

Probing CRISPR–Cas9 Specificity Using LUMICKS’ C-Trap[®] Dymo Platform

Mechanical modulation of DNA reveals critical insights into CRISPR–Cas9 behaviour that are not accessible through conventional approaches. The study shows that structural perturbations in DNA expose recurrent off-target binding sites, while relaxed conformations restore targeting specificity. It also highlights that DNA cleavage is not purely sequence-driven but modulated by physical forces acting on the substrate. Together, these observations offer a more nuanced view of Cas9 performance under conditions that better reflect the complexity of cellular environments.

Keywords or phrases:

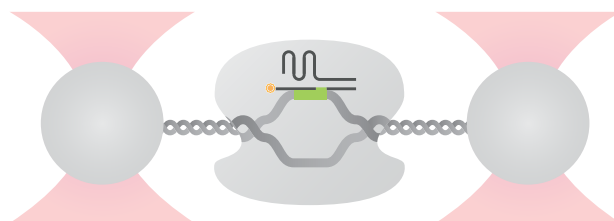
CRISPR–Cas9, single-molecule analysis, optical tweezers, DNA mechanics, off-target effects, binding specificity

INTRODUCTION

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, success has been hindered due to off-target effects of the Cas9 machinery in vivo. This is a major challenge for the development of Cas9 therapeutics as off-target effects can cause potentially lethal genetic mutations or lead to severe diseases such as cancer or auto-immune diseases. To battle these undesired Cas9 effects in vivo it is crucial to better understand its properties, which cannot be achieved with the bulk in vitro assays currently used by scientists.

Bulk assays lack detailed insights into the mechanism and only focus on the DNA sequence but miss out on the effect of local DNA structures that can be found in vivo. This could result in misinterpretation of data and false assumptions about the mechanism, which could potentially have devastating effects on clinical outcomes. Directly manipulating and visualizing the interaction between the Cas9 machinery and DNA would enable scientists to see the precise sequence of events that take place during Cas9 gene-editing, but also any potential unwanted interactions. This could help in the development of improved Cas9 therapeutics with minimal off-target effects and better clinical outcomes.

This application note introduces a new dynamic single-molecule analysis solution that enables you to study the binding and cleaving properties of Cas-related complexes and other gene-editing tools, in real time. With dynamic single-molecule analysis you can simultaneously manipulate and visualize single-molecule interactions in real time, allowing you to reveal the crucial and dynamic interactions taking place at the molecular level and uncover the root cause of disease development in response to gene editing. This note shows results from a recent publication from the laboratory of Prof. David Rueda at the Imperial College in London, UK1, which were obtained using LUMICKS' C-Trap® Dymo.



METHODS

DNA tethering and manipulation with optical tweezers. The C-Trap Dymo system used in this study to capture Cas9–DNA interactions is equipped with high-resolution optical tweezers. Optical tweezers enable you to trap 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 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 a force of at least 20 pN. These bubbles enable the assessment of Cas9 binding and cleaving properties on different structural conditions.

Cas9 preparation and DNA targeting

To study Cas9 positioning on a DNA molecule, you can combine catalytically active or dead Cas9 (dCas9) with fluorescently labelled crRNAs, a 20-nucleotide long sequence that is complementary to the target DNA site.

The labelled complexes are then loaded into a channel of the C-Trap Dymo 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 labelled with cyanine dyes (Cy3 or Cy5).

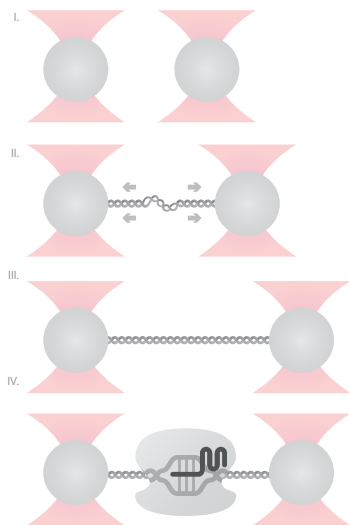


Figure 1a: A schematic illustration depicting the process of stretching DNA to then enable the assessment of Cas9. Samples are prepared in the laminar microfluidics channels: I. Bead Channel II. DNA Channel III. Buffer-only Channel IV. Imaging Channels



Figure 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.

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 labelled proteins on a trapped molecule, such as Cas9 on a DNA.

RESULTS

Using the C-Trap Dymo optical tweezers, the researchers were able to manipulate the DNA structure by applying different stretching forces. Following fluorescently labelled 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 2a, top panel). Under this condition, they found no observable off-target binding. The investigators then induced the formation of DNA bubbles (Figure 2b) by stretching the DNA molecule with a force of 20 pN and found that the established condition caused the fluorescently labelled dCas9-complex to bind to several off-target sites (Figure 2a, bottom panel). Interestingly, reducing the force 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 were then able to quantify force-dependent off-target binding by stretching the DNA molecule with

increasing forces (between 20 and 50 pN). While a force of 20 pN 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, while reducing the stretching-forces to 5 pN re-established the on-target specificity.

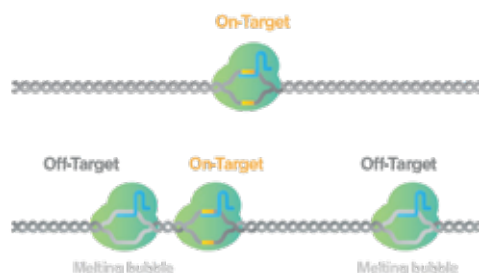


Figure 2a: A schematic illustration depicting the process of stretching DNA to form bubbles.

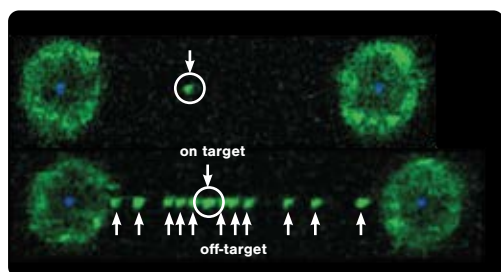


Figure 2b: A DNA molecule trapped between two beads with a fluorescently labelled dCas9-complex (green) bound to the target site (top). The dCas9-complex binds to increasing numbers of off-target sites upon stretching with 20 pN forces or higher (bottom). Adapted from Prof. David Rueda's YouTube Video: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

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 (Figure 3). 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.

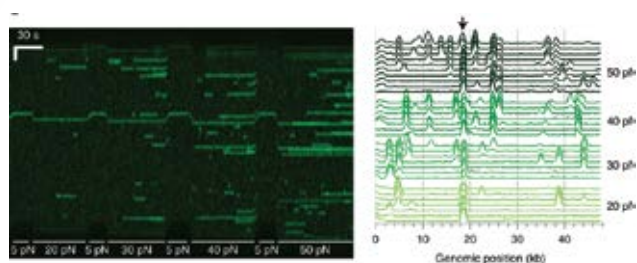


Figure 3: Cas9 binding events on force-stretched DNA. Binding events of Cas9 (green) on DNA stretched with forces between 5 and 50 pN, with off-target binding >20 pN. Time-binned intensity histogram (right) shows force dependence and genomic off-target binding locations (arrow marks on-target site).

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 4). 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.

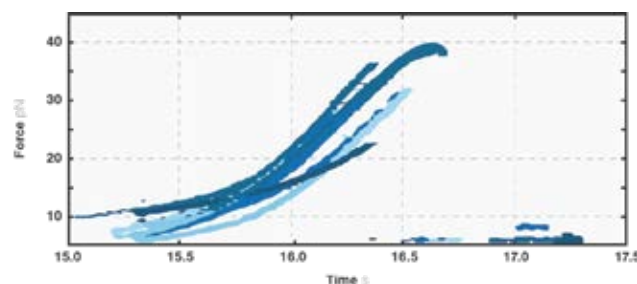


Figure 4: Graph representing the force-extension curve showing forces applied to the DNA after cleavage and the corresponding DNA lengths and ruptures.

CONCLUSION

Gene-editing using the Cas9 machinery is a valuable tool that has the potential to cure genetic diseases by removing, adding, or altering sections of DNA.

However, this potential is dampened by the devastating side-effects that can occur from Cas9 off-target binding. To study the Cas9 machinery up close and develop solutions that will allow for more effective CRISPR-Cas9 gene therapies without the side effects, better investigative tools than the ones used by scientists up until now are necessary. With dynamic single-molecule analysis that offers you the opportunity to explore and visualize in real time the molecular interactions between the Cas9 complex and DNA, it is now possible to fully understand and obtain direct evidence for the on/off-target binding mechanism of Cas9. This note showed that dynamic single-molecule analysis enabled researchers to localize and quantify features associated with target binding of Cas9-complexes upon manipulation of the targeted DNA. It is important to note that dynamic single-molecule measurements can be applied to study a wide range of gene editing tools besides Cas9, including other CRISPR derived systems (e.g., Cpf1 or Cas8) or unrelated systems (for example, TALENs or zinc-finger nucleases).

Significantly, by applying this technology to other DNA-binding proteins besides Cas9, it is possible to

investigate in real time, at the nanoscale level, the molecular mechanisms involved in DNA organization, replication, repair, editing and transcription.

The LUMICKS C-Trap Dymo, a powerful tool for dynamic single-molecule measurements, offers a unique way to expand on the existing findings provided by conventional bulk population or other conventional assays. With its seamless combination of highly dynamic and sensitive measurements with a variety of microscopy techniques, it provides scientists with the much-needed missing tool from their toolbox to accelerate gene-editing research and therapy development by proving their molecular mechanisms.

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