Detection of DNA replication by 5-ethynyl-2'-deoxyuridine

Protocol for fluorescence microscopy including Image cytometry

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.

**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.*

Protocol

1. Incubate the cells with 10 µM 5-ethynyl-2'-deoxyuridine (EdU) for the required time.
2. Wash with 1× PBS
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 min in total, room temperature).

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

4. EdU detection

A. Wash in 1× PBS (3×).

B. Prepare fresh EdU labelling solution 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 the buffer from the samples and immediately add the prepared EdU labelling mixture.

**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 provides enough cells for the analysis of the cell cycle using image cytometry.*

**Use 30 µl per circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.*
**1 ml of the mixture is enough for Petri dish with diameter 3.5 cm.*

D. Remove the EdU labelling mixture.

E. Wash with 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl (3×, 15 minutes in total).

F. Incubate with DAPI (final concentration: 1 - 10 µM) in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (30 min, room temperature).

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

G. Wash the samples four times in 25 mM Tris–HCl, pH 7.5 and 150 mM NaCl (15 minutes in total).

H. 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.

I. 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.