GRADIENTECH

PROTOCOL
Chemotaxis protocol for co-cultured human polymorphonuclear neutrophils (PMNs) and human platelets in CellDirector 2D

CELLS AND REAGENTS:

- Whole blood samples
- Acid citrate
- EDTA
- ACD buffer
- Collagen A, Typ I acid-soluble
- PBS+/- (with/without Ca^{2+}/Mg^{2+})
- CellTracker™ solutions
- RPMI-1640
- IL-8

Note: *All media should be equilibrated before use (overnight at 37 °C in an incubator) to avoid bubble formation during the experiment.*

Preparation of cells from whole blood

1. Whole blood was drawn from healthy donors and anticoagulated with acid citrate. PMNs were isolated with double-density centrifugation using the Percoll method.
2. Whole blood was drawn from healthy donors and anticoagulated with EDTA. Platelet rich plasma was isolated and an anticoagulant (ACD buffer) added to prevent platelet activation.

Preparation of CellDirector 2D

1. Coat with Collagen A 1:1 mixed with PBS+, pH3.
2. Incubate 2 hours at room temperature.
3. Wash the CellDirector 2-3 times with PBS-.

Staining protocol

Important: *All steps should be performed at 4 °C for PMNs, and at room temperature for thrombocytes.*

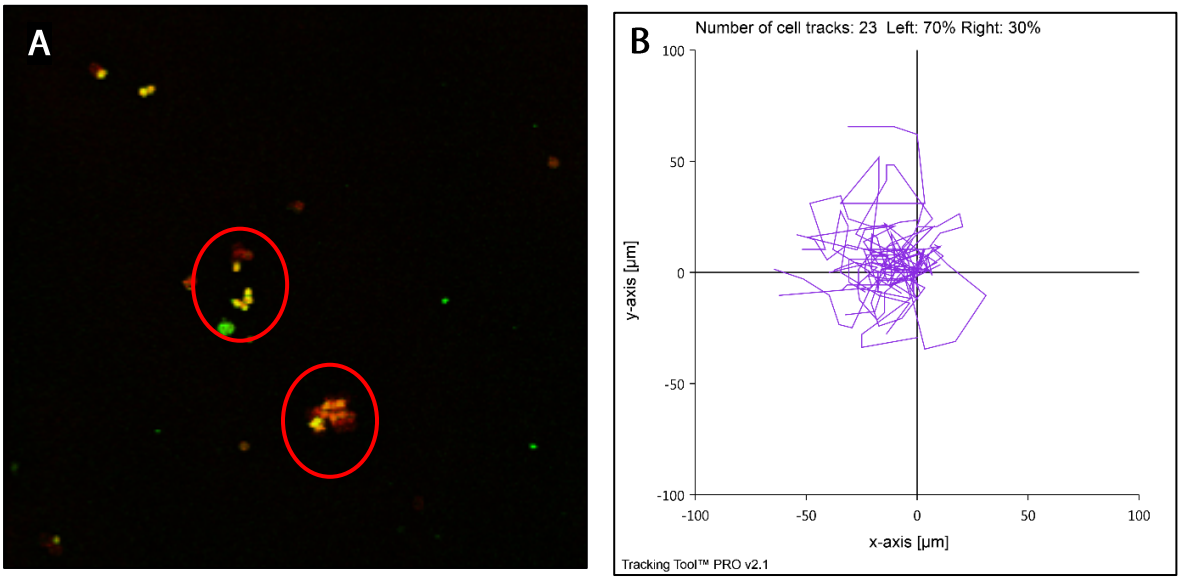
1. Harvest cells by centrifugation and aspirate the supernatant. Resuspend the cells gently in pre-warmed CellTracker™ Working Solution. Stain PMNs with *CellTracker Red CMTPX* and platelets with *CellTracker Green CMFDA*.
2. Incubate 30 minutes under growth conditions appropriate for the particular cell type.
3. Centrifuge the cells and remove the supernatant of CellTracker™ Working Solution.
4. Wash the cell suspension 2 times with PBS- to remove remaining CellTracker™ Solution.
5. Add culture media without supplements.

Pre-activation of PMNs

1. Spin your neutrophils suspension at 3500 rpm for 10 min.
2. Pre-activate the neutrophils by resuspending them in 50 ng/ml recombinant IL-8 (diluted in RPMI-1640) for 5 min at 37 °C.
3. Spin your neutrophil suspension at 3500 rpm for 10 min.
4. Resuspend the neutrophils in RPMI-1640 to a final concentration of $0.25x10^6$ cells/ml.

Cell Loading

1. Mix neutrophils and platelets to a final concentration of $0.25x10^6$ neutrophils and $1.25x10^6$ platelets in 1 ml RPMI-1640
2. With a pipette, slowly inject 200 µl of the cell suspension into CellDirector 2D.
3. Place a wet napkin and the loaded CellDirector 2D with the green side facing upwards in a 10 cm Petri dish to avoid excessive evaporation, and place in the incubator for 45 min at 37 °C. Verify adhesion under a microscope.
4. Fill syringe 1 with equilibrated RPMI-1640. Fill syringe 2 with equilibrated RPMI-1640 containing your chemoattractant.
5. Start the CellDirector 2D experiment as described in the manual.



A: Platelet-neutrophil complexes in CellDirector 2D. Red: neutrophils, green: platelets.
B: Tracking results of 90 min live cell imaging of neutrophil-platelet-complexes in an IL-8 gradient. Tracking was performed using Tracking Tool PRO.

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