Aug 09, 2022

Mouse Ischemia Experiment

gagandeep kaur[1], Irfan_Rahman[1]

[1]University of Rochester Medical Center

dx.doi.org/10.17504/protocols.io.ceyitfue

Dongmei_Li

ABSTRACT

The objective of this protocol is to understand the effect of cold ischemia time on cellular senescence in mouse lung and heart tissues.

The sampling of lung and heart tissues will be done based on equal weights. 6 pieces weighing around 40-50mg will be cut and put in cold preservative to conduct the time-controlled experiment.

As a positive control for C_{12}FDG a technical control of cells treated with EtOH (500µM) will be used.

DOI

dx.doi.org/10.17504/protocols.io.ceyitfue

PROTOCOL CITATION

gagandeep kaur, Irfan_Rahman 2022. Mouse Ischemia Experiment. **protocols.io** https://protocols.io/view/mouse-ischemia-experiment-ceyitfue

KEYWORDS

ischemia, cellular senescence, lung tissue

CREATED

Aug 07, 2022

LAST MODIFIED

Aug 09, 2022

PROTOCOL INTEGER ID

68330

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue

MATERIALS TEXT

Lung Tissue Dissociation

Materials, Equipment & Reagents including Formulations

1. Liberase: (Cat# 5401127001; Roche)
2. DNase I (Cat# 4380-096-06; R&D)

3. Serum free DMEM

5. Disposables:
i) C-tubes for GentleMACS (Cat# 130-093-237, Miltenyi Biotec)
ii) MACS SmartStrainers 70µm (Cat# 130-098-462, Miltenyi Biotec)
iii) Sterile serological pipets; 50, 25,10, 5, 2, 1 mL size
iv) Sterile pipet tips; 1000, 200 and 20 µl
v) Sterile 15mL or 50mL conical polypropylene tubes
vi) Test tube racks for 15mL or 50mL conical tubes
vii) LD columns (Cat# 130-042-901; Miltenyi Biotec)

6. Equipment:
i) GentleMACS Tissue Octo Dissociator (Miltenyi Biotec Inc San Diego, CA)
ii) MACSmix tube rotator
iii) Table Top Centrifuge
iv) Biological Safety Cabinet, Class II
v) Dissecting forceps and scalpel

Measure of senescence (C12FDG) by flow cytometry

Reagents:

-Bafilomycin A1
o10 ug – Sigma Catalog number B1793

-C12FDG – 5 mg – Invitrogen Catalog number D2893

Reagent Setup:

-0.1 mM bafilomycin A1: first reconstitute 100 ug in 160.56 ul DMSO for a final concentration of 1 mM. Use this intermediate dilution to make aliquots at a concentration of 0.1 mM (1 ul + 9 ul DMSO).
-2 mM C12FDG: Dilute 5mg of C12FDG in 0.3 ml of DMSO to make 20mM stock. Dilute the 20 mM stock solution of C12FDG 1:10 with fresh cell culture medium to make a 2 mM working solution just before its addition into the cell culture.

Co-immunolabelling

Reagents:

- CD326 (Biolegend; Cat# 118213)
- CD140a (Invitrogen; Cat# 12-1401-81)
- CD31 (Biolegend; Cat# 102528)

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue

1 1) Mince tissue finely, place into 50ml GentleMACS C-tube with liberase enzymatic cocktail
a. For 50mg tissue use 0.5 ml liberase + 2ml DMEM + 2ul 5 unit/ul DNase I)

2 Immediately transfer the tubes to MACS Tissue Dissociator and run user-defined program named m_lung_01_02 for mouse lungs and m_heart_01.01 for mouse heart.

3 Thereafter, place 50ml conical with digestion into an incubating rocker for 30 minutes at 37 degrees C.
NOTE: If you still see chunks of lung tissue in the tubes after this step then transfer to the MACS Tissue Dissociator and run user-defined program named m_lung_02_01 for mouse lungs for 10-15 sec.

4 Remove conical from rocker and strain the cell suspension through 70 micron into 50mL tube

5 Add 3 ml DMEM (10% FBS) through the strainer to collect the remaining cell suspension

6 After straining, centrifuge at 300g for 5 minutes at 4degree C.

7 Remove supernatant, add 500 µl of red blood cell lysis to cell pellet. Incubate the cell suspension on ice for 5 min.

8 After incubation add 4.5 mL of DMEM (10% FBS) to the suspension and centrifuge at 300g for 5 minutes at 4degree C.

9 Remove supernatant, re-suspend pellet in 2 mL DMEM (10%FBS)

10 Count cells and viability using AO/PI method, confirm reading under microscope

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue

Treatment for positive control

11 After cell counting, divide the cell fractions into three equal parts. For each sample take 1 million cell and pool the remaining cells to make a heterogeneous cell population. Divide this heterogeneous cell population into three fractions. One for positive control and other as negative control and one as a single stained control for C12FDG. Make up the final volume in each tube as 5mL.

12 For positive controls, treat the cell fraction with 500 µM EtOH, and leave the rest of the two fractions as is. EtOH stock = 17.3M; For treatment first make a first dilution of 500mM EtOH in 1 mL media (29.4 µL in 970 µl of DMEM). Add 5 uL of the first dilution (500mM) to 5mL volume of cell suspension in positive control tube to make the final concentration of 500µM.

13 Incubate all the tubes in CO2 incubator for 2 hrs. (NOTE: All this could be done in a 15 mL tube. Make sure the tube caps are loosened when placed in the incubator).

Measure of senescence (C12FDG) by flow cytometry

14 **C12FDG (FITC channel):**

1. Treat the cells with a final concentration of 100 nM of bafilomycin A1. For this add 5µL of 0.1 mM dilution to each tube with 5mL cell suspension. Incubate 1 h at 37°C, 5% CO2
PS: Bafilomycin A must be added to the negative control tube as well.

15 2. Add C12FDG to cells at a final concentration of 20 uM. For this add 50uL of 2mM of C12FDG stock solution to each 5mL cell suspension tube (except negative control). Incubate for 1.5 h at 37°C, 5% CO2. Leave one fraction of cells untreated. This will act as our negative control for C12FDG treatment.

16 3. Centrifuge the cells at 300 g for 5 min and wash 2x with FACS buffer.

17 4. Discard the supernatant by inversion and resuspend the cell pellet in the remain volume (≈ 100 ul).

18 5. Perform a co-immunolabeling to further characterize the population of interest.

Co-immunolabelling

19 PS: from this point keep the samples out of direct light.
1. Prepare the blocking reagent by making a 1:10 dilution (1µL in 100µL of PBS) of CD16/32.

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue

Incubate at 4^0C for 10 minutes.

20 2. Thereafter add 10 µL of the blocking buffer to each sample tube, vortex and incubate at 4^0C for 10 minutes

Following incubation, centrifuge the cells at 500g for 5 min at 4^0C and wash once with 1 mL PBS. Finally, resuspend in 75uL of FACS buffer. Add 25ul of the 75ul of each sample to the unstained tube.

21 3. Create bead and single stained cell controls. For bead controls one drop beads with 1µL of each antibody to make single stained controls. DO NOT forget to make unstained bead controls.
Likewise, can make for cells suspension as well.

22 4. Master Mix for Samples (markers change depending upon the experiment):

Antibody	**Dilution**	**Volumes to be added in 1mL PBS**
CD326 (APC-Cy7)	1:1000	1 µL
CD140a (PE)	1:750	1.5 µL
CD31 (APC)	1:1000	1 µL
CD26/DPP4	1:750	1.5 µl

Add 50ul to control and sample tubes. Do not add any master mix to unstained control tubes or C12FDG only tube.
PS: Do not add the antibodies to the single stained tube for C12FDG

23 5. Vortex gently & incubate @ 4°C (place in frig) for 20 minutes.

24 6. Wash with 1ml 1xPBS & centrifuge @ 500g for 5 min @ 4 degrees

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue

25 7. Repeat 1xPBS wash a second time & centrifuge @ 500g for 5min @ 4 degrees.

26 8. Resuspend in 150ul 1x FACs buffer and run samples on flow-cytometer.

The Capture of the cells must be done at both 10000 and 100,000 counts for gating.

Citation: gagandeep kaur, Irfan_Rahman Mouse Ischemia Experiment https://dx.doi.org/10.17504/protocols.io.ceyitfue