Detection of ALT Associated Promyelocytic Leukemia Nuclear Bodies (APBs) by Immunofluorescence-FISH (IF-FISH)

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[Abstract] The activation of functions that counteract the physiological shortening of telomeres in rapidly proliferating cell is prerequisite for the progression of cancer cells to full malignancy (Collado *et al.*, 2007). In most human cancers, the length of telomere is maintained through up-regulation of telomerase whereas a telomerase-independent pathway, termed Alternative Lengthening of Telomeres (ALT) is active in about 10-15% of cancers (Johnson and Broccoli, 2007; Heaphy *et al.*, 2011). One characteristic feature of ALT is the formation of ALT-associated Promyelocytic Leukemia nuclear bodies (APBs) (Lang *et al.*, 2010; Yeager *et al.*, 1999). APBs contain Promyelocytic Leukemia nuclear bodies (PML-NB) components such as PML, SP100 and SUMO, telomeric DNA and telomere associated proteins including the shelterin components TRF1, TRF2, POT1, TIN2, TPP1 and Rap1 (Yeager *et al.*, 1999). In addition, APBs contain proteins involved in DNA repair. In particular, the presence of components of the homologous recombination machinery suggests that APBs may promote telomere elongation by facilitating the homologous recombination of telomeric templates (Nabetani *et al.*, 2004; Stavropoulos *et al.*, 2002). This is also supported by the requirement of the homologous recombination-associated MRN complex for APB formation (Wu *et al.*, 2000). Furthermore, APBs are suggested to be active sites of ATM and ATR dependent DNA repair (Nabetani *et al.*, 2004). Finally, the number of APBs increases in G2 phase of the cell cycle when recombination is mainly active (Grobelny *et al.*, 2000). We have shown that infection of normal and malignant B lymphocytes with the human oncogenic herpesvirus Epstein-Barr virus (EBV) is associated with the induction of APBs and with numerous signs of chromosomal and genomic instability (Kamranvar *et al.*, 2007; Kamranvar and Masucci, 2011; Kamranvar *et al.*, 2013).

Here we describe a method for detection of APBs in human B-lymphocytes. The method can be applied with minor modifications to different cell types including adherent, suspension and primary cells.

Materials and Reagents

1. Cells (suspension cells)

2. Formaldehyde (Merck KGaA, catalog number: K43634203 228)
3. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
4. BSA (Sigma-Aldrich, catalog number: A7906)
5. Blocking reagent (Roche Diagnostics, catalog number: 11096176001)
6. Maleic acid (Sigma-Aldrich, catalog number: M0375)
7. Deionized formamide (Merck KGaA, catalog number: K25761484 902)
8. Tris-HCl
9. Green-fluorescent Alexa Fluor® 488 (Life Technologies, Invitrogen™, catalog number: A-11034) or Red-fluorescent Alexa Fluor® 594 (Life Technologies, Invitrogen™, catalog number: A-11005)
10. Ethanol (Kemetyl, catalog number: 200-578-6)
11. Cy3-TelG (PANAGENE, catalog number: F1006)
12. FITC-TelC (PANAGENE, catalog number: F1009)
13. DAPI (Vector Laboratories)
14. Fixation buffer (see Recipes)
15. Permeabilization buffer (see Recipes)
16. IF blocking buffer (see Recipes)
17. FISH blocking solution (see Recipes)
18. Maleic acid buffer (see Recipes)
19. PNA probes (see Recipes)
20. Hybridizing solution (see Recipes)
21. Washing solution (see Recipes)
22. PML antibody (see Recipes)
23. Secondary antibody (see Recipes)
24. Dehydration solution (see Recipes)
25. Mounting medium (see Recipes)

Equipment

1. Microscope glass slide (76 x 26 mm)
2. Coverslip (preferably circular 19 mm diameter)
3. Cytospin or slide centrifuge (Cytospin3 SHANDON)
4. Cytospin funnel with white filter card
5. Metal holder
6. Hydrophobic barrier pen (ImmEdge Pen, model: H-4000)
7. Hot plate preheated to a temperature 80 °C
8. Coplin jar

9. Moist chamber

Procedure

1. Wash the suspension cells once with PBS and prepare a dilution of 0.5 x 10^6 cells per ml of PBS at RT.
2. Pre-label the slides and make a small hydrophobic barrier circle (~1.3 cm diameter) with ImmEdge pen around the area where the cells will be placed and let it dry before cytospin. (Hydrophobic circle will be used to fill with the buffers for fixation, permeabilization and blocking buffer.)
3. Prepare the slides mounted with the filter card and cytospin funnel in the metal holder as shown in the Figure 1.

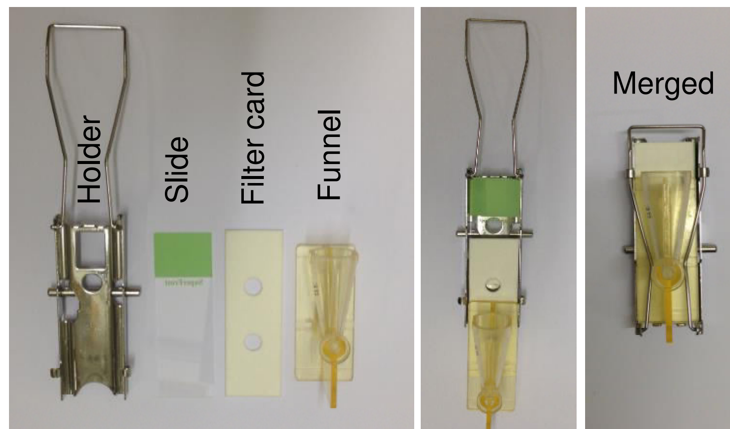

Figure 1. The figure illustrates the preparation of a slide for cytospin

4. Load up to 100 µl of the prepared cells in each funnel and spin for 1 min at 600 rpm.
Note: If the method will be applied to growing adherent cells on coverslip, the steps 1-4 can be skipped.
5. Remove the slides from the holders and let the moisture of the cells dry out for a few sec before fixation with the fixation buffer for 10 min at RT.
6. Wash the cells 2 x 5 min each with PBS.
7. Permeabilize the cells with permeabilization buffer for 5 min at RT.
8. Remove the permeabilization buffer and incubate the cells for one hour with the IF blocking buffer in moist chamber.
9. Remove the IF blocking buffer and incubate the cells in moist chamber with the fresh IF blocking buffer containing PML antibody for 2 h at RT or overnight at 4 °C.
10. Wash the cells gently 3 x 10 min each with PBS on a shaker. (Washing steps can be done in any glass or plastic microscope slide staining Jars.)

Note: The slides should be protected from light in the next following steps.

11. Incubate the cells in moist chamber for 30 min with the secondary antibody.
12. Wash the cells gently 3 x 10 min each with PBS on a shaker.
13. Re-fix the cells for 5 min with fixation buffer at RT.
14. Wash the cells 3 x 10 min each in PBS.
15. Dehydrate the cells in coplinjar containing ethanol series, consecutively 70%, 95%, 100% EtOH, for 5 min each.
16. Aspirate the ethanol completely and let the slides dry for a couple of minutes in the dark.
17. Place a drop of hybridizing solution containing the PNA probe on each slide and cover it with a coverslip.
18. Denature the probes in hybridizing solution for 10 min at 80 °C by placing the slides on a pre-warmed hot plate.
19. Incubate the slides in moist chamber for 2 h at RT.
20. Wash the slides 2 x 15 min in washing solution.
21. Wash the slides 3 x 5 min in PBS.
22. Air-dry the slides at RT for 10 min.
23. Mount the slides with one drop of Vectashield and cover with a coverslip.
24. Seal the coverslip edges with clear nail polish and let it dry.
25. Keep the slides at 4 °C in dark until microscopy.
26. Visualize the APBs using a confocal microscope as fluorescence signals from PML bodies co-localized with telomere signals.
27. A Cell containing two or more APBs can be scored as a ALT positive cell (Figure 2).

Representative data

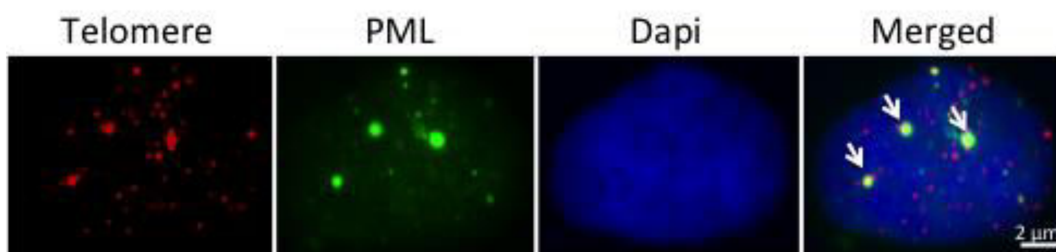

Figure 2. Representative micrograph illustrating APBs positive U2OS cell in interphase. The arrows show three APB foci in which the green PML signal co-localizes with the red telomere signal. In some cell lines the PML antibody gives a weak diffuse fluorescent background that is easily distinguished from the bright fluorescence of the PML bodies.

Notes

1. Rapidly proliferating cancer cells often have shorter telomeres and higher protein expression compared to slow growing primary cells. This may cause high background in immunofluorescence and weak telomere signals in FISH. To improve the method, the incubation time with the primary PML antibody should be reduced and less photobleaching telomeric probes should be used. Cy3-TelG usually last longer than FITC-TelC and Alexa Fluor® 488 produces less background than Alexa Fluor® 594. In cells with short telomeres and high background signals, Cy3-TelG and Alexa Fluor® 488 are recommended.

Recipes

1. Fixation buffer
   3.7% formaldehyde in PBS (10-fold dilution of 37% commercial stock solution)
   Adjust the PH to 7.5
   The fixative should be freshly prepared for each experiment
2. Permeabilization buffer
   0.5% Triton X-100 in PBS
3. IF blocking buffer
   1% BSA and 0.1% Triton X-100 in PBS
4. FISH blocking solution
   10% blocking reagent in maleic acid buffer: 10 g of blocking reagent should be dissolved in 100 ml of maleic acid buffer
   Adjust the pH to 7.5 with NaOH and store at 4 °C
5. Maleic acid buffer
   100 mM maleic acid and 150 mM NaCl in dH_2O
6. PNA probes
   Cy3-TelG: Cy3-OO-TTAGGGTTAGGGTTAGGG 3'
   Stock concentration: 53 µM in dH_2O
   FITC-TelC: FITC-OO-CCCTAACCCTAACCCTAA 3'
   Stock concentration: 111 µM in dH_2O
   Store the stocks at 4 °C in the dark
7. Hybridizing solution
   70% deionized formamide
   0.5% FISH blocking reagent (from 10% stock)
   10 mM Tris-HCl (pH 7.2)

PNA telomeric probe: FITC-TelC (1:500-1,000 dilution from stock) or Cy3-TelG (1:3,000-5,000 dilution from stock)
Prepare it freshly and protect it from light

8. Washing solution
   70% formamide
   10 mM Tris-HCl (pH 7.2) in dH_2O
9. PML antibody
   Use 1:100-200 dilution in the IF blocking buffer
   Monoclonal or polyclonal PML Antibodies can be used
10. Secondary antibody
    Green-fluorescent Alexa Fluor® 488 or red-fluorescent Alexa Fluor® 594 diluted 1:1,000 in IF blocking buffer
11. Dehydration solution
    Ethanol series 70%, 95% and 100%
12. Mounting medium
    Vectashield embedding medium containing DAPI

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