Simultaneous detection of DNA replication using 5-ethynyl-2'-deoxyuridine and 5-bromo-2'-deoxyuridine

Protocol for fluorescence microscopy including Image cytometry

This protocol allows the performance of 5-ethynyl-2'-deoxyuridine (EdU) detection and 5-bromo-2'-deoxyuridine (BrdU) revelation in one step.

Stock solutions to be prepared:

Solution A **- 8 mM $CuSO_4$ and 200 mM NaCl**.

Dissolve $CuSO_4$ in deionized water, add an appropriate volume of 1 M NaCl (stock solution) and fill with deionized water to the final volume. Store at 4°C.

Solution B **- 20 mM sodium ascorbate and 40 mM glycine**.

Dissolve sodium ascorbate and glycine in deionized water and fill with deionized water to the final volume. Store at −20°C.

10 0M azide dye solution (e.g. FAM azide)

Dissolve azide dye in DMSO. Store at −20°C.

100 mM 2-azidoethanol

Dissolve 2-azidoethanol in deionised water. Store at −20°C.

**As an aqueous solution of sodium ascorbate is the subject of quick air oxidation, prepare the aliquots in Eppendorf tubes and seal them with parafilm.*

**2-azidoethanol is necessary only if the used anti-BrdU antibody reacts also with EdU as after the reaction with azide dye there is still large amount of EdU in DNA that can react with the anti-BrdU antibody.*

Protocol

1. Incubate the cells with 10 μM EdU and then with 10 μM BrdU for the required times.

**Do not reverse this order if the pulses follow immediately one by one or thymidine is used during the chase. Otherwise, the EdU signal will be suppressed significantly as EdU is much less willingly incorporated into DNA than BrdU or thymidine.*

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2. Wash the samples with 1× PBS (3×).
3. Fixation/permeabilization

A. *Formaldehyde fixation*: Fix the samples with 1-2% formaldehyde in 1× PBS (10 min, room temperature), wash with 1× PBS and permeabilise with 0.2% Triton X-100 (10 min, room temperature). Then, wash samples with 1× PBS (3x, 15 minutes in total, room temperature).

B. *Ethanol fixation*: Fix samples with 70% ethanol (30 min - several weeks, -20°C)

4. EdU and BrdU detection

A. Wash samples with 1× PBS (3×).

B. Prepare fresh EdU labelling and DNA cleavage mixture from stock solutions consisting of copper(I) solution with azide dye (the final concentration of azide dye is 10 μM):

Add one volume of the solution A, 1/500 of volume of azide dye and at last, add one volume of the solution B. Mix on vortex for 5 s. Prepare the mixture just before labelling.

**Pre-warm solution A and solution B to 25°C before use.*

C. Remove 1× PBS from the samples and immediately add the mixture prepared in the previous step. Incubate for 5 minutes at 25°C.

**In the case of cells grown on coverslips, perform this step in a Petri dish. Do not perform it on drops of prepared mixture as it protects the sample from oxygen.*

**1 ml of mixture is enough for Petri dish with a diameter of 3.5 cm.*

**Use one coverslip per one Petri dish; otherwise, it is necessary to check the position of the coverslips during the next step to protect their mutual sliding resulting in stacking.*

**Although 25°C is the optimal temperature, usually room temperature provides similar results*

D. Put the samples on the shaker for 10 minutes, 300 rpm, 25 °C.

**The solution should gradually turn light blue without the formation of any precipitate.*

**DNA is cleaved during this step.*

**Azide dye reacts with EdU during step C and step D.*

E. Remove the mixture from samples.

F. Wash with 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl (3x, 20 min in total).

G. Incubate the samples with the solution containing primary anti-BrdU antibody, exonuclease III (final concentration: 0.4 U/µl) and 1× buffer for exonuclease III for at least 30 min at 37°C).

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

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

**Use anti-BrdU clone MoBu-1 as its cross-reactivity with EdU is minimal. If clones that cross-react with EdU are used, add a blocking step by 2-azidoethanol (Liboska et al., 2012, PLoS One 7(12): e51679) after step F. In that case, incubate samples for 20 minutes in the same mixture as in step C containing 20 mM 2-azidoethanol instead of azide dye. Do not shake the solution as no additional DNA cleavage is necessary. Then, continue with step F. If necessary, this suppression of EdU reactivity can be repeated. The suppression procedure can be combined also with other protocols for simultaneous detection of EdU and BrdU.*

**2-Azidoethanol is a sufficiently small, non-fluorescent molecule that can efficiently react with EdU in DNA. The product of the reaction does not react with the anti-BrdU antibody.*

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

H. Shortly wash with 1× PBS (2×, 1 min or less in total). Incubate with 0.2% formaldehyde in 1× PBS (10 min, room temperature).

**This step serves for the stabilization of the BrdU-antibody complex. Otherwise, the BrdU-antibody complex can dissociate quickly (Ligasová et al., 2015, PLoS ONE 10(7): e0132393; Ligasová et al., 2017, PLoS One 12(3): e0174893).*

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

**The quick wash followed by the stabilisation step is important in ethanol-fixed cells only for some antibodies. This step is necessary e.g. for antibody MoBu-1 and Bu20a, however not for clone B44. The data about some other clones are available in Ligasová et al., 2017, PLoS One 12(3): e0174893. On the contrary, for the formaldehyde-fixed cells, this step is important for the majority of the antibodies tested.*

**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.*

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

**It is possible to perform only one wash without significant effect on the secondary antibody reaction.*

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

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

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

L. 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.

M. 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 these sources:

For the detection of BrdU using this protocol:

Ligasová et al., 2012, PLoS ONE 7(12): e52584
Ligasová et al., 2015, PLoS ONE 10(7): e0132393
Ligasová et al., 2017, PLoS One 12(3): e0174893

For the suppression of EdU reactivity with anti-BrdU antibodies:

Liboska et al., 2012, PLoS One 7(12): e51679