

***In vivo 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)
- 2% paraformaldehyde in PBS, prepare fresh and keep on ice
- Microinjection buffer (100 mM BrUTP (Sigma), 140 mM KCl, 2 mM Pipes, pH 7.4), store in aliquots at  $-20^{\circ}\text{C}$
- 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  $\alpha$ -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.
2. Plate the cells such that they reach 50-70% confluency at the day of experiment.<sup>a</sup>
3. Take the first dish out of tissue culture incubator and place onto the stage of fluorescence microscope equipped with a microinjection system.<sup>b</sup>
4. Inject the injection buffer into the cytoplasm of cells.<sup>c</sup>
5. Place the dish back in the tissue culture incubator and incubate 5 min at  $37^{\circ}\text{C}$ .<sup>d</sup>
6. Take the dish out of tissue culture incubator and gently add 1 ml of PBS while lowering the tilted dish.
7. Remove the PBS completely by tilting the dish and aspirating the PBS with a glass capillary pipette.

8. Fix the cells by adding 3 ml of 2% paraformaldehyde and incubate for 15 min at room temperature.
9. Remove the paraformaldehyde by aspirating it with a glass capillary pipette.
10. Process all of the samples for immunofluorescence microscopy.

<sup>a</sup> This also applies to transiently transfected cells.

<sup>b</sup> Keep the cells in the growth medium. The growth media change or PBS wash is not necessary at this stage.

<sup>c</sup> Inject always less than 5% of total cell volume. One indication that the cells are injected with more volume than necessary is the apparent change in cell morphology. Particularly, the edges of cells become uneven and cells lose their round shape. This can also be observed easily by staining the cells with DAPI after the paraformaldehyde fixation (step 8). To optimize the microinjection technique, replace the BrUTP with a fluorescent dye such as Rhodamine in the injection buffer and inject different volumes of buffer into cytoplasm, while observing the cells by fluorescence microscopy.

<sup>d</sup> The duration of incubation may vary depending upon the cell type used and has to be optimized. A good starting point is to use four different incubation times (5, 15, 30 and 60 min). Once the transcription sites are labeled successfully on a regular basis, it is important to further optimize the labeling period until transcription site labeling is achieved with minimum labeling time.

- 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.