# Preparation of non-neural embryonic fibroblasts.

1. Set up timed pregnancies, embryos should be harvested between day 13.5-14.5d of gestation.
2. Euthanize pregnant female by cervical dislocation
3. Remove E13.5-E14.5 embryos from uterine horns and place in a 10cm tissue culture dish filled with HBSS.
4. Wash embryos twice with 20 mL HBSS and place in a fresh 10 cm tissue culture dish
5. Under a dissection microscope, remove arms and legs of embryo with surgical scissors and pincers. Be careful to not include any tissue above he shoulder and hip joints. Place limbs in a few drops of HBSS in a 15cm tissue culture dish.
6. Place limbs of 3-4 mice on a 15cm tissue culture dish and mince thoroughly until homogenously using curved scissors for at least 2-3 min. Thorough disruption of the tissue is essential for generating a single cell suspension.
7. Add 1ml of trypsin solution and incubate at 15min at 37°C.
8. Briefly triturate dissociated tissue using a 10 mL pipette filled with 9 ml MEF media for 5-10 times.
9. Place cells in a 37°C incubator.
10. When cells become confluent (typically 2-4 days later), remove MEF media, rinse once with HBSS, and add 3 mL of 0.25% trypsin. Incubate for 3 minutes in 37°C incubator. Quench trypsin by adding 3 mL MEF media. Transfer detached cells into a 15 mL falcon tube.
11. Spin down solution at 1000 rpm for 3 minutes. Remove supernatant and resuspend cell pellet in MEF media.
12. Add cells to 15cm tissue culture dishes containing 20 mL of MEF media. One 15cm plate can be replated onto 3-4 15 cm plates (i.e. a 1:3 or 1:4 split).
13. When cells become confluent, remove MEF media, rinse once with HBSS, and add 3 mL of 0.25% trypsin. Incubate for 3 minutes in 37 degree incubator. Quench trypsin by adding 3 mL MEF media.
14. Spin down solution at 1000 rpm for 3 minutes. Remove supernatant and resuspend cell pellet in 2 mL MEF media per plate of cells. Add an equal volume of 2X freezing media and mix well.
15. Quickly aliquot cell suspension into cryotubes. Immediately place cryotubes in an insulated container and store at -80 degrees overnight.
16. Transfer cells to liquid nitrogen for long term storage.

**MEF Tissue culture media:**

DMEM (12430) 435ml

Sodium Pyruvate (100x) 5ml

Pen/Strep (100x) 5ml

FBS 50ml

Non essential amino acids (100x) 5ml

**2x Freezing media (Volume = 50ml):**

DMSO 10ml

DMEM 10ml

FBS 30ml