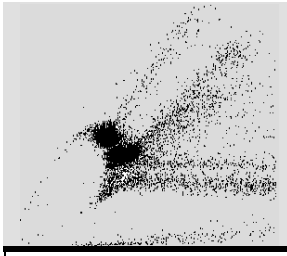

THE GOODELL LABORATORY

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Title	**Bone marrow isolation, crushing technique**	
Introduction	This protocol describes the isolation of whole bone marrow from whole mice using a bone crushing technique. This protocol may be particularly useful to isolate large numbers of bone marrow cells, or to isolate bone marrow from mice for which a bone flushing technique is difficult [i.e. young or small mice].	
Materials	1. Dissecting tools, including scissors, various sized tweezers. 2. Mortar and pestle, sterilized. 3. HBSS+: Hanks Balanced Salt Solution (from Gibco) + 2% Heat-inactivated Fetal Bovine Serum + 10mM Hepes + Pen/Strep. 4. PBS and PBS+: PBS + 2% Heat-inactivated Fetal Bovine Serum. 5. Collagenase/Dispase [Roche cat# 11097113001]. 6. 50mL conical centrifuge tubes. 7. 40 micron sterile cell strainers. 8. Table top centrifuge capable of spinning falcon tubes at ~500xg. 9. Tissue culture dishes.	
Protocol		*Notes*
1.	Prepare bone marrow from mice starting by dissecting tibias, femurs and iliac crests, cleaned of all muscle and connective tissue. Place intact bones into a tissue culture dish on ice with PBS+. Continue by dissecting the arms and scapulas, again cleaning away all muscle and connective tissue. Dissect sternum and spine away from ribs, ensuring spine is also devoid of spinal cord.	*We do not typically dissect ribs, however any bones maybe used in this protocol, so long as they are dissected free from all soft tissue.*
2.	Transfer cleaned bones to sterile mortar, containing 1mL PBS+ per 3 bones.	*After trisecting the spine, we find most mice give rise to ~18 bones, so we generally use 6mL PBS+ per mouse.*
3.	Using the pestle, crush bones into bone fragments, releasing marrow from within.	*Bones should not be crushed into a fine powder. We generally crush until no major "crunching" is heard from large bone fragments.*

4.	Collect supernatant from mortar and filter into a 50mL conical tube using a 40micron cell strainer.	
5.	Rinse the remaining bone fragments with an equal volume of PBS+ and filter into the same 50mL conical tube using the same 40micron strainer. This tube may be topped off with PBS+ and placed on ice.	
6.	Make up Collagenase/Dispase enzyme solution as follows: Add 10mL sterile ddH2O to one vial of 500mg Collagenase/Dispase [concentration is 50mg/mL]. Swirl to dissolve. Next dilute an aliquot 10 fold with PBS to make a 5mg/mL solution to use for separating bone marrow cells from crushed bone fragments. Save the 50mg/mL stock at 4deg.	
7.	Transfer bone fragments from mortar to a 50mL conical centrifuge tube. Add 1mL of the enzyme solution per 3 bones.	*Again, this usually equates to about 6mL enzyme solution per mouse dissected.*
8.	Shake the tube containing the bone fragments and enzyme solution using a shaker set at 250rpm for 15 minutes at 37degC.	*We have excellent results when incubating for 15min, though some sources suggest a shorter incubation to preserve cell surface markers.*
9.	After the incubation, add 15mL PBS to the tube and shake vigorously for 15 seconds. Filter the supernatant using a new 40micron cell strainer into a new 50mL conical tube.	
10.	Add an additional 15mL PBS to the bone fragments, shake vigorously again for 15 seconds, and filter into same 50mL conical tube with same 40micron cell strainer. Top the tube with filtered bone marrow with PBS+ and place on ice.	*After the entire isolation procedure, bones will appear bright white.*

11.	Centrifuge 50mL conical tubes containing bone marrow using table top centrifuge at 400xg for 8 minutes at 4degC. Decant supernatant.	
12.	Resuspend cells in desired media, such as Hank's+, at an appropriate volume. Once cells are resuspended, perform a viable cell count using both trypan blue and red blood cell lysis buffer.	*Cells may now be used for specific utility or staining.*
13.	If cells are to be used for SP staining [see Hoescht staining protocol], we recommend using manual MACS columns rather than the AutoMACS, to prevent clogging of tubing due to any contaminating "bone dust."	

References.

1. Millipore Bone Marrow Harvesting and Hematopoietic Stem Cell Isolation Kit protocol.