

Classes 1.

Methods for isolation of stem cell populations

Introduction

Stem cells (SC) due to ability to self-renewal and wide differentiation potential (into cells targeted to specific developmental lines) represent the desired material from the viewpoint of regenerative medicine. One of the major challenges resulting from the use of SC in regenerative medicine is the development of optimal protocols for their isolation and expansion. The most important isolation methods include: Magnetic Assisted Cell Sorting (MACS), Fluorescence Activated Cell Sorting (FACS) as well as SC ability to adherence to negative charged surfaces of culture vessels.

MACS technology uses paramagnetic particles (MicroBeads) that are conjugated to specific antibodies against a particular antigen on the cell surface. Due to the small size, MicroBeads do not activate cells. During separation, the unlabeled cells pass through while the magnetically labeled cells are retained within the column. The flow-through can be collected as the unlabeled cell fraction. After a short washing step, the magnetically labeled cells are eluted from the column. MACS sorting is maintainly used as a pre-selection stage proceeding isolation based on the multi-antigenic profile.

FACS technology uses monoclonal antibodies conjugated with colored fluorochromes. This technology allows a single cell to be measured for a variety of characteristics and can be used to select and purify a specific subset of cells within a population. In this technology, the cell suspension is entrained in the center of a narrow stream of liquid. A vibrating mechanism causes the stream of cells to break into individual droplets containing no more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. Thus, the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through electrostatic deflection system that diverts droplets into containers based upon their charge. The table below contains a comparison of isolation by using MACS and FACS sorting.

MACS	FACS
Sort based on paramagnetic properties of cells	Sort based on immunofluorescent properties of cells
Sort mainly based on one cellular marker	Sort generally based on multi-antigenic cellular profile
Presence of antigen- positive selection Lack of antigen- depletion (negative selection)	The simultaneously presence of a marker/s or his/ their absence
Relatively low purity	High purity
Method used as pre-selection stage of SC sorting	Method used in the final sorting of SC populations

Flow cytometry platform provides information about size (FSC- *forward scatter*), granularity (SSC- *side scatter*) of cells as well as fluorescence emitted by these cells. Flow cytometry data are displayed as dot plots or histograms. Dot plot displays use two parameters to graph the data generated by flow analysis, with each dot representing the passage of one cell through the detector. The X- and Y-axes measure the different emissions, displaying a dot for each of the cells that show that particular emission. Histograms can also be used to display data from flow cytometry experiments. In

these plots the X-axis shows the intensity of the detected signal and the Y-axis measures the number of events (cells) counted.

The most important source of mesenchymal stem cells (MSC) is bone marrow. These cells represent adherent fraction of bone marrow obtained by removing of non-adherent fraction at 72h following seeding of whole BM into culture vessels. Primary culture flasks are dedicated to primary cell culture. These flasks are fabricated from modified polystyrene that enhance adhesiveness of cells.

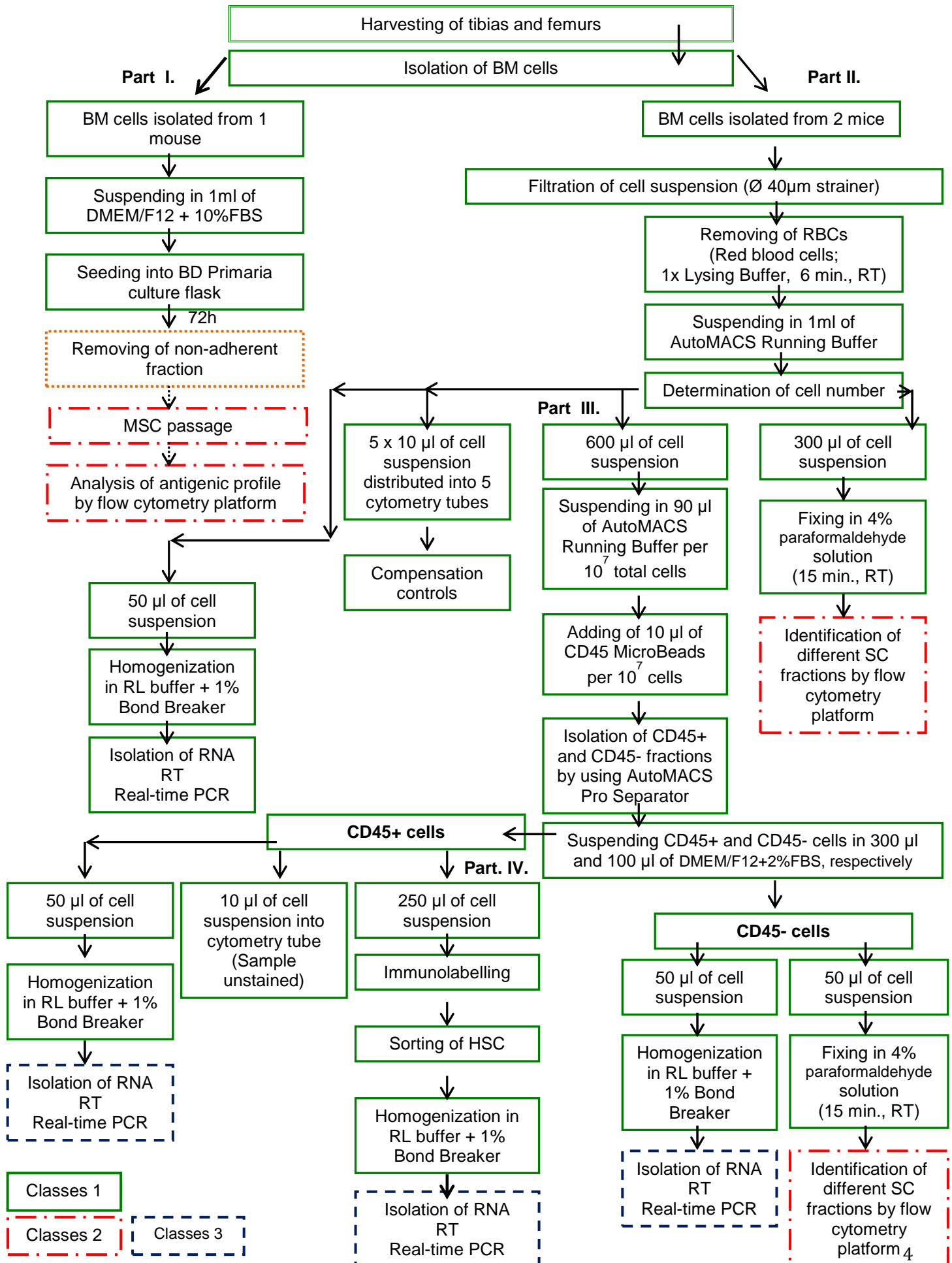
Aim of classes:

Aim of the first classes is to isolate different SC fractions based on antigenic profile by MACS and FACS technologies as well as based on adhesive properties.

Experimental layout:

Work in two people teams. Each team will receive three mice, which will be used according with the table given below.

No. of mice	Destiny
1.	Isolation and culture of MSC
2 i 3.	Isolation and analysis of SC fractions by MACS and FACS technologies



Part I. Isolation and culture of BM-derived Mesenchymal Stem Cells (MSC):

1. Isolate tibias and femurs from one mouse into Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12.
2. Cut off the epiphysis of bones and flushed bones using 1ml syringe into new Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12. Homogenize cell suspension using 1 ml syringe.
3. Remove cell suspension into 15 ml tube and centrifuge at 1800 rpm. for 7 min at RT.
4. Discard supernatant and suspend cell pellet in 1 ml of culture medium supplemented with 10% of FBS (DMEM/F12+10%FBS).
5. Add 4 ml of DMEM/F12+10%FBS and seed whole cell suspension into BD Primaria culture flask (25cm²).
6. Culture MSC in cell culture incubator (37°C, 5% CO₂).
7. Remove non-adherent fraction of BM cells after 72h following seeding and add 5ml of DMEM/F12+10%FBS.
8. Passage MSC during the next classes.

Part II. Preparation of whole BM cells for isolation of hematopoietic and non-hematopoietic SC by MACS and FACS sorting procedure

1. Isolate tibias and femurs from two mice into Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12.
2. Cut off the epiphysis of bones and flushed bones using 1ml syringe into new Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12. Homogenize cell suspension using 1 ml syringe.
3. Filter cell suspension through Ø 40 µm strainer into 15 ml tube. Fulfill tube to the volume of 10 ml by culture medium DMEM/F12.
4. Centrifuge at 1800 rpm for 7 min at RT.
5. Discard supernatant and suspend cell pellet in 1 ml of Lysing Buffer (1x BD PharmLyse) to remove red blood cells. Add 1 ml of Lysing Buffer. Incubate for 6 min at RT.
6. Fulfill tube to the volume of 10 ml by culture medium DMEM/F12 and centrifuge at 1800 rpm for 7 min. at RT.
7. Discard supernatant and suspend cell pellet in 1 ml of AutoMACS Running Buffer.
8. Determine cell numer.

Attention! The next steps are intended to protect the material for 2nd and 3rd classes!

9. Remove 50 µl of cell suspension into new Eppendorf tube. Add 100 µl of RL buffer containing 1% of Bond Breaker (β-merkaptoethanol). Vortex. Homogenize using 1 ml syringe. Store at -80°C until isolation of RNA (material for 3rd classes).
10. Remove 300 µl of cell suspension into new 5 ml (cytometry) tube. Add 200 µl of DPBS and 500 µl of 4% paraformaldehyde solution. Fix for 15 min at RT. Add 2 ml of DPBS and centrifuge at 1800 rpm for 7 min at RT. Discard supernatant and resuspend cell pellet in 1 ml of DPBS. Store at +4°C until next classes (material for 2nd classes).

11. Add 100 μ l DMEM/F12+2%FBS and 10 μ l of cell suspension into each of four 5 ml tubes (compensation controls, look at p. IV.2). Store samples on ice.

Part III. MACS separation:

12. Resuspend cells in 90 μ l of AutoMACS Running Buffer (+4°C) per 10^7 total cells.
13. Add 10 μ l of CD45 MicroBeads per 10^7 total cells. Mix well and incubate for 15 min. at 4-8°C. (*Higher temperatures and/or longer incubation times lead to non-specific cell labelling*).
14. Wash cells by adding 1 ml of AutoMACS Running Buffer per 10^7 cells and centrifuge at 1400 rpm for 10 min. Pipette off supernatant completely.
15. Resuspend up to 10^8 cells in 500 μ l of AutoMACS Running Buffer.
16. Separate CD45+ cells and CD45- by AutoMACS Pro Separator (Program Possels).
17. Centrifuge at 1500 rpm for 7 min.
18. Resuspend CD45+ and CD45- pellets in 300 μ l and 100 μ l DMEM/F12+2%FBS, respectively.
19. Remove 50 μ l of cell suspensions into new Eppendorf tubes. Add 100 μ l of RL buffer containing 1% of Bond Breaker (β -merkaptoetanol). Vortex. Homogenize using 1 ml syringe. Store at -80°C until isolation of RNA (material for 3rd classes).

Part IV. FACS sorting:

1. Add 100 μ l DMEM/F12 + 2% FBS into two 5 ml tubes.
2. Immunolabel CD45+ cells with antibodies listed below according with scheme given in the table.

Sample	Type of cells	List of antibodies applied for immunolabelling
CD45+	CD45+	<ol style="list-style-type: none"> 1. Hematopoietic lineage markers (Lin): PE Rat anti- mouse Ly-6G and Ly-6C; PE Rat anti- mouse CC11b; PE Rat anti- mouse CD45R/B220; PE Rat anti- mouse Ter-119; PE Hamster anti- mouse $\gamma\delta$ T- Cell Receptor; PE Hamster anti- mouse TCR β Chain <u>Add 1.5 μl of each = 9 μl of Lin mix per sample</u> 2. PE/Cy5 anti- mouse Ly-6A/E (Sca-1) = 5 μl per sample 3. FITC Rat anti-mouse CD45 = 7 μl per sample
CD45+/ Empty	CD45+	Sample unstained
Comp./ Empty	BM cells (without erythrocytes)	Sample unstained
Comp./ PE		Hematopoietic lineage markers (Lin) = 2 μ l
Comp./ PE-Cy5		PE/Cy5 anti- mouse Ly-6A/E (Sca-1) = 2 μ l
Comp./ FITC		FITC Rat anti-mouse CD45 = 2 μ l

3. Stain for 20 min., on ice (protect samples from light).
4. Wash cells by adding 2 ml of DPBS (without Mg^{2+} , Ca^{2+} ions) and centrifuge at 1800 rpm for 7 min at RT.
5. Discard supernatant and resuspend pellets in 300 μ l of DMEM/F12 + 2% FBS. Mix by pipetting.

6. Sort HSC (CD45+/Lin-/Sca-1+) by BD FACS Aria III cell sorter (material for 3rd classes).

The scope of material that will be prepared to classes:

1. General principles of flow cytometry.
2. Immunolabelling cells for analysis by fluorescent microscopy and flow cytometry techniques.
3. Features characteristic for stem cells.

Recommended materials:

1. <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Flow-Cytometry/Flow-Cytometry-Technical-Resources.html?icid=fr-flow-4>
2. Lin KK, Goodell MA. Detection of hematopoietic stem cells by flow cytometry. *Methods Cell Biol.* 2011;103:21-30.
3. Ratajczak MZ, Zuba-Surma EK, Machalinski B, Kucia M. Bone-marrow-derived stem cells--our key to longevity? *J Appl Genet.* 2007;48(4):307-19.
4. Zuba-Surma EK, Ratajczak MZ. Analytical capabilities of the ImageStream cytometer. *Methods Cell Biol.* 2011;102:207-30.