F38. Organ culture protocol for mouse bone and skeletal development (Liang Chen, 10/15/2009; commented by TCH)

1. Organ culture (e.g., metatarsal bones) were prepared by dissecting out bone and skeletal tissues from **newborn mice** (Days 0-3 after birth) or mouse embryos (**E18.5-E21** Days after the pregnancy of the mother) under sterile conditions.

2. The isolated fetal or neonatal bones (usually contained with soft tissue) are incubated in DMEM containing 0.5% Bovine Serum Albumin, 50ug/ml Ascorbic Acid, 1mM b-glycerol phosphate and 100ug/ml Penicillin-Streptomycin solution at 37^{o}C in humidified air with 5% CO_2 for up to 14-30 days.

- Medium Containing:

  0.5% Bovine Serum Albumin (BSA, Sigma A4161)
  50ug/ml Ascorbic Acid
  1mM -Glycerophosphate
  100ug/ml Penicillin-Streptomycin solution (Mediatech)
- Culture Condition: 37^{o}C in humidified air with 5% CO_2
- Cultured tissue should be observed in Days 1, 4, 7 and 10 under Microscope.

4. For fluorescent labeling of new bone formation, Calcein (Sigma-Aldrich; at 0.5-1.0% in DMEM) can be added at 24h prior to tissue processing or added to the medium for the whole culture duration.

5. Metatarsals were fixed in 4% paraformaldehyde or 10% formalin for 4-6 hours at RT and stored in 70% ethanol.

6. Bones were embedded in paraffin blocks and sectioned.

7. Stained with Hematoxylin & Eosin, Trichrome or with Alizarin Red was performed after Hydrating slides (see below).

Alizarin Red S Solution:

Alizarin Red S	2 gm
Distilled Water	100 ml

Mix the solution and adjust the pH to 4.1-4.3 using 0.5% ammonium hydroxide. The pH is critical - make fresh.

Acetone – Xylene:

Acetone	25 ml
Xylene	25 ml

Make fresh.

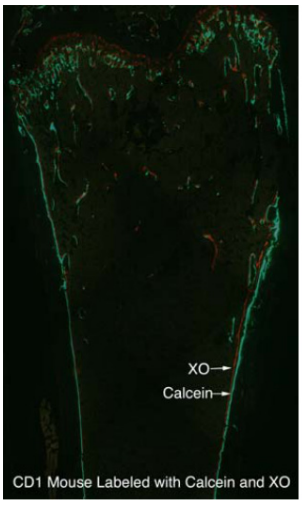
Procedure:.

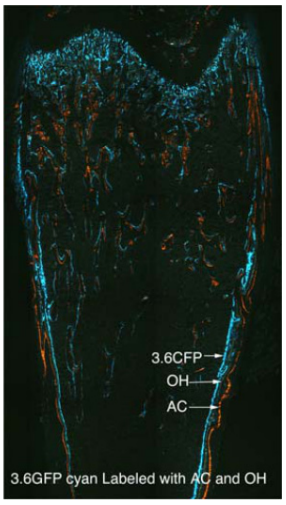
1. Bones were Fixed in 4% paraformaldehyde or 10% formalin for 4-6 hours at 4^{o}C and stored in 70% ethanol.

2. Bones were embedded in paraffin blocks and sectioned.
3. Hydrating slides. Rinse slides rapidly with distilled water.
4. Alizarin red S solution, 30 sec to 3 min, checking microscopically for the orange-red color.
5. Shake off excess dye.
6. Acetone:- 20 dips.
7. Acetone-xylene:- 20 dips.
8. Clear in xylene, mount in permount.

Fluorescent Label in Bone (in vivo):

Some fluorescent compounds, which are fixed in newly formed calcified tissue, are used to label bone deposition. Such labels help to determine the time sequence of bone growth. The label may be given single or double label by intraperitoneal injection.





Agents	Sigma Cat #	Color	Dosage (mg/kg)	Stock Con. (mg/ml)	Diluents
Calcein	C-0875	Green	10	3	2%NaHCO3 PH7.4
Xylenaol Orange(XO)	X-0127	Red	90	30	2%NaHCO3 PH7.4
Alizarin Complexone(AC)	A-3882	Red	30	10	2%NaHCO3 PH7.4
Oxytetracycline Hydrochloride(OH)	O-5875	Yellow	30	10	20% EtOH

Age	First Injection Before Sacrificing (days)	Second Injection Before Sacrificing (days)
<3 months	10	2
>3 months, <6 months	15	5
> 6 months	20	8