Methods for characterizing, applying, and teaching CRISPR-Cas systems

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Bacteria and archaea possess fascinating defense systems comprised of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) arrays and CRISPR-associated (Cas) proteins whose characterization is driving a rapid and widespread technological revolution. In their natural form, CRISPR-Cas systems confer adaptive immunity against mobile genetic elements through a rich diversity of mechanisms and functions. This functional diversity and the role of easy-to-design guide RNAs (gRNAs) led to the facile translation of these systems into versatile tools impacting applications ranging from genome editing and gene regulation to diagnostics, antimicrobials, and imaging agents. Ongoing progress in applications such as human gene therapy and gene drives in turn has spurred heated ethical debates, underscoring an urgent need to inform the wider population about these technologies and their ramifications.

This special issue in *Methods* comprises two broad reviews and seven original methods representing a broad sampling of current methodologies. Below is a brief overview of each article grouped based on their primary relevance to the fundamental characterization of CRISPR-Cas immune systems, implementation of CRISPR technologies specifically in bacteria, and teaching CRISPR through hands-on modules intended for the classroom.

**Methods for characterizing the biology of CRISPR-Cas systems.** Five articles in the special issue entail methodologies for identifying and characterizing the biomolecular properties of CRISPR-Cas systems and their related components. Alkhnbashi and coworkers provide a comprehensive review of available bioinformatics tools [ref1]. The review principally describes tools for identifying and annotating CRISPR-Cas immune systems, including finding CRISPR arrays and cas genes within genomic sequences, properly orienting the arrays, identifying targets of the encoded CRISPR RNAs (crRNAs, the natural form of gRNAs), and classifying the type and sub-type of each system. Furthermore, available design tools for gRNAs relevant to CRISPR technologies are also described. The bioinformatics tools for identifying CRISPR-Cas systems are particularly useful given the
ubiquity of these systems in prokaryotes and their extensive diversity that is still being understood and mined for technologies.

In the second review, Behler and Hess describe common approaches for interrogating the mechanisms underlying crRNA biogenesis, with resulting insights across CRISPR-Cas types and subtypes [ref2]. Prior work has shown that these mechanisms can vary widely, requiring a broad set of techniques to pinpoint each mechanism and the responsible genetic factors. The authors describe how these techniques helped reveal the different ways in which a transcribed CRISPR array can be converted into individual crRNAs and aspects of crRNA biogenesis that remain to be explored.

The first of the methods articles transitions the special issue from crRNA biogenesis to target cleavage by the CRISPR effector nuclease responsible for immune defense. Creutzburg and coworkers report a medium-throughput method to evaluate DNA cleavage activity in vitro by the Type V-A nuclease Cas12a [ref3]. This single-effector nuclease offers unique advantages over canonical Cas9 nucleases, such as staggered DNA cleavage or the ability to generate crRNAs without accessory factors. As part of the method, the authors targeted linear DNA with biotin on one end and a fluorophore on the other end, allowing the facile measurement of the solution’s fluorescence following the addition and removal of streptavidin beads. The method was used to detect nuclease concentration and cleavage rates, although it could be further applied to elucidate potential protospacer-adjacent motifs (PAMs) or the impact of guide-target mismatches.

The next method from Gramelspacher and coworkers addresses a unique feature of some Cas9 nucleases: the capacity to target RNA [ref4]. The authors detail how to characterize RNA cleavage activity under in vitro conditions, with a focus on evaluating the extent of cleavage, mapping the cleavage site, and determining whether the cleaved target remains bound to Cas9. They also describe the purification of an anti-CRISPR protein (Acr) and testing its ability to inhibit RNA cleavage by Cas9 in vitro. The method should be useful when evaluating the extent to which other Cas9 nucleases exhibit this behavior as well as the impact of different Acrs.
The final method devoted to CRISPR biology takes a unique approach to the characterization of Acrs. Acrs are thought to exist against virtually every CRISPR-Cas system, yet Acrs have only been associated to-date with a small fraction of all CRISR-Cas subtypes. Wandera and coworkers report the use of an \textit{E. coli}-based transcription-translation system (TXTL) to rapidly and scalably characterize different Acrs, using Acrs against Cas9 as a case study \[\text{ref5}\]. In particular, they show how to modify a previously published assay to minimize non-specific inhibition of gene expression associated with some Acrs.

\textbf{Methods for implementing CRISPR technologies in bacteria and archaea.} The special issue then turns from CRISPR biology to CRISPR technologies—specifically, programmable gene editing and repression in prokaryotes \[\text{ref6}\]. Diallo and coworkers developed an inducible two-plasmid system for genome editing with Cas9 for industrial strains of the bacterial genus Clostridia. One of the plasmids encoded the recombineering template, allowing for repair in coordination with Cas9 cleavage. The authors used this strategy to delete a sporulation gene or insert a fungal cellobiohydrolase gene in one strain of \textit{Clostridium beijerinckii} followed by phenotypic characterization. The strategy was further applied in another strain of \textit{C. beijerinckii} to delete a gene conferring thiamphenicol resistance and remove an endogenous plasmid, together allowing greatly improved transformation of a plasmid selected with thiamphenicol.

Next, Depardieu and Bikard detail how to achieve programmable gene repression with a catalytically-dead Cas9 (dCas9) in the bacteria \textit{Escherichia coli} and \textit{Staphylococcus aureus} \[\text{ref7}\]. This mode of gene repression has been extremely effective for single or multiplexed gene knock-down and has been easier to implement across bacterial species than genome editing. Beyond showing how to generate and apply the basic constructs, the authors incorporated origins-of-replication with broad host ranges. They also devised a clever high-throughput screen to determine the optimal expression level of dCas9.
expression that maintains silencing activity without inducing a cytotoxic “bad seed” effect observed previously by the authors.

Finally, Albers and coworkers describe an entirely distinct approach for achieving CRISPR-based gene repression in archaea [ref8]. Rather than relying on dCas9 or another heterologous nuclease, the authors co-opted the CRISPR-Cas system endogenous to the archaeon Haloferax volcanii. The system was devoid of the nuclease-encoding subunit Cas3 as well as the processing enzyme Cas6; as a result, expressing a mature version of a designed crRNA allowed the authors to achieve targeted gene silencing without competition by endogenous crRNAs. Through this work, the authors provided basic design rules for efficient gene silencing and demonstrated silencing of chromosomal and plasmid-encoded genes, providing a strong foundation for CRISPR-based repression in other archaea.

Methods for using CRISPR in the classroom. The final article of the special entails how to communicate the basic properties of genome editing with CRISPR technologies in a classroom setting. Here, Ziegler and Nellen report a set of experiments to illustrate DNA cleavage in E. coli [ref9]. They provide detailed protocols and instructions as well as lecture materials, and the entire module can be completed in a day using simple and low-cost materials and equipment. They also provided one round of student assessments indicating positive reception as well as feedback to further improve the module.

Collectively, this special issue covers available methods for characterizing the rich diversity of CRISPR biology, implementing a cross-section of current CRISPR technologies, and porting CRISPR into the classroom. I hope you enjoy this issue and find it useful for your own interests and pursuits.

References

1. Voss [review] – Bioinformatics tools
2. Hess [review] – Characterizing crRNA biogenesis and resulting insights
3. van der Oost [method] – Characterizing cleavage by Cas12a in vitro
5. Beisel [method] – Acr characterization using TXTL
6. Diallo [method] – Inducible editing with Cas9 in Clostridia
8. Marchfelder [method] – CRISPRi in archaea