I. Immunofluorescence – Triton

Triton is a stronger detergent than Saponin. It solubilizes cellular membranes without disturbing protein-protein interactions.

Updated 12/22/05, Rewritten 9/28/05 – Wandinger-Ness lab

Reagents

PBS(+): Dulbecco’s PBS (Gibco 450-1600EL), with the addition of 0.9mM Ca$^{2+}$ and 1mM Mg$^{2+}$. Add Ca$^{2+}$ and Mg$^{2+}$ slowly with stirring, otherwise they will precipitate out and give you a cloudy solution.

PBS(+): containing 50mM NH$_4$Cl

PBS(+): containing 0.2% gelatin

PBS(-): containing 0.2% gelatin and 0.1% Triton X-100 (no Ca$^{2+}$ and Mg$^{2+}$) and 5µl/ml of Hoechst 33258 stain (stock = 0.5mg/ml in water, sterile filtered and kept dark at 4°C)

3% Paraformaldehyde (PFA) in PBS(+):
- Dissolve 3g PFA in 100ml of PBS(-)
- Heat to 80°C in a hood with stirring until dissolved, then cool to room temp.
- While stirring, add 10µl 1M CaCl$_2$ and 10µl MgCl$_2$
- Filter through a 0.45µm Millipore filter
- Freeze in 10ml aliquots and store at -20°C (use aliquots only once, do not refreeze).

Mowiol Mounting Medium:
- Add 6g glycerol (AR grade from Merck) to a 50ml conical centrifuge tube
- Add 2.4g Mowiol 4-88 (Calbiochem) and vortex
- Add 6ml dH$_2$O and leave at room temperature for 2 hours
- Add 12ml 0.2M Tris, pH 8.5 and incubate at 53°C overnight until Mowiol is dissolved
- Clarify by centrifugation at 4000RPM for 20 minutes
- Store as 1ml aliquots at 4°C or -20°C

- Add 6.0g glycerol
- Add 3.6g Mowiol 4-88 (Calbiochem)
- Add 6.0ml H$_2$O to a 50ml conical centrifuge tube
- Leave at room temperature for 3 hours
- Add 12.0ml 0.2M Tris, pH 8.5
- Incubate at 50°C for 1-3 hours
- Centrifuge at 5000 RPM for 15 minutes to clarify
- Aliquot and store at -20°C until ready for use
Procedure

A. Cell Growth

1. Autoclave coverslips in a glass Petri Dish between layers of filter paper.
2. Sterilize a pair of fine tipped forceps with ethanol and flame. Then use them to transfer sterile coverslips to individual wells of a multi-well plate.
3. Trypsinize 1 confluent 75mm² Falcon flask of cells. Resuspend cells in 10ml of medium. Add 3.3ml of resuspended cells to 30ml of medium, mix. Plate 2.5ml of resuspended cells on each well.
4. Allow cells to grow at 37°C for 16-20 hours to desired confluence.
5. Perform experiment

B. Fixation and Staining (all steps at room temperature)

Fixation

1. Wash cells 3x with 2ml PBS(-).
2. Add 1ml 3% Paraformaldehyde and fix for 20 minutes, can be left overnight at 4°C. (After PFA treatment, do not allow cells to dry – even for a few seconds!)
3. Wash cells 3x with 2ml PBS(-).
4. Quench with 2ml of 50mM NH₄Cl/PBS(+) for 10 minutes.
5. Wash cells 3x with 2ml PBS(-).
6. For permeabilization, add 2ml of 0.1% Triton X-100 in PBS(+)/0.2% gelatin for 2 minutes.
7. Wash cells 3x with 2ml PBS(-).

Staining

1. Dilute antibodies to proper concentration in PBS(+)/gelatin in Eppendorf tubes, keep on ice. Just before using, centrifuge 1 minute in microfuge.
2. Aspirate PBS and put 25µl of diluted first antibody on coverslip, incubate for 20 minutes.
3. Wash cells 2x with 2ml PBS(+)/gelatin for 5 minutes.
4. Wash cells 2x with 2ml PBS(+) for 5 minutes.
5. Do a quick spin of diluted secondary antibody to remove clumps and stain with the secondary antibody (25µl)/Hoechst 33258 (1:1000) as in step 2.
6. Wash cells 2x with 2ml PBS(+)/gelatin for 5 minutes.
7. Wash cells 2x with 2ml PBS(+) for 5 minutes.

Mounting

1. Label microscope slides, place cotton swab in distilled H₂O and tear pieces of filter paper into quarters.
2. Pick up coverslip with jewelers forceps, wash non-cell side off with cotton swab, dry with filter paper, and mount coverslip on slide using one drop of Mowiol Mounting Medium.
3. View slides on fluorescent microscope.