

Figure S1 Epi-fluorescence imaging of GFP-NPCs recognized by Alexa Fluor 647 labeled anti-GFP nanobodies at micro-molar concentration and single-molecule photon collection and precision for nanobodies binding GFP-Nups at 0.1 nM by SPEED microscopy. (A) On the left is a cartoon representation of GFP-Pom121 proteins bound and incorporated into a single NPC. Pom121 is represented as purple with GFP shown as its beta-barrel structure (green). Anti-GFP nanobodies (blue) are shown conjugated to Alexa Fluor 647 (red). These nanobodies are shown binding firmly to GFP-Pom121 or freely diffusing through the NPC. (B) Epi-fluorescence radial view (bottom-view) imaging of the nucleus is shown in green and red channels respectively for GFP-Pom121 and 0.5- μ M Alexa Fluor 647 labeled nanobodies. A merge of these two channels (yellow) suggests that nanobody nearly saturated the available GFP-Pom121 binding sites in the NPCs at its high micro-molar concentrations. For Epi-fluorescence axial-view (side-view) imaging at the equator of the nucleus: red and green channels are similarly shown before and after merging. (C-D) Shown are six consecutive frames captured by SPEED microscopy. Each frame represents 50 ms of recording time for the fluorescence obtained from an Alexa Fluor 647- conjugated anti-GFP nanobody bound to a GFP-Nup within a single NPC. N represents the total photon count for each frame and σ represents the localization precision obtained for each frame, as shown in Equation (1) of Materials and Methods. The six frames are merged together reaching a photon count of 23,643 photons and correspondingly 1.6-nm localization precision.

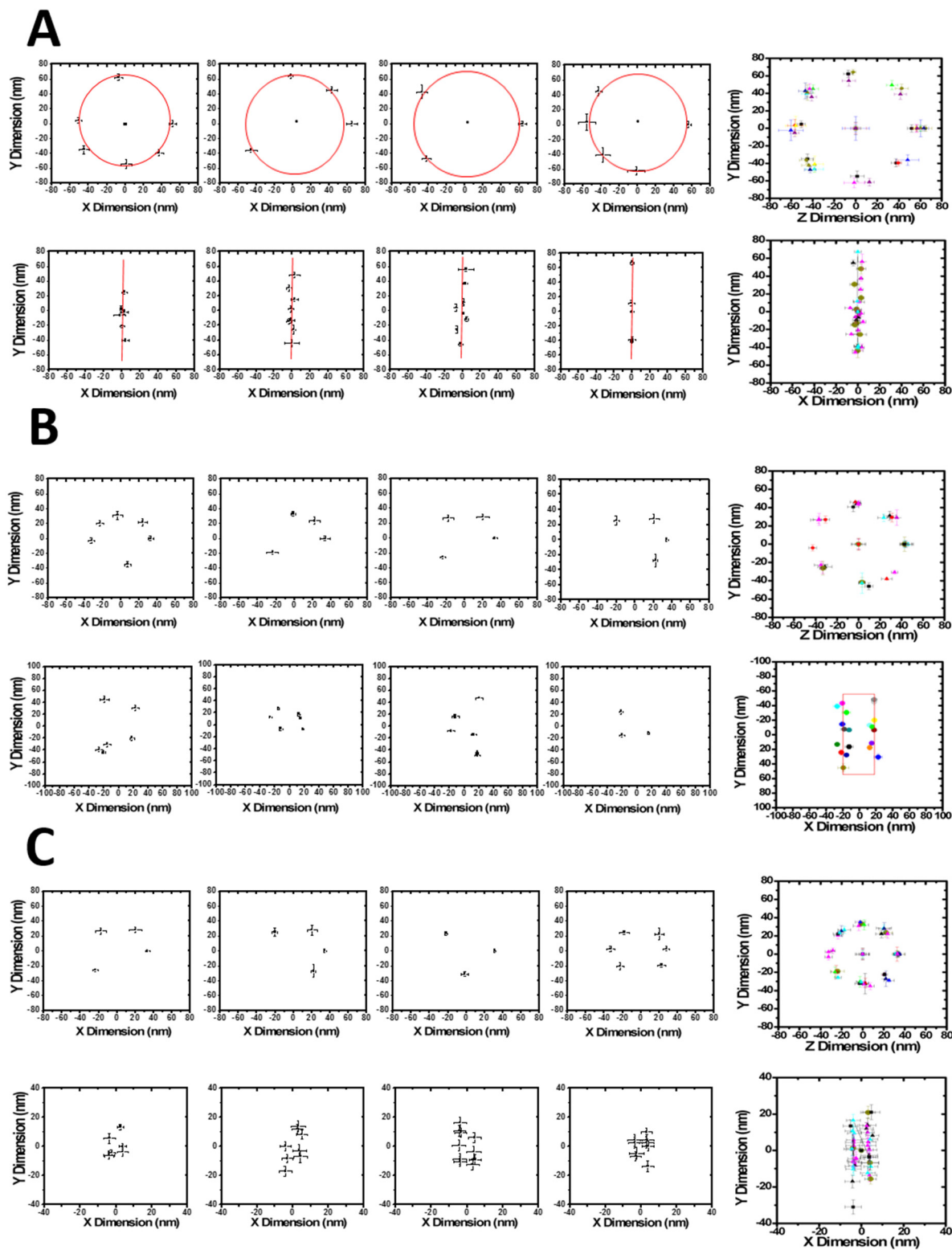


Fig. S2. Montage and reconstruction of spatial positions obtained from individual NPCs for scaffold nups. (A) Data obtained from four randomly selected individual NPCs for Pom121 are used to demonstrate our analysis process. Above shows the radial distribution, while below shows the axial distribution. In the radial dimension, the obtained spatial data points are fit along a circle function (red cycle). As described by Equation (3-5) in the Materials and Methods, the center of the circle is calculated based on a permutation of all possible centers between each three or more data points. Once the center is calculated, the data point with the highest precision is rotated to be located at the 0- or 360-degree position along the circle. In the axial dimension, the data points are fit to one or more lines (red line). Data along the axial dimension could distribute as 1, 2, or 4 isolated lines in the Y direction according to models shown in Fig. S3. The final data for each dimension is seen on the right side. (B) Data obtained from four randomly selected individual NPCs for Nup 37. Above shows the radial distribution, while below shows the axial distribution. The final data for each dimension is seen on the right side. (C) Data obtained from four randomly selected individual NPCs for Nup 35. Above shows the radial distribution, while below shows the axial distribution. The final data for each dimension is shown on the right side.

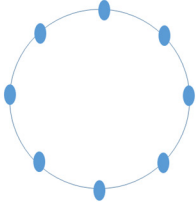

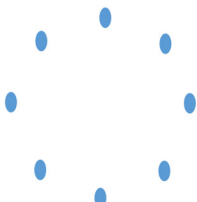

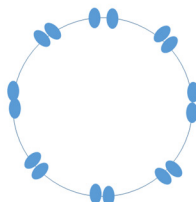
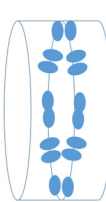
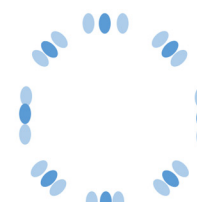

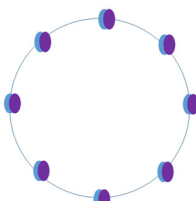



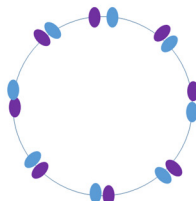



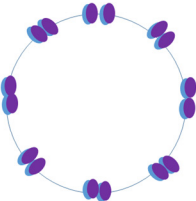
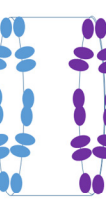


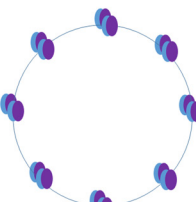



	Model		Experiment		Total Number
	Radial View	Axial view	Radial view	Axial view	
(i)					8
(ii)					16
(iii)					16
(iv)					16
(v)					32
(vi)					32

Fig. S3. Possible models for Nups distributions in the NPC. Model (i) represents a single layer of 8-fold rotational symmetry yielding a total copy number of 8. Model (ii) represents a single layer of 16 copies of a Nup grouped in pairs of 8-fold symmetry. This formation could possible cause three locations for each paired Nup when determined the location experimentally depending on the distance between the paired Nups. Model (iii) represents two layers of 8 fold symmetry along the axis of the NPC yielding a final copy number of 16. Model (iv) represents a similar distribution to (iii) except the two layers of rotational symmetry have some offset in regard to angular positioning. Model (v) represents a Nup distribution in which the Nup is present as two layers of 16 nups grouped in pairs yielding a final Nup count of 32. Model (vi) represents another possible formation for Nups present at 32 copies per NPC with four layers each with 8 Nups.

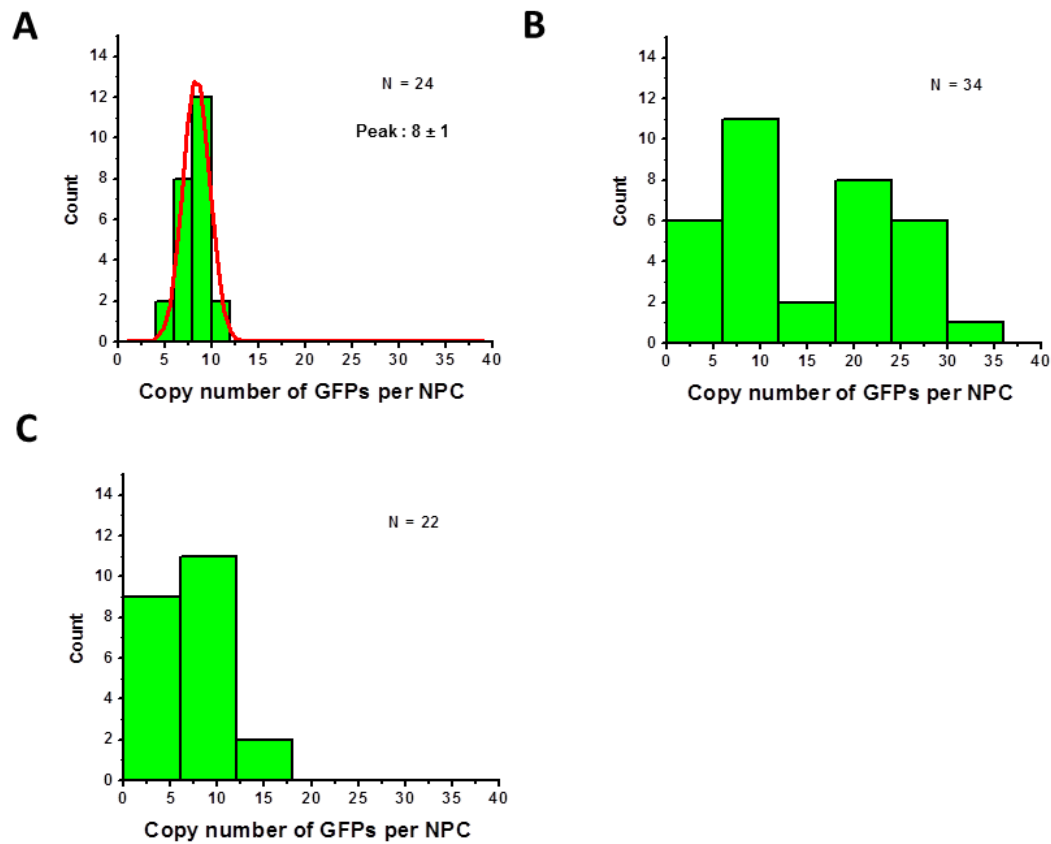


Fig. S4. Copy number of GFP-Nup per NPC The copy number of GFPs present per NPC is shown for cells expressing each individual tested scaffold GFP-Nup. Copy number is determined by the intensity recorded for illuminated GFPs within individual NPCs. Data was normalized by the intensity of a single-GFP for each condition. N represents number of NPCs and cells. (A) Data is shown for the copy number of GFP for the stable cell line containing GFP-Pom121. Peak position is 8 ± 1 nm. (B) Data is shown for the copy number of GFP from the transiently transfected cell line containing Nup37. (C). Data is shown for the copy number of GFP for the transiently transfected cell line containing Nup35.