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Spatial and temporal organization of the genome: current state and future aims of the 4D Nucleome Project

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Declaration of Interest

J.D. is on the Scientific Advisory Board of Arima Genomics (San Diego, CA), and Omega Therapeutics (Cambridge, MA).
F.A. is a shareholder of EarlyDiagnostics, Inc.
S.A. is a member of the Chao-Ting Wu laboratory, and holds or has patent filings pertaining to imaging and has held a sponsored research agreement with Bruker Inc.
L.B. is a co-founder and scientific advisor of Stylus Medicine (Cambridge, MA).
W.L. is a co-founder of Hub Biosciences.
B.R. is a co-founder of and member of the Scientific Advisory Board of Arima Genomics (San Diego, CA), and a co-founder of Epigenome Technologies (San Diego, CA).
J.S. is a Scientific Advisory Board member, consultant and/or co-founder of Cajal Neuroscience, Guardant Health, Maze Therapeutics, Camp4 Therapeutics, Phase Genomics, Adaptive Biotechnologies, Scale Biosciences, Sixth Street Capital, Pacific Biosciences and Prime Medicine.
X.S. is a co-founder of Granule Therapeutics (San Francisco, CA).
S.Z. is a founder and board member of Genemo, Inc (San Diego, CA).
B.J.B, B.G.B, A.S.B., A.B., R.C., C.M.D., D.M.G., T.G., A.S.H., B.H., D.H., R.K., C.S.L., Y.L., J.M., W.S.N., P.J.P., J.E.P-C., K.S.P., S.M.R., Y.R., Y.S-T., Y.S., C.S.D.C, A.V., H.Z. declare no competing interests.

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Summary

The 4-Dimensional Nucleome (4DN) consortium studies the architecture of the genome and the nucleus in space and time. We summarize progress by the consortium and highlight development of technologies for (1) mapping genome folding and identifying roles of nuclear components and bodies, proteins, and RNA, (2) characterizing nuclear organization with time- or single-cell resolution, and (3) imaging of nuclear organization. With these tools, the consortium has provided over 2,000 public datasets. Integrative computational models based on these data are starting to reveal connections between genome structure and function. We then present a forward-looking perspective and outline current aims to (1) delineate dynamics of nuclear architecture at different timescales, from minutes to weeks as cells differentiate, in populations and in single cells, (2) characterize cis-determinants and trans-modulators of genome organization, (3) test functional consequences of changes in cis- and trans-regulators, and (4) develop predictive models of genome structure and function.

Introduction

The 4-Dimensional Nucleome (4DN) consortium is funded by the National Institutes of Health Common Fund to study the spatial architecture of the cell nucleus in 4 dimensions: 3-dimensional space and in time. After completing its first phase in 2020, the consortium is currently in its second phase that will run till 2025.

In the first phase, the consortium developed, validated, and benchmarked technologies to determine genome and nuclear organization in time and space, and the interplay between nuclear organization and regulation of gene expression[1,2]. These technologies include (1) biochemical approaches to reveal and quantify the 3-dimensional (3D) interaction frequencies between genomic loci and between genomic loci and protein and RNA

regulators of genome organization; (2) tools to reveal the 3D architecture of the nucleus, including the spatial arrangement of the genome at and around nuclear bodies in 3D; and (3) imaging approaches to measure dynamics and variation in nuclear organization in live single cells. Leveraging these technologies, 4DN has released 1,922 experimental datasets generated from 4,611 experiments to the public. All the data, experimental protocols, data analysis tools, and software generated by 4DN are accessible through the 4DN web portal (https://4dnucleome.org).

In the second phase, 4DN is focusing on several fronts. First, 4DN analyzes chromatin dynamics in live mammalian cells, including primary tissues and organoids, e.g., as cells change the folding of their genome during the cell cycle, and as cells change their state, e.g., during differentiation, stress response, senescence, and pathogenic processes (Figure 1). The focus is on investigating dynamics on different length scales (individual loci to entire nuclei) and on different time scales (minutes to hours, across a single cell cycle, and across multiple cell cycles during cell differentiation). Second, 4DN will model the spatial and temporal organization of the nucleus by integrating imaging and genomic datasets, with the goals of building genome-wide navigable reference maps in 4 dimensions, and deriving models that connect the reference maps and cellular variability to genome functions. Third, the consortium will test the predictive models in the contexts of mammalian development and human disease. 4DN will achieve these goals by iterations of the characterization of the nuclear organization (data generation) at population and single cell resolution, model building and refinement, and validations including manipulations and perturbations of nuclear organization.

4DN technologies

Here, we highlight subsets of 4DN technologies and the major findings enabled by these technologies. Some of these technologies were already in use during the first phase of the project[1] or were developed as part of phase I, and some were brought into the consortium during the current phase. The first set of technologies, which we will refer to as nucleome mapping tools, help expand our understanding of the 3D organization of the genome (Hi-C 3.0, Micro-C, GAM, PLAC-seq, in situ ChIA-PET, ChIA-Drop, and SPRITE), the local transcriptome (iMARGI) and proteome (C-BERST) of any genomic locus, and the spatial proximity of genomic loci to a variety of nuclear bodies (TSA-seq, GPSeq).

Nucleome mapping tools

Hi-C 3.0

The Hi-C technology is one of the most widely used methods for mapping genome-wide chromatin interactions. During Phase I of the 4D Nucleome project, a major benchmarking effort led to the development of an improved Hi-C protocol that combines two different cross-linking agents, with pools of multiple restriction enzymes[3]. This protocol, Hi-C 3.0, produces chromatin interaction maps with improved signal-to-noise ratios that allow better quantification of chromosome folding features such as compartments and chromatin loops. For instance, for HFFc6 cells, Hi-C 3.0 detected 28,922 loops, as compared to 13,867

loops detected with the conventional Hi-C procedure using formaldehyde fixation and DpnII digestion.

Micro-C

Micro-C combines MNase digestion with Hi-C to generate genome-wide nucleosome-resolution chromatin interaction maps. First developed for *Saccharomyces cerevisiae*[4], micro-C was adapted for mammalian cells during the first phase of the 4DN project[5,6]. Micro-C was shown to be the most effective of all Hi-C-based genome-wide methods for detecting loop-extrusion mediated features such as CTCF-CTCF loops[3].

Genome Architecture Mapping (GAM)

GAM is a ligation-free method for analysis of chromatin organization[7]. It works by sequencing DNA obtained from thin sections of nuclei that are sliced at random orientations, and then measuring the co-occurrence probabilities of loci in these slices. It provides a complementary view of the genome organization compared to chromosome conformation capture assays such as Hi-C by measuring spatial proximities independent of ligation frequencies and providing estimates of the distance between loci. GAM has uncovered super-long range chromatin contacts in different brain cell types that specify expression of cell-identity genes[8]. Polymer models have been built using GAM data to predict effects of DNA deletions, insertions, and other structural variants on chromatin architecture[9].

Chromatin Interaction Analysis in Droplet (ChIA-Drop)

ChIA-Drop is a new strategy for multi-way chromatin interaction analysis via droplet-based and barcode-linked sequencing. In ChIA-Drop, a crosslinked and fragmented chromatin sample or ChIP-enriched chromatin sample is directly loaded onto a microfluidics device without proximity ligation or DNA purification. Each chromatin complex is partitioned into a droplet that contains unique DNA oligonucleotides and reagents for linear amplification and barcoding. The barcoded amplicons with droplet-specific indices are then pooled for high-throughput sequencing, and the sequencing reads with identical barcodes are computationally assigned to the same origin of the chromatin complex. Mapping the sequencing reads to the reference genome identifies which genomic loci are in close spatial proximity with one another, and multiplex chromatin interactions can therefore be inferred. ChIA-Drop experiments have shown that chromatin structures predominantly consist of multiplex chromatin interactions with high heterogeneity. In addition, RNAPII-enriched ChIA-Drop data revealed promoter-centered multivalent interactions, which provide insights into the connections between long-range chromatin interactions and transcription[10].

Proximity-ligation assisted ChIP-seq (PLAC-seq) and in situ ChIA-PET

Chromatin interaction analysis by paired end tag sequencing (ChIA-PET) was one of the first genome-wide approaches for mapping long-range chromatin interactions[11]. It combines chromatin immunoprecipitation (ChIP) with proximity ligation, thereby detecting chromatin interactions for loci bound to specific proteins of interest. This strategy has been improved as reflected in HiChIP[12], PLAC-seq[13] and *in situ* ChIA-PET[14] for detecting chromatin contacts at kilobase pair resolution and with reduced number of input cells. These methods

have enabled fine mapping of enhancer-promoter contacts across diverse tissues and cell types, significantly enhancing our ability to predict the target genes of distal enhancers and associated transcription factors[15,16].

Split-pool recognition of interactions by tag extension (SPRITE)

The SPRITE method uses extensive cross-linking so that spatially proximal groups of loci, and RNA molecules, are covalently linked. DNA is then fragmented with nucleases, and individual clusters of linked molecules are barcoded through a split-pool barcoding strategy. Barcoded molecules are then identified through sequencing[17]. This approach allows identification of multi-way higher order interactions between sets of loci. SPRITE was found to be particularly powerful for detecting chromosome compartments, revealing key roles for nuclear bodies such as nuclear speckles and nucleoli, as interaction hubs for spatially organizing the genome. More recently SPRITE has been extended to include analysis of DNA-RNA interactions[17].

In situ mapping of RNA-genome interactions (iMARGI)

The iMARGI technology is an all-vs-all method to identify chromatin-associated RNAs (caRNA) and each caRNA's associated genomic sequence[18]. It works by carrying out proximity ligation of RNA and DNA through a linker. iMARGI revealed thousands of previously uncharacterized caRNAs transcribed from coding and non-coding genes as well as from intergenic sequences[19,20]. iMARGI led to the characterization of a biogenesis pathway of RNA fusions, where the association of Gene A's transcripts, i.e. the caRNA, with Gene B's genomic sequence provides the necessary spatial proximity for Gene B's nascent transcripts to be spliced with the caRNA, to create a fusion RNA. This RNA fusion pathway explains the majority of the approximately 4,000 fusion RNAs that do not have corresponding fusion genes, which were detected from The Cancer Genome Atlas (TCGA) program's approximately 10,000 tumor samples in 33 cancer types[20]. iMARGI also revealed that stress-induced interchromosomal RNA-DNA interactions are responsible for activation of inflammatory genes in the progressively dysfunctional vascular endothelium in diabetic patients[21]. iMARGI will be used for further elucidating RNA's roles in epigenetic gene regulation.

Local proteome of specific genomic loci (C-BERST)

The dCas9–APEX2 biotinylation at genomic elements by restricted spatial tagging (C-BERST) technology maps the local proteome at and around specific genomic loci[22]. C-BERST can be used to identify sub-nuclear proteomes and relate these to chromosome folding and nuclear organization. C-BERST was validated through analysis of proteomes at centromeres and telomeres, identifying many known centromeric and telomeric proteins as well as factors not previously reported to be associated with these genomic loci[22].

TSA sequencing (TSA-seq)

TSA-Seq estimates the cytological distances of any chromosome loci to particular nuclear compartments, for example nuclear speckles[23]. By converting sequencing reads into actual calibrated physical distances, TSA-seq provides cytological-scale mapping of the genome

relative to nuclear locales[23,24]. TSA-seq data led to the concept that distance to nuclear speckles is a key correlate with levels of gene expression, surpassing previous measures such as radial position and distance from the nuclear lamina, with small shifts towards or away from nuclear speckles highly correlated with increased versus decreased gene expression, respectively[23,24].

GPSeq

GPSeq is based on the different kinetics with which chromatin within fixed nuclei can be digested with a restriction enzyme: chromatin at the periphery is digested faster than more internally located loci[25]. By sequencing the cut sites one can map the relative radial positions of loci genome-wide. GPSeq was developed by the Bienko group, outside of the 4D Nucleome Consortium, and later adopted by the project.

Time-resolved and single-cell tools

The second set of 4DN approaches that we highlight here provide the capabilities of mapping the 4D nucleome with time or single-cell resolution. This set includes time-resolved DamID, Liquid Chromatin Hi-C (LC-Hi-C), single-cell combinatorial indexing Hi-C (sci-Hi-C), multi-contact 3C (MC-3C), single-cell Repli-seq (scRepli-seq) and high resolution Repli-seq.

Time-resolved Dam-ID

The time-resolved DamID (in vivo protein-genome interactions using tethered DNA adenine methyltransferase) overcomes the previous time resolution limitations of conventional DamID[26]. Time-resolved DamID helped to demonstrate the local loss of nuclear lamina interactions of LADs by transcription[27]. Time-resolved DamID and TSA-seq collectively furthered our understanding of the nucleus beyond the binary division of the genome into A or B compartments, allowing the identification of multiple interior active and multiple interior and peripheral repressed chromosome spatial states with distinctive gene densities, gene expression, and chromatin modification features which, at least in some cell types, are organized with a high degree of nuclear polarity[23,28]

Liquid Chromatin Hi-C (LC-HI-C)

LC-Hi-C measures chromatin interaction dynamics genome-wide[29]. LC-Hi-C revealed the forces that drive chromosomal compartmentalization by quantifying chromatin interaction dissociation rates upon *in situ* chromatin fragmentation. Dissociation rates of genomic interactions fluctuate along chromosomes in relation to compartment status and chromatin state[29].

Single-cell combinatorial indexing-based genomic assays

4DN developed exponentially scalable single cell molecular profiling methods based on the concept of “combinatorial indexing”. These “sci-” assays can profile chromatin accessibility (sci-ATAC-seq), gene expression (sci-RNA-seq), chromosome conformation (sci-Hi-C), genome sequence, protein binding sites (sci-TIP)[30] and DNA methylation, and can be combined as sci-co-assays to jointly profile features[31,32]. sci-Hi-C maps chromatin

interactomes in a large number of single cells by adding combinatorial cellular indexing to DNase Hi-C, which replaces the previously used restriction enzymes with a non-specific DNA nuclease[33]. sci-Hi-C captures cell-to-cell variability in 3D genome folding and reveals the association of such variability with cell-cycle states[33].

Multi-contact 3C (MC-3C)

MC-3C detects strings of co-occurring chromatin interactions in single cells that represent three-dimensional step-by-step walks (percolation paths) through the folded genome[34,35]. Multi-contact data can be used to determine clusters of co-associated loci. In addition, combined with polymer simulations and computational methods, MC-3C data can be used to determine the extent to which different chromatin domains are intermingled in single cells. Such intermingling can in turn be used to infer the topological state of chromosomes, i.e., the presence of entanglements such as catenanes and interlinks. Surprisingly, the genome, in interphase cells, was found to be largely free of entanglements[34].

Single-cell and high resolution Repli-seq

Repli-seq determines the sequence in which chromosomes replicate their DNA during S phase (replication timing; RT). 4DN investigators developed tools to measure RT in single-cells and for individual homologous chromosomes within cells[36], and at high temporal (16 time intervals of S phase) and spatial (50 kb) resolution[37]. These methods are starting to relate replication timing programs to the 4D nucleome. For instance, it was demonstrated that different segments of the genome have different degrees of cell-to-cell heterogeneity in their time of replication, that replication timing is directly linked to maintenance of the epigenome and appropriate compartmentalization through action of the RIF1 proteins[38], and that cohesin is required to confine the initiation of replication to the borders of a particular class of TADs[39].

Multiplexed FISH for single-cell chromatin structure measurements

Specific genomic sequences can be visualized by Fluorescence In Situ Hybridization (FISH) using fluorescently-labeled DNA probes, and their location in individual cells can be imaged using either traditional microscopy techniques (eg. widefield or confocal)[40–42] reviewed by[43–46], or super resolution microscopy[47–50], as reviewed in[51,52].

As a logical evolution of more traditional techniques, by adding an automated microfluidics system to the microscope and, crucially, novel bioinformatics platforms and array-based synthesis to design targeting oligonucleotide (hereafter referred to as oligo) probes[53–56] including Oligopaints[57–60], sequential rounds of hybridization and imaging are performed, allowing the detection of multiple and ideally all DNA locations across the genome (see recent reviews:[52,59,61–64]. Collectively these technologies can be called interchangeably Multiplexed FISH or FISH Omics, which emphasize the visualization of multiple or ideally *all* genomic targets, respectively. A variety of protocols have been developed in the past few years, and they can be divided into two main categories depending on whether the targeted genomic segment is visualized as a centroid (i.e., fitting the imaged spot of a detected

fluorescent signal) or a cloud of single-molecule localizations (i.e., often rendered as a volume; Figure 2).

In the end, all of these techniques map the physical localization of specific genomic DNA segments that can be either contiguous or separated by varying genomic distances and importantly they can be combined with the detection of other chromatin molecular components such as RNA and proteins[61,65–71]. These data are used for tracing the 3D path of chromatin fibers across genes, TADs, or entire chromosomes, for evaluating the spatial distribution and compaction of specific loci, or for interrogating specific 3D associations such as enhancer-promoter interactions, interchromosomal interactions, and intranuclear positioning[67,69,72–75].

A major focus of Phase II of the 4D Nucleome Project is on mapping the dynamics of chromosome and nuclear organization, and the variation in this organization between single cells. Imaging has become a major approach in the consortium and below we describe several of the methods that are being used.

Ball-and-Stick Chromatin Tracing

The distinguishing feature of Ball-and-Stick Chromatin Tracing techniques is that the centroid of an imaged spot corresponds to the entirety of the targeted genomic segment (2–100 kb) during each hybridization round (i.e., “there is an actual correspondence between the number of targeted genomic segments and the number of imaged spots”), and the 3D chromatin folding is assembled by connecting the centroids of the spots imaged in each successive round. Multiple groups have developed protocols to do this “walking” across the genome at different step sizes[69]. In particular, Ball-and-Stick Chromatin Tracing methods can be further subdivided in two general sub-groups depending on whether different segments are imaged sequentially using a different fluorophore for each imaging step (i.e., there is an actual 1:1 correspondence between targeted genomic segments and imaged spots), versus targeted genomic segments are distinguished by a unique combination of fluorophores (i.e., barcoding).

Optical Reconstruction of Chromatin Architecture (ORCA)[66], high-throughput, high-resolution, high-coverage, microscopy-based (Hi-M)[76], and Multiplexed Imaging of Nucleome Architectures (MINA)[67], belong to the first sub-group of Ball-and-Stick Chromatin Tracing techniques in addition to the method developed by Wang et al.[69]. The advantage of these techniques is that they enable the reconstruction of chromatin traces that fall within the same diffraction-limited volume, as is often the case for single TADs.

On the other hand, techniques that belong to the second Ball-and-Stick Chromatin Tracing sub-group enable genome-wide mapping by leveraging parallel multiplexing methods in which many genomic regions are imaged in parallel and their genomic identities are decoded at the end of the experiment based on barcodes that indicates the presence (1) or absence (0) of fluorescent signal in each round at that position (e.g., 10010 for five rounds) or the presence of a given color combination (e.g., Blue-Green-Red-FarRed vs. Blue-Green-FarRed). This approach allows the detection of 2^N genomic regions, where N is the number of imaging rounds. Different protocols for DNA detection and barcode

encoding have been developed: DNA Sequential Fluorescence In Situ Hybridization (DNA seqFISH/seqFISH+)[70,71,77], Oligopaint Fluorescent In Situ Sequencing (OligoFISSEQ)[78], DNA Multiplexed error-robust fluorescence in situ hybridization (DNA-MERFISH)[68], and In-situ Genomic Sequencing (IGS)[79].

Volumetric Chromatin Tracing

Another application of barcode-based DNA FISH methods are technologies such as Oligopaint Stochastic Optical Reconstruction Microscopy (OligoSTORM), and Oligopaint DNA-based Point Accumulation for Imaging in Nanoscale Topography (OligoDNA-PAINT)[47,73,75,80,81]. These techniques have been utilized in species as diverse as humans and Drosophila and have been shown to spatially resolve genomic structures labeled with Oligopaints with nanoscopic precision. The distinguishing feature of these techniques is that they produce data in which each targeted genomic segment is visualized as a “cloud” of tens to thousands of single molecular localizations and thus enable the computation and analysis of the occupied volumes, and overlap fractions for each step. For this reason, we propose here to collectively call these methods as Volumetric Chromatin Tracing.

Standards and tools for imaging-data documentation, exchange, and integration with sequencing data

A major goal of the 4D Nucleome project is to share all data it produces in an unrestricted manner with the larger community and public. Public sharing of sequencing-based genomic data is well established through depositing standard file formats (e.g., fastq files) in public databases such as Gene Expression Omnibus (GEO), and the 4DN consortium has generally adopted genomic data file standards developed by ENCODE and other international efforts such as the Open Chromosome Collective (Open2C)[82]. It is likely that in the future additional file standards for long-read sequencing assays will be needed (e.g., for PacBio and Oxford Nanopore generated data). Sharing microscopy-based datasets has been more complicated due to the size of the data files and the wide array of file formats. Recent years have seen a rapid expansion of FISH omics methods, which quantify the spatial organization of DNA, RNAs, and proteins in individual cells and provide an expanded understanding of how higher-order chromosome structure relates to transcriptional activity and cell state. Despite this progress, FISH image-based data is not yet routinely made available through public repositories because of the lack of community specifications for data quality control, documentation, and exchange. This challenge is experienced across the bioimaging community. As a result, solutions built, tested, and proven in 4DN can have a broad impact across the scientific community.

4DN in collaboration with the Open Microscopy Environment (OME) initiative is actively engaged in important efforts to support the FAIR (Findable, Accessible, Interoperable, and Reusable)[83] reuse and exchange of multiplexed FISH Omics data.

This work includes two complementary aspects, the first of which centers around the development of the tiered system of Microscopy Metadata specifications[84] that scale with experimental intent and complexity, and make it possible for

scientists to create comprehensive records of imaging experiments. These specifications were developed in partnership with BioImaging North America (BINA, https://www.bioimagingnorthamerica.org/qc-dm-wg/) to extend the OME Data Model[85] and make it possible to faithfully interpret scientific claims, foster reproducibility and reuse, and promote the integration of imaging with omics datasets[86]. In order to streamline the collection of 4DN-BINA-OME compliant microscopy metadata, 4DN has developed the Micro-Meta App[87] and integrated it into the 4DN-Data Portal[88,89]. Micro-Meta App provides a visual interface that greatly facilitates building comprehensive descriptions of the hardware specifications, image acquisition settings and quality control metrics associated with the production of microscopy datasets and can be further employed for educational and training purposes.

In addition to the development of microscopy metadata specifications and tools, the 4DN has spearheaded the development of the 4DN multiplexed FISH Omics Format - Chromatin Tracing (FOF-CT)[90], a community data format designed for capturing and exchanging the results of chromosome imaging experiments that could serve as the basis of shared data management and analysis pipelines. FOF-CT is directly compatible with several Ball-and-Stick Chromatin Tracing techniques[69], including ORCA, MINA, Hi-M, OligoFISSEQ, DNA seqFISH/seqFISH+, DNA-MERFISH, and IGS[66–68,71,76–79]. In addition, the format is designed to be consistent with the ongoing development of extensions that will encompass Volumetric Chromatin Tracing methods, such as OligoSTORM and OligoDNA-PAINT[73,75,80,81].

The systematic dissection of the functional implications of nuclear spatial architecture in health and disease requires the effective integration of sequencing-based (e.g., Hi-C) and imaging-based (e.g., Multiplexed FISH) omics data from multiple different tissue types, biological statuses (e.g., cell cycle, cell differentiation, response to stimuli and stress), conditions, and laboratories. In turn, because of this variability and complexity, the interpretation of 4DN results requires to directly address the functional significance of the *nuclear topography* on nuclear function. For this reason, the 4DN is in the process of developing a standardized nuclear reference coordinate system to be integrated into the FOF-CT. Such a Common Coordinate Framework (CCF)[91,92] would allow anchoring the location of imaged chromosome segments in the nuclear context, thus promoting data integration, facilitating predictive modeling and making 4DN-produced imaging data more valuable for computational scientists and for the community at large.

Multiplexed FISH Omics experiments generate complex and multidimensional sets of images. This complexity makes it clear that addressing reproducibility and sharing challenges is beyond the scope of individual efforts and instead requires concerted global action.

As such, the efforts of the 4DN Consortium are conducted in close collaboration with similar endeavors to improve reproducibility, data quality, and re-use value for imaging experiments carried out by several international bioimaging initiatives including OME, BINA, the EMBL-European Bioinformatics Institute (EBI)[93,94], Global Bioimaging[95], the Image Data Resource (IDR)[96,97], Quality Assessment and Reproducibility for Light

Microscopy (QUAREP-LiMi)[98,99], and involving all major community stakeholders including microscope users, custodians and manufacturers, standards organizations, journals, and funders.

Determinants and modulators of the nuclear architecture

4DN has started to delineate general principles on how sequence elements, trans-factor, and environmental modulators affect genome organization.

Sequence determinants of genome organization

4DN adopted an emerging multiplexed high throughput DNA FISH technique to study the relationship between topologically associating domain (TAD) boundaries and enhancer-blocking insulator elements. The results showed that TAD boundaries do not completely prevent enhancer-driven transcriptional activation of target genes[100]. Further, the strength of insulation by TAD boundaries depends on both the number and sequence contexts of CTCF binding sites. Taking a complementary approach, consortium members built models that predict Hi-C or Micro-C contact maps from sequence and/or epigenetic features[101,102]. By analyzing these models and using them to perform high-throughput computational mutagenesis screens (e.g., deletions, scrambling regulatory motifs, etc.), they identified sequence determinants of TAD boundaries, loop anchors, and other features of the contact maps. These analyses highlighted the importance of clusters of CTCF motifs at TAD boundaries, but also revealed a complex grammar of sequence determinants of 3D genome folding, including known transcription factor binding sites, active promoters (with or without CTCF), and many motifs of unknown function.

Repetitive DNA's role in nuclear organization

Less studied sequence determinants of the 3D genome are repetitive DNA elements. Repetitive DNA comprises more than 50% of the human genome and is central to several critical tasks in the life cycle of the cell including housing the DNA component of the centromere necessary for chromosome segregation. In the first phase of the 4DN effort, the consortium found profound impacts of repetitive DNA on the 3D folding of chromosomes in interphase, including the macroscale partitioning of the human inactive X chromosome into two "megadomains"[103], the tendency of short tandem repeat regions to serve as 3D chromatin domain boundaries that are susceptible to disruption in disease[104], and the formation of liquid condensates that cluster the telomeres from many chromosomes to facilitate the Alternative Lengthening of Telomeres (ALT) pathway in cancer[105]. In the second phase, an unbiased genome-wide screen using the Akita model[101] identified several families of retrotransposons and small RNA repeats as some of the most important sequence determinants of 3D genome folding[106]. In the coming years, 4DN plans to leverage the first "telomere to telomere" human genome[107] and collaborate with the Human Pangenome Reference Consortium to expand the investigations on the roles of repetitive DNA in regulating nuclear architecture, genome stability, and other the DNA transactions, and to understand the functional roles of RNA products made from repetitive DNA.

Trans modulators of the genome organization

Phase separation

Phase separation is an intrinsic property of many biological molecules. It underlies the formation of several sub-compartments in the nucleus that can shape genome organization. For example, chromatin microphase separation leads to chromatin compaction and domain formation, heterochromatin protein phase separation drives the formation of heterochromatin regions[108,109], and protein-RNA co-phase separation creates nuclear bodies that can pull interacting chromatin together or push non-interacting chromatin apart to control genome organization[110].

In this second phase, 4DN aims to clarify how phase separation regulates genome organization and subsequently cellular functions, and how alterations of those phase separation processes can in some cases be linked to disease. Towards these goals, 4DN is developing techniques capable of probing and modulating such dynamic processes at different scales. One project aims to profile the complex effects of transcription factor phase separation on gene expression[111]. Another project tests the hypothesis that aberrant phase separation underlies the formation of abnormal nuclear features such as mislocalization of PML nuclear bodies and clustering of telomeres in telomerase-free cancer cells that use a homology-directed pathway for telomere maintenance[105]. The outcomes of these efforts could help develop strategies to ameliorate or reverse aberrant phase separation-mediated nuclear organization in disease.

RNA

Evidence has emerged that multiple types of RNAs, including coding and noncoding RNAs can influence chromatin structure and nuclear organization. Examples include the noncoding RNA (ncRNA) XIST that induces silencing and condensation of the X chromosome, the repeat-containing ncRNA TERRA that helps telomere condensation, and the ncRNA MALAT that facilitates formation of speckles[112–115]. In phase 1, 4DN released high resolution maps of chromatin-associated RNAs (caRNA) and their associated genomic sequences in three human cell types[116]. 4DN also released caRNA-DNA contact maps in genome-edited human cells where specific TAD boundaries were removed[116]. Comparisons of RNA-DNA association maps before and after deleting a TAD boundary revealed that a TAD boundary can insulate not only crossover DNA-DNA contacts but also crossover RNA-DNA contacts, evidencing an impact of genome architecture to the spatial distribution of RNA in the nucleus[116]. Conversely, those caRNA that are associated with genomic sequences located between anchors of a chromatin loop suppresses this loop’s strength, pointing to roles for caRNAs in modulating 3D genome organization[116].

During the second phase, 4DN will expand its scope from genome organization to include RNA’s expression, spatial distribution, interactions with the folded genome and with nuclear organelles. 4DN will prioritize coordinated efforts combining sequencing and live-cell imaging approaches to map the dynamics in the spatial distributions and interaction partners of selected RNA species in the context of the dynamic nuclear organization, with an

emphasis on clarifying the regulatory role of RNA during organismal development and cell differentiation.

Cohesin and condensin complexes

Cohesin and condensin complexes are currently the best characterized molecular machines that fold chromatid through a process of ATP-dependent loop extrusion. The process of loop extrusion and its contribution to chromosome folding, nuclear organization, and gene expression are extensively studied in the 4DN consortium. A key focus is on studying loop formation in real time in living cells (Figure 3, papers from Hansen[117] and Giorgetti[118]), and to determine the roles of cis-elements, including CTCF-bound sites, promoters, enhancers, etc., and trans factors that control the activity of loop extruding complexes along the genome.

Embracing time, the 4th dimension

One of the central missions of the 4DN consortium is to study the three-dimensional (3D) chromatin organization with temporal dynamics (3D + time = 4D)[1,2]. There are at least three relevant timescales for studying chromatin dynamics (Figure 1). First, at the scale of multiple cell divisions, e.g., as cells differentiate, the 4D nucleome is modulated in correlation, and possibly through causal relationships, with changes in chromatin state (e.g., histone modification and factor binding profiles along chromosomes) and gene expression. This time scale can cover several hours, days, or weeks. Second, during a single cell cycle, the 4D nucleome is drastically reorganized to facilitate gene expression in interphase, DNA replication in S-phase, and chromosome condensation and sister chromatid segregation during mitosis. This time scale is in the order of hours and up to 1 day. Finally, at the time scale of minutes and hours, chromatin is dynamic even within a single G1 phase of the cell cycle. Such dynamics includes formation and solidification of compartmental interactions, i.e., the formation of preferred interactions between chromatin domains of similar type (e.g., inactive and active chromatin domains), and the dynamic association of domains with the nuclear lamina. All these events occur over a timescale of several hours after cells enter G1[119–122]. At even shorter timescales, loops are formed through SMC-complex driven loop formation[123]. These loops, e.g., formed by cohesin in interphase, form at the scale of tens of minutes and are dissolved again when cohesin dissociates from chromatin[117,118]. Here we outline some examples of ongoing work within 4DN phase 2 to study chromatin dynamics at these different timescales.

4D Nucleome dynamics during differentiation

Human cell lineage specification is an ideal process for studying this temporal dimension. Guided differentiation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) towards many specific lineages is now established, and can be applied to hPSCs from both healthy individuals or patients with diseases[124]. Compared to primary tissues, hPSC-based models are more accessible, scalable, reproducible, and amenable to manipulation. In addition, new tools for (epi)genetic editing, perturbation, and large-scale CRISPR screening in hPSCs have allowed rapid progress, facilitating functional studies of 3D chromatin and (epi)genome

organization[125]. These raise opportunities for measuring 3D chromatin dynamics in human development with high temporal and cell-lineage resolutions, permitting mechanistic studies to dissect how 3D genome topology and temporal dynamics may impact both health and disease. Genetic changes of NIPBL serve as an illustrative example. As a chromatin loader of cohesin, NIPBL alteration can cause Cornelia de Lange Syndrome (CdLS) with multiple-organ abnormality[126]. Interrogating NIPBL functions in relevant hPSC-derived cell types will be critical to understand how its defect changes chromatin topology and contributes to disease phenotypes, and discover both shared and distinct mechanisms across diverse tissue types.

Focusing on hPSCs and their differentiation, the 4DN consortium will make cross-differentiation-platform comparisons regarding epigenome mapping (at both bulk and single cell levels), (epi)genome editing and perturbation, and large-scale CRISPR screening. We aim to develop and improve protocols as applicable in hPSC systems and share standard operating procedures (SOPs) for hPSC gene editing and guided differentiation (e.g., hPSC differentiation to definitive endoderm, neurons, and cardiomyocytes) to benefit 4DN members as well as the larger scientific community. We will also share key experimental reagents (e.g., engineered hPSC lines, plasmids etc.), datasets, computational tools, and analytical results for collaborative and integrative analyses. The deliverables of this effort are that our work will benchmark human cell differentiation systems, identify robust mapping method(s) and/or data type(s) for predictive modeling, develop effective and broadly relevant computational methods, and discover both generalizable and cell lineage-specific principles that govern the 4D nucleome organization in development and disease.

Chromatin dynamics during the cell cycle

There are two periods of the cell cycle that are highly disruptive for the human 4DN. During mitosis, the nucleus including the envelope and likely all phase separated nuclear bodies are completely disassembled, transcription is nearly shut down, chromatin condenses and many chromatin proteins are removed or post-translationally modified. Mitotic exit then, is a window of opportunity to observe the 4DN re-assemble in real time, and is the exclusive period when cell fate changes can occur, permitting one to relate 4DN structure to function during the establishment of transcription and replication programs that either maintain or alter cell fate. By applying computational approaches to data from mapping and perturbation studies, 4DN aims to create an interactive visual representation of nuclear re-assembly that will provide mechanistic insights into the function of the 4D nucleome in development, tissue homeostasis, and disease.

To study how cells re-establish cell type-specific 3D genome features and alter them in ways that are important for their lineage specification, new experimental approaches are being developed that allow for longitudinal tracking of genome structures across cell division and along lineages. However, as genome-wide 4D nucleome technologies require fixed cells, longitudinal tracking remains a challenge. To address this problem, 4DN aims to leverage genomic barcoding technologies wherein heritable stochastic mutations create a trace of every cell's lineage history in its genome[127,128]. These barcodes can be used to decipher the time that has elapsed since two cells' most recent common ancestor underwent mitosis[129],

thus providing insights into mitotic stability and/or alterations of genome structure features over cell divisions[130].

The second disruptive period of the cell cycle is S phase, during which the entire structure of chromatin is disassembled to the nucleotides and re-assembled. This is done by replicating segments of chromosomes in a cell-type specific temporal sequence (RT) that reflects their location in the nucleus. 4DN investigators have developed methods to perturb RT, demonstrating that RT contributes to maintenance of the epigenome, likely by assembling different types of chromatin at different times during S phase[38]. 4DN is interested in what directs different types of chromatin to be assembled at different times and locations in the nucleus and how that influences cell fate.

4DN has discovered dramatic alterations in long range interactions involving cell-type specific classes of heterochromatin that occur specifically during S phase[38,131]. The T2T human genome build has revealed a new set of domains that replicate as large spatial clusters at the end of S phase and may form novel heterochromatic spatial compartments. Indeed, 4DN investigators are exploiting the spatio-temporal organization of DNA replication to relate genomics measurements of the proximity of sequences to each other and to nuclear landmarks to direct imaging measurements of the dynamic changes in sub-nuclear position of DNA replication sites.

Cells also must make the choice to withdraw from the cell cycle, either temporarily (quiescence) or permanently (senescence). Quiescence and senescence are vital to maintain tissue homeostasis, to respond to loss of tissue, and to combat cancer. 4DN investigators are applying Hi-C, TSA-seq and Oligopaint imaging to investigate the changes in nuclear organization in senescent cells. Some of these changes have already been characterized, such as the loss of heterochromatin and dissociation from the nuclear lamina in senescent cells. Higher resolution Hi-C map will provide information about changes in sub-compartments and loops; TSA-seq will be used to study changes in chromatin’s association with nuclear bodies; Oligopaints will be used to study how chromosome territories are altered during quiescence and cellular senescence.

Chromatin Dynamics and Function

Causal links between chromosome conformation and transcription and in particular how dynamic changes in chromatin structure contribute to reliable and robust control of gene activity are still strongly disputed[132,133]. In its second phase, 4DN is developing microscopy methods that enable investigation of 3D genome organization at the single cell level as well as changes in chromatin and nuclear organization in living cells. These approaches are geared to further our understanding of chromatin structure and function, and to facilitate the validation of predictive models derived from chromatin contact measurements. The developed next-generation technologies will enable the biomedical research community to explore changes of nuclear organization in real time, and to investigate how changes in the spatial and temporal organization of the genome relate to cell function and dysfunction.

Achieving real time measurements of chromatin structure and dynamics faces many challenges. These include balancing fundamental tradeoffs in spatial and temporal resolution, the lower throughput of such intensive imaging in the number of cells, chromatin loci, and conditions that can be analyzed, and the sheer size of microscopy data, often many terabytes per experiment (see above). By bringing together geneticists, data scientists, microscopists, and physicists, 4DN aims to tackle these challenges. For example, three recent studies[117,118,123] used complex, polymer model-guided inference approaches (polymer physics and computation) to address limitations in measurement error and uncertainty in single-particle tracking in live cells (enabled by advanced microscopy) to study cis-interactions on the TAD scale, and measure how these dynamics are affected by acute depletion of key architectural proteins (genetics). Expanding such interdisciplinary teams, coupled with new advances in instrumentation, software, and new dissemination approaches of these enabling technologies, promise that progress in understanding dynamics of chromatin organization in the next several years will be likely.

Inflammation and stress

Inflammatory stresses, namely fever and pro-inflammatory cytokines, are evolutionarily conserved mechanisms to eliminate harmful pathogens. Inflammation is a well-orchestrated response and, when misregulated, underlies a number of conditions such as cardiovascular disorder, cancer, metabolic syndrome, neurologic disorders, etc.[134]. Inflammatory stresses activate well-established signal transduction pathways that culminate in gene expression and cell function modifications. Nevertheless, the processes leading to tissue dysfunction during inflammation remain poorly understood. Whether acute and chronic inflammatory stresses alter the chromatin architecture in all or specific cell types and at what level of chromatin organization remains elusive.

Within the 4DN consortium, an number of projects are focused on studying 3D chromatin dynamics over time (the 4th dimension), such as immune response, heat stress, DNA damage, and viral infections[21,135,136], thus providing a one-of-a-kind platform to initiate data collection from different stress conditions and affected levels of chromatin organization. This provides a unique opportunity to obtain a comprehensive view of the impact of inflammatory and other stresses on 3D chromatin organization. The ultimate goal is to create a data collection that would reveal the effects of stress on chromatin architecture. Current challenges include limited data with diverse experimental systems and stress conditions, making direct comparisons impossible. Accumulating more data, including data from similar experimental systems or stresses, will be instrumental in overcoming these temporal limitations to foster more collaborative work.

Mouse development

In Phase 2, 4DN consortium will address the question of how the 4D nucleome contributes to mammalian development. 4DN is generating systematic datasets on nuclear morphology in mammalian tissues and cell types in the context of the premier model system for mammalian development, the mouse. The approach focuses on following nuclear structure, chromatin and gene expression changes at a “whole organism” scale, using a combination

of scalable single cell profiling applied to embryos collected at tight intervals during mouse embryonic development to derive detailed trajectories of nuclear architecture, chromatin accessibility and gene expression. The novel visual cell sorting (VCS) assay[137] allows us to directly connect molecular features of each nucleus to chromatin and organelle features derived from imaging data. In addition, new spatial genomic approaches provide crucial information about cell position within the embryos[138]. The ultimate goal is to generate a publicly available, high-resolution 4DN atlas of mouse embryogenesis. The different types of data generated by different 4DN groups are being integrated, including cross-species imputation to integrate with human data, as well as to generate models and user-navigable maps applied to pathways relevant to mammalian development[139].

Computational modeling of the 4D nucleome

Computational modeling can help to obtain mechanistic understanding of nuclear structure and function, to reveal the complementarity of different data modalities, and to unveil the impact of nuclear structure in a wide variety of cellular processes in health and disease. 4DN developed methods for the integration of multiple experimental data to generate physical higher-order predictive models of the 3D nuclear genome organization and reveal the functional implications of different genome structural organizations. Here, we highlight a subset of these models and the knowledge that can be derived from them.

Machine learning-based integrative models for nuclear organization

Machine learning models can harness the information from multimodal datasets to provide new insights into genome structure and function. For example, a recent graph-based neural network model[140] combines 3D chromatin interaction and 1D epigenomic signals as well as DNA sequence features to predict gene expression. Graph-based machine learning models have also been developed to achieve a comprehensive view of large-scale chromatin organization in the nucleus. SPIN[28] uses a probabilistic graphical model to provide a more refined view of chromosome spatial localization relative to multiple functional nuclear bodies, by integrating Hi-C data with DamID[141] and TSA-seq data[23]. In K562 cells, SPIN identifies ten spatial compartmentalization states (“SPIN states”) relative to nuclear speckles, nuclear lamina and putative nucleoli, stratifying various structural and functional genomic features. In addition to gene transcription, machine learning models have also been developed to connect genomic and epigenomic properties to other important nuclear genome functions. For example, a recently developed method CONCERT[142] captures long-range spatial dependency of sequence features to predict replication timing domains. Together, machine learning models are expected to integrate a wide range of 4DN datasets to comprehensively reveal the relationship between nuclear structure and function.

Physical principle-based models to assess mechanisms of nuclear structure formation

Physical principle based models assume that chromatin folding is driven by a priori known or postulated mechanisms. Such mechanistic bottom-up models based on polymer physics are important to explain the experimental data and shed new light onto the formation principles of nuclear organization. In particular, loop extrusion and phase separation are two major types of models used to explain the observed nucleome data and understand

the intrinsic structure and dynamics of the nucleus. Recent polymer physics modeling approaches have demonstrated how mechanisms of loop extrusion and phase separation combine to drive major aspects of nuclear and chromosome organization[9,143].

Population-based integrative genome modeling

Data-driven integrative structure modeling provides complementary perspectives compared to machine learning and polymer-physics based models. Population-based Genome Structure (PGS) is a probabilistic approach for deconvoluting ensemble Hi-C data into a model population of 3D genome structures, thereby incorporating the stochastic nature of chromosome conformations and allowing a detailed analysis of alternative chromatin structure states across a population of single cells[144–146]. An application of PGS revealed the chromosome-specific centromere clustering as a driving force in shaping genome structure organization in human lymphoblastoid cells[144]. Another application of PGS revealed heterochromatic supercontraction in the folding of the genome in human neutrophils during neutrophil differentiation[147]. More recently developed Integrative Genome Modeling platform (IGM) provides a systematic integration of multimodal data from both genomic and imaging data types, e.g., Hi-C, Lamin B1 DamID, SPRITE and HIPMap data to produce populations of single-cell genome structures that are highly predictive for nuclear locations of genes and nuclear bodies, chromatin folding patterns, and spatial segregation of functionally related chromatin domain[148,149]. IGM allowed for characterizing chromatin folding patterns with respect to nuclear bodies to define the subnuclear microenvironment of genomic regions and their variability across a population of cells. A genomic loci's nuclear microenvironment was found to be a strong descriptor of its functional potential in terms of transcription and replication timing[149].

In Phase 2, 4DN prioritizes its modeling development efforts on: 1) Predictive modeling to identify roles of sequence and epigenomic signals on nuclear structural features and their collective, cell type-specific impact on genome functions (e.g., transcription, DNA replication, recombination). 2) Predictive modeling to reveal a realistic single-cell nuclear structure and dynamics by integrating multimodal data types, including genomic and imaging data. 3) Predictive modeling to guide experimental design and prioritize perturbations that maximize the efficiency of discovery.

We anticipate computational methods will be developed to produce concrete predictions of sequence properties and epigenomic signatures that modulate chromosome folding and nuclear compartmentalization. The models will help establish the interplay between sequence features and epigenomic signals in different cell types and biological contexts[140]. Predictive models will guide specific perturbation experiments to validate important sequence elements and epigenetic regulators, which can in turn improve the predictive power of the models. In addition, models will be developed to predict genome functions from 3D genome structures, thereby modeling structure-function relationships of nuclear chromatin architecture. To further connect 3D genome architecture and human disease, models will be developed to harness widely available data on disease-associated genetic variation and identify causal variants, elements, and genes in their corresponding cell types or tissues to shed light on mechanisms underlying genome-wide association studies (GWAS) through the

lens of 3D genome structure and function. Finally, to connect structure to function, 4DN will identify functional structural units, such as frequent higher-order spatial chromatin clusters of genes within and between chromosomes to establish a structure-function atlas of genomes that link specific 3D genome conformations to nuclear processes[150].

Multi-modal single nucleus time-course analysis of differentiation

Finally, the consortium has embarked on a consortium-wide collaborative effort, with most groups actively participating, to build a clear long-term legacy of insight, data, algorithms that reveal the role for higher-order chromatin folding as a fundamental contributor to the properties of life. Gene expression is a genome function which drives a myriad of complex developmental and physiological programs in humans. The functional role for chromatin folding in governing transcription through the spatial proximity of enhancers with promoters has long been hypothesized. However, unequivocal models for how multiscale 3D genome structures, including A/B compartments, TADs, subTADs, subnuclear bodies, and loops, govern gene expression are lacking.

During phase II of the 4DN project, an important consortium-wide initiative involves the establishment of a collaborative project that catalyzes technology-driven insight into the genome's structure-function relationship. We will focus on the function of gene expression and create predictive models of its control by genome folding and other epigenetic features such as DNA methylation at the granularity of the single cell. Centering on model systems representing the human heart and brain, we aim to produce three key deliverables. First, we aim to produce single-cell reference data sets using the leading edge technologies of (1) multi-modal single nucleus methyl 3C seq to assess DNA methylation and genome folding in the same single cell[151], (2) DNA FISH oligopaints with imaging of megabase separated bins to observe large scale folding patterns genome-wide in single cells[68,70], and (3) sequential oligopaints with imaging of tiled 5–20 kb-sized adjacent bins to observe smaller scale structures including TADs, subTADs, and loops at pre-selected genomic loci[66,73,81]. Second, we will develop algorithms for identifying genome folding patterns in single-cell genomics and imaging data sets and through integration with other single cell and bulk data sets[16,152,153]. Third, we will generate insight into how genome folding patterns are linked to single cell transcriptional changes over time. Unified by common assays, in stage 1 we will focus on the model systems of human brain (including iPSC-derived neurons, iPSC-derived organoids, and adult brain tissue) and heart (iPSC-derived cardiomyocytes, heart tissue). Once the technology development is established, in stage 2 we aim to pan out to multiple development and differentiation mammalian model systems. Together, we will strive to demonstrate structure-function connections of single-cell 4D Nucleome in a wide range of biological systems.

Data availability and resources

A major goal of the 4DN consortium is to provide a high-quality data resource for the community in addition to developing innovative technologies and algorithms. The 4DN Data Coordination and Integration Center (DCIC) has collected all data, both genomic and image-based, along with relevant metadata in a searchable repository (http://data.4dnucleome.org),

hosted on a cloud platform. Currently, there are nearly 2000 experimental sets across more than 40 assays, with the largest being more than 300 *in situ* Hi-C datasets. To ensure data quality, members of a 4DN working group reviews and approves the experimental protocols and bioinformatic analysis steps for each genomic assay. To qualify for deposition, sufficient sequencing depth and concordance of two replicate experiments are required for most assays. All data are centrally processed using the standard pipelines and all steps and parameters are recorded to enable full reproducibility; Docker images of the pipelines are also available for other investigators to run on their own data. The web portal also hosts a number of external datasets that are of high interest to the 4DN community and has visualization tools that allow the user to explore the data without downloading them. More details about the data and the platform are described in a recent paper[88]. In addition, to further enhance user experience with accessing these reference maps based on multimodal datasets, 4DN developed the Nucleome Browser for integrative and interactive visualization of genomic, microscopy, and modeling data sets[154].

Outlook

After a highly productive Phase I of the project that had been focused on technology development and the generation of the first detailed chromatin state and chromosome conformation maps of several key cell types[2], Phase II will place emphasis on measuring and quantifying chromatin dynamics, determining the cell-to-cell variation in chromosome folding and nuclear organization, and on establishing roles of the 4D nucleome in controlling genome functions such as gene expression, and on relating these to health and disease.

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The 4D Nucleome Project aims to develop an understanding of how the genome works and is regulated through analysis of its dynamic spatial organization. Dekker et al. summarize progress made by the Project since its start in 2015, and present an outline of current and future goals.

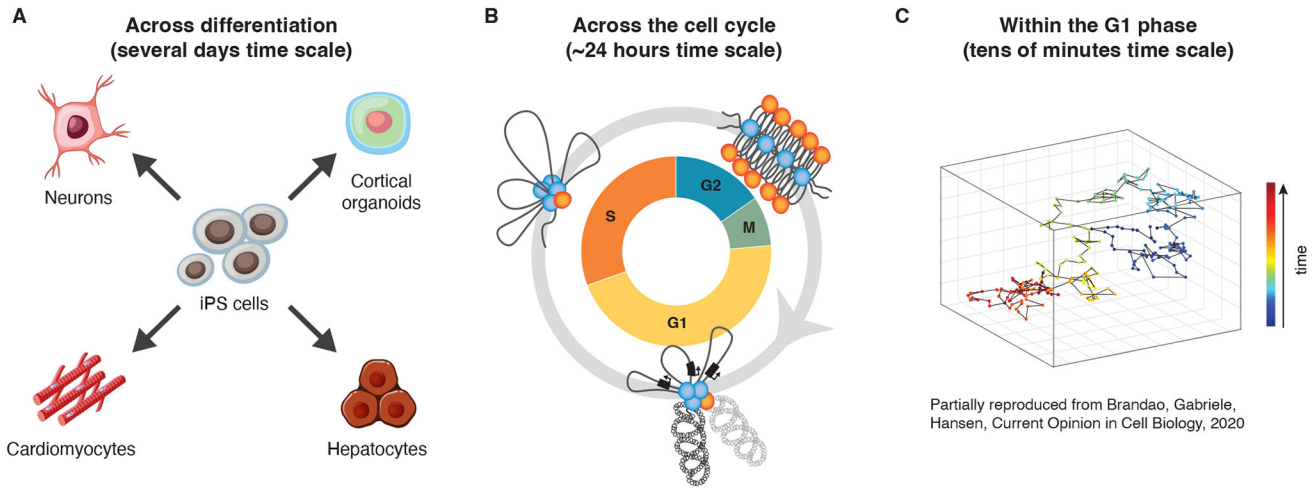

Figure 1.

Chromatin dynamics at different timescales. (A) Across differentiation, on a time scale involving several cell cycle stages and many days with iPS cells differentiating towards neurons, cortical organoids, cardiomyocytes, and hepatocytes. (B) Across the cell cycle on a scale of hours (~24 hours as a typical cell cycle). (C) Within the G1 phase (tens of minutes time scale), where an individual locus is traced in space over time to observe loop extrusion for example.

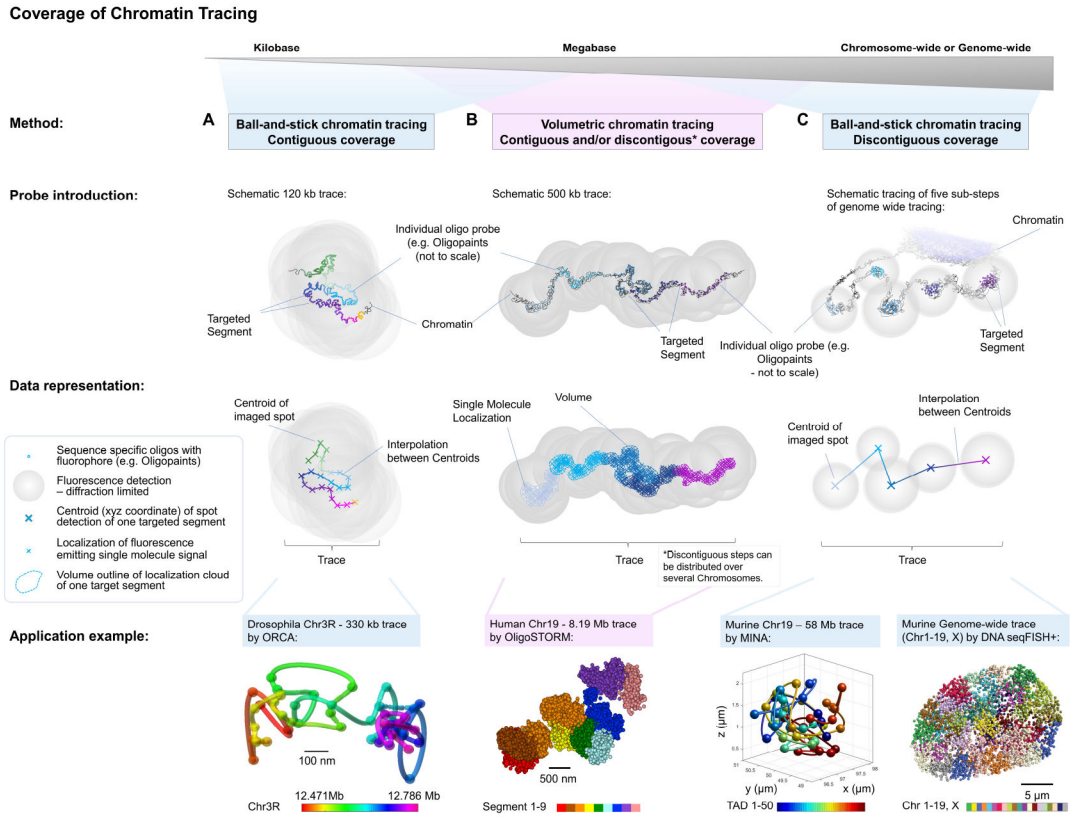

Figure 2. FISH Omics methods can be utilized to map chromatin structures across multiple genomic scales.

FISH Omics methods (also known as Multiplexed FISH) can be subdivided into two general categories, Ball-and-Stick (**A** and **C**) and Volumetric (**B**) Chromatin Tracing. In the former, a targeted genomic segment is represented by the centroid of an imaged spot and reflects the totality of fluorescence from multiple fluorophores. Instead, in Volumetric Chromatin Tracing a targeted genomic segment is visualized as a cloud of localizations each representing one emission event of a single-molecule fluorophore and outlining the volume that is occupied by the entire chromatin trace. As a whole, the two approaches can be used to dissect genomic chromatin structure across multiple genomic scales that range from kilobases to genome-wide (top row). (**A**) When Ball-and-Stick Chromatin Tracing is performed to target genomic segments that are contiguous to one another along the chromosome (i.e., contiguous coverage), it is typically used to map structural features that unfold at the kilobase scale (e.g., Promoter-Enhancer interactions). (**B**) When Volumetric Chromatin Tracing is employed to map chromatin structures in the megabase range in either a contiguous[73] or discontinuous manner[78] it is typically used to understand chromatin compaction[73] or intermingling of neighboring gene domains[75]. Finally, (**C**) when Ball-and-Stick Chromatin Tracing is used with discontiguous coverage it can be employed to map the structure of full chromosomes[67] as well as the organization of chromosome compartments across the whole genome[70]. Presented here from top to bottom are typical mapping coverage (top row), schematic representations of the strategy of probe introduction (second row from the top), and the way in which data is typically displayed (second row from bottom). The bottom row displays example applications for each of the three representative cases

as follows (left to right): contiguous coverage with 3 Kb target segments, 52 rounds of hybridization, 100 nm scale bar, adapted from[66]; contiguous coverage with 0.36–1.8 Mb target segments, 9 rounds of hybridization, 500 nm scale bar, adapted from[73]; discontiguous coverage with 100 Kb target segments (50 TADs), 40 rounds of hybridization, adapted from[67]; discontiguous coverage with 25 Kb target segments, 80 rounds of hybridization (each targeting 2460 segments), 5 μm scale bar, data from Cell 102, adapted from[70].

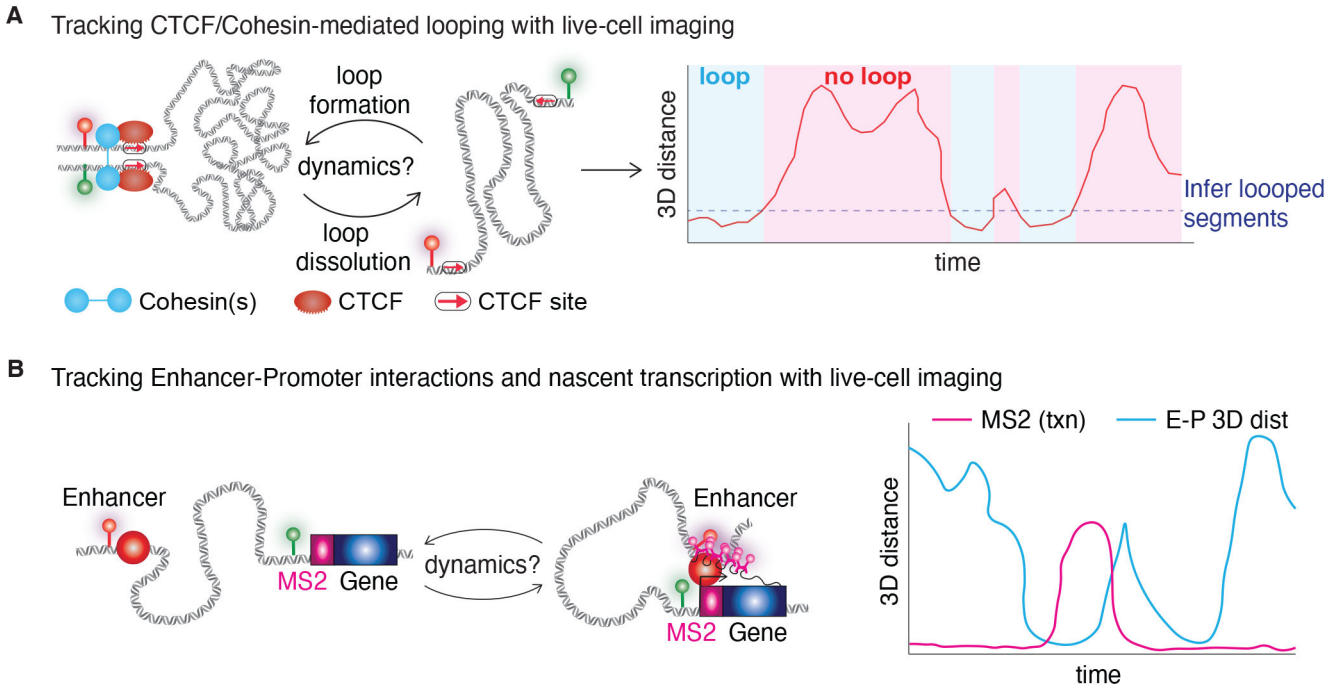

Figure 3. Tracking chromatin looping, enhancer-promoter interactions, and nascent transcription in real-time using live-cell imaging.

(**A**) Tracking and quantifying the dynamics of CTCF/cohesin-mediated loops with live-cell imaging. By fluorescently labeling the two CTCF sites that hold together a TAD or CTCF/cohesin loop, it is possible to estimate the duration and looped fraction from live-cell imaging using 3D distance as read-out as recently demonstrated in[117,118]. (**B**) Tracking Enhancer-Promoter interactions and nascent transcription with live-cell imaging. Similarly, by fluorescently labeling an enhancer and a promoter and by using the MS2/PP7 systems to visualize nascent transcription, it is also possible to track enhancer-promoter interactions with live-cell imaging to understand their relationship with transcription as illustrated in 132,133.