

# Illuminating the structure and dynamics of chromatin by fluorescence labeling

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**BACKGROUND:** Visualization of chromosomal loci location and dynamics is crucial for understanding many fundamental intra-nuclear processes such as DNA transcription, replication, and repair.

**OBJECTIVE:** Here, we will describe the development of fluorescence labeling methods for chromatin imaging, including traditional as well as emerging chromatin labeling techniques in both fixed and live cells. We will also discuss current issues and provide a perspective on future developments and applications of the chromatin labeling technology.

**METHODS:** A systematic literature search was performed using the PubMed. Studies published over the past 50 years were considered for review. More than 100 articles were cited in this review.

**RESULTS:** Taking into account sensitivity, specificity, and spatiotemporal resolution, fluorescence labeling and imaging has been the most prevalent approach for chromatin visualization. Among all the fluorescent labeling tools, the adoption of genome editing tools, such as TALE and CRISPR, have great potential for the labeling and imaging of chromatin.

**CONCLUSION:** Although a number of chromatin labeling techniques are available for both fixed and live cells, much more effort is still clearly required to develop fluorescence labeling methods capable of targeting arbitrary sequences non-intrusively to allow long-term, multiplexing, and high-throughput imaging of genomic loci and chromatin structures. The emerging technological advances will outline a next-generation effort toward the comprehensive delineation of chromatin at single-cell level with single-molecule resolution.

**Keywords** chromatin structure and dynamics, FROS, FISH, TALE, CRISPR/Cas9, single-guide RNA, Suntag, super-resolution imaging

## Introduction

Beyond its primary sequence, chromatin is known to package into higher order structures that play important roles in gene regulation in both prokaryotes and eukaryotes (Meldi and Brickner, 2011). At the chromosomal level, chromosomes in interphase are found to occupy discrete regions named chromosome territories (CTs). Although there is no apparent chromatin intermingling between neighboring chromosomes, the inter-territory contact provides a space for some gene regulation processes, such as translocation and transcription-dependent association. Interestingly, the position of individual CTs are non-random with gene-poor chromosomes

localizing to the nuclear periphery and gene-rich chromosomes to the interior (Meaburn and Misteli, 2007). Several studies have suggested that the transcriptional activity of specific genes is correlated with their nuclear positioning (Kumaran et al., 2008; Zuleger et al., 2013; Therizols et al., 2014; Shachar et al., 2015). Within a CT, active genes often localize on the surface of a territory while repressed genes often hide in the interior (Cremer and Cremer, 2001). At the megabase scale, recent sequencing studies have revealed that chromosomal loci, some of which are genomically distant, may undergo preferential interaction to organize the loci into distinct modules called topologically associating domains (TADs) (Dixon et al., 2012; Nora et al., 2012). Chromatin organization at the TAD level plays a fundamental functional role in transcription (Tang et al., 2015) and DNA replication (Pope et al., 2014).

On the other hand, chromatin is a dynamic structure and altering its structural organization is closely related to the regulation of its functions (Hubner and Spector, 2010),

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including DNA transcription, replication, and repair (Lotterberger et al., 2015; Dekker and Mirny, 2016). For instance, upon external environmental stimulation or differentiation, specific gene regulation is often accompanied by rearrangement or repositioning of the genes relative to the nuclear envelope or other nuclear marker structures (Aizer et al., 2008; Noordermeer et al., 2011; Deng et al., 2012; Fabre et al., 2016).

Investigating chromatin structure and dynamics requires direct visualization of chromosomal loci *in situ* (Hubner and Spector, 2010). When taking sensitivity, specificity, and spatio-temporal resolution into account, fluorescent labeling and imaging techniques have been the most prevalent approaches for chromatin visualization. In the last decade, fluorescence microscopy techniques have been developed for chromatin imaging in a wide range of organisms, including bacteria (Viollier et al., 2004), yeast (Verdaasdonk et al., 2013), and mammals (Saad et al., 2014). This has made it possible to visualize and track specific genomic loci, chromosomal segments, single chromosomes, or even whole genomes to gather information regarding their positions and dynamics under different situations, such as mitosis and embryo development (Marshall et al., 1997; Tsukamoto et al., 2000; Chuang et al., 2006; Masui et al., 2011; Lucas et al., 2014; Chacon et al., 2016; Wijchers et al., 2016). However, these observations are highly dependent on the development of specific labeling methods, including fluorescent marker proteins, fluorescence *in situ* hybridization (FISH), fluorescent repressor and operator systems (FROS), and fluorescent programmable, sequence-specific DNA binding proteins. Herein, we review the development of different chromatin labeling methods and highlight emerging methods based on programmable, sequence-specific DNA binding proteins (Table 1). In addition, we discuss challenges facing these methods and provide a perspective on future developments and applications for chromatin labeling technology in the post-genome era.

## Fluorescent labeling of chromatin in fixed cells

Although fluorescent labeling methods, including the use of DAPI (Kapusinski, 1995) and fluorescent dNTPs (Bick and Davidson, 1974; Gratzner, 1982; Waldman et al., 1991; Salic and Mitchison, 2008), have contributed greatly to the study of chromatin DNA, they offer little or no specificity. Site-specific labeling methods are in high demand for situations where the relationship between DNA sequences and their relative positions need to be explored. FISH was developed several decades ago for the purpose of labeling specific genomic sequences using probes complementary to the target sequence (Gall and Pardue, 1969; Langer-Safer et al., 1982). By concentrating multiple FISH probes at the target site, the signal is amplified to produce a high signal-to-background

ratio (Fig. 1A). Since its invention, FISH has undergone numerous modifications and optimizations to increase its specificity, sensitivity, multiplexing, and throughput (Levsky and Singer, 2003). Nowadays, FISH is used in a wide variety of applications, from single genomic loci labeling (Tagarro et al., 1994) to multicolor whole genome painting (Ried et al., 1998) (Fig. 1B). Owing to its high specificity and relative ease of implementation, FISH has remained the gold standard for chromatin DNA labeling.

FISH probe detection was originally based upon radioactive signals (Gall and Pardue, 1969), but quickly turned to fluorescence signal detection using fluorophores such as cyanine, Alexa Fluor, and quantum dots. Fluorophores are usually attached to the probe nucleotide by the hydroxyl-amino reaction or biotin-streptavidin conjugation. The high quality and low cost of probes are key features for FISH, with three methods currently utilized for probe preparation. The first method is direct synthesis of nucleic acid oligomers or peptide nucleic acids (PNAs), which are suitable for repetitive sequences such as telomeres and centromeres. The second method is nick translation, which prepares FISH probes from cloned genomic regions, such as cosmids (30–40 kb), P1-artificial chromosomes (PAC, 130–150 kb), bacterial artificial chromosomes (BAC, 100–1000 kb) (Simonis et al., 2004), yeast artificial chromosomes (YAC, 100–1000 kb), and a flow-sorted chromosomes (Cremer et al., 2000). Nick translation is marginally less expensive and more convenient to operate, but bears lower efficiency.

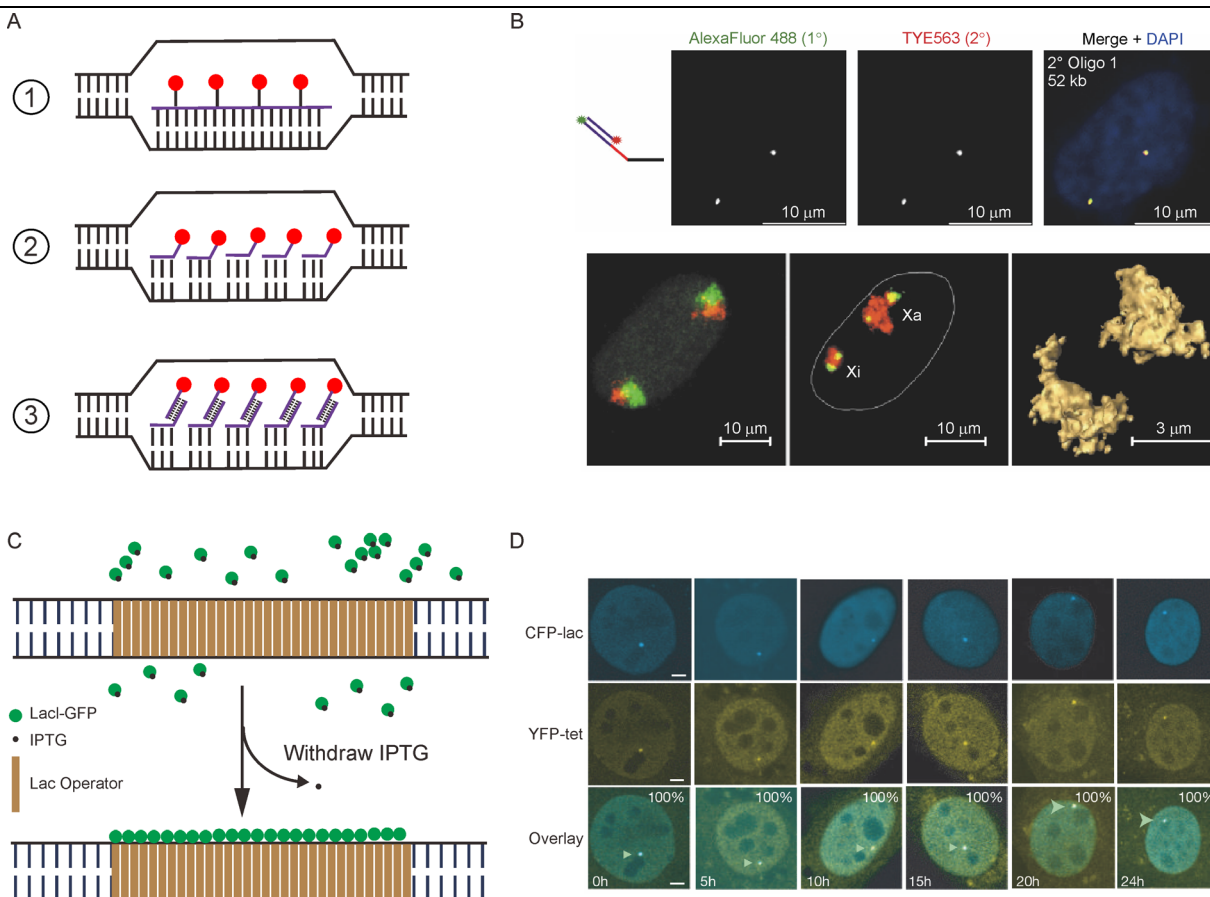
The third probe preparation method is based on PCR and is usually conducted in the following three ways: (i) Degenerate oligonucleotide primed PCR (DOP-PCR)-based FISH probes (Cremer et al., 2000; Bolzer et al., 2005), which are based on a random six nucleotide (nt) sequence on the 3' end of the degenerate oligonucleotide primer that can potentially bind anywhere along the genomic DNA sequence and thereby amplify multiple loci or even the whole chromosome; (ii) a versatile genome-scale PCR-based pipeline for high-definition DNA FISH probes (Bienko et al., 2013), which are based on 200–220 nt amplicons. This method provides a database of over 4.8 million primer pairs targeting the human genome that are readily usable for the rapid and flexible generation of probes. While this method has high labeling efficiency, it suffers from high cost and complexity; and (iii) in order to improve probe labeling efficiency and throughput, Beliveau et al. developed a new method, named “Oligopaints.” This method uses a complex oligo pool as a renewable source of FISH probes that carry only 32 bases of homology to the genome and are labeled by PCR with fluorophore-conjugated PCR primers (Beliveau et al., 2012). In 2015, a modified strategy was reported that introduced secondary oligonucleotides (oligos) to produce and enhance the fluorescence signal. This modified method was then applied to single-molecule super-resolution imaging of chromosomes (Beliveau et al., 2015). In 2016, the Wu laboratory further optimized the “Oligopaints” strategy by utilizing a unique pair of index

**Table 1** Comparisons of different chromatin labeling methods

Labeling system	Origin	Multiplexing capacity	Multi-color capacity	Advantages	Drawbacks	Applications	Reference
Intrusive							
DNA dye	Exogenous	No	No	Easy to implement	Unspecific; Bulk labelling	Any cell type; Labeling the nucleus DNA	Kapuscinski, 1995
FISH (fluorescence <i>in situ</i> hybridization)	Exogenous	Great	Yes	Sequence specific; Highly parallel, library level; Allow super-resolution imaging; Signal amplification	Fixed cell	Any cell type; Single gene localization; Chromatin interaction; Chromosome painting	Gall and Pardue, 1969; Pardue and Gall, 1969; Langer-Safer et al., 1982; Tagarro et al., 1994; Ried et al., 1998; Cremer et al., 2000; Levsky and Singer, 2003; Bolzer et al., 2005; Simonis et al., 2006; Beliveau et al., 2012; Nora et al., 2012; Bienko et al., 2013; Chen et al., 2013; Beliveau et al., 2015; Deng et al., 2015; Boettiger et al., 2016; Wang et al., 2016
Histones (H2B-GFP etc.)	Exogenous	No	No	Live cell; Photoactivatable fusion allows activating individual chromosomes; Easy to stably express	Unspecific; Bulk labelling	Any cell type; Chromosome dynamics during cell cycle	Abney et al., 1997; Kanda et al., 1998; Held et al., 2010; Ricci et al., 2015
Fluorescent dNTPs	Exogenous	No	Yes	Fixed cell; Allow super-resolution maging; Transfection or injection	Unspecific; Semi-Bulk labelling	Any cell type; Replication region Labeling	Bick and Davidson, 1974; Grazner, 1982; Waldman et al., 1991; Salic and Mitchison, 2008
FROS (Fluorescent repressor Operator system)	<i>E. coli</i> (lactose); Tn10 transposon (tetracycline gene); Phage lambda; ParB-INT system	No	Yes	Robust; Live cell; Bright; High signal- to- noise ratio; Long term tracking; Ease to stably express	Replication blockage (fragile sites); Potential chromatin disruption; Interference with transcription	Yeast, difficult in metazoans; DNA repair and manipulation	Gilbert and Muller-Hill, 1966; Hillen et al., 1982; Robinet et al., 1996; Viollier et al., 2004; Soutoglou et al., 2007; Masui et al., 2011; Verdaasdonk et al., 2013; Roukos et al., 2013; Backlund et al., 2014; Lucas et al., 2014

(Continued)

Labeling system	Origin	Multiplexing capacity	Multi-color capacity	Advantages	Drawbacks	Applications	Reference
Non-intrusive	Endogenous	No	No	Any cell type; Live cell; Single component; Easy to stably express	Limited number available	Original cells; Telomere and centromere	Wang et al., 2011; Kepten et al., 2015
ZFN (Zinc-finger nuclease)	Eukaryotes	No	Yes	Live cell; Sequence specific; Single component; Easy to stably express	Transcription block; Hard to construction	Any cell type; Repetitive Sequences	Segal et al., 1999; Lindhout et al., 2007
TALE (transcription activator-like effector)	Xanthomonas oryzae transcription activator	Little	Yes	Live cell; Sequence specific; Single component; Easy to stably express	Transcription block; Hard to construction	Any cell type; Repetitive Sequences	Segal et al., 1999; Lindhout et al., 2007; Boch et al., 2009; Ma et al., 2013; Miyazari et al., 2013, 2014; Pederson, 2014; Thanisch et al., 2014; Wu et al., 2015; Hu et al., 2017; Ren et al., 2017
dCas9 based CRISPR clustered regulatory interspaced short palindromic repeat)	Streptococcus pyrogenes (SP), NM, ST1, SA	Good	Yes	High throughput; Live cell; Sequence specific; Easy to construct	Transcription block; Local DNA unwinding/triple helix formation; Multiple components; Hard to stably express	Any cell type; Repetitive or non-repetitive	Horvath and Barrangou, 2010; Chen et al., 2013; Esvelt et al., 2013; Fanucchi et al., 2013; Fujita and Fujii, 2013; Mali et al., 2013; Levine, 2014; O'Connell et al., 2014; Ma et al., 2015; Ran et al., 2015; Zalatan et al., 2015; Chen et al., 2016; Nelles et al., 2016
sgRNA based CRISPR	Streptococcus pyrogenes (SP), NM, ST1, SA	Great	Yes	Any cell type; High throughput; Sequence specific; Live cell; Long term observation; Easy to construct	Transcription block; Local DNA unwinding/triple helix formation; Multiple components; Hard to stably express	Repetitive or non-repetitive	Bertrand et al., 1998; Daigl and Ellenberg, 2007; Larson et al., 2011; Strack et al., 2013; Shechner et al., 2015; Cheng et al., 2016; Shao et al., 2016



**Figure 1** Conventional intrusive labeling of chromatin loci in fixed and living mammalian cells. (A) Several commonly used FISH methods. ①: probes each contains several dye molecules were generated by nick translation or PCR. ②: probes each contains one dye molecule were synthesized by commercial companies. ③: Oligopaint, a new technology that uses secondary oligo nucleotides to produce and enhance fluorescence signals. (B) Upper: a 52 kb DNA fragment in diploid human WI-38 cells was labeled by oligopaint with both primary and secondary probes. Lower: X chromosomes were painted by multiple probes covering the whole chromosome. (C) Working mechanism of the FROS system. 256x LacO array were inserted into the targeted chromosome loci and GFP fused LacI can be recruited to the site to accumulate the fluorescent signals by removing IPTG. (D) Living cells tracking of the double strand break (DSB) repair process by inserting LacO-*I*SceI-TetO into the chromosome.

primers in a PCR reaction so as to selectively amplify the probe set of interest from a complex pool of custom, array-derived oligos. This modification was then applied in visualizing chromatin structures (Boettiger et al., 2016; Wang et al., 2016).

In traditional FISH, probe hybridization requires harsh treatments using heat and formamide to denature global DNA that could also distort cell morphology and DNA structure. Recently, two novel approaches have been developed based on the highly specific and efficient enzymatic DNA binding ability of CRISPR/Cas9 under mild conditions. In 2013, Chen et al. applied modified FISH to verify CRISPR/Cas9 DNA labeling signals by transfecting living cells with CRISPR/Cas9 expression plasmids, and then using FISH probes to bind a complementary sgRNA binding strand in fixed cells at 37°C (Chen et al., 2013). In 2015, Deng et al. reported the development of CASFISH in which Cas9 and sgRNA complexes were able to be constituted *in vitro* as probes to label sequence-specific genomic loci without global DNA

denaturation (Deng et al., 2015). The rapid, less intrusive, cost-effective, and convenient method of CASFISH has great potential for future application in the study of chromatin structure.

Although this technology has made significant contributions to illuminating the organization of the crowded nucleus, and elucidating the sophisticated structure of chromatin folding, the inextricable limitation with FISH-based methods remains their incompatibility with living cells. This limitation restricts the application of this method in its ability to monitor the dynamic processes of chromatin.

## Fluorescent labeling of chromatin in living cells

### Fluorescent marker proteins

A straightforward approach to labeling and imaging chromatin in living cells is by tagging chromatin binding proteins

with fluorescent proteins. This approach allows the non-intrusive labeling of whole chromatin or certain special chromosomal segments, such as centromeres and telomeres (Belmont, 2001). For example, as a constitutive component of nucleosomes, histone H2B fused with GFP facilitated the fluorescence imaging of chromosomes without perturbation of chromosomal structure and function (Kanda et al., 1998). This histone labeling method was also applied to study chromosomal dynamics during mitosis, including condensation, pairing, and decondensation (Held et al., 2010). Moreover, this method was adapted to provide the first insight into chromatin fiber movement during interphase using fluorescence recovery after photobleaching (FRAP) of a small region in the nucleus (Abney et al., 1997). When coupled with photo-convertible fluorescent proteins for super-resolution imaging, nucleosome density along the chromatin fiber in single nuclei can be visualized and characterized quantitatively at ultra-high spatial resolution. Using this approach, nucleosomes were found to assemble in heterogeneous groups called “clutches,” which are interspersed with nucleosome-depleted regions. The compaction and number of nucleosomes inside individual clutches varied in a cell-type-specific manner (Ricci et al., 2015).

However, several drawbacks limit the application of histone-based chromatin labeling methods. First, fluorescent histones are indistinctly incorporated into all chromosomes with no sequence or chromosome information. Second, all fluorescent protein tagged histones are exogenously expressed by transient transfection or random integration, which can introduce labeling artifacts. For example, over-expressed fluorescent protein-histone may change the packing density of native histones and thus the unbound fraction may contribute to false-positive localization. Even with the advanced genome editing technology available nowadays, the multi-copy nature of histone genes in the genome makes tagging of the endogenous gene challenging.

Besides histones, other DNA binding proteins can also be used to label special regions of chromatin. As examples, telomeres and centromeres can be respectively labeled by TRF1/2 and CENPA in mammal cells (Kepten et al., 2015). In *Escherichia coli*, chromosome organization can be studied by labeling nucleoid-associated proteins, H-NS or HU (Wang et al., 2011).

### Fluorescent Repressor and Operator System (FROS)

To circumvent the constraints caused by the non-specificity of fluorescent histones and limited number of specialized chromatin binding proteins, the Fluorescent Repressor and Operator System (FROS) method was developed for in situ DNA sequence localization (Robinett et al., 1996). FROS takes advantage of the high binding affinity and specificity of repressor proteins to operator sequences, which are inserted in the genome in either a targeted or random manner. The most commonly used FROS systems are the Lac operator (LacO),

from the lactose operon (Gilbert and Muller-Hill, 1966), and the Tet operator (TetO), from the tetracycline operon, of *E. coli* (Hillen et al., 1982). To amplify the fluorescent signal, as many as 256 tandem repeats of the operators are inserted into the genome. In practice, a higher number of tandem repeats of the operators may be integrated into the genome as multiple plasmid copies tend to insert at the same site. FP-fused lac repressor expressed in live cells allows direct in vivo visualization of targeted chromosomal loci dynamics (Fig. 1C). More importantly, the high orthogonality of many FROS systems can be utilized simultaneously in a single living cell without crosstalk, allowing multiple loci to be tagged with different fluorescent proteins (Backlund et al., 2014).

FROS has been widely applied in studies of the highly advanced genetic manipulation systems in bacteria and yeast, which allow for the insertion of long tandem repeats in any desired location of the chromosome. Viollier et al. constructed 112 *Caulobacter crescentus* strains, each with a LacO repeat inserted at an individual locus dispersed over the circular chromosome. Tracking results demonstrated that each locus had a specific subcellular address in living cells, and individual chromosomal loci move rapidly and sequentially to specific subcellular locations during bacterial DNA replication (Viollier et al., 2004). Similar experiments have also been conducted in budding yeast, where chromosome motion was indicated to vary in a predicted manner along the length of the chromosome in such a way that local mobility was established to be a function of distance from the tether (Verdaasdonk et al., 2013).

However, the application of FROS in mammalian cells has been restrained by the lack of efficient genome editing tools for locus-specific knock-in of operator array long tandem repeats. Current methods are also deemed unreliable for their tendency to introduce repeat truncations during homologous recombination. In the past few years, several studies have employed embryonic stem (ES) cells owing to their relatively high efficiency for homologous recombination. For example, Masui et al. integrated the TetO/TetR system into the Xic loci by gene targeting to investigate homologous pairing events controlling asymmetric Tsix expression, and to visualize the dynamics of Xic and other genomic loci during X chromosome inactivation of ES cells. Results from this study found the Xic loci to show markedly reduced movements during pairing (Masui et al., 2011). In a separate study, Lucas et al. explored the three-dimensional (3D) trajectories adopted by the coding and regulatory DNA elements of the immunoglobulin gene, during B lymphocyte development, by inserting the TetO array into the IgH locus, using the ES cell gene targeting technique (Lucas et al., 2014).

Another beneficial means by which to use the FROS system is based on random integration. An elegant study, aimed at visualizing and quantitatively analyzing the dynamics of single double strand breaks (DSBs) in living mammalian cells, was carried out by randomly inserting 256

× LacO-ISceI-96 × TetO arrays into NIH3T3 cells randomly (Fig. 1D). By selecting a cell colony with only one integration site and inducing ISceI restriction endonuclease expression to cut the *ISceI* locus and thereby generate a DSB, the broken ends were found to be constrained in space before undergoing translocation with neighboring chromosomes (Soutoglou et al., 2007; Roukos et al., 2013).

In summary, although it is a powerful tool to visualize and track chromosomal loci in all living organisms, FROS suffers some intrinsic constraints that prevent it from becoming the routine method for genomic loci labeling in live mammalian cells. First, the gene targeting process is labor-intensive and time-consuming thus limiting the throughput for library-level investigation. Second, the introduction of long exogenous fragments (~10 kb) may inadvertently alter the local chromosome structure (Guo et al., 2015). Further efforts are therefore required to develop versatile and robust methods that can achieve site-specific chromosomal loci labeling methods in live cells.

### Programmable, sequence-specific DNA binding proteins

Several new, endogenous genomic labeling approaches, based on gene targeting techniques, have recently been developed for use in living cells. These new techniques include zinc-finger nucleases (ZFNs), transcription activator-like effectors (TALE), and the clustered regulatory interspaced short palindromic repeats (CRISPR/Cas9) system. These non-intrusive imaging techniques can be visualized by programmable, sequence-specific DNA binding modules fused to fluorescent proteins by various linkers (Badique et al., 2013; Chen et al., 2013; Hsu et al., 2014; Li et al., 2015; Shalem et al., 2015; Wan et al., 2015; Wu et al., 2015).

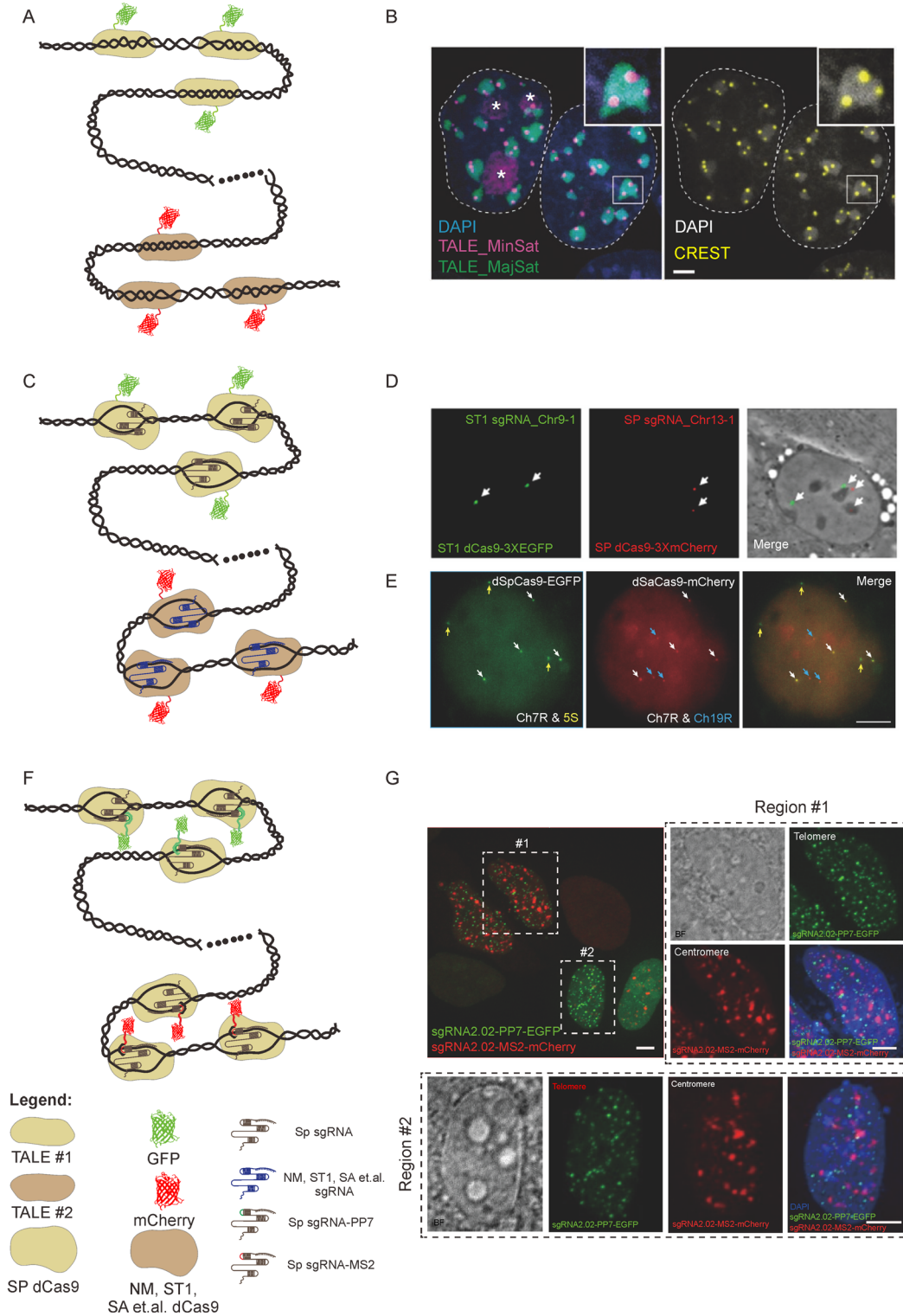
Zinc fingers (ZF) are artificial DNA binding proteins containing Cys<sub>2</sub>His<sub>2</sub> domains that recognize trinucleotides (Segal et al., 1999). A stretch of more than 10 nucleotides can be specifically targeted by constructing a fusion protein consisting of multiple ZF domains, such that ZF domain order determines the length of the DNA recognition site. In 2007, Lindhout et al. developed a ZF-based chromatin labeling method and applied it to live cell visualization of endogenous genomic sequences in both *Arabidopsis thaliana* and mouse cells (Lindhout et al., 2007). However, the complete recognition code for all 64 possible trinucleotide combinations did not possess the same affinity and specificity, thereby restricting its application in chromatin labeling. In contrast, TALE, a new generation of programmable DNA binding proteins, emerged with robust performance in chromatin labeling. TALE consists of 34 amino acid long tandem repeats that are nearly identical, except for two variable amino acids referred to as repeat-variable diresidues (RVDs). RVDs define the base-recognition specificity of each unit (Boch et al., 2009). An array of different repeat unit combinations therefore allows for the generation of TALEs with user-defined specificity for chromatin imaging (Fig. 2A).

In 2013, Miyanari et al. applied TALEs to visualize endogenous repetitive genomic sequences in mouse cells. This technology, referred to as TAL effector-mediated genome visualization (TGV), allows labeling of specific repetitive sequences and tracking of nuclear remodeling through mitosis in living cells (Fig. 2B). Additionally, by taking advantage of single-nucleotide polymorphisms (SNPs), the parental origin of chromosomes could specifically be resolved (Miyanari et al., 2013). Since targeting specificity is determined by TALE repeat order, multicolor labeling of different loci can be easily visualized by fusing individual TALEs with different fluorescent proteins (Ma et al., 2013; Miyanari, 2014; Pederson, 2014; Thanisch et al., 2014). To further improve the signal-to-background ratio of TGV, Hu et al. combined TGV with bimolecular fluorescence complementation (BiFC) to develop a new technique named BiFC-TALE (Hu et al., 2017). Ren et al. also recently discovered that conventional TALEs tend to aggregate in live cell imaging, and thus fused TALEs with thioredoxin to improve TGV specificity and accuracy (Ren et al., 2017).

However, the repetitive building block assembly process of TALE is labor-intensive and costly, thus making the technique suitable for labeling of repetitive sequences only. In contrast, the CRISPR/Cas9 system recognizes target DNA, using a short guide RNA sequence through Watson-Crick base pairing. This approach makes it easier to perform gene targeting in a high-throughput manner.

CRISPR/Cas9 is an innate immunity system used by most bacteria and archaea to protect themselves against exogenous plasmid and virus invasion. Adaptation of the CRISPR system to genome editing toolkits in mammal cells has picked up momentum since its working mechanism was established. The type II CRISPR system, the simplest and thus most popular, consists of an endonuclease termed Cas9, which cuts DNA, and a short RNA sequence referred to as the single guide RNA (sgRNA), which determines targeting specificity. Any genomic sequence can generate a DSB if it possesses a trinucleotide (NGG, where N can be any base) called a proto-spacer adjacent motif (PAM) (Horvath and Barrangou, 2010). An induced DSB can be repaired endogenously via the error-prone non-homologous end-joining (NHEJ) pathway, which can induce insertion or deletion mutations (indels) for knockout applications, or the homologous recombination (HR) pathway, which integrates exogenous repair templates into the cutting site for precise knock-in applications (Mali et al., 2013). This technology has since been rapidly extended to almost all model organisms for genome engineering applications.

In addition to using the catalytic activity of Cas9, a mutated nuclease-deficient Cas9, termed dCas9, enables the repurposing of the system for targeting genomic DNA without cleaving it. A huge body of recent work suggested that the engineered dCas9, acting as a flexible, site-specific RNA-guided DNA recognition platform, enabled precise, reprogrammable, and robust transcription regulation (Zalatan et al.,



**Figure 2** Emerging non-intrusive multicolor labeling of repetitive chromatin loci in living cells. (A) Schematic diagram of dual-color labeling of two different loci by TALE. Two TALE modules were generated to recognize two chromosomal repetitive regions, each fused with a different fluorescent protein. (B) Mini satellite and major satellite were labeled simultaneously by transiently expressing TALE-mRuby2 (magenta) and TALE-mClover (green) in living mouse cells. (C) Schematic diagram of dual-color labeling of two different loci by Cas9-based CRISPR imaging. Two orthogonal dCas9s fusing with different fluorescent proteins and two distinct sgRNAs each targeting one repetitive region were coexpressed in the same cells. (D) Two repetitive sequences in human chromosome 9 and 13 were labeled by ST1-dCas9 and SP-dCas9. (E) Three different loci can be labeling using combination of dSpCas9 and dSaCas9. (F) Schematic diagram of dual-color labeling of two different loci by sgRNA-based CRISPR imaging. The sgRNAs were modified by inserting aptamers in tetraloop or loop2. Dual-color labeling is achieved with one Cas9 and two modified sgRNAs and two coat proteins. (G) Telomeres and centromeres were labeled by sgRNA2.02-PP7-EGFP and sgRNA2.02-MS2-mCherry. Two zoom-in regions were showed.

2015), DNA and RNA imaging (Chen et al., 2013; Nelles et al., 2016), and chromatin and RNA pull-down (O'Connell et al., 2014; Fujita and Fujii, 2013). By transfecting cells with dCas9, tagged with a fluorescent protein, and the corresponding sgRNA targeting the desired locus, both repetitive and non-repetitive DNA sequences have been successfully labeled (Chen et al., 2013). This powerful imaging technique allowed live cell tracking of the diffusion behavior of telomeres and genes with repetitive sequences, which enabled the comparison of telomere length under different conditions, counting of gene copy number, and calculation of the physical distance between two loci along the same chromatin fiber with different genomic distances (Chen et al., 2013).

To date, *Streptococcus pyogenes* (SP) has been favored by almost all biology scientists owing to its simplicity and ease of implementation. Meanwhile, since the targeting specificity of the CRISPR system is solely determined by sgRNA base pairing instead of the Cas9 protein, different chromosomal loci cannot be resolved using multiple fluorophores that are dependent on SP Cas9 alone. To further expand its application in probing chromatin organization and dynamics, such as spatial resolving of individual alleles (Miyanari et al., 2013), DSB-induced translocations (Soutoglou et al., 2007; Roukos et al., 2013), and promoter-enhancer looping (Fanucchi et al., 2013; Levine, 2014; Lucas et al., 2014), capacity for multicolor imaging with the CRISPR system is desired. One possible solution is to use orthogonal CRISPR systems originating from different species (Fig. 2C), which guarantees no crosstalk is present when visualizing inter- and intra-chromosomal repetitive sequences (Ma et al., 2015; Chen et al., 2016) (Fig. 2D and 2E). However, unlike SP Cas9, the efficiency of NM (*Neisseria meningitidis*), ST1 (*Streptococcus thermophilus*), and SA (*Staphylococcus aureus*) Cas9 requires further optimization (Esvelt et al., 2013; Ran et al., 2015). Additionally, the required PAM sequences of NM, ST1, and SA Cas9 are more complicated than that of SP Cas9, making it difficult to design a pool of sgRNAs for the target regions (Esvelt et al., 2013; Ran et al., 2015). Another solution is to load different fluorescent proteins with different sgRNAs, since they determine targeting specificity (Fig. 2F and 2G).

Shechner et al. developed a technique called 'CRISPR-display', which permitted the recruitment of long non-coding RNA to a special chromatin locus via the sgRNA insertion (91). Using a similar concept, RNA aptamers, such as MS2 (Bertrand et al., 1998), PP7 (Larson et al., 2011),  $\lambda$ N (Daigle and Ellenberg, 2007), com (Daigle and Ellenberg, 2007), and spinach (Strack et al., 2013), could also be inserted in the 5', 3', tetraloop, or loop2 regions to recruit functional effectors to the destination. Interestingly, compared with the dCas9-based labeling method, the fast exchange kinetics of fluorescent effectors with RNA aptamers makes this modified sgRNA method more suitable for long-term tracking of chromosomal dynamics, particularly for short repeats or non-repetitive sequences, which would be more prone to suffering from

photobleaching under continuous exposure (Shao et al., 2016). Moreover, the engineered multi-functional sgRNAs can be used to regulate gene expression and simultaneously monitor gene position and dynamics. To bypass the limited number of well-characterized RNA aptamers, Cheng et al. invented a versatile CRISPR-Cas9-Pumilio hybrid technique, termed Casilio, for gene regulation and genomic labeling (Cheng et al., 2016). In addition to multicolor CRISPR imaging, tracking multiple genomic loci in single cells can be achieved by combining CRISPR imaging with sequential DNA FISH (Guan et al., 2017; Takei et al., 2017).

## Challenges and prospects

By and large, most quantitative measurements of chromatin structure and dynamics have primarily relied on the implementation of fluorescence labeling and imaging techniques. Imaging-based technologies have several advantages over traditional biochemistry- or sequencing-based technologies for the study of chromatin structure and dynamics. First, imaging-based techniques can provide critical spatial and temporal information. Second, conclusions obtained from optical imaging experiments are based on the interpretation of multiple single cell data, thereby avoiding the averaging effects of cell-population-based measurements.

However, many challenges remain for imaging-based single cell studies. For example, it is generally difficult to simultaneously achieve both high spatial and temporal resolution while minimizing laser phototoxicity to cells (Waldchen et al., 2015). Moreover, low throughput may restrict the application of imaging-based techniques in investigating whole genome organization. More importantly though, gaps between different spatial and temporal scales remain open and undefined. For example, both chromosome territories and chromosomal loci have been explored, yet little knowledge has been gained regarding the architecture in-between. Additionally, chromosomal loci movements have been tracked over both short (~20 ms) (Levi et al., 2005) and extended time scales (~h) (Chuang et al., 2006), but further efforts are needed to accurately combine these two scales. Last, but not least, most imaging experiments were based on a phenomenon observed in cultured cell lines *ex vivo* and it is difficult to visualize sub-cellular images in living organisms owing to the scattering of light. The culture and acclimation of tissue cells in culture dishes may also cause dramatic alteration of nuclear rigidity and chromosome organization, which could lead to a misinterpretation of the relationship between structure and function.

To explore the particular scientific questions and technical challenges in the nucleus, the US National Institutes of Health (NIH, USA) initiated the four-dimensional (4D) nucleome project in December 2014 (Dekker et al., 2017). Representing the emerging field of chromatin research, the project aims "to understand the principles behind the 3D organization of the nucleus in space and time (the 4th dimension), the role

nuclear organization plays in gene expression and cellular function, and how changes in the nuclear organization affect normal development as well as various diseases.” Considering the current stages of studying each of the methods described above, we offer a few prospects regarding the future development of chromatin labeling methods.

### Reduced cellular perturbation

A critical consideration when choosing a labeling method is to confirm that the natively existing structure is truly being investigated instead of artifacts caused by the labeling method. Taking FISH as an example, some of the finest cellular details may be destroyed by the harsh treatments of heat denaturation and formamide required to obtain single stranded target DNA for fluorescent probe hybridization. This makes little difference when solely examining the nuclear position of a specific locus or when calculating the distance between two loci (Cremer et al., 2008; Solovei and Cremer, 2010); however, if the packing morphology of a chromatin segment was to be painted, the denaturation treatments may disrupt local chromatin integrity and attenuate the association with its interaction partners. To circumvent this limitation of FISH, the CASFISH technique mentioned above takes advantage of the CRISPR-based mechanism for rapid DNA hybridization to label genomic loci in fixed cells (Deng et al., 2015). Additional artifacts may also come from the cell fixation process, causing a significant chromatin shrinkage effect. However, Boettiger et al. demonstrated that using osmotically balanced, methanol-free formaldehyde in PBS can largely preserve native structure when fixing cells (Boettiger et al., 2016).

Minimal perturbation of chromatin labeling and imaging makes use of genetically encoded fluorescent proteins in living cells, including fluorescent histones, FROS, TALE, and CRISPR. For FROS, insertion of operator sequences into the integration site may disturb the interaction of local chromatin structures. Taken together, the CRISPR labeling system would be the most suitable and promising method for the non-intrusive study of chromatin structure and dynamics.

### Long-term tracking

The 4D nucleome project highlights the application of the dimension of time since it is essential to measure chromatin dynamics, such as chromatin condensation and decondensation, chromatin loci diffusion, and pre-mRNA splicing and transport in this dimension. One of the most formidable tasks in live cell imaging is to carry out long-term tracking, as most of live-cell compatible fluorescent probes tend to rapidly photobleach. Several strategies, both in probes and imaging methodologies, have been developed to extend the duration of live cell imaging. An important development for probes is the signal amplification system based on the principle of protein multimerization. Recently, Tanenbaum et al. developed the

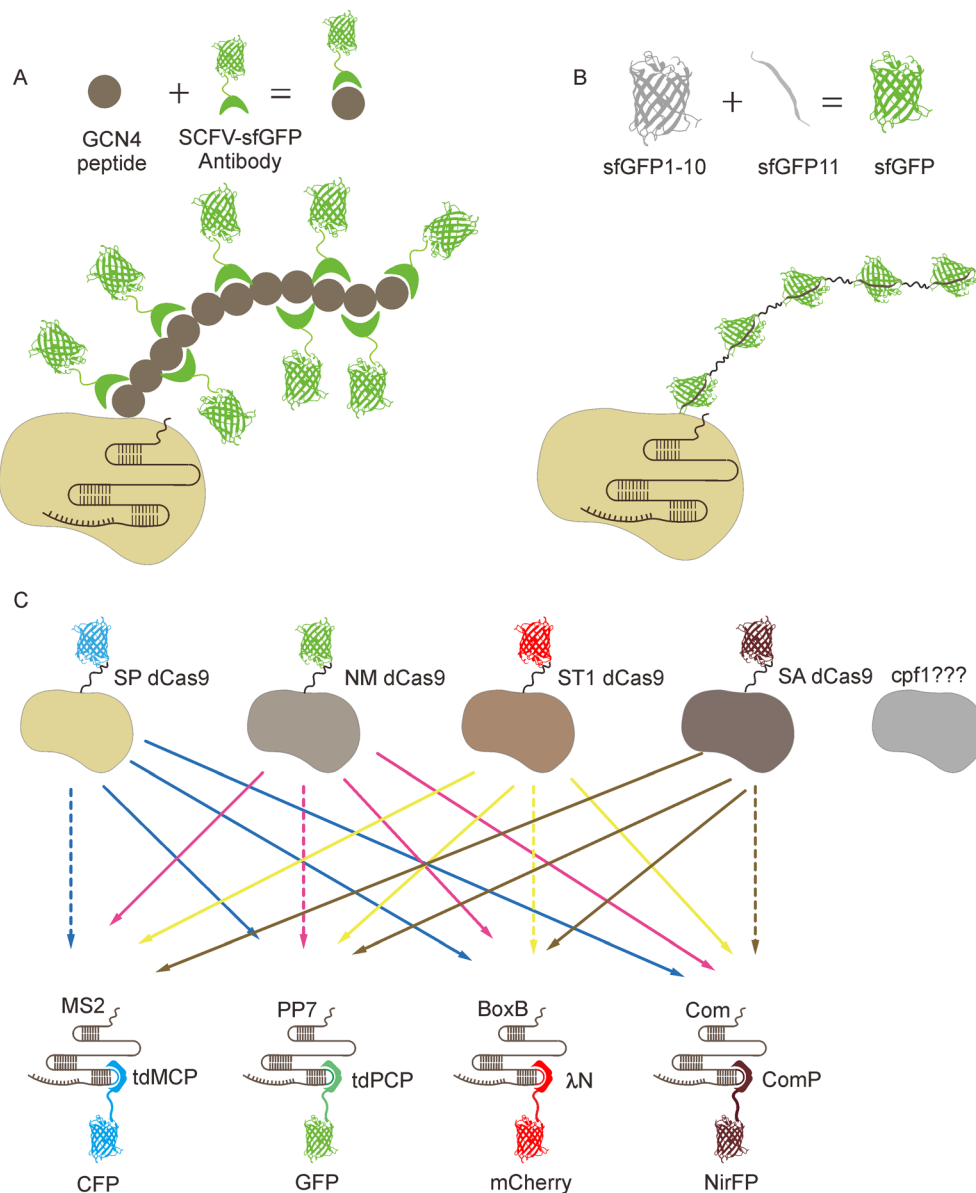
SunTag array consisting of 24 copies of a small peptide epitope (GCN4) that can recruit as many as 24 cognate single-chain variable fragment antibodies (scFV) fused to a sfGFP (super fold GFP) (Tanenbaum et al., 2014). Coupling the SunTag system with dCas9 established that telomeres could be labeled with a nearly 20-fold higher intensity compared to those labeled by dCas9 directly, without altering telomere mobility (Tanenbaum et al., 2014) (Fig. 3A). These signal amplification systems allow for both a lower laser power and shorter exposure time to be used, while maintaining the same image quality. This enables long-term tracking of probes with reduced photobleaching and phototoxicity side effects.

Another strategy is based on background fluorescence reduction, which is often implemented by bimolecular fluorescence complementation (BiFC). As an example, Kamiyama et al. developed a protein tagging system using split sfGFP, which reduces background fluorescence since the diffusive FP fragments are non-fluorescent (Kamiyama et al., 2016) (Fig. 3B). Interestingly, continuous re-supplementation of fluorescent probes to the site of interest also enables long-term imaging. Our previous work has also demonstrated a chromatin labeling method based on modified sgRNA, which exhibits significant resistance to photobleaching owing to the fast exchange rate of RNA aptamers and its target binding proteins (Shao et al., 2016). Based on these results, multiple MS2/PP7 stem loops have been inserted into modified sgRNA to amplify the fluorescent signal (Qin et al., 2017).

An additional strategy employs genetically encoded self-labeling tag proteins that can be coupled to cell-permeable synthetic dyes inside living cells. This results in brighter and more photostable reporters than fluorescent proteins alone (Grimm et al., 2015). In addition to probe optimization, the development of novel imaging methods can also improve long-term tracking performance. The newly developed methods of reflecting light-sheet microscopy and lattice light-sheet microscopy have demonstrated significant potential in improving both spatial and temporal resolution, while reducing specimen phototoxicity. This has, in turn, allowed for rapid 3D scanning of large fields as well as long-term tracking with negligible photobleaching outside of the focal plane, at extremely low excitation intensities (Gebhardt et al., 2013; Chen et al., 2014).

### High spatial resolution

As a result of light diffraction, the spatial resolution of conventional fluorescence microscopy is limited to a scale comparable to the wavelength of light, making it unable to resolve structures smaller than  $\sim 200$  nm. This scale, which is smaller than chromosome territories but larger than chromosomal loci and some functional domains (TAD, LAD), is insufficient for studying chromatin ultrastructure. With the advent of super-resolution microscopy in the last decade, several techniques, including STORM, PALM, STED, SIM, and RESOLFT, have been developed to “break” the



**Figure 3** Future development of the non-intrusive CRISPR-based chromatin labeling: signal amplification and multiplexing. (A) With the signal amplification device SunTag system, one dCas9 molecule is able to recruit more effectors at target site with one sgRNA. The SunTag array, which consists of 24 copies of a small peptide epitope (GCN4), can recruit as much as 24 cognate single-chain variable fragment antibody (scFV) fused to a sfGFP (scFV–sfGFP). (B) By splitting super-fold green fluorescent proteins into 1st-10 th and 11th  $\beta$ -strand and multimerizing the 11th  $\beta$ -strand in a tandem manner, a dCas9 can also recruit multiple sfGFP molecules with one sgRNA. Both (A) and (B) can be utilized to label the non-repetitive sequence. (C) Schematic diagram of a multiplexing chromosomal loci labeling strategy combining the modified sgRNAs and the orthogonal Cas9s. Increasing the SNR of labeling by fusing the same fluorescent protein with dCas9 and the modified sgRNA (dashed arrows). Combination of the orthogonal dCas9 proteins and the modified sgRNAs with different fluorescent proteins allows live-cell high multiplexing labeling by color coding (solid arrows).

diffraction limit to nanometer resolution. Multicolor-SIM was the first super resolution technique used to study the relations between chromatin and lamina, nuclear pore complex and nuclear compartmentalization (Schermettelet al., 2008), and X chromosome inactivation (Smeets et al., 2014). PALM and STORM have both been used to resolve the spatial organization of chromatin by labeling core histones with fluorescent protein or antibody (Bohn et al., 2010; Ricci et al.,

2015). One of the most exciting results came from a combination of the high spatial resolution of STORM with the high specificity of FISH techniques to systematically investigate the folding of chromatin in different epigenetic states (Boettiger et al., 2016).

However, to date, only static information has been obtained from these super-resolution imaging analyses. Imaging the dynamic regulation of chromatin structures at the sub-

chromosome scale would provide an enhanced understanding as to how chromatin folding affects its functional output. In particular, as the modular basis for higher-order chromosomal structures, TADs have only been detected by population-average Hi-C or FISH studies in fixed cells. A fascinating research area would be to visualize TADs in single, live cells and investigate in real time how or whether TADs form and disassemble dynamically. This goal will need further imaging technique development that could give consideration to both spatial and temporal resolution. Considering the techniques available nowadays, specific labeling by CRISPR-SunTag, coupled with light-sheet based super-resolution imaging, would be the best choice for uncovering the dynamics of chromatin ultrastructure in living cells (Li et al., 2015).

### Non-repetitive sequence labeling in living cells

Most genes contain non-repetitive sequences; however, the most promising non-intrusive chromatin labeling methods, based on TALE and CRISPR, have primarily been limited to repetitive sequence labeling. Although the non-repetitive part of *MUC4* has been successfully targeted with multiple sgRNAs targeting the genomic locus of interest in a cell line with minimal dCas9-EGFP expression (Chen et al., 2013), there have been no reports of other genes with non-repetitive sequences that could be labeled and imaged in live cells. The major problem is likely to be poor transfection efficiency as multiple sgRNAs need to be simultaneously transfected into one cell to reach a sufficiently high labeling density. Using an ultra-high titer lenti-virus library, Zhou et al. painted one chromosome in live cells with CRISPR imaging (Zhou et al., 2017). However, current co-transfection sgRNA co-expression systems remain poorly efficient and virus-based transfection approaches are relatively costly and labor intensive. We thus argue that it would be more feasible to label non-repetitive sequences by combining multiple sgRNA co-expression and signaling amplification systems, such as with SunTag (Cremer et al., 2008). Recently, we established a vector-independent method allowing multiple sgRNA expression cassettes to be assembled in series into a single plasmid. This synthetic biology-based strategy excels in its efficiency, controllability, and scalability. In combination with the SunTag system, we successfully labeled several non-repetitive genomic loci (Shao et al., 2017).

### Multi-functional imaging

For the study of chromatin structure and nuclear dynamics, the relationship between gene transcription and genomic position remains a complicated question (Chakalova and Fraser, 2008). In particular, when nuclear positioning affects gene expression, do genes move to specific functional compartments to be activated or silenced, or is relocation a consequence of the process of being activated or silenced?

To reach a casual conclusion, it is essential to measure

transcriptional activity and simultaneously monitor the nuclear position and dynamics of endogenous genes. To answer such questions, a multi-functional system needs to be constructed to allow the manipulation of a specific gene to any desired destination inside the nucleus and monitor transcription in real time. The 'Real-time Observation of Localization and Expression' (ROLEX) system has been established to enable the detection of sub-genome-wide mobility changes that depend on the state of Nanog transactivation in embryonic stem cells (Ochiai et al., 2015).

In 2008, three groups developed similar systems based on the random insertion of LacO repeats to support the inducible tethering of genes to the inner nuclear membrane and measurements of transcription output. However, the conclusions reached were not in agreement (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008), with the random, intrusive insertion potentially disturbing the local structure and thereby leading to the inconsistent conclusions.

In contrast, the chromatin imaging system, based on modified sgRNAs, has offered an admirable opportunity to address these questions because of the system's polyvalency. Imaging of gene position and dynamics can be achieved using the dCas9 channel, while perturbation or manipulation can be loaded to the sgRNA channel. owing to the ease of implementation, any gene that is targetable by the CRISPR system can be investigated using this multi-functional imaging system. We believe this platform will provide important insights to the questions discussed above.

### Multiplexing toward imagomics

Most current studies probe only one set of specific loci at a time. Global chromosome organization and dynamics thus remain unclear. To fill this gap, it is essential to fully automate the entire workflow to achieve high-throughput, time-resolved characterization, including imaging acquisition and data analysis of a large set of genomic loci dynamics in single cells. Shachar et al. constructed a FISH-based, high-throughput platform that could determine the spatial position of a gene in the 3D nuclear space and discover protein factors that determine genome organization (Shachar et al., 2015). However, data from fixed cells cannot provide information about the dynamic regulation of gene localization and transcription. Currently, high-throughput live cell genomic loci labeling largely remains difficult due to the aforementioned problem of non-repetitive sequence labeling.

Strategically speaking, high-throughput genomic loci labeling can be achieved by multiplexing labeling approaches in single cells. By inserting different RNA aptamers in the same sgRNA, Ma et al. developed a technology called CRISPRainbow, which allowed six chromosomal loci to be imaged simultaneously (Ma et al., 2016). Taken together, all current multicolor chromosomal CRISPR labeling methods are complementary to each other. Combining orthogonal Cas9 and modified sgRNA methods to achieve

the capabilities of higher levels of multiplexing labeling of multiple chromosomal loci simultaneously in single, living cells is of significant potential (Fig. 3C).

### Combining imaging and sequencing

Although imaging techniques are powerful for understanding the structure and dynamics of chromatin, their application has apparently been constrained by insufficient throughput. In contrast, the newly developed sequencing-based chromosome conformation capture techniques have been able to reveal paired interaction maps of any given genomic locus and thus provided global views of chromatin topology at the genome-wide level. The low throughput of imaging can be compensated to some extent by the combination of sequencing methods.

A multitude of elegant works have demonstrated the advantage of coupling imaging and sequencing. To name a few, the discovery and verification of lamina associated domains (LADs), by a powerful tool termed DamID, helped map the binding of chromatin DNA to the nuclear lamina in mammalian cells (Vogel et al., 2007). However, the spatial distribution and temporal dynamics of LADs were unknown until the development of imaging techniques based on the ‘contact and memory’ <sup>m6</sup>A-tracer. The imaging results showed that LADs remain constrained to the nuclear periphery in interphase and their positions are not detectably inherited, but instead are stochastically reshuffled upon mitosis (Kind et al., 2013). To further verify the existence of internal TAD domain organization, individual TADs have been visualized by multicolor 3D FISH with sets of differentially labeled TAD specific probes in combination with super-resolution microscopy (Fabre, et al., 2016). Further, the combination of super-resolution imaging and the ChIP-seq technique helped uncover the influence of histone epigenetic modification on the 3D folding of local chromatin DNA (Boettiger et al., 2016).

### Concluding remarks

In the post-genome era, the 4D nucleome project aims to understand fundamental intra-nuclear processes with high spatiotemporal sensitivity. To this end, live cell genomic loci labeling and imaging are of crucial importance. Although a number of traditional, and emerging, chromatin labeling techniques are available for both fixed and live cells, much more effort is still clearly required to develop fluorescence labeling methods capable of targeting arbitrary sequences non-intrusively to allow long-term, multiplexing, and high-throughput imaging of genomic loci and chromatin structures. The new generation of genome editing tools, such as TALE and CRISPR, have begun to offer such opportunities. In conjunction with the development of new probes and imaging methods, it would not be too far to reach our current goals. It

is also worth noting that rapid advances in super-resolution imaging and genome sequencing techniques will further open the possibilities for studying chromatin structure and dynamics. Taken together, these emerging technological advances will outline a next-generation effort toward the comprehensive delineation of chromatin at single-cell level with single-molecule resolution.

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### Compliance with ethics guidelines

#### Conflict of Interest

Shipeng Shao, Lei Chang, Yingping Hou, and Yujie Sun declare that they have no conflicts of interest.

#### Human and animal rights, and informed consent

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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