F-24. Bone Histology Protocols from David Rowe Lab (UCONN)

http://skeletalbiology.uchc.edu/30_ResearchProgram/304_gap/index.htm

Protocol for Frozen section of GFP Bone:

1. Fix the bone in 4% Paraformaldehyde at 4 ℃ under constant agitation for 3 days.
2. Decalcification in 14% EDTA solution at 4 ℃ or RT under constant agitation for 3~5 days (change fresh 14% EDTA solution every 24 hours).
3. Wash in PBS for 2 hours.
4. Soak in 30% Sucrose in PBS at 4 ℃ under constant agitation overnight.
5. Embed bone in OCT compound.
6. Cut 5µm thick frozen sections using cryostat and allow the sections to dry (keep in dark).
7. Rinse in PBS 5min x 2.
8. Soak in 1mM MgCl2 in PBS for 30min.
9. Rinse in PBS 5min.
10. Mount coverslide using 50% Glycerol in PBS.
11. Examine by fluorescence microscopy.

Solution:

4% Paraformaldehyde in 0.1M PBS:

- Heat PBS to ~ 65 ℃ to dissolve Paraformaldehyde,then cool it and adjust pH to 7.4. (use fresh solution)

Decalcification solution:

- 280 g EDTA
- ddwater 1.5 L
- 180 ml Ammonium Hydroxide
- Mix well and adjust pH to 7.1 with Ammonium Hydroxide
- Add water to 2 L.

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Embedding Bones for Frozen Section:

1. Fill an ice bucket with dry ice pellets and place a 500ml beaker in the middle.
2. Put approximately 200ml of 2-MethylButane in the beaker and cover the bucket. (The MethylButane will get extremely cold, allowing you to ‘flash freeze’ your samples).
3. Label your molds and fill the molds with Frozen Embedding Medium (Thermo Shandon, Pittsburgh, PA).
4. Immerse bone in embedding medium and adjust right position as needed.
5. Test 2-MethylButane with dry ice pellets, if it does not boil, it is ready.
6. Use a forceps to keep the embedding mold horizontaly for few seconds until the embedding medium is frozen in pre-cold Methybutane and allow it to sit to the bottom of the beaker for at least 2 minutes to ensure it is frozen all the way through.

7. Frozen samples can be placed on dry ice in the bucket until all samples are done. Wrap your samples in aluminiumfoil.
8. Place in a sealed container and store your samples at -20 筷 or -80 筷.

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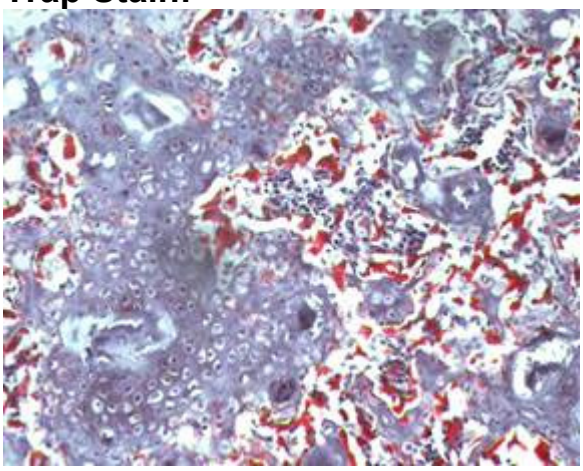
Trap Stain:



Buffer I Solution:

Sodium Acetate Anhydrous (Sigma S-2889)	9.2g
L- (+)Tartaric Acid (Sigma T-6521)	11.4g
Distilled Water	950ml
Glacial Acetic Acid	2.8ml

- Dissolve and adjust pH to 4.7-5.0 with 5M Sodium Hydroxide
- Bring total volume to 1L
- (5M NaOH: Sodium Hydroxide Pellets 50g, Distilled Water—250ml)

Buffer II Solution:

Naphthol AS-BI Phosphate (Sigma N-2125) store at –20?/td>	0.1g
Ethylene Glycol Monoethyl Ether (Sigma E-2632)	5ml

Buffer III Solution:

Sodium Nitrite (Sigma S-2252)	1g
Distilled Water	20ml

Buffer IV Solution:

Pararosaniline Chloride (Sigma P-3750)	1g
2N HCL	20ml

- Heat to 60? C for 5 min do not boil.
- Filter through kimwipe.
- (2N HCL: HCL, 83ml; Distilled water, 417ml)

Staining Procedure:

1. Preheat to 37°C 2 Coplin jars with 50ml of Buffer I Solution each.
2. Take one Coplin jar and add 0.5 ml of Buffer II Solution, add slides and incubate at 37°C for 45 minus.
3. A few min before the time is up, mix 1 ml of Buffer III Solution and 1 ml of Buffer IV solution for 30 sec and let it sit for 2 min.
4. Add this mixed solution to the other Coplin jar of 50 ml Buffer I Solution, mix and add the slides without rinsing.
5. Incubate at room temp ~5 min.
6. Rinse, counterstain with hematoxylin for 40 sec and put slides in 0.05% Ammonia water.
7. Dehydrate through alcohol, clear in xylene and mount.

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Alizarin Red S Staining - Calcium

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

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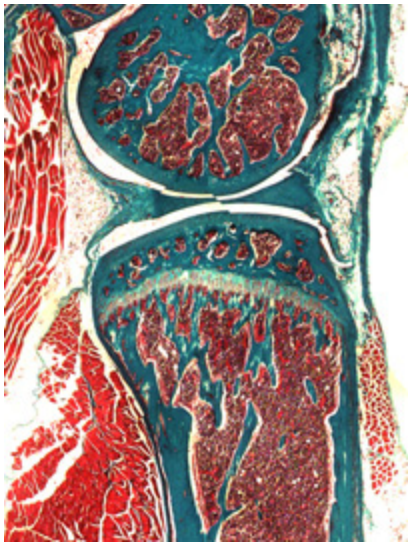
Goldner Trichrome Staining



Weigert Hematoxyline:

Solution A	
Hematoxyline	5 g
95% Alcohol	500 ml
Solution B	5 g
Ferric chloride	5.8 gl
Deionized water	500 ml
Concentrated Chlorhydric Acide	5.0 ml

- 24 hours before use, mix solutions A and B in equal amounts Stable for 8 days.

Fushine-Ponceau:

Fushine acid	0.167 g
Ponceau S	0.667 g
Deionized water	500 ml
Acetic Acid (glacial)	1.0 ml

- Shake well and filter.

Orange G:

Phospho-molybdic Acid	25 g
Deionized water (Shake well)	500 ml
Add Orange G	10 g

- Shake well and filter.

Light Green:

Light green	1.5 g
Deionized water	500 ml
Acetic Acid (glacial)	1 ml

- Shake well and filter.

Acetic Acid 1%

Staining Procedure:

1. Put slides in deionized water.
2. Mordant in Bouin's Fixative (Poly Scientific Inc. Catalog# s129-16oz) at room temperature overnight.
3. Rinse with deionized water frequently for 10 minutes.
4. Weight Hematoxyline 15 minutes.
5. Rinse with deionized water frequently for 10 minutes.
6. Fushine-ponceau 30 minutes.
7. Rinse with 1% acetic acid rapidly.
8. Orange G 8 minutes.
9. Rinse with 1% acetic acid rapidly.
10. Light Green 20 minutes
11. Rinse with 1% acetic acid rapidly.
12. Dehydrate through alcohol, clear in xylene and mount.

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Fluorescent Immunostaining for BrdU:

1. .

- Wash frozen sections with PBS 2x.
- 0.1%Pepsin in 0.1N HCL in PBS, incubate sections at 37 ? for 50mi
- Incubate with 2N HCL in distilled water 30 min at 37 ?.
- Rinse with PBS 5x.
- 5% normal donkey serum in PBS with 1% BSA at RT for 1 hour.
- Mouse anti-BrdU (Singa) diluted to 1:1000 with 1% NDS, 1%BSA in incubate at 4 ? overnight.
- Rinse with PBS 3x.
- Donkey anti-Mouse IgG conjugated-Tritc 1:100 (Jackson ImmunoRes Inc. Pennsylvania) in 1% NDS, 1%BSA in PBS, incubateat RT for 1 h overnight at4 ?.
- Rinse with PBS 3x.
- Mount with 50% glycerol in PBS

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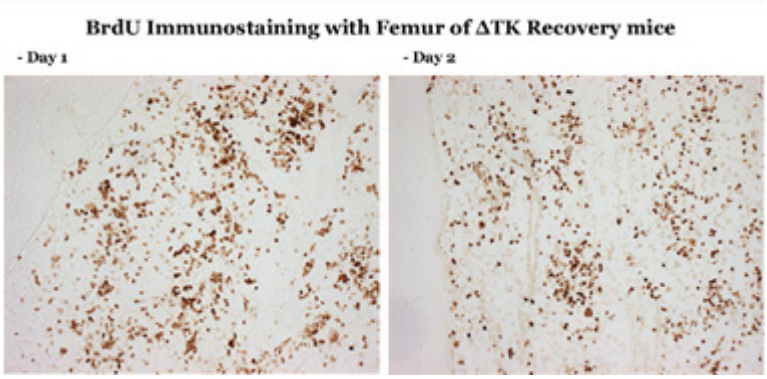
Enzyme Immunostaining for BrdU:



1. Wash frozen sections with PBS 2x.
2. Put them in 0.1% Pepsin in 0.1N HCL (in PBS) at 37 筭 for 50min.
3. Then in 0.3% hydrogen peroxide in PBS, 30min. Then rinse with PBS 3x.
4. Then in 5% normal goat serum in PBS with 1% BSA at RT for 1 hour.
5. Mouse anti-BrdU diluted to 1:1000 with 1% NGS and1%BSA in PBS, incubateat 4 筭 overnight.
6. Rinse with PBS 3x.
7. Goat Biotinylated anti-Mouse IgG 1:200 in 1% NGS, 1%BSA in PBS, incubate at RT for 1 hour. Rinse in PBS 3x.
8. Incubate with ABC reagent in PBS containing 0.1% Tween 20 at RT for 1 hour.. (ABC reagent solution: 5ml PBS/0.1% Tween 20 with 1 drop A and 1 drop B, mix well).
9. Rinse with PBS 3x.
10. DAB reaction: 2.5ml of distilled water + 1 drop Buffer + 2 drop DAB solution + 1 drop hydrogen peroxide. ~Needs about 8 min to stop DAB reaction.
11. Rinse with distilled water 3x. Counterstain and mount.

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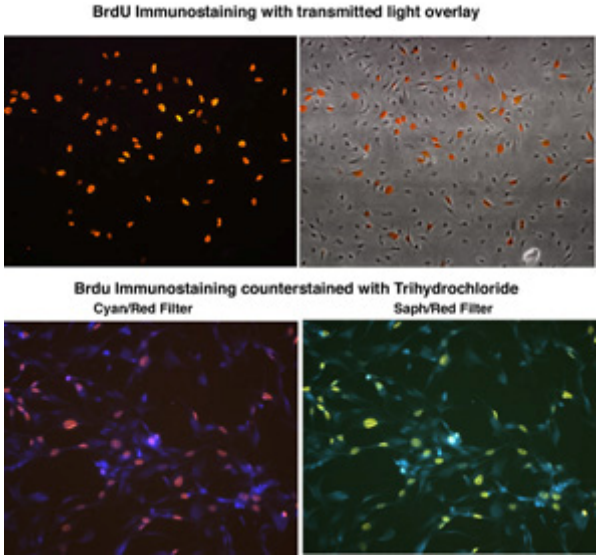
Culture Cell Fluorescence Immunostaining for BrdU:



1. Wash plates with PBS 3x.

2. Fix them in 4%PFA, 20 min at RT.
3. Wash them in PBS 3x.
4. 0.5% Triton X-100 in PBS, 15min at 4 筭
5. Wash slides in PBS 3x.
6. 2N HCL(in Distilled water) 30min at 37 筭(DNA denaturation).
7. Wash in PBS 5x
8. Incubate slides with 1%BSA in PBS at RT for 30min.
9. Mouse anti-BrdU diluted to 1:1000 with 1% NDS and1%BSA in PBS, incubate at RT for 2hrs.
10. Wash with PBS 3x.
11. 1: 100 dilution of Rhodamine(Tritc) conjugated Donkey anti mouse IgG with 1% NDS,1%BSA in PBS, incubate at RT for 1 hr.
12. Wash in PBS 3x
13. Mount with 50% glycerol in PBS.

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Culture Cell Enzyme Immunostaining for BrdU:

1. Wash plates with in PBS 3x.
2. Put 4%PFA 20 min at RT.
3. Wash with PBS 3x.
4. 0.5% Triton X-100 in PBS, 15min at 4 筭
5. Wash with PBS 3x.
6. Incubate with 2N HCL(in Distilled water) 30min at 37 筭(DNA denaturation).
7. Wash with PBS 5x
8. 1% BSA ,incubate for 30min at RT.
9. Mouse anti-BrdU diluted to 1:1000 with 1% NGS,1%BSA in PBS, incubate at RT for 2hrs.
10. Wash withPBS 3x.
11. Dilute Goat Bionytilated anti-Mouse IgG to 1:200 with1% NGS and 0.1% BSA in PBS, incubate at RT for 1 hour. Rinse with PBS 3x.
12. ABC at RT for 1 hour. Rinse in PBS 3x.
    (ABC solution: 5ml PBS with 1 drop A and 1 drop B, mix well)
13. DAB reaction: 2.5ml of distilled water + 1 drop Buffer + 2 drop DAB solution+1 drop hydrogen peroxide. Needs about 8 min to stop DAB reaction.
14. Rinse with distilled water 3x.
15. Countstain with Mayer's Hematoxylin

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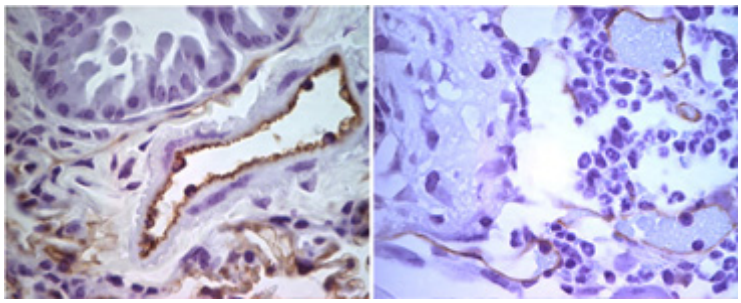
Frozen Section- Enzyme Immunostaining for CD31 (endothelium):



1. Rinse slides 3 times with PBS.
2. Put them in 3% hydrogen peroxide in Distill water, 30min. Then rinse in PBS 3x.
3. Incubate with 1xPower Block at RT for 20min.
4. Rinse 3 times with PBS.
5. Put them in Rat anti-Mouse CD31 diluted to 1:100 with 1% NRS in 0.1%BSA+ PBS, incubate at 4C overnight. Rinse in PBS x3.
6. Then in Ribbit Bionytilated anti-Rat IgG 1:200 in PBS with 1% NRS and 0.1% BSA, incubate at RT for 1 hour. Rinse in PBS 3x.
7. ABC at RT for 1 hour. Rinse in PBS 3x. (ABC solution: 5ml PBS with 1 drop A and 1 drop B, mix well)
8. DAB reaction: 2.5ml of distilled water + 1 drop Buffer + 2 drop DAB solution + 1 drop hydrogen peroxide. ~Needs about 8 min to stop DAB reaction.
9. Rinse in distilled water 3x.
10. Countstain with Mayer's Hematoxylin.

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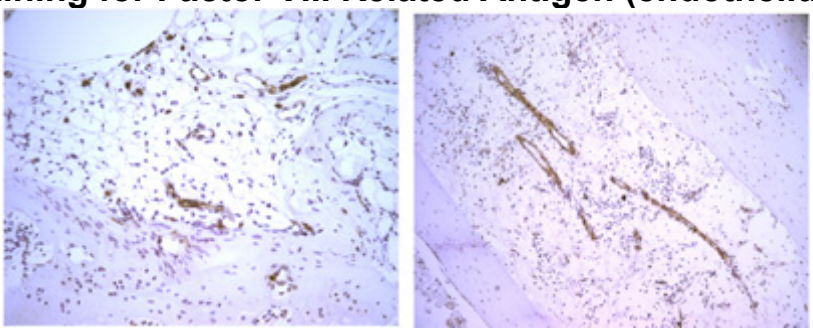
Immunostaining for Factor VIII Related Antigen (endothelium):



1. Deparaffinize and rehydrate tissue sections. Rinse 3 times with PBS.
2. 0.1%Pepsin in 0.1N HCL in PBS incubated sections at 37°C for 40min.
3. Rinse with PSA 3x
4. 0.3% hydrogen peroxide in PBS, 30min. Then rinse in PBS 3x
5. 5% normal goad serum in PBS at RT for 1 hour.
6. Rabbit anti-Human Factor VIII Related Antigen diluted to 1:500 with 1% NGS in PBS, incubated at 4°C overnight. Rinse in PBS x3.
7. Goat Bionitylated anti-Rabbit IgG 1:200 in 1% NGS in PBS, incubated at RT for 1 hour. Rinse in PBS 3x.
8. ABC at RT for 1 hour. Rinse in PBS 3x. (ABC solution: 5ml PBS with 1 drop A and 1 drop B, mix well)
9. DAB reaction: 2.5ml of distilled water + 1 drop Buffer + 2 drop DAB solution + 1 drop hydrogen peroxide. ~Needs 8 min to stop DAB reaction.
10. Rinse in distilled water 3x.

11. Counterstain and mount.

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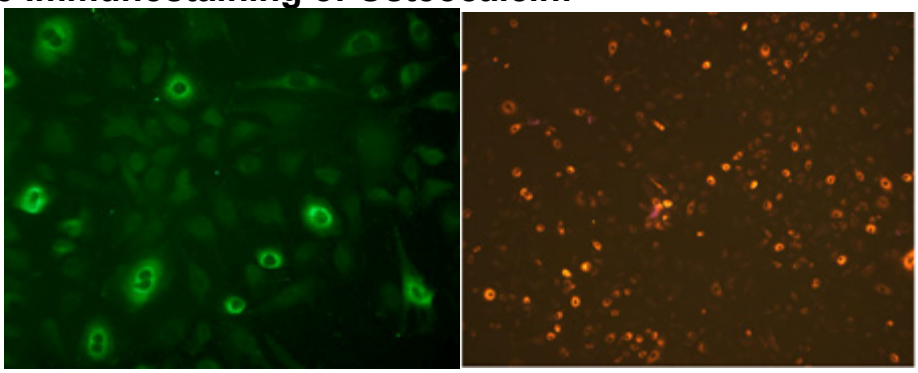
Cell Culture Immunostaining of Osteocalcin:



1. Wash with PBS 3x.
2. Fixed in 4%PFA, 20 min at RT.
3. Wash with PBS 3x.
4. 0.5% Triton X-100 in PBS, 15 min at 4 ⁰C.
5. Wash with PBS 3x
6. 1% BSA incubated for 30min at RT.
7. 1:100, 1: 200, 1:300 dilution of Goat anti- osteocalcin, incubated at 4 ⁰C, overnight.
8. Wash with PBS 3x
9. 1: 100 dilution of Rhodamine(Tritc) conjugated Donkey anti Goat IgG with 1%normal donkey serum+1%BSA in PBS, incubated at RT for 1 hr.
10. Wash with PBS 3x
11. Mount with 50% glycerol in PBS.

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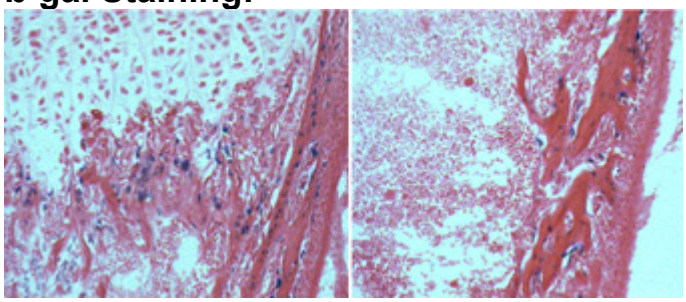
ß-gal Staining:



Reagents:

0.1M Phosphate Buffer (pH 7.3):

0.1M Sodium Phosphate monobasic	115ml
0.1M sodium phosphate dibasic	385ml
Total Volume	**500 ml**

Fix solution:

0.5% Gluteraldehyde:

25% gluteraldehyde	0.4 ml
100mM EGTA pH 7.3	2.5 ml
1M magnesium chloride	0.1 ml
0.1 M sodium phosphate pH 7.3	47.0 ml
Total Volume	**50.0 ml**

4% Paraformadehyde:

paraformadehyde	20 g
1M magnesium chloride	1ml
100mM EGTA	25ml
~500 ml PBS	

- Prepare fresh each time

Wash buffer:

1M magnesium chloride	0.4 ml
1% deoxycholate	2.0 ml
2% Nonidet-P40	2.0 ml
0.1M sodium phosphate pH 7.3	195.6 ml
Total Volume	**200.0 ml**

X-gal Staining:

25mg/ml x-gal stock dissolved in di-methyl formamide	2.0 ml
potassium ferrocyanide (Sigma P-9387)	0.106 g
potassium ferricyanide (Sigma P-8131)	0.082 g
wash buffer	48.0 ml
Total Volume	**50 ml**

- This buffer can be reused, filter after use and store in the dark.
- Also note, crystal from due to di-methyl formamide. If these crystal are a problem, prepare X-gal stock in dimethyl sulfoxide.

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X-gal Staining of Bone:

Whole mount staining:

1. Dissect bone out of mice.
2. Fix at tissue in fixative for 30min-2h at RT or overnight in 4 ℅ with 4% paraformadehyde.
3. Rinse with PBS.
4. Soak tissue in X-gal staining solution for 4 hours at 37 ℅.
5. Pour off the staining solution, replace with wash buffer, 2 times.
6. Sock tissue in 30% sucrose in PBS.
7. Embed and cut frozen section.

Frozen section staining:

1. Bone after fixed and briefly rinse with PBS.
2. Soak with decal solution. Gently rock at 4 ℅ for 1-3 days.
3. Soak with 30% sucrose in PBS + 2mM MgCl2 at 4 ℅ for overnight.
4. Embed in OCT.
5. Cut frozen section. Place slides in wash solution.
6. Incubate sections with x-gal solution at 37 ℅ for 1-3 hours (monitor staining every 30 min. It usually takes 2 hours).
7. Rinse slides in PBS, 3 times.
8. Rinse with 95% EtoH and stain with Eosin 20 secs for counterstain.
9. Dehydrate and coverslip

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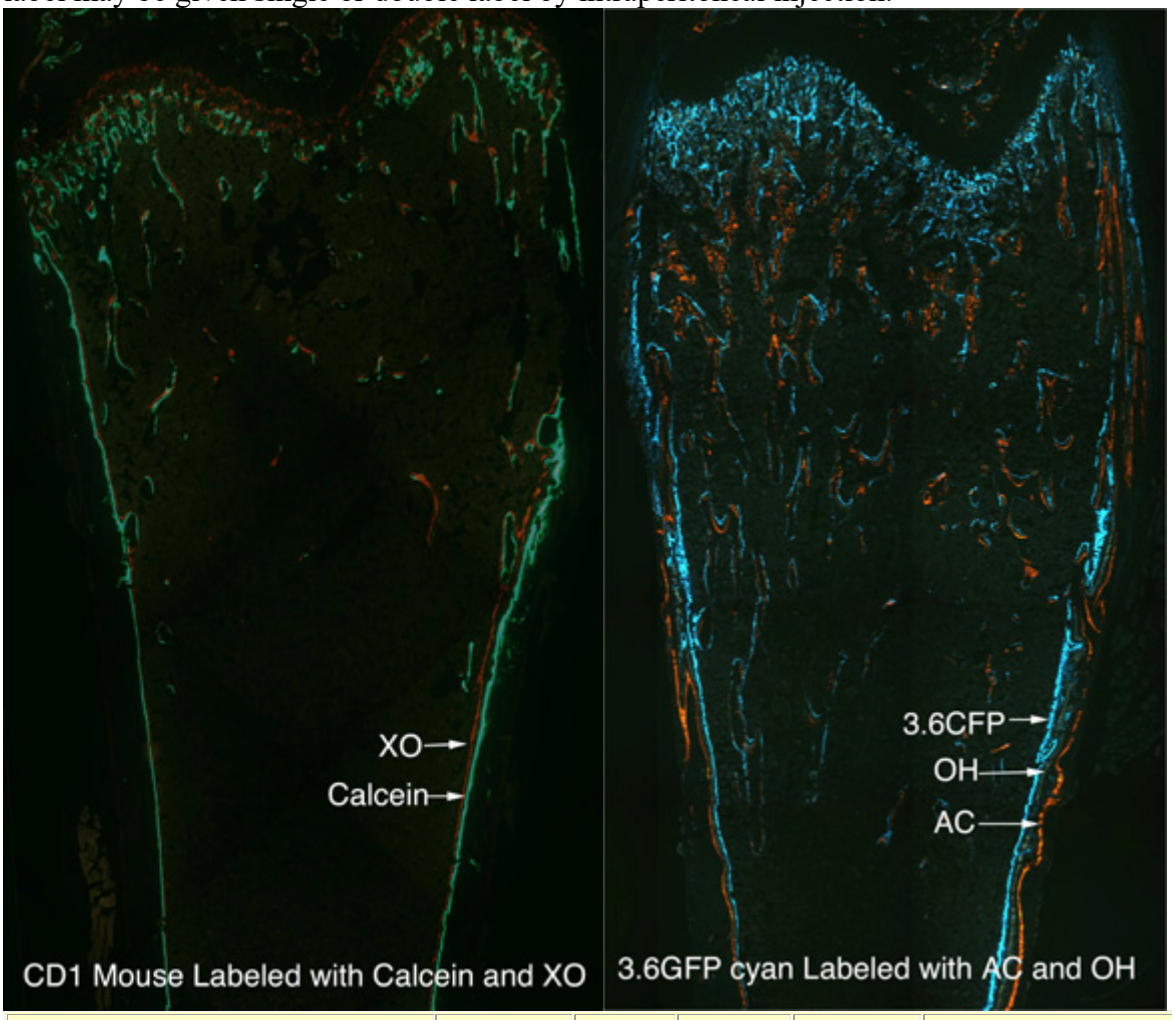
Fluorescent Label in Bone

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

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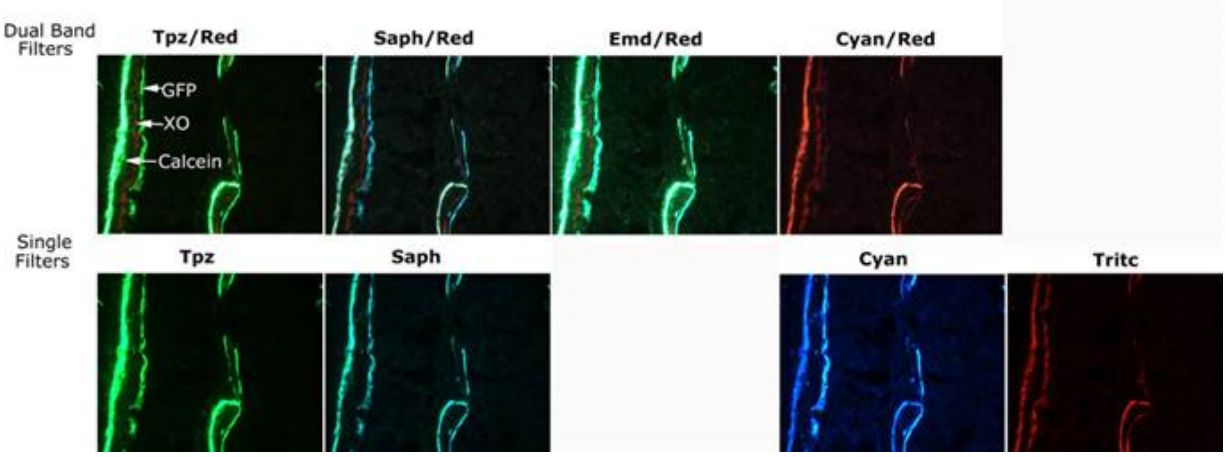
Viewing GFP in bone

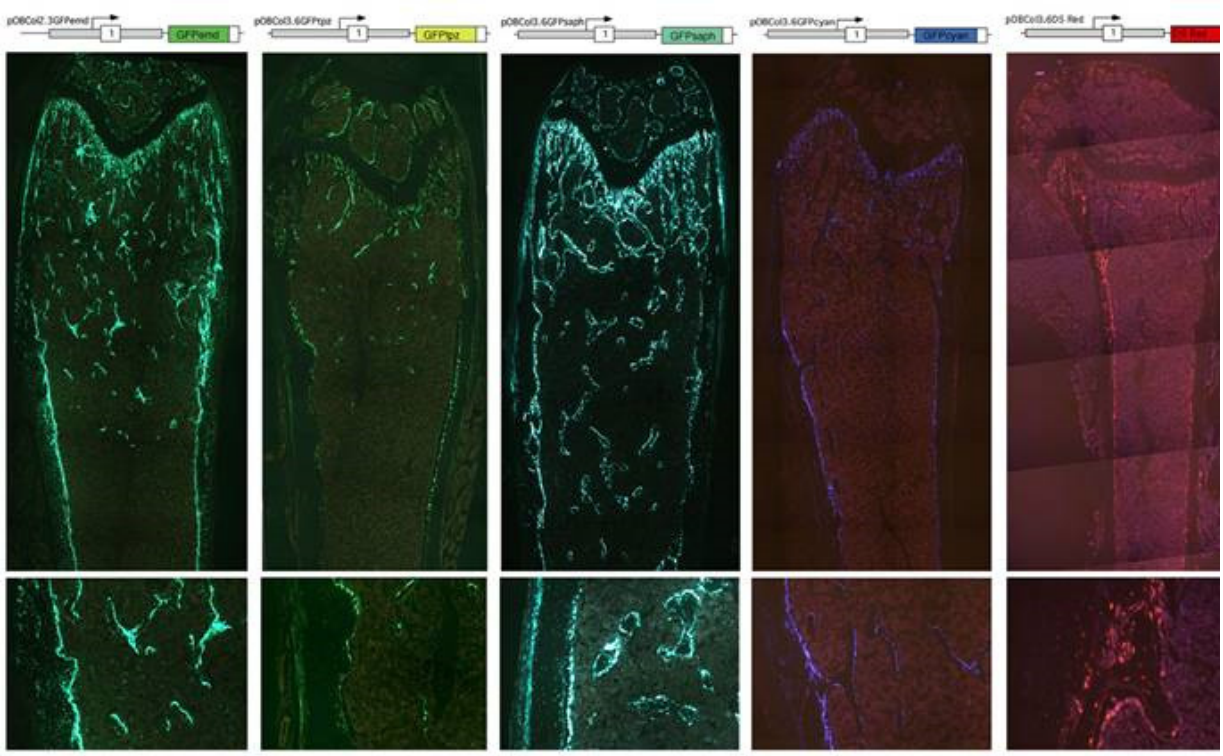
The optics have been developed for standard mercury arc epifluoresence and single filter cubes that should be available on most fluorescent microscopes. It was the discovery that a dual filter cube allowed the GFP signal to be distinguished from the autofluorescent color of bone marrow that made the imaging feasible. The figure below contrasts the image using a single or dual filter cube (for the GFP and red shifted for the marrow). The images are captured on a color digital camera which immediately reveals the distinction between each color. Although the images should be visable using a greyscale camera with pseudocoloring, the color camera with sufficient sensitivity to the color ranges make the process effortless.

Filter Selection: The table gives the filter cubes that are listed in the Chroma catalogue that we are currently using to distinguish different colors of GFP in bone. There are optimized for a specific GFP spectrum with minimal spillage into other colors.

		For GFP			For Tissue Bkgd and ds Red		
GFP	Cat No.	Excitation	Dichroic	Emission	Excitation	Dichroic	Emission
Cyan(CFP)	51018	410/30	425	470/20	530/20	555	625/40
Saph	51021	405/20	415	505/45	545/15	560	595/60
EGFP	51019	475/25	495	525/45	565/25	580	620/60
Topaz(YFP)	51004v2	470/10	485	510/15	555/40	575	600/80

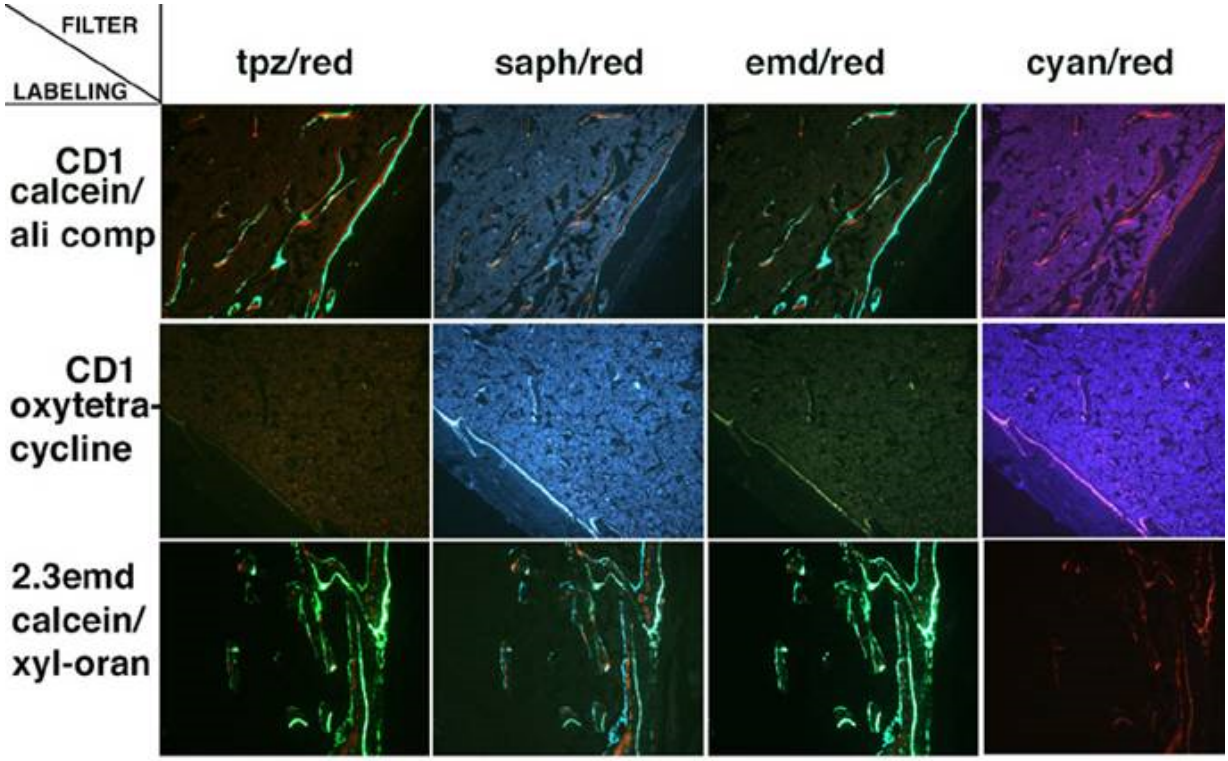


All of these filters have a dual filter design. The red-based second filter has two functions. First, it displaces the autofluoresence of the bone marrow away from the blue/green spectrum of the cyan, saph, eGFP and topaz colors so that they can be detected from a pink background. Without the second red filter, it is difficult to distinguish the GFP color from back ground autofluoresence. The second advantage it the ability to detect red-based second antibodies and mineral ization stains (xylenol orange and alizarin complexone) against the GFP color. Unlike the tissue culture set, eGFP can be distinguished from cyan (still some spill over) but cannot be used with saph or topaz.



Colors of GFP in bone – The figure contrast the 5 colors of GFP driven by a strong bone restricted type I collagen fragment that have been used to develop transgenic mouse lines. The details of each line and their availability can be seen at xxx link. The figure shows the distribution of the GFP signal in a low power composite image and a higher power view from the came composite image.

Metabolic labeling - We surveyed various methods for single or dual labeling of bone to determine to optimal combination with colors of GFP. The figure below show a series of double labels of non-transgenic bone given 10 and 2 days before sacrifice in 4 month old mice viewed with the 5 filter set listed in the table above.


FILTER
LABELING
tpz/red
saph/red
emd/red
cyan/red
CD1 calcein/ ali comp
CD1 oxytetra-cycline
2.3emd calcein/ xyl-oran