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Whole bone Immunostaining using Murray's clear method

References:

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Half bone whole-mount tissue preparation for imaging.

1. Fix freshly dissected femur from 8-12 week old mice in ice cold 4% PFA in PBS (Affymetrix) for 7-8 h at 4°C while shaking in an amber colored epi tube.
2. Wash femur with 1x PBS to remove PFA and cryoprotect in 30% sucrose PBS solution overnight at 4°C with shaking.
3. Submit the bones in 30% sucrose to histology for embedding in OCT and cryo processing. A Leica cryostat is used to longitudinally bisect the bones.
4. Request to trim bone longitudinally till marrow is seen and the bone is almost half.
5. Wash Intact half bone in 1x PBS to remove OCT then process for staining, clearing and imaging.

Whole-mount immunostaining.

1. All staining procedures are performed in Eppendorf tubes on a rotator at room temperature. The staining solution contain 10% dimethyl sulfoxide, 0.5%IgePal630 (Sigma), and 5% donkey serum (Jackson Immuno) in 1x PBS.
2. Block Half bone in staining solution containing and 1% BlokhenII (Aves Labs) overnight at room temperature.
3. After blocking, immuno-stain the whole mount for three days at room temperature with primary antibodies in staining solution.
4. Wash the bone multiple times (at least 4-5 times) at 30 min interval in 1 x PBS at room temperature.
5. Now place the bone in staining solution containing secondary antibodies and 1 x DAPI for three days.
6. Wash the bone multiple times (at least 4-5 times) at 30 min interval in 1 x PBS at room temperature.

The fixation time of the tissue, using 0.5% IgePal630 and 10% DMSO in the staining solution, and incubation of the tissue for 3 days in both primary

and secondary antibodies are critical factors for efficient deep penetration of antibodies for the whole mount tissue.

Tissue clearing

1. Perform clearing of half bones in Eppendorf tubes on a rotator at room temperature.
2. Subject immuno- stained tissues, washed in 1 x PBS, to a series of graded ethanol concentration to dehydrate as shown in table below.

Solution	Incubation time
50% Ethanol	10 min
70% Ethanol	10 min
80% Ethanol	10 min
95% Ethanol	10 min
100% Ethanol	O/N

3. Exchange alcohol with ***freshly prepared BABB*** (1:2 Benzyl Alcohol: Benzyl Benzoate) and changed fresh solutions every 20-30 minutes till the bone is clear.

4. Place the whole mount bone on May-tek dish containing BABB with marrow side facing downwards.

5. Image the bone in LSM 780 DS confocal microscope.