

Peer Review File

Manuscript Title: Integrated spatial genomics reveals global architecture of single nuclei

Redactions – Third Party Material

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Recent studies observing the heterogeneity among cells in a population emphasize the need for technologies that provide whole cell readouts of DNA, RNA, and protein with single-cell resolution. Here, Takei and colleagues build upon their previously described RNA seqFISH+ and DNA seqFISH technologies to image DNA in situ, mapping an unprecedented number (up to 3,660) of loci in mouse embryonic stem cells. This is equivalent to visualizing a ~25 Kb locus every megabase. In addition, they trace twenty 25 Kb regions sequentially along each chromosome. Notably, they also combine this seqFISH+ iteration with RNA seqFISH as well as sequential immunofluorescence to provide multimodal views of single cells. Thus, by simultaneously mapping genomic loci, 70 RNAs, and 17 chromatin marks, they identify nuclear zones with specific properties related to nuclear function. They end the manuscript with an interrogation of nuclear structure in clonal cells. Overall, the achievements of this manuscript include a significantly enhanced version of DNA seqFISH, application of the improved technology as well as published technologies for RNA and protein imaging to achieve a milestone leap in genome coverage and integration of epigenomic information, and several biological observations pertaining to 3D genome and epigenomic architecture. Complicating assessment of the impact of the manuscript, however, is a surprisingly insufficient consideration of prior publications in the field, making it difficult to determine the extent of the novelty of the work. More detailed comments are as below:

- 1) Pg 1: The introduction to the field is too short, and the citations are incomplete and occasionally oddly selective. For example, Dixon et al. (2012) is usually cited along with Nora et al. (2012) and Sexton et al. (2012) for TADs, while Bonev et al. seems an odd choice to complete the set. As for nuclear bodies, the references do not cover the many that are known. Similarly, regarding DNA FISH, there are several publications other than 11 and 12 that would be important to cite. As for recent imaging papers, references 20-24 seem to be a standard set, although the spelling of oligo-STORM is OligoSTORM (Boettiger & Murphy 2020 PMID: 32007290), and, as all five papers address TADs and heterogeneity, all five should be cited for these topics. Finally, as the paper focuses on heterogeneity, this topic deserves a more in-depth treatment, with better citation of recent studies. (Other lapses in referencing are mentioned, below, in order of appearance.)
- 2) Pg 2: While the manuscript refers DNA seqFISH and intron seqFISH, it does not explain how these technologies work, so the transition to paragraph 3 is too great a leap. However, based on the legends, Methods, and cited literature, it seems that the oligos used are commonly known as Oligopaints. Thus, please use this term and include a cartoon of the structure of the oligo probe, along with its barcoding positions, in the main text. This would be immensely helpful to the readers.

- 3) Pg. 2: The manuscript cites RNA seqFISH+, but refers to a paper that names the technology intron seqFISH. Please clarify. Also, please include a citation to MERFISH (Chen et al. 2015 PMID: 25858977 PMID: PMC4662681), as this technology seems quite similar in concept.
- 4) Pg 2: Please provide a clear explanation of what is being hybridized to what in the 16 rounds of hybridization and how the barcodes were being visualized. Please also make clear in the main text how much time it took to conduct 80 rounds of hybridization, with 3 colors imaged in each round and how many species of fluorescent read out probes were used, in total, as well as for each iteration of the experiment. As fluorescently labeled oligos can be expensive, please indicate in the main text the ballpark cost of the labeled oligos and whether they were labeled in-house. This information will help readers assess whether they can use the method.
- 5) Pg 1: The abstract mentions that over 3,660 loci were mapped? However, the text on pg. 2, mentions 2,460 targets spaced 1 Mb apart + 1,200 targets traced, which gives a sum of 3,660 targets. What is the basis of "over"?
- 6) Pg 1, abstract, and throughout the manuscript: The phrase "genome-level super-resolution" should be defined, as it will otherwise be confused with the use of "super-resolution" in terms of microscopy. If not possible to explain clearly in the abstract, we recommend avoiding use of the term until after it is explained in the main body of the text. Similarly, "deterministic super-resolution" and "super-resolved image" should be defined.
- 7) Pg. 2: With respect to the 80 hybridizations, are the targets separated by 1 Mb and the 25 kb steps imaged simultaneously during hybes 1-60, such that hybes 61-80 are then used to complete the imaging of the remainder of the 1 Mb resolved targets? What, then, is the percentage (including \pm range) of the mESC genome that was successfully imaged in any single cell?
- 8) Pg. 2: Some readers will be puzzled as to why the detection efficiency of loci spaced 1 Mb apart is only \sim 50%. Thus, please include a brief mention of possible explanations.
- 9) Pg. 2, "Overall, 80 rounds of hybridization are used for the DNA seqFISH+ experiment (Extended Data Fig. 2a-c).": Should this be Extended Data Fig. 1a-c?
- 10) Pg. 2, Extended Fig. 1: Please incorporate a description of padlocking into the main text and supplement the mention of padlocking in the supplementary material with quantitation of the effectiveness of padlocking under varying conditions. As padlock probes have been used in many other studies, please also add citation to these other studies.
- 11) General comment: There does not seem to be any clear reporting of total cells imaged for the DNA seqFISH+ experiments with or without RNA FISH and IF.
- 12) Pg. 3, "Using pairwise contacts as a metric for comparison, single cell Hi-C measurements detected between 10,000 to 1×10^6 median pairwise contacts per cell (Extended Data Fig. 2h). DNA seqFISH+ measures effectively 5 million median pairwise contacts per cell and contains higher order contact information intrinsic to the natively imaging data.": The basis for this comparison is questionable, as Hi-C detects physical pairwise contacts whereas DNA seqFISH+ measures all pairwise distance relationships, independent of physical contact. To avoid misinterpretation, please remove the first of the two sentences.
- 13) Pg. 3 and elsewhere: What is meant by "chromosomal alleles"? Please doublecheck use of "alleles" as well as "sister chromosomes".

- 14) Pg. 3: Analogous studies have addressed power law relationships between genomic and physical distance. Thus, the authors are encouraged to provide the plots of genomic vs. physical distance at 1Mb and 25 kb resolution.
- 15) Pg. 3: As written, it seems as if conjugation of oligos to antibodies was developed in this study. Please provide references to prior art directly in the main text wherever needed.
- 16) Pg. 3, "This optimization allowed us to profile these different modalities without a major loss of signal and accurate alignment between IF and DNA FISH images for over 130 rounds of hybridizations on an automated confocal microscope (Extended Data Fig. 2a-e).": Please provide quantitation of this optimization, preferably with variation of the conditions so that it is clear that the "optimal" conditions have been obtained.
- 17) Pg 4 & 15, Fig. 2b: The authors show IF reconstructions as blobs/rounded structures. Please include description of the algorithm used to generate these shapes. In particular, were filters applied and, if so, how were the cut-offs determined?
- 18) Pg 4, Fig. 2b: Are these images displaying only a subset of the chromosomes and, similarly, only a subset of the various epigenetic marks? If so, please make this clear along with any parameters or cut-offs used. Mouse rDNA is also located on several other chromosomes. Please comment on these other locations and whether they also appeared significantly associated with the nucleolus.
- 19) Pg. 4, "To determine whether loci with similar chromatin profiles aggregated in single cells, we first grouped the loci into four clusters based on the IF data (Fig. 2e).": Was any clustering conducted without input of IF data? For example, was k-means clustering of the loci conducted as an independent assessment?
- 20) Pg. 5: Only some readers will know that mouse centromeres are located at the ends of chromosomes and thus, the significance of Figure 2h will not be evident. Please provide background for this and, also, place all observations in the context of other published studies. Broadening to the paragraph beginning with "The contact maps generated from DNA seqFISH+" and ending with "did not completely recapitulate global trends", which makes broad statements about the 3D organization of the mouse genome, please place these findings in the perspective of other published studies (Hi-C, imaging, etc.).
- 21) Pg. 5: Please put observations about lamin association in the context of published studies, and also comment on which loci might have been found to be enriched in the nuclear lamina.
- 22) Pg. 5: Regarding the recognition of zones, were the analyses carried out solely with IF data, and did they also include input from DNA imaging? Furthermore, were any analyses conducted with a smaller subset of primary antibodies? Please comment on the potential for artifacts arising from the simultaneous visualization of 17 IF targets.
- 23) Pg. 5: As UMAP is a relatively new method, please provide background and rationale for its application. Please explain its strengths and its weaknesses and discuss the observations in this context. How are markers in multiple zones mapped?
- 24) Pg. 5, section subtitled "Active loci are enriched in the interfaces between zones": While the concepts are straightforward, the figures are difficult to follow, with a number of instances where the text and figures do not align. For example, "active sites are close to the nuclear speckle zone" refers to Figure 3e, which does not show this. There is similar confusion about Figure 3e. Please go through

this paragraph and provide clearer explanations of the conclusions, including a quantitative definition for the interface between zones. Also, please provide P values for enrichments and depletions. Finally, please put the findings in the context of published studies, especially what is known about nuclear bodies and zonation.

25) Pg. 5: Is there any specific reason why IF was done after DNA imaging? How would the zonation look if IF were performed first?

26) Pg. 6: Please explain why Pou5f1 (Oct4) and Dazl were selected for special comment. Are they typical or outliers with respect to their behavior?

27) Pg. 6, "To determine whether loci with similar zone assignments are more likely to be spatially close regardless of genomic distance, we examined the likelihood that pairs of loci are found within a 500 nm radius sphere in single cells as a function of genomic distance": What is the size distribution of the overall chromosome territory for each chromosome? What was the rationale behind the choice of 500 nm radius? If the territories have highly variable size, a uniform 500 nm search radius might not be appropriate to calculate the return probability. Again, please put these observations into the perspective of published studies. Please also consider the rich literature on compartments, including their sizes and organization, as well as various nuclear bodies.

28) Pg. 6: Please explain why the RNA seqFISH+ information was not used in the correlations between loci, zones, and RNA-seq data. Also please make clear which findings are new discoveries and which findings are confirmation of previously published correlations.

29) Pg. 7 & 8/Fig. 4: "These data suggest that chromosome contacts are preserved across one cell cycle between sisters, which supports previous observations that the global arrangement of chromosomes is heritable for one generation but are then rapidly lost after 2 generations." Please mention other published studies in this area, including those that are contradictory.

30) Pg. 8: Please integrate more references into the Discussion. For example, with respect to the proposal that zones and interfaces suggest a "new layer of regulation", please consider this proposal in the context of published hypotheses about compartments, domains, nuclear bodies, chromosome territories, etc., as it is unclear whether the concepts presented in the Discussion are truly novel.

31) Pg. 15, Fig. 2d: How many cells were used for this figure? Please provide data for single cell comparisons.

32) Pg. 33: In the legend of Fig. S7, the reference to panels c and d seem erroneous.

33) Pg. 34, Extended Fig. 8: Panels a and b are swapped.

34) Pg. 36, Extended Fig. 9: Why is the H3S10 plot in panel e not enriched?

35) Pg. 43: Does storing cells in 70% at -20°C have an effect on chromatin organization?

36) Pg. 47: What is the rationale behind using a 1.73-pixel search radius? How was this value chosen? Is it same for both the 1Mb spaced loci and 60 consecutive loci?

37) Pg. 49: Does the presence of 3 or more copies imply aneuploidy? What fraction of cells demonstrates this feature?

Referee #2 (Remarks to the Author):

This manuscript, Global architecture of the nucleus in single cells by DNA seqFISH+ and multiplexed immunofluorescence by Takei et al., presents the results of a powerful new method, DNA seqFISH+. The method uses super-resolution imaging to detect thousands of chromosomal loci spaced throughout the genome in single mouse embryonic stem cells (mESCs). The authors have combined this with sequential immunofluorescence detection of several chromatin marks, nuclear bodies/landmarks and expression profiles of 70 RNAs, in the same cells. Consequently, their data is capable of simultaneous reports on global genome architecture, genome function and genome associations with nuclear bodies and the nuclear lamina. The method and results are exciting and will be of great interest across a wide readership, and especially for those working in the 3D genome fields.

Comments:

1. The authors go to great lengths to present their data in the context of previously published Hi-C studies, including unfortunately, adopting some of the terminology used in describing Hi-C results. This is a major concern about this work.
2. The authors describe the use of DNA seqFISH+ results to create a "pairwise contact map". These pairwise contacts are created by designating all loci within half a micron of a target locus as engaged in "pairwise contacts". This is quite a stretch since there is little evidence that two loci within 500nm of each other could be considered in contact. In molecular terms this is a large distance. Separation distances of a few tens of nanometers might be acceptable as evidence of possible contact, but using 500nm is an unrealistic and incorrect inflation.
3. A comparison of proximities with Hi-C heatmaps would be OK if the authors simply wanted to compare the proximities measured to standard Hi-C matrices but the suggestion that they are measuring "contacts" is a misnomer and misleading, simply serving to muddy the already muddied water of what a "Hi-C contact" is -- actual contact or in fact just a ligation pair. Hi-C ligation implies proximity but is not proof of contact, and in this manuscript with the distances used (500nm), proximity certainly does not provide evidence of contact. The authors go on to incorrectly use the "contact" terminology throughout the manuscript to describe what are actually spatial proximities. This is wrong and should be removed.
4. Referring to these as contacts weakens the power of the authors data. With FISH, seeing is believing. One can measure the actual physical distance between two loci. With Hi-C on the other hand, it is not actually known what ligation between two loci means in physical terms. Obviously two sequences have to be close enough together to ligate in the nucleus, but an actual separation distance or range of distance is not known, nor is whether they need to be (or are) in contact or simply in proximity. This is where the authors miss a huge opportunity in the presentation of their data and its usefulness to future studies. Rather than try to mimic Hi-C by inaccurately referring to their measured proximities as contacts, they could do the entire field a great service by using their 3D physical measurements in an attempt to do what Hi-C cannot do, to calibrate Hi-C data in physical terms. For example, by comparing the frequency of ligation events between two genomic regions to the range of 3D measurements between those two regions obtained by DNA seqFISH it should be possible, through the tens of thousands of measurements made, to come up with some metric of physical distance and frequency that could be applied to Hi-C ligation frequencies. Given that the number of labs that are likely to have the setups required for DNA seqFISH is low, and that the number of labs using Hi-C is comparatively enormous, such a metric would seem to be a tremendously useful output from this work.

5. The authors attempt to 'one-up' the Hi-C community with an inflated "5 million median pairwise contacts per cell", when in fact they haven't measured any. This type of prejudicial comparison isn't necessary for a reader to see the value in their work, and basically only puts more smoke in the air. For example, if "pairwise contacts" were inferred in a similar way from the type of single cell 3D reconstructions made by Tan et al., Science 361, 924-928 (2018), the number would far exceed 5 million per cell.

In summary, this technique and the data generated are potentially of great use to the field. My advice to the authors is in addition to all the useful results on proximities to nuclear bodies and the nuclear lamina, to present their work in a straightforward way as to provide the greatest utility to the field, since the true value of this work will be measured in how often it is cited, rather than through acceptance from those in the field who are not savvy enough to know that their terminology is inaccurate and their comparisons over-inflated.

Referee #3 (Remarks to the Author):

Takei et al present a multi-modal imaging method to simultaneously visualize specific DNA loci, transcribed RNAs, and proteins. This is a heroic effort and an impressive technical achievement. The authors apply the method to study heterogeneity among cultured mouse embryonic stem cells, including clonal dynamics. Building on previous expertise, they scale up genome imaging from ~70 to ~3700 loci (at a resolution of 1 Mb genome-wide, and 25 kb for distinct regions), scale up immunofluorescent sequential detection of proteins to 17 and combine this with expression values obtained with mRNA and intron FISH. Integrative analyses of these measurements reveal that DNA loci, in particular active ones, are enriched at presumed "interfaces" between nuclear zones and they identify ~7 cellular states based on clustering of global combinatorial chromatin/protein levels, which show partial overlap with transcriptional states. Finally, clonal expansion experiments reveal the inheritance of H3K27me3 states over few generations.

Single-cell multimodal methods are essential to start understanding the principles that govern gene regulation and cellular specification. The technological achievements presented in this manuscript represents an important contribution towards achieving this goal. However, the novelty of this manuscript is, while impressive, largely technical. Implementation of the proposed method is deemed non-trivial for the larger audience and therefore not of broad interest as a resource. More importantly, the biological insights garnered are not sufficiently groundbreaking to warrant publication in Nature, in our opinion.

General textual comments

Although lengthy technical descriptions are included -for which we do commend the authors-general readability can be improved. Thorough revision of all figure panels, legends and textual references to figures is necessary. For completion, we recommend including some references on (multi-modal) single-cell sequencing methods in paragraph 1 of the Main Text.

General content-related comments

Much emphasis is placed on so-called "interfaces" between nuclear "zones". However, not much detail is given on how these interfaces are calculated. It seems to me that, with such purported high resolution, a DNA locus would not commonly find itself at the interface of two (or more) IF-classified zones. Regardless, it would be useful to quantify the number of loci associating with the number of zones (one, two, or more). Transcriptome profiling presented here does not recapitulate the complete reported heterogeneity in mESCs (while the authors do make such claims in their comparisons with

literature). For instance, it is curious that Oct4 is only (and lowly) expressed in a few cells, and these cells are spread across nearly all the clusters called on mRNA. (The authors do report that the Oct4 locus is predominantly associated with active DNA zones.)

Figure-by-figure comments

Fig. 1i-j. It is not clear what is plotted on the y-axis. Median spatial distance for the same probe across single-cells or for multiple consecutive probes along the linear chromosome? With the current presentation, it is difficult to tell whether the heterogeneity comes from the difference between chromosomes (as claimed) or from variations in measurements between cells (also interesting). Also, these figures require a measure for the spread of the data.

Fig. 2. In general. The validation of integrated DNA seqFISH+ and IF requires a much more thorough and systematic quantification (i.e. genome-wide, for all chromatin marks with correlation coefficient's). We find anecdotal displays of 120 Mb for two examples (2d) and references to one representative 3D reconstruction (2b) insufficient.

Fig. 2b (bottom left) and Extended data Fig. 5a. From our experience and others, H3K9Ac is broadly distributed in the nucleus. This is also apparent from ED Fig. 5a. Yet, the modelling of the signal as defined globules depicted in Fig. 2b does not reflect this at all. We can see how you would obtain such structures from Fibrillarin staining, yet not H3K9Ac. The authors should explain how these visualizations were derived and why they appear at odds with the IF images. Otherwise, sentences like: "Chromosomes 2, 4 and 5, which appear in the active hubs in the SPRITE data, were spatially close to H3K9ac-enriched regions in single cells" lose validity.

Fig. 2c and Extended data Fig. 6a. The distance of 1 μ M in defining contact probability seems arbitrary. We would like to see contact probability as a function of distance to determine a proper cutoff.

Extended data Fig. 6e. These findings do not seem in complete agreement with literature. Especially in the cases of constitutive heterochromatin, the IF signal does not fall convincingly in compartment B versus A. Do the authors have an explanation for this observation?

Extended data Fig. 6g. Shows two example cells of observed versus shuffled A and B compartments. Is there additional evidence to support the claim that A/B compartments intermingle in single cells?

Fig. 2g. The patterns of clusters 2 (nucleolus) and 4 (lamin) do not follow the stereotypical sub-nuclear localization of these proteins (see also comment below). This should be clarified.

Fig. 2i and extended data Fig. 6i. Show anecdotal examples of intercellular variation. At present, the claim that single cells do not recapitulate global trends is overstated without proper systematic quantification.

Fig. 3c. A general comment on this work is that it is difficult to discern true biological events/observations from outcomes related to technical limitations. This is especially true for the experiments and analyses described for Fig. 3. This is also in part related to the next point below about textual editing. One example to illustrate this are the three Lamin classifications in Fig. 3c (zones 10, 11 and 12). Especially zones 10 and 12 appear to be mixtures of active and inactive marks. We are cautioned by the spatial lamina distribution assigned to loci in Fig.2g. and the relatively poor correspondence in contact maps of extended data Fig. 5d. The spatial distribution of the lamina-assigned spots (green) are not (at all) exclusively localized towards the nuclear periphery. Yet, to our knowledge (and as shown in extended data Fig. 5a.), Lamin B1 is found exclusively at the nuclear periphery. Such, "off-peripheral" assignments would generate a mixture of signals which may be

interpreted as separate states. Of note, the authors ignore lamin state 11, even though this state appears to be the most stereotypical for LADs. Studies on LADs and H3K9me3 in many species have shown that typically these genomic regions encompass uniform types of chromatin. Therefore, the observed classifications in Fig. 3c require further scrutiny. E.g. for reference, a zone of 1 micron underneath the nuclear periphery should best reflect the true combinatorial chromatin state associated with the regions of the genome that contact the lamina.

Fig. 3d-e. The entire paragraph 2 under "Active loci are enriched in the interfaces between zones." (referring mainly to 3d-e and ED 7d,e) would benefit from textual editing to better guide the reader through the figures. It requires considerable effort from the reader to first understand which part of the graph to look at, and subsequently match the written text to an interpretation of the presented data.

- From the text it is unclear how interfaces are defined
- From Fig. 3e it is not immediately clear that "loci are enriched at interfaces"
- The selection of zone interfaces depicted in Fig. 3e appears rather arbitrary

It is also unclear to what extent such interphases are the result of the limitations in resolution. The paragraph would benefit if the authors would elaborate on these points more.

Fig. 3d and ED7d. Sentences such as "loci appear at the edges, rather than the center, of RNAPII dense regions in the nuclei" would benefit from quantification rather than visual representation of a few nuclei.

Fig. 3f. Related to the arbitrary selection of zones and interphases. Why are different interphases displayed in this figure compared to Fig. 3e? Based on what qualifications?

Fig. 3f. We do not find the contact probability per locus (distributed over different zones and interfaces) an intuitive measure of intercellular variability, since each locus can appear in multiple pairwise interactions (rows, in these heatmaps).

Fig. 3g. To gain true insight into (and make claims about) what is happening at a single-cell level, it is imperative to show the distribution of return probabilities across all the cells. What we see now is that, on average, zone 1 and the interface between zones 6 and 7 maintain more contacts at large distances compared to the whole chromosome.

Fig. 3h-i. The authors claim that gene expression and nuclear localization in single cells is not correlated. The authors continue to say that highly expressed genes are always close to active zones, while lowly expressed genes are always close to the nuclear lamina and heterochromatin. This follows and is assumed from the data, but the relationship is not displayed. Perhaps show, per mRNA, the correlation between expression level and zone localization).

Fig. 4b. We are unsure how to biologically interpret overall intensity levels of histone PTMs without information on genomic positional information or subnuclear localization patterns. E.g. cells in clusters 4 and 5 have overall high H3K27me3 & mH2A.1 or H3K9me2 & H3K9me3 levels respectively, yet how should we see this biologically? Do the cells in these clusters overall have lower expression output? Are the levels of these histone PTMs more elevated at their "normal" genomic locations, or do these cells in general have higher levels of these modifications throughout the genome? Without genomic positional information, and without associating differential chromatin signatures to transcriptional output in the same cell, the meaning of these classifications remains rather elusive. This data requires cross-referencing with single-cell transcriptomics datasets -or other- to ensure that these states are 1)

biologically meaningful and 2) can be observed in other datasets.

The clustering based on mRNA as presented in Extended Fig. 9a could have been revealing towards identifying the biological significance of these chromatin assignments, yet in our opinion the almost uniform mixing of the chromatin states over the 5 transcriptional states indicates that these chromatin states have little predictive value and may have little biological significance. The exception is the transcriptional "mESC naïve ground state" #1 which appears to be associated with the high H3K27me3 and mH2A.1. However, as far as we recall, naïve ground state mESCs are supposed to be associated with low H3K27me3 levels rather than high (e.g. Marks et al., 2012).

Fig. 4d-h. The clonal analyses performed in the second part of Fig. 4 are potentially very interesting. Combinatorial single-cell measurements of different modalities as presented in this work are pivotal for meaningful interpretation of the inheritance of transcriptional states over one or multiple generations. Especially, the inheritance of H3K27me3 states over few generations is striking. However, this experiment could have been performed with single-modal, non-sequential IF imaging, and as such does not take advantage of the proposed method. This is a missed opportunity in our opinion, since without the combinatorial information, this finding is merely an interesting observation. This finding should be further substantiated and requires additional investigations to assign functional relevance to the inheritance of the H3K27me3 states.

Minor points

- Reference to Fig. 3e in the text should read Fig. 3d.
- ED Fig. 6j is described in the legend but the letter annotation does not exist.
- ED Fig. 7 legend letters need to be adjusted.
- ED Fig. 8 legend letters and references to this figure in the main text need to be adjusted.

Author Rebuttals to Initial Comments:

Point-by-point Response:

We thank all the reviewers for providing valuable detailed comments as well as recognizing the technological and conceptual innovations in the manuscript. We revised the manuscript to improve clarity and discussed the findings in the context of existing literature. In addition, we performed new analyses and revised figures accordingly. In particular, there are three areas of major revision.

1. We performed additional analysis of DNA loci-chromatin mark/protein association in single cells. In the original manuscript, we showed that spatial association of DNA loci with chromatin marks matched ChIP-seq results when averaged over many cells. In the revision, we report that many of these associations were observed consistently in single cells. For example, the Oct4 locus appears to be spatially close to H3K9ac globules in 77.2% of the cells. Speckles and other active or repressive marks similarly have these fixed loci that behave consistently across cells. Chromosome 4, as an example, contains a number of fixed loci for H3K9me3 and SF3a66. Correspondingly, in single cells, we observed chromosome 4 spanning heterochromatic globules and nuclear speckles (Figure 2f-h). Similarly, chromosome 19 has fixed loci for both speckles and nucleolus and appears to connect these nuclear bodies in 96%

of the cells. More 3D images are also shown in Extended Data Figure 6. These results illustrate that there are underlying constraints on how nuclear bodies and chromosomes are organized in the nucleus, despite the variability in chromosomes structure and nuclear body positioning in single cells.

2. We modified Figure 3 to illustrate how mRNA and intron expression levels relate to the chromosomal positions of those loci. Many gene loci appear to be pre-positioned to active zones regardless of the mRNA and intron expression levels in single cells. We included single cell reconstructions and detailed analysis to support this conclusion. We added further quantitation to show that active loci preferentially appear at the exterior of RNAP globules (Figure 3).

3. We performed network analysis of global chromatin states with respect to RNA profiles to gain biological insights into these ES chromatin states. We found that global H3K27me3 levels correlated with Tbx3 high states, which correspond to a more naïve mESC state, while global mH2A1 high states correlated with Essrb and Nanog high states. An additional pseudotime analysis showed that H3K27me3 and Tbx3 levels decay rapidly along pseudotime trajectories from pluripotency to differentiation. These new analyses are shown in Figure 4.

We hope this revision highlights the new biological concepts in addition to the technological innovations. This dataset of single cells with thousands of DNA loci, 17 epigenetic markers and 70 mRNAs and introns can be a resource for the nucleome community for additional data mining and discoveries. We highlighted major changes in the revision in yellow.

Referee #1 (Remarks to the Author):

Recent studies observing the heterogeneity among cells in a population emphasize the need for technologies that provide whole cell readouts of DNA, RNA, and protein with single-cell resolution. Here, Takei and colleagues build upon their previously described RNA seqFISH+ and DNA seqFISH technologies to image DNA in situ, mapping an unprecedented number (up to 3,660) of loci in mouse embryonic stem cells. This is equivalent to visualizing a ~25 Kb locus every megabase. In addition, they trace twenty 25 Kb regions sequentially along each chromosome. Notably, they also combine this seqFISH+ iteration with RNA seqFISH as well as sequential immunofluorescence to provide multimodal views of single cells. Thus, by simultaneously mapping genomic loci, 70 RNAs, and 17 chromatin marks, they identify nuclear zones with specific properties related to nuclear function. They end the manuscript with an interrogation of nuclear structure in clonal cells. Overall, the achievements of this manuscript include a significantly enhanced version of DNA seqFISH, application of the improved technology as well as published technologies for RNA and protein imaging to achieve a milestone leap in genome coverage and integration of epigenomic information, and several biological observations pertaining to 3D genome and epigenomic architecture. Complicating assessment of the impact of the manuscript, however, is a surprisingly insufficient consideration of prior publications in the field, making it difficult to determine the extent of the novelty of the work. More detailed comments are as below:

1) Pg 1: The introduction to the field is too short, and the citations are incomplete and

occasionally oddly selective. For example, Dixon et al. (2012) is usually cited along with Nora et al. (2012) and Sexton et al. (2012) for TADs, while Bonev et al. seems an odd choice to complete the set. As for nuclear bodies, the references do not cover the many that are known. Similarly, regarding DNA FISH, there are several publications other than 11 and 12 that would be important to cite. As for recent imaging papers, references 20-24 seem to be a standard set, although the spelling of oligo-STORM is OligoSTORM (Boettiger & Murphy 2020 PMID: 32007290), and, as all five papers address TADs and heterogeneity, all five should be cited for these topics. Finally, as the paper focuses on heterogeneity, this topic deserves a more in-depth treatment, with better citation of recent studies. (Other lapses in referencing are mentioned, below, in order of appearance.)

We thank the reviewer for these suggestions. We now included Nora et al. (2012) and Sexton et al. (2012) for TADs, revised the citations for nuclear bodies and multicolor DNA FISH including recent reviews (Dekker et al. 2017; Kempfer and Pombo 2020) for sequencing and imaging technical advances in nuclear organization, and corrected the spelling of oligo-STORM as OligoSTORM. In addition, we revised the sentences to introduce the recent imaging studies and their findings with particular focus on heterogeneity of chromosome organization in single cells.

2) Pg 2: While the manuscript refers DNA seqFISH and intron seqFISH, it does not explain how these technologies work, so the transition to paragraph 3 is too great a leap. However, based on the legends, Methods, and cited literature, it seems that the oligos used are commonly known as Oligopaints. Thus, please use this term and include a cartoon of the structure of the oligo probe, along with its barcoding positions, in the main text. This would be immensely helpful to the readers.

Thank you for this suggestion. We expanded our explanation of the concept and technical advancement of seqFISH. In addition, we clarified that our primary probes are designed using the Oligopaint technologies (Beliveau et al. 2012) in the main text, and added a cartoon of the structure of the oligo probe with its barcoding positions in Figure 1a.

3) Pg. 2: The manuscript cites RNA seqFISH+, but refers to a paper that names the technology intron seqFISH. Please clarify. Also, please include a citation to MERFISH (Chen et al. 2015 PMID: 25858977 PMID: PMC4662681), as this technology seems quite similar in concept.

We now reference both RNA seqFISH+ and intron seqFISH. We also added a reference to MERFISH (Chen et al. 2015).

4) Pg 2: Please provide a clear explanation of what is being hybridized to what in the 16 rounds of hybridization and how the barcodes were being visualized. Please also make clear in the main text how much time it took to conduct 80 rounds of hybridization, with 3 colors imaged in each round and how many species of fluorescent read out probes were used, in total, as well as for each iteration of the experiment. As fluorescently labeled oligos can be expensive, please indicate in the main text the ballpark cost of the labeled oligos and whether they were labeled in-house. This information will help readers assess whether they can use the method.

We clarified the details for the barcoding implementation, experimental time, fluorescently labeled readout probe preparation in the main text and methods as below.

“In two of the fluorescent channels (Alexa Fluor 647 and Cy3B), we used 16 rounds of serial hybridization of fluorescently-labeled readout probes to primary probes to generate a super-resolved image with 16 pseudocolors, which was repeated for a total of 5 barcoding rounds with 80 rounds of hybridization to barcode up to 2,048 barcodes with 2 round error corrections (Fig. 1a-c, Extended Data Fig. 1b, Supplementary Table 2).”

“Overall, 80 rounds of hybridization were performed with three fluorescent channels using a total of 240 fluorescently-labeled readout probes for the DNA seqFISH+ experiments (Extended Data Fig. 1b, c, see Methods),”

“The each readout probe hybridization and stripping routine took approximately 30 minutes. Imaging time per position took around 2.5-6 minutes at each hybridization round with our microscope setup and imaging conditions described above, and we typically imaged for 30 minutes per hybridization round with 5-10 positions. In total, it took approximately 80 hours to complete the 80 rounds of the hybridization and imaging routine for the DNA seqFISH+ experiments.”

“The 5' amine-modified DNA oligonucleotides (Integrated DNA Technologies) with the readout probe sequences were conjugated in-house to Alexa Fluor 647-NHS ester (Invitrogen A20006) or Cy3B-NHS ester (GE Healthcare PA63101) or Alexa Fluor 488-NHS (Invitrogen A20000) as described before (Shah et al. 2018; Eng et al. 2019), or fluorophore conjugated DNA oligonucleotides were purchased from Integrated DNA Technologies. In total, 240 unique readout probes (Eng et al. 2019) were designed and synthesized for DNA seqFISH+ experiments, and subsets of those readout probes were used for RNA seqFISH experiments. The probes were purchased for approximately \$15,000 but can be used over hundreds of reactions. “

5) Pg 1: The abstract mentions that over 3,660 loci were mapped? However, the text on pg. 2, mentions 2,460 targets spaced 1 Mb apart + 1,200 targets traced, which gives a sum of 3,660 targets. What is the basis of “over”?

Thank you for catching this. We have removed the term “over” from the abstract.

6) Pg 1, abstract, and throughout the manuscript: The phrase “genome-level super-resolution” should be defined, as it will otherwise be confused with the use of “super-resolution” in terms of microscopy. If not possible to explain clearly in the abstract, we recommend avoiding use of the term until after it is explained in the main body of the text. Similarly, “deterministic super-resolution” and “super-resolved image” should be defined.

We clarified this to just “super-resolution imaging” in the abstract. We further explained “deterministic super-resolution” and “super-resolved image” in the text.

7) Pg. 2: With respect to the 80 hybridizations, are the targets separated by 1 Mb and the 25 kb steps imaged simultaneously during hybes 1-60, such that hybes 61-80 are then used to complete the imaging of the remainder of the 1 Mb resolved targets? What, then, is the percentage (including \pm range) of the mESC genome that was successfully imaged in any single cell?

The 2,460 loci at 1 Mb resolution are imaged in hyb 1-80 in the 640 nm and 561 nm fluorescent

channels, while 1,200 loci at 25 kb resolution (60 loci for 1.5 Mb region per chromosome) are imaged individually in hyb 1-60 and then decoded in hyb 61-80 in the 488 fluorescent channel. We observed $3,636.0 \pm 1,052.6$ (median \pm standard deviation) dots for the 1 Mb targets per cell and $5,616.5 \pm 1,551.4$ (median \pm standard deviation) dots including the 25 kb resolution targets (Extended Data Fig. 2h). Because we imaged cycling mESCs, cells can contain between 2-4 alleles for each chromosome depending on the replication phase, so we can only provide an estimation with a range of efficiency as $50.7 \pm 14.0\%$ (median \pm standard deviation) per cell. We added the additional discussion in the Methods section under “*Estimation for DNA seqFISH+ detection efficiency*”.

8) Pg. 2: Some readers will be puzzled as to why the detection efficiency of loci spaced 1 Mb apart is only ~50%. Thus, please include a brief mention of possible explanations.

Although our targeting loci are 1 Mb apart, we only targeted 25-kb genomic regions per locus with 150-200 probes due to the probe cost. This could cause an incomplete denaturation or hybridization of primary probes at the 25-kb sites, preventing the detection of loci. In addition, dropout of barcodes can contribute to false negative detection. We have explained these points in the main text under “*DNA seqFISH+ images global chromosome conformation in single cell*” and clarified the details in the Methods section.

9) Pg. 2, “Overall, 80 rounds of hybridization are used for the DNA seqFISH+ experiment (Extended Data Fig. 2a-c).”: Should this be Extended Data Fig. 1a-c?

Yes, we apologize for the confusion and thank you for pointing this out. We have changed the reference in the manuscript to Extended Data Fig. 1b, c.

10) Pg. 2, Extended Fig. 1: Please incorporate a description of padlocking into the main text and supplement the mention of padlocking in the supplementary material with quantitation of the effectiveness of padlocking under varying conditions. As padlock probes have been used in many other studies, please also add citation to these other studies.

We now describe the padlocking of primary probes in the main text with associated citations (Nilsson et al. 1994; Rouhanifard et al. 2018). In addition, we have quantified the padlocking efficiency under different conditions in Extended Figure 2b.

11) General comment: There does not seem to be any clear reporting of total cells imaged for the DNA seqFISH+ experiments with or without RNA FISH and IF.

Thank you for raising this point - we imaged 446 cells in two replicates with RNA FISH and IF and DNA seqFISH+. 172 cells were imaged with introns and IF. Another 170 cells were imaged in the clonal tracing experiments with RNA, IF and DNA seqFISH+. We clarified this point in the main text and figure caption.

12) Pg. 3, “Using pairwise contacts as a metric for comparison, single cell Hi-C measurements detected between 10,000 to 1×10^6 median pairwise contacts per cell (Extended Data Fig. 2h). DNA seqFISH+ measures effectively 5 million median pairwise contacts per cell and contains higher order contact information intrinsic to the natively imaging data.”: The basis for this comparison is questionable, as Hi-C detects physical pairwise contacts whereas DNA seqFISH+ measures all pairwise distance relationships, independent of physical contact. To

avoid misinterpretation, please remove the first of the two sentences.

Thank you for this comment. We removed the quantitative comparison to scHi-C in order to avoid the potential misinterpretation.

13) Pg. 3 and elsewhere: What is meant by “chromosomal alleles”? Please doublecheck use of “alleles” as well as “sister chromosomes”.

We use “alleles” to refer to different homologous chromosomes in single cells. We clarified their usage in the text.

14) Pg. 3: Analogous studies have addressed power law relationships between genomic and physical distance. Thus, the authors are encouraged to provide the plots of genomic vs. physical distance at 1Mb and 25 kb resolution.

We extended the analysis for the genomic and physical chromosomal distance in Figure 1j, k, and added the Extended Data Figure 4c, d with power-law curve fitting on individual chromosomes at 1 Mb and 25 kb resolution.

15) Pg. 3: As written, it seems as if conjugation of oligos to antibodies was developed in this study. Please provide references to prior art directly in the main text wherever needed.

Thank you. We added the citations (Söderberg et al. 2006; Agasti et al. 2017) for the conjugation of oligos to antibodies in the main text.

16) Pg. 3, “This optimization allowed us to profile these different modalities without a major loss of signal and accurate alignment between IF and DNA FISH images for over 130 rounds of hybridizations on an automated confocal microscope (Extended Data Fig. 2a-e).”: Please provide quantitation of this optimization, preferably with variation of the conditions so that it is clear that the “optimal” conditions have been obtained.

To clarify this point, we added the quantification for DNA FISH signal retention across 4 different conditions in Extended Data Figure 2b, changes of fiducial marker intensity for 80 rounds in DNA seqFISH+ experiments in Extended Data Figure 2d, and antibody signal retention and localization precision in Extended Data Figure 2g. Those additional quantifications can support the claim in the main text and are now referenced in the sentence.

17) Pg 4 & 15, Fig. 2b: The authors show IF reconstructions as blobs/rounded structures. Please include description of the algorithm used to generate these shapes. In particular, were filters applied and, if so, how were the cut-offs determined?

To clarify this point, we added Extended Data Figure 5c, illustrating how the 3D reconstruction in Fig. 2b was generated. Pixels with Z-score above 2 were used to capture the puncta visible in the raw image shown in Fig. 2a. The details for the software and algorithm are described under “*Visualization of seqFISH data*” in the Methods section.

18) Pg 4, Fig. 2b: Are these images displaying only a subset of the chromosomes and, similarly, only a subset of the various epigenetic marks? If so, please make this clear along with any parameters or cut-offs used. Mouse rDNA is also located on several other chromosomes. Please comment on these other locations and whether they also appeared significantly associated with the nucleolus.

To ensure visual clarity, we only showed a subset of the chromosomes and a select group for epigenetic marks in Figure 2b. We highlighted a few interactions that corresponded to known epigenetic marks and chromosomes in the existing literature. We now clarify the parameters and thresholds used in the main text as well as in the figure caption. Systematic comparisons for all the loci and chromatin marks are provided in Figure 2d. We have provided additional examples of the IF images overlaid with DNA loci in Extended Data Fig. 5d. We found all fixed loci for the nucleolar marker, Fibrillarin in the chromosomes, which were previously characterized as rDNA array containing chromosomes, ($n = 39, 1, 22, 30$ and 41 loci for chromosome $12, 15, 16, 18$ and 19) with less enrichment on chromosome 15 (Fig. 2h). Importantly, previous studies using the allele of the 129 mouse strain reported the loss of rDNA or nucleolar enrichments on chromosome 15 ([Kurihara et al. 1994](#); [Strongin et al. 2014](#); [Quinodoz et al. 2018](#)), consistent with our observation with E14 cells derived from $129/Ola$ mouse strain. We now further discussed these points in the main text and methods under “DNA loci to IF marker interactions”.

19) Pg. 4, “To determine whether loci with similar chromatin profiles aggregated in single cells, we first grouped the loci into four clusters based on the IF data (Fig. 2e).”: Was any clustering conducted without input of IF data? For example, was k-means clustering of the loci conducted as an independent assessment?

This analysis was performed by using ensemble-averaged loci and IF marker enrichment profiles in Figure 2d, which includes the input of IF data. We then mapped these cluster assignments to loci in single cells to visualize the spatial distribution. These panels are moved to Extended Data Fig. 7a-e.

20) Pg. 5: Only some readers will know that mouse centromeres are located at the ends of chromosomes and thus, the significance of Figure 2h will not be evident. Please provide background for this and, also, place all observations in the context of other published studies. Broadening to the paragraph beginning with “The contact maps generated from DNA seqFISH+” and ending with “did not completely recapitulate global trends”, which makes broad statements about the 3D organization of the mouse genome, please place these findings in the perspective of other published studies (Hi-C, imaging, etc.).

We revised this panel and moved it to Extended Data Fig. 4e-i, including the cartoon for the mouse chromosomes as well as systematic quantification of the variability across individual cells. We have now removed this discussion in the main text.

21) Pg. 5: Please put observations about lamin association in the context of published studies, and also comment on which loci might have been found to be enriched in the nuclear lamina. We added the direct comparison of DamID traces ([Peric-Hupkes et al. 2010](#)) with our IF imaging data in Figure 2c and Extended Data Fig. 6a, which shows good agreement. We further characterized the association of lamin in each type of lamina associated domains (LADs) ([Meuleman et al. 2013](#)) in Extended Data Fig. 6c. We added the following discussion in the main text.

“Notably, these chromatin profiles were strongly correlated with ChIP-seq (Shen et al. 2012), DamID (Peric-Hupkes et al. 2010), and SPRITE (Quinodoz et al. 2018) datasets (Extended Data Fig. 6a, b) with Pearson correlation coefficient of 0.90 (H3K9ac), 0.82 (H3K27ac), 0.49 (Lamin B1), 0.75 (SF3a66) and 0.77 (Fibrillarin). We further confirmed the enrichment of Lamin B1 IF signal at loci that were previously characterized as lamina-associated domains (LADs) in mESCs (Peric-Hupkes et al. 2010; Meuleman et al. 2013) (Extended Data Fig. 6c).”

22) Pg. 5: Regarding the recognition of zones, were the analyses carried out solely with IF data, and did they also include input from DNA imaging? Furthermore, were any analyses conducted with a smaller subset of primary antibodies? Please comment on the potential for artifacts arising from the simultaneous visualization of 17 IF targets.

The nuclear zone analysis was performed solely with IF data without the input of DNA seqFISH loci data. We downsampled the number of antibodies and found the zone assignments can be recapitulated even with a smaller number of markers (Extended Data Fig. 8d), because many markers are correlated (or anticorrelated). We also showed there are few experimental artifacts from simultaneous imaging of 17 IF targets. Extended Data Fig. 2f shows that there are little image shifts in IF taken before and after heating for DNA FISH. Extended Data Figure 5a also shows there is almost no crosstalk between different channels.

23) Pg. 5: As UMAP is a relatively new method, please provide background and rationale for its application. Please explain its strengths and its weaknesses and discuss the observations in this context. How are markers in multiple zones mapped?

We used hierarchical clustering to define zones from the multiplexed immunofluorescence data. The marker enrichment profiles for each zone were calculated directly from the clusters. UMAP was only used for visualization. We have clarified UMAP as a nonlinear dimensionality-reduction technique in the main text and added in the figure caption that “UMAP projection is used for visual clarity.”.

24) Pg. 5, section subtitled “Active loci are enriched in the interfaces between zones”: While the concepts are straightforward, the figures are difficult to follow, with a number of instances where the text and figures do not align. For example, “active sites are close to the nuclear speckle zone” refers to Figure 3e, which does not show this. There is similar confusion about Figure 3e. Please go through this paragraph and provide clearer explanations of the conclusions, including a quantitative definition for the interface between zones. Also, please provide P values for enrichments and depletions. Finally, please put the findings in the context of published studies, especially what is known about nuclear bodies and zonation.

Thank you. We significantly revised this section and matched the figure with the text. We now show transcription active sites measured by introns along with zones separately in Fig. 3e for clarity and provided quantification of the transcription active sites with respect to the Pol2 hotspots in Fig. 3f. Similarly, we showed in Fig. 3d and Extended Data Fig 8j that the fraction of loci from transcription active sites associated with zone 1, 2 and interfaces are twice as high as those expected from random positioning. We also provided P-values for Figure 3d with Supplementary Table 5. We cited additional references on nuclear condensates and nuclear bodies in the text.

“nuclear “zones” (Fig. 3b, c), corresponded to known nuclear bodies such as the nuclear speckles ([Spector and Lamond 2011](#)) (zone 1), enriched with the splicing factor SF3a66, the nucleolus ([Pederson 2011](#)) (zone 8 and

9) enriched with Fibrillarin, a key nucleolar protein, and nuclear lamina ([van Steensel and Belmont 2017](#)) (zone 10 and 11) enriched with Lamin B1 together with the histone modification H3K9me2 (zone 11), which was previously found at the nuclear periphery and found to be enriched in LADs ([Guelen et al. 2008](#); [Peric-Hupkes et al. 2010](#); [Kind et al. 2013](#); [van Steensel and Belmont 2017](#)). In addition, zone 2 enriched in active marks (RNAPII ser5 phosphorylation and histone acetylation marks) formed contiguous regions in the nucleus that often surrounded the nuclear speckles ([Spector and Lamond 2011](#)) (Fig. 3b, c).”

25) Pg. 5: Is there any specific reason why IF was done after DNA imaging? How would the zonation look if IF were performed first?

We imaged IF after DNA FISH so that the IF data can be registered to the DNA seqFISH+ loci at high resolution. To check that the IF defined zones are not affected by DNA FISH, we examined the zones definitions from the multiplexed IF data in the 1k intron experiment, which did not contain DNA seqFISH+. When we analyzed the immunofluorescence from that dataset, we found that the relative abundance of each type of zones agreed well with the dataset with DNA FISH, shown in Extended Data Figure 8c, indicating that IF zones are not substantially affected by DNA imaging.

26) Pg. 6: Please explain why Pou5f1 (Oct4) and Dazl were selected for special comment. Are they typical or outliers with respect to their behavior?

We commented on Pou5f1 (Oct4) as it is a commonly known master regulator of pluripotency. We commented on Dazl as it is genomically adjacent to Pou5f1. We have included additional loci for visualization in the revised Figure 3c. Full list for the normalized association frequencies of individual zones and interfaces on each of the 3,660 DNA loci is available as Supplementary Table 4.

27) Pg. 6, “To determine whether loci with similar zone assignments are more likely to be spatially close regardless of genomic distance, we examined the likelihood that pairs of loci are found within a 500 nm radius sphere in single cells as a function of genomic distance”: What is the size distribution of the overall chromosome territory for each chromosome? What was the rationale behind the choice of 500 nm radius? If the territories have highly variable size, a uniform 500 nm search radius might not be appropriate to calculate the return probability. Again, please put these observations into the perspective of published studies. Please also consider the rich literature on compartments, including their sizes and organization, as well as various nuclear bodies. To improve clarity, we removed the discussion of the “return probability” from the main text and figures in the revision. The size distributions for different chromosomes appear to be fairly consistent, as seen in the genome vs physical scaling relationship in (Fig 1i). We chose a search radius to be 500 nm because the mean displacement for loci 1 Mb apart (Fig 1j and k) was 500 nm.

28) Pg. 6: Please explain why the RNA seqFISH+ information was not used in the correlations between loci, zones, and RNA-seq data. Also please make clear which findings are new discoveries and which findings are confirmation of previously published correlations.

The comparison of RNA seqFISH data to DNA seqFISH+ and nuclear zones was previously shown in Figure 3i, j in the original manuscript. To further clarify those results, we extended those parts to Figure 3h-l and added the discussion under the “*Active loci are pre-positioned at the interfaces*” section in the main text. We cited previous references showing correlations between LAD, nucleolar and nuclear speckle regions with transcription. We clarified that the new finding in this work is that DNA loci appear to be prepositioned to active zones regardless of whether the loci is transcribed or bursting in single cells. We showed specific examples for Tfcp2l1 mRNA and Bdnf introns in Figure 3i and j, as well as the full analysis of mRNAs in Figure 3k and l, and Extended Data Fig. 8m, n. Furthermore, to compare our RNA seqFISH results to the published bulk and single-cell RNAseq studies, we added Extended Data Figure 9a-c and a discussion in the main text, showing a good agreement of RNA counts per cell as well as transcriptionally distinct subpopulations in mESCs found by both RNA seqFISH and RNA-seq methods.

29) Pg. 7 & 8/ Fig. 4: “These data suggest that chromosome contacts are preserved across one cell cycle between sisters, which supports previous observations that the global arrangement of chromosomes is heritable for one generation but are then rapidly lost after 2 generations.” Please mention other published studies in this area, including those that are contradictory.

Thank you. We added multiple published studies including the contradictory ones (Gerlich et al. 2003; Walter et al. 2003; Thomson et al. 2004) in the main text.

30) Pg. 8: Please integrate more references into the Discussion. For example, with respect to the proposal that zones and interfaces suggest a “new layer of regulation”, please consider this proposal in the context of published hypotheses about compartments, domains, nuclear bodies, chromosome territories, etc., as it is unclear whether the concepts presented in the Discussion are truly novel.

We significantly revised the discussion and incorporated additional references. While previous works have proposed compartments and condensates as mechanisms to compact DNA and perform regulatory function, to the best of our knowledge, no one has shown that DNA loci are positioned on the surfaces of these nuclear bodies and that many loci are consistently associated with these nuclear bodies in single cells in a systematic fashion. In addition, the good agreement between the chromatin mark-DNA associations in single cells and ChIPseq results shows that these associations can account for a significant proportion of the interactions observed in ChIPseq data at 1Mb level. We have clarified this further in the text and discussions.

31) Pg. 15, Fig. 2d: How many cells were used for this figure? Please provide data for single cell comparisons. We now indicate the number of cells in the figure caption (n = 446 cells). In addition, we added Figure 2c for the single cell comparison and the section “*Many DNA loci associated with IF marks consistently in single cells*” in the main text which discusses the single cell data.

32) Pg. 33: In the legend of Fig. S7, the reference to panels c and d seem erroneous. Thank you. We corrected the labels in the figure caption.

33) Pg. 34, Extended Fig. 8: Panels a and b are swapped. We removed those figures for clarity.

34) Pg. 36, Extended Fig. 9: Why is the H3S10 plot in panel e not enriched?

We now present the cell cycle pseudotime course as a heatmap in Extended Data Figure 9f, where the enrichment of H3pSer10 was observed in G2/M phase cells. The same enrichment can be found in Extended Data Figure 9e in the original manuscript, however due to a few outlier cells with high normalized intensity, the range of y-axis of H3pSer10 was larger than the other markers.

35) Pg. 43: Does storing cells in 70% at -20°C have an effect on chromatin organization?

The 70% ethanol preparation is a common protocol for FISH experiments. The minimum effect of the permeabilization on 2D distance between the loci was examined in a previous study (Figure S2 in Finn et al., 2019). We have now added this citation in the Methods section under “Cell culture and preparation”.

36) Pg. 47: What is the rationale behind using a 1.73-pixel search radius? How was this value chosen? Is it same for both the 1Mb spaced loci and 60 consecutive loci?

For the 1 Mb resolution data image processing, the 1.73-pixel (square root of 3) search radius was taken to search spots across different barcoding rounds for 1 pixel in each of the xyz directions. On the other hand, for the 25 kb resolution data for the 60 consecutive loci, the spots are only found once in hybridization 1-60, and do not require a search radius. The rounded pixel locations of the spots were then compared to the chromosome paint intensities in hybridization 61-80 instead. Those points are clarified in the Methods section under “Image Analysis”.

37) Pg. 49: Does the presence of 3 or more copies imply aneuploidy? What fraction of cells demonstrates this feature?

Because of cell cycle progression, it is possible to have 3 or 4 copies of loci at S, G2 and M phase cells. We cannot draw any conclusions about aneuploidy based on the current data in mESCs.

Referee #2 (Remarks to the Author):

This manuscript, Global architecture of the nucleus in single cells by DNA seqFISH+ and multiplexed immunofluorescence by Takei et al., presents the results of a powerful new method, DNA seqFISH+. The method uses super-resolution imaging to detect thousands of chromosomal loci spaced throughout the genome in single mouse embryonic stem cells (mESCs). The authors have combined this with sequential immunofluorescence detection of several chromatin marks, nuclear bodies/landmarks and expression profiles of 70 RNAs, in the same cells. Consequently, their data is capable of simultaneous reports on global genome

architecture, genome function and genome associations with nuclear bodies and the nuclear lamina. The method and results are exciting and will be of great interest across a wide readership, and especially for those working in the 3D genome fields.

Comments:

1. The authors go to great lengths to present their data in the context of previously published Hi-C studies, including unfortunately, adopting some of the terminology used in describing Hi-C results. This is a major concern about this work.

We thank the reviewer for the clarification. We have now removed the term “contact” for our measurement and used “spatial proximity”.

2. The authors describe the use of DNA seqFISH+ results to create a “pairwise contact map”. These pairwise contacts are created by designating all loci within half a micron of a target locus as engaged in “pairwise contacts”. This is quite a stretch since there is little evidence that two loci within 500nm of each other could be considered in contact. In molecular terms this is a large distance. Separation distances of a few tens of nanometers might be acceptable as evidence of possible contact, but using 500nm is an unrealistic and incorrect inflation.

Thank you. We no longer use “pairwise contacts” to describe our results and clarify the quantifications are the probability of loci within a certain search radius in the related figures. In addition, to evaluate the robustness of the analysis, we added a comparison of different search radii to generate the probabilities in Extended Data Figure 2k and 3a, b.

3. A comparison of proximities with Hi-C heatmaps would be OK if the authors simply wanted to compare the proximities measured to standard Hi-C matrices but the suggestion that they are measuring “contacts” is a misnomer and misleading, simply serving to muddy the already muddied water of what a “Hi-C contact” is -- actual contact or in fact just a ligation pair. Hi-C ligation implies proximity but is not proof of contact, and in this manuscript with the distances used (500nm), proximity certainly does not provide evidence of contact. The authors go on to incorrectly use the “contact” terminology throughout the manuscript to describe what are actually spatial proximities. This is wrong and should be removed.

We thank the reviewer for raising this point. We carefully revised the manuscript and removed the “contact” terminology throughout the manuscript and figures.

4. Referring to these as contacts weakens the power of the authors data. With FISH, seeing is believing. One can measure the actual physical distance between two loci. With Hi-C on the other hand, it is not actually known what ligation between two loci means in physical terms. Obviously two sequences have to be close enough together to ligate in the nucleus, but an actual separation distance or range of distance is not known, nor is whether they need to be (or are) in contact or simply in proximity. This is where the authors miss a huge opportunity in the presentation of their data and its usefulness to future studies. Rather than try to mimic Hi-C by inaccurately referring to their measured proximities as contacts, they could do the entire field a great service by using their 3D physical measurements in an attempt to do what Hi-C cannot do, to calibrate Hi-C data in physical terms. For example, by comparing the frequency of ligation

events between two genomic regions to the range of 3D measurements between those two regions obtained by DNA seqFISH it should be possible, through the tens of thousands of measurements made, to come up with some metric of physical distance and frequency that could be applied to Hi-C ligation frequencies. Given that the number of labs that are likely to have the setups required for DNA seqFISH is low, and that the number of labs using Hi-C is comparatively enormous, such a metric would seem to be a tremendously useful output from this work.

We appreciate the helpful comments. To compare the Hi-C data to the physical distance obtained by our DNA seqFISH+ measurements, we added a whole chromosome comparison in Figure 1i, as well as a chromosome by chromosome systematic comparison in Extended Data Fig. 3f, g between the HiC and our datasets for both 1 Mb and 25 kb resolution. This provides a calibration of ligation frequency with physical distance. To visualize the physical distances between pairs of loci at 1 Mb and 25 kb resolution, we added heatmaps in Extended Data Fig. 3h, i. To compare the relationship between genomic and spatial distance, which is unique to the imaging data, we added a chromosome by chromosome comparison of power-law scaling of spatial distance as a function of genomic distance in Extended Data Fig. 4c, d.

5. The authors attempt to ‘one-up’ the Hi-C community with an inflated “5 million median pairwise contacts per cell”, when in fact they haven’t measured any. This type of prejudicial comparison isn’t necessary for a reader to see the value in their work, and basically only puts more smoke in the air. For example, if “pairwise contacts” were inferred in a similar way from the type of single cell 3D reconstructions made by Tan et al., *Science* 361, 924–928 (2018), the number would far exceed 5 million per cell.

Thank you for this comment. We removed the quantitative comparison between DNA seqFISH+ and single cell Hi-C measurements.

In summary, this technique and the data generated are potentially of great use to the field. My advice to the authors is in addition to all the useful results on proximities to nuclear bodies and the nuclear lamina, to present their work in a straightforward way as to provide the greatest utility to the field, since the true value of this work will be measured in how often it is cited, rather than through acceptance from those in the field who are not savvy enough to know that their terminology is inaccurate and their comparisons over-inflated.

Referee #3 (Remarks to the Author):

Takei et al present a multi-modal imaging method to simultaneously visualize specific DNA loci, transcribed RNAs, and proteins. This is a heroic effort and an impressive technical achievement. The authors apply the method to study heterogeneity among cultured mouse embryonic stem cells, including clonal dynamics. Building on previous expertise, they scale up genome imaging from ~70 to ~3700 loci (at a resolution of 1 Mb genome-wide, and 25 kb for distinct regions), scale up immunofluorescent sequential detection of proteins to 17 and combine this with expression values obtained with mRNA and intron FISH. Integrative analyses of these measurements reveal that DNA loci, in particular active ones, are enriched at

presumed “interfaces” between nuclear zones and they identify ~7 cellular states based on clustering of global combinatorial chromatin/protein levels, which show partial overlap with transcriptional states. Finally, clonal expansion experiments reveal the inheritance of H3K27me3 states over few generations.

Single-cell multimodal methods are essential to start understanding the principles that govern gene regulation and cellular specification. The technological achievements presented in this manuscript represents an important contribution towards achieving this goal. However, the novelty of this manuscript is, while impressive, largely technical. Implementation of the proposed method is deemed non-trivial for the larger audience and therefore not of broad interest as a resource. More importantly, the biological insights garnered are not sufficiently groundbreaking to warrant publication in Nature, in our opinion.

We thank the reviewer for recognizing the technical breakthrough achieved in this manuscript. In the revision, we show that many loci appear to be associated with either active or repressive nuclear bodies in a highly deterministic fashion across many cells. This result shows that despite the variability in chromosome structures in single cells, there is consistent order in chromosome organization in the nucleus. We believe this simple take-home message will be of interest to a broad audience and that the dataset generated here will be a resource for the community for further discoveries.

We also included additional analyses to uncover how chromosome structures in single cells relate to gene expression states. In particular, we show that many loci are positioned near active zones (zone 1, 2) in single cells, regardless of the mRNA levels and bursting activity measured by intron expression. We provide quantitation to show that the active loci are close to the surfaces rather than the center of the RNAP globules. Lastly we provide a network analysis of global chromatin levels and RNA levels to understand the biological roles of these chromatin profiles in distinct ES states.

General textual comments

Although lengthy technical descriptions are included -for which we do commend the authors- general readability can be improved. Thorough revision of all figure panels, legends and textual references to figures is necessary. For completion, we recommend including some references on (multi-modal) single-cell sequencing methods in paragraph 1 of the Main Text.

We carefully revised the manuscript including the text, figures, legends, textual references to figure and citations. In addition, we have included references on multi-modal single cell sequencing techniques (Kelsey et al. 2017; Ludwig and Bintu 2019; Zhu et al. 2020) in the first paragraph of the main text.

General content-related comments

Much emphasis is placed on so-called “interfaces” between nuclear “zones”. However, not much detail is given on how these interfaces are calculated. It seems to me that, with such purported high resolution, a DNA locus would not commonly find itself at the interface of two (or more) IF-classified zones. Regardless, it would be useful to quantify the number of loci

associating with the number of zones (one, two, or more). Transcriptome profiling presented here does not recapitulate the complete reported heterogeneity in mESCs (while the authors do make such claims in their comparisons with literature). For instance, it is curious that Oct4 is only (and lowly) expressed in a few cells, and these cells are spread across nearly all the clusters called on mRNA. (The authors do report that the Oct4 locus is predominantly associated with active DNA zones.)

We determined that a DNA locus is present at the interface if pixels belonging to two or more zones are found within a 300 nm search radius of the locus. This search radius is chosen because the IF images are taken at the diffraction limit. We clarified this in the main text. For each locus in single cells, we assign it either a zone or an interface (2 or more zones). We provide the quantification for the probability of each locus associating with a zone or an interface from the single cell measurements in Extended Data Fig. 8h-i and Supplementary Table 4. On average, loci are associated with an interface 79% of the time, and in single zones only 21% of the time (Extended Data Fig. 8j, Supplementary Table 4, 5). Revised Figure 2e-g shows that many loci are present at the exterior of IF mark territories, which are reflected in the loci being present close to interfaces because zones are defined by the combinatorial profiles of the IF markers and exterior of IF markers correspond to interfaces between zones.

We apologize for the confusion on the transcriptome data. In Extended Data Fig. 9a of the original manuscript, we showed the scaled expression values rather than the absolute expression levels. Indeed the level of Oct4 (Pou5f1) mRNA represents one of the highest in our measurements (530 ± 184 counts per cell with mean \pm std for $n = 326$ cells), and shows little variability among cells. Thus, the scaled values are mostly close to zero. We clarified this point by labeling the figures for RNA seqFISH as “scaled expression” and by adding a sentence in the figure caption. In addition, we compared our RNA seqFISH counts per cell with bulk RNA-seq data, which confirmed a good agreement between the two datasets with a Pearson correlation coefficient of 0.94. Furthermore, we merged our RNA seqFISH data with scRNA-seq data, and performed integrated analysis. We confirmed the existence of subpopulations with similar transcriptional profiles between datasets, which is now represented as a UMAP projection in Extended Data Figure 9b as well as with similar marker gene expression patterns in Extended Data Figure 9c. We hope the analyses provided here have now clarified that our RNA seqFISH data match well with those in existing studies.

Figure-by-figure comments

Fig. 1i-j. It is not clear what is plotted on the y-axis. Median spatial distance for the same probe across single- cells or for multiple consecutive probes along the linear chromosome? With the current presentation, it is difficult to tell whether the heterogeneity comes from the difference between chromosomes (as claimed) or from variations in measurements between cells (also interesting). Also, these figures require a measure for the spread of the data.

We apologize for the lack of clarity, the y-axis is the median distance between any pairs of loci at a certain genomic distance bin (x-axis). We presented the scatter plots for all pairs of loci and the power-law fits for each chromosome in Extended Data Fig. 4c. As shown in Extended

Data Fig. 4d, the heterogeneity in the 25 kb data is due to the differences between the chromosomal regions that were examined (as claimed) and not due to single cell variabilities, which is averaged out in these population-averaged results from 446 cells..

Fig. 2. In general. The validation of integrated DNA seqFISH+ and IF requires a much more thorough and systematic quantification (i.e. genome-wide, for all chromatin marks with correlation coefficient's). We find anecdotal displays of 120 Mb for two examples (2d) and references to one representative 3D reconstruction (2b) insufficient.

We provided a systematic comparison between our dataset and other studies in Extended Data Figure 6a-c, and added the following discussion in the main text. We also added more comparisons in Figure 2 as well as additional reconstructions showing the fixed points in both Figure 2g and in Extended Data Fig. 6f, g.

“Notably, these chromatin profiles were strongly correlated with ChIP-seq (Shen et al. 2012), DamID (Peric-Hupkes et al. 2010), and SPRITE (Quinodoz et al. 2018) datasets (Extended Data Fig. 6a, b) with Pearson correlation coefficient of 0.90 (H3K9ac), 0.82 (H3K27ac), 0.49 (Lamin B1), 0.75 (SF3a66) and 0.77 (Fibrillarin). We further confirmed the enrichment of Lamin B1 IF signal at loci that were previously characterized as lamina-associated domains (LADs) in mESCs (Peric-Hupkes et al. 2010; Meuleman et al. 2013) (Extended Data Fig. 6c).”

Fig. 2b (bottom left) and Extended data Fig. 5a. From our experience and others, H3K9Ac is broadly distributed in the nucleus. This is also apparent from ED Fig. 5a. Yet, the modelling of the signal as defined globules depicted in Fig. 2b does not reflect this at all. We can see how you would obtain such structures from Fibrillarin staining, yet not H3K9Ac. The authors should explain how these visualizations were derived and why they appear at odds with the IF images. Otherwise, sentences like: “Chromosomes 2, 4 and 5, which appear in the active hubs in the SPRITE data, were spatially close to H3K9ac-enriched regions in single cells” lose validity.

We clarified the 3D reconstructions for the H3K9ac mark in Figure 2b and Extended Data Figure 5c. We agree that H3K9ac signals may appear to be broadly distributed in the nucleus, but the high intensity value pixels tend to aggregate at certain parts of the nucleus as shown in both the raw images in Extended Data Fig. 5a, b and the heatmaps in Extended Data Figure 5c with intensity value Z-score above 2. The Z-score cut-offs for the 3D reconstructions were added in the Figure 2 caption. In addition, our H3K9ac enrichment data on individual loci (Figure 2c, d) match those from ChIP-seq with Pearson's correlation coefficient of 0.90. Many of the same loci are observed near these H3K9ac globules consistently across all cells, indicating these globules could serve functional roles in organizing the nucleus.

Fig. 2c and Extended data Fig. 6a. The distance of 1 μ M in defining contact probability seems arbitrary. We would like to see contact probability as a function of distance to determine a proper cutoff.

We now compare the different search radii in Extended Data Fig. 7e, j, showing a conservation of the trends across a wide range of search radii.

Extended data Fig. 6e. These findings do not seem in complete agreement with literature.

Especially in the cases of constitutive heterochromatin, the IF signal does not fall convincingly in compartment B versus A. Do the authors have an explanation for this observation?

To address this point, we examined the existing Hi-C literatures in mESCs (Bonev et al. 2017; Nagano et al. 2017) and found that none show a clear enrichment of heterochromatin marks (e.g. H3K9me3 and H3K27me3) in compartment B compared to compartment A as shown in the figure below. Furthermore, this trend can be seen in human cells with B subcompartments B1-B3 (Rao et al. 2014). In contrast, the literature shows the decrease of active marks in compartment B versus A, consistent with our study.

Supporting Figure 1 [redacted]

Supporting Figure 1 (A) Comparison of A/B compartments with active H3K4me3 and repressive H3K27me3 marks in mESCs reproduced from Nagano et al. 2017 Extended Data Fig. 3e. (B) Comparison of A/B compartments with active H3K4me1 and constitutive heterochromatin H3K9me3 marks in mESCs from Bonev et al. 2017 Figure S1I. (C) Comparison of A/B subcompartments with epigenetic profiles in human GM12878 cells from Rao et al. 2014 Figure 2D. Note that subcompartment B4 spans only 0.3% of the human genome.

Extended data Fig. 6g. Shows two example cells of observed versus shuffled A and B compartments. Is there additional evidence to support the claim that A/B compartments intermingle in single cells?

We compare the A/B compartment intermingling with different search radius in ED Figure 7j. We observe similar behavior across all search radii indicating that the intermingling occurs at all length scales.

Fig. 2g. The patterns of clusters 2 (nucleolus) and 4 (lamin) do not follow the stereotypical sub-nuclear localization of these proteins (see also comment below). This should be clarified.

In the original manuscript, the clusters for DNA loci were generated from the ensemble-averaged enrichment profiles in Figure 2c. In single cells, those DNA loci enriched with one of the clusters can localize to other regions of the nucleus. Similar results with nuclear-lamina associated domain localization in the nuclear interior were seen in Kind et al. 2013 (doi: 10.1016/j.cell.2013.02.028). We moved this figure into Extended Data Fig. 7a-e. In the revised Figure 2, we focused the presentation on the loci that show deterministic association patterns that are consistent across many cells. We further addressed this comment in the response for Fig. 3c below.

Fig. 2i and extended data Fig. 6i. Show anecdotal examples of intercellular variation. At present, the claim that single cells do not recapitulate global trends is overstated without proper systematic quantification.

To address this point and quantify the variability across single cells, we quantified the mean probabilities in Extended Data Figure 4f, the coefficient of variation (CV) across individual cells in Extended Data Figure 4g, and corresponding probabilities in single cells in Extended Data

Figure 4h, highlighting the contrast of the probability values at the ensemble-averaged level and the single-cell level. We removed the statement that single cells do not recapitulate global trends in the main text.

Fig. 3c. A general comment on this work is that it is difficult to discern true biological events/observations from outcomes related to technical limitations. This is especially true for the experiments and analyses described for Fig. 3. This is also in part related to the next point below about textual editing. One example to illustrate this are the three Lamin classifications in Fig. 3c (zones 10, 11 and 12). Especially zones 10 and 12 appear to be mixtures of active and inactive marks. We are cautioned by the spatial lamina distribution assigned to loci in Fig. 2g, and the relatively poor correspondence in contact maps of extended data Fig. 5d. The spatial distribution of the lamina-assigned spots (green) are not (at all) exclusively localized towards the nuclear periphery. Yet, to our knowledge (and as shown in extended data Fig. 5a.), Lamin B1 is found exclusively at the nuclear periphery. Such, “off-peripheral” assignments would generate a mixture of signals which may be interpreted as separate states. Of note, the authors ignore lamin state 11, even though this state appears to be the most stereotypical for LADs. Studies on LADs and H3K9me3 in many species have shown that typically these genomic regions encompass uniform types of chromatin. Therefore, the observed classifications in Fig. 3c require further scrutiny.

E.g. for reference, a zone of 1 micron underneath the nuclear periphery should best reflect the true combinatorial chromatin state associated with the regions of the genome that contact the lamina.

We thank the reviewer for these comments and clarified these points in the revision. Our results are actually consistent with the literature. Kind et al. 2013 showed that LADs can interconvert between associating with the nuclear periphery or with the interior of the nucleus presumably with heterochromatic regions, and only ~30% of LADs are positioned at the nuclear periphery in each nucleus, as shown in the figure below. This observation is consistent with “off-peripheral” localization of some of our Lamin B1 enriched loci in Fig. 2g in the original manuscript. Furthermore, we added a comparison between loci in different LAD categories obtained from published studies in mESCs (Peric-Hupkes et al. 2010; Meuleman et al. 2013) and Lamin B1 enrichment in Extended Data Fig. 6c, validating that our measurement of Lamin B1 enriched loci agrees with the literature.

Supporting Figure 2 [redacted]

Kind et al., 2013.

Supporting Figure 2 Live cell imaging of LADs in the nucleus reproduced from Kind et al., 2013. After labeling the LADs (green) at the nuclear periphery (top left panel) in the nucleus (blue), stochastic shuffling can occur upon mitosis, distributing LADs to both the nuclear periphery and interior (bottom left panel).

In addition, the nuclear zones are defined by the combinatorial IF signals at each pixel, independent of DNA loci information. Thus, Lamin B1 enriched nuclear zones (10, 11 and 12)

reflect Lamin B1 localization and appear at the nuclear periphery (Fig. 3c, Extended Data Fig. 8e, f). In addition, zone 11 is also enriched with H3K9me2, which was observed around nuclear periphery (Wu et al. 2005 10.1091/mbc.e04-11-0997; Kind et al., 2013), consistent with the literature. On the other hand, the LADs and the Lamin B1 enriched loci were obtained from population-averaged data. Although those genomic regions are on average associated more frequently with Lamin B1, it may not necessarily appear at the nuclear periphery around Lamin B1 in single cells as shown above.

Fig. 3d-e. The entire paragraph 2 under “Active loci are enriched in the interfaces between zones.” (referring mainly to 3d-e and ED 7d,e) would benefit from textual editing to better guide the reader through the figures. It requires considerable effort from the reader to first understand which part of the graph to look at, and subsequently match the written text to an interpretation of the presented data.

We significantly revised this section and matched the figure with the text. We now show transcription active sites measured by introns along with zones separately in Fig. 3e for clarity and provided quantification of the transcription active site with respect to the Pol2 hotspots in Fig. 3f. Similarly, we showed in Fig. 3d and Extended Data Fig 8j that the fraction of loci from transcription active sites associated with zone 2, 3 and interfaces are twice as high as those expected from random positioning.

- From the text it is unclear how interfaces are defined We added sentences to clarify the definition of interfaces.

- From Fig. 3e it is not immediately clear that “loci are enriched at interfaces”

To clarify this point, we corrected the figure legend in Figure 3d, and clarified in the main text as described below.

“Many loci were enriched at interfaces between zones (Fig. 3c, d, f, Extended Data Fig. 8e, j and Supplementary Table 5), consistent with the observation of loci near the exterior of nuclear bodies (Fig. 2e, g). For example, DNA loci are 46.3% more likely to be detected at interfaces 2/3 than random chance (Fig. 3d).”

- The selection of zone interfaces depicted in Fig. 3e appears rather arbitrary

The zones were ordered according to the probability of occupancy, which we now state in the caption. We have moved this figure to Extended Data Figure 8h-i. The full lists of loci and zone interfaces are shown in Supplementary Table 4 and 5. This figure also illustrates zones and interfaces with different enrichment patterns. For example, transcription active sites are enriched in interface 2/3, while intrachromosomal pairs are enriched in interface 5/7. In the main text, we clarified this.

“DNA loci are 46.3% more likely to be detected at interfaces 2/3 than random chance (Fig. 3d). Furthermore, pairs of interchromosomal loci were enriched at the active interfaces 2/3 while pairs of intrachromosomal loci were enriched at the heterochromatic interfaces 5/7 and nucleolus interfaces 8/9 (Figure 3d).”

It is also unclear to what extent such interphases are the result of the limitations in resolution.

The paragraph would benefit if the authors would elaborate on these points more.

The IF imaging is diffraction limited, so it is likely that even more granularity will be observed with higher resolution imaging. Analogous to geographical boundaries become fractal like at higher resolution (e.g. Coastline paradox), we expect the nuclear body boundaries to be similarly complex. We added a discussion of this in the main text.

Fig. 3d and ED7d. Sentences such as “loci appear at the edges, rather than the center, of RNAPII dense regions in the nuclei” would benefit from quantification rather than visual representation of a few nuclei.

We appreciate this suggestion. We added the quantification of the distance between the nascent transcription active sites to the center and edge of RNAPII dense regions, showing the closer localization of the sites at the exterior of the PolIII dense globules in Figure 3f.

Fig. 3f. Related to the arbitrary selection of zones and interphases. Why are different interphases displayed in this figure compared to Fig. 3e? Based on what qualifications?

The zones and interfaces in Figure 3f in the original manuscript were sorted according to the probability of occupancy. There were 12 zones and 66 interfaces in total and we could not show all of them in the main figure. We clarified this point in the figure caption. In addition, a full list of the quantification associated with this figure can be found in Supplementary Table 4.

Fig. 3f. We do not find the contact probability per locus (distributed over different zones and interfaces) an intuitive measure of intercellular variability, since each locus can appear in multiple pairwise interactions (rows, in these heatmaps).

In each cell, one locus is assigned to a single zone or interface. Thus, this figure shows the single cell heterogeneity in spatial proximity of loci to zones across all cells measured. We clarified this in the text and captions.

Fig. 3g. To gain true insight into (and make claims about) what is happening at a single-cell level, it is imperative to show the distribution of return probabilities across all the cells. What we see now is that, on average, zone 1 and the interface between zones 6 and 7 maintain more contacts at large distances compared to the whole chromosome.

Return probability measured the fraction of loci pairs from single cell data that are close in physical space as a function of genomic distance. Since this metric appears to be confusing, we removed it from the revised manuscript.

Fig. 3h-i. The authors claim that gene expression and nuclear localization in single cells is not correlated. The authors continue to say that highly expressed genes are always close to active zones, while lowly expressed genes are always close to the nuclear lamina and heterochromatin. This follows and is assumed from the data, but the relationship is not displayed. Perhaps show, per mRNA, the correlation between expression level and zone localization).

Figure 3h,i and j in the original manuscript may have been too condensed, but were intended to illustrate this point with each of the dots in Figure 3i as a single gene and its correlation with the active zones. We expanded them into Figure 3h-l and clarified this point with additional

visualization and reconstructions. We also included individual gene comparison in Extended Data Fig. 8m,n.

Fig. 4b. We are unsure how to biologically interpret overall intensity levels of histone PTMs without information on genomic positional information or subnuclear localization patterns. E.g. cells in clusters 4 and 5 have overall high H3K27me3 & mH2A.1 or H3K9me2 & H3K9me3 levels respectively, yet how should we see this biologically? Do the cells in these clusters overall have lower expression output? Are the levels of these histone PTMs more elevated at their “normal” genomic locations, or do these cells in general have higher levels of these modifications throughout the genome? Without genomic positional information, and without associating differential chromatin signatures to transcriptional output in the same cell, the meaning of these classifications remains rather elusive. This data requires cross-referencing with single-cell transcriptomics datasets -or other- to ensure that these states are 1) biologically meaningful and 2) can be observed in other datasets.

To address the question of the functional relevance of the H3K27me3 states, we performed a network analysis of the histone mark and RNAs to understand how H3K27me3 relates to the other pluripotency markers. As shown in Figure 5d, H3K27me3 states are correlated with Tbx3 and Aebp2, while mH2A1 high states are correlated with Nanog and Esrrb. This is further shown in the pseudotime analysis in Figure 5c, where H3K27me3 levels follow Tbx3 expression states. We did not observe a significant difference in the single cell “chromatin profile,” as shown Fig 2c, from each of the clusters. It is likely thousands or more cells are needed to identify loci with distinct profiles above statistical significance and is beyond the scope of the current manuscript. Additional comprehensive transcriptome and epigenetic analysis integrated in single cells is needed to fully characterize the differences in molecular states and needs to be obtained in future experiments.

The clustering based on mRNA as presented in Extended Fig. 9a could have been revealing towards identifying the biological significance of these chromatin assignments, yet in our opinion the almost uniform mixing of the chromatin states over the 5 transcriptional states indicates that these chromatin states have little predictive value and may have little biological significance. The exception is the transcriptional “mESC naïve ground state” #1 which appears to be associated with the high H3K27me3 and mH2A.1. However, as far as we recall, naïve ground state mESCs are supposed to be associated with low H3K27me3 levels rather than high (e.g. Marks et al., 2012).

Marks et al., 2012 showed an enrichment of H3K27me3 modification in serum-grown ESC population compared to more naïve 2i-grown ESC population. However, in this paper, their observations were restricted to the individual genomic regions with ChIP-seq profiling and did not assess the differences of the total H3K27me3 levels in the nuclei. In a follow up paper (van Mierlo et al., 2019), Marks and co-authors showed that the global levels of H3K27me3 was higher in 2i treated cells, which is consistent with our observations. In addition, imaging- based study (Tosolini et al., 2018) has compared the number of heterochromatic clusters in the nuclei among 2i-grown ESCs, serum-grown ESCs and EpiSCs (primed state cells), and found the enrichment of H3K27me3 heterochromatin clusters in the more naïve cell states as well as the

enrichment of H3K9me3 heterochromatin clusters in the more primed cell states. This observation is consistent with our pseudo-time course results (from naive to primed states) with serum-grown ESCs in Extended Data Fig. 5c, and those points were added in the main text. Overall chromatin state heterogeneity is largely unexplored and thus the functional relevance remains elusive. Our network and pseudotime analysis suggest that they may be functionally relevant and need to be further tested in differentiation experiments.

Fig. 4d-h. The clonal analyses performed in the second part of Fig. 4 are potentially very interesting. Combinatorial single-cell measurements of different modalities as presented in this work are pivotal for meaningful interpretation of the inheritance of transcriptional states over one or multiple generations. Especially, the inheritance of H3K27me3 states over few generations is striking. However, this experiment could have been performed with single-modal, non-sequential IF imaging, and as such does not take advantage of the proposed method. This is a missed opportunity in our opinion, since without the combinatorial information, this finding is merely an interesting observation. This finding should be further substantiated and requires additional investigations to assign functional relevance to the inheritance of the H3K27me3 states.

We analyzed the combinatorial states as a function of time in the clonal tracing experiment. The decay in the correlation of the combinatorial chromatin states is shown in Fig. 4g in the revised manuscript. It may be possible to discretize the data and map the transition probability between different chromatin cluster states, but we will need thousands of cells to capture all the possible states and transitions. This is possible in a future experiment when we image the IF states without DNA seqFISH+ to capture a large number of cells. The chromatin state to state correlation as well as correlation between chromatin and mRNA level are shown in Extended Data Fig. 9h. Similarly, the decay in mRNA state and chromosome structure within the clone are shown in Figure 4g.

Minor points

-Reference to Fig. 3e in the text should read Fig. 3d. We have now corrected the reference.

-ED Fig. 6j is described in the legend but the letter annotation does not exist. We have removed the figure.

-ED Fig. 7 legend letters need to be adjusted. We have corrected the letters in the figure caption.

-ED Fig. 8 legend letters and references to this figure in the main text need to be adjusted. We have corrected the letters in the figure caption and text.

Reviewer Reports on the First Revision:Referee #1 (Remarks to the Author):

The authors have addressed the majority of our comments, including additional data and analyses. We also appreciate the effort the authors have made to cite prior publications and put their findings in the context of published studies. However, we have a few remaining considerations, which we have numbered according to the numbering system in the authors' response to reviewers:

4) The authors state that the probes were purchased for approximately \$15,000, but they also mention that probes were both purchased as 5'amine-modified DNA oligonucleotides and then conjugated in-house to fluorophores as well as purchased already conjugated to fluorophores. Thus, will the authors please provide additional clarity by a) estimating the cost if all 240 probes had been purchased as 5'amine-modified DNA oligonucleotides and conjugated in-house and b) estimating the cost if all 240 probes had been purchased fully labeled. This information will be important for readers wishing to gain a concrete sense of whether the technology is compatible with their budgets.

6) Unfortunately, the authors' use of "super-resolution" continues to be problematic, with significant potential to confuse readers. Specifically, while diffraction-limited light microscopy was able to resolve closely positioned targets, it did not provide structural information about any of the targets, themselves. This is a critical issue, and we urge the authors to reassess their use of "super-resolution/resolved". Hence, the phrase in the abstract, "Here, we report the super-resolution imaging of 3,660 chromosomal loci..." will mislead readers into thinking that the authors are providing super-resolved structures of 3,660 chromosomal loci. The authors could write, instead, "Here, we report the super-resolved positioning of 3,660 chromosomal loci...". As for the title of Figure 1, we would recommend removing the word "super-resolution".

In thinking about this issue of terminology, we consulted the three papers that pioneered the use of sequential hybridization and diffraction-limited light microscopy to achieve super-resolved tracings of chromosomal pathways (cited as references 31, 33, and 34 in the revised manuscript). All three papers avoid the use of "super-resolution" to describe their images obtained with diffraction-limited light microscopy, with reference 31 also explicitly explaining the limits of diffraction-limited light microscopy. Thus, reference 31 describes traces and/or conformations, but not individual targets, as super-resolved. As the current manuscript uses the same approach, though scaled to more targets, we would ask that the authors similarly limit their use of "super-resolution/resolved".

6a) In researching the issue of super-resolution terminology before responding to the authors, we were struck even more by the similarity in approach between the current manuscript and the technologies described by references 31, 33, and 34 as Hi-M (reference 33) and ORCA (reference 31 and 34), respectively. What sets the current manuscript apart is, of course, the significant scaling in the number of genomic targets. Nevertheless, as an essential part of this scaling is the strategy demonstrated by Hi-M and ORCA for the genome, we would ask the authors to explicitly mention the technologies of Hi-M and ORCA with respect to genome imaging along with mention of the authors' prior work with respect to RNA imaging. Finally, we suggest citing the early work of Christoph Cremer's group on spectral precision distance microscopy (SPDM), which demonstrated the capacity diffraction-limited light microscopy to break the diffraction limit of light, thus setting the stage for temporal discrimination of targets (Esa et al. *J Microsc.* 2000; 199:96-105. doi: 10.1046/j.1365-2818.2000.00707.x; Cremer et al. *Handbook of Computer Vision and Applications*, ed. by B. Jähne, H. Haußecker, P Geißler, Academic Press, 1999; 3:839-857; *Principles of Spectral Precision Distance Confocal Microscopy for the Analysis of Molecular Nuclear Structure*).

6b) Continuing on the theme of confusing terminology in the field of super-resolution, we believe that the abbreviation, OligoSTORM, represents the combination of Oligopaint (not oligo) and stochastic optical reconstruction microscopy. Thus, the authors should also make this correction. (See Beliveau et al. *Methods Mol Biol.* 2017;1663:231-252. doi: 10.1007/978-1-4939-7265-4_19.)

22) The significance of Extended Data Fig. 8d is not clear. Please explain the implications of a gradual increase along the y axis with an increase in the number of IF marks. It would be nice to see some representative images when IF marks are down-sampled.

29) With respect to the heritability of chromosome positioning, we would suggest adding one more reference, this one to Essers, et al. *Molec. Biol. Cell* 2005;16:769-75. doi: 10.1091/mbc.e04-10-0876.

30) We are still not fully convinced about the novelty of the biological findings, as the authors' conclusions about biology seem, overall, to be reinforcements and extensions of previously published observations. However, we very much appreciate their imaging of a milestone number of genomic loci simultaneously with that of chromatin marks and transcripts inside a single cell.

Referee #2 (Remarks to the Author):

My main concerns have been addressed by the authors.

Referee #3 (Remarks to the Author):

While the manuscript has significantly improved in general readability, terminology, and (analytical) structure, I stand by my earlier recommendation that this work deserves a place in an impactful technical journal, with more elaborate attention for the achievements in the method.

Interfaces

My previous comments on interface calculation remain. The authors have clarified their methods, but that does not address the underlying limitation of resolution.

Response by the authors: "The IF imaging is diffraction limited, so it is likely that even more granularity will be observed with higher resolution imaging. Analogous to geographical boundaries become fractal like at higher resolution (e.g. Coastline paradox), we expect the nuclear body boundaries to be similarly complex. We added a discussion of this in the main text."

Yes, finer granularity would be observed, or the concept of interfaces might disappear, and loci would simply be assigned to zones. Besides resolution, also the IF-chromatin assignments need careful scrutiny (see comment on Lamin B1 below) as this directly impacts on the biological interpretation of the data (including the concept of interfaces).

Transcriptional heterogeneity

While the good correlation between seqFISH and bulk RNA-seq counts is encouraging, it does not address my concern of little variability across cells (especially when intending to make biological claims on the metastable states found in mESCs). The additional analyses integrating RNA seqFISH and scRNA-seq are valuable, yet do not entirely explain why e.g. Oct4 shows no differential expression as it does in the data used for the integration (Kolodziejczyk 2015).

Chromatin zones

I commend the authors for their additional efforts to place their data in context of the current literature on nuclear bodies and zonation (as also suggested by the other referees). However, the

conclusions to be drawn from this manuscript are not necessarily strengthened. The analyses as presented in Fig 2c and ED Fig 6d (single-cell chromosome heatmaps) give a good indication of the locus-specific enrichment across cells. Much emphasis is placed on the comparison with Lamin B1 data, and good agreement is claimed between DamID and "chromatin profiles" generated in the current study, but that is not apparent from the figure (and correlation coefficients are much worse than for the other markers mentioned). Looking at Fig. 2a and especially ED Fig. 5d it is clear that the Lamin B1 signal is not exclusively peripheral as it should be (this is generally expected in the field). This explains the identification of "mixed" zones such as zone 12 in Fig. 2b. This is apparent from Fig. 2c where the assigned zone 12 regions are all off-peripheral with a distance of at least 1micron from the edge of the nucleus. The loci found in these zones are thus not true Lamin B1 associated genomic regions. The source of the interior Lamin B1 signal may be related to the use of a poor-quality antibody (the antibody used in this study is not commonly used), the experimental procedure or image processing. Judging from Fig. 2a and the clear explanation by the authors about the image processing, it is most likely related to the quality of the IF. Regardless, this explains 1) the existence of the mixed zone #12, 2) the poor correlation with LADs (DamID) and 3) why no fixed loci were found for Lamin B1 imaging (Fig. 2c). By single-cell DamID a percentage of loci were previously identified to contact the lamina in near 100% of the cells (Kind et al., 2015; Rooijrs et al., 2019). Those loci should at least have been identified in this work (Fig. 2c). Collectively, these results therefore do not assuage my concerns raised in the first revision round; instead, additional questions arise.

In the Response to reviewers, the authors cite Kind 2013 as additional evidence for lamina-marked loci found in the nuclear interior. These loci are deemed variable between single cells, and the population-averaged nature of the cited DamID sequencing data (Peric-Hupkes 2010, Meuleman 2013) is given as a reason that the genomic regions in the current study do not necessarily appear at the nuclear lamina. These arguments are lacking for two reasons. 1) My comments pertain to the imaging of Lamin B1 protein (and loci being assigned to its periphery within 300 nm). LADs do become reshuffled upon mitosis, with a proportion of LADs indeed not returning to the nuclear lamina, but those LADs do NOT "retain an interaction" with Lamin B1. That is, I think, exactly the power of this DamID-Tracer imaging approach, in contrast to measurement of endogenous DNA-protein proximities. 2) To circumvent the caveats of population-based Lamin B1 DamID, the authors could have performed a comparison with single-cell Lamin B1 DamID (see figure below for KBM7 cells). Even visual inspection would have shown that Lamin B1 does, in fact, come into close proximity with highly specific regions of the genome, with patterns similar to the population-averaged LADs. Altogether, I find that the presented analyses do not sufficiently justify the claimed functional consequences of what are considered novel findings. I propose to at least 1) remove the Lamin B1 data, or 2) repeat this dataset with a good Lamin B1 antibody (e.g. AB16084 is cited 625 times according to the Abcam website).

Comments from Reviewer 3 on a preliminary rebuttal to these reports:

I think the work is overall of high quality and the manuscript improved considerably upon revisions, yet the claim by Cai and colleagues that Lamin B1 is also present in a nucleoplasmic pool goes against decades of work in the field. Lamin B1 has a farnesylated tail that integrates the protein into the inner-nuclear membrane. For Lamin A/C the tail is cleaved off which results in nucleoplasmic localizations in addition to predominant peripheral signal. The images provided in the rebuttal are selective and the interpretation of the SR-image is incorrect. There is a "fuzzy" surface, yet the entire thickness of the lamina is still less than 100nm (see Fig.S5b). The images they refer to display oblique sections and therefore the lamina appears much thicker. With their analyses, only genomic regions that are within 300nm spatial distance (plus ~100nm lamin meshwork) to the nuclear periphery

should be considered true lamina-associated regions. All other regions (like zone 12) are not (by their definition). Therefore, all off-peripheral lamin "contacts" are in my opinion misassigned.

Author Rebuttals to First Revision:

Referee #1 (Remarks to the Author):

The authors have addressed the majority of our comments, including additional data and analyses. We also appreciate the effort the authors have made to cite prior publications and put their findings in the context of published studies. However, we have a few remaining considerations, which we have numbered according to the numbering system in the authors' response to reviewers:

4) The authors state that the probes were purchased for approximately \$15,000, but they also mention that probes were both purchased as 5'amine-modified DNA oligonucleotides and then conjugated in-house to fluorophores as well as purchased already conjugated to fluorophores. Thus, will the authors please provide additional clarity by a) estimating the cost if all 240 probes had been purchased as 5'amine-modified DNA oligonucleotides and conjugated in-house and b) estimating the cost if all 240 probes had been purchased fully labeled. This information will be important for readers wishing to gain a concrete sense of whether the technology is compatible with their budgets.

[We clarified the costs for the probes further in the methods section.](#)

6) Unfortunately, the authors' use of "super-resolution" continues to be problematic, with significant potential to confuse readers. Specifically, while diffraction-limited light microscopy was able to resolve closely positioned targets, it did not provide structural information about any of the targets, themselves. This is a critical issue, and we urge the authors to reassess their use of "super-resolution/resolved". Hence, the phrase in the abstract, "Here, we report the super-resolution imaging of 3,660 chromosomal loci..." will mislead readers into thinking that the authors are providing super-resolved structures of 3,660 chromosomal loci. The authors could write, instead, "Here, we report the super-resolved positioning of 3,660 chromosomal loci...". As for the title of Figure 1, we would recommend removing the word "super-resolution".

In thinking about this issue of terminology, we consulted the three papers that pioneered the use of sequential hybridization and diffraction-limited light microscopy to achieve super-resolved tracings of chromosomal pathways (cited as references 31, 33, and 34 in the revised manuscript). All three papers avoid the use of "super-resolution" to describe their images obtained with diffraction-limited light microscopy, with reference 31 also explicitly explaining the limits of diffraction-limited light microscopy. Thus, reference 31 describes traces and/or conformations, but not individual targets, as super-resolved. As the current manuscript uses the same approach, though scaled to more targets, we would ask that the authors similarly limit their use of "super-resolution/resolved".

[We used the terminology "super-resolved positioning" or "localized" instead of "super-resolved imaging" as the reviewer suggested in the revised manuscript.](#)

6a) In researching the issue of super-resolution terminology before responding to the authors, we

were struck even more by the similarity in approach between the current manuscript and the technologies described by references 31, 33, and 34 as Hi-M (reference 33) and ORCA (reference 31 and 34), respectively. What sets the current manuscript apart is, of course, the significant scaling in the number of genomic targets. Nevertheless, as an essential part of this scaling is the strategy demonstrated by Hi-M and ORCA for the genome, we would ask the authors to explicitly mention the technologies of Hi-M and ORCA with respect to genome imaging along with mention of the authors' prior work with respect to RNA imaging. Finally, we suggest citing the early work of Christoph Cremer's group on spectral precision distance microscopy (SPDM), which demonstrated the capacity diffraction-limited light microscopy to break the diffraction limit of light, thus setting the stage for temporal discrimination of targets (Esa et al. *J Microsc.* 2000; 199:96-105. doi: 10.1046/j.1365-2818.2000.00707.x; Cremer et al. *Handbook of Computer Vision and Applications*, ed. by B. Jähne, H. Haußecker, P Geißler, Academic Press, 1999; 3:839-857; *Principles of Spectral Precision Distance Confocal Microscopy for the Analysis of Molecular Nuclear Structure*).

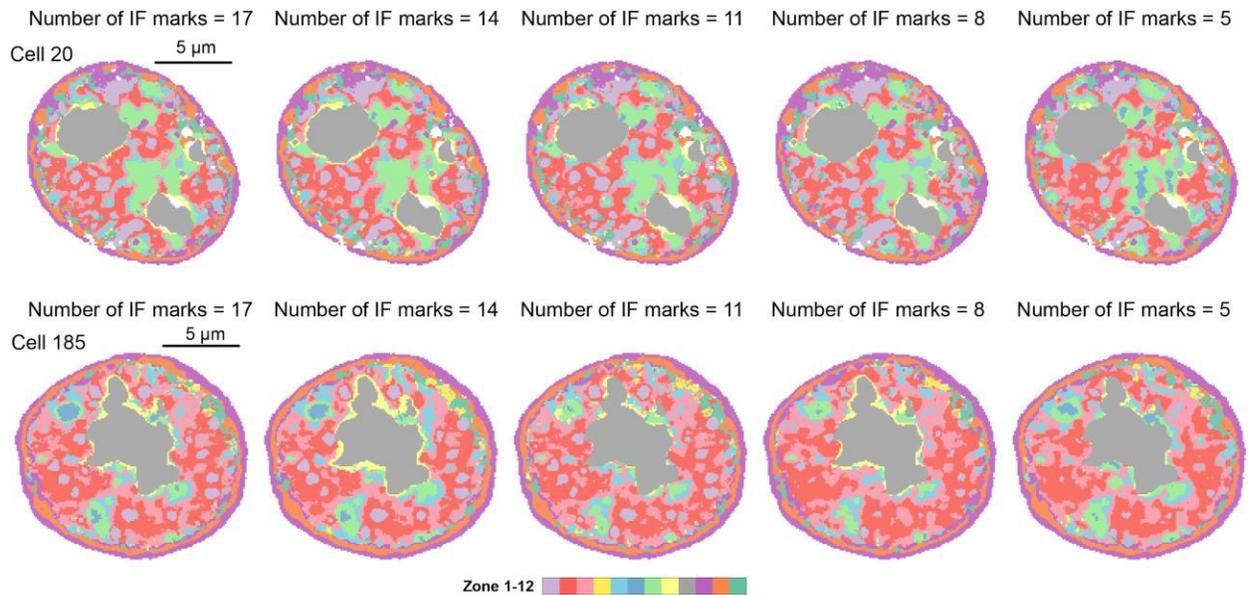
We added a sentence to describe Hi-M and ORCA for the simultaneous imaging of genome structures and transcription. In addition, we added the citations for the early work of multicolor DNA FISH.

6b) Continuing on the theme of confusing terminology in the field of super-resolution, we believe that the abbreviation, OligoSTORM, represents the combination of Oligopaint (not oligo) and stochastic optical reconstruction microscopy. Thus, the authors should also make this correction. (See Beliveau et al. *Methods Mol Biol.* 2017;1663:231-252. doi: 10.1007/978-1-4939-7265-4_19.)

We made this correction.

22) The significance of Extended Data Fig. 8d is not clear. Please explain the implications of a gradual increase along the y axis with an increase in the number of IF marks. It would be nice to see some representative images when IF marks are down-sampled.

This extended data figure shows that zone assignments become more accurate as the number of IF markers used increases. At the same time, even few IF marks can recapitulate the major zone assignments because many marks are correlated with each other. Representative cell reconstructions with downsampled IF are shown below.



Caption: Visualization of downsampling of IF marks for nuclear zone assignments in single cells. The number of IF marks used to generate each image is labeled above. The zone classifiers are trained using the number of IF marks indicated using the training data, and then propagated to the entire cell.

29) With respect to the heritability of chromosome positioning, we would suggest adding one more reference, this one to Essers, et al. *Molec. Biol. Cell* 2005;16:769–75. doi: 10.1091/mbc.e04-10-0876.

We added this citation.

30) We are still not fully convinced about the novelty of the biological findings, as the authors' conclusions about biology seem, overall, to be reinforcements and extensions of previously published observations. However, we very much appreciate their imaging of a milestone number of genomic loci simultaneously with that of chromatin marks and transcripts inside a single cell.

Referee #2 (Remarks to the Author):

My main concerns have been addressed by the authors.

Referee #3 (Remarks to the Author):

While the manuscript has significantly improved in general readability, terminology, and (analytical) structure, I stand by my earlier recommendation that this work deserves a place in an impactful technical journal, with more elaborate attention for the achievements in the method.

Interfaces

My previous comments on interface calculation remain. The authors have clarified their methods, but that does not address the underlying limitation of resolution.

Response by the authors: "The IF imaging is diffraction limited, so it is likely that even more

granularity will be observed with higher resolution imaging. Analogous to geographical boundaries become fractal like at higher resolution (e.g. Coastline paradox), we expect the nuclear body boundaries to be similarly complex. We added a discussion of this in the main text.”

Yes, finer granularity would be observed, or the concept of interfaces might disappear, and loci would simply be assigned to zones. Besides resolution, also the IF-chromatin assignments need careful scrutiny (see comment on Lamin B1 below) as this directly impacts on the biological interpretation of the data (including the concept of interfaces).

In the revision, we showed that DNA fixed loci are present on the surface of nuclear bodies (Figure 2). This finding supports the idea that many DNA loci are located near the interfaces between zones (Figure 3) because surfaces of nuclear bodies correspond to the interfaces. Thus, even at higher resolution, it is likely that we will observe an enrichment of DNA loci near interfaces.

Transcriptional heterogeneity

While the good correlation between seqFISH and bulk RNA-seq counts is encouraging, it does not address my concern of little variability across cells (especially when intending to make biological claims on the metastable states found in mESCs). The additional analyses integrating RNA seqFISH and scRNA-seq are valuable, yet do not entirely explain why e.g. Oct4 shows no differential expression as it does in the data used for the integration (Kolodziejczyk 2015).

We explicitly showed that similar subpopulations in mESCs were observed in both mRNA seqFISH and scRNAseq in the revision in Extended Data Figure 9b, c. In addition, it is well known that Oct4 is expressed homogeneously in mESCs (Kumar et al. 2014; Singer et al. 2014). The CV for the homogeneous genes such as Oct4 and Sall4 were low: 0.35 vs 0.50 for Oct4, and 0.40 vs 0.47 for Sall4 in our data vs Singer et al. 2014 smFISH data, while CV for stochastically expressed genes were higher such as Tbx3 (3.0 vs 2.1 in our data vs Singer et al. 2014). We also found a good agreement of pairwise relationships between mRNA expressions in single cells between our data and Singer et al. 2014. For example, we found relatively low Pearson's r of 0.32 (our data) and 0.20 (Singer et al. 2014) for Oct4 and Zfp42, while relatively high Pearson's r of 0.63 (our data) and 0.70 (Singer et al. 2014) for Esrrb and Nanog. Thus, mRNAseqFISH accurately captures mRNA expression in single cells.

Chromatin zones

I commend the authors for their additional efforts to place their data in context of the current literature on nuclear bodies and zonation (as also suggested by the other referees). However, the conclusions to be drawn from this manuscript are not necessarily strengthened. The analyses as presented in Fig 2c and ED Fig 6d (single-cell chromosome heatmaps) give a good indication of the locus-specific enrichment across cells. Much emphasis is placed on the comparison with Lamin B1 data, and good agreement is claimed between DamID and “chromatin profiles” generated in the current study, but that is not apparent from the figure (and correlation coefficients are much worse than for the other markers mentioned). Looking at Fig. 2a and especially ED Fig. 5d it is clear that the Lamin B1 signal is not exclusively peripheral as it should be (this is generally expected in the field). This explains the identification of “mixed” zones such as zone 12 in Fig. 2b. This is apparent from Fig. 2c where the assigned zone 12 regions are all off-peripheral with a distance of at least 1 micron from the edge of the nucleus. The loci found in these zones are thus not true Lamin B1 associated genomic regions. The source of the interior Lamin B1 signal may be related to the use

of a poor-quality antibody (the antibody used in this study is not commonly used), the experimental procedure or image processing. Judging from Fig. 2a and the clear explanation by the authors about the image processing, it is most likely related to the quality of the IF. Regardless, this explains 1) the existence of the mixed zone #12, 2) the poor correlation with LADs (DamID) and 3) why no fixed loci were found for Lamin B1 imaging (Fig. 2c). By single-cell DamID a percentage of loci were previously identified to contact the lamina in near 100% of the cells (Kind et al., 2015; Rooijrs et al., 2019). Those loci should at least have been identified in this work (Fig. 2c). Collectively, these results therefore do not assuage my concerns raised in the first revision round; instead, additional questions arise.

In the Response to reviewers, the authors cite Kind 2013 as additional evidence for lamina-marked loci found in the nuclear interior. These loci are deemed variable between single cells, and the population-averaged nature of the cited DamID sequencing data (Peric-Hupkes 2010, Meuleman 2013) is given as a reason that the genomic regions in the current study do not necessarily appear at the nuclear lamina. These arguments are lacking for two reasons. 1) My comments pertain to the imaging of Lamin B1 protein (and loci being assigned to its periphery within 300 nm). LADs do become reshuffled upon mitosis, with a proportion of LADs indeed not returning to the nuclear lamina, but those LADs do NOT “retain an interaction” with Lamin B1. That is, I think, exactly the power of this DamID-Tracer imaging approach, in contrast to measurement of endogenous DNA-protein proximities. 2) To circumvent the caveats of population-based Lamin B1 DamID, the authors could have performed a comparison with single-cell Lamin B1 DamID (see figure below for KBM7 cells). Even visual inspection would have shown that Lamin B1 does, in fact, come into close proximity with highly specific regions of the genome, with patterns similar to the population-averaged LADs. Altogether, I find that the presented analyses do not sufficiently justify the claimed functional consequences of what are considered novel findings. I propose to at least 1) remove the Lamin B1 data, or 2) repeat this dataset with a good Lamin B1 antibody (e.g. AB16084 is cited 625 times according to the Abcam website).

Reviewer’s main objection to our results is that LaminB1 should be exclusively on the nuclear periphery. As we pointed out in the previous rebuttal letter, literature results show that LaminB1 is NOT exclusively at the periphery. Even the antibody that the reviewer recommended does not show exclusive periphery localization (image below). Furthermore, Lamin-GFP labels also show that laminB1 is enriched at the nuclear envelope, but not exclusive to the periphery, with puncta and intensities in the nucleus. We attached the following images from the literature to show that they are consistent with our Lamin immunofluorescence images in Fig 2a and Extended Data Fig. 5a for multiple cells, which also showed Lamin B1 signals were observed not exclusively at the nuclear periphery but enriched at the periphery.

[redacted]

LaminB1-GFP fusion in U2OS cells.

<https://www.sigmaaldrich.com/catalog/product/sigma/cll1033?lang=en®ion=US>

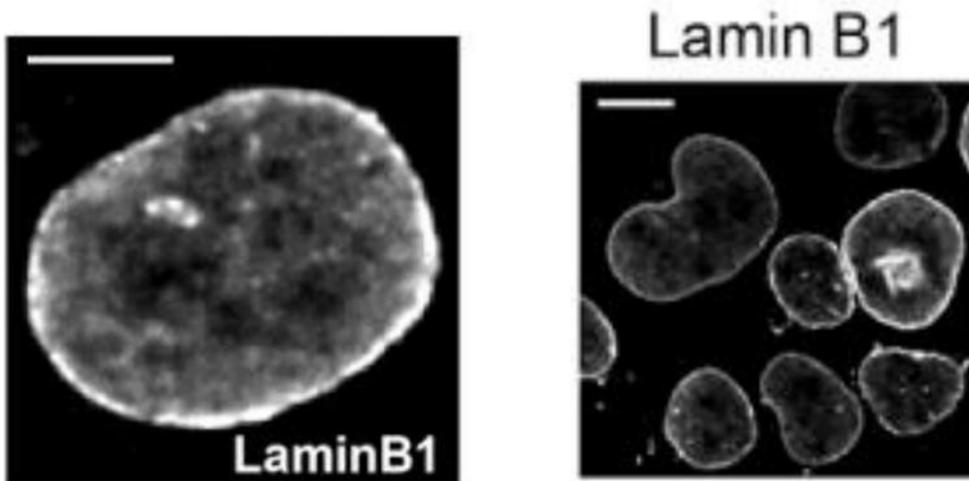


Figure 2a

ED Fig 5a

Lamin B1 staining in our data. Zoomed in cell from Figure 2a as well as multiple cells from ED Fig 5a showing enriched lamin staining at the nuclear periphery, but also staining inside the nucleus. Scale bars are 5um in the left images and 10um in the right image.

[redacted]

Immunohistochemistry on frozen sections of human kidney showing nuclear lamina staining in epithelial and connective tissue cells.

<https://www.abcam.com/lamin-b1-antibody-119d5-f1-nuclear-envelope-marker-ab8982.html>

[redacted]

Human and mouse cells stained with ab16048 (1/500). The cells were fixed and permeabilized in 4% formaldehyde, 0.2% Tritonm X100 for 10 minutes at room temperature, then washed 3x in PBS.

- A: HeLa cells + ab16048 (green)
- B: HeLa cells counterstained with DAPI (blue)
- C: 3T3 cells + ab16048 (green)
- D: 3T3 cells counterstained with DAPI (blue)

LaminB1 antibody from Abcam AB16084 that the reviewer recommended, where a gradient of Lamin intensity is observed from the peripheries in Hela cells and heterogeneous staining in 3T3 cells. <https://www.abcam.com/lamin-b1-antibody-nuclear-envelope-marker-ab16048.html>

[redacted]

Figure 6 in Kind et al 2015 showing LaminB1 (red) staining interior of the nucleus, along with LADs (labeled with m6A-Tracer). Scale bars represent 500 nm.

Kind et al 2015 further states:

“It is noteworthy that the edge of the LmnB1 signal at the NL is less sharply defined at the nucleoplasmic side than at the inner nuclear membrane side (Figures 6A and 6B). This could indicate that lamin filaments extend to varying degrees into the nuclear interior, creating a somewhat fuzzy surface. Interestingly, the m6A-Tracer signals that abut the NL often appear partially embedded in this LmnB1 meshwork (Figure 6A, top). Oblique sections show that m6A-Tracer signals tend to occupy small pockets in the Lamin B1 signals (Figure 6A, bottom).

..... Together, these results show that contact of LADs with the NL often involves embedding of chromatin in the relatively fuzzy nucleoplasmic surface of the NL.”

This description matches closely with those observed in our IF and zone data.

We similarly observed with the 300nm search radius, that we used uniformly for all IF data, there were only a few fixed loci associated with nuclear lamina, while a larger number of fixed loci for H3K9me3, H3K9Ac and Sf3 were found. This likely indicates a “fuzzier” organization of the lamina than the “tighter” DNA to nuclear bodies interactions for heterochromatin and speckles. Overall, we kept the search radius and other parameters constant for all IF analysis for uniformity as well as to reflect the real biological differences in the DNA loci proximity to each of the IF marks.

It is worth noting the technical differences between DamID and our Lamin B1–DNA loci measurements to further address the reviewer’s concerns about our Lamin B1 results. In order to label LADs, DamID methods induce Dam–LmnB1 protein for 12–15 hours (Kind et al., 2015; Rooijers et al., 2019), which integrates the LmnB1–LADs interactions over hours. As a result, any LADs that were in contact with LaminB1 sometime in the past 12–15 hours would be detected even if those LADs are not currently colocalized with LaminB1 signal. On the other hand, our measurements captured spatial proximity (<300 nm) between Lamin B1 and DNA loci as a snapshot of the moment we fixed cells, which is a more stringent measurement.

Consistent with this, Figure 2 in Kind et al. 2015 showed that even loci with high interaction scores with the lamin were often observed at the center of the cell instead of at the nuclear periphery. Second, although the reviewer mentioned “*By single-cell DamID a percentage of loci were previously identified to contact the lamina in near 100% of the cells (Kind et al., 2015; Rooijers et al., 2019).*”, the contact frequencies in those studies were calculated differently from our fixed loci (fraction of loci within 300 nm from Lamin B1) and 100% of the contact frequencies do not necessarily mean those loci are within 300 nm from Lamin B1 high signals in 100% of the cells. In addition, Kind et al. 2015 used a human haploid cell line versus mESCs in our data. These technical differences could explain the lower fixed loci fractions from our data compared to the contact frequencies from single cell DamID, while the overall trends between our measurements and DamID are similar (Fig 2C).

Finally, regarding reviewer's comment on zone 12:

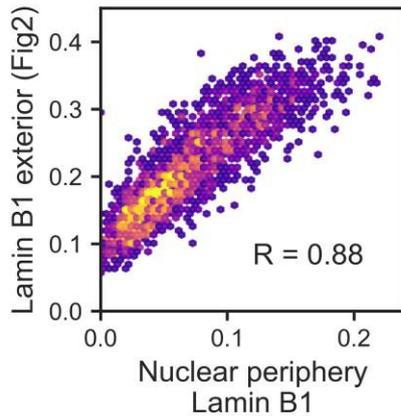
"This is apparent from Fig. 2c where the assigned zone 12 regions are all off-peripheral with a distance of at least 1micron from the edge of the nucleus. The loci found in these zones are thus not true Lamin B1 associated genomic regions."

Zone 12 has mixed active and nuclear lamina marks interior to the outermost lamin layer (zone 10, Figure 3), which is similar to the fuzzy off-peripheral region of the nuclear lamina that Kind et al. 2015 described. We did not discuss the DNA loci associated with zone 12 in the context of Lamin B1 associated regions. All the Lamin associated DNA loci are calculated directly from the LaminB1 data in Figure 2.

Additional comments from reviewer 3 and responses. 9/27/20

I think the work is overall of high quality and the manuscript improved considerably upon revisions, yet the claim by Cai and colleagues that Lamin B1 is also present in a nucleoplasmic pool goes against decades of work in the field. Lamin B1 has a farnesylated tail that integrates the protein into the inner- nuclear membrane. For Lamin A/C the tail is cleaved off which results in nucleoplasmic localizations in addition to predominant peripheral signal. The images provided in the rebuttal are selective and the interpretation of the SR-image is incorrect. There is a "fuzzy" surface, yet the entire thickness of the lamina is still less than 100nm (see Fig.S5b). The images they refer to display oblique sections and therefore the lamina appears much thicker. With their analyses, only genomic regions that are within 300nm spatial distance (plus ~100nm lamin meshwork) to the nuclear periphery should be considered true lamina-associated regions. All other regions (like zone 12) are not (by their definition). Therefore, all off-peripheral lamin "contacts" are in my opinion misassigned.

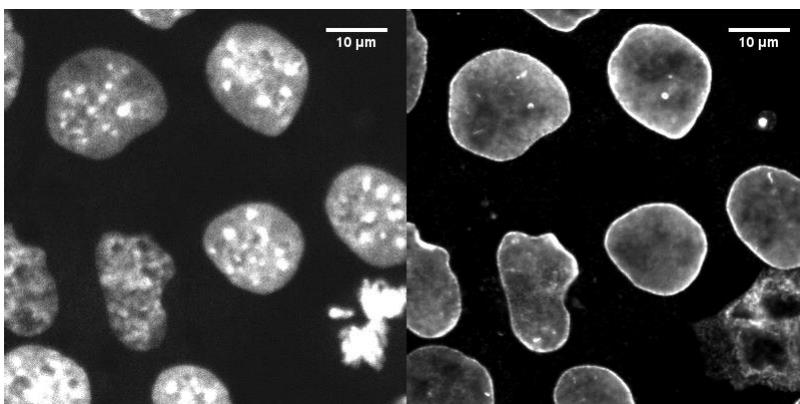
Following the reviewer's suggestion, we performed the lamin association analysis using only lamin signal at the nuclear periphery, by taking only the lamin signal close to the convex hull of all pixels in the nucleus and excluding the bright lamin puncta and the general nucleoplasmic lamin signal in the nucleus from the analysis. We observe highly similar loci association frequency using only the nuclear peripheral lamin as the one shown in Fig 2c (shown below). We hope this addresses the reviewer's concern by using only the thin periphery lamin layer to perform the loci association analysis.



Caption: comparison of fraction of loci within 300 nm from Lamin B1 exterior (shown in Figure 2 in the manuscript) to that from nuclear periphery Lamin B1, showing similar results of enrichment of specific loci. R represents Pearson's correlation coefficient.

One potential source of the confusion about “off-periphery” contacts may be Extended Figure 7a-c, which was part of a main figure in the original manuscript but is no longer discussed in the main text and figures in the revision. In those analyses, we clustered DNA loci based on their population average IF profiles across all markers. Each group of DNA loci was previously labeled by the descriptive labels “active, inactive, nucleolus and nuclear lamina,” based on which combinations of markers were enriched in each group. Some of the loci in the cluster 4 are “off-peripheral” not because they are associated with nucleoplasmic lamin B1 in that cell, but because those loci are enriched in lamin marks on average across all cells and happened to be in the nucleoplasm in that particular cell. In contrast, in Figure 2C, we directly measure which loci are in proximity with each individual marker in single cells. To avoid confusion with the single-cell lamin B1-DNA loci analysis in Fig 2, we now removed the descriptive labels and just label clusters in Extended Data Figure 7 by their numbers (i.e. 1-4) in the current manuscript.

We also have performed additional immunostaining using the anti-Lamin B1 antibody (Abcam ab16084) that the reviewer suggested and observed the same results as the previous antibody used in the manuscript. We also found the similar Lamin B1 localization pattern in literature with mouse ES cells ¹.



Our experiment: Example DAPI staining (left) and immunofluorescence (right) images using anti-Lamin B1 antibody (Abcam ab16084) in E14 mouse ES cells.

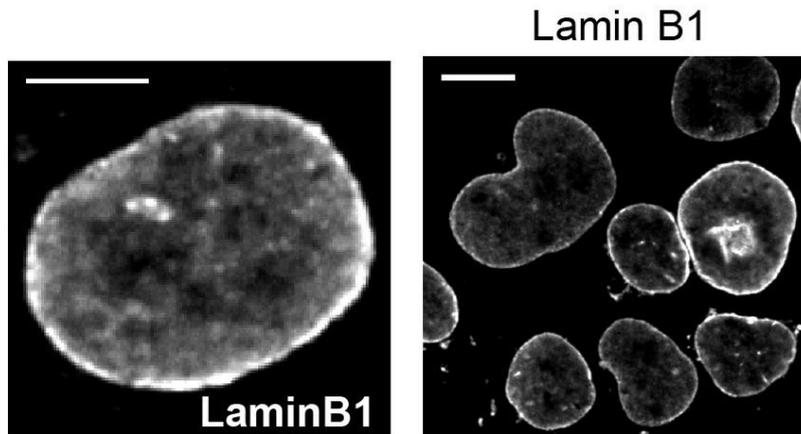


Figure 2

Extended Data Figure 5

Lamin B1 staining in our manuscript (Abcam ab220797) for comparison. Scale bars are 5um in the left images and 10um in the right image.

[redacted]

Literature: Example immunofluorescence images using anti-Lamin B1 antibody (Abcam ab16084) in mouse ES cells in a published study ¹.

There are literature reports of nuclear membrane invagination ^{2,3} and cell cycle dependence of nucleoplasmic lamin B1 ^{4,5}. These literature findings are consistent with the lamin B1 puncta observed inside the nucleus in our IF staining.

[redacted]

Literature: Example 3D STORM image of Lamin B1 immunofluorescence in human foreskin fibroblasts. Arrowheads indicate tube-like invaginations ³.

[redacted]

Literature: Example images of GFP-lamin B1 signals in daughter PAM cells after cell division ⁵, showing the non-peripheral lamin B1 signals.

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1. Chen, C.-K. *et al.* Xist recruits the X chromosome to the nuclear lamina to enable chromosome-wide silencing. *Science* **354**, 468–472 (2016).
2. Jorgens, D. M. *et al.* Deep nuclear invaginations are linked to cytoskeletal filaments – integrated bioimaging of epithelial cells in 3D culture. *Journal of Cell Science* vol. 130 177–189 (2017).
3. Schoen, I., Aires, L., Ries, J. & Vogel, V. Nanoscale invaginations of the nuclear

envelope: Shedding new light on wormholes with elusive function. *Nucleus* **8**, 506–514 (2017).

4. Moir, R. D., Montag-Lowy, M. & Goldman, R. D. Dynamic properties of nuclear lamins: lamin B is associated with sites of DNA replication. *J. Cell Biol.* **125**, 1201–1212 (1994).
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Reviewer Reports on the Second Revision:

Referee #1 (Remarks to the Author):

The revised version meets all our concerns. We appreciate the authors' efforts to mitigate the confusion around terminologies in the field.

Referee #3 (Remarks to the Author):

I think the work is overall of high quality and the manuscript has considerably improved upon revisions. However, my previous concern about the off-peripheral staining of Lamin B1 still stands. The lamina meshwork is less than 100nm thick, thus any signal measured at a distance >300-500nm (considering diffraction barriers and detection limitations etc) from the nuclear periphery should not be considered in contact with the lamina. In e.g. cell #20 in Fig. 3c zones 11 and 12 are at a distance further than 500nm from the nuclear periphery, yet they are considered enriched for Lamin B1 in Fig. 3b. This Lamin B1 enrichment is the result of the hazy off-peripheral Lamin B1 signal observed in Fig. 2a. In my strong opinion, this signal does not reflect true Lamin B1 localizations, and it should be regarded as background signal and not be included in any of the analyses. E.g. the mixed zone 12 is the result of this off-peripheral Lamin B1 patterns and I question its biological relevance. I am a bit puzzled why the authors stand their ground that Lamin B1 isn't an exclusively nuclear peripheral protein and it surprises me that they continue to make this argument. Any general review on Lamins will tell them otherwise and it is generally accepted in the field that Lamin B1 is exclusively localized to the nuclear periphery (as opposed to Lamin A/C).

To potentially resolve this issue, what I would like to suggest is the authors redo the analyses that involve lamin with Lamin B1 associations determined only based on a) lamin signal close to the convex hull and/or b) lamin signal with a more stringent threshold on the intensity values (to remove the nuclear interior signal). For the latter it is OK to retain the nucleoplasmic signal corresponding to membrane invaginations since they present genuine lamin signal (this signal is equally bright as the peripheral lamin signal).

Finally, it is easy to spot off-target signal in Lamin B1 immuno-detections because of the exclusive peripheral localization. However, for other factors with less defined spatial nuclear distribution patterns background signal may largely go unnoticed. Hence, because of Lamin b1, I am generally cautious about the potential contribution and interference of background signal present in the

other IF channels. Judging from the quality of the data (benchmarking and conclusions in line with previous findings), my feeling is that Lamin B1 is most affected of all stainings, yet I would like to advise to include a cautionary note stating something general about background signal that is - perhaps inevitably- associated with such integrated methods.

Author Rebuttals to Second Revision:

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We thank the reviewer for providing detailed comments on the multiplexed IF and zone analysis. We incorporated reviewer's suggestion regarding using only lamin IF signal at the nuclear periphery. We calculated the lamin association frequencies with only the Lamin B1 signal close to the convex hull, the convex hull itself, and the high intensity threshold (3 standard deviations away from the mean). All three are shown in Extended Data Figure 6c and e, replacing the Lamin B1 association frequency figure in the main text.

Finally, it is easy to spot off-target signal in Lamin B1 immuno-detections because of the exclusive peripheral localization. However, for other factors with less defined spatial nuclear distribution patterns background signal may largely go unnoticed. Hence, because of Lamin b1, I am generally cautious about the potential contribution and interference of background signal present in the other IF channels. Judging from the quality of the data (benchmarking and conclusions in line with previous findings), my feeling is that Lamin B1 is most affected of all stainings, yet I would like to advise to include a cautionary note stating something general about background signal that is -perhaps inevitably- associated with such integrated methods.

To incorporate the reviewer's concern, we added a section in the zone analysis to clarify the caveat with the resolution of the IF data.

“We note that the zone assignments are based on the combinatorial chromatin marks at each diffraction limited pixel. So the resolution and the boundary of the zones are also diffraction limited, which could contribute to some of the mixed zones detected. For example, we cautiously note that previous super-resolution imaging ([Nmezi et al. 2019](#)) showed that Lamin B1 meshwork is around 100 nm thick at the nuclear periphery, while our zone analysis showed Lamin B1 enriched zone 11 and mixed zone 12 were typically found at the pixels further than 100 nm from the nuclear periphery (Fig. 3b, Extended Data Fig. 8f-h), possibly due to the limitation of the resolution. In addition, we note that background signals of the multiplexed IF could also affect the nuclear zone distribution patterns. Future works with super-resolution microscopy may resolve the mixed regions at finer resolution.”

We have also added a sentence in the text stating that “We note that IF images and zone assignments were limited by diffraction and background, and that even finer granularity would be observed with super-resolution imaging of the IF markers (see Methods).”

In Extended Data Figure 6c and e, where the Lamin B1 figure is now shown, we added that “To take into account only Lamin B1 staining at the nuclear periphery, we calculated the distances between the DNA loci and the Lamin B1 signal near the convex hull of the nucleus as well as with different intensity thresholds.”

Correspondingly, in the methods section, we added “For Lamin B1, we calculated the distances from DNA loci to Lamin B1 signals with two and three standard deviations away from the mean intensity, as well as using only Lamin B1 signals at the nuclear periphery (as determined from the convex hull of the nuclear pixels) and the nuclear periphery pixels. Similar Lamin B1 or nuclear periphery association profiles were observed for all analysis in correlation plots (Extended Data Fig. 6c) across DNA loci (Extended Data Fig. 6e).”

Reviewer Reports on the Third Revision:

Referee #3 (Remarks to the Author):

The revised version meets my concerns. I appreciate the author's efforts to address my points about the zone assignments (10-12) in figure 2 and to include several lines of discussion on the topic of the technical limitations of the approach.