INTRAVITAL IMAGING WORKSHOP
APRIL 27-MAY 1, 2015

INDIANA CENTER FOR BIOLOGICAL MICROSCOPY

NIH O'BRIEN NATIONAL CENTER FOR RENAL MICROSCOPY

Outline

Preparation/ Surgery

Positioning

Landmarks: Unstained/ Stained

- Visual Indicators Determine Problems

Fluorescent Markers: Dye Characterization

Microscope Objectives

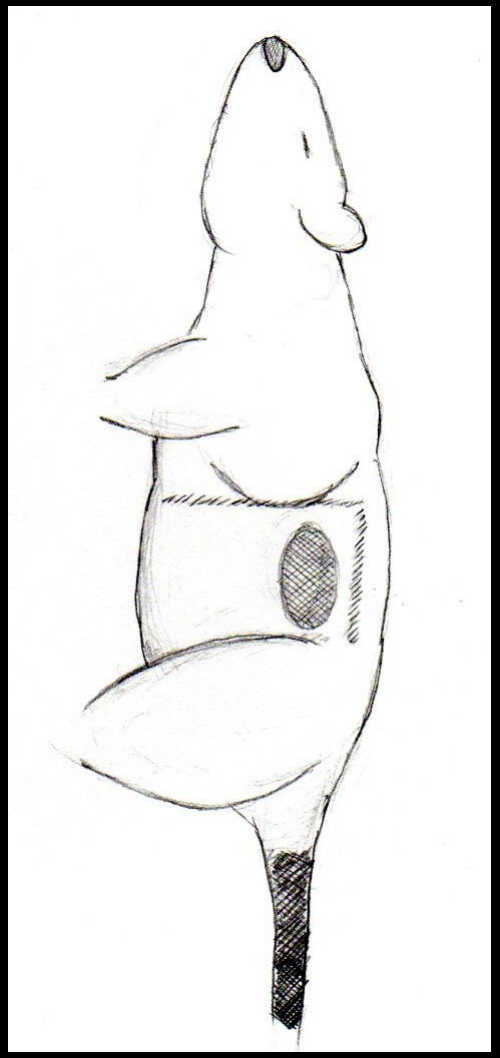
Detector Settings

Surgical Preparation

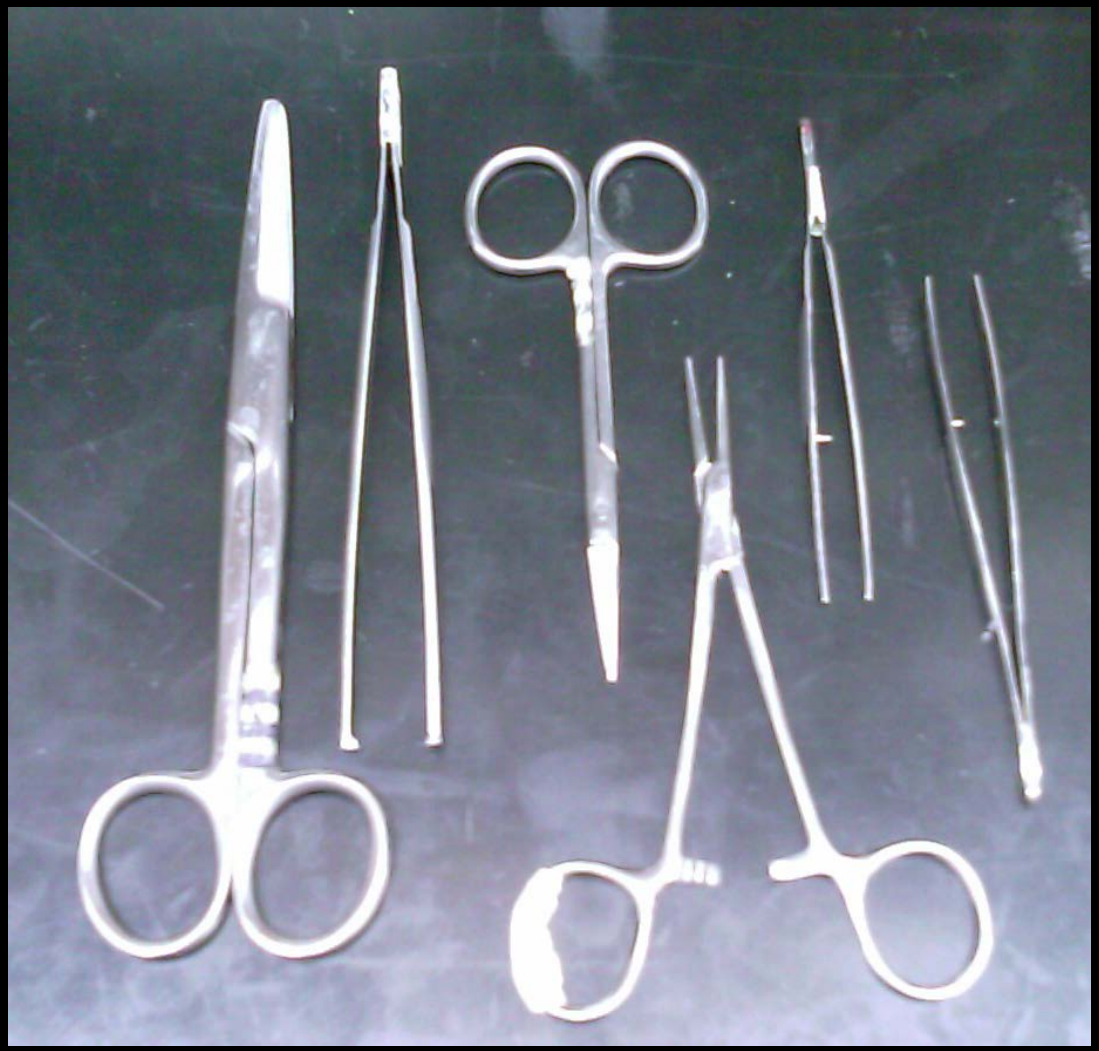
Short Term Imaging (less than 30')
Venous access:
Jugular- Infusion of probes
short line to minimize dead space
Rectal Temp Probe

Long Term Imaging (greater than 30-45')
Venous/Arterial access:
Femoral venous- continuous infusion of
0.9% Saline at ~1.5cc/Hr
Femoral arterial- BP/ HR monitoring, long line
Temp Probe in Saline dish, monitor fluid bath temp

During prep/ probe infusion: Receive ~ ¾ to 1.5 cc 0.9% Saline



Surgical Preparation



Hemostat
Crushing outer skin and muscle layers to prevent bleeding

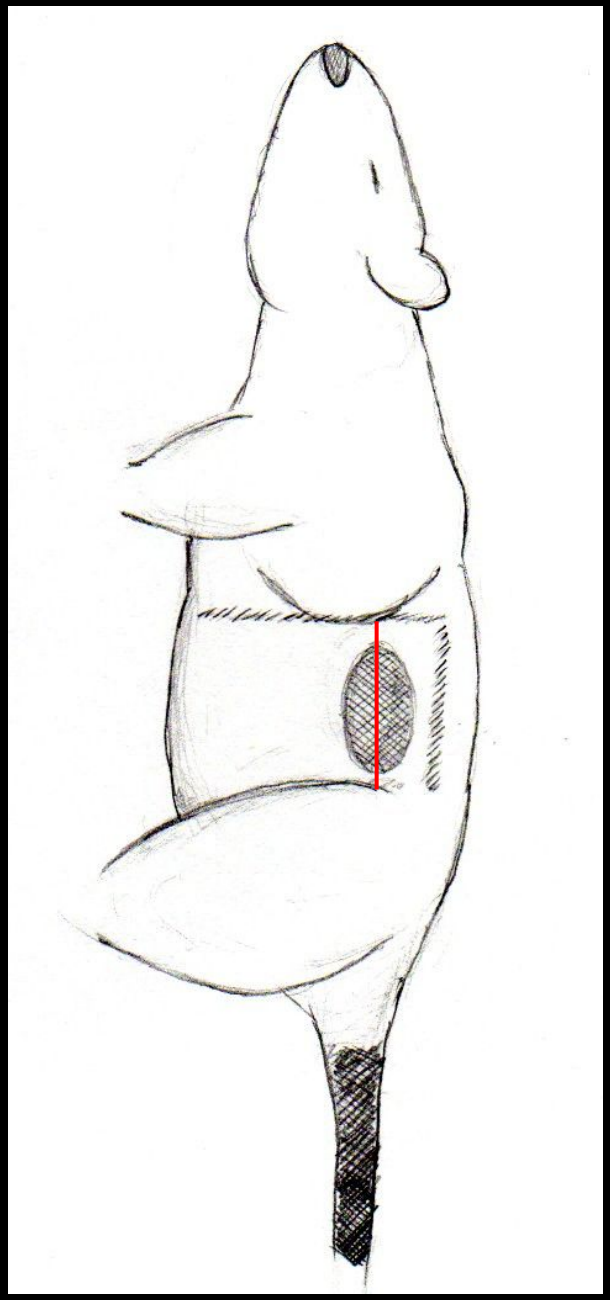
Long Forceps, w/ teeth
hold outer skin during cutting

Large Scissors
cutting outer skin

Small Scissors
cutting inner muscle layers

2 small forceps, blunt tipped w/ no Teeth to handle the fat/capsule surrounding the kidney

Surgical Preparation



1). Lay rat perfectly on its side w/ left side facing you.

2). Palpate flank gently to find kidney, draw line down flank if necessary (make this line as large as necessary).

3). Lift skin w/ large forceps, crush tissue w/ hemostats, hold for 5-10 seconds.

4). Using large scissors, cut across crushed tissue line, there should be no bleeding.

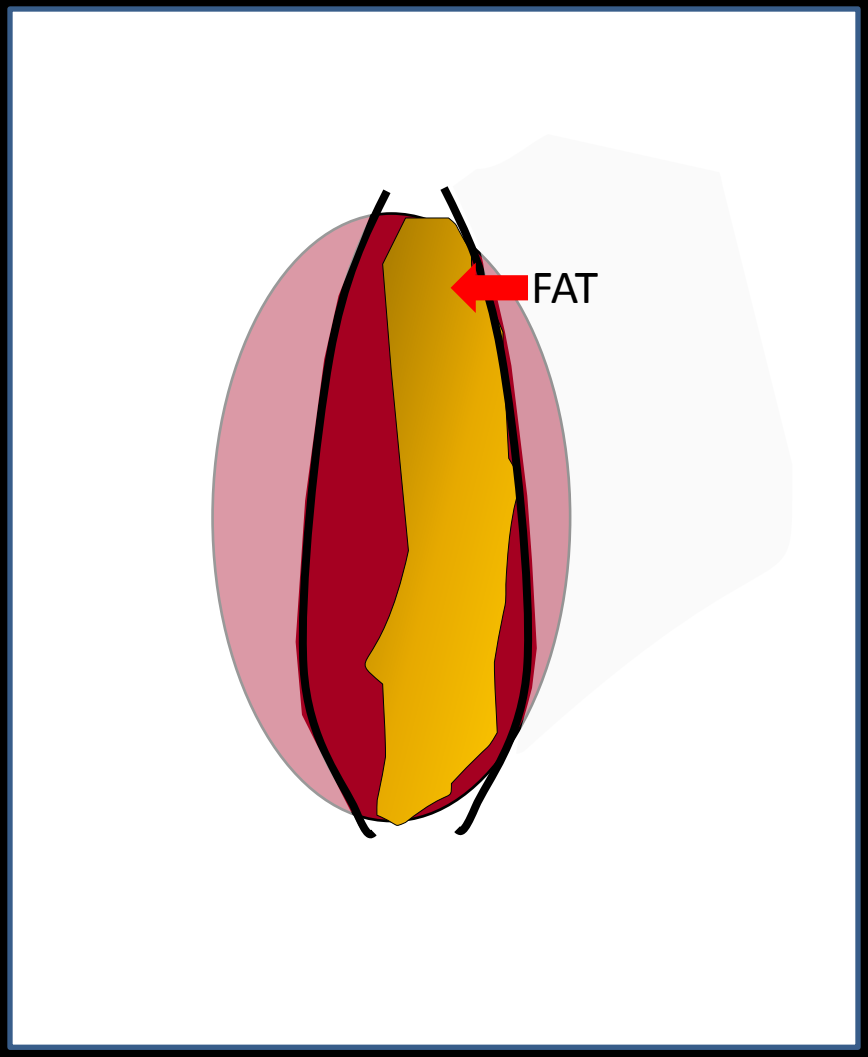
5). Repeat 3 & 4 w/ outer muscle layer, except use the small scissors

6). Crush the tissue on the 2nd muscle layer

7). Cut a small incision to visualize the kidney; a large incision will not hold the kidney out of the body.
It is easier to make another small cut than to stitch.

Surgical Preparation

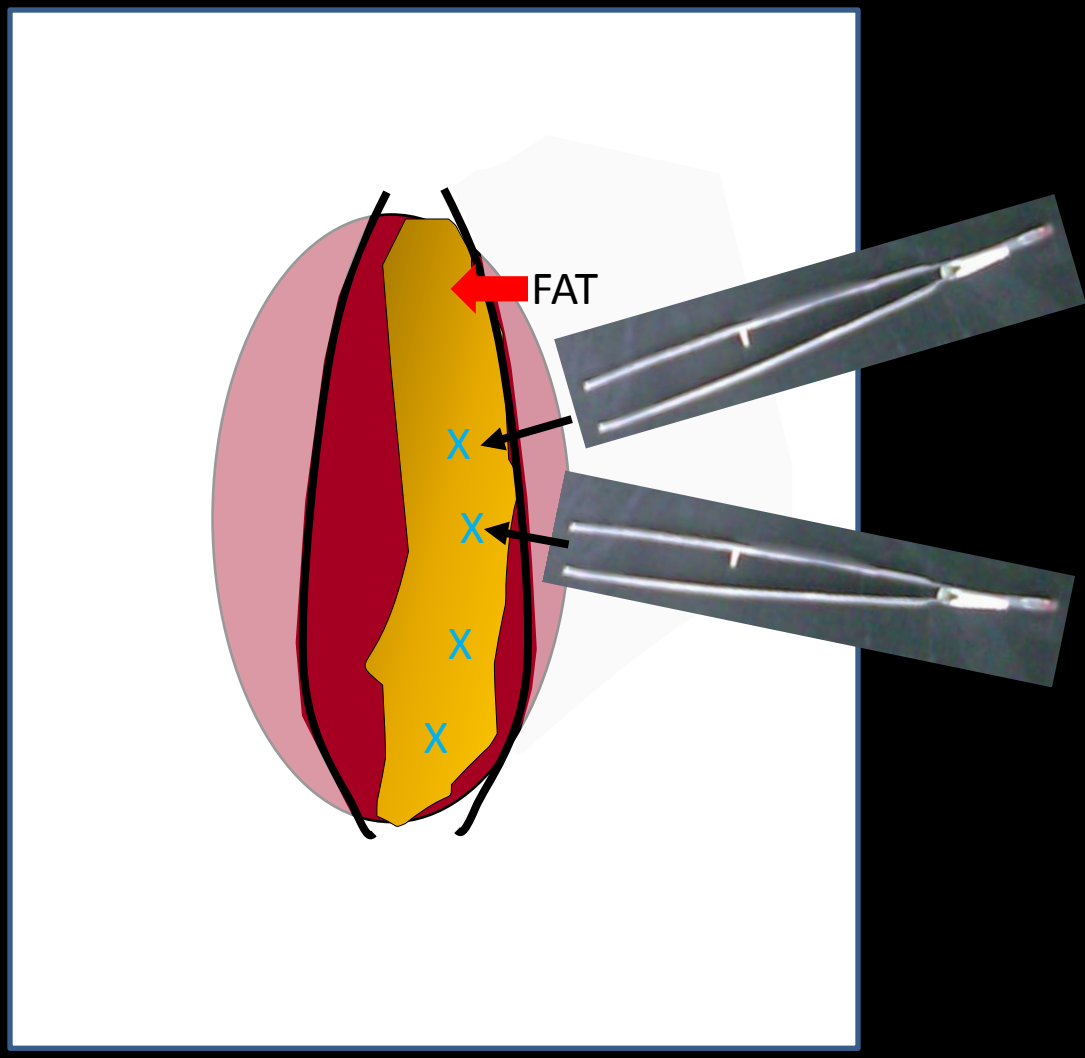
Head



Tail

Surgical Preparation

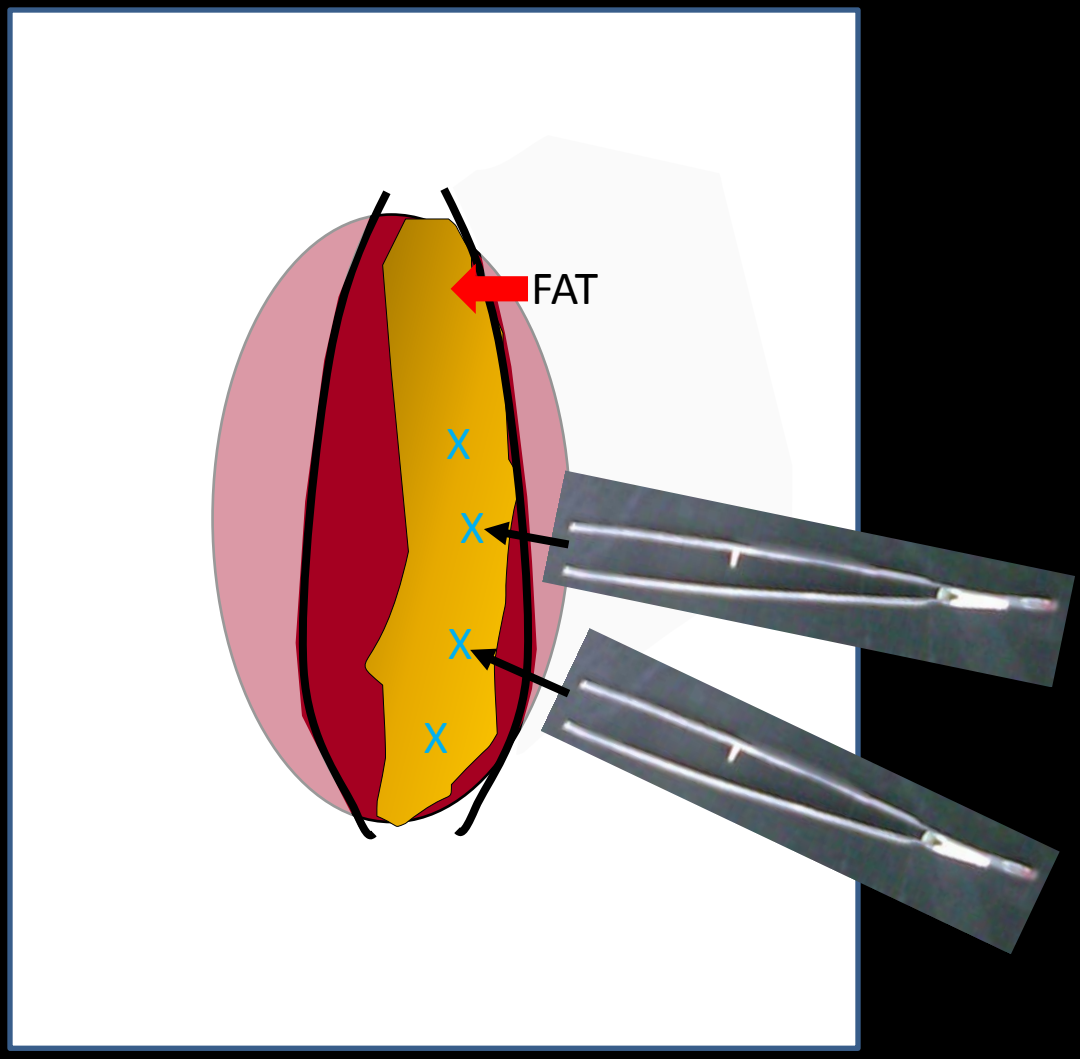
Head



Tail

Surgical Preparation

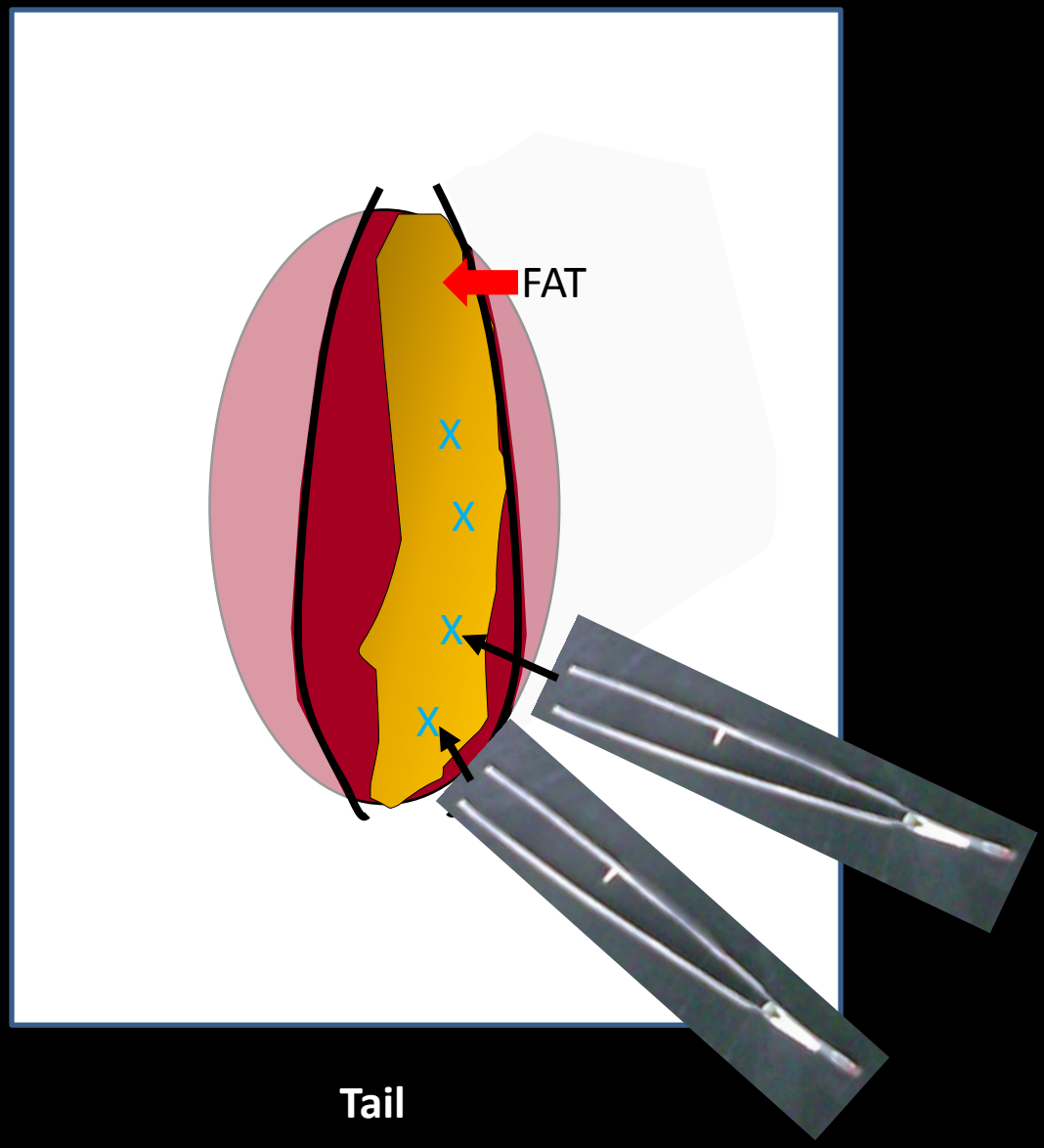
Head



Tail

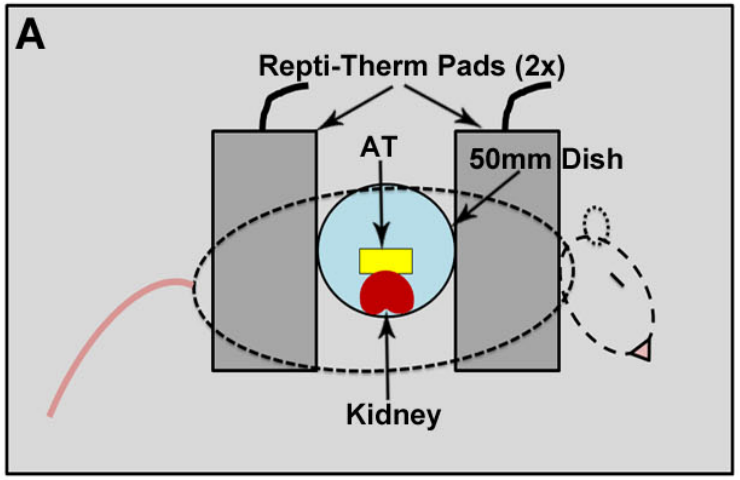
Surgical Preparation

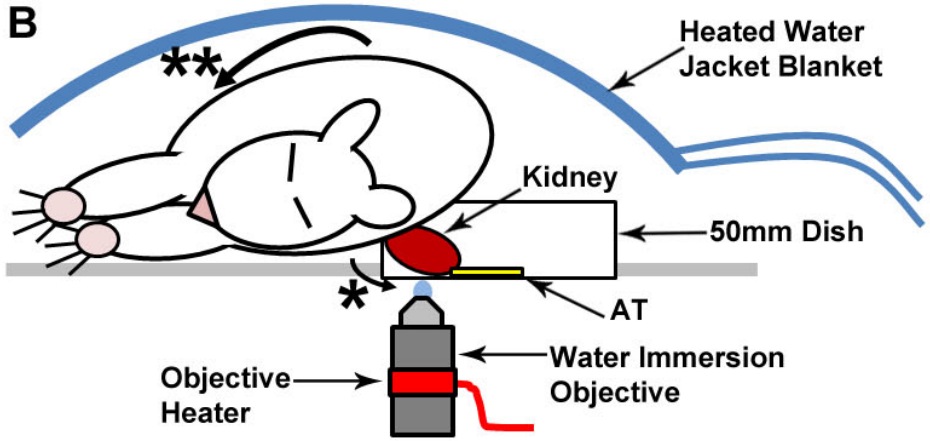
Head



Tail

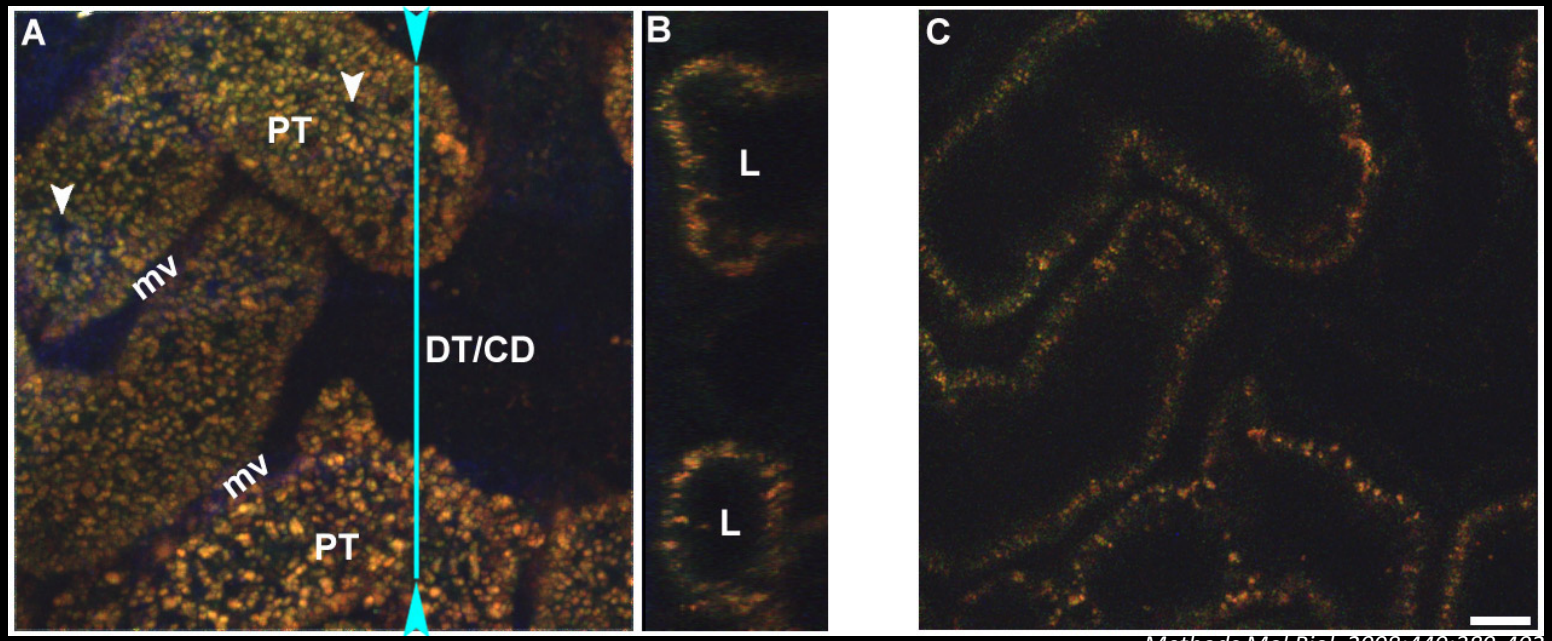
Placement on Stage



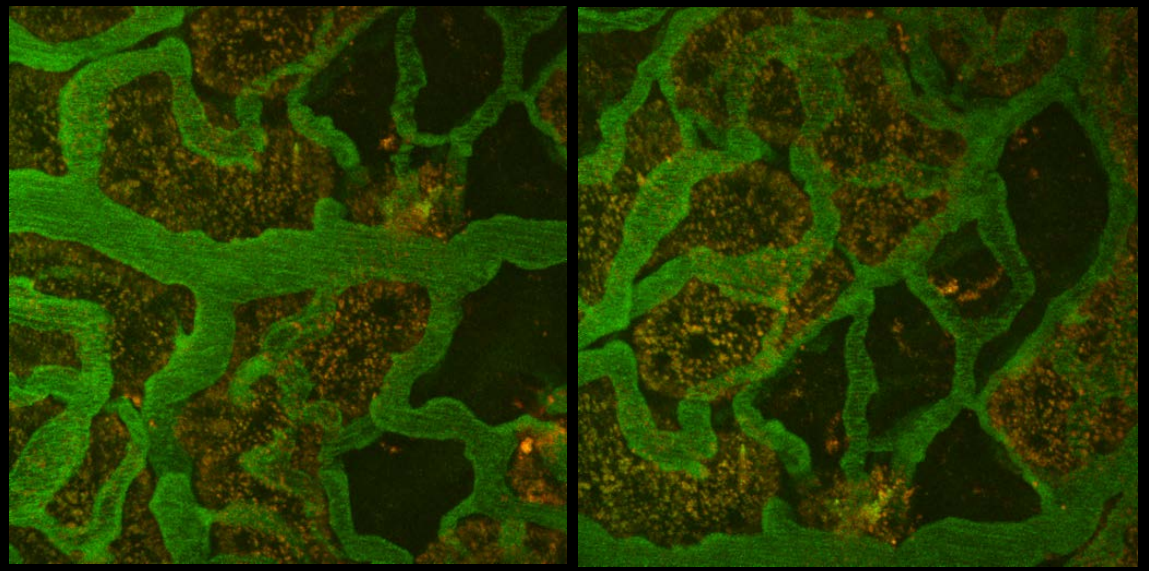


AT= Autoclave Tape

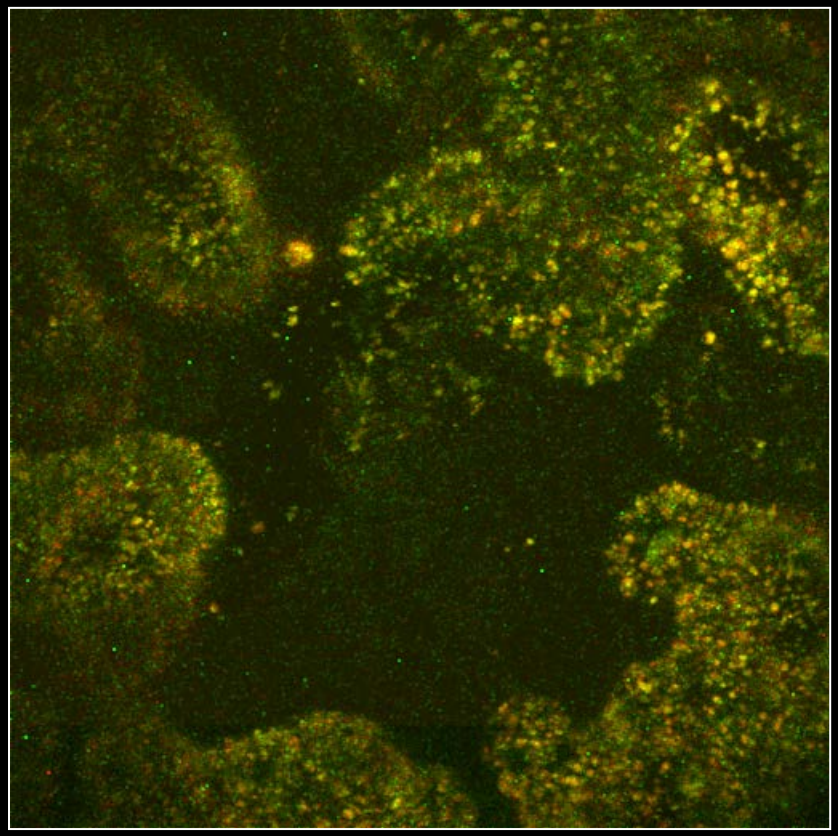
Landmarks

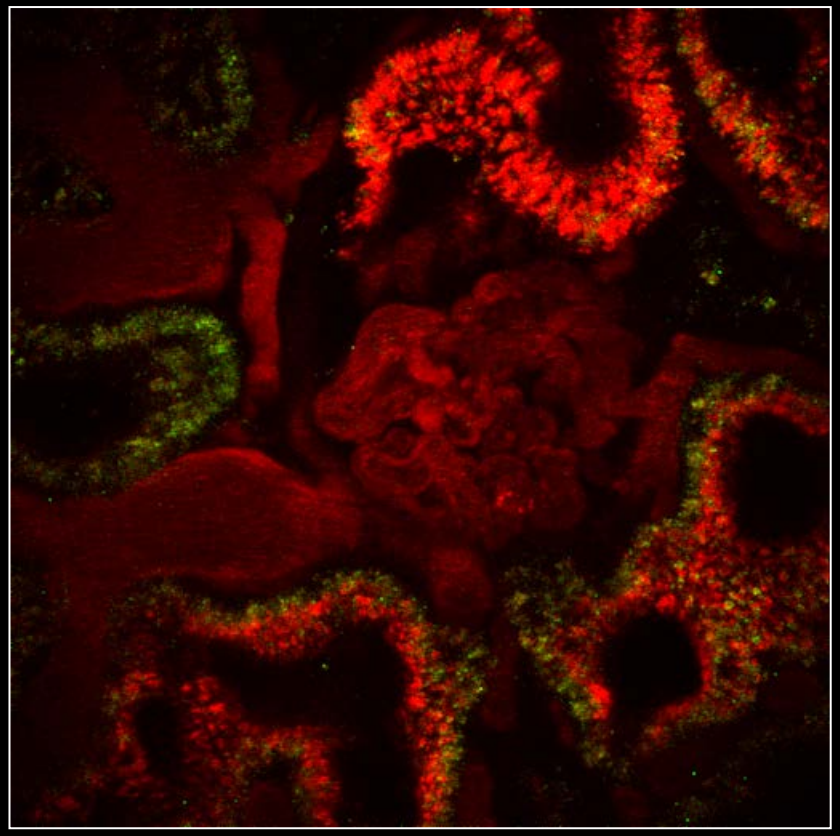


Methods Mol Biol. 2008;440:389-402



Landmarks

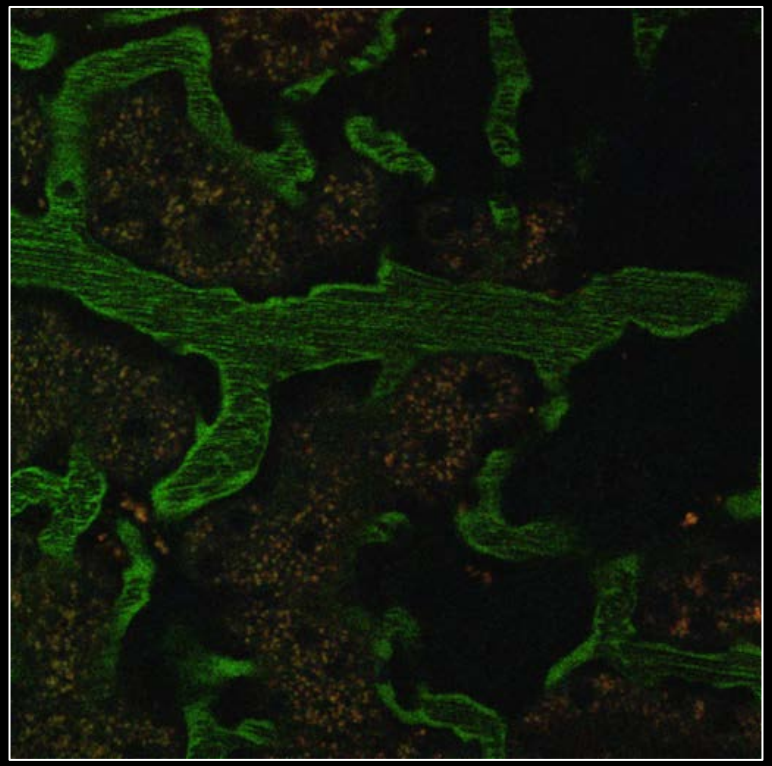


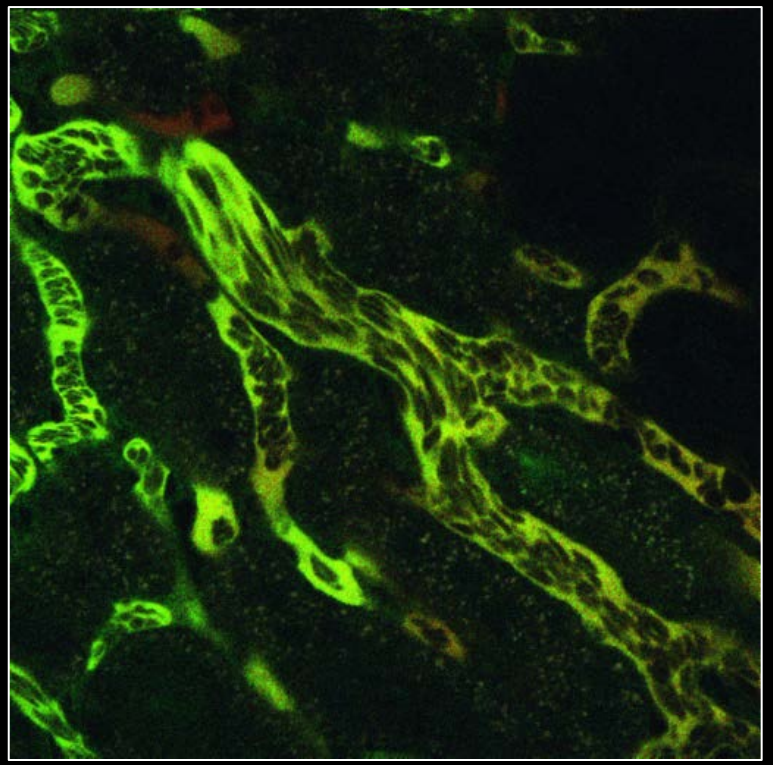


Landmarks/ Anomalies

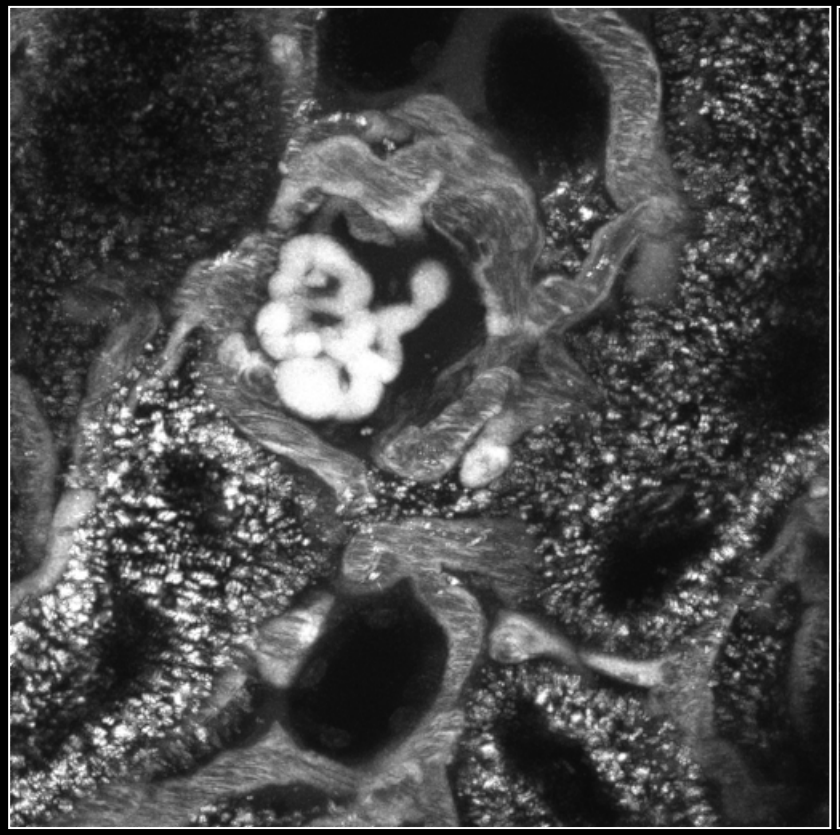
Normal Infusion:
1). Should see dye in bloodstream within 5-7 seconds
2). Appearance in field is fairly uniform
3). RBC's should appear as streaks across straight blood vessels

Alterations:
1). Appearance of dye exceeds 10 seconds
2). Appearance within field is staggered among vessels
3). Shape of RBC's is discernible, abundant plasma in vessels

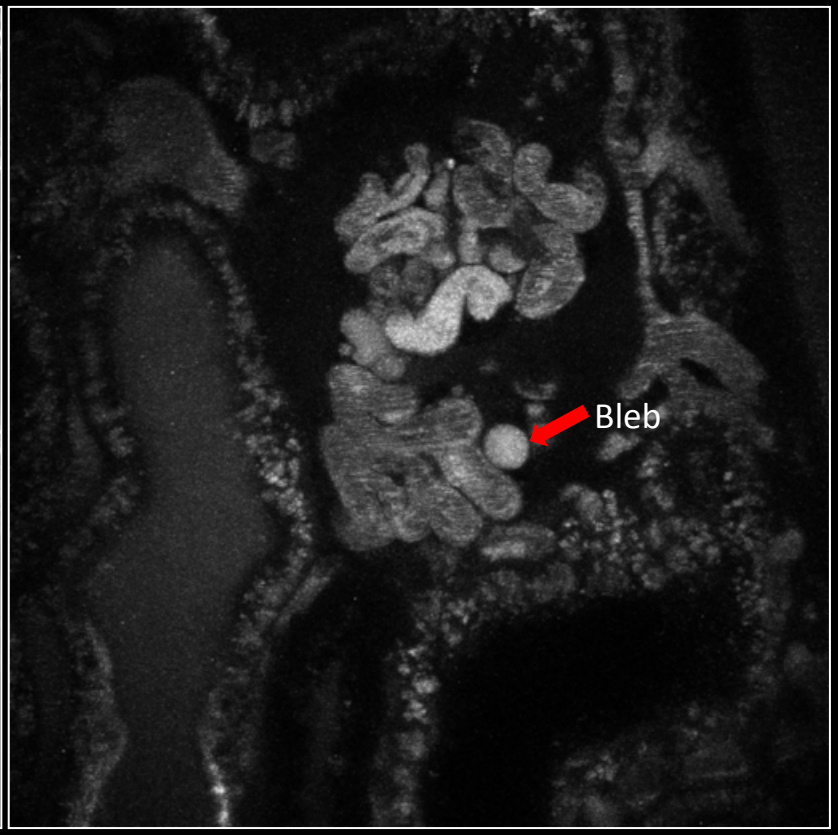




Landmarks/ Anomalies

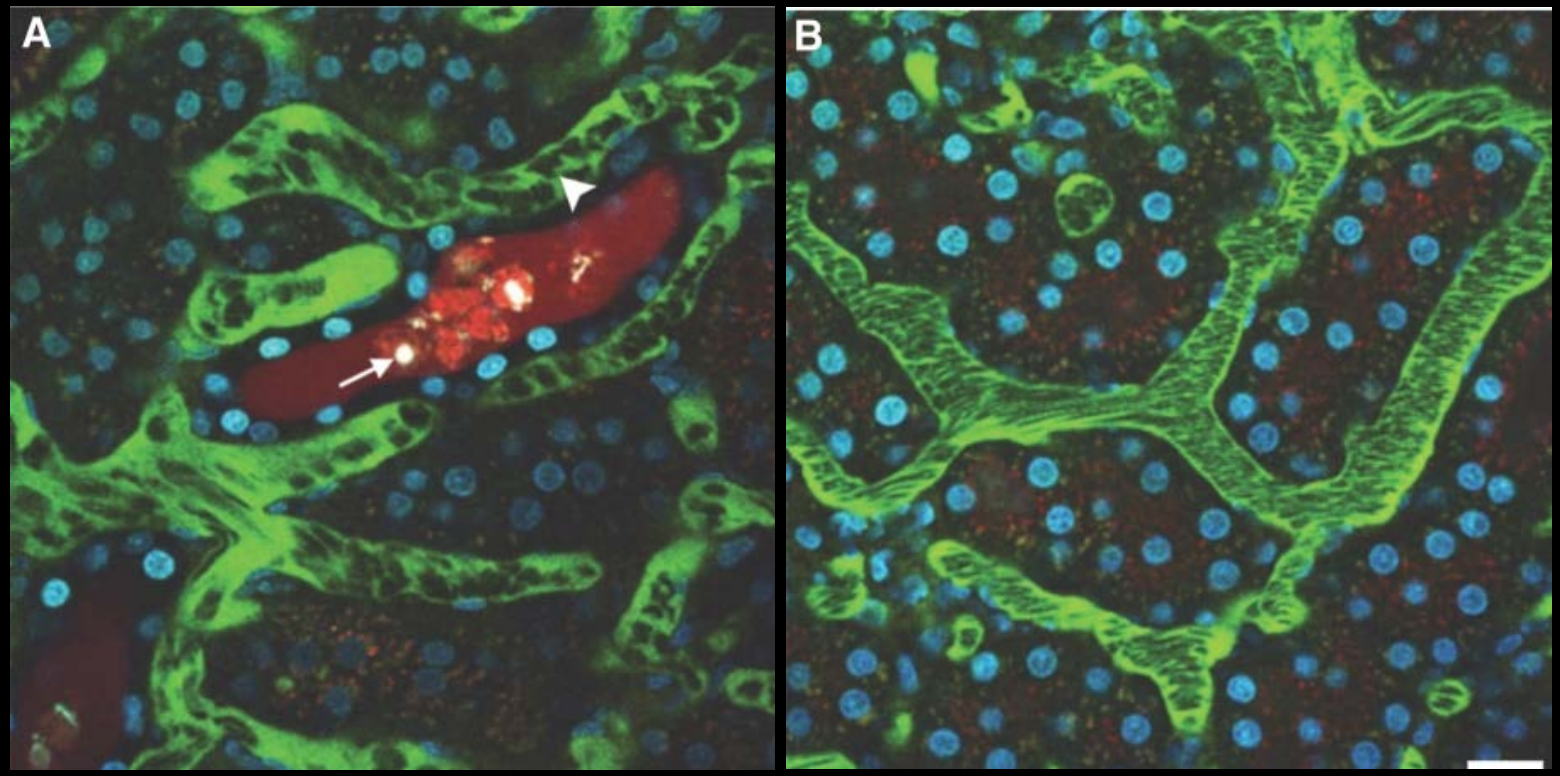


Sclerotic Glomerulus



Damaged Glomerulus

Landmarks/ Anomalies



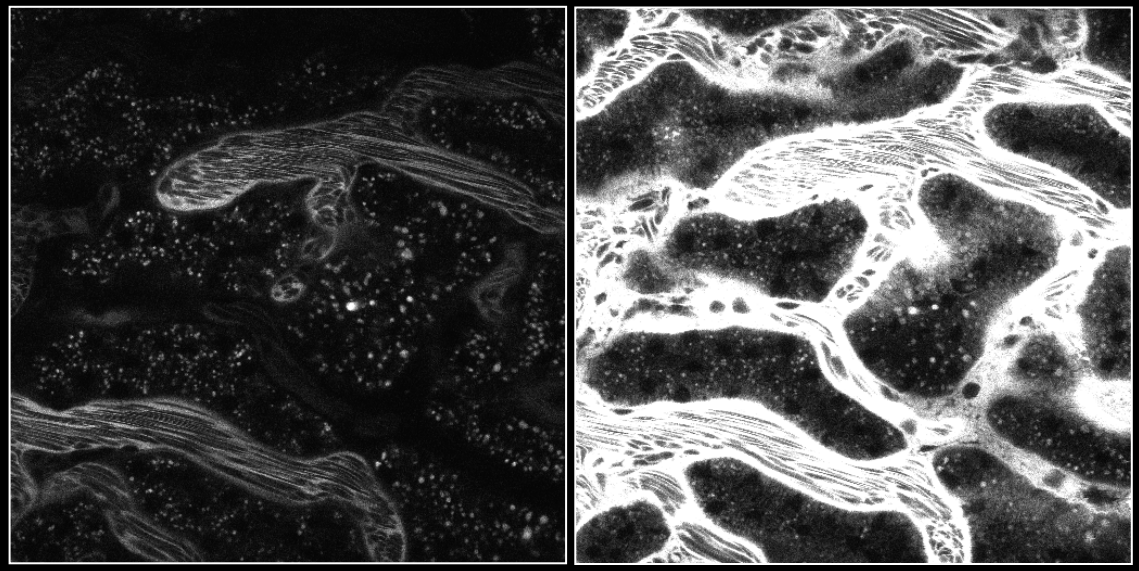
Soluble thrombomodulin protects ischemic kidneys. Sharfuddin AA et al, *J Am Soc Nephrol*. 2009 Mar;20(3):524-34.

Flow Anomolies
Proximal Tubule anomalies
Cast material

Fluorescent Markers: *in vitro* calibration

Important when setting up experiment to collect quantitative data.

Involving collection of images that will later be used to determine and subtract background values.



Fluorescent Markers: *in vitro* calibration
Prior use in cell culture

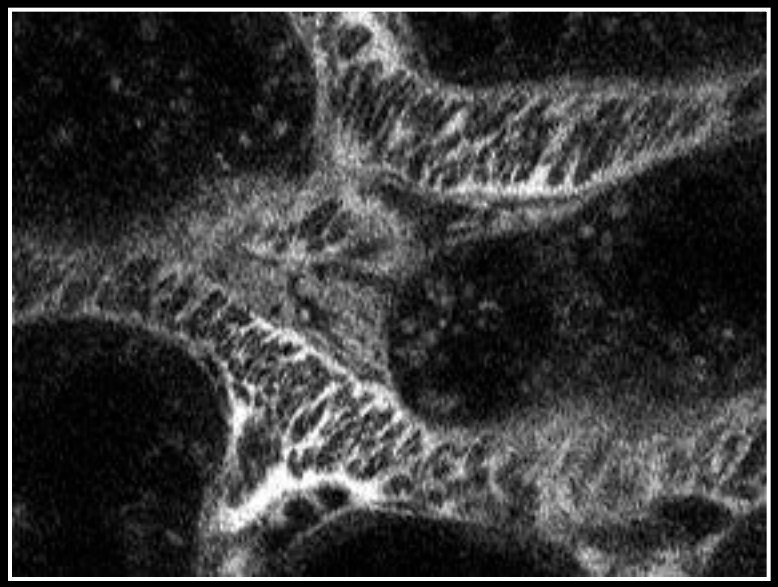
1.) Determine Cell Culture Concentrations (500ug/mL)

2.) Determine Plasma Volume of Rat (Typically ~3.5%; dyes do not cross into RBC's)

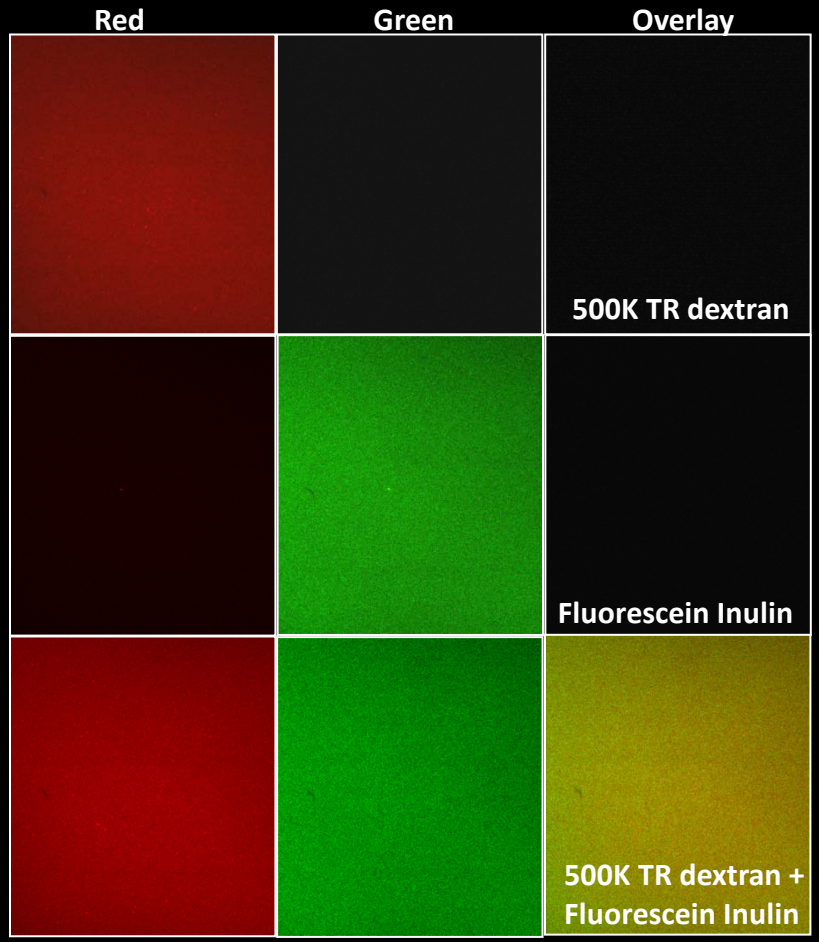
250g rat = 8.75 mL of plasma

Dose = 4.3mg; Add ~20%, final Dose= 5.16mg

3). Load syringe w/ 2-3 doses, carefully monitor fluorescence during infusion



Fluorescent Markers: *in vitro* calibration



1). Carry out a dilution series of your stock marker in PBS

2). Calculate a dilution factor of the stock solution (1:150, etc) that best uses the dynamic range of the microscope.

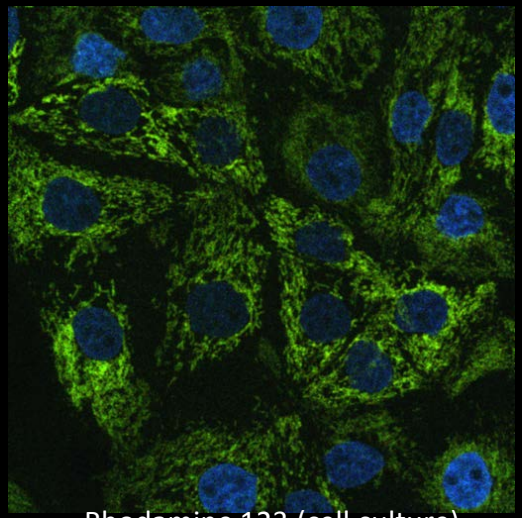
3). Calculate approximate plasma volume of rat bases on body weight (250g)= 8.75mL

4). Divide Plasma Volume (in uL)/ Dilution Factor) 8750/150= ~58.333uL stock

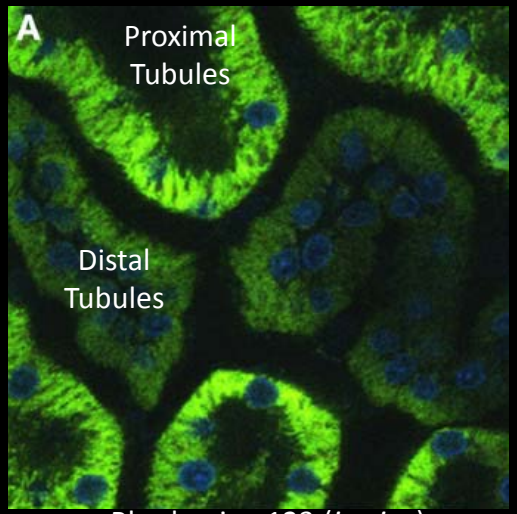
5). Add at least 20% extra (light scatter through tissue, dead space in venous line)

6). Dilute into at least 500-750uL of Normal Saline, watch monitor as you infuse to assure you do not saturate your specimen.
*Crucial probes retained in the blood.

Fluorescent Markers: *in vitro* characterization

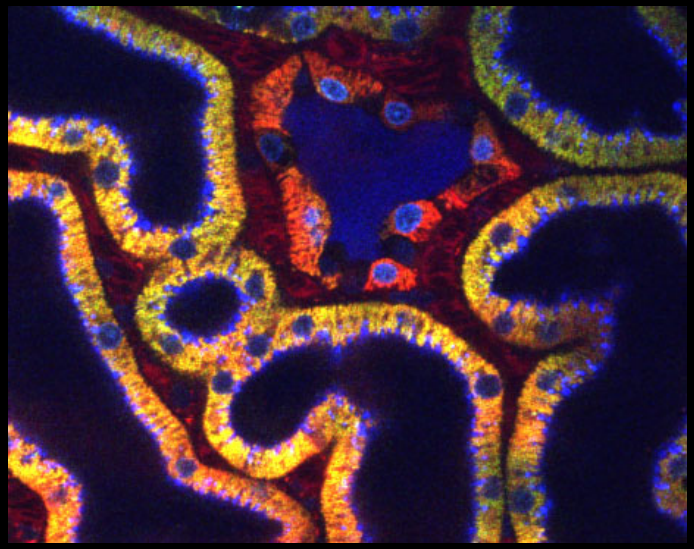


Rhodamine 123 (cell culture)

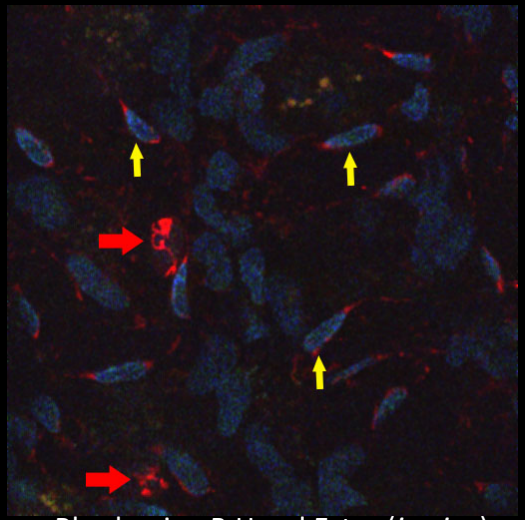


Rhodamine 123 (*in vivo*)

Adv Drug Deliv Rev. 2006 Sep 15;58(7):809-23 2006 Aug 15. Review



Rhodamine 123 (green)/TMRM (red)



Rhodamine B Hexyl Ester (*in vivo*)
White Blood Cell (red arrows)
Endothelial Cell (yellow arrows)

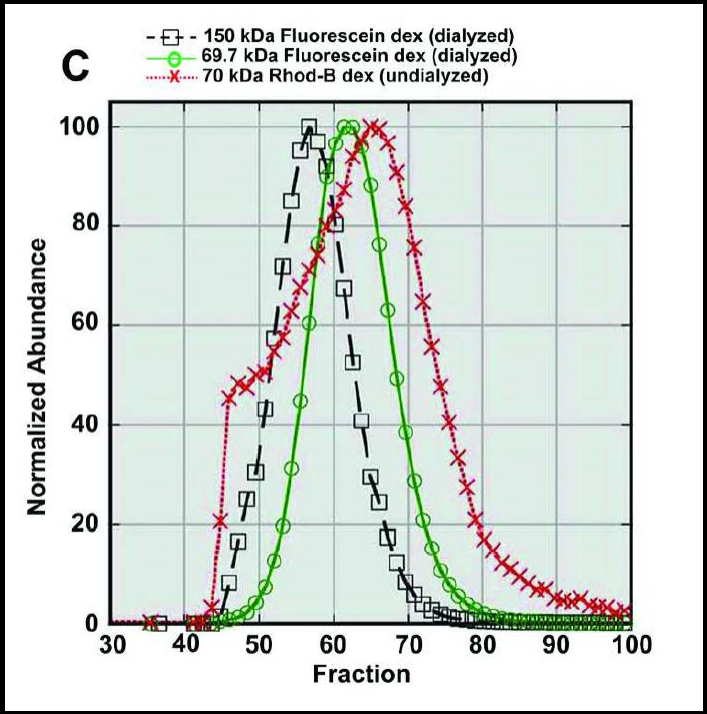
Adv Drug Deliv Rev. 2006 Sep 15;58(7):809-23 2006 Aug 15. Review

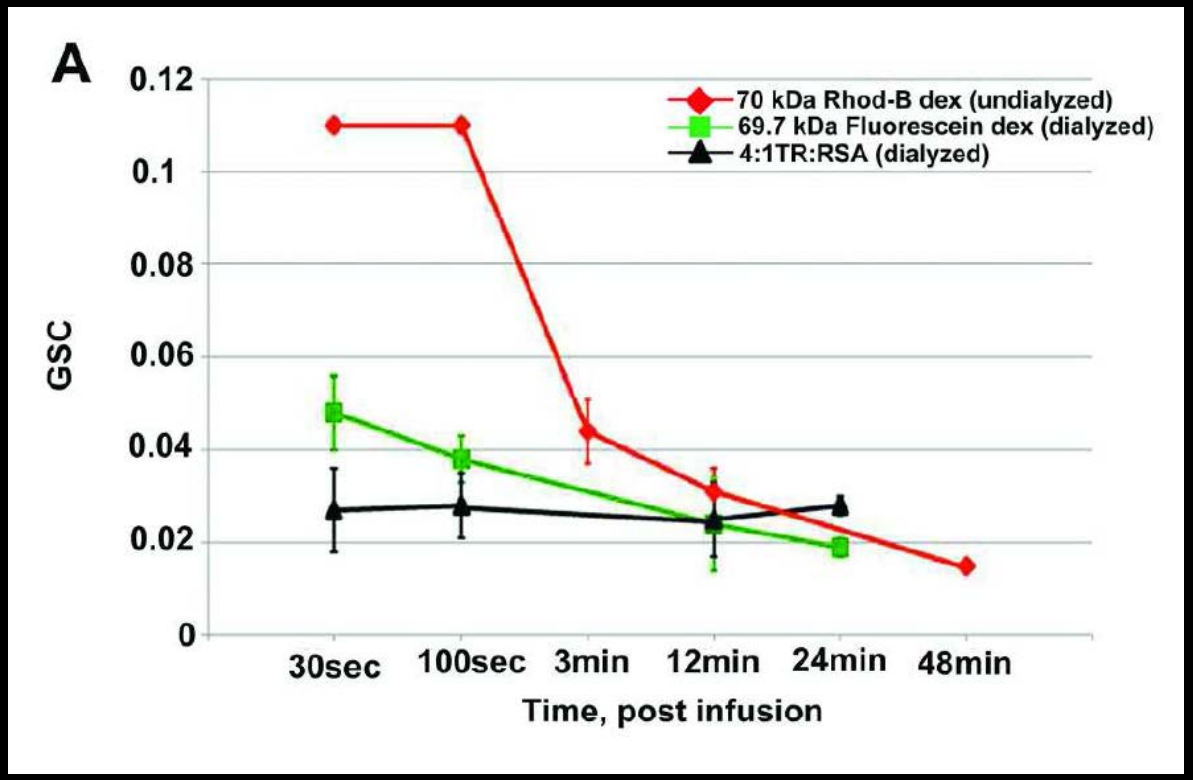
Fluorescent Markers: Dextrans

SUPPLIERS:

Invitrogen/ Molecular Probes	TdB Consultancy (Uppsala)
More Expensive	Less Expensive
Greater # of Fluorophores to Dextran	Dimmer probes
Size dispersion not well characterized	Better quality in size dispersion (Typically no need to dialyze)
Smaller MW probes (3,000 and 10,000) are OK	Larger MW probes (40,000+) are of better quality (this is key in correct interpretation of any type of permeability studies, vascular or glomerular)

Fluorescent Markers: Dextrans

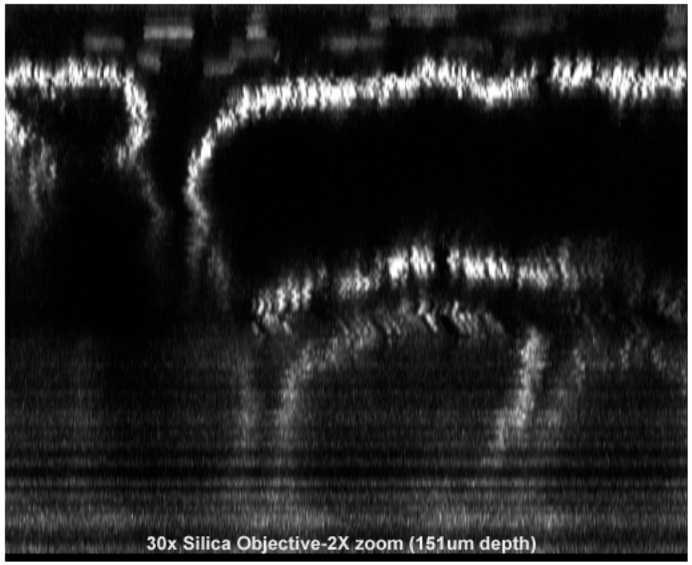


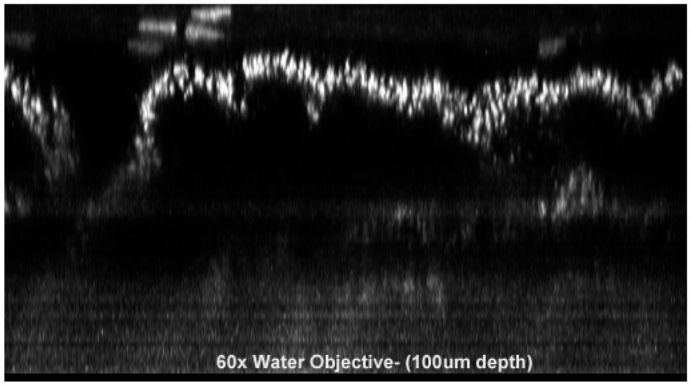


Microscope Objectives:

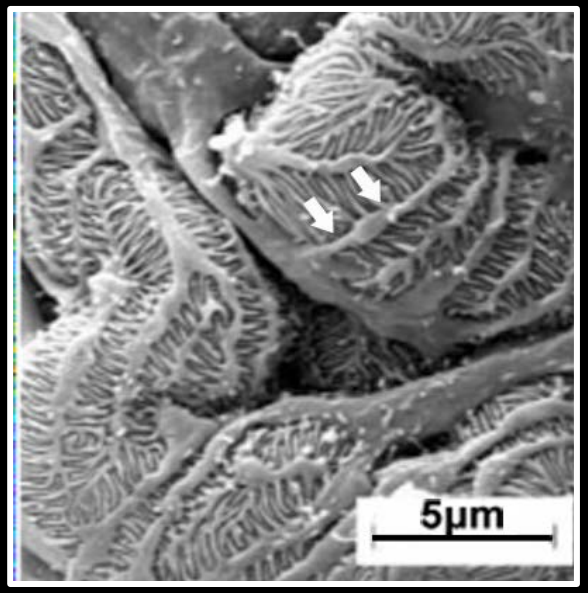
20x/60x Water	30x Silica oil	100x Oil
NA 0.7/ 1.2	NA 1.05	NA 1.4
Typically Scan w/ 20x Acquire w/ 60x	**Scan at 30x, Zoom to 1.5 or 2.0x to acquire**	**High resolution work acquire at 2.0 to 3.0x zoom**
Good balance between resolution/ penetration depth	**Good resolution/ best penetration depth, 80+ um (due to 1.33 R.I. oil)**	**Best resolution lowest penetration depth ~25-30um**

Microscope Objectives:

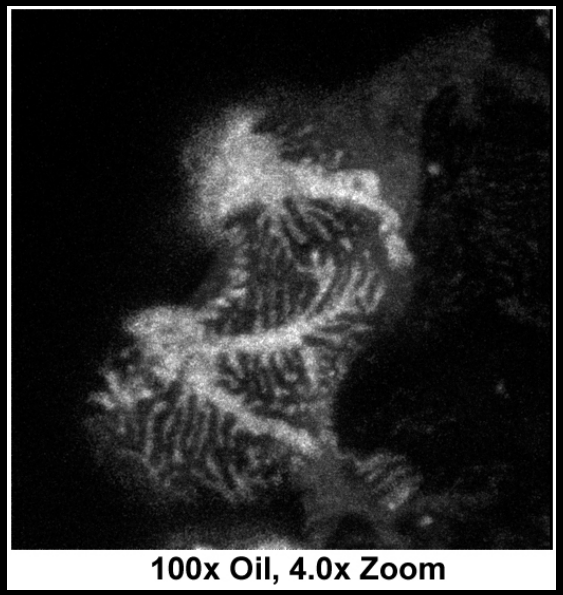




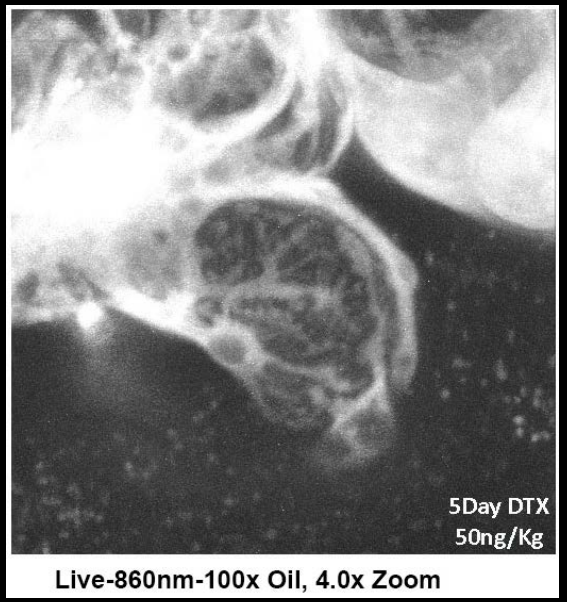
SEM



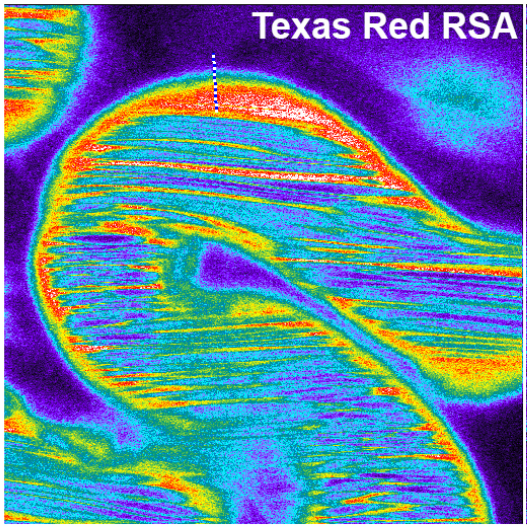
100x Oil-1P, Fixed, Zoomed

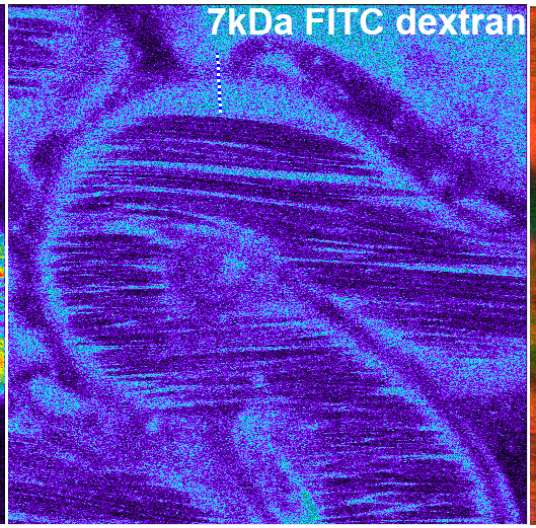


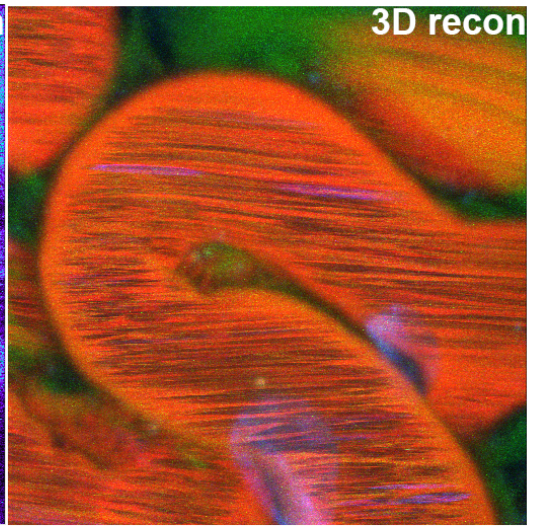
100x Oil-2P, Intravital, Zoomed

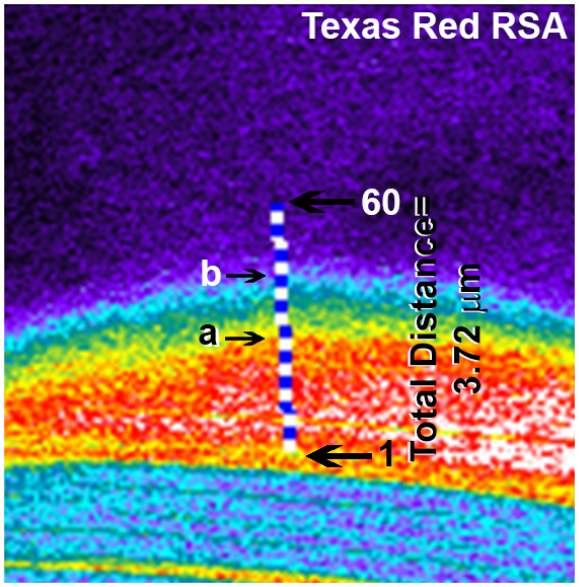


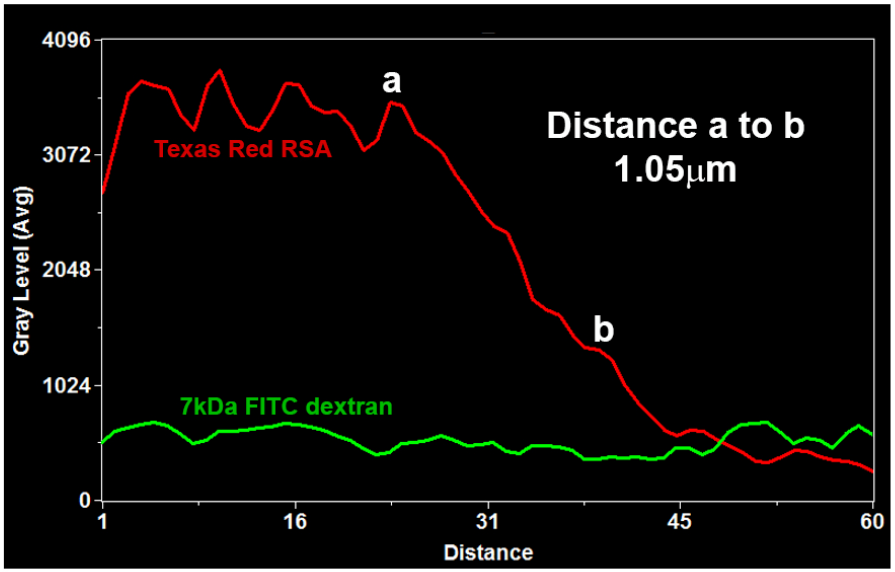
Microscope Objectives:



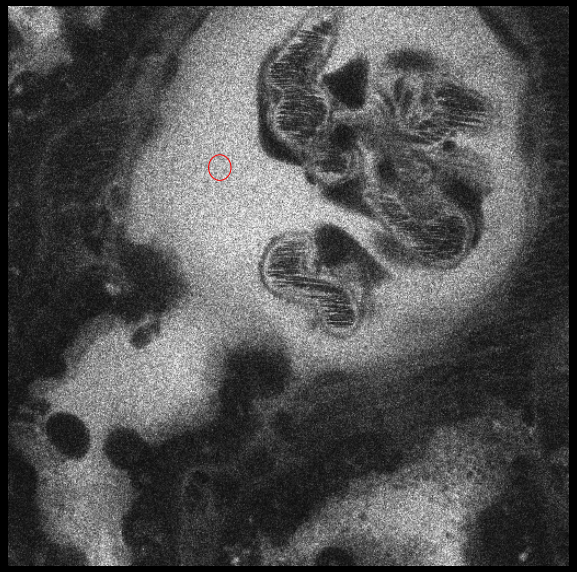




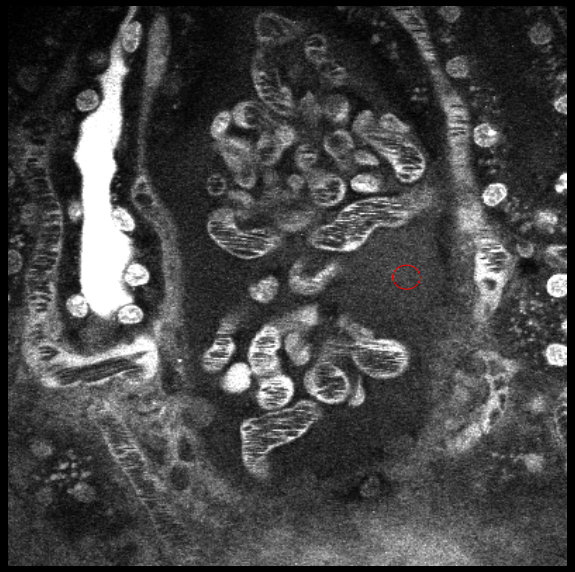




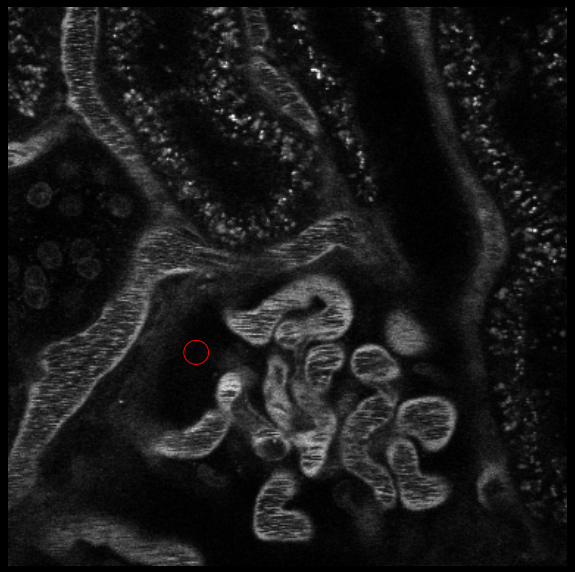
Microscope Settings:
Gain and Black Level



FITC Inulin
(continuous infusion)

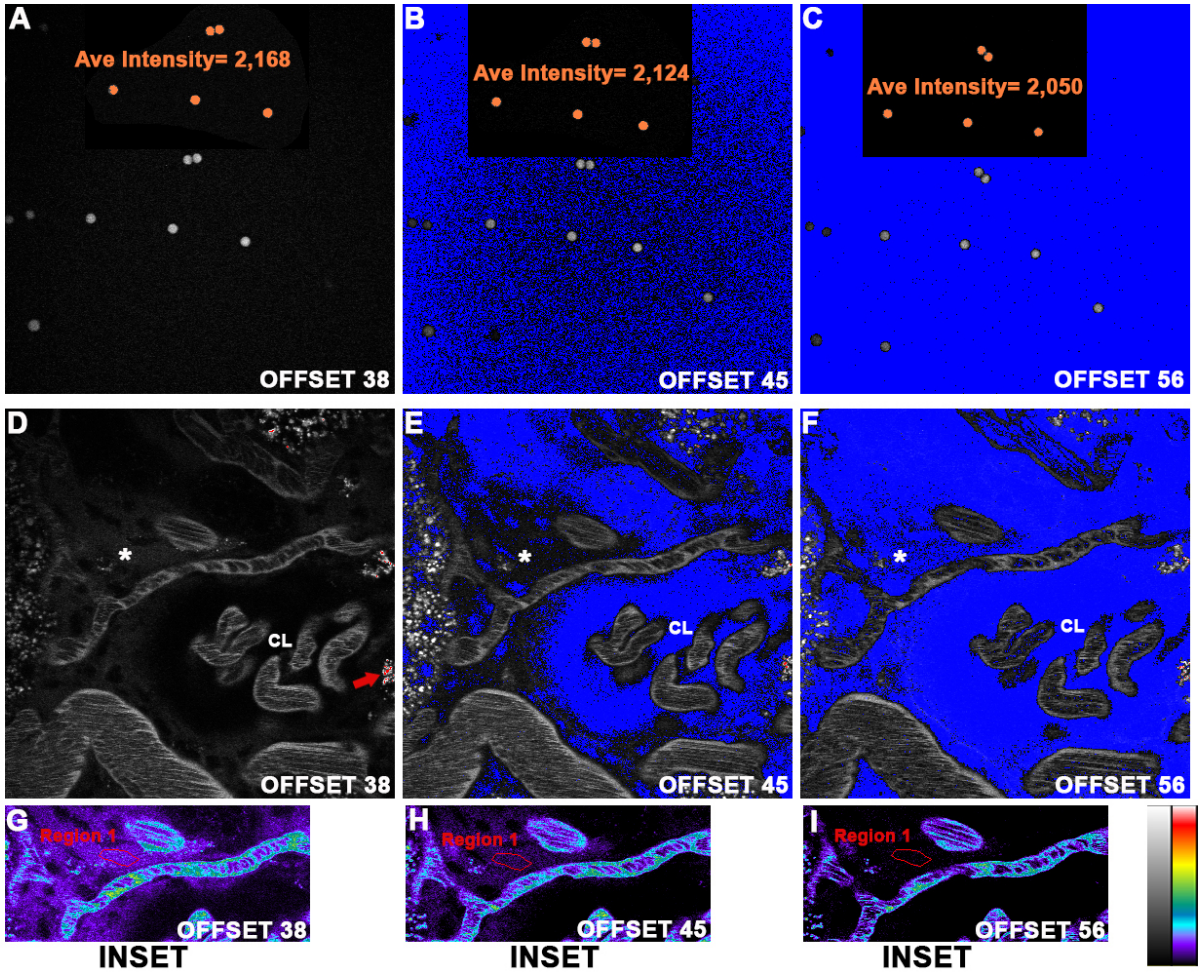


40kDa Ficoll
(bolus infusion)



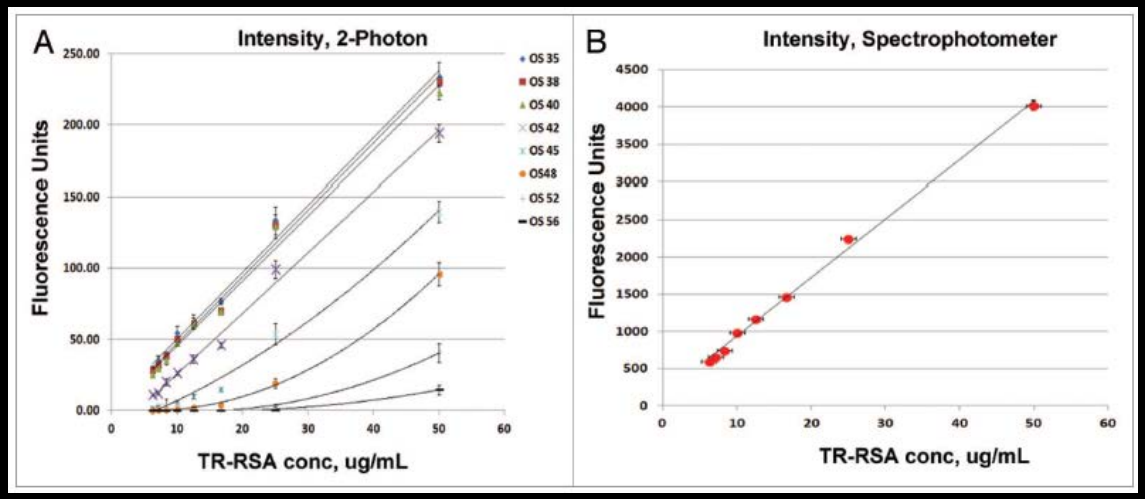
Fluorescent Albumin
(bolus infusion)

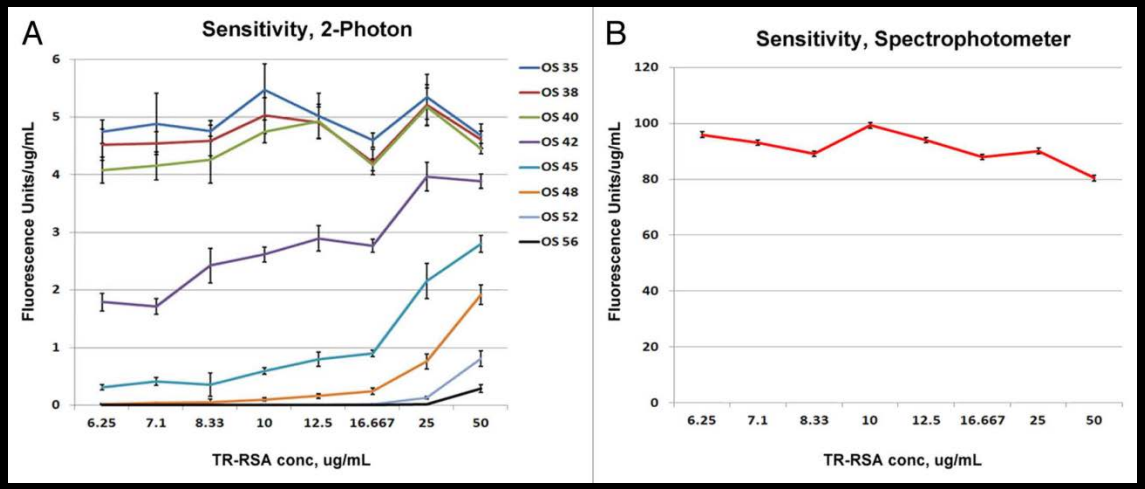
Microscope Settings:
Black Level



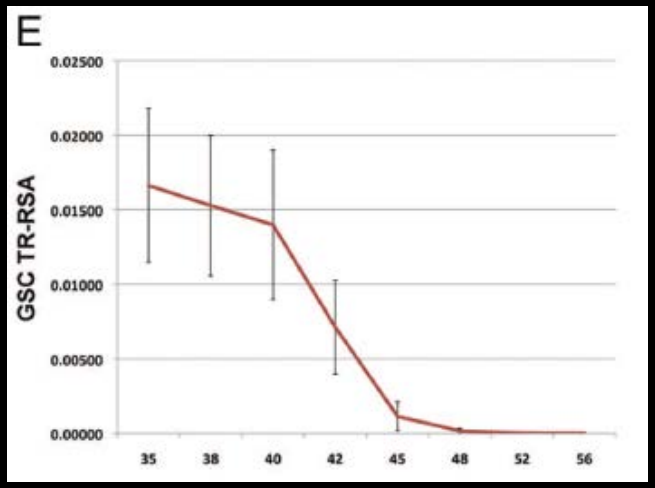
	BACKGROUND VALUES		RAW VALUES		CORRECTED VALUES		GSC		REGION 1 (Panel G, H & I)			Reduction
OFFSET	CapLoops	BowSpace	CapLoops	BowSp-Ave 3	CapLoops	BowSp	TR-RSA	OFFSET	Background	Raw	Corrected	in intensity
38	87	48	2007	69.667	1920	21.667	0.011285	38	50	487	437	
45	6	0	2035	0.430	2029	0.43	0.000212	45	2	169	167	62%
56	0	0	1630	0.000	1630	0	0.000000	56	0	26	26	94%

Microscope Settings:
Black Level





in vitro



in vivo

Acknowledgements

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Gosia Malgorzata
Seth Winfrees
Jeff Clendenon (Bloomington)
Jason Byars (New Mexico)

Original image courtesy of T. Hato & P.C. Dagher

Disclosures

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FAST Biomedical, Indianapolis