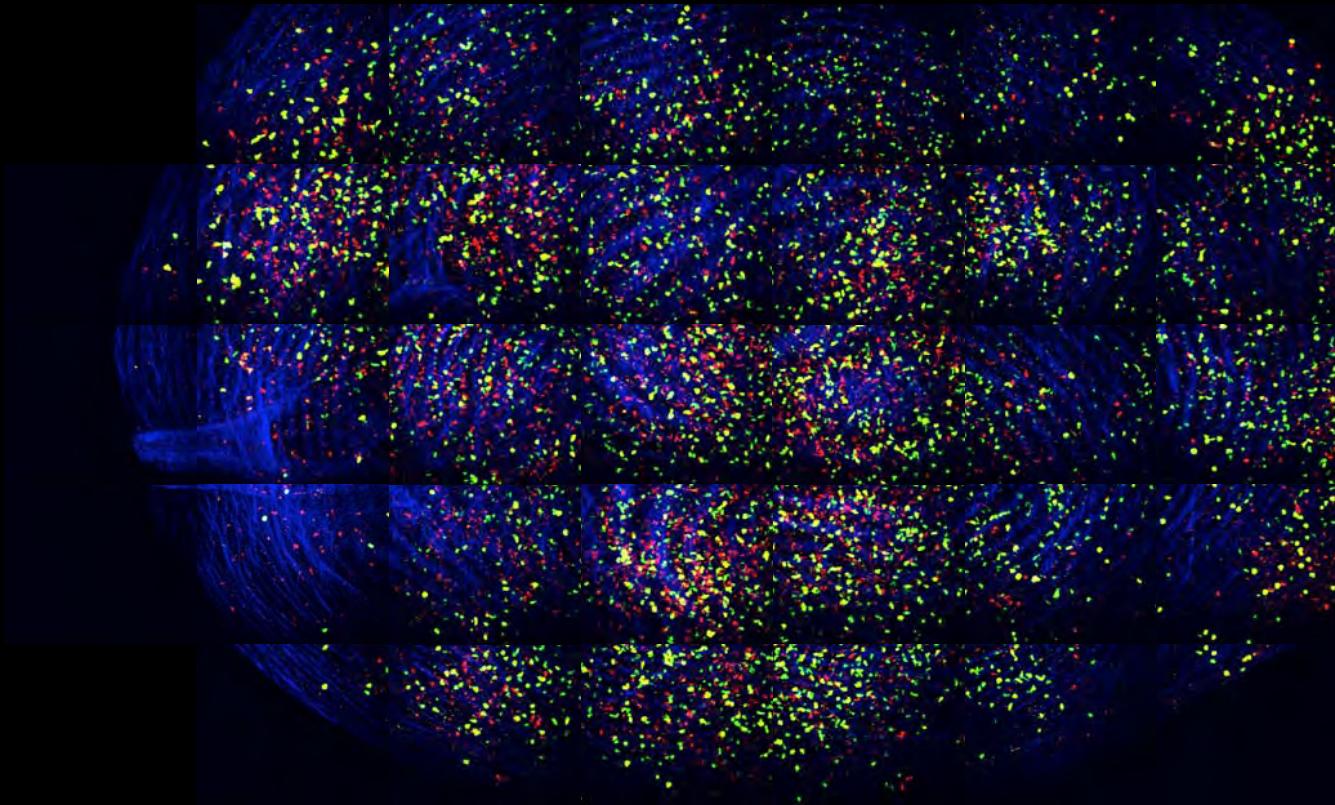
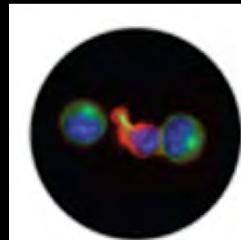


Multiphoton Imaging *in vivo*.

PIBS Microscopy Course



Krummel Lab, Department of Pathology



Biological
Imaging
Development
Center

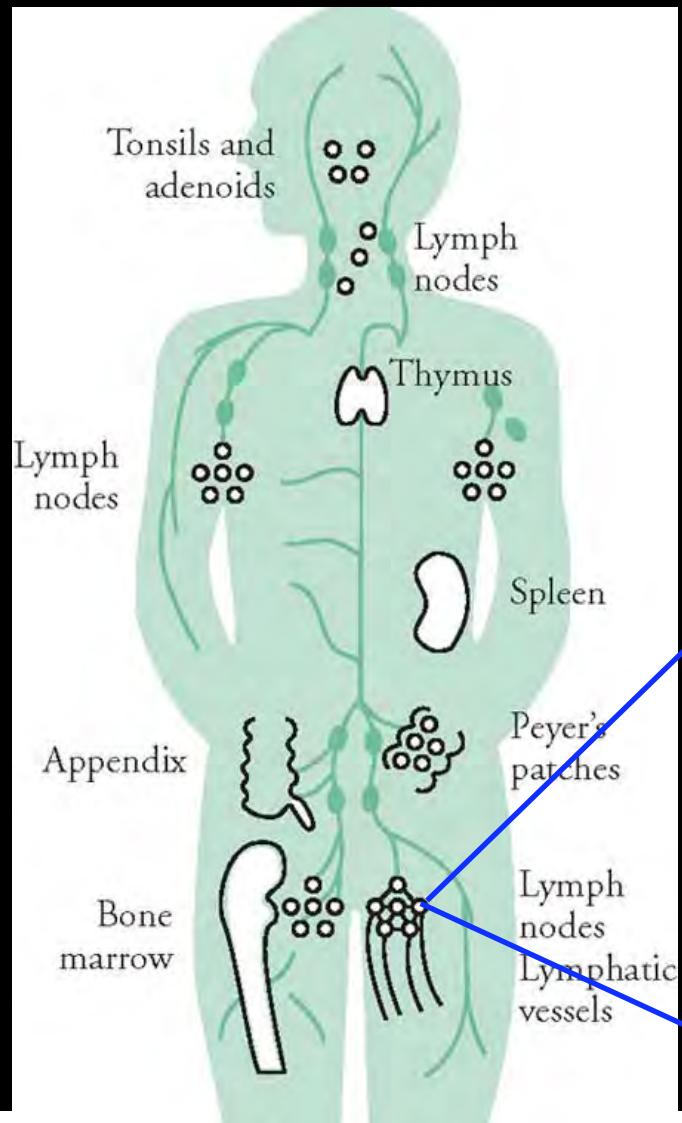
at

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.]

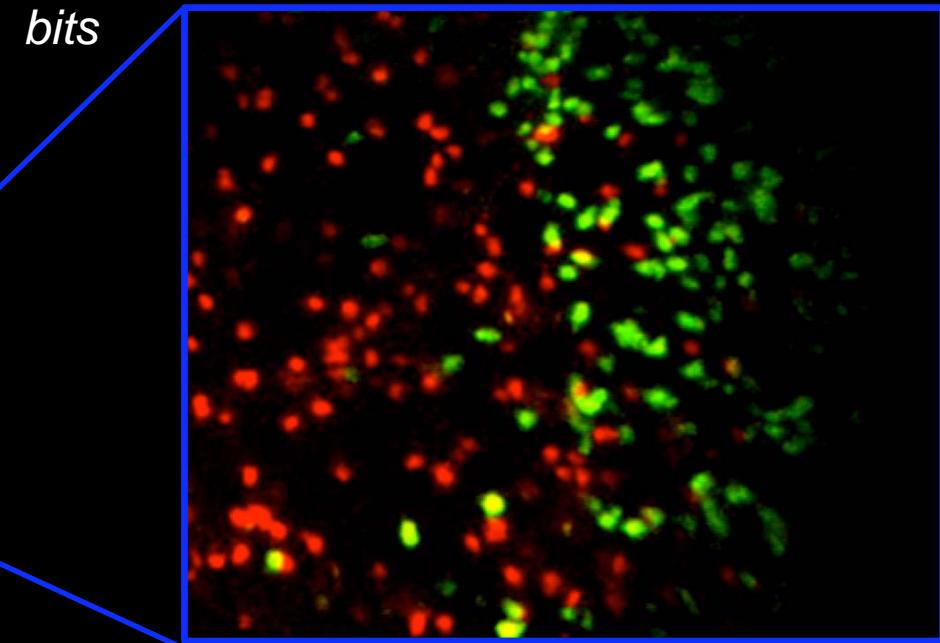


Lymphocyte Communication Does not Obey at least one Aristotelian Ideal: “Broadcast”

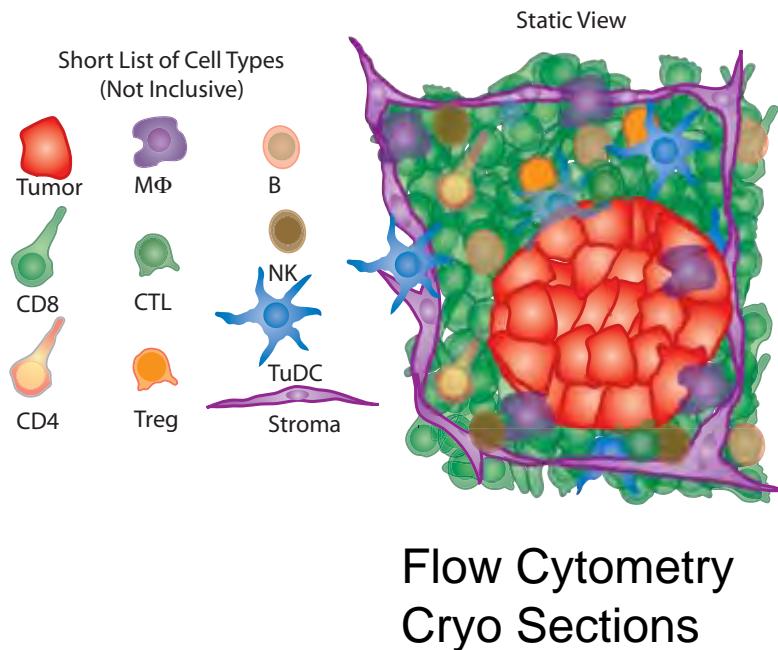
An entire city should be of a sufficiently small size that all citizens would be able to hear a single herald in peace or a single general in war [Politics VIII]

Rather, more akin to “Memetic”.

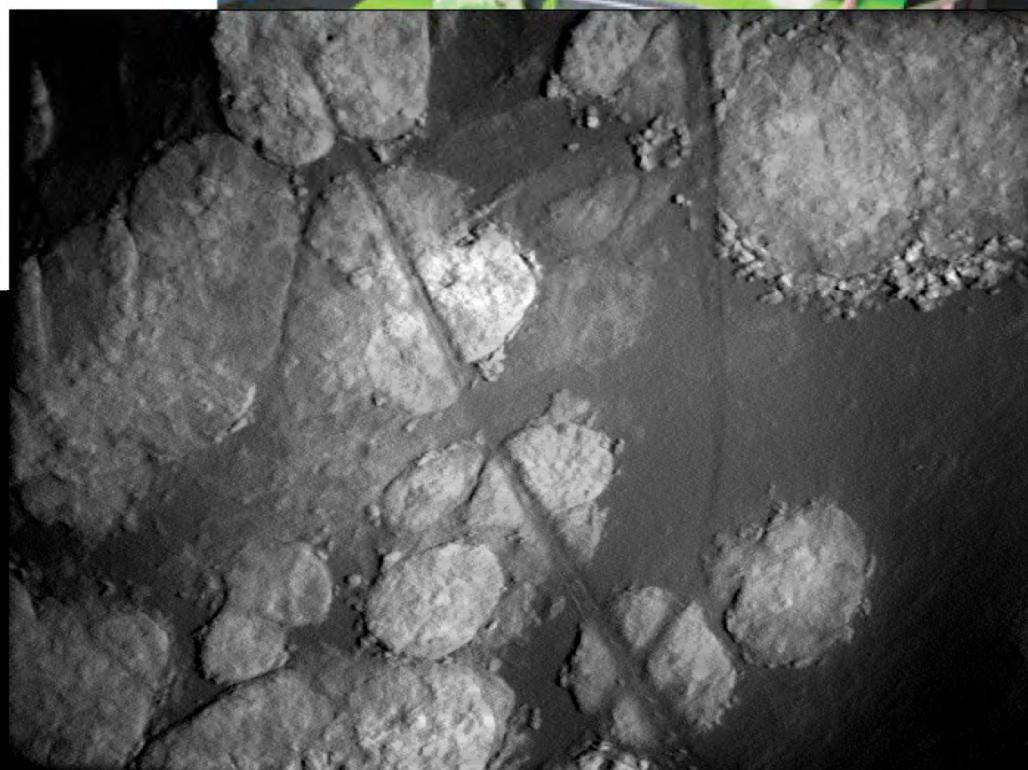
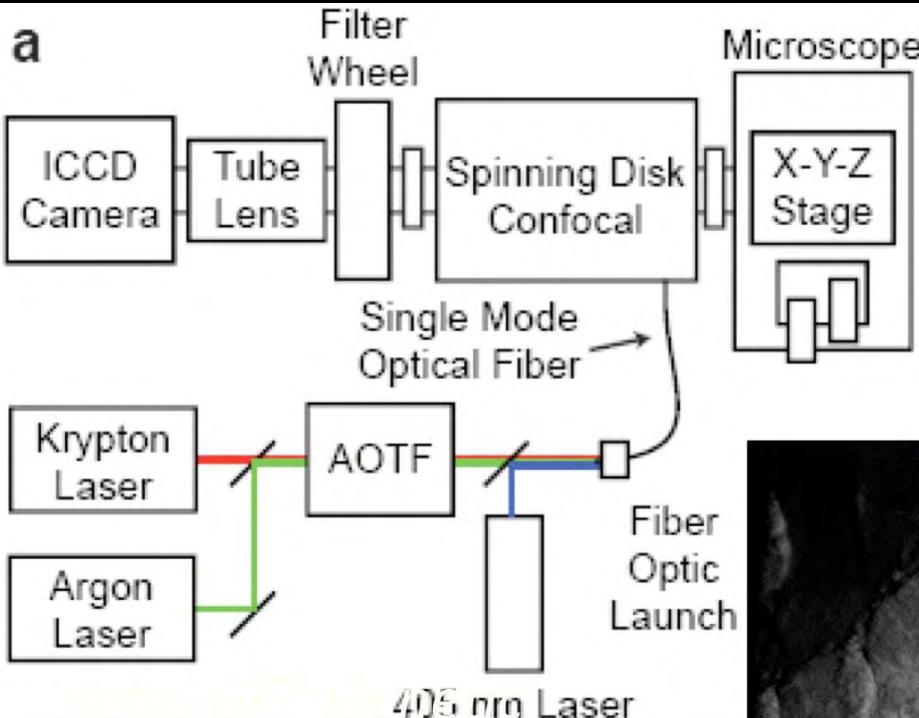
Each Cell Acts to gather and disseminate information bits



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



Imaging the PyMT Model of Breast Cancer



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

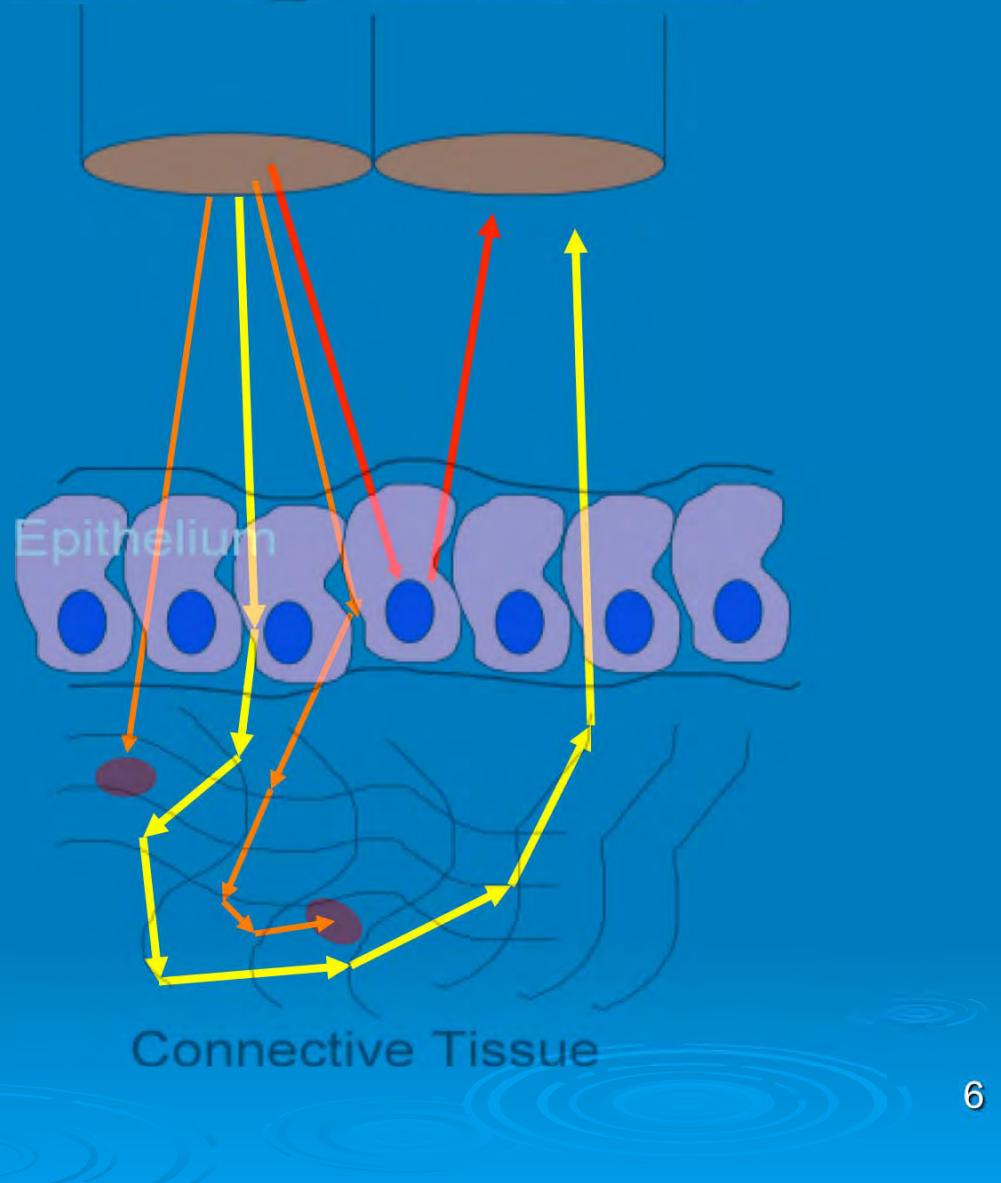
00:00

Issues & limitations of microscopy in tissues & organs

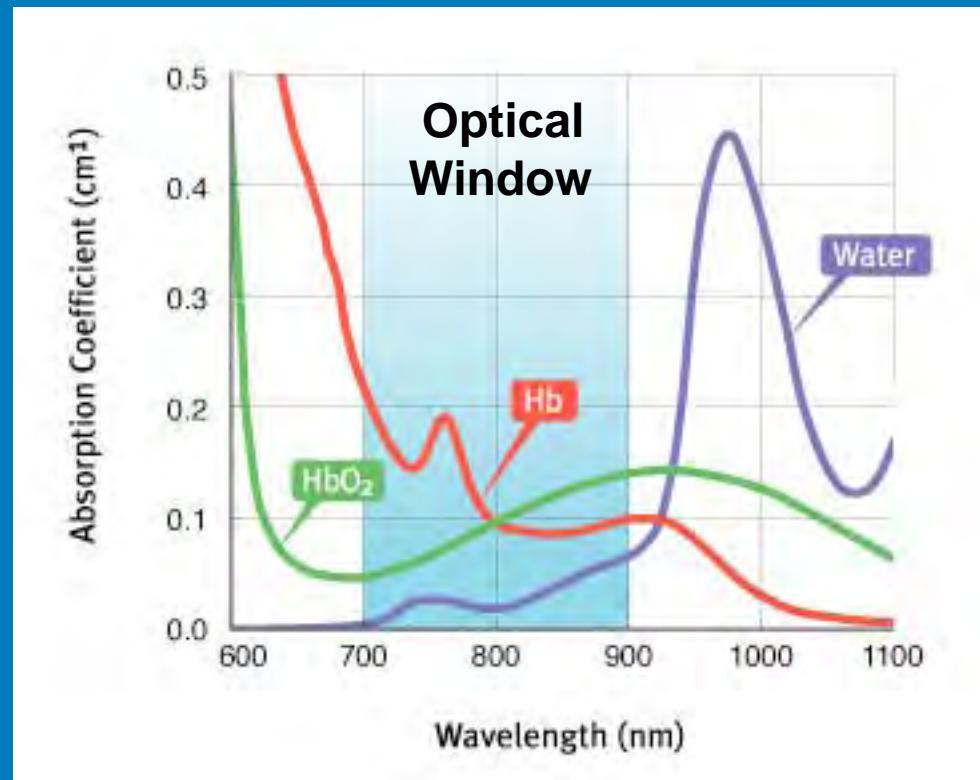
Tissues absorb & scatter light

- wavelength dependent

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

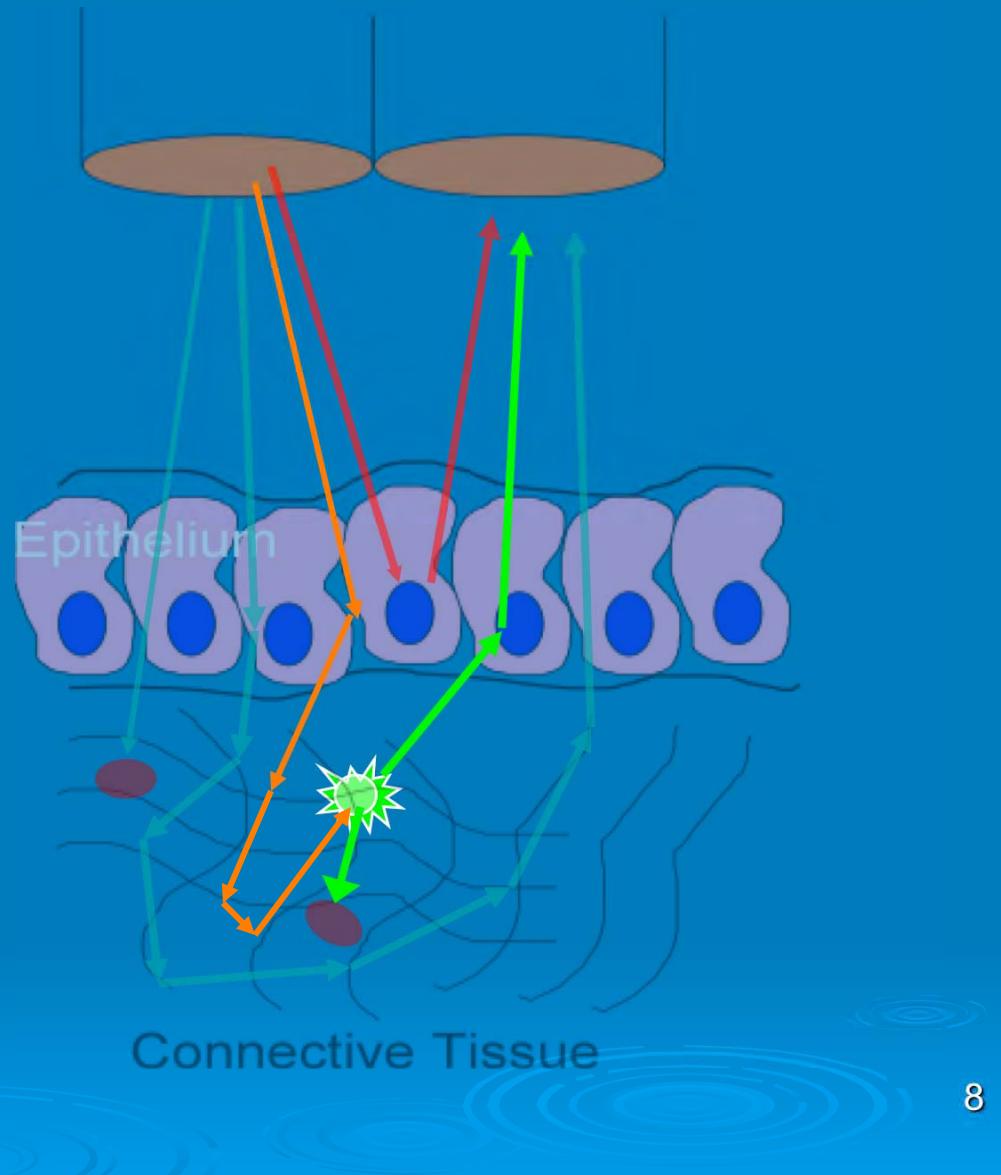


Optical window for imaging with min. absorption & scatter



Tissues are Auto-fluorescent

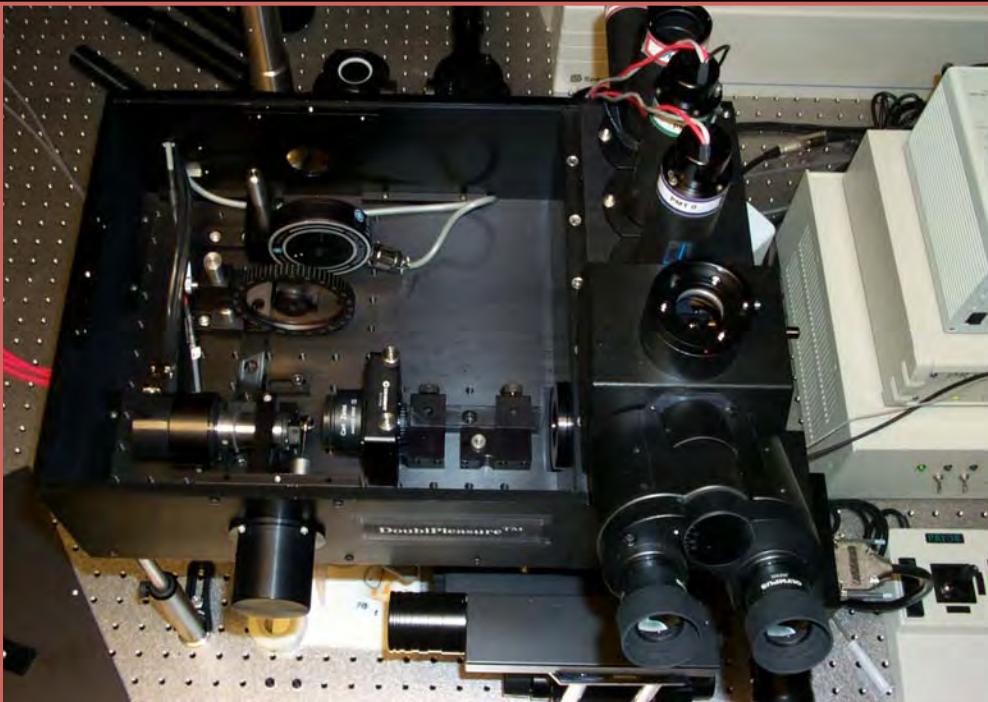
- scattering
 - elastic scattering
 - multiple scattering
 - single scattering
- absorption
- fluorescence



Mechanical issues limit physical access in situ in some cases

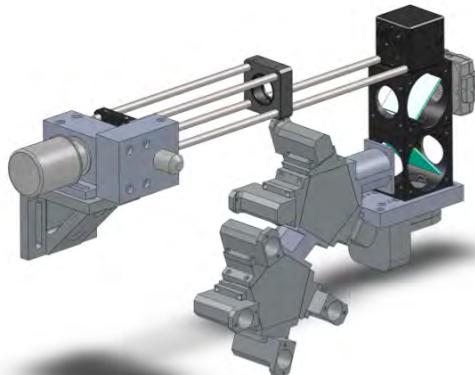


Custom 2P Instrumentation



3-30fps
6 emission PMTs

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



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

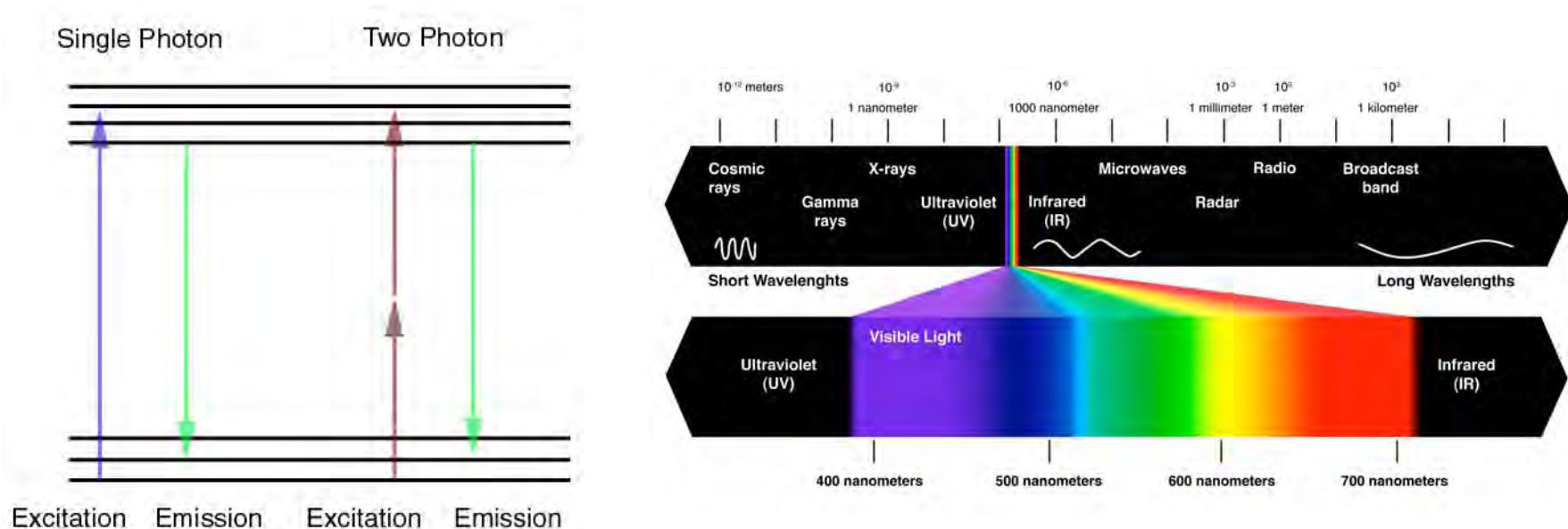
at
UCSF

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 ($2 \times 0.5 = 1x$) to drive fluorophore to activated state. (NOTE: This requires very high powered, very expensive lasers).

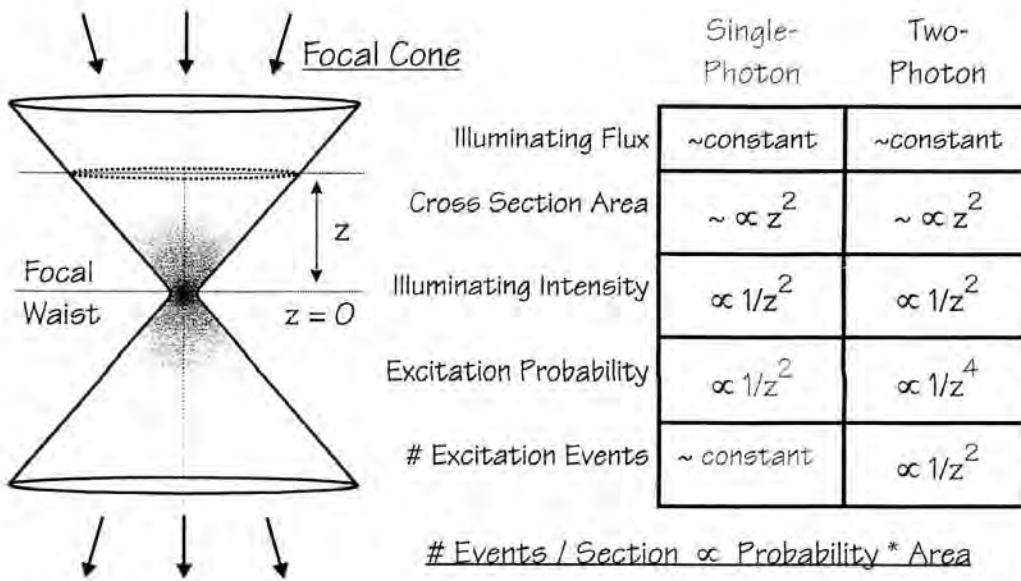


Why: 2 Photons only coincide with high frequency precisely at the focal point.

- No absorption of light except at focal point (since single photons are of a power that is not absorbed by proteins, DNA, water, lipids ~800nm)
- 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

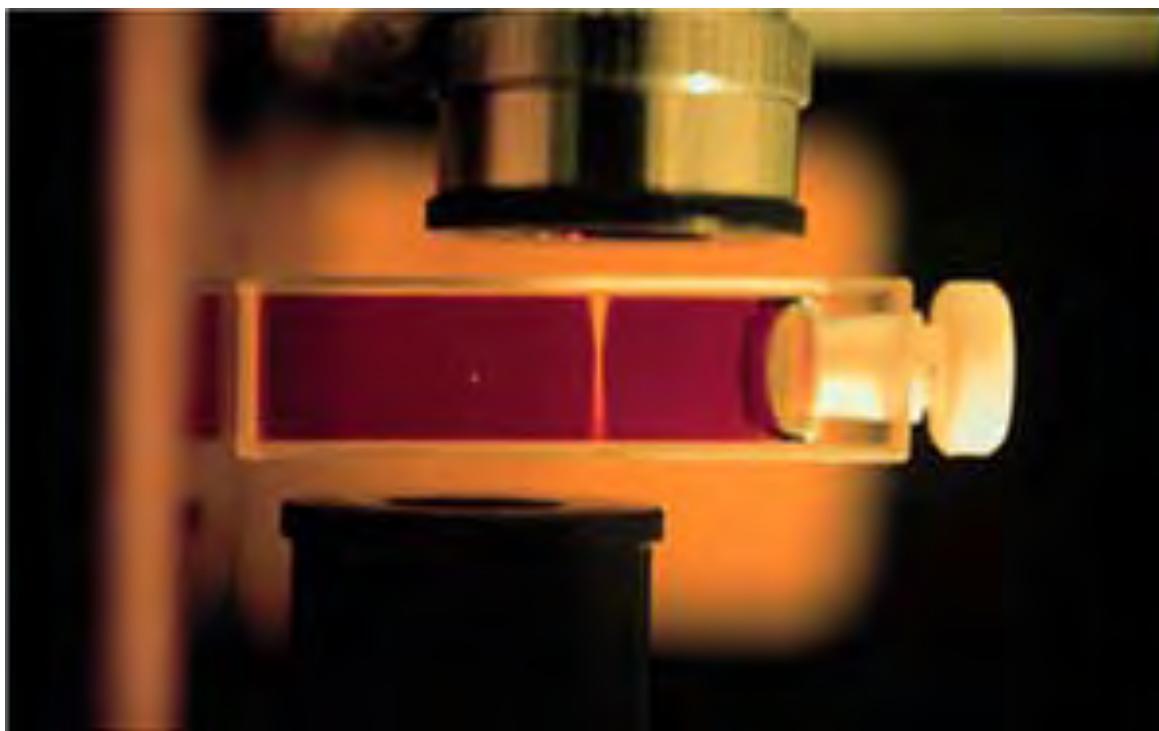


Single-Photon (Confocal) vs. Two-Photon Imaging:

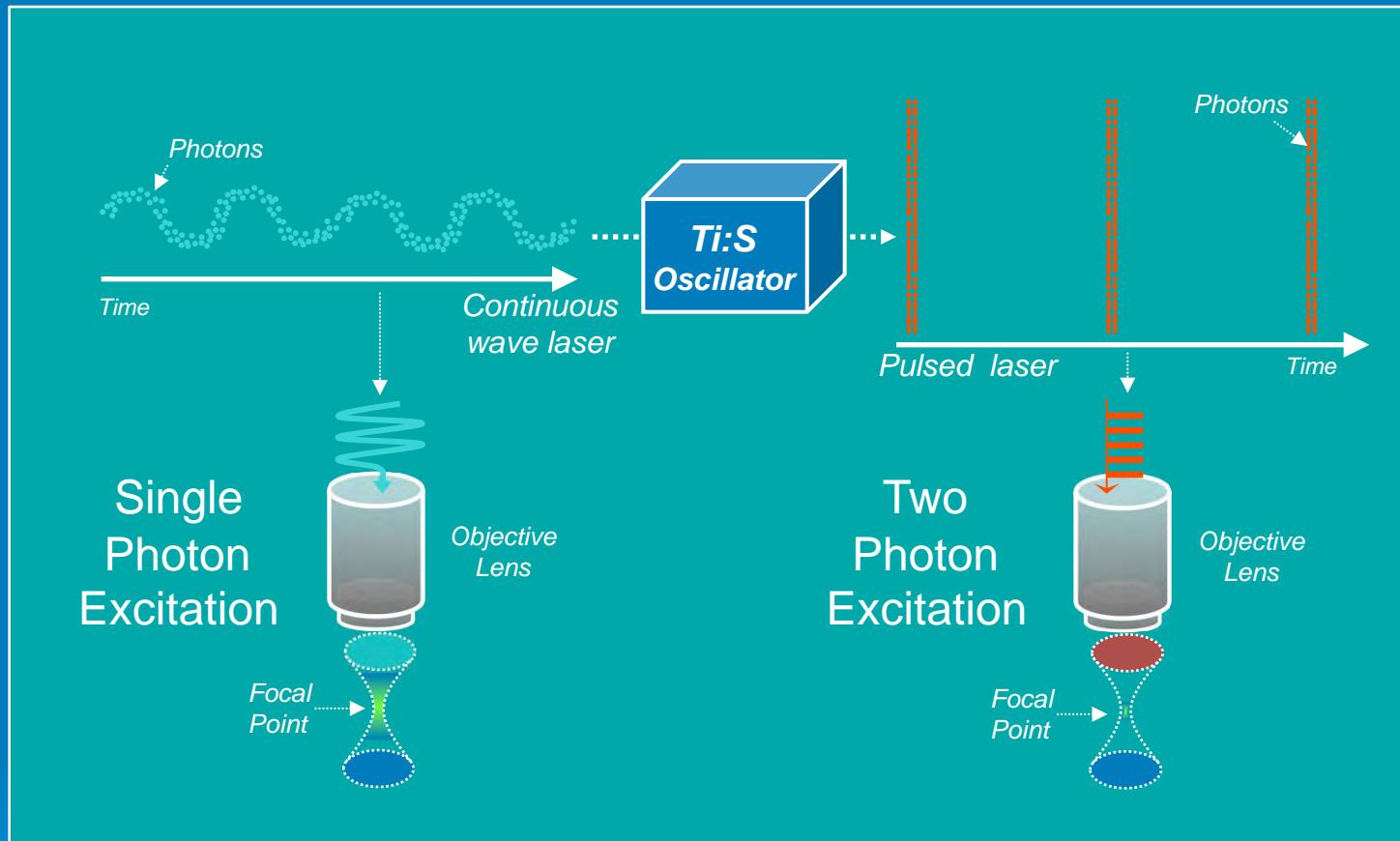
Single-Photon process produces excitation (and photodamage) throughout specimen volume. Optical sectioning requires imaging and spatial filtration of emission light, which is inherently inefficient and is highly susceptible to light scattering losses.

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..

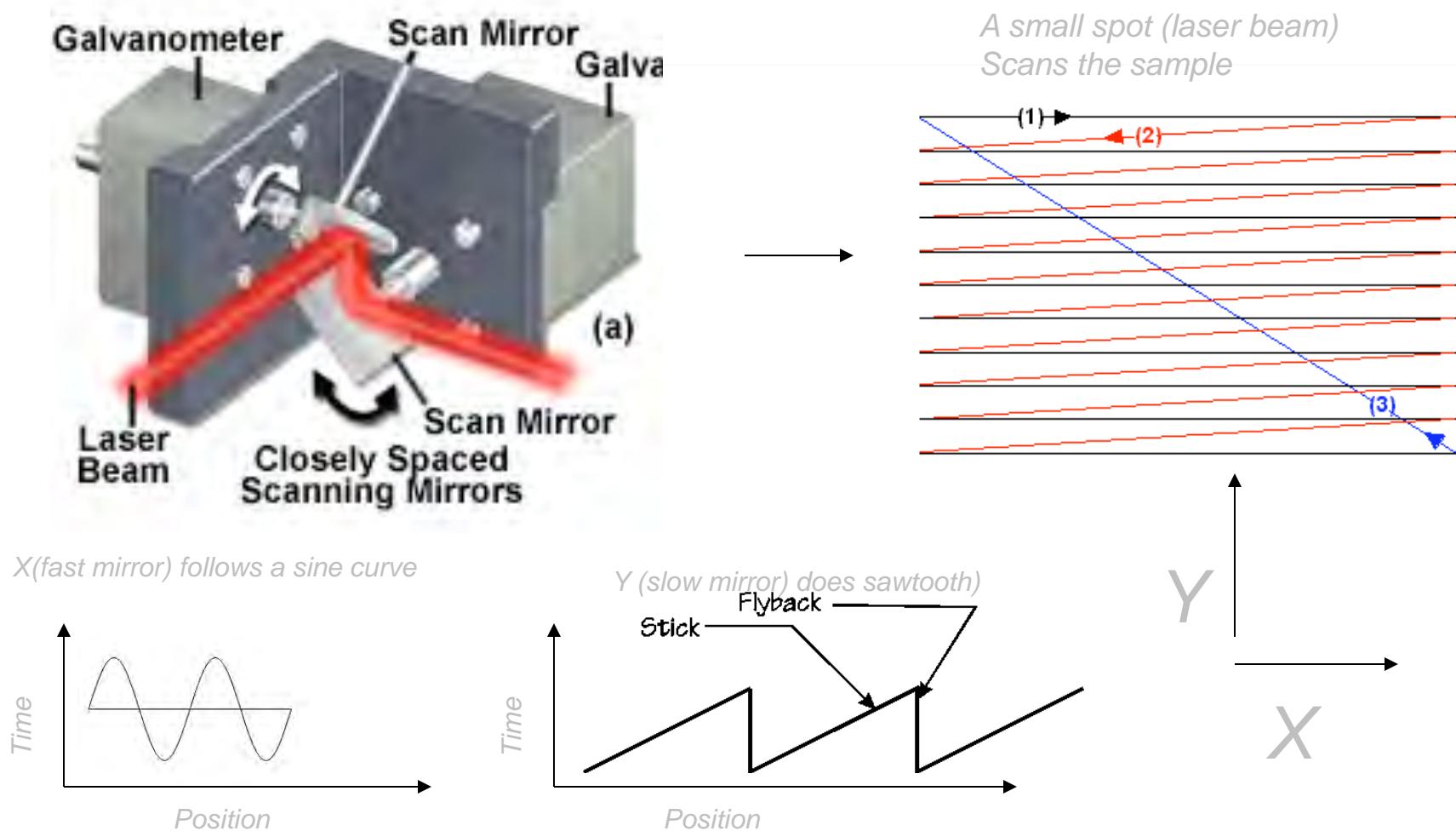
Multi-photon Microscopy: Example of Precision of Excitation



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

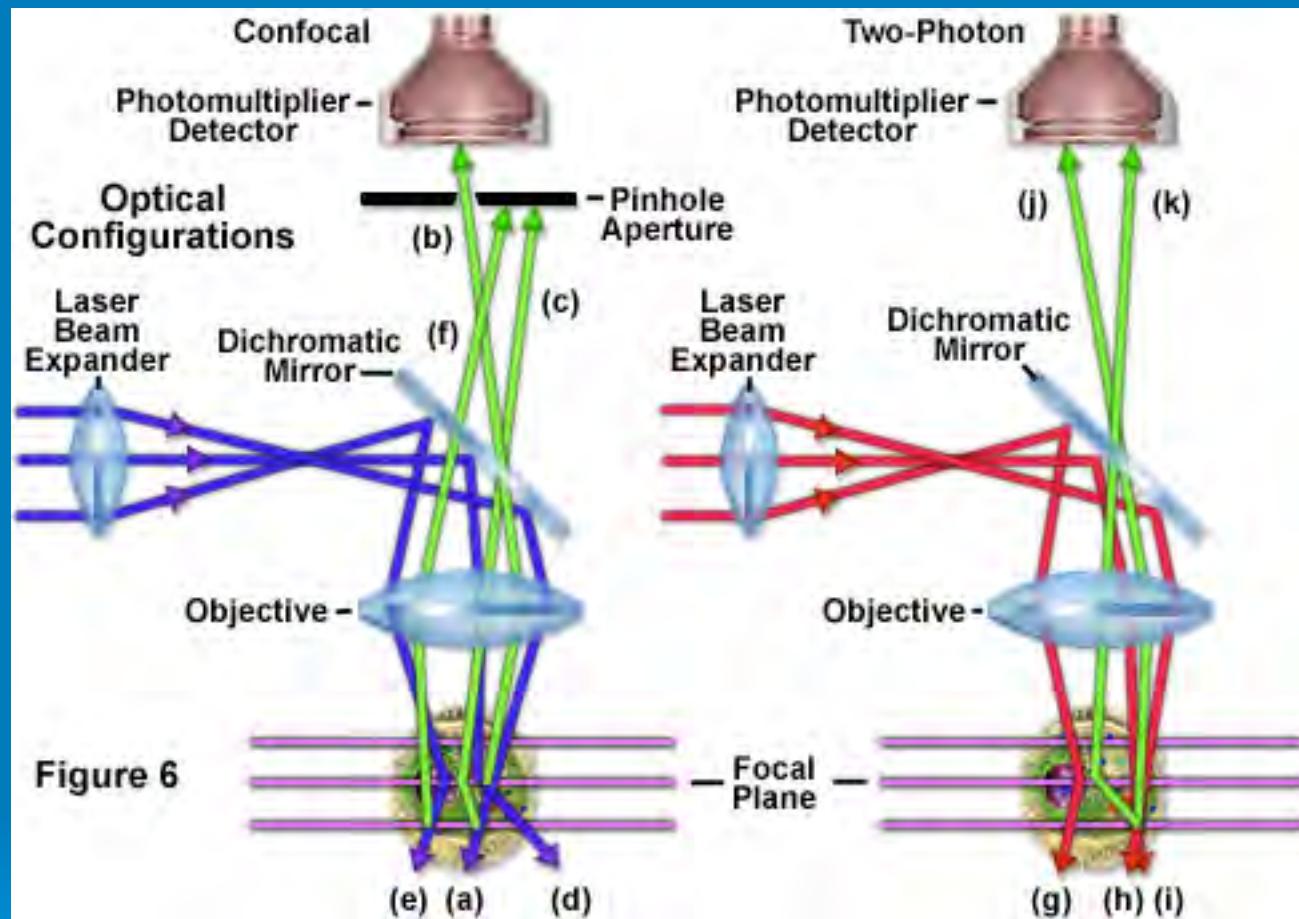


Recall that this is achieved via: The Raster Scan

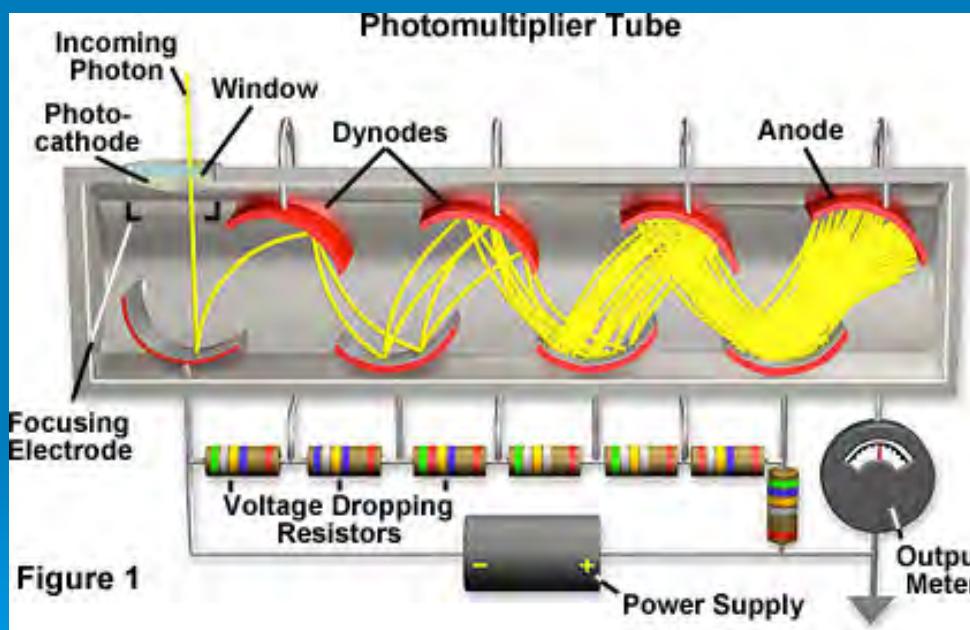
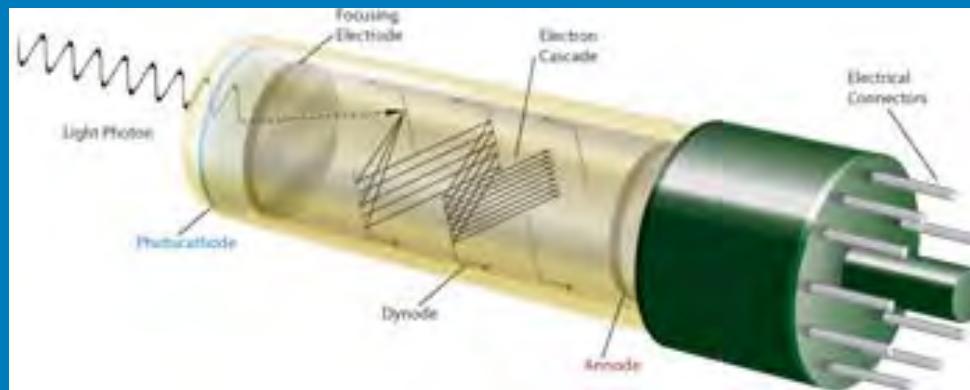


Collect All Emission at detector (regardless of path)

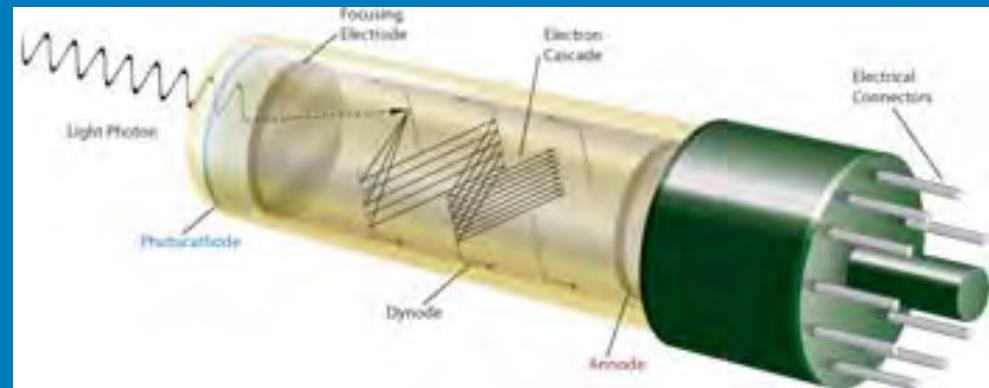
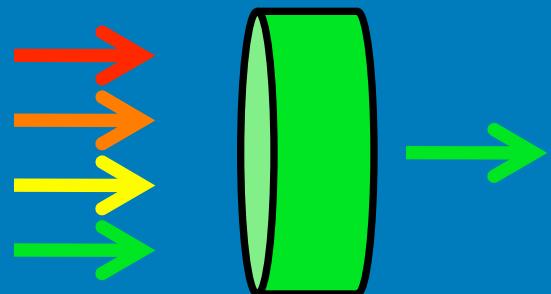
2P microscopy employs non-descanned detectors



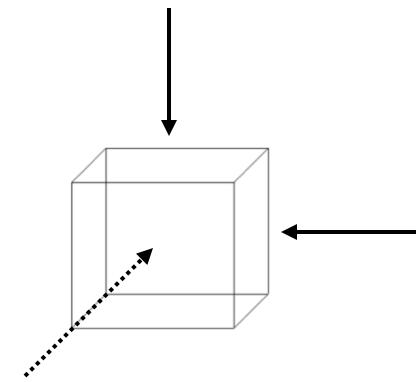
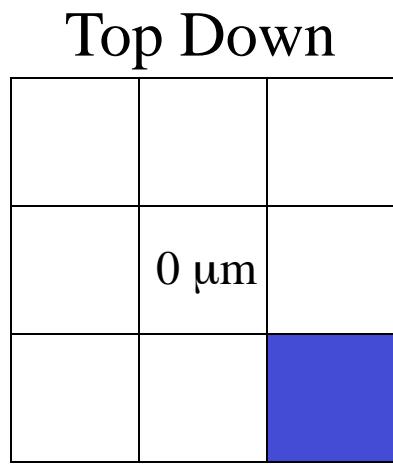
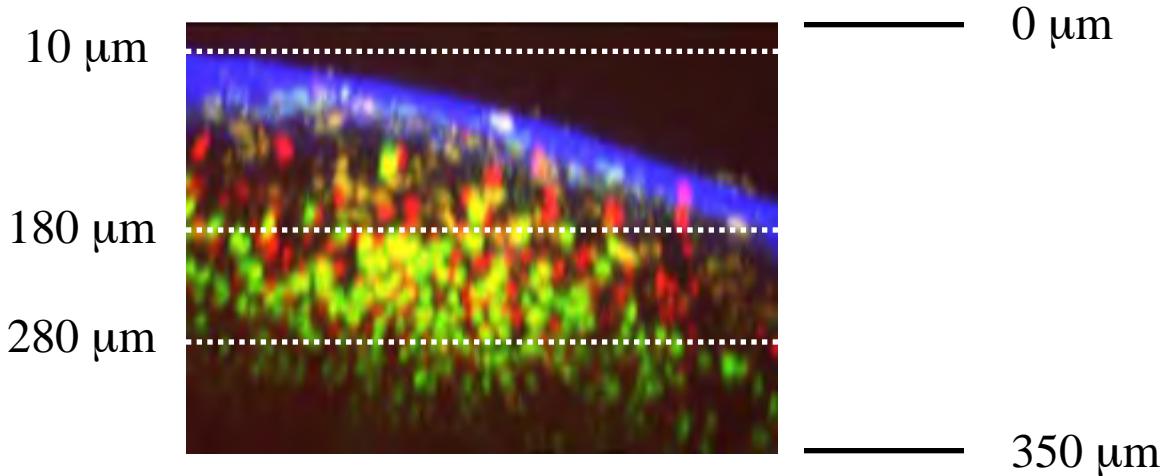
2P scopes use PMTs as detectors



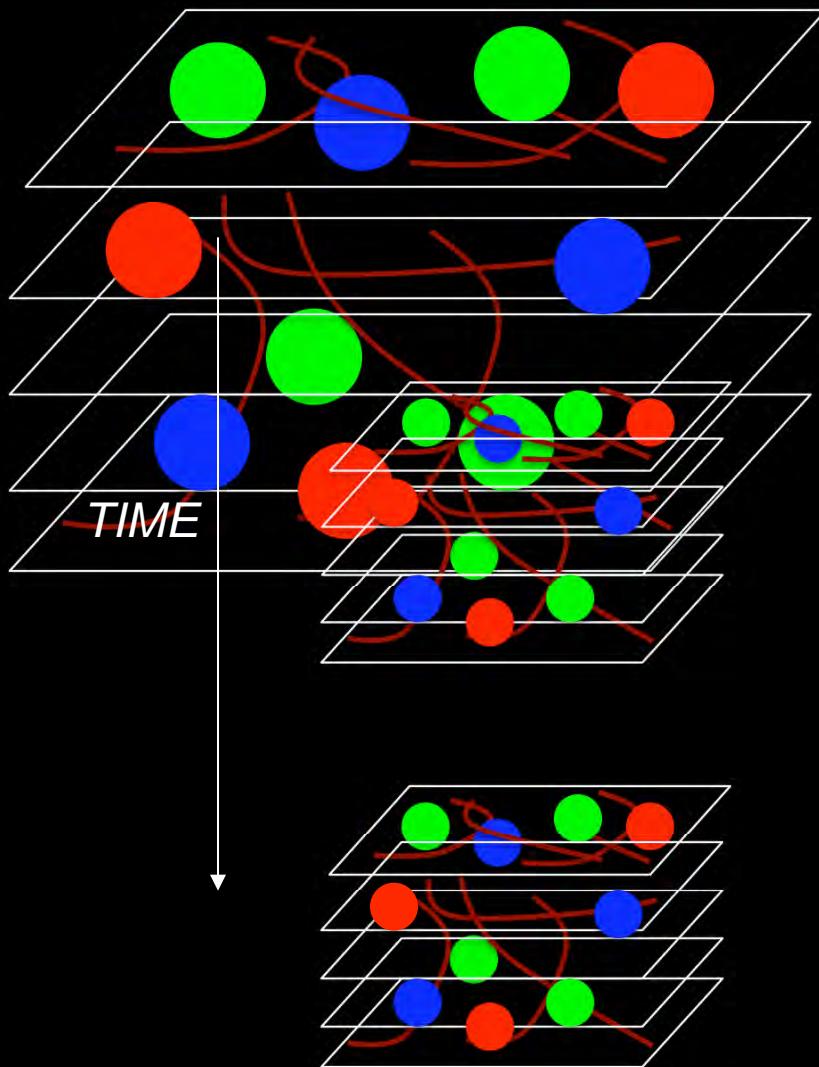
PMTs report intensity for single color based on fixed filter



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

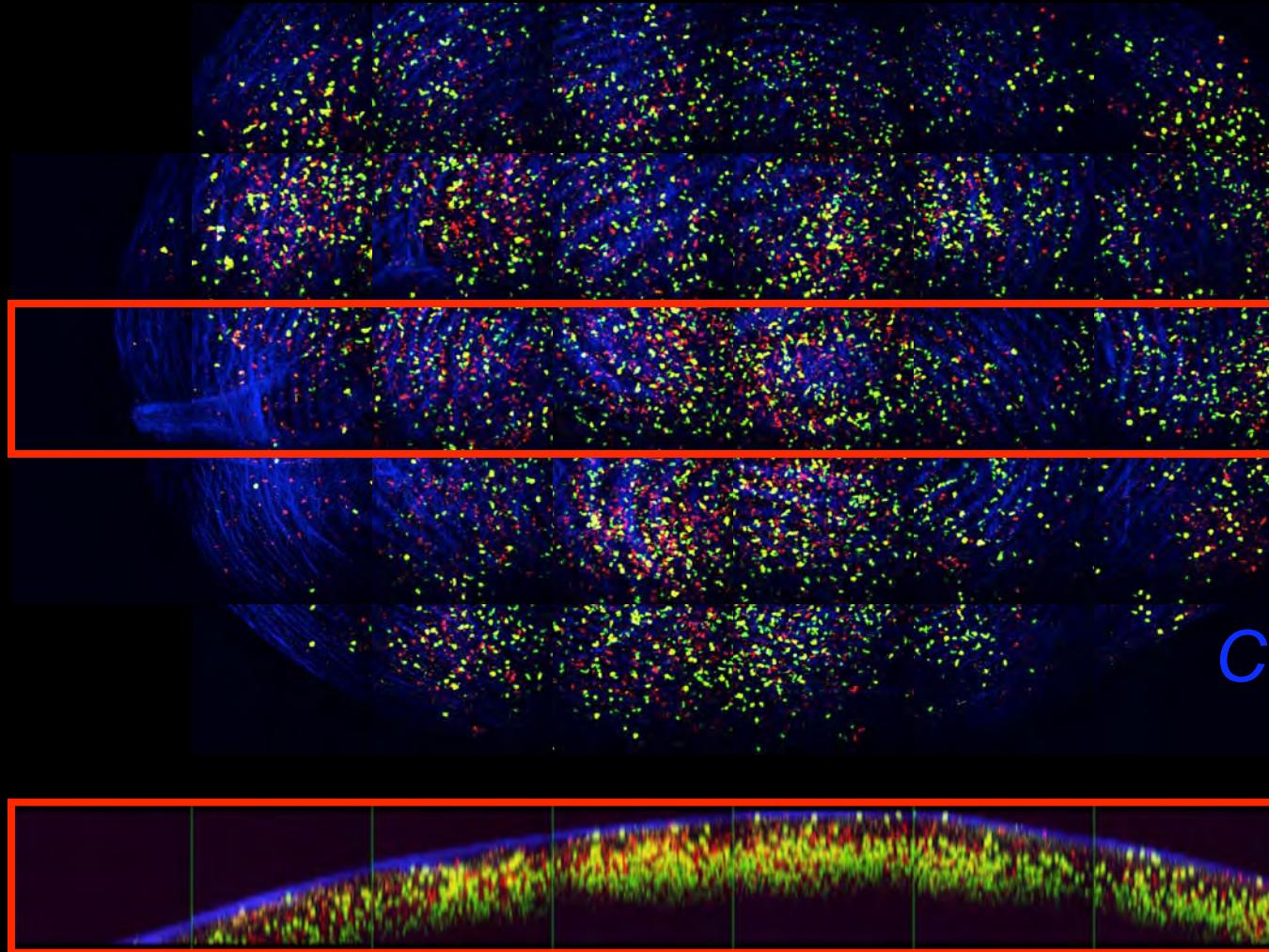


A 5D 2-Photon Platform

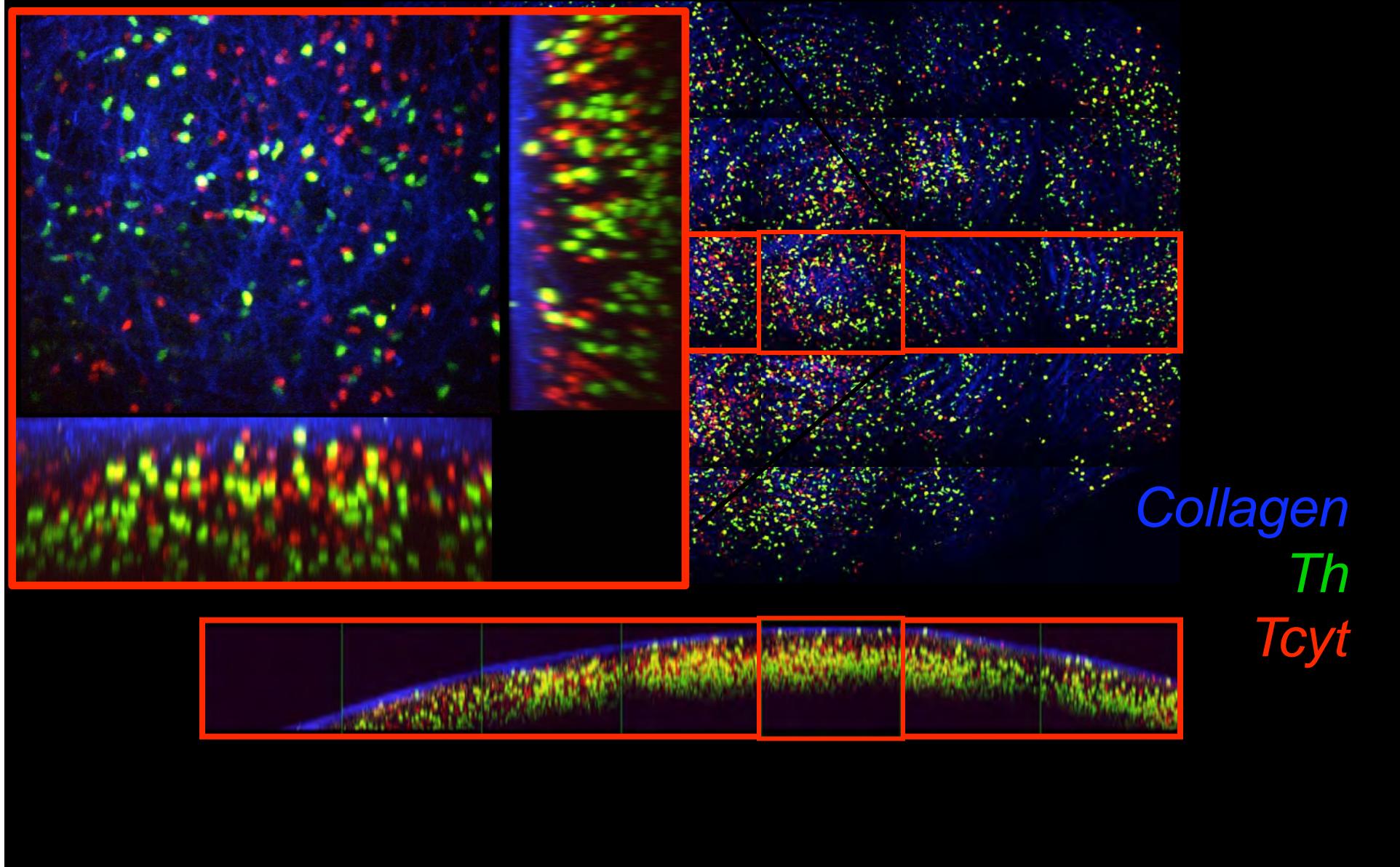


Analyze in:
Matlab
ImageJ
Metamorph
Imaris
IDT

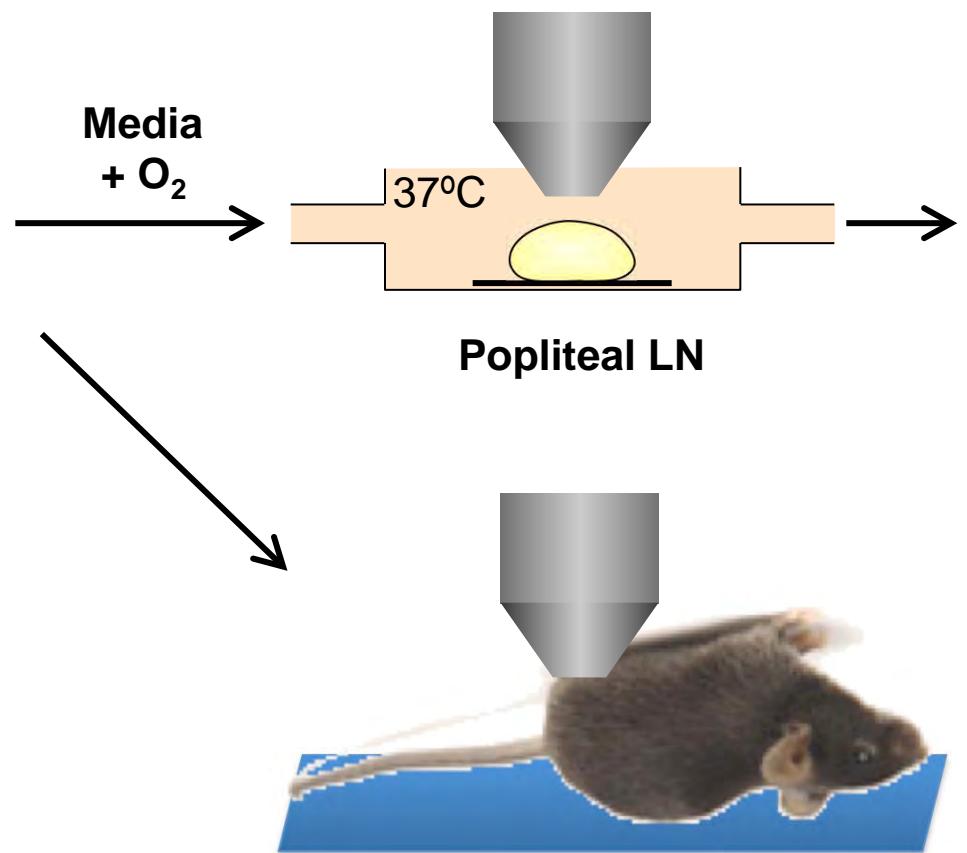
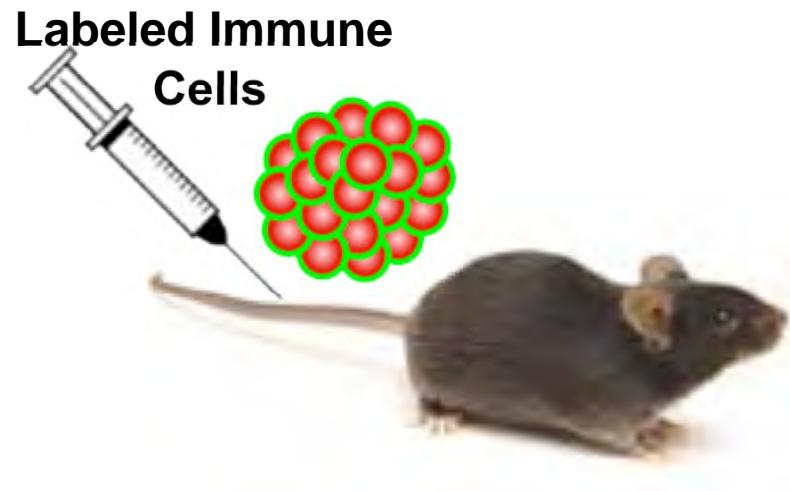
A 5D 2-Photon Platform



A 5D 2-Photon Platform



Organ Explant and intravital system for 2-photon imaging

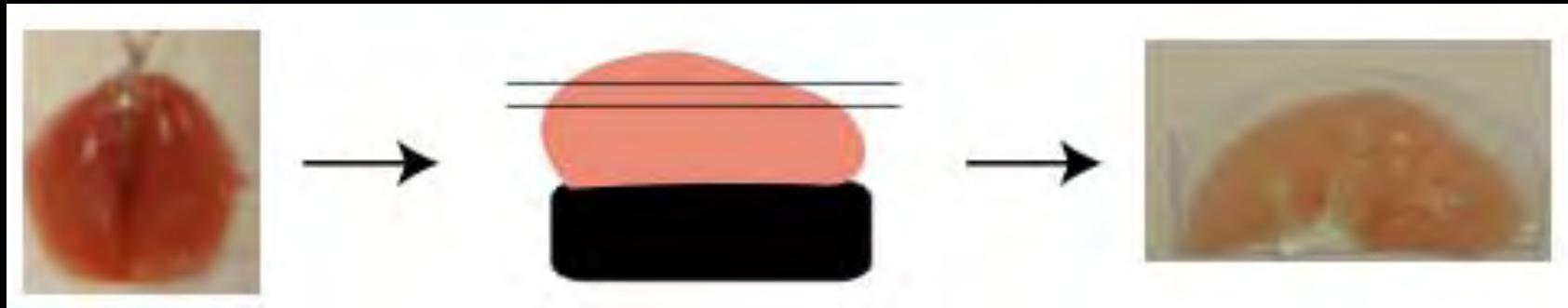


Model System for Asthma/Allergy Imaging

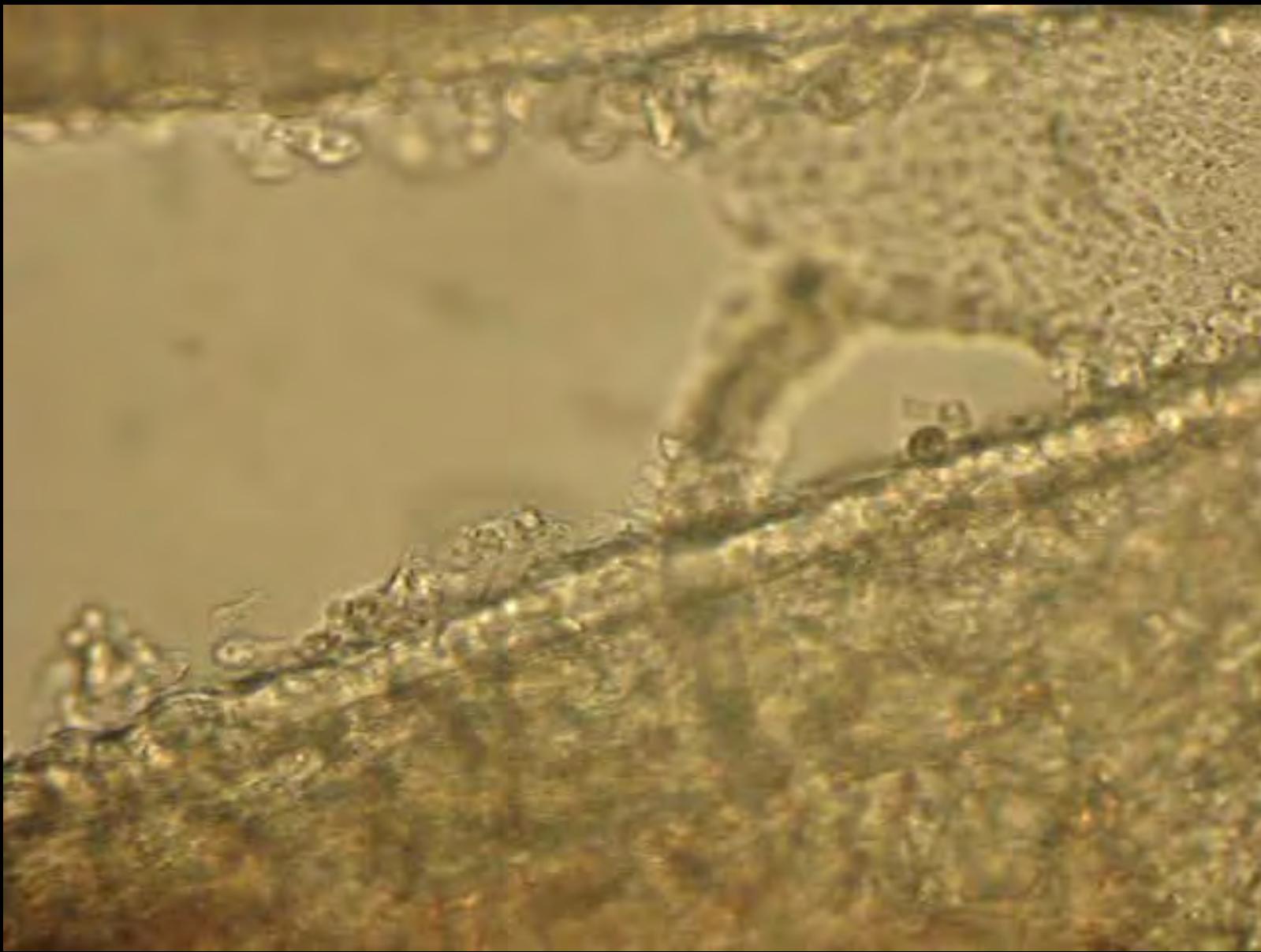


Sensitize with ova-alum IP

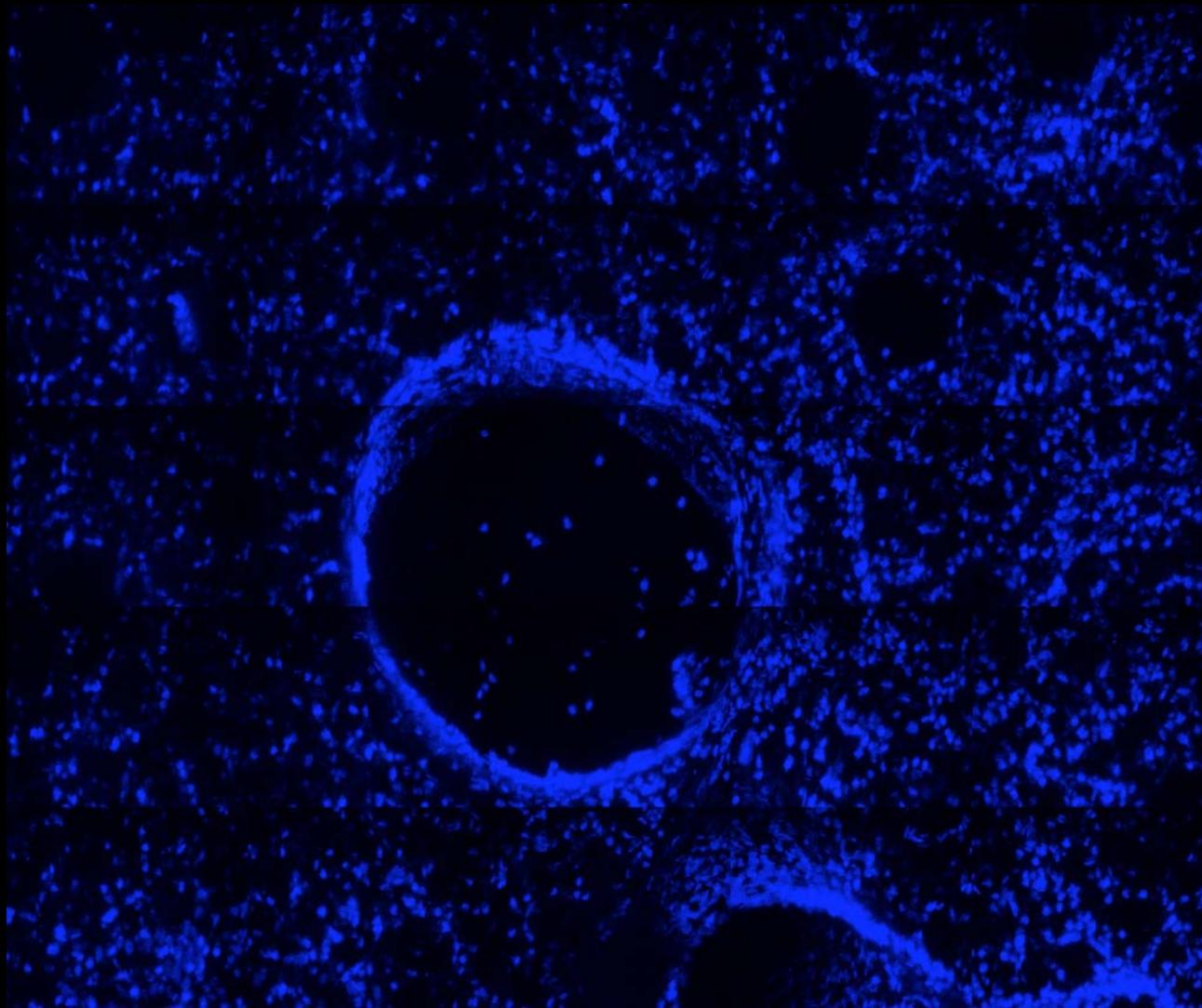
Challenge with ova IN



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

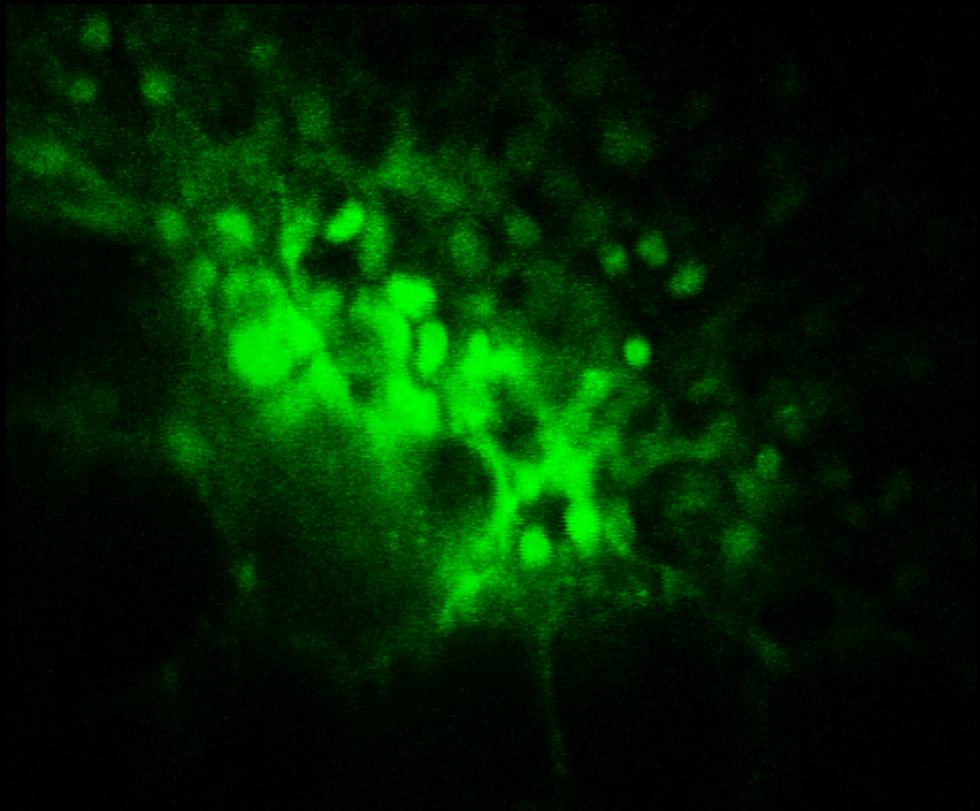


How to find your way around?. Vital dyes...



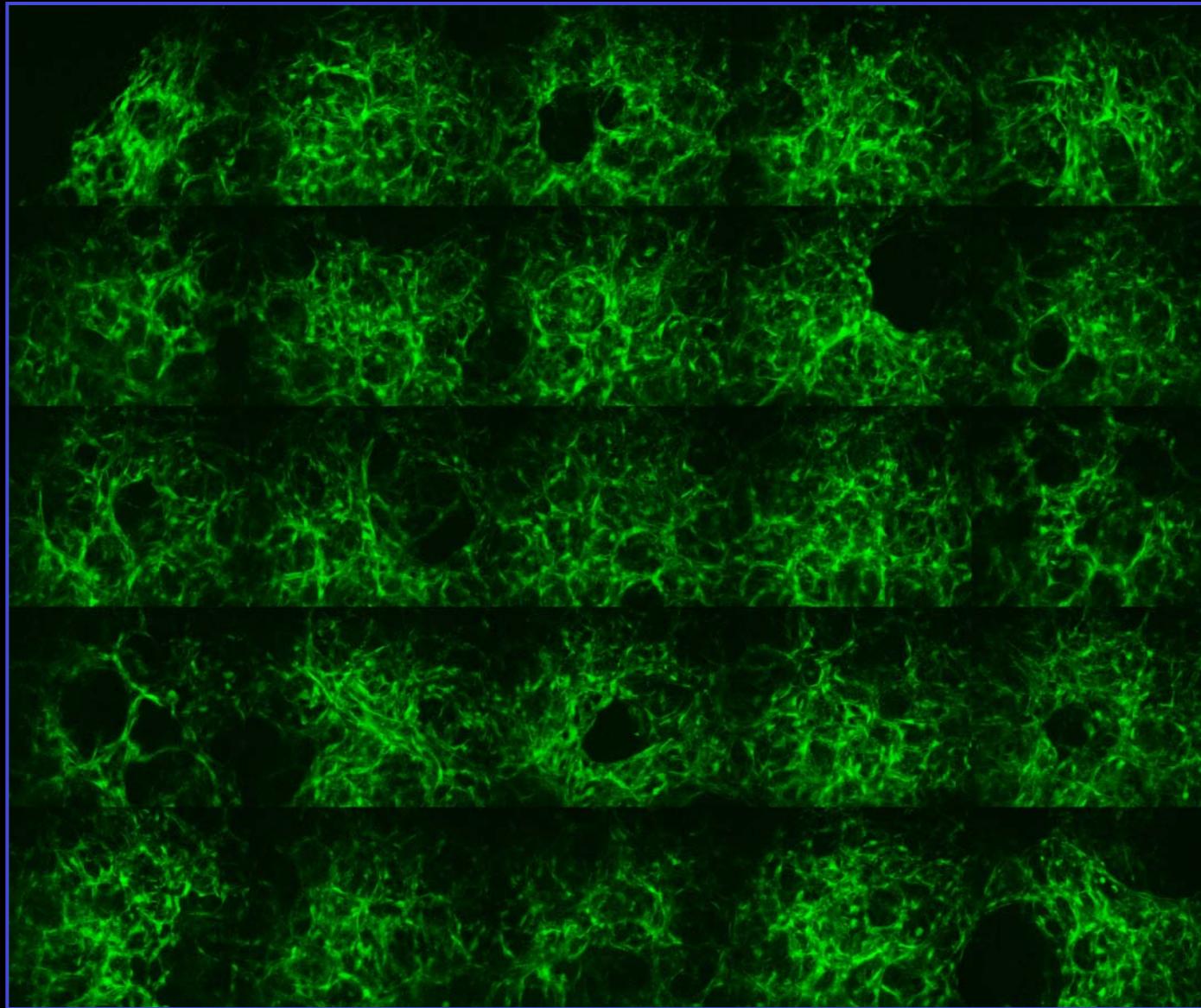
Small wt airway-hoechst

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



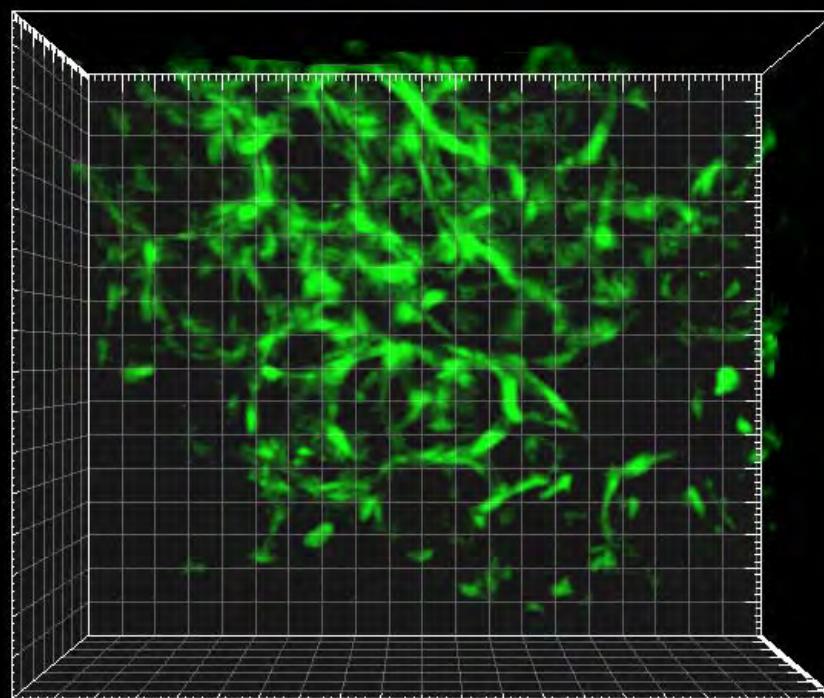
Small Ova airway → alveoli :: CFSE

GFP Fusions: Sca-1 GFP



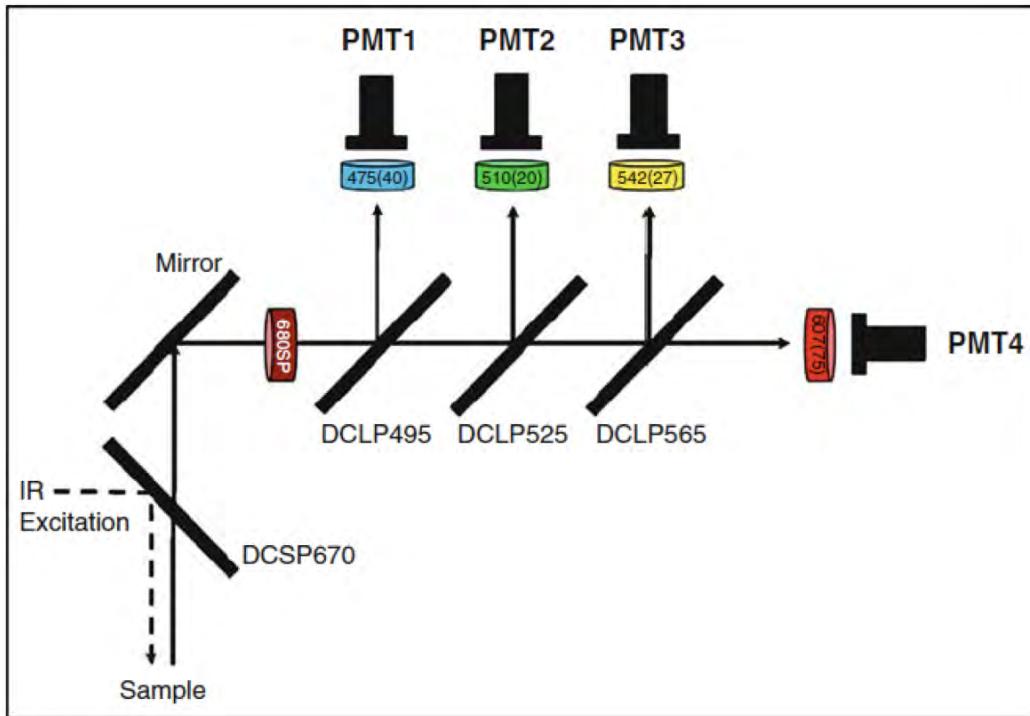
Jonathan Alexander/Chapman

GFP Fusions: Sca-1 GFP



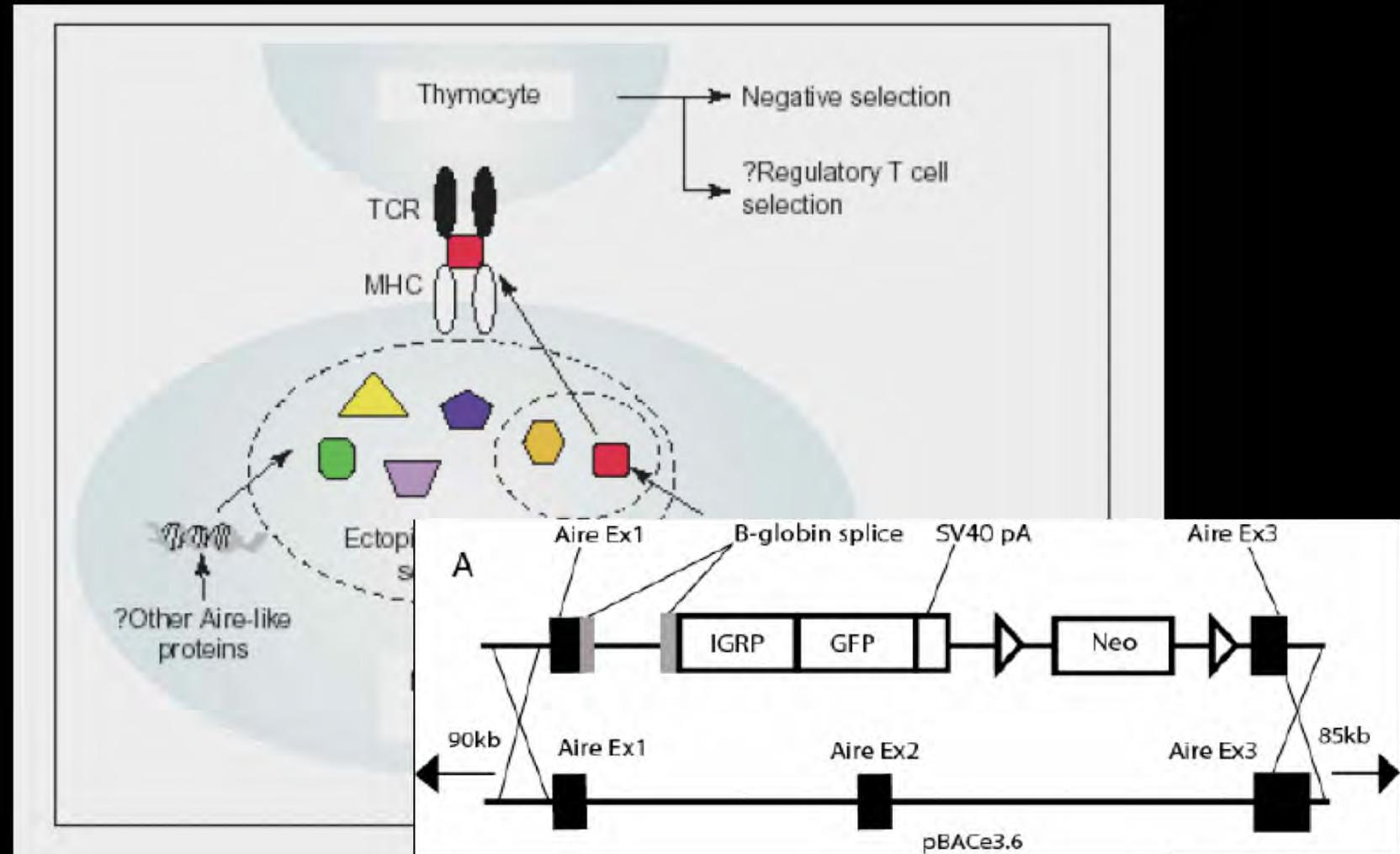
14:06:21.562

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. Opinion in Immunology* 2004 Dec; 16(4): 400-406

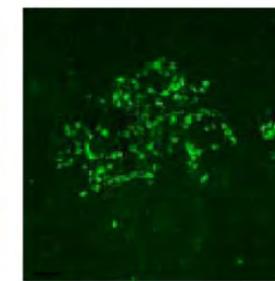
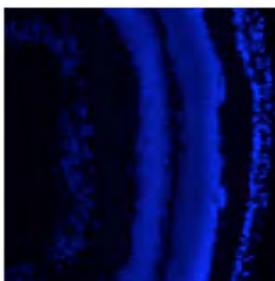
Extrathymic IGRP-GFP+ Cells Subsequently Detected in Tissue Survey



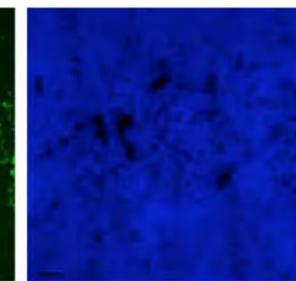
liver



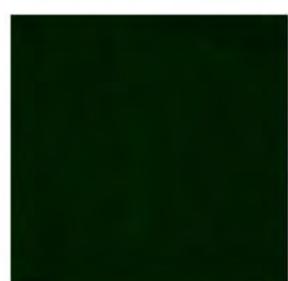
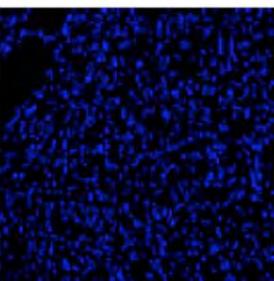
retina



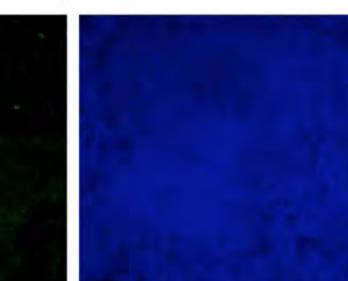
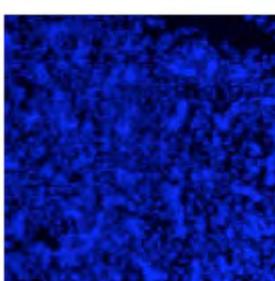
thymus



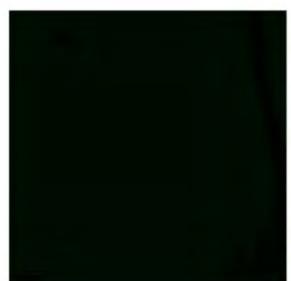
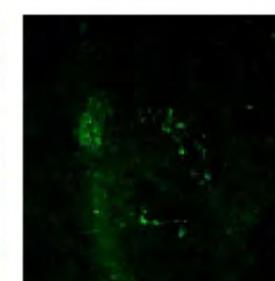
heart



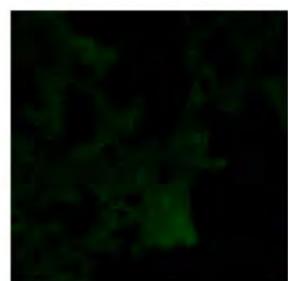
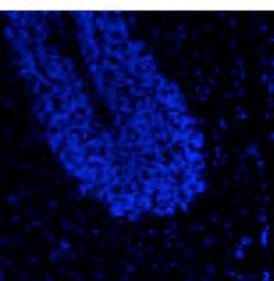
salivary



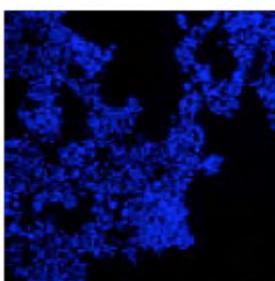
spleen



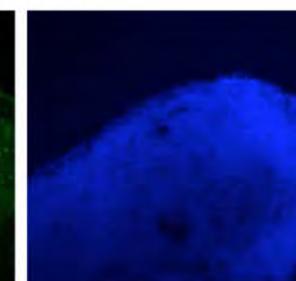
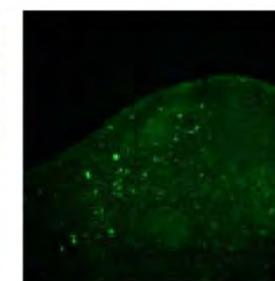
cerebellum



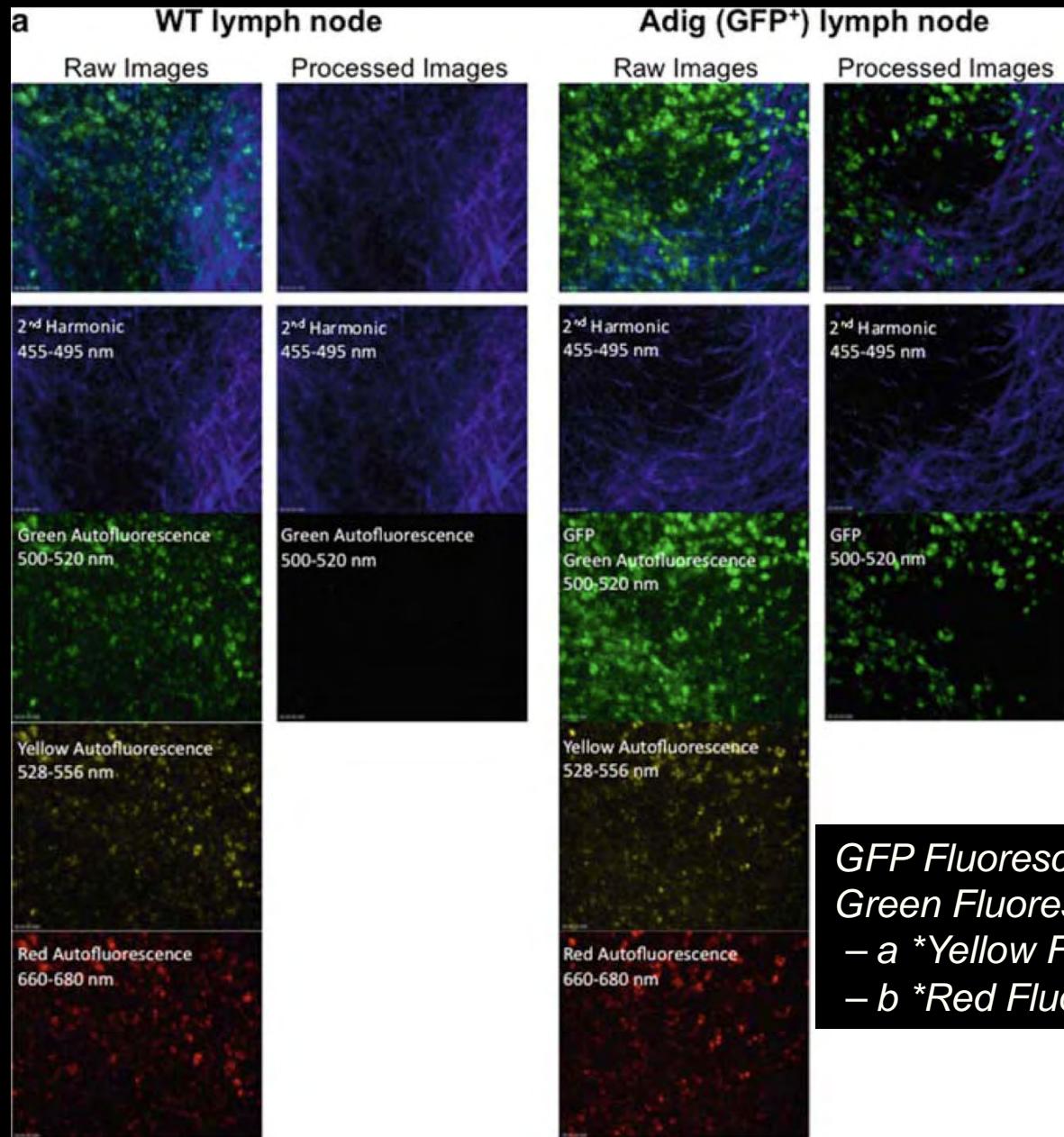
lung



lymph node



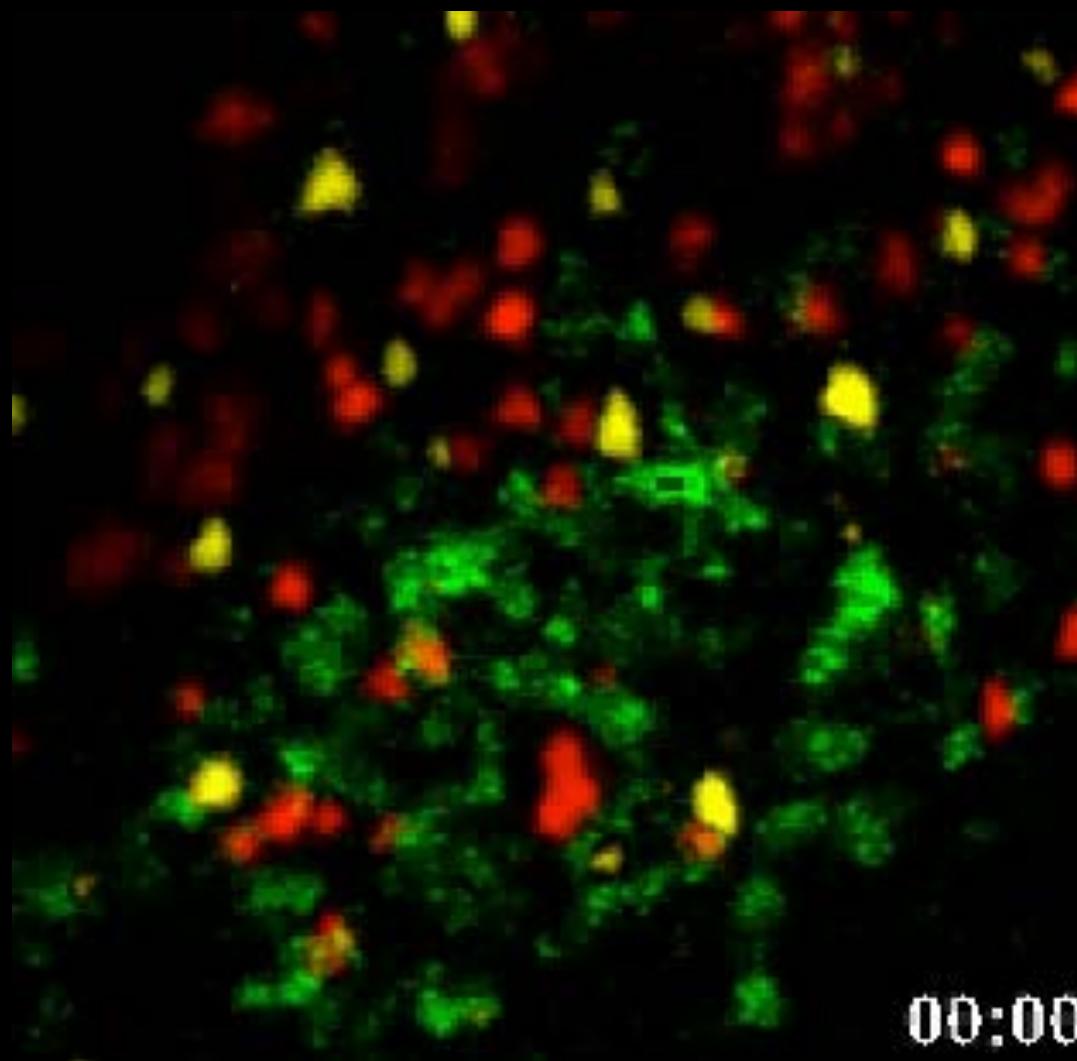
Using Multiple Channels to Remove Autofluorescence



$$\begin{aligned} \text{GFP Fluorescence} = \\ \text{Green Fluorescence} \\ - a * \text{Yellow Fluorescence} \\ - b * \text{Red Fluorescence} \end{aligned}$$

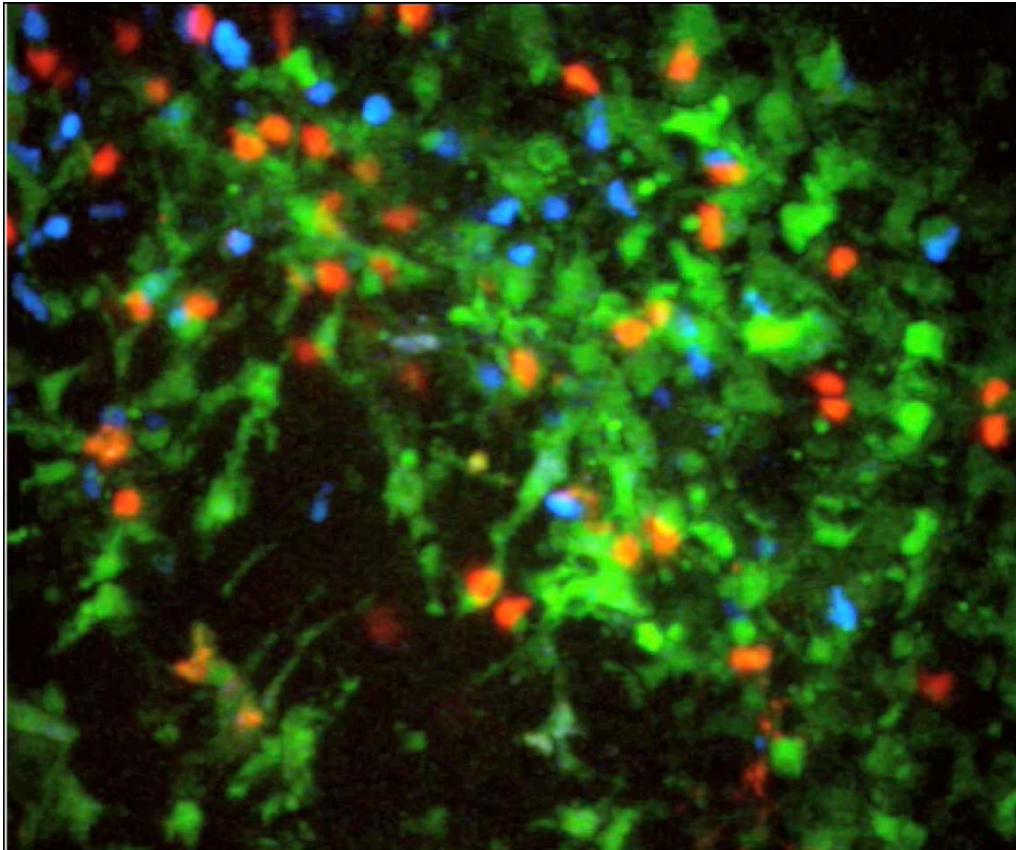
T cell interactions with Peripheral AIRE expressing cells (PAECs) in the LN

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



00:00

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



Kinetics of Cellular Behaviors

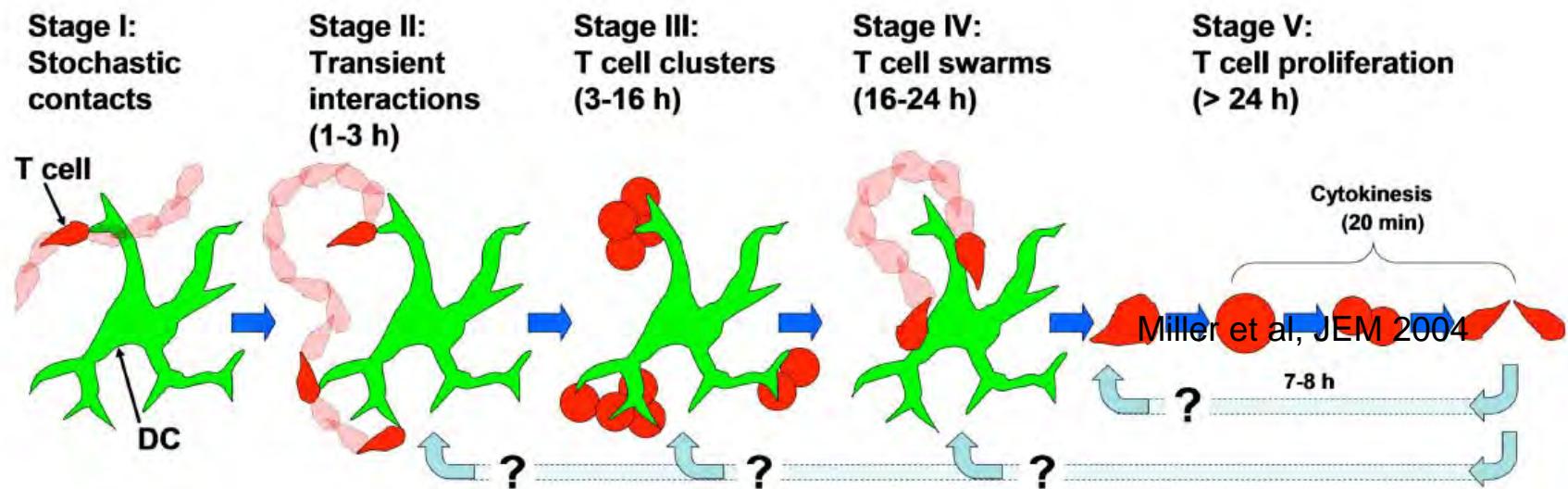
Cell-cell interactions

Cellular morphology

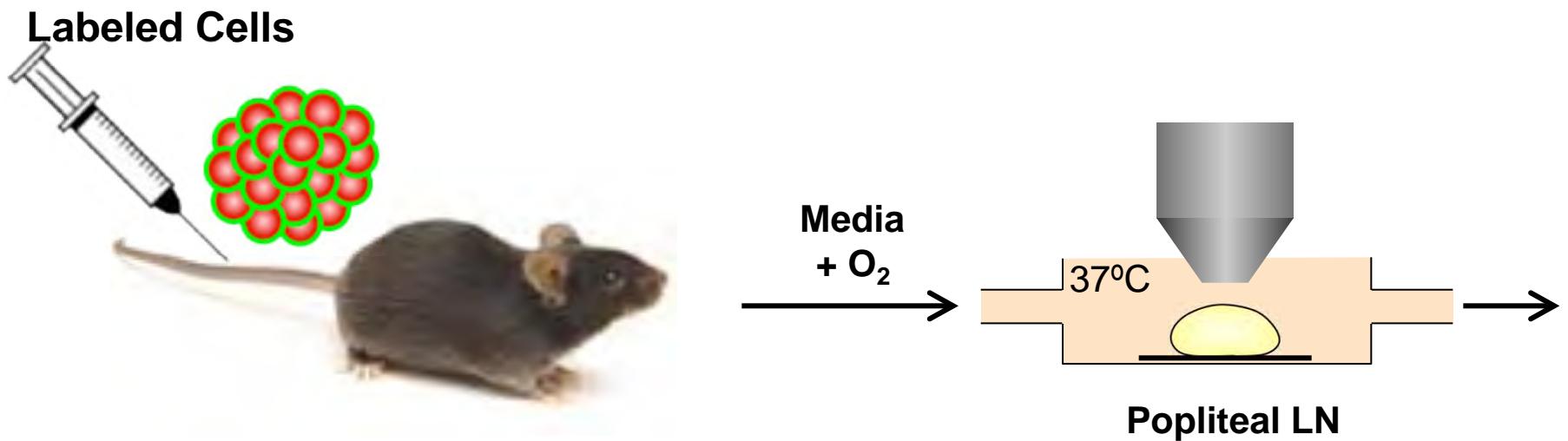
Speed/direction/confinement

Cell death

In vivo T cell activation



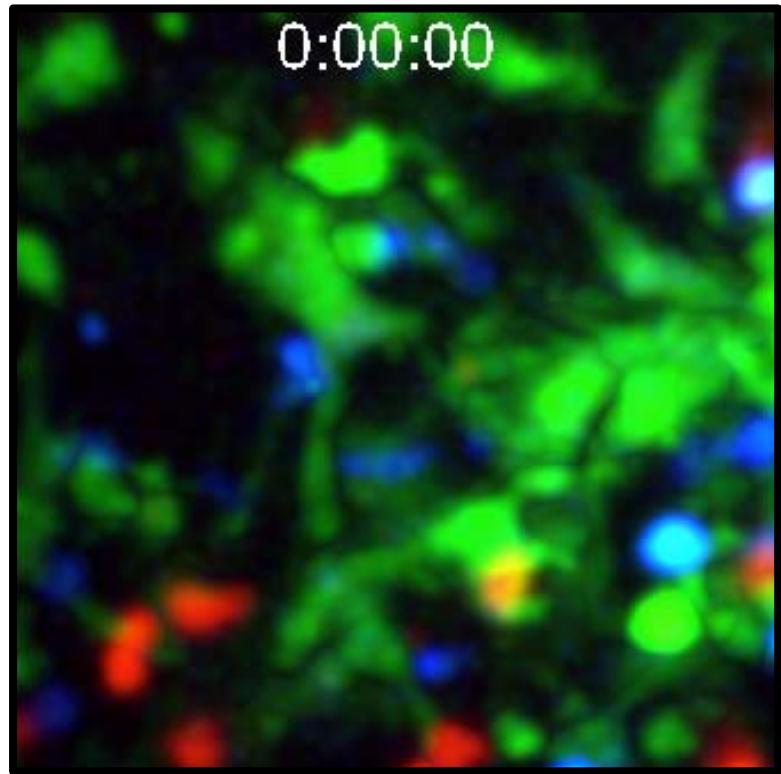
Experimental system for 2-photon imaging



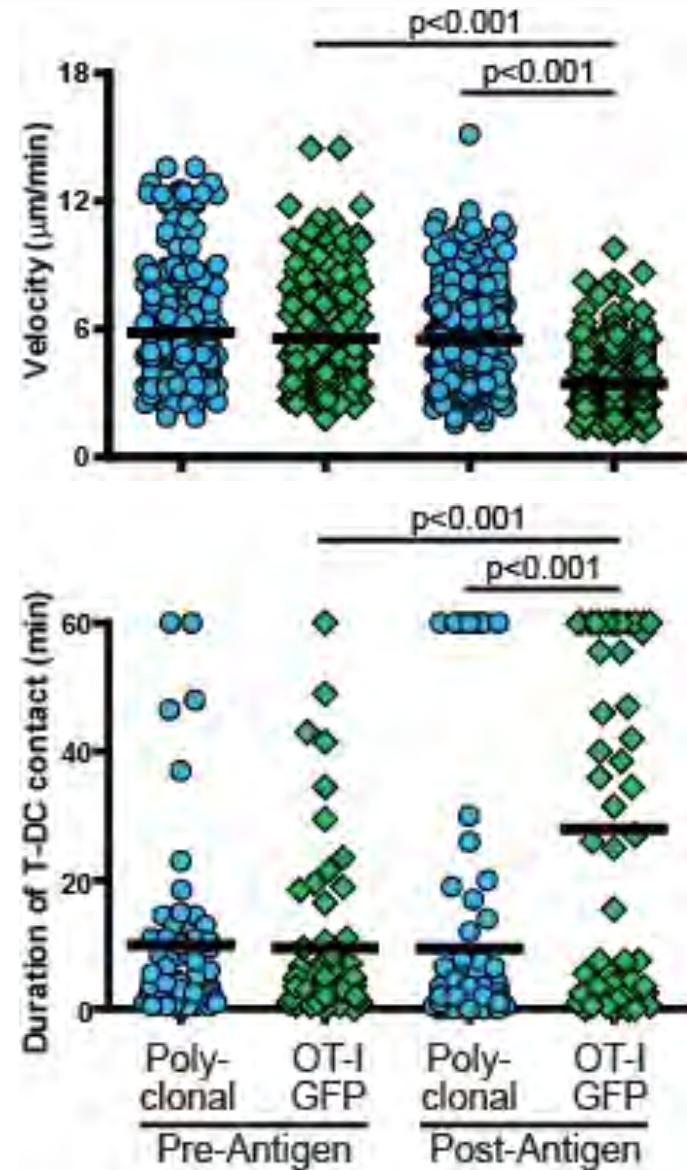
Host:

- 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

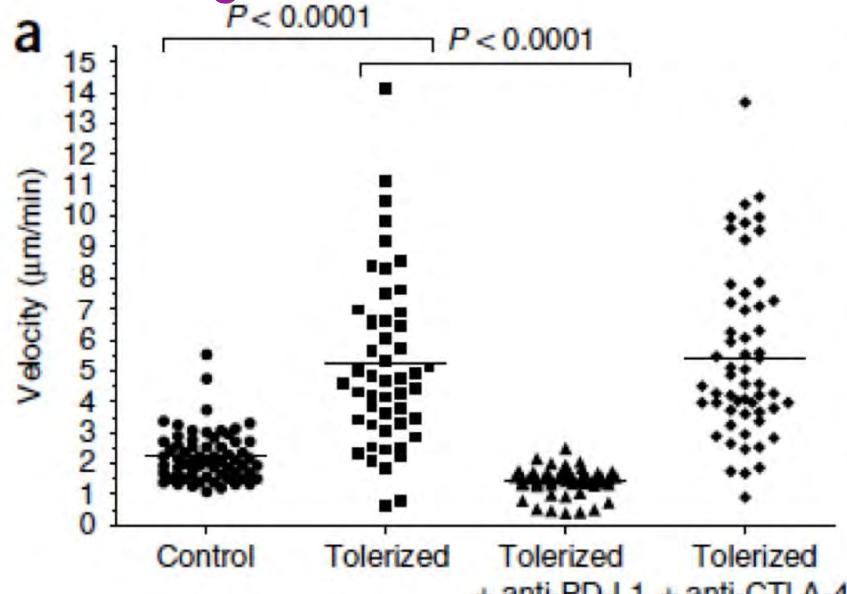


CD11c-YFP DCs
OT-I-GFP T cells
Actin-CFP+ Polyclonal T cells

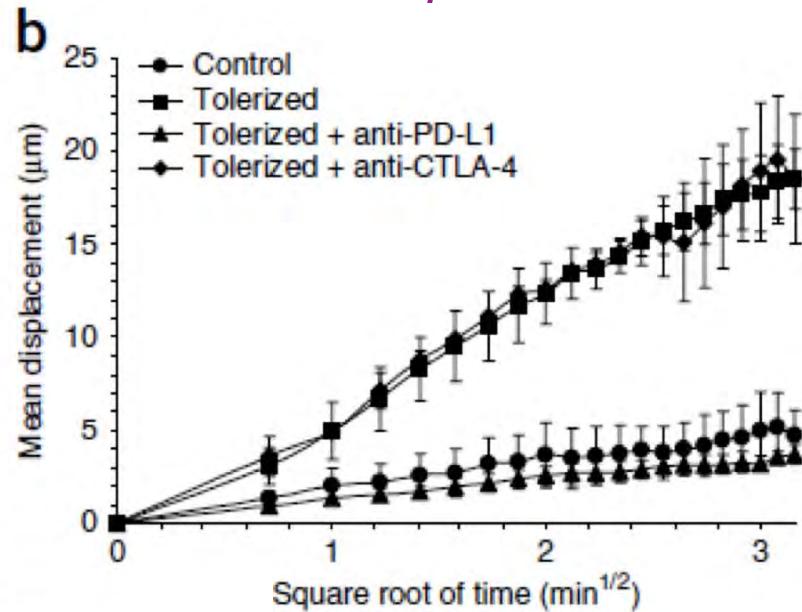


Data Analysis: Motility

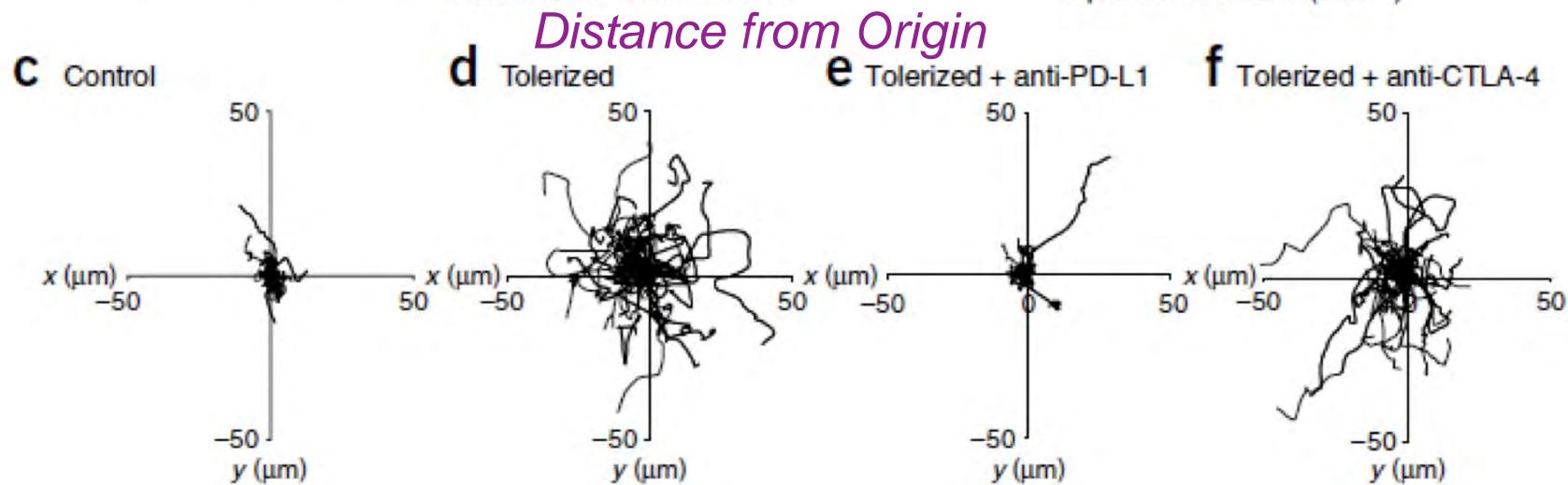
Avg Velocities



RMS Displacement



Distance from Origin



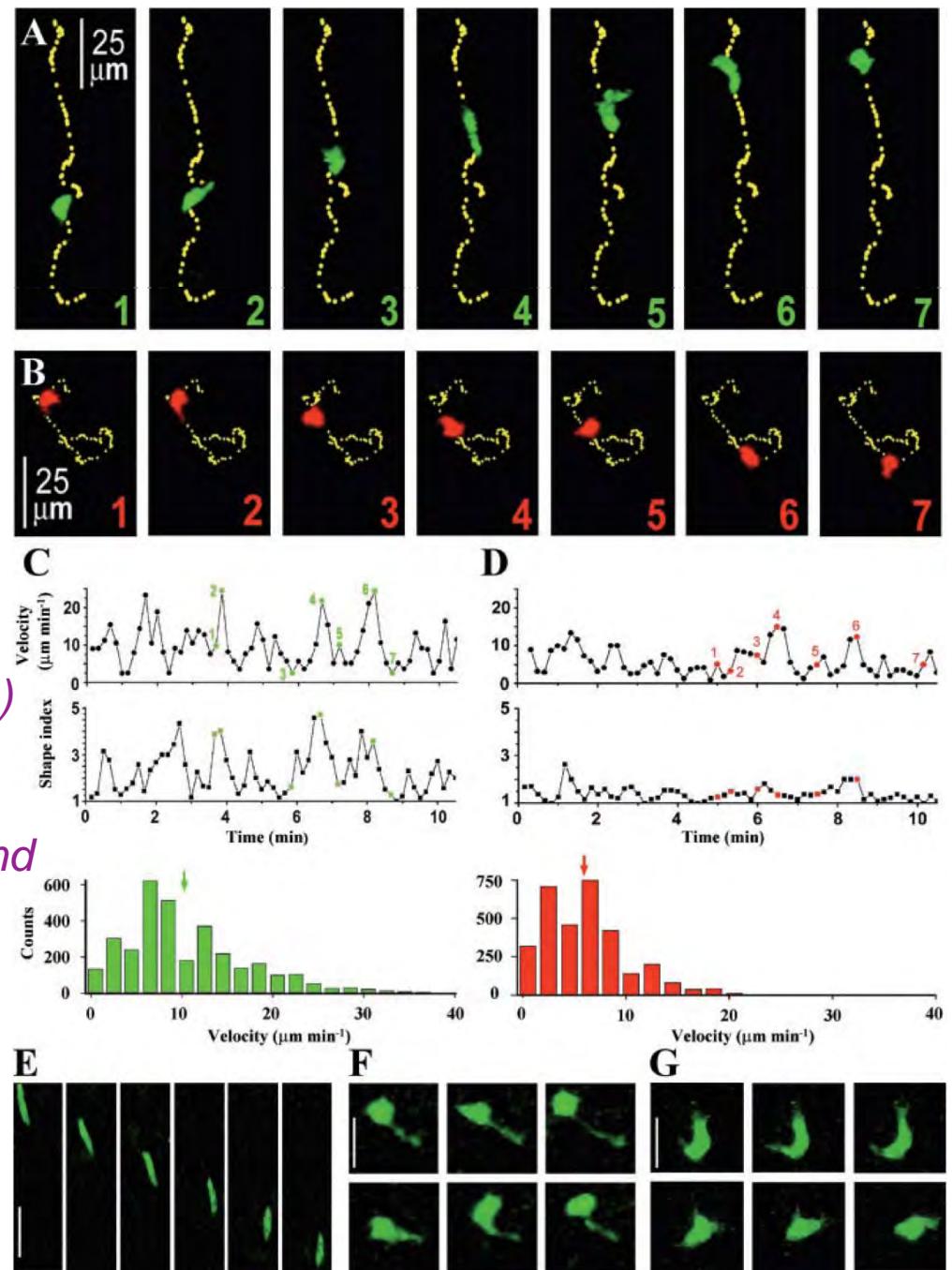
Morphology:

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



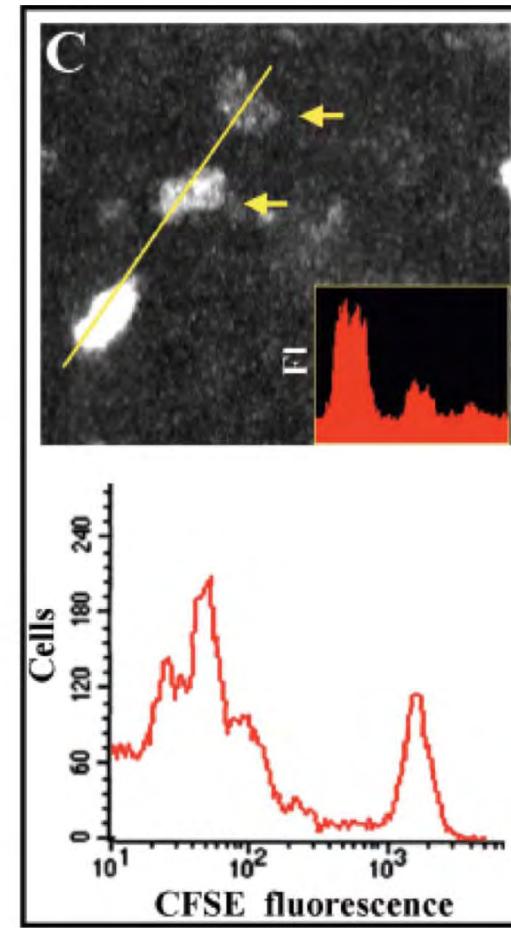
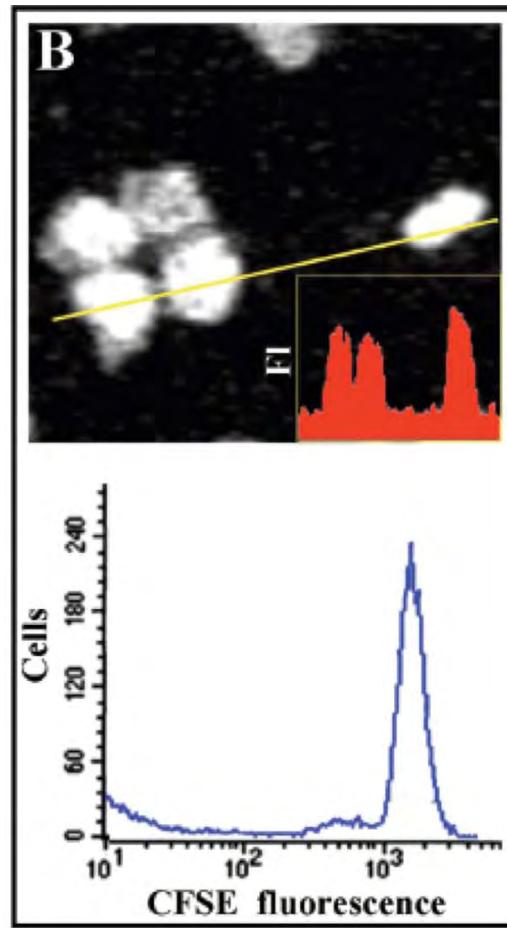
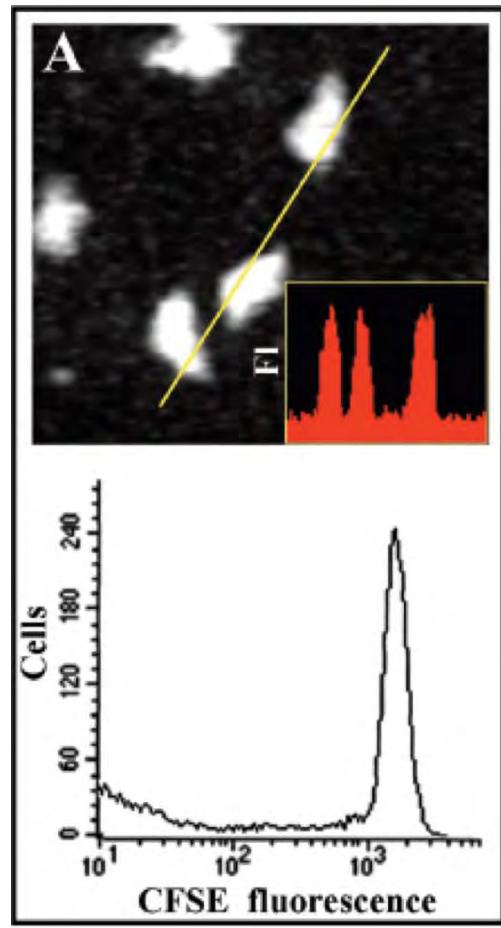
*Ratio of longest and
perpendicular chords (1 for circle)*

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

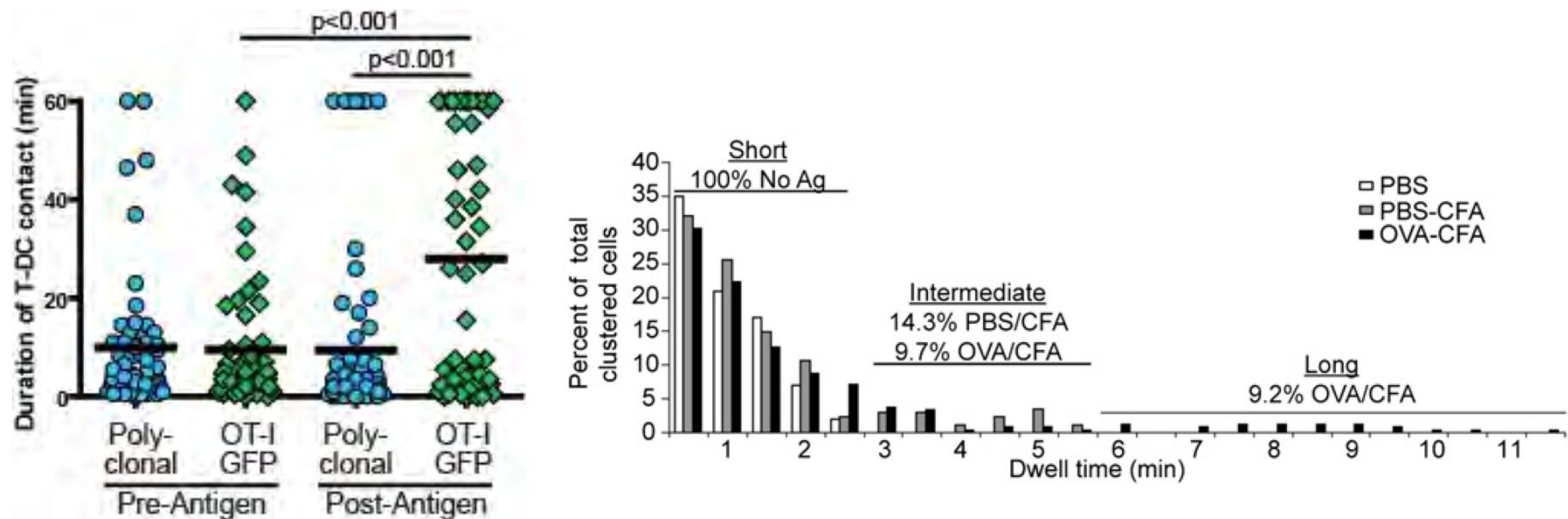


Cahalan.

Cell Division:

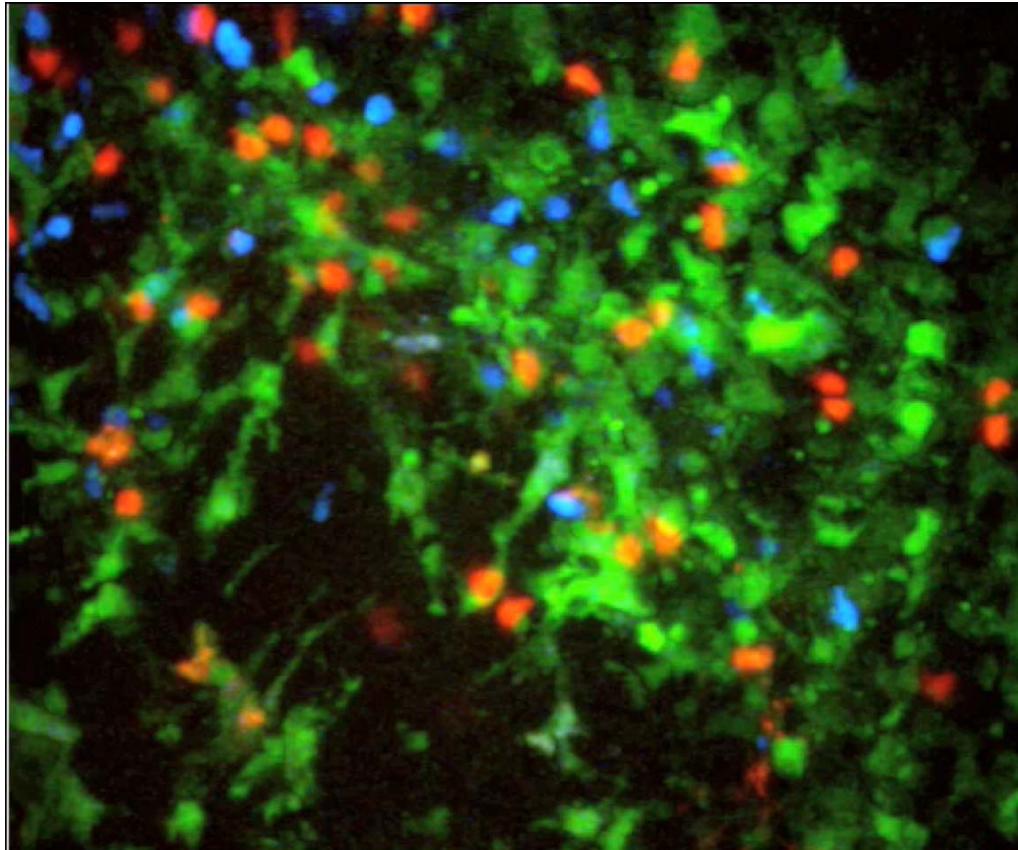


Data Analysis: Cell-Interaction



*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

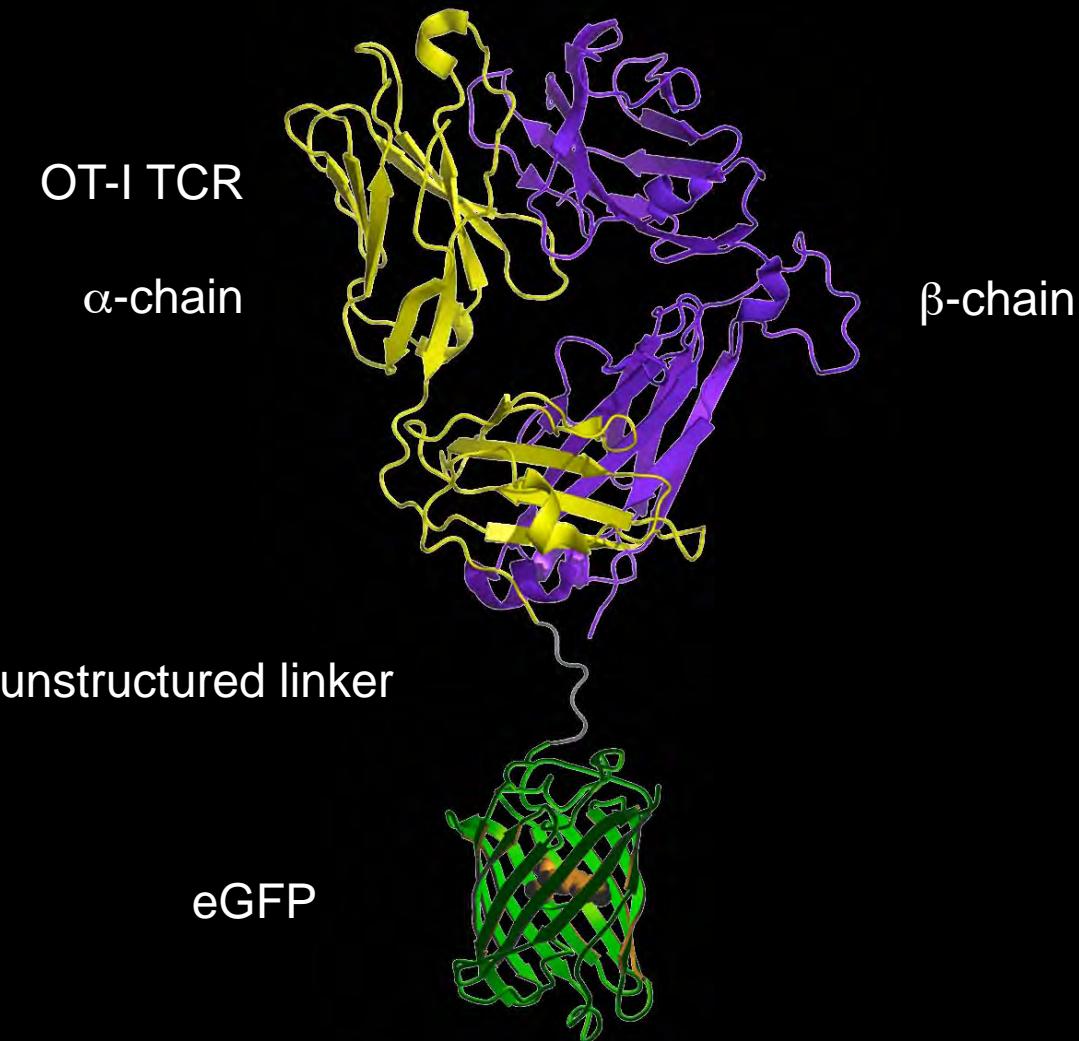
Cellular morphology

Speed/direction/confinement

Cell death

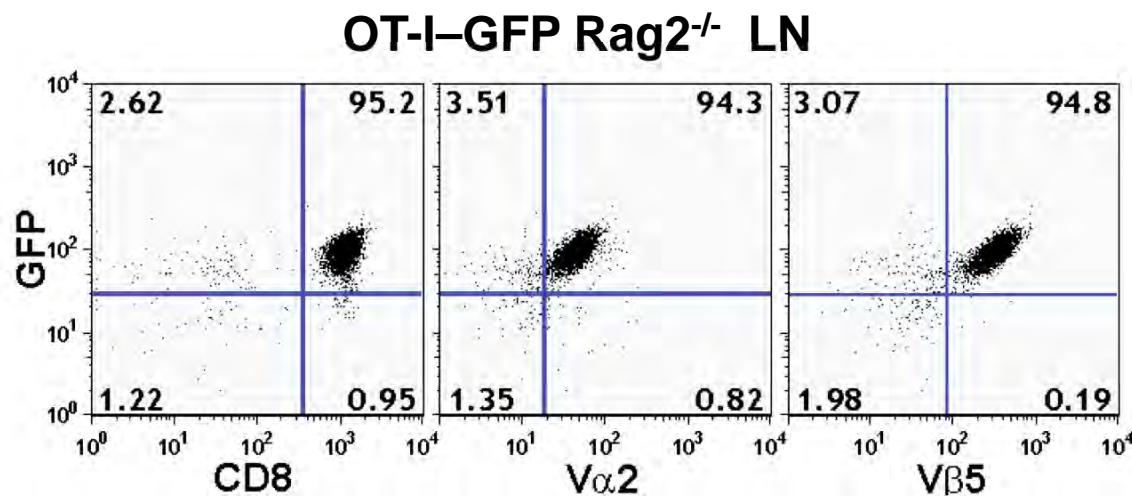
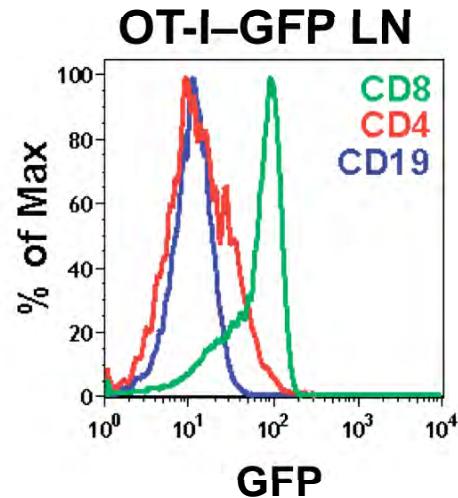
How can we gain more information from *in situ* imaging?

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



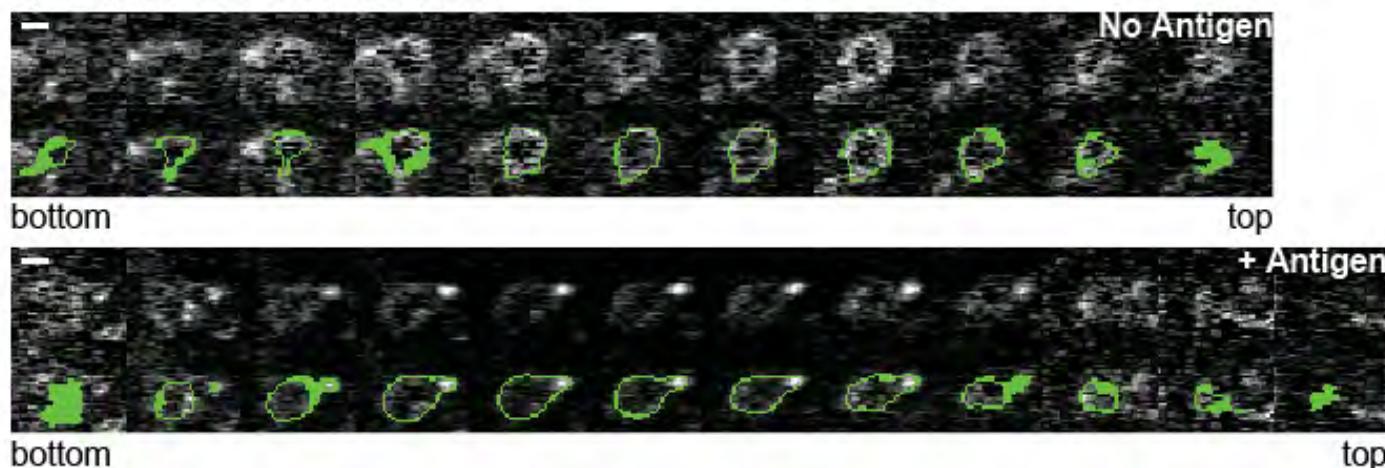
TCR structure by David K. Cole
GFP structure from Roger Tsien

Surface expression of OT-I-GFP

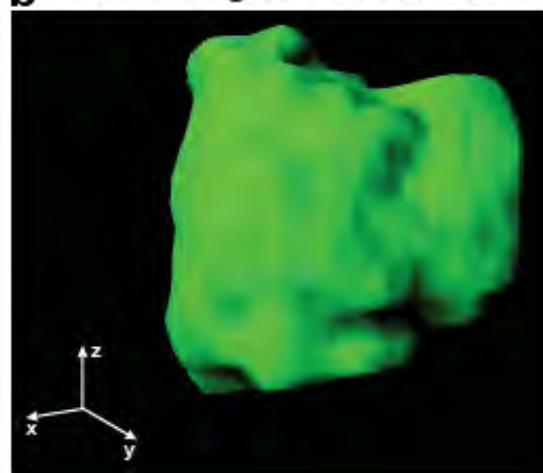


GaAs Detectors Are Great, But... (Estimation of Signal Limit)

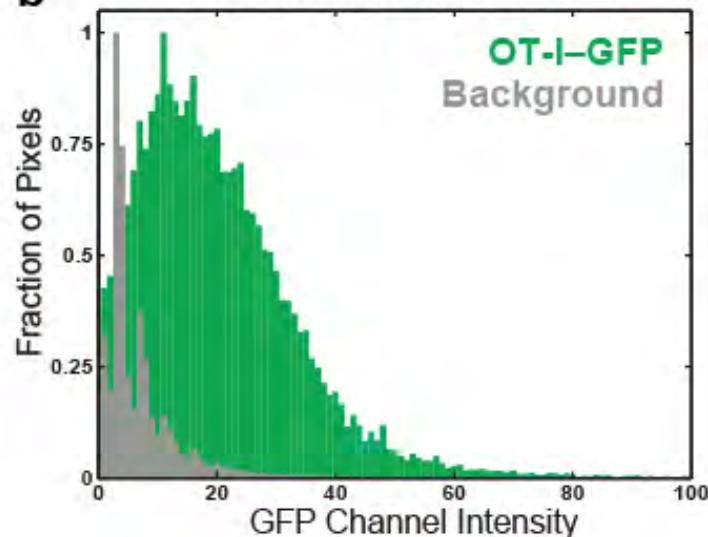
a Cell surface identification



b Rendering of Cell surface



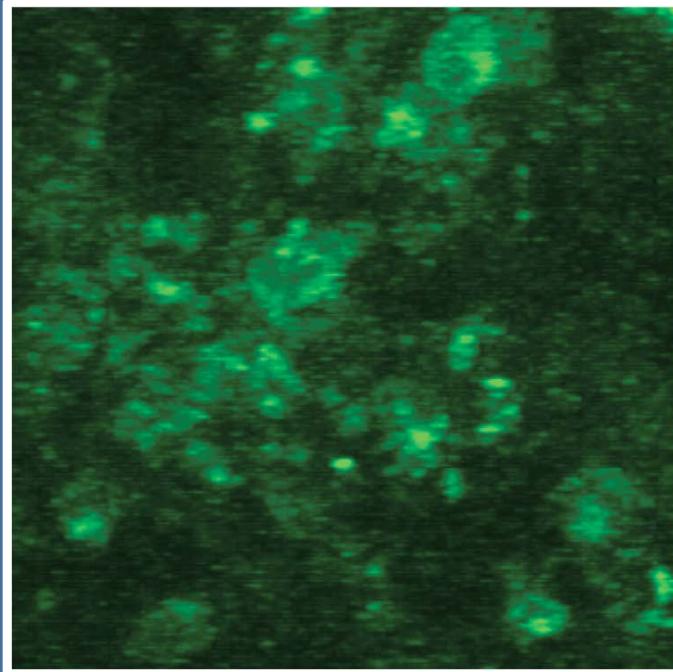
b Cell Surface Fluorescence



For a shell calculation of TCRs, we voxelize and we can magnitude ~ = 1 IU per bound 2.5GFPs/voxel

Autofluorescence obscures OT-I-GFP fluorescence

Problem

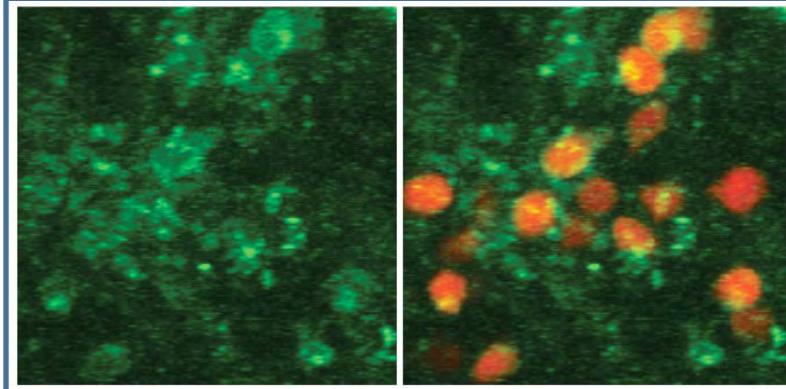


OT-I-GFP & Autofluorescence

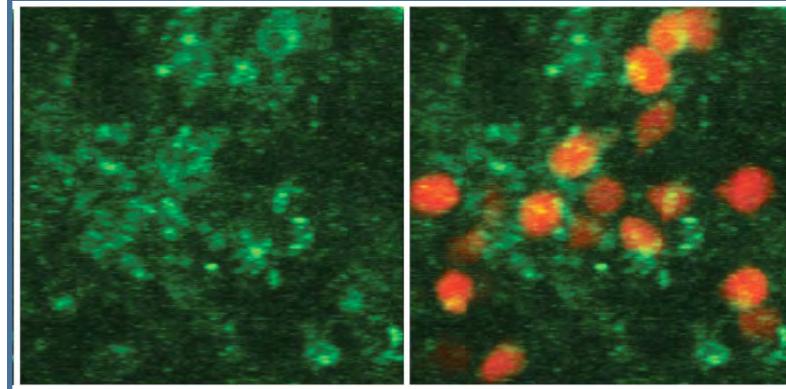
CMTMR co-labeled Cells

Image processing to eliminate autofluorescence

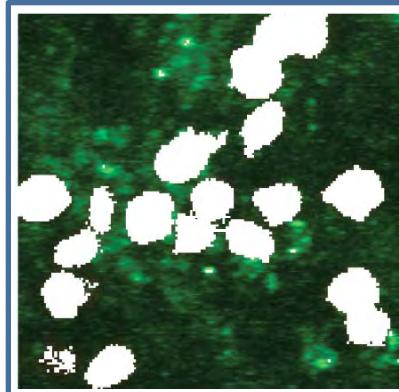
Unprocessed Image



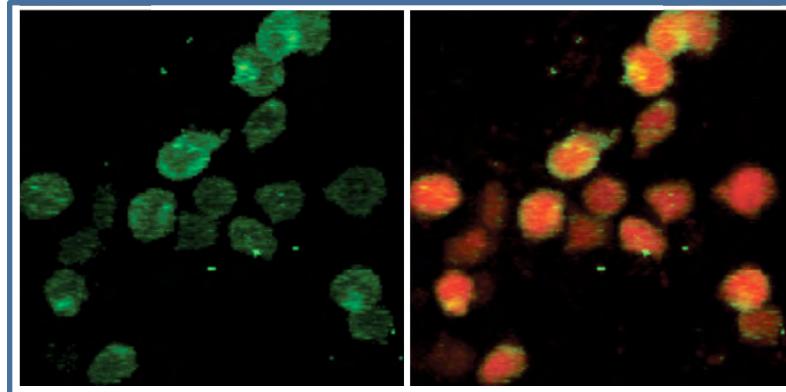
Linear Unmixing



Volumetric Mask

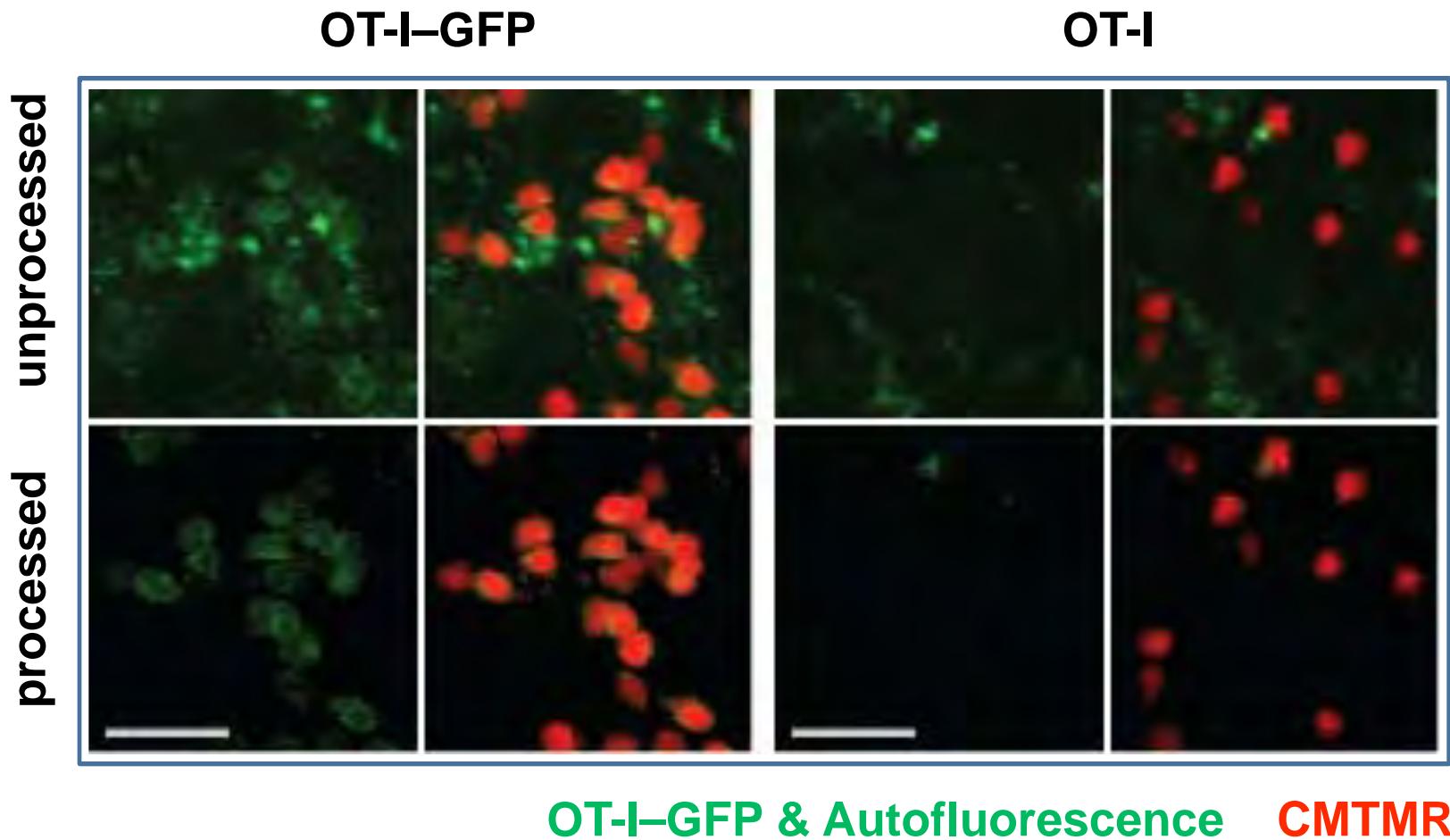


Processed Images

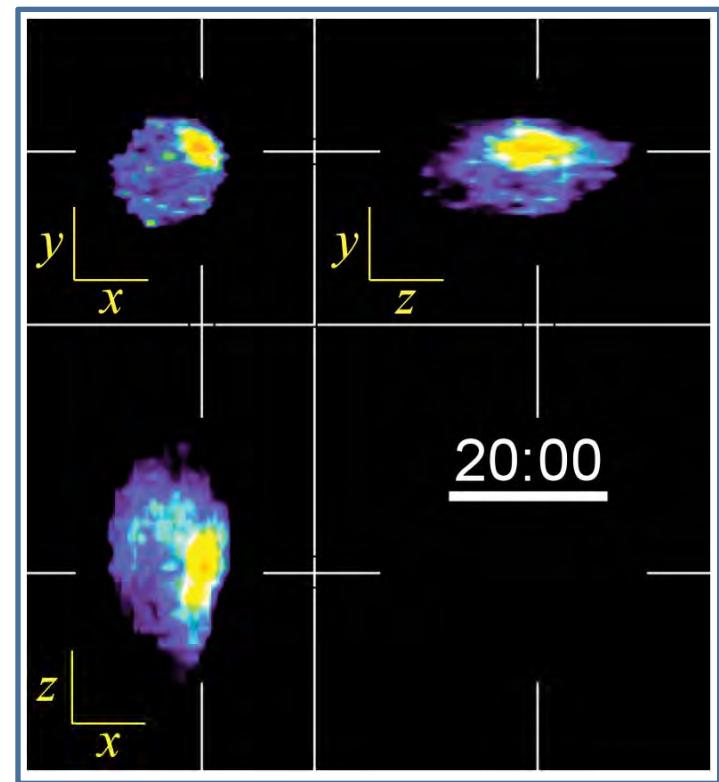
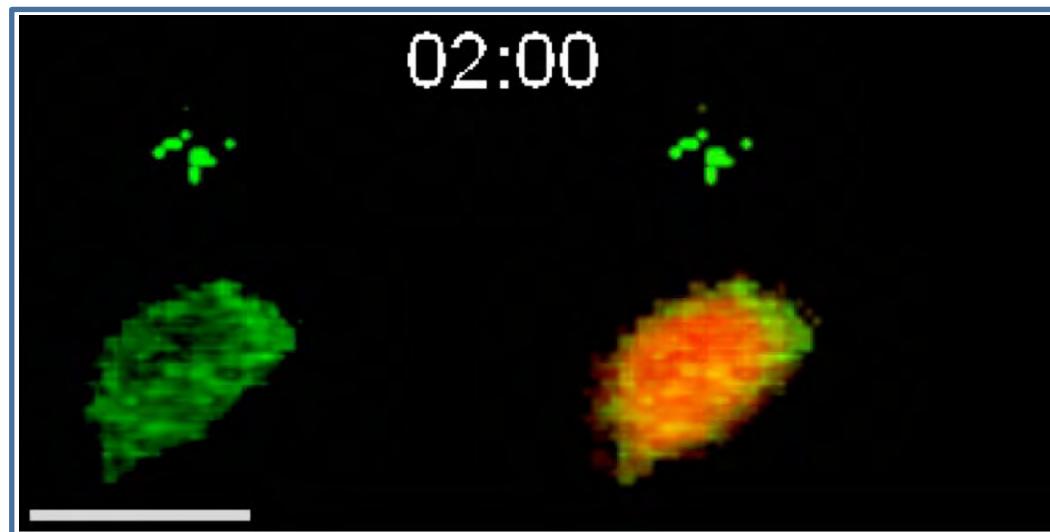


OT-I-GFP & Autofluorescence CMTMR

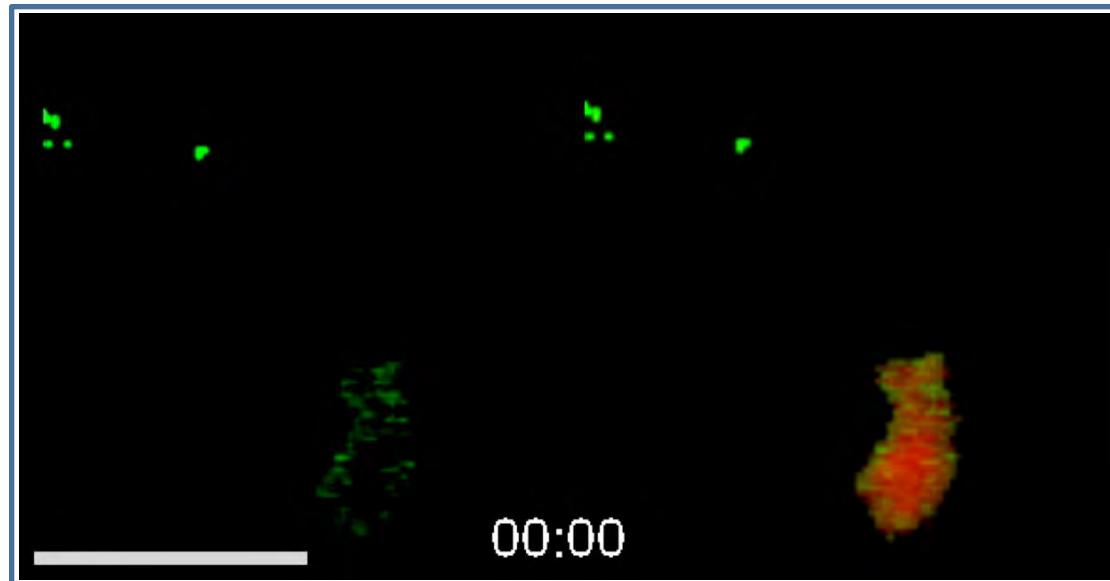
Image processing effectively isolates OT-I-GFP specific fluorescence



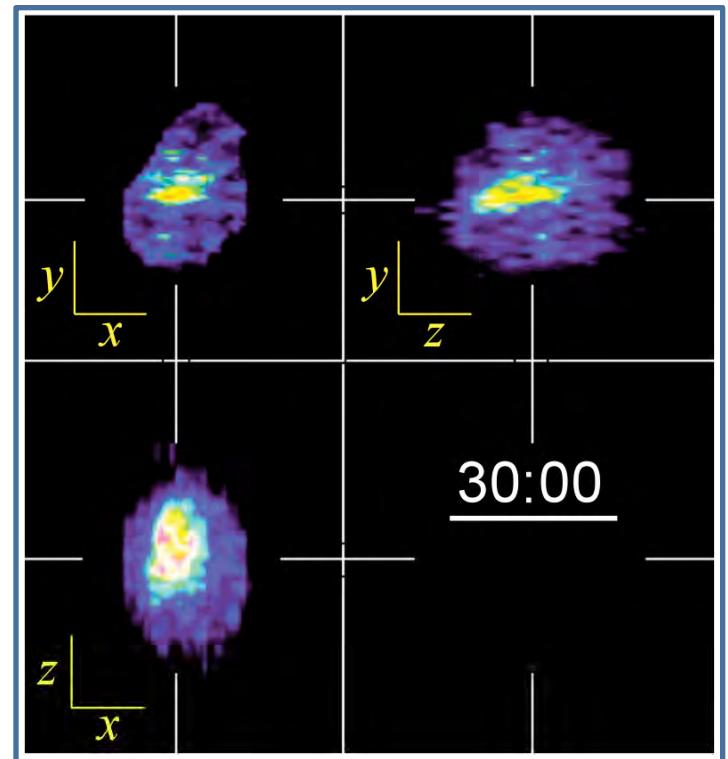
Rapid antigen-dependent TCR clustering



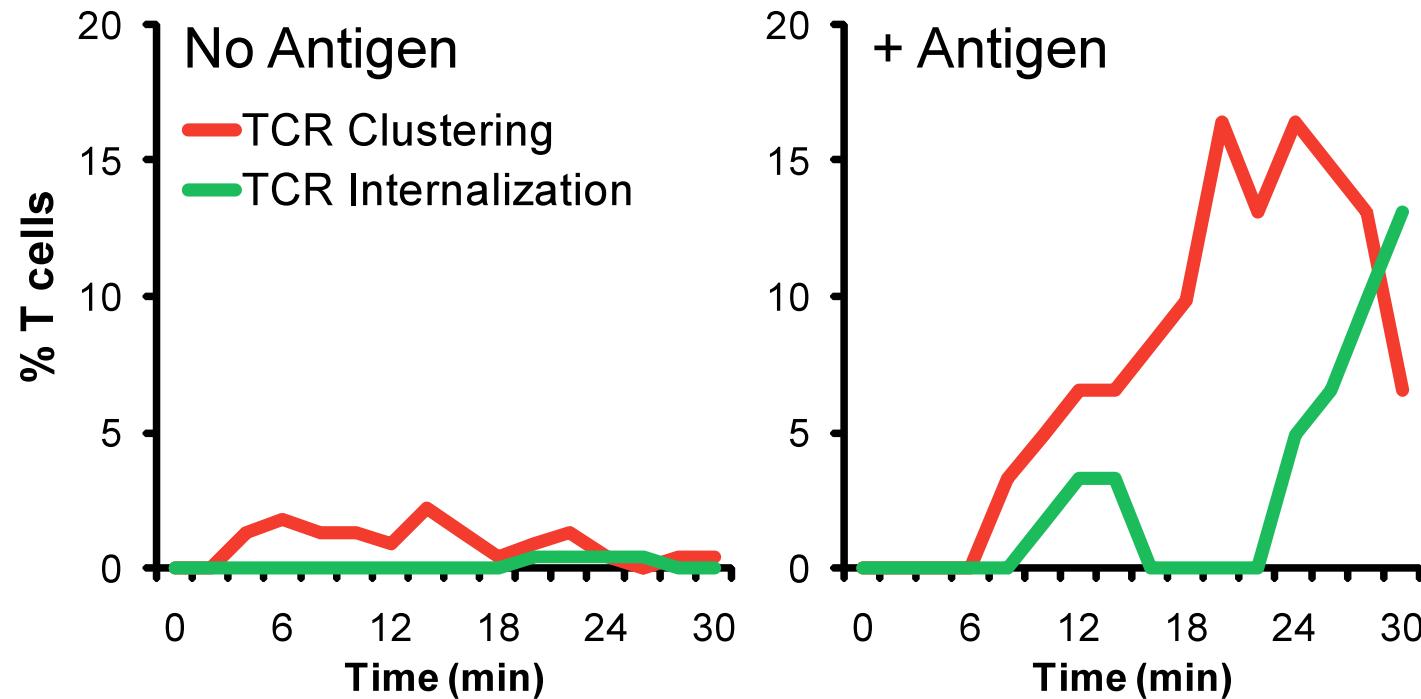
Rapid antigen-dependent TCR internalization



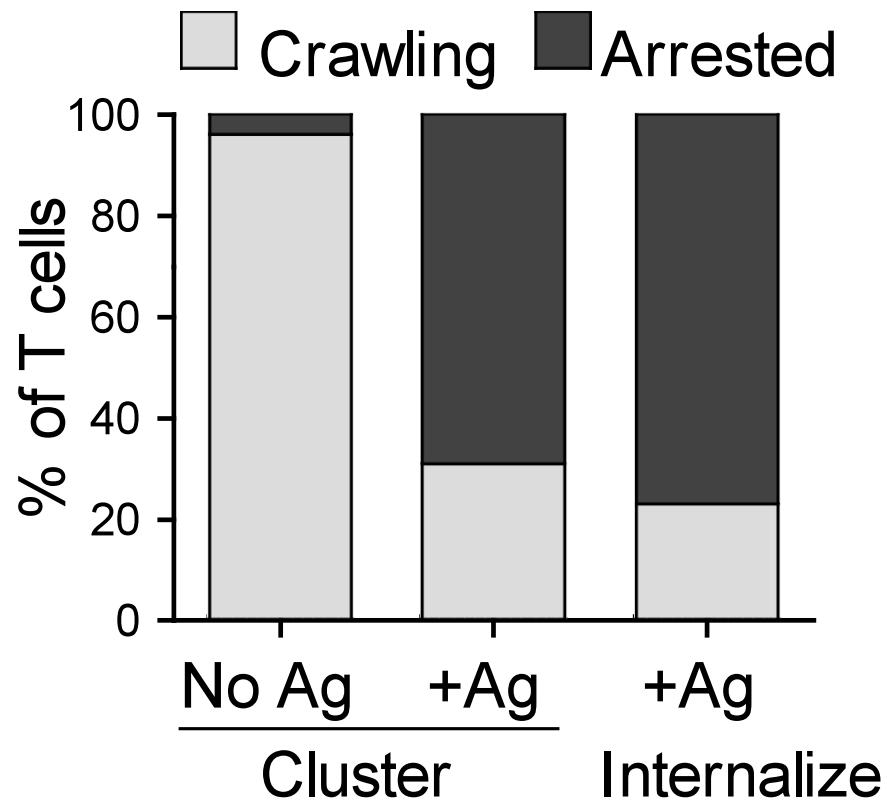
TCR CMTMR



In vivo TCR clustering and internalization

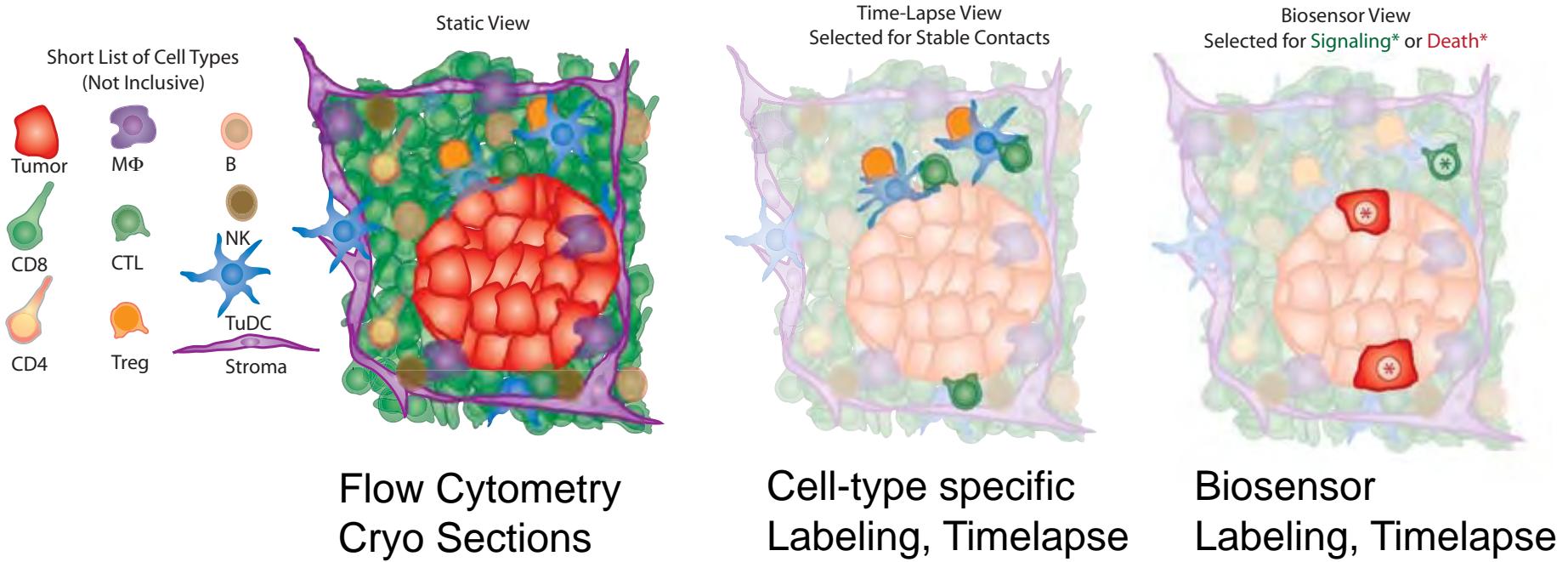


Arrest is Not Required for TCR signaling as assessed by internalization



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