Chapter 27

I-SceI-Based Assays to Examine Distinct Repair Outcomes of Mammalian Chromosomal Double Strand Breaks

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Abstract

Chromosomal double strand breaks (DSBs) can be repaired by a number of mechanisms that result in diverse genetic outcomes. To examine distinct outcomes of chromosomal DSB repair, a panel of human cell lines has been developed that contain *GFP*-based reporters with recognition sites for the rare-cutting endonuclease I-SceI. One set of reporters is used to measure DSB repair events that require access to homology: homology-directed repair, homology-directed repair that requires the removal of a nonhomologous insertion, single strand annealing, and alternative end joining. An additional reporter (EJ5-GFP) is used to measure end joining (EJ) between distal DSB ends of two tandem I-SceI sites. These Distal-EJ events do not require access to homology, and thus are distinct from the repair events described above. Indeed, this assay provides a measure of DSB end protection during EJ, via physical analysis of Distal-EJ products to determine the frequency of I-SceI-restoration. The EJ5-GFP reporter can also be adapted to examine EJ of non-cohesive DSB ends, using co-expression of I-SceI with a non-processive 3′ exonuclease (Trex2), which can cause partial degradation of the 4 nucleotide 3′ cohesive overhangs generated by I-SceI. Such co-expression of I-SceI and Trex2 leads to measurable I-SceI-resistant EJ products that use proximal DSB ends (Proximal-EJ), as well as distal DSB ends (Distal-EJ). Therefore, this co-expression approach can be used to examine the relative frequency of Proximal-EJ versus Distal-EJ, and hence provide a measure of the fidelity of end utilization during repair of multiple DSBs. In this report, the repair outcomes examined by each reporter are described, along with methods for cell culture, transient expression of I-SceI and Trex2, and repair product analysis.

Key words: I-SceI, End joining, NHEJ, alt-EJ, Homologous recombination, Double strand break

1. Introduction

Efficient repair of chromosomal double strand breaks (DSBs) is critical for genome stability and promotes cellular resistance to clastogenic cancer therapeutics (1). Understanding the factors and pathways that influence the efficiency and fidelity of DSB repair will provide insight into cancer etiology and therapeutics. Here, methods are described for a series of reporter cell lines to quantify

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the relative frequency of diverse repair outcomes with distinct mutagenic consequences.

A panel of human cell lines (HEK293 and U2OS) has been generated, each of which harbors a chromosomally integrated copy of an individual reporter. Each reporter contains an inactive expression cassette for green fluorescent protein (GFP) that is interrupted by one or more recognition sites for the rare-cutting endonuclease I-SceI (2, 3). I-SceI generates a defined DSB within its 18 base pair (bp) recognition sequence, resulting in DSB ends with 4 nucleotide (nt) 3′ cohesive overhangs (4). The individual reporters are designed such that a defined DSB repair outcome leads to restoration of a *GFP* expression cassette. Accordingly, the *GFP*-marked repair outcome is measured by transiently expressing I-SceI, culturing the cells to allow completion of repair, then determining the percentage of *GFP*+ cells by fluorescence-activated cell sorting (FACS) analysis. The duration of cell culture is determined by the number of days required to achieve maximal repair frequencies, which for these cell lines is 3 days post-transfection (2, 3). Some of the reporter assays also involve physical analysis of repair products using PCR amplification with primers that flank the I-SceI recognition site(s) (2, 3).

Beginning with DR-GFP, DRins-GFP, SA-GFP, and EJ2-GFP, these reporters are designed to examine a series of repair outcomes that utilize homology (Fig. 1a–e). DR-GFP and DRins-GFP are used to quantify two distinct homology-directed repair (HDR) events, whereas SA-GFP and EJ2-GFP are used to measure single strand annealing (SSA), and alternative end joining (alt-EJ), respectively (2, 3, 5, 6). With DR-GFP, an I-SceI-induced DSB in the upstream *SceGFP* cassette, followed by HDR that uses the downstream homologous template (*iGFP*) to prime nascent DNA synthesis, restores the *GFP*+ cassette (5) (Fig. 1a). The derivative reporter, DRins-GFP, includes a nonhomologous 464 bp insertion in the *SceGFP* cassette that is removed during HDR (3) (Fig. 1b). The SA-GFP reporter contains a 5′ *GFP* fragment separated 2.7 kilobases (kb) from a 3′ *GFP* fragment that contains an I-SceI recognition site (6). The two *GFP* fragments share 266 nt of homology that can bridge the I-SceI-induced DSB during SSA, thereby restoring a functional *GFP*+ cassette (Fig. 1c). While this repair outcome could also be caused by HDR with crossing-over (HDR/CO), the low frequency of HDR/CO (7, 8) indicates that these rare events do not significantly contribute to the formation of the *GFP*+ product in SA-GFP. Finally, EJ2-GFP contains an I-SceI site flanked by 8 nt of homology that can bridge the I-SceI-induced DSB during alt-EJ, thereby restoring a functional *GFP*+ cassette, causing a short 35 nt deletion, and generating an XcmI recognition site (2) (Fig. 1d).

The terms HDR, SSA, and alt-EJ are used in this context to describe a defined repair outcome, while also invoking the mechanism

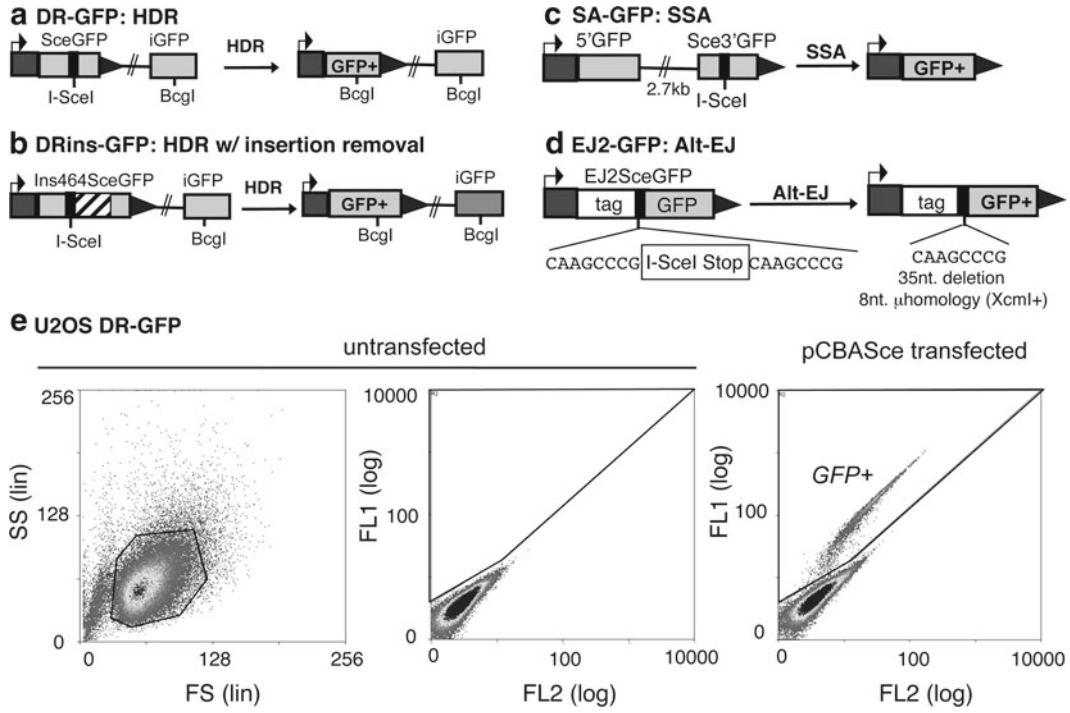

Fig. 1. I-SceI-based reporters for repair outcomes that involve access to homology. (**a**) DR-GFP contains the *SceGFP* cassette that is interrupted by a single I-SceI site, along with a 5′ and 3′ truncated fragment of GFP (*iGFP*). Homology-directed repair (HDR) of the I-SceI-induced DSB, using *iGFP* as the template, can lead to a GFP+/BcgI+ product. (**b**) DRins-GFP is similar to DR-GFP, with the exception of a 464 nt insertion (hatched box within *Ins464SceGFP*) that must be removed during HDR to generate the GFP+/BcgI+ product. (**c**) SA-GFP contains a 5′ fragment of GFP (*5′GFP*), and a 3′ fragment of GFP (*Sce3′GFP*) that contains an I-SceI site. The *GFP* fragments are separated by 2.7 kb and share 266 nt of homology. Repair of the DSB in *Sce3′GFP* by single-strand annealing (SSA) will lead to a *GFP+* product. (**d**) EJ2-GFP contains an expression cassette for a tagged version of *GFP* that is interrupted by an I-SceI site and a series of stop codons, which is flanked by 8 nt of homology. Repair by alternative end joining (alt-EJ) that deletes the stop codons, restores the *GFP* coding frame, and bridges the 8 nt of flanking homology, leads to a *GFP+* cassette that is marked with an XcmI recognition site. (**e**) Representative FACS profiles of the U2OS DR-GFP cell line. Shown (*left*) is a plot of untransfected cells using forward scatter (FS, *x*-axis) and side scatter (SS, *y*-axis), which is used to generate a gate of cells (within polygon). A logarithmic plot of these gated cells is shown (*middle*) using FL2 (orange fluorescence, *x*-axis) and FL1 (green fluorescence, *y*-axis). Also shown (*right*) is an FL2/FL1 plot for pCBASce transfected cells that were gated using a FS/SS plot, as for the untransfected sample. Autofluorescence is detected on the diagonal, where cells showing increased green fluorescence above autofluorescence are gated to determine the percentage of *GFP+* cells. Items in this figure are reproduced from Bennardo et al. (2009) with permission from *PLoS Genetics.*

of repair. Repair outcome alone is not sufficient to assign mechanism; however, these repair outcomes show distinct genetic requirements that allow some degree of mechanistic classification. For instance, the strand exchange factor Rad51 specifically promotes HDR, which is consistent with the unique requirement for HDR of nascent DNA synthesis using a homologous template (9). In contrast, Rad51 suppresses SSA and is dispensable for alt-EJ (2, 6). Also, alt-EJ is independent of Xrcc4 and Ku70 (2, 3, 10, 11), which are two factors integral to the classical nonhomologous end joining (c-NHEJ) pathway that mediates V(D)J recombination

(12). In fact, these two c-NHEJ factors suppress each of the repair events that require access to homology (alt-EJ, SSA, and HDR) (2, 3). Conversely, Nbs1 and CtIP, which are factors that appear important for the initiation of end resection (13–15), promote these repair events (alt-EJ, SSA, and HDR) (2, 3). Finally, SSA and HDR of the DRins-GFP reporter show some distinct genetic requirements (e.g., Ercc1) (3), which likely reflect the greater degree of end processing required for these repair outcomes.

Examining end joining (EJ) events that do not require access to homology is more complex because a major EJ product is likely restoration of the I-SceI site, which is indistinguishable from a site that was never cut. To circumvent this complication, EJ can be measured effectively using linearized plasmid substrates (16). However, such plasmid EJ events might have substantial mechanistic distinctions from repair of DSBs within a chromosome, and do not allow an examination of how correct ends are matched during repair of multiple chromosomal DSBs. As another approach, chromosomal substrates with tandem I-SceI sites allow an examination of these aspects of EJ repair.

One such EJ reporter, EJ5-GFP, contains a promoter that is separated from the rest of a *GFP* expression cassette by a marker gene (*puro*) that is flanked by two tandem I-SceI sites (2) (Fig. 2a). EJ that uses the distal DSB ends (Distal-EJ) restores the *GFP* expression cassette. Because the two I-SceI sites in EJ5-GFP are in the same orientation, one possible Distal-EJ product is the restoration of an I-SceI site using the 3′ cohesive DSB ends. Such I-SceI restoration can be quantified by PCR amplification of the Distal-EJ repair junction of *GFP*+ sorted cells, followed by I-SceI digestion analysis (Fig. 2a, b, primers p1 and p2). Furthermore, the pattern of deletions/insertions during EJ can be examined by subcloning the I-SceI-resistant amplification products for sequence analysis.

Factors that protect DSB ends during EJ are likely important for I-SceI-restoration. Accordingly, the c-NHEJ factors Ku70 and Xrcc4, as well as Brca1, are each important for I-SceI-restoration; whereas Nbs1 is important for loss of the I-SceI site (Fig. 2b) (2, 3, 10, 11). Thus, examining the frequency of I-SceI restoration, and the repair junctions via sequencing analysis, can provide a complementary method to EJ2-GFP for identifying factors or growth conditions that influence the degree of end protection during EJ. Notably, the overall frequency of Distal-EJ using the EJ5-GFP reporter is not substantially affected by genetic loss of Ku70 or Xrcc4 (2, 3, 17). These findings indicate that alt-EJ pathways are sufficient to generate Distal-EJ products with an intact *GFP* expression cassette, albeit with larger deletions. In summary, the EJ5-GFP reporter can be used to examine the frequency of EJ repair that is independent of access to homology, which provides a useful contrast to the other reporters. Furthermore, examining the Distal-EJ repair junctions by PCR analysis can provide insight into the degree of end protection during EJ.

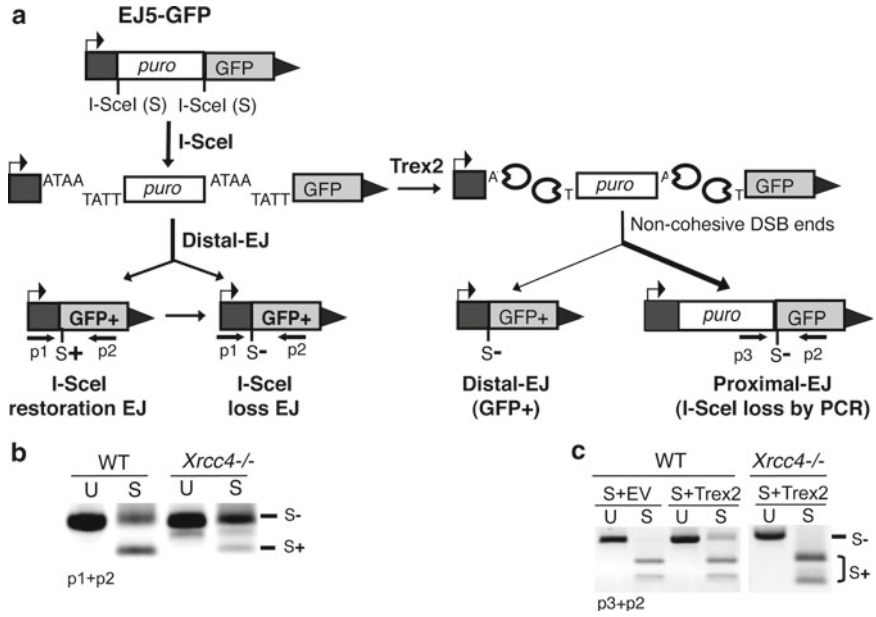

Fig. 2. A reporter for end joining between tandem I-SceI-induced DSBs. (**a**) The EJ5-GFP reporter system for measuring multiple aspects of end joining (EJ). EJ5-GFP contains the pCAGGS promoter separated from the rest of a *GFP* expression cassette by a puromycin selection cassette (*puro*) flanked by I-SceI sites. Shown (*left*) are Distal-EJ events following expression of I-SceI alone, which lead to restoration of the *GFP* cassette. Such Distal-EJ can also restore an I-SceI site if the DSB ends are protected during EJ, which can be quantified by PCR amplification with primers p1 and p2, followed by I-SceI digestion analysis. Also shown (*right*) are EJ events following co-expression of I-SceI and Trex2. Again, Distal-EJ is marked with restoration of the *GFP* cassette, but in this case, all Distal-EJ products show loss of the I-SceI site (S-), due to Trex2 activity. Also, Trex2 expression leads to siginificant loss of the I-SceI site (S-) during Proximal-EJ, which can be quantified by PCR amplification using p3 and p2, and subsequent I-SceI digestion analysis. (**b**) Xrcc4 is important for I-SceI restoration during Distal-EJ. Wild type (WT) and Xrcc4−/− mouse ES cells with the EJ5-GFP reporter were transfected with the I-SceI expression vector, and subsequently *GFP*+ cells (Distal-EJ events) were enriched using FACS sorting. Shown are amplification products from these sorted samples (primers p1/p2) that were uncut (U) or I-SceI digested (S). (**c**) Co-expression of I-SceI and Trex2 leads to significant levels of I-SceI-resistant Proximal-EJ products, which are promoted by Xrcc4. The cell lines described in (**b**) were transfected with I-SceI in conjunction with either a Trex2 expression vector, or empty vector (EV). Shown are Proximal-EJ products that were amplified (p3/p2) and left uncut (U) or digested with I-SceI (S). Items in this figure are reproduced from Bennardo et al. (2009), Bennardo and Stark (2010), with permission from *PLoS Genetics*.

Finally, the EJ5-GFP reporter can also be used to examine EJ outcomes that use correct DSB ends versus incorrect DSB ends (Proximal-EJ and Distal-EJ, respectively) (17). Proximal-EJ is difficult to measure with I-SceI expression alone because EJ that restores the I-SceI site cannot be differentiated from the uncut reporter. To address this limitation, the EJ5-GFP reporter system has been adapted by co-expressing I-SceI with a non-processive 3′ exonuclease (Trex2) (18, 19), which can cause partial degradation of the 4 nt 3′ cohesive overhangs generated by I-SceI (3, 17). Co-expression of I-SceI with Trex2 leads to a high level of I-SceI-resistant Proximal-EJ products that show short deletions of the I-SceI overhang region (3). Thus, Proximal-EJ that leads to loss of the I-SceI site can be quantified by PCR amplification and subsequent I-SceI digestion analysis (Fig. 2a, c, primers p2 and p3).

Notably, the I-SceI-resistant Proximal-EJ products that result from I-SceI/Trex2 co-expression require a number of c-NHEJ factors (Fig. 2c) (3, 17), which is consistent with the role of this pathway during EJ repair of non-cohesive DSB ends (12). Furthermore, the Distal-EJ products resulting from co-expression of I-SceI and Trex2 are also completely I-SceI-resistant (3, 17). Thus, this co-expression approach can be used to measure the frequency of two different I-SceI-resistant products from a single sample: Proximal-EJ that uses correct DSB ends, and Distal-EJ that uses incorrect DSB ends (Fig. 2a). As an example using this method, cells deficient in either of two DNA damage response factors (ATM or Nbs1) show elevated levels of Distal-EJ, but not Proximal-EJ, indicating that these factors are important for the fidelity of end utilization during repair of multiple DSBs (17). In summary, methods are presented for using a panel of I-SceI-based reporters to examine a number of DSB repair outcomes with diverse mutagenic consequences.

2. Materials

2.1. Plasmids and Primer Sequences

1. Expression vectors: I-SceI (pCAGGS-I-SceI, called pCBASce), Trex2 (pCAGGS-Trex2), GFP (pCAGGS-NZEGFP), and empty vector (pCAGGS-BSKX) (3, 5). Each vector contains an ampicilin-resistant gene, and can be amplified in *E. coli* using Circlegrow with ampicilin, purified using a Qiagen QiaFilter plasmid preparation, and dissolved in sterile TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0).
2. Primer sequences for examining EJ5-GFP repair products. Distal-EJ product analysis: p1 (KNDRF) 5′-CTGCTAACCAT GTTCATGCC, p2 (KNDRR) 5′-AAGTCGTGCTGCTTCAT GTG (2, 20). Proximal-EJ product analysis (3′ I-SceI site): p3 (EJ5PurF) 5′-AGCGGATCGAAATTGATGAT and p2 (3).

2.2. Cell Lines

1. A panel of HEK293 cell lines (clone HEK293A7 from New England Biolabs), each with an integrated reporter (DR-GFP, SA-GFP, EJ2-GFP, or EJ5-GFP), were described previously (2).
2. A panel of U2OS cell lines (HTB-96, ATCC, Manassas), each with an integrated reporter (DR-GFP, DRins-GFP, SA-GFP, EJ2-GFP, or EJ5-GFP), were generated using the same integration protocol as for the HEK293 cells (2).

2.3. Growth Media, Transfection, and Fixation

1. Growth medium: 500 ml DMEM high glucose with L-glutamine, 55 ml fetal bovine serum, and 6 ml Pen/Strep solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycin). Including 8 μg/ml plasmocin and 2 μg/ml puromycin can promote cell line stability during long-term culturing. For

antibiotic-free medium, omit Pen/Strep and plasmocin. For the entire transfection experiment, omit puromycin.

2. Freezing medium: 50 % Growth media, 40 % fetal bovine serum, 10 % dimethylsulfoxide.
3. Trypsin: 1× Trypsin-EDTA in Hanks Salts.
4. Poly-lysine: 0.01 % Solution.
5. Transfection reagents: Lipofectamine 2000, RNAiMAX, and Optimem without phenol red (Invitrogen).
6. Formaldehyde solution: 10 % Formaldehyde (w/v in aqueous phosphate buffer).
7. Sterile tissue culture plates: 10 cm plate, 2 cm^2 vessel (typical well of a 24-well plate), 4 cm^2 vessel (typical well of a 12-well plate).

2.4. Physical Analysis of I-SceI-Resistant Repair Products

1. P-lysis buffer: 20 mM Tris pH 8.0, 85 mM NaCl, 15 mM EDTA, 0.5 % SDS.
2. RNase: DNase-free RNase from bovine pancrease, 500 μg/ml.
3. Proteinase K: Recombinant proteinase K, 14–22 mg/ml, PCR grade.
4. Phenol: Saturated phenol pH 6.6 ± 0.2.
5. Chloroform.
6. 70 % Ethanol in water.
7. Isopropanol.
8. 0.1× TE: 1 mM Tris pH 8, 0.1 mM EDTA pH 8.
9. PCR SuperMix High Fidelity (Invitrogen).
10. Column for PCR purification: Illustra GFX PCR DNA and Gel Band Purification Kit (GE).
11. I-SceI enzyme and incubation buffers (New England Biolabs).
12. Agarose gel: Low EEO agarose, 1× TBE gel running buffer (90 mM Tris-borate, 2 mM EDTA).
13. Loading buffer: 15 % Ficoll in gel running buffer, 30 % glycerol, 0.01 % bromophenol blue, 50 mM EDTA.

3. Methods

3.1. Culturing HEK293 and U2OS Cells, and siRNA Transfection

1. U2OS cells are grown as adherent cells directly on tissue culture plastic, using growth medium, in a tissue culture incubator (37 °C and 5 % CO_2). HEK293 cells adhere more efficiently if plates are first coated with Poly-lysine: add 1–3 ml per 10 cm plate to coat the growth surface and remove prior to adding growth medium. Poly-lysine solution can be reused to coat several plates.

2. Keep stocks of cells between 20 and 80 % confluent. To passage, remove growth medium, add 1 ml of trypsin per 10 cm plate to cover the growth surface, wait 3–5 min, dilute with growth medium, and add to a fresh plate with growth medium.
3. For shRNA-mediated disruption of individual factors in HEK293 cell lines, infect with lentiviral shRNA expression cassettes prior to I-SceI transfection. Such infection is beyond the scope of this review.
4. For use of siRNA in U2OS cells, treat with siRNA 48 h prior to I-SceI transfection. Reverse transfect the U2OS cells by plating 1×10^5 cells in antibiotic-free media per 2 cm^2 culture vessel that already contains preformed siRNA transfection complexes (e.g., 10 pmol siRNA duplex mixed with 1.8 μl RNAiMAX in 100 μl Optimem for 25 min). Let the transfection proceed for 20 h and passage to 4 cm^2 cutlure vessel (see Note 1). 48 h after the siRNA transfection, transfect with I-SceI (or I-SceI and Trex2) with 10 pmol of siRNA duplex (see below).

3.2. Transient I-SceI Expression with or Without Trex2

1. 24 h pre-transfection, plate 1×10^5 cells per 4 cm^2 culture vessel using 2 ml growth medium. Disperse the cells thorougly by pipetting, add the cells to the growth medium in the culture vessel, then spread the cells by shaking the plate gently and repeatedly (see Note 1).
2. The day of transfection, aspirate the medium, wash with 1 ml antibiotic-free medium or PBS, and add 1 ml fresh antibiotic-free medium. Incubate for 2 h before adding transfection complexes.
3. Prepare transfection complexes. In one tube, dilute nucleic acid into 100 μl Optimem. Suggested: 0.8 μg of I-SceI expression vector along with 10 pmol siRNA and/or 0.4 μg secondary expression vector (e.g., Trex2 expression vector, or internal transfection control plasmid, see Note 2). In a second tube, dilute 3.6 μl Lipofectamine 2000 into 100 μl Optimem, and mix by pipetting. Invert Lipofectamine 2000 several times before use, and do not vortex any of the above solutions. Let diluted Lipofectamine sit for 5 min at room temperature. Next, add diluted Lipofectamine 2000 to the diluted nucleic acid, mix by pipetting, and incubate for 25 min at room temperature. Add 200 μl of transfection complexes to the cells in the 4 cm^2 culture vessel, without changing the media. Shake the plate back and forth several times to mix, and return to the tissue culture incubator for 3 h. Aspirate off transfection complexes, add 1 ml of antibiotic-free medium, shake gently and aspirate to wash off residual transfection reagent. Add 4 ml growth medium (containing antibiotics) and culture for 3 days (approximately 72 h) prior to end-point analysis (see Note 2).

4. Chemical treatment. To address the effect of a chemical on these assays, add the compound right after transfection, at the 3 h media change. Subsequently, leave the cells alone until preparation for FACS (3 days). Removal of the transfection reagents prior to adding the chemical is likely important to avoid transfection of the chemical, which could cause high intracellular levels of the compound.

3.3. FACS Analysis

1. Three days (approximately 72 h) after transfection, analyze the cells by FACS.
2. Aspirate medium from each of the transfected wells, and add 200 μl trypsin per well. Incubate at room temperature 3–5 min. After incubation, add 200 μl growth medium to each transfected well and disperse cells by pipetting.
3. Immediatedly after dispersing the trypsinized sample, mix 2:1 with 10 % formaldehyde (e.g., 400 μl sample added to 200 μl 10 % formaldehyde). Vortex immediately for 2–3 s at medium speed. Samples are ready for FACS analysis, and are stable up to 4 h at room temperature or on ice (see Note 3).
4. FACS analysis. Use a plot of forward scatter versus side scatter to gate for events that are consistent with individual cells. Analyze these gated cells in a plot for green fluorescence (FL1) on the *y*-axis, and orange or red fluorescence (FL2 or FL3) on the *x*-axis. This plot allows a distinction between cells with high autofluorescence versus green fluorescent cells. Namely, cells expressing GFP show an increase in the FL1 signal above autofluorescence (Fig. 1e).

3.4. PCR Analysis to Examine I-SceI-Resistant Products

1. Cell lysate for genomic DNA (gDNA). For Proximal-EJ analysis, on the day of FACS, trypsinize the cells as described above, use half the sample for fixation and FACS, and use the other half for gDNA extraction. For Distal-EJ analysis, sort live *GFP*+ cells using FACS, culture to expand the population of sorted cells, and collect approximately 10^6 cells using trypsinization. Spin cells at 150 × *g* for 5 min to pellet the cells, pour off the aqueous, and add 500 μl PBS. Repeat spin and pour off aqueous, keeping approximately 50 μl of the PBS. Vortex to disperse pellet, then add 500 μl P-lysis buffer. Samples are stable in this buffer for at least 1 month at room temperature.
2. Preparing gDNA via phenol/chloroform extraction. Add 1 μl RNase to the sample in 500 μl P-lysate buffer, invert 25×, and incubate 5 min at room temperature. Next, add 5 μl proteinase K, invert 25×, and incubate at room temperature for 1 h. Add 30 μl 5 M NaCl. Add 500 μl saturated phenol, shake tubes gently, but thoroughly, for 10 min at room temperature, then centrifuge at 10,000 × *g* for 5 min. Transfer the aqueous (top) layer to a fresh microcentrifuge tube and repeat this step twice

more, for a total of three phenol extractions. Extract once using 500 µl chloroform, using the same procedure as the phenol extraction. Transfer the aqueous layer to a fresh microcentrifuge tube, and add 500 µl isopropanol to precipitate gDNA. Shake for 10 min and centrifuge at 10,000 × *g* for 5 min. After this spin, there should be a visible pellet at the bottom of the tube. Carefully aspirate without disturbing the pellet, and add 1 ml 70 % ethanol. Spin again at 10,000 × *g* for 5 min, and aspirate the ethanol carefully before air-drying the pellet (≤10 min). Add 50 µl 0.1× TE to the pellet, and heat samples at 55 °C for 30–60 min with regular vortexing to resuspend the pellet. Store at 4 °C, but reheat at 55 °C for 30 min prior to PCR.

3. PCR conditions. Analysis of Distal-EJ junctions from *GFP*+ sorted cells can be performed with p1 and p2. Analysis of 3′ Proximal-EJ can be performed with p3 and p2. Create a Primer Mix with each primer at 10 µM in sterile water. Next, add 1.5 µl Primer Mix and 1 µl gDNA (approximately 200 ng) to 22.5 µl PCR HiFi Supermix. Thermocycler conditions: 94 °C 3 min, cycle 32 times (94 °C 45 s, 63 °C 45 s, 68 °C 1 min 45 s), 68 °C 7 min, store at 4 °C.
4. Product purification and quantitative I-SceI digestion. Column purify PCR product prior to analysis according to manufacturer's instructions. Elute purified PCR product in 22 µl 10 mM Tris pH 8. Add 3 µl 10× I-SceI buffer, 3 µl 10× BSA, and 1 µl I-SceI enzyme (5 U/µl), then incubate at 37 °C for 1 h. Add an additional 1 µl I-SceI and incubate for a second hour, and repeat for a total of 3 µl I-SceI and 3 h. Add 5 µl loading buffer to digested samples, and run samples on a 2 % agarose gel for 120 min at 100 V. Stain the gel evenly with 0.5 µg/ml ethidium bromide in gel running buffer for 15 min prior to capturing image.
5. Determine the percentage of I-SceI-resistant products by quantifying the relative intensity of the I-SceI-resistant and I-SceI-sensitive products within the same sample (see Note 4). The Distal-EJ product that restores the I-SceI site (EJ5-GFP, p1 and p2, Fig. 2a, b) is 380 bp, and is digested by I-SceI into two products 281 and 99 bp (calculated from the middle of the 3′ overhang generated by I-SceI). Determine the relative staining intensity values for the 380 and 281 bp fragments using gel imaging software. To account for the smaller size of the 281 bp fragment, multiply the staining value by 1.35 (normalized 281 bp fragment). Divide the value of the 380 bp fragment by the sum of the values for the 380 bp and normalized 281 bp fragment, and multiply by 100 to determine the percentage of I-SceI site loss. Similarly, the Proximal-EJ product that restores the I-SceI site (p1 and p3, Fig. 2a, c) is 748 bp,

and is digested by I-SceI into two products, 467 and 281 bp (calculated as above). In this case, determine the relative staining intensity values for the 748 and 467 bp fragments, and multiply the value of the 467 bp fragment by 1.6 to generate the normalized 467 bp fragment. Divide the value of the 748 bp fragment by the sum of the values for the 748 bp and normalized 467 bp fragment, and multiply by 100 to determine the percentage of I-SceI-resistant Proximal-EJ products.

3.5. Analysis of Repair Junctions

1. The I-SceI-resistant PCR products can be isolated from the gel for sequence analysis using the same kit as for purification of the PCR product. These isolated fragments can be used directly for ligation into a cloning/sequencing vector (e.g., TA cloning vector pCR2.1, Invitrogen), according to the manufacturer's instructions. Incubation of the purified fragments with polymerase immediately before ligation (e.g., 5 μl purified fragment and 20 μl PCR HiFi Supermix, 68 °C 20 min) can substantially improve ligation efficiency.

4. Notes

1. Even dispersal of the cells. Suggested cell densities are based on even dispersal of the cells to allow sufficient surface area for growth during the experiment. At a minimum, gently shake each plate back and forth, and then side to side, a dozen times each. Optimally, return the plate to the incubator and allow the cells to settle for 10 min, and subsequently remove for another set of shaking. Repeat the incubation and shaking an additional three times.
2. Controlling for transfection efficiency. Since the frequency of any repair event is directly affected by the efficiency of I-SceI-induced DSBs, as well as the survival of transfected cells, it is important to control for variations in these parameters. For example, it is important to determine whether a change in the frequency of *GFP*+ cells using EJ2-GFP reporter, following treatment with a specific RNAi reagent, is due to an effect on alt-EJ repair, rather than I-SceI activity and/or survival after transfection. Parallel transfections using a GFP expression vector (e.g., pCAGGS-NZEGFP), or co-expressing another fluorescent marker with I-SceI (e.g., co-transfection of expression cassettes for mCHERRY or dsRED), can control for such alterations in transfection efficiency or survival. Since transfection efficiency with these cell lines show very little variation between replicates, a parallel transfection with the GFP

expression vector is usually sufficient. Alternatively, the I-SceI protein expressed from pCBASce includes a hemagglutinin (HA) tag, which can be used to quantify expression levels by immunoblotting with commercially available anti-HA antibodies. In addition to the above transfection controls, focusing on RNAi reagents, chemicals, or cellular growth conditions that differentially affect a subset of repair outcomes is suggested. For example, a reagent that causes a decrease in the frequency of *GFP*+ cells with the DR-GFP reporter, but not with the EJ5-GFP reporter, cannot be readily explained by changes in transfection or I-SceI activity.

3. Formaldehyde fixation before FACS. Analysis can also be performed on live cells resuspended in media without fixation, though cells can eventually start to aggregate. Accoringly, fixation is suggested immediately after trypsinization and resuspension to avoid aggregation. Should *GFP*+ cells be absent in the analysis, ensure that the correct concentration of formaldehyde is being used (10 %, w/v, in PBS stock solution, 3 % final). To analyze fixed cells more than 4 h after fixation and up to 2 days later, wash out the fixative. Specifically, incubate the cells in fixative for 30 min, spin at 150 × *g* for 5 min to pellet the cells, remove the fixative, and resuspend in 500 μl PBS. Repeat spin and pour off aqueous, resuspend in 500 μl PBS, and store at 4 °C in the dark.
4. Quantitative I-SceI digestion. Be sure to include a control for complete cutting by I-SceI, usually gDNA from untransfected cells. For the analysis of Distal-EJ products (p2 and p3), use untransfected cells and the Proximal-EJ primers (p1 and p2). Ensure that the amount of PCR amplification product from the control digestion is equivalent or greater than the transfected samples. If the control sample is not completely digested, digest a smaller amount of the PCR amplification products, and ensure that each addition of I-SceI enzyme includes pipetting to mix thoroughly. These assays involve quantifying the relative ratio of two products within the same sample, which remains consistent during amplification. In any case, defined mixtures of control and transfected cells can be used to ensure the assay is quantitative within twofold (5, 17).

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