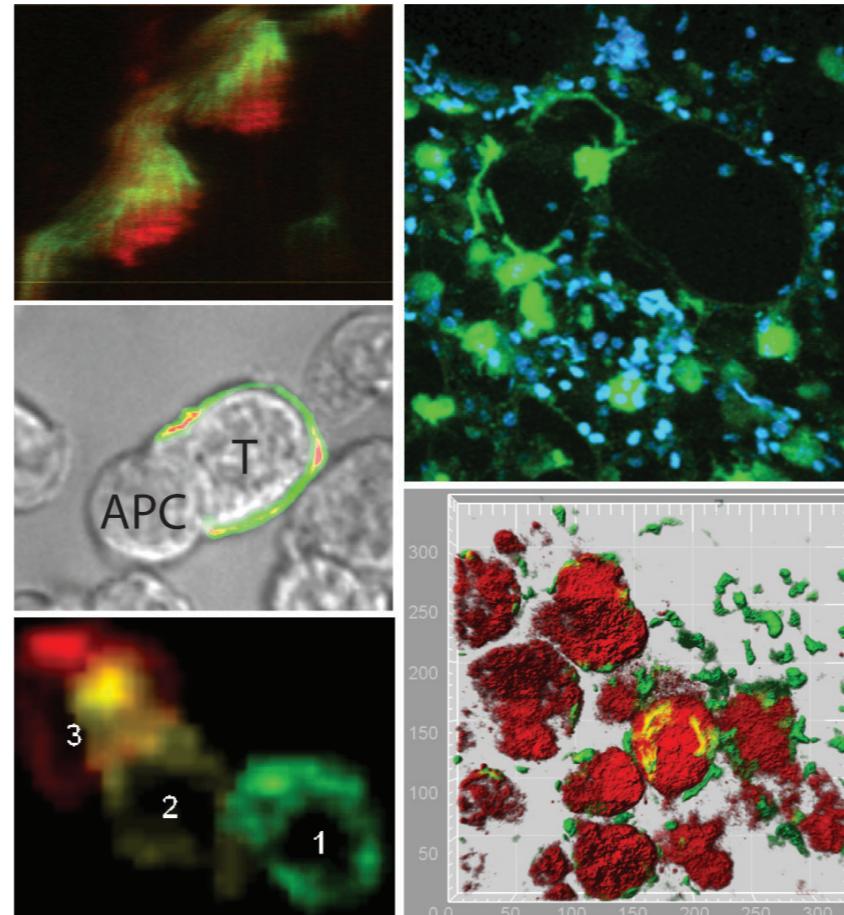
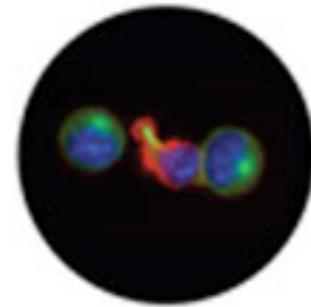


# Principles, Benefits, and Applications of Multiphoton Microscopy

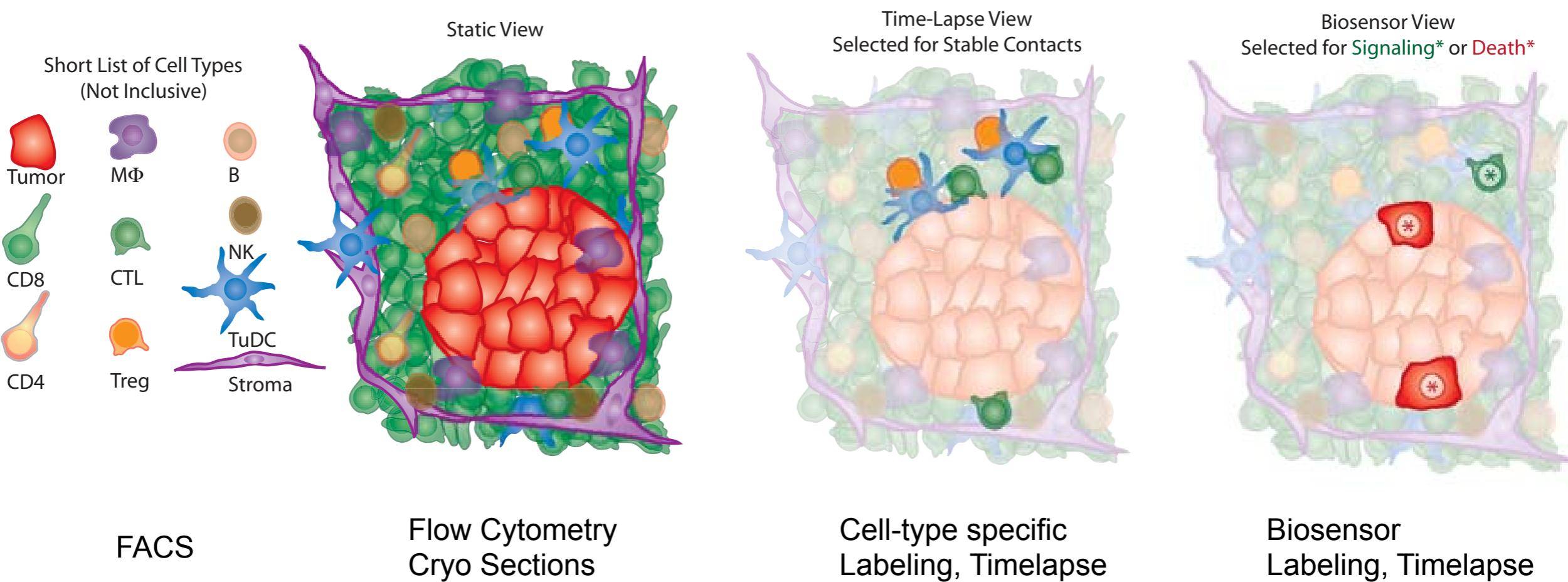


Bijan Boldajipour, PhD  
Krummel Lab, Department of Pathology



Biological  
Imaging  
Development  
Center  
at  
**UCSF**  
University of California  
San Francisco

# Live Imaging to Identify Significant Systems Events in Complex Tissues



# Issues with Imaging in Complex Tissues

“2-Photon Microscopy”

Also commonly

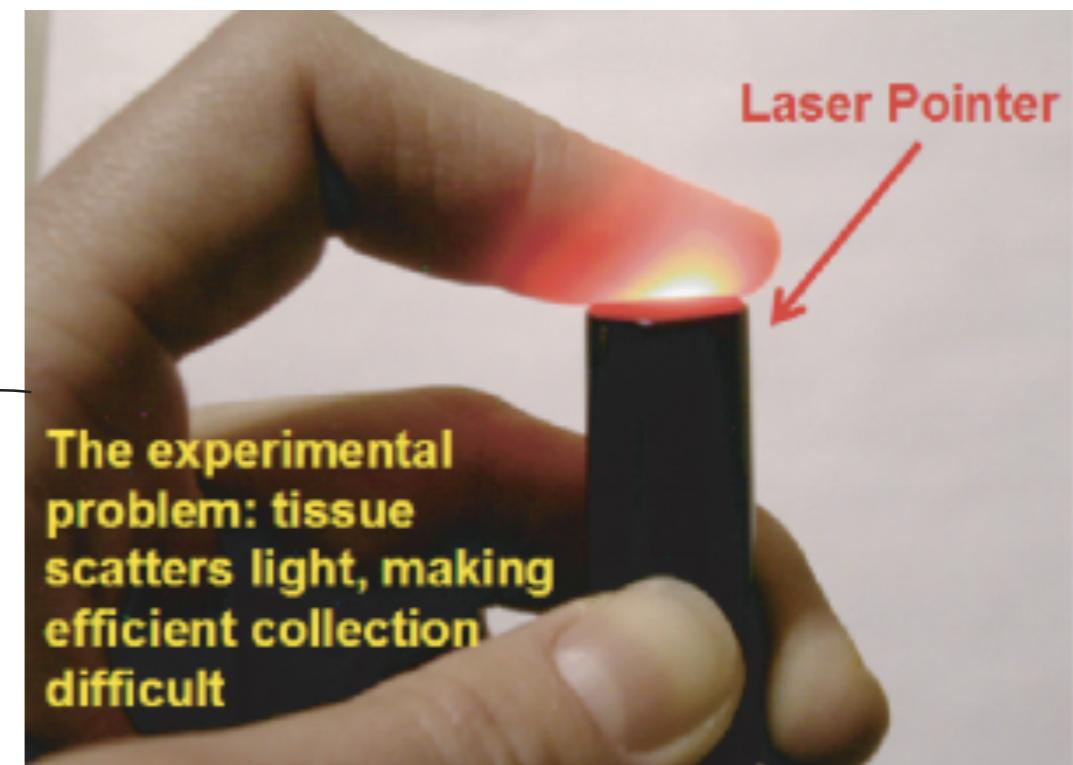
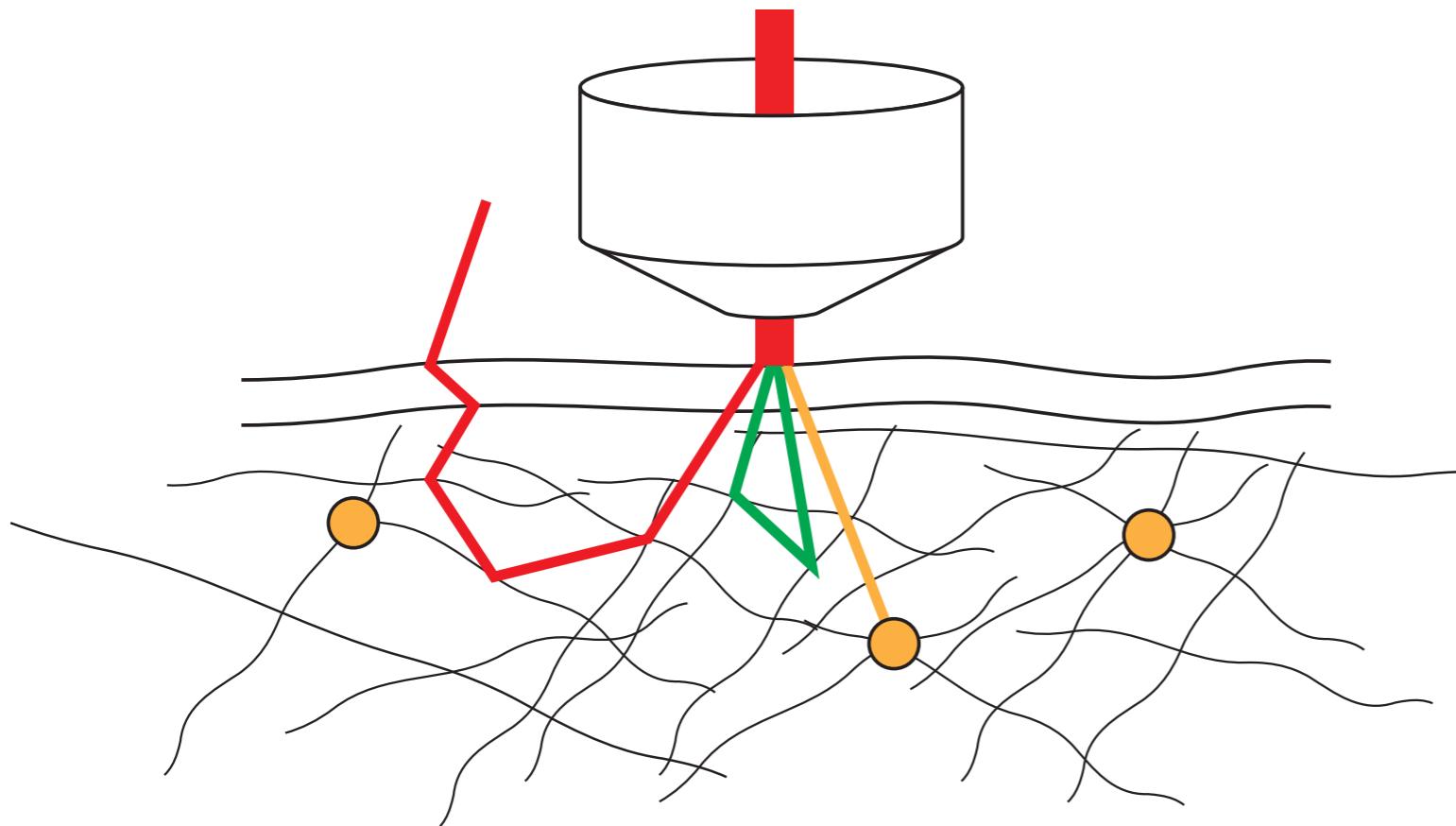
“Multiphoton Microscopy”

# Lecture Outline

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

## 1. The Root Reason for 2P Microscopy

# Tissues absorb and scatter light

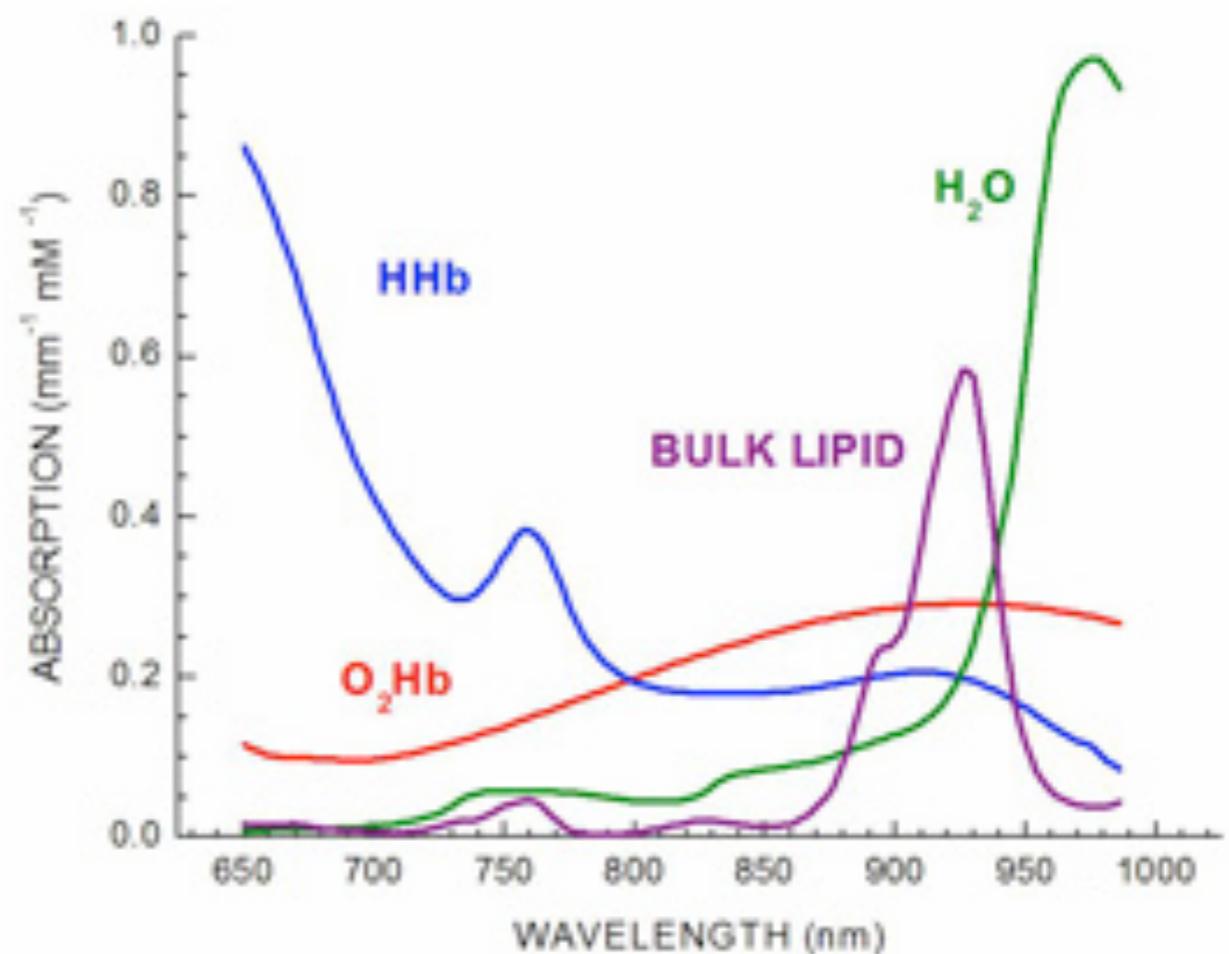
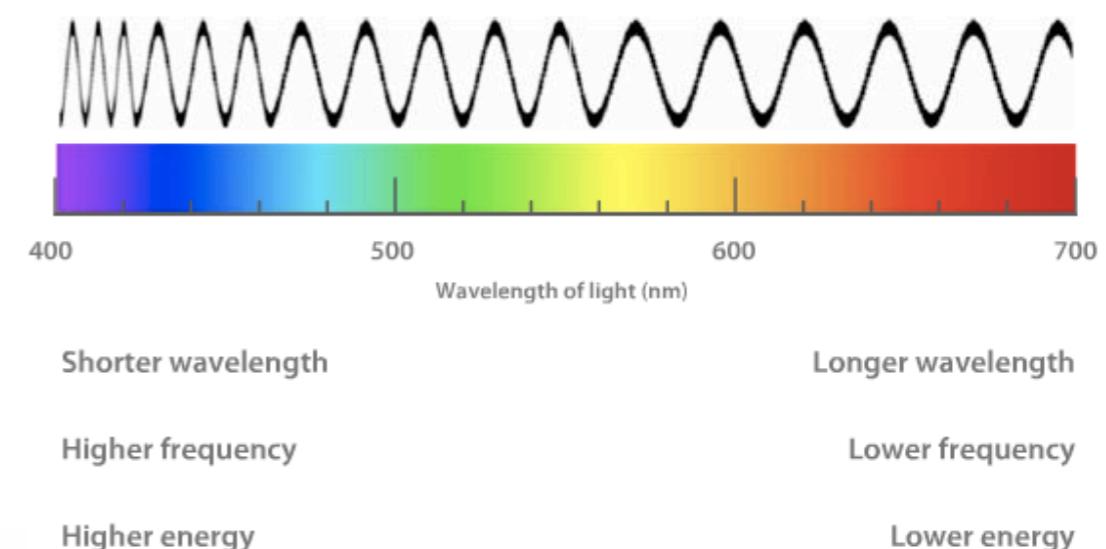


- elastic scattering: single/multiple
- absorption

## 1. The Root Reason for 2P Microscopy

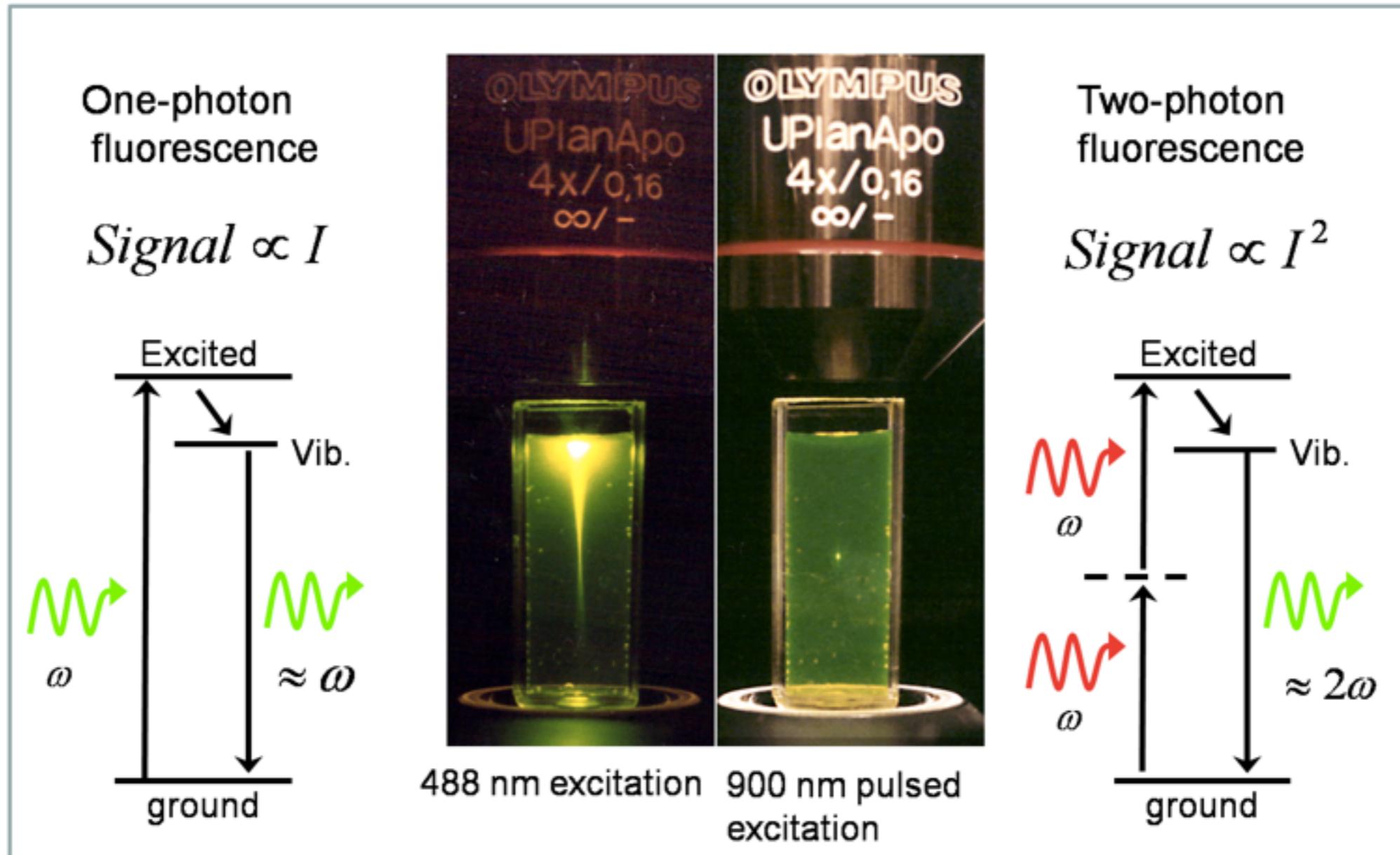
# Avoiding Tissue absorption

- Different tissue components absorb at different wavelength
- A good optical window exists 700nm-1000nm with lowest tissue absorption
- Can this range be used without developing a new set of dyes and tools?



## 2. The Principle

# 2-Photon excitation: How and Why



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

### 3. Fluorophore excitation and the 2nd Harmonic

# 2P excitation: the $2\lambda$ rule

- Quantum mechanics:  
Most dyes do not optimally excite exactly at  $2\lambda$  of their single-photon excitation
- Difficult to predict

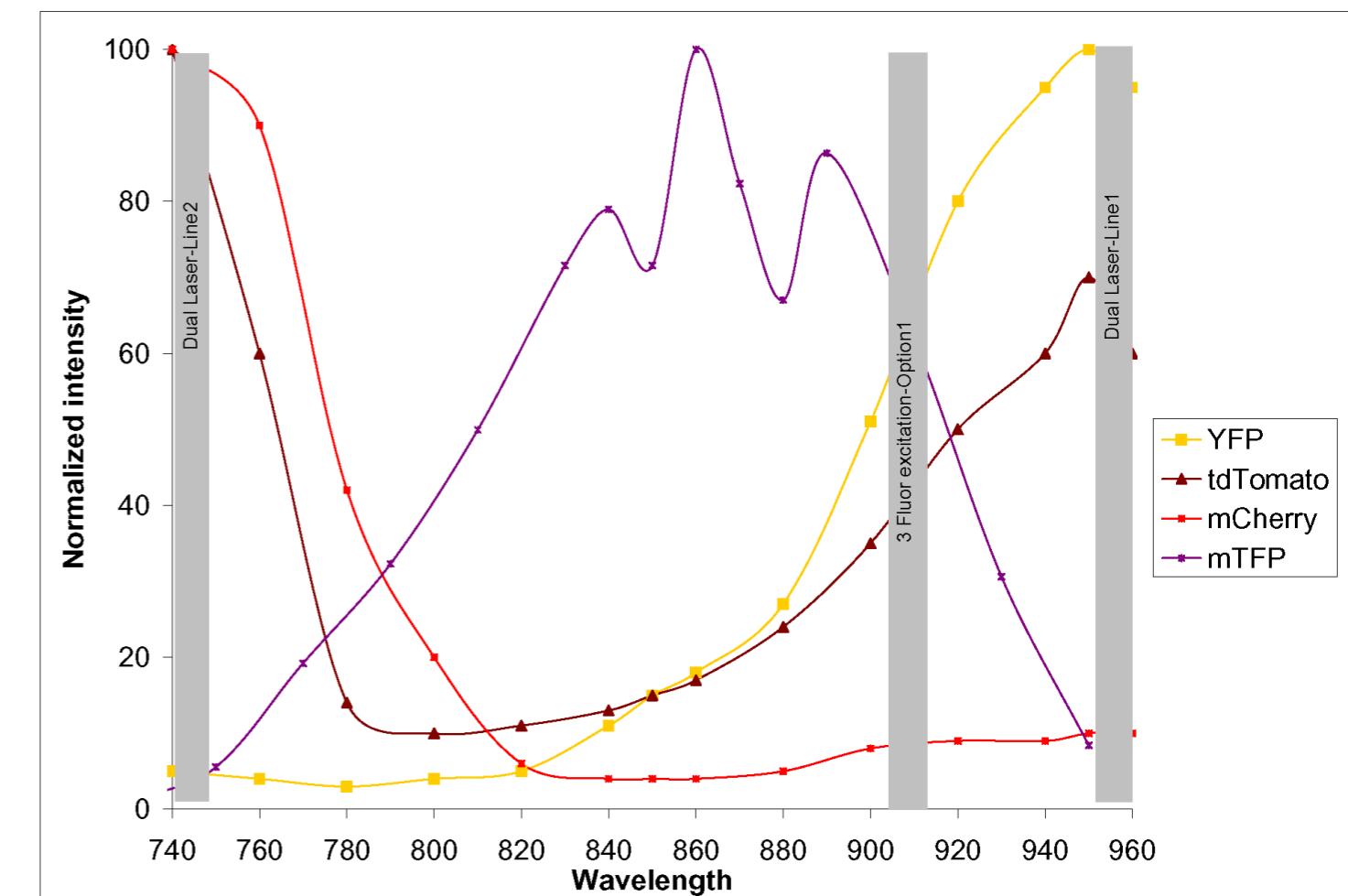
**Table 1. Selected applications of fluorescent probes for two-photon excitation (TPE) microscopy**

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	$\text{Ca}^{2+}$ -insensitive, neuronal tracer *	<i>Neuron</i> (2002) 33: 439–452; <a href="http://www.stke.org/cgi/content/full/sigtrans;2004/219/pl5">www.stke.org/cgi/content/full/sigtrans;2004/219/pl5</a>
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 <sub>2</sub> 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 $\text{Ca}^{2+}$ concentration dynamics in dendrites and dendritic spines	<i>Neuron</i> (2002) 33:439–452; <a href="http://www.stke.org/cgi/content/full/sigtrans;2004/219/pl5">www.stke.org/cgi/content/full/sigtrans;2004/219/pl5</a>
Fura-2	780 nm	Detection of GABA-mediated $\text{Ca}^{2+}$ transients in rat cerebellar Purkinje neurons	<i>J Physiol</i> (2001) 536:429–437

### 3. Fluorophore excitation and the 2nd Harmonic

# 2P excitation: the $2\lambda$ rule

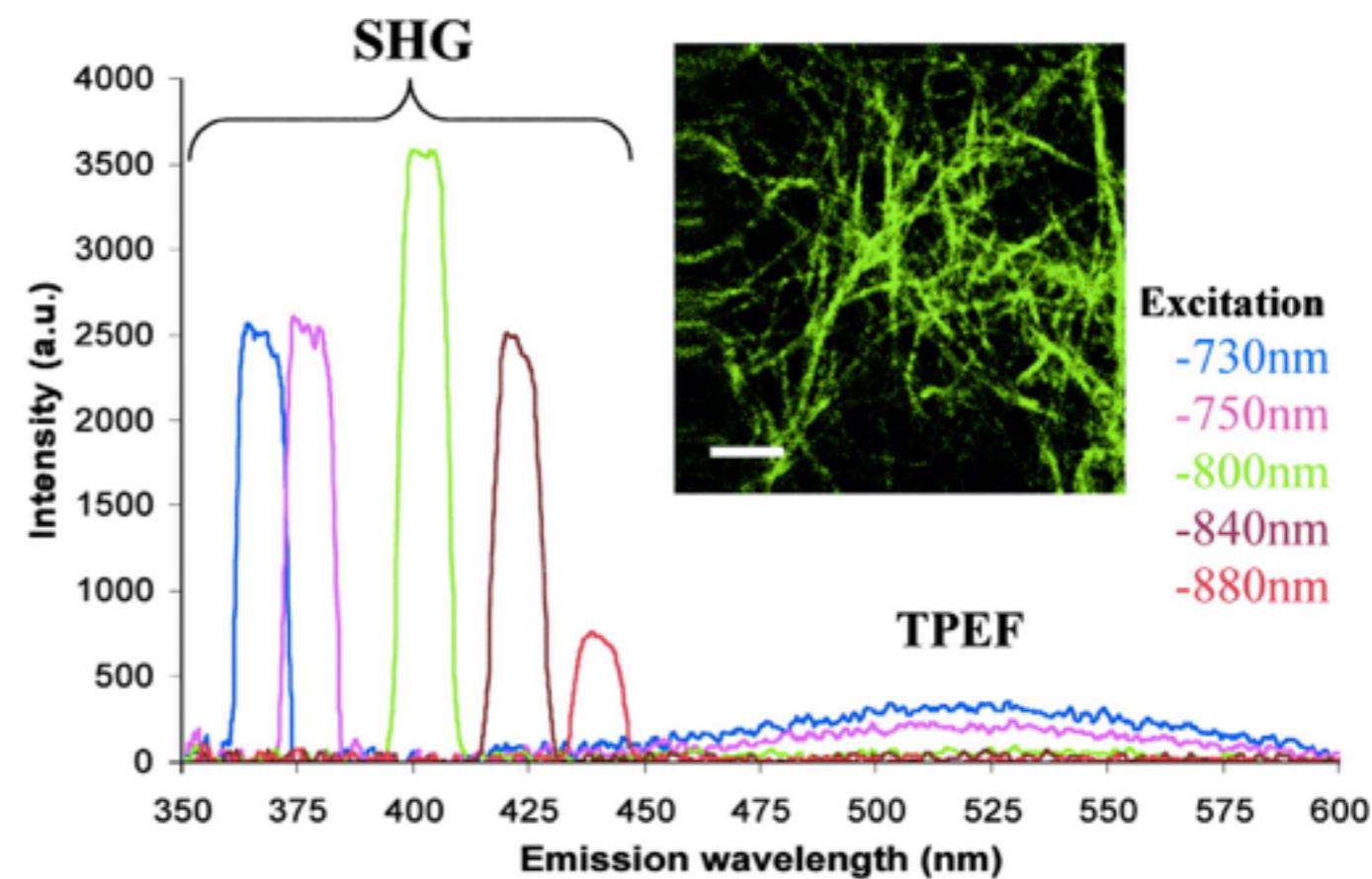
- Doubling of excitation broadens excitation range: many dyes can be hit by same wavelength
- Exceptions to  $2\lambda$  rule: Some fluors with similar emission can be distinguished by different excitation (eg FITC/GFP, GFP/YFP, tdTomato/mCherry)



### 3. Fluorophore excitation and the 2nd Harmonic

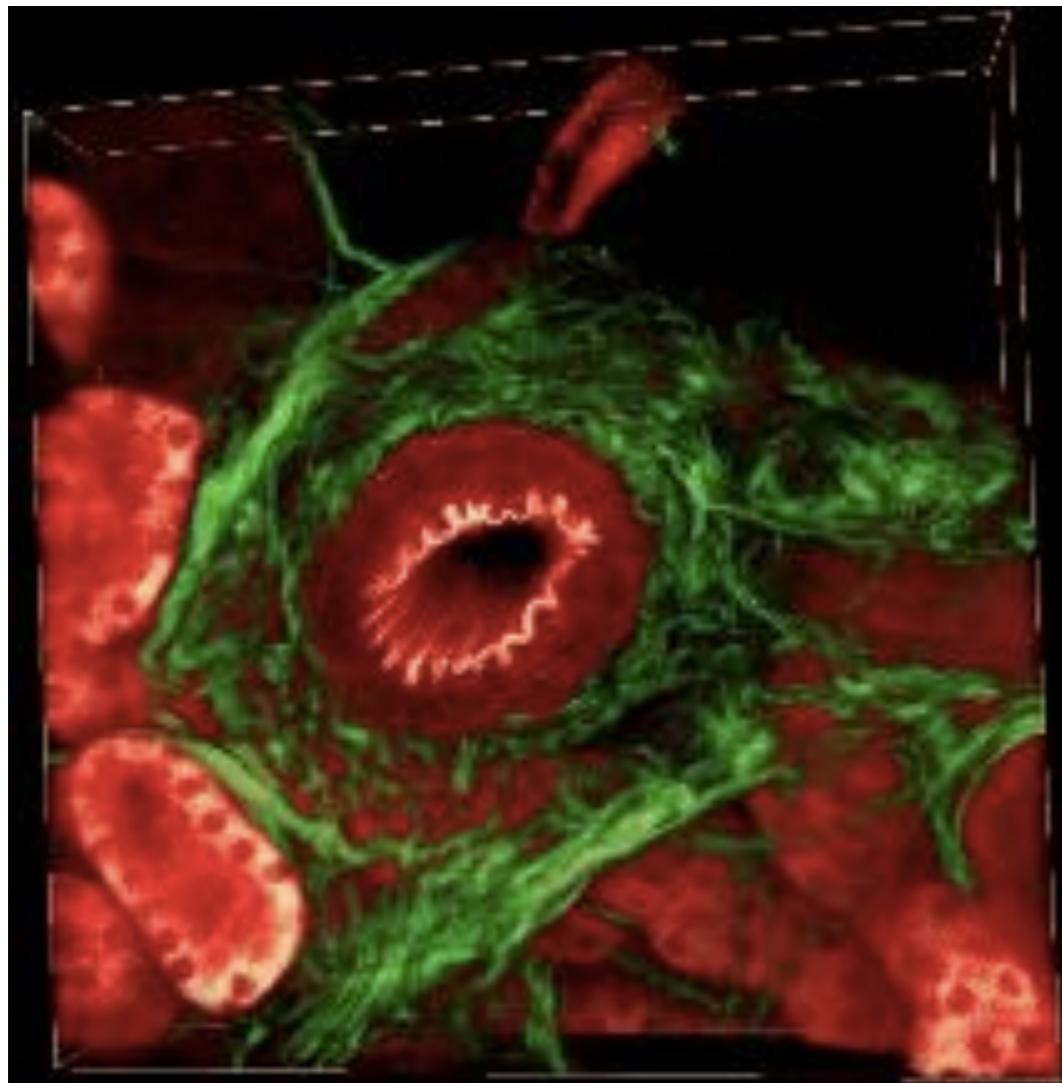
# 2nd Harmonic signals

- 2nd Harmonic generation: Two photons of same frequency interact with a non-linear material and are combined into one photon of twice the energy (double frequency or half the wavelength)
- Only non-centrosymmetric molecules (e.g. helical fibers) can do this: Collagen, Cellulose, some membrane dyes
- These molecules light up at  $\lambda/2$  of the excitation wavelength

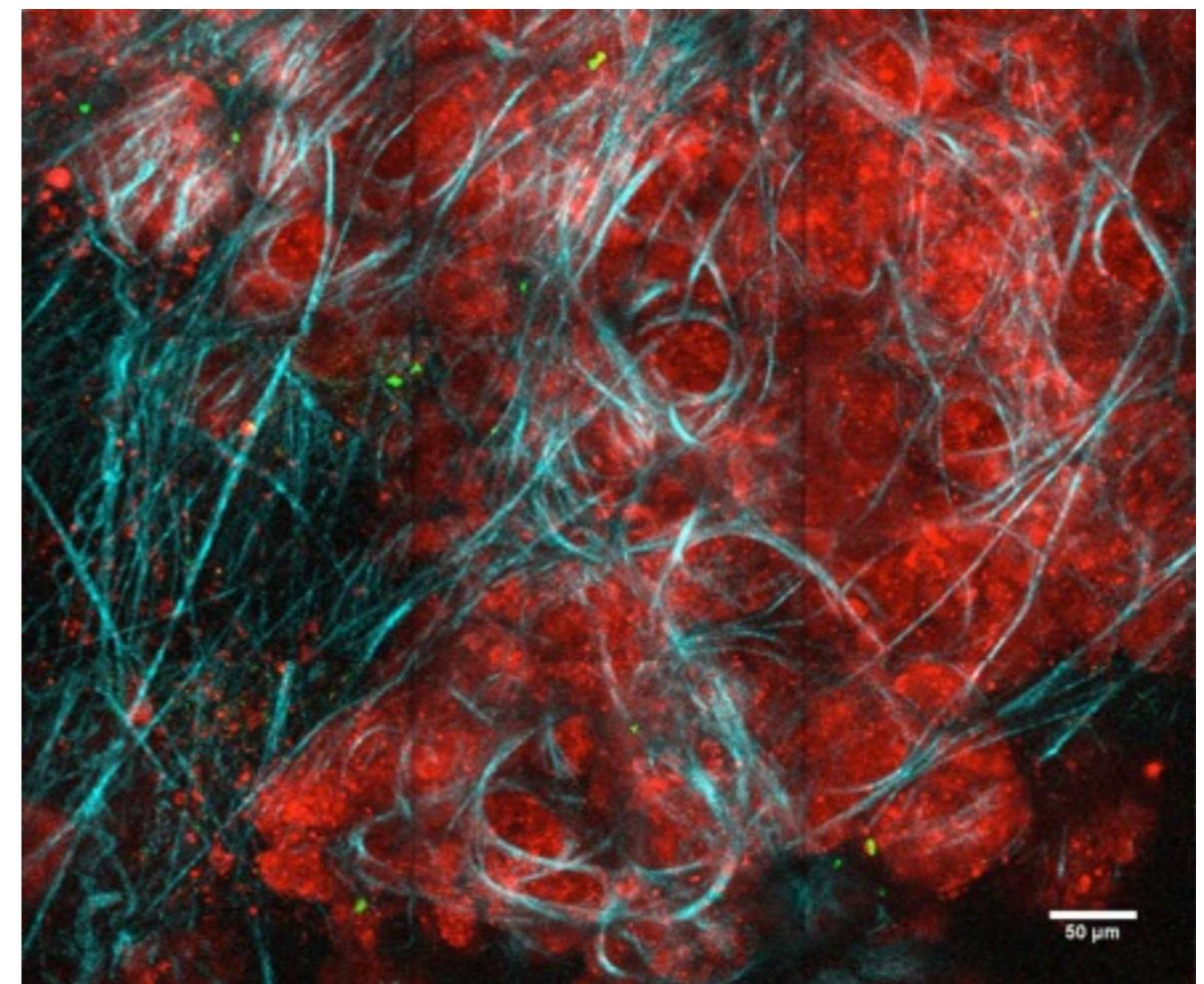


### 3. Fluorophore excitation and the 2nd Harmonic

# 2nd Harmonic signals



fibrotic murine kidney  
Laboratory for Optics & Biosciences  
École Polytechnique, France



collagen in breast tumors  
Krummel Lab, UCSF

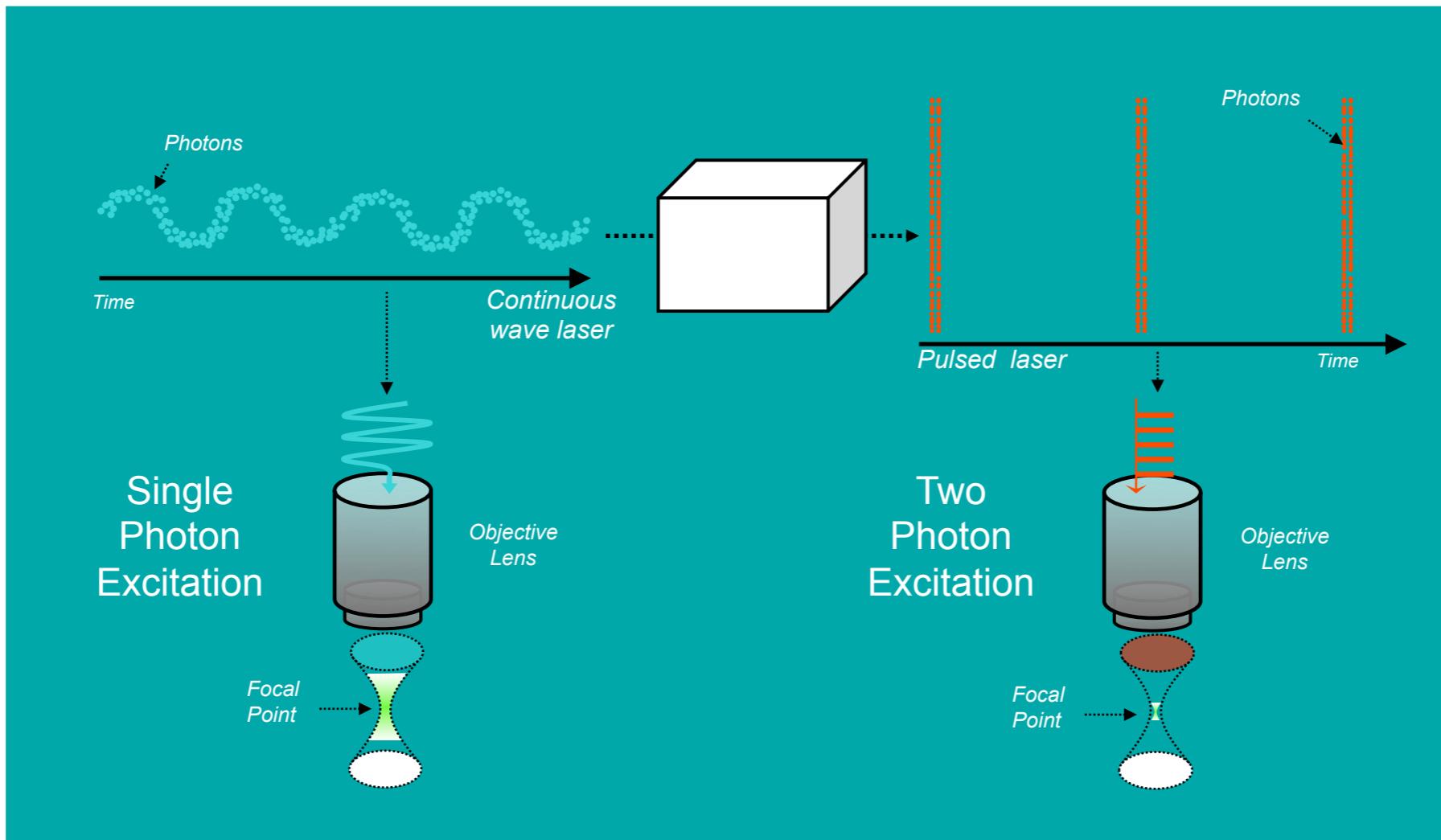
#### 4. That's a Mighty Big Laser You've Got There

# 2P excitation requirements

- 2P occurs only when two photons coincide in time and space (simultaneous absorption with <0.5fs)  
→ laser with extremely high photon density required
- Why can't we use a regular continuous wave laser as we do in confocal microscopy?

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

# Pulsed lasers and power



- Current Ti:Sapphire lasers: Tunable (720-1100nm), expanded emission with optical parametrical oscillators (OPO)
- Cost is \$100k-250k → commercial setups \$450k-700k

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

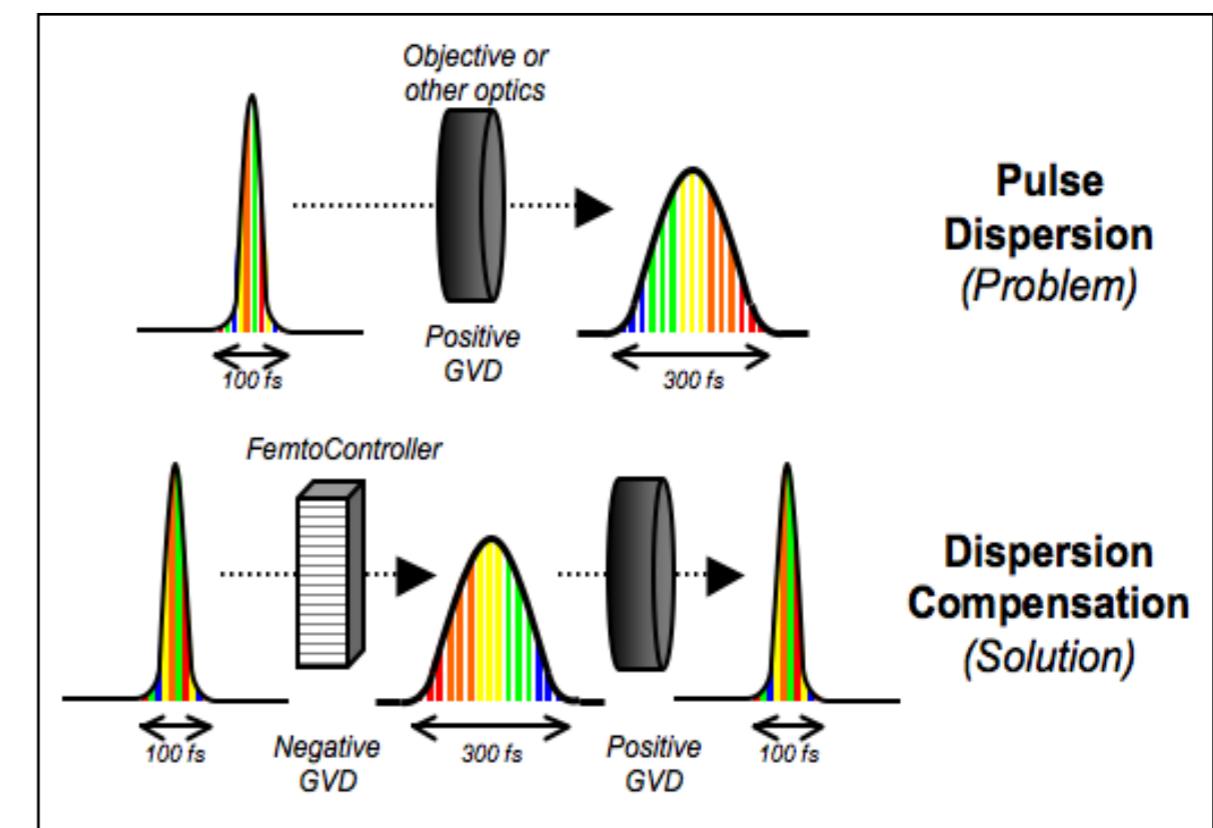
# Pulsed lasers and power

- A Soldering Iron typically consumes/concentrates 25W of power to an area approx. 2mm in diameter giving a power density of **~8W/mm<sup>2</sup>**
- 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 **~5MW/mm<sup>2</sup>**
- However it has a duty cycle of  $\sim 10^{-5}$  (the ‘duty cycle’ is pulselwidth x rep rate). Typical pulselwidths 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  $5 \times 10^{11}$ W/mm<sup>2</sup> or **~10<sup>11</sup> times the power density compared to a soldering iron.**

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

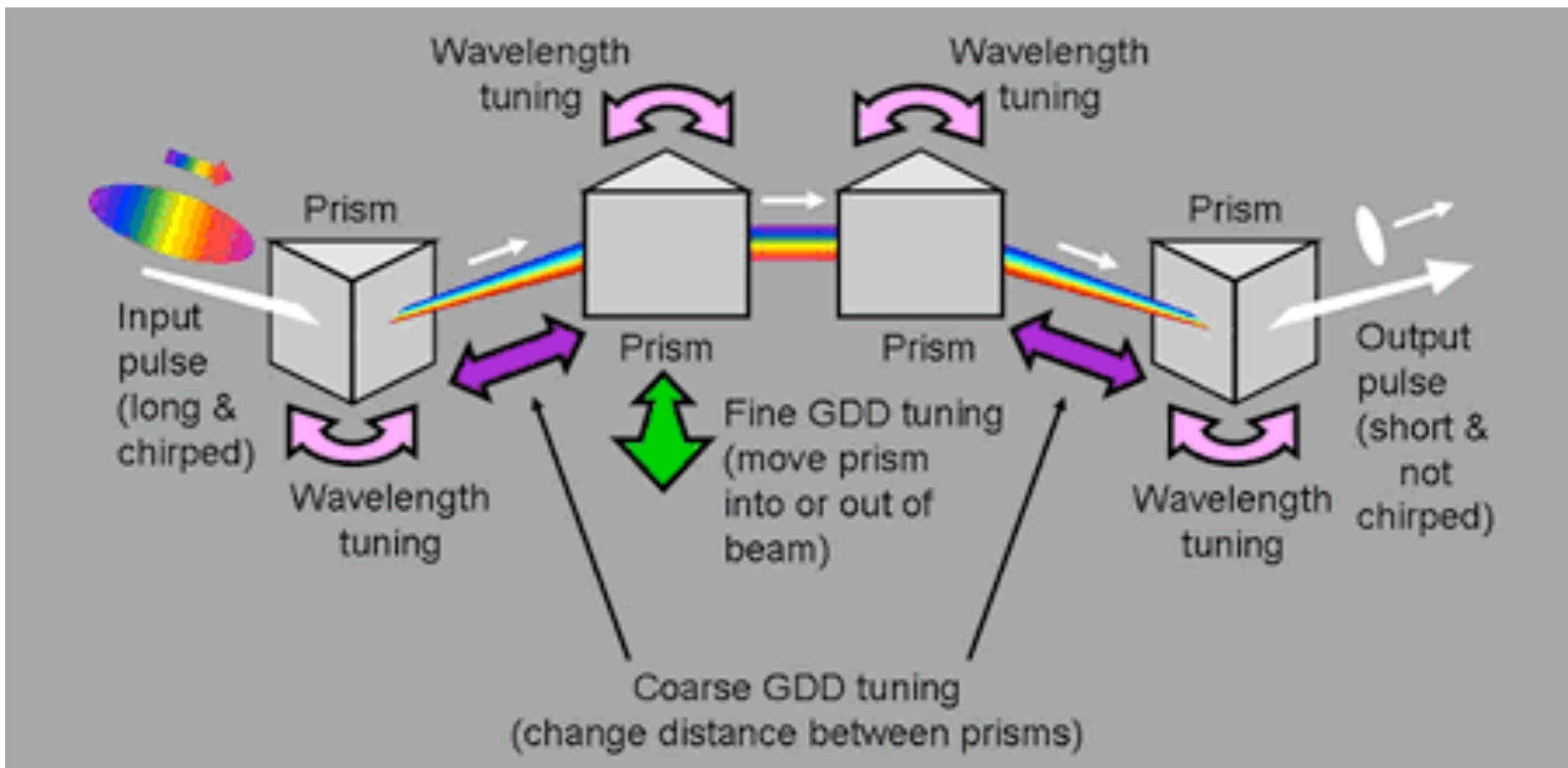
# Pulsed laser dispersion

- When light passes through optic materials there will be dispersion: short pulses become longer
- Pulse dispersion in 2P microscopy means less efficient 2P excitation
- Compensation of pulse dispersion is known as “chirping”
- Lasers can either be “pre-chirped” (MaiTai ‘DeepSee’ and Chameleon ‘Vision II’) or a component can be added into the light beam path (APE FemtoController)



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

# Pulsed laser dispersion



## 5. Non-Descanning Detection

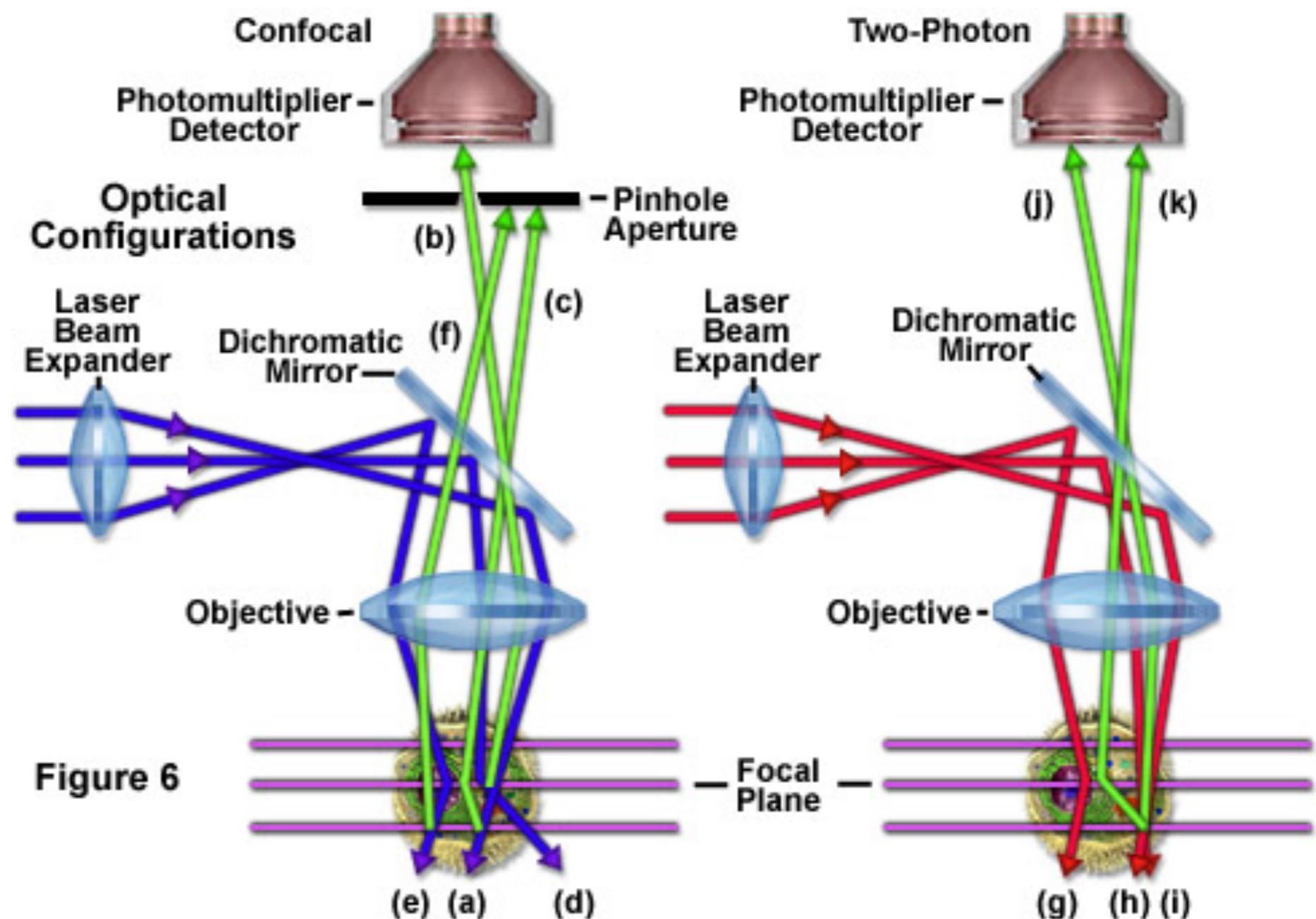
# Fluorescence detection in 2P

- 1P excitation creates cone of light → elimination of out-of-focus excitation using pinhole before detection PMT
- 2P excitation only illuminates at focal plane → no need to eliminated scattered light
- BUT: Point illumination → time-resolved scanning detection only

## 5. Non-Descanning Detection

# 2P offers maximum sensitivity

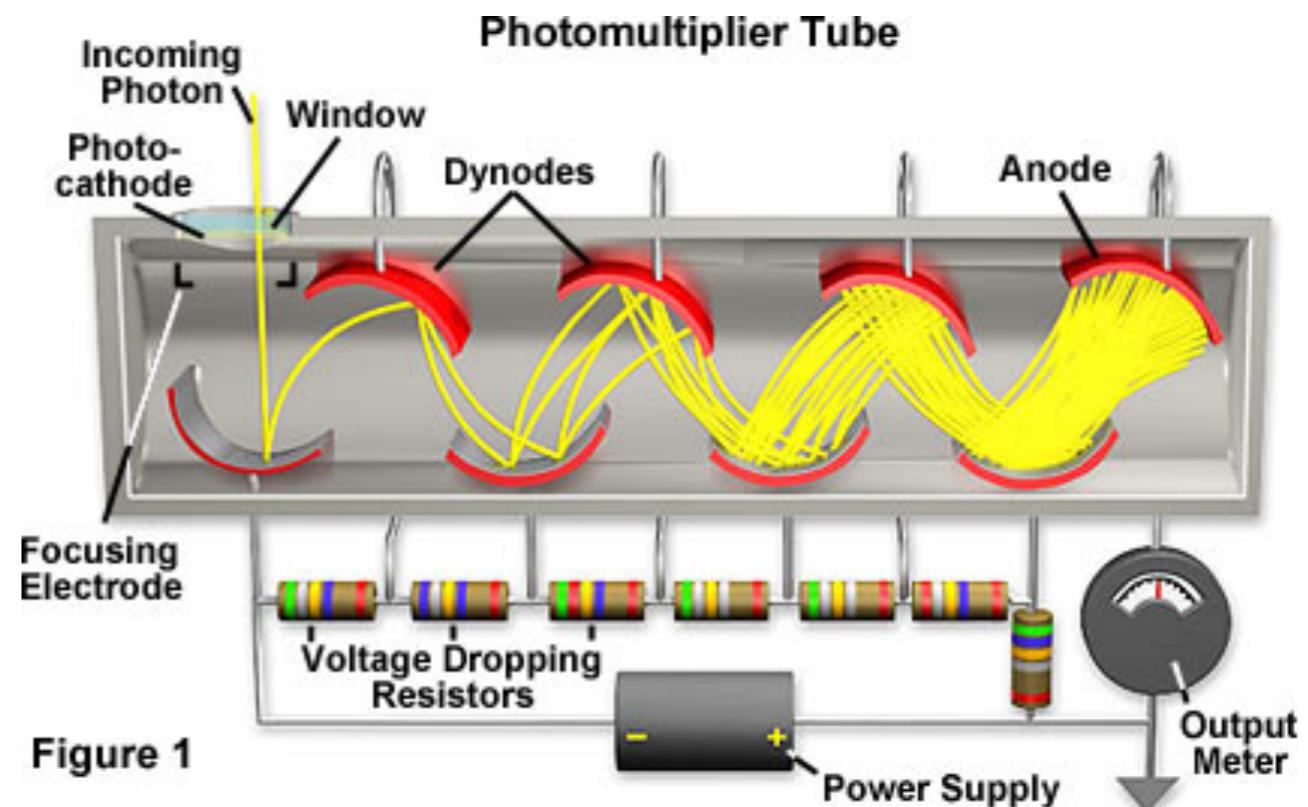
- Excitation can only occur at focal point
- Reduction of light damage in surrounding tissue
- Collection of all emitted light increases sensitivity



## 5. Non-Descanning Detection

# 2P require PMTs as detectors

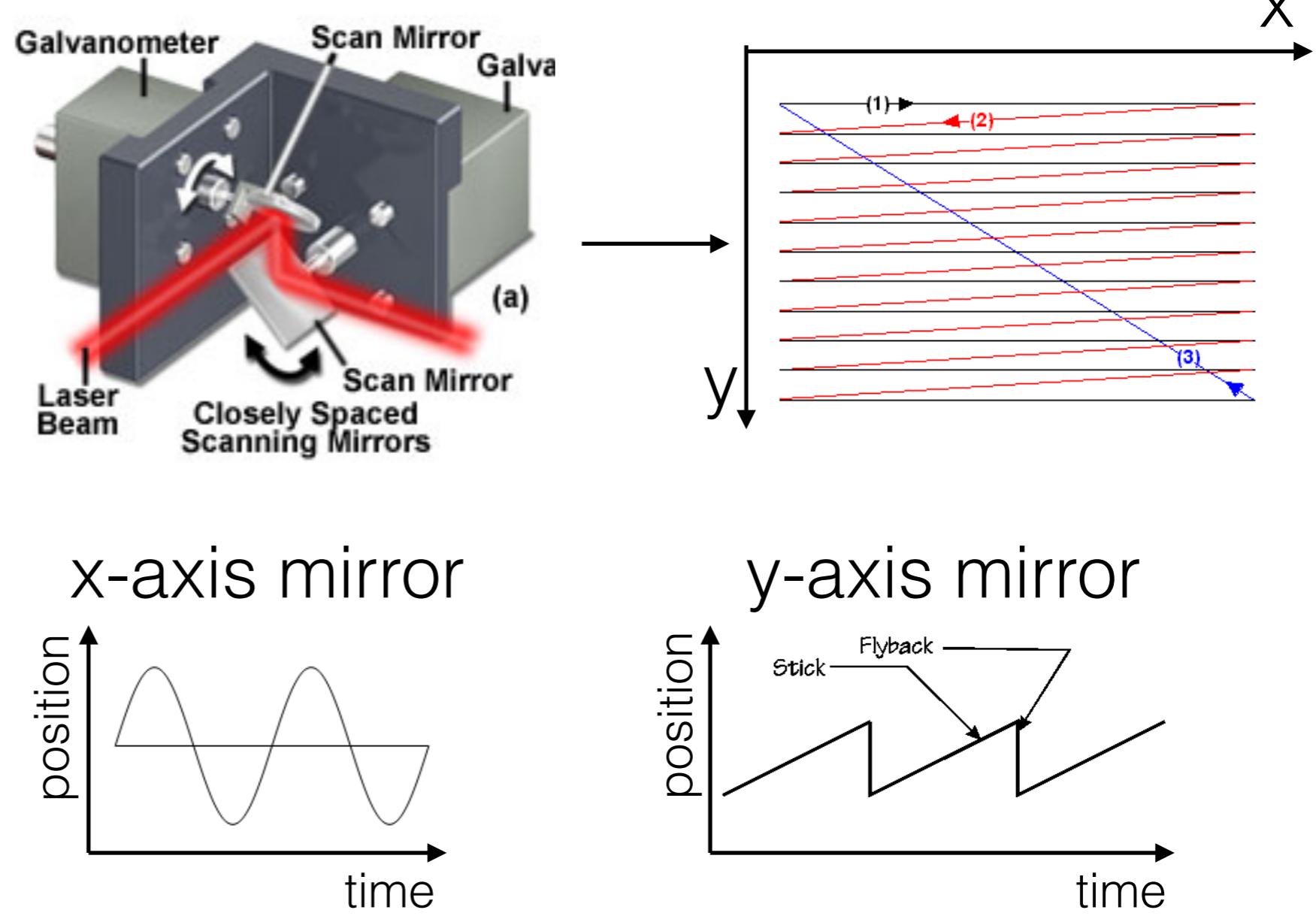
- 2P requires time-resolved detection (no cameras!)
- Highly sensitive PMTs, but noisy at high gain
- Low QE and differential wavelength sensitivity



## 6. Resonant versus Galvo-based Scanning

# Assembling the image

- Focal illumination requires rapid scanning across the specimen
- xy-position on specimen is determined by elapsed time from beginning of scan



## 6. Resonant versus Galvo-based Scanning

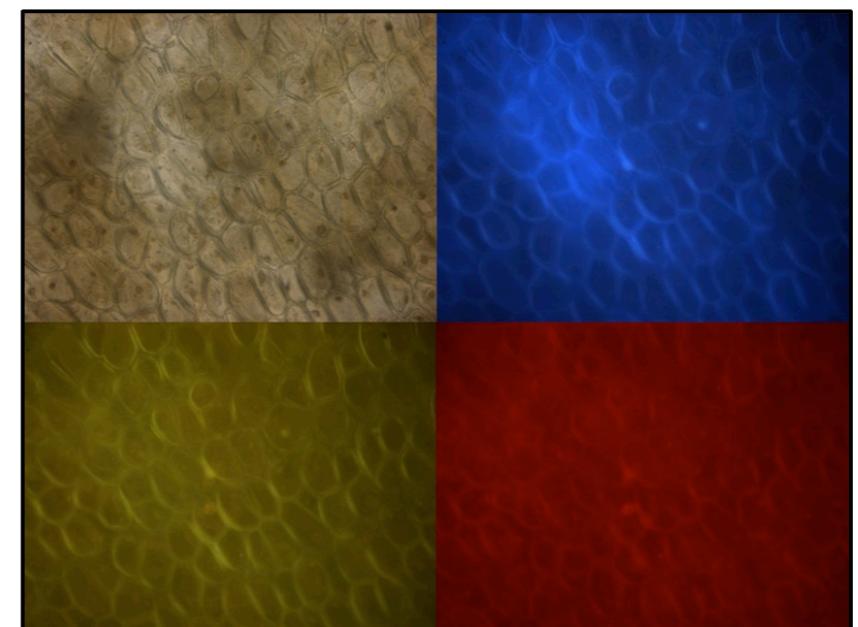
# Resonant vs Galvo Scanning

- 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), 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).

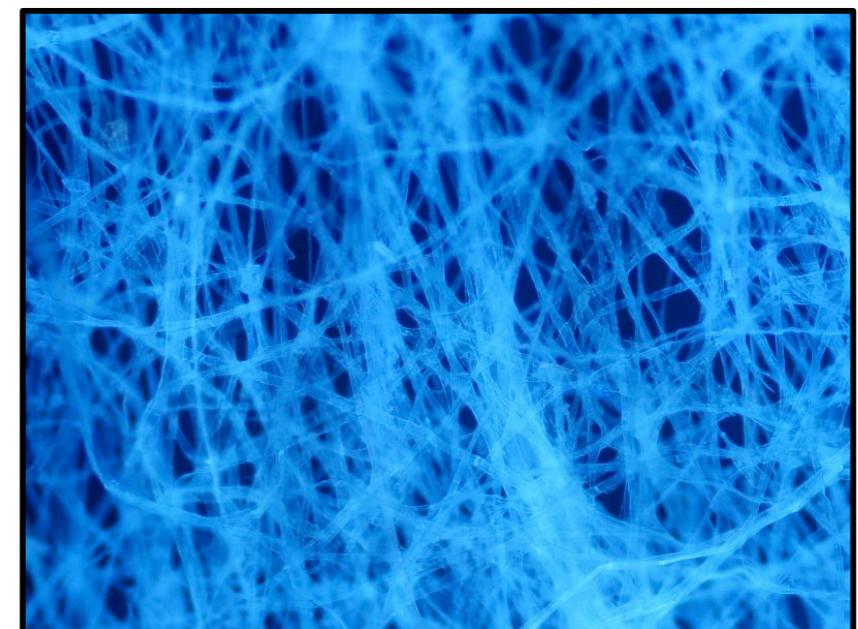
## 7. Autofluorescence - the continual scourge

# Dealing with autofluorescence

- Naturally occurring molecules in specimens gives them 'autofluorescence' in short wavelengths
- To avoid:
  - Use long-wavelength fluorophores (not always possible)
  - Filter out during acquisition (difficult, broad emission spectra)
  - Postprocessing/Linear unmixing
  - Chemically remove it (fixed only)



Banana Autofluorescence (Wikipedia)



Paper Autofluorescence (Wikipedia)

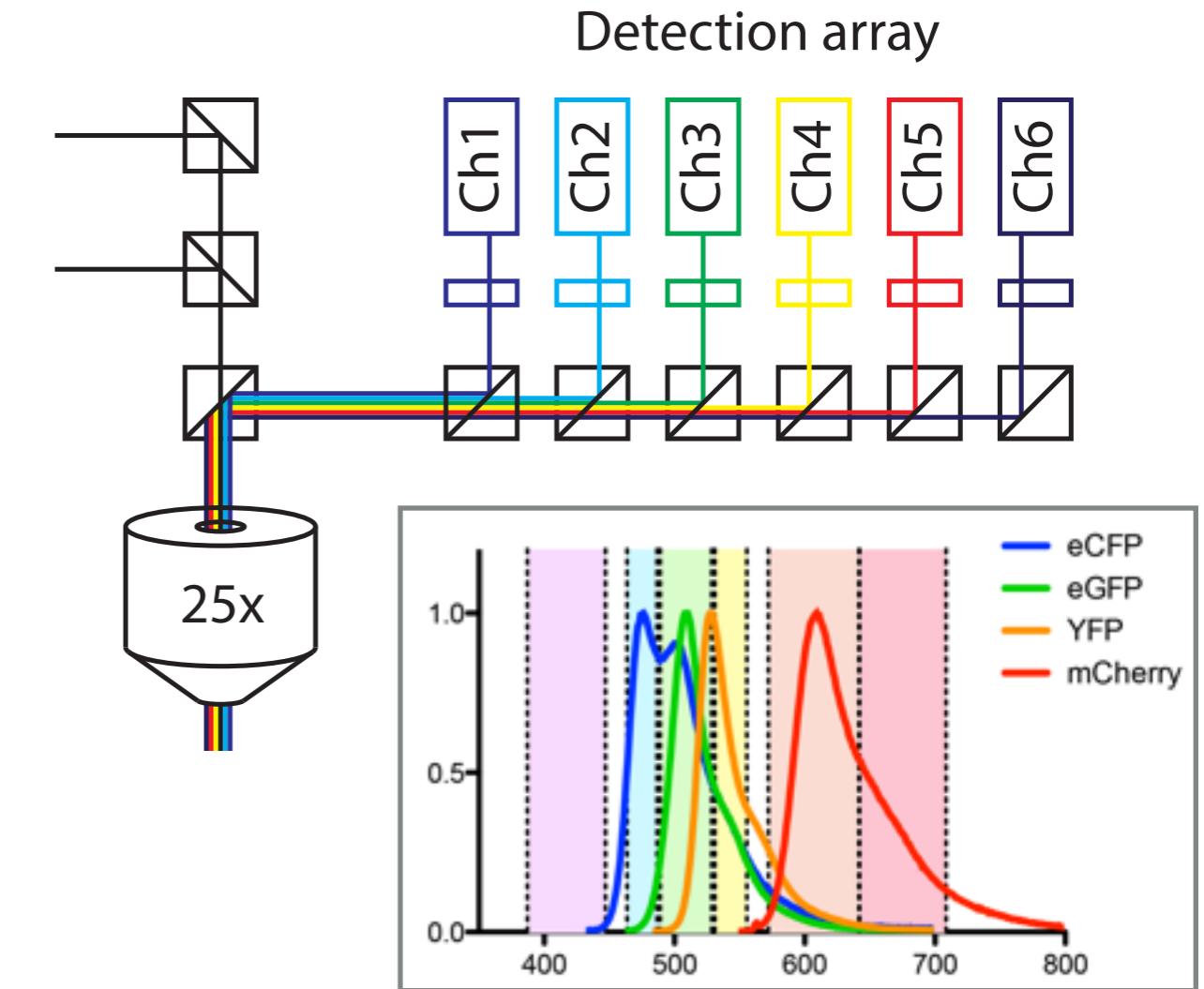
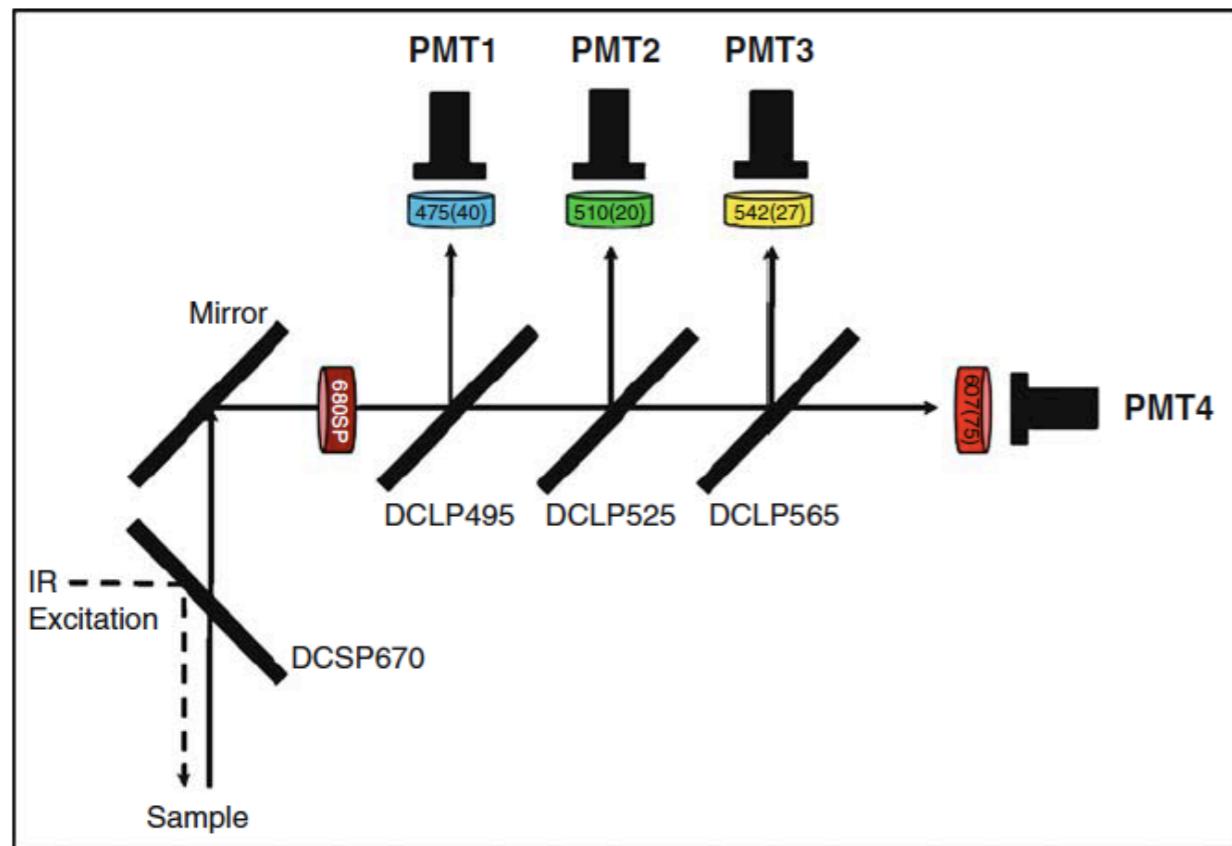
## 7. Autofluorescence - the continual scourge

# Dealing with autofluorescence

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]

## 7. Autofluorescence - the continual scourge

# Acquire more channels and do channel maths ('unmixing')



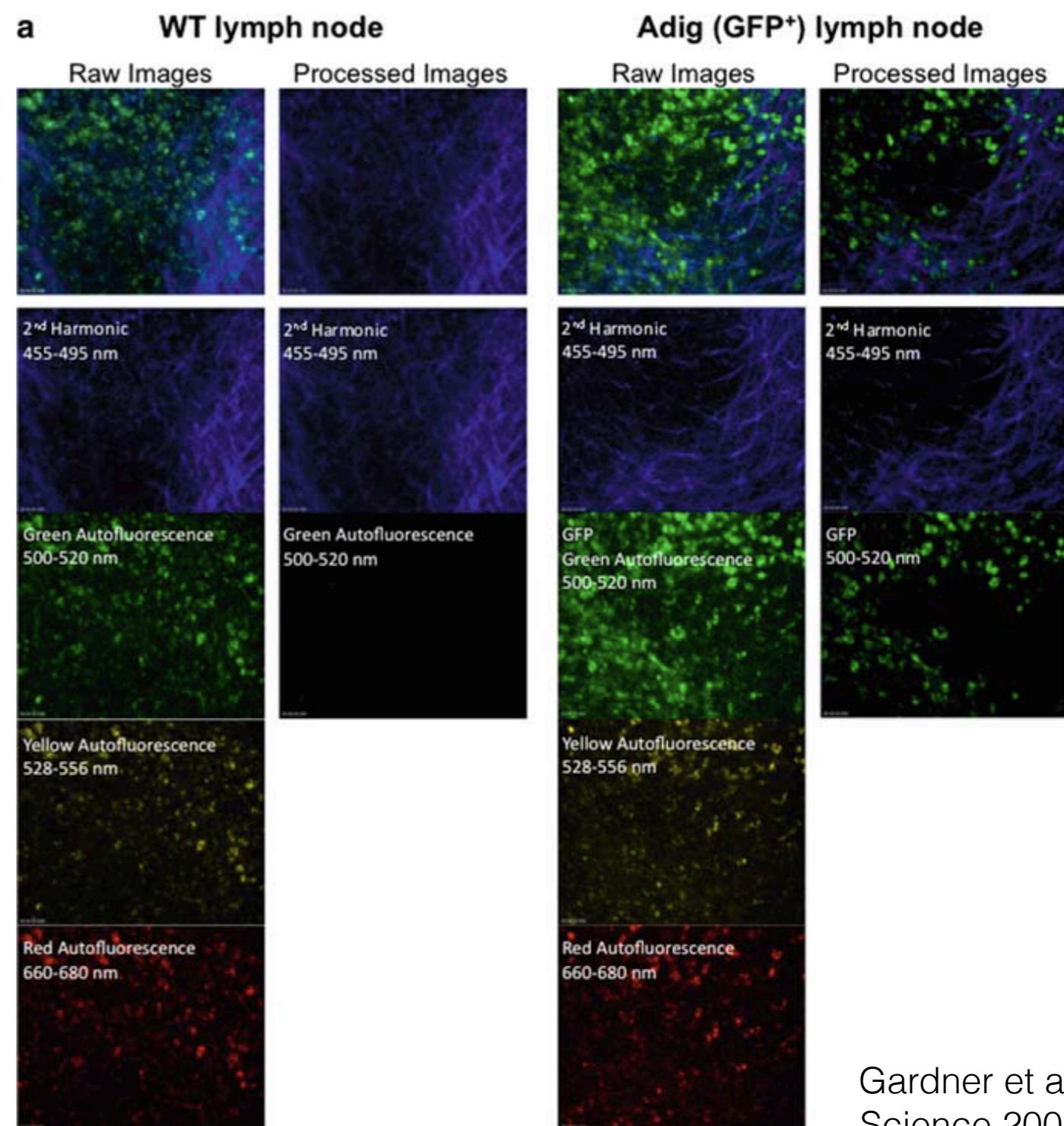
Generation 1

Generation 3

## 7. Autofluorescence - the continual scourge

# Example: Aire+ cells in peripheral tolerance

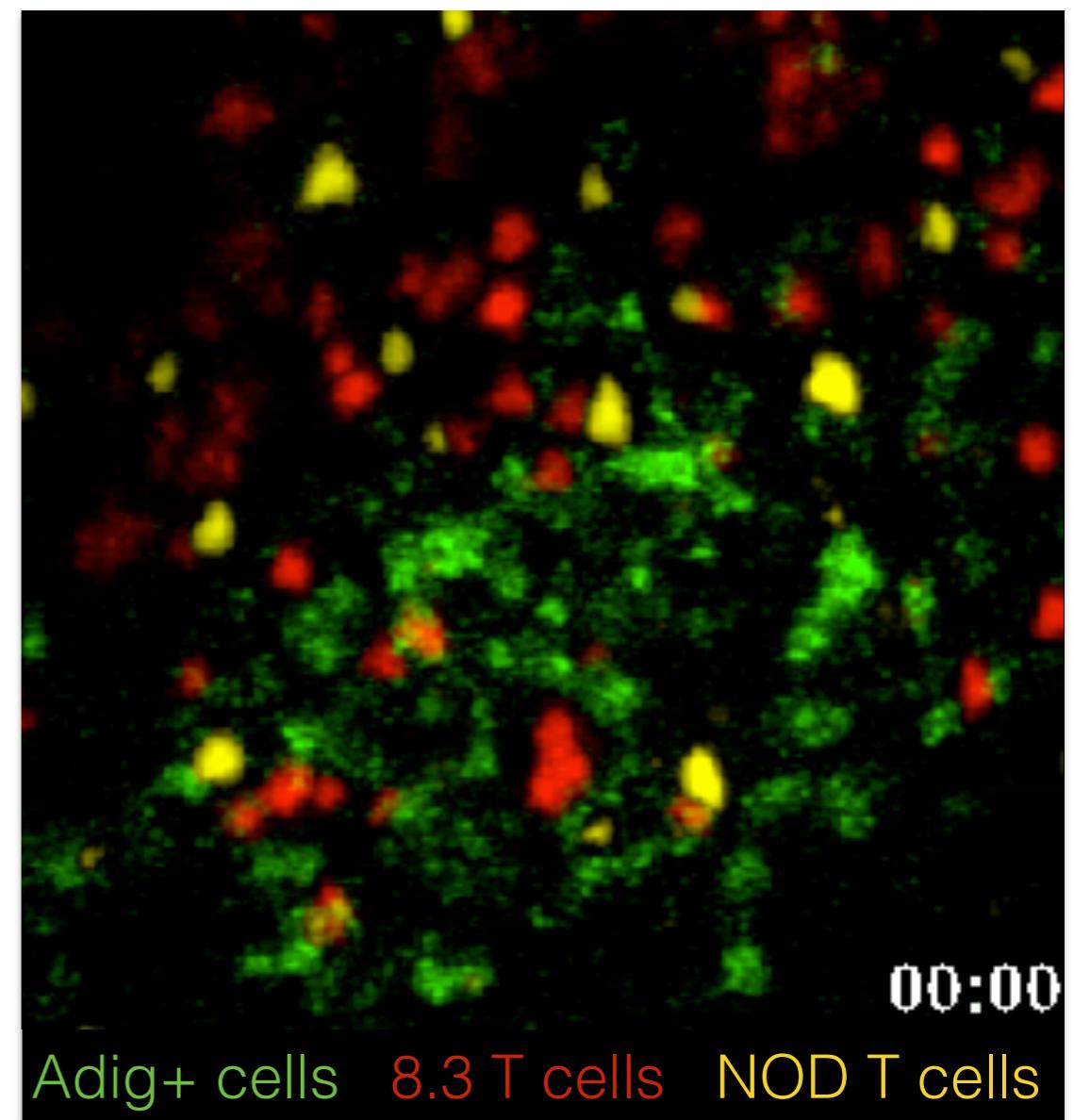
- GFP expression by Aire-expressing cells
- High autofluorescence in all channels prevented identification of cells
- Identification of real GFP:  
Green fluorescence =
  - a × Yellow
  - b × Red



## 7. Autofluorescence - the continual scourge

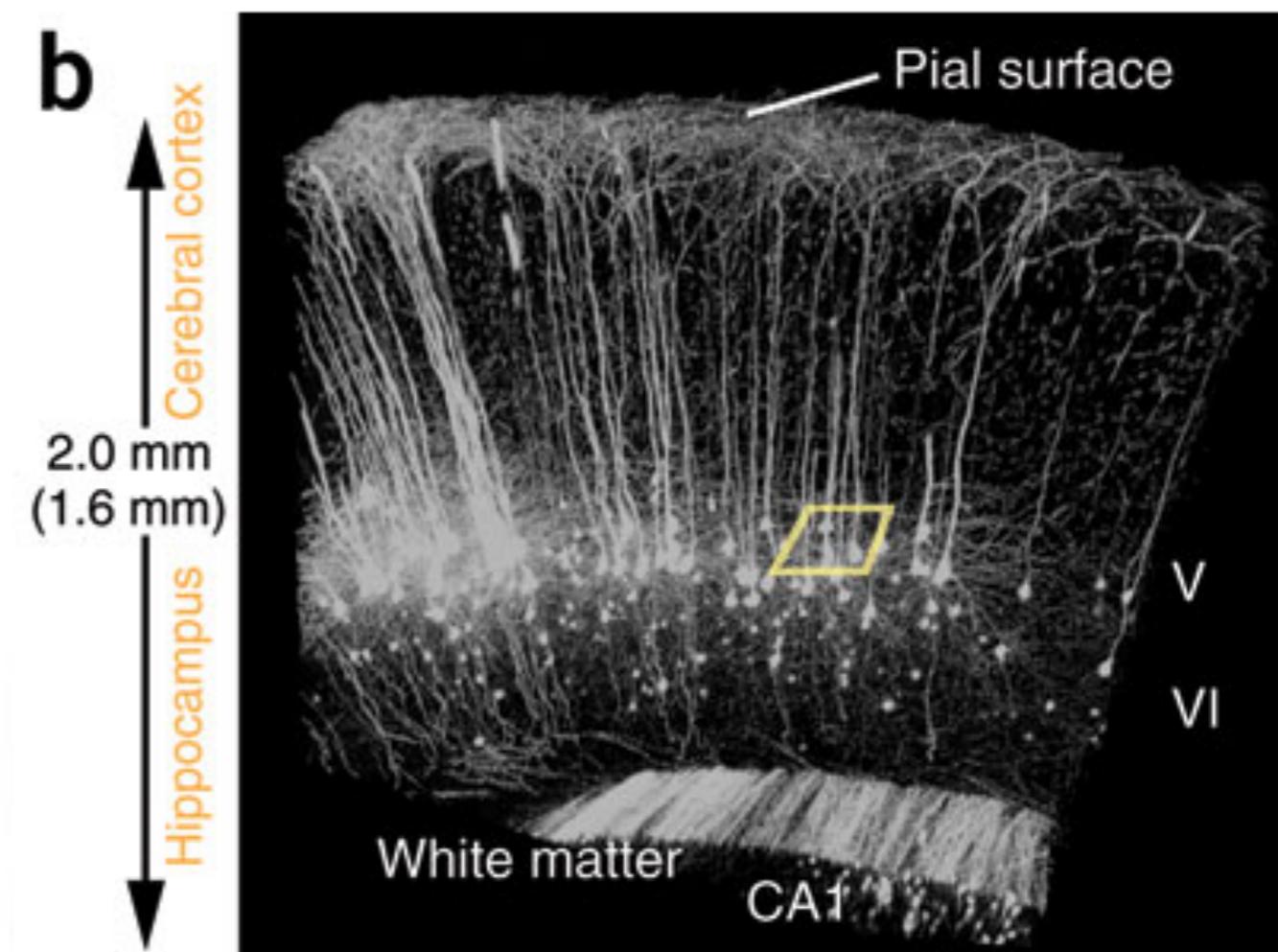
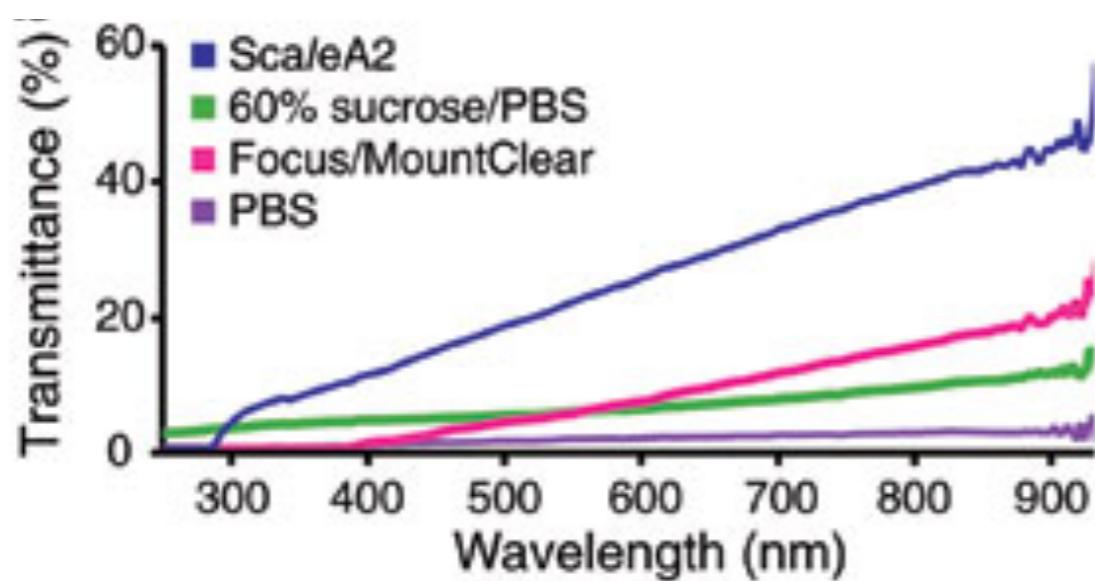
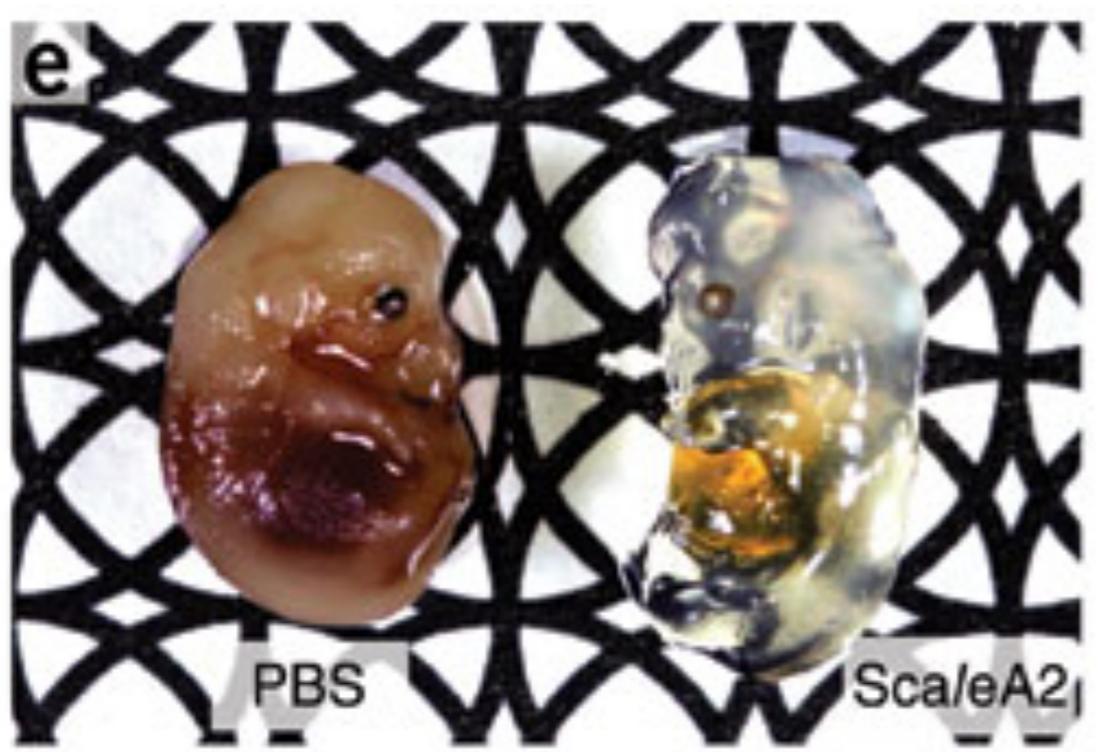
# Example: Aire+ cells in peripheral tolerance

- GFP expression by Aire-expressing cells
- High autofluorescence in all channels prevented identification of cells
- Identification of real GFP:  
Green fluorescence =
  - a  $\times$  Yellow
  - b  $\times$  Red



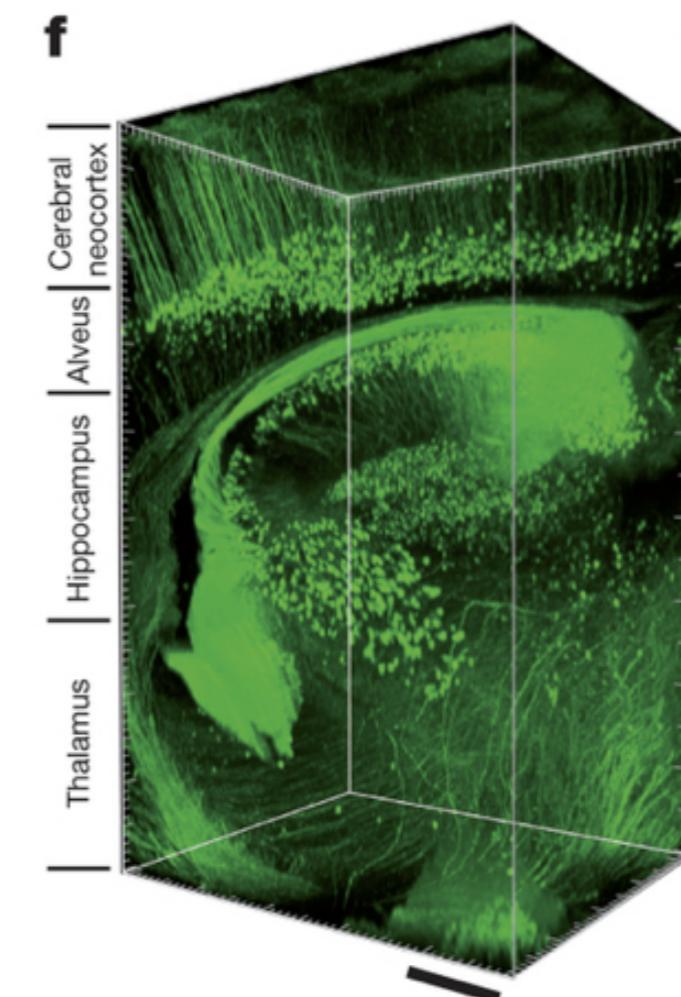
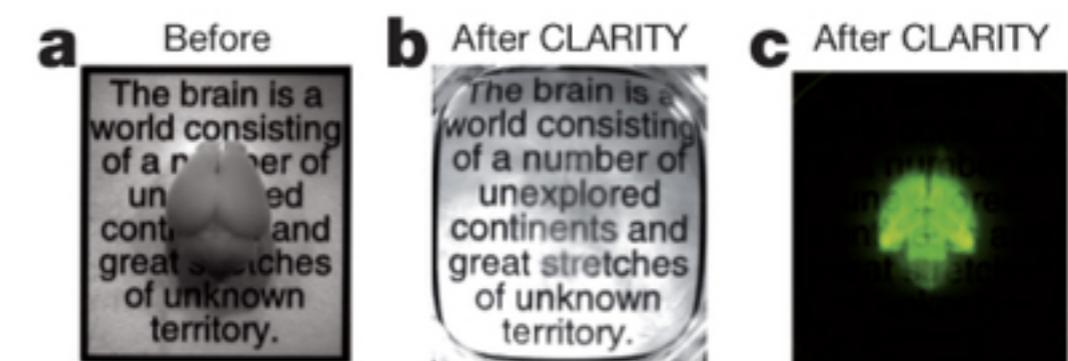
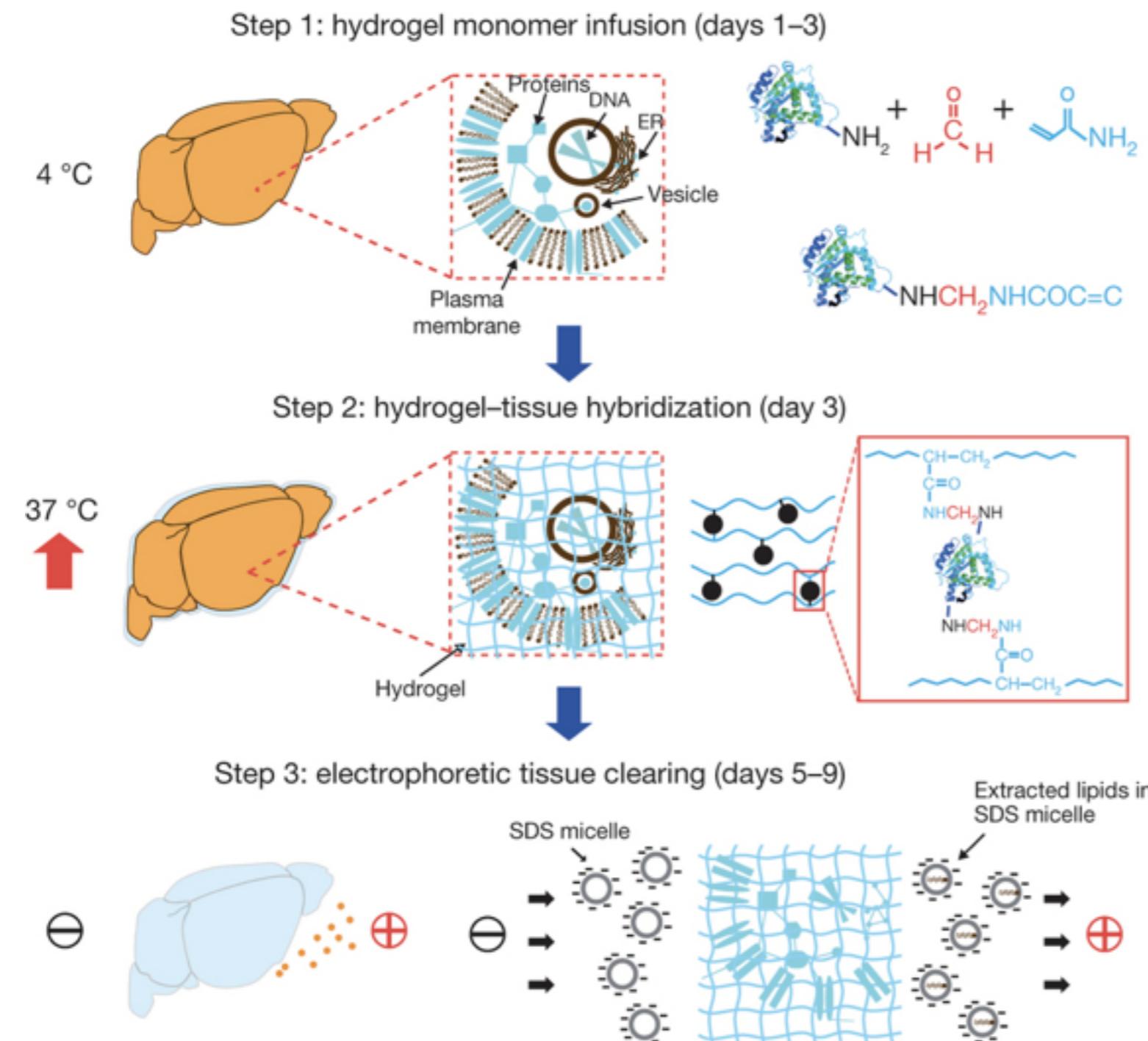
## 7. Autofluorescence - the continual scourge

# Clearing agents: ScaleA2



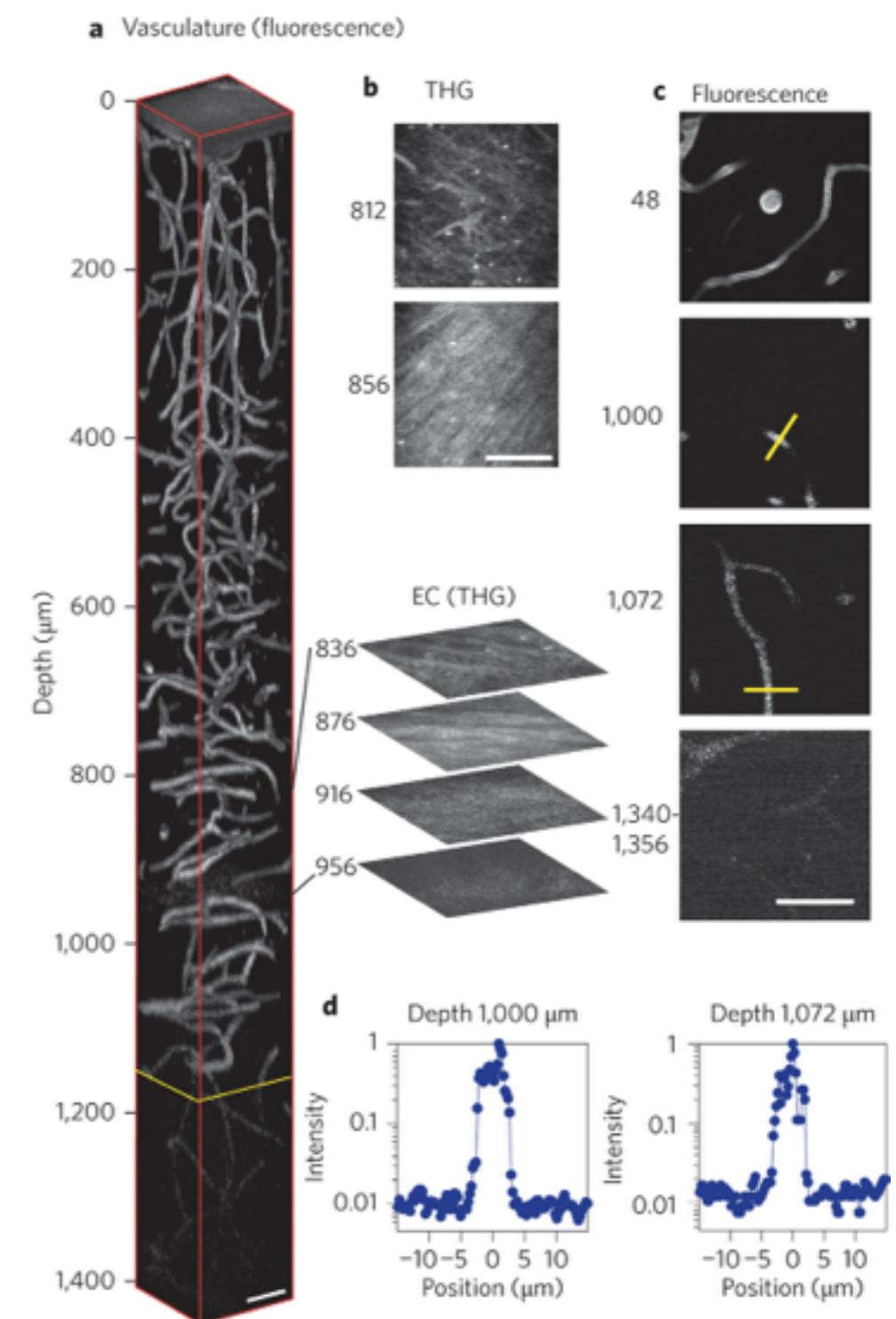
## 7. Autofluorescence - the continual scourge

# Clearing agents: CLARITY

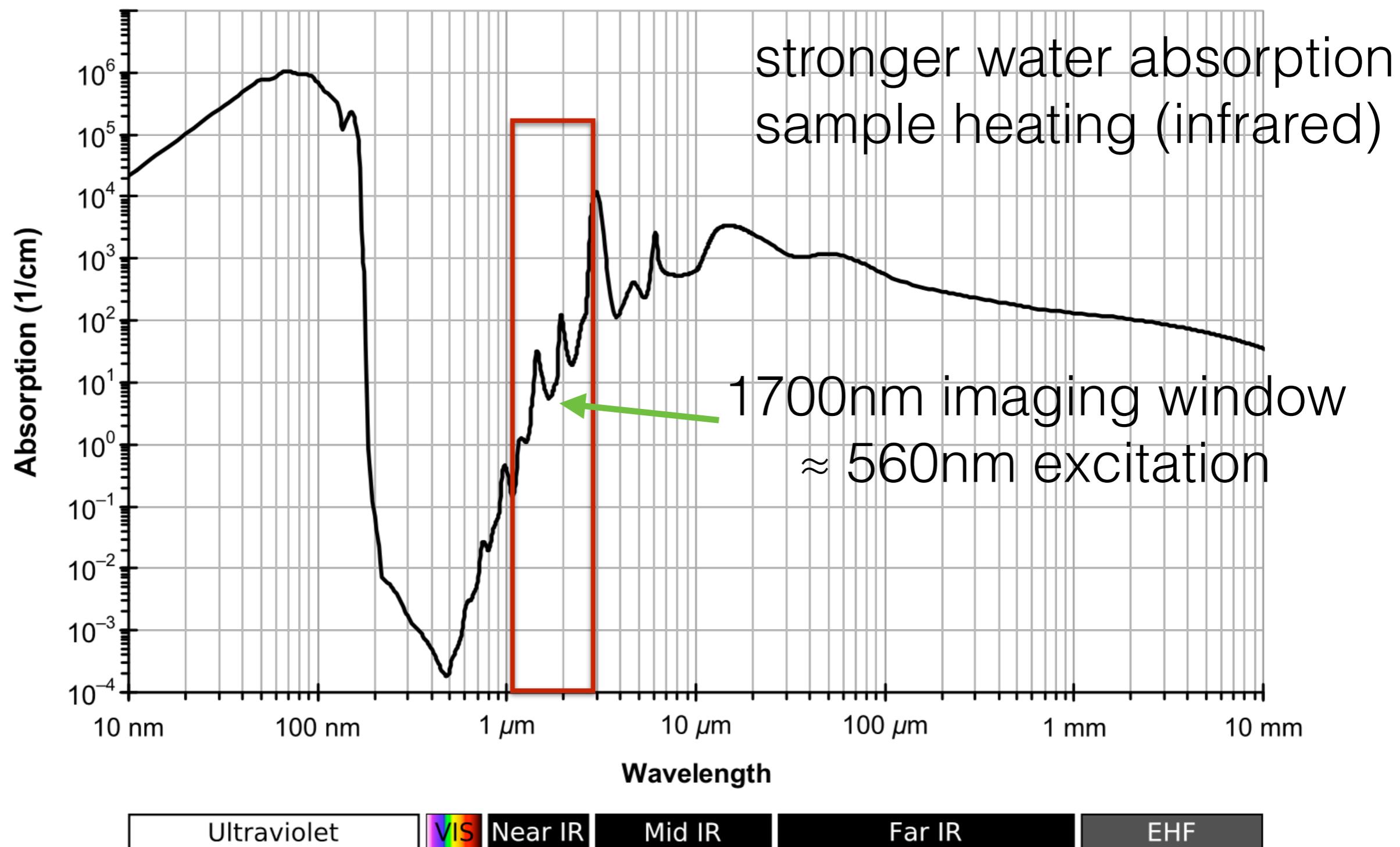


# 3P and beyond

- **MULTI**photon microscopy:  
more than two photons?
- All phenomena apply also for  
3 or more photon excitation
- 3P promises even higher  
penetration depth
- Constraints: Excitation  
probability, laser pulse  
intensity, tissue heating

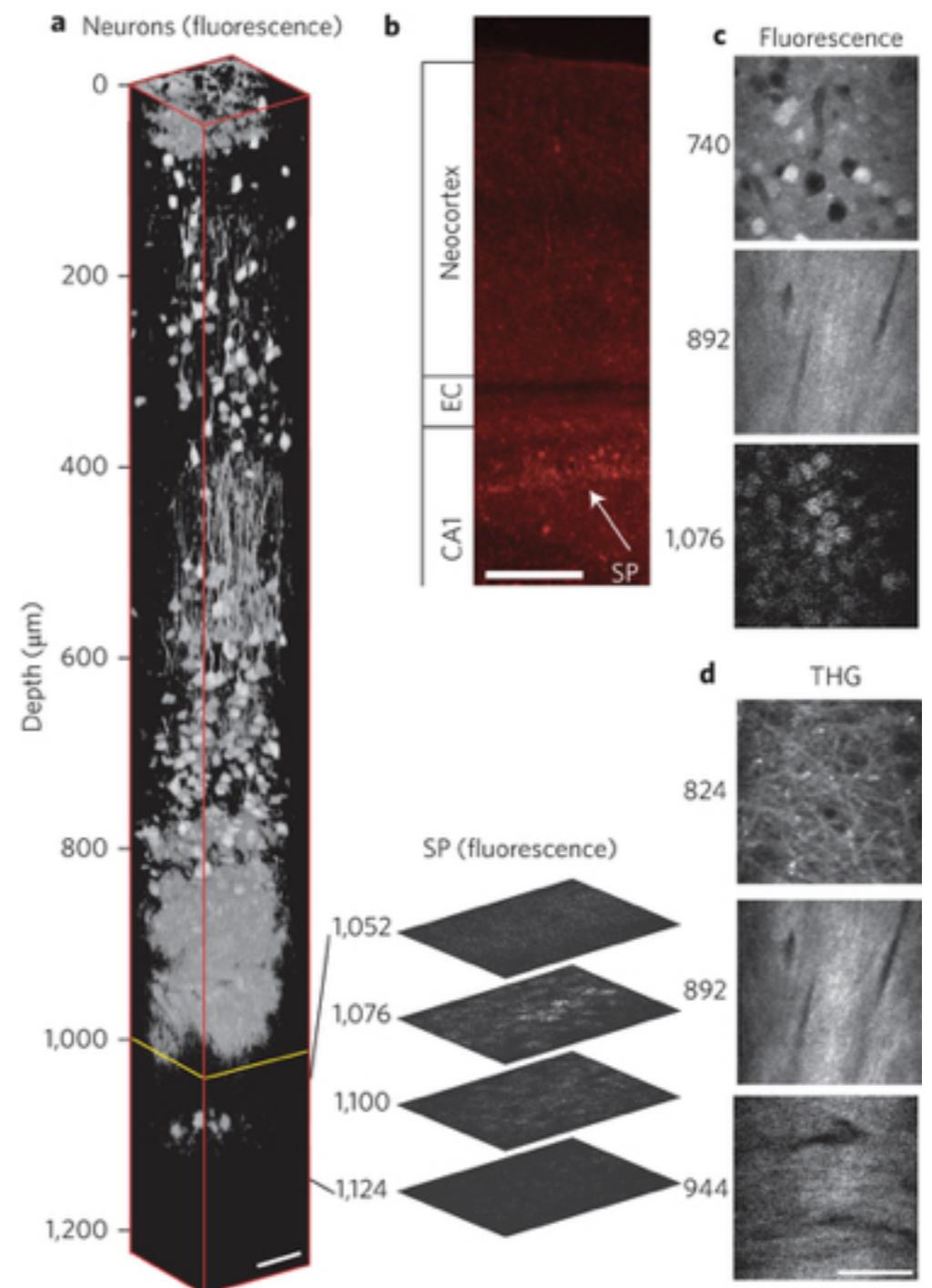


# Absorption of water



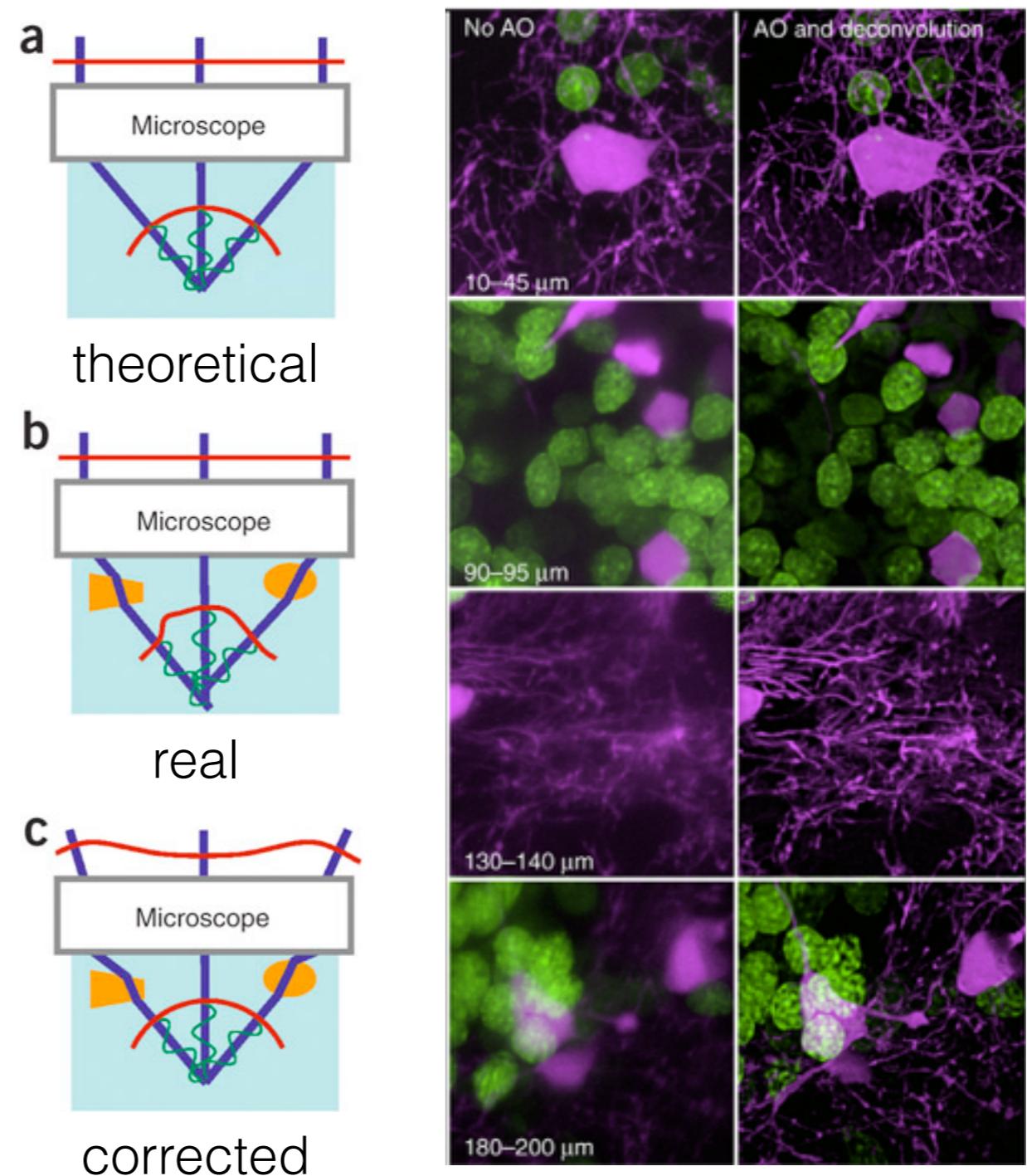
# 3P Imaging

- Novel high-energy laser system with very short pulse (65fs) and high power (67nJ) at 1650nm (pulse is 5fold stronger than Coherent's Chameleon Vision I at 800nm)
- Low duty cycle (1MHz) prevents tissue burning
- Requires long imaging times (8-20s per frame) and high laser power
- Works with red-fluorescent probes



# Adaptive Optics

- Light path through tissue is not uniform for all angles, causing deformation of the 2P focal point
- A deformable mirror allows shaping of the light paths to correct for the tissue



# Further Reading

## **Nikon Microscopy U**

[http://www.microscopyu.com/articles/fluorescence/  
multiphoton/multiphotonintro.html](http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html)

## **Kurt Thorn's iBiology 2P lecture**

<https://www.youtube.com/watch?v=CZifB2aQDDM>

## 9. The Labs

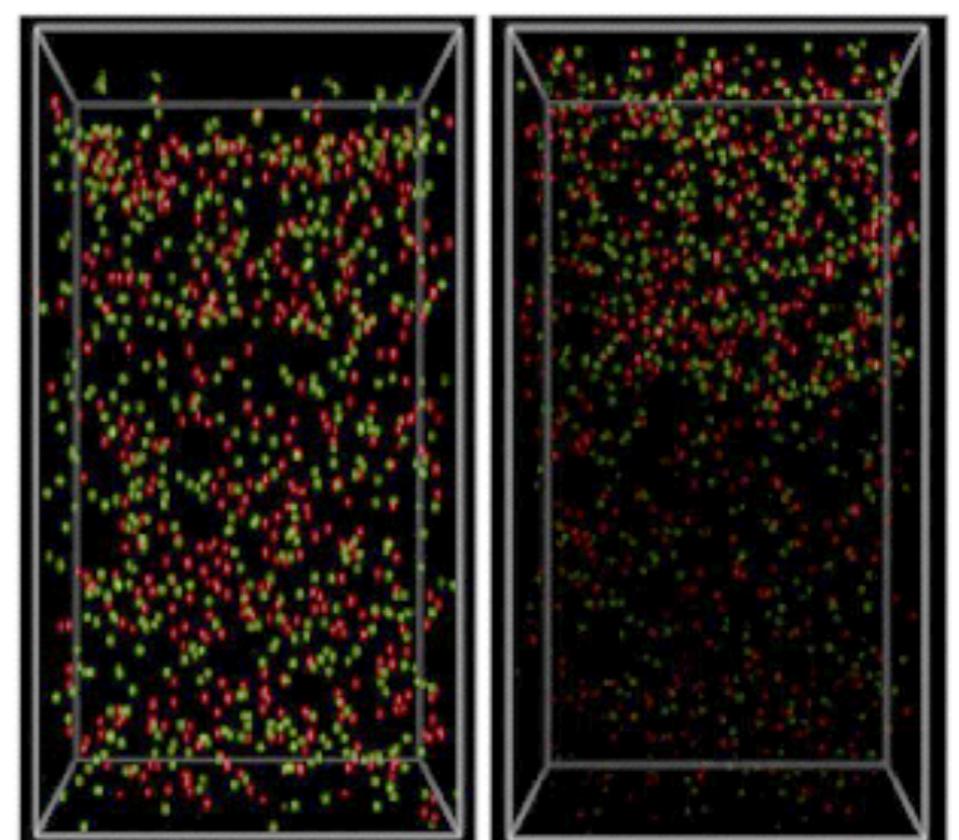
# The Labs

## **Comparison depth penetration 1P vs 2P**

Dispersive beads mimic biological specimens

Compare 1P vs 2P performance

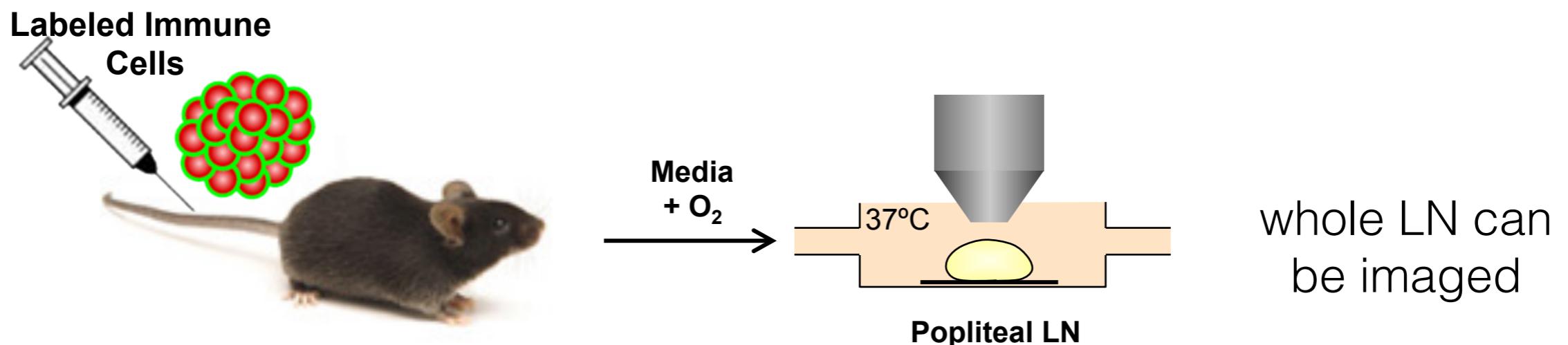
Possibly: Commercial vs custom-built microscopes



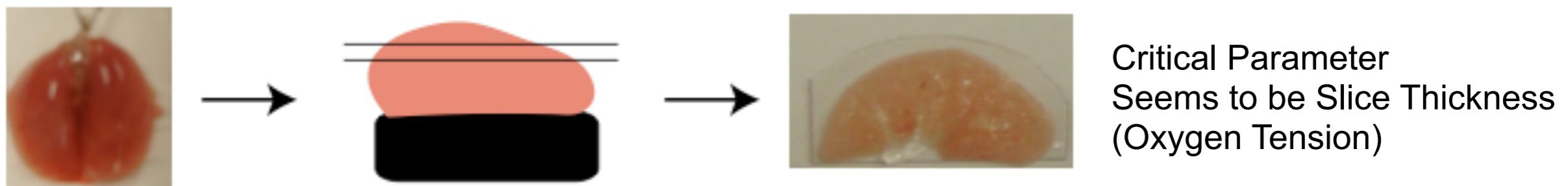
## 9. The Labs

# The Labs

## Imaging of biological specimens ex vivo



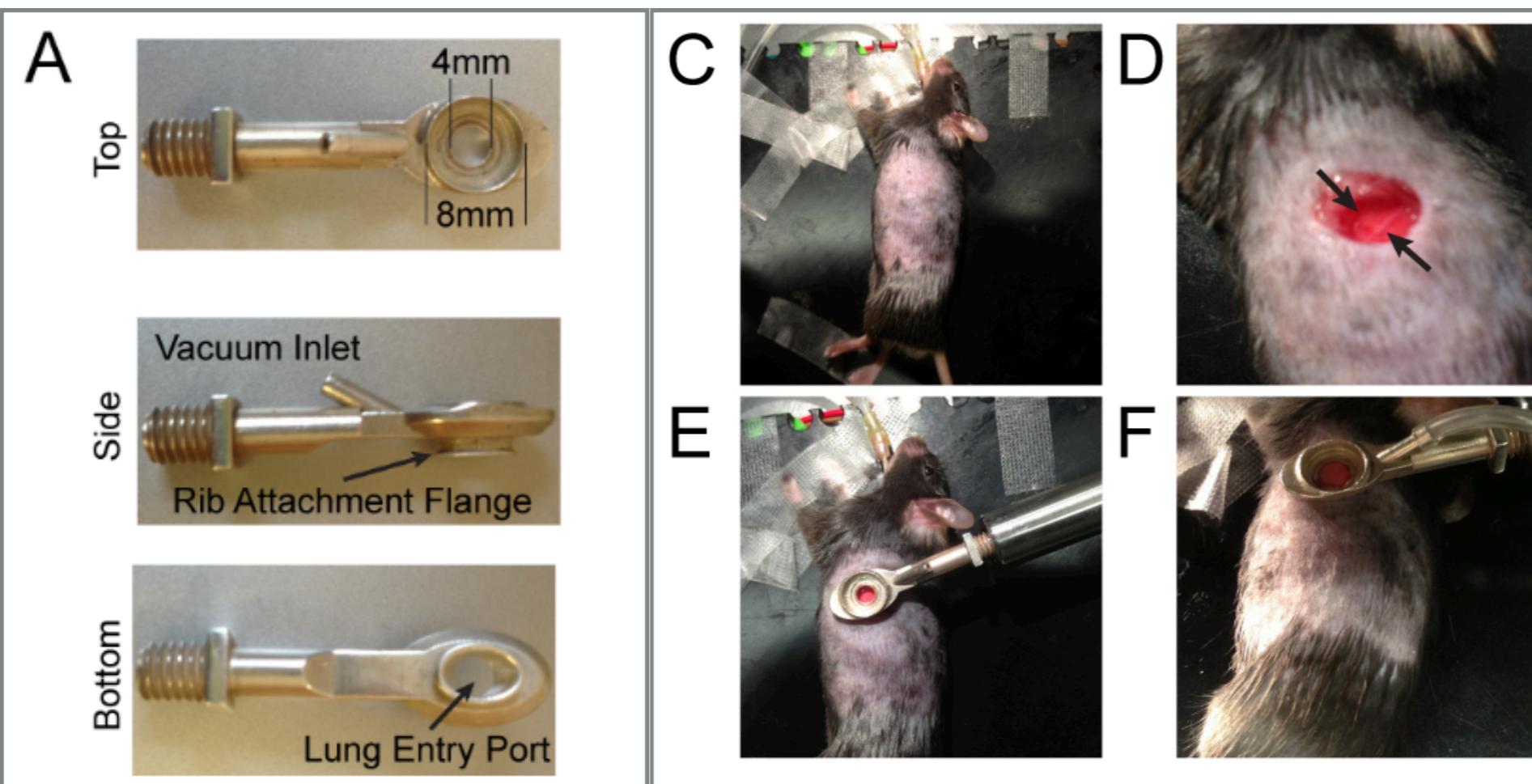
**Slices of larger organs are viable:** Brain, lung, pancreatic islets (not whole pancreas), tumors



## 9. The Labs

# The Labs

## Imaging of biological specimens *in vivo*



Lung imaging



breast/tumor  
imaging

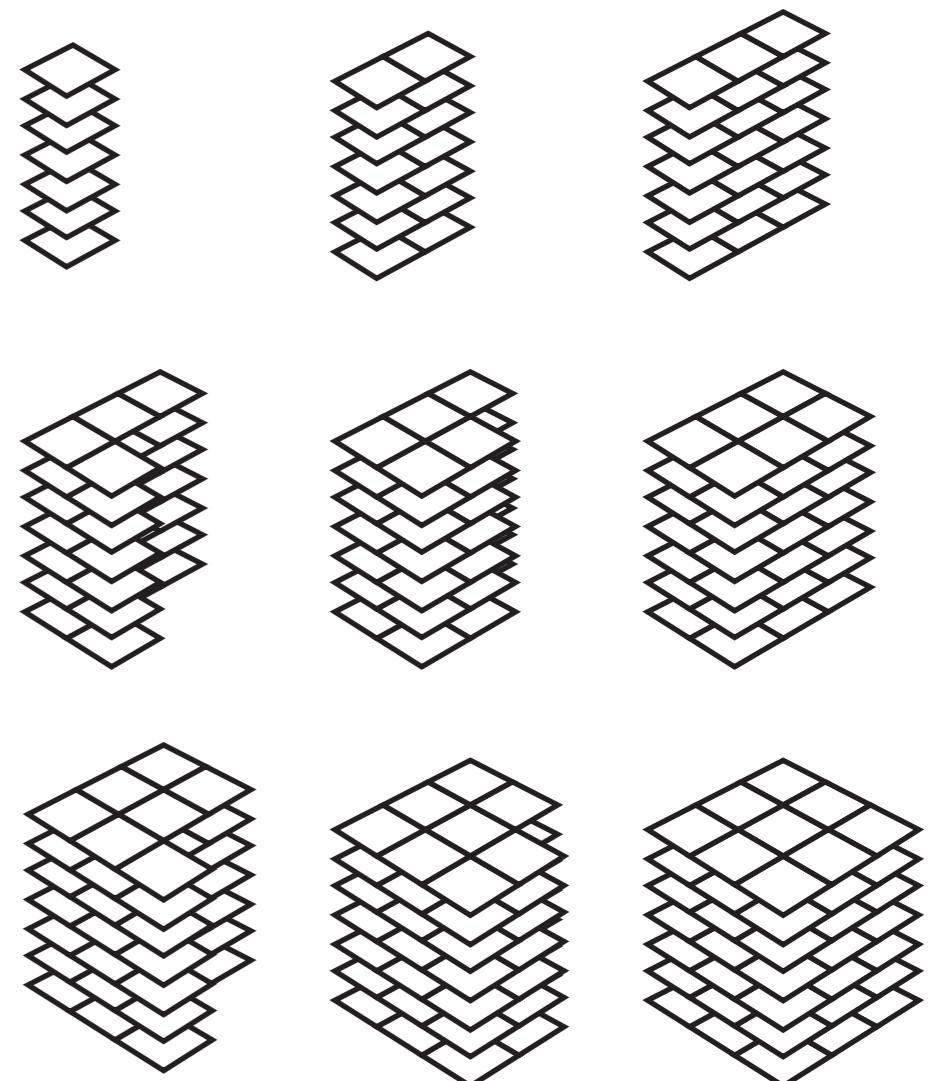
## 9. The Labs

# The Labs

## Imaging of biological specimens

Larger datasets are composed of smaller cubes of data that are stitched together

For fast acquisition usually z planes before a new xy position

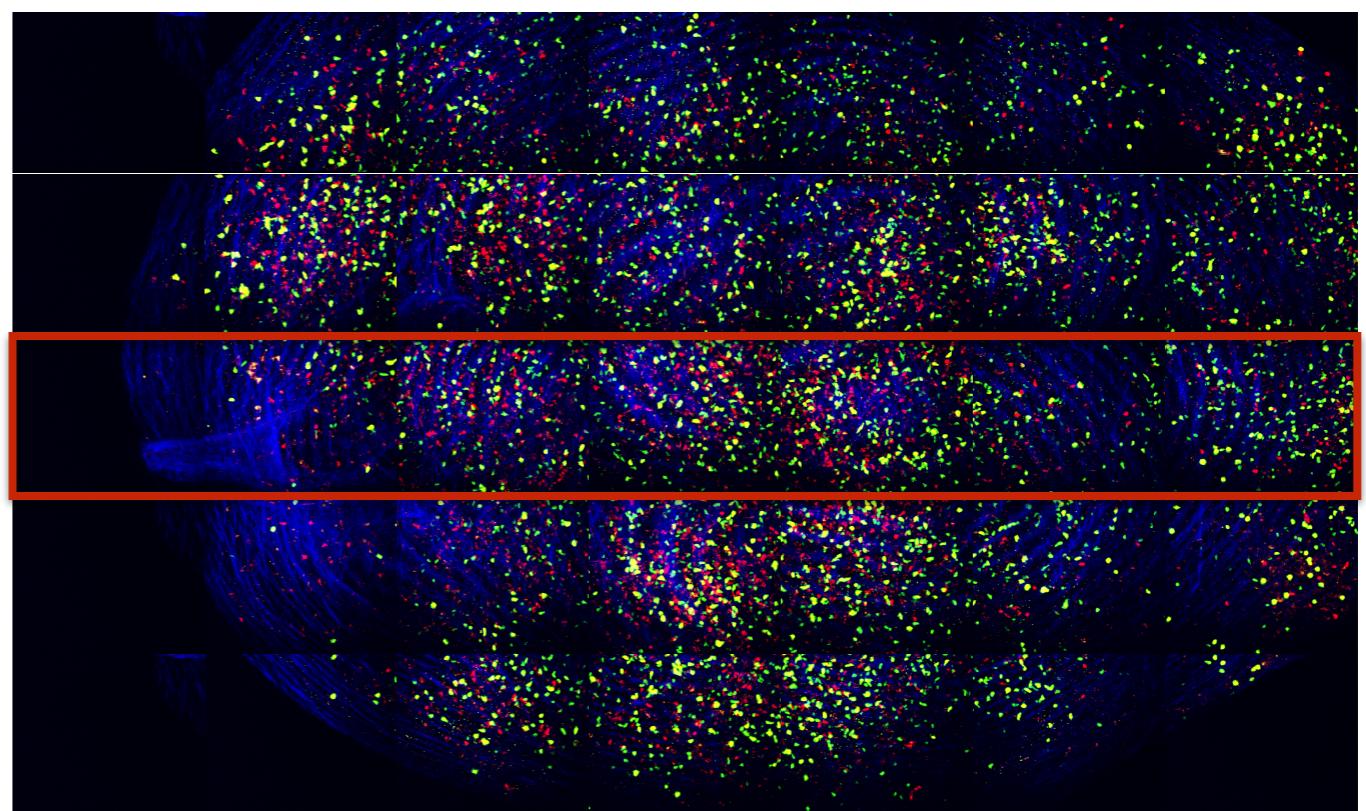


## 9. The Labs

# The Labs

## **Imaging of biological specimens**

Image acquisition usually begins with surveys to identify areas of interest



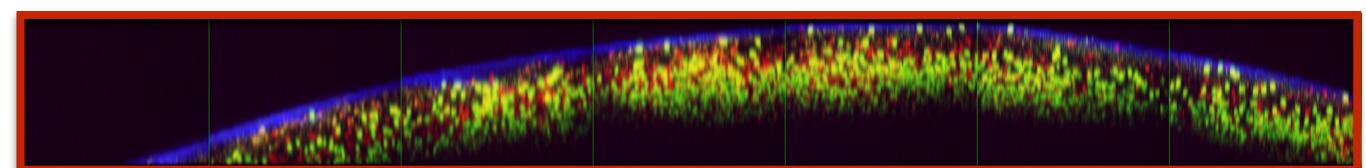
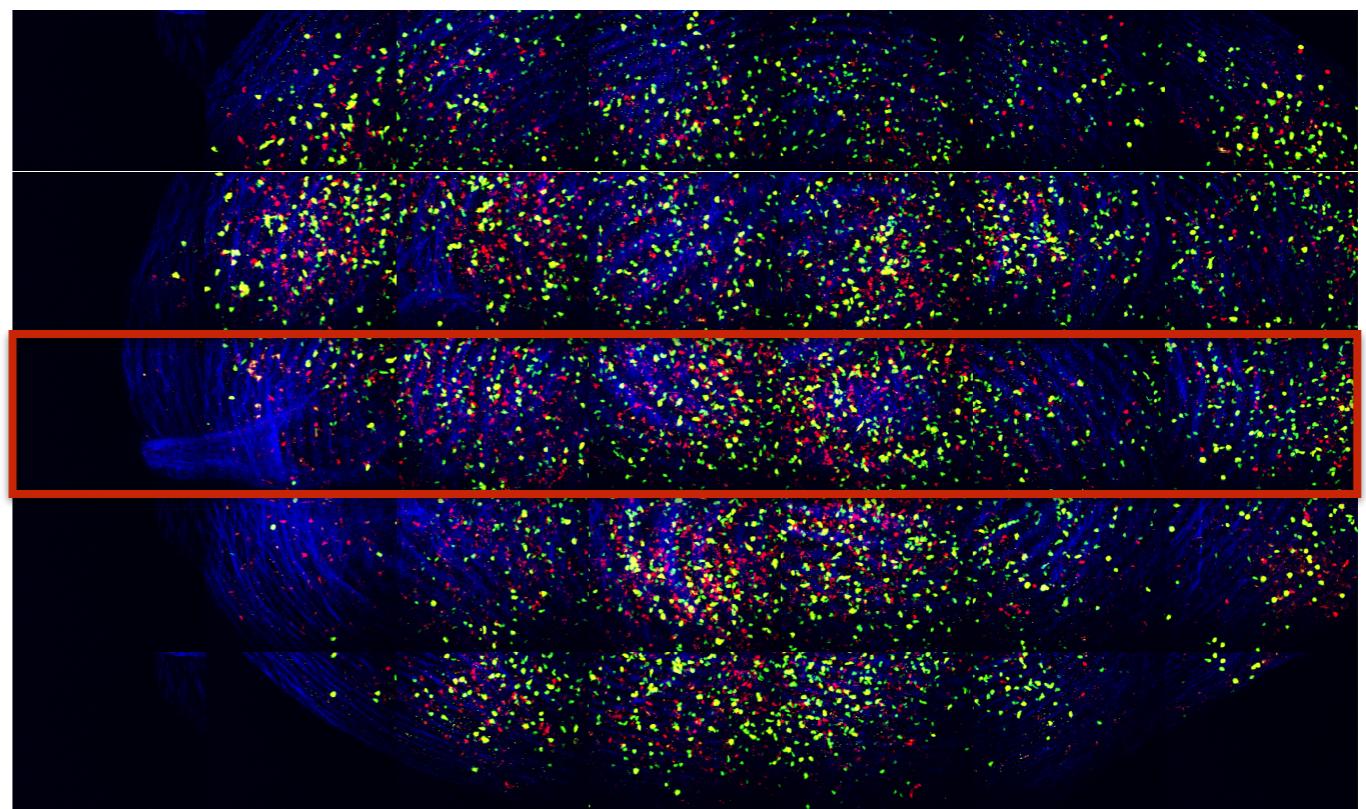
## 9. The Labs

# The Labs

## Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest

Then identify the z depth of a possible stack



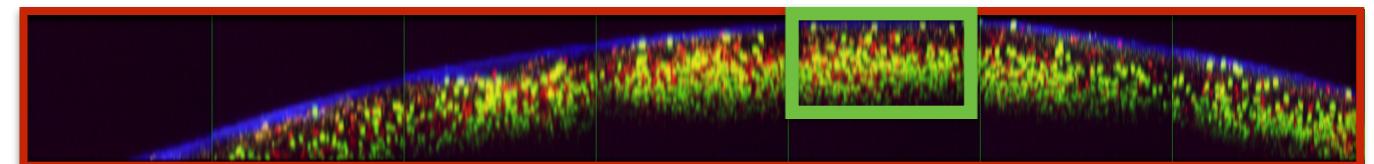
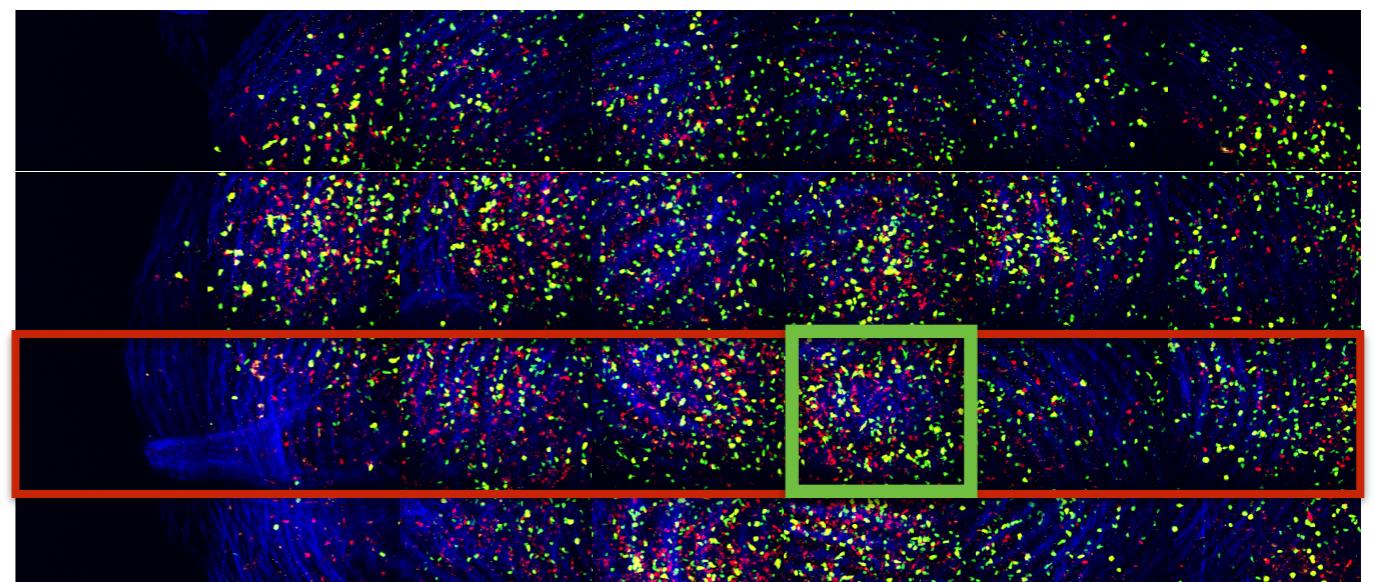
## 9. The Labs

# The Labs

## Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest

Then select specific xy and z boundaries for a time-lapse acquisition



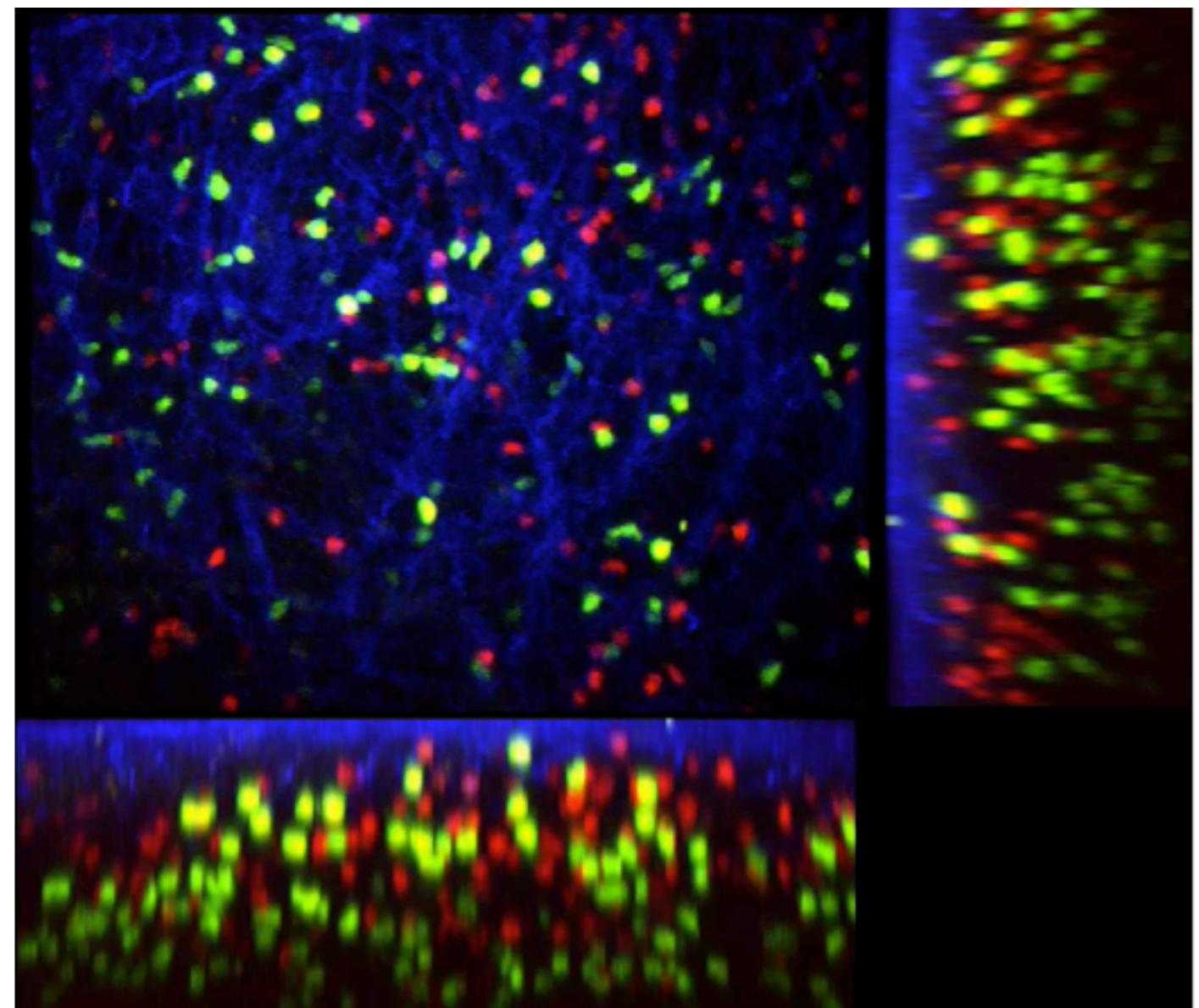
## 9. The Labs

# The Labs

## Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest

Then select specific xy and z boundaries for a time-lapse acquisition



## 9. The Labs

# The Labs

## **Analysis of Data**

ImageJ/Fiji: free, lots of tools, open source

IDT: proprietary, microscope control, limited analysis, Matlab integration

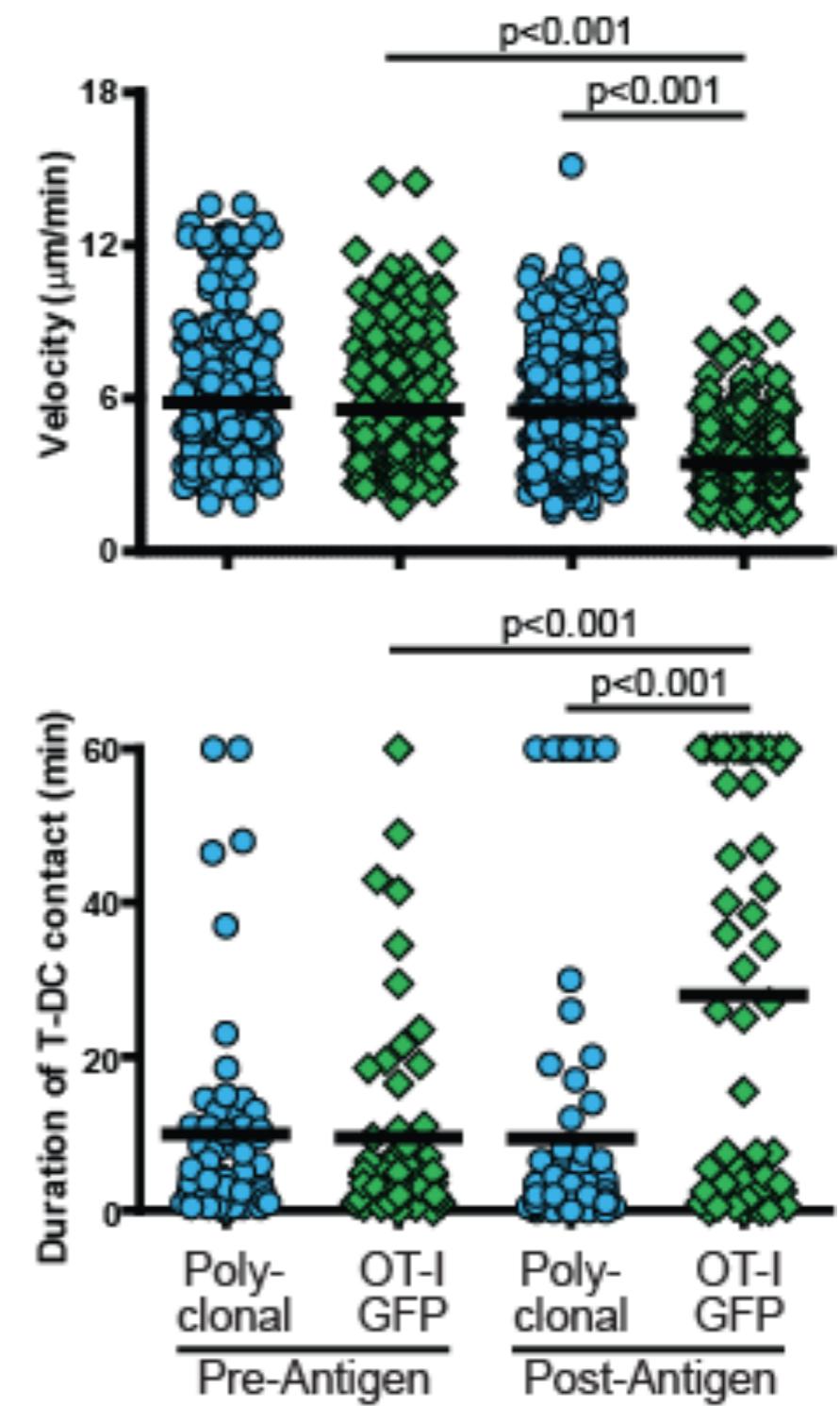
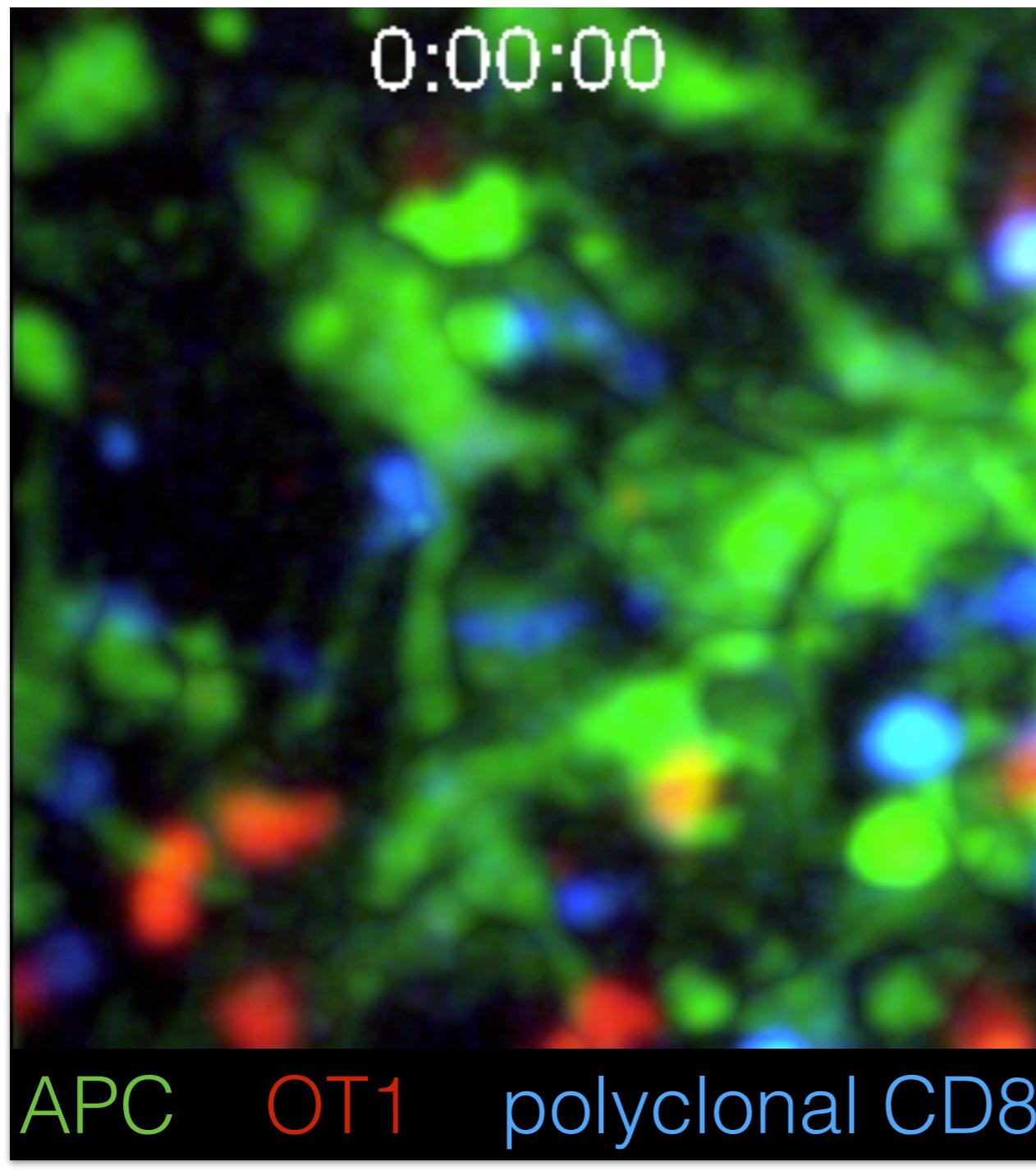
Metamorph: proprietary, microscope control, some analysis function

Imaris: proprietary, no hardware control, 5D analysis, Matlab/ImageJ integration

Matlab: proprietary, programming software, only in conjunction with ImageJ/IDT, required for extensive analysis

## 9. The Labs

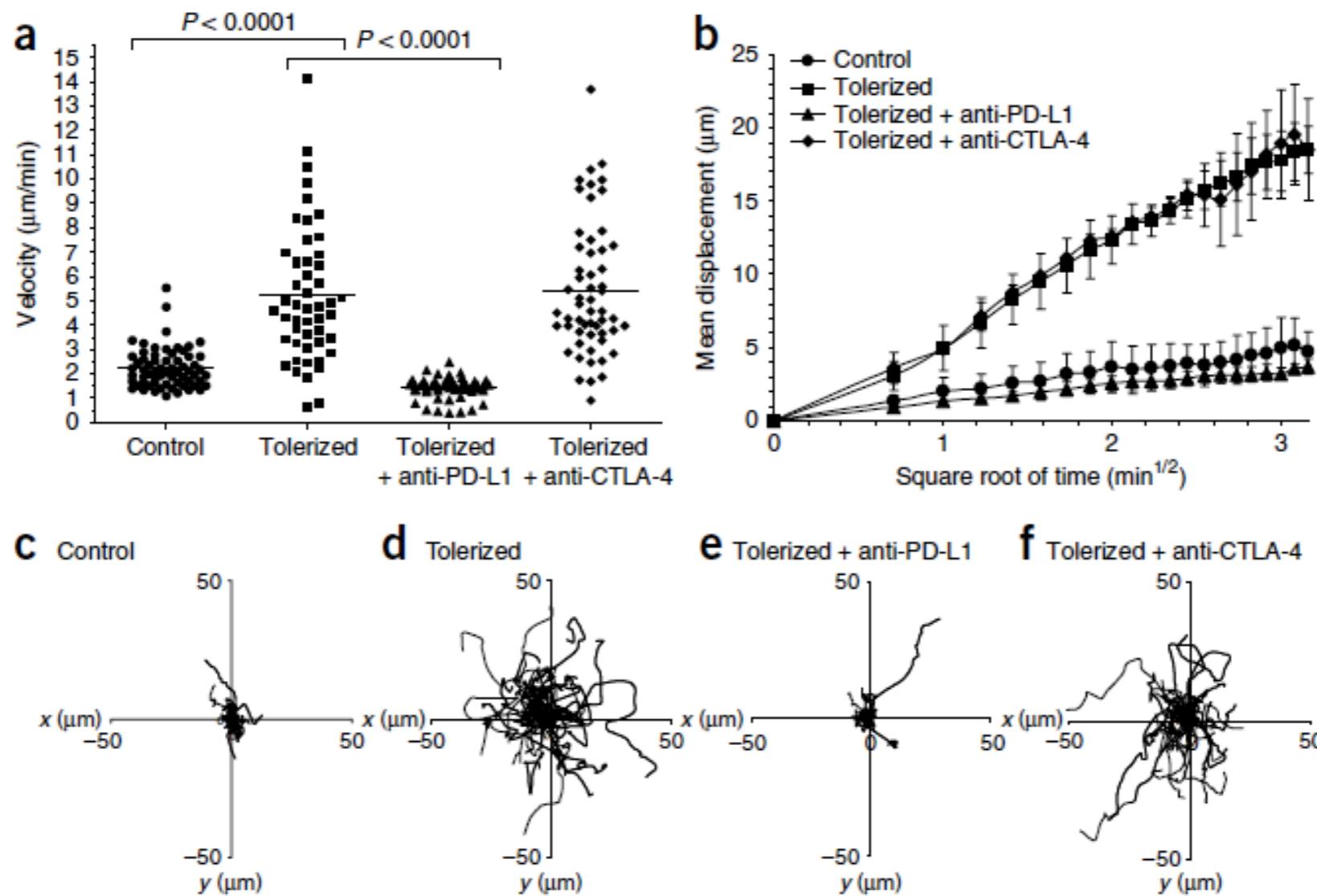
# The Labs



## 9. The Labs

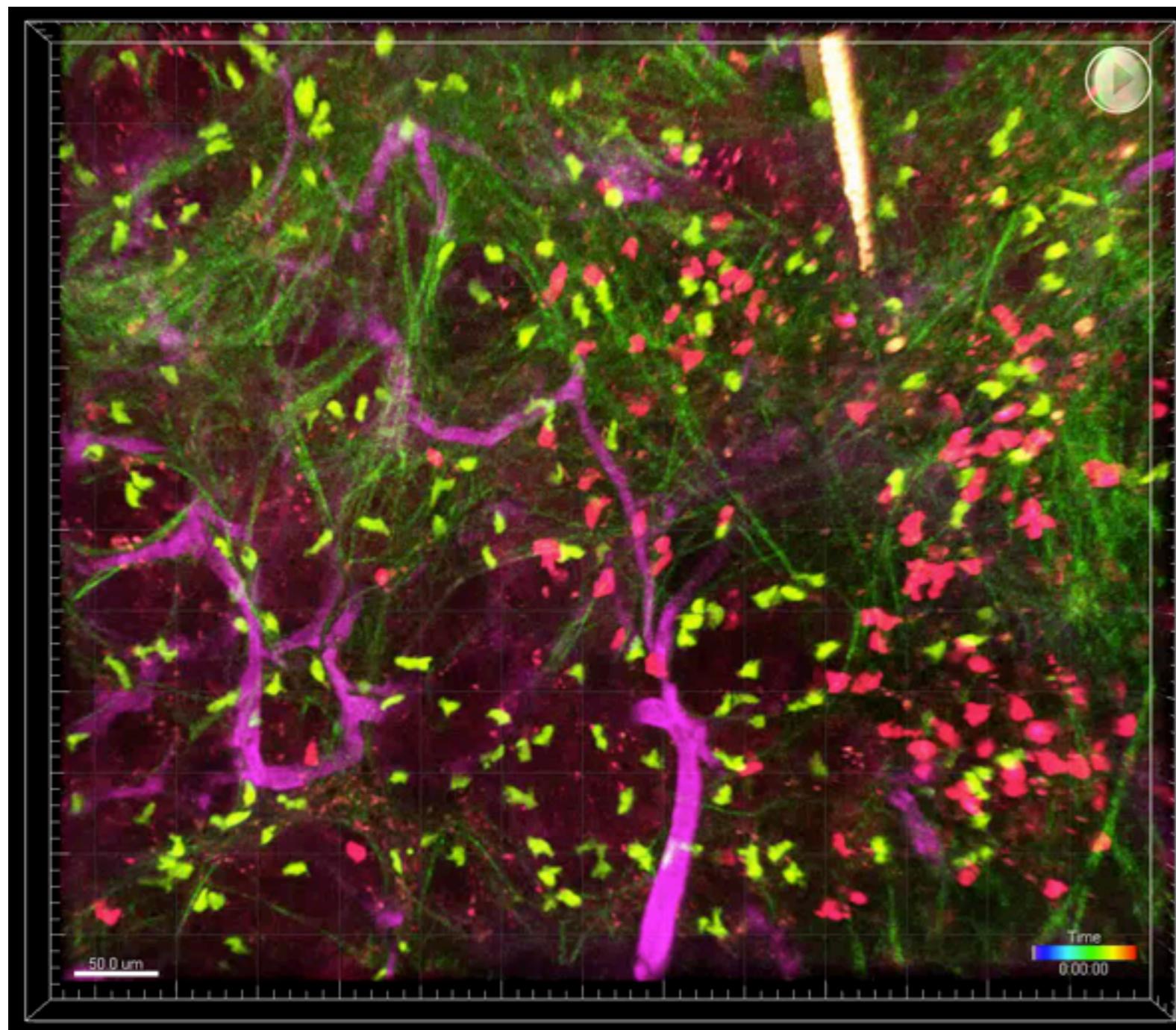
# The Labs

**Migration measurements:** Speed, displacement, track angles, distance from origin, arrest coefficients



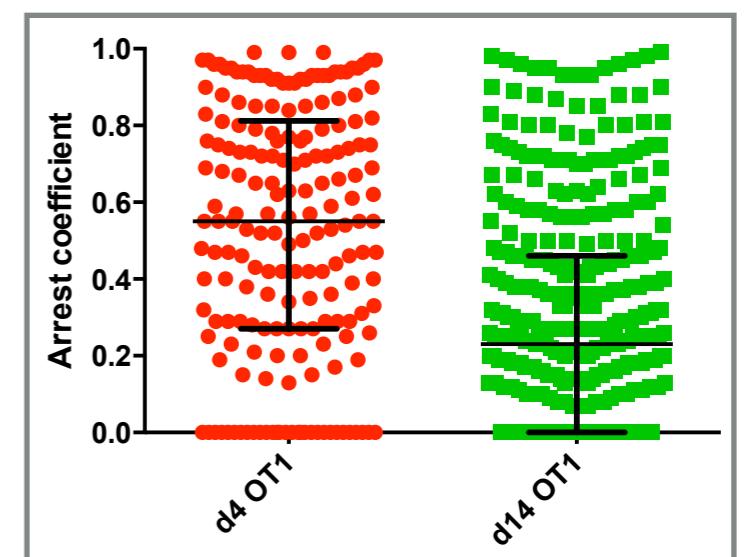
## 9. The Labs

### EXAMPLE: T CELL MATURATION IN TUMORS



OT-I T cells (d14)   T cells (d4 OTI)   Evans Blue (blood)

- Direct comparison of newly activated (red) and experienced T cells (green) reveals that T cells undergo maturation at tumor site

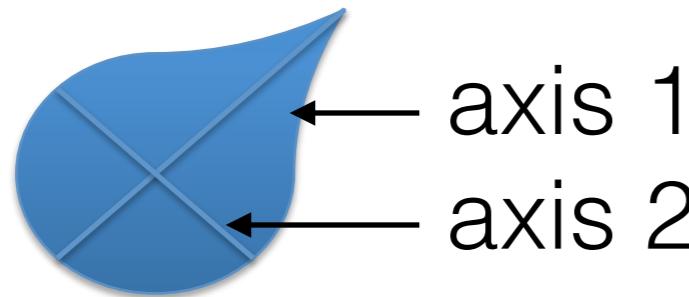


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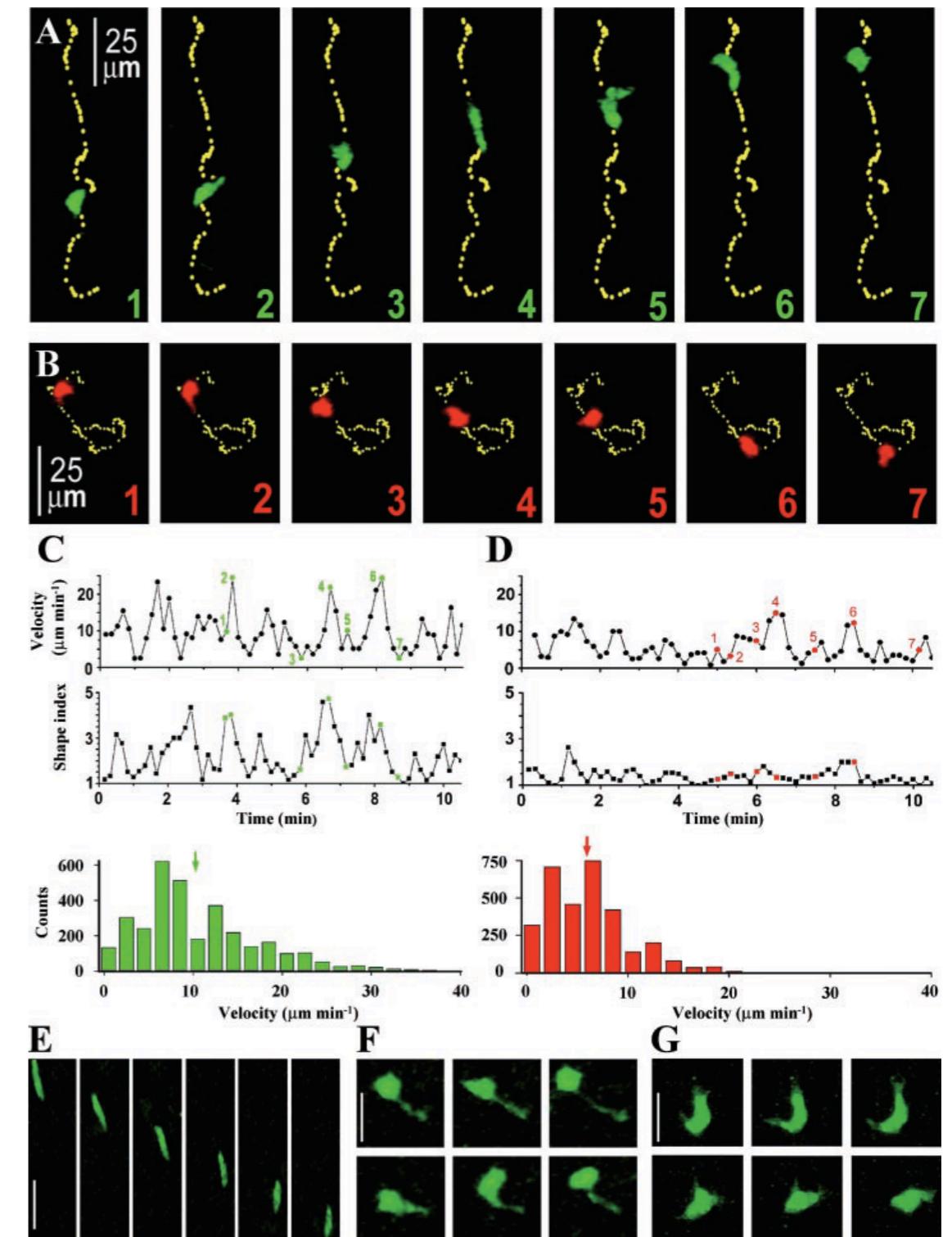
# The Labs

## Morphology measurements



Shape factors correlate with motility vs signaling

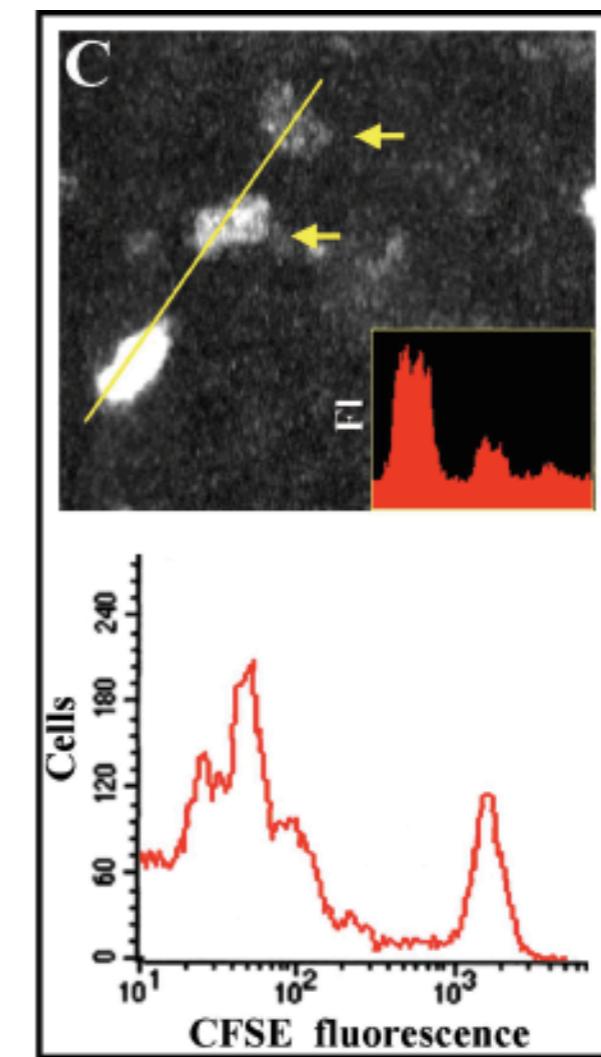
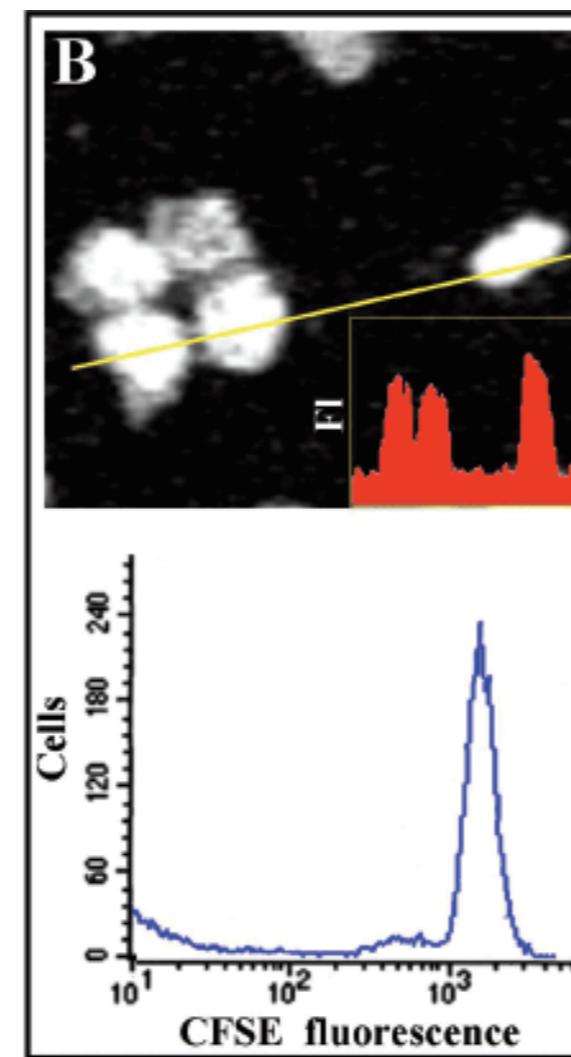
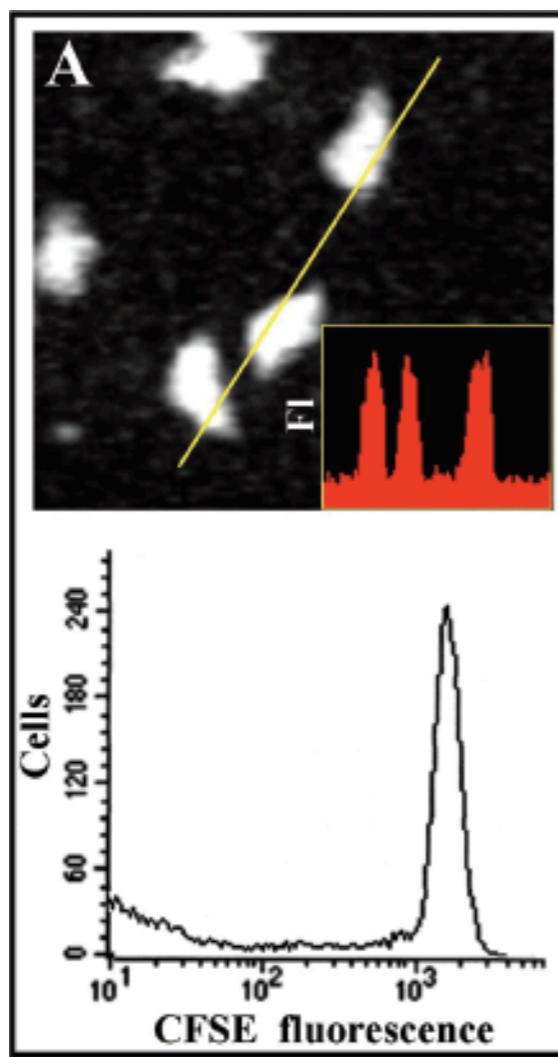
Roundedness:  
axis1/axis2 (=1 for circle)  
perimeter/radius ( $=2\pi r$ )



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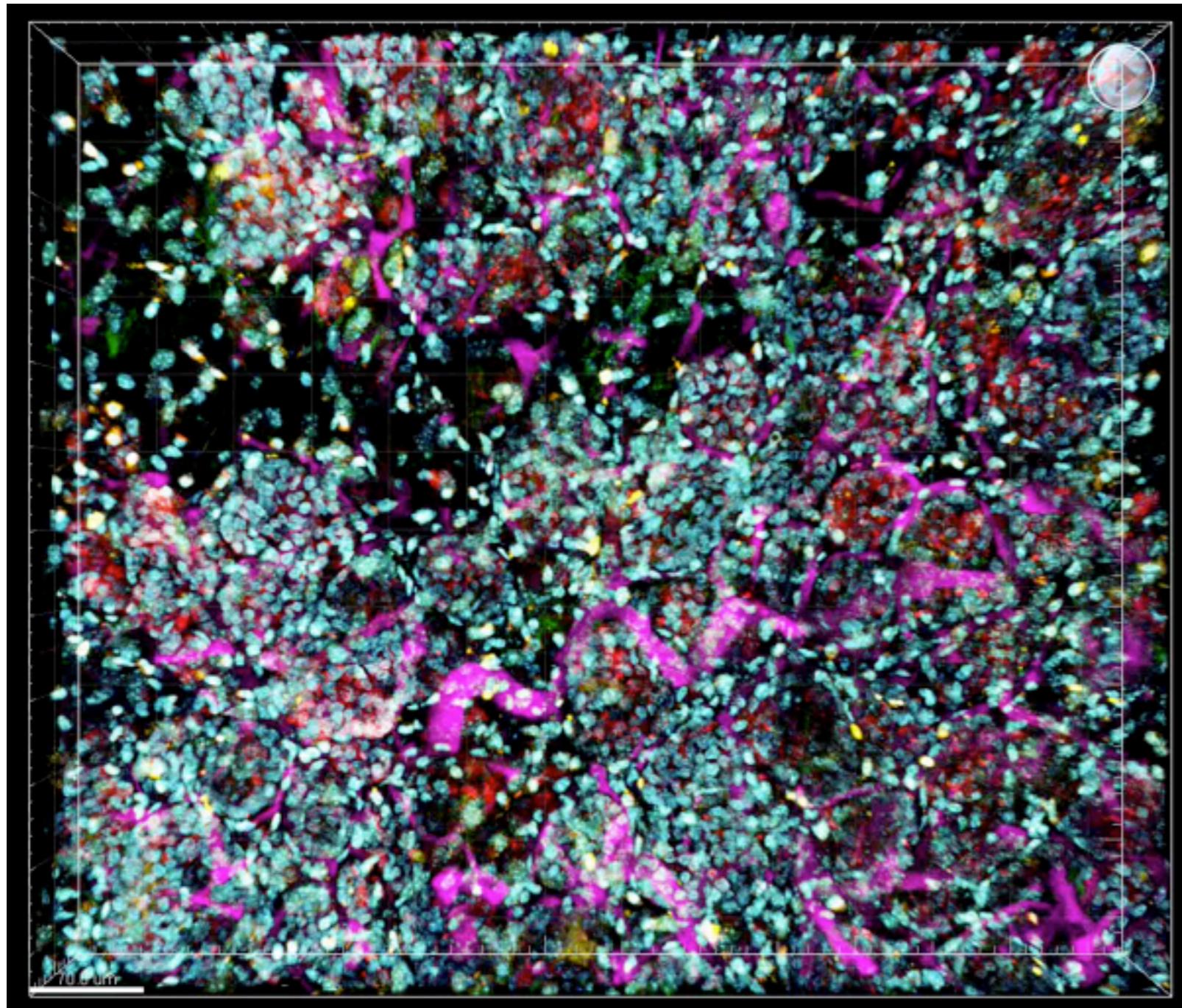
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**Cell activity:** Dilution of a dye to asses cell division



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# EXAMPLE: ASSESSING CTL ACTIVITY IN VIVO



Hoechst    cleaved Caspase 3/7  
Cherry (tumor host)    Evans Blue (blood)

- Use intravital microscopy to assess whether and where CTL activity occurs
- Soaking of exposed mammary gland with cell-permeable apoptosis dyes before imaging
- Visualizing dying cells *in vivo* reveals that tumor cells rarely die

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**Cell interactions:** Manual or via Matlab scripts

