reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines, as highlighted below:

1) In particular, as highlighted by Rev#2 in comments below, we would ask that you please explicitly discuss the traffic jam concept in the manuscript text, even if that is not a favored hypothesis, in the interest of a balanced discussion and because we feel that readers in and outside of the field would benefit from hearing more of your thoughts on this point.

2) Please note that, prior to publication, we will ask that you please upload the step-by-step protocols used in this manuscript to the Protocol Exchange. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol sharing platform of their choice; this is encouraged for all articles but mandatory for a Technical Report. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can found at https://protocolexchange.researchsquare.com/protocol-exchange/about.
When uploading your protocol to the Protocol Exchange, please include a sentence in the protocol abstract stating that it is related to your Nature Cell Biology paper. Once uploading is complete, please email protocol.exchange@nature.com to alert them that your protocol is associated with your Nature Cell Biology paper, and to send them the license to publish form (http://www.nature.com/licenceforms/npg/mpl-ltp-cc-by-nocharge.pdf) that is required for publication on the Protocol Exchange. Please also ensure that the protocol is made public to coincide with publication of your Nature Cell Biology paper. The protocol should be cited in the Methods and included in the reference list of your Nature Cell Biology paper, as in the following NCB publication: http://www.nature.com/ncb/journal/v17/n4/full/ncb3138.html. We will provide the DOI number.

3) As you will see in their comments below, Reviewer #2 asked that I put you in touch with them via email, and you will thus receive a follow-up email from me to you and Rev#2 both. Please note that, for publication, editorially we will not require the analyses of fixed samples to determine precision that Reviewer #2 favors. We will leave it to you to decide the best way to resolve this.

4) **The current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex) as we can not proceed with PDFs at this stage.** Please do so as soon as possible so that we can proceed with the next phase of checks:

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Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewer #2 (Remarks to the Author):

This is a very nice revision. I highly recommend publication.

I do however still disagree on using fixed beads in agarose to define a preciison estimate. This really just recovers an ideal precision, since in the cell there are other factors lowering precision.

Since many people do this like them, it does not seem fair to obstruct publication as is. I would however be happy if the editor brings us in direct contact to discuss this prior to publication.

In a literature highly polluted from overstimated precision estimates as e.g. done for SPEED microscpy, a

more conservative estimate would be beneficial.

In reviewer 1, comment 6, the authors state the following:
While the traffic jam concept is a reasonable explanation for trajectories that cross through the pore to the other side, over 40% of 3+ point trajectories never penetrate the pore, suggesting a cluster of binding sites rather than a reflection of necessary waystations on the way to/from the pore. This uncertainty is now more completely discussed (lines 158-167, 287-296).

In the text, they do not mention the traffic jam concept in the provided lines, but explain in more detail why they think that the most likely explanation might be FG repeat binding sites, but also non-NPC binding sites such as cytoskeletal structures. They could also add the traffic jam explanation, with relevant citations but explain why they think it might be unlikely.

Reviewer #3 (Remarks to the Author):

The authors have fully addressed all my previous queries. Importantly, the additional data using an alternative transport factor and cargo reporter, shown in Figs. 5 and 6 and appropriately discussed, adds considerable weight to the manuscript and its conclusions. I note in Fig. 6 that the Imp B density seems a little skewed towards the nuclear side – could this be a consequence of the higher affinity FG docking sites in the vicinity of the nuclear basket (e.g. Nup50, Nup153)? Apologies, I did indeed mean angular preferences, and this is also now completely addressed. In summary, I think this is an important advance in our understanding of nuclear transport, and recommend publication.

Author Rebuttal, first revision:

REVIEWER 1 (as commented by Reviewer 2):

In reviewer 1, comment 6, the authors state the following:

While the traffic jam concept is a reasonable explanation for trajectories that cross through the pore to the other side, over 40% of 3+ point trajectories never penetrate the pore, suggesting a cluster of binding sites rather than a reflection of necessary waystations on the way to/from the pore. This uncertainty is now more completely discussed (lines 158-167, 287-296).

In the text, they do not mention the traffic jam concept in the provided lines, but explain in more detail why they think that the most likely explanation might be FG repeat binding sites, but also non-NPC binding sites such as cytoskeletal structures. They could also add the traffic jam explanation, with relevant citations but explain why they think it might be unlikely.

The phrase 'traffic jam' was raised by Reviewer 1 at the original review – it was not mentioned in the first or revised version of the manuscript. This is not a citable hypothesis or ongoing discussion in the literature. The question at hand is the explanation for the numerous localizations near, but at some distance from, the pore openings on both sides of the nuclear envelope. In the first version of the manuscript, we argued that "large clouds near both the cytoplasmic and nucleoplasmic openings…are also consistent with molecules converging on a pore through which they transit". This statement (largely retained in the revised manuscript) likely prompted the original traffic jam comment from the Reviewer. In reassessing, however, we feel that this picture is not a realistic possibility at the molecular level, as molecular

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diffusion would prevent any accumulation at the NPC openings in the absence of binding. We therefore eliminated this statement from the text (paragraph corresponding to p. 8, lines 160-168). As such, we feel that further discussion of a 'traffic jam' is no longer warranted.

REVIEWER 2:

This is a very nice revision. I highly recommend publication.

I do however still disagree on using fixed beads in agarose to define a preciison estimate. This really just recovers an ideal precision, since in the cell there are other factors lowering precision.

Since many people do this like them, it does not seem fair to obstruct publication as is. I would however be happy if the editor brings us in direct contact to discuss this prior to publication.

In a literature highly polluted from overstimated precision estimates as e.g. done for SPEED microscpy, a more conservative estimate would be beneficial.

We are glad that the Reviewer liked the revised manuscript and thank the Editor for connecting us to the Reviewer directly. Having discussed this issue with the Reviewer, we have cleared up the confusion as to what we were being asked to do. In the manuscript, we defined the precision as the standard deviation of 100 position measurements of stationary beads. The Reviewer asked us to obtain precision measurements on stationary dye molecules by fixing permeabilized cells. Due to photobleaching, one cannot measure the position of stationary dye molecules at high photon levels for 100 frames. Instead, however, one can construct histograms of jump distances between pairs of measurements – the standard deviation of such measurements (fit according to Eq. 3 in the manuscript) would provide the average experimental precision. While we understand the desire to obtain measurements under conditions more closely approximating the experiment, the approach we used is substantially more informative. The jump distance approach is necessarily an average. With beads, we measured the precision at different photon levels and at different *z*-heights to quantitatively estimate different the *x*, *y*, and *z* precisions as these parameters varied (Fig. 1f). These relationships were essential for simulating the localizations arising from the fluorescently tagged NPCs, and, more specifically, for addressing the role of tilt and jiggle in the particle distributions obtained (Fig. 3). This analysis simply would not be possible with the average precision values that would be obtained from jump distance histograms. As to whether the precision estimates are sufficiently 'conservative', we point out that the simulations indicate relatively low jiggle values (< 9 nm in *x* and *y*, and < 6 nm in *z*) and these ultimately force limits on what the precision must be to obtain the distributions observed. If our precisions are substantially worse than our estimates, than the jiggles must necessarily be less, implying an even more stable system. This is not something we would expect. Finally, the suggested dye approach relies on the assumption that all measurements are obtained on fixed molecules. Dyes are typically attached via flexible tethers (of non-negligible length). Since motion can be expected on surface-affixed structures, this would need to be carefully checked. Also, oriented dipoles of restricted dyes can yield significant polarization dependent effects. Consequently, interpretation is not expected to be as straightforward as it might initially seem.

REVIEWER 3:

The authors have fully addressed all my previous queries. Importantly, the additional data using an alternative transport factor and cargo reporter, shown in Figs. 5 and 6 and appropriately discussed, adds considerable weight to the manuscript and its conclusions. I note in Fig. 6 that the Imp B density seems a little skewed towards the nuclear side – could this be a consequence of the higher affinity FG docking sites in the vicinity of the nuclear basket (e.g. Nup50, Nup153)? Apologies, I did indeed mean angular preferences, and this is also now completely addressed. In summary, I think this is an important advance in our understanding of nuclear transport, and recommend publication.

It seems to us that the Imp β density may be a little skewed to the cytoplasmic side. Is the Reviewer perhaps confused by the orientation of Figure 6a,b, cytoplasm on the bottom and nucleoplasm on the top? We recognize that the normal orientation is the opposite, but the orientation used corresponds to the laboratory orientation of the experiment. To avoid confusion, we have marked the nucleoplasm (N) and cytoplasm (C) in Fig. 6b. With the current data, however, the robustness of this skewness is uncertain, and we recommend further investigation to draw more firm conclusions.

Once again, thank you for your efforts in handling this manuscript. We hope that you find the current version of this manuscript suitable for publication in *Nature Cell Biology*.

Please contact me if you have any questions, or need additional information.

Sincerely,

Siegfried Musser

Siegfried M. Musser

Final Decision Letter:

Dear Dr Musser,

I am pleased to inform you that your manuscript, "Super-resolved 3D Tracking of Cargo Transport Through Nuclear Pore Complexes", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With kind regards,

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