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Advancements in mapping 3D genome architecture

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1. Introduction

For many decades, microscopy-based techniques revealed important aspects of nuclear organization, specifically with regard to nuclear bodies. Additionally, the labeling of specific RNA or DNA sequences using fluorescence in situ hybridization has been instrumental for studying the localization of specific loci and understanding variability across individual cells of a population. More recently, application of high-throughput sequencing approaches to map the physical organization of DNA molecules in cells has revolutionized the study of genome organization. The ability to generate genome-wide measurements with high-resolution sequence information has greatly advanced our understanding of chromatin architecture. Despite their power, these sequencing-based Chromosome Conformation Capture (3C) assays have significant limitations, which we will discuss further.

Here we review our current understanding of 3D genome organization and the 3C technologies that have informed this knowledge. Then we discuss two new methods, termed SPRITE and Trac-looping, and how they allow for deeper investigation of the biological processes that shape the 3D genome.

2. Features of genome organization

In order for the genome to function properly, packaging of genetic material into the nucleus must allow for dynamic DNA-templated processes like transcription, replication, and repair. A variety of studies conducted in different cellular and organismal contexts have begun to define the principles of genome packaging [1–3]. These principles include the identification of fundamental units of genome organization, as well as knowledge of the molecular mechanisms that form them [4,5]. While models and terminology in the field continue to evolve, the relationship between transcription and architectural proteins is becoming increasingly clear.

The field of genome organization has moved rapidly toward the dual goals of classification of specific DNA structures and understanding their relevance to the control of gene expression and other biological processes. Units of genome organization have been defined based on features such as size, physical properties like the ability to block other DNA interactions, and functional outputs such as gene expression or chromatin state. The terminology that has emerged includes Topological Associating Domains (TADs), contact domains, ordinary domains, compartment domains, subcompartments, gene loops, DNA loops, and more. A hierarchical model has generally been used to explain how these structures relate to one another, with smaller-sized structures being contained within progressively larger structures. However, the sizes and biological mechanisms driving these structures are sometimes discordant. Additionally, most methods used to examine genome organization are performed on populations of cells, and as a result, it is unclear if these ensemble structures exist in any single cell. With deeper sequencing, single cell analyses, and microscopy-based approaches, these structures may be reclassified in the future.

Here, we follow an emerging view in the field regarding the definition of structural features of genome organization. Several groups now describe the genome as being organized into two major units of structure: compartmental domains, and DNA loops formed by Cohesin and CTCF proteins (Fig. 1A–C) [6,7]. First, compartmental domains are a feature associated with transcription and chromatin state. A-type compartmental domains are generally correlated with active transcription and chromatin state, whereas B-type compartmental domains are generally correlated with inactive transcription and a repressive chromatin state (Fig. 1B). Individual compartmental domains tend to interact with others of the same type, which is visualized as a plaid pattern in HiC heat maps. Compartmental domains can be larger or smaller than DNA loops. The second major unit of genome structure is a DNA loop mediated by the proteins CTCF and Cohesin (Fig. 1C). Each DNA loop is formed by a physical interaction between a pair of CTCF-occupied sites in the genome. Cohesin ring complexes assist in the pairing of two distal CTCF-occupied sites thus forming a DNA loop. Despite their importance in genome organization and function, the mechanisms controlling the formation of compartmental domains and DNA loop structures, and their interdependencies, are not fully understood.

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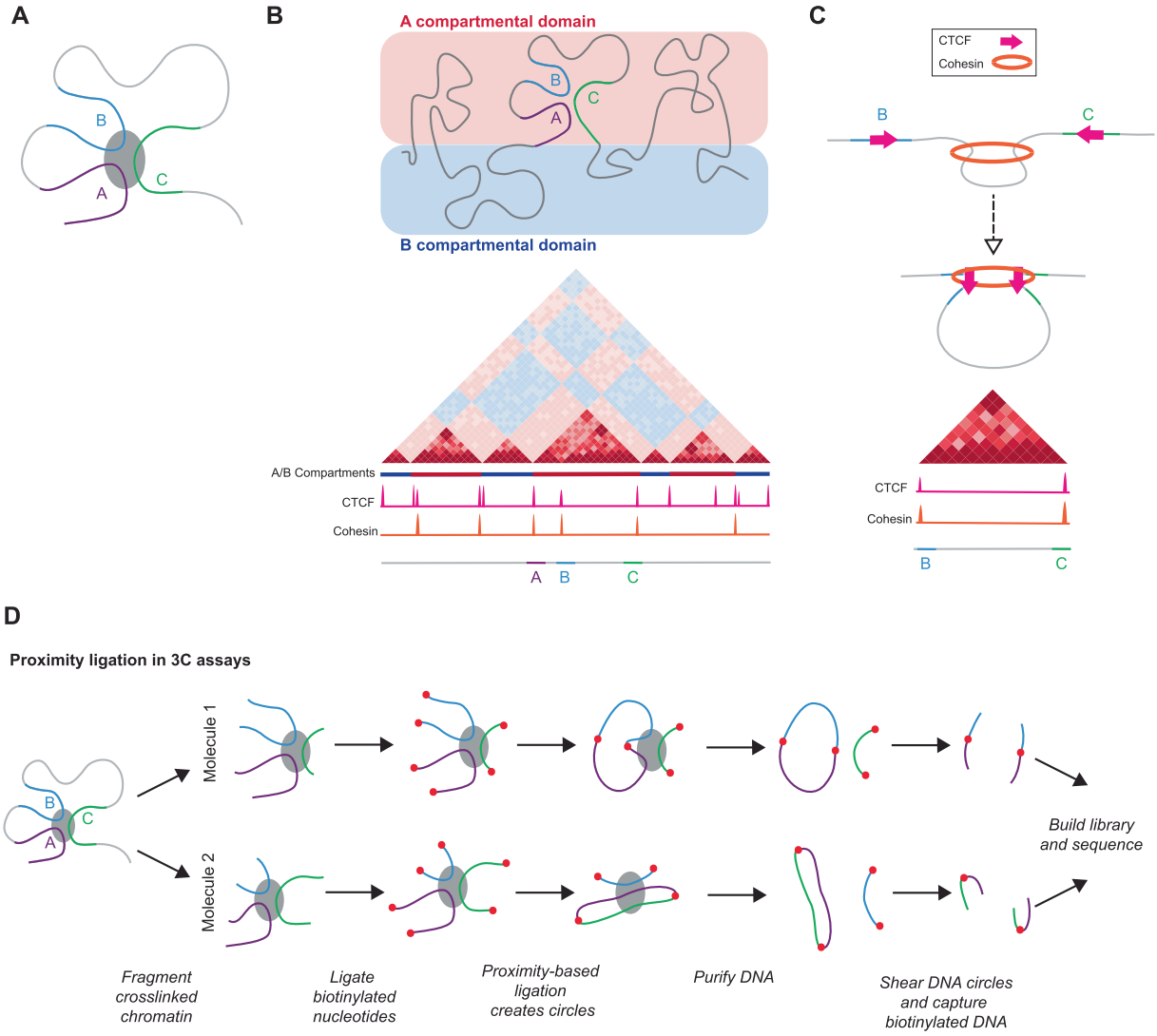

Fig. 1. Detection of compartmental domains and DNA loops mediated by Cohesin and CTCF. A. A chromatin molecule with three distal DNA sequences (A, B, and C) brought into close physical proximity by a protein of interest (grey). B. A diagram showing interaction frequency data obtained from HiC that identifies A and B compartmental domains (red and blue) across a window of the genome. Compartmental domains are identified in the red/blue track and can be observed in the HiC heatmap as a plaid pattern that stretches far off the linear distance axis. Binding of the architectural proteins Cohesin and CTCF is based on ChIP-seq data. C. An example DNA loop formed by Cohesin and CTCF proteins. Two convergently oriented CTCF bound sites are brought into proximity by an extruding Cohesin ring. This loop appears as a punctate spot at the top of a triangle in the HiC heatmap. D. Schematic of proximity-based ligation assays. Fragmentation of crosslinked chromatin is achieved by enzymatic or mechanical shearing. Biotinylated nucleotides or oligos are ligated to the ends and DNA is circularized with a ligation step. DNA is purified, sheared, and biotinylated fragments are captured by affinity purification to enrich for ligation junctions (not shown). From these fragments a library is created and high-throughput sequencing is performed to identify regions of the genome that are in close proximity in 3D space. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Current models posit that the 3D genome is shaped by the action of several different biological processes and properties: transcription, Cohesin-mediated extrusion of DNA, and phase separation. First, compartmental domains are closely correlated with transcription, suggesting an active role for transcriptional complexes and/or chromatin modifications in driving physical interactions along chromosomes. Concordantly, inhibition of transcription decreases compartmental domain formation [8], and the onset of zygotic transcription in early embryos often coincides with the appearance of compartmental domains [9]. Thus, compartmental domains may be a consequence of transcription. It will be important to determine the relative contribution of individual components of the transcriptional machinery and the role of specific chromatin modifications in compartmental domain formation in future studies. The second process that shapes genome organization is Cohesin-mediated extrusion of DNA [10]. Cohesin is a ring-shaped protein complex thought to translocate along DNA, thereby assisting the pairing of two CTCF-occupied genomic sites. The mechanistic details of Cohesin translocation are not well understood, including whether the process is driven by motor activity from the two ATPase domains of Cohesin or instead relies on a gradient of diffusing Cohesin rings. Interestingly, the two CTCF sites that anchor a DNA loop tend to be in a convergent orientation, with respect to the consensus binding motif [11,12]. The mechanisms by which the architectural proteins Cohesin and CTCF dynamically assemble, maintain, and disassemble DNA loop structures remain unclear. Several studies show that loss of CTCF or Cohesin causes loss of DNA loops and these perturbations do not phenocopy one another [13–15]. While more studies are needed to fully elucidate these mechanisms, it is clear that perturbing transcription or Cohesin-mediated extrusion of DNA can change the pattern of genome organization in different ways. A third property that shapes the 3D genome is phase separation. Regions of active and inactive chromatin may be, in part, segregated into discrete locations

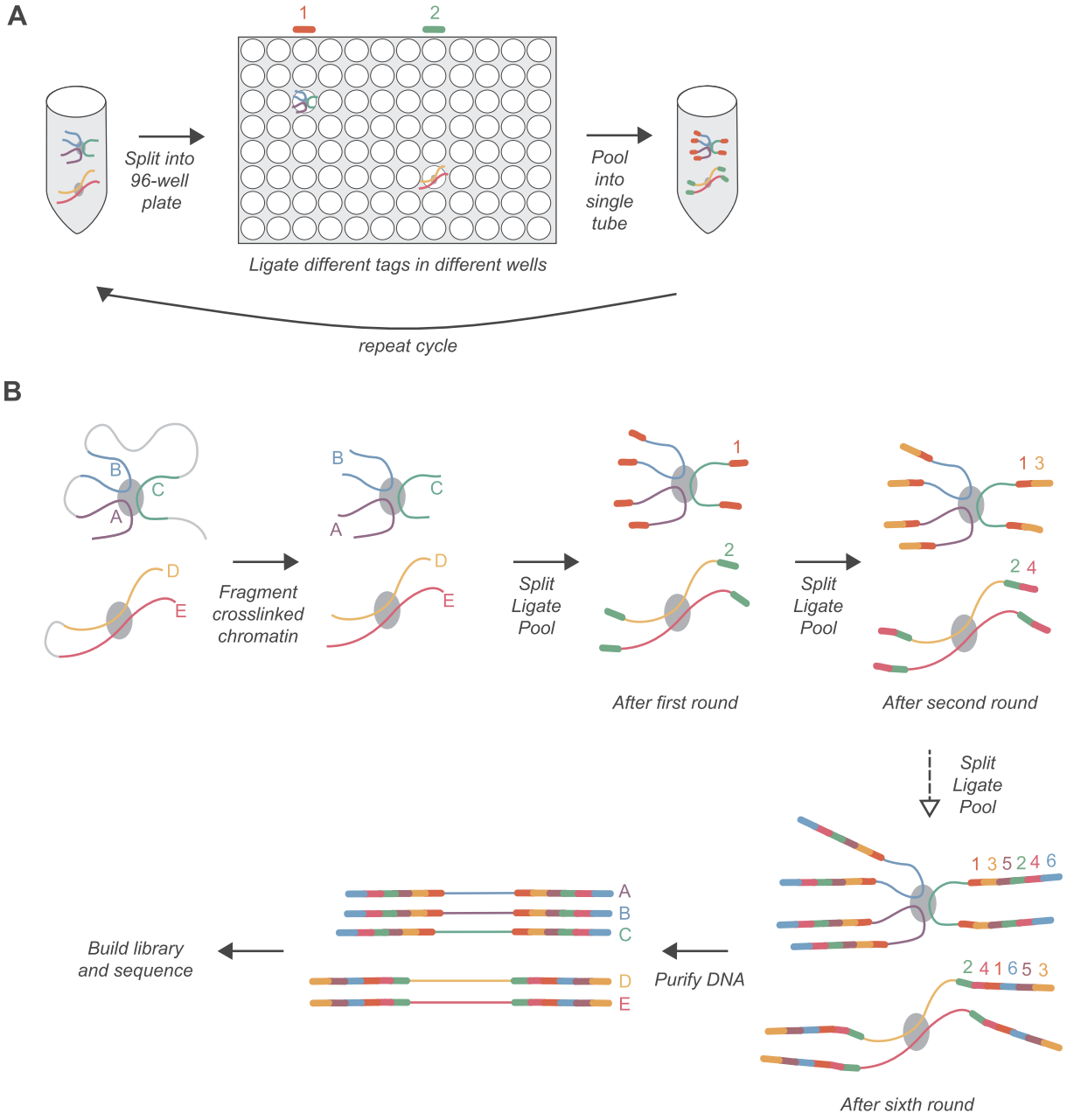

Fig. 2. Schematic of SPRITE protocol. A. Diagram of the split/ligate/pool strategy for building a unique 36 bp barcode on the ends of chromatin fragments. Chromatin is divided across a 96-well plate and one of six 6 bp barcodes is ligated onto the ends. The chromatin is then pooled together in a single tube before being split across another 96-well plate. This cycle of split/ligate/pool is performed a total of six times to build a 36 bp barcode. B. Crosslinked chromatin is fragmented with mechanical and enzymatic shearing. The chromatin is split across a 96-well plate, 6 bp tags are ligated, and all samples are pooled together. Five additional cycles of splitting, ligating and pooling yield 36 bp barcodes that are identical for all covalently-linked DNA fragments (termed "clusters"). DNA is purified, a library is built, and high-throughput sequencing is performed. This approach allows for efficient capture and identification of multiple simultaneously occurring DNA interactions that are informative of 3D genome organization.

via their biophysical properties. In living cells, liquid-liquid phase separation occurs when a homogenous solution has de-mixed forming droplets that are enriched for specific molecules relative to the surrounding environment. The formation of liquid droplets is driven by an increasing concentration of a nucleating factor, such as proteins or nucleic acids. Liquid droplets appear to organize or facilitate biological activities within a cell through selective permeability of factors [16]. Recently, phase separation has been implicated in both heterochromatin formation and transcriptional control. HP1 forms liquid phase-separated droplets that correspond to heterochromatin domains [17]. Transcription factors and transcriptional activators like MED1 and BRD4 participate in phase separation at super-enhancers and appear to concentrate the transcription apparatus at highly transcribed genes [18,19]. These biomolecular condensates appear to underlie or reinforce aspects of genome organization and function. Future work will reveal the molecular mechanisms and extent to which phase separation regulates compartmental domains, DNA loops, and the function of the genome.

3. Measuring 3D genome contacts with chromosome conformation capture

Current methods of mapping chromatin structure use high-throughput sequencing based assays to measure the frequency of long-

range DNA interactions that are captured via ligation due to their close physical proximity (Fig. 1D). These assays generally begin with fixing chromatin structures in place through covalent crosslinking with formaldehyde. After fragmentating chromatin, a key step relies on ligation of two DNA ends that are labeled with biotinylated nucleotides and are located in close spatial proximity inside the nucleus or in dilute conditions in a tube. Examples of these Chromosome Conformation Capture (3C) methods include HiC, HiChIP, Plac-seq, ChIA-PET, 5C, 4C, 3C, Capture-C, and many others [20–27]. While fundamentally similar in determining regions of the genome that are in close proximity in 3D space, these methods differ from one another in two major ways: 1) detection of all chromatin interactions versus the subset of interactions involving a protein of interest that has been targeted with an antibody, or 2) use of oligo-based methods to focus detection to a smaller and specific region(s) of the genome versus genome-wide interrogation. These assays have been comprehensively reviewed by others [21,24].

Despite the power of sequencing-based assays, these methods possess several limitations that prohibit a complete understanding of genome architecture. They sometimes fail to identify established architectural features revealed by microscopy-based approaches (e.g., nuclear bodies), suggesting they don't provide a comprehensive picture of genome organization. In addition, these assays generally require millions of cells, and therefore yield a population-averaged ensemble genome structure. Several groups have recently suggested that these averaged patterns are an emergent property that may not describe actual structure in any individual cell. Rather, microscopy-based approaches have recently shown that genome organization is remarkably heterogenous at the single cell level, with few examples of individual cells exhibiting the population averaged structures [28,29]. While approaches like single cell HiC aim to resolve this issue, it can be technically challenging and prohibitively expensive for labs to generate sufficiently deep sequencing data for large numbers of single cells [30,31]. Another challenge relates to potential biases introduced by proximity-based ligation assays. It has been hard to assess whether the act of capturing a pairwise interaction may preclude the capture of other potential interactions among a cluster of DNA fragments and, therefore, bias the data. For example, if site A becomes ligated to site B, then neither site A nor site B are available for ligation to site C, even though all three sites are in close physical proximity in the nucleus and otherwise compatible. Finally, it has been hard to ascertain biases in the capture of DNA interactions during traditional 3C methods. It is possible that certain DNA fragment characteristics might prevent or favor capture in a proximity-based ligation assay, including the spatial distance between two DNA molecules, their fragment lengths, rigidity, position on the crosslinked chromatin complex, interactions with RNA, etc. While it is hard to assess the significance of this lack of information, recently developed alternative methods should provide insight into this issue and overcome such challenges.

4. Recent developments in mapping genome structure

4.1. Sprite

A method called SPRITE (split-pool recognition of interactions by tag extension) was recently described for measuring genome organization in the nucleus [32] (Fig. 2A and B). SPRITE does not utilize proximity ligation, but instead uses 6 cycles of splitting crosslinked and fragmented chromatin into separate tubes, ligating short 6 bp tags onto the DNA fragments, and then pooling the chromatin together again. The 6 cycles of split/pool ligations yield groups of DNA fragments in a single chromatin complex that are all labeled with the same unique 36 bp tag, or barcode, which can be identified with paired end high-throughput sequencing. The odds of chromatin complexes that are not covalently linked acquiring the same 36 bp barcode is exceedingly small. This key feature is the major strength of the SPRITE method.

In this method, cells are serially crosslinked first with disuccinimidyl glutarate, then with formaldehyde. Subsequently, nuclei are isolated and mechanically sheared with a needle-tip sonicator followed by DNAse digestion to yield DNA fragments of 150–1000 bp size. These DNA-protein complexes are bound to NHS-activated magnetic beads (proteins are coupled via amide linkages) and the 6 cycles of splitting, ligating of 6 bp tags, and pooling are performed. The six rounds of ligation are reported to be efficient and the tags contain alternating 5′ and 3′ overhangs, which increases the efficiency of ligation and construction of the full barcode, while minimizing multimerization of individual tags. High-throughput sequencing yields reads that contain a barcode and genomic sequence. Computational analysis identifies and classifies DNA fragments based on their barcodes. The average fragment size in the library is 200–300 bp and ~1.5 billion reads are sequenced per mammalian sample. About 1/3 of the reads have a barcode that is not shared by any other reads; these correspond to linear DNA fragments that aren't informative of higher-order structure. The remaining 2/3 of the reads have barcodes that are shared with other reads, which represent a SPRITE cluster, and lead to the identification of many higher-order DNA interactions [32].

Similar to 3C technologies, SPRITE identifies general features of higher-order genome structure, including the previously described chromosome territories, compartmental domains, topologically associating domains, and DNA loops. Importantly, SPRITE is more sensitive than HiC at detecting long-range interactions. In addition, SPRITE identifies hubs of many interactions that are associated with nuclear bodies, an aspect of genome structure that has been lacking in tradition 3C datasets.

SPRITE has several advantages over traditional proximity ligation-based assays. First, it can detect multiple simultaneous interactions, where site A interacts with both site B and site C simultaneously in a single nucleus. Techniques like HiC can only detect pairwise interactions between sites A and B or B and C and cannot determine whether A and C were in close physical proximity in a single cell, if an A-C interaction is not directly captured. Therefore, SPRITE overcomes a major challenge inherent to proximity-ligation of pairs of DNA fragments. However, despite this advantage, the number of times a three-way A-B-C interaction is detected is still subject to population effects (e.g., a strong interaction found in 50% of cells in a population produces a signal similar to a weaker interaction found in 100% of the cells). Second, the multi-way interactions captured in SPRITE are particularly rich in long distance interactions and have, therefore, provided insight into DNA organization around nuclear bodies and other structural features inside the nucleus. Capturing a more diverse array of interacting DNA fragments in SPRITE suggests that proximity-based ligation assays have limited sensitivity that has prevented a complete understanding of higher-order nuclear organization. Third, the frequency of SPRITE contacts is strongly correlated with 3D spatial distances measured by DNA FISH. This is both a validation of the SPRITE assay and its ability to recapitulate spatial information, as well as an advantage over proximity-ligation based assays which are limited by inherent features of DNA fragments and the capture of pairwise interactions.

Perhaps the most exciting advantage of the SPRITE protocol is that it can be adapted to capture interactions between DNA and RNA molecules by employing an RNA-specific adaptor and a single-stranded RNA ligase. This novel ability has revealed new insight about the relationship between RNA molecules and DNA structure at two RNA-rich nuclear bodies: nuclear speckles and the nucleolus. Quinodoz et al. report the majority of interactions between chromosomes segregate into one of two distinct hubs that do not overlap [32]. One hub is associated with active transcription, which was determined to correspond to nuclear speckles, sites of mRNA processing and splicing. The second hub is correlated with inactive transcription and is close to rDNA regions of the genome. By mapping RNA-DNA interactions with SPRITE, the authors showed that rRNA is in physical proximity to DNA in the inactive hub. This inactive hub is in contact with the nucleolus, the site long known to be important for rRNA transcription and processing.

These results suggest that the genome is largely partitioned into two hubs that form at either the nucleolus or nuclear speckles, depending on the level of RNA polymerase II transcription occurring nearby. While individual genes can contradict this strong trend, it does appear to be a global feature of genome organization inside the nucleus.

The SPRITE method provides a powerful new tool for future studies of large-scale spatial organization inside the nucleus. The novel ability to map RNA-DNA interactions inside the nucleus allows for deeper investigation of various biological problems, including the emerging role of membrane-less compartments or phase-separated liquid droplets that facilitate essential biochemical and biophysical processes in a localized environment. While the initial SPRITE method uses commercially available NHS-activated magnetic beads that nonspecifically bind protein, in the future it could be possible to substitute antibody coated magnetic beads in order to focus on the interactions mediated by a specific protein of interest. This makes SPRITE a tool of choice for studying the role of lncRNAs and eRNAs made at enhancer sequences in mediating cooperative DNA interactions and genome structure important for transcriptional control. Other prospective studies include investigating whether SPRITE can be modified to work in a nucleus. Previous studies have shown improved data quality and reproducibility for in situ rather than in-dilution ligation in HiC [33,34]. Finally, it is worth mentioning another new technique that, like SPRITE, also aims to resolve several issues with traditional 3C. Genome Architectural Mapping (GAM), involves collecting thin cryosections of a nucleus and sequencing to identify DNA interactions in that slice [35]. While the technical and analytical methods of this approach are different from HiC and SPRITE, GAM captures multi-way interactions from small numbers of cells without the need for ligation.

4.2. Trac-looping

Trac-looping (transposase-mediated analysis of chromatin looping) is a newly described method for measuring simultaneous DNA interactions between regions of accessible chromatin [36] (Fig. 3). Trac-looping does not require chromatin fragmentation or proximity-based ligation since it relies on the activity of a modified Tn5 transposase to cleave DNA and label the ends with a specific DNA sequence (i.e., “proximity-based transposition”).

In the Trac-looping assay a Tn5 transposase, Tnp, is expressed and purified. The protein is then mixed with a pair of annealed oligos to create functional transposase complexes. In this method, the double stranded DNA, termed the “bivalent ME linker”, consists of two 19-bp mosaic end sequences separated by a short 30-bp linker. Since a dimer of Tn5 forms at each mosaic end sequence, the bivalent ME linker contains a tetrameric complex. When these tetramers are incubated with formaldehyde-crosslinked chromatin, transposition into the genome can occur in *cis* or in *trans*. These two possibilities can be distinguished by mapping the sequences that flank the inserted DNA. *Cis* insertion informs on chromatin accessibility and is similar to ATAC-seq data. Integration of the tetramer in *trans* results in bridging of two distal sites, yielding information on long-range interactions. The next step is enzymatic fragmentation of DNA followed by capture of DNA fragments containing the transposed sequence. The fragments are circularized, amplified and high-throughput sequencing is performed.

A Trac-looping dataset typically contains 50–60% *cis* interactions that are < 150 bp in size, which reflect the accessibility of chromatin. Around 20–30% of paired-end tags (PETs) are from 150 bp to 1 kb which provide information on secondary chromatin structure. Another 15% of PETs range from 1 kb to 200 kb and these DNA fragments are enriched for interactions between *cis*-regulatory elements such as enhancers and promoters. About 5% of PETs span a distance greater than 200 kb and reflect higher-order genome organization [36].

Trac-looping identifies established features of genome organization. The long distance (> 200 kb) PETs reveal A/B compartmental domains and Cohesin and CTCF-mediated DNA loops. The short distance PETs

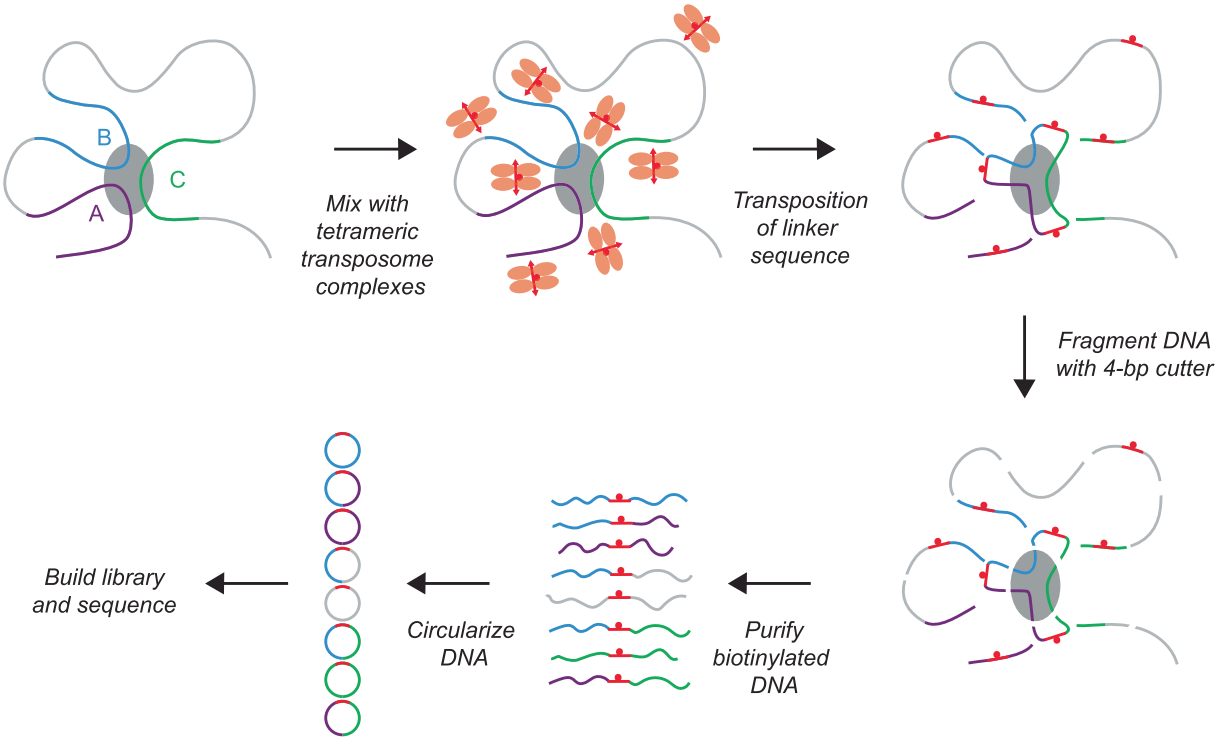

Fig. 3. Schematic of Trac-looping protocol. Crosslinked chromatin is mixed with assembled tetrameric transposome complexes to insert defined DNA sequences into genomic sites. Insertion into a DNA site in *cis* tends to occur in open chromatin and is used to measure accessibility while insertion in *trans* links two different DNA molecules together based on their proximity revealing information about 3D DNA organization. Chromatin is enzymatically fragmented, DNA is purified by affinity capture, and a ligation creates circularized molecules. Finally, a library is built and sequenced. This approach is a ligation-free method of capturing DNA interactions between regions of accessible chromatin.

(< 150 bp) recapitulate chromatin accessibility maps generated by ATAC-seq. Importantly, the Trac-looping method efficiently detects dynamic enhancer-promotor interactions in two model systems: resting versus stimultated CD4 + T cells and recruitment of the chromatin effector dCas9-KRAB to several enhancers of the *IL2RA* gene [36,37]. Lai et al. note that this method identifies 40–70% of active chromatin marked by histone modifications H3K27ac and H3K4me1/2/3, however it does not efficiently capture inactive chromatin since only 3–5% of H3K27me3 and H3K9me3 modified histones are captured [36]. Enrichment for active chromatin is likely due to the preferential integration of Tn5 transposase into sites of open chromatin.

Trac-looping has multiple advantages over traditional assays for measuring DNA interactions. First, it does not require prior chromatin fragmentation by mechanical or enzymatic means. Second, it does not require proximity-based ligation, but rather uses a Tn5 transposase enzyme to cut and insert a short sequence at accessible DNA sites. Third, it simultaneously provides information about DNA interactions and chromatin accessibility, which other methods do not. A potential disadvantage of Trac-looping arises from a preference for the Tn5 transposase to cut open chromatin rather than closed chromatin. Because of this bias, Trac-looping does not provide information on DNA loops unless the interacting sites are both open. Consequently, the dynamic loss of chromatin accessibility, in comparisons between two cell states, will result in loss of a detectable loop, regardless of whether the loop itself is dynamic. In the future, different length linkers can be tested for their ability to enhance capture of longer-range DNA interactions. It is also conceivable that bead-based selection of DNA fragment sizes can be used to enrich for a targeted subset of DNA interactions. Commercially available transposases would be a convenient tool for researchers that would allow for mixing with different DNA linkers to create custom transposome complexes. Trac-looping allows for detection of interactions between open chromatin regions and will be a powerful tool for future study of the relationship between chromatin accessibility, DNA interactions, and transcriptional regulation.

5. Conclusions

High-throughput sequencing has become the method of choice for mapping 3D genome organization. SPRITE allows for mapping multiple simultaneous DNA interactions in a single nucleus. It also makes possible the detection of RNA-DNA interactions. Finally, it enables mapping of spatial distances, similar to microscopy-based methods. The method can be modified to allow for study of protein-RNA and protein-DNA interactions with the use of barcoded antibodies. This makes SPRITE an especially attractive assay for studying the transcriptional regulation of genes, the role of RNA in organizing membraneless organelles, and the function of microRNAs, lncRNAs, eRNAs, and more in the context of genome organization. Because of its advantages, we predict that SPRITE could overtake the use of HiC in the future. Trac-looping enables the simultaneous detection of chromatin accessibility and DNA interactions. By employing a Tn5 transposase, it avoids common sources of variability and generates high-resolution information on DNA interactions. The strengths of the Trac-looping assay make it a top choice for studies of enhancer function in gene expression, as well as interactions in the open chromatin landscape.

While SPRITE and Trac-looping don't fully resolve all questions about single cell vs. population effects, they do address many limitations of Chromosome Conformation Capture methods. These assays represent important advances in the mapping of 3D genome architecture and will enable a more complete understanding of how the genome is structured and operates inside the nucleus. Future technical refinements to the Trac-looping and SPRITE protocols (crosslinking conditions, linker length, etc.) may lead to capture of even more diverse DNA interactions, and move the field closer to achieving the goal of a comprehensive view of genome organization.

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