

Advanced Methods Flow Cytometry

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Lecture: April 27, 2010 – 4pm SR 4th floor

Practical part: April 28 + 29, 2010 – 9.30am – 5.30pm

Day 1

We will be divided into 4 groups of 2-3 people each.

Two groups will stain cells to look at cell death in bone marrow derived macrophages, and two groups will do the staining to look at the cell cycle status of bone marrow derived macrophages. Both sets of groups will have the opportunity to see both samples run on the flow cytometer.

Examining cell death in bone marrow derived macrophages

Reagents and solutions needed:

PI 100X solution

PBS 2mM EDTA

PBS

Annexin V binding buffer

0.01M HEPES/NaOH, pH7.4

0.14 M NaCl

2.5 mM CaCl₂

Annexin V FITC

4ml FACS tubes

You will each be given 2 dishes of macrophages. One dish has been treated with 1 ug/ml puromycin for 18 hours to induce apoptosis, the other dish has been left untreated.

- 1) As many dead cells will not be attached, transfer supernatant to a tube and put on ice.
- 2) Lift macrophages by washing cell once with 10 mLs of PBS (combine the PBS wash in the same tube as the initial removed supernatant) and putting 10 mL cold PBS/EDTA on cells.
- 3) Incubate cells at 4 degrees for about 10-15 minutes until they round up and start to lift cells off plate.
- 4) If the macrophages do not detach they can be GENTLY scraped with a cell scraper.

- 5) Spin cells at 1300 RPM in an eppendorf centrifuge for 3 minutes.
- 6) Resuspend cells in 10 mL of PBS
- 7) Count cells
- 8) Put 1 million cells from each treatment into 5 different 4 ml plastic tubes. Put 1 million cells in each tube and label the tubes 1-5.
- 9) Spin tubes in eppendorf centrifuge at 1300 RPM
- 10) Meanwhile, dilute Annexin V 1:50 in Annexin V binding buffer.
- 11) Resuspend cells in the following
 - Tube 1: Add 100 ul of Annexin V diluted in Annexin V binding buffer
 - Tube 2: Add 100 ul of Annexin V diluted in Annexin V binding bufer
 - Tube 3: Add just Annexin V binding buffer (we will add PI later)
 - Tube 4: Add just Annexin V binding buffer
 - Tube 5: Add just Annexin V binding buffer
- 12) Incubate at room temperature for 20 minutes in the dark (DO NOT spin afterwards)
- 13) Add 400 ul of annexin V binding buffer to each tube and put on ice
- 14) Just before running samples add 5 ul of diluted PI to tubes 1 and 3 for a final concentration of 1 ug/ml and mix well.
- 15) Run samples on flow cytometer

Examining cell cycle in bone marrow derived macrophages

Reagents needed

PI solution

- PI 40 ul of 500 ug/ml stock
- RNase 10 ul of 10mg/ml stock
- PBS Ca⁺⁺ Mg⁺⁺ free 450 ul
- 1 ml of 70 % EtOH

PBS 2mM EDTA

PBS

FACS buffer (PBS, 1% BSA, 0.01% NaAzide)

70% ethanol

4ml FACs tubes

You will each be given two dishes of macrophages. One has been deprived of growth factors to induce cell cycle arrest. The other has been growing normally.

To analyze cell cycle of these cells follow the next steps:

- 1) As many dead cells will not be attached transfer supernatant to a tube and put on ice
- 2) Lift macrophages by washing cell once with 10 mL PBS (combine the PBS wash in the same tube as the initial removed supernatant) and putting 10mL cold PBS/EDTA on cells.
- 3) Incubate cells at 4 degrees for about 10-15 minutes until they round up and start to lift cells off plate.
- 4) If the macrophages do not detach they can be GENTLY scraped with a cell scraper.
- 5) Spin cells at 1300 RPM in an eppendorf centrifuge for 3 minutes.
- 6) Resuspend cells in 10 mls of PBS
- 7) Count cells
- 8) Put cells of each treatment into 3 different 4 ml plastic tubes. Put 1 million cells in each tube and label the tubes 1-3.
- 9) Spin tubes in eppendorf centrifuge at 1300 RPM and remove supernatant
- 10) Fix cells by adding dropwise with gentle vortex to disrupt pellet and clumps 500 ul of ice cold 70% ethanol.
- 11) Incubate at 4 degrees for 60 minutes
- 12) Wash cells twice with PBS
- 13) Meanwhile prepare PI solution (**see above**).
- 14) Add 300 ul of PI solution and incubate for 30 minutes at 37 in the dark
- 15) Immediately analyze by flow cytometry (within one hour)

Day 2

Four color staining and analysis of mouse splenocytes

Reagents needed:

Mouse spleens

PBS

RBC lysis buffer

Complete DMEM

FACs Buffer

Anti-CD19 FITC

Anti-CD4 PE

Anti-CD3 Percp

Anti-CD8a APC

V-bottom plates and lids

Tinfoil

Eppendorf tubes and racks

Each group will be given a mouse spleen, will make single cell suspensions from the mouse spleen, stain the cells with markers for B cells and T cells, and analyze these cells by flow cytometry.

- 1) Make a single cell suspension of the spleen by mashing it with the end of a syringe into the cell strainer over a 50 ml conical.
- 2) Rinse cell strainer 2 times with 10 mls each time of PBS
- 3) Centrifuge cells for 5 minutes at 1300 rpm in an eppendorf centrifuge.
- 4) Remove supernatant
- 5) Resuspend pellet in 1 ml of Red blood cell lysis buffer
- 6) Incubate cells for 1 minute at room temperature.
- 7) Add 10 mls of complete media and spin cells.
- 8) Resuspend pellet in 10 mls of FACs buffer.
- 9) Count cells
- 10) Add 500,000 cells each to 8 wells of a V-bottom plate (use one plate for two groups).
- 11) Spin plates at 1300 rpm for 3 minutes.
- 12) Dilute antibodies (1:100) into 5 eppendorf tubes containing 100 ul of FACS buffer FACS buffer.
 - Tube 1: anti-CD19 FITC, anti-CD4 PE, anti-CD3 Percp, anti-CD8a PE
 - Tube 2: anti-CD19 FITC
 - Tube 3: anti-CD4 PE
 - Tube 4: antiCD3 Percp
 - Tube 5: anti-CD8a PE
- 13) Add 25 ul of the antibody solutions to the cells in wells 1-5 add FACS buffer to the remaining wells. Make sure cells are resuspended. Add FACS buffer only to the remaining wells.
- 14) Cover the plate with lid and tinfoil (to block out light) and put on ice for 30 minutes
- 15) Add 200 ul of FACs buffer to each well.
- 16) Spin plates at 1300 rpm for 3 minutes.
- 17) After spin remove liquid from plates by swift flicking into the sink. Check for pellet first.
- 18) Add 200 ul of buffer and spin again, flick buffer.
- 19) Add 200 ul of buffer and transfer to micro titre tubes.
- 20) Run samples on flow cytometer.