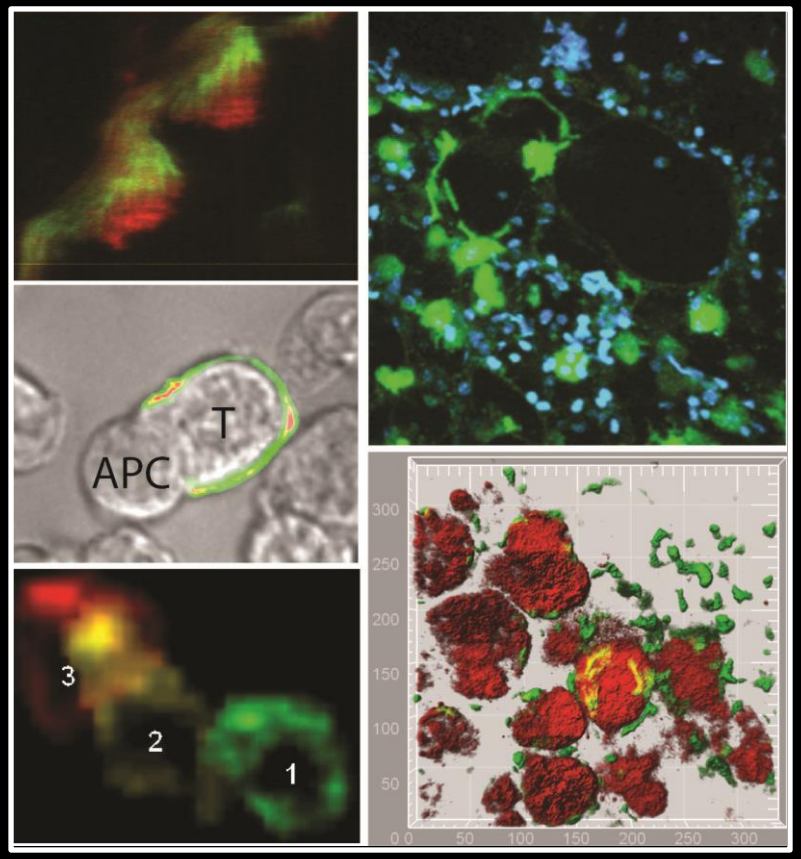
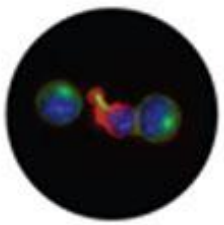
Principles, Benefits and Application of Multi-photon Microscopy .

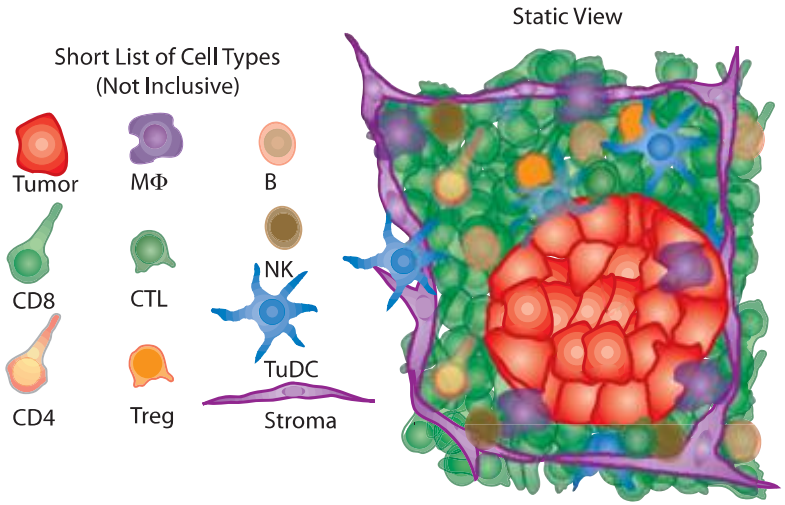


Krummel Lab, Department of Pathology



Biological Imaging Development Center at UCSF University of California San Francisco

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

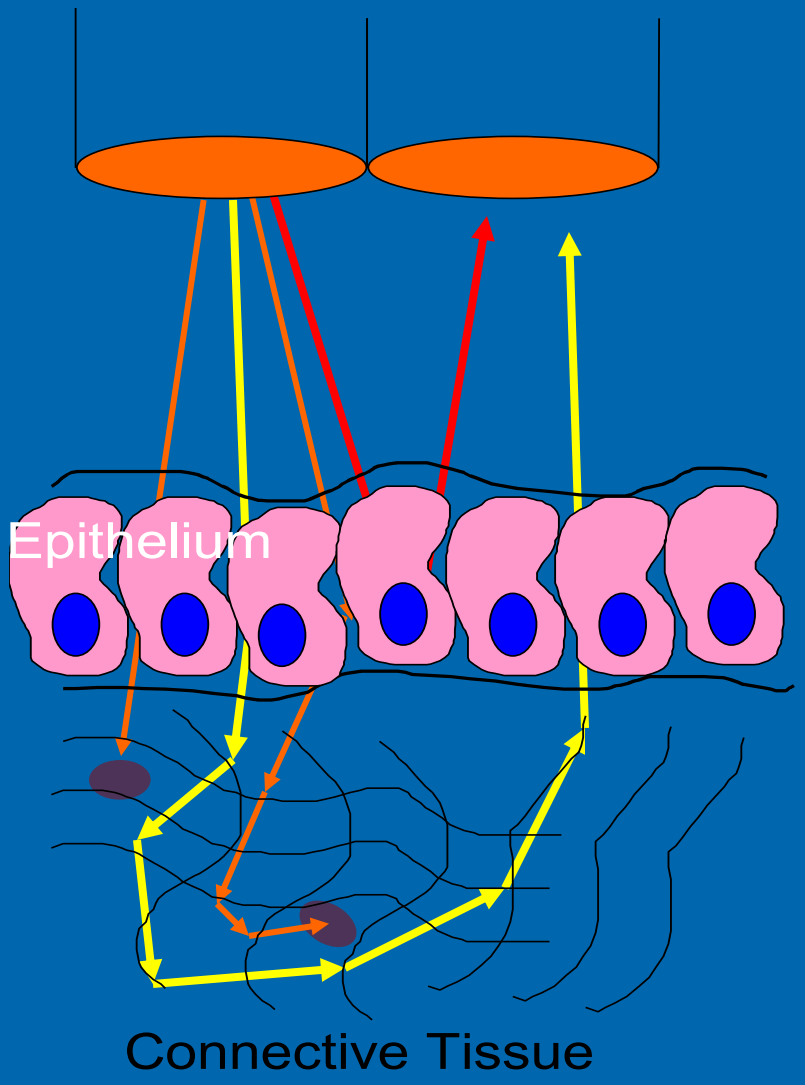
“Multiphoton Microscopy”

Lecture Outline

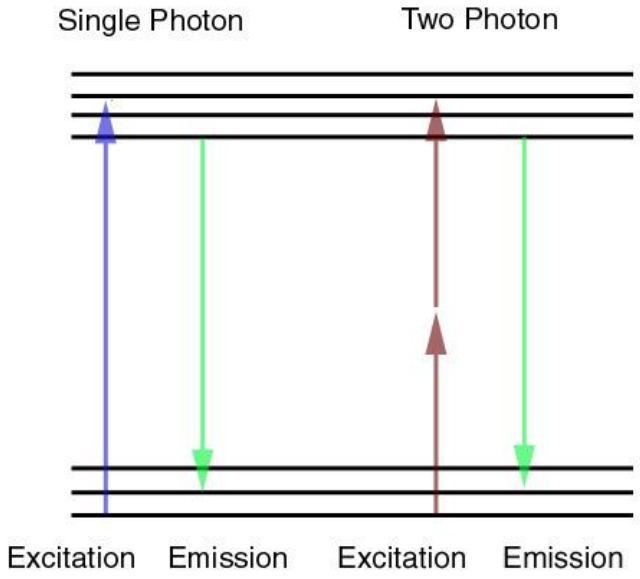
1. The Root Reason for 2P Microscopy
2. The Principle.
3. Fluorophore excitation and the 2nd Harmonic
4. Non-Descanning Detection.
5. That's a Mighty Big Laser You've Got There.
6. Resonant versus Galvo-based Scanning.
7. Autofluorescence—the continual scourge.
8. 3P and beyond
9. To the Deep: Adaptive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

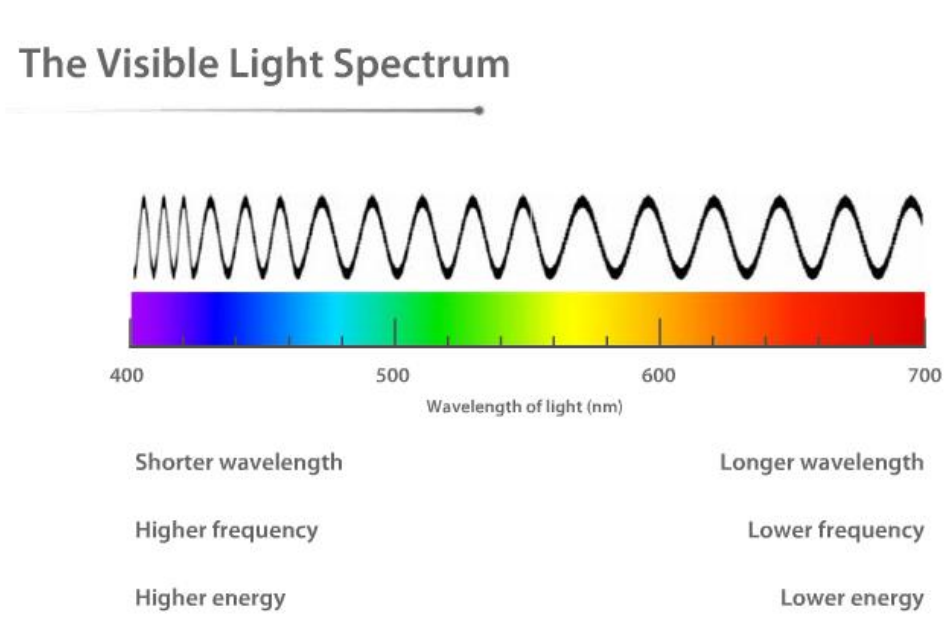
Tissues absorb & scatter light

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



2-Photon Fluorescence: How and Why?

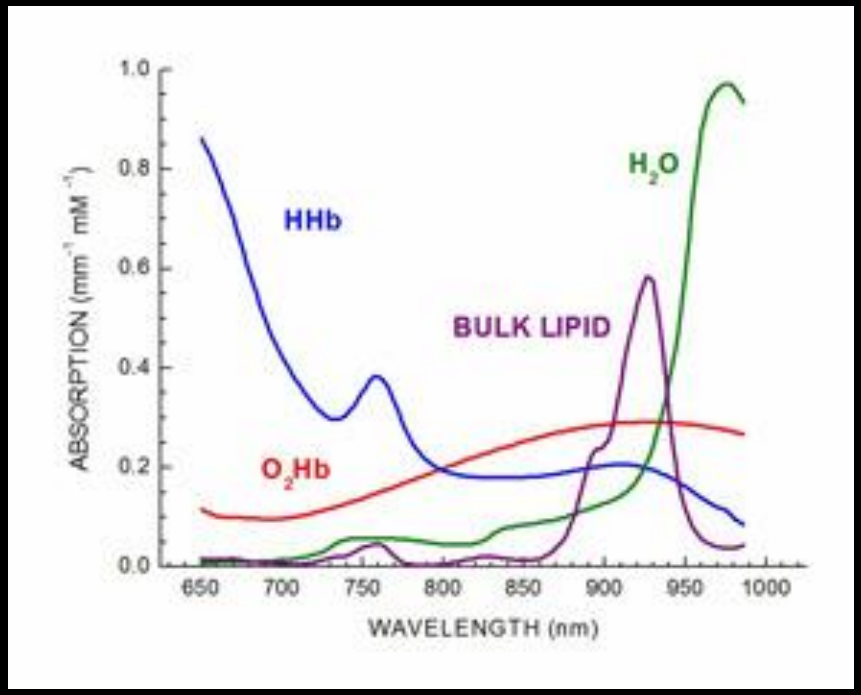




How: Two Photons of half the energy (twice the wavelength), if temporally concident, sum their energies (2x0.5=1x) to drive fluorophore to activated state. (NOTE: This requires very high powered, very expensive lasers).

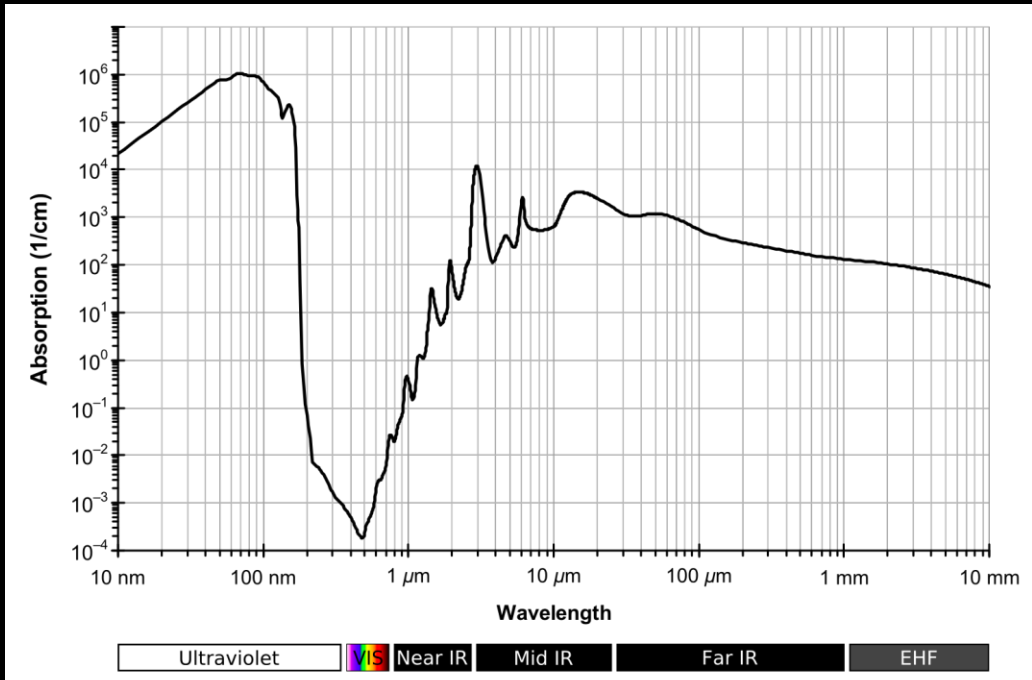
Why? Thought experiment:
Flashlight/you as kid/ Camping

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

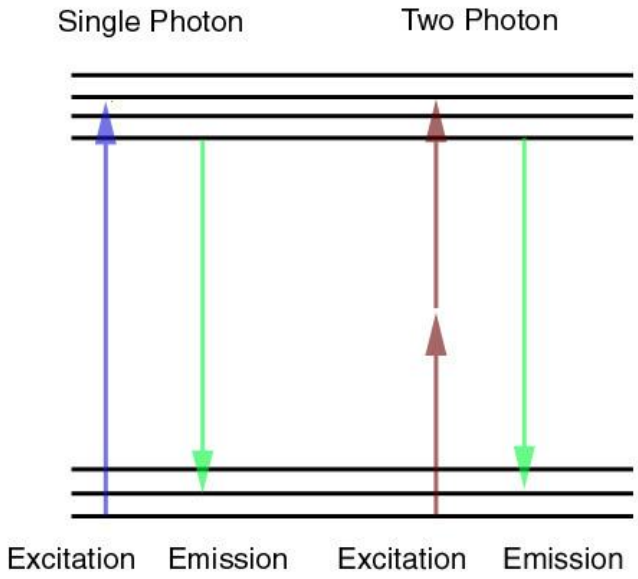


Adipose does tend to burn up… crispy!

Water Spectra



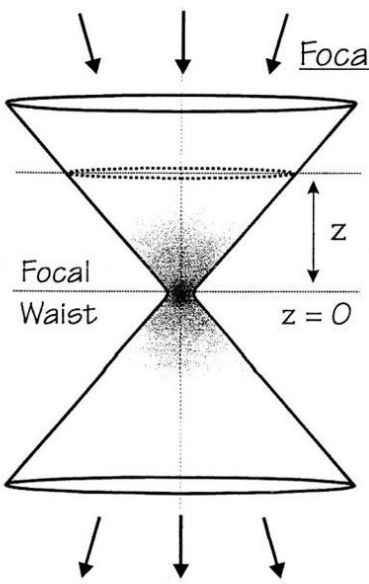
2-Photon Fluorescence: How and Why?



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

- No absorbtion 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	Two-Photon
Illuminating Flux	~constant	~constant
Cross Section Area	$\sim \propto z^2$	$\sim \propto z^2$
Illuminating Intensity	$\propto 1/z^2$	$\propto 1/z^2$
Excitation Probability	$\propto 1/z^2$	$\propto 1/z^4$
# Excitation Events	~ constant	$\propto 1/z^2$

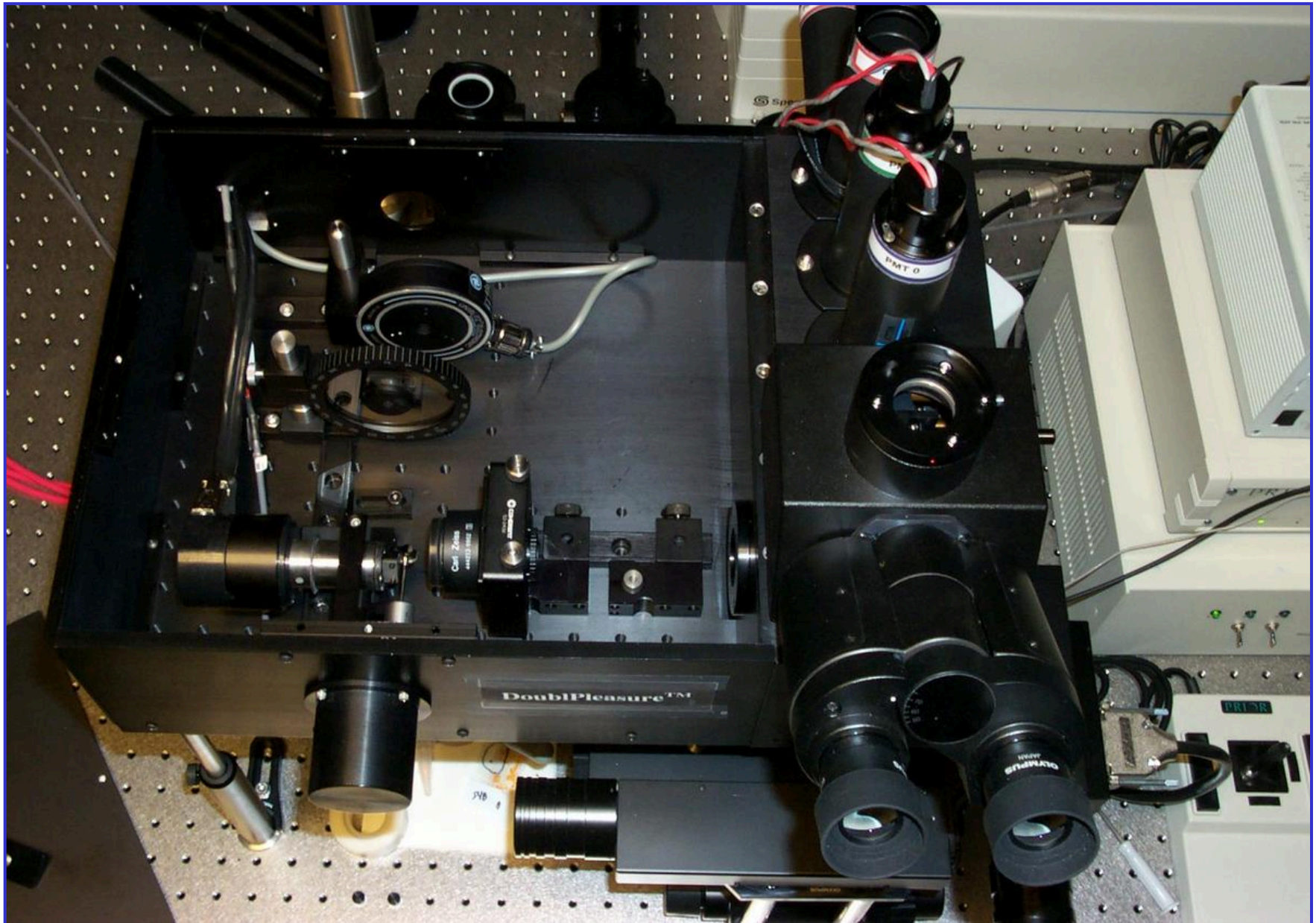
Events / Section $\propto$ Probability * Area

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

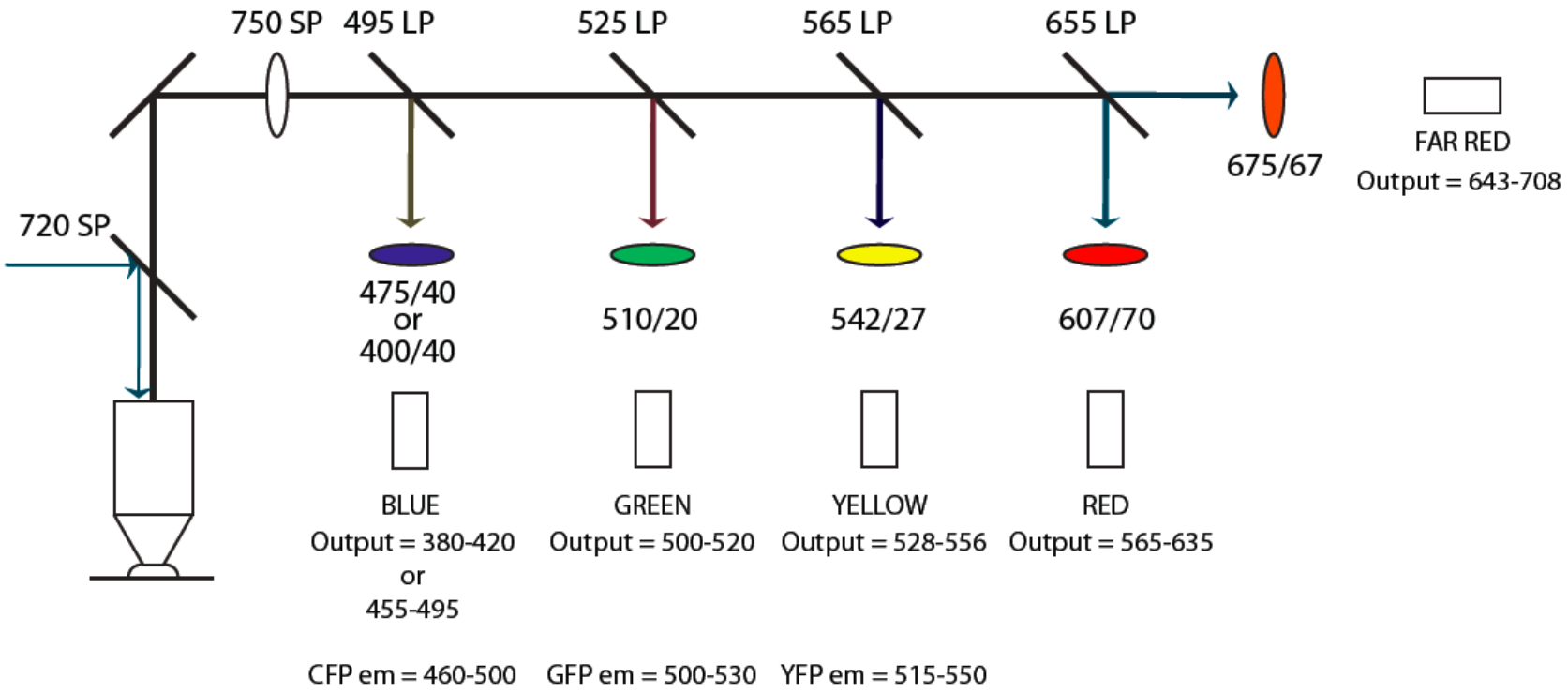
Single-Photon process produces excitation (and photodamage) throughout specimen volume. Optical sectioning requires imaging and spatial filtration of emission light, which is inherently inefficient and is highly susceptible to light scattering losses.

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..

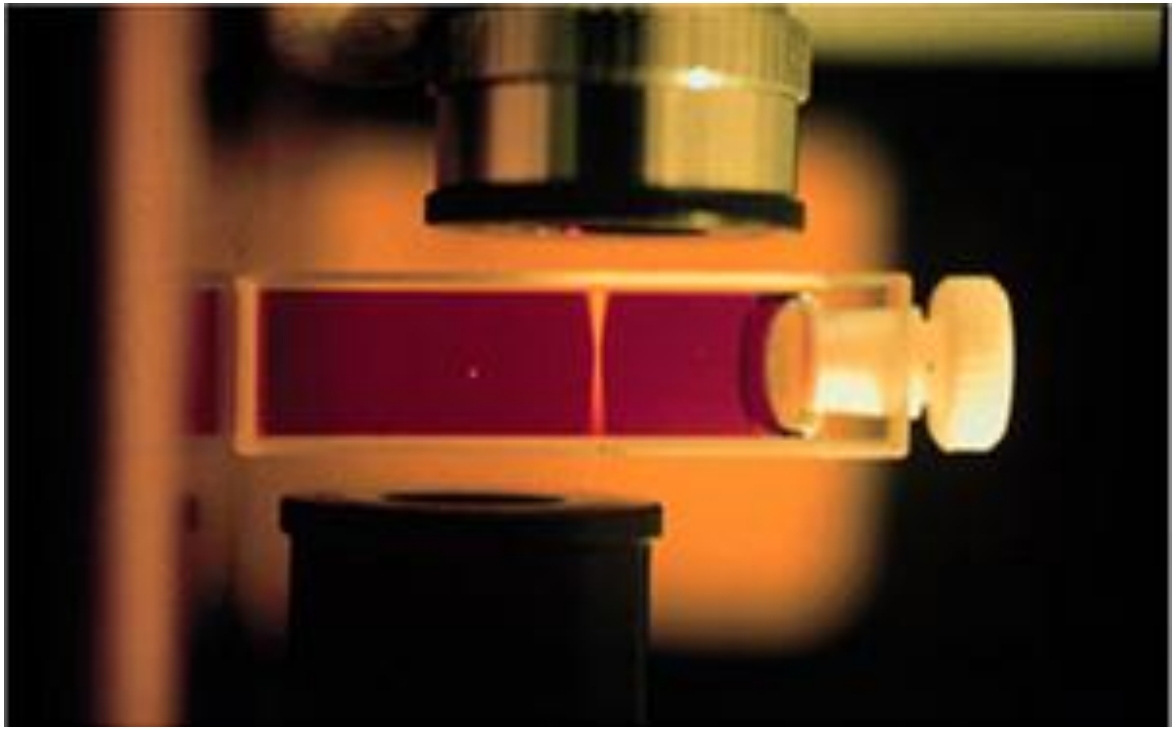
This can be done quite simply…



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

Peaks

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	*J Biol Chem* (2004) 279:37544–37550
Alexa Fluor 594 hydrazide	810 nm	Ca^{2+}-insensitive, neuronal tracer *	*Neuron* (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	*J Neurosci* (2003) 23:2212–2217
CFSE, CMTMR	820 nm	Tracking T and B lymphocytes and dendritic cell motility patterns in intact mouse lymph nodes †	*Science* (2002) 296: 1869–1873; Proc Natl Acad Sci U S A (2004) 101: 998–1003
CM-H_2DCFDA	740 nm	Detection of localized reactive oxygen species release in cardiomyocytes ‡	*J Biol Chem* (2003) 278: 44735–44744
DAPI, Hoechst 33342	740 nm	Imaging DNA in nuclei and isolated chromosomes	*Micron* (2001) 32:679–684; *Histochem Cell Biol* (2000) 114:337–345
DiD	817 nm	Intravital imaging of mouse erythrocytes	*Proc Natl Acad Sci U S A* (2005) 102:16807–16812
FM 1-43	840 nm	Monitoring synaptic vesicle recycling in rat brain slices	*Biotechniques* (2006) 40:343–349
Fluo-5F §	810 nm	Imaging Ca^{2+} concentration dynamics in dendrites and dendritic spines	*Neuron* (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	*J Physiol* (2001) 536:429–437

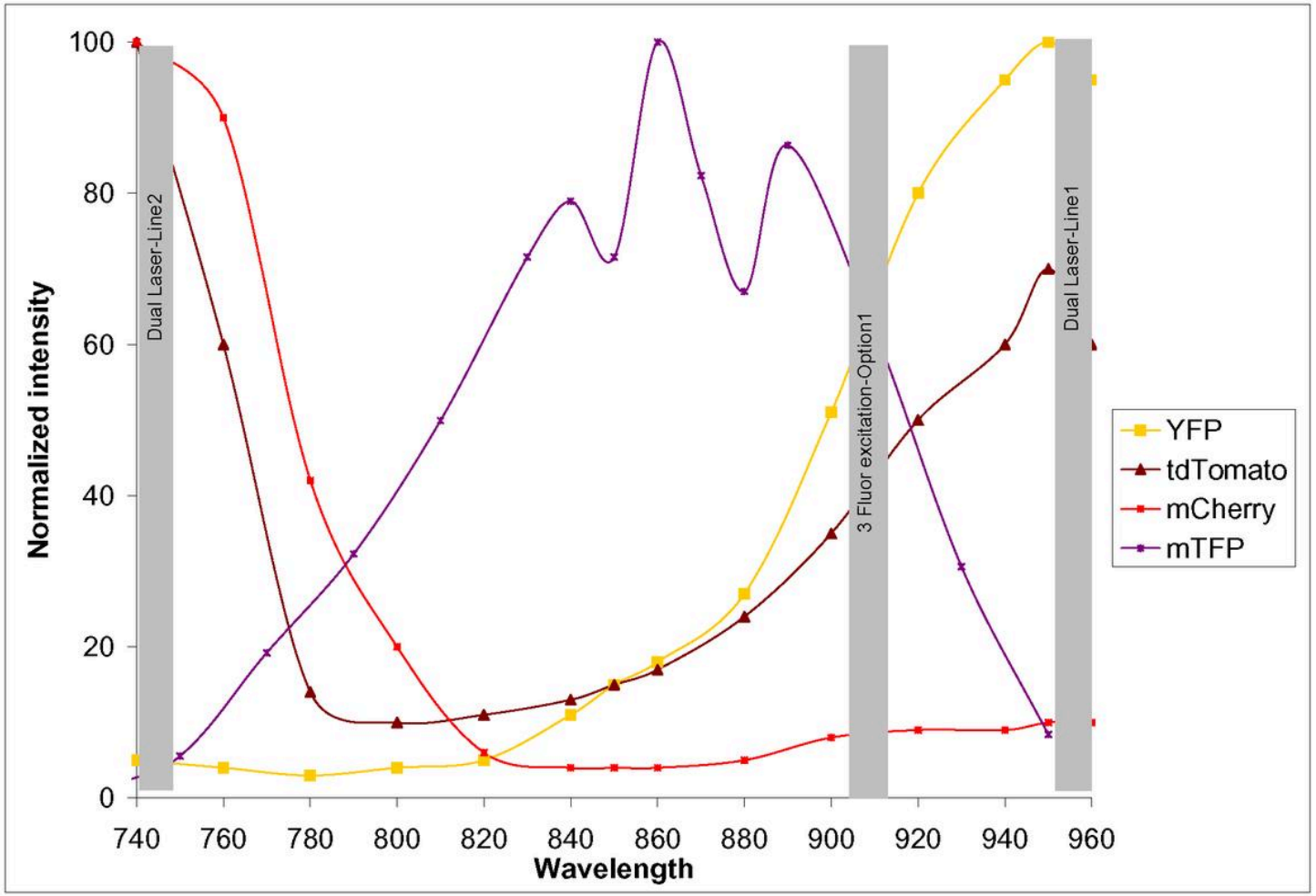
Excitation is not exactly 2x 1P λ

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

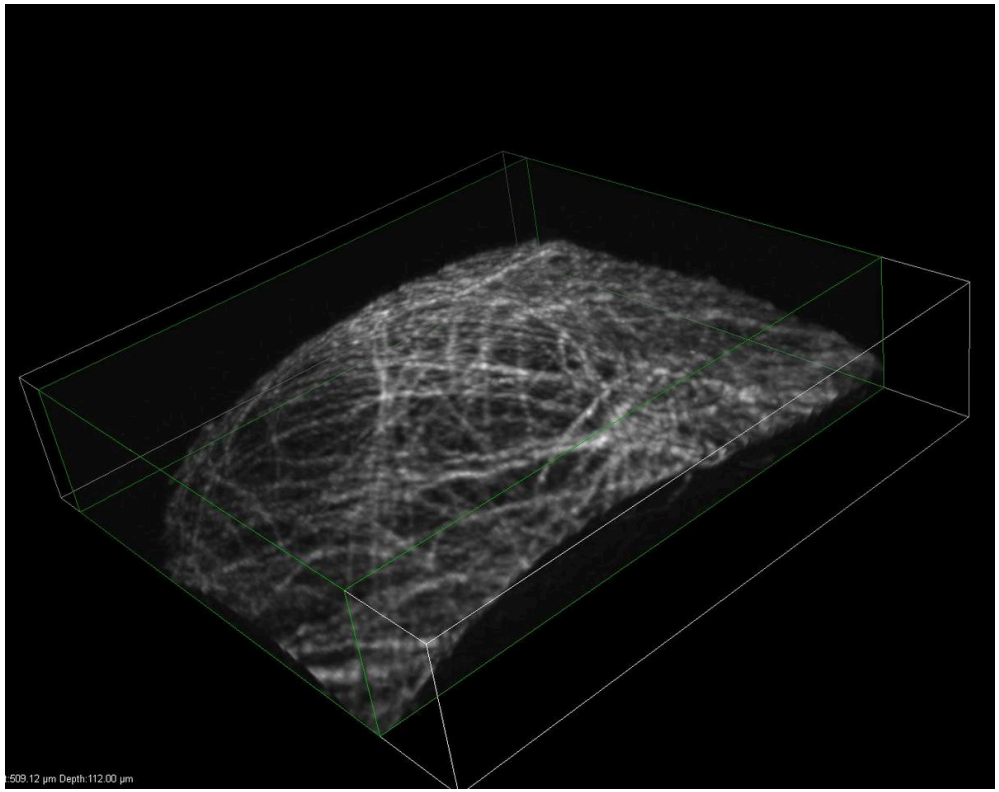


2nd Harmonic Signal: Fiducials for 'Free'

Some anisotropic molecules like collagen will frequency-double light and thereby generate an emission with ½ of the incident λ

Collagen I and IV
Cellulose
Some membrane dyes

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



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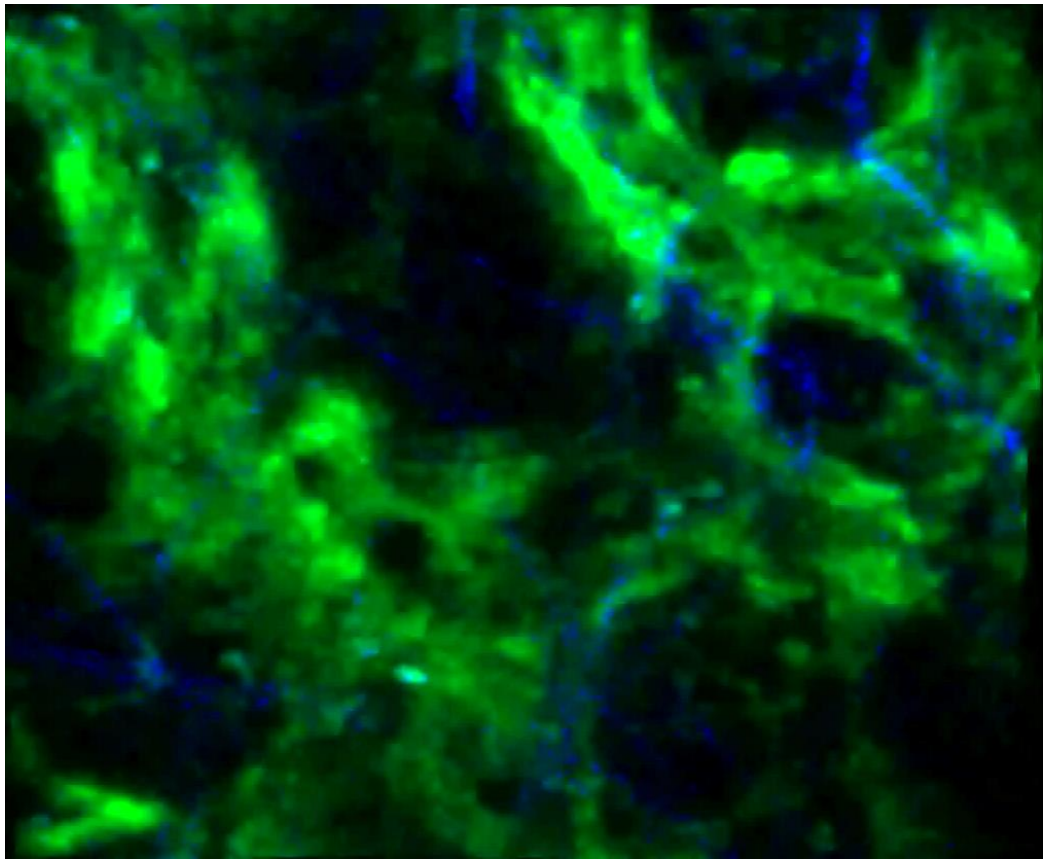
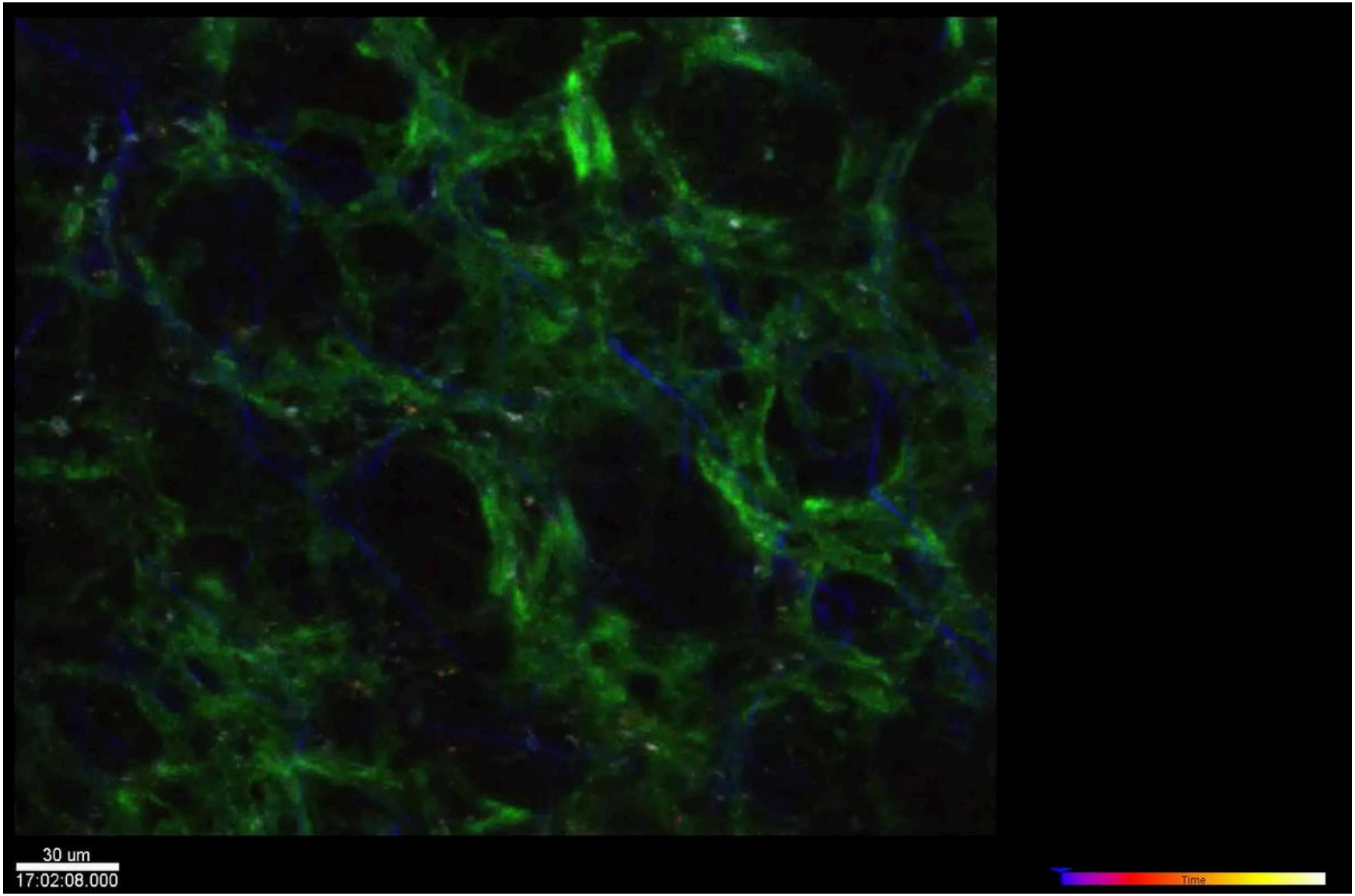
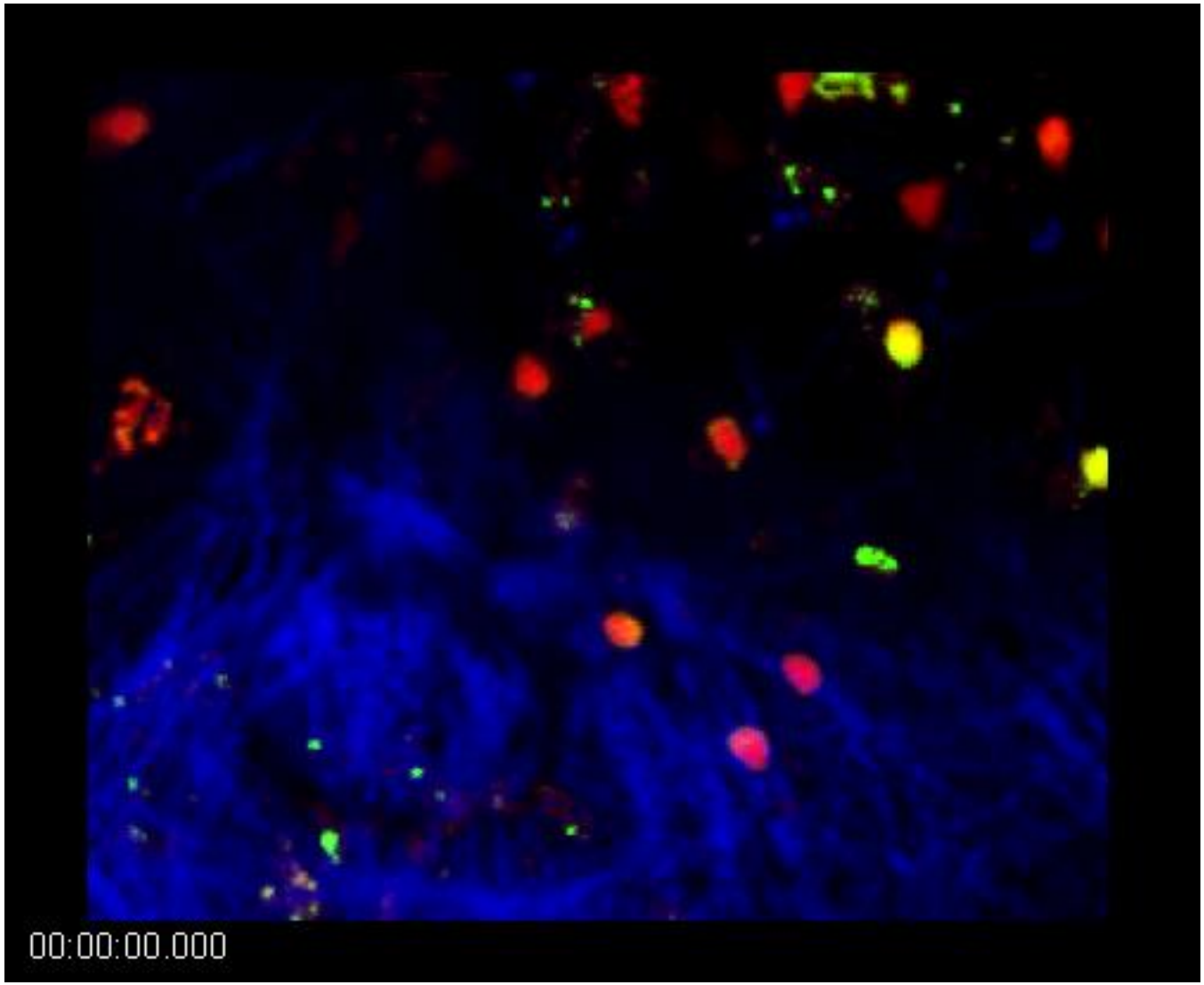


2nd Harmonic Signal: Fiducials for 'Free'

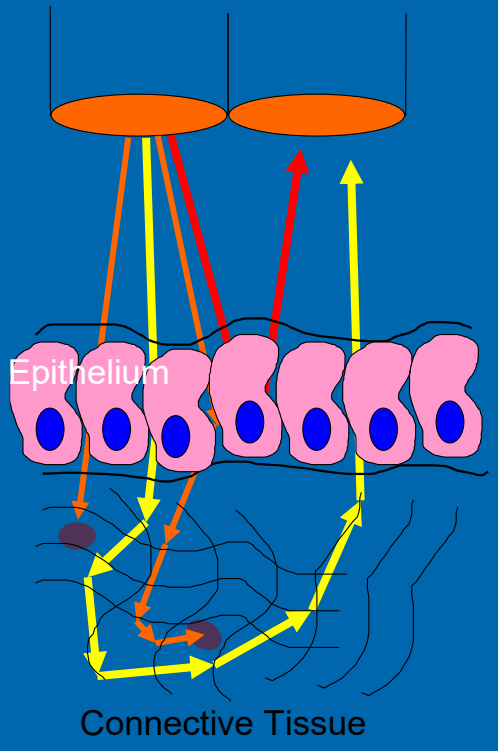


2nd Harmonic: LN Capsule

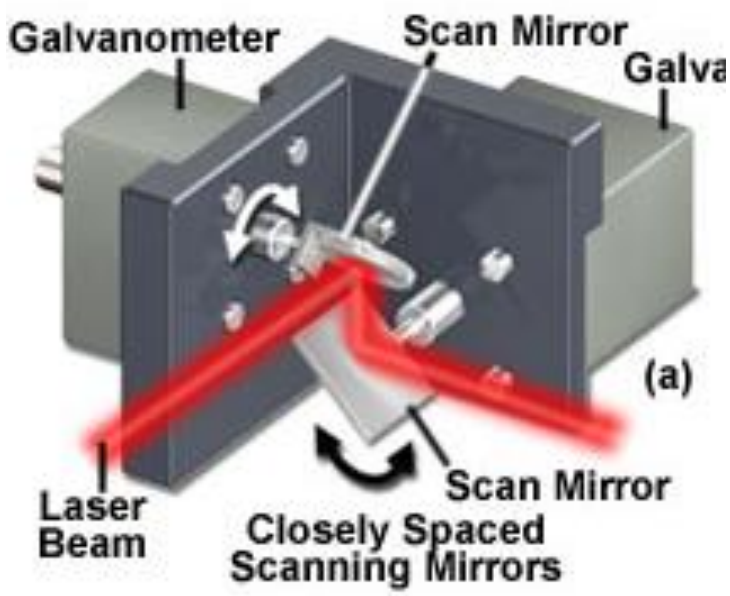


Returning to the issue: Tissues absorb & scatter light

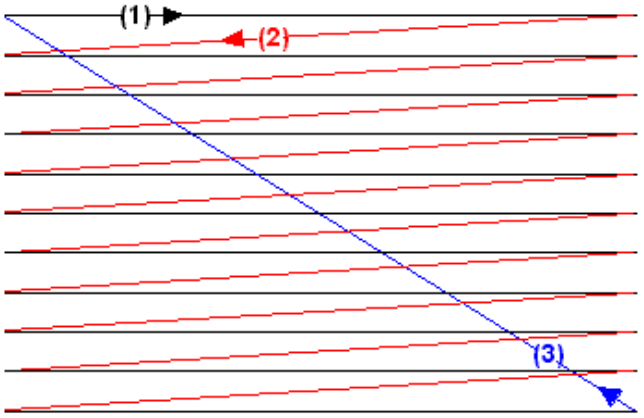
Should a sensitive 2-Photon Collect Without a Pinhole?



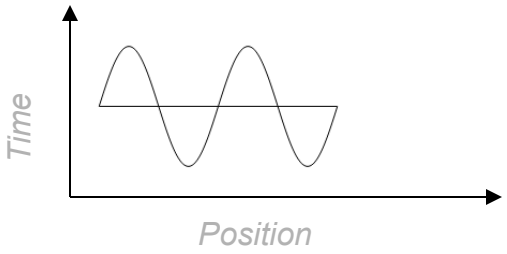
Recall that this is achieved via:
The Raster Scan.
This allows temporal assignment of all light coming from an excited spot.



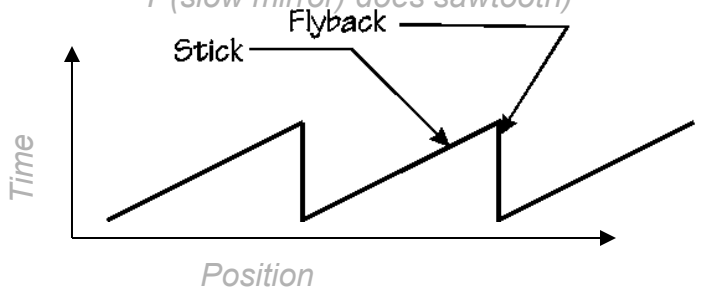
A small spot (laser beam)
Scans the sample

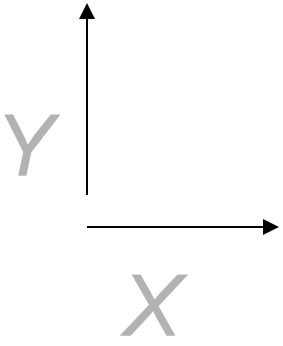


X(fast mirror) follows a sine curve



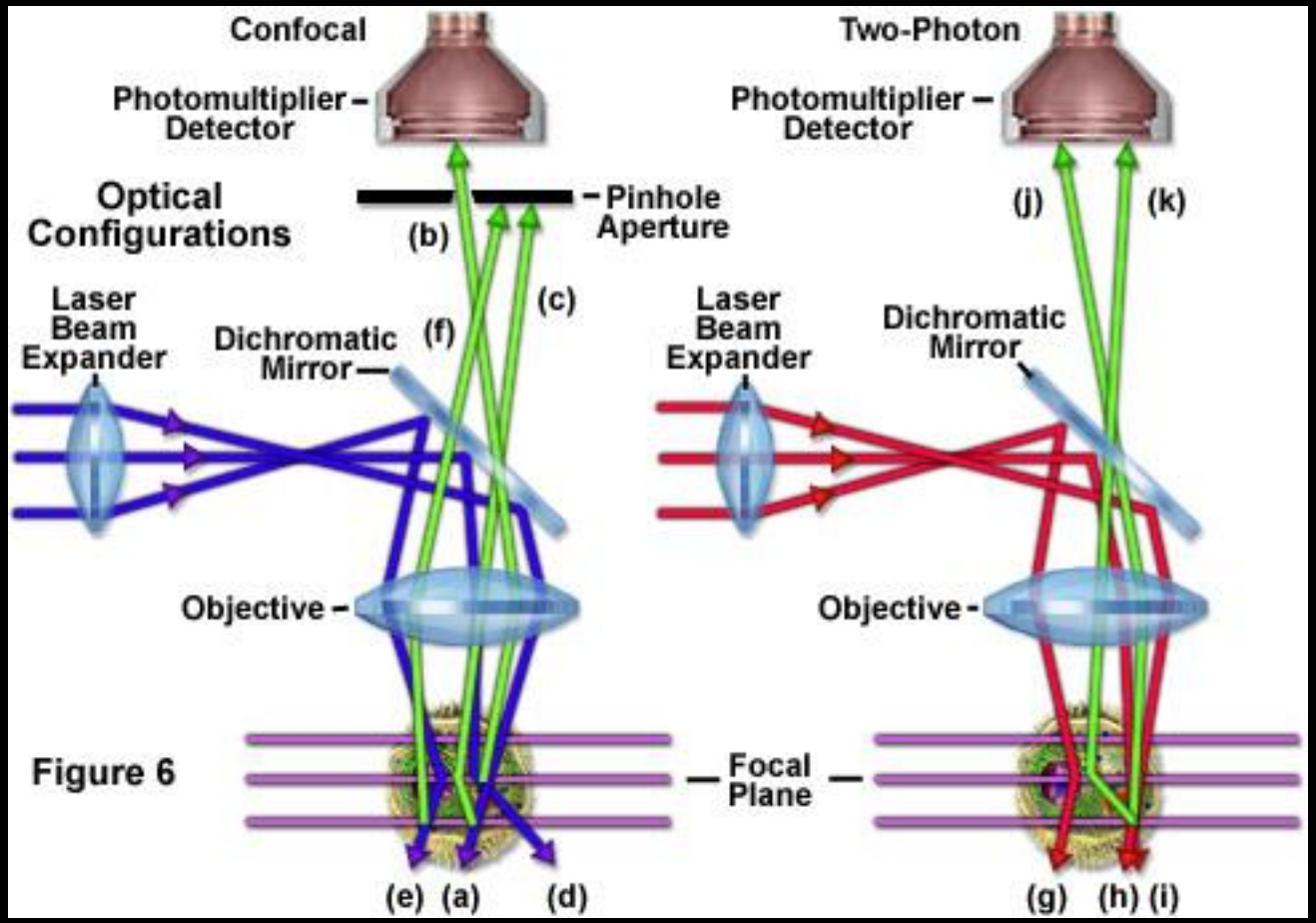
Y (slow mirror) does sawtooth)



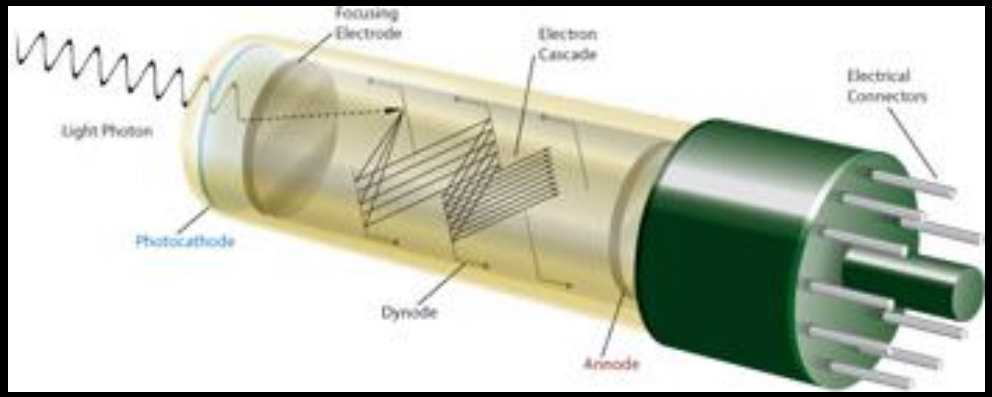


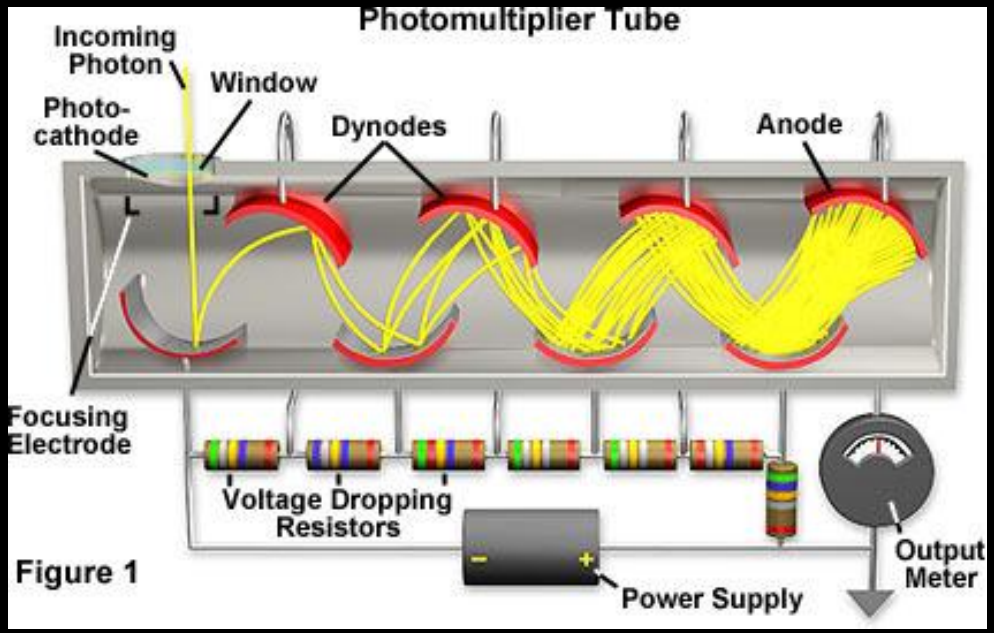
Ergo: Collect All Emission at detector (regardless of path)

The Most sensitive 2P microscopy employs non-descanned detectors



2P scopes use PMTs as detectors



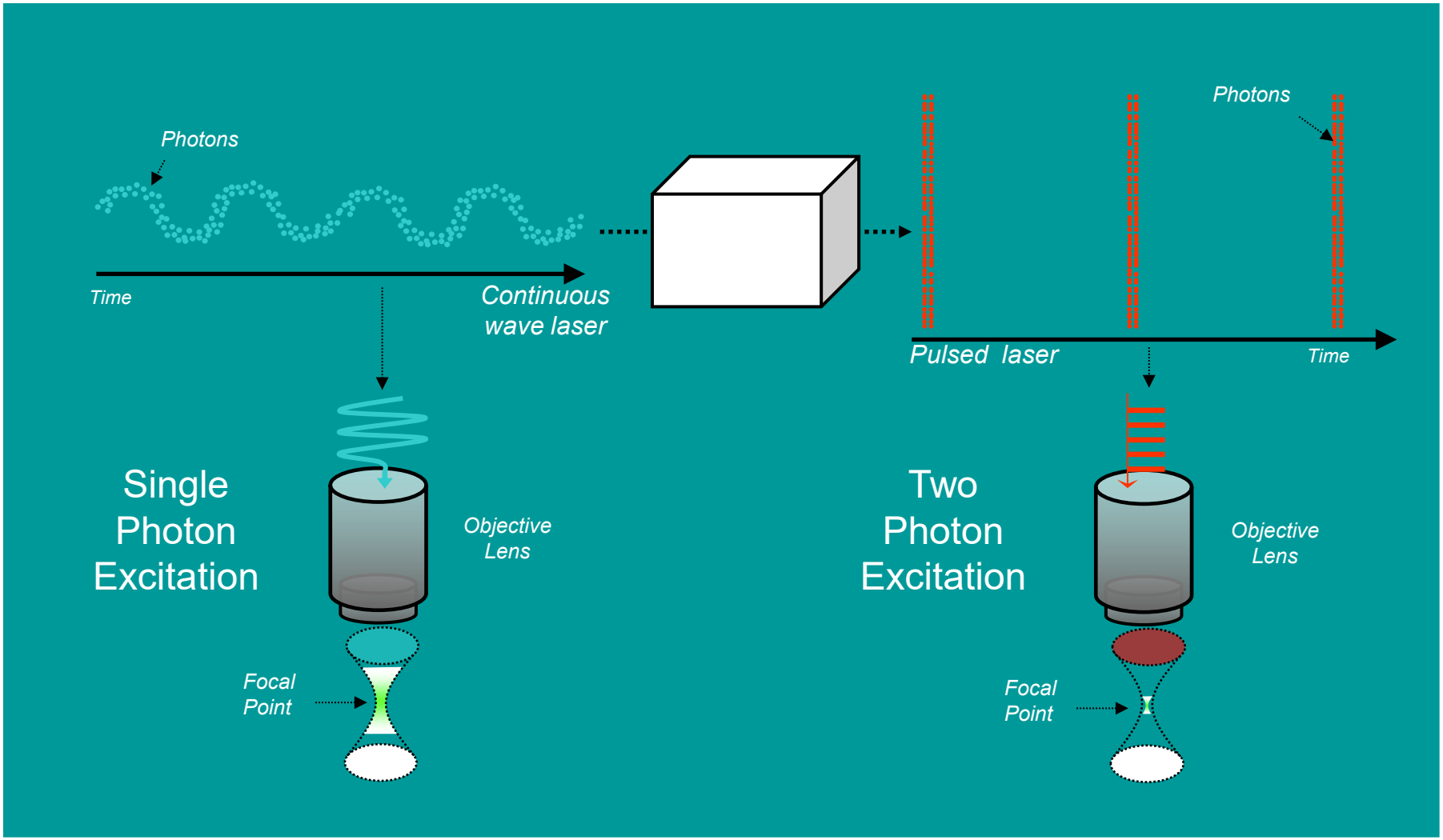


Extra Credit:

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

Why don't we use them?

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/mm^2***

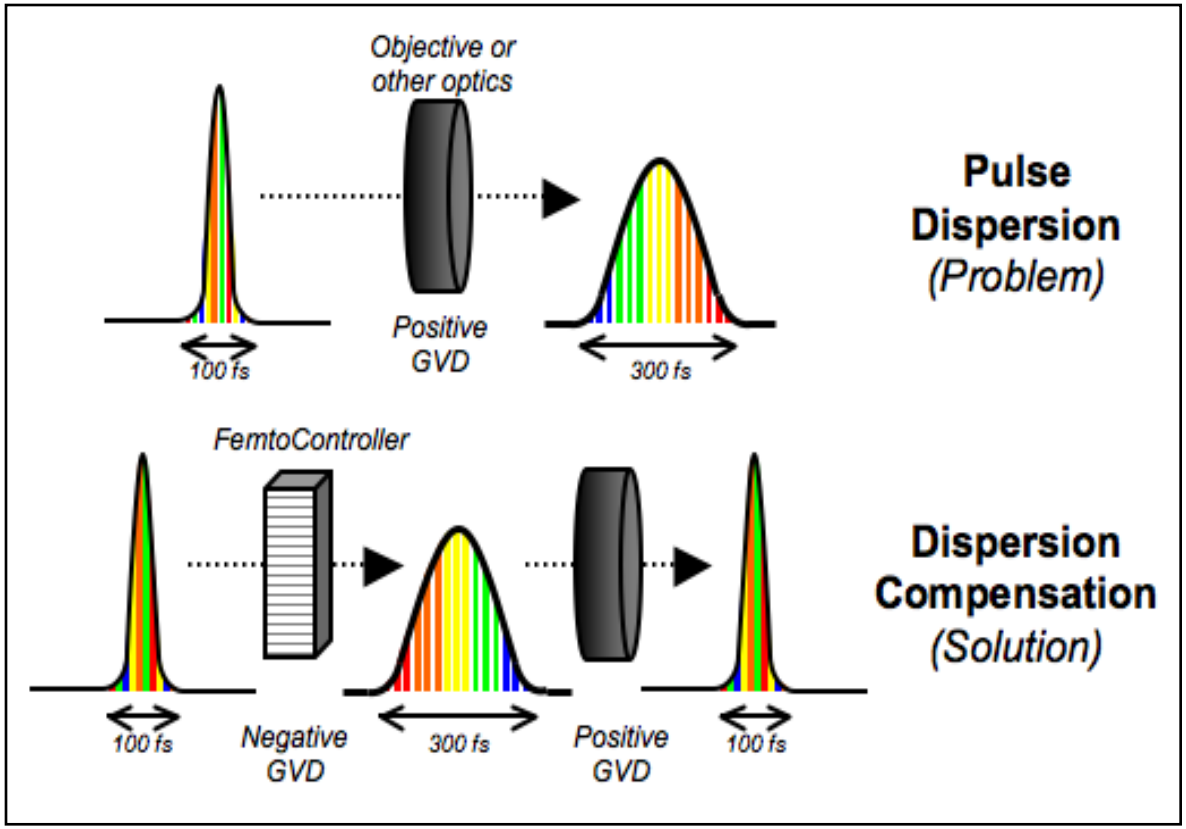
A 2P laser is pumped by a 5-20W pump resulting up to 1W of pulsed power at the objective which is focused to a spot size of ~0.5um diameter. This is a power density of ***~5 MW/mm^2***

However it has a duty cycle of ~10^{-5} (the 'duty cycle' is pulsewidth x rep rate).
The pulsewidths are around 70-200fs (fs = 10^{-15}s) 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* ***5x10^{11}W/mm^2***
or ***~10^{11}x*** *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

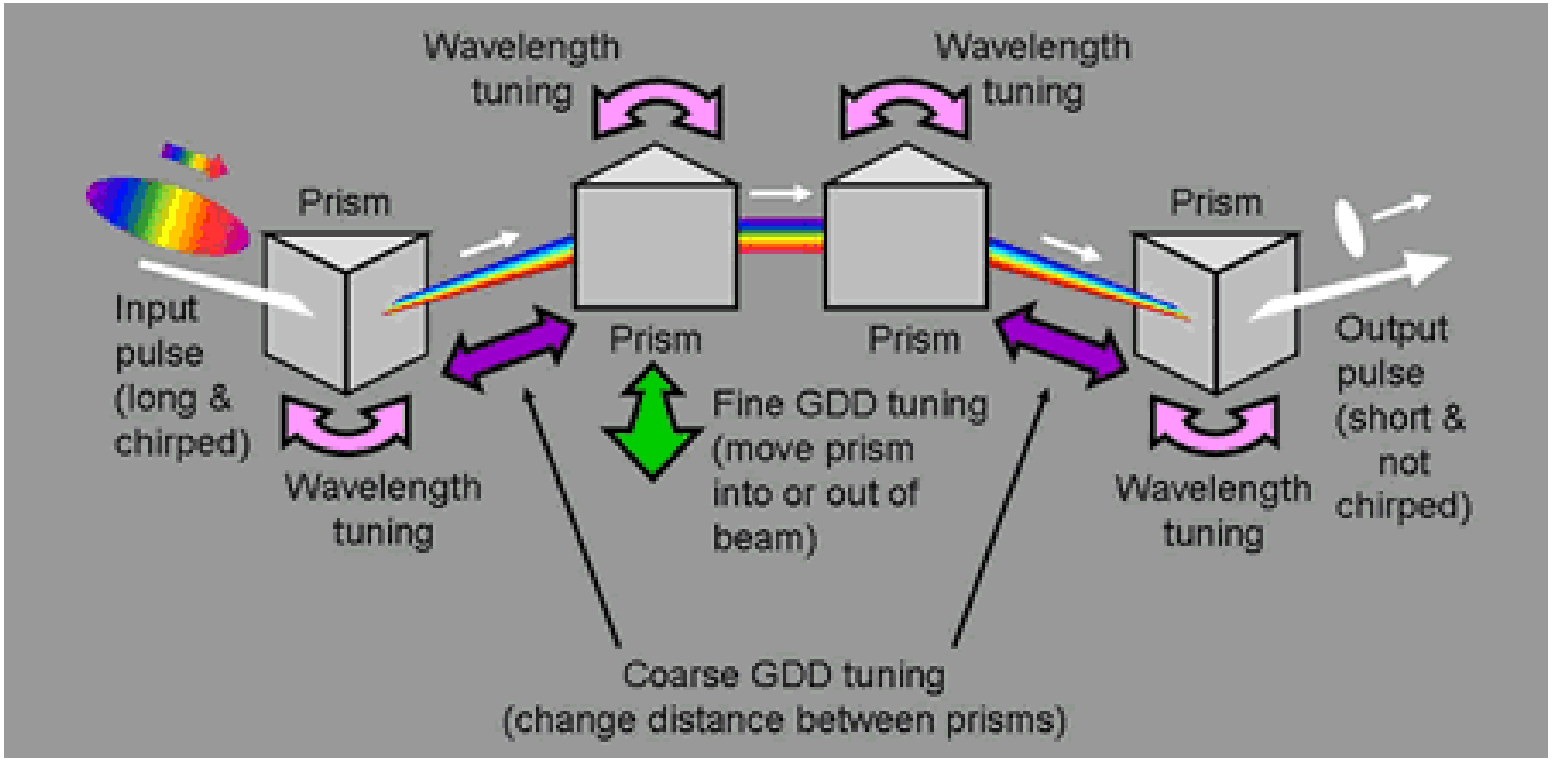


These can be bundled in the laser:
e.g
S/P MaiTai ‘DeepSee’
Chamelion ‘Vision II’

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



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.

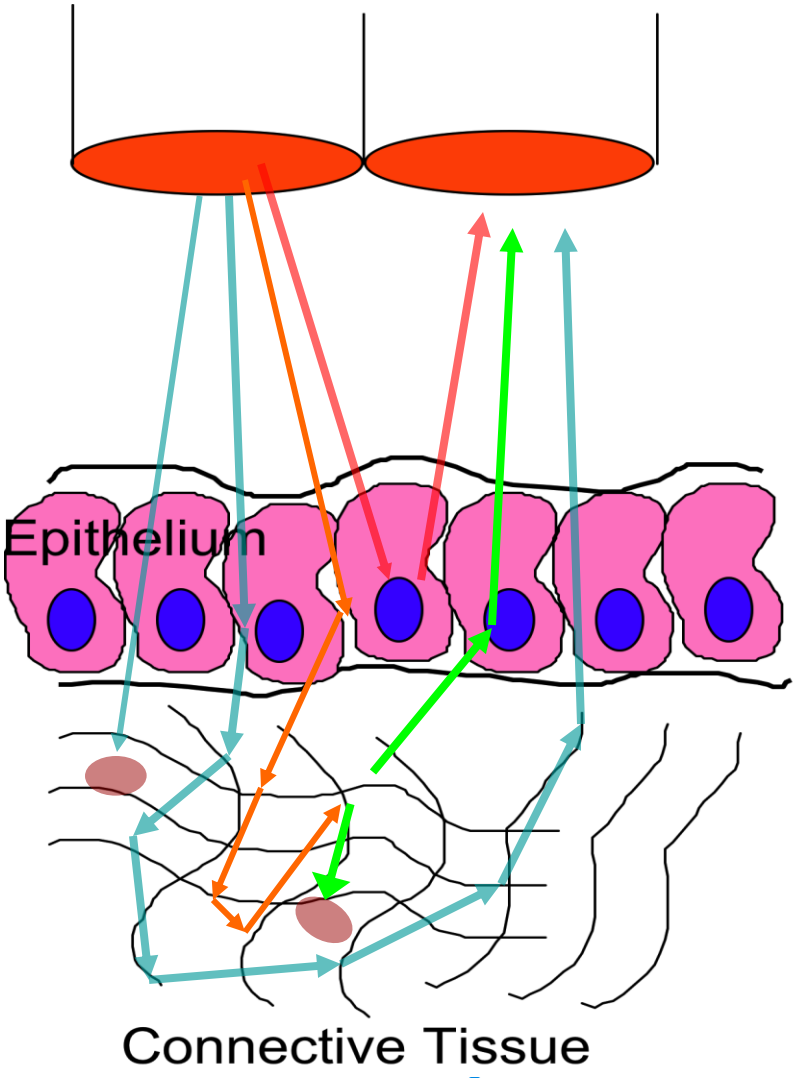
Since pulses are so bright, the pixel dwell time can be important.
Molecules like GFP can bleach if illuminated too closely in succession, when in the relaxed state post-activation

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'.

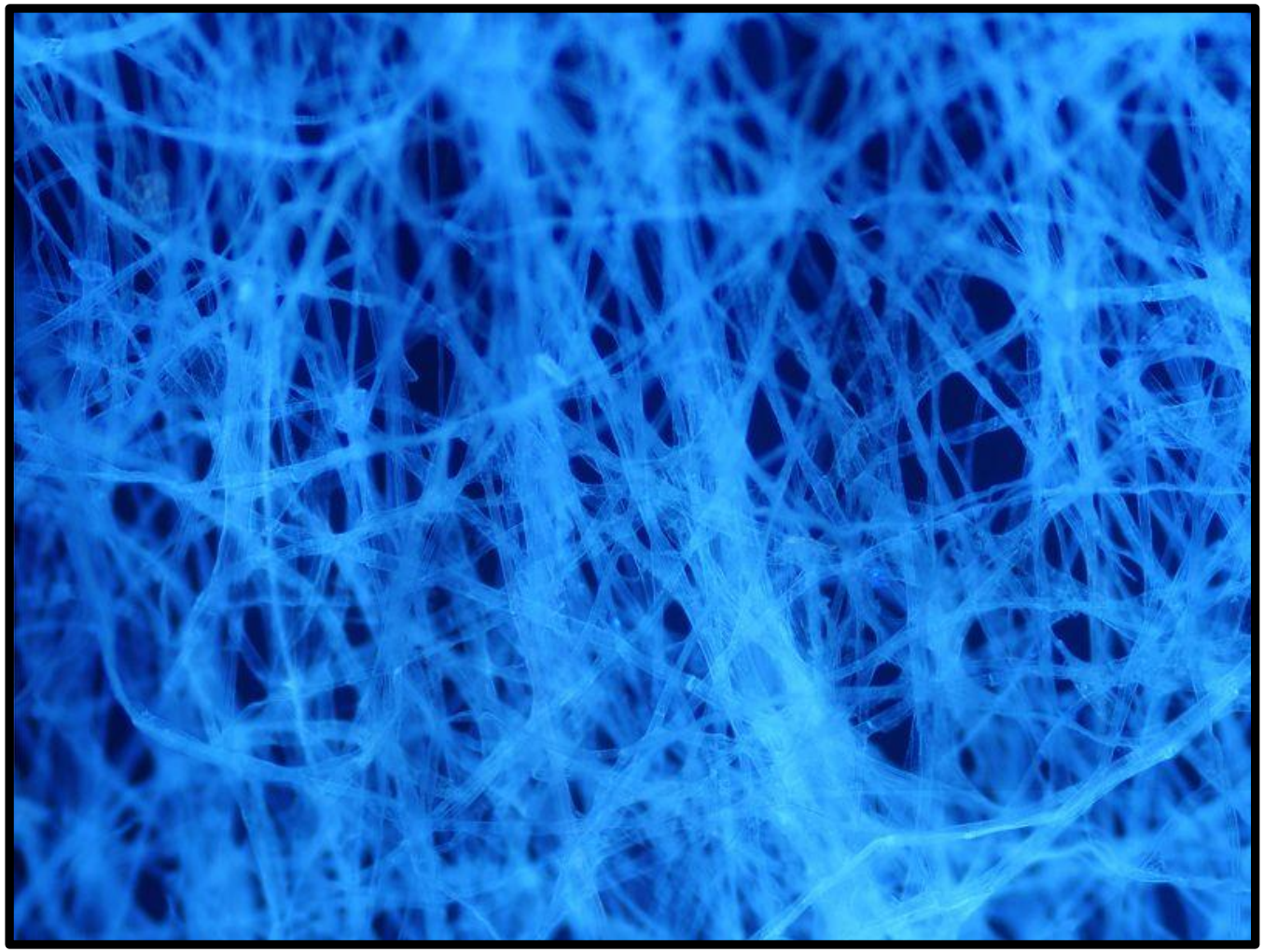
In a galvo system, you may also have a 'dwell time' of 1us but may be tempted to dwell longer to achieve the same S/N. →Bleaching? (You guys may test this).

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)

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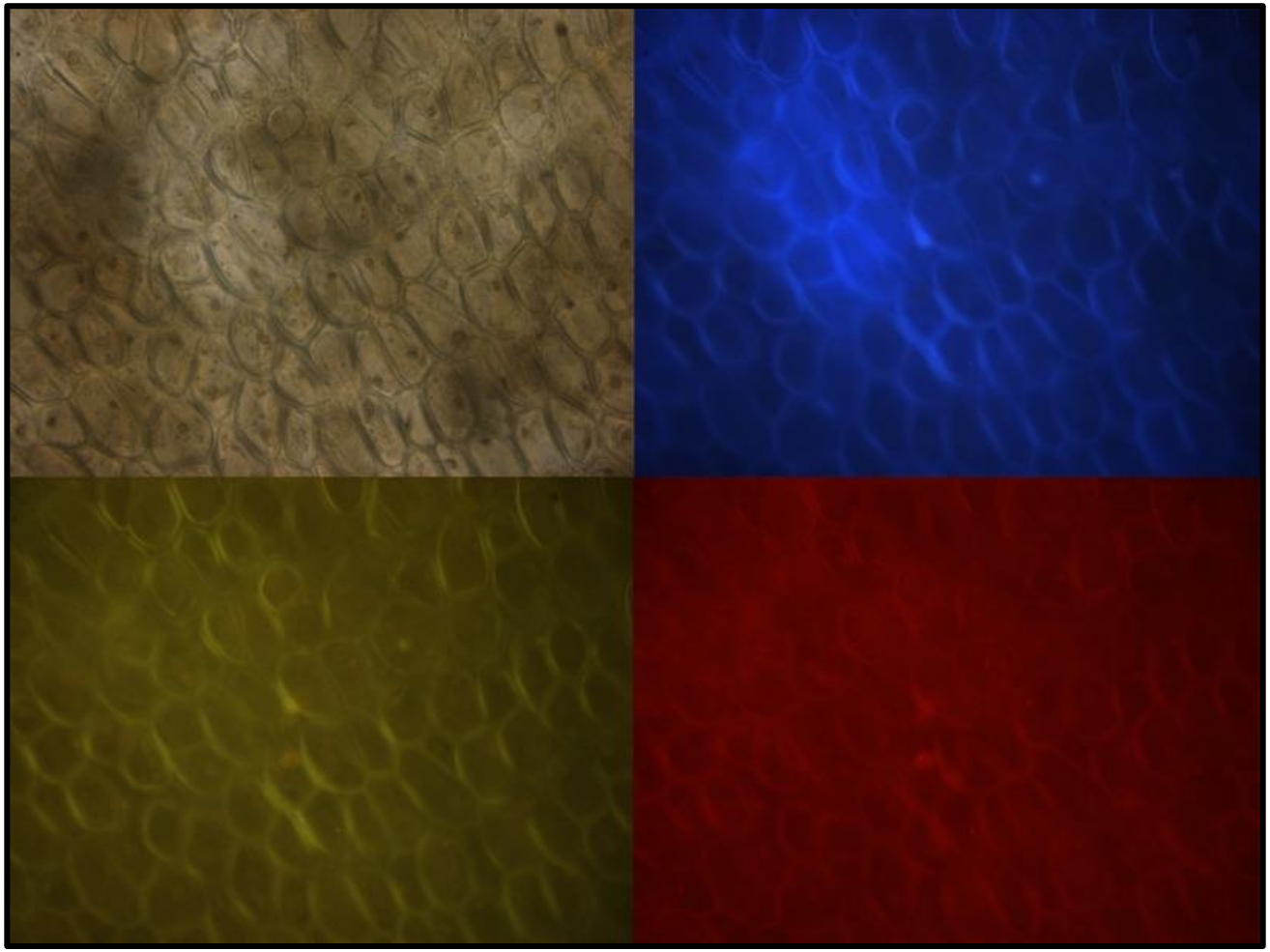


Bananas Autofluorescence (Wikipedia)

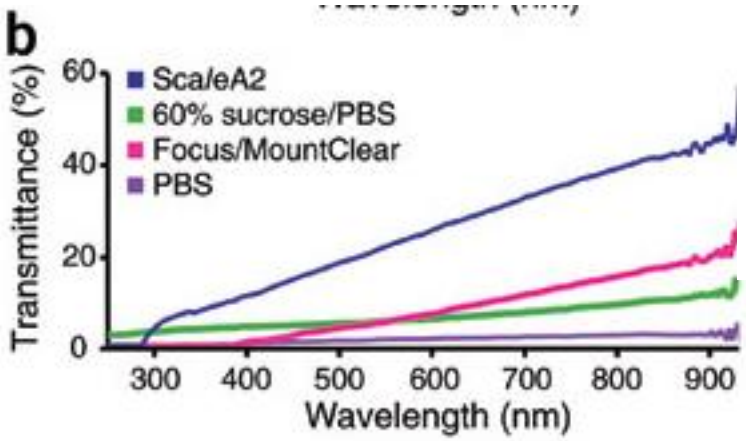
Autofluorescence

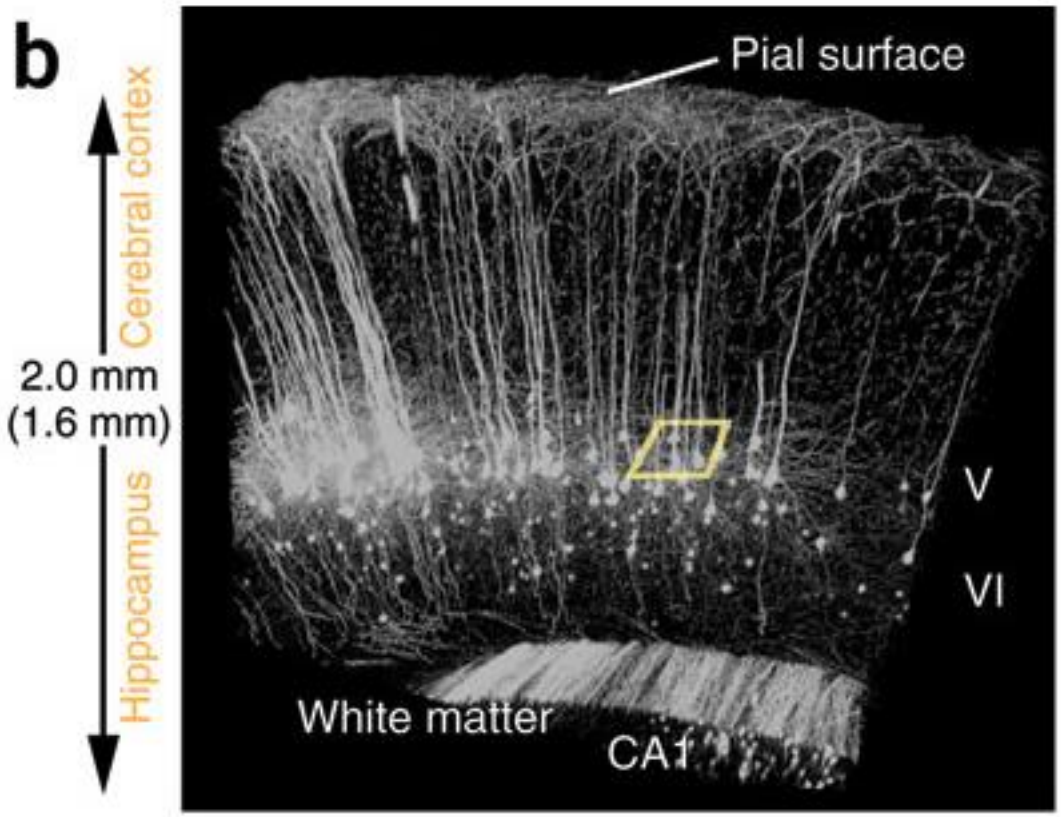
Autofluorescent molecules

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]

(Wikipedia)

Some autofluorescence and scatter can be ‘cleared’



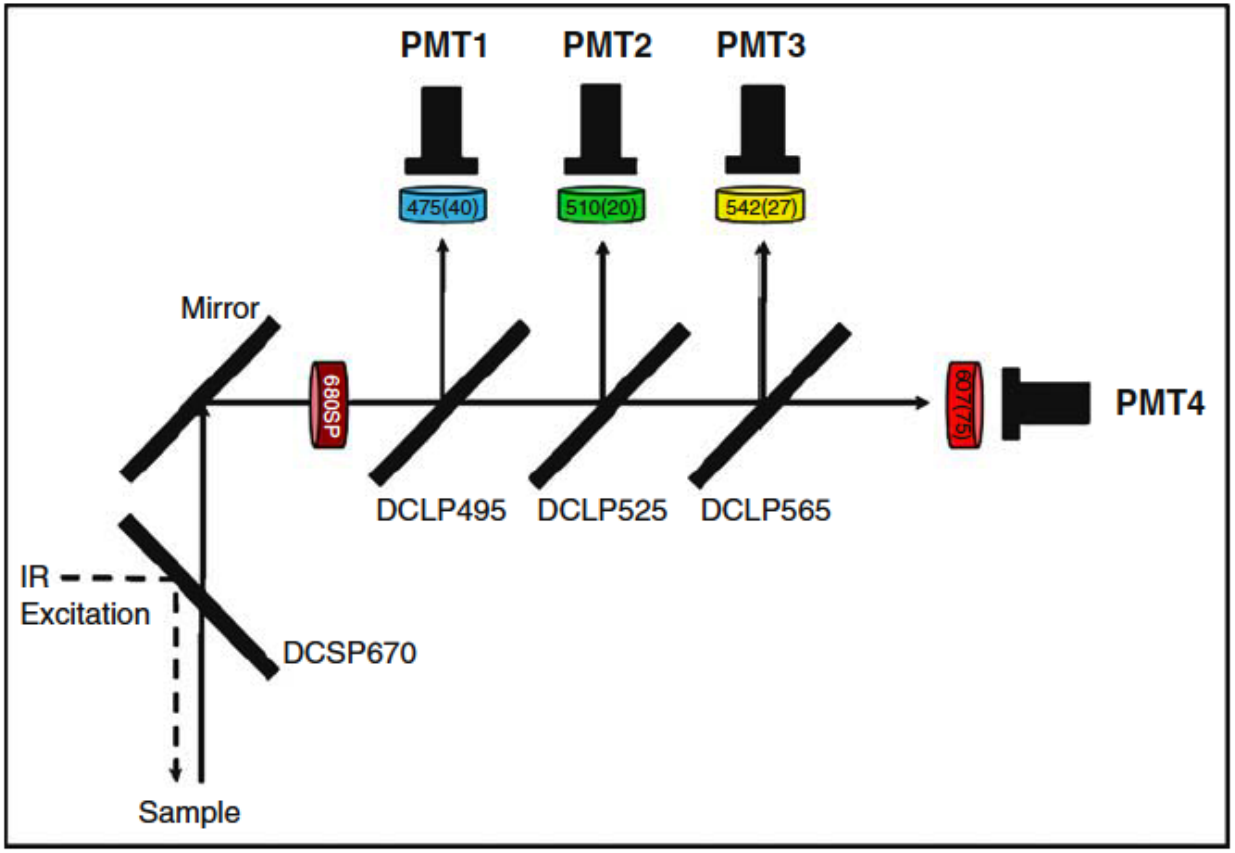


Clearing Agent should leave fluorophore intact

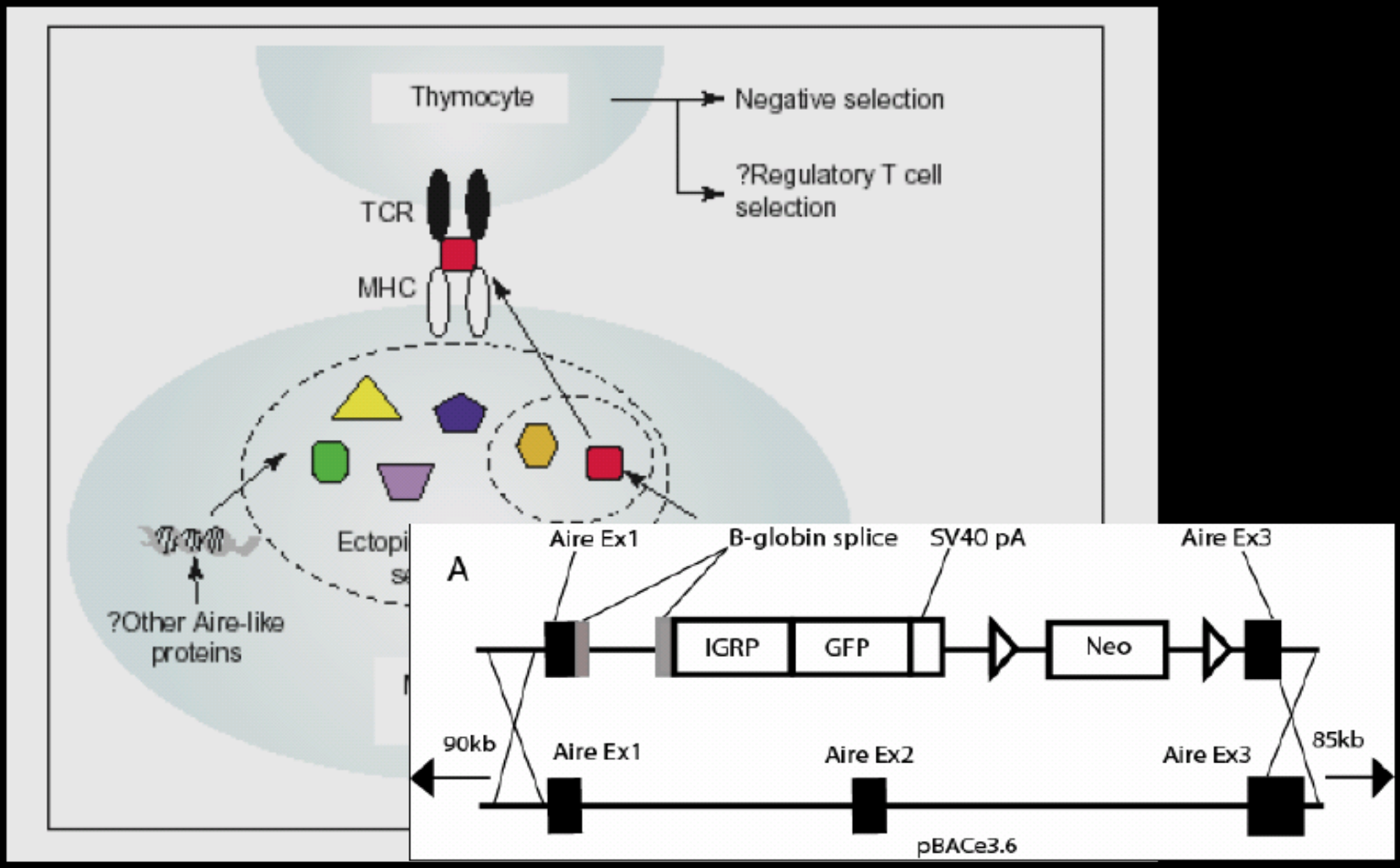
Miywaki et al.

Collecting lots of channels can help with autofluorescence.

GFP Autofluorescence



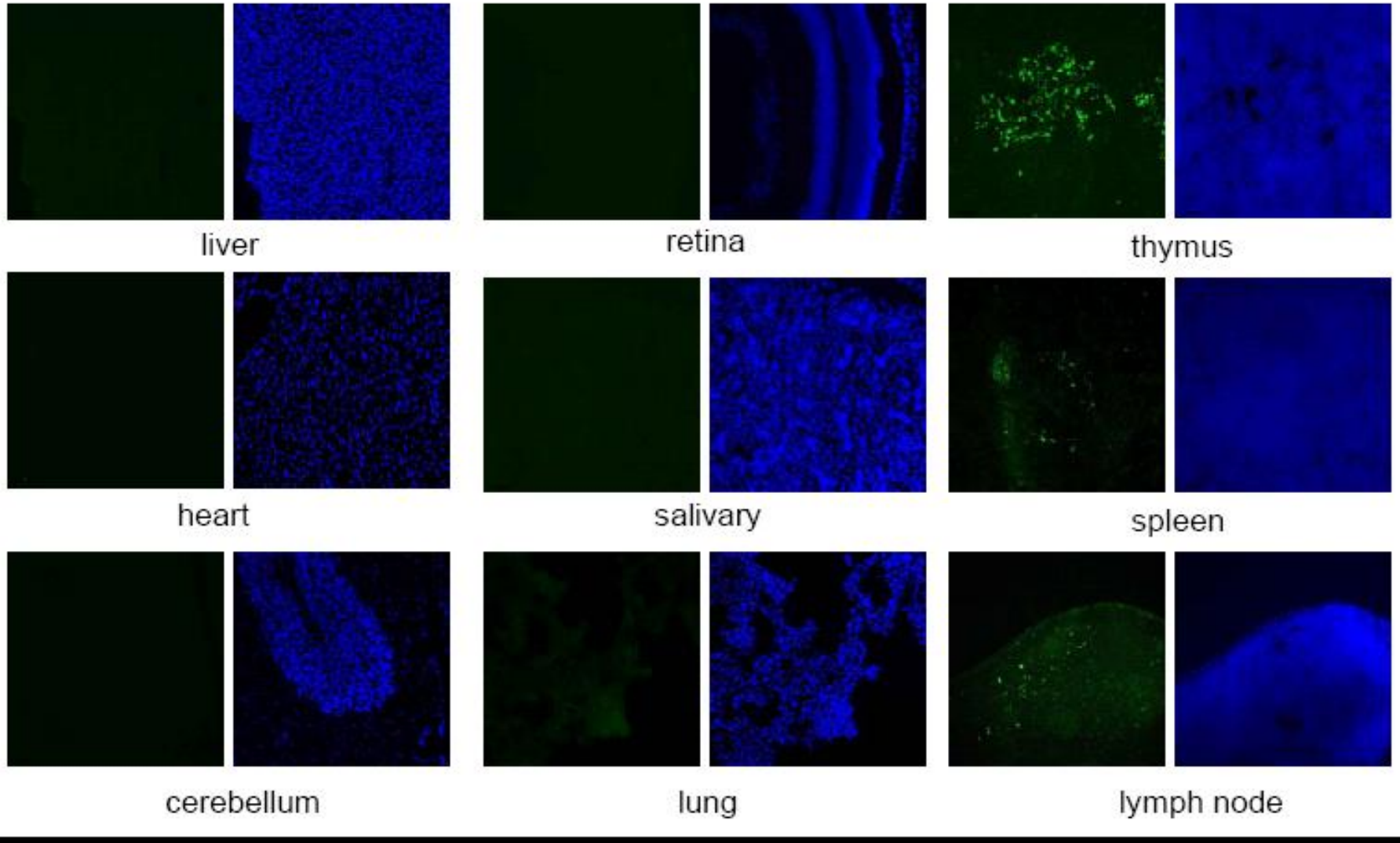
Imaging AIRE-expressing 'Adig' mice to study peripheral tolerance.



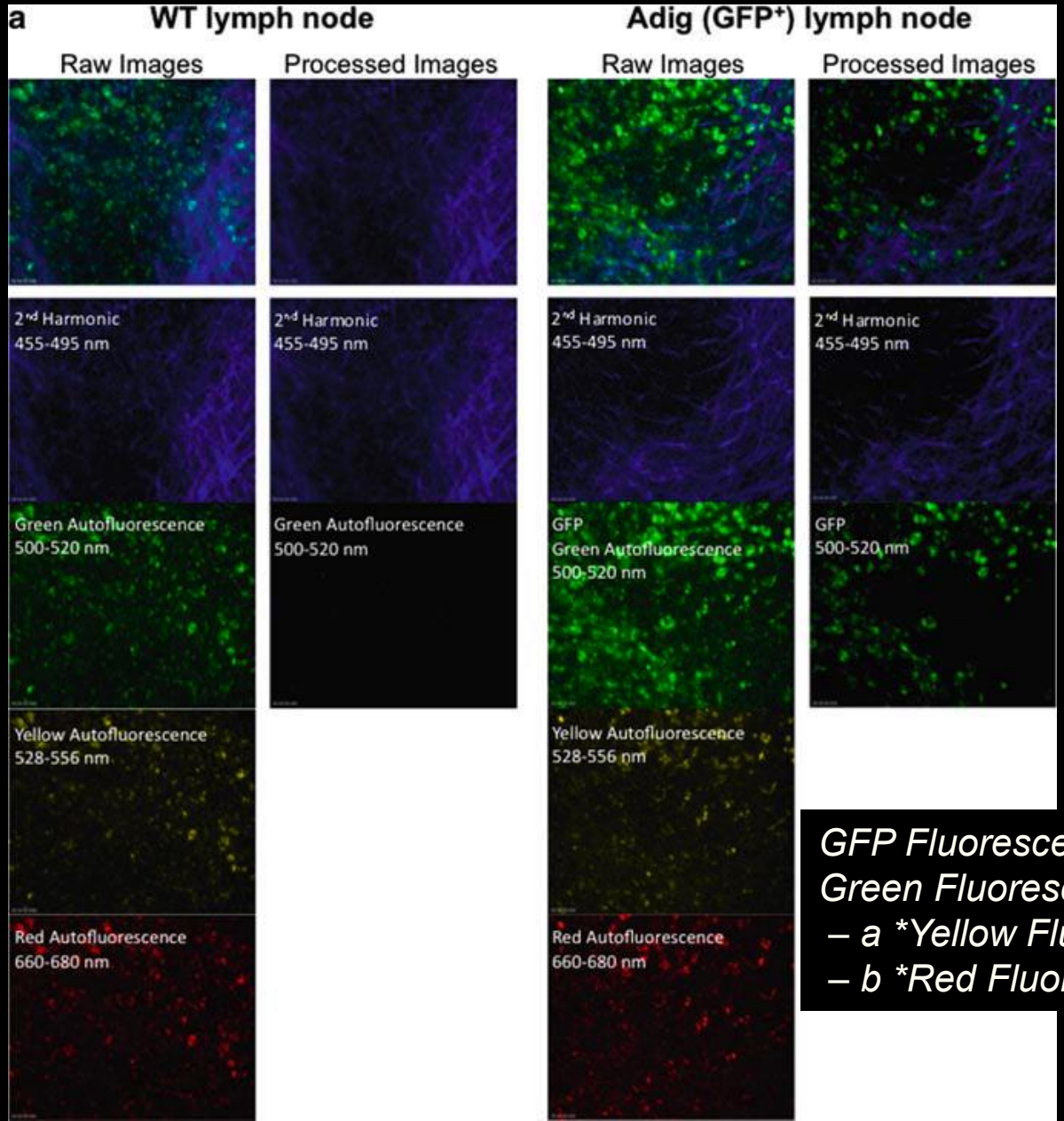
Su, M. and Anderson, M. *Curr. Opinion in Immunology* Dec 2004

Jay Gardner, Mark Anderson

Extrathymic IGRP-GFP+ Cells Subsequently Detected in Tissue Survey

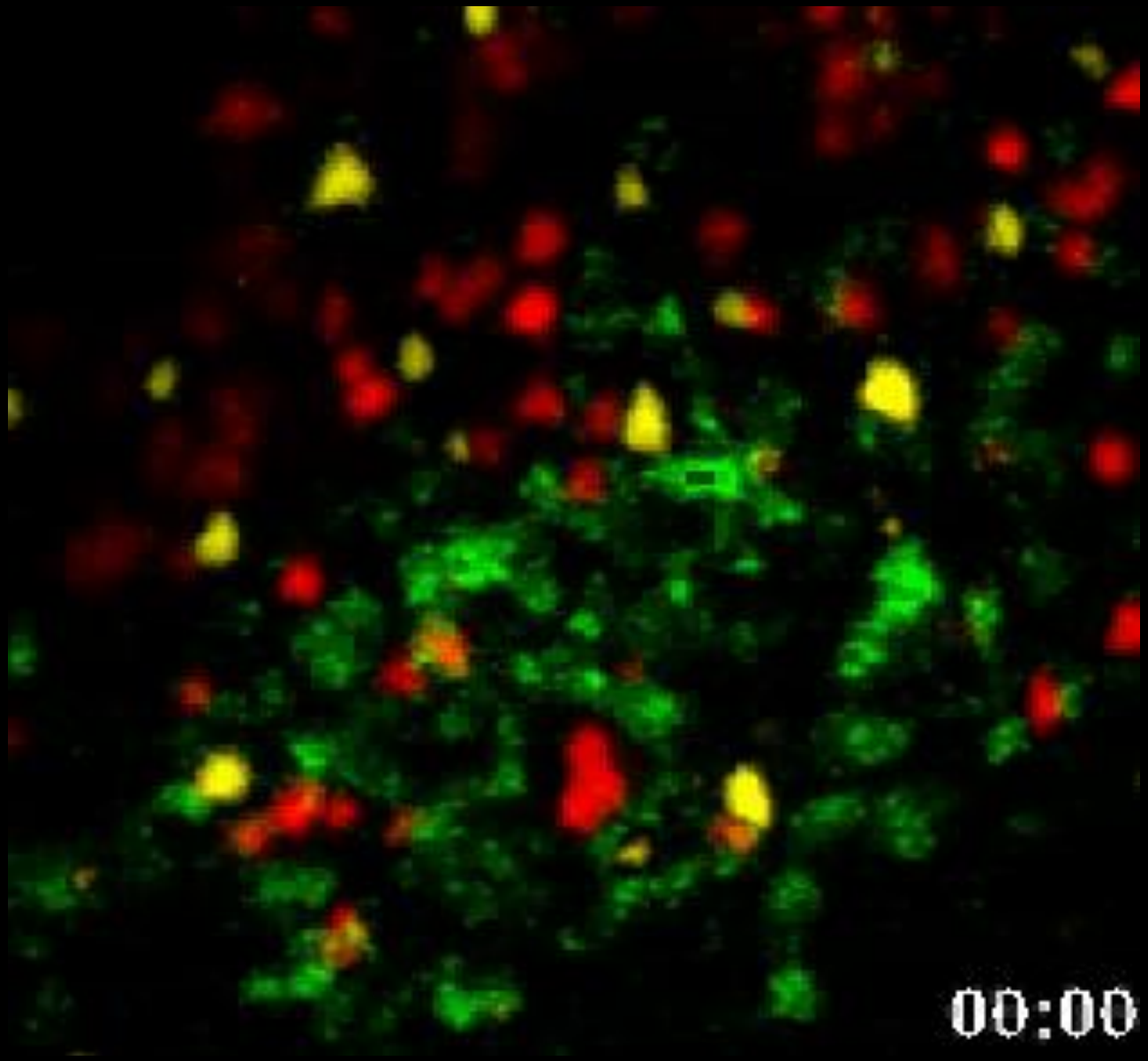


Using Multiple Channels to Remove Autofluorescence



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

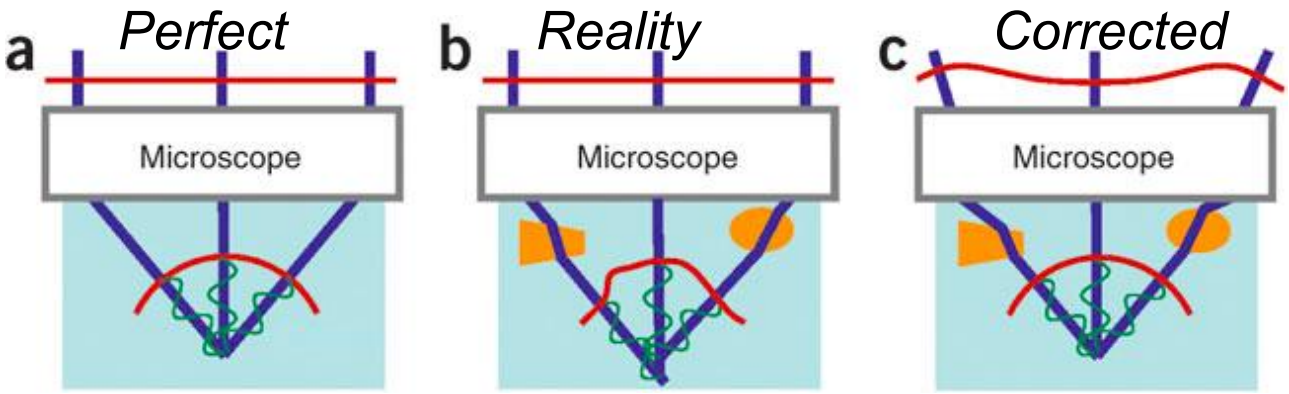
Note: Why stop at 2P.
We call it Multiphoton, right?

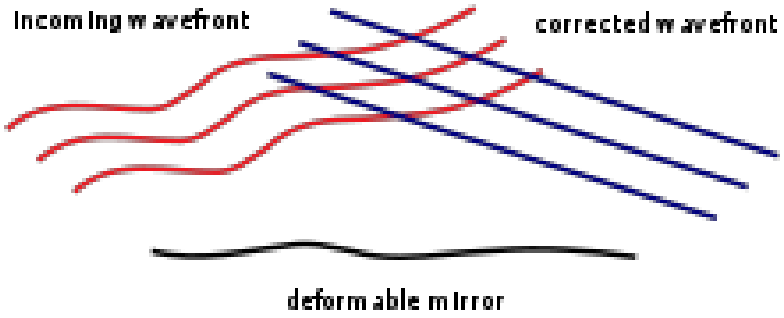
3 photons can coincide and do the same thing
Now excitation cone is even tighter.

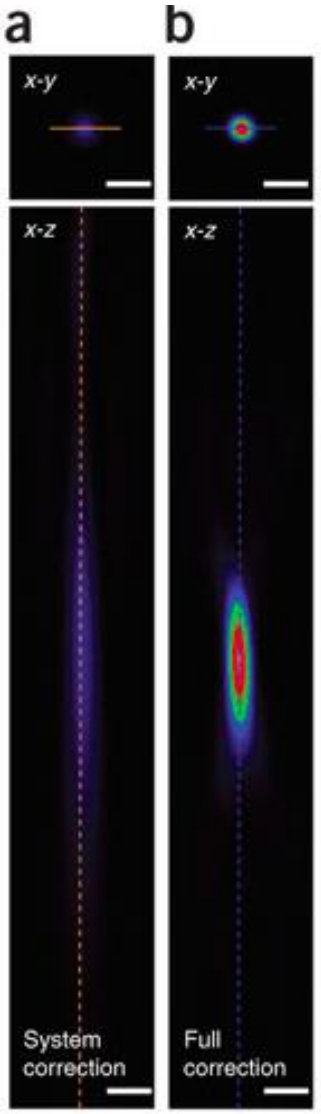
What are some downsides of trying to do this?

(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 ‘DISPERSIVE’ BEADS VS. NOT.

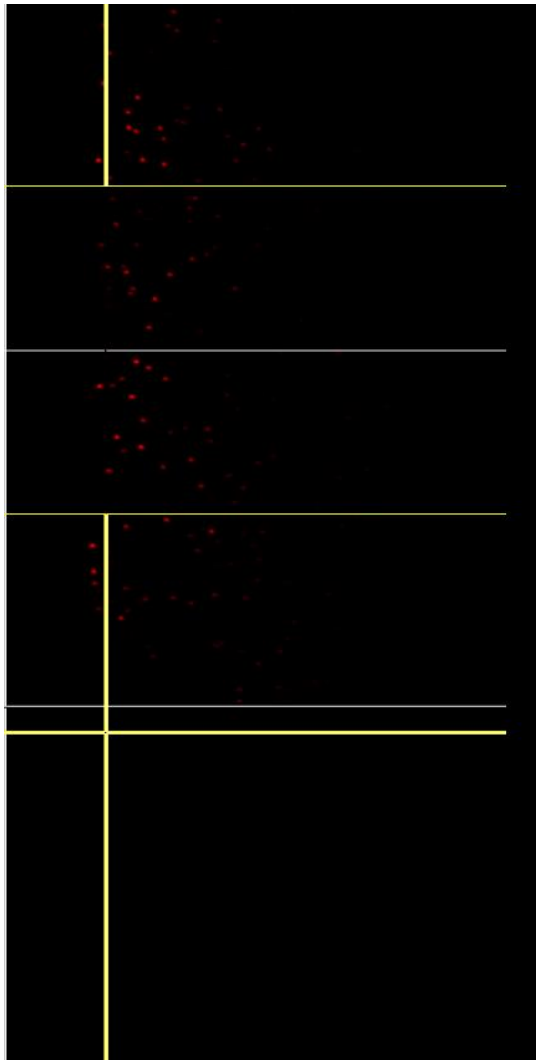
•IMAGING MYSTERY EARS—SEE IF YOU CAN FIGURE OUT WHAT CELLS MIGHT BE BEING LABELED.

•SOME OTHER THINGS THAT MAY COME UP:
- •RESONANT VERSUS GALVO
- •HOME-GROWN VERSUS COMMERCIAL.

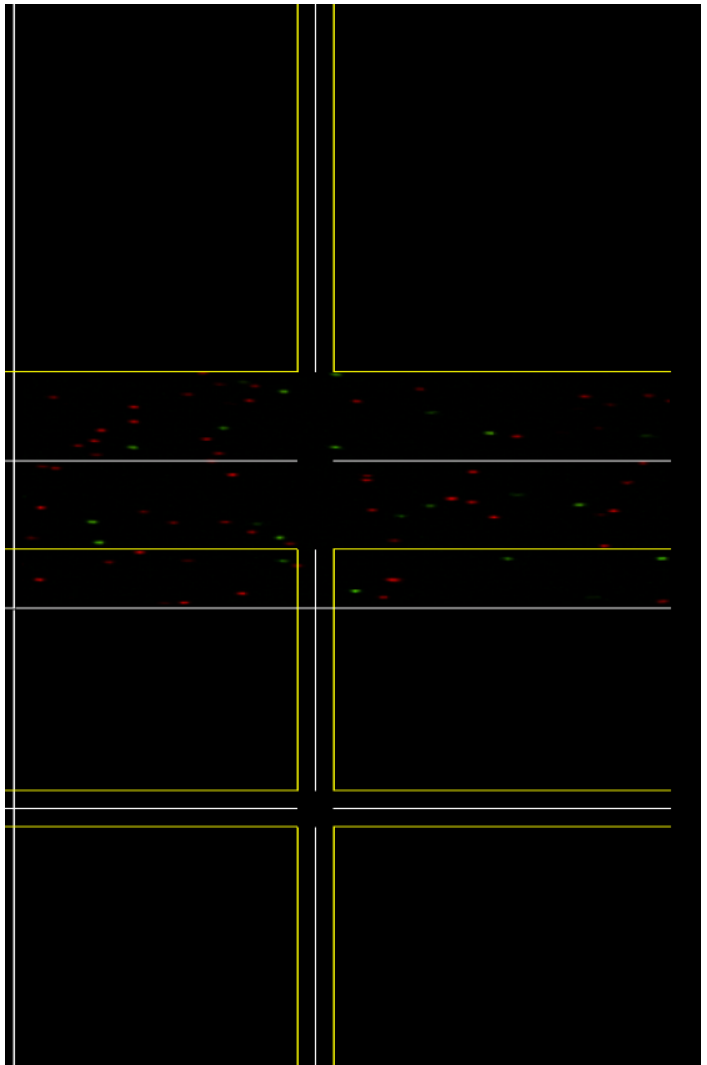
Phantoms

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

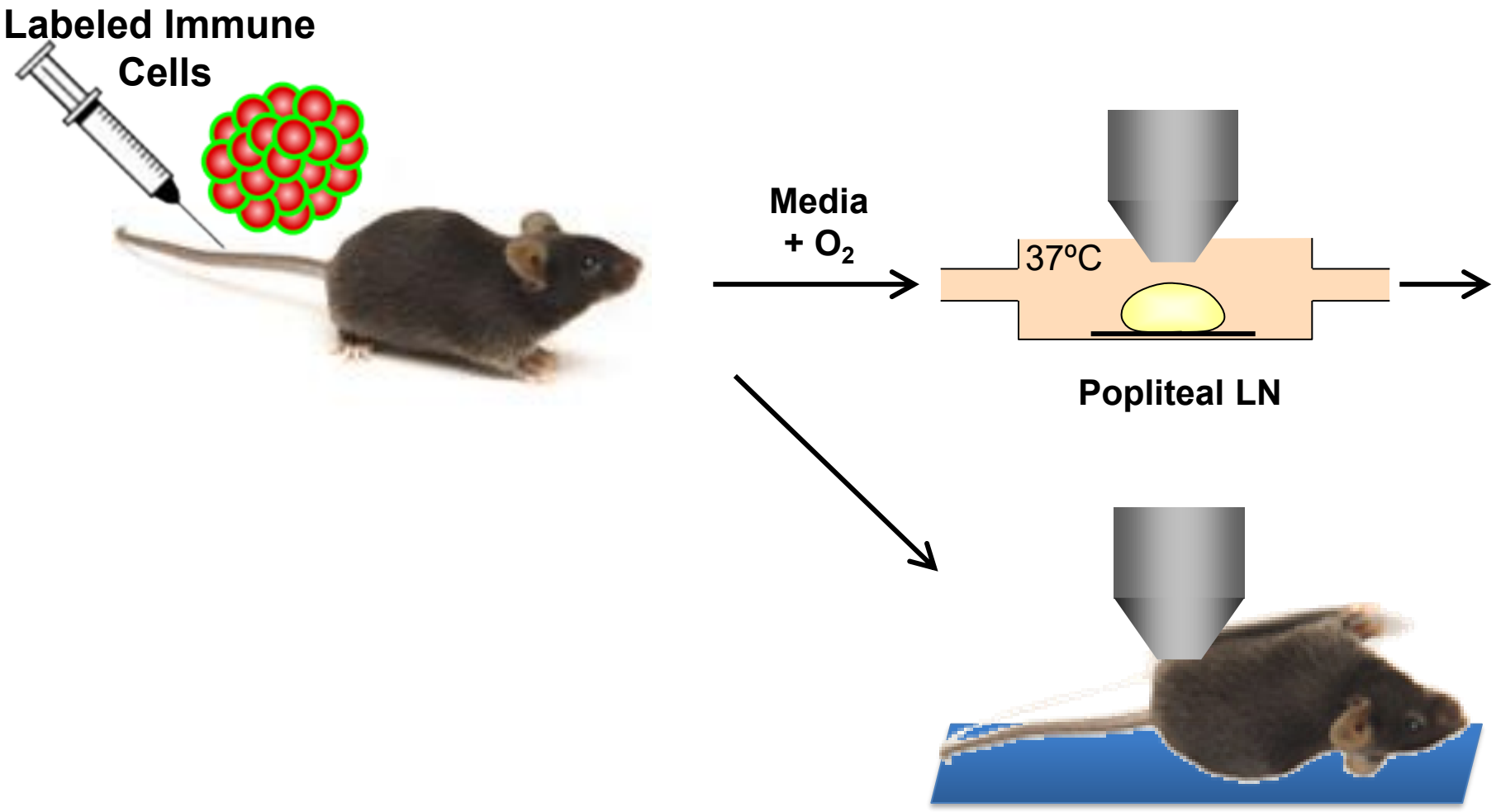
→ z



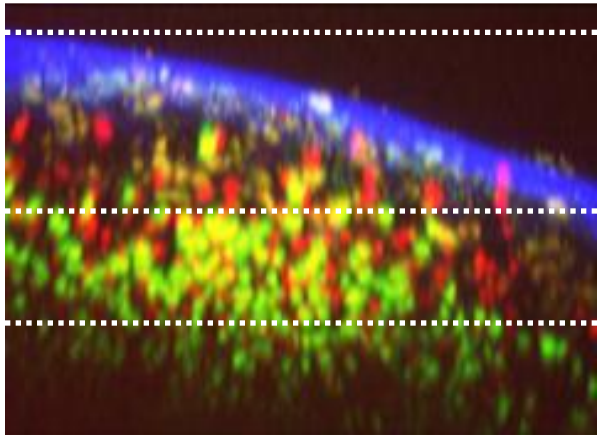
→ z



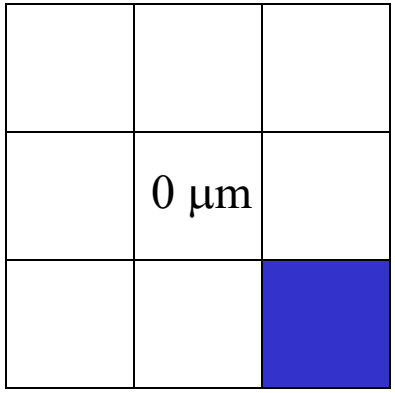
Organ Explant and intravital system for 2-photon imaging

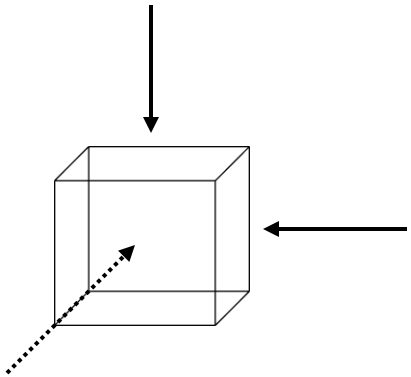


Tiled Cubes of Data to ‘Survey’ an Organ: e.g. Inguinal Non-draining Lymph Node

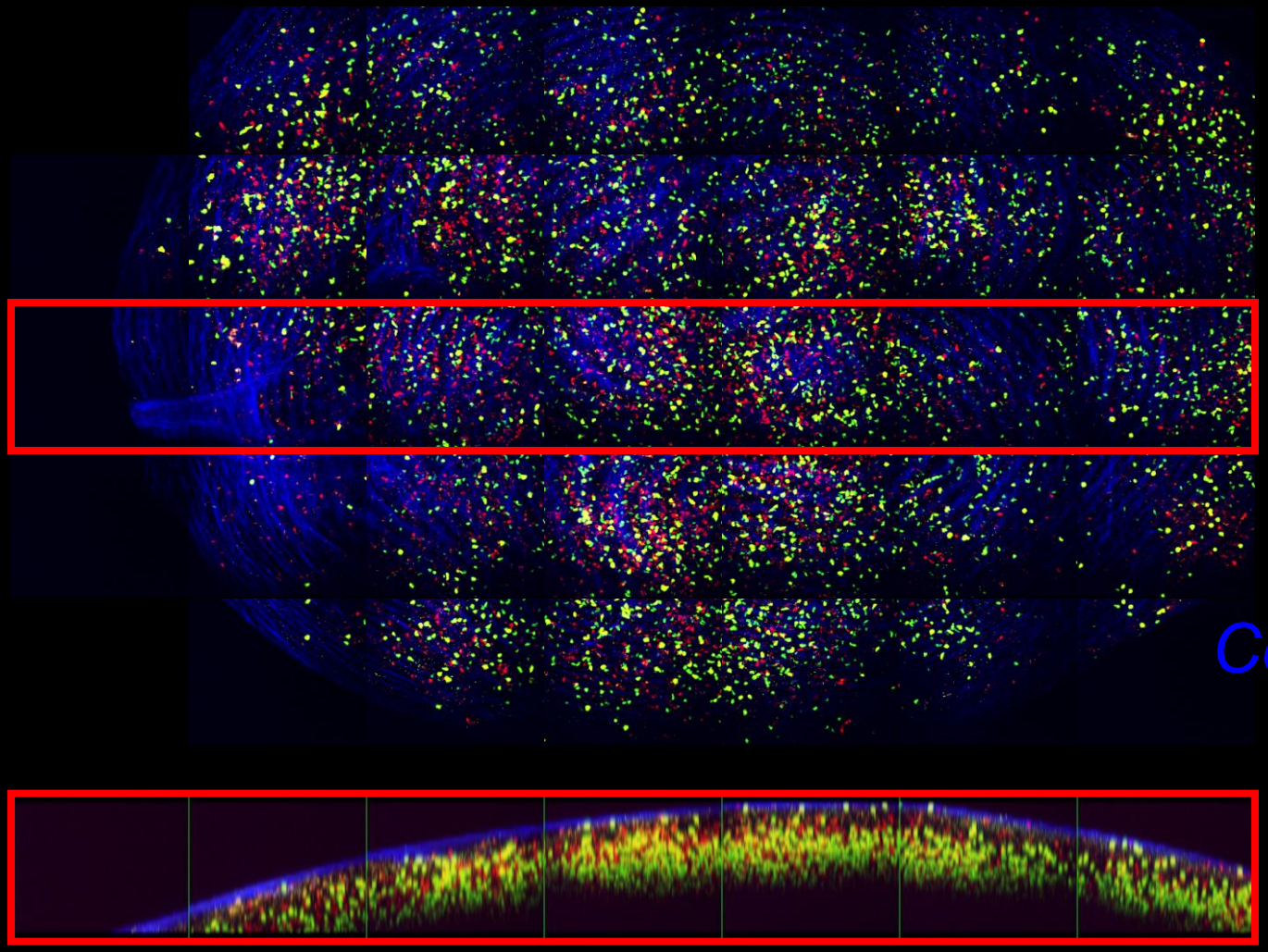


Top Down



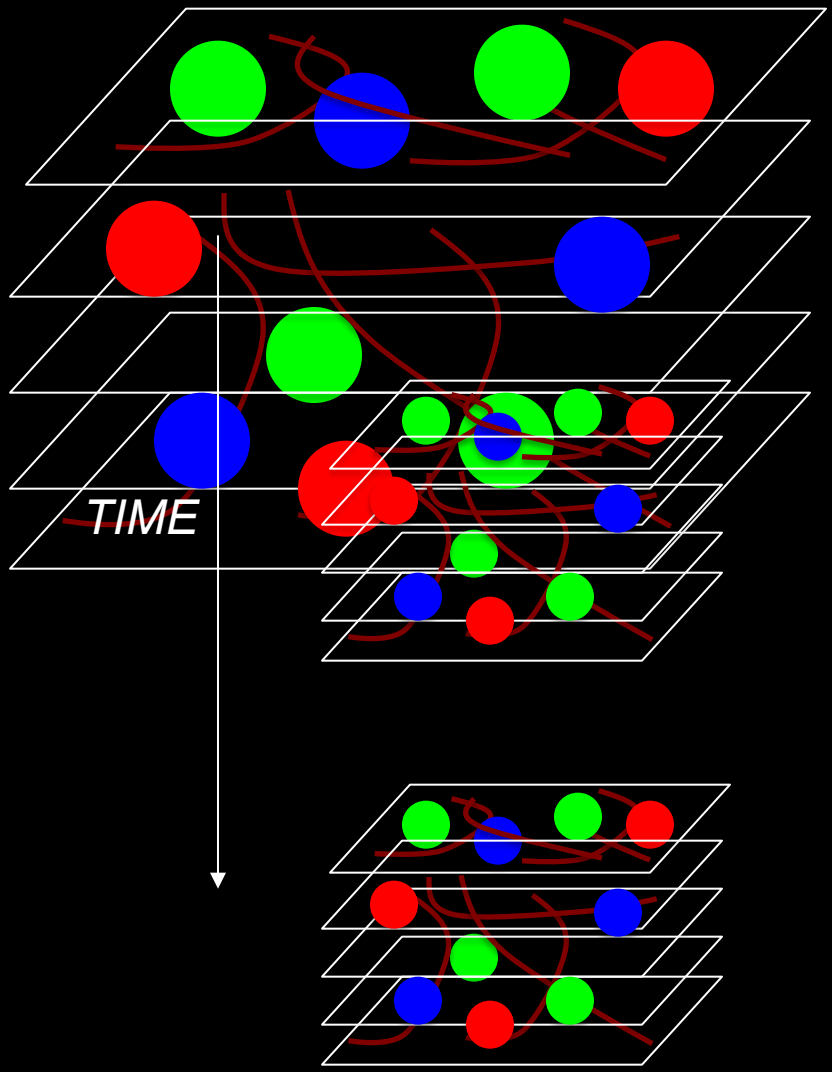


Typical First Step: The Survey



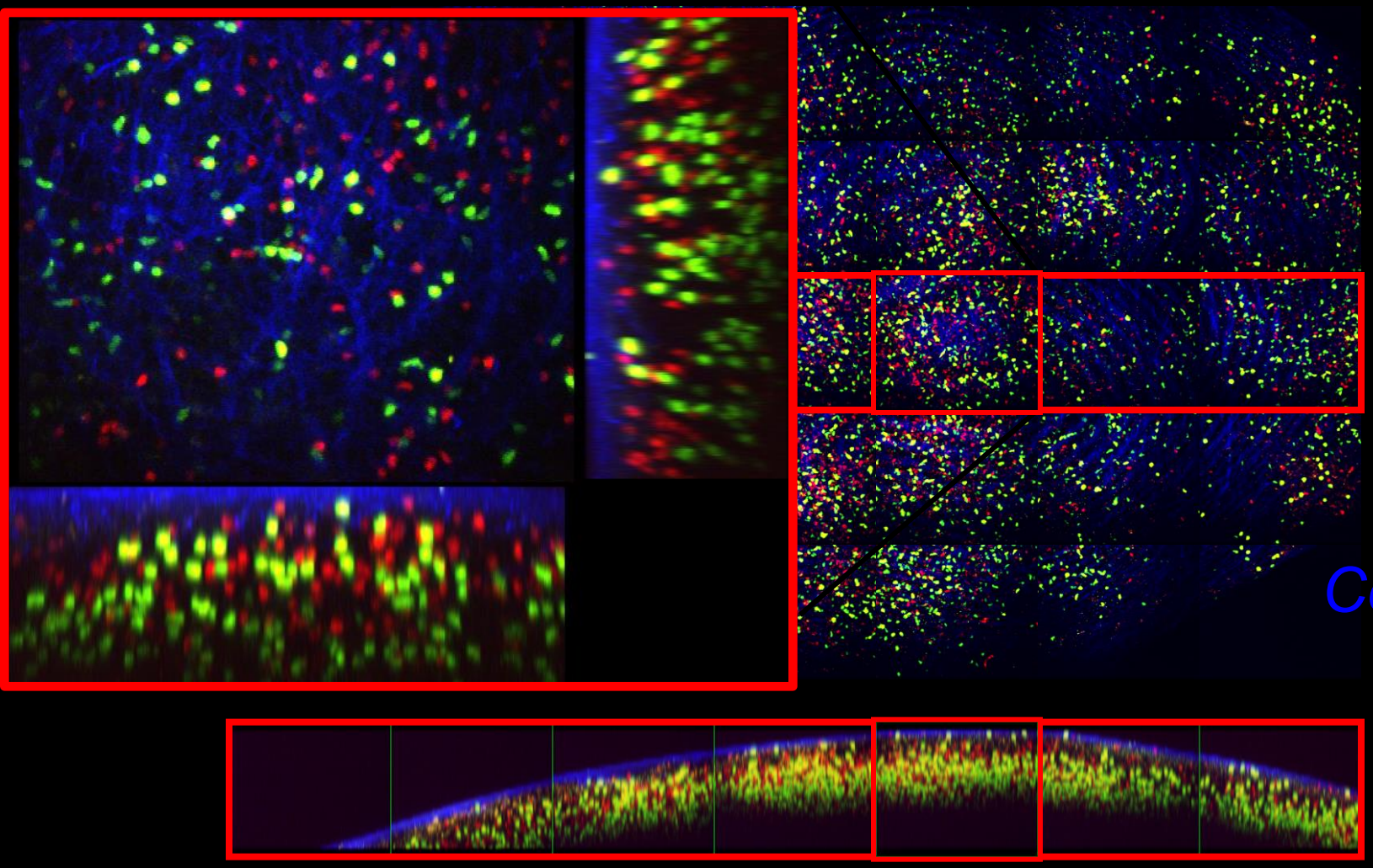
Collagen
Th
Tcyt

A 5D 2-Photon Platform

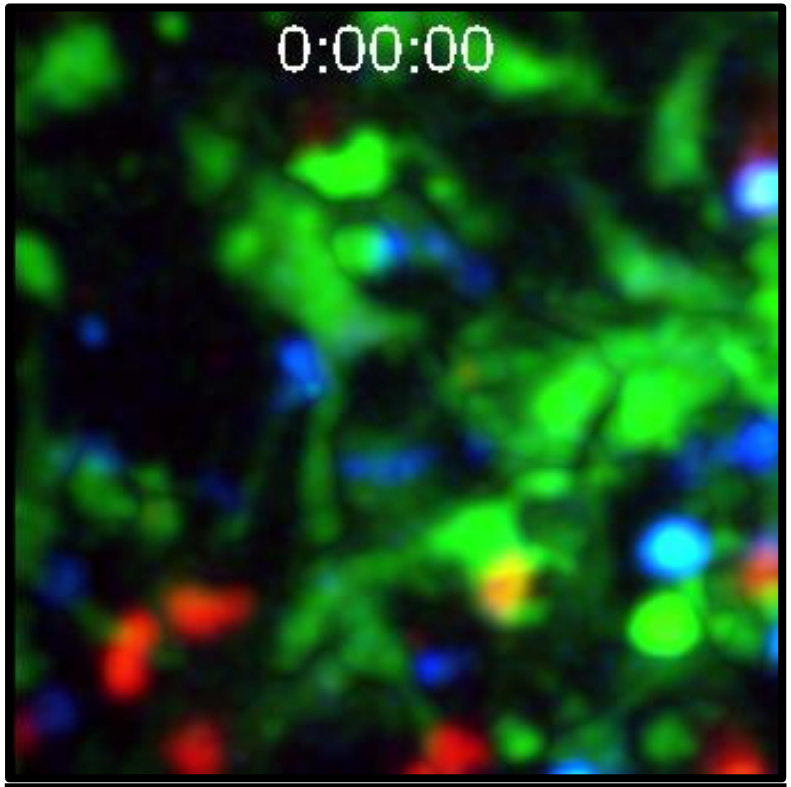


Analyze in:
Matlab
ImageJ
Metamorph
Imaris
IDT

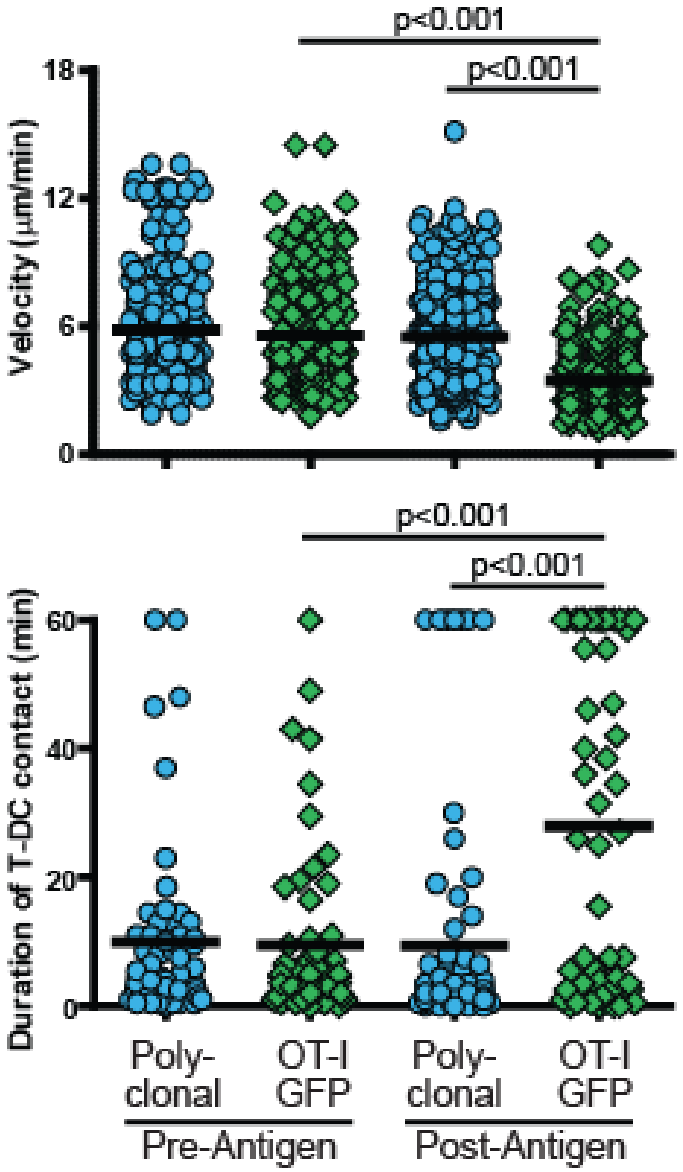
Hone in On and Area and Collect Timelapse



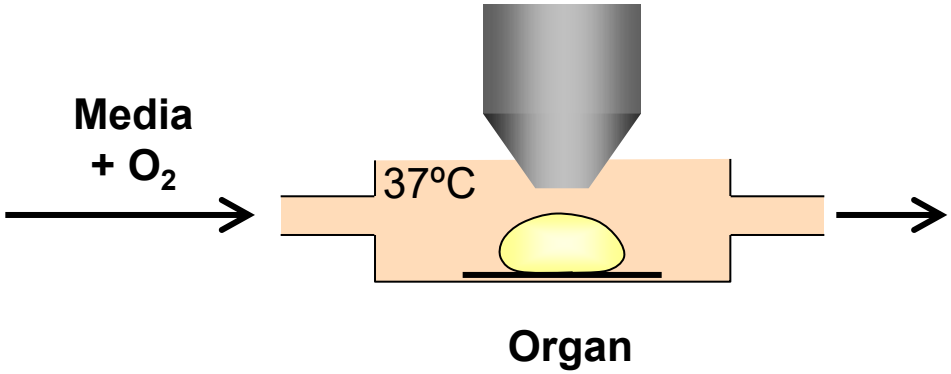
T cell–DC interactions before & after antigen addition



CD11c-YFP DCs
OT-I–GFP T cells
Actin-CFP+ Polyclonal T cells



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



Critical Parameter
Seems to be Slice Thickness
(Oxygen Tension)

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

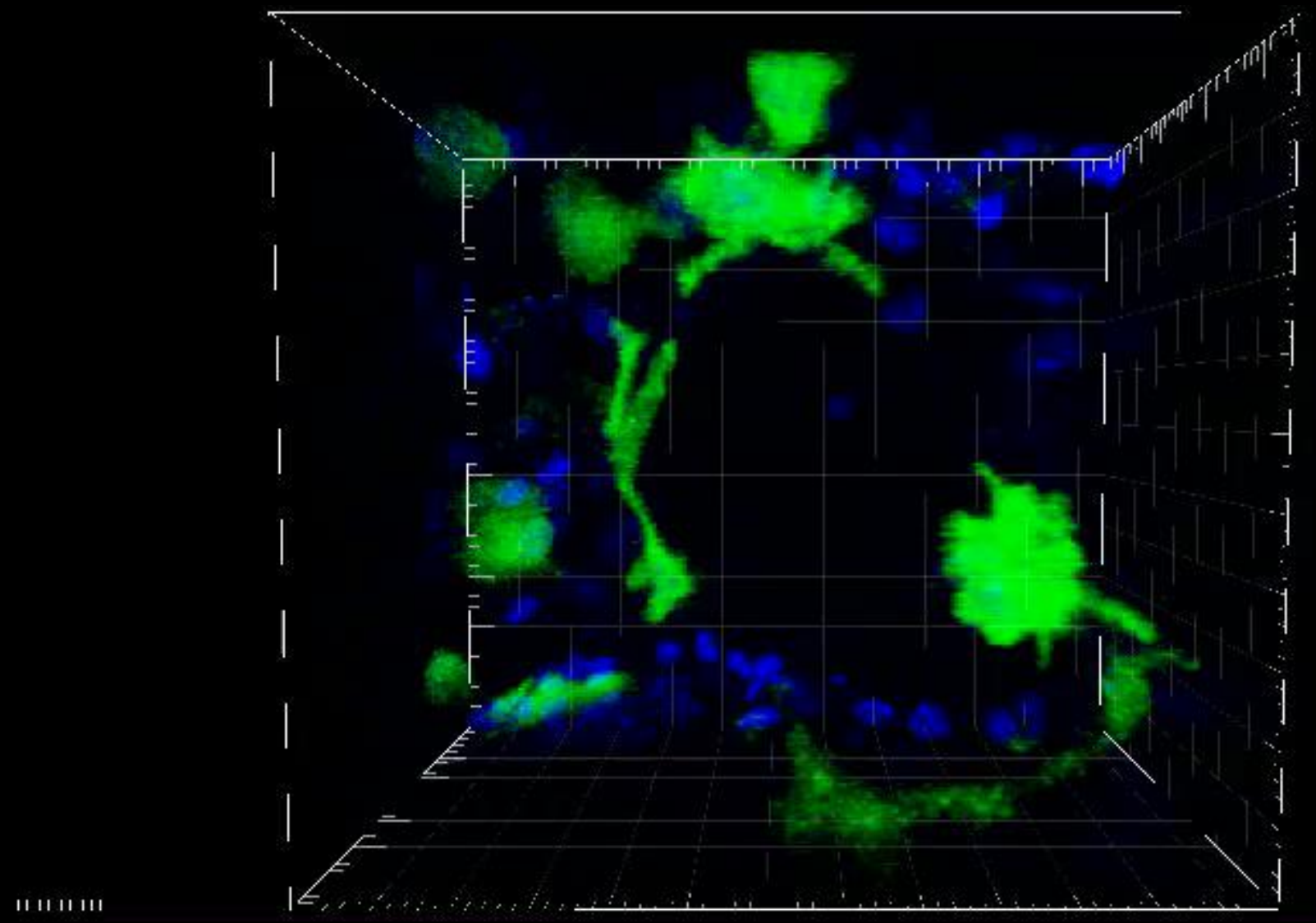
---

e.g. Lung

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

Movie of Cilia at 3 hrs post sac

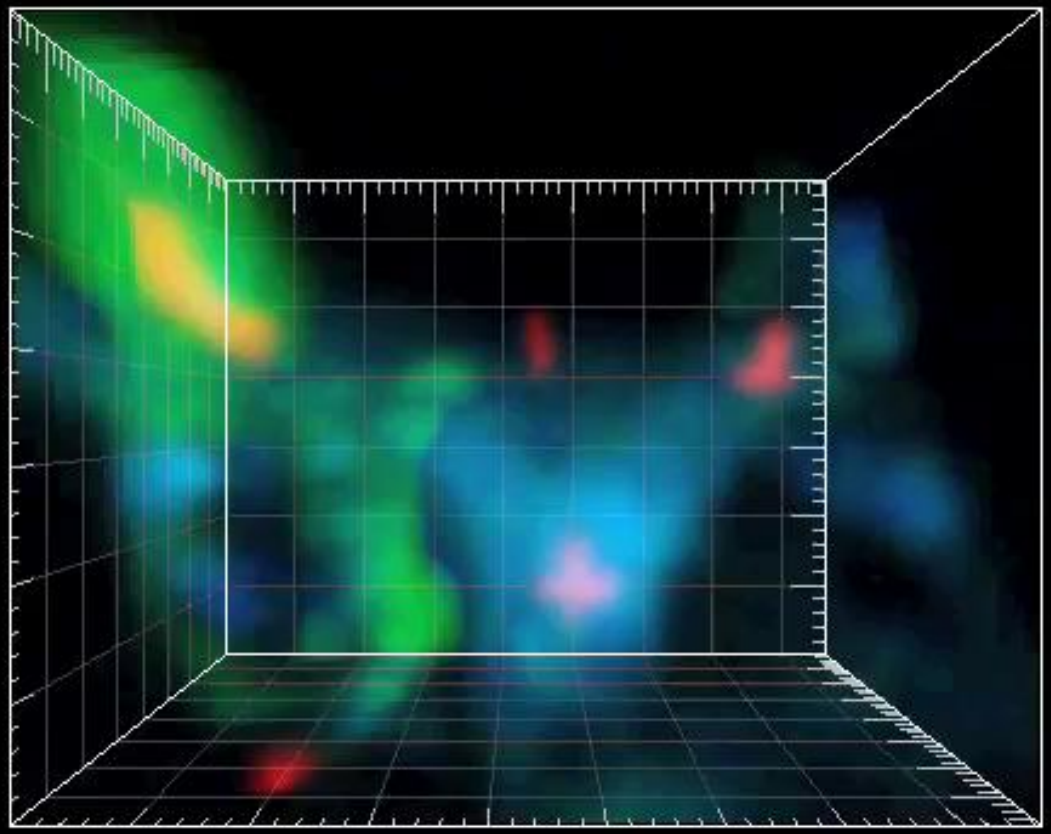
DC Projections into the Alveolar Atrium and Mucosal Scanning



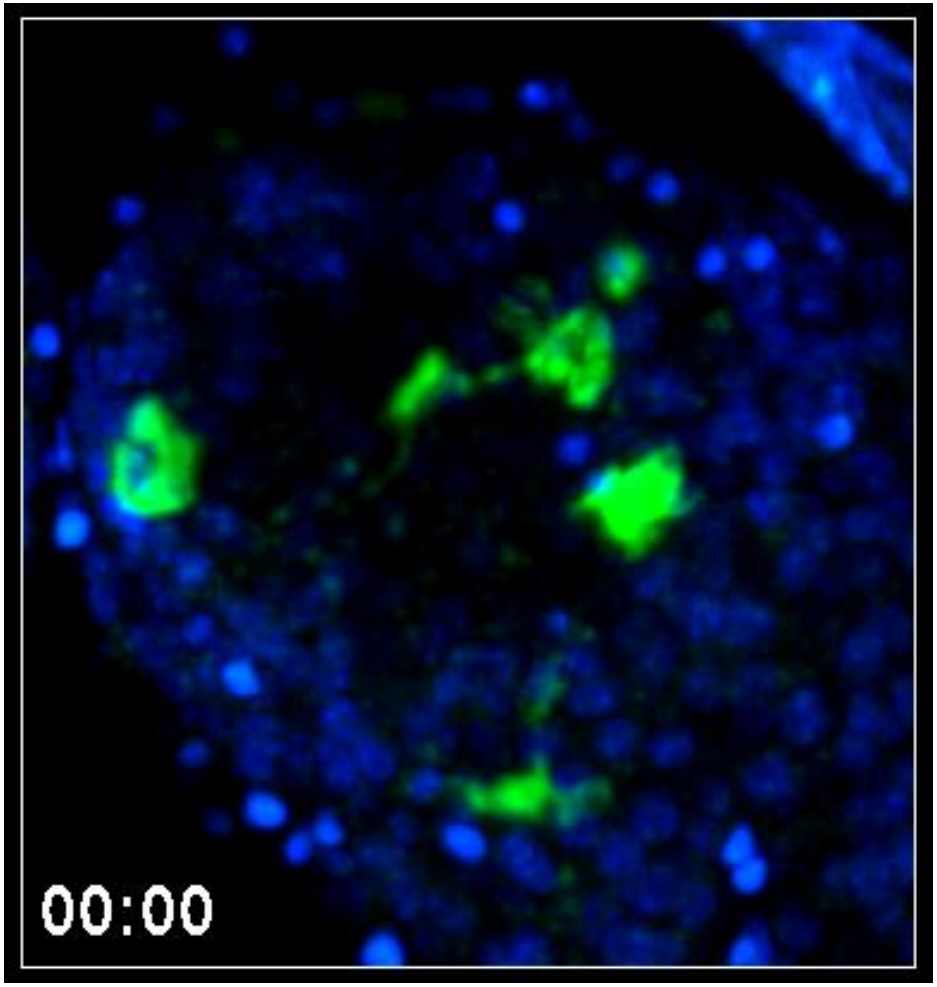
CD11c YFP
Hoechst

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

Cfms GFP
Actin CFP
Inhaled Beads

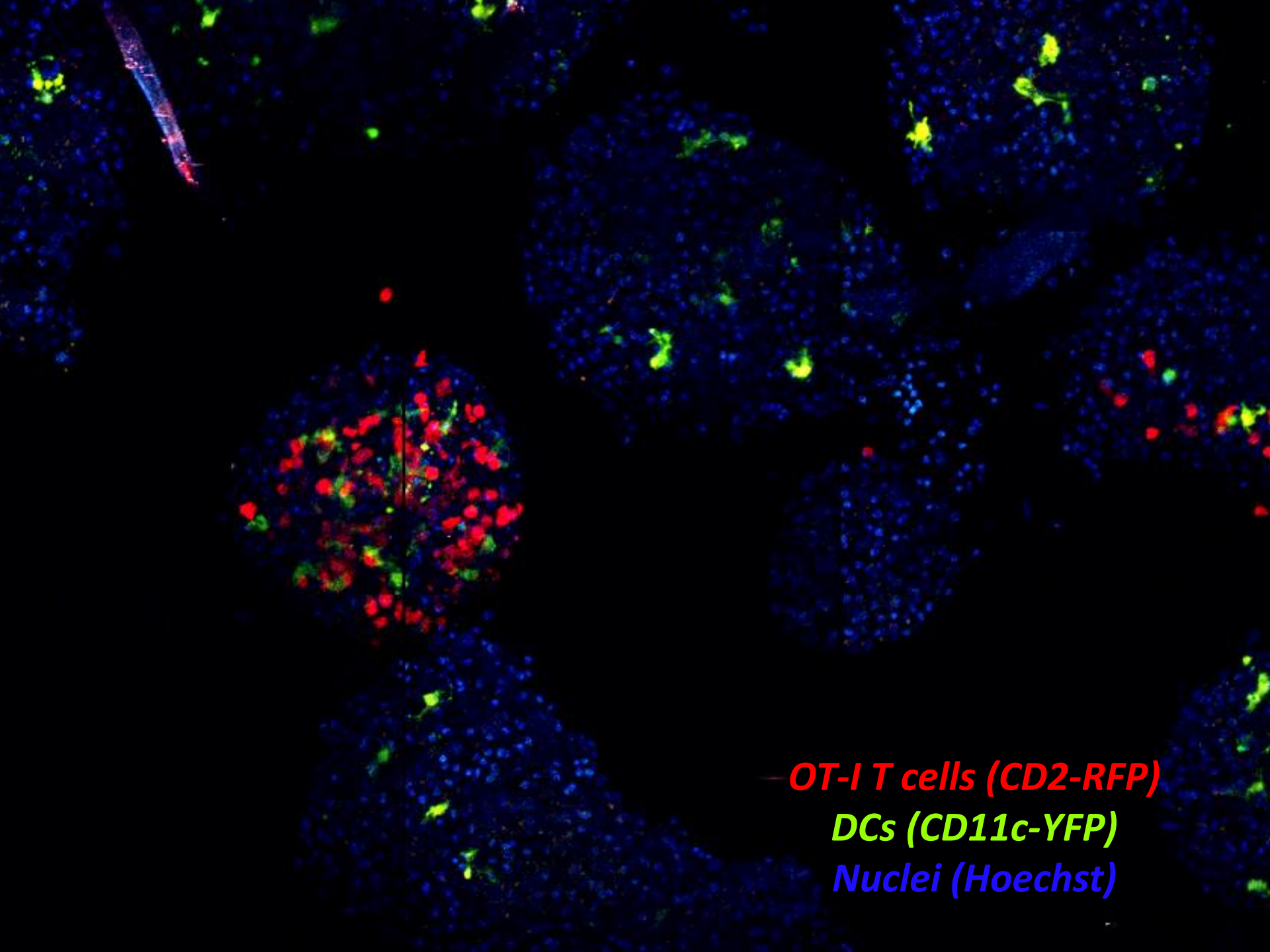


Surveillance of islets by DCs in the steady-state

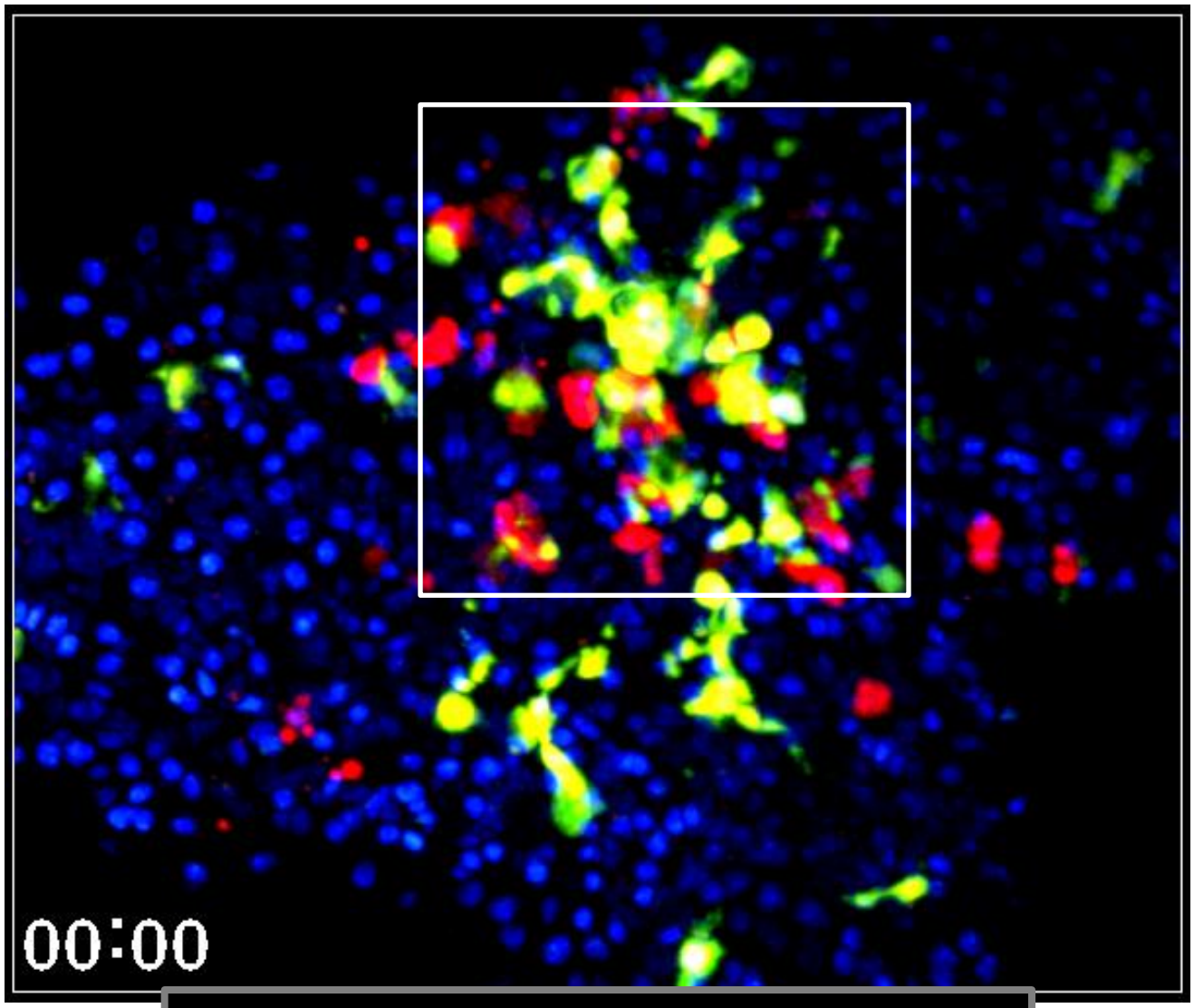


DCs (CD11c-YFP)
Nuclei (Hoechst)

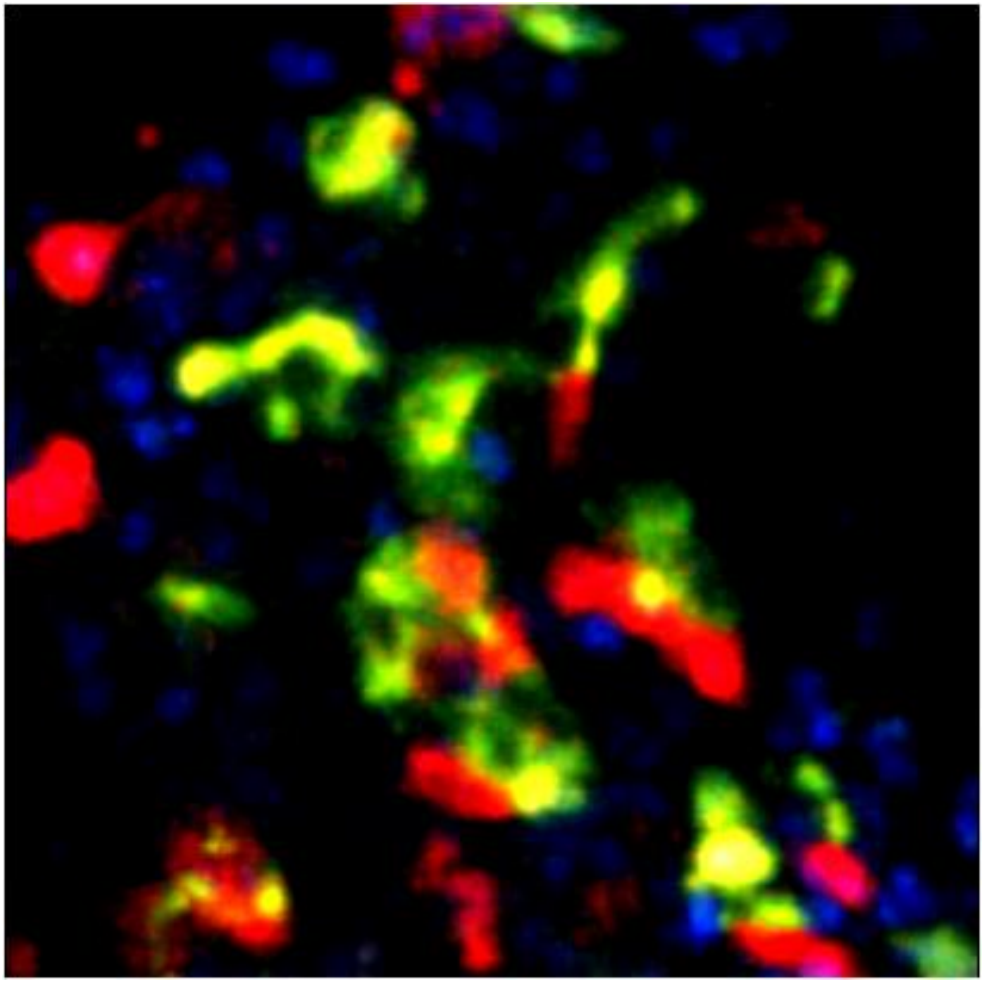
How do intra-islet DCs affect infiltrating T cells?


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

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

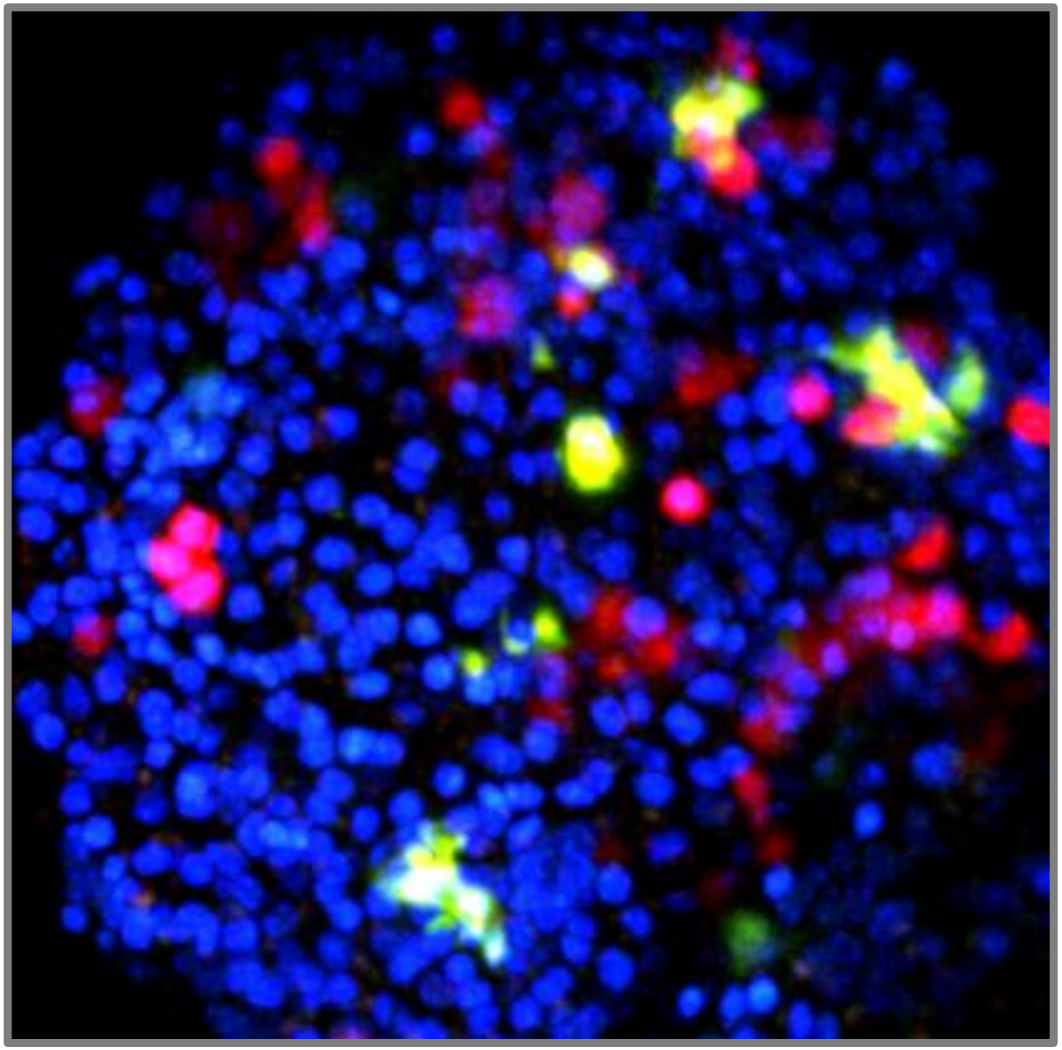


T cells form transient & sustained interactions with intra-islet DCs



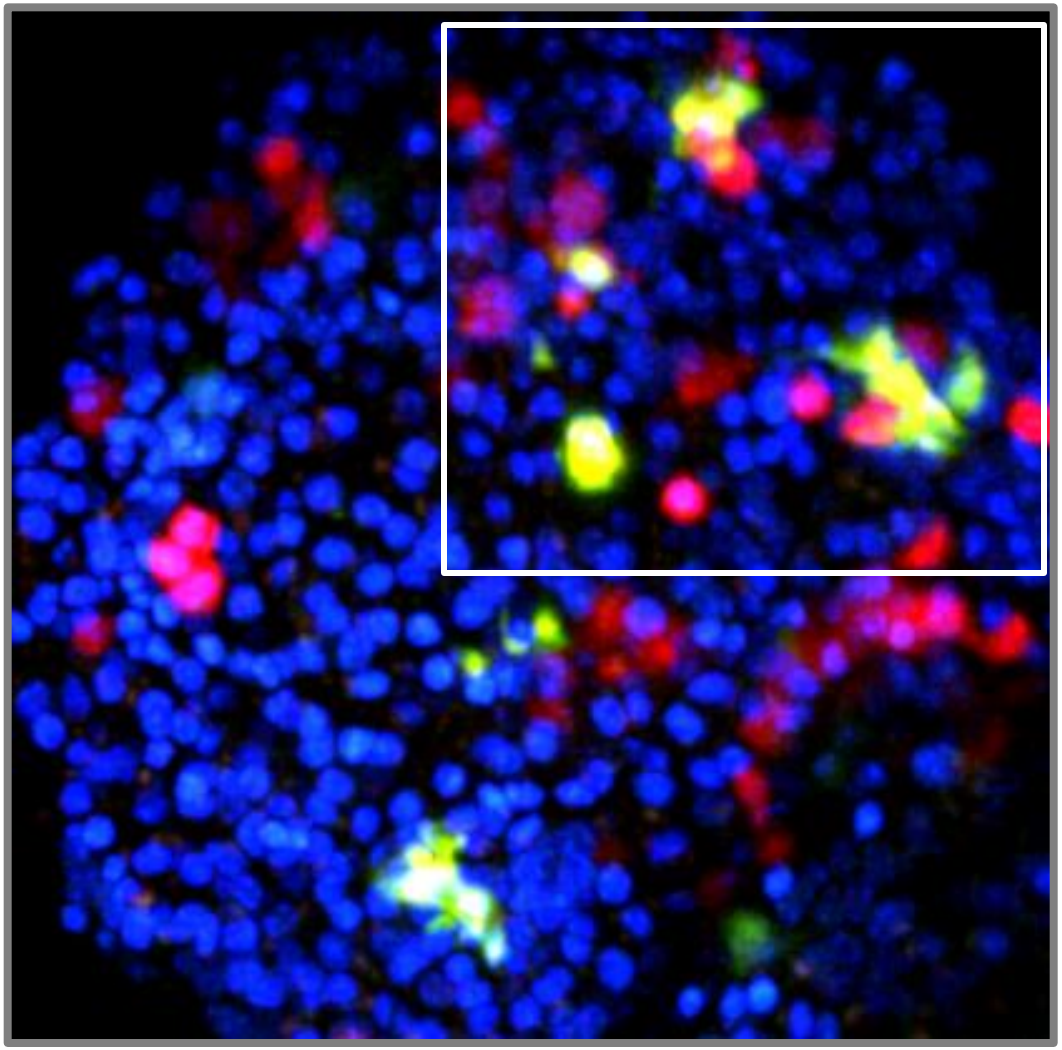
OT-I T cells ***DCs*** ***Nuclei***

T cells can induce DC lysis



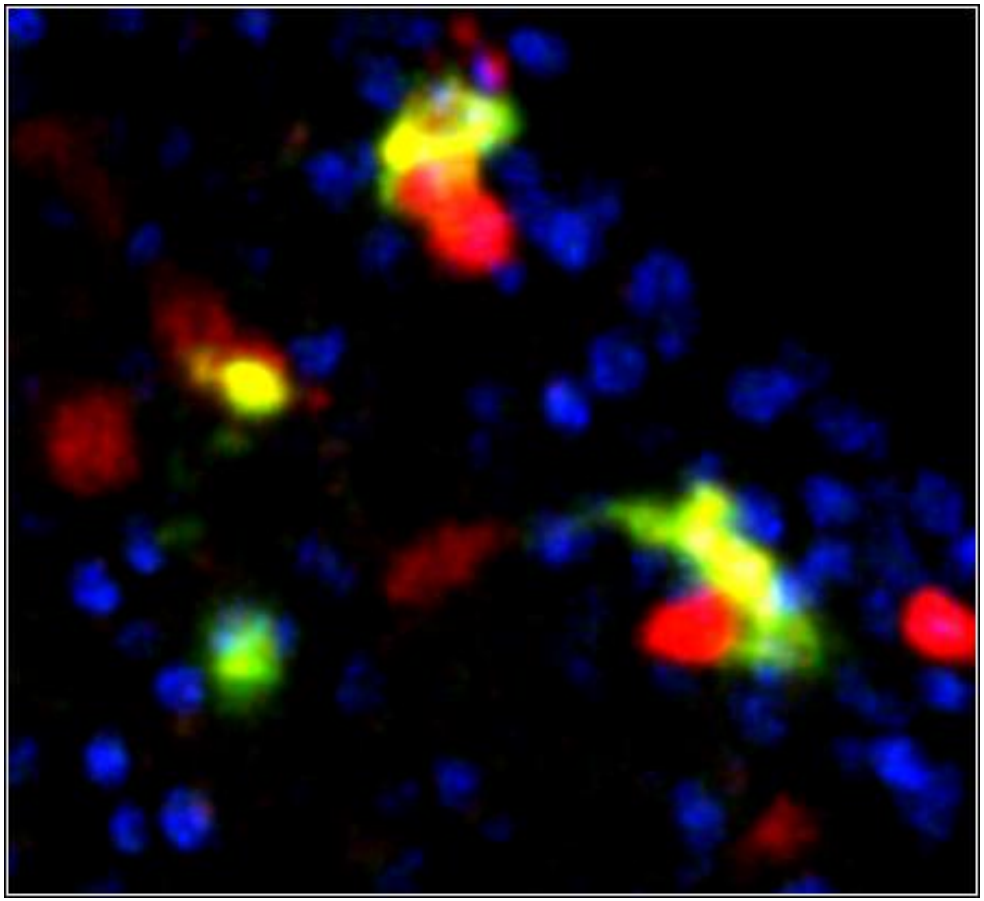
OT-I T cells DCs Nuclei

T cells can induce DC lysis



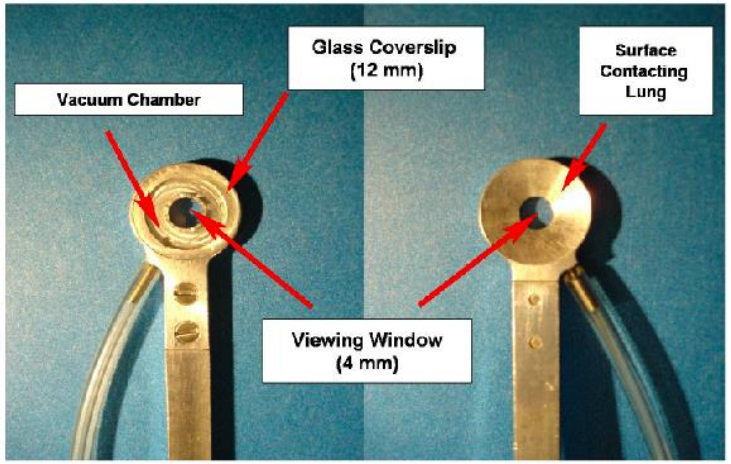
OT-I T cells ***DCs*** ***Nuclei***

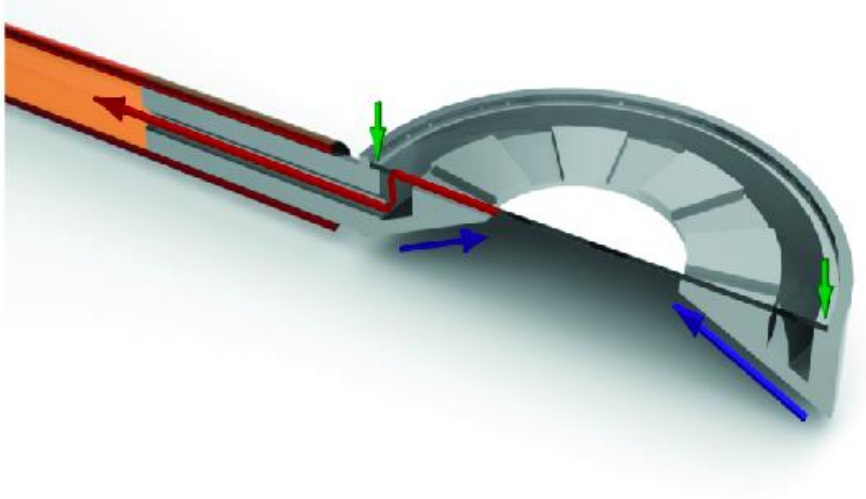
T cells can induce DC lysis

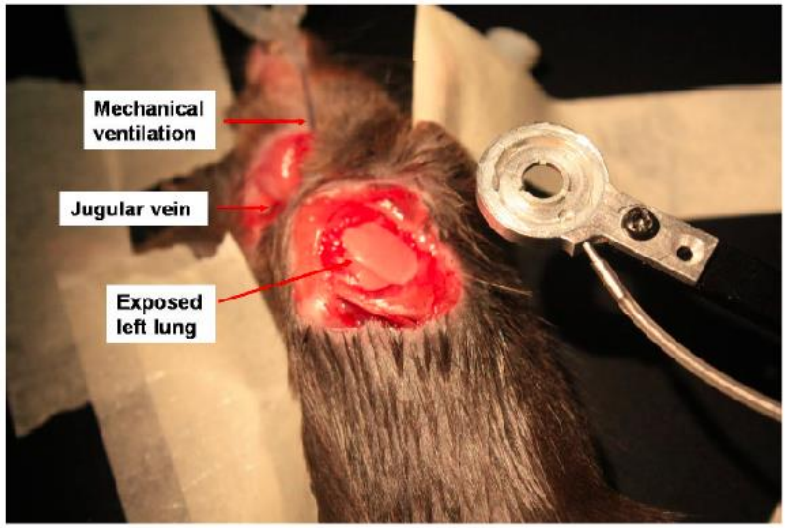


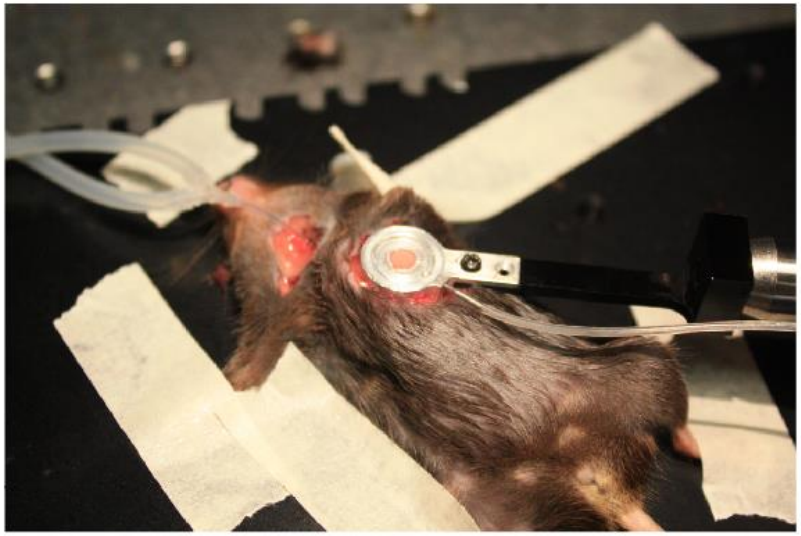
OT-I T cells ***DCs*** ***Nuclei***

Taking it ‘Live’

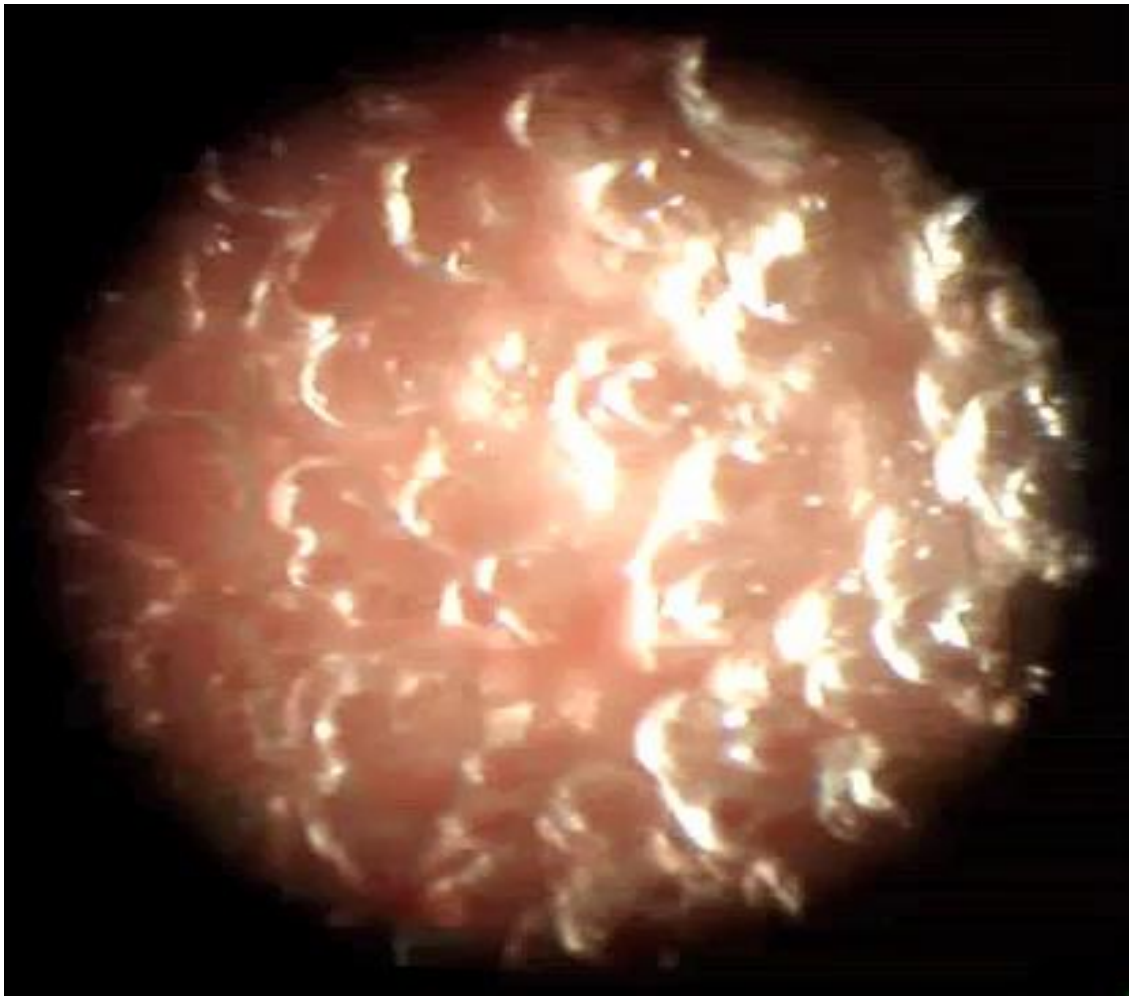






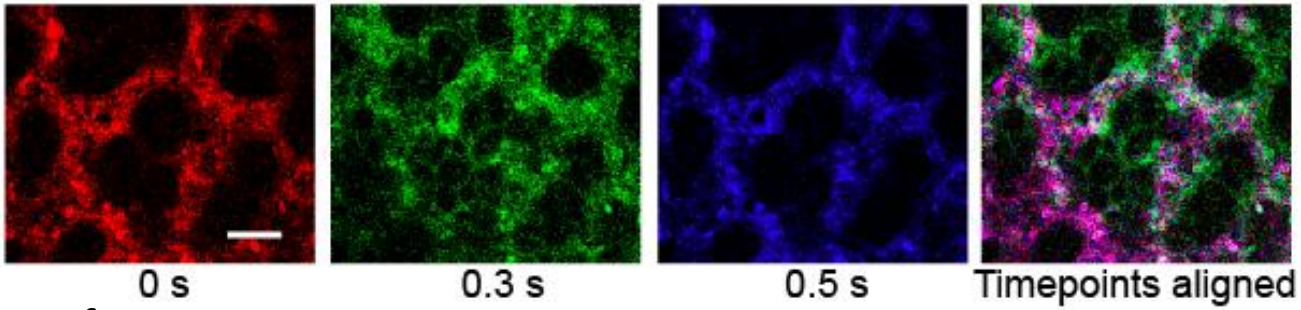


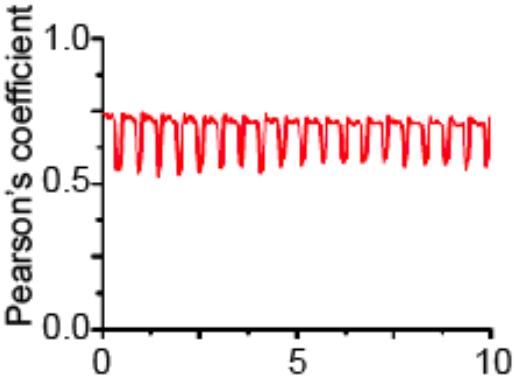
Vacuum window isolates and stabilizes the lung



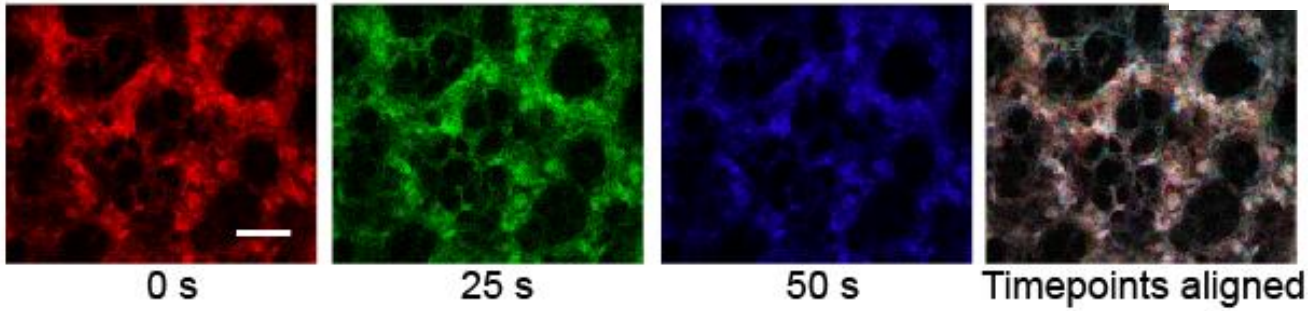
Window allows for inflation of alveoli under suction

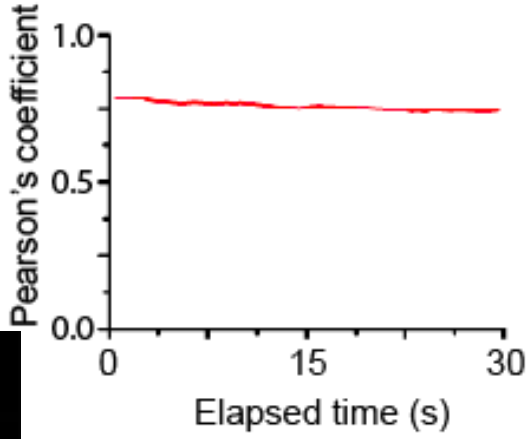
Real-time imaging

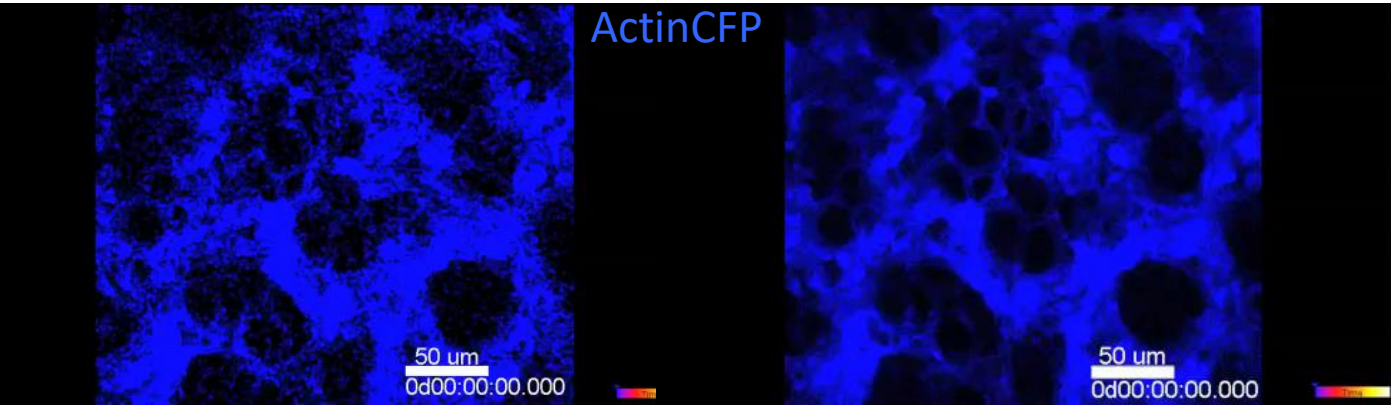




15 frame averages

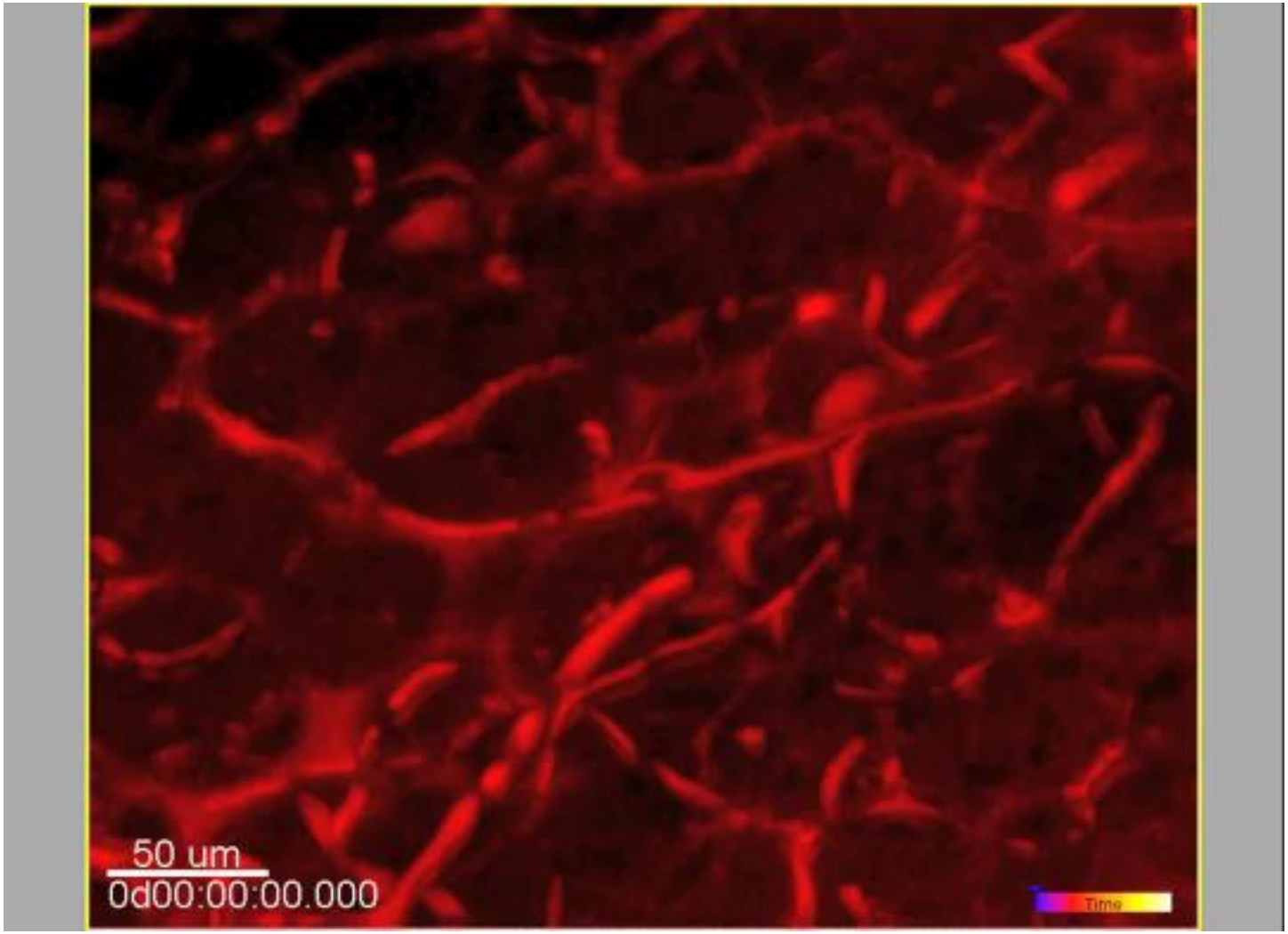






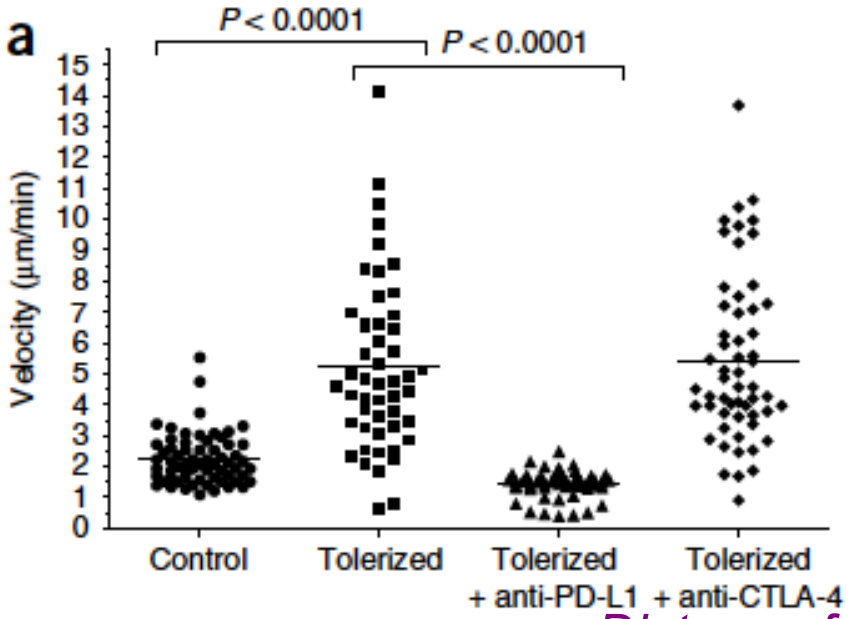
Averaging or frame deletion can provide a very stable image

Intact Blood Flow-I

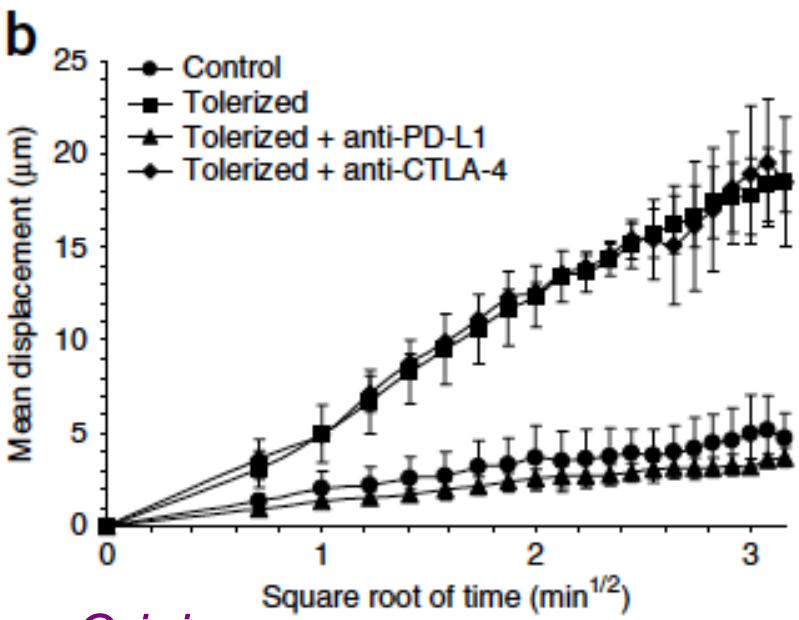


Data Analysis: Motility

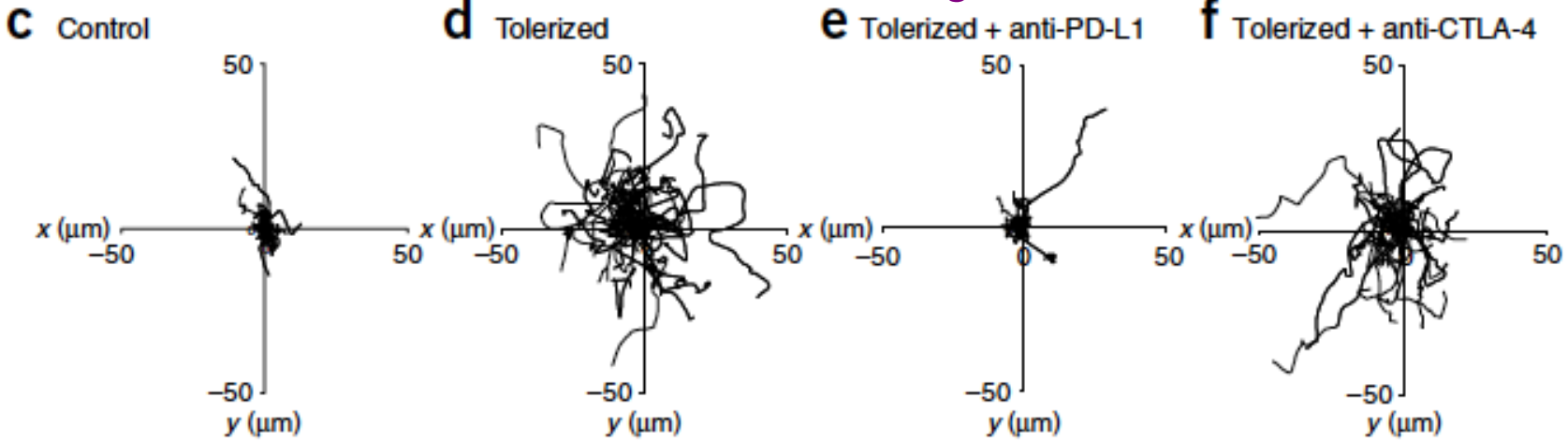
Avg Velocities



RMS Displacement



Distance from Origin

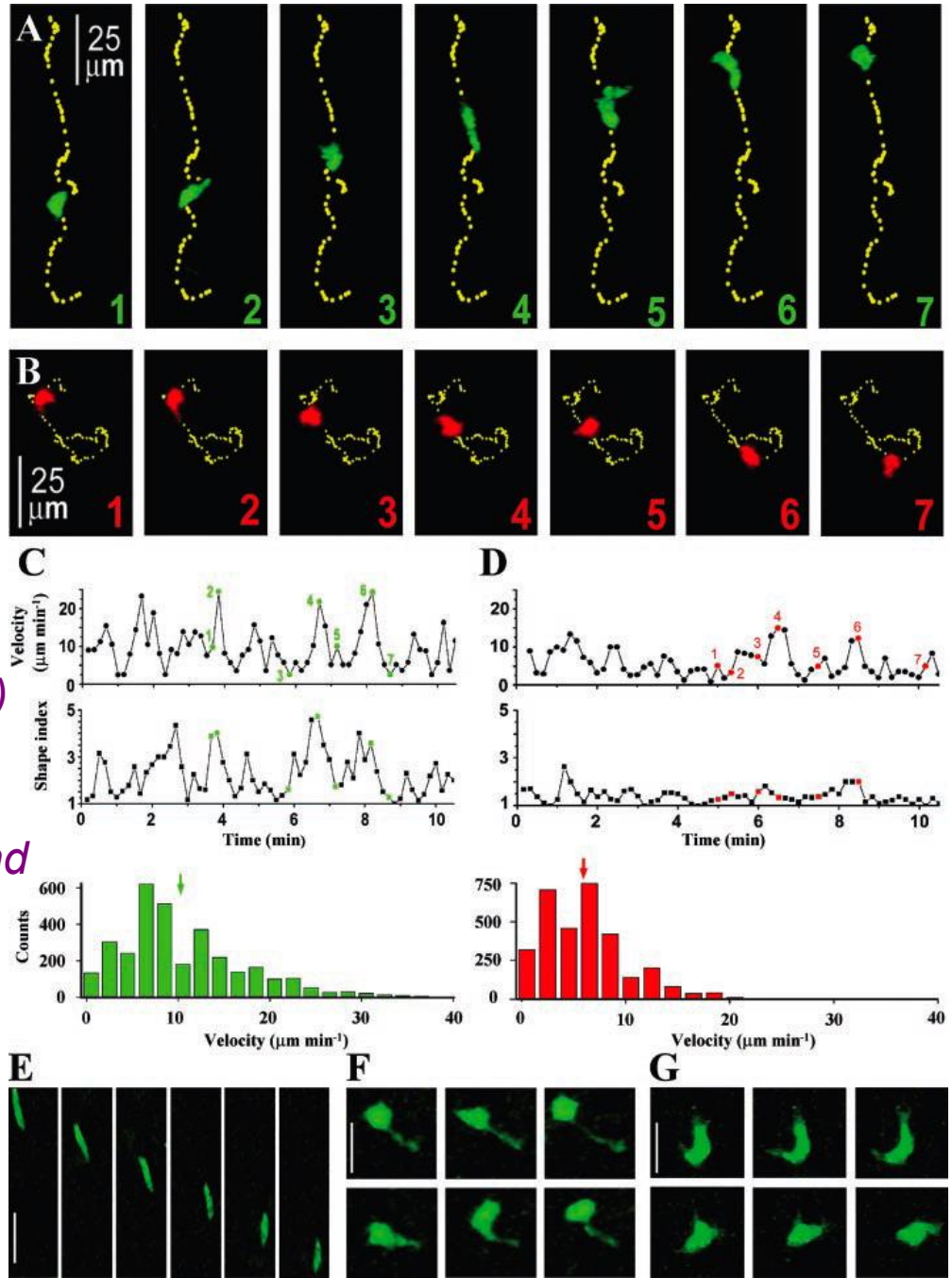


Morphology:

Shape Factors:
Often Correlate with
Motility vs. signaling

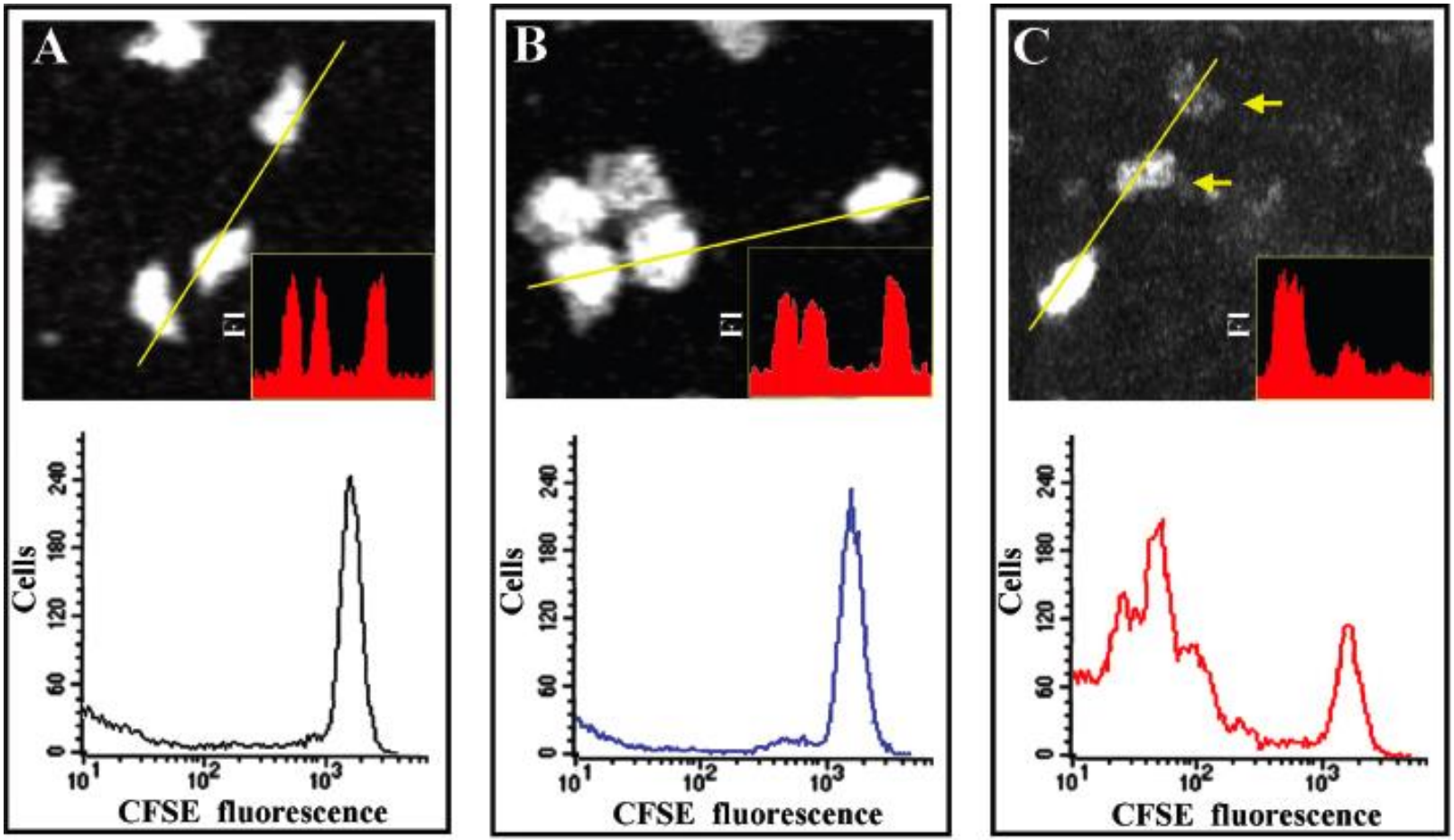
Ratio of longest and perpendicular chords (1 for circle)

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

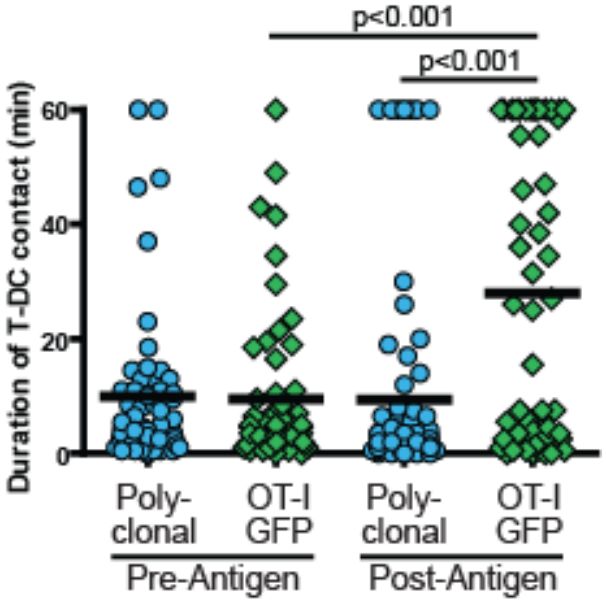


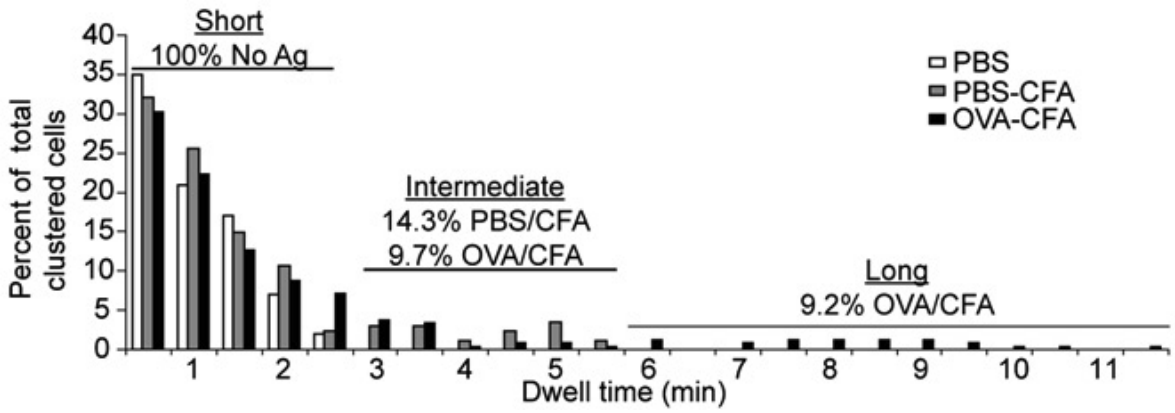
Cahalan.

Cell Division:



Data Analysis: Cell-Interaction





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