In situ labeling of newly-synthesized RNA with 5-Bromouridine 5'-Triphosphate and immunodetection of transcription sites by microscopy

Equipment and reagents

- Tissue culture dishes (35 mm)
- Glass coverslips (22X22 mm, No: 1.5) stored in 75% (v/v) ethanol
- Phosphate-buffered saline (PBS)
- Trypan Blue Dye 0.4% (Sigma)
- 2% paraformaldehyde in PBS, prepare fresh and keep on ice
- Permeabilization buffer [20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 5-40 :g/ml digitonin (Calbiochem) or 0.02%-0.1% Triton X-100 (Calbiochem), 1 mM PMSF, recombinant RNasin (Promega) ribonuclease inhibitor 20 units/ml], prepare fresh, add the last two ingredients before use and keep at room temperature
- Transcription buffer [100 mM KCl, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 2 mM ATP (Roche Molecular Biochemical), 0.5 mM CTP (Roche Molecular Biochemical), 0.5 mM GTP (Roche Molecular Biochemical), 0.5 mM BrUTP (Sigma), 1 mM PMSF, Recombinant RNasin (Promega) ribonuclease inhibitor 20 units/ml], prepare fresh, add the last two ingredients before use and keep at room temperature
- Monoclonal mouse antibodies to bromodeoxyuridine; clone BR-3 (Caltag) or clone BMC-9318 (Roche Molecular Biochemical) or clone Bu-33 (Sigma)
- Actinomycin D (Calbiochem) and α-amanitin (Sigma)
- RNase A (Sigma)
- DAPI Nucleic Acid Stain (Molecular Probes)

Method

1. Remove coverslips from the 75% ethanol solution, flame to burn the ethanol off and place them into the 35 mm tissue culture dishes.[a]
2. Plate the cells such that they reach 50-70% confluency at the day of experiment.[b]
3. Put the transcription buffer into the tissue culture incubator at 37°C.[c]

4. Take the first dish out of tissue culture incubator and wash the cells twice with PBS at room temperature.[d]
5. Remove the PBS completely by tilting the dish and aspirating the PBS with a glass capillary pipette.
6. Gently add 2 ml of permeabilization buffer while lowering the tilted dish and incubate 3 min at room temperature.[e]
7. Remove the permeabilization buffer completely by tilting the dish and aspirating it with a glass capillary pipette.
8. Gently add 1.5 ml of transcription buffer while lowering the tilted dish and incubate 5 min at 37°C.[f]
9. Remove the transcription buffer completely by tilting the dish and aspirating it with glass capillary pipette.
10. Gently add 1 ml of PBS while lowering the tilted dish.
11. Remove PBS completely by tilting the dish and aspirating it with a glass capillary pipette.
12. Fix the cells by adding 3 ml of 2% paraformaldehyde and incubate for 15 min at room temperature.
13. Process the next sample (step 4), while cells are in the fixation solution.
14. Remove the paraformaldehyde by aspirating it with a glass capillary pipette.
15. Process all samples for IF.[g]

[a] It is generally more convenient to process only 6 dishes per experiment

[b] This also applies to transiently transfected cells.

[c] It is best to do the rest of the protocol on a laboratory bench very close to this tissue culture incubator.

[d] Process one dish at a time through steps 4-12.

[e] The cells may easily come off from coverslips so take great care between steps 4-14. The necessary concentration of detergent for a slight permeabilization will vary depending upon the cell type and has to be optimized in order to have a successful labeling. A good starting point is to monitor the level of permeabilization using trypan blue exclusion. Simply mix 100-200 :l 0.4% trypan blue and 2 ml of permeabilization

buffer containing 5-40 :g/ml digitonin or 0.02%-0.1% Triton X-100 and add to the coverslips. Incubate 3 min at room temperature and count the dark blue cells by light microscopy. Determine the detergent concentration that permeabilizes 50%-75% of cells. Once the transcription sites are labeled successfully on a regular basis, it is very important to further optimize the detergent concentration until transcription site labeling is obtained with a minimum concentration of detergent.

f The duration of labeling may also vary depending upon the cell type and has to be optimized in order to have a successful labeling. As initial labeling times, we suggest using 5, 15 and 30 min both at 37°C and room temperature. Once the transcription sites are labeled successfully on a regular basis, it is very important to further optimize the labeling period until transcription site labeling is achieved with minimum labeling time. A large and intense nucleolar staining is a good indication for the necessity of shorter labeling periods. The BrUTP concentration can also be lowered to 0.2 mM with identical results.

g Since digitonin does not permeabilize the nuclear membrane, if cells are being processed for indirect IF, they have to be permeabilized with Triton X-100 in order for BrUTP antibody to gain access to the nucleus interior.

The sensitivities of the commercially available monoclonal mouse antibodies to bromodeoxyuridine vary. The antibodies from Caltag and Roche Molecular Biochemical are far more sensitive than the antibody from Sigma under the conditions of these protocols. Good starting antibody dilutions for use in indirect IFM are for the antibody from Caltag 1:200 in PBS, the antibody from Roche Molecular Biochemical 1:50 in PBS and the antibody from Sigma 1:100 in PBS. These dilutions are optimized for use with the minimal cross-reactive secondary antibodies from Jackson ImmunoResearch Laboratories.

- When these protocols are used in conjunction with IF or in situ hybridization techniques, one can simultaneously study the spatial relationships between transcription sites and a protein, a gene, or a nuclear domain of interest. It is also possible to determine whether the gene of interest is active or inactive. Both

protocols can also be applied to GFP-fusion proteins, since the GFP fluorescence signal remains stable throughout the transcription procedure.

- Several controls must be performed to verify whether the observed sites are genuine transcription sites:

  (a) Do an experiment with UTP in the transcription and microinjection buffers instead of BrUTP. There should be no labeling detected by indirect immunofluorescence microscopy demonstrating the specificity of the antibody.

  (b) Do a labeling with transcription and microinjection buffers containing 1-10 :g/ml ∀-amanitin or 5-10 :g/ml actinomycin D. If used in these concentrations, ∀-amanitin specifically inhibits the transcription by RNA polymerase (RNA pol) II. Thus, only the nucleolar transcription sites, but not the nuclear transcription sites should be detected. Actinomycin D at the above concentration inhibits transcription by all three RNA polymerases.

- If no labeling is observed

  (a) Repeat the experiment by using either one of the other mouse monoclonal antibodies.

  (b) Increase the duration of labeling.

  (c) Do the labeling at 37°C.

  (d) Increase the concentration of BrUTP to 1 mM.

  (e) Optimize the concentration of detergent.

  (f) Use a biotin-streptavidin fluorescent signal enhancement system (e.g. Biotin-conjugated anti-mouse IgG and Texas Red dye-conjugated Streptavidin) in indirect IF. These options can be used individually or in combination.