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Probing when dCas9 tolerates DNA mismatches

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Sensing changes in ionic current as barcoded DNA translocates through solid-state nanopores allows the study of how nucleotide sequences alter the DNA-binding specificity of the catalytically inactive Cas9 ribonucleoprotein complex.

Solid-state nanopores can be used to assess binding events for DNA, RNA and proteins in their folded and functional states by measuring alterations in ionic current as the molecules translocate a nanopore in the presence of an applied electric field. However, the sensing specificity of solid-state nanopores is insufficient for multiplexed-sensing applications. Now, reporting in *Nature Biomedical Engineering*, Ulrich Keyser and colleagues report a multiplexable nanopore-sensing system for the detection of binding events between DNA and the inactive version of the clustered regularly interspaced short palindromic repeats (CRISPR) ribonucleoprotein complex Cas9 (also known as dead Cas9; dCas9)[1].

As with the quipu system used by the Inca civilization, where knots in cords were tied in specific patterns as a way to record and transmit information[2], Keyser and colleagues' nanopore-sensing system leverages barcoded DNA nanostructures containing multiple binding sites for the dCas9 ribonucleoprotein. Unique barcodes allowed the authors to distinguish individual DNA nanostructures in solution as they translocated through a glass nanopore about 10 nm in diameter, and hence to detect multiple dCas9 complexes in a single measurement. The authors had developed this idea for the detection of different antibodies of the same isotype[3].

Keyser and co-authors first evaluated the use of DNA nanostructures for the detection of the binding of the dCas9 complex to a specific double-stranded DNA (dsDNA) overhang sequence. The DNA nanostructures consisted of a single-stranded DNA backbone with complementary staple oligos binding along its length. During translocation through a nanopore, the sections of the nanostructure that were entirely complementary appeared as a current drop, indicating the presence of dsDNA (Fig. 1). The authors designed the DNA with two essential components: a five-bit barcode portion, where each bit can be set to either '0' (no spike in current) or '1' (a spike in current), allowing for the detection of dCas9 binding to a particular target dsDNA overhang sequence; and a sensing region containing the dsDNA overhang, which can be programmed with any DNA sequence of interest. When the dCas9 complex does not bind to the target dsDNA in the overhang region, the spike from the overhang sequence is absent in the current trace, yet the five-bit barcode set of spikes in it remains. By applying a simple thresholding algorithm around the expected location for the spike from the overhang sequence, the authors determined the presence or absence of bound dCas9.

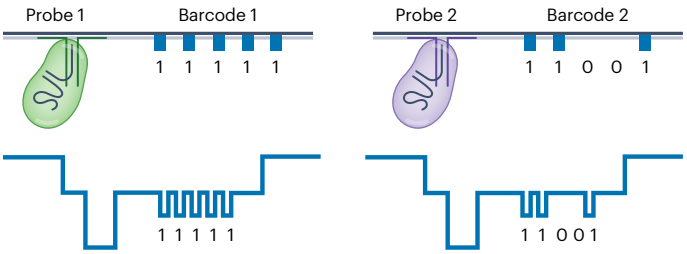

Fig. 1 | Assessing the binding specificity of dCas9 to target sequences via barcoded DNA translocating through a solid-state nanopore. Barcoded DNAs with dCas9 probes bound to a specific overhang sequence (top) and schematic of the corresponding current traces from the nanopore (bottom). Figure adapted with permission from ref. 1, Springer Nature Ltd.

To study the relationship between DNA sequence and the DNA-binding specificity of the dCas9 complex, and to assess its tolerance to DNA mismatches, Keyser and colleagues conducted experiments targeting a specific DNA region with a single point mutation in the *katG*315 gene of *Mycobacterium tuberculosis*. This gene is associated with antibiotic resistance to the small-molecule drug isoniazid in 64.2% of resistant cases. The authors evaluated the binding efficiency and specificity of two dCas9 complexes by creating two nanostructures, each with a unique barcode and overhang sequences. One nanostructure represented the wild-type genome of *katG*315, whereas the other carried the mutation and thus represented the drug-resistant genome. Both dCas9 complexes exhibited high specificity to their perfectly matched target overhangs. Indeed, more than 94.7% (for the wild-type nanostructure) and 94.0% (for the mutation-carrying nanostructure) of the binding events were accurately classified. The authors postulate that false positives probably resulted from knots in the DNA during translocation that produced signals similar to those generated by a bound dCas9 complex. They also show that the relative concentration of the DNA nanostructures correlated with the ratio of binding events between each nanostructure and the corresponding dCas9 complex (at equimolar concentrations of the two complexes).

Moreover, Keyser and colleagues designed DNA nanostructures with three distinct overhangs so as to investigate how the binding specificity of the dCas9 complex is influenced by changes in the position of a mutation and by the specific base pair mutated. Specifically, they introduced single-base-pair changes in the overhangs at the protospacer adjacent motif (PAM) site and at various distances from it. They found that mutations located one, two and three base pairs proximal to the PAM site exhibited the lowest binding efficiencies, suggesting that this region displayed the highest specificity for the dCas9 complex. As the mutations approached the distal region of the PAM, there

was a steady increase in dCas9 binding. They also found that the most tolerated base pairings were the result of slight shifts in the position of the nucleotides, owing to the flexibility of the DNA backbone (this is known as 'wobble' base pairing). An assessment of the most common mismatched DNA–RNA base pairings indicated that the wobble base pairs were dominant from the PAM site to position 5. This finding suggests that, when near to the PAM site, mismatches in base pairing are tolerated owing to the flexibility of the DNA backbone, which allows for nucleotide misalignment without hindering DNA–RNA base pairing. These findings align with those of previous work[4].

To illustrate the limitations of the computational design of guide RNAs, the authors show that, for two dCas9 complexes with different scores for cleavage efficacy (obtained from the computational tool CHOPCHOP), the probe with a lower score had higher binding efficiency. They also show that the binding efficiency of the dCas9 complex depends on the guide RNA used, and that the introduction of single-base-pair mismatches in the DNA overhang and in the guide RNA at the same location led to different binding efficiencies. Overall, these findings highlight the limitations of relying exclusively on computation for the design of CRISPR–Cas systems, and emphasize the need for robust and reliable experimental approaches to assess the specificity of dCas9.

Keyser and colleagues' integration of nanopore sensing with the design of DNA nanostructures offers a single-molecule-sensing technique for evaluating the DNA-binding efficiency and specificity of dCas9 enzymes and for identifying potential off-target sites, and could also be used to examine the specificity of other CRISPR-associated enzymes before their use in diagnostic assays[5]. The programmable, multiplexable and single-base-sensitive nature of the technique holds promise for accelerating the development of CRISPR-associated enzymes. In this respect, the technique should be optimized to reduce false positives, and to increase its throughput (this has long been a challenge for nanopore sensing[6]). Also, the system is currently limited to the detection of dCas9 binding. A nanopore-based technique that would allow for the assessment of how mismatches in the target region of DNA affect the cleavage activity of various CRISPR enzymes, particularly Cas12 (ref. 7) and Cas13 (ref. 8), would unlock opportunities for the development of high-fidelity CRISPR-based diagnostics and safe gene regulation.

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Competing interests

The author declares no competing interests.