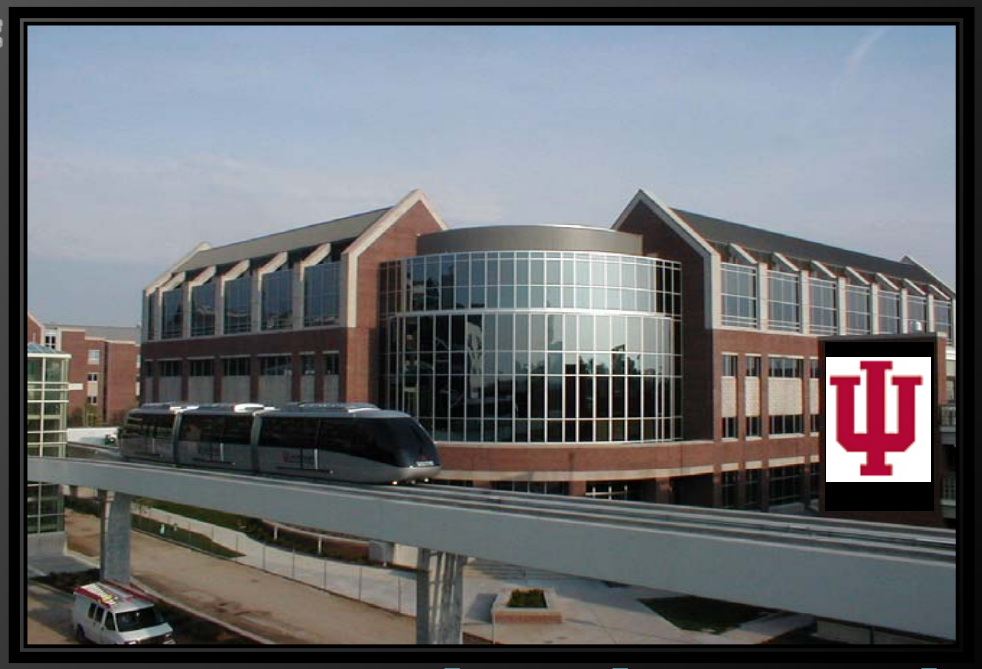
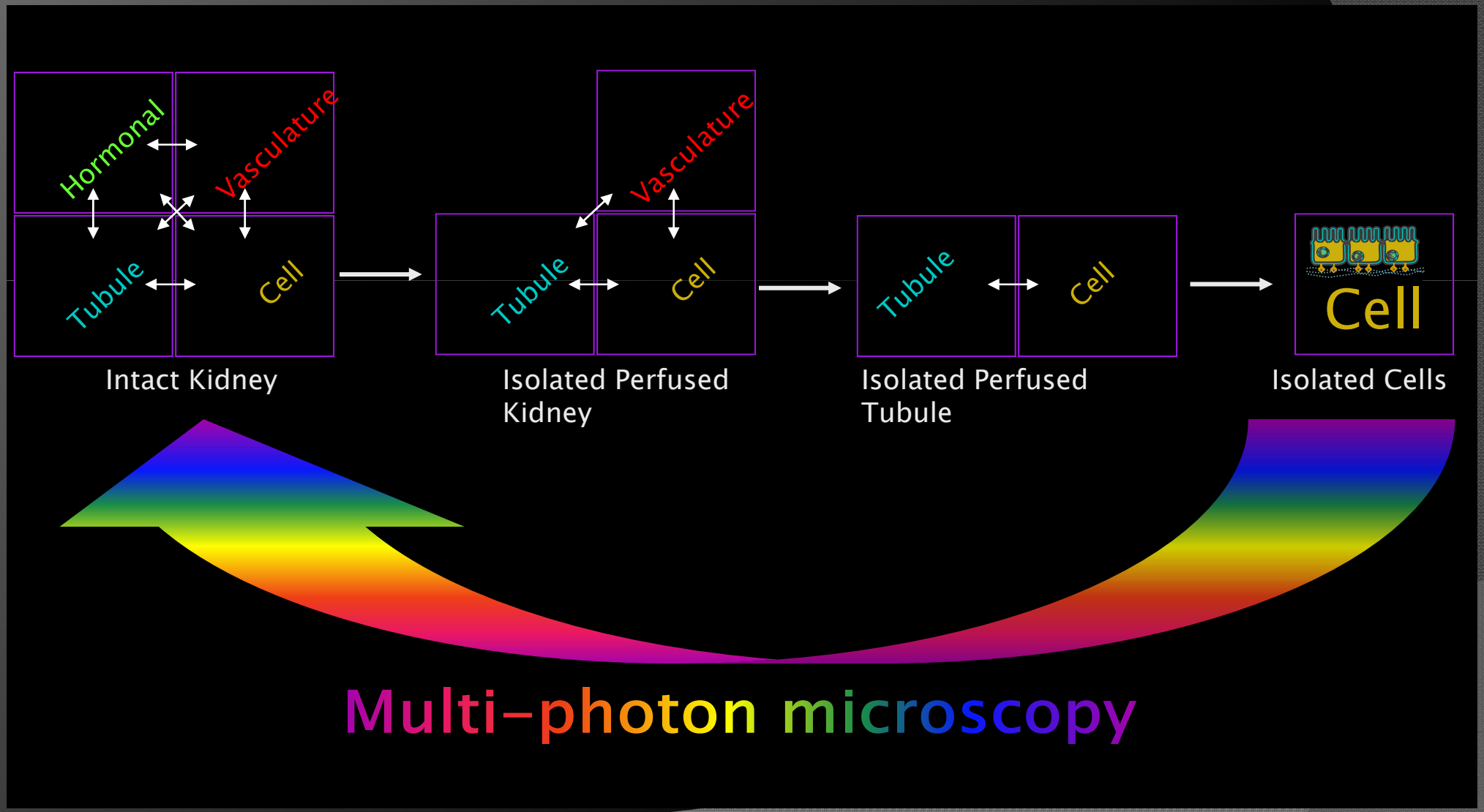


Advancing Nephrology Through 2-Photon Microscopy

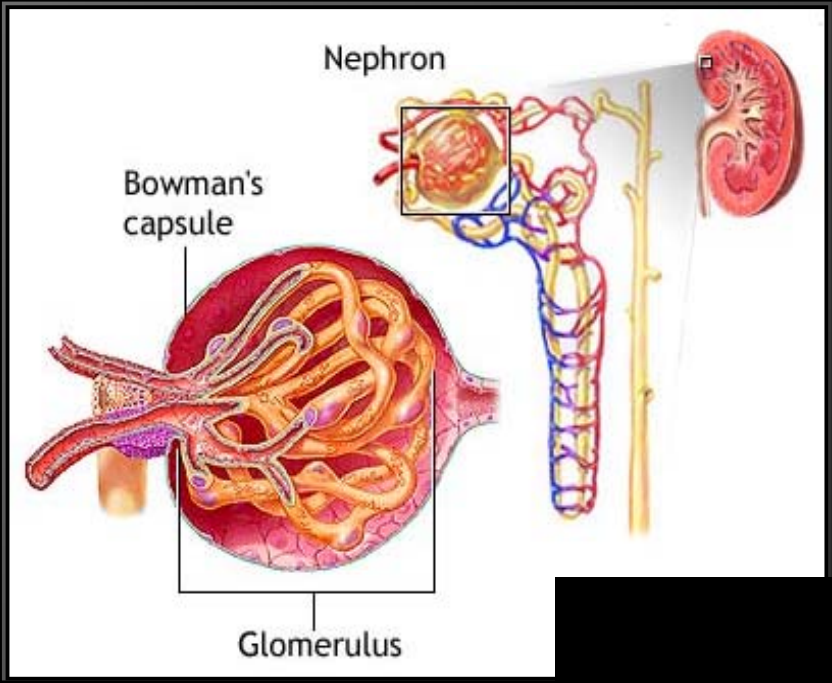
Bruce A. Molitoris
Department of Medicine
Indiana Center for Biological Microscopy
Indiana University School of Medicine

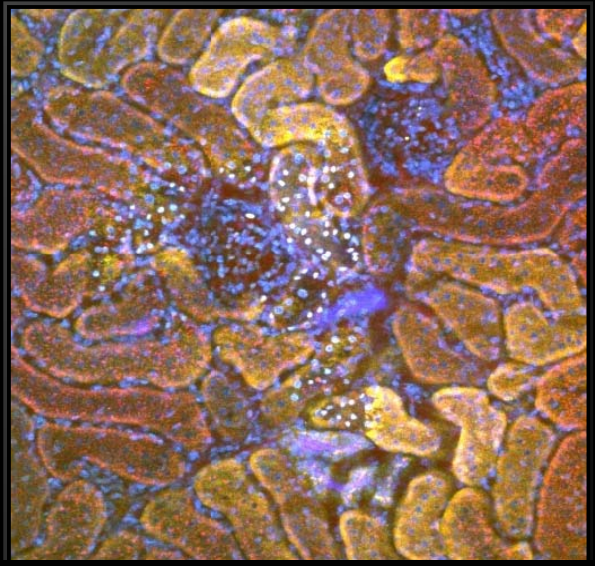
Reversing Reductionism

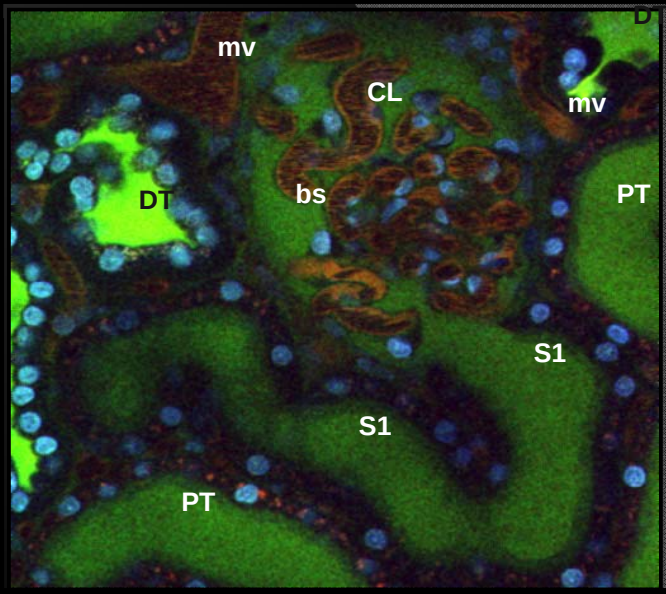


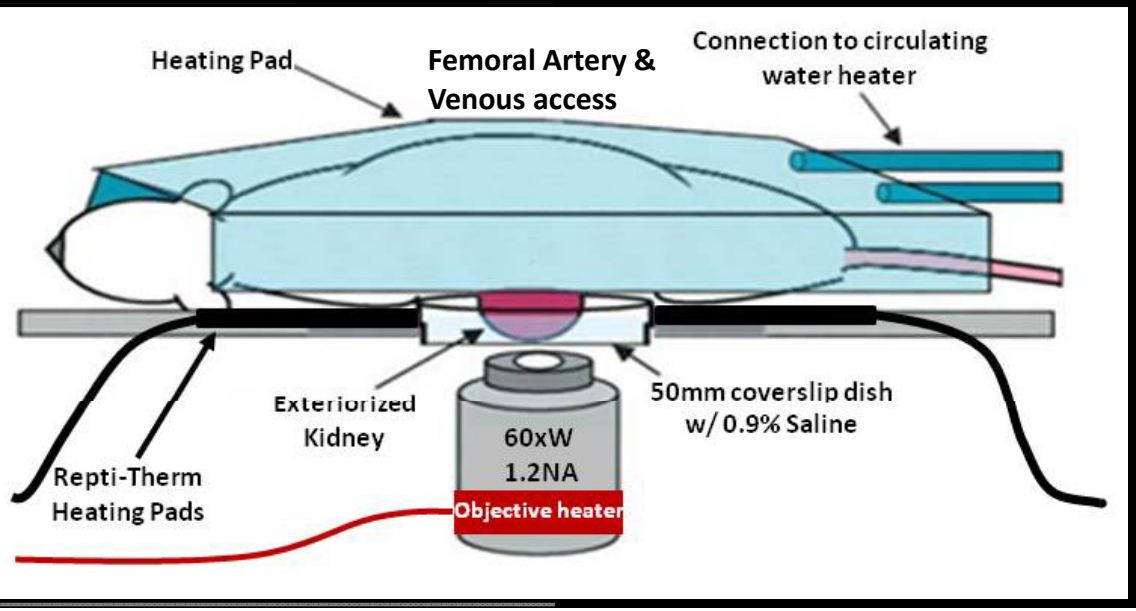
Sandoval et.al. AJP 2005

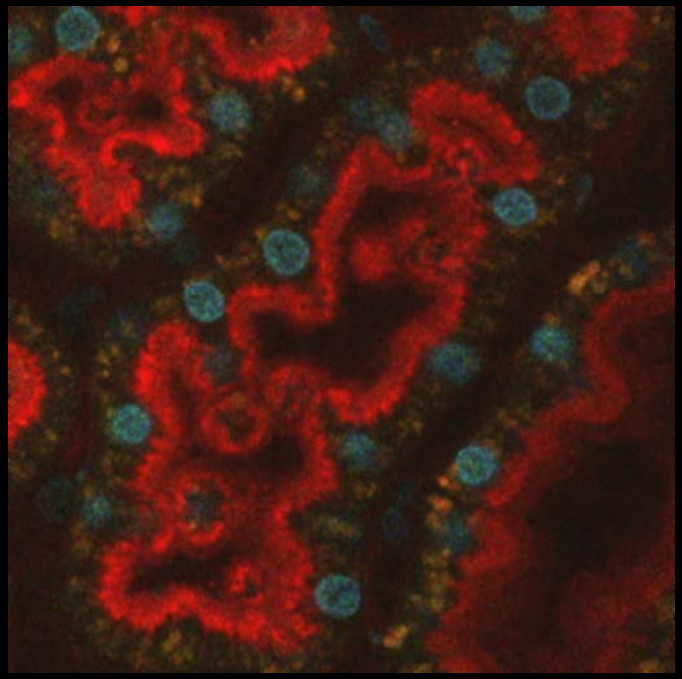
Visualizing Glomerular & Nephron Function



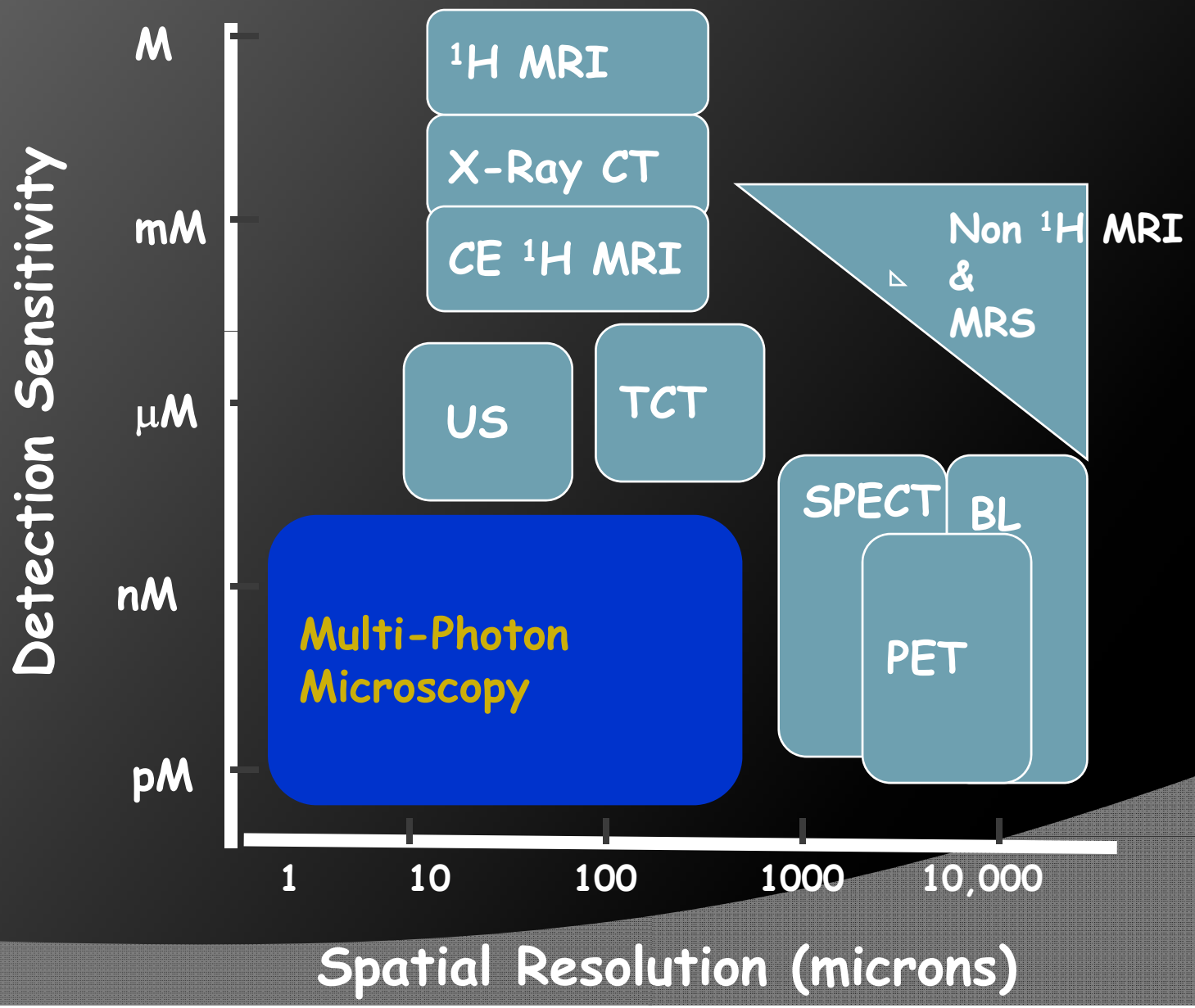




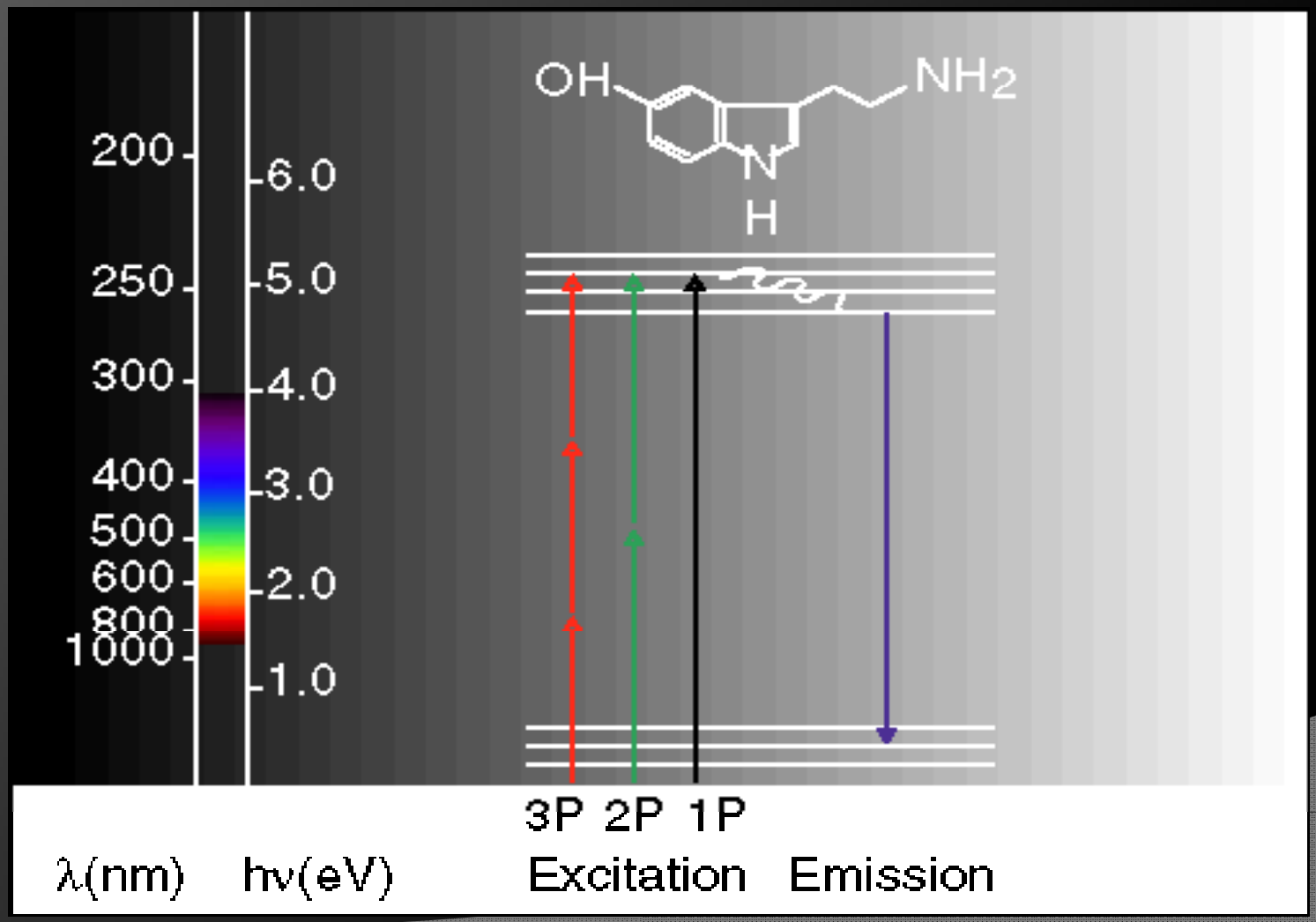




Intra-Vital Imaging Sensitivity vs Resolution



TWO-PHOTON MICROSCOPY PRINCIPLE:



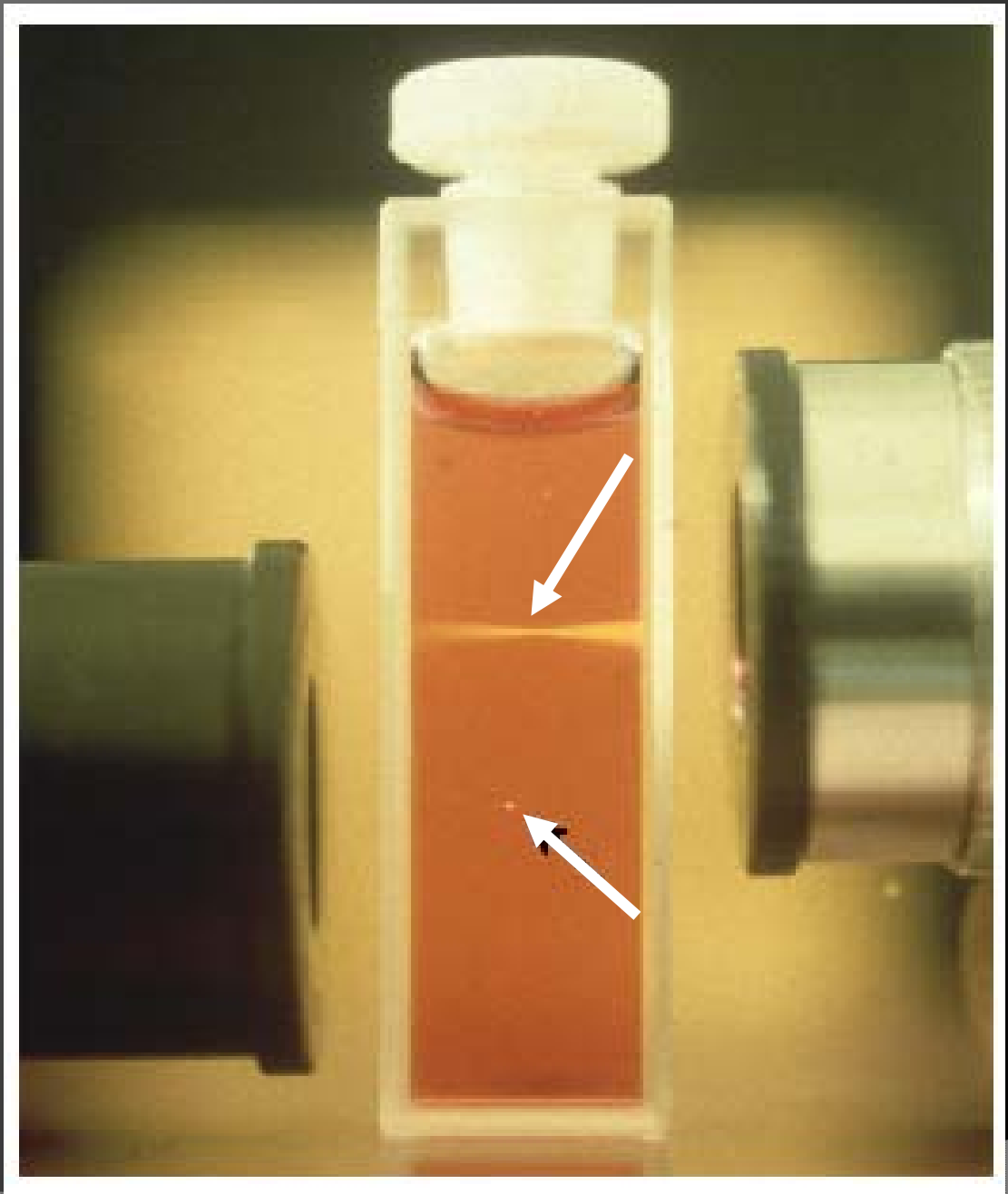
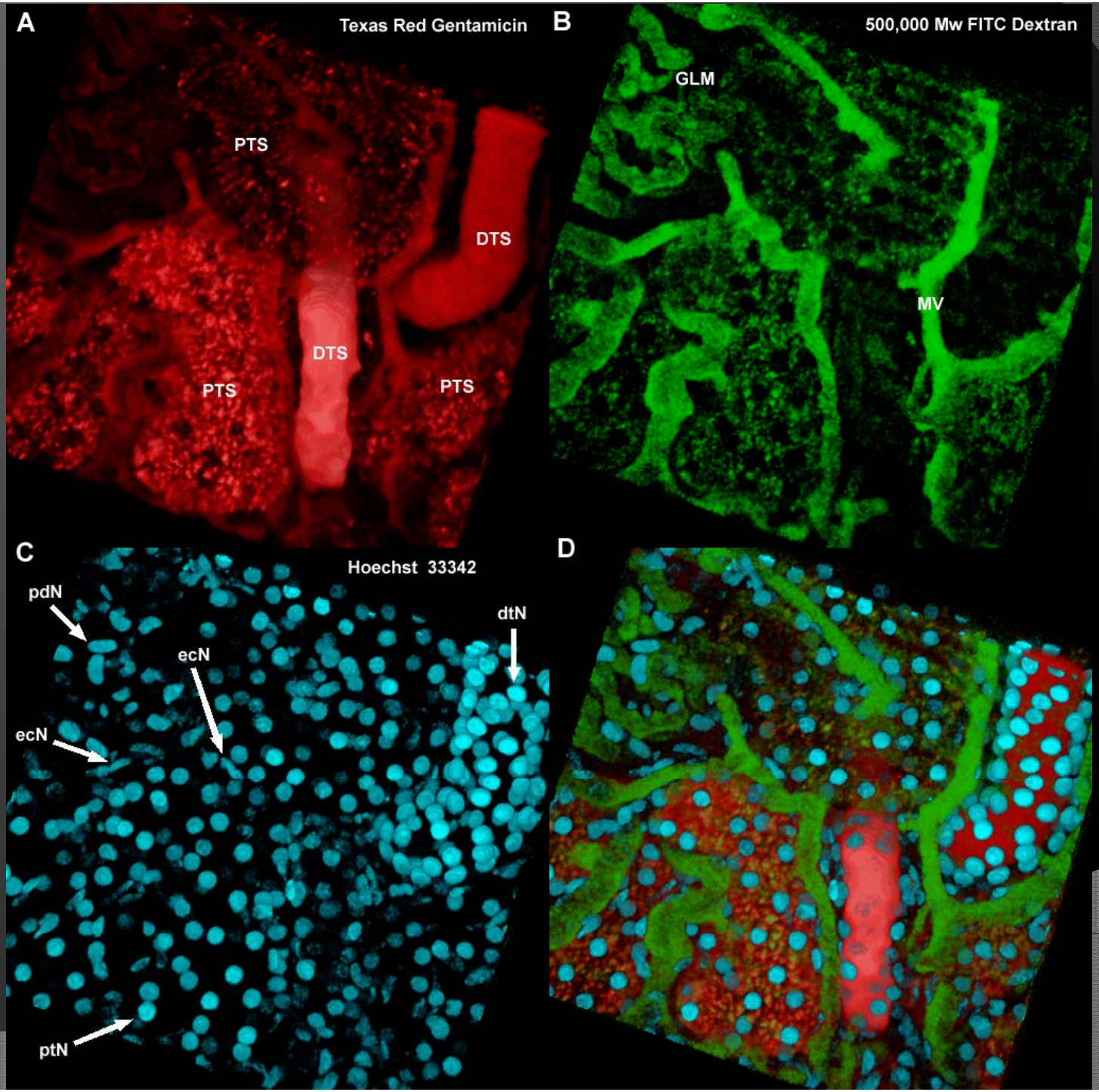
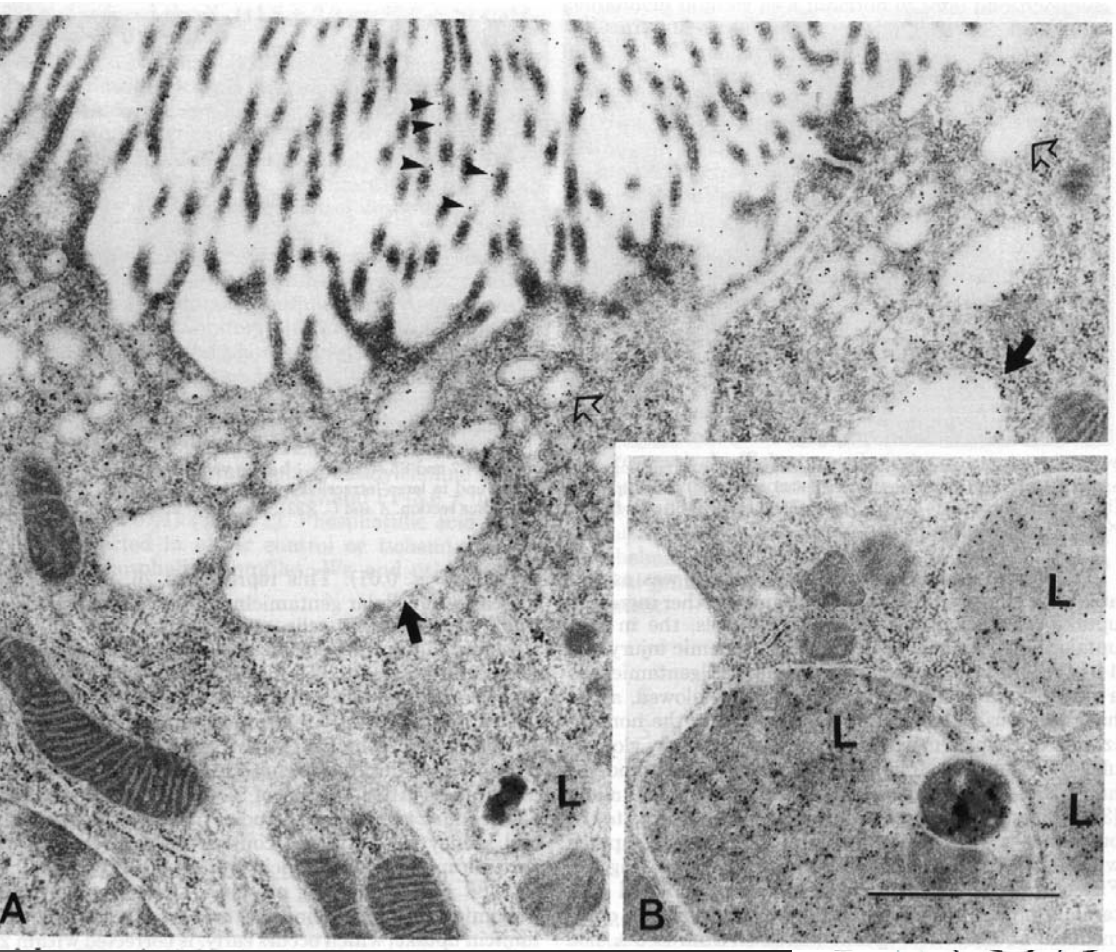


Figure courtesy of Brad Amos, MRC Laboratory

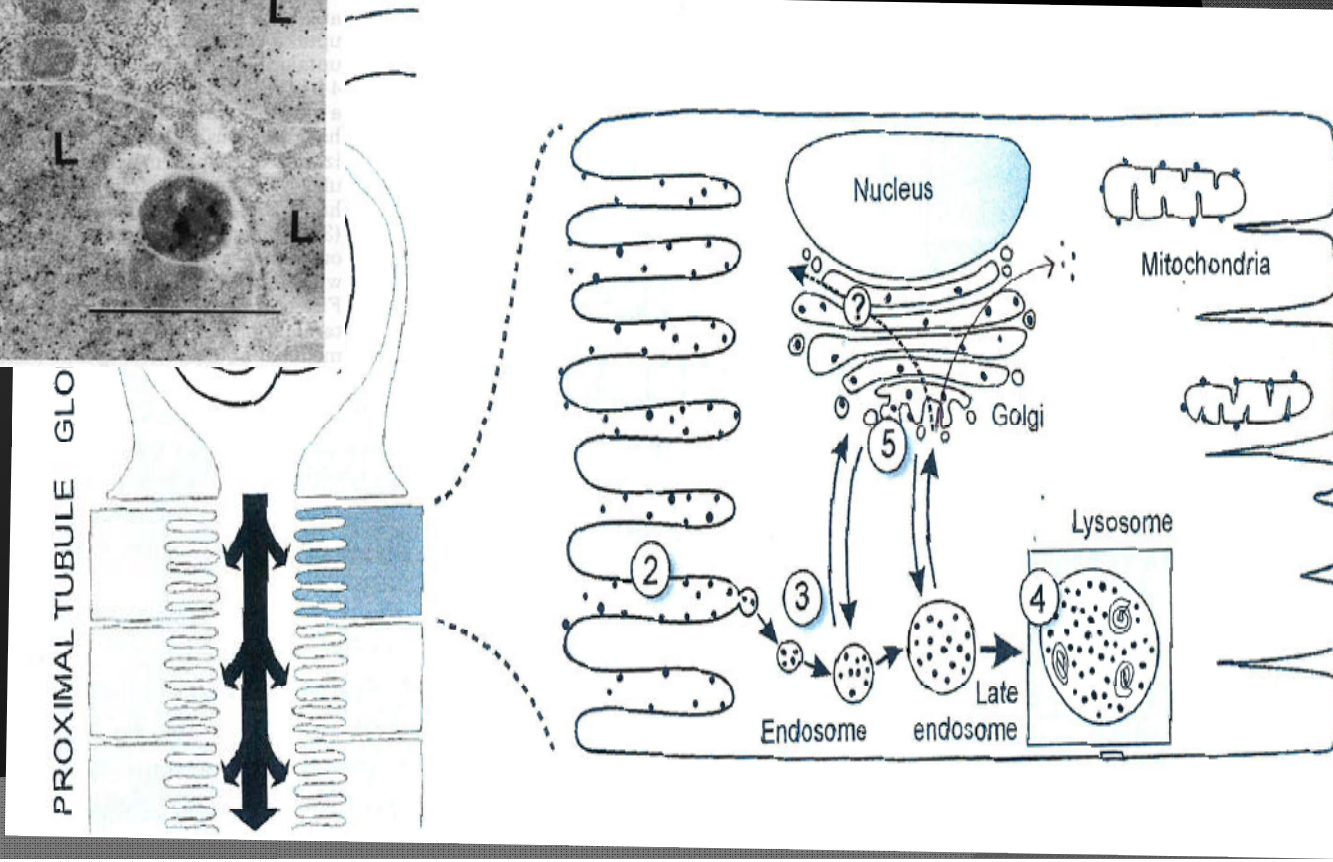
Volume of fluorescence excitation –

Confocal versus 2-photon microscopy


A
Texas Red Gentamicin
PTS
DTS
DTS
PTS
PTS
B
500,000 Mw FITC Dextran
GLM
MV
C
Hoechst 33342
pdN
ecN
dtN
ecN
ptN
D

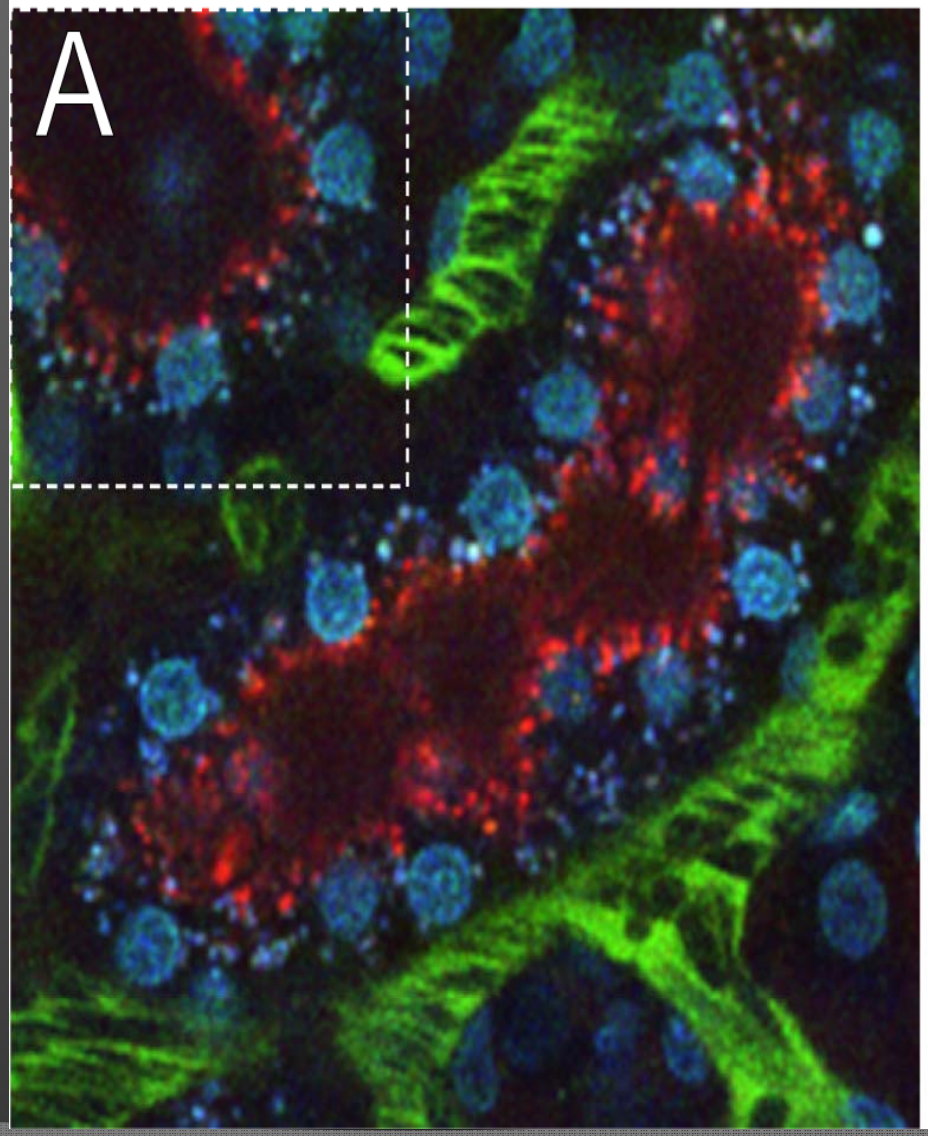


High Oxygen
Aerobic metab.
Minimal anaerobic metab
Fatty acids, acetoacetate
No glycogen
Fluid Phase and Receptor Mediated Endocytosis
Sensing environment, TLR
Long lived cell



Modified from Kidney & Urinary Tract Eighth Edition

Figure 4



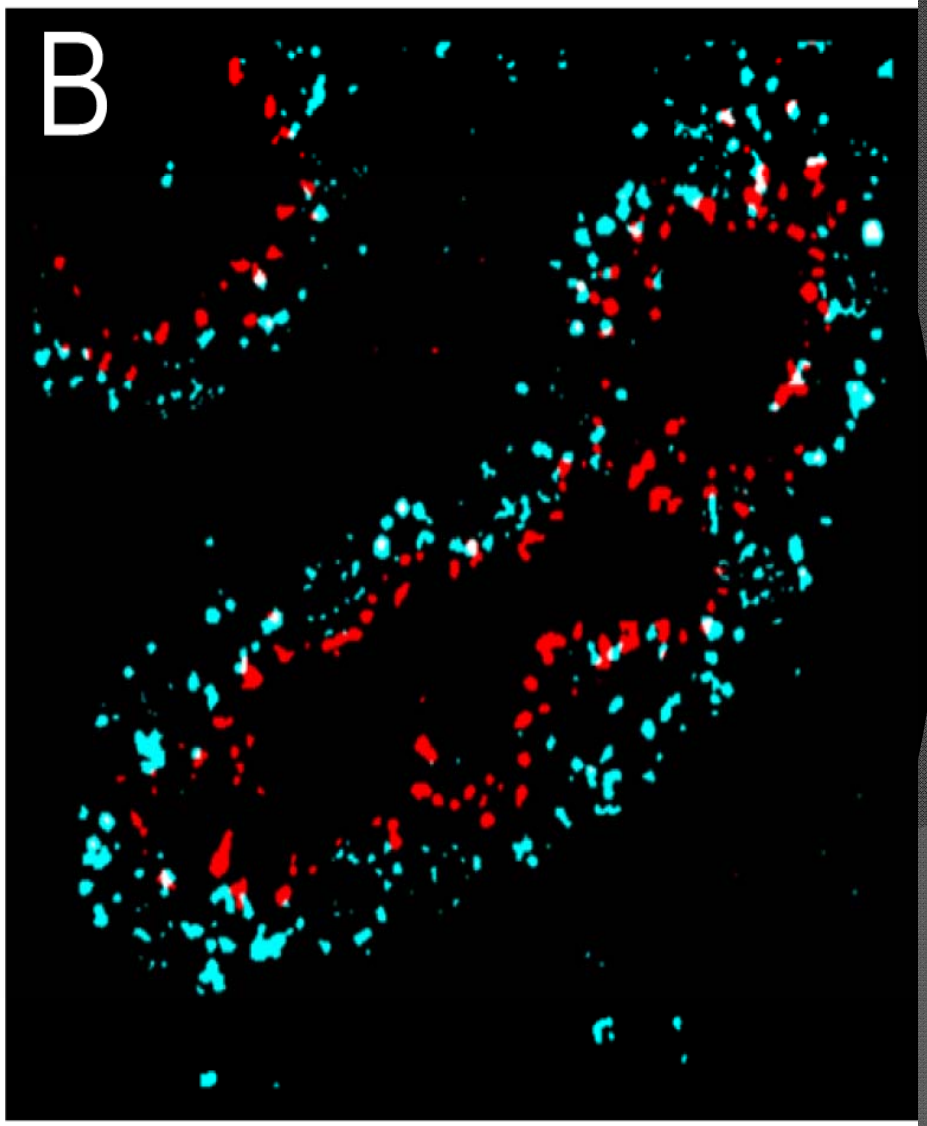
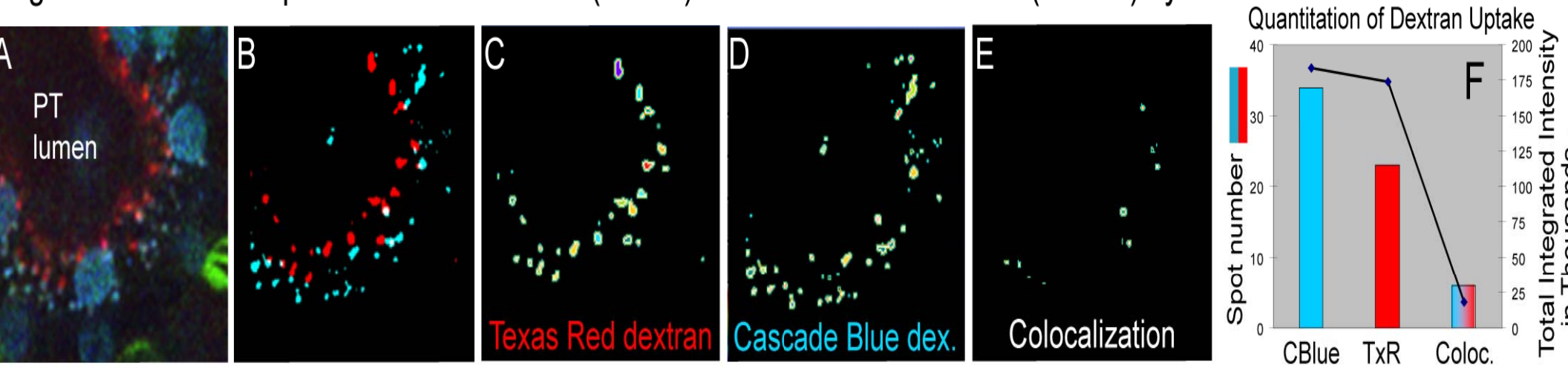
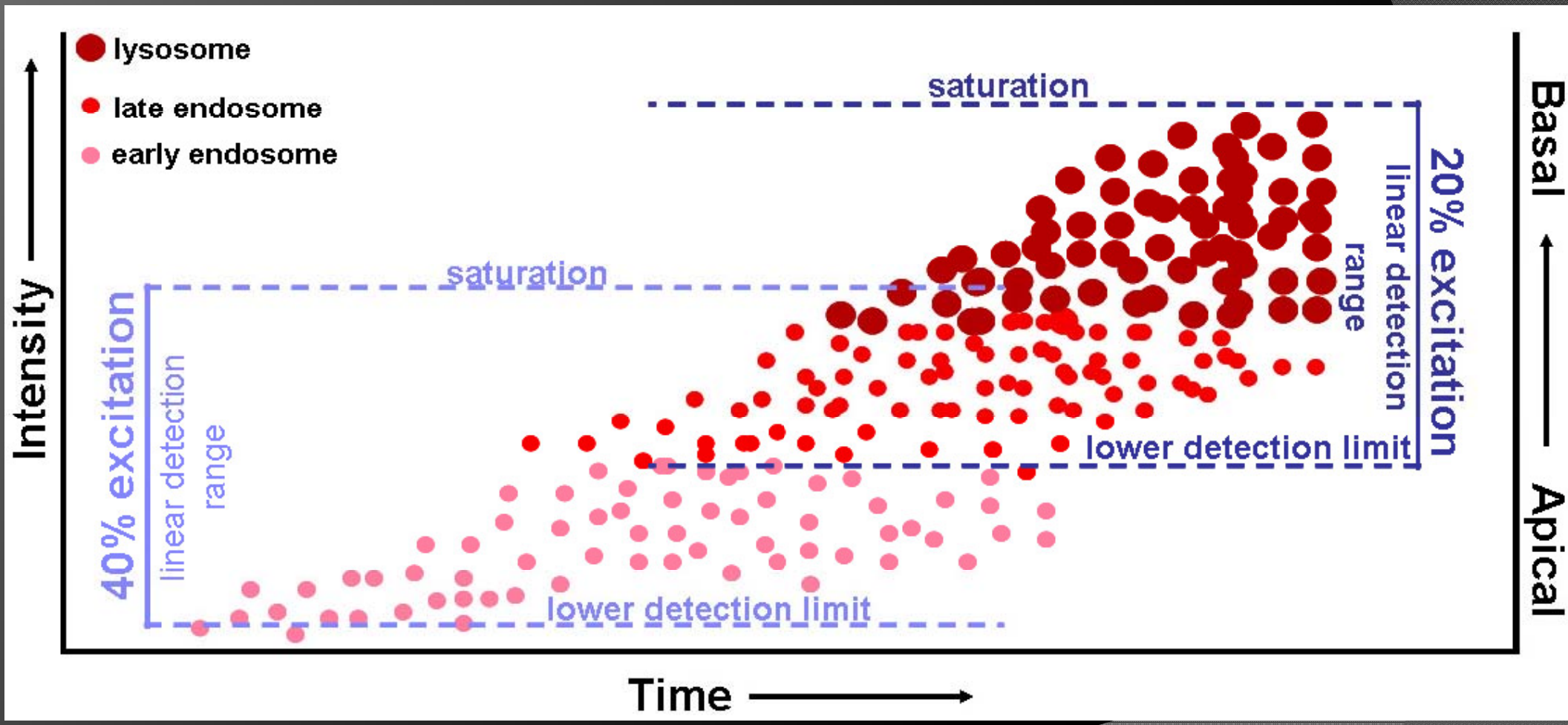
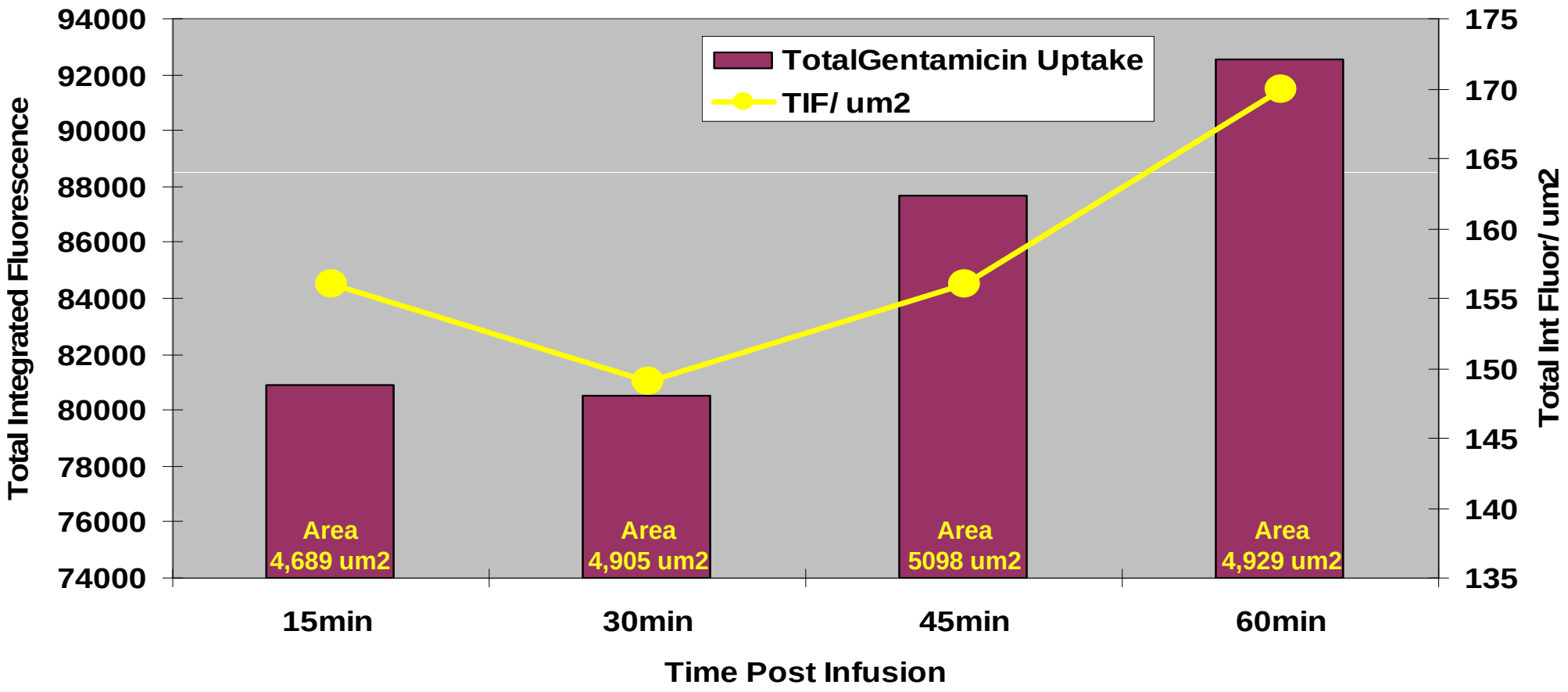


Figure 33 In vivo uptake of Cascade Blue (24 hrs) and Texas Red dextrans (15 min) by PTCs

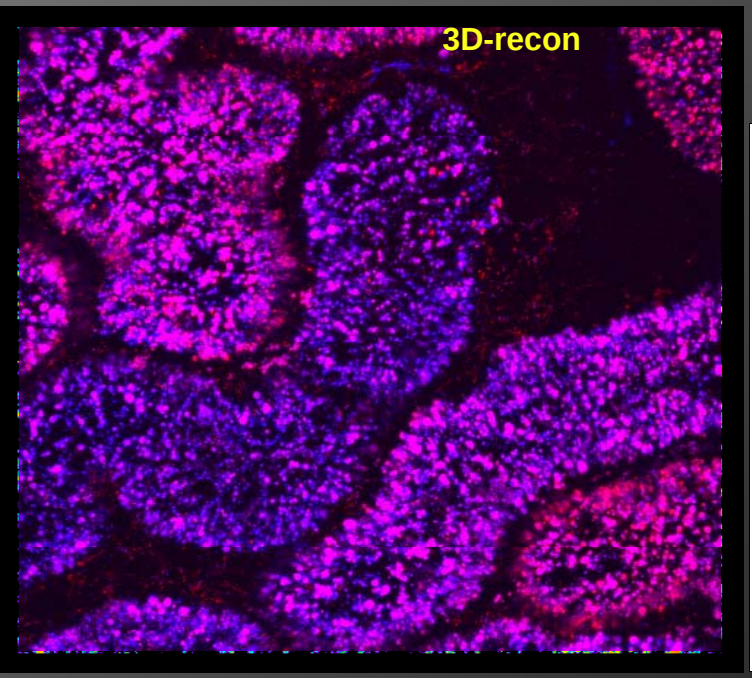


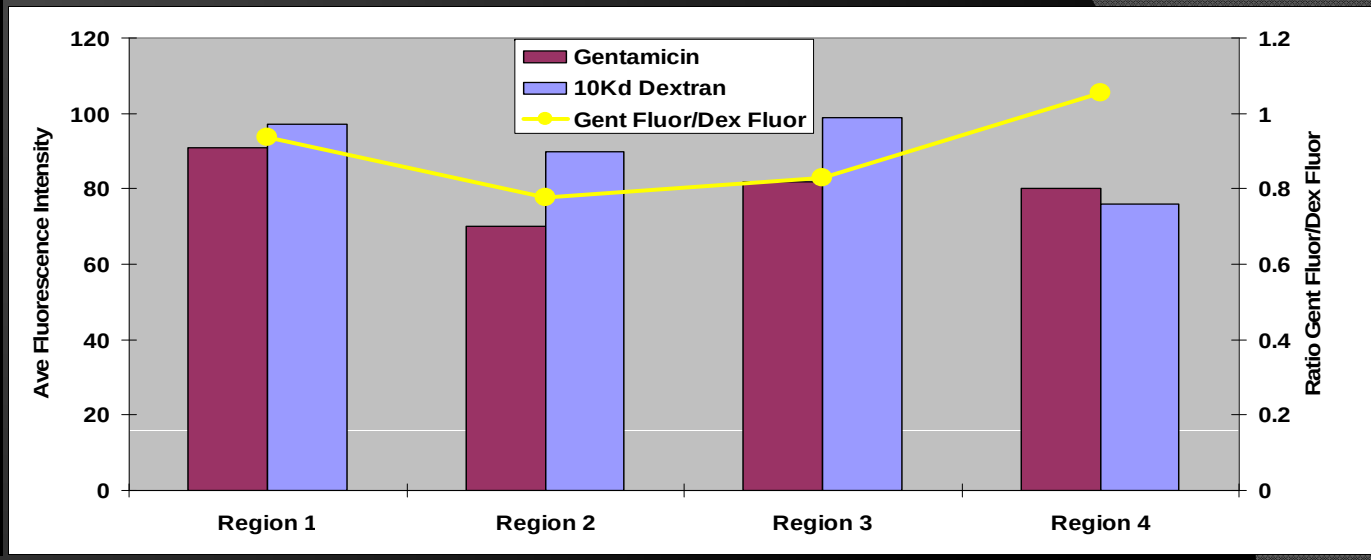

lysosome
late endosome
early endosome
saturation
20% excitation
linear detection range
lower detection limit
saturation
40% excitation
linear detection range
lower detection limit
Intensity
Time
Basal
Apical

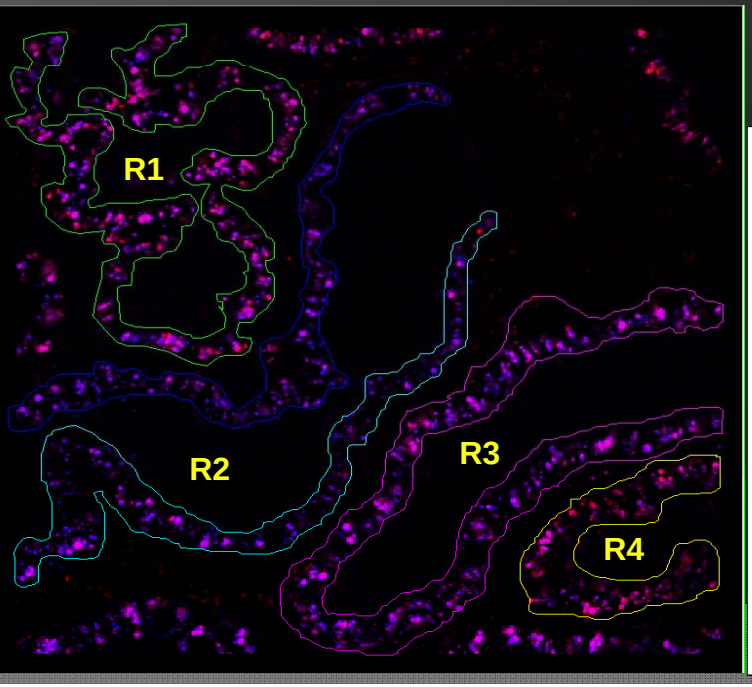
Total Texas Red Gentamicin Uptake-Day 1

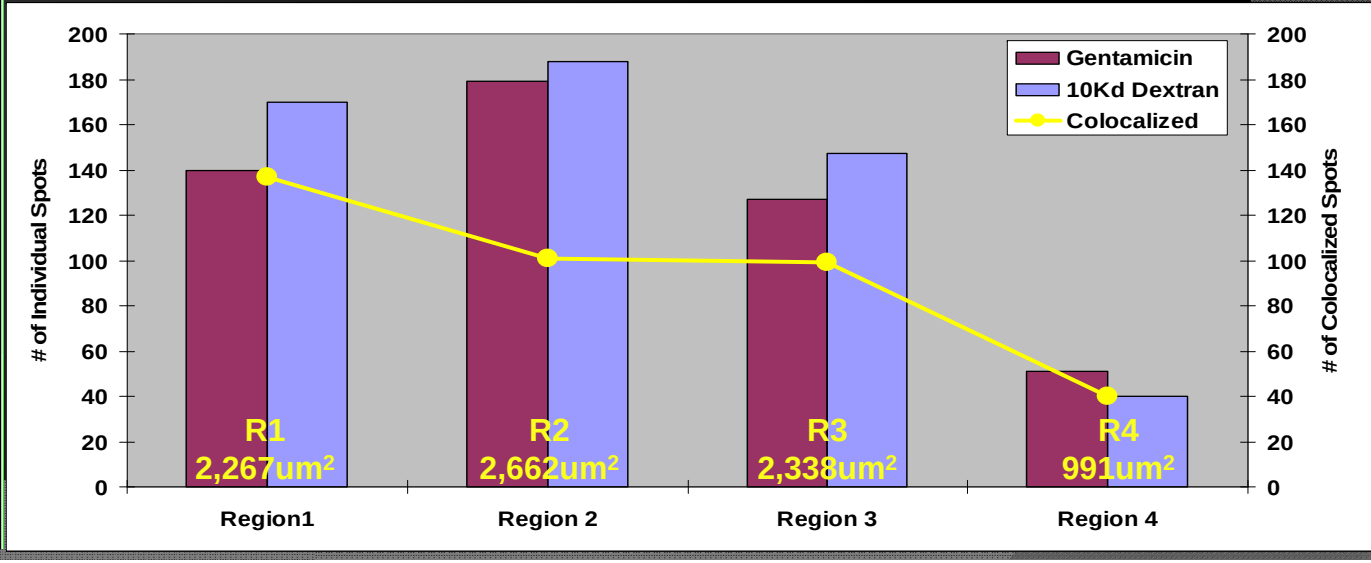


Texas Red Gentamicin, 10,000 MW Cascade Blue Dextran
24 hr post injection

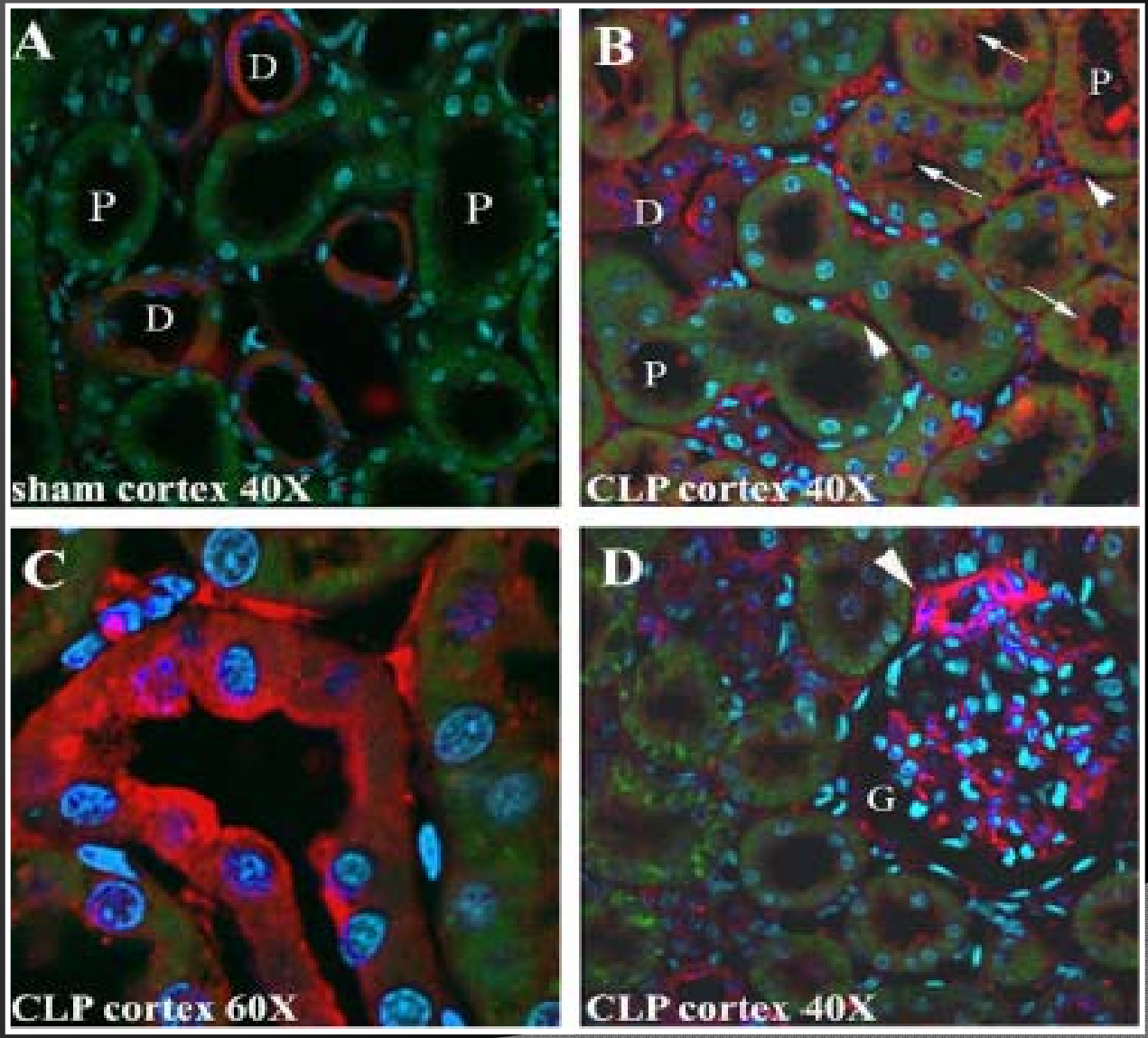








CLP Induces TLR4 Expression in Proximal Tubule Cells



El-Achkar TM, et al. *Am J Physiol Renal Physiol.* 2006

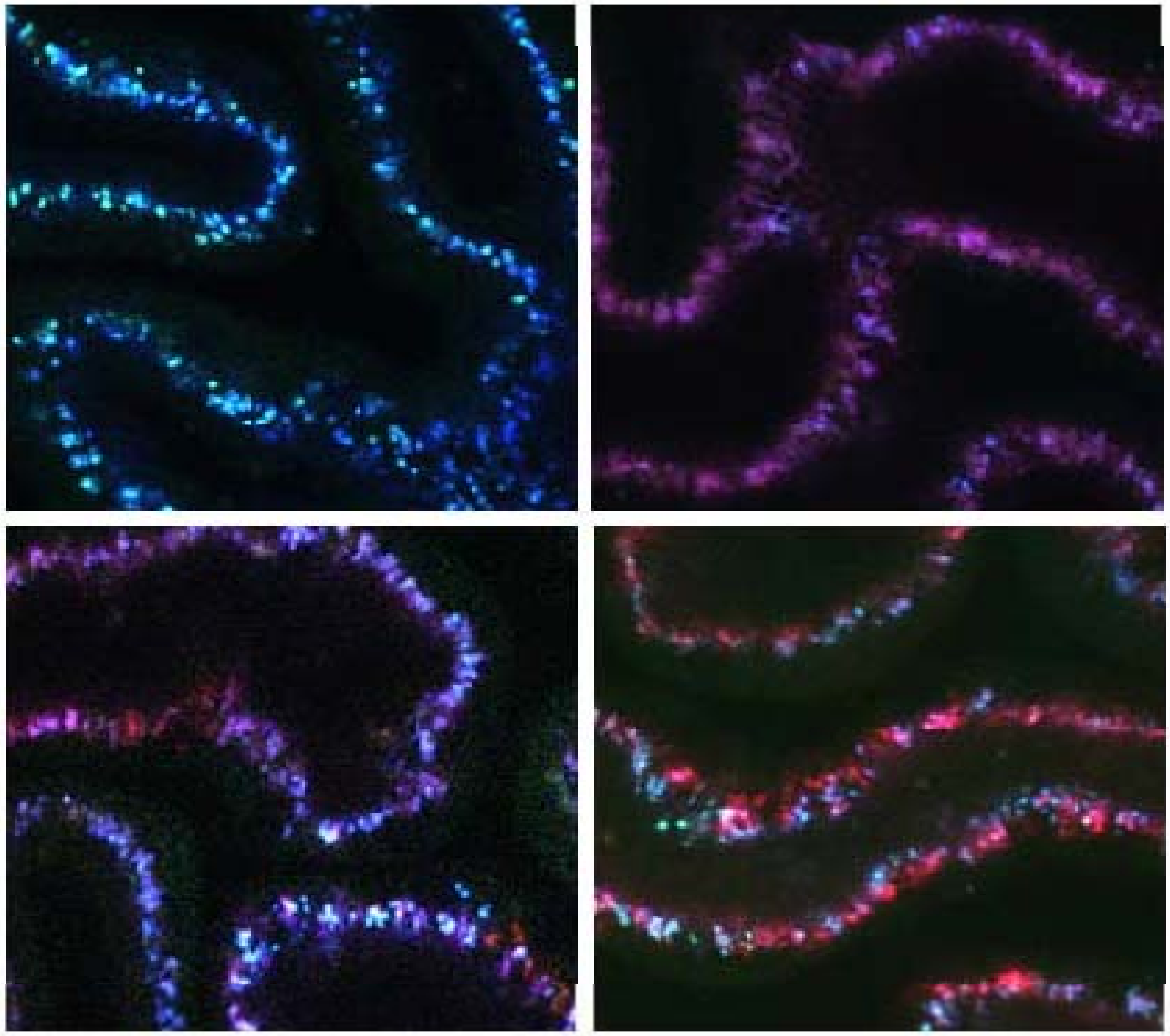
LPS Endocytosis is Receptor Mediated

Blue: cascade blue LMWD, fluid phase

Red: Alexa 568-LPS

Pre exposure: 0.25 mg/Kg unlabeled LPS 24 hr pre to upregulateTLR4

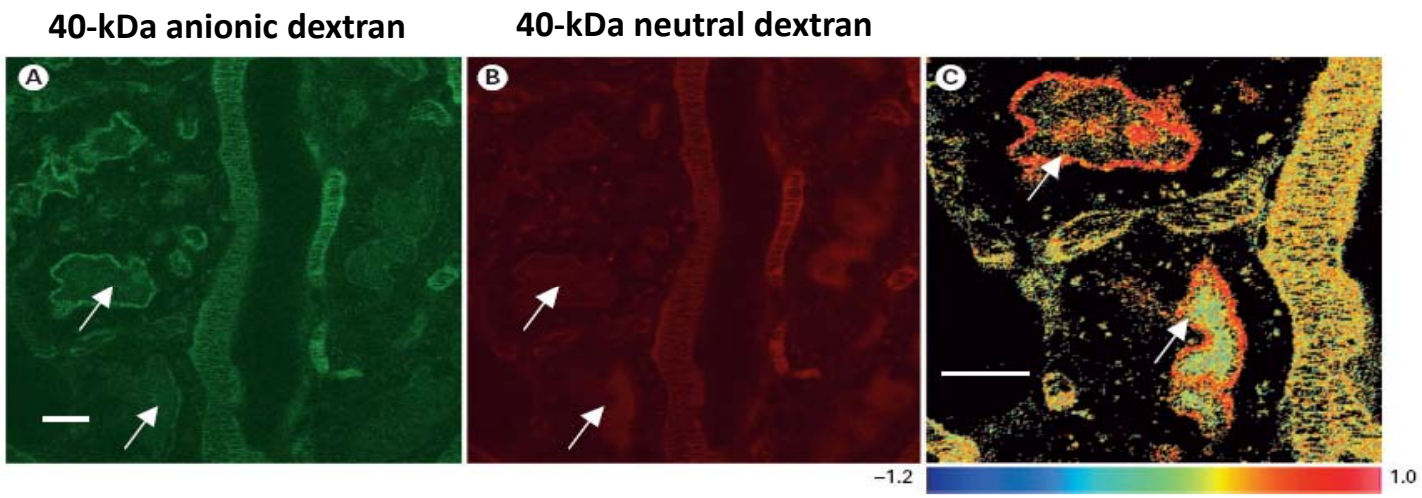
LMWD WT or TLR4 KO **TLR4 KO**

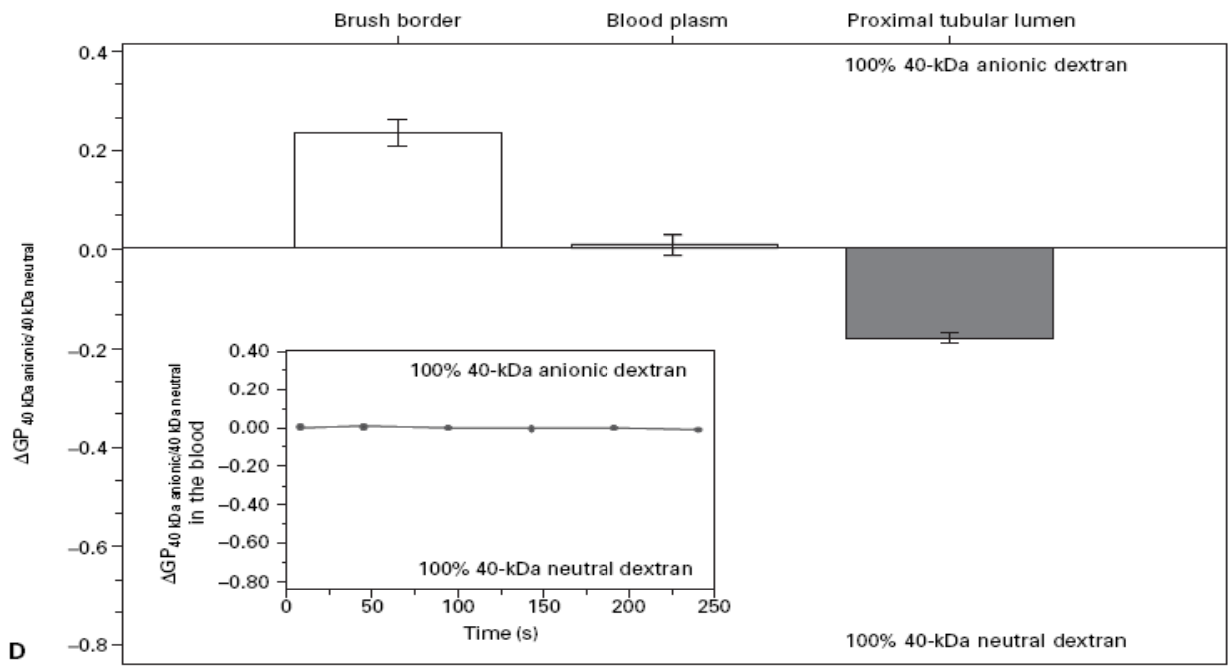


WT **WT pre exposed**

P. Dagher unpublished observation

Proximal Tubule Uptake Explains Differential Filtration

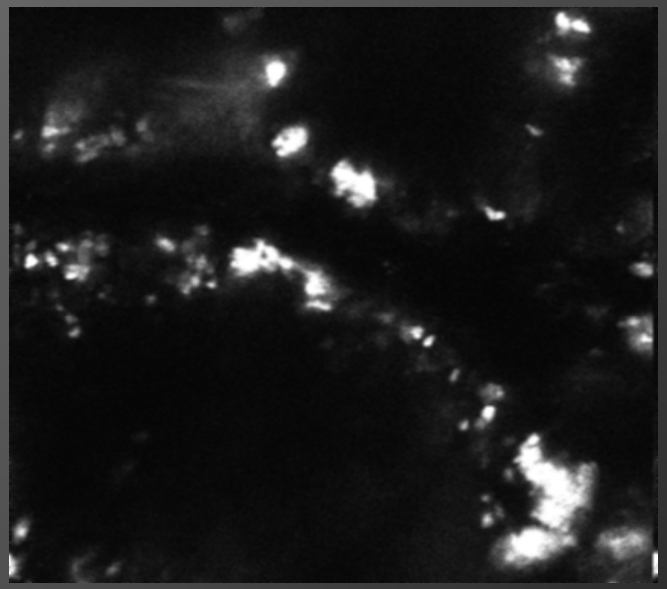




Yu et.al AJP 2005 and *Nephron Physiol 2006*

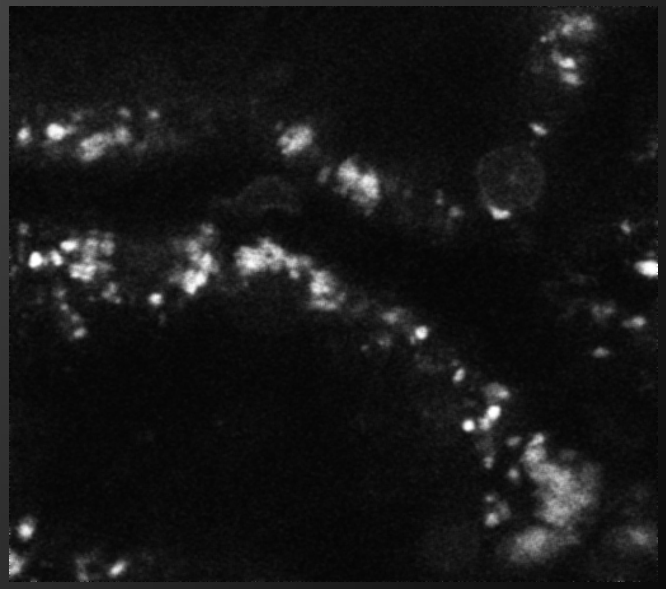
Evaluating for Functional Impairment

Red Channel Alone



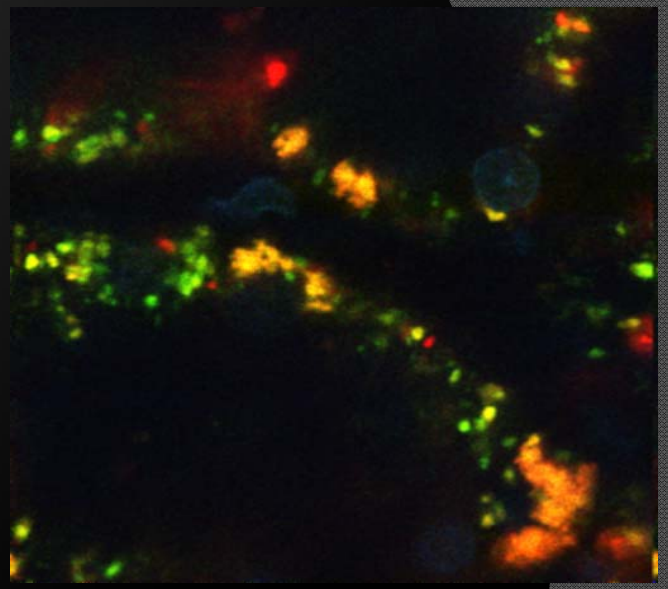
TAMRA Oligo (red)

Green Channel Alone

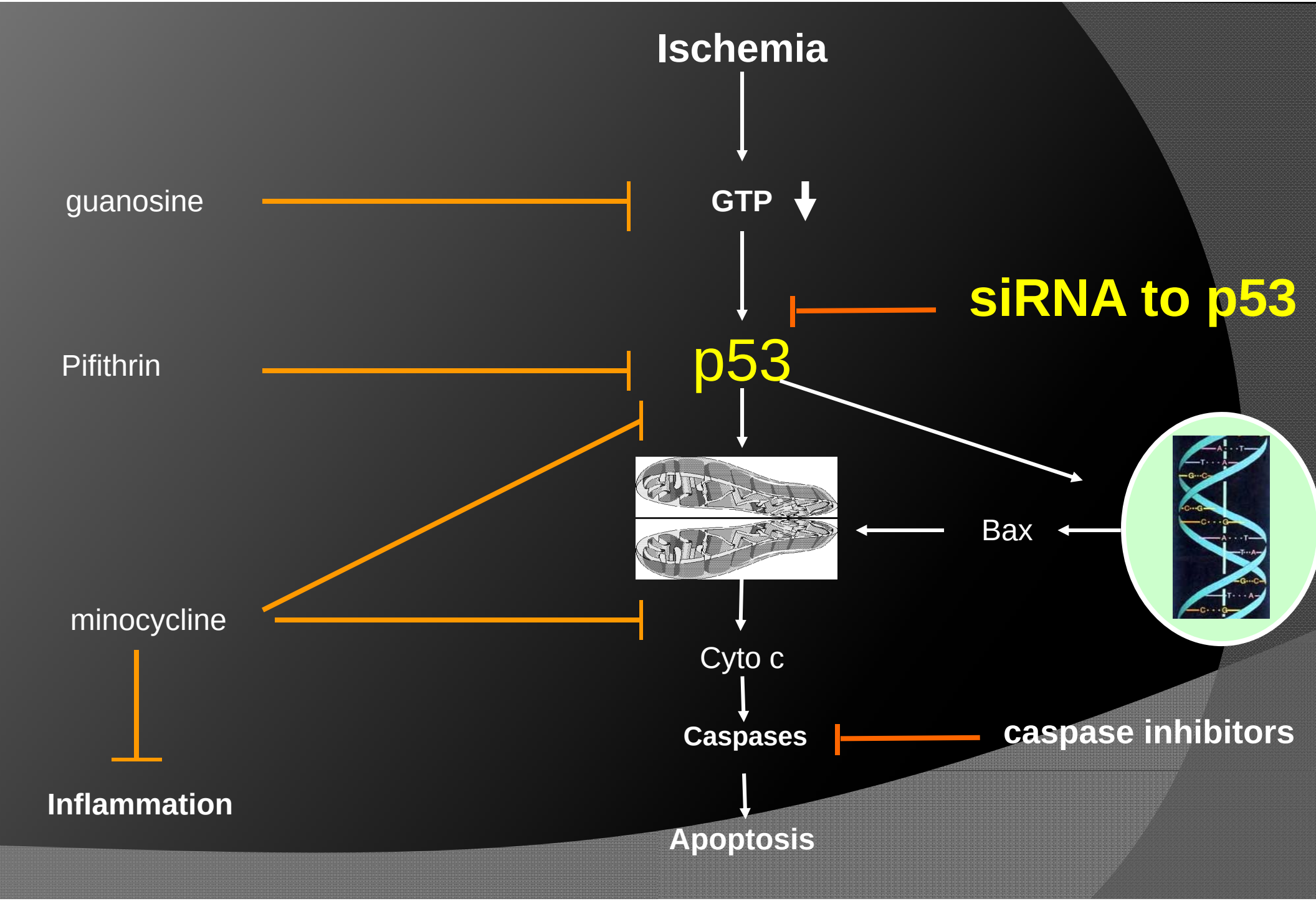


Beta-2-microglobulin (green)

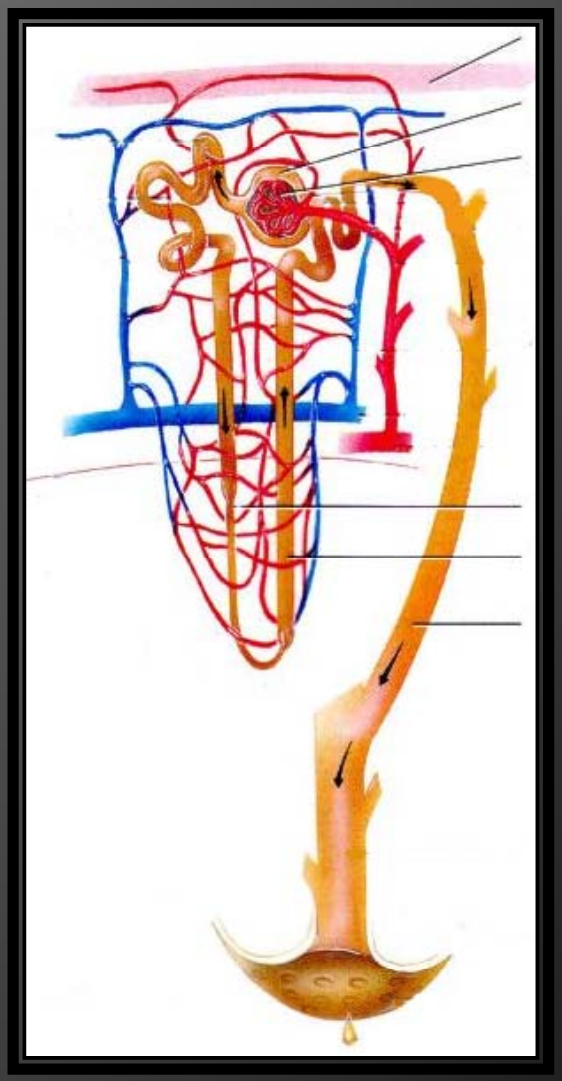
Color Combine

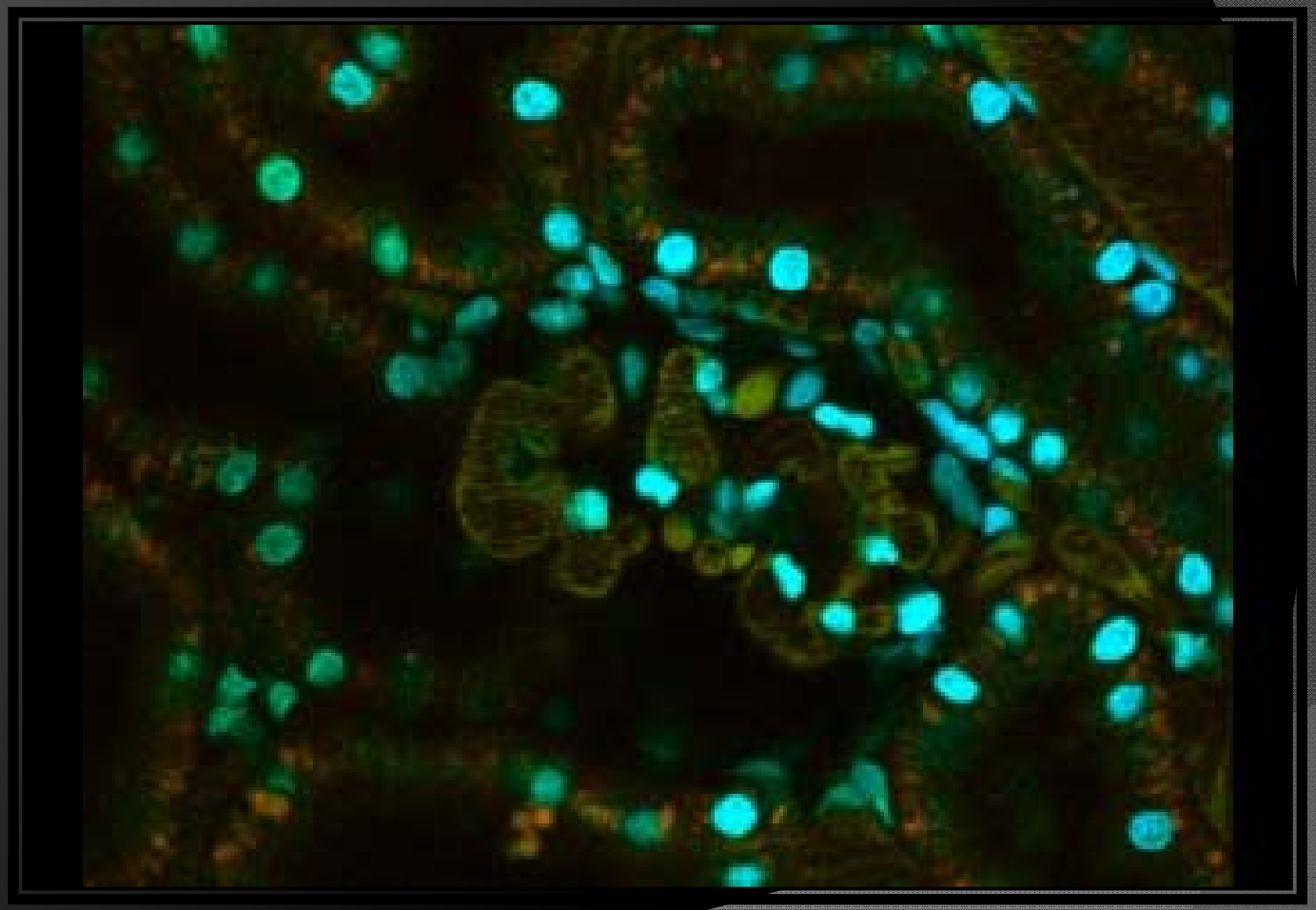


Long Term 25mg/Kg 10% TAMRA 24Hrs Post Injection of ß2M


Ischemia
GTP
guanosine
siRNA to p53
Pifithrin
p53
Bax
minocycline
Cyto c
Caspases
caspase inhibitors
Inflammation
Apoptosis

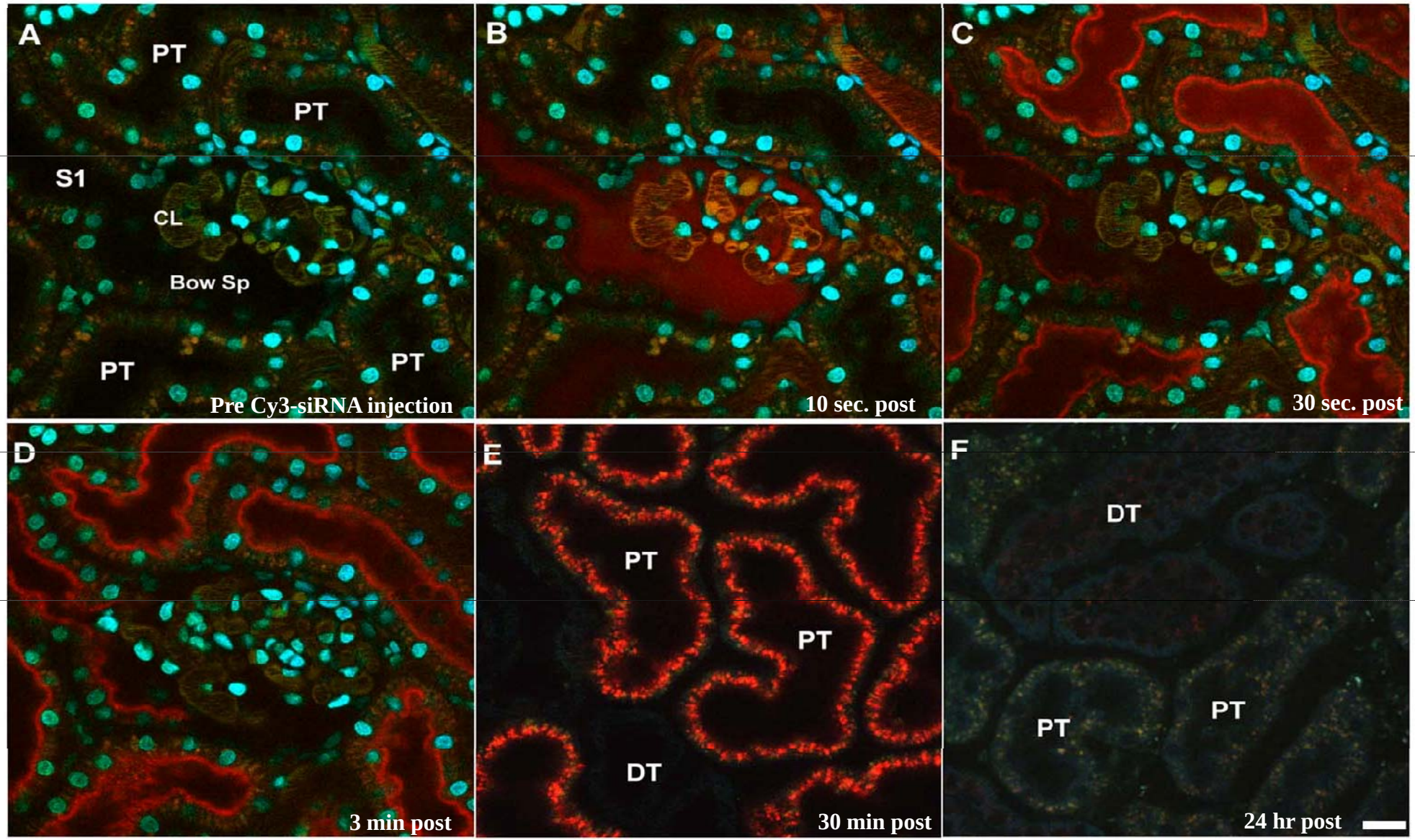
Cy3-siRNA Filtration and Reabsorption by PTCs

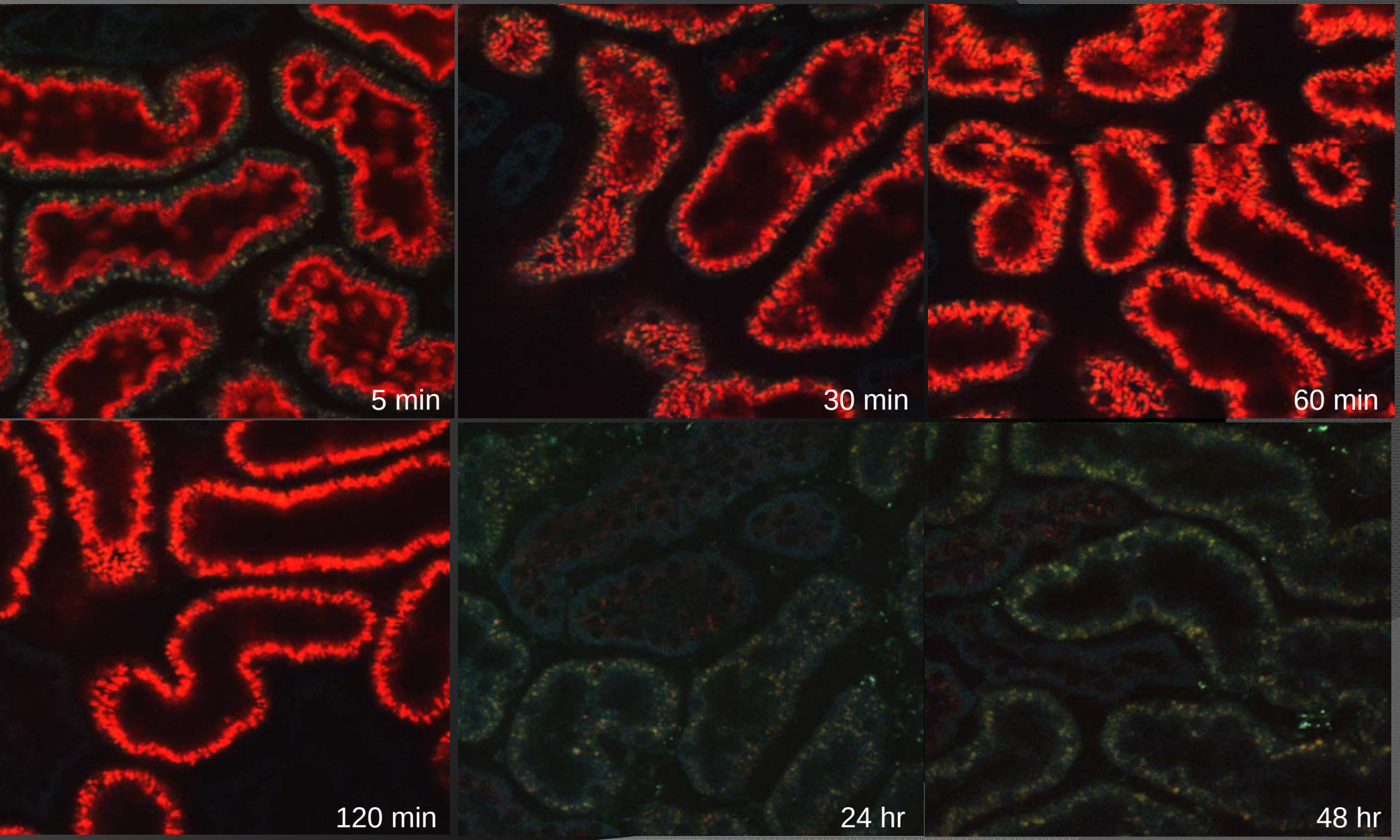




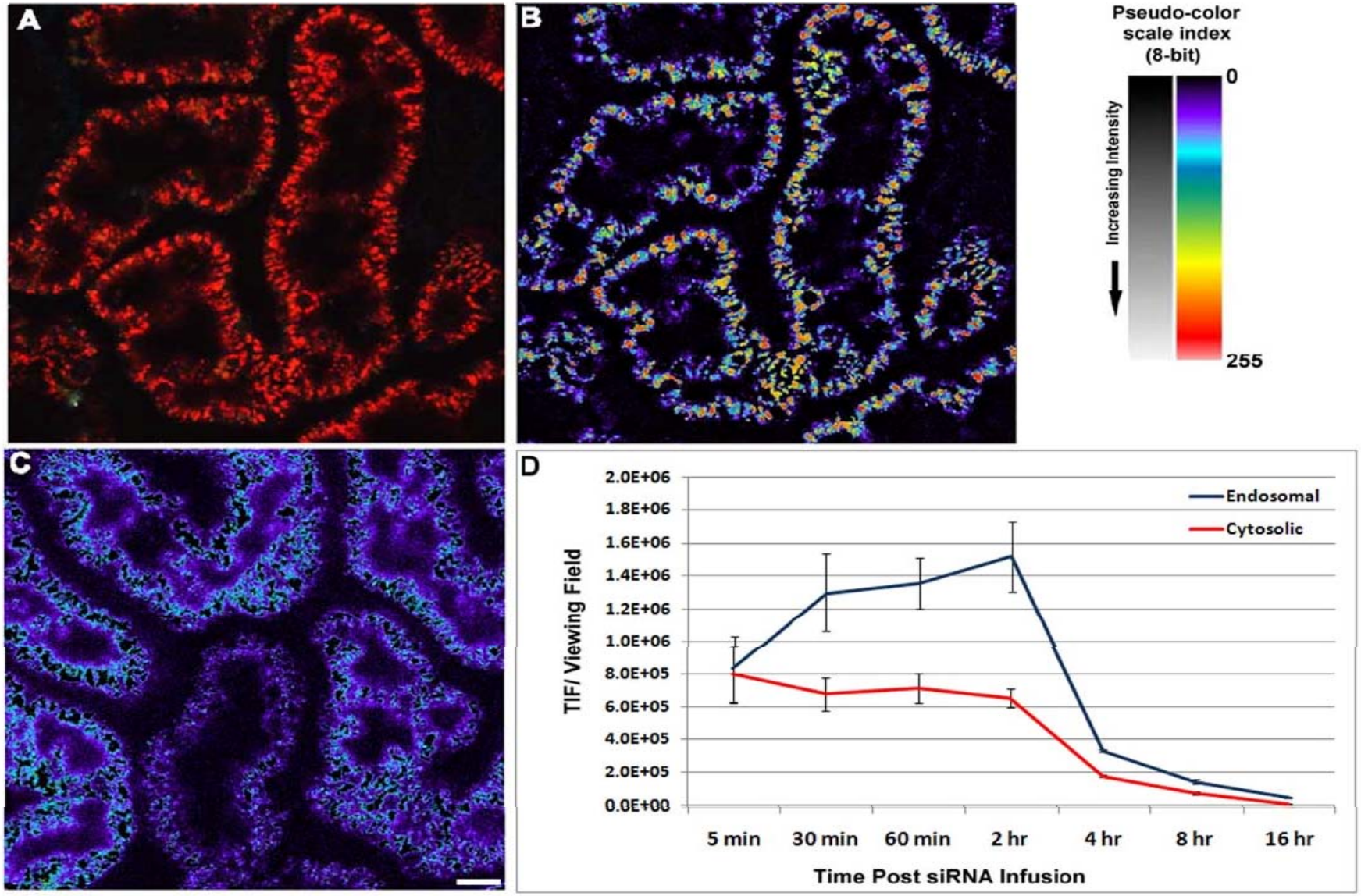
Molitoris et.al. JASN 2009

PTC Uptake and Metabolism of Cy3-siRNA



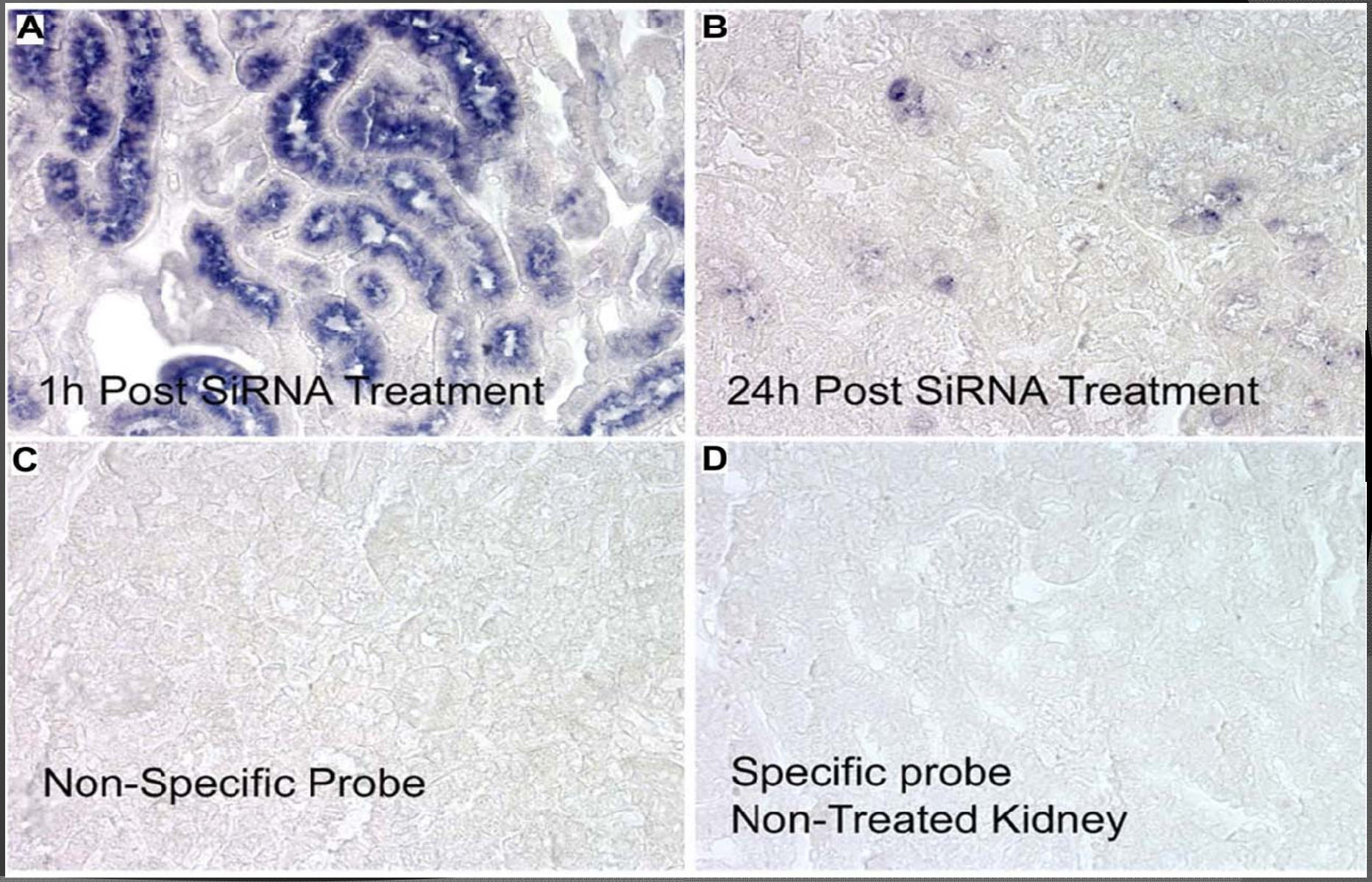

5 min
30 min
60 min
120 min
24 hr
48 hr

Quantifying Vesicular vs Cytosolic Cy3-siRNA in PTCs

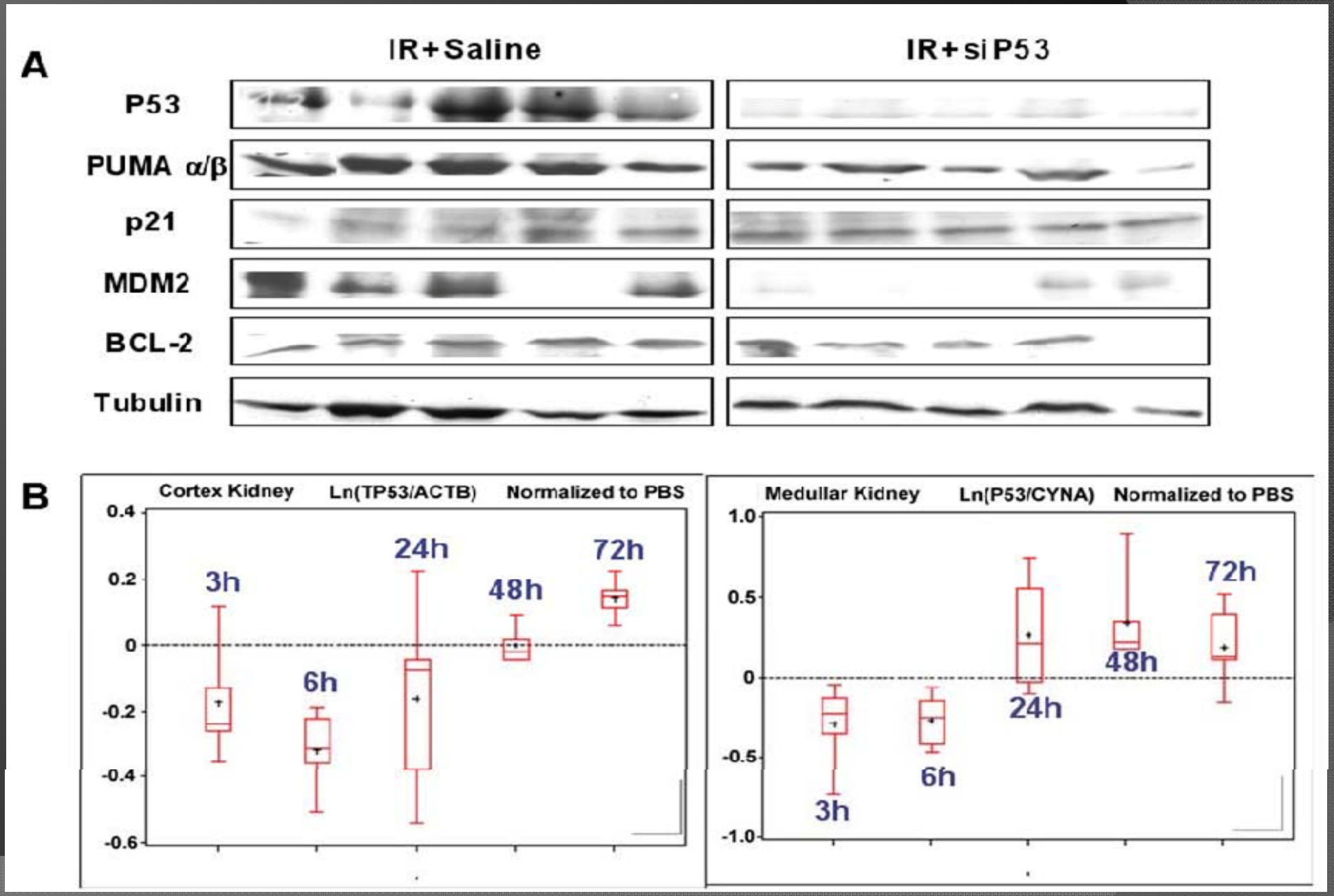


Molitoris et. al. JASN 2009

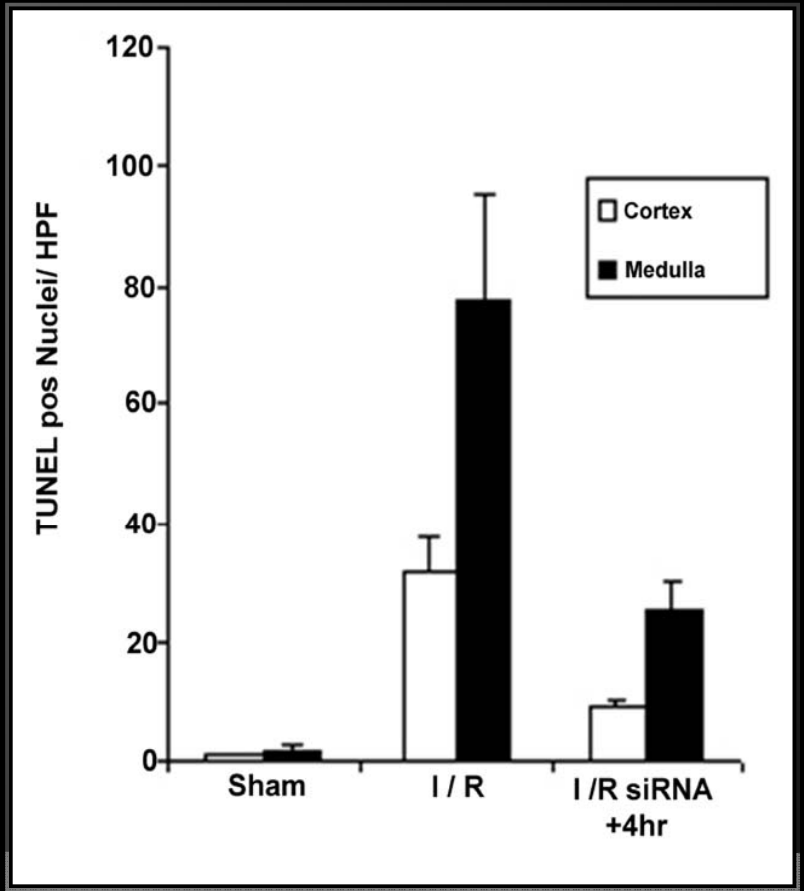
Rapid Metabolism of siRNA in PTC by In situ Hybridization

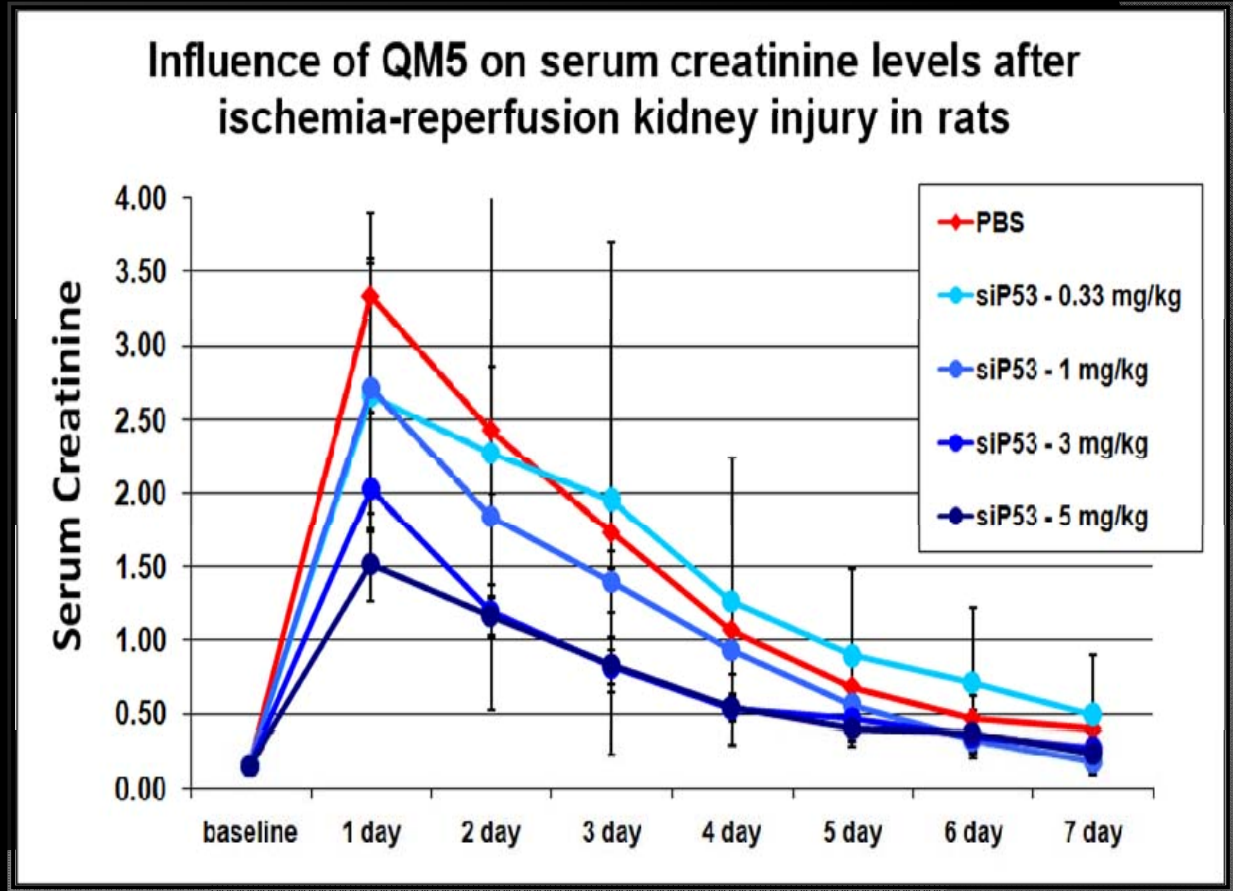


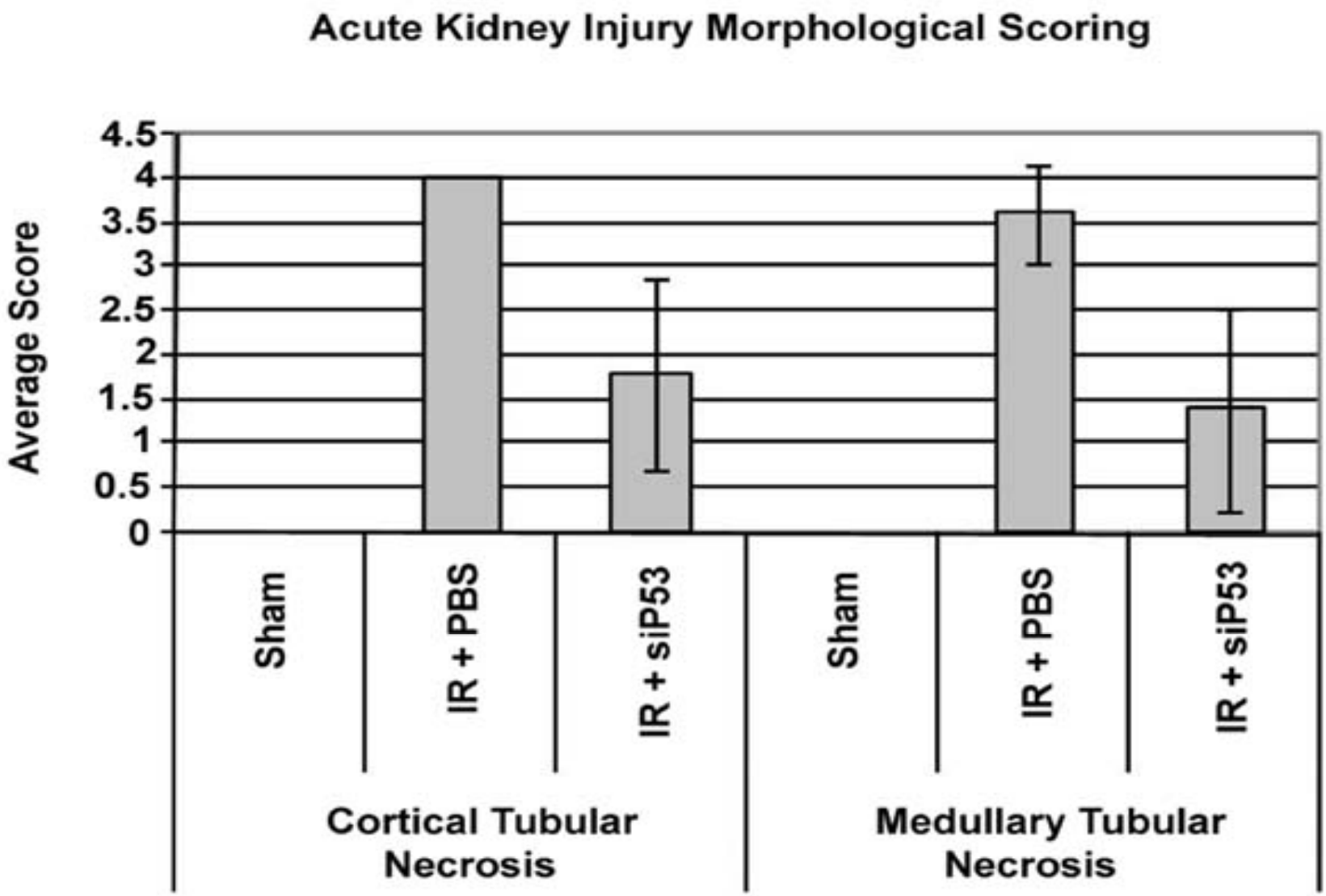
Molitoris et.al JASN 2009


A
IR+Saline
IR+siP53
P53
PUMA α/β
p21
MDM2
BCL-2
Tubulin
B
Cortex Kidney
Ln(TP53/ACTB)
Normalized to PBS
0.4
0.2
0
-0.2
-0.4
-0.6
3h
6h
24h
48h
72h
Medullar Kidney
Ln(P53/CYNA)
Normalized to PBS
1.0
0.5
0
-0.5
-1.0
3h
6h
24h
48h
72h

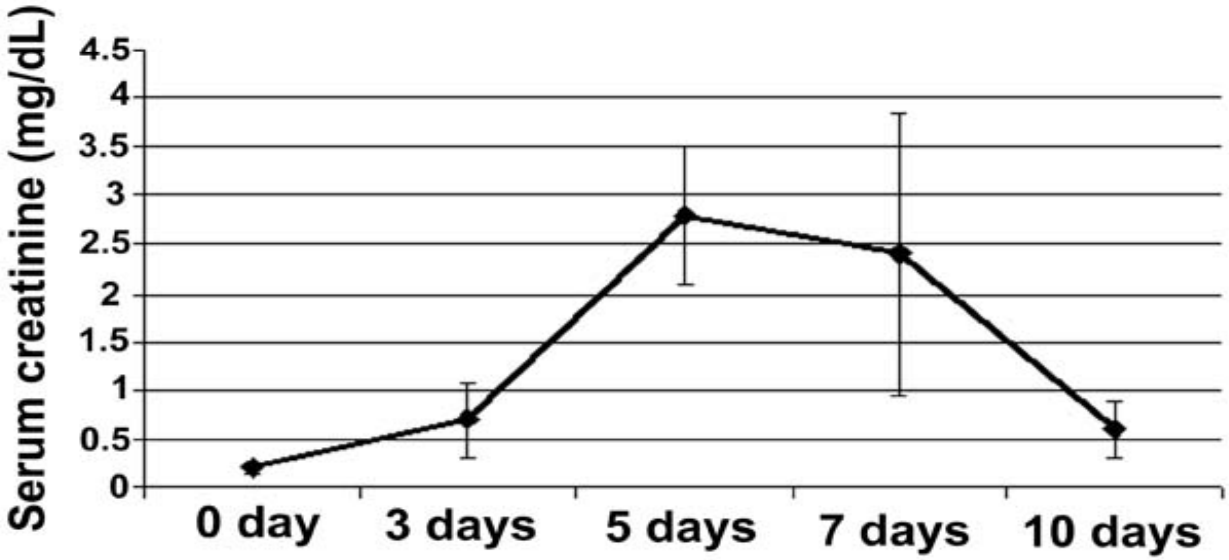
Effect of siRNA to P53 on Expression, Apoptosis and Kidney Function



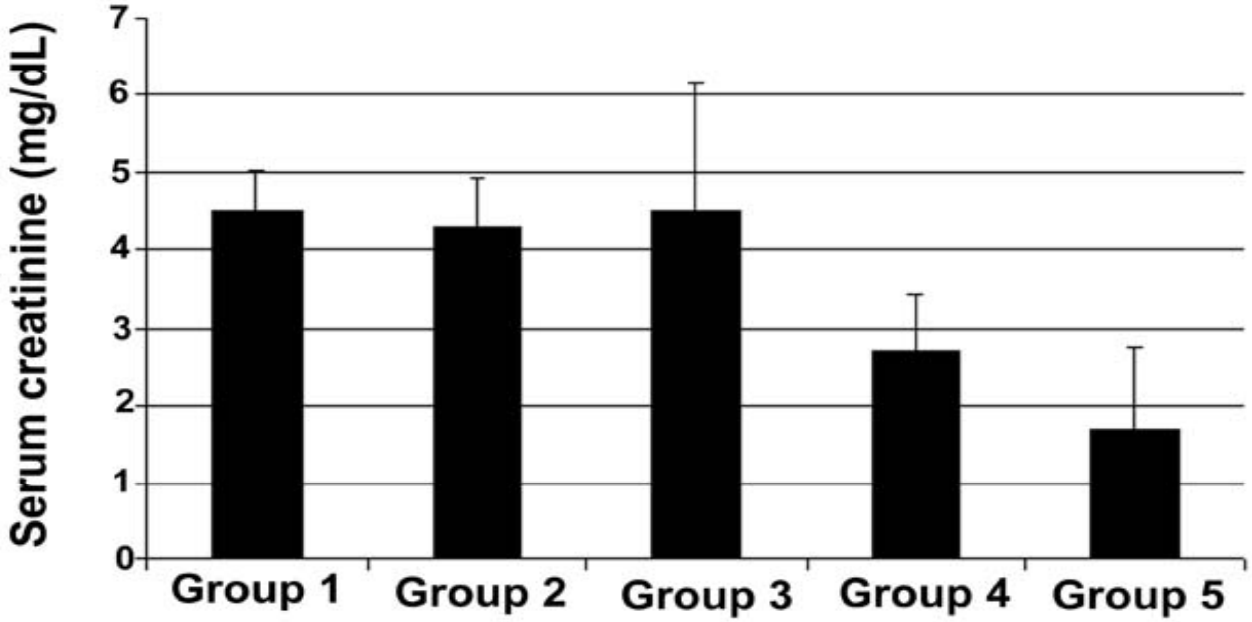



Acute Kidney Injury Morphological Scoring
Average Score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Sham
IR + PBS
IR + siP53
Sham
IR + PBS
IR + siP53
Cortical Tubular Necrosis
Medullary Tubular Necrosis

siP53 Protects Against Cisplatin Induced Kidney Injury



Time Course of Injury Without Therapy

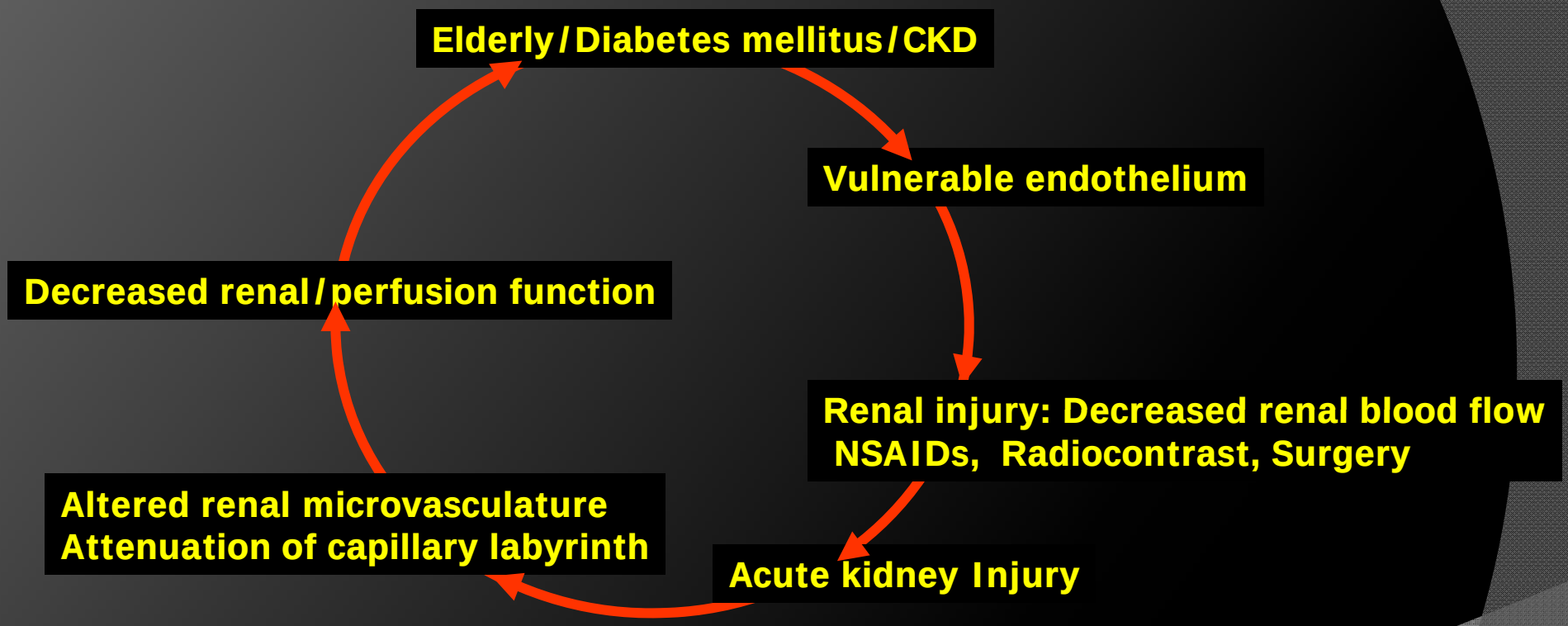


Effect of siP53 Therapy at Day 5

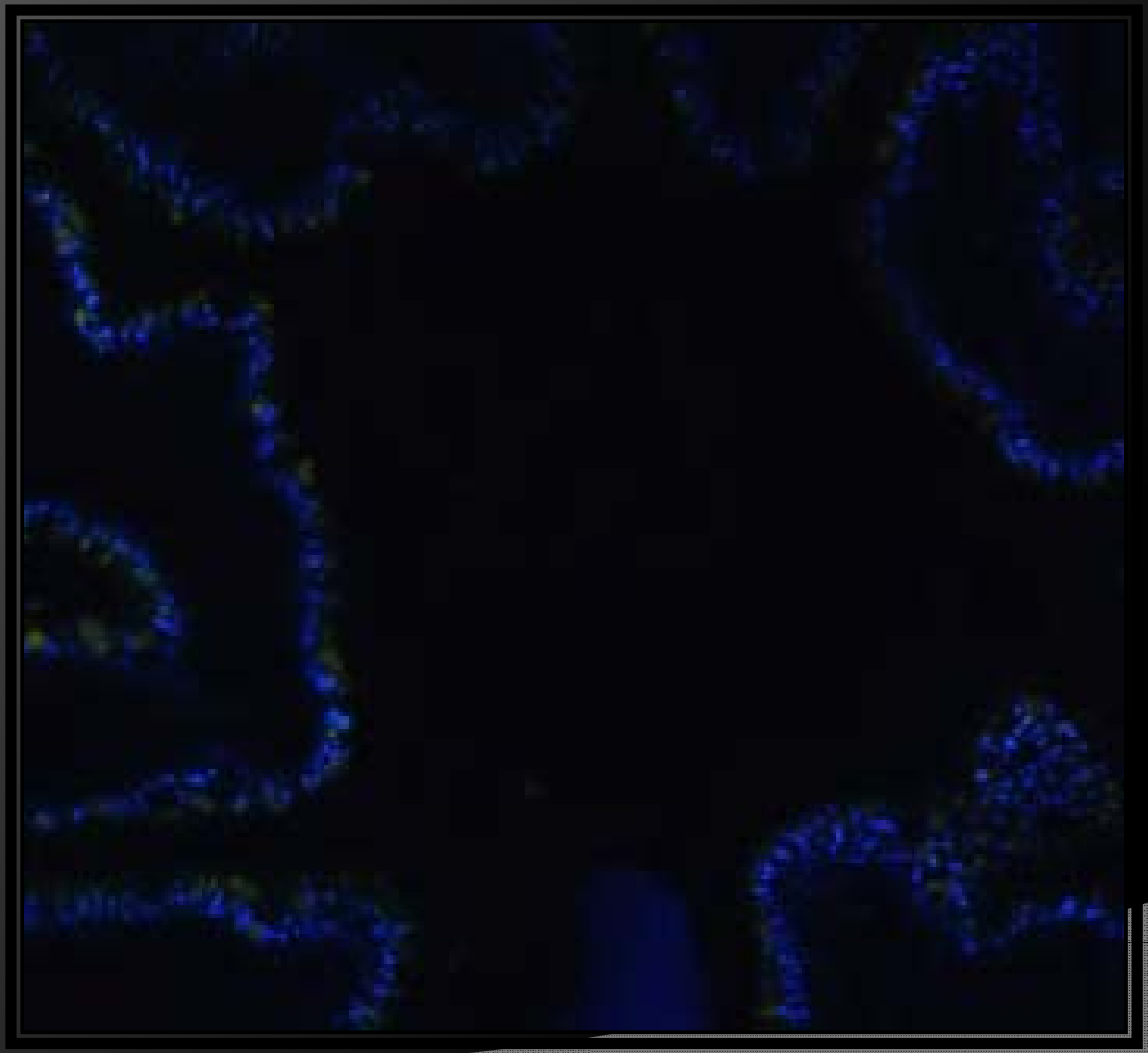
Group 1 No Therapy
Group 2 12 mg/Kg 30 prior to cisplatin
Group 3 siP53 4 hours post cisplatin
Group 4 Group 2 plus Doses on Days 2
Group 5 Group 3 plus Doses on Days 2

Molitoris et.al. JASN 200

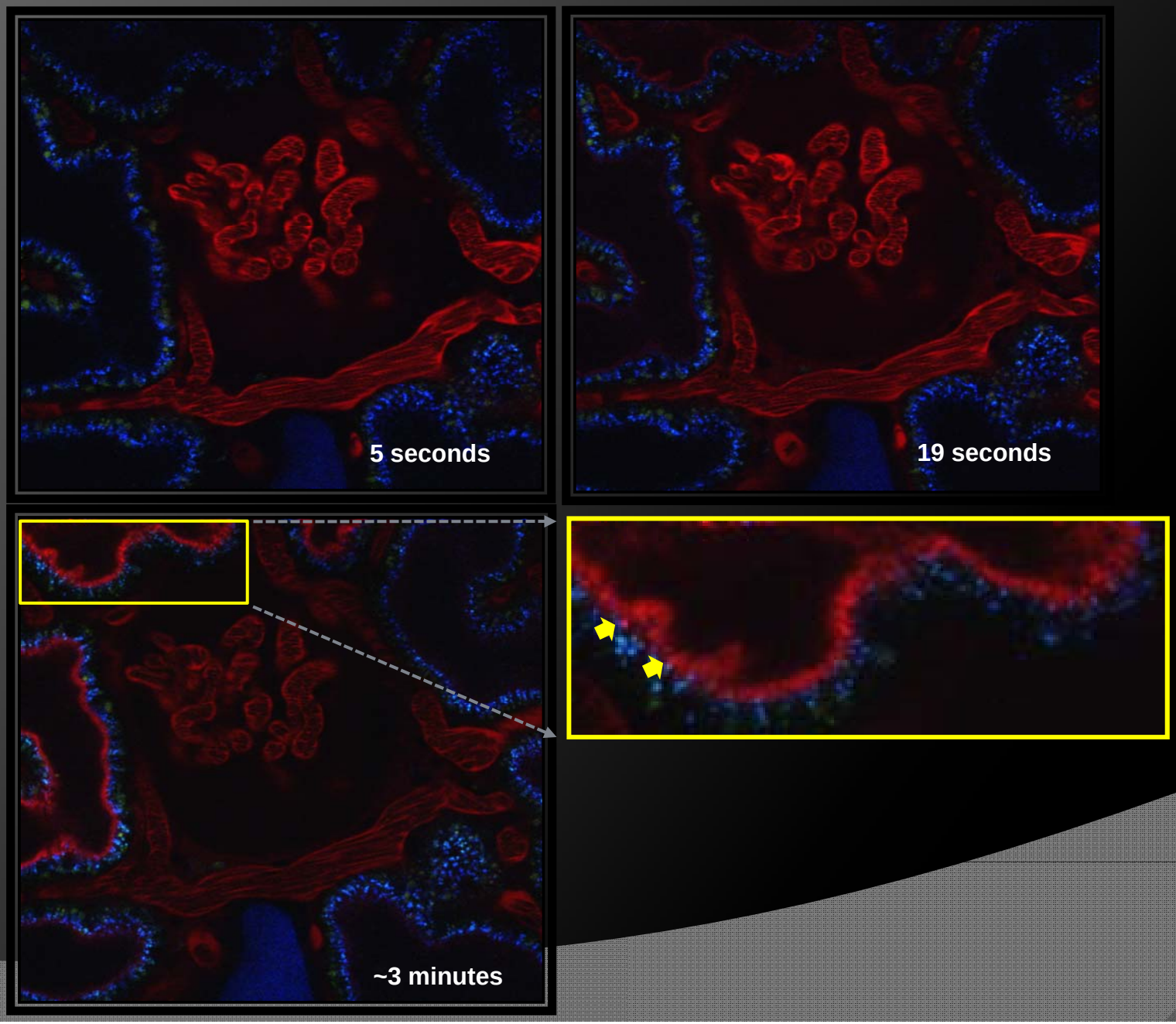
A vicious cycle



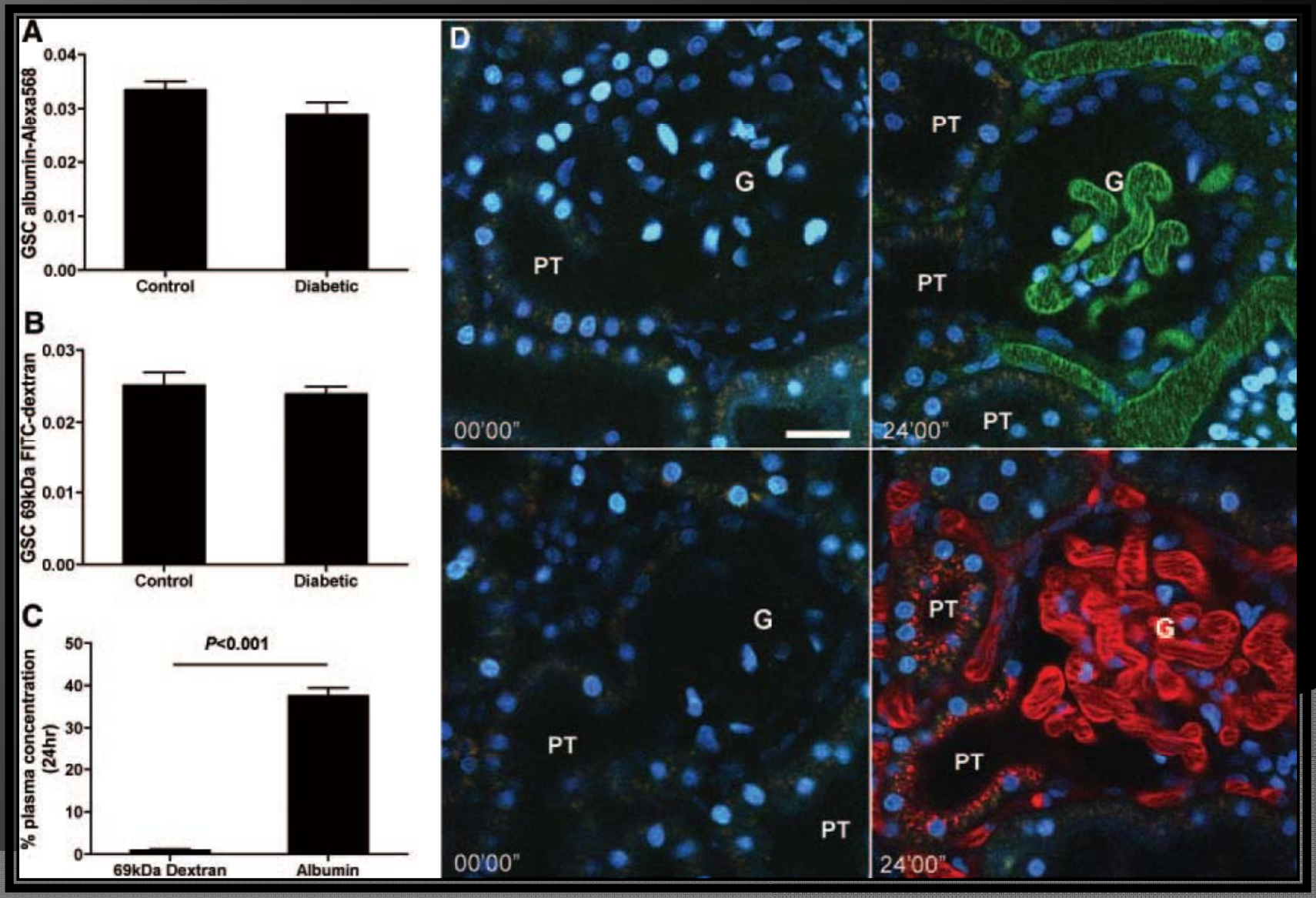
Albumin Filtration and Reabsorption in the Rat



Albumin Filtration and Reabsorption in the Rat



Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption

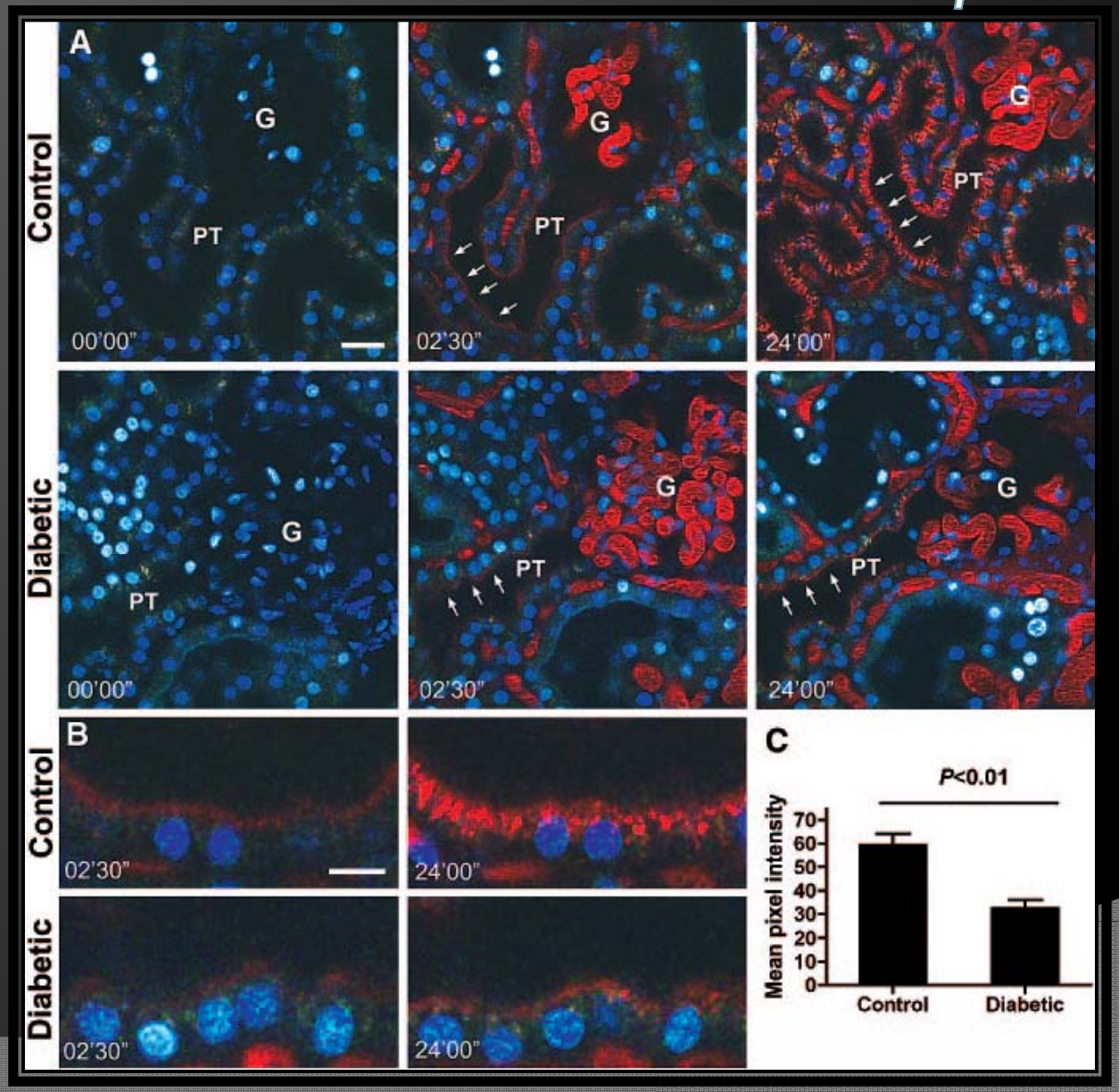


69 kDa
FITC -Dextran

ALEXA 586
Albumin

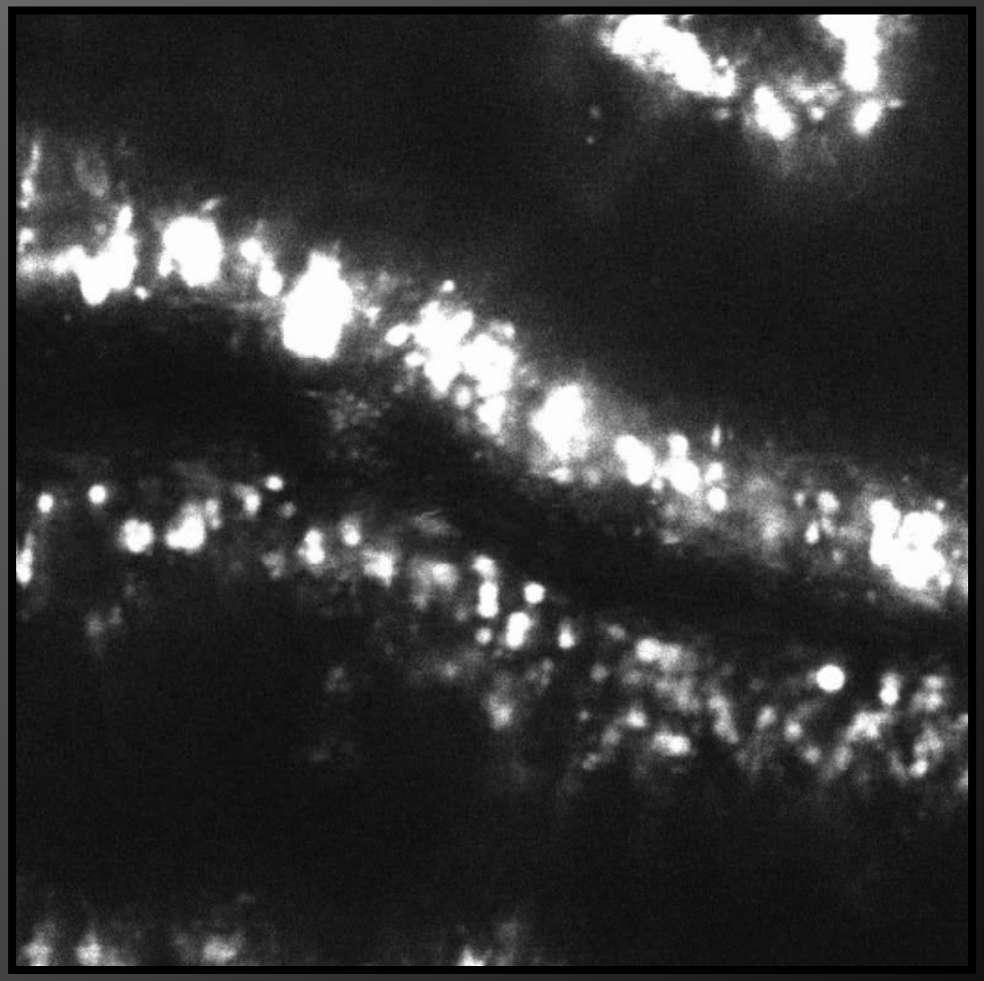
Russo et.al. JASN 2009

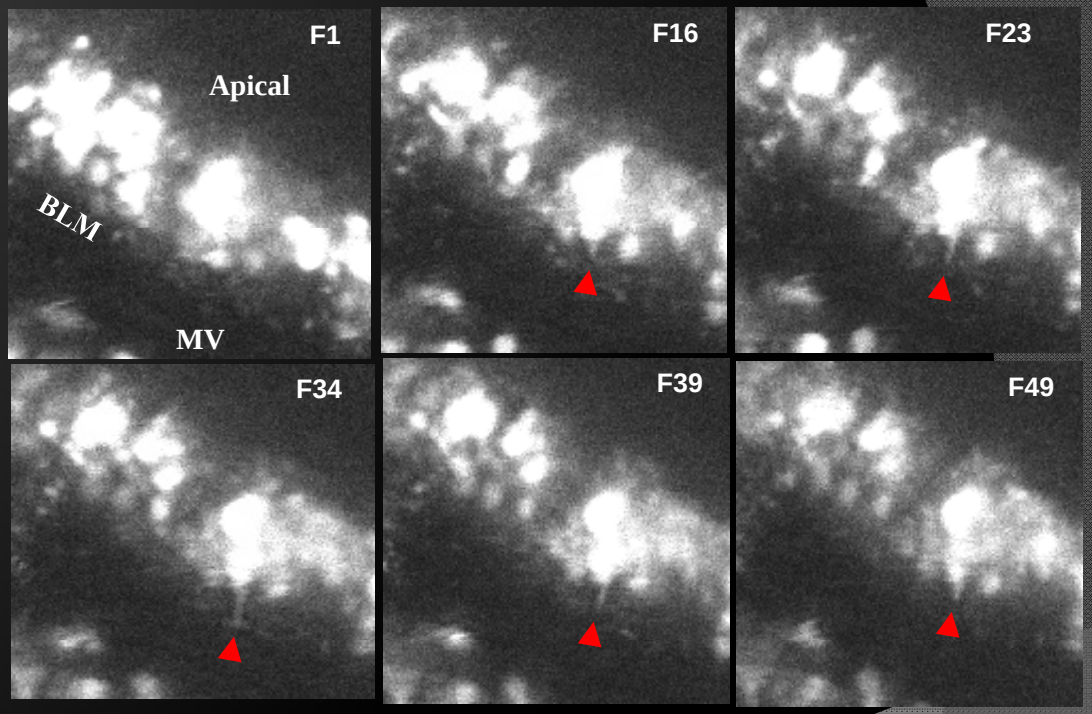
Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption



Russo et.al. JASN 2009

PTC Albumin Transcytosis





Summary

The Proximal Tubule cell is a long lived cell with avid endocytosis

Endocytosis is necessary for recycling filtered materials

Unfortunately, this includes toxins that accumulate and cause cell injury

RNAi therapy is perhaps best applied to the Proximal Tubule

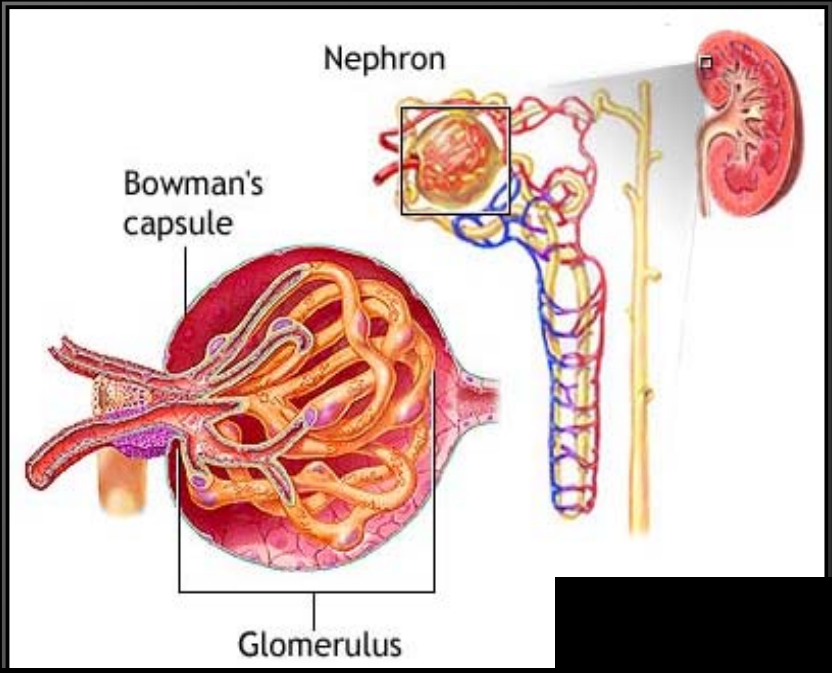
Presently it is possible to inhibit upregulation of specific proteins

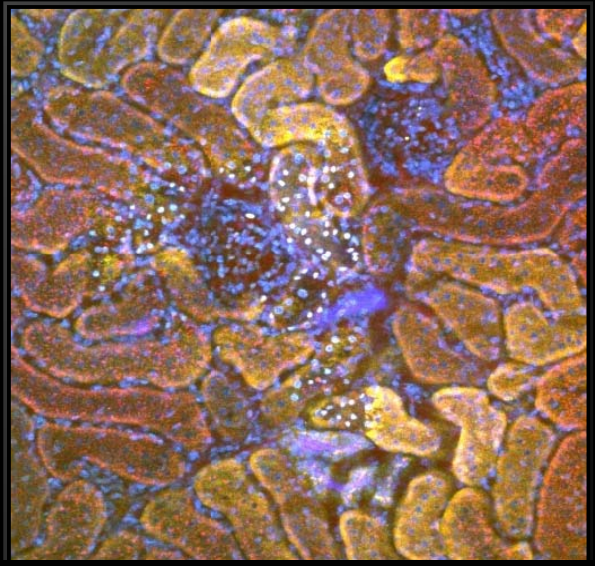
It is also possible to down regulate specific proteins

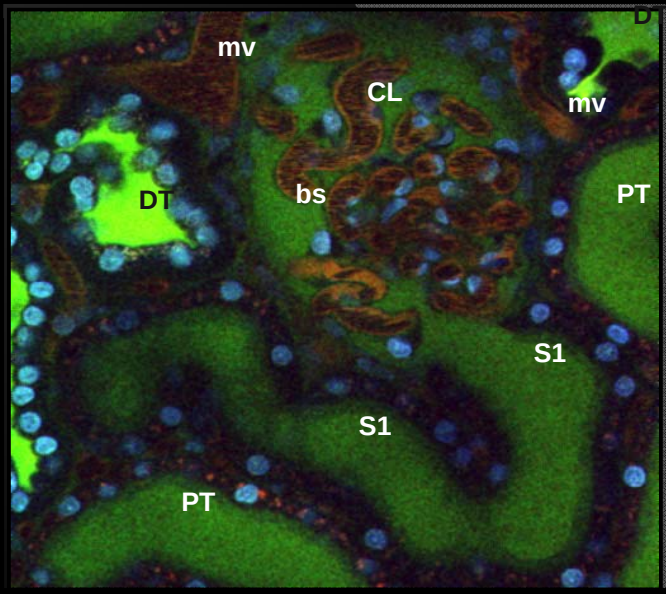
Clinical trials are underway for both uses of RNAi in Proximal Tubule cells

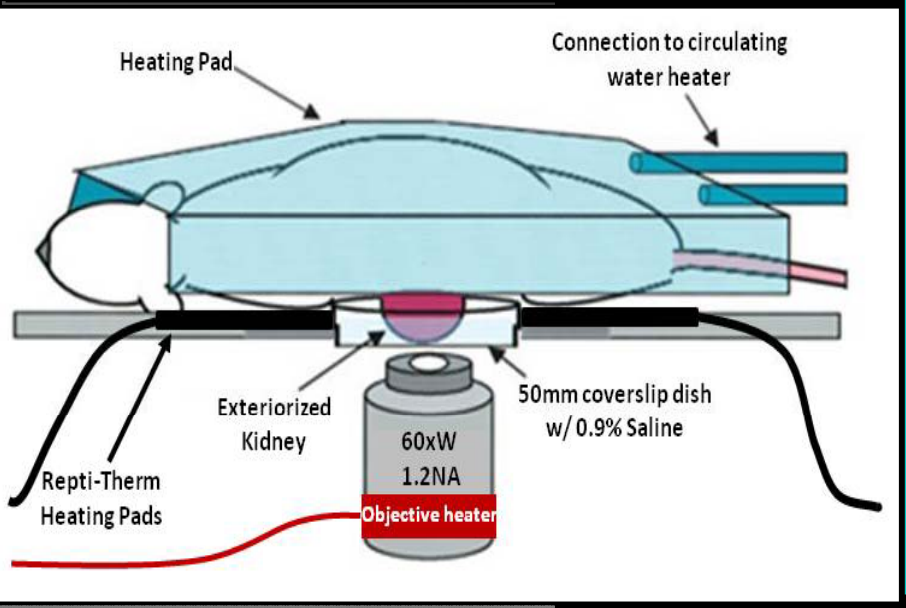
There are many untested potential targets for endocytic processes in PTCs

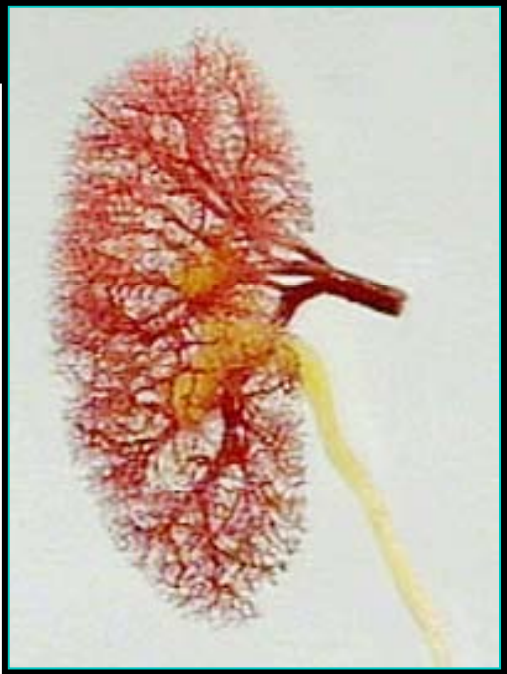
Visualizing Vascular, Glomerular & Nephron Function

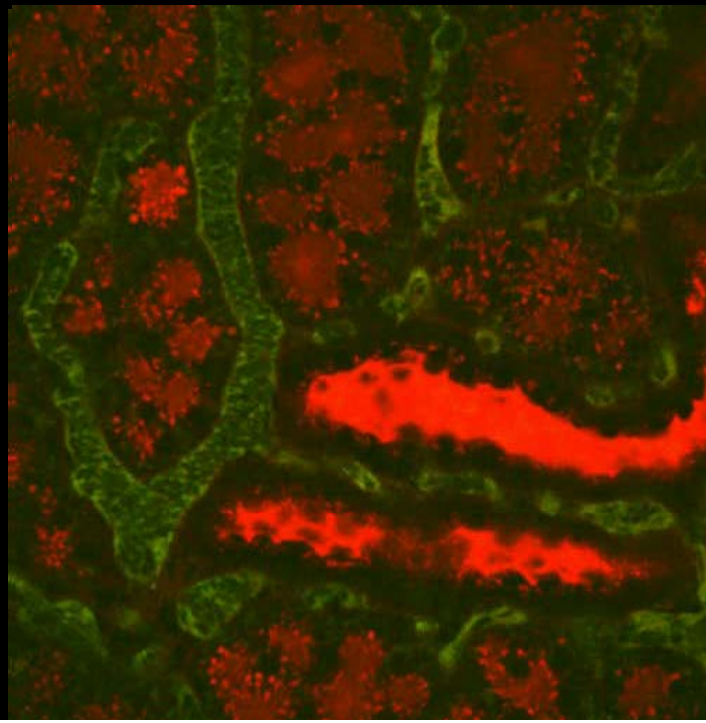


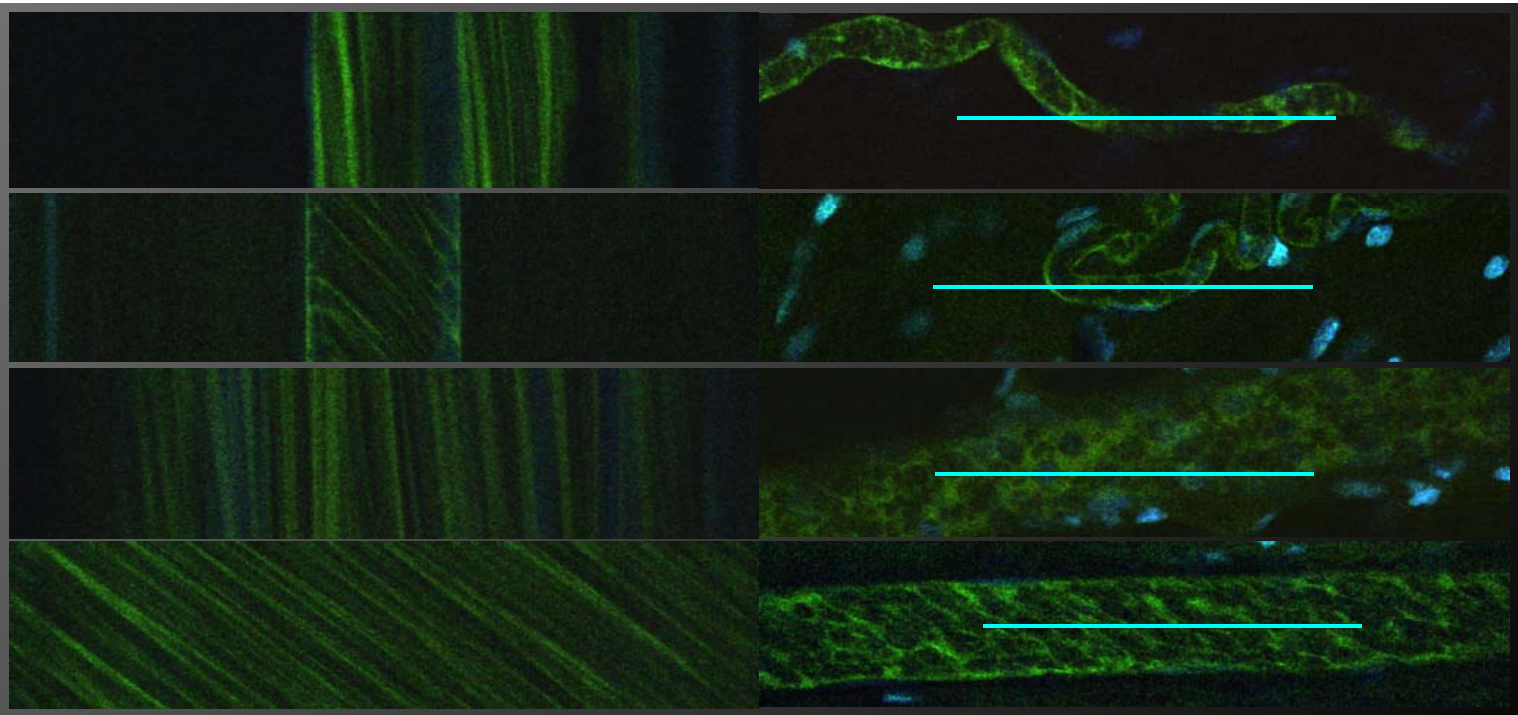










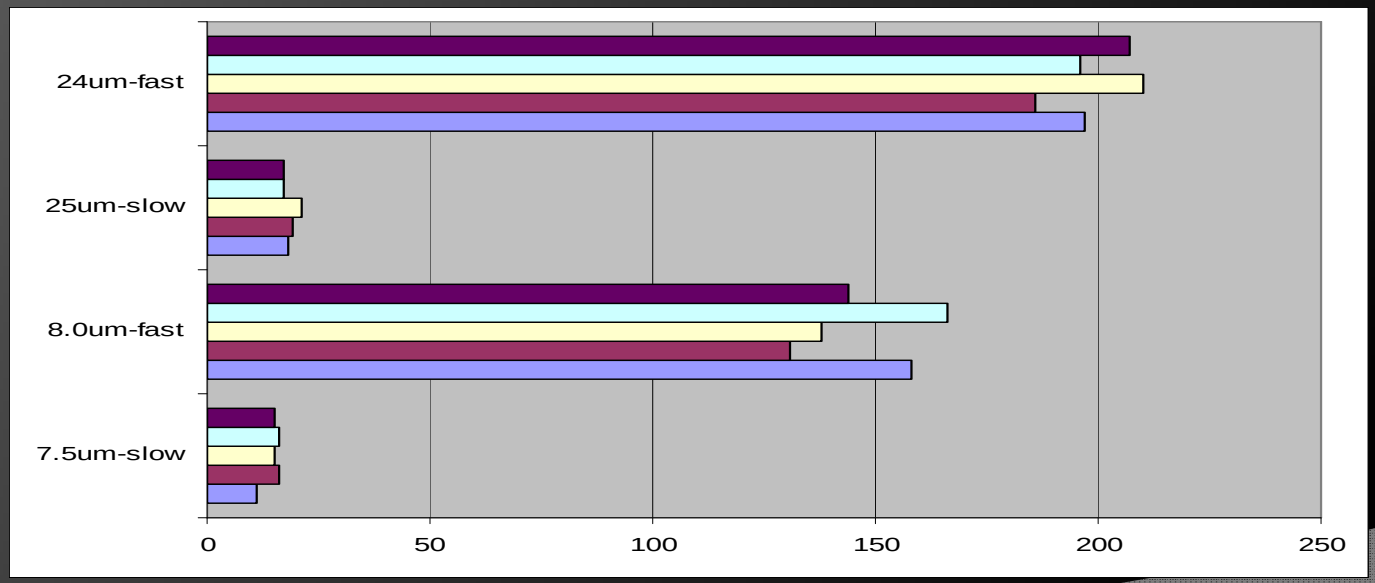


Vessel Diam.=7.5 um
Ave.Speed=14um/sec

Vessel Diam.=8 um
Ave Speed=147um/sec

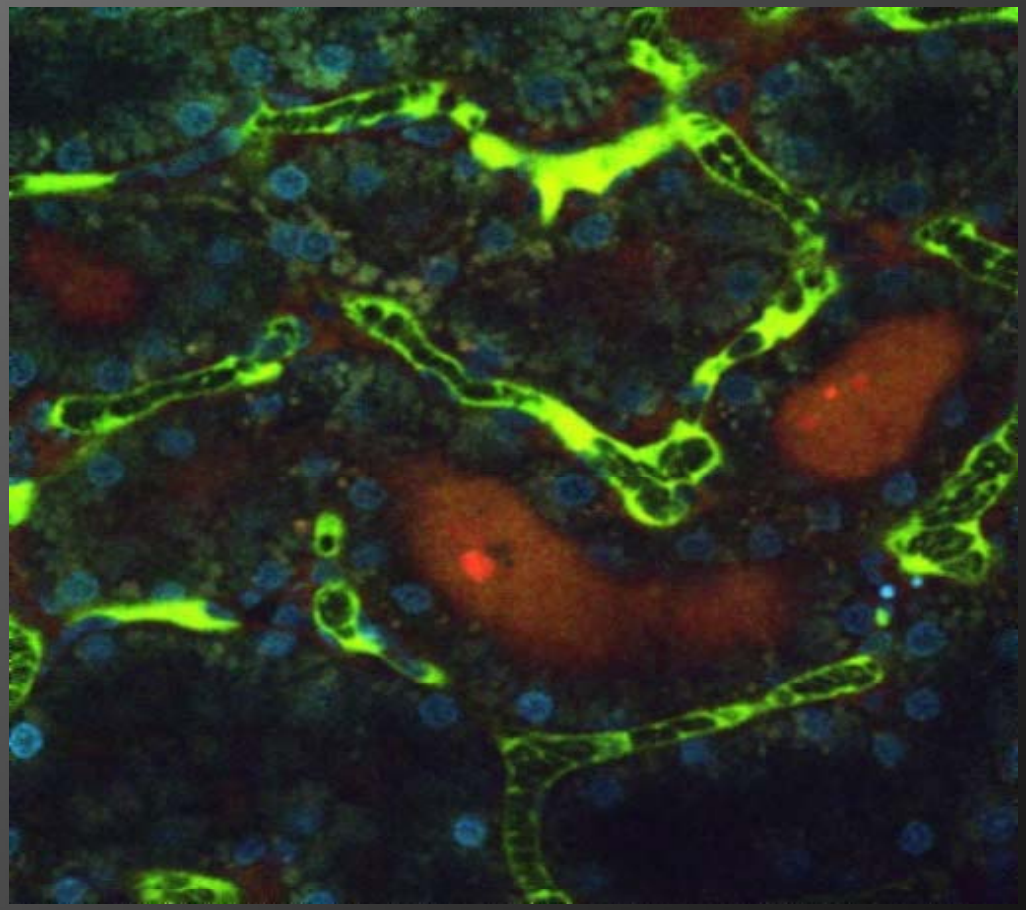
Vessel Diam.=23 um
Ave Speed=18um/sec

Vessel Diam.=24 um
Ave Speed=199um/sec

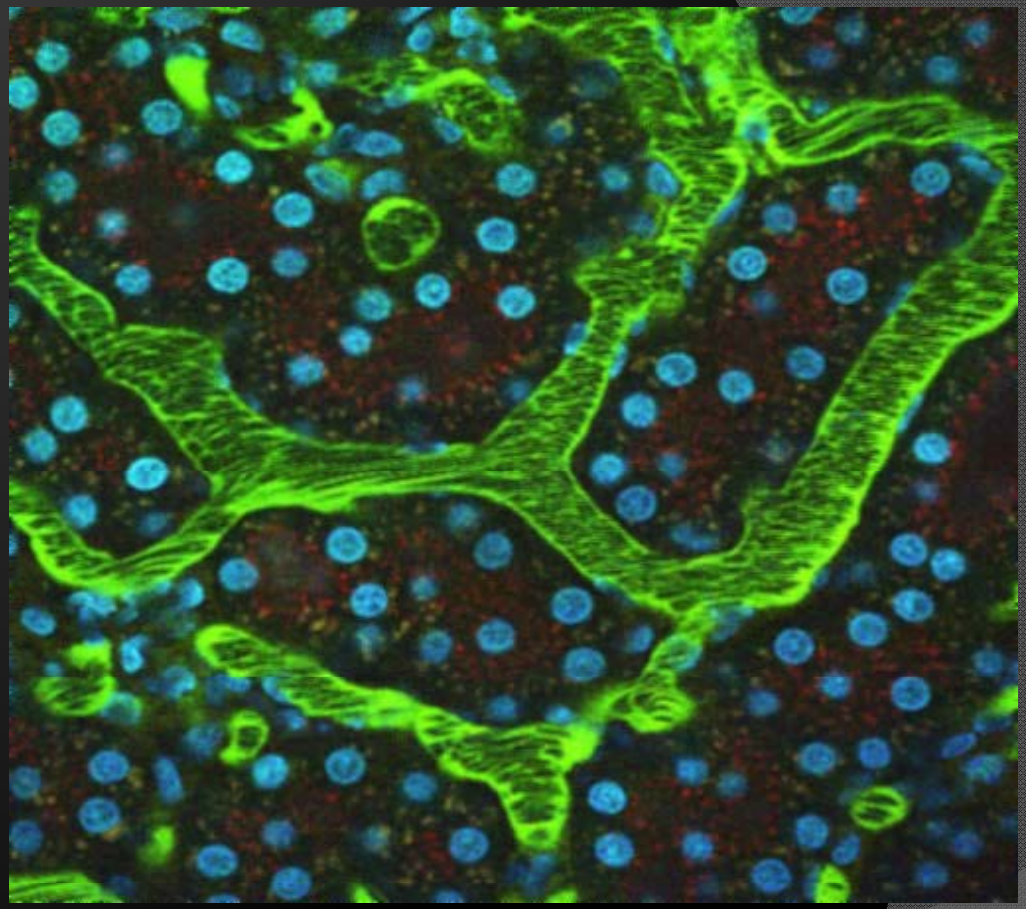


Vessel Diam. relative speed	Ave Speed in um/sec	St. Dev
7.5um-slow	14.6	2.07364414
8.0um-fast	147.4	14.3805424
25um-slow	18.4	1.67332005
24um-fast	199.2	9.5760117

Microvascular Blood Flow at 24h Post Ischemia Effect of sTM



Saline treated



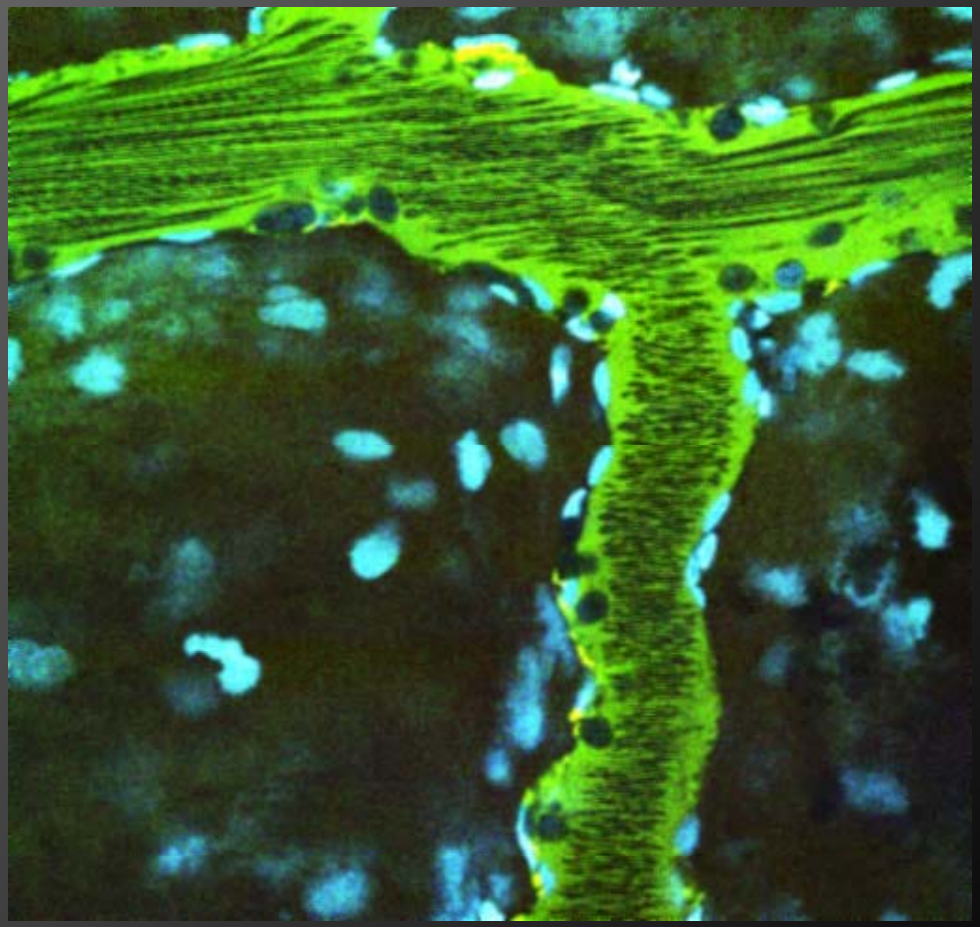
sTM treated

Blood Flow velocity (µm/sec)	253.36+/-95.01	786.75 +/- 280.75 *

*P < 0.05

Sharfuddin et.al JASN 2009

Leukocyte-Endothelial Interactions – Intra-Vital 2-Photon



Ischemic – Saline treated rat at 24h

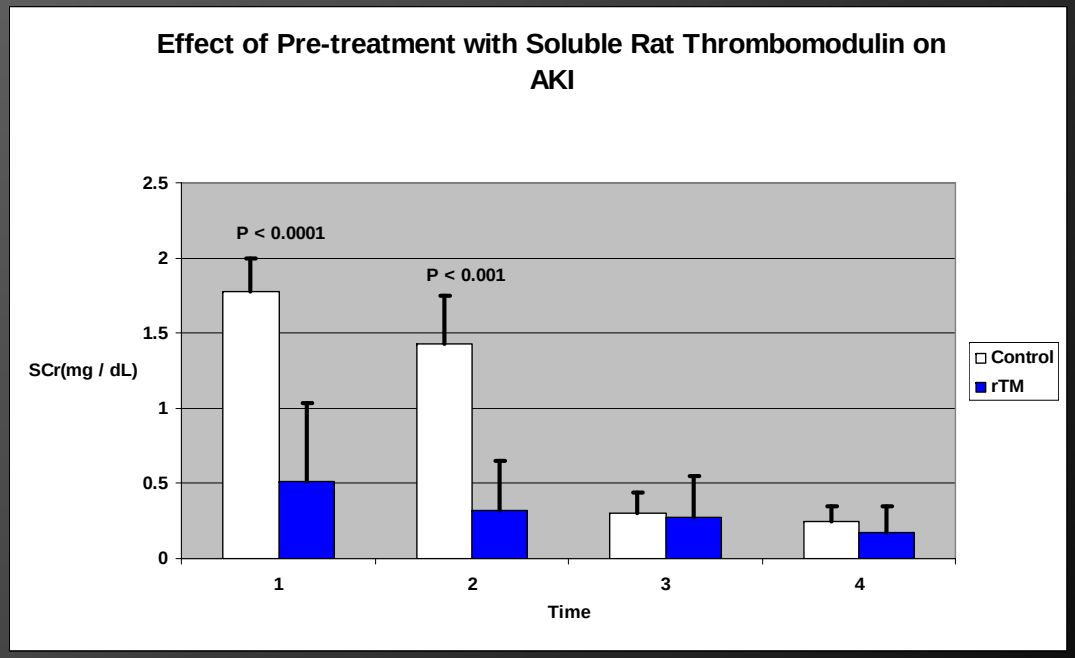
	Saline	sTM treated
Flowing (%)	69.5	88.3 *
Rolling (%)	18.2	8.3 *
Static (%)	12.9	3.3 *

* $p<0.05$

Sharfuddin et.al. JASN 2009

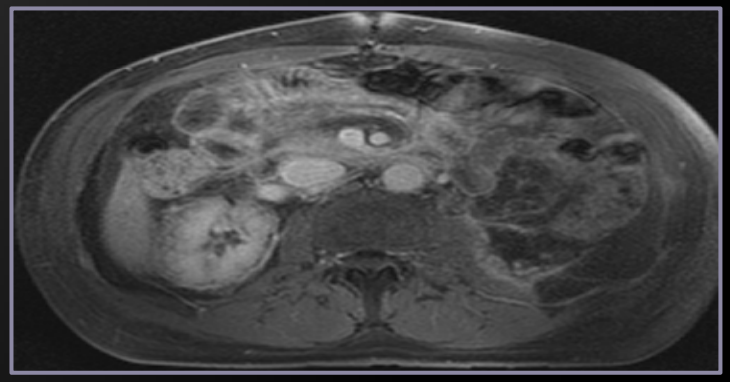
with/without sTMGross Specimens

Effect of sTM Therapy on Kidney Function in Acute Kidney Injury

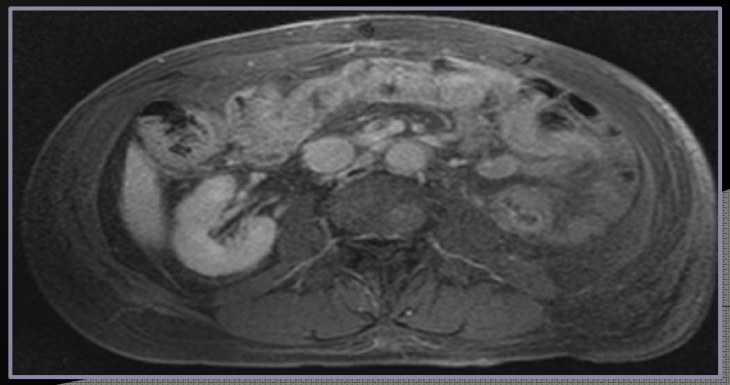


Sharfuddin et.al. JASN 2009

NMR Prior to Kidney Donation



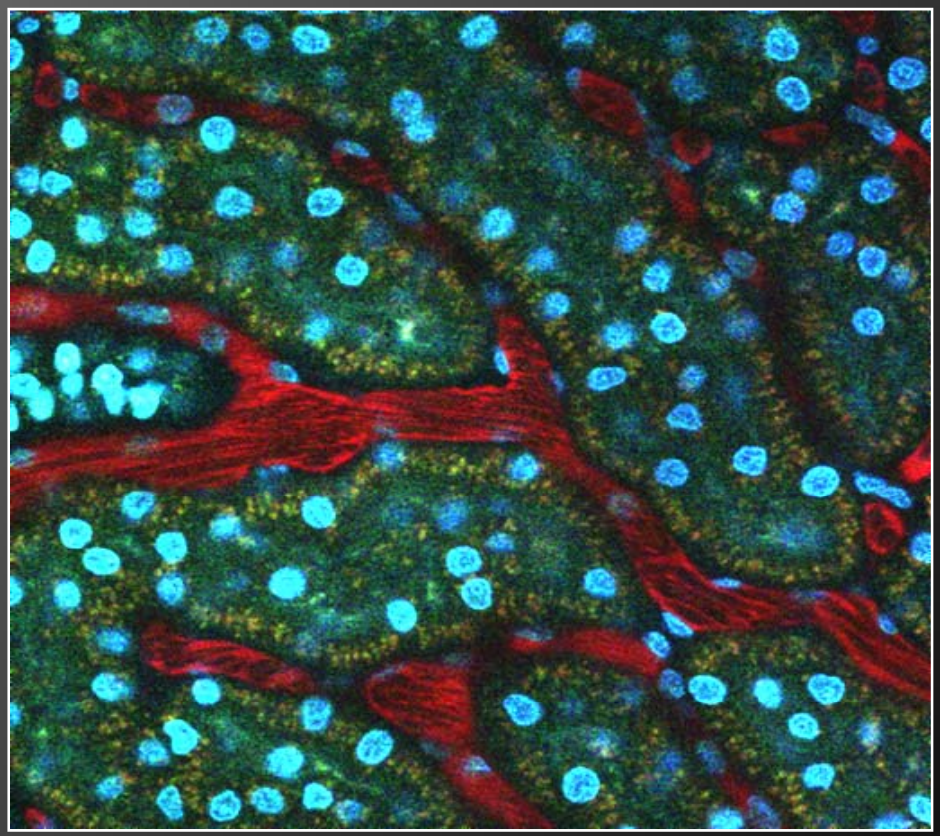
Acute Kidney Injury



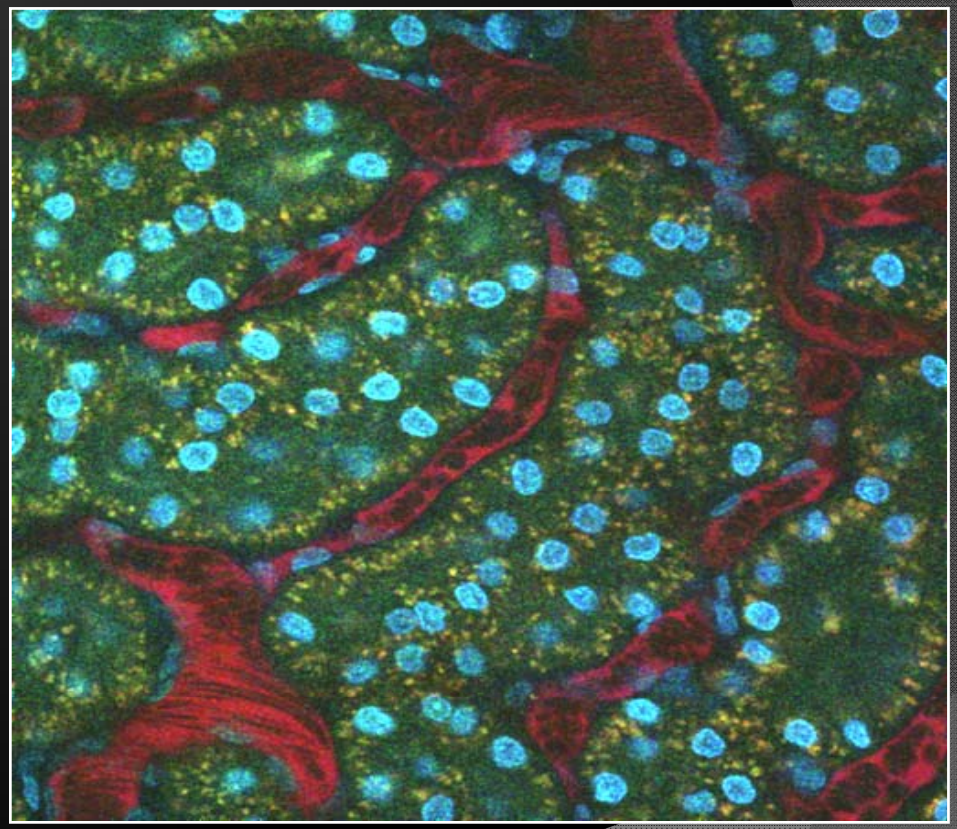
Resolution of AKI

Rosenthal et.al JASN, 2003

Microvascular Flow in CLP

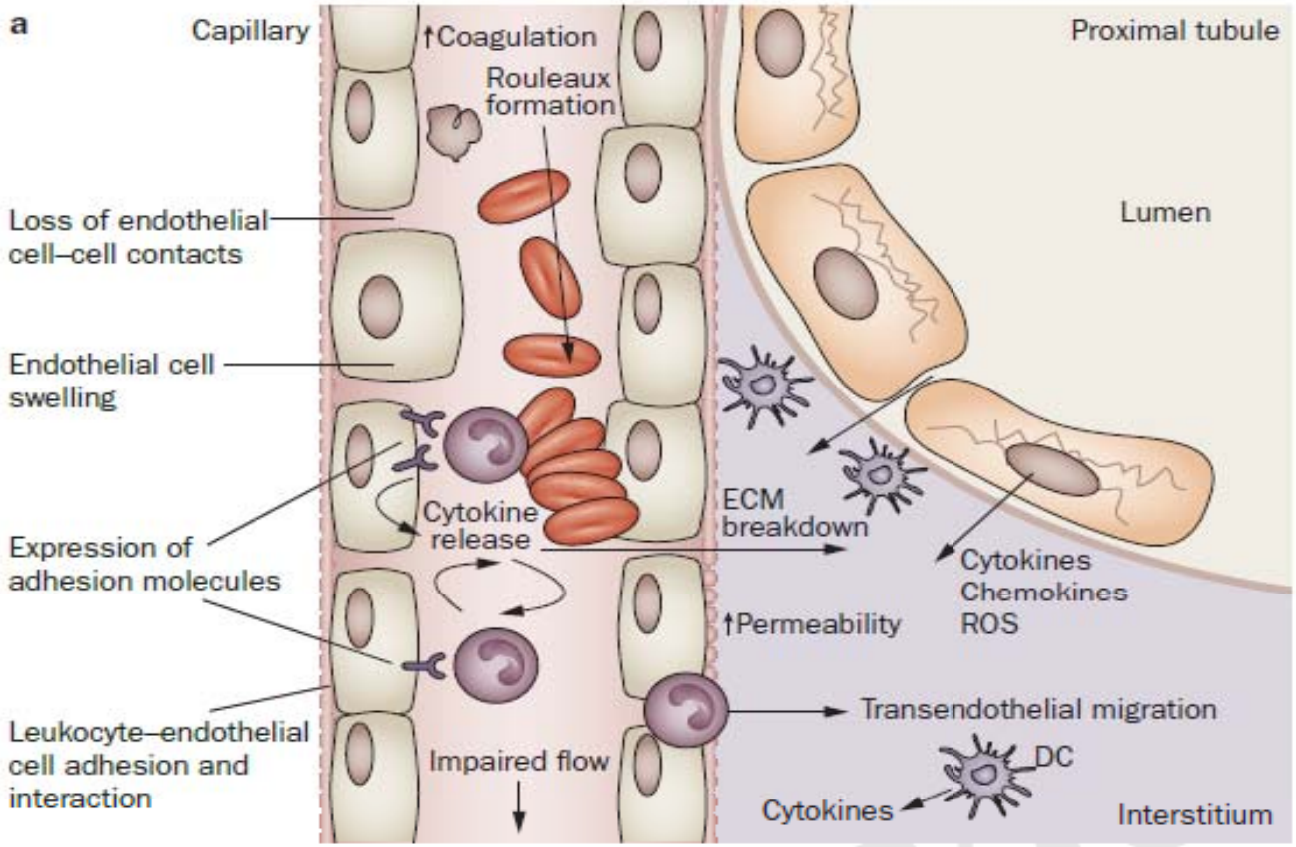


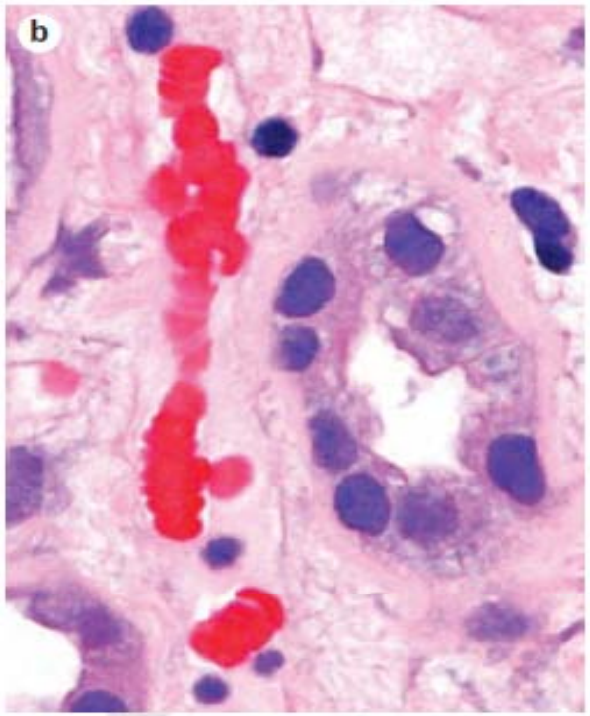
4Hr CLP



24Hr CLP

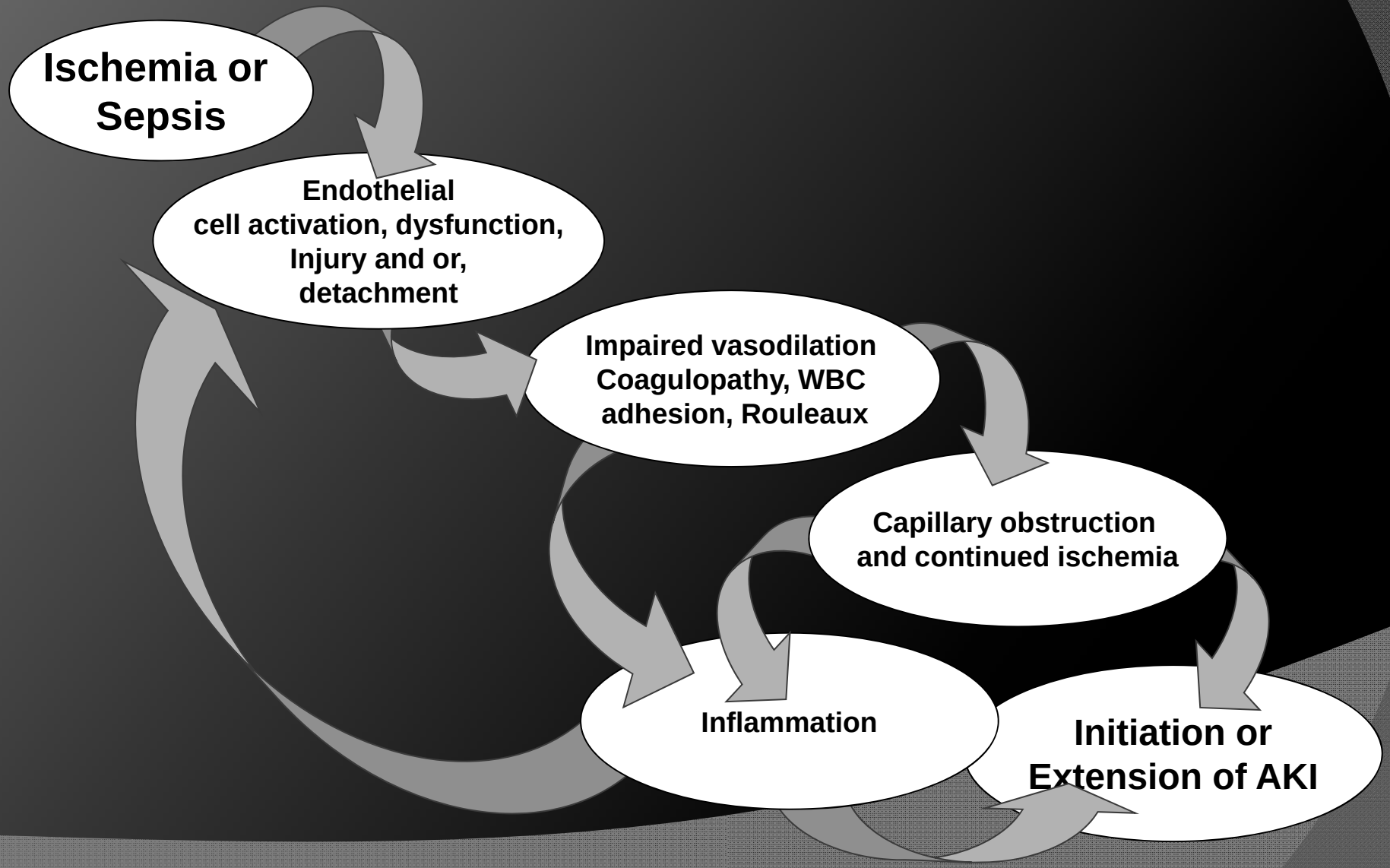
Endothelial Pathophysiologyic Events in AKI



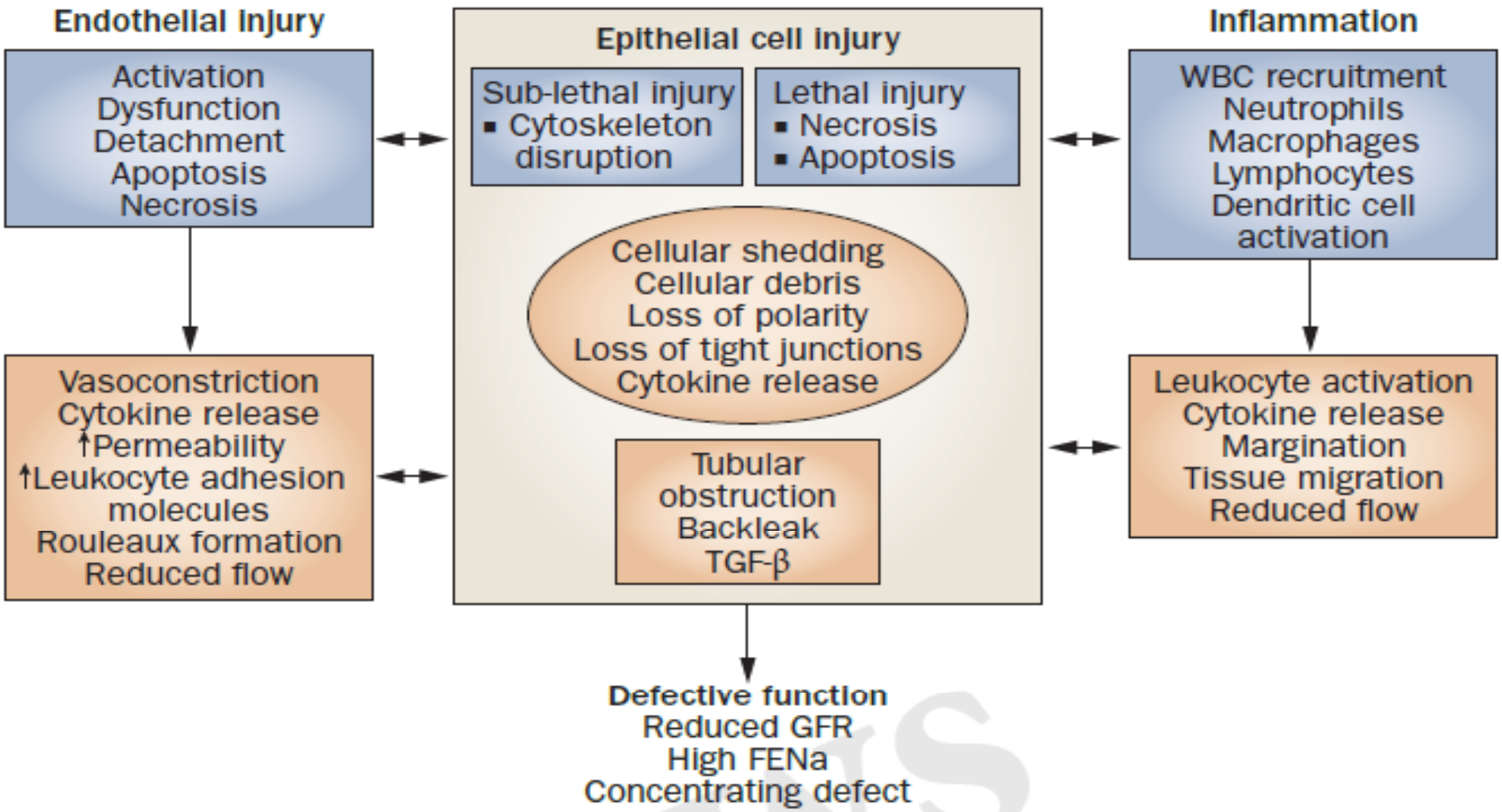


Sharfuddin and Molitoris Nature Neph Reviews 2011

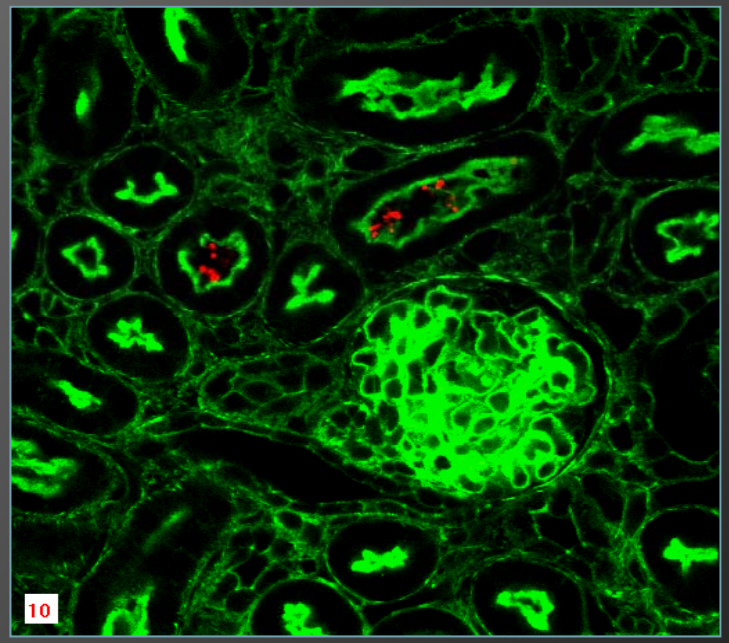
Small Vessel Injury in Acute Kidney Injury

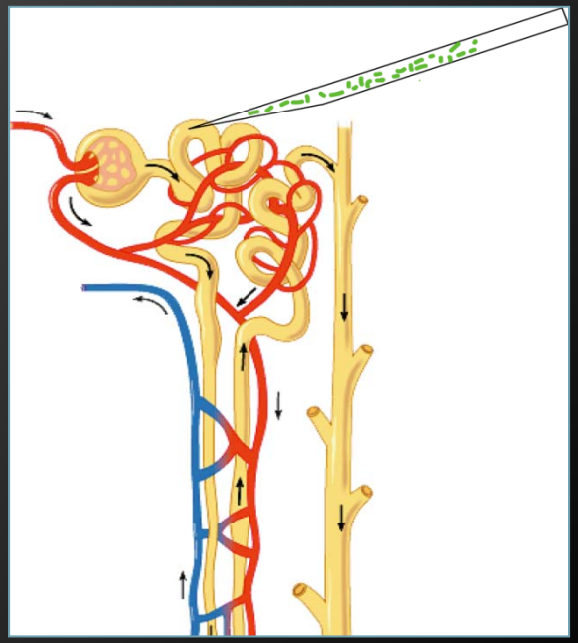


Major Cellular Components and Physiologic Effects of AK



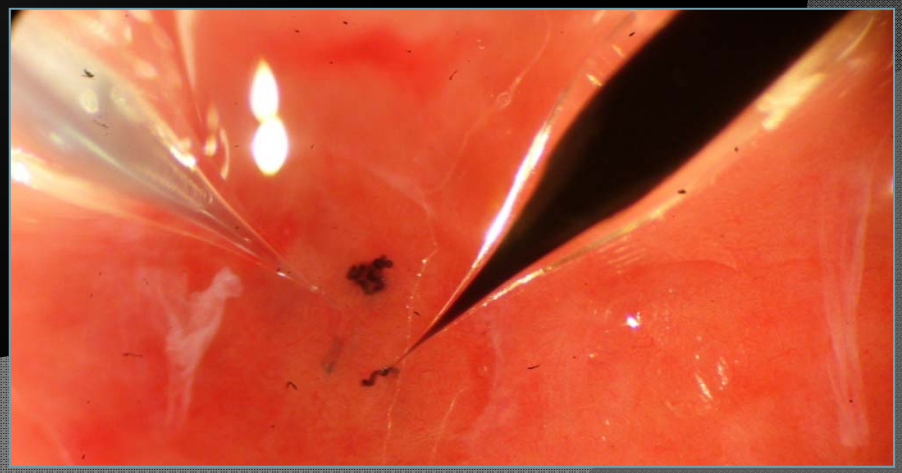
Spatial Specificity Achieved by Micro-Infusion of Bacteria into Proximal Tubules





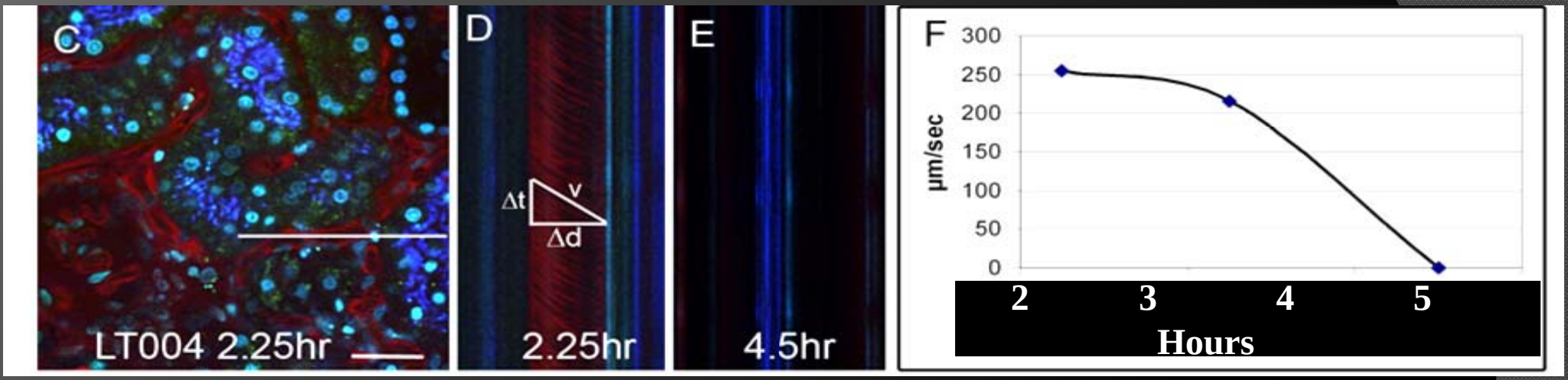
- 10^5 cfu UPEC GFP$^+$
- 0.1 to 0.7 μl injected

Agneta Richter-Dahlfors,
Lisa E. Mansson and Keira Melican
Karolinska Institutet

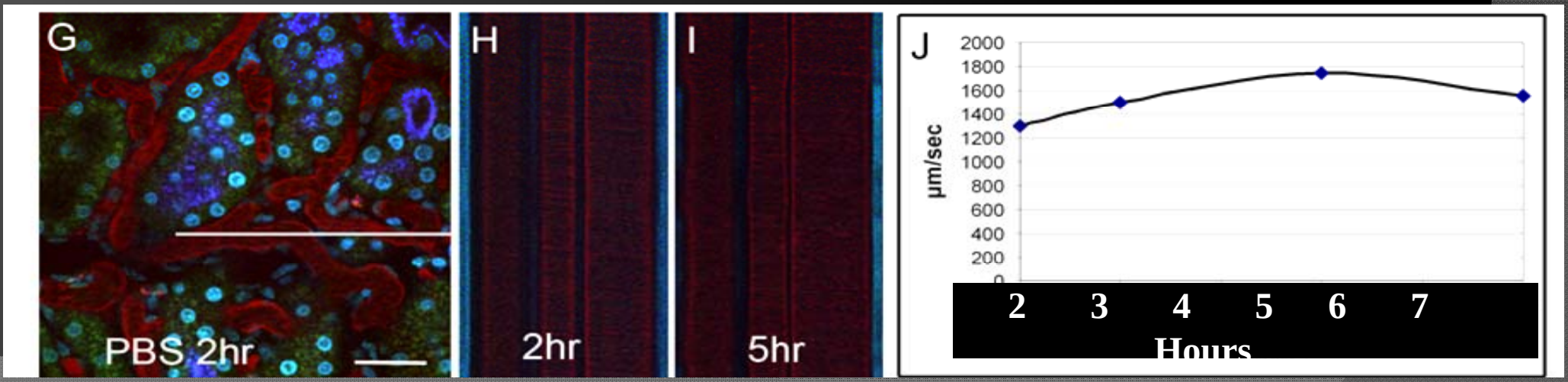


Determining blood flow rates *in vivo*

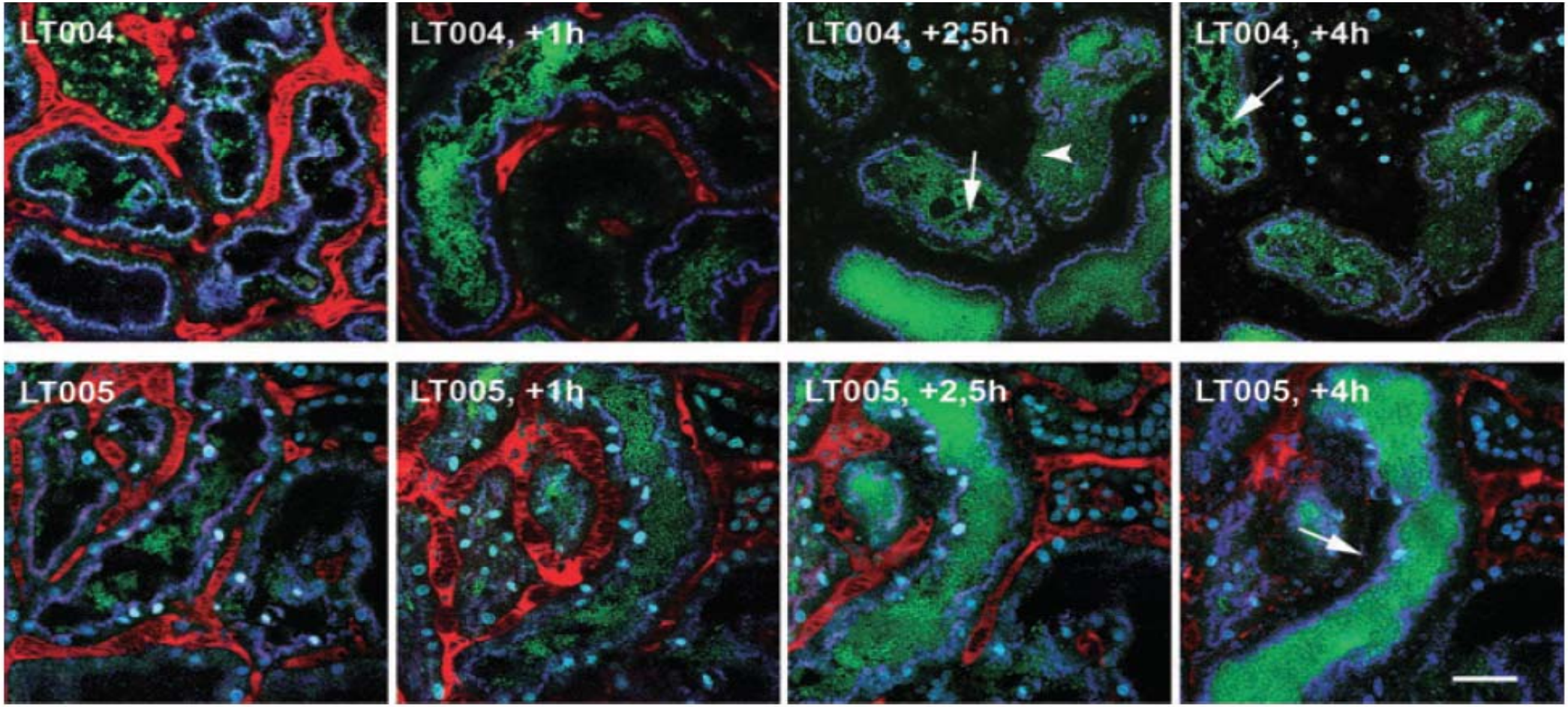
UPEC wt



PBS

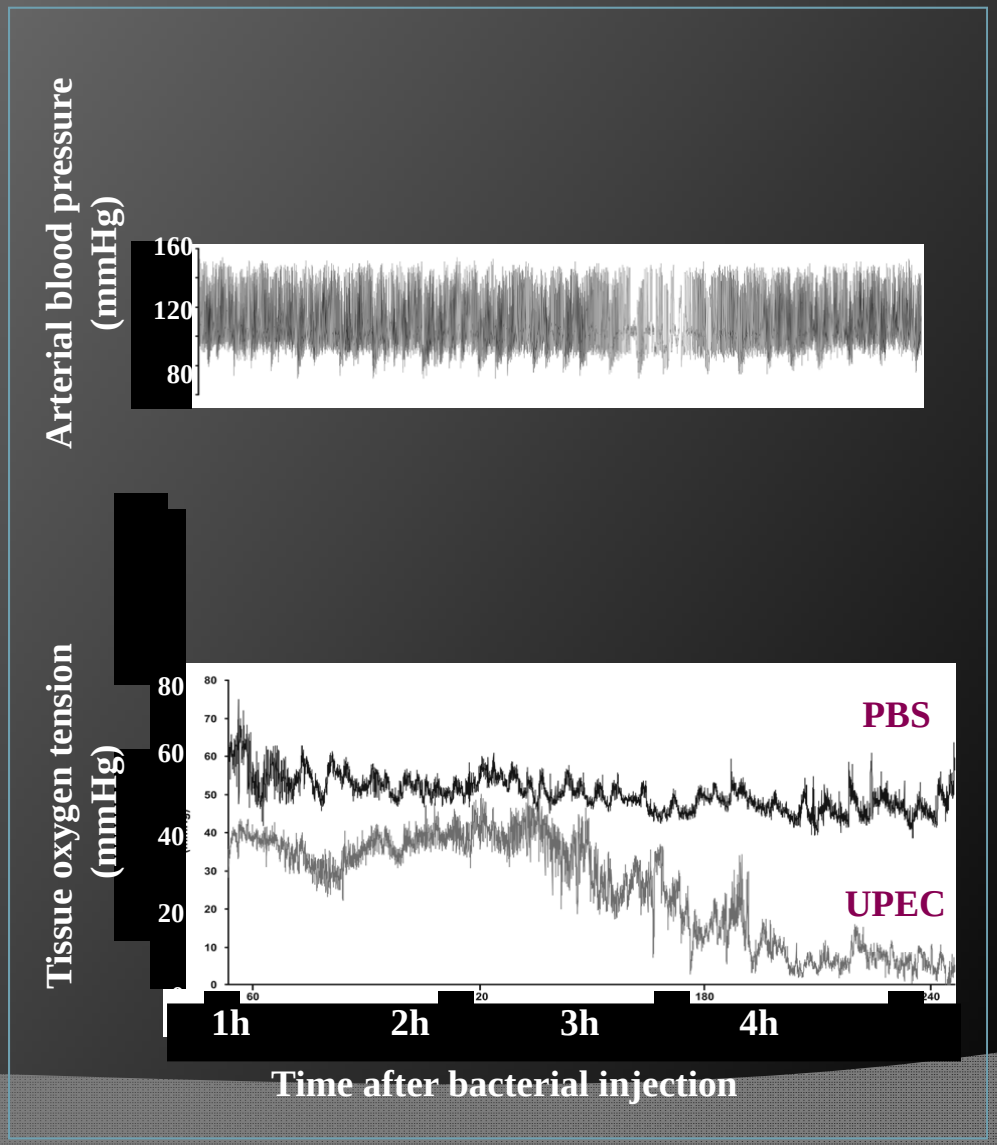


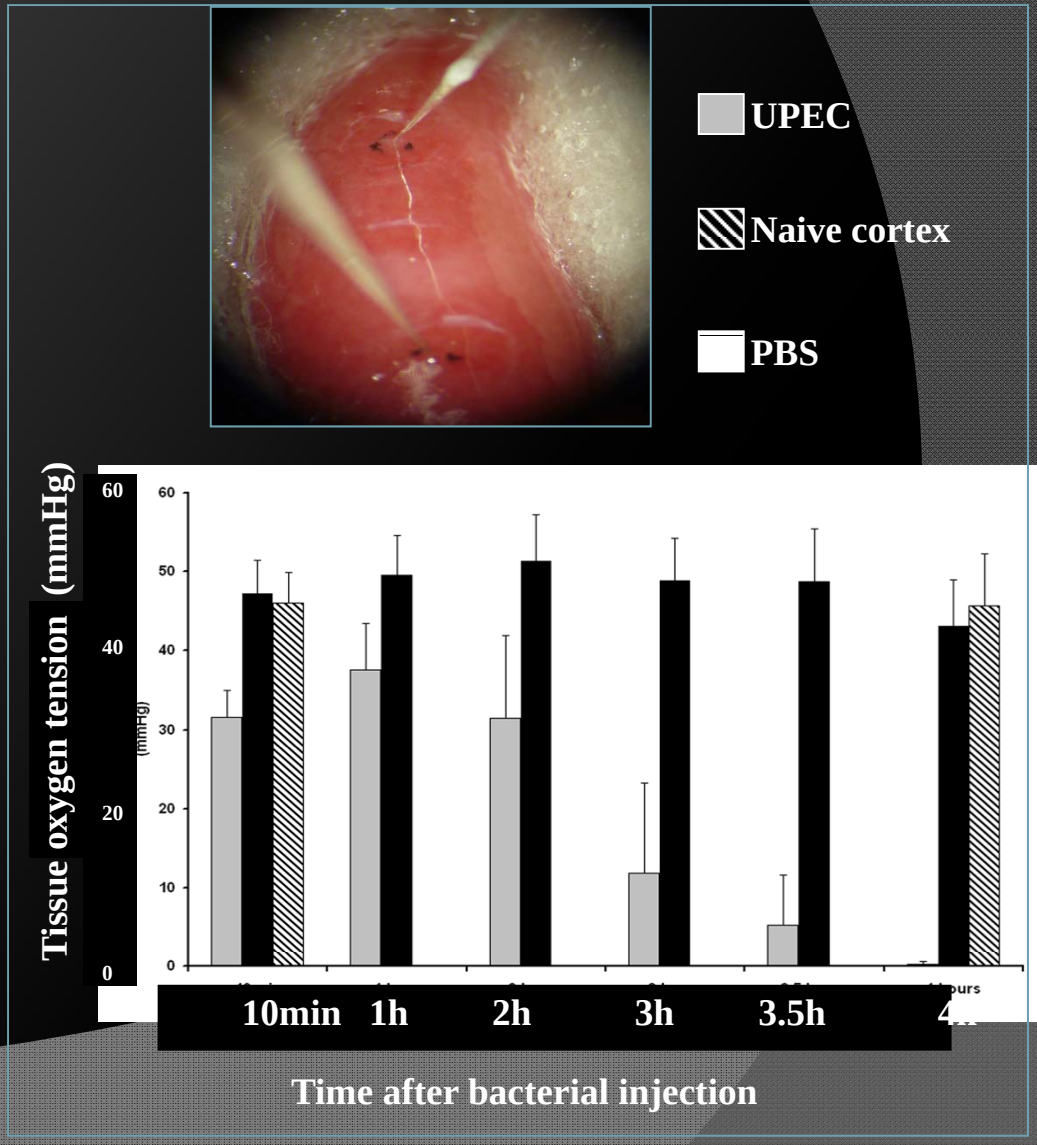
Proximal Tubule E. coli Infection: Effect of Virulence Factor



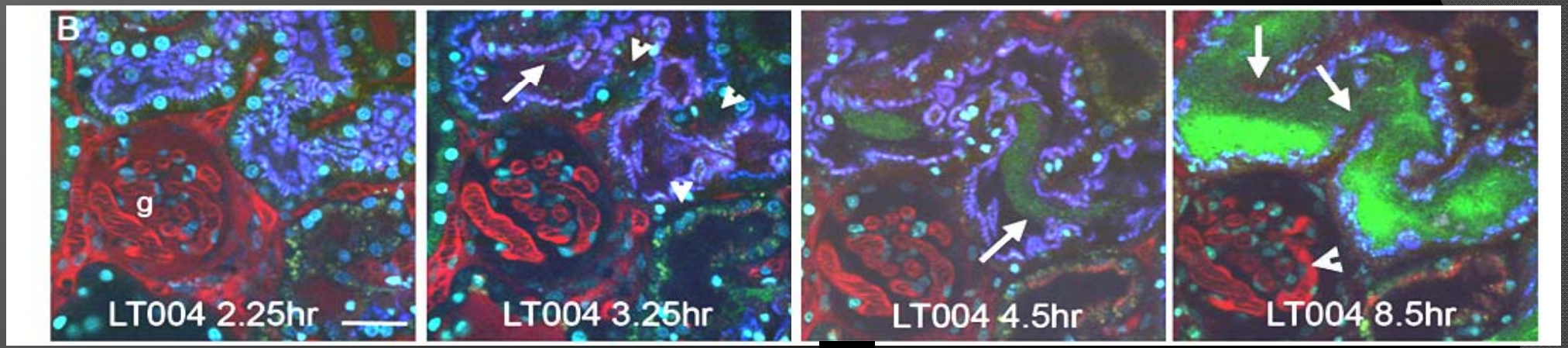
LE Månsson et al, Cell Microbiol 2007 Feb; 9(2) 413-24

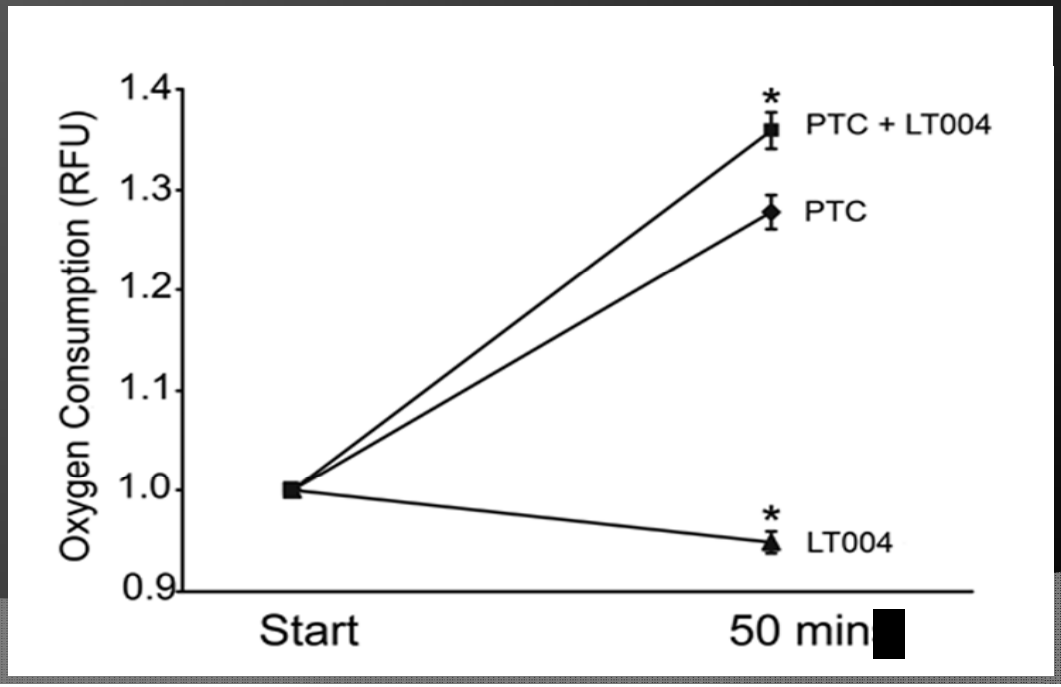
Bacterial Infection Causes Rapid Drop in Tissue Oxygen Tension (pO_2)

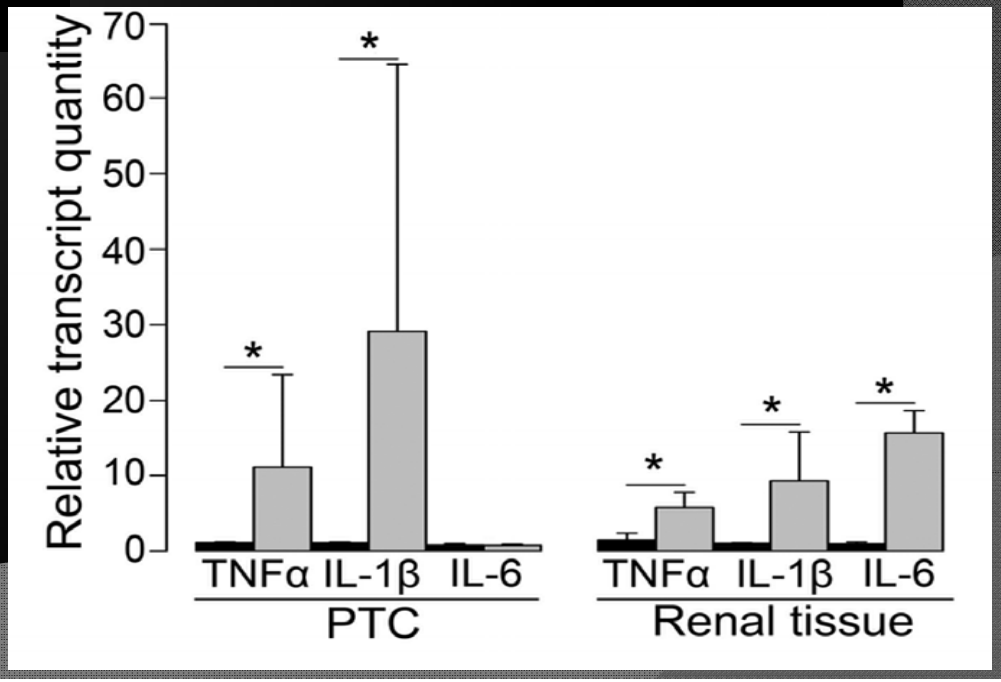




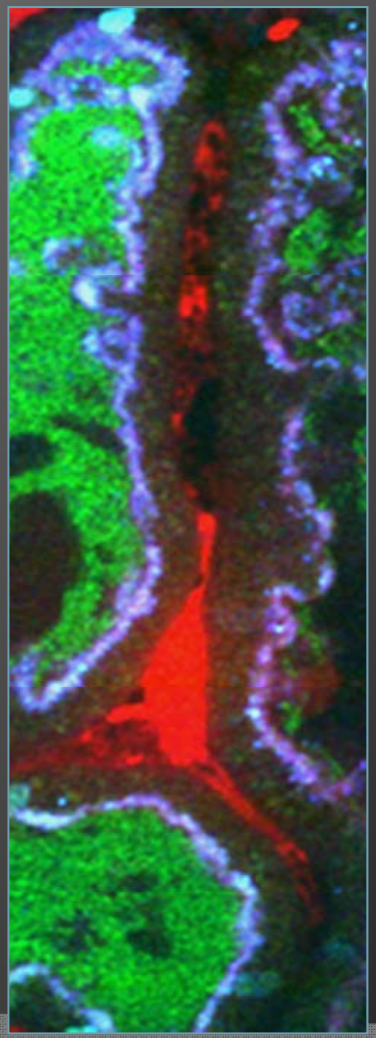
Infection Triggers Increased Oxygen Consumption in Renal Cells

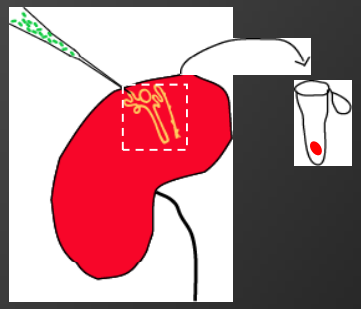




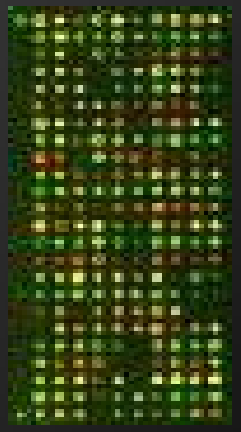


Clotting Cascade Genes are Up-Regulated in Infected Kidneys

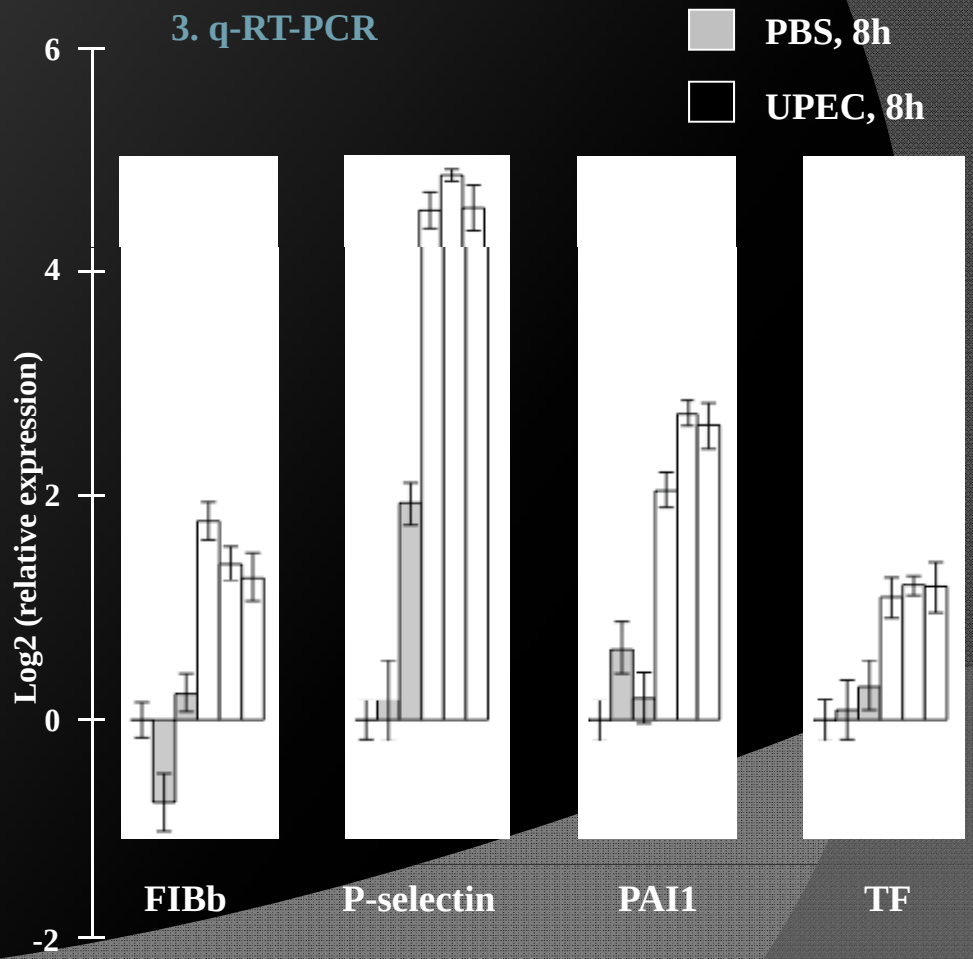




1. Precise dissection to enrich for local mRNA

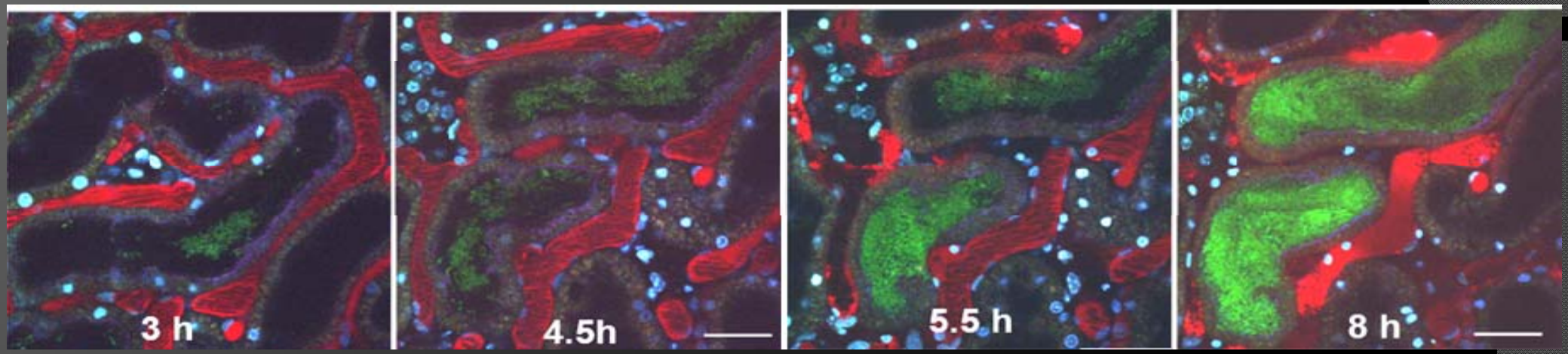


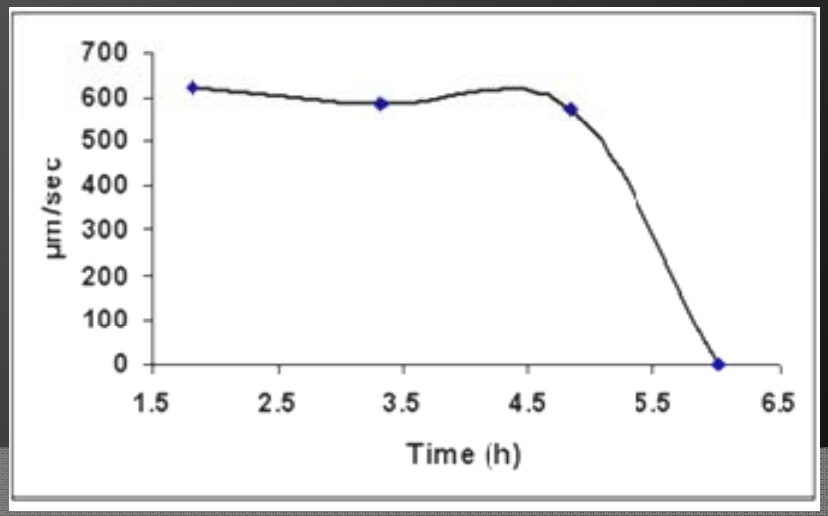
2. Gene expression array

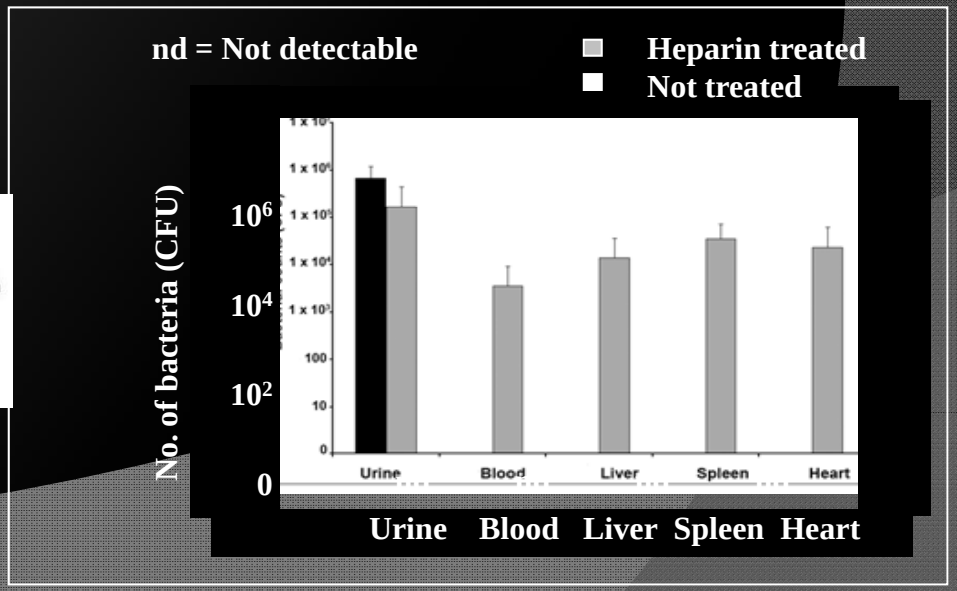


Heparin-Treatment Causes Systemic Bacterial Spread, Rats Die from Sepsis

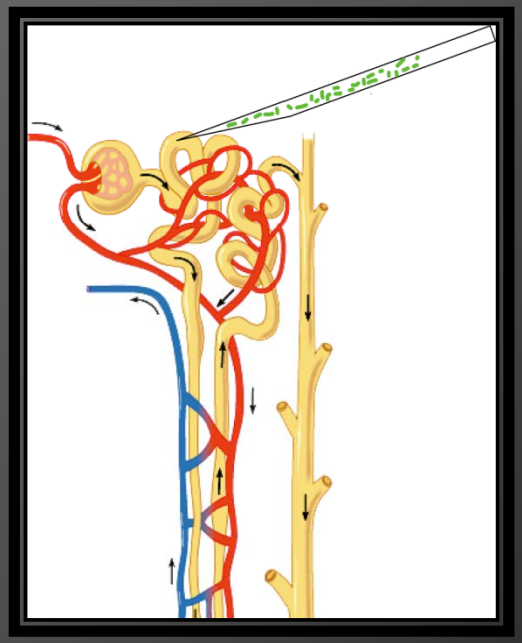
Animals treated with heparin (400 U/kg) to prevent clotting

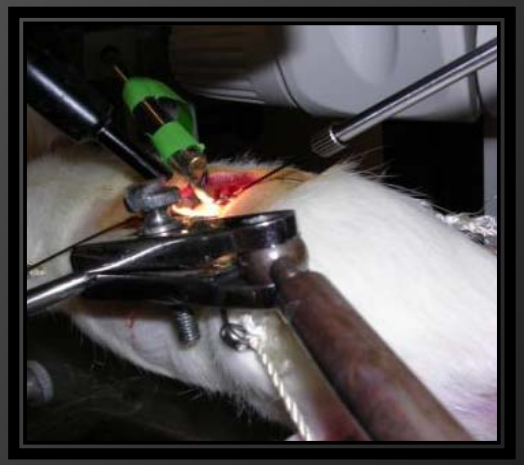


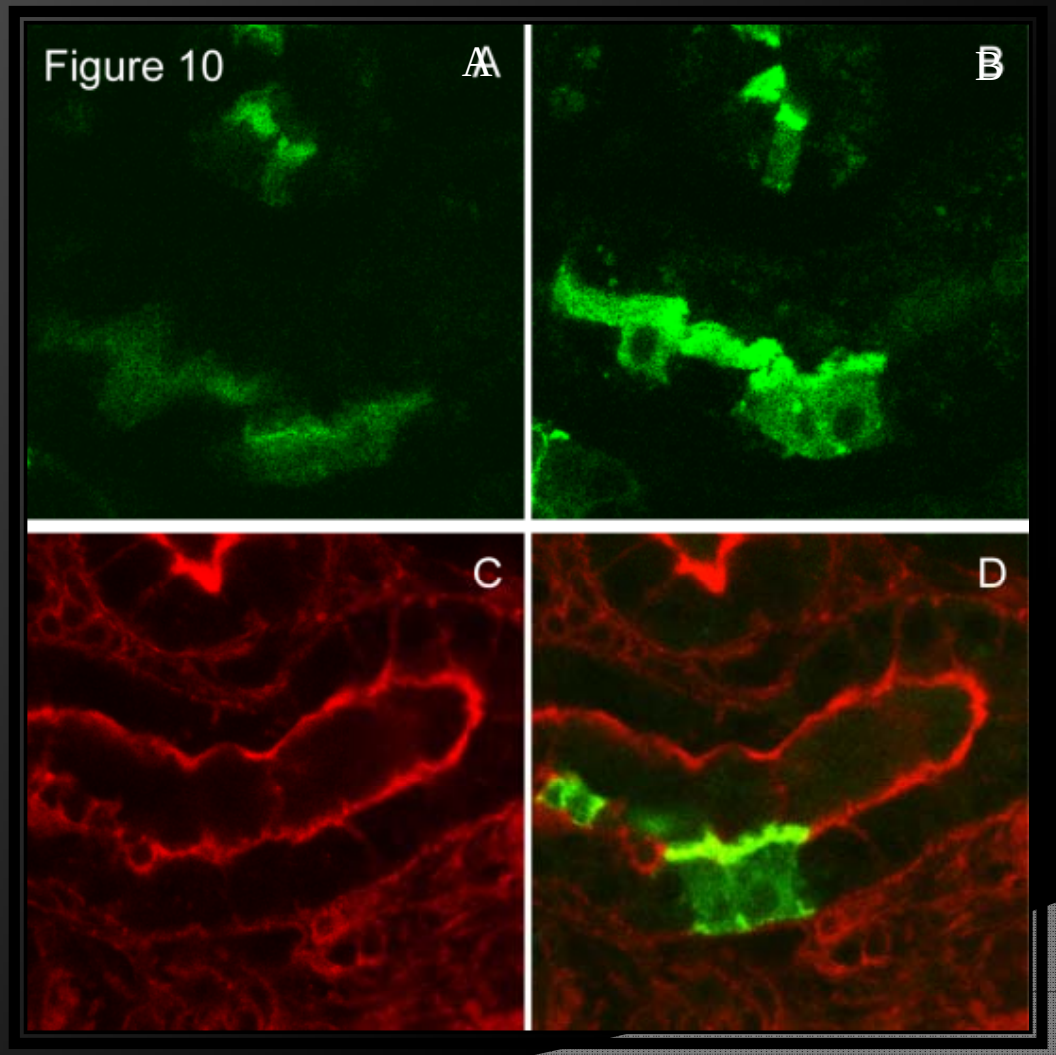




Micropuncture Delivery of Adeno-eGFP Actin





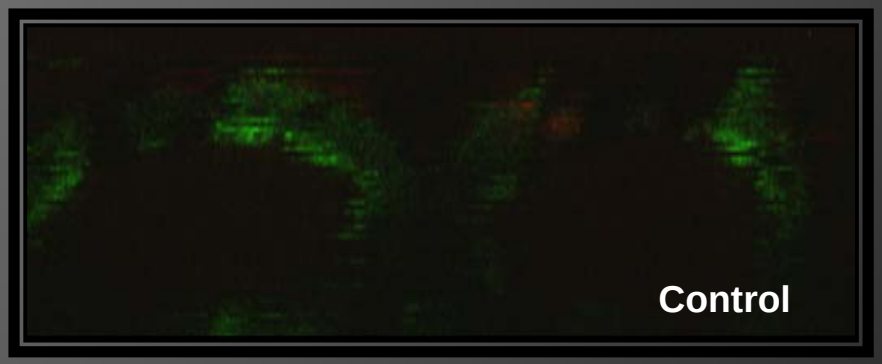


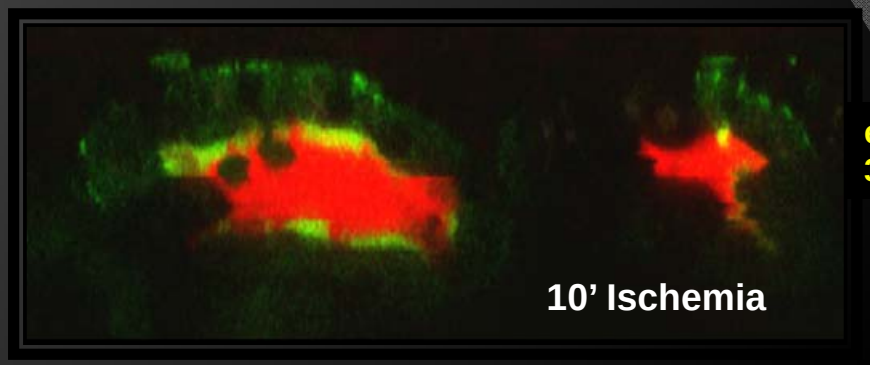
Proximal Tubules 48 hr post Viral Injection

Proximal Tubules Post Fixation and rhodamine Phalloidin Staining;.

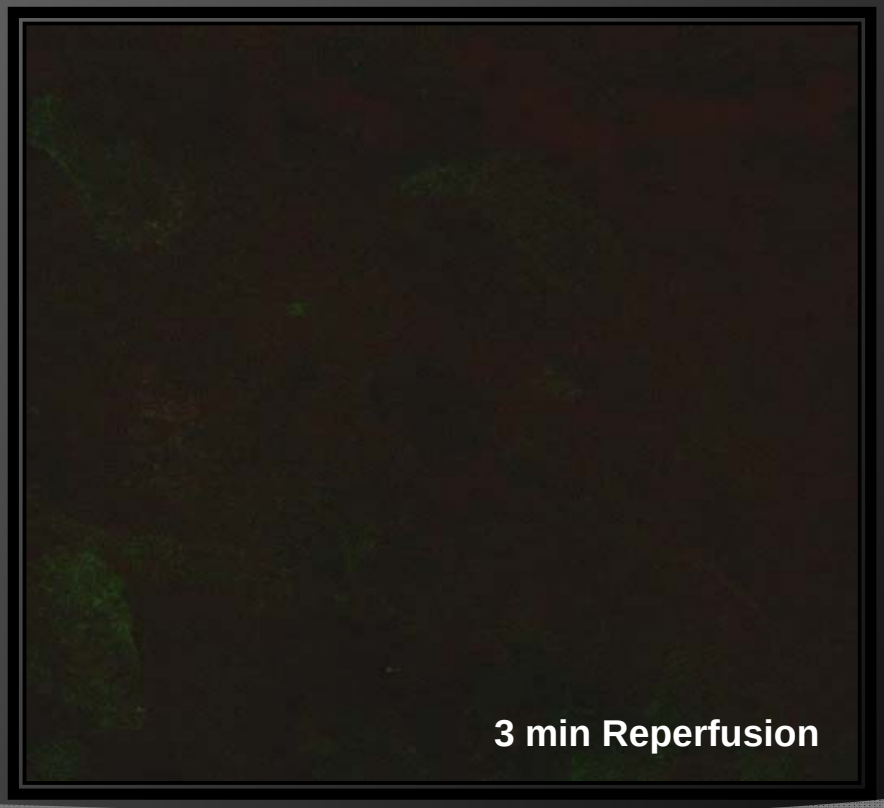
Tanner et.al. *AJP-Renal* 2005

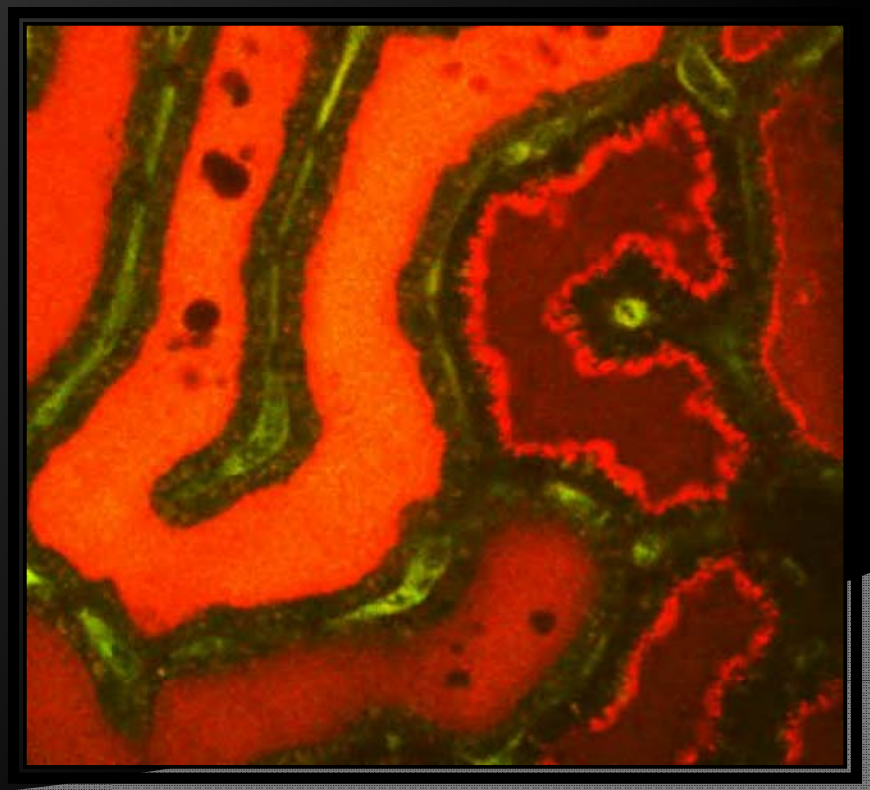
Apical Membrane Bleb and Tubular Cast Formation in Ischemia





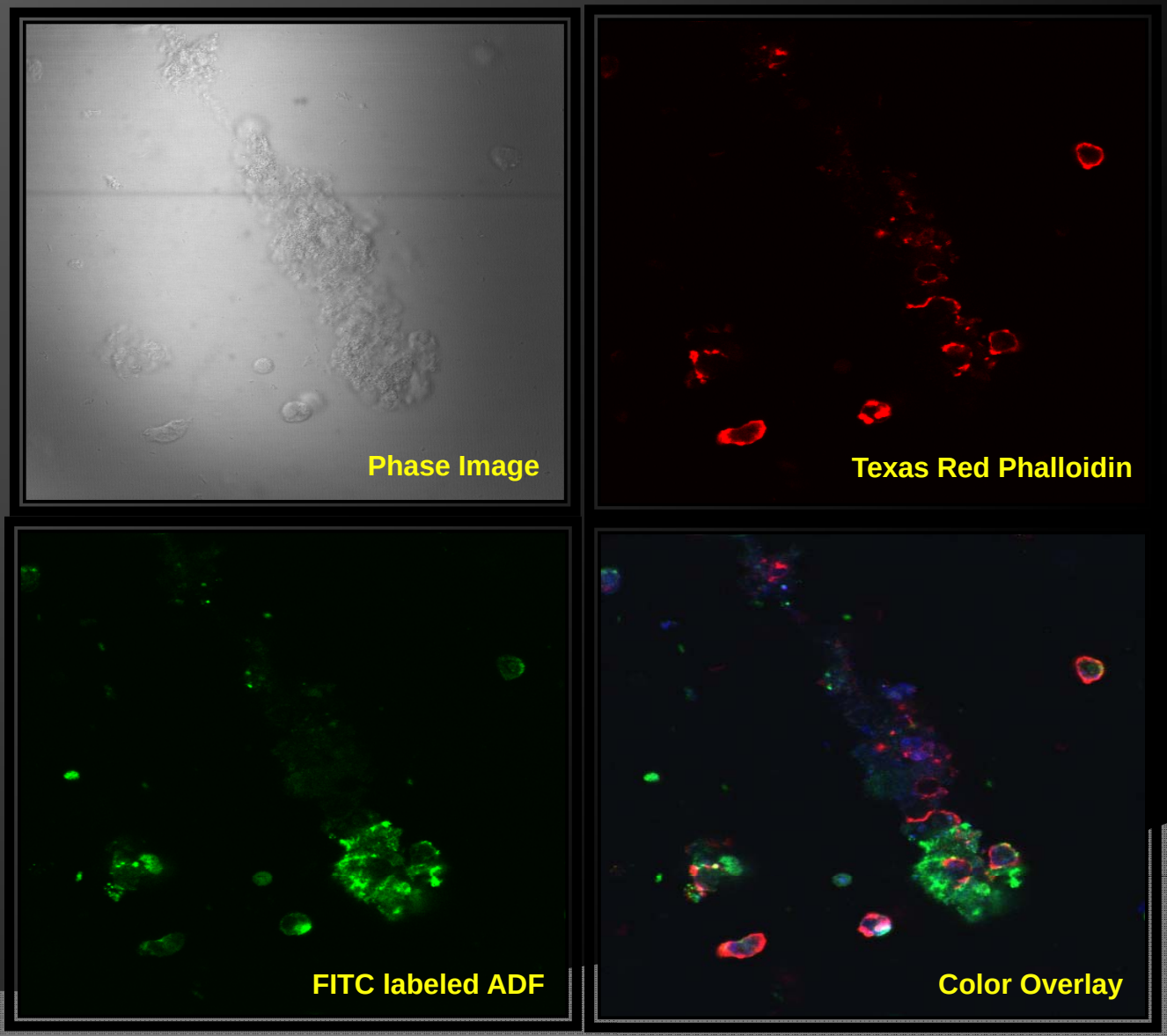
eGFP-Actin and
3kDa TR Dextran





Ashworth et.al. *Kidney Int*. 2007

Actin Components of a Urinary Cast in Acute Renal Failure



Molitoris , *Kidney Int*. 200

Table 1. Investigational uses for multi-photon microscopy

Glomerular
Size/volume
Permeability/filtration
Fibrosis/sclerosis
Microvasculature
RBC flow rate
Endothelial permeability
WBC adherence/rolling
Vascular diameter
Cellular uptake
Cell type-specific uptake
Site – apical vs. basolateral membrane
Mechanism – endocytosis vs. carrier/transporter mediated
Cellular trafficking
Intracellular organelle distribution
Cytosol localization
Cellular metabolism
Fluorescence decay over time
Cell toxicity
Cell injury in necrosis, apoptosis
Surface membrane/blebbing
Mitochondrial function
Glomerular filtration rate determination