CHAPTER SIX

Recovery of Alternative End-Joining Repair Products From *Drosophila* Embryos

Terrence Hanscom, Varandt Y. Khodaverdian, Mitch McVey[1]
Tufts University, Medford, MA, United States
[1]Corresponding author: e-mail address: mitch.mcvey@tufts.edu

Contents

Abstract

In this chapter, we describe a method for the recovery and analysis of alternative end-joining (alt-EJ) DNA double-strand break repair junctions following I-*Sce*I cutting in *Drosophila melanogaster* embryos. Alt-EJ can be defined as a set of Ku70/80 and DNA ligase 4-independent end-joining processes that are typically mutagenic, producing deletions, insertions, and chromosomal rearrangements more frequently than higher-fidelity repair pathways such as classical nonhomologous end joining or homologous recombination. Alt-EJ has been observed to be upregulated in HR-deficient tumors and is essential for the survival and proliferation of these cells. Alt-EJ shares many initial

Methods in Enzymology, Volume 601
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2017.11.027

processing steps with homologous recombination, specifically end resection; therefore, studying alt-EJ repair junctions can provide useful insight into aborted HR repair. Here, we describe the injection of plasmid constructs with specific cut sites into *Drosophila* embryos and the subsequent recovery of alt-EJ repair products. We also describe different analytical approaches using this system and how amplicon sequencing can be used to provide mechanistic information about alt-EJ.

ABBREVIATIONS

alt-EJ	alternative end joining
c-NHEJ	classical nonhomologous end joining
CRISPR	clustered regularly interspaced short palindromic repeats
ddH_2O	distilled, deionized water
DSB	double-strand break
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate

1. INTRODUCTION

DNA end-joining mechanisms are a set of double-strand break (DSB) repair processes that can be used throughout the cell cycle, preventing improper recombination and loss of heterozygosity (Ceccaldi, Rondinelli, & D'Andrea, 2016; Lieber, 2010; Lieber, Gu, Lu, Shimazaki, & Tsai, 2010; Shrivastav, De Haro, & Nickoloff, 2008). End-joining repair can be separated into two main classes. The first, known as classical nonhomologous end joining (c-NHEJ), occurs when the Ku70/80 heterodimer binds DNA ends with high affinity, ends are juxtaposed and processed, and the DNA ligase 4/XRCC4/XLF complex completes the reaction by ligating the DNA backbone (Chiruvella, Liang, & Wilson, 2013; Lieber, 2010). Because it does not require the presence of a homologous template, c-NHEJ is the dominant DNA repair pathway outside of S and G2 phases. Ku and ligase 4-independent end joining, also referred to as alternative end joining (alt-EJ), is kinetically slower than c-NHEJ, occurs throughout the cell cycle, and can happen even when c-NHEJ is available (Chang, Pannunzio, Adachi, & Lieber, 2017). Alt-EJ repair is generally imprecise, leading to larger insertions and deletions than are typically observed with c-NHEJ repair (Frit, Barboule, Yuan, Gomez, & Calsou, 2014; Rodgers & McVey, 2016).

One well-characterized alt-EJ mechanism, known as microhomology-mediated end joining, utilizes 5′–3′ resection to expose short, generally 1–20 nucleotide, single-stranded microhomologies on both ends of the break (Seol, Shim, & Lee, 2017; Sfeir & Symington, 2015). This dependence on resection highlights one of the aspects common between alt-EJ and homologous recombination (Truong et al., 2013). Annealing of the complementary bases in these microhomologies, followed by flap trimming, fill-in synthesis, and ligation, leads to deletions of varying sizes. Microhomology-bearing junctions are commonly found at sites of chromosomal translocations, implicating alt-EJ in the formation of genomic rearrangements and mutations associated with cancer (Soni, Siemann, Pantelias, & Iliakis, 2015; Wood & Doublie, 2016).

Drosophila melanogaster has proven to be an excellent model in which to study alt-EJ. The *Drosophila* genome lacks many c-NHEJ proteins which are found in various other organisms, including DNA-PKcs, Artemis, and the X family polymerases mu and lambda (Sekelsky, 2017). Furthermore, flies lacking the c-NHEJ component DNA ligase 4 are able to carry out alt-EJ with high efficiency (McVey, Radut, & Sekelsky, 2004). Recently, we have utilized *Drosophila* to investigate the precise mechanisms of alt-EJ and how sequence context may influence the spectrum of alt-EJ repair products (Khodaverdian et al., 2017; Yu & McVey, 2010). Here, we describe our recently developed, rapid, high-throughput methodology for the recovery, and analysis of alt-EJ products in a variety of sequence contexts.

1.1 A Comparison of Methods for Recovering Alt-EJ Repair Products

Researchers studying DSB repair utilize various methods to induce site-specific DSBs, including endonucleases such as I-*Sce*I and I-*Ppo*I, zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR) systems (Gaj, Gersbach, & Barbas, 2013). Site-specific DSBs offer advantages over ionizing radiation and chemical agents that cause multiple nonspecific DSBs, with the biggest advantage being the ability to easily discern sequence changes that occur during repair. We have previously worked with an I-*Sce*I recognition sequence integrated via *P*-element transposition on the second chromosome of *D. melanogaster* (Rong & Golic, 2003). By using standard genetic crosses to introduce the I-*Sce*I endonuclease into male flies with the recognition site, multiple independent DSB repair events can be recovered from these flies' germ lines and sequenced. However, there are several drawbacks

to using the chromosomal I-*Sce*I site. One is the amount of time that the process requires; induction of DSBs and recovery requires multiple crosses, each of which takes approximately 2 weeks. Another issue is that repair product identification requires a number of downstream steps, including recovery of genomic DNA, polymerase chain reaction (PCR) to amplify the DNA surrounding the break, and either direct sequencing of the PCR product or cloning of the product into a suitable vector for Sanger sequencing.

Because of the time and labor required with the chromosome-based system, we have recently developed a more efficient, plasmid-based injection system. Advantages of this system include significantly reduced time to isolate and sequence repair products and the ability to apply next-generation amplicon sequencing to sequence hundreds of thousands of repair junctions. In addition, early-stage *Drosophila* embryos contain high levels of maternally deposited DNA repair enzymes, including proteins such as DNA polymerase theta and DNA ligase III that have been implicated in alt-EJ (Beagan & McVey, 2016; Paul et al., 2013; Sharma et al., 2015). By utilizing mutant embryos that lack DNA ligase 4, a protein essential for c-NHEJ, we can specifically study alt-EJ repair. Importantly, we have found that alt-EJ repair events recovered from plasmids injected into embryos are similar in type and frequency to those recovered from a chromosomal-based system, suggesting that alt-EJ operates similarly in both systems (Khodaverdian et al., 2017; Yu & McVey, 2010).

2. PREPARING AND INJECTING PLASMIDS INTO EMBRYOS

2.1 Overview of the Process

Plasmids containing an I-*Sce*I recognition site, referred to here as I-*Sce*I repair constructs, are injected into *Drosophila* embryos expressing the I-*Sce*I endonuclease. After a 4-h incubation, during which time the plasmids are cut and repaired, plasmid DNA is extracted and subjected to individual Sanger or high-throughput amplicon sequencing. The available strategies for injection and recovery are broadly outlined in Fig. 1. One strategy involves transformation of the recovered plasmids into bacteria, which results in colonies containing independent repair events. DNA from these colonies can be extracted and subjected to in vitro I-*Sce*I digest to identify inaccurate repair products. These are then Sanger sequenced, and the repair junctions are analyzed. Alternatively, the recovered plasmids can be pooled

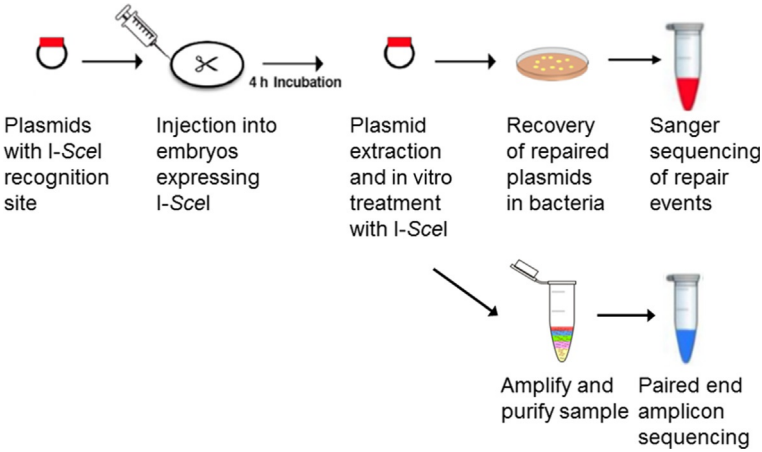

Fig. 1 Injection, recovery, and characterization of alt-EJ repair events. Transgenic embryos expressing I-*Sce*I are injected with plasmids containing the endonuclease recognition site. Following cutting and repair, inaccurately repaired plasmids are preferentially recovered following in vitro treatment with I-*Sce*I, transformed into bacteria, and Sanger sequenced. Alternatively, repair junction sequences are amplified by PCR and deep sequenced.

and the DNA sequence flanking the I-*Sce*I site can be amplified via PCR. After appropriate indexes are added, the libraries are sequenced using next-generation amplicon sequencing. The use of amplicon sequencing produces several million reads per run, of which hundreds of thousands are inaccurate alt-EJ repair products.

2.2 Reagent Preparation

2.2.1 Plasmid Preparation

1. To generate plasmids containing an I-*Sce*I recognition site, amplify the site from a suitable template using PCR. We used Taq polymerase to amplify the I-*Sce*I site and flanking sequence from the *Iw7* chromosomal locus (Rong & Golic, 2003) and used TA cloning to insert the PCR fragment into the pGem-T Easy vector, but any sequence with an I-*Sce*I recognition site is suitable.
2. Transform the plasmids into a suitable *Escherichia coli* strain, plate on antibiotic-containing medium, and incubate at 37°C overnight.
3. Inoculate 50 mL of LB broth with a single colony, incubate overnight, and purify the DNA using a Macherey-Nagel NucleoBond Xtra Midi kit or other suitable method for obtaining high-purity DNA.

4. Prior to injection, subject a sample of the purified plasmid to sequencing to validate the presence of the I-*Sce*I site.
5. If changes to the DNA sequence flanking the I-*Sce*I site are desired, these can be engineered using site-directed mutagenesis.

2.2.2 Injection Mix Preparation

1. Make a 0.1-*M* sodium phosphate (pH 6.8) solution using 0.1 *M* NaH_2PO_4 (monobasic) and 0.1 *M* Na_2HPO_4 (dibasic). Make a 0.1-*M* potassium chloride solution. Combine these to create a 10-mL stock solution of 1 m*M* sodium phosphate and 50 m*M* potassium chloride. Filter sterilize using a 0.22-μm filter.
2. Bring plasmids to a standard concentration of 500 ng/μL within the injection buffer to create the final injection mix.
3. Store 10 μL aliquots of the injection mix at −20°C. Thaw immediately before using.

2.3 Embryo Collection

The success of this protocol depends upon the injection of I-*Sce*I-bearing plasmids into early-stage embryos (<2 h old) that have not yet cellularized. To do this, females must be primed so that they are healthy and laying large quantities of eggs that can be collected in a short period of time. Egg collection conditions must be optimized to facilitate rapid recovery and processing of embryos.

Additional resources describing effective methods for collecting, preparing, and injecting embryos with plasmid DNA can be found in Kiehart, Crawford, and Montague (2007) and O'Brochta and Atkinson (2004).

2.3.1 Procedure

1. Obtain a stock of flies expressing the I-*Sce*I endonuclease. We use a stock expressing I-*Sce*I under control of a ubiquitin promoter with genotype: w^{1118}; *P{Ubiq::I-SceI}*, *Sp/CyO*, *P{Ubiq::I-SceI}* (Preston, Flores, & Engels, 2006). To recover inaccurate repair events in a c-NHEJ deficient genetic background, an isogenic stock with the $lig4^{169a}$ mutation can be used (McVey et al., 2004). Stocks are maintained in culture bottles containing cornmeal agar in a 25°C incubator on a 12-h light–dark cycle.
2. To prepare grape agar, combine 400 mL distilled, deionized water (ddH_2O), 90 mL frozen grape juice concentrate, 11 g agar, 29 g dextrose, 14.5 g sucrose, and 1.25 mL 10 N sodium hydroxide in a 1-L Pyrex bottle.

3. Microwave until the agar is melted. Monitor carefully as it boils over easily. Set aside to cool.
4. In a 50-mL conical tube, create acid solution by mixing 20.9 mL propionic acid, 2.08 mL phosphoric acid, and ddH_2O to bring the volume to 50 mL.
5. Add 5.6 mL of acid solution to the cooling bottle of agar when it reaches a temperature of 65°C. Store remainder of the acid mix at room temperature. Mix the agar well and pour into 55 mm plastic Petri plates, filling about half full. Plates will last several months stored in plastic bags at 4°C.
6. To create yeast paste, cover the bottom of a 100-mL beaker with active yeast pellets. Add ddH_2O intermittently, 1 mL at a time, and stir until a thick paste is formed. Store excess yeast paste at 4°C.
7. Prepare embryo collection cages (Fig. 2A) by fitting the grape agar plate within the lip of a 100-mL plastic beaker. Secure the plate by wrapping a rubber band around the collection cage.

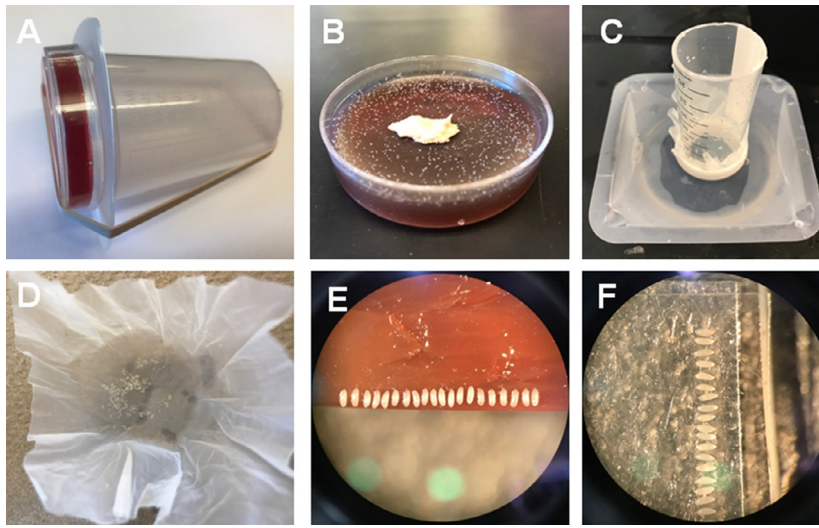

Fig. 2 Apparatus for embryo collection and injection. (A) Embryo collection cage, with rubber band securing a grape agar plate to a tricorner plastic beaker. (B) Grape agar collection plate with yeast paste in the center. A collection of 100 well-fed females will lay hundreds of embryos on the plate in less than an hour. (C) Nylon mesh secured to conical tube cut in half, for dechorionation and washing of embryos. (D) Nylon mesh with embryos after dechorionation and washing is complete. (E) Embryos aligned in a row on a grape agar slab, ready for transfer to a coverslip. (F) Embryos after transfer to double-stick tape attached to a glass coverslip, covered in halocarbon oil.

8. Collect embryos according to the following schedule:

   Day 1: Sort ~100–200 newly eclosed flies (a mixture of females and males) into a bottle/large vial containing standard cornmeal- or molasses-based fly food and a few active yeast pellets on the food surface. The yeast promotes egg laying.

   Day 2: Make fresh yeast paste. Warm a grape plate to 25°C, blot dry, and apply a ~1-cm diameter dab of yeast paste to the center of the plate. Anesthetize the bottle of flies with CO_2 and move them into the embryo collection cage. Cover the cage with the grape plate and wrap tight with a rubber band to secure the plate (Fig. 2A). Lay the cage on its side at 25°C and incubate overnight.

   Day 3: In the morning remove the old grape agar plate and swap with a new plate warmed to 25°C with fresh yeast paste in the center (Fig. 2B). This is best accomplished by tapping the cage on a benchtop, grape plate up, to knock the flies down to the bottom while you change the plates. Alternatively, flies can be anesthetized using CO_2, although this typically decreases egg laying for a period of time. Wrap the plastic beaker of the cage with tin foil, as females generally lay better in the dark. Place the cage on its side at 25°C and incubate for 1 h. It is important to keep the cage on its side so that the flies have enough surface area to walk, can easily access the grape plate (especially important if they cannot fly), and do not get stuck in the yeast paste. Repeat the plate changing process as needed for additional collections. Grape plates can be reused by rinsing them and blotting them dry immediately after rinsing. Flies will generally lay well in a cage for up to 5 days. When finished with the cage, freeze it overnight and dispose of flies.

Note: If the females are not laying sufficient eggs, ensure that the grape plates are warmed to 25°C before placing in the cages, are dry, and the yeast paste is fresh. Additionally, certain fly stocks do not lay as well as others. If using a mutant with low fecundity, add more flies to the cage. Disturbing the flies too frequently is detrimental to the egg-laying process and should be minimized.

2.4 Dechorionation of Embryos

2.4.1 Materials Needed

Squirt bottles
Soft-bristled paint brush
50% Bleach

10 × Embryo Wash Stock: (7% NaCl, 0.7% Triton X-100; dilute to 1 × working concentration)
Razor blade
50 mL Conical tube with the bottom half removed, and a 1-cm hole cut in the center of the cap
Genesee Scientific Mesh, Nitex Nylon 50 μ*M* screen, Item # 57–106 (cut into 4 × 4 cm pieces)
Glass slides
Glass coverslips (1 in. square)
Paper towels
Plastic weighing dishes
3M double-sided tape

2.4.2 Procedure

1. Perform these manipulations at a sink, preferably with a ddH_2O water faucet. Stretch the mesh screen over the top of the conical tube and screw the cap over the top of it. The screen should be stretched tight at the cap opening. Place the conical tube cap-down in the plastic weighing dish.
2. Using a spray bottle filled with ddH_2O, gently dispense water onto the grape plate, and using the bristles of the paint brush, separate the embryos from the grape agar. Break up the yeast paste to recover any embryos that were laid within. While the embryos are floating in the water, carefully pour them into the conical tube and onto the screen. Repeat as necessary until all embryos are in the conical tube (Fig. 2C).
3. Use the ddH_2O water from the faucet to gently wash the embryos in the conical tube, allowing all yeast paste and residual grape agar to flow through the screen until only embryos remain.
4. Submerge and agitate embryos for 1 min in a shallow container (a weighing dish works well) containing bleach diluted to 50% concentration in water (Fig. 2C). Make sure all embryos are covered. If embryos get stuck on side walls, use a spray bottle of 50% bleach solution to get them back onto the screen. After 1 min, remove the embryos from the bleach and rinse them for at least 2 min using ddH_2O from the faucet. The amount of time necessary to remove all of the chorion will vary depending on the strength and age of the bleach and should be adjusted accordingly.
5. Wash the embryos with embryo wash solution by submerging the bottom of the conical in a weighing dish containing 1 × embryo wash.

Agitate for 4 min. If embryos stick to the sides of the conical tube, use a squirt bottle of embryo wash to get them back onto the screen. Rinse the embryo wash from the conical using a squirt bottle containing ddH_2O for at least 2 min. Using the squirt bottle of ddH_2O, gently spray the walls of the conical tube to collect the embryos in the center of the screen. Place aside (Fig. 2D).

2.5 Positioning Embryos for Injection

1. Gently remove the grape agar from the plate and flip upside down on a paper towel. Using a razor blade, cut out a flat, rectangular piece of agar and place on a microscope slide. Pat dry with a paper towel. If any water remains, the surface tension on the agar slab will make it extremely difficult to position the embryos in the next step.
2. With embryos in the center of the screen, pick up the conical tube and grip the excess screen against the outside of the tube. Slowly, unscrew the cap, ensuring that the screen remains pressed against the tube and does not move. Gently blot off any residual water on a paper towel, remove the conical tube, and place the screen down on a dry paper towel. With dry gloves, grip the edges of the screen, gently flip over, and press the embryos onto the grape agar until all embryos are stuck to the agar. This may require several gentle taps.
3. Embryos should be approximately 1.5 h old at this point and should remain uncellularized throughout the injection process. Younger embryos are opaque, bright white, and firm while older, unusable embryos are more translucent. When lining up embryos for the injection process, do not select embryos older than 2 h or those that have a mushy-looking consistency, as they have been overbleached and will rupture when injected.
4. Place the slab of grape agar under a microscope and align at least 60 embryos with a metal-tipped probe. Line them up, side by side, with approximately one-half of an embryo's width between each (Fig. 2E). If you are right-handed, sort the embryos from left to right on the grape agar. If left-handed, sort from right to left. This will prevent disturbing the embryos once they are aligned on the agar.
5. Cut a 1 cm piece of double-sided tape lengthwise down the middle and apply it to the edge of a glass coverslip, cut-side out. The cut side of the tape has a clean edge that is better suited for embryo adhesion and injection.

6. Remove the top adhesive cover of the tape and press the taped coverslip down on the aligned embryos, lightly at first and then adding slightly more pressure. Gradually raise the coverslip off of the grape agar slab (Fig. 2F). Do not slide the tape off the edge as this can cause the embryos to fall off the tape.
7. Place the coverslip in a desiccating chamber for 1–2 min to dry the embryos. This prevents rupturing during the subsequent injections.

2.6 Microinjection of Embryos

2.6.1 Materials Needed

Borosilicate glass capillary tubes pulled to make injection needles
Halocarbon oil 700 (Sigma Aldrich)
Anhydrous calcium sulfate desiccate (Drierite)
Injection microscope (Zeiss injection scope and Parker-Hannifin Picospritzer II)
2 μL Pipettor with long gel loading tips (like Eppendorf GELoader)
Plasmid injection mix (prepared as described earlier)
1% Sodium dodecyl sulfate (SDS) solution in a 0.7% sodium chloride buffer

2.6.2 Preparing Needles

1. Pulling of microinjection needles is best done using an instrument such as the Sutter Instrument Co. Model P-87 Flaming/Brown Micropipette Puller. For this instrument, our settings are as follows: heat: 848; pull: 125; velocity: 70; time: 250 ms; pressure: 200.
2. Pulled injection needles do not have an opening and must be cut at the ends with a razor blade. Use the razor blade to cut off just the tip of the needle at a 45-degree angle. The needle bore should be extremely narrow, wide enough to aspirate the droplet of injection mix, but small enough that the embryos do not rupture when punctured. This will require some trial and error, so it helps to pull more needles than necessary for a single round of injections.
3. Load 1–2 μL of injection mix into several needles using a 2-μL pipettor with a long pipette tip. Extra filled needles can be placed on rolled pieces of clay or colored lab tape in a humid Petri dish at 4°C.

2.6.3 Injecting the Embryos

The injection procedure can be adapted according to the particular injection apparatus that is used. We use a Picospritzer II injector connected to

pressurized nitrogen gas, with a foot pedal that releases air in pulses of 25 ms when depressed.

1. Cover embryos with a thin coat of halocarbon oil 700, placing an additional drop on the distal end of the coverslip to be used for air aspiration and test injections to adjust the size of the injection mix droplet. Put a drop of halocarbon oil on a glass slide and position the coverslip on top for stability.
2. Place the loaded needle into the aspirator apparatus and the slide onto the microscope stage. Focus the microscope on the halocarbon oil drop. Using the Picospritzer, dispense any air in the tip of the needle into the oil droplet and adjust the pulse time so that the volume of plasmid mix being injected is approximately 1/20th the size of the embryo (you should be able to see the liquid when it is injected into the embryo).
3. Prior to injection, examine each embryo individually to determine its approximate age. Young embryos should have a clear outer edge and a brown oval center (Fig. 3). Embryos with well-defined, cellularized contours at their periphery are too old.
4. Align the exterior edge of the embryo in the same plane as the injection needle. Puncture the tip of the embryo with the needle and insert the needle so that its tip is in the middle of the embryo. Inject the plasmid mix and immediately remove the needle from the embryo. Some small leakage of cytoplasm may occur, but if leaking continues more than 1 s,

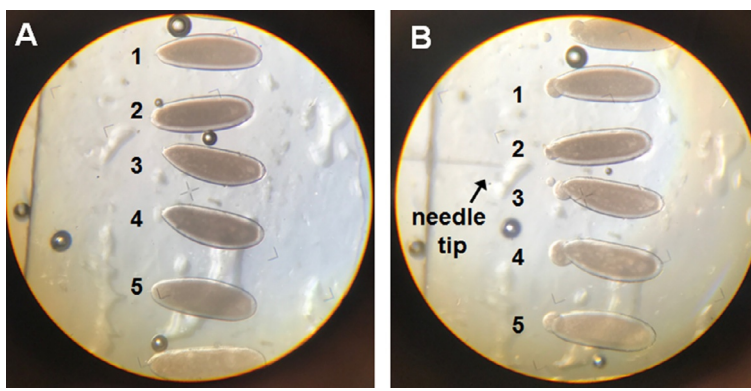

Fig. 3 Injection of embryos. (A) Embryos prior to injection. Embryos 1, 2, and 4 are approximately 2 h old but have not yet cellularized. Embryos 3 and 5 are younger and are surrounded by a translucent ring smaller than that observed in older embryos. (B) Embryos after injection. Some cytoplasmic leakage can be observed at the injection site. This is normal but should be minimized as much as possible.

the needle may be too large. Note: While embryos do not have to survive to hatching, as is required for transgenic procedures (O'Connor & Chia, 1993), their structural integrity should remain intact until plasmid isolation several hours later.

5. As injections proceed down the row of embryos, the stage may need to be adjusted so that both the needle and the embryo edge are focused in the same plane. This avoids such issues as needle breakage, pushing the embryos off the tape, or aspirating injection mix outside of the embryos.
6. Should the needle become clogged, move the needle to the distal droplet of halocarbon oil and disperse the clog with rapid aspirations or by increasing the pulse time. If the needle cannot be cleared, then begin again with a new needle.
7. Maintenance tip: Regularly clean the stage of the injection microscope for smooth movements of the slide, as halocarbon oil can cause sticking. Ensure all gaskets in the injector are sealing correctly, especially those that make a direct seal with the needle. If air is escaping from the needle input junction during injection bursts, droplet size will be inconsistent. Further, a loss of pressure can cause a vacuum in the needle which can result in reuptake of the injection mix and embryo contents.
8. After the injections, place the coverslip of embryos into a small covered container (e.g., Petri dish) and incubate at 25°C for 4 h to allow for cutting of the construct and subsequent repair. Incubation time can be increased or decreased according to preference. Following the incubation, DNA can immediately be extracted or the embryos can be stored at −20°C for up to 2 weeks. The halocarbon oil will freeze over the embryos.
9. Following the injections, the needles can be saved if they are free of clogs and handled carefully. To do this, remove the needle from the injection apparatus and place in a protected container at −20°C.

3. PLASMID RECOVERY AND REPAIR PRODUCT SEQUENCING

Here, we describe the recovery and purification of the repaired plasmid constructs. Following plasmid DNA isolation, the repair constructs can either be transformed into bacteria for Sanger sequencing (protocol 3.3) or amplified for next-generation amplicon sequencing (protocol 3.4).

3.1 Recovery of Injected Embryos

1. If starting with frozen embryos, remove the coverslips from the freezer. The halocarbon oil will melt.
2. Using a dissecting microscope, use a razor blade to carefully scrape excess halocarbon oil off of the double-sided tape without disturbing the embryos or the adhesive.
3. Using a bulb, gently squirt a 1% SDS solution over the embryos to remove any remaining halocarbon oil. The embryos should remain stuck to the adhesive, but stop rinsing if they start to fall off.
4. Wash away the SDS by gently agitating the coverslip in a Petri dish containing ddH_2O.
5. In one smooth motion, scrape a clean razor blade down the row of embryos as if running a spatula underneath them. Avoid scraping up the adhesive as much as possible. The embryos will collect in a pile on the edge of the razor blade.

3.2 Plasmid DNA Recovery by Alkaline Lysis

3.2.1 Reagents

P1 solution: 50 m*M* Tris–HCl, pH 7.5; 10 m*M* EDTA
P2 solution: 0.2 N NaOH, 1% SDS
P3 solution: 1.32 *M* KOAc, pH 4.8. Should be kept at 4°C
Plastic pestles (for homogenization)
95% and 70% Ethanol

3.2.2 Procedure

1. Use a plastic pestle to transfer the embryos from the razor blade to a 1.5-mL tube containing 25 μL of P1 solution.
2. Using the pestle, homogenize the embryos so that any that did not rupture while being scraped up are fully disrupted. Ensure the no embryos remain on the pestle. Vortex thoroughly to promote further lysis.
3. Add 25 μL P2 solution and shake. Mixture should clear immediately, indicating lysis, but can stand up to 5 min for best yields. Incubation for longer than 5 min can cause contamination with genomic DNA.
4. Add 25 μL of cold P3 solution and shake. A white precipitate containing cell debris and proteins should form. Spin in a microcentrifuge for 7 min at maximum speed.
5. Transfer the supernatant to a new tube with 200 μL 95% ethanol and mix by inverting several times. Incubate on ice for at least 10 min.

6. Centrifuge for 5 min at maximum speed. Discard the supernatant.
7. Wash pellet with 200 μL of 70% ethanol to remove excess salts. Centrifuge for 2 min at maximum speed.
8. Discard the ethanol and dry the pellet at room temperature for 10 min or until no ethanol remains in the tube.
9. Resuspend in 20 μL of ddH_2O water. Incubate at 37°C for 30 min with occasional gentle vortexing. Store at 4°C.
10. Optional step: We have found that the percentage of plasmids that are cut and inaccurately repaired is low (on the order of 10%), compared to the percentage of plasmids that are repaired perfectly or not cut. To mitigate this, recovered plasmid samples can be treated with I-*Sce*I endonuclease to reduce the number of uncut or perfectly repaired plasmids and to prevent them from being included in the downstream analysis. Incubate the recovered plasmid DNA, 2 μL of I-*Sce*I buffer, and 1 μL of I-*Sce*I restriction enzyme (NEB) in a 20-μL total reaction volume at 37°C for 1 h. Inactivate the I-*Sce*I by incubating 65°C for 20 min.

3.3 Bacterial Transformation and Sanger Sequencing

1. Use chemically competent *E. coli* XL10 or other suitable bacterial strain. Allow the frozen cells to thaw on ice for 10–15 min.
2. Add 5 μL of DNA directly to the cells. To promote high transformation efficiency, do not mix by pipetting. Cells must remain on ice at all times.
3. Incubate for 30 min on ice.
4. In a heat block, incubate at 42°C for 30–45 s.
5. Place the cells back on ice for 2 min.
6. Add 200 μL of sterile Luria broth and incubate at 37°C for 20 min.
7. Pipette 200 μL of each sample on antibiotic-containing plates that have been prewarmed to 37°C and incubate at 37°C overnight. Use the injection mix as a positive control for transformation efficiency.
8. Each colony that forms will contain an independent repair event. Inoculate a portion of each bacterial colony in 2 mL of Luria broth plus the appropriate antibiotic. Grow overnight at 37°C.
9. Transfer the culture into a 1.5-mL Eppendorf tube. Centrifuge at maximum speed for 10 s to pellet the cells. Discard the supernatant and proceed with a standard alkaline lysis miniprep protocol (described earlier, but increasing the volume of solutions fourfold).

10. Perform Sanger sequencing on the plasmid DNA using sequencing primers that anneal to plasmid sequence about 200 bp on either side of the I-*Sce*I site.
11. Alternative method: To eliminate the need to grow each colony in liquid culture, colony PCR can be performed using cells obtained from each independent colony. The PCR products can then be purified and sequenced using the Sanger method.

3.4 Next-Generation Amplicon Sequencing

3.4.1 Materials Needed

Agencourt AMPure beads (Protocol 000601v024)
Primers for the region flanking the I-*Sce*I recognition site
High-fidelity DNA polymerase (Q5, New England Biolabs)
Nextera sequencing index kit
Fragment analyzer or other equipment to analyze PCR product size
Illumina MiSeq Reagent Kit v3 600-cycle (MS-102-3003)

3.4.2 Amplicon Sequencing Approach

1. Following DNA extraction and enrichment of the inaccurately repaired plasmids, use PCR to amplify ~200 bp of DNA flanking the I-*Sce*I recognition site. To add variation to the amplicon PCR pool and promote efficient sequencing on the Illumina MiSeq platform, we use a set of pooled primers containing adapter sequences and 1–4 nucleotide random sequences at the 5′ end. Use a high-fidelity polymerase such as Q5 (New England Biolabs) and limit the amplification to 15–20 cycles to promote diversity of inaccurately repaired junctions and to prevent overrepresentation of the most common repair constructs.
2. Perform an AMPure bead purification to remove all PCR products less than 100 bp.
3. Perform a second, eight-cycle PCR with Nextera sequencing indexes (Illumina) for amplicon sequencing.
4. Perform a secondary AMPure bead purification step to remove all products less than 100 bp.
5. Use a fragment analyzer or other diagnostic tool to assess the consistency of fragment sizes. Ideally, 99%–100% of the samples should match the desired fragment size ±5 bp.
6. Sequence the amplicons using a paired-end sequencing kit. We use the Illumina MiSeq platform, which we find typically generates 8–10 million

paired-end reads per sequencing run. PhiX genomic DNA should be spiked into the sequencing reactions at concentrations of 5%, or higher if a pooled primer set is not used in step 1.

4. DATA ANALYSIS

Data analysis using either the Sanger or amplicon sequencing can be used to address a number of questions (Fig. 4):

(1) Did the repair process remove DNA (a deletion), add new DNA (an insertion), or both (an indel)?

(2) Did the repair process involve annealing at microhomologous sequences prior to synthesis and ligation?

(3) If there are inserted sequences, were these fully or partially templated from sequences flanking the I-*Sce*I cut site?

(4) Could the repair process utilize secondary-structure-forming sequencing as primers for synthesis of new DNA?

To conduct our sequence analysis, we typically utilize a number of open-source and commercially available sequence analysis tools, together with custom-designed programs in Python and R. Individual sequences from Sanger sequencing can be aligned to the original reference sequence using BLAST or various proprietary software tools. Below, we briefly describe our workflow for analyzing amplicon sequencing data.

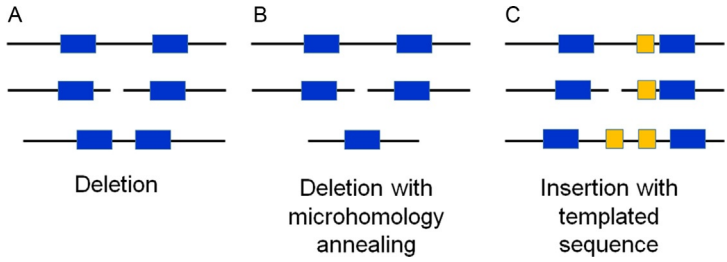

Fig. 4 Types of end-joining repair. (A) Deletion of sequences flanking the I-*Sce*I site. This can occur via both classical and alternative end joining. (B) Deletion of sequences via microhomology annealing (microhomologies are represented by *dark blue boxes*). This type of junction is frequently associated with alt-EJ. (C) Insertion of sequences that are templated from DNA flanking the I-*Sce*I cut site (represented by *light orange boxes*). This type of junction typically results from alt-EJ repair.

4.1 Analyzing Reads From Amplicon Sequencing

1. Raw reads from paired-end amplicon sequencing runs should be trimmed to the amplicon primer sequence. This includes trimming the random primer sequences at the ends of the amplicon. Junctions lacking 10 bp of reference sequence at both the 5′ and 3′ ends of each amplicon should be removed as PCR artifacts as they fail to span both sides of the break.
2. Overlapping read pairs can then be merged into single consensus reads as FASTQ files, using a program such as CLC Bio Genomics Workbench.
3. The reads can be mapped to the reference sequence corresponding to the original repair construct using a program such as Geneious and exported as SAM files for analysis.
4. The structure of repair junctions can be determined by using the CIGAR string to identify sequences matching the 5′ and 3′ ends of the amplicon, deleted sequences relative to the I-*Sce*I cut site, and inserted sequences. More detailed information about potential analytical parameters is described in chapter "High-throughput analysis of DNA break-induced chromosome rearrangements by amplicon sequencing" by Brown et al., in this volume.
5. After removing reads that match the reference sequence exactly (and represent either uncut or perfectly repaired plasmids), the percentage of reads corresponding to each junction can be calculated by dividing the number of reads per junction by the total number of inaccurate reads in the sequencing run. This provides a rough estimate of the overall representation of different repair junctions in each sample. We have found good correlation between repair junction frequency using Sanger and amplicon deep sequencing methods (Khodaverdian et al., 2017).

5. SUMMARY AND CONCLUSION

Alt-EJ is now recognized as a bona fide DSB repair mechanism that operates both in the presence and absence of classical end-joining repair. Elucidation of the mechanisms of alt-EJ will provide insight into the etiology of deletions, templated insertions, and chromosome translocations that are frequently associated with cancer and other human diseases. Here, we have described a process for the rapid identification and recovery of large numbers of alt-EJ repair products, using a high-throughput in vivo embryo injection system in *Drosophila*. While we have focused on the repair of

double-stranded breaks induced by the I-*Sce*I endonuclease, the protocol can easily be adapted for other types of DSBs, including those induced by CRISPR–Cas9, which produces blunt-ended cuts that may promote a different spectrum of alt-EJ repair products.

ACKNOWLEDGMENTS

We would like to thank Amy Yu and Juan Castaneda for their help in developing and documenting these protocols.

REFERENCES

Beagan, K., & McVey, M. (2016). Linking DNA polymerase theta structure and function in health and disease. *Cellular and Molecular Life Sciences*, *73*(3), 603–615. https://doi.org/10.1007/s00018-015-2078-9.

Ceccaldi, R., Rondinelli, B., & D'Andrea, A. D. (2016). Repair pathway choices and consequences at the double-strand break. *Trends in Cell Biology*, *26*(1), 52–64. https://doi.org/10.1016/j.tcb.2015.07.009.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews. Molecular Cell Biology*, *18*(8), 495–506. https://doi.org/10.1038/nrm.2017.48.

Chiruvella, K. K., Liang, Z., & Wilson, T. E. (2013). Repair of double-strand breaks by end joining. *Cold Spring Harbor Perspectives in Biology*, *5*(5), a012757. https://doi.org/10.1101/cshperspect.a012757.

Frit, P., Barboule, N., Yuan, Y., Gomez, D., & Calsou, P. (2014). Alternative end-joining pathway(s): Bricolage at DNA breaks. *DNA Repair (Amst)*, *17*, 81–97. https://doi.org/10.1016/j.dnarep.2014.02.007.

Gaj, T., Gersbach, C. A., & Barbas, C. F., 3rd. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, *31*(7), 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004.

Khodaverdian, V. Y., Hanscom, T., Yu, A. M., Yu, T., Mak, V., Brown, A. J., et al. (2017). Secondary structure forming sequences drive SD-MMEJ repair of DNA double-strand breaks. *Nucleic Acids Research*, *22*, 12848–12861. https://doi:10.1093/nar/gkx1056.

Kiehart, D. P., Crawford, J. M., & Montague, R. A. (2007). Collection, dechorionation, and preparation of Drosophila embryos for quantitative microinjection. *CSH Protocols*, *2007*. pdb prot4717 https://doi.org/10.1101/pdb.prot4717.

Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the non-homologous DNA end-joining pathway. *Annual Review of Biochemistry*, *79*, 181–211. https://doi.org/10.1146/annurev.biochem.052308.093131.

Lieber, M. R., Gu, J., Lu, H., Shimazaki, N., & Tsai, A. G. (2010). Nonhomologous DNA end joining (NHEJ) and chromosomal translocations in humans. *Sub-Cellular Biochemistry*, *50*, 279–296. https://doi.org/10.1007/978-90-481-3471-7_14.

McVey, M., Radut, D., & Sekelsky, J. J. (2004). End-joining repair of double-strand breaks in Drosophila melanogaster is largely DNA ligase IV independent. *Genetics*, *168*(4), 2067–2076. https://doi.org/10.1534/genetics.104.033902.

O'Brochta, D. A., & Atkinson, P. W. (2004). Transformation systems in insects. *Methods in Molecular Biology*, *260*, 227–254. https://doi.org/10.1385/1-59259-755-6:227.

O'Connor, M., & Chia, W. (1993). P element-mediated germ-line transformation of Drosophila. *Methods in Molecular Biology*, *18*, 75–85. https://doi.org/10.1385/0-89603-245-0:75.

Paul, K., Wang, M., Mladenov, E., Bencsik-Theilen, A., Bednar, T., Wu, W., et al. (2013). DNA ligases I and III cooperate in alternative non-homologous end-joining in vertebrates. *PLoS One*, *8*(3), e59505. https://doi.org/10.1371/journal.pone.0059505.

Preston, C. R., Flores, C. C., & Engels, W. R. (2006). Differential usage of alternative pathways of double-strand break repair in Drosophila. *Genetics*, *172*(2), 1055–1068. https://doi.org/10.1534/genetics.105.050138.

Rodgers, K., & McVey, M. (2016). Error-prone repair of DNA double-strand breaks. *Journal of Cellular Physiology*, *231*(1), 15–24. https://doi.org/10.1002/jcp.25053.

Rong, Y. S., & Golic, K. G. (2003). The homologous chromosome is an effective template for the repair of mitotic DNA double-strand breaks in Drosophila. *Genetics*, *165*(4), 1831–1842.

Sekelsky, J. (2017). DNA repair in Drosophila: Mutagens, models, and missing genes. *Genetics*, *205*(2), 471–490. https://doi.org/10.1534/genetics.116.186759.

Seol, J. H., Shim, E. Y., & Lee, S. E. (2017). Microhomology-mediated end joining: Good, bad and ugly. *Mutation Research*, *17*, 30041–30046. https://doi.org/10.1016/j.mrfmmm.2017.07.002.

Sfeir, A., & Symington, L. S. (2015). Microhomology-mediated end joining: A back-up survival mechanism or dedicated pathway? *Trends in Biochemical Sciences*, *40*(11), 701–714. https://doi.org/10.1016/j.tibs.2015.08.006.

Sharma, S., Javadekar, S. M., Pandey, M., Srivastava, M., Kumari, R., & Raghavan, S. C. (2015). Homology and enzymatic requirements of microhomology-dependent alternative end joining. *Cell Death & Disease*, *6*, e1697.https://doi.org/10.1038/cddis.2015.58.

Shrivastav, M., De Haro, L. P., & Nickoloff, J. A. (2008). Regulation of DNA double-strand break repair pathway choice. *Cell Research*, *18*(1), 134–147. https://doi.org/10.1038/cr.2007.111.

Soni, A., Siemann, M., Pantelias, G. E., & Iliakis, G. (2015). Marked contribution of alternative end-joining to chromosome-translocation-formation by stochastically induced DNA double-strand-breaks in G2-phase human cells. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis*, *793*, 2–8. https://doi.org/10.1016/j.mrgentox.2015.07.002.

Truong, L. N., Li, Y., Shi, L. Z., Hwang, P. Y., He, J., Wang, H., et al. (2013). Microhomology-mediated end joining and homologous recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(19), 7720–7725. https://doi.org/10.1073/pnas.1213431110.

Wood, R. D., & Doublie, S. (2016). DNA polymerase theta (POLQ), double-strand break repair, and cancer. *DNA Repair (Amst)*, *44*, 22–32. https://doi.org/10.1016/j.dnarep.2016.05.003.

Yu, A. M., & McVey, M. (2010). Synthesis-dependent microhomology-mediated end joining accounts for multiple types of repair junctions. *Nucleic Acids Research*, *38*(17), 5706–5717. https://doi.org/10.1093/nar/gkq379.