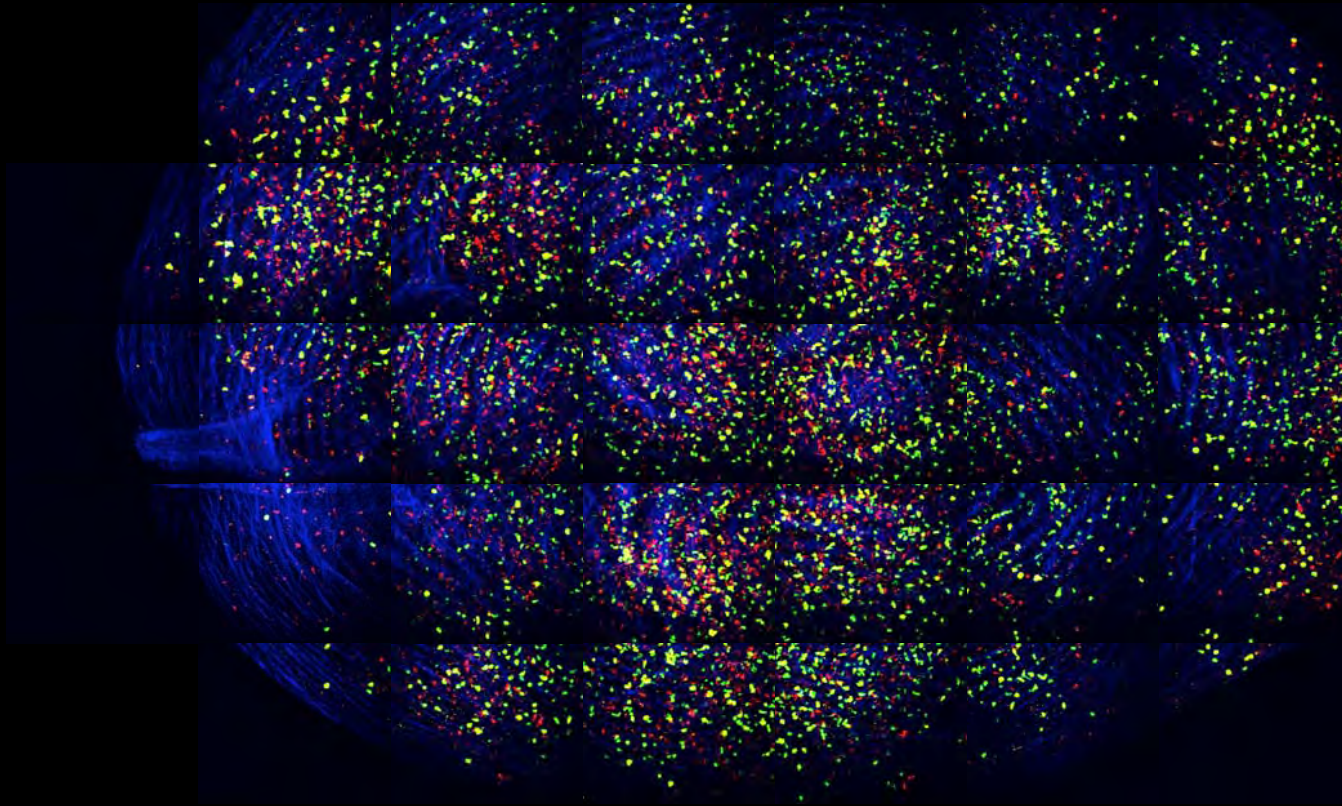
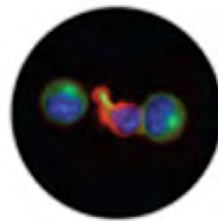


# Multiphoton Imaging in vivo. PIBS Microscopy Course



Krummel Lab, Department of Pathology



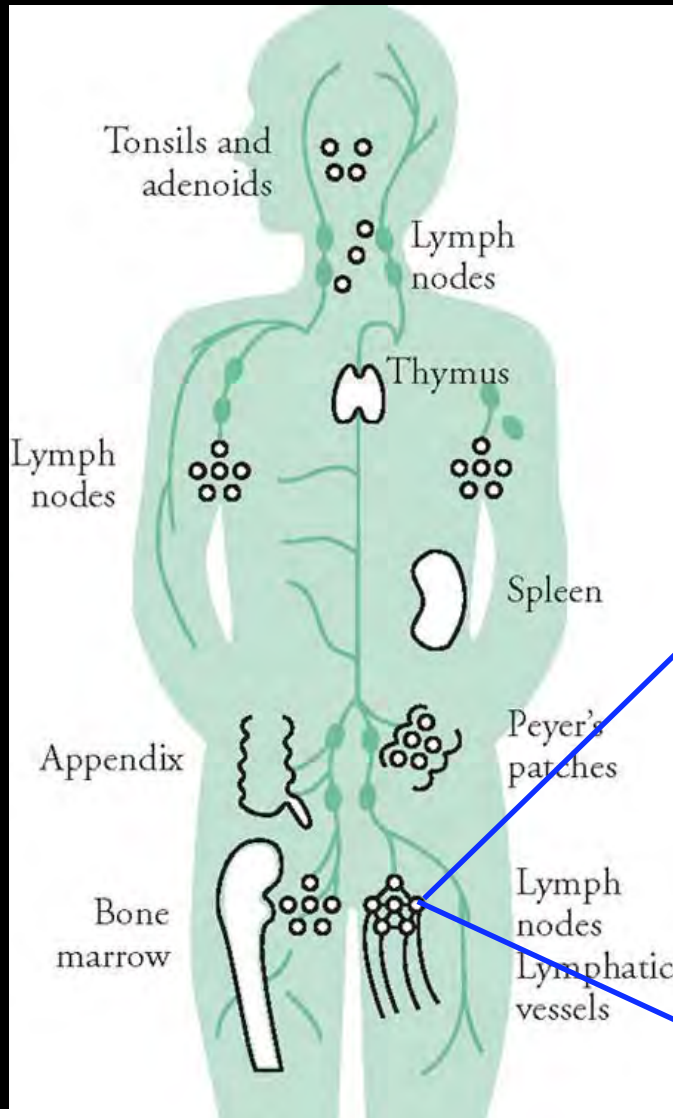
**B**iological  
i  
maging  
**D**evelopment  
**C**enter

at

**UCSF**  
University of California  
San Francisco

# A Challenging Spatial Landscape for Communication/for Systemic Responses

[A complex community of T cells, B cells, NK, DC monocytes, Neutrophils, etc.]

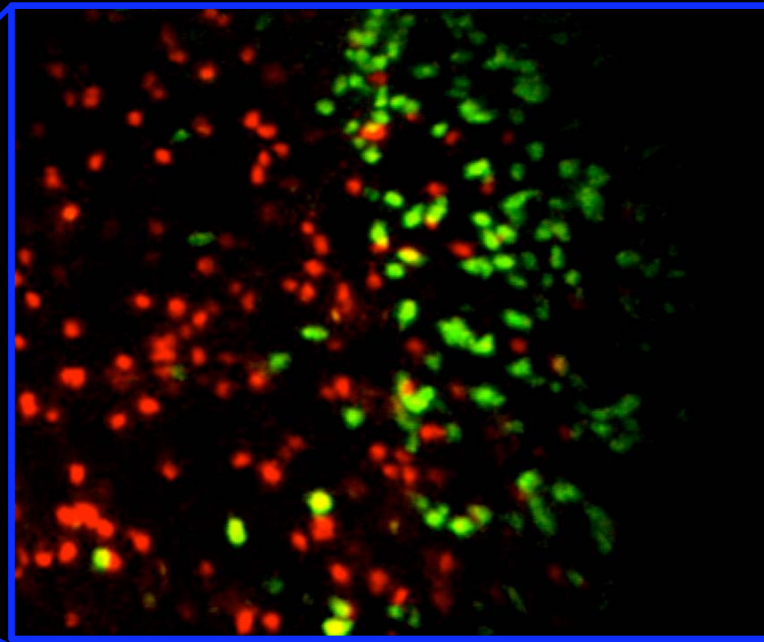


Lymphocyte Communication Does not Obey at least one Aristotelian Ideal: "Broadcast"

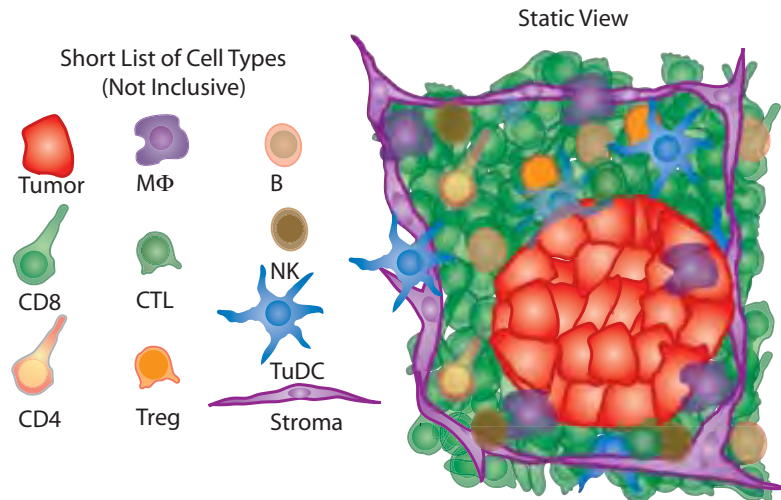
*An entire city should be of a sufficiently small size that all citizens would be able to hear a single herald in peace or a single general in war [Politics VIII]*

Rather, more akin to "Memetic".

*Each Cell Acts to gather and disseminate information bits*

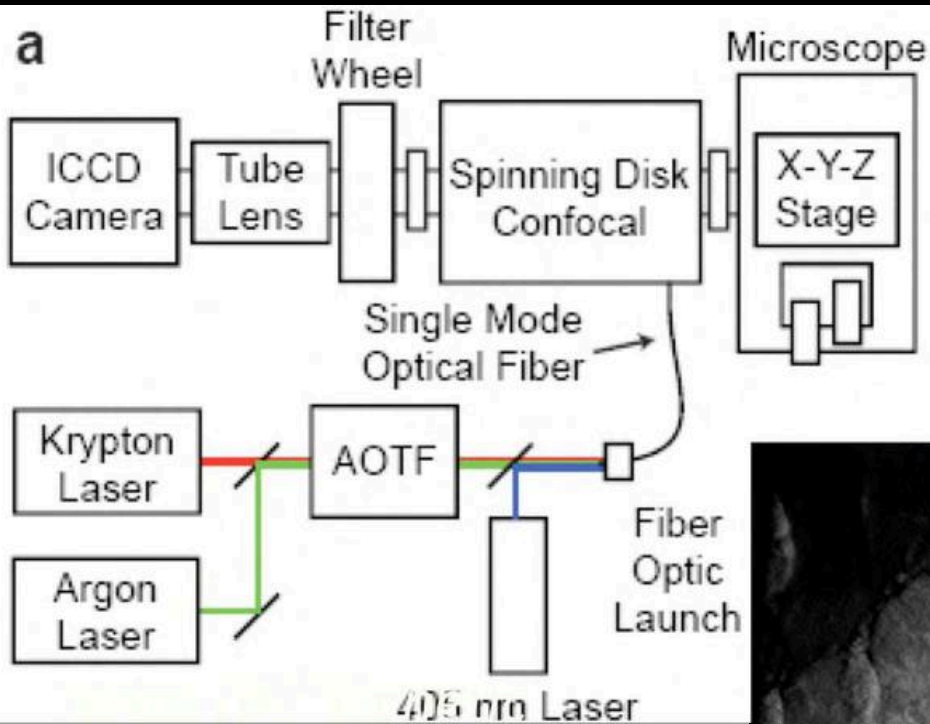


# How can Live-Imaging Help Mine out Significant Systems Events in Complex Tissues?



Flow Cytometry  
Cryo Sections

# Imaging the PyMT Model of Breast Cancer



*Background: → Highlights Tumor AND Immune Cells nicely but not definitively.*

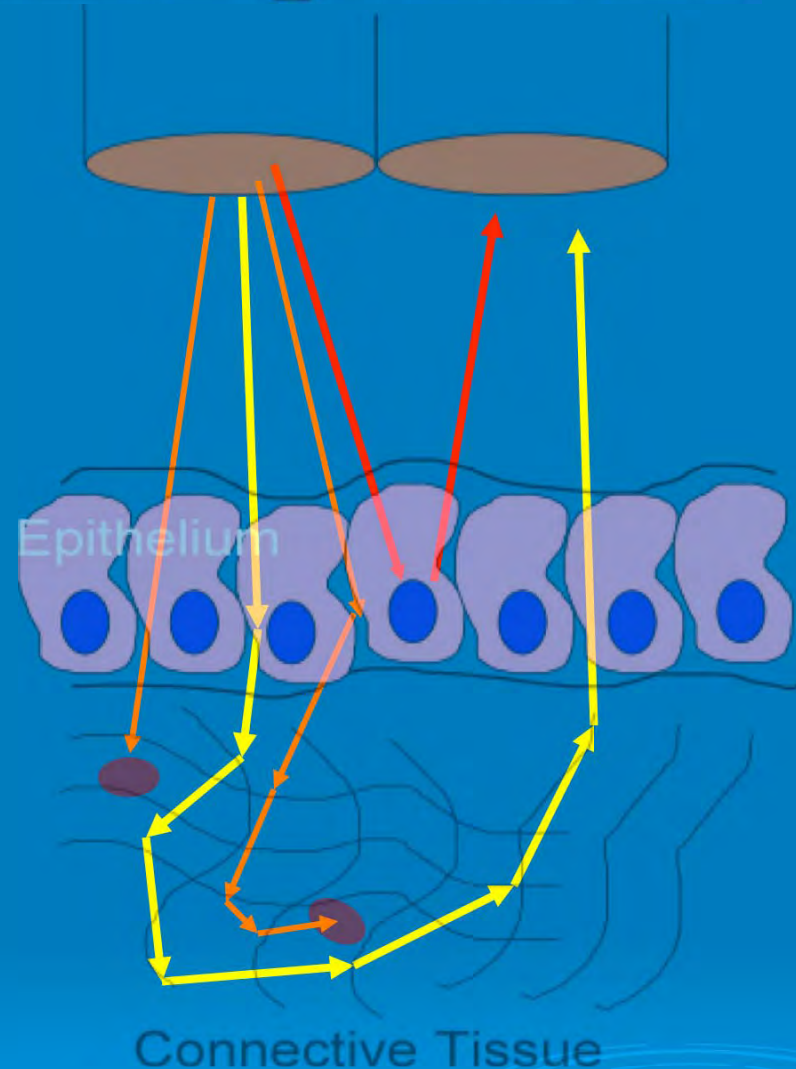


00:00

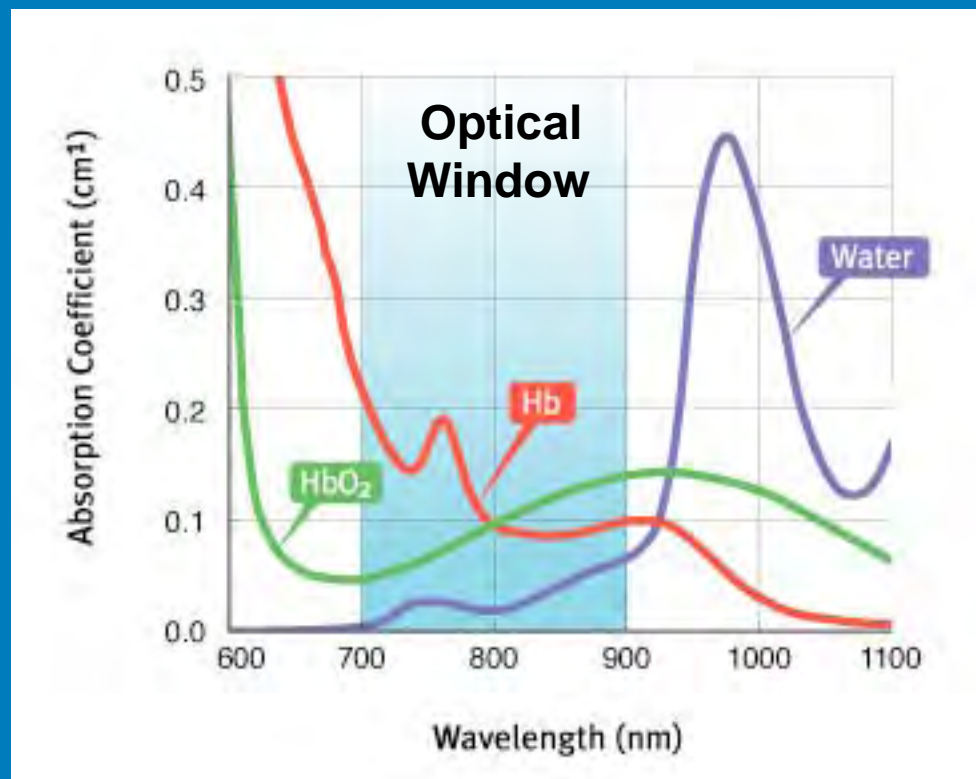
# Issues & limitations of microscopy in tissues & organs

# Tissues absorb & scatter light - wavelength dependent

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

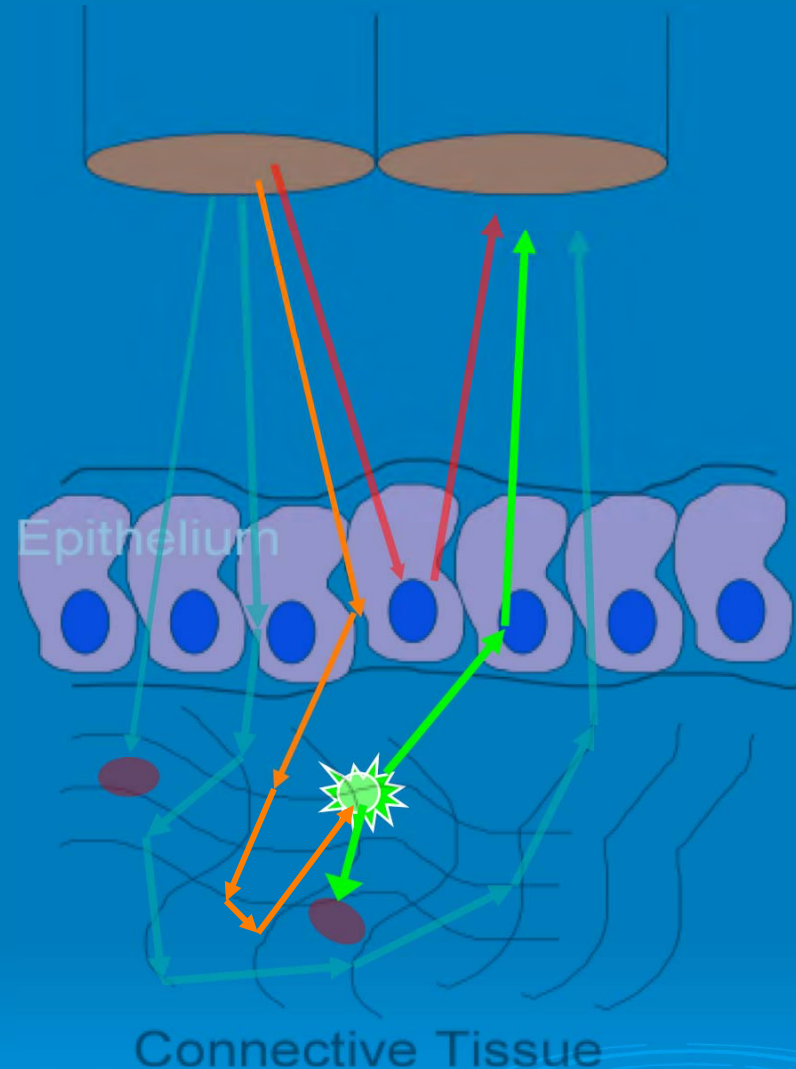


# Optical window for imaging with min. absorption & scatter



# Tissues are Auto-fluorescent

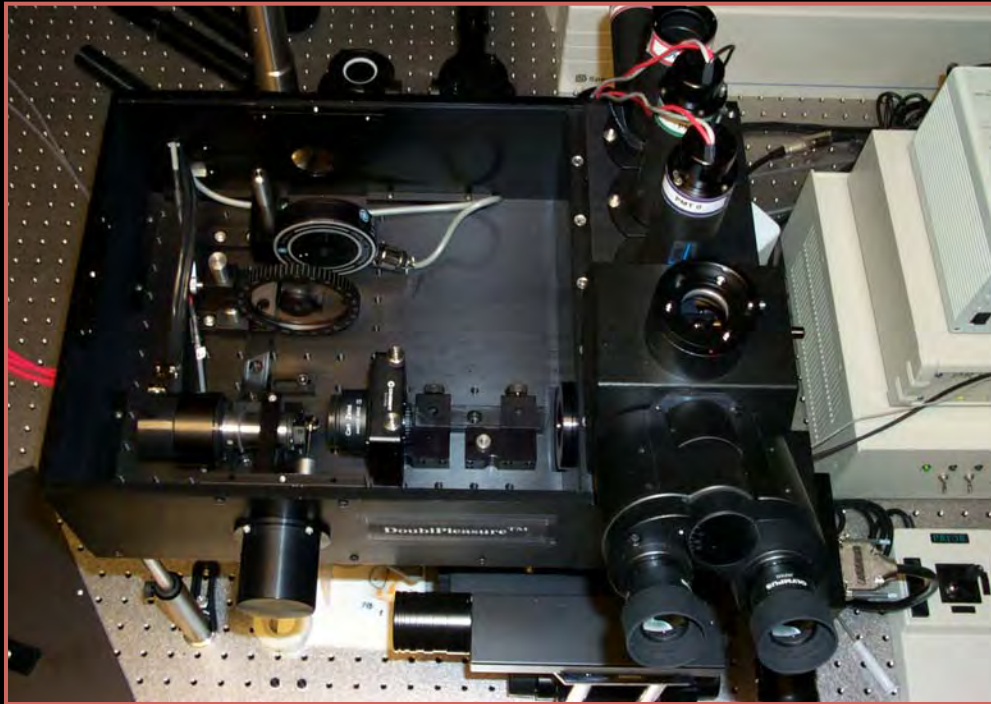
- scattering
  - elastic scattering
    - multiple scattering
    - single scattering
- absorption
- fluorescence



# Mechanical issues limit physical access in situ in some cases

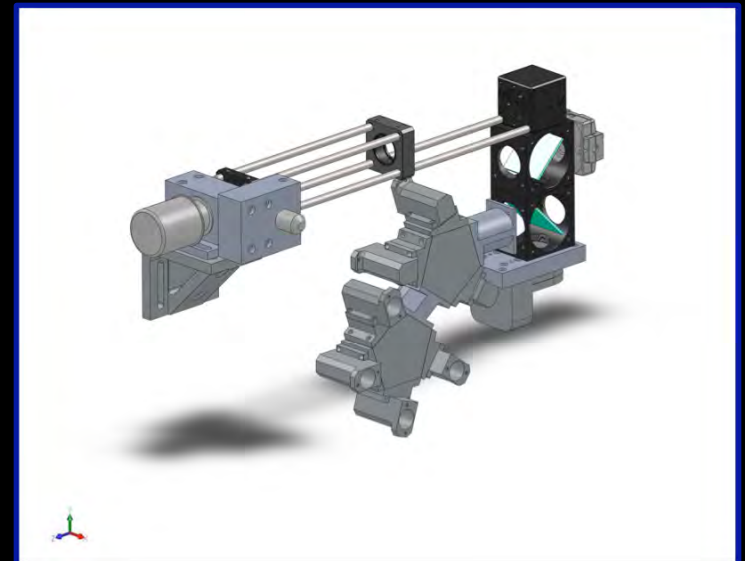


# Custom 2P Instrumentation



3-30fps  
6 emission PMTs

Dual exc. laser (Gen3 only)  
(6-12 channels overall)



**B**iological **I**maging **D**evelopment **C**enter

at

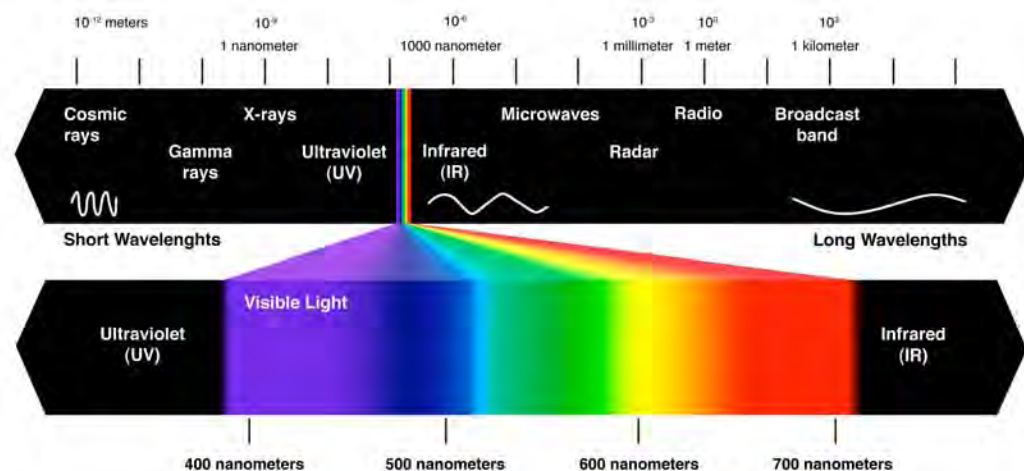
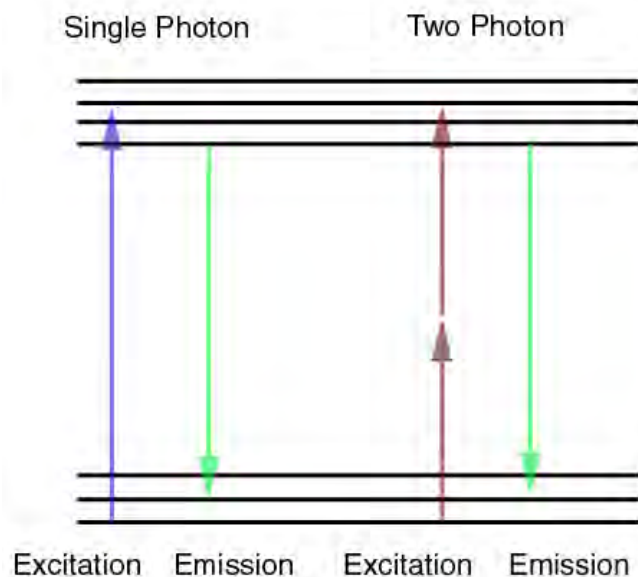
**UCSF**

University of California  
San Francisco

Home of the Strategic Asthma Basic REsearch Imaging Center

# 2-Photon Fluorescence: How and Why?

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

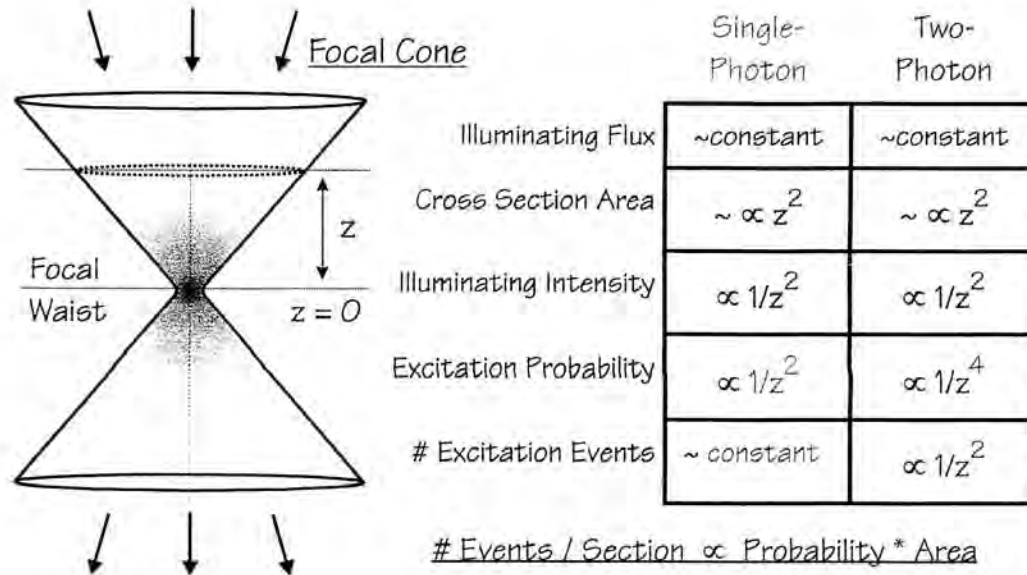


**Why:** 2 Photons only coincide with high frequency precisely at the focal point.

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

*Thought experiment:  
Flashlight/Camping*

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

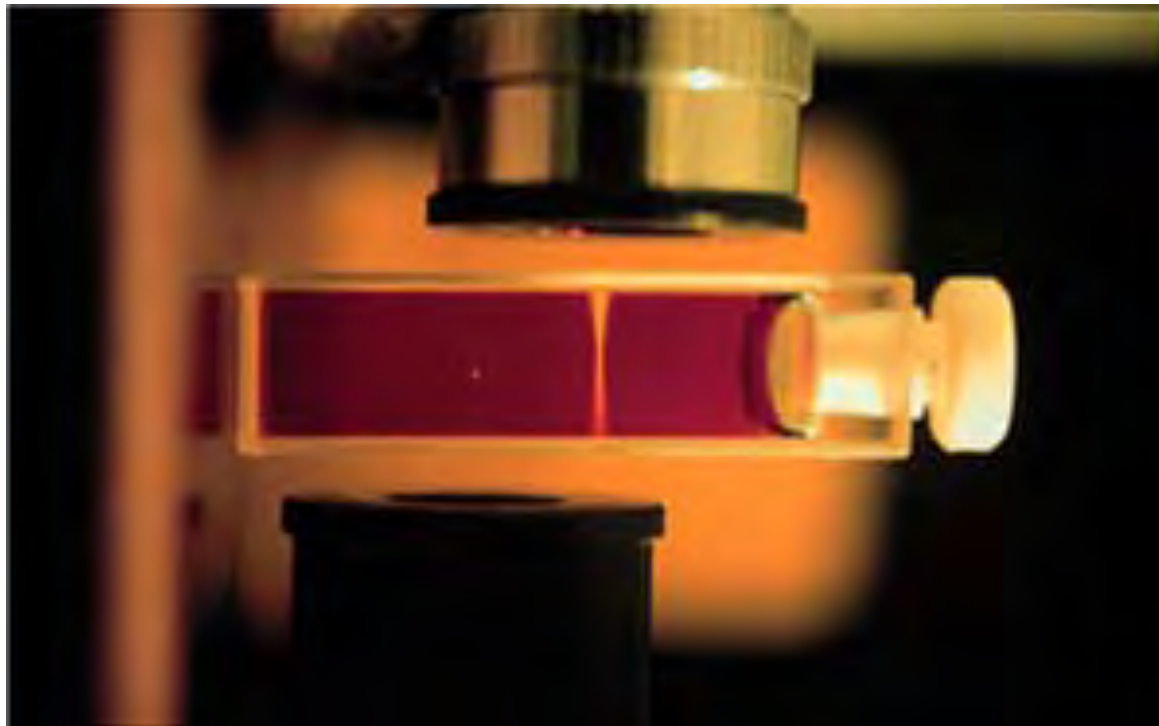


## Single-Photon (Confocal) vs. Two-Photon Imaging:

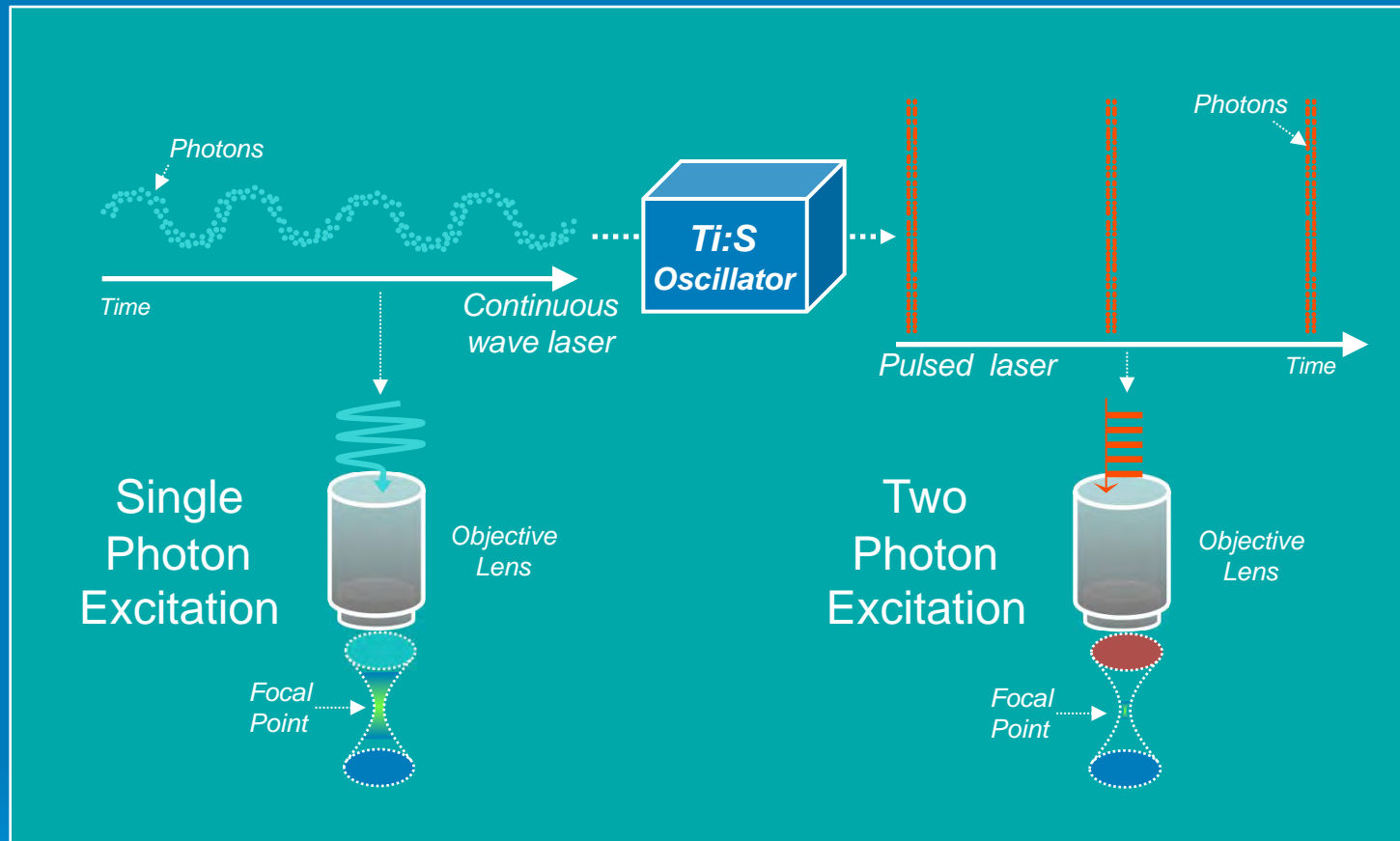
Single-Photon process produces excitation (and photodamage) throughout specimen volume. Optical sectioning requires imaging and spatial filtration of emission light, which is inherently inefficient and is highly susceptible to light scattering losses.

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..

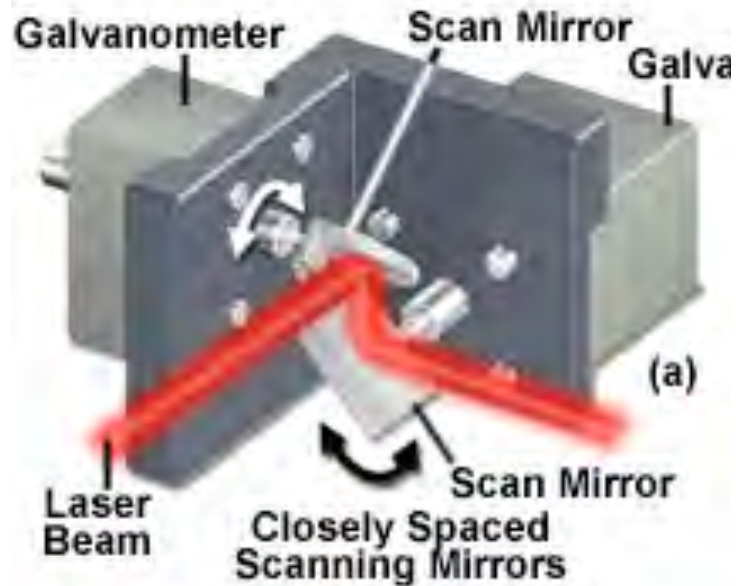
## Multi-photon Microscopy: Example of Precision of Excitation



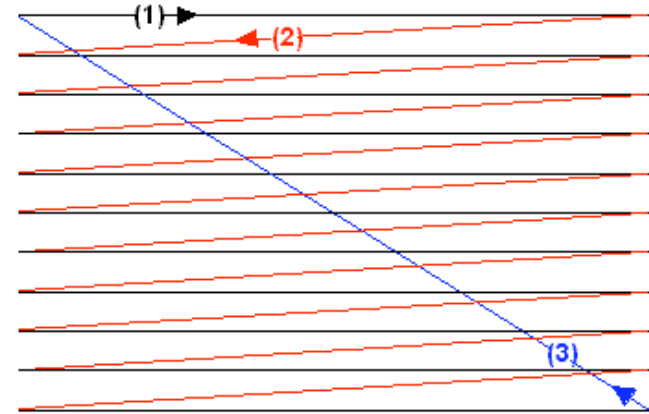
# 2P excit. requires concentration of photons in space & time



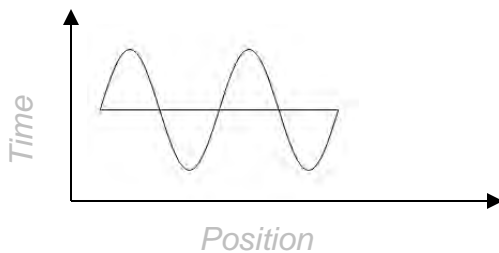
# Recall that this is achieved via: The Raster Scan



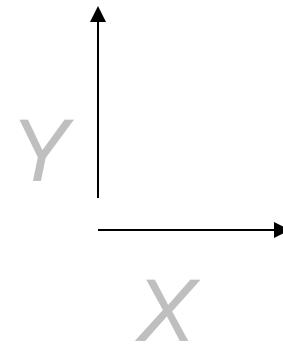
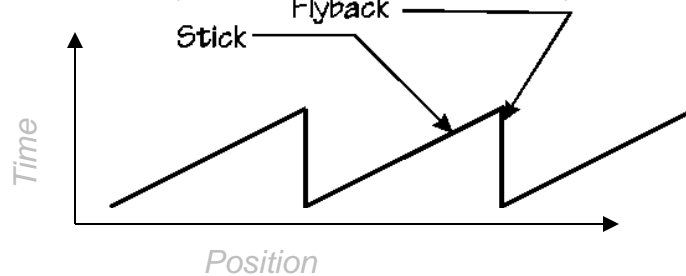
*A small spot (laser beam)  
Scans the sample*



*X (fast mirror) follows a sine curve*

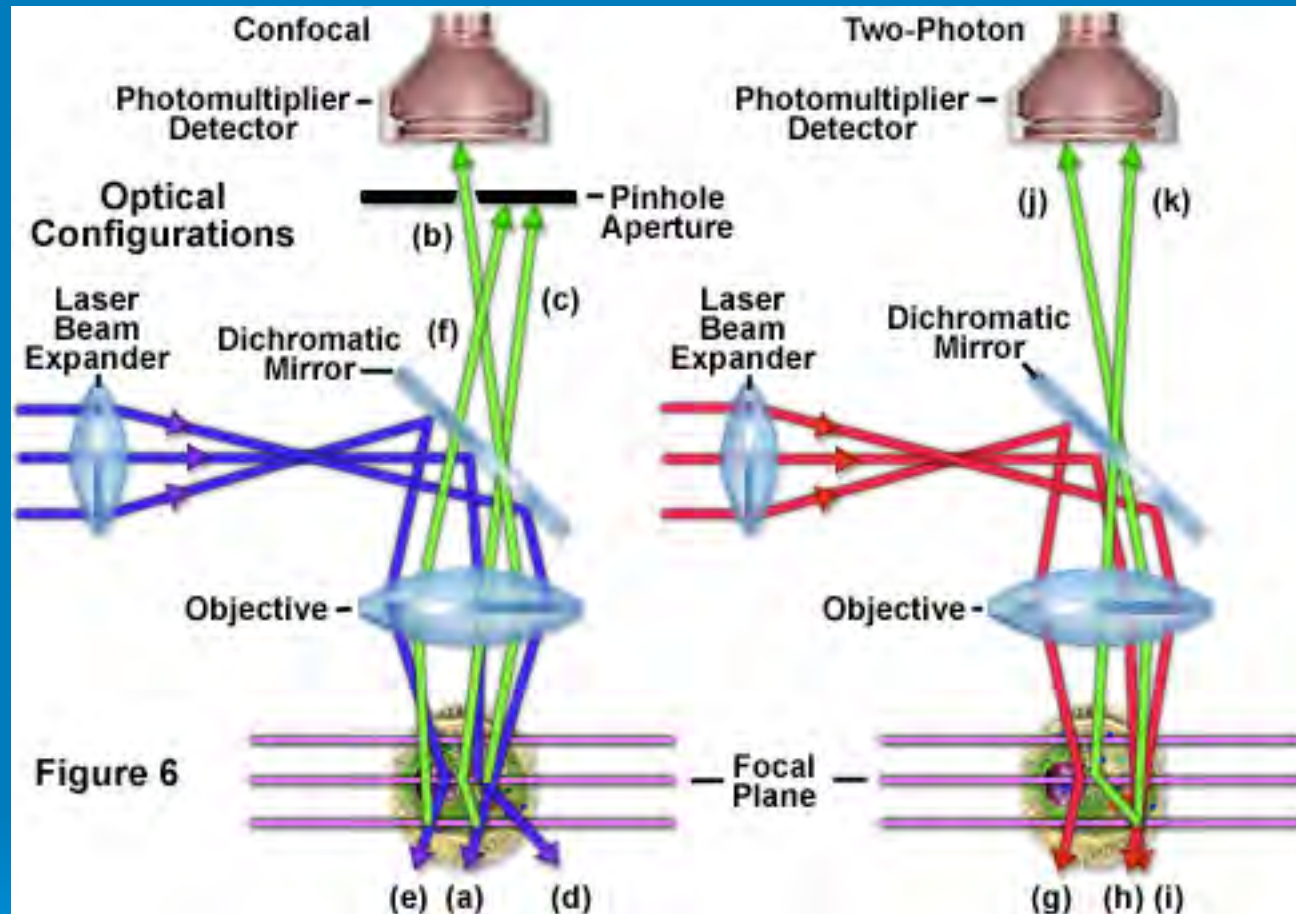


*Y (slow mirror) does sawtooth*

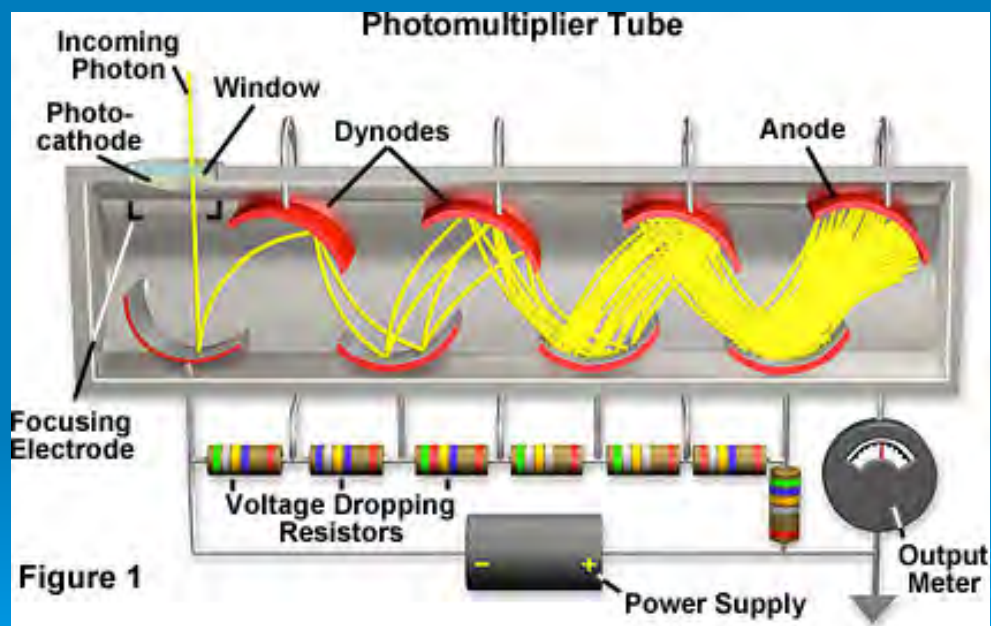
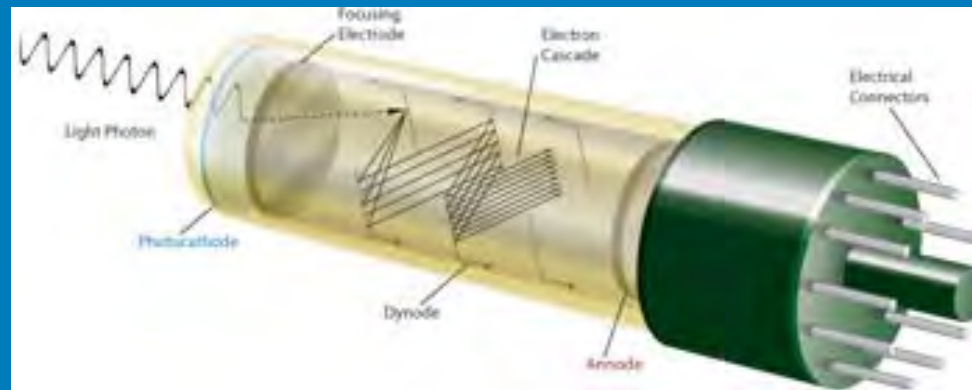


*Collect All Emission at detector (regardless of path)*

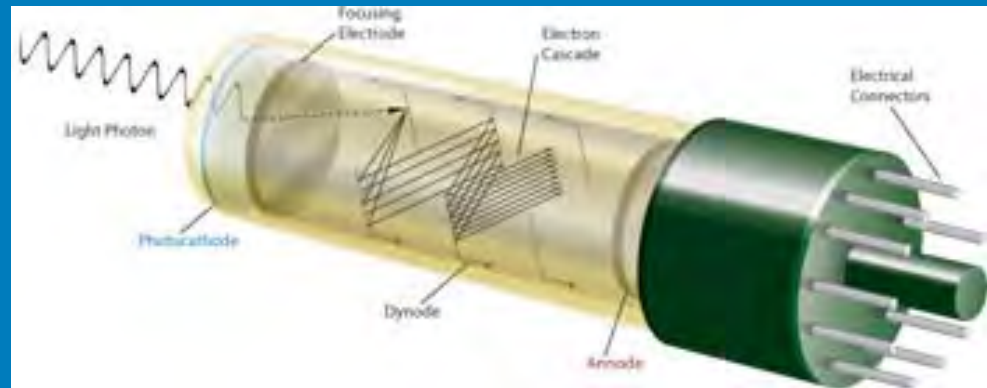
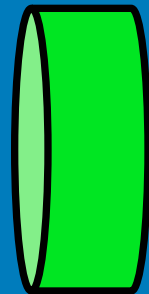
# 2P microscopy employs non-descanned detectors



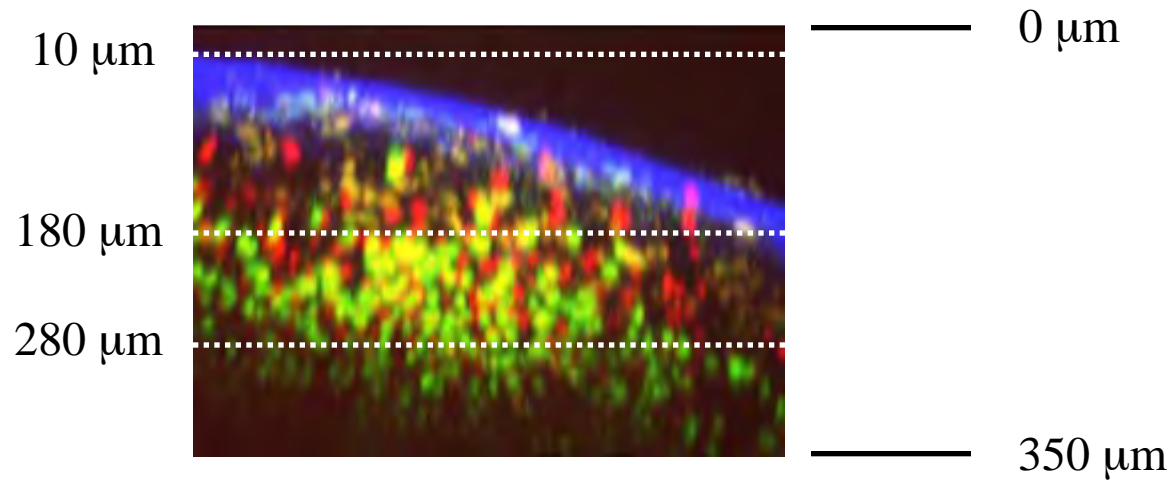
# 2P scopes use PMTs as detectors



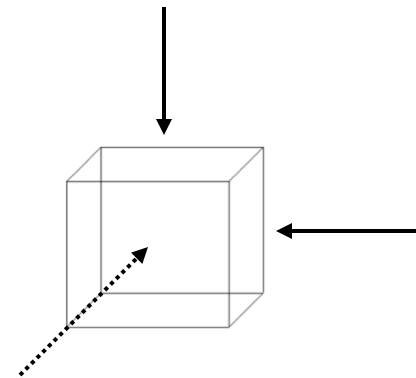
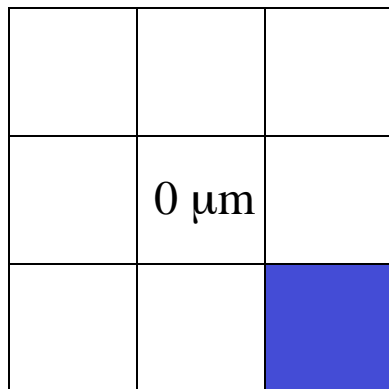
# PMTs report intensity for single color based on fixed filter



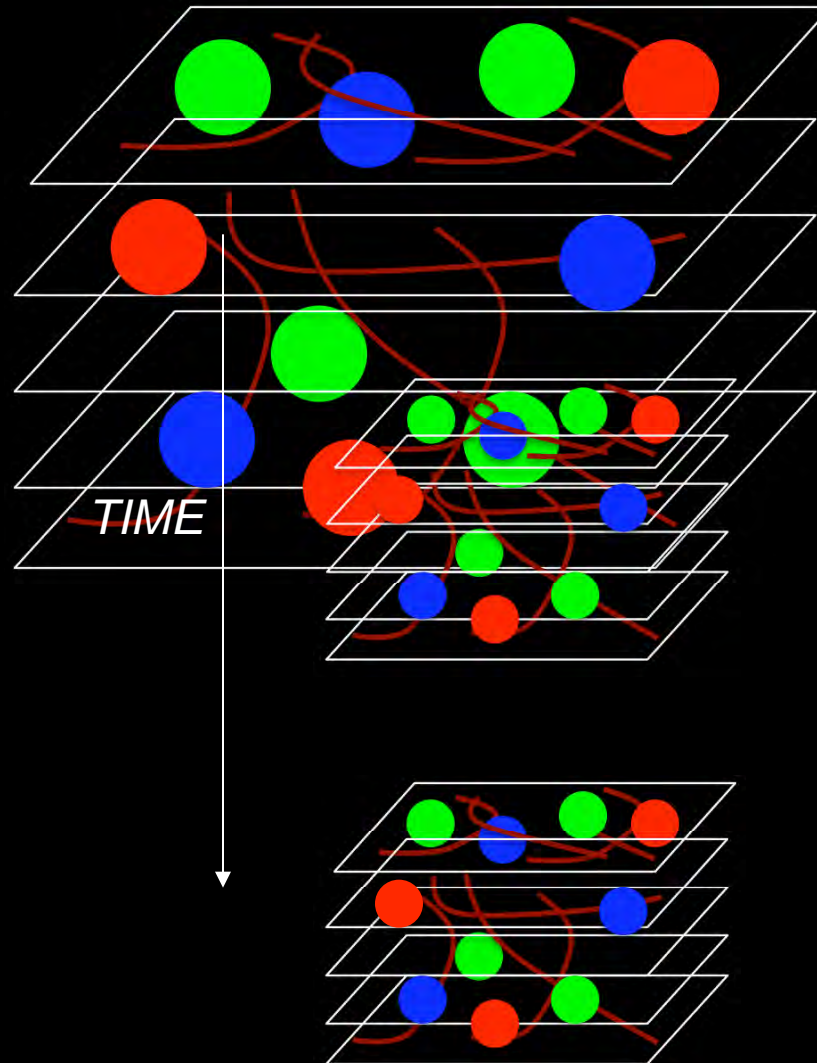
# Tiled Cubes of Data to 'Survey' an Organ: e.g. Inguinal Non-draining Lymph Node



Top Down

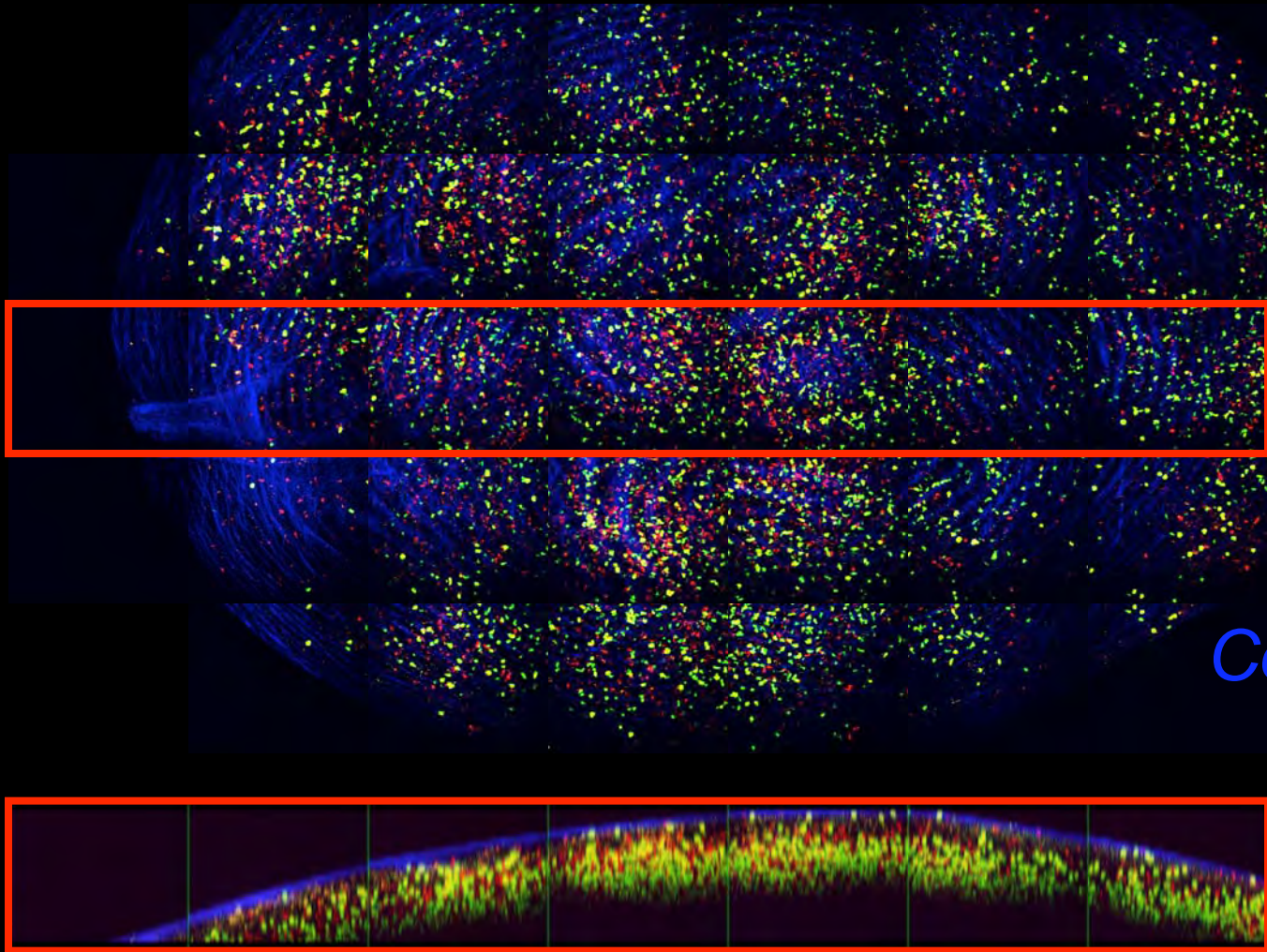


# *A 5D 2-Photon Platform*

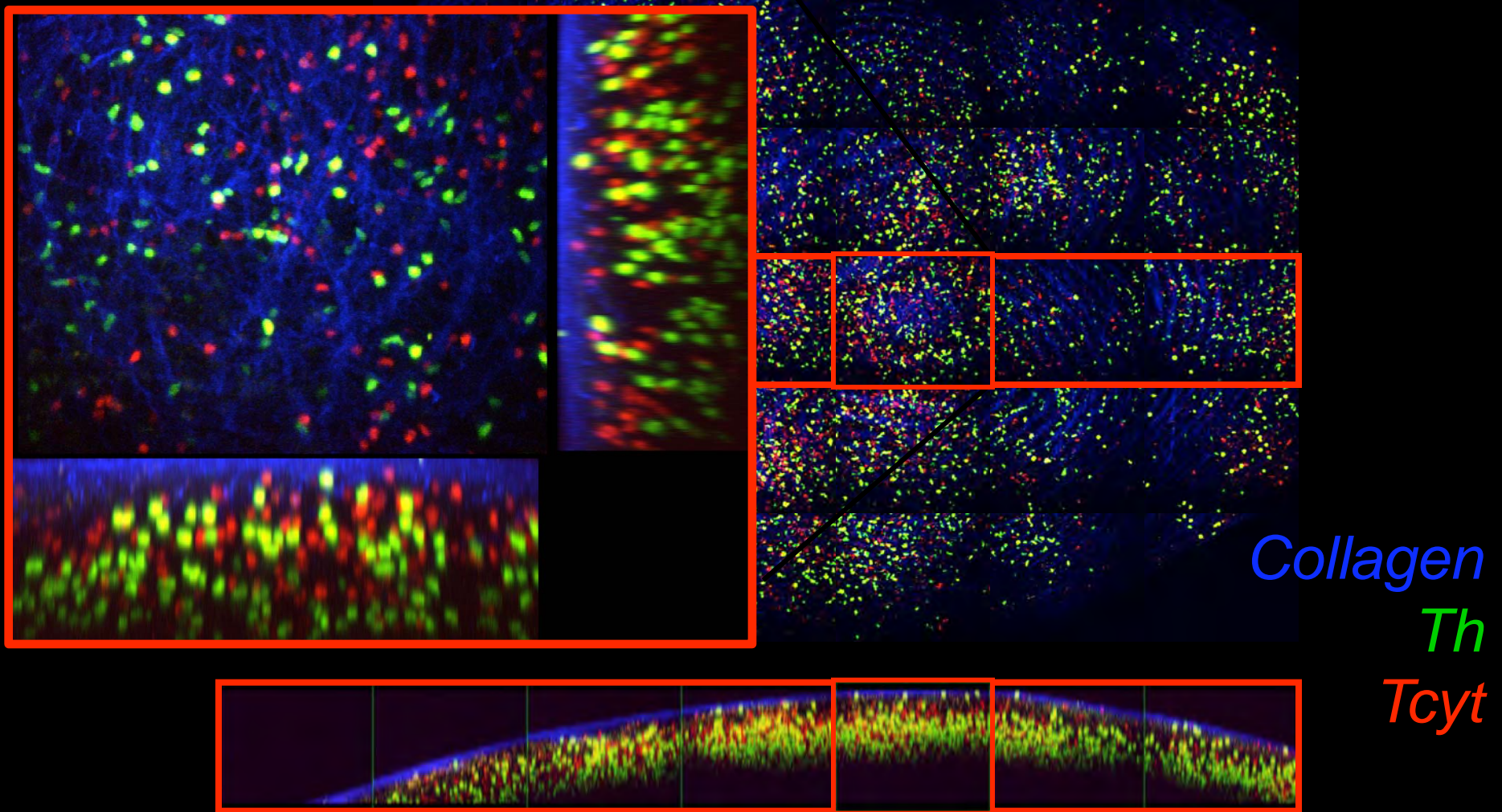


*Analyze in:*  
*Matlab*  
*ImageJ*  
*Metamorph*  
*Imaris*  
*IDT*

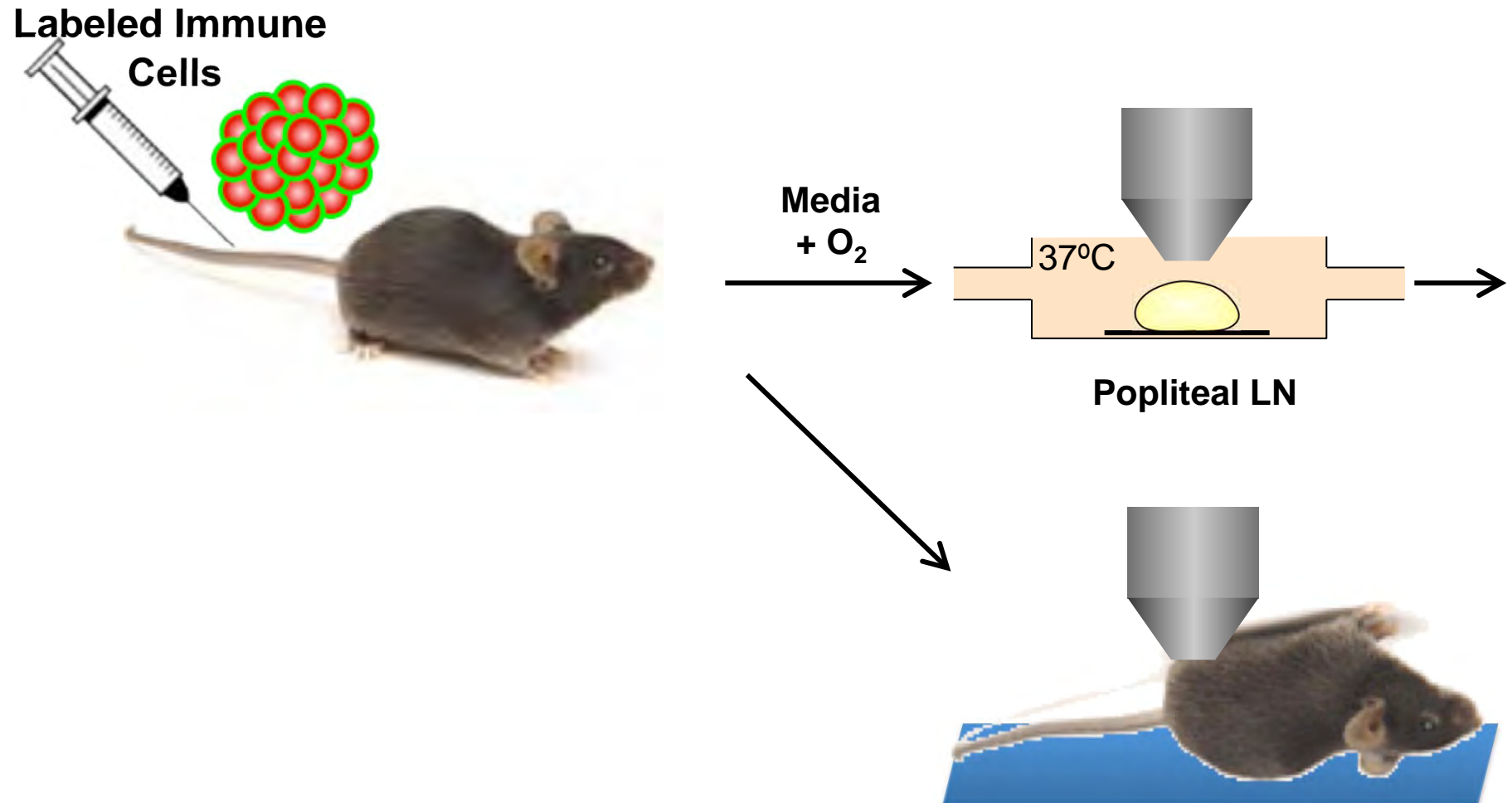
# *A 5D 2-Photon Platform*



# A 5D 2-Photon Platform



# Organ Explant and intravital system for 2-photon imaging

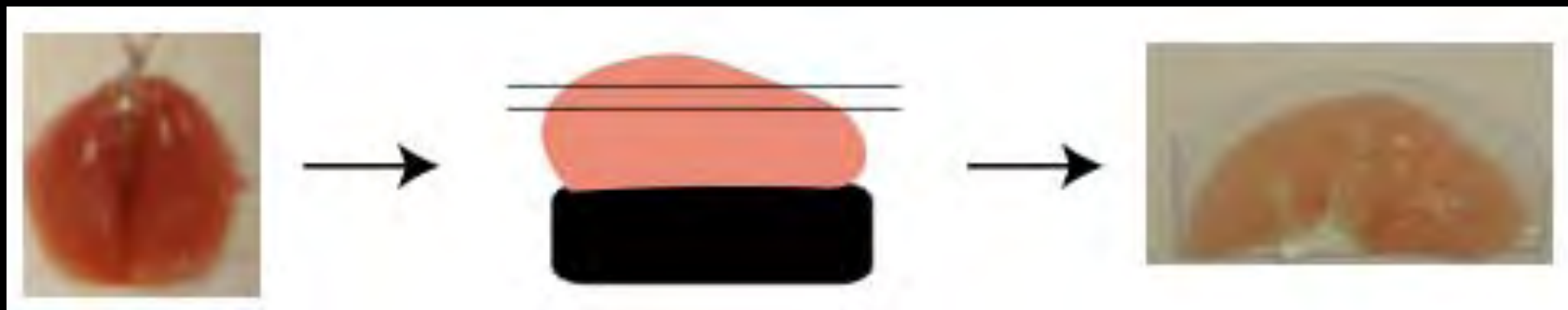


# Model System for Asthma/Allergy Imaging



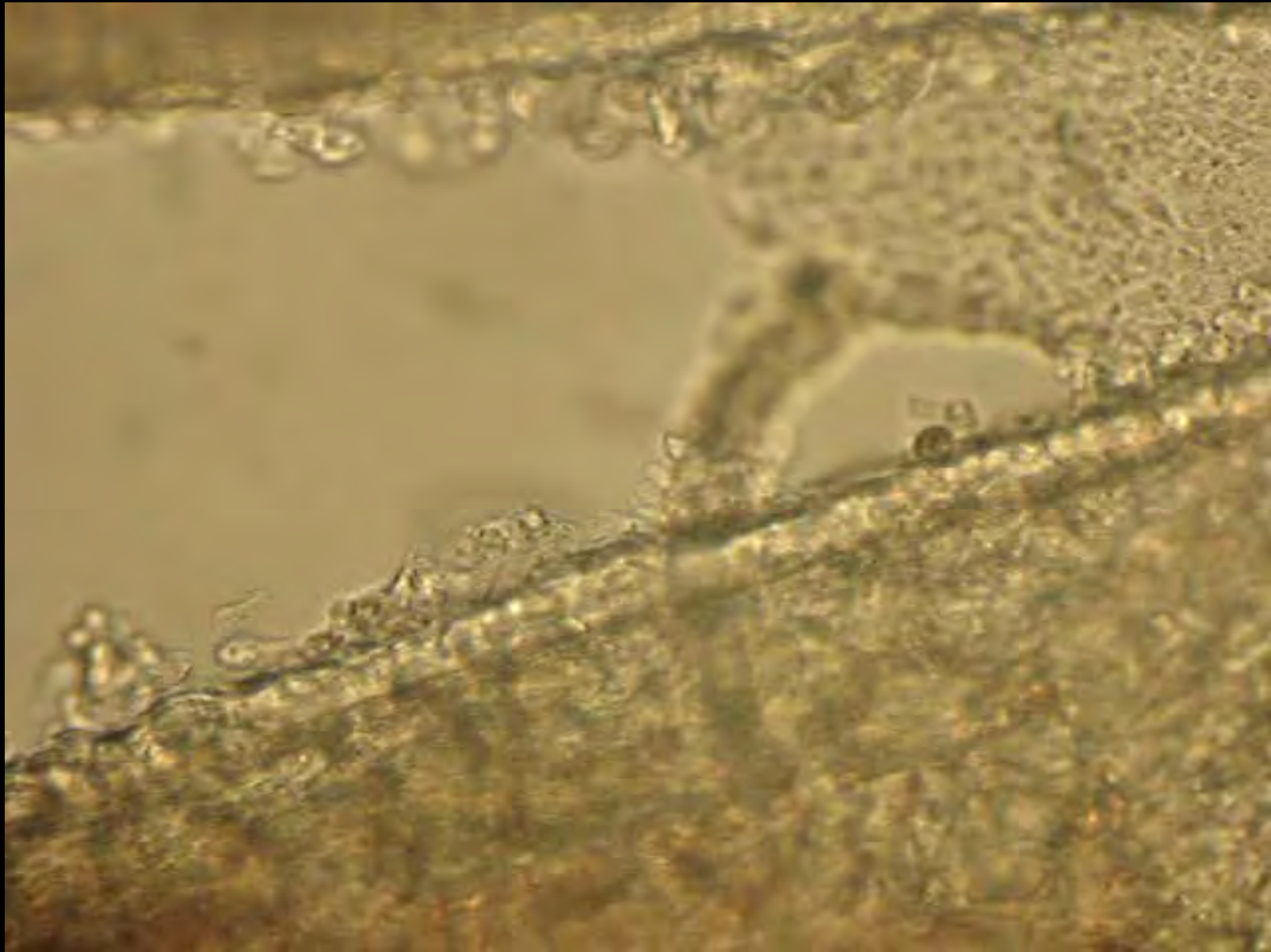
Sensitize with ova-alum IP

Challenge with ova IN

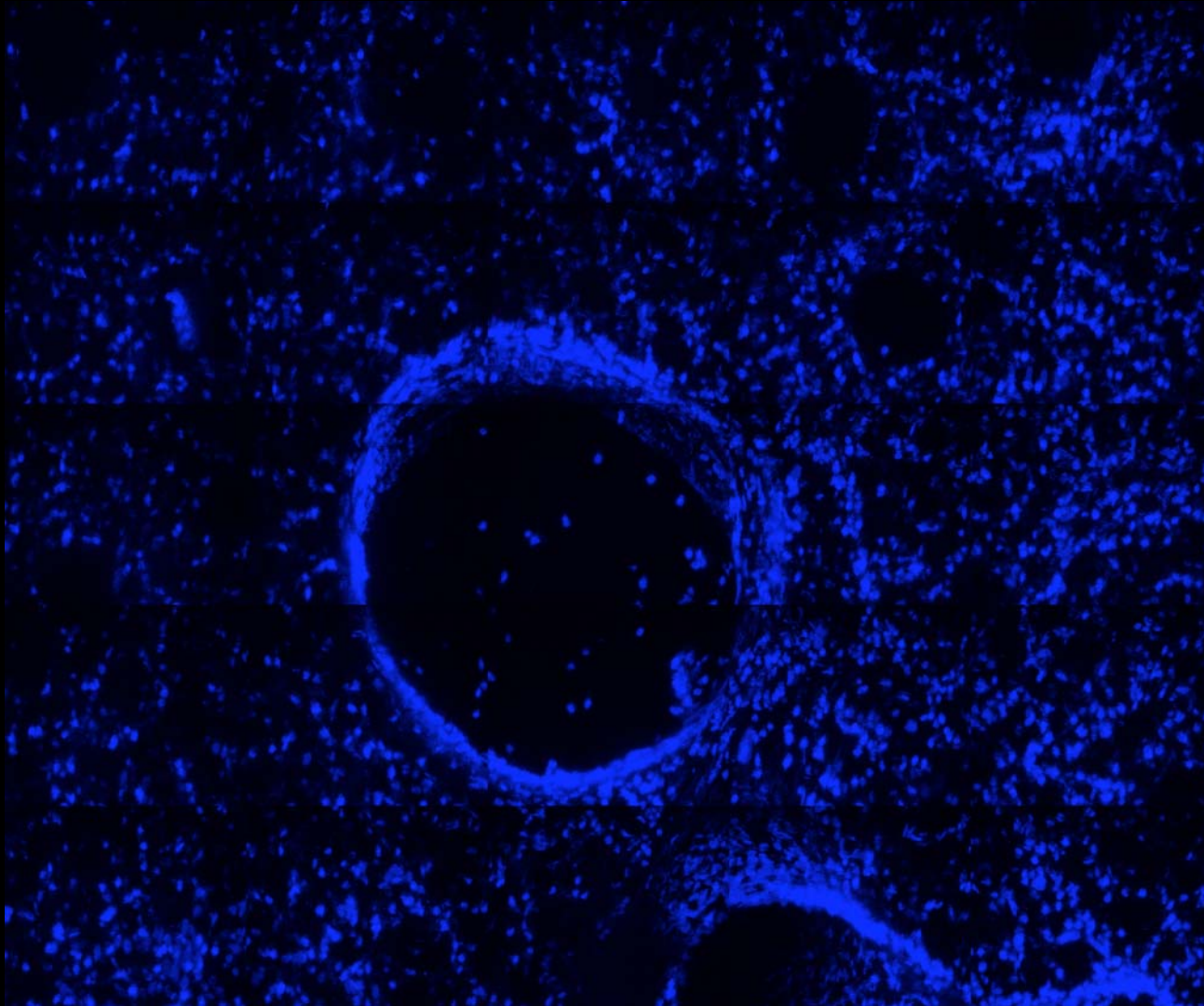


Confirming Tissue Viability:  
Movie of Cilia at 3 hrs post sac

---

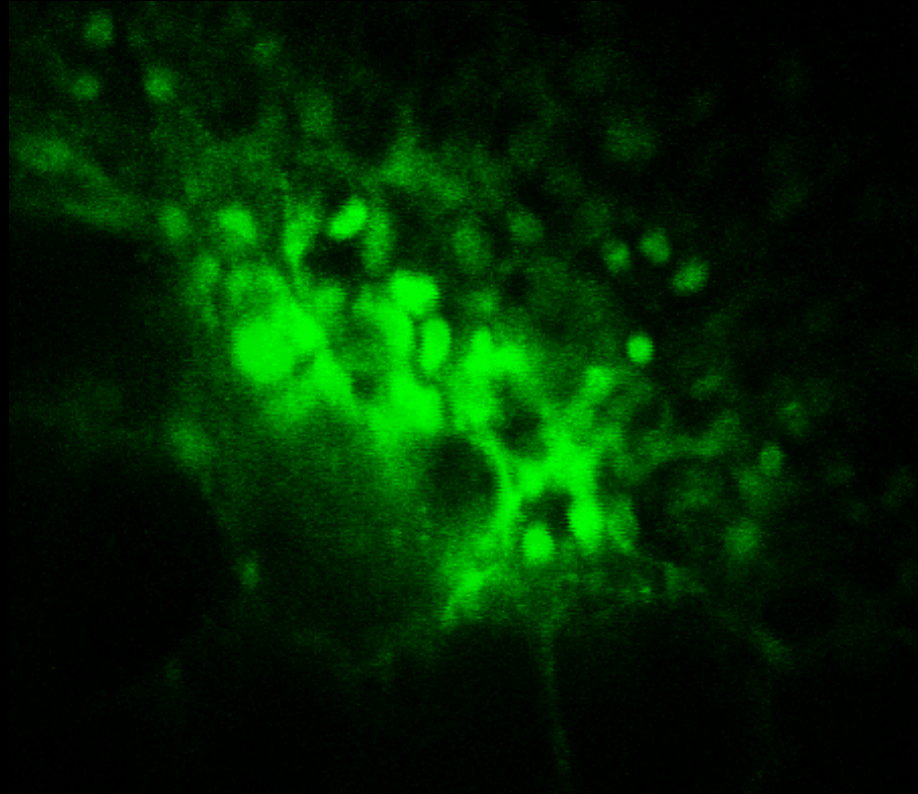


How to find your way around?. Vital dyes...



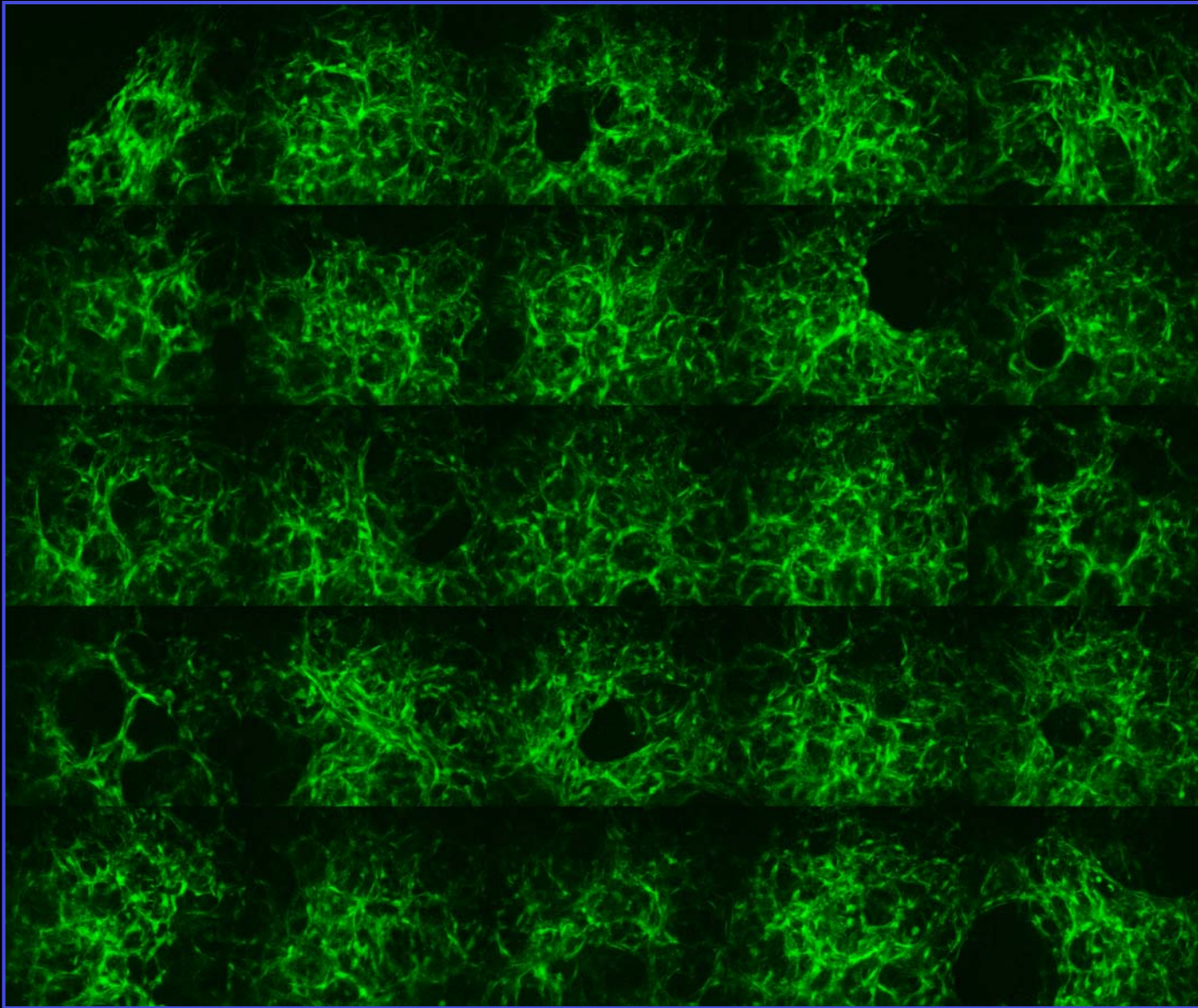
Small wt airway-hoechst

Cytoplasmic Vital Dyes also permit live-imaging.  
Further support that tissue is 'viable'.

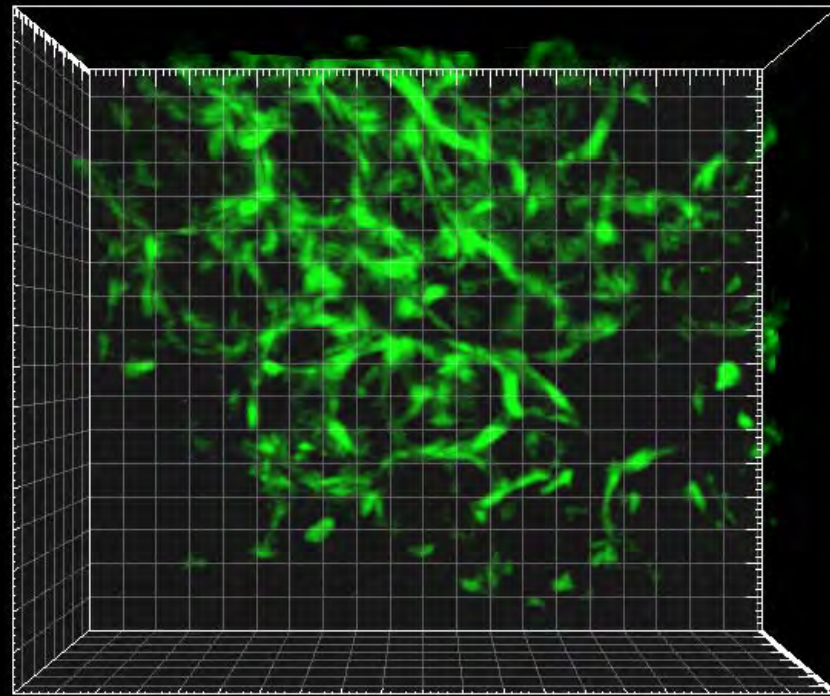


Small Ova airway → alveoli :: CFSE

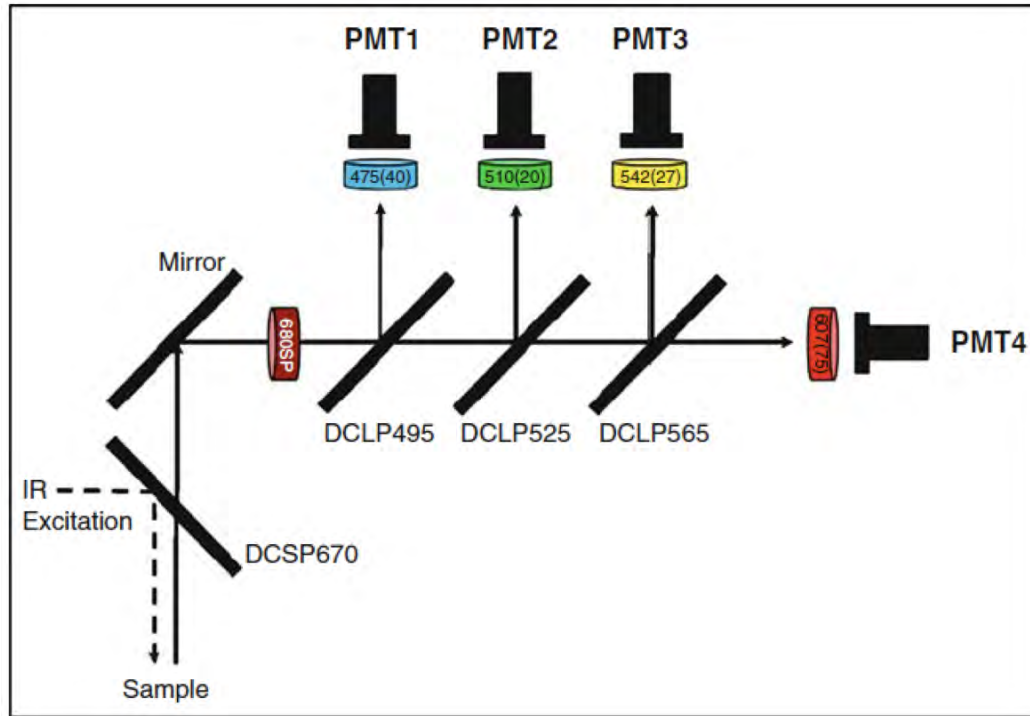
# GFP Fusions: Sca-1 GFP



# GFP Fusions: Sca-1 GFP

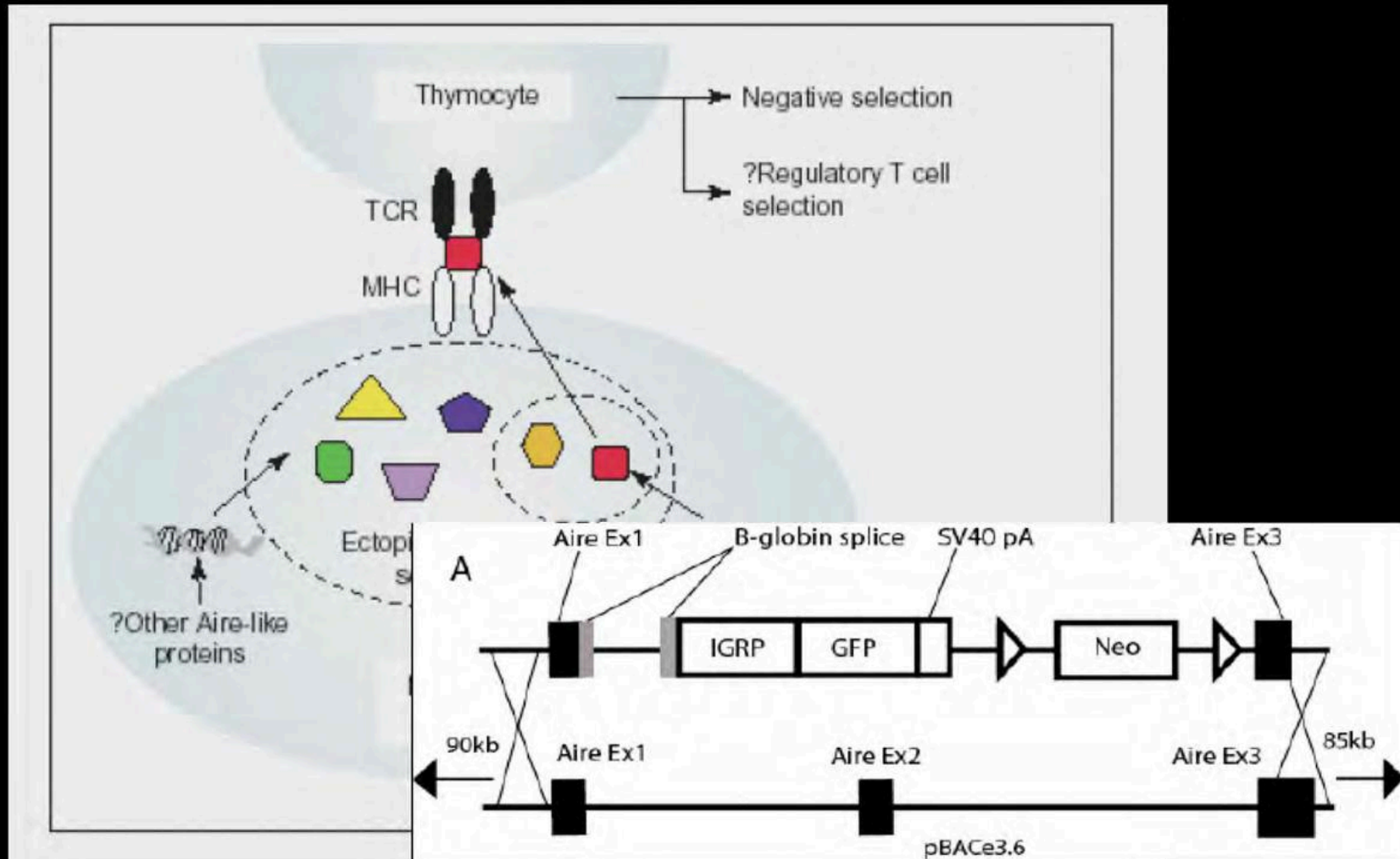


# Why so many detectors?



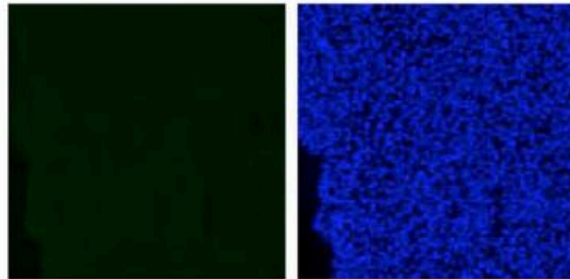
- 1. In 2P, non-linearity allows that two green fluorophores with nearly identical emission can be distinguished.  
e.g. GFP excites 920nm (emits ~510nm)  
FITC excites 800nm (emits 510nm)***
- 2. Collect a lot of parameters***
- 3. Defeat Autofluorescence.***

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

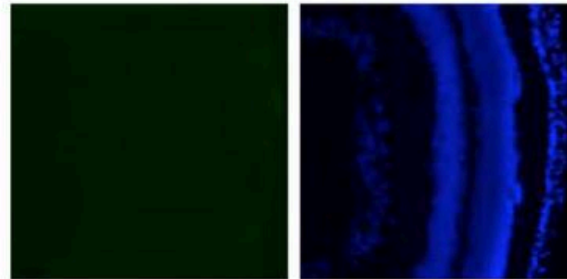


Su, M. and Anderson, M. *Curr. Opin. Immunol.* 2004, 16, 200-204

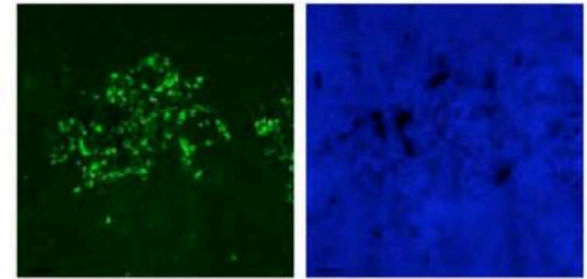
# Extrathymic IGRP-GFP+ Cells Subsequently Detected in Tissue Survey



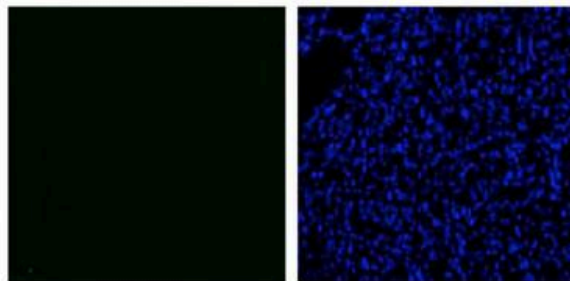
liver



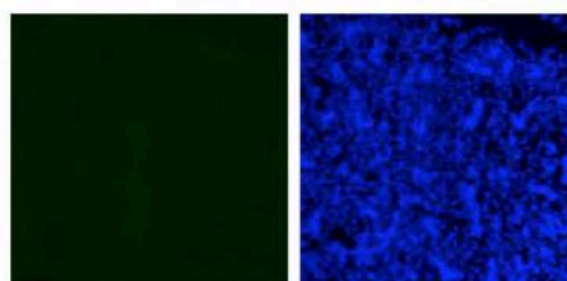
retina



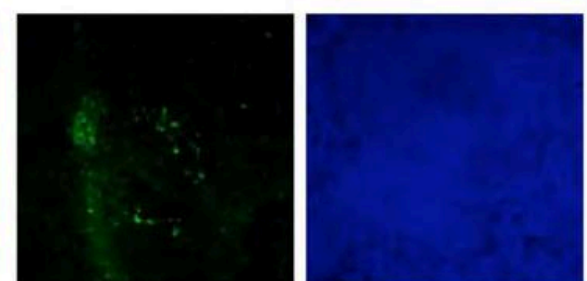
thymus



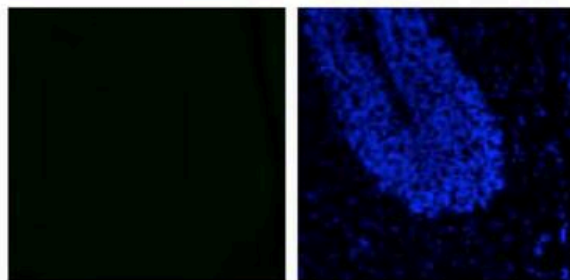
heart



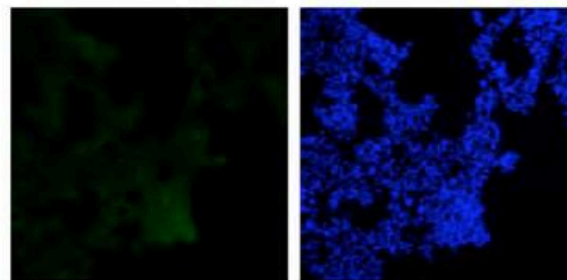
salivary



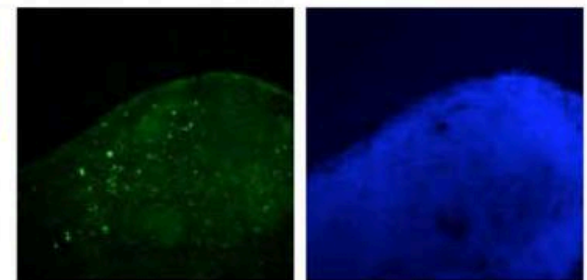
spleen



cerebellum

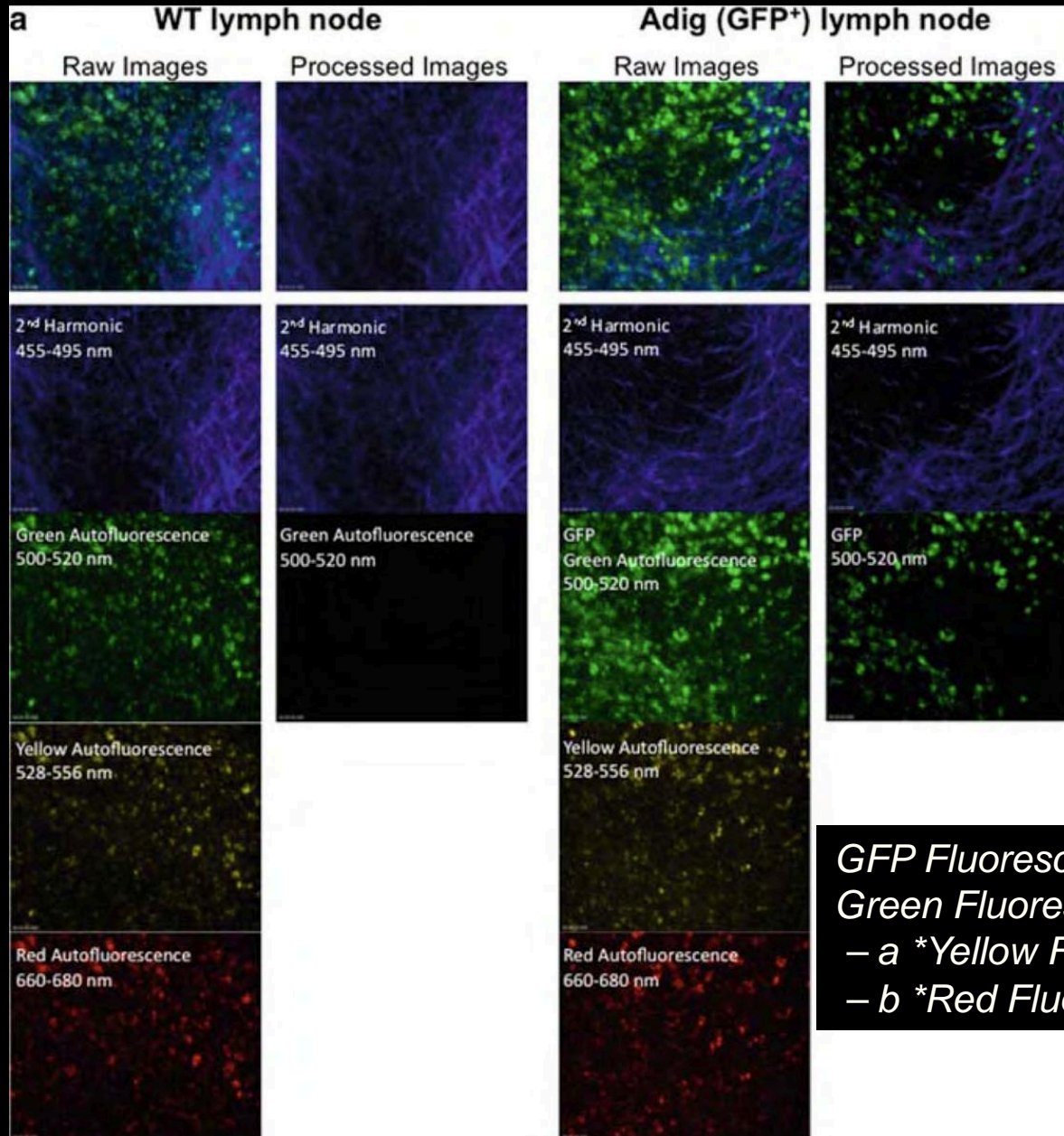


lung



lymph node

# Using Multiple Channels to Remove Autofluorescence



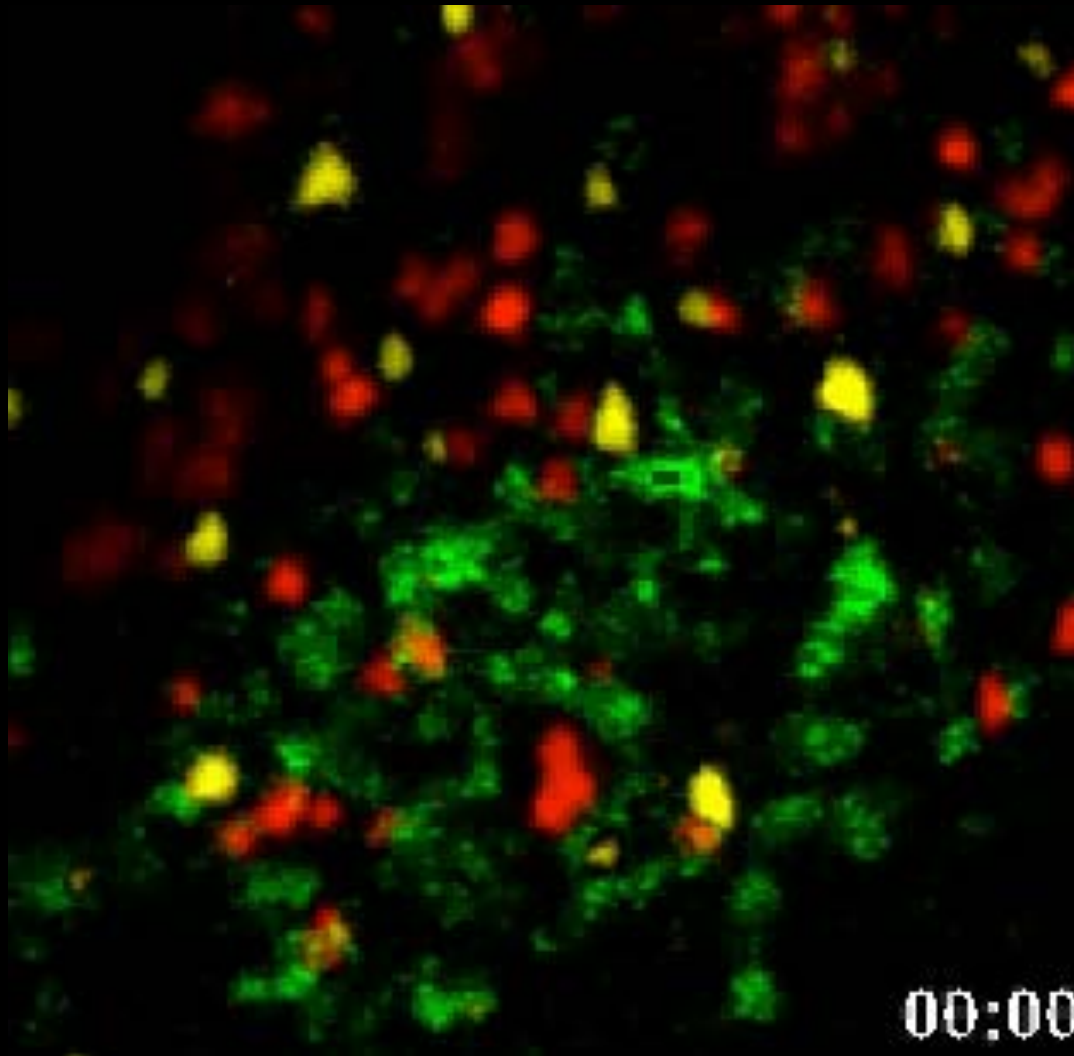
*GFP Fluorescence =  
Green Fluorescence  
– a \*Yellow Fluorescence  
– b \*Red Fluorescence*

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

AIRE: IGRP-GFP

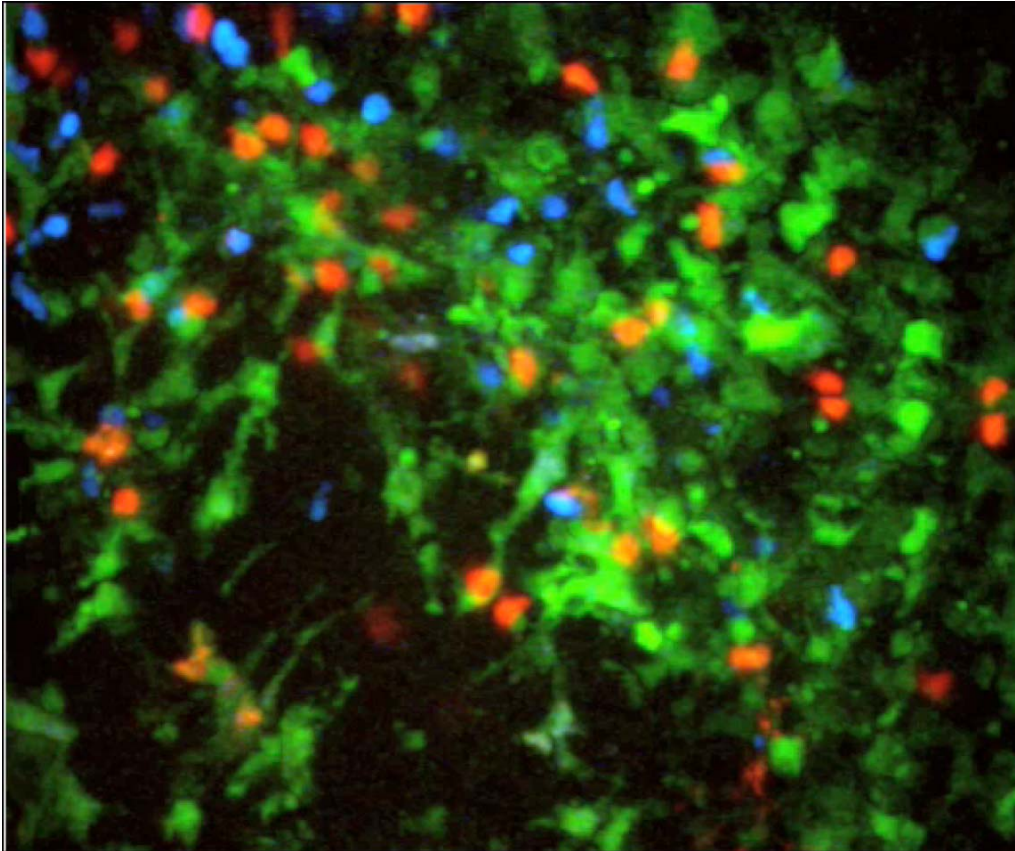
8.3 T cells

Polyclonal NOD T cells



# What can live *in situ* imaging tell us about biology?

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## Kinetics of Cellular Behaviors

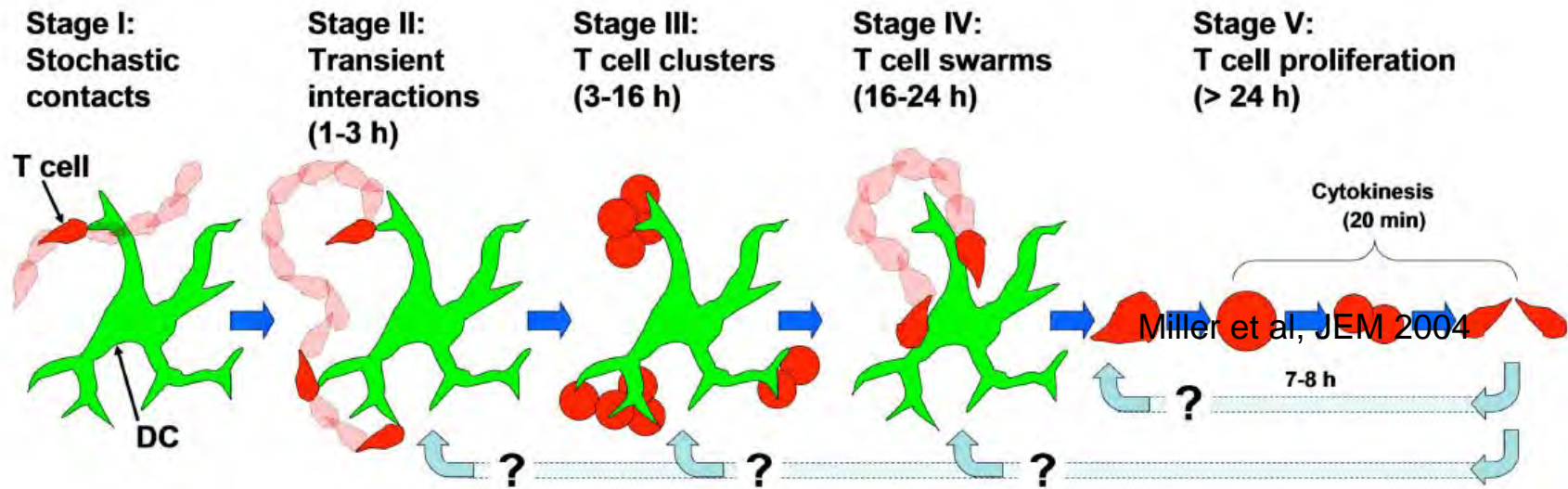
Cell-cell interactions

Cellular morphology

Speed/direction/confinement

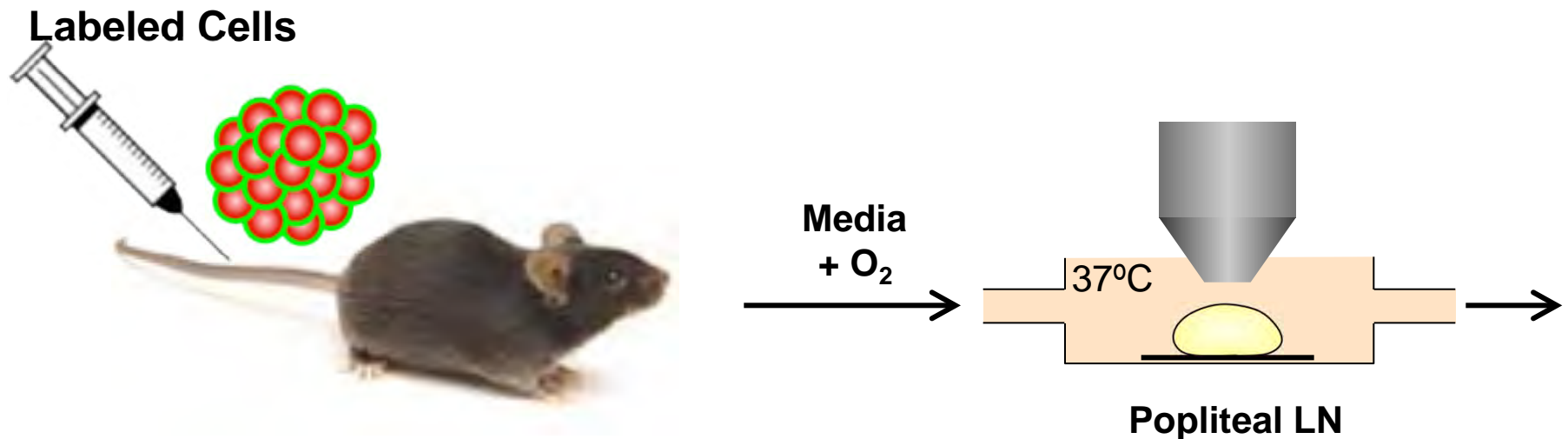
Cell death

# *In vivo* T cell activation



# Experimental system for 2-photon imaging

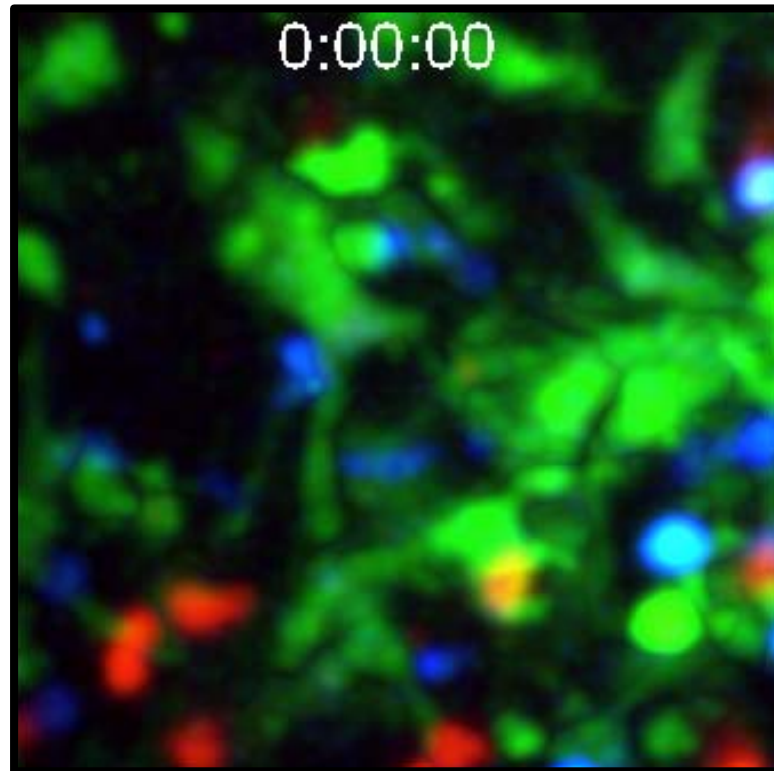
---



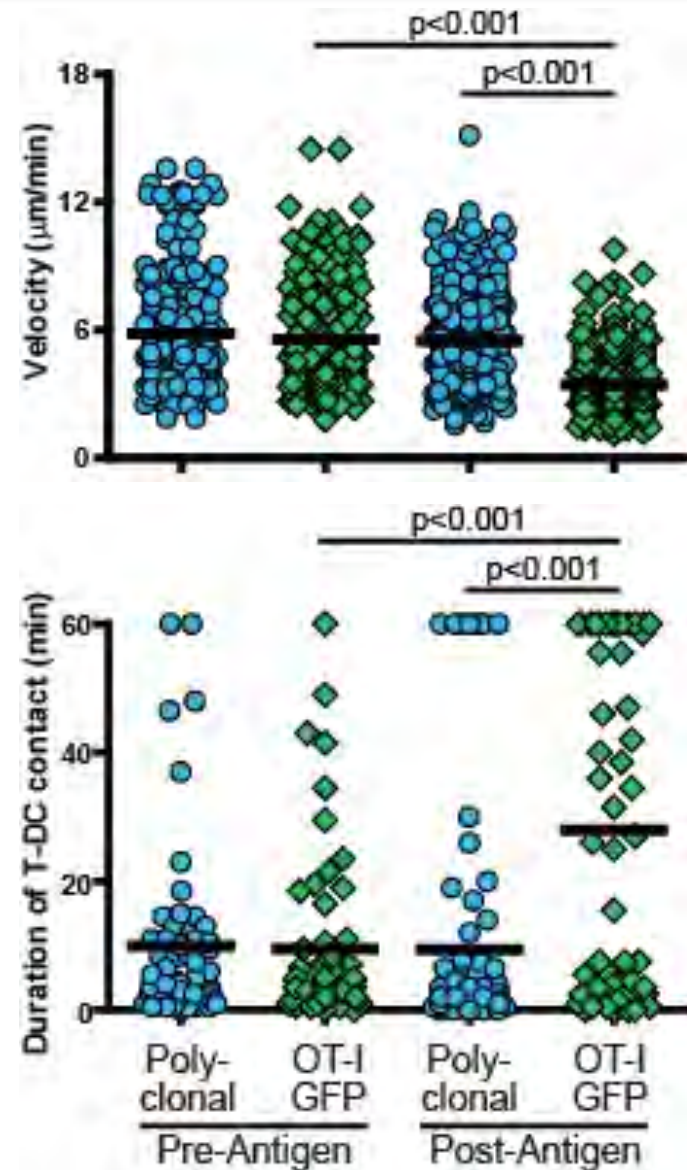
## Host:

- wt or genetically marked (e.g. CD11cYFP) mouse
- with or without labeled antibody injection to mark cell types
- Vascular markers or Dextran to mark vessels
- Hoechst or vital dyes to mark nuclei.

# T cell-DC interactions before & after antigen addition

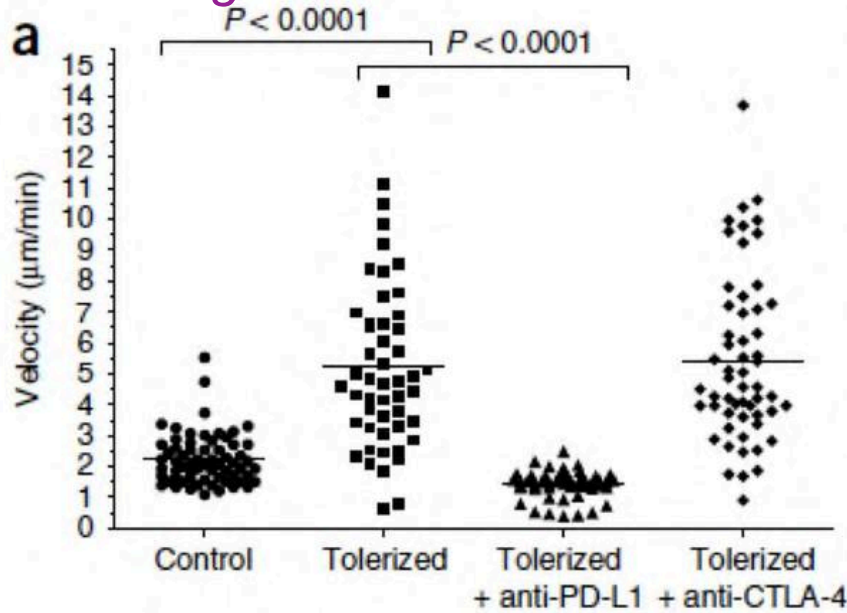


**CD11c-YFP DCs**  
**OT-I-GFP T cells**  
**Actin-CFP+ Polyclonal T cells**

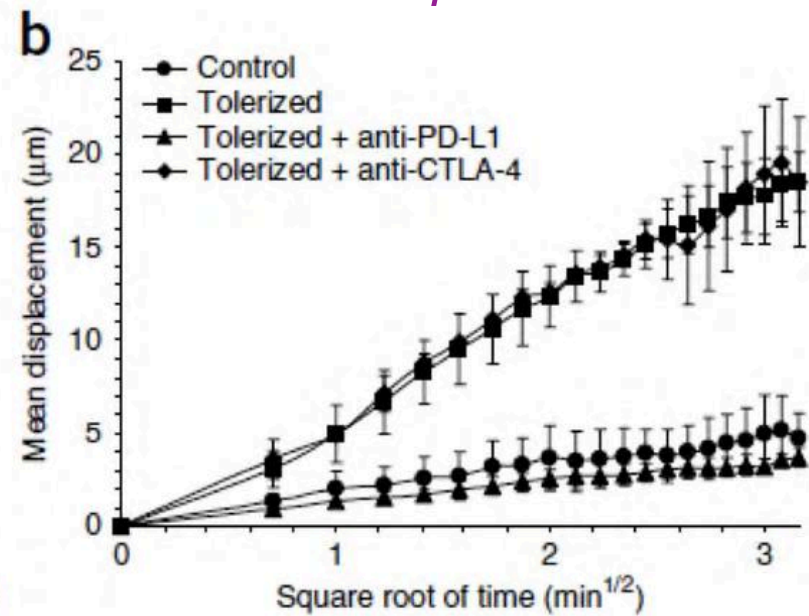


# Data Analysis: Motility

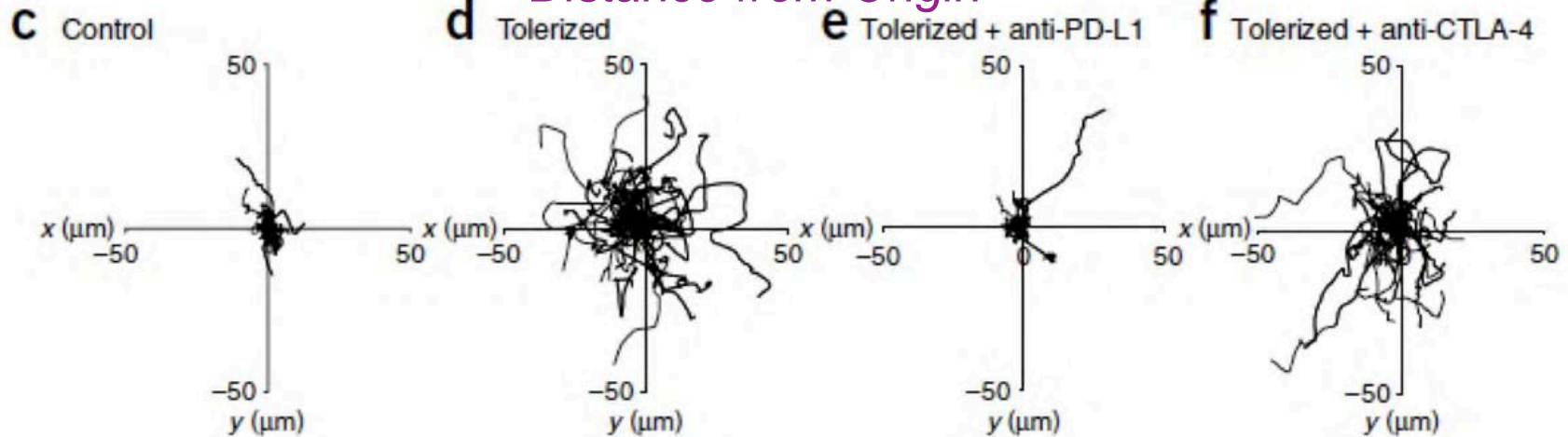
*Avg Velocities*



*RMS Displacement*



*Distance from Origin*



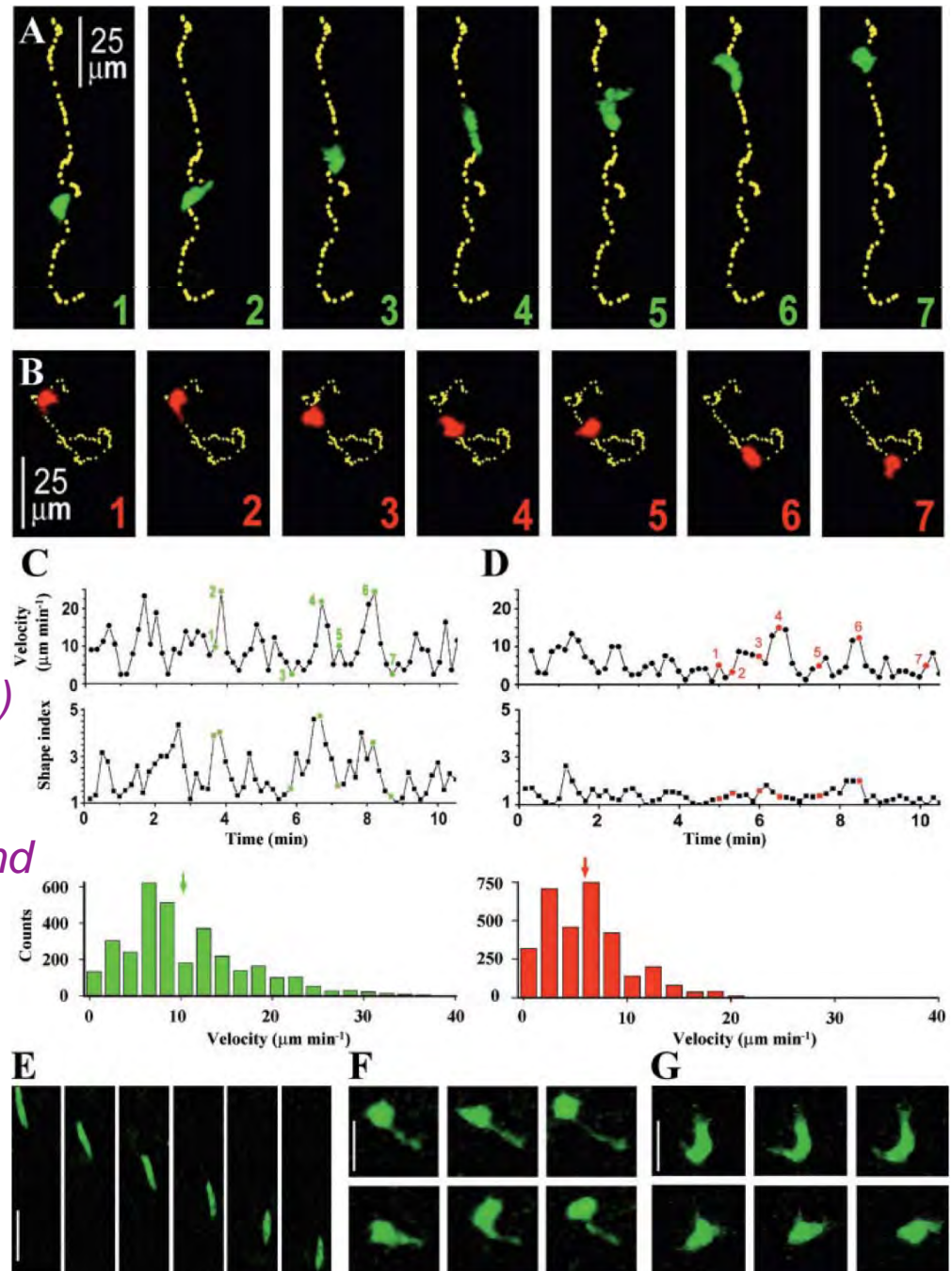
# Morphology:

Shape Factors:  
Often Correlate with  
Motility vs. signaling



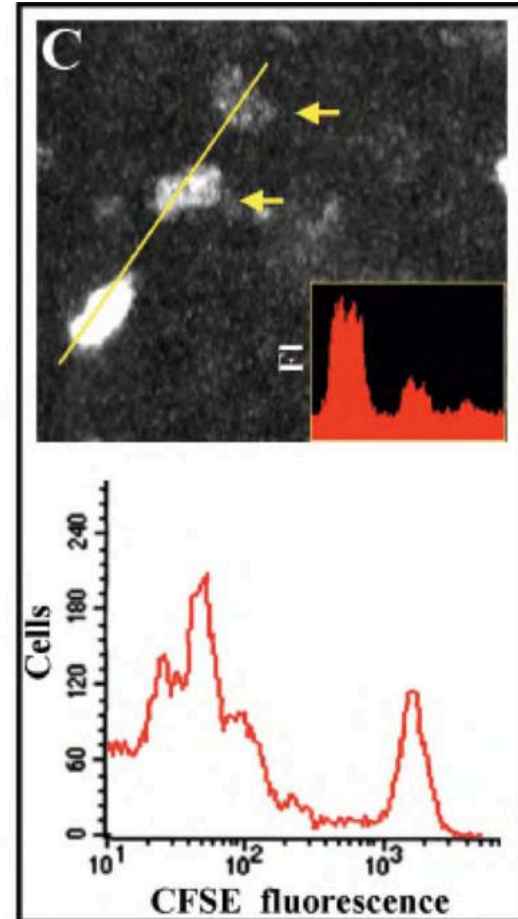
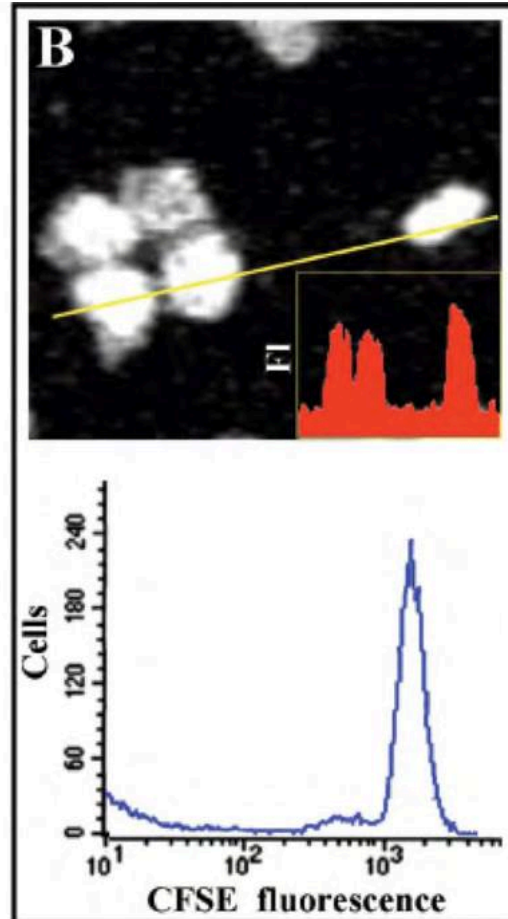
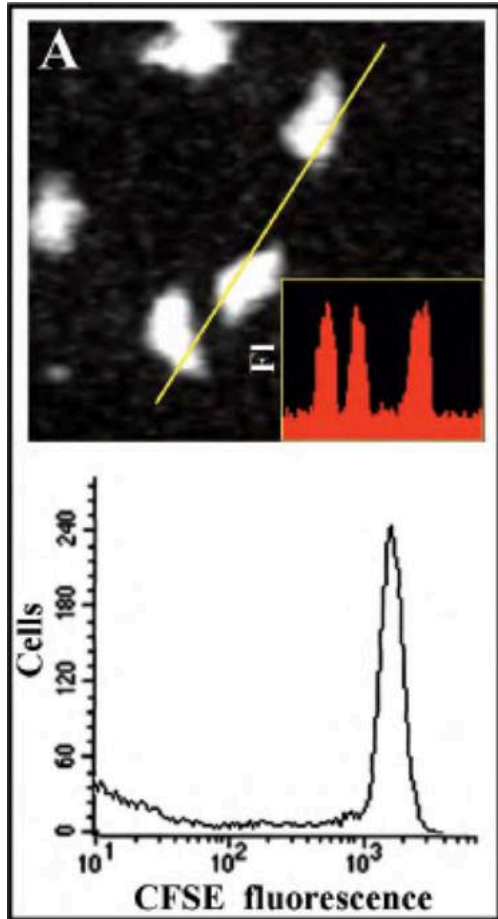
Ratio of longest and  
perpendicular chords (1 for circle)

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

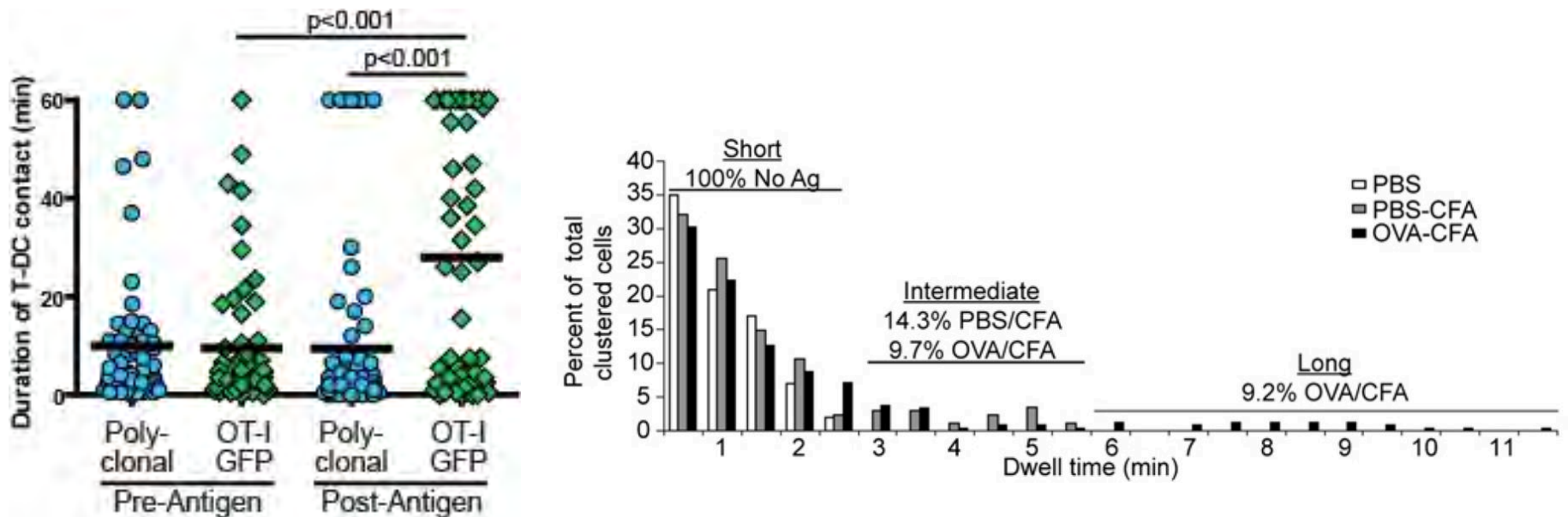


Cahalan.

# Cell Division:



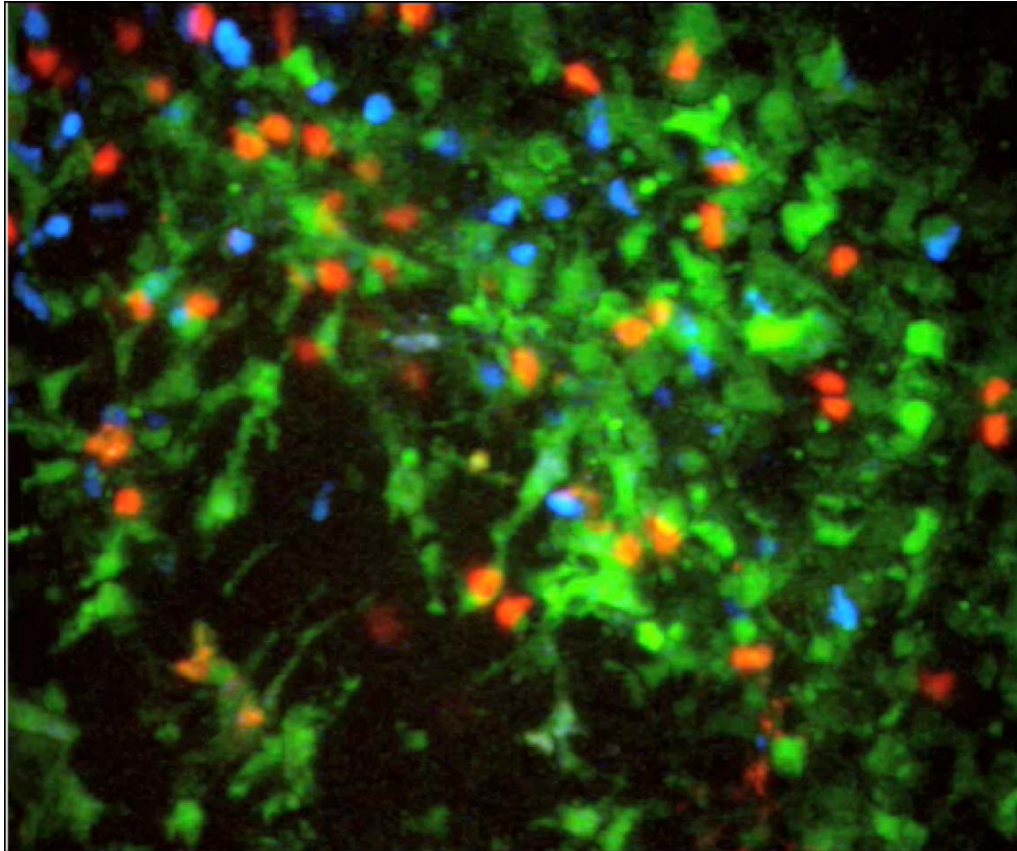
# Data Analysis: Cell-Interaction



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

# What can live *in situ* imaging tell us about biology?

---



## Kinetics of Cellular Behaviors

Cell-cell interactions

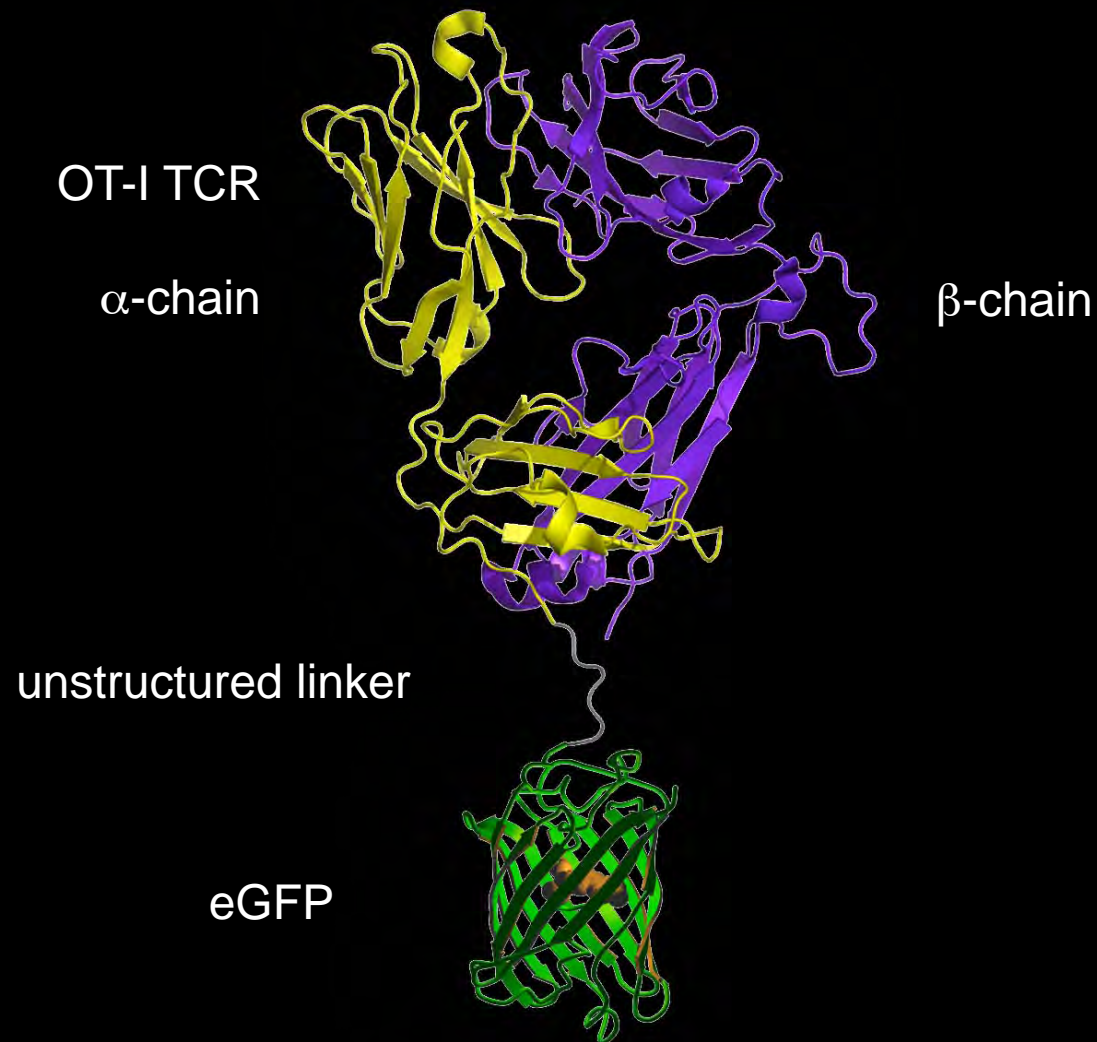
Cellular morphology

Speed/direction/confinement

Cell death

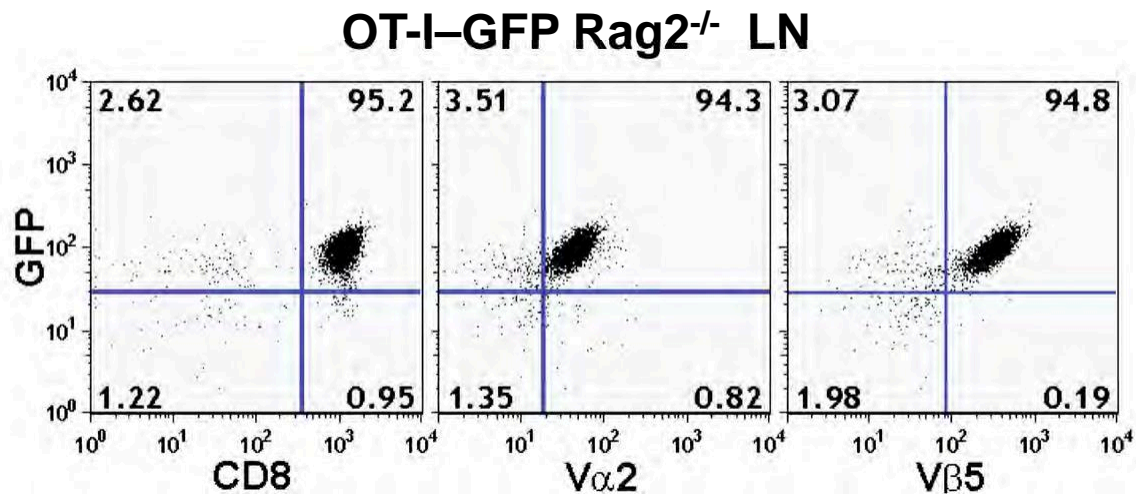
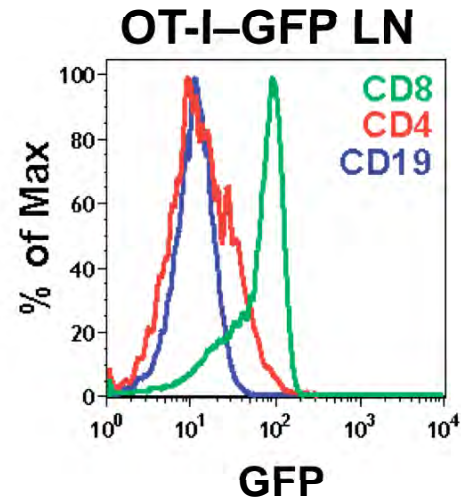
How can we gain more information from *in situ* imaging?

# Biosensors: Fuse T cell receptors with GFP to make Transgenic Mice



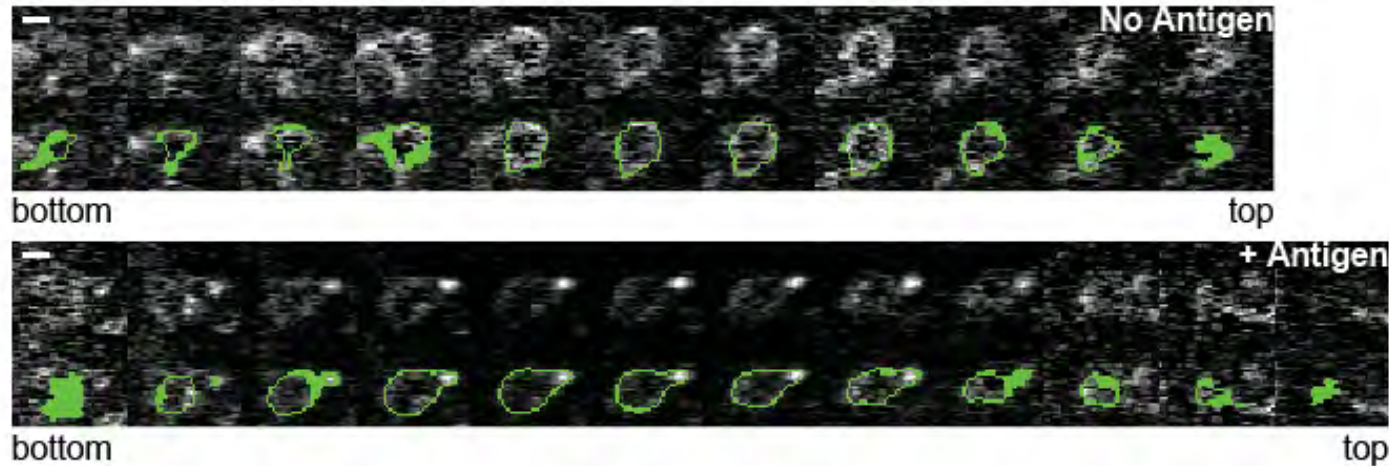
TCR structure by David K. Cole  
GFP structure from Roger Tsien

# Surface expression of OT-I-GFP

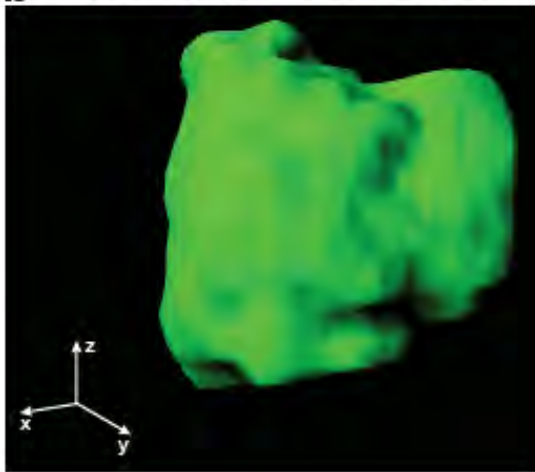


# GaAs Detectors Are Great, But... (Estimation of Signal Limit)

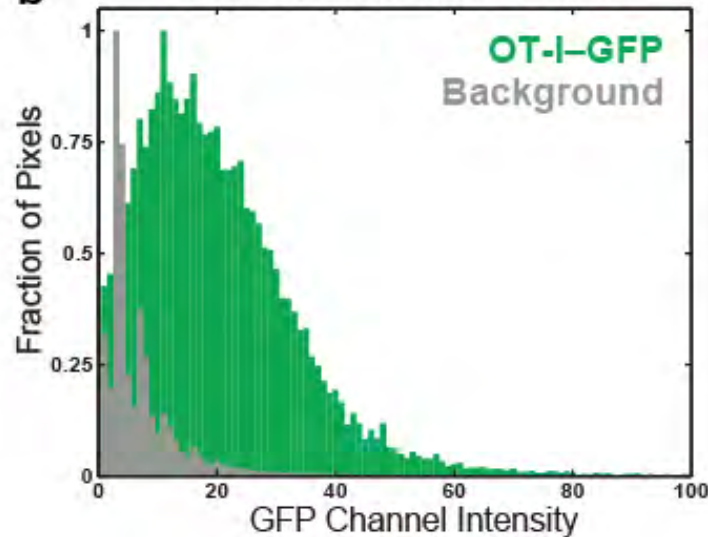
**a** Cell surface identification



**b** Rendering of Cell surface



**b** Cell Surface Fluorescence

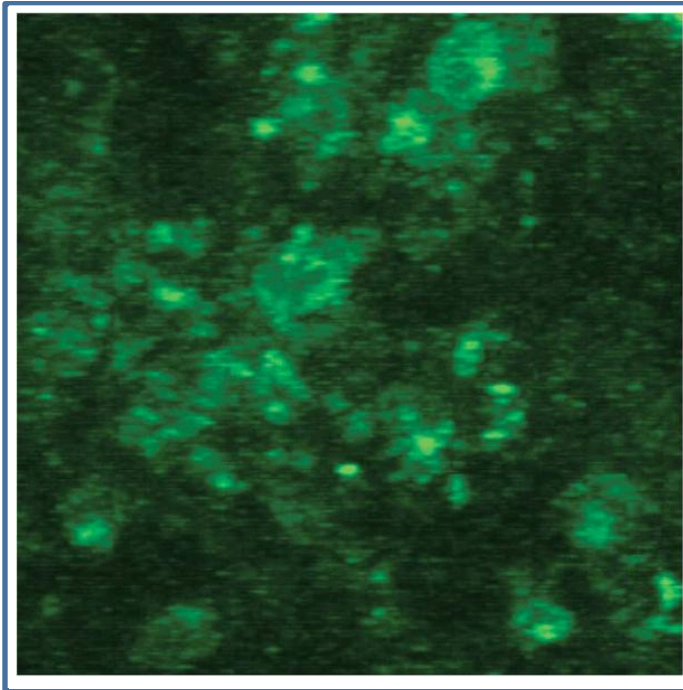


...e-shell calculation  
...ation of TCRs, we  
...voxel and we can  
...gnitude  $\sim 1$  IU per  
...ound 2.5GFPs/voxel

# Autofluorescence obscures OT-I-GFP fluorescence

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*Problem*



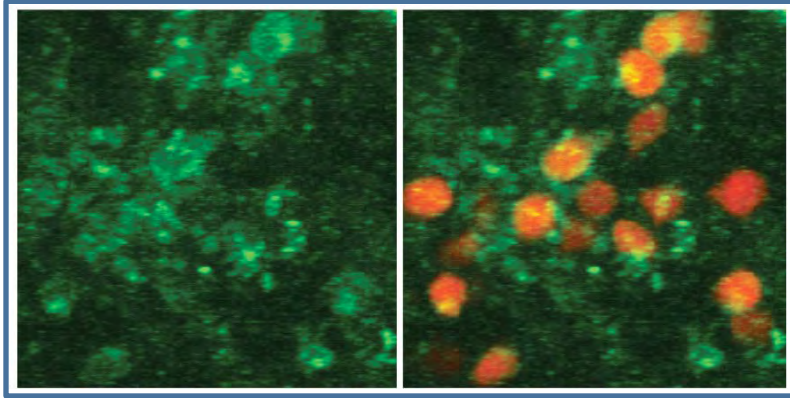
**OT-I-GFP & Autofluorescence**

**CMTMR co-labeled Cells**

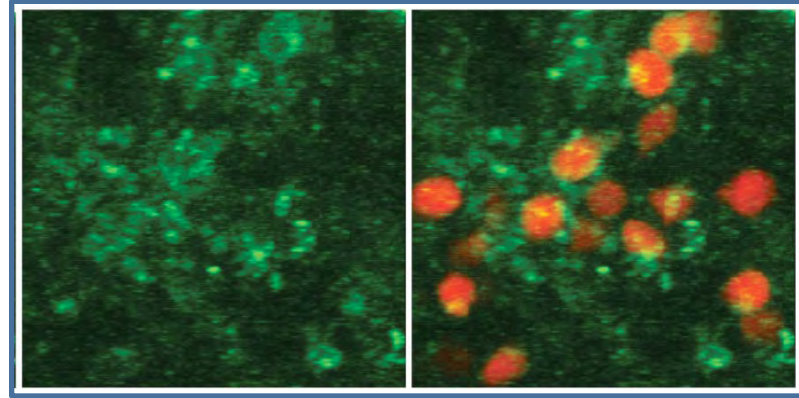
# Image processing to eliminate autofluorescence

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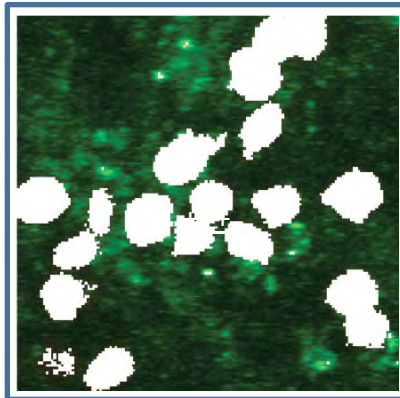
**Unprocessed Image**



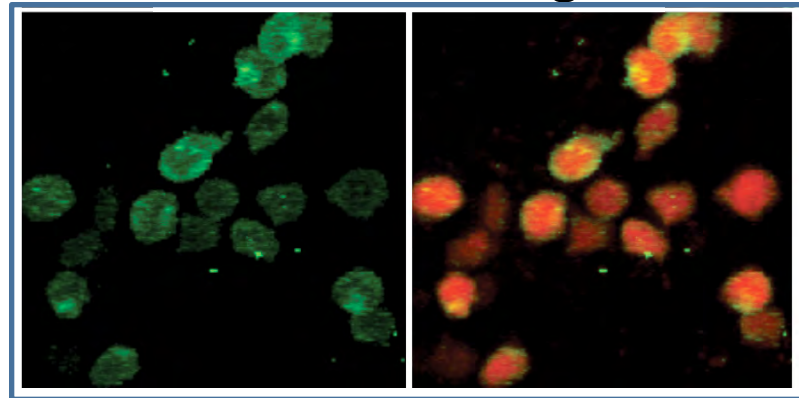
**Linear Unmixing**



**Volumetric Mask**



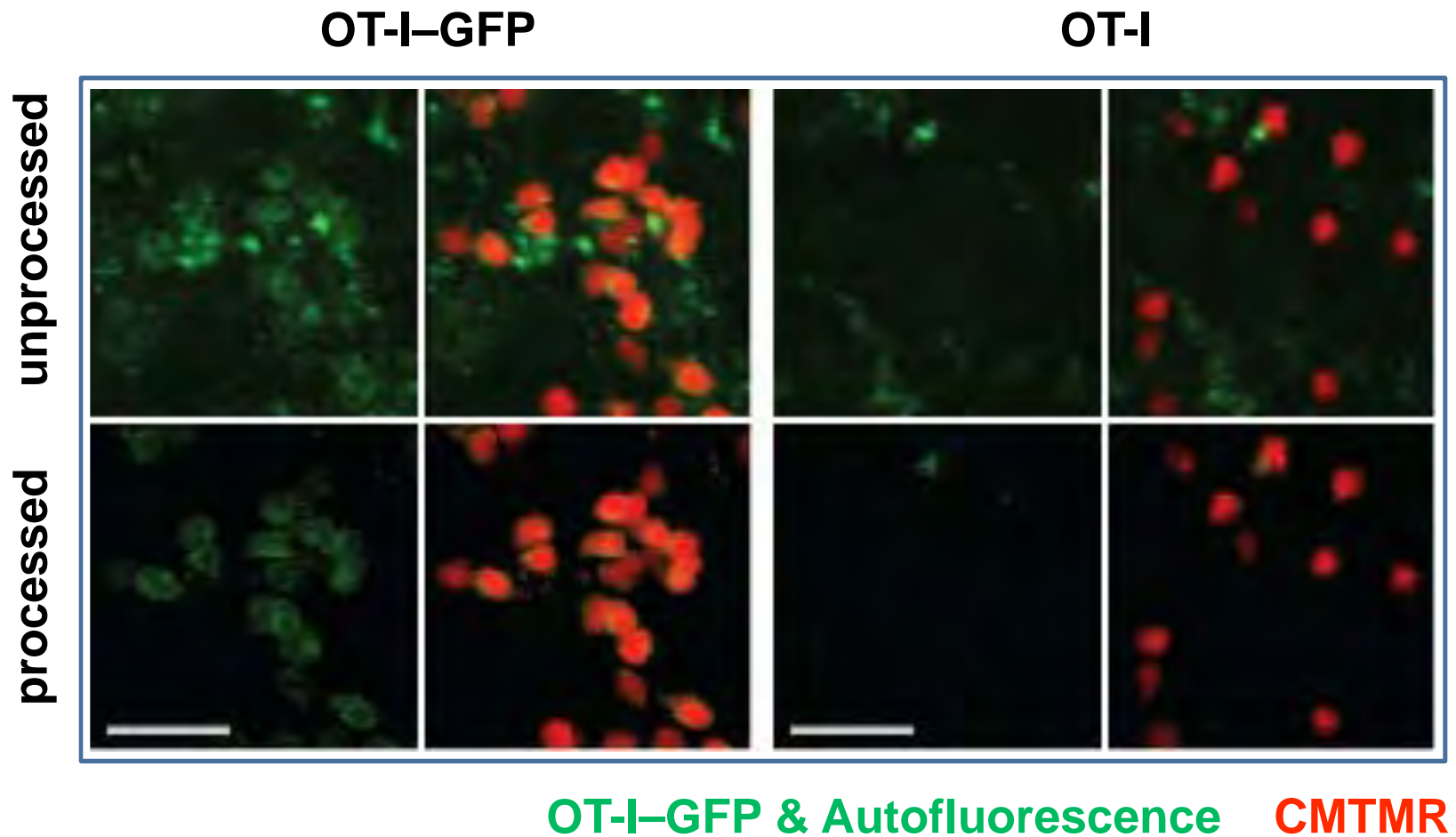
**Processed Images**



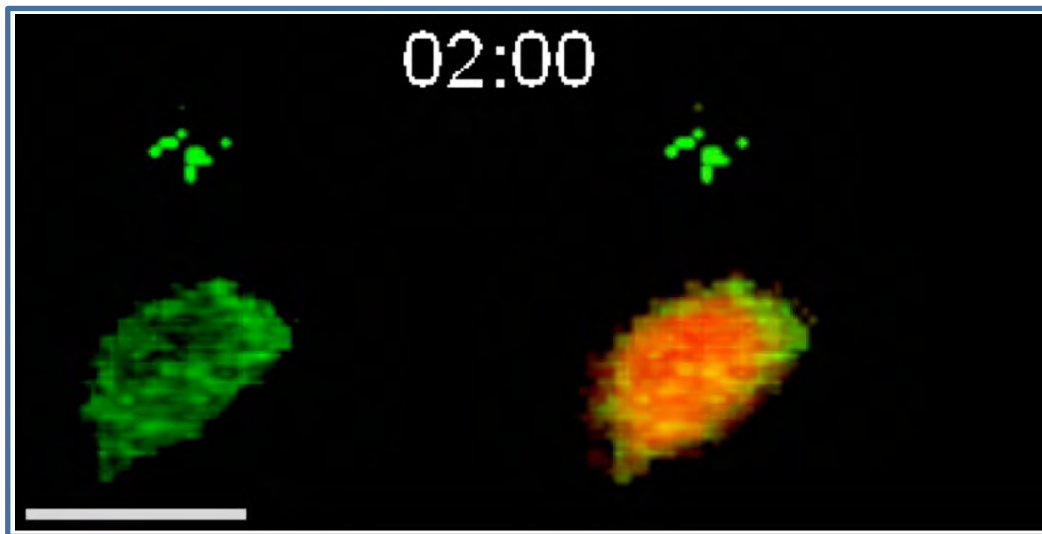
**OT-I-GFP & Autofluorescence**    **CMTMR**

# Image processing effectively isolates OT-I–GFP specific fluorescence

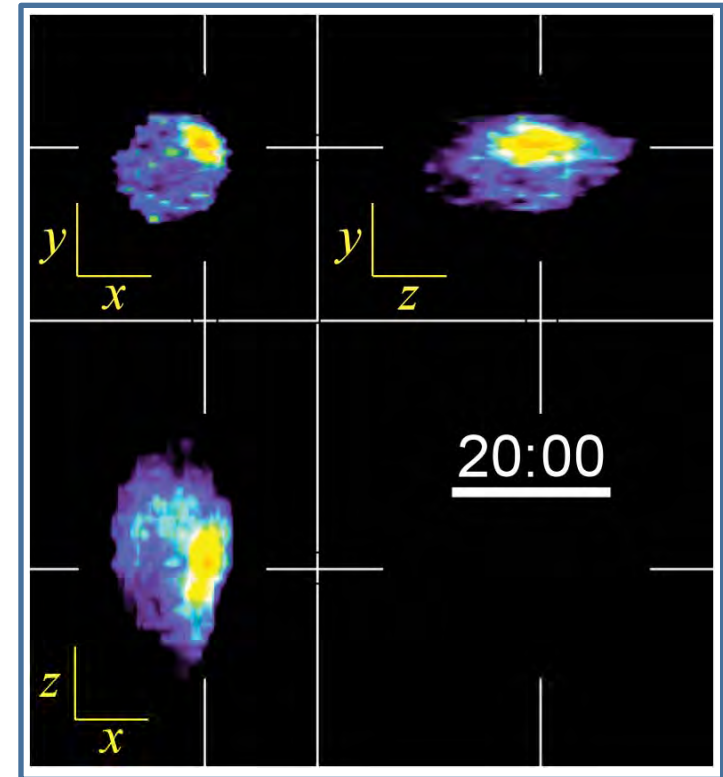
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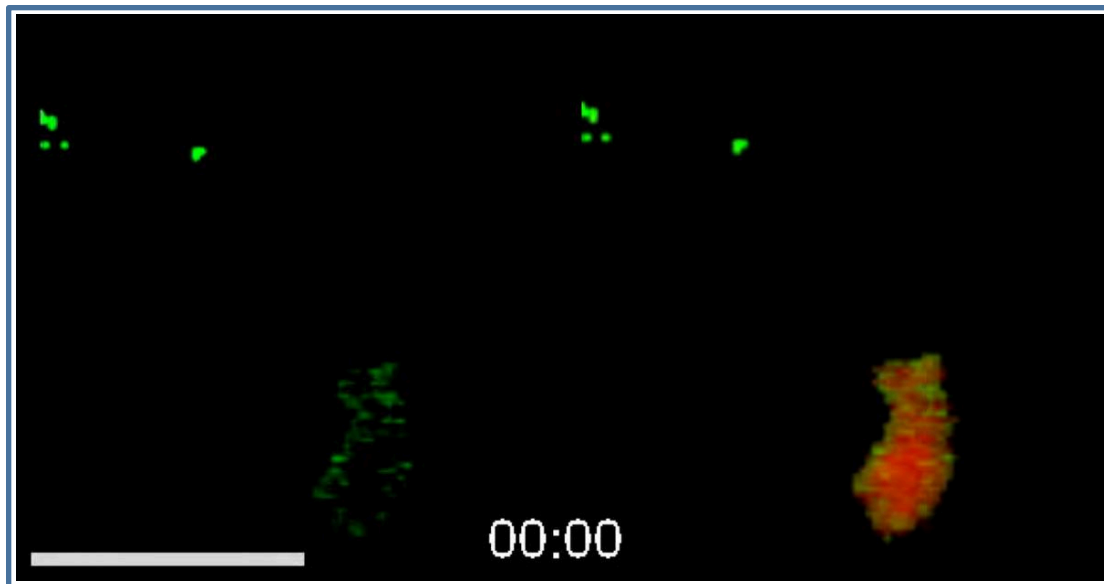
# Rapid antigen-dependent TCR clustering



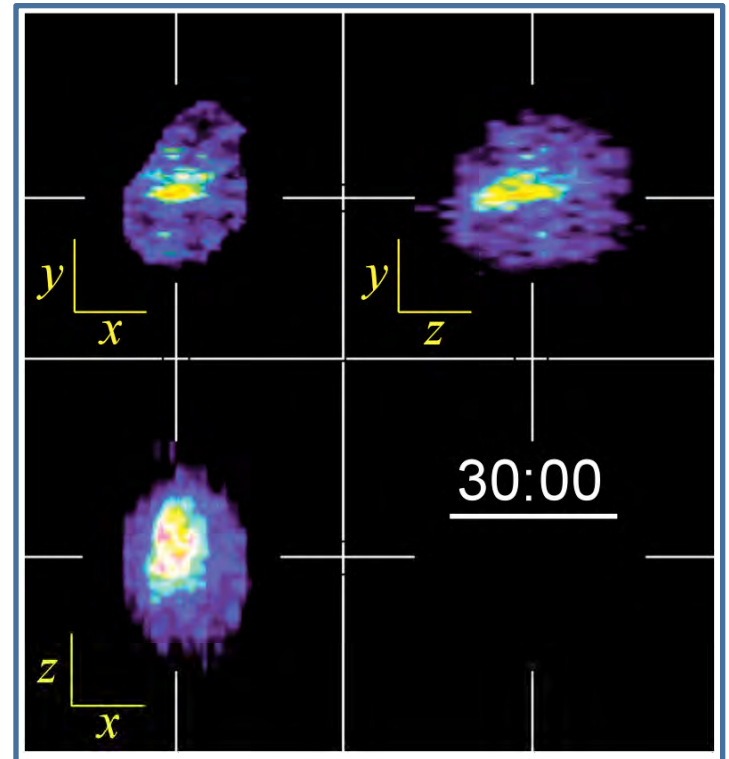
**TCR**   **CMTMR**



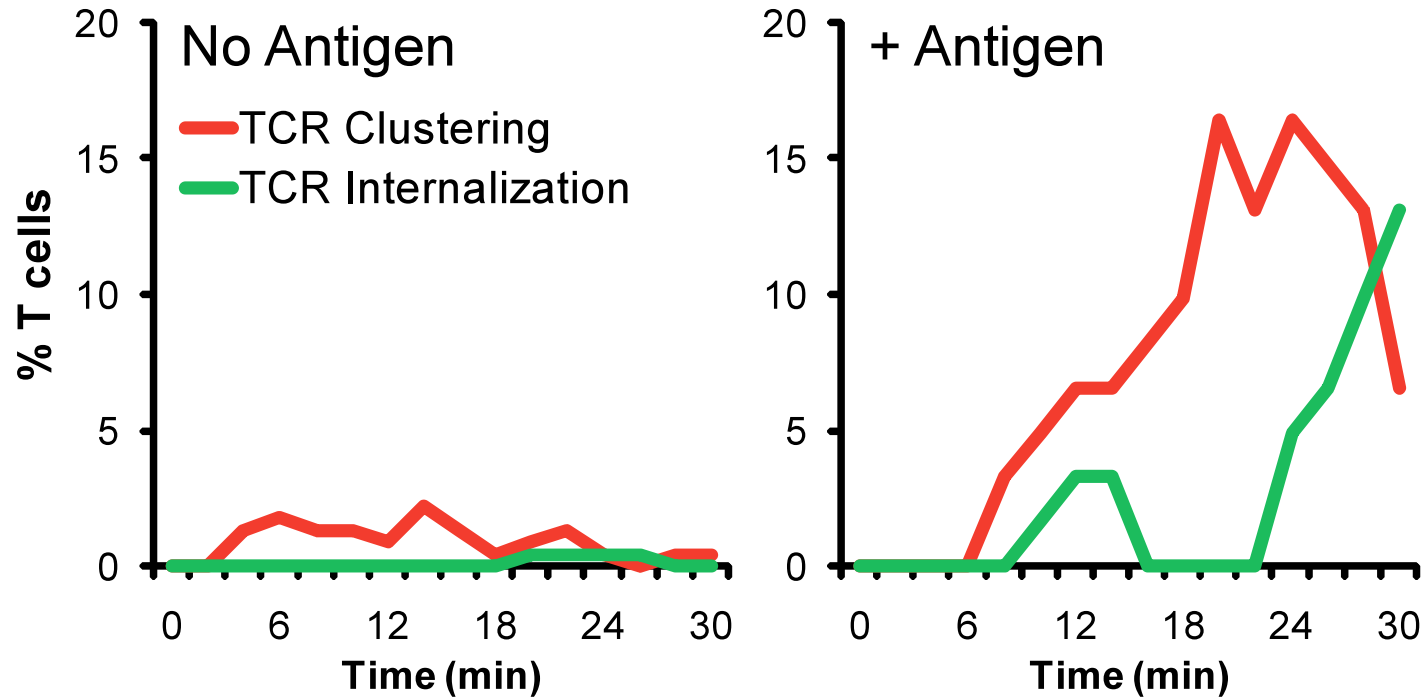
# Rapid antigen-dependent TCR internalization



**TCR**   **CMTMR**

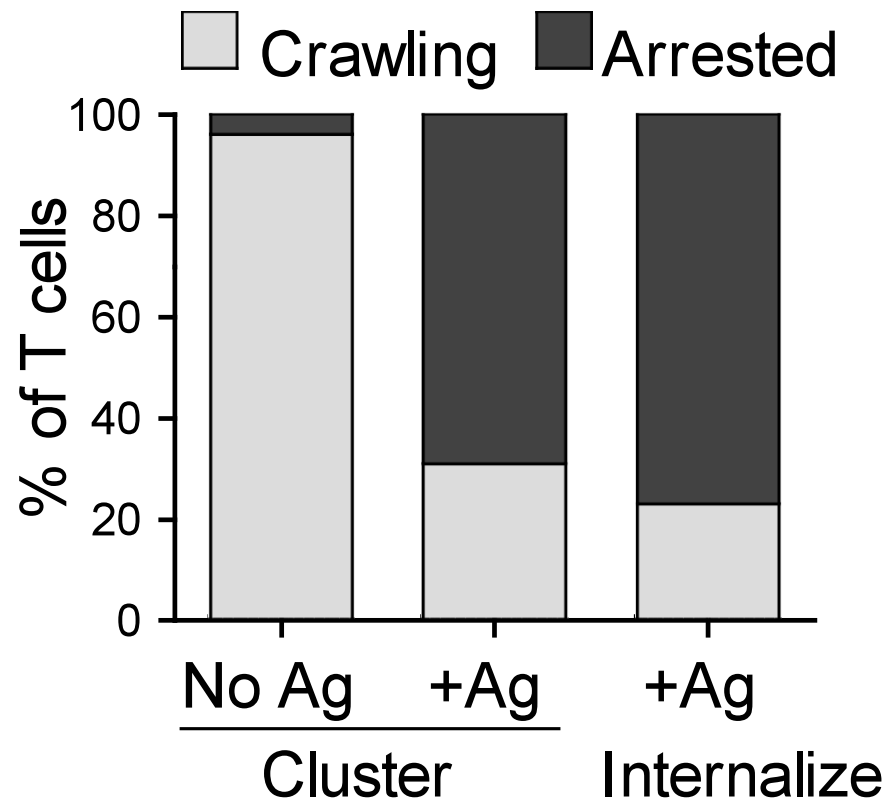


## *In vivo* TCR clustering and internalization



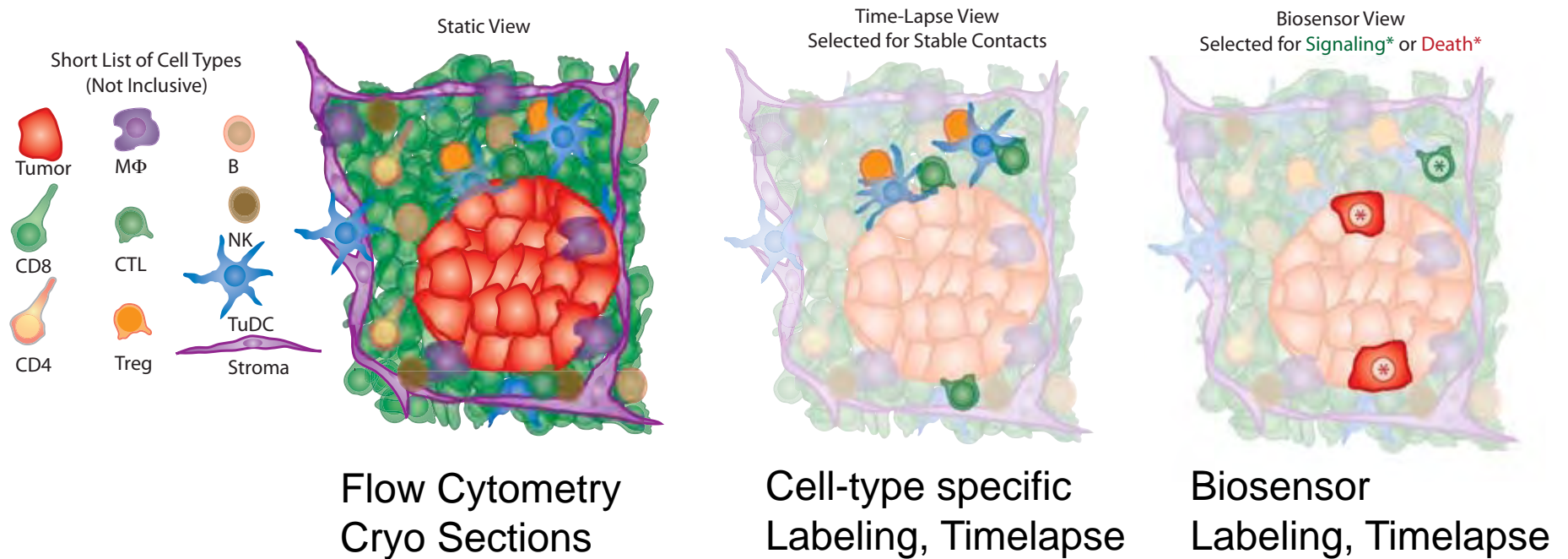
## Arrest is Not Required for TCR signaling as assessed by internalization

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Real-time analysis allows distinction of “average” and the range of individual kinetics

# The State of the Art.



## Ongoing Developments

Multicolor Collection to Defeat Autofluorescence Variations

Multicolor Labeling/Collection to Mask ('Gate') Data

Use of Biology to 'Label' Cells in Situ, with Tracking

## Future Needs

Improved Toolkit of Biosensors to readout signaling in situ

Improved computational algorithms to study patterns of behavior

Continuous 'rechecking' of models against human disease.

# Topics Covered

- Tissue Penetration/Autofluorescence issues
- Mechanics of multiphoton microscopy
- Options for access to living tissues
- Options for labeling
- Unmixing and Masking
- Parameters that are easily accessible.
- Future benefits of biosensors