

Visualizing Nucleic Acid Loci with the CRISPR-Cas System: Current Approaches

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Abstract. The visualization of genomic loci in living cells is crucial for detecting mutations and observing the spatial proximity of DNA regions in the 3D nuclear environment. One of the primary applications of the clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9 (CRISPR/Cas9) system is the non-invasive, real-time labeling of DNA loci in living cells, enabled by its unique characteristics. Visualization of genomic loci has been made possible by the use of an endonuclease-inactive form of the Cas9 protein (dCas9) and sgRNA in combination with fluorescent molecules. However, using CRISPR/Cas9 for targeting DNA regions has certain limitations, the most significant being suboptimal signal-to-noise ratio, the need for multiplexed labeling, and the large size of the Cas fluorescent reporter system, which impacts the complex's functionality and complicates its delivery. Current variations of the method using CRISPR/dCas9 overcome these limitations in different ways. This review examines the evolution of genome locus visualization methods based on CRISPR/Cas9 from the initial use of the system to the present. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: CRISPR/Cas9; genome locus visualization; endonuclease-inactive Cas9 protein; nucleic acids.

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1 Introduction

The function of the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated (Cas) system is based on the ability of the Cas protein to bind with a guide RNA that is complementary to a specific target DNA sequence. Additionally, the protein recognizes a specific sequence of 3-6 nucleotide adjacent to the target DNA, enhancing the overall specificity of the system [1]. While the primary function of the CRISPR-Cas system is to induce double-strand breaks in target DNA, leading to deletions or replacements in the genome, it has been adapted for many other applications. Many of these are based on the mutagenic inactivation of the endonuclease activity of the Cas protein, known as dCas [2]. Despite losing the ability to induce DNA breaks, dCas retains its capacity to bind to guide RNA and target

DNA. Proteins linked to dCas via a linker (dCas-X) are known to maintain their activity, thereby expanding their range of applications, such as methylation and demethylation of specific DNA loci [3, 4], activation (CRISPRa) and inactivation (CRISPRi) of transcription in specific DNA regions [5, 6], base editing using adenosine deaminases [7], and analysis of chromatin remodeling factors via biotinylation (CAPTURE - CRISPR affinity purification in situ of regulatory elements, CasID) [8–10]. However, one of the earliest methods using dCas9 is the visualization of genomic loci with molecules, where dCas9 is fused to fluorescent protein (dCas9-FP). Today, this method has undergone numerous modifications aimed at optimizing labeling for key tasks such as marking loci in living cells and tracking them in real time. This is essential for better understanding the molecular mechanisms underlying chromatin dynamics. Beyond its

role in fundamental research, this technique also shows significant potential in medical diagnostics, particularly for detecting hereditary disorders. Currently, diseases caused by microdeletions and microduplications are predominantly diagnosed using Multiplex Ligation-dependent Probe Amplification (MLPA) – a technically challenging, multi-step technique requiring sample denaturation. Moreover, interpreting MLPA results can be complicated by factors such as mosaicism, contamination with normal tissue, and mutation heterozygosity [11]. Therefore, the CRISPR-Cas9 system offers a transformative alternative, enabling direct visualisation of genomic loci, overcoming many of the limitations of traditional diagnostic methods.

2 Visualization of Genomic Loci in Living Cells Using the CRISPR-Cas System

The non-invasive introduction of CRISPR-Cas components into cells, without the need for DNA fixation or denaturation, enables the dynamic visualization of genomic loci throughout the cell's life cycle. Currently, there are four CRISPR-Cas systems that can potentially be used for visualizing DNA or RNA loci. These include Class 2 CRISPR/Cas systems: CRISPR/Cas9, CRISPR/Cas12a, CRISPR/Cas13, and CRISPR/Cas12f (also known as CRISPR/Cas14). The Cas proteins in these systems have the ability to bind to guide RNA and target DNA, as well as introduce breaks in nucleic acids (NA). This makes them applicable in higher eukaryotic cells since these systems require fewer components than Class 1 CRISPR/Cas systems, simplifying their delivery [12].

At present, endonuclease-inactive forms of the Cas protein (dCas) have been developed for all these systems, with mutations in the HNH and RuvC domains, preserving all functions except for introducing breaks in NA [13, 14]. Comparing the mechanisms of different systems within Class 2, we see that CRISPR/Cas9 and CRISPR/Cas12a differ in two key aspects that are crucial for their application in higher eukaryotic cells. Specifically, Cas9 recognizes GC-rich protospacer adjacent motif (PAM) sequences and creates double-strand breaks in DNA, leaving blunt ends. In contrast, Cas12a requires recognition of AT-rich PAM sequences, resulting in the formation of sticky ends in DNA [15, 16]. Therefore, when visualizing DNA loci, the only relevant difference lies in the nucleotide composition of the PAM, which can be effectively addressed by using various Cas9 orthologues or modifying its PI domain. The other two Class 2 CRISPR/Cas systems show greater functional diversity. For instance, Cas13 recognizes and cleaves RNA sequences [17], making it suitable for labeling RNA molecules. Cas12f, discovered in 2018 and initially named Cas14, is distinct from previously identified Class 2 CRISPR/Cas proteins. The main distinguish due to its significantly smaller size (400–700 amino acids, compared to 1,250, 1,300, and 1,368 amino acids for Cas13, Cas12a, and Cas9, respectively) and operates as a dimer to introduce double-strand breaks in DNA, although its monomeric form can cleave single-stranded

DNA [18–20]. Additionally, Cas12f does not require recognition of a PAM sequence when targeting a specific DNA fragment using a 36-nucleotide guide RNA. Despite these advantages, Cas12f remains the least studied system. Therefore, the only two CRISPR/Cas systems currently used for labeling nucleic acid sequences are Cas9 and Cas13.

3 DNA Labelling

The first DNA locus labeling using the dCas9 protein fused to a fluorescent protein (EGFP) was demonstrated by Chen et al. [21]. This study showcased the method's capability for real-time analysis of chromatin behavior and conformation, offering potential insights into telomere length, gene copy number, and interactions between different loci. However, the existing limitations – such as the inability to simultaneously labeling of multiple loci in a single living cell, the need to introducing numerous guide RNAs, and a low signal-to-noise ratio (SNR) – emphasized the need for further method optimization.

The first issue (labeling multiple NA loci simultaneously) was addressed by using several dCas9 orthologs, each recognising different PAM sequences and targeting distinct DNA loci, along with fluorescent proteins of different colours. For instance, Chen et al. used two Cas9 orthologs (SpdCas9 and SadCas9) to label two loci at once [22], and Ma et al. utilised NmdCas9 and StdCas9 [23]. One key drawback of the dCas9-FP system was its SNR. To tackle this, researchers increased the number of fluorescent components in the reporter system. Tanenbaum et al. introduced the SunTag peptide scaffold, which can bind to an scFv antibody fused with GCN4 peptide and EGFP, allowing recruitment of up to 24 EGFP molecules. Using this system to label telomeres significantly increased signal intensity [24].

Later, Ye et al. [25] applied the SunTag approach with a brighter fluorescent protein, Neon Green, reducing the number of guide RNAs needed for labeling. However, issues remained, particularly with the size of the construct and high background fluorescence levels. To mitigate background fluorescence, Ye et al. [26] proposed a system where fluorescence is only activated in the complete ribonucleoprotein complex (dCas9-sgRNA). They enhanced the signal by attaching multiple copies of fluorescent protein fragments to the system's components, enabling the simultaneous visualization of up to three DNA loci. The individual components were fused to the N- and C-terminal parts of the yellow fluorescent protein Venus, as described previously. Through bimolecular fluorescence complementation (BiFC), Venus regained its full structure upon the formation of the ribonucleoprotein complex. This method eliminated background fluorescence from free dCas9 protein bound to the fluorescent molecule or guide RNA recruiting fluorescent proteins. Additionally, this study demonstrated signal enhancement by using alternative fluorescent proteins, such as mCherry and Clover, to label genomic loci, reducing background noise and improving signal strength. However, several limitations remain, primarily due to the bulkiness of the construct. Currently, signal enhancement

can be achieved not only by increasing the number of fluorescent molecules but also by improving their brightness and photostability. Organic dyes exhibit significantly greater brightness and photostability compared to the fluorescent proteins typically used in dCas systems. Furthermore, some of them have the added advantage of being able to penetrate cells. Grimm and colleagues were the first to create fluorescent dyes that maintained their spectroscopic characteristics while being capable of cell penetration by modifying tetramethylrhodamine [27]. This structural alteration of the dye allowed Deng et al. [28] to use Halo Tag labels linked to dCas9 proteins and fluorescent dyes as markers for visualising genomic loci in the nucleus. Additionally, molecular beacons (MBs), oligonucleotides complementary to target sequences equipped with fluorescent tags and quenchers, present a viable alternative. The quencher is attached in such a way that it suppresses fluorescence when the beacon is not bound to its target sequence. Wu et al. applied MBs in conjunction with the CRISPR-Cas9 system for DNA visualization, where MBs bind to guide RNA loops, thereby enhancing the signal while minimizing background fluorescence by preventing emission from unbound MBs [29]. Mao et al. further modified sgRNAs to interact with two distinct molecular beacons simultaneously, thereby expanding the diversity of color labels available for targeting specific DNA sequences [30].

All the methods discussed significantly improve the SNR and enable the labeling of various DNA sequences using fewer guide RNAs. However, in recent years, systems based on the Casilio method have shown even better results. This method uses Pumilio RNA-binding proteins that recognize specific 8-mer PUF-binding sequences (PBS), which are typically integrated into the guide RNA. By extending the guide RNA, more fluorescent proteins can be recruited, enabling complex multimerization and labeling of non-repetitive sequences. This system employs proteins such as Clover and Ruby, which are significantly brighter than commonly used GFP and mCherry, reducing the required number of guide RNAs to just one per non-repetitive sequence [31]. However, in this system, increasing signal intensity also raises background fluorescence due to the recruitment of fluorescent proteins by the tag molecule. This issue was mitigated by using the t-Deg peptide, which makes fluorescent proteins fluorogenic. These proteins are normally degraded through the proteasomal pathway via ubiquitination, but when a Pepper RNA aptamer is present, the t-Deg peptide binds to it, preventing degradation. By integrating this mechanism with the CRISPR-Cas9 system, Zhang et al. developed a fluorogenic CRISPR system that incorporates the Pepper aptamer into the guide RNA, recruiting a fluorogenic protein through a degron [32]. Since all fluorescent proteins not bound to the aptamer are degraded, this system significantly reduces background fluorescence and effectively visualizes both high-copy (e.g., telomeres) and low-copy DNA sequences. Additionally,

the system can label sequences using two fluorescent proteins simultaneously, enhancing the signal by recruiting multiple fluorescent proteins, one of which is tagged with the tDeg peptide. Interestingly, each guide RNA can recruit up to 12 fluorescent proteins to both of its loops. These proteins do not fluoresce when attached individually because the system removes 1–10 β -strands from the fluorescent proteins attached to one loop and the 11th β -strand from the proteins bound to the second loop. This approach enhances the signal without increasing background fluorescence and allows for the labeling of non-repetitive DNA sequences [33]. Compared to the Casilio method and two other new approaches, which also aim at visualising non-repetitive DNA sequences, this method features simpler guide RNA engineering and a less bulky fluorescent reporter system. For example, the CRISPR FISHer ((CRISPR-mediated) fluorescence *in situ* hybridisation amplifier) method utilises a system for local signal amplification by binding sgRNA through two PP7 aptamers to a foldon (C-terminal domain of T4 fibrin) complex made up of GFP, the PCP coat protein, and the trimeric motif of T4 fibrin. It was shown that attaching up to eight aptamers to the guide RNA and adding purified foldons to the system led to exponential complex assembly and local accumulation at the guide RNA targeting locus [34]. Peng et al. developed the Simultaneous Imaging and Manipulation of genomic loci by Biomolecular Assemblies (SIMBA) method to enhance signal strength during genomic locus labeling by leveraging multivalent interactions between HP1 α and SunTag repeats. In this approach, FRB-mCherry-HP1 α molecules are recruited to dCas9, which is fused to 24 SunTag repeats, and activated using rapamycin. To reduce background fluorescence, the Venus fluorescent protein is split across different guide RNA loops, while signal enhancement is achieved through the recruitment of large numbers of FRB-mCherry-HP1 α molecules [35]. These methods not only reduce background fluorescence but also enable the labeling of non-repetitive sequences and can even detect DNA breaks. This opens up the possibility of using these approaches to observe chromatin integrity and its dynamic changes in living cells. Additionally, they hold potential for monitoring genome editing processes by active Cas proteins. Nearly all the cited studies emphasise that background fluorescence affects target point detection and, consequently, any associated measurements – typically involving fluorescence intensity, photostability, and fluorophore lifetime. Several factors contribute to background fluorescence, including the nature of the effector molecule, the induction mechanism for transgene expression, and the delivery method. Studies highlight the advantages of lentiviral transduction over transient transfection for delivering constructs, as well as the impact of viral titer ratios on the various components involved. Excessive amounts of fluorescent products or bulky constructs can induce cytotoxic and phototoxic effects in cells. To mitigate transgene toxicity, some methods rely on inducible systems such as Tet-On,

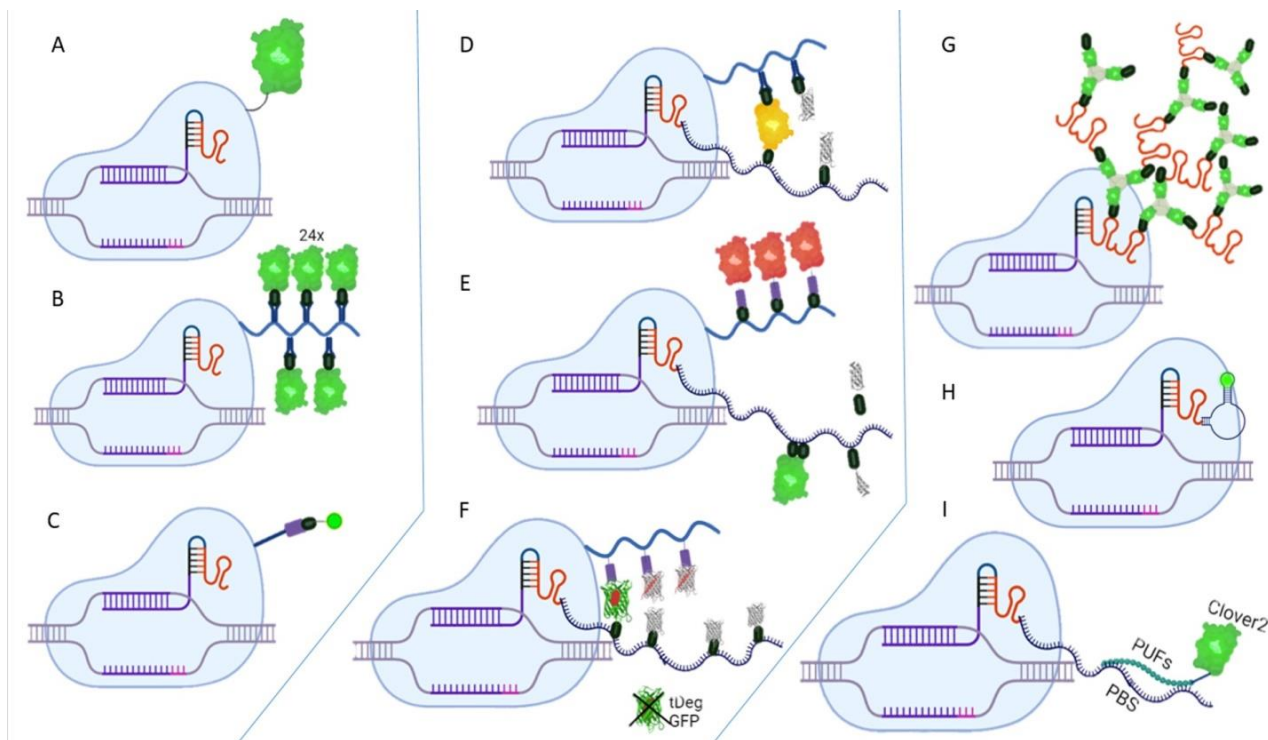


Fig. 1 Methods for labeling DNA loci using the CRISPR/Cas9 system. Methods based on the modification of dCas9: (A) dCas9-FP, (B) dCas9-SunTag, (C) dCas9-HaloTag. Methods based on the modification of both dCas9 and sgRNA: (D) dCas9-BiFC-sgRNA, (E) SIMBA, (F) dCas9-tDeg-sgRNA-Pepper. Methods based on the modification of sgRNA: (G) FISHer, (H) sgRNA-aptamer, (I) Casilio.

where delayed component introduction or fluorescent protein degradation mechanisms are often employed. Additionally, splitting fluorescent proteins into two non-fluorescent parts can help reduce background noise.

Therefore, among the methods described, the CRISPR-Cas9 approach utilizing fluorogenic split proteins remains the most promising for DNA labeling in living cells. Fig. 1 provides an overview of the techniques used for labeling genomic loci with these methods.

4 RNA Labelling

Even before the discovery of dCas13 and its ability to label RNA sequences, dCas9 was modified to recognise RNA sequences instead of DNA [36]. This was achieved by replacing the PAM-recognition domain with an oligonucleotide called PAMmer, which hybridises with the target RNA, allowing the new RdCas9 to target sequences in RNA. This protein is also smaller, making it easier to deliver the coding sequences compared to the original constructs used for DNA labelling. In subsequent work [37], Batra and colleagues demonstrated that RdCas9-EGFP could not only visualise microsatellite expansions of repeats but also eliminate such repeats in human RNA, leading to positive molecular changes in the cell. This could be considered a potential therapeutic approach for treating certain diseases.

A few years later, Sun et al. combined the RNA locus labelling system RdCas9-EGFP with the SunTag approach to label non-repetitive sequences in RNA [38]. They developed a fluorescent RNA aptamer called

Pepper, which has high photostability and brightness compared to its analogs. This monomeric aptamer features a wide range of emission maxima from blue to red, allowing for reliable visualisation of RNA in living cells. The study also demonstrated the ability to target the Pepper aptamer using the CRISPR/Cas9 system [39].

Subsequent approaches for visualising RNA loci have primarily relied on the CRISPR-Cas13 system, which targets RNA. Similar to Cas9, an endonuclease-inactive form of Cas13 was created and later fused with a fluorescent protein [40]. This method is user-friendly, does not require complex genetic manipulations, and effectively visualises both repetitive and non-repetitive RNA regions.

Further development led to the dCas13a-NF (Negative-Feedback) approach, which incorporates the KRAB repression domain. This modification reduces background fluorescence and allows for precise targeting of non-repetitive sequences [41]. Similar results have been observed when combining the dCas13 system with RNA aptamers [42].

Tang and his team achieved more accurate visualisation of RNA loci by using dCas13 linked to the Tat peptide, which interacts with fluorescent RNA aptamers via the trans-activation response (TAR) hairpin structure [43]. This design is flexible, smaller, and provides pinpoint signals, optimising the signal-to-noise ratio. Today, this method is considered the most effective for multiplexed visualisation of non-repetitive RNA sequences.

As seen in the Table 1, various DNA locus labeling systems have been developed to visualise highly repetitive sequences (such as telomeric, centromeric, and pericentromeric regions, as well as the exon of the MUC4 oncogene) and repetitive sequences (e.g., the intron of the MUC4 oncogene and the 5S rDNA gene). However, only a few studies have optimized these systems to label unique sequences and improve the signal-to-noise ratio (Table 1). This often increases the complexity of the fluorescent tag, complicating its delivery or functionality. Additionally, it is important to note that the main method for visualisation remains confocal fluorescence microscopy.

Table 1 Advantages and Limitations of CRISPR/Cas-Based Visualisation Methods.

Visualization method	Cas protein	Fluorescent reporter system	Target	Advantages	Limitations	Microscopy method	Ref.
Visualization of chromatin/genomic loci							
Fluorescent Protein Based	dCas 9	dCas9-EGPF	Telomeres*, MUC4 gene**	A simple and versatile tool for DNA visualization	Low signal-to-noise ratio (SNR), low labeling efficiency.	Fluorescent confocal microscopy	[21]
	dCas 9	dSaCas9-EGFP, dSpCas9-mCherry	Telomeres*, centromeres*, MUC4* gene*, 5S rDNA gene** and unique tandem repeats on chromosomes 1, 7, and 17**	Two-Color Image of CRISPR	Relatively weak fluorescence signal and low signal-to-noise ratio	Fluorescent Widefield Microscopy	[22]
	dCas 9	dSt1Cas9-EGFP, dSpCas9-EGFP, dNmCas9-mCherry	Telomeres*	Tet-on expression, highly specific detection based on the fluorescent protein's lifetime	A preliminary assessment of the dynamics of inducible expression of labeled orthologs is required	Fluorescent confocal microscopy	[44]
	dCas 9	dCas 9/sgRNA-aptamer-FP	Telomeres* and unique tandem repeats on chromosomes 1, 3, 7, 13, 14, and X**	Simultaneous labeling of six genetic loci	Complex sgRNA design; an effective delivery system is required	Fluorescent Phase-Contrast Microscopy	[45]
	dCas 9	dCas9-SunTag-scFv-GCN4-sfGFP	Telomeres*	Enhancing fluorescence intensity improves SNR, enabling the labeling of rare motifs	The large size of the reporter molecule impacts functionality of Cas9	Fluorescent confocal microscopy	[24]

Table 1 Cont.

	dCas 9	dCas9-SunTag-FRB-mCherry-HP1 α	Telomeres*, MUC4 gene**, MUC4.1gene****, IL-1B gene*****	Enhancing fluorescence intensity improves SNR, enabling the labeling of rare motifs	The large size of the reporter molecule impacts functionality of Cas9 and gene activity	Fluorescent confocal microscopy	[35]
	dCas 9	dCas9/sgRN A-PP7-PCP-T4 fibrin foldon-GFP	Telomeres*, Chr3Rep*, Chr13q34*, PPP1R2 locus****, SOX1 gene****, TOP3 gene****, TOP1gene****, BAGE gene****, TPTE gene****	Signal amplification enhances detection directly at the recognition site, improves the SNR and enables labeling of unique sequences	The large size of the reporter molecule complicates the its delivery.	Fluorescent confocal microscopy	[34]
Based on the BiFC Fluorescent Protein system	dCas 9	dCas9-N-Venus/sgRN A-C-Venus	Telomeres*, MUC4*, repetitive sequence on chromosome 3**, EMX-1 gene****	Improved signal-to-noise ratio, use of smaller amounts of guide RNA, high specificity	Low SNR, complex sgRNA design, and too large reporter molecule for labeling non-repetitive sequences	Fluorescent confocal microscopy	[26]
Based on organic dyes	dCas 9	dCas9-HaloTag/sgRNA	Telomeres*, centromeres*, major satellites*	Cost-effective, efficient	Altered cell physiology	Fluorescent confocal microscopy	[28]
	dCas 9	sgRNA-MTS-MB	Telomeres*, satellites*	Flexible combinations of fluorophore/cleavage agent pairs and MB/MTS sequences	MBS are costly	Fluorescent confocal microscopy	[29]
	dCas 9	dCas 9/3sgMUC4-dual-MTS-MB	Telomeres*, MUC4 gene*	Dynamic visualisation of unique genomic motifs using only three distinct sgRNAs. High sensitivity	MBS are costly. Simultaneous visualization of multiple genomic motifs is impossible	Fluorescent confocal microscopy	[30]
Fluorescent Protein Based	dCas 9	dCas9/SgRN A-Pepper-tdTomato-Tdeg	Telomeres*, centromeres*, MUC4 gene**, sequences on chromosomes 13*, 3, 9, 13, and 19*.	Efficient labeling of two repetitive sequences and labeling of a unique sequence with a single guide RNA. Reduced background signal	Complex sgRNA design, background signal retention, and use of multiple guide RNA	Fluorescent confocal microscopy	[32]

Table 1 Cont.

Fluorescent Protein Based	dCas9/SgRNA-Pepper-split12xGFP-Tdeg		Telomeres*, centromeres*, sequence on chromosome 3*, IDR1 **, IDR3** (intergenic DNA regions (IDRs)), FBN3 gene**, IL-1B gene***	Efficient labeling of two repetitive sequences and labeling of a unique sequence with a single guide RNA. Reduced background signal	Complex sgRNA design	Fluorescent confocal microscopy	[33]
RNA Visualization							
Fluorescent Protein Based	dCas 9	dCas9-FP	(CTG) repeats***, (CCTG) repeats**, and (CAG) repeats***	RNA Visualization	Limited visualization of low abundance mRNA	Fluorescent confocal microscopy	[37]
	dCas 9	dCas9-EGFP	HBG1 mRNA transcripts	Enables efficient visualization of mRNA	The large size of the reporter tag	Fluorescent confocal microscopy	[38]
Organic dyes based	dCas 9	dCas9/sgRNA-Pepper530	CAP31 and TMED2 mRNA transcripts	High fluorescence intensity and its rapid induction	The detailed structure-activity relationship of Pepper is poorly studied	Fluorescence confocal microscopy, two-photon excitation microscopy, Structured Illumination Microscopy (SIM)	[39]
Fluorescent Protein Based	dCas13a	dCas13a-EGFP/sgRNA	Transcripts of the SatIII, MUC4, GCN4, and NEAT1 genes*	RNA Visualization	Currently, no established guidelines exist for designing effective gRNAs targeting the RNA of interest	Fluorescence confocal microscopy, two-photon excitation microscopy, Structured Illumination Microscopy (SIM)	[40]
Fluorescent Protein Based	dCas13a	dCas13a-msfGFP-ZF-KRAB	Transcripts of the KRAS and CXC4 genes*	Optimised SNR	Visualization of intranuclear RNA is currently not possible	Fluorescent confocal microscopy	[41]
Fluorescent Protein Based	dCas13b	CRISPR-TRA-tag	Transcripts of the GCN4, MUC4, and SatIII lncRNA genes*	Flexibility of the modular design, smaller size and more precise fluorescent signal	Complex tag design?	Fluorescent confocal microscopy	[43]

* Highly repetitive sequences (350–500 binding sites).
** Repetitive sequences (40–100 binding sites).
*** Unique sequences (1–20 binding sites).
**** Unique sequences in heterochromatin regions.

5 Labeling DNA Sequences in Cells of Other Organisms

In addition to labelling sequences in human cells, research has also been conducted in other organisms. For example, dynamic labelling of DNA loci using various CRISPR/dCas9 methodologies has been demonstrated in mice, plants, and viruses. The systems used for different eukaryotic organisms remain unchanged; for example, dCas9 fused with GFP [46] and dCas9 with the Halo-ligand system [27, 47] have been employed for labelling DNA loci in mouse cells. Meanwhile, the first signal amplification method, which utilised three GFPs fused with dCas9 [48], and the tagging of two loci with two orthologs of dCas9 and a second red fluorescent protein, Ruby2 [49], were used for visualising DNA fragments in plant cells. Since both mouse and plant cells are eukaryotic, the principle of the system remains unchanged; only the delivery method varies, which subsequently affects the plasmid vector carrying the necessary construct and the promoter used for expression in specific organisms or their tissues. Thus, repeated telomeric and pericentric sequences have been visualised in mouse and plant cells, leading to a likely focus on human cells in further studies.

An intriguing approach involves labelling the nucleic acids of the pseudorabies virus (PRV) using quantum dots. These quantum dots are characterised by their high brightness, photostability, and unique emission properties, making them among the best fluorescent markers available [50]. The quantum dots were fused with dCas, which binds to the target nucleic acid via sgRNA, and were subsequently encapsulated within the virus during virion assembly [51]. This method opens up possibilities for visualising the nucleic acids of various viruses and for in-depth studies of the infection processes they induce in cells. However, it is essential to note the need for further research in this area to enhance signal compactness and improve the signal-to-background ratio.

6 Challenges in Using CRISPR/Cas Systems for Visualizing Genomic Loci in Living Organisms

One of the primary limitations of employing the CRISPR/Cas system in living organisms is the low signal brightness and inadequate signal-to-noise ratio. These issues can hinder the detection of fluorescent labels throughout entire organisms or within specific organs. One potential solution is to generate stable cell lines that express all components of the CRISPR system, which can then be introduced into the organism. However, it is crucial to consider the necessity of specific animal models and the immunogenicity of these interventions. Additionally, careful selection of tag molecules with detectable fluorescence is vital for successful visualisation [52].

7 Visualization Techniques

Confocal microscopy is the primary method for detecting fluorescent signals generated by CRISPR-Cas-based imaging techniques. There are two main types of confocal microscopes: laser-scanning and spinning-disk. Both systems enable optical sectioning of samples but rely on different technologies. Laser-scanning confocal microscopes generate images by sequentially capturing individual points along the Z-plane within the selected region, compiling them into a complete image. In contrast, spinning-disk confocal microscopes utilise a high-speed rotating disk perforated with multiple pinholes to scan the sample, providing a full image in real time. Both laser-scanning and spinning-disk microscopy enhance signal-to-noise ratios and facilitate visualisation of deeper cellular and tissue layers. However, spinning-disk systems offer superior speed, quantum efficiency, and signal-to-noise ratios while requiring lower laser power. Wide-field fluorescence microscopy is another commonly used method for visualisation. This technique captures all light emitted by the sample, regardless of focus, which can increase background fluorescence and complicate imaging of samples thicker than 50 μm [53]. Additionally, phase-contrast microscopy is employed for imaging, offering high-contrast visualisation by creating significant phase differences between sample structures. However, a notable limitation of this method is the appearance of bright halos, which can obscure adjacent structures [54]. Advanced imaging techniques, such as two-photon microscopy, structured illumination microscopy (SIM), and fluorescence lifetime imaging microscopy (FLIM), are particularly useful for detecting fluorescent labels in live cells and organisms and monitoring their dynamics. Two-photon microscopy uses near-infrared fluorescent proteins, which reduce light scattering in tissues, making it ideal for deep-tissue imaging [55]. SIM improves both lateral and axial resolution by enabling optical imaging near the diffraction limit [56]. FLIM measures the spatial distribution of fluorescent labels based on their fluorescence lifetime, effectively distinguishing target fluorescence from autofluorescence and reducing background interference [57]. Together, these methods provide high-resolution detection of fluorescent labels in live cells and organisms while minimising background fluorescence.

8 Conclusion

The development of new nucleic acid locus labelling methods using the CRISPR/Cas system opens unique opportunities not only for visualising the dynamics of chromatin loci but also for studying the interactions between specific genomic regions. These methods can facilitate the correction of genetic errors associated with the expansion of short repeats, which can have severe consequences for the organism, manifesting in various neurodegenerative and inherited disorders, such as Huntington's chorea, Kennedy disease, and others [37].

Using the CRISPR/Cas method, it is possible to determine the length of repetitive sequences [32] and identify different types of mutations (beyond single-nucleotide substitutions) [31]. These advancements hold significant potential for both fundamental research into the four-dimensional structure of the nucleome and practical medical applications, including the diagnosis of hereditary diseases, offering an alternative to the existing MLPA (Multiplex Ligation-dependent Probe Amplification) method, which has its own limitations [33]. Thus, the application of CRISPR/Cas can greatly improve the accuracy and reliability of

diagnostics, which is especially critical in the context of inherited diseases.

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Disclosures

The authors declare no conflict of interest.

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