HubLE | Methods

Isolation and culture of primary rodent osteoblasts

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The *in vitro* culture of calvarial osteoblasts from neonatal rodents represents a widely used technique for studying osteoblast function **(Fig. 1)**[1,2]. This HubLE Method describes the protocol for isolation and culture of these cells.

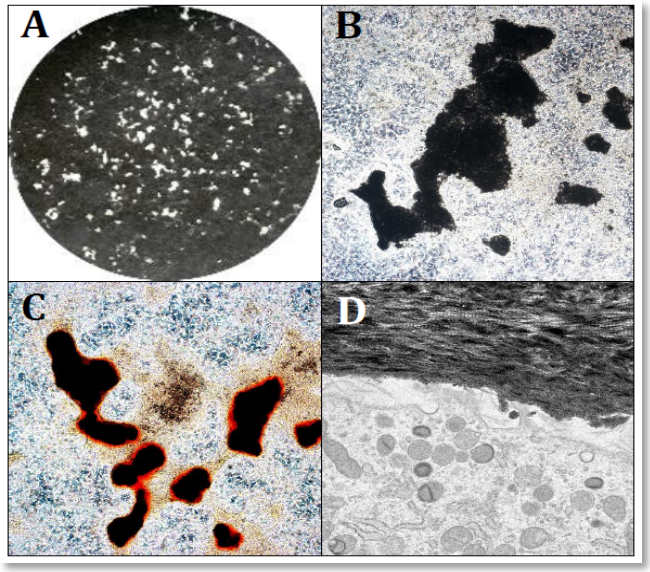

Fig. 1. (A) Whole well scan and phase contrast microscopy images of **(B)** unstained and **(C)** alizarin red stained mouse osteoblasts. **(D)** Transmission electron microscopy image of mineralized matrix formation in mouse osteoblasts.

Materials

- Animals *[**Tip No. 1**]*
- Phosphate buffered saline (PBS)
- Alpha modified essential medium (αMEM) *[**Tip No. 2**]*
- Osteogenesis αMEM (osMEM) *[**Tip No. 3**]*
- Trypsin-EDTA (0.25%)
- Type II collagenase from Clostridium histolyticum (0.2%)
- Glutaraldehyde (2.5%)
- Alizarin Red (1%)

Method

Update

All procedures should be performed under sterile conditions.

1. Carefully isolate the calvaria, taking care to remove all excess tissue and cartilage.
2. Cut in half and place in a flat bottomed tube, rinse with phosphate buffered saline (PBS).
3. Incubate in 500µl of 0.25% trypsin for 10 minutes at 37°C.
4. Wash with αMEM, discard solution
5. Incubate in 500µl of 0.2% type II collagenase for 30 minutes at 37°C. Steps 3-5 remove non-osteoblastic cells from the sample.
6. Remove collagenase, discard and replace with fresh collagenase solution for a further 60 minutes.
7. Keep the final digest and spin at 1,500g for 5 minutes.
8. Resuspend in 1ml αMEM per calvaria and transfer cell suspension to 75cm² flask containing αMEM. Rat cells = 1 calvaria/flask, Mouse cells = 3 calvaria/flask.
9. Incubate at 37°C/5% CO_2 until confluent (~ 4 days).
10. Once confluent, remove medium, wash with PBS and incubate with 0.25% Trypsin for 5-10 minutes to detach cells.
11. Spin at 1,500g for 5 minutes and resuspend in αMEM (1ml per flask).
12. Perform a cell count and seed cells in tissue culture trays in osMEM *[**Tip No. 4 & No. 5**]*.
13. Culture for up to 21 days with half medium changes every 2-3 days.
14. Fix with 2.5% glutaraldehyde for 5 minutes before staining with 1% alizarin red to visualise mineralized nodules *[**Tip No.6**]*.

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Tips

Update

1. **Animals**: cells can be obtained from neonatal rats (2-3 days) or mice (3-6 days). After expansion, the following cell yields per calvaria can be expected: rat (~$7x10^6$ cells) and mouse (~$4x10^6$).
2. **αMEM**: Add 10% foetal calf serum (FCS) and AB/AM (100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin). Rat cells can also be cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FCS, AB/AM and 2mM L-glutamine.
3. **Osteogenesis αMEM:** to add αMEM add 50μg/ml ascorbate, 2mM β-glycerophosphate and, for rat cells only, 10nM dexamethasone. Always make fresh on the day of use.
4. **Tissue culture plates:** Rat osteoblasts can be cultured successfully in a number of well plate formats, whilst mouse osteoblasts will only form abundant mineralized nodules in 12 or 6 well plates. Culture in 24 well trays will result in significant peeling prior to mineralization. Typical seeding densities per well: $2.5x10^4$ (24-well), $5x10^4$ (12-well), 10^5 (6-well). Plate all cells at this stage: do not passage primary osteoblasts as it will result in a significant loss of phenotype leading to longer cultures and delayed, if any, mineralization.
5. **β-glycerophosphate:** This method uses a low concentration of β-glycerophosphate (2mM) since culture with 5-10mM results in reduced cell viability, alkaline phosphatase expression and causes widespread, non-specific mineral deposition that differs from true bone formation.
6. **Alizarin Red staining:** Mineralised bone nodules can be stained with alizarin red; however, it is also possible to perform image analysis and obtain good quality images on unstained cell layers (**Fig 1A-1B**).

References

Update

1. Perpetuo IP, Bourne LE, Orriss IR (2019). Isolation and generation of osteoblasts. Methods Mol Biol, 1914:21-38.

2. Orriss IR, Hajjawi MOR, Heusa C, MacRae V, Arnett TR (2014). Optimisation of the differing conditions required for bone formation in vitro by primary osteoblasts from mice and rats. In J Mol Med, 34:1201-1208.

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