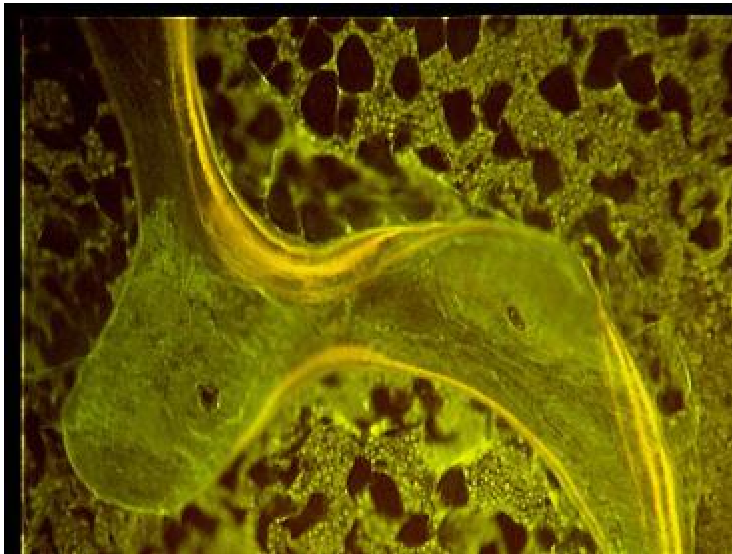
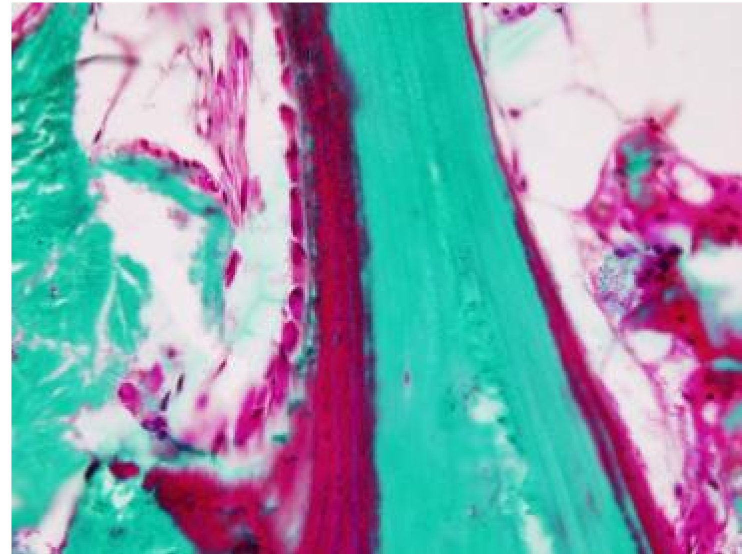
# What's new in Bone Histology and Histomorphometry





Deborah Novack, MD/PhD
Director, Histology and Morphometry Core
Musculoskeletal Research Center
Division of Bone and Mineral Diseases
Departments of Medicine and Pathology

Washington University in St. Louis
School of Medicine

Musculoskeletal Research Center at Washington University

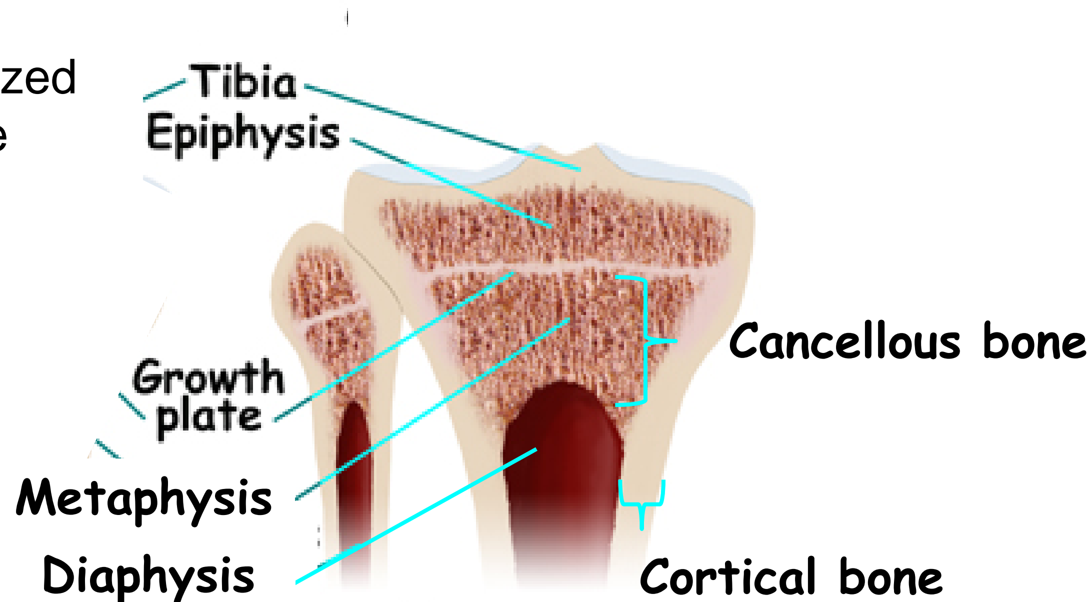
# Role of Bone Histomorphometry

- histological assessment of bone phenotypes
- directly visualize bone cells in relation to tissue, at baseline or in disease models
- “quantitative” nature allows comparisons between groups even when differences are not visually “obvious”
- most useful when interpreted in the context of other data such as structural analysis (microCT, DEXA), serum markers of bone turnover, and/or disease scoring

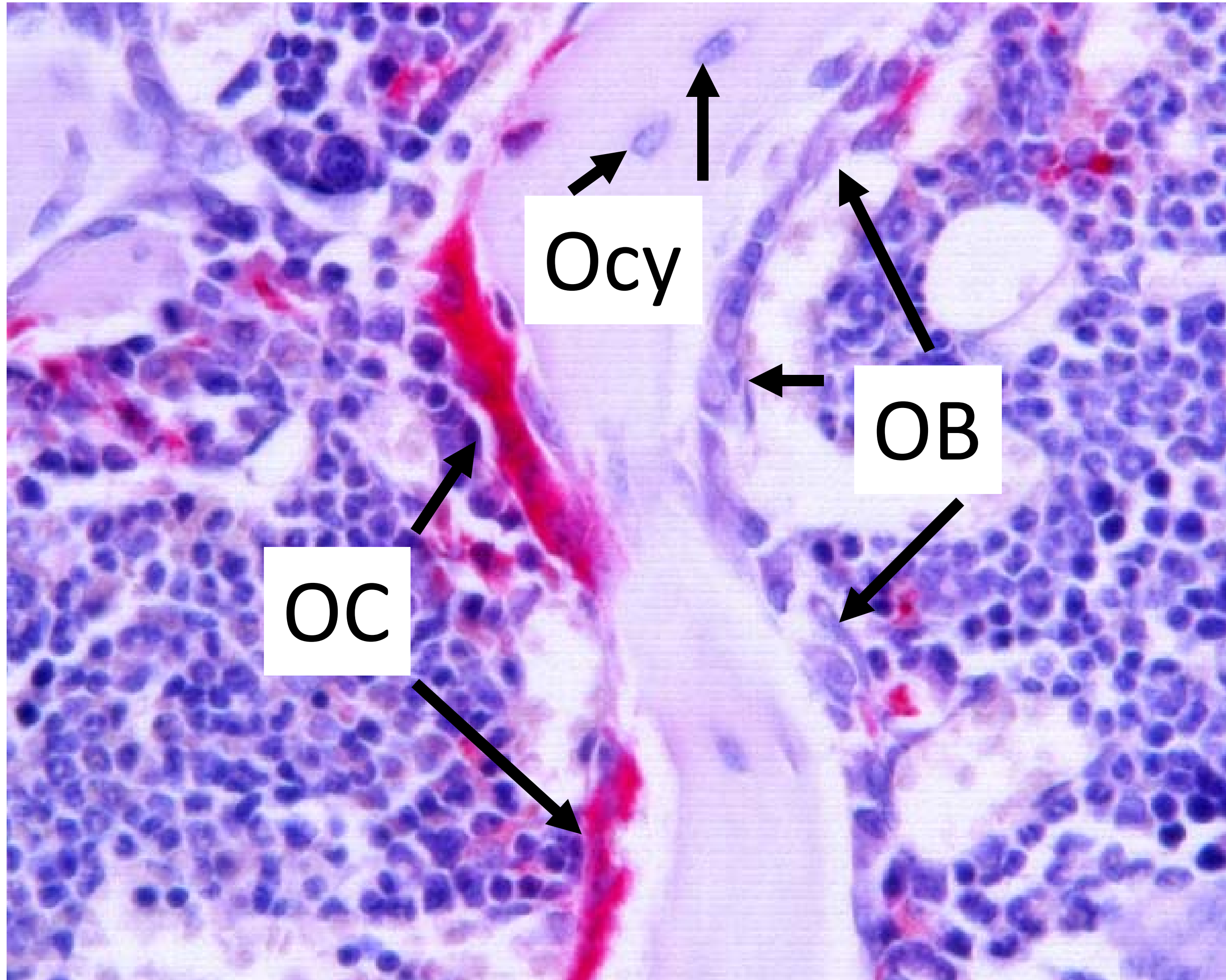
# Terminology

**Bone**: includes mineralized and unmineralized bone matrix, but not marrow

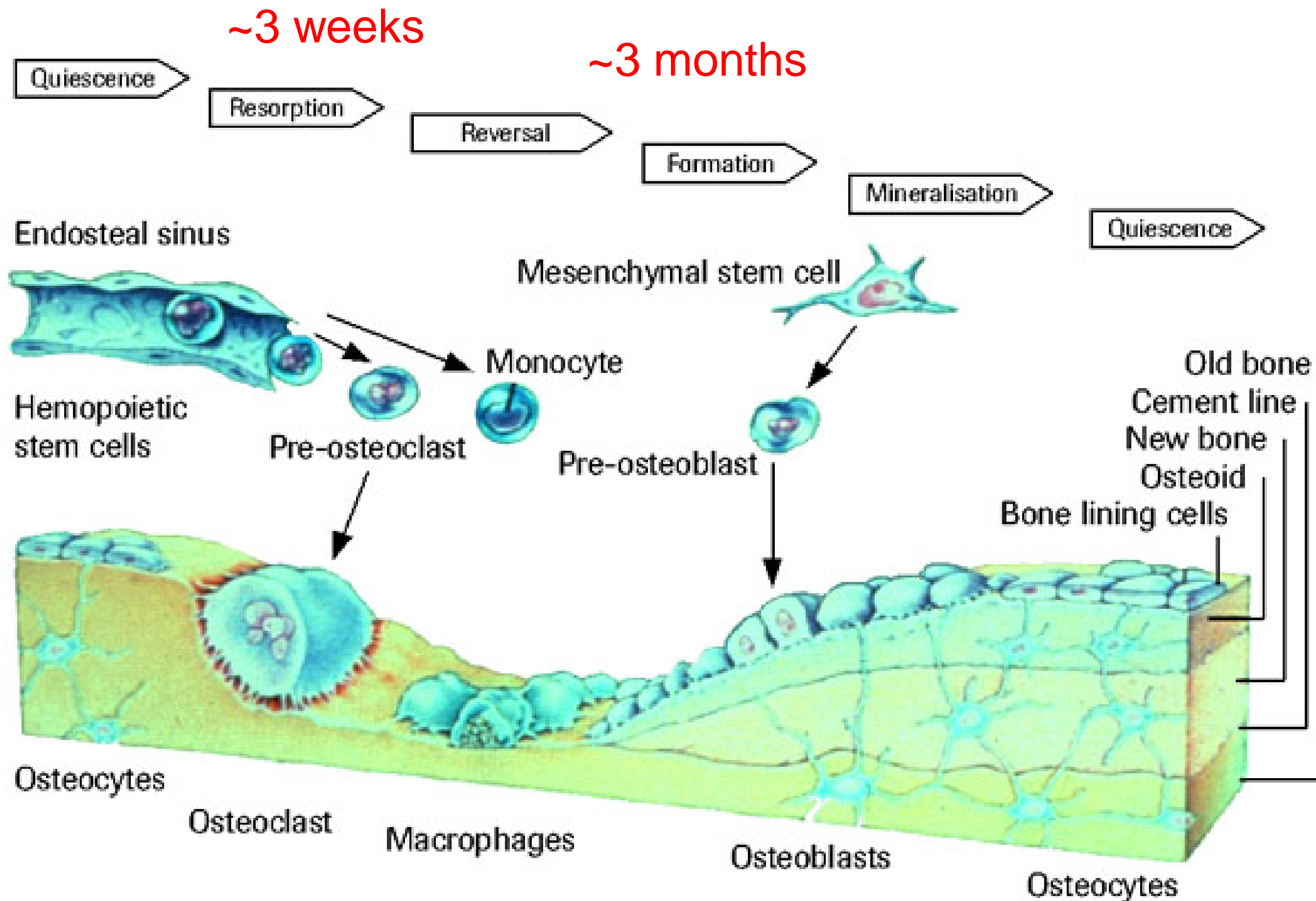
**Bone tissue**: includes marrow and other associated soft tissue




**Trabecula**/trabecular refers to an individual structural element of cancellous bone (ie a rod or plate), but should not be used to refer to the type of bone (although we/I often do this)


Ocy
OB
OC

# Bone Remodeling Cycle



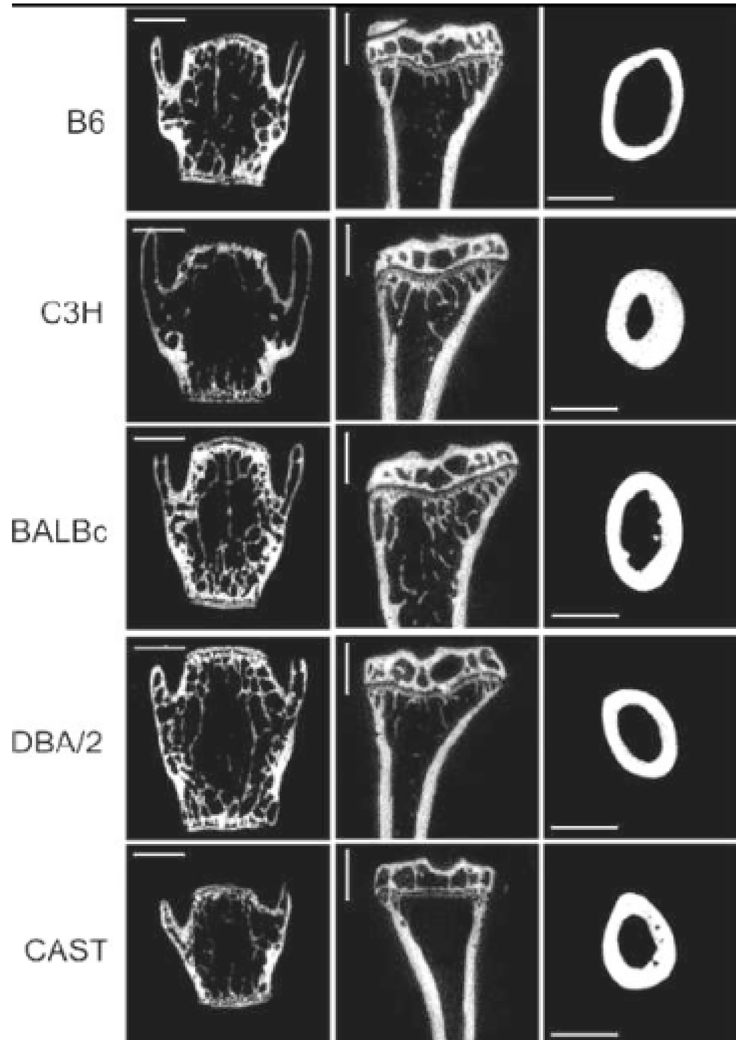

For humans

http://www.roche.com/pages/facets/11/ostedefe.htm

# Decide what analysis you want to do BEFORE you start your experiment!!

- There are many options for histology, and some affect how you handle the tissue when you harvest – plan ahead!
  - ✓ Fixation
  - ✓ Embedding medium
  - ✓ Orientation/plane of section
  - ✓ Type of stain

# Choice of controls




Mouse strains vary a lot
C57/Bl6 may not be the "best" since bone mass is relatively low, but it is what we often have to use

-littermates are best
-age/sex matched
-same strain from different suppliers are not the same in bone mass, so beware of buying controls

# A quick editorial

- Although histomorphometry gives you numbers with standard deviation, p values, etc, the parameters that you measure are often not black and white, and thus are somewhat subjective
- This lack of objectivity can be especially problematic if the one measuring is biased, either consciously or subconsciously
- Therefore, you should always be blinded
  - have a labmate relabel your slides and keep the key

    or
  - have a labmate take photos for you and code them
- The quality of your sections DEPENDS on your treatment of the tissue prior to processing for histology
  - Think about this as you plan your experiment, not on the day of harvest

# Choice of fixation

- 10% neutral buffered formalin is the standard
  - Necessary for paraffin embedding
    - 48 hours max for TRAP stains (to see OCs), then wash in PBS and put through 30% and 50% ethanol prior to 70% for storage at 4$^{o}$C
  - PFA 4% gives similar results
  - Optional for plastic/MMA embedding
    - 24 hours max if you have calcein/alizarin labels
  - Usually not used for frozen sections
- 70% ethanol
  - Samples for plastic/MMA can go directly into this
- Always use at least 10x volume of your bone!
- Excess muscle inhibits fixation

# Choice of embedding method

- Plastic (methylmethacrylate) vs. paraffin
  - plastic is used for mineralized bone, paraffin requires demineralization (eg. with EDTA)
  - bone formation (calcein labels) can only be analyzed in plastic sections
  - stains that differentiate mineralized bone from osteoid (vonKossa, trichrome) can only be done on plastics
  - BUT, plastic takes longer and is more expensive

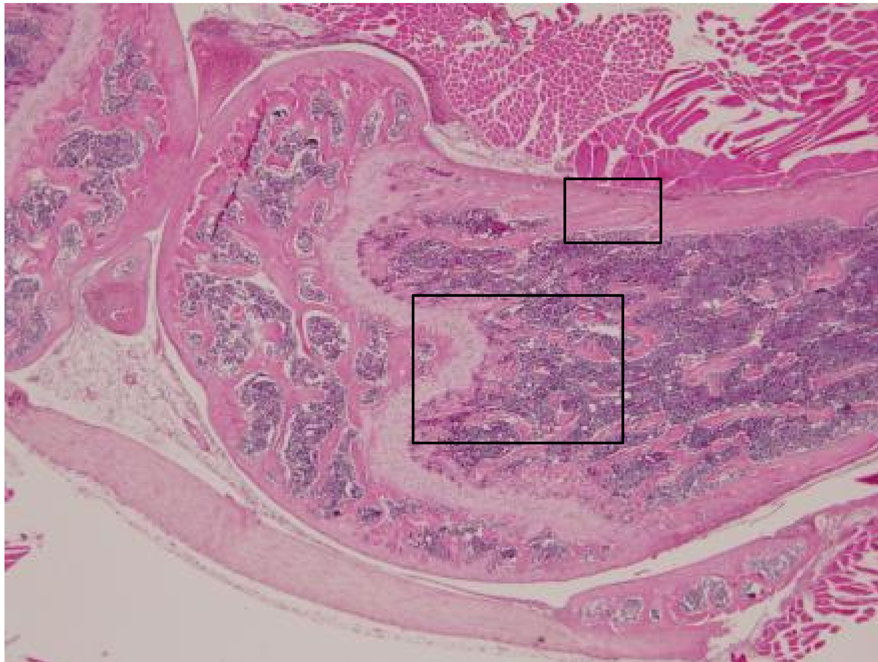
Frozen section options (see later) are not used for standard histomorphometry

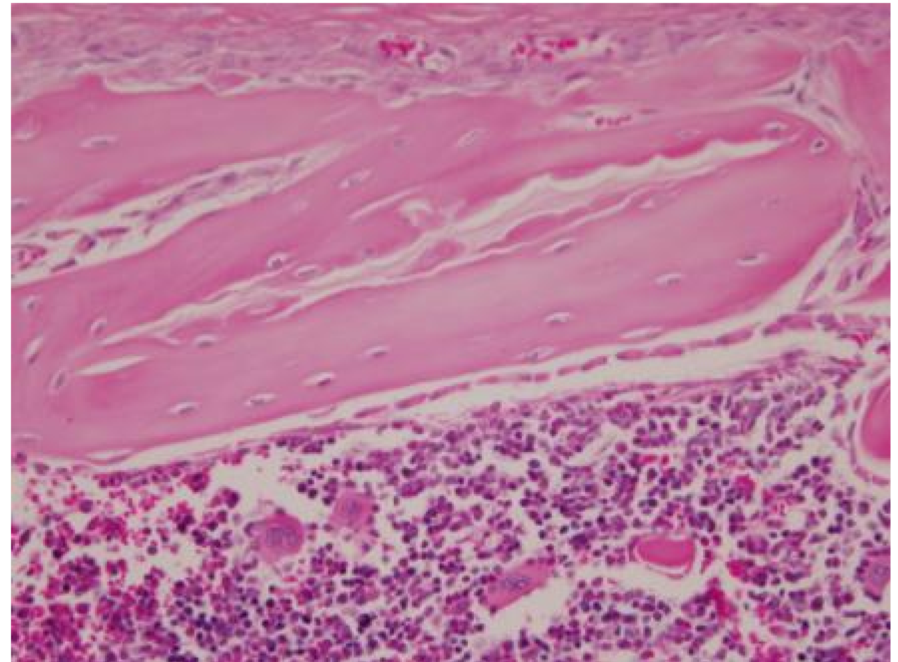
# Tissue orientation

- Since histology gives you a 2-D image, you need to think about exactly what you are trying to capture
- If you need a very particular plane of section, it is useful to provide a low power image of some sort (can even be a uCT slice) to help guide the sectioning

# Choice of stain – H&E

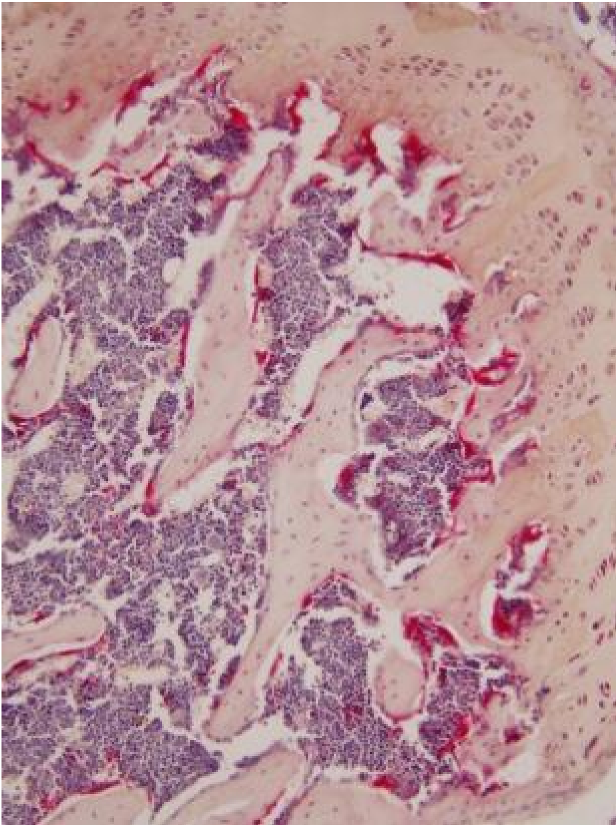
- The “bread and butter” of standard histology
- good for seeing bone vs cartilage (growth plate) and seeing OBs, but not OCs, also good to see tumor or inflammation

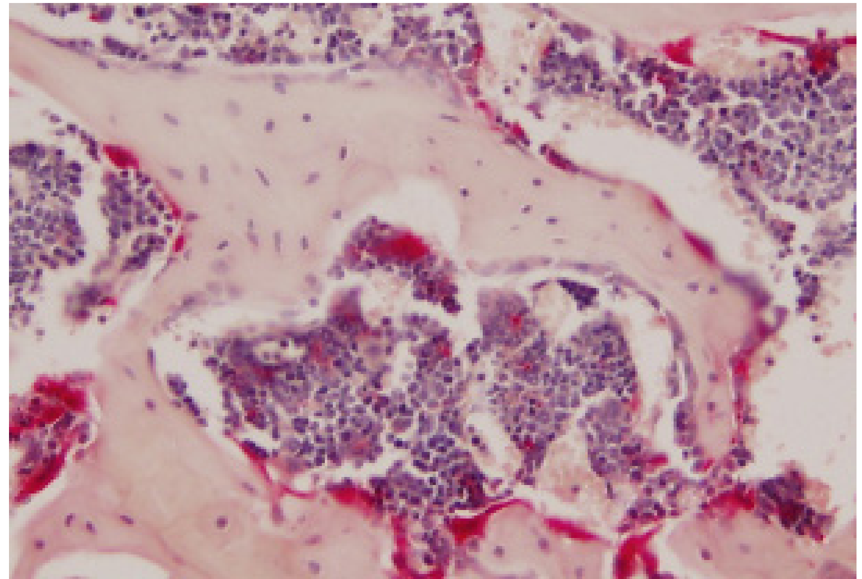




# Choice of stain – TRAP

- highlights OCs, still OK for bone vs cartilage, OBs usually identifiable, often used for histomorph
- Works on paraffin or plastic

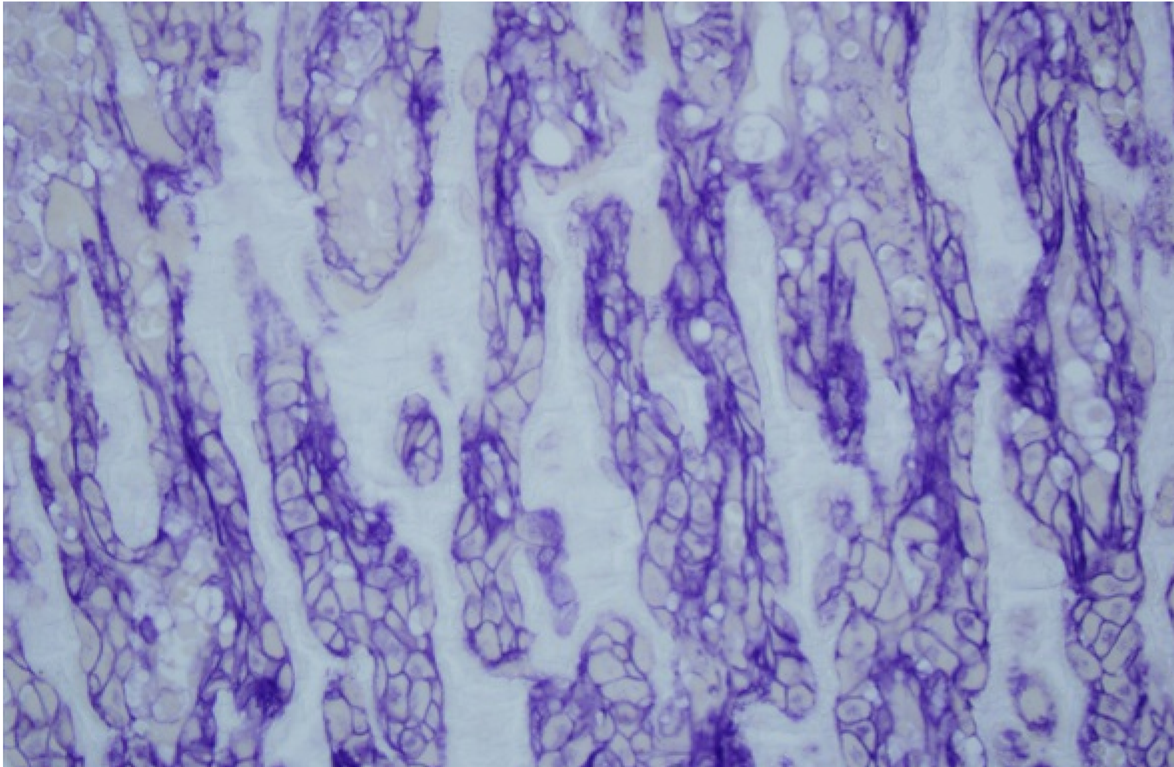




OCs are often defined as TRAP+ cells on bone surface with > 1 nucleus, but sometimes they have 1 or none in a section. ASBMR says to state criteria you use.

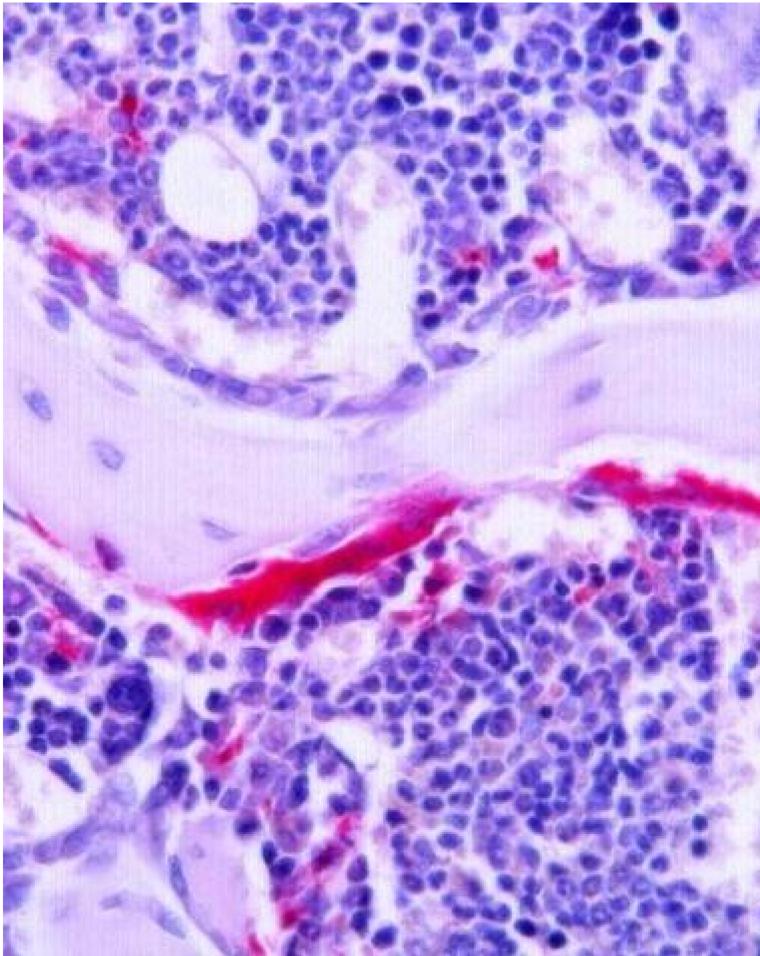
# Choice of stain – Alkaline phosphatase

- Highlights OBs, can't see OCs, decreased by formalin fixation



Not currently offered routinely in our core; being developed for MMA and frozen sections

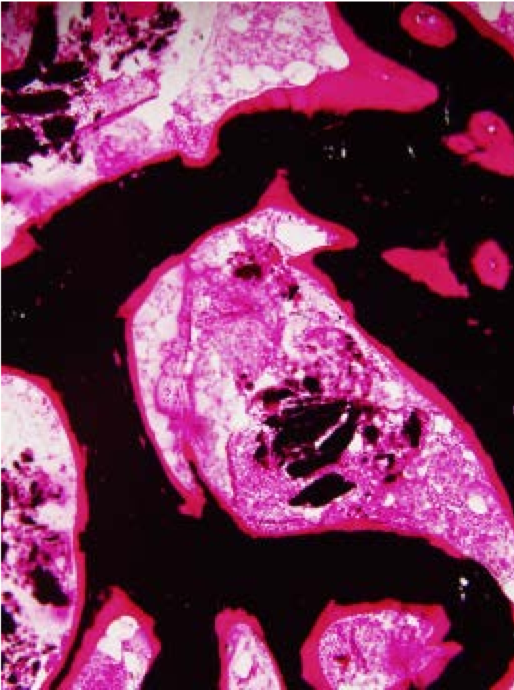
# A word on counting osteoblasts



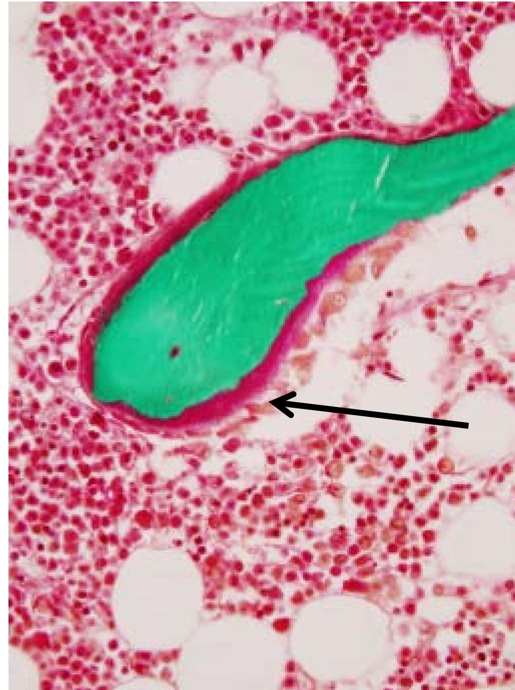
- ASBMR taskforce says that term Ob should be restricted to active cells, and not flat lining cells (which are also of Ob lineage)
- With our without an AP stain, Obs should be cuboidal in shape
- Counting Obs often feels very subjective – try to be consistent
- Back up counts with calcein labels and/or serum markers

# Identifying osteoid

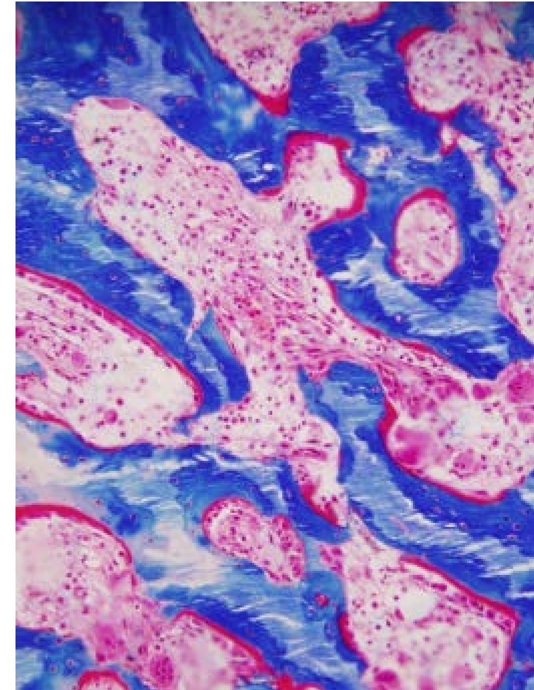
- vonKossa – shows osteoid vs bone, not good for cellular detail
- trichrome (Masson or Goldner) – shows osteoid, and better for cellular detail



vonKossa



Goldner trichrome

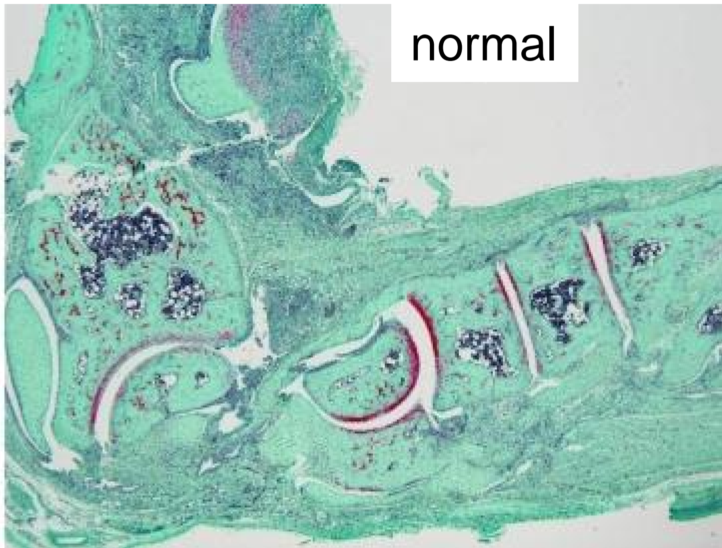


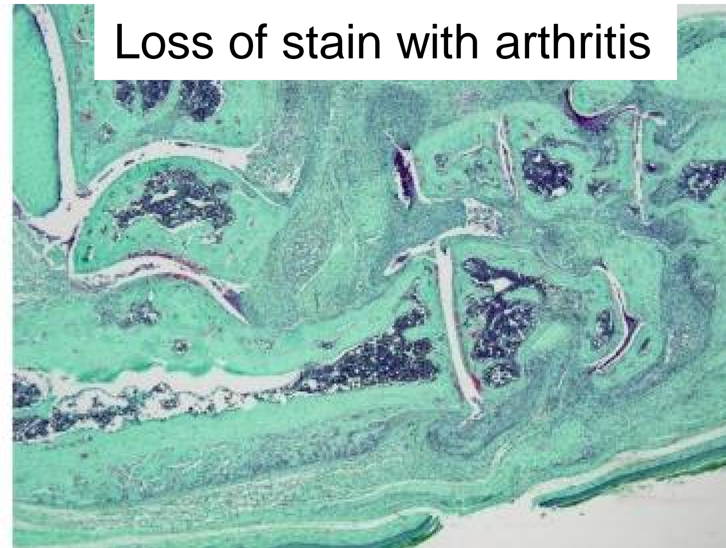
Masson trichrome

# Identifying cartilage

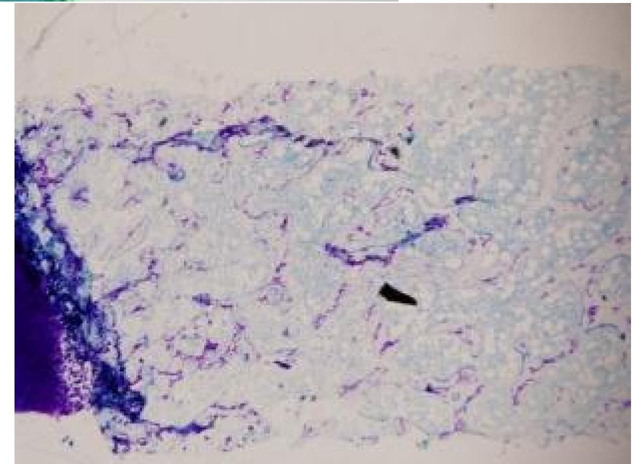
- safranin O – red with methyl green counterstain
- toluidine blue – deep blue/purple

safranin O




toluidine blue



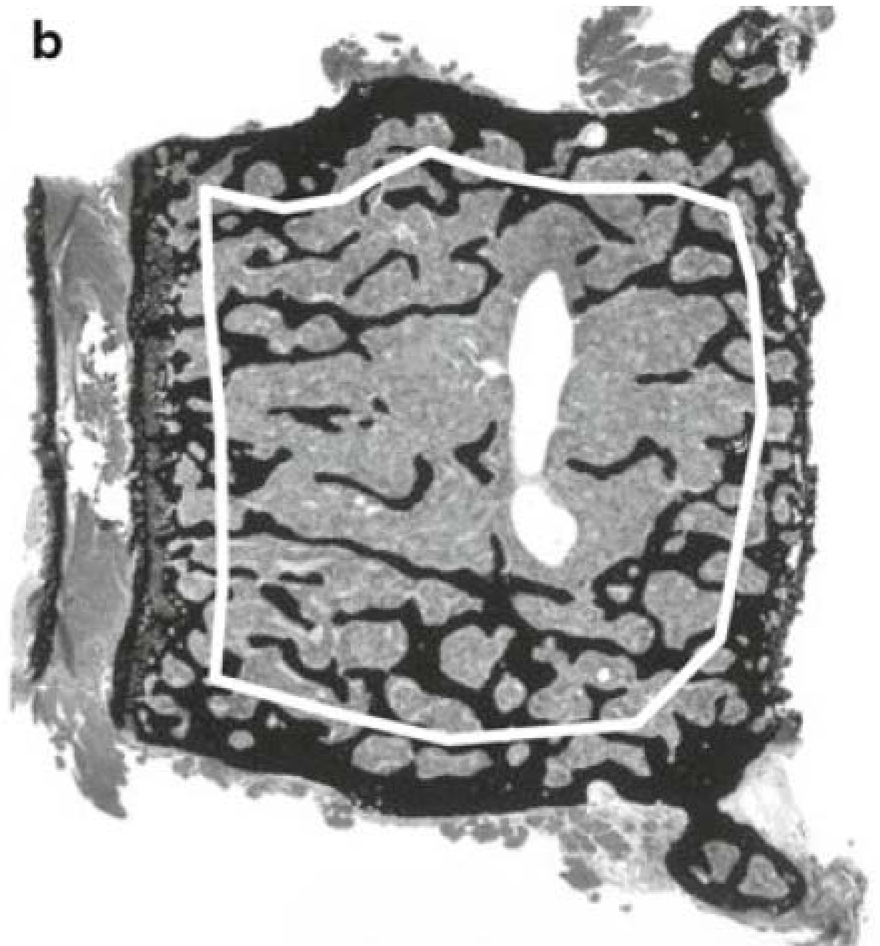
# A brief advertisement…

- This histology core (aka Core C) provides a number of services including various embedding and staining options
- Crystal is not only skilled with the various techniques, but can be an important resource during both the planning and troubleshooting phases of your projects
  - Understanding the histochemical basis for the stains, she can help choose fixative to work with a stain, or the right embedding procedure
  - Looking in the microscope, she can help determine if problems are due to tissue preparation or staining

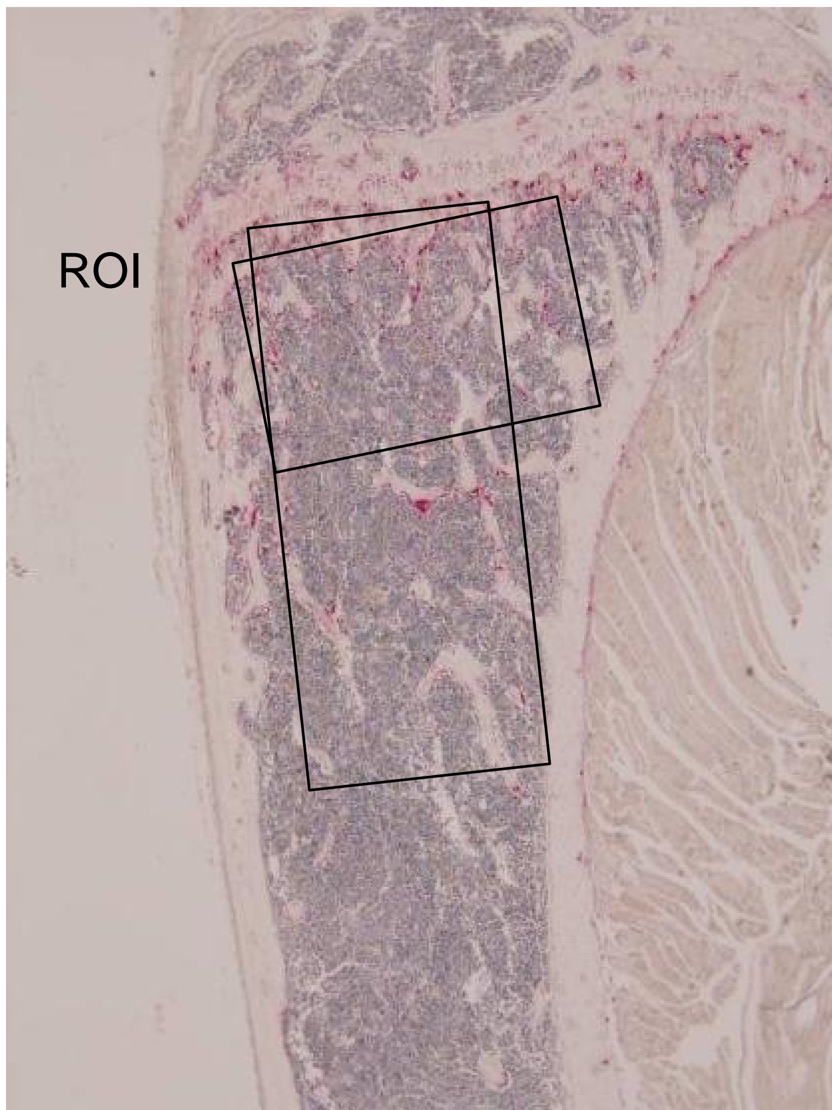
# So I have stained slides, now what?

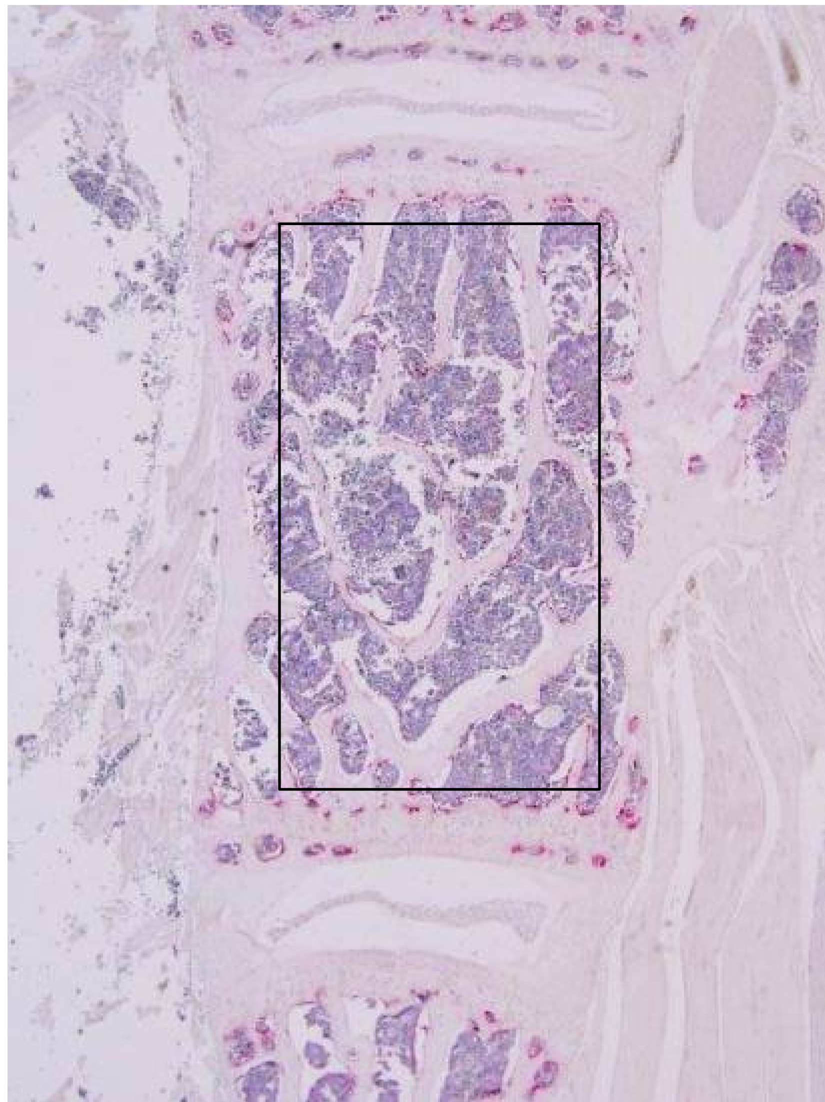
- Prior to beginning analysis with BioQuant (histomorphometry), browse your sections
  - Are they all adequate or do you need some recuts?
  - Which regions do you need to analyze?
  - Which parameters are most important?
    - Are you defining a basal phenotype or looking at a disease model?
- Keep in mind that the ASBMR documents were designed primarily for basal phenotypes and osteoporosis, but the BioQuant software can accommodate many other situations

# Define your region of interest



# Define your region of interest

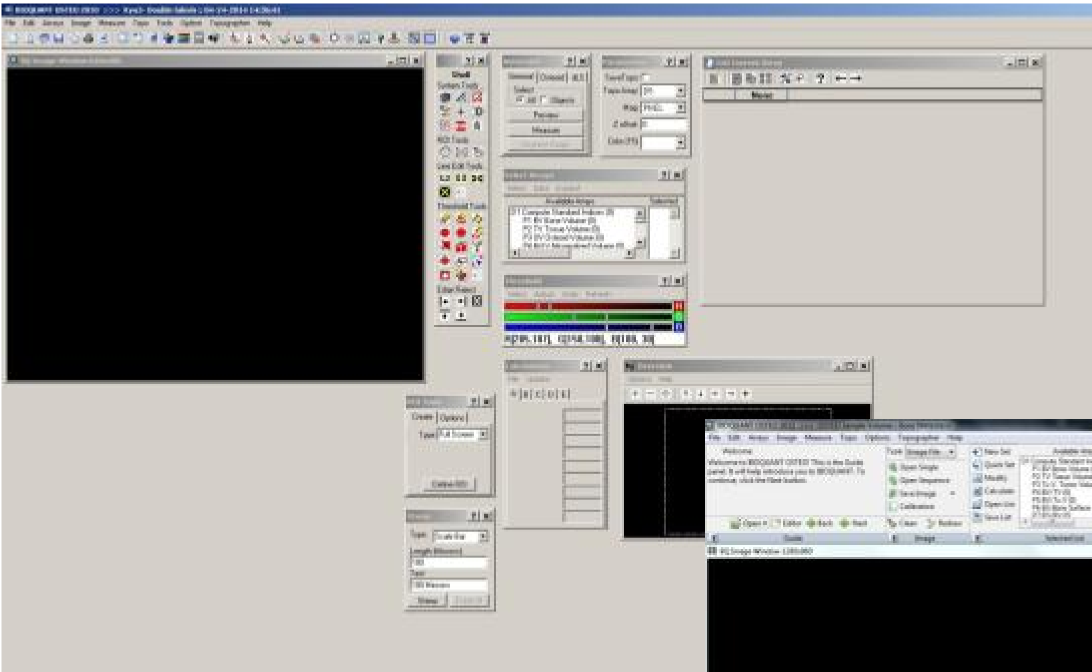


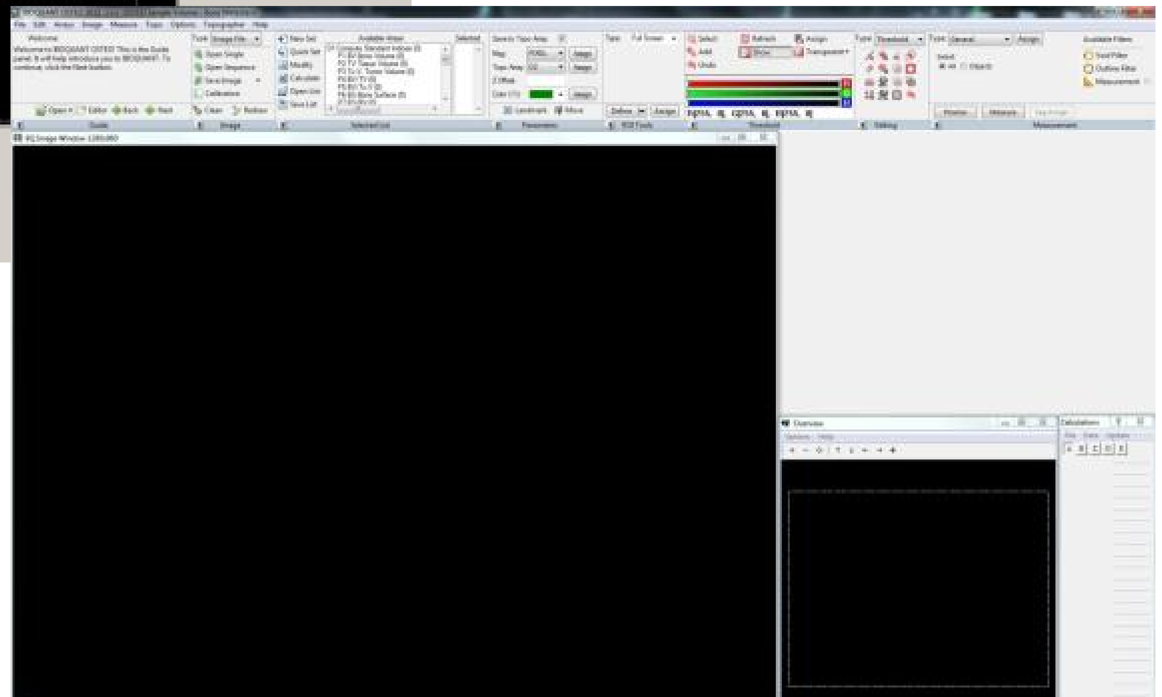
# A brief digression

# -- New Bioquant upgrade

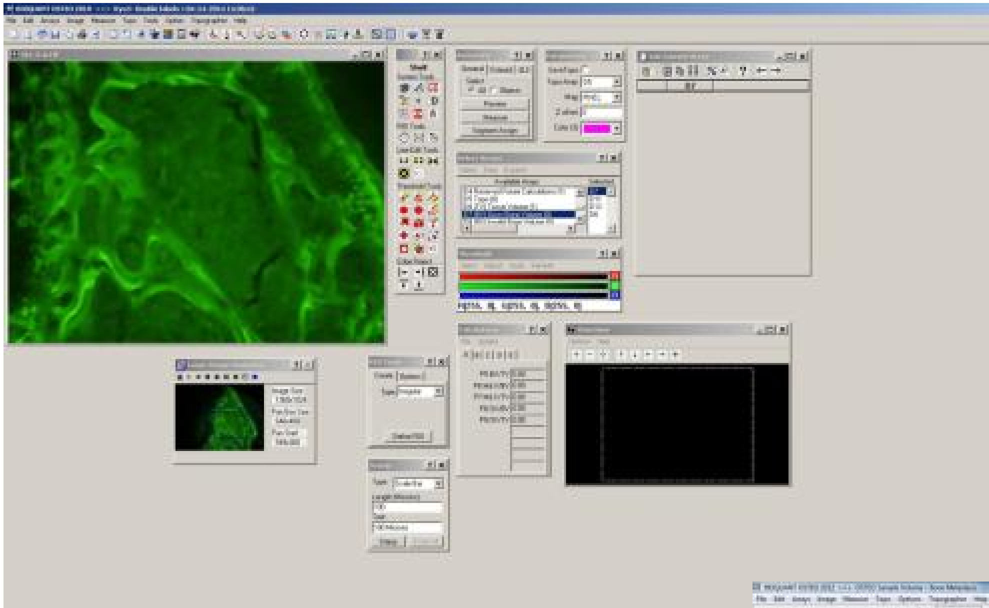
Old version



New version with task bar at top

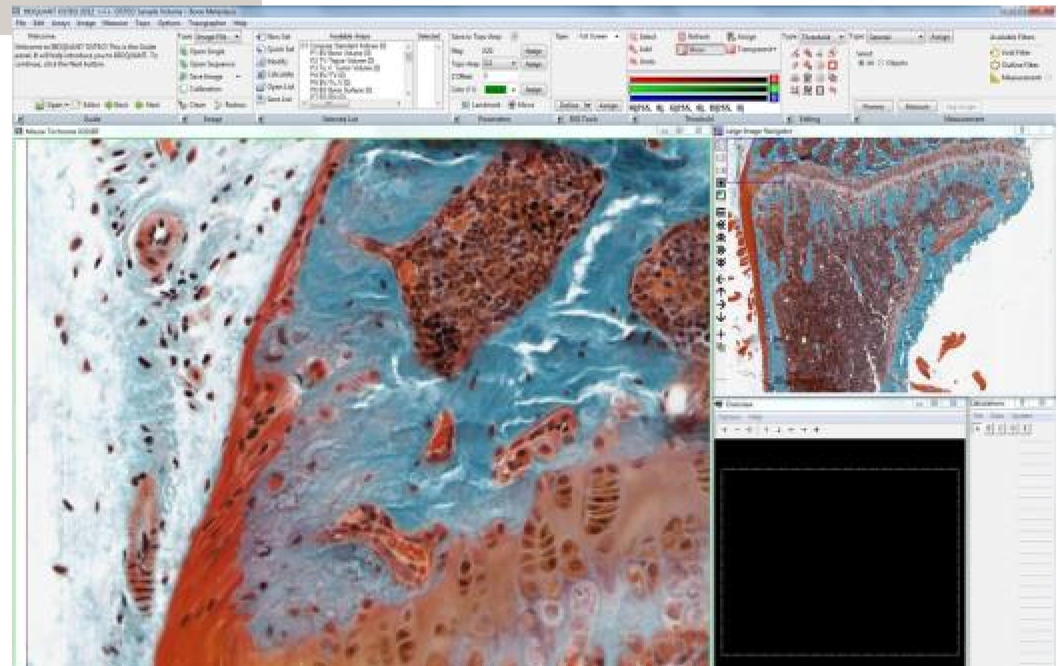


Old version: Image Window only 640x480 pixels.



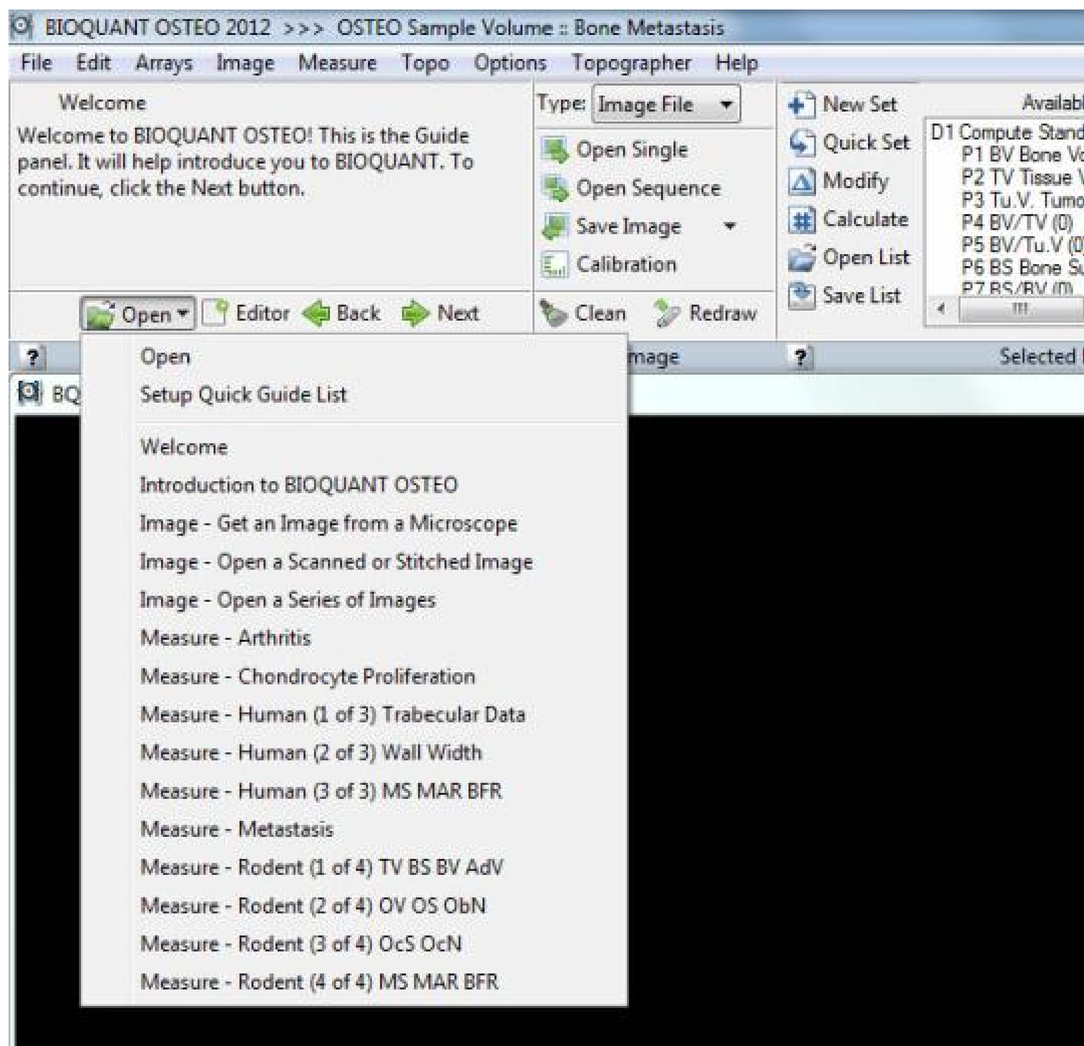
Step-up magnification (keyboard Z button) to measure features directly in Image Window. No need to move around smaller ROI box to cover your overall RO1.

New version:
Image Window up to 1280x960 pixels.



4. Detailed tutorial is provided in 2012 version

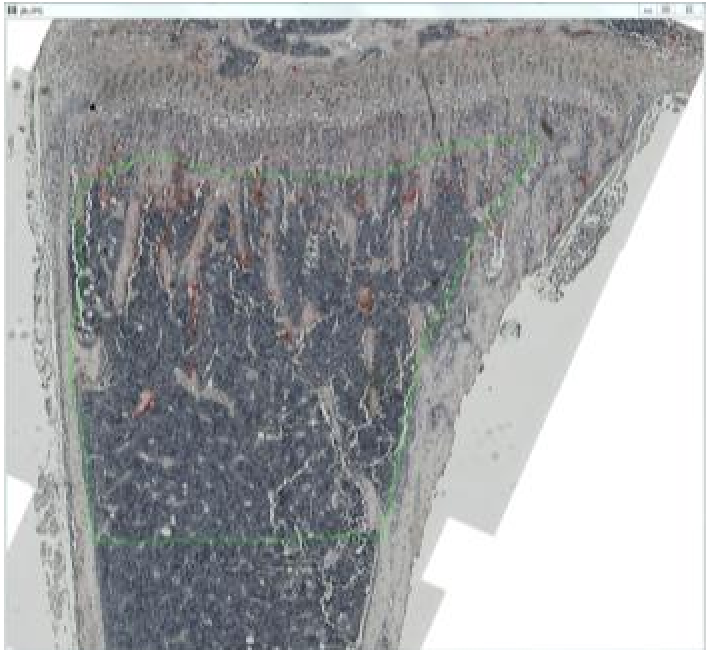
Tutorials to walk you through most the common analyses



# Additional upgrades in new Bioquant

- Assign buttons to fix the unique settings in current analysis, such as magnification, ROI type, manual threshold, etc. No need to reset between samples.
- One array (L3) finishes both BV and BS measurement.
- Automatic thresholding still only useful with a high contrast stain like trichrome, not TRAP or H&E

Unmagnified photo in Image Window



2x magnification in Image Window

# Standard nomeclature

- ASBMR taskforce has a list of individual terms
  - All except BMU (basic multicellular unit) are 1 or 2 letters, the first being capitalized and second lower case
- Standard format = Source – Measurement/Referent; period between 2 terms if double letter
  - Eg. Cn-N.Oc/BS means cancellous Oc number per bone surface
  - If same source (cancellous or cortical) is used repeatedly, don't need to keep indicating
  - The referent (TV, BV, BS, or OS most commonly) is very important and should never be omitted

**Table 2.** Sources and Referents in Bone Histomorphometry

| Sources | | Referents | |
|---|---|---|---|
| Name | Abb. | Name | Abb. |
| Total core | Tt | Bone surface | BS |
| Cortical bone tissue | Ct | Bone volume | BV |
| Cancellous bone tissue | Cn | Tissue volume | TV |
| Endocortical surface | Ec | Core volume | CV |
| Periosteal surface | Ps | Osteoid surface | OS |
| Transitional zone | Tr.Z | Bone interface | BI |
| Diaphyseal bone | Dp | Eroded surface | ES |
| Metaphyseal bone | Mp | Mineralized surface | Md.S |
| Epiphyseal bone | Ep | Osteoblast surface | Ob.S |
| Medullary bone | Me | Osteoclast surface | Oc.S |

Abb. = abbreviation.

Those listed will cover most situations in both human and nonhuman studies, but neither list is exhaustive. Combinations of source terms may be needed, such as Dp.Ec for diaphyseal bone, endocortical surface.

# Slides are 2-dimensional but most reported histomorphometric parameters are 3-D

- The goal of histomorphometry is to understand 3-dimensional reality
  - Stereology and mathematics provide approaches to convert data to 3-D, but most rely on assumptions such as isotropy which are not strictly true for bone
  - ASBMR recommends that 3-D terminology be used for volume, surface, and thickness, although numbers of cells remains in 2-D (ie. Referent is a surface, not a volume)
  - 2-D perimeter/area ratios are converted to 3-D surface/volume by multiplying by $4/\pi$ or 1.2
    - The Bioquant software does this for you

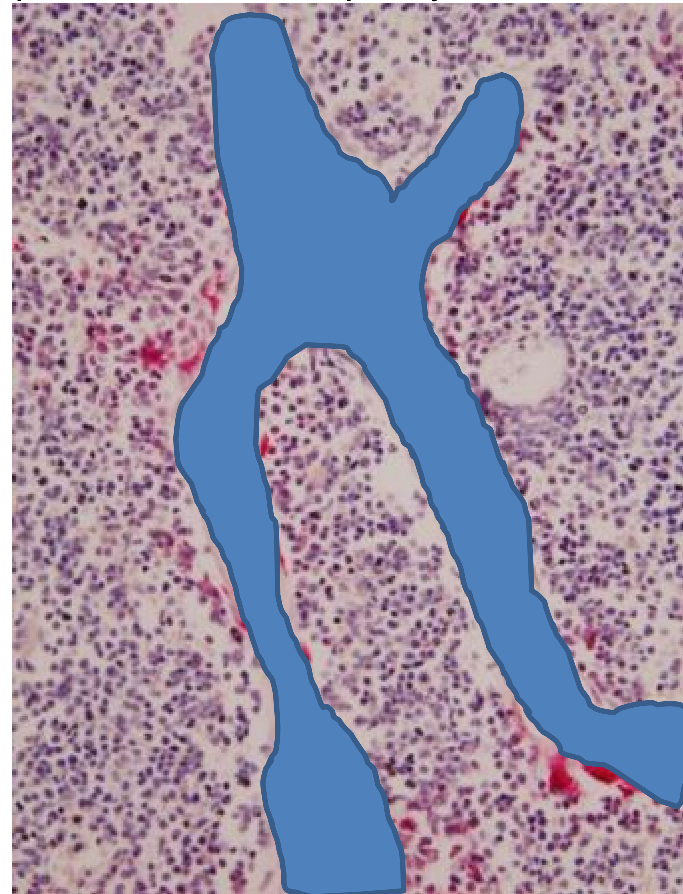
**Table 3.** Primary Measurements in Bone Histomorphometry

| Type of measurement | Name of measurement | Abbreviations | |
|---|---|---|---|
| | | 3D | 2D |
| Area | Bone volume[a] | BV | B.Ar |
| | Osteoid volume | OV | O.Ar |
| | Mineralized volume | Md.V | Md.Ar |
| | Void volume | Vd.V | Vd.Ar |
| | Marrow volume | Ma.V | Ma.Ar |
| | Fibrosis volume | Fb.V | Fb.Ar |
| | Canal volume[b] | Ca.V | Ca.Ar |
| | Cell volume[b,c] | Ce.V | Ce.Ar |
| | Cytoplasmic volume[b,d] | Cy.V | Cy.Ar |
| | Nuclear volume[b,d] | Nc.V | Nc.Ar |
| Length | Bone interface[e] | BI | B.Bd |
| | Bone surface[f] | BS | B.Pm |
| | Osteoid surface | OS | O.Pm |
| | Eroded surface | ES | E.Pm |
| | Quiescent surface[g] | QS | Q.Pm |
| | Mineralized surface[h] | Md.S | Md.Pm |
| | Osteoblast surface | Ob.S | Ob.Pm |
| | Single-labeled surface[i] | sLS | sL.Pm |
| | Double-labeled surface[i] | dLS | dL.Pm |
| | Osteoclast surface | Oc.S | Oc.Pm |
| | Reversal surface[j] | Rv.S | Rv.Pm |
| Distance[k] | Cortical thickness[l] | Ct.Th | Ct.Wi |
| | Wall thickness | W.Th | W.Wi |
| | Mineralized thickness | Md.Th | Md.Wi |
| | Osteoid thickness | O.Th | O.Wi |
| | Label thickness | L.Th | L.Wi |
| | Trabecular thickness[m] | Tb.Th | Tb.Wi |
| | Interstitial thickness | It.Th | It.Wi |
| | Trabecular diameter[n] | Tb.Dm | —[o] |
| | Canal radius | Ca.Rd | —[o] |
| | Cell height[c] | Ce.Ht | —[o] |
| | Nuclear height[d] | Nc.Ht | —[o] |
| | Erosion depth | E.De | —[o] |
| Number[p] | Osteoblast number | — | N.Ob |
| | Osteoclast number | — | N.Oc |
| | Osteocyte number | — | N.Ot |
| | Adipocyte number | — | N.Ad |
| | Nuclear number[d] | — | N.Nc |
| | Canal number | — | N.Ca |
| | Seam number | — | N.Sm |
| | Erosion number | — | NE |
| | Profile number | — | N.Pf |

In rodent models, additional parameters such as thickness of growth plate or articular cartilage might be useful, and the ASBMR “lexicon” provides appropriate symbols

# Things you measure directly

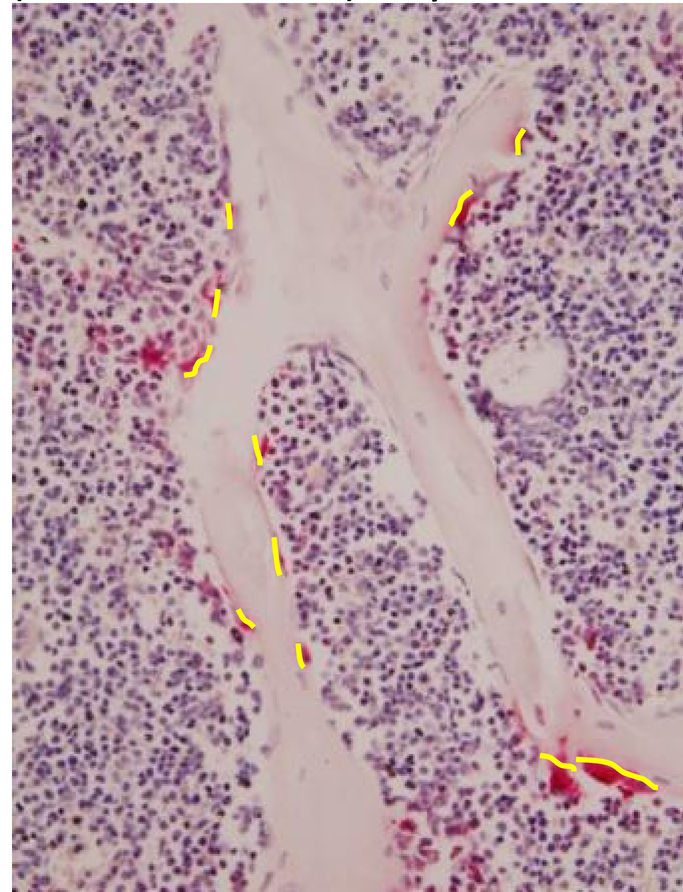
- Area (volume) – outlines a closed space
  - bone volume (BV ) = amount of bone in your ROI
  - tissue volume (TV) = amount of tissue (bone+ marrow) in your ROI
  - osteoid volume (OV)
- Length - lines
  - bone surface (BS)
  - osteoblast surface (Ob.S)
  - single labeled surface (sLS)
- Distance – between 2 lines
  - osteoid thickness (O.Th)
  - interlabel distance (Ir.L.Th)
- Number - dots
  - osteoblast number (N.Ob)
  - osteoclast number (N.Oc)



The so-called primary indices are then calculated from these – eg BV/TV or N.Ob/BS

# Things you measure directly

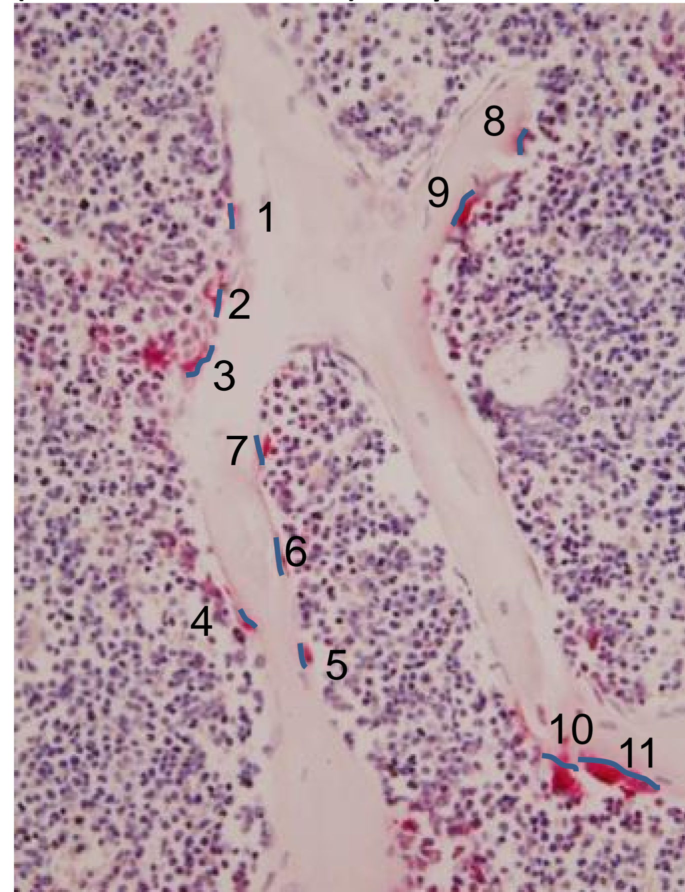
- Area (volume) – outlines a closed space
  - bone volume (BV ) = amount of bone in your ROI
  - tissue volume (TV) = amount of tissue (bone+ marrow) in your ROI
  - osteoid volume (OV)
- Length - lines
  - bone surface (BS)
  - osteoclast surface (Oc.S)
  - single labeled surface (sLS)
- Distance – between 2 lines
  - osteoid thickness (O.Th)
  - interlabel distance (Ir.L.Th)
- Number - dots
  - osteoblast number (N.Ob)
  - osteoclast number (N.Oc)



The so-called primary indices are then calculated from these – eg BV/TV or N.Ob/BS

# Things you measure directly

- Area (volume) – outlines a closed space
  - bone volume (BV ) = amount of bone in your ROI
  - tissue volume (TV) = amount of tissue (bone+ marrow) in your ROI
  - osteoid volume (OV)
- Length - lines
  - bone surface (BS)
  - osteoclast surface (Oc.S)
  - single labeled surface (sLS)
- Distance – between 2 lines
  - osteoid thickness (O.Th)
  - interlabel distance (Ir.L.Th)
- Number - dots
  - osteoblast number (N.Ob)
  - osteoclast number (N.Oc)




The so-called primary indices are then calculated from these – eg BV/TV or N.Ob/BS

# A note on eroded surface (ES)

- eroded surface is the "new" surface of the bone generated by an active OC, and appears "crenated" or "lacunar"

  - ES can be considered Oc+ if a cell is present, or Oc-

In mice, however, it is often hard to see nice pits since the OCs seem to sit on a flat bone surface. I consider ES in mice to be very subjective, compared to Oc.S (which still involves some judgment calls).

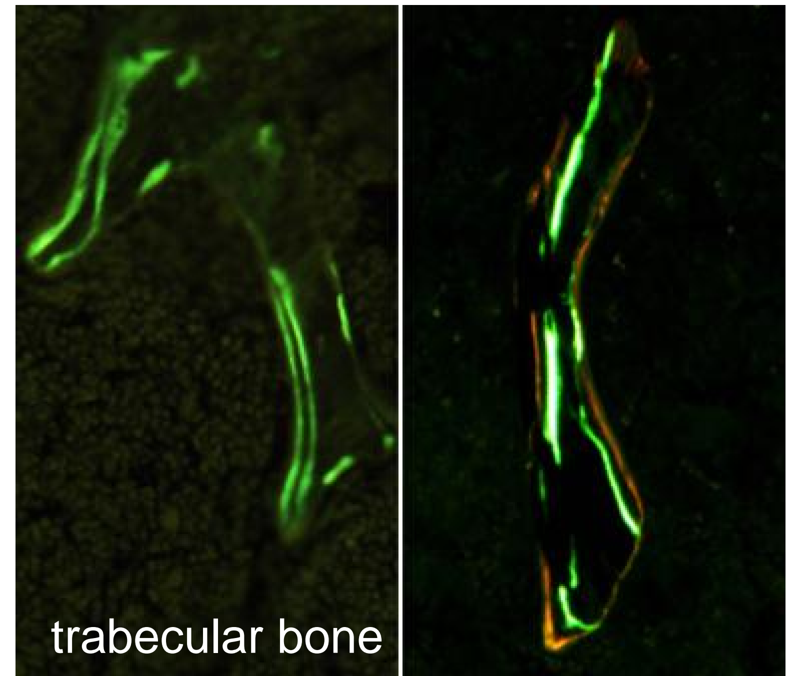
# Derived structural indices (things that get calculated from your primary measurements based on some assumptions)

- trabecular number Tb.N=(BV/TV)/Tb.Th
- trabecular separation Tb.Sp=(1/Tb.N)-Tb.Th

- These, as well as BV/TV, are better assessed by microCT
- However, in order to get values for cell counts and surfaces, you always have to measure the bone area and bone perimeter on your sections
  - Which then get converted to BV and and BS

# Dynamic histomorphometry

- measurement of bone formation parameters
- mice are given 2 doses of label several days apart, by IP injection
- calcein (green) and/or alizarin red (red) incorporate into newly calcifying bone
- interval depends on age of mice
  - 2-4 week mice, 3-4d
  - 6-12 week mice, 5-7d
  - older mice, 7-10d
- easier to measure in calvaria or endocortical surface than trabecular bone
  - Always should indicate where measurement was taken
  - Sometimes get different results from different sites

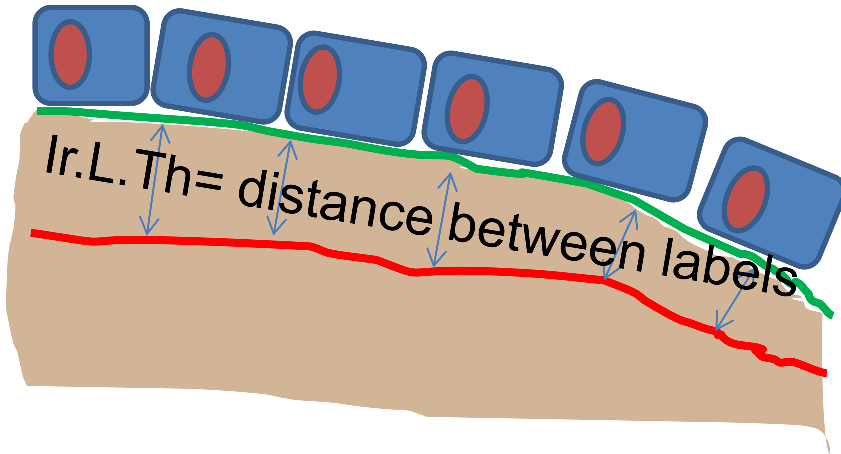


# Derived kinetic indices

- Mineralizing surface MS=(dLS + sLS/2)/BS
  - the extent of bone surface actively mineralizing
- Mineral apposition rate MAR = Ir.L.Th/Ir.L.t
  - distance between the labels divided by time between labels
- Bone formation rate BFR=MAR * (MS/BS)
  - multiplies the MAR by the fraction of bone surface that is labeled

# MAR vs BFR

## MAR = Ir.L.Th/Ir.L.t




Ir.L.t = time between injection of labels

MAR is the rate at which OBs are making matrix, which calcifies at a constant rate and incorporates the labels. Thus, it measures the average activity of individual OBs in your section.

## BFR=MAR * (MS/BS)

BFR takes into account how much of the bone surface is actively mineralizing, which depends on the number of OBs that are active. It multiplies the average work of each OB by the fraction of bone surface with active OBs, giving a “team” output.

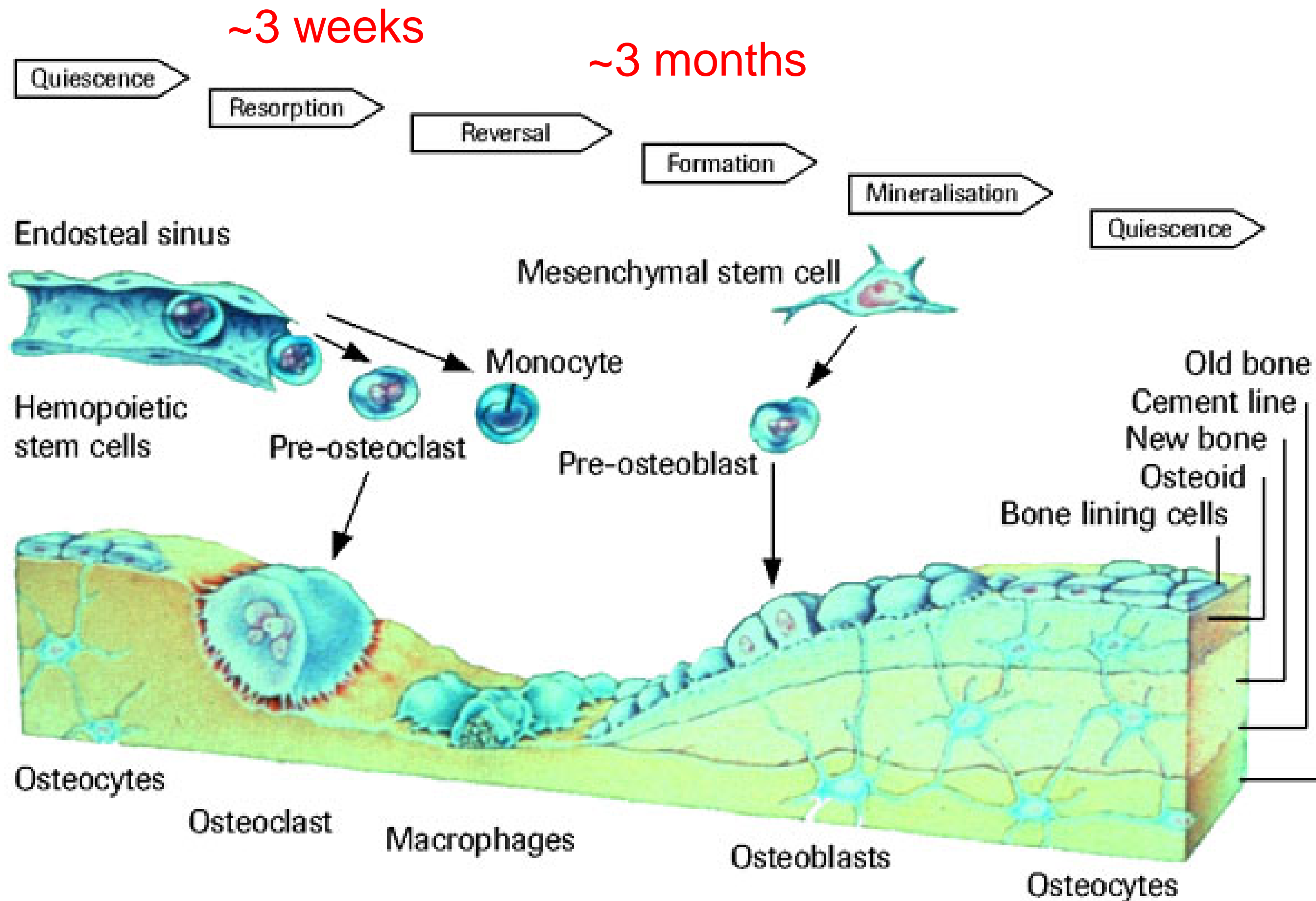
# Bone formation rates

- There is a BFR for every possible referent (OS, BS, BV, TV) but usually we use BS
- ASBMR taskforce paper says
  - BFR/BS seems most logical when considering hormonal effects on bone remodeling
  - BFR/BV is equivalent to bone turnover rate, which determines bone age and various age-dependent properties of bone
  - BFR/TV seems most logical when considering biochemical markers of bone remodeling because the entire tissue is perfused and contributes its products to circulation

# Bone Remodeling Cycle

~3 weeks

~3 months




For humans

http://www.roche.com/pages/facets/11/ostedefe.htm

# Issues related to low bone turnover

- Recent studies of potent bisphosphonates and denosumab have run into issues where the dynamic labeling is very low, with no double labels with which to calculate BFR or MAR
- Need to state # samples with double labels, only single, or no labels, and the method of dealing with single labels
  - MAR can be recorded as missing data or a value assigned (imputed)
    - Either 0.1 μm/d (lowest measured MAR) or 0.3 μm/d (lowest average MAR)
    - How you include the samples with no double label has a big effect on the group averages

# Effects of Denosumab on Bone Histomorphometry: The FREEDOM and STAND Studies

J Bone Miner Res. 2010 Oct;25(10):2256-65. doi: 10.1002/jbmr.149.

Ian R Reid,[1] Paul D Miller,[2] Jacques P Brown,[3] David L Kendler,[4] Astrid Fahrleitner-Pammer,[5] Ivo Valter,[6] Katre Maasalu,[7] Michael A Bolognese,[8] Grattan Woodson,[9] Henry Bone,[10] Beiying Ding,[11] Rachel B Wagman,[12] Javier San Martin,[11] Michael S Ominsky,[11] and David W Dempster[13] on behalf of the Denosumab Phase 3 Bone Histology Study Group

If the first unstained section did not show tetracycline fluorescence, then every tenth section was examined until tetracycline fluorescence was found. This section was used as the index slide, and the three groups of slides just described were prepared at 250-µm intervals. Examination at 50-µm intervals was continued throughout the entire biopsy, if necessary, to identify tetracycline labels (extended label search).

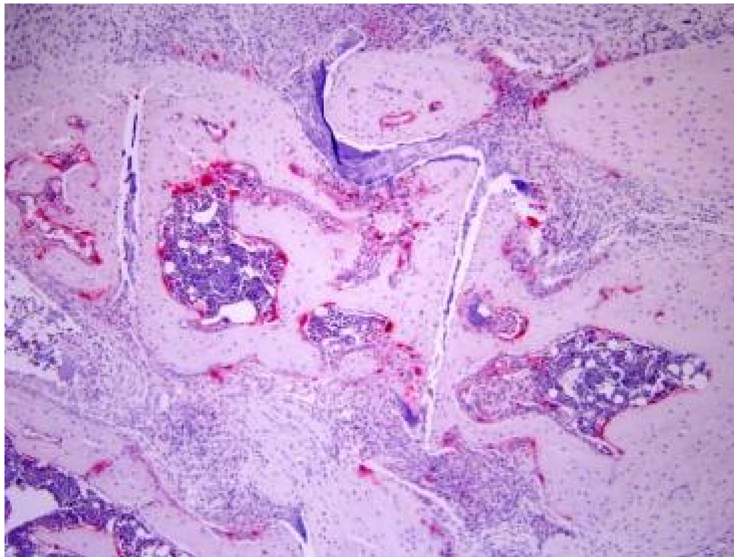
Analysis included data from all subjects who received one or more doses of investigational product and had one or more evaluable biopsies during the study. For biopsies with only single labels in trabecular bone within the measurement field of 20 $mm^2$, a value of 0.3 µm/day was imputed for mineral apposition rate and used to derive other dynamic parameters, as recommended by Hauge and Foldes.[18,19] Biopsies with single and/or double labels that were located only in cortical bone or were outside the measurement field in trabecular bone were excluded from these analyses. Between-group treatment differences for histomorphometric and µCT variables were evaluated using the Wilcoxon rank-sum test.

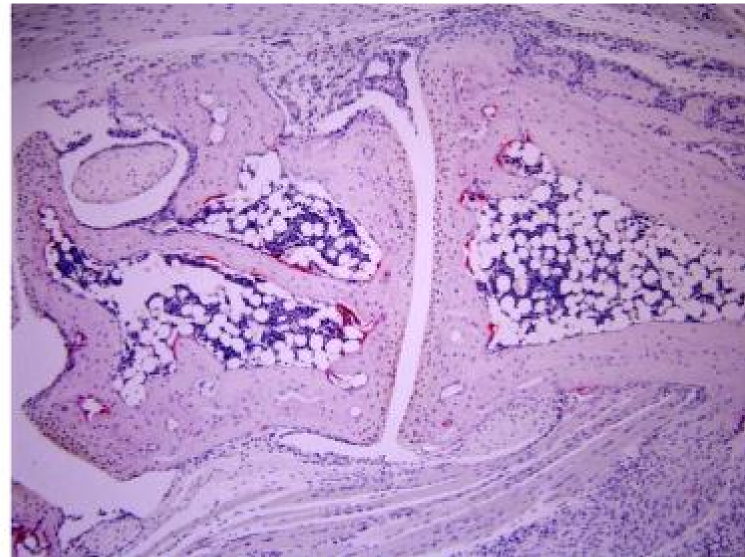
# Examples of histomorphometry in disease models

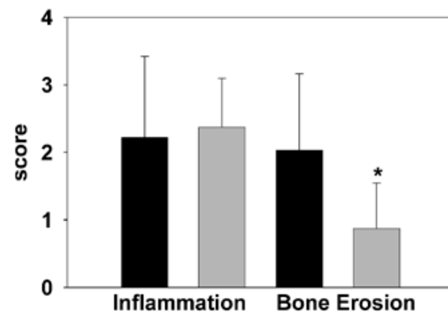
# Histomorphometry in Inflammatory Arthritis

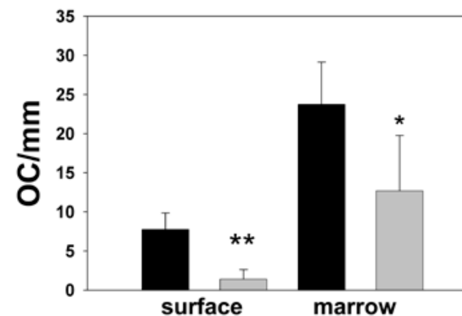
WT

$NIK^{-/-}$





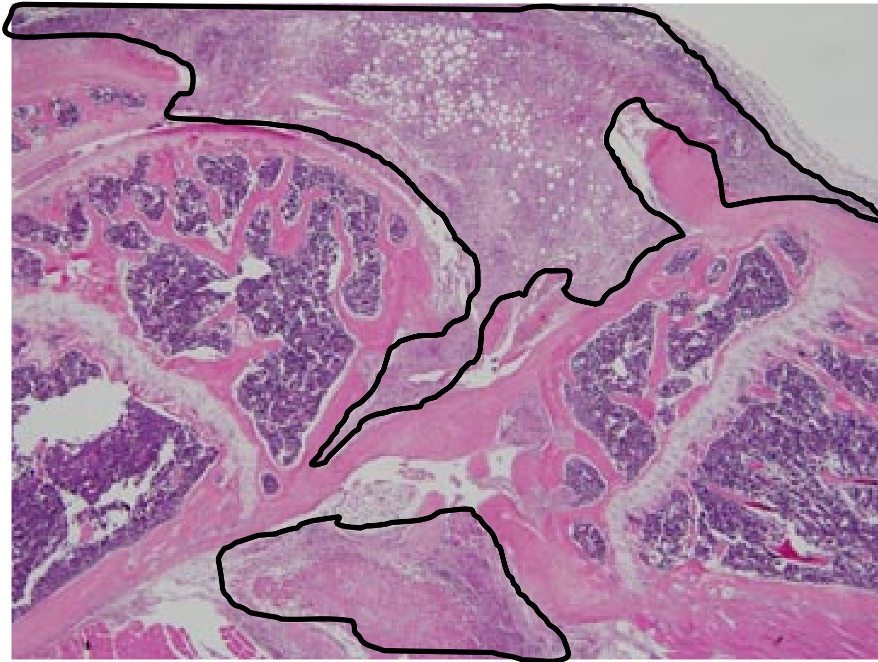


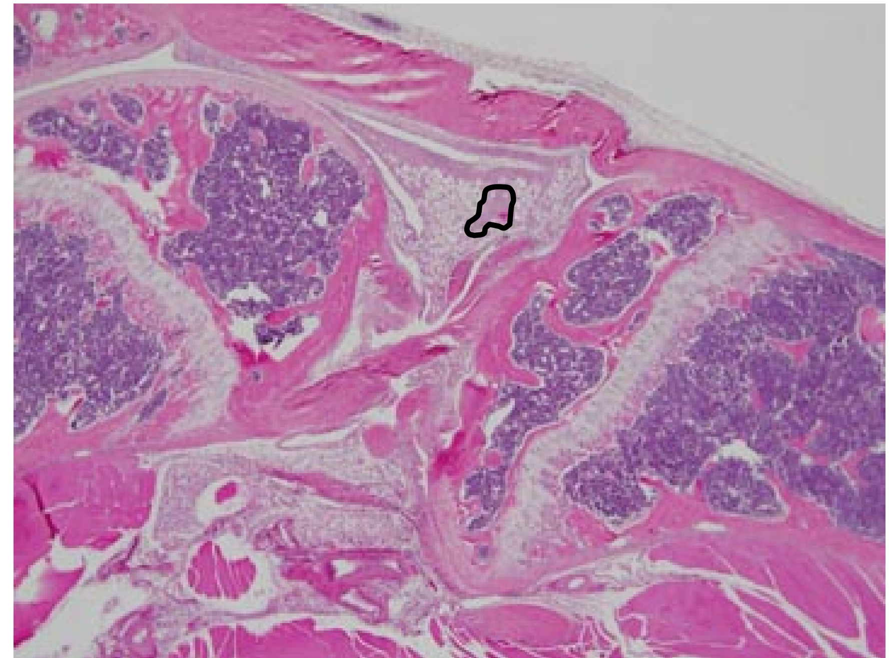

Aya JCI 115: 1848

# Antigen-induced arthritis

WT



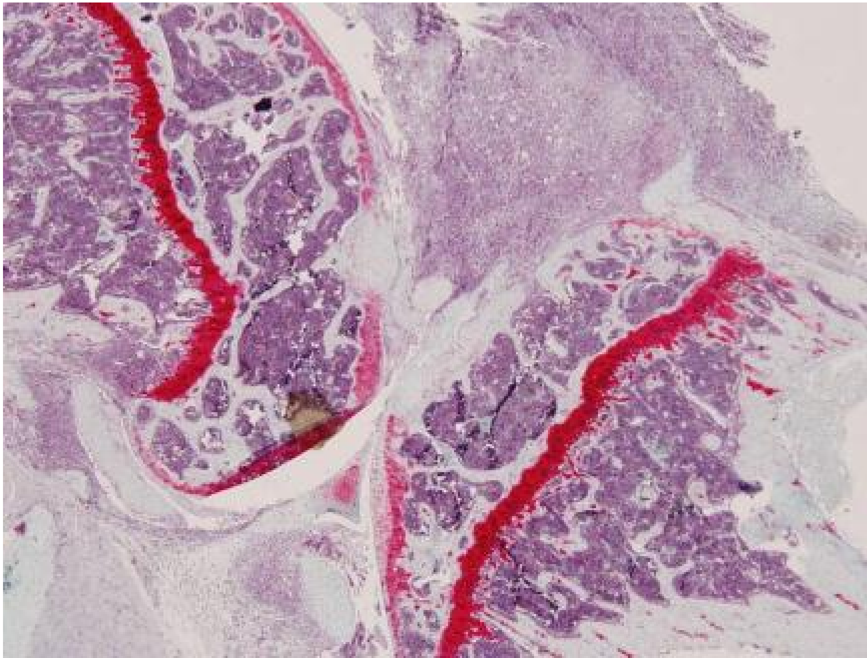
KO



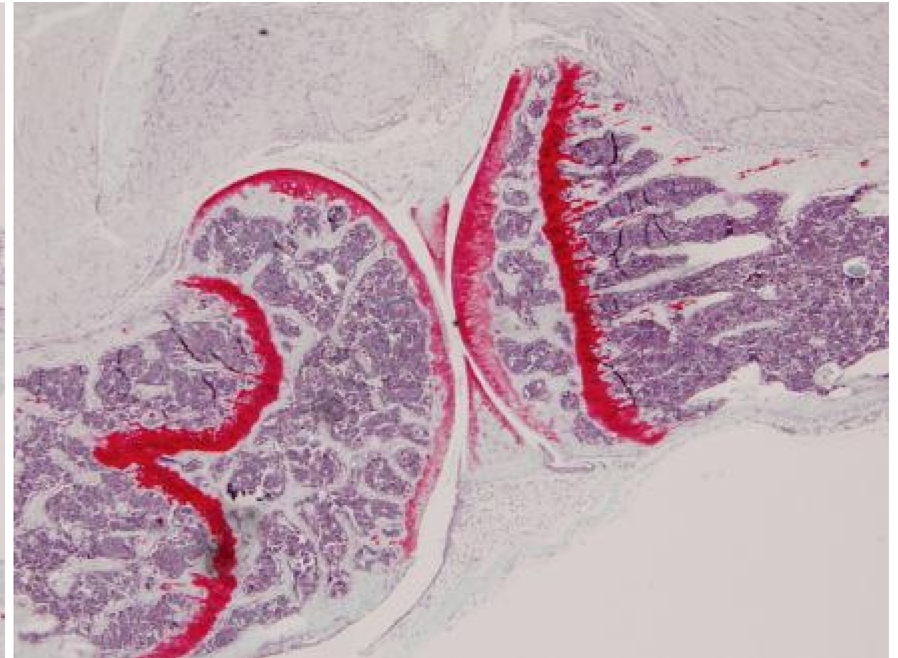
quantify area with inflammation

# Antigen-induced arthritis

WT

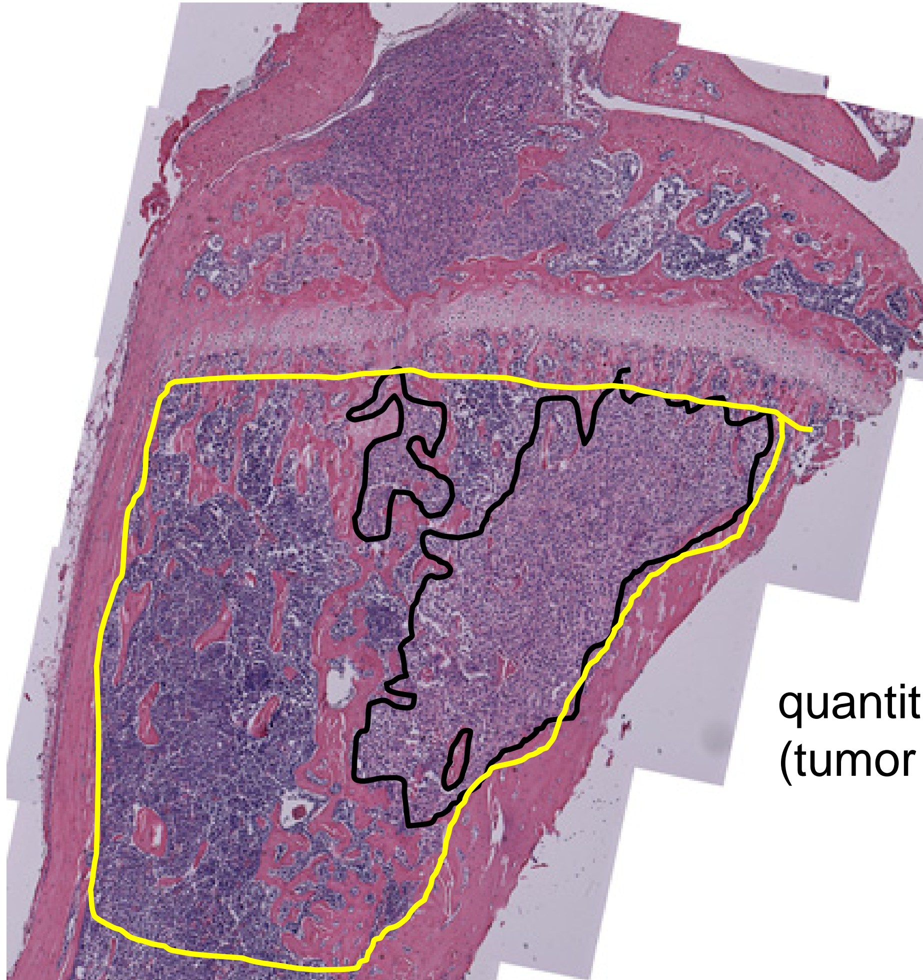


KO



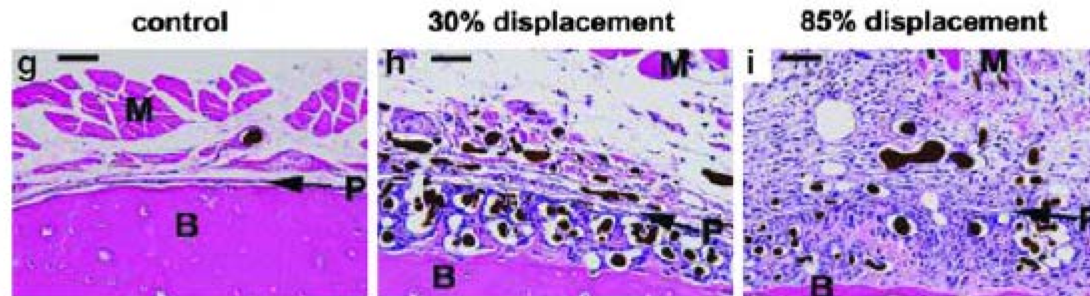
quantitate extent of safO staining on articular surface

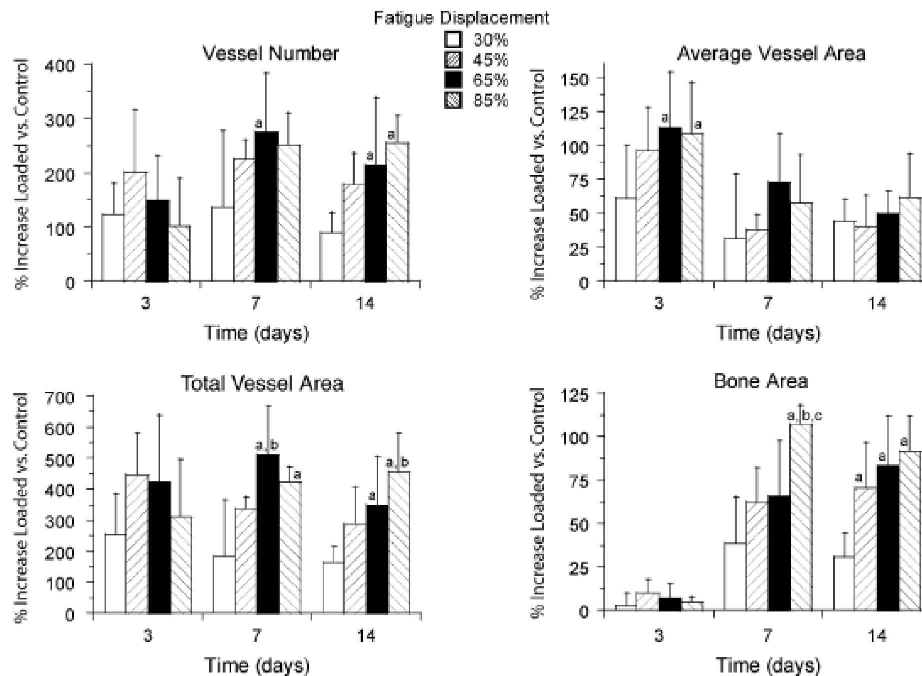
# Bone metastasis



quantitate tumor area/tissue area
(tumor volume/tissue volume)

# Analysis of fracture model looking at vessels




Silva group

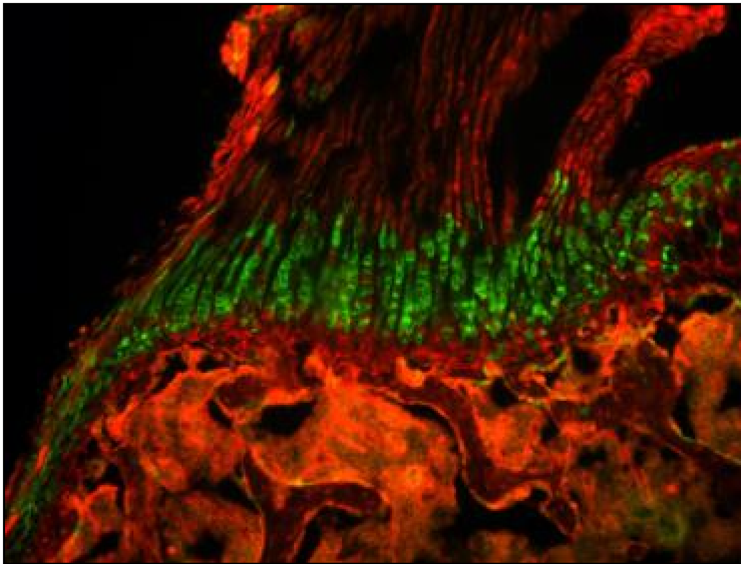
Calcif Tissue Int (2007) 80:391–399

# Newer options – frozen sections

- Cryojane tape transfer system (Leica)
  - Tape is applied to tissue block and knife cuts behind the tape, leaving your section adherent to the tape
  - Tape is rolled onto a special adhesive slide and tissue is transferred to slide with a zap (or several) of UV
  - Nondecalcified tissue does not transfer well, so this is best for (at least partially) decalcified samples
  - Can be used for histo stains, IHC, or IF
- Cryofilm tape (Section labs)
  - Film is applied to block and knife cuts behind
  - Film is then glued to a regular slide, tissue side up
  - Tissue can be stained, then coverslipped
  - Special LCM film is available for laser capture, with special slides (with a hole in the middle)

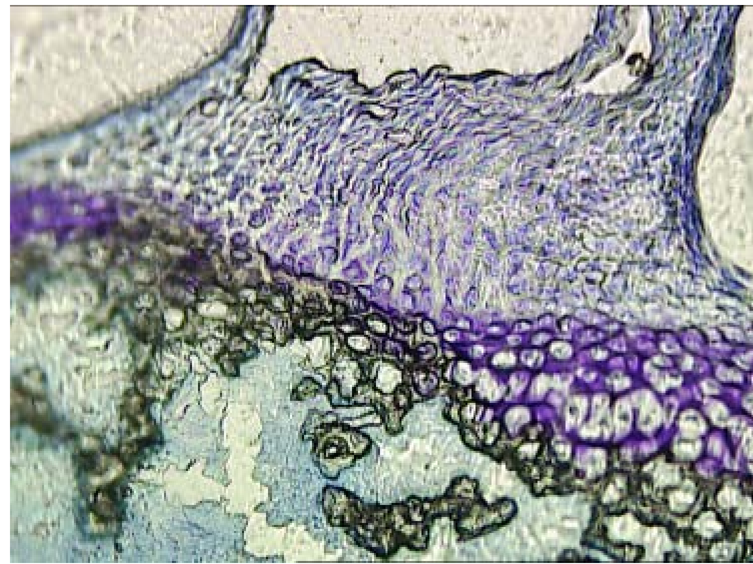
# Cryojane/cryofilm

Cryojane



Frozen section of fixed, decalcified and frozen tissue observed without further staining under fluorescence, mTmG reporter

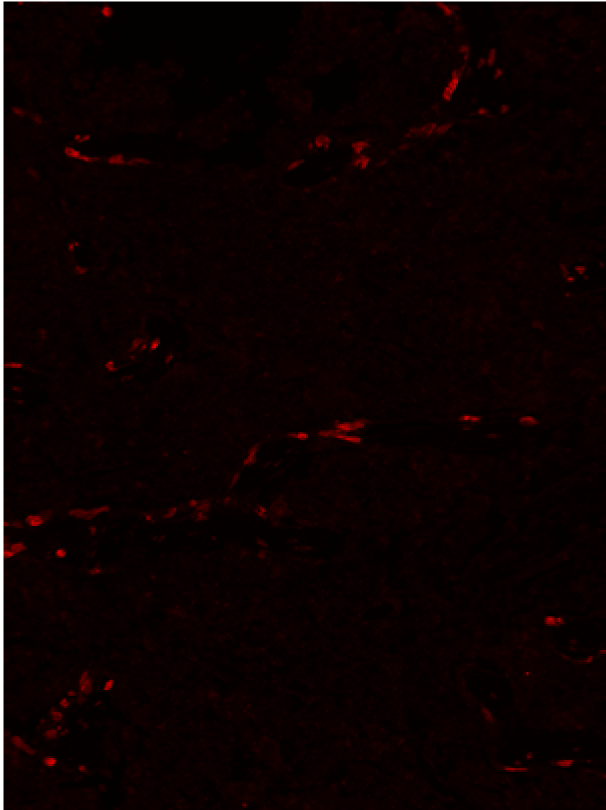
Cryofilm



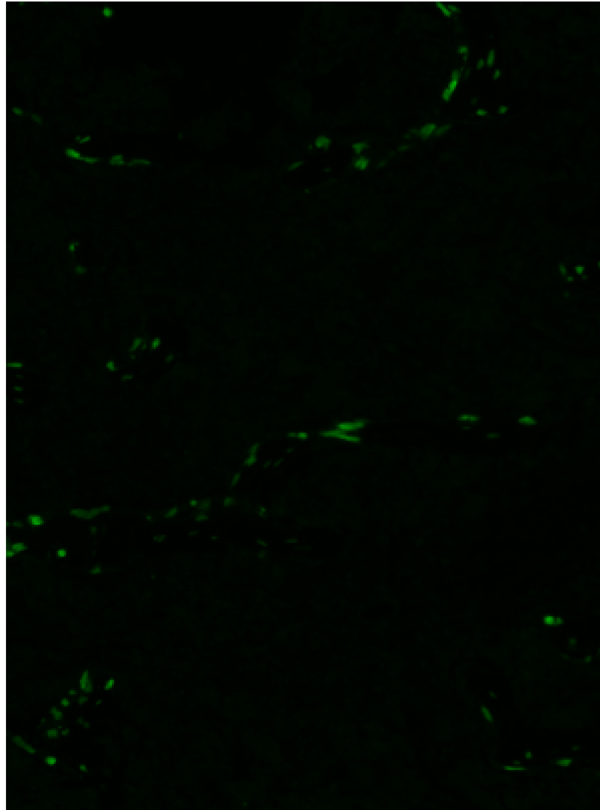
Unfixed, nondecalcified frozen section, toluidine stain

From Megan Killian

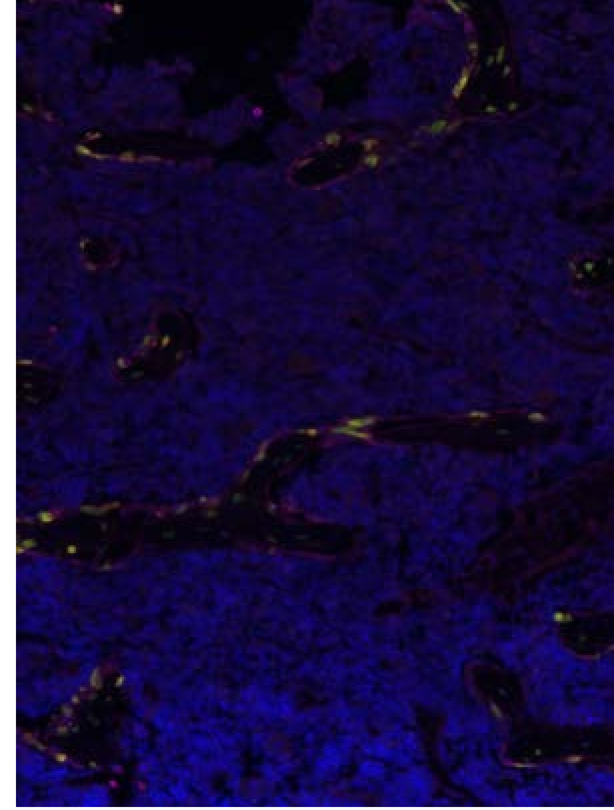
# Cryofilm example



IF, osteocalcin



GFP transgene, no stain

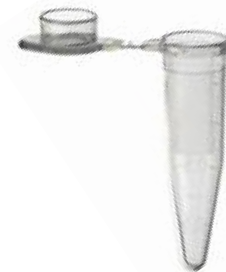


Overlay, with Hoechst

From Stewart lab

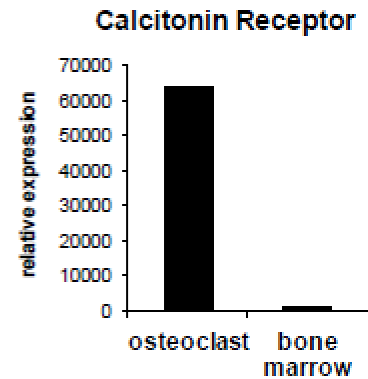
# Laser Capture Microdissection

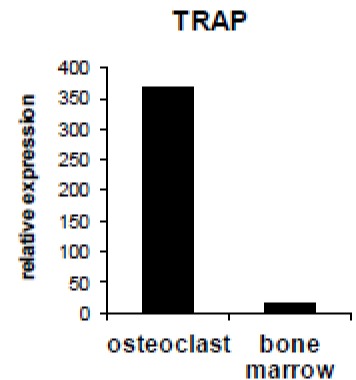
Laser beam cuts tissue and it falls into cap



Brief TRAP stain

# Draw a contour, and cut with laser




RNA suitable for qPCR
Testing for RNAseq now

From Chang Yang

# Useful references

**Standardized Nomenclature, Symbols, and Units for Bone Histomorphometry: A 2012 Update of the Report of the ASBMR Histomorphometry Nomenclature Committee**

David W Dempster,[1,2] Juliet E Compston,[3] Marc K Drezner,[4] Francis H Glorieux,[5] John A Kanis,[6] Hartmut Malluche,[7] Pierre J Meunier,[8] Susan M Ott,[9] Robert R Recker,[10] and A Michael Parfitt[11]

J Bone Miner Res. 2013 January ; 28(1): 2–17. doi:10.1002/jbmr.1805.

**Histomorphometry in Rodents**

**Reinhold G. Erben and Martin Glösmann**

Miep H. Helfrich and Stuart H. Ralston (eds.), *Bone Research Protocols*, Methods in Molecular Biology, vol. 816,
DOI 10.1007/978-1-61779-415-5_19, © Springer Science+Business Media, LLC 2012

# Thank You !!

# Less commonly used terms

- More relevant in clinical studies

# Adjusted apposition rate

- Aj.AR = MAR*MS/OS
  - Represents the mineral apposition rate averaged over the osteiod surface
- In a steady state and in the absence of osteomalacia (mineralization defect), Aj.AR is the best estimate of the mean rate of osteoid apposition, and should be the same as the rate of mineral apposition
- The mineralization lag time (how long it takes to mineralize a newly deposited hunk of osteoid) is the osteoid thickness divided by the Aj.AR
  - Mlt = O.Th/Aj.AR
  - Osteomalacia has been defined as Mlt > 100 d

# Wall width (W.Wi) and Formation period (FP)

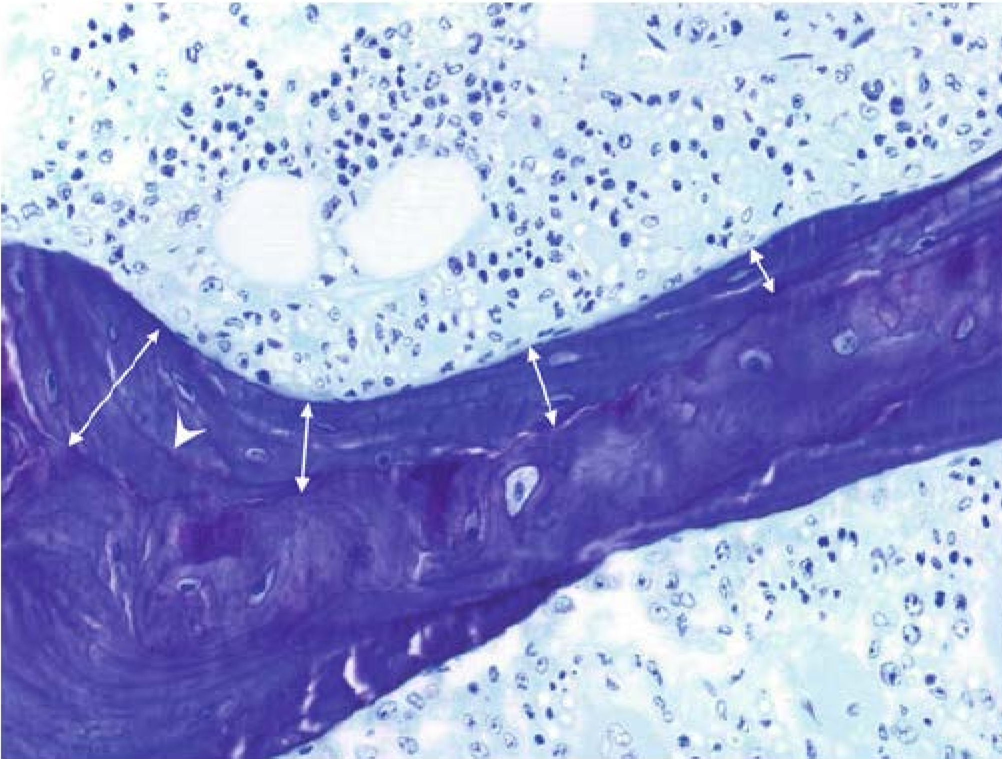


Fig. 7. Measurement of wall width for remodeling-based histomorphometric parameters. Wall width is defined as the mean distance between the bone surface and the scalloped reversal line in individual completed remodeling units. A smooth arrest line, indicating a temporary stop in osteoblastic bone formation within a remodeling unit, is marked by an arrowhead. Five-micron-thick section of a rat lumbar vertebra surface-stained with cement line stain. Original magnification × 400.

- Wall width is used to determine the amount of bone added during a remodeling cycle
- The idea is that the surface extent of a certain activity is proportional to the time occupied by the activity
- Measure at least 15 units/sample
- Convert to thickness (W.Th in 3-D) by multiplying by $\pi/4$
- FP=W.Th/MAR in rodents or W.Th/Aj.AR for humans

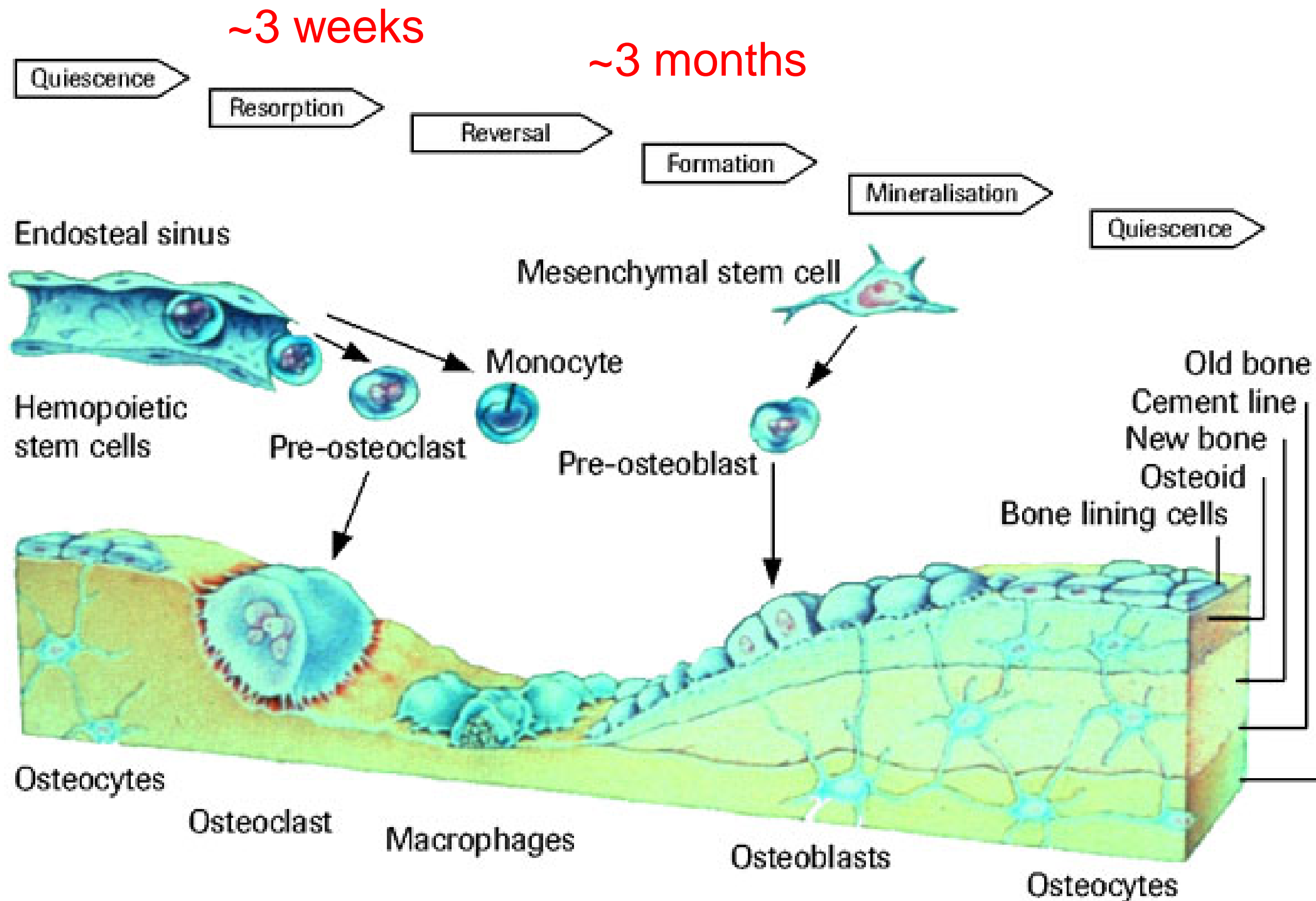
# Activation frequency (Ac.f)

- The Ac.f is the probability that a new cycle of remodeling will occur at any point on the surface by the event of activation
  - It is defined as 1/Tt.P (total period) or the reciprocal of the total time it takes for a remodeling cycle
  - Tt.P = FP*(BS/OS)
  - We don’t have a direct way of measuring the resorption or reversal periods, although there are formulas based on Oc.S and ES

# Bone Remodeling Cycle

~3 weeks

~3 months




For humans

http://www.roche.com/pages/facets/11/ostedefe.htm