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Methods to Monitor DNA Repair Defects and Genomic Instability in the Context of a Disrupted Nuclear Lamina

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Abstract

The organization of the genome within the nuclear space is viewed as an additional level of regulation of genome function, as well as a means to ensure genome integrity. Structural proteins associated with the nuclear envelope, in particular lamins (A- and B-type) and lamin-associated proteins, play an important role in genome organization. Interestingly, there is a whole body of evidence that links disruptions of the nuclear lamina with DNA repair defects and genomic instability. Here, we describe a few standard techniques that have been successfully utilized to identify mechanisms behind DNA repair defects and genomic instability in cells with an altered nuclear lamina. In particular, we describe protocols to monitor changes in the expression of DNA repair factors (Western blot) and their recruitment to sites of DNA damage (immunofluorescence); kinetics of DNA double-strand break repair after ionizing radiation (neutral comet assays); frequency of chromosomal aberrations (FISH, fluorescence in situ hybridization); and alterations in telomere homeostasis (Quantitative-FISH). These techniques have allowed us to shed some light onto molecular mechanisms by which alterations in A-type lamins induce genomic instability, which could contribute to the pathophysiology of aging and aging-related diseases.

Keywords

Genomic instability; DNA repair; DNA damage response; Western blot; Immunofluorescence; Neutral comet assay; Q-FISH; Nuclear lamina

1 Introduction

Efficient DNA repair is intimately linked to the integrity of the nuclear lamina, a meshwork of intermediate filaments under the inner nuclear membrane that also extends throughout the nucleoplasm. The nuclear lamina is formed by A-type lamins (lamin A/C), B-type lamins (lamin B1/B2), and lamin-associated proteins. A whole body of evidence indicates that cells with a disrupted nuclear lamina due to mutation or loss of lamins exhibit genomic instability [1–4].

Many studies have focused on the characterization of how expression of unprocessed prelamin A or mutant lamin A proteins affect DNA repair. A landmark study demonstrated delayed recruitment of DNA repair factors 53BP1 and RAD51 to sites of DNA damage

4 Notes

³We develop our Western blots with the Syngene PXi. We like this method because you can view the blot as it is developing. However, a cheaper alternative is to use autoradiography films which also work well. The developing method might also impact ECL choice.

(γ H2AX foci) induced by radiation in cells with a disrupted nuclear lamina [5]. Other studies showed aberrant accumulation of the repair protein XPA at DNA lesions, which activates ATM- and ATR-dependent signaling cascades contributing to proliferation arrest in cells deficient in A-type lamins [6]. Additional suggested mechanisms include reduction of DNA-PK holoenzyme, a key factor in non-homologous end-joining (NHEJ) repair [7], delayed recruitment to DNA double-strand breaks (DSBs) of factors of the MRN complex (NBS1 and MRE11), which are necessary for homologous recombination (HR) [8], and defects in chromatin-modifying activities such as the NuRD complex and the histone acetyltransferase Mof [9, 10]. Recently, increased H3K9me3 levels due to higher activity of histone methyltransferase Suv39h1 has been linked to genomic instability and premature senescence [11].

Our studies have also revealed mechanisms behind DNA repair defects in lamin A/C-deficient cells. We found that loss of lamin A/C leads to activation of cathepsin L-mediated degradation of 53BP1 and reduced expression of BRCA1 and RAD51 [12–17]. We also showed that loss of lamin A/C leads to altered distribution of telomeres in the 3D nuclear space, which is accompanied by telomere shortening. Moreover, an increase in aneuploidy and in the frequency of chromosome and chromatid breaks was observed in lamin A/C-deficient cells. Altogether, these studies identified a plethora of factors whose levels and recruitment to sites of DNA damage are altered in cells with a disrupted nuclear lamina, leading to defects in the two main mechanisms of DNA DSB repair: HR (homologous recombination) and NHEJ (non-homologous end-joining). As a consequence, cells exhibit a permanent checkpoint activation that induces proliferation arrest.

Here, we describe methods utilized in lamin A/C-deficient cells to monitor expression of DNA repair factors (Western blot), and their recruitment to sites of DNA damage (immunofluorescence), as well as the kinetics of DNA DSB repair (neutral comet assays after ionizing radiation). Alterations in the lamina can also induce shortened and dysfunctional telomeres, which can be assessed by quantitative-fluorescence in situ hybridization (Q-FISH). FISH also allows the monitoring of genomic instability (aneuploidy, chromosome end-to-end fusions, chromosome and chromatid breaks, and other chromosomal aberrations) in metaphase spreads.

2 Materials

2.1 Western Blotting Components

All techniques, with the exception of Western blotting, require a fluorescence microscope with camera.

1. RIPA buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.5 % SDS. Prepare 50 mL by mixing 2.5 mL 1M TRIS-HCl (pH 8.0), 1.5 mL 5 M NaCl, 500 μ L NP-40, 2.5 mL DOC 10 %, 2.5 mL 10 % SDS, and 40.5 mL deionized water. Store at 4 °C.
2. Fresh RIPA Solution: 2 mL RIPA buffer, 20 μ L PMSF, 4 μ L DTT, 20 μ L 100 $\times$ protease and phosphatase inhibitor cocktail. Prepare fresh for each Western blot (*see* **Note 1**).

3. 1 mL syringes.
4. Needles: 26 G $\times$ 1/2 in; 30 G $\times$ 1/2 in.
5. Bovine Gamma Globulin Standard (BGG).
6. Protein Assay Dye Reagent: Dilute Protein Assay Dye Reagent Concentrate 1:4 with deionized water.
7. 4 $\times$ Laemmli buffer.
8. Tris-glycine running buffer (10 $\times$): Add 29 g TRIS base, 144 g glycine, 10 g SDS and bring final volume to 1 L with deionized water.
9. Precision Plus Protein™ Kaleidoscope™ Standards.
10. Gel 4–15 % Criterion™ TGX™ (18 wells of 30 μ L).
11. Trans-Blot® Turbo™ Transfer Starter System.
12. Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Kit.
13. Transfer buffer: Mix 40 mL 5 $\times$ transfer buffer and 40 mL ethanol, and bring to 200 mL with deionized water.
14. Ponceau S solution.
15. PBS-T: Add 800 μ L Tween™ 20 to 1 L PBS.
16. Blotting buffer: Add 3 g of blotting grade buffer to 100 mL of PBS-T.
17. Antibodies: 53BP1, BRCA1, vinculin, RAD51, γ H2AX, donkey anti-rabbit IgG-HRP, bovine anti-goat IgG-HRP, goat anti-mouse IgG-HRP.
18. Boxes or trays suitable for incubation of blotting membranes.
19. Clarity Western ECL Substrate (*see Note 2*).
20. Membrane development: multi-application gel imaging system or classical autoradiography film and cassette (*see Note 3*).

2.2 Components for Detection of DNA Repair Foci

1. Unifrost microscope slides 75 $\times$ 25 $\times$ 1 mm.
2. Glass coverslips 9 $\times$ 9 mm. Coat coverslips with HistoGrip following the manufacturers' instructions. Histogripped cover-slips can be stored after preparation in a sterile 50 mL conical tube (*see Note 4*).
3. 60 mm (p60) tissue cell culture plate.

¹While the RIPA buffer can be prepared and stored for months at 4 °C, make the RIPA working solution, with PMSF, DTT, and protease & phosphatase inhibitors fresh before each blot.

²ECL used for ideal signal can vary depending upon protein of interest and the amount of protein loaded. Clarity Western ECL is a good starting point. If the signal is weak, Immobilon Western Chemiluminescent HRP Substrate is stronger and works well for less abundant proteins. If the Clarity Western ECL signal is too strong, Pierce™ ECL Western Blotting Substrate produces a weaker signal and works well for more abundant proteins, like vinculin.

⁴We previously coated coverslips with poly-L-lysine. However, we found that HistoGrip works much better than poly-L-lysine, especially for cells that do not normally attach tightly to coated surfaces.

4. Fine point micro tweezers for handling coverslips.
5. 10 % Triton X-100 solution: Dilute Triton X-100 1:10 in PBS.
6. 10 % BSA solution: Dissolve 10 g of bovine serum albumin in PBS to a final volume of 100 mL. Mix solution until BSA is entirely dissolved in solution. Make aliquots and store them at -20°C .
7. Fixing buffer: 3.7 % formaldehyde, 0.2 % Triton X-100 in PBS. Use 4 mL for each p60. For 20 mL, mix 2 mL 36.5–38 % formaldehyde solution, 400 μL Triton X-100 10 %, and 17.6 mL PBS.
8. Blocking solution: 2 % BSA, 0.1 % Triton X-100 in PBS. For 20 mL, mix 4 mL BSA 10 %, 200 μL Triton X-100 10 %, and 15.8 mL PBS.
9. Antibody diluting solution: 1 % BSA, 0.1 % Triton X-100 in PBS. For 20 mL, mix 2 mL BSA 10 %, 200 μL Triton X-100 10 %, and 17.8 mL PBS.
10. Antibodies: γH2AX , 53BP1, BRCA1, RAD51, goat anti-rabbit secondary antibody Alexa Fluor[®] 488 conjugate, goat anti-mouse IgG secondary antibody Alexa Fluor[®] 594 conjugate.
11. DAPI Mounting Medium: Mix two drops of VECTASHIELD Mounting Medium with one drop of VECTASHIELD Mounting Medium containing DAPI.

2.3 Components for Neutral Comet Assay

1. PBS and Lysis Solution. Bring to 4°C before use.
2. Low-Melting Agarose. Prepare 1 mL aliquots. One hour before use, melt aliquots at 95°C and then immediately move to thermal block at 37°C (see **Note 5**).
3. TBE electrophoresis buffer: Prepare $1\times$ Tris–Borate–EDTA buffer by diluting $5\times$ concentrate in deionized water. Bring to 4°C before use.
4. Staining dish and slide staining rack.
5. SYBR^{00AE}; Gold Nucleic Acid Gel Stain (10,000 $\times$ Concentrate in DMSO). For use, dilute concentrate in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA). Diluted stock is stable for several weeks if stored at 4°C in dark.
6. Comet assay analysis by CometScore program.

2.4 Components for Q-FISH

1. Colcemid—10 $\mu\text{g}/\text{mL}$.
2. Potassium chloride. Prepare 0.56 % KCl by adding 0.56 g to 100 mL of deionized water.

⁵Be cautious not to keep agarose at 95°C for too long. Agarose that is too hot can impact comet tail length.

3. Methanol–acetic acid fixing solution: Mix methanol with acetic acid at 3:1 and keep at -20°C . Make fresh for each Q-FISH.
4. Acidified pepsin: Mix 200 mg pepsin, 200 mL deionized water, and 168 μL of concentrated HCl.
5. 4 % formaldehyde fixing solution: For 800 mL, mix 94.4 mL of 35 % formaldehyde with 705.6 mL PBS.
6. Telomere probe mix (Table 1):
7. Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl (pH 7.5). Adjust with NaOH. For 200 mL, mix 6 mL 5 M NaCl, 2 g maleic acid and bring to 200 mL with water. Aliquot and store at -20°C .
8. BM 10 %: 10 g blocking reagent in 100 mL maleic acid buffer pH 7.5. Dissolve on a heating block or microwave. Aliquot and store at -20°C .
9. MgCl_2 buffer: 25 mM MgCl_2 , 9 mM citric acid, 82 mM Na_2HPO_4 , pH 7.0. For 100 mL, mix 2.5 mL of 1 M MgCl_2 , 9.0 mL 0.1 M citric acid, 8.2 mL of 1 M Na_2HPO_4 , and 80.3 mL deionized water.
10. Formamide–BSA wash solution (Table 2):
11. TBS–Tween 20 wash solution: Make 10 $\times$ TBS stock, with final concentration 1.5 M NaCl and 1 M TRIS pH 7.0–7.5. Dilute stock 10 $\times$ TBS 1:10 in deionized water and add Tween-20 (0.08 %).
12. Staining dish and slide staining rack.
13. DAPI Mounting Medium: Mix two drops of VECTASHIELD Mounting Medium with one drop of VECTASHIELD Mounting Medium with DAPI.
14. Nail polish.
15. TFL-Telo computer program. This is an application program developed by Peter Lansdorp and used to estimate the length of telomeres from captured images of metaphases that have been stained with a telomere probe for quantitative in situ hybridization analysis [18]. With the program, the integrated fluorescence intensity value for each telomere, which is proportional to the number of hybridized probes, is calculated.

3 Methods

3.1 Western Blotting for DNA Repair Factors

Day 1

1. Collect at least 600,000 cells in conical tube. Centrifuge at $300 \times g$ for 5 min. Discard supernatant and resuspend pellet in PBS. Centrifuge at $300 \times g$ for 5 min. Discard supernatant and collect the cell pellet.
2. Add fresh RIPA solution, according to size, to each cell pellet. The amount of RIPA added ranges between 60 and 150 μL , although this depends upon

the number of pelleted cells. Generally, 10^6 cells will be suspended in ~100 μ L to obtain an adequate protein concentration. Mix cell lysates well by pipetting up and down.

3. Transfer lysates to Eppendorf tubes. Sonicate with “High” setting for 7.5 min at 4 °C.
4. Shear lysates with ten passes through a 26-gauge needle. Then, shear samples with ten passes through a 30-gauge needle (*see Note 6*).
5. Determine protein concentration by Bradford assay, as per manufacturer’s instructions. Read absorbance at 595 nm.
6. Prepare samples for gel loading. Load ~50 μ g in 30 μ L per well in 4–15 % Criterion™ TGX™ gel with 18 wells per gel (*see Note 7*). Mix 50 μ g of protein, 7.5 μ L 4× Laemmli buffer, and take the volume to 30 μ L with RIPA buffer.
7. Heat tubes to 90 °C for 5 min.
8. Vortex samples well and centrifuge.
9. Set up electrophoresis chamber: place gel in chamber and add 1× Tris-Glycine running buffer to compartment; remove comb and load Kaleidoscope™ Standards ladder and samples.
10. Run gel with voltage set anywhere from 90 V to 180 V. Run gel as far as needed to allow adequate separation of proteins of interest for investigation.
11. After electrophoresis is complete, remove cartridge and open on sides by using spatula.
12. Assemble transfer cassette for Trans-Blot® Turbo™ Transfer Starter System. Place membrane stack in transfer buffer and add first to cassette. Place nitrocellulose membrane in transfer buffer and add to stack. Place gel on membrane (*see Note 8*). Place another membrane stack in transfer buffer and add to cassette. Close cassette and insert into Trans-Blot® Turbo™ Transfer Starter System.
13. Run Transfer System at 1.3 A and 25 V for 25 min.
14. Remove membrane from cassette and place in tray. Add Ponceau-S for ~30 s (*see Note 9*).

⁶After sonication and processing with needles, the cell lysate should have a similar viscosity to water, whereby it can be easily pipetted dropwise. If the sample is too viscous, more RIPA buffer should be added.

⁷The ideal amount of protein to load depends upon the protein being investigated. It is imperative that the amount of protein loaded falls within the linear and quantitative dynamic range for the protein of interest given the experimental conditions. This linear range must be determined by performing a dilution series of protein and determining if the signal generated from the blot corresponds to the dilution.

⁸As each successive component is added to the cassette, it is crucial to ensure that everything is flat by using a roller. When adding the gel to the cassette, make sure there is ample buffer on the membrane to allow easy repositioning of the gel if necessary. After adding the gel to the membrane, ensure that there are no air bubbles between the gel and the membrane, as this could prevent transfer in those areas. Before closing the cassette, dump excess liquid from the cassette. Too much extra liquid could reduce efficiency of transfer.

15. Wash off Ponceau-S with deionized water and cut membrane. Place various sections of membrane in Western blot boxes.
 16. Wash membranes in blotting buffer for 15 min.
 17. Incubate samples overnight, shaking at 4 °C, in primary antibody dissolved in blotting buffer.
- Day 2
18. Wash membranes 3× with PBS-T, 10 min each time.
 19. Incubate samples for 1 h with secondary antibodies dissolved in blotting buffer.
 20. Wash membranes 3× with PBS-T, 10 min each time.
 21. Develop membranes by incubating membrane with Clarity Western ECL Substrate or ECL of choice (*see Note 2*).
 22. Visualize blot using either gel imaging system or autoradiography film in dark room. An example of results obtained by Western blotting is shown in Fig. 1.

3.2 Formation and Resolution of DNA Repair Foci

Day 1

1. HistoGrip coverslips following manufacturer's instructions.
2. Drop 8 coverslips coated with HistoGrip in a p60 for each set of conditions to be investigated, as shown in Fig. 2 (*see Note 10*).
3. Plate cells at 70–80 % confluency in p60. Ensure that after cells are plated, all coverslips are attached to bottom of plate and are not floating. Leave overnight for cells to attach.

Day 2

4. Add treatment/irradiate all p60s together with desired level of treatment/dosage (*see Note 11*).
5. After treatment/irradiation, place cells back in incubator and allow given amount of time to repair DNA damage. If there is a 0 min time point for irradiation, irradiate those cells on ice or place them on ice immediately after irradiation.

⁹Ponceau-S staining reveals success for protein transfer. If transfer occurred correctly, protein bands should be evident, including high molecular weight bands. When staining, take precaution that the membrane does not become dry for an extended period of time.

¹⁰Prepare one p60 for each set of conditions being investigated. For example, to investigate a particular DNA repair protein in wild-type and knock-out cells at 0 min, 30 min, 1 h, 6 h, 12 h, and 24 h after irradiation, plate 12 plates total, six for the wild-type and six for the knock-out. Pick time points based upon cell type and protein of interest, as has previously been done [17, 19].

¹¹Since the goal is to monitor kinetics of DNA repair, the amount of damage should be enough to induce damage but not kill the cell. When irradiating, we normally irradiate between 0.5 Gy and 3 Gy for human normal fibroblasts. However, the optimal dose of irradiation will need to be determined for each cell type.

6. When the desired amount of repair time has elapsed, aspirate media and wash cells with PBS. Then, immediately fix cells with fixing buffer for 10 min at room temperature (see **Note 11**).
 7. Wash coverslips 3× with PBS, 5 min each time. After washing, cells can be left at 4 °C in PBS until all other time points have been fixed.
 8. Incubate coverslips for 1 h at 37 °C in blocking solution.
 9. Wash coverslips 3× with PBS, 5 min each time. At this point, cells can be stored in PBS at 4 °C for up to a week before proceeding. For longer storage, keep coverslips in PBS with 0.02 % sodium azide.
 10. Prepare microscope slides for immunofluorescence (Fig. 2): put Parafilm over a microscope slide to cover it. Pipette 25–40 µL of the diluted antibody in two separate spots on Parafilm. Our typical antibody dilutions, diluted in antibody diluting solution, are: 1:50 (γH2AX), 1:100 (BRCA1), 1:200 (RAD51), 1:1000 (53BP1). Use tweezers to lower the cover-slip (cell side down) onto the droplets (see **Notes 12 and 13**).
 11. Incubate for 1 h in primary antibody at 37 °C in humidity chamber made by placing a wet paper towel into a sealed plastic box, together with the slide(s) (Fig. 2).
 12. Transfer coverslips from slide to a new p60 containing PBS, keeping coverslip cell side up. Wash coverslips 3× with PBS, 10 min each time. Meanwhile, prepare slide with new piece of Parafilm and two drops of secondary antibody. We use 1:1000 secondary antibody dilutions.
 13. Incubate coverslips in secondary antibody for 1 h in humidity chamber.
 14. Transfer the coverslips from slide to a p60 containing PBS, keeping coverslip cell side up. Wash coverslips 3× with PBS, 10 min each time. To reduce background, leave PBS on cover-slips overnight at 4 °C in the dark.
 15. After washing, obtain new slide. Add 7 µL of DAPI mounting medium directly to slide. Add coverslip to slide, cell side down. Pipette off excess liquid. Fix coverslip to slide with fingernail polish by outlining edge of coverslip. Leave slides in dark over-night at 4 °C (see **Note 14**).
- Day 3
16. Visualize staining in microscope and take pictures (Fig. 3).
 17. Quantitate staining in each slide. There are two ways to quantitate the IF (see **Note 15**). One way is to count the number of foci. In each cell

¹²When washing cells, be careful to minimize detachment of cells. If any solution is added to p60 too quickly, cells can detach from coverslips, hindering your ability to perform immunofluorescence. The Parafilm prevents the antibody from spreading out on the slide and allows the coverslip to float on the antibody solution.

¹³We usually have at least two coverslips per condition. That way, if one coverslip breaks, there is another coverslip to use. Also, there are more cells to analyze this way.

¹⁴There is usually a moderate amount of background present if you look at the cells immediately after fixing the coverslips to the slide. Letting the coverslips sit overnight before viewing usually increases the quality of the immunofluorescence.

nucleus, when examining DNA repair proteins (53BP1, BRCA1, RAD51) or markers of DNA damage (γ H2AX), there should be foci at areas of DNA damage (*see* **Note 16**). Another way is to quantitate the total fluorescence signal in a cell nucleus (*see* **Note 17**).

3.3 Neutral Comet Assay

Day 1

1. Plate ~600,000 cells in a p60 and add treatment interested in investigating. Prepare 4–6 plates per sample (*see* **Note 18**).
2. Irradiate sample with 8 Gy. Write down the exact time the plates came out of irradiator and the subsequent time they are to be harvested, depending upon the amount of time to be given to repair (*see* **Note 19**).
3. Trypsinize cells to a single cell suspension. Dilute approximately 1:1 in PBS. Do not use media! Immediately place cell suspension on ice (*see* **Note 20**).
4. Place slides on thermal block at 37 °C.
5. Mix 10 μ L cell suspension and 100 μ L melted agarose in Eppendorf tube. Mix well and drop 50 μ L in each window on the comet assay slide (*see* **Note 21**).
6. Place the slides immediately at 4 °C. Let them cool down for 10–30 min (*see* **Note 22**).
7. Run electrophoresis at 35 V for 30 min using TBE buffer.
8. Wash slides 2 $\times$ with deionized water, 10 min each time.
9. Wash slides 1 $\times$ with ethanol for 5 min. Keep slides in a dry box until the next day.

¹⁵Generally, count at least 200 cells/condition when quantitating.

¹⁶One method for quantitating immunofluorescence for DNA repair proteins involves quantitating the number of foci present in the cell nucleus. This is usually done in two ways: count the number of foci per cell and determine average number of foci per cell; alternatively, set a cut-off point and determine whether the cells have more foci (termed positive) or less foci (termed negative) than the cutoff point. Then, determine the percentage of cells that are positive or negative. Generally, we use five as our cut-off point when determining whether cells are positive or negative for a given DNA repair protein, although this cut-off can be changed depending on the given situation.

¹⁷The intensity of the immunofluorescence signal can be quantitated in each situation. Programs like ImageJ can measure the intensity of signal for a given area, and DAPI staining can allow for determination of the specific area of the nucleus.

¹⁸The neutral comet assay will monitor kinetics of repair for DNA double strand breaks. Consequently, for each treated and untreated sample, we usually have plates for repair times of 0, 30, 60, 90, 120, and 150 min.

¹⁹Induce a large amount of damage for neutral comet assays. However, the amount irradiated will vary based on cell type. If there is no repair by 120 min, reduce the dose of irradiation. Also, we usually irradiate our samples so that they can all be embedded on the slide at the same time. Thus, we irradiate our 150 min repair sample first. 30 min later, we irradiate our 120 min sample, etc. Cells for the 0 time point are trypsinized before irradiation and irradiated on ice.

²⁰Choose a dilution so that you will have cells on your slide, but not so many that it will be hard to analyze. Confluency will need to be optimized to determine the correct dilution of trypsin:PBS to determine adequate cell plating without overplating.

²¹Once the suspension is added to the slide, quickly distribute suspension with pipette tip throughout slide. Be sure that agarose is secured to edge of slide windows to ensure that electrophoresis will work properly. Add suspension right against the edge and work it up onto red border to ensure that the edges are sealed as suspension is being spread across the slide.

²²It is important that the slides are thoroughly cooled. If electrophoresis is begun with warm TBE or slides where the agarose is not cool, the agarose may melt or dissociate from the slide.

Day 2

10. Stain slides with SYBR Gold.
11. Wash membranes 2× with deionized water, 10 min each time.
12. Take pictures of at least 20–25 comets for each experimental condition using fluorescence microscope (*see Note 23*).
13. Analyze images with CometScore™, a free software that measures a number of parameters such as Olive tail moment, tail percentage intensity, and tail length, which overall serve to determine the extent of unrepaired DNA damage (double-strand breaks if the comet assay is performed under neutral conditions). Olive tail moment is used most frequently to monitor DNA repair kinetics, and to identify cells that are deficient in DNA repair (Fig. 4).

3.4 Q-FISH

Day 1: Preparation of Metaphases

1. Add colcemid (100 ng/mL) to cells in culture. Incubate for 2–4 h at 37 °C (*see Note 24*).
2. Collect 10 mL of media from cells and transfer to a conical vial. Wash cells with 5 mL PBS and combine with 10 mL media (*see Note 25*).
3. Trypsinize cells. Inactivate trypsin with the medium in the conical tube and collect cells by centrifugation for 8 min.
4. Aspirate supernatant, leaving 1 mL in the tube; resuspend cells by tapping the tube.
5. Add dropwise, while gently vortexing, 9 mL of 0.56 % KCl preheated to 37 °C.
6. Keep in water bath at 37 °C for 10–12 min.
7. Add 3 drops of fresh methanol–acetic acid fixing solution at 4 °C (*see Note 26*).
8. Sediment cells by centrifugation for 8 min.
9. Aspirate supernatant until only 1 mL remains.

²³The best pictures are taken from the center of the slide windows. Pictures should reveal a comet-shaped DNA pattern as a result of electrophoresis (Fig. 4). The comet head will contain high molecular weight and intact DNA, while the tail will contain the leading ends of migrating fragments.

²⁴Colcemid arrests cells in metaphase. Since telomeres are best analyzed during metaphase, it is best to arrest many cells in metaphase. The amount of cells arresting with a given colcemid treatment is a function of how quickly cells are cycling. Thus, slower cycling cells will need to be kept in colcemid for a greater amount of time. As a starting point, we keep lymphocytes and immortalized cells in colcemid for 2 h. Fibroblasts and primary cells are kept in colcemid for 4 h. This time may need to be lengthened if very few cells arrest in metaphase.

²⁵Cells in mitosis do not attach well to the plate. It is important to keep the media and the PBS wash, since it might contain mitotic cells.

²⁶It is important that fixative is made fresh every time. Also, cover tube label with transparent tape to ensure that methanol–acetic acid does not drip on outside and remove label while vortexing.

10. Add 2 mL fresh methanol–acetic acid fixing solution while gently vortexing. Add another 9 mL (*see Note 27*).
11. Repeat **steps 8–10**. Samples can be kept at –20 °C until preparation of metaphases.
12. Sediment cells by centrifugation for 8 min.
13. Aspirate supernatant until only 1 mL remains.
14. Resuspend cells and add 10 mL fresh methanol–acetic acid fixing solution while vortexing.
15. Sediment cells.
16. Aspirate supernatant until only 1 mL or less remains, depending on size of cell pellet (*see Note 28*).
17. Aspirate cells with a Pasteur pipette where a capillary end has been created.
18. Wet a glass slide in 45 % Acetic acid and drain. Let some drops of the cell solution fall on the slide from the maximum height possible (*see Note 29*).
19. Let slides dry overnight. Check for metaphases in the microscope (*see Note 30*).

Day 2: Metaphase Hybridization

20. Prepare acidified pepsin and incubate for 15 min at 37 °C.
21. Wash slides in PBS for 15 min in shaker (*see Note 31*).
22. Fix cells in 4 % formaldehyde fixing solution for 2 min.
23. Wash slides 3× with PBS, in shaker, 5 min each time.
24. Digest with preheated pepsin 10 min at 37 °C in water bath.
25. Wash slides 2× with PBS, in shaker, 5 min each time.
26. Fix cells in 4 % formaldehyde fixing solution for 2 min.
27. Wash slides 3× with PBS, in shaker, 5 min each time.
28. Dehydrate slides: wash 5 min each in 70 %, 90 % and 100 % ethanol.
29. Air-dry for 5–20 min.
30. Prepare telomere probe mix.

²⁷If there are too many tubes, add 2 mL to all of them first, and then add the rest of the solution. If there are only a few tubes, add 11 mL to each tube.

²⁸As you become familiar with Q-FISH, you will figure out the best cell density for your experiments. However, start by leaving 1 mL, and if this is too dilute, use smaller volumes in future trials.

²⁹Drop metaphases from the maximum height possible. Extend one arm as far down as possible, and place the pipette bulb near your eye so that you can look down on your slide as you drop metaphases. There will be a greater separation of meta-phases if dropped from a greater height. Place two drops per slide.

³⁰If there are not enough metaphases, re-drop metaphases from a smaller starting volume in **step 16**.

³¹Perform all washes and other treatments of slides in staining dish.

31. Add two drops (10–15 μ L) of probe mix to a long cover slide. Turn slide upside down onto the cover, so that the probe extends by diffusion (*see Note 32*).
32. Denature at 80 °C for 3 min (*see Note 33*).
33. Make a wet chamber by covering the walls of a big cylinder with wet paper towels. Put slides into the chamber with covers facing down. Seal cylinder with Saran Wrap. Incubate in dark for 2 h at room temperature.
34. Wash 2 $\times$ 15 min each with formamide–BSA wash solution, while vortexing. If covers do not separate from slides after 5 min, use tweezers to remove them.
35. Wash slides 3 $\times$ 5 min with PBS in shaker.
36. Dehydrate slides: wash 5 min each in 70 %, 90 % and 100 % ethanol.
37. Air-dry slides.
38. Add two drops (10–15 μ L each) of DAPI mounting medium to a long coverslip; place slides over the coverslip; let dry for 5 min.
39. Seal cover to slides with nail polish.
40. Keep samples at 4 °C in the dark.

Day 3: Microscopy and Analysis

41. Take pictures of metaphases (Fig. 5a).
42. Analyze pictures: Analyze telomere length TFL-Telo following software instructions. Analyze metaphases for genomic instability by looking for telomere loss, chromatid breaks, chromosome breaks, gaps, fusions, and other markers of genomic instability (Fig. 5b).

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³²Take care not to make bubbles. If bubbles are present, use a pipette tip to tease them out. However, take care not to move the coverslip.

³³The exact time here is important. Use a timer to ensure slides are on the thermal block for exactly 3 min. When we do this with many slides, we add slides to the thermal block every 5 s to ensure we are able to collect them all after they have been at 80 °C for exactly 3 min.

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Fig. 1.

Detection of DNA repair factors by immunoblotting. Immunoblots show changes in several DNA repair factors upon depletion of A-type lamins (lamin A/C) via lentiviral transduction with specific shRNAs in mouse embryonic fibroblasts (MEFs). Decreased levels of BRCA1 and RAD51 are associated with defects in homologous recombination (HR), and decreased 53BP1 levels with defects in non-homologous end joining (NHEJ)

Fig. 2.

Immunofluorescence of cells growing on coverslips. **(a)** Place eight coverslips in each 60 mm plate (p60) for immunofluorescence (IF). **(b)** Plate cells on top of coverslips. **(c)** When ready to fix cells, aspirate media carefully. **(d)** Perform incubation with antibodies of interest as in figure: (1) obtain microscope slides; (2) cover slide with Parafilm; (3) add 25–40 μ L drops of diluted antibodies; (4) place coverslips up-side down on antibody drop; (5) repeat for all coverslips. **(e)** Place slides in a humidity chamber adding a wet paper towel, cover, and incubate at 37 °C

Fig. 3. Detection of DNA repair foci by immunofluorescence. Cells were irradiated with 3 Gy and processed for immunofluorescence with antibodies recognizing the DNA repair factors 53BP1, RAD51, and BRCA1. All proteins form foci at sites of radiation-induced DNA damage. We score cells as positive for DNA repair foci when they show at least 5 foci

Fig. 4. DNA repair deficiencies in lamin A/C-deficient cells. **(a)** Representative images of individual DNA comets from *Lmna*^{+/+} and *Lmna*^{-/-} MEFs, indicating deficiencies in DNA double-strand break (DSB) repair in *Lmna*^{-/-} cells. **(b)** Graph shows average olive moments over a period of time in *Lmna*^{+/+} and *Lmna*^{-/-} MEFs. *Lmna*^{+/+} cells show the characteristic biphasic mode of DNA DSB repair. Note how *Lmna*^{-/-} cells exhibit lower kinetics of repair

Fig. 5. Monitoring chromosomal instability. (a) Metaphase spread processed for FISH with a telomere probe (*yellow*). (b) Examples of chromosomal aberrations that can be monitored in metaphase spreads to determine the extent of genomic instability in different cells

Table 1

Composition of the telomere probe mix

Stock	10 slides (μL)	20 slides (μL)
Tris 1M pH 7.4	2.5	5.0
MgCl ₂ buffer	21.4	42.8
Deionized formamide	175.0	350.0
Probe 25 μg/mL (telomeric)	5.0	10.0
BM 10 % (Blocking reagent)	12.5	25.0
Deionized water	33.6	67.2

Table 2

Composition of the formamide–BSA solution

Stock	400 mL	800 mL
Formamide	280 mL	560 mL
TRIS 1M, pH 7.2	4 mL	8 mL
BSA 10 % (in water)	4 mL	8 mL
Deionized Water	112 mL	224 mL