Isolation of adipocyte progenitors from adipose tissues: Explant method

Before collection:

1. Check for sufficient amounts of BSA (A12 fridge), RBC lysis buffer (10X Biolegend Cat 420301 (A3 fridge)) and Liberase TL (Roche 05 401 020 001 (-20 freezer A12 glass chamber), and Pre-Adipocyte media (A12 fridge).
2. Place KRBB in water bath for 15 mins.
3. Place the remaining KRBB in the original container back in a water bath to keep warm for later. Head to collect samples.

Supplies

1. Fresh adipose tissue (Collected in KRBB supplemented with A/A and Gentamicin MP CAS number: 1405-41-0
2. Dissecting instruments including 1 small forceps, 1 large scissors, 1 small scissors.
3. 50mL tubes used for digestion.
4. Pipettors and tips, 10mL to 20mL and 1000µL.
5. Incubator at 37°C. A rise in temperature of more than 0.5°C will inevitably result in a loss of responsiveness to hormones in adipocytes.

KRBB+ A/A+Gentamicin solution (1L)

Compound	Con [ ]	Weight
NaCl	135 mM	7.88g
KCl	5 mM	0.373g
MgSO4	1 mM	0.246g
KH2PO4	0.4 mM	0.070g
Glucose (Sigma G6152)	5.5 mM	0.991g
HEPES	20 mM	4.766g
Antibiotic/Antimicotic (Cellgro 30- 04CL)	10 mL	
Gentamicin	10mg/ml	5mL

Add to 1L of MilliQ H2O. Adjust pH to 7.4

Pre-Adipocyte media (1L)

Ingredient	Concentration	Amount
D-MEM/F-12		1 pouch (15.6 g)
Sodim Bicarbonate (84.007 g/mol)	44.05 mM	3.701 g
Ascorbic acid (176.12 g/mol)	100 µM	17.61 mg (two 8.81 mg aliquots)
Biotin (244.31 g/mol)	33 µM	8.06 mg (two 4.03 mg aliquots)
Pantothenate (219.23 g/mol)	17 µM	3.73 mg (two 1.86 mg aliquots)
FBS	10%	100 mL
Antibiotic/Antimycotic		10 mL
HEPES (1 M)	20 mM	5 mL
Milli-q water		Bring up to 1 L

D-MEM/F12 contains L-glutamine (2.5mM/L), HEPES (15mM/L), Phenol red (0.021mM/L)

MesenPro RS™ Complete Medium

Prepare 50ml of MesenPro medium according to the following:

- Add 50ml of MesenPro RS Basal medium (fridge 4°C)
- Add 1ml aliquot of MesenPro Growth Supplement (-20°C)
- Add 500µL of L-Glutamine

Protocol

1. Transport the samples in 5ml of KRBB+A/A.
2. Wash the samples three times with KRBB+A/A.
3. Mince the tissue in sections (1mm^2 size) and then transfer the samples into a petri dish
4. Holding the small piece of AT with a forceps, touch the petri dish in dry areas with the tissue to dry it out.
5. Place four pieces of PVAT in each well of a 24-well plate.
6. Put the plate in the incubator for <3 mins.
7. Add 250µl of MesenPro complete slowly into the wall of the well. This avoids detachment of the AT from the bottom.
8. Add 250µl of MesenPro complete slowly.
9. Replace MesenPro Complete media the following day.
10. Incubate cells in the incubator at 37C.
11. Replace MesenPro Complete media every two days.
12. Once cells have proliferated (~7 days after), remove media and wash with 500µL of PBS.
13. Remove PBS and add 200µL of Trypsin 0.05%, incubate for 2 mins at 37C.
14. Rock slightly the well-plate to complete the detachment of cells.
15. Recover cells with 500µl of Preadipocyte media in each well.
16. Suspension of cells is transferred to a 15mL tube.
17. Centrifuge at 300g for 5 mins at RT.
18. Resuspend in 10ml of MesenPro Complete and plated in a T25 for further passage.

Transferring Cells to Larger Flasks

1. Check flasks under microscope; if 80% confluent, place pre-adipocyte media, trypsin (according to figure below), and 1x PBS in water bath (aliquot pre-adipocyte media and 1x PBS into 50mL tubes and label if not already done).
2. Gather autopipette, 10mL stripettes, 25mL stripettes, waste bin, Pasteur pipettes, tube racks, new flasks (one size larger than current), and permanent marker. Ethanol and place under sterilized hood.
3. Label new flasks with sample ID, pass number, and date.
4. Gather confluent flasks, 1x PBS, trypsin, and pre-adipocyte media. Ethanol and place under hood.
5. Using Pasteur pipettes, remove media from current cell flasks using aspirator. Add 1x PBS, aspirate completely, and follow with trypsin:
    a. T25: 5mL of PBS and 2 mL of Trypsin
    b. T75: 10mL and 3 mL of Trypsin
    c. T175: 20mL of PBS and 4 mL of Trypsin
6. Place Trypsinized flasks in the incubator for 2 minutes.

7. After two minutes of incubation, place flasks on a bench near the microscope. Tap each flask on the bench edge to mechanically detach cells from the flask wall. Check under microscope that cells are detached; cells should float when the flask is moved (it should look like confetti).
8. Turn off the microscope and return the flasks to the hood. Add the necessary volume of pre-adipocyte media to current and new flasks as follows:
    a. T25: Total volume 10mL
    b. T75: Total volume 15mL
    c. T175: Total volume 25mL
9. Add media to new flasks using stripette and pre-adipocyte media aliquots prior to adding cells from the current flask. (This prevents cell clumping and encourages better distribution across the flask cell growth surface.)
10. Using a 10mL stripette, gather and rinse cells from the current flasks and carefully add them to the new flask. To avoid contamination and bubbles, gently add to the neck of the new flask. Screw on the flask cap.
11. Upon completion of all necessary transfers, return new flasks to incubators and dispose of old flasks in the biohazard bin. Return leftover media, 1x PBS, and trypsin to the glass refrigerator in A12. Dispose of glass Pasteur pipettes in the glass biohazard bin. Remove long stripes from the waste bin and pour the remaining liquid down the sink drain. Dispose of the remaining materials in the biohazard bin.

Adipogenesis and Lipogenesis induction

Rat Maintenance Media

Prepare ***Pre-Adipocyte Media*** **as previously** and add the following components before filtration of media:

Product	Concentration	Volume	Location
Insulin	5 µg/mL	250µL	A12 4C
T3	50mL	5µL of 20µM SS	A12 -20C
Rosiglitazone	0.5µM	50µL of 5mM	A12 -20C
Transferrin	10µg/ml	1 aliquot	A12 -20C

Rat Induction Media

Take **100ml of Rat Maintenance Media** and add the following components before filtration of media:

Product	Concentration	Volume	Location
Dexametasone	0.5µM	5µL of 10mM SS	A12 -20C
IBMX	0.5mM	100µL of 10mM SS	A12 -20C
Glucose	To reach 25mM	135.3mg	A12 RT storage cabinet

1. Count your cells in suspension and plate cells at 40.000 cells/cm2.
2. Allow the cells to reach confluency (Proliferation) and after 2 days of plating replace to Rat Induction Media (Day 0 post-induction)
3. After 2 days of induction, replace 100% of media with Rat Maintenance Media (Day 2 post-induction)

4. Replace 60% of maintenance media every two days and evaluate adipogenesis (Day 4 post-induction).
5. To evaluate lipogenesis change to Rat maintenance media every two days and evaluate (Day 7 post-induction).

Adipogenesis and Lipogenesis with Incucyte

Bodipy

1. Preparing Bodipy in sterile DMSO: 10mg of Bodipy 493/503 in 763µL of DMSO (**50mM Stock Sol**)
2. Add 2µL of 50mM Bodipy 493/503 to 10mL of media or 5uL in 25ml (**10µM working solution**) – use media to make treatments

Nuclei staining

1. Add 5 µL of Verapamil to 10ml of culture medium (10µM Verapamil solution)
2. Dilute NucSpot Live 650 1000X in DMSO to a final concentration of 1X in cell culture medium (1.5µL of NucSpot per 1mL of medium)

Notes:

- Avoid creating bubbles in the wells when changing the media.
- Add the nuclei staining near the collection day (d 4 or 7).
- Allow the NucSpot (Nuclei) staining to incubate for 20 mins and then schedule the measurement.
- Remove media on the last day and take the pictures using IncuCyte live imaging software (reduces background).