Neutrophil Extracellular Traps PROTOCOL

This is an immunofluoresence protocol on round coverslips suitable for NETs preparation.

- Place a round coverslip in a 24-well plate well. Seed ~200.000 neutrophils in 500 μl **RPMI medium (containing 2% FCS)** per well and incubate for *1 hour* in CO_2 at 37 °C.
- Add 100 nM **PMA** (stock concentration 1.62 mM, 1/100 dilution in PBS add 3μl) in the wells. Incubate for *15 min – 4 h (3.5 h)* in CO_2 at 37 °C.
- Fix cells in **4% PFA** (paraformaldehyde). Add 500μl 8% PFA so that your final concentration in the well is 4%. Incubate for *2-4 h* at room temperature.
- Remove coverslip carefully using a bent needle and a pair of bent forceps. Put coverslip upside down on a drop of **PBS 1x**. Wash 3 times for *5 min* at RT.
- Incubate coverslip in the same manner in a drop of **0.5% Triton X-100** for *1 min* at room temperature.
- Wash 3 times in **PBS 1x**.
- Prepare a humid chamber with parafilm and wet tissue. Lay the coverslip upside down on a drop of blocking buffer (**5% rabbit serum in PBS**) and incubate for *30 min* at 37 °C.
- Dilute primary antibody in blocking buffer (PBS +5% rabbit serum). Transfer coverslip in the humid chamber directly from blocking buffer onto a drop of primary antibody and incubate for *1 h* at 37 °C.
- Wash 3 times in **PBS 1x**.
- Dilute secondary antibody in blocking buffer. Transfer coverslip into the humid chamber on a drop of secondary antibody and incubate for *1 h* at 37 °C.
- Wash 3 times in **PBS 1x**.
- Counterstain with **DAPI**. Incubate with 300 nM DAPI for *1-5 min* in the dark. Wash twice with distilled water. Drain slides.
- Set a 20 μl drop of **Mowiol** onto a glass slide and a mount coverslip upside down.

NOTES: For immersion visualization let the slide dry for at least 1 hour at room temperature in dark, in order for the mowiol to dry. For long terms storage place the slide at 4 °C in dark.

Purification of NETs Proteins

This is a method to isolate protein from NETs by digestion.

- After neutrophils stimulations and NETs formation wash twice with 1 ml of fresh and pre-warmed **RPMI** medium (37 °C for 10 min). Pippetting in the wall of the well.
- Wash with **RPMI medium with 10 U/ml (or 5 U/ml) DNase I** digestion for *20 min* at 37 °C.
- DNase I was stopped with 5mM **EDTA**.
- Centrifuge at 300 x g to remove whole cells *10-15 min*
- Centrifuge at 1,000 x g for *20 min* to remove debris.

Isolation of NETs for Stimulations

This is an undigested NETs method to collect supernatants from NETs to use them as stimuli for other cell types. We use 6 well plates.

- Seed cells in 1ml **RPMI** medium per well and incubate for *4 h* in CO_2 in 37 °C.
- Remove RPMI very carefully.
- Wash with 1 ml **RPMI** medium (pre-warmed in room temperature for *10 min*).
- Vigorous agitation by pippetting in the wall of the well.
- Collect NETs in a 15-ml conical tube (each plate in a different tube).
- Centrifuge in 20 x g for *5 min*.
- Collect supernatants from NETs.

MATERIALS :

1. RPMI + FCS 2% & RPMI + 10 (or 5) U/ml DNase I
2. PMA (PHORBOL 12-MYRISTATE 13-ACETATE)
3. PFA 4 % (50 ml)

   5ml PBS 10x

   5ml 37 % PFA (1.85gr PFA, 3.5ml ddH_2O, 10µl 10N KOH)

   40 ml ddH_2O

Mix well, divide into aliquots of 5-10ml and store at -20°C.

Use each aliquot once and avoid freeze-thaw cycles.

4. PBS 10x (pH 6.8) → PBS 1x = 10 ml PBS 10x in 90 ml dH_2O (pH 7.4)

   80gr NaCl,

   2gr KCl,

   17.8gr Na_2HPO_4,

   2.7gr KH_2PO_4,

   1L dH_2O

   Autoclave or filter sterilize and store at room temperature.

5. DAPI
6. Mowiol

   2.4gr Mowiol

   6gr glycerol

   6ml ddH_2O

   12 ml 0.2M Tris pH 8.5

   Place glycerol in a 50ml conical tube.

   Add Mowiol and stir thoroughly.

   Add dH_2O and leave 2h at room temperature. Stir occasionally.

   Clarify by centrifuge at 4.000 rpm for 20 min at room temperature.

   Collect and aliquot the supernatant into 1 ml each.

   Store at -20 °C. Once defrosted, store at 4 °C.

7. EDTA