

The Sister Chromatid Exchange (SCE) Assay

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Abstract

A fully optimized staining method for detecting sister chromatid exchanges in cultured cells is presented. The method gives reproducibly robust quantitative results. Sister chromatid exchange is a classic toxicology assay for genotoxicity and for detecting alterations to the biochemistry underlying cellular homologous recombination. Growth of cells in the presence of 5'-bromo-deoxyuridine for two rounds of DNA replication followed by collecting metaphase spreads on glass slides, treatment with the UV-sensitive dye Hoechst 33258, long-wave UV light exposure, and Giemsa staining gives a permanent record of the exchanges.

Key words Sister chromatid exchange (SCE), Homologous recombination (HR), Genotoxicity

1 Introduction

Homologous recombination (HR) is an important pathway for genomic repair of many forms of DNA damage including chromosomal double-strand breaks (DSBs), interstrand cross-linking damage, and collapsed replication forks [1]. HR is usually considered to be “error-free” repair because it uses the available, identical sequence from the sister chromatid to repair the DSB, thereby preserving DNA sequence information. Mitotic HR is a complex, varied, and tightly regulated process, and defects in several of the components of HR have long been associated with cancer (reviewed in refs. 2 and 3). The clinical importance of recombination as a mechanism for influencing overall genomic integrity in human disease is illustrated by the manner in which defects in the HR tumor suppressor genes *BRCA-1* or *BRCA-2* lead to cancer susceptibility [4, 5]. Importantly, the defective HR status of cancers in *BRCA1/2* mutation carriers can also be exploited therapeutically through chemical inhibition of the poly(ADP-ribose) polymerase (PARP), which is involved in the base excision repair pathway (BER) [6]. PARP inhibitors induce the accumulation of DNA single-strand

breaks (SSBs), which are synthetically lethal in cells that are already defective in *BRCA1/2*-mediated HR, but which are efficiently repaired in cells with functional HR.

The most established means of detecting dysregulated homologous recombination, whether in cells with defective/deficient HR capacity, or in response to damage, is the sister chromatid exchange assay (SCE) which differentially stains sister chromatids, allowing for microscopic detection of the physical exchange of DNA which occurs with crossover HR [7]. The SCE assay has been in use since the 1970s for the purpose of identifying potential “chromosomal mutagenicity” of chemical agents [8]. Chemicals that generate interstrand cross-links, such as mitomycin C, are potent inducers of SCE, since HR is required to repair the resultant blockage during replication [9]. Conditions and drugs which increase the number of SSBs also increase the number of SCEs, presumably by overburdening the BER pathway such that unrepaired SSBs remain, become DSBs during replication, and elicit repair by HR [7]. Mutation or knockdown of BLM, the genetic determinant of Bloom syndrome which is involved in DSB repair, causes a tenfold elevation in SCE [10].

The protocol described below utilizes 5-bromo-2'-deoxyuridine (BrdU) incorporation and fluorescence plus Giemsa (FPG) staining to make exchanges between sister chromatids visible [11, 12]. BrdU is a nucleoside analog that resembles thymidine and is efficiently incorporated into replicating DNA. Since DNA replication is semi-conservative, after BrdU has been made available to cells, it is incorporated as the nascent strand is elongated. After two rounds of replication, paired metaphase sister chromatids differ in the amount of BrdU each contains. One sister has one strand of non-BrdU-substituted DNA and one strand with BrdU substitution. In the other sister, both DNA strands contain BrdU substitutions. Subsequent incorporation of the intercalating ultraviolet (UV) light-absorbing Hoechst 33258 dye into the DNA, followed by UV light exposure, causes “bleaching” of the DNA in proportion to amount of incorporated BrdU in the double-stranded molecule, likely due to free-radical mediated damage. Subsequent staining of the UV-treated chromosomes with Giemsa makes this differential bleaching apparent by light microscopy [7], where doubly substituted chromosomes stain much more faintly than hemi-substituted chromosomes. See Fig. 1 for a schematic of the procedure and expected results. SCEs following either the first or second round of DNA replication in the presence of BrdU lead to visible exchanges after the staining procedure.

Other methods for measuring SCE following differential BrdU incorporation include fluorescent imaging and the use of either acridine orange in place of UV treatment followed by Giemsa staining [13], or an anti-BrdU antibody with either propidium iodide or DAPI counterstain for bulk DNA [7, 12, 14]. Rapid photobleaching is a potential pitfall, however, especially with the use of acridine

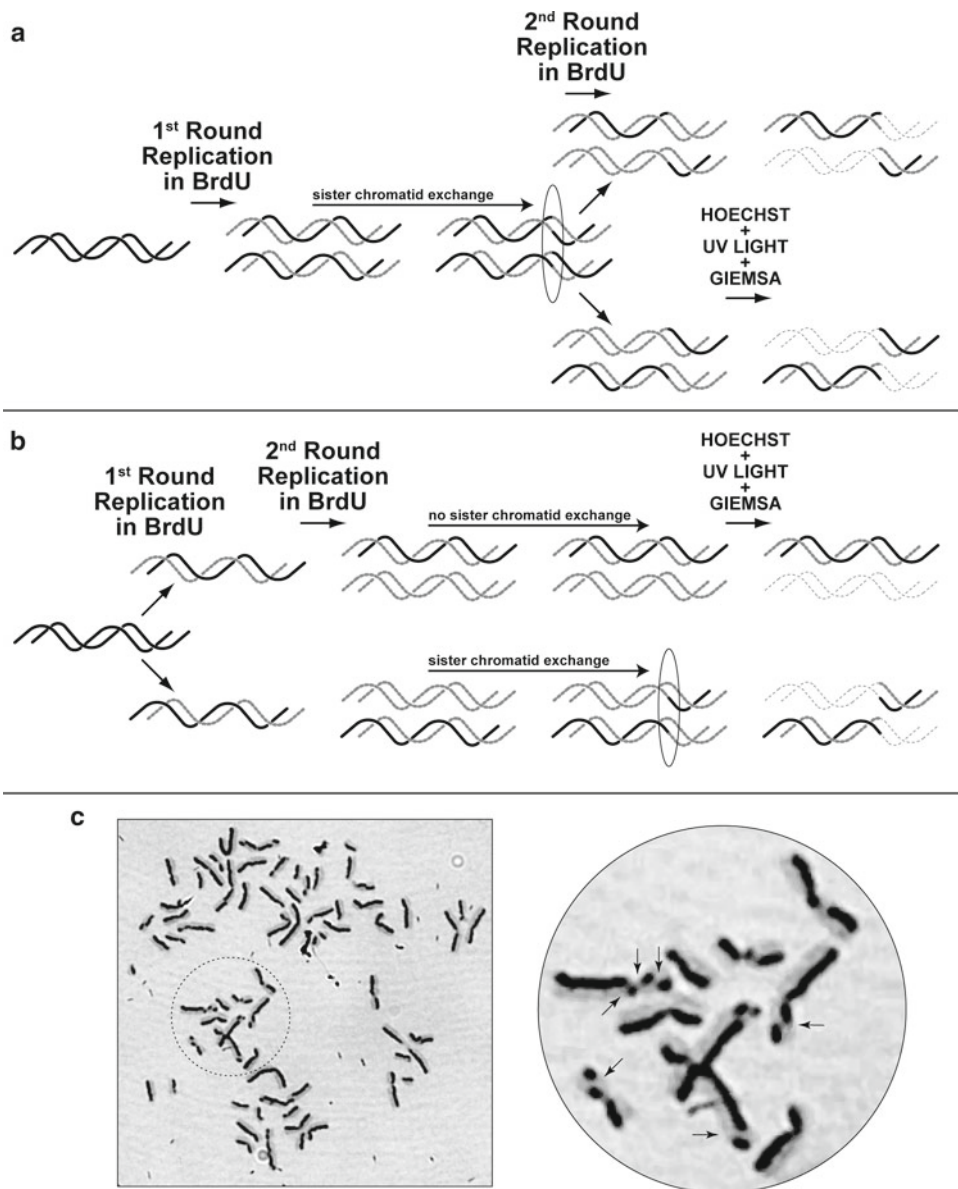


Fig. 1 Schematic of sister chromatid staining using two rounds of BrdU incorporation followed by Hoechst 33258 staining, exposure to UV light, and staining with Giemsa. *Solid black lines*: unsubstituted DNA single strand; *dotted gray lines*: BrdU substituted DNA single strand; *ellipse*: point of physical SCE. **(a)** Schematic of the effect of a SCE following the first round of DNA synthesis in the presence of BrdU. **(b)** Schematic of the effect of a SCE following the second round of DNA synthesis in the presence of BrdU. Without an exchange, one sister stains uniformly dark and the other uniformly light (*top*). With exchange, light and dark staining regions switch at the point of exchange (*bottom*). **(c)** Results of the full staining procedure on K562 cells. The *magnified circular inset* has exchanges indicated by *arrows*

orange, and fluorescent filters are required. We have achieved excellent results with optimized FPG staining (described in this work), and prefer the convenience of bright-field microscopy and the permanence of this staining method, including the possibility for repeated and extended viewing of the same spread.

This protocol has been tested in a wide variety of human cancer cell lines that have been immortalized to undergo unlimited rounds of replication. We have not tested it in primary cell lines, plant cells, or non-cultured tumor cells extracted from donors. It is not effective for cells that are not actively dividing, due to the requirement for differential BrdU incorporation.

2 Materials

2.1 Growing Cells

1. Latex or nitrile gloves.
2. 10-cm Tissue culture plates (or tissue culture flasks for non-adherent cells).
3. Well-characterized line of adherent or non-adherent mammalian cells.
4. Tissue culture incubator.
5. Laminar flow hood with standard tissue culture setup, including serological pipets, micropipets, and vacuum aspiration apparatus.
6. Phase-contrast inverted light microscope with 4×, 10× and 25× objective lenses with 10× eyepieces for viewing plates of growing cells.
7. Tissue culture medium such as Dulbecco's Modified Eagle medium or RPMI-1640.
8. 95 % reagent grade ethanol.
9. Fetal bovine serum (FBS).
10. Penicillin–streptomycin–L-glutamine 100× solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, 200 mM L-glutamine).
11. 25 mg/mL Plasmocin (for mycoplasma prophylaxis).
12. Complete culture medium: Medium appropriate for in vitro growth of cells. We typically use MEM or RPMI supplemented with 5–10 % final concentration FBS, with 1× penicillin–streptomycin–L-glutamine, and a 1:10,000 dilution of the Plasmocin stock solution.
13. 70 % Ethanol: 95 % Reagent grade ethanol diluted to 70 % with sterile distilled water.
14. Trypsin–EDTA (0.05 % Trypsin, 0.53 mM EDTA).
15. 15-mL Conical centrifuge tubes.
16. Clinical centrifuge.

2.2 Cellular BrdU Incorporation

1. 10 mM BrdU stock aliquots: dissolve BrdU (Fisher Bioreagents, Pittsburgh, PA) powder in water. Make 200 µL aliquots and store at –20 °C. The 10 mM stock solution should be made in the dark and stored in 1.5 mL Eppendorf tubes which have been covered with aluminum foil (*see Note 1*).

2. Sterile distilled water.
3. Aluminum foil.

2.3 Harvesting Cells for Metaphase Spreads

1. Colcemid: demecolcine solution 10 µg/mL in Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, MO).
2. Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS): 200 mg/L KCl, 200 mg/L KH₂PO₄, 8 g/L NaCl, 2.16 g/L Na₂HPO₄·7H₂O (Invitrogen, Carlsbad, CA).
3. Potassium chloride.
4. Sodium citrate.
5. Methanol.
6. Glacial acetic acid.
7. Hypotonic solution: 46.5 mM KCl, 8.5 mM Na-Citrate.
8. 3:1 Methanol-acetic acid fixative: add 1 vol glacial acetic acid to 3 vol reagent grade ethanol. *MAKE FRESH BEFORE EACH USE!!*

2.4 Preparing and Storing Slides for Metaphase Spreads

1. Microscope slides.
2. Glass Coplin jars or other glass container suitable for holding a rack with at least a dozen slides.
3. Slide racks and containers for staining, such as EasyDip™ slide staining system (Fisher), which allows for easy transfer of an entire rack of slides from one solution to another.
4. Refrigeration at 4 °C for chilling and holding slides.
5. Small rubber suction bulbs.
6. 5" glass Pasteur pipets.
7. Slide warmer with adjustable temperature.

2.5 Differential SCE with Giemsa

1. Hoechst 33258 98 % (Acros Organics [Fisher]).
2. Na₂HPO₄.
3. KH₂PO₄.
4. NaCl.
5. Long-wave UV light source: two 20-W F20T10BLB/RS-type blacklight/blue bulbs (Sanyo-Denki, Torrance, CA).
6. Shaker, hybridization oven, or warm room that can be maintained at 50 °C.
7. Concentrated Giemsa stain solution (50 % Giemsa in methanol and glycerin), (Acros Organics [Fisher]).
8. 1 mg/mL Hoechst 33258 in H₂O (protect from light), stored at 4 °C (*see Note 2*).
9. Sorensen phosphate buffer (0.1 M, pH 6.8): mix equal vol 0.1 M Na₂HPO₄ and 0.1 M KH₂PO₄.

10. 20× Salt sodium citrate buffer (SSC): 3 M NaCl and 300 mM sodium citrate in water.
11. 10 % Giemsa in Sorensen buffer: Add concentrated Giemsa stain solution to Sorensen buffer such that the concentration of Giemsa stain solution (not total concentration of Giemsa itself) comprises 10 % of the total volume. It is convenient to mix this 100 mL at a time and store in a 100 mL glass bottle protected from light. The stain solution can be poured back into the bottle after staining and reused several times.
12. Cytoseal-60 low viscosity mounting medium.
13. Coverslips for slides: 24×50×1 mm.

3 Methods

3.1 Thawing Cryopreserved Cells

1. Wear latex or nitrile gloves. Wipe down the working surface of the laminar flow hood with 70 % EtOH. Add 8 mL of tissue culture medium to a 10-cm tissue culture dish or flask for non-adherent cells and warm for 15 min in a tissue culture incubator to equilibrate temperature and pH.
2. Remove a vial of cryopreserved cells from liquid nitrogen freezer. In the laminar flow hood, thoroughly wipe down the outer surface of the vial and gloves to limit bacterial contamination. Unscrew the cap of the cryovial to equalize gas pressure inside the vial with normal atmospheric pressure. Screw the lid back down to seal the vial. Hold the vial in your gloved hand to rapidly warm the contents until partially thawed.
3. Remove the tissue culture plate or flask from the incubator and place it in the tissue culture hood. When the cells are sufficiently thawed that the still frozen portion moves freely in the tube, invert the tube vigorously to break up any cell pellet that may have formed during the freezing process, then unscrew the cap and dump the entire contents onto the tissue culture plate. Quickly replace the lid to the tissue culture plate and GENTLY agitate the plate so that the frozen pellet thaws and is evenly distributed in the pre-warmed medium. Place the plate in the humidified tissue culture incubator and leave overnight at 37 °C and 5 % CO₂. Non-adherent cells should be stored in the incubator with either a breathable filtered cap or with a non-breathable cap in place but not screwed tight, to permit appropriate gas and humidity equilibration.
- 4a. FOR ADHERENT CELLS: The next day, vacuum-aspirate the medium to remove the cryopreservative and dead cells. Replace with room-temperature complete culture medium (containing FBS).

- 4b. FOR NON-ADHERENT CELLS: The next day, titurate cells to ensure a good cell suspension, then transfer the cells and medium from the flask to a 15-mL conical tube and centrifuge at $200\times g$ for 5 min to pellet the cells. Aspirate medium and replace with fresh, pre-warmed complete medium: first, add 1 mL and pipet up and down to disperse, then add 7 mL more. Transfer cell suspension to a new flask and put back in the incubator.
- 5a. FOR ADHERENT CELLS: Visualize the plate with the $10\times$ objective on an inverted microscope to estimate survival. Cells should be checked under the microscope at least once a day to verify the morphology as that of a healthy, dividing culture.
- 5b. FOR NON-ADHERENT CELLS: Flasks or plates may be viewed on an inverted microscope. Cells from healthy non-adherent cultures have a round surface and bright, glowing edges on phase contrast. Dead cells and debris are generally irregularly shaped and dark under phase contrast. Trypan blue, and propidium iodide exclusion techniques are also commonly used to determine the viability of a non-adherent culture, either in a hemocytometer, or in a flow cytometer, respectively [15].

3.2 Expanding the Cell Population and Establishing Doubling Time

1. Cells usually need at least 1 day to recover from thawing and begin to divide. Depending on how many cells survived freezing, expansion may be required in concert with removal of dead cells and cryopreservative; or, depending upon the health of the culture and the number of cells originally frozen, cells may require a day or two before they are ready for expansion.
- 2a. FOR ADHERENT CELLS: A culture is ready for expansion when it is at about 80 % confluence on the plate. If, the day after thawing, there are only a few cells adhering to the plate, another vial should be thawed. It should not require more than a day or two after thawing for cells to reach 80 % confluence. Ideally, at least 50 % of the frozen cells should survive thawing.
- 2b. FOR NON-ADHERENT CELLS: The phenol red pH indicator in the medium will begin to turn from rosy-peach to yellow as the pH becomes more acidic, the result of metabolic wastes being released into the medium. For a healthy culture, the medium will begin to turn during the first 48 h after thawing. Upon microscopy, the population of healthy cells should have significantly increased in proportion to the debris observed upon thawing. Medium that is still peach-colored 2 days after thawing indicates that most or all of the culture is dead. Check for viability via propidium iodide or trypan blue exclusion staining. It may be necessary to thaw another frozen stock, or put the cells into a smaller volume container (such as an individual well of a 6-well plate) to concentrate the suspension, as some cell lines fare better when grown in a dense culture. Expand the population by dividing the

dense culture into five plates, wells, or flasks, and adding warmed complete medium to the appropriate volume. Skip to **step 4**.

3. Once the cells have reached 80 % confluence they are ready for expansion. Aspirate medium and add 2 mL of 0.05 % trypsin–EDTA to detach cells. Trypsin works by cleaving proteins that attach cells to the plate and to each other. After about 2 min, the bottom of the plate will appear a little cloudy to naked-eye visual inspection, indicating that the cells are beginning to “ball up” and disengage themselves from the plate. This is visible under the microscope, as the cells become circularized and show brightly glowing edges on phase-contrast microscopy. Detachment time may vary depending upon how tightly the cells adhere to the plate, but should not be more than 10 min. Once the cells have begun to ball up, tap the plate gently against your palm or carefully but firmly shake it to detach the cells. Pipeting up and down is also helpful to ensure full detachment. Tilt and swirl the plate to coax the cells off of the bottom of the plate.
4. Once the cells are completely detached, add 5 mL complete culture medium including at least 5 % FBS to “stop” the action of the trypsin–EDTA. Leaving the cells for an extended period of time in trypsin–EDTA is undesirable and decreases cellular viability.
5. Using a 5-mL serological pipette, remove the detached cells to a 15-mL conical centrifuge tube and centrifuge for 5 min at $200\times g$ to pellet the cells.
6. During centrifugation, prepare five different 10-cm diameter tissue culture plates with 7 mL each of room-temperature complete culture medium.
7. Use vacuum apparatus to aspirate the medium overlaying the centrifuged cell pellet, taking care not to aspirate the pellet itself. Leave a small volume of medium on top of the pellet in order to minimize the risk of aspirating the pellet.
8. Using a P1000 variable volume pipette (e.g., PIPETMAN, Gilson, Middleton, WI), add 1 mL of fresh complete culture medium and gently pipette up and down to break up and resuspend the pellet. Add 4 mL of medium and mix by gentle inversion in the conical tube.
9. Pipet 1 mL of resuspended cells onto each of the five plates and swirl to evenly distribute. Each of the plates should contain approximately the same number of cells.
10. While the cells are still in suspension, and BEFORE they have had a chance to settle to the bottom of the plate, remove a few microliters from at least three of the plates and determine the initial cell density using a hemocytometer or flow cytometer

with volumetric counting to count live cells. The density values should not be significantly different from one plate to the next. Average cell densities to approximate the initial cell number and concentration of cells in each of the plates. This is merely to get an idea of the number of cells on each plate and verify that there is equal distribution amongst the plates.

11. After 24 h, harvest the cells from ONE of the plates as before as a quantification control. Count the number of cells from this plate as in **step 10**. Record this value as the density at T_0 (time = zero) for the remaining uncounted plates. Remember that it takes the cells several hours to recover from plating. The input cell number should not be used as the value for T_0 .
12. The remaining plates should be harvested and counted at convenient intervals of less than 24 h, such as +18, +36, +48, and +60 h. Record these cell densities and plot them on a semi-log curve (log total cell number vs. linear time elapsed) to determine how quickly the cells are dividing. Calculate the amount of time required for cells to reach a population of approximately twice the density at T_0 . You will use this doubling time to determine when to harvest cultures after BrdU has been added. The process for establishing doubling time is inexact. The objective is to get a general idea, within a few hours, of how quickly the cells are dividing, in order to optimize the differential incorporation of BrdU. Most immortalized human cells double about once every 24 h in culture and adequate results may therefore often be obtained by adding BrdU 48 h before harvest. Some cell lines may proliferate substantially more rapidly or more slowly. The effects of having added BrdU too soon or too late will not be apparent until the procedure is complete, however; so, especially for a new or unfamiliar cell line, it is worth investing the time to establish how fast a particular cell line is actually dividing in culture under your specific laboratory conditions.

3.3 BrdU Incorporation

1. After having established the doubling time for the cell line of interest, split cells into a fresh plate for growth at approx. 10–20 % confluency.
2. When cells appear to be 80 % confluent, split and expand the culture by dividing the cells evenly across five plates prepared as in Subheading 3.2, **step 6**. Incubate overnight at 37 °C in the tissue culture incubator to allow the cells to recover.
3. The next day, thaw an aliquot of 10 mM BrdU stock. The tube should be protected from direct light while it is thawing and throughout the procedure. Turning off the nearest room lights and the fluorescent lamp in the laminar flow hood is usually sufficient.

4. Add BrdU to each of the four plates to a final concentration of 20 μM (1:500 dilution from 10 mM stock). Gently swirl the medium to hasten the even distribution of BrdU. Cover the plates LOOSELY with aluminum foil to block out ambient light and place in the incubator.
5. Have a backup-plate in case something goes wrong: the fifth plate from the expansion should remain in culture until it is 80 % confluent. It may then be frozen. Alternatively, the fifth culture may be carried until the procedure is complete and satisfactory results have been obtained, in order to avoid the delay of having to thaw, grow, and split a new culture in the event of assay failure.

3.4 Arresting Cells in Metaphase

Cells should be harvested after two rounds of cell division. Approx. 4 h before harvest is scheduled, add Colcemid to a final concentration of 0.02 $\mu\text{g}/\text{mL}$ and swirl to distribute. This should be performed using aseptic technique in laminar flow hood, in the dark, as above. Replace the aluminum foil and return the plates to the incubator. In general, about 5 % of the cells in an actively dividing culture are in metaphase at any given time. Colcemid works by inhibiting mitotic spindle formation, preventing cells from segregating chromosomes. The prescribed concentration of Colcemid in the medium is sufficient to arrest cells in metaphase and prevent them from continuing through the cell cycle; thus, time in the presence of Colcemid allows for enrichment of the percentage of the population of cells in metaphase (*see Note 3*).

3.5 Harvesting Cells for Metaphase Spreads

- 1a. FOR ADHERENT CELLS: Remove cells from the incubator and aspirate medium as usual. Add 2 mL 0.05 % trypsin-EDTA, wait for cells to round up, then fully detach all cells by gentle pipeting, adding back complete medium followed by centrifugation to recover a cell pellet, as described in Subheading 3.2, steps 3–9 (*see Note 4*).
- 1b. FOR NON-ADHERENT CELLS: No harvest with trypsin-EDTA is necessary. Centrifuge cell suspension to pellet and go directly to **step 2**.
2. Aspirate medium. Gently flick the sides of the tube with your finger to mechanically loosen the pellet, then add 1 mL hypotonic solution. Pipet up and down gently with a P1000 PIPETMAN to completely and homogeneously resuspend the pellet in the hypotonic solution.
3. Add 7 mL more hypotonic solution, mix by gentle inversion, and place in the incubator for 12 min. The hypotonic solution causes water to diffuse into cells by osmosis, making them swell. This change in morphology is visible under the microscope. The amount of time is approximate. About 12 min is usually sufficient. Leaving the cells in hypotonic solution for too long will cause delicate cells to lyse (*see Note 5*).

4. Add 2 mL freshly prepared 3:1 methanol–acetic acid fixative and invert the tube a few times to mix and fully resuspend the cells. If the cells have lysed due to an overlong exposure to hypotonic solution, it will be apparent at this time, since DNA released from lysed cells will appear “goosey” and will not resuspend.
5. Centrifuge for 5 min at $200 \times g$ to pellet the swollen, partially fixed cells.
6. Remove the hypotonic solution–methanol–acetic acid by aspiration, taking care not to aspirate the pellet.
7. Add 5 mL of 3:1 methanol–acetic acid fixative and resuspend the pellet. Cells will appear opaque white and the pellet will only require gentle inversion of the tube for complete resuspension after this step. The protocol may be stopped at this point and the cells stored in the fixative solution at 4 °C indefinitely.

3.6 Preparing and Storing Slides for Metaphase Spreads

We find that a very slight “roughening” and degreasing of the slide surface with dilute HCl helps cells adhere better to the slide upon dropping. This process should be completed the day before dropping to ensure that slides are completely chilled.

1. Fill a slide rack with new, dry microscope slides.
2. Completely immerse the slides in a solution of 0.1 N HCl in 95 % EtOH at room temperature for 20 min.
3. Remove the rack and completely immerse in a container filled with 95 % EtOH.
4. Remove the rack, discard the EtOH, replace with fresh 95 % EtOH, and immerse the slides again.
5. Repeat for a total of three rinses in EtOH.
6. Follow with three rinses in fresh distilled H₂O.
7. Store the slides completely submerged in distilled H₂O at 4 °C. It takes several hours for the water and the slides to chill to 4 °C. Although the process may be hastened by chilling in a –20 °C freezer, there is a risk for freezing, container breakage, and slide breakage. We find it most convenient to prepare the slides at least a day in advance of dropping. Unused slides can be stored indefinitely, submerged in distilled H₂O, at 4 °C.

3.7 Preparing Metaphase Spreads

1. Use 3:1 methanol–acetic acid fixative made fresh on the day that cells are to be dropped onto the slides. According to some protocols, this procedure works best on wet days, when the room is air particularly humid due to the manner in which atmospheric conditions affect the speed of evaporation of the fixative solution on a microscope slide. We have not found normal room air humidity to be problematic. This procedure works well at normal humidity, 28–35 %. It does seem to work better, even on sunny days, if the room temperature is slightly high, around 27 °C.

2. Centrifuge swollen/fixed cells at $200\times g$ for 5 min, aspirate fixative, resuspend in 5 mL fresh fixative.
3. Repeat for a total of three aspirate/resuspend/centrifuge cycles.
4. Resuspend cells in the minimal amount of fixative that causes the suspension to have a slightly translucent, milky appearance similar to Scotch tape.
5. Remove a slide from the container of chilled water. Prop one end on a disposable 10 mL serological pipette so that the slide angles slightly downward.
6. Attach a rubber suction bulb to a 1 mL glass Pasteur pipet with a 5" barrel. Squeeze the bulb, then insert the pipet into the cell suspension and gently release the bulb to draw up the cells. Position the end of the pipet about 5 in above the slide and quickly distribute 7–10 drops of cell suspension along the length of the still-wet slide (*see Note 6*).
7. Allow most of the liquid to pool at the bottom of the slide, and quickly follow with 7 or 8 drops of 3:1 methanol–acetic acid fixative distributed over the slide.
8. Once this has also pooled at the bottom of the slide, blot the excess liquid from the bottom edge of the slide using a paper towel. Hold the slide about an inch from your open mouth and gently exhale a single breath (Do not blow! The goal is to temporarily increase the local humidity and temperature), then fan the slide once back and forth in the air. Set the slide vertically upright at approx. 90° to the benchtop, leaning against a vertical surface (such as an empty test tube rack).
9. After about 30 s, pick up the slide and watch for the fixative to begin to recede from around the edges of the slide as it evaporates. When this occurs, place the slide on a slide warmer at 42°C and allow to completely dry. If the slides are too dry, the chromosomes will be too close to each other to allow for appropriate staining and scoring. If the slides are too wet, individual chromosomes will float away from one another and not form obvious spreads which can be visualized in a single microscope field.
10. Repeat this process to make several slides from each cell suspension. The cells will settle relatively rapidly in the tube. Handle them carefully, inverting gently to remix. Vigorous pipeting will cause cells to rupture while still in the tube.
11. Once the slides are dry (about 10 min on the slide warmer at 42°C), metaphases can be located using a 25x objective on the inverted microscope with 10x eyepieces for a total of 250 \times power. The vast majority of cells will be round and intact (predominantly non-metaphase cells), but occasionally, there

should be easily visible, tiny, X-shaped chromosomes arranged in small spreads. These are the metaphases that will be stained and scored in subsequent steps. Screen each slide to ensure that there are at least 20–30 metaphases. Allow the slides to dry overnight in the open air at room temperature on the benchtop.

3.8 Differential Sister Chromatid Staining with Giemsa

1. Immerse slides with metaphases in 10 µg/mL (1/100th vol of 1 mg/mL stock) Hoechst 33258 in water for 20 min. Hoechst 33258 is a UV sensitizer.
2. Rinse by immersing slides in Sorensen buffer (*see Note 7*).
3. Remove each slide from the rack, quickly pipet a few drops of Sorensen buffer along the length of the slide, and immediately add a coverslip (0.15-mm average thickness) to prevent slides from drying out (*see Note 8*).
4. Expose slides coverslip-side-up on a 55 °C slide warmer to long wavelength (approximately 365 nm) UV light (for example, at a distance of 5–10 cm from two 20 W blacklight-blue bulbs) for 20–30 min. Exposure to UV preferentially degrades DNA more highly incorporated with BrdU, preventing it from accepting Giemsa stain later in the procedure.
5. Carefully remove and discard the coverslips and place the slides back in an empty rack. Immerse the rack in 1× SSC and incubate for 1 h at 50 °C. We use an EasyDip container immobilized in a shaker oven, but a regular hybridization oven or warm room would work equally well.
6. Remove the slides from SSC and immerse the slides in 10 % Giemsa made up in Sorensen buffer for 30 min at room temperature.
7. Rinse by quickly immersing the slides in distilled H₂O and allowing them to drain on a paper towel. Two or three quick rinses in water may be required to remove the Giemsa solution, although it is not necessary for all of the Giemsa stain to be completely rinsed away. The slides will retain a purplish tint at the end of this procedure. Only rinse the slides sufficiently to keep droplets of relatively concentrated Giemsa solution from collecting and drying on the slide surface, which would obscure the results.
8. Allow the slides to dry face up without a coverslip on the benchtop overnight at room temperature.

3.9 Preparing Slides for Visualization

Place one or two drops of Cytoseal-60 low viscosity mounting medium onto slide and add coverslip. Ideally, slides should be left face-up overnight to allow the Cytoseal-60 to dry completely. Although the medium is characterized as fast drying, we have

3.10 Visualizing and Digital Imaging of Chromosomes

experienced some difficulty with coverslip displacement when the slides were not allowed to dry completely before microscopy.

1. Observe SCEs using bright-field microscopy and a 63× or 100× high-quality oil-immersion objective lens. A microscope with good optics is absolutely required. At a minimum, a 63× plan apochromatic oil-immersion objective lens with a numerical aperture of 1.4 is necessary. Otherwise, the differentially stained sister chromatids will fail to resolve, and the lighter staining chromatid will appear washed out to the point of invisibility. This assay is impossible with inferior quality optics. High-quality digital imaging equipment and interface is also essential for effective and efficient data acquisition. Adjust the exposure and gain settings to capture as optimal an image as possible. Use the microscope/camera's imaging software and manufacturer's instructions to save captured images as tiff or high-quality .JPEG files. Images may be saved as grayscale.
2. After capture and saving, open the image in image processing software such as Adobe Photoshop or Macintosh Preview and adjust the magnification, contrast, and grayscale levels to maximize the differential staining of the sister chromatids.

3.11 Scoring SCEs and Analyzing Data

Well-stained metaphase spreads are straightforward to score for SCEs (*see Note 9*). It is convenient to print a high-resolution hardcopy of the image file and score the number of exchanges and chromosomes on the paper printout, then input the numbers into a spreadsheet program such as Microsoft EXCEL. A properly differentially stained chromatid pair with no SCEs will appear as one black stripe (the mono-strand BrdU-substituted sister) and one lighter gray stripe (the doubly BrdU-substituted sister). A single exchange is any time the dark and light staining on the chromatids switches places. The BrdU itself induces a low level of SCEs so it is rare to find an entire spread with no exchanges whatsoever. Count the total number of exchanges in the spread and also the total number of scorable chromosomes in a spread. If the change happens at the centromere, this may be due to the chromosome being twisted and does not represent a true SCE. Do not include these chromosomes in your scoring. If the image is ambiguous and it is not possible to determine with reasonable certainty that an exchange has taken place (*i.e.*, on a partially stained chromosome) do not include that chromosome in the count. For each metaphase spread, divide the number of exchanges by the total number of scorable chromosomes in the spread. This is the number of exchanges per chromosome. Scoring 20 metaphases should provide a general idea of the level of stability (*see Note 10*).

4 Notes

1. BrdU must be protected from light at all times in order to avoid UV-light mediated free radical decomposition.

2. Dilute Hoechst 33258 works equally well dissolved in PBS instead of water, however, concentrated Hoechst 33258 is well soluble in water but only sparingly soluble in PBS, so the powder should first be dissolved in water and then subsequently diluted in PBS if so desired.
3. Adding a higher concentration of Colcemid WILL NOT increase the number of cells in metaphase. The amount of time the cells are left in Colcemid will influence the number of metaphases, however, and should be adjusted depending on how quickly the cells divide. The longer the time in Colcemid, the higher the percentage of cells in metaphase will be. However, if cells are arrested in metaphase by the Colcemid for too long, the chromosomes will condense and shrink somewhat, rendering them suboptimal for staining and scoring later in the procedure. We have found that for a cell line which doubles approximately every 24 h, 4 h in Colcemid is sufficient for enriching the number of metaphases with minimal numbers of shrunken chromosomes.
4. Some investigators prefer to enrich for metaphase cells from adherent cultures by using dilute trypsin-EDTA to slow the detachment process so that cells can be recovered by agitation as they begin to ball up. Theoretically, the metaphase cells will preferentially round up on the plate and become gently mechanically detachable first, enabling non-metaphase cells to be left behind. Many protocols include this step for preferential harvest of cells in metaphase. We do not recommend this method, since we find it difficult to perform reproducibly, and to generally result in a much smaller cell yield without significantly increasing the proportion of metaphases. By this method, the overall size of the cell pellet is reduced by 80 or 90 %, and even though the pellet may contain a much larger percentage of cells in metaphase, the pellet itself becomes physically difficult to work with and is easily lost during repeated rinses in fixative and/or dropping onto slides. By harvesting the entire plate, the non-metaphase cells add volume to the pellet, making it easy to work with. Using four confluent plates of cells and a dense suspension eliminates the need for further metaphase enrichment by preferential harvest.
5. Depending on membrane characteristics, some cell lines are more amenable to this procedure than others. It may be useful to practice making metaphase spreads using relatively easy to grow and manipulate cells such as HeLa or K562 first.
6. Ensure that the steps from removing the slide from the chilled water to dropping the swollen/fixed cells onto the slide are done as rapidly as possible, to ensure that the slide is still very wet with distilled water when the cells are dropped onto it. It may be helpful to fill the pipet with cell suspension and rest it in the centrifuge tube. Remove the slide from the H₂O

container with one hand and rest it on the serological pipette. IMMEDIATELY drop the cells using the other hand.

7. Some protocols call for using McIlvaine's buffer at this step, but in our hands, McIlvaine's solution tends to precipitate and leave debris on the slide. We get excellent results with the use of Sorensen buffer throughout the procedure.
8. Do not seal the coverslip to the slide.
9. If only one chromatid is visible after staining or if chromosomes appear extremely desiccated, try decreasing the UV exposure time. Overexposure to UV will essentially obliterate the sister doubly substituted with BrdU, and render it invisible, rather than very lightly stained. If both chromatids appear lightly stained, but there is no differentiation, or if there is only partial staining of one chromatid, decrease the amount of time between BrdU and Colcemid treatment/cell harvest. Allowing more than two rounds of division in BrdU will cause incorporation into both of the sisters, preventing differential staining. Conversely, if both chromatids appear darkly stained, try increasing the time between adding BrdU and harvesting. Insufficient incorporation of BrdU will cause the sister chromatids to accept Giemsa equally well, resulting in no differential staining.
10. If there is a great deal of variability between the scores of each spread, or if you need to detect small differences in levels of SCEs between different cell lines, 50 or even 100 metaphases may be required to provide adequate statistical power.

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