

High-Speed multiplexed DNA-PAINT imaging of nuclear organization using an expanded sequence repertoire

Corresponding Author: Dr Mahipal Ganji

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors describe DNA-PAINT enabling 10-plex imaging on DNA-Origami and 9-plex imaging in the nuclear environment with a partially new set of speed-optimized probes. They show sequential imaging with “molecular resolution” and characterization of relative spatial distribution of many proteins in the nucleus, demonstrating a reorganization of the chromatin landscape upon transcription inhibition.

The data is technically sound and analyzed carefully but could be presented with more quantitative details in the main text/figure captions as remarked in my more detailed comments below. The conclusions that the authors draw are well supported. The work is an extension of previous work by Jungmann et al. (ref10 “100-fold speed-up.. paper”), providing an extension/almost doubling of straightforward to use repetitive sequence DNA-PAINT probes for faster imaging compared to first reported “standards” (e.g. ref.6). The design principles used are in my opinion not new in themselves. I agree that the probes are indeed much easier to use than recently published (ref 11+12) unlimited multiplexing DNA-PAINT approaches, thus very useful for the biophysics/life science community.

I like combination of quantification/evaluation with origami followed by a very extensive bioimaging in cells, multiplexing the 9 different structures/proteins in the cell nucleus. The figures are very clear, good information transmission.

More detailed comments that should be addressed before publication are the following:

I am missing quantitative parameters in the abstract! Like how fast is the imaging/kinetics, what localization precision/resolution (FRC or decorrelation analysis) is achieved etc..this is also missing in the text and needs to be added.

They should state clearly what is different/same/similar in their design compared to the previous 100-fold speed-up.. paper..

How long was data acquisition for origami exchange-PAINT rounds, number of frames and exposure time – please mention this in the text/caption and how does it compare to previous experiments with speed-enhanced probes? What was the imager strand concentration for all experiments, I cannot find it many times neither in text nor in caption? Is it comparable to previous experiments with speed-enhanced probes?

Generally, their new strands are still fast due to shortened dark time, but they have a considerably longer binding time/bright time than the previously proposed set (see binding time kinetics Fig. 1).

They should comment on what the large bright time means for the speed of DNA-PAINT imaging and where it comes from based on the sequences. How much is this really still speed-enhanced DNA-PAINT?

Which sequences should be preferred for which purpose/experiment (speed vs. resolution)?

I find the dark time quantification as important, can this be added in the main manuscript?

What is described as specific and unspecific binding Fig 1e? what is the number used as a threshold for classification and what are the numbers underlying the classification? Can the authors add a similar quantification of sequence orthogonality as in 100-fold speed-up.. paper Extended data Fig. 4? Couldn't this cross-binding be expected when overlaying the

sequences?

Can they comment what previous highly multiplexed cellular/tissue DNA-PAINT experiments there are in the literature and compare?

What does Actinomycin D do, I am missing this explanation when the experiment is introduced.

The correlation methods used to quantify the cell imaging data should be very briefly introduced/explained in the text, and more so in the methods part with references to show that these are appropriate.

Reviewer #2

(Remarks to the Author)

The authors introduce an interesting resource of DNA-PAINT sequences for high-speed multiplexed DNA-PAINT. This work seems a complement to the work described in the Brief Communication by Strauss and Jungmann in 2020, providing 6 additional sequences albeit some of them are not compatible so up to 10 might be used in a single experiment. While expanding the space of DNA sequences to allow for more complex Exchange-PAINT experiments is useful and the manuscript shows that they extensively tested them – especially on DNA origamis –, the work could benefit from some more innovative approaches to DNA-PAINT, and by itself constitutes more of a resource than an original piece of work. In any case, we value that the authors are trying to make additional sequences available for fast exchange-PAINT experiments as we agree that FLASH-PAINT and SUM-PAINT experiments, that depend on several steps of strand hybridization and disassociation, can be exceptionally complex.

While the results in DNA origamis seem good, we have some major concerns about the controls used to prove the validity of the experiment in vivo, especially given the number and complexity of nuclear targets. Supplementary 4 shows that the authors used Lamin B1 as a control, and kept the sequences that produced reasonable lamin B1 distributions, namely all of them except R3, that was ruled out because it also produce signal in the nucleoli. Can the authors provide an explanation to why this might be happening? R5, R7 and R8 also display weird spots in the images, is this just an artefact or something the authors should consider?

We are also concerned that only using Lamin B1 as a control for the DNA PAINT strands might result in a partially biased result, given the exceptional intensity of the lamin B1 signal in the nuclear periphery that might increase the signal to noise ratio to levels that are unachievable when targeting any other nuclear protein. Can the authors provide negative controls for other targets in the nucleus, particularly some of the histone marks? That is, performing DNA-PAINT without including the primary antibody. As described by Lucinskaite et al. (<https://www.biorxiv.org/content/10.1101/2024.06.03.597143v1>), imaging nuclear targets through DNA-PAINT is specifically challenging because of non-specific signal in the cell nucleus. We wonder how the proposed DNA sequences deal with this phenomenon, as the non-specific signal is often extremely similar to that produced by proteins that are more homogeneously distributed in the nucleus than Lamin B1.

We are also surprised by the Lamin B1 redistribution identified upon actinomycin D-mediated transcription inhibition and fear that it could be a result of a not good DNA-PAINT experiment. Can the authors justify, or at least hypothesize, why such phenotype could occur?

We are also very concerned by the fact that while Lamin B1 seems homogeneously distributed in the cell nucleus upon transcription inhibition in Figure 2 (right), there is still a significant amount of signal in the nuclear periphery in Supplementary Fig. 7. Is this a consequence of different colors used in the representation, or the experiments in an Exchange-PAINT experiment using the proposed sequences can differ that much to the ones aiming to target a single protein? This is a major issue the authors should clarify.

The spatial correlation viewpoint analysis presented in Figure 3 and Supplementary Figures 8 and 9 seems weak and some of the conclusions extracted not sustained. For example, H3K27ac shows increased correlation with all other targets excepting SC35 upon transcription inhibition (Supplementary Fig. 9), including those that were pertaining to the same euchromatin fraction. How do the authors explain this instead of a general blending of interactions?

What's the criterion to decide when the enrichment is high and when it isn't? The difference in enrichment in untreated cells of H3K27me3 with respect to lamin B1 and SC35 in Fig. 3 c (top) is less than 3% (18.34% vs 15.55%, respectively), but the authors only claim that the enrichment occurs between H3K27me3 and lamin B1 contacts. The authors should think of a metric to measure the significance of these changes to be able to state that such differences exist.

Additionally, in Fig. 3c (bottom), in spite of the stated redistribution of lamin B1, the colocalization of such target with the heterochromatin markers H3K9me3 and H3K27me3 increases. How does this agree with a general redistribution of the protein?

Apart from this, we consider that the manuscript could benefit from several clarifications, including those referring to:

- Supplementary Figure 1 a: using 4 nt blocks could make it clearer to compare with 1 c
- Supplementary Figure 1 d: further clarification is needed to understand the mismatches between imaging strands.
- Figure 3 b: additional explanations are required to understand the figure, it is extremely obscure as it is.
- Figure 3c: how does this analysis differ from the one done in Supplementary figures 8 and 9?

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

General comment:

I am satisfied with the revisions in response to my comments, and the additions to the main text are very useful. I share the sentiment of the additional referees that this paper is a useful additional resource for multiplexing using the same concept introduced before in ref 10, but does not constitute a new strategy for fast DNA-PAINT.

Response to the more detailed comments in the revision (N.A. means that the revision/response is fine by me):

1. N.A.
2. N.A.
3. Please double-check that the SI tables are also referenced somewhere in the manuscript.
4. This addition to the main text is mostly very clear. Why is the sampling in Figure 2c for low sampling not also lower for the last timepoint?
5. N.A.
6. N.A.
7. N.A.
8. N.A.
9. N.A.

Reviewer #2

(Remarks to the Author)

We want to thank the authors of the paper for addressing our comments. Nevertheless, we still have some major concerns about the quality of the experiments and the validity and relevance of the biological conclusions extracted from them.

The authors provide negative and positive controls for each one of the nuclear targets shown in Figure 3 in Supplementary Figures 8-10. Observing the nucleolar shape and observed structures (for example when imaging SC35 and H3K4me3) it seems that the cell in the positive control is the same one displayed in Figure 3b. Are the authors using the same images / cell shown there as positive controls? If this is the case, why is the signal intensity so different in Supplementary Figure 10h (H3K9me3) and the H3K9me3 channel in Figure 3a? In the latter, the signal seems much more intense, even if the nucleolus shape is the same.

Moreover, many of the positive controls (antibody with nanobody) in Supplementary Figures 8-10 show extremely unspecific signal outside of the nucleus, which was not visible in Figure 3 because the nuclei were cropped out. For example, Supplementary Figure 8a shows almost the same signal within the nucleus and in the cytoplasm for Ser2P. It is hard to extract significant conclusions in the images. Similar noisy signals are observed in Supplementary Figure 8b (Ser5P), Supplementary Figure 9f (CTCF). Supplementary Figure 24a (ser2P, mock) and Supplementary Figure 25e (H3K4me3, mock and ActD) also show very noisy signals. This is specially worrying when imaging histones, given that the signal within the nucleus should be much more intense than in the cytoplasm. As mentioned in the initial review of the manuscript, Supplementary Figure 5 shows that the lamin signal is quite strong in the nucleus periphery but also it also shows a lot of noise in the cytoplasm (see R1, R2, R4, R9, R10). When imaging targets that do not accumulate that clearly in specific regions of the nucleus (such as histone marks), many of the sequences may not have enough quality to draw valid conclusions.

Some other images show inconsistencies. For example, Supplementary Figure 15a does not seem to show the nucleolar structure that is seen in the channels for H3K9me3, H3K27me3 and CTCF channels. In the other images (before transcription inhibition) all channels seem to display nucleoli.

In line 201, authors say that H3K27me3 and H3K9me3 show negative correlation with nuclear speckles and RNA polymerase marks (S2P and S5P). The plot in Figure 4a does not appear to show this and instead shows these histone marks are positively correlated with S5P. Similarly, in line 222 the authors claim that nuclear speckles positively correlate with euchromatin marks in untreated cells, but Figure 4a shows otherwise, being the highest correlation with H3K27ac in a color that seems close to 0, at best.

The authors include a new analysis in Figure 4, but it is poorly described in the text making hard its interpretation. Specifically, Figure 4c is very sparsely commented, and many of the results extracted from it are not referenced to any specific panel or section of the figure. For example, can the authors comment results from the Lamin B1 viewpoint? Are the results referring to the mock or ActD case? In this current state it's very hard to interpret, especially when one of the few conclusions made contradicts what is shown in Figure 4b.

The authors should also specify which of these analyses are done on just one cell or in many cells, indicating number of cells analyzed for each experiment. If experiments are carried out for only one cell, the authors should try to find a metric that can group the results from many cells to improve robustness.

Some of the biological changes observed upon transcription inhibition are not novel, such as the reshaping of nuclear speckles (reviewed in <https://doi.org/10.1242/jcs.259594>), limiting relevance of the functional study.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

you can find my assessment in the attached and uploaded file.

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REVIEWER COMMENTS

Black text: Reviewers comments

Green text: Our response to reviewers' comments

Red text: New text added to the manuscript based on reviewers' suggestions. Black text in between red text is from initial manuscript.

Reviewer #1 (Remarks to the Author):

The authors describe DNA-PAINT enabling 10-plex imaging on DNA-Origami and 9-plex imaging in the nuclear environment with a partially new set of speed-optimized probes. They show sequential imaging with “molecular resolution” and characterization of relative spatial distribution of many proteins in the nucleus, demonstrating a reorganization of the chromatin landscape upon transcription inhibition. The data is technically sound and analyzed carefully but could be presented with more quantitative details in the main text/figure captions as remarked in my more detailed comments below. The conclusions that the authors draw are well supported. The work is an extension of previous work by Jungmann et al. (ref10 “100-fold speed-up.. paper”), providing an extension/almost doubling of straightforward to use repetitive sequence DNA-PAINT probes for faster imaging compared to first reported “standards” (e.g. ref.6). The design principles used are in my opinion not new in themselves. I agree that the probes are indeed much easier to use than recently published (ref 11+12) unlimited multiplexing DNA-PAINT approaches, thus very useful for the biophysics/life science community.

I like combination of quantification/evaluation with origami followed by a very extensive bioimaging in cells, multiplexing the 9 different structures/proteins in the cell nucleus. The figures are very clear, good information transmission.

We thank the reviewer for reviewing our work and for their appreciation of our work. We have performed additional experiments and analysis to answer all the concerns raised by the reviewer. Please see our point-by-point answers to the comments below.

More detailed comments that should be addressed before publication are the following:

1. I am missing quantitative parameters in the abstract! Like how fast is the imaging/kinetics, what localization precision/resolution (FRC or decorrelation analysis) is achieved etc..this is also missing in the text and needs to be added.

We have extended the abstract to include quantitative parameters such as localization precision and imaging speed. Specifically, we now report the localization precision obtained from DNA-PAINT imaging on DNA origami, measured by Gaussian fitting of the localization spread around each docking strand. Under standard imaging conditions, we achieve a localization precision below 5 nm. Additionally, we have added a corresponding discussion in the main text to highlight these quantitative performance metrics.

Abstract:

“By implementing Exchange-PAINT protocol, we demonstrate 12-plex super-resolved imaging of docking strand patterned DNA origami nanostructures within four hours with

a localization precision of 3 to 5 nm. Using these sequences, we demonstrate 9-plex super-resolution imaging of diverse nuclear targets within four hours.”

Main Text:

“Each species was imaged for 1000 seconds, with washes entailing 300 seconds between each round, taking less than 4.5 hours for 12-plex imaging. As expected, all 12 species of DNA origami nanostructures were clearly visible in the merged data with a localization precision lower than 5 nm”

2. They should state clearly what is different/same/similar in their design compared to the previous 100-fold speed-up.. paper..

The previous study designed six speed-optimized sequences but did not explore the full possible sequence space. In contrast, we systematically searched through the complete sequence space of all building blocks. This enabled us to generate both concatenated sequences for increased imaging speed and mutual orthogonality between sequences, all while keeping the imager length at 7 or 8 nucleotides. In the initial manuscript, we have included the design rules for generating the sequences (page 2, line 49 onwards). We have clarified this distinction further in the manuscript for better understanding.

Main Text:

To outline sequences that could be used for speed-optimized DNA-PAINT, we systematically explored all combinations of orthogonal imager-docking strand pairs in the entire sequence space that support faster imaging.”

“The presence of non-complementary nucleotides in these sequences preclude any secondary structures, ensuring that the DNA strands remain in an open configuration. This conformation facilitates rapid and efficient binding to the complementary strand. Concatenation of such blocks in the docking strand further improves binding rates by increasing the number of potential binding sites for the imager.¹

3. How long was data acquisition for origami exchange-PAINT rounds, number of frames and exposure time – please mention this in the text/caption and how does it compare to previous experiments with speed-enhanced probes? What was the imager strand concentration for all experiments, I cannot find it many times neither in text nor in caption? Is it comparable to previous experiments with speed-enhanced probes?

We thank the reviewer for their suggestion on adding this crucial information in the main text and figure caption. They are not any different than previous experimental results obtained with speed-optimized imagers. We have added this information at appropriate places along the text. We also provide all the imaging conditions including the concentration of imager, imaging duration, exposure time, laser power, and localization precision (NeNA) in the Supplementary Table 2.

Main Text:

Each species was imaged for 1000 seconds, with washes entailing 300 seconds between each round, taking less than 4.5 hours for 12-plex imaging. As expected, all 12 species of DNA origami nanostructures were clearly visible in the merged data with a localization precision lower than 5 nm (Figure 1d and Supplementary Figure 2).

4. Generally, their new strands are still fast due to shortened dark time, but they have a considerably longer binding time/bright time than the previously proposed set (see binding time kinetics Fig. 1). They should comment on what the large bright time means for the speed of DNA-PAINT imaging and where it comes from based on the sequences. How much is this really still speed-enhanced DNA-PAINT? Which sequences should be preferred for which purpose/experiment (speed vs. resolution)?

We thank the reviewers for these insightful comments. We have now included quantitative data demonstrating the advantages of longer-binding imagers compared to the shorter-binding ones. Specifically, we compared imagers with distinct binding times (~0.3 s vs ~0.7 s) and show that longer binding times lead to better resolution. These results, presented in the new Figure 2, highlighting that longer-binding imagers enable improved imaging quality and more efficient sampling of target sites. We have incorporated these findings and the corresponding discussion into the revised manuscript. From this data, we suggest that longer-binding imagers can be used when higher resolution is required, especially under limited resources, such as low laser powers. We have extensively discussed this in the main text. However, we are not completely sure where the longer binding times for certain imagers stem from. So, we decided not to speculate much on this aspect.

Main Text:

Long binding imagers have distinct advantages

Five of the six (R8 – R12) newly proposed imagers show longer binding times as compared to the other speed-optimized sequences (Figure 1f and Supplementary Figure 4c). These imagers have a few notable advantages over the shorter binding imagers. Firstly, with long binding imagers, one can achieve better imaging resolution (localization precision) owing to greater number of frames with binding events spanning the entire frame as compared to those showing shorter binding times (Figure 2a). The full frame events have better signal-to-noise ratio compared to partial frame events and thus improving the localization precision. We compared the ability of two imagers with different binding times: R1 with an average time of 282 milliseconds, and R8 with an average time of 678 milliseconds to resolve the 12 docking strand extensions (4x3 grid) separated by 20 nanometers arranged on a DNA origami nanostructure. At a low laser power density, imaging with R1 does not resolve the individual docking strands. On the other hand, the R8 imager resolves the 4x3 grid with $\sigma = 5.71$ nm, the standard deviation of the fitted Gaussian distribution to the localization signal, representing localization precision. At a high laser power density, R1 is able to resolve the grids with $\sigma = 5.16$ nm, whereas R8 resolves them with a considerably better precision of $\sigma = 3.79$ nm (Figure 2b). This showcases the advantages of longer binding imagers in achieving better resolution under both lower and higher laser power densities.

The long binding imagers provide additional flexibility by allowing imaging at lower laser power densities and slower frame rates (longer exposure). This allows the camera to collect more photons per binding event resulting in a localization precision comparable to that obtained with a short binding imager at ~ 8 -fold higher laser power density. This is crucial as higher laser powers often lead to laser induced damage and loss of docking strands over time.² Additionally, long binding imagers would generate a greater number of localizations at the same imaging frame rate upon each binding event. This would improve target sampling and thus require shorter acquisition times to adequately sample the targets at similar depths. We compared the target sampling on DNA origami nanostructures carrying 12 docking strands in a 4×3 grid. Considering a threshold of 20 localizations to sample a given target site, we observe that R8 shows faster sampling as compared to R1 while having comparable dark times (Figure 2c). Individual origami heatmaps confirm this trend across many origami structures (Figure 2d) making longer binding imagers faster at target sampling.

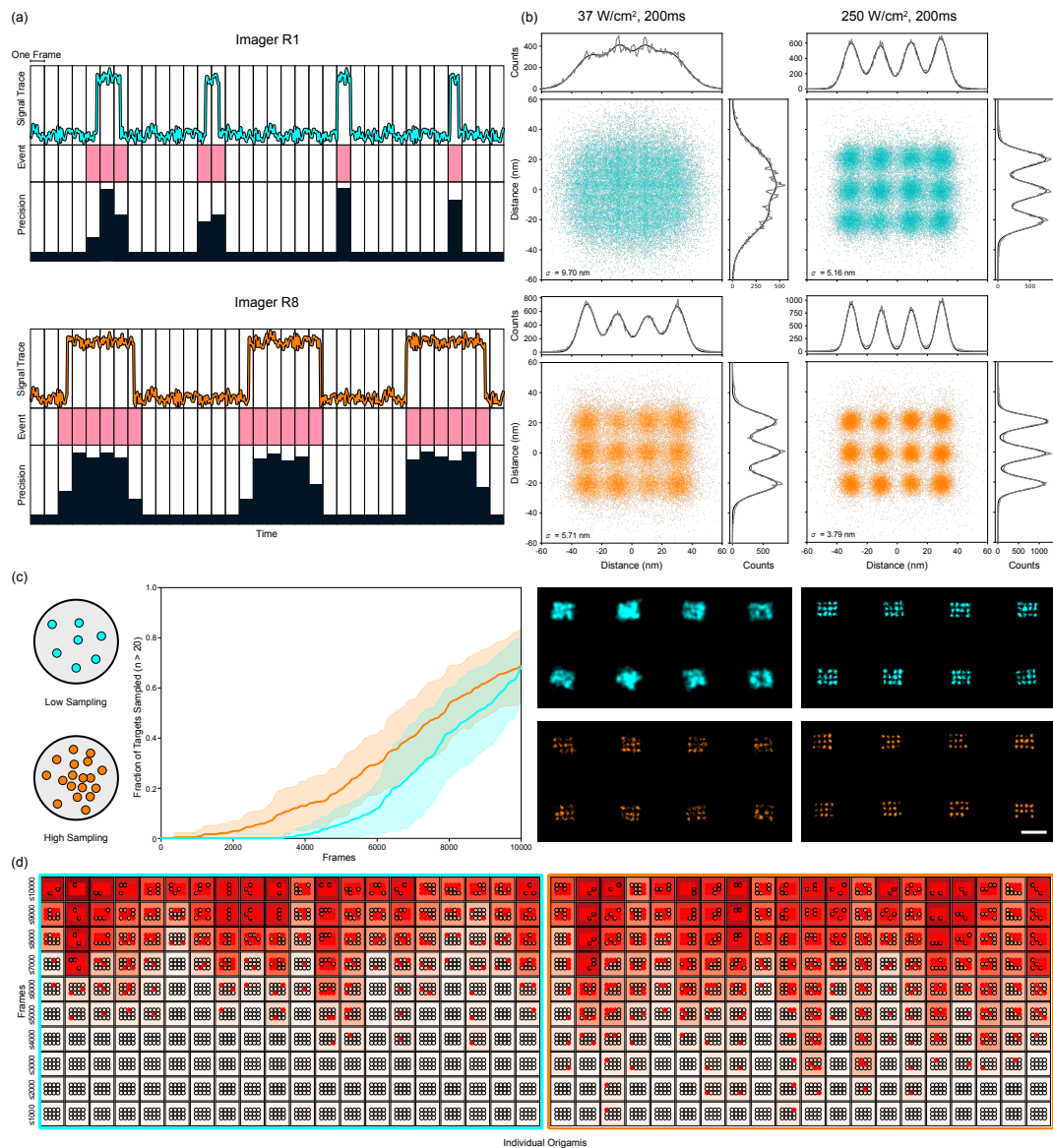


Figure 2: Longer binding imagers provide better localization precision and target sampling. (a) Schematic binding time traces for short (top) and long (bottom) binding

imagers. (b) Top: Average of patterned DNA origami nanostructure imaged with R1 (cyan) and R8 (orange) at 37 W/cm² (left) and 250 W/cm² (right). Bottom: Representative origami nanostructures imaged with R1 (cyan) and R8 (orange) at 37 W/cm² (left) and 250 W/cm² (right). (c) Fraction of targets sampled over time for R1 (cyan) and R8 (orange). (d) Time evolution heat maps of example origamis, left is R1 and right is R8. Solid red dots indicate a complete sampling with more than 20 localizations. (Scale: (b) 100nm)

5. I find the dark time quantification as important, can this be added in the main manuscript?

We thank the reviewer for showing interest in the dark times of these imagers. Although, it is a fundamental property of a DNA imager-docking strand sequence pair, we can tune the dark times by 1) varying the number of repeat blocks in the docking strand and 2) varying imager concentration. We have discussed the range of dark times showcased by these imagers in the main text and the data was already presented in Supplementary Figure 4 which we have now referenced in the main text.

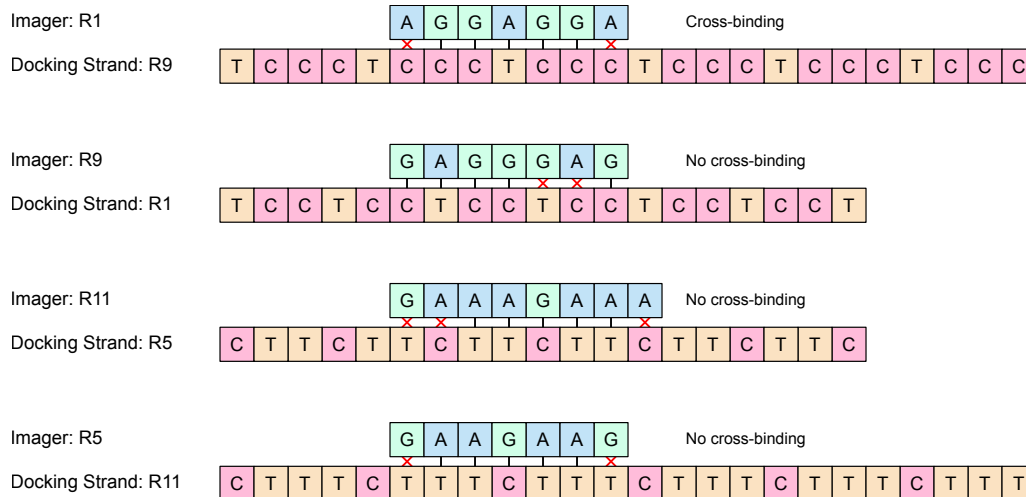
Main Text:

The speed of DNA-PAINT imaging is determined by the frequency of hybridization events between the imager and its corresponding docking strand, a parameter that we systematically analyzed. We observed that the average time between two consecutive binding events (dark time) of these imagers ranged between 10² to 10³ seconds on a single docking strand repeat at 1 nM imager concentration which is in agreement with the previous report (Supplementary Figure 4c). The dark times can be further tuned by modulating the length of concatenated docking strands. Short dark times allow for a higher frequency of binding events at lower imager concentrations reducing fluorophore background while concurrently improving sampling and the resolution of the image.

6. What is described as specific and unspecific binding Fig 1e? what is the number used as a threshold for classification and what are the numbers underlying the classification? Can the authors add a similar quantification of sequence orthogonality as in 100-fold speed-up.. paper Extended data Fig. 4? Couldn't this cross-binding be expected when overlaying the sequences?

We apologize for the lack of clarity in the text and captions. The numbers used as the thresholds were buried in the analysis code. All the necessary annotations and update to the methods section have also been performed to make this clearer. We have computed the average number of binding events on each DNA origami across all 144 combinations (12 imagers × 12 origamis) and normalized the number with the correct target origami. After visual inspection of all the data, we could clearly see that R1 shows signal on R9 origami and R2 shows signal on R10. Based on the visual inspection analysis, we decided to call these as non-specific signals which we did not observe for any other pairs. The fraction of non-specific localizations for R1 on R9 is 31.4% and for R2 on R10 is 14.4%. Based on these numbers, we have taken 10% as cut off for calling a signal as non-specific. As rightly pointed out by the reviewer, we plotted the distribution of the number of localizations per origami with a cut off as 10% for calling non-specific binding. We have added this plot as a supplementary figure (Supplementary Figure 3).

We are surprised to notice that the sequences showing crosstalk have a complimentary stretch of only five-nucleotides (four of which are G-C pairs). Interestingly, not all five-nucleotide complementarity show detectable cross-binding (see below image). This behavior is still a mystery and would need a deeper understanding on DNA hybridization to explain it.



Main Text:

...with sufficient stability allowing for detection of a binding event. This unexpected non-specific binding further highlights the limitations of secondary probe-based multiplexing approaches where longer probes are more prone to unpredictable non-orthogonality.

Methods:

All origamis were picked from the entire field of view and analyzed for the number of localizations coming from the same region during all imaging rounds. Random picks were generated at locations not closer than 260 nm from any other picks. Strip plots depicting the number of localizations arising from each channel over each origami were plotted to understand any obvious cross-talks. Average localization counts greater than 10% of the correct target counts was considered a cross talk if it arises from an orthogonal target. A heatmap for the same was plotted. This was performed using custom python codes.

7. Can they comment what previous highly multiplexed cellular/tissue DNA-PAINT experiments there are in the literature and compare?

Previous highly multiplexed experiments were performed using slow DNA-PAINT imagers or by using complex secondary probe-based strategies. As suggested by the reviewer, comparison of the same would be valuable as it would highlight the advantages and limitations of different approaches. Text for the same has been added in the relevant section. We have referenced following publications:

- 1) Rahman, F. *et al.* Mapping the nuclear landscape with multiplexed super-resolution fluorescence microscopy. *Nature Communications* **16**, 6042 (2025). <https://doi.org/10.1038/s41467-025-61358-0>

- 2) Agasti, S. S. *et al.* DNA-barcoded labeling probes for highly multiplexed Exchange-PAINT imaging. *Chemical Science* **8**, 3080-3091 (2017). <https://doi.org/10.1039/C6SC05420J>
- 3) Unterauer, E. M. *et al.* Spatial proteomics in neurons at single-protein resolution. *Cell* **187**, 1785-1800.e1716 (2024). <https://doi.org/https://doi.org/10.1016/j.cell.2024.02.045>
- 4) Schueder, F. *et al.* Unraveling cellular complexity with transient adapters in highly multiplexed super-resolution imaging. *Cell* **187**, 1769-1784.e1718 (2024). <https://doi.org/https://doi.org/10.1016/j.cell.2024.02.033>
- 5) M. S. Schröder, M.-L. I. E. Harwardt, J. V. Rahm, Y. Li, P. Freund, M. S. Dietz and M. Heilemann, Imaging the fibroblast growth factor receptor network on the plasma membrane with DNA-assisted single-molecule super-resolution microscopy, *Methods* 2021 Vol. 193 Pages 38-45. <https://doi.org/10.1016/j.ymeth.2020.05.004>

Introduction:

.....Although recent approaches using secondary³ or adapter DNA probes⁴ allow near-unlimited multiplexing capabilities, they require complex strand **design to avoid non-orthogonality along with tedious and often inefficient** hybridization and dissociation schemes.⁵ Thus, expanding the sequence space to enable faster imaging with greater multiplexing-ability remains a key challenge.

Main Text:

The true power of multiplexing can be exploited only when many cellular targets are visualized to map their spatial distribution within the cell. Earlier strategies to build a super-resolved map of many proteins were performed using non-optimized DNA-PAINT sequences, showcasing slow on-rates, thus requiring massive acquisition times. With the development of new speed-optimized sequences, we can now push the boundaries of multiplexed cellular imaging to more than six targets in shorter time scales.

8. What does Actinomycin D do, I am missing this explanation when the experiment is introduced.

We agree that this was missing in the text. We have added more text to outline the effects of Actinomycin D on cellular transcription. Here is the text: “We treated cells with Actinomycin D (Act D), a known drug that stably intercalates with the genomic DNA and inhibits transcription elongation.”

Main Text: We treated cells with Actinomycin D (ActD), a known drug that stably intercalates in the genomic DNA and inhibits transcription.

9. The correlation methods used to quantify the cell imaging data should be very briefly introduced/explained in the text, and more so in the methods part with references to show that these are appropriate.

We thank the reviewer for pointing out the incoherence in text with the two broad correlation analysis tools we have used to better understand the vast data generated with our Imaging modality. We have added relevant text in the sections to better explain the two types of correlation analysis.

In the main text under figure 4, we mentioned about global correlation: To quantitatively interpret our data, we employed a coarse-grained correlation analysis between target pairs (see Methods) comparing the number of localizations in each pixel across different protein pairs.

In addition, we also added following text describing Spearman's spatial correlation: This analysis involves finding the Spearman's correlation between the density of two targets encompassed by concentric donuts centered over a given localization. This measurement, also called DoC (Degree of Colocalization), provides a suitable measure of spatial correlation that can be attributed to each localization

More elaborated explanation is provided in the Methods sections.

Reviewer #2 (Remarks to the Author):

The authors introduce an interesting resource of DNA-PAINT sequences for high-speed multiplexed DNA-PAINT. This work seems a complement to the work described in the Brief Communication by Strauss and Jungmann in 2020, providing 6 additional sequences albeit some of them are not compatible so up to 10 might be used in a single experiment. While expanding the space of DNA sequences to allow for more complex Exchange-PAINT experiments is useful and the manuscript shows that they extensively tested them – especially on DNA origamis –, the work could benefit from some more innovative approaches to DNA-PAINT, and by itself constitutes more of a resource than an original piece of work. In any case, we value that the authors are trying to make additional sequences available for fast exchange-PAINT experiments as we agree that FLASH-PAINT and SUM-PAINT experiments, that depend on several steps of strand hybridization and disassociation, can be exceptionally complex.

We would like to thank the reviewer for their positive assessment of our manuscript. The answers to specific concerns are detailed below.

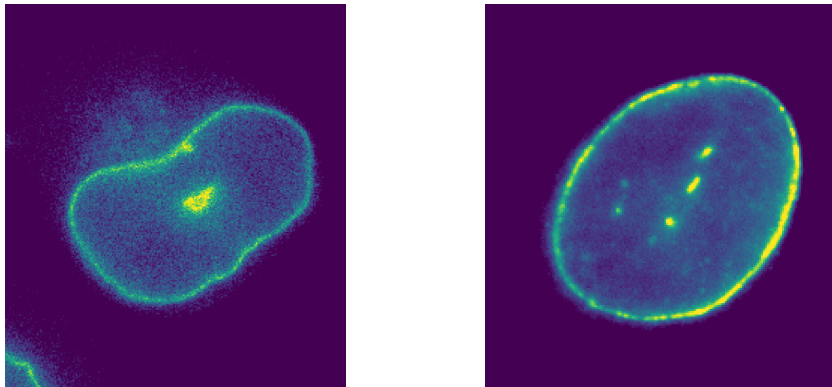
1. While the results in DNA origamis seem good, we have some major concerns about the controls used to prove the validity of the experiment in vivo, especially given the number and complexity of nuclear targets. Supplementary 4 shows that the authors used Lamin B1 as a control, and kept the sequences that produced reasonable lamin B1 distributions, namely all of them except R3, that was ruled out because it also produce signal in the nucleoli. Can the authors provide an explanation to why this might be happening?

We don't really have a reasonable explanation why R3 was showing non-specific binding within the nucleoli. We note that it is not binding on RNA as RNase treatment of cells did not really cause any change in R3 binding (Data not shown). However, we now confirm that it is binding on nucleoli by imaging R3 alone with DNA-PAINT and immunofluorescence staining of Fibrillarin which is marker for nucleolus. Corresponding figure is added in the supplementary information (Supplementary Figure 7).

Supplementary Figure 7: R3 imager shows off target binding to the nucleolus. (a) DNA-PAINT image with R3 imager alone (10000 frames; 1000 s); (b) Immunofluorescence image of Fibrillarin using ATTO647N labelled secondary antibody; (c) Overlay of (a) and (b); Scale bar: 10 μ m

2. R5, R7 and R8 also display weird spots in the images, is this just an artefact or something the authors should consider?

These are expected signals for Lamin. This is there in literature and for further supporting the view, we have done immunofluorescence imaging of Lamin B1. We observe signals within the nucleus as expected, confirming this is not due to any artifacts of DNA-PAINT.



Left: 1st anti-Lamin B1 Antibody and -2nd Anti-rabbit antibody immunofluorescence data. Right: 1st anti-Lamin B1 Antibody and -2nd Anti-rabbit NANOBODY data. Lamin B1 Z-projections

3. We are also concerned that only using Lamin B1 as a control for the DNA PAINT strands might result in a partially biased result, given the exceptional intensity of the lamin B1 signal in the nuclear periphery that might increase the signal to noise ratio to levels that are unachievable when targeting any other nuclear protein. Can the authors provide negative controls for other targets in the nucleus, particularly some of the histone marks? That is, performing DNA-PAINT without including the primary antibody.

We agree that the controls were absent. We have now performed all the controls as suggested by the reviewer. There is no unexpected signal that we see other than homogenous non-specific binding of imager across nucleus. We performed negative controls as suggested by the reviewer without including the primary antibodies. We are happy to report that we saw minimal nuclear localizations for all imagers used in our multiplexing assay. As a positive control, we performed DNA-PAINT imaging of each of the individual targets and observe a clear nuclear signal for all targets in presence of the primary antibody (Supplementary Figure 8-10)

Main Text:

A significant challenge in conventional immunofluorescence-based imaging of nuclear targets is the diffuse signal obtained for the target because of their distribution across the entire nuclear volume. We strategized to exploit these sequences to delineate the nanoscale spatial distribution of various nuclear features. The nuclear environment

poses an unavoidable challenge to DNA-PAINT due to the abundance of chromatin polymer which can often lead to spurious off-target binding events resulting in a homogenous artefact in the data. Due to the propensity of R3 imager to generate off-target nucleolar localizations, it was omitted from the multiplexing imager pools. We tested all the imagers for their propensity to generate off-target nuclear localizations. In absence of a corresponding primary antibody, the docking strand-conjugated nanobody alone controls showed sparse nuclear localizations. In contrast, the presence of primary antibodies showed prominent nuclear localizations. (Supplementary Figure 8, 9 and 10). These data support the applicability of imager sequences for multiplexed imaging in the challenging environment of the nucleus.

As described by Lucinskaite et al. (<https://www.biorxiv.org/content/10.1101/2024.06.03.597143v1>), imaging nuclear targets through DNA-PAINT is specifically challenging because of non-specific signal in the cell nucleus. We wonder how the proposed DNA sequences deal with this phenomenon, as the non-specific signal is often extremely similar to that produced by proteins that are more homogeneously distributed in the nucleus than Lamin B1.

We are unsure what was causing such unexpected non-specific binding in the above preprint. In our hands, we do not really observe as much background as they reported (Supplementary figure 8-10). We note that the preprint utilized earlier DNA sequences. This could be because of the sequence of choice. For instance, we did observe R3 binding non-specifically on nucleolus. However, we refrain to comment on this as this might have stemmed from any other reason including buffer conditions or sample preparation.

3. We are also surprised by the Lamin B1 redistribution identified upon actinomycin D-mediated transcription inhibition and fear that it could be a result of a not good DNA-PAINT experiment. Can the authors justify, or at least hypothesize, why such phenotype could occur? We are also very concerned by the fact that while Lamin B1 seems homogeneously distributed in the cell nucleus upon transcription inhibition in Figure 2 (right), there is still a significant amount of signal in the nuclear periphery in Supplementary Fig. 7. Is this a consequence of different colors used in the representation, or the experiments in an Exchange-PAINT experiment using the proposed sequences can differ that much to the ones aiming to target a single protein? This is a major issue the authors should clarify.

We fully agree with the reviewers and appreciate this valuable observation, which prompted us to re-evaluate our data. Through additional control experiments, we realized that the imagers exhibited non-specific binding across the nucleus, which had compromised our initial analysis. Although the cause of this behavior was unclear, we found that including cysteamine, a reducing agent commonly used in STORM imaging, in the imaging buffer completely eliminated the non-specific binding. We subsequently repeated all experiments, including controls, using cysteamine-containing buffers and replotted the data. The corrected results show no detectable redistribution of Lamin B1 upon actinomycin D treatment.

We do, however, observe certain chromatin reorganizations upon transcription inhibition. Specifically, nuclear speckles lose contacts with chromatin and adopt a more circular

morphology. Additionally, the signals corresponding to H3K4me3 and H3K27Ac are markedly reduced, approaching baseline levels comparable to imager-only controls. These findings were confirmed through independent control imaging experiments and are consistent with previously reported observations (Supplementary Figure 25).

Main Text:

We detected profound nanoscale changes in the chromatin organization upon ActD treatment. Notably, nuclear speckles appeared more circular compared to the amorphous structures observed under mock conditions. The amorphous speckles arise in the untreated cells via chromatin tethering to nuclear speckles because of co-transcriptional splicing⁶. However, transcription inhibition by ActD halts new RNA synthesis, disrupting the chromatin tethering, hence the nuclear speckles morphology appears as circular (Figure 3c, Supplementary Figures 17 to 23).

Interestingly, inhibition of transcription by ActD leads to reduction in overall levels of H3K27ac and H3K4me3^{7,8} among all the targets imaged. We confirmed this result by individual targets imaging after ActD treatment (Supplementary Figures 24 to 26). We therefore omitted H3K27ac and H3K4me3 for further correlation analysis for ActD treated cells (Fig 4a-top diagonal, Supplementary Figures 17 to 23). While the nuclear speckles positively correlated with euchromatin marks in untreated cells, inhibition of transcription lead to negative correlation between speckles and all chromatin modifications (Figure 4a-top diagonal, Supplementary Figures 17 to 23). For instance, heterochromatin marks displayed increased overall positive correlation with the nuclear lamina. These results establish the robustness of the coarse-grained analysis in capturing the chromatin redistributions in a single cell.

5. The spatial correlation viewpoint analysis presented in Figure 3 and Supplementary Figures 8 and 9 seems weak and some of the conclusions extracted not sustained. For example, H3K27ac shows increased correlation with all other targets excepting SC35 upon transcription inhibition (Supplementary Fig. 9), including those that were pertaining to the same euchromatin fraction. How do the authors explain this instead of a general blending of interactions?

We provide fresh data obtained using modified buffer conditions and the pairwise cross correlation analysis (PCCF). The new data is consistent with existing literature. We have extensively described about it in the text and cite published literature where required.

Main Text:

Interestingly, inhibition of transcription by ActD leads to reduction in overall levels of H3K27ac and H3K4me3^{7,8} among all the targets imaged. We confirmed this result by individual targets imaging after ActD treatment (Supplementary Figures 24 to 26). We therefore omitted H3K27ac and H3K4me3 for further correlation analysis for ActD treated cells (Fig 4a-top diagonal, Supplementary Figures 17 to 23). While the nuclear speckles positively correlated with euchromatin marks in untreated cells, inhibition of transcription lead to negative correlation between speckles and all chromatin modifications (Figure 4a-top diagonal, Supplementary Figures 17 to 23). For instance, heterochromatin marks displayed increased overall positive correlation with the nuclear

lamina. These results establish the robustness of the coarse-grained analysis in capturing the chromatin redistributions in a single cell.

Because nucleus is a highly dense and inhomogeneous compartment containing diverse nuclear bodies, global correlation analysis produces averaged correlations that obscure specific local interactions. To overcome this limitation, we performed spatial correlation analysis between each pair of targets. For this, we computed the pairwise cross-correlation function (PCCF) between target pairs. The PCCF is a measure of the probability of finding a target at a certain distance with another target when compared to a random distribution. A PCCF value of one signifies a random distribution of the target with respect to the other target used as the viewpoint. A value higher than one at a given spatial distance signifies the spatial correlation or interaction between the two targets. A value lower than one indicates a negative spatial correlation or mutual exclusion between the targets. We observed a clear positive correlation between all four euchromatin markers and SC35. Concurrently, a negative correlation was observed for heterochromatin markers and nuclear lamina from SC35 viewpoint (Figure 4b-SC35 viewpoint). On the other hand, from the lamina point of view, heterochromatin marks displayed a positive correlation, whereas both speckles and euchromatin showed diminished interactions with it (Figure 4b-Lamin viewpoint)."

"We note that the DoC analysis contradicts with PCCF analysis where SC35 and euchromatin showed positive correlations. This apparent contradiction likely arises from the peripheral arrangement of active transcription and chromatin around nuclear speckles.⁹ This arrangement leads to decreased localizations of SC35 in the concentric donuts around the periphery of speckles with a corresponding increase in localizations of the active transcription and chromatin marks, resulting in the negative Spearman's correlation or DoC between them.

6. What's the criterion to decide when the enrichment is high and when it isn't? The difference in enrichment in untreated cells of H3K27me3 with respect to lamin B1 and SC35 in Fig. 3 c (top) is less than 3% (18.34% vs 15.55%, respectively), but the authors only claim that the enrichment occurs between H3K27me3 and lamin B1 contacts. The authors should think of a metric to measure the significance of these changes to be able to state that such differences exist.

We appreciate the reviewer's suggestion. Following this comment, we performed an additional quantitative analysis using the Pairwise Cross-Correlation Function (PCCF), which provides a clearer measure of interaction specificity. This analysis robustly captures the preferential associations between heterochromatin and the nuclear lamina, and between euchromatin and nuclear speckles, under both untreated and transcription inhibition conditions.

Main Text:

Because nucleus is a highly dense and inhomogeneous compartment containing diverse nuclear bodies, global correlation analysis produces averaged correlations that obscure specific local interactions. To overcome this limitation, we performed spatial correlation

analysis between each pair of targets. For this, we computed the pairwise cross-correlation function (PCCF) between target pairs. The PCCF is a measure of the probability of finding a target at a certain distance with another target when compared to a random distribution. A PCCF value of one signifies a random distribution of the target with respect to the other target used as the viewpoint. A value higher than one at a given spatial distance signifies the spatial correlation or interaction between the two targets. A value lower than one indicates a negative spatial correlation or mutual exclusion between the targets. We observed a clear positive correlation between all four euchromatin markers and SC35. Concurrently, a negative correlation was observed for heterochromatin markers and nuclear lamina from SC35 viewpoint (Figure 4b-SC35 viewpoint). On the other hand, from the lamina point of view, heterochromatin marks displayed a positive correlation, whereas both speckles and euchromatin showed diminished interactions with it (Figure 4b-Lamin B1 viewpoint).

7. Additionally, in Fig. 3c (bottom), in spite of the stated redistribution of lamin B1, the colocalization of such target with the heterochromatin markers H3K9me3 and H3K27me3 increases. How does this agree with a general redistribution of the protein?

We sincerely apologize to the reviewers for our initial data that showed unexpected redistribution of Lamin B1 upon transcription inhibition which was because of imager non-specific binding. The improved buffer conditions helped us to abolish these spurious localizations. However, the new data shows that there is a general trend in positive spatial correlation of Lamins and heterochromatin marks but Lamin and euchromatin marks correlate negatively.

Main Text:

We detected profound nanoscale changes in the chromatin organization upon ActD treatment. Notably, nuclear speckles appeared more circular compared to the amorphous structures observed under mock conditions. The amorphous speckles arise in the untreated cells via chromatin tethering to nuclear speckles because of co-transcriptional splicing⁶. However, transcription inhibition by ActD halts new RNA synthesis, disrupting the chromatin tethering, hence the nuclear speckles morphology appears as circular (Figure 3c, Supplementary Figures 17 to 23).

Interestingly, inhibition of transcription by ActD leads to reduction in overall levels of H3K27ac and H3K4me3^{7,8} among all the targets imaged. We confirmed this result by individual targets imaging after ActD treatment (Supplementary Figures 24 to 26). We therefore omitted H3K27ac and H3K4me3 for further correlation analysis for ActD treated cells (Fig 4a-top diagonal, Supplementary Figures 17 to 23). While the nuclear speckles positively correlated with euchromatin marks in untreated cells, inhibition of transcription lead to negative correlation between speckles and all chromatin modifications (Figure 4a-top diagonal, Supplementary Figures 17 to 23). For instance, heterochromatin marks displayed increased overall positive correlation with the nuclear lamina. These results establish the robustness of the coarse-grained analysis in capturing the chromatin redistributions in a single cell.

.....We observed a clear positive correlation between all four euchromatin markers and SC35. Concurrently, a negative correlation was observed for heterochromatin markers and nuclear lamina from SC35 viewpoint (Figure 4b-SC35 viewpoint). On the other hand, from the lamina point of view, heterochromatin marks displayed a positive correlation, whereas both speckles and euchromatin showed diminished interactions with it (Figure 4b-Lamin B1 viewpoint).

8. Apart from this, we consider that the manuscript could benefit from several clarifications, including those referring to:

- Supplementary Figure 1 a: using 4 nt blocks could make it clearer to compare with 1 c

We added this extra schematic to Supplementary Figure 1

- Supplementary Figure 1 d: further clarification is needed to understand the mismatches between imaging strands.

These are five-nt block repeat docking strands. We intended to show that they will have non-specific interactions with imagers against four- or three-nt block repeats with one or two mismatches. Hence, more speed-optimized docking strands are not possible than we already present in the manuscript.

- Figure 3 b: additional explanations are required to understand the figure, it is extremely obscure as it is.

We agree with the reviewer that the figure is difficult to understand. We removed the figure to avoid any confusion as the same information is carried from PCCF analysis.

- Figure 3c: how does this analysis differ from the one done in Supplementary figures 8 and 9?

Figure 3C is now Figure 4C and 4D. The figure 4C and 4D are analyzed at selected regions of the nucleus that is highlighted in the figure itself. Supplementary figures 8 and 9 show full nucleus viewpoint analysis. We now provide the individual nucleus analysis for all nuclei.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We would like to thank you for the insightful review. We addressed all the concerns raised.

- 1 Hertel, S. *et al.* The stability and number of nucleating interactions determine DNA hybridization rates in the absence of secondary structure. *Nucleic Acids Research* **50**, 7829-7841 (2022). <https://doi.org/10.1093/nar/gkac590>
- 2 Blumhardt, P. *et al.* Photo-Induced Depletion of Binding Sites in DNA-PAINT Microscopy. *Molecules* **23**, 3165 (2018).

- 3 Unterauer, E. M. *et al.* Spatial proteomics in neurons at single-protein resolution. *Cell* **187**, 1785-1800.e1716 (2024).
<https://doi.org/https://doi.org/10.1016/j.cell.2024.02.045>
- 4 Schueder, F. *et al.* Unraveling cellular complexity with transient adapters in highly multiplexed super-resolution imaging. *Cell* **187**, 1769-1784.e1718 (2024).
<https://doi.org/https://doi.org/10.1016/j.cell.2024.02.033>
- 5 Lycas, M. D. & Manley, S. DNA-PAINT adaptors make for efficient multiplexing. *Cell Reports Methods* **4** (2024). <https://doi.org/10.1016/j.crmeth.2024.100801>
- 6 Raina, K. & Rao, B. J. Mammalian nuclear speckles exhibit stable association with chromatin: a biochemical study. *Nucleus* **13**, 58-73 (2022).
<https://doi.org/10.1080/19491034.2021.2024948>
- 7 Martin, B. J. *et al.* Transcription shapes genome-wide histone acetylation patterns. *Nature Communications* **12**, 210 (2021).
- 8 Rybtsova, N. *et al.* Transcription-coupled deposition of histone modifications during MHC class II gene activation. *Nucleic acids research* **35**, 3431-3441 (2007).
- 9 Bhat, P. *et al.* Genome organization around nuclear speckles drives mRNA splicing efficiency. *Nature* **629**, 1165-1173 (2024).

REVIEWER COMMENTS

Black text: Reviewers comments

Green text: Our response to reviewers' comments

Red text: New text added to the manuscript based on reviewers' suggestions.

Blue text is from initial manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

General comment:

I am satisfied with the revisions in response to my comments, and the additions to the main text are very useful. I share the sentiment of the additional referees that this paper is a useful additional resource for multiplexing using the same concept introduced before in ref 10 but does not constitute a new strategy for fast DNA-PAINT.

We thank the reviewer for the agreeing with revisions made to our manuscript. Indeed, FLASH-PAINT is identical to our strategy in terms of acquisition rates when coupled with speed sequences. However, our approach avoids the additional complexity of designing and verifying orthogonal transient adaptor probes.

“Although recent approaches using secondary or adapter DNA probes allow near-unlimited multiplexing capabilities, they require complex strand design to avoid non-orthogonality along with tedious and often inefficient hybridization and dissociation schemes”

“This expanded pool of speed-optimized imager-docking strand sequences will unlock the full potential of DNA-PAINT imaging's multiplexing capabilities. Additionally, the adapter-strand-based “unlimited” multiplexing can be done in batches of ten instead of six targets per labeling round which would drastically reduce the time required for multiplexing.”

“We achieved similar imaging speeds as compared to FLASH-PAINT¹, and better than SUM-PAINT² that utilized existing speed-optimized imagers. However, this is significantly faster compared to Exchange-PAINT modality using the classical imager-docking strand pairs³⁻⁵.”

Response to the more detailed comments in the revision (N.A. means that the revision/response is fine by me):

1. N.A.

2. N.A.

3. Please double-check that the SI tables are also referenced somewhere in the manuscript.

Supplementary Table 1 has been referenced in Main Text. 2, 3 and 4 have been referenced in Methods.

4. This addition to the main text is mostly very clear. Why is the sampling in Figure 2c for low sampling not also lower for the last timepoint?

In our analysis, we have defined 20 localizations at each docking strand on the 4x3 DNA origami nanostructure as the minimum for identifying it as sampled strand. Long binding imagers generate a greater number of localizations for each binding event at the same acquisition rate, thus reaching the threshold sooner. In contrast, shorter binding imagers generate fewer localizations, thus taking longer time to reach the threshold. Over longer imaging durations, the shorter binding imagers would eventually attain the sampling threshold. This is what is clearly seen towards the end of our imaging duration.

“Number of localizations on every origami at each docking strand was counted across frames. A complete target sampling was considered only when more than 20 localizations are present on the docking site. Target sampling was measured over time and was plotted. Heatmaps for 20 exemplary origamis representing the sampling evolution over every 1000 frames were plotted.”

5. N.A.

6. N.A.

7. N.A.

8. N.A.

9. N.A.

Thank you again for your valuable time in reviewing our manuscript. We appreciate your efforts.

Reviewer #2 (Remarks to the Author):

We want to thank the authors of the paper for addressing our comments. Nevertheless, we still have some major concerns about the quality of the experiments and the validity and relevance of the biological conclusions extracted from them.

We appreciate the reviewer's detailed feedback but believe there may be some misunderstanding regarding our data presentation and conclusions. To clarify: our primary contribution is the development of new speed-optimized DNA-PAINT imager sequences. As a proof-of-concept, we applied these alongside existing imagers for

multiplexed Exchange-PAINT imaging of euchromatin, heterochromatin, and transcription marks under mock and transcription-inhibition conditions.

Our custom data analysis pipelines robustly capture the expected chromatin rearrangements upon transcription inhibition, as shown in the data. We do not introduce novel biological conclusions; rather, we demonstrate that our approach reliably detects established organizational changes in the chromatin organization. We addressed remaining concerns below.

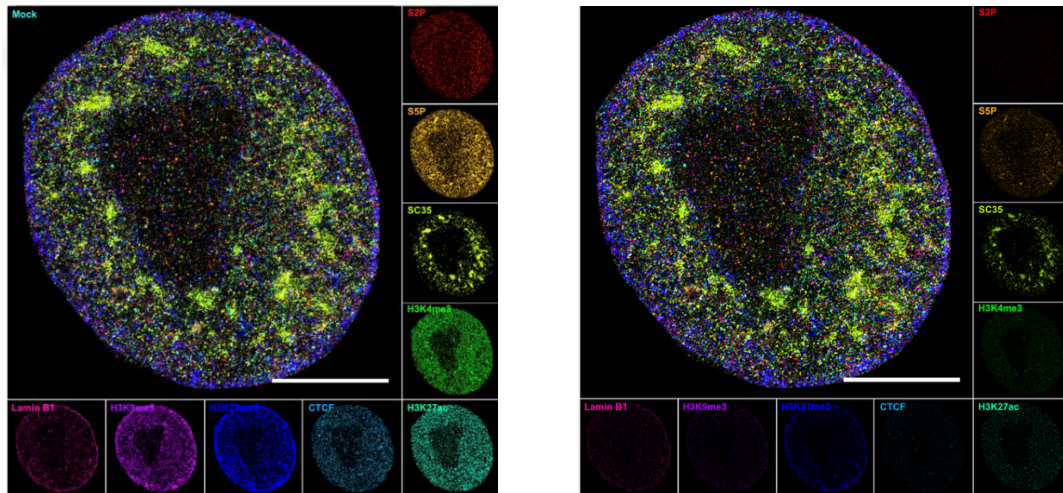
1. The authors provide negative and positive controls for each one of the nuclear targets shown in Figure 3 in Supplementary Figures 8-10. Observing the nucleolar shape and observed structures (for example when imaging SC35 and H3K4me3) it seems that the cell in the positive control is the same one displayed in Figure 3b. Are the authors using the same images / cell shown there as positive controls? If this is the case, why is the signal intensity so different in Supplementary Figure 10h (H3K9me3) and the H3K9me3 channel in Figure 3a? In the latter, the signal seems much more intense, even if the nucleolus shape is the same.

We understand the concern raised by the reviewer. We are indeed using the same cell as a positive control in Supplementary Figure 8-10. The imaging parameters for the negative control experiment using nanobody alone are identical to the cell in Figure 3b. This allows for direct comparison and unbiased visualization of the data for the presence of any off-target artefacts. To better compare the nanobody controls, we utilized the same color map across all channels and displayed all targets with the same contrasts, enabling a one-to-one comparison. In multiplexed images (e.g., Fig. 3b with cell masking), however, each channel requires adjusted contrast and color schemes to clearly visualize all targets, particularly weaker signals. As a common practice in fluorophore-based multi-target imaging, one uses different contrast levels across different color channels to make all targets visually comparable.

Similarly, in DNA-PAINT, the number of localizations acquired depends on a myriad of factors including target abundance, labelling efficiency, imager concentration, and imager binding times. As these all parameters vary across different targets, imagers and imaging rounds, one must relatively scale the visual display to portray all targets at human perceivable contrasts to be able to visually compare them. All the analysis on the other hand is performed on the raw localization counts that are unaffected by these display settings. We have attached a visual render of the cell shown in Figure 3b without scaling the relative intensities of individual targets below. This image clearly shows that it is not possible to visually appreciate the differences in the organization of these targets if they all are viewed with the same contrast.

Using identical imaging parameters, we ensured that our negative controls could be displayed identically to provide a better visualization of the specific labelling of the targets in the nucleus. This can be clearly observed in Supplementary Figures 8-10 where

the display parameters have been kept identical between the positive and negative controls to display the specific nuclear signal for all nine nuclear targets.



Left: Figure 3b from manuscript with different contrasts for each panel for visualization. **Right:** The same panels with same contrast for all the panels. This led to poor visual clarity. Hence, we adopted Left figure for the manuscript.

- Moreover, many of the positive controls (antibody with nanobody) in Supplementary Figures 8-10 show extremely unspecific signal outside of the nucleus, which was not visible in Figure 3 because the nuclei were cropped out. For example, Supplementary Figure 8a shows almost the same signal within the nucleus and in the cytoplasm for Ser2P. It is hard to extract significant conclusions in the images. Similar noisy signals are observed in Supplementary Figure 8b (Ser5P), Supplementary Figure 9f (CTCF). Supplementary Figure 24a (ser2P, mock) and Supplementary Figure 25e (H3K4me3, mock and ActD) also show very noisy signals. This is specially worrying when imaging histones, given that the signal within the nucleus should be much more intense than in the cytoplasm. As mentioned in the initial review of the manuscript, Supplementary Figure 5 shows that the lamin signal is quite strong in the nucleus periphery but also it also shows a lot of noise in the cytoplasm (see R1, R2, R4, R9, R10). When imaging targets that do not accumulate that clearly in specific regions of the nucleus (such as histone marks), many of the sequences may not have enough quality to draw valid conclusions.

We would like to note that it is not completely unexpected to see cytoplasmic signals for nuclear targets. We want to bring the reviewer's attention to the published data which always show signal in the cytoplasm of varying degrees depending on the target being imaged. This could be because the target might be present outside the nucleus or antibody adsorption. We are sure that the non-nucleus signal is not stemming from nanobody staining or imager non-specific sticking as clearly demonstrated with our control experiments (Supplementary Figure 8-10). Here is the list of a few recent publications (PMID: 34297911, 40593784, 40773232) where cytoplasmic localizations

were visible for nuclear targets. Since we want to analyze the interactions of targets inside the nucleus only, we adopted the widely used practice to generate nuclear masks to avoid analyzing cytoplasmic localizations (See the citations mentioned above).

Although the nuclear signal of Ser2P in the positive control appears weak in Supplementary Figure 8a, we see a clear nuclear enrichment of the same target in mock cells as seen in Supplementary Figure 24a. Note that we analyzed seven replicates for the Ser2P signal with varying degrees of nuclear enrichment. The variation is likely due to the regular cell-to-cell variability across each replicate.

We would again like to emphasize the observation that in the absence of the primary antibody, there are minimal off-target localizations for each of the imagers used for cellular multiplexing, confirming that the cytoplasmic signal is likely because of antigen presence or antibody adsorption but not due to nanobody or imager non-specificity.

3. Some other images show inconsistencies. For example, Supplementary Figure 15a does not seem to show the nucleolar structure that is seen in the channels for H3K9me3, H3K27me3 and CTCF channels. In the other images (before transcription inhibition) all channels seem to display nucleoli.

We agree that the nucleolus is not clearly visible in some of the target images. But upon a closer look, one can observe the void in the signal where the nucleolus is present. The varied visibility is likely due to the cell-to-cell variability. We want to note that we are not drawing any conclusions based on any single cell data.

4. In line 201, authors say that H3K27me3 and H3K9me3 show negative correlation with nuclear speckles and RNA polymerase marks (S2P and S5P). The plot in Figure 4a does not appear to show this and instead shows these histone marks are positively correlated with S5P. Similarly, in line 222 the authors claim that nuclear speckles positively correlate with euchromatin marks in untreated cells, but Figure 4a shows otherwise, being the highest correlation with H3K27ac in a color that seems close to 0, at best.

We appreciate this detailed comment. Indeed, the global correlation analysis provided in Figure 4a does not fully capture the interactions of target species. As a result, in the revised version of our manuscript we adopted alternative analysis strategies which capture the local interactions more robustly. We were perhaps not very clear in the revised manuscript about the same. Now, we have added text to emphasize that the global correlation is not perfect which led us to provide PCCF and DoC. We have extensively discussed these analysis pipelines in the manuscript.

“Because nucleus is a highly dense and inhomogeneous compartment containing diverse nuclear bodies, global correlation analysis produces averaged correlations that obscure specific local interactions. This is especially noticeable in the observed apparent positive correlation between RNAPolIII-S5P and the transcriptionally silent

heterochromatin marks and the lack of a clear positive correlation between nuclear speckles and the euchromatin marks (Figure 4a (below diagonal)). Additionally, since the coarse-grained approach derives information by dividing the sample into grids of a fixed dimension, it fails to accurately capture the relative spatial patterning of the targets at longer length scales in the complex nuclear environment. To overcome this limitation, we performed spatial correlation analysis between each pair of targets to better understand their relative organization in the nuclear volume.”

5. The authors include a new analysis in Figure 4, but it is poorly described in the text making hard its interpretation. Specifically, Figure 4c is very sparsely commented, and many of the results extracted from it are not referenced to any specific panel or section of the figure. For example, can the authors comment results from the Lamin B1 viewpoint? Are the results referring to the mock or ActD case? In this current state it's very hard to interpret, especially when one of the few conclusions made contradicts what is shown in Figure 4b.

We thank you for raising this important concern regarding lack of in depth discussion over analysis of the data which we have elaborated in the manuscript.

“A value lower than one indicates a mutual exclusion between the targets. This analysis further supports the trends seen in the global correlation analysis where heterochromatin interactions with the nuclear lamina are minimally perturbed by transcription inhibition (Figure 4b- Left). Importantly, this analysis unravels a more detailed picture of interactions between the nuclear speckles and other euchromatin marks, showing higher enrichment among them, which were obscured in the global correlation analysis. We observed a clear local enrichment between all four euchromatin marks and SC35 emphasizing the spatial correlation between actively transcribed euchromatin and the splicing machinery. Concurrently, nuclear speckles were mutually excluded from the heterochromatin markers and nuclear lamina (Figure 4b-SC35 viewpoint). During the transcription inhibition, the PCCF analysis, similar to global correlation analysis, captures the loss of enrichment of all chromatin marks with nuclear speckles (Figure 4b-SC35 viewpoint).”

“This measurement, also called DoC (Degree of Colocalization), provides a suitable measure of spatial correlation that can be attributed to each localization⁶. DoC reveals the fraction of localizations from each target showing positive (>0.7) and negative (<0.7) correlation with the localizations of the target set as the viewpoint. A higher fraction of positively correlated localizations signifies spatial co-existence of the targets while a higher fraction of negatively correlated localizations suggests mutual exclusion. Such spatial correlation maps enable us to discern regions where interactions are significantly higher and correlate better with the target of interest.”

“Interestingly, SC35 displayed a higher fraction of localizations showing negative correlation with the euchromatin markers as was also observed in the coarse-grained

analysis (Figure 4c center –SC35 Viewpoint). This observation contradicts with PCCF analysis where SC35 and euchromatin displayed clear positive enrichment. This apparent contradiction likely arises from the peripheral arrangement of active transcription and chromatin around nuclear speckles.⁷ This leads to a gradual decrease in localizations of SC35 in the concentric donuts around the periphery of speckles with a corresponding increase in localizations of the active transcription and chromatin marks. Consequently, a greater fraction of these localizations appears to be negatively correlated with the localizations of SC35. Such interactions where the targets that are mutually excluded from each other but peripherally interact such as nuclear speckles and euchromatin marks are robustly captured by PCCF analysis.”

“ We note that global correlation analysis provides overall picture of interactions among all the targets, however masks local interactions which could be efficiently captured with DoC or PCCF analysis. Both DoC and PCCF analyses emphasize distance-dependent relative distribution of target pairs providing a comprehensive understanding of their spatial organization and redistribution upon perturbation of cellular processes.”

6. The authors should also specify which of these analyses are done on just one cell or in many cells, indicating number of cells analyzed for each experiment. If experiments are carried out for only one cell, the authors should try to find a metric that can group the results from many cells to improve robustness.

Thank you for pointing this oversight from our side. Figure 4a and 4b are derived from seven mock and eight ActD treated cells. Figure 4c and 4d are on single nucleus at the specified region. All DoC analysis is done on individual cells. Additionally, we provided single nucleus analysis for each cell in the Supplementary information. The metric we used for this analysis is the mean of all the cells, both for global correlation and PCCF, which is now mentioned in the figure captions.

7. Some of the biological changes observed upon transcription inhibition are not novel, such as the reshaping of nuclear speckles (reviewed in <https://doi.org/10.1242/jcs.259594>), limiting relevance of the functional study.

We would like to clarify that we were not claiming the novelty in our observation of alterations in speckle morphology. We rather wanted to emphasize that our multiplexed imaging robustly captures the expected changes in the chromatin organization upon transcription inhibition. To this extent, we have cited existing literature (<https://www.tandfonline.com/doi/full/10.1080/19491034.2021.2024948#d1e322>). We have now cited the review suggested alongside other publications.

“Earlier studies support that amorphous speckles arise in the untreated cells via chromatin tethering to nuclear speckles via co-transcriptional splicing allowing for constant exchange of material between the splicing factories and chromatin^{8,9}. However, transcription inhibition by ActD halts new RNA synthesis, disrupting the chromatin

tethering, hence making the nuclear speckles' morphology appear round (Figure 3c, Supplementary Figures 17 to 23)⁸⁻¹⁰. “

We hope we have addressed all the concerns satisfactorily. We really would like to thank you for your invaluable efforts and keen attention to detail. Because of that, we believe the manuscript is vastly improved in its readability and brings in better clarity.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for your valuable time in reviewing our manuscript. We greatly appreciate your efforts. We hope we have addressed them all.

- 1 Schueder, F. *et al.* Unraveling cellular complexity with transient adapters in highly multiplexed super-resolution imaging. *Cell* **187**, 1769-1784.e1718 (2024). <https://doi.org/https://doi.org/10.1016/j.cell.2024.02.033>
- 2 Unterauer, E. M. *et al.* Spatial proteomics in neurons at single-protein resolution. *Cell* **187**, 1785-1800.e1716 (2024). <https://doi.org/https://doi.org/10.1016/j.cell.2024.02.045>
- 3 Rahman, F. *et al.* Mapping the nuclear landscape with multiplexed super-resolution fluorescence microscopy. *Nature Communications* **16**, 6042 (2025). <https://doi.org/10.1038/s41467-025-61358-0>
- 4 Fischer, L. S. *et al.* Quantitative single-protein imaging reveals molecular complex formation of integrin, talin, and kindlin during cell adhesion. *Nature Communications* **12**, 919 (2021). <https://doi.org/10.1038/s41467-021-21142-2>
- 5 Schlichthaerle, T., Ganji, M., Auer, A., Kimbu Wade, O. & Jungmann, R. Bacterially Derived Antibody Binders as Small Adapters for DNA-PAINT Microscopy. *Chembiochem* **20**, 1032-1038 (2019). <https://doi.org/10.1002/cbic.201800743>
- 6 Xu, J. *et al.* Super-Resolution Imaging of Higher-Order Chromatin Structures at Different Epigenomic States in Single Mammalian Cells. *Cell Reports* **24**, 873-882 (2018). <https://doi.org/https://doi.org/10.1016/j.celrep.2018.06.085>
- 7 Bhat, P. *et al.* Genome organization around nuclear speckles drives mRNA splicing efficiency. *Nature* **629**, 1165-1173 (2024).
- 8 Raina, K. & Rao, B. J. Mammalian nuclear speckles exhibit stable association with chromatin: a biochemical study. *Nucleus* **13**, 58-73 (2022). <https://doi.org/10.1080/19491034.2021.2024948>
- 9 Faber, G. P., Nadav-Eliyahu, S. & Shav-Tal, Y. Nuclear speckles – a driving force in gene expression. *Journal of Cell Science* **135** (2022). <https://doi.org/10.1242/jcs.259594>

- 10 Lamond, A. I. & Spector, D. L. Nuclear speckles: a model for nuclear organelles. *Nature Reviews Molecular Cell Biology* **4**, 605-612 (2003).
<https://doi.org/10.1038/nrm1172>

Please note that the reference numbers here and main text are different.

Point-by-point response to reviewer's comments:

Our response is provided in [blue color](#)

Reviewer-1

I am satisfied with the revisions in response to my comments, and the additions to the main. I am fine with there not being new biology in the paper, as long as the experiments serve the purpose of validating the new probes. As the authors put it [“We do not introduce novel biological conclusions; rather, we demonstrate that our approach reliably detects established organizational changes in the chromatin organization.”](#)

[We are happy to learn that the reviewer is satisfied with our revised manuscript.](#)

Reviewer-2

Reviewer#2 1: I think the argumentation of the authors makes sense.

Reviewer#2 2 - likely unspecific signals in positive controls, ie. some signal outside nuclei with antibody staining that should theoretically localise only within the nucleus:

My comment 2: First, I agree that there is indeed unspecific antibody independent signal and antibody dependent signal outside the nucleus in the positive controls, which the authors also acknowledge.

For unspecific antibody independent signal:

- _there is unspecific sticking of the nanobody/ sequences on nanobodies or unspecific sticking of imager sequences - this is inspected with negative control experiments supplementing primary Ab with PBS in the otherwise identical protocol (authors added this to the SI sample preparation description)
- _there is unspecific sticking of the imager sequences – this is inspected with negative control experiments only the imagers (for R3 imager authors show there is indeed sticking to the nucleolus S7)

“in the absence of primary antibody there is little unspecific (off-target) localizations” . The question is, is it negligible compared to specific binding? This is S8-S10 left side compared to right side. Qualitatively, I see some unspecific binding everywhere in the cell and it seems random without any substructure indicating that there is no off-target binding – maybe in S8-Ser5P there is some substructure in the image but this is speculation when looking at just one image.

For antibody dependent signal outside the nucleus:

- _there is unspecific sticking of the antibody used in the positive control – a problem of the antibody
- _the signal is actually due to protein also localized outside of the nucleus – this should be specifically stained in all experiments with antibody and nanobody present, is in principle possible can be seen in other experiments that authors mention

In all cases shown in S8-S10 it seems evident that there is more signal in the nucleus than outside of it, although the difference is not always very apparent.

The noisy images mentioned by reviewer #2 are similarly due to both new and old DNA-PAINT sequences:

Figure 8a - Ser2P-R7 new, Figure 8b (Ser5P)-R1 old, Figure 9f (CTCF)-R5 old, 24a (ser2P, mock)-R7 new, Figure 25e (H3K4me3, mock and ActD)-R4 old. To me that indicates that the new probes are no better or worse than the previously reported ones.

Thus, it boils down to the question if one can conclude on the quality of nanobodies/sequences on nanobodies vs quality of antibodies/ target in the cytosol used in the positive controls. To me S8-S10 says that this additional higher signal outside of the nucleus on the right is due to either of the latter, thus likely not a problem specific to the new sequences. Without additional experiments with another antibody this might not be possible to say which of the latter it is, or might not be a clear answer possible..this goes too far for this context.

Can the nanobodies/sequences on nanobodies be used to do specific staining in principle? Lamin controls in S5, 6, 7. From my own experience with Lamin staining (primary + secondary Ab-Alexa647 for dSTORM), I often see very strong signal on the nuclear lamina and additional cytoplasmic staining. Now what we see or don't see with our human eyes might also be due to contrast and colors used to represent the different imagers in S5 – I see as much unspecific staining in the new set of probes (second half) as in the old, already known repeat sequence probes (first half). To me that means again the new probes are no better or worse than the previously reported ones for Lamin.

Reviewer#2 4 - issue regarding some conclusions on the correlation between markers in the nucleus:

My comment 4: I think the reply of the authors to 4 and 5 clarifies that indeed the global analysis is not capable of capturing the local correlations, which is why they have included different measures. The authors now added a much better explanation of these more local measures, clarifying the advantage and disadvantage of their approaches.

We are happy to have answered the reviewer's comments with satisfaction.

We have added a sentence explaining that the cytoplasmic signal likely arising from either antibody non-specificity or due to the presence of nuclear epitopes in the cytoplasm. Here is the text that we added '*Any stray cytoplasmic staining of nuclear targets is likely from antibody non-specificity or the presence of nuclear target epitopes in sparing amounts in the cytoplasm, but unlikely due to the non-specific binding of imager sequences as the same doesn't appear in the control imaging experiments with nanobody-docking strands alone (Supplementary Figure 8, 9 and 10 (left)).*'

General comment:

I am satisfied with the revisions in response to my comments, and the additions to the main. I am fine with there not being new biology in the paper, as long as the experiments serve the purpose of validating the new probes. As the authors put it *"We do not introduce novel biological conclusions; rather, we demonstrate that our approach reliably detects established organizational changes in the chromatin organization."*

I have been asked to provide my thoughts on technical aspects previously raised by referee#2, see below:

Response to the more detailed comments in the re-revision:

Reviewer#2 1: *I think the argumentation of the authors makes sense.*

Reviewer#2 2 - likely unspecific signals in positive controls, ie. some signal outside nuclei with antibody staining that should theoretically localise only within the nucleus:

My comment 2: First, I agree that there is indeed unspecific antibody independent signal and antibody dependent signal outside the nucleus in the positive controls, which the authors also acknowledge.

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