



Advancing Nephrology Through 2-Photon Microscopy

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Goals

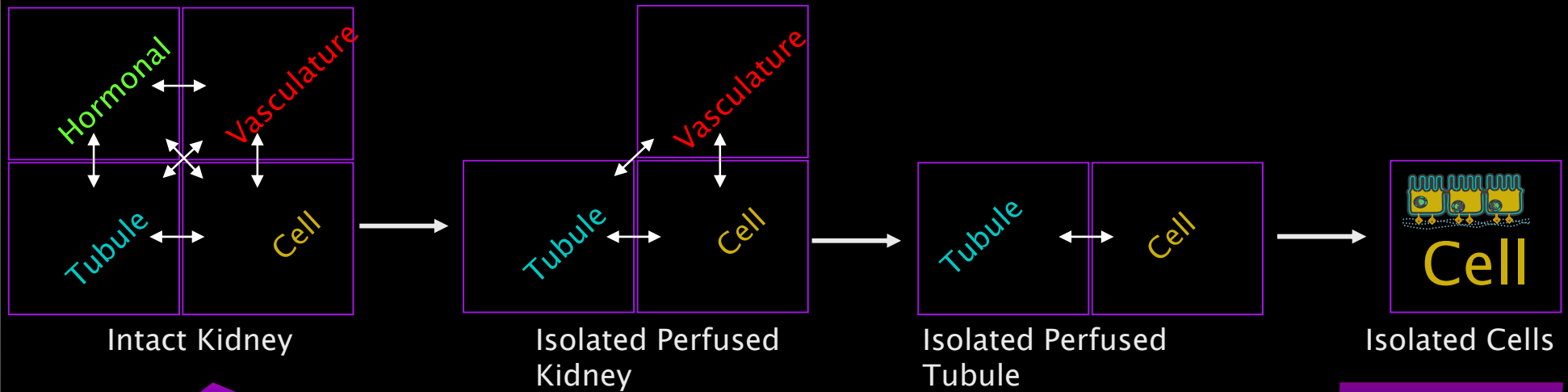
Introduce the power of 2-photon microscopy

Demonstrate some of what we learned along the way

Discuss challenges and traps that face you

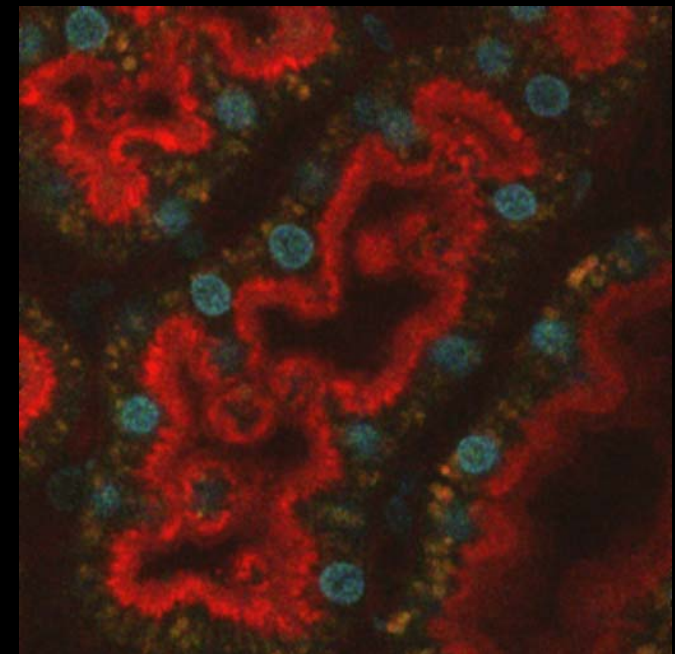
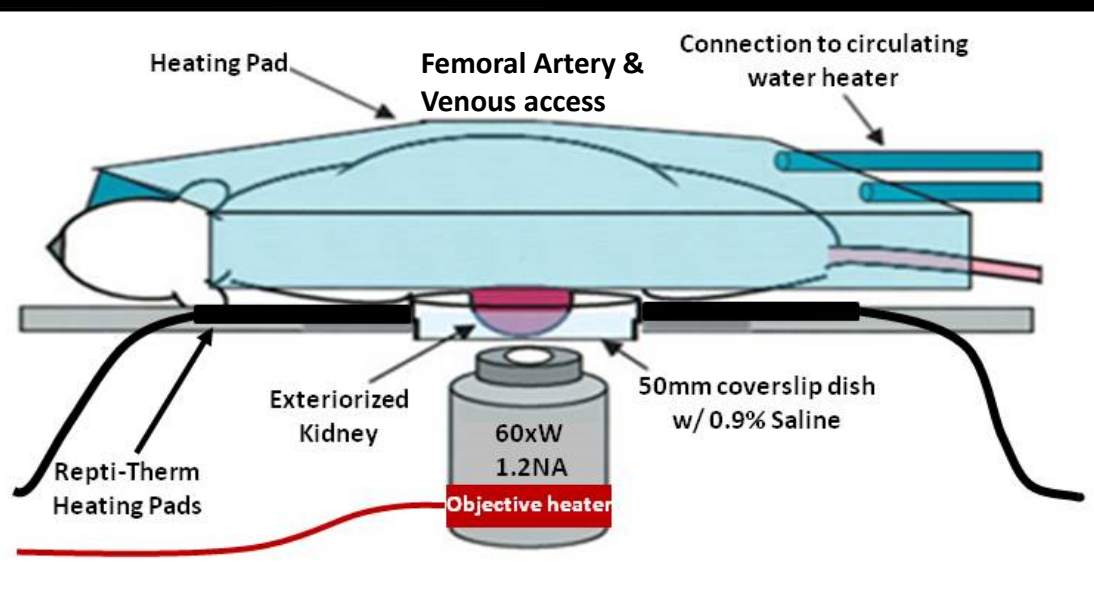
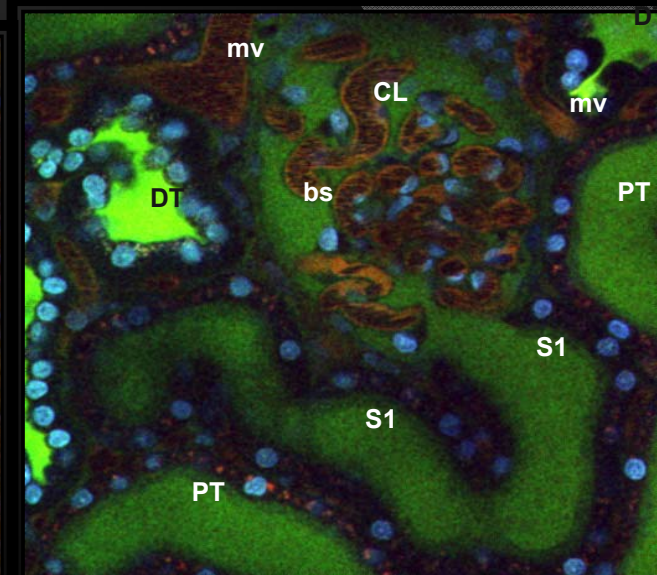
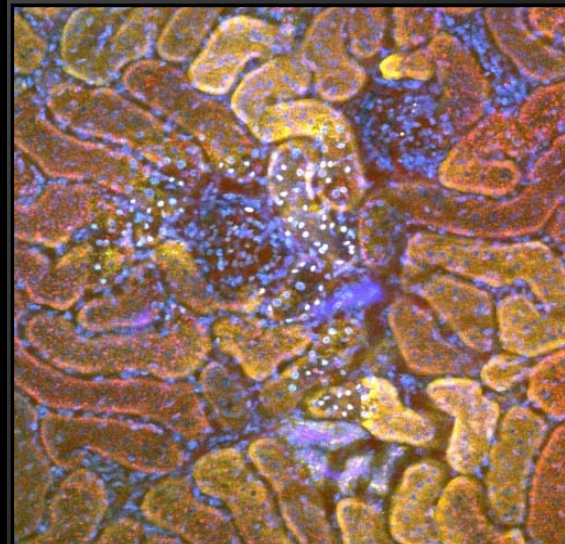
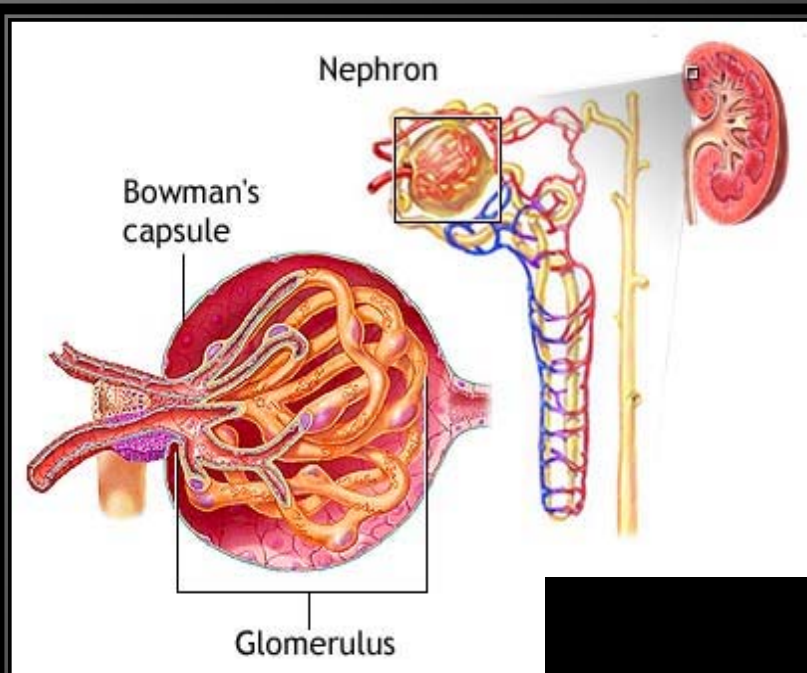
Discuss challenges facing the field

Visualizing Subcellular Organ Biology

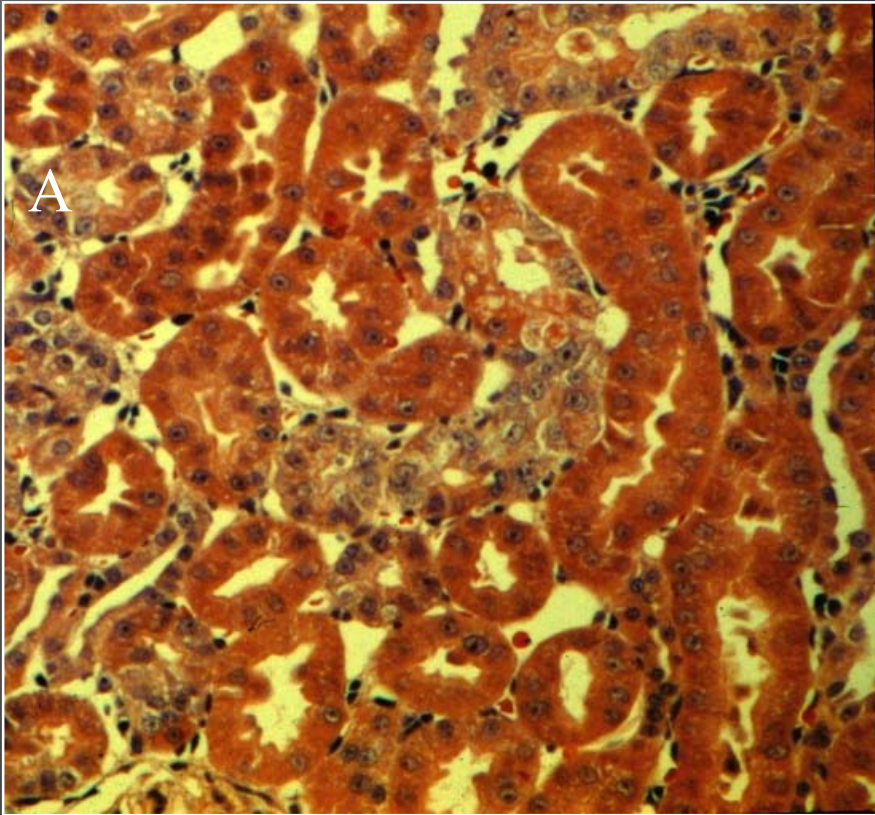


Multi-photon microscopy

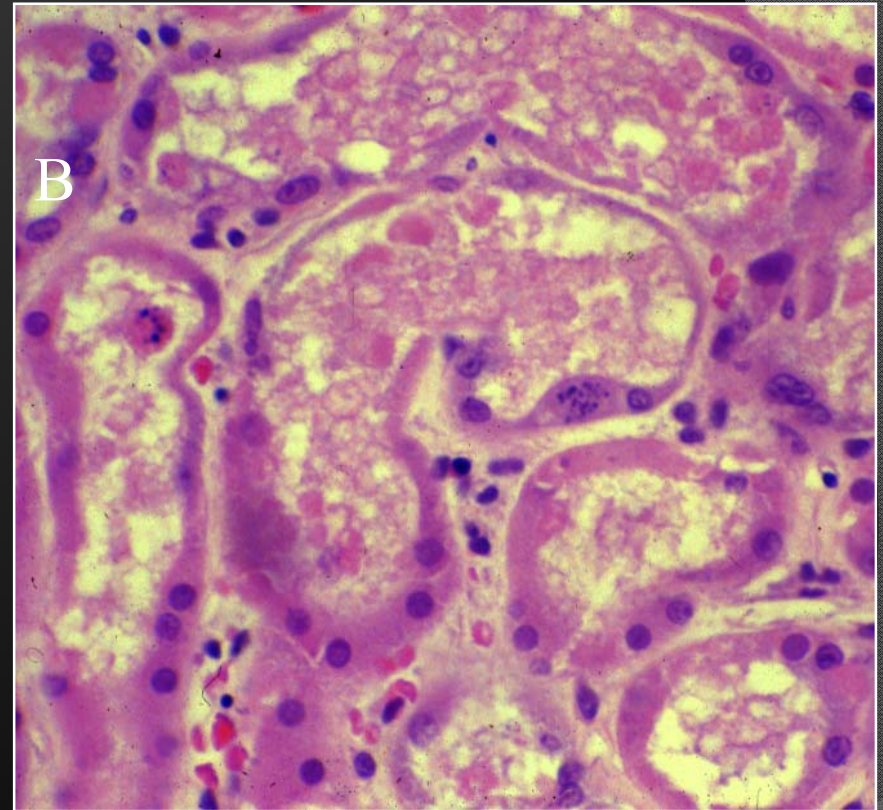
Visualizing Glomerular & Nephron Function



Human Renal Ischemia



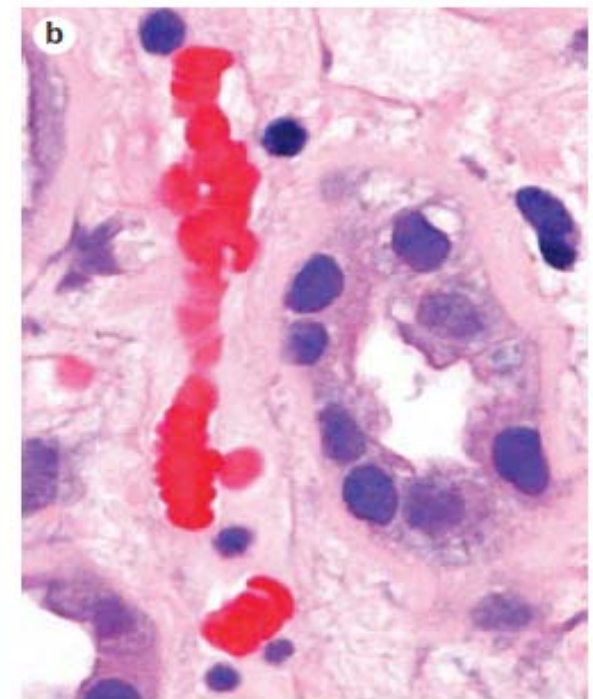
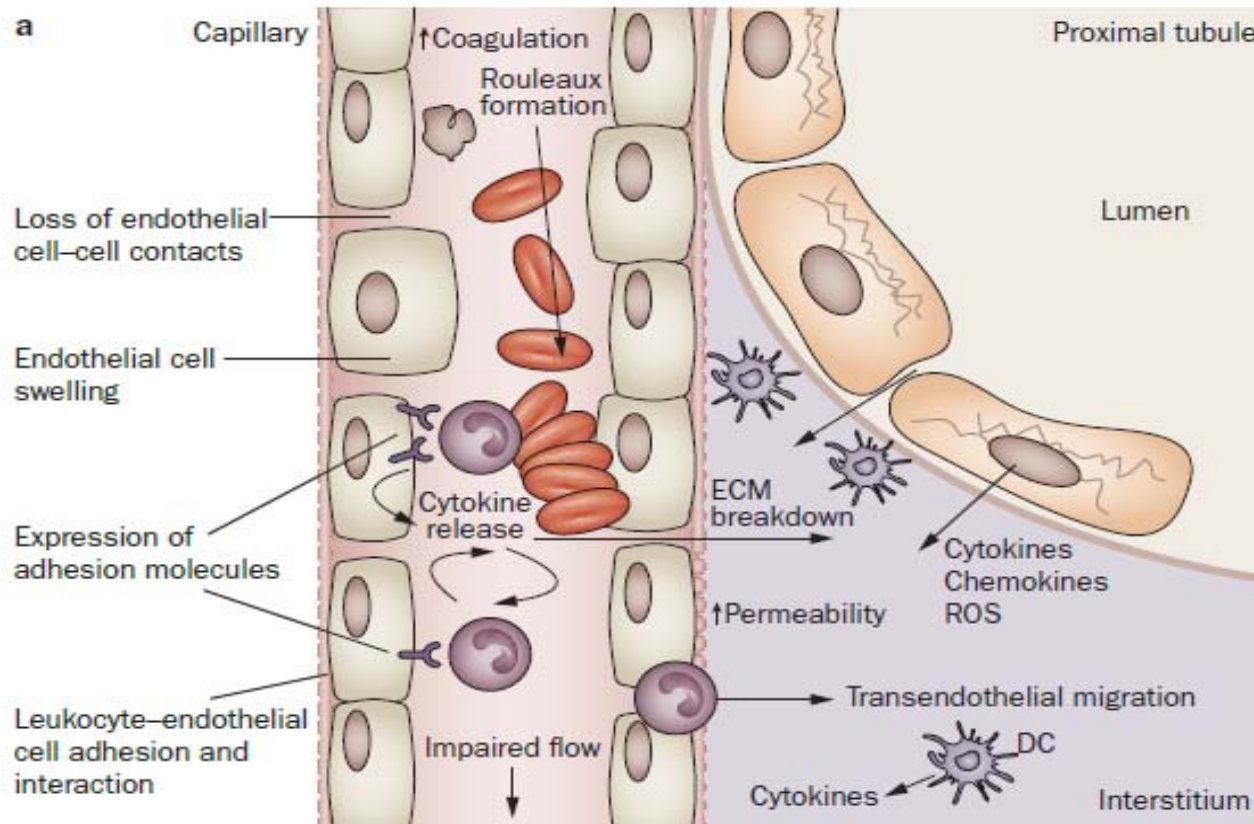
Control



Ischemic

**Limited information regarding mechanisms,
structure functions relationships, chronology
No information of therapeutic mechanisms**

Endothelial Pathophysiologic Events in AKI



Insights Gained Along the Way

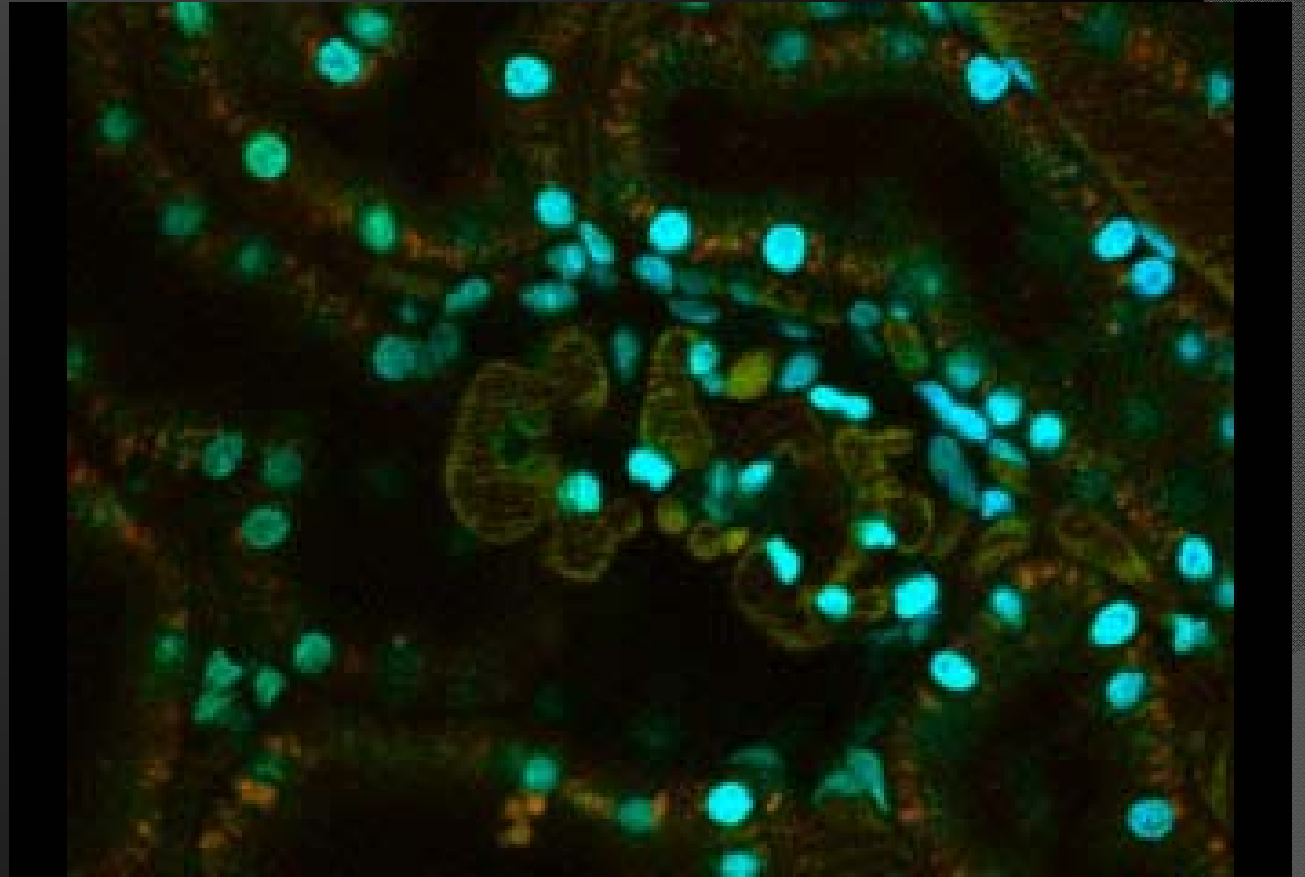
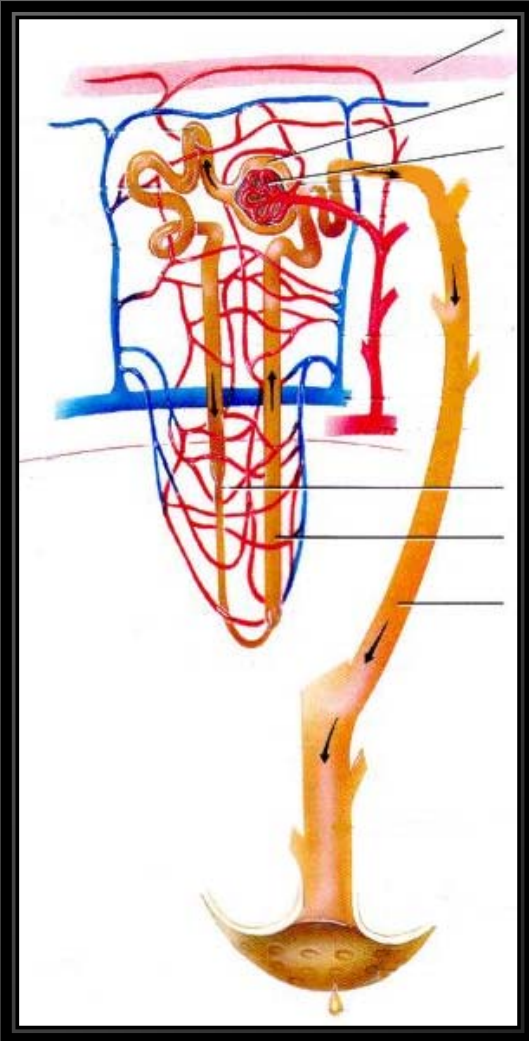
Developing techniques is required, but not an end in themselves.

You must have a biologic question that microscopy gives a unique perspective too

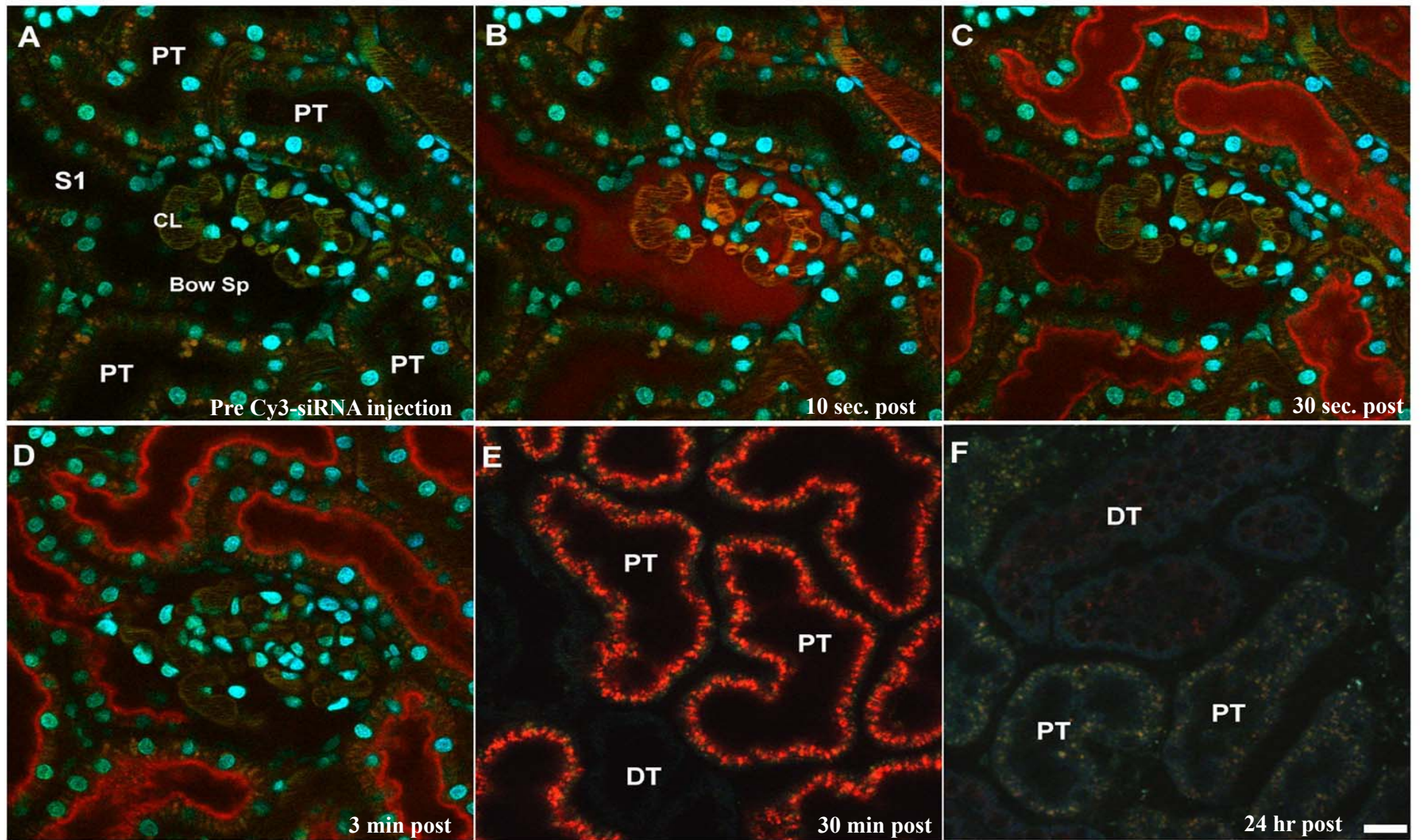
Quantitative analysis is required and the most difficult aspect

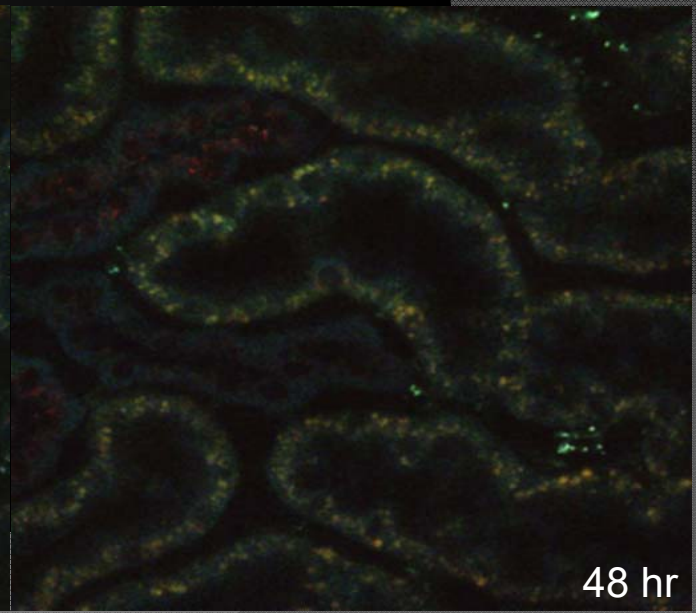
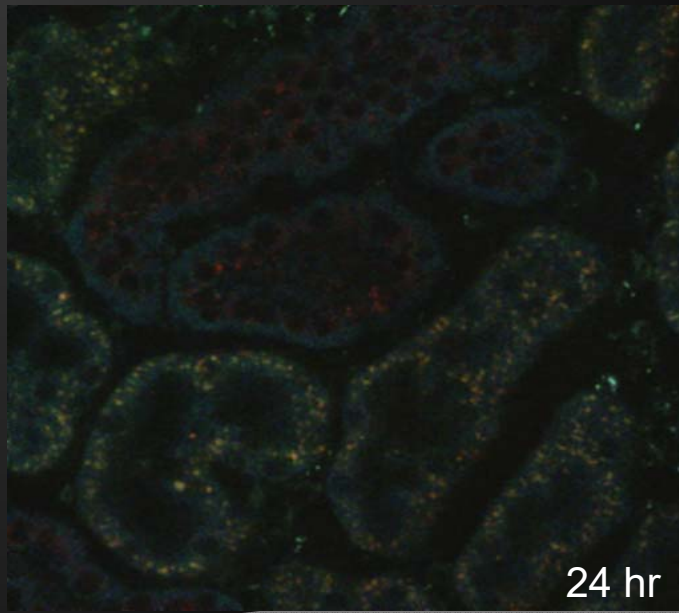
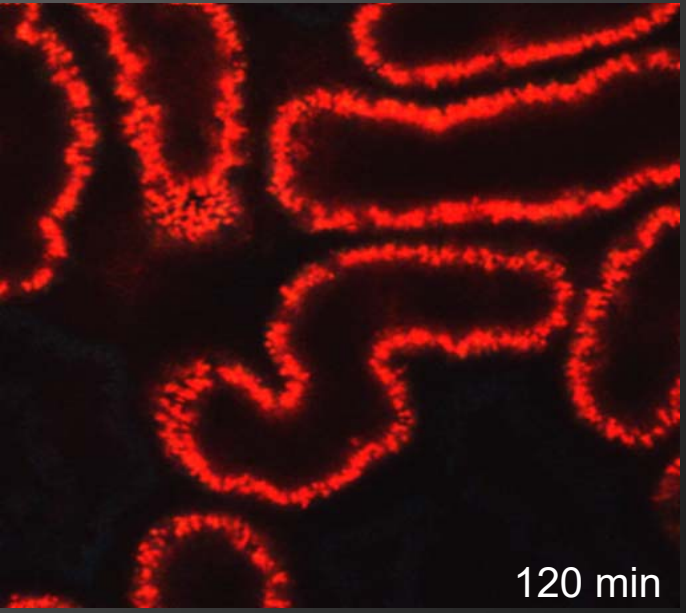
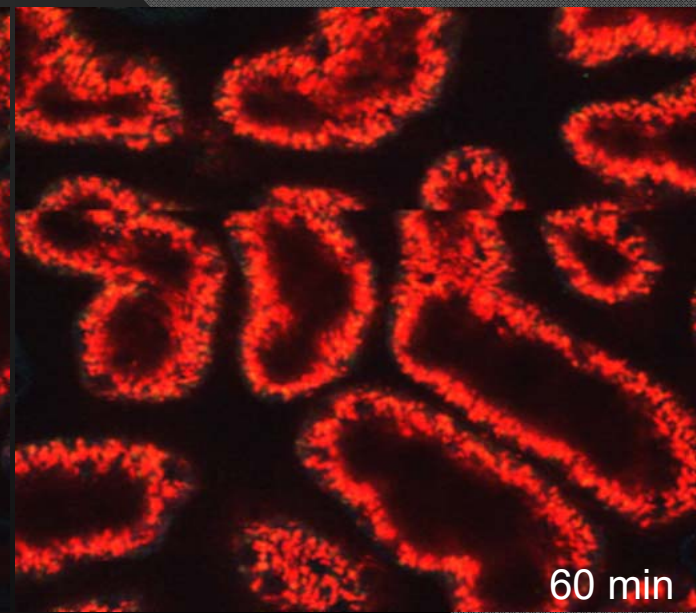
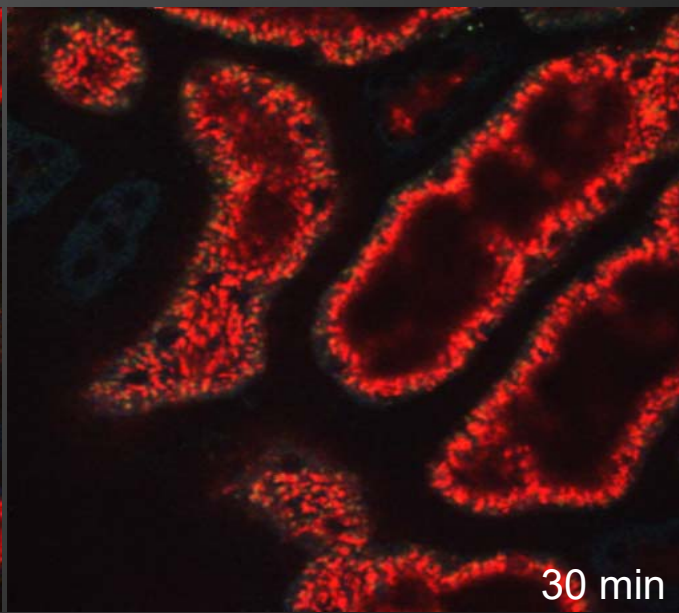
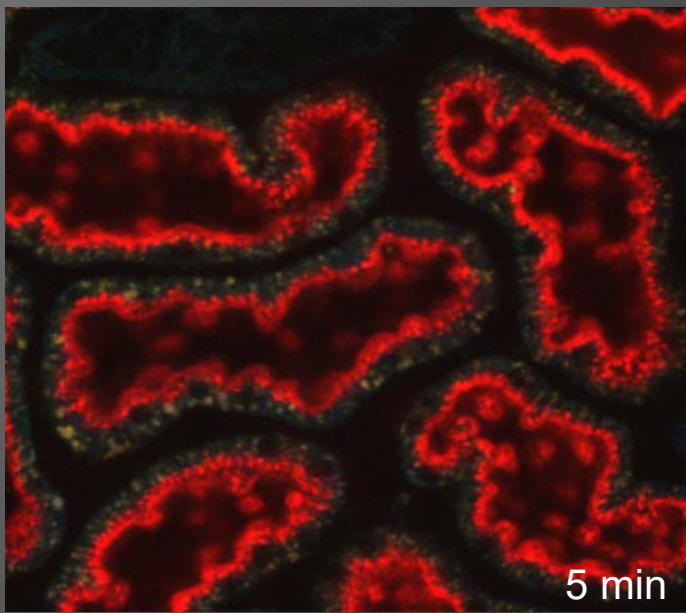
Given this is a Disruptive Technology you must validate the data

Cy3-siRNA Filtration and Reabsorption by PTCs

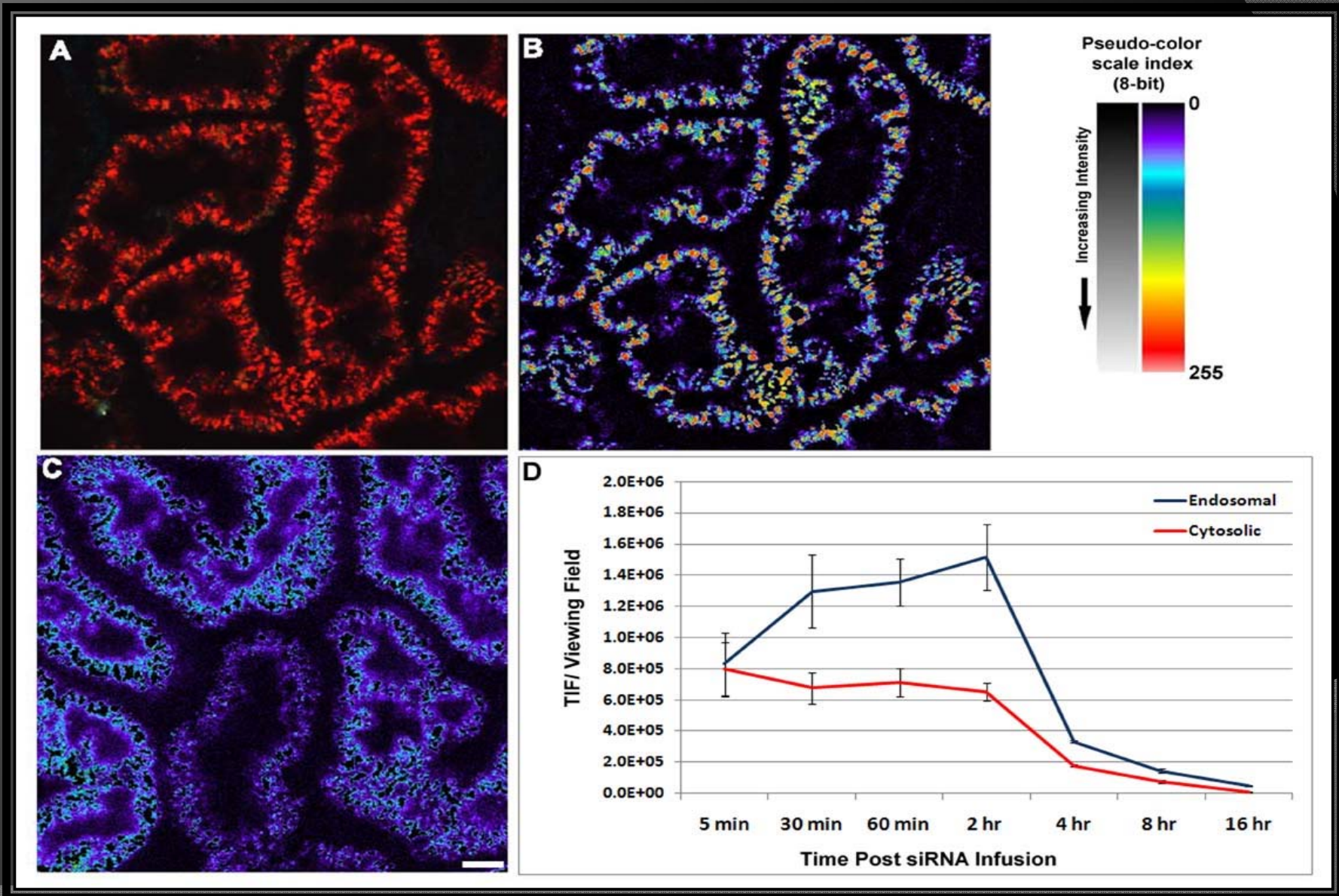


PTC Uptake and Metabolism of Cy3-siRNA

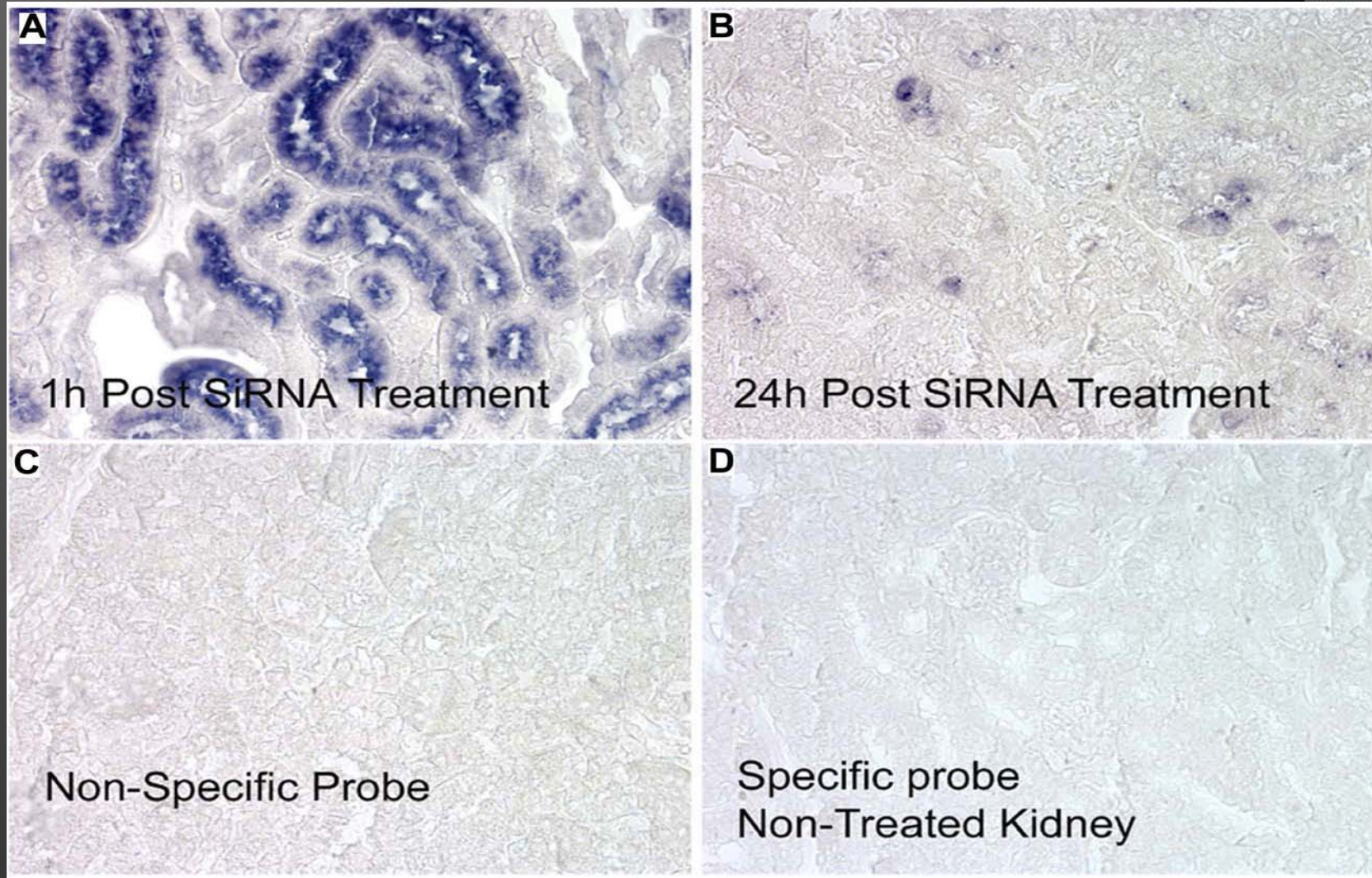




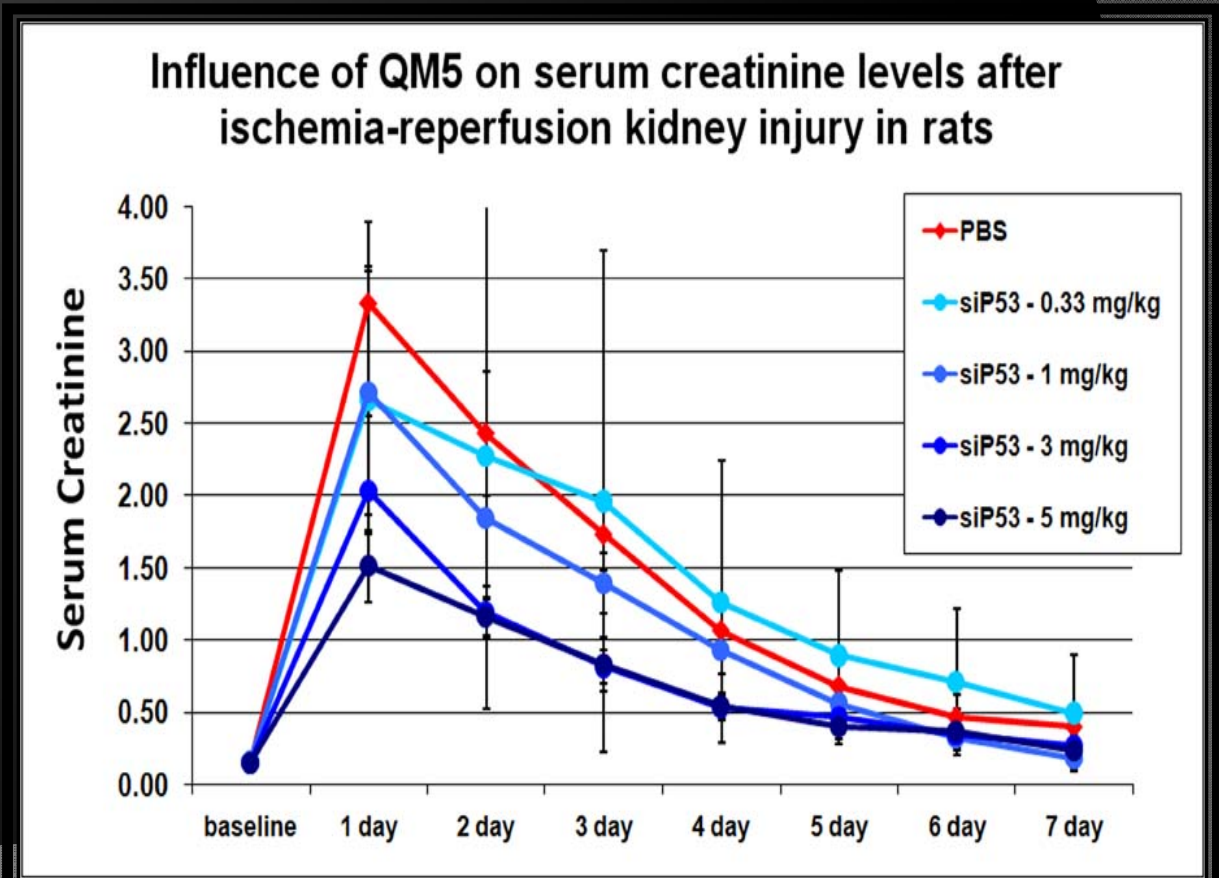
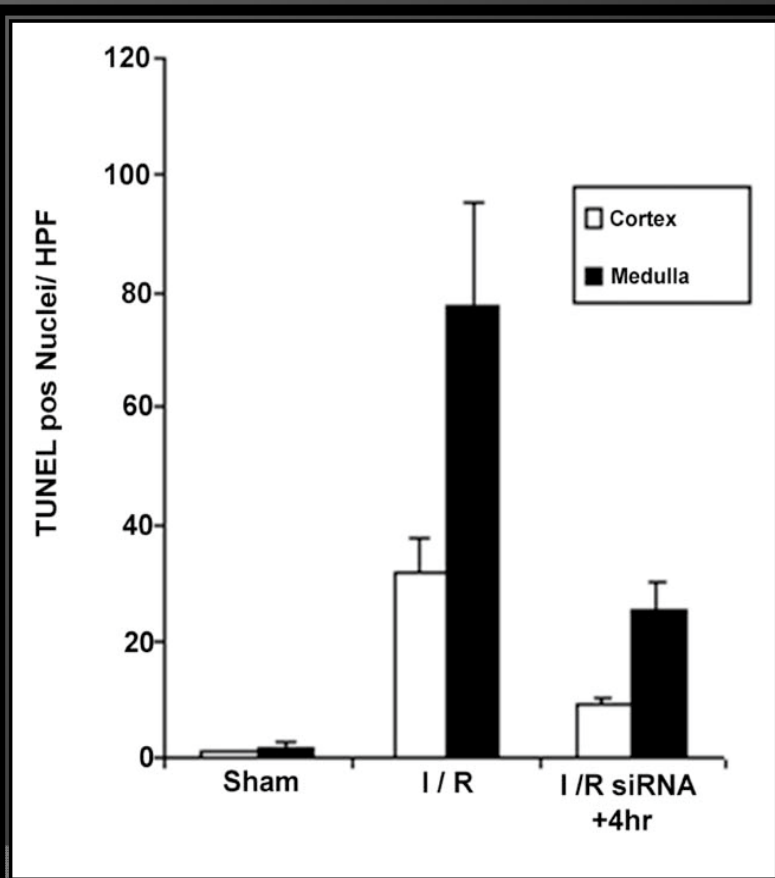
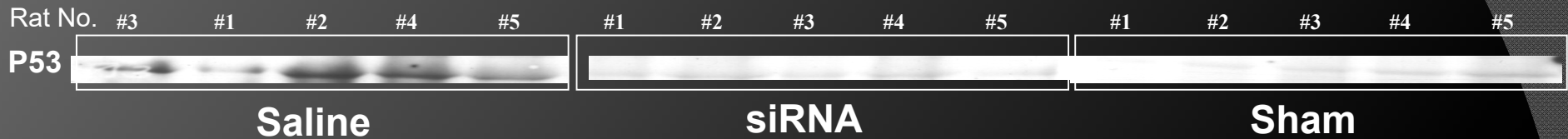
Quantifying Vesicular vs Cytosolic Cy3-siRNA in PTCs



siRNA in PTC by In situ Hybridization: Verifying 2-photon Data



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



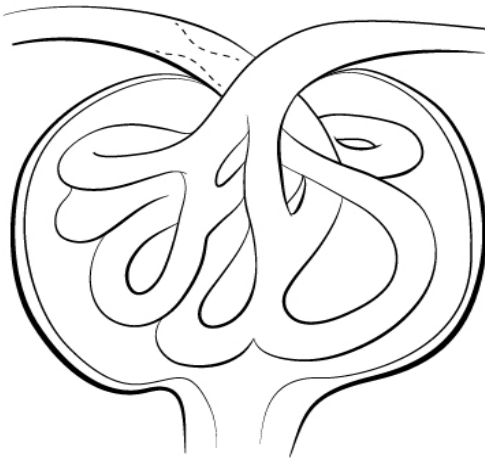
Series and Parallel Resistors to Vascular Blood in the Kidney

A. Macro-vasculature



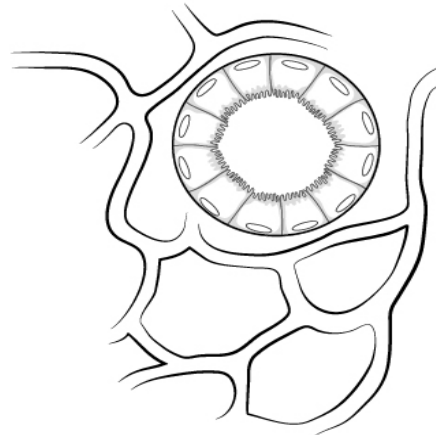
**Volume Depletion, CHF
Liver Disease, NSAIA
Renal Artery Stenosis
Thrombosis, Sepsis with
Reduced PVR**

B. Glomerular



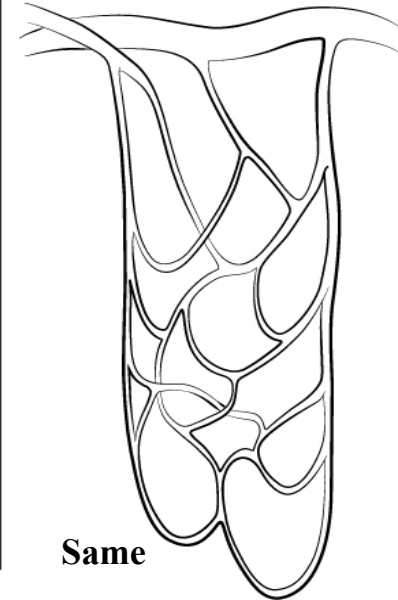
**Vasculitis, Inflammation
Sclerosis, Fibrosis**

C. Peritubular



**Endothelial Dysfunction,
Coagulopathy, Sepsis,
Ischemia, Hyperviscosity**

D. Vaso Recti

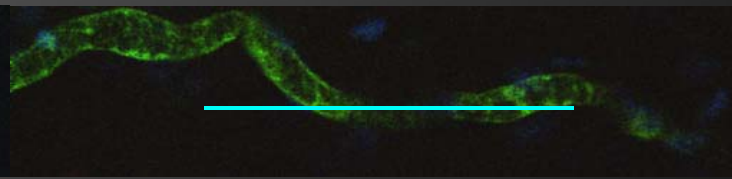
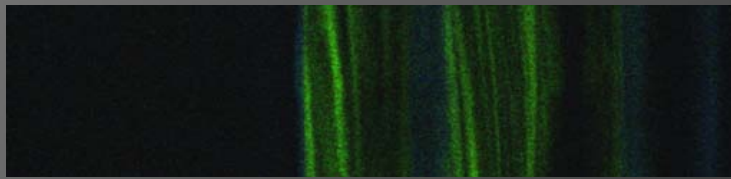


Same

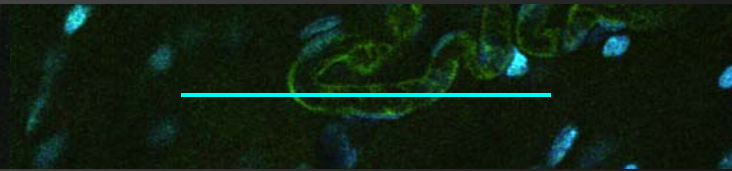
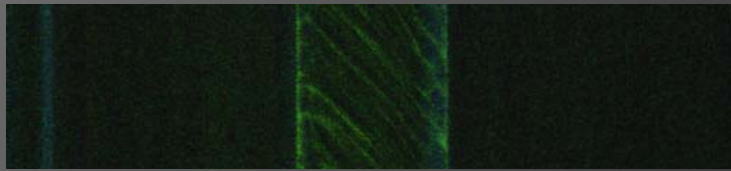
D. Venous



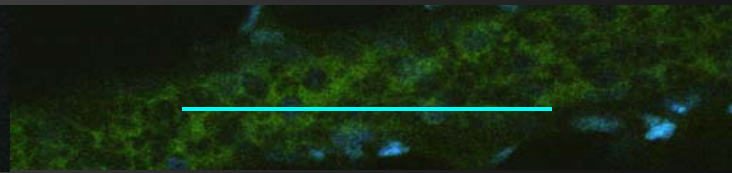
**High Venous
Pressure
CHF, ACS
Thrombosis**



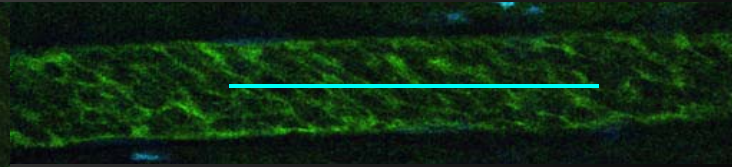
Vessel Diam.=7.5 μm
Ave.Speed=14 $\mu\text{m}/\text{sec}$



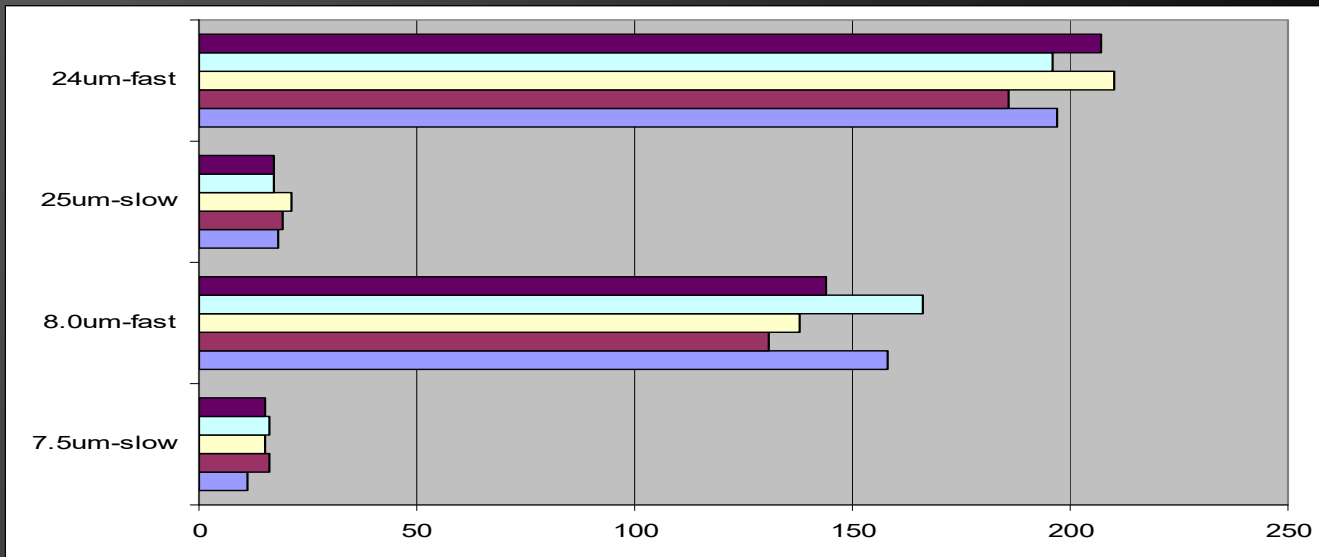
Vessel Diam.=8 μm
Ave Speed=147 $\mu\text{m}/\text{sec}$



Vessel Diam.=23 μm
Ave Speed=18 $\mu\text{m}/\text{sec}$

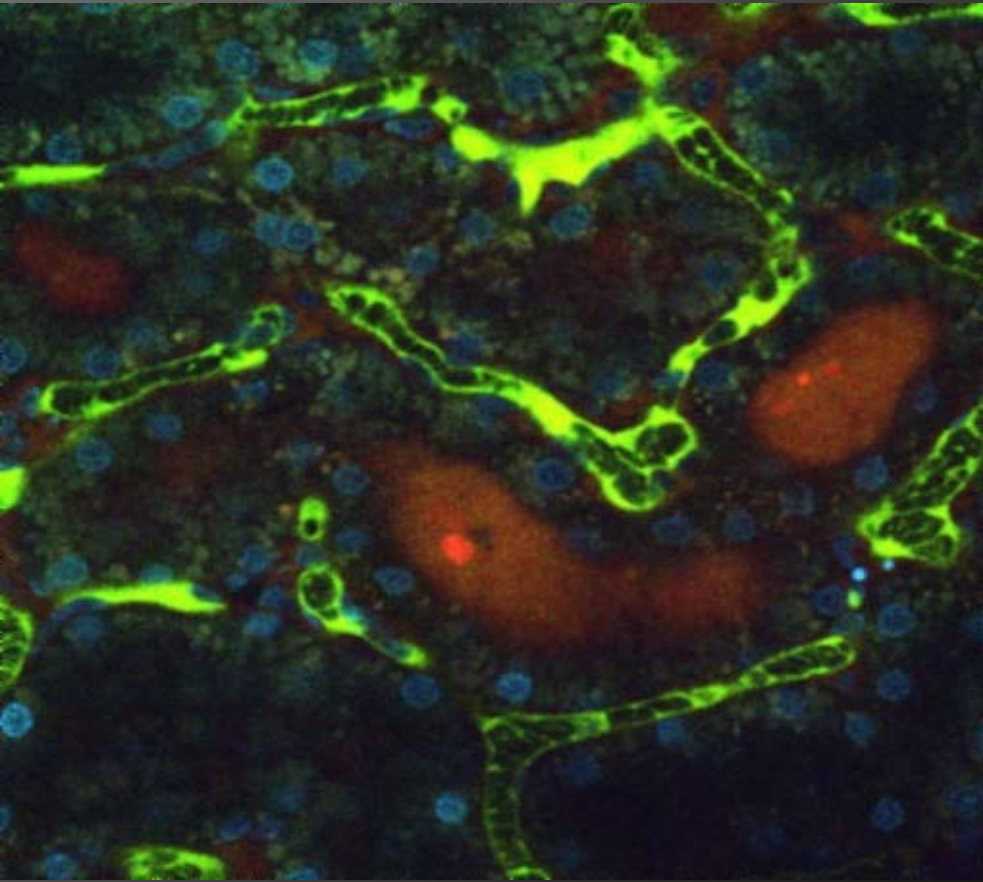


Vessel Diam.=24 μm
Ave Speed=199 $\mu\text{m}/\text{sec}$

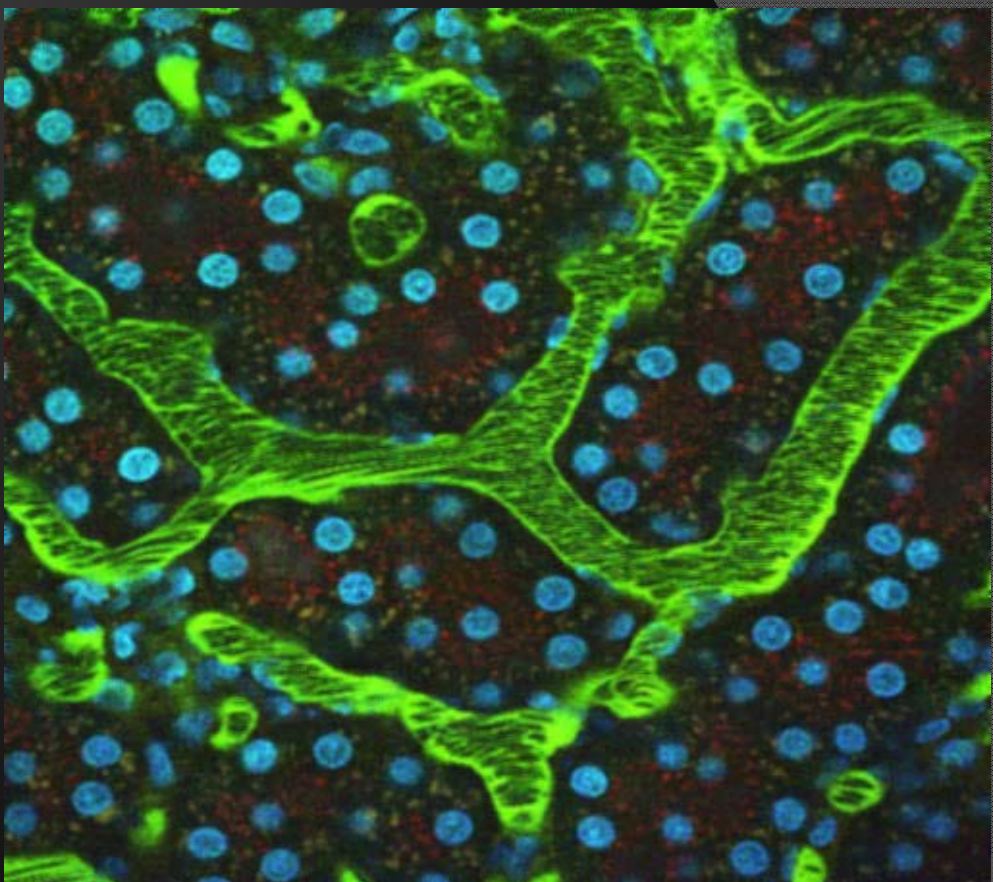


Vessel Diam.	Ave Speed	St. Dev
7.5um-slow	14.6	2.07364414
8.0um-fast	147.4	14.3805424
25um-slow	18.4	1.67332005
24um-fast	199.2	9.5760117

Microvascular Blood Flow at 24h Post Ischemia Effect of sTM



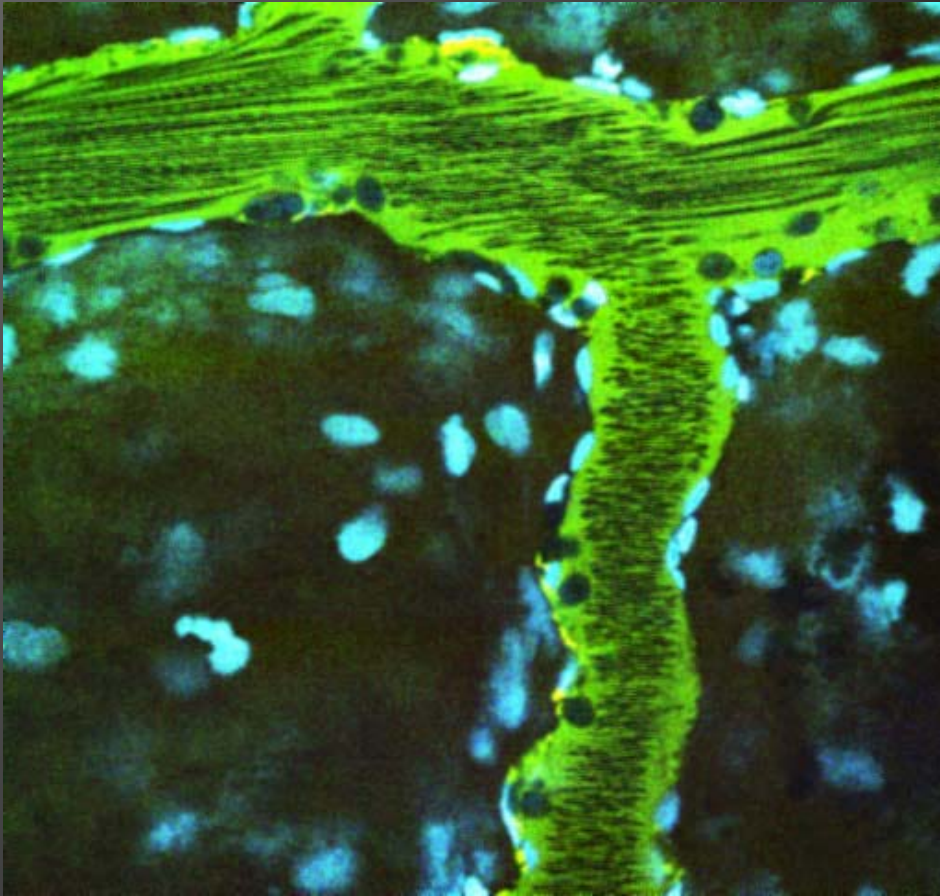
Saline treated



sTM treated

Blood Flow velocity ($\mu\text{m}/\text{sec}$)	253.36 \pm 95.01	786.75 \pm 280.75 *
		*P < 0.05

Leukocyte-Endothelial Interactions – Intra-Vital 2-Photon



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

* $p < 0.05$

Ischemic – Saline treated rat at 24h

Insights Gained Along the Way

Often techniques must be combined to yield the necessary data

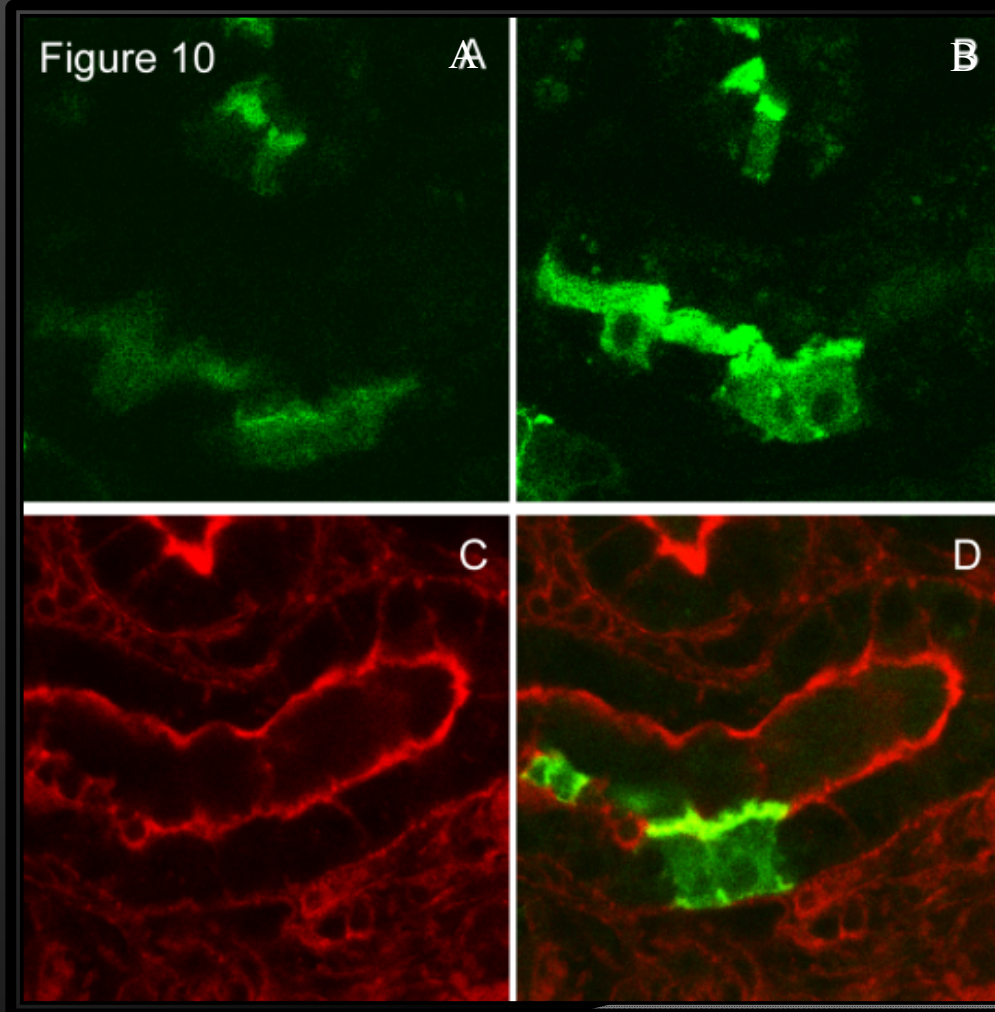
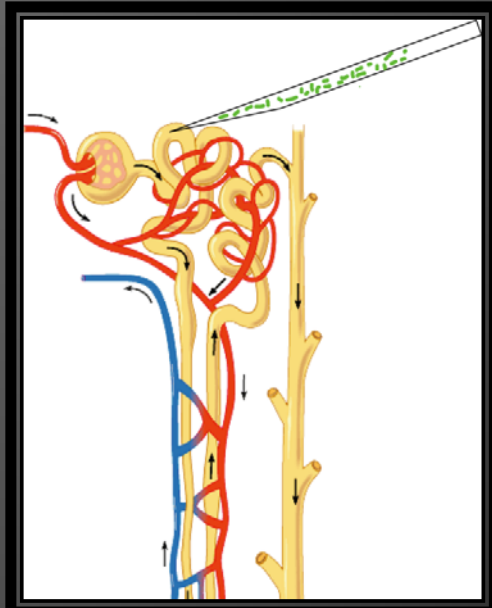
Maintaining normal physiology is essential for data interpretation

**Quantifying glomerular-tubular, tubular-vascular,
vascular-interstitial.... is possible and can only be accomplished
this way**

Resolution, Resolution, Resolution

Sometime you get lucky if you are thinking

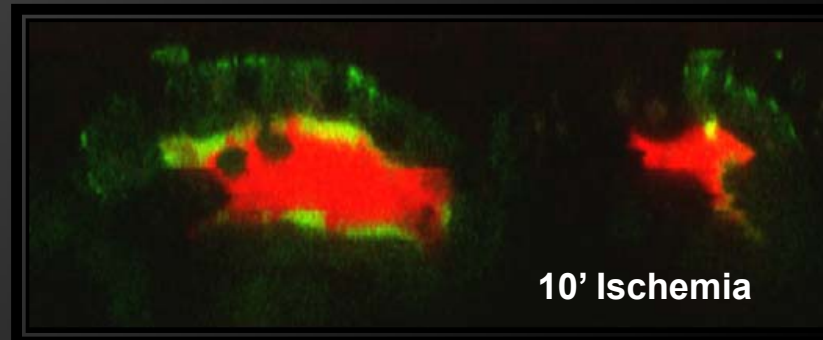
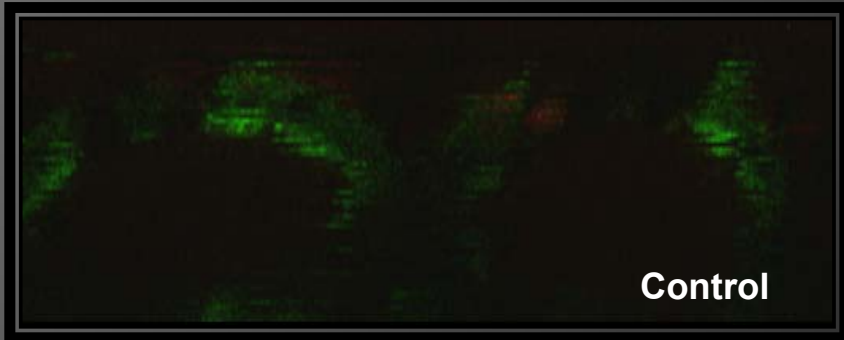
Micropuncture Delivery of Adeno-eGFP Actin



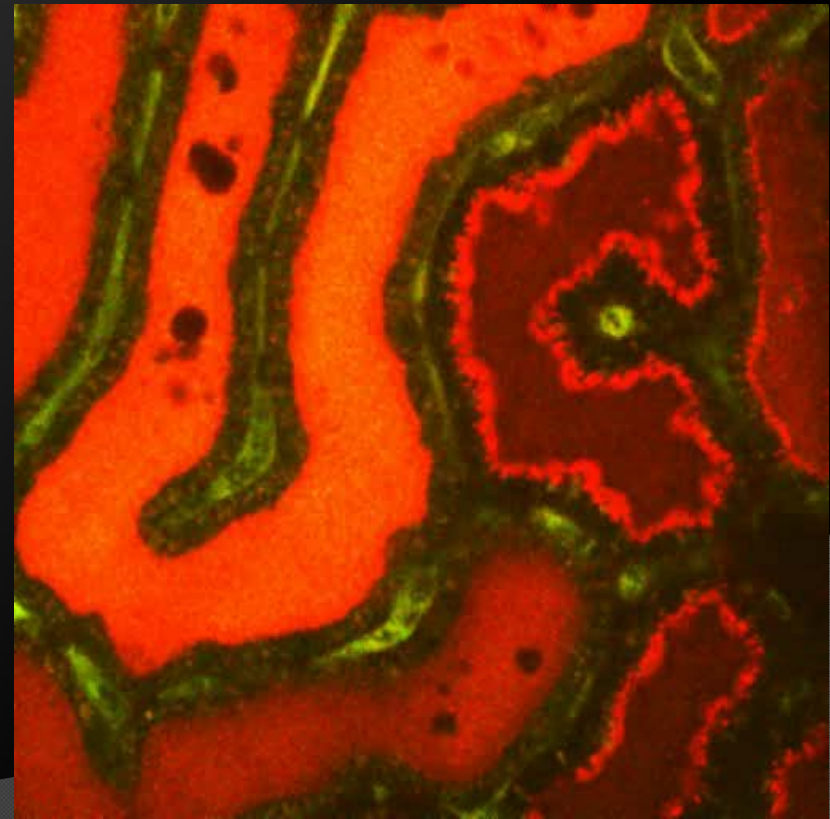
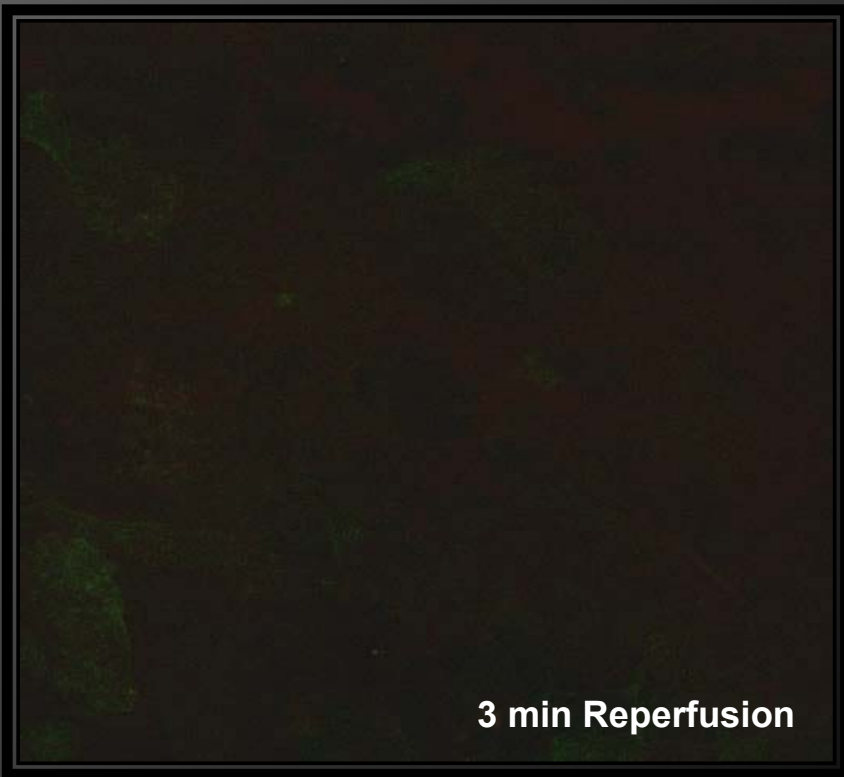
Proximal Tubules
48 hr post Viral
Injection

Proximal Tubules
Post Fixation and
rhodamine Phalloidin
Staining;

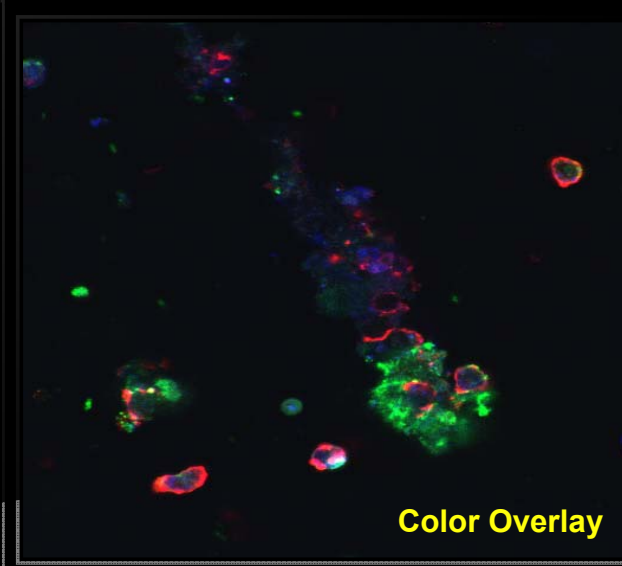
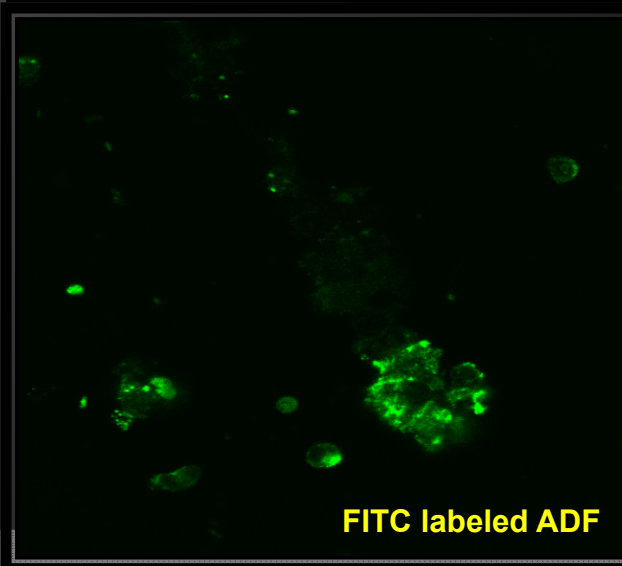
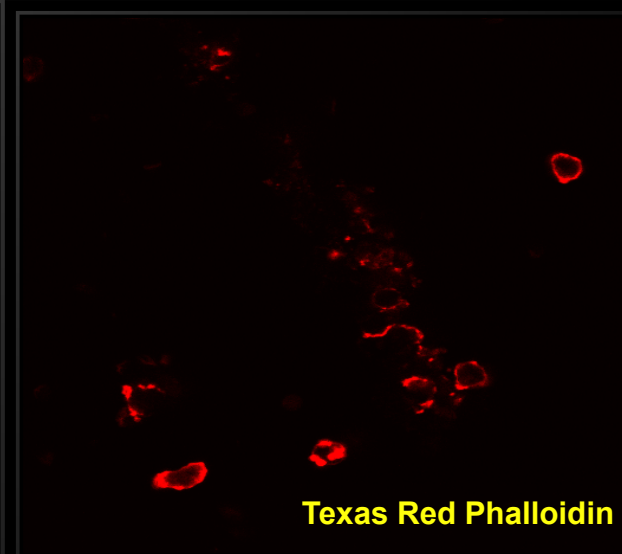
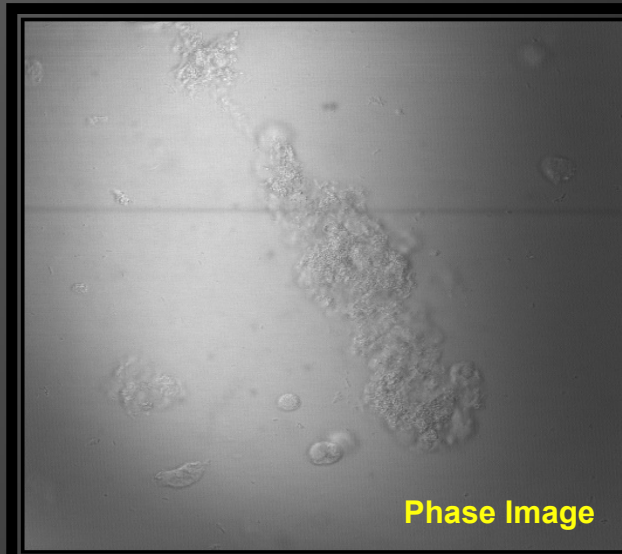
Apical Bleb and Tubular Cast Formation in Ischemia



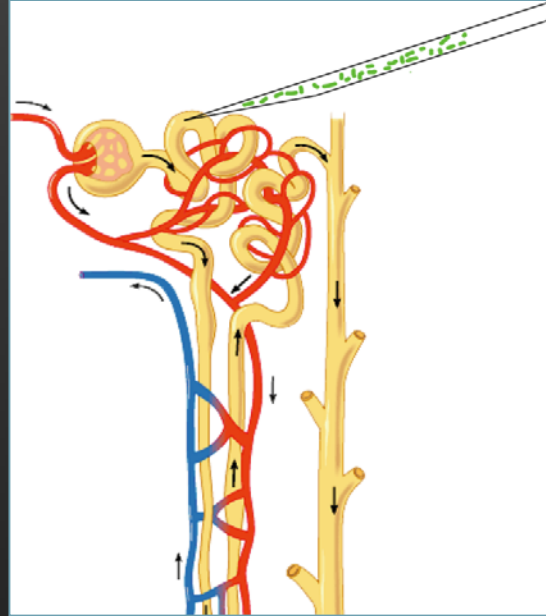
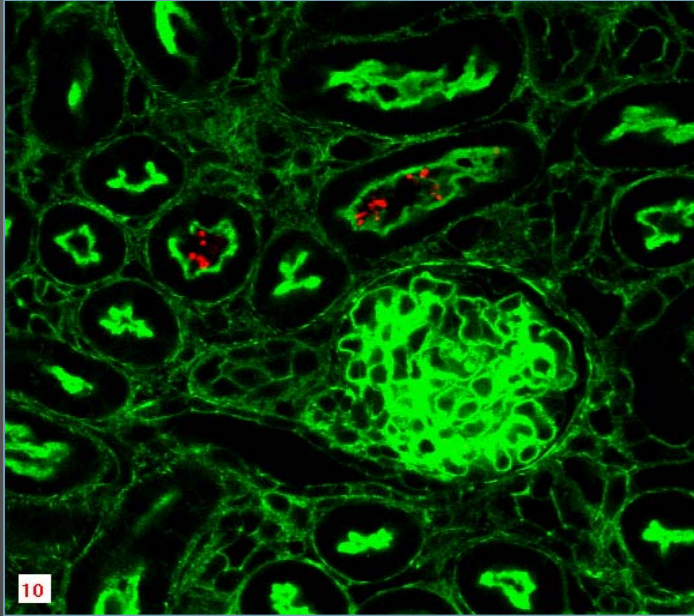
eGFP-Actin and
3kDa TR Dextran



Actin Components of a Urinary Cast in Acute Renal Failure

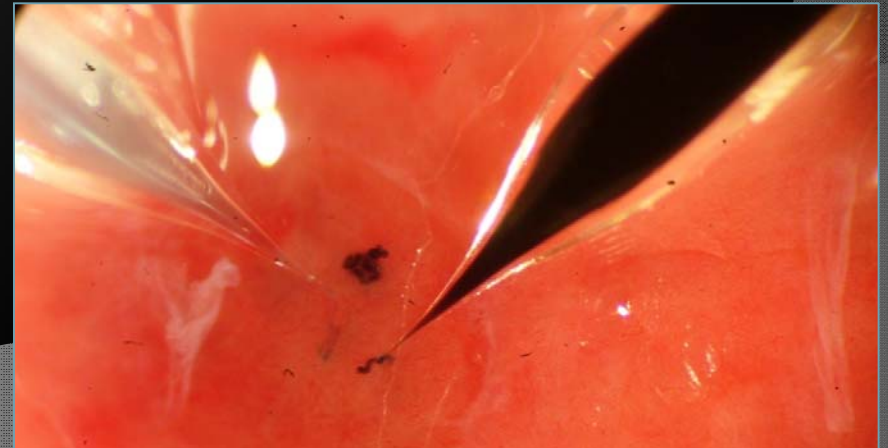


Spatial Specificity Achieved by Micro-Infusion of Bacteria into Proximal Tubules

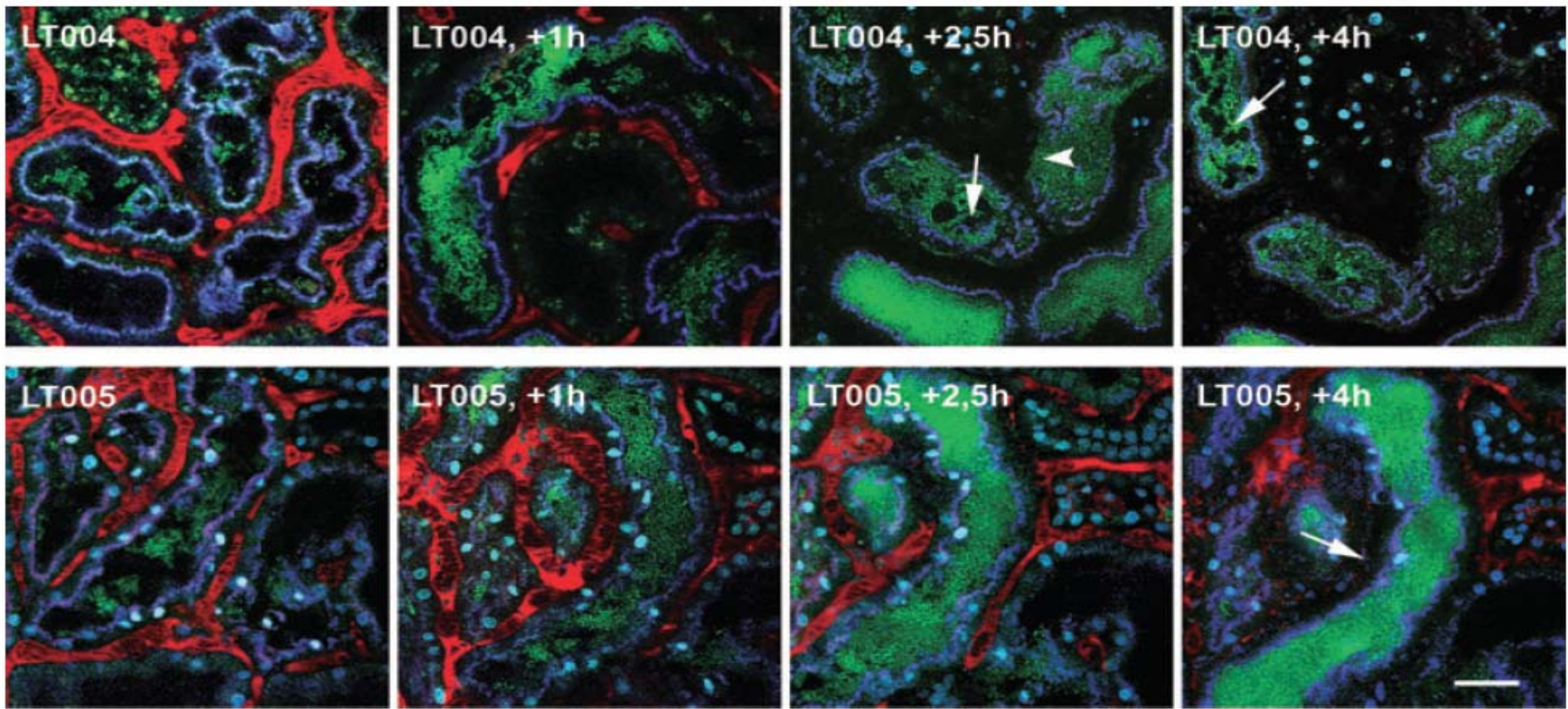


- 10^5 cfu UPEC GFP⁺
- 0.1 to 0.7 μ l injected

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

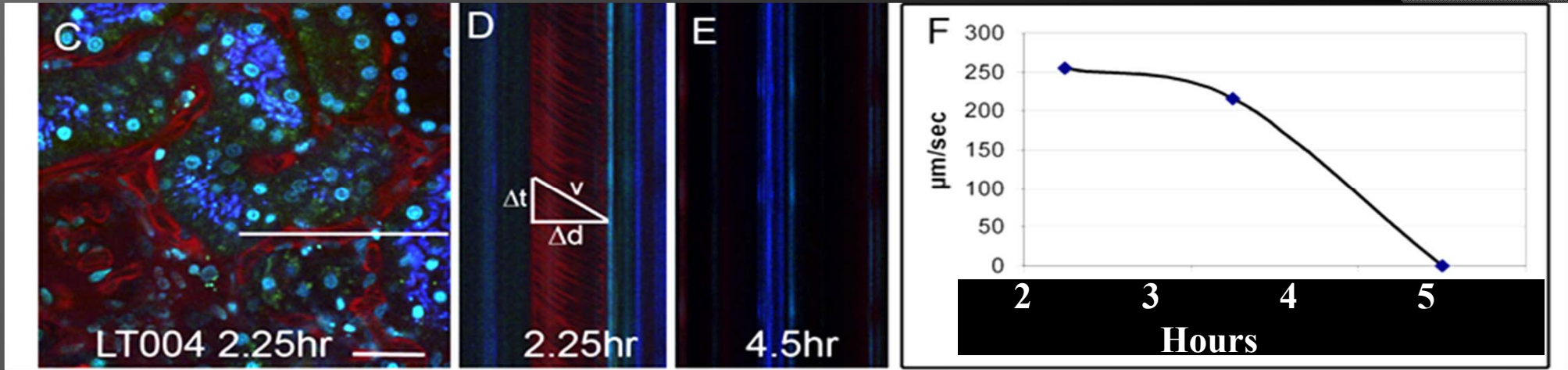


Proximal Tubule *E. coli* Infection: Effect of Virulence Factor

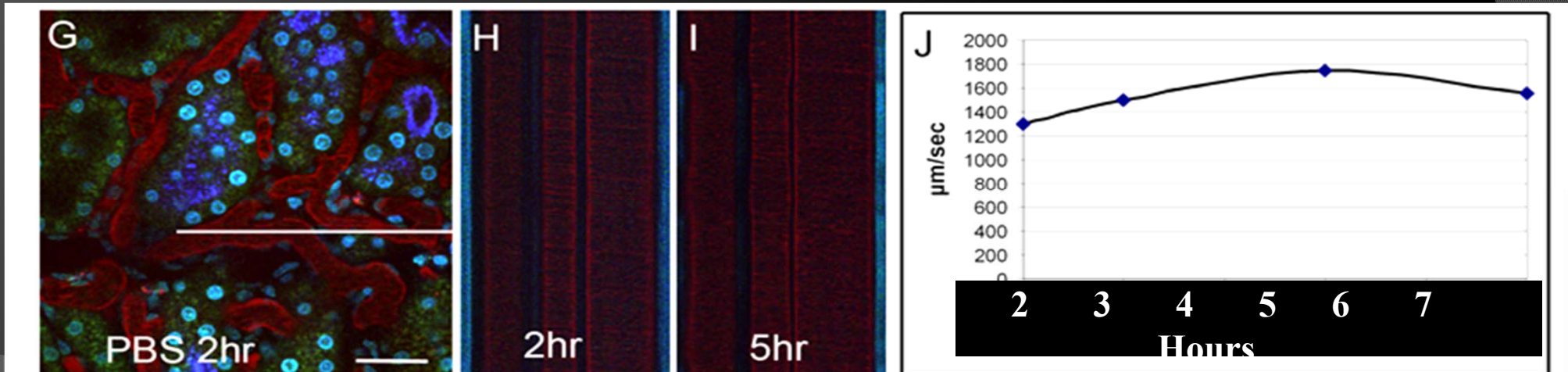


Determining blood flow rates *in vivo*

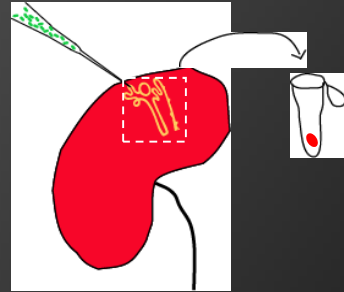
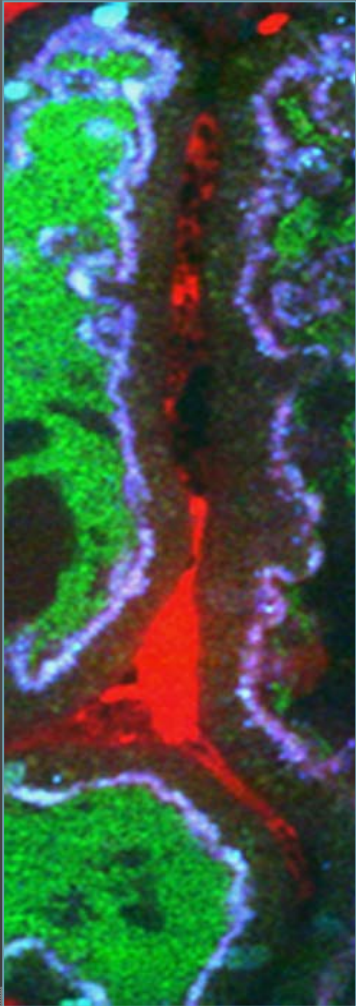
UPEC wt



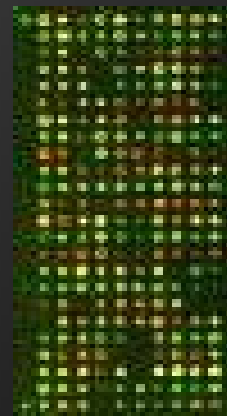
PBS



Clotting Cascade Genes are Up-Regulated in Infected Kidneys

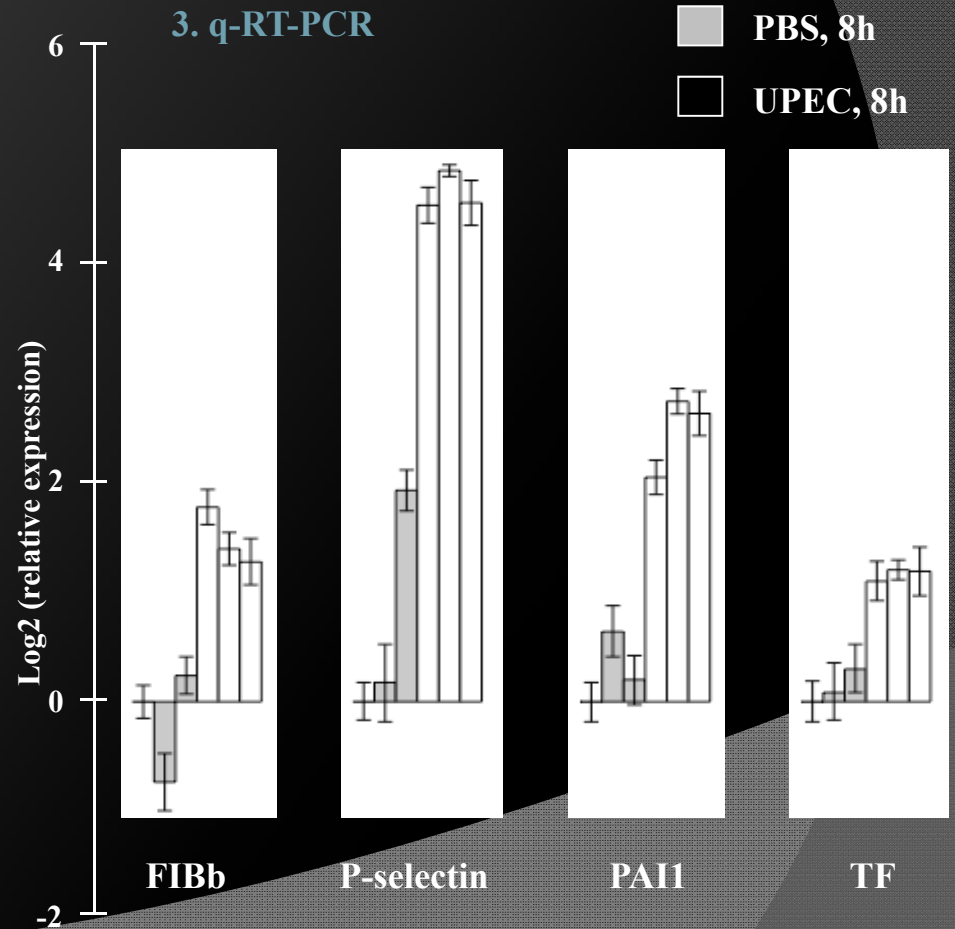


1. Precise dissection to enrich for local mRNA



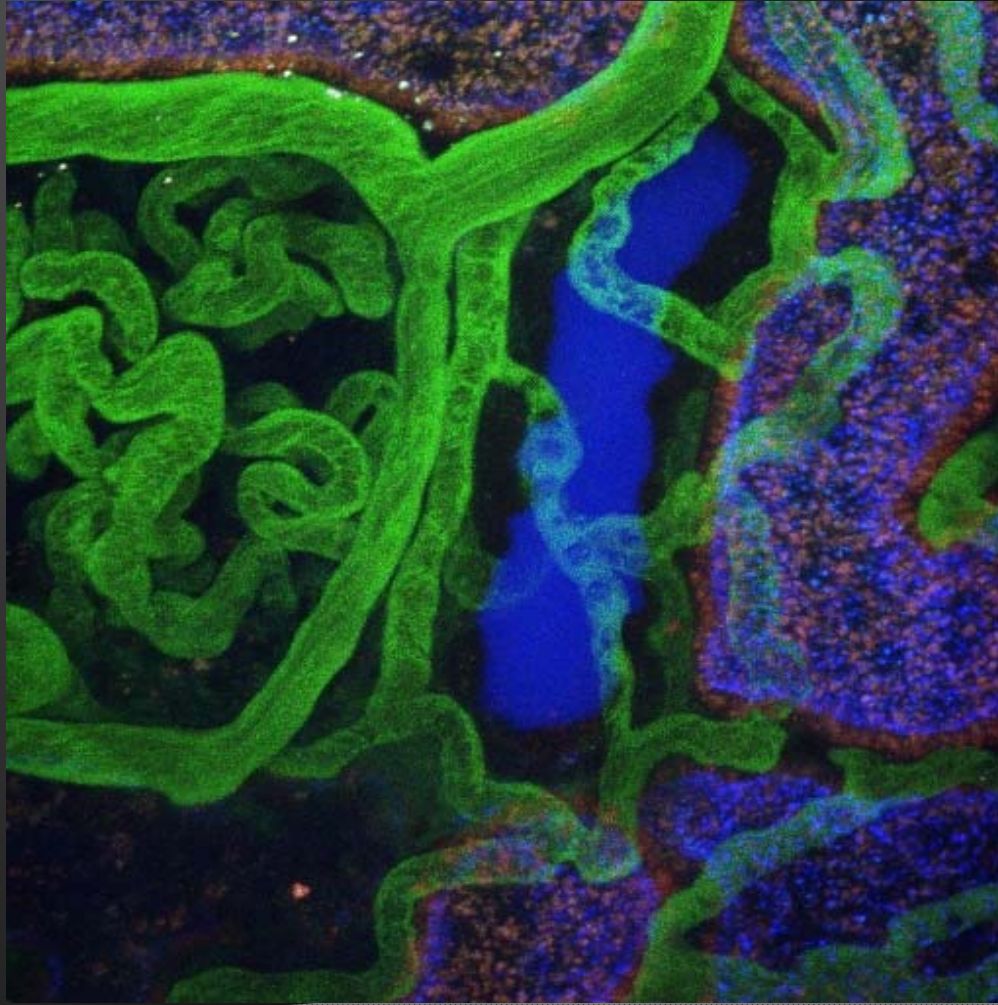
2. Gene expression array

3. q-RT-PCR

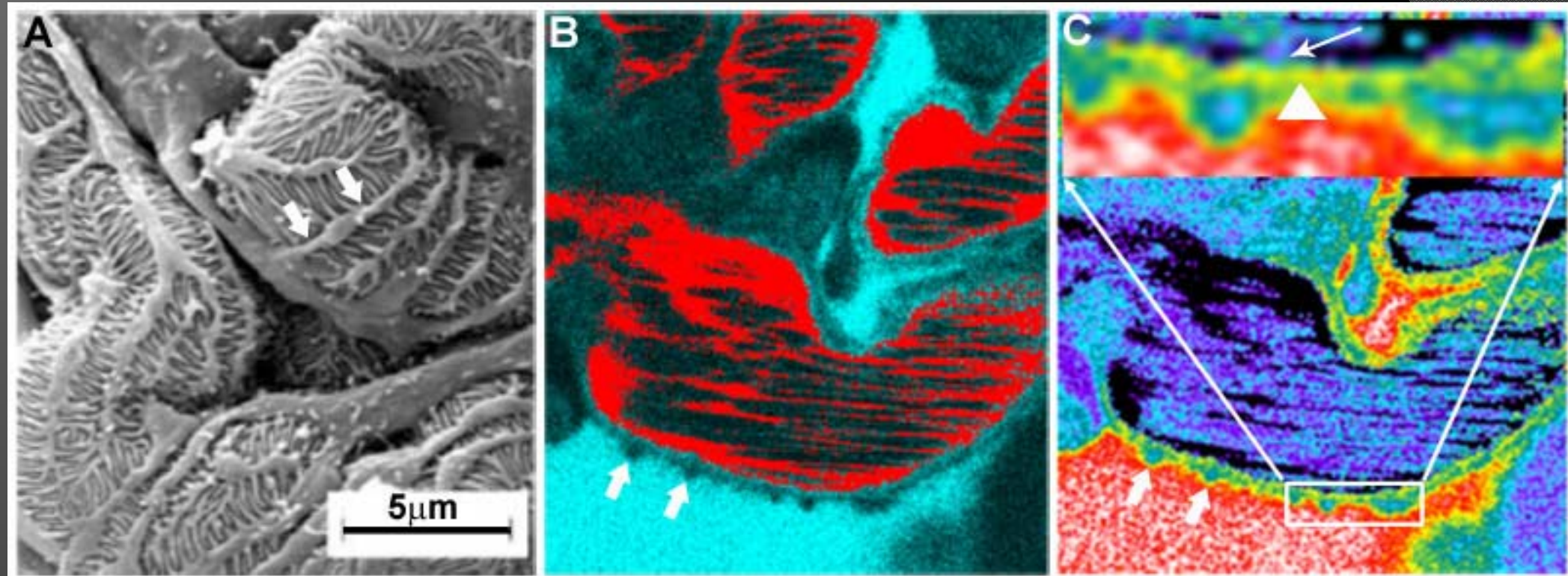


Separate movie

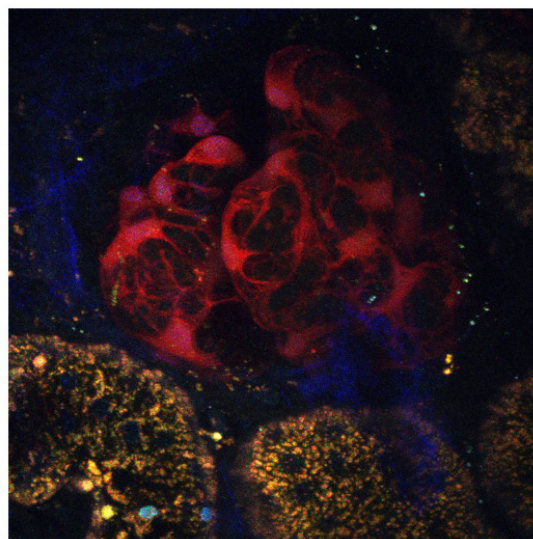
4-D Rat Glomerulus



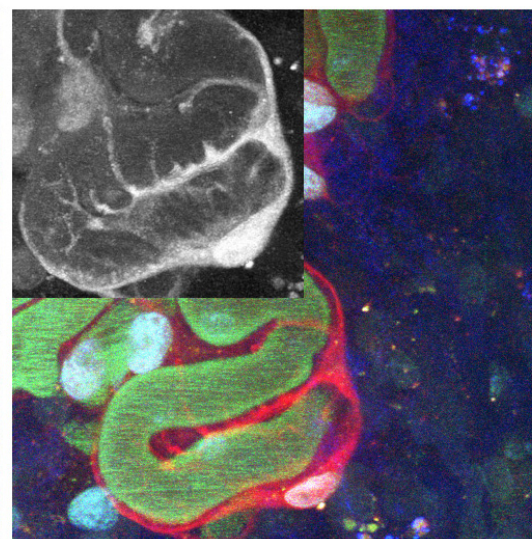
2-Photon Resolution of Glomerular Filtration



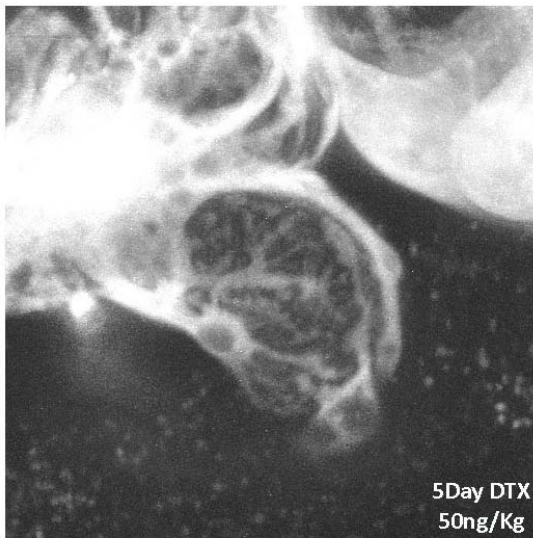
DS Red Labeled Podocytes *in vivo*



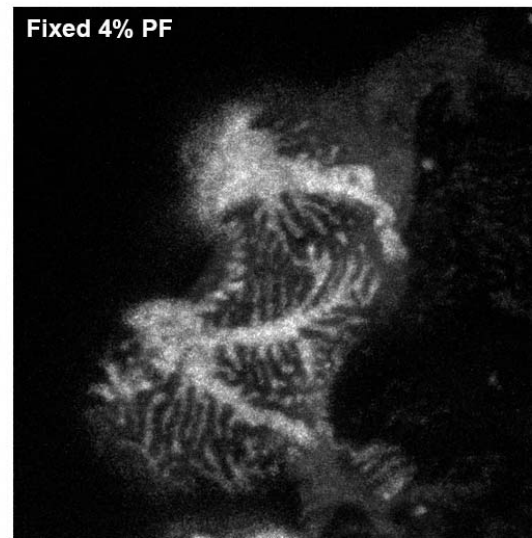
Live-860nm Hoechst, 60x Water-1x Zoom



Live-800nm, 60x water-2x
150K FITC dextran, inset B/W podocyte



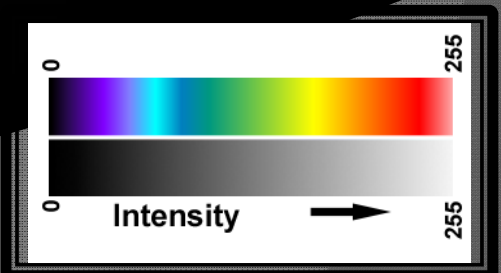
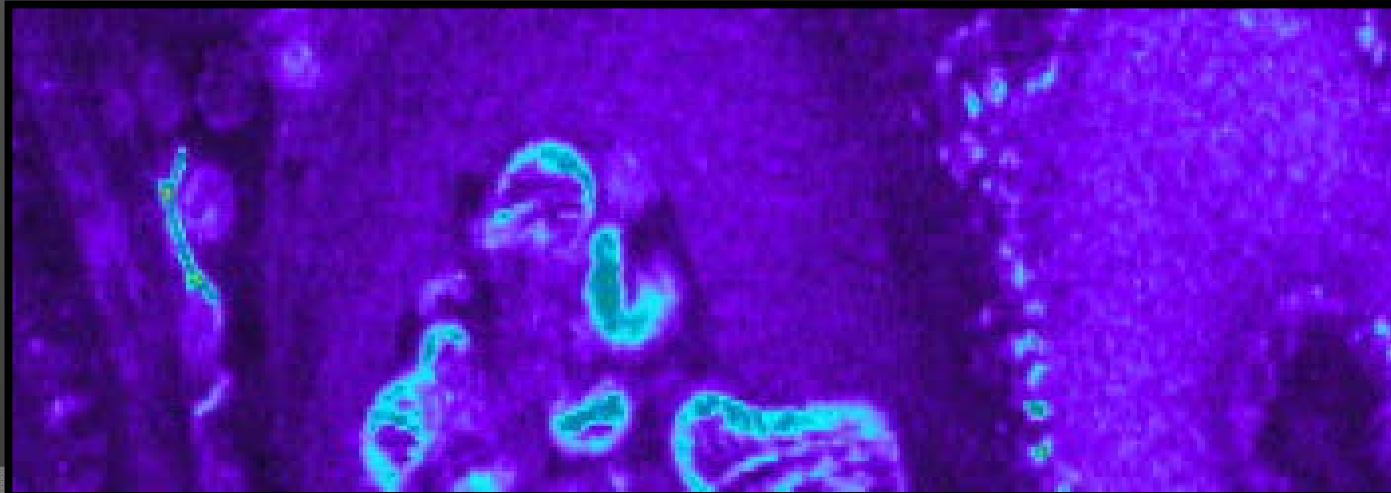
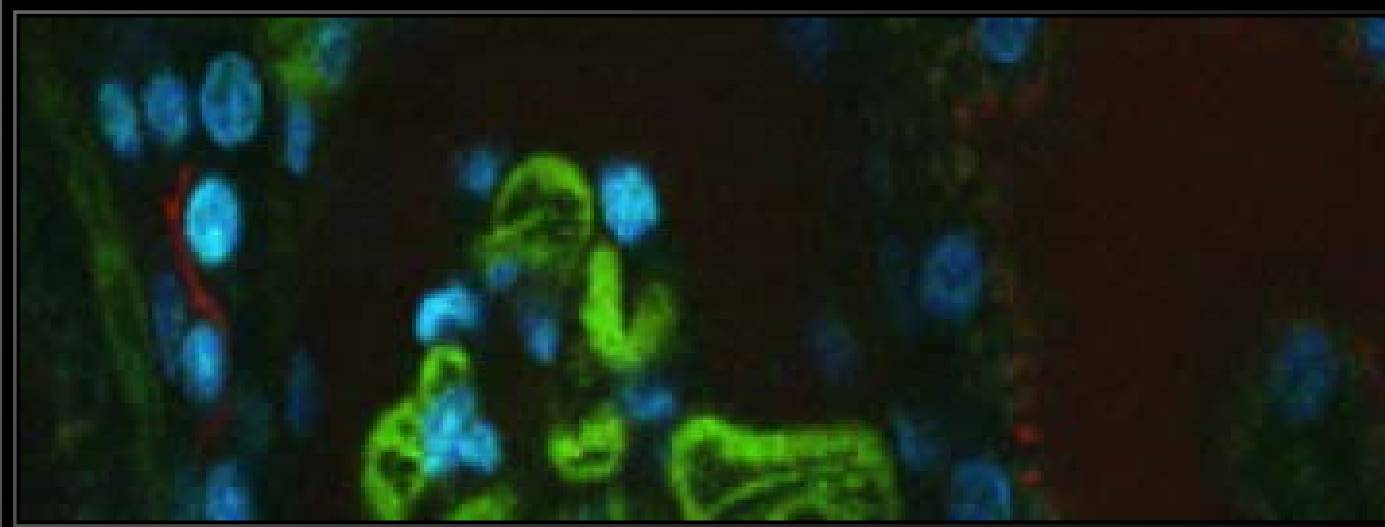
Live-860nm-100x Oil, 4.0x Zoom



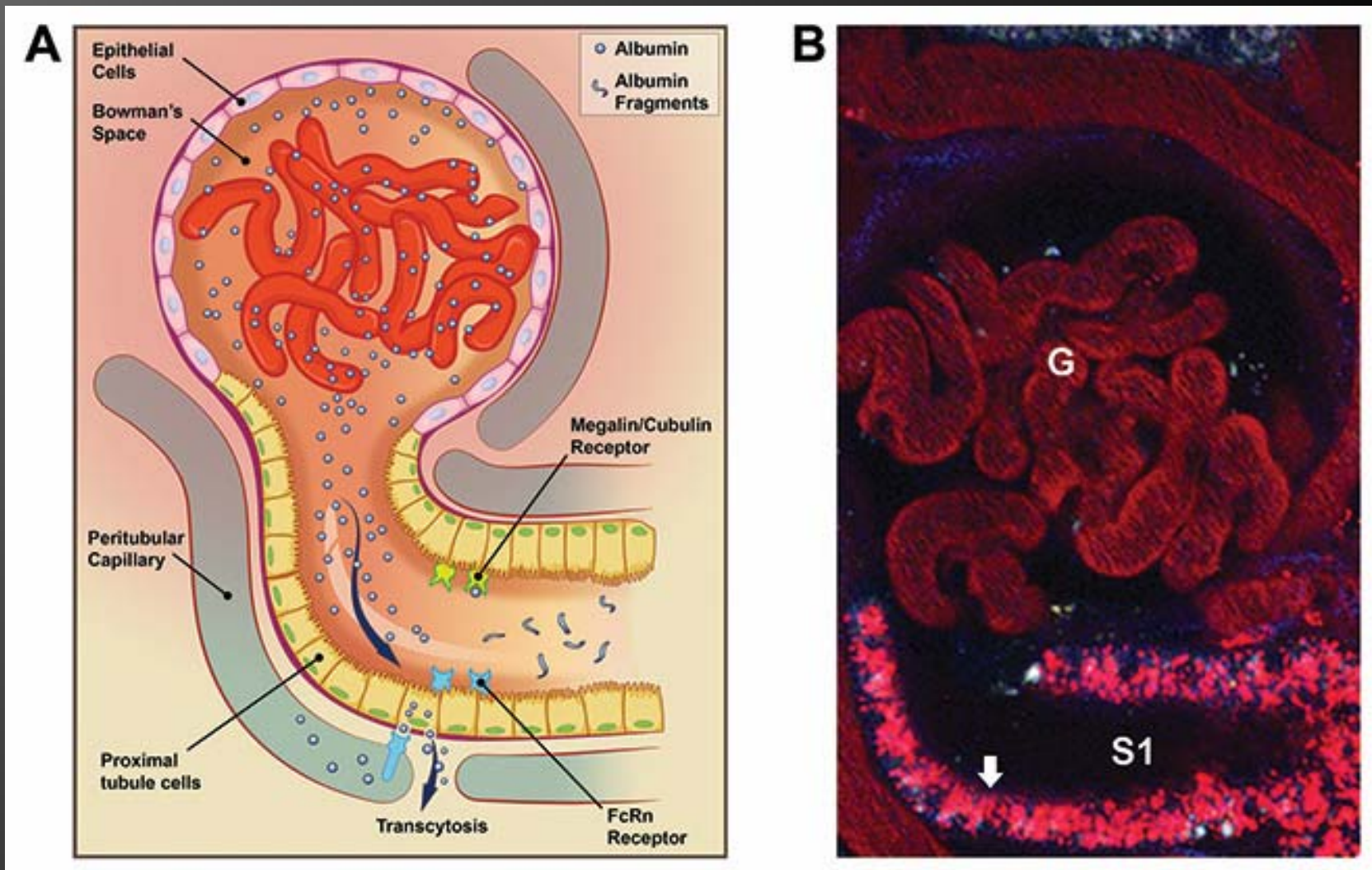
Fixed 4% PF
100x Oil, 4.0x Zoom

Reducing Scan Size

5 Frames/sec
500kDa FITC Dextran with 3kDa
TR Dextran Injection



Renal Handling of Albumin by the PCT

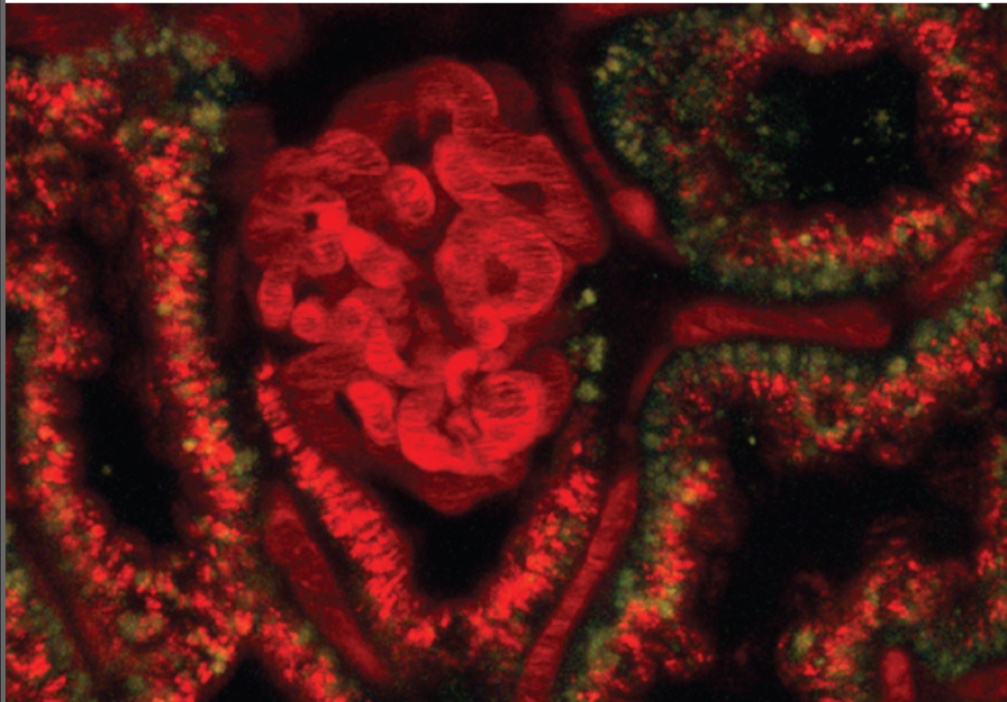


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kidney

INTERNATIONAL



VOLUME 71 | ISSUE 6 | MARCH (2) 2007
<http://www.kidney-international.org>

Albumin filtration
Classification of
lupus nephritis
Peritoneal dialysis
solutions

Things Learned Along the Way

◎ Challenges occur

Dogma, Assumptions, Reagents, Sensitivity

Quantitative Analysis without Gold Standards

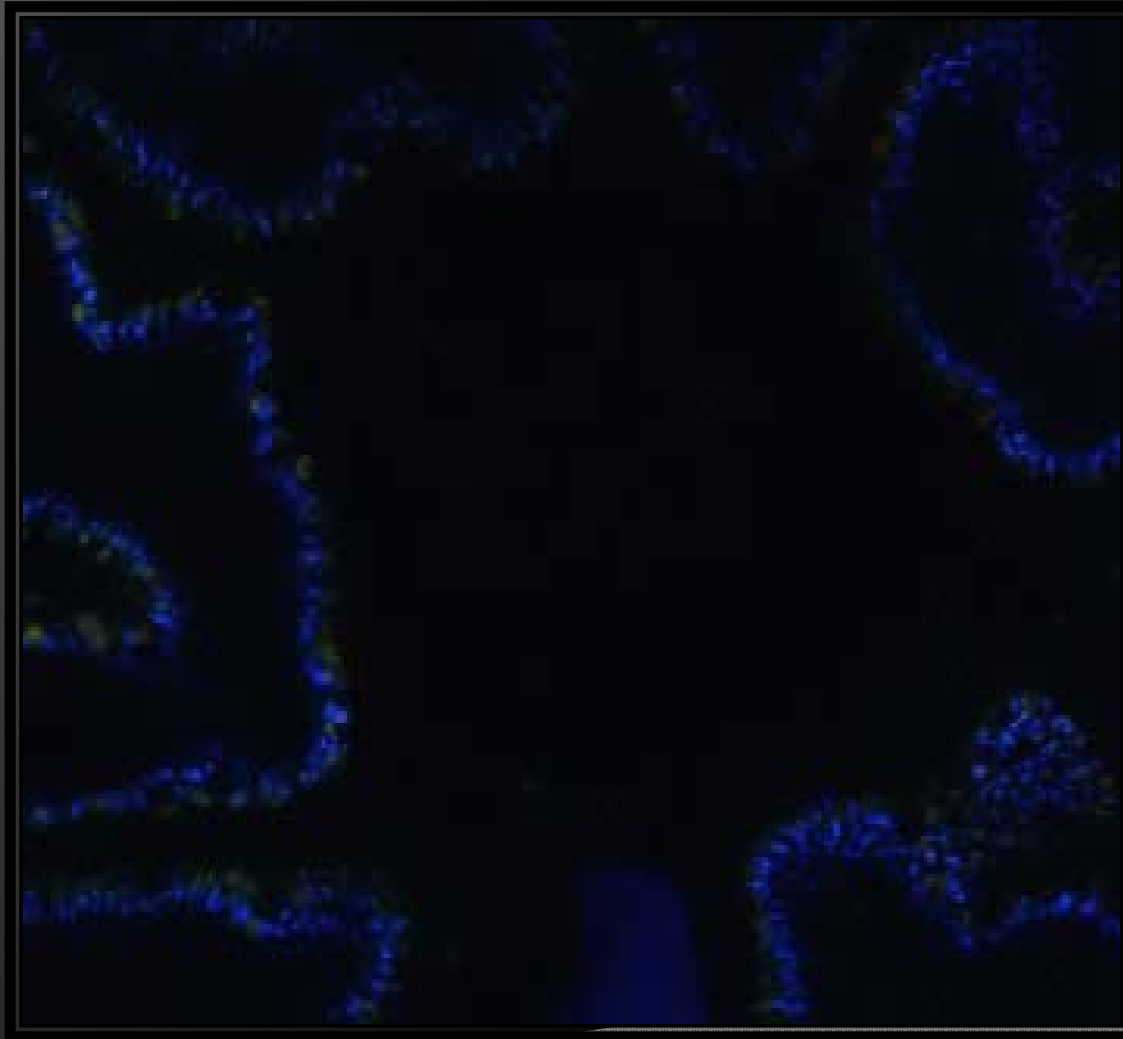
You See What you are Looking For

Correcting for Depth of Field

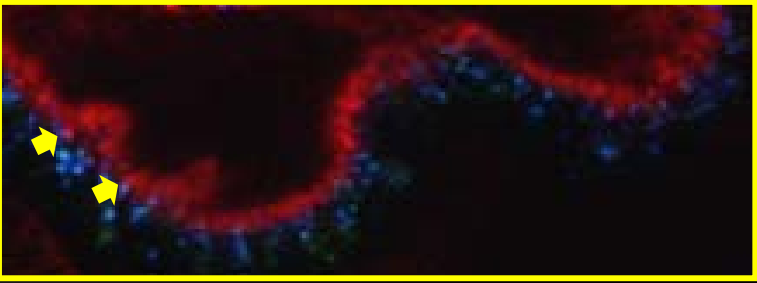
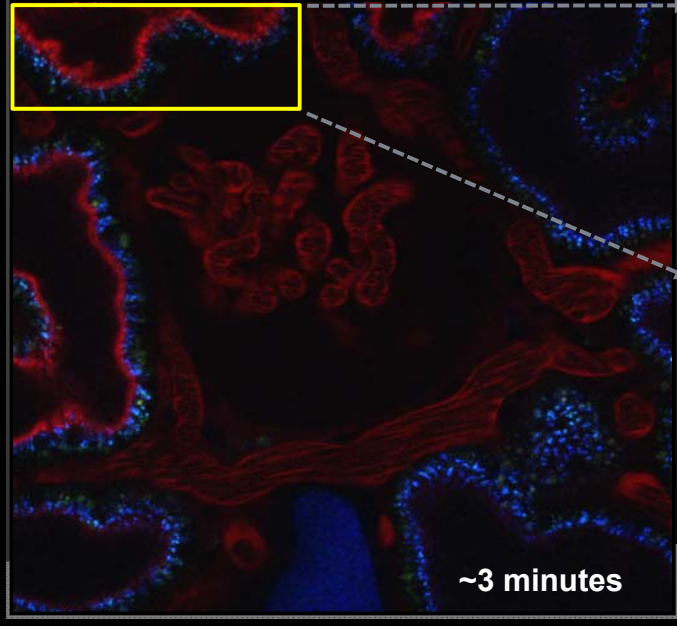
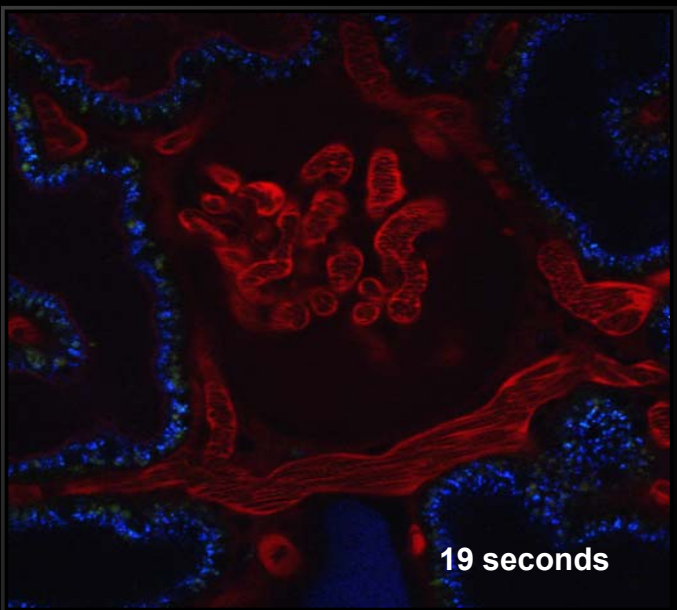
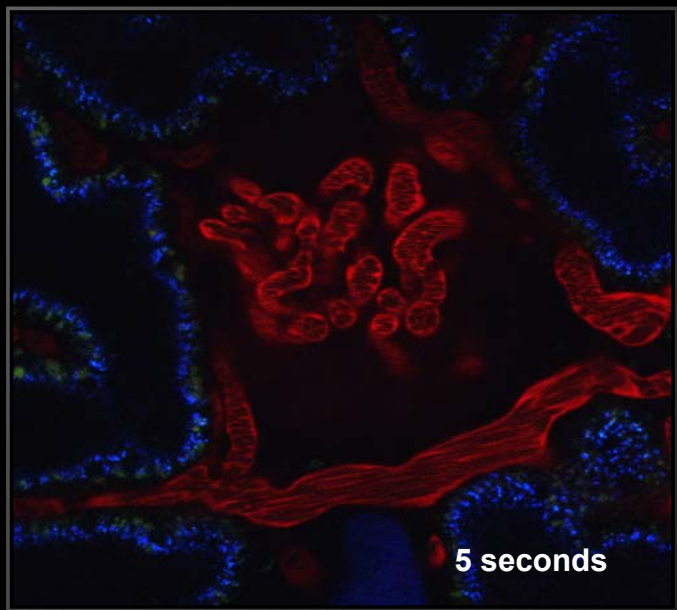
Out of Focus Fluorescence

Physiologic state of the rat

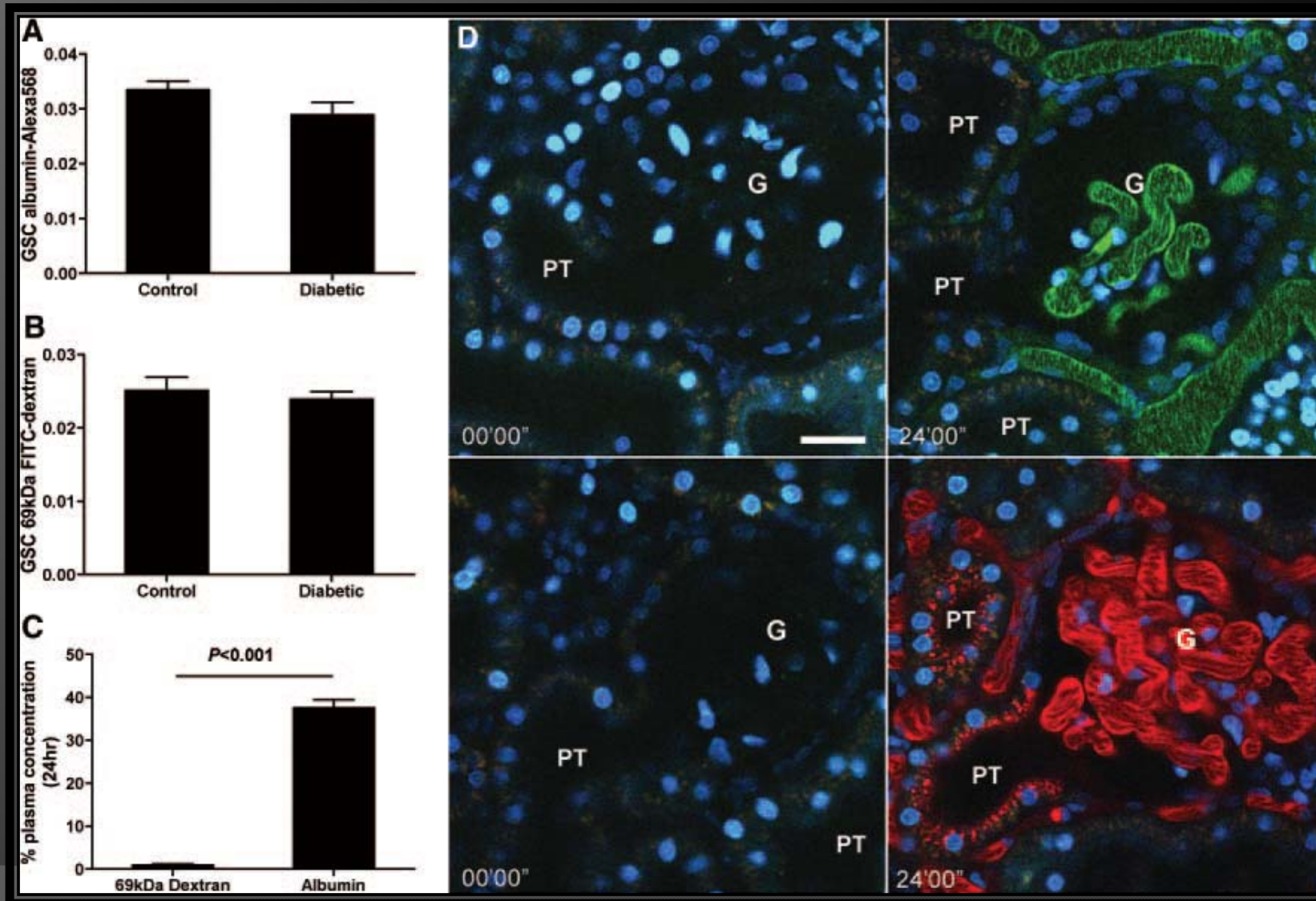
Albumin Filtration and Reabsorption in the Rat



Albumin Filtration and Reabsorption in the Rat



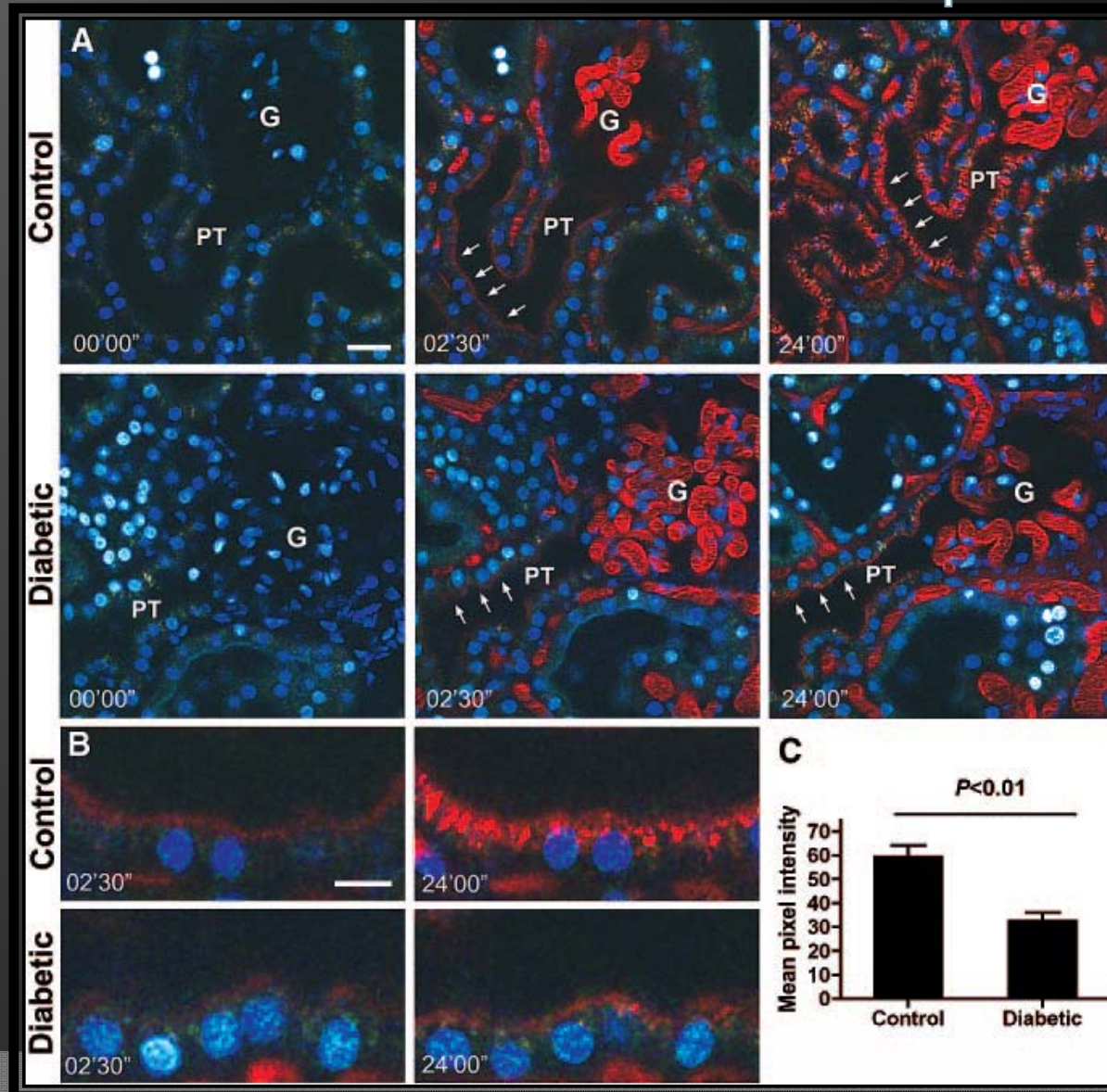
Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption



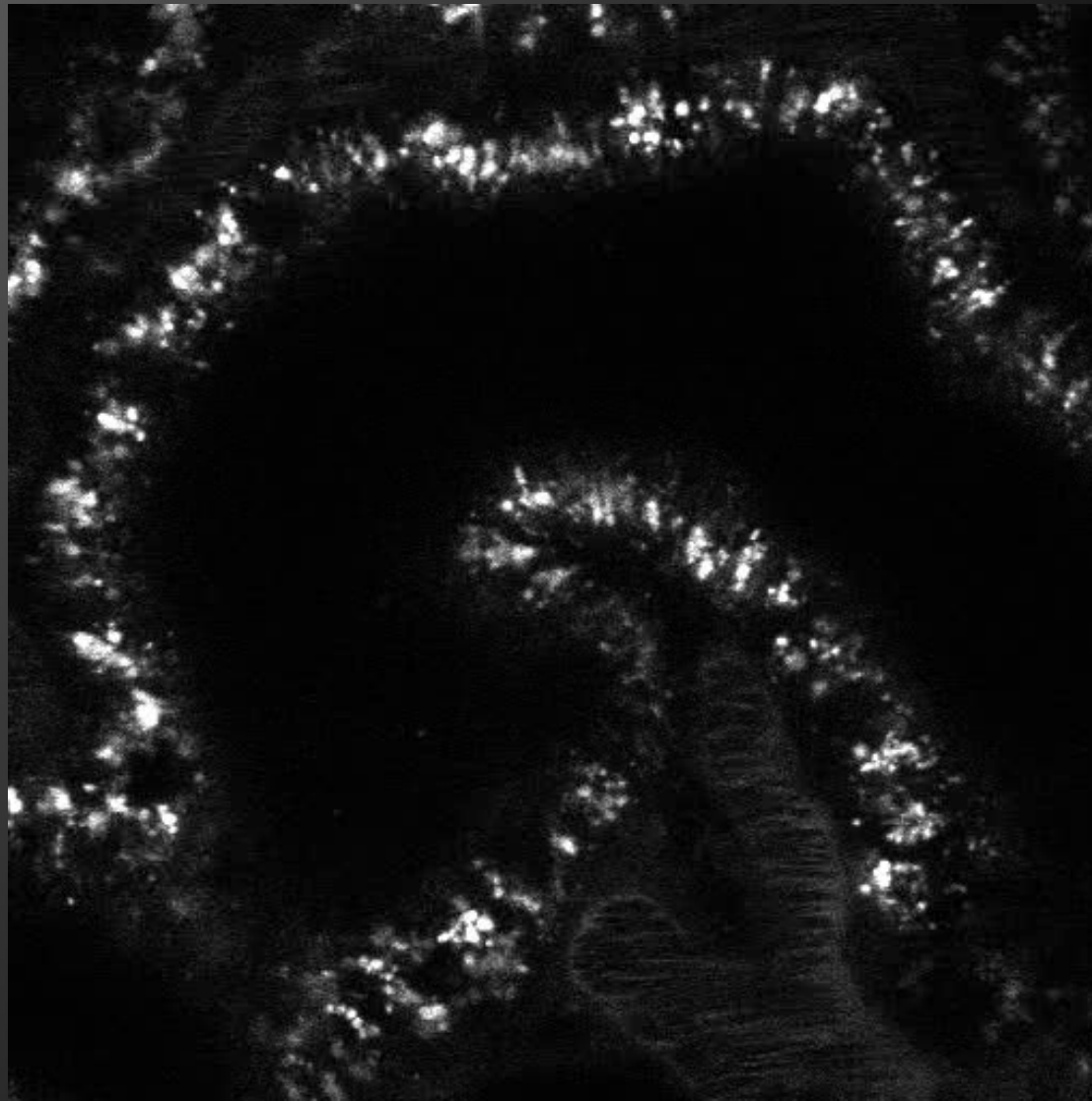
69 kDa
FITC -Dextran

ALEXA 586
Albumin

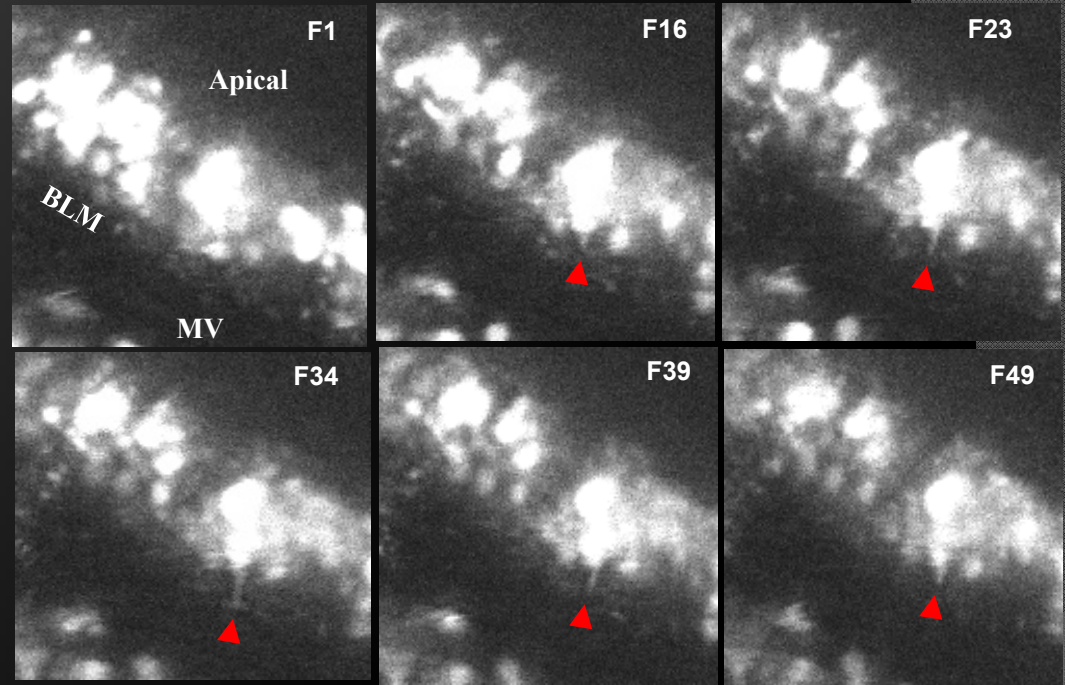
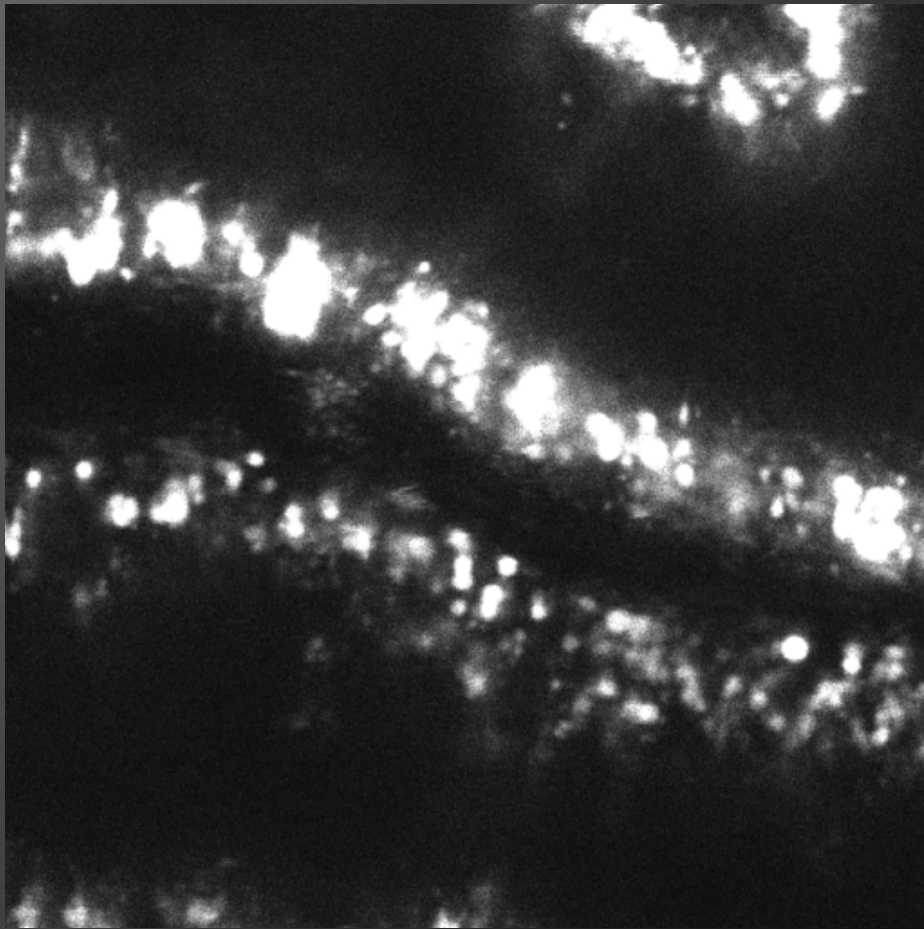
Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption

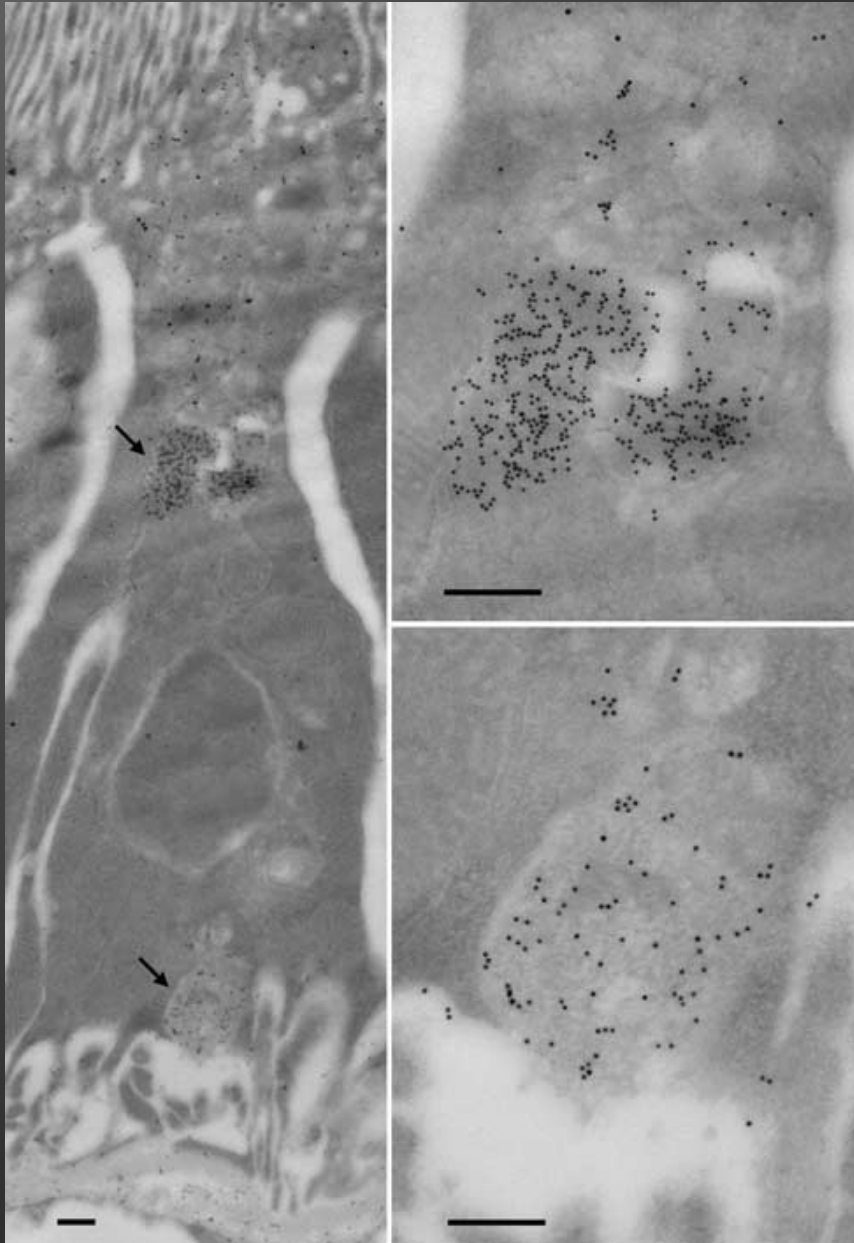


Albumin Transcytosis



PTC Albumin Transcytosis

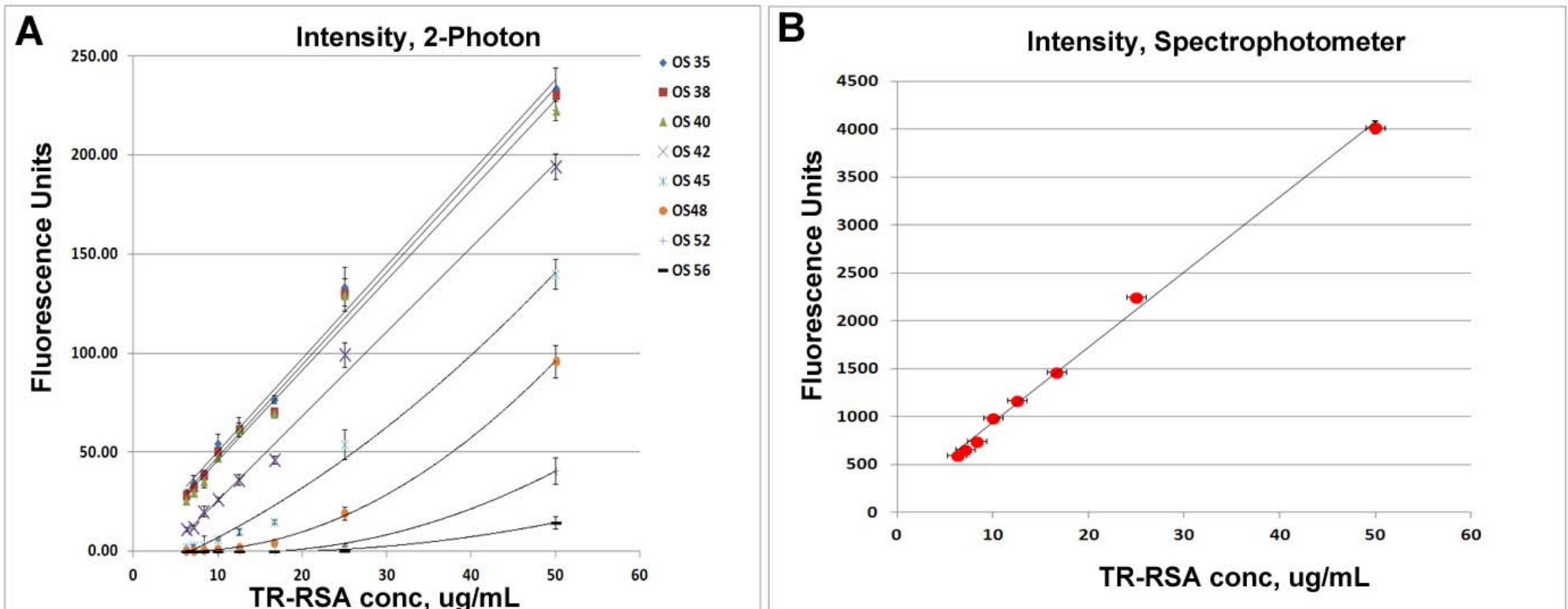


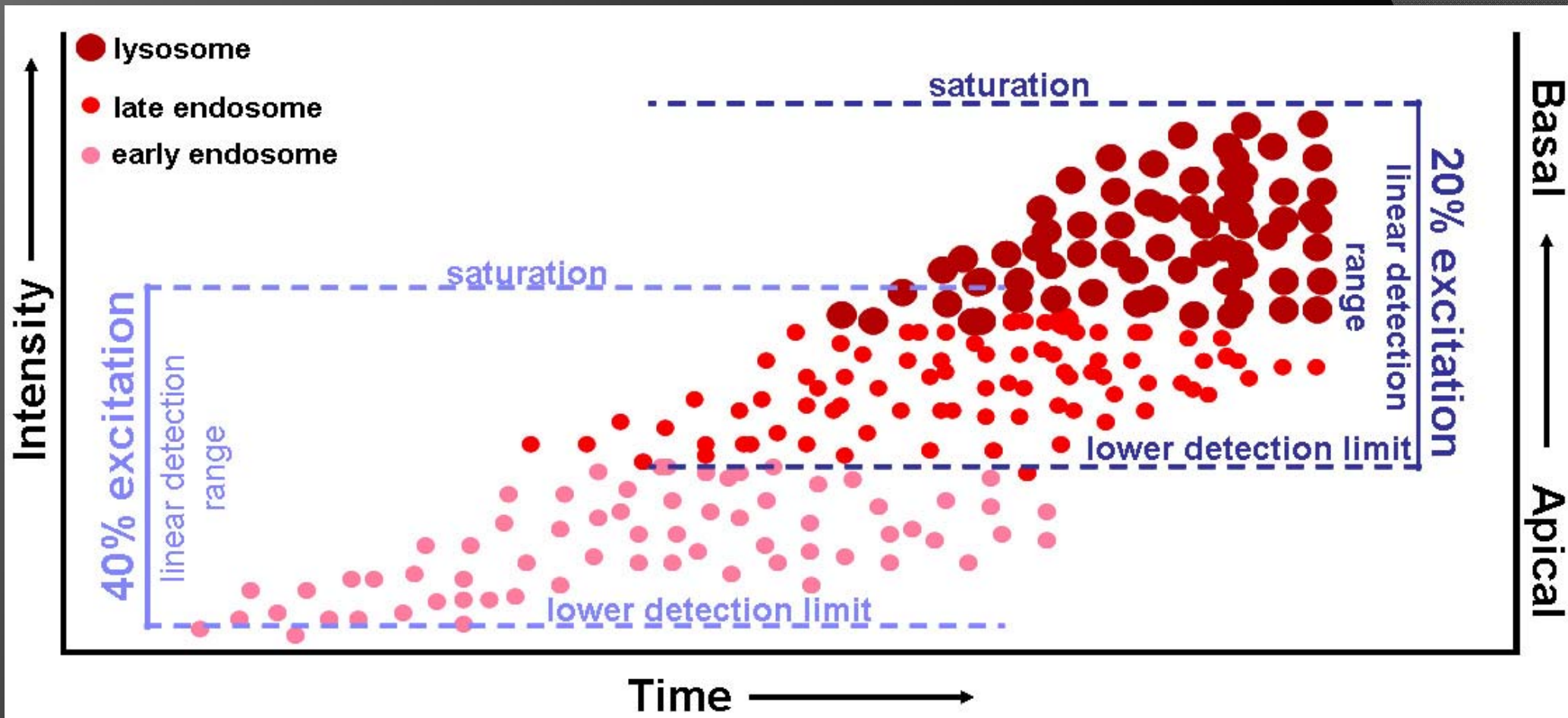


EM Gold Visualization of RSA Endocytosis and Transcytosis in a Rat PTC

Setting Background Level Determines Sensitivity

Figure 2

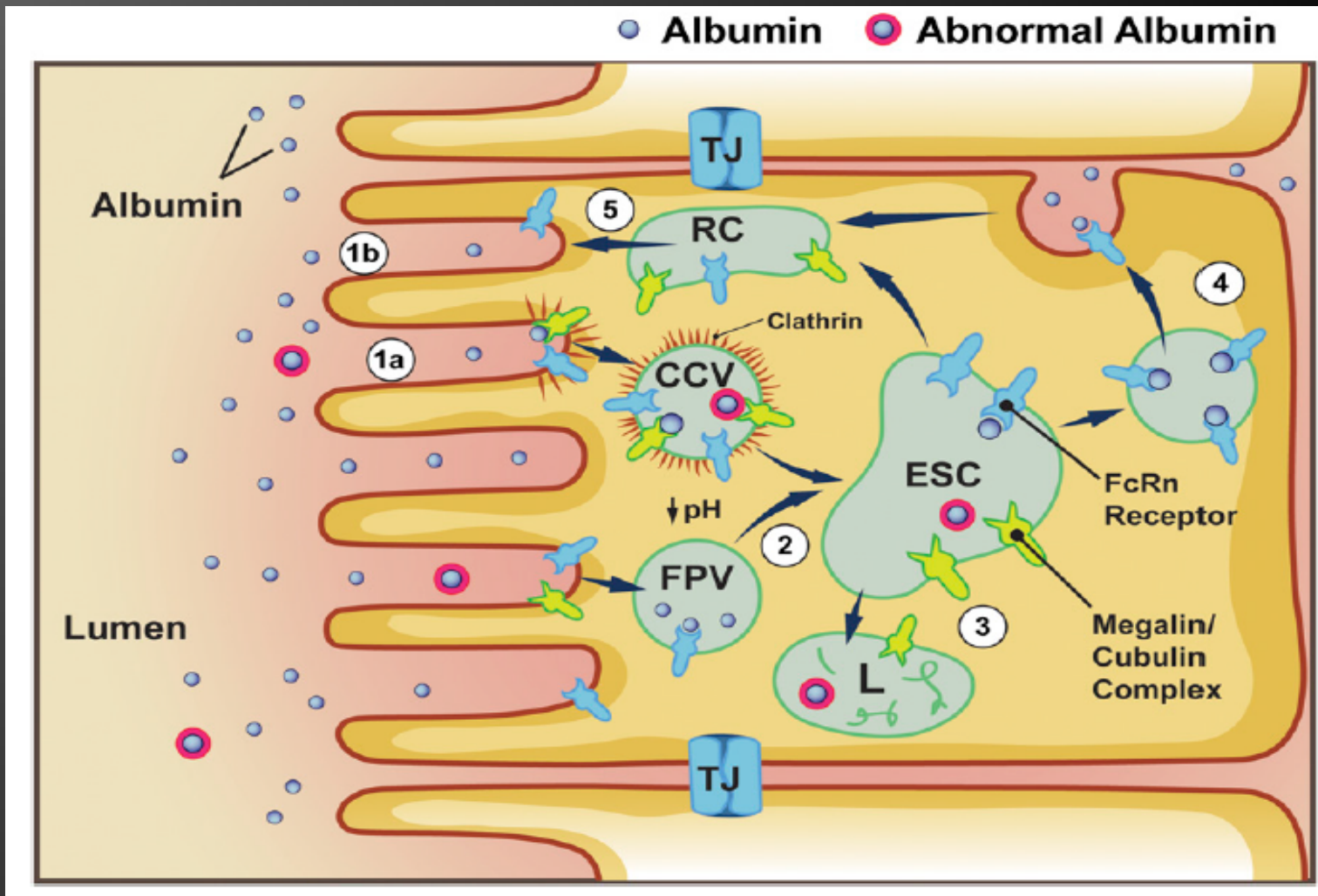




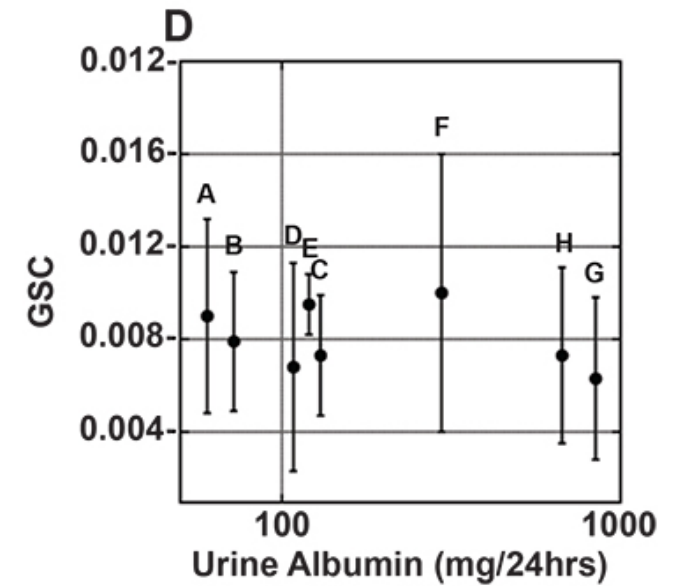
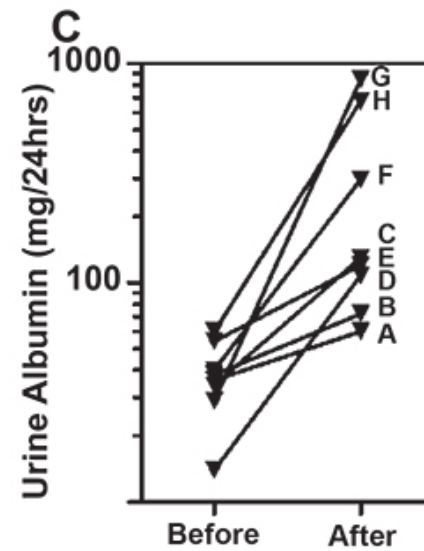
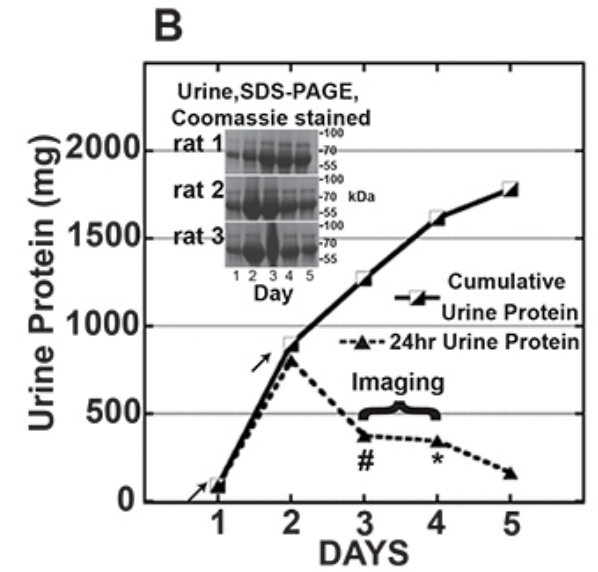
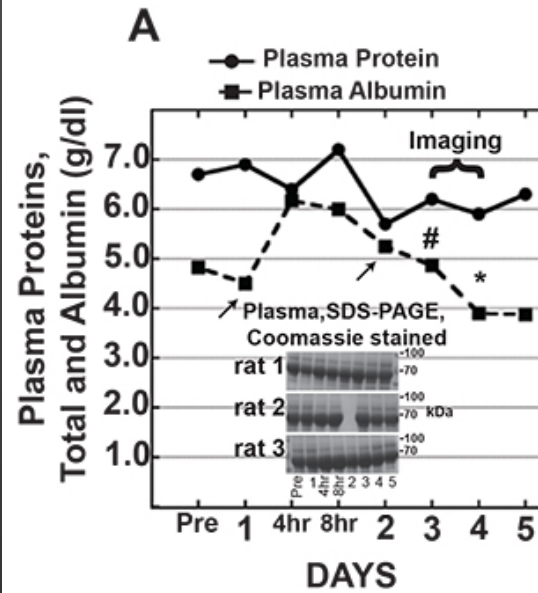
Albumin Is Recycled from the Primary Urine by Tubular Transcytosis.

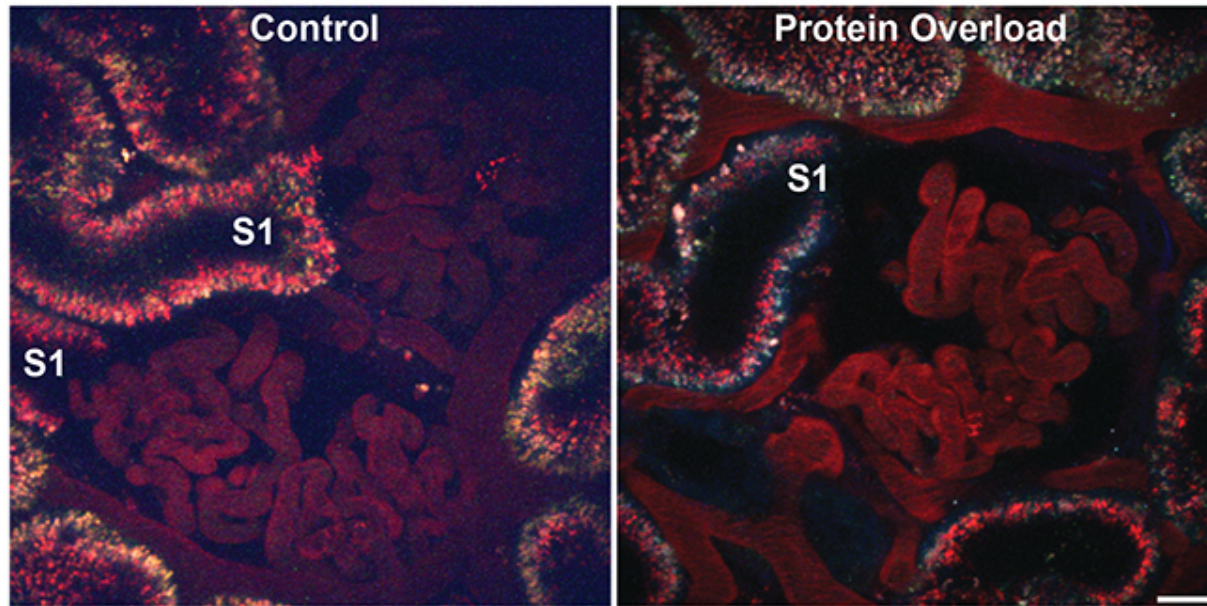
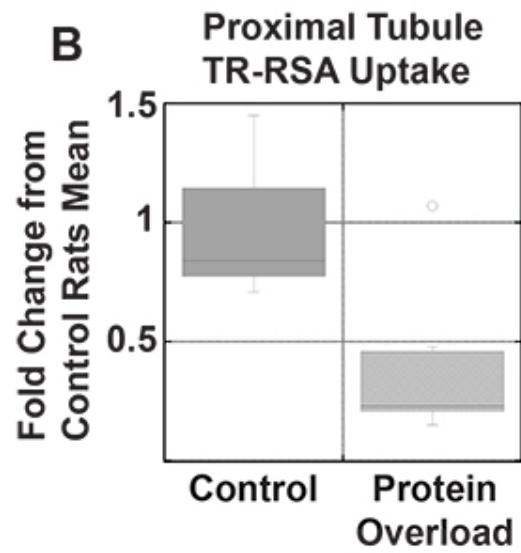
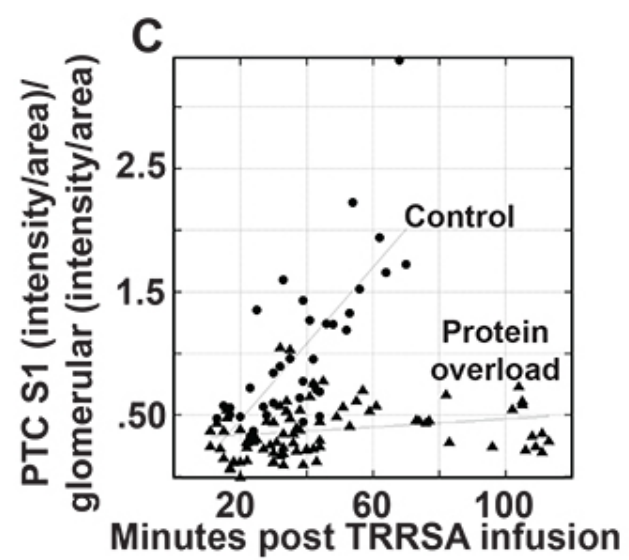
Tenten V, Menzel S, Kunter U, Sicking EM, van Roeyen CR, Sanden SK, Kaldenbach M, Boor P, Fuss A, Uhlig S, Lanzmich R, Willemsen B, Dijkman H, Grepl M, Wild K, Kriz W, Smeets B, Floege J, Moeller MJ.

J. Am. Soc. Nephrol., Aug 2013

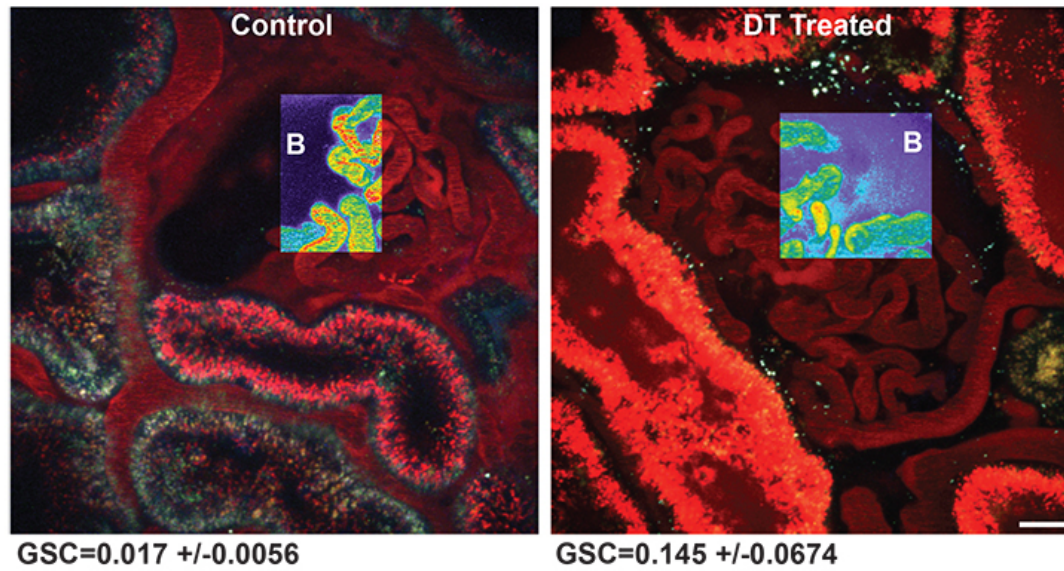


Albumin Overload Model of Albuminuria

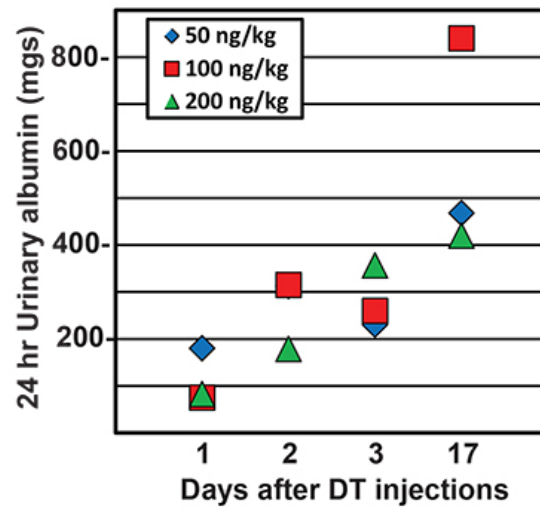


A**B****C**

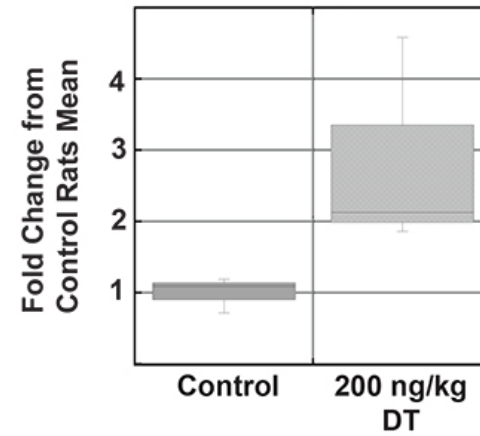
A



B



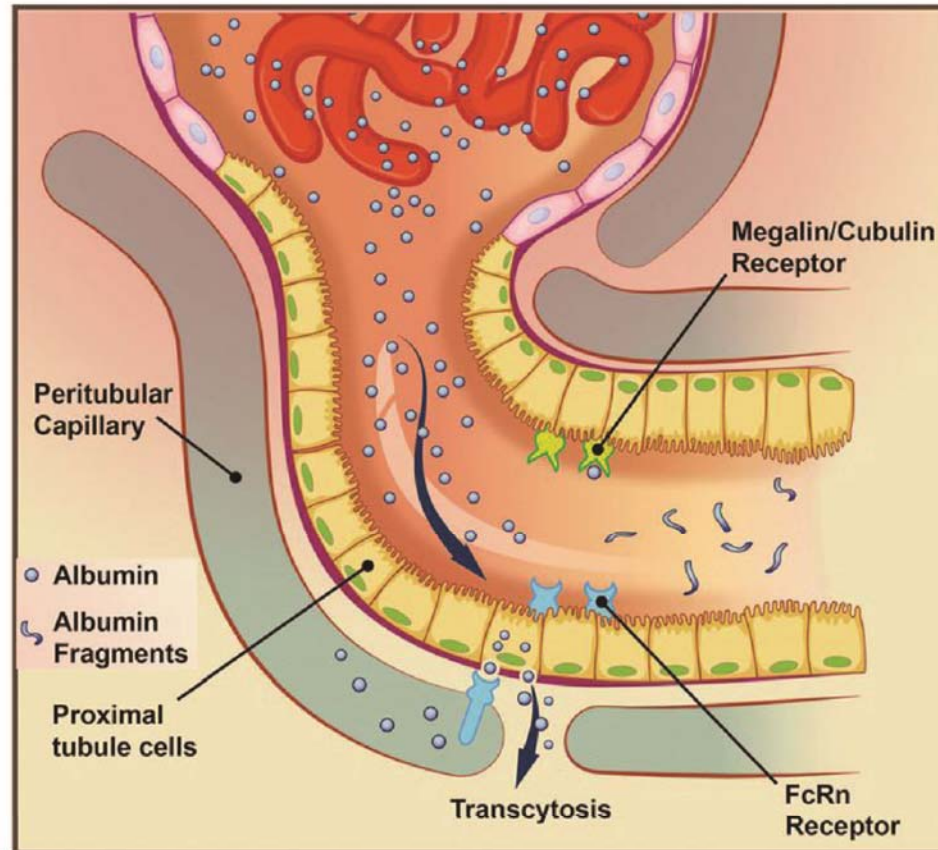
C
Proximal Tubule
TR-RSA Uptake



Wagner et.al. JASN 2015

Serum Albumin 2.0 mg/dl

Figure 5



	GFR (ml/min)	Serum Alb (mg/ml)	GSC_A	Albumin Filtered (mg/24hr)	PT Uptake (%)	Urine Alb Calculated (mg/24hr)	Urine Alb Observed (mg/24hr)
Control	1.4	45	0.008	725	95	X	38
Protein Overload	1.4	45	0.008	725	37	457	329

Summary

2-photon microscopy is a disruptive technology

We are just beginning to explore what can be accomplished

You must have sound biological questions

Combining techniques is extremely powerful

**Your data are only as good as your reagent, sensitivity,
resolution and quantitative techniques.**