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Bone marrow, spleen and tumor collection for flow cytometry analysis V.1

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Materials

MATERIALS

DMEM, High Glucose **Life Technologies** **Catalog #**11965-092

Troubleshooting

BONE MARROW

1 Kill the animal by cervical dislocation and disect into a tissue culture hood. Clean the animal by spraying with 70% EtOH.

00:30:00 Duration

2 Take the ventral midline incision to the hips and then divide the cut to both legs until you reach both uncles. Remove as much skin and fur as you can and completely peel the animal from hips down.

3 Cut both legs from the hipbones. If you want to preserve the material, immerse it in a 15ml conical tube with enough PBS to cover it and avoid drying. Legs can be preserved for several hours on ice.

4 In a 100 mm or 150 mm plate place the leg and start removing the flesh covering femur and tibia using forceps, scissors or a scalpel.

5 Twist and distort the femur from the knee and tibia and separate them using forceps. Prevent the bone fracture of damage of the bone marrow will be affected.

6 Using strong scissors, cut the metaphysis and epiphysis (head of the femur from hip and knee respectively) and allow the inside of the shaft to be visible, it is red and rounded.

7 Holding the bone by the middle shaft take 1-2 ml of sterile PBS with a 2 ml syringe and a 23g needle and introduce it in the shaft. Bend the dish to keep all the material in a corner and flush the content down and repeat it 3-4 times until the shaft turns white. The PBS should turn reddish or a red strain should be visible (that is the bone marrow).

8 Disaggregate flushing up and down with the syringe and when the PBS have been homogenised filter to a 50ml conical tube with a 100µm cell strainer.

9 Repeat the procedure with the other leg and add a few ml of fresh PBS to avoid drying.

10 Centrifuge the cells 300g 5 minutes RT.

00:05:00

11 Re-suspend in 10 ml of DMEM and count the cells using a hemocytometer Neubauer chamber. Count $4 \cdot 10^5$ cells per ml and plate, depending on the total media that you will plate in the petri dish or multiwell.

SPLEEN

12 Kill the animal by cervical dislocation and dissect into a tissue culture hood. Clean the animal by spraying with 70% EtOH.

13 Take the ventral midline incision, extract the spleen and remove all the fat tissue around. If part of the spleen is needed for other analysis, cut it with a scalpel and maintain at least a half of the spleen in 1mL of cold collagenase in a 15mL conical tube, until extract the spleen of all animals, for flow cytometry. Spleen can be preserved for several hours on cold collagenase because the enzyme acts at 37ºC.

Note

The longer it takes to preserve the samples the worst the outcome will be

1 mL Collagenase

14 Put a 70µm cell strainer on a bacterial plate and place the spleen on the cell strainer without media or collagenase. Do the mechanical disaggregation crushing it with a plastic plunger of a 3mL syringe. Triturate the spleen until there is just connective tissue on the cell strainer.

15 Clean the cell strainer and the bacterial plate with the 1mL cold collagenase where the spleen was maintained to have as many cells as it was possible in the cold collagenase. Put the same cell strainer in a 50mL conical tube, clean it with the cell suspension (in collagenase) that was left in the bacterial plate and after it, clean again with another 1mL of cold collagenase. Maintain on ice until disaggregate all the spleens.

16 To do the enzymatic disaggregation, put the cell suspensions into at 37ºC bath for 1 hour, shaking every 10 minutes. Mechanical and enzymatic disaggregation will allow us to obtain single-cell suspension.

01:00:00 Shake from time to time

17 Prepare a new 50mL conical tube with a new 70µm cell strainer.

18 Add 3mL of RPMI-10 to the cell suspension. Pass through a 5mL syringe with a 19.5 g needle 3-4 times and pass the cell suspension through the new cell strainer in the 50mL conical tube.

3 mL RPMI

19 Centrifuge 5 minutes at 300g.
00:05:00

20 Remove the supernatant, resuspend with 5mL of Red Blood Lysis Buffer and incubate 4 minutes at room temperature.
5 mL ACK Buffer (Red blood lysis buffer)

21 Centrifuge 10 minutes at 300g.
00:10:00

22 Remove the supernatant and resuspend with 5-10 mL of DPBS (depending if the cell suspension comes from a half or a whole spleen and according to the pellet).
10 mL DPBS

23 Count the number of cells using trypan blue.
10 µL Trypan blue

TUMOR

24 Depending on the tumor origin proceed differently and with caution. This protocol has been stablished for B16F1 mouse melanoma and LLC carcinomas.

25 Remove the tumour without taking any part of skin and/or connective tissue or fat.

26 Depending on the nature of the tumour it may need a thorough mechanical disassociation, thus, proceed with with caution within a clean surface as in a cell culture plate with small scissors and/or a blade and dissagregate the piece as smooth as you can
To do the enzymatic disaggregation, incubate the sample in cold collagenase

FLOW CYTOMETRY

27 The following description is valid for the samples aquired previously, Bone marrow, spleen and tumor cell suspension.

Note

The description of the antibodies can be applied to every reagent that you have in the laboratory but it should be assay-dependen to take that into consideration.

28 Incubate 1×10^{-6}cells per FACS tube. For each sample will be needed 2 tubes to do a triple staining: CD3-CD45R-CD11b and CD11b-F4/80-Gr1. For one sample of each group will be also needed 5 tubes to do a simple staining (CD3, CD45R, CD11b, F4/80 and Gr1) and another one to do the negative control (without antibody).

29 Centrifuge 5 minutes at 300g.

30 Add 7AAD (1:100) and blocking 2.4G2 (1:100) solutions in FACS buffer in a total volume of 50µL and pipette vigorously. Incubate 5 minutes on ice.

31 Add the antibodies to the incubated tube (1:50 in a total volume of 50 µL to use them at a final dilution of 1:100 in a total volume of 100µL) and incubate 25 minutes on ice. 50µL of FACS buffer without antibody will be added to the negative control tube.

32 Add 1mL of FACS buffer and centrifuge 5 minutes at 300g.

33 Resuspend the stained cells (without pippeting) in 500µL of FACS buffer.

34 Cell suspension is ready to be analysed by flow cytometry!