Hypotonic introduction of modified deoxynucleotides into cells

Example of the detection of transcription and replication using 5-bromouridine 5'-triphosphate and biotin-16-2'-deoxyuridine 5'-triphosphate, respectively in cells grown on coverslips

Stock solutions to be prepared:

10× KHB (stock solution)

300 mM KCl
100 mM Hepes

Add three volumes of 1M KCl and one volume of 1 M Hepes (pH 7.4) to 6 volumes of deionized water. Filter the prepared solution in the flow box, aliquot and store at -20°C.

**This protocol can be used for the introduction of other small molecules that do not enter cells efficiently as well. For example, see Koberna et al 1999, Chromosoma, 108(5): 325-335.*

Protocol

1. Warm up cell culture medium to 37°C.
2. Prepare sterile 1× KHB (e.g. mix 1 ml of sterile 10× KHB with 9 ml of sterile deionized water) and warm it up to 37°C.
3. Prepare the hypotonic solution consisting of 1× KHB and 20 mM 5-bromouridine 5′-triphosphate (BrUTP) for the detection of transcription or 0.2 mM biotin-16-2'-deoxyuridine 5'-triphosphate (biotin-16-dUTP) for the detection of replication.

**Usually, 20 µl of hypotonic solution is sufficient per coverslip with a diameter of 12 mm.*

**The nucleotide concentration used are close to the minimal effective concentration for HeLa cells. For other cell lines or pieces of tissues, the concentrations should be tested.*

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4. Put 10 ml of 1× KHB solution in the sterile Petri dish (e.g. 60 mm in diameter).
5. Prepare a new empty sterile Petri dish for the coverslips.
6. Remove the first coverslip from the culture Petri dish by tweezers, aspirate the culture medium using filter paper and quickly wash the coverslip in the prepared 1× KHB in Petri dish by brief dipping.
7. Aspirate carefully the rest of 1× KHB solution and place the coverslip in the prepared empty sterile Petri dish (the cells are on the upper side).
8. Cover carefully the whole upper side of the coverslip with 20 µl of the prepared hypotonic solution.
9. After preparation of the required number of coverslips, close the Petri dish and put it in the incubator. Incubate cells for 10 min.

**The molecules enter the cells during this step.*

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10. Remove the Petri dish with the samples from incubator and add warmed culture medium (to 37°C). Put the Petri dish back to the incubator and incubate the samples for the required time, e.g. 30 minutes.
11. Remove the samples from the incubator, wash them three times with pre-warmed 1× PBS.

12. Fix the samples with 2% formaldehyde in 1× PBS (10 min, room temperature).
13. Wash samples with 1× PBS and permeabilise them with 0.2% Triton X-100 in 1× PBS (10 min, room temperature).
14. Wash samples with 1× PBS (3×).

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

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15. Incubate the samples with primary anti-BrdU or anti-biotin antibody dissolved in 1× PBS (60 min, room temperature).

**Use 20 µl per circular coverslip with a diameter of 12 mm.*

**In the case of the detection of additional cellular components by specific antibodies, add the required primary antibody to the mixture described in step 15 and secondary antibody to the mixture described in step 17.*

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16. Wash the samples with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (3× 5 min).
17. Incubate with a secondary antibody and DAPI (final concentration: 1 - 10 µM) in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (30 min, room temperature).

**Use 20 µl per circular coverslip with a diameter of 12 mm.*

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18. Wash the samples in 25 mM Tris-HCl, pH = 7.5 and 150 mM NaCl (3× 5 min).
19. 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.
20. Use nail polish to seal the coverslips and to protect the medium from drying. The coverslips can be stored at −20°C.

Please, if you use this protocol, cite this source: **Koberna, K., et al. (1999) Chromosoma, 108(5):325-335**