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Trends in Genetics

Review

Expanding horizons of CRISPR applications beyond genome editing

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Clustered regularly interspaced short palindromic repeats (CRISPR) technologies have rapidly evolved beyond genome editing, transforming fields such as molecular diagnostics, biosensing, transcriptional regulation, molecular imaging, protein interaction mapping, and single-cell analysis. Emerging CRISPR-based diagnostics harness the collateral cleavage activity of CRISPR-associated (Cas) enzymes for rapid nucleic acid detection. Advanced biosensors extend CRISPR's capabilities to detect ions, metabolites, and proteins by integrating synthetic biology components. Catalytically inactive Cas proteins enable precise gene regulation and live-cell imaging of nucleic acids, whereas CRISPR-guided proximity labeling has revolutionized the mapping of biomolecular interactions. Recent single-cell CRISPR screens provide unprecedented resolution of cellular heterogeneity. Future research will focus on overcoming current limitations. The integration of CRISPR technologies with artificial intelligence (AI), spatial omics, and microfluidics is expected to further amplify their impact.

Highlights

Clustered regularly interspaced short palindromic repeats (CRISPR) systems now enable rapid, field-deployable nucleic acid diagnostics leveraging CRISPR-associated (Cas) enzymes Cas12, Cas13, and Cas14 collateral cleavage.

Engineered CRISPR-based biosensors integrate synthetic biology modules to detect diverse non-nucleic acid targets, including metabolites, ions, and proteins.

Catalytically inactive Cas proteins facilitate precise, programmable control of gene expression without DNA editing.

CRISPR-guided molecular imaging methods provide live-cell, multiplexed visualization of genomic loci, RNA dynamics, and chromatin rearrangements.

CRISPR-guided proximity labeling technologies enable spatially resolved mapping of transient protein interactions in living cells.

Single-cell CRISPR screening technologies significantly advance the resolution and scale of functional genomic analyses.

Emerging CRISPR technologies beyond genome editing

The discovery of CRISPR systems as programmable nucleases revolutionized genome editing, fundamentally transforming biological research and therapeutic development. More recently, CRISPR systems have rapidly expanded their application repertoire beyond genome editing, having unprecedented capabilities in molecular diagnostics, biosensing, gene regulation, molecular imaging, proteomic mapping, and single-cell functional genomics (Figure 1, Key figure). The rapid pace of recent innovations – such as **collateral cleavage** (see Glossary) diagnostics, living-cell imaging, and programmable **proximity labeling** – has opened up new avenues that are reshaping both fundamental biology and practical biotechnology. A comprehensive understanding of these emerging technologies is critical not only for advancing current applications but also for guiding future innovations and addressing unresolved technical challenges.

CRISPR diagnostics: rapid and field-deployable nucleic acid detection

The deployment of CRISPR-Cas systems in molecular diagnostics represents one of the most striking examples of how genome-editing tools have been repurposed for completely new applications (Table 1). The key to this transformation lies in the collateral cleavage activities exhibited by class 2 effectors such as Cas12, Cas13, and Cas14 (now renamed Cas12f [1]). Upon recognition of a specific target sequence, these enzymes activate indiscriminate nucleic acid cleavage: Cas12 [2] and Cas14 [3] cleave single-stranded DNA (ssDNA), while Cas13 cuts ssRNA [4]. These unique properties have been harnessed to develop highly sensitive, rapid, and cost-effective diagnostic platforms [3,5,6] (Figure 2).

Cas12-based HOLMES (one-hour low-cost multipurpose highly efficient system) [7] and DETECTR (DNA endonuclease-targeted CRISPR trans reporter) [6], along with Cas13-based

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Key figure

Expanding horizons of clustered regularly interspaced short palindromic repeats (CRISPR) applications beyond genome editing

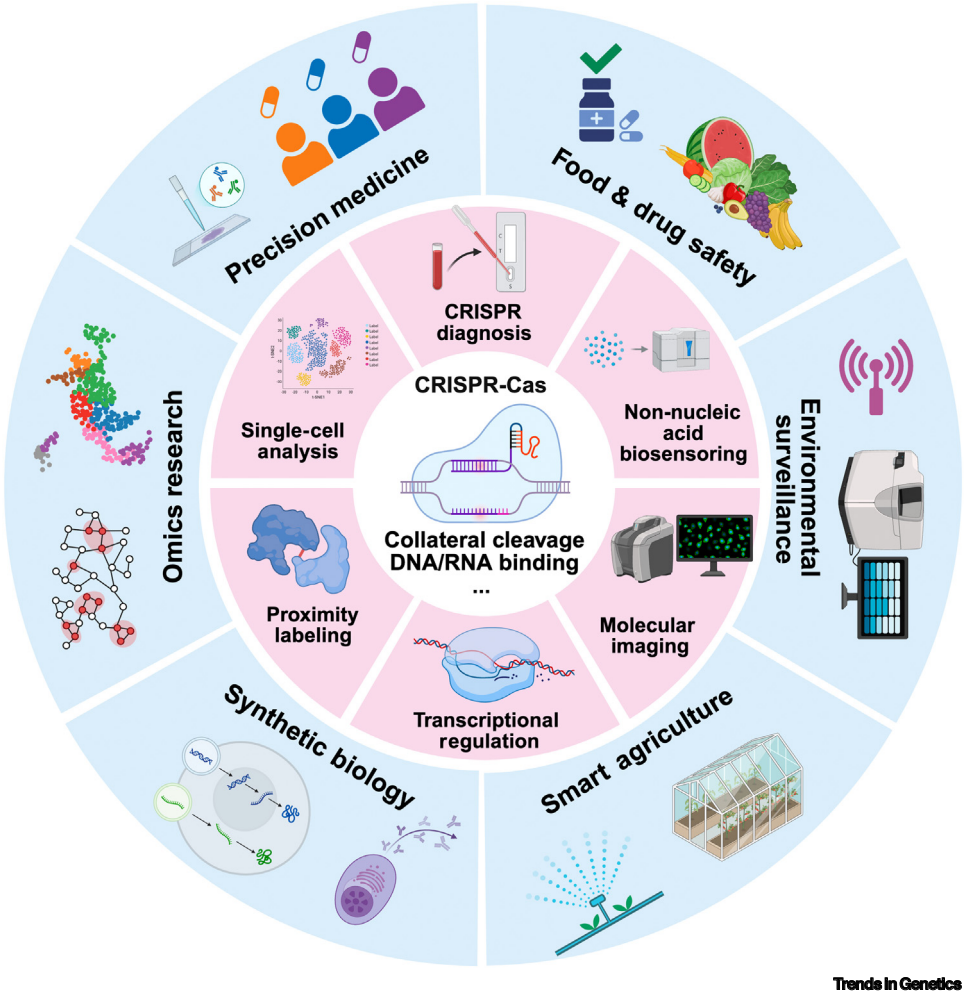

Figure 1. CRISPR systems, originally developed for genome editing, have rapidly evolved into a versatile molecular toolkit with wide-ranging applications beyond DNA cleavage. This conceptual overview illustrates the expansion of CRISPR-based technologies into six major domains: (i) diagnostics, leveraging Cas12, Cas13, and Cas14 trans-cleavage activity for rapid and field-deployable nucleic acid detection; (ii) non-nucleic acid biosensing, integrating aptamers, DNAzymes, and synthetic biology circuits to enable detection of ions, small molecules, and proteins; (iii) programmable gene regulation using dCas9 and dCas12 effectors fused to transcriptional repressors or activators for tunable control of gene expression; (iv) live-cell molecular imaging, applying dCas-fusion proteins and fluorescence strategies for real-time visualization of nucleic acids and RNA dynamics; (v) CRISPR-guided proximity labeling, employing APEX2, BioID, or TurboID enzymes to map chromatin- and transcript-associated proteomes *in situ*; and (vi) single-cell functional genomics, combining CRISPR perturbations with single-cell RNA sequencing (scRNA-seq) [101] and multi-omic profiling to dissect gene functions with unprecedented resolution. Together, these platforms reframe CRISPR systems as modular information processors for interrogating and reprogramming biological systems. Six domains are functionally connected to real-world applications of precision medicine, food and environmental safety, smart agriculture, synthetic biology, cell engineering, and omics research.

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Table 1. Representative CRISPR-based platforms for diagnostics, biosensing, gene regulation, imaging, proximity labeling, and single-cell functional genomics

Applications	Target type	Detection target	Cas effector	Platform	Refs
Nucleic acid targets sensing	dsDNA	Methylated DNA	Cas9	CAS-EXPAR	[108]
		mtDNA SNV		CasPLA	[109]
		cfDNA, ctDNA	Cas9, Cas12a	CUT-PCR	[110]
		HPV	Cas12a	DETECTR	[111]
		DNA SNP, PRV, JEV		HOLMES	[7]
		HPV-16, PB-19		E-CRISPR	[112]
		miRNA, *Acinetobacter baumannii*		With DSN, aptamers	[113]
		HPV-16, *Escherichia coli* plasmid		CRISPR Cas12a-gFET	[34]
		CTCs		With aptamers	[114]
		HPV, ABO, SNP, cfDNA	Cas12b	CDetection	[115]
		SNP, JEV, circRNA, DNA methylation		HOLMESv2	[15]
		SARS-CoV-2		CASdetec	[116]
		SARS-CoV-2		STOPCovid	[16]
		Staphylococcus aureus, *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*	CasΦ (Cas12j)	TCC	[117]
	ssDNA	SNP	Cas14a	Cas14a-DETECTR	[3]
	ssRNA	SARS-CoV-2	Cas13a	SHINE	[118]
		SARS-CoV-2, ZIKV, KSHV		PEARL	[119]
		SARS-CoV-2		CREST	[120]
		ZIKV, DENV, 16S rRNA V3, SNP, cfDNA		SHERLOCK	[5]
		ZIKV, DENV, YFV, WNV		HUDSON	[11]
		LCMV, IAV, VSV		CARVER	[121]
		SARS-CoV-2		With Cas-HRP	[122]
		SARS-CoV-2	Cas13a, Csm6	FIND-IT	[123]
Non-nucleic acid targets sensing	Ions	Pb^{2+}	Cas12a	With DNAzymes	[113]
		Pb^{2+}		With DNAzymes	[28]
		Hg^{2+}		With TSDR	[32]
		Na^{+}		With DNAzymes	[30]
		F^{-}	Cas13a	FRITCas13a	[31]
		Zn^{2+}, Cu^{2+}, F^{-}		SPRINT	[124]

Glossary

Aptamers: short synthetic oligonucleotides (DNA or RNA) designed to bind specific molecular targets such as ions, proteins, or small molecules. Widely used in biosensors due to their high specificity and programmability.
Collateral cleavage: a secondary, non-specific cleavage activity activated in some CRISPR effectors (Cas12, Cas13, Cas14) after target recognition, allowing signal amplification in molecular diagnostics.
CRISPR activation (CRISPRa): a technique utilizing catalytically inactive Cas proteins (dCas9/dCas12a) to modulate gene expression without DNA editing. CRISPRa activates transcription via recruitment of transcriptional activators.
CRISPR interference (CRISPRi): a technique utilizing catalytically inactive Cas proteins (dCas9/dCas12a) to modulate gene expression without DNA editing. CRISPRi represses transcription by steric hindrance.
dCas9 (dead Cas9): a catalytically inactive variant of Cas9 that retains programmable DNA-binding activity without introducing double-stranded breaks, commonly used for gene regulation, molecular imaging, and proximity labeling.
DNAzyme: a catalytic DNA sequence capable of cleaving target nucleic acids upon specific ion or molecular activation, widely used in biosensors to transduce chemical stimuli into nucleic acid signals for CRISPR detection.
Perturb-seq: a method combining pooled CRISPR screens with single-cell RNA sequencing (scRNA-seq) to measure the functional effects of genetic perturbations at single-cell resolution, enabling detailed mapping of gene networks and cellular heterogeneity.
Proximity labeling: techniques utilizing enzyme tags (such as BioID, TurboID, or APEX2) fused to targeting molecules (e.g., dCas9, dCas13) to covalently tag nearby proteins, enabling spatial mapping of protein–protein or protein–nucleic acid interactions within cells.
Riboswitch: an RNA regulatory element that modulates gene expression by changing its secondary structure upon binding specific small-molecule ligands, commonly integrated into synthetic circuits and biosensors to control CRISPR activation signals.
Single-cell CRISPR screening: technologies integrating CRISPR

Table 1. (continued)

Applications	Target type	Detection target	Cas effector	Platform	Refs
	Proteins	Streptavidin/biotin ADIG/DIG	Cas12a	With small molecule modified activator DNA	[125]
		TGF-β1		E-CRISPR	[112]
		Exosomes		With aptamers	[126]
		SAA1, FV	Cas12a and Cas13a	DNAzyme walkers with Cas12a/Cas13a	[25]
		Creatine kinase MB	Cas14a	With aptamers	[127]
	Small molecules/metabolites	Uric acid, p-HBA, tetracycline	Cas12a	CaT-SMelor	[39]
		Cocaine		With aptamers	[128]
		Melamine		With aptamers	[40]
		17β-estradiol		With aptamers	[129]
		Kanamycin		With aptamers	[130]
		Tobramycin		Sensor-ss, Sensor-ds	[34]
		Tetracycline, doxycycline, oxytetracycline		With aTFs	[33]
		ATP		With aptamers	[30]
		ATP		With aptamers	[122]
		AFP		With aptamers	[131]
		AFP		With aptamers	[128]
		ATP, GTP, SAM, FAD, FMN, 5HTP, hypoxanthine, serotonin, SAM, SAH, 5HTP, adenine, guanine, tetracycline, rifampicin, immucillin-H	Cas13a	SPRINT	[124]
		Ampicillin	Cas14a	With aptamers	[132]
Transcriptional regulation	CRISPRi	–	dCas9	dCas9-VP64, dCas9-p65AD	[58]
				dCas9-KRAB-MeCP2	[133]
				Multiplexed CRISPRi	[134]
				Multistable and dynamic CRISPRi	[135]
			dCas12a, dCas12e, dCas12j	dCas12-KRAB	[136]
	CRISPRa	–	dCas9	dCas9-VP64	[137]
				dCas9-KRAB	[58]
				SunTag	[61]
				dCas9-VPR	[60]
				dCas9-TV	[138]
				scRNA	[139]
				SAM	[62]

(continued on next page)

perturbations (gene knockouts, knockdowns, or activations) with single-cell transcriptomics or epigenomics to decode gene function and regulatory mechanisms at single-cell resolution (e.g., Perturb-seq, ECCITE-seq, CRISPR-sciATAC).

Toehold switch: a synthetic RNA regulator designed to alter its secondary structure upon binding a complementary trigger RNA sequence, used in biosensors to produce or reveal CRISPR activators.

Table 1. (continued)

Applications	Target type	Detection target	Cas effector	Platform	Refs
				CRISPR-Act1.0	[140]
				CRISPR-Act2.0	[141]
				CRISPR-Act3.0	[63]
			dCas12a	dCas12-VPR	[142]
			dCas12a, dCas12e, dCas12j	dCas12-VPR	[136]
Molecular imaging	–	–	dCas9	With optimized sgRNA and EGFP	[69]
				With SunTag	[61]
				With optimized sgRNA and CRISPRainbow	[70]
				CRISPR/Casilio	[143]
				CRISPR/Pepper-tDeg	[144]
				With PAMmers and dCas9-GFP/mCherry	[145]
				CRISPR-Sunspot	[75]
			dCas9, dCas13b	With optimized sgRNA and fluorescent proteins (FPs)	[73]
			dPspCas13b	CasFAS	[146]
				CRISPR-TRAP-tag	[147]
			dEcCas6	Cas6FC	[76]
			dCsm3	smLiveFISH	[77]
Proximity labeling	–	–	dCas9	C-BERST	[86]
				GLoPro	[148]
				CARGO-BioID	[87]
			dCas13	CRUIS	[90]
				eCRUIS	[91]
			dCasRx	CARPID	[92]
Single-cell analysis	–	–	dCas9	Direct-capture Perturb-seq	[98]
			Cas9	CRISPR-sciATAC	[100]
			Cas9/dCas9	ECCITE-seq	[99]
			Cas9/dCas9	Perturb-FISH	[102]
			RfxCas13d	CaRPool-seq	[97]

SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) [5], represent the most widely adopted CRISPR-based diagnostic platforms. Cas14-based DETECTR [3], due to its miniature size, offers potential advantages in delivery and point-of-care integration, but remains an emerging system requiring further validation. HOLMES and Cas12-based DETECTR utilize Cas12a's collateral activity on single-stranded DNA (ssDNA) reporters following isothermal amplification, enabling rapid detection of human papillomavirus (HPV) genotypes [6] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from clinical samples with visual or fluorescent readouts [8]. SHERLOCK combines isothermal amplification with Cas13a-mediated cleavage of RNA

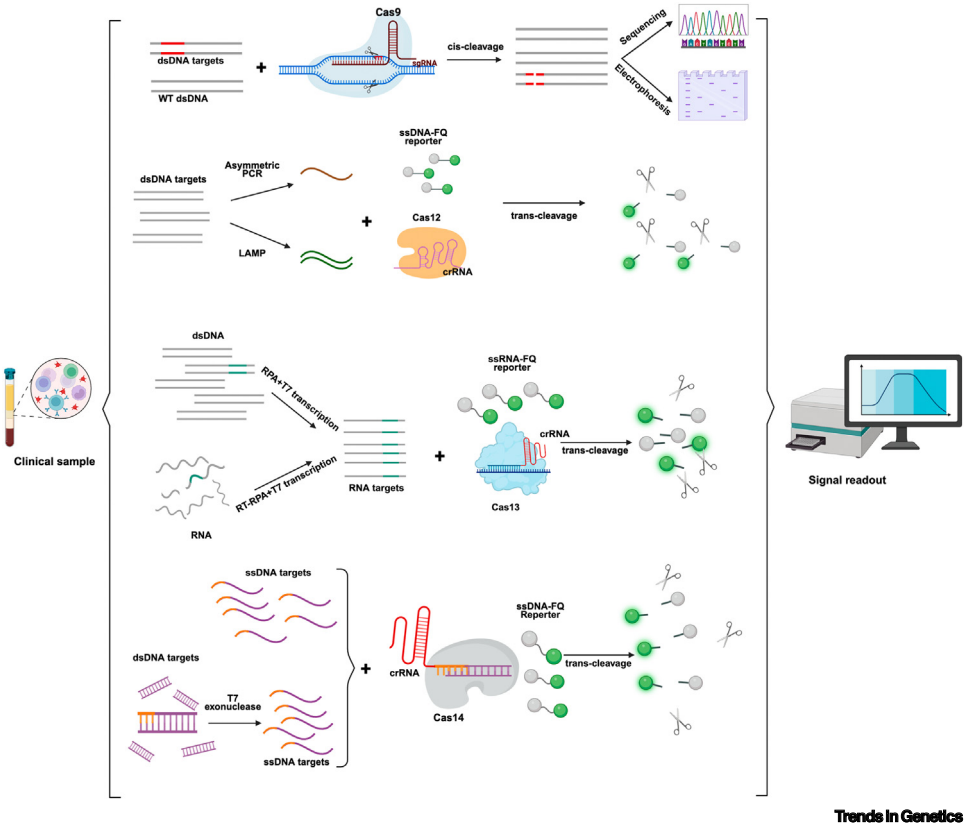


Figure 2. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems for rapid and field-deployable molecular diagnostics. This schematic summarizes key CRISPR-based platforms for nucleic acid detection. While Cas9 lacks trans-cleavage activity, it has been adapted for diagnostics via protospacer-adjacent motif (PAM)-dependent recognition (e.g., NASBACC [17]) or dead Cas9 (dCas9)-mediated binding in microfluidic and fluorescent biosensors. By contrast, Cas12, Cas13, and Cas14 exhibit robust collateral cleavage, enabling signal amplification upon target recognition. Cas13-based SHERLOCK [5] detects RNA via guide-directed recognition and RNA reporter cleavage, supporting fluorescence and lateral flow readouts. Cas12-based DETECTR [6] and HOLMES [7] targets DNA and cleaves single-stranded DNA (ssDNA) reporters post-amplification for rapid detection of pathogens such as human papillomavirus (HPV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). STOPCovid [16] further simplifies workflows by coupling reverse transcription loop-mediated isothermal amplification (RT-LAMP) with Cas12b in a single reaction. Cas14, a miniature nuclease without PAM constraints, enables detection of fragmented DNA, including cell-free nucleic acids. Emerging platforms incorporate orthogonal Cas effectors, lateral flow devices, and amplification-free designs. Upstream tools such as HUDSON [11] enable direct analysis from unextracted samples. These CRISPR diagnostic systems are positioned as practical alternatives to PCR for rapid pathogen detection in clinical settings. Abbreviations: aTFs, allosteric transcription factors; RNA Pol, RNA polymerase.

reporters, achieving attomolar sensitivity and single-base resolution [5,9,10]. It has been successfully applied to the detection of viral pathogens (e.g., Epstein–Barr virus, Zika virus, Dengue virus, SARS-CoV-2), tumor mutations, and environmental targets [11–14].

To enhance multiplexing capabilities and usability, second-generation platforms such as HOLMESv2, one of the first systems integrating RT-LAMP (reverse transcription loop-mediated isothermal amplification) and Cas12b for one-pot nucleic acid detection [15], and SHERLOCKv2, which introduced orthogonal Cas enzymes and lateral-flow detection [9], while HUDSON (heating unextracted diagnostic samples to obliterate nucleases) enabled RNA preservation without the need for nucleic acid extraction [11]. For DNA targets, Cas12-based systems have demonstrated high sensitivity through platforms such as HOLMES [7] and STOPCovid [16].

Although Cas9 lacks trans-cleavage activity, it has been repurposed for diagnostic applications through selective cleavage or binding. NASBACC (nucleic acid sequence-based amplification CRISPR cleavage) was among the first platforms to use Cas9 to distinguish Zika virus lineages via protospacer-adjacent motif (PAM)-dependent recognition [17]. The FLASH (finding low abundance sequences by hybridization) method combined programmable Cas9 cleavage with next-generation sequencing (NGS) for multiplex detection of antimicrobial resistance genes [18]. Catalytically inactive **dCas9 (dead Cas9)** has also been incorporated into microfluidic biosensors and fluorescent readouts to improve real-time detection and portability [19].

Cas14, a miniature endonuclease from the DPANN archaea (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota and Nanohaloarchaeota), has introduced new possibilities for compact diagnostics [3]. Its ability to cleave ssDNA without PAM constraints offers a significant advantage in detecting fragmented DNA in degraded or complex samples, such as cell-free DNA or environmental specimens. Cas14-DETECTR and related systems provide high specificity and flexibility in target selection [3,20].

Despite their promise, CRISPR-based diagnostic systems [21] face several limitations, including reliance on preamplification steps, variable performance in complex matrices, and the risk of off-target activation. In addition, RNA-targeting systems struggle with stability due to RNase contamination in field settings. To overcome these challenges, researchers are developing amplification-free formats using engineered Cas effectors with higher intrinsic activity, synthetic guide RNA (gRNA) designs for improved specificity, and microfluidic platforms that enable sample-in, answer-out platforms [22].

Taken together, CRISPR-Cas-based diagnostics represent a paradigm shift in nucleic acid testing, offering field-deployable alternatives to conventional PCR-based assays. As ongoing efforts aim to broaden target range, minimize user intervention, and enable quantitative readouts, these systems are poised to become indispensable tools for infectious disease surveillance, precision oncology, and public health preparedness [23].

CRISPR-based biosensors for non-nucleic acid targets

Beyond nucleic acids, recent advances in CRISPR systems have enabled the detection of a wide array of non-nucleic acid targets (NNTs) (Table 1), including metal ions, small molecules, metabolites, and proteins, significantly expanding their diagnostic potential. Since Cas12, Cas13, and Cas14 effectors are inherently activated only by nucleic acid triggers, engineering CRISPR biosensors for NNT detection requires a signal conversion strategy that transduces the presence of the target molecule into a detectable nucleic acid intermediate.

The most common approach involves coupling CRISPR-Cas systems with **aptamers** [24], **DNAzymes**, or **riboswitches** – nucleic acid-based molecular recognition elements that undergo conformational changes upon binding to specific targets. These elements can be engineered to release a trigger strand, activate transcription, or alter the structure of a CRISPR-activating reporter upon ligand binding. This design enables the indirect activation of Cas12, Cas13, and Cas14 collateral cleavage, which can then be measured using fluorescence, colorimetric, electrochemical, or lateral-flow readouts [25,26] (Figure 3). For example, DNAzyme-mediated Pb^{2+} detection has been integrated with CRISPR-Cas12a systems to achieve sensitive and selective sensing of lead ions in environmental water samples [27], and in drinking water with a limit of detection (LOD) as low as 0.48 nM [28]. In another case, ATP-binding aptamers have been used to control transcription templates or strand displacement cascades that initiate Cas12a activity, enabling detection of femtomolar-level Pb^{2+} [29]. Similar constructs have been adapted for a wide variety of targets, including ions such as Na^{+} [30], F^{+} [31], and Hg^{2+} [32];

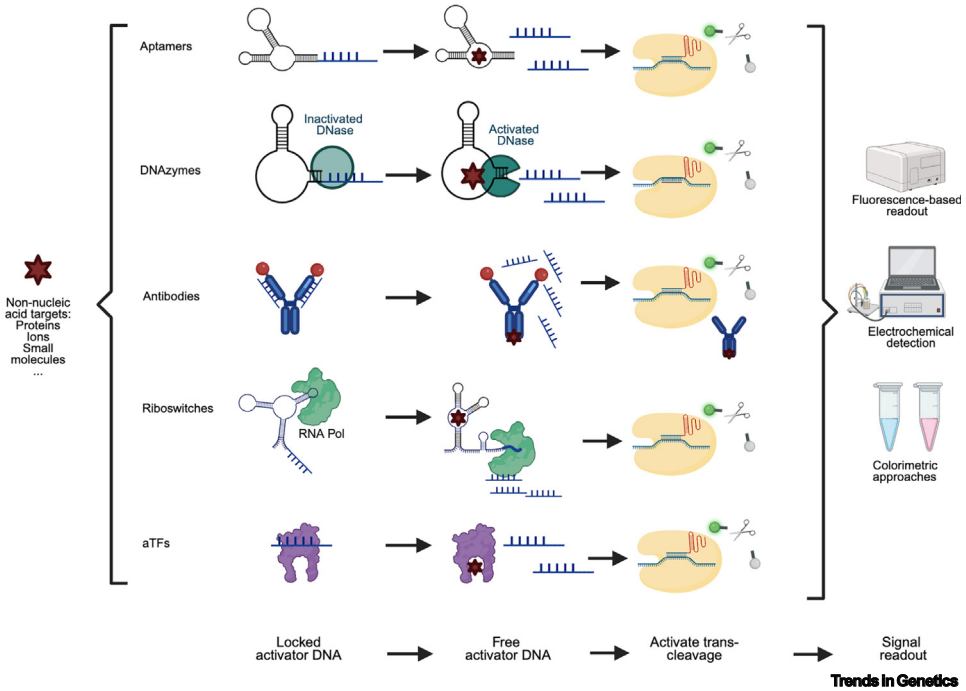


Figure 3. Signal transduction strategies for clustered regularly interspaced short palindromic repeats (CRISPR)-based detection of non-nucleic acid targets (NNTs). To enable CRISPR detection of NNTs such as small molecules, metal ions, and proteins, biosensors utilize signal conversion mechanisms that produce a nucleic acid intermediate capable of activating Cas effectors. Aptamers undergo conformational changes upon ligand binding to release a trigger strand; DNAzymes cleave inhibitory sequences in the presence of specific ions (e.g., Pb2+ , Hg2+); and anti-bodies can initiate strand displacement reactions or structural rearrangements that liberate activators. Riboswitches and al-losteric transcription factors (aTFs) modulate transcriptional output or DNA accessibility in response to their cognate ligands. These triggers initiate collateral cleavage by CRISPR-associated enzymes Cas12 and Cas13, generating fluorescent, color-imetric, or electrochemical signals. Additionally, Cas14, distinguished by its small size and lack of protospacer-adjacent motif (PAM) constraints, has been increasingly utilized in NNT detection due to its compatibility with fragmented DNA and highly compact biosensor designs. Together, these modular strategies make CRISPR-based biosensors hold potential for broad environmental monitoring, clinical diagnostics, and food safety applications. Abbreviations: FP, fluorescent protein; gRNA, guide RNA; ScFv, single-chain variable fragment; sgRNA, single-guideRNA.

antibiotics such as tetracycline [33], kanamycin [19], tobramycin [34], and ampicillin [35]; and common metabolites such as ATP [36], NAD^+ [37], glucose [38], and uric acid [39].

Food safety testing has also benefited from CRISPR-Cas-enabled biosensing. For instance, melamine – a toxic additive once implicated in milk powder contamination – has been detected using aptamer-controlled Cas12a sensors [40]. Similarly, methamphetamine and cocaine have also been detected using CRISPR-based assays [41]. Moreover, Cas13-based platforms have been developed for detecting microRNAs (miRNAs) and exosome-derived RNA from biological fluids, with potential applications in early cancer screening and prognosis monitoring [42].

Despite these advances, several challenges limit the robustness and field deployment of CRISPR NNT biosensors. First, the complexity of signal conversion cascades can compromise sensitivity and reproducibility, particularly in complex biological matrices. Unlike direct nucleic acid detection, these systems rely on multistep biochemical logic circuits, which introduce variability, potential false positives, and timing constraints. Second, many aptamer–ligand interactions exhibit

moderate binding affinities or poor selectivity under physiological conditions, which may reduce diagnostic accuracy. To address these limitations, researchers are exploring strategies to improve biosensor modularity and scalability. One promising direction involves integrating cell-free synthetic biology systems – such as **toehold switches** [43] or transcription-based amplification cascades [44] – with CRISPR-Cas readouts, enabling tunable and multiplexed detection schemes. Additionally, machine-learning-guided aptamer selection [45], improved DNAzyme libraries [46], and *in vitro* selection technologies such as SELEX (systematic evolution of ligands by exponential enrichment) technologies [47] are expected to improve the performance of molecular recognition elements used in CRISPR biosensors.

Another emerging avenue involves coupling CRISPR-based sensors with wearable and portable platforms [48]. Innovations such as microfluidic integration [49], paper-based diagnostics [50], and smartphone-based readouts [51] are enabling the development of real-time, point-of-care tools suitable for home, field, or low-resource settings. These features are particularly relevant for applications such as food-contaminant monitoring, detection of drug residues in agriculture, and environmental surveillance.

In summary, CRISPR-based biosensors for NNTs are unlocking new opportunities for molecular diagnostics beyond genomics. By bridging synthetic biology circuits with programmable nucleases, these systems offer powerful, customizable, and field-deployable tools for detecting a wide range of analytes.

Programmable gene regulation with CRISPR

In addition to sensing applications, CRISPR systems have also been repurposed for targeted gene regulation, offering powerful tools to manipulate gene expression without altering DNA sequences (Figure 4). This is primarily achieved using catalytically inactive variants of Cas – most commonly dCas9 and dCas12a – which retain DNA-binding capability but lack endonuclease activity. These dead Cas proteins serve as programmable DNA-binding scaffolds to deliver transcriptional repressors or activators to specific genomic loci, enabling precise modulation of gene expression without altering the coding sequences [52].

CRISPR interference (CRISPRi) (Figure 4A) functions by sterically blocking transcription initiation or elongation. In its simplest form, dCas9 alone can inhibit transcription by targeting promoter regions or early coding sequences. Enhanced repression is achieved by fusing dCas9 to transcriptional repressors such as KRAB (Krüppel-associated box) [53], SID4X [54,55], or methyltransferases [56], which recruit chromatin silencing complexes. Besides dCas9, other CRISPR effectors such as ddCas12a (formerly known as ddCpf1) have also been used successfully [57]. CRISPRi has been successfully applied across bacterial, yeast, plant, and mammalian systems to repress both endogenous genes and exogenous reporters [58]. CRISPRi exhibits strand-dependent activity within transcribed regions in both prokaryotic and eukaryotic systems, favoring the non-template strand for effective repression. However, when targeting promoter regions, CRISPRi generally functions in a strand-independent manner. Conversely, **CRISPR activation (CRISPRa)** relies on the recruitment of transcriptional activators to gene promoters or enhancers (Figure 4B). Several architectures have been developed for this purpose. The dCas9–VP64 fusion was among the first systems capable of activating silent or weakly expressed genes [59], followed by more potent activators such as VPR (VP64–p65–Rta), which combines multiple activation domains into a single polypeptide [60]. The SunTag system amplifies transcriptional activation by fusing dCas9 to a repeat peptide array that recruits multiple single-chain variable fragment (scFv)-tagged activators (e.g., VP64, p300), enabling enhanced multimerization and signal amplification [61].

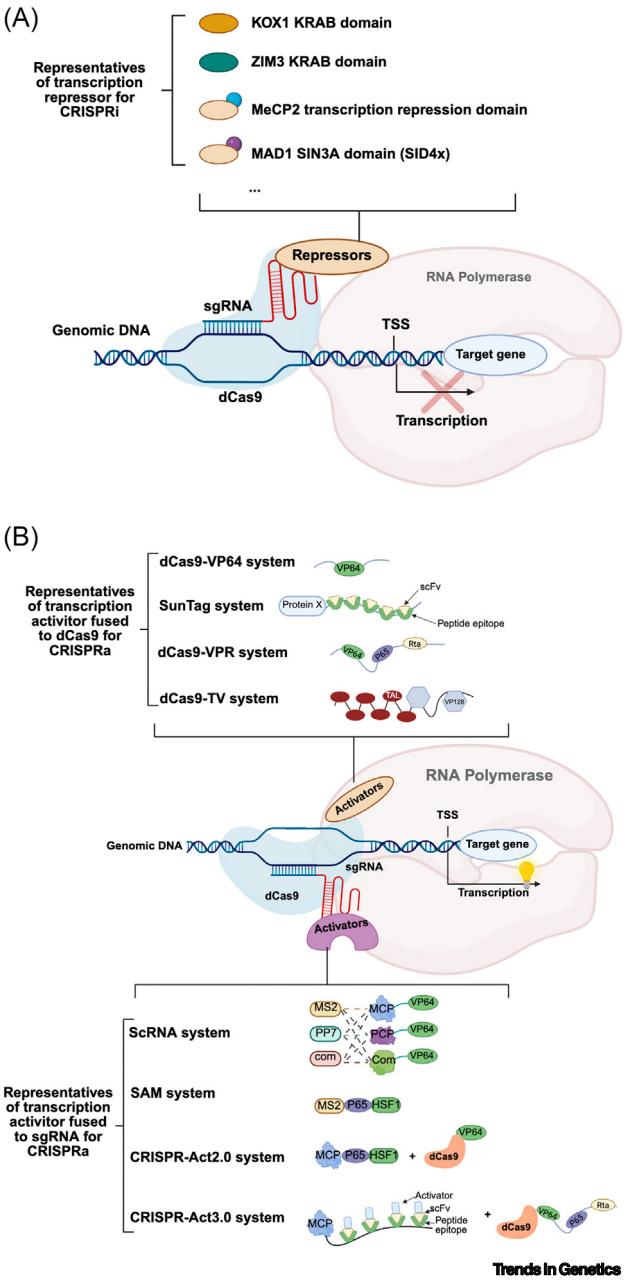


Figure 4. Clustered regularly interspaced short palindromic repeats (CRISPR) interference (CRISPRi) and CRISPR activation (CRISPRa) systems for programmable transcriptional regulation. (A) CRISPRi uses catalytically inactive dCas9 proteins to repress gene transcription by blocking RNA polymerase at promoters or transcription start sites (TSSs). Repression efficiency is enhanced by fusing dCas9 with repressor domains such as KRAB (KOX1 or ZIM3), MeCP2, or SID4x (MAD1-SIN3A). (B) CRISPRa upregulates gene expression by recruiting transcriptional activators to target loci. These can be fused directly to dead CRISPR-associated protein 9 (dCas9) (e.g., VP64, VPR, TV), or tethered via scaffold RNA systems. Representative scaffold-based systems include scRNA (with MS2, PP7, or com aptamers), SAM (synergistic activation mediator), and CRISPR–Act2.0/3.0, which combine dCas9 with multimeric peptide arrays (e.g., SunTag) or aptamer-binding activator modules. Together, these tools enable programmable, multiplexed, and tunable regulation of endogenous gene networks in diverse organisms. Abbreviation: WT, wild type.

A more modular approach is exemplified by the SAM (synergistic activation mediator) system, which uses modified gRNAs containing MS2 RNA hairpins that recruit MS2 coat protein (MCP)-fused activators. This design enables independent tuning of DNA binding (via dCas9) and effector recruitment (via MS2), offering greater flexibility and multiplexing potential [62]. Similarly, the scaffold RNA (scRNA) and CRISPR-Act3.0 platforms expand on this concept by integrating multiple RNA aptamers to recruit diverse regulators [63].

CRISPR-based transcriptional regulation has been widely adopted in functional genomics, synthetic biology, and metabolic engineering. Genome-scale CRISPRi/a screens have been

used to map gene essentiality, identify drug resistance mechanisms, and annotate enhancer–gene relationships [64]. In industrial strains, CRISPRi has been used to tightly repress competing metabolic fluxes, thereby improving product yields and reducing byproduct formation [65]. By contrast, the application of CRISPRa in bacteria has been relatively limited, primarily due to the scarcity of well-characterized transcriptional activator domains and the compact nature of bacterial promoters. Recent advances, however, have demonstrated that engineered synthetic activators – such as dCas9 fused with prokaryotic σ factor fragments, heterologous transcriptional activators, or scaffolded recruitment systems – can enable programmable upregulation of endogenous and heterologous genes [66], offering new opportunities for metabolic pathway optimization and synthetic circuit construction in microbial cell factories.

Importantly, dCas12a has emerged as an alternative to dCas9 for transcriptional control. Its inherent ability to process crRNA arrays enables streamlined multiplex targeting without requiring multiple promoters or single-guide RNA (sgRNA) constructs. Recent studies have demonstrated that dCas12a–VPR or dCas12a–KRAB fusions can effectively modulate endogenous loci in mammalian cells and plants, expanding the repertoire of orthogonal regulators for combinatorial design strategies [67].

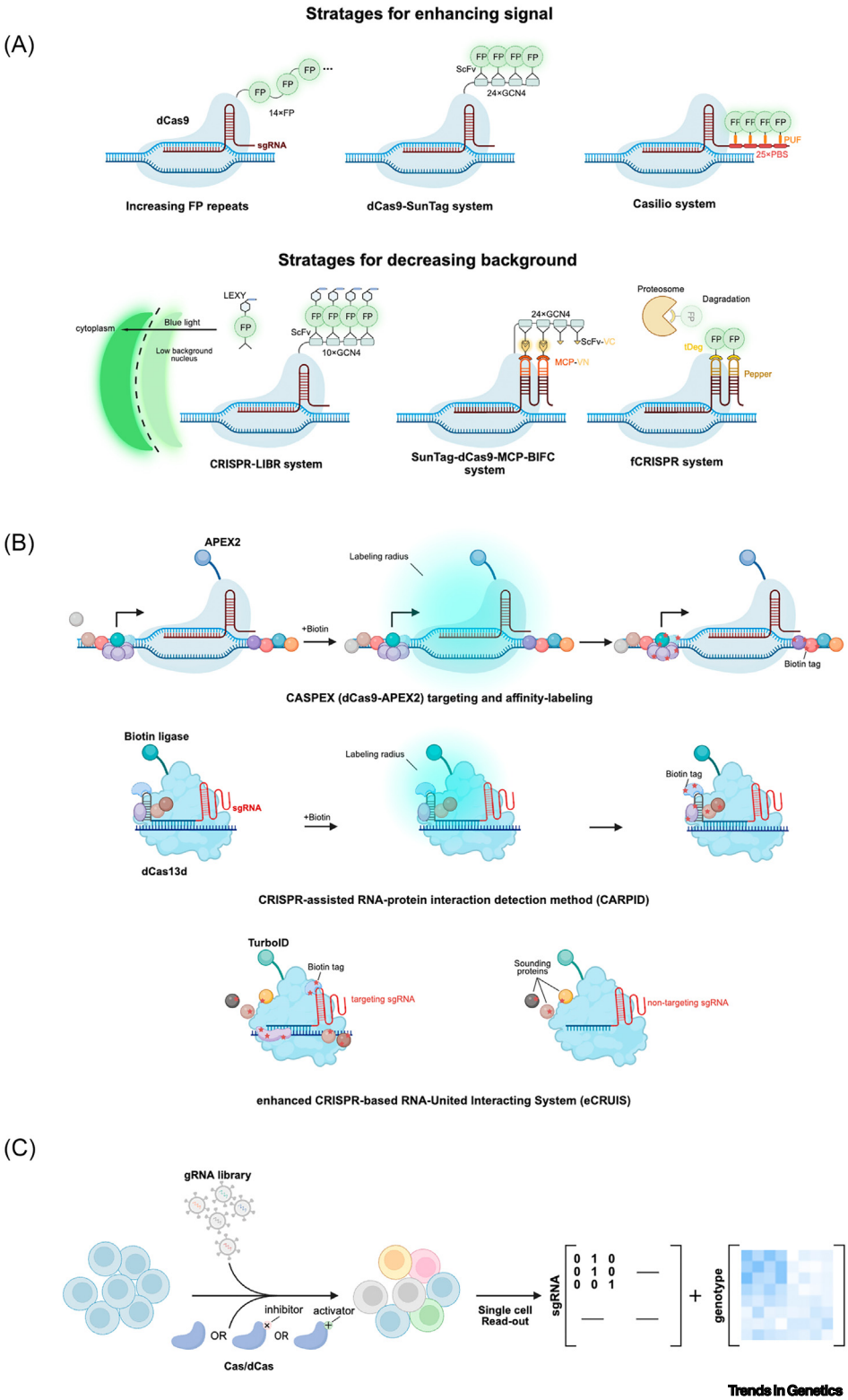
Nonetheless, challenges remain in achieving robust and tunable regulation across diverse cell types. Factors such as chromatin accessibility, transcriptional context, and sgRNA positioning influence efficacy. To address these issues, machine-learning models are being developed to predict optimal gRNA sites for CRISPRa/i, and synthetic logic gate architectures are being introduced for programmable gene control. Additionally, temporal regulation using inducible or optogenetic dCas fusions enables dynamic modulation of gene expression in response to environmental or experimental stimuli [68].

Altogether, CRISPR-based transcriptional regulation provides a scalable and programmable alternative to traditional gene overexpression or RNA interference (RNAi) knockdown strategies. As novel effectors and delivery systems continue to emerge, these tools will play an increasingly important role in cell engineering, developmental biology, and therapeutic applications.

Live-cell molecular imaging via CRISPR

The programmable DNA or RNA-binding capability of catalytically inactive Cas proteins not only revolutionizes gene regulation but also provides a versatile platform for live-cell molecular imaging. CRISPR-based imaging tools enable real-time visualization of nucleic acids in living cells with unprecedented precision, offering dynamic insights into genome organization, gene expression, and RNA localization. By repurposing catalytically inactive Cas proteins fused to fluorescent or proximity-labeled moieties, researchers have developed a versatile molecular toolbox for real-time tracking of specific DNA or RNA loci (Figure 5A).

Genomic imaging was the first major application of dCas-based visualization. In early studies, dCas9 fused to enhanced green fluorescent protein (eGFP) was guided by sequence-specific sgRNAs to repetitive genomic regions, such as telomeres or centromeres, enabling their spatial localization within the nucleus [69]. To address the challenge of imaging non-repetitive regions, which often yield low signal-to-noise ratios, multiple strategies were developed, including tiling arrays of sgRNAs to a target locus and multimerizing fluorescent signals through engineered scaffolds [70]. One such approach is SunTag (SuperNova Tag), in which dCas9 is fused to an array of peptide epitopes (e.g., GCN4), each capable of recruiting a fluorescent protein via an scFv. This amplification strategy enables highly sensitive detection of low-abundance targets and supports



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long-term tracking without photobleaching [61]. Multicolor imaging has been achieved using orthogonal RNA hairpins or Cas orthologs, as exemplified by the CRISPRainbow platform, which can label up to six genomic loci simultaneously using distinct fluorophores [71].

RNA imaging poses additional challenges due to the transient nature of RNA and its lack of repetitive structure. CRISPR–Cas13 effectors, which naturally bind and cleave RNA, have been adapted for fluorescent RNA imaging by the disabling of their nuclease activity. Fluorescent dCas13a or dCas13b fusions have been used to label mRNAs, long non-coding RNAs (lncRNAs), and viral RNAs in living cells [72,73], enabling the monitoring of transcriptional bursts, RNA transport, and subcellular localization with single-molecule sensitivity. However, dCas13-based imaging systems face several limitations, including elevated background signals, competition with endogenous RNA-binding proteins (RBPs), and delivery challenges due to the large size of Cas13 effectors.

Alternative RNA imaging strategies involve dCas9–PAMmer systems, in which a short DNA oligo (PAMmer) hybridizes with RNA targets, enabling dCas9 binding and visualization [74]. Although less widely used than Cas13, this method benefits from dCas9's well established delivery platforms and modularity. Emerging technologies, such as CRISPR-Sunspot, which combines CRISPR targeting with spatially controlled signal amplification, and Cas6-based BiFC (bimolecular fluorescence complementation) systems, are improving imaging resolution while minimizing background fluorescence [73,75,76]. Additionally, smLiveFISH, a CRISPR-Csm-based platform, enables real-time tracking of unmodified endogenous mRNAs at single-molecule resolution in live cells across various cell types [77].

Key advances in CRISPR-based imaging include the dynamic tracking of chromatin architecture during cell division or environmental responses, visualization of subnuclear positioning of regulatory elements, real-time monitoring of RNA metabolism, and multiplexed nucleic acid labeling using orthogonal Cas proteins. Despite this progress, challenges remain. In DNA imaging, targeting non-repetitive regions requires extensive sgRNA design and can cause signal dilution. In RNA imaging, background fluorescence and competition with endogenous RNA-binding proteins may reduce fidelity. Furthermore, delivering large dCas–fusion constructs into primary or post-mitotic cells remains technically challenging.

To overcome these issues, ongoing efforts [78] focus on optimizing sgRNA scaffolds for improved binding affinity, engineering split-fluorescent systems to minimize background signal, and integrating organic fluorophores or quantum dots for higher resolution. The development of miniature Cas effectors (e.g., CasMINI, Cas14) may also facilitate delivery into hard-to-

Figure 5. Advanced clustered regularly interspaced short palindromic repeats (CRISPR)-based tools for imaging, interactome mapping, and single-cell functional screening. Innovative strategies shown here significantly improve the precision and resolution of CRISPR-based imaging and interactome mapping. (A) Strategies to enhance signal and reduce background in CRISPR-based live-cell imaging. Signal enhancement approaches include increasing fluorescent protein (FP) repeats, using dCas9–SunTag scaffolds, or Casilio systems with RNA aptamer arrays. Background reduction is achieved via light-inducible localization (CRISPR-LIBR), split-fluorescent complementation (SunTag-MCP-BiFC), or degradation-based noise suppression (fCRISPR system). (B) CRISPR-guided proximity labeling systems for DNA-and RNA-centered protein interactome mapping. GLoPro (dCas9–APEX2) [148] targets specific genomic loci for biotinylation of nearby proteins. CARPID [92] uses dCas13d to identify RNA-binding partners via proximity labeling. eCRUIS [91] improves labeling efficiency through dCas–TurboID fusions, enabling detection of low-abundance or transient RNA–protein interactions. (C) Single-cell CRISPR screening platforms. Pooled guide RNA (gRNA) libraries introduce targeted perturbations (via Cas/dCas effectors), and phenotypic outcomes are measured at single-cell resolution using single-cell RNA sequencing (scRNA-seq) or multi-omic readouts. This enables the reconstruction of gene function, regulatory networks, and context-specific cellular responses. Abbreviations: ScFv, single-chain variable fragment; sgRNA, single-guide RNA; TSS, transcription start site.

transfect cell types. In parallel, synthetic logic gates and inducible imaging systems are being developed to control the timing and context of imaging events.

Altogether, CRISPR-based imaging has evolved into a powerful platform for spatial genomics and transcriptomics. Its compatibility with live-cell analysis, multiplexing capabilities, and customizable targeting makes it an ideal tool for unraveling the dynamic architecture of the genome and transcriptome in life science and medical research. Complementary approaches such as CRISPR-Chip [79] and CRISPR-GO [80] have also been developed to interrogate genome architecture and nuclear organization, expanding the toolkit for spatial genomics.

Delivery strategy is another critical consideration. Plasmid-based delivery is convenient but may lead to prolonged background expression. Viral vectors such as adeno-associated virus (AAV) and lentivirus support stable delivery but face size constraints, especially for dCas13 fusions. Alternatively, nanoparticles and electroporation can deliver ribonucleoprotein (RNP) complexes transiently with reduced background, although often at the cost of lower efficiency. Choosing appropriate delivery methods is essential for optimizing imaging fidelity and temporal resolution.

CRISPR-guided mapping of protein interactions

CRISPR-based imaging provides spatial and temporal insights at the nucleic acid level; similarly, proximity labeling techniques now extend this spatial resolution to protein interaction networks. Deciphering the molecular interactions surrounding DNA or RNA loci is essential to understanding cellular function and gene regulation. Traditional protein–protein interaction (PPI) mapping techniques – such as co-immunoprecipitation or yeast two-hybrid systems – often lack the spatial or temporal resolution needed to probe chromatin states or RNA interactomes in living cells. CRISPR-guided proximity labeling tools have emerged as powerful alternatives by combining the sequence-specific targeting capability of dCas proteins with proximity-labeling enzymes such as APEX2 [81], BioID [82], or TurboID [83].

The core concept involves fusing catalytically inactive Cas9 or Cas13 to a labeling enzyme, which biotinylates nearby proteins within a radius of 10–20 nm upon activation. These biotinylated proteins can then be purified via streptavidin enrichment and identified using quantitative mass spectrometry, enabling unbiased profiling of local proteomes in their native context [84,85] (Figure 5B).

DNA-centric platforms are designed to identify proteins associated with specific genomic loci. One of the earliest systems, C-BERST, utilizes dCas9–APEX2 to label proteins in proximity to CRISPR-targeted chromatin regions in mammalian cells [86]. Similarly, dCas9–TurboID fusions have enabled *in situ* biotinylation at telomeres, centromeres, and gene regulatory elements with reduced labeling time and cytotoxicity [87]. These approaches allow for dynamic assessment of chromatin states under various conditions, such as differentiation, stress, or drug exposure. Integration with techniques such as assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) [88] or CUT&RUN [89] further enhances the interpretability of proximity-labeled proteome data. However, off-target labeling remains a concern due to the intrinsic promiscuity of labeling enzymes, necessitating careful gRNA design and inclusion of appropriate negative controls for robust data interpretation.

RNA-centric proximity labeling has rapidly advanced with the advent of CRISPR-Cas13 systems. dCas13 effectors can be programmed to bind endogenous RNAs without sequence alteration or genetic tagging, making them ideal scaffolds for transcript-specific labeling. The CRUIS (CRISPR RNA-united interacting system) framework, which couples dCas13 with APEX2, has been used to profile the interactome of lncRNAs and mRNAs in human cells [90].

Subsequent optimization led to eCRUIS, which enhances signal specificity by employing two orthogonal gRNAs targeting adjacent regions of the same transcript. This spatial co-targeting strategy improves enrichment of bona fide RNA-associated proteins and reduces background [91]. Another notable platform, CARPID (CRISPR-assisted RNA–protein interaction detection), utilizes a dCasRx–BioID fusion to biotinylate proteins in proximity to targeted RNAs. When applied to lncRNA HOTAIR and MALAT1, CARPID identified both known and novel interactors with high sensitivity and reproducibility [92].

These RNA-centric systems have distinct advantages: they operate in live cells, do not require prior knowledge of RNA localization or structure, and can resolve cell-type-specific or condition-dependent interactions. The high resolution of Cas13-based tools makes them particularly well suited for dissecting dynamic RNA regulatory networks in development, stress response, and disease progression.

Nonetheless, several caveats remain. Labeling efficiency is influenced by factors such as local transcript abundance, gRNA binding stability, and subcellular compartmentalization. Furthermore, the bulky nature of dCas13–enzyme fusions may sterically hinder native interactions or affect RNA structure. To address these concerns, ongoing efforts include the development of smaller Cas variants, split-labeling enzymes, and inducible systems for more refined control and minimal perturbation.

The future of CRISPR-guided proteomics lies in multi-omic integration. Combining proximity labeling with single-cell RNA-seq, spatial transcriptomics, or chromatin conformation capture will offer a more comprehensive understanding of how nucleic acids and proteins coordinate to regulate gene expression. Modular CRISPR platforms using multiple orthogonal Cas enzymes could simultaneously map DNA, RNA, and protein dynamics at single-locus or single-cell resolution.

In summary, CRISPR-guided proximity labeling systems represent a transformative approach for interrogating nucleic-acid-centric molecular environments. As these tools continue to evolve, they promise to deliver deep insights into gene regulation, RNA metabolism, and cellular signaling with unmatched spatial and temporal precision.

Proximity labeling also requires careful delivery optimization. Plasmid expression may cause sustained background labeling, while inducible viral systems or RNP-based delivery can offer better temporal control. For large constructs like dCas13-TurboID, packaging size limits in viral vectors may necessitate split or dual-vector strategies [93].

Single-cell functional genomics using CRISPR

While proximity labeling elucidates molecular interactions within populations of cells, combining CRISPR perturbations with single-cell sequencing technologies, **single-cell CRISPR screening** enables unprecedented resolution of cellular heterogeneity and functional genomics. The integration of CRISPR-mediated gene editing or regulation with single-cell sequencing platforms provides powerful strategies for dissecting complex biological networks with unprecedented granularity (Figure 5C).

The foundational platform in this space is **Perturb-seq** [94], which pairs pooled CRISPR screening with single-cell RNA sequencing (scRNA-seq). In Perturb-seq, cells are transduced with sgRNA libraries targeting genes of interest, and the identity of each sgRNA is captured alongside the transcriptome of each cell. Early implementations used polyadenylated sgRNAs with capture sequences for sequencing. This approach allowed researchers to cluster cells by perturbation

and observe changes in gene expression profiles, uncovering both direct and indirect effects of gene knockouts or knockdowns [95].

Subsequent optimization of Perturb-seq [96,97] have enhanced sgRNA capture efficiency and scalability. For example, modified expression vectors now allow simultaneous expression of sgRNAs and transcript barcodes, improving linkage fidelity between perturbation and phenotype. Strategies such as direct-guide barcoding and expressed UMI-embedded sgRNAs enable robust perturbation assignment even under high multiplicity of infection (MOI) conditions [98], making it feasible to interrogate combinatorial perturbations and synthetic genetic interactions.

One of the most comprehensive platforms to date is ECCITE-seq (expanded CRISPR-compatible cellular indexing of transcriptomes and epitopes by sequencing) [99]. ECCITE-seq extends the capabilities of standard CITE-seq by capturing sgRNAs, mRNAs, surface protein markers, and sample identity tags (hashtag oligonucleotides) in a single assay. This multimodal design enables a holistic view of how CRISPR perturbations influence the transcriptome, surface protein expression, and cell lineage. ECCITE-seq has been applied to investigate immune cell activation, T cell exhaustion, and viral response dynamics at single-cell resolution.

Another emerging frontier is the integration of CRISPR perturbation with single-cell chromatin accessibility profiling, as exemplified by CRISPR-sciATAC [100]. This method combines pooled CRISPR editing with single-cell ATAC-seq, enabling researchers to link genetic perturbations to changes in chromatin landscape. CRISPR–sciATAC has revealed how transcription factor deletions reshape enhancer usage, nucleosome positioning, and cell state transitions.

Complementary methods – such as sci-Plex, a high-throughput platform for multiplexed scRNA-seq with small-molecule or CRISPR perturbations using combinatorial barcoding [101], and Perturb-FISH, which employs direct guide barcoding for RNA-based readout of perturbation effects in primary cells [102] – have opened new avenues for pooled CRISPR screening in hard-to-transfect systems or *ex vivo* tissues.

Despite their versatility, single-cell CRISPR platforms face several technical and analytical challenges. Efficient delivery of perturbation libraries, especially to primary cells or organoids, remains a bottleneck. Moreover, assignment of perturbations can be confounded by sgRNA dropout, mosaic editing, or variable Cas9 activity. To mitigate these, researchers have adopted orthogonal strategies such as barcode redundancy, Cas9 fusion to regulatory domains, and direct RNA expression profiling of Cas9 activity.

From a computational standpoint, large-scale single-cell CRISPR screens require sophisticated pipelines for guide-to-cell mapping, batch correction, and causal inference. New algorithms such as MUSIC [103], MIMOSCA [94], MAUDE [104], and scMAGeCK [105] are being developed to infer gene regulatory networks and identify synergistic perturbations from sparse and noisy data.

Looking ahead, combining CRISPR-based perturbations with spatial transcriptomics, single-cell proteomics, and lineage tracing will further enhance the resolution and interpretability of functional genomics. As scalable delivery systems, improved barcoding chemistries, and multi-omic readouts continue to mature, single-cell CRISPR technologies are poised to become essential tools for dissecting cellular circuits in development, disease, and therapeutic contexts.

Concluding remarks

The CRISPR revolution began with precise genome editing but has rapidly evolved into a versatile molecular platform that extends far beyond DNA cleavage. By engineering Cas effectors and gRNAs into programmable components, researchers have developed a suite of powerful tools for diagnostics, biosensing, gene regulation, molecular imaging, protein interactome profiling, and single-cell functional screening (Table 1).

Together, these emerging applications redefine CRISPR systems not merely as genome editors, but as modular information processors capable of detecting, decoding, and rewiring cellular states. The unifying principle behind these innovations is programmability: whether for targeting nucleic acids in diagnostics, modulating transcription, or recruiting effectors for spatial and temporal analysis of molecular events. Importantly, these functions are being realized in live cells, in real time, and with single-cell or even subcellular resolution.

Looking forward, several frontiers are expected to shape the next phase of CRISPR innovation. First, the discovery and engineering of compact and PAM-free Cas variants (e.g., CasMINI, Cas14, CasΦ) will enhance accessibility, delivery efficiency, and targeting flexibility across a wide range of biological systems. While compact Cas effectors offer advantages in delivery and packaging, their cleavage activity and target specificity are generally lower than those of Cas9 and Cas12a, presenting trade-offs that must be considered when selecting systems for genome or diagnostic applications. Second, improvements in gRNA design, inducible expression systems, and orthogonal Cas combinations will enable precise, multiplexed control of complex phenotypes. Third, the integration of CRISPR systems with multi-omic readouts, spatial transcriptomics, high-content imaging, and AI-guided design platforms such as AlphaFold 3 [106]-inspired Cas protein modeling, DeepCRISPR [107] for sgRNA efficiency prediction, and DeepAptamer [45] for optimized biosensor construction will drive systems-level insights in development, disease, and synthetic biology.

Moreover, efforts to reduce background noise, improve *in vivo* delivery, and streamline data integration will be essential for translating CRISPR technologies into real-world applications such as point-of-care diagnostics, programmable therapeutics, and responsive biosensors. As Cas technologies converge with advancements in microfluidics, nanomaterials, and machine learning, CRISPR is poised to become a central node in the next generation of bioengineering platforms.

We conclude this review with a selection of open questions (see Outstanding questions) that highlight both the promise and current limitations of CRISPR systems beyond genome editing.

Outstanding questions

How can the specificity of CRISPR-based diagnostics be further improved to reliably distinguish closely related pathogenic strains or variants, minimizing false-positive or false-negative results in clinical and environmental settings?

What novel strategies can simplify CRISPR diagnostic workflows by eliminating or minimizing preamplification steps without compromising assay sensitivity?

Can robust CRISPR biosensors be developed for non-nucleic acid analytes that effectively function in complex biological fluids (e.g., blood, saliva) with minimal sample preprocessing?

Which design principles will enable dCas-based transcriptional regulation systems to achieve predictable, stable, and tunable expression levels across diverse cell types and conditions?

How can CRISPR-based molecular imaging technologies overcome current limitations, such as background signal, limited multiplexing capability, and large Cas protein size, to enable real-time, high-resolution tracking of multiple genomic or transcriptomic targets simultaneously?

What advancements are required for CRISPR-guided proximity labeling methods to reliably capture transient or low-affinity protein interactions in living cells, and how can these techniques integrate effectively with single-cell or spatial proteomics platforms?

How can single-cell CRISPR screening methods be optimized to reduce noise, improve scalability, and integrate seamlessly with multi-omic datasets, particularly with emerging spatial transcriptomics and proteomics techniques?

How will artificial intelligence and computational modeling drive future innovations in CRISPR systems design, gRNA prediction, specificity optimization, and high-throughput screening analysis?

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Declaration of interests

The authors declare no competing interests.

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