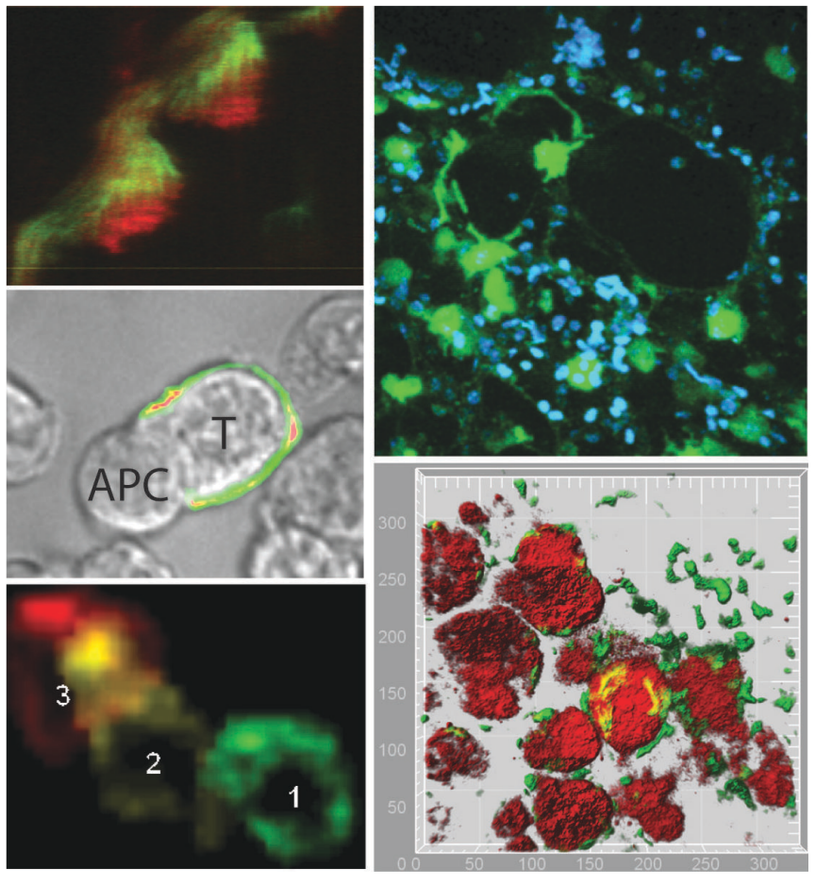
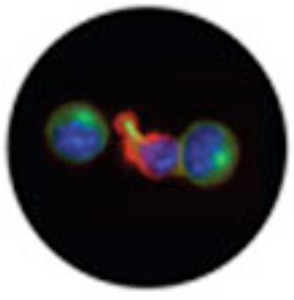
Principles, Benefits, and Applications of Multiphoton Microscopy

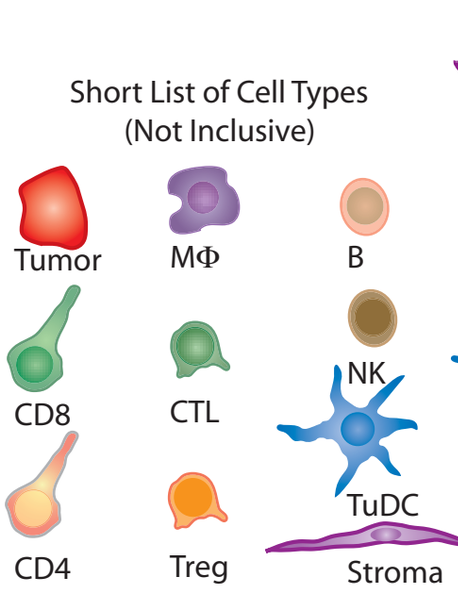


Bijan Boldajipour, PhD
Krummel Lab, Department of Pathology

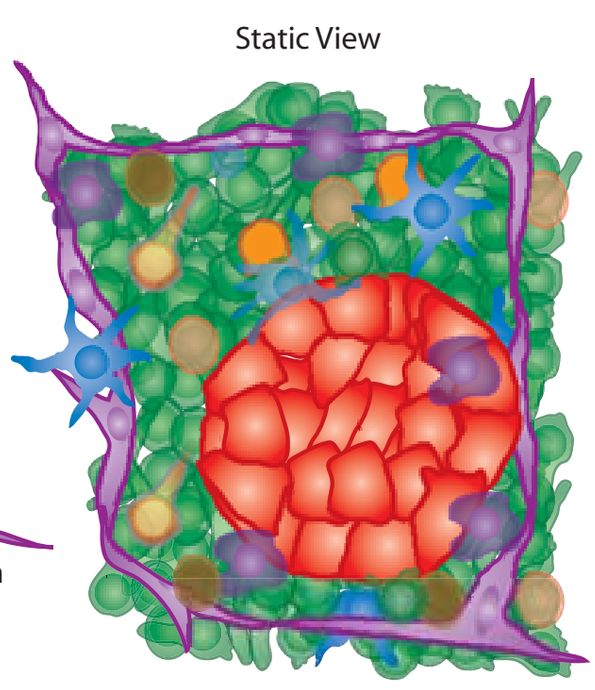


Biological Imaging Development Center at UCSF University of California San Francisco

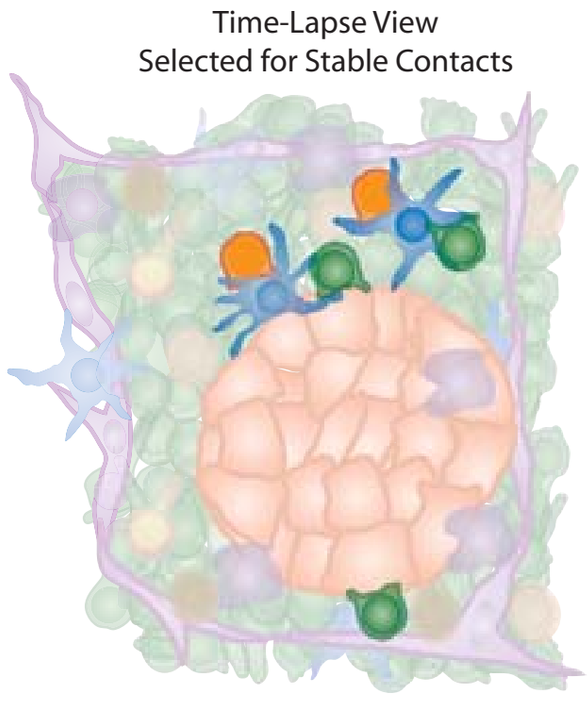
Live Imaging to Identify Significant Systems Events in Complex Tissues



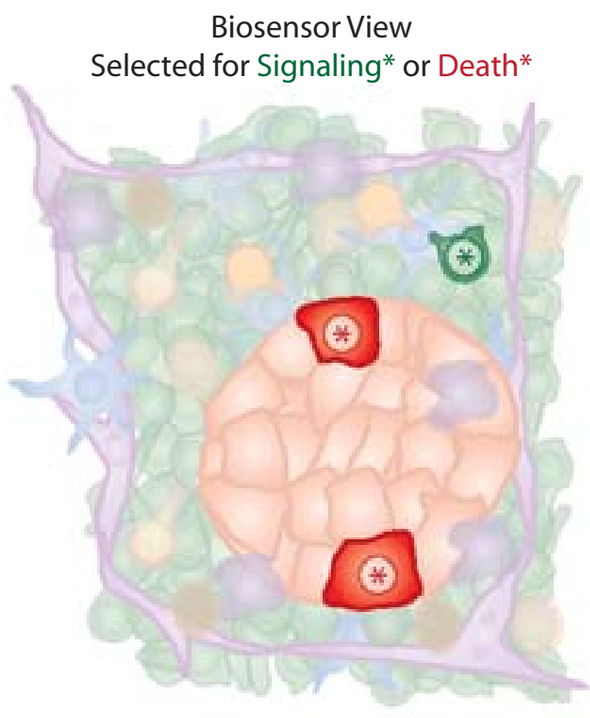
FACS



Flow Cytometry
Cryo Sections



Cell-type specific
Labeling, Timelapse



Biosensor
Labeling, Timelapse

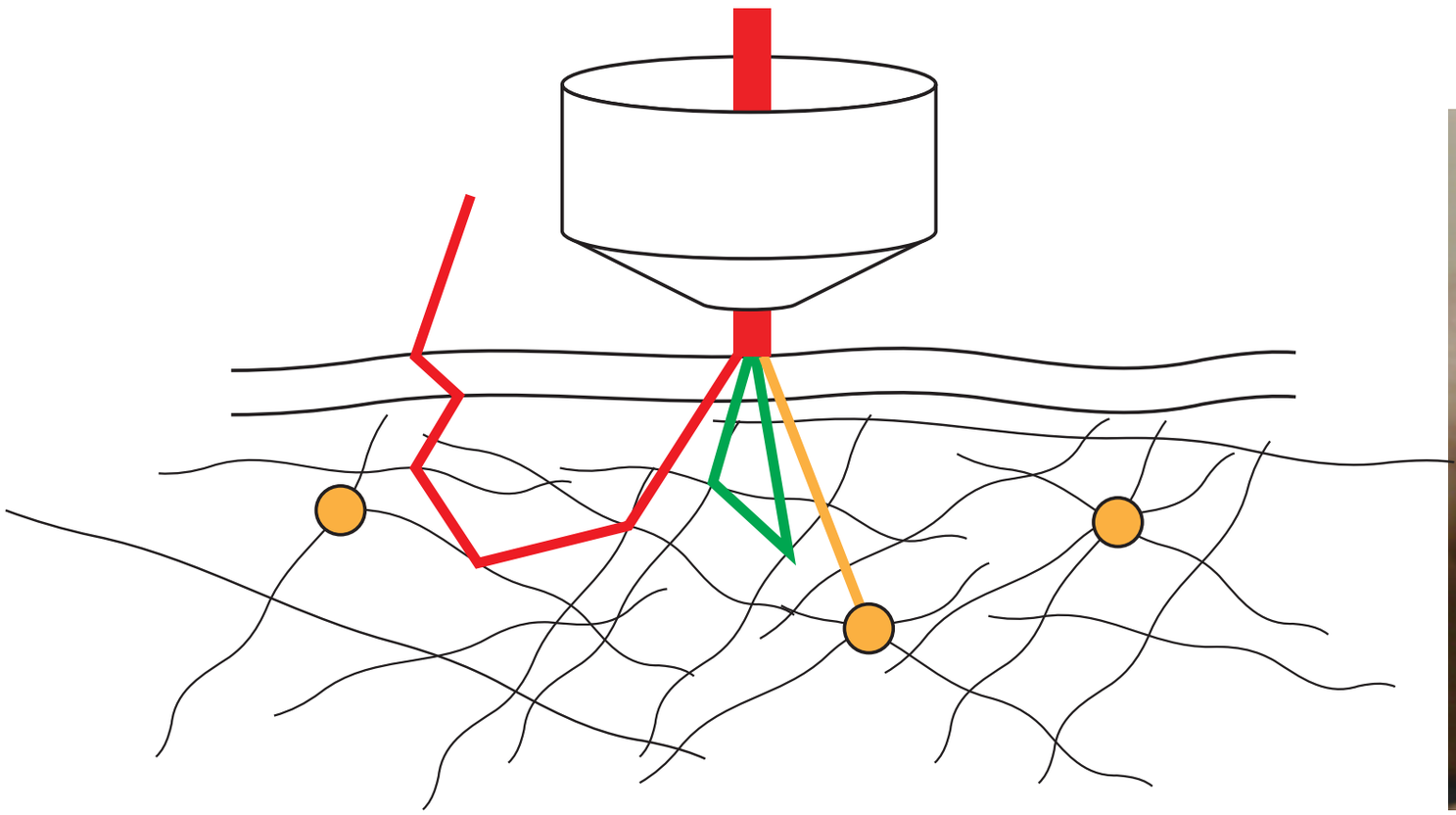
Issues with Imaging in Complex Tissues

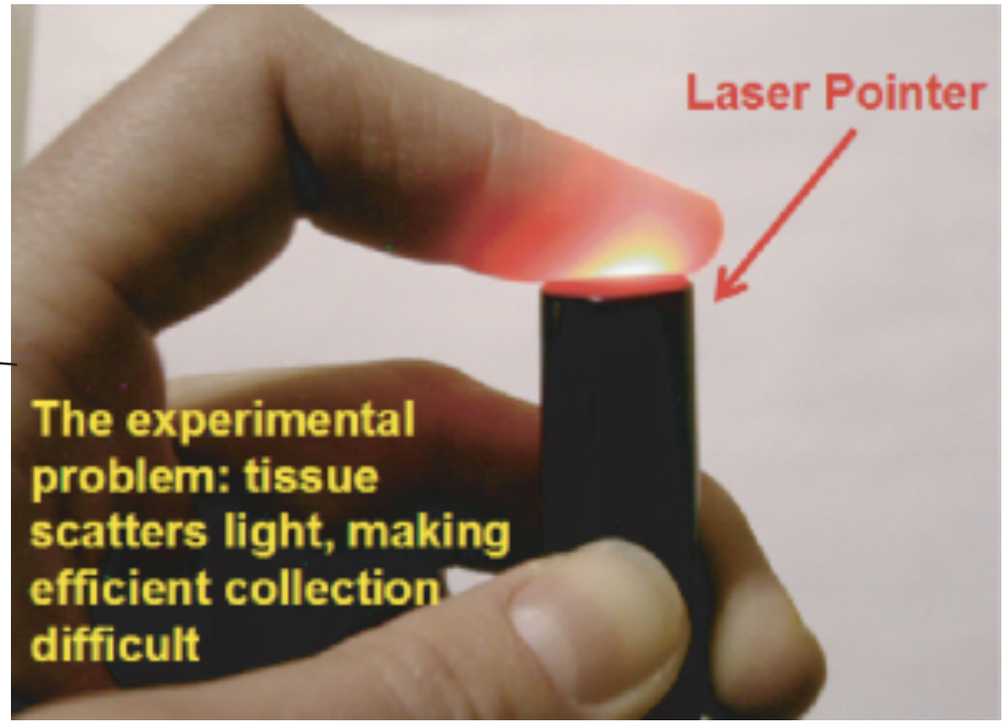
"2-Photon Microscopy"
Also commonly
"Multiphoton Microscopy"

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

Tissues absorb and scatter light

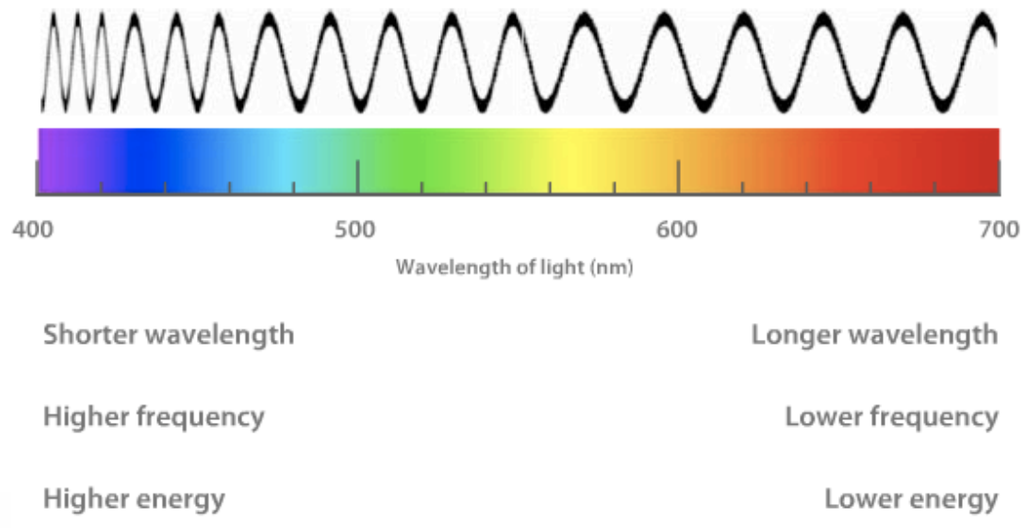




- elastic scattering: single/multiple
- absorption

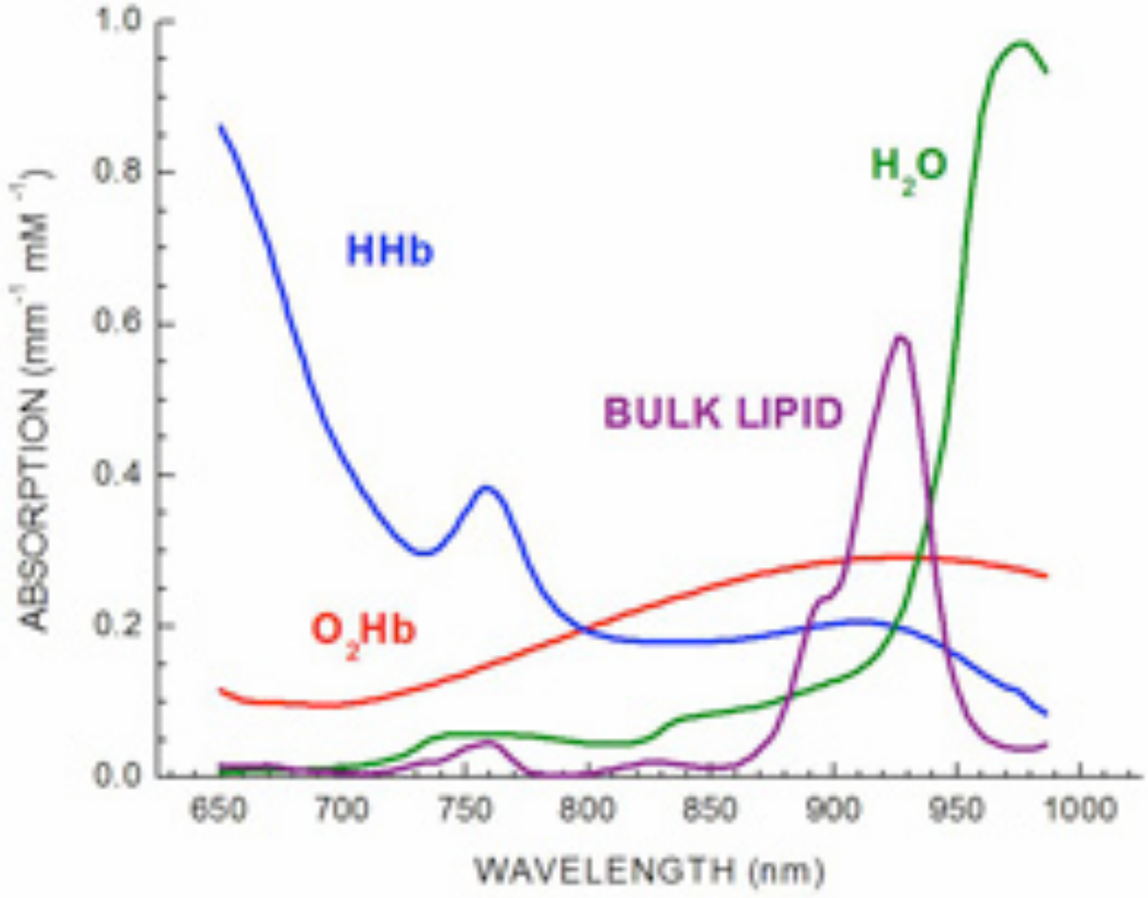
Avoiding Tissue absorption

- Different tissue components absorb at different wavelength

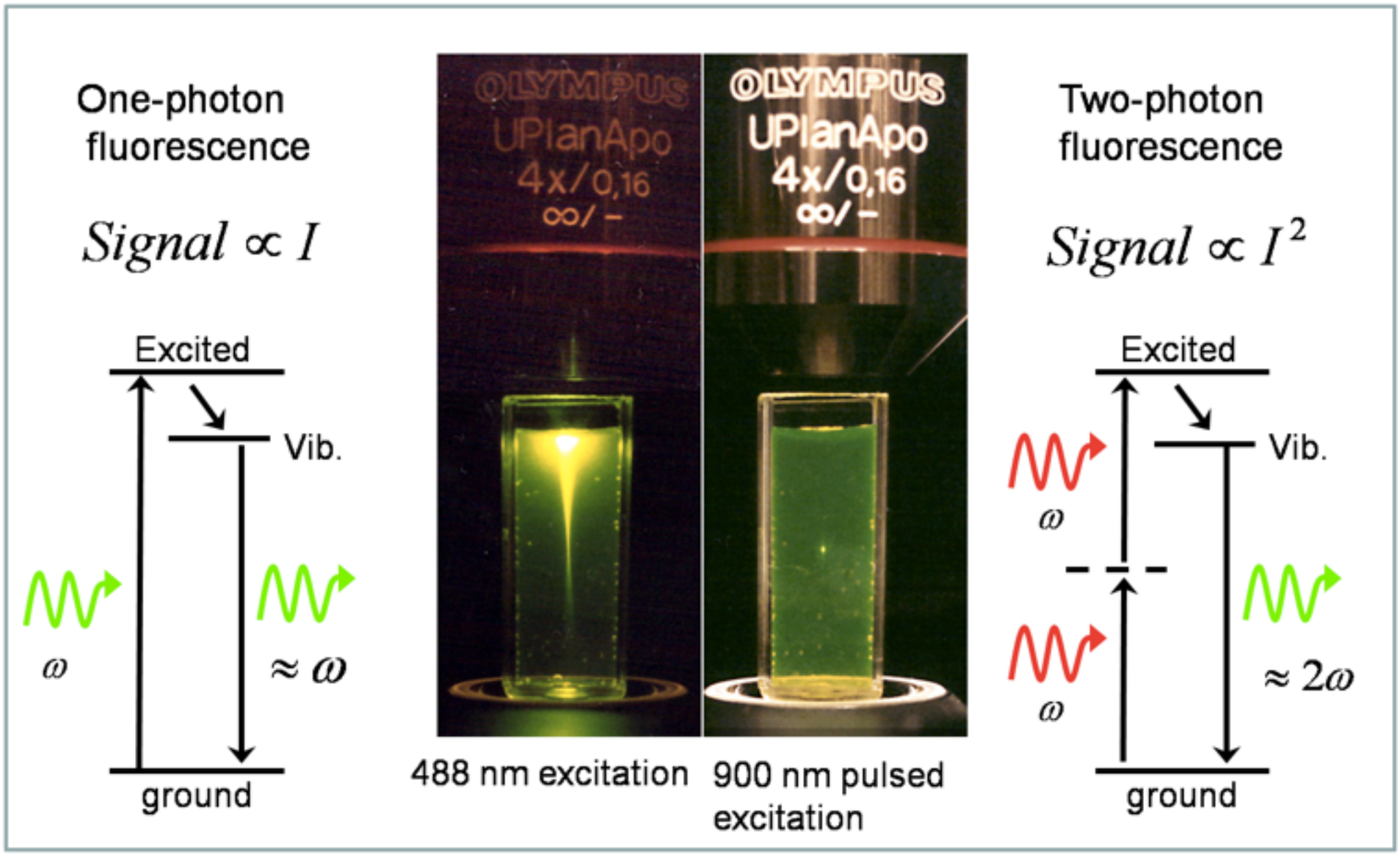


- A good optical window exists 700nm-1000nm with lowest tissue absorption

- Can this range be used without developing a new set of dyes and tools?



2-Photon excitation: How and Why



How: Two Photons of half the energy (twice the wavelength), if temporally coincident, sum their energies (2x05=1x) to drive fluorophore to activated state (NOTE: This requires very high powered, very expensive lasers)

2P excitation: the 2λ rule

- Quantum mechanics: Most dyes to not optimally excite exactly at 2λ of their single-photon excitation

- Difficult to predict

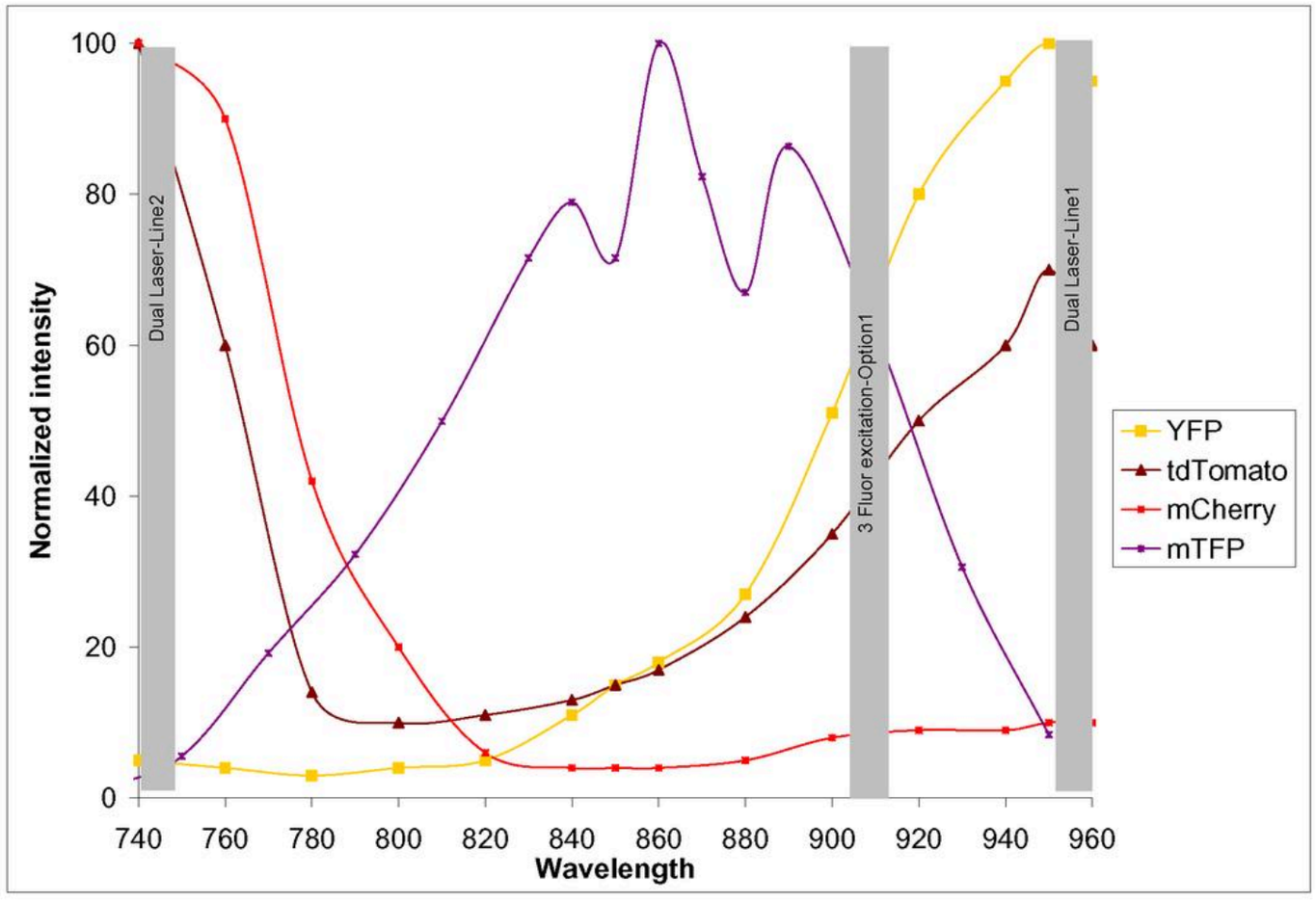
Table 1. Selected applications of fluorescent probes for two-photon excitation (TPE) microscopy

Probe	TPE Excitation Wavelength	Application	References
Alexa Fluor 488 phalloidin	720 nm or 830 nm	Imaging F-actin organization in pancreatic acinar cells	*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

2P excitation: the 2λ rule

- Doubling of excitation broadens excitation range: many dyes can be hit by same wavelength

- Exceptions to 2λ rule: Some fluors with similar emission can be distinguished by different excitation (eg FITC/GFP, GFP/YFP, tdTomato/mCherry)

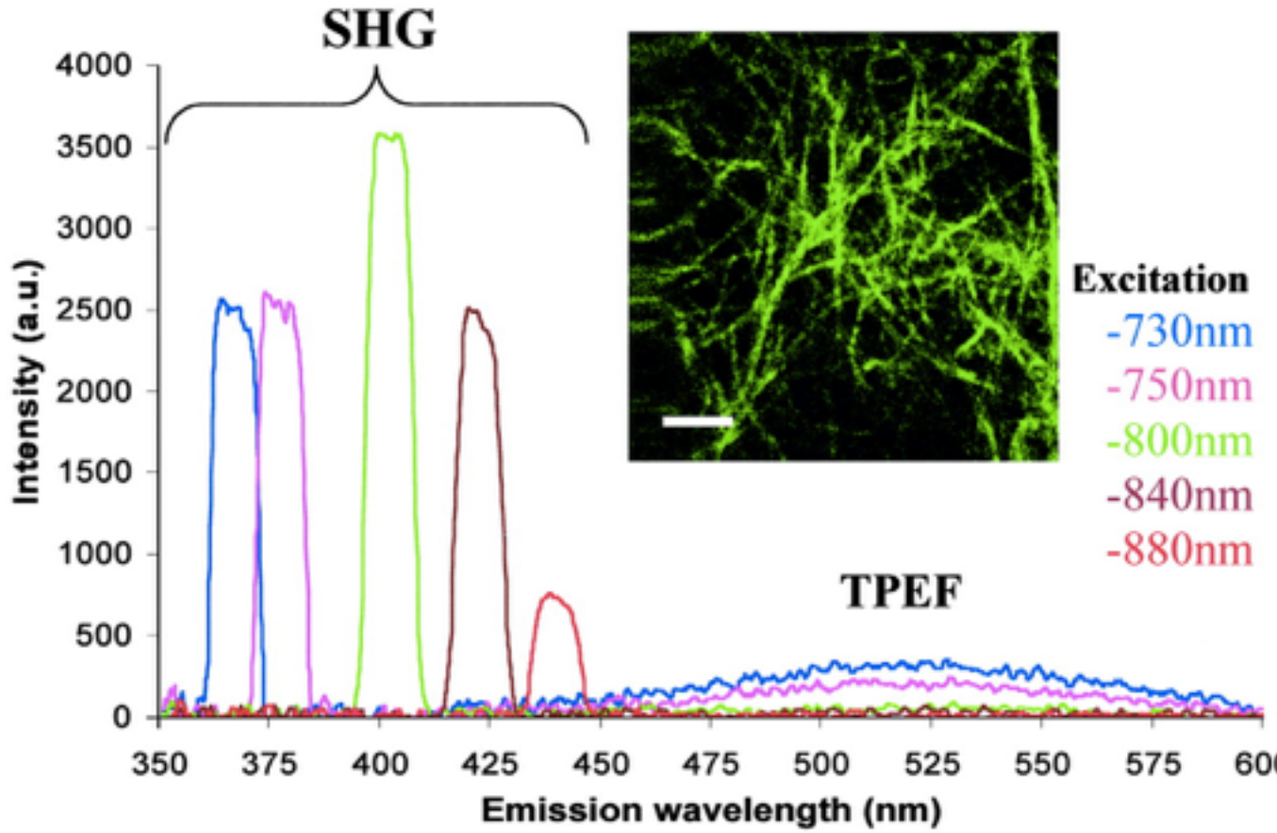


2nd Harmonic signals

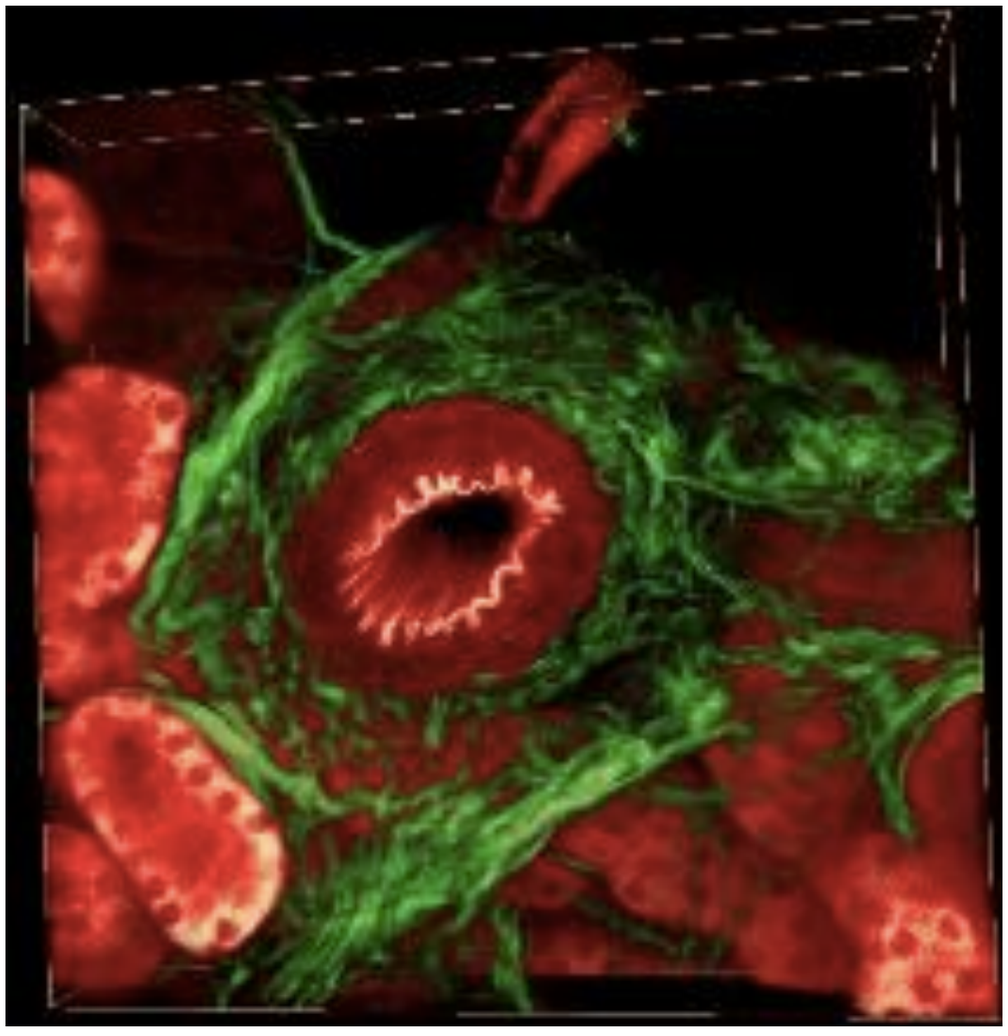
- 2nd Harmonic generation: Two photons of same frequency interact with a non-linear material and are combined into one photon of twice the energy (double frequency or half the wavelength)

- Only non-centrosymmetric molecules (e.g. helical fibers) can do this: Collagen, Cellulose, some membrane dyes

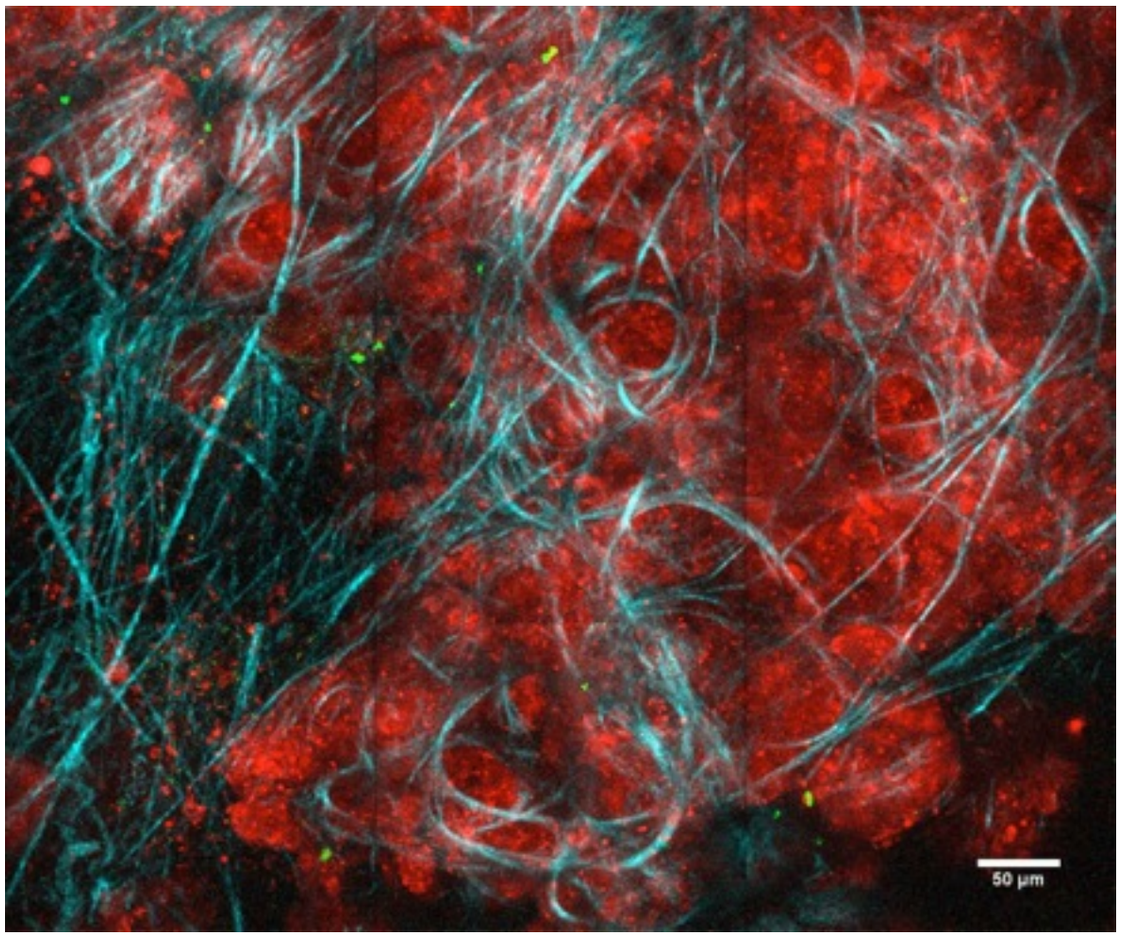
- These molecules light up at λ/2 of the excitation wavelength



2nd Harmonic signals



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



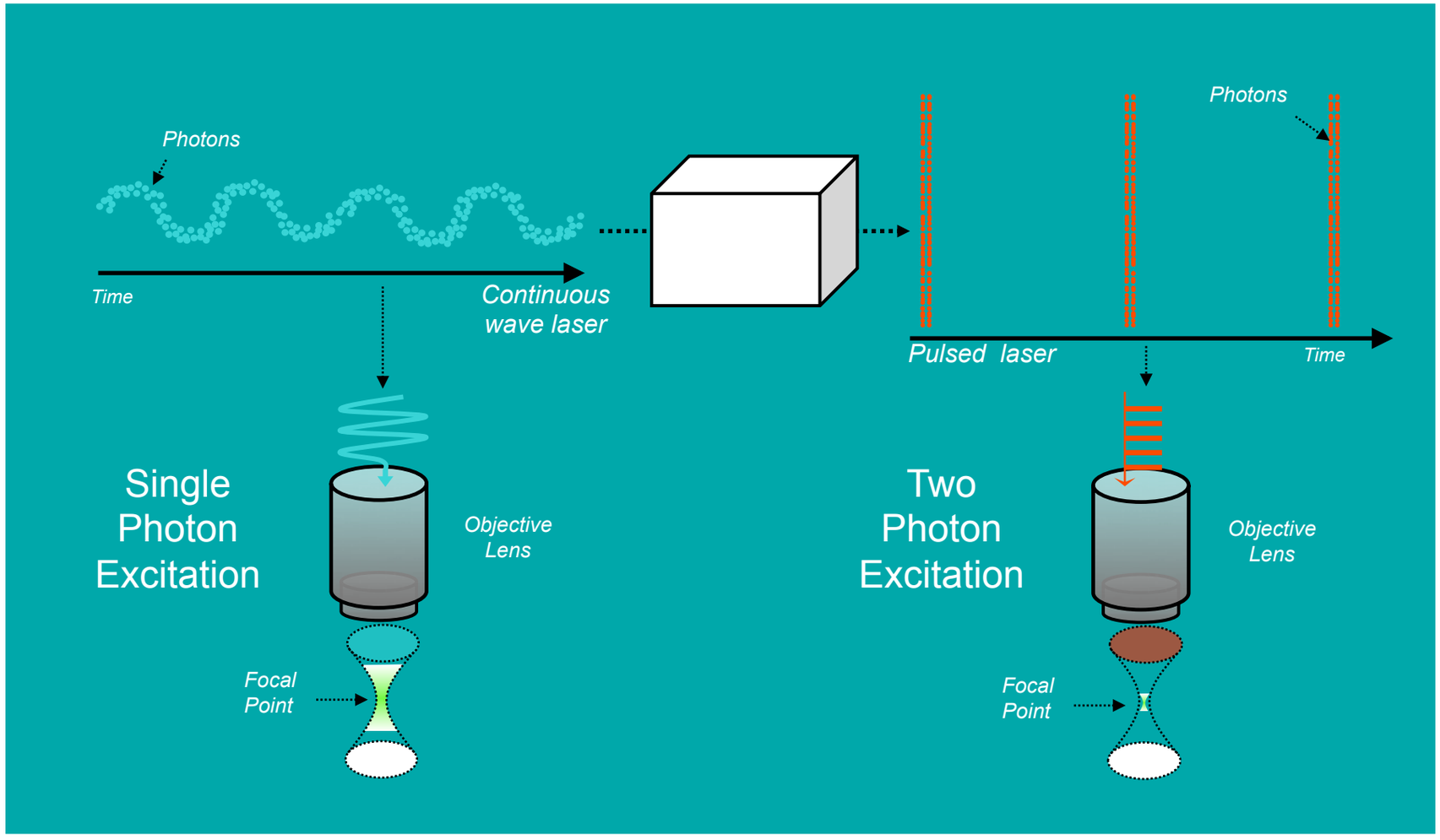
collagen in breast tumors
Krummel Lab, UCSF

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?

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

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/mm2**

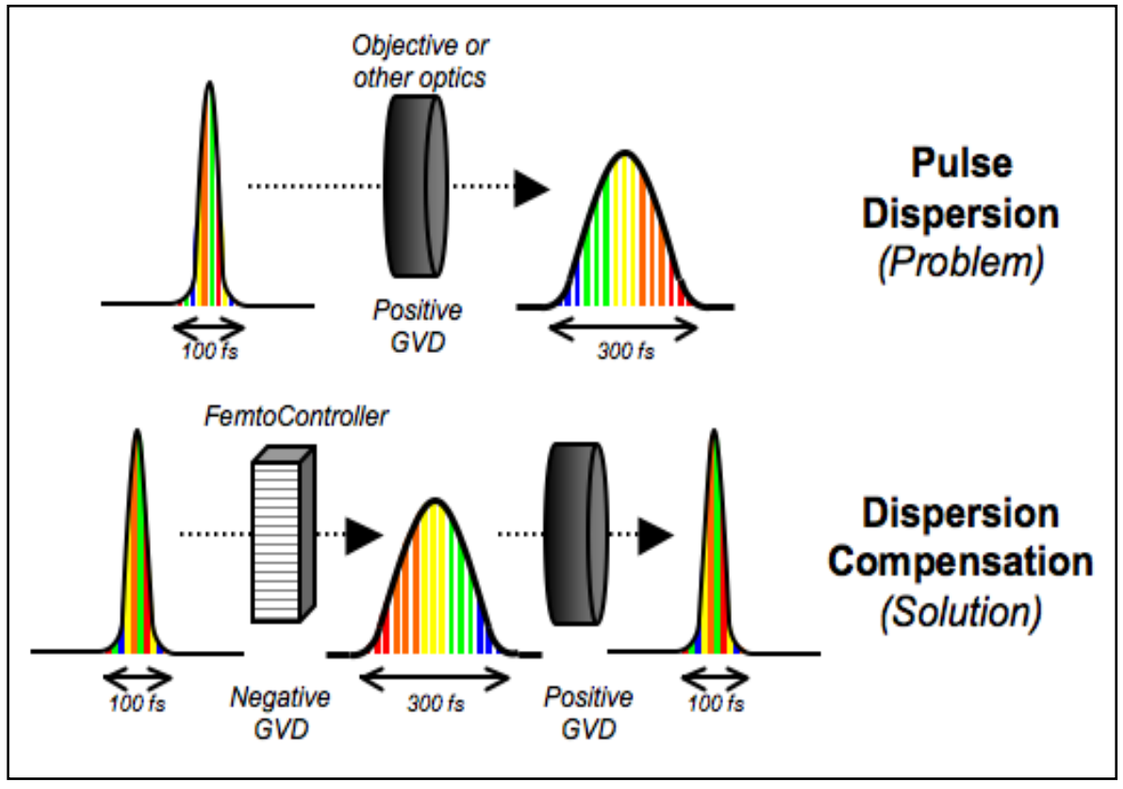
- A 2P laser is pumped by a 5-20W pump resulting up to 1W of pulsed power at the objective which is focused to a spot size of ~0.5um diameter. This is a power density of **~5MW/mm2**

- However it has a duty cycle of ~10^{-5} (the 'duty cycle' is pulsewidth x rep rate). Typical pulsewidths are around 70-200fs (fs = 10^{-15}s) and the rep-rate is around 80-100MHz.

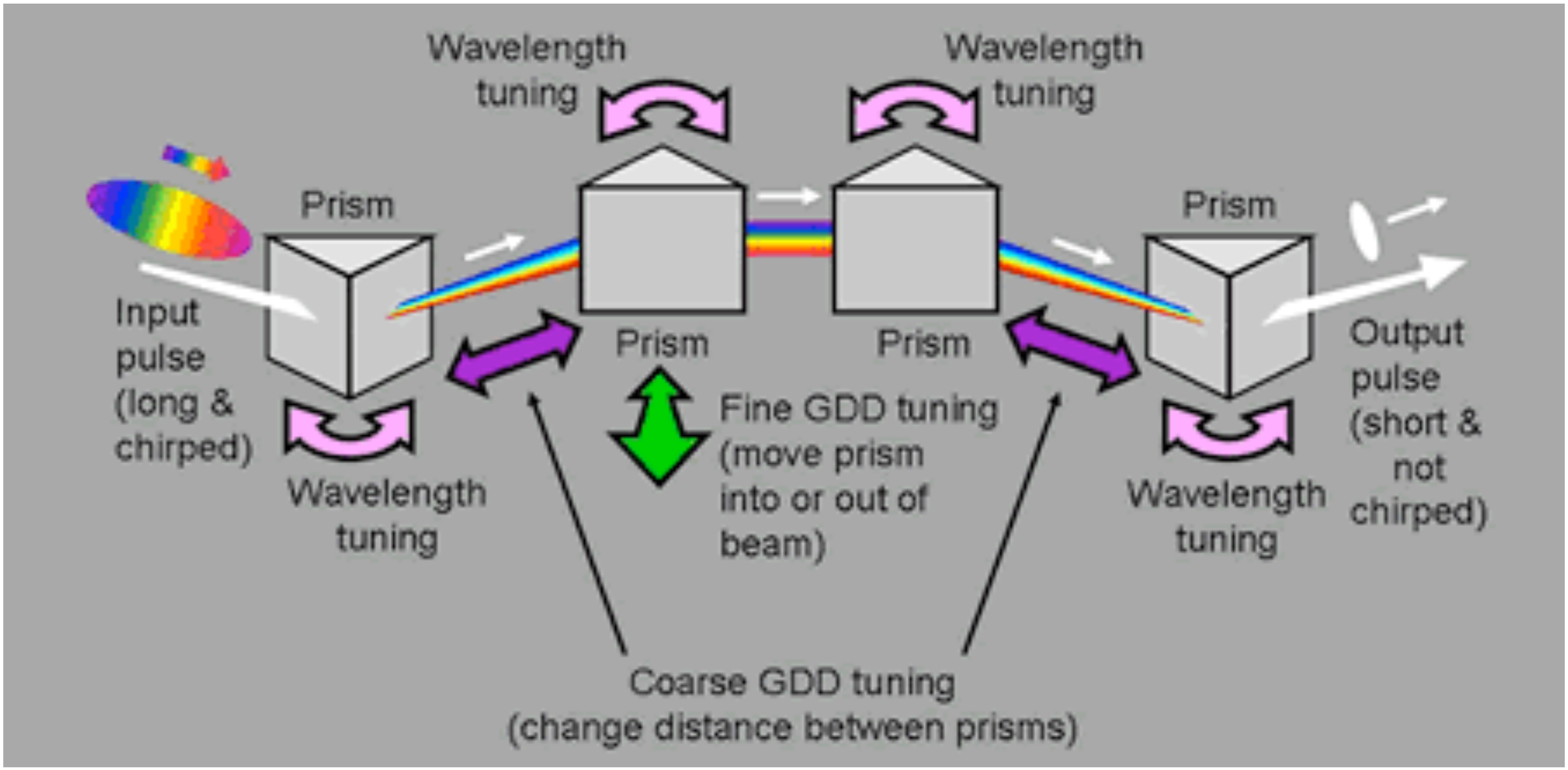
- If this were on 'continuous' it would consume 100kW, equating to consumption of about 100 homes worth of power. More impressive, it would be putting out 5x10^{11}W/mm2 or **~10^{11} times the power density compared to a soldering iron.**

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)



Pulsed laser dispersion



Fluorescence detection in 2P

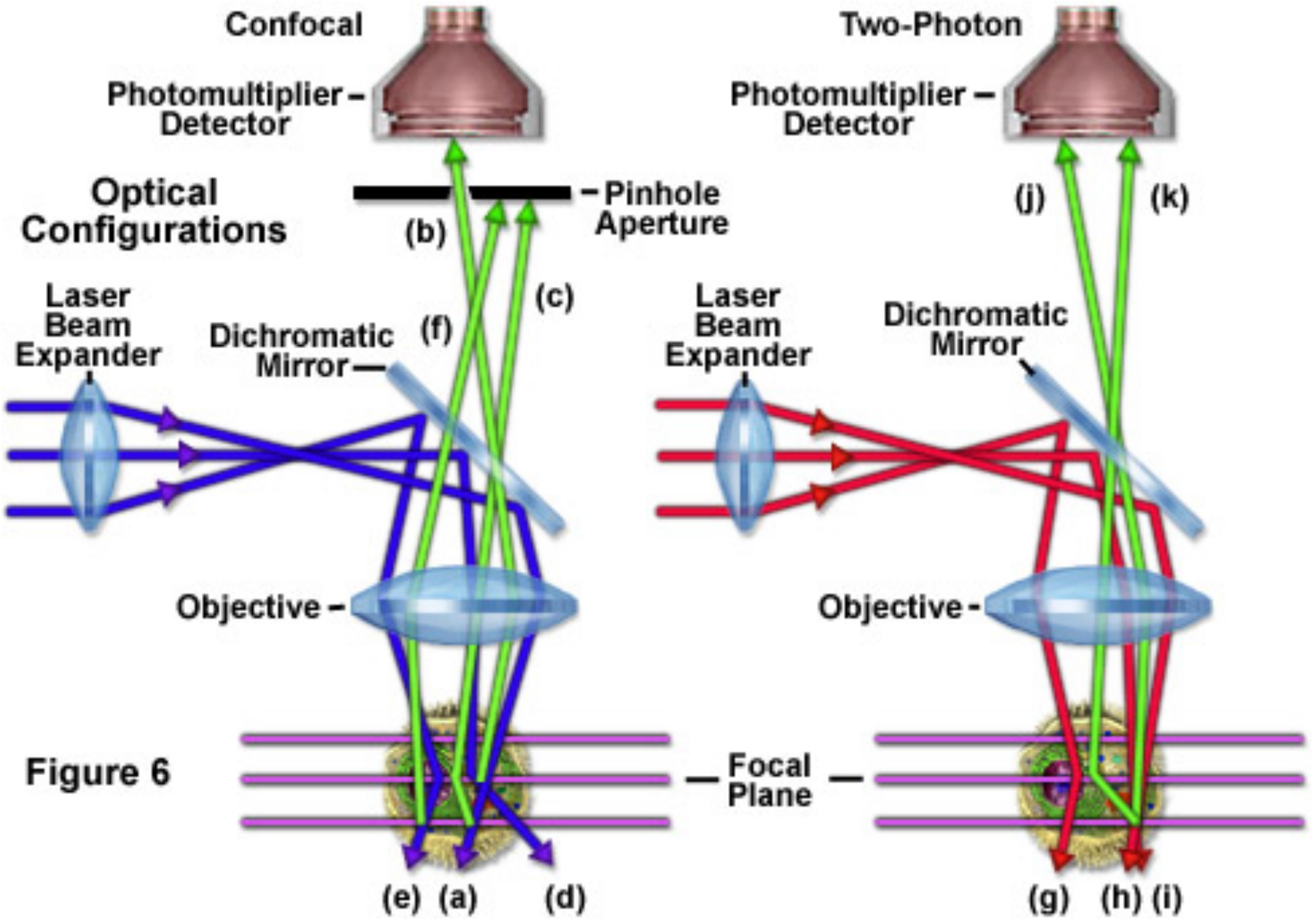
- 1P excitation creates cone of light → elimination of out-of-focus excitation using pinhole before detection PMT

- 2P excitation only illuminates at focal plane → no need to eliminated scattered light

- BUT: Point illumination → time-resolved scanning detection only

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



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

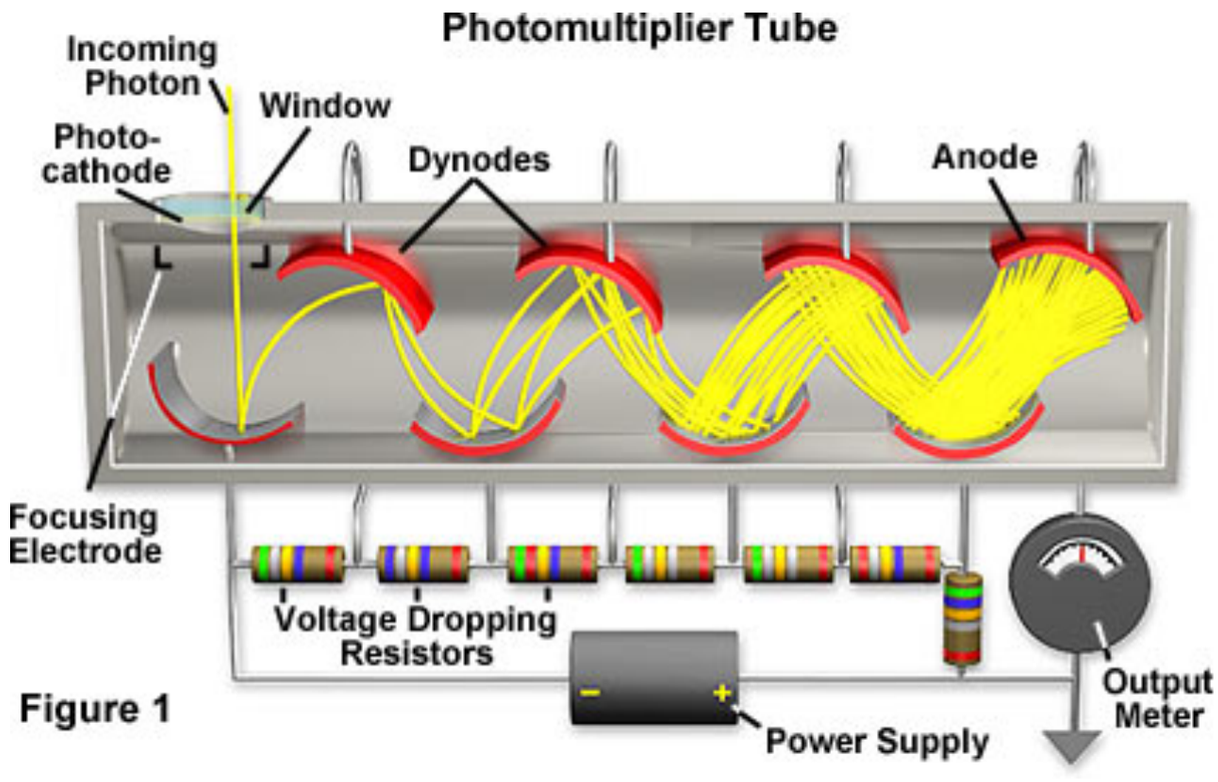
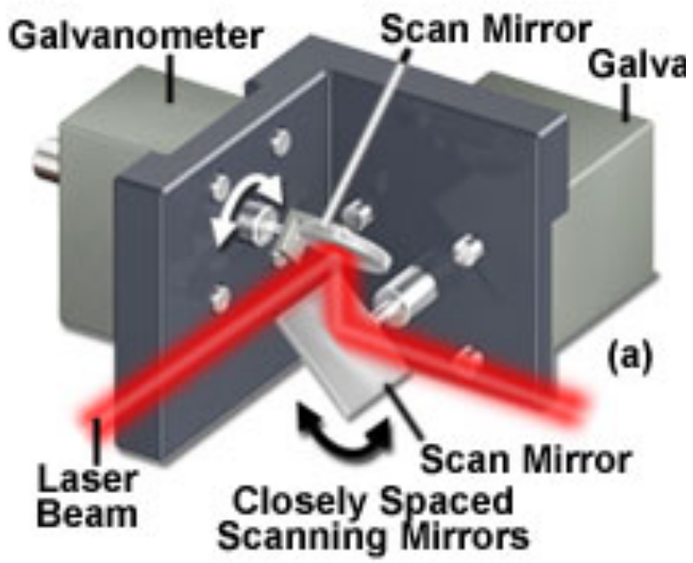
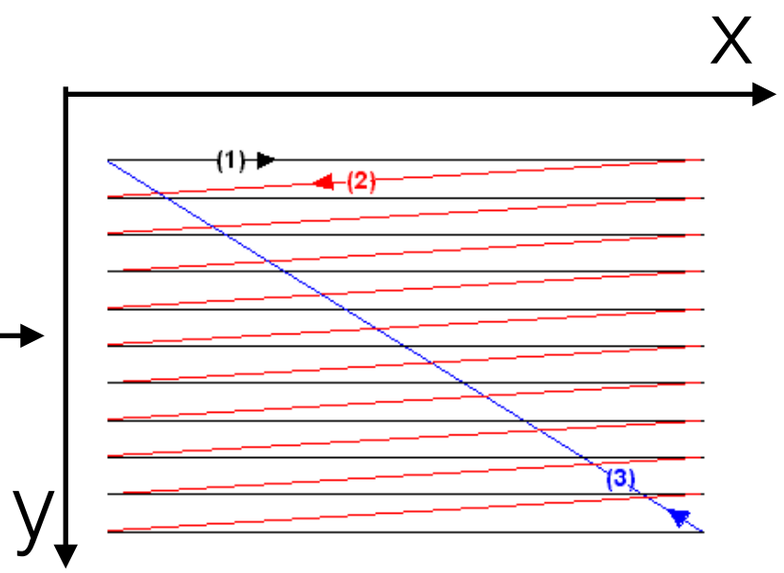


Figure 1

Assembling the image

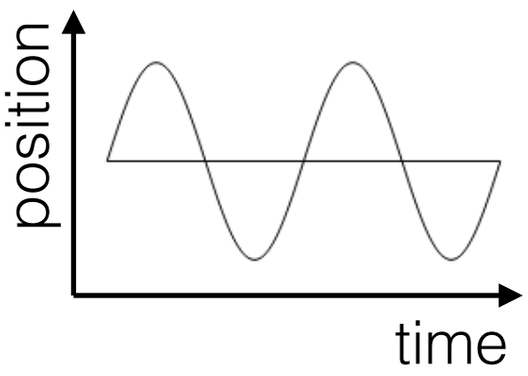
- Focal illumination requires rapid scanning across the specimen



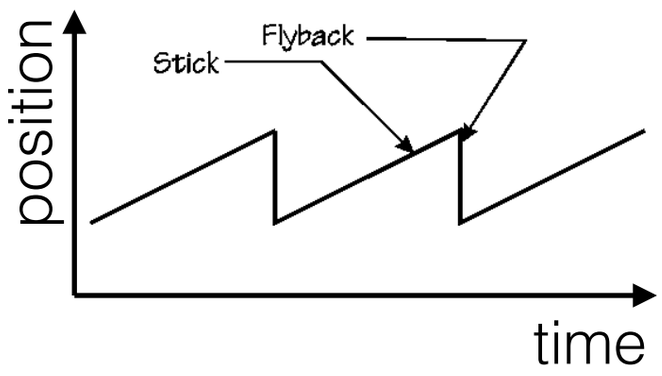


- xy-position on specimen is determined by elapsed time from beginning of scan

x-axis mirror



y-axis mirror



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

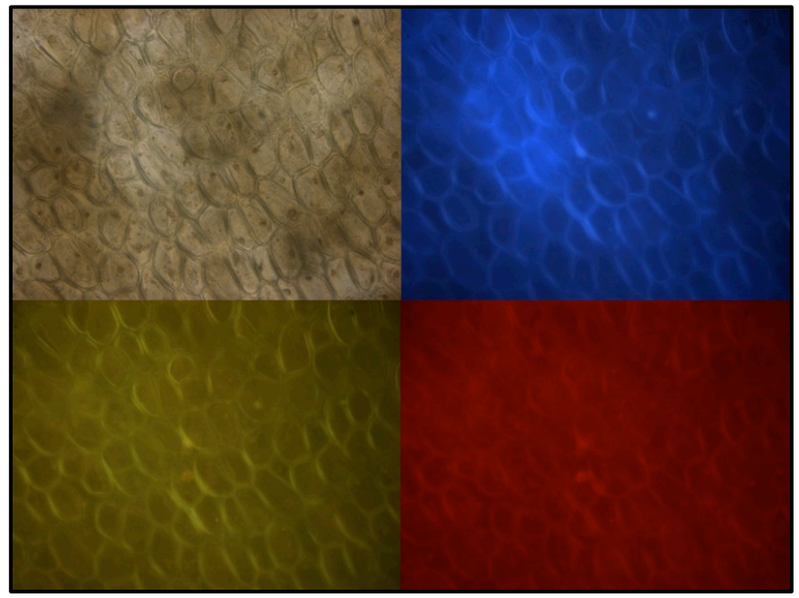
- In a resonant scanning system we built (30fps), a 0.6um pixel integrates about 7 pulses 'on the fly' (as the laser sweeps through this voxel which takes about 1us). We then may integrate 10 frames (sweeps) that each give the sample 1/30th of a second to 'recover'.

- 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).

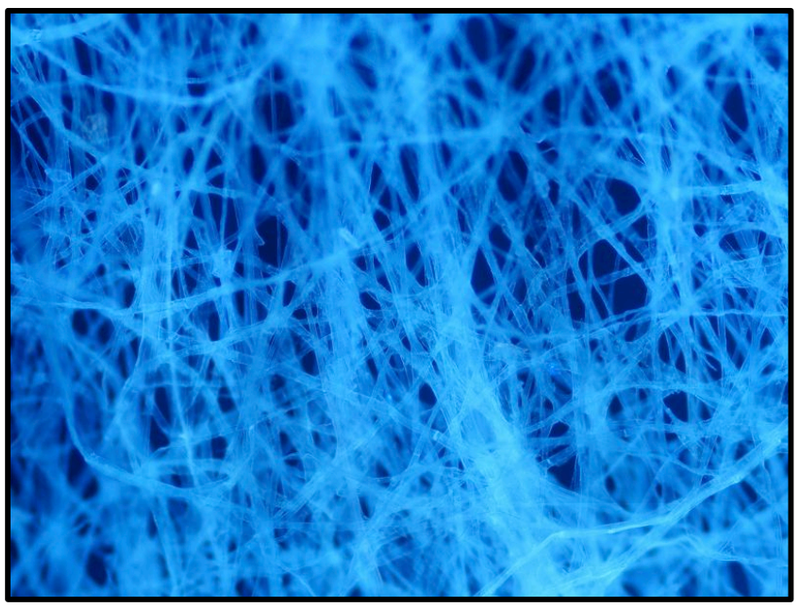
Dealing with autofluorescence

- 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)



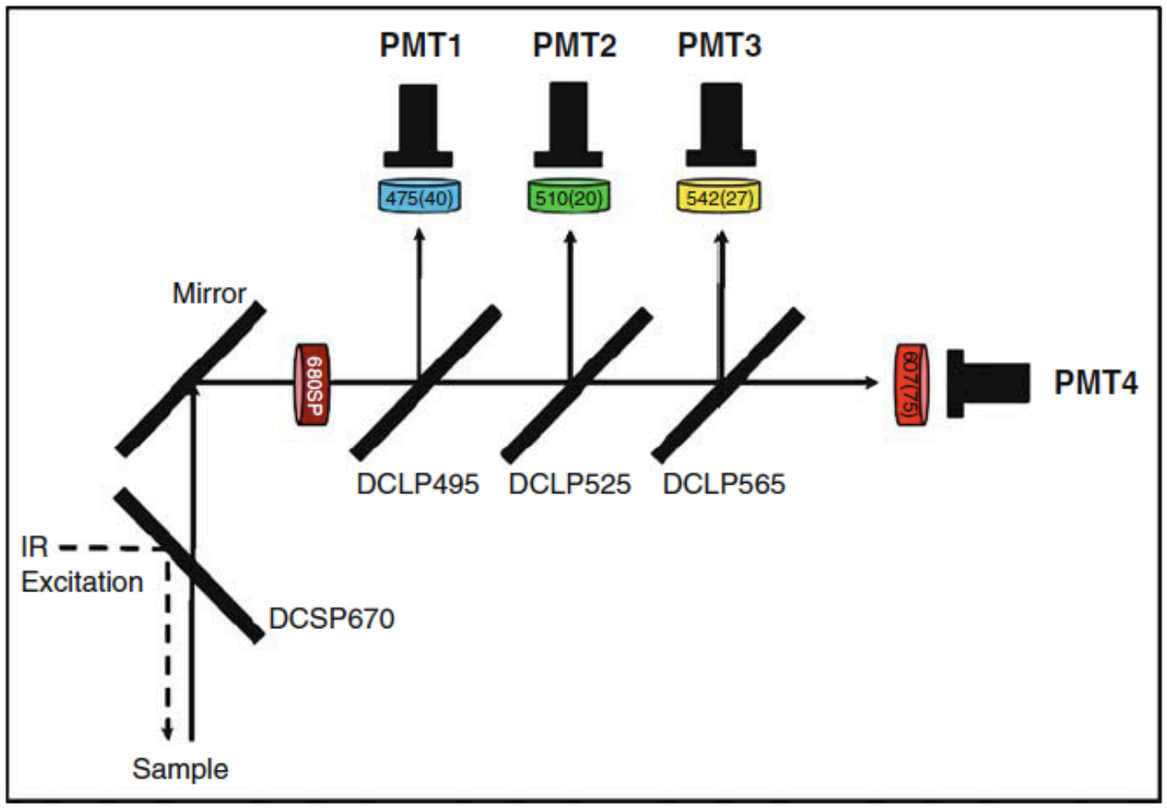
Paper Autofluorescence (Wikipedia)

Dealing with autofluorescence

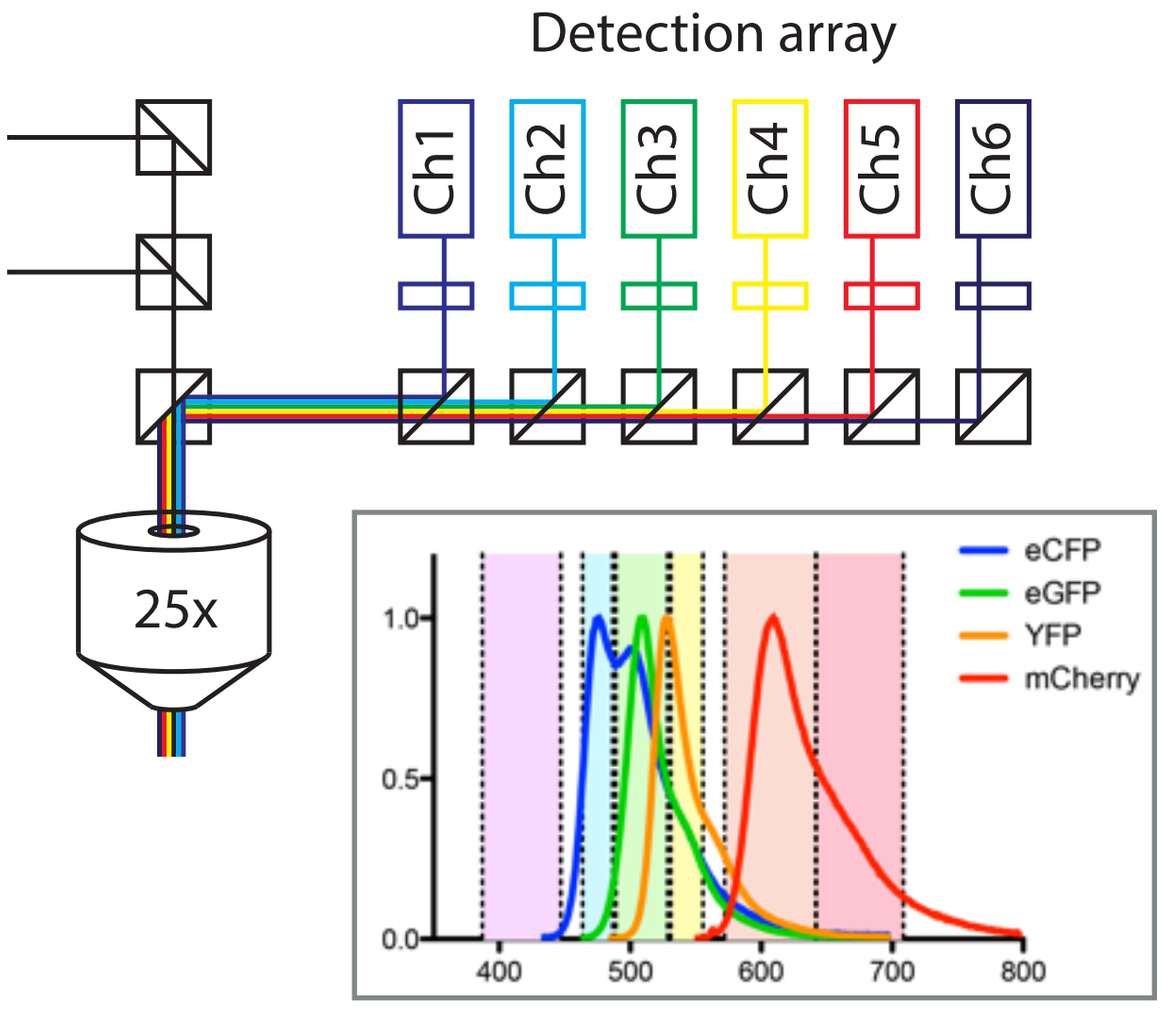
Molecule	Excitation (nm)	Fluorescence (nm)	Organisms	Reference
NAD(P)H	260	450	All	[6]
Chlorophyll	465, 665	673, 726	Plants	
Collagen	270-370	305-450	All	[6]
Retinol		500	Animals & bacteria	[7]
Riboflavin		550	All	[7]
Cholecalciferol		380-460	Animals	[7]
Folic acid		450	All	[7]
Pyridoxine		400	All	[7]
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]

Wikipedia

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



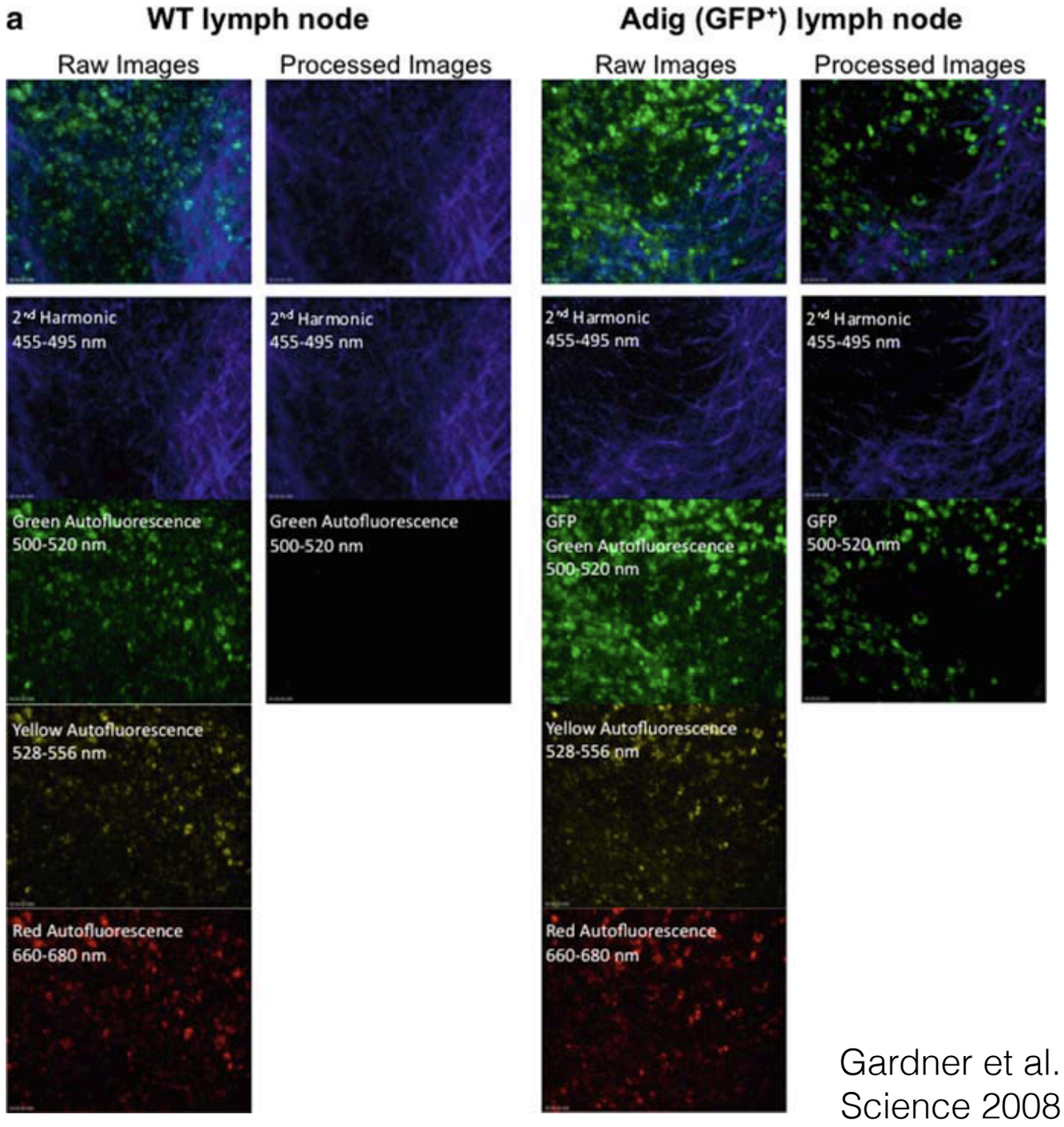
Generation 1



Generation 3

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



Gardner et al. Science 2008

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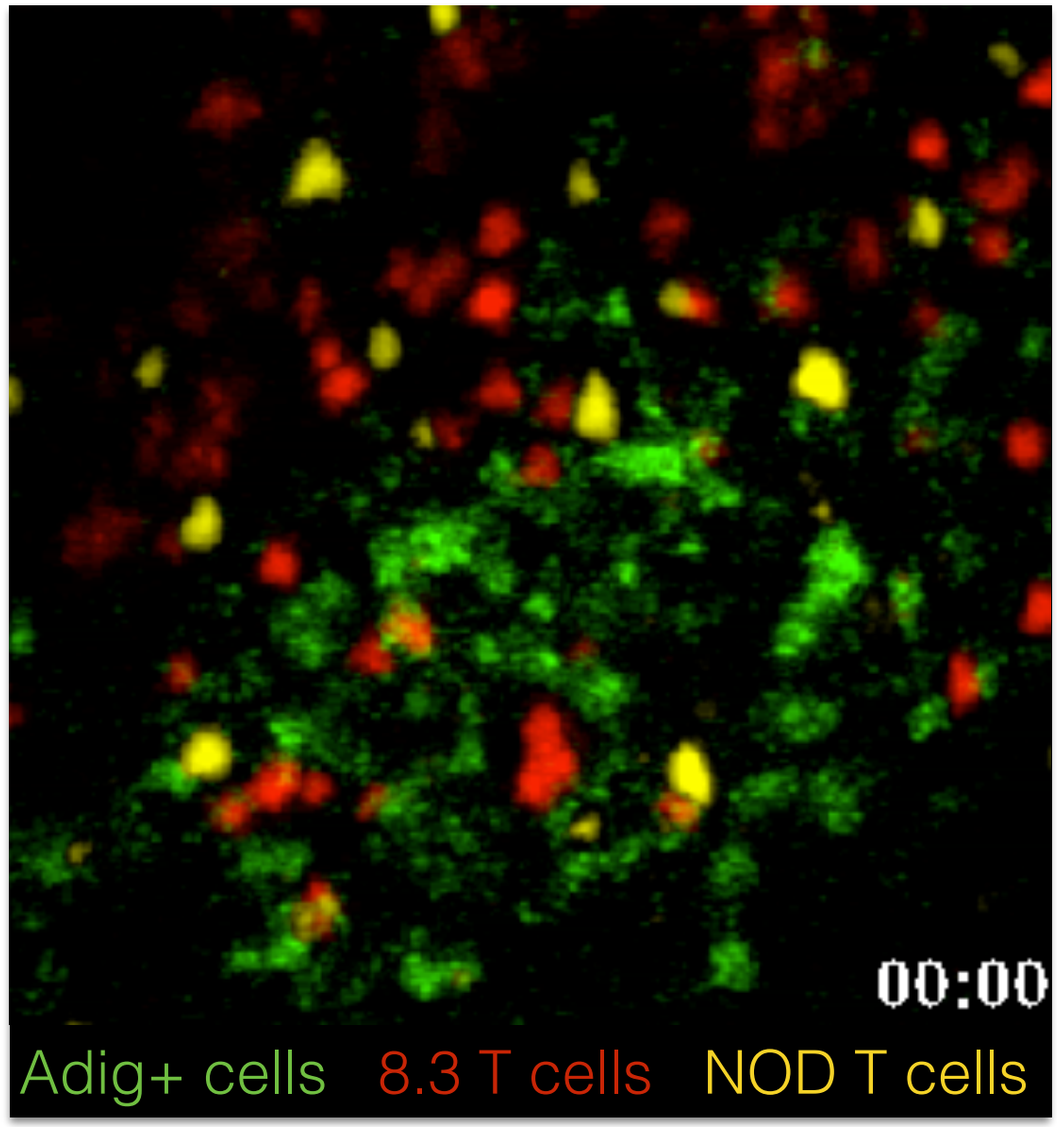
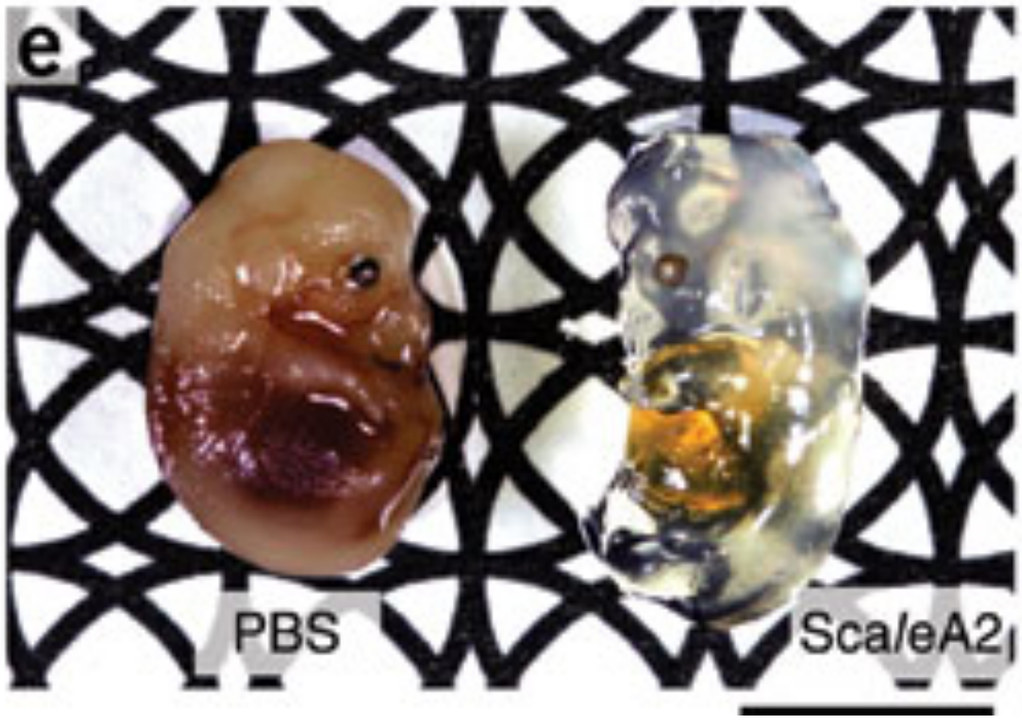
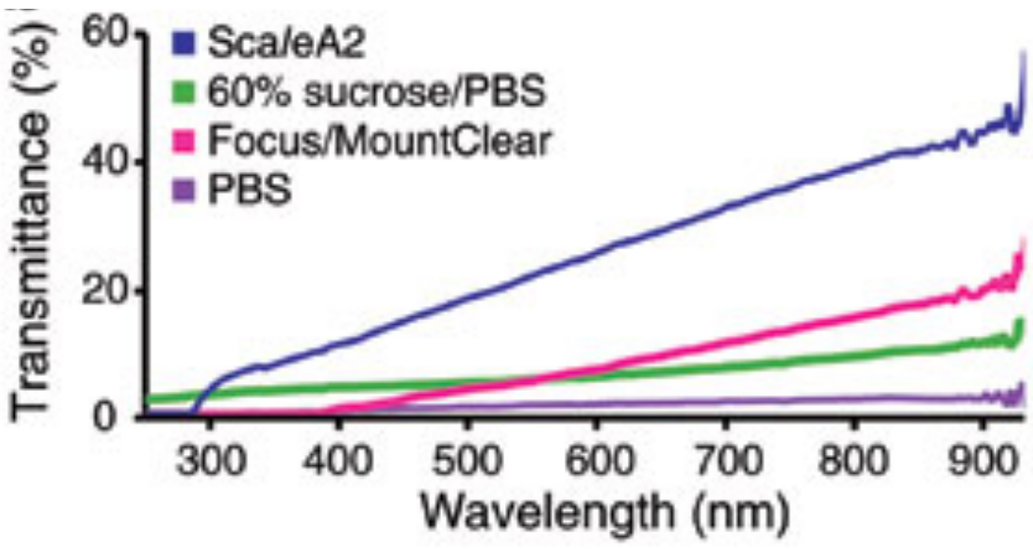
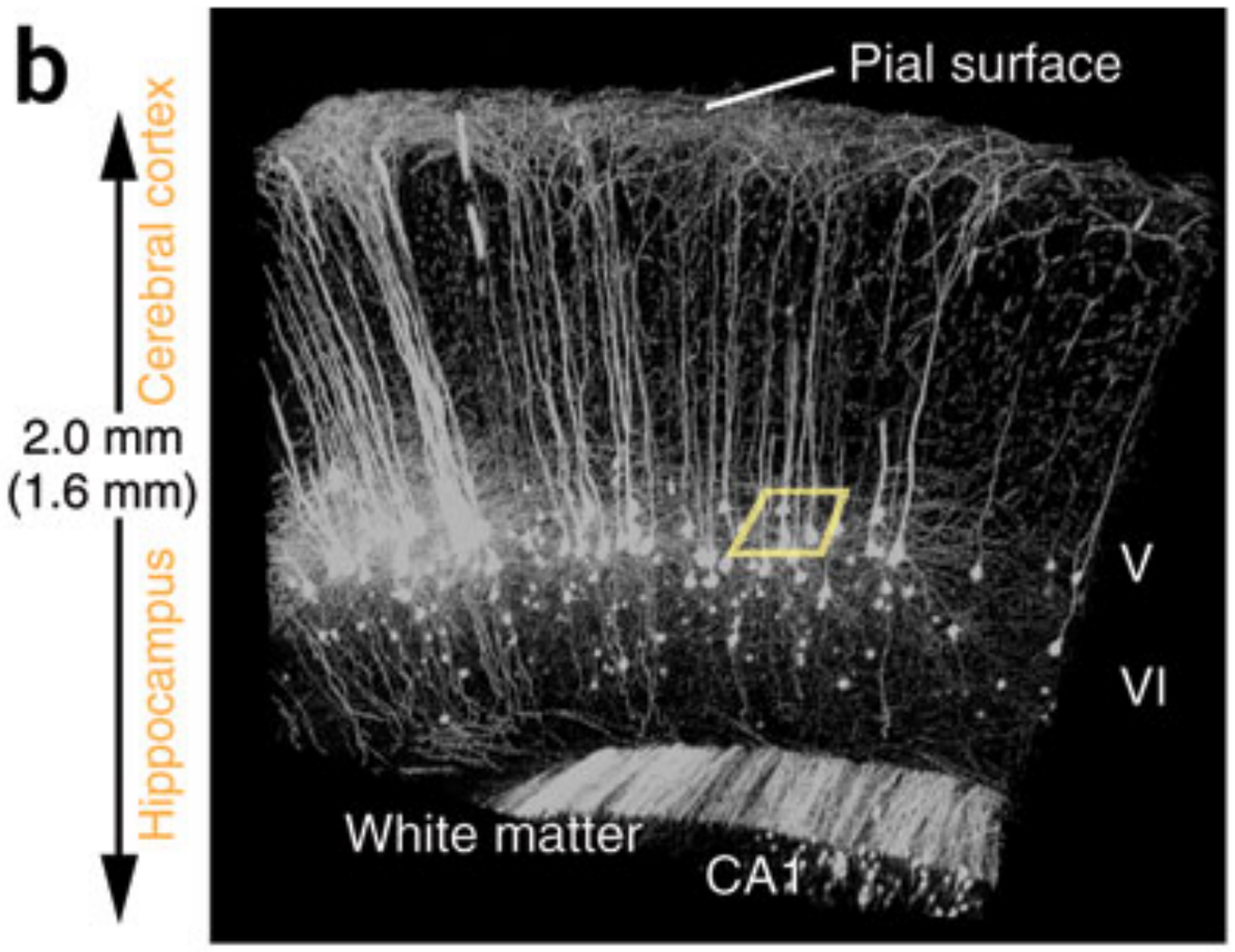


Gardner et al.
Science 2008

Clearing agents: Sca*l*eA2

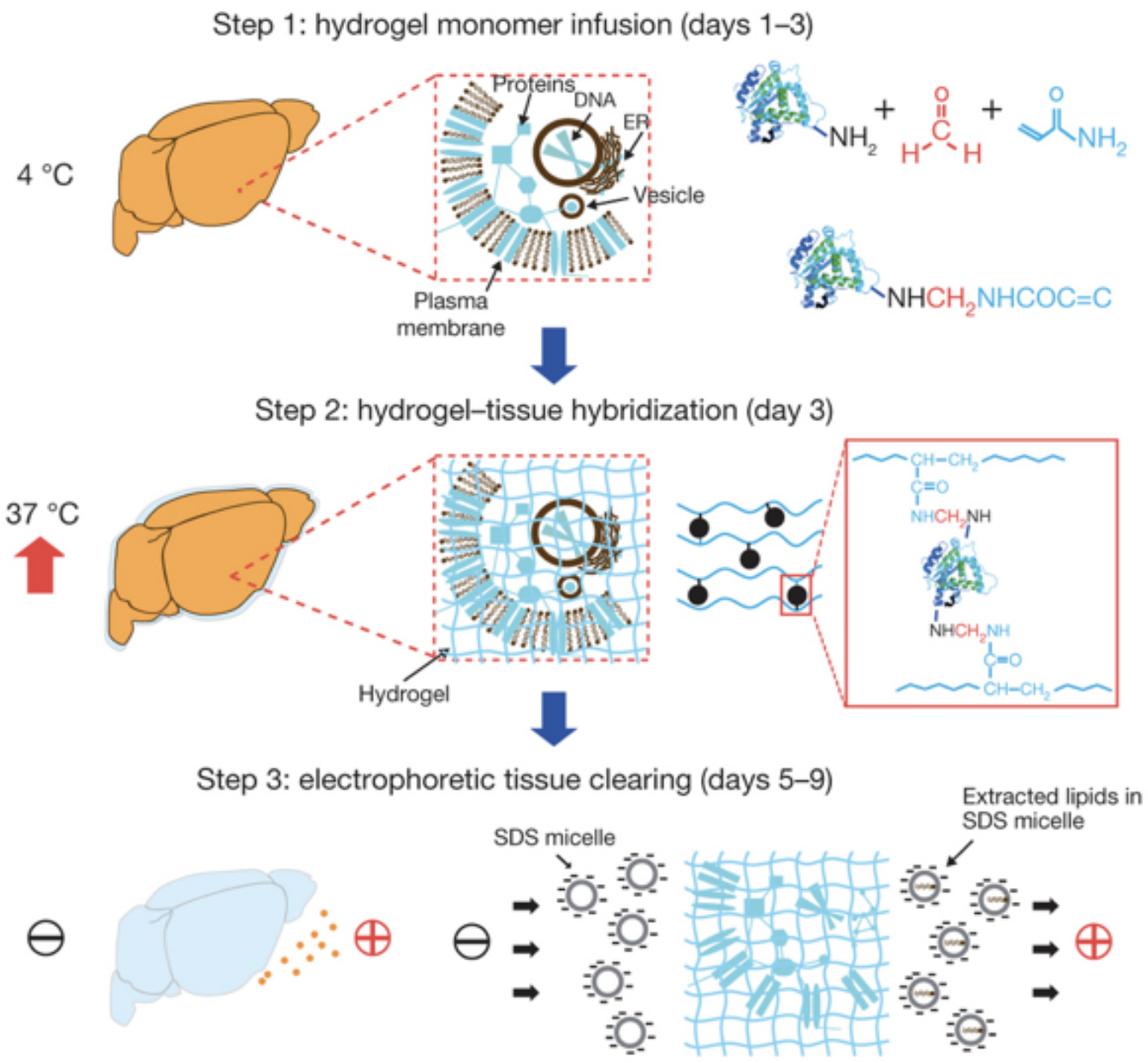


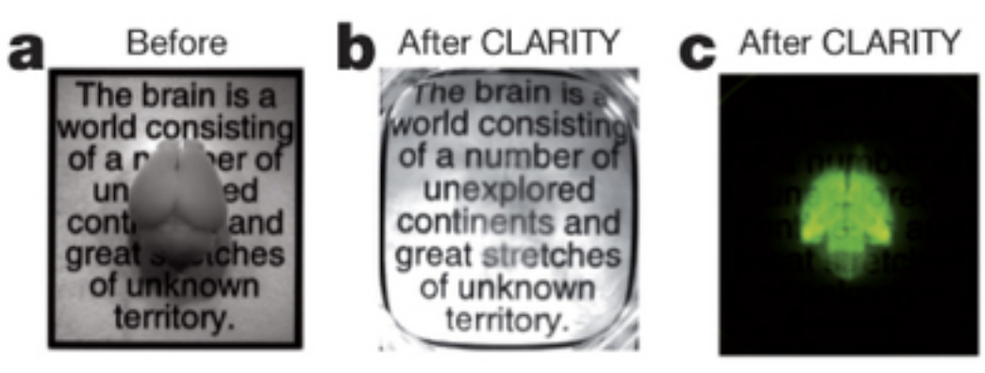


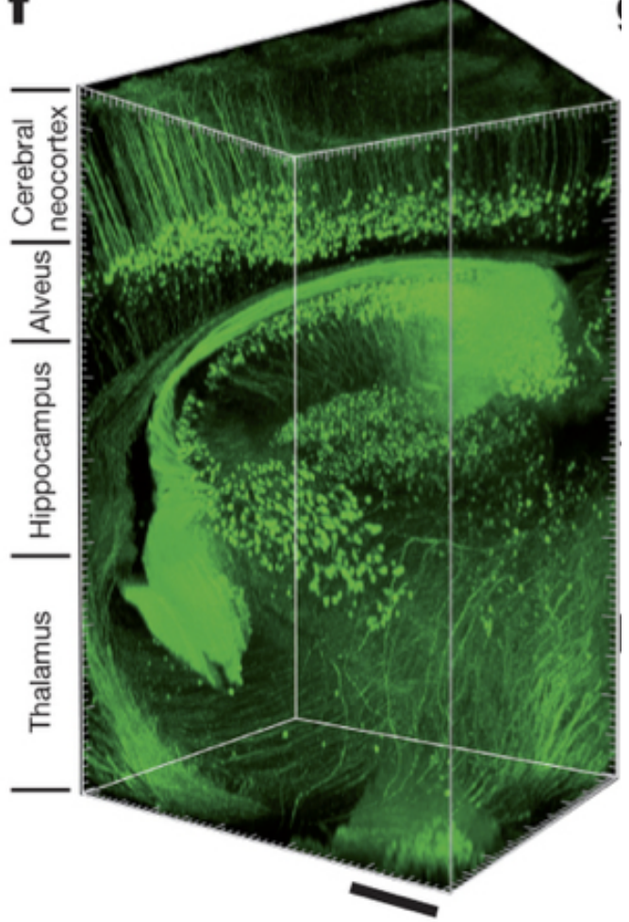


Miywaki et al., Nature Neuroscience 2011

Clearing agents: CLARITY



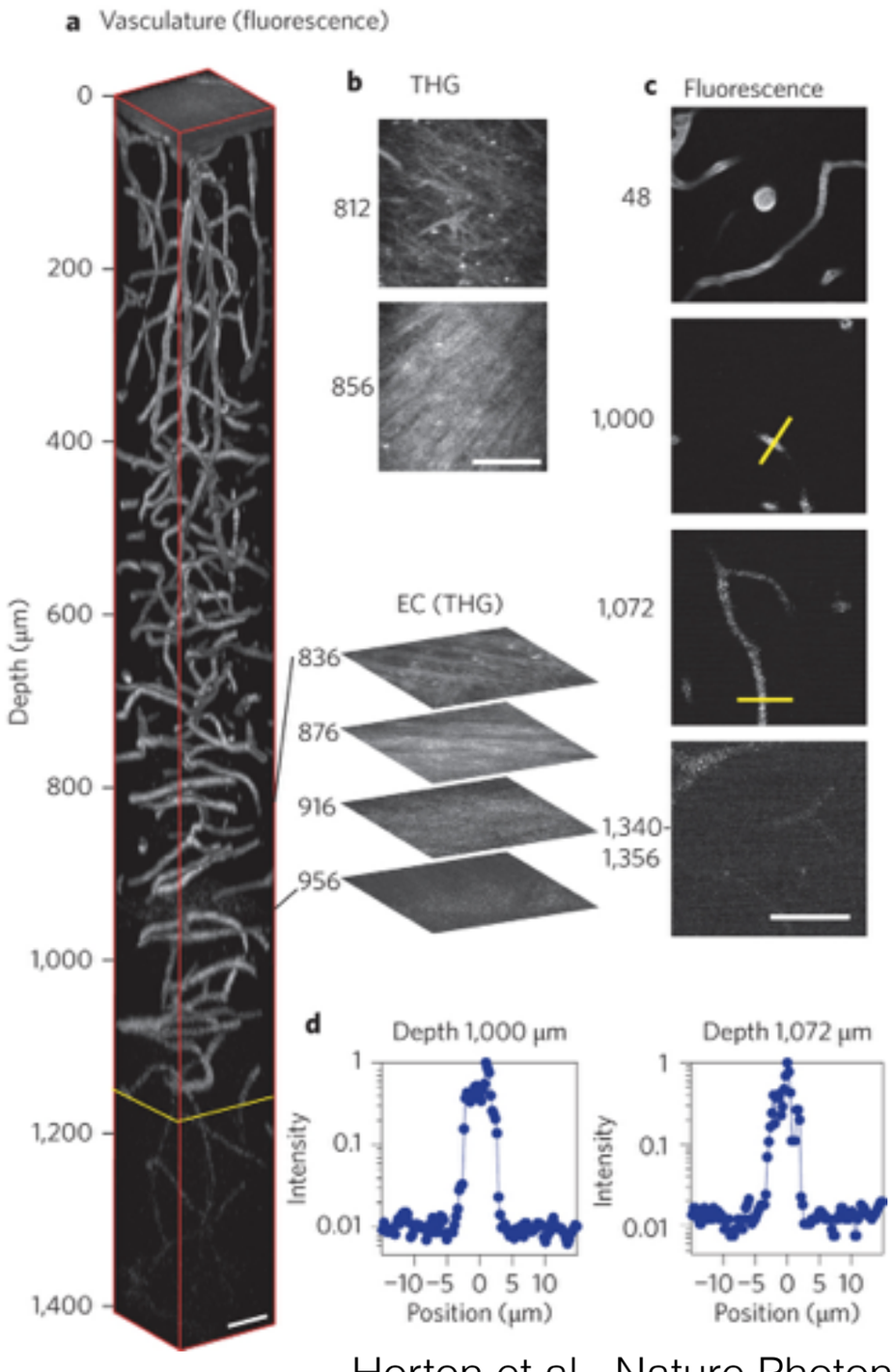




Chung et al., Nature 2013

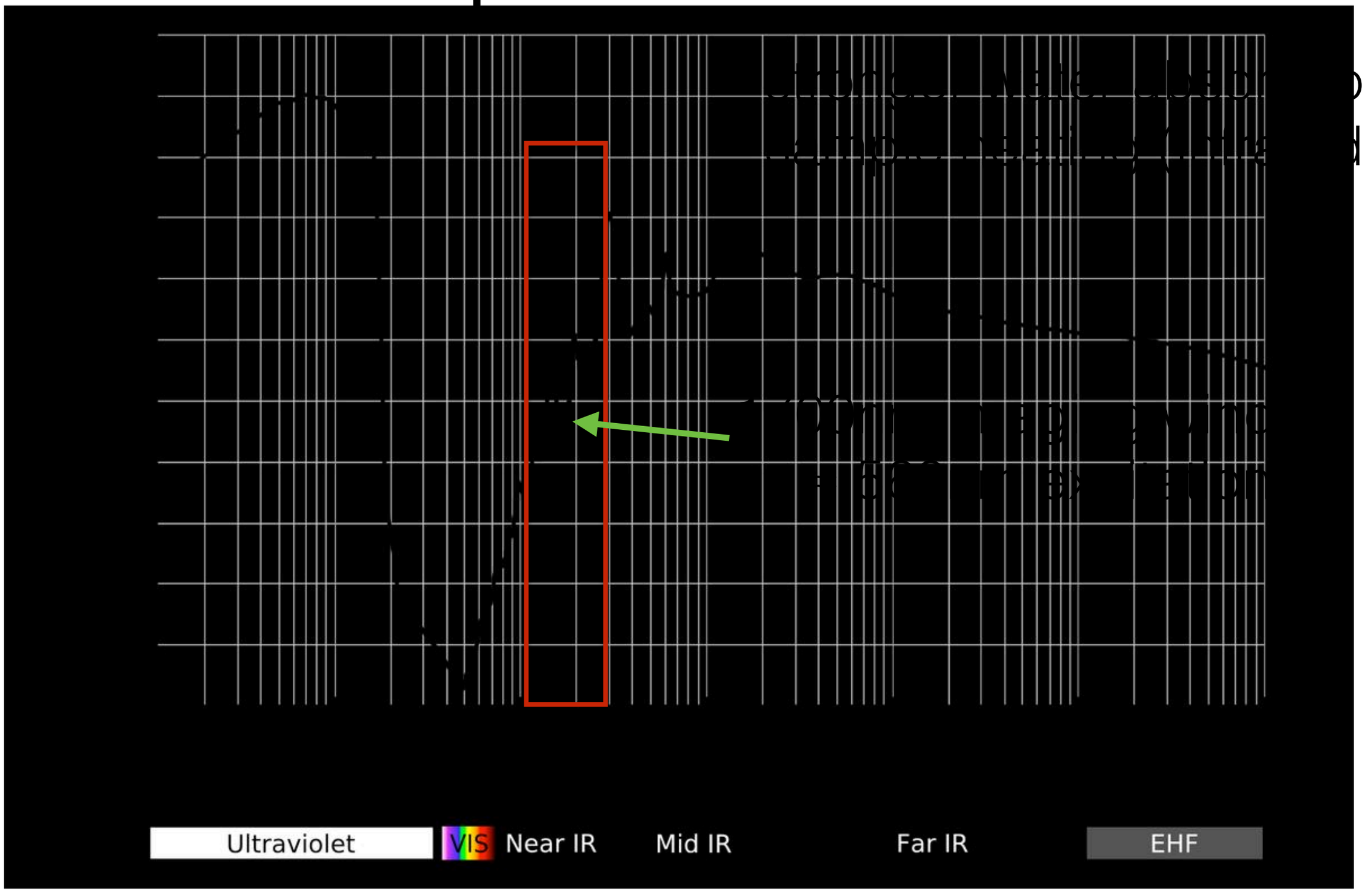
3P and beyond

- **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



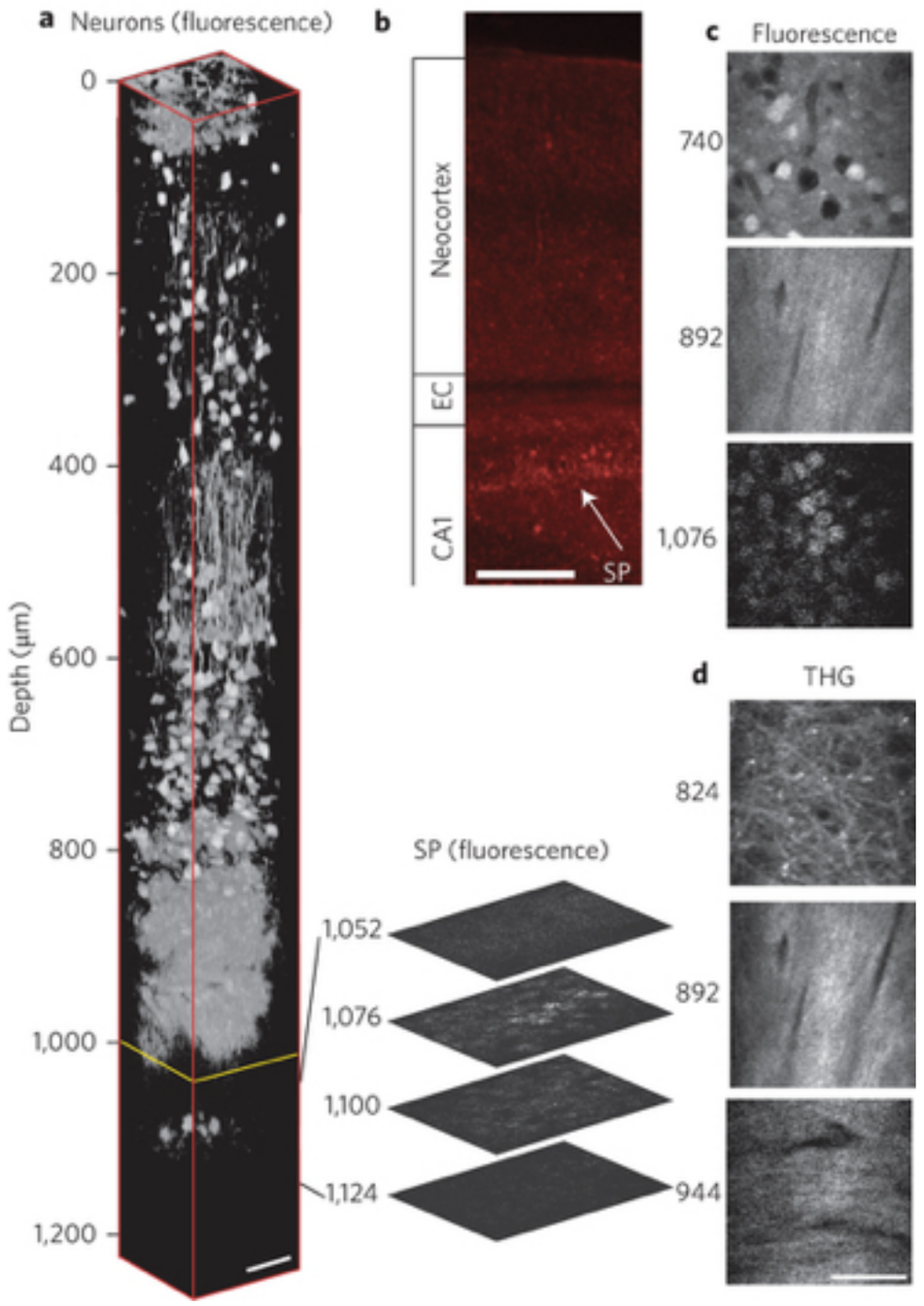
Horton et al., Nature Photonics 2013

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

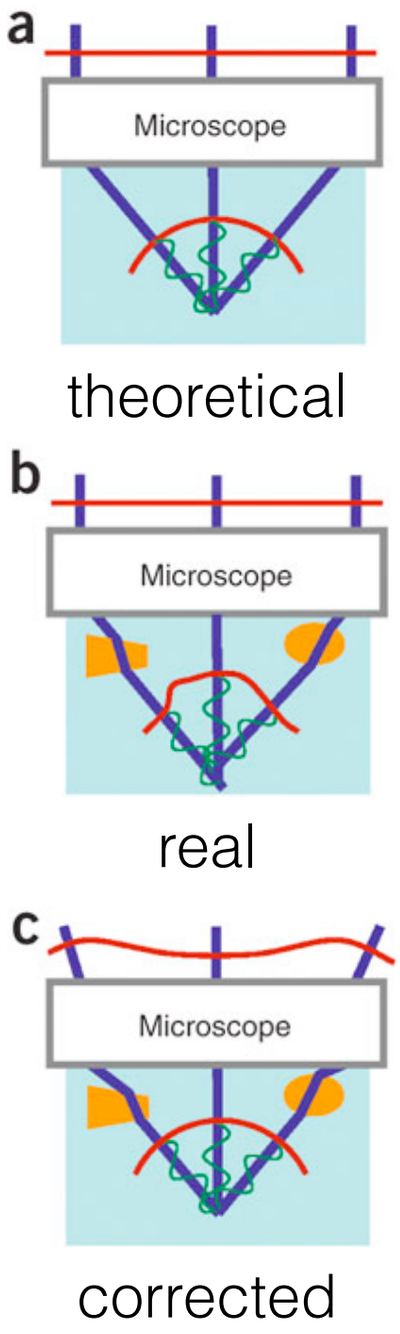


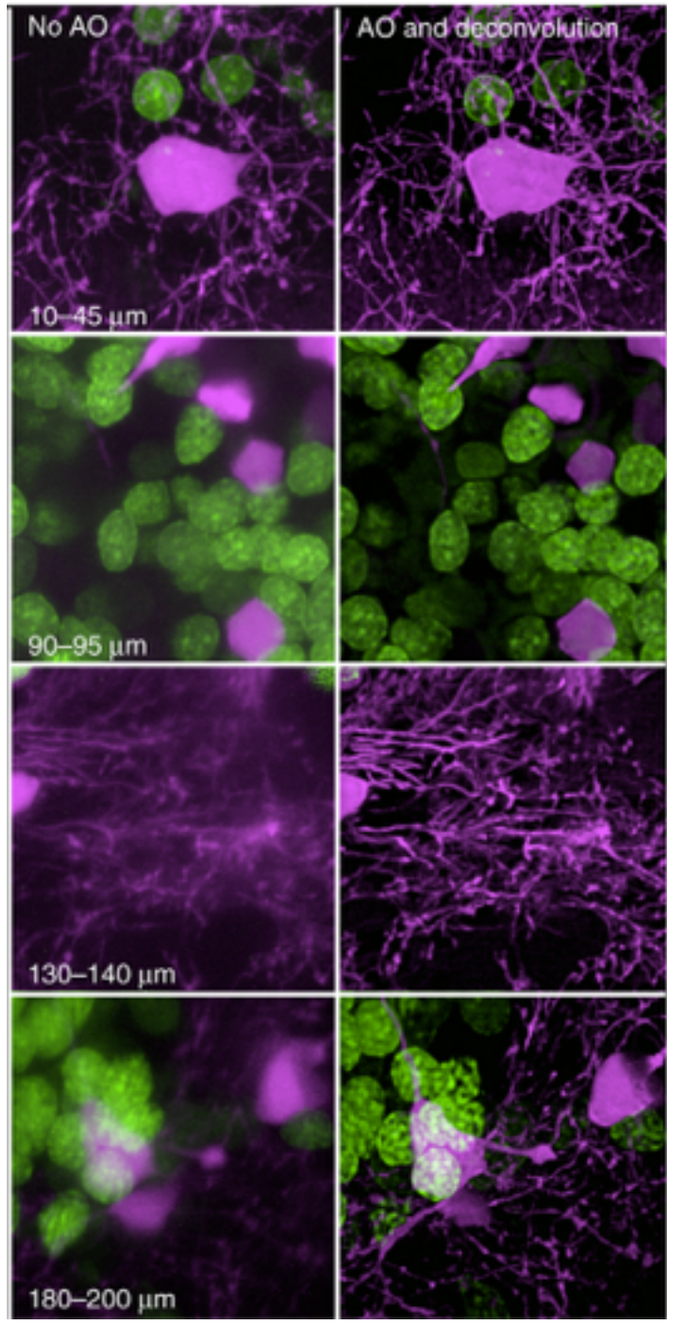
Horton et al., Nature Photonics 2013

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





Wang et al., Nature Methods 2014

Further Reading

Nikon Microscopy U
http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html

Kurt Thorn's iBiology 2P lecture
https://www.youtube.com/watch?v=CZifB2aQDDM

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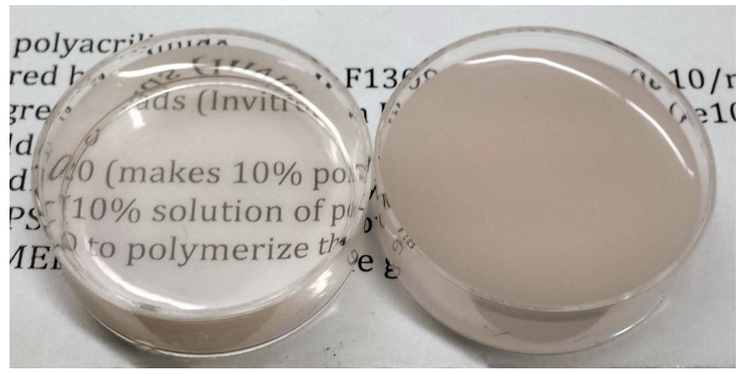
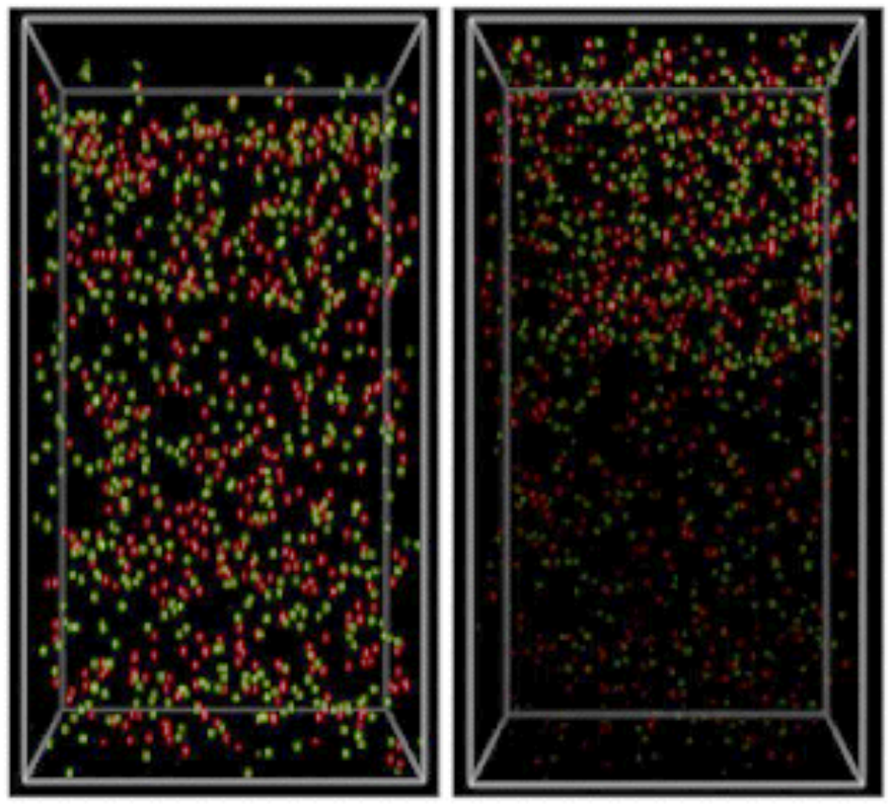
Comparison depth penetration 1P vs 2P

Dispersive beads mimic biological specimens



Compare 1P vs 2P performance

Possibly: Commercial vs custom-built microscopes



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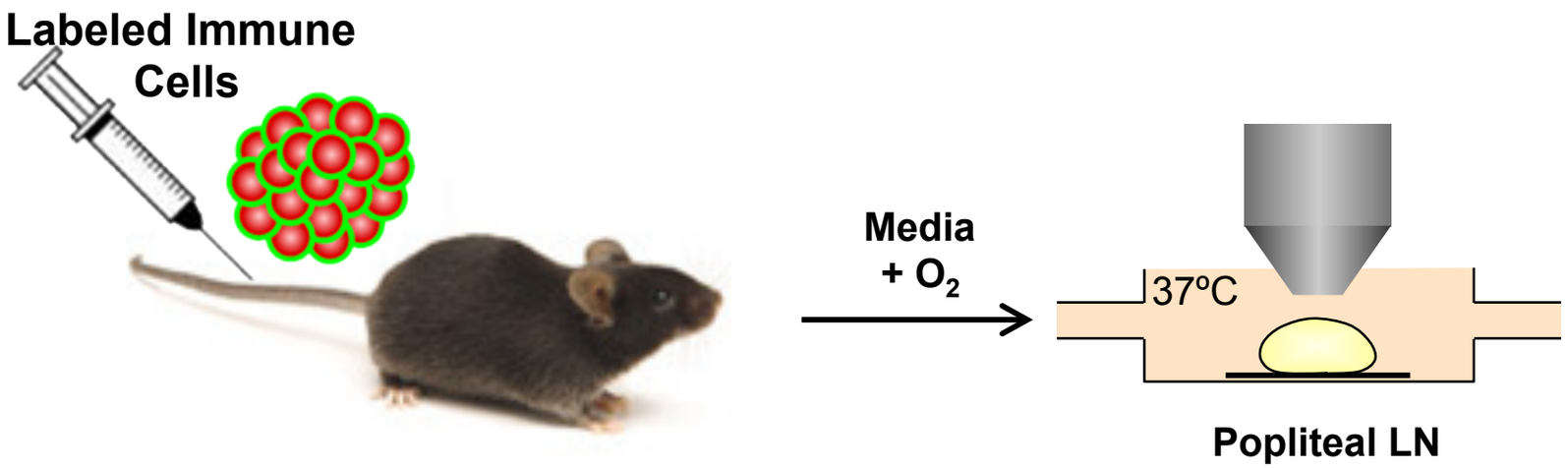
Imaging of biological specimens ex vivo



whole LN can be imaged

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

Critical Parameter
Seems to be Slice Thickness
(Oxygen Tension)

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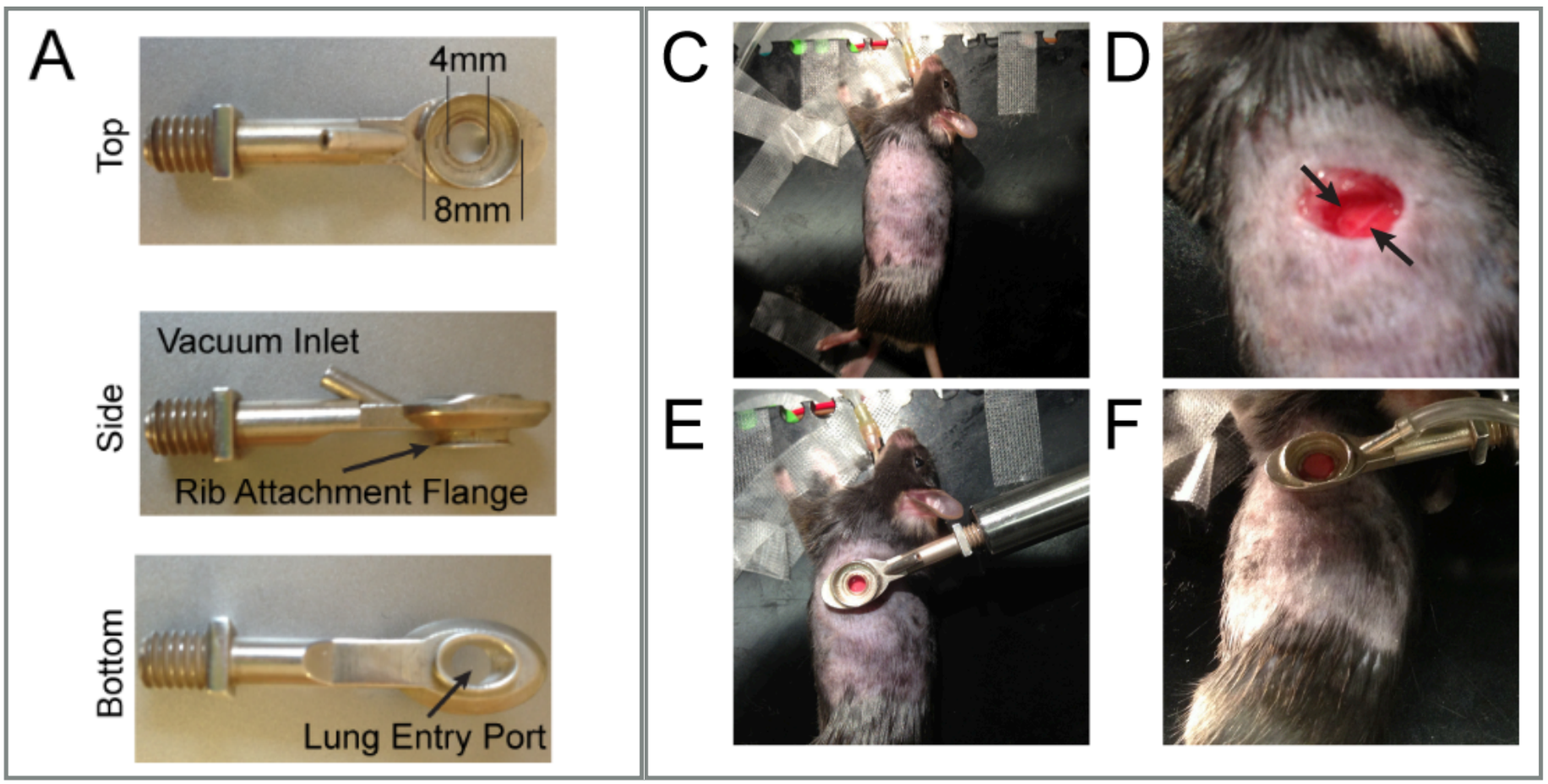
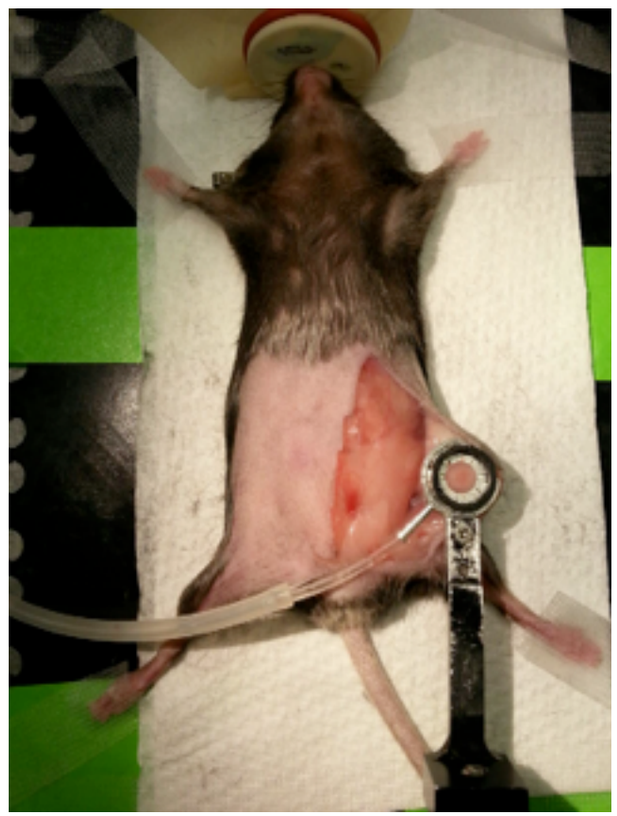
Imaging of biological specimens in vivo



Lung imaging



breast/tumor imaging

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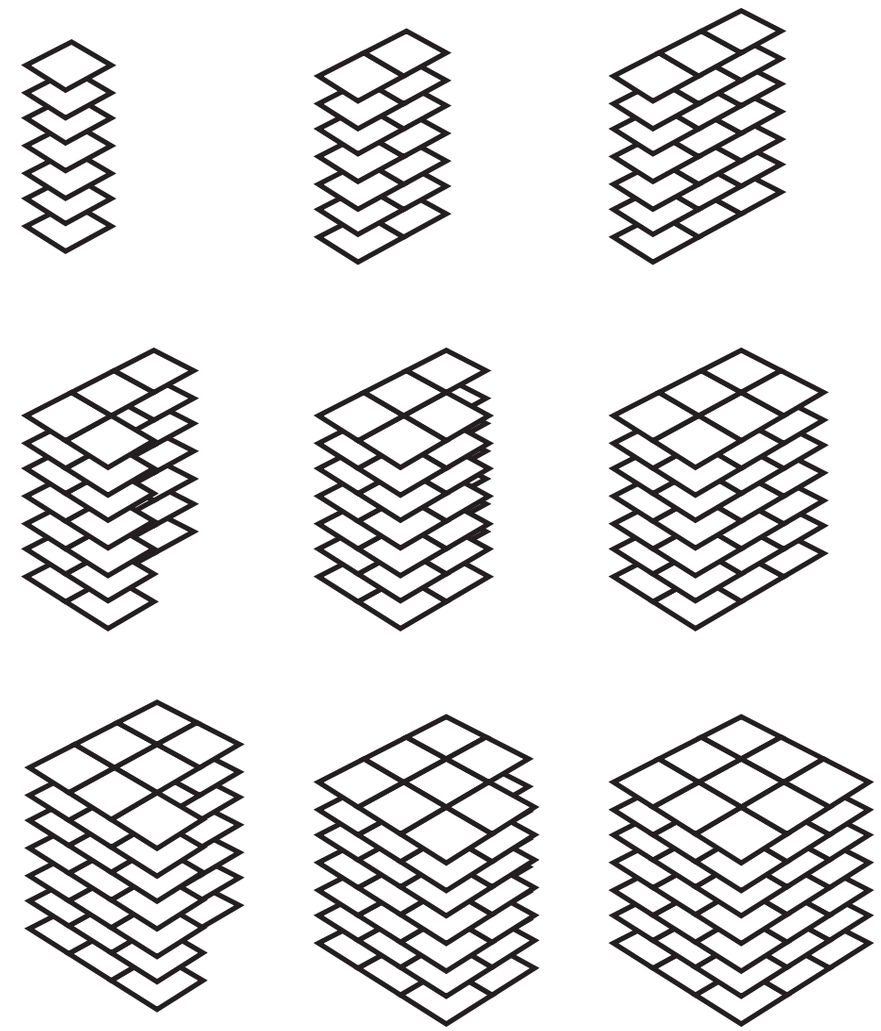
Imaging of biological specimens

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

For fast acquisition usually z planes before a new xy position



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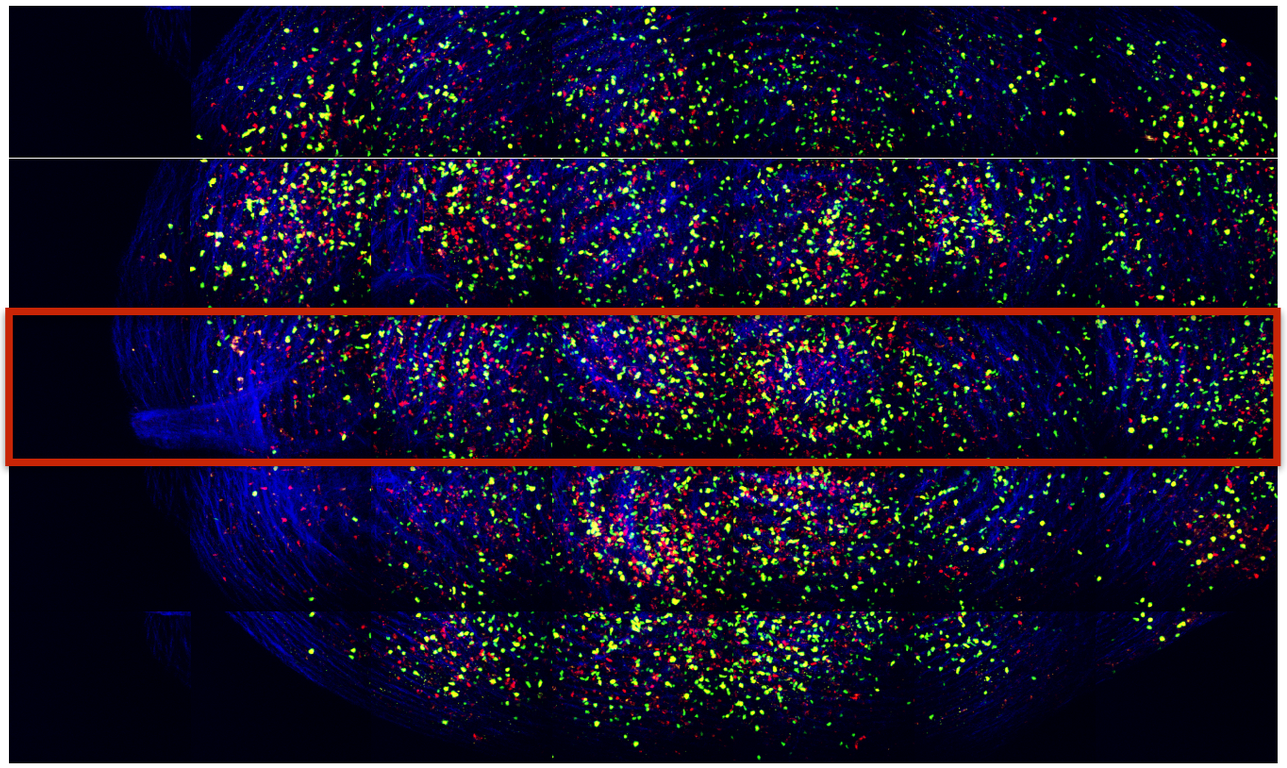
Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest



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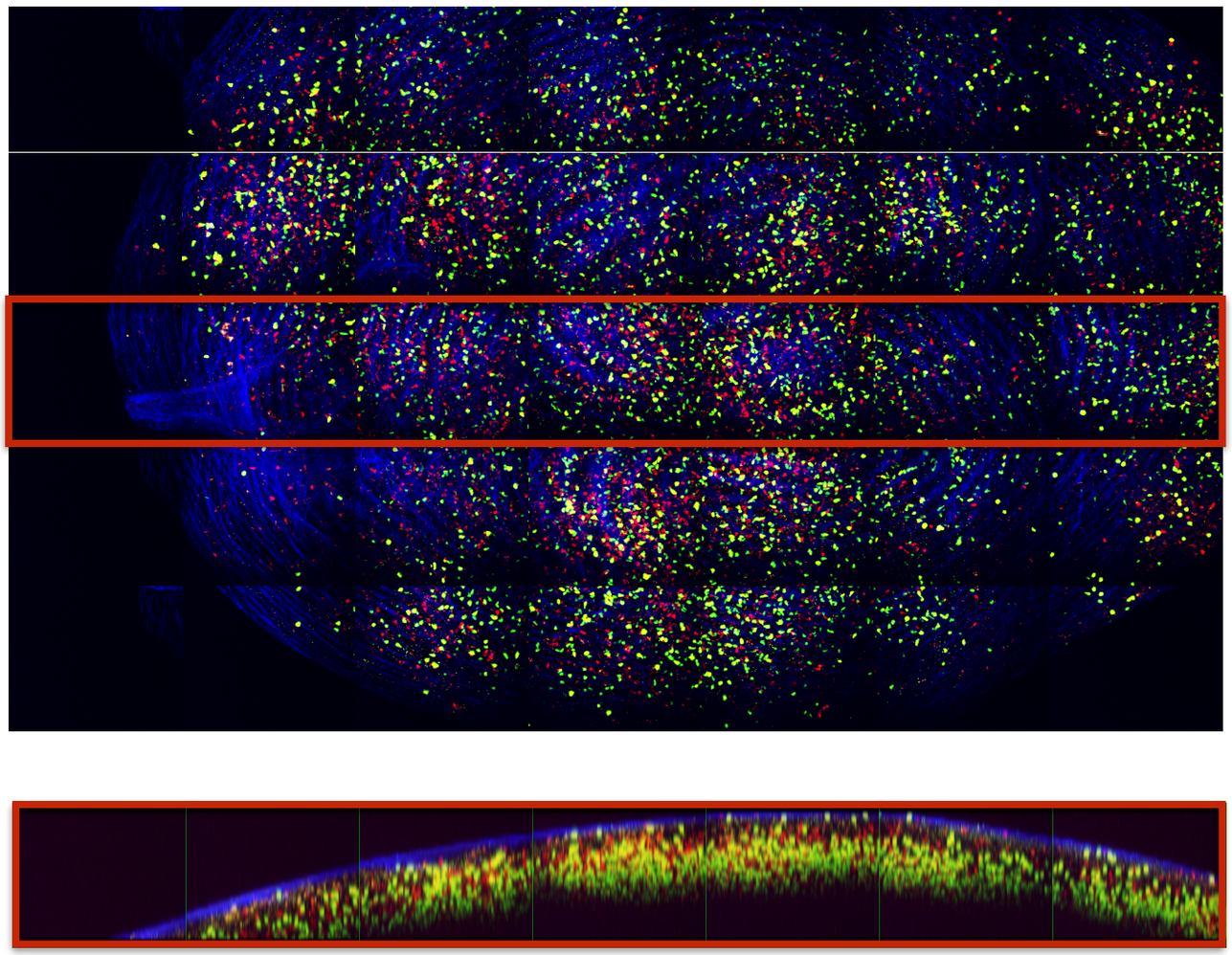
Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest

Then identify the z depth of a possible stack



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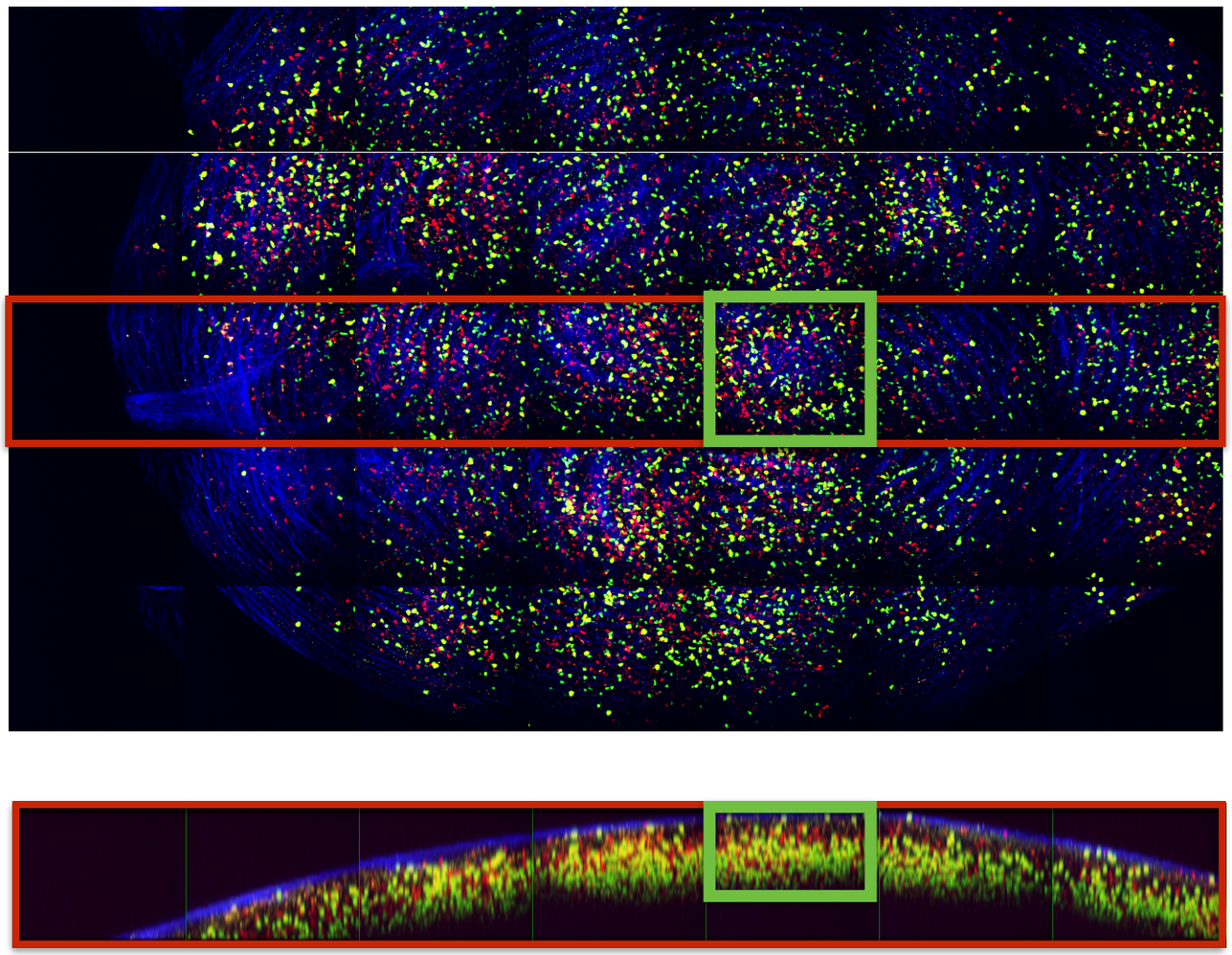
Imaging of biological specimens

Image acquisition usually begins with surveys to identify areas of interest

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



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Imaging of biological specimens

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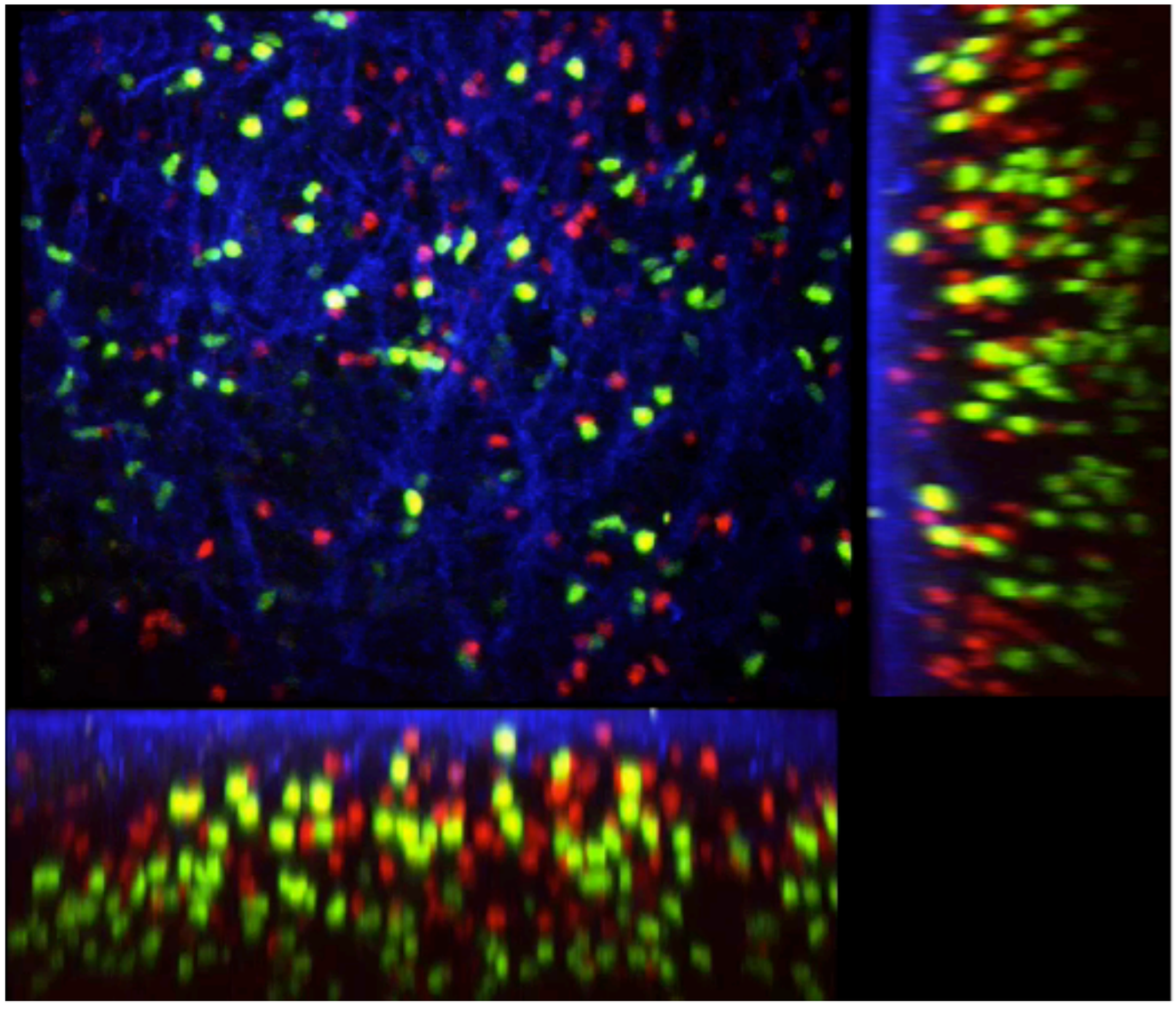

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Analysis of Data

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

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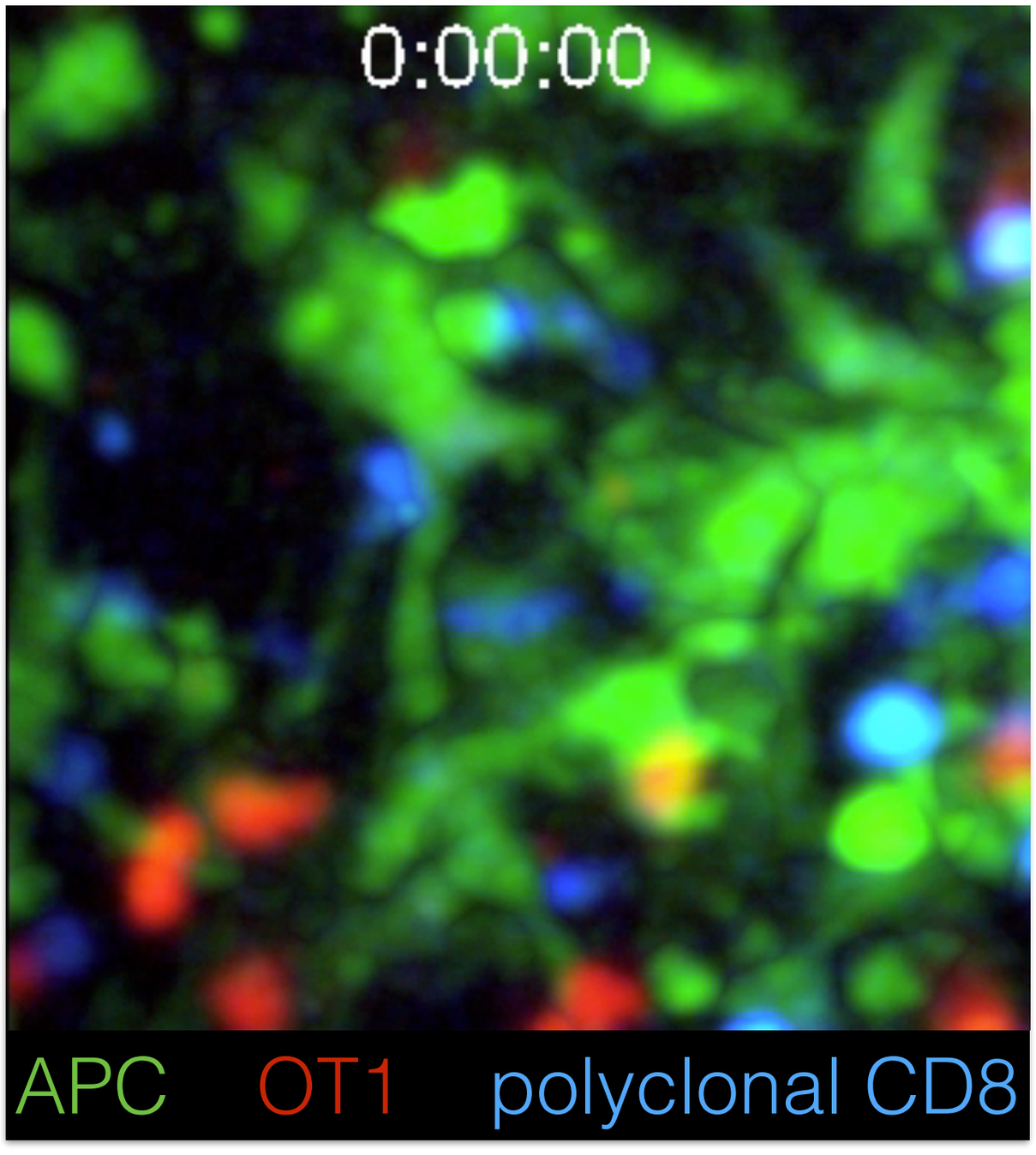
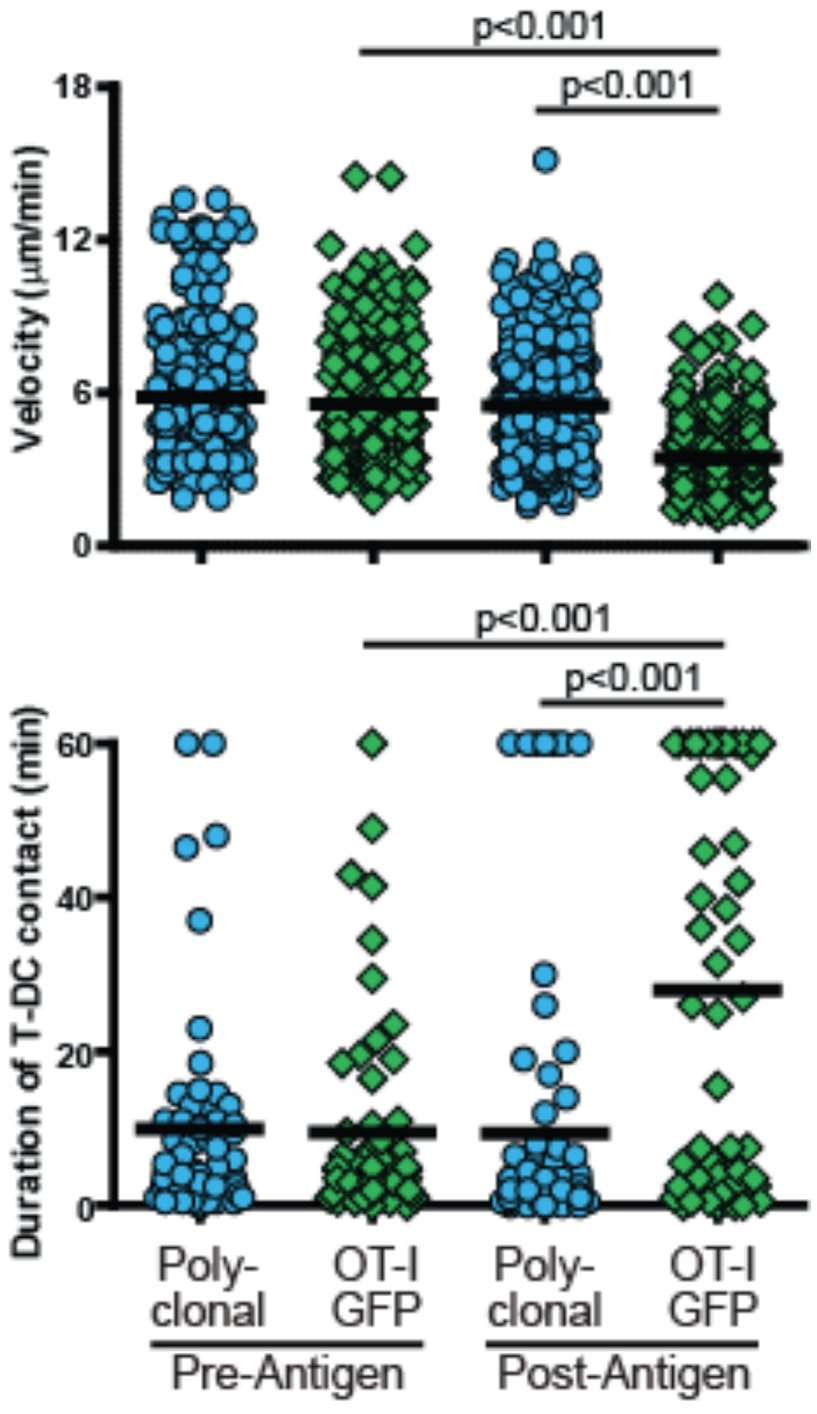

0:00:00
APC
OT1
polyclonal CD8


p<0.001
p<0.001
Velocity (μm/min)
18
12
6
0
p<0.001
p<0.001
Duration of T-DC contact (min)
60
40
20
0
Poly-clonal
OT-I GFP
Poly-clonal
OT-I GFP
Pre-Antigen
Post-Antigen

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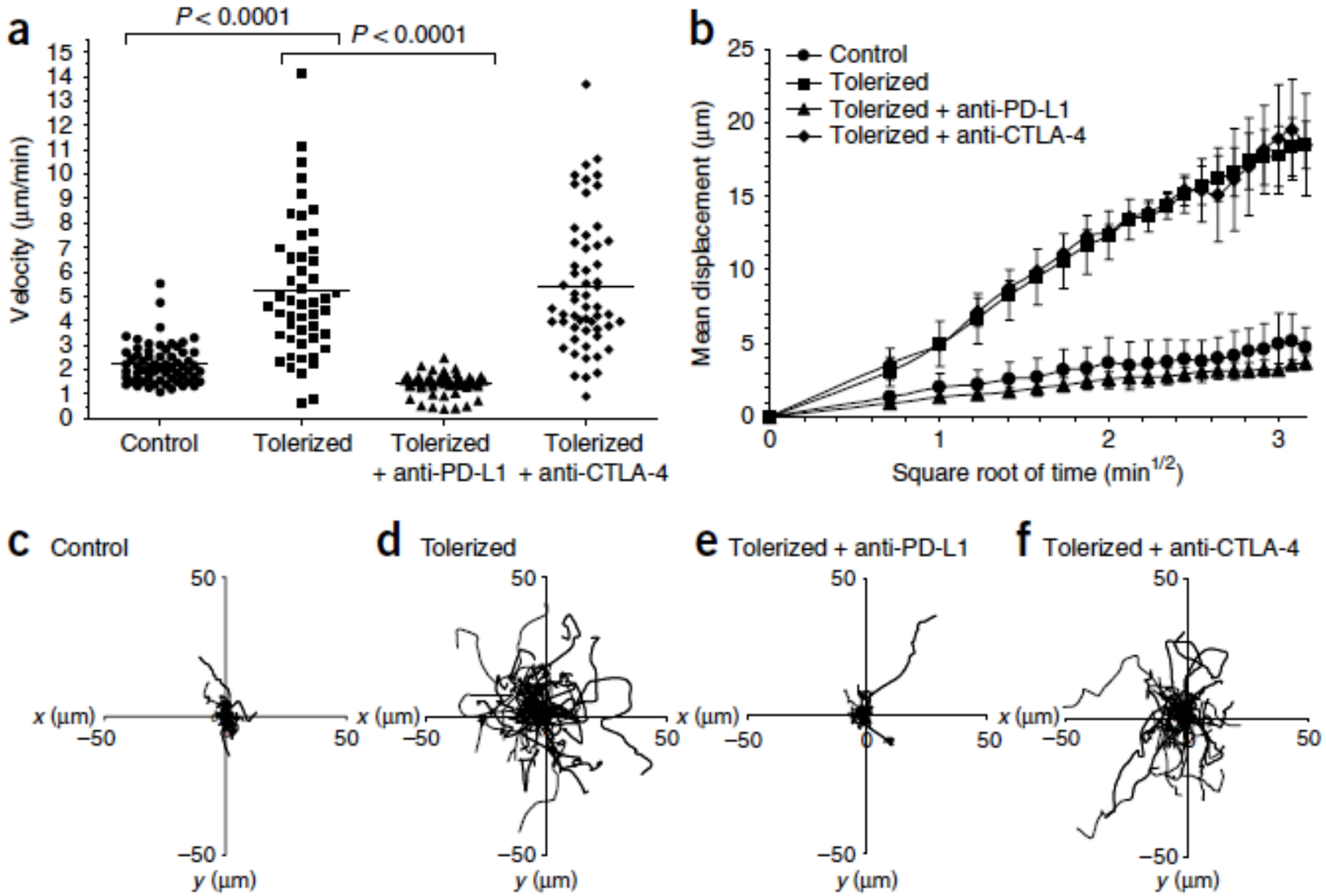
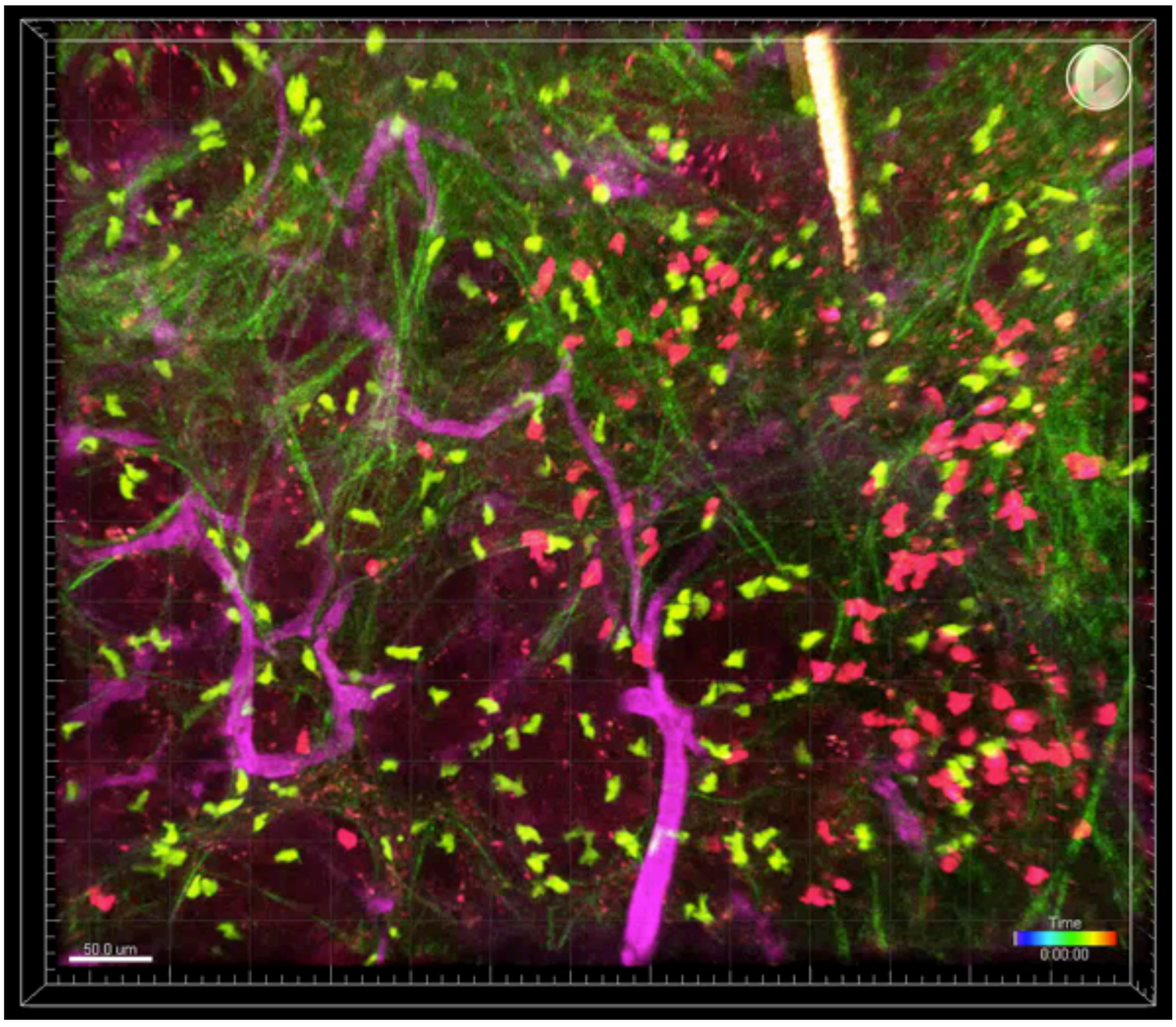
Migration measurements: Speed, displacement, track angles, distance from origin, arrest coefficients

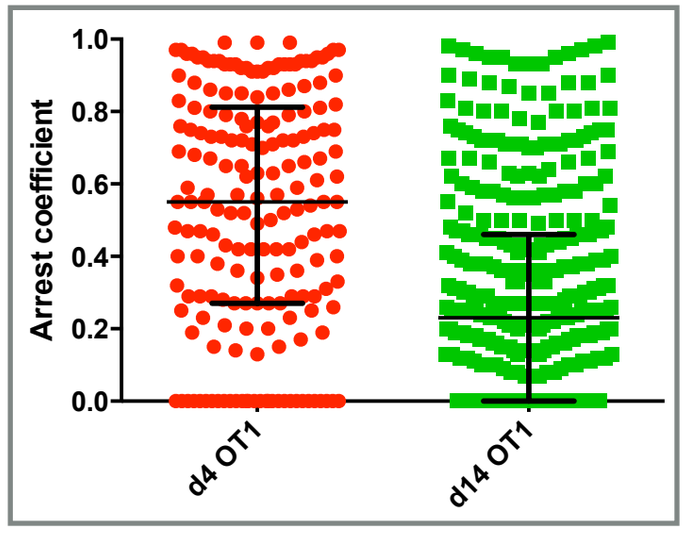


EXAMPLE: T CELL MATURATION IN TUMORS



OT-I T cells (d14) T cells (d4 OT1) Evans Blue (blood)

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



Boldajipour et al., in preparation

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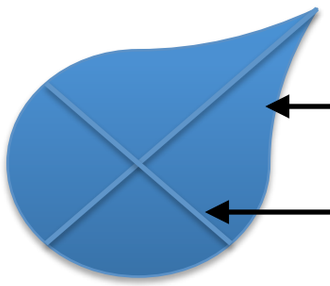
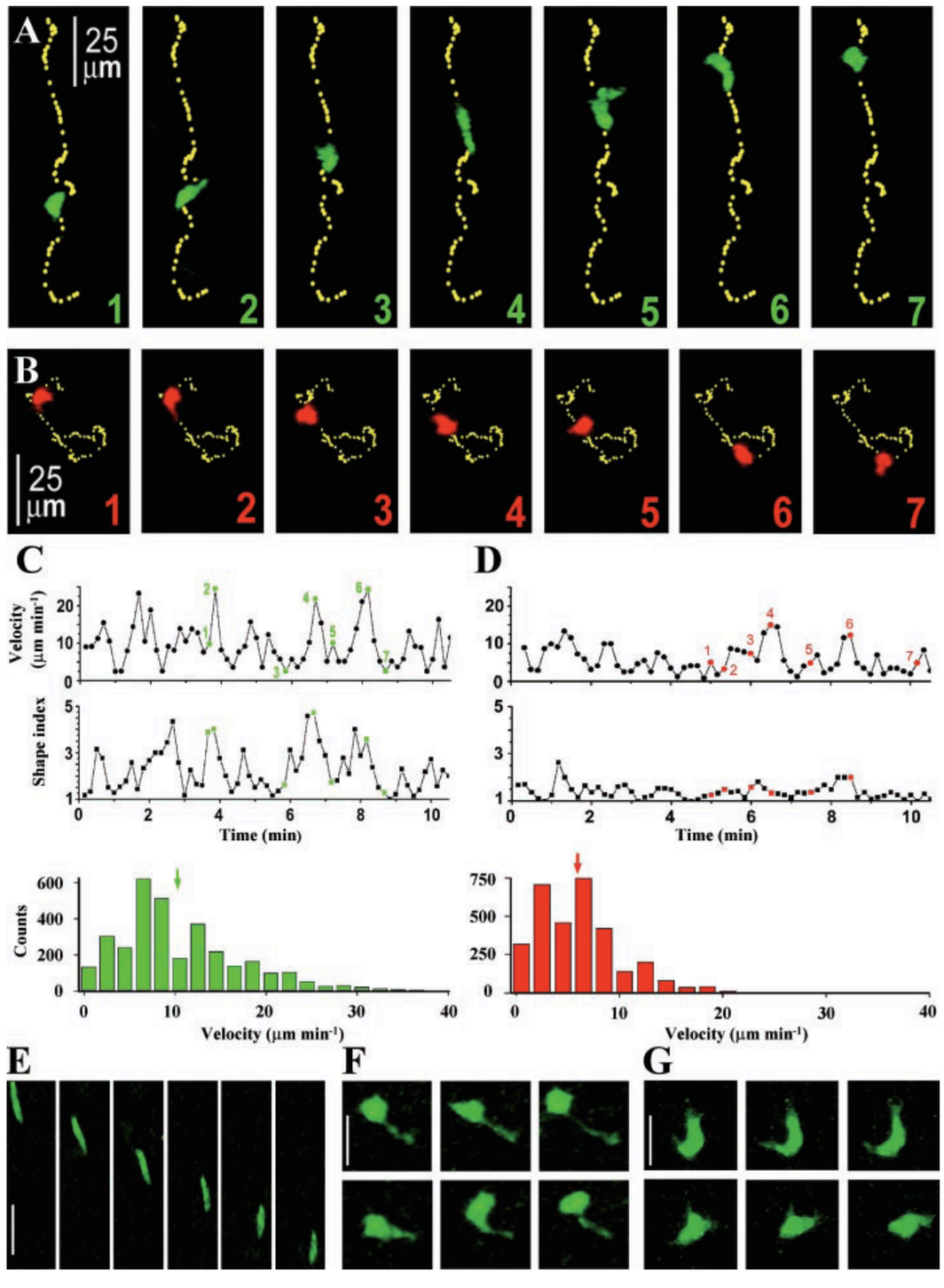
Morphology measurements



Shape factors correlate with motility vs signaling

Roundedness:
axis1/axis2 (=1 for circle)
perimeter/radius (=2πr)



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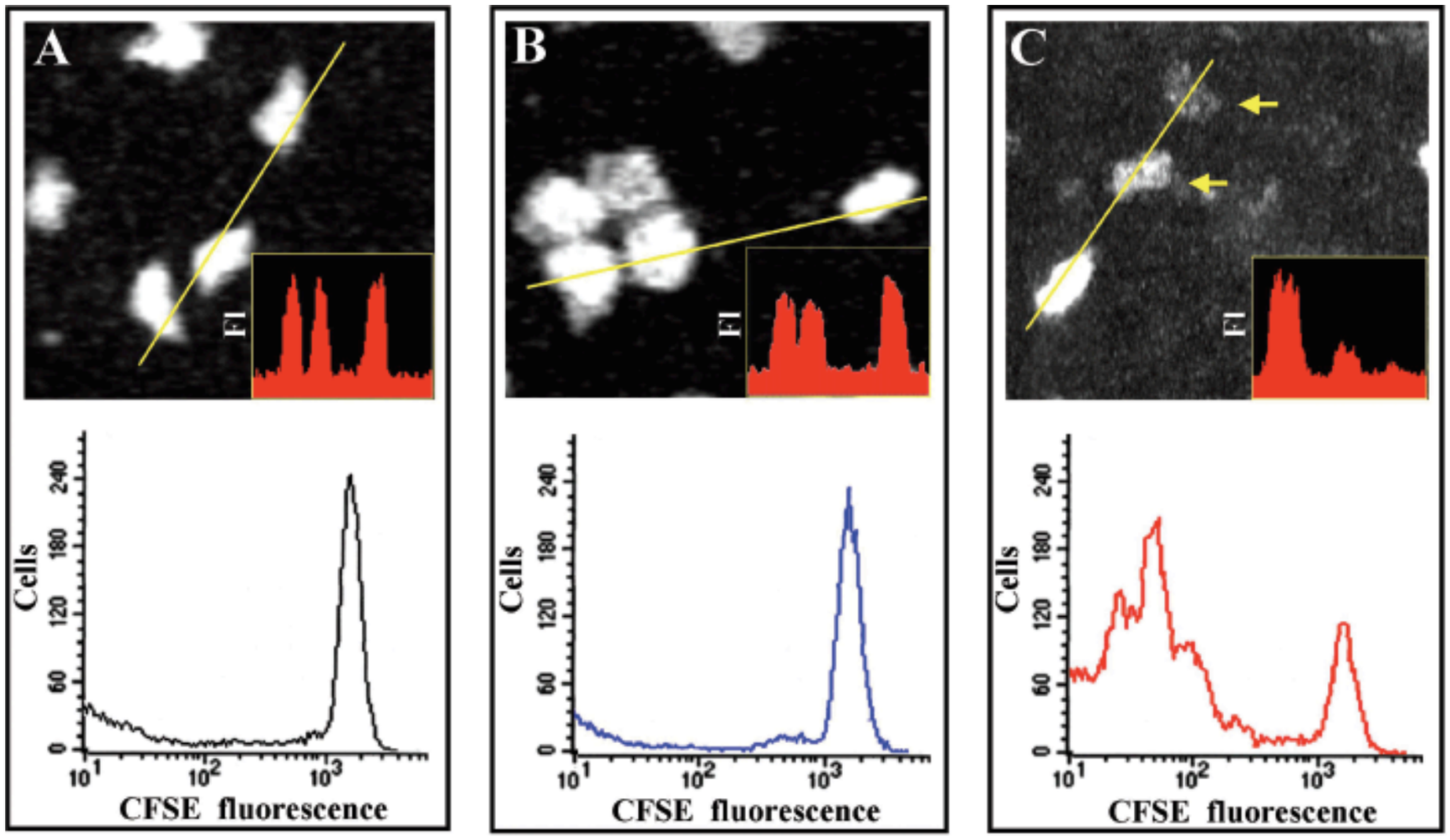
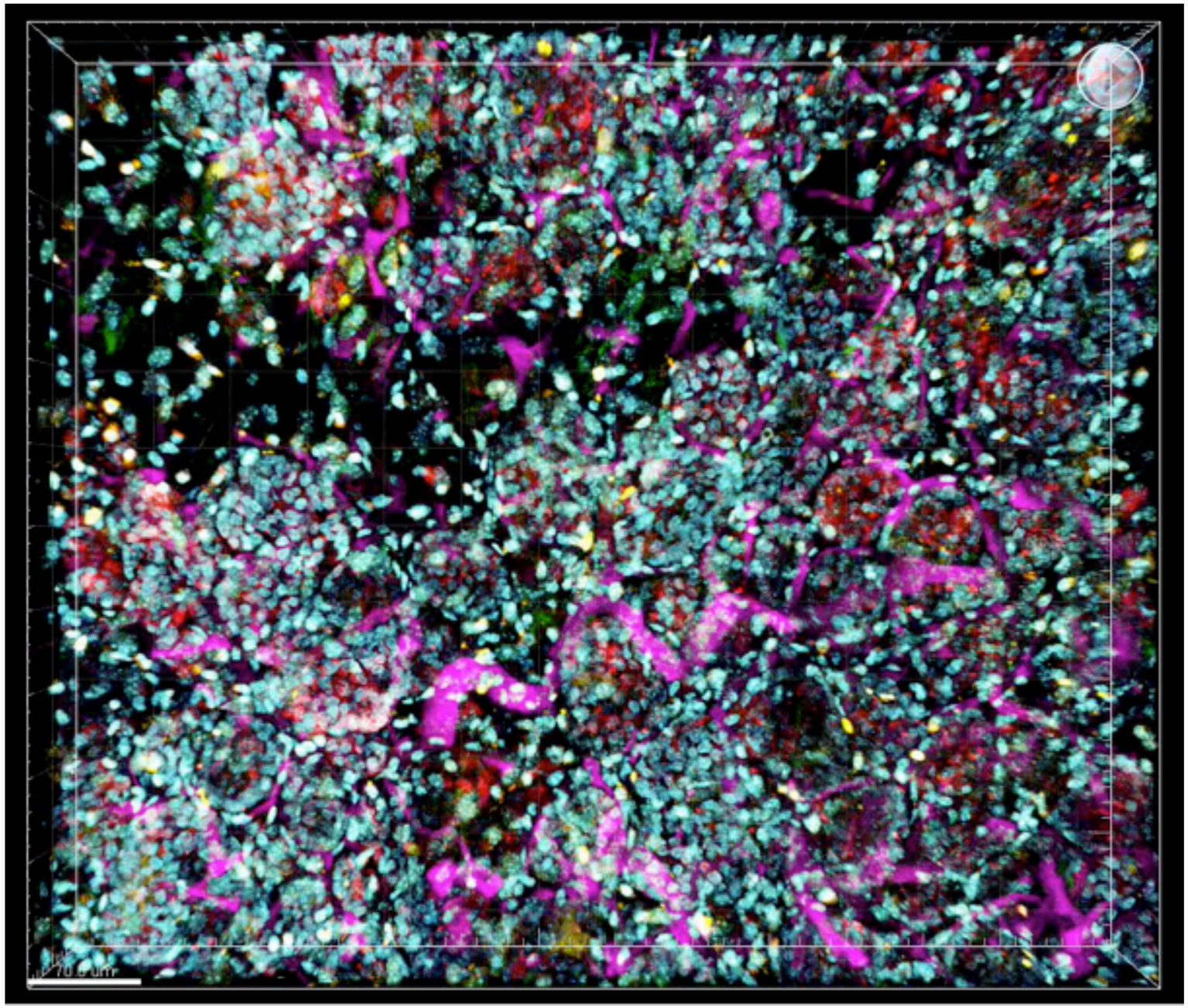
Cell activity: Dilution of a dye to asses cell division



EXAMPLE: ASSESSING CTL ACTIVITY IN VIVO



- Use intravital microscopy to asses 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

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

Boldajipour et al., in preparation

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Cell interactions: Manual or via Matlab scripts