Published in final edited form as:

Nat Protoc. 2010 July ; 5(7): 1362–1377. doi:10.1038/nprot.2010.102.

Chromosome orientation fluorescence *in situ* hybridization (CO-FISH) to study sister chromatid segregation *in vivo*

Ester Falconer¹, Elizabeth Chavez¹, Alexander Henderson¹, and Peter M. Lansdorp^{1,2}

¹Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

²Division of Hematology, Department of Medicine, University of British Columbia, 675 West 10th Avenue, Vancouver BC, Canada, V5Z 1L3

Abstract

Previously, assays for sister chromatid segregation patterns relied on incorporation of BrdU and indirect methods to infer segregation patterns after two cell divisions. Here we describe a method to differentially label sister chromatids of murine cells and directly assay sister chromatid segregation patterns following one cell division *in vitro* and *in vivo* by adaptation of the well-established CO-FISH (chromosome orientation fluorescent *in situ* hybridization) technique. 5-bromo-2 -deoxyuridine (BrdU) is incorporated into newly-formed DNA strands, followed by photolysis and exonuclease digestion to create single-stranded sister chromatids containing parental template DNA only. Such single-stranded sister chromatids are differentially labeled using unidirectional probes to major satellite sequences coupled to fluorescent markers. Differentially-labeled sister chromatids in post-mitotic cells are visualized using fluorescence microscopy and sister chromatid segregation patterns can be directly assayed after one cell division. This procedure requires four days for *in vivo* mouse tissues, and two days for *in vitro* cultured cells.

INTRODUCTION

To test the proposed biological roles for non-random segregation of sister chromatids^{1,2}, techniques that can distinguish sister chromatids and follow their segregation are necessary, especially for studies of asymmetric cell divisions *in vivo*. We recently described non-random segregation of sister chromatids in a subset of murine colon crypt epithelial cells by directly labeling and assessing the distribution of sister chromatids in post-mitotic colon cells using CO-FISH³. The approach uses the injection of the nucleotide analog 5-bromo-2 -deoxyuridine (BrdU) into mice for the duration of one DNA replication cycle to incorporate BrdU exclusively into newly-formed DNA strands. All BrdU-incorporated newly formed DNA is then removed to leave single-stranded chromosomes composed of parental template DNA only. The invariant orientation of major satellite repeats⁴ is utilized to differentially label single-stranded sister chromatids by hybridizing with unidirectional probes recognizing major satellite repeats coupled to fluorescent dyes, and directly assaying sister chromatid segregation patterns. Here we describe in detail the steps and adaptations of the CO-FISH procedure necessary for identification of sister chromatids in mouse chromosomes and for

Correspondence and requests for materials should be addressed to P.M.L.: plansdor@bccrc.ca.

AUTHOR CONTRIBUTIONS: E.F. wrote the paper and created the figures; E.C. performed most of the CO-FISH and image acquisition for the original study and proofread the manuscript; A.H. performed the animal experiments in the original study and proofread the manuscript; P.M.L. originally developed this procedure, verified the protocol and helped write this paper. We thank S. Poon for helping proofread the manuscript.

COMPETING INTERESTS STATEMENT: The authors declare that they have no competing financial interests.

following sister chromatid segregation in cultured murine cells *in vitro* and mouse tissues *in vivo*.

Non-random sister chromatid segregation and CO-FISH

Previous evidence for asymmetric sister chromatid segregation has been based on long-term radiolabel retention by some cells in the epithelial crypt⁵, or the selective retention of unlabeled chromatids through successive divisions by undifferentiated neural, muscle or epithelial stem cells in pulse-chase experiments^{6–8}. In these studies, BrdU is incorporated into newly-formed DNA strands, and unlabeled template strands are followed through two rounds of cell division^{6–8}. This method indirectly infers sister chromatid segregation patterns from the first cell division based on the distribution of BrdU label in the four granddaughter cells following the second cell division. The granddaughter cell containing no BrdU label is the presumptive stem cell because it is expected not to incorporate BrdU in these assays. However, this type of indirect analysis becomes challenging *in vivo*, where it may be difficult to confidently distinguish daughter cells from granddaughter cells or infer segregation patterns based on the absence of a label. To directly study sister chromatid segregation patterns, they must be visually differentiated in order to follow their distribution to daughter nuclei. This is problematic since the genetic sequence of both sister chromatids is expected to be identical and molecular methods to distinguish one sister from the other have not been available. Most often, the Immortal Strand Hypothesis (ISH) is cited to explain selective retention of specific sister chromatids² in which some stem cells are proposed to selectively retain all sister chromatids containing original parental template strands in order to prevent accumulation of replication errors that may result in genomic instability or cancer⁹.

Recently, we proposed an alternative to ISH, the “Silent Sister Hypothesis”, in which non-random segregation of specific sister chromatids that differ in different epigenetic marks is a mechanism involved in cell fate specification¹. To directly test whether some sister chromatids indeed segregate non-randomly, we have adapted the CO-FISH technique to differentially label murine sister chromatids *in vivo* and directly assay sister chromatid segregation patterns following one cell division³. CO-FISH is a well-established technique that was first developed as a strand-specific *in situ* hybridization protocol to map and orient repetitive regions on chromosomes¹⁰. A full discussion of the evolution of CO-FISH is beyond the scope of this paper. Instead, we refer to the excellent review by Bailey *et al.*¹¹ which details the origin and variations of the CO-FISH technique including studies on the orientation of repetitive regions such as pericentric satellite repeats and telomeres to detect inversions and assay replication timing.

Adaptation of CO-FISH to assay non-random segregation of sister chromatids

We have adapted the CO-FISH method to differentially label sister chromatids and directly assay whether sister chromatids segregate randomly to daughter cells in higher eukaryotes, in one cell division³. First we observed that all mouse chromosomes have a fixed polarity, based on the observation that the uniform head-to-tail orientation of major satellite sequences^{12–14} are fixed relative to chromosome ends (Fig. 1a)³. As a result each complementary chromosomal DNA strand will have either A-rich or T-rich major satellite DNA which can be used to distinguish DNA strands, which we arbitrarily labeled Watson and Crick (Fig. 1b). During replication, these two DNA strands serve as a template for DNA synthesis, giving rise to sister chromatids with identical genetic sequence but each containing one of the original complementary template strands from the parental DNA molecule. By removing newly-formed DNA from sister chromatids, *in situ* hybridization with fluorescently-labeled strand-specific probes recognizing the A-rich and T-rich major satellite repeats, we can distinguish the remaining template strands and thus sister

chromatids from each other. Because the orientation of repetitive major satellite sequences is invariant in all chromosomes of C57BL/6J mice (with the exception of small inversions on chromosomes 4 and 18)^{3,15}, the sister chromatids of all chromosomes in a mitotic cell are distinguished by being directly labeled with fluorescent-coupled probes specific for A-rich or T-rich template strands. Following cell division, the patterns of sister chromatid distribution can be assayed by the patterns of major satellite sequences detected by fluorescent microscopy (Fig. 1c–d).

The approach described was used to study sister chromatid segregation patterns in murine colon epithelial cells. The technique could potentially test sister chromatid segregation patterns in other tissues, however the time course of BrdU administration must be experimentally determined (see section on BrdU incorporation, below). We have also performed CO-FISH on cultured murine embryonic stem cells and fibroblast cells, and describe how to modify the technique for other cultured murine cell types.

Our approach adopts the basic principle of CO-FISH which is to generate single stranded DNA for strand-specific hybridization. However, a number of adaptations allow this procedure to become an assay for sister chromatid segregation. In addition, CO-FISH performed on fixed and sectioned tissues requires a number of modifications to previously published protocols, which we discuss in detail. Since our application of CO-FISH is a quantitative assay for sister chromatid segregation patterns, we discuss all proper control experiments and troubleshooting to allow the correct interpretation of probe fluorescence patterns as sister chromatid segregation patterns. With the proper controls, this approach could be adapted for answering questions about chromatid segregation patterns in other murine cell types *in vivo*.

Limitations of CO-FISH to study sister chromatid segregation patterns

This is the first method that can differentiate between sister chromatids and directly study segregation patterns after one cell division, both *in vivo* and *in vitro* cultured cells. As a result, it is difficult to compare our approach with previously published methods, which indirectly infer asymmetric segregation based on the absence of an incorporated nucleotide analog in one of four granddaughter cells after two cell divisions^{6–8}. In addition, this is the first quantitative technique to assay and compare segregation patterns between different cell types. As discussed, our approach is the only one suited to study segregation patterns *in vivo*, where positional information and niche environments are maintained, without effects of *in vitro* culture. However, the CO-FISH technique is complicated and there are a number of criteria that must be met in order to accept the distribution of template strand fluorescence in post mitotic cells as an accurate reflection of sister chromatid segregation. Some limitations stem from relying on BrdU to incorporate into tissues *in vivo*. First, we cannot rule out that BrdU incorporation affects normal segregation patterns. In addition, BrdU metabolism *in vivo* requires continuous administration via hourly injections. Proper control experiments must also determine that the time course of BrdU administration is restricted to one DNA synthesis phase (S-phase). If these parameters are not met, interpretation of sister chromatid segregation patterns are complicated by the failure to properly label all parental template DNA strands (Fig. 2).

In order to correctly quantify fluorescence values within a particular pair of daughter cells, there must be no overlap with surrounding cells. Nuclear boundaries must be clearly identifiable and isolated from other cells, which limits this technique to tissues where such cells are abundant, and is therefore not suitable for very dense tissue. In addition, care must be taken to ensure that the quantified daughter nuclei are completely encompassed within the sectioned tissue. Any nuclei that are cut due to sectioning will complicate analysis because the entire complement of major satellite sequences in one cell may not be included

in the quantification. In our previous study, we used criteria of reciprocal Watson and Crick fluorescence ratios to exclude such cells from statistical analysis³. In addition, *in situ* hybridization is difficult to combine with immunofluorescent techniques to label and identify cells of interest.

The other major limitation of this approach stems from the fact that random segregation of chromosomes can still result in fluorescence patterns in post-mitotic cells that seem asymmetric. A certain number of apparently asymmetric events are expected from a normal distribution of Watson-template or Crick-template sister chromatids segregated to daughter cells. Since our CO-FISH method differentiates between sister chromatids based on a molecular feature common to all murine chromosomes and does not provide information about specific chromosomes, selective segregation of a small number of chromosomes cannot be detected. Therefore, this approach is only suitable to detect major deviations from random segregation patterns. The Silent Sister Hypothesis predicts that cells could selectively segregate a subset of chromosomes, depending on differential epigenetic modifications that would be required to direct cell fate after cytokinesis¹. Importantly in our original study, we did not observe completely asymmetric segregation in any mitotic colon cells³.

Experimental Design

Generation of single-stranded chromosomes containing template DNA only

The generation of single-stranded chromosomes to which fluorescently labeled probes are hybridized, relies on incorporation of the thymidine analog BrdU into newly-formed DNA strands for one round of cell division, while the unmodified template strands are not BrdU-substituted and are essentially left intact^{4,10,11}. During DNA synthesis, BrdU is administered either by injection *in vivo*, or by addition to tissue culture medium. Following replication and cell division, the newly-formed DNA is removed by staining with the DNA dye Hoechst 33258 which binds the minor groove and sensitizes BrdU-substituted DNA to nicking by UV irradiation¹⁶. Following UV photolysis, digestion by Exonuclease III results in removal of the nicked DNA, leaving single-stranded, intact chromosomes comprised of original parental DNA template strands only. Such single stranded chromosomes are a suitable target for hybridization with unidirectional fluorescently labeled probes such as PNA probes^{10,11,17}.

Probe design and hybridization

Peptide nucleic acid (PNA) probes have higher affinity and hybridize more stably to single-stranded DNA than regular DNA or RNA oligonucleotide probes¹⁸. We designed strand-specific PNA probes (18-mers) complementary to either the A-rich or T-rich repetitive sequences within murine major satellite DNA³. Such PNA probes were directly coupled to Cy3 or Cy5 fluorophores, respectively (Table 1). These probes allow differential fluorescent labeling of single-stranded sister chromatids. Three fluorescent probe molecules hybridize within each 234 bp major satellite repeat¹⁹ representing 240 kb to greater than 2000 kb in length per mouse chromosome²⁰. This results in thousands of fluorescent molecules hybridized to major satellite repeats during CO-FISH, allowing for easy detection and quantification in sister chromatid segregation assays. The total quantified fluorescence in each daughter cell after CO-FISH corresponds roughly to the total number of Watson or Crick template strands in each cell to indicate sister chromatid segregation patterns during mitosis. Note that not all chromosomes have the same amount of major satellite DNA³ and such variation needs to be taken into account in modeling and statistical analysis.

Prior to hybridization of the PNA probes DNA is denatured at 80 °C in 70% formamide solution. These conditions minimize non-specific hybridization and ensure denaturation of

any complementary PNA probe interactions. Hybridization is achieved overnight at room temperature (21 °C to 24 °C) in a low ionic strength hybridization buffer containing 70% formamide, which favors PNA probe hybridization to single-stranded targets but prevents re-annealing of complementary DNA sequences¹⁸. These conditions can be used to determine whether BrdU incorporation resulted in single-stranded chromosome targets for PNA probe hybridization, or whether double stranded DNA sequences remain. The presence of overlapping fluorescence signals from PNA probes indicates that both PNA probes hybridized to double-stranded DNA at major satellite sequences, suggesting that BrdU incorporation, subsequent Hoechst incorporation, UV treatment or exonuclease digestion was incomplete (see Troubleshooting). Conditions that favour double stranded DNA re-annealing over probe hybridization would prevent assessment of whether single stranded chromosome preparation was complete. Hybridization is followed by washes in 70% formamide to remove unbound probe, and cells or chromosomes are counterstained with DAPI to visualize DNA.

BrdU incorporation

The success of differentially-labeling sister chromatids using unidirectional major satellite probes depends entirely on the generation of single-stranded chromosomes containing only parental template strand DNA¹¹. One critical variable is complete and continuous incorporation of the BrdU nucleotide analog into newly-formed DNA strands during S-phase. If BrdU is not incorporated for the entire duration of S-phase, newly-formed DNA will not be completely removed, as regions without BrdU incorporation will remain annealed to the template strands (Fig. 2a). Since major satellite repeats are part of pericentric heterochromatin and replicate in mid to late S-phase²¹, decreasing BrdU incorporation at the tail end of DNA synthesis will result in double-stranded major satellite repeat regions remaining after the CO-FISH procedure, and thus overlapping probe signals. Similarly, administering BrdU for more than one cell cycle will result in two rounds of BrdU incorporation into DNA and will also complicate analysis of sister chromatid segregation. Therefore, it is critical to determine the length of one cell cycle in the cells to be assayed, by performing a BrdU time-course experiment. For mitotic colon epithelial cells, we injected mice with BrdU hourly for 8, 12 and 16 hours as well as a single injection of colcemid to arrest mitotic cells at metaphase 1 hour prior to tissue harvest. To determine length of one cell cycle, we performed CO-FISH and assayed metaphase cells for overlapping (incomplete BrdU incorporation), paired (one complete S-phase) or unpaired (two rounds of BrdU incorporation) probe signals (Fig. 2b). Paired, non-overlapping probe signals in all metaphase chromosomes indicate one complete round of DNA replication in the presence of BrdU (Fig. 2b, 12 hours BrdU). Overlapping probe signals indicates that newly-formed DNA strands were not completely removed likely due to incomplete incorporation of BrdU for the entire duration of S-phase (Fig. 2b, 8 hours) while unpaired red or green probe signals at a subset of chromosomes indicates that BrdU was incorporated for two cell divisions, and results in both newly-formed and template strands removed during CO-FISH procedure. This means that only one template strand is available for labeling with PNA probes (Fig. 2b, 16 hours). In 100 metaphases tested, there were no instances of overlapping or unpaired probes when 12 hours of BrdU was administered (data not shown). Therefore, for colon epithelial cells, 12 hours approximates one complete cell cycle. Similar control experiments will have to be performed when adapting this CO-FISH technique to other cells or tissues.

In our studies, BrdU was injected into mice at hourly intervals for the entire duration of S-phase in colon epithelial cells (12 hours) in order to ensure continuous and consistent incorporation of BrdU during S-phase³. Injected BrdU is rapidly metabolized and unless BrdU is injected hourly and at a relatively high dose range of 20–30 mg kg⁻¹ body weight

(per injection)²², BrdU incorporation into DNA will be variable and removal of newly formed DNA during CO-FISH will be incomplete. In our hands, 3 injections of BrdU at 4 hour intervals resulted in overlapping probe signals on chromosomes in post-mitotic daughter cells indicating incomplete incorporation of BrdU into newly formed DNA (Fig. 3, top panels). Similarly, BrdU administered via drinking water did not result in sufficient incorporation of BrdU in bone marrow cells and does not allow fine-tuning of BrdU dosing (data not shown). An alternative approach which has not been explored is continuous BrdU infusion using a pump.

Visualizing post-mitotic cells

Assaying sister chromatid segregation patterns requires acquisition of images using a fluorescence microscope (coupled with a suitable camera) that is equipped with the appropriate filters to detect the specific emissions from the fluorescence-coupled PNA probes. For colon crypt epithelial cells, post-mitotic cells appeared as distinct paired cells projecting into the colon crypt, separate from non-mitotic epithelial tissue (Fig. 1d, boxed). To easily find post-mitotic cell pairs with non-overlapping probes, we scan slides manually through the ocular lens using the Cy3 emission filter. Post-mitotic cells projecting into the colon crypt generally contain brighter Cy3 fluorescence than the surrounding non-mitotic tissue (data not shown). Switching to the Cy5 emission filter confirms whether probe fluorescence territories are distinct, and cell pair could be digitally captured for quantification. It is important to note that cell pairs must not overlap with surrounding non-mitotic tissue (Fig. 1d top left panel, yellow arrowhead) in order to prevent fluorescence due to unrelated cells from being included in the quantification. In addition, daughter cells must not overlap with each other. Refer to Anticipated Results section for typical CO-FISH images of post-mitotic nuclei, and Limitations of CO-FISH section to determine which tissues are suitable for this assay.

Quantification of PNA probe fluorescence to assay sister chromatid segregation

To generate digital images suitable for quantification, high-quality, high numerical aperture lenses are recommended (e.g. Plan Apochromat 60 $\times$ /1.4 or equivalent) in order to maximize the detection of the fluorescent probes. We acquired images on a Coolsnap HQ digital camera attached to an inverted microscope (IX70 Olympus) fitted to an imaging system (DeltaVision RT, Applied Precision) and equipped with filter sets to detect DAPI, Cy3, Cy5, Texas Red and FITC. For tissue sections, a stack of images at 0.2 μ m intervals is acquired to cover the entire thickness of the section and maximize signal resolution in the Z-plane. It is critical to ensure that the fluorescence signal from any focal plane image does not approach saturation. Since focal planes are projected into a single image, any pixel saturation will affect signal quantification and therefore interfere with interpretation of chromatid segregation patterns. In addition, since multiple focal planes are acquired for each image, it is important to prevent photobleaching by limiting light exposure for any given focal plane. For our studies, fluorescence signals are projected onto a single plane using ISIS (Metasystems) or SoftWoRx (Applied Precision) software with or without deconvolution.

We quantified specific fluorescence from each probe in each daughter cell using dedicated software^{3,23}. The image manipulation steps and specific software requirements for quantifying fluorescence signals are beyond the scope of this paper. For a more detailed discussion of CO-FISH fluorescence measurements and subsequent statistical tests for non-random segregation, we refer to our previous discussion of image acquisition and signal quantification¹⁸ and our original manuscript and its Supplementary Information³. Briefly, to assess chromatid segregation patterns we normalized measured fluorescence values based on the assumption that for each chromosome the Watson template sister chromatid will be segregated to one daughter while the Crick template chromatid will be segregated to the

other daughter (Fig. 1c). Therefore, the measured fluorescence from each probe in each daughter will be a fraction of 1. Sister chromatid segregation patterns can be assessed and compared to values expected by chance using simulated random segregation³.

Adapting CO-FISH technique for cultured cells

Using the CO-FISH technique to assay sister chromatid segregation patterns in cultured cells includes a number of modifications. BrdU is added to culture medium at a final concentration of 40 μM for the duration of one S-phase (this must be experimentally determined, to exclude incomplete BrdU incorporation or granddaughter cells from complicating analysis of segregation patterns). For murine embryonic stem cells and cultured lung fibroblasts, BrdU was added to the culture medium for 12 hours prior to harvest of cells. In order to allow daughter nuclei to remain adjacent to each other in a background of single cells, post-mitotic binucleated cells are prepared by preventing cytokinesis with 3 $\mu\text{g ml}^{-1}$ (final concentration) of cytochalasin B for 2 hours prior to harvesting cells. Cells are spun onto slides using a cytopsin apparatus prior to fixation. The details for the CO-FISH procedure for fixed cultured cells appear in the Procedure below.

Controls

We recommend performing anti-BrdU immunofluorescence staining on a set of slides to ensure BrdU was incorporated into tissues and cultured cells³. Because of the difficulty combining immunofluorescence with *in situ* hybridization, we recommend that anti-BrdU immunofluorescence be performed on a section of tissue directly adjacent to those undergoing CO-FISH. We also recommend including a set of slides from a mouse or cultured cell line which did not undergo BrdU treatment. This allows comparison to post-mitotic cells in which chromosomes are not single-stranded. Though the proportion of post-mitotic cells or tissues may be low depending on the tissue or cell line studies, BrdU-treated cells or tissues are expected to show some cells which have distinct non-overlapping probe signal. These two controls determine whether BrdU was incorporated, and whether the PNA probes successfully hybridized to single stranded DNA targets. Further troubleshooting advice is given in Table 2.

MATERIALS

REAGENTS

- Experimental animals: The rate of cell division will vary among cells, tissues and species of laboratory animals. The procedures described here were tested with colon epithelial cells in *Mus musculus* (strain C57BL/6J) mice aged 8 to 12 weeks. Assessment of sister chromatid segregation patterns was performed using 6 μm sections of paraffin-embedded formaldehyde fixed colon. Please see Experimental Design for adaptation of techniques for other tissues. All *in vivo* experiments must be performed using facility-approved guidelines and regulations.
- Cultured cells: The rate of cell division will vary among tissues and cell lines. The procedures described here work for *Mus musculus* wild-type embryonic stem cells (C2, C57BL/6NTac background and C1^{+/+}, 129S1 background)³ and *Mus musculus* fetal lung fibroblasts. Please see Experimental Design for how to adapt technique for other cell lines.
- Dulbecco's Modified Eagle's Medium (DMEM, StemCell Technologies, cat. no. 36250)
- Fetal Calf Serum (FCS, HyClone Cosmic Calf Serum, cat. no. SH30087-04)
- Trypsin in Citrate Saline, 0.25% (StemCell Technologies, cat. no. 07400)

- Distilled, deionized water (ddH₂O)
- Concentrated hydrochloric acid (Fisher Scientific, cat. no. 351278-212) for pH adjustments. **CAUTION:** Concentrated HCl is destructive to respiratory tract, eyes and skin. Wear appropriate gloves and safety glasses.
- Sodium citrate (Sigma-Aldrich, cat. no. S1804-500g)
- Citric acid (EMD, cat. no. CX1723-1 500g)
- NaCl (EMD Biosciences, cat. no. 7760)
- Trizma base (Sigma-Aldrich, cat. no. T1503-5kg)
- RNase A Type III-A (Sigma-Aldrich cat. no. R5215-250mg)
- Sodium acetate (Sigma-Aldrich, cat. no. S-8750) for preparing RNase A stock solution
- Bovine serum albumin (BSA, Sigma-Aldrich, cat. no. A2153-50g)
- Peptide nucleic acid probes (PNA) coupled to suitable fluorescent dyes (in-house synthesis or commercial source, e.g., from Panagene (www.panagene.com))
- Hoechst 33258 (Sigma-Aldrich, cat. no. 861405-100mg). **CAUTION:** Hoechst 33258 is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.
- Formamide (99.5 %, EMD Biosciences, cat. no. FX0425-3) for DNA denaturation and wash steps. **CAUTION:** Formamide is a poison and a mutagen; prevent contact with skin and eyes. Wear gloves and work in a hazardous fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to institutional rules and regulations.
- Ultra-pure formamide (99 %, Invitrogen, cat. no. 15515-026) Must be deionized prior to use in hybridization mixture; see REAGENT SETUP. **CAUTION:** Formamide is a poison and a mutagen; prevent contact with skin and eyes. Wear gloves and work in a hazardous fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to institutional rules and regulations.
- Mixed bed resin for deionizing formamide (Bio-Rad, cat. no. AG 501-X8(D))
- 5-bromo-2 -deoxyuridine (BrdU, 99 %, Sigma, cat. no. B9258) **CAUTION:** BrdU may cause skin irritation and may be harmful if absorbed through the skin or inhaled. Work with appropriate personal protective gear.
- Colcemid (Gibco, cat. no. 1510-040 10ml) **CAUTION:** Colcemid is toxic to cells. Wear gloves and protect eyes and skin from contact.
- Cytochalasin B (Sigma-Aldrich, cat. no. C2743) **CAUTION:** Colcemid is toxic to cells. Wear gloves and protect eyes and skin from contact.
- 10% formalin, acetate-buffered (Fisher Scientific, cat. no. SF99-4L). **CAUTION:** Formalin is toxic. Avoid inhalation and contact with skin. Wear gloves and work in hazardous fume hood. Formalin waste must be disposed of properly in accordance with institutional guidelines.
- 1× phosphate buffered saline (PBS, without Ca²⁺ and Mg²⁺, StemCell Technologies, cat. no. 37350) for BrdU injections.

- 10× phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+} , StemCell Technologies, cat. no. 37354). Working solutions for washes will be prepared fresh by diluting 10× PBS with ddH₂O.
- Xylenes (Fisher Scientific, cat. no. X16-4). **CAUTION:** Xylenes are toxic organic solvents. All work with Xylenes must be carried out in hazardous fume hood. Wear gloves and protect from contact with skin and eyes. Xylenes waste must be disposed of properly in accordance with institutional guidelines.
- Absolute (100 %) ethanol. **CAUTION:** Flammable. Avoid inhalation.
- Pepsin (Sigma-Aldrich, cat. no. P7000-100g)
- Exonuclease III (New England Biolabs, cat. no. M0206L)
- Tween-20 (Polyoxyethylenesorbitane monolaurate, Sigma-Aldrich, cat. no. P1379-500ml). **CAUTION:** May cause eye and skin irritation.
- Blocking reagent (Perkin-Elmer, cat. no. FP-1012)
- Magnesium Chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, cat. no. M9272)
- 4 ,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, cat. no. D9542-10mg). **CAUTION:** DAPI is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.
- 1,4 Diazabicyclo-[2,2,2]-octane (DABCO, Sigma-Aldrich, cat. no. D2522) to prepare antifade mounting medium. **CAUTION:** DABCO is toxic. Wear gloves when handling powdered format. Avoid inhalation
- Glycerol (99.5 %, Sigma-Aldrich, cat. no. G789-3)
- Methanol (EMD Biosciences, cat. no. MX0485-7). **CAUTION:** Methanol is toxic and very flammable. Wear gloves when handling and avoid inhalation. Use of hazardous fume hood is recommended.
- Acetic acid, glacial (Fisher Scientific, cat. no. 351271-212). **CAUTION:** Glacial acetic acid is volatile and destructive to eyes, skin and respiratory tract. Wear gloves and handle in hazardous fume hood.
- Formaldehyde solution, 37 % (VWR, cat. no. VW3408-1). **CAUTION:** Formaldehyde is toxic and destructive to skin, eyes and respiratory tract. Wear gloves and use in hazardous fume hood only. Dispose of waste formaldehyde properly in accordance with institutional regulations.

Equipment

- Whatman Grade 1 filter paper, 15 cm diameter (VWR, cat. no. 28450-150)
- Dissecting tools
- 1ml syringes
- 26G 1-inch needles
- 23G blunt needles
- plastic petri dishes or other suitable dishes for temporarily holding dissected tissues
- 15 ml polypropylene conical tubes, 17 × 120 mm (BD Falcon, cat. no. 352096)
- 50 ml polypropylene conical tubes, 30 × 115 mm (BD Falcon, cat. no. 352070)
- lint-free tissues (Kimwipes, Kimtech Science, cat. no. 34120)

- Vibratome/Microtome or facility dedicated to embedding and sectioning biological samples
- Slide warmer or heating plate with thermostat
- slide holders
- 50 ml white polypropylene screw-top Coplin staining jars (VWR, cat. no. 47751-792). **CRITICAL:** Standard Coplin staining jars contain slots for 5 slides, but 9 slides may be placed in a zigzag pattern and allow sufficient interaction between tissue sections and buffer components, for all steps except for denaturation (Steps 34 and 44) during which a maximum of 5 slides per jar is recommended. It is important not to crowd slides together, therefore slide staining chambers with very closely spaced slots for slides are not recommended for any part of this protocol.
- Wheaton glass slide staining chambers, 100 ml capacity (VWR, cat. no. 25455-000)
- UV oven (Stratalinker 1800)
- Bacterial incubator (37 °C)
- Hybridization oven (80 °C) with aluminum plate equilibrated to 80 °C
- Water Baths (37–80 °C; two water baths must be available to use at 37 °C and 72 °C simultaneously during antigen retrieval)
- Shaker (Cole-Parmer Rotatest model no. 51500-10)
- Microscope slides 25 × 75 × 1 mm (VWR, Superfrost Plus White, cat. no. 48311-703)
- Cover-glass forceps (VWR, cat. no. 82027-390)
- Parafilm-M sealing film (VWR, cat. no. 52858-000)
- Micro cover-glasses 22 × 60 mm No.1 (VWR, cat. no. 16004-330)
- Cytospin (Shandon Cytospin 4, Shandon Scientific)
- Fluorescent microscope/camera/digital image processing software

REAGENT SETUP

- Prepare **5 M NaCl**. In a clean 100 ml graduated cylinder, add 29.2 g NaCl and ddH₂O up to 100 ml. Add magnetic stir bar and stir until dissolved, heating if necessary. Store at room temperature up to one year.
- Prepare **ultrapure deionized formamide**. In hazardous fume hood, carefully pour 500 ml ultra pure (≥99.5%) formamide into 1 l glass Erlenmeyer flask. Mix 25 g mixed bed resin with 25 ml of formamide. Swirl, discard formamide and add the washed resin to the 500 ml ultra pure formamide. Stir with magnetic stir bar for approximately 2 h, in hazardous fume hood. Leave at room temperature overnight. Filter twice with Whatman Grade 1 filter paper. Aliquot in 50 ml tubes and store at –20 °C for up to 1 year. **CAUTION:** Formamide is a poison and a mutagen; prevent contact with skin and eyes. Wear gloves and work in a hazardous fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to institutional rules and regulations.
- Prepare **20× SSC Buffer** (3 M NaCl, 300 mM sodium citrate). Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 ml ddH₂O. Adjust pH to 7.0 with few drops

of concentrated HCl. Adjust volume to 1 L. Sterilize by autoclaving. Store at room temperature up to one year. **CAUTION:** Concentrated HCl is destructive to respiratory tract, eyes and skin. Wear appropriate gloves and safety glasses, and handle in hazardous fume hood.

- Prepare **1 M Tris-HCl pH 7.6**. Dissolve 121.1 g Trizma Base in 800 ml ddH₂O. In hazardous fume hood, slowly add approximately 60 ml concentrated HCl. Allow to cool to room temperature to make final adjustments to pH. Adjust volume to 1 L with ddH₂O. Sterilize by autoclaving. Store at room temperature for several months. **CAUTION:** Concentrated HCl is destructive to respiratory tract, eyes and skin. Wear appropriate gloves and safety glasses, and handle in hazardous fume hood.
- Prepare **100 mM Tris-HCl pH 6.5**. Dissolve 1.21 g Trizma Base in 80 ml ddH₂O. In hazardous fume hood, slowly add concentrated HCl while closely monitoring pH. Adjust volume to 100 ml with ddH₂O and sterilize by autoclaving. Store aliquots at -20 °C up to one year. Allow to cool to room temperature to make final adjustments to pH. Adjust volume to 1 l with ddH₂O. Sterilize by autoclaving. Store at room temperature for several months. **CAUTION:** Concentrated HCl is destructive to respiratory tract, eyes and skin. Wear appropriate gloves and safety glasses, and handle in hazardous fume hood.
- Prepare **1 M sodium acetate buffer**, pH 5.2. Dissolve 13.61 g sodium acetate into 80 ml ddH₂O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 100 ml with ddH₂O, sterilize by autoclaving. Store at room temperature up to one year. **CAUTION:** Glacial acetic acid is volatile and destructive to eyes, skin and respiratory tract. Wear gloves and handle in hazardous fume hood.
- Prepare **100 mM Citric Acid Buffer** for use in MgCl₂ hybridization buffer component. Dissolve 1.92 g citric acid (anhydrous) in 80 ml ddH₂O. Adjust volume to 100 ml. Filter-sterilize using 0.4 µm membrane. Store at room temperature up to one year.
- Prepare **1 M Na₂HPO₄ buffer** (sodium phosphate). Dissolve 14.2 g Na₂HPO₄ (sodium phosphate, dibasic) into 80 ml ddH₂O with aid of magnetic stir bar. Adjust volume to 100 ml. Filter-sterilize with 0.4 µm membrane. Store at room temperature up to one year.
- Prepare **1 M MgCl₂**. Dissolve 20.33 g MgCl₂·6H₂O in 80 ml of ddH₂O. Adjust volume to 100 ml. Filter-sterilize using 0.4 µm filter unit. Dispense into small aliquots and freeze at -20 °C up to one year.
- Prepare stock solution of **MgCl₂ hybridization buffer component** for use in hybridization (82 mM Na₂HPO₄, 9 mM citric acid, 20 mM MgCl₂). To 8.08 ml ddH₂O, add 820 µl of 1M Na₂HPO₄, 900 µl of 100 mM citric acid and 200 µl of 1 M MgCl₂. Mix by inversion. Dispense into small aliquots and freeze at -20 °C up to one year.
- Prepare **100 mM Citrate Buffer** (100 mM sodium citrate, 100 mM Citric Acid). Dissolve 19.2 g Citric Acid and 29.41 g Sodium Citrate in 800 ml ddH₂O. Adjust volume to 1 l. Store at room temperature up to one year. Buffer will be warmed to 80 °C prior to antigen retrieval step (Step 17).
- Prepare **acidified (pH 2.0) ddH₂O** by slowly adding appropriate amount of Hydrochloric Acid to ddH₂O, while monitoring with pH meter. Store at room temperature for up to one year. Allow to cool to room temperature to make final adjustments to pH. Adjust volume to 1 l with ddH₂O. Sterilize by autoclaving.

Store at room temperature for use several months. **CAUTION:** Concentrated HCl is destructive to respiratory tract, eyes and skin. Wear appropriate gloves and safety glasses, and handle in hazardous fume hood.

- Prepare stock solution of **10 % (wt/vol) BSA** in ddH₂O. Add 10 g BSA to 80 ml ddH₂O, and make up to 100 ml. Stir with magnetic stir bar, heating if necessary to dissolve. Filter with 0.22 µm filter and store aliquots at -20 °C up to one year.
- Prepare stock solutions of **100 mg ml⁻¹ DNase-free RNase A** in ddH₂O. To make 100 mg ml⁻¹ RNase A, add 2.25 ml of 10 mM sodium acetate (pH 5.2) directly to 250 mg vial of lyophilized RNase A Type III-A (vial from manufacturer). Mix by inversion on rotator at room temperature. Place vial in container of water and boil for 15 minutes to destroy residual DNase activity. Let vial cool to room temperature and neutralize with 250 µl 1 M Tris-HCl, pH 7.4. Aliquot stock solutions into 1.5 ml microcentrifuge tubes and freeze at -20 °C up to one year.
- Prepare stock solutions of **10mg ml⁻¹ Hoechst 33258** in ddH₂O. Add 3 ml of ddH₂O to 100 mg vial (from manufacturer). Mix well using vortexer. Transfer to 15 ml polypropylene tube and make up volume to 10 ml with ddH₂O. Mix well. Freeze aliquots at -20 °C up to one year. Working solutions will be freshly diluted to 1 mg ml⁻¹ in ddH₂O. Ensure that all stock and working solutions are protected from light. **CAUTION:** Hoechst 33258 is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.
- Prepare **2.5% (wt/vol) Blocking Reagent** stock solution. Add 2.5 g Blocking Reagent to 100 ml nuclease-free ddH₂O. Stir gently overnight, heating to 60 °C to facilitate dissolving; filter using a 0.4 µm membrane. Store at -20 °C in aliquots for up to one year. Store working aliquot at 4 °C for up to four weeks
- Prepare stock solution of **10 mg ml⁻¹ DAPI** and working solution of **1 mg ml⁻¹ DAPI**. To make 10 mg ml⁻¹ DAPI, add 1 ml ddH₂O directly to 10 mg vial of lyophilized DAPI (vial from manufacturer). Mix by inversion on rotator at room temperature, protecting from light. To make 1 mg ml⁻¹ working solution, mix 100 µl of 10 mg ml⁻¹ DAPI solution with 900 µl ddH₂O in 1.5 ml microcentrifuge tube. Mix by inversion, store at 4 °C up to four weeks, protected from light. Split remainder of 10 mg ml⁻¹ stock solution into 100 µl aliquots in 1.5 ml microcentrifuge tubes. Freeze aliquots at -20 °C for up to one year. **CAUTION:** DAPI is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.
- Prepare **Formamide/2× SSC denaturation solution**. For each set of 5 slides, a Coplin jar of 50 ml formamide/2× SSC denaturation solution must be prepared fresh and preheated to 72 °C prior to denaturation (Steps 33 and 43). In a hazardous fume hood, for each Coplin jar, mix 10 ml ddH₂O, 5 ml 20× SSC, 35 ml formamide (50 ml total). Mix by retropipetting. Place in 72 °C water bath to equilibrate to temperature. **CAUTION:** Formamide is a poison and a mutagen; prevent contact with skin and eyes. Wear gloves and work in a hazardous fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to institutional rules and regulations.
- Prepare **70%, 90% (vol/vol) and 100% ethanol** in 50 ml Coplin jars, place in -20 °C freezer to chill until ice-cold.
- Prepare **70%, 90% (vol/vol) and 100% ethanol** in 500 ml plastic bottles for room temperature dehydration steps. Store at room temperature for several weeks.

- Prepare working dilutions of **PNA probes** (20–50 $\mu\text{g ml}^{-1}$) in ddH₂O and store at 4° C for up to four weeks. Ensure fluorescent-coupled probe solutions are protected from light.
- Prepare **PNA hybridization mixture**. For each slide, prepare 50 μl : In microcentrifuge tube, mix 5 μl 100 mM Tris-HCl pH 6.5 (10 mM final), 2.5 μl MgCl₂ buffer, 5 μl Blocking Reagent (0.25% final), and each PNA probe to a final concentration of 1 $\mu\text{g ml}^{-1}$. Add ddH₂O to make up to 15 μl . In hazardous fume hood, add 35 μl deionized ultrapure formamide (70% final). This solution should be made fresh just prior to hybridization step. **CRITICAL:** Deionized formamide must be added last, in hazardous fume hood. Warm working solutions of PNA probes (20–50 $\mu\text{g/ml}$) in 72 °C water bath if precipitates are noticed. Spin tubes briefly prior to adding probes to hybridization mixture, to prevent transfer of probe aggregates. It's important to protect probes and hybridization mixture from light.
- Prepare **formamide wash solution** for post-hybridization washes. Two sequential formamide washes are necessary (Step 51), therefore prepare two Coplin jars for each set (up to 9) of experimental slides. To each Coplin jar in hazardous fume hood, add 14 ml ddH₂O, 500 μl 1M Tris pH 7.4 (10mM final), 500 μl 10% BSA (0.1% final) 35 ml Formamide (70% final). Add formamide last, mix contents by retropipetting. Prepare fresh just prior to use. **CAUTION:** Formamide is a poison and a mutagen; prevent contact with skin and eyes. Wear gloves and work in a hazardous fume hood to avoid inhaling fumes. Formamide waste should be collected and disposed of according to institutional rules and regulations.
- Prepare **TNT wash solution** fresh prior to use. Three TNT washes are required for each experiment. For 150 ml, (sufficient for 3 washes of 50 ml each) mix 129.3 ml ddH₂O, 15 ml 1M Tris-HCl (pH 7.4, 100 mM final), 4.5 ml 5M NaCl (150 mM final) and 1.2 ml of Tween-20. Mix well by inversion. Discard any unused wash buffer.
- Prepare **DAPI counterstain solution** fresh before use. Dilute 50 μl of 1mg ml^{-1} DAPI working solution into 50 ml 1× PBS. Mix well, transfer to Coplin staining jar. Protect DAPI stock solution and working solution from light. **CAUTION:** DAPI is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.
- Prepare **DABCO mounting medium**. In 15 ml screw-top polypropylene tube, dissolve 233 mg DABCO in 800 μl ddH₂O by vortexing. Add 200 μl Tris-HCl pH 8.0 and 9 ml 100% glycerol. Mix gently by inversion overnight. Aliquot into 1.5 ml microcentrifuge tubes and freeze at –20 °C for up to one year. Store aliquots in use at 4 °C for up to four weeks.
- Prepare **3:1 methanol:acetic acid fixative solution**. Must be prepared fresh for fixing cytospin-prepared cells for CO-FISH. To make 150 ml of fixative, mix 112.5 ml methanol and 37.5 ml glacial acetic acid in a glass bottle. Mix by inversion. Discard any unused fixative according to institutional regulations. **CAUTION:** Methanol is toxic and very flammable. Wear gloves when handling and avoid inhalation. Use of hazardous fume hood is recommended. Glacial acetic acid is volatile and destructive to eyes, skin and respiratory tract. Wear gloves and handle in hazardous fume hood.
- Prepare **formaldehyde fixative** for fixing cultured cells spun onto slides. In Coplin staining jar, prepare 4% (vol/vol) formaldehyde solution in 1× PBS. To 39.6 ml ddH₂O, add 5 ml 10× PBS and 5.4 ml 37% formaldehyde solution. Mix by retropipetting. This solution should be prepared fresh each day. Store at room

temperature in hazardous fume hood until needed. Discard at end of day.

CAUTION: Formaldehyde is toxic and destructive to skin, eyes and respiratory tract. Wear gloves and use in hazardous fume hood only. Dispose of waste formaldehyde properly in accordance with institutional regulations.

- Prepare **DABCO+DAPI mounting medium** for counterstaining and mounting slides, only when performing CO-FISH on cultured cells spun on slides. In 15 ml screw-top polypropylene tube, dissolve 233 mg DABCO in 800 μ l ddH₂O by vortexing. Add 200 μ l Tris-HCl pH 8.0 and 2 μ g DAPI (2 μ l of 1 mg ml⁻¹ DAPI solution) and mix well. Add 9 ml 100% glycerol. Cover tube with foil to protect from light and mix gently by inversion overnight. Aliquot into 1.5 ml microcentrifuge tubes and freeze at -20 °C up to one year. Store aliquots in use at 4 °C up to four weeks, protected from light. **CAUTION:** DAPI is a known mutagen. Handle with gloves and dispose safely in accordance with institutional regulations.

PROCEDURE

BrdU Incorporation

- 1 For BrdU incorporation, follow option A for *in vivo* tissues, and option B for cultured cells.

- A Intraperitoneal injections of BrdU into mice

TIMING: 11 hours for injections

- i. Hand-warm aliquots of 20 mM BrdU solution in PBS. Fill 1 ml syringe fitted with 26-gauge needle with warmed solution.
- ii. Prepare mouse for intraperitoneal injections according to approved protocols from animal facility. Isolate lower abdominal cavity. Tilt the mouse with its head slightly toward the ground to shift abdominal viscera cranially and to prevent accidental puncture of abdominal organs at the site of injection.
- iii. Inject 100 μ l of BrdU solution into each mouse. **CRITICAL STEP:** This gives a range of 20 to 30 mg BrdU kg⁻¹ body weight (per injection), and results in sufficient incorporation of BrdU into colon epithelial cells to prepare single-stranded chromosomes for the CO-FISH procedure. For other tissue types, BrdU dose will need to be determined experimentally.
- iv. If target tissue is colon epithelial cells, repeat injections hourly, giving a total of 11 injections over a 12 hour period. This results in a total dose of 220 – 330 mg BrdU kg⁻¹ body weight. **CRITICAL STEP:** Timing and dosage of BrdU injections must be consistent to maintain a steady level of BrdU in the mouse. For other tissues, timing and dosage may be determined experimentally. At end of injection protocol, proceed immediately to harvesting of tissue and fixation (Step 2).

- B BrdU treatment of cultured cells.

TIMING: 12 HOURS (generally performed overnight)

- i. Culture cells according to accepted protocols specific for cell type. For our studies, feeder-free murine embryonic stem cells were cultured on gelatin-coated plastic dishes in DMEM/20% FCS and 100 ng ml⁻¹ of leukaemia inhibitory factor²⁴. Murine fetal lung fibroblasts were grown in DMEM containing 10% FCS.
- ii. To semi-confluent cultures, add 40 μ M (final) BrdU for a total of 12 hours prior to harvesting cells. Total length of BrdU incorporation will depend on length of S-

phase, and must be determined experimentally for each cell type, as described in Experimental Design.

- iii. To obtain binucleated cells, add $3 \mu\text{g ml}^{-1}$ (final) Cytochalasin B to cultures for final 2 hours prior to harvesting cells.

Harvest and Fixation of BrdU-incorporated Tissues and Cells

- 2 For harvesting and fixation procedure, follow option A for *in vivo* tissues, and option B for *in vitro* cultured cells.
- A Harvesting and fixation of mouse tissues.

TIMING: 1 hour for preparation of tissues plus overnight for fixation

- i. At end of 12-hour BrdU injection protocol (Step 1Aiv), sacrifice mouse by CO₂ asphyxiation or other facility-approved method.
- ii. With proper dissecting tools, isolate colon. Sever the ascending colon at the junction to the caecum, leaving rest of colon intact. Flush the faeces from the colon with a 5 ml syringe fitted with a 16 gauge blunt-end needle, filled with ice-cold 1× PBS. Flush by inserting needle into the intact rectum, flushing through the newly severed ascending colon opening.
- iii. Dissect the descending colon from the rectum and place flushed colon section into petri dish containing ice-cold 1× PBS.
- iv. Fill 50 ml polypropylene tube with 20 ml buffered 10% formalin fixative solution. Cut the colon longitudinally. While holding one end of the colon with forceps, lightly dry sectioned colon on a lint-free tissue. Still holding with forceps, pierce other end of the colon with a 30 G needle and roll the colon longitudinally around the end of the forceps. Pierce the needle entirely through the roll to maintain rolled structure. Drop the needle and roll into 50 ml tube containing fixative.
- v. Incubate in fixative at 4 °C overnight (12 to 16 hours). Needle will be removed from colon prior to paraffin-embedding and sectioning. **PAUSE POINT:** Overnight fixation. Next day, proceed directly to paraffin embedding and sectioning (Step 3). **CRITICAL STEP:** Some tissues and organs may need different fixation times, or be partially dissected to allow penetration of fixative into internal structures.

- B Harvesting and fixation of *in vitro* cultured cellsM

TIMING: 2 hours plus overnight air-drying

- i. Harvest cells according to accepted protocols. For the protocol used in our studies, wash cells with 1× PBS, and incubate with pre-warmed (37 °C) trypsin for 2–3 minutes, until adherent layer of cells has detached from surface of tissue culture plates and floats as a suspension of cells. Quench harvested cells in trypsin suspension in 1× PBS containing 10% fetal calf serum, and centrifuge at 300 g for 5 min at room temperature to pellet cells.
- ii. Centrifuge cells onto microscope slides using Cytospin according to manufacturer's directions. For our studies, we used a Shandon Cytospin 4.
- iii. Prepare 3 Coplin jars with freshly-prepared 3:1 methanol:acetic acid fixative solution (see REAGENT SETUP). Fix slides three times, 5 min each at room temperature.

- iv. Air-dry slides overnight at room temperature. **PAUSE POINT:** Overnight air-drying.
- v. Proceed directly to Rehydration and Pepsin Treatment (Step 13).

Paraffin Embedding and Sectioning of Fixed *in vivo* Mouse Tissues

TIMING: Variable, depending whether performed in-house or dedicated facility

- 3 For our studies, paraffin-embedding and sectioning was performed by a dedicated facility. The success of the CO-FISH procedure requires quality 6 μm sections of uniform thickness. **CRITICAL STEP:** To ensure tissues have been properly fixed, stain every tenth section with haematoxylin and eosin (H&E) to check for preservation of tissue morphology. This protocol was optimized for 6 μm sections of mouse colon. All incubation times and concentrations apply to this thickness. Significantly thinner or thicker sections will require optimization of steps to ensure proper digestion of newly-formed DNA and optimal hybridization of PNA probes. The nuclear diameter of tissues to be tested is also a factor for section thicknesses, as complete sister nuclei should be captured within each section.
- 4 Store paraffin-embedded tissue sections at 4 °C. **PAUSE POINT:** Fixed and embedded tissue sections can be stored at 4 °C indefinitely.

Deparaffinizing Fixed *in vivo* Mouse Tissue Sections

TIMING: Overnight for baking plus 90 minutes for deparaffinizing, without air-drying pause point (Step 9)

- 5 Transfer slides from 4 °C storage and lay flat on slide warmer set to 60 °C. Bake overnight. **PAUSE POINT:** slides may be baked for 8–16 hours, ensuring that paraffin has sufficiently melted.
- 6 Set water bath to 80 °C and pre-warm 50 ml of 100 mM Citrate Buffer (see REAGENT SETUP) in screw-top Coplin jar. **CRITICAL STEP:** Prepare one jar for every nine slides to be processed for CO-FISH. Standard Coplin staining jars contain slots for 5 slides, but 9 slides may be placed in a zigzag pattern and allow sufficient interaction between tissue sections and buffer components, for all steps except for denaturation (Steps 29 and 38), during which a maximum of 5 slides per jar is recommended. It is important not to crowd slides together, therefore slide staining chambers with very closely spaced slots for slides are not recommended for any part of this protocol.
- 7 In hazardous fume hood immerse slides in large volume Xylenes, in glass slide staining chambers, at room temperature. Incubate at room temperature 3 times, 15 min each, replacing with fresh Xylenes between incubations. Discard used Xylenes according to institutional practices. **CRITICAL STEP:** Do not overcrowd slide holder. We use 100 ml Xylenes per 5–9 slides, ensuring adequate spacing between slides..
- 8 Dehydrate slides with absolute (100%) ethanol. Incubate at room temperature (without shaking) 2 times, 10 min each, replacing with fresh ethanol between incubations.
- 9 Air dry slides. **PAUSE POINT:** Slides may be stored at room temperature for several hours, or may proceed directly to antigen retrieval (Step 10) when slides have dried.

Antigen Retrieval of Deparaffinized *in vivo* Mouse Tissue Sections

TIMING: 60 minutes plus overnight for optional air-drying (Step 12)

- 10 Transfer slides to pre-warmed 100 mM citrate buffer (from Step 6) and incubate in 80 °C water bath for 45 min.
- 11 Wash slides in 1× PBS (pH 7.4) two times, 5 min each, on a shaker at room temperature. **CRITICAL STEP:** Use flat cover-glass forceps to transfer slides between jars. Take care not to scratch tissue sections by contact from other glass slides or forceps. Set shaker to 100 rpm to avoid tissue sections detaching from glass surface due to excessive forces.
- 12 Wash slides in ddH₂O for 5 min, on a shaker. **PAUSE POINT:** Slides can be air-dried and stored at room temperature overnight; however Steps 11 and 12 (PBS and ddH₂O washes) must be repeated prior to commencing with pepsin treatment. Alternatively, one may proceed directly to pepsin treatment (Step 13) after ddH₂O wash, without air-drying and rehydration. **CRITICAL STEP:** Once pepsin treatment is initiated, protocol must be followed until end of hybridization (Step 49) with no overnight breaks.

Rehydration, Pepsin and RNase A Treatment

TIMING: 70 minutes for tissue sections, 90 minutes for cultured cells

- 13 Prepare 50 ml of 1mg ml⁻¹ pepsin in acidified ddH₂O (pH 2), transfer to Coplin jar and pre-warm in 37 °C water bath. **CRITICAL STEP:** Pepsin must be prepared fresh prior to each use, and allowed to equilibrate to 37 °C.
- 14 To rehydrate slides, use Option A for *in vivo* tissue sections and Option B for *in vitro* cultured cells. If *in vivo* tissue section slides have not been air dried after Step 12, proceed directly to pepsin treatment (Step 15), ensuring that prepared pepsin solution has equilibrated to 37 °C.
 - A. Rehydration of air dried *in vivo* tissue sections
 - i. To rehydrate air dried *in vivo* tissue sections, repeat Steps 11 and 12.
 - B. Rehydration and fixation of air dried *in vitro* cultured cells.
 - i. Rehydrate slides (from Step 2Bv) by washing in Coplin jar containing 1× PBS at room temperature 15 min, shaking. **CRITICAL STEP:** Use flat cover-glass forceps to transfer slides between jars. Take care not to scratch surface of slides by contact from other glass slides or forceps. Set shaker to 100 rpm to avoid cells detaching from glass surface due to excessive forces.
 - ii. Fix slides by immersing in Coplin jar containing 4% Formaldehyde fixative solution (see REAGENT SETUP) in PBS, at room temperature for 2 min.
 - iii. Wash slides in 1× PBS (pH 7.4) three times, 5 min each, on a shaker at room temperature.
- 15 Transfer slides of rehydrated tissue sections or cells to pre-warmed pepsin (from Step 13), incubate at 37 °C for 15 min for tissue sections and 10 min for cells. Timing must be exact.

- 16** Meanwhile, prepare RNase A solution in PBS according to Option A for tissue sections and Option B for cultured cells..
- A.** Preparation of RNase A solution for tissue sections
 - i.** Prepare 10 mg ml⁻¹ RNase A in PBS (see REAGENT SETUP). For tissue sections, RNase treatment will be repeated in Step 33, therefore 200 µl of 10 mg ml⁻¹ RNase A in PBS should be prepared for each slide at this step, with the remainder stored on ice or at 4 °C until needed.
 - B.** Preparation of RNase A solution for cultured cells
 - i.** Prepare 100 µl of 0.1mg ml⁻¹ RNase A in PBS for each slide. **CRITICAL STEP:** RNase A solution for tissue sections is 100× the concentration used for cultured cells
- 17** Wash slides in 1× PBS two times, 5 min each, on a shaker at room temperature. For tissue sections, proceed to 2× SSC wash (Step 18). For cultured cells, skip 2× SSC wash and proceed to RNase treatment (Step 19).
- 18** For tissue sections, dip slides briefly in 2× SSC.
- 19** Shake excess 2× SSC or 1× PBS from slides, and place them flat on tray lined with wet paper towel. Pipette 100 µl of prepared RNase A solution in PBS (from Step 16) onto each slide, directly on top of tissue sections, or on middle of slides for cultured cells. **CRITICAL STEP:** Removing excess 2× SSC or PBS from surface of slide prevents dilution of RNase A. However, do not let section air dry prior to pipetting of RNase A onto slide surface.
- 20** Carefully lay strip of Parafilm on top of each slide to distribute RNase A, taking care not to create air bubbles directly over tissue sections or on slide surface. Cover slides with a box to prevent air-drying. Transfer tray with covered slides to bacterial incubator at 37 °C for 10 min.
- 21** Remove Parafilm. Wash slides in 1× PBS two times, 5 min each, on a shaker at room temperature.

Removal of Newly-formed DNA Strands

TIMING: 2.5 hours for tissue sections, 3 hours for cultured cells, without optional air-drying pause points

- 22** Shake excess PBS from slides, and place on tray lined with wet paper towel. In hazardous fume hood, pipette 50 µl of 1mg ml⁻¹ Hoechst 33258 (diluted in ddH₂O) onto each slide, directly on top of tissue sections or slide surface.
- 23** Carefully lay strip of Parafilm on top of each slide, taking care not to create air bubbles directly over tissue section or on slide surface. Cover slides with dark box to shield from light. Incubate at room temperature, in hazardous fume hood for 15 min.
- 24** Remove Parafilm using tweezers; discard Hoechst-covered Parafilm according to institutional practices.
- 25** Dip slides briefly in 2× SSC to remove remaining Hoechst 33258, and place flat on a tray. Pipette 200 µl 2× SSC directly onto slides, and carefully cover with 26 × 60 mm cover-glass. **CRITICAL STEP:** To prevent air bubbles from forming on surface of tissue sections or on slides, place short edge of cover-glass at one

end of the slide and create a slight curve in the glass prior to laying cover-glass down at an angle. It is also critical to ensure that only one cover-glass is placed on each slide, as a double layer will affect penetrance of ultraviolet energy (Step 26) and prevent complete photolysis of newly-formed DNA strands.

- 26 Irradiate slides with UV light in Stratalinker 1800 for 30 min (calculated dose $5.4 \times 10^3 \text{ J m}^{-2}$).
- 27 Meanwhile, prepare 70% formamide/2× SSC buffer in screw-top Coplin jar (see REAGENT SETUP), and preheat in a water bath to 72 °C for tissue sections, or 70 °C for cells. **CRITICAL STEP:** Allow plenty of time to equilibrate buffer to 72 °C or 70 °C in anticipation of denaturation (Step 29 for tissue sections or Step 38 for cultured cells).
- 28 Remove cover glass and wash slides in 2× SSC two times, 5 min each, shaking at room temperature. **CRITICAL STEP:** To safely remove cover-glass prior to washes, place slides vertically in Coplin jar filled with 2× SSC. Gently dip slide in 2× SSC until cover-glass slides off. Take care not to scratch surface of tissue section when cover-glass is removed. For tissue sections, continue with DNA denaturation (Step 29). For cultured cells, let slides air dry up to several hours, or proceed directly to rehydration (Step 32) once slides are dry.
- 29 For tissue sections, denature DNA. In hazardous fume hood, transfer slides to pre-heated 70% formamide/2× SSC buffer (from Step 27). Place back in 72 °C water bath and incubate for a total time of 2 min. **CRITICAL STEP:** The timing of this DNA denaturation step must be exact. Start timer immediately after placing slides into buffer, and have ice-cold ethanol washes ready in fume hood to transfer slides into (Step 30) exactly at the end of the 2 min incubation. **CRITICAL STEP:** Do not overcrowd Coplin jars during denaturation steps. Use a maximum of 5 slides per Coplin jar, preparing several jars if necessary.
- 30 In hazardous fume hood, serially dehydrate slides in ice-cold (−20 °C) 70%, 90% and 100% ethanol for 2 min each. **CRITICAL STEP:** Transfer slides into 70% ethanol immediately at end of 2 min incubation time. Replace ethanol with fresh dilutions at end of each incubation and replace containers in −20 °C freezer to chill ethanol in preparation for Step 39. Replace 70% formamide solution back into 72 °C water bath for second denaturation (Step 38).
- 31 Air dry slides. **PAUSE POINT:** Slides may be kept at room temperature for several hours. Alternatively, proceed directly to Step 32 once slides are dry.
- 32 Rehydrate slides (from Steps 28 and 31) in 1× PBS for 10 min, on a shaker at room temperature.
- 33 For tissue sections, repeat RNase A treatment and PBS washes from Steps 19–21 prior to exonuclease digestion in Step 34. For cultured cells, proceed directly to exonuclease digestion (Step 34).
- 34 For each slide, prepare 50 µl of 3 Units µl^{−1} Exonuclease III in 1× NEB Buffer 1 (dilute 10× NEB Buffer 1 from manufacturer to 1× using ddH₂O). Shake excess PBS from each slide, and place flat on tray lined with wet paper towel.
- 35 Pipette 50 µl of prepared Exonuclease III onto slides, directly on surface of tissue sections or on slide surface for cultured cells.
- 36 Carefully lay strip of Parafilm on top of each slide, taking care not to create air bubbles directly over tissue section. Cover slides with a box to prevent air-drying. Transfer tray to bacterial incubator at 37 °C for 10 min.

- 37 Wash slides in 2× SSC three times, 5 min each, shaking at room temperature.
- 38 In hazardous fume hood, transfer slides into pre-warmed 70% formamide denaturation solution (prepared in Step 27) for 1 min at 72 °C for tissue sections, or 70 °C for cultured cells. Note that this is the second of two denaturation steps for tissue sections, and the only denaturation step for cultured cells. **CRITICAL STEP:** Do not overcrowd Coplin jars during denaturation steps. Use a maximum of 5 slides per Coplin jar, preparing several jars if necessary. **CRITICAL STEP:** This denaturation step is only one minute long, but timing must be exact. Start timer immediately after placing slides in warm buffer, and have ice-cold ethanol washes ready at the end of the one minute incubation time. In order to maintain denaturation temperature, jar must be placed back into water bath for duration of incubation, taking care to remove jar and place in hazardous fume hood in preparation for the following dehydration step prior to end of one minute.
- 39 In hazardous fume hood, serially dehydrate slides in ice-cold (−20 °C) 70%, 90% and 100% ethanol for 2 min each. **CRITICAL STEP:** Transfer slides into 70% ethanol immediately at end of 1 min denaturation time.
- 40 Air dry slides. **PAUSE POINT:** Slides may be kept at room temperature for several hours, or proceed once slides are dry. For tissue sections, proceed directly to hybridization (Step 46). For cultured cells, proceed with rehydration and formaldehyde fixation (Step 41).
- 41 For cultured cells, rehydrate slides in 1× PBS for 10 min, on a shaker at room temperature.
- 42 Fix slides by immersing in Coplin jar containing 4% formaldehyde fixative solution in PBS (see REAGENT SETUP), at room temperature for 2 min.
- 43 Wash slides in 1× PBS (pH 7.4) three times, 5 min each, on a shaker at room temperature.
- 44 Dehydrate slides in 70%, 90% and 100% ethanol, 5 min each, at room temperature with shaking. **CRITICAL STEP:** Please note that this dehydration series uses room temperature ethanol dilutions.
- 45 Air dry slides. **PAUSE POINT:** Slides may be kept at room temperature for several hours. Alternatively, proceed directly to hybridization (Step 46) once slides are dry.

Hybridization with PNA probes

TIMING: 30 minutes plus overnight for tissue sections, 1.5 hours for cultured cells

- 46 Prepare 50 µl hybridization mixture per slide for tissue sections, 20 µl per slide for cultured cells (see REAGENT SETUP).
- 47 In hazardous fume hood, pipette 50 µl of hybridization mix onto each slide (from Steps 40 or 45), directly adjacent to tissue sections or 20 µl onto slide surface for cultured cells. Carefully cover with 26 × 60 mm cover-glass to distribute hybridization mixture directly over tissue section. **CRITICAL STEP:** To prevent air bubbles from forming on surface of tissue sections or on slide surface, place short edge of cover-glass at one end of the slide (toward same side as probe droplets were placed) and create a slight curve in the glass prior to laying down at an angle.

- 48 Place slides in 80 °C oven to denature probes. Incubate for 3 min for tissue sections, 2 min for cultured cells. **CRITICAL STEP:** Timing must be exact. Protect slides from light to prevent photobleaching of coupled fluorescent molecules.
- 49 Place slides in plastic slide holder containing moist paper towel. Store upright in sealed slide holder at room temperature in a dark place. Incubate overnight for tissue sections, 1 hour for cultured cells. **PAUSE POINT:** Overnight or one hour incubation.

Washes and Counterstaining

TIMING: 2 hours for tissue sections, 1.5 hours for cultured cells, plus time required for microscopy

- 50 In hazardous fume hood, prepare formamide wash solution in two separate Coplin jars (see REAGENT SETUP).
- 51 Transfer slides from slide box to first formamide wash solution. **CRITICAL STEP:** To safely remove cover-glass, place slides vertically in Coplin jar filled with wash solution. Gently dip slide in until cover-glass slides off. Take care not to scratch surface of tissue section or adhered cells when cover-glass is removed. In addition, slides must be protected from light to prevent photobleaching of hybridized probes.
- 52 Wash slides in formamide wash solution two times, 15 min each at room temperature with no shaking, transferring slides to fresh formamide wash solution between washes.
- 53 Wash slides in freshly-made TNT wash buffer (see REAGENT SETUP) three times, 5 min each at room temperature. **CRITICAL STEP:** First TNT wash should be in hazardous fume hood because of formamide residue on slides, with no shaking. Second and third TNT washes are on shaker, at room temperature.
- 54 For tissue sections wash slides in 1× PBS, 5 min, shaking at room temperature. Proceed with counterstaining (Step 55). For cultured cells, proceed directly to dehydration (Step 57).
- 55 Counterstain tissue sections in DAPI counterstain solution (see REAGENT SETUP), 5 min, with shaking at room temperature.
- 56 Wash tissue sections in 1× PBS three times, 5 min each, with shaking at room temperature.
- 57 Dehydrate slides (from Steps 54 or 56) in 70%, 90% and 100% ethanol, 5 min each, at room temperature with shaking. **CRITICAL STEP:** Please note that this dehydration series uses room temperature ethanol dilutions.
- 58 Air dry slides. **CRITICAL STEP:** Protect slides from light.
- 59 Mount slides using antifade reagent appropriate for the fluorophores coupled to the probes. For Cy-5 coupled probes, use approximately 10 µl DABCO mounting medium per slide, for tissue sections. For cultured cells, mount in approximately 10 µl DABCO + DAPI mounting medium (see REAGENT SETUP). Cover with cover-glass. **CRITICAL STEP:** It is easy to introduce bubbles into glycerol-based mounting media. Pipette carefully to ensure no bubbles are introduced.

- 60** Visualize after 10 minute incubation at room temperature. Protect slides from light during incubation. **TROUBLESHOOTING:** See Anticipated Results for typical CO-FISH images. Since the success of CO-FISH is only evident during visualization by microscopy, many steps in the procedure could affect final outcome. See Troubleshooting and Table 2 for advice.

TIMING

The timing of the entire procedure for studying chromatin segregation patterns for *in vivo* mouse tissues is approximately 4 days, excluding paraffin embedding and sectioning (Step 3), the timing of which can vary depending on whether these procedures are performed in-house or by a dedicated facility. For cultured cells, the procedure takes 2 days to complete, following overnight BrdU incubation.

Steps 1–9: BrdU incorporation and tissue preparation. 2.5d for *in vivo* tissues (including 12 hourly BrdU injections, overnight fixation, overnight baking and Xylene deparaffinization); 2d for *in vitro* cultured cells (including overnight administration of BrdU and additional overnight air-drying). Note that preparation of *in vitro* cultured cells skips steps 3–9.

Steps 10–12 Antigen retrieval of *in vivo* tissue sections. 1h plus optional air-drying step. Alternatively, may proceed directly to pepsin treatment (Step 13) after without air-drying and rehydration. However, once pepsin treatment is initiated, protocol must be followed until end of hybridization (step 49) with no overnight breaks. Note that preparation of *in vitro* cultured cells skips steps 10–12.

Steps 13–21: Rehydration and pepsin treatment. 70 minutes for tissue sections, 1.5h for cultured cells.

Steps 22–45: Removal of newly-formed (BrdU substituted) DNA strands. 2.5h for tissue sections, 3h for cultured cells, without optional pausing during air-drying (Steps 31, 40, 45).

Steps 46–49: Hybridization with PNA probes. 30 minutes plus overnight incubation for *in vivo* tissue sections, 1.5 h for *in vitro* cultured cells.

Steps 50–60: Washes and counterstaining. 2h for tissue sections, 1.5 h for cultured cells.

ANTICIPATED RESULTS

Assaying sister chromatid segregation patterns by CO-FISH depends on continuous incorporation of BrdU into newly formed DNA for exactly one round of DNA replication, followed by complete removal of BrdU substituted strands and hybridization of unidirectional PNA probes to resultant single stranded sister chromatids to allow quantitative analysis of probe fluorescence. Typical differential labeling of sister chromatids in post-mitotic colon crypts cells using CO-FISH appears as distinct regions of non-overlapping probe fluorescence signals (Fig. 1d **top panels**, Fig. 3 **bottom panels**). Post-mitotic cells must be spatially separated from the surrounding tissue (Fig. 1d, top left panel, boxed) in order to allow quantitative analysis of probe fluorescence. Mitotic cells embedded in surrounding non-mitotic tissue (Fig. 1d, top left panel, yellow arrowhead) cannot be accurately quantified. Typical CO-FISH labeling of sister chromatids in post-mitotic cultured cells appears in Fig. 1d, bottom panels. Overlapping probe signals from non-mitotic cells are easily distinguished (bottom left panel, white arrowhead).

If the criteria for generating completely single stranded chromatids are not met (either by difficulties of BrdU incorporation, photolysis, exonuclease digestion or hybridization; see Introduction), then probes will hybridize to double-stranded major satellite sequences and

give overlapping FISH rather than CO-FISH signals (Fig. 3, top panels). Overlapping probe signals are expected in non-mitotic cells (Fig. 1d, **left panel**, white arrowhead) as there are no BrdU-substituted DNA strands for photolysis and exonuclease digestion. Overlapping probe signals in mitotic cells (example in Fig. 3, **top panels**) indicate that any of the steps in the procedure were not completed correctly and produced remaining double-stranded DNA (see Troubleshooting). Importantly, the BrdU time course (and thereby the cell cycle length) of the cells or tissues to be studied must be experimentally determined in order to ensure that quantified probe signals reflect sister chromatid segregation patterns (see Introduction).

TROUBLESHOOTING

The success of CO-FISH for differential labeling of sister chromatids becomes evident only at the end of the procedure, when the cells or tissues are visualized by fluorescence microscopy. It is not possible to troubleshoot the procedure without visually assaying probe signal territories in pairs of post-mitotic cells. There are three main problems that become apparent upon visualization: overlapping FISH probe signals instead of single-stranded CO-FISH signals (inability to distinguish sister chromatids), poor or weak probe signals, and high background fluorescence. Because the CO-FISH technique is complex with the final outcome dependent on numerous manipulations, these few problems may arise at many steps throughout the procedure. Troubleshooting advice is provided in Table 2.

References

1. Lansdorp PM. Immortal strands? Give me a break. *Cell*. 2007; 129 (7):1244–1247. [PubMed: 17604711]
2. Rando TA. The immortal strand hypothesis: segregation and reconstruction. *Cell*. 2007; 129 (7): 1239–1243. [PubMed: 17604710]
3. Falconer E, et al. Identification of sister chromatids by DNA template strand sequences. *Nature*. 463(7277):93–97. [PubMed: 20016487]
4. Meyne J, Goodwin EH. Strand-specific fluorescence in situ hybridization for determining orientation and direction of DNA sequences. *Methods Mol Biol*. 1994; 33:141–145. [PubMed: 7894576]
5. Potten CS, Hume WJ, Reid P, Cairns J. The segregation of DNA in epithelial stem cells. *Cell*. 1978; 15 (3):899–906. [PubMed: 728994]
6. Karpowicz P, et al. Support for the immortal strand hypothesis: neural stem cells partition DNA asymmetrically in vitro. *J Cell Biol*. 2005; 170 (5):721–732. [PubMed: 16115957]
7. Shinin V, Gayraud-Morel B, Gomes D, Tajbakhsh S. Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. *Nat Cell Biol*. 2006; 8 (7):677–687. [PubMed: 16799552]
8. Smith GH. Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. *Development*. 2005; 132 (4):681–687. [PubMed: 15647322]
9. Cairns J. Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. *Proc Natl Acad Sci U S A*. 2002; 99 (16):10567–10570. [PubMed: 12149477]
10. Goodwin E, Meyne J. Strand-specific FISH reveals orientation of chromosome 18 alphoid DNA. *Cytogenet Cell Genet*. 1993; 63:126–127. [PubMed: 8467711]
11. Bailey SM, Goodwin EH, Cornforth MN. Strand-specific fluorescence in situ hybridization: the CO-FISH family. *Cytogenet Genome Res*. 2004; 107 (1–2):14–17. [PubMed: 15305050]
12. Goodwin EH, Meyne J, Bailey SM, Quigley D. On the origin of lateral asymmetry. *Chromosoma*. 1996; 104 (5):345–347. [PubMed: 8575246]
13. Garagna S, et al. Pericentromeric organization at the fusion point of mouse Robertsonian translocation chromosomes. *Proc Natl Acad Sci U S A*. 2001; 98 (1):171–175. [PubMed: 11136254]

14. Lin MS, Davidson RL. Centric fusion, satellite DNA, and DNA polarity in mouse chromosomes. *Science*. 1974; 185 (4157):1179–1181. [PubMed: 4137570]
15. Alves P, Jonasson J. New staining method for the detection of sister-chromatid exchanges in BrdU-labelled chromosomes. *J Cell Sci*. 1978; 32:185–195. [PubMed: 81211]
16. Limoli CL, Ward JF. A new method for introducing double-strand breaks into cellular DNA. *Radiat Res*. 1993; 134 (2):160–169. [PubMed: 7683818]
17. Lansdorp PM, et al. Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet*. 1996; 5 (5):685–691. [PubMed: 8733138]
18. Poon SS, Lansdorp PM. Quantitative fluorescence in situ hybridization (Q-FISH). *Curr Protoc Cell Biol*. 2001; Chapter 18(Unit 18):14.
19. Horz W, Altenburger W. Nucleotide sequence of mouse satellite DNA. *Nucleic Acids Res*. 1981; 9 (3):683–696. [PubMed: 6261227]
20. Vissel B, Choo KH. Mouse major (gamma) satellite DNA is highly conserved and organized into extremely long tandem arrays: implications for recombination between nonhomologous chromosomes. *Genomics*. 1989; 5 (3):407–414. [PubMed: 2613229]
21. Wu R, Singh PB, Gilbert DM. Uncoupling global and fine-tuning replication timing determinants for mouse pericentric heterochromatin. *J Cell Biol*. 2006; 174 (2):185–194. [PubMed: 16831888]
22. Allen JW, Latt SA. Analysis of sister chromatid exchange formation in vivo in mouse spermatogonia as a new test system for environmental mutagens. *Nature*. 1976; 260 (5550):449–451. [PubMed: 1256591]
23. Poon SS, Martens UM, Ward RK, Lansdorp PM. Telomere length measurements using digital fluorescence microscopy. *Cytometry*. 1999; 36 (4):267–278. [PubMed: 10404142]
24. Gertsenstein M, Lobe C, Nagy A. ES cell-mediated conditional transgenesis. *Methods Mol Biol*. 2002; 185:285–307. [PubMed: 11768997]

Figure 1. Uniform orientation of mouse chromosomes as shown by four-colour CO-FISH

a, The orientation of A-rich (green) and T-rich (pink) major satellite DNA repeats is uniform in all mouse chromosomes, relative to C-rich (yellow) and G-rich (red) telomeric repeats. The T-rich major satellite is always adjacent to C-rich telomeric repeats on the short arm of the chromosome. As a result, the T-rich satellite repeats marks the same template strand in all mouse chromosomes. Adapted with permission from ref. 3. **b**, Based on this orientation, we have arbitrarily named the T-rich template strand Crick and the A-rich template strand Watson. Adapted with permission from ref. 3. **c**, Watson and Crick template strands can be used to follow sister chromatid segregation patterns in mouse tissues *in vivo*. BrdU is

injected into mice and incorporates exclusively into newly-formed DNA. UV photolysis and exonuclease digestion removes the BrdU-substituted newly-formed DNA strands, leaving the Watson and Crick template strands intact, and creating single-stranded chromosomes as targets for probe hybridization. The unique orientation of major satellite repeats in Watson and Crick strands serve as a molecular feature to distinguish the template strands, and thus sister chromatids. The patterns of the fluorescent-coupled probes in post-mitotic cells reflects the distribution of sister chromatids at anaphase. Adapted with permission from ref. 3. **d**, Typical CO-FISH image of colon crypt epithelial cells (top panels) and *in vitro* cultured murine embryonic stem (ES) cells (bottom panels). Watson and Crick template strand fluorescence patterns from post-mitotic daughter nuclei (boxed) can be imaged and subsequently quantified. Only isolated sister nuclei are suitable for quantitative analysis in tissue sections (top panels) as fluorescence from non-mitotic cells (white arrowhead) can complicate analysis of cells embedded within tissues (yellow arrowhead). For *in vitro* cultured cells (bottom panels), non mitotic cells (white arrowhead) are easily distinguished from post-mitotic cells due to overlapping probe signals. Adapted with permission from ref. 3.

Figure 2. Time-course experiments to ensure BrdU incorporation is for one complete cell cycle
a, Patterns of probe hybridization in metaphase-arrested cells reveals whether BrdU incorporation was complete, or exceeded more than one cell cycle. **b**, For colon crypt epithelial cells, 12 hours of BrdU incorporation encompasses one cell cycle, as revealed by paired, non-overlapping probe fluorescence. Adapted with permission from ref. 3.

Figure 3. Hourly injections of BrdU are necessary to ensure single-stranded chromosome targets for strand-specific hybridization

For *in vivo* analysis, BrdU must be injected hourly to ensure complete incorporation. Longer intervals between BrdU injections results in incomplete incorporation and overlapping probes (top panels), whereas hourly injections of the same total dose result in single-stranded chromosomes and non-overlapping CO-FISH fluorescence signals (bottom panels).

Table 1

Sequences of unidirectional PNA probes recognizing Watson or Crick template strands

PNA probe sequence	target
Cy3 - GACGTGGAATATGGCAAG	Crick strand (major T-rich)
Cy5 - CTTCAGTGTGCATTCTC	Watson strand (major A-rich)

Table 2

Troubleshooting

Steps	Problem	Possible Reason	Solution
60	Overlapping probe signals due to incomplete uptake of BrdU.	Precipitates in BrdU stock solution (Step 1)	Warm stock solution to 37 °C and vortex prior to injecting or adding to cell culture medium.
		Dead space in syringe prior to injections into mice (Step 1 A. iii.)	Withdraw more than 100µl of BrdU stock solution; inject 100 µl according to markings on side of 1 ml syringe.
		Injection protocol too short for <i>in vivo</i> tissues (Step 1 A. iv.)	Determine length of S-phase experimentally as described; increase number of hourly injections accordingly
		Hoechst 33258 too dilute (Step 22)	Ensure Hoechst is not diluted by excess PBS remaining on sections after washes. Shake excess PBS from slides, taking care that sections do not dry.
		Hoechst 33258 did not penetrate evenly (Step 23)	Ensure there are no air bubbles created when Parafilm is placed on top of tissue section or slide.
	Overlapping probe signals due to incomplete removal of newly-formed strands.	UV did not penetrate tissues (Step 26)	Ensure only one cover-glass is placed on slide. Thickness of glass affects penetration of UV radiation.
		Denaturation of DNA was incomplete (Step 29 tissues; Step 38 for both tissues and cells)	Ensure 70% formamide/2× SSC denaturation solution is pre-warmed to 72 °C (tissues) or 70 °C (cells) prior to use. Ensure slides denature for full time indicated prior to ethanol dehydration.
		Exonuclease digestion was incomplete (Step 35).	Ensure exonuclease enzyme is within expiration date. Ensure enzyme is not diluted by excess PBS remaining from wash. Ensure there are no air bubbles created when Parafilm is placed on top of tissue section.
		Low fluorescence signal due to poor probe penetration.	Ensure fixation is no longer than 16 hours, and is at 4 °C. Ensure that paraffin-embedding is arranged to coincide with end of fixation period.
			Ensure sections are 6 µm thick. Alternatively, optimize probe concentration, denaturation time and incubation time to improve signal.
			Ensure slide warmer is set to 60 °C for complete melting of paraffin. In addition, do not overcrowd slides during incubation in Xylenes. Use a large volume of Xylenes to ensure complete deparaffinization. Check sections under light microscope after dehydration and air-drying (Step 9).
	High background fluorescence signal	Incomplete denaturation prior to hybridization (Step 48).	Ensure oven is at 80 °C and metal plate on which slides are placed has equilibrated to temperature. Ensure incubation time is for a full time indicated.
		Air bubbles between tissue section and cover-glass during room temperature probe hybridization (Step 47).	To prevent air bubbles from forming on tissue sections, create a slight curve in cover-glass prior to laying on slides.
		Antifade compound not used or not suitable	Ensure mounting medium contains DABCO or other suitable antifade compound to prevent photobleaching of fluorescent molecules used.
		RNA not removed completely (Steps 10 and 33).	Ensure RNase A dilution is made fresh, to indicated final concentration. Ensure no air bubble created when Parafilm placed on top of RNase solution on tissue section. Ensure incubation at 37 °C is for full time indicated.
		Incomplete washing of unbound probes (Step 52).	Ensure formamide washes are complete. Use fresh wash solution for both washes. Shake slide jars on shaker at 100–150 rpm if necessary.
		Lenses not suitable for detection of signal	Ensure only high numerical aperture lenses are used.