Use of the CRISPR-Cas9 System in *Drosophila* Cultured Cells to Introduce Fluorescent Tags into Endogenous Genes

Justin A. Bosch,[1] Shannon Knight,[1,2] Oguz Kanca,[3,4] Jonathan Zirin,[1,2] Donghui Yang-Zhou,[1,2] Yanhui Hu,[1,2] Jonathan Rodiger,[1,2] Gabriel Amador,[1,2] Hugo J. Bellen,[3,4,5,6] Norbert Perrimon,[1,2,7] and Stephanie E. Mohr[1,2,8]

[1]Department of Genetics, Harvard Medical School, Boston, Massachusetts
[2]Drosophila RNAi Screening Center, Harvard Medical School, Boston, Massachusetts
[3]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas
[4]Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, Texas
[5]Department of Neuroscience, Baylor College of Medicine, Houston, Texas
[6]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas
[7]Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts
[8]Corresponding author: *Stephanie_Mohr@hms.harvard.edu*

The CRISPR-Cas9 system makes it possible to cause double-strand breaks in specific regions, inducing repair. In the presence of a donor construct, repair can involve insertion or 'knock-in' of an exogenous cassette. One common application of knock-in technology is to generate cell lines expressing fluorescently tagged endogenous proteins. The standard approach relies on production of a donor plasmid with ~500 to 1000 bp of homology on either side of an insertion cassette that contains the fluorescent protein open reading frame (ORF). We present two alternative methods for knock-in of fluorescent protein ORFs into Cas9-expressing *Drosophila* S2R+ cultured cells, the single-stranded DNA (ssDNA) Drop-In method and the CRISPaint universal donor method. Both methods eliminate the need to clone a large plasmid donor for each target. We discuss the advantages and limitations of the standard, ssDNA Drop-In, and CRISPaint methods for fluorescent protein tagging in *Drosophila* cultured cells. © 2019 by John Wiley & Sons, Inc.

Basic Protocol 1: Knock-in into Cas9-positive S2R+ cells using the ssDNA Drop-In approach
Basic Protocol 2: Knock-in into Cas9-positive S2R+ cells by homology-independent insertion of universal donor plasmids that provide mNeonGreen (CRISPaint method)
Support Protocol 1: sgRNA design and cloning
Support Protocol 2: ssDNA donor synthesis
Support Protocol 3: Transfection using Effectene
Support Protocol 4: Electroporation of S2R+-MT::Cas9 *Drosophila* cells
Support Protocol 5: Single-cell isolation of fluorescent cells using FACS

Keywords: cell culture • CRISPaint • CRISPR • *Drosophila* • fluorescent protein tagging • gene tagging • GFP fusion • knock-in • ssDNA Drop-In

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INTRODUCTION

Tagging endogenous proteins by insertion of a fluorescent protein open reading frame (ORF) into a gene is a common application of CRISPR knock-in technology, as it facilitates visualization of the cellular and subcellular distribution of the resulting fusion protein in live or fixed cells. In *Drosophila* cell lines, for example, introduction of a fluorescent protein ORF has been applied to generate a resource of *Drosophila* cell lines in which various organelles and sub-cellular compartments have been tagged with mCherry (Neumuller et al., 2012). The efficiency of introduction of tags into *Drosophila* cells is dramatically improved by introduction of the CRISPR-Cas9 system as a tool to facilitate insertion or 'knock in' of an insertion cassette into a specific locus. Indeed, CRISPR approaches have been successfully used to generate *Drosophila* cells in which endogenous loci are tagged by GFP fusion, e.g., Bosch, Colbeth, Zirin, & Perrimon, 2019; Bottcher et al., 2014; Kanca et al., 2019; Kunzelmann, Bottcher, Schmidts, & Forstemann, 2016; Wang et al., 2016.

The standard protocol involves production of a plasmid donor with ~500- to 1000-bp homology arms (Housden & Perrimon, 2016). Alternative approaches, as presented here, can accelerate the CRISPR knock-in workflow, for example by making it easier to obtain or prepare donor constructs (Bosch et al., 2019; Kanca et al., 2019). Specifically, we present, as alternatives to the standard approach, a single-stranded DNA (ssDNA) "Drop-In" method based on in vitro synthesis of an ssDNA donor (Kanca et al., 2019; Basic Protocol 1) and a "CRISPaint"-based approach that relies on universal donors

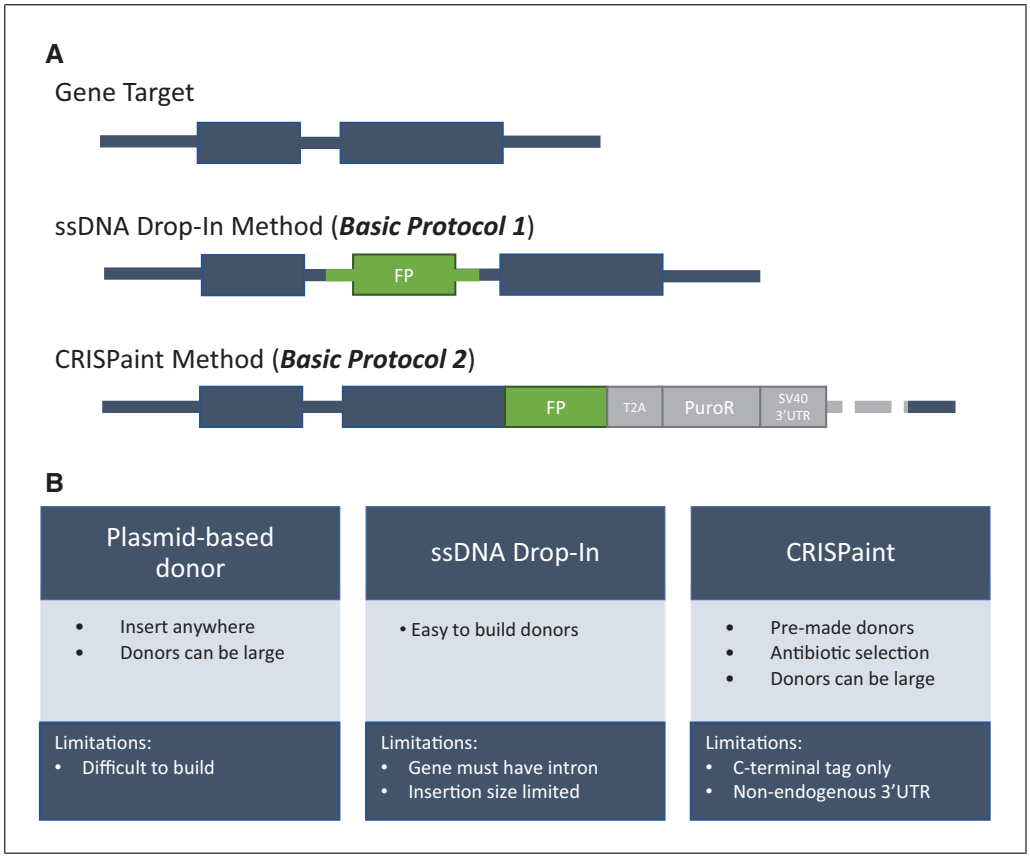

Figure 1 Comparison of CRISPR knock-in methods for introduction of fluorescent protein tags into *Drosophila* cultured cells. (**A**) Diagram of a theoretical gene target and results of knock-in using the ssDNA Drop-In method (Basic Protocol 1) and CRISPaint method (Basic Protocol 2). FP, fluorescent protein open reading frame (ORF); T2A, self-cleaving peptide ORF; PuroR, puromycin resistance ORF. (**B**) Comparison of standard plasmid-based donor method for tagging with a fluorescent protein ORF with ssDNA Drop-In and CRISPaint methods.

(Bosch et al., 2019; Schmid-Burgk, Honing, Ebert, & Hornung, 2016; Basic Protocol 2). For all three approaches, starting with a Cas9-positive cell line increases efficiency. The protocols described here are both based on use of the S2R+-MT::Cas9 cell line, which is described in Viswanatha, Li, Hu, & Perrimon (2018) and available from the *Drosophila* Genomics Resource Center (DGRC #268; *https://dgrc.bio.indiana.edu*).

The standard plasmid-based donor, ssDNA Drop-In, and CRISPaint methods have different strengths and limitations (Fig. 1). A standard donor plasmid provides the most flexibility, allowing for insertion of GFP or another sequence into any region of the *Drosophila* genome with a single guide RNA (sgRNA) target site in close proximity (i.e., effectively, any genomic region). With the ssDNA Drop-In method (Basic Protocol 1), the donor construct is built using PCR followed by an *in vitro* digestion reaction to remove one of the two strands. Gene-specific regions are included in the design of the synthetic oligos used as primers in the PCR step, and there is no need for sub-cloning or propagation of donor plasmids in bacteria. These improve donor production efficiency; however, the size of the ssDNA insertion cassette is limited compared to the standard approach due to the way in which the ssDNA is generated (Support Protocol 2). The specific ssDNA Drop-In protocol described here corresponds to the research report by Kanca et al., (2019) and is based on insertion of sfGFP as an artificial exon (Basic Protocol 1). With the CRISPaint method (Basic Protocol 2), there are no gene-specific regions in the donor; however, because the donor plasmid is linearized and integrated in full into the target locus, the CRISPaint method is only useful for C-terminal tagging. The specific CRISPaint protocol described here is based on the research report by Bosch et al. (2019) for insertion of mNeonGreen and a puromycin selection marker that contributes to efficient isolation of insertion events (Basic Protocol 2). The nature of the gene target(s) and scale of the project are among the considerations that go into choosing an optimal method for a given project (see Strategic Planning).

A workflow for both protocols is shown in Figure 2. For either protocol, transfection with the donor and sgRNA constructs can be performed by chemical transfection, such as with Qiagen Effectene (Support Protocol 3), or by electroporation, such as with the Lonza Nucleofect system (Support Protocol 4). Moreover, for both approaches, fluorescence-activated cell sorting (FACS) is used to identify and perform single-cell isolation of putative fluorescent protein–tagged cells, and this can be followed by image analysis to observe GFP or mNeonGreen in the cells, for example using Cell Profiler (Carpenter et al., 2006) and taking advantage of the fact that the S2R+-MT::Cas9 cell line expresses mCherry (Neumuller et al., 2012; Viswanatha et al., 2018). The mCherry signal can be used to identify cells and can be compared with the signal from the knock-in tag.

STRATEGIC PLANNING

Which Method is Best for my Target?

When deciding among the standard plasmid donor method, the ssDNA Drop-In method, and CRISPaint method to fluorescently tag an endogenous protein, important planning considerations include the following—(a) the size of the insertion cassette, (b) the intron-exon structure of the target gene, and (c) the desired position of the insertion relative to the coding sequence—as these will determine which strategy or strategies match with the project goals (Fig. 1). Insertion cassette size is limited for the ssDNA Drop-In method but not for the standard or CRISPaint methods. The ssDNA Drop-In method provides the fluorescent protein ORF as an artificial exon, such that an appropriate intron must be present in the target gene. The standard method allows for insertion of a cassette anywhere, and the CRISPaint method is useful for C-terminal tagging. For all approaches, the expression level of the gene in *Drosophila* cells must be sufficient for detection of the fluorescently tagged protein. Expression levels of a target gene(s) in any

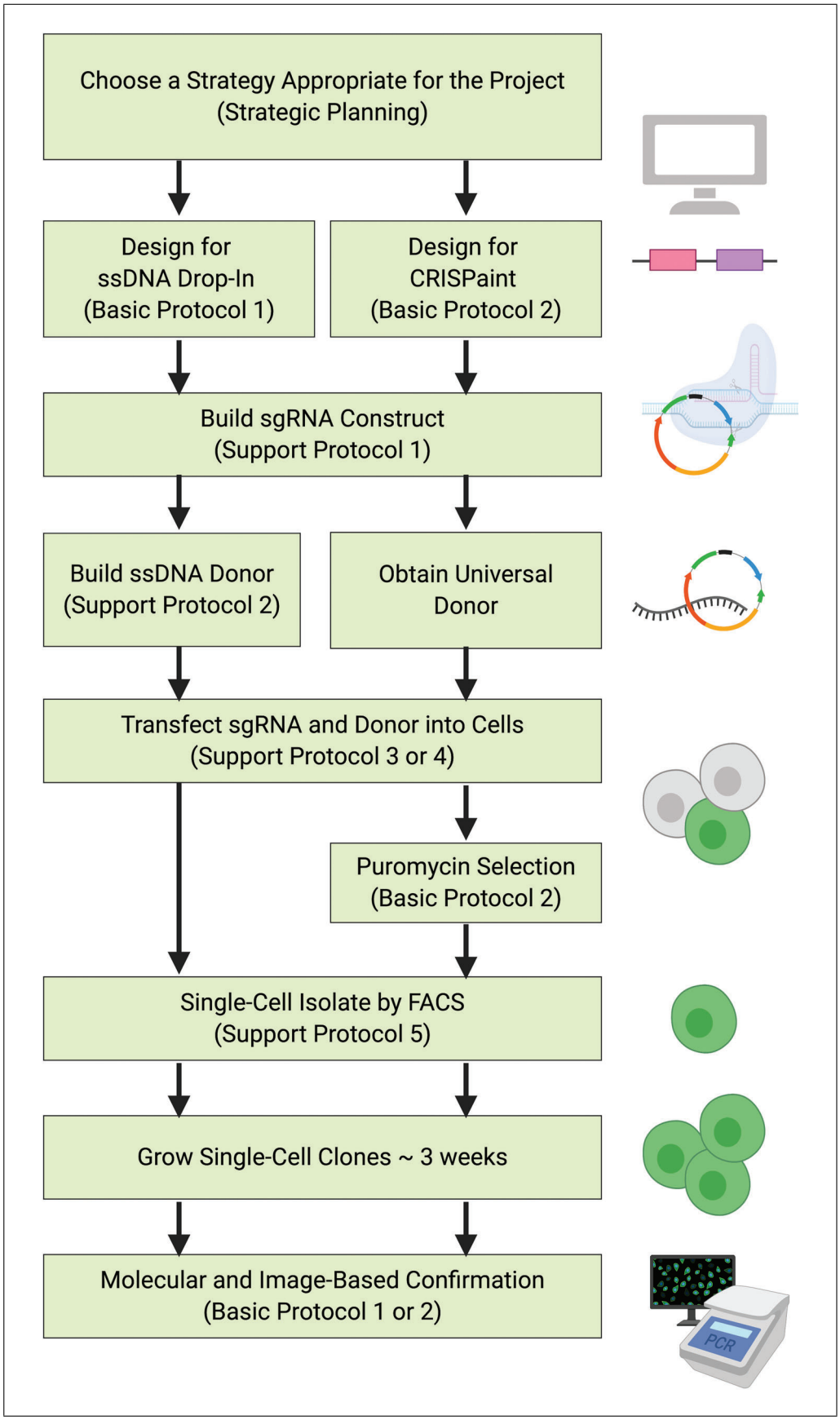

Figure 2 Workflow for ssDNA Drop-In and CRISPaint approaches to knock-in of fluorescent protein tags into *Drosophila* cultured cells. With both methods, production and validation of single-cell clones positive for the knock-in takes about 2 months.

of several *Drosophila* cell lines can be queried based on modENCODE *Drosophila* cell line transcriptomics data sets (Cherbas et al., 2011), for example, using the *Drosophila* Gene Expression Tool (DGET; *https://www.flyrnai.org/dget*; Hu, Comjean, Perrimon, & Mohr, 2017). We note that Kanca et al. (2019) report isolation of GFP-tagged cell lines using the ssDNA Drop-In approach for some targets expressed at moderate or low levels.

Rationale—ssDNA Drop-In Method (Basic Protocol 1)

Why single-stranded donors?

For ssDNA homology donors, short homology arms, typically 100 nucleotides (nt), are used to facilitate integration (Beumer, Trautman, Mukherjee, & Carroll, 2013; Gratz et al., 2013; Wissel et al., 2016). These are short enough to be included in PCR primers as 5′ overhangs, rather than requiring PCR amplification and cloning of homology arms, which is a requirement for the standard method. Moreover, PCR conditions do not change from gene to gene since the PCR template does not change. Thus, as compared with making standard donors, making donors using the ssDNA Drop-In method is faster and more scalable. In addition, different donor constructs can be amplified with the same primers. The method we use to generate ssDNA homology donors was modified from the ssDNA production method described in Higuchi & Ochman (1989).

Why provide the fluorescent tag as an artificial exon?

In our experience, integration of donor cassettes is not always precise. With an artificial exon approach, small indels are unlikely to affect function because the ssDNA Drop-In cassette is inserted into an intron (i.e., would not affect the resulting mature mRNA). Moreover, a comparison of data from an intronic tagging effort as reported in Nagarkar-Jaiswal et al. (2015) to data from a C-terminal tagging effort as reported in Sarov et al. (2016) suggests that intronic tagging leads to a slightly higher percentage of functionally tagged proteins (75% of intronically tagged proteins versus 67% of C-terminally tagged proteins were functional). Selecting introns that do not bifurcate functional domains will likely increase the chance of obtaining a functional tagged protein. Based on our own analysis of the *Drosophila* reference genome at FlyBase (Thurmond et al., 2019), about 40% of *Drosophila* protein-coding genes contain an intron with suitable sgRNA sites and of sufficient size (i.e., at least ∼150 nt) to support the ssDNA Drop-In artificial exon approach.

Rationale—CRISPaint Method (Basic Protocol 2)

Whereas the standard and ssDNA Drop-In approaches rely on homology-directed repair (HDR) to integrate donor DNA with homology arms, the CRISPaint method uses an NHEJ mechanism to insert a universal donor plasmid into a target gene (Schmid-Burgk et al., 2016). This accelerates up-front molecular steps by eliminating the need for PCR amplification of long homology donor arms (as for the standard approach) or generating ssDNA. Furthermore, publicly available universal donor plasmids containing different insert sequences provide flexibility (e.g., the CRISPaint Gene Tagging Kit; Addgene #1000000086). As mentioned, one trade-off is that the entire plasmid will insert into the locus, so, for fluorescence tagging, this approach is only useful for C-terminal tagging.

What cell line should I start with?

Different cell lines will be optimal for different targets, as different cell lines express different subsets of *Drosophila* genes. As mentioned in the Introduction, above, the protocols described here are based on use of a Cas9-expressing S2R+ cell line, generated as described in Viswanatha et al. (2018), and made available through the DGRC (#268). For other cell lines, Cas9 could be provided transiently via co-transfection with a Cas9 expression vector, or a Cas9-expression cell line could be established. For stable

transfection protocols, see Santos, Jorge, Brillet, & Pereira (2007) and *https://fgr.hms.harvard.edu/stable-fly-cell-lines*.

Is your goal to tag any allele or all alleles?

In the protocols presented, we make the assumption that generating any tagged allele will result in a cell clone useful for the project. In some cases, however, the goal might be to isolate a cell line in which the fusion protein is the only source of the protein. This could be achieved either by isolating cell clones in which all alleles were converted to the knock-in allele, or in which the non-knock-in alleles were disrupted by NHEJ-induced indels. Given that *Drosophila* S2R+ cells are polyploid, isolation of cells in which the tagged protein is the only source of the protein can be challenging. This would likely require additional molecular analyses not described here to identify and characterize non-tagged alleles (e.g., PCR amplification of the non-tagged alleles and next-generation sequencing of the product to detect indels), and might require a multi-step approach in which remaining non-tagged alleles are targeted for CRISPR knockout following successful isolation of a knock-in event.

What about knock-in of other types of sequences?

Knock-in of other sequences, including non-fluorescent tags, could be approached using protocols similar to those presented here. With the introduction of a fluorescent tag or reporter, FACS can be used to identify and isolate the subset of single cells that are positive for the fluorescent marker from a live cell population. However, for most or all non-fluorescent tags or other knock-in events, it would not be possible to use live-cell FACS to identify and isolate single cells positive for the insertion. Instead, detection of non-fluorescent tags would require screening single-cell clones for tag expression by methods such as immunoblot or molecular analysis. In this case, antibiotic enrichment of correct insertion events, as is possible using the CRISPaint method, could make identification of positive cells much more feasible by enriching for successful insertion events prior to single-cell isolation and analysis.

BASIC PROTOCOL 1

KNOCK-IN INTO Cas9-POSITIVE S2R+ CELLS USING THE ssDNA DROP-IN APPROACH

This protocol describes a method for CRISPR-mediated knock-in of a fluorescent protein that relies on an ssDNA donor to provide the fluorescent protein ORF as an artificial exon, referred to as the ssDNA Drop-In method (Kanca et al., 2019). Following design of the knock-in and corresponding ssDNA and sgRNA, these molecular reagents are generated and transfected into cells. Cells are then single-cell-isolated by FACS and grown to form colonies, and individual colonies are tested using imaging and molecular analysis. The most effective method for molecular confirmation is PCR amplification of each junction site using a genomic-specific primer and an insertion cassette-specific primer, followed by sequencing. The protocol takes approximately 2 months to complete.

Materials

Cas9+ *Drosophila* cells, such as S2R+-MT::Cas9 (Viswanatha et al., 2018) (DGRC #268)
Schneider's medium (see recipe)
GFP flanking primer R1: 5′ ACCCTGAAGTTCATCTGCAC 3′
GFP flanking primer F2: 5′ GCATCACCCTGGGCATGGAT 3′
Genomic DNA Extraction Kit (e.g., Zymo Quick-DNA MiniPrep Kit; Zymo Research #D3024)
PCR polymerase and buffer such as High Fidelity Phusion Polymerase (NEB #M0530) and 5× buffer
QIAquick Gel Extraction Kit (Qiagen #28704)

DNA editing software program (e.g., SnapGene; *http://www.snapgene.com*)
Fluorescence microscope
Image analysis software such as CellProfiler (Carpenter et al., 2006)
Microcentrifuge tubes (e.g., Eppendorf)
25-cm^2 (T-25) tissue culture flasks
Tabletop centrifuge (low speed with standard rpm settings)
Thermal cycler (PCR machine)

Additional reagents and equipment for sgRNA cloning (Support Protocol 1), ssDNA donor synthesis (Support Protocol 2), transfection (Support Protocol 3 or 4), isolation of single cells (Support Protocol 5), measuring DNA concentration (see Current Protocols article: Gallagher, 2004), PCR (see Current Protocols article: Kramer & Coen, 2001), agarose gel electrophoresis (see Current Protocols article: Voytas, 2000), DNA sequencing (see Current Protocols article: Shendure et al., 2011), and immunoblotting (see Current Protocols article: Ni, Peng, & Xu, 2016)

Target selection, knock-in design, and isolation of single-cell clones

1. Obtain the gene structure from FlyBase GBrowse or from NCBI with the sequence accession number and import to a DNA editing software program such as SnapGene.
2. Choose a target intron. If there are multiple suitable introns, select the introns shared in all annotated transcripts and do not divide known or putative functional domains (e.g., using the SMART database, *http://smart.embl-heidelberg.de/*; Letunic & Bork, 2018).
3. Scan the selected intron for sgRNAs. Select sgRNA target sites that are >50 nt away from endogenous splice donor/splice acceptor sites to ensure proper splicing of the artificial exon in the mature transcript. Also apply general sgRNA design principles (Support Protocol 1).
4. Clone the sgRNA (Support Protocol 1).
5. Generate the ssDNA donor (Support Protocol 2).
6. Transfect cells with the ssDNA donor and sgRNA plasmid (Support Protocol 3 or 4).
7. *Optional*: View the cells using a fluorescence microscope (40× or 60× objective). Some GFP-positive cells might be detectable.

   Visualize cells with a 40× or 60× microscope objective. If S2R+-MT::Cas9 cells are used, then all cells will be positive for the mCherry signal. We see a range of percent positive cells with this approach, and even in cases where a signal is not obvious, GFP-positive cells might be identified, so we continue the workflow. For knock-in cell lines reported in Kanca et al. (2019), we observed a range of ∼0.5% to 7%, depending on the target.

8. Grow the cells to confluency in Schneider's medium.
9. Isolate single cells and grow to form colonies (Support Protocol 5).

Validation of single-cell clones

10. With aliquots of cells isolated in step 9, identify strong GFP-expressing clones using fluorescence microscopy and an image analysis software package such as CellProfiler (Carpenter et al., 2006). Also see Basic Protocol 2, step 5. The mCherry signal present in parental S2R+-MT::Cas9 cells can be used to define all cells and can be compared with the GFP signal to determine brightness and localization.

    For the work described in Kanca et al. (2019), we identified the three brightest clones using CellProfiler version 2.1.1 (see Internet Resources) and selected these for follow-up.

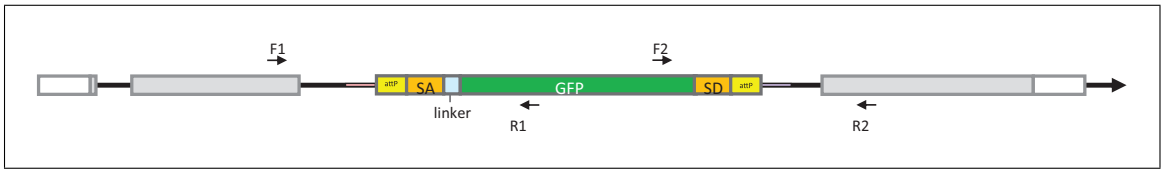

Figure 3 Example design for ssDNA Drop-In into fibrillarin. The position of the ssDNA donor and location of the primers used to amplify the 5′ and 3′ insertion sites for molecular validation are shown.

Table 1 PCR Conditions for Amplification of the Junctions Between the ssDNA Drop-In Cassette and the Intron Into which it has Inserted

Component	Volume (µl)	
Nuclease-free water	12.4	—
5× Phusion HF	4	—
2.5 mM dNTPs	0.4	—
10 µM F1 or F2	1	—
10 µM R1 or R2	1	—
100 ng/µl genomic DNA	1	—
Phusion DNA polymerase	0.2	—
Step	Temperature	Time
Initial denaturation	98°C	30 s
35 Cycles	98°C	10 s
	54°C	30 s
	72°C	30 s
Elongation	72°C	10 min
Hold	4°C	Indefinite

You can cryopreserve the remaining cells, either individually or as a pool, so that they are available for testing if the initial candidates fail validation.

11. Grow each candidate clone in a 25-cm^2 (T-25) tissue culture flask until the cells have reached confluency. Resuspend the cells in medium and aliquot 1 ml into a microcentrifuge tube.

12. Prepare genomic DNA. If using a Zymo gDNA Miniprep Kit, spin the cells for 5 min at 45 × *g* at room temperature, discard supernatant, and resuspend the pellet in 1000 µl of Genomic Lysis Buffer (from the kit), then follow the rest of the manufacturer's protocol to isolate genomic DNA. Measure the DNA concentration of the sample.

    One alternative to the Zymo kit is Lucigen QuickExtract DNA Extraction Solution (see Basic Protocol 2).

13. Design a forward primer that amplifies ~300 bp upstream of the insert sequence in the target locus using the GFP flanking primer R1 (5′ ACCCTGAAGTTCATCTGCAC 3′) as the reverse primer. This will be Flanking Primer F1. See Figure 3.

14. Design a reverse primer that amplifies ~300 bp downstream of the insert sequence in the target genome using the GFP flanking primer F2 (5′ GCATCACCCTGGGCATGGAT) 3′ as the forward primer. This primer will be Flanking Primer R2.

15. Run a PCR reaction (see Current Protocols article: Kramer & Coen, 2001) following the parameters set in Table 1, and assess the products by agarose gel electrophoresis (see Current Protocols article: Voytas, 2000; purify DNA from gel using QIAquick

gel extraction kit) and Sanger or next-generation sequencing (see Current Protocols article: Shendure et al., 2011).

16. *Optional*: Detect GFP fusion proteins by immunoblotting (also see Current Protocols article: Ni et al., 2016).

    Grow cell lines in 6-well plates until confluent, resuspend cells, and transfer 1 ml of resuspended cells into a 1.5-ml microcentrifuge tube. Centrifuge 10 min at 250 × g at room temperature, to pellet the cells. Aspirate the supernatant and gently resuspend cells in 1 ml of ice-cold 1× PBS. Centrifuge 10 min at 250 × g at room temperature, to pellet the cells. Lyse and denature the cell pellet by boiling in 250 µl of 2× SDS sample Buffer for 5 min. Load 10 µl of protein onto an SDS-PAGE gel, transfer to blotting paper, and detect GFP fusion proteins using an anti-GFP antibody at an appropriate dilution.

17. For successful clones, further expand and cryopreserve the cells according to standard protocols such as those found at *https://dgrc.bio.indiana.edu/include/file/FreezingCells.pdf*.

KNOCK-IN INTO Cas9-POSITIVE S2R+ CELLS BY HOMOLOGY-INDEPENDENT INSERTION OF UNIVERSAL DONOR PLASMIDS THAT PROVIDE mNeonGreen (CRISPaint METHOD)

BASIC PROTOCOL 2

This protocol describes CRISPR/Cas9 knock-in using the CRISPaint approach (Bosch et al., 2019; Schmid-Burgk et al., 2016), which employs an NHEJ mechanism to insert a universal donor plasmid into a target gene (Fig. 4). To tag proteins with mNeonGreen in S2R+-MT::Cas9 cells (Viswanatha et al., 2018), you will first need to design and clone sgRNA-expressing plasmid(s) that target your gene(s) of interest. Next, for each target, you will transfect the target-specific sgRNA plasmid along with two publicly available plasmids, a frame-selector sgRNA plasmid and the mNeonGreen universal donor plasmid. After transfection, puromycin selection can be used to enrich for in-frame insertions, followed by single-cell isolation, visualization of the tagged protein, and molecular confirmation.

Materials

Cas9+ *Drosophila* cells, such as S2R+-MT::Cas9 (Viswanatha et al., 2018) (DGRC #268)
Frame selector plasmids (*pCFD3-frame_selector_*(0,1,or 2) (Addgene #127553-127555; DGRC # 1482-1484)
CRISPaint donor plasmid(s) (see Addgene Kit #1000000086; *pCRISPaint-mNeonGreen-T2A-PuroR* cannot be distributed through Addgene and is available directly from Hornung lab; Schmid-Burgk et al., 2016)
Optional control sgRNA, *pCFD3-Act5c* (Addgene #130278; DGRC #1492)
Schneider's medium (see recipe) with 2 µg/ml puromycin (see recipe)
Genomic DNA extraction reagent, such as QuickExtract DNA Extraction Solution (Lucigen, #QE09050)
PCR polymerase and buffer such as High Fidelity Phusion Polymerase (NEB #M0530) and 5× buffer

DNA analysis software such as Lasergene DNAstar
Fluorescence microscope, inverted
6-well and 96-well culture plates
96-well PCR plates or strip tubes
Thermal cycler (PCR machine)
Image analysis software such as CellProfiler (Carpenter et al., 2006)
Eppendorf tubes
Tabletop centrifuge
Spectrophotometer, such as a NanoDrop microvolume spectrophotometer
Standard agarose gel electrophoresis apparatus

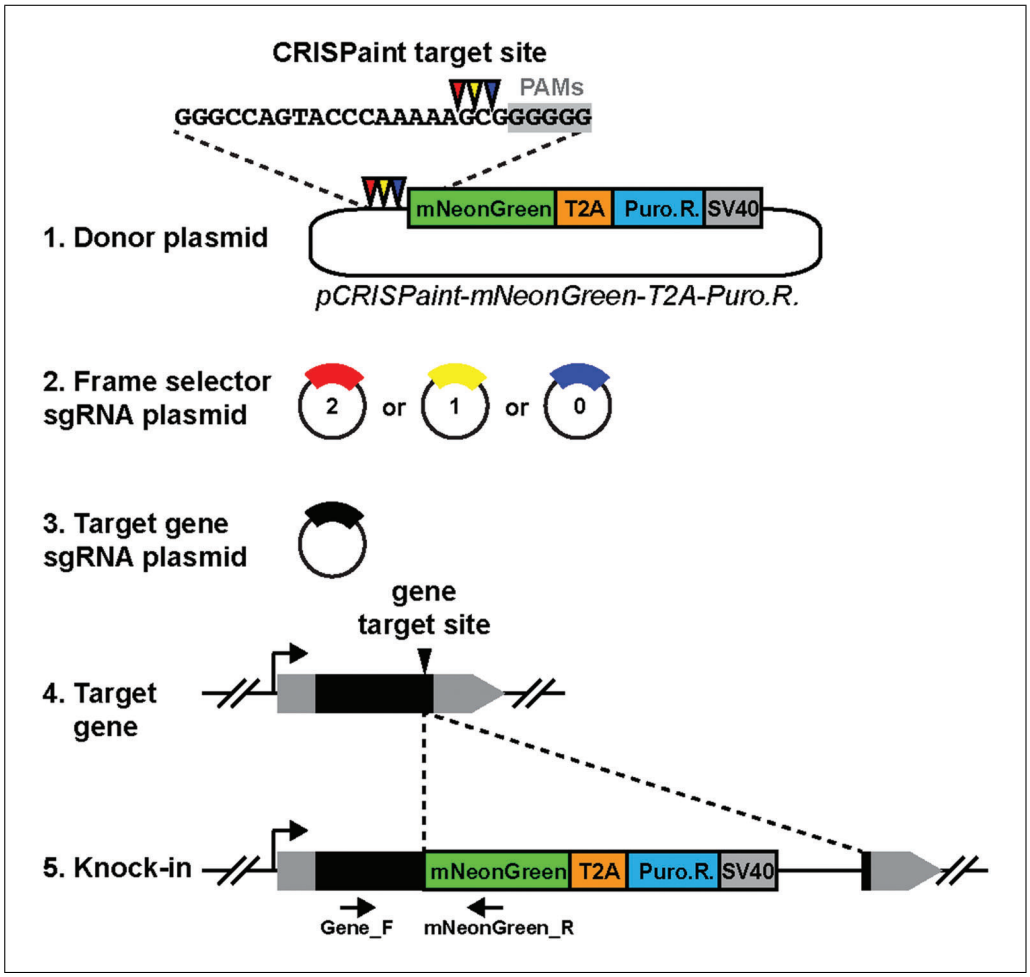

Figure 4 Stepwise schematic of mNeonGreen-T2A-PuroR knock-in using homology-independent insertion. mNeonGreen-T2A-PuroR is inserted into 3′ coding sequence. From Bosch et al. (2019); used with permission.

Additional reagents and equipment for sgRNA cloning (Support Protocol 1), transfection (Support Protocol 3 or 4), isolation of single cells (Basic Protocol 5), PCR (see Current Protocols article: Kramer & Coen, 2001), agarose gel electrophoresis (see Current Protocols article: Voytas, 2000), DNA sequencing (see Current Protocols article: Shendure et al., 2011), and immunoblotting (see Current Protocols article: Ni et al., 2016)

Target selection, knock-in design, and isolation of single-cell clones

1. For each target gene, identify an sgRNA target site in the 3′ coding sequence. The sgRNA should be as close to the stop codon as possible (<100 bp away) and follow general rules for sgRNA design (Support Protocol 1).

2. For each target cut site, identify a matching frame-selector sgRNA (named frame 0, 1, or 2; Figs. 4 and 5). The frame-selector sgRNA is used to cut and linearize the donor plasmid. Matching the cutting frame of the donor plasmid with the target gene improves the chances of generating seamless in-frame insertions (see Schmid-Burgk et al., 2016). Choose an appropriate frame-selector sgRNA by analyzing the location of the target gene sgRNA DNA cleavage site relative to the reading frame. Note that the frame-selector sgRNA numbers are reversed relative to the traditional coding frame numbering system (Fig. 5).

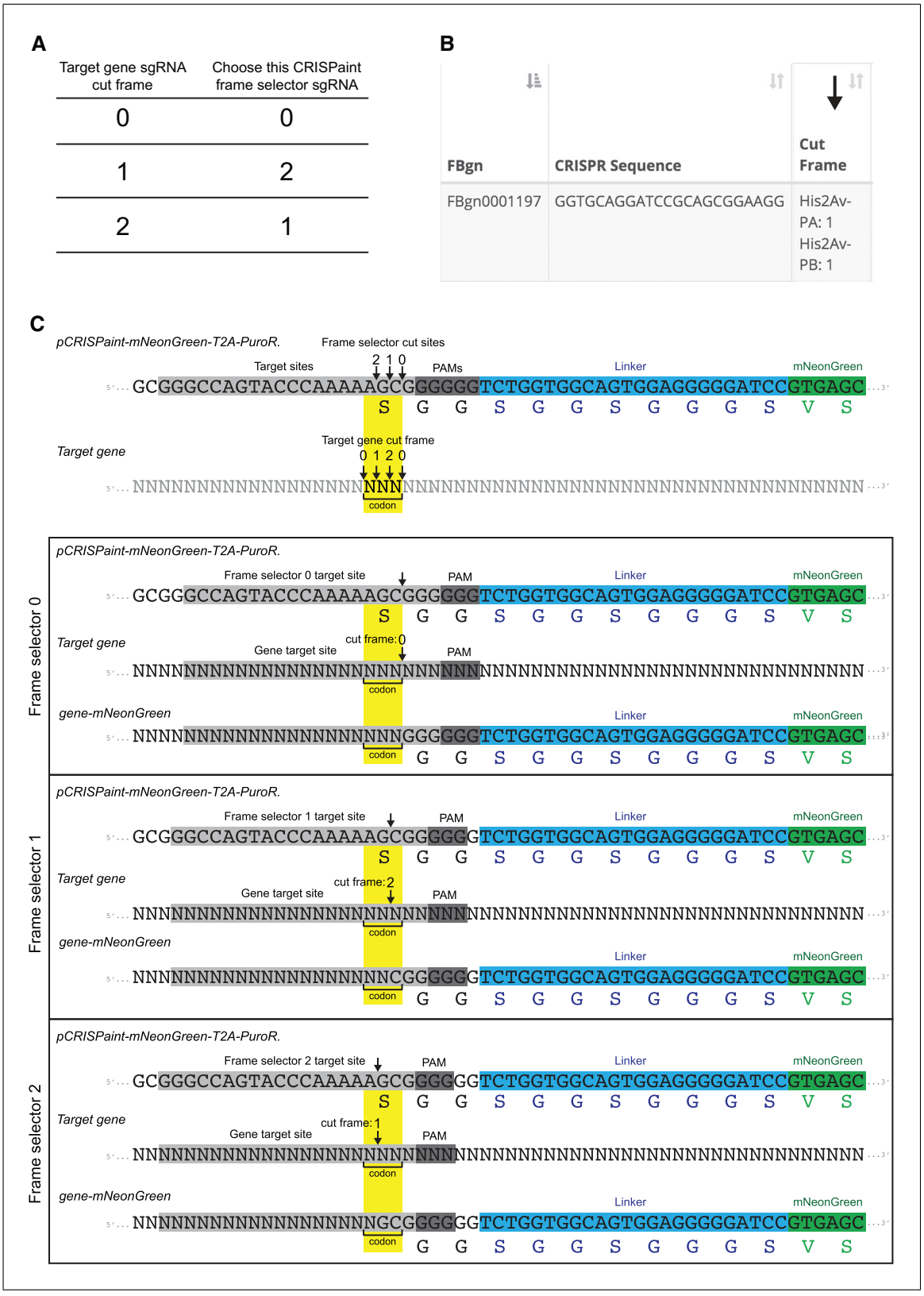

Figure 5 Diagram to help determine the appropriate CRISPaint frame-selector. (**A**) Conversion table for target gene cut frame and CRISPaint frame selector sgRNA. (**B**) Screenshot of output of the Find CRISPRs tool (*http://www.flyrnai.org/crispr3/web*). Arrow: column in a search results table showing the target gene sgRNA cut frame. The example sgRNA shown was used to generate His2Av-mNeonGreen knock-in cell line in Bosch et al. (2019). (**C**) Schematic of gene targeting when using the three CRISPaint frame selector sgRNAs (0, 1, 2).

We analyze the genomic sequence of the target gene using Lasergene DNAstar software, although other DNA analysis programs are available. This helps locate and annotate the sgRNA target site, predicted DNA cleavage site, and amino acid reading frame. To help this analysis, we recommend using the online sgRNA prediction tool CRISPR3 (http://www.flyrnai.org/crispr3/web), which reports the cutting frame of the sgRNA in the target gene (Fig. 5). The orientation of the target gene sgRNA site (5′ or 3′) does not matter.

3. Clone the target-specific sgRNA (Support Protocol 1). Plasmids for *Drosophila* expression of the frame-selector sgRNAs can be obtained from Addgene or the DGRC (see Materials list above for catalog numbers).

4. Transfect donor and sgRNA plasmids into cells (Support Protocol 3 or 4).

   The work described in Bosch et al. (2019) used Effectene (Support Protocol 3). The experimental transfection mix will contain a donor plasmid (pCRISPaint-mNeonGreen-T2A-PuroR), the appropriate frame-selector sgRNA plasmid, and the target gene sgRNA plasmid. As a positive control for knock-in, you can use pCFD3-Act5c, frame selector 2, and the pCRISPaint-mNeonGreen-T2A-PuroR donor plasmid.

5. *Optional*: Visualize cells on an inverted fluorescence microscope. Cells should be proliferating and some might be noticeably fluorescent.

   Visualize cells using a 40× or 60× microscope objective. If S2R+-MT::Cas9 is used as the starting cell line, then all cells will be positive for mCherry signal. The number of knock-in tagged fluorescent cells is dependent on the transfection and knock-in efficiency, and the level of fluorescence in cells is dependent on the expression of the target gene. Act5c-mNeonGreen positive control integration events should be visible at this stage, with ∼3% cells expected to be positive for mNeonGreen signal.

6. At a time point 3 to 4 days after transfection, split cells into new 6-well plates at a 1:6 dilution with fresh Schneider's medium with puromycin at a final concentration of 2 µg/ml (1:5000 dilution of 10 mg/ml puromycin stock; see Reagents and Solutions). Incubate plates at 25°C.

   It is possible that genes with lower expression levels may require lower concentrations of puromycin, or a longer recovery period after transfection before puromycin treatment. We use the DGET tool from the Drosophila RNAi Screening Center (DRSC; https://www.flyrnai.org/tools/dget/web/) to determine gene expression levels in S2R+ cells from RNA-seq data. We also note that you could skip the puromycin selection step and go to step 8 (FACS isolation).

7. Every 3 to 5 days, gently replace the medium with fresh Schneider's medium with 2 µg/ml puromycin. Monitor the growth of cells on an inverted fluorescence microscope. Cultures should become confluent after 12 to 16 days at 25°C.

   To avoid disturbing the adherent cells when changing the medium, we use vacuum aspiration to remove the medium and cell debris and add fresh Schneider's medium to the side of the well. Clones of adherent mNeonGreen+ cells may be observed using an inverted microscope even after a few days of puromycin selection. Use positive control wells (e.g., pCFD3-Act5c) and negative control wells (e.g., untransfected) to help determine the success of the puromycin selection. If cells are growing but not yet confluent after 12 to 16 days, continue replacing puromycin and monitoring every 3 to 5 days.

8. Perform single-cell isolation of cells positive for the fluorescent tag (Support Protocol 5).

Validation of single-cell clones

9. Examine mNeonGreen localization in single-cell cloned lines using a confocal microscope or inverted fluorescence microscope. Retain cell lines that exhibit correct localization and robust growth. See Figure 6 for representative results.

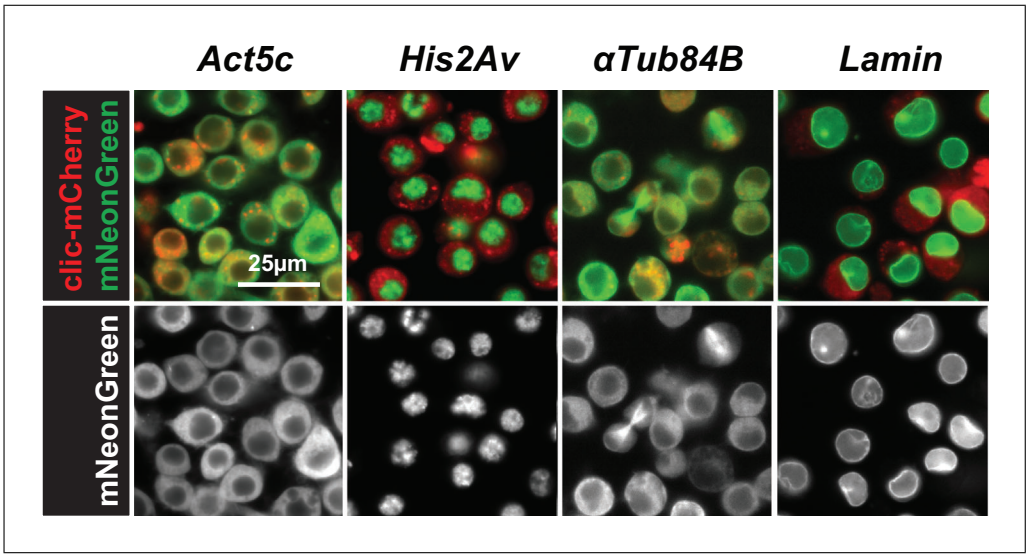

Figure 6 Confocal images of live mNeonGreen-expressing single-cell cloned S2R+ lines. Results with targeting four genes are shown. Images show fluorescence from Clic-mCherry (red), which is present in the parental Cas9-positive cell line, and mNeonGreen (green). Scale bar, 25 μm. Modified from Bosch et al. (2019); used with permission.

To image live cell lines with high resolution and in a high-throughput manner, we transfer cell lines to a glass-bottom 384-well plate and obtain images on an In Cell 6000 microscope using a 60× objective.

10. Remove the medium from cells growing in 96-well plates (Support Protocol 5) and add 100 μl of QuickExtract solution (Lucigen) to each well. Pipette up and down to resuspend and lyse the cells, then transfer the solution to a 96-well PCR plate or 8-well PCR strip tube. Incubate at 65°C for 15 min, then at 98°C for 2 min, in a thermal cycler. Store genomic DNA at 4°C.

    We typically prepare a 96-well plate containing replicate cultures of each cell line in an organized layout to facilitate downstream PCR analysis. We allow the cells to adhere to the plate for at least 2 hr before harvesting. Genomic DNA can be extracted from suspensions between 1×10^6 and 1×10^7 cells/ml. One alternative to using the QuickExtract solution for genomic DNA isolation is the Zymo Quick-DNA MiniPrep Kit, as noted for Basic Protocol 1.

11. Design a gene-specific forward primer upstream of the insertion site, to be used with the mNeonGreen_R reverse primer in a PCR reaction.

    We design the gene-specific forward primer using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to result in an amplified DNA fragment size of 300 to 1000 bp when used with mNeonGreen_R.

12. Run a PCR reaction following the parameters in Table 1 (also see Kramer & Coen, 2001). See Figure 7.

    The successful amplification of a DNA fragment indicates that the cell line contains at least one correct-orientation insertion of mNeonGreen into the target cut site.

13. Perform agarose gel electrophoresis of the PCR products (Voytas, 2000). Purify amplified DNA fragments from agarose gels using QIAquick gel extraction kit and submit for Sanger sequencing to determine the sequence of the mNeonGreen insertion site. See Figure 5 for representative data.

    We use QIAquick columns to purify DNA fragments from agarose gels. We use the same primers for sequencing as were used for PCR amplification. We analyze the sequence of the insertion site using Lasergene software. First, using SeqBuilder, we create a DNA

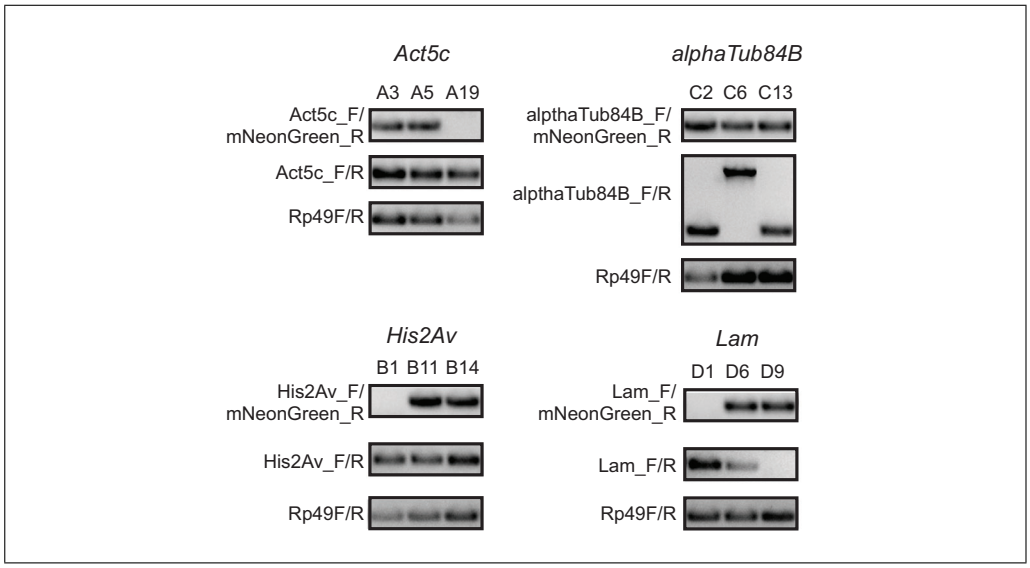

Figure 7 Agarose gel with PCR fragments amplified from knock-in (Gene_F/mNeonGreen_R) and non-knock-in loci (Gene_F/R). Positive control bands were amplified from Rp49 genomic sequence. From Bosch et al. (2019); used with permission.

sequence file representing the hypothetical seamless mNeonGreen insertion site. Next, we align the chromatogram sequences to this reference file using SeqMan. Finally, we use SeqBuilder to annotate any differences between the two. If indels are present at the insertion site, the predicted amino acid sequence is analyzed to determine if mNeonGreen is in coding frame with the target gene. If more than one type of insertion allele is present (double peaks in Sanger sequencing data), a different method will have to be used to resolve the sequences. This can be done by TOPO cloning of the PCR fragment and sequencing individual plasmids to identify the different allele sequences, or using next-generation sequencing.

14. *Optional*: PCR amplify (Kramer & Coen, 2001) the non-insertion locus using the gene-specific forward primer and a gene-specific reverse primer that flanks the insertion site. Analyze DNA fragments by gel imaging and sequencing as described above.

    If more than one indel allele is present, users will have to TOPO clone the PCR fragment and sequence individual plasmids to identify the different allele sequences, or use next-generation sequencing.

15. *Optional*: Detect mNeonGreen fusion proteins by immunoblotting (also see Current Protocols article: Ni et al., 2016).

    Grow cell lines in 6-well plates until confluent, resuspend cells, and transfer 1 ml of resuspended cells into a 1.5-ml centrifuge tube. Centrifuge 10 min at 250 × g, 4°C, to pellet the cells. Aspirate the supernatant and gently resuspend cells in 1 ml of ice-cold 1× PBS. Centrifuge 10 min at 250 × g, 4°C, to pellet the cells. Lyse and denature the cell pellet by boiling in 250 µl of 2× SDS sample Buffer for 5 min. Load 10 µl of protein onto an SDS-PAGE gel, transfer to blotting paper, and detect mNeonGreen fusion proteins using mouse anti-mNeonGreen antibody at 1:1000 concentration. See figure number 2F in Bosch et al. (2019) for representative data.

16. For successful clones, further expand and cryopreserve (see, for example, *https://dgrc.bio.indiana.edu/include/file/FreezingCells.pdf*).

SUPPORT PROTOCOL 1

sgRNA DESIGN AND CLONING

Factors relevant to sgRNA design include (a) position of the sgRNA relative to the target, with the specific approach in mind (see Basic Protocols 1 and 2), (b) predicted

effectiveness of the sgRNA, and (c) the presence of single-nucleotide polymorphisms (SNPs) in the target region of the cell line being used. Online resources such as DRSC Find CRISPRs or CRISPR Optimal Target Finder can be used to identify and evaluate appropriate sgRNAs (see below and Internet Resources). Cloning of the sgRNA is straightforward.

Materials

pCFD3 plasmid (Addgene #49410)
Chemically competent *E. coli, such as* TOP10 cells (Invitrogen #C404010)
*Bbs*I-HF enzyme (New England Biolabs #R3539)
T4 DNA ligase (New England Biolabs #M0202)
Lysogeny broth (LB) (Sigma #L3022-1KG)
LB agarose plates with 50 μg/ml carbenicillin (Sigma #C1389-10G)
LB with ampicillin at 100 μg/ml (Roche #10835269001)
QIAprep Spin Miniprep Kit (Qiagen #27104)
Oligos to anneal for sgRNA (user specific)
Shaking incubator at 37°C (Multitron #MS012T6)

14-ml culture tubes (VWR #60818-703)
42°C water bath (Precision #182)

Additional reagents and equipment for molecular cloning (see appropriate articles of *Current Protocols in Molecular Biology*)

1. Use a *Drosophila* sgRNA design resource such as the DRSC Find CRISPRs tool or CRISPR Optimal Target Finder to select sgRNA target sites (see Internet Resources). For the DRSC Find CRISPRs tool, optimal designs have a seed score of 12 or 13 and an efficiency score of >5. When possible, you should exclude target sites that overlap known genomic variation in the cell line. The DRSC Find CRISPRs tool displays whole-genome variation data for the S2R+ Cas9 cell line. Users working with cell lines that have not had their genomes sequenced should PCR-amplify and sequence the target region to determine if the region contains variants that would affect sgRNA effectiveness.

2. Evaluate cutting efficiency of sgRNAs that do not have any predicted off-target sites for cutting using CRISPR Efficiency Predictor (*https://www.flyrnai.org/evaluateCrispr/*). If available, choose sgRNAs with efficiency scores of >5.

   It is important to avoid possible U6 Terminator (TTTT) in the sgRNA sequence, since the pCFD3 plasmid uses the U6 promoter.

3. Clone the sgRNA-expressing plasmid(s) according to the appropriate articles in *Current Protocols in Molecular Biology*. For each sgRNA target site, design and order two complementary oligonucleotides (IDT or equivalent company) encoding the sgRNA. Anneal each pair of oligos, creating overhanging sticky ends, and ligate the annealed oligos into a sgRNA expression plasmid backbone.

   We clone single sgRNAs into pCFD3 (Port, Chen, Lee, & Bullock, 2014), which contains the Drosophila U6:3 promoter (see https://www.crisprflydesign.org/grna-expression-vectors/). Briefly, this involves digesting pCFD3 with BbsI-HF, ligating annealed oligos into the digested pCFD3 backbone using T4 DNA ligase, transforming ligated plasmid into chemically competent TOP10 bacteria using a 42°C heat shock, and plating cells onto LB carbenicillin agar plates for incubation overnight at 37°C. Single bacterial colonies are cultured in LB ampicillin shaking overnight at 37°C. Plasmids are isolated from cultures using a miniprep kit (Qiagen or equivalent) and sequenced by Sanger sequencing using the pCFD3 sequencing primer. We typically resuspend final plasmids in

water (or Qiagen EB) at a final concentration of 200 ng/µl. Alternatively, sgRNAs can be cloned into alternative plasmid backbones or synthesized and ordered from a company.

SUPPORT PROTOCOL 2

ssDNA DONOR SYNTHESIS

This protocol describes amplification of the ssDNA Drop-In method cassette using primers that recognize the donor vector and add gene-specific homology arms, and subsequent production of ssDNA from the amplified fragment.

Materials

Drop-In *SA-sfGFP-SD* donor template plasmid (Kanca et al., 2019)
Q5 Hot Start High Fidelity 2× master mix (NEB #M0494)
QIAQuick PCR Purification Kit (Qiagen #28106)
Lambda exonuclease (NEB #M0262) and corresponding buffer
Monarch PCR and DNA Cleanup Kit (NEB #T1030)
Optional: 2× QX RNA denaturation solution (Qiagen #929607)

0.2-µl thin-walled PCR tubes
Thermal cycler (PCR machine)
NanoDrop microspectrophotometer

Additional reagents and equipment for PCR (Kramer & Coen, 2001) and agarose gel electrophoresis (see Current Protocols article: Voytas, 2000)

1. Design PCR primers with gene-specific homology arms as 5′overhangs. See Figure 8.

   Primer binding sites in the template vector are 5′-GAATTCTGTAAAACGACGGCCAGT GG-3′ for forward primer and 5′-GCCCCCAGGAAACAGCTATGACGG-3′ for reverse primer. Example gene specific primers would be:

   Forward primer: 5′ 100 nt left homology arm: GAATTCTGTAAAACGACGGCCAGT GG 3′

   Reverse primer: 5′ 100 nt right homology arm (reverse complement): GCCC-CCAGGAAACAGCTATGACGG 3′.

   Left homology arm is 100 nt upstream of 3 nt prior to protospacer adjacent motif (PAM) and right homology arm is 100 nt downstream of 3 nt prior to PAM. An easy way to design oligos is to integrate the template construct (from 5′ primer binding site to the 3′ primer binding site) in silico to the targeted region (3 nt prior to PAM sequence) and design the primers on this in silico generated sequence. The SA-sfGFP-SD construct is directional. It is important to know the orientation of the gene relative to the reference genome sequence while designing the knock-in approach. We work on in silico files that are orientated according to the orientation of the gene of interest (i.e., if the gene of interest is transcribed on the minus strand of the genomic reference sequence in FlyBase, then we use the reverse complement of the sequence for in silico design steps). In addition, you should PCR amplify and sequence the homology arm region using genomic DNA from your target cell type, to make sure that there are no SNPs in this region as compared with the reference genome.

2. Order high-quality oligo primers, such as Ultramers from Integrated DNA Technologies (IDT). One of the primers should be ordered with the 5′ phosphorylation modification. This will ensure that one of the resulting PCR strands is 5′ phosphorylated and the other strand not phosphorylated, which is critical for production of ssDNA from the PCR product.

3. Spin down the lyophilized primers briefly and dissolve in 40 µl distilled water at 50°C for 15 min.

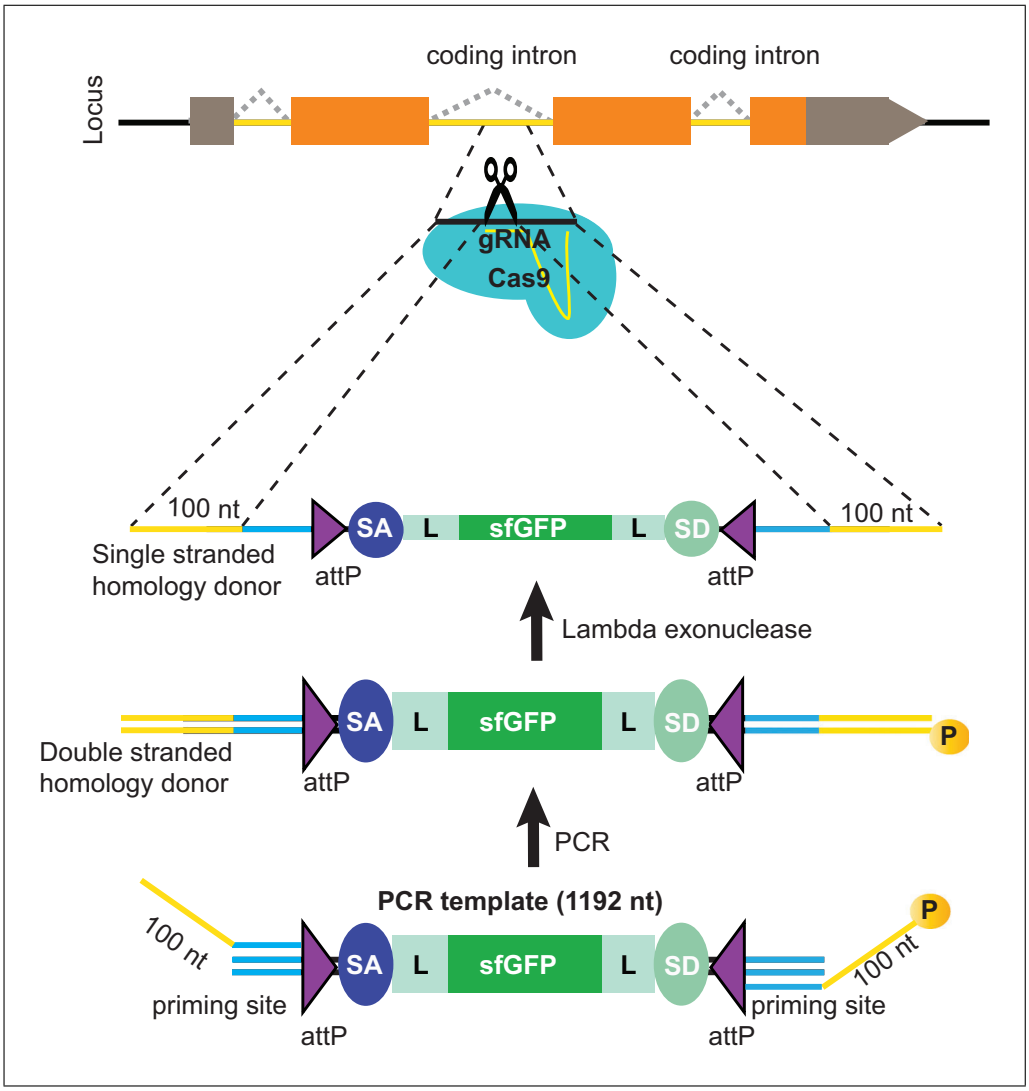

Figure 8 Schematic of PCR-based generation of drop-in ssDNA constructs to tag genes with sfGFP using an artificial exon. Gray boxes, UTRs; orange boxes, coding exons; yellow line, coding introns; black line, outside coding introns and exons. sfGFP: superfolderGFP; SA: Splice Acceptor of mhc; SD: Splice Donor of mhc; L: flexible linker that consists of four copies of Gly-Gly-Ser. Adapted from Kanca et al. (2019).

4. Set up four 50-µl PCR reactions (see Current Protocols article: Kramer & Coen, 2001) per construct using Q5 Hot Start High Fidelity 2× Master Mix (NEB #M0494). A master mix of the reactions should be set up with 1 µl of each primer, 4 µl of template (diluted to 1 ng/µl), 94 µl distilled deionized H_2O, and 100 µl of 2× master mix. Distribute 200 µl reaction to four PCR tubes. PCR conditions:

Step	Temperature	Time
Initial denaturation	98°C	30 s
35 cycles	98°C	10 s
	70°C	30 s
	72°C	40 s
Elongation	72°C	2 min
Hold	12°C	

The template should be selected according to the last codon of preceding exon in order to avoid frameshift mutations. There are three donor template vectors: phase 0, phase 1, and phase 2. If the splice donor is situated at position 0 at a codon (i.e., if the last codon in the exon before splice donor is not divided), phase 0 PCR template is used. If the last

codon in the preceding exon is divided in position one (one nucleotide in preceding exon and two nucleotides in following exon), then phase 1 template is used. If the last codon is divided in position 2 (two nucleotides in preceding exon, one nucleotide in the following exon), then phase 2 template is used.

5. PCR reactions result in 1392 nt amplicons. Pool the resulting PCR reactions and run 10 µl on an agarose gel (see Current Protocols article: Voytas, 2001) to confirm the band size.

   This results in a single strong band at 1392 nt and a weak band of a smaller size that we assume is composed of primer dimers.

6. Distribute the remaining PCR products into two tubes and isolate amplicons using two Qiaquick PCR purification kit columns. Elute each column in 50 µl elution buffer (from the QIAquick kit) and mix the two isolated samples. Measure DNA concentration in a NanoDrop microspectrophotometer using 1 µl sample with a dsDNA protocol.

   A typical yield is 100 µl of dsDNA in solution at a concentration of ~100 to 200 ng/µl.

7. Set up two Lambda exonuclease reactions using 4 to 6 µg of DNA, 10 µl of 10× Lambda exonuclease buffer, 8 µl of Lambda exonuclease, and distilled deionized H_2O to 100 µl.

   We distribute the two samples in a total of four PCR tubes (50 µl per tube) and do the reactions in a thermal cycler for convenience.

8. Incubate at 37°C for 1 hr, then 10 min at 75°C, and hold at 4°C.

9. Pool the reaction results in two 100-µl samples and isolate using the Monarch PCR and DNA Cleanup Kit employing the ssDNA isolation protocol in two columns. Elute each column in 10 µl of 55°C elution buffer from the Monarch kit per column. Pool the eluates and measure DNA concentration with NanoDrop using ssDNA protocol.

   We typically achieve a yield of ssDNA at ~100 to 150 ng/µl.

10. *Optional*: Denature the ~200 to 300 ng ssDNA by mixing ssDNA with an equal volume of 2× QX RNA denaturation solution and incubating at 70°C for 2 min, and then on ice 1 min. Load samples on 2% agarose gel along with untreated double-stranded PCR products to confirm the band size (see Current Protocols article: Voytas, 2001).

    The single-stranded donor construct runs at a smaller size than the double stranded controls (~650 to 850 bp).

SUPPORT PROTOCOL 3

TRANSFECTION USING EFFECTENE

This protocol describes transfection using the chemical transfection reagent Effectene (Qiagen), the method used in Bosch et al. (2019), and follows closely the protocol provided by the manufacturer. Another commercial transfection reagent shown to be effective for *Drosophila* cultured cells could be used, following the manufacturer's protocol.

Materials

Drosophila Cas9-expressing S2R+ cells (S2R+-MT::Cas9, DGRC Stock #268)
Schneider's medium (see recipe)
Effectene transfection reagent (Qiagen #301427)
sgRNA plasmid for each target/design (see Basic Protocol 1 or 2 for design and Support Protocol 1 for synthesis)

ssDNA donor (see Basic Protocol 1, Support Protocol 2) or CRISPaint donor plasmid (see Basic Protocol 2)
For Basic Protocol 2, frame selector plasmid (*pCFD3-frame_selector_*(0, 1,or 2) (Addgene #127553-127555)
Optional control sgRNA for Basic Protocol 2, *pCFD3-Act5c* (GP07595, Addgene #130278)

6-well tissue culture plates (Costar #3516)
25°C incubator

1. Plate *Drosophila* Cas9-expressing S2R+ cells into 6-well dishes at a concentration of 1.8×10^6 cells/ml (2 ml volume each well, 3.6×10^6 cells/well) in Schneider's medium. Incubate the plates at 25°C for at least 4 hr to allow the cells to adhere to the bottom of the plate.

   We grow cells for at least two passages after thawing before using them for transfections, at which point cells should be growing robustly. We also avoid using cells that have gone through more than 30 passages.

2. Prepare transfection mixture with sgRNA plasmid(s) and donor. As a negative control, use untransfected cells or omit the gene-targeting sgRNA plasmid from the transfection mix.

   For the work described in Bosch et al. (2019), we used Qiagen Effectene transfection reagent, though similar products are available. Briefly, 400 ng of each plasmid are diluted in EC buffer up to a total volume of 90.4 µl and mixed by brief vortexing. Diluted plasmids are mixed with 9.6 µl of Enhancer, vortexed, and incubated for 2 to 5 min. 15 µl Effectene is added to the mixture, vortexed, and incubated for 10 to 15 min.

3. Add transfection mixture to cultured cells prepared as described in step 1. Gently transfer 600 µl of medium from a 6-well culture well to the transfection mix, mix by pipetting up and down, and add the medium plus transfection mix back to the same culture well in a gentle dropwise manner.

ELECTROPORATION OF S2R+-MT::Cas9 *Drosophila* CELLS

SUPPORT PROTOCOL 4

This protocol describes electroporation of *Drosophila* S2R+ cells using the Nucleofect system (Lonza V4XC-2032). This method was used to generate the ssDNA Drop-In tagged cell lines reported in Kanca et al. (2019).

Materials

Drosophila Cas9-expressing S2R+ cells (S2R+-MT::Cas9, DGRC Stock #268) at ~70% confluency
Schneider's medium (see recipe)
SF Cell Line Solution 4D-NucleofectorTM X Kit S (Lonza #V4XC-2032)
sgRNA plasmid for each target/design (see Basic Protocol 1 or 2 for design and Support Protocol 1 for synthesis)
ssDNA donor (see Basic Protocol 1, Support Protocol 2) or CRISPaint donor plasmid (see Basic Protocol 2)
For Basic Protocol 2, frame selector plasmid (*pCFD3-frame_selector_*(0,1,or 2) (Addgene #127553-127555)
Optional control sgRNA for Basic Protocol 2, *pCFD3-Act5c* (GP07595, Addgene #130278)

48-well flat-bottom plates (Corning #29442-952)
Standard bright field microscope or equivalent (such as the Leica DM IL)
Bright-Line hemocytometer with coverslip (Sigma-Aldrich #Z359629)
15-ml conical tubes (Falcon #352097)

Centrifuge
PCR tubes
Electroporation cuvettes
Lonza 4D Nucleofector electroporation device (Lonza #AAF-1002B)

Additional reagents and equipment for counting cells (see Current Protocols article: Phelan & May, 2017)

1. Fill the wells of a 48-well plate with 500 µl of room temperature Schneider's medium per reaction and set aside for after the cells are electroporated.

   Even though the outermost wells will not be used with cells, they should be filled with medium to help maintain local humidity. The cells have difficulty growing after electroporation, and humidity encourages growth.

2. Grow cells to ~70% confluency prior to electroporation.

   The cells should not reach full confluency before being electroporated. If they do, fewer viable cells will be recovered post-electroporation.

3. Gently resuspend ~70% confluent *Drosophila* S2R+-MT::Cas9 cells in Schneider's medium. Load 10 µl of the cell suspension onto a hemocytometer and count the cells (see Current Protocols article: Phelan & May, 2017).

4. For each reaction, load 4×10^5 cells in a 15-ml conical tube and centrifuge 10 min at $88 \times g$ at room temperature. Aspirate the medium.

5. Gently resuspend the cells in 17 µl of SF Cell Line Nucleofactor Solution and aliquot the reactions into separate PCR tubes.

6. Add sgRNA(s) and donor to the cell solution for a final volume of 20 µl.

   For Kanca et al. (2019), we used 1 µl of sgRNA (~100 ng/µl) and 2 µl of the ssDNA donor (~100 ng/µl).

7. Add the entire reaction volume to an electroporation cuvette by pipetting into the space between the electrode plate.

   Avoid making bubbles by pipetting slowly. If bubbles are present or if solution is unevenly placed between the electrodes, gently tap the cuvette on the counter a few times to settle the solution.

8. Load the electroporation cuvette into the Lonza 4D Nucleofector electroporation device, select the cuvette wells to be electroporated, and run Program DS-137.

9. Remove the cells from the cuvette immediately following transfection and place into the individual wells of the prepared 48-well plate (see step 1).

10. Observe the cells daily for health and growth. After 2 days, remove half of the medium along with any floating cells and replace with fresh room temperature medium.

    Fluorescence from the knock-in tag should be visible after 7 to 10 days.

SUPPORT PROTOCOL 5

SINGLE-CELL ISOLATION OF FLUORESCENT CELLS USING FACS

This protocol describes use of FACS to isolate single cells. If FACS is not available, serial dilution can be used as an alternative method for single-cell isolation (see Internet Resources). Single cells will not survive in Schneider's medium alone. To support growth, use conditioned medium (see Reagents and Solutions) or feeder cells (see Critical Parameters).

Materials

Transfected (Support Protocol 3) or electroporated (Support Protocol 4) cells
Schneider's medium (see recipe)
Conditioned medium (see recipe)
Plastic box with lid to create a humidity chamber (see annotation to step 5)
12-well flat bottom plate (Corning #29442-040)
6-well flat bottom plate (Corning #29442-042)
T-25 Falcon tissue culture treated flasks (VWR #29185-300)
T-75 tissue-culture-treated flasks (Genesee Scientific #25-209)
Fluorescence microscope
96-well flat bottom plate (Corning #29442-056)
Bright-Line hemocytometer with coverslip (Sigma-Aldrich #Z359629)
40-µm pore-size filter
FACS machine, such as the FACSAria II (BD Biosciences)
Bright-field microscope

Additional reagents and equipment for counting cells (see Current Protocols article: Phelan & May, 2017)

1. Monitor growth of the cells after transfection/electroporation. Expand the cells by resuspending them when confluent and transferring them to plates/flasks of increasing size: 12-well plate, 6-well plate, T-25 flask, and T-75 flask. Use an aliquot of each cell population to document the GFP fluorescence using a fluorescent microscope.

   Confluent cells have a fast growth rate. You should be able to expand cells to a larger vessel each day. Check expanded cells the next morning and expand further if confluent.

2. Prepare conditioned medium as described in Reagents and Solutions at least 2 to 3 days before single-cell sorting of the clones. Conditioned medium can be stored at 4°C for a few weeks. Prepare several 96-well plates filled with 100 µl of conditioned medium per well.

   To ensure the recovery of sufficient numbers of single cell clones, we prepare three 96-well plates per knock-in experiment. Do not sort cells into the outer wells. Fill the outer wells of the 96-well plates with 200 µl of regular medium instead of conditioned medium. This is extremely important, as the 96-well plates are to be left alone for ~20 days, and filling the outer wells with extra medium will provide a mini humidity chamber that will last the 20 days.

3. Grow cells to near confluency (~80%) in a T-75 flask, resuspend the cells in medium, and count the cells on a hemocytometer (see Current Protocols article: Phelan & May, 2017). Remove at least 10^5 cells and filter through a 40-µm filter into a 15-ml conical tube. If necessary, add regular medium to bring the total volume in the tube to 1 ml. Include a sample of untransfected S2R+-MT::Cas9 cells as a negative control for GFP.

4. Pass the samples through a FACS machine (such as the BD Aria II), using the untransfected S2R+-MT::Cas9 cells to set the gates as shown in Figure 9. Sort the cells that are positive for GFP (Gate P4) into the inner wells of the 96-well plates filled with conditioned medium.

5. Wrap each 96-well plate and lid with Parafilm and incubate at 25°C.

   To further prevent the plates from drying out, we place plates inside a plastic box containing wet strips of paper towels. Alternatively, plates can be kept in an incubator with 33% relative humidity. Users can also keep plates at room temperature in a dark drawer.

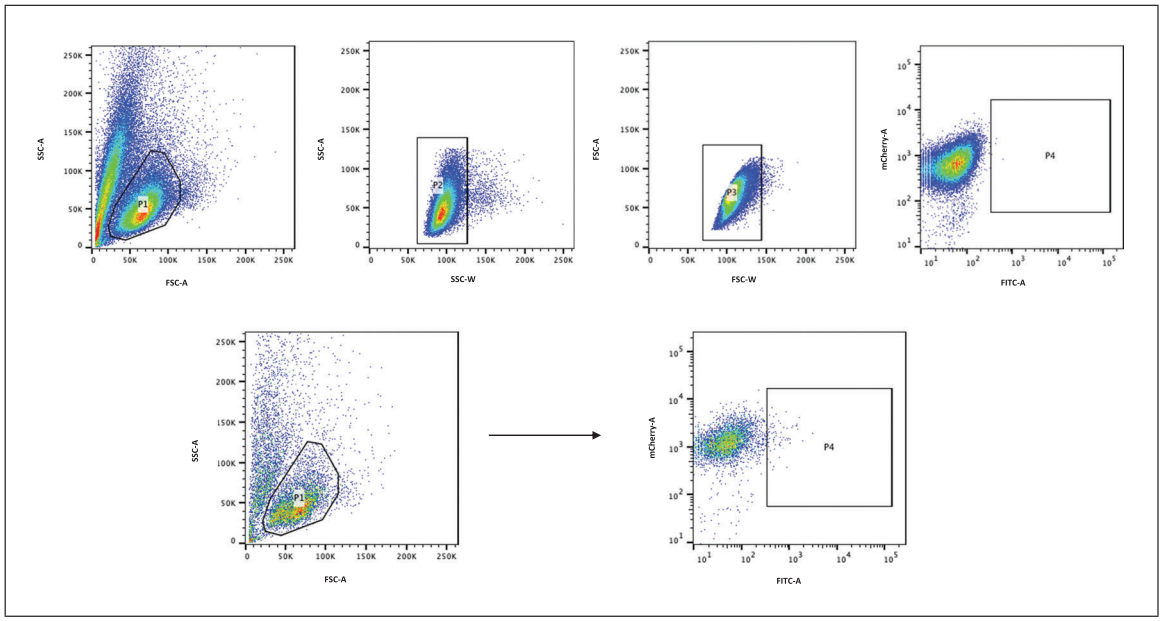

Figure 9 FACS gating to identify potential GFP-positive cell populations. Demonstration of the three different gates used to separate living and healthy cells from debris and dead cells (Gates P1-P3). Healthy cells are then gated by the presence of GFP (Gate P4). The top row shows the S2R+-MT::Cas9 cells used to set up the gates that are then applied to the electroporated populations. The bottom row is an electroporated population showing the initial gating of the entire population (Gate P1) and then the GFP positive cells (Gate P4). Cells in this gate are then single-cell sorted.

6. About 3 weeks after FACS isolation of single cells, look at the wells of the 96-well plate under a bright-field microscope to find growing single-cell clones.

   Clones that are growing well are usually visible by eye as tiny specks in the well. Under the microscope, such a clone should look similar to the example shown in Figure 10. However, it is important to look at all of the wells under the microscope, as some clones might be present but not large enough to be seen by eye. Cells in small clusters (5 cells or less) have a much lower chance of surviving. Clusters of ~6 cells or more are likely to grow to confluence; they will just take a little longer to grow.

7. Resuspend the clump of cells in the medium to spread out the cells and encourage growth to confluency. Check the cells in a few hours or on the following day for confluency. If confluent, expand the cells as previously described using regular medium.

   We find it helpful to assign unique identifiers to each clone to keep track of the targeted gene and the number of clones. We typically obtain single cell cloning efficiencies of ~10% to 30%. In our experience, some individual wells might contain mold. If this is widespread, consult the FACS facility or review sterile techniques. If this is rare, we recommend continuing the workflow but avoiding these wells for selection of colonies.

REAGENTS AND SOLUTIONS

Conditioned medium

This is Schneider's medium (see recipe) conditioned by the growth of cultured cells, e.g., *Drosophila* S2R+ cells. Inoculate 175-cm^2 (T-175) flasks with 1×10^6 cells/ml S2R+ cells and collect the conditioned medium 1 week later, when the cultures are confluent. Gently pour off medium from a culture flask containing adherent confluent cells into a conical centrifuge tube, centrifuge 10 min at $250 \times g$ at room temperature, to pellet any remaining cells, and pass the supernatant through a 0.2-µm filter. We store conditioned medium at 4°C and use it for as long as 1 month.

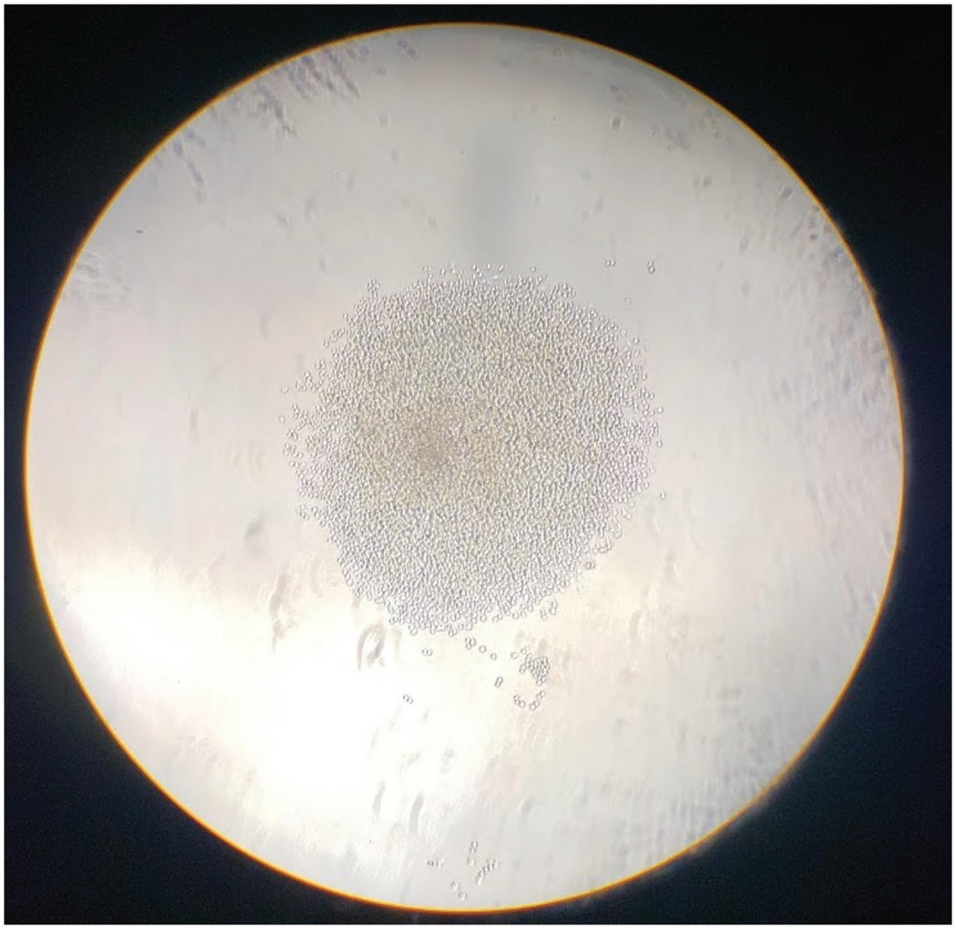

Figure 10 Example clone in a 96-well plate well 20 days after single-cell isolation. A clone of this size can be expected to expand easily after transfer to Schneider's medium in a flask.

Also see Housden, Nicholson, & Perrimon, 2017 and https://fgr.hms.harvard.edu/single-cell-isolation.

Schneider's medium

1× Schneider's *Drosophila* medium (ThermoFisher Scientific #21720024)
10% FBS (Sigma #A3912)
1× penicillin/streptomycin (ThermoFisher Scientific #15070-063)

Prepare Schneider's medium by adding FBS (10% final) and penicillin and streptomycin (final 500 U/ml) to Schneider's Drosophila medium. Filter sterilize using a 0.2-µm filter. Store up to 1 month at 4°C.

Schneiders' medium with 2 µg/ml puromycin

Dilute a 10 mg/ml puromycin (Calbiochem #540411) stock solution to a final concentration of 2 µg/ml (1:5000) in Schneider's medium (see recipe).

COMMENTARY

Critical Parameters

Location of the tag

Before attempting a knock-in, it is important to predict whether a fluorescent protein fusion will impact the target protein localization or function. This can be accomplished using a combination of literature review and bioinformatic tools. For example, users should search for publications in which their target protein (or homolog) was previously tagged, making note of the location of the tag (i.e., N-terminus, C-terminus, or internal) and

whether the tagged protein was functional. Users should also analyze the amino acid sequence of their target protein for insights into whether the location of a tag is acceptable. For example, users should tag a protein in a region that is less evolutionarily conserved, that is unstructured, or that does not disrupt a protein domain. Unlike the standard knock-in approach, which has the most flexibility, the ssDNA Drop-In knock-in method is limited to tagging at an internal protein location defined by an intron, and the CRISPaint knock-in method is limited to C-terminal tags. Furthermore, the CRISPaint method will delete a small portion of C-terminus from the final target protein. For all methods, when possible, users should compare their final tagged protein localization to the localization of the untagged protein using antibody staining.

Gene expression in cells

Another important consideration is the expression levels of the target gene, because isolation of the knock-in cell line is dependent on expression of the inserted tag; fluorescence from the endogenously tagged protein is detected by FACS and used to sort single cells. See Strategic Planning and DGET (Hu et al., 2017) in Internet Resources. Furthermore, when selecting knock-in cells by antibiotic resistance (standard and CRISPaint methods), co-expression of the antibiotic-resistance gene is required. Note that we have not empirically determined the lower limit of gene expression to obtain a knock-in. If target gene expression is off or low, but induced by a biological or chemical factor (such as a signaling ligand or drug), we recommend inducing expression of the gene before FACS or antibiotic selection.

sgRNA design

A functional sgRNA is critical to generate knock-in events. We recommend using established protocols and online resources to design sgRNAs with efficient binding to their target site (see Support Protocol 1 and Internet Resources). In addition, the exact location of the sgRNA target site is an important decision. For the standard and ssDNA Drop-In methods, the sgRNA binding site should be close to the homology arms (Paquet et al., 2016). Moreover, for the ssDNA Drop-In method, the chosen intron(s) must be of sufficient length, and the sgRNA binding site should not disrupt noncoding regulatory elements. For the CRISPaint method, the sgRNA binding site in 3′ coding sequence must be as close to the stop codon as possible, to limit the removal of C-terminal amino acids. To increase the chances of obtaining a knock-in cell line, users may want to perform parallel knock-ins using independent sgRNAs. For the standard and Drop-In methods, donor DNAs corresponding to each sgRNA would have to be constructed. In contrast, for the CRISPaint method, the same universal donor can be used with multiple sgRNAs.

Transfection

The efficiency of introducing DNA into the cells (via electroporation or transfection) is also an important variable. We strongly recommend including positive controls to ensure that multiple steps in the protocol are performed correctly. For the CRISPaint method, *pCFD3-Act5c* can be used as a positive control, where ∼3% of cells are expected to be positive for the knock-in.

Single-cell isolation and growth

Drosophila cultured cells can be difficult to grow following single-cell isolation. As presented here, we use conditioned medium to support growth of cells following FACS. There are alternative methods for support of cell growth following single-cell isolation, such as use of irradiated feeder cells. Available methods are reviewed in Luhur, Klueg, & Zelhof (2019), and feeder cells are available from the DGRC (*https://dgrc.bio.indiana.edu/cells/FeederCells*).

Troubleshooting

Table 2 below describes common problems and corresponding troubleshooting advice.

Understanding Results

Both protocols described in this article can result in successful fusions. Example results, including information about molecular and image-based validation of successful knock-in events, are presented in Kanca et al. (2019) and Bosch et al. (2019). If fluorescent protein–tagged knock-in events are not obtained, a number of steps can be looked at, including sgRNA design and cell health following transfection, FACS, and growth (see Troubleshooting). For some targets, reconsideration of the design might be needed, for example in the rare case that the fusion generates a dominant negative mutation that prevents successful events from being isolated. Moreover, successful detection of a fusion protein does not ensure that it is localized properly. For proteins with an expected localization based on antibody detection or other data, confirmation that the knock-in results in the expected localization

Table 2 Troubleshooting

Problem	Potential causes	Solutions
No fluorescent cells (or, for Basic Protocol 2, no puromycin-resistant cells)	Target gene sgRNA does not cut efficiently	Confirm sgRNA can cut target site using a T7 endonuclease assay; design and clone an alternate sgRNA if necessary; test cutting efficiency prior to attempting the knock-in
	SNP in the sgRNA target region	Check for SNPs; design and clone an alternate sgRNA if necessary
	Target protein not expressed or expressed at low levels	Check RNAseq or qPCR data for the target; detect fluorescent tagged protein using an anti-tag antibody by immunostaining or immunoblot
Cells are positive by FACS (or for Basic Protocol 2, cells are puromycin resistant) but do not appear fluorescent by live-cell imaging	Target protein expressed at low levels	Check by qRT-PCR for the target; detect fluorescent tagged protein using an anti-tag antibody by immunostaining or immunoblot; check for the presence of the fluorescent ORF transcript by qRT-PCR
Unexpected fusion protein localization within cells	Fusion protein affects protein localization	If possible, target the tag to different location in the protein, or a different protein in the complex or organelle
	Unidentified on- or off-target genome changes	Screen multiple single cell cloned lines for expected localization; bioinformatics analysis of off targets

pattern is relatively easy. When an antibody is not available and localization data has not been reported, it can be more difficult to have confidence that the correct localization pattern is observed. In addition, for tagged proteins with the expected subcellular distribution, there is no guarantee that the fusion protein is functional. Additional testing would be required to determine if that is the case.

As mentioned in Strategic Planning, the protocols presented here describe methods for tagging any allele, not all alleles. For many assays, such as when the tagged protein is being used to visualize an organelle or compartment, the fact that wild-type alleles are present does not matter. For other assays, however, it might be important that only the fusion protein be expressed. In those cases, additional molecular analysis would be necessary to determine if the knock-in is present in all alleles or a subset, and if any remaining non-tagged alleles are wild-type or modified, e.g., by an NHEJ-mediated indel. If antibodies against the target protein are available, immunoblotting could be used to determine if both fusion and wild-type proteins are present. Finally, we have not observed loss of the fusion protein in culture, but if multiple types of alleles are present, then this could occur, such as by gene conversion. Thus, cells should be monitored during repeated passages to ensure that the fusion protein is retained.

Time Considerations

Both protocols take about 2 to 2.5 months from start to finish.

Timeline for the ssDNA Drop-In method (Basic Protocol 1):

design, sgRNA cloning, and ssDNA synthesis, 4 days

transfection and growth, 7 days

FACS, single-cell cloning and characterization, 30 days.

Timeline for the CRISPaint method (Basic Protocol 2):

design and sgRNA cloning, 4 days

transfection and growth, 7 days

selection in puromycin-containing medium, 20 days (optional)

single-cell cloning and characterization, 30 days.

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Internet Resources

FlyBase

http://flybase.org/

Information on fly genes, bioinformatics tools, and fly genome browser. Described in Thurmond et al. (2019).

DRSC Find CRISPR online resource, updated version

https://www.flyrnai.org/crispr3/web/

Useful for sgRNA design for Drosophila melanogaster. Includes ability to check if there are SNPs in the sgRNA region in the genome sequence of the S2R+-MT::Cas9 cell line. With this online resource, you can also view the frame of the cut site for a given sgRNA (see Fig. 4B).

CRISPR Optimal Target Finder

http://targetfinder.flycrispr.neuro.brown.edu/

Alternative online resource for design of sgRNAs for Drosophila melanogaster.

DRSC Efficiency Predictor

https://www.flyrnai.org/evaluateCrispr/

Useful for evaluation of sgRNA designs for Drosophila melanogaster made using any design tool. Described in Housden et al. (2015).

Drosophila Gene Expression Tool (DGET)

https://www.flyrnai.org/tools/dget/web/

Useful for query of modENCODE transcriptomics data for S2R+ and other cell lines, for confirmation that a target gene is expressed at levels likely to be sufficient for detection of a fluorescent fusion protein. Described in Hu et al. (2017).

DRSC protocols for Drosophila cell culture

https://fgr.hms.harvard.edu/fly-cell-culture

Step-by-step protocols and media formulations for Drosophila cell culture.

DGRC protocols for Drosophila cell culture

https://dgrc.bio.indiana.edu/Protocols?tab=cells

Step-by-step protocols and media formulations for Drosophila cell culture. See Luhur et al. (2019).

DGRC cell catalog

https://dgrc.bio.indiana.edu/Protocols?tab=cells

Catalog of cell lines available, including S2R+-MT::Cas9, fluorescence-tagged cell lines generated using the approaches presented in Bosch et al. (2019); Kanca et al. (2019); and many other Drosophila cultured cell lines. See Luhur et al. (2019).

CRISPR Fly Design website

https://www.crisprflydesign.org

Online resource for Drosophila CRISPR protocols and information, including cloning of sgRNAs into the pCFD3 plasmid vector. See Port et al. (2014).

CellProfiler image analysis software user manual resources

http://cellprofiler-manual.s3.amazonaws.com/CellProfiler-3.0.0/modules/objectprocessing.html#identifyprimaryobjects

Details how to identify Primary Objects (for these protocols, mCherry-positive cells) and Secondary Objects (for these protocols, the GFP or mNeonGreen-tagged fusion proteins) from fluorescent micrographs of cells. See Carpenter et al. (2006).

http://cellprofiler-manual.s3.amazonaws.com/CellProfiler-3.0.0/modules/measurement.html#measureobjectintensity

Details how to measure the intensity of the targeted object of interest (for these protocols, brightness of the GFP or mNeonGreen fusion proteins). See Carpenter et al. (2006).

Addgene protocol, "Isolating a Monoclonal Cell Population by Limiting Dilution"

https://www.addgene.org/protocols/limiting-dilution/

Details how to generate a monoclonal cell culture from a heterogeneous population of cells using serial dilution in 96-well plates.

Key References

Viswanatha et al. (2018). See above.

Describes generation of Cas9-expressing S2R+ cells (S2R+-MT::Cas9, DGRC #268).

Kanca et al. (2019). See above.

First demonstration of the use of the ssDNA Drop-In method in Drosophila cells (and use of related technologies in vivo).

Bosch et al. (2019). See above.

First demonstration of the use of the CRISPaint method in Drosophila cells (and in vivo).

Schmid-Burgk et al. (2016). See above.

First demonstration of homology-independent insertion in mammalian cells using the CRISPaint system. Generated CRISPaint donor plasmids that are compatible in Drosophila cultured cells.

Higuchi and Ochman (1989). See above.

First demonstration of production of ssDNA following PCR amplification.

Port et al. (2014). See above.

Describes the pCFD3 plasmid vector.

Housden and Perrimon (2016). See above.

Describes production of long homology arm donors for the standard knock-in approach.