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Nuclei-Segregated DNA Fiber (NuSeF) Assay Protocol

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We use this protocol and it's working

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Abstract

Micronuclei have recently been shown to be associated with a complex genomic rearrangement mechanism known as chromothripsis. Although DNA replication has been observed within micronuclei, it is often less efficient as compared to primary nuclei. We developed a method known as Nuclei-Segregated DNA Fiber (NuSeF) Assay, which allows us to investigate replication stress within micronuclei and compare the DNA replication events between micronuclei and primary nuclei.



Materials

1. Complete culture medium: DMEM (gibco 41966-29); 10% FBS; 1% penicillin/streptomycin
2. Lysis buffer I, pH 8.5 (10mM Tris-HCl; 2mM Magnesium acetate; 3mM Calcium chloride; 0.32M sucrose solution; 0.1mM EDTA; 0.1% NP-40)
3. Lysis buffer II (200mM Tris-HCl pH 7.4, 50mM EDTA, 0.5% SDS)
4. 1.8 M sucrose buffer, pH8 (10mM Tris-HCL; 5mM Magnesium Acetate; 0.1mM EDTA; 1.8M sucrose solution)
5. 1.6 M sucrose buffer, pH8 (10mM Tris-HCL; 5mM Magnesium Acetate; 0.1mM EDTA; 1.6M sucrose solution)
6. Blocking solution (2% BSA; 0.1% Tween-20; PBS; 0.22mm filtered)
7. PBS
8. Protease inhibitor (Roche cOmplete, EDTA free 1187358001)
9. Spermidine (Sigma S0266-1G)
10. Spermine (Sigma S4264-1G)
11. Cytochalasin B (Enzo BML-T108-00)
12. Reversine (MCE HY-14711/CS-1523)
13. MitoView Green (Biotium 70054-50ug)
14. Hoechst 33342 (Life Technologies H3570)
15. CldU (Sigma C6891-100MG)
16. IdU (Sigma I7125-5G)
17. CldU antibody (rat anti-BrdU antibody; abcam ab6326)
18. IdU antibody (mouse anti-BrdU; BD Biosciences 347580)
19. ssDNA antibody (DSHB) – labelled with FlexAble 2.0 CoraLite Plus 488 Antibody labeling kit
20. ProLong Gold Antifade gold Mountant (Invitrogen P36930)

Materials/reagents prepared right before use:

- 1
 1. Working lysis buffer I, pH 8.5 – add the following ingredients to the buffer: 10µg/ml Cytochalasin B; 1mM DTT; 0.15mM Spermine; 0.75 mM Spermidine; 1X protease inhibitor
 2. Working 1.8M sucrose buffer: 1mM DTT; 0.15mM Spermine; 0.75mM Spermidine
 3. Working 1.6M sucrose buffer: 1mM DTT; 0.15mM Spermine; 0.75mM Spermidine
 4. Dissolve 4 tablets of protease inhibitor in 2ml of PBS (100X protease inhibitor); 0.2µm filtered

Experimental Protocol

2 Treatment of cells to induce micronuclei formation

- 2.1 Prepare 10 × 15cm culture plates with each seeding 1 million HEK293T cells per 15cm culture plate on Day 0
- 2.2 On Day 1, add 0.5 µM of reversine to the cells and incubate for 48 hours
- 2.3 On Day 3, the cells should reach roughly 60–70% of confluence

Note: The number of starting cells needs to be optimized for each cell line, depending on if the cells are prone to micronuclei formation.

3 Labelling of cells with CldU and IdU

- 3.1 On Day 3, prepare complete medium containing CldU (4 µM) and IdU (34 µM), pre-warm at 37 °C
- 3.2 Discard the culture medium from the cells, then add complete medium containing CldU. Incubate the cells for exactly 30 minutes at 37 °C, 6% CO₂.

Note: Due to the high number of plates, especially working with different conditions, the plates should be marked in sequence to ensure the same order is applied for nucleoside addition and washing
- 3.3 Remove the medium and wash the cells with 20ml of pre-warmed PBS

Note: This step should be performed as fast as possible to reduce the size of the gap between CldU and IdU.

- 3.4 Add complete medium containing IdU. Incubate the cells for exactly 30 minutes at 37 °C, 6% CO₂.
- 3.5 Remove the medium and wash the cells with 20ml of pre-warmed PBS
- 3.6 Trypsinized the cells by adding 5ml of trypsin per plate and incubating at 37 °C for 5 minutes
- 3.7 Stop the trypsin activity by adding 10 ml of the complete medium and transfer all cells to 50 ml falcon tubes
- 3.8 Wash the plates with additional 10 ml of complete medium and transfer to 50 ml falcon tubes
- 3.9 Centrifuge the cells at 1200 rpm for 3 minutes and discard the supernatant
- 3.10 Pool all the cells from the 10 plates into a single 50ml falcon and resuspend the cells with DMEM without FBS with a final volume of 20ml
- 3.11 Perform a cell count. Typically, the total number of cells from 10 plates with confluence around 60% would be around 1.2×10^8 .
- 3.12 Wash the cells again with 20 ml of DMEM without FBS and remove the supernatant.
- 3.13 Store the cell pellet on ice and proceed immediately to the nuclei isolation steps.

4 Separation of primary nuclei and micronuclei-enriched fractions

- 4.1 Resuspend the cell pellet in 5ml of ice-cold working lysis buffer.
- 4.2 Transfer the cell suspension to a 15ml douncer (ideally use a loosely-fitting pre-chilled douncer)

- 4.3 On ice, gently apply 10 strokes to rupture the cell membrane
- 4.4 Transfer all the solution back to the original 50ml falcon tube
- 4.5 Add 5ml of working 1.8M sucrose buffer and add 1X of protease inhibitor
- 4.6 Pre-cool the centrifuge to 4 degrees
- 4.7 Prepare sucrose gradient: pipette 15ml of working 1.6M sucrose buffer to the bottom of a fresh 50ml falcon tube; then carefully layer 20ml of working 1.8M sucrose on the top (use the minimum speed of the pipette boy for this step)
- 4.8 Transfer 10ml of the sample/1.8M sucrose buffer mixture on top of the sucrose gradient from step 23 (use the minimum speed of the pipette boy for this step)
- 4.9 Centrifuge the sucrose gradient at 950g for 20 minutes (use reduced acceleration and deceleration to avoid disruption of the gradient e.g. use 5 for both for Eppendorf Centrifuge 5810 R)
- 4.10 Transfer the falcon tube without disrupting the gradient to a bench
- 4.11 Carefully pipette the uppermost 2 mL of the gradient and discard
- 4.12 Transfer the next 5ml of the gradient to a new 50ml falcon tube and mark as MN-enriched fraction (MN)
- 4.13 Transfer the next 5ml of the gradient into a new 50ml falcon tube and label as PN-enrich fraction 1 (PN1)
- 4.14 Then transfer the next 5ml of the gradient into a new 50ml falcon tube and label as PN-enriched fraction 2 (PN2)
- 4.15 For each of the MN, PN1, and PN2 tube, add 20 ml of cold PBS with 1X protease inhibitor

- 4.16 Centrifuge the tubes at 1500g for 20 minutes
- 4.17 Discard the top 24ml of the solution and resuspend the pellet with the remaining 1ml of the buffer
- 4.18 Keep the samples on ice and proceed to the flow cytometry for nuclei separation

5 **Separation of micronuclei from primary nuclei by flow cytometry**

- 5.1 Setup the flow cytometer for the separation of primary and micronuclei. This protocol is optimized for both BD FACSAria III Cell Sorter and BD FACSsymphony S6 Cell sorter.
- 5.2 Use the following conditions for sorting: 100µm nozzle; threshold of 500; 4-way purity mask
- 5.3 Prepare collection tubes with PBS supplemented with 1X protease inhibitor
- 5.4 Add Hoechst 33342 at 1:10,000 and MitoView Green at 1:5,000 to the sample 10 minutes before sorting
- 5.5 Setup the gating by plotting FSC-A vs. Hoechst 33342; then gate on Hoechst 33342 positive but MitoView Green negative population; primary nuclei and micronuclei can be separated by size via FSC-A
- 5.6 The MN-enriched fraction (MN) is abundant with micronuclei and typically gives rise to 2 million or more sorted micronuclei from 1.2×10^8 cells.
- 5.7 The PN-enriched fractions (PN1 and PN2) are abundant with primary nuclei and typically give rise to 4 millions or more sorted events from 1.2×10^8 cells.
- 5.8 Sort samples with a chilling sample holder and add 1X protease inhibitor right after sorting
- 5.9 Centrifuge the sorted samples at 3000g at 4 °C for 10 minutes

- 5.10 Remove the supernatant and keep only 200µl of the buffer. Keep the samples on ice until use.

6 Spreading and denaturing of DNA

- 6.1 Transfer the samples to 1.5ml Eppendorf tubes and centrifuge at 3000g at 4 °C for 10 minutes
- 6.2 Discard the majority of the supernatant, but keep the volume as indicated:
- Primary nuclei: 10 µl per 10⁵ events
 - Micronuclei: 10 µl per 10⁶ events
- 6.3 Use the remaining buffer for resuspending the nuclei pellet
- 6.4 Label the frosted glass slides for DNA spreading
- 6.5 Pipette 3µl of the sample close to the frosted end of the glass slide (two spots can be prepared on a single glass slide)
- 6.6 Add 7µl of lysis buffer II into each sample spot and gently mix 5 times by pipetting. Note: Avoid generating air bubbles in the sample spot
- 6.7 Start the timer when the sample is mixed. Leave enough time between sample spots, typically 1 minute
- 6.8 Leave the slide horizontal and incubate for 5 minutes at room temperature for PN samples, and 9 minutes for MN samples (if room temperature is higher, the incubation time may need to be shortened to avoid drying of the sample)
- 6.9 Tilt the slide manually at an angle of 30-45° to allow the sample to run slowly down the glass slide to spread the DNA
- 6.10 Air dry the slides completely and fix with methanol/acetic acid (3:1) for 20 minutes at room temperature
- 6.11 Rehydrate the slides in PBS for 3 minutes, and repeat the step once

6.12 Denature DNA in 2.5M HCl for 1 hour at room temperature

6.13 Wash the slide 5 times with PBS, with 3 minutes of incubation time for each exchange of PBS

7 Immunofluorescence staining of CldU and IdU

7.1 Block the slides with blocking buffer for 40 minutes at room temperature

7.2 Prepare primary antibody in blocking solution: rat anti-CldU (1:400) and mouse anti-IdU (1:80).

7.3 Use 65µl of the antibody mixture per slide. Use cover slides to help spread the antibody solution throughout the whole glass slide

7.4 Incubate the slides at 4°C overnight in a humidified chamber

7.5 On the next day, wash the slides 5 times with PBS supplemented with 0.2% Tween-20, incubate for 3 minutes at room temperature for each exchange

7.6 Prepare secondary antibody mixture: anti-rat AF555 1:200 and anti-mouse AF647 1:400

7.7 Dip the slides 3 times in blocking solution and drain the excess liquid from the slides using tissue

7.8 Pipette 65µl of the antibody mixture per slide. Use cover slides to help spread the antibody solution throughout the whole glass slide

7.9 Incubate the slides at room temperature for 2 hours in a humidified chamber

7.10 Wash the slides 5 times with PBS supplemented with 0.2% Tween-20, incubate for 3 minutes at room temperature for each exchange

7.11 Dilute ssDNA antibody in blocking solution (1:100)

- 7.12 Dip the slides 3 times in blocking solution and drain the excess liquid from the slides using tissue
- 7.13 Pipette 65µl of the antibody mixture per slide. Use cover slides to help spread the antibody solution throughout the whole glass slide
- 7.14 Incubate the slides at room temperature for 1 hour in a humidified chamber
- 7.15 Wash the slides 5 times with PBS supplemented with 0.2% Tween-20, incubate for 3 minutes at room temperature for each exchange
- 7.16 Wash once with PBS
- 7.17 Mount the slides with 25µl of antifade gold mounting medium (Invitrogen)
- 7.18 Keep the slides in the dark at 4°C and allow the slides to set before imaging

8 Image acquisition and DNA fiber quantification

- 8.1 Use the ssDNA to track the location of the spread DNA. This is particularly helpful for MN samples.
- 8.2 Take images using a high magnification (typically with 63x oil immersion objectives)
- 8.3 Acquire representative images from different regions of the spread DNA
- 8.4 Quantify the length of CldU and IdU using ImageJ.



8.5 Measure at least 100 DNA fibers for comparison

Protocol references

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