

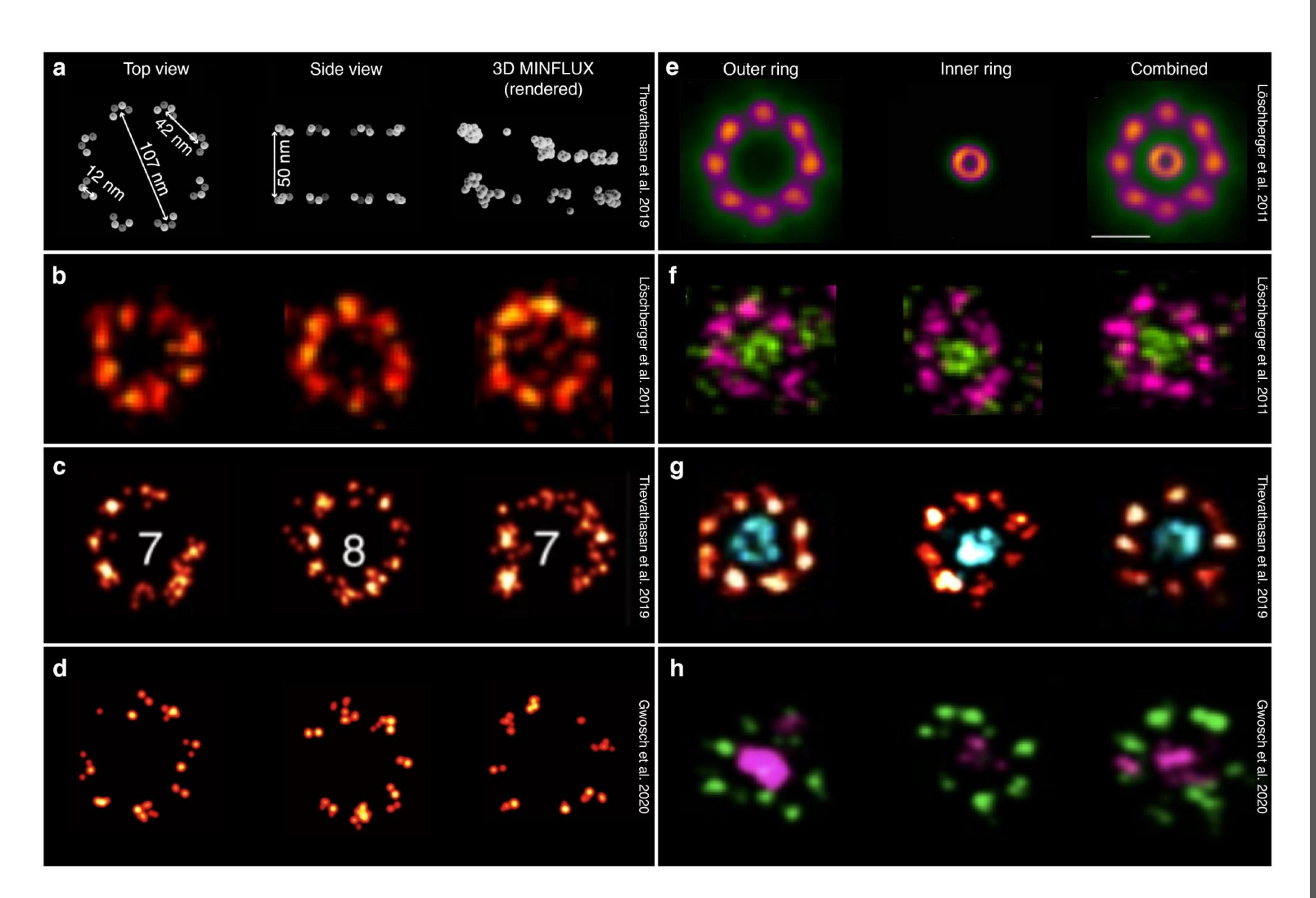
Is MINFLUX the right method to explore biology at the nanoscale?

Not yet!

While MINFLUX can achieve very high localization precision, quantitative analysis of reported results leads us to dispute the resolution claim and question reliability for imaging sub-100-nm structural features.



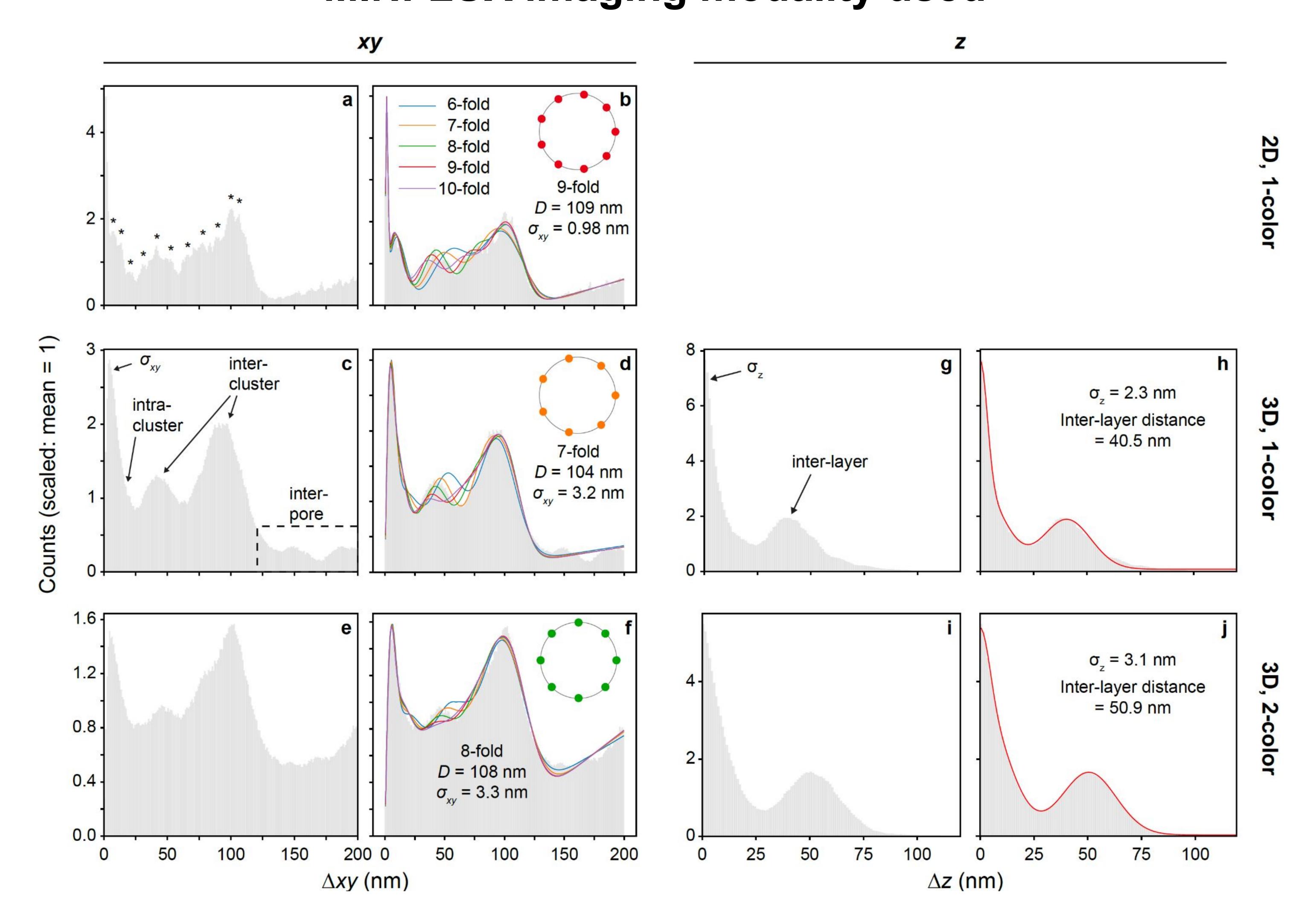
Standard dSTORM setups outperform MINFLUX



(a) A schematic of the Nup96 complex (Thevathasan et al. 2019). (b) dSTORM imaging of membrane protein gp210 (Loeschberger et al. 2012). (c) SMLM imaging of U2OS cell expressing Nup96-SNAP (Thevathasan et al. 2019). (d) MINFLUX imaging of U2OS cell expressing Nup96–SNAP (Gwosch et al. 2020). (e) Average images of gp210 and WGA (Loeschberger et al 2012). Scale bar: 100 nm. (f) dSTORM images of WGA (green) and gp210 (pink) in amphibian oocytes (Loeschberger et al. 2012). (g) 2-color SMLM image of Nup96-SNAP (red) and WGA (cyan) in U2OS cell lines (Thevathasan et al. 2019). (h) 2-color MINFLUX image of Nup96-SNAP (green) and WGA (pink) in U2OS cell lines (Gwosch et al. 2020).

In layman's terms: Standard dSTORM setups consistently show 7- and 8-subunit nuclear pores (b-c). In contrast, the MINFLUX data is sparse, and none of the representative pores show all 8 subunits (d). Moreover, the inner WGA ring of the nuclear pores which is clearly resolved by SMLM setups (f-g), appears as a blob in MINFLUX images (h).

Nuclear pore diameter and inter-layer distances depend on MINFLUX imaging modality used



Histograms of xy- and z-distances (Δxy , Δz) between localizations, bin-width 1 nm. Δxy distribution for the Nup96 localizations of Gwosch et al. (2020) Fig. 2a (a), 3f (c) and 5c (e) and fits to them (b, d, f,) of nuclear pore models from 6to 10-fold symmetry, including repeated single-molecule localizations (Δxy), intra- and inter-cluster distances within an NPC, and background~inter-pore distances (Curd et al., 2020). Symmetry, nuclear pore diameter (D) and σ_{yy} for the model selected by AICc (Curd et al., 2020) in each experiment (b, d, f). Indications of resolved intra-cluster substructure in a (*). ∆z distribution for the data in Gwosch et al. (2020) Fig. 3f (g) and 5c (i) and fit with a model including two layers of localizations and repeated single-molecule localizations (σ_{2}) (h, j).

In layman's terms: the nuclear pore parameters depend on the MINFLUX modality used (full field of view analysis)-1. Pore diameter and subunits - 109 nm, 9-fold (2D, 1-color); 104 nm, 7-fold (3D, 1-color); 108 nm, 8-fold (3D, 2-color) 2. Inter-layer distance - 40.5 nm (3D, 1-color); 50.9 nm (3D, 2-color)

One of the possible reasons?

A min localisation density is needed to get meaningful result

True molecular events

4,586 (79.7%), 435 µm⁻²

14,778 (54.3%), 1,204 µm⁻²

38 (17.9%), 119 µm⁻²

Published data

Background events

1,171 (20.3%)

12,408 (45.7%)

175 (82.1%)

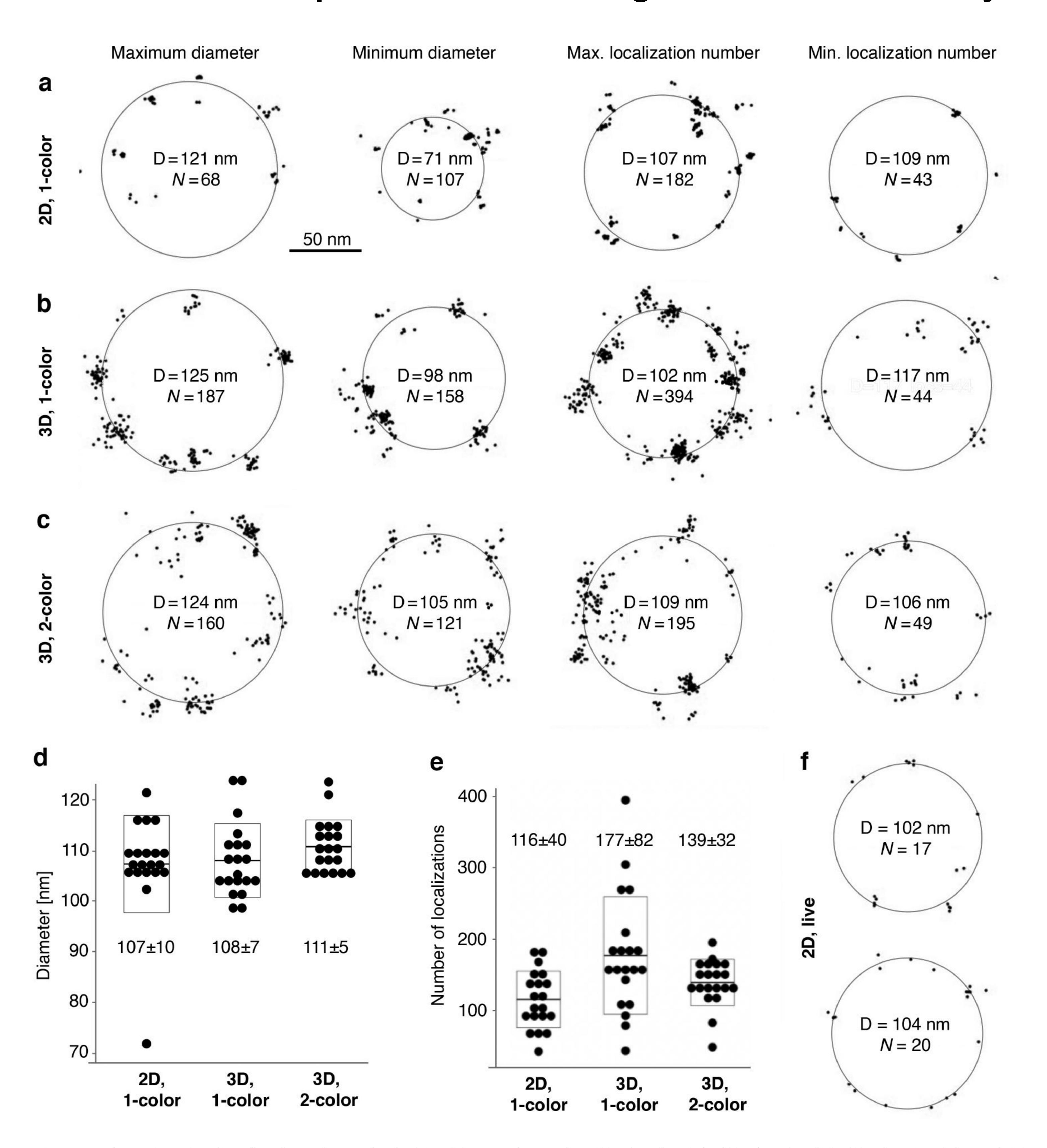
Molecular emission events

5,757 (total)

27,186 (total)

213 (total)

What's happening? 1-nm localisation precision but still high structural variability?



Scatter plots showing localizations from single Nup96 complexes for 2D, 1-color (a); 3D, 1-color (b); 3D, 2-color (c); and 2D, live (f) MINFLUX datasets. We segmented N = 20 NPCs for each dataset (a–c) and show those with minimum/maximum diameter and minimum/maximum number of localizations for the outer rings of Nup96. The distributions of the fitted diameter (d) and the number of localizations (e) among the NPCs. Two Nup96 complexes were only visible in the live data (f).

In layman's terms: the nuclear pore parameters depend on which MINFLUX imaging one does (per-pore analysis): 1. Pore diameter - 107± 10 nm (2D, 1-color); 108 ± 7 nm (3D, 1-color); 111 ± 5 nm (3D, 2-color) 2. Number of localisations - 116 ± 40 nm (2D, 1-color); 177 ± 82 nm (3D, 1-color) or 139 ± 32 nm (3D, 2-color)

Note the high variability in number of localisations/subunit and compare it to the rendered image in the top-left panel above.

The ROYAL MARSDEN

NHS Foundation Trust

Kirti Prakash^{1, 2, *}

¹National Physical Laboratory, TW11 0LW Teddington, UK ²Integrated Pathology Unit, Centre for Molecular Pathology, The Royal Marsden Trust and Institute of Cancer Research, SM2 5NG Sutton, UK

*Email: kirtiprakash2.71@gmail.com; kirti.prakash@icr.ac.uk

National Physical Laboratory



References:

1. Prakash and Curd. Assessment of 3D MINFLUX data for quantitative structural biology in cells. bioRxiv (2021) (Primary reference)

defined by the total molecular emission events before filtering. Scale bar: 500 nm (a, c), 200 nm (b), 50 nm (d).

Scatter plots for 2D, 1-color (a); 3D, 1-color (b); 3D, 2-color (c); and 2D, live (d) unfiltered and filtered MINFLUX datasets.

In layman's terms: the localisation density and filtered localisations drastically change as the imaging complexity increases:

2. Localisation density: 435 μm⁻² (2D, 1-color); 631 μm⁻² (3D, 1-color); 1204 μm⁻² (3D, 2-color); 119 μm⁻² (live imaging)

The MINFLUX raw data comes in a tabular format, with a Boolean flag indicating that localization was assigned as background

event or a true molecular event (Gwosch et al. (2020). For the final data, we give the density of true localizations over the FOV

Only 3D, 2-color MINFLUX data has a reasonable localisation density and provides accurate estimates of pore diameter and inter-layer

distance. In contrast, note the localisation density and the highly undersampled configuration of the pores in the live MINFLUX data.

- 2. Gwosch et al. Assessment of 3D MINFLUX data for quantitative structural biology in cells revisited. bioRxiv (2022)
- 4. Balzarotti et al. Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. Science (2017)
- 5. Gwosch et al. MINFLUX nanoscopy delivers 3d multicolor nanometer resolution in cells. Nature methods (2020)
- 7. Thevathasan et al. Nuclear pores as versatile reference standards for quantitative superresolution microscopy. Nature methods (2019) 8. Curd et al. Nanoscale pattern extraction from relative positions of sparse 3d localizations. Nano letters (2020)

9. I thank Lothar Schermelleh, Rainer Heintzmann, Johannes Hohlbein, Alistair Curd and Carlas Smith for the feedback on the poster.

I. Filtering of localisations: 20% (2D, 1-color); 37% (3D, 1-color); 45% (3D, 2-color); 82% (live imaging)

3. Prakash. At the molecular resolution with MINFLUX?. Philosophical Transactions of the Royal Society A (2022)

6. Löschberger et al. Super-resolution imaging visualizes the eightfold symmetry of gp210 proteins around the nuclear pore. JCS (2012)