Topic Introduction

Cas9-Mediated Genome Engineering in *Drosophila melanogaster*

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The recent development of the CRISPR–Cas9 system for genome engineering has revolutionized our ability to modify the endogenous DNA sequence of many organisms, including *Drosophila*. This system allows alteration of DNA sequences in situ with single base-pair precision and is now being used for a wide variety of applications. To use the CRISPR system effectively, various design parameters must be considered, including single guide RNA target site selection and identification of successful editing events. Here, we review recent advances in CRISPR methodology in *Drosophila* and introduce protocols for some of the more difficult aspects of CRISPR implementation: designing and generating CRISPR reagents and detecting indel mutations by high-resolution melt analysis.

INTRODUCTION

The process of genome engineering relies on the generation of a double-strand break (DSB) at a specific, user-defined locus in the genome. Following this event, endogenous cellular mechanisms repair the DNA damage primarily through the nonhomologous end joining (NHEJ) or homologous recombination (HR) pathways (Jeggo 1998; van Gent et al. 2001). NHEJ is an error-prone repair mechanism, often resulting in the generation of short insertion or deletion (indel) mutations at the DSB site. This can be exploited for the generation of gene knockouts by inducing frameshift mutations in coding sequence. Alternatively, by providing a donor construct with homology to the target locus, which can serve as a template for repair by the HR machinery, the repair process can be used to produce more precise alterations to the genomic sequence. For example, single-nucleotide changes, insertions, deletions, or substitutions are possible using HR.

Several existing technologies have been developed to generate DSBs at specific genomic loci (e.g., TALENs [transcription-activator-like effector nucleases] and zinc-finger nucleases) (Bibikova et al. 2003; Joung and Sander 2013; Carroll 2014); however, CRISPR has been widely adopted because of the simplicity of its use and its high efficiency of DSB generation. Since the first demonstrations of genome engineering with CRISPR (Jinek et al. 2012, 2013; Cong et al. 2013; Mali et al. 2013b), the technology has been used in a wide range of organisms, including *Drosophila* (Bassett et al. 2013; Gratz et al. 2013; Kondo and Ueda 2013; Ren et al. 2013; Yu et al. 2013), for a rapidly expanding repertoire of applications.

CRISPR requires two components. The first is a nonspecific DNA nuclease protein called Cas9. The second is an RNA molecule capable of binding to Cas9, which provides customizable specificity to a DNA sequence (Jinek et al. 2012). Although the endogenous CRISPR system in *Streptococcus pyogenes* uses two RNA molecules, these have been combined into a single guide RNA (sgRNA) to

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further simplify the use of the system in other organisms (Jinek et al. 2012). A 20-bp region of the sgRNA provides specificity to the complementary DNA sequence. The target site can be determined simply by recoding this region. Therefore, CRISPR can be directed to different genomic loci just by expressing different sgRNAs. This is in contrast to previous genome engineering technologies based on TALENs and zinc-finger nucleases, which require production of new proteins for each target site. In addition, the only targeting requirement in the CRISPR system is the presence of a PAM (protospacer-adjacent motif) sequence (NGG) 3′ of the 20-bp target site (Jiang et al. 2013); thus, possible target sequences are common. Because CRISPR enables rapid and efficient genome modification with minimal effort required to reprogram target site specificity, it is a highly flexible and robust system for genome engineering.

CRISPR IN *Drosophila*

The use of CRISPR for any application in *Drosophila* requires several steps. First, sgRNA design is critical to achieving the desired editing event at high frequency and with high specificity. Second, CRISPR reagents must be delivered efficiently. Finally, an appropriate method must be available to detect the desired editing event. Here we summarize important factors for the experimental design.

Design of sgRNAs

CRISPR has been shown to function with high efficiency both in cultured *Drosophila* cells and in vivo. Detailed protocols for many aspects of its use have been described elsewhere (Bassett et al. 2013, 2014; Gratz et al. 2013; Kondo and Ueda 2013; Ren et al. 2013; Yu et al. 2013; Bassett and Liu 2014; Beumer and Carroll 2014; Bottcher et al. 2014; Housden et al. 2014). However, despite the widespread adoption of CRISPR methodologies in *Drosophila*, there remain several unanswered questions regarding an optimal experimental design. For example, off-target (OT) mutations have been shown to occur often in mammalian systems, both at predicted OT sites and at other loci not predicted by any current sgRNA design algorithm (Carroll 2013; Fu et al. 2013; Hsu et al. 2013; Mali et al. 2013a; Pattanayak et al. 2013; Tsai et al. 2015). In contrast, OT mutations have yet to be reported in *Drosophila*, perhaps because of reduced genome complexity. In addition, although mutation efficiency varies widely between different sgRNAs, investigations into the difference in efficiencies have produced inconsistent results (Doench et al. 2014; Ren et al. 2014; Wang et al. 2014). It is therefore advisable to design sgRNAs carefully using the available online tools (see Protocol: **Design and Generation of *Drosophila* Single Guide RNA Expression Constructs** [Housden et al. 2016]) and, where possible, perform experiments using multiple sgRNAs in parallel.

Delivery of CRISPR Reagents

The method used to deliver CRISPR reagents into flies can greatly affect the efficiency of genome editing. Delivery methods have been discussed in detail elsewhere (Ren et al. 2013; Housden et al. 2014), so we will provide only a brief summary here. One option is to generate Cas9 mRNA or protein in vitro and inject it together with synthesized sgRNA directly into fly embryos (Gratz et al. 2013; Lee et al. 2014b). This approach can be laborious because of the need to generate high-quality protein or mRNA and generally results in low efficiency. However, one advantage is that the reagents are short-lived and so the chance of generating OT mutations or somatic mutations is reduced, resulting in lower toxicity. An alternative approach is to inject an sgRNA-expressing plasmid into flies that express Cas9 in the germline, which results in higher efficiency compared with direct mRNA injection (Ren et al. 2013). Finally, both sgRNA and Cas9 can be expressed from transgenes. By crossing flies that each express one of these components, very high modification rates can be achieved (Kondo and Ueda 2013). The disadvantages of this approach are the increased time required to generate the transgenic lines and the challenge of delivering a donor for homologous recombination.

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Delivery of CRISPR reagents into cultured cells can be problematic, as many *Drosophila* cell lines suffer from low transfection efficiency. One option is to encode both sgRNA and Cas9 on a single plasmid (see Protocol: **Design and Generation of *Drosophila* Single Guide RNA Expression Constructs** [Housden et al. 2016]), and transfect the cell type of interest to generate indel mutations. This method works with relatively high efficiency, although when mutations cause cell viability effects, the population can rapidly revert to wild type. Including an antibiotic selection cassette in the CRISPR expression plasmid allows enrichment for transfected cells (Bassett et al. 2014), thereby increasing the proportion of successfully modified cells. However, because of the accumulation of nonframeshift mutations, these populations can also revert to wild type. One solution to this issue is to generate clonal mutant populations by selecting homozygous mutant cells from the mixed population and growing new cultures from individual cells, yet this approach is also problematic because of the low survival rate of single cells in culture.

To stabilize a genomic change in cells, a donor construct or oligo can be cotransfected with the CRISPR expression plasmid to insert a selection cassette into the genome and simultaneously disrupt the gene of interest (see Protocol: **Design and Generation of Donor Constructs for Genome Engineering in *Drosophila*** [Housden and Perrimon 2016a]), leading to more direct selection of the modification event. However, whether donors can integrate nonspecifically, and the frequency of such events, is not yet clear. Although it is possible to include a selection cassette in the HR product, a major limitation is that there is currently no method to ensure all copies of a gene are modified. This is a particular problem in *Drosophila* cell culture because the majority of cell lines are polyploid or aneuploid (Lee et al. 2014a).

Detection of Editing Events

Several methods are available to detect genome-editing events and the approach taken will depend on the type of genomic modification induced. For example, when generating insertions using donor constructs, it is often possible to include a selection marker such as *mini-white* in flies or an antibiotic resistance gene in cells. An alternative approach is to detect insertions using polymerase chain reaction (PCR) assays to amplify fragments present only in the modified flies or cells. In addition, deletions generated using a donor construct can be detected by PCR assays using primers flanking the deletion site.

Detection of small indel mutations can be more challenging because the exact genomic change can be unpredictable and no markers can be included. In this case, molecular assays such as surveyor assays, restriction profiling, or high-resolution melt analysis (HRMA) must be used. Detailed methods for surveyor assays and restriction profiling have been described elsewhere (Bassett et al. 2013; Cong et al. 2013; Housden et al. 2014). We provide a method for HRMA in Protocol: **Detection of Indel Mutations in *Drosophila* by High-Resolution Melt Analysis (HRMA)** (Housden and Perrimon 2016b).

A final consideration for the experimental design is the background in which CRISPR is used. For example, as described above, DSBs can be repaired using NHEJ or HR, with the resulting modification being dependent on which of these pathways is used. To improve the chances of recovering the desired modification, repair pathway choice can be biased. For example, DSBs induced in the background of a *ligase4* mutation have been shown to increase the recovery of HR repair events in vivo and in cells by inhibiting the NHEJ pathway (Beumer et al. 2008; Bozas et al. 2009; Bottcher et al. 2014; Gratz et al. 2014). In addition, small molecules were recently developed to inhibit either HR or NHEJ in pluripotent stem cells (Yu et al. 2015) and thus increase the rate of NHEJ or HR events, respectively. Although these inhibitors have not yet been tested in *Drosophila*, this may provide a useful mechanism to increase efficiency in cell culture.

In summary, the CRISPR system provides a robust and simple method to induce modifications into the *Drosophila* genome. With careful design of the experimental approach and reagents, genome engineering can be achieved with unprecedented efficiency and accuracy.

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