5-bromo-2'-deoxyuridine detection using DNase I and Exonuclease III

Protocol for fluorescence microscopy including image cytometry

Stock solutions to be prepared:

10× Buffer for exonuclease III

660 mM Tris, pH 8, 30°C
6.6 mM $MgCl_2$
Dissolve in deionised water, aliquot and store at -20°C.

Protocol

1. Incubate the cells with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for the required time.

**If necessary another halogenated nucleoside analogues can be used including 5-trifluoromethyl-2'-deoxyuridine (TFdU).*

2. Wash with 1× PBS
3. Fixation/permeabilization

A. *Formaldehyde fixation*: Fix the samples with 1 - 2% formaldehyde in 1× PBS (10 min at room temperature), wash with 1× PBS and permeabilise with 0.2% Triton X-100 (10 min at room temperature). Then, wash the samples with 1× PBS (3×, 1 hr in total, at room temperature).

**Generally, a longer wash step is not critical, but its shortening leads to a decrease of the signal.*

B. *Ethanol fixation*: Fix the samples with pre-cooled 70% ethanol - put the samples into the freezer and incubate at least 30 min.

4. BrdU detection

A. Wash with 1× buffer for exonuclease III (3×).

**In the case of cells grown on coverslips this and all additional steps can be performed on drops of solutions.*

**The circular coverslips with a diameter of 12 mm provide enough cells for the analysis of the cell cycle using image cytometry.*

B. Incubate samples in a mixture of primary anti-BrdU antibody, exonuclease III (0.4 U/µl), DNase I (10 U/ml in the formaldehyde-fixed cells, 2 - 10 U/ml in the ethanol-fixed cells), 0.1 mM $CaCl_2$ and 1× buffer for exonuclease III (incubate 30 min at 37°C).

**If RNA has to be removed (e.g. if propidium iodide is used for DNA labelling), add RNase A (100 µg/ml) to the mixture.*

**For the antibody clone B44 the optimal concentration is 0.25 - 0.5 µg/ml, for Bu20a 2.5 - 5 µg/ml (for other anti-BrdU antibodies, test the optimal concentration).*

**Use 30 µl of the mixture per one circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.*

**Too long incubation in this mixture and/or high concentration of DNase I can result in the lowering of DNA-derived signal after its staining with the specific dyes. Higher concentration of exonuclease III can be used without the risk of lowering of DNA- or BrdU-derived signals.*

**In the case of the detection of additional cellular components using specific antibodies, add the required primary antibody to the mixture described in step B and secondary antibody to the mixture described in step E.*

C. Briefly wash with 1× PBS (2×, 1 min or less in total) and then incubate with 0.2% formaldehyde in 1× PBS (10 min at room temperature).

**This step serves for the stabilization of an antibody with incorporated BrdU, otherwise, the antibody-BrdU complex can dissociate quickly (Ligasová et al., 2017, PLoS One 12(3): e0174893).*

**It is possible to use a methanol-stabilised stock solution of formaldehyde or a solution prepared from paraformaldehyde in 1× PBS.*

**If the stabilisation step is not used, the signal can be improved by a quick reaction with the secondary antibody although the stabilisation step usually allows faster and more effective stabilisation of the antibody.*

D. Wash with 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (3× 5 min).

E. Incubate the samples with the secondary antibody (dilution according to the manufacturer or testing) and DAPI (final concentration: 1 to 10 µM) in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (30 min at room temperature).

**Use 30 µl of the mixture per one circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.*

F. Wash in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (3× 5 min).

G. In the case of coverslips, remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on the filter paper. Apply drops of the mounting medium on the glass slide (3 – 5 µl) and carefully lay the coverslips onto the mounting medium. Avoid the creation of any air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 µl of the mounting media per well.

H. Use nail polish to seal the coverslips and to protect the medium from drying. The well plates and coverslips can be stored at−20°C. In the case of well plates, seal them with parafilm to protect the medium from drying.

Please, if you use this protocol, cite this source: **Ligasová et al. 2017, PLoS One 12(3): e0174893**