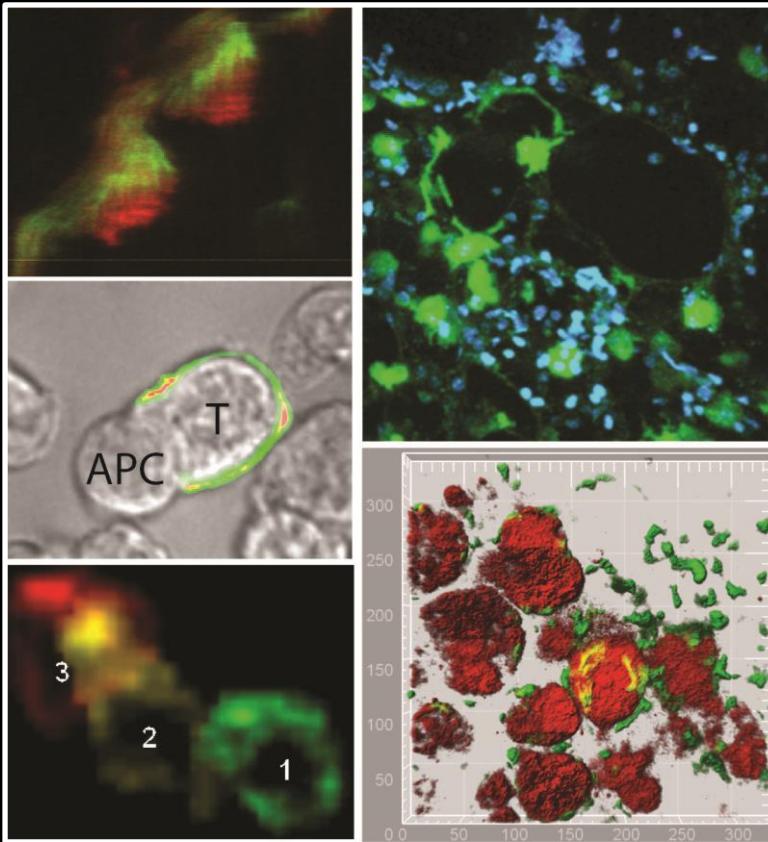
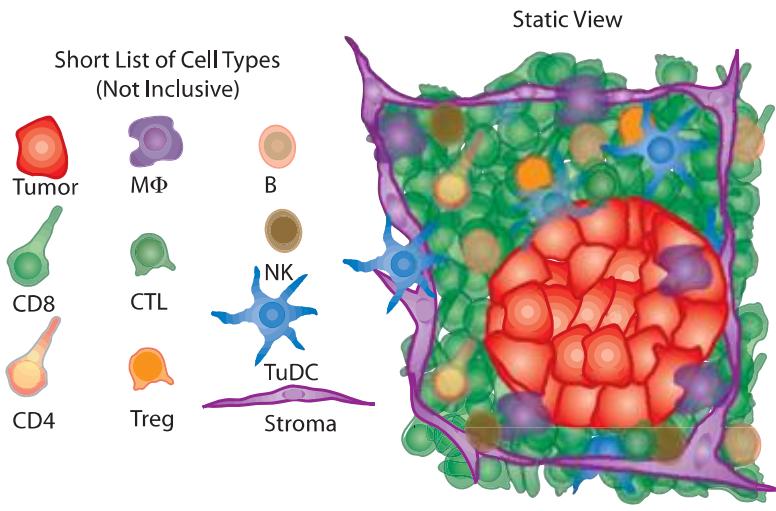


Principles, Benefits and Application of Multi-photon Microscopy .



Krummel Lab, Department of Pathology

How can Live-Imaging Help Mine out Significant Systems Events in Complex Tissues?



Flow Cytometry
Cryo Sections

Issues with Imaging in Complex Tissues



“2-Photon Microscopy”
Also commonly
“Multiphoton Microscopy”

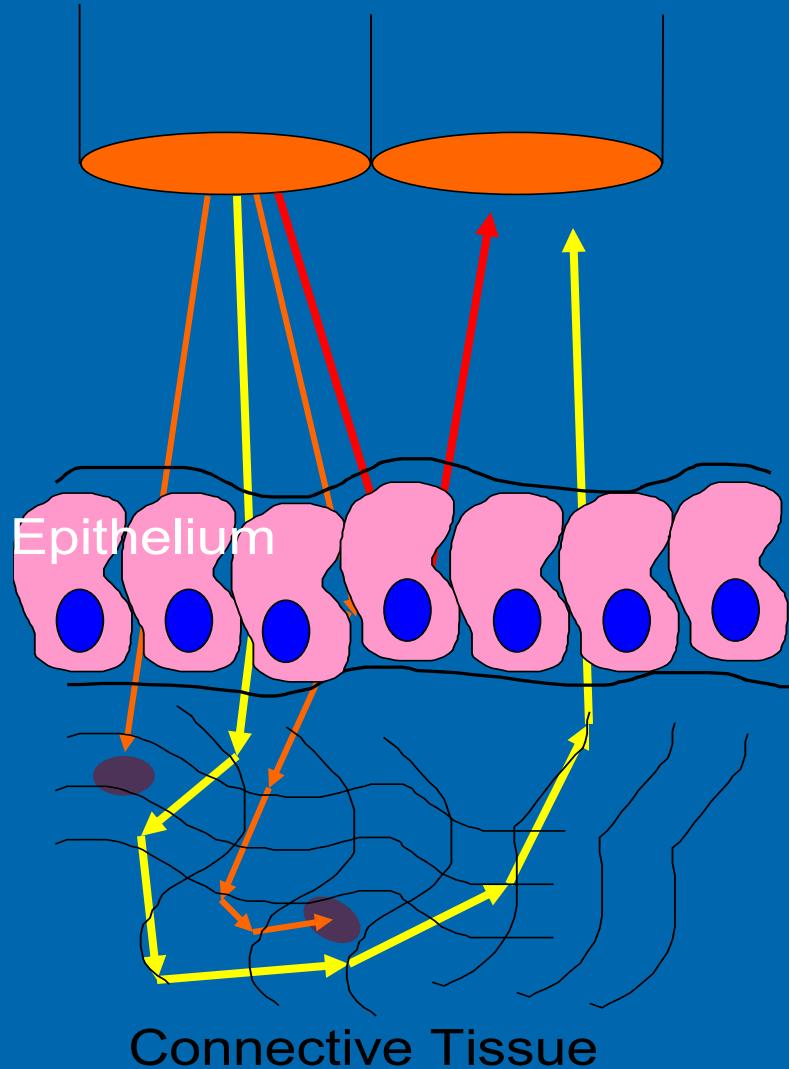
Lecture Outline

1. The Root Reason for 2P Microscopy
2. The Principle.
3. Fluorophore excitation and the 2nd Harmonic
4. Non-Descanning Detection.
5. That's a Mighty Big Laser You've Got There.
6. Resonant versus Galvo-based Scanning.
7. Autofluorescence—the continual scourge.
8. 3P and beyond
9. To the Deep: Adaptive Optics
10. The Labs

1. The Root Reason for 2P Microscopy

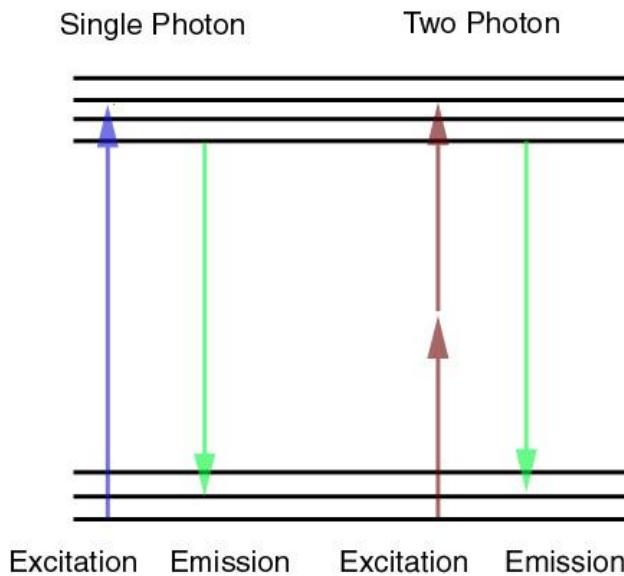
Tissues absorb & scatter light

- scattering
 - elastic scattering
 - multiple scattering
 - single scattering
- absorption

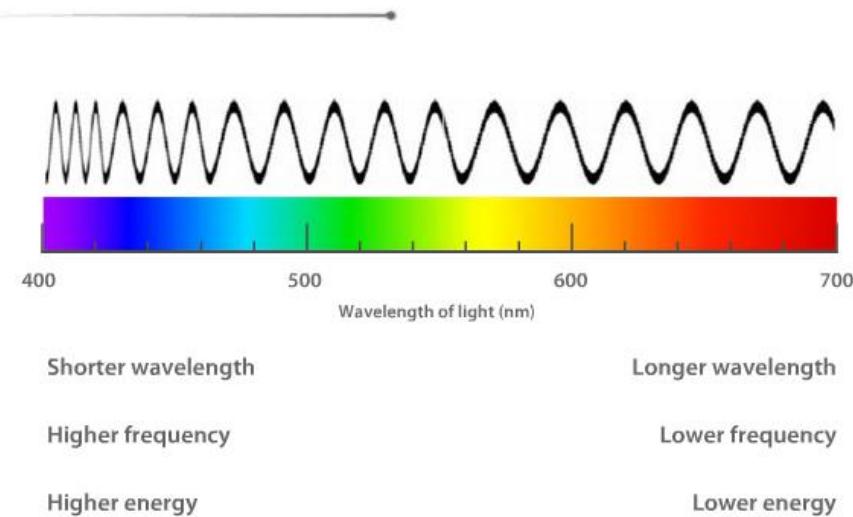


2. The Principle

2-Photon Fluorescence: How and Why?



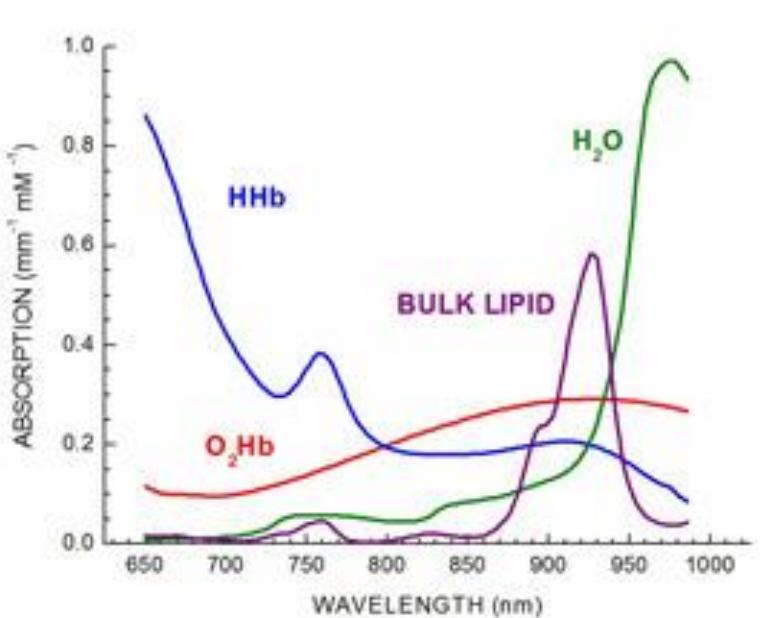
The Visible Light Spectrum



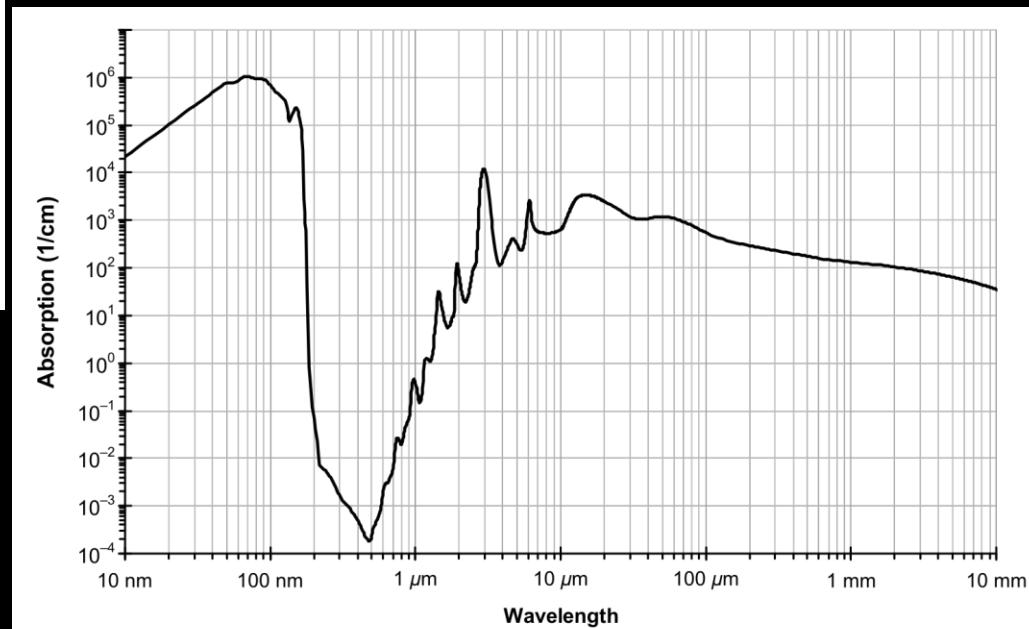
How: Two Photons of half the energy (twice the wavelength), if temporally coincident, sum their energies ($2 \times 0.5 = 1x$) to drive fluorophore to activated state. (NOTE: This requires very high powered, very expensive lasers).

*Why? Thought experiment:
Flashlight/you as kid/ Camping*

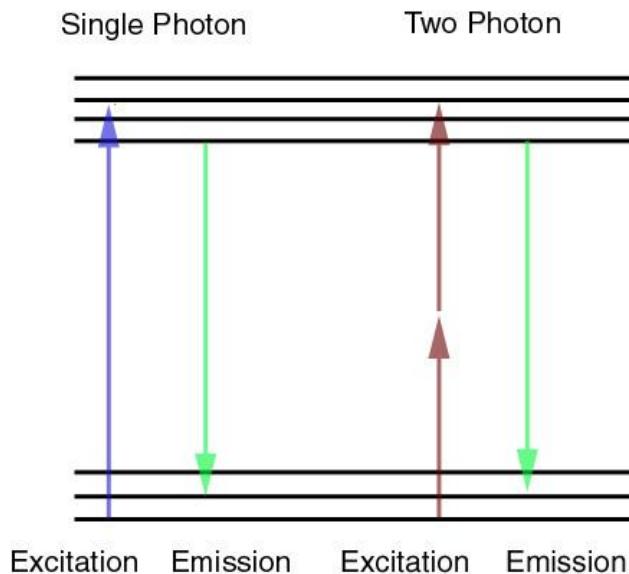
Near IR is a special optical window for imaging with minimal absorption & scatter



*Adipose does
tend to burn up...
crispy!*



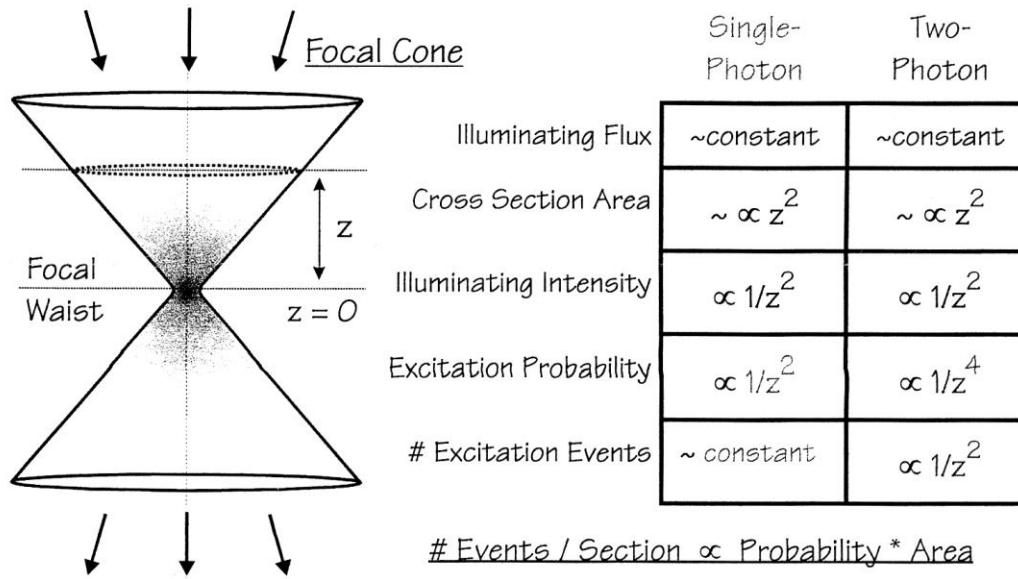
2-Photon Fluorescence: How and Why?



Why: 2 Photons only coincide with high frequency precisely at the focal point.

- No absorption of light except at focal point (since single photons are of a power that is not absorbed by proteins, DNA, water, lipids ~800nm)
- Less Bleaching in out of plane
- Superior Z-axis resolution.

Fluorescence Excitation in Illuminating Focal Cone by Single Photon and Two-Photon Processes

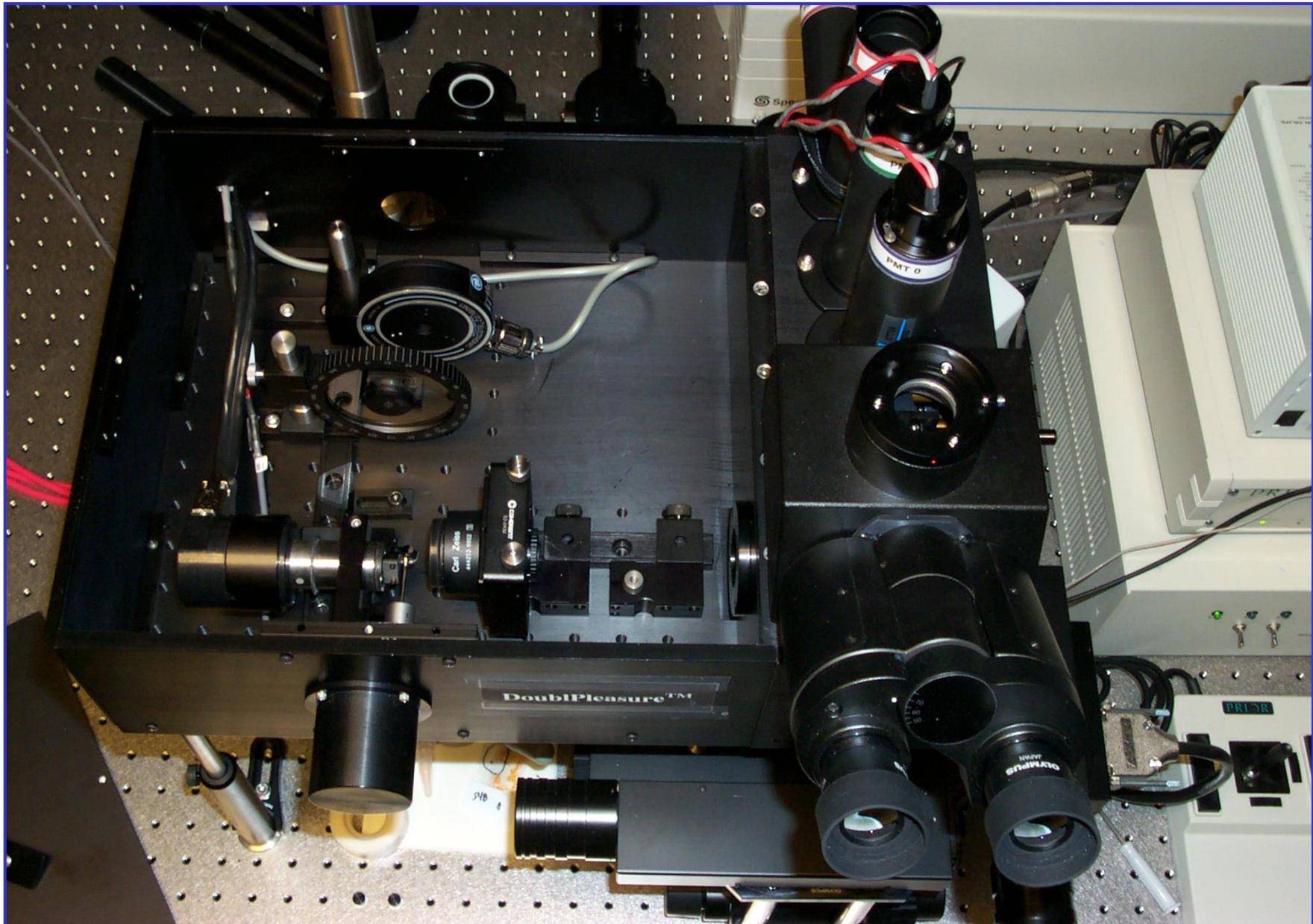


Single-Photon (Confocal) vs. Two-Photon Imaging:

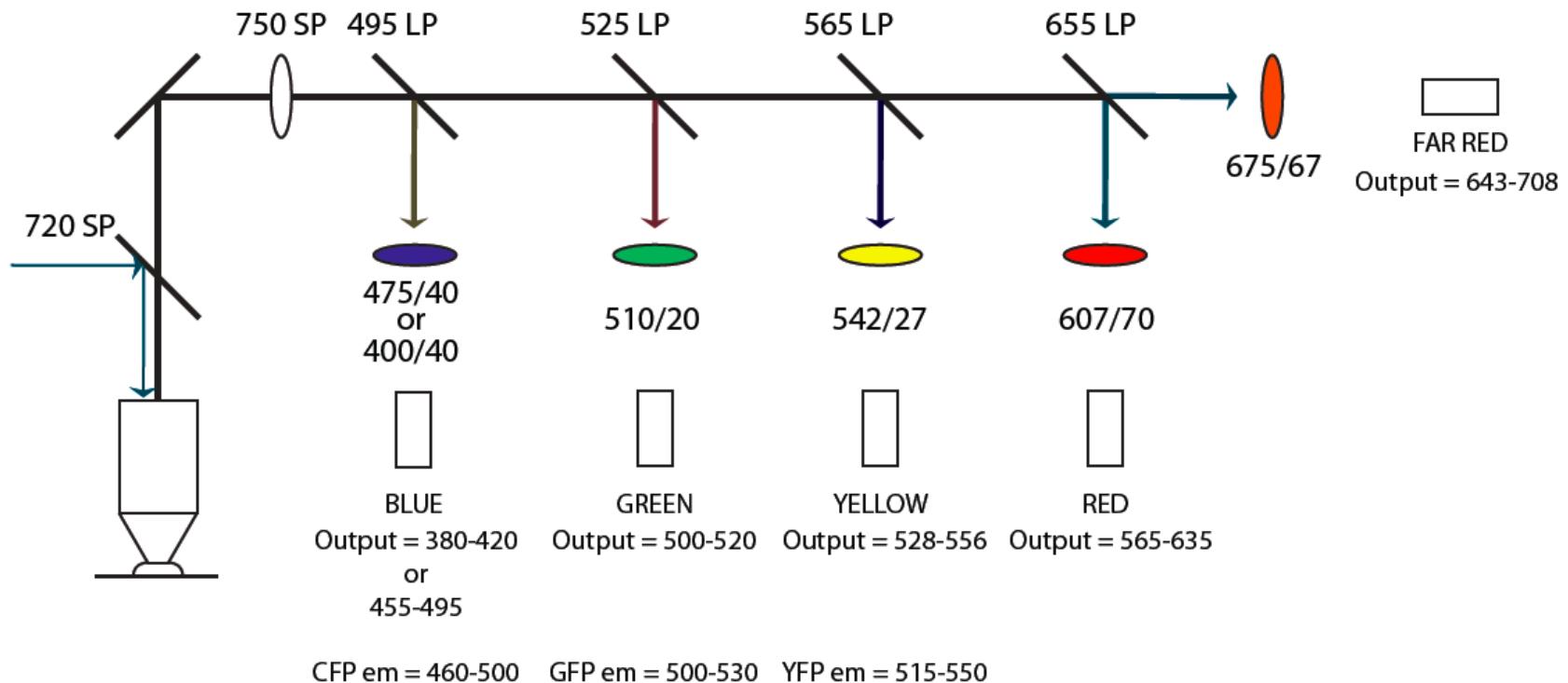
Single-Photon process produces excitation (and photodamage) throughout specimen volume. Optical sectioning requires imaging and spatial filtration of emission light, which is inherently inefficient and is highly susceptible to light scattering losses.

Two-Photon excitation is restricted to small focal volume near $z = 0$. Resolution and optical sectioning are thus inherent in excitation process. All emission photons usable for imaging! High efficiency not diminished by light scattering in thick or turbid specimens..

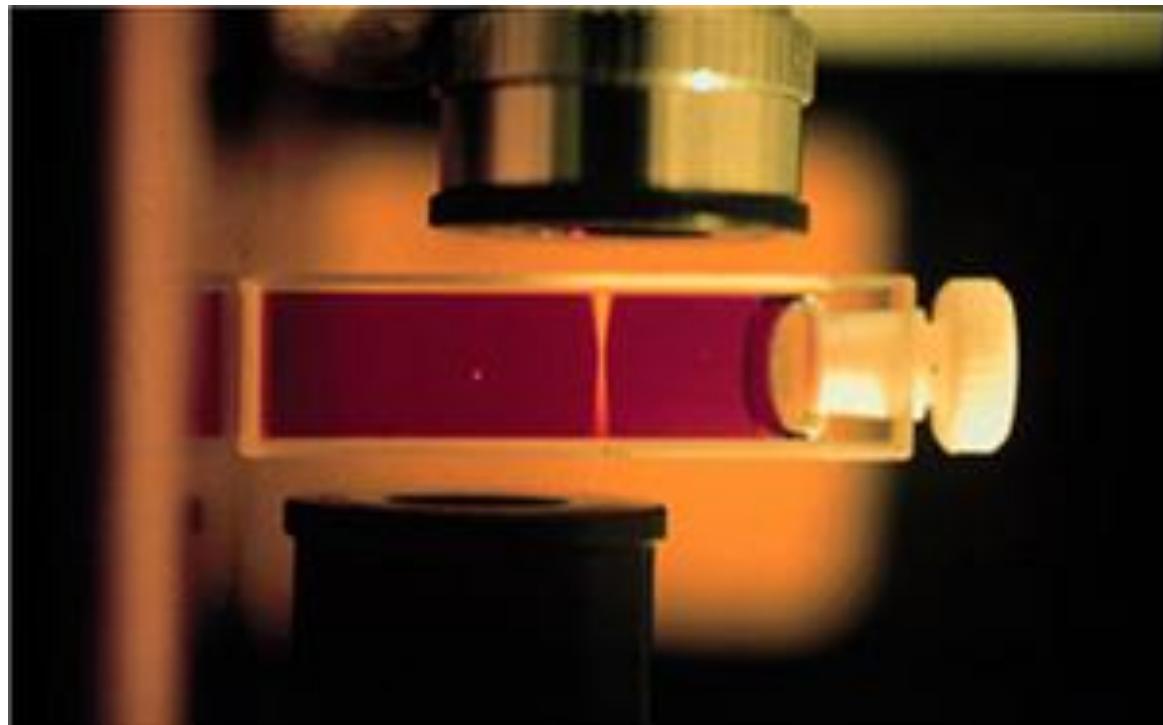
This can be done quite simply...



*Output path—many channels (colors)
collected simultaneously...*



Multi-photon Microscopy: Example of Precision of Excitation



3. Fluorophore excitation and the 2nd Harmonic

Excitation is not exactly 2x 1P λ

Somewhat unpredictable

But broad, meaning many fluors can be hit by one λ

Table 1. Selected applications of fluorescent probes for two-photon excitation (TPE) microscopy

Probe	TPE Excitation Wavelength	Application	References
Alexa Fluor 488 phalloidin	720 nm or 830 nm	Imaging F-actin organization in pancreatic acinar cells	<i>J Biol Chem</i> (2004) 279:37544–37550
Alexa Fluor 594 hydrazide	810 nm	Ca ²⁺ -insensitive, neuronal tracer *	<i>Neuron</i> (2002) 33: 439–452; www.stke.org/cgi/content/full/sigtrans;2004/219/pl5
Amplex Red reagent	750 nm or 800 nm	Detection of reactive oxygen species (ROS) associated with amyloid plaques	<i>J Neurosci</i> (2003) 23:2212–2217
CFSE, CMTMR	820 nm	Tracking T and B lymphocytes and dendritic cell motility patterns in intact mouse lymph nodes †	<i>Science</i> (2002) 296: 1869–1873; <i>Proc Natl Acad Sci U S A</i> (2004) 101: 998–1003
CM-H ₂ DCFDA	740 nm	Detection of localized reactive oxygen species release in cardiomyocytes ‡	<i>J Biol Chem</i> (2003) 278: 44735–44744
DAPI, Hoechst 33342	740 nm	Imaging DNA in nuclei and isolated chromosomes	<i>Micron</i> (2001) 32:679–684; <i>Histochem Cell Biol</i> (2000) 114:337–345
DiD	817 nm	Intravital imaging of mouse erythrocytes	<i>Proc Natl Acad Sci U S A</i> (2005) 102:16807–16812
FM 1-43	840 nm	Monitoring synaptic vesicle recycling in rat brain slices	<i>Biotechniques</i> (2006) 40:343–349
Fluo-5F §	810 nm	Imaging Ca ²⁺ concentration dynamics in dendrites and dendritic spines	<i>Neuron</i> (2002) 33:439–452; www.stke.org/cgi/content/full/sigtrans;2004/219/pl5
Fura-2	780 nm	Detection of GABA-mediated Ca ²⁺ transients in rat cerebellar Purkinje neurons	<i>J Physiol</i> (2001) 536:429–437

Peaks

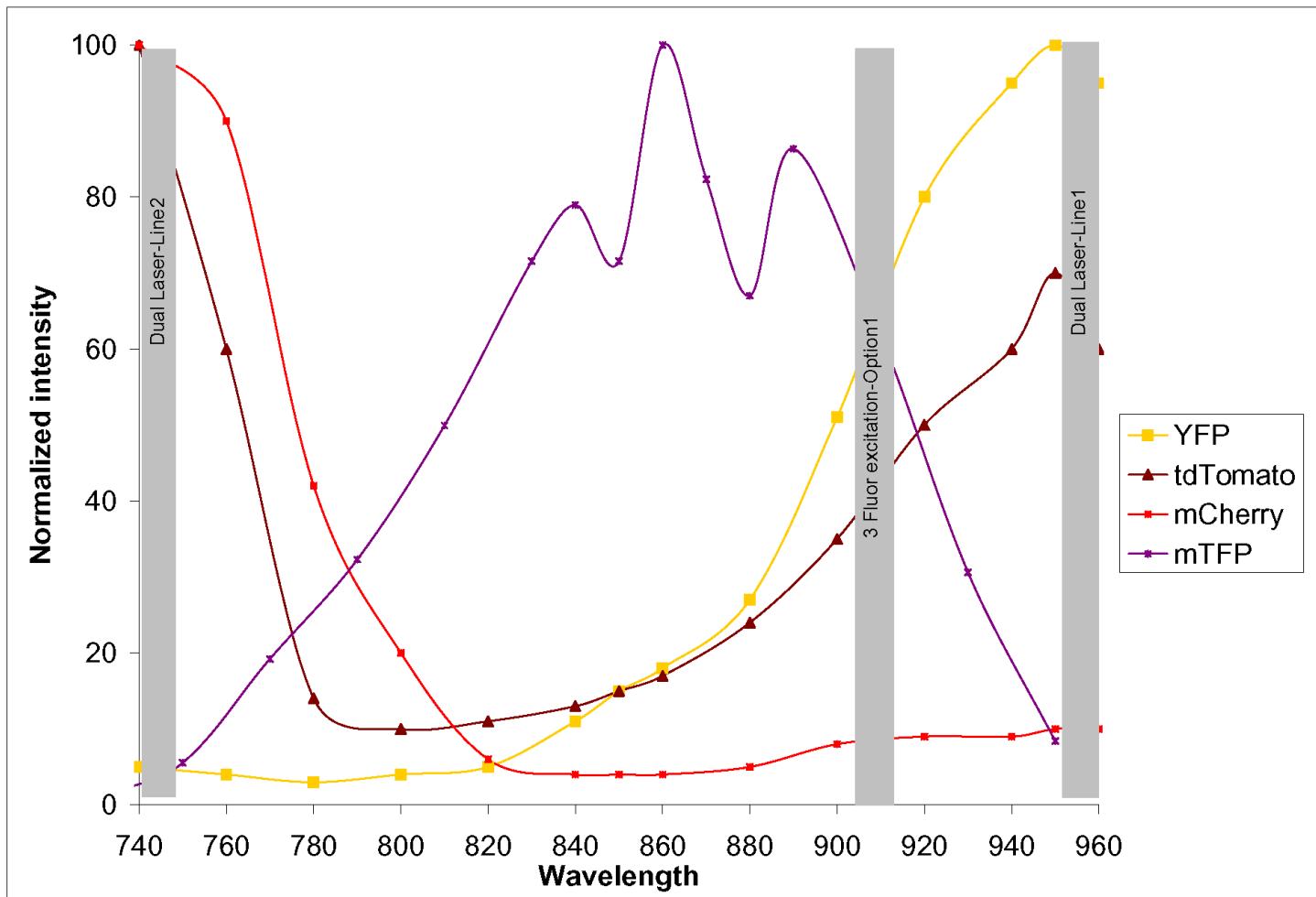
Excitation is not exactly $2 \times 1P \lambda$

Somewhat unpredictable

But broad, meaning many fluors can be hit by one λ

Note: Reds in the plot below... Also (not shown) GFP is ~900, FITC is ~800

Broad



2nd Harmonic Signal: Fiducials for ‘Free’

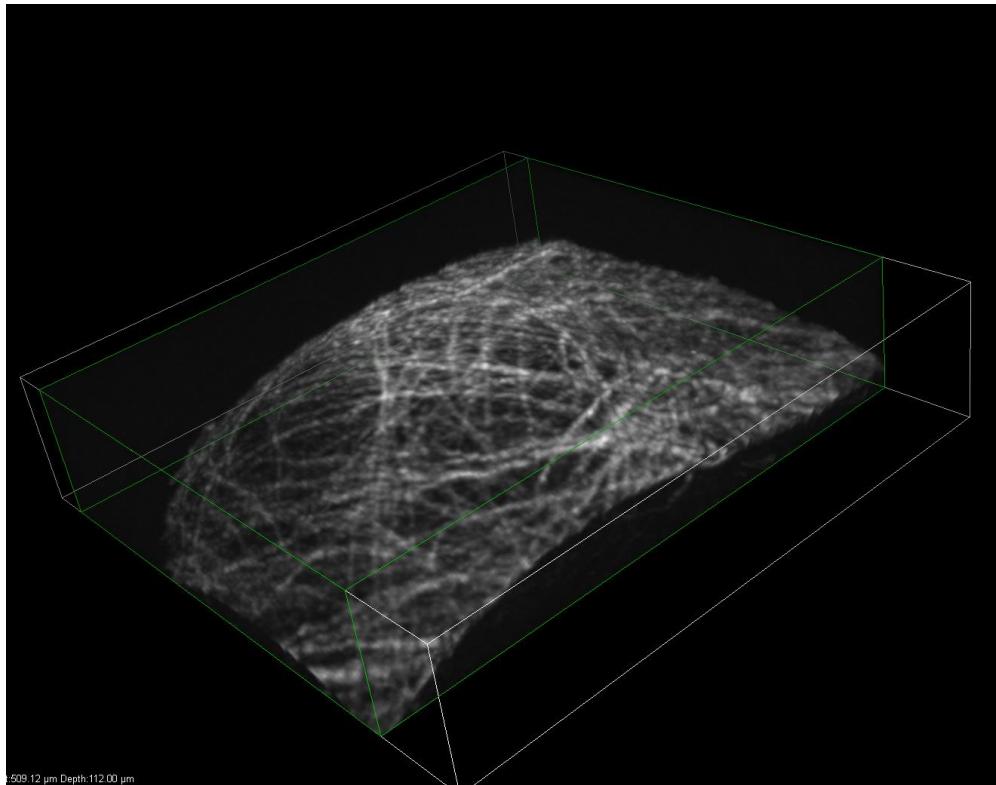
Some anisotropic molecules like collagen will frequency-double light and thereby generate an emission with $\frac{1}{2}$ of the incident λ

Collagen I and IV

Cellulose

Some membrane dyes

A non-linear effect of increasing laser excitation (“non-linear optics”)



2nd Harmonic Signal: Fiducials for ‘Free’

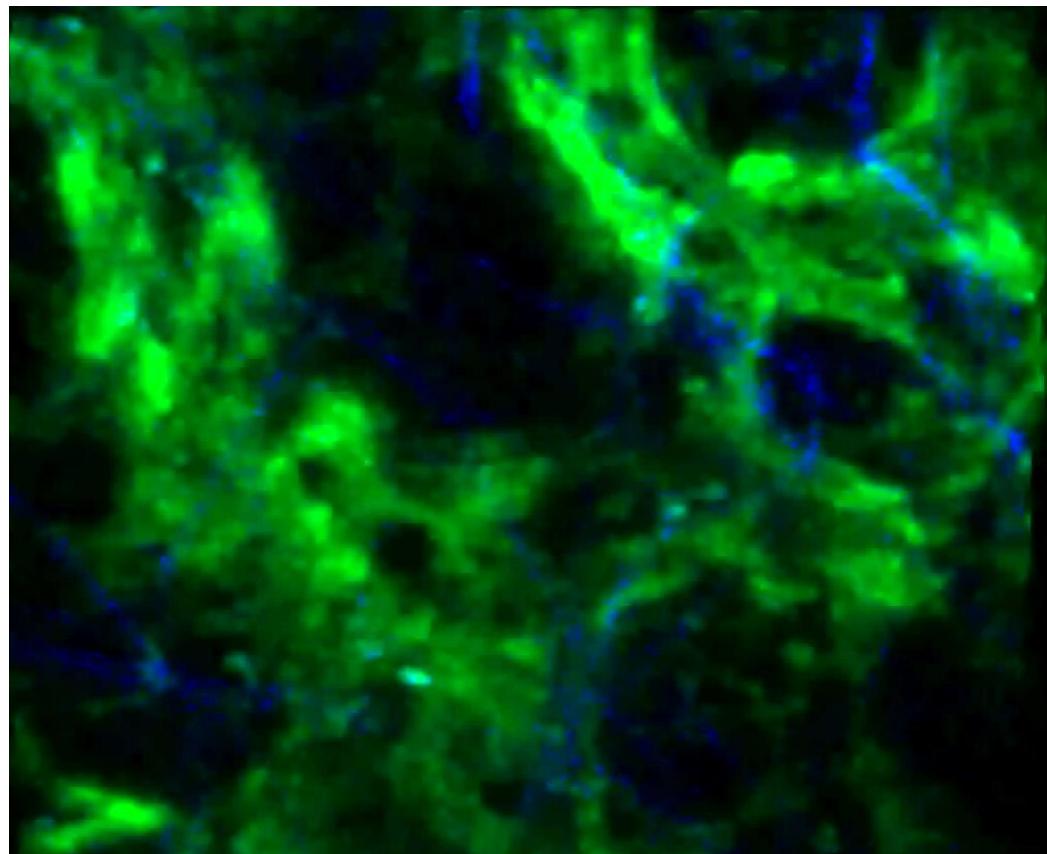
Some anisotropic molecules like collagen will frequency-double light and thereby generate an emission with $\frac{1}{2}$ of the incident λ

Collagen I and IV

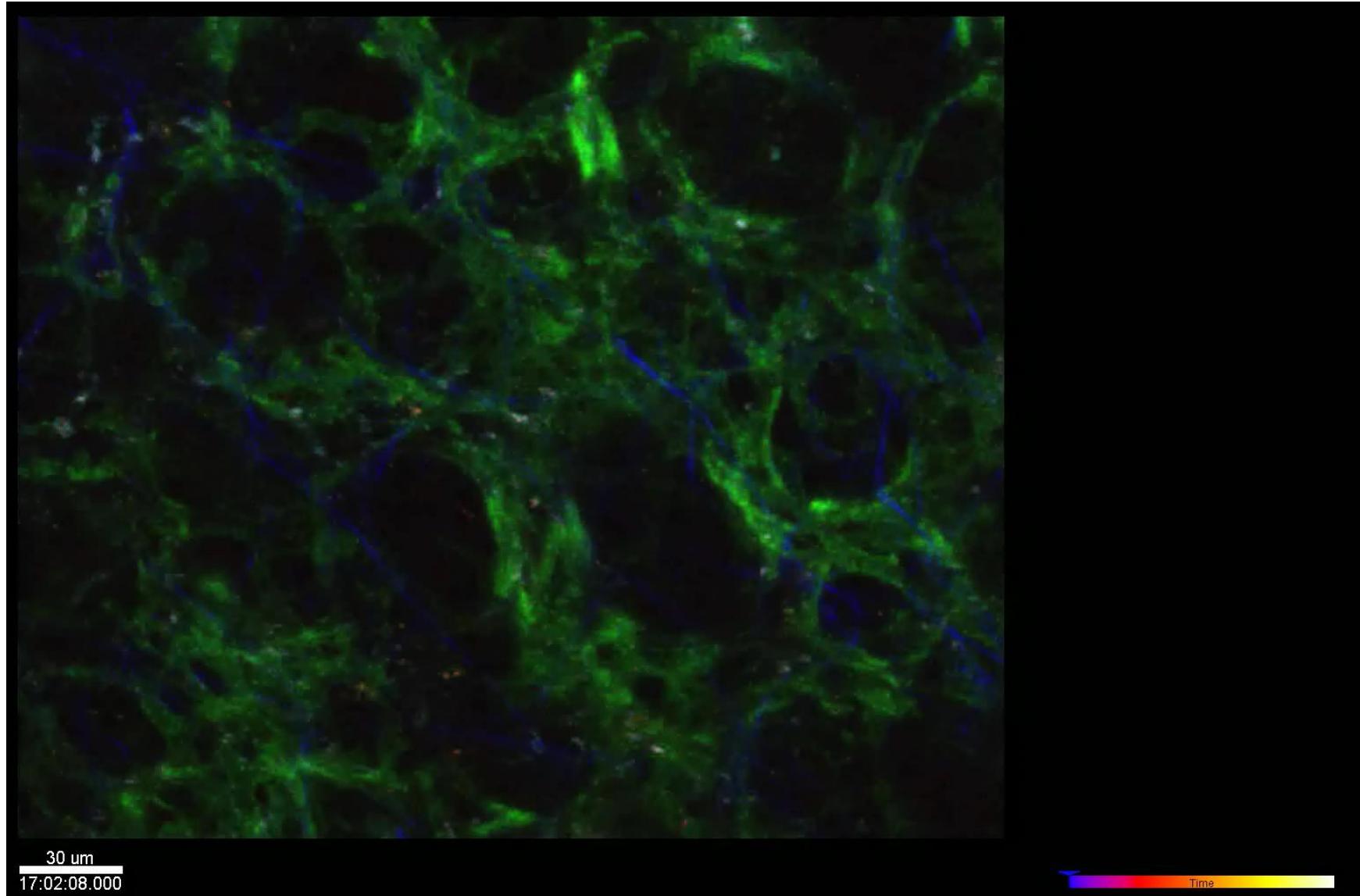
Cellulose

Some membrane dyes

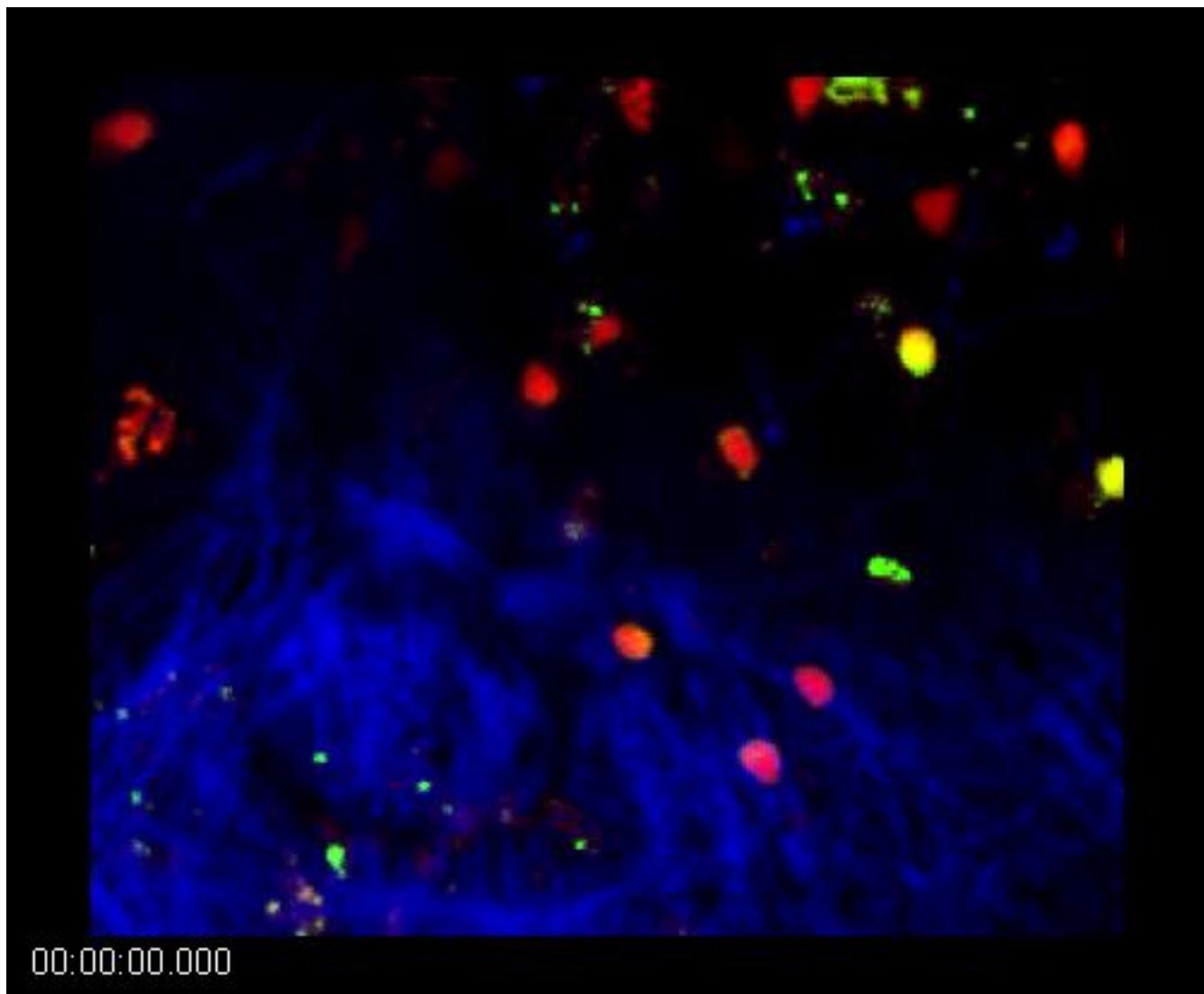
*A non-linear effect of increasing laser excitation
 (“non-linear optics”)*



2nd Harmonic Signal: Fiducials for ‘Free’

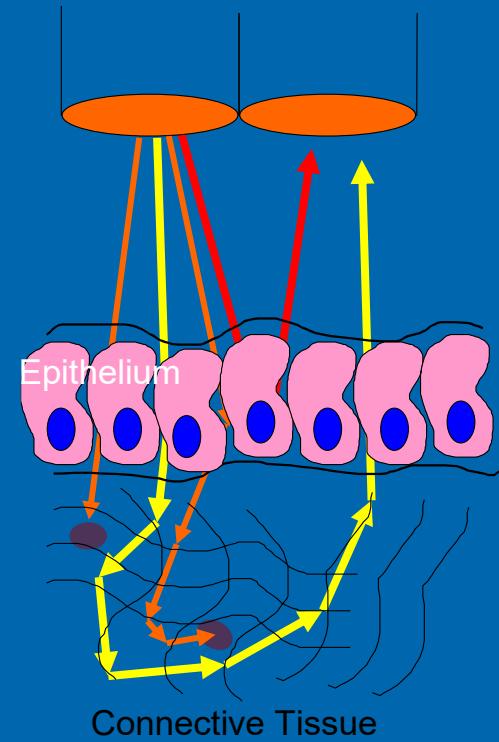


2nd Harmonic: LN Capsule



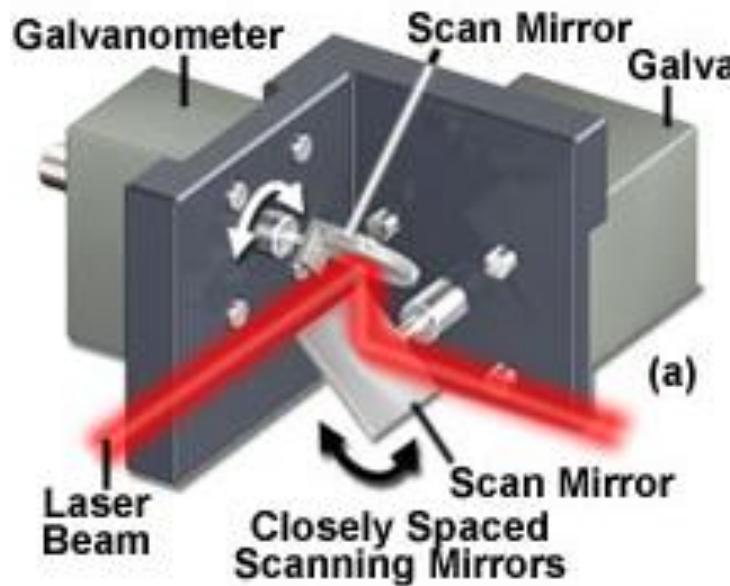
Returning to the issue: Tissues absorb & scatter light

Should a sensitive 2-
Photon Collect Without
a Pinhole?

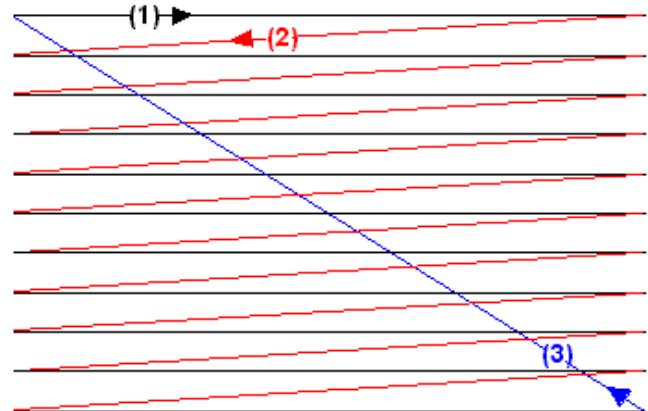


Recall that this is achieved via:
The Raster Scan.

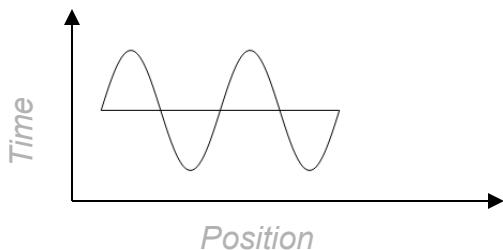
This allows temporal assignment of all light coming from an excited spot.



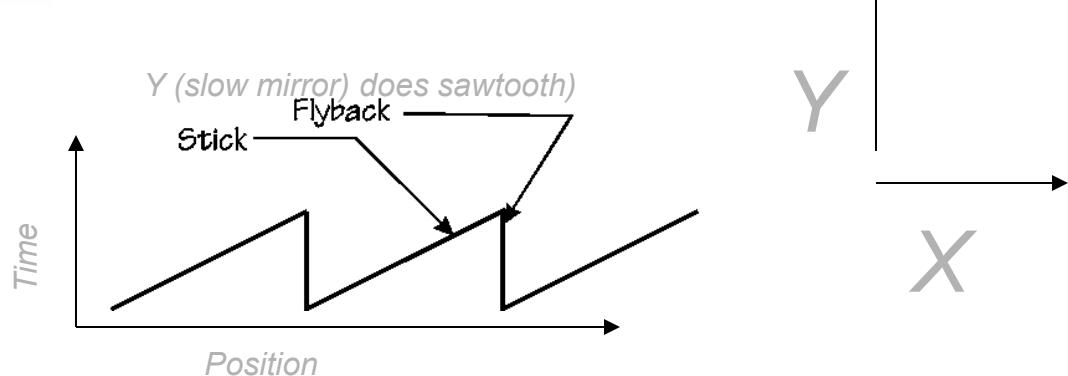
*A small spot (laser beam)
Scans the sample*



X(fast mirror) follows a sine curve

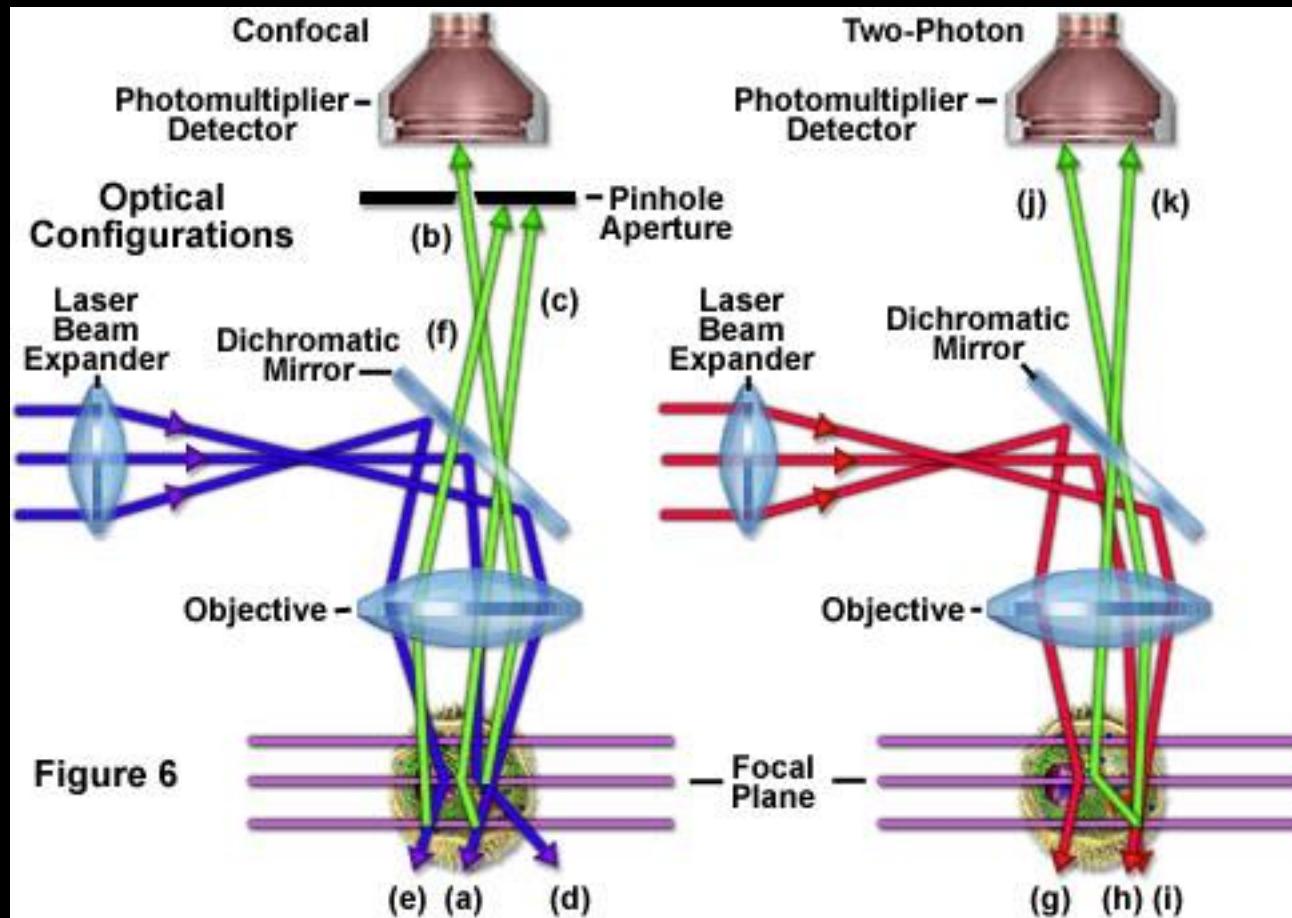


Y (slow mirror) does sawtooth

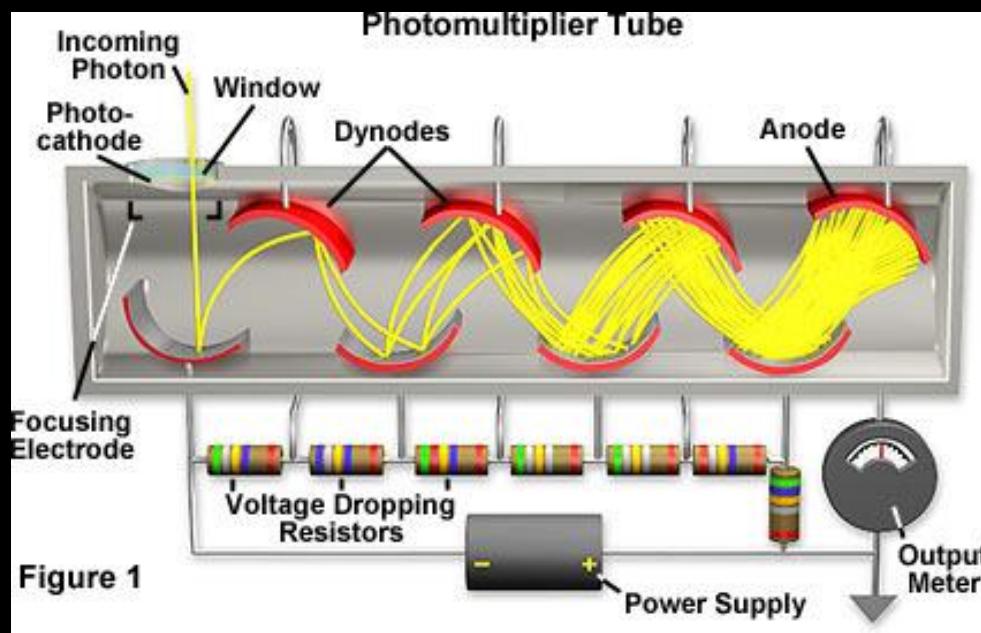
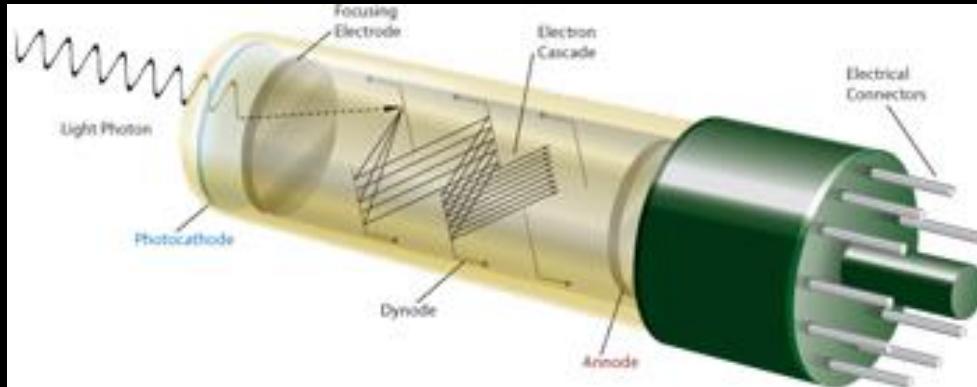


Ergo: Collect All Emission at detector (regardless of path)

The Most sensitive 2P microscopy employs non-descanned detectors



2P scopes use PMTs as detectors



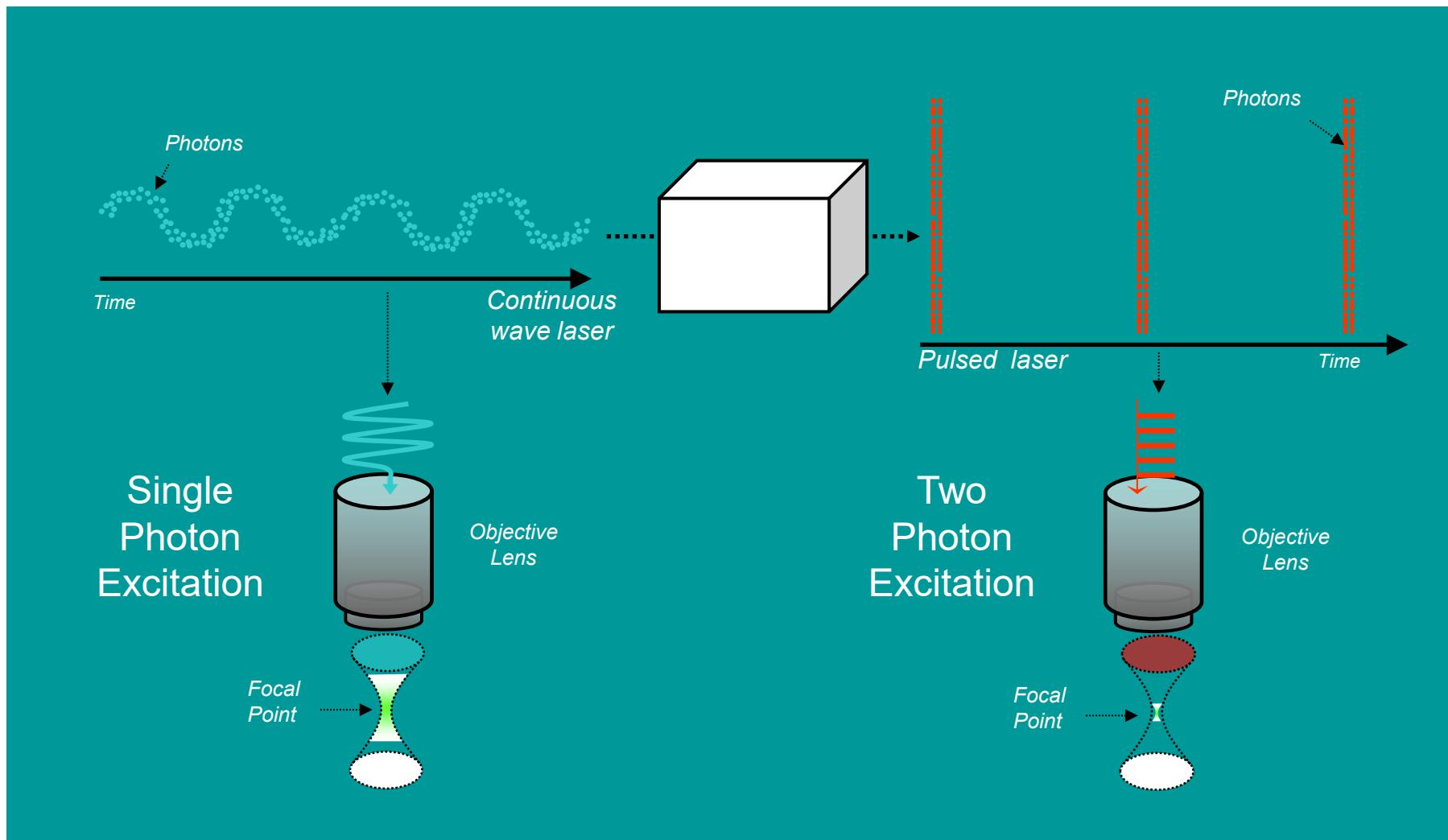
Extra Credit:

“But”, you say, “We know CCDs have better QE and less noisy signal gain. “

Why don’t we use them?

5. That's a Mighty Big Laser You've Got There.

2P excit. requires concentration of photons in space & time



Tell me about your laser?

A Soldering Iron typically consumes/concentrates 25W of power to an area approx. 2mm in diameter giving a power density of ~8W/mm²

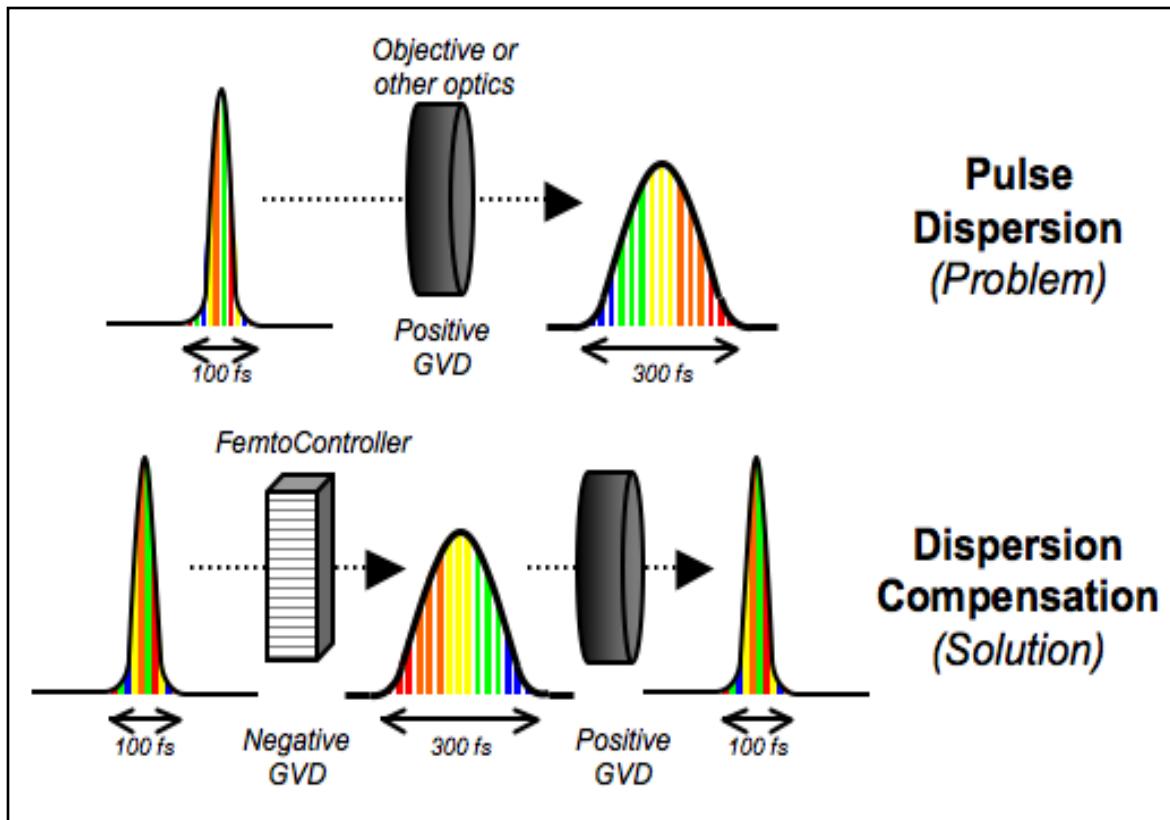
A 2P laser is pumped by a 5-20W pump resulting up to 1W of pulsed power at the objective which is focused to a spot size of ~0.5um diameter. This is a power density of ~5 MW/mm²

*However it has a duty cycle of ~10⁻⁵ (the ‘duty cycle’ is pulselwidth x rep rate). The pulselwidths are around 70-200fs (fs = 10⁻¹⁵s) and the rep-rate is around 80-100MHz) If this were on ‘continuous’ it would consume **100kW**, equating to consumption of about 100 homes worth of power. More impressive, it would be putting out **5x10¹¹W/mm²** or ~10¹¹x the power density compared to a soldering iron.*

Current commercial models of Ti-sapphire lasers cover the wavelength range of 720 to 980 nanometers through easily implemented computer control. Switching typically takes around 2-5sec although newer lasers do this sub-second. Further improvement in the ease of use and versatility of laser illumination systems is likely to continue in the foreseeable future and wavelengths to 1080 are achievable (>1200nm with an optical parametric oscillator (OPO)

Cost is \$100-250K (So commercial scopes are ~\$400-750K)

Dispersion Compensation ("Pre-chirping") To Minimize Pulsewidth



These can be bundled in the laser:

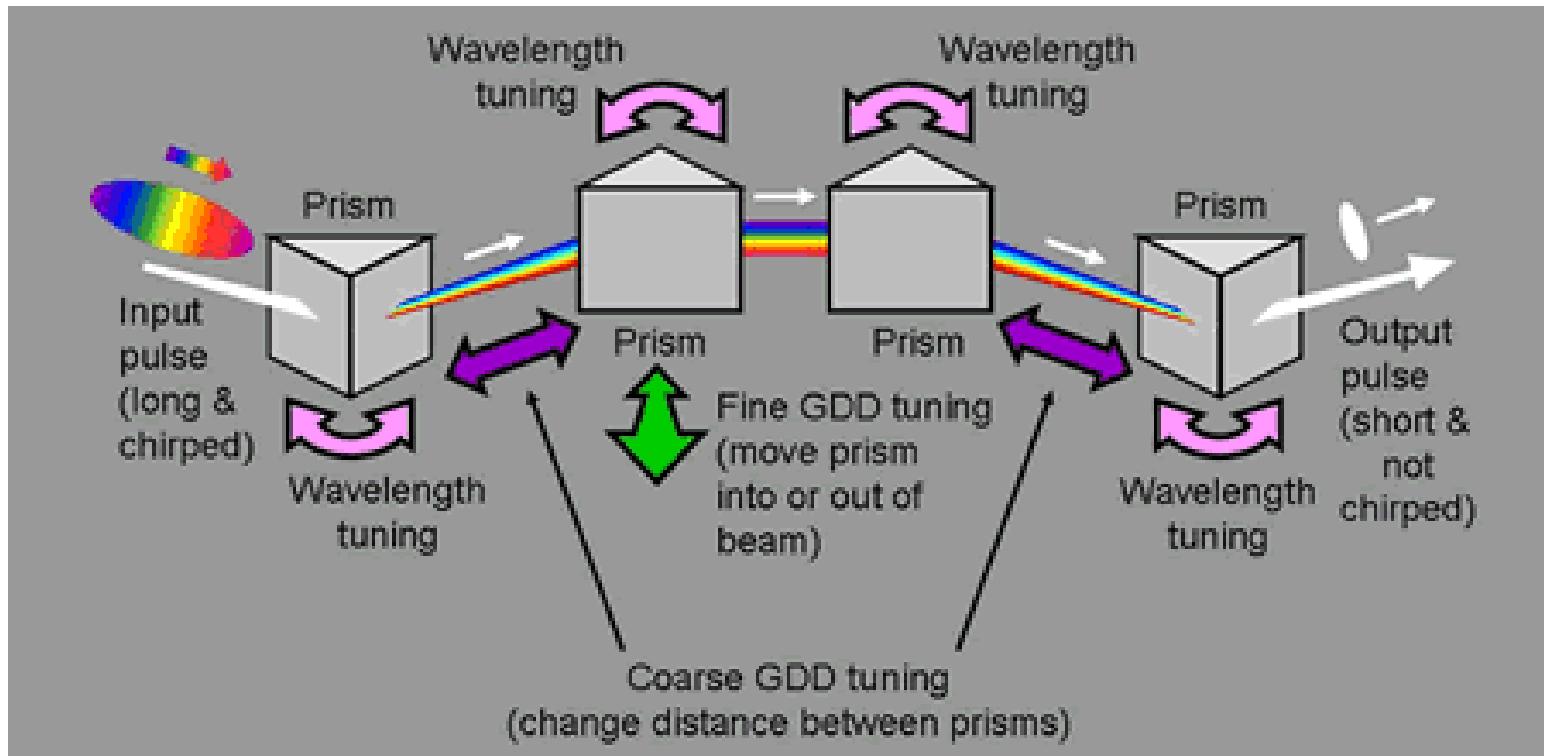
e.g.
S/P MaiTai 'DeepSee'
Chameleon 'Vision II'

Or this can be added in the beampath:

e.g.
FemtoController (APE)

AOTF are particularly bad for this.

Dispersion Compensation ("Pre-chirping") To Minimize Pulsewidth



6. Resonant versus Galvo-based Scanning.

Resonant versus Galvo based scanning.

A resonant scanning system oscillates back/forth and can achieve faster frame rates whereas a Galvo based system can be programmed to move in many types of increments but generally can't be driven hard/fast for long without overheating.

Since pulses are so bright, the pixel dwell time can be important.

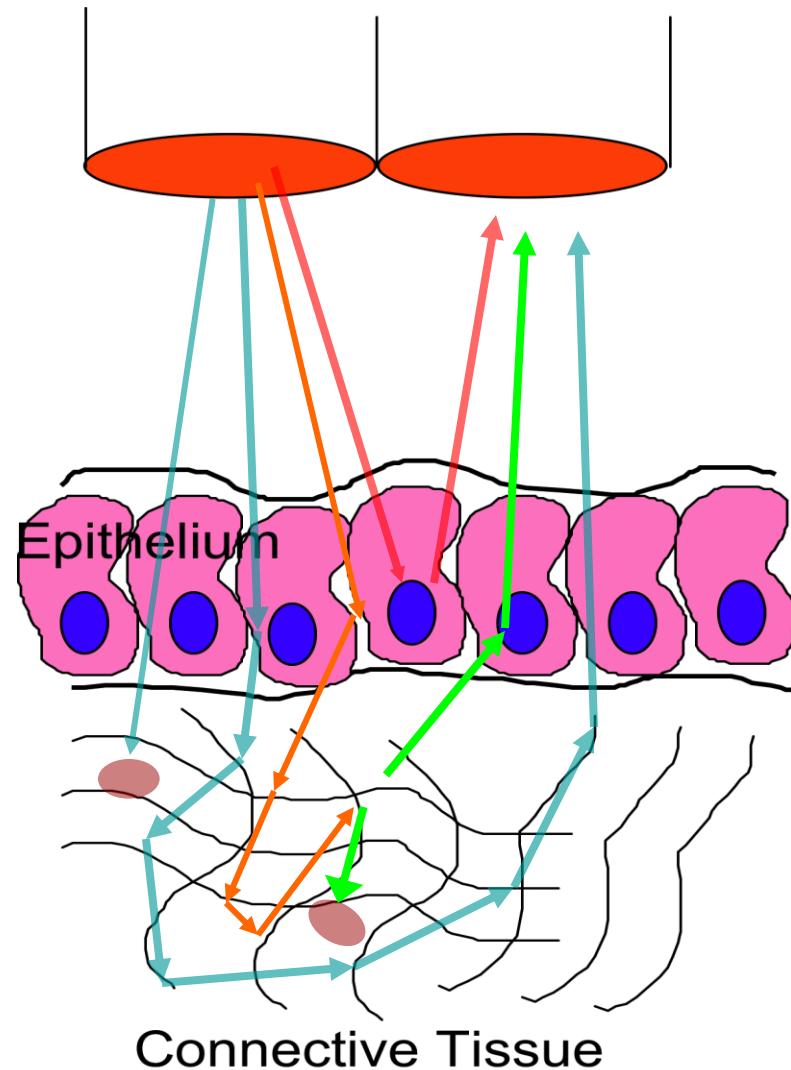
Molecules like GFP can bleach if illuminated too closely in succession, when in the relaxed state post-activation

In a resonant scanning system we built (30fps at , a 0.6um pixel integrates about 7 pulses ‘on the fly’ (as the laser sweeps through this voxel which takes about 1us). We then may integrate 10 frames (sweeps) that each give the sample 1/30th of a second to ‘recover’.

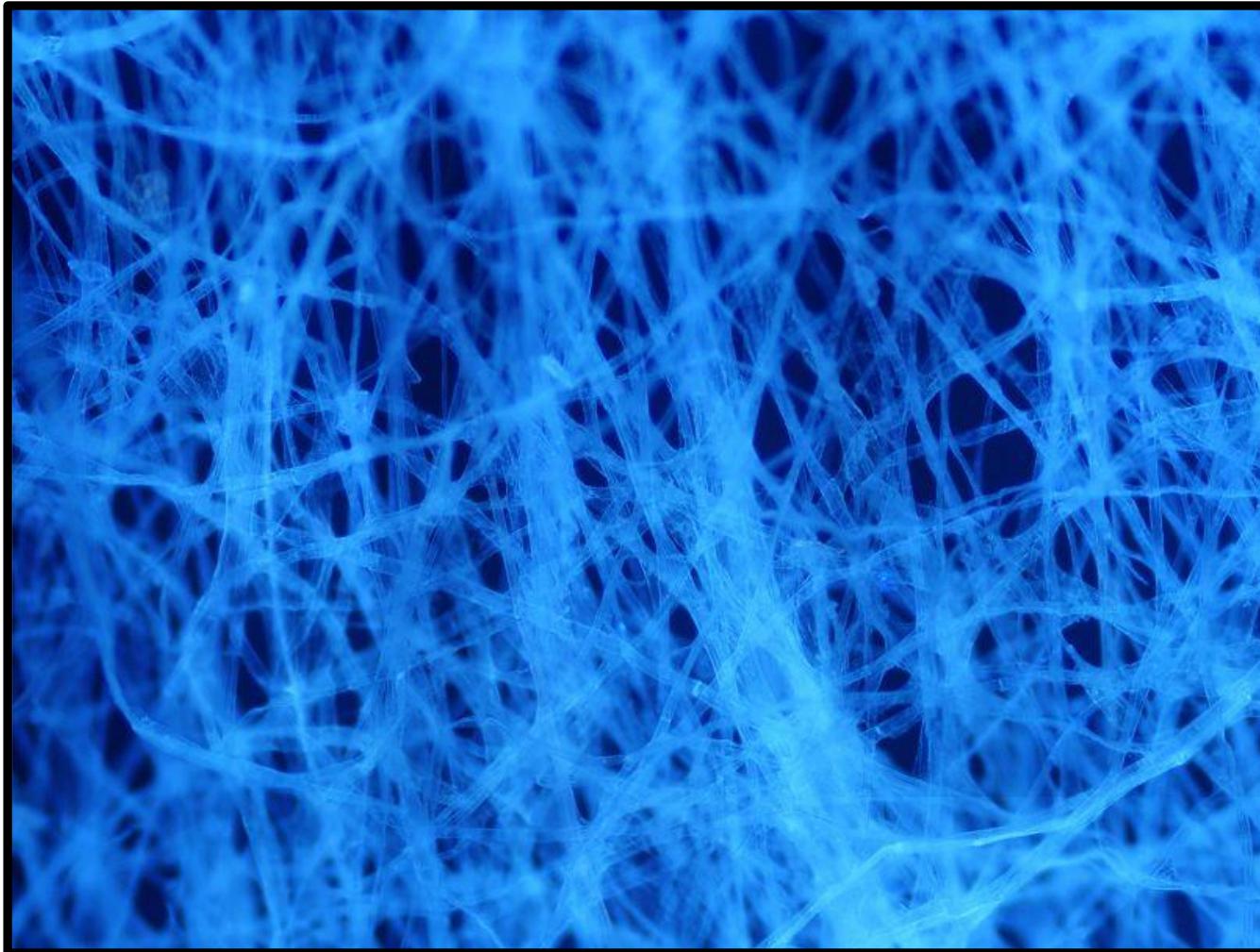
In a galvo system, you may also have a ‘dwell time’ of 1us but may be tempted to dwell longer to achieve the same S/N. →Bleaching? (You guys may test this).

Recall Tissues are Auto-fluorescent

- scattering
 - elastic scattering
 - multiple scattering
 - single scattering
- absorption
- fluorescence

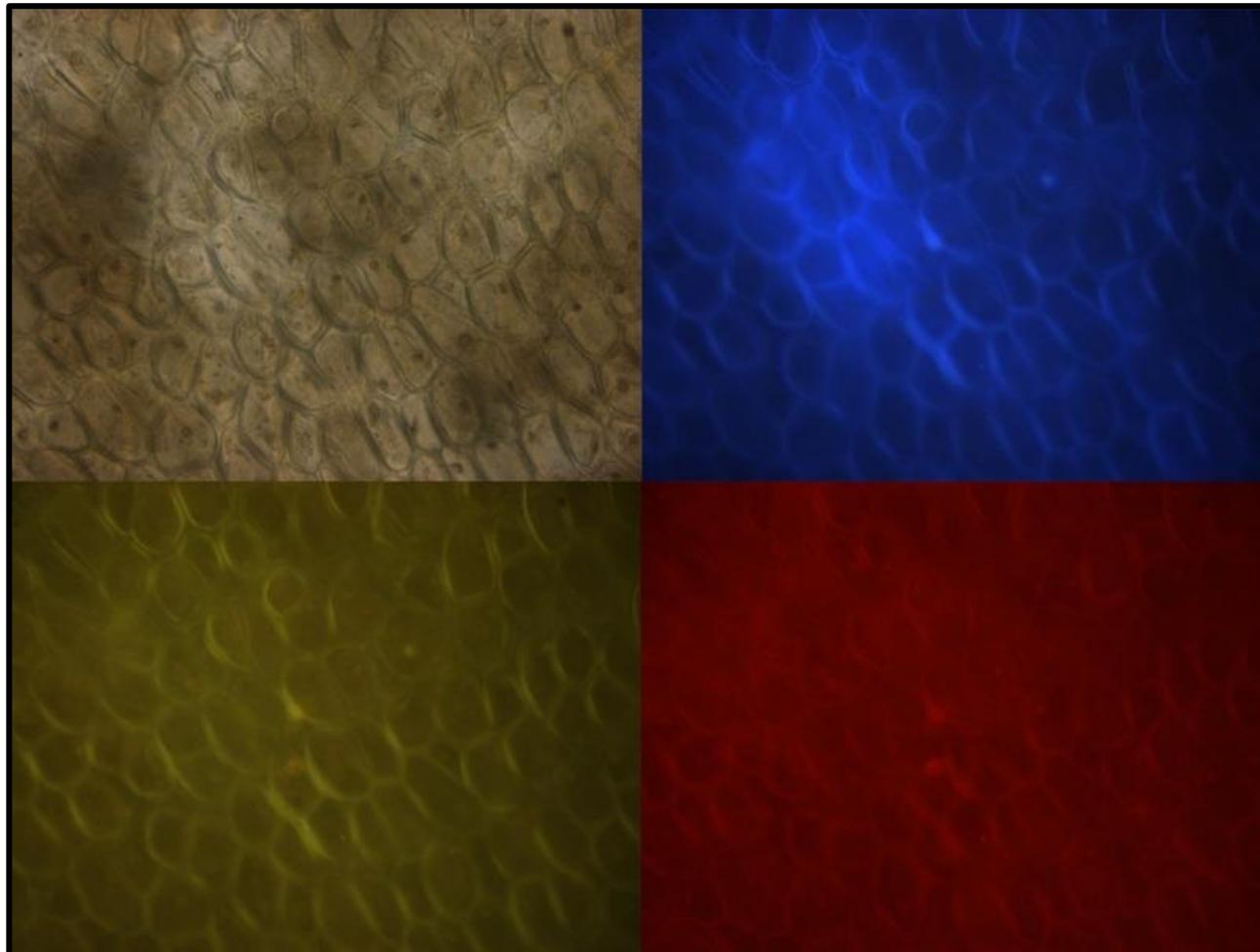


Autofluorescence is, of course
'natural' or 'native' fluorescence



Paper Autofluorescence (Wikipedia)

Autofluorescence is, of course
'natural' or 'native' fluorescence



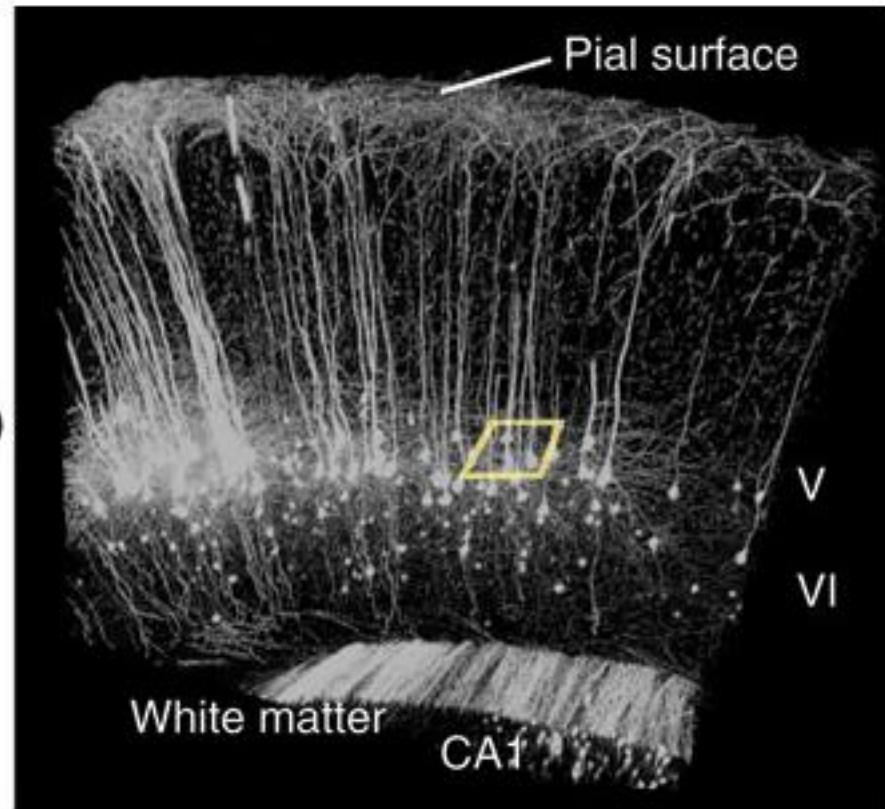
Bananas Autofluorescence (Wikipedia)

Autofluorescence

Autofluorescent molecules

Molecule	Excitation (nm)	Fluorescence (nm)	Organisms	Reference
NAD(P)H	260	450	All	[6]
Chlorophyll	465, 665	673, 726	Plants	
Collagen	270-370	305-450	All	[6]
Retinol		500	Animals & bacteria	[7]
Riboflavin		550	All	[7]
Cholecalciferol		380-460	Animals	[7]
Folic acid		450	All	[7]
Pyridoxine		400	All	[7]
Tyrosine	270	305	All	[2]
Dityrosine	325	400	Animals	[2]
Excimer-like aggregate	270	360	Animals	collagen [2]
Glycation adduct	370	450	Animals	[2]
Indolamine			Animals	
Lipofuscin	410-470	500-695	Eukaryotes	[8]
Polyphenol			Plants	
Tryptophan	280	300-350	All	
Melanin	340–400	360–560	Animals	[9]

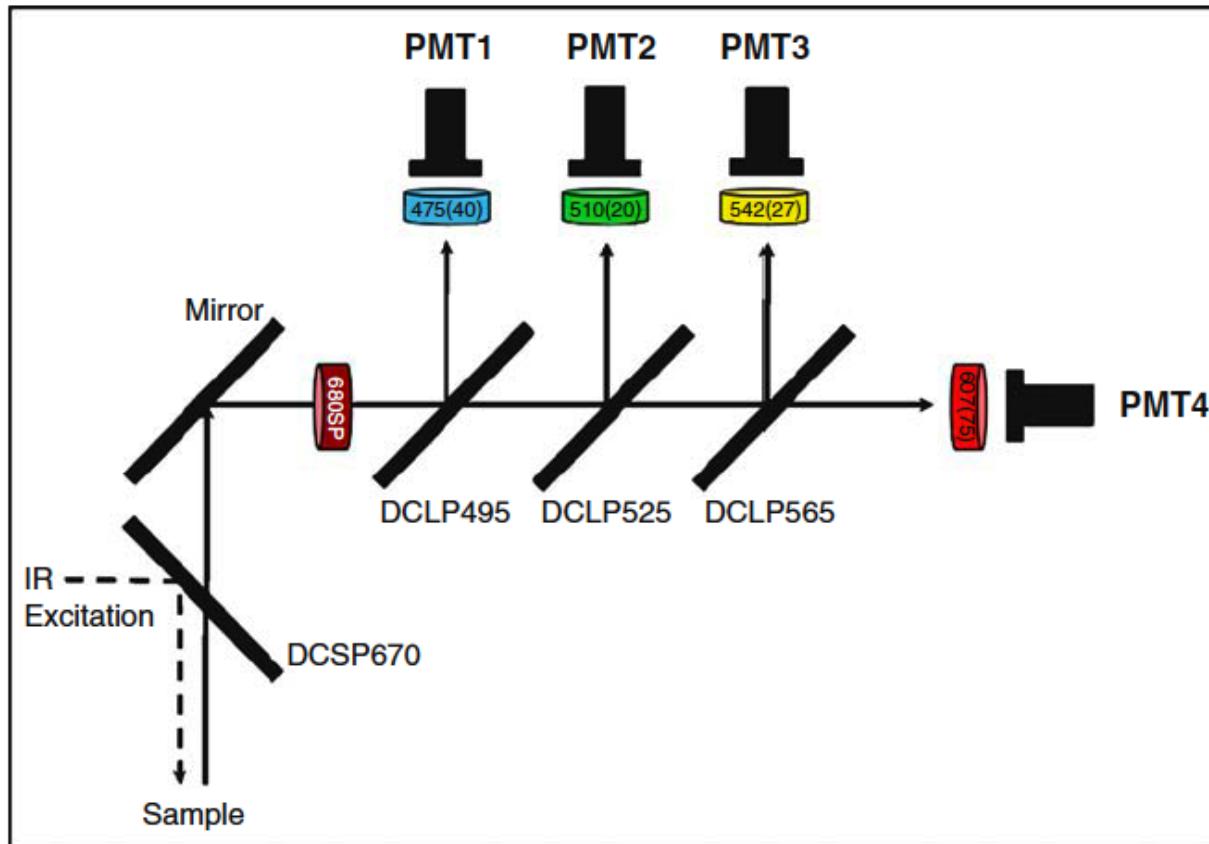
Some autofluorescence and scatter can be ‘cleared’



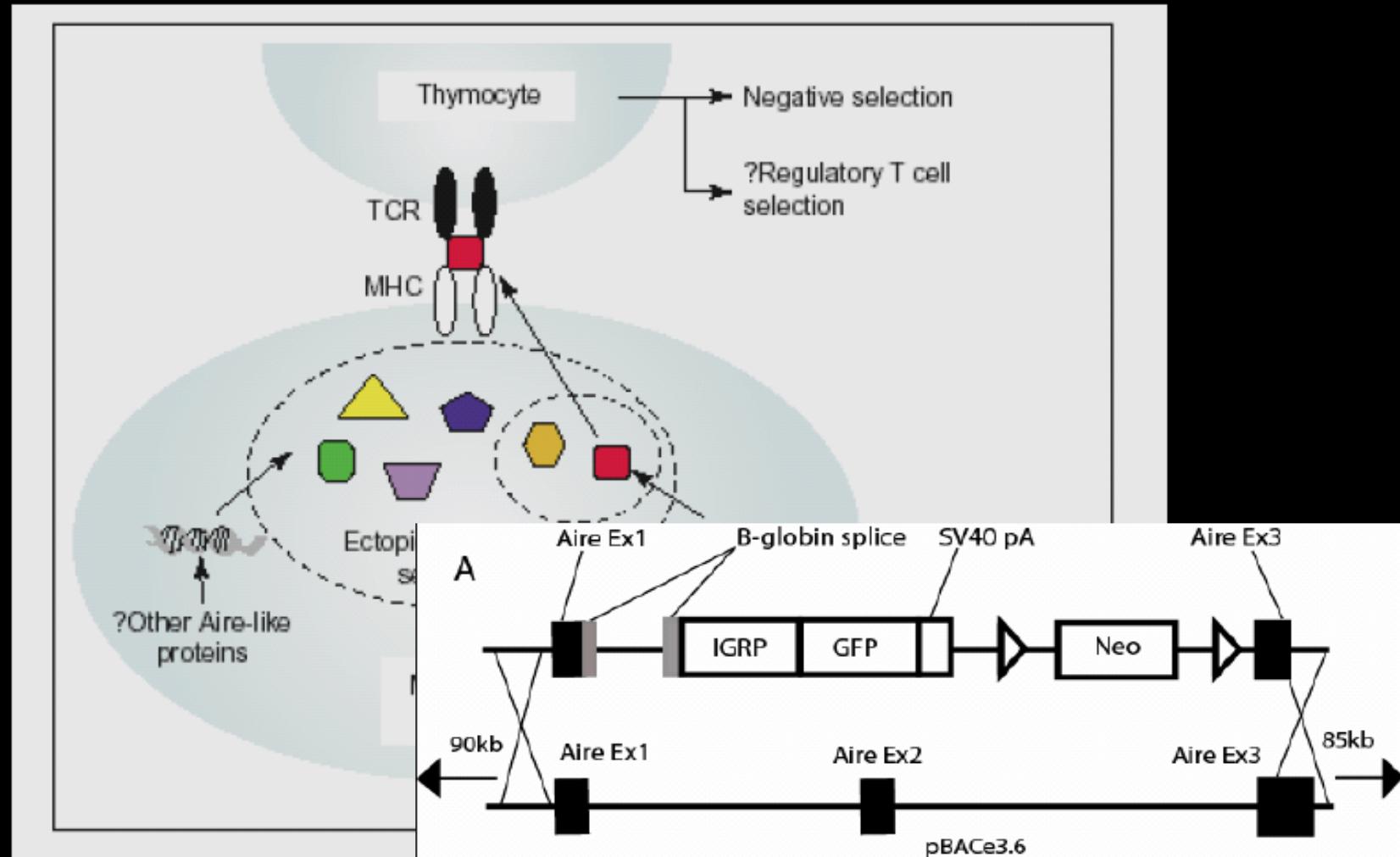
Clearing Agent should leave fluorophore intact

Collecting lots of channels can help with autofluorescence.

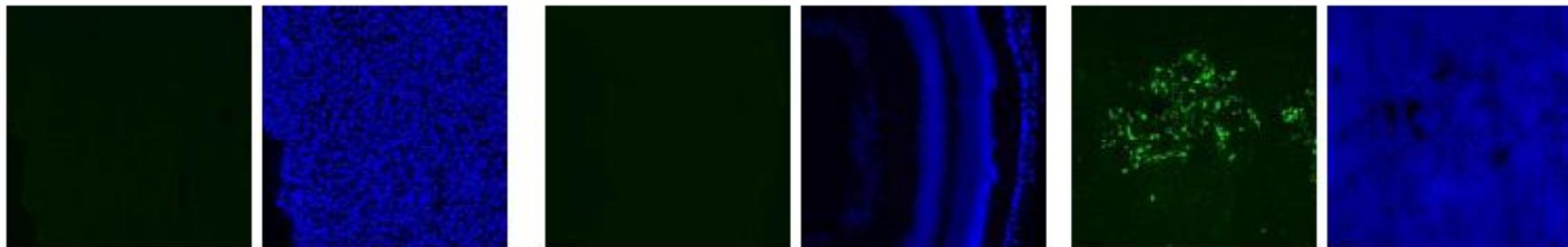
GFP Autofluorescence



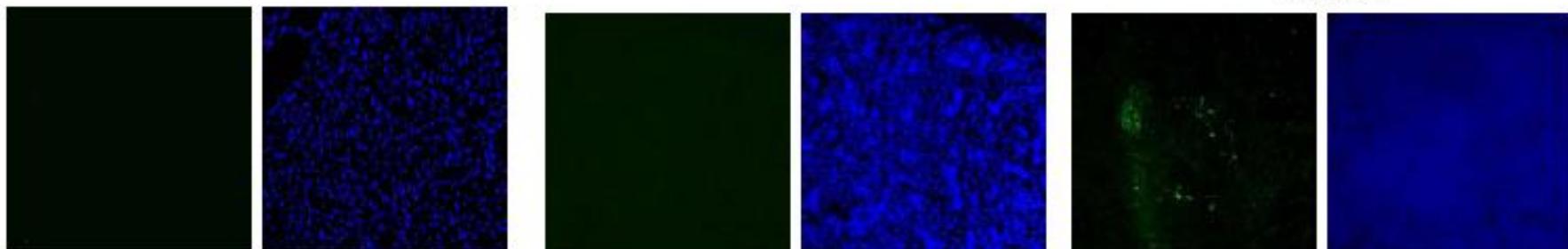
Imaging AIRE-expressing 'Adig' mice to study peripheral tolerance.



Extrathymic IGRP-GFP+ Cells Subsequently Detected in Tissue Survey



liver



retina

thymus

heart

salivary

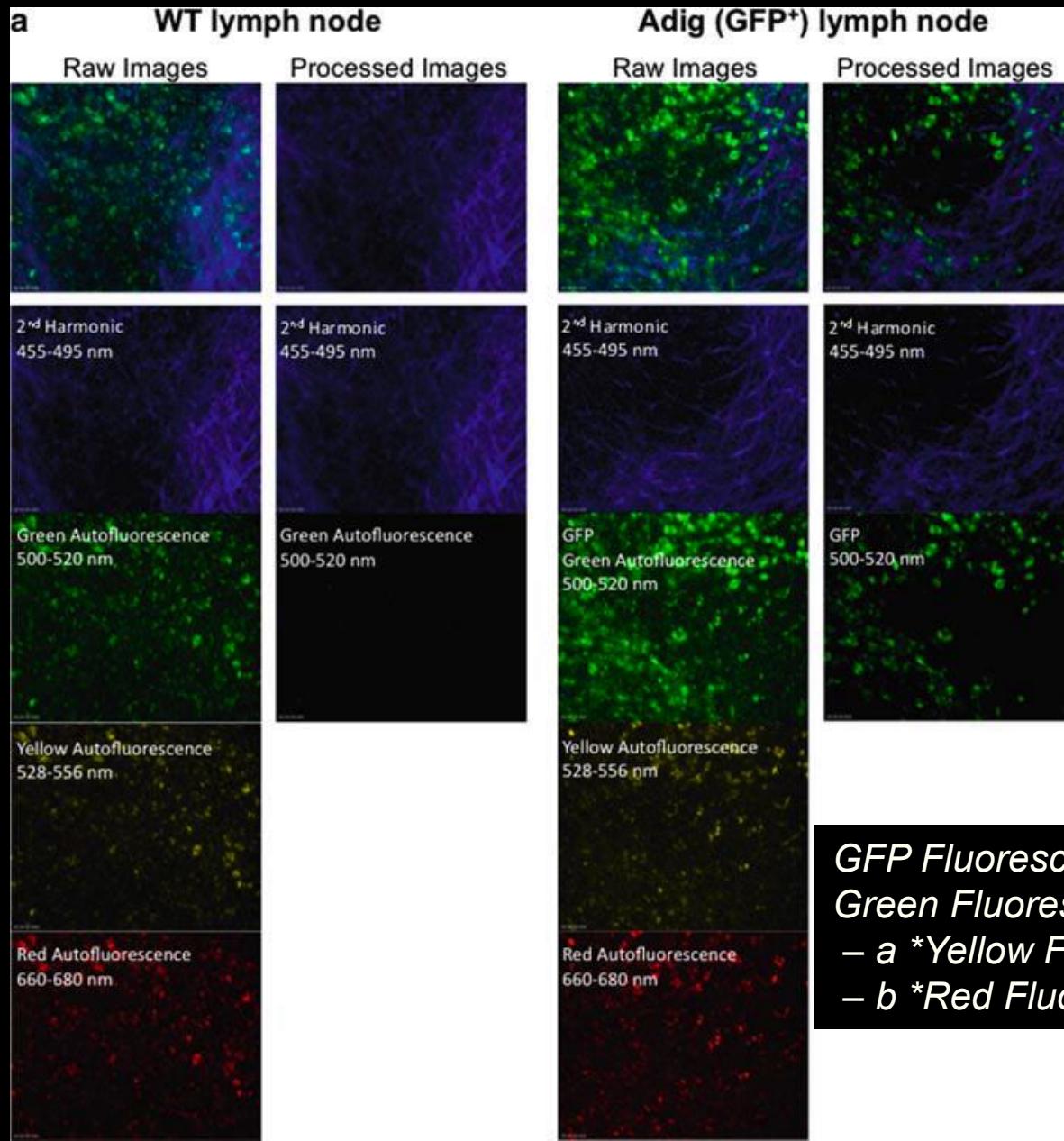
spleen

cerebellum

lung

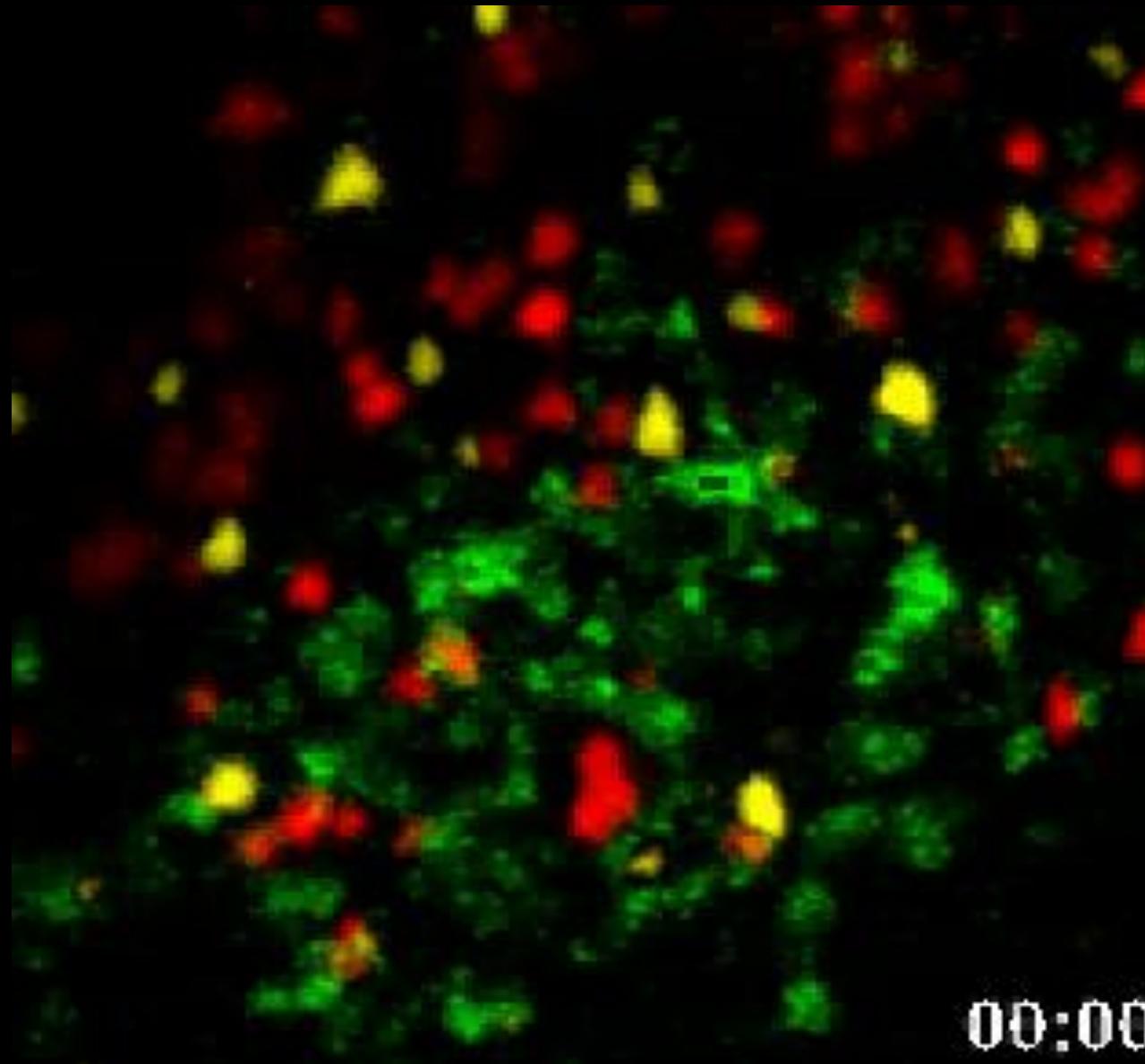
lymph node

Using Multiple Channels to Remove Autofluorescence



*GFP Fluorescence =
Green Fluorescence
– a *Yellow Fluorescence
– b *Red Fluorescence*

T cell interactions with Peripheral AIRE expressing cells (PAECs) in the LN



AIRE: IGRP-GFP
8.3 T cells
Polyclonal NOD T cells

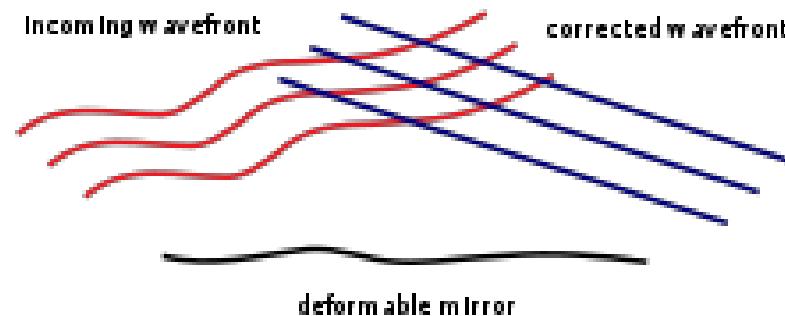
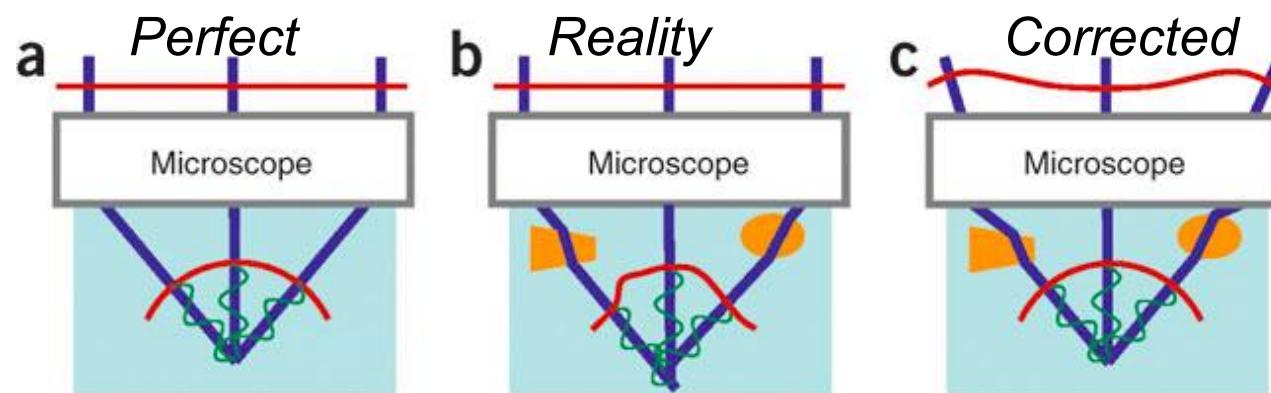
00:00

Note: Why stop at 2P.
We call it Multiphoton, right?

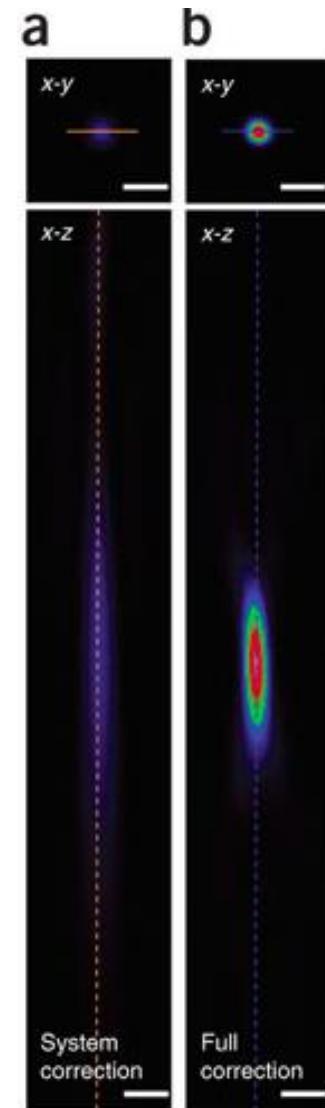
*3 photons can coincide and do the same thing
Now excitation cone is even tighter.*

*What are some downsides of trying to do this?
(Laser power? What wavelength?)*

Adaptive Optics can help get through tissues that distort the incoming wavefront.



Betzig and Ji



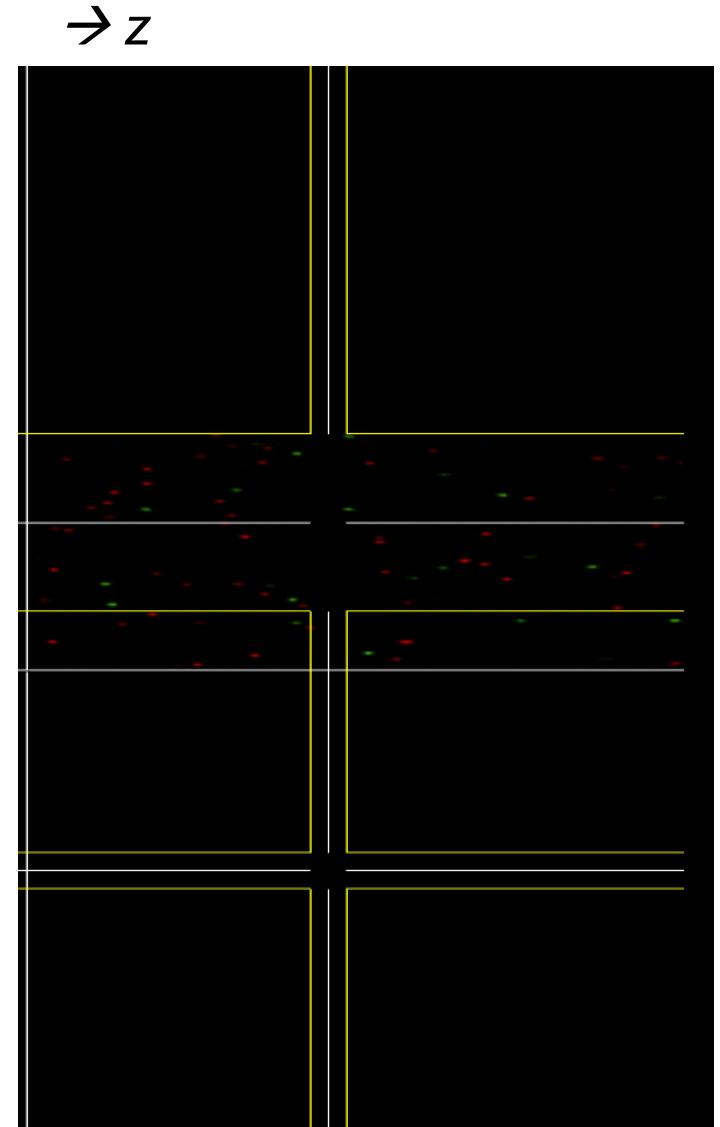
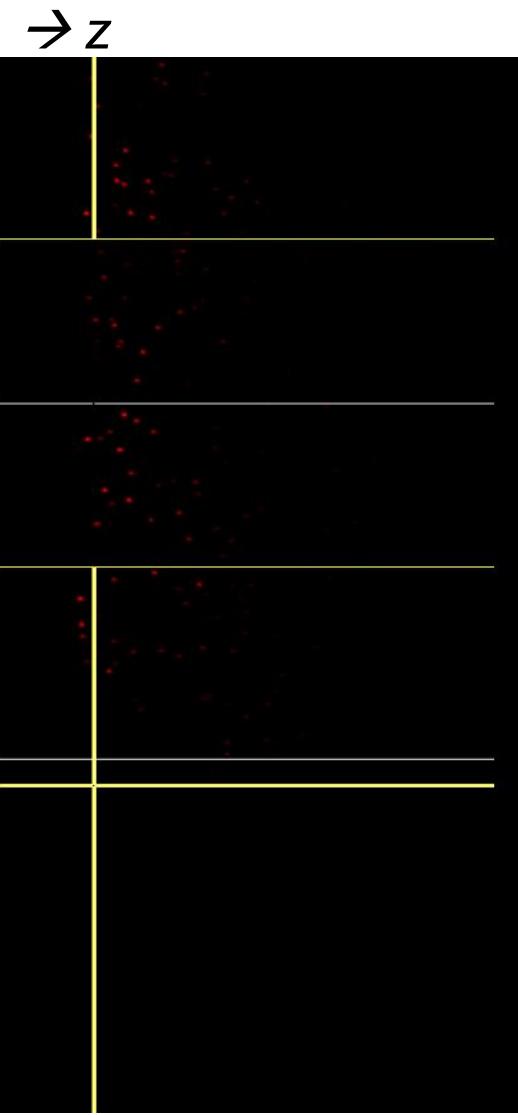
The labs

Phantoms and Ears

- CONFIRMING DEPTH IS BETTER WITH 2P: PHANTOM SAMPLES OF BEADS WITH EMBEDDED ‘DISPERSEIVE’ BEADS VS. NOT.
- IMAGING MYSTERY EARS—SEE IF YOU CAN FIGURE OUT WHAT CELLS MIGHT BE BEING LABELED.
- SOME OTHER THINGS THAT MAY COME UP:
 - RESONANT VERSUS GALVO
 - HOME-GROWN VERSUS COMMERCIAL.

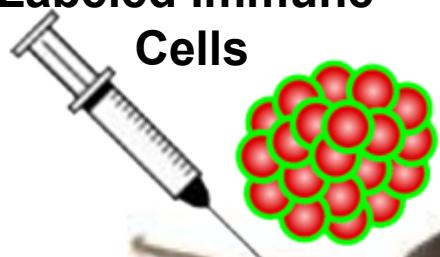
Phantoms

*Polyacrylamide with fluorescent beads
w/ or w/out dispersive beads spiked in*

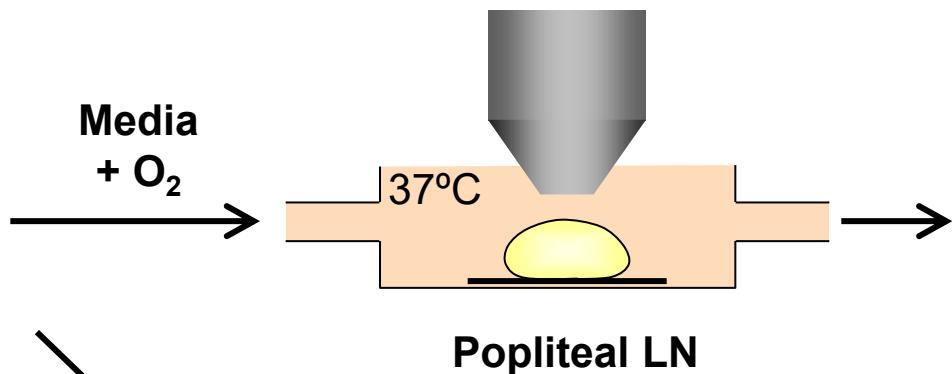


Organ Explant and intravital system for 2-photon imaging

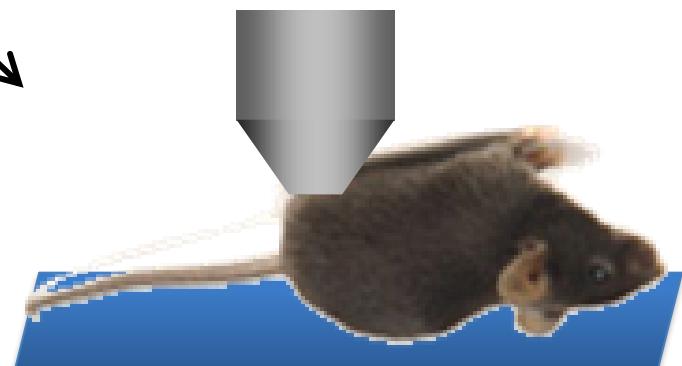
Labeled Immune Cells



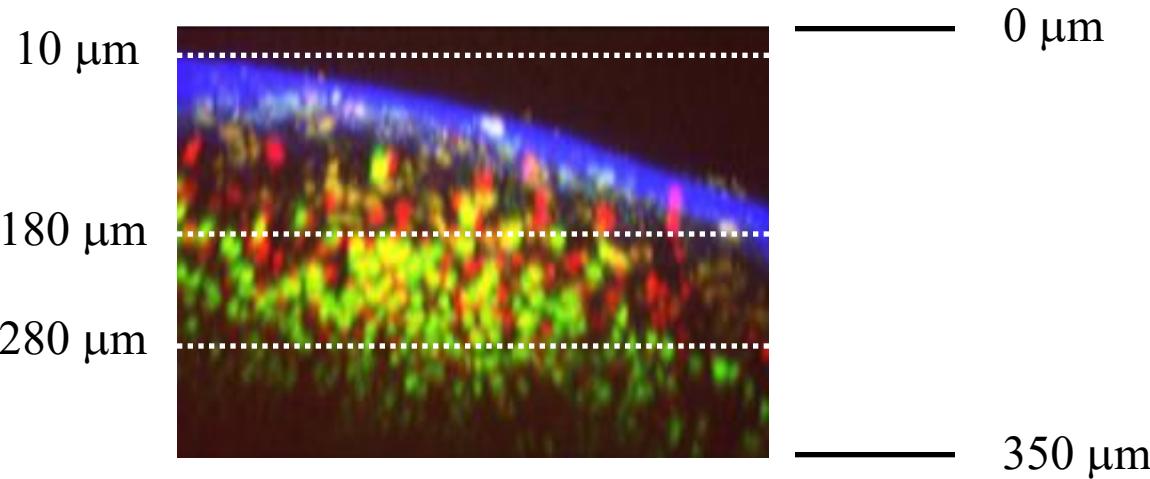
Media
+ O₂



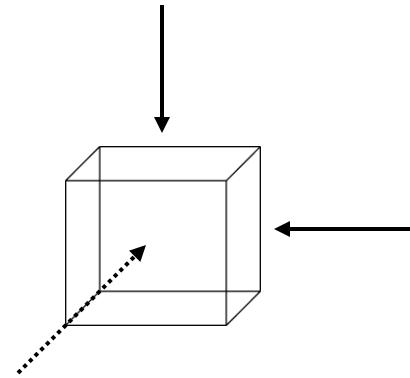
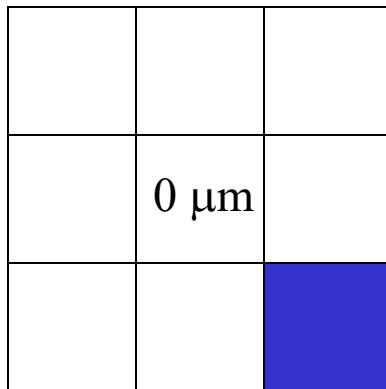
Popliteal LN



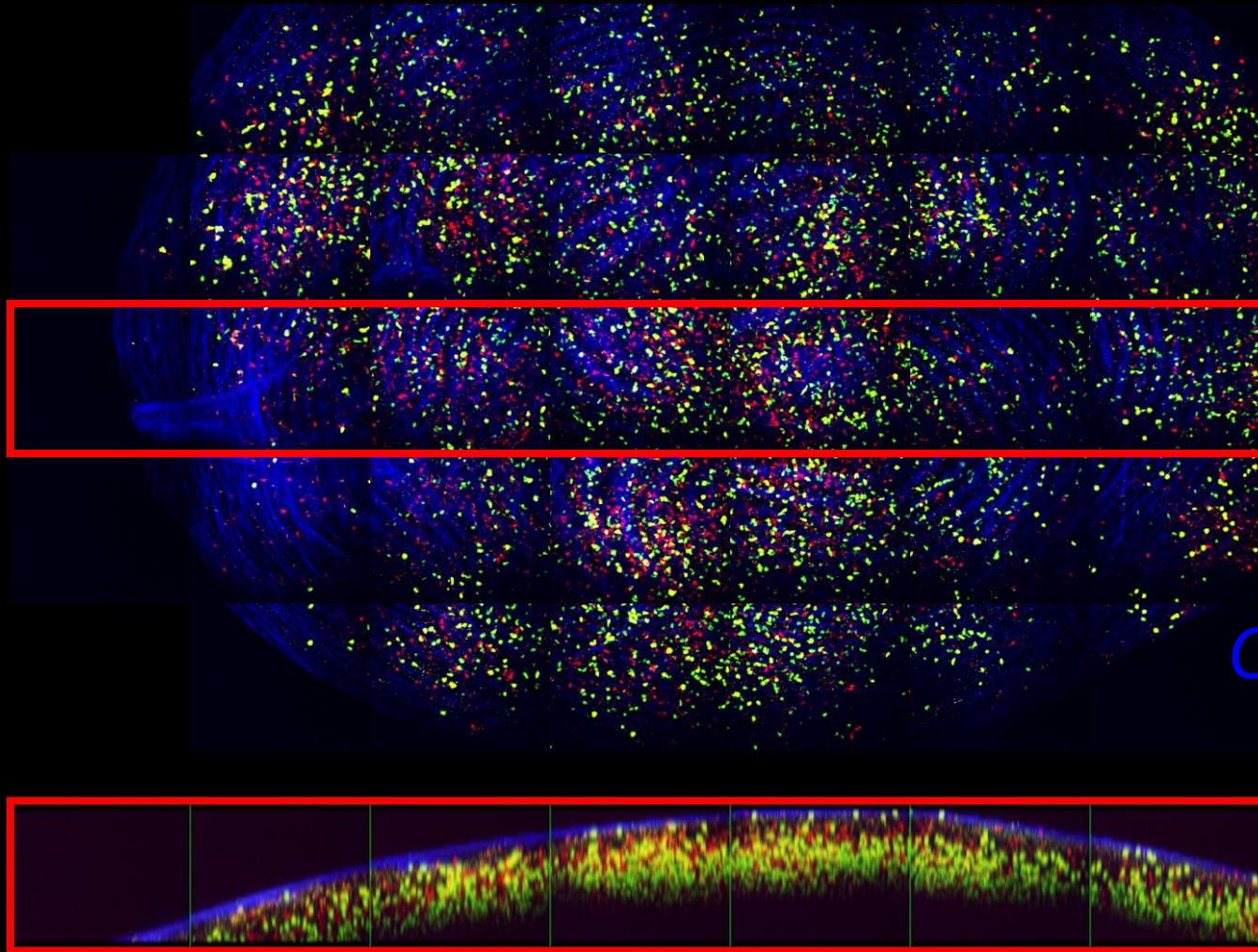
Tiled Cubes of Data to ‘Survey’ an Organ: e.g. Inguinal Non-draining Lymph Node



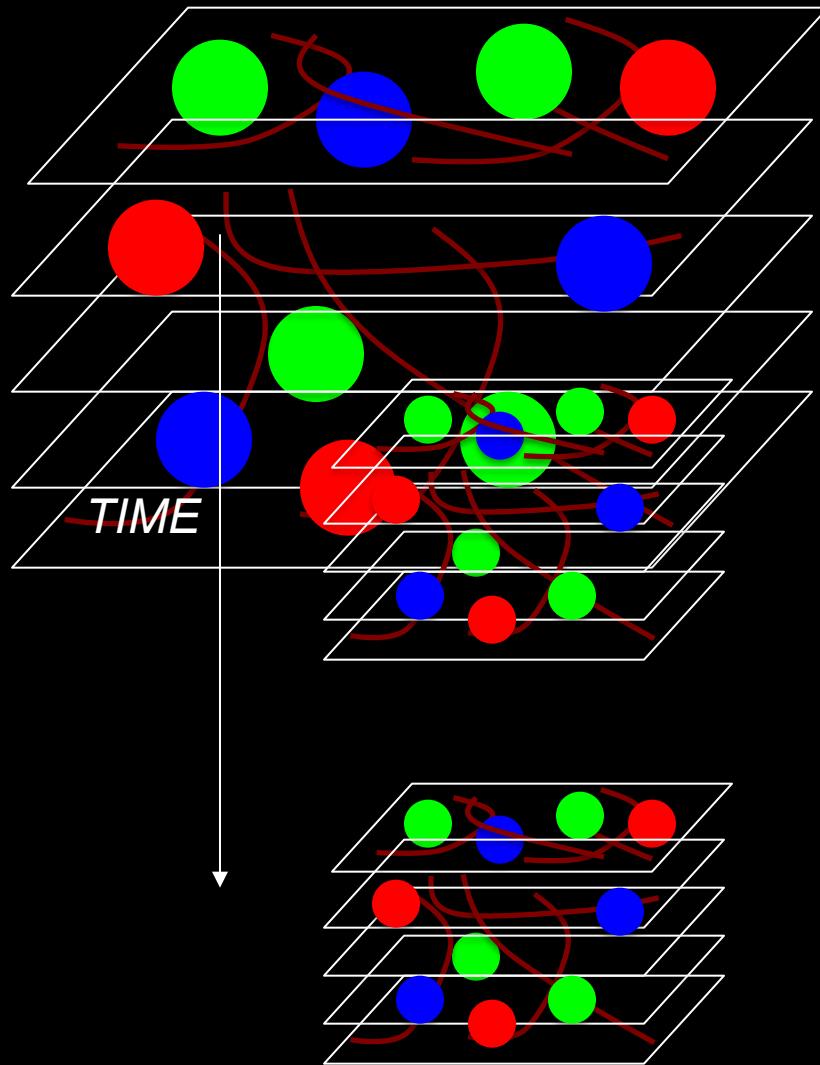
Top Down



Typical First Step: The Survey

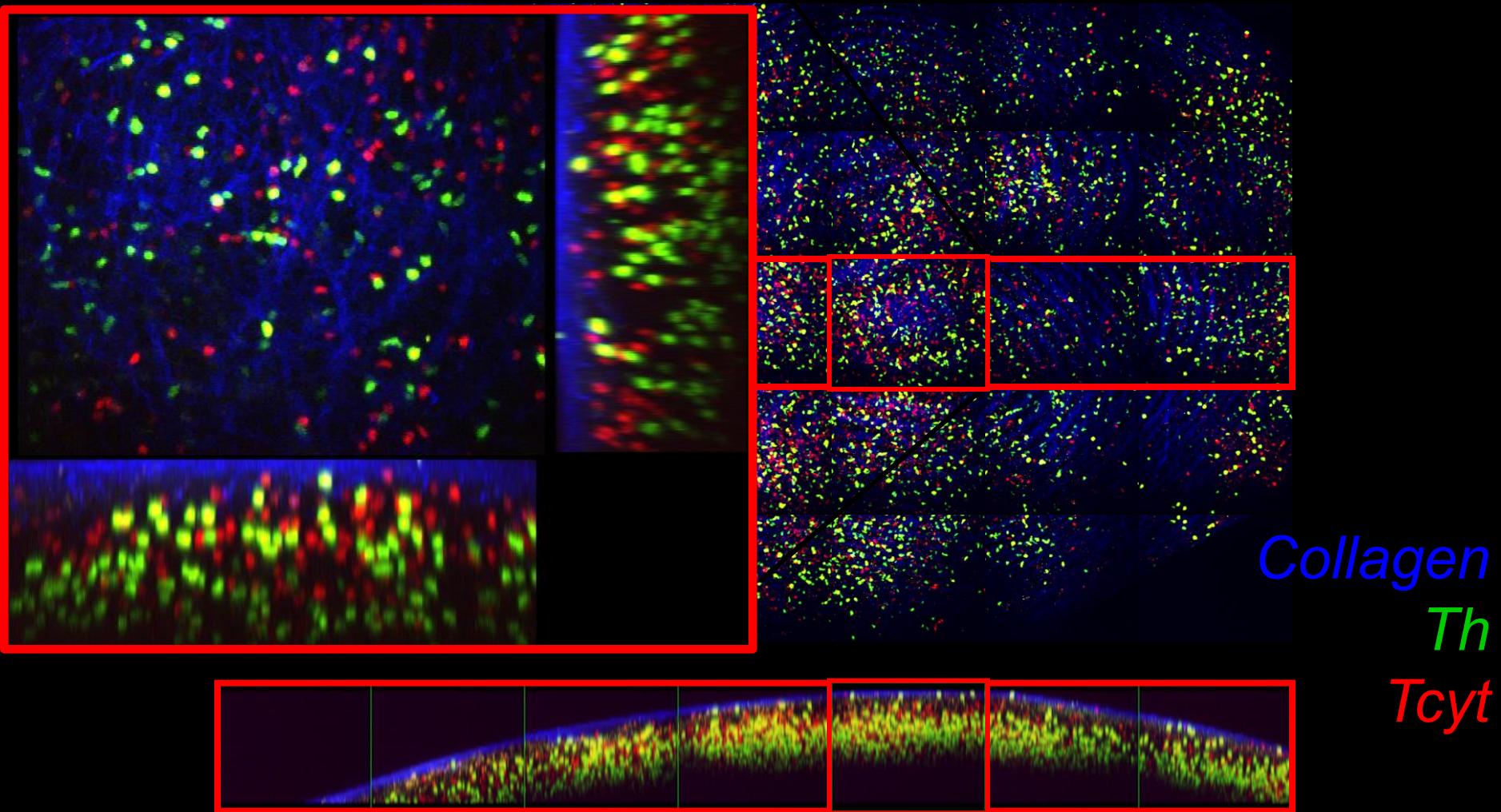


A 5D 2-Photon Platform

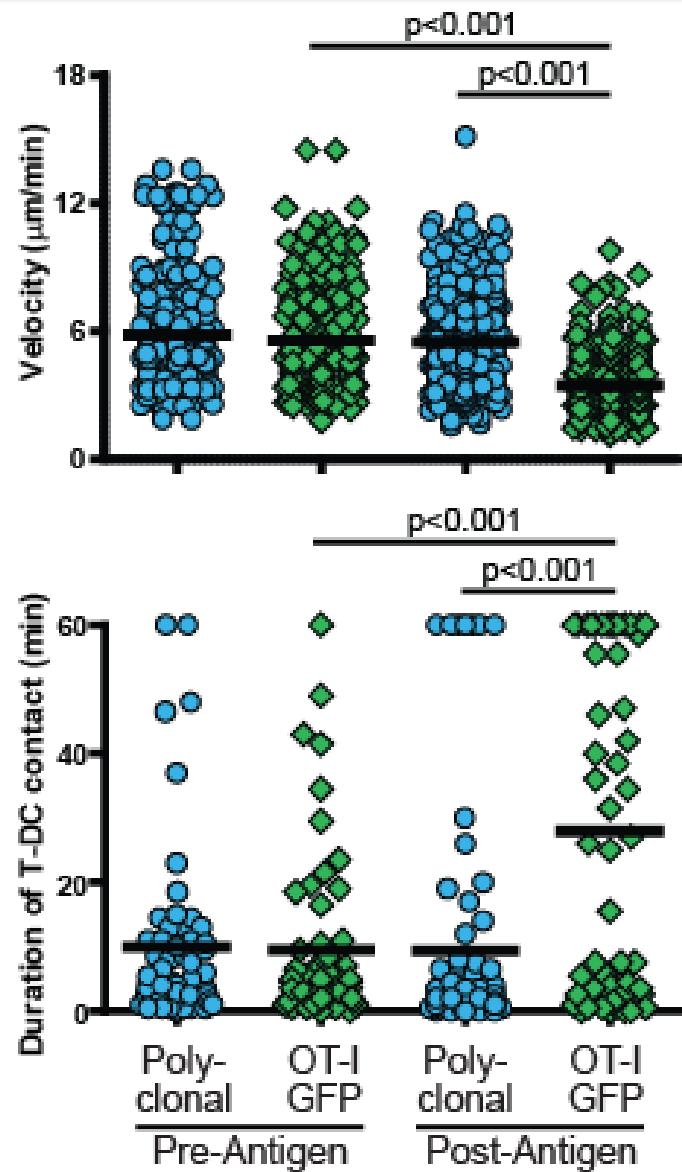
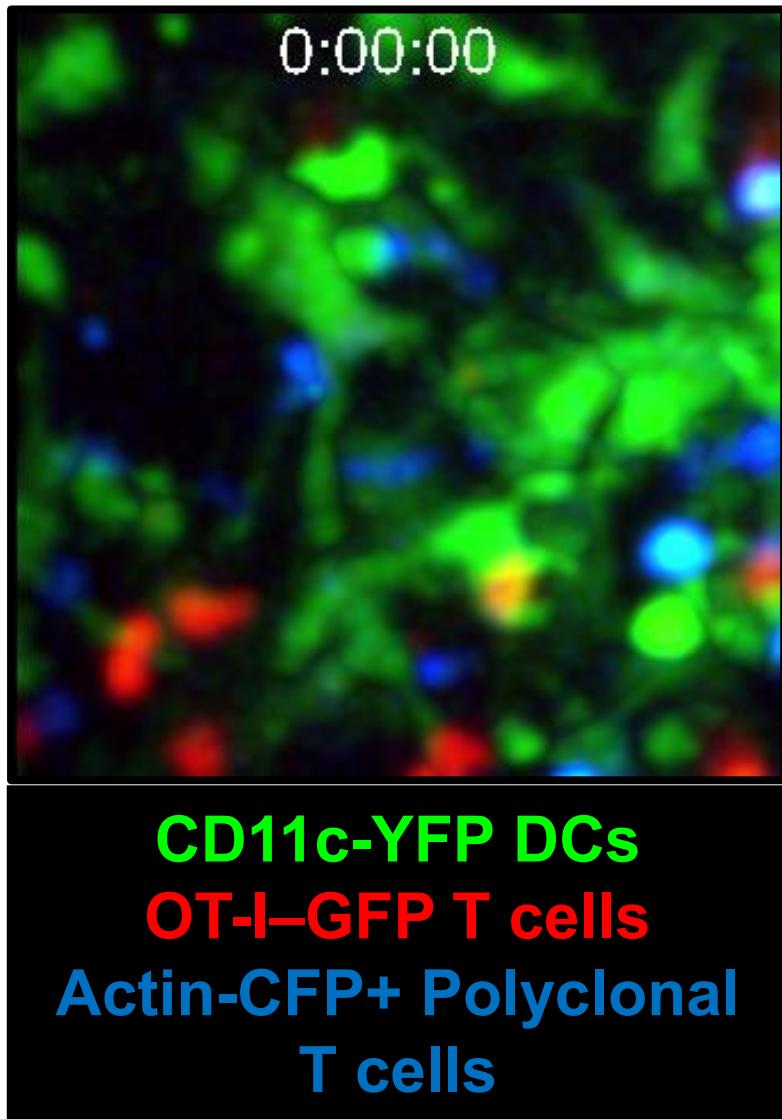


Analyze in:
Matlab
ImageJ
Metamorph
Imaris
IDT

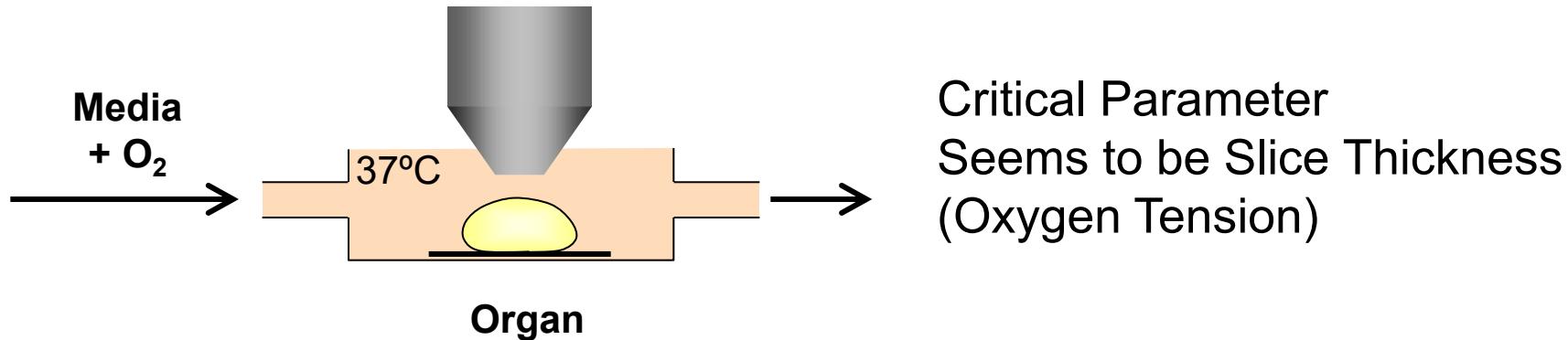
Hone in On and Area and Collect Timelapse



T cell–DC interactions before & after antigen addition

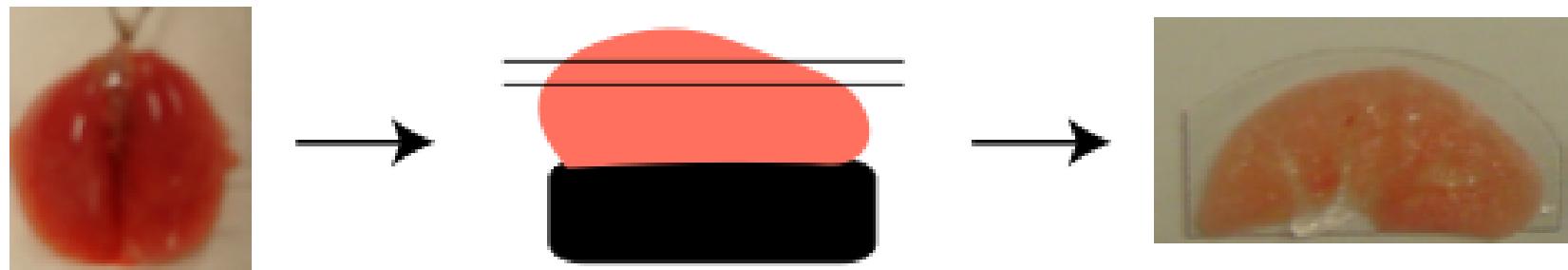


Quite a Few tissues are happy if sliced and kept under oxygenated media



Brain, LN, Pancreatic Islets (but not whole pancreas), etc.

e.g. Lung

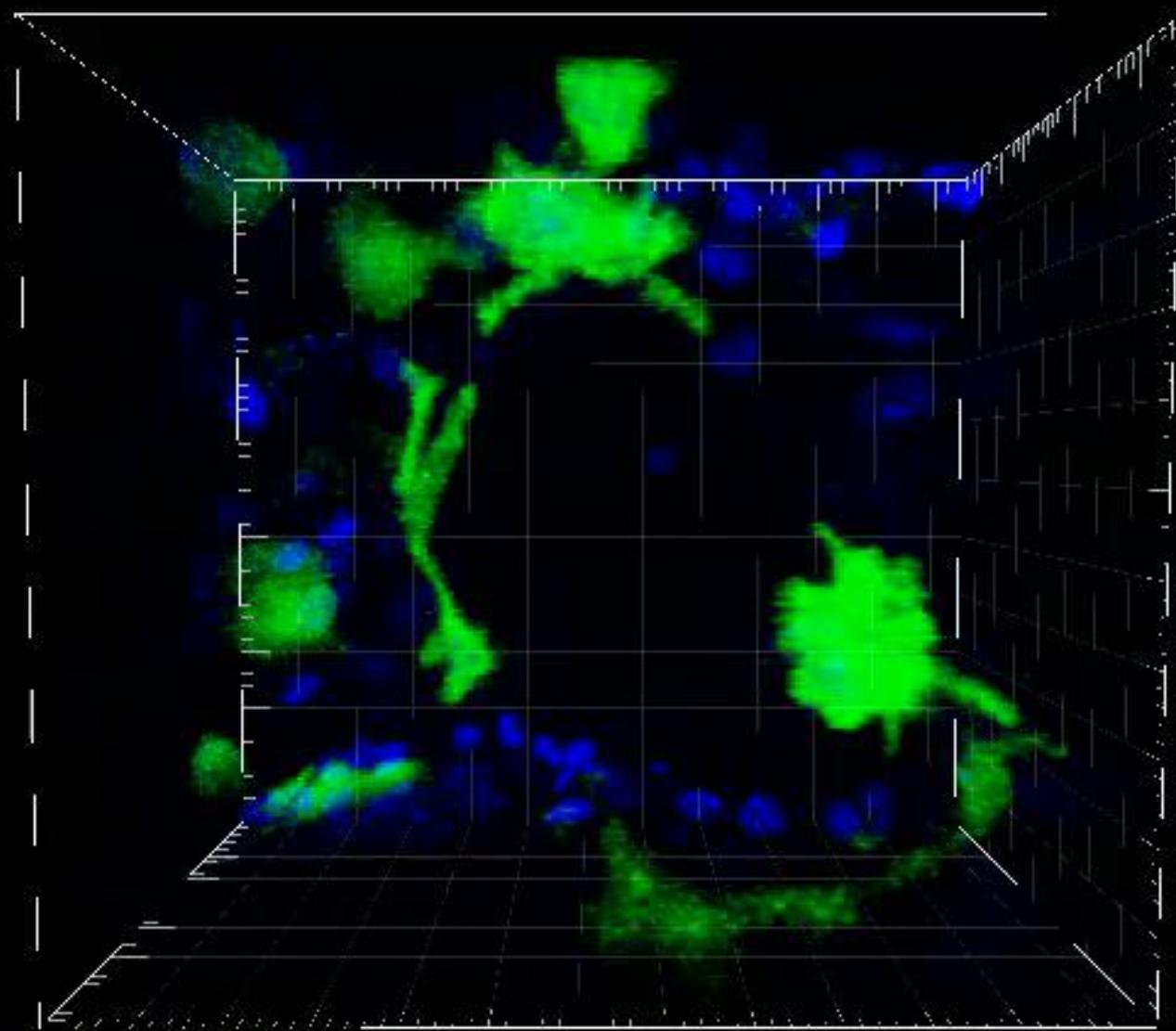


Viability of Tissue is a Critical Parameter and
must be independently monitored.

Movie of Cilia at 3 hrs post sac



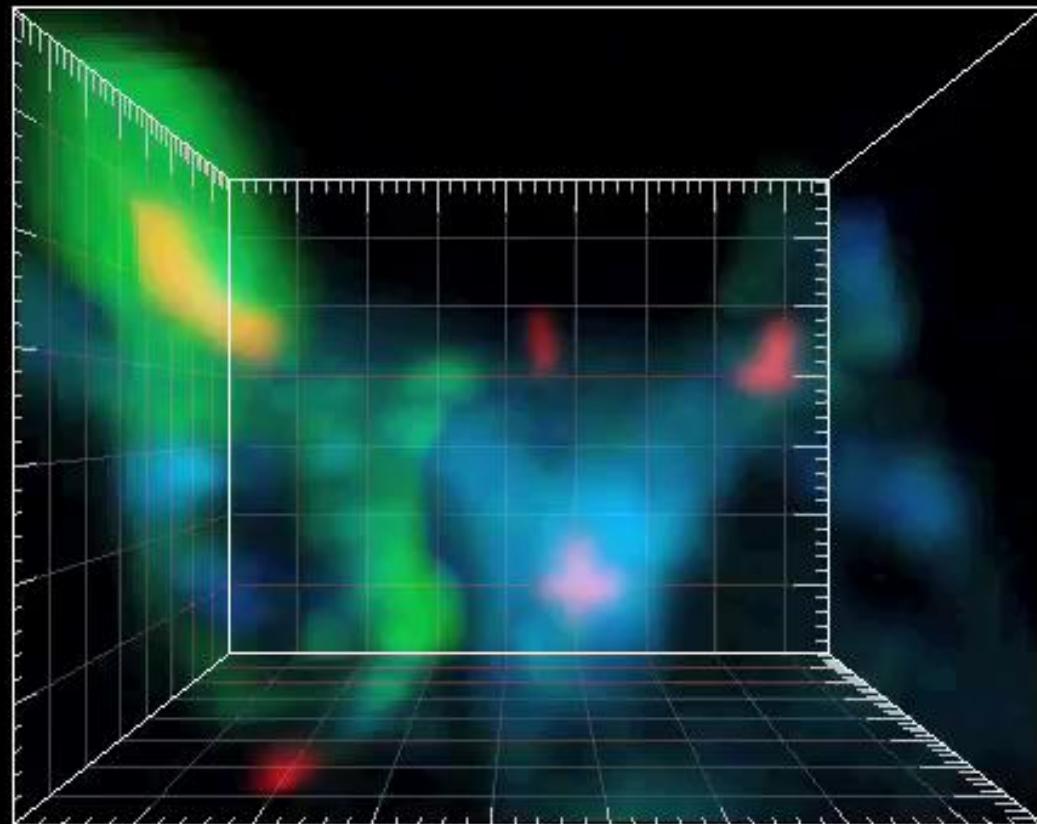
DC Projections into the Alveolar Atrium and Mucosal Scanning



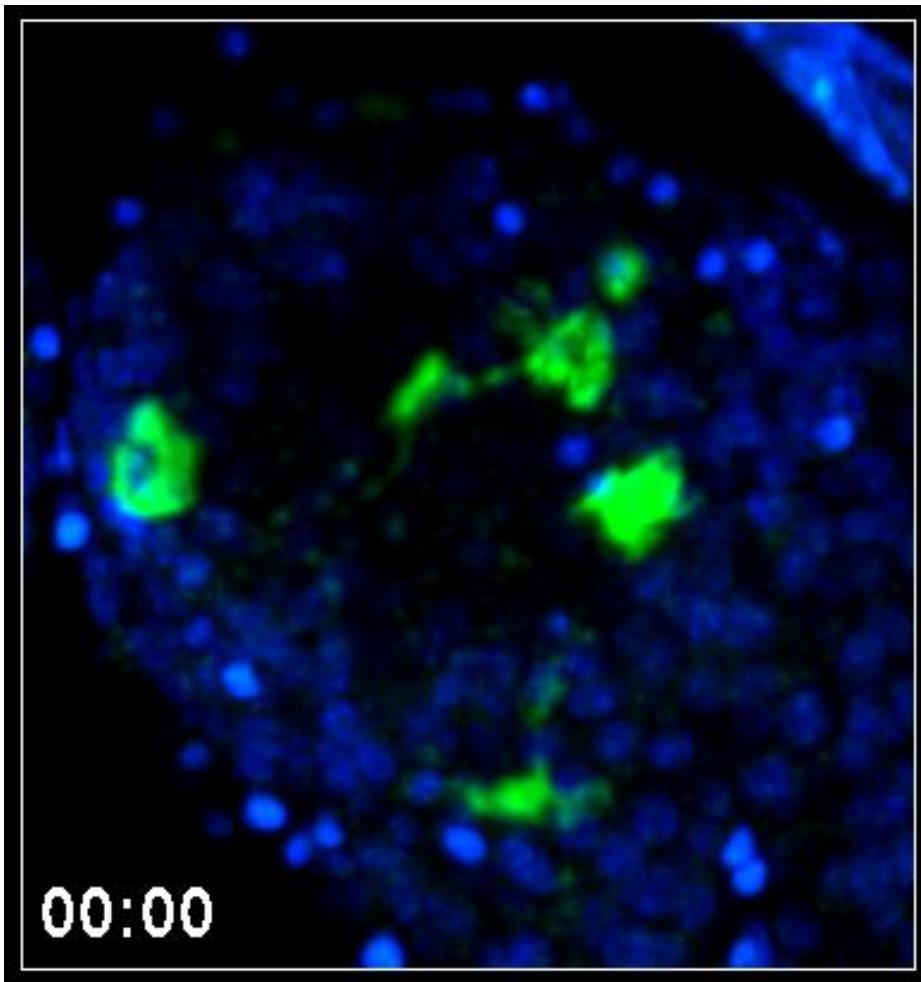
CD11c YFP
Hoechst

“Allergens” are Taken Up Across the Alveoli by Dendritic Scavenging

Cfms GFP
Actin CFP
Inhaled Beads

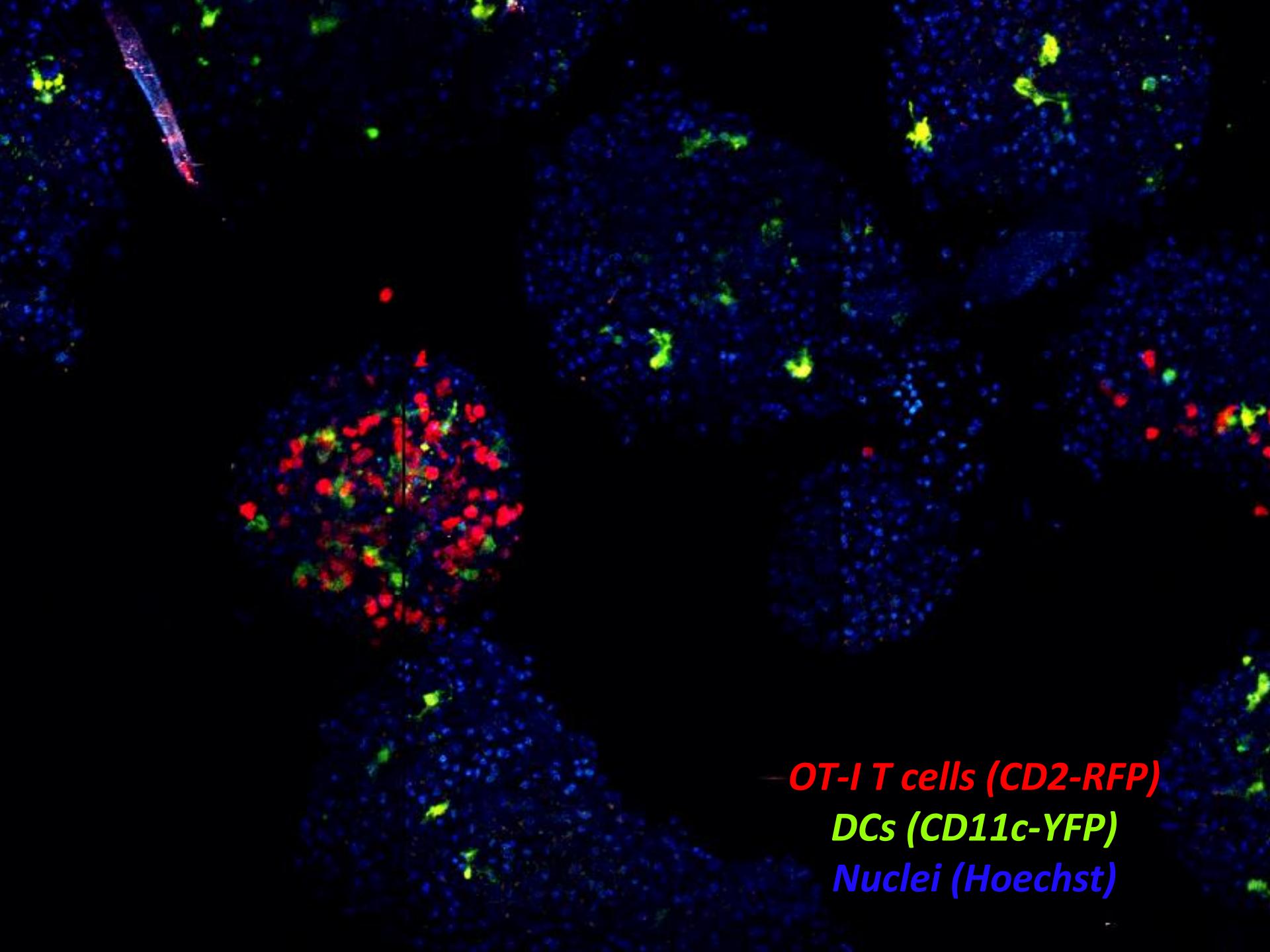


Surveillance of islets by DCs in the steady-state



DCs (CD11c-YFP)
Nuclei (Hoechst)

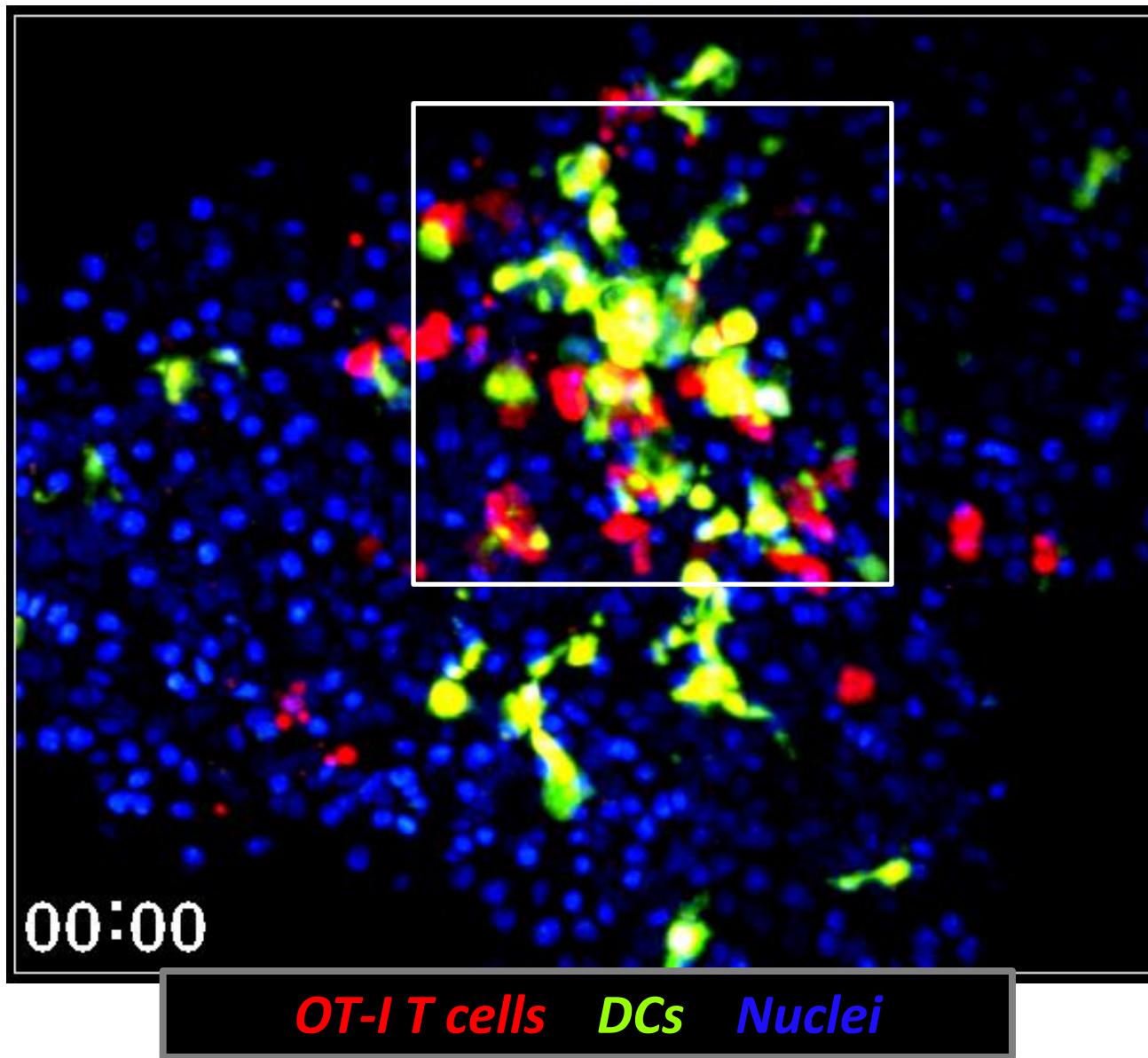
How do intra-islet DCs affect infiltrating T cells?



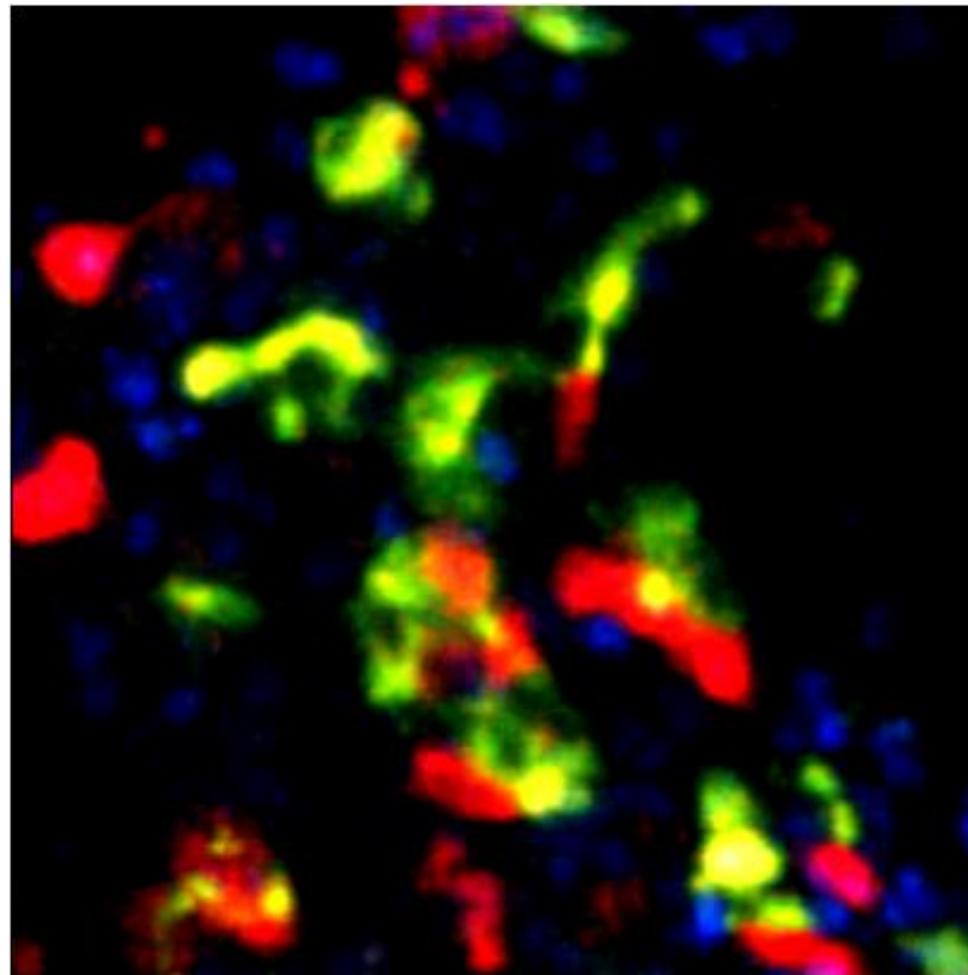
A fluorescence microscopy image showing a tissue section. The image is stained with three markers: CD2-RFP (red), CD11c-YFP (green), and Hoechst (blue). The red signal highlights OT-I T cells, the green signal highlights DCs, and the blue signal highlights the nuclei. A cluster of red and green cells is visible in the lower-left quadrant, while a more diffuse distribution of green and blue cells is seen across the rest of the field.

OT-I T cells (CD2-RFP)
DCs (CD11c-YFP)
Nuclei (Hoechst)

T cells form transient & sustained interactions with intra-islet DCs

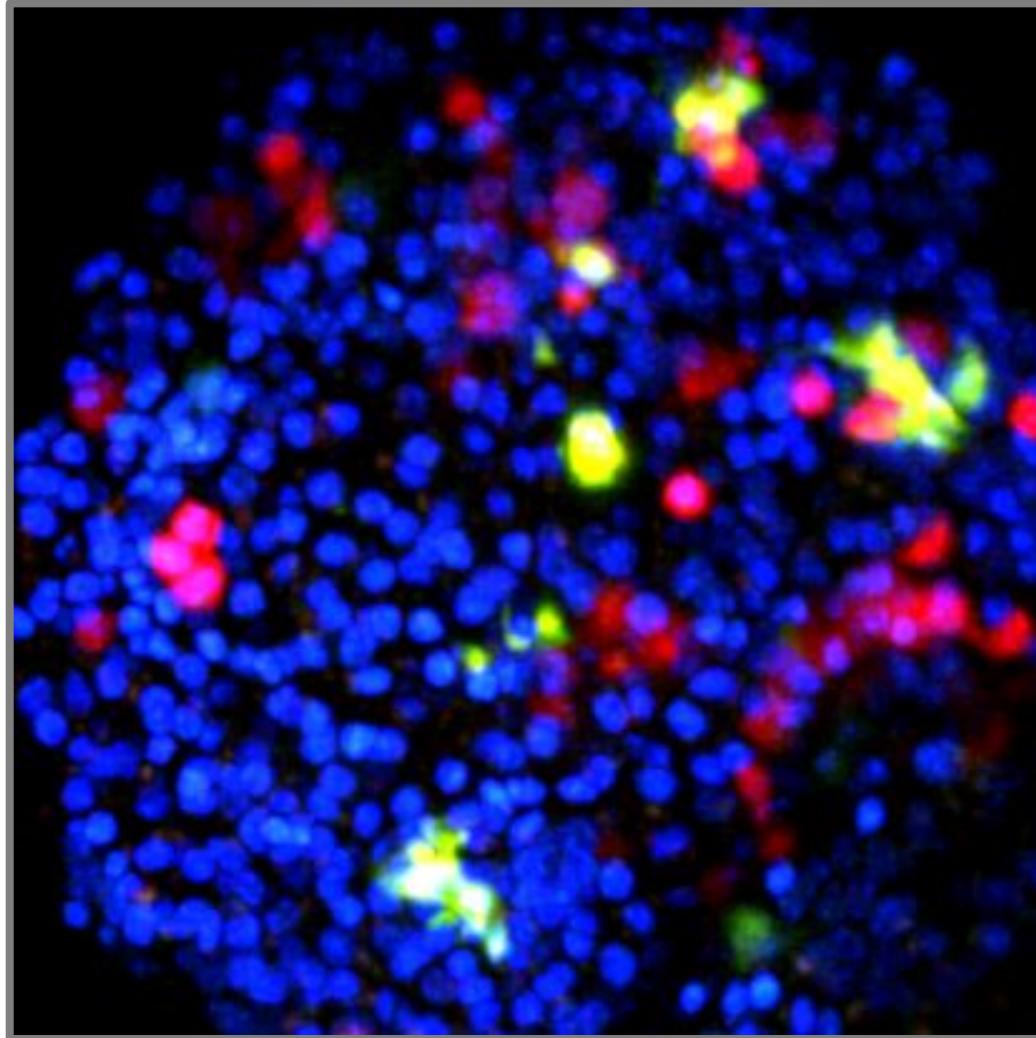


T cells form transient & sustained interactions with intra-islet DCs



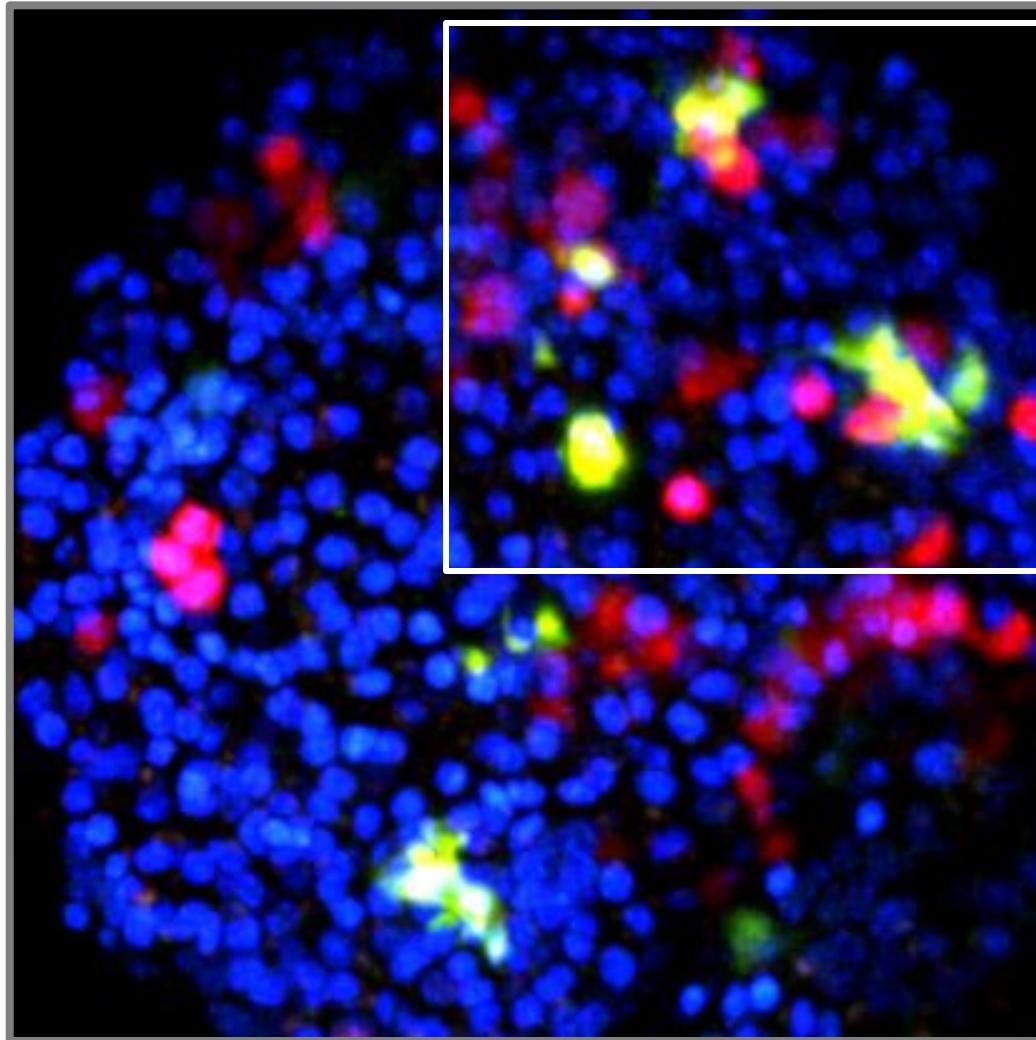
OT-I T cells DCs Nuclei

T cells can induce DC lysis



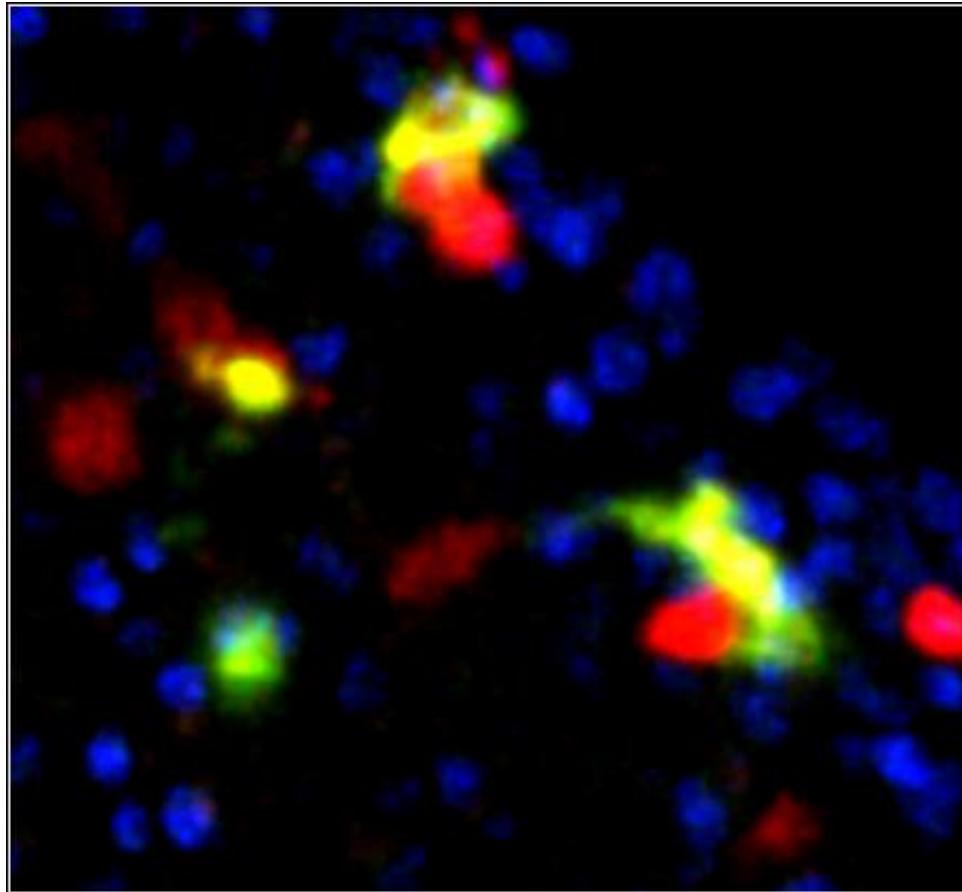
OT-I T cells DCs Nuclei

T cells can induce DC lysis



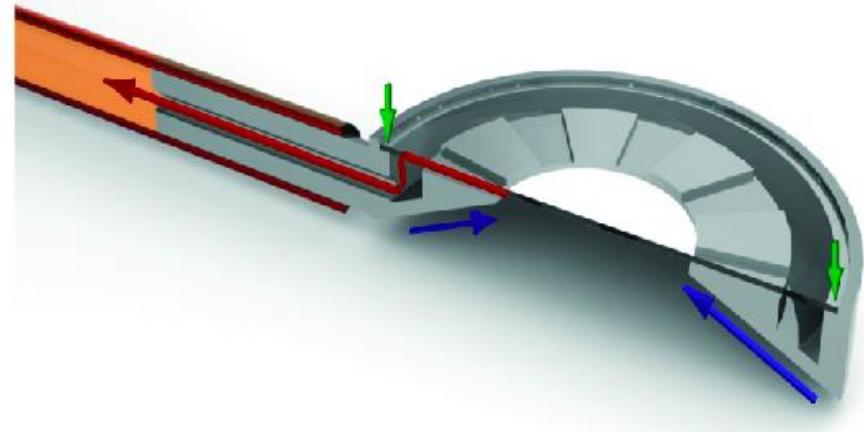
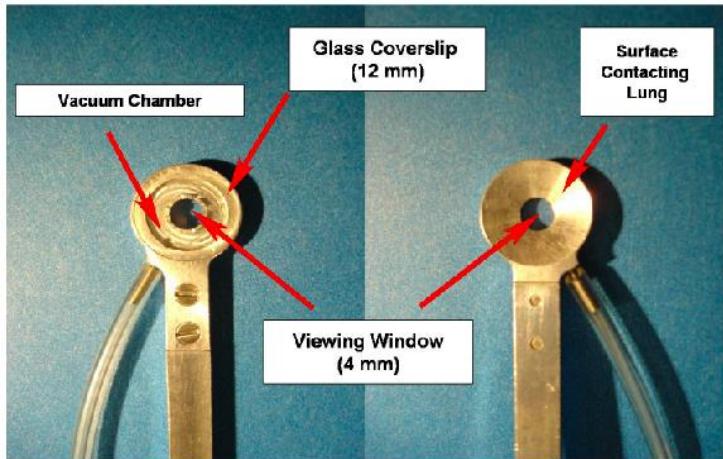
OT-I T cells DCs Nuclei

T cells can induce DC lysis

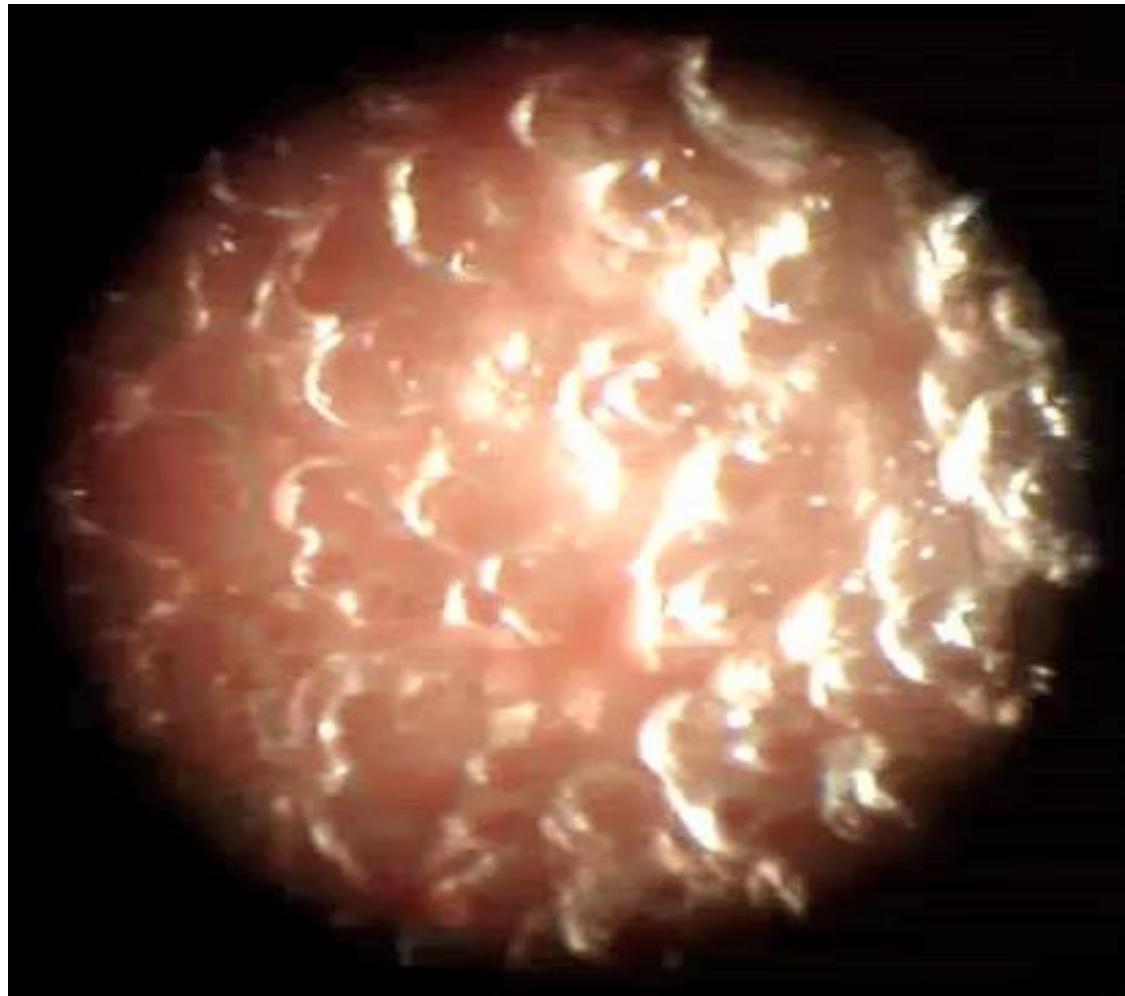


OT-I T cells DCs Nuclei

Taking it 'Live'

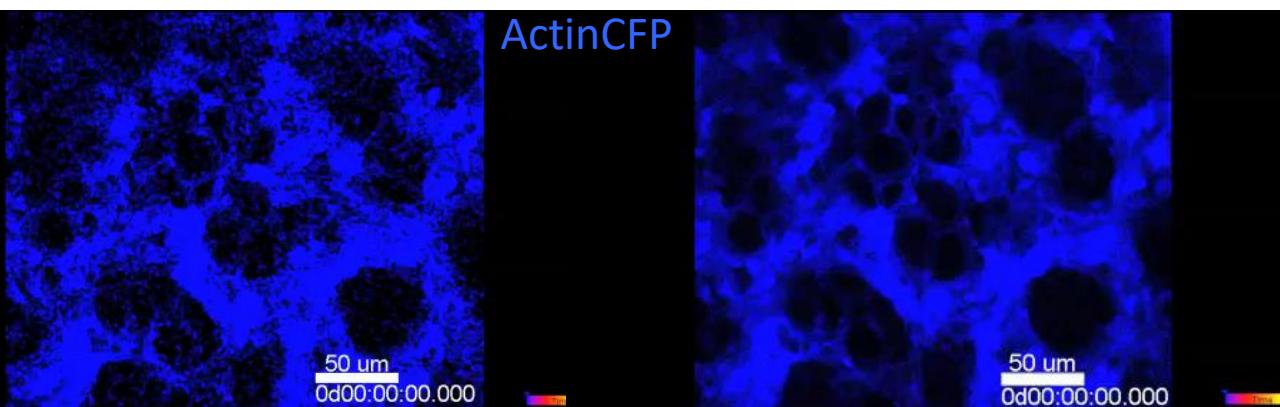
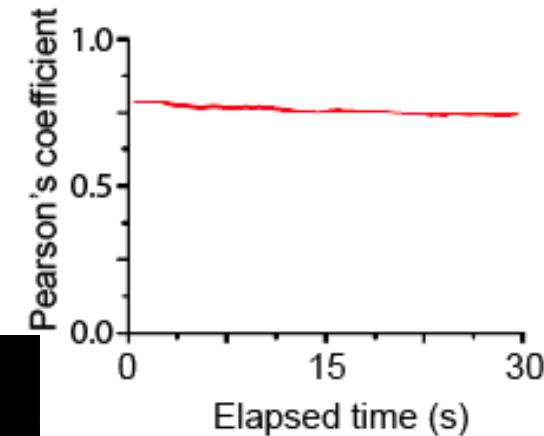
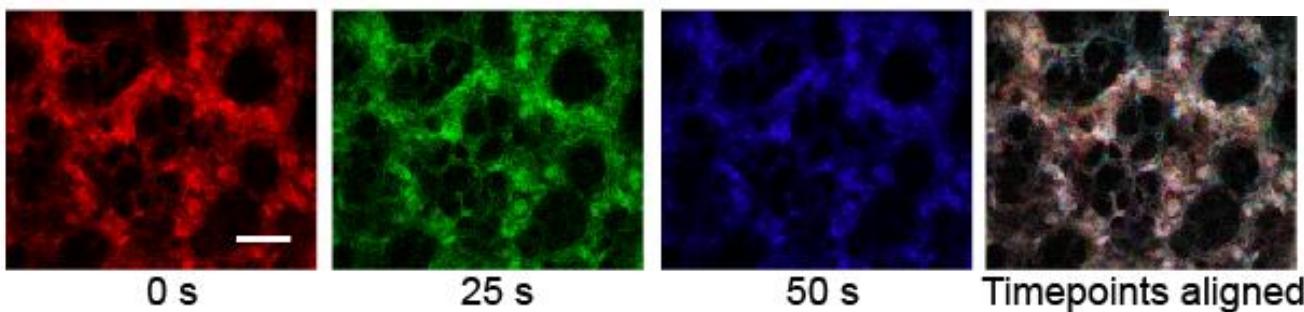
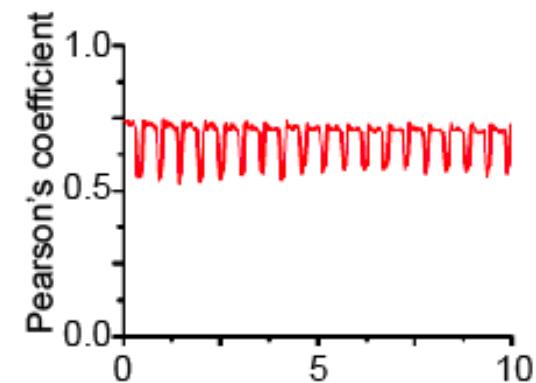
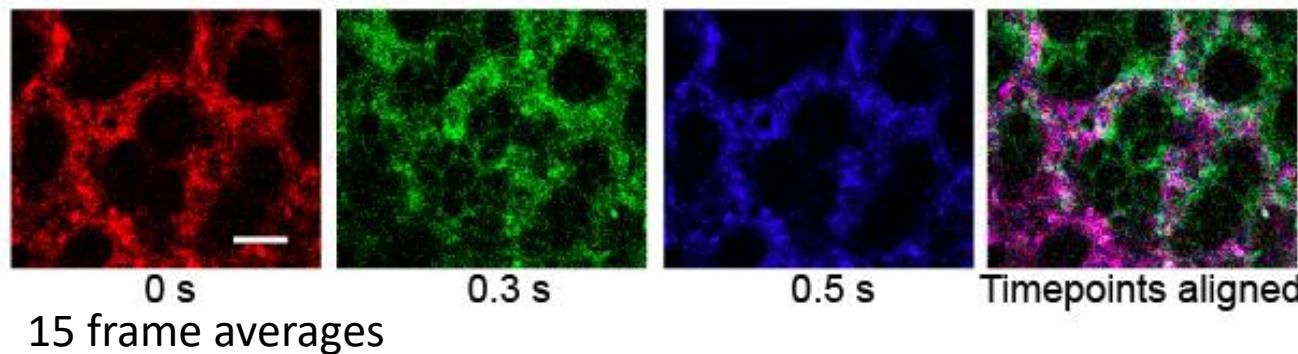


Vacuum window isolates and stabilizes the lung



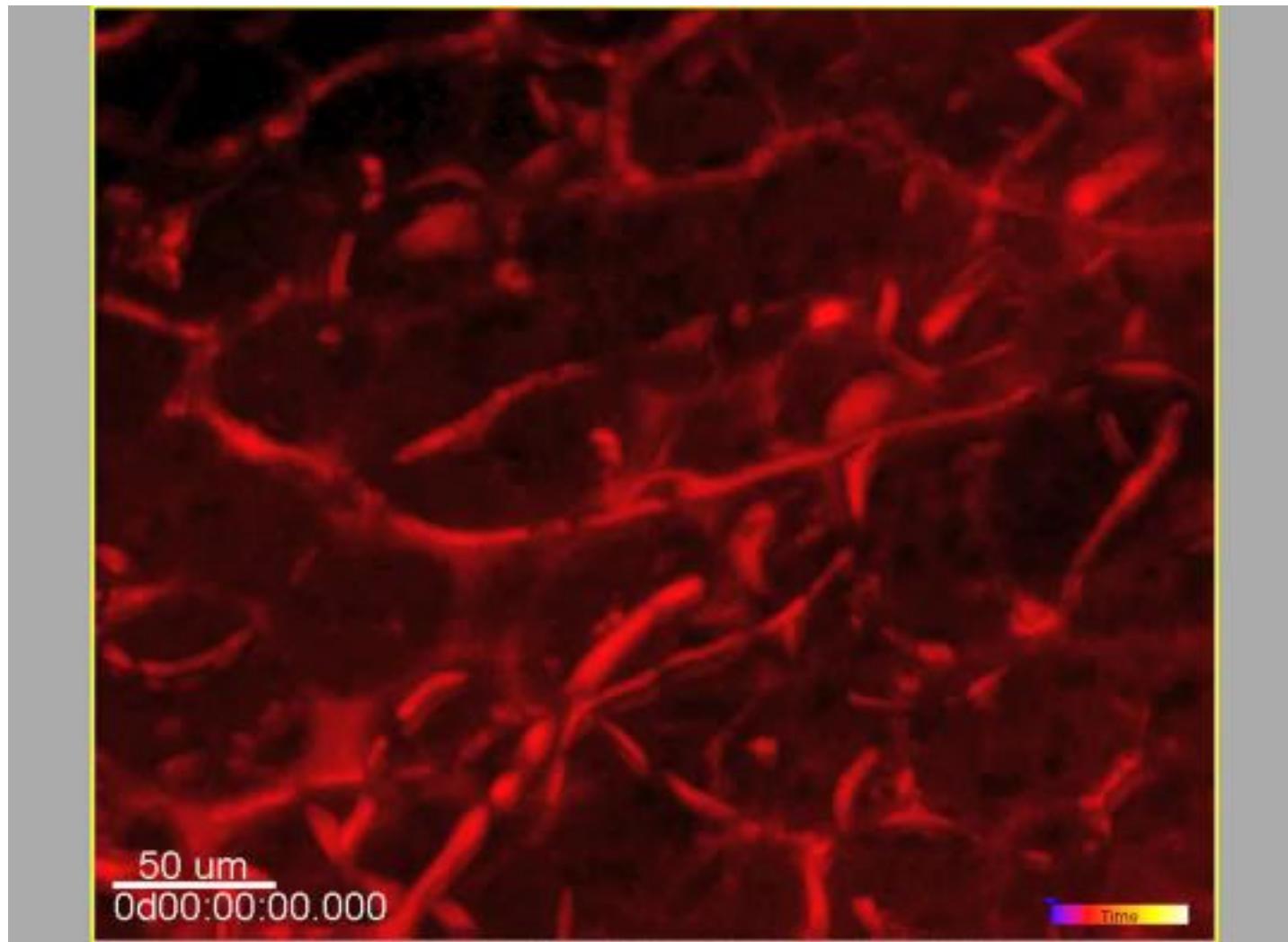
Window allows for inflation of alveoli under suction

Real-time imaging



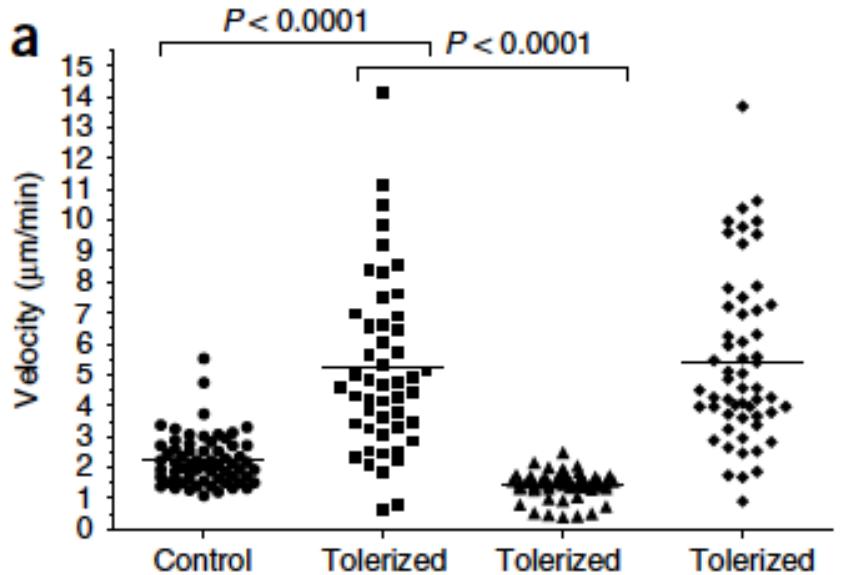
Averaging or frame deletion can provide a very stable image

Intact Blood Flow-I

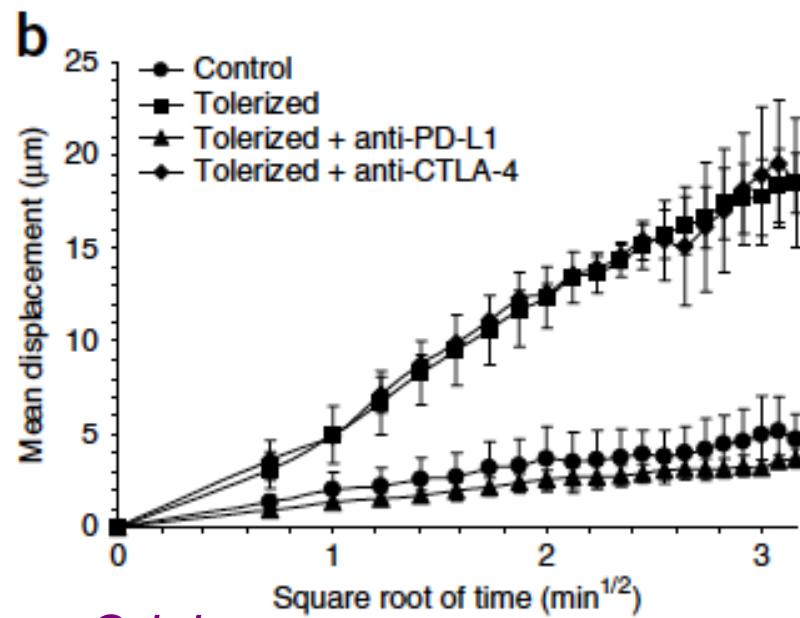


Data Analysis: Motility

Avg Velocities



RMS Displacement



Distance from Origin

c Control

x (μm)

y (μm)

d Tolerized

x (μm)

y (μm)

e Tolerized + anti-PD-L1

x (μm)

y (μm)

f Tolerized + anti-CTLA-4

x (μm)

y (μm)

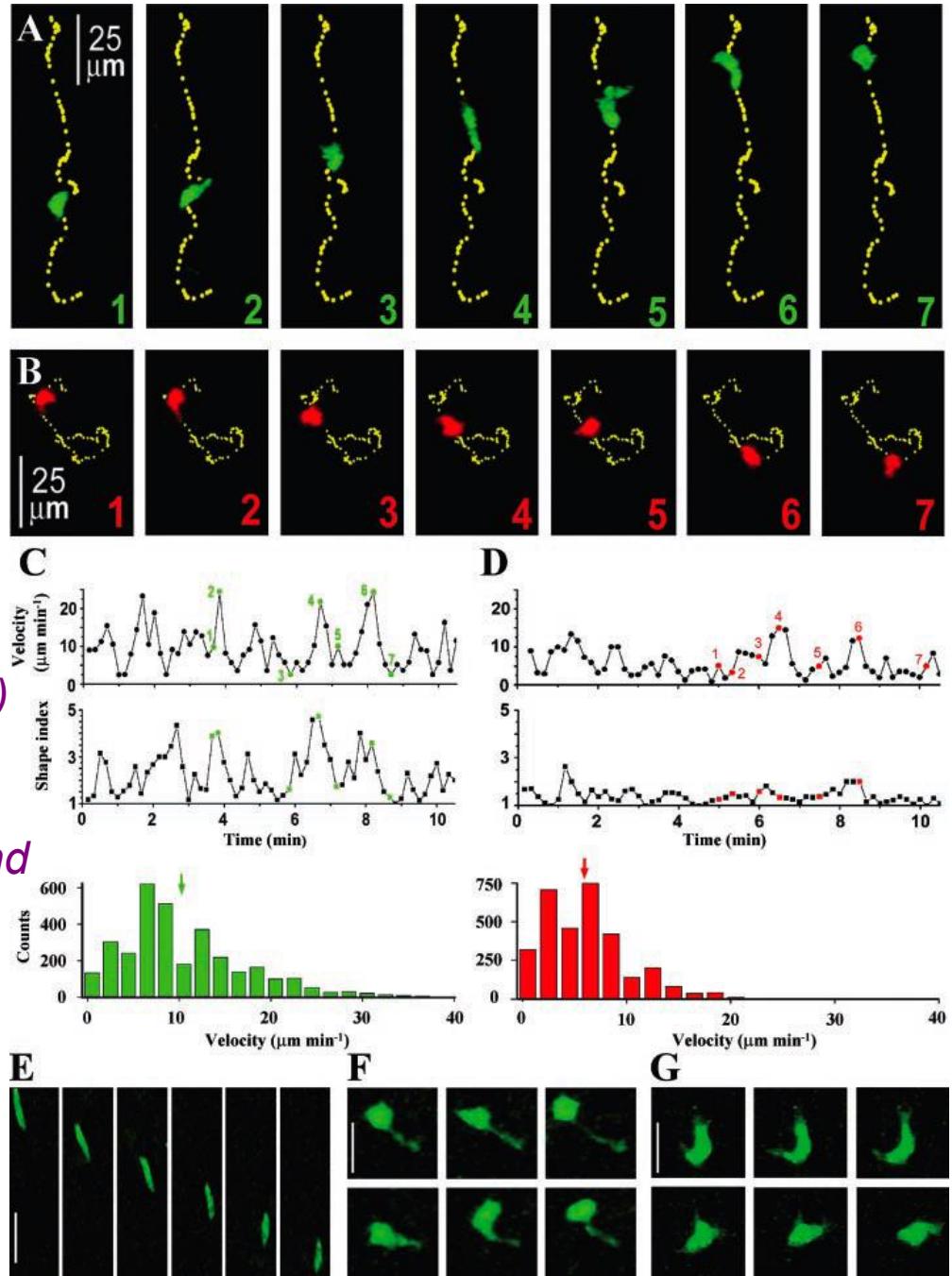
Morphology:

*Shape Factors:
Often Correlate with
Motility vs. signaling*

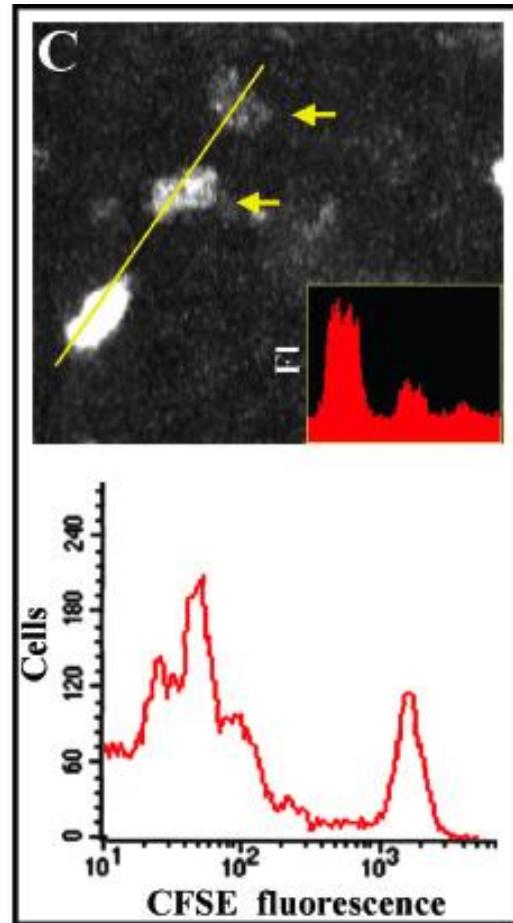
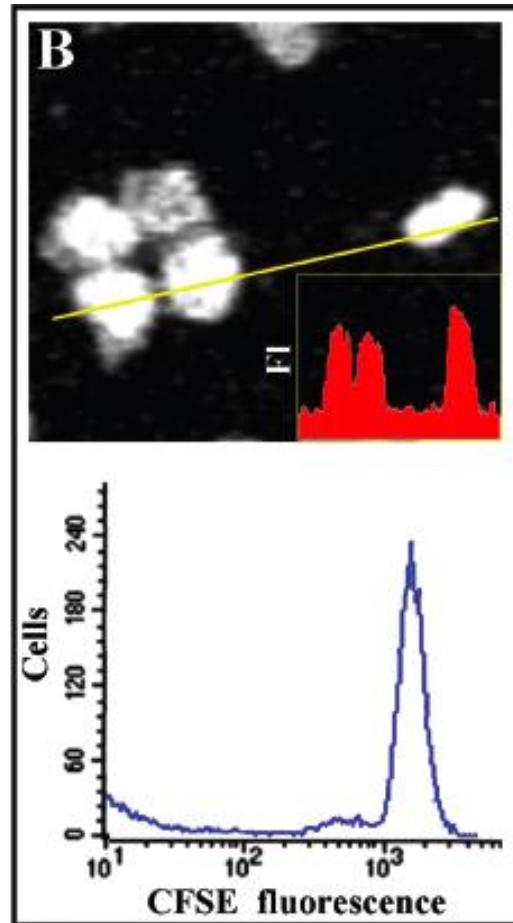
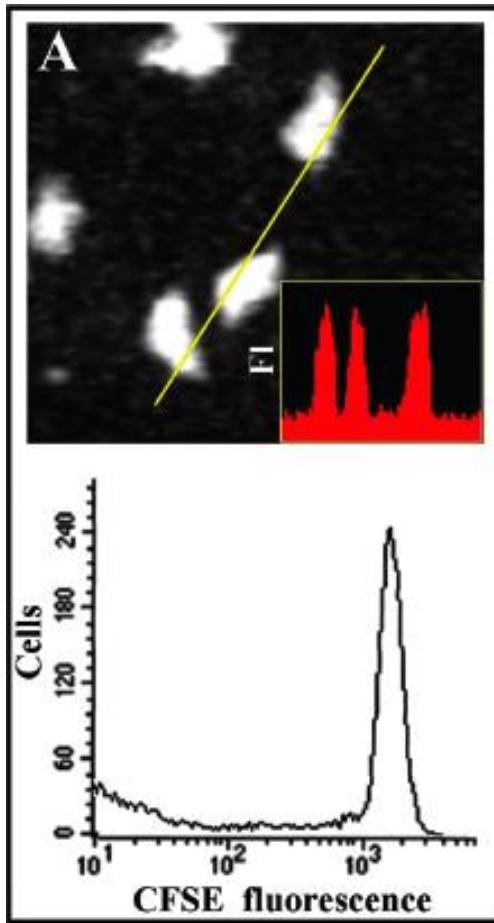


*Ratio of longest and
perpendicular chords (1 for circle)*

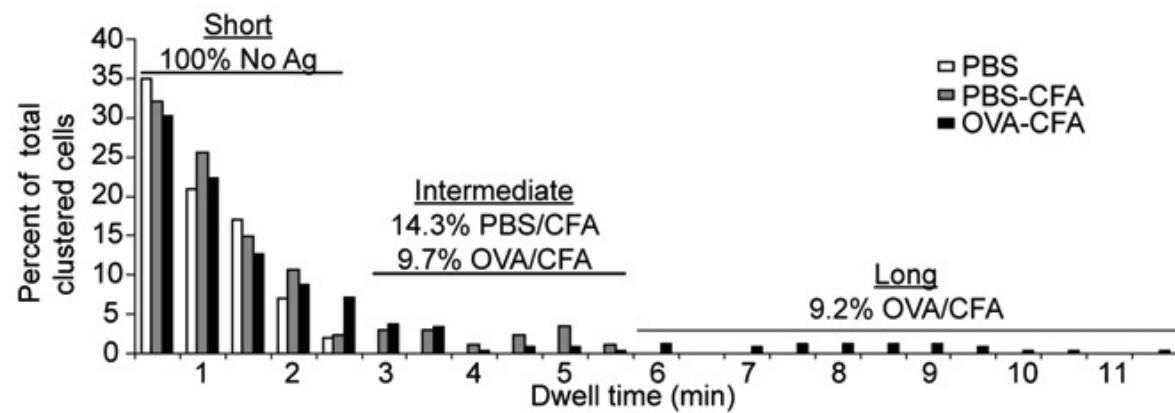
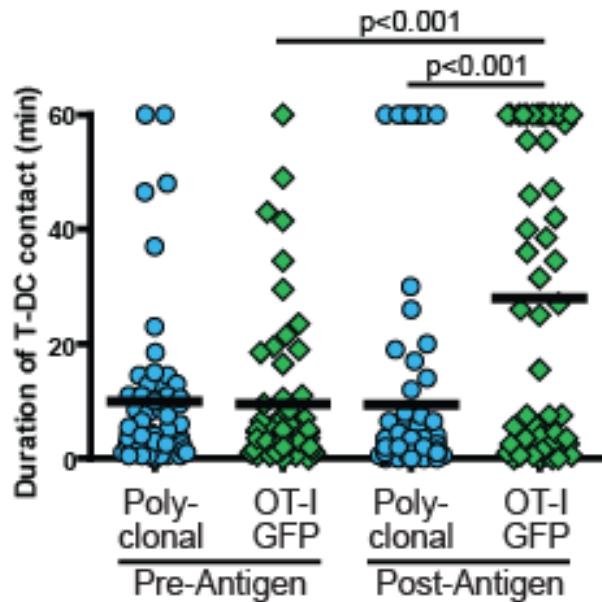
*relationship between perimeter and
radius ($2\pi r$ for circle)*



Cell Division:



Data Analysis: Cell-Interaction



*Can be done manually OR through
MATLAB or in some cases Imaris*