Visualizing the genome in high resolution challenges our textbook understanding

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Abstract

In the recent decade, new methods have been developed that probe the 4D folding of the genome in space and time with unprecedented resolution. These methods, including chromosome conformation capture and high resolution light and electron microscopy, are shedding new light onto genome architecture and function. We review the emerging picture on genome organization revealed by super-resolution and live-cell imaging. We compare and contrast population based chromosome conformation capture approaches and imaging based approaches and highlight the future challenges ahead.

Introduction

Human DNA is 2 meters long. How this DNA undergoes enormous compaction to fit into the tiny space of a cell's nucleus (~20 μm in diameter) has been among the major mysteries of cell biology. Repeating units of nucleosomes (146 bp of DNA wrapped around a core histone octamer) organize into the 10 nm beads-on-a-string¹. However, further compaction is needed to fit the DNA into the nucleus. The long standing model posited that chromatin folds into higher order structures in a hierarchical manner, including the folding of the 10 nm fiber into the 30 nm fiber by linker histone H1 and subsequently into larger structures².

Histones, in addition to compacting DNA, can also actively control gene expression through post-translational modifications to their tails that collectively generate an epigenetic "histone code"³. Specific histone marks correlate with active versus silenced regions of the genome and hence are thought to give rise to different levels of chromatin compaction and higher order structure³. That being said, a portion of the genome seems to contain unmodified histones^{4,5} and repressive histone marks can sometimes also be found in active promoters⁶, underscoring the need for better tools to visualize and map the precise relationship between histone marks, genome structure and function.

With the advent of new imaging and genomic technologies, we are now at an exciting time in history, in which the *in vivo* organization of the genome is being visualized at an unprecedented level of detail, overwriting our existing textbook models.

Genome organization using chromosome conformation capture

Elucidating the physical structure of chromosomes is challenging because the relevant length scales are small. Nucleosomes are only 10 nm and less than 200 bp in size, highly compacted fibers postulated by the textbook model are not much larger and individual genes can be as small as few kb, containing only tens of nucleosomes. Our microscopic understanding of chromosome organization in interphase nucleus hence has been limited by the low spatial resolution of a light microscope (250-400 nm) compared to the size of a nucleosome (~10 nm). Hence, until recently, our light microscopic understanding of genome organization in intact nuclei was limited to the observation that individual chromosomes occupy specific regions within the nucleus, so

called chromosome territories (micron length scales) (Figure 1)⁷. In addition, heterochromatic and gene poor chromosomes are enriched at the nuclear periphery and around nucleoli^{8,9}, whereas gene rich chromosomes tend to be within the nuclear interior⁹. Upon activation, genomic regions decondense and loop out of chromosome territories¹⁰. These observations suggested that chromosomes are not randomly organized inside the nucleus and nuclear organization is important for gene activity, yet deciphering the 3D genome folding at length scales relevant for gene activity was hindered by the lack of appropriate tools.

The recent development of chromosome conformation capture (3C)¹¹ as well as methods like ChIP-Seq¹² has therefore been revolutionary, allowing us to map DNA contacts, epigenetic modifications and chromatin binding proteins genome-wide and relate these to the 3D genome organization at high resolution. The working principles of these genomic methods have been reviewed elsewhere^{11,12}. The resolution of the 3C methods depends on several factors including crosslinking efficiency, frequency of restriction cutting and sequencing depth and can range from 1 kb to 1 Mb^{13,14}. These methods have confirmed many of the observations from fluorescence microscopy such as the existence of chromosome territories^{14,15}. Additionally, Hi-C combined with deep sequencing has revealed several levels of organizational units of the genome including A/B compartments corresponding to active eu-chromatin and silenced heterochromatin, Topologically Associating Domains (TADs) - megabase regions of the genome with increased intra-TAD interactions compared to inter-TAD interactions (Figure 1), sub-TADs and loops - self-associating physical domains at smaller length scales¹⁶. These organizational units in part help to bring promoters in close contact with distant cis-regulatory elements (i.e. enhancers) and these promoter-enhancer contacts are thought to be important in regulating gene activity¹⁶. Several “architectural” proteins have been identified that are responsible for organizing and defining the boundaries of these domains. In particular, Hi-C revealed that TADs and loops are dependent on the cohesin complex and on CTCF¹⁷⁻²⁰. Cohesin is a multi-subunit protein complex that forms a ring shaped structure. CTCF (CCCTC-binding factor) is a highly conserved DNA-binding protein. Depletion of cohesin subunits or CTCF leads to loss of all TADs and loops¹⁹⁻²³. The prevailing model is that cohesin rings embrace DNA, loops are extruded through the rings and loop extrusion stops when cohesin encounters two CTCF proteins bound on either end of the DNA in a convergent orientation^{24,25}. Although the exact details of this model are debated, *in vitro* experiments have shown that the cohesin loader NIBPL and cohesin together can extrude DNA loops in the presence of ATP^{26,27}.

While 3C methods have dramatically improved our understanding of 3D genome organization, they only provide an average view over millions of cells obscuring details of single cell heterogeneity and analysis of rare cell populations. In addition, these methods do not preserve the relation of genomic regions to important nuclear landmarks including the nuclear lamina or the nucleoli. Finally, the nucleosome level structure of organizational units uncovered by Hi-C remain unresolved, although this

limitation may be overcome by a recently developed method called micro-C, which uses MNase rather than restriction enzymes to cut DNA at hypersensitive sites in between nucleosomes²⁸. The remaining limitations and questions are further starting to be addressed with the recent revolution in light microscopy and labeling technologies that are now allowing us to visualize the smallest organizational units of chromosomes *in vivo*.

Electron microscopic view of genome organization

Given the high density of nucleic acids and proteins inside the nucleus, it has been difficult to use electron microscopy to visualize chromosome structure in intact cells due to low contrast. As a result, a large number of early electron microscopy studies of higher order chromatin folding was carried out on *in vitro* reconstituted chromatin fibers, in which nucleosomes were assembled together with plasmids containing strong nucleosome positioning sequences and further compacted by adding linker histones under specific ionic strength conditions²⁹. Alternatively, electron micrographs of nuclei isolated from hypotonically lysed cells and treated with $MgCl_2$ were used³⁰. These studies converged on the model that chromatin folds into a 30-nm fiber. However, cryo-electron microscopic imaging of chromosomes *in situ* revealed a homogenous and grainy texture containing features at the size scale of 11 nm and no super-structure or periodic order^{31,32}. Further, 3D electron tomography of *Xenopus* egg extracts revealed 30-40 nm domains consisting of nucleosome clusters³³. Finally, electron spectroscopic imaging of mouse somatic cells were consistent with the open and closed chromatin being made up of 10-nm rather than 30-nm fibers³⁴. All these experiments put into doubt the hierarchical folding model of chromatin and the existence of a regular 30-nm fiber structure. Most recent evidence against the hierarchical folding and 30-nm fiber model came from super-resolution imaging of nucleosomes in intact nuclei (see more below)³⁵ and subsequently electron tomographic imaging of DNA stained with an EM-compatible dye that resolved the contrast problem (ChromEMT)³⁶.

Imaging the global genome organization at high resolution with light microscopy

Super-resolution microscopy extended the spatial resolution of a light microscope from ~250 to 10-30 nm length scales, breaking a fundamental limit. The underlying principles behind various super-resolution microscopy methods have been reviewed elsewhere^{37,38}. These technologies, including structured illumination microscopy (SIM), single molecule localization microscopy (SMLM including STORM, PALM and DNA-Paint) and stimulated emission depletion microscopy (STED) have been applied to visualize features of nuclear organization that are smaller than the diffraction limit. Combined with the development of new labeling technologies, these super-resolution methods are providing a second revolution in parallel to 3C methods in our understanding of the genome organization.

STORM was used to visualize the global organization of nucleosomes inside the nucleus (Figure 2)³⁵. These images revealed structures at varying length scales ranging from tens to hundreds of nanometers (Figure 2A-C)³⁵. The striking aspect of these early images was the absence of a prominent 30 nm fiber like structure. Instead, nucleosomes were shown to form heterogeneous groups, which were named nucleosome clutches (Figure 1, Figure 2B)³⁵. Based on their spatial size (~30-50 nm) and the number of nucleosomes they contain, clutches correspond to a genomic length scale of few kilobases, which is at the current limit of the spatial resolution of STORM. Clutches were interspersed with nucleosome poor regions and multiple clutches clustered in close proximity forming larger “clutch domains” in the size range of several hundred nanometers (tens of kilobases to megabases genomic scale) (Figure 1, Figure 2A-C)³⁹. It would be interesting in the future to determine if these clutch domains correspond to TADs and loops identified using Hi-C (Figure 1). Clutch size and density was found to inversely correlate with cell pluripotency, suggesting that despite heterogeneity, clutch organization is cell-type specific³⁵. The heterogeneous organization of nucleosomes in the form of nucleosome clutches uncovered by STORM is in stark contrast to the textbook model of ordered, hierarchical folding of the chromatin fiber. In line with the super-resolution results on nucleosomes, STORM imaging of individual chromosomes in which DNA was labeled with EdU, showed small clustered nanodomains organized into larger domains (Figure 2D), suggesting that DNA organization mirrors nucleosome organization⁴⁰. Live cell super-resolution imaging of nucleosomes also showed globular domains in the size range of hundreds of nanometers, resembling the clustered organization of multiple clutches (i.e. clutch domains)⁴¹.

Multi-color super-resolution imaging was further used to correlate specific epigenetic modifications to chromatin compaction. These images revealed that silencing histone marks (H3K27me3) formed larger nanodomains that localized to more compacted DNA regions compared to active marks (H3K9ac, H3K4me3)⁴². These results are in line with the earlier work, which showed that inhibition of the histone deacetylase enzyme (HDAC) to induce hyperacetylation gives rise to smaller and less dense nucleosome clutches^{35,39}. To further analyze the impact of histone tail acetylation on chromatin organization, nucleosomes were imaged together with DNA. This work showed that the compaction level of DNA within nucleosome clutches was independent of clutch size and instead depended on the level of acetylation of the histone tails³⁹. In hyperacetylated cells, histone clutches were not only smaller but they compacted DNA to a lesser extent compared to similar sized clutches in wild type cells³⁹. Interestingly, the biggest change in DNA compaction in hyperacetylated cells was observed within the clutch domains³⁹. Combined together these results suggest that transcriptional activity and DNA accessibility is likely regulated at multiple levels: (i) at the level of clutch size, with the DNA within smaller clutches being more accessible, (ii) at the level of DNA compaction within the nucleosome clutches, with acetylated histones leading to less compaction and more accessibility of the underlying DNA, and (iii) at the level of clutch

domains that unfold and become more dispersed in hyperacetylated cells leading to increased DNA accessibility.

Imaging specific genomic regions at high resolution

Nucleosome and DNA labeling coupled with high resolution imaging provides a global view of chromatin organization with near nucleosome level spatial resolution, but it does not provide sequence specificity and hence it cannot be used to study the organization of specific genomic regions. Fluorescence in situ hybridization (FISH) has long been used to visualize the spatial positioning of genomic loci and revealed that genes tend to occupy specific regions within the nucleus, with silenced genes often being closer to the nuclear lamina compared to active genes^{9,43}. More recent high throughput imaging has revealed a lot of heterogeneity also in gene positioning⁴⁴. While FISH correlates with Hi-C: i.e. regions with higher contact frequency in Hi-C on average tend to be closer in space, at a single cell level these regions can be as far apart as regions with no enhanced contact frequency⁴⁴. These results are compatible with the heterogeneity in genome folding that is observed at genome-wide level by super-resolution microscopy and ChromEMT.

FISH has been limited to visualizing a subset of relatively small genomic regions ranging in size between 50-300 kb. This challenge was overcome with the advent of Oligopaints, small (32-mer) oligonucleotides with genomic homology (Figure 3A)^{45,46}. Combined Oligopaint and STORM imaging (OligoSTORM) revealed that genomic regions with different epigenetic states have distinct folding⁴⁷. In this work, the genome was separated into active regions (enriched in H3K4me2 and H3K79me3 marks), repressed regions (enriched in H3K27me3 mark or polycomb group proteins) and inactive regions (lacking H3 modifications, transcription factors or polycomb proteins). Oligopaint probes were designed for each state and the volume occupied in space by each region was measured⁴⁷. Repressed and inactive regions were more compacted and occupied a smaller volume compared to active regions. Thus, folding of the chromatin correlates with epigenetic state, although interestingly large regions of the genome depleted of specific histone H3 marks (denoted as inactive regions) also showed distinct folding and compaction from active and repressed regions, suggesting that factors other than epigenetic modifications also play a role in 3D genome folding.

OligoPaint can be used to walk along chromosomes by sequentially hybridizing, imaging and extinguishing oligo probes⁴⁸⁻⁵¹. This approach helps trace the folding of genomic DNA through 3D space. The step size of the walk can be tuned to either walk along megabase domains with larger steps and hence lower spatial resolution⁵¹ or the step size can be made finer to walk along smaller kilobase domains with much higher spatial resolution⁴⁹. This approach was first used to label subsequent TADs within a chromosome to determine their spatial organization⁵¹. Later, megabase regions containing multiple TADs and sub-TADs were labeled in finer steps of 30 kb and imaged sequentially using OligoSTORM⁴⁸. Remarkably, these images revealed globular TAD-like domains in single cells (Figure 2E), consistent with super-resolution images of

TADs in *Drosophila*⁵². The centroid to centroid distance between each 30kb segment was measured. Finally, a spatial interaction heatmap was created from the distance scores⁴⁸. These heatmaps, when averaged over hundreds of cells, were highly compatible with Hi-C heatmaps of the same region, revealing TADs with strong boundaries at CTCF and cohesin sites⁴⁸. Surprisingly, however, heatmaps generated from individual cells showed highly heterogeneous boundaries, in which a boundary had a non-zero probability of being positioned at any genomic site but with a slight bias towards being positioned at cohesin/CTCF sites⁴⁸. Strikingly, depletion of cohesin did not eliminate globular-TAD like structures in single cells but made their boundary positions more variable, with no preference for CTCF sites.

Walking along much larger regions with larger steps and imaging with STORM also revealed that chromosome folding at these large length scales is highly variable even between two alleles of the same chromosome⁵⁰. While on average there is a correlation between the active A-compartments and the inactive B-compartments assigned by Hi-C and the spatial entanglement or separation of these compartments in the imaging data, there is also a high amount of heterogeneity in the spatial organization of A/B compartments⁵⁰. Therefore, tracing the path of genomic regions with varying step sizes using sequential FISH shows on average remarkable agreement with genomic methods revealing domains and interactions within single cells. These chromosome walk approaches interestingly further illustrate the variability of domain boundaries at the level of single cells and clearly show that cohesin is not so critical for the maintenance or formation of these clustered domains.

Recently, the resolution of “chromosome walks” was pushed to kilobase scale by walking with much smaller step sizes (2-10 kb) in an impressive method called optical reconstruction of chromatin architecture (ORCA)⁴⁹. ORCA was used to reconstruct genomic regions in cryosections of *Drosophila* embryos, and match genome architecture to cell type specific gene expression profiles by combining the DNA imaging with highly multiplexed RNA FISH⁴⁹. Perhaps most surprisingly, this approach revealed that while the physical proximity of a promoter to its enhancer was somewhat predictive of active transcription, the correlation was weak and many active promoters were not in physical proximity of their enhancers⁴⁹. These results suggest that the promoter-enhancer interactions are likely highly dynamic rather than forming stable loops (see more below).

There are remaining challenges with this type of “domain painting” approaches, including efficient multiplexing of DNA and protein labeling. The denaturing conditions used in OligoPaint can be detrimental to several proteins, making it hard to label them with high enough efficiency for subsequent high-resolution imaging. In addition, these methods currently remain as specialist tools accessible to a limited number of labs. Finally, low efficiency of hybridization of the Oligopaint probes, which becomes more problematic as the probe size becomes smaller, can amount to many segments missing from the imaged regions. Addressing these challenges will enable better tracing of the

conformation of the chromatin fiber within specific genomic loci and provide valuable information for computational modeling of chromatin (see below).

Computational modeling of chromosome structure

Computational models play an important role in enriching our understanding of chromosome structure especially in the current era where the field is trying to interpret rich experimental datasets obtained from 3C and imaging approaches. These models have been reviewed extensively elsewhere⁵³⁻⁵⁶ and hence here we only aim to provide a broad overview of the general classes of models and discuss the future challenges. Models can range in scale from all-atom molecular dynamics simulations of individual nucleosomes, mesoscale coarse-grained models of oligonucleosome fibers consisting of tens or hundreds of nucleosomes to even coarser polymer and continuum models of megabase size regions⁵⁵. Coarse-grained models of oligonucleosome fibers maybe compared to super-resolution images of nucleosome clutches whereas polymer and continuum models may better capture domain features at the scale of TADs.

The models can further be categorized based on whether they incorporate experimental data as input parameters. Data-driven models use Hi-C maps or in some cases imaging data to put constraints on pairwise distances between sequences to convert contact maps into distance maps and to finally generate a single consensus structure or an ensemble of most probable structures⁵³. De novo models, on the other hand, use a set of physical principles to simulate an ensemble of structures and the compatibility of these structures with Hi-C or imaging data is determined afterwards⁵³. This class of models include the fractal globule model and loop extrusion models^{24,57}. De novo models inform our mechanistic understanding of the folding principles of the genome, making valuable predictions that can potentially be experimentally tested. However, these models often recapitulate genome folding at a specific length scale but may fail at a different length scale⁵³. Data driven models have their own pitfalls. For example, they are often guided by Hi-C maps, which only provide an average spatial map from a large population of cells, or insufficient resolution in terms of abundance of contacts. While some models have incorporated constraints from imaging data⁵⁰, currently there is not sufficient high throughput and high resolution FISH based imaging data to guide data driven models. In the future, integrating data from both genomic and imaging studies may help enrich the input data and produce more precise models of chromosome structure.

Live cell imaging of genome dynamics

The main drawback of FISH-based approaches is that they can only be used in fixed cells and hence cannot reveal information about genome dynamics. Visualizing specific genomic regions in living cells requires a labeling system that does not rely on denaturing conditions coupled with the accumulation of sufficient fluorescent molecules at the genomic site over the background to enable efficient detection. Artificial gene

arrays have been a useful tool that satisfy these criteria. In particular, the *lac* operator (LacO) / *lac* repressor (LacI) system from *E. coli* has been widely used to visualize chromatin dynamics and DNA-protein interactions (Figure 3B)⁵⁸. However, while a useful tool, this system has important drawbacks, specifically the high repeats present in the LacO sequence can disrupt chromatin organization/function and importantly this system is often inserted in the genome in multiple copies at random locations and is not suitable for visualizing endogenous genomic sites. Alternatively, the Anchor (ParB/*parS*) system takes advantage of protein oligomerization at a site specific locus, rather than a repetitive gene array to achieve the signal amplification needed to visualize this locus (Figure 3C)⁵⁹. Despite not using a large repetitive gene array, the *parS* sequence still needs to be incorporated into the genome and doing so at a specific locus without perturbing the locus dynamics, organization and function may be challenging.

More recently, methods that can label endogenous genomic loci, without the need for incorporating non-native sequences into the genome have been developed. It is, however, important to mention that some of these methods (e.g. Crispr) may still perturb the gene locus structure since they may lead to disruptions of the DNA-duplex upon guide RNA (gRNA) hybridization. These methods rely on DNA binding proteins such as Transcription activator-like effector (TALE) domain or the enzymatically deactivated version of Crispr associated protein 9 (dCas9) (Figure 3D, E)^{60,61}. Both approaches have been used successfully to label genomic regions that contain highly repetitive sequences like centromeres or telomeres. The specificity of the CRISPR/dCas9 to the genomic region is mediated solely by gRNAs making this approach much simpler than TALEs. Hence, recent effort has focused on optimizing and multiplexing CRISPR/dCas9 for genome visualization⁶²⁻⁶⁵. For example, the use of orthogonal dCas9 systems or the incorporation of RNA aptamers into the gRNA enabled multi-color imaging of different genomic regions^{63,65}.

A main challenge has been in adapting this approach to label non-repetitive regions of the genome. Initial work showed that to accumulate detectable signal at the locus, ~30 gRNAs were required to tile along the genomic region⁶⁰. Delivering these gRNAs into the cell requires transfections with multiple plasmids, markedly decreasing labeling efficiency. Several groups have undertaken this challenge to both boost the number of gRNAs that can be delivered using a single plasmid and to also use amplification methods to enhance the signal at the gene locus. Enhanced signal has been achieved by combining CRISPR/dCas9 with SunTag as well as incorporating repetitive aptamers into the gRNA (Figure 3E)⁶⁴⁻⁶⁶. SunTag labeling for example enabled super-resolution imaging of low repeat regions and estimation of telomere length in different cell types⁶⁴. Crispr-Tag used Crispr targetable repeats from *C. Elegans* incorporated into the gene locus as a universal, efficient tag for labeling genomic regions⁶². In parallel, chimeric array of gRNA oligonucleotides (CARGO) was developed to deliver 12 or more gRNAs in a highly multiplexed fashion using a single plasmid⁶⁷. Using this approach, it was possible to label gene promoters and cis-regulatory elements (enhancers) with enough signal to background ratio to visualize their dynamics in live cells⁶⁷. These studies

showed that the mobility of promoters and enhancers was linked to their transcriptional state, with higher transcriptional activity leading to greater mobility⁶⁷. These results suggest that RNA PolII may induce “stirring” and increased genomic mobility⁶⁷. Such a mechanism may ensure robust transcription by increasing the frequency by which enhancers bump into their promoters. Although, recent super-resolution chromatin tracing⁴⁹ (described above) and live cell imaging of Sox2 and its control region⁶⁸ revealed that transcriptional activation of the promoter can happen in the absence of physical proximity between the promoter and the enhancer. Further work is needed to reconcile the enhanced loop formation between active enhancers and promoters detected using Hi-C and the lack of correlation between enhancer-promoter proximity and transcriptional activation observed using super-resolution and live cell imaging in single cells.

Single molecule tracking of chromatin-protein interactions

While our ability to tag and visualize endogenous genomic loci in living cells is relatively new, live-cell imaging of chromatin interacting proteins, including transcription factors, architectural proteins and polymerases has made a huge impact in our understanding of protein dynamics inside the nucleus. The protein of interest is typically tagged with a Halo- or SNAP-tag, under-labeled using the Halo/SNAP substrate such that single proteins can be visualized sparsely and their motion inside the nucleus tracked^{69,70}. The residence time of transcription factors on DNA typically show bi-exponential kinetics with a short (hundreds of milliseconds) and long-lived (tens of seconds) binding time interpreted as non-specific and specific DNA interactions⁷⁰. These experiments overall posited that transcription factors likely find their target sites through a 3D diffusion dominated model in which they spend most of their time diffusing through the nucleus and sampling many non-specific sites before engaging with a specific target site⁷⁰. These studies were extended to live-cell super-resolution imaging to visualize the dynamics and spatial organization of RNA PolII in the nucleus⁷¹. RNA PolII formed transient clusters, whose lifetime correlated with the mRNA output⁷². Follow up work showed that at least some of the RNA PolII clusters were phase separated liquid droplet condensates⁷³. Mediator, a transcriptional co-activator was also shown to form phase separated liquid droplets that co-localized with PolII droplets at super-enhancers^{73,74}. The enhancer may thus not need to physically contact the promoter but rather be present inside a phase separated transcriptional droplet to activate the promoter. These phase separated droplets can be several hundreds of nanometers in size and their presence may explain the lack of correlation between enhancer-promoter proximity and transcriptional activation. Tracking architectural proteins like cohesin and CTCF, further showed that these proteins, rather than forming stable complexes, are in fact highly dynamic⁷⁵. These results suggest that the loops observed in Hi-C maybe much more dynamic, forming and breaking apart often.

It would be very interesting, in the future, to combine the CRISPR/dCas9 based gene labeling with imaging of transcription factors, polymerases, co-activators as well as

imaging of the transcriptional output to further dissect the hierarchies in promoter-enhancer positioning, transcription factor/transcriptional machinery binding and transcriptional activation.

Mechanisms that organize the genome

The high resolution and live cell imaging studies have been uncovering several underlying determinants of genome organization. First, as described above, genome packing and nucleosome organization depend on the epigenetic state with repressed regions showing high level of folding and compaction that increases with genomic length⁴⁷. Acetylation marks correspond to small nucleosome clutches with lower DNA compaction^{35,39,42}. The epigenetic state of the histones alone can likely influence nucleosome packing by modulating the electrostatic charges and impacting nucleosome-nucleosome and nucleosome-DNA interactions. In addition, these modified histones may be bound by additional repressive factors such as the Polycomb group proteins, that can further modify chromatin folding and compaction.

Second, Hi-C showed that the compartmentalization of the genome into TADs and loops is dependent on architectural proteins cohesin and CTCF, which demarcate TAD and loop boundaries. However, super-resolution imaging and chromatin tracing revealed a more complex picture of TAD architecture in single cells with highly variable boundaries that persisted after cohesin depletion⁴⁸. More recent studies also revealed a similar picture in which neighboring TADs intermingled with each other extensively⁷⁶. Even more surprisingly, depletion of cohesin lead to a reduction in the intermingling between neighboring TADs rather than inducing ectopic interactions as would be expected from the global loss of TADs observed in Hi-C⁷⁶. Overall, architectural proteins like cohesin and CTCF do seem to play an important role in genome organization, however the precise principles of how these proteins cooperate to organize the genome are still being uncovered. Particularly, the imaging work is showing a more complicated picture than Hi-C and these proteins do not seem to form stable loops and TADs with well-defined boundaries in single cells.

Third, at a larger scale, the genome is compartmentalized and spatially segregated into active eu-chromatin and inactive heterochromatin, although this binary classification is likely over-simplistic and there are different flavors of hetero- and eu-chromatin with different levels of compaction and spatial organization. The epigenetic marks likely play a role at this level of organization as well, as TADs with similar histone marks tend to organize within the same compartment^{14,51}. Phase separation is also emerging as a major player of hetero and eu-chromatin segregation. In addition to transcriptional machinery forming phase separated droplets, it has been shown that linker histone H1 and the heterochromatin protein HP1 can phase separate⁷⁷ and this phase separation may play a role in the formation of heterochromatin since nucleosomes seem to preferentially partition into the HP1 droplets but transcription factors show no preference⁷⁷. In addition, transcription itself may create domains of high nascent RNA density around the transcriptionally active regions and may help organize eu-chromatin

around these domains through phase separation⁷⁸. Hence, while the organization of genome is an important determinant of transcription, transcription itself can also shape genome organization. Finally, nucleosome arrays themselves phase separate *in vitro* under physiological salt concentrations and the liquid droplet formation by these arrays is enhanced by linker histone H1 and disrupted by histone acetylation⁷⁹. The *in vivo* relevance of phase separation in the formation of nucleosome and chromatin domains observed at multiple length scales remains to be determined.

Conclusions, Open Questions and Future Perspectives:

We are at an exciting time in which development of new methods including light and electron microscopy as well as genomic approaches are elucidating long standing questions about how the folding of the genome relates to gene activity. Single cell imaging is more targeted and lower throughput than genomic approaches such as Hi-C, allowing visualization of only a few genomic regions at a time in a small population of cells. However, these methods are inherently well-suited for studying the heterogeneity in genome folding as well as genome organization in rare cell populations such as cells undergoing reprogramming to pluripotency. The information obtained from imaging and genomic approaches has often been consistent, however, there have also been cases in which the two approaches generated seemingly contradictory results⁸⁰. This discrepancy may be due to the fact that the single cell view from microscopy and the population average view from genomics are difficult to compare considering cell to cell heterogeneity and dynamics of genome folding. This challenge is particularly exasperated by the fact that genomics methods capture sequences especially when they are in very close contact, whereas microscopy methods capture the spatial distance of these sequences with the same frequency whether they are in close contact or not. In order to unify the view from these two approaches, it is important to more clearly define what contact frequency in 3C corresponds to in terms of spatial distance in imaging. Typically, a distance threshold (~200 nm) is used to define contact in imaging data, however this may be too large for actual contact as measured by genomic approaches.

Further, it is essential to bridge the gap in the throughput of the imaging and genomics methods. Recent advances that combine barcoding and high resolution imaging have enabled multiplexed visualization of RNA transcripts at the level of the entire transcriptome⁸¹. Similar barcoding approaches combined with multiplexed multi-color imaging⁸²⁻⁸⁴ and the use of microfluidics for automated in situ sample handling may in the near future also enable visualization of many genes simultaneously in a large population of cells, making these approaches more compatible with genome-wide analysis. State-of-the art examples in which genomic regions were visualized in thousands of cells at high resolution^{44,47,50} already demonstrated how the heterogeneity of TADs in single cells can be reconciled with the well-defined TAD boundaries observed at the population level in Hi-C⁴⁸. Similar approaches, in which the single cell view can be compared to the population average view in a high-content imaging dataset

of thousands or millions of cells will help better understand how emergent properties of genome folding can arise from seemingly heterogeneous single cell data. Finally, integration of genomic and imaging data through data-driven modeling and the development of de novo models compatible with both types of datasets can further guide our understanding of the mechanisms that shape genome organization.

Another interesting future challenge would be to determine whether the heterogeneity in genome folding observed in single cells has a functional consequence for transcriptional activation. Elucidating the exact mechanisms involved in shaping genome organization and the interplay between the various proposed mechanisms (architectural proteins, epigenetic modifications, phase separation) is another important challenge.

Despite the heterogeneity, chromatin organization at the nanoscale level is highly cell-type specific and correlates with cell pluripotency³⁵. It would be interesting to determine how the folding of the genome is actively and dynamically remodeled during differentiation and cellular reprogramming, what are the molecular players involved in the remodeling and whether this remodeling indeed is determinant of gene expression profiles and cell fate. Such studies may enable direct manipulation of cell fate through manipulating genome folding, potentially leading to enhanced reprogramming. For example, during in vivo reprogramming, in the oocyte-to-zygote transition in mice, it was shown that chromatin conformation is reorganized and it is distinct in paternal and maternal pronuclei. TADs and loops, but not compartments, were shown to be present in zygotic maternal chromatin⁸⁵. Another exciting prospect is to study how disruptions in the spatial organization of the genome may be pathological and lead to diseases including cancer or neurological disorders. Indeed, Hi-C showed that short term repeats that become expanded in diseases like Fragile X Syndrome are found at domain boundaries of TADs and these boundaries are severely disrupted in disease states, uncovering correlation between chromatin architecture and repeat expansion diseases⁸⁶. Oligopaint imaging showed that reduced intermixing of TADs upon cohesin loss affects the transcriptional bursting of genes near domain boundaries, which can potentially explain gene expression changes observed in the cohesinopathy Cornelia de Lange syndrome⁷⁶.

While we have advanced in our ability to visualize DNA, RNA and proteins such as transcription factors in cells, we still don't have an easy-to-use, plug-and-play type unified platform that enables simultaneous visualization of all these components at high resolution in living cells. Such a tool can potentially revolutionize our understanding of the hierarchies involved in activating and repressing transcription. Similarly, tools that enable manipulating and rewiring the genome at will are at their infancy^{51,87}. New methods that can disrupt or form specific loops upon stimulation⁸⁷, change the nucleosome level compaction of chromatin fiber at specific loci, dissolve or reform new chromatin domains will open a new window, allowing us to go beyond correlations and study the cause and effect relationship between genome organization and transcriptional activation.

Overall, we are in an exciting time in which development of new tools is driving huge progress in our understanding of genome architecture. It will be exciting to see how existing and new cutting-edge biochemical and imaging tools will advance our understanding of the link between genome architecture and gene activity in physiology and disease states.

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