Immunoflourescence – Whole Cell and In situ fractionation

Adam Leman

Materials:

Cytoskeleton buffer:

10mM PIPES pH 6.8
100mM NaCl
300mM Sucrose
3mM $MgCl_2$
1mM EGTA
0.5% Triton X-100

Cytoskeleton Stripping buffer:

10mM Tris-HCl pH 7.4
10mM NaCl
3mM $MgCl_2$
1% Tween-20
0.5% Sodium Deoxycholate
(reagents added in order, and
Tween fully suspended prior to DOC addition)

Permeabilization Buffer:

100mM Tris-HCl pH 7.4
50 mM EDTA
0.5% Triton X-100

Method:

Plate ~$2x10^5$ cells in a 30mm containing a 18x18mm glass coverslip

Wait 48 hrs

For In situ fractionation:

1. Wash cells with ice cold 1x PBS (2 x 4min).
2. Incubate in Cytoskeleton Buffer for 5min on ice.
3. Incubate in Cytoskeleton Stripping Buffer for 5min on ice.
4. Wash cells with ice cold 1x PBS (2 x 4min).

For whole cell preparation:

1. Wash cells with RT 1x PBS for 4 min.

Fixation:

1. Incubate cells in 1x PBS containing 3.7% formaldehyde (made just before use) for 15min at RT.
   -Can skip steps 2 and 3-
2. Wash cells with RT 1x PBS (3 x 2min).
3. Incubate cells in 1x PBS containing 50mM Ammonium Chloride (made just before use) for 15min at RT.
4. Wash cells with RT 1x PBS (3 x 4min). *–good stopping point*

Permeabilization and Blocking:

1. Whole cell preparations must be permeabilized by incubation with Permeabilization buffer for 15min at RT.
2. Wash whole cell preparations with RT 1x PBS (3 x 4min).
3. Incubate cells in 1x PBS containing 5% Fetal Calf Serum (FCS) for 1hr.

Primary Antibody:

1. Add primary antibody, diluted in 1x PBS containing 5% FCS (~50-100μl per coverslip).
2. Add dH_20 to the space between wells, wrap the 6-well with parafilm, and store at 4°C O/N.

Next Day

Secondary Antibody:

1. Wash cells with RT 1x PBS (5 x 10min).

2. Add secondary antibody (florescent-conjugated), diluted in 1x PBS containing 5% FCS (~50-100μl per coverslip).
3. Move cells to dark area at RT, keep space between wells wet, and let incubate for 2 hrs in the dark (from this point on, keep light to the absolute minimum).
4. Wash cells with RT 1x PBS (5 x 10min).

Curing coverslips on glass slides:

1. Remove coverslip from well with forceps, dip briefly in dH_20, and touch the side of the coverslip to paper towel to remove water.
2. Add ~20μl of ProLong Gold + DAPI to the surface of a clean microscope slide (the droplet of ProLong **must be free from bubbles**).
3. Mount Coverslip, cells down, onto microscope slide, allowing the coverslip to gently fall onto the ProLong.
4. Store the microscope slide in a dark box O/N at RT.
5. If not viewing the slides the next day, move the dark box to -20°C.

Protocol modified from:

Gonzales *et al.* J. Cell Biol. 1993 **122**, 1089-1101
-and-
Mirzoeva and Petrini. Mol Cell Biol. 2001 **21**, 281-288.