Published in final edited form as:

Nat Protoc. 2014 October ; 9(10): 2476–2492. doi:10.1038/nprot.2014.167.

Generation of cell-based systems to visualize chromosome damage and translocations in living cells

Vassilis Roukos, Rebecca C Burgess, and Tom Misteli

National Cancer Institute, US National Institutes of Health, Bethesda, Maryland, USA.

Abstract

Traditional methods for the generation of DNA damage are not well suited for the observation of spatiotemporal aspects of damaged chromosomal loci. We describe a protocol for the derivation of a cellular system to induce and to visualize chromosome damage at specific sites of the mammalian genome in living cells. The system is based on the stable integration of endonuclease I-SceI recognition sites flanked by bacterial *LacO/TetO* operator arrays, coupled with retroviral-mediated integration of their fluorescent repressors (LacR/TetR) to visualize the *LacO/TetO* sites. Expression of the I-SceI endonuclease induces double-strand breaks (DSBs) specifically at the sites of integration, and it permits the dynamics of damaged chromatin to be followed by time-lapse microscopy. Sequential LacO-I-SceI/TetO-I-SceI integrations in multiple chromosomes permit the generation of a system to visualize the formation of chromosome translocations in living cells. This protocol requires intermediate cell culture and molecular biology skills, and it is adaptable to the efficient derivation of any integrated clonal reporter system of interest in ~3–5 months.

INTRODUCTION

Maintaining the integrity of genetic information is essential for the survival of cells. Mechanisms that counteract DNA damage ensure cellular homeostasis, suppress mutagenic events and prevent genome rearrangements that may lead to disease¹. Recent reports have highlighted the role of higher-order chromatin structure, chromatin dynamics and the nonrandom organization of the genome in the maintenance of genomic integrity^{2–4}. These studies explored the biological implications of chromatin dynamics by following intact and damaged chromatin in living cells. Most currently available methodologies used for these studies are indirect and involve tracking of repair foci formed by fluorescently tagged repair proteins after DNA damage^{5–9}, the incorporation of labeled deoxy-NTP (dNTP) analogs during replication^{10–12} or the expression of core histones tagged with photoactivatable fluorescent proteins after laser-induced DNA damage¹³. Although considerable insights into repair dynamics have emerged from these studies, these methods are limited in their ability

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

Correspondence should be addressed to T.M. (mistelit@mail.nih.gov) or V.R. (roukosv@mail.nih.gov).

AUTHOR CONTRIBUTIONS V.R. developed the protocol. V.R. and R.C.B. wrote the manuscript. T.M. supervised the study and edited the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper

to probe the dynamics of specific gene loci or damaged chromosome sites in the cell nucleus.

Here we present a protocol for the generation of a cell-based system that can be used to induce and to visualize DSBs in specific chromosomal sites in mammalian cells for the exploration of dynamics in various chromatin states and genomic environments. The approach is based on the generation of cell lines that contain, stably integrated into their genomes, the 18-nt recognition site for the yeast endonuclease I-SceI, which is not present in mammalian cells. The I-SceI site serves as a means to induce a DSB in a controlled manner by the introduction of the I-SceI restriction enzyme by exogenous expression. The I-SceI site is flanked by bacterial *LacO/TetO* operator array sequences, which serve to visualize the chromosome ends after *in vivo* cutting with I-SceI (Fig. 1). The DNA arrays can be visualized as discrete dots owing to the binding of fluorescently tagged LacR and/or TetR repressor proteins to their cognate *LacO* and *TetO* arrays^{14,15}.

The protocol describes the preparation of *LacO/TetO* repeat-containing plasmids and the generation of cell lines that carry stably integrated *LacO/TetO* repeats (Steps 1–36). Procedures are described for effective integration and expression of the fluorescent repressors, which require the generation and selection of optimized cell lines for microscopy using bicistronic retroviral vectors (Steps 37–56). The protocol also provides guidelines for the controlled and efficient formation of DSBs at specific chromosome sites by transient expression of the endonuclease (Steps 57–76). The visualization and tracking of chromosome ends in space and in time is then possible by time-lapse fluorescence microscopy (Steps 77–86). We have successfully used the protocol outlined here to generate cell-based systems to probe the dynamics of chromosome ends and to visualize the formation of chromosome translocations in living mammalian cells^{16,17}.

Applications of the protocol

Integration of endonuclease sites flanked by the Lac or Tet operator/ repressor system has been the basis of several models to probe the spatial dynamics of DNA repair in the eukaryotic nucleus and to visualize genomic loci in yeast, fly, *Caenorhabditis elegans* and mammalian cells^{17–26}. For instance, tracking the chromosome ends flanking an engineered DSB suggested that broken ends are positionally stable in mammalian cells¹⁷. This strategy is also particularly useful for the analysis of the motion properties of specific intact and damaged loci⁴. Moreover, it was recently used to generate a cellular system in which distinct DSBs marked by Lac and Tet operator/repressors on different chromosomes were tracked to reveal the spatial dynamics of chromosome translocations in living cells by high-throughput microscopy¹⁶. Similarly, in yeast *Saccharomyces cerevisiae*, engineered integrations of *LacO* and *TetO* operator arrays in the vicinity of an inducible DSB and on the corresponding unbroken site on the homologous chromosome have been used to detect increased motion of yeast chromosome breaks engaged in homology search for recombinational repair partners^{27,28}.

This protocol outlines the steps in the generation of cell lines to visualize chromosome breaks, but the approach is readily adaptable to all applications that require visualization of chromosomal loci within the mammalian genome^{29–31} or the integration of any fluorescent

reporter of interest^{32–35}. Furthermore, this approach can be used to specifically target and tether proteins of interest (expressed as chimeric fusions with the LacR/TetR repressors) to operator sites integrated in different genomic locations with different chromatin characteristics^{36–39}. This methodology has been successfully used to target repair factors to chromatin, and it has revealed the hierarchy of protein interactions within the DNA repair cascade and suggested the presence of amplification signaling loops^{36,37}. Similarly, targeting of the VP16 acidic activation domain via the *LacO/LacR* system has been shown to result in transcriptional activation and re-distribution/expansion of chromatin^{32,40}, whereas HP1 tethering to chromatin has been shown to cause condensation⁴¹. Further applications are the *de novo* creation of nuclear bodies^{42,43}, or the *in vivo* testing of protein-protein interactions^{44,45}. Recently, this approach was used to tether the FokI endonuclease to a *LacO* operator array to induce multiple DSBs within the locus and reveal that DNA damage silences transcription in *cis*³⁴.

Limitations of the protocol

Control of I-SceI expression.—In the described protocol, DSBs are induced after transfection of the endonuclease I-SceI by nucleofection. Although transient transfection of I-SceI induces DSBs with high efficiency, the downside of this approach is the uncertainty of the timing of DSB induction because of the nonsynchronized expression of the I-SceI protein, thus limiting its usefulness for kinetic studies that require the coordinated formation of DSBs. This limitation can be overcome by the expression of I-SceI fused to portions of the glucocorticoid receptor (GR-I-SceI), which retains the fusion protein in the cytoplasm until it is stimulated with glucocorticoid receptor-binding steroid hormone. Upon hormone binding, the fusion translocates rapidly into the nucleus, which results in the synchronized induction of breaks in a time-controlled manner¹⁷. Once it is induced, however, the enzyme is constitutively active, and it continues cutting intact sites as soon as they are repaired, thus making tracking of repair kinetics difficult. This limitation may eventually be overcome by the expression of I-SceI as a chimera with a degron, allowing its controlled degradation⁴⁶.

Insertion site location.—This protocol describes the steps for random integrations of the array plasmids into the genome, which precludes analysis of preselected genomic sites. However, the location of integration sites may be controlled by the use of established gene-targeting methods using sequence-specific targeted cleavage events such as transcription activator-like effector nucleases⁴⁷ and clustered regularly interspaced short palindromic repeats-associated nucleases (CRISPR)⁴⁸. These methods enable the targeted formation of DSBs at defined genome sites of interest, substantially increasing the efficiency of recombination^{47,48}.

Array size.—The manipulation of the *LacO/TetO* long repeated sequences requires diligence due to their intrinsic susceptibility to recombination and instability¹⁸. We provide here important information for the development and propagation of repeat-containing plasmids of up to ~20 kb in size. Larger transgene arrays with long genomic DNA inserts require different cloning strategies and manipulations using bacterial artificial chromosomes⁴⁹.

Imaging requirements.—We provide an outline of the steps required for visualization of the broken chromosome ends by high-throughput time-lapse microscopy. We describe a protocol using a high-end imaging system (Perkin-Elmer OPERA), and although automated imaging is required to capture the rare events of chromosome translocations in living cells¹⁶, less advanced instruments equipped with an environmentally controlled chamber and a typical epifluorescence unit or confocal setup are sufficient to follow DSBs in time and sufficient for many other applications of tagged genomic loci. The motion dynamics of DSBs or intact chromosome loci can then be assessed by calculating the mean squared displacement, as described in detail elsewhere²².

Comparison with other methods

Other methods to induce DNA damage and to visualize spatial dynamics of DNA repair include irradiation with alpha-particles^{6,50}, UV damage⁵¹, laser-induced damage^{52–55}, radiomimetic drugs^{56,57} or gamma-irradiation^{5,58–60}. In contrast to the protocol described here, those methods produce damage indiscriminately throughout the genome, which complicates the assessment of the effects of spatial genome organization and the local chromatin environment in the DNA damage response. Although DSBs can be induced in a recurrent and site-specific manner by taking advantage of the presence of endogenous I-PpoI⁶¹ or the AsiSI⁶² restriction sites after the expression of the respective endonucleases in mammalian cells, it is currently technically impossible to mark and track multiple distinct chromosome loci in living cells using these approaches. The high-affinity binding of the LacR and TetR to their cognate operators ($K_d^{\text{Lac}} \sim 10$ pM; ref. 63) allows highly specific detection, and it enables the efficient visualization of single integrations even when live-cell imaging for several hours is required. For example, time-lapse imaging for up to 24 h was recently used to track the motion paths of individual DSBs marked by *LacO* and *TetO* arrays toward the formation of chromosome translocations in living cells¹⁶. This protocol specifically details the steps that are required for optimized repressor expression and maximum detection rate.

Recent studies have successfully applied transcription activator-like effector nuclease and CRISPR/Cas9 technologies to visualize endogenous repeated genomic sequences^{64,65}. Visualization of endogenous repetitive loci by these methods coupled with the induction of DSBs in the same loci could in theory be used to assess the spatial dynamics of endogenous gene loci bearing repetitive sequences. Furthermore, using modifications of the CRISPR system and arrays of single guide RNAs (sgRNAs) tiled along a specific chromosomal target locus, recent work enabled the visualization of nonrepetitive genomic sequences for the first time, allowing for the possibility of visualizing any genome location in living cells⁶⁴. Although these tools have tremendous potential, they have not been applied to the study of DNA damage, and the current detection sensitivity is insufficient for live-cell imaging⁶⁴.

Experimental design

Manipulation of plasmids containing repeated Lac and/or Tet operator (*LacO*/*TetO*) sequences adjacent to the I-SceI endonuclease site.—The propagation and manipulation of long repeated array sequences requires careful handling of the plasmids carrying the repeats owing to the high instability of the repeated sequences during

replication in *Escherichia coli*. We have successfully used Stbl2 competent cells to transform plasmids containing array sequences. The instability of the plasmids is minimized by propagating bacteria at 30 °C and by selecting smaller-sized colonies from LB agar plates. As high yields of these plasmids are required for ensuing steps in the protocol, large-scale plasmid purification must be achieved. We have noticed increased instability of these constructs during large-scale preparations, and thus highly recommended testing the integrity of the propagated plasmid by restriction fragment analysis at each step (Fig. 2). Alternatively, multiple smaller preps (minipreps) that have been verified by restriction analysis may be pooled together to achieve sufficiently large quantities of DNA. Standard commercial DNA plasmid preparation kits can be used to extract the plasmid DNA, and typically a minimum of 20 µg of each plasmid is required for the subsequent protocol steps. If additional cloning steps are required (e.g., addition of a reporter assay cassette and so on), it is highly recommended to use the plasmids containing the repeated sequences as the template to insert the sequence of interest, as we have empirically found that this decreases the probability of array recombination after transformation of the ligation products.

Generation and selection of stable cell lines with *LacO/TetO* integrated arrays.

—To facilitate the identification and selection of clones with integrations of the *LacO/TetO* I-SceI plasmids, we cotransfect the cells of interest with a second plasmid encoding a gene conferring resistance to an antibiotic (pSV40-Zeocin/ pTRE2-hygromycin, respectively). To increase the percentage of cells bearing integrations of both the array plasmid and the selectable marker, cells are transfected with the respective plasmids at a 10:1 ratio, both linearized to increase plasmid integration efficiency. It is essential to carefully titrate the antibiotic dose for each cell line to determine the minimum lethal dose. For plasmid delivery, we routinely use electroporation-based methods (Amaza nucleofection) or standard cationic lipid-based methods. By using the former, ~10–20% resistant clones that are positive for *LacO/TetO* integrations can typically be isolated, but this may substantially vary depending on the cell line, plasmids and selection drugs. Optimal plating density of the transfected cells in the presence of the selection drug will ensure the formation and isolation of monoclonal cell lines (Supplementary Fig. 1). The integration of *LacO/TetO* plasmids can be confirmed by the transient expression of fluorescently tagged LacR/TetR repressors using conventional cationic lipid-based methods to deliver the plasmids. Positive clones can then be isolated on the basis of the stability, position and number of the integrations after examination under a standard epifluorescence or confocal microscope (Fig. 3a). In case of polyclonal cells (mixed population of cells), replating of cells at a low density may allow isolation of monoclonal lines. Clones of interest are further expanded, and stocks are frozen during early passages. If the integration of different arrays at distinct chromosome sites in the same cell line is desired, sequential transfection and establishment of stable cell lines by repeating the stable cell line production protocol for each integrant is recommended (Fig. 3b).

Stable integration of the fluorescent LacR/TetR repressors to visualize the *LacO/TetO* operators and selection of optimal cell lines.

—To develop cell lines that stably express the LacR and/or TetR repressors, we used bicistronic retroviral expression vectors (pQCXIP and pQCXIN, respectively) that were designed to express the

GFP-LacR and TetR-mCherry along with different selection markers (puromycin and neomycin, respectively; Supplementary Fig. 2a). These plasmids are co-transfected into the packaging cell line Platinum GP (Plat-GP) with a plasmid encoding the viral envelope VSV-G protein. Plat-GP cells stably express the retroviral structure proteins encoded by *gag* and *pol* genes and show high transfectability if they are transfected at optimal confluency (Supplementary Fig. 2b). We routinely achieve high transfection efficiency (70–90%) with the nonliposomal vehicle FuGENE HD. Depending on the investigator's expertise and preference, other transfection methods such as cationic lipid-based methods or calcium phosphate transfection may also be tested⁶⁶. The fluorescent LacR or TetR repressors and the puromycin or neomycin resistance genes are cotranscribed by a cytomegalovirus (CMV) immediate-early promoter as a bicistronic message via an internal ribosome entry site, and thus they confer long-term resistance only to the cells expressing the repressors. The selection process is very efficient, and typically 6 ml of the retroviral vector-containing supernatant produced by the packaging cell line from a 10-cm plate (2 d after transfection) is sufficient to transduce $\sim 1.2 \times 10^6$ NIH3T3 cells plated in three wells of a six-well plate. It is essential to carefully titrate the antibiotics in any cell line that is used to determine the minimum lethal dose. As the strong binding of the LacR and TetR repressors to the respective operators may interfere with cellular functions such as replication and transcription⁶⁷, it is essential that the clones be maintained in the presence of isopropyl β -D-1-thiogalactopyranoside (IPTG) and doxycycline to prevent the binding of the LacR and TetR to their respective operators. Washing out these drugs will then permit the binding of the fluorescent repressors and the detection of the arrays by microscopy (Fig. 4a). In general, low expression of the fluorescent repressors is required for optimal detection of the integrated arrays (Supplementary Fig. 3). Selection of the desired expression levels can be achieved either by flow cytometry or by manually selecting sparsely plated clones showing optimal expression.

Expression of the endonuclease I-SceI to induce DSBs and time-lapse microscopy.

—To induce DSBs specifically at the locations where the I-SceI restriction sites have been integrated, the yeast I-SceI endonuclease can be expressed using different strategies. Transient transfection by electroporation (nucleofection) permits the expression of I-SceI as soon as 4–6 h later, and it can be achieved with high transfection efficiency. Typically, up to 80% of cells express I-SceI after electroporation, as judged by the detection of an immunofluorescence signal 24 h later. We routinely use a version of I-SceI tagged with the hemagglutinin (HA) epitope⁶⁸, which permits the detection by immunofluorescence microscopy of the cells expressing the endonuclease (Fig. 4b). By using this protocol, DSBs can be specifically induced with high efficiency at specific genome sites, as judged by the recruitment of repair factors, such as 53BP1, only in cells expressing the enzyme (Fig. 4b). To follow the dynamics of the formed DSBs, time-lapse fluorescence microscopy can be used. To capture the rare formation of translocations within the mammalian nucleus, we follow individual DSBs over time by high-throughput time-lapse microscopy, acquiring a total of 840 time-lapse movies with 1-h intervals for up to 24 h (ref. 16; Fig. 5).

MATERIALS

REAGENTS

- Cells: this protocol uses mouse NIH3T3 cells, but other cell lines (e.g., human U2OS) have been successfully used to generate cell-based systems to visualize chromosome damage
- Plasmids used in this protocol (Supplementary Table 1)
- Dulbecco's PBS (DPBS, 1×) without calcium or magnesium (Gibco/Life Technologies, cat. no. 14190–144)
- Paraformaldehyde (PFA), 16% (wt/vol) solution (electron microscopy (EM) grade, cat. no. 19208, Electron Microscopy Sciences) ! **CAUTION** PFA is a known human carcinogen. It is toxic by inhalation.
- Triton X-100 (Sigma-Aldrich, T9284)
- Tween-20 (Sigma-Aldrich, P7949)
- BSA (Sigma-Aldrich, A3294)
- Rabbit polyclonal antibody anti-53BP1 (Novus Biologicals, cat. no. NB 100–304)
- Rat monoclonal antibody anti-HA 3F10 (Roche, cat. no. 11867431001)
- Alexa Fluor 647 chicken anti-rat IgG H+L (Life Technologies, cat. no. A21472) ! **CAUTION** The product is light-sensitive; protect it from light.
- Alexa Fluor 405 goat anti-rabbit IgG H + L (Life Technologies, cat. no. A31556) ▲ **CRITICAL** The product is light-sensitive; protect it from light.
- DMEM with 4.5 g/l D-glucose (Gibco/Life Technologies, cat. no. 11960)
- FBS (Gibco/Life Technologies, cat. no. 26140–079)
- L-Glutamine (Gibco/Life Technologies, cat. no. 25030)
- Pen/Strep (Gibco/Life Technologies, cat. no. 15140)
- Trypsin-EDTA 0.25%, 1× (Gibco/Life Technologies, cat. no. 25200–072)
- MAX Efficiency Stbl2 competent cells (Invitrogen, cat. no. 10268–019) ▲ **CRITICAL** Competent cells that are designed for cloning unstable inserts are required.
- Ultrapure agarose (Invitrogen, cat. no. 16500)
- Nuclease-free water (Ambion, cat. no. AM 9937)
- LB agar plates with 100 µg/ml ampicillin (Qbiogene, cat. no. 3014–224)
- Super-optimal broth with catabolite repression (SOC) medium (Invitrogen, cat. no. 15544–034)
- Ampicillin sodium salt (Sigma-Aldrich, cat. no. A9518–25G)

- LB medium (Crystalgen, cat. no. 226–082-05)
- Hi-Lo DNA marker (Fisher Scientific, NC9829338)
- Tris-Acetate-EDTA (TAE), 10× (Crystalgen, cat. no. 221–197-01)
- GelRed, nucleic acid stain, 10,000× in DMSO (Biotium, cat. no. 41002)
- Glycerol, 100% (Invitrogen, cat. no. 15514–011)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Nucleofector kit R (Lonza, cat. no. VCA 1001) or appropriate kit for the cell line used
- Xylene cyanol (Sigma-Aldrich, cat. no. X4126)
- Restriction endonucleases, buffers and 10 mg/ml (100×) BSA solution (New England Biolabs)
- Opti-MEM (Gibco/Life Technologies, cat. no. 11058–021)
- Hygromycin (Gibco/Life Technologies, cat. no. 10687–010) ▲ **CRITICAL** Protect it from light.
- Zeocin (Gibco/Life Technologies, cat. no. R250–01)
- Puromycin (Gibco/Life Technologies, cat. no. A11138) ▲ **CRITICAL** Protect it from light.
- G418 (Invivogen, ant-gn-5) Note that resistance to G418 is conferred by the neomycin resistance gene (neo)
- Blasticidin S HCL (Gibco/Life Technologies, A1113903) ▲ **CRITICAL** Protect it from light.
- Isopropyl β-D-1-thiogalactopyranoside, IPTG (Sigma-Aldrich, cat. no. I6758)
- Doxycycline (Sigma-Aldrich, cat. no. D-9891)
- FuGENE HD (Promega, cat. no. E2311)
- Platinum GP (Plat-GP) cells (Cell Biolabs, cat. no. RV-103)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668–019)
- Hoechst dye, bisBenzimide H 33258 (Sigma-Aldrich, cat. no. B2883)
- Vectashield mounting medium for fluorescence (Vector Laboratories, cat. no. H-1000)
- Milli-Q ultrapurified water

EQUIPMENT

- Biosafety Level II laboratory with biosafety cabinet for tissue culture
- Tissue culture incubator at 37 °C, 5% CO₂ (Sanyo MCO-18AIC or similar)

- Inverted phase-contrast microscope (Nikon Eclipse TS100 or similar)
- Tissue culture dishes, 150 × 25 mm (BD Biosciences, cat. no. 353025)
- Tissue culture dishes, 100 mm (BD Biosciences, cat. no. 353003)
- Tissue culture plates, six-well (Corning, Costar, cat. no. 3506)
- Tissue culture plates, 24-well (Corning, Costar, cat. no. 3524)
- 384-Well plates (PerkinElmer, Cell Carrier, cat. no. 6007460)
- Conical tubes, 15 ml and 50 ml (BD Biosciences, cat. nos. 352097 and 352070)
- Tissue culture/clinical centrifuge (International Equipment IEC-HNSII or similar)
- Disposable plastic serological pipettes, individually wrapped; 1, 2, 5, 10, 25 and 50 ml (Corning Costar, cat. no. CLS4487, 4101, 4489 and 4490)
- Microcentrifuge tubes, 1.5 ml (Corning Costar, cat. no. 3620)
- Water baths at 37 and 42 °C (Precision 280, or similar)
- Agarose gel electrophoresis apparatus and power source (Bio-Rad MiniSub Cell GT and 1,000 power source, or similar)
- Hemocytometer, 0.0025 mm² (Labor Optik, Neubauer), or preferred cell counter
- Multichannel pipette, 200 µl (Rainin 200 LTS, or similar)
- Solution Basin, 55 ml, polystyrene, sterile (Heathrow Scientific, cat. no. HS20521C)
- OPERA high-content screening microscopy system (PerkinElmer) ▲
CRITICAL Although a high-content microscope is essential to obtain the high number of images required to capture rare translocation events, instruments equipped with an environmentally controlled chamber and a standard epifluorescence unit or confocal setup are sufficient to follow the chromatin dynamics experimentally using cell lines generated by following this protocol.
- Acapella High Content Imaging and Analysis Software (PerkinElmer)
- Computer: minimum Pentium 4 with 3.2GHz or Core 2 with 2 GHz, >2GB RAM
- Gel documentation system, Molecular Imager Gel Doc XR plus system with Image Lab software (Bio-Rad)
- Microcentrifuge (Eppendorf, centrifuge 5,424 or similar)
- Sterile pipette tips with aerosol barrier (any vendor)
- NanoDdrop spectrophotometer (Thermo Scientific, cat. no. ND-1,000 or similar)
- RC-5C plus centrifuge or similar (Sorvall)
- Microscope cover glass (Fisher Scientific, cat. no. 12–545-80)
- Freezing container (Nalgene, cat. no. 5100–0001)

- Cryovials, 1.2 ml (Nalgene, cat. no. 5000–0012)
- Liquid nitrogen tank (any style)
- Frosted microscope slides, precleaned (Fisher Scientific, cat. no. 12–550-343)
- Falcon 2059 tubes, 17 × 100 mm (BD Falcon, cat. no. 352059)
- Propylene bottles, 500 ml (any vendor)
- Qiaex II gel extraction kit (Qiagen, cat. no. 20051)
- Nucleofector 2b device (Lonza)

EQUIPMENT SETUP

Opera high-content screening system The Opera high-throughput microscopy system used in these studies is equipped with four diode lasers for sample illumination (405, 488, 560 and 640 nm). It is also equipped with three high quantum efficiency cooled charge-coupled device (CCD) cameras for simultaneous acquisition of multiple fluorescence channels, to prevent bleed-through between the channels. The cameras are located in the light path behind the Nipkow spinning disc unit, providing confocality for the captured digital image data.

REAGENT SETUP

DMEM FBS 10% (vol/vol), 2 mM L-glutamine, 100 units/ml Pen and 100 µg/ml Strep Mix 500 ml of DMEM with 50 ml of FBS, 5 ml of L-glutamine and 5 ml of Pen/Strep. This solution can be stored at 4 °C for up to 1 month.

Triton X-100, 0.3% (vol/vol) in PBS Mix 0.75 ml of Triton X-100 with 249.25 ml of PBS to prepare 0.3% Triton in PBS. This solution can be stored at room temperature (RT, 25 °C) for at least 1 month.

Tween-20, 0.1% (vol/vol) in PBS Mix 0.5 ml of Tween-20 to a final volume of 500 ml of PBS. Store it at RT for up to 1 month.

Blocking buffer for immunofluorescence Dilute 0.3 g of BSA into 9 ml of 1× PBS. Add 1 ml of FBS and mix it. This buffer can be stored at 4 °C for 3–7 d.

PFA fixation solution Dilute 10 ml of 16% PFA solution into 30 ml of PBS to prepare 4% PFA fixation solution. ! **CAUTION** This solution is harmful if it is inhaled. It is to be used only in a ventilated hood. ▲ **CRITICAL** Make the solution freshly on the day of the experiment; the solution can be divided into aliquots and stored frozen for up to 1 year.

Plat-GP cell medium Add 50 ml of FBS, 5 ml of L-glutamine and 5 ml of 100× penicillin-streptomycin in a 500-ml bottle of DMEM supplemented with 10 µg/ml blasticidin. Store the medium at 4 °C for up to 1 month.

Hoechst DNA staining solution Dilute 10 µg of Hoechst bisBenzimide H 33258 in 1 ml of water and store it at - 20 °C.

Loading dye (6×) Loading dye is 30% (vol/vol) glycerol, 0.25% (wt/vol) bromophenol blue and 0.25% (wt/vol) xylene cyanol FF; store it at 4 °C.

Isopropyl β-D-1-thiogalactopyranoside Dilute 10 g of IPTG in 41.96 ml of sterile water to achieve a final concentration of 1 M. Store it at - 20 °C for up to 1 year.

Doxycycline Dilute 10 mg of doxycycline to 10 ml of sterile Milli-Q water to reach a final concentration of 1 mg/ml. Store it at - 20 °C for up to 1 year.

PROCEDURE

Plasmid preparation and propagation ● TIMING 4 d

1. | *Day 1.* Thaw three vials of Stbl2 MAX Efficiency Stbl2 chemically competent *E. coli* cells on ice, and transfer 100 µl of cells to three chilled 17 mm × 100 mm Falcon 2059 tubes on ice.
2. | To each tube, add 5 µl (~50 ng) of plasmid DNA containing arrays of repeated sequences adjacent to the endonuclease I-SceI recognition site (e.g., LacOI-SceITet0, LacOI-SceI and TetOI-SceITet0). Move the pipette up and down while dispensing. Gently tap the tube to mix the contents.
▲ **CRITICAL STEP** Use sterile reagents and equipment, and take careful measures against contamination in this step and in all subsequent steps.
3. | Incubate the cells on ice for 30 min.
4. | Heat-shock the cells in a 42 °C water bath for 25 s.
▲ **CRITICAL STEP** Do not shake the cells.
5. | Place the tubes on ice for 2 min.
6. | Add 0.9 ml of SOC medium at RT and shake it at 225 r.p.m. at 30 °C for 90 min.
7. | Spread 100 µl from each tube onto an individual LB agar plate containing 100 µg/ml ampicillin. Incubate the plates at 30 °C for ~16–20 h. As bacteria grow at a slower rate at 30 °C, incubation for additional time may be required. Frequently check the size and number of colonies to determine the optimal incubation period.
8. | *Day 2.* Pick 5–10 single colonies from each transformation and inoculate them in 2–3 ml of LB medium containing 100 µg/ml ampicillin. Incubate the colonies at 30 °C while shaking at 225 r.p.m. for 12–16 h.
▲ **CRITICAL STEP** Pick small colonies, as there is a higher probability that they contain intact, nonrecombined plasmids (which are more difficult to replicate, and thus grow at a slower rate).
9. | *Day 3.* Use 1 ml of each bacterial culture for plasmid DNA extraction using the Qiagen Plasmid Miniprep Kit according to the manufacturer's instructions. Elute with 50 µl of nuclease-free water.

10. | Use 2 µl of each miniprep to calculate the DNA concentration by using a NanoDrop spectrophotometer according to the manufacturer's instructions. Resuspend in the appropriate volume of water to obtain a concentration of 200 ng/µl.

? TROUBLESHOOTING

11. | Verify the identity of all plasmids by restriction enzyme digestion using appropriate enzymes; XhoI and SacII are suitable for Lac0I-SceITet0, Lac0I-SceI and Tet0I-SceITet0. For each plasmid, assemble the following 10-µl reaction in a microcentrifuge tube. Incubate the reaction at the appropriate temperature for 1–3 h; the appropriate temperature for XhoI/SacII digests is 37 °C.

Component	Amount (per reaction)	Final
Plasmid DNA (200 ng/ml)	2 µl	400 ng
Buffer 10×	1 µl	1×
Restriction enzyme, XhoI (20 U/µl)	0.5 µl	10 U
Restriction enzyme, SacII (20 U/µl)	0.5 µl	10 U
BSA, 100×	0.1 µl	1×
Nuclease-free H ₂ O	5.9 µl	—

12. | Add to each digestion 2 µl of the 6× loading dye and load into the wells of a 1% (wt/vol) agarose gel (in 1× TAE) supplemented with 1× GelRed. Analyze the restriction fragments by electrophoresis in 1× TAE buffer at 5 V/cm for 20–40 min. Visualize and capture the gel image on a gel documentation system. Mark the DNA minipreps that show the expected restriction fragments (Fig. 2a,b).
13. | Inoculate 0.5 ml of any bacterial culture (from Step 8) showing the expected restriction pattern (Step 12) into 250 ml of LB medium supplemented with 100 µg/ml ampicillin in a 1,000-ml flask. Incubate the culture at 30 °C while shaking at 225 r.p.m. for 16–20 h.
14. | *Day 4.* Transfer 0.75 ml of each bacterial culture into a microcentrifuge tube and add 0.25 ml of 50% (vol/vol) glycerol to prepare a glycerol stock. Store it at –80 °C.

■ **PAUSE POINT** Glycerol stocks of recombinant *E. coli* can be stored at –80 °C indefinitely, and they can be used to initiate bacterial cultures for subsequent plasmid preparations.

15. | Transfer the remainder of the bacterial cultures into polypropylene bottles and pellet the bacteria by centrifugation at 6,000g for 15 min at 4 °C. Discard the supernatant.

■ **PAUSE POINT** Bacterial pellets can be stored at –20 °C for up to 1 month for plasmid DNA extraction at a later time.

16. | Proceed with plasmid DNA extraction using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. Elute DNA with 200 μ l of nuclease-free water and resuspend it to a final concentration of 1 μ g/ml.
- **PAUSE POINT** Plasmid DNA can be stored at -20°C indefinitely or at 4°C for several months.
17. | Repeat Steps 10–12 to verify the integrity of the DNA preps by restriction endonuclease analysis.

Generation and selection of cell lines with stable *LacO/TetO* array integrations

● TIMING 26 d

18| Day 1. Linearize the plasmid of interest (e.g., TetOI-SceITetO) and a plasmid conferring resistance to a drug (e.g., pTRE2Hyg) by using restriction enzymes that do not cut within the TetOI-SceITetO sequence (e.g., ScaI) and the hygromycin expressing cassette (e.g., BamHI), respectively. For each plasmid, assemble the following reaction in a microcentrifuge tube. Incubate at 37°C for 5 h to ensure a complete digestion.

Component	Amount (per reaction)	Final
Plasmid DNA (1 μ g/ml)	20 μ l	20 μ g
Buffer 10 $\times$	10 μ l	1 $\times$
Restriction enzyme, e.g., ScaI (20 U/ μ l) or BamHI (20 U/ μ l)	5 μ l	100 U
BSA, 100 $\times$	1 μ	1 $\times$
Nuclease-free H ₂ O	64 μ l	—

19| Perform phenol/chloroform extraction (Box 1). Alternatively, run digestions (load each digestion in three separate wells to prevent overloading) on a 1% (wt/vol) agarose gel (in 1 $\times$ TAE) supplemented with 1 $\times$ GelRed at 5 V/cm for 20–40 min. Check digestions and expected restriction fragments on a gel documentation system and cut linearized plasmids. Gel-extract using the Qiagen Qiaex II Gel extraction kit according to the manufacturer's instructions, and resuspend the products in water.

20| Transfect linearized TetOI-SceITetO and pTRE2Hyg plasmids at a 10:1 ratio by using the Amaxa Nucleofector according to the manufacturer's instructions for the cell line of interest. For integrations into NIH3T3 cells, electroporate using the program U-030 a total of 1×10^6 cells, resuspended in 100 μ l of the R solution, with 3 μ g of TetOI-SceITetO and 0.3 μ g of pTRE2Hyg, and plate the electroporated cells in three wells of a six-well culture dish containing DMEM at a final volume of 2 ml.

Note that incorporation of the resistance cassette (conferring hygromycin resistance) into the TetOI-SceITetO plasmid using additional cloning steps will remove the need for co-transfection, and it will increase the efficiency of the selection.

21| Day 2. Wash the cells with 1 $\times$ PBS twice, trypsinize them using 0.25% (wt/vol) trypsin-EDTA and plate the cells in a 150×25 mm tissue culture dish in DMEM in a

total volume of 25 ml. Add the drug for selection. Plate the same number of untransfected cells to use as a positive control for the drug selection.

▲ **CRITICAL STEP** Cell density and homogeneous dispersion in this plating step are important to enable recovery and screening of single-cell colonies.

▲ **CRITICAL STEP** Different cell lines show distinct resistance to drugs. In pilot experiments, determine the optimal lower lethal dose for each cell type by titrating the drug concentration.

? TROUBLESHOOTING

22| Days 3–15. Wash the cells with 1× PBS and replenish the DMEM supplemented with the selection drug every 3–4 d. During the next 7–12 d, the surviving resistant cells will form colonies.

The time after drug selection until single colonies are formed is dependent on the cell type and the type and concentration of the selection drug used. Check the size of the forming colonies under an inverted phase-contrast microscope every 1–2 d to avoid mixed populations (supplementary Fig. 1).

23| Day 16. When the colonies reach the optimal size (supplementary Fig. 1), use the P200 pipette at 200-μl volume and P200 tips to simultaneously scratch and draw up individual colonies into the tip, and immediately transfer them into individual wells of 24-well plates containing 1 ml of DMEM supplemented with the selection drug. Incubate the mixture at 37 °C, 5% CO₂.

The number of the isolated colonies may vary according to the efficiency of the procedure. Typically, 30–50 colonies are isolated and processed further.

24| Days 17–20. Wash the cells with 1× PBS, trypsinize with 50 μl of 1× trypsin-EDTA 0.25% (wt/vol) and mix well by shaking strongly forward and backward and side-to-side. Add 1 ml of fresh medium and incubate it at 37 °C, 5% CO₂. Replenish the medium every 3 d until the cells reach confluency. The time required to reach confluency may vary owing to distinct proliferation rates of the different clones.

25| Autoclave the circular coverslips and place one into each well of a 24-well plate in the biosafety cabinet. When cells from Step 24 reach confluency, trypsinize and transfer half of the cells into individual wells of a six-well plates and half to individual wells of the 24-well plate containing coverslip ($\sim 7.5 \times 10^4$ NIH3T3 cells in each well). Supplement with 2 ml and 1 ml of DMEM per well, respectively. Mark with a pen the wells in the six-well and 24-well plates that contain cells from the same colony to maintain identity between plates.

26| Day 21. Verify the integration of the TetOI-SceITet0 plasmid in individual cell clones plated in individual wells of the 24-well plate by transiently transfecting a fluorescent version of the Tet repressor (pCAGGS-TetRmCherry⁶⁹) using Lipofectamine. Use two 15-ml falcon tubes to assemble the reactions as given below:

Component	Amount per reaction		Master mix for 50 reactions (50 clones)	
	Tube 1	Tube 2	Tube 1	Tube 2
Opti-MEM	250 μ l	250 μ l	12.5 ml	12.5 ml
Lipofectamine	10 μ l	—	500 μ l	—
TetRmCherry plasmid	—	0.3 μ g	—	15 μ g

27| Mix the contents of tube 1 and tube 2 together, and incubate them at RT for 10 min.

28| Aspirate the medium from the wells and add 500 μ l of the reaction in a dropwise manner on top of cells or coverslips. Incubate the mixture at 37 °C, 5% CO₂ for 6 h. Replace it with fresh medium and incubate overnight at 37 °C, 5% CO₂.

29| Day 22. Aspirate the medium and wash the cells twice with 1 $\times$ cold PBS. Fix the cells by adding 1 ml of 4% PFA per well at RT for 10 min.

30| Wash the cells three times using 1 $\times$ PBS, by shaking the plate for 5 min during each wash.

31| Remove the wash buffer and add 1 ml of Hoechst solution to the wells by incubating in the dark for 15 min at RT to stain the nuclei.

32| Wash away the excess stain using 1 $\times$ PBS.

33| Dispense 10 μ l of mounting medium on the top of a microscope slide. Use forceps to place each coverslip on top of the mounting medium in a way that cells are in contact with the mounting medium and the microscope slide, without introduction of air bubbles. Seal the edges of the coverslips on the microscope slides by applying clear nail polish. Allow it to dry for at least 1 h protected from light.

34| Day 23. Examine the slides using a standard epifluorescence or confocal microscope. Search for clones containing TetOI-SceITet0 integrated arrays visualized as distinct spots (Fig. 3a).

? TROUBLESHOOTING

35| Days 23–25. Further expand the desired cell clones from the six-well plate (from Step 25) by trypsinizing and plating to individual 10-cm dishes. Continue to expand the selected clones by trypsinizing and replating in 150 mm $\times$ 20 mm tissue culture dishes. Before plating, freeze the aliquots of 1×10^6 cells as follows: pellet the desired number of cells by centrifugation at 500g at RT for 5 min. Discard the supernatant. Resuspend the cells in freshly prepared 90% (vol/vol) FBS and 10% (vol/vol) DMSO. Immediately add 1 ml per cryovial, place the cryovials in a freezing container and store them overnight at - 80 °C. Transfer the cryovials the next day into a liquid nitrogen storage tank.

■ PAUSE POINT Frozen cells can be stored indefinitely in liquid N₂, but viability decreases with prolonged storage. Do not store at - 80 °C for more than a week.

36| Day 26. If integration of a second array, such as Lac0, is required, repeat Steps 18–35 by transfecting the Tet0I-SceITet0 stable cell line with linearized Lac0I-SceI plasmid, and a plasmid conferring resistance to Zeocin (pSV40Zeocin), and select positive clones by transfecting cells with a plasmid expressing the repressor GFP-LacR⁴² (Fig. 3b). Both Lac0I-SceI and pSV40Zeocin plasmids can be linearized with ScaI. For NIH3T3 cells, Zeocin should be added at 300 μ g/ml for selection.

▲ CRITICAL STEP Stable *Lac0/Tet0* cell lines can be maintained in culture even in the absence of selection¹⁶. Split cells at a 1:3 ratio when the cells reach 90% confluency. It is recommended to initially prepare several frozen aliquots of low-passage cells for future use.

Generation of *Lac0/Tet0* cell lines stably expressing LacR and TetR

fluorescent repressors ● TIMING 15 d—▲ CRITICAL This section describes the generation and use of infectious retroviral particles; proper safety precautions for working with live virus should be practiced.

37| Day 1. Thaw a vial of Plat-GP cells quickly at 37 °C. Transfer the contents into a 15-ml conical tube containing 10 ml of Plat-GP cell medium. Centrifuge the cells at 500g at RT for 5 min, and discard the supernatant. Resuspend the cells in 10 ml of Plat-GP medium, transfer them into a 100-mm dish and incubate them at 37 °C, 5% CO₂ for 24–48 h until the cells reach 80–90% confluency.

▲ CRITICAL STEP Low-passage Plat-GP cells need to be used for higher efficiency of viral production. It is recommended to initially prepare several frozen aliquots of low-passage cells in good condition for future use, as the growth rate of Plat-GP cells is inversely correlated with passage.

38| Day 2. Aspirate off the medium, wash the cells gently once with 1× PBS and trypsinize by adding 1 ml of trypsin-EDTA. After the cells visibly detach, add 5 ml of Plat-GP cell medium and thoroughly pipette up and down using a 10-ml pipette until a single-cell suspension is achieved.

39| Replate 3 ml in each of two 100-mm dishes, and add 7 ml of Plat-GP cell medium. To homogeneously disperse the cells, move the dishes forward and backward and side-to-side on a flat surface and incubate them at 37 °C, 5% CO₂ for ~24 h or until the cells reach 80–90% confluency (Supplementary Fig. 2b). Freeze down the cells of one of the plates as in Step 35.

▲ CRITICAL STEP In order to reach maximum transfection efficiency, Plat-GP cells should not be plated too sparsely or to the point of overconfluence (Supplementary Fig. 2b). Optimal density for high transfection efficiency is 80–90%, which can be typically reached 24 h after splitting 80–90% confluent cells at a 1:2 ratio.

40| Day 3. Inspect the cells to ensure 80–90% confluency. Gently replace the medium with 10 ml of Plat-GP cell medium prewarmed to 37 °C for each 100-mm dish, and incubate it at 37 °C, 5% CO₂ for 1 h before transfection.

41| Prepare the plasmid-transfection reagent complex for each 100-mm plate of Plat-GP cells by mixing 490 μ l of Opti-MEM with 2 μ g of pVSV-G, 4 μ g of pQCXIP-GFP_{LacR} and 4 μ g of pQCXIN-TetR-mCherry in a microcentrifuge tube and incubate it at RT for 5 min.

42| Add 30 μ l of FuGENE HD, gently mix it once and allow the complexes to form for 20 min at RT.

43| Dispense 500 μ l of the complex solution dropwise on top of Plat-GP cells + medium, and gently move the dishes forward and backward and side-to-side on a flat surface to mix well in the medium. Incubate the dishes at 37 °C, 5% CO₂ for 16 h.

! CAUTION Note that Plat-GP cells produce live virus; use appropriate biosafety level practices from this point forward.

44| Day 4. Check the transfection efficiency of Plat-GP cells with the fluorescent repressor plasmids on an inverted fluorescence microscope equipped with the appropriate filters.

? TROUBLESHOOTING

45| If the transfection efficiency is optimal (70–90%), aspirate off the medium and replace it gently with 6 ml of prewarmed P_{Lac}-GP medium per plate. Incubate the plates at 37 °C, 5% CO₂ for ~24 h to allow virus production. The formation of syncytia by fusion of Plat-GP cells is normal owing to the VSV-G protein expression.

46| Trypsinize the selected stable *LacO/TetO* array cell line generated from Step 36. Count 1.2×10^6 cells and plate them into three wells of a six-well plate at confluencies in the range of 2×10^5 , 4×10^5 and 6×10^5 cells. Supplement DMEM to a final volume of 2 ml per well.

▲ CRITICAL STEP The final nuclear expression of LacR and TetR repressors is crucial for the optimal visualization of the *LacO* and *TetO* arrays. Titration of cell confluency will provide infected cells with variable expression of the repressors, allowing subsequent selection of the optimal expression levels. Typically, low expression of the fluorescent repressors is essential for discrete visualization of arrays over the free nucleoplasmic fraction (Supplementary Fig. 3).

47| Day 5. Collect the virus-containing supernatant and replace it with 6 ml of prewarmed P_{Lac}-GP medium per plate and incubate it at 37 °C, 5% CO₂ for ~24 h.

48| Filter the supernatant through a sterile 0.45- μ m filter. Aspirate off the medium from the *LacO/TetO* cells and replace it with 2 ml of the filtered supernatant. To prevent binding of the expressed LacR and TetR repressors to the respective operators, supplement each well with 5 mM IPTG (10 μ l of 1 M stock) and 1 μ g/ml doxycycline (2 μ l of 1 mg/ml stock), and incubate at 37 °C, 5% CO₂ for ~24 h.

▲ CRITICAL STEP IPTG is unstable. Replenish the medium supplemented with IPTG every 2–3 d.

49| Day 6. Inspect the infected cells on an epifluorescence or a confocal microscope. Cells expressing GFP-LacR and TetR-mCherry at low levels in the nucleus should be

detected. Replace the supernatant with fresh medium supplemented with 5 mM IPTG and 1 µg/ml doxycycline.

? TROUBLESHOOTING

50| Days 7–12. Trypsinize the cells and plate them in a 150 × 25 mm culture dish supplemented with 30 ml of DMEM. Add 2 µg/ml puromycin and 600 µg/ml G418 to select for cells that are positive for both GFP-LacR and TetR-mCherry stable integrations. Supplement the medium with 5 mM IPTG (150 µl of 1 M stock) and 1 µg/ml doxycycline (30 µl of 1 mg/ml stock). Replenish selection drugs and IPTG and doxycycline every 2–3 d.

▲ CRITICAL STEP It is essential to supplement and replenish the IPTG and doxycycline every 2–3 d to prevent the binding of the repressors to the respective operators and a possible interference with replication or transcription.

? TROUBLESHOOTING

51| Days 12–15. Further expand the selected clones by replating them into 150 × 20 mm tissue culture dishes, and freeze the aliquots as in Step 29.

Selection of monoclonal cell lines with optimal expression of LacR and TetR fluorescent repressors ● TIMING 25 d

52| Days 1–10. Wash polyclonal *Lac0/Tet0* cells expressing the GFP-LacR and TetR-mCherry repressors plated in a 150 × 20 mm tissue culture dish with PBS and trypsinize by adding 3 ml of trypsin-EDTA. When the cells visibly detach, add 9 ml of DMEM and thoroughly pipette the cells up and down against the bottom of the dish with a 10-ml pipette to generate a single-cell suspension. Plate ~200 cells (typically 5–10 µl of the cell suspension) in a new 150 × 20 mm tissue culture dish supplemented with medium, the selection drugs, 5 mM IPTG and 1 µg/ml doxycycline, and incubate it at 37 °C, 5% CO₂ for 6–10 d or until colonies from single cells are formed.

▲ CRITICAL STEP It is essential to supplement and replenish the IPTG and doxycycline every 2–3 d.

▲ CRITICAL STEP It is essential that cells be plated sparsely to avoid mixed cell populations. It is recommended that different amounts of cells be plated on individual plates to ensure optimal density.

53| Days 11–16. Pick ~50 individual colonies and process them as described in Steps 23–25. Supplement each well of the clones plated in the six-well plates with 5 mM IPTG and 1 µg/ml doxycycline to prevent repressor binding. Do not add any drugs to the clones plated on coverslips in the 24-well plates to allow the binding of the repressors to their respective operators. Incubate the six-well and 24-well plates at 37 °C, 5% CO₂ for 2 d.

▲ CRITICAL STEP Wash the individual clones extensively with PBS (four times) before trypsinization and plating in six-well and 24-well plates. Extensive washing is necessary to completely remove doxycycline to allow the binding of the TetR

repressor to the *TetO* operator. After IPTG and doxycycline have been removed by washing, the *LacO* and *TetO* arrays can be visualized within 6–10 h and up to 2 d, respectively.

54| *Day 17.* Fix cells from the 24-well plates and mount them on a microscope slide as described in Step 33. Examine the slides using a standard epifluorescence or confocal microscope. Select clones in which both *LacO* and *TetO* arrays are clearly visible (Fig. 4a).

55| *Days 18–21.* Expand the selected cell clones from the six-well plate (from Step 53) to a 100-mm dish.

56| *Days 22–25.* Further expand the selected clones to 150 × 20 mm tissue culture dishes. Freeze the cells and store them as described in Step 35.

DSB induction and detection ● TIMING 4 d

57| *Day 1.* Wash the cells three times with 1× PBS, and trypsinize and resuspend them in 10 ml of medium. Collect the cells and transfer them into a 15-ml conical tube. Centrifuge the mixture at 500*g* for 5 min at RT.

▲ CRITICAL STEP To visualize *TetO* arrays, the doxycycline must be thoroughly washed away. The centrifugation step ensures that the excess doxycycline is removed before the cells are plated to a new dish.

58| Discard the supernatant, resuspend the cell pellet in 9 ml of DMEM and plate 3 ml in 150 × 20 mm tissue culture dishes in the presence of 5 mM IPTG (150 µl of 1 M IPTG stock in 30 ml of DMEM). Incubate the cells at 37 °C, 5% CO₂ for 2 d, or until they reach 90% confluency.

59| *Day 3.* Wash the cells three times with 1× PBS to remove the IPTG; supplement the cells with DMEM and incubate them for 5 h at 37 °C, 5% CO₂.

60| Autoclave circular coverslips and place them into 24-well plates in the tissue culture biosafety cabinet.

61| Trypsinize and count the cells using a hemocytometer. Electroporate 1 × 10⁶ cells, resuspended in 100 µl of the R solution (for *LacO/TetO* stable NIH3T3 cells), with 10 µg of the HA-I-SceI plasmid using the Amaxa Nucleofector (program U-030), according to the manufacturer's instructions. As a control, transfect cells with 15 µg of the plasmid expressing the catalytically inactive I-SceID44A.

62| Add to the transfected cells 1 ml of DMEM and plate 250 µl each in four wells of a 24-well plate containing a coverslip. Add DMEM to a final volume of 1 ml and incubate the cells at 37 °C, 5% CO₂ for 12 h.

63| Aspirate off the medium and wash cells twice with 1× cold PBS.

64| Fix the cells by adding 1 ml of 4% PFA per well at RT for 15 min.

65| Wash the cells 3 times using 1× PBS (by shaking the plate for 5 min during each wash).

66| Permeabilize the cells by adding 1 ml of 0.3% (vol/vol) Triton X-100 and incubate them at RT for 5 min.

67| Wash the cells three times using 1× PBS (shaking the plate for 5 min during each wash), and add 1 ml of blocking buffer for 1 h at RT.

68| Dilute (1:500) the rat monoclonal antibody against the HA epitope of the I-SceI and the rabbit polyclonal antibody against 53BP1 (1:1,000) in blocking buffer.

69| Transfer the coverslips from the 24-well plates using forceps to a layer of Parafilm within a 150 × 20 mm tissue culture dish with cells facing up.

70| Remove any excess blocking buffer and dispense 100 µl of the antibody dilution on top of the coverslips. Incubate the mixture for 1–2 h at RT or leave it overnight at 4 °C.

▲ CRITICAL STEP Place a wet paper wipe in the dish to create a humidified chamber and to avoid evaporation.

71| Place each coverslip, cell side up, in a well of a six-well plate supplemented with 2 ml of 0.1% (vol/vol) Tween-20, and wash it three times (by shaking the plate for 5 min during each wash).

72| Prepare the secondary antibody dilutions. Use Alexa Fluor anti-rat 647 for detecting HA diluted in blocking buffer (1:500) and Alexa Fluor anti-rabbit 405 for detecting anti-53BP1 (1:500 in blocking buffer). Use forceps to transfer the coverslips back to the chamber, dispense 100 µl of the secondary antibody dilution and incubate for 1 h at RT protected from light.

▲ CRITICAL STEP Different combinations of primary/secondary antibodies can be used according to the particular needs of the experiment, the primary/secondary antibody availability and the detection capacity by fluorescence microscopy.

▲ CRITICAL STEP Secondary antibody dyes are light-sensitive; protect them from light using aluminum foil at all subsequent steps.

73| Place each coverslip in a well of a six-well plate containing 2 ml of 0.1% (vol/vol) Tween-20 and wash it three times (by shaking the plate for 5 min during each wash).

74| Place 10 µl of fluorescence mounting medium on a microscope slide. Use forceps to place each coverslip on top of the mounting medium such that the cells are in contact with the mounting medium and the microscope slide, without introducing air bubbles. Seal the edges of the coverslips on the microscope slides by applying clear nail protector. Allow it to dry for 1 h, protected from the light.

75| Examine the slides using a standard epifluorescence or confocal microscope. Determine the percentage of cells that are positive for the active form of the enzyme (HAI-SceI) or the inactive mutant (HAI-SceID44A), and show colocalization of the *LacO* or *TetO* arrays with the formed 53BP1 repair foci (Fig. 4b).

? TROUBLESHOOTING

76| Select and expand the clonal cell lines from Step 56, which show a colocalization pattern, as shown in Figure 4b.

High-throughput live-cell imaging ● TIMING 1 d

77| By using expanded clonal cell lines from Step 76, repeat Steps 57–61. Place the electroporated cells in 3.2 ml of warmed DMEM contained in a solution basin. Mix well and use a multichannel pipette to plate 50 μ l (~3,000 cells per well) in each of 64 wells of a 384-well plate. Incubate the cells at 37 °C, 5% CO₂ for 6 h.

78| Switch on the OPERA high-content screening system and use the climatization control software to equilibrate the incubator to 37 °C. Wait for ~30 min until the temperature is reached, and set it at 60% humidity and 5% CO₂.

▲ **CRITICAL STEP** Keep the temperature at 37 °C while the humidity is turned on to avoid condensation of water and instrument damage.

79| Start the Perkin-Elmer OPERA software. Open the CONFIGURATION tab and select from the plate type menu the OPERA adjustment plate and the appropriate objective, typically the 40× 0.9-NA (numerical aperture) water objective lens. Activate the 488-nm and 561-nm lasers. Open the MICROSCOPE tab and click TABLE IN/OUT, insert the OPERA adjustment plate and perform optics correction (skewcrop analysis and take reference images) according to the manufacturer's instructions.

80| In the CONFIGURATION tab, change to the 384-well cell carrier plate type. Click on TABLE IN/OUT and remove the OPERA adjustment plate before loading the 384-well plate containing the cells. Select one of the wells containing cells and define the optimal exposure parameters: select two exposures and use the check box to activate the 488-nm laser and camera 1 and the 561-nm laser and camera 2. Under the MICROSCOPE tab, select the appropriate filters for the optical path (camera 1: 520/35, camera 2: 600/40, Detect_dichro: 568, Primary_dichro: 405/488/561/640). For each exposure acquisition, select the optimal best focus height and click the 'take height' button when satisfied. Determine the optimal laser intensity for each different exposure by clicking at EXPOSE, and adjust the laser power and exposure time as desired. Save the exposure parameters.

81| Create the plate layout marking the desired wells that you want to image. Open the NAVIGATE tree, right-click on the experimental folder and select CREATE LAYOUT. Follow the layout wizard to select the desired wells, and click on FINISH. Similarly create the well sublayout by selecting CREATE SUBLAYOUT in the NAVIGATE tree and select the number and location of the fields per well that will be acquired. For a typical high-throughput experiment to visualize chromosome translocations¹⁶, 14 adjacent fields per well of a total of 60 wells are sampled; typically, $\sim 1.5 \times 10^4$ cells are captured in 840 time-lapse movies.

82| Click on CREATE STACK in the NAVIGATE tree to define the number and the interval of the acquired z stacks. Choose three z stacks with 2- μ m interval.

83| Under the EXPERIMENTAL DEFINITION tab, drag and drop all the experimental conditions defined in Steps 80–82 and save them by right-clicking on the experimental folder selecting *save current: Experiment*.

84| Switch the Perkin-Elmer OPERA to REMOTE mode. Open the Remote Control Interface (RCI) Scheduler software, press configure and open a text file defining the interval time of the time-lapse experiments. For a 1-h interval, load a text file containing the experiment directory, a barcode for the specific experiment and the desired time intervals as follows:

Experiment directory\experiment_name.exp; barcode_name; interval time.

For example, C:\Opera\OperaDB\user1\live_timelapse
 \live_timelapse.exp;BC_140228_live_timelapse; 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h, 1 h.

85| On the RCI Scheduler software press START. The microscope will acquire images of 3 z stacks per field, of 14 fields per well in a total of 60 wells (3 z stacks per field of 840 fields in total) and every 1 h it will repeat the imaging procedure 16 times.

86| Open the Perkin-Elmer ACAPELLA High-Content Imaging and Analysis Software and use the Interactive Video script to assemble, maximum project and export the acquired time-lapse movies for analysis.

The exported movies can be visually inspected or automatically processed using image analysis scripts, which are generated in Matlab or other advanced image analysis software¹⁶. Sufficient storage capability to accommodate the large amount of generated images is required.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–17, plasmid preparation and propagation: 4 d

Steps 18–36, generation and selection of cell lines with stable Lac0/Tet0 array integrations: 26 d

Steps 37–51, generation of Lac0/Tet0 cell lines stably expressing LacR and TetR fluorescent repressors: 15 d

Steps 52–56, selection of monoclonal cell lines with optimal expression of LacR and TetR fluorescent repressors: 25 d

Steps 57–76, DSB induction and detection: 4 d

Steps 77–86, high-throughput live-cell imaging: 1 d

Box 1, phenol/chloroform extraction of the linearized plasmids: 4 h

Although a total of 10–15 weeks are theoretically sufficient to perform this protocol when NIH3T3 cells are used, the requirement to optimize various steps, troubleshooting, repeats of experimental steps and the determination of the optimal lethal dose for the several of the used drugs will most likely extend the duration to 3–5 months.

ANTICIPATED RESULTS

By using the protocol described here, propagation of plasmids containing large unstable repeated sequences can be achieved (Steps 1–17). We have applied this method to propagate plasmids up to ~20 kb in size containing arrays of the *LacO* and *TetO* operators adjacent to the restriction endonuclease site I-SceI for the development of stable cell lines in which DSBs can be induced and tracked in space and time¹⁶. We are able to isolate NIH3T3 cell lines with stable *LacO/TetO*I-SceI array integrations with efficiency of up to 20% (Steps 18–36). In our recent study¹⁶, a cell line with three integrations of a *TetO*I-SceI array in distinct chromosomes was isolated and used for the subsequent integration of a *LacO*I-SceI plasmid. By using this protocol, we then could generate cell lines that stably express the fluorescently tagged LacR and TetR repressors to visualize the arrays with high efficiency¹⁶. Through the stable integration of bicistronic retroviral vectors that express the LacR and TetR repressors along with genes conferring resistance to antibiotics, as described in this protocol, ~80–90% of the cells stably expressed the LacR and TetR repressors during the initial passages after selection (Steps 37–51). However, we noticed the propensity of cells to lose the GFP-LacR expression with time, which can be overcome by single-cell subcloning or sorting by flow cytometry. Moreover, despite single-cell picking, in some cases, cells expanded from an individual colony typically consist of a mixture of two or more clones with variability in the number of the *LacO/TetO* integrations and/or the LacR and TetR expression levels. These mixed cell populations can be separated by a second round of single-cell subcloning, using the same procedure described in Steps 52–56. These steps allow the generation of monoclonal cell lines with defined numbers of array integrations and homogeneous LacR and TetR expression levels (Fig. 4a).

By following the protocol outlined here (Steps 57–76), the expression of the endonuclease I-SceI can specifically induce DSBs at chromosome sites marked by the *LacO* and *TetO* operators. By using electroporation, we routinely achieve up to 80% transfection efficiency of the endonuclease I-SceI, as determined by immunofluorescence. It is anticipated that the vast majority (70–100%) of *LacO/TetO* arrays colocalize with repair foci in cells expressing the enzyme, suggesting the efficient formation of DSBs (Fig. 4b). We have successfully used this protocol to develop a cell-based system to induce and track DSBs during the formation of chromosome translocations in living cells by high-throughput microscopy¹⁶.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Work in the Misteli Laboratory is supported by the Intramural Research Program of the US National Institutes of Health (NIH), National Cancer Institute (NCI), Center for Cancer Research. We thank E. Soutoglou and C. Schmidt for the optimization of specific steps of this protocol, and we thank T. Karpova (NCI Fluorescence Imaging Microscopy Facility) for help with microscopy.

References

1. Ciccia A & Elledge SJ The DNA damage response: making it safe to play with knives. *Mol Cell*. 40, 179–204 (2010). [PubMed: 20965415]
2. Misteli T & Soutoglou E The emerging role of nuclear architecture in DNA repair and genome maintenance. *Nat. Rev. Mol. Cell Biol.* 10, 243–254 (2009).
3. Soria G, Polo SE & Almouzni G Prime, repair, restore: the active role of chromatin in the DNA damage response. *Mol. Cell* 46, 722–734 (2012). [PubMed: 22749398]
4. Dion V & Gasser SM Chromatin movement in the maintenance of genome stability. *Cell* 152, 1355–1364 (2013). [PubMed: 23498942]
5. Jakob B, Splinter J, Durante M & Taucher-Scholz G Live cell microscopy analysis of radiation-induced DNA double-strand break motion. *Proc. Natl. Acad. Sci. USA* 106, 3172–3177 (2009). [PubMed: 19221031]
6. Aten JA et al. Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains. *Science* 303, 92–95 (2004). [PubMed: 14704429]
7. Krawczyk PM et al. Chromatin mobility is increased at sites of DNA double-strand breaks. *J. Cell Sci.* 125, 2127–2133 (2012). [PubMed: 22328517]
8. Agarwal S et al. ATP-dependent and independent functions of Rad54 in genome maintenance. *J. Cell Biol.* 192, 735–750 (2011). [PubMed: 21357745]
9. Mehta IS, Kulashreshtha M, Chakraborty S, Kolthur-Seetharam U & Rao BJ Chromosome territories reposition during DNA damage-repair response. *Genome Biol.* 14, R135 (2013). [PubMed: 24330859]
10. Zink D, Bornfleth H, Visser A, Cremer C & Cremer T Organization of early and late replicating DNA in human chromosome territories. *Exp. Cell Res.* 247, 176–188 (1999). [PubMed: 10047460]
11. Zink D et al. Structure and dynamics of human interphase chromosome territories *in vivo*. *Hum. Genet.* 102, 241–251 (1998). [PubMed: 9521598]
12. Gotoh E Visualizing the dynamics of chromosome structure formation coupled with DNA replication. *Chromosoma* 116, 453–462 (2007). [PubMed: 17503067]
13. Kruhlak MJ et al. Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. *J. Cell Biol.* 172, 823–834 (2006). [PubMed: 16520385]
14. Michaelis C, Ciosk R & Nasmyth K Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35–45 (1997). [PubMed: 9335333]
15. Robinett CC et al. *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/ repressor recognition. *J. Cell Biol.* 135, 1685–1700 (1996). [PubMed: 8991083]
16. Roukos V et al. Spatial dynamics of chromosome translocations in living cells. *Science* 341, 660–664 (2013). [PubMed: 23929981]
17. Soutoglou E et al. Positional stability of single double-strand breaks in mammalian cells. *Nat. Cell Biol.* 9, 675–682 (2007).
18. Belmont AS, Li G, Sudlow G & Robinett C Visualization of large-scale chromatin structure and dynamics using the lac operator/lac repressor reporter system. *Methods Cell Biol.* 58, 203–222 (1999). [PubMed: 9891383]
19. Gonzalez-Serricchio AS & Sternberg PW Visualization of *C. elegans* transgenic arrays by GFP. *BMC Genet.* 7, 36 (2006). [PubMed: 16759392]
20. Lassadi I & Bystricky K Tracking of single and multiple genomic loci in living yeast cells. *Methods Mol. Biol.* 745, 499–522 (2011). [PubMed: 21660713]

21. Lisby M, Mortensen UH & Rothstein R Colocalization of multiple DNA double-strand breaks at a single Rad52 repair centre. *Nat. Cell Biol.* 5, 572–577 (2003). [PubMed: 12766777]
22. Meister P, Gehlen LR, Varela E, Kalck V & Gasser SM Visualizing yeast chromosomes and nuclear architecture. *Methods Enzymol.* 470, 535–567 (2010). [PubMed: 20946824]
23. Nagai S et al. Functional targeting of DNA damage to a nuclear pore- associated SUMO-dependent ubiquitin ligase. *Science* 322, 597–602 (2008). [PubMed: 18948542]
24. Rohner S, Gasser SM & Meister P Modules for cloning-free chromatin tagging in *Saccharomyces cerevisiae*. *Yeast* 25, 235–239 (2008). [PubMed: 18302313]
25. Strukov YG & Belmont AS Development of mammalian cell lines with lac operator-tagged chromosomes. *Cold Spring Harb. Protoc.* 2008 pdb prot4903 (2008).
26. Vazquez J, Belmont AS & Sedat JW The dynamics of homologous chromosome pairing during male *Drosophila* meiosis. *Curr. Biol.* 12, 1473–1483 (2002). [PubMed: 12225662]
27. Dion V, Kalck V, Horigome C, Towbin BD & Gasser SM Increased mobility of double-strand breaks requires Mec1, Rad9 and the homologous recombination machinery. *Nat. Cell Biol.* 14, 502–509 (2012). [PubMed: 22484486]
28. Mine-Hattab J & Rothstein R Increased chromosome mobility facilitates homology search during recombination. *Nat. Cell Biol.* 14, 510–517 (2012). [PubMed: 22484485]
29. Chubb JR, Boyle S, Perry P & Bickmore WA Chromatin motion is constrained by association with nuclear compartments in human cells. *Curr. Biol.* 12, 439–445 (2002). [PubMed: 11909528]
30. Thomson I, Gilchrist S, Bickmore WA & Chubb JR The radial positioning of chromatin is not inherited through mitosis but is established *de novo* in early G1. *Curr. Biol.* 14, 166–172 (2004). [PubMed: 14738741]
31. Vazquez J, Belmont AS & Sedat JW Multiple regimes of constrained chromosome motion are regulated in the interphase *Drosophila* nucleus. *Curr. Biol.* 11, 1227–1239 (2001). [PubMed: 11525737]
32. Chuang CH et al. Long-range directional movement of an interphase chromosome site. *Curr. Biol.* 16, 825–831 (2006). [PubMed: 16631592]
33. Janicki SM et al. From silencing to gene expression: real-time analysis in single cells. *Cell* 116, 683–698 (2004). [PubMed: 15006351]
34. Shanbhag NM, Rafalska-Metcalf IU, Balane-Bolivar C, Janicki SM & Greenberg RA ATM-dependent chromatin changes silence transcription in *cis* to DNA double-strand breaks. *Cell* 141, 970–981 (2010). [PubMed: 20550933]
35. Tsukamoto T et al. Visualization of gene activity in living cells. *Nat. Cell Biol.* 2, 871–878 (2000). [PubMed: 11146650]
36. Soutoglou E & Misteli T Activation of the cellular DNA damage response in the absence of DNA lesions. *Science* 320, 1507–1510 (2008). [PubMed: 18483401]
37. Bonilla CY, Melo JA & Toczyski DP Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. *Mol. Cell* 30, 267–276 (2008). [PubMed: 18471973]
38. Bergmann JH et al. Epigenetic engineering shows H3K4me2 is required for HJURP targeting and CENP-A assembly on a synthetic human kinetochore. *EMBO J.* 30, 328–340 (2011). [PubMed: 21157429]
39. Luijsterburg MS et al. A new non-catalytic role for ubiquitin ligase RNF8 in unfolding higher-order chromatin structure. *EMBO J.* 31, 2511–2527 (2012). [PubMed: 22531782]
40. Tumber T & Belmont AS Interphase movements of a DNA chromosome region modulated by VP16 transcriptional activator. *Nat. Cell Biol.* 3, 134–139 (2001). [PubMed: 11175745]
41. Verschure PJ et al. *In vivo* HP1 targeting causes large-scale chromatin condensation and enhanced histone lysine methylation. *Mol. Cell Biol.* 25, 4552–4564 (2005). [PubMed: 15899859]
42. Kaiser TE, Intine RV & Dundr M *De novo* formation of a subnuclear body. *Science* 322, 1713–1717 (2008). [PubMed: 18948503]
43. Shevtsov SP & Dundr M Nucleation of nuclear bodies by RNA. *Nat. Cell Biol.* 13, 167–173 (2011). [PubMed: 21240286]

44. Zolghadr K et al. A fluorescent two-hybrid assay for direct visualization of protein interactions in living cells. *Mol. Cell Proteomics* 7, 2279–2287 (2008). [PubMed: 18622019]
45. Zolghadr K, Rothbauer U & Leonhardt H The fluorescent two-hybrid (F2H) assay for direct analysis of protein-protein interactions in living cells. *Methods Mol. Biol.* 812, 275–282 (2012). [PubMed: 22218866]
46. Aymard F et al. Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks. *Nat. Struct. Mol. Biol.* 21, 366–374 (2014). [PubMed: 24658350]
47. Joung JK & Sander JD TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* 14, 49–55 (2013). [PubMed: 23169466]
48. Cong L et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823 (2013). [PubMed: 23287718]
49. Khanna N, Bian Q, Plutz M & Belmont AS BAC manipulations for making BAC transgene arrays. *Methods Mol. Biol.* 1042, 197–210 (2013). [PubMed: 23980009]
50. Stap J et al. Induction of linear tracks of DNA double-strand breaks by alpha-particle irradiation of cells. *Nat. Methods* 5, 261–266 (2008). [PubMed: 18309310]
51. Zotter A et al. Recruitment of the nucleotide excision repair endonuclease XPG to sites of UV-induced dna damage depends on functional TFIIH. *Mol. Cell Biol.* 26, 8868–8879 (2006). [PubMed: 17000769]
52. Bekker-Jensen S et al. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J. Cell Biol.* 173, 195–206 (2006). [PubMed: 16618811]
53. Kim JS, Krasieva TB, LaMorte V, Taylor AM & Yokomori K Specific recruitment of human cohesin to laser-induced DNA damage. *J. Biol. Chem.* 277, 45149–45153 (2002). [PubMed: 12228239]
54. Mari PO et al. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proc. Natl. Acad. Sci. USA* 103, 18597–18602 (2006). [PubMed: 17124166]
55. Roukos V et al. Dynamic recruitment of licensing factor Cdt1 to sites of DNA damage. *J. Cell Sci.* 124, 422–434 (2011). [PubMed: 21224399]
56. Haaf T, Golub EI, Reddy G, Radding CM & Ward DC Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. *Proc. Natl. Acad. Sci. USA* 92, 2298–2302 (1995). [PubMed: 7892263]
57. Stathopoulou A et al. Cdt1 is differentially targeted for degradation by anticancer chemotherapeutic drugs. *PLoS ONE* 7, e34621 (2012). [PubMed: 22479651]
58. Nagy Z & Soutoglou E DNA repair: easy to visualize, difficult to elucidate. *Trends Cell Biol.* 19, 617–629 (2009). [PubMed: 19819145]
59. Nelms BE, Maser RS, MacKay JF, Lagally MG & Petrini JH *In situ* visualization of DNA double-strand break repair in human fibroblasts. *Science* 280, 590–592 (1998). [PubMed: 9554850]
60. Polo SE & Jackson SP Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev.* 25, 409–433 (2011). [PubMed: 21363960]
61. Berkovich E, Monnat RJ, Jr. & Kastan MB Assessment of protein dynamics and DNA repair following generation of DNA double-strand breaks at defined genomic sites. *Nat. Protoc.* 3, 915–922 (2008). [PubMed: 18451799]
62. Iacovoni JS et al. High-resolution profiling of gammaH2AX around DNA double strand breaks in the mammalian genome. *EMBO J.* 29, 1446–1457 (2010). [PubMed: 20360682]
63. Sadler JR, Sasmor H & Betz JL A perfectly symmetric lac operator binds the lac repressor very tightly. *Proc. Natl. Acad. Sci. USA* 80, 6785–6789 (1983). [PubMed: 6316325]
64. Chen B et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155, 1479–1491 (2013). [PubMed: 24360272]
65. Miyanari Y, Ziegler-Birling C & Torres-Padilla ME Live visualization of chromatin dynamics with fluorescent TALEs. *Nat. Struct. Mol. Biol.* 20, 1321–1324 (2013). [PubMed: 24096363]
66. Papapetrou EP & Sadelain M Generation of transgene-free human induced pluripotent stem cells with an excisable single polycistronic vector. *Nat. Protoc.* 6, 1251–1273 (2011). [PubMed: 21886095]

67. Jacome A & Fernandez-Capetillo O Lac operator repeats generate a traceable fragile site in mammalian cells. *EMBO Rep.* 12, 1032–1038 (2011). [PubMed: 21836640]
68. Rouet P, Smih F & Jasin M Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. USA* 91, 6064–6068 (1994). [PubMed: 8016116]
69. Masui O et al. Live-cell chromosome dynamics and outcome of X chromosome pairing events during ES cell differentiation. *Cell* 145, 447–458 (2011). [PubMed: 21529716]

Box 1 |**Phenol/chloroform extraction of the linearized plasmids ● TIMING 4 h**

1. Add to the digestions (Steps 18 and 36) 400 µl of nuclease-free water to a final volume of 500 µl.
2. Add one volume of cold phenol/chloroform mix. Vortex and spin it down briefly. Transfer the supernatant to a new 1.5-ml centrifuge tube.
3. Add one volume of chloroform. Vortex and spin it down briefly at RT. Transfer the supernatant to a new 1.5-ml centrifuge tube.
4. Precipitate DNA by adding 1/10 volume of sodium acetate, pH 5.2 and 2 volumes of ice-cold absolute ethanol and incubate at -20°C for 1 h. Spin down at full speed (20,000g) for 10 min at 4°C in a microcentrifuge.
5. Aspirate off the supernatant and wash the pellet with cold 70% (vol/vol) ethanol. Aspirate off the liquid and either let it air-dry or use a SpeedVac.

▲ CRITICAL STEP Do not overdry the pellet, as this makes it difficult to dissolve.
6. Resuspend the pellet in 20 µl of nuclease-free water, and measure the DNA quantity in a spectrophotometer. Check the linearization by running 500 ng of each sample on an agarose gel as in Step 19 and proceed with Step 20.

Figure 1 |.

Overview of the protocol. The cell line of interest is sequentially transfected with the TetOI-SceI/TetO and LacOI-SceI vectors together with plasmids conferring resistance to antibiotics, and cell clones containing both integrations are isolated (Steps 18–36). Stable cell lines that emerge are transduced with retroviral vectors expressing fluorescent versions of the LacR (green) and TetR (red) repressors (Steps 37–51), and clones are selected on the basis of optimal LacR/TetR expression detected by microscopy (Steps 52–56), from top to bottom: cells with overabundant LacR expression but optimal TetR expression (green nucleus, red dot), cells with optimal LacR and TetR expression (light yellow nucleus, green and red dots), cells with overabundant LacR and TetR expression (bright yellow nucleus, no dots visible) and cells with overabundant TetR expression but optimal LacR expression (red nucleus, green dot). The selected clones are tested for their ability to induce DSBs by colocalization analysis of the *LacO/TetO* arrays with the recruitment of a repair protein (blue dot) after the expression of the endonuclease I-SceI (Steps 57–76) and used to assess DSB dynamics (Steps 77–86).

Figure 2 |.

Characterization of the *LacO/TetO* plasmids. **(a)** Maps of the plasmids containing arrays of the *LacO* operators (256× repeats, 10 kb) and the *TetO* operators (96× repeats, 4 kb) adjacent to the restriction site of the endonuclease I-SceI used to generate cell lines to visualize DSBs. **(b)** Restriction fragment analysis using the XhoI and SacII restriction enzymes, used to verify the integrity of the arrays in the plasmids.

Figure 3 |.

Selection of clones containing various numbers of array integrations. **(a)** Isolated clones with one, two or three TetOI-SceITetO integrations (arrows). The *TetO* operators were visualized by transient expression of the TetR repressor tagged with mCherry. Nuclei were stained with Hoechst. Scale bars, 10 μ m. **(b)** A clone with three TetOI-SceITetO (red) and a single LacOI-SceI (green) integration. *LacO* and *TetO* operators were visualized by transient expression of the GFP-LacR and TetR-mCherry repressors. Nuclei were stained with Hoechst. Scale bar, 10 μ m.

Figure 4 |.

A cell-based system to visualize chromosome damage in living cells. **(a)** The *LacO/TetO* operator/repressor system marks distinct genome sites of integrated recognition sequences of the endonuclease I-SceI. Clones with stable expression of the GFP-LacR and TetR-mCherry repressors, optimal for detection by microscopy, are isolated. **(b)** Cells left untreated or transfected with the endonuclease I-SceI or the inactive form I-SceID44A for 12 h were fixed and immunofluorescence was performed to detect cells that were positive for I-SceI/I-SceID44A expression (anti-HA staining, magenta) and to visualize 53BP1 repair foci (cyan). The percentage of cells with colocalization of the *LacO* arrays (green arrows) and *TetO* arrays (red arrows) with 53BP1 repair foci was calculated and plotted. Scale bars, 10 μ m.

Figure 5 |.

High-throughput time-lapse microscopy to follow DSB dynamics. Cells described in Figure 4a were transfected with the I-SceI plasmid, and 6 h later time-lapse microscopy was performed to follow DSBs marked by the *LacO* (green dots) and *TetO* (red dots) for up to 20 h. Maximal projected image sequences are shown. Scale bar, 10 μ m.

TABLE 1 |

Troubleshooting table.

Step	Problem	Possible reason	Solution
10	Low plasmid yield	Contamination of bacterial cultures	Repeat procedure starting from Step 8. Replate in freshly made LB agar plates supplemented with the antibiotic and inoculate single colonies into liquid cultures
21	No colonies resistant to the antibiotic emerge	Low quality or low expression of the plasmid conferring resistance to the antibiotic	Check the quality of the plasmid expressing the resistance gene. Increase the quantity of the transfected vector
		Excessively high concentration of the selection antibiotic was used	Recalibrate the optimal lower lethal dose of the antibiotic for utilized cell lines
34	Resistant colonies without <i>LacO/TetO</i> integrations	Low quality or recombination of <i>LacO/TetO</i> plasmid	Verify the <i>LacO/TetO</i> plasmid integrity by restriction analysis (Fig. 2)
		Sub-optimal transfection ratio of the plasmids conferring resistance to the antibiotic and the <i>LacO/TetO</i> plasmid	Transfect the <i>LacO/TetO</i> and the plasmid expressing the resistance gene at a 10:1 ratio. If problem persists, transfect at 20:1 ratio
44	No expression of the GFP-LacR/TetR-mCherry in the packaging cell line	Poor quality of the transfection reagent	Repeat with new transfection reagent
		Poor quality of packaging cell line	Check the quality of Plat-GP cells. Exclude contamination by mycoplasma. Check their growth rate/passage. Thaw new vials from earlier passage
49	Lack of GFP-LacR/TetR-mCherry expression in infected cells	Low-titer virus produced	Check transfection efficiency in Plat-GP cells (Step 44, see previous comment). Check the quality of the pVSV-G plasmid
		<i>LacO/TetO</i> cells plated at high confluency	Check <i>LacO/TetO</i> cell plating. Allow cells to grow for one more day and recheck. Restart from Step 37 if necessary
50	Binding of the LacR/TetR at <i>LacO/TetO</i> during cell line maintenance	Did not replenish IPTG and doxycycline (it is required at minimum every 3 d)	The high-affinity, long-term binding of the LacR/TetR repressors to their respective operators may contribute to local instability and the formation of traceable fragile sites. Discard this plate and thaw a new stock
75	Unable to visualize the <i>LacO/TetO</i> arrays	Poor IPTG/doxycycline washes	Repeat protocol. Wash three times with 1× PBS and replat cells in a new plate (add 5 mM IPTG) 2 d before transfection to ensure doxycycline removal. Wash again three times with 1× PBS 5 h before transfection to ensure IPTG removal and transfect
	Low I-SceI expression	Poor I-SceI transfection efficiency	Check the quality of the HAISceI plasmid. If low, ethanol precipitate and re-transfect. High number of electroporated cells. Optimize according to manufacturer's recommendations. Ensure the proper functioning of the Nucleofector machine
	Low viability of transfected cells	Low number of electroporated cells or electroporation of high quantity of the endonuclease ISceI	Optimize the transfected cell number and plasmid quantity taking account manufacturer's recommendations for the cell line used.
		Time of nucleofection exceeded 30 min	After trypsinization complete procedure within 30 min