Unconventional uses of CRISPR/Cas

Reviewing applications of CRISPR/Cas beyond conventional genetic engineering purposes in biomedical sciences

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Preface

This thesis was written in October and November 2019 in the context of my Master Biomedical Sciences at the University of Groningen. Supervision was provided by Christy Hong and Floris Foijer, whom I would like to thank for the opportunity.

Summary

CRISPR/Cas is an antiviral immune system in prokaryotes that has been repurposed to serve as a genetic engineering tool. Conventional use of this repurposed CRISPR/Cas system aims to induce physiological changes to cells by altering the way cells are able to interact with their genome. However, CRISPR/Cas has other applications in biomedical sciences which are perhaps overshadowed by the vast amounts of research that use CRISPR/Cas in a conventional manner. Hence, this thesis aims to review unconventional uses of CRISPR/Cas. Seven different uses are described: chromatin pull-down assays, genome imaging, programmable genome organization, RNA-tracking, recording, and storage of information, molecular diagnostics, and smart materials. Concluded is that while these CRISPR/Cas-based tools are not unique in their purpose, they have many advantages compared to their traditional counterparts aimed to fulfill the same purpose.

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Introduction

A brief history of CRISPR/Cas

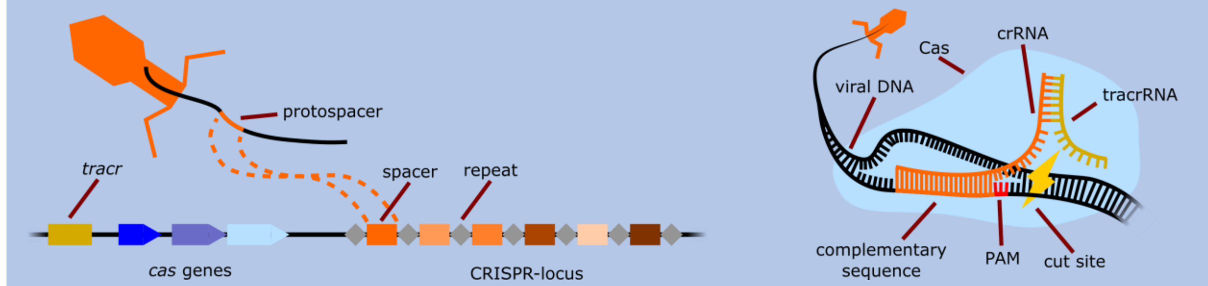
The now (in)famous acronym CRISPR was coined in 2002 by Jansen et al. after finding clustered regularly interspaced short palindromic repeat (CRISPR) loci in different species of prokaryotes[1]. Jansen et al. were not able to identify a function of the CRISPR-loci and CRISPR associated (*cas*) genes, nor were they likely able to foresee the incredible developments in biological sciences its elucidation put in motion. The first paper suggesting a role for CRISPR as part of an immune system was published in 2005 by Mojica et al[2]. This hypothesis was confirmed by Barrangou et al. in 2007[3]. Although the CRISPR/Cas system is a clever antiviral immune system in prokaryotes[4] (Box 1), it is not this discovery that led to its fame. In 2008, Marraffini and Sontheimer were first to propose the utilization of the CRISPR/Cas system outside of its native environment[5]. This was first conducted by Sapranauskas et al. in 2011, managing to transfer a functional CRISPR/Cas system into *Escherichia coli*[6]. The breakthrough paper that started the CRISPR/Cas mania was published in 2013 by Cong et al, who harnessed the CRISPR/Cas system for genome editing by reprogramming the target sequence of the guide RNA[7]. It should be stressed that genome editing itself was already possible for years, utilizing tools using DNA-binding proteins such as zinc-finger nucleases (ZFNs) or transcription activator-like effector-based nucleases (TALEN). However, CRISPR/Cas-based genome editing is considered to be a vastly superior tool, because of the simplicity of only having to change the guide RNA sequence for the system to work (Box 2). Consequently, its introduction has substantially facilitated research in biological sciences[8,9].

Conventional applications of CRISPR/Cas in biomedical sciences

In biomedical sciences, CRISPR/Cas is used extensively to study the effects of genetic modifications (e.g. gene knockouts) in cell lines or animal models (Figure 1A). Other widely used applications use dead Cas (dCas).

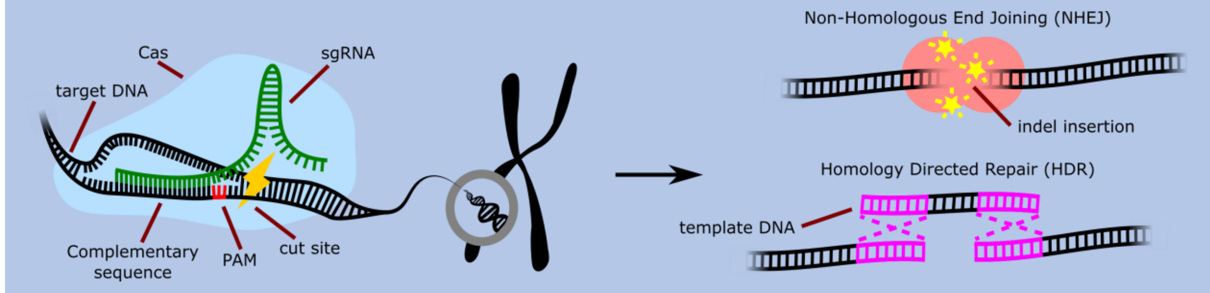
Box 1 | **CRISPR/Cas, an antiviral immune system in prokaryotes**

Some prokaryotes can turn injected viral genomic information against the invader. This is achieved by incorporating a small piece of the viral DNA, called a protospacer, into a specific locus in the genome where it is called a spacer. This locus can have multiple spacers which are separated by palindromic repeats. This locus was initially identified by these repeats and was hence named Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)[1]. Depending on the type of the CRISPR/Cas system, the transcript of the locus, called pre-crRNA, is processed into mature crRNAs. CRISPR-associated protein (*cas*) genes are usually located in the proximity of the CRISPR locus. Cas proteins are responsible for the spacer acquisition and are able to form a 'search and destroy' complex by binding to a crRNA. In the complex, the crRNA binds to complementary DNA sequences, originating from a new viral infection. When bound, Cas cuts the DNA, 'disarming' the infection mechanism. A short and specific DNA sequence not found adjacent to the spacer in the CRISPR-locus must be recognized by Cas before it can bind and thus, cut. This so-called Protospacer adjacent motif (PAM) sequence depends on the type of Cas and prevents self-cutting. Perhaps the most famous Cas is *Streptococcus pyogenes* Cas9 (spCas9). Being a type II CRISPR/Cas system, maturation of crRNA and complex forming with Cas9 requires *trans*-activating crRNA (tracrRNA). For a more detailed description, see Hiller et al[4].



Box 2 | **CRISPR/Cas as a tool for genome editing**

The 'search and destroy' mechanism of CRISPR/Cas is used as an antiviral immune system in prokaryotes (Box 1) but is more famously known for its utilization in genome editing. Cas9 is most frequently used in genome editing by CRISPR/Cas. Here, the tracrRNA and crRNA are combined into a single guide RNA (sgRNA). By introducing Cas and sgRNA into a cell, any location of interest in a genome can be cut. While pre-complexed Cas/sgRNA can be introduced by transfection, the more conventional approach is the transfection of an expression vector for Cas and the sgRNA. After induction of a double-strand break (DSB) at the desired location, the DSB can be repaired by non-homologous end-joining (NHEJ), inducing mutations at the cleavage site. An alternative is homologous directed repair (HDR) by co-transfection of a DNA template. For a more detailed description, see Wang et al[9].



Although dCas can bind to any genomic location using its complexed guide RNA, it lacks endonuclease activity. By fusing a transcriptional repressor or activator to dCas, gene expression can be lowered (e.g. knockdown) or enhanced. These applications are called CRISPR interference (CRISPRi) and activation (CRISPRa), respectively (Figure 1B). An additional tool to regulate gene expression is epigenetic editing by using methyl- or acetyltransferases fused to dCas (Figure 1C)[8]. Despite the described differences in these specific applications of CRISPR/Cas, they all aim to induce physiological changes to the cells by altering the way cells are able to interact with their genome. For the sake of clarity, all such applications of CRISPR/Cas will be considered and referred to in this thesis as conventional applications of CRISPR/Cas.

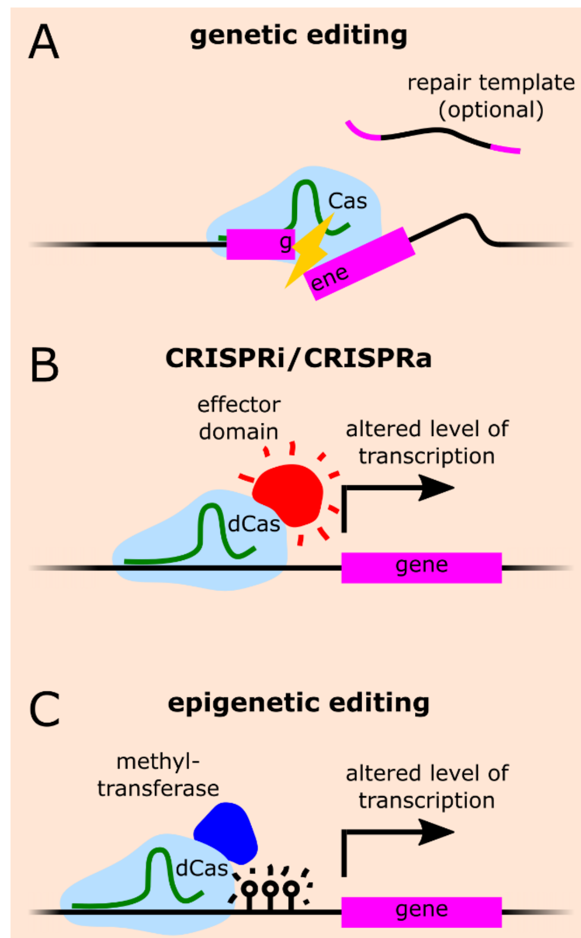


Figure 1 | Conventional applications of CRISPR/Cas. In genetic editing using CRISPR/Cas, a target of interest in the genome (e.g. a gene) is cut (A). Alternatively, dCas can be utilized to alter the level of gene transcription by fusing it to a transcriptional effector domain (B) or to an enzyme which modifies the epigenetic elements in the proximity of the target (C).

CRISPR/Cas, overshadowed applications?

Molecular biologists are eager to improve and expand a newly given toolbox. An example is the invention of the Polymerase Chain Reaction (PCR) in 1983[10], a now indispensable tool in biomedical sciences. Ten years later, quantitative PCR (qPCR) was introduced[11]. Still, developments in PCR had far from stopped. In 2006, PCR-based bridge-

amplification was introduced[12]. This ingenious technique was utilized in the Solexa Genome Analyzer released in 2006, of which its technical principle is unchanged in the now widely used Illumina sequencers[13]. Although Kary Muller, the inventor of PCR, said to "have used plenty of LSD" (which he said to have aided him in his invention)[14], it is difficult to imagine him foreseeing such a specific evolution of his invention at that time. In comparison to PCR, CRISPR/Cas is a youngster in the toolbox used by molecular biologists. The future will tell how the CRISPR/Cas toolbox will develop over time. Currently, much effort is taken into improving targeting specificity, with a long-term goal being to apply CRISPR/Cas in the clinic in a safe manner[15]. Although these are both exciting and controversial developments indeed, CRISPR/Cas has more useful applications than the previously described conventional uses. A respectable number of papers have been published describing such out-of-the-box applications, but these are perhaps overshadowed by the vast numbers of papers using CRISPR/Cas for conventional purposes. Although unconventional uses of CRISPR/Cas are mentioned in reviews, these reviews either 1) discuss only one specific unconventional use[16–19], or 2) mention unconventional uses briefly, having a large focus on the conventional uses[8,9,20]. In addition, considering recent developments, these reviews are often outdated.

Research questions

To our knowledge, there is currently no review specifically focused on (according to the definition in this thesis) unconventional uses of CRISPR/Cas. This thesis aims to provide such a review, with the research questions being what types of unconventional applications of CRISPR/Cas there are, how they work, and how they compare to more traditional tools or strategies serving similar purposes.

Unconventional applications of CRISPR/Cas that will be reviewed have been divided into seven types.

Chromatin pull-down assays

Chromatin pull-down assays have been around since 1984 with the introduction of ChIP (chromatin immunoprecipitation) and have, like PCR, been improved and expanded upon[21]. In traditional use, ChIP aims to elucidate protein-DNA interactions by isolating a protein of interest and identifying the bound DNA-sequence (e.g. by ChIP-sequencing)[22]. It is also possible to couple mass spectrometry to ChIP (ChIP-MS), first performed by Wang et al. in 2013. Here, a tagged protein of interest binds DNA with other bound proteins which are subsequently identified by MS[23]. A limitation of such an approach is the dependency on the availability of known endogenous DNA-binding molecules to reveal the chromatin composition there where it binds the DNA. In an approach called reverse ChIP, however, a DNA probe is used to analyze chromatin there where the probe binds[24]. This allows chromatin identification independent of using an endogenous protein known to bind at the sequence of interest. Because dCas is a modular DNA-binding

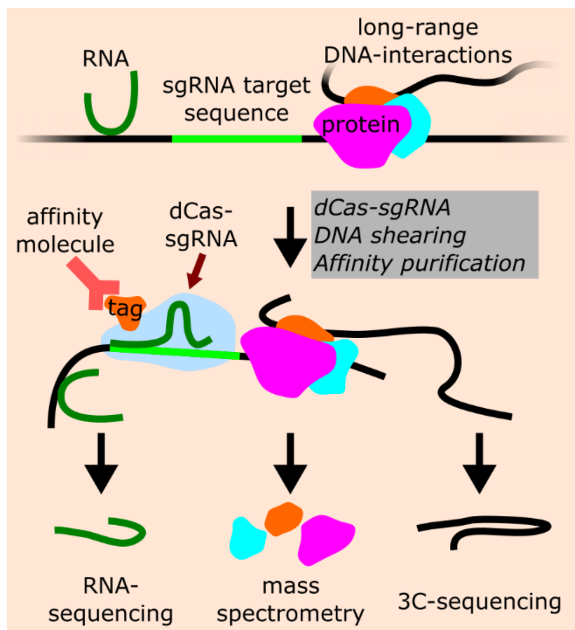


Figure 2 | Chromatin pull-down assays using CRISPR/Cas. *Chromatin composition is analyzed by using a dCas-sgRNA complex binding to a locus of interest. By introducing a tag to dCas, chromatin complexes can be purified (e.g. by immunoprecipitation) and analyzed. Chromatin associated RNA can be analyzed by RNA-sequencing. Bound proteins can be identified by mass spectrometry. Finally, long-range DNA interactions can be analyzed by chromosome conformation capture (3C) sequencing. Image adapted from Liu et al[27].*

molecule thanks to its guide RNA, researchers were quick to repurpose the CRISPR/Cas system for reverse ChIP. To our knowledge, the first paper to utilize CRISPR/Cas beyond conventional genetic engineering introduces such a reverse ChIP tool, published half a year after the introduction of the CRISPR/Cas system as a genome editing tool[7,25]. In this paper by Fujita et al. in 2013, enCHIP-MS (engineered DNA-binding molecule-mediated chromatin IP mass spectrometry) using the CRISPR/Cas system is introduced. The workflow of CRISPR/Cas mediated enCHIP-MS is similar to ChIP-MS. First, the chromatin of cells expressing epitope-tagged dCas and sgRNA targeting a region of interest is crosslinked, after which the DNA is sheared. By performing IP, complexed dCas is isolated, after which the bound protein can be identified by MS[25,26]. A more recent paper introduced CAPTURE (CRISPR affinity purification *in situ* of regulatory elements) which aims to provide more detailed information compared to enCHIP[27]. In CAPTURE, biotinylated dCas allows affinity purification by streptavidin (SA). Such affinity purification is superior over antibody-mediated purification because, among other things, it is both more sensitive and specific. In addition to bound-protein analysis by MS, CAPTURE also includes analysis chromatin-associated RNA (e.g. long non-coding RNA) by RNA-sequencing. Finally, long-range DNA-interactions (e.g. chromatin loops) are analyzed by chromosome conformation capture (3C) sequencing (Figure 2). Taken together, CAPTURE allows for a less biased and more detailed analysis of chromatin interactions compared to enCHIP[27].

Fujita et al. also showed that reverse ChIP can be done using TALEN-based enCHIP[25]. Prior to enCHIP, insertional CHIP (iChIP) introduced in 2009, allowed reverse ChIP. Here, a sequence on which a known exogenous DNA-binding protein (e.g. LexA) is introduced in a locus of interest (LOI) and subsequently used as bait[28].

Genome imaging

The spatial-temporal organization of the genome is involved in many cellular processes such as differentiation and gene transcription. Hence, the ability to image the genomic structure to study it is of much interest in biomedical sciences[16]. Genome imaging using dCas (henceforth referred to as CRISPR-imaging) was introduced by Chen et al. in 2013[29]. This was achieved by fusing dCas to a fluorescent protein (dCas-FP) (Figure 3A). Hence, CRISPR-imaging tools can be used to study genome dynamics in live cells. In addition to monitoring repetitive sequences such as telomeres in time, imaging nonrepetitive genomic sequences such as genes is also possible. Although this can be achieved by using multiple sgRNAs targeting different parts of an LOI to ensure a sufficient signal, this is a complex strategy[29]. Hence, multiple alternative CRISPR-imaging strategies have been developed to enhance the

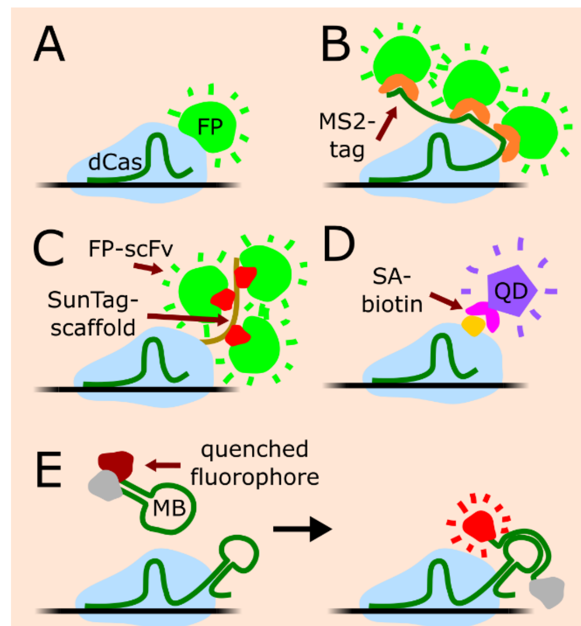


Figure 3 | CRISPR-imaging. *By fusing a fluorescent protein (FP) to dCas, a genomic region of interest can be monitored in live cells (A). To enhance the signal, either an extended sgRNA (MS2-tagging) (B) or a dCas fusion protein can serve as a platform for multiple FP-fusion proteins to bind (SunTag) (C). In nanoparticle-based CRISPR-imaging, a system such as the biotin-streptavidin (SA) system can be used to target a Quantum Dot (QD) to dCas (D). A novel dye-based CRISPR-imaging system is CRISPR/Molecular Beacon (MB) in which a quencher is released from a fluorophore as soon as the RNA molecule they are coupled to binds to an extended sgRNA (E). Image adapted from Wu et al[34].*

signal in CRISPR-imaging[30]. An extended sgRNA scaffold can serve as a platform for multiple co-expressed RNA-binding FP fusion proteins (e.g. CRISPR-Sirius; Figure 3B)[31]. Alternatively, dCas can be fused to a protein scaffold, binding multiple co-expressed FP fusion proteins (SunTag system; Figure 3C)[32]. Although FP-based CRISPR-imaging has already been used extensively since its introduction, it is not the only CRISPR-imaging method. An alternative is nanoparticle-based using QDs (Quantum Dots). This can be achieved by using biotinylated dCas and SA-coupled QDs (Figure 3D). QDs have a substantially higher brightness compared to FP. However, major drawbacks in nanoparticle-based CRISPR-imaging is that QDs get trapped in the endolysosomal system where they aggregate, resulting in background signal as well as possibly changing the physiology of the cells. In addition, the cellular delivery of QDs is difficult due to their large size[30,33]. Another approach is organic dye-based CRISPR-imaging, also providing a brighter signal than FP-based CRISPR-imaging. A recently introduced organic dye-based CRISPR-imaging tool providing a high signal-to-noise ratio (SNR) is the CRISPR/MB (Molecular Beacon) system. Here, a dye and a quencher are conjugated to a looped RNA-molecule, quenching the signal. Only when the RNA-molecule hybridizes with the sgRNA complexed in Cas, the dye released from the quencher[30,34] (Figure 3E).

Before the introduction of CRISPR-imaging, FISH (Fluorescent In Situ Hybridization) was already being used extensively for decades to study the genome. In FISH, fixed cells are labeled with a fluorophore coupled DNA or RNA molecules after denaturing the genomic DNA[35]. Later, live-cell imaging was made possible by using ZFN and TALEN-based technologies[36–38].

Programmable genome organization

Besides visualizing the spatial-temporal organization of the genome described previously, it is also possible to alter the genome organization to study the resulting effects on the cellular phenotype. This can be achieved by using dCas to reposition loci of interest to a specific nuclear region (e.g. nuclear envelope or nuclear bodies) in a tool called CRISPR genome organization (CRISPR-GO), developed by Wang et al. in 2019[39]. In CRISPR-GO, the abscisic acid (ABA) inducible ABI/PYL1 system is used. In this system, dCas targeting an LOI by its sgRNA is fused to ABI, while a nuclear region associated protein (NRAP) is fused to PYL1. An example of an NRAP is emerin, which is located at the inner nuclear membrane. When the cells are treated with ABA, ABI and PYL1 dimerize, repositioning the LOI to the nuclear site of interest (Figure 4), This repositioning often represses expression of the genes located in the LOI. Remarkably, genes are affected at a longer distance (30-600 kb) compared to CRISPRi, which only acts locally[39].

CRISPR-GO is not the first system that enables the repositioning of an LOI. In 2008, Reddy et al used a LacI-LacO system to move an LOI to the nuclear periphery. In this system, a *LacO* site is integrated at an LOI and LacI was fused to emerin. Because isopropyl β-d-1-thiogalactopyranoside (IPTG) binds to LacI which blocks it from binding *LacO*, IPTG blocks repositioning of the LOI[40].

RNA-tracking

The ability to track RNA allows studying the spatial-temporal behavior of the cellular transcriptome. This includes localization to specific organelles and transport of RNAs out

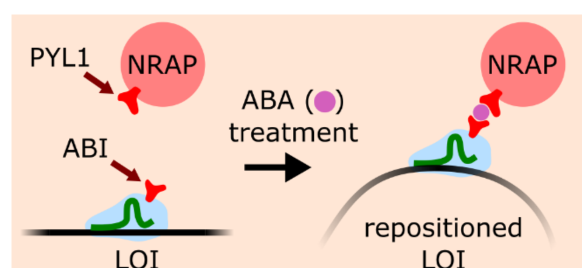


Figure 4 | CRISPR genome organization (CRISPR-GO). *ABI is fused to dCas targeting a locus of interest (LOI) using its sgRNA while PYL1 is fused to a nuclear region associated protein (NRAP). Treatment with abscisic acid (ABA) induces dimerization of ABI and PYl1, repositioning the LOI to a nuclear region of interest. Image adapted from Wang et al*[39].

of the cell (e.g. by exosomes), all processes known to be involved cell physiology and as well as many pathologies[41]. The CRISPR/Cas system can also be repurposed to target RNAs, which are usually single-stranded. However, dCas9, frequently used in CRISPR-imaging, requires recognition of the PAM-sequence before it can bind, which is on the opposite strand of the target strand. By providing a PAMmer, a separate oligonucleotide that hybridizes to the RNA of interest and provides a PAM-sequence, dCas9 is able to bind to the RNA. By using a mismatched PAM-sequence, dCas only binds to the RNA and not the corresponding encoding DNA in the genome[42–44] (Figure 5A). In 2016, Nelles et al. utilized this strategy to track specific endogenous mRNAs in living cells by using FP-dCas9, without altering the abundance of the corresponding translated protein. In addition, because a nuclear localization signal (NLS) was fused to dCas9-FP, it only leaves the nucleus when a bound target RNA leaves the nucleus. This subsequently results in reduced background signal[44]. A year later, Abbudayyeh et al. tracked RNA using dCas13a, which omits the requirement of a PAMmer, simplifying the method[45] (Figure 5B). Although different FPs could be fused to Cas, the enzyme itself is unable to distinguish between different sgRNAs. Hence, an expression-based multiplexed RNA-tracking method has not been developed yet.

Similar to genome imaging, before the introduction of CRISPR/Cas-based tools, FISH was already used to localize RNAs. Still, FISH does not provide temporal information[35]. A live-cell expression-based alternative used before CRISPR/Cas-based RNA-tracking uses the MS2 system. Here, a specific exogenous part is inserted in the gene of interest. The resulting stem-loop in the RNA transcript of interest is subsequently targeted by the MS2 coat protein fused to a FP, allowing expression-based live-cell imaging[46]. A live-cell RNA-tracking method independent of gene editing is the transfection of RNA-targeting MBs similar to those used in genome imaging (Figure 3E).

Recording and storage of information

Because a natural purpose of DNA is to encode information, it is potentially a great storage system to record cellular events of interest. Such events can range from biological events (e.g. presence of intracellular molecules) to environmental events (e.g. changes in temperature)[17]. By storing such events in the genome of the cells aimed to study, reading the genome (e.g. by NGS) will provide the recorded information of interest. In 2016, Shipman et al. were the first to repurpose the CRISPR/Cas system to record and store such events. Instead of using sgRNA and Cas endonuclease, they used overexpressed endogenous Cas1 and Cas2 in *E. coli*, which are responsible for spacer acquisition. By electroporating different arbitrary oligos over time and sequencing the CRISPR-loci afterward, they were able to trace back the order of the electroporated oligos, since spacers are integrated unidirectionally[47] (Figure 6A). A year later, the same researchers used this method of information storage to encode a digital movie in a CRISPR-locus, with each oligonucleotide providing information pixel information[48]. However, Sheth et al. showed that cellular information could also be stored in a similar method they named TRACE (temporal recording in arrays by CRISPR expansion)[49]. TRACE makes use of a copy number-inducible trigger plasmid (pTrig) as a template for spacer integration. By comparing plasmid spacer integration with stable background spacer integration (e.g. from own genome), they showed that a chemically induced increase in pTrig copy number also

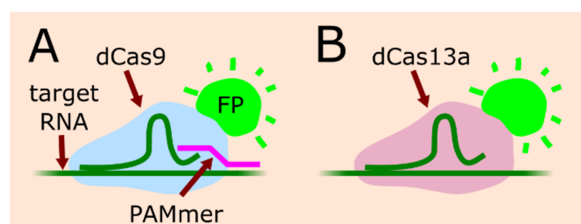


Figure 5 | RNA-tracking using CRISPR/Cas. *dCas9-FP can target and thus visualize an RNA of interest when a PAMmer is provided. Because the PAM sequence in the PAMmer mismatches, only the RNA is targeted (A). dCas13a-FP can also track an RNA of interest but binds independent of a PAM and thus a provided PAMmer (B).*

increased the relative spacer integration derived from pTrig (Figure 6B). In addition, they were able to use multiple chemical inducers for different pTrig plasmids, allowing multiplexed recording[49]. A radically different approach recording cellular events, developed by Perli et al. in the same year as TRACE and is called mSCRIBE (Mammalian Synthetic Cellular Recorder Integrating Biological Events). mSCRIBE uses a self-targeting guide RNA (stgRNA) which, as the name implies, targets its own corresponding gene. Doing so creates mutations in the gene and thus the sgRNA. Thus, in time, the sgRNA sequence which serves as barcode will accumulate indels. By letting its expression depend on a stimulus, the number of indels corresponds to the intensity of the stimulus[50] (Figure 6C). In a comparable system called MEMOIR (mutagenesis with optical in situ readout), Cas9 targets a given 'scratchpad' gene instead of its own sgRNA. This genome integrated sequence contains multiple barcodes which are targets for different FISH-probes to bind, with each induced DSB deleting one pseudorandom target. By using sequential FISH targeting the unique combination of mRNA transcripts of every cell transcribed

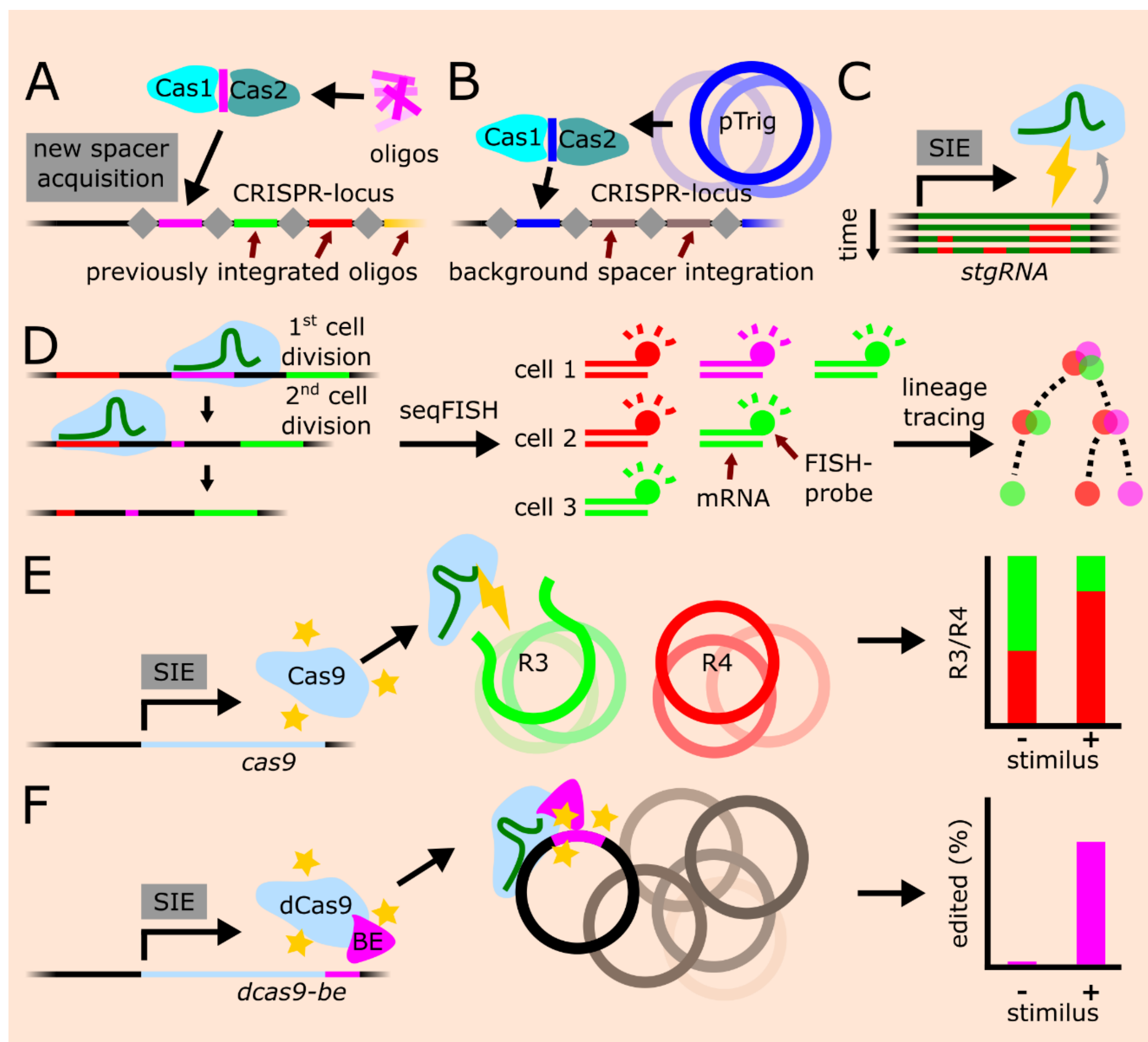


Figure 6 | Recording and storage of information using CRISPR/Cas. *By directed spacer acquisition by Cas1 and Cas2, information of interest is stored in a CRISPR-locus which can be retrieved by sequencing the locus (A). In TRACE, spacers are derived from a high-copy plasmid (pTrig). By comparing the number of pTrig-derived spacers with spacers acquired from background integration, the copy number of plasmids present in the cell in time can be traced back (B). In SCRIBE, a self-targeting guide RNA (stgRNA) creates indels in its corresponding gene in time (C). In MEMOIR, a 'scratchpad' sequence is targeted by Cas. In time, transcript targeted by specific FISH-probes are lost, allowing lineage tracing (D). In CAMERA1 two high-copy plasmids (R3 and R4) are maintained in a bacterial cell. Stimulus induced expression (SIE) of Cas9 results in the deletion of R3 plasmids, resulting in a decrease in R3/R4 ratio (E). In CAMERA2, however, there is only one targeted plasmid, which is base edited after SIE of dead Cas9 fused to a base editor (dCas-BE) (F).*

from the scratchpad, cells can be distinguished based on their unique composition of binding FISH-probes. When sgRNA expression is dependent on endogenous Wnt-signaling, cell division results in a reduction in barcodes. Hence, subsequent FISH-seq can reveal lineage information of the cells[51]. The *in situ* approach of MEMOIR is in contrast to GESTALT (Genome Editing of Synthetic Target Arrays for Lineage Tracing), developed by McKenna et al. in 2016. Here, a genomic barcode is uniquely modified by Cas during proliferation of cells but is instead read by a Next-Generation Sequencing (NGS) approach[52]. The approach of GESTALT was combined with single-cell RNA-sequencing in LINNAEUS (lineage tracing by nuclease-activated editing of ubiquitous sequences), introduced by Spanjaard et al. in 2018, providing information of the cell type[53]. One of the latest types of recording systems utilizing the CRISPR/Cas-system was introduced in 2018 by Tang & Liu and is called CAMERA (CRISPR-mediated analog multi-event recording apparatus)[54]. In CAMERA1, two different types of high-copy 'recording' plasmids are stably maintained in the same ratio for multiple days. These two plasmids, R3 and R4, are highly similar and share the same origin of replication. The only difference is that unlike R4, R3 is targeted by a sgRNA. Because Cas expression is dependent on a stimulus (e.g. induction by IPTG or cell division), the ratio of the two plasmids changes after this stimulus, because the resulting DSB in R3 'deletes' the plasmid. The resulting decrease in R3/R4 ratio can be measured by NGS (Figure 6D). Additionally, it is possible to reset CAMERA1 by inducing expression of a separate sgRNA which targets the references recording plasmid R4. This restores the ratio because although reference plasmids will be deleted, the cell will replicate both recording plasmids, restoring the high-copy numbers. CAMERA2, however, uses only one recording high-copy plasmid and dCas9 fused to a base editor dCas9-BE of which its expression is again dependent on a stimulus of interest. Co-expression of a sgRNA targeting the recording plasmid results in a base change in the plasmid which, similar to CAMERA1, can be measured by NGS (Figure 6E)[54].

One of the latest cellular recording technologies before the introduction of CRISPR/Cas is SCRIBE (Synthetic Cellular Recorders Integrating Biological Events), which more resembles the CAMERA2 strategy than mSCRIBE. In SCRIBE, ssDNA is expressed after an input signal (e.g. cellular event), which is recombined into specific genomic loci[55].

Molecular diagnostics

Traditional methods to detect pathogens or perform genotyping typically rely on bacterial culturing or antibody-based assays (e.g. ELISA). Although detection by analyzing nucleic acids (e.g. by PCR and/or NGS) is, in general, quicker and more accurate than the previously described methods, it requires expensive and complex equipment[56]. Because of the ability of Cas to identify specific nucleotide sequences, researchers were quick to envision CRISPR-based diagnostic tools. The first paper that introduced such a tool was published in 2017 by Gootenberg et al, naming this tool SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing)[57]. In SHERLOCK, the DNA or RNA of interest is first amplified by RPA (recombinase polymerase amplification). Unlike PCR, RPA is an isothermal process and thus requires no thermal cycler. Next, added T7 polymerase transcribes the total amplified DNA to RNA. Finally, RNA-targeting Cas13a, as well as quenched fluorescent RNAs, are added. Cas13a is a *trans* RNA cutting enzyme, meaning that it will cut any RNA molecule when its sgRNA finds a match. When the Cas13a-sgRNA complex finds a match in the sample, Cas13a cuts reporter RNAs, separating the quencher from the fluorophore[57] (Figure 7A). Later, SHERLOCK version 2 (SHERLOCKv2) was introduced, which used orthologues of the Cas13a used in SHERLOCK. Having differences in cleavage specificity, they were able to use these specific orthologs to cleave specific RNA-reporters, allowing multiplexed detection of specific sequences. In addition, SHERLOCKv2 also

introduced quantitative measurements by using a relatively low concentration of primers in the RPA. This unsaturated the reaction, making the amount of fluorescent signal proportional to the input[58]. A highly similar approach to SHERLOCK, developed by Li et al. in 2018 and named HOLMES (an one-HOur Low-cost Multipurpose highly Efficient System) uses Cas12a. Like Cas13a, Cas12a has *trans* endonuclease activity but instead targets ssDNA instead of RNA. Hence, in HOLMES, a reporter ssDNA with a quencher and fluorophore is used[59]. Only three days later, Chen et al. introduced a highly similar tool called DETECR (DNA endonuclease-targeted CRISPR trans reporter) that also used Cas12a[60]. Interestingly, in addition to detecting specific nucleotide sequences, *trans* cutting CRISPR/Cas systems can also be used as a signal output system to detect molecules besides nucleic acids. Liang et al. were able to detect small molecules such as uric acid in blood samples utilizing the strategy of DETECR as signal output in a tool called CaT-SMElor (CRISPR/Cas12a and aTF-mediated small molecule detector) developed in 2019[61]. In bacteria, these small molecules can bind to corresponding allosteric transcription factors (aTFs) which, due to a conformational change, are subsequently unable to bind to their respective binding sites on DNA. Liang et al. repurposed aTFs to bind a target DNA sequence for a sgRNA complexed with Cas12a. Subsequent binding of a small molecule to aTF releases the target DNA. Similar in DETECTR and HOLMES, when Cas12a matches with targeted DNA using its sgRNA, *trans* cleavage of additionally added reporter DNA molecules allows detection by measuring fluorescence[61] (Figure 7B). A method to detect specific nucleic acid sequences independent of *trans* cleavage was developed by Hajian et al. in 2019. In this system called CRISPR-chip, dCas9 complexed with a sgRNA targeting a sequence of interest is linked to a graphene transistor with a flowing current. When a DNA sample is provided and matches the sgRNA, the natural negative charge of the DNA in the proximity of the graphene leads to a measurable change in the current[62] (Figure 7C). A strategy that both combines electrical readout and *trans* cleavage by Cas was developed by English et al. in 2019. Here, ssDNA is crosslinked with electricity conducting carbon back (CB), creating a CB-DNA gel. By providing Cas12a, the CB-DNA gel degrades by *trans* cleavage of the linker ssDNA when the sgRNA finds a match in a provided DNA-sample. When

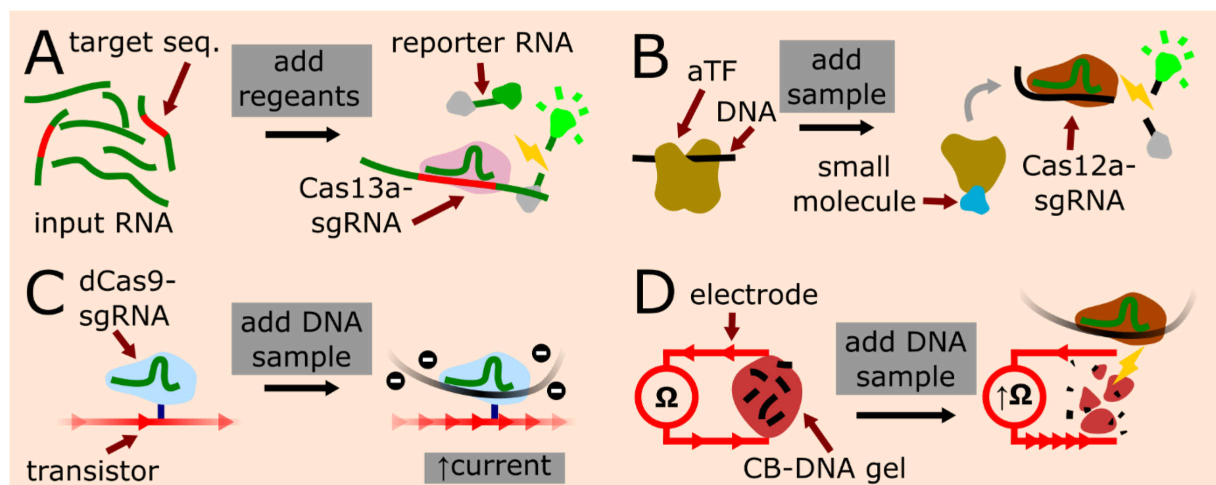


Figure 7 | Molecular diagnostics using CRISPR/Cas. *In SHERLOCK, the genetic material of interest is amplified and transcribed to RNA. If added Cas13a and sgRNA targeting a sequence of interest find a match, a detectable fluorescent signal is released by the trans cleavage of added reporter RNA (A). In CaT-SMElor, DNA is released from allosteric transcription factors (aTFs) if a corresponding small molecule of interest binds. Cas12a will subsequently bind to the target DNA using its sgRNA, resulting in trans cleavage of added reporter DNA and thus a detectable fluorescent signal (B). In CRISPR-chip, dCas9 is coupled to a graphene transistor. If, after adding a DNA sample, dCas binds to a sequence of interest using its sgRNA, negatively charged molecules are brought in the proximity of the transistor, resulting in a detectable increase in the current (C). By using an electricity-conducting carbon-black (CB) ssDNA-gel as a fuse, a detectable increase in resistance (Ω) is measured when the gel is degraded by trans cleavage of the ssDNA in the gel by Cas13a after its sgRNA finds a complementary sequence in a provided DNA-sample (D).*

electricity is run through the CB-DNA gel, which acts as an electrical fuse, subsequent degradation leads to a measurable increase in resistance[63] (Figure 7D).

Smart materials

The 'trigger' mechanism in *trans* cutting CRISPR/Cas systems is not limited to serve as a signal output system in diagnostic tools. In 2019, English et al. used Cas12a activation to induce changes in material properties. By coupling cargo molecules to a hydrogel using ssDNA as a linker, cargo release by *trans* cleavage by Cas12a could be induced by providing DNA molecules specifically targeted by its sgRNA (Figure 8A). In addition, by linking polymers of hydrogel together using ssDNA, degradation of the hydrogel could be induced by a similar strategy as the cargo release (Figure 8B). By using the latter strategy, English et al. were able to release nanoparticles and larger objects including cells encapsulated in an ssDNA crosslinked hydrogel. Such DNA-mediated controlled release of molecules or larger objects from hydrogels has great therapeutic potential, such as in regenerative medicine, where hydrogels are frequently used[63].

It should be stressed that DNA-responsive hydrogels were introduced before the introduction of CRISPR/Cas. These systems relied on adenosine-induced structural changes in the crosslinked DNA[64] or strand displacement, requiring a relatively high concentration of trigger DNA[65].

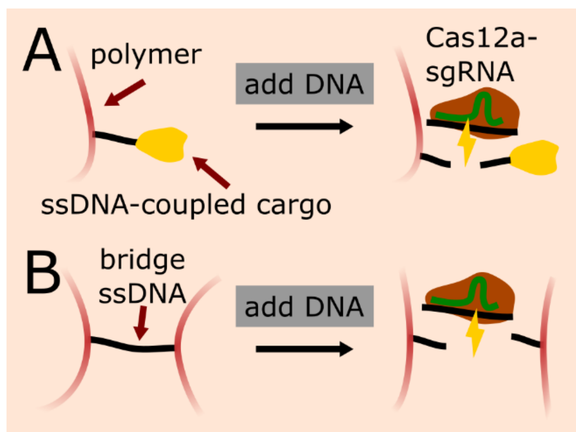


Figure 8 | CRISPR/Cas in smart materials. *| Cargo molecules can be crosslinked to a polymer-based hydrogel using ssDNA. If 'trigger' DNA molecules are added complementary to the sgRNA, trans cleavage by Cas12a will result in release of the cargo (A). Using a similar strategy, hydrogel degradation can be induced when the polymers themselves are crosslinked (B).*

Discussion

CRISPR/Cas is often associated with the editing of the genome to induce physiological changes in cells. Although this association is understandable, the CRISPR/Cas system has been repurposed for many other applications. This thesis aims to give an overview of these more unconventional uses of the CRISPR/Cas system in biomedical sciences. A summary of all reviewed unconventional uses of CRISPR/Cas is provided (Table 1).

CRISPR/Cas-based reverse ChIP such as enCHIP makes use of dCas and a sgRNA targeting an LOI to pull-down and analyze bound chromatin. As a reverse ChIP approach, it is independent of the requirement of an endogenous molecule known to bind at the sequence of interest. A major advantage of CRISPR/Cas-based reverse ChIP over iChIP is that there is no need to knock-in a target sequence for an exogenous DNA-binding protein. In addition, Watson-Crick base-pairing by the sgRNA is generally stronger than DNA binding by proteins such as in iChIP or TALEN-based reverse ChIP[66]. Finally, only having to change the sgRNA to target another locus makes CRISPR/Cas-based reverse ChIP highly modular in comparison to iChIP or TALEN-based reverse ChIP. One advantage of iChIP is the use of exogenous DNA-binding proteins resulting in high binding specificity. Still, the sgRNA sequence can be designed to limit and can be tested for off-target binding. The second advantage of iChIP is that it allows allele-specific reverse ChIP when only one allele is modified to include the exogenous targeted sequence, something impossible to perform using a CRISPR/Cas-based system[19].

Genome imaging using CRISPR/Cas-based systems use dCas and its bound sgRNA to trace a specific (repetitive) sequence in the genome, allowing visualization by e.g. fusing FP to dCas. The obvious advantage of CRISPR/Cas-, ZNF- or TALEs-based genome imaging over FISH is

that it allows live-cell imaging. However, the shared advantage of FISH and CRISPR/Cas-based genome imaging is its modularity in changing the target sequence. In addition, the strength of Watson-Crick base pairing allows imaging of nonrepetitive genomic sequences[29]. Hence, CRISPR/Cas-based genome imaging combines the best of both worlds, allowing live-cell imaging while binding strongly to a target sequence that is highly modular. Additionally, there is no need to denature the DNA in CRISPR-imaging which could potentially damage the genome architecture aimed to study[16,30].

CRISPR-GO is a powerful programmable tool for inducible genome reorganization which uses dCas as bait to move the bound LOI within the nucleus. Compared to the older LacO-LacI system which serves a similar purpose, CRISPR-GO is far more modular for the same reason when comparing iChIP to enChIP (omitting the need of a knock-in sequence at the LOI)[67]. Still, to our knowledge, there are no papers published describing the use of CRISPR-GO or a similar tool to answer a biological question which raises the question of what the demand for such tools is. For instance, although long-distance gene knock-down in an LOI is unique, it is difficult to imagine a research case where this would be interesting to perform.

By using RNA-targeting dCas-FP, RNA can be tracked in live cells with advantages similar to that of genome imaging when comparing it to its FISH-based counterpart. Although using the MS2-system for RNA tracking also allows live-cell RNA-tracking, it is not a modular approach since it requires the insertion of an exogenous sequence in a gene of interest. In addition, extending the RNA molecule might influence its behavior and thus, that of the cell[41]. Alternatively, transfection of RNA-targeting MBs allows multiplexed live-cell imaging[68]. However using MBs is not an easy approach, as they are relatively difficult to produce[69]. In addition, an expression-based approach would omit the need for transfection, which would simplify the method as well as protect the cell from resulting physiological changes. Although Abudayyeh et al. made the expression-based CRISPR/Cas-based RNA-tracking considerably easier by omitting the need for a PAMmer[45], the technique remains limited to tracking one RNA per cell. Multiplexed CRISPR/Cas-based RNA-tracking in living cells is difficult because of the inability of Cas to distinguish between sgRNAs. As suggested by George et al, a possible solution to this would be pre-complexing Cas with different sgRNAs in separate reactions and transfecting them into the cells[41], but this is not an expression-based system. However, when interested in tracking two distinct RNAs at the same time in living cells, I propose that one could extend one targeting sgRNA to contain an MS2 stem-loop. By co-expressing MS2 protein fused to YFP, only one of the two sgRNAs has YFP bound, allowing fluorescence resonance energy transfer (FRET) when CFP is fused to dCas13a. Although both RNAs will have CFP signal, only one RNA has YFP signal. By subtracting the CFP signal which colocalizes with YFP signal from the total CFP signal, the two RNAs can be tracked separately. To not break the tradition to give such newly developed techniques cringeworthy names, I would like to name this method STUFF (Subtraction Tracking Using Förster's FRET) (Figure 9).

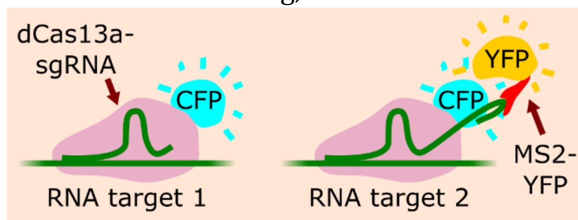


Figure 9 | Subtraction Tracking Using Förster's FRET (STUFF). *STUFF could allow live-cell tracking of two different RNAs by the extension of one sgRNA to include a MS2-loop. Expressed MS2-YPF should only target one sgRNA and thus visualize one RNA target by FRET. By subtracting the CFP signal that colocalizes with the YFP signal from the total CFP signal, one could track the other RNA target.*

CRISPR/Cas-based systems used to store information from cellular events can broadly be separated into two types. One type makes use of spacer acquisition by Cas1/Cas2 in a CRISPR-locus to store information (e.g. TRACE) in bacteria, while the other uses Cas

endonucleases to edit a plasmid or genomic region to record information (e.g. CAMERA). Although each type of recording system has its own qualities, a substantial advantage of CAMERA over all other systems (including the non-CRISPR/Cas-based system mSCRIBE) is that it is independent of genomic modification such as integration of a scratchpad sequence or CRISPR-locus while also being applicable in mammalian cells[54]. Although LINNAEUS and similar tools to study cell lineage have applications in fields such as developmental biology[53], tools such as CAMERA have, to our knowledge, yet to be used independently to answer a biomedical research question. Like CRISPR-GO, this raises the question what the demand for such tools is. For example, while recording a movie into a genome is unique, it therefore is not useful per se.

Methods such as SHERLOCK cleverly use *trans* cutting Cas endonucleases to target reporter RNA or DNA molecules after their bound sgRNA finds a match. This has obvious application in the clinic, as this could be used to detect pathogens or perform genotyping. Although nucleic acid detection is not unique, the main advantage of SHERLOCK and similar tools is its simplicity, portability, speed, and low costs, all while having no need for specialized equipment such as a thermal cycler[18]. The fluorescent output signal mediated by *trans* cleaving Cas was cleverly applied in CaT-SMElor to detect small molecules that are otherwise quantified by tools such as high-performance liquid chromatography after purification steps. Hence, CaT-SMElor shares the same earlier described advantages. The main advantage of digital CRISPR-Chip technology over SHERLOCK and similar tools is that it does not rely on an initial amplification step, speeding up the detection process considerably[61]. However, the immobilization of dCas-sgRNA complexes to graphene chips is a relatively difficult procedure. Still, one can imagine large-scale production of such chips for diagnostic purposes, with customers also being able to choose their own complexed sgRNA. Such large-scale production could also be applied to electrodes with a CB-DNA gel acting as a fuse.

By crosslinking DNA to hydrogels, degradation of the gel or release of coupled cargo can be induced by *trans* cleavage of Cas. Controlled cargo-release has much potential in fields such as regenerative medicine, where gradual release of (stem cell) factors can benefit the regeneration process. The CRISPR/Cas-based system is more modular in comparison to adenosine-induced structural changes in the linker DNA. This is because in the CRISPR/Cas-based system any DNA-molecule with a specific sequence can serve as a trigger molecule. Because *trans* cleavage is induced after providing a small amount of trigger DNA, it is also advantageous over strand displacement-based approaches, since in this approach high concentrations of trigger DNA is required[70].

Although genetic engineering was possible before the introduction of CRISPR/Cas, its addition to the toolbox of molecular biologists substantially facilitated biological research because of its ease to use compared to traditional ZNF- or TALEs-based approaches. Although unconventional applications of CRISPR/Cas have been developed more recently, the same applies to these tools. For instance, the ability to track RNA or perform reverse ChIP was not introduced by CRISPR/Cas. Instead, using a CRISPR/Cas-based tool is often easier, cheaper and more modular while providing a more accurate result than when using its traditional counterpart. Hence, these CRISPR/Cas-based tools will likely gradually replace their traditional counterparts, ultimately making them not so unconventional after all.

Table 1 | Overview of reviewed unconventional applications of CRISPR/Cas. *A selection of papers is provided that review the unconventional use of CRISPR/Cas, as well as papers that have applied these tools to answer a biomedical research question. NA, Not available/applicable.*

Unconventional CRISPR/Cas application	Brief description	First introduced by	Specifically reviewed in	Applied in biomedical research in
Chromatin pull-down assays	Use of tagged dCas coupled to sgRNA to target an LOI with subsequent IP targeting the tag to study chromatin at the LOI.	Fujita et al. August 2013[25]	[19,71]	[27,72–74]
Genome imaging	Use of dCas and coupled sgRNA to recruit fluorophores such as FP to the LOI, enabling live-cell imaging of (non-)repetitive sequencing.	Chen et al. December 2013[29]	[16,30]	[75,76]
Programmable genome organization	Use of tagged dCas and coupled sgRNA to move an LOI to a nuclear protein specific to a nuclear region of interest.	Wang et al. October 2018[39]	[67]	[39]
RNA-tracking	Use of RNA-targeting FP-dCas coupled to a sgRNA to track the movement of specific RNAs in live cells.	Nelles et al. March 2016[44]	[41,77]	[44]
Recording and storage of information	Use of the Cas1/Cas2 system to store information in CRISPR-locus or use of Cas endonuclease to modify and record information in genome or plasmid.	Shipman et al. July 2016[47]	[17,78]	[53]
Molecular diagnostics	Use of Cas which, after finding a match using its coupled sgRNA, outputs a detectable signal.	Gootenburg et al. April 2017[57]	[18,79,80]	NA
Smart materials	Use of *trans* cutting Cas to degrade linker DNA in hydrogels after binding 'trigger' DNA by its sgRNA, subsequently degrading the hydrogel or releasing coupled cargo molecules.	English et al. September 2019[63]	[70]	NA

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