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# 1 Understanding 3D genome organization by multidisciplinary methods

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## 6 7 8 **Abstract**

9 Understanding how chromatin is folded in the nucleus is fundamental to understanding its function. Although 3D  
10 genome organization has been historically difficult to study owing to lack of relevant methodologies, major  
11 technological breakthroughs in genome-wide mapping of chromatin contacts and advances in imaging technologies  
12 in the 21<sup>st</sup> century considerably improved our understanding of chromosome conformation and nuclear architecture.  
13 In this Review, we discuss methods of 3D genome organization analysis, including **sequencing-based techniques**,  
14 such as Hi-C and its derivatives, micro-C, DamID and others; **microscopy-based techniques**, such as super-resolution  
15 imaging coupled with fluorescent *in situ* hybridization (FISH), multiplex FISH, *in situ* genome sequencing and live  
16 microscopy methods; and **computational and modeling approaches**. We describe the most commonly used  
17 techniques and their contribution to our current knowledge of nuclear architecture and, finally, we provide a  
18 perspective on up-and-coming methods that open possibilities for future major discoveries.

## 19 20 Introduction

21 Euchromatin, heterochromatin and the hypothesis of individual-chromosome territories in the nucleus have been  
22 observed and suggested already at the end of the 19<sup>th</sup> and the beginning of the 20<sup>th</sup> century using light microscopy  
23 and chromatin dyes <sup>1-3</sup>. However, by the middle of 20<sup>th</sup> century, the chromosome territories hypothesis was largely  
24 abandoned as conventional electron microscopy failed to confirm it. It was not until the 1980s and the development  
25 of a novel imaging technique named fluorescence in-situ hybridization (FISH), that the chromosome territories  
26 hypothesis was finally validated, thereby instigating the study of nuclear architecture as we know it today <sup>4-6</sup>. The  
27 FISH method demonstrated the existence of chromosome territories and chromosome intermingling at territory  
28 edges, and also indicated that chromosomal regions rich in active genes largely reside at the nuclear interior,  
29 whereas chromosomal regions rich in inactive genes largely reside at the nuclear periphery <sup>7-16</sup>.

30 About two decades later, completion of sequencing of the human genome and the subsequent genome-wide  
31 characterization of genetic variations and epigenetic transcription regulation propelled the study of nuclear  
32 architecture into a new era. One breakthrough was the development of new techniques based on the principles of  
33 ligation of linearly-distal genomic regions that come into 3D spatial proximity in order to map genome organization  
34 and, at the same time, assay the functionality of this 3D organization <sup>17,18</sup>. However, despite the tremendous  
35 advancements made in this relative short time, the full complexity of the biophysical principles underlying the strong

36 3D compaction of the long linear genomic DNA into a micrometer-sized nucleus remain to be elucidated.  
37 Furthermore, we do not know the rules by which the structures imposed by general biophysical laws can be modified  
38 at specific genes in order to drive changes in gene expression programs that underlie cell fate and plasticity. These  
39 major challenges stimulate methodological improvements and invention of new experimental techniques and  
40 modeling approaches. On the other hand, fast and numerous technical developments generate confusion in the field  
41 of genome organization research, since it is unclear what information can each method provide, and the scarce  
42 comparison of concurrent methods hampers progress. Given the technological challenges and limitations of each of  
43 technology, the multiscale nature of genome organization and the multifaceted regulation of genome function, only  
44 the deployment of the full discourse of experimental and theoretical genome analysis approaches will allow us to  
45 reach a complete appreciation of genome function and the capacity to harness it in order to deliver not only  
46 fundamental knowledge but also valuable biomedical applications.  
47 In this Review, we discuss techniques for high-throughput chromatin contacts analysis and highly multiplexed, super-  
48 resolution and live-imaging methods. The applicability of these techniques is intimately linked with computational  
49 tools, including machine learning and mathematical modeling based on **first principles** or driven by quantitative data.  
50 We discuss relevant technological progress, provide a view of the current state of 3D genome organization research,  
51 and discuss promising future developments.

## 52 **Studying the multilayered 3D genome**

53 The idea of the nucleus as a highly organized organelle existed for over a century<sup>1-3</sup>. However, so far, we grasp only  
54 a part of the principles that govern nuclear organization, and the emergence of new evidence is tightly connected  
55 with the development of new methods.

56 A major breakthrough in chromatin biology was the establishment of chromosome conformation capture (3C) — a  
57 nuclear ligation assay in conjunction with PCR, which marked the beginning of the era of high-throughput next-  
58 generation sequencing-based techniques for the investigation of chromosome conformation<sup>17,18</sup>. Indeed, a series  
59 of 3C derivatives (from here on defined as C-based techniques) were developed to assay contact frequency between  
60 multiple genomic loci, including circular 3C (4C)<sup>19</sup>, which measures interaction frequencies of one locus with many  
61 loci ('one-vs-many'); many-vs-many assays (3C carbon copy (5C)<sup>20</sup>, Capture-C<sup>21-25</sup>, Capture-Hi-C<sup>26</sup>, etc.) and  
62 genome-wide, all-vs-all assays<sup>27,28</sup> (Hi-C) (Box 1)<sup>29</sup>. Progressively, these techniques were tweaked to allow for  
63 enrichment of specific contacts driven by proteins of interest (many-vs-all), including chromatin immunoprecipitation  
64 (ChIP-loop<sup>30</sup>), chromatin interaction analysis with paired-end tag (ChIA-PET<sup>31</sup>), HiChIP<sup>32</sup> and proximity ligation  
65 assisted ChIP-seq (PLAC-seq<sup>33</sup>); or of contacts focused on selected genomic locations (Capture-C<sup>21-25</sup>, Capture-Hi-C  
66 <sup>26</sup>)<sup>29</sup>

67 Simultaneously with the development of the C-based techniques, ligation-independent techniques were invented  
68 to assay not only chromosome conformation in general, but also the nuclear position of chromatin contacts  
69 (tyramide signal amplification (TSA), DNA adenine methyltransferase identification (DamID), split-pool recognition  
70 of interactions by tag extension (SPRITE)) and **multi-way contacts** (SPRITE and genome architecture mapping (GAM)),  
71 which are not assayed effectively using ligation-based techniques<sup>34–42</sup>. Finally, the recent advancement of super-  
72 resolution microscopy and imaging techniques allowed us to investigate chromatin conformation of single cells at  
73 extremely high resolution and at a higher throughput than ever before<sup>12,14,15,43–48</sup>. In addition to improvements in  
74 spatial resolution, live-imaging in combination with genome-engineering using CRISPR–Cas9 systems facilitated and  
75 improved the study of chromatin-contact dynamics<sup>49–51</sup>.

76 Owing to these methodological and technological advancements, it is not surprising that the past decade has  
77 provided major revelations in 3D genome organization and function. Most notably is the finding that chromosomes  
78 in interphase predominantly fold into two compartments, A and B, which respectively consist of predominantly  
79 gene-active and gene-inactive regions<sup>27</sup> (Figure 1). Furthermore, parts of compartments, from the same or different  
80 chromosomes, can come together and create hubs, which are connected by multiple chromatin interactions, thereby  
81 sharing a common function (for example, gene repression) and coalescing around different nuclear bodies such as  
82 **nuclear speckles**<sup>35,37,52</sup>. On a scale below the compartments, chromatin interactions were found to be enriched  
83 within domains of 100 kb to 1 Mb in length termed topologically associating domains (TADs); these partially insulated  
84 domains are subdivided into smaller chromatin nanodomains (CNDs)<sup>43,53–57</sup> (Figure 1). Both of these layers of  
85 organization — compartments and TADs/CNDs — were confirmed to be genomic features present across cell lines  
86 and species, but the principles that govern their folding are just beginning to be elucidated<sup>58–66</sup>. Chromatin-loop  
87 extrusion has been shown to be one mechanism responsible for folding at Mb scale. In interphase it is mediated by  
88 cohesin complex which can be blocked by CCCTC-binding factor (CTCF) bound to sequence motifs in convergent  
89 orientation, thereby demarcating TAD boundaries<sup>58,59,72–75,61,62,64,67–71</sup> (Figure 1). Importantly, these features are not  
90 only of structural nature, but are functional as well, as compartments are quite homogeneously comprised of gene-  
91 active or gene-inactive regions and TADs can facilitate the formation of enhancer–promoter contacts within their  
92 borders (Box 2; Figure 1). Although it is not entirely clear what the relationship is between TAD boundaries, insulation  
93 and disease, structural variations perturbing TAD boundaries and changes in CTCF binding and insulation can alter  
94 gene expression and lead to developmental defects and disease<sup>76–84</sup> (Box 2). These exemplary findings clearly  
95 demonstrate the importance of method development and choice in studying 3D genome organization. Below, we  
96 discuss established and more recently-developed methods in detail.

## 97 **2 Sequencing-based techniques**

98 The most common sequencing-based approach to assay chromatin architecture involves crosslinking of spatially  
99 proximal chromatin fragments followed by their isolation and sequencing, which is then used as a proxy to estimate



100 contact frequency. Some sequencing-based techniques allow the investigation of chromatin conformation genome-  
101 wide (non-enrichment methods), whereas others depend on isolation and identification of a subset of contacts  
102 (enrichment methods). Another important distinction is between methods based on ligation of formaldehyde cross-  
103 linked chromatin fragments (C-based) and methods that do not involve ligation of cross-linked fragments (non-C-  
104 based).

## 105 **2.1 Non-enrichment methods**

106 Non-enrichment methods assay chromatin conformation at once across entire genomes. The first techniques used  
107 ligation to retain together spatially proximal fragments prior to their isolation and sequencing. Subsequently,  
108 ligation-free techniques have been developed as well. Both types of approaches capture the majority of 3D features,  
109 but they do not deliver identical information due to their inherent technical differences.

### 110 **C-based methods: Hi-C and Micro-C**

111 Hi-C is frequently used to identify 3D chromatin contacts genome-wide<sup>27,85</sup> (Figure 2). The original Hi-C protocol  
112 included dilution during proximity-ligation, a step introduced originally in 3C and 4C in order to favor ligation of  
113 intramolecular chromatin contacts to reduce artifacts. However, this dilution was not very effective, as it was shown  
114 that around 60% of contacts originate from inter-chromosomal interactions<sup>85-87</sup>. This problem was circumvented by  
115 the omission of the SDS treatment just before ligation, which allowed permeabilization of the nuclear membrane  
116 and thus chromatin digestion and ligation *in situ*<sup>85,86</sup>. This modification is justified by the fact that ligation occurs in  
117 fixed nuclei, where molecular diffusion is virtually absent, and by the fact that the cross-linking reaction occurs  
118 between reactive moieties that are located in the nanometer range. Therefore, *in situ* ligation enabled a more  
119 efficient capture of true contacts, thereby delivering higher resolution for the same sequencing depth. After this  
120 methodological improvement, the omission of SDS treatment was adopted in virtually all subsequent C-based  
121 techniques. *In situ* chromatin processing allowed further development of single-cell Hi-C, which was the first  
122 sequencing-based single-cell chromatin analysis technique to be successfully established<sup>87</sup>.

123 Although Hi-C is suitable for the detection of compartments and TADs, its resolution is intrinsically linked with the  
124 use of restriction enzymes and to sequencing depth. Therefore, even if Hi-C libraries are sequenced by the billions  
125 of reads, the unbiased detection of local interactions, like enhancer–promoter contacts in the sub-TAD range, largely  
126 depends on the distribution of restriction sites, which in turn depends on the underlying sequence composition and  
127 thus is not uniform across the genome. This issue was first addressed concomitantly in two techniques, DNase Hi-C  
128 and Micro-C, both based on chromatin fragmentation without using restriction enzymes<sup>88-91</sup>. Micro-C introduced  
129 double cross-linking and replaced the restriction enzymes used in Hi-C with micrococcal nuclease digestion<sup>91-94</sup>  
130 (Figure 2). This produces a fairly uniform fragmentation down to the nucleosome level, which increases local  
131 resolution. In addition, Micro-C (and theoretically DNase Hi-C) also retains information on nucleosome positioning,  
132 which can be jointly analyzed with chromatin contact information from a single data set. However, although this  
133 technique is a promising improvement for the study of local chromatin topology, according to a recent preprint

134 article, it is less efficient in capturing long-distance and inter-chromosomal contacts compared with Hi-C <sup>95</sup>.  
135 Therefore, careful framing of the research questions is needed to accurately select between different non-  
136 enrichment methods.

### 137 **Non-C-based methods: SPRITE and GAM**

138 Although C-based methods have been extremely successful; they have intrinsic limitations and potential sources of  
139 bias. First, like many other chromatin analysis methods, they rely on mild formaldehyde cross-linking, which is  
140 powerful but potentially limited in capturing interactions of proteins with short residence time on chromatin or  
141 containing a low fraction of amino acids that can be crosslinked<sup>96,97</sup>. Second, these methods require ligation of  
142 genomic fragments prior to sequencing, a procedure that is only partially efficient. Third, they depend on short  
143 paired-end sequencing, which provides information only on bipartite interactions, whereas multipartite *in vivo*  
144 chromatin interactions escape the analysis.

145 Several methods that do not rely on ligation of chromatin fragments allow the detection of dual or multiple  
146 interactions. In SPRITE, crosslinked nuclei are isolated and fragmented, then individual crosslinked pieces of  
147 chromatin are uniquely barcoded using multiple cycles of a split-and-pool strategy; after high-throughput  
148 sequencing, reads carrying the same combination of barcodes represent genomic sites that are a part of the same  
149 crosslinked cluster (Figure 3). Since the method does not select for specific sequence sizes, it yields bipartite as well  
150 as multipartite contacts. SPRITE has been further adapted to facilitate the capture of DNA–DNA, RNA–DNA and RNA–  
151 RNA interactions, thereby allowing to determine whether RNAs of interest are associated with a subset of genome  
152 interactions and what the relationship of the RNA is to nuclear landmarks <sup>37</sup>. Finally, a single cell version of SPRITE  
153 allows the study of multi-way contacts in individual cells <sup>36</sup>. Future work applying this method to a broad range of  
154 cell types and analyzing two-way and multi-way contacts to a deeper level is required in order to fully exploit its  
155 advantages and appreciate its limitations <sup>52</sup>. GAM is an orthogonal method that can also provide frequencies of  
156 multivalent interactions<sup>35</sup>. In GAM, fixed cells are embedded in sucrose, frozen and cryo-sectioned, and the DNA is  
157 extracted and sequenced from each section<sup>41,98</sup> (Figure 3). Loci that are closer to each other in the nuclear space are  
158 co-sequenced more frequently than distant loci. Since sections are taken from multiple nuclei sliced at random  
159 orientations, the co-segregation of all possible pairs of loci among a large collection of nuclear section profiles is  
160 used to generate a matrix of inferred locus proximities. GAM matrices produce maps similar to Hi-C maps but require  
161 fewer cells — few hundred nuclei produce maps that approximate those obtained from large populations of cells in  
162 Hi-C. Like SPRITE, GAM can identify multiple interactions, thereby enabling the direct study of multivalent enhancer-  
163 promoter interactions and of higher-order chromatin structures.

## 164 **2.2 Enrichment methods**

165 The above-described techniques detect chromatin contacts present in the nucleus irrespective of genomic location,  
166 nuclear topography or the underlying protein binding. Yet, to fine-scale map chromatin folding and understand some  
167 of its functional aspects, it is necessary to detect specific contacts using enrichment approaches, thereby amplifying

168 the contacts signal in a specific genomic region of interest (Capture-C, capture Hi-C (cHi-C)) or for a specific protein  
169 of interest (ChIA-PET, ChIP-loop, HiChIP, PLAC-seq, DamID, DamC, tyramide signal amplification (TSA-  
170 seq))<sup>21,22,38,39,42,99–101,23–26,30,32–34</sup> (Figure 2).

#### 171 **C-based methods: HiChIP, Capture-C and cHi-C**

172 The first C-based enrichment-dependent techniques to be developed combined proximity-ligation with ChIP, for  
173 example the low-throughput ChIP-loop and later, with higher-throughput, ChIA-PET, which was subsequently  
174 improved to allow for more efficient mapping and detection of single-nucleotide polymorphism<sup>30,31,102</sup>. However,  
175 proximity-ligation in these techniques was performed in non-optimal conditions, with ChIP and sonication done  
176 preceding ligation, possibly affecting the accuracy of captured interactomes. These shortcomings were addressed in  
177 the next generation of protein-enrichment C-based techniques, HiChIP and PLAC-seq, in which the C-technique is  
178 performed first and *in situ*, thereby creating an optimal environment for proximity-ligation, followed by protein  
179 enrichment<sup>32,33,102</sup>. HiChIP and PLAC-seq allow the identification of a subset of interactions forming in association  
180 with specific protein binding, but can only be used as a proxy, not a definitive proof, that a protein of interest  
181 mediates the captured chromatin contacts. In addition to protein-mediated enrichment, chromatin contacts can also  
182 be enriched for a specific genomic location(s) using techniques such as Capture-C and cHi-C<sup>21–26</sup> (Figure 2). In these  
183 techniques, a Hi-C library is first generated and then hybridized to specifically designed baits (RNA or DNA)  
184 corresponding to either one large region (several Mbs) of interest in the case of cHi-C, or to multiple specific sites in  
185 the genome (for example, a collection of specific gene promoters) in the case of Capture-C. This allows mapping of  
186 contacts in fine detail, which would normally require 20–50 fold more sequencing without enrichment<sup>26,76,103</sup>. In the  
187 future, capture approaches could be combined with other techniques to adjust them for specific needs.

#### 188 **Non-C-based methods: TSA and DamID**

189 Ligation-independent techniques such as TSA-Seq and DamID can also enrich for contacts associated with specific  
190 proteins and map the nuclear topology<sup>34,38,39,42,100</sup>. TSA-Seq relies on **tyramide signal amplification**<sup>34,42</sup>. Cells are first  
191 crosslinked, stained with a primary antibody against a protein of interest and then with a horseradish peroxidase  
192 (HRP)-conjugated secondary antibody<sup>34,42</sup>. HRP catalyzes the formation of tyramide–biotin free radicals, which  
193 diffuse and covalently link to nearby proteins, DNA and RNA. The biotin moiety can be used to stain nuclei as well as  
194 purify and sequence the associated DNA. Since the amount of tyramide–biotin signal decreases with increasing  
195 distance from the antibody localization source, TSA-Seq read frequencies can be transformed into 3D distances from  
196 nuclear landmarks of interest upon appropriate calibration. A second and orthogonal, cross-linking-independent  
197 technique is DamID, which involves tethering *Escherichia coli* DNA adenine methyltransferase (Dam) to a chromatin  
198 protein; the Dam moiety methylates adenines at GATC consensus DNA sites surrounding the chromatin protein of  
199 interest<sup>38</sup>. Application of DamID to proteins with distinct nuclear compartmentalization allows identifying genomic  
200 domains associated to nuclear landmarks, such as the **lamina associated domains (LADs)**<sup>40</sup>. More recent applications  
201 of DamID also enable identifying LADs in single cells and to simultaneously quantify protein–DNA contacts and RNA  
202 expression in the same cell<sup>100,104</sup>. Similar to DamID, DamC has been established as a cross-linking and ligation free

203 technique that can replace 4C; in DamC, a fusion protein of Dam and reverse tetracycline receptor (rTetR) is recruited  
204 to Tet operator sequences (TetOs) ectopically inserted at a genomic site of interest<sup>101</sup>. Methylated DNA is then  
205 detected by high-throughput sequencing, and scoring of the methylated Dam target sites around the TetOs allows  
206 quantifying chromatin contacts. DamC may be of great interest for low cell number or tissue-specific applications.

### 207 3 Super-resolution microscopy methods

208 In DNA FISH, DNA probes are hybridized to cognate genomic regions of interest and visualized by fluorescence  
209 microscopy, which allows measuring localization, shape and inter-probe distances<sup>4-6</sup>. However, the study of  
210 chromosome conformation and of individual chromatin contacts under the microscope has been limited by the low  
211 number of loci that can be probed simultaneously owing to the low number of available independent fluorescence  
212 channels, and by the limited spatial resolution of traditional light microscopes. These limitations have been removed  
213 thanks to major technological advancements in light microscopy applications.

214 Light emitted by any point source is diffracted such that the point will appear in an image as a so-called **airy**  
215 **diffraction pattern**, the size of which is proportional to the wavelength. In practice, this property of light, together  
216 with aberrations of optical systems and light scattering, limits the resolution (the minimal distance at which two  
217 signals can be distinguished), following a formula derived by the physicist Ernst Abbe, to approximately 250 nm in  
218 the x and y axes (lateral resolution) and 600 nm in the z axis (axial resolution), even when using the best confocal  
219 microscopes and image processing software. For decades, the resolution of light microscopy was believed to be  
220 intrinsically limited by diffraction, but imaging technologies have progressed at a remarkable speed, allowing the  
221 detection of increasing number of nuclear components at a spatial resolution surpassing the Abbe limit. These  
222 methods, collectively called super-resolution microscopy, increase spatial resolution mainly in three different ways,  
223 and here we discuss their applications in 3D genome organization research (Figure 4a). Detailed descriptions of these  
224 methods can be found in more specialized microscopy reviews<sup>105,106</sup>.

225 Structured illumination microscopy (SIM) is an approach to super-resolution microscopy that increases resolution by  
226 a factor of two in each axis by exploiting a non-uniform illumination pattern: the sample is serially exposed to light  
227 from different angles and different axial phases<sup>107-109</sup> (Figure 4a). This illumination pattern interferes with the  
228 sample in a manner that can be conveniently analyzed in the Fourier mathematical space to improve resolution.  
229 Despite its complexity, the advent of commercially available SIM microscopes and software and the fact that the  
230 technology is compatible with standard fluorophores, labeling procedures and multi-color imaging has stimulated  
231 the widespread application of SIM. SIM has allowed to assess unprecedented details of chromatin and nuclear  
232 organization, such as the relations between chromatin and the nuclear periphery or the physical structure of TADs  
233 in single cells<sup>43,53,110</sup>. In particular, the analysis of mammalian TADs using SIM has revealed that TADs are subdivided  
234 into the smaller CNDs: as CND boundaries vary from cell to cell, ensemble Hi-C experiments blur their identification,  
235 thereby illustrating the power of single-cell, super-resolution imaging to illuminate 3D genome organization features  
236 that are inaccessible to cell-population-based technologies<sup>57,111</sup>.

237 A second family of super-resolution microscopy methods is called single molecule localization microscopy (SMLM);  
238 it includes stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM)  
239 and fluorescence photo-activated localization microscopy (FPALM) <sup>112–114</sup>. All three methods use fluorophores that  
240 can be converted from a fluorescent (or activated) state to a dark (or inactivated) state (and vice versa) and rely on  
241 the stochastic excitation and detection of spatially separated single fluorophores. The spatial separation is achieved  
242 by making sure that only a small fraction of the total population of fluorophores in the sample can emit light, so that  
243 individual emitters do not overlap in a single imaging frame <sup>115</sup>. Sequential imaging using cycles of activation and  
244 inactivation of the fluorophores, followed by the precise localization of the center of emission of the individual  
245 fluorophores and on the superposition of all imaging cycles, generates super-resolution images that can reach a  
246 lateral resolution of 20 nm in biological samples ((3D images can be obtained using various methods)<sup>115</sup> (Figure 4a).  
247 The application of these methods has provided crucial insights into the fundamental folding of chromatin in the  
248 nucleus. Nucleosomes were shown to transiently interact to form clutches of various sizes interspersed with  
249 nucleosome-depleted regions <sup>116</sup>. At a higher scale of organization, nucleosomes were found to form CNDs, i.e.,  
250 aggregates of a diameter of ~160 nm, within which individual nucleosomes display highly correlated motion in live  
251 cells <sup>117</sup>. This organization suggests that CNDs may arise from a coordinated behaviour that might reflect multiple,  
252 dynamic nucleosome interactions, consistent with a recent analysis of fixed chromatin by SIM <sup>57,111</sup>. It will be  
253 interesting to study whether CNDs might regulate genome functions such as gene expression (in particular, the  
254 frequency of Enhancer-Promoter contacts) or DNA replication.

255 The third approach to super-resolution microscopy is stimulated emission depletion (STED). This technique uses a  
256 configuration similar to confocal microscopy, but with an additional laser called the depletion beam, which  
257 illuminates the sample in a donut shape that has zero intensity at the center of the excitation laser <sup>118,119</sup>. This  
258 illumination provokes the depletion of emissions in the periphery and only allows emission in the center, thereby  
259 generating a **sub-diffractive point spread function**. In most applications, STED reaches a lateral resolution of 30–50  
260 nm and high axial resolution can also be obtained (Figure 4a). The drawbacks of this method are its strong laser  
261 intensity and the requirement for specific fluorophores. A modified version called reversible saturable optical  
262 fluorescence transitions (RESOLFT) allows using lower beam intensities <sup>120</sup>. Importantly, SMLM and STED do not have  
263 a theoretical resolution limit and a combination of the two methods has achieved axial and lateral resolutions below  
264 3 nm in cells <sup>121</sup>. Therefore, imaging applications are quickly enabling to investigate chromatin and nuclear  
265 architecture at the macromolecular scale.

266 A remaining limitation of these super-resolution microscopy methods is throughput, as they are limited to using  
267 two or three colors, which restricts the number of loci that can be analyzed simultaneously. Recently, however,  
268 oligonucleotide-based FISH protocols called oligopaints were coupled with microfluidics to allow multiple cycles of  
269 hybridization<sup>122</sup> (Figure 4b, 4c). This conjunction of methods enabled sequential probe hybridization and assaying  
270 multiple different loci with high precision in the 3D nuclear space <sup>12,15,44–48,123</sup>. These techniques allowed obtaining  
271 distance distribution maps among all imaged points that recapitulate the maps obtained from Hi-C experiments and

272 provide 3D trajectories of chromatin molecules at high resolution and in thousands of cells, something which is  
273 unattainable even in single-cell Hi-C (Figure 4c, 4d). These techniques include Multiplex FISH imaging, which helps  
274 establish high resolution tracing of chromatin folding of megabase-size genomic domains by labeling several tens of  
275 locations in the genome simultaneously<sup>44,46,48,123</sup> optical reconstruction of chromatin architecture (ORCA)<sup>47</sup>; Hi-M  
276<sup>45</sup>, which is a multiplexed, sequential imaging approach; and oligopaint fluorescence *in situ* sequencing (OligoFISSEQ)  
277<sup>14</sup> (Figure 4b, 4c, 4d). Importantly, OligoFISSEQ is a method combining barcoded Oligopaint to *in situ* sequencing  
278 technology, which is used to read out the barcode carried by the oligos (Figure 4d). This makes OligoFISSEQ a  
279 powerful high-multiplexing technology<sup>14</sup>. *In situ* sequencing can also be coupled to Tn5 transposase-mediated  
280 random insertion of DNA-sequencing adapters into hundreds of positions of fixed genomic DNA. *In situ* amplification  
281 can then be used to insert unique molecular identifiers that are sequenced *in situ* prior to *ex situ* sequencing in order  
282 to identify the genomic region of Tn5 insertion. This allows obtaining the 3D location of hundreds of loci per cell, a  
283 powerful alternative to hybridization-based imaging methods<sup>16</sup>.

284 Currently, it is possible to simultaneously visualize thousands of DNA loci, hundreds of different RNA molecules  
285 and several proteins or histone modifications, thereby enabling high-throughput structure–function analyses in  
286 thousands of single cells and truly inaugurating the field of spatial imaging-based 3D genomics<sup>12,15</sup>. These light  
287 microscopy methods are accompanied by developments in electron microscopy, with electron-microscopy  
288 tomography allowing the study of chromatin at nanometer resolution<sup>124</sup>. Each of these methods has advantages  
289 and limitations. For instance, methods with very high spatial resolution are typically not optimal for the description  
290 of architectures of large domains owing to a slow acquisition process and to the intrinsic noise in the images that  
291 are obtained. However, advanced OligoSTORM imaging provides powerful information on 3D genome organization  
292 that is complementary to molecular techniques such as Hi-C, thereby enabling the investigation of genome  
293 architecture and function to a degree that was unthinkable a decade ago<sup>14</sup>.

#### 294 **4 Computational analysis and modelling**

295 Although the methods discussed above provided important insights into 3D genome organization and function, they  
296 are still limited in their ability to describe how the chromatin fiber folds in the 3D space of the nucleus and they  
297 cannot predict structural changes that would result from perturbations such as mutations in genes or in gene-  
298 regulatory components. Evaluating the impact of architecture on genome function remains even more inaccessible  
299 at present. All these limitations have stimulated computational analyses and the development of mathematical  
300 modelling which, in conjunction with experiments, might help achieve a quantitative and predictive understanding  
301 of chromosome architecture and function.

#### 302 4.1 Analysis of Hi-C data

303 The advent of Hi-C and related technologies has raised strong interest in the development of matching  
304 computational analysis tools, owing to the inherent complexity of Hi-C data. The achievable spatial resolution of Hi-  
305 C is affected by sequencing depth, library complexity and the DNA-cutting frequency of the enzyme used for  
306 chromatin fragmentation. Since the number of possible chromatin-fragment interactions is extremely high ( $> 10^{14}$  in  
307 the human genome, when using 4-base cutter restriction enzymes) and the sequencing depth of a typical experiment  
308 is limited, Hi-C matrices are sparse, that is, many entries in the matrices are 0, if they are not represented at the  
309 appropriate resolution. This makes it impossible to distinguish between genuine absence of contacts and absence  
310 of contacts owing to low sequencing depth. Furthermore, the different sizes of restriction fragments across the  
311 genome, differences in mappability between regions with high or low density of repetitive elements, and the decay  
312 of interaction frequencies with increase in genomic distance make Hi-C matrices typically very heterogeneous in  
313 terms of contacts at different genomic locations or across different distances. All these factors limit the resolution  
314 with which one can call contact regions or domain boundaries, and generate difficulties in defining the precise  
315 locations of compartments, TADs and chromatin loops. The first Hi-C study<sup>27</sup>, which used a 6-base cutter and  
316 achieved low sequencing depth, produced reliable matrices at the resolution of 100kb and identified the  
317 compartments. To this end, Hi-C matrices were normalized by genomic distance, converted to correlation matrices  
318 and subjected to principal component analysis, which distinguished the active (A\_ and inactive (B) compartment  
319 types. Later algorithms additionally applied clustering steps like Gaussian hidden Markov modelling to Hi-C maps  
320 based on much deeper sequencing, leading to further specification of epigenetic compartment signatures and more  
321 detailed stratification of the A compartment into two sub-compartments and B compartment into three sub-  
322 compartments<sup>85,125–129</sup>.

323 The computational identification of TADs required high resolution maps, which were published three years after the  
324 first low-resolution HiC experiment<sup>53–55,130</sup>. Although nowadays TAD calling is done routinely, there is no clear  
325 consensus method but rather numerous TAD callers that are based on different principles. Initial computational  
326 approaches such as insulation score and directionality index determined TAD boundaries by defining a one  
327 dimensional linear score of a bin-fractionated genome, where the bin of the local minima (for the insulation score)  
328 or the one between local minima and maxima (for the directionality index) would determine the boundary position  
329<sup>55,131,132</sup>. These approaches, however, could not inform on TAD hierarchy and missed identifying nested TADs.  
330 Subsequently, other computational approaches were developed to address this issue either by further developing  
331 the linear score approach (Matryoshka<sup>133</sup>), by clustering contact map data (ICFinder<sup>134</sup>, TADpole<sup>135</sup>) or by using  
332 graph theory-based algorithms that identify nested TADs as contact subnetworks connecting to form larger TADs  
333 (3DNetMod<sup>136</sup>). Furthermore, numerous other computational tools have been developed that combine these  
334 approaches with different efficiencies at different scales and resolutions<sup>137–140</sup>.

335 The third major feature of Hi-C data are chromatin contacts and loops, which, like TADs, became detectable as the  
336 data became more resolute. Specific chromatin contacts are defined as statistically significant increases in contact

337 detection in comparison with a general background model. This is the basis of Fit-Hi-C, a computational tool that  
338 assigns a statistical confidence to a contact by using random polymer modeling while accounting for known Hi-C  
339 biases such as genomic distance; an adapted version, HiC-DC, additionally accounts for sparsity and over-dispersion  
340 and yields a more conservative statistical significance estimate <sup>141,142</sup>. However, a locally enriched contact — a  
341 chromatin loop — might elude algorithms that use only a general background model to estimate statistical  
342 significance of enrichment. HiCCUPS, one of the first loop-dedicated algorithms, identifies a chromatin loop as the  
343 most enriched bin in comparison to its immediate neighborhood while using a high-resolution 5kb Hi-C data as an  
344 input <sup>85</sup>. This algorithm helped drive the discovery of a specific subtype of loops, CTCF loops, and contributed to the  
345 development of the loop-extrusion model, thereby demonstrating the importance of specialized algorithm  
346 development in order to grasp the full biological significance of the experimental data. In-depth reviews and  
347 websites have compiled and compared available compartment, TAD and loop callers <sup>139,143–147</sup>. The increasing  
348 robustness of primary computational tools to identify 3D genome features has led to a blooming of applications  
349 aimed to identify DNA sequences of biological significance linked with genomic regions in spatial proximity as  
350 mapped by Hi-C techniques. These applications have enabled associating genes with putative enhancers based on  
351 contact frequency, epigenomic and DNA features; identifying novel regulatory elements from genome-wide  
352 association studies; and assigning a potential role for short tandem repeats in genome organization <sup>148–155</sup>.  
353 Although these computational tools can provide precious information on genome structure and function, the cost  
354 of obtaining high-resolution Hi-C maps can become prohibitive, particularly when many experimental conditions  
355 need to be compared. In order to tackle this problem, a machine learning approach based on deep convolutional  
356 neural networks has been used to impute higher-coverage Hi-C maps from low-coverage data in order to increase  
357 the resolution with which loops or TAD borders can be defined <sup>156</sup>. This function is important in order to identify  
358 genomic features that might be involved in the regulation of these structures even if the data is not sufficiently  
359 resolute.

## 360 **4.2 3D chromatin modelling**

361 The computational tools discussed above provide new information about regulatory elements and their function,  
362 but they do not inform on 3D architecture. Three main types of modelling strategies have been used to infer 3D  
363 genome folding, although some methods can blur this classification as they have characteristics belonging to more  
364 than one type <sup>137,157–160</sup>. The first modelling strategy is the bottom-up approach of polymer modelling, which  
365 attempts to infer and understand chromosome architecture from first principles, typically modelling chromosomes  
366 as self-avoiding polymers moving in a confined space that represents the nucleus (Figure 5a). Applications aim to  
367 identify components regulating 3D folding, and thus they try to reduce the number of variable parameters describing  
368 the polymer behavior while maximizing the fit between experimental data, typically Hi-C contact matrices, and  
369 analogous matrices that are derived from measuring contacts in snapshots taken at given times after starting  
370 polymer motion simulations. This modelling strategy has been applied to rationalize the decay in contact frequencies



371 between chromatin regions as a function of the linear distance separating them<sup>161,162</sup>. More recently, these models  
372 have offered an explanation for the formation of domains of active and inactive chromatin, the generation of TADs  
373 through loop extrusion and the contribution of epigenetic features such as chromatin types to the formation  
374 of TADs and compartments<sup>58,67,128,163–166</sup>. The combined role of loop extrusion and of active and inactive  
375 compartments in the determination of global chromosome organization has also been studied<sup>68</sup>. Furthermore,  
376 in addition to intra-chromosomal contact frequencies, this strategy allowed the investigation of inter-  
377 chromosomal contacts<sup>167</sup>. A current limitation of these models is that they can typically reproduce and predict  
378 some, but not all the features of 3D chromosome folding and in particular, they usually do not perform equally  
379 well at different scales (loops, TADs, compartments, chromosome territories)<sup>137,138</sup>. This is partly due to the  
380 considerable computational time required for the iterative simulation processes that are involved in generating  
381 the models, and interesting ongoing developments involve accelerating computation<sup>168</sup>.

382 An alternative, physics-based bottom-up modelling strategy does not aim at minimizing the number  
383 of parameters to describe polymer behavior. Instead, it models chromosomal regions as polymers, in which  
384 each monomer represents a genomic region of fixed size and can interact with any other monomer with a  
385 specific energy. Each of the interaction energies can be adjusted until the configuration ensemble of the  
386 polymer produces a contact matrix that resembles the Hi-C data matrix. This approach allows searching for the  
387 monomers that have the most crucial role in driving the specific 3D configuration defining the genomic region  
388 of interest<sup>169–171</sup>. Furthermore, it can also be used to study the 3D path of the chromatin fiber in the resulting  
389 polymer models and to compare it with 3D data such as those provided by imaging methods in order to relate  
390 contacts to 3D architectural features of the region of interest<sup>43</sup>.

391  
392 The second type of modelling strategy is the top-down approach of restraint modelling, starting from data,  
393 which are often derived from Hi-C maps and sometimes integrated by maps of chromatin  
394 –nuclear lamina interactions, in order to infer the 3D architecture of genomic loci, entire chromosomes or the  
395 whole nucleus (Figure 5b). In some applications, the contact maps are used to set restraints that the models  
396 must satisfy in setting the 3D folding paths of chromatin fibers. The modelling result can be a consensus  
397 genome structure, or an ensemble of structures, which reflects the structural variability among cells or during  
398 time<sup>28,172–179</sup>. This type of modelling can also deliver information about chromatin folding dynamics, provided  
399 Hi-C data from time-course experiments are available. This is possible by interpolating the restraints through  
400 each of the time points<sup>180</sup>. Another interesting data-driven modelling uses a population deconvolution  
401 approach, in which Hi-C data are used to generate a large population of structures which, together, reproduce  
402 the experimental interaction patterns. This approach has been extended to incorporate chromatin–lamina  
403 interaction and imaging data<sup>46,181,182</sup>.

404 The recent progress in computation speed led to the deployment of another cohort of 3D chromosome folding  
405 prediction tools, which are based on machine learning methods that use epigenomic and chromosome

406 conformation information as input and display the predicted 3D architecture as output <sup>171,183–188</sup>. Such  
407 computational tools rely on input data obtained from several different cell lines to train their models and identify  
408 the minimum necessary signature to accurately predict an enhancer–promoter pair, promoter–promoter pair and  
409 CTCF loops as well as for contact quantification <sup>183,184,187,188</sup>. Recently, two tools, Akita and DeepC, used convolutional  
410 neural networks to predict 3D folding solely on the basis of DNA sequence <sup>185,186</sup>. These kinds of computational tools  
411 could become very important to enable making predictions from experimental samples, for which the full epigenome  
412 datasets are not available because of sample-quantity limitations, for example in the case of patient samples.

413

414 A third type of modelling strategy, which combines both top-down data driven models and bottom-up physical  
415 models has been described <sup>189</sup>. This model uses parameters derived entirely from a Hi-C experiment as input,  
416 but also factors in a polymer’s energy function like in bottom-up approaches. However, in this case the energy  
417 function is designed strictly from biological factors that have been demonstrated to have a role in 3D genome  
418 organization, which ensures biological relevance while simultaneously allowing for mechanical investigation.  
419 With monomers of 1 Mb in size, this model successfully reconstituted the radial positioning of entire  
420 chromosomes and uncovered previously unknown contributions of distinct biological processes (separation of  
421 A and B compartment, centromere clustering, inter-chromosomal contacts). However, the large monomer size  
422 limited resolution and prevented the capture of more local features such as sub-TAD or loop structures, but  
423 the currently available computational power could allow decreasing the monomer size and testing whether  
424 mechanistic insights into more local structures can be correctly modelled.

425 Improved algorithms and the ever-increasing computation power will soon allow modelling the dynamics of  
426 whole-genome folding at high spatial and temporal resolution, making computational methods crucial  
427 complementary tools to the experimental methods.

## 428 5 Emerging genome structure technologies

429 Many outstanding questions remain in the research of nuclear architecture, and it is therefore not surprising that  
430 new sequencing-based methods, microscopy-based methods and computational methods are continually being  
431 developed.

432 One outstanding question in the field is how to address single-cell variability while not compromising high-  
433 throughput. Sequencing-based techniques address this through the adaptation of C-based techniques and C-  
434 independent techniques for single cells <sup>36,87,190</sup>. The first of these single-cell adaptations was single-cell Hi-C (scHi-C),  
435 which revealed high inter-cell contact and TAD variability and indicated that TADs are highly stochastic domains <sup>87</sup>.  
436 This finding, however, put into question whether this major Hi-C feature, TADs, represents actual physical structures  
437 or reflects statistical average rather than a physical reality. Later, microscopy studies settled this controversy by  
438 showing that TADs do correspond with physical domains, but also that they have highly variable structures, clearly

439 emphasizing the need to focus on techniques that provide information on large numbers of single cells<sup>43,44,57</sup>. Indeed,  
440 an increasing number of chromatin analysis techniques are being developed into single-cell applications to address  
441 this issue and study chromatin conformation stochasticity and inter-cell variability<sup>36,190–193</sup>.

442 Another major goal pertaining to C-based methods, is to overcome the resolution limit and potential cross-linking  
443 bias. Although alternative fragmentation techniques (Micro-C, DNase-Hi-C) successfully deal with the resolution  
444 problem, every technique that relies on crosslinking is inherently biasing fragmentation towards open-chromatin  
445 regions<sup>88,91</sup>. A recently published method, Cap-C, approaches this problem by exchanging the standard  
446 formaldehyde crosslinking with **dendrimer crosslinking**. By using three differently sized molecules, Cap-C allows  
447 homogeneous crosslinking of open and closed chromatin, thereby achieving more uniform fragmentation and higher  
448 resolution<sup>88–91,194</sup>. Since this is a straightforward change to current C-based protocols, it has the potential to be  
449 widely implemented.

450 The question of how to obtain spatial coordinates of the chromatin in the nucleus in a high-throughput manner and  
451 how to integrate chromosome conformation data with the spatial position has been difficult to answer using  
452 sequencing-based techniques. Recently, two new methods to study nuclear topology and higher-order organization  
453 based on ligation-free methodology were published, genomic loci positioning by sequencing (GPSeq)<sup>195</sup>, and  
454 chromatin interaction analysis by droplet based genomic analysis (ChiA-Drop)<sup>192</sup>, which provides information on  
455 multivalent interactions (similar to SPRITE). In ChiA-Drop, cross-linked and fragmented chromatin is loaded onto a  
456 microfluidics device so that individual cross-linked molecules are partitioned into droplets that contain unique  
457 barcoding reagents (Figure 3). After pooling, high-throughput sequencing and identification of reads carrying the  
458 same barcodes, putative 3D interactions are identified. In *Drosophila melanogaster*, ChiA-Drop was performed using  
459 less than 10,000 cells and thus it could be suitable for analyzing rare cell types. Furthermore, the possibility to enrich  
460 for interactions that depend on specific proteins allows to infer the relative position of the interacting regions  
461 relative to nuclear bodies or landmarks<sup>192</sup>. GPSeq primarily focuses on the study of radial chromosomal positions in  
462 the nucleus by performing restriction enzyme digestion in a time course *in situ*, which allows the capture of the most  
463 nuclear-periphery-adjacent chromatin following short-term digestion, whereas the longer digestion times capture  
464 progressively more interior parts of the genome. However, to infer distances to the periphery correctly it is necessary  
465 to perform a YFISH (in which a Y-looking FISH adapter is ligated on the restriction enzyme overhang while the other  
466 side of the adapter interacts with FISH probes) coupled with super-resolution imaging in a time course. Using this  
467 approach, it is possible to investigate not only the radial position of chromosomes but also the radial positions of  
468 DNA replication, double-stranded DNA breaks (DSBs) and mutations<sup>195</sup>. Recently, a computational method called  
469 SPIN (spatial position inference of the nuclear genome) has been developed to predict genome-wide spatial  
470 positioning in the nucleus. The method integrates spatial multi-omics data including TSA-seq, DamID and Hi-C in a  
471 computational framework based on Hidden Markov random field to localize clusters of chromatin contacts relative  
472 to nuclear bodies such as nuclear speckles or the lamina<sup>196</sup>. This complementary tool of experimental and

473 computational multi-omics methods might provide the essential missing components in the nuclear organization  
474 research toolbox.

475 Finally, the last outstanding questions in the field we shall discuss are how to achieve higher spatial resolution in  
476 microscopy, and how to assay chromatin dynamics of individual genomic loci. In order to achieve spatial resolution,  
477 one has to be able to image beyond the **diffraction limit**. In addition to super resolution microscopy, several recent  
478 publications reported an alternative approach called expansion microscopy (ExM), in which the sample is embedded  
479 in a polyelectrolyte gel that expands 4–5 times when immersed in water<sup>197–199</sup>. ExM offers imaging of structures  
480 that are beyond the diffraction limit using conventional microscopy, and according to a recent preprint article, when  
481 combined with super-resolution microscopy, ExM achieved resolution of 5 nm<sup>198</sup>. Furthermore, ExM can be  
482 extremely powerful for spatially precise positioning of RNA species *in situ* and has been recently used in combination  
483 with FISSEQ technology to perform RNA *in situ* sequencing in an unbiased manner<sup>200</sup>. This technology  
484 conglomeration offers great promise for the future, as it combines different principles to achieve sub-diffractive  
485 resolution and multiplexing. One could easily imagine extending its applications to multiplex DNA FISH or *in situ*  
486 sequencing methods in order to analyze the traces of chromosomes, chromosome domains or individual loci at high  
487 resolution. However, it is important to keep in mind that sample expansion can alter the ultrastructure of the  
488 chromatin and it will be necessary to ensure that the structure remains preserved under standard conditions.

489 There are two major bottlenecks to studying chromatin contact dynamics of individual loci. First, in order to visualize  
490 such contacts, cells usually had to be subjected to heavy genome engineering to insert either lac-O or tet-O arrays  
491<sup>50,201–203</sup>. Second, the signal must be sufficiently strong in order to visualize individual loci. Multiple different methods  
492 have been developed to reach this goal<sup>49,50,204–207</sup>. Chimeric array of gRNA-oligo (CARGO) and CRISPR–Cas-mediated  
493 Live FISH are examples of two independently developed live-imaging techniques that addressed these limitations by  
494 using the CRISPR–Cas9 system. CARGO uses multiple guide RNAs (gRNAs) targeting nuclease-dead Cas9 (dCas9)-  
495 EGFP to certain genomic locus to achieve efficient fluorescence-signal amplification and circumvent the need for  
496 strenuous genome engineering<sup>49</sup> (Figure 6a). This method enabled the detection of *cis*-regulatory element mobility  
497 during cell differentiation in relation to their expression, and offers great promise as it is relatively simple<sup>49</sup>. Similarly,  
498 CRISPR–Cas-mediated Live FISH utilizes dCas9 to target a region of interest, but here the gRNAs are fluorescently  
499 labeled, thereby amplifying the signal more than fourfold<sup>206</sup> (Figure 6b). Furthermore, the use of catalytically active  
500 Cas9 together with dCas9 allowed the simultaneous visualization of DSBs and fluorescent DSB-repair proteins,  
501 practically creating a live Immuno-FISH<sup>206</sup>. Finally, Live FISH was further expanded by coupling the dCas9–gRNAs  
502 with the dCas13–gRNA system, thereby granting visualization of both DNA and nascent RNA transcripts in live cells  
503<sup>206,207</sup>. Together, these and similar dCas9-based techniques might become valuable for studying chromatin and  
504 transcription dynamics in live cells, and open venues for application ranging from basic science to diagnostics<sup>50,208</sup>.

505 **6 Future directions**

506 Genome architecture as a field of research has come a long way in a remarkably short period of time thanks to multi-  
507 disciplinarity that was driven by technological advancements. In this Review, we discussed major discoveries in  
508 chromatin conformation and nuclear topology through a technical prism. However, there is still so much we do not  
509 understand and that is not accessible to us owing to methodological shortcomings.

510 The next decade will likely see a continued expansion of imaging-driven techniques with a strong emphasis on  
511 multiplexing and on live microscopy, especially in conjunction with sub-diffractive resolution. As already now we see  
512 the implementation of Live FISH, further development and specialization of these methods could possibly help to  
513 study enhancer–promoter dynamics in respect to transcriptional output<sup>206,207</sup>. Furthermore, live-microscopy-based  
514 techniques could be well suited to study the kinetics of transcription factor binding to chromatin, a subject that is  
515 poorly understood. In addition to live microscopy, the throughput of the super-resolution FISH-based methods will  
516 likely further increase and help study inter-cell variability. However, these predictions are based on the premise that  
517 the hardware necessary for these techniques will become more available and affordable.

518 Regarding sequencing-based methods, there is already a strong tendency to use different methods on the same  
519 sample, for example nucleosome occupancy and Hi-C (Micro-C, DNase-Hi-C) or bisulfite sequencing and Hi-C, in order  
520 to obtain different types of information from the same sample, but also to expand the amount of information that  
521 can be extracted from limited material such as patient or rare-cell samples<sup>91–93,191</sup>. This trend will likely continue,  
522 and new multifaceted approaches will emerge allowing the collection of complex data. Furthermore, existing  
523 techniques are constantly being adapted to extend their applicability. For instance, two recent Hi-C-based  
524 techniques and a SIM imaging-based technique have been described that allow inspection of sister chromatid  
525 topology at the cell population level<sup>73,74,209</sup>. These techniques mark a significant milestone because, unlike other  
526 sequencing-based techniques, they allow the study of chromatin conformation during S phase of the cell cycle. It is  
527 possible that the implementation of these techniques will fuel discoveries related to S phase and DNA replication  
528 that were unattainable with the previously available techniques.

529 An important open question is the causality between topological insulation and transcription, which currently is  
530 difficult to properly address. Ideally, a single-locus proteomics approach would be appropriate to investigate the  
531 underlying proteome of a TAD border or a local chromatin insulator region in order to identify candidate insulator  
532 factors and analyze whether they play a causal role to determine insulation. Existing single-locus proteomics  
533 techniques to study topological insulation and transcription are available, but they are incredibly laborious and  
534 complicated to implement<sup>210–212</sup>. A major breakthrough in proteomics or in wet lab protocols to decrease input  
535 material is required for single-locus proteomics techniques to become widely applicable. However, it is an exciting  
536 lane of research that will certainly help explain the functional aspect of chromatin conformation.

537 Finally, we argue that not only methods driven by a technological boom, but also different and unconventional points  
538 of view should coalesce to invent new approaches and fuel milestone discoveries in genome architecture research.  
539 The importance of the interdisciplinary approaches described above will become even more prominent with future  
540 technological developments. Indeed, in order to re-orientate our field of research in the interdisciplinary direction,  
541 large consortia are being organized with the purpose of connecting different expertise and points of view. We believe  
542 that such developments should be highly encouraged and adopted even in individual laboratories, as they might  
543 promote individual projects and, in turn, in the field itself.

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1069 **Figure legends:**

1070 **Figure 1 Multiple levels of genome organization and the methods to study them.**

1071 DNA folds at multiple scales (indicated in the left) to build chromosomes. DNA winding around histones  
1072 forms nucleosomes, which are organized into clutches, each containing ~1–2 kb of DNA<sup>116</sup>. Nucleosome  
1073 clutches form chromatin nanodomains (CNDs) of ~100 kb in size, where most enhancer–promoter (E–P)  
1074 contacts take place<sup>43,57,117</sup>. At the scale of ~1 Mb, CNDs and CTCF–cohesin-dependent chromatin loops  
1075 form topologically associating domains (TADs)<sup>43,57,58,67,116,213,214</sup>. On the higher scale of up to 100s of Mbs,  
1076 chromatin segregates into gene active and inactive compartments (A and B, respectively) and into  
1077 compartment-specific contact hubs (not shown). At the highest topological level, the nucleus is organised  
1078 into chromosome territories<sup>27,35</sup>. Different techniques can be used to study different genome organization  
1079 levels, and some techniques can be used to study different organization levels. Although Hi-C, genome  
1080 architecture mapping (GAM) and other sequencing-based techniques can be used to detect chromosome  
1081 territories, 3D fluorescent *in situ* hybridization (FISH) is most useful to study this level of organization, as  
1082 it provides direct spatial information<sup>7,27,41</sup>. Compartments and hubs are usually studied with Hi-C, GAM,  
1083 split-pool recognition of interactions by tag extension (SPRITE) and several multiplexed super-resolution



1084 FISH techniques<sup>14,15,123,27,35,41,44-48</sup>. At the more functional, 1 Mb scale, most informative are capture Hi-C  
1085 (cHi-C), Capture-C, micrococcal nuclease chromosome conformation assay (Micro-C) and super-resolution  
1086 FISH approaches<sup>21-26,91</sup>. On the other hand, protein-driven enrichment techniques such as Hi-C chromatin  
1087 immunoprecipitation (HiChIP), chromatin interaction analysis by paired-end tag sequencing (ChIA-PET),  
1088 proximity ligation assisted chromatin immunoprecipitation (PLAC-seq) and chromatin-interaction analysis  
1089 via droplet-based and barcode-linked sequencing (ChIA-Drop) can be used to study different levels of  
1090 folding depending on whether the protein is associated with relatively local folding (e.g., MED12 in E-P  
1091 contacts), more long-range contacts (e.g., Polycomb proteins) or inter-chromosomal hubs (e.g., LHX2,  
1092 LDB1)<sup>31-33,102,192,215-218</sup>. CNDs have been discovered only recently. Owing to their stochastic nature and to  
1093 inter-cell variability, only super-resolution microscopy FISH has so far been able to detect CNDs<sup>43,57,117</sup>.  
1094 DNA adenine methyltransferase identification (DamID), Genomic loci positioning by sequencing (GPSeq),  
1095 Tyramide signal amplification (TSA).

1096

1097 **Figure 2 Main C-based methods for interrogation of 3D genome organization.**

1098 For the application of every chromosome conformation capture (C)-based method, chromatin must first  
1099 be cross-linked, either with one cross-linker (in most methods) or with two cross-linkers in the case of  
1100 micrococcal nuclease C (Micro-C) and chromatin interaction analysis by paired-end tag sequencing (ChIA-  
1101 PET)<sup>85,91,102</sup>. Hi-C derivatives then use digestion by restriction enzymes (RE) to fragment the chromatin, fill-  
1102 in fragment ends with biotin and perform ligation, all *in situ*. The sample is then sonicated, de-crosslinked  
1103 and enriched for informative fragments through biotin pulldown; the resulting chromatin-fragments  
1104 library is subjected to amplification and sequencing<sup>85</sup>. Hi-C chromatin immunoprecipitation (HiChIP)  
1105 differs from this standard protocol by introducing an immunoprecipitation (enrichment) step just after  
1106 ligation, whereas capture Hi-C (cHi-C) includes an enrichment step involving hybridization to RNA baits  
1107 that represent a genomic region of interest, followed by pulldown that is performed on the final Hi-C  
1108 library<sup>26,32</sup>. The resulting libraries will therefore be enriched either for all genomic contacts (in the case of  
1109 Hi-C), for chromatin contacts at genomic regions where the protein of interest binds (HiChIP), or for  
1110 contacts at a specific region of interest (cHi-C). Micro-C follows an almost identical procedure as Hi-C, with  
1111 distinctions in the steps of fixation and of digestion, in which the restriction enzymes used in the Hi-C  
1112 protocol are replaced with micrococcal nuclease (MNase)-mediated chromatin fragmentation<sup>91</sup>. This  
1113 digestion modification results in a more uniform fragmentation of the genome, thereby allowing Micro-C  
1114 to achieve higher resolution of local contacts. ChIA-PET provides similar information to HiChIP, but in ChIA-  
1115 PET immunoprecipitation is performed immediately after fixation and sonication; and while the sample is

1116 still on the pull-down beads, a linker with biotin is added and ligation is performed on-beads in order  
1117 to reduce the amount of random ligation products<sup>102</sup>. Concomitant with adapter addition, the sample is de-  
1118 crosslinked and fragmented using a transposase (Tn5). Finally, the sample is enriched for informative  
1119 fragments through biotin pulldown and undergoes library amplification and sequencing. DSG,  
1120 disuccinimidyl glutarate; EGS, ethylene glycol bis(succinimidyl succinate).

1121

1122 **Figure 3 Main ligation-independent methods for interrogation of 3D genome organization.**

1123 Ligation-independent methods have been developed in order to study multi-way contacts that are  
1124 inaccessible to ligation-based methods. The most common ligation-independent techniques are split-pool  
1125 recognition of interactions by tag extension (SPRITE), chromatin-interaction analysis via droplet-based and  
1126 barcode-linked sequencing (ChIA-Drop) and genome architecture mapping (GAM)<sup>35,41,192</sup>. In all three,  
1127 nuclei are fixed. Then, SPRITE and ChIA-Drop proceed with sonication and chromatin digestion by DNase  
1128 I. From this point on, the two techniques take advantage of different approaches to retaining information  
1129 on multi-way contacts. SPRITE utilizes a split-and-pool strategy, in which every sample is split, barcoded  
1130 and pooled together five times. This results in unique barcoding of all fragments that crosslinked together  
1131 and thus to the identification of DNA sequences that were involved in the same multi-way contacts<sup>35</sup>.  
1132 ChIA-Drop uses microfluidics to produce a droplet carrying a unique barcode, adapters and material for  
1133 DNA amplification reactions used to label a single chromatin-interaction knot, thereby allowing the  
1134 identification of all DNA sequences that have been crosslinked together. GAM utilizes a completely  
1135 different strategy to assay genome architecture and is suitable for investigating multi-way contacts,  
1136 higher-order chromatin structures as well as more local contacts. In GAM, the sample is fixed, embedded  
1137 in sucrose and cryo-sectioned to obtain thin slices, from which individual nuclear slices are laser micro-  
1138 dissected. Genomic DNA is then extracted from a single-nucleus slice followed by whole-genome  
1139 amplification and sequencing. The data obtained from the different sections of a nucleus are pulled  
1140 together and interactions are identified as DNA sequences that co-segregate more often than others.

1141

1142 **Figure 4 Microscopy and FISH-based methods for 3D genome investigation.**

1143 **a)** Super-resolution microscopy techniques overcome the diffraction limit using different approaches. In  
1144 structured illumination microscopy (SIM), the sample is exposed to a series of non-uniform illumination  
1145 from different angles and axial phases<sup>107-109</sup>. The resulting light pattern is analysed by Fourier  
1146 transformation to achieve a final mathematical reconstruction of the image, which improves lateral and  
1147 axial resolution by two-fold. Single molecule localization microscopy (SMLM) uses low excitation energy

1148 that causes a stochastic excitation of photoswitchable fluorophores and allows for the precise localization  
1149 of the centre of emission<sup>112–115</sup>. Sequential images of the sample are taken, in which fluorophores turn  
1150 either bright or dark and the final image is created by a superposition of all imaging cycles. In most  
1151 practical applications, this method yields a lateral resolution of up to 20 nm. Stimulated emission  
1152 depletion (STED) uses stimulated emission depletion through the combination of two lasers: an excitation  
1153 laser illuminates the sample in the middle and a doughnut-shaped depletion beam depletes the  
1154 surrounding signal<sup>118,119</sup>. In practice, STED reaches lateral resolution of about 50 nm and axial resolution  
1155 of about 80 nm to 600 nm. **b)** Oligopaints are fluorescently labelled synthetic DNA oligonucleotides that  
1156 can be combined with fluorescence *in situ* hybridization (FISH) to label, visualize and measure the  
1157 distances between genomic regions<sup>122</sup>. **c)** Oligopaints can be further modified to allow sequential imaging  
1158 by incorporating sample bleaching or automatized microfluidics with microscopy. Following every round  
1159 of imaging, the oligonucleotides are washed out before proceeding with a new round of hybridization and  
1160 imaging<sup>44–48,123</sup>. Distant genomic regions are imaged simultaneously using different detection  
1161 fluorophores while their neighbouring regions are imaged in the next round, thereby allowing chromatin  
1162 tracing. **d)** In oligo fluorescence *in situ* sequencing (OligoFISSEQ), oligonucleotides contain barcodes that  
1163 can be read through hybridization with a set of specific primers, to which fluorescently labelled  
1164 dinucleotides are ligated. The fluorescent signal is then imaged and cleaved off. This process is repeated  
1165 until the barcode is read in full (inferred from the specific combination of fluorophores)<sup>14,15,219</sup> gDNA,  
1166 genomic DNA; PSF, **point spread function**; SPDM, spectral position determination microscopy.

1167

### 1168 **Figure 5. Computational modelling of the 3D genome.**

1169 Two main computational strategies have been devised to investigate chromosome folding and the  
1170 mechanisms driving it: a bottom-up strategy (polymer modelling) and a top-down strategy (restraint-  
1171 based modelling)<sup>137,157,158</sup>. **a)** Polymer modelling mimics the physical behaviour of the chromatin fibre that  
1172 is represented as series of monomers (beads) on a string and in which attraction or repulsion forces can  
1173 be attributed based on first principles. The behaviour of the polymer is influenced by non-specific  
1174 restraints, e.g. imposing that the beads must be self-avoiding, by specific restraints, such as cohesin-  
1175 dependent loop extrusion that is blocked at convergent CTCF sites, and by specific interactions, such as  
1176 attraction between beads sharing the same epigenetic modifications. Such strategy can produce virtual  
1177 contact maps that can be compared with Hi-C interaction matrices. This process is reiterated until the  
1178 input parameters can reconstitute a simulation that optimally recapitulates experimental maps. Polymer  
1179 modelling allows to infer the mechanisms and estimate the forces that are necessary or sufficient to

1180 achieve the chromatin conformation closest to the experimental data<sup>159</sup>. Beads of different colours  
1181 indicate regions carrying different epigenetic modifications **b)** Restraint-based modelling uses  
1182 experimental data such as Hi-C contact maps or fluorescent in situ hybridization (FISH)-measured nuclear  
1183 distances to infer all the spatial restraints necessary to determine the structure of the genomic region of  
1184 interest. The resulting model is an optimally reconstructed 3D folded chromatin fibre that gives  
1185 information on spatial positioning of chromatin regions.

1186  
1187 **Figure 6. Live microscopy using CRISPR-dCas9 to study the 4D genome.**

1188 Live-cell microscopy is one of the few approaches that informs on the dynamics of chromatin contacts.  
1189 Multiple different methods allow the study of chromatin in 4D (changes of 3D chromatin structure over  
1190 time)<sup>50,204–207</sup>. Here, we illustrate two such methods based on nuclease-dead Cas9 (dCas9) recruitment  
1191 to the chromatin: chimeric array of gRNA oligonucleotides (CARGO) and Live FISH. **a)** CARGO utilizes a  
1192 single plasmid encoding multiple guide RNA (gRNAs) targeting the same genomic region of interest, for  
1193 example an enhancer, in order to amplify the fluorescence provided by dCas9-EGFP<sup>49</sup>. The region of  
1194 interest can be imaged over time to study its spatial dynamics. **b)** Live FISH utilizes two fluorescently  
1195 labelled gRNAs (one red (Cy3) and one green (A488)) targeting different regions of interest<sup>206</sup>. The two  
1196 regions can be labelled and imaged simultaneously, which allows tracking the dynamics of the regions  
1197 over time. D, distance.

1198

1199 **Box 1. Overview of proximity-ligation-based methods for mapping chromatin interactions**

1200 The development of chromosome conformation capture (3C), which detects pair-wise interactions  
1201 between select loci ('one-vs-one') through nuclear-proximity ligation in combination with semi-  
1202 quantitative PCR, marks the onset of the eponymous C-based techniques in the early 2000s<sup>17,18,29</sup>. Using  
1203 3C, the locus control region of the  $\beta$ -globin locus was shown for the first time to form chromatin loops  
1204 with and thus to activate its promoter, and to form an active chromatin hub that dynamically follows  
1205 transcription during differentiation and is stabilized by transcription factors<sup>220–223</sup>. However, 3C is low-  
1206 throughput and cannot successfully detect long-distance contacts. Circular 3C (4C) overcame these  
1207 limitations by using primers in order to detect genome-wide contacts formed by a single 'viewpoint' to  
1208 (one-vs-all)<sup>19,224,225</sup>. Later, 4C was combined with next generation sequencing, and was used to describe  
1209 the dynamics of chromatin contacts during development<sup>226–229</sup>. However, the most influential technique  
1210 in 3D genome organization research is Hi-C, in which the DNA interactome of the entire genome is assayed  
1211 (all-vs-all)<sup>27</sup>. Hi-C led to the identification of genomic compartments and topologically associating

1212 domains and to the development of the **loop-extrusion model**<sup>27,53,130,54,55,58,59,61,64,67,85</sup>. Finally, several  
1213 techniques were developed, which combined Hi-C with chromatin immunoprecipitation, thereby allowing  
1214 the interrogation of chromatin contact frequencies based on the presence of a specific protein<sup>31–33</sup>. It is  
1215 important to note that in all C-based techniques that include protein enrichment, the mapped contacts  
1216 are probabilistic rather than deterministic features and it is impossible to predict how and whether these  
1217 contacts will translate into function.

1218 Together, proximity-ligation based techniques fueled most of the discoveries in 3D genome organization  
1219 research during the past fifteen years. The ease of application of these techniques is anti-correlated with  
1220 the richness of data obtained from them, which is likely the reason why so many different C-based  
1221 technique adaptations exist. With the recent development of microscopy-related techniques, the  
1222 proximity ligation-based data is even more valuable as it will offer an imaging-complementary information  
1223 that is invaluable for achieving a better understanding of genome folding.

1224

## 1225 **Box 2 Manipulation of genome architecture**

1226 A fundamental question that has been very difficult to address in the past is whether genome architecture  
1227 changes that are detected in different cellular conditions are a cause or a consequence of changes in gene  
1228 expression<sup>65,230</sup>. Use of Hi-C in *Drosophila melanogaster* lines that carry a set of known genomic  
1229 alterations has allowed to address this question. Intriguingly, genome topology changes stemming from  
1230 chromosomal inversions or other mutations can be buffered to a large extent, such that gene expression  
1231 remains robust in most, although not all genes<sup>231</sup>. Whereas this analysis used pre-existing mutant lines,  
1232 CRISPR–Cas technology has also been used extensively to manipulate genome architecture. Inducing  
1233 specific mutations at critical genome architecture regulatory regions has shown that genome organization  
1234 into topologically associating domains with specific boundaries contributes to correct cell-type and tissue-  
1235 specific gene regulation<sup>213,232</sup>. In *D. melanogaster*, the deletion of specific chromatin-loop anchor  
1236 sequences or the insertion of boundary elements that prevent loop formation, showed that **Polycomb-**  
1237 dependent genomic loops can contribute to gene silencing during development<sup>233</sup>. The same approach  
1238 showed that CTCF-binding sites are required for correct insulation of gene expression and that their  
1239 deletion activates an oncogenic gene-expression program<sup>234</sup>.

1240 CRISPR–Cas was used not only to edit the genome, but also the epigenome, as in the case of using a fusion  
1241 of nuclease-dead Cas9 (dCas9) with DNA (cytosine-5)-methyltransferase 3A, which targets DNA  
1242 methylation to specific regions and displaces CTCF binding<sup>235</sup>. Another powerful experimental tool  
1243 combines CRISPR–Cas with optogenetics to induce chromatin looping upon stimulation with blue light and

1244 study its functional consequence<sup>236</sup>. Finally, the CRISPR-genome organization (CRISPR-GO) system enables  
1245 inducible and reversible repositioning of dCas9-targeted genomic regions in the nucleus; CRISPR-GO was  
1246 shown to reposition a locus of interest to the nuclear lamina, thereby perturbing its function, and to **Cajal**  
1247 **bodies [G]** and **PML bodies**<sup>237</sup>. This system could be used to target loci to other nuclear compartments in  
1248 order to study the functional consequences of their relocation. Combined with the many experimental  
1249 tools that enable measuring the effects of perturbation of genome structure and function, these  
1250 techniques will be crucial for differentiating between cause and consequence of 3D genome organization  
1251 and gene regulation.

1252  
1253  
1254

## **GLOSSARY**

### 1255 **First principles**

1256 Basic building blocks of knowledge that cannot be deduced from any other preposition used for  
1257 mathematical modeling of polymer behaviors.

### 1258 **Airy diffraction pattern**

1259 A diffused circle surrounded by rings of decreasing intensity generated when a laser passes through a  
1260 circular opening.

### 1261 **Cajal bodies**

1262 Nuclear bodies of 0.3 to 1 $\mu$ m in size, containing RNAs and proteins and involved in RNA-related metabolic  
1263 processes.

### 1264 **Dendrimer crosslinking**

1265 A procedure in which formaldehyde crosslinking can be followed or replaced by crosslinking with  
1266 dendrimers, i.e. highly ordered, branched polymeric molecules of different sizes.

### 1267 **Diffraction limit**

1268 The points where two Airy patterns are too close to be distinguishable.

### 1269 **Lamina associated domains**

1270 Chromosome domains associated to the nuclear lamina in the 3D nuclear space.

### 1271 **Loop extrusion model**

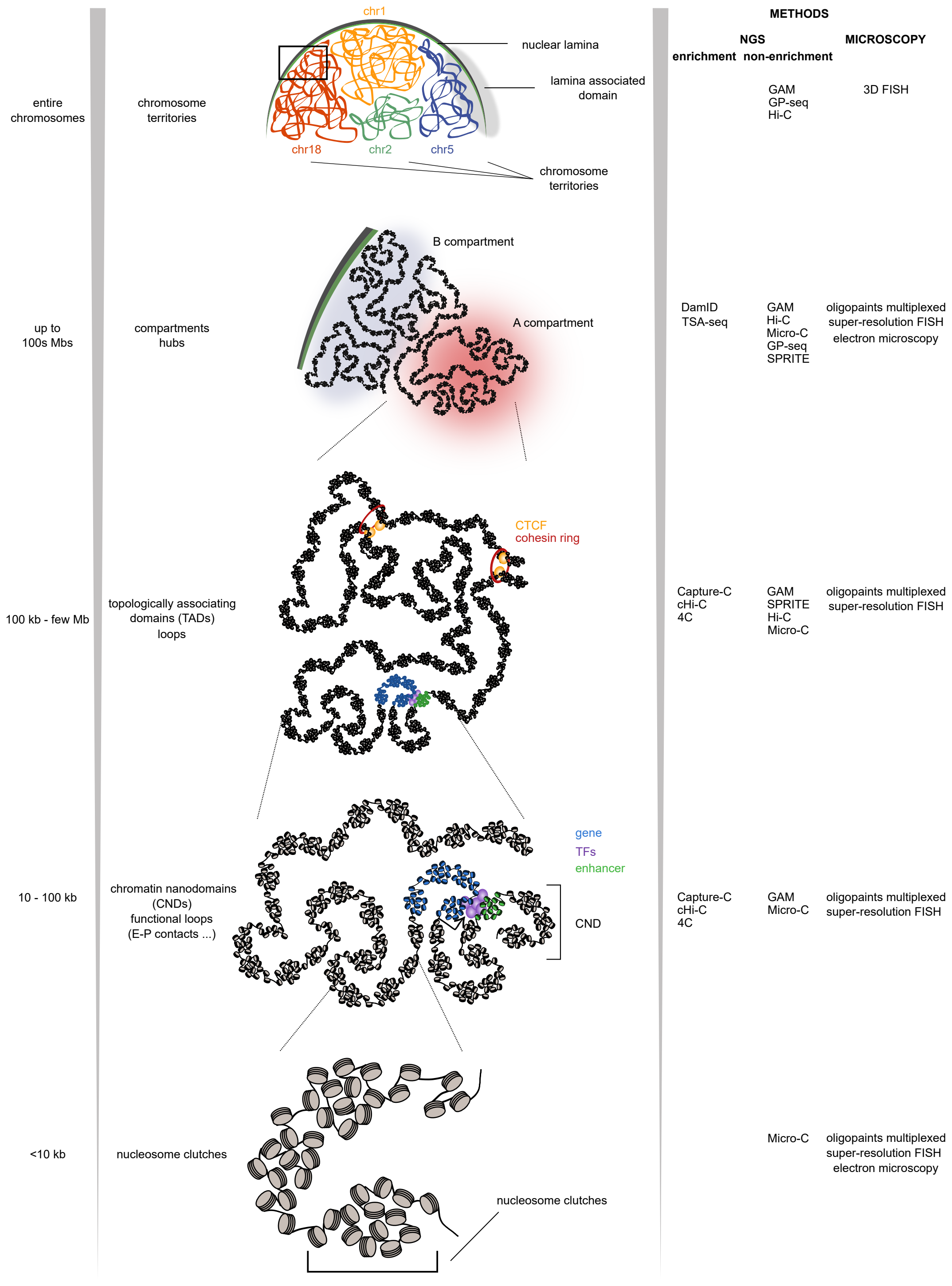
1272 Model suggesting that motors such as cohesin or condensin form a ring around chromatin and use the  
1273 energy of ATP to slide through it while extruding the intervening region.

### 1274 **Multi-way contacts**

1275 Chromatin contacts involving more than two chromatin fragments.

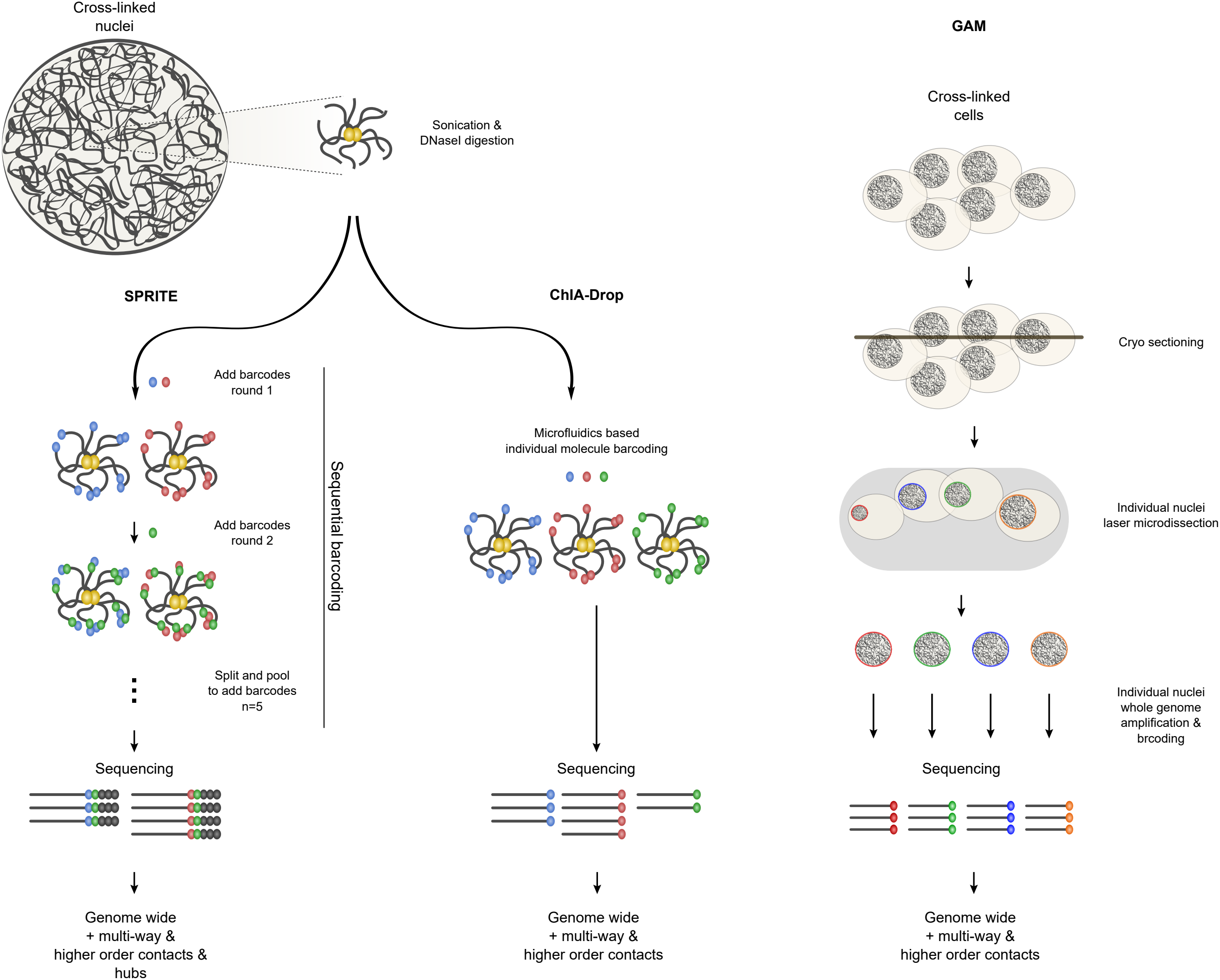
### 1276 **Nuclear speckles**

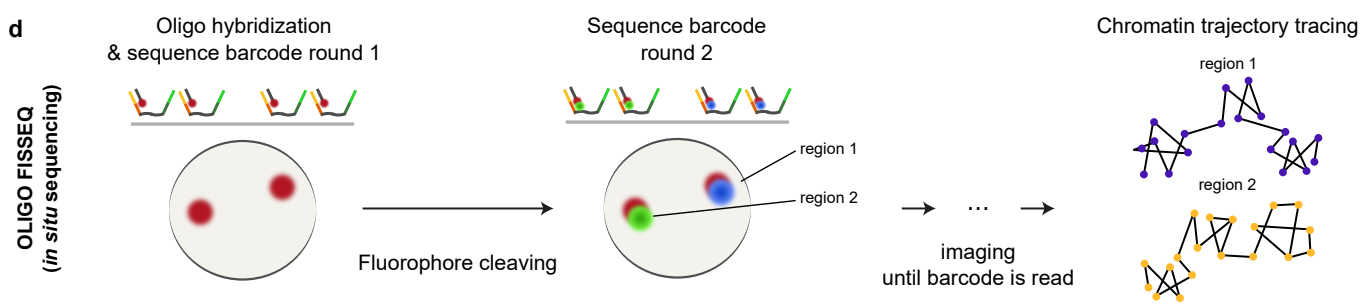
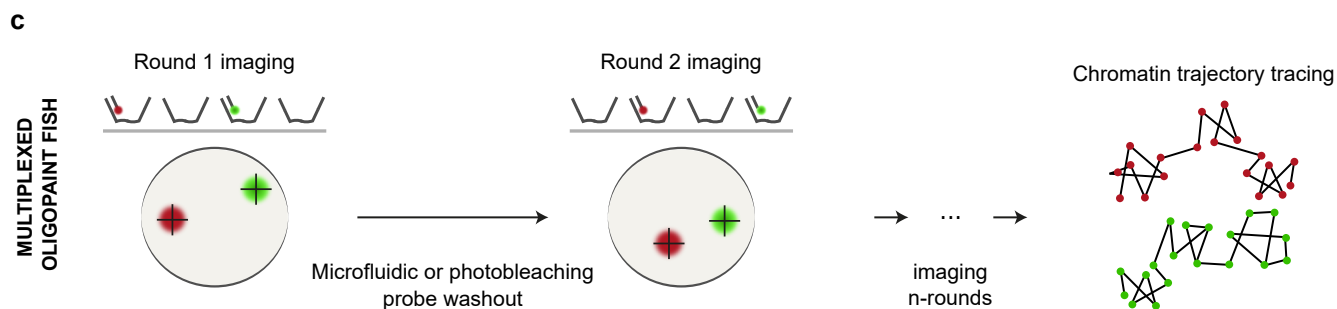
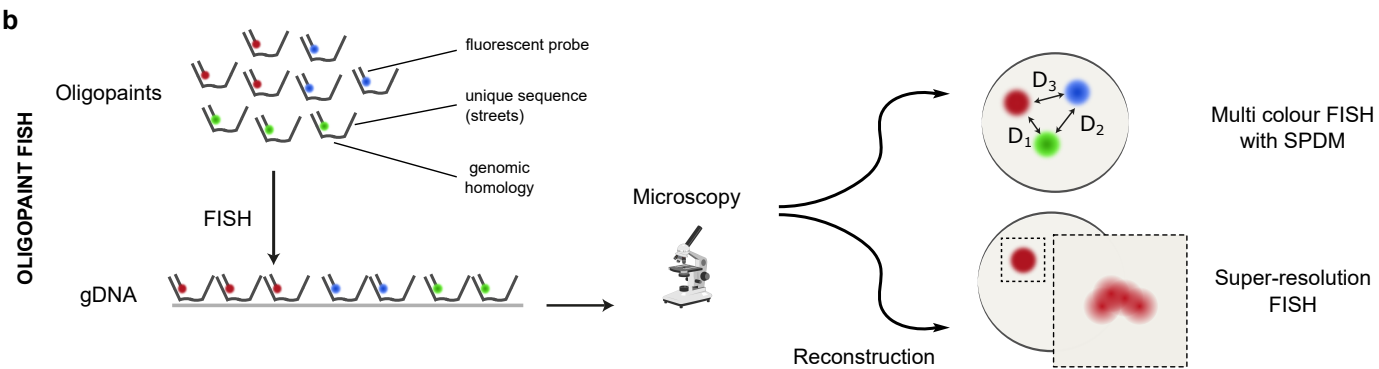
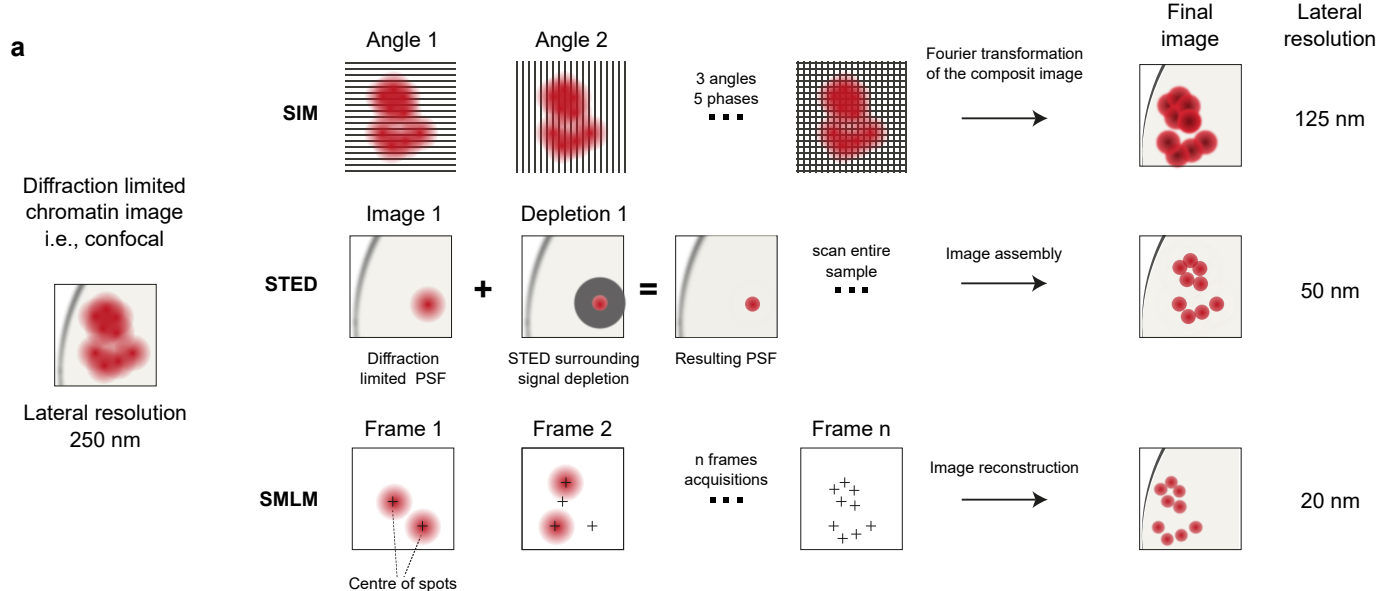
- 1277 Nuclear foci enriched in pre-mRNA splicing factors located in the nucleoplasm of eukaryotic cells.
- 1278 **Point spread function (PSF)**
- 1279 The response of an imaging system to a point object. If the object is below the microscope resolution it  
1280 will appear larger than it really is..
- 1281 **PML bodies**
- 1282 Nuclear bodies of 0.1 to 1 $\mu$ m in size, containing many components, including the promyelocytic leukemia  
1283 protein (PML) and frequently associated to Cajal bodies.
- 1284 **Polycomb**
- 1285 An evolutionarily conserved group of proteins involved in the regulation of a large group of target genes.
- 1286 **Sub-diffractive point spread function**
- 1287 A point spread function of smaller size than that generated by diffraction-limited systems.
- 1288 **Tyramide signal amplification**
- 1289 A method enabling sensitive detection of low-abundance molecules in fluorescent immunocytochemistry  
1290 applications.





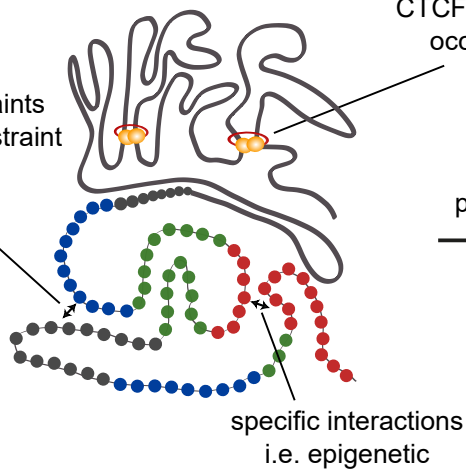
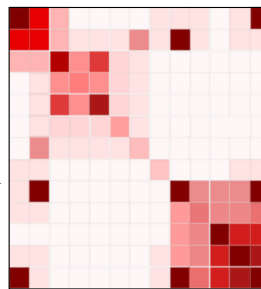
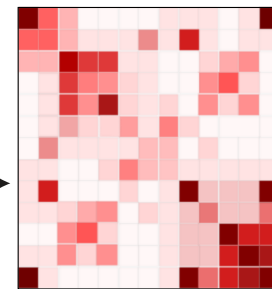






**a**

POLYMER MODELING

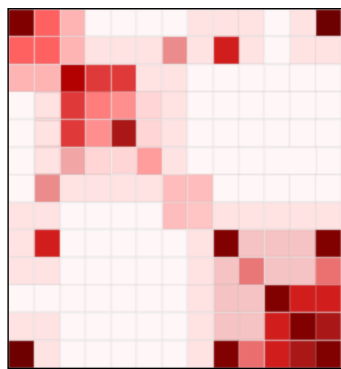
non-specific restraints  
i.e. topological constraintspecific restraints  
CTCF & cohesin  
occupancysimulation of  
polymer foldingcomparison  
with experiment

repeat to find the conditions that best recapitulate the experiment

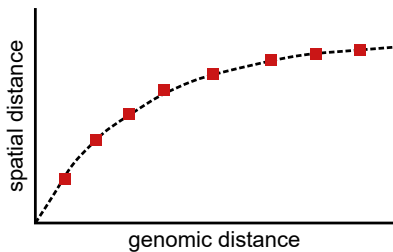
**b**

RESTRAINT MODELING

HiC data



FISH data

input experimental  
constraints  
i.e. contacts, distances

conformational ensemble

