molecular systems biology

Cell type-specific nuclear pores: A case in point for context-dependent stoichiometry of molecular machines

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Review timeline:

Submission date:	13 December 2012
Editorial Decision:	13 January 2013
Revision received:	02 February 2013
Accepted:	17 February 2013

Editor: Thomas Lemberger

Transaction Report:

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

1st Editorial Decision 13 January 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who accepted to evaluate the study. As you will see, the referees find the topic of your study of potential interest and are supportive. They raise however a series of concerns and make suggestions for modifications, which we would ask you to carefully address in a revision of the present work.

One important issue is to examine, if possible, the distribution of NPC compositions within a cell (ref #2) using the available images. Raw data should also be provided for the super-resolution microscopy results. This could be submitted as 'source data' (http://www.nature.com/msb/authors/index.html#a3.4.3) associated Figure 1B.

The reviewers were confused by the section on stoichiometric variation of complexes and were unclear about the methodology used and the validity of the conclusions.

Thank you for submitting this paper to Molecular Systems Biology.

Referee reports:

Reviewer #1 (Remarks to the Author):

In the current manuscript by Ori et al. a variety of cell biology, proteomics and microscopy technologies are applied (and integrated) to present a fresh look at the composition of the nuclear pore complex (NPC). The NPC is a very large multi-protein complex that is essential to biology.

Here, the authors use carefully controlled cell biology procedures to isolate highly purified nuclear envelopes/NPCs. These isolation are then quantified using state of the art proteomics (both targeted and shotgun). super-resolution microscopy is used in parallel to interrogate purified nuclei and to obtain novel insights into context-dependent stochiometry of the NPC over various cancer cell lines.

I have carefully read this manuscript including the large amount of supplementary data, figures, tables and methods. Overall this is a very interesting manuscript and to the best of my knowledge all described technologies are state-of-the-art. Although at the moment it is still quite descriptive, the data presented here could potentially provide the bases for novel mechanistic/structural investigation of the NPC in the context of general biology and tissue-spcificity and disease.

The technological aspect of this paper is very strong. This reviewer could not really find any major flaws in that context.

Some points should be address to further clarify:

1) Figure 1 is an essential part of this manuscript and in combination with Supplemental Figure 1 makes a strong case for the quality of the isolated nuclei/NPCs and the MRM data.

On the other hand the some details on the MRM assays are missing.
How much NPC digest was loaded on the nano-columns for each MRM analyses? how much synthetic peptides were spiked? how many assays were multiplexed for each analyses? how were the assays optimized and what are the assay performance criteria? I couldn't find any of this information.
the large supplemental table2 (second work sheet) needs a legend. I have looked at enough MRM data an still was not completely clear what all the columns are?
also please provide a table with the absolute abundance, for each peptide since they were measured.

2) Although in general I found most of the data well described and presented it should be mentioned in the main manuscript that results from Figure 4 are based on public microarray data (this was not clear until one really digs into the supplemental methods).

3) The one part of the paper that this reviewer did not particularly like was this data in Figure 5. If I understand correct the authors performed shotgun proteomics of nuclear lysates and performed relative quantification based on MaxQuant. somehow this data was then correlated back to large nuclear complexes to somehow infer stable or dynamic complexes? I read this section several times and am still not completely clear what was actually done? the authors didn't really isolate complexes so how can they actually make an inference on the dynamics of nuclear complexes?

Overall a strong paper that has the potential to provide a fresh look at the dynamic composition of the NPC.

Reviewer #2 (Remarks to the Author):

The work presented by Ori et al. is of high interest to the nuclear pore community. It combines proteomic and imaging methods in an innovative way to resolve details about the copy number of individual nucleoporins in the nuclear pore complex (NPC) in different cell types, resulting in the unique finding that variations in NPC composition between different cell types might exist that have been previously unexpected. While this conclusion is exciting and well documented the other major conclusion of this work is that the current estimates of copy numbers of nucleoporins, shown on the example of Nup107, is to low, in other words that instead of 16 copies 32 copies are used to assemble the NPC.

I recommend to ask the authors for improving the manuscript prior to publishing.

This reviewer is not an expert on proteomics but rather on imaging. Hence a detailed review of the proteomics data is not given. Both, the proteomics work and the super-resolution microscopy data is well described. The proteomics work is done using multiple controls and verification steps. Viewed from a distance the proteomics data seem convincing, but if they have the "resolution" to draw conclusions on the exact copy number (16 or 32 per NPC) seems questionable, based on the large

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number of estimates (~3400 +/- 1300 NPCs per nucleus, the counting error of nuclei in the preparation and estimated 125,000 +/- 6000 Nup107 per nucleus). Also, having demonstrated variability between cell types, one could imagine a situation were two classes of NPC exist, one with 16 and one with 32 copies, which would bias any ensemble measurement.

Presenting the super-resolution measurements could address this concern of "resolution". The authors use a imaging method that is based on mEos2, a fluorescent protein. Compared to the proteomic work less controls were done and while a good documentation of the image processing is provided I would feel more comfortable with the data if some raw data and verification of the achieved localization precision and resolution would be presented. In the current version the two figures dedicated to the imaging do show little, if any, raw data, and are hard to interpret to understand the clustering and estimates being done. The estimates being made fall into two categories 1) resulting in underestimating copy numbers, 2) assigning and reconstructing the image. While the first category is well discussed and presented the second is laking detail. As mentioned above, the localization precision is simply stated and the resulting resolution is not further described or calculated.
Ultimately it seems the preparation of the nuclei should also support the use of a method like d-Storm, which by means of titration could address the issue of labeling density more quantitatively than a microRNA approach. L^schberger et al. 2012 (PMID: 22389396) showed impressive results of d-Storm imaging on NPCs.
Finally, using imaging of individual NPC composition the authors should be able to analyze the distribution of NPC compositions within a given nuclei to great detail, providing insights on NPC variability within the individual cell.

Suggested improvements:

1) The provided tables could benefit form a short description and explanation at each header that allows the reader to easily relate the information to the main and or supplemental material and Method used. It would also help to define the labels used in the tables upfront (many tables extend over several pages).
2) Provide detailed analysis of localization precision and resolution achieved.
3) Show raw data as movies and still-images in the supplement.
4) Improve the presentation of verification of the clustering algorithm used.
5) Detail the number of nuclei and NPCs per nuclei being imaged.
6) Analyze NPC composition variation within individual nuclei.

Reviewer #3 (Remarks to the Author):

This is very challenging study with high technical quality attempting to determine absolute stoichiometry of the nuclear pore complex. The study utilizes integrated approaches of targeted proteomics and super resolution microscopy, both of which are rapidly developing exciting technologies.

To determine the absolute abundance of NPC components, the authors use nuclear envelope isolated from HeLa cells through subcellular fractionation. In general view, this is rather an audacious approach because one can expect that Nups fall off from NPC structure during the fractionation. On the other hand, use of isolated nuclear envelope is the only reasonable approach for quantifying each Nups within the assembled NPCs directly, because accumulating evidences from recent reports show that many Nups have functions besides nuclear transport and localizes not only at NPC but also within the nucleus or other cellular compartment. The result presented in FigS1A show most Nups are stably attached to the isolated nuclear envelope, verifying the integrity of NPC structure, and this structure was quantified in the manuscript. Approach taken in this manuscript is very straight ford, which I think is adequate and acceptable.

Stoichiometries of Nups presented in Figure1 is very informative, providing new insights to our knowledge on NPC structure that was never seen with any of the previously reported studies 7 components of Nup107 complex are present in assembled NPC structure with the same stoichiometry while 3 components (Elys, Sec13 and Seh1) show lower abundance, indicating these components are not associated tightly with other Nup107 complex components at interphase NPC, which agree with some of previous reports. The result is interesting also from different aspects: for

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example, interaction between Nup107 and Elys is crucial for post mitotic NPC assembly, but some population of Elys could be dissociating from Nup107 complex after completion of nuclear envelope formation (which agree that there exist significant intranuclear pool of Elys in interphase). The stoichiometry of Nup93 subcomplex components indeed implicates the presence of two different components, as authors pointed out. Pom121 was shown to be essential for interphase NPC formation, however, its abundance is lower than scaffold Nups (Nup107 complex or Nup93 complex), which was shown to interact with Pom121. This implies that Pom121 is not essential for anchoring scaffold Nups to nuclear membrane, but may be playing different roles in NPC structure/function. Nup98 and Nup62 was reported/considered as abundant Nup in NPC structure. Data in Figure 1 support this argument.

One of the most important finding/argument of this manuscript is a copy number of Nup107 (copy numbers of other Nups are calculated as ration of Nup107). Nup107 is one of the most important scaffold Nups. Therefore, how Y-structure complex (Nup107 complex) is organized within the NPC is current critical argument for understanding NPC architecture. A copy number of Nup107 affects argument of Y-complex organization within NPC, and therefore ultimately help our understanding of NPC architecture. The authors carefully examined copy number of Nup107 complex by integrating different imaging approaches with quantification: super-resolution microscopy for examining signals of ectopically expressing mEos2-Nip107 in endogenous Nup107 knockdown cell lines. The authors also use TIRF microscopy to quantify copy number of Nup107 per NPC with isolated nuclei. All the obtained results show copy number of Nup107 per NPC is above 16, allowing the authors to conclude that Nup107 exist 32 copies instead of 16 copies, which has been previously considered. All these experiments are employing new technologies, and have been performed as carefully as possible. New result was obtained through employing new approaches. The result would trigger arguments in the field. In my opinion, whether the provided number is definitely correct or not is not a critical issue at this moment as far as a way of analysis is correct, which I appreciate with the present manuscript. Important issue is that the data presented in this paper would trigger arguments in the field on this important topic, providing opportunity for people in the field to re-consider various evidences that exist at this moment.

In the final part of the manuscript, the authors examine the cell-type specific compositional variation of Nups by examining 5 different cell lines in combination with proteomics including shotgun and gene expression data. The results show about 38% of the complexes that was examined, including NPC, have stoichiometric variation in within only 5 cell lines examined, which are all cancer human cell lines.

I have one concern in the last part of the results of this manuscript, regarding "stoichiometric variation of complexes examined in 5 cell lines". What are the purposes of selecting these 5 cell lines? Are the authors arguing that 38% of complexes (even with under-estimated) with varied stoichiometry would vary among any of selected cell lines? What about the rest of complexes that showed similar stoichiometry? Such complexes might show similar stoichiometry among human cancer cell lines, but they might vary when compared with normal cell lines (i.e. TIG or normal fibroblast). These issues (purpose of comparing complexes within 5 human cancer cell) should be mentioned and discussed, and the arguments should be clarified.

Other minor comments:
1) Although authors can put the information regarding the integrity of nuclear envelope shown in FigS1C, I don't think this is important information: upon fractionation process, lipid membranes of either outer nuclear membrane and/or inner nuclear membrane can be partly disrupted, which would loose the "integrity" of nuclear envelope, while keeping NPC structure intact. Therefore, it does not matter, particularly in this report, whether the isolated nuclear envelope excludes dextran or not. I would not point this strongly as one of evidence arguing integrity of NPC structure (p3, result section). It can be indicated as supporting evicence if authors want to.

1st Revision - authors' response 02 February 2013

Response to the reviewer's comments:

We want to thank all the reviewers for their effort and very constructive criticism, which was very helpful to improve our manuscript.

The comments of the editor and reviewers one and three made us realize that the last part of our manuscript about stoichiometric variations of several nuclear protein complexes was most probably not very well explained. The motivation for this analysis was to demonstrate that the NPC is not a unique case but that many protein complexes undergo cell-type specific stoichiometric variations. We believe that this is a very important point since protein complex composition was not yet systematically studied across human cell types on a global scale. In the revised version of our manuscript we made a strong effort to describe the motivation, scope, underlying caveats and thus justified conclusions more clearly.

As suggested by reviewer two we describe the super-resolution microscopy experiments and the respective data analysis workflow in much more detail. We also included computer simulations that address the question if single cell stochiometric variations of the scaffold structure might underlie our data. In this context, we want to point out that already in the previous version of the manuscript we had clearly stated that our mass spectrometric data comprise averages over multiple cells and in that sense we do not disagree with the reviewer.

We have made two additional improvements to the manuscript that were suggested to us by colleagues.

1. Sec13 was one of the four nucleoporins that were marked with a star as lower confidence identification in Figure 1, indicating that it was absolutely quantified only with a single AQUA peptide. Going back to the original data we realized that a second peptide for Sec13 had been detected, which we had spiked into the samples but previously overlooked and thought it was below the detection limit. Although both Sec13 peptides deviate considerable more from each other as this is the case for most of the other Nups, we felt that this data must be included for consistency and adjusted the respective figures and tables accordingly. Consequently, the star has been removed from Sec13.

2. Since Gp210 is one of the very few nucleoporins for which a relatively short mean residence time at the NPC has been previously reported (Rabut et al. Nat Cell Biol 2004), we performed immune fluorescence staining of our nuclear envelopes to check if Gp210 remains associated with nuclear pores throughout the preparations, which it was: These data were included in Figure S1 and show the typical punctuate NPC pattern, further confirming the integrity of our preparations. This is not necessarily surprising, because Gp210 is one of the three nucleoporins that contain a transmembrane domain. This property will render a dissociation of Gp210 from nuclear envelopes as virtually impossible.

Our detailed point by point response follows below.

Reviewer #1 (Remarks to the Author):

"In the current manuscript by Ori et al. a variety of cell biology, proteomics and microscopy technologies are applied (and integrated) to present a fresh look at the composition of the nuclear pore complex (NPC). The NPC is a very large multi-protein complex that is essential to biology.

Here, the authors use carefully controlled cell biology procedures to isolate highly purified nuclear envelopes/NPCs. These isolation are then quantified using state of the art proteomics (both targeted and shotgun). Super-resolution microscopy is used in parallel to interrogate purified nuclei and to obtain novel insights into context-dependent stochiometry of the NPC over various cancer cell lines.

I have carefully read this manuscript including the large amount of supplementary data, figures, tables and methods. Overall this is a very interesting manuscript and to the best of my knowledge all described technologies are state-of-the-art. Although at the moment it is still quite descriptive, the data presented here could potentially provide the bases for novel mechanistic/structural investigation of the NPC in the context of general biology and tissue-specificity and disease.

The technological aspect of this paper is very strong. This reviewer could not really find any major flaws in that context.

Some points should be address to further clarify:

1) Figure 1 is an essential part of this manuscript and in combination with Supplemental Figure 1 makes a strong case for the quality of the isolated nuclei/NPCs and the MRM data.

On the other hand the some details on the MRM assays are missing.
How much NPC digest was loaded on the nano-columns for each MRM analyses? How much synthetic peptides were spiked? How many assays were multiplexed for each analyses? How were the assays optimized and what are the assay performance criteria? I couldn't find any of this information. "

We are grateful to the reviewer for pointing out the missing information regarding MRM measurements. In the revised version, we provide all the required information, in detail:

- Between 2,000 and 5,000 nuclei or NE equivalents (corresponding approximately to 300-700 ng protein) were loaded on the nano-LC for MRM analyses. These figures are provided in Supplementary Materials and Methods, Sample preparation for mass spectrometry paragraph, Page 9.
- AQUA peptides were spiked at 1 pmol per $1x10^6$ nuclei or NEs extract. This information is now provided in Supplementary Material and Methods, LC-MRM paragraph, Page 12.
- MRM assays were generally split into 3 or 4 scheduled MRM runs, corresponding to an average of 38 assays (380 transitions) per run. This information is now provided in Supplementary Material and Methods, LC-MRM paragraph, Page 11.
- MRM assays were manually validated by the detection of co-eluting light and heavy traces in spiked-in samples (now stated in Supplementary Material and Methods, LC-MRM paragraph,

Page 11). Assays performance was evaluated using the discrimination score of mProphet (d_score) that take into account various parameters including peak shape, co-elution, deviation from predicted retention time, etc. (for details see Reiter et al. Nat Methods 2012).

"The large supplemental table2 (second work sheet) needs a legend. I have looked at enough MRM data a still was not completely clear what all the columns are? Also please provide a table with the absolute abundance, for each peptide since they were measured."

We believe reviewer 1 refers to Table S1, which contains the MRM data. We have revised the sheets containing information about MRM assays and provide a legend for all the columns. Column headers are compliant with the Peptide Atlas format for SRM methods. We also included an additional sheet in Table S1 were we list the quantification values for each peptide in each replicate.

"2) Although in general I found most of the data well described and presented it should be mentioned in the main manuscript that results from Figure 4 are based on public microarray data (this was not clear until one really digs into the supplemental methods)."

We have now explicitly stated the data source of the gene expression analysis in the legend of Figure 4.

"3) The one part of the paper that this reviewer did not particularly like was this data in Figure 5. If I understand correct the authors performed shotgun proteomics of nuclear lysates and performed relative quantification based on MaxQuant. Somehow this data was then correlated back to large nuclear complexes to somehow infer stable or dynamic complexes? I read this section several times and am still not completely clear what was actually done? The authors didn't really isolate complexes so how can they actually make an inference on the dynamics of nuclear complexes"

Since the nuclear pore is certainly an unconventional protein complex, we wanted to ask if cell-type specific variations of protein complexes are a more generic concept or special to the NPC. We believe that this is a very exciting question since only very few studies, e.g. about the proteasome or BAF complexes report cell-type specific protein complex composition (e.g., Ho et al PNAS 2009). To approach this question, we analyzed the same nuclear extracts of 5 different cancer cell lines in shotgun mode (Figure 5) that were generated in order to investigate the compositional dynamics of the nuclear pore in targeted mode (Figure 3). We than focused the analysis on a set of previously well-characterized, stable and non-overlapping protein complexes. MaxQuant was used to estimate their absolute abundance across the 5 cell lines as median of their components. The individual components of the complexes generally display a similar expression profiles across those 5 cell lines (protein complex abundance is highly regulated). To deduce potential stoichiometric changes underlying such abundance variations an additional 'complex-centered' normalization step had to be introduced, which revealed that dynamic subunit expression across cell-types occurs for a certain fraction of the analyzed complexes.

We agree with the reviewer that the ultimate proof would be to isolate those protein complexes from the different cell types, which we have not done. However, as we demonstrate in Figure 5B, such

analysis can recapitulate, at least to some extent, the stoichiometric variations of the NPC for which we have performed all the conceivable controls and used more accurate targeted measurements. A disadvantage of affinity purification is that, at least in some cases, the isolation of specific subspecies might be favored. Our method definitely reveals a dynamic, cell-type specific expression of subunits that are thought to be stably integrated into well-characterized protein complexes. Of course this does not necessarily imply a stoichiometric change: Conceivable alternatives are subunit switches or the integration into another, yet uncharacterized protein complex (as we aim to illustrate in Figure 6). Nevertheless, we believe that our complex-oriented way of looking at large-scale proteomics data provides useful insights into the dynamic behavior of protein assemblies and the description of proteomes variation between different cell types.

In the revised version of the manuscript, we made a strong effort to revise the respective section for more clarity. Specifically we explain:

1. Why we chose to analyze these 5 cell lines.
2. How our analysis is different from conventional methods to look at large scale proteomics data.
3. That a stoichiometric change of the respective protein complexes is a possible, in our view likely conclusion, but has to be proven by isolation these complexes in the future. On Page 11:

"Shotgun proteomics confirmed the cell-type specific variation of NPCs and revealed that more than one third of the well-characterized molecular machines analyzed here show a dynamic expression of at least one subunit across the respective five cell lines in which we studied nuclear pore composition. This finding indicates that these complexes also might similarly undergo compositional rearrangements as a function of the cell type (Figure 5), although this remains to be ultimately proven by their biochemical isolation from the different cell types in the future."

"Overall a strong paper that has the potential to provide a fresh look at the dynamic composition of the NPC."

We appreciate this comment!

Reviewer #2 (Remarks to the Author):

"The work presented by Ori et al. is of high interest to the nuclear pore community. It combines proteomic and imaging methods in an innovative way to resolve details about the copy number of individual nucleoporins in the nuclear pore complex (NPC) in different cell types, resulting in the unique finding that variations in NPC composition between different cell types might exist that have been previously unexpected. While this conclusion is exciting and well documented the other major conclusion of this work is that the current estimates of copy numbers of nucleoporins, shown on the example of Nup107, is too low, in other words that instead of 16 copies 32 copies are used to assemble the NPC.

I recommend t ask the authors for improving the manuscript prior to publishing.

This reviewer is not an expert on proteomics but rather on imaging. Hence a detailed review of the proteomics data is not given. Both, the proteomics work and the super-resolution microscopy data is well described. The proteomics work is done using multiple controls and verification steps. Viewed from a distance the proteomics data seem convincing, but if they have the "resolution" to draw conclusions on the exact copy number (16 or 32 per NPC) seems questionable, based on the large number of estimates (~3400 +/- 1300 NPCs per nucleus, the counting error of nuclei in the preparation and estimated 125,000 +/- 6000 Nup107 per nucleus). Also, having demonstrated variability between cell types, one could imagine a situation were two classes of NPC exist, one with 16 and one with 32 copies, which would bias any ensemble measurement.

Presenting the super-resolution measurements could address this concern of "resolution". The authors use an imaging method that is based on mEos2, a fluorescent protein. Compared to the proteomic work less controls were done and while a good documentation of the image processing is provided I would feel more comfortable with the data if some raw data and verification of the achieved localization precision and resolution would be presented. In the current version the two figures dedicated to the imaging do show little, if any, raw data, and are hard to interpret to understand the clustering and estimates being done. The estimates being made fall into two categories 1) resulting in underestimating copy numbers, 2) assigning and reconstructing the image. While the first category is well discussed and presented the second is laking detail. As mentioned above, the localization precision is simply stated and the resulting resolution is not further described or calculated.
Ultimately it seems the preparation of the nuclei should also support the use of a method like d-Storm, which by means of titration could address the issue of labeling density more quantitatively than a microRNA approach. Löschberger et al. 2012 (PMID: 22389396) showed impressive results of d-Storm imaging on NPCs.

The reviewer is right that dStorm could be an interesting alternative and indeed we have thought about this before starting this project. However, also this approach faces several hurdles, and how and if they can be overcome remains to be tested. First of all, the impressive data generated by Loschberger was done by staining chemically fixed cells with antibodies coupled to synthetic fluorophores. Naturally, working with fixed specimen and synthetic dyes is expected to yield a much higher resolution.

Nevertheless, antibody labeling has also its drawbacks: 1. The cross-reactivity of many antibodies. 2. It is very difficult to assess if the labeling ever gets close to completeness. Particularly in case of the NPC, it is conceivable that epitope species exist that are buried in the structure and not accessible. There is thus no guarantee that an antibody based approach could ever be quantitative. As a consequence, we decided to focus on genetically engineered mEos2 fusion protein, which photo-characteristics have been are well described.

Finally, using imaging of individual NPC composition the authors should be able to analyze the distribution of NPC compositions within a given nuclei to great detail, providing insights on NPC variability within the individual cell.

Suggested improvements:

1) The provided tables could benefit form a short description and explanation at each header that allows the reader to easily relate the information to the main and or supplemental material and Method used. It would also help to define the labels used in the tables upfront (many tables extend over several pages)."

The description of each table provided at the beginning of the Supplementary Information has been improved, including references to the related figures, and Table legends describing the headers are provided within the table files.

"2) Provide detailed analysis of localization precision and resolution achieved."

We estimated a localization-based resolution at full width half maximum (FWHM) of 37 nm. We have now included this information in the Supplementary Material, Measurement of Nup107 copies per NPC by super-resolution microscopy, Page 16.

Please note that - since the nuclei imaged in our study were not chemically fixed - the actual experimental resolution might be influenced by additional factors that are difficult to estimate, e.g. minor diffusion movements of individual nuclear pores within the nuclear envelope plane during that data acquisition. However, the ring structure of ~ 90 nm in diameter is clearly resolved, while the 8-fold rotational symmetry (in-subunit distance ~45 nm) appears unresolved.

"3) Show raw data as movies and still-images in the supplement."

We have now included images for all the nuclei (Figure 2A and Figure S4A-D). We additionally provide an illustrative movie for the nucleus displayed in Figure 2A. A single original data set (movie) takes 1.2 GB of disk space. In order to make the movie available as supplementary file, the original movie was compressed, frame rate was changed and only 1,000 out of 10,000 frames are shown. The original raw movies for all the imaged nuclei are available upon request.

"4) Improve the presentation of verification of the clustering algorithm used."

In order to validate the entire image analysis workflow, including the clustering algorithm, we performed Monte Carlo simulations to generate synthetic images of NPCs. These simulations take into account a stochastic NPC position within the NE, a stochastic labeling density across individual NPCs, a rotational distribution of fluorophores around the central channel as well as the photo-conversion, blinking and bleaching of mEos2 (explained in detail in Supplementary Materials and Methods, Monte-Carlo simulation of super-resolution images of NPCs, Page 17). The simulated data are in astonishing agreement with our experiments in terms of both, visual appearance (Figure S4F) and quantification of fluorophores. When our clustering algorithm and consolidation scheme are applied to the simulated data, they recover the input parameters, i.e. the fluorophore copy number per NPC, with very high accuracy (>90%).

"5) Detail the number of nuclei and NPCs per nuclei being imaged."

The final copy number frequency distribution was obtained from 172 clusters imaged from 5 nuclei. We have included this statement in Figure 2 legend and provided images for all the 5 nuclei in Figure 2 and Figure S4.

"6) Analyze NPC composition variation within individual nuclei."

Using the simulation approach described above, we generated synthetic data of a mixed population containing an equal number of NPCs with 16, 32 or 64 copies of Nup107. It became obvious that our experimental data are the most consistent with a monodisperse distribution of 32 copies (Figure S4G) and that, if one of the other scenarios contributes to the experimental data, it must comprise a very minor fraction. We believe that the synthetic data nicely underline the appropriateness of our data analysis procedure and data interpretation. We are thus very thankful to the reviewer for pointing us at doing such analysis.

We also want to point out that these findings are very consistent with previous studies by electron microscopy, which nicely demonstrated that major variations of the structural scaffold comprise only a minor fraction, likely because they are less stable: Various studies visualized nuclear pores of mostly consistent structure across single particles (see e.g. Beck et al, Science 2004; Elad et al, COSB 2009), which is very consistent with a large number of electron tomograms that we have acquired of our samples. We are aware of only two exceptions. Hinshaw and Milligan (JSB 2003) have shown that nine and tenfold rotationally symmetric NPCs occur in Xenopus nuclear envelopes but with a very low frequency of less than 2.5% as compared to the 'conventional' 8-fold symmetric structure. Maeshima et al (NSMB 2010) have presented data that indicate that ~5% of all NPCs have a significantly smaller diameter. These might comprise assembly intermediates that are populated at the specific stage of the cell cycle (G1/S phase) investigated by this study. For both above cases, we cannot exclude that such variations underlie our data because they comprise such a minor fraction that would remain undetected. However, since they are very rare, our data account for the major fraction of structural species.

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Stoichiometries of Nups presented in Figure1 is very informative, providing new insights to our knowledge on NPC structure that was never seen with any of the previously reported studies : 7 components of Nup107 complex are present in assembled NPC structure with the same stoichiometry while 3 components (Elys, Sec13 and Seh1) show lower abundance, indicating these components are not associated tightly with other Nup107 complex components at interphase NPC, which agree with some of previous reports. The result is interesting also from different aspects: for example, interaction between Nup107 and Elys is crucial for post mitotic NPC assembly, but some population of Elys could be dissociating from Nup107 complex after completion of nuclear envelope formation (which agree that there exist significant intranuclear pool of Elys in interphase). The stoichiometry of Nup93 subcomplex components indeed implicates the presence of two different components, as authors pointed out.

Pom121 was shown to be essential for interphase NPC formation, however, its abundance is lower than scaffold Nups (Nup107 complex or Nup93 complex), which was shown to interact with Pom121. This implies that Pom121 is not essential for anchoring scaffold Nups to nuclear membrane, but may be playing different roles in NPC structure/function. Nup98 and Nup62 was reported/considered as abundant Nup in NPC structure. Data in Figure 1 support this argument.

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microscopy to quantify copy number of Nup107 per NPC with isolated nuclei. All the obtained results show copy number of Nup107 per NPC is above 16, allowing the authors to conclude that Nup107 exist 32 copies instead of 16 copies, which has been previously considered. All these experiments are employing new technologies, and have been performed as carefully as possible. New result was obtained through employing new approaches. The result would trigger arguments in the field. In my opinion, whether the provided number is definitely correct or not is not a critical issue at this moment as far as a way of analysis is correct, which I appreciate with the present manuscript. Important issue is that the data presented in this paper would trigger arguments in the field on this important topic, providing opportunity for people in the field to re-consider various evidences that exist at this moment.

In the final part of the manuscript, the authors examine the cell-type specific compositional variation of Nups by examining 5 different cell lines in combination with proteomics including shotgun and gene expression data. The results show about 38% of the complexes that was examined, including NPC, have stoichiometric variation in within only 5 cell lines examined, which are all cancer human cell lines.

I have one concern in the last part of the results of this manuscript, regarding "stoichiometric variation of complexes examined in 5 cell lines". What are the purposes of selecting these 5 cell lines? Are the authors arguing that 38% of complexes (even with under-estimated) with varied stoichiometry would vary among any of selected cell lines? What about the rest of complexes that showed similar stoichiometry? Such complexes might show similar stoichiometry among human cancer cell lines, but they might vary when compared with normal cell lines (i.e. TIG or normal fibroblast). These issues (purpose of comparing complexes within 5 human cancer cell) should be mentioned and discussed, and the arguments should be clarified."

This is a fair point. As we already explained in the response to reviewer one above, the 3 cell lines (RKO, SK-MEL-5 and K562) were selected using gene expression data because they displayed very pronounced variations in Nup mRNA expression levels, in order to analyze stoichiometric variations of the NPC. We additionally included the HeLa cell line used for the stoichiometry determination and the non-cancer derived HEK293 line. To investigate on a more global scale if also other protein complexes might undergo compositional changes, we analyzed the very same samples with in shotgun mode. We included a sentence in the result section to clarify this point (Result, Page 8).

We defined the complexes as dynamic if their composition varied in at least one of the selected lines. As correctly pointed out by the reviewer, if more cell lines/tissues/conditions would be analyzed variations in the composition of the other complexes that we defined as stable could be observed. This is why we mentioned a possible underestimation. Alternatively, one might argue that only specifc cellular function require a fine tuning in different cell types such as the nuclear transport, RNA processing and chromatin remodelling system, while core functions such as RNA/DNA polymerization, translation, etc. remain stable. We have pointed this out more clearly in the discussion on Page 11.

"Other minor comments:

1) Although authors can put the information regarding the integrity of nuclear envelope shown in FigS1C, I don't think this is important information: upon fractionation process, lipid membranes of either outer nuclear membrane and/or inner nuclear membrane can be partly disrupted, which would loose the "integrity" of nuclear envelope, while keeping NPC structure intact. Therefore, it does not matter, particularly in this report, whether the isolated nuclear envelope excludes dextran or not. I would not point this strongly as one of evidence arguing integrity of NPC structure (p3, result section). It can be indicated as supporting evicence if authors want to."

Agreed, we adjusted the main text accordingly (Results, Page 4). As suggested by the reviewer, the data were left in the Supplementary information as supporting evidence.