

A 3D molecular model of a Cas9 protein (pink and orange) bound to a DNA double helix (purple). The background is a solid blue color. The Cas9 protein is shown in a complex, multi-domain structure, and the DNA is shown as a standard double helix.

Analyze Cas9 binding and cleavage properties in real-time while manipulating DNA structures

Gene editing
Application note - C-Trap[®] Dymo
2019

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An introduction to gene editing.

The field of gene editing has increased in popularity and applicability since the adaptation of the bacterial CRISPR-Cas systems to research; most notably the Cas9 system. The success of the Cas9 technology rests on its simplicity, allowing researchers to seamlessly edit DNA sequences by combining a sequence-identifying guide RNA (gRNA) with the Cas9 endonuclease enzyme. The approach has enabled researchers to target almost any genomic site, in all types of living cells, and manipulate gene-sequences, transcription products, or protein functions.

Although the Cas-systems are widely used in pre-clinical and clinical research, we need a better and more detailed understanding of its properties. In particular, with the advancements of gene-editing tools and their introduction into the clinic, understanding target specificity and off-target properties is high priority.

This application note introduces you to a new dynamic single-molecule analysis approach that enables you to study the binding and cleaving properties of Cas-related complexes and other gene-editing tools. We show results from a recent publication from the laboratory of Prof. David Rueda at the Imperial College in London, UK [1], which were obtained using the C-Trap® Dymo.

Highlights

This application note shows how you can use correlated optical tweezers with fluorescence microscopy to retrieve high-resolution and single-molecule based dynamic data of Cas9 binding and cleavage mechanisms.

We introduce you to five experiments using the C-Trap Dymo that evaluate Cas9 binding and enzymatic properties upon structural DNA changes:

- Genomic binding specificity.
- Off-target dwell-times.
- Sequence-dependent binding properties.
- DNA structure dependent binding properties.
- Cleavage kinetics.



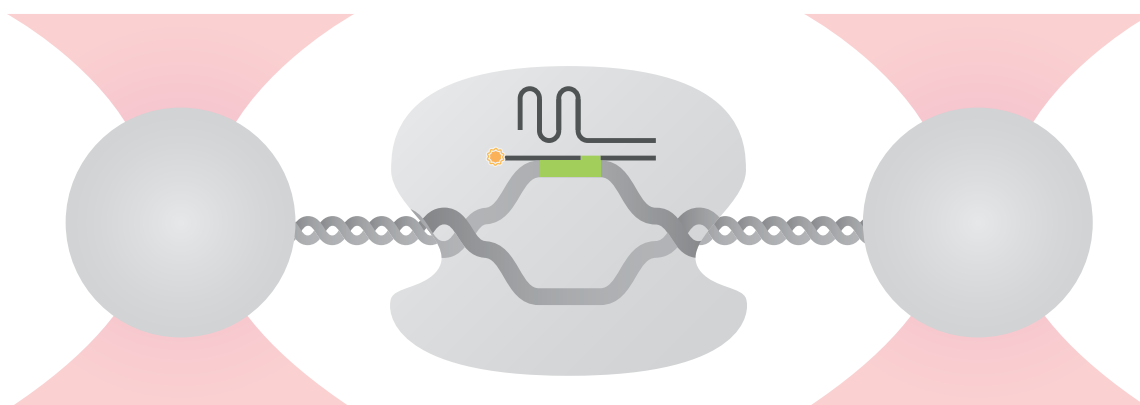
Sample & Data courtesy

Prof. Dr. David Rueda at the Imperial College in London



Read more

Newton et al. (2019)
Nat Struct Mol Biol.



Challenges and solutions

The challenges

Although Cas9 is a widely used gene-editing system, much of the conducted research aiming to understand Cas9 mechanisms and properties rely on bulk population studies and retrospective readouts. Examples of such include reporter assays, Sanger or next-generation sequencing, protein quantification, or gel visualization.

While valuable to study the outcomes of Cas9 activity, these commonly used approaches do not offer high-resolution or real-time measurements of Cas9 processes. Instead, gene-editing assessment in bulk cell populations often relies on population averages and retrospective genotypic or phenotypic changes. On the other hand, gene reporter assays enable you to follow the Cas9 activity in real time but often require mutation events and do not offer you a detailed read-out of the actual Cas9 cleaving events.

Also, since specific (on-target) binding and cleavage by Cas9 is crucial for reliable gene editing – especially because its great potential as a clinical strategy – extensive research aims to understand features associated with unintended off-target binding and cleavage. Several online tools enable researchers to design target-specific complexes by scoring targeting fidelity of the designed gRNAs. However, recent studies have shown that the designing tools miss several off-target sites that cannot be explained by sequence alone [2-4], but possibly structural DNA changes during Cas9 binding or cleavage. Here we present a new method to find off-target sites with high accuracy and spatiotemporal resolution.

The solutions

Dynamic single-molecule analysis using the C-Trap Dymo enables you to study Cas9-associated binding and cleavage in real time. The system's high spatiotemporal resolution allows you to detect transient events in detail while simultaneously manipulating the conformation of the DNA using stretching forces at the piconewton scale.

Thus, it enables you to complement and extend your findings extracted from conventional bulk assays.

The C-Trap Dymo integrates three main functions that make it ideal to evaluate gene-editing tools in different genomic conditions:

1. Optical tweezers that can manipulate the structure and features of a single DNA molecule before endonuclease targeting.
2. High-resolution multicolor fluorescence and label-free microscopy to track endonucleases and other relevant DNA-binding proteins.
3. An advanced microfluidics system that speeds up your workflow.



How do we do it?

DNA tethering and manipulation

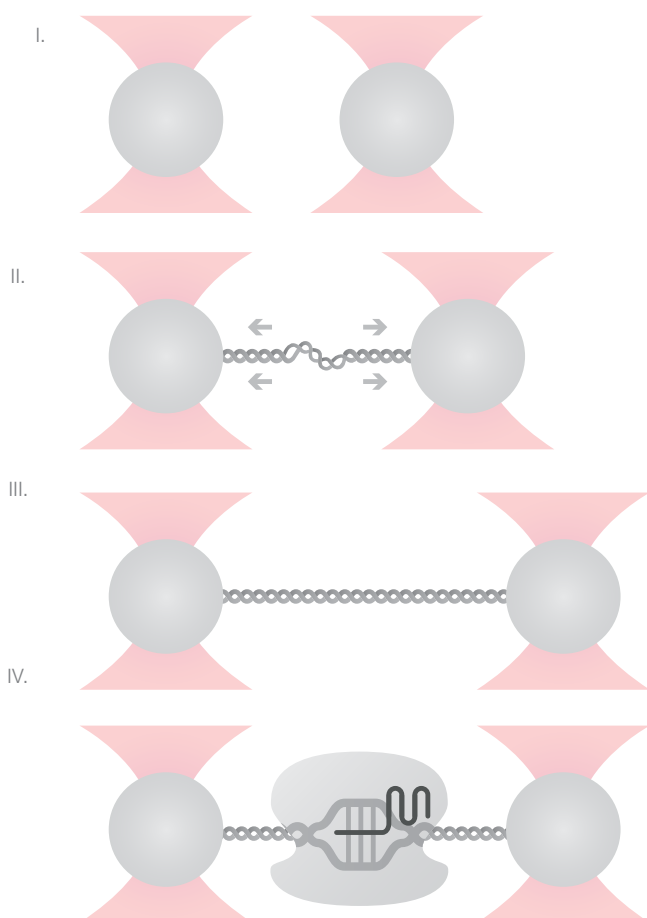
Optical tweezers enables you to trap a micron-sized particle, for example a bead, with the radiation pressure from a laser beam. Once trapped, the bead position can be manipulated by simply controlling the direction and force of the beam. You can use this principle to study a single molecule, such as a DNA, by tethering the ends of the molecule between two polystyrene beads. Next, you can stretch the molecule with low piconewton (pN) forces to reach the so-called contour length of the DNA, which is the maximum achieved length while extended, without disrupting base-pairing (**Figure 1a**).

To create DNA bubbles that are structures also formed during cellular processes, such as DNA replication or transcription, you can stretch the DNA molecule with forces of at least 20 pN. These bubbles enable the assessment of Cas9 binding and cleaving properties on different structural conditions.

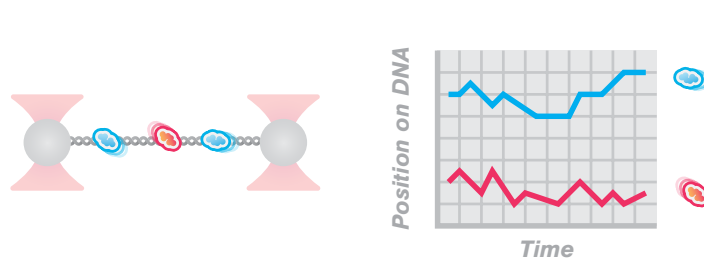
Cas9 preparation and targeting

In order to study Cas9 positioning on a DNA molecule, you can combine catalytically active or dead Cas9 (dCas9) with fluorescently labeled crRNAs, a 20-nucleotide long sequence that is complementary to the target DNA site. The labeled complexes can next be loaded into a channel of the microfluidics system, ultimately allowing you to follow the nuclease-complex on the DNA in real time.

In this study, the researchers used two independent crRNAs targeting different sequences on the same DNA molecule, to validate their findings in separate target sequences. They used catalytically active Cas9 to assess target specificity and cleavage properties, and dCas9 to study binding specificity. For visualization purposes, they assembled Cas9 with crRNAs labeled with cyanine dyes (Cy3 or Cy5).



1a A schematic illustration depicting the process of stretching DNA to then enable the assessment of Cas9. Samples are prepared in the laminar microfluidics channels: I. Bead Channel II. DNA Channel III. Buffer-only Channel IV. Imaging Channel



1b A schematic illustration describing Cas9's binding by time-binning the intensity of the cyanine-derived fluorescence on the DNA molecule using data from a kymograph.

The readout

In these experiments, researchers localized Cas9 binding by time-binning the intensity of the cyanine-derived fluorescence on the DNA molecule using data from a kymograph (Figure 1b), which is a graphical representation of the position of the dye (y-axis) over time (x-axis). The kymograph gives you a real-time representation of the localization of fluorescently-labeled proteins on a trapped molecule, such as Cas9 on a DNA.

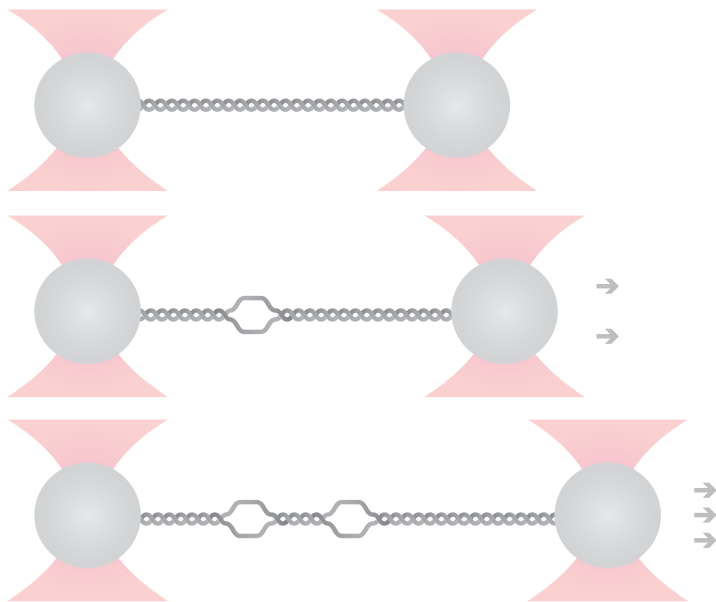
Experiments

1. Off-target binding upon induction of DNA bubbles

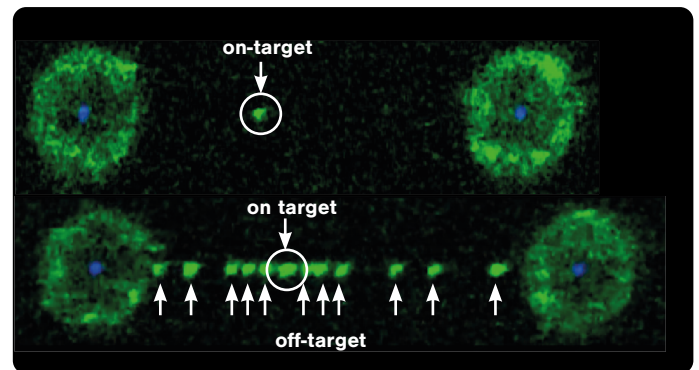
Through the use of the C-Trap optical tweezers, the researchers could manipulate the DNA structure by applying different stretching forces. Following fluorescently labeled dCas9 complexes on the C-Trap's integrated confocal microscope enabled them to observe single on-target binding events on the DNA at contour length (**Figure 2, top panel**). At this condition, they found no observable off-target binding. The investigators next induced the formation of DNA bubbles (**Figure 3**) by stretching the DNA molecule with 20 pN forces and found that the established condition caused the fluorescently labeled dCas9-complex to bind several off-target sites (Figure 2, bottom panel). Interestingly, reducing the forces back to 5 pN – hence re-establishing the contour length – caused dCas9 to dissociate from all off-target sites, maintaining only the single on-target binding event.

The researchers could next quantify force-dependent off-target binding by stretching the DNA molecule with increasing forces (between 20 and 50 pN). While 20 pN forces resulted in six observable off-target binding sites, higher stretching-forces increased the number of off-target sites in a force-dependent manner. Importantly, many of the off-target locations were recurrent and reducing the stretching-forces to 5 pN re-established the on-target specificity.

“How do DNA conformational changes affect the number of dCas9 on-target and off-target events?”



3 A schematic illustration depicting the process of stretching DNA to form bubbles.



2 A DNA molecule trapped between two beads with a fluorescently labeled dCas9-complex (green) bound to the target site (top). The dCas9-complex binds to increasing numbers of off-target sites upon stretching with 20 pN forces or higher (bottom). Adapted from Prof. David Rueda's YouTube Video: <https://www.youtube.com/watch?v=rm1TfNgMOXI>

2. Dwell-times of off-target binding events

The team assessed the dwell times for hundreds of off-target binding events established by one dCas9-complex. Using the same stretching forces as in Experiment 1 (between 20 and 50 pN), they found two populations associated with off-target binding modes: a fast (short-lived binding) and a slow (long-lived binding) population (**Figure 4**). Interestingly, analysis of the respective populations revealed that both populations comprised almost constant binding lifetimes (mean times, fast = 9.0 s and slow = 61.0 s).

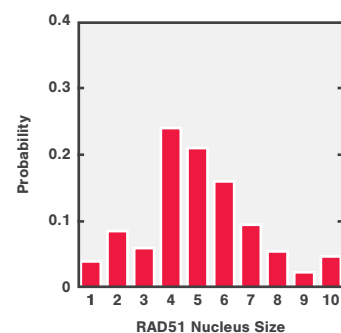
Of note, while 20 pN only established fast dCas9 off-target binding, higher forces (30, 40, or 50 pN) resulted in both fast and slow off-target binding.

“For how long is dCas9 bound to its off-target site?”

Summary Cas9 Binding

Understanding Cas9 binding, independent from cleavage, has several advantages to understand different Cas9 applications, including transcription control and gene editing. We show here how you can use C-Trap to analyze Cas9-binding mechanisms and:

- Visualize binding sites (on-target and off-target) without disrupting the DNA sequence.
- Compare Cas9 binding on single-stranded and double-stranded DNA.
- Study how DNA structures affect Cas9 binding.



4 Plot showing the two off-target populations (slow and fast) and their respective binding times at the off-target site.



What can you do with the C-Trap® Dymo?

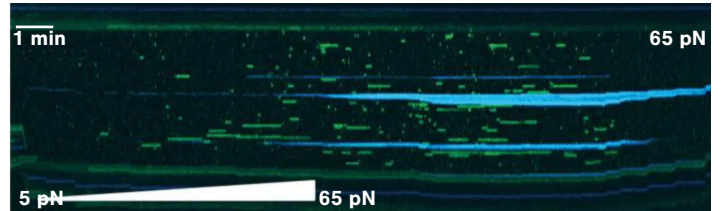
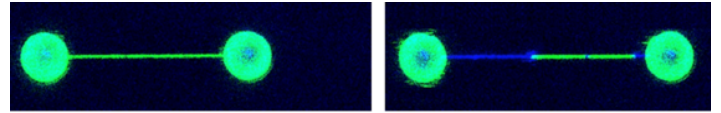
- Study how DNA-binding proteins affect Cas9 binding.
- Evaluate if Cas9 binding impairs the association of other DNA-binding proteins.
- Study how epigenomic states affect Cas9 binding.
- Measure dwell-time differences between crRNA specific Cas9-complexes.

Experiments

3. DNA strand properties of off-target events

A competition experiment between dCas9 and an eGFP-labeled human replication protein A (hRPA), exclusively binding single-stranded DNA, could test dCas9 binding selectivity towards the DNA template. Researchers used the multiple-color integrated confocal microscopy on the C-Trap to distinguish and localize both proteins on the DNA. Stretching-forces between 5 and 65 pN induced the formation of increasing areas of single-stranded DNA regions. At the lower force ranges, hRPA bound a small number of single-stranded DNA sites at the nicks, while at highest ranges (60-65 pN) it bound larger regions. Importantly, hRPA and dCas9 never colocalized, demonstrating that neither nicks nor single-stranded DNA induced dCas9 off-target binding (Figure 5).

“Does the Cas9-complex bind to single-stranded or double-stranded DNA specifically?”



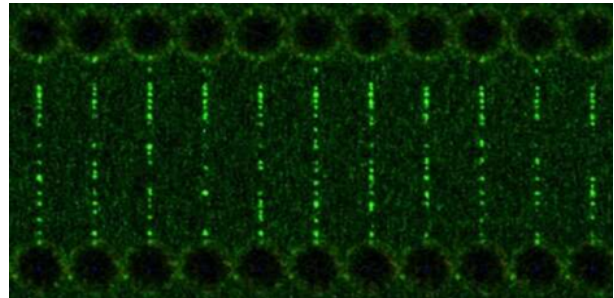
5 Top panels show a double-stranded DNA molecule stained with Sytox (green) trapped between two beads and stretched with minimal tension. Upon overstretching, hRPA (blue) binds to an increasing area of the DNA. Bottom panel shows a kymograph of a competition experiment with a DNA molecule in the presence of fluorescently labeled dCas9 (green) and hRPA (blue). Both proteins are mutually exclusive. Bottom figure obtained from Newton et al., Supplementary materials.

4. Measuring sequence specificity of off-target events

To measure off-target binding features, researchers pulled the tethered DNA three consecutive times with 40 pN forces in the presence of the dCas9 complex. The kymograph results showed recurrent binding-locations after each pull. Also, lowering the stretching-forces to 5 pN – thus re-establishing the contour length of the DNA – restored the single on-target binding corresponding to the respective crRNAs.

Notably, every 40 pN pull resulted in site-specific and recurrent Cas9 off-target binding sites, suggesting that the off-target binding is highly dependent on intrinsic features of the DNA molecule. Interestingly, comparison of the observed binding sites with common off-target prediction tools showed no overlap (Figure 6), suggesting that current prediction tools may miss several off-target locations.

“Are the off-target binding events at DNA bubbles sequence-specific?”



6 Cas9 binding-events on force-stretched DNA.

5. Kinetics of catalytically active Cas9

Using the laminar microfluidics system, researchers moved the DNA-nuclease complex from medium containing EDTA to a channel containing Mg^{2+} , thus activating the Cas9 nuclease activity. Under this condition, they tested Cas9 induced cleavage upon stretching of the DNA molecule with increasing forces (from 5 to 55 pN).

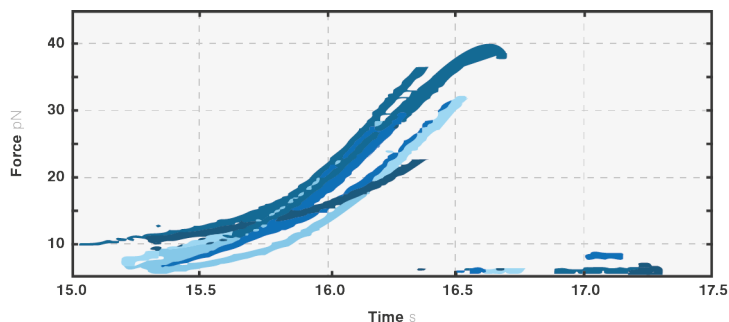
While low stretching forces (5 pN) did not cause DNA disruption within ten seconds, increasing the force to 40 pN induced rupture of the DNA molecule at the cleavage site (**Figure 7**). Interestingly, the Cas9 remained bound to the DNA after rupture, suggesting that, while the actual DNA-cleavage occurs early after complex-binding, disassembly of the complex requires extensive mechanical force.

“What forces are associated with DNA disruption and Cas9 binding post-cleavage?”



What can you do with the C-Trap® Dymo?

- Test if nuclease-DNA interactions impair association of other DNA-binding proteins (e.g., DDR proteins).
- Test if DNA-bound proteins impair nuclease binding.
- Measure how epigenomic states affect nuclease-induced DNA cleavage.
- Evaluate how DNA conformations (hairpins, supercoil, bubbles) affect cleavage.
- Time the cleavage induced by the nuclease upon complex-binding.

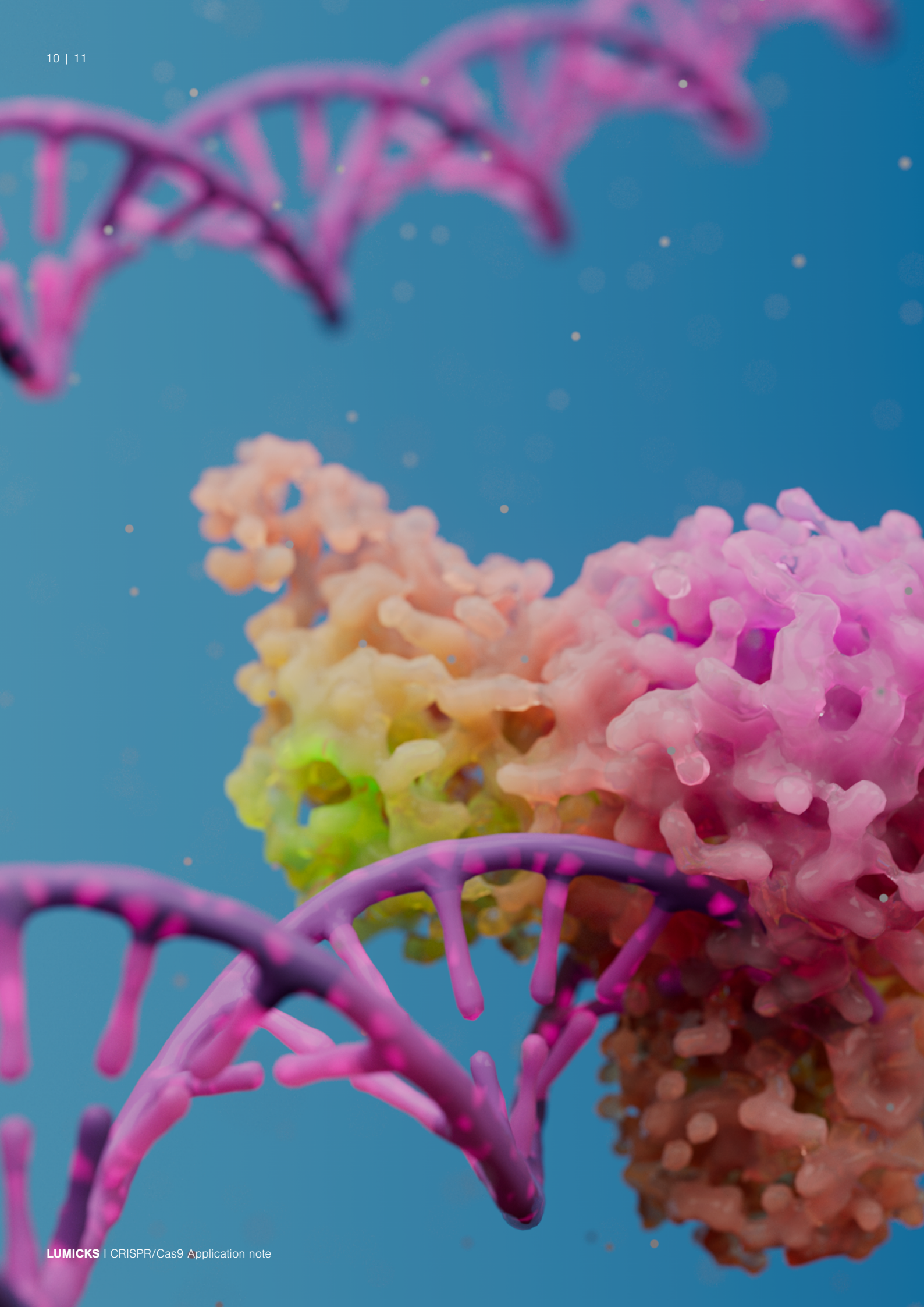


7 Graph representing the force-extension curve showing forces applied to the DNA after cleavage and the corresponding DNA lengths and ruptures.

Summary Cas9 Binding

Most research with Cas9 or other gene-editing tools depends on the catalytic activity of the respective nuclease. Understanding the kinetics of nucleases enables the optimization of the tools. We show here how you can use C-Trap to improve your understanding of Cas9 cleaving and:

- Test if your nuclease (e.g., Cas9) is catalytically active.
- Measure nuclease dwell-times after DNA cleavage.
- Measure the forces required for DNA disruption or nuclease dissociation after cleavage.



Conclusions

Here, the C-Trap Dymo was used to localize and quantify features associated with target binding of Cas9-complexes upon manipulation of the targeted DNA. It is important to note that measurements with the C-Trap can be applied to study your preferred gene-editing tool, whether it is a CRISPR-derived system (for example, Cas9, Cpf1 or Cas8) or unrelated systems (for example, TALENs or zinc-finger nucleases).

Although highly advanced, gene editing is still an open field with much more mechanistic insights to explore. The C-Trap Dymo is a powerful tool to complement and expand on the existing findings provided by conventional bulk population or other conventional assays.

The C-Trap[®] Dymo seamlessly combines highly dynamic and sensitive measurements with a variety of microscopy techniques. You can choose between widefield, confocal, and stimulated emission depletion (STED) microscopy to visualize any gene-editing tool on its target molecule (RNA or DNA). On top of that, the system supports up to three channels of fluorescent excitation, which allows you to identify different proteins and their co-localization on the targeted molecule.

Remarks

All experiments presented in this application note can be found in Newton et al. 2019, published in Nature Structural & Molecular Biology.

Collaborators

Rueda Lab, Imperial College, London, UK

References

- [1] Newton MD, et al. DNA stretching induces Cas9 off-target activity. *Nat Struct Mol Biol* 26(3):185-92. 2019.
- [2] Kim D, et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 12(3):237-43, 1 p following 243. 2015
- [3] Tsai SQ, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 33(2):187-97. 2015
- [4] Tsai SQ, et al. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods* 14(6):607-14. 2017

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