Two-photon microscopy

Emily Thornton

16 November 2020

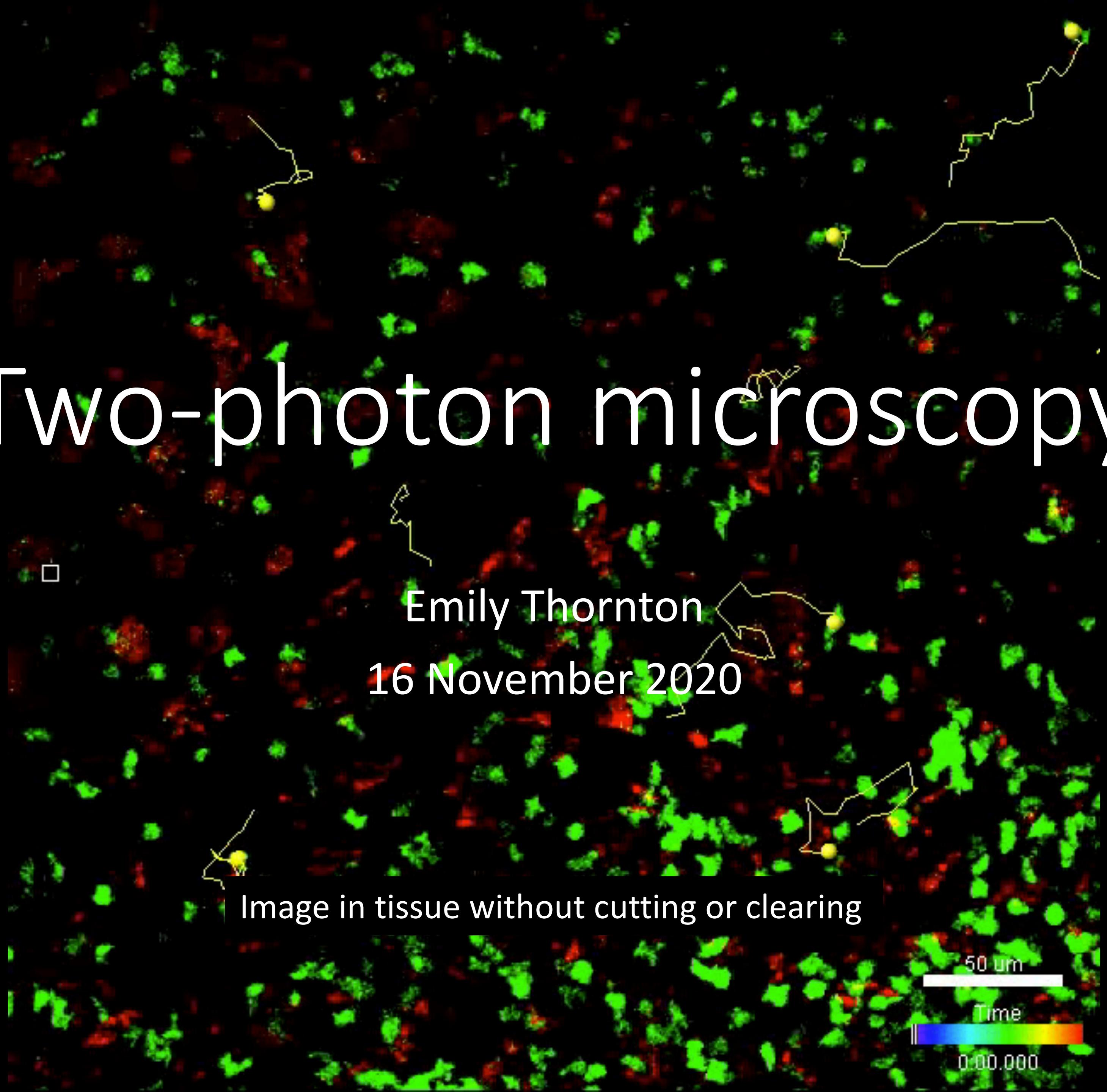
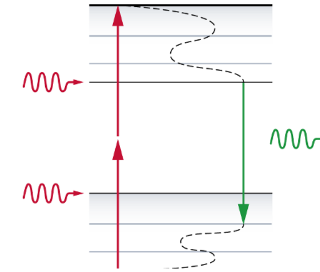
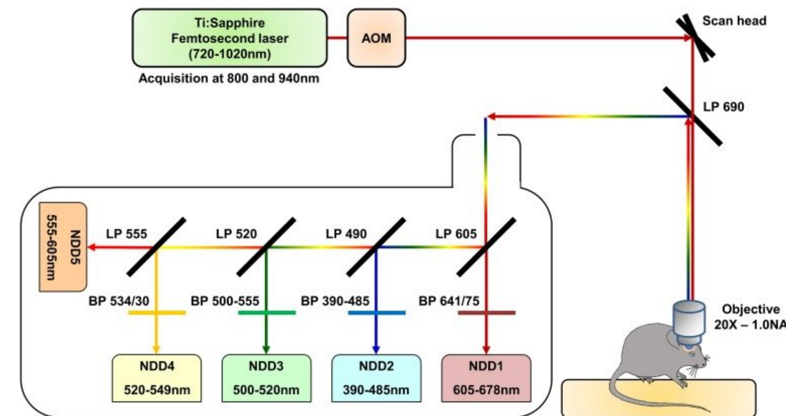


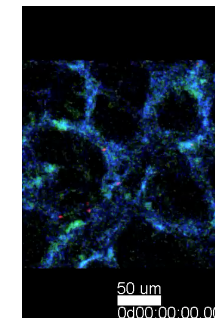
Image in tissue without cutting or clearing

Outline

- General two-photon concepts
- Two-photon lasers
  - Femtosecond pulse
  - Tunable wavelength
- Excitation of fluorophores
  - 2P excitation
  - Second harmonic generation
- Detection
  - Light path
  - Non-descanned detectors
  - Spectral detection
- Applications
  - Explants
  - Intravital
  - Photoactivtion/photoconversion

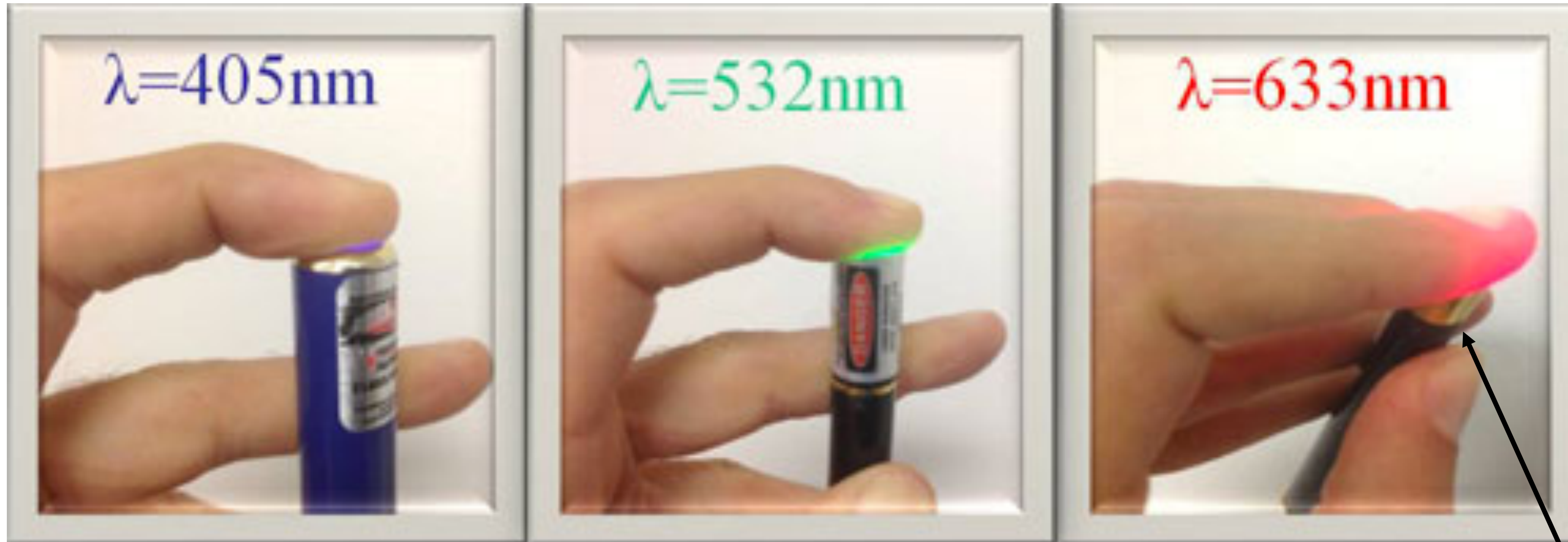






Limits of tissue imaging

Tissue absorbs light

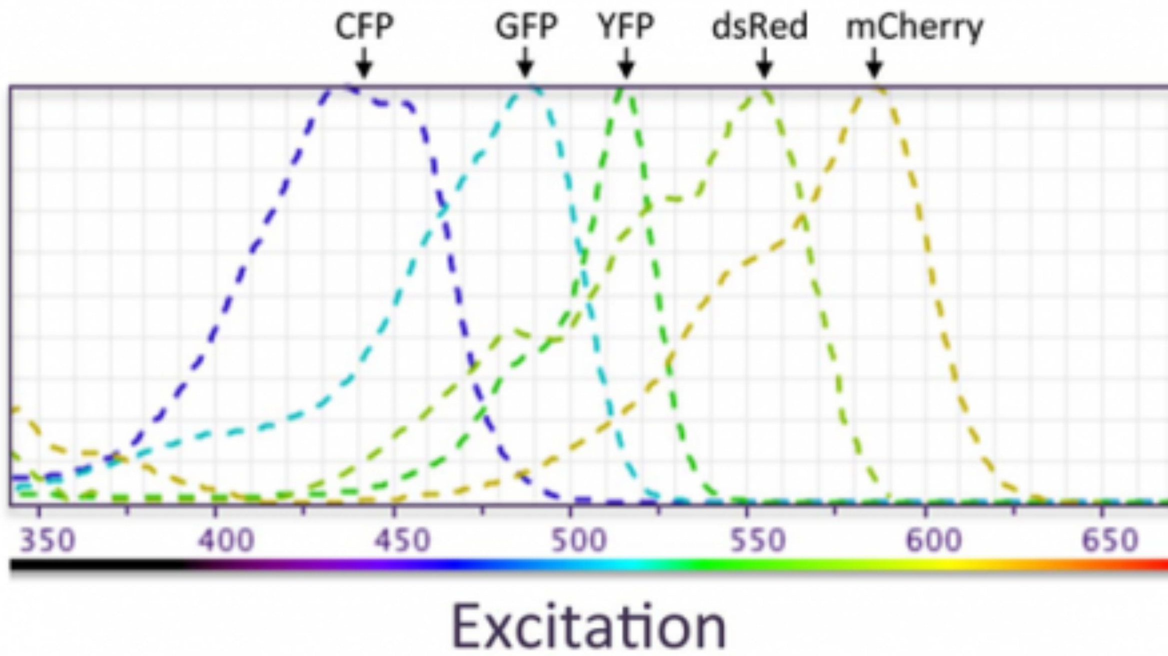


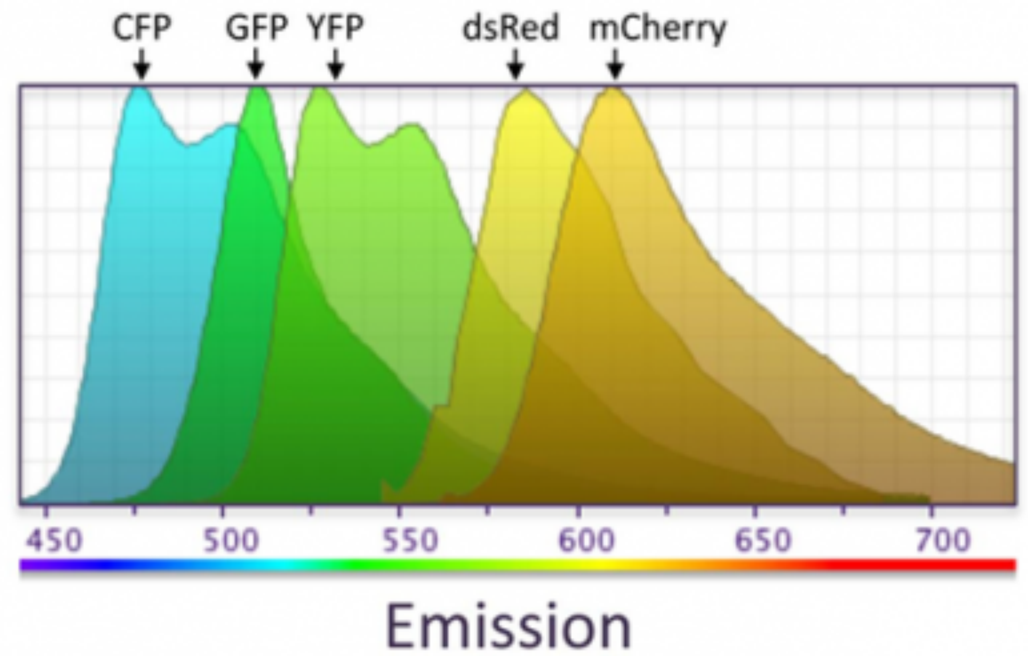
Tissue scatters light $\frac{1}{\lambda^4}$

How do we use longer wavelengths to image?

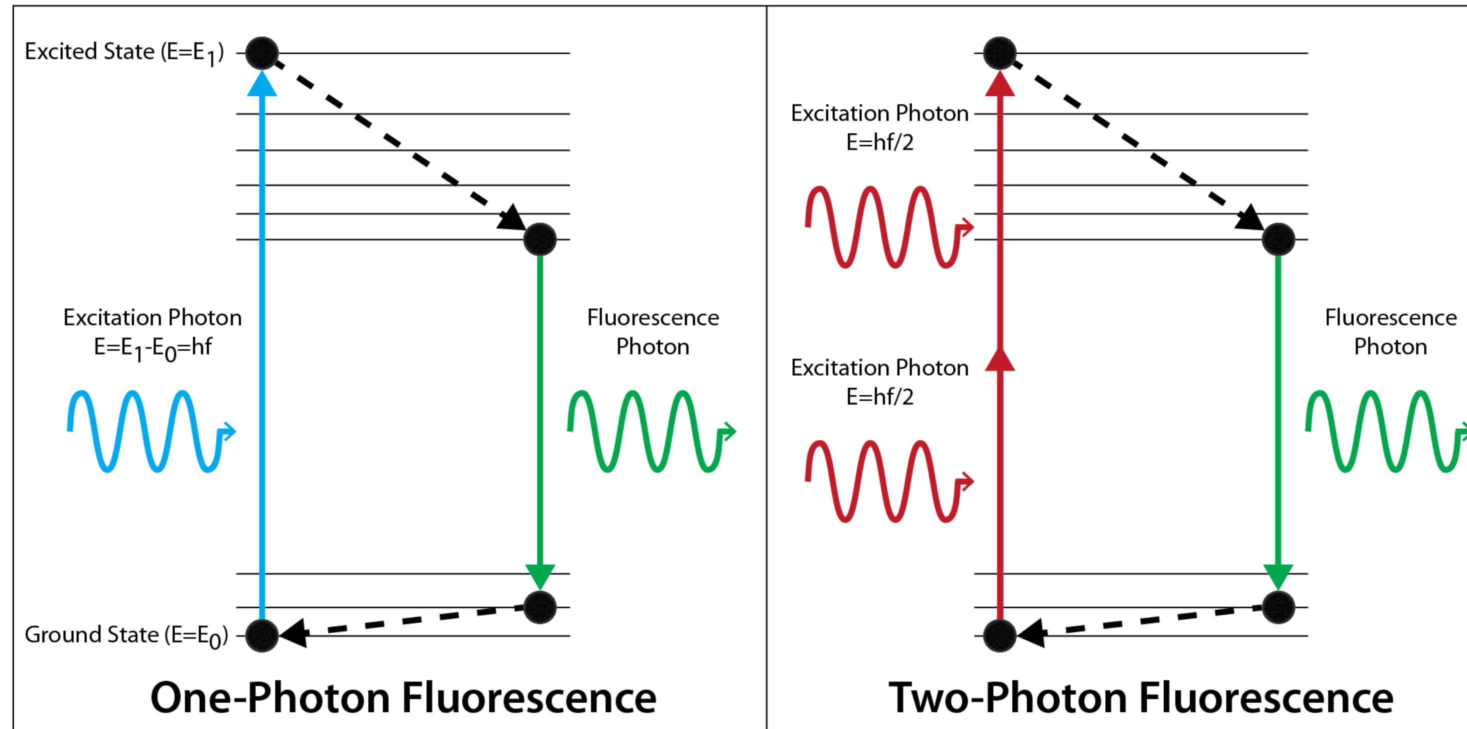
http://www.nanowerk.com/spotlight/spotid=32606.php

Far red dyes





Single-photon vs Two-photon excitation



This is very unlikely to happen by chance

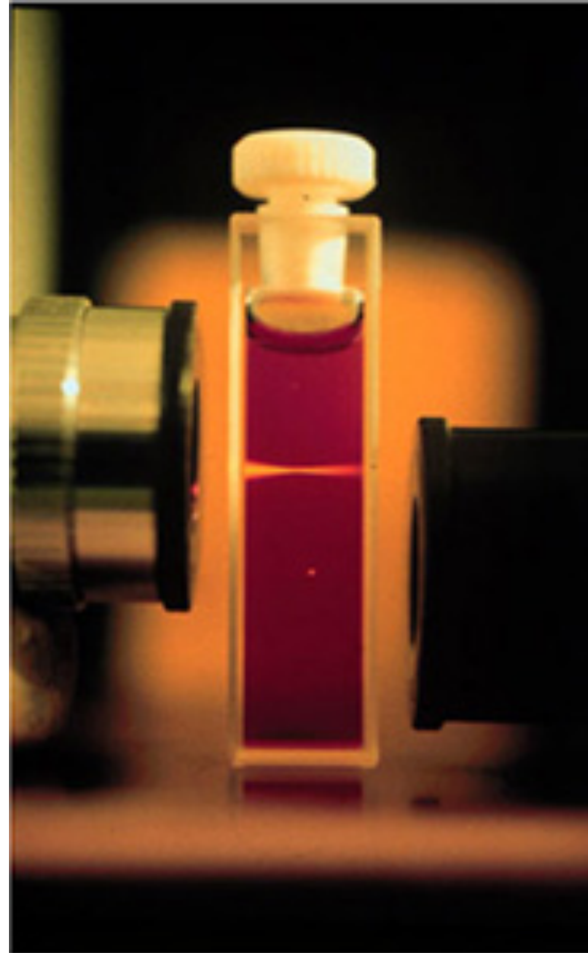
http://sites.middlebury.edu/durst/research/

Confocal vs Two-photon

Confocal:

Excitation occurs out of focus

Pinhole eliminates out of focus illumination



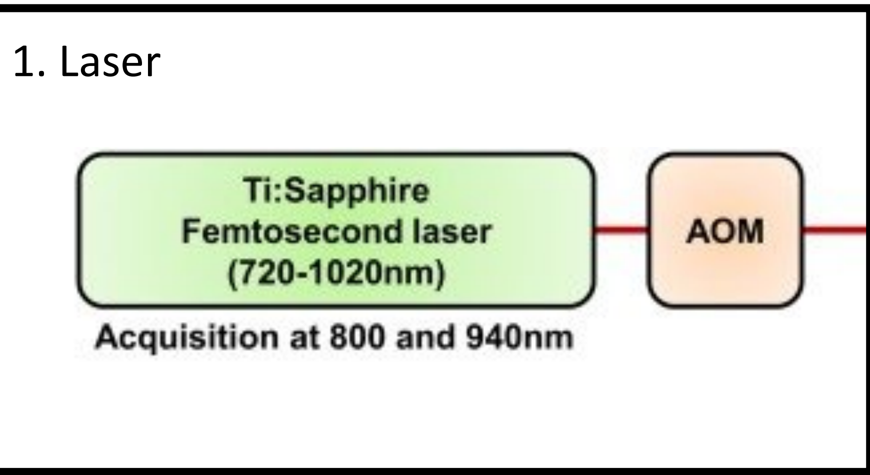
Two-photon:

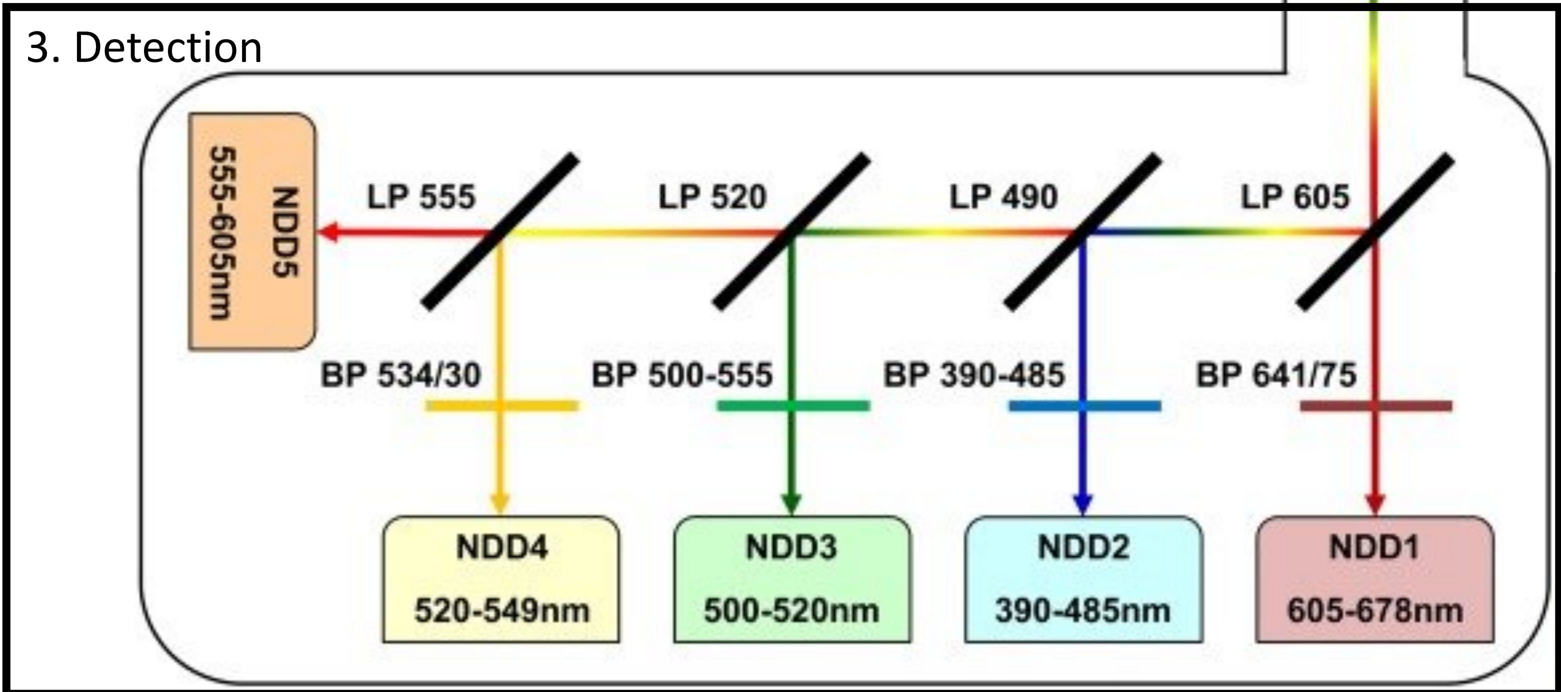
Excitation only occurs in the focal point

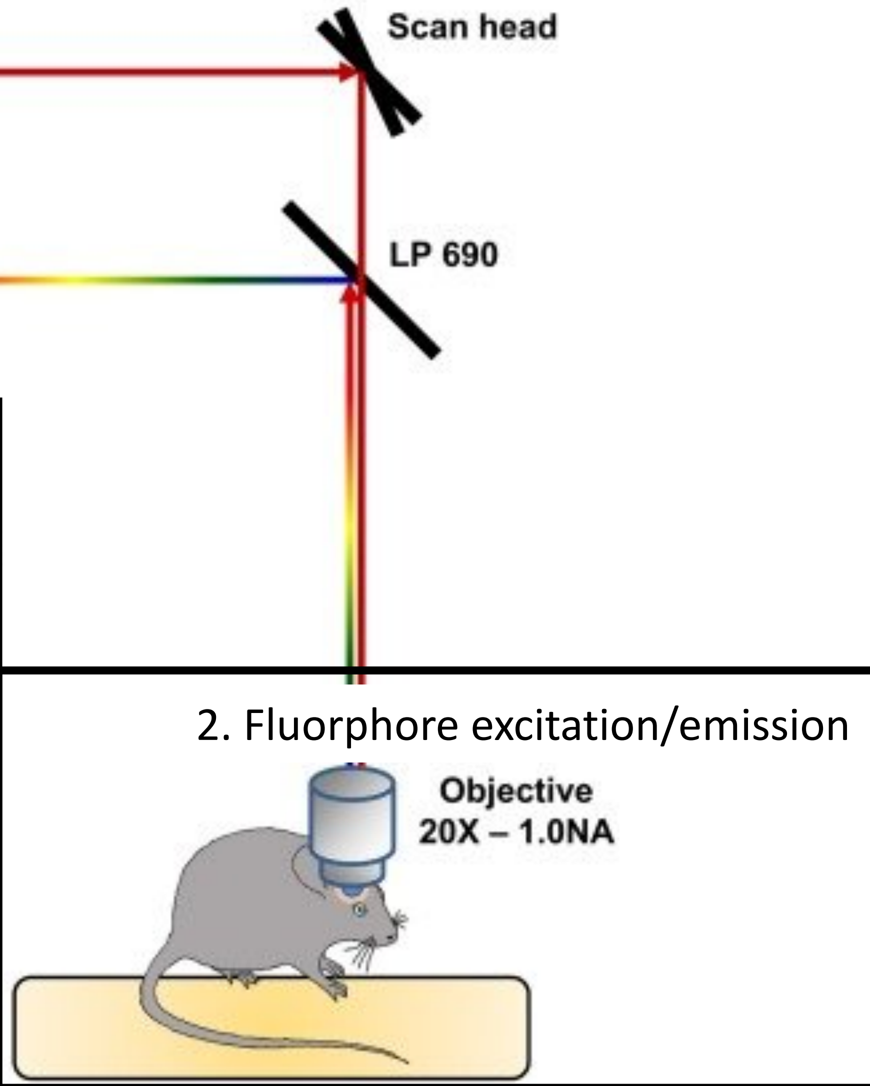
No need for a pinhole

Less tissue damage

Brad Amos MRC, Cambridge

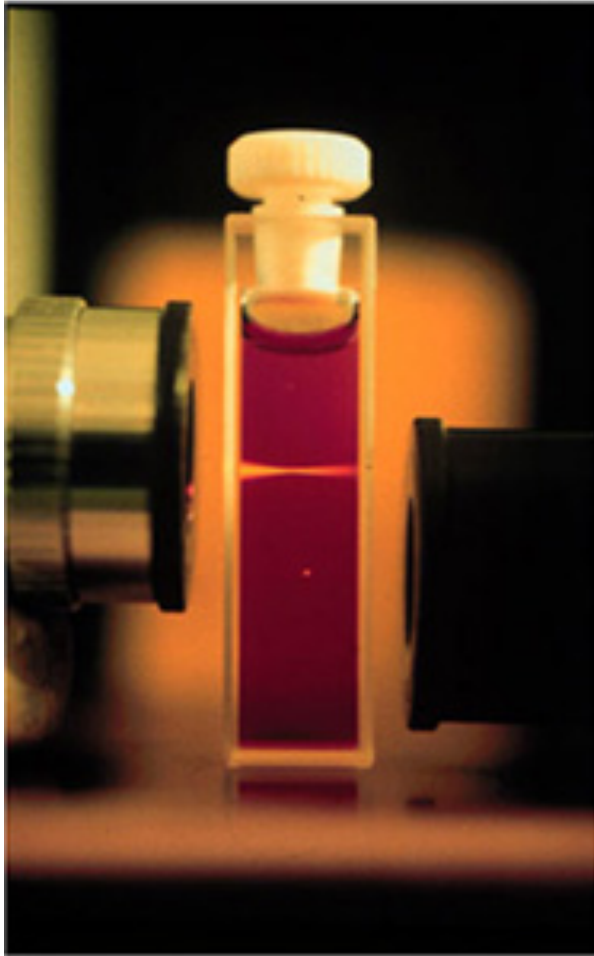






Ricard et al 2014

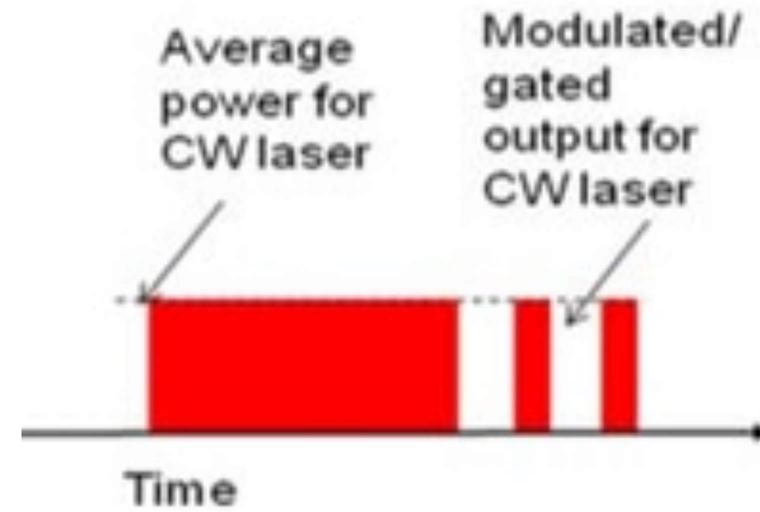
1. Laser



How do hit a fluorophore with two photons at once?

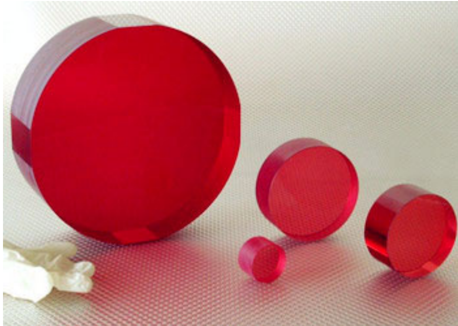
Brad Amos MRC, Cambridge

Femtosecond Lasers



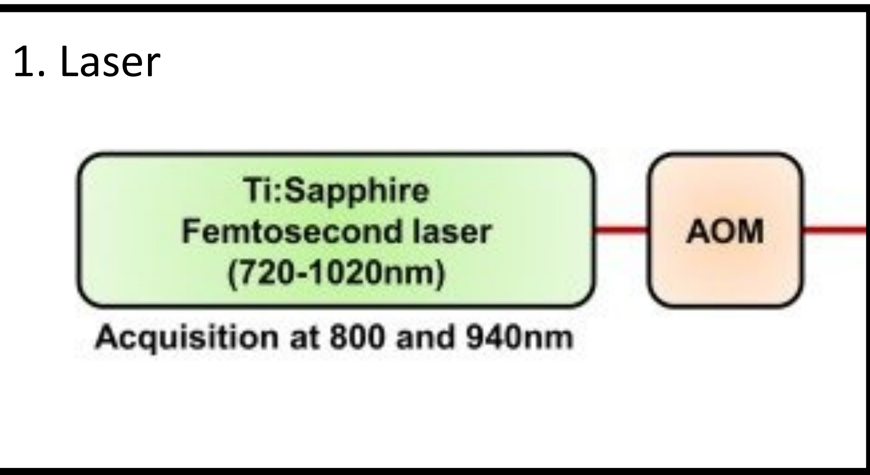
https://www.ebindustries.com/cw-and-pulsed-laser-welding/

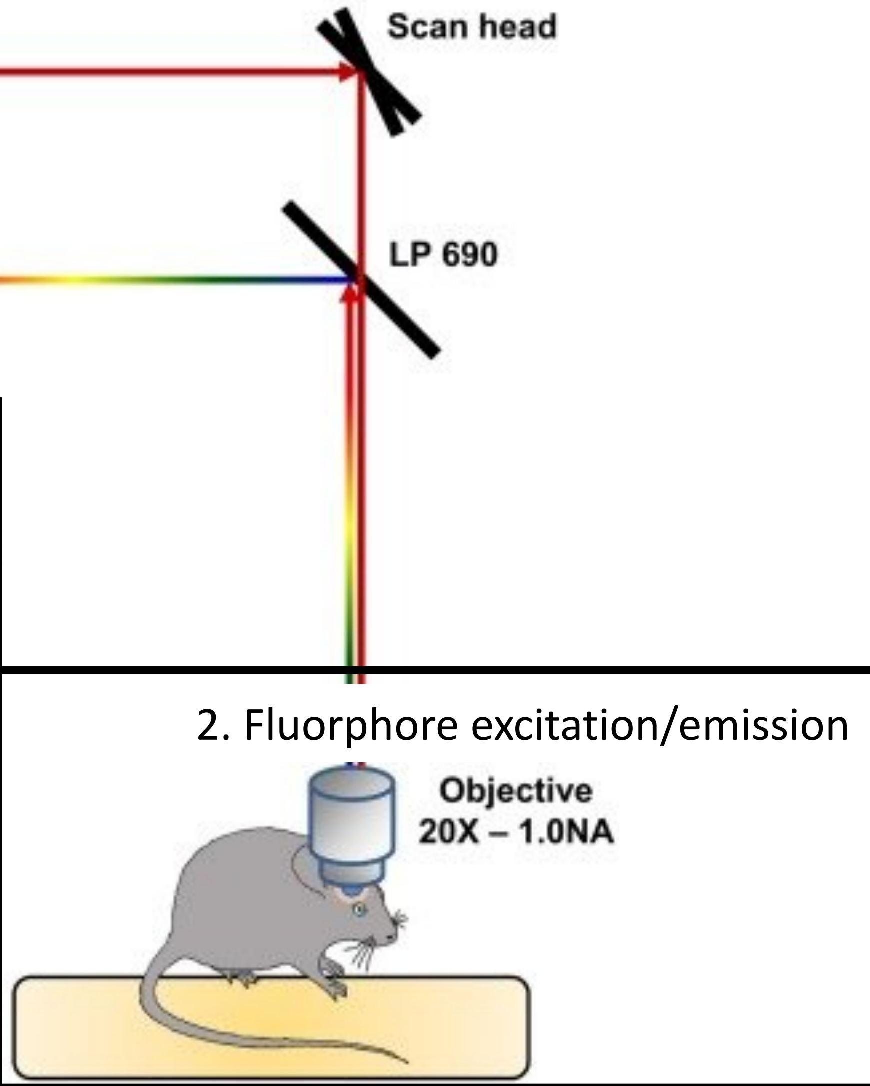
Ti-Sapphire laser (the expensive part)

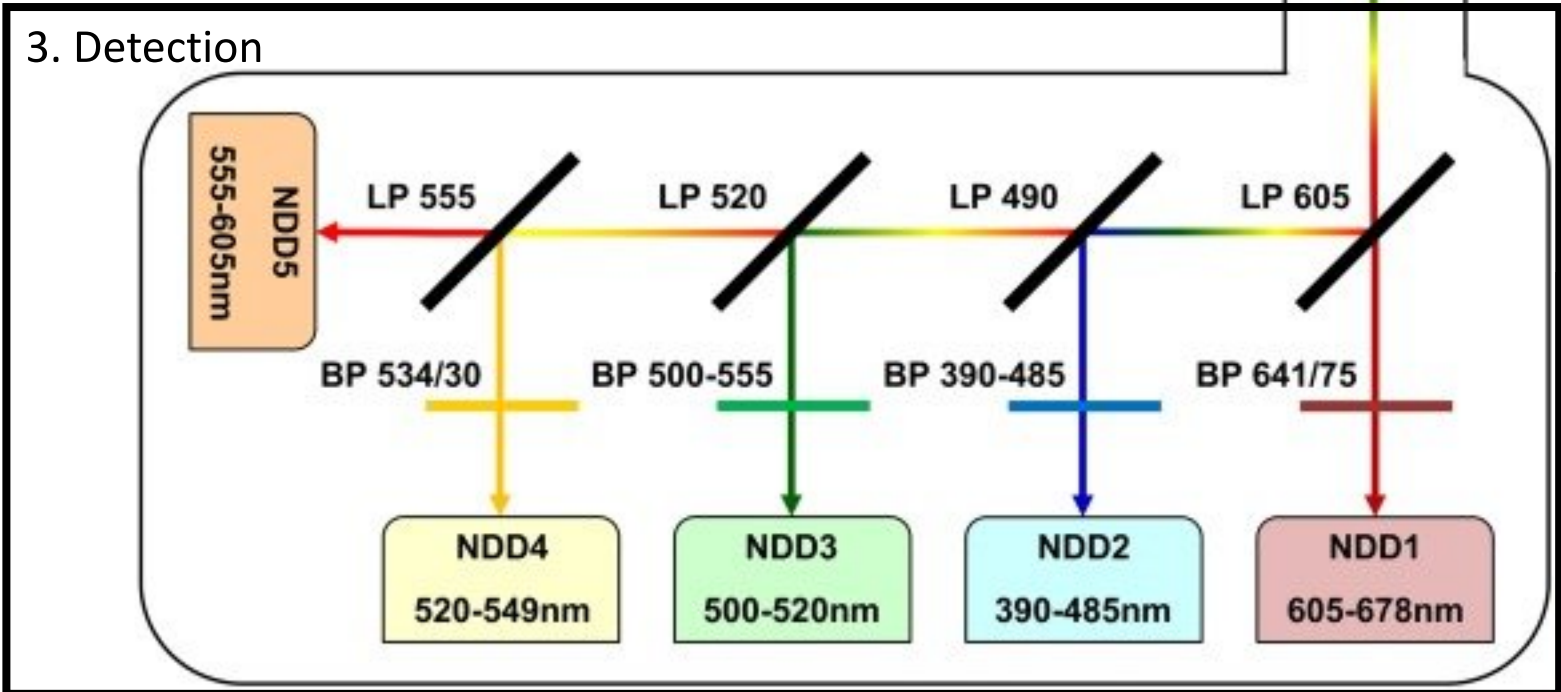


Example: Mai tai deep see

- Pulse width <70 fs (10^-15)
- 680-1040 nm tunable range
  - 690 nm >56 kW at
  - 710 nm >217 kW at
  - 920 nm >217 kW at
  - 1040 nm >34 kW at
- Multiple lasers are required to excite at two wavelengths simultaneously

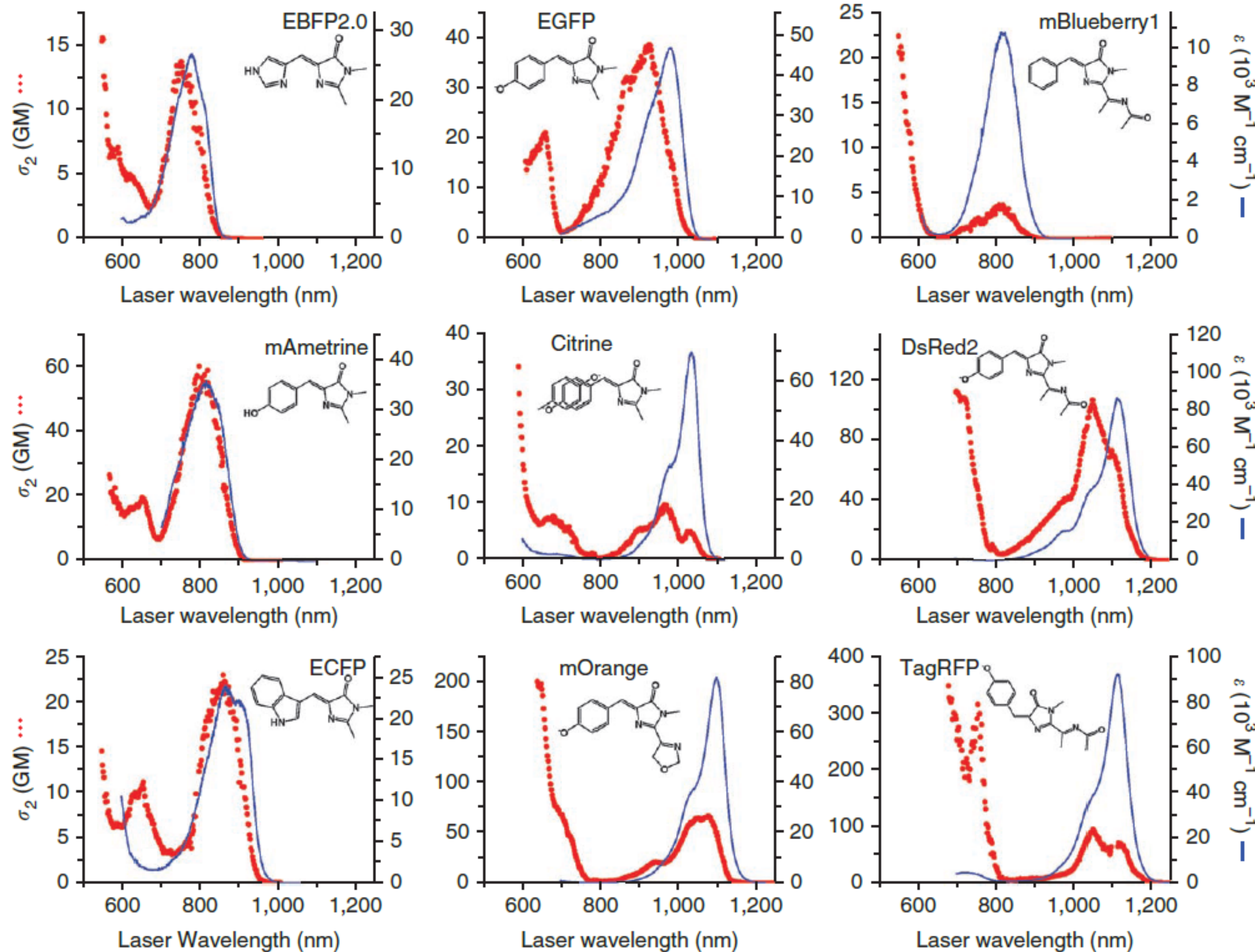






Ricard et al 2014

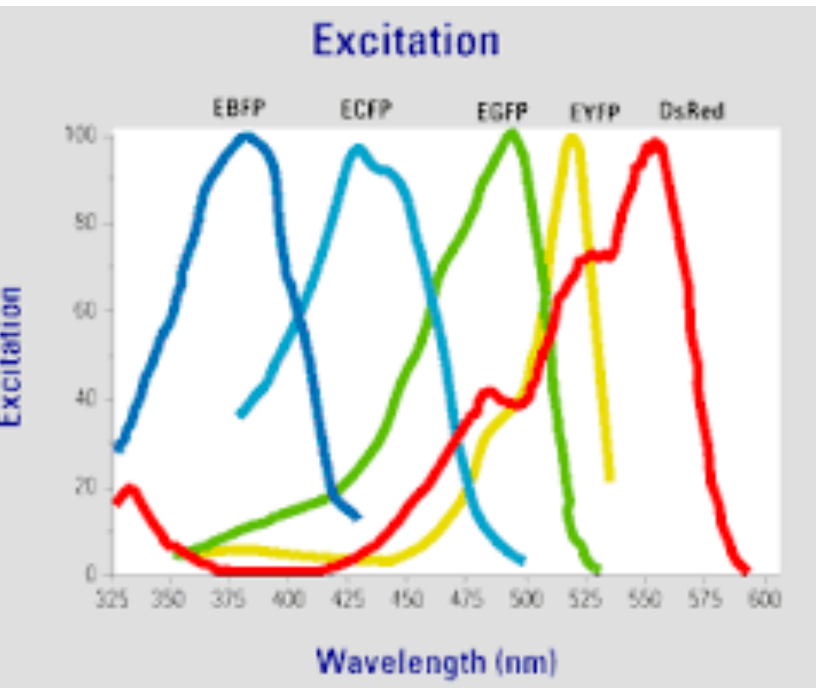
Excitation wavelengths

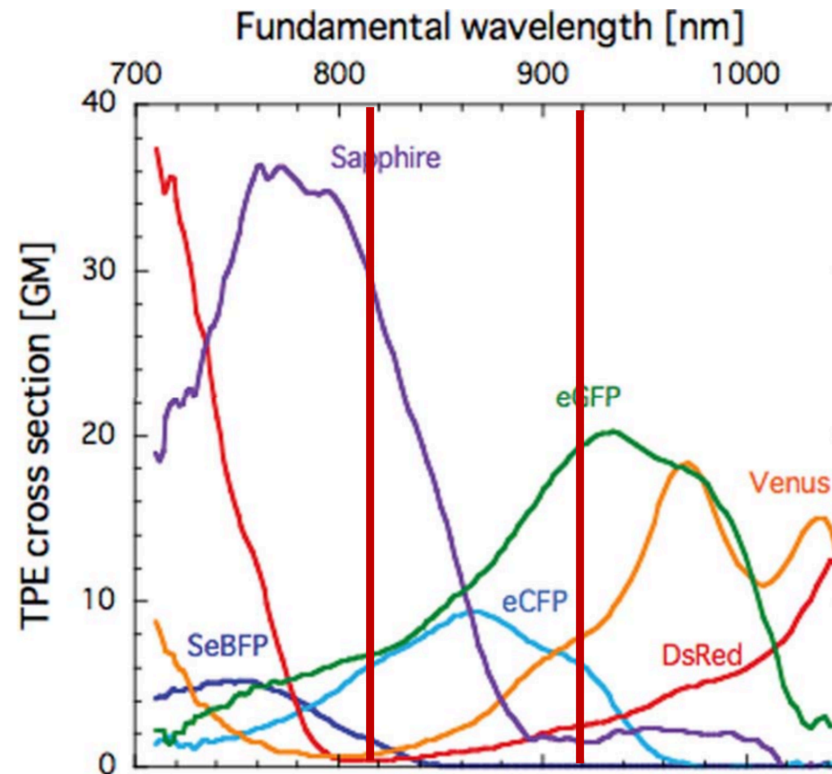


- Almost double 1P excitation
- Wide curve of excitation
- Determine optimal wavelength empirically

Drobizhev,2011

Simultaneous excitation of multiple fluorophores





Where would you tune your laser?

910nm laser could excite, eCFP, eGFP, Venus, and DsRed +second harmonic generation

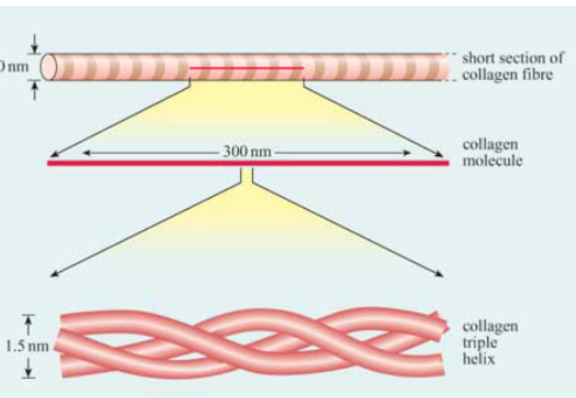
Moving excitation wavelength can optimize excitation of samples with varying brightness

A second excitation laser can dramatically expand the number of fluorophores you can image at once

Hashimoto, 2010

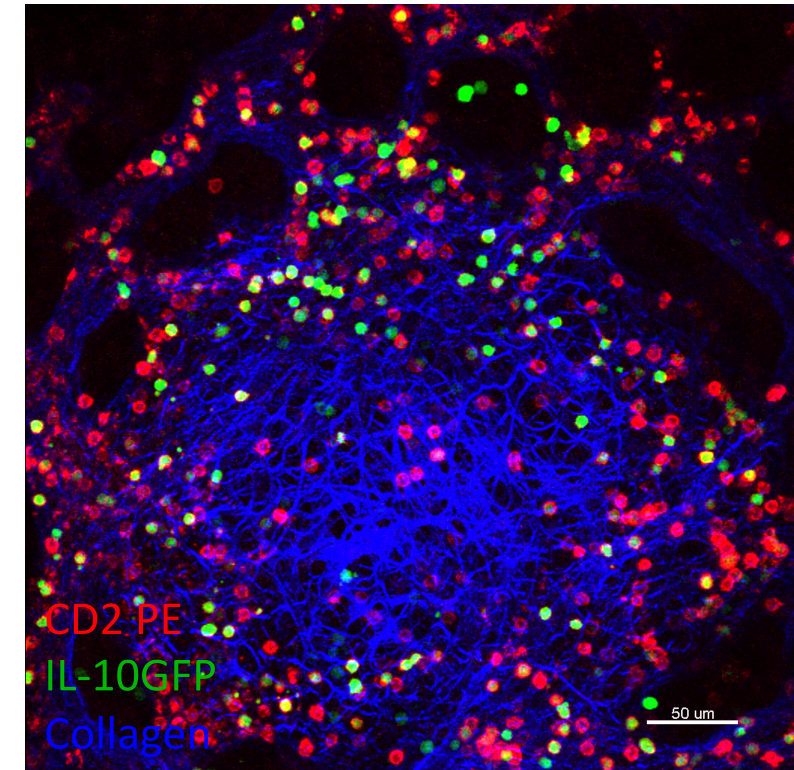
Second harmonic generation

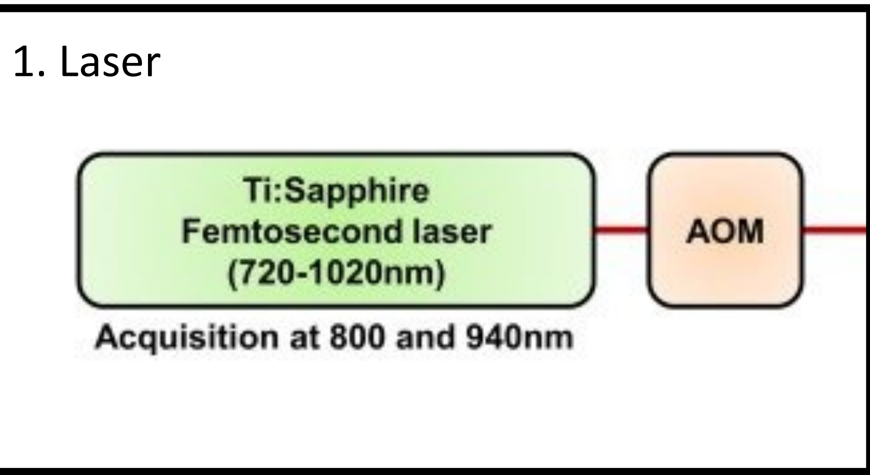
Two photons interact to create a new photon with twice the frequency and half the wavelength

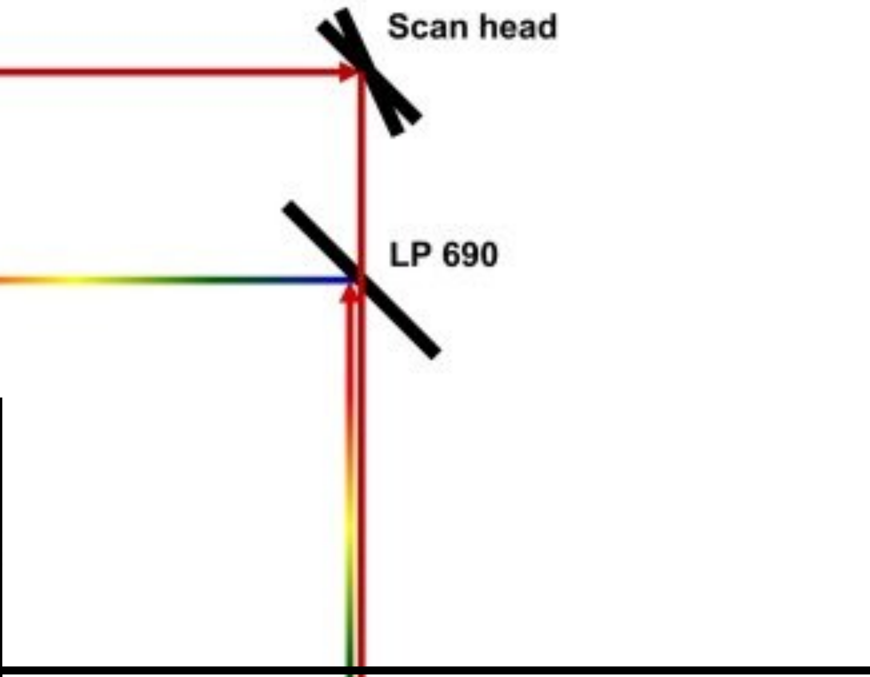


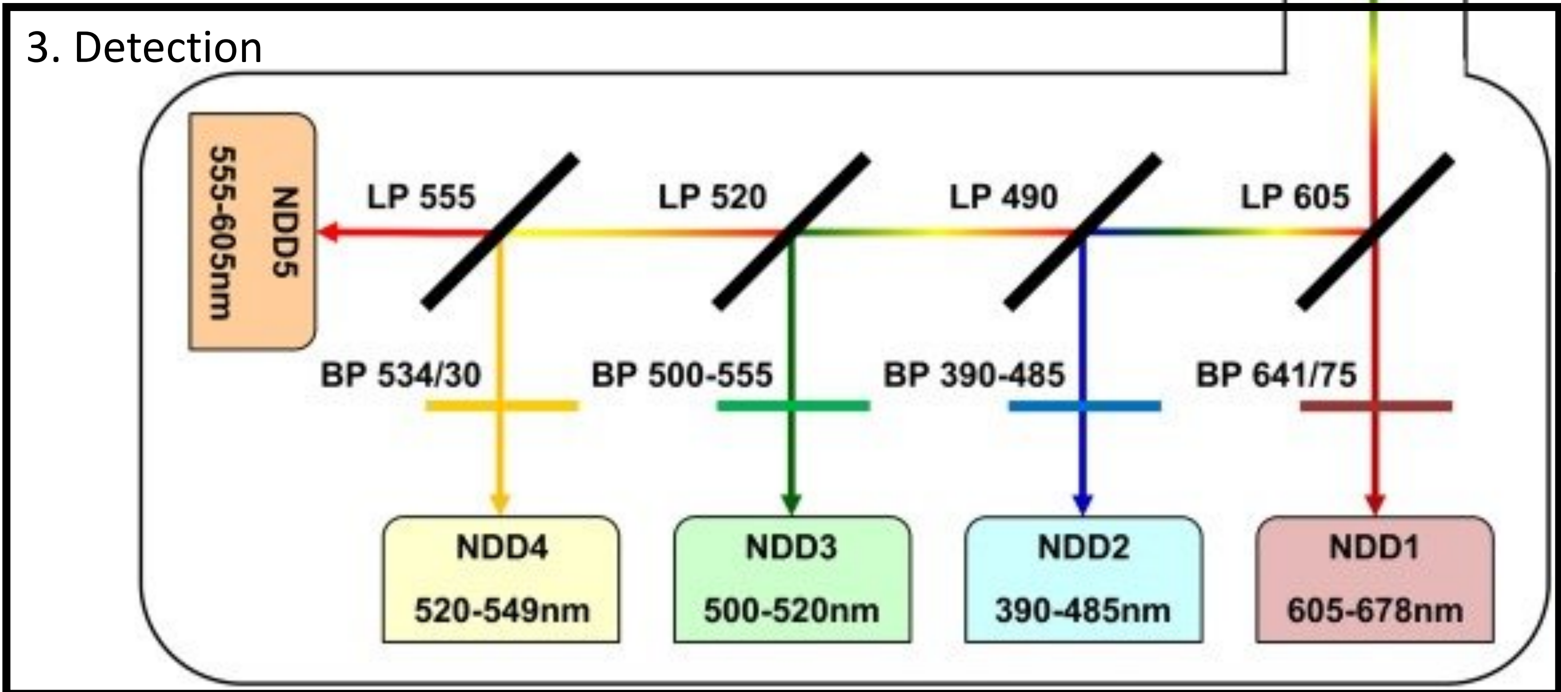
Excitation wavelength: 810	Excitation wavelength: 910
Collagen fibrils: Second Harmonic Generation	Collagen fibrils: Second Harmonic Generation
Detection: 405	Detection: 455

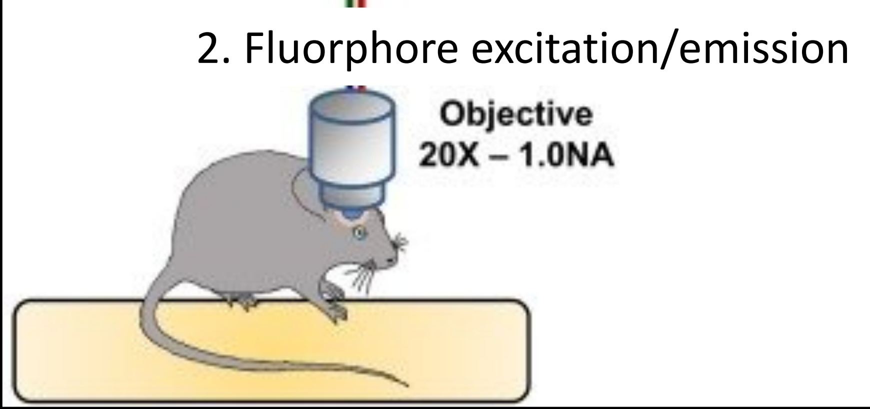
What would the emission be at 910nm?











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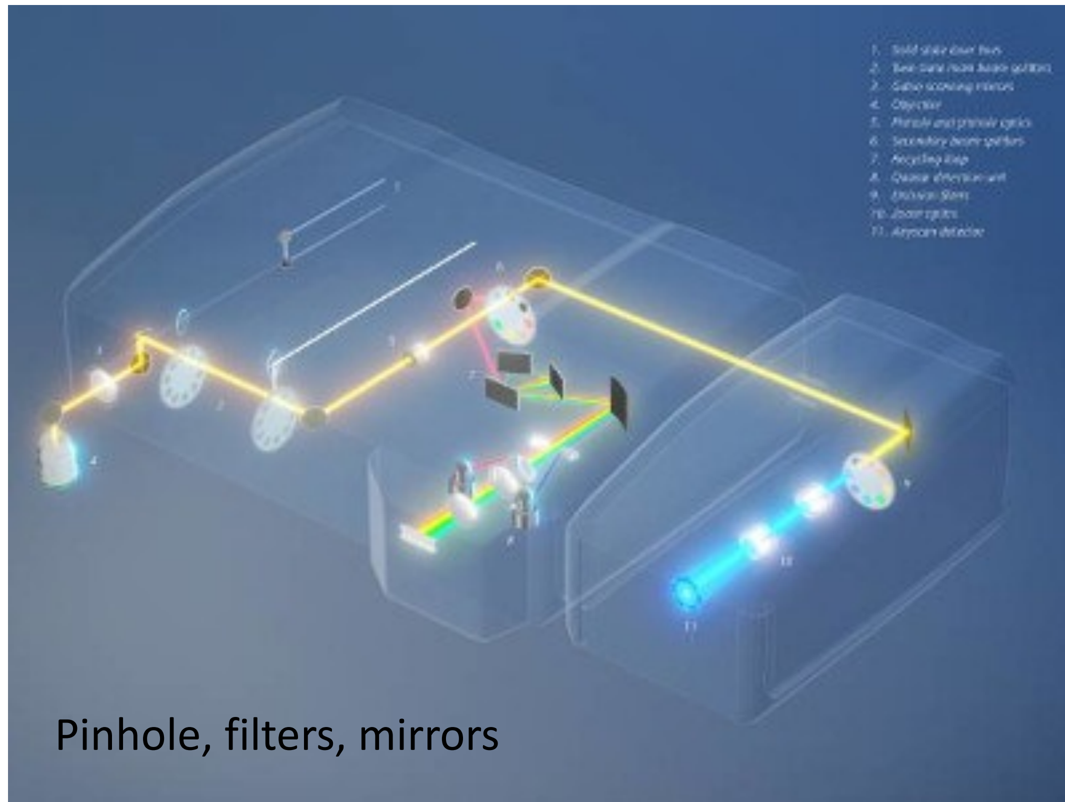
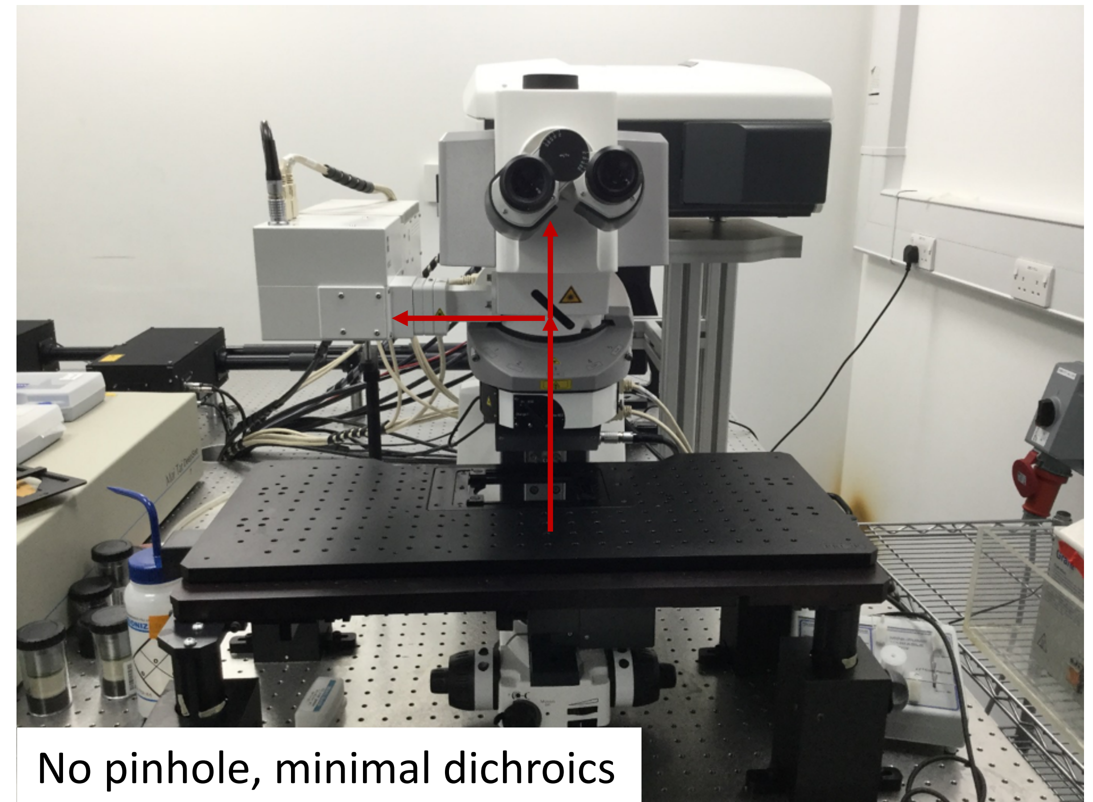
Light path optimization

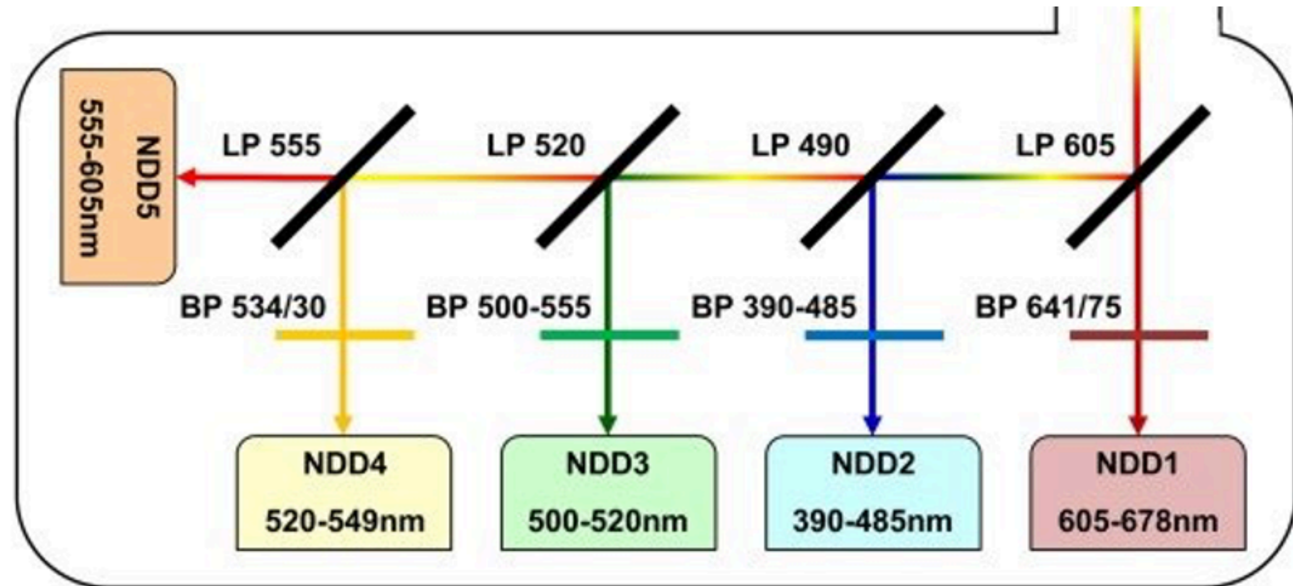
Confocal



2P NDD array

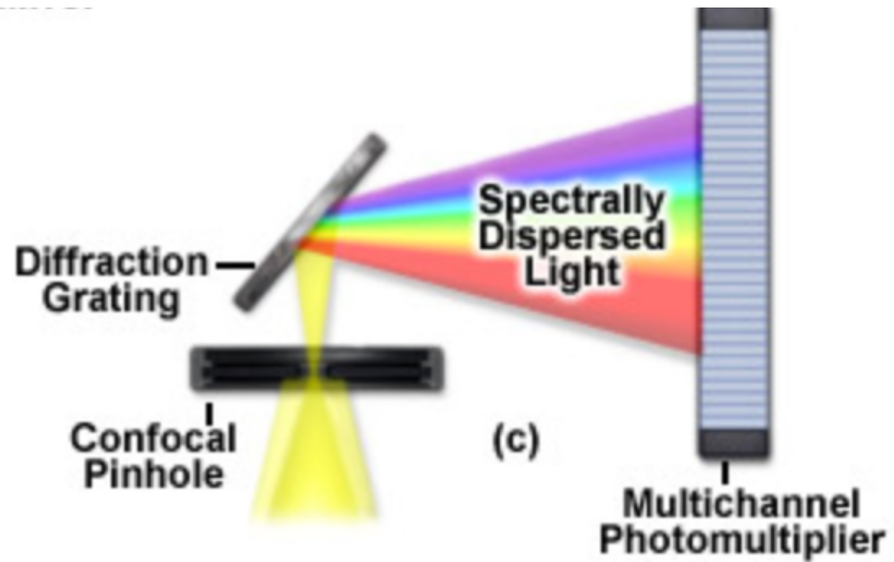


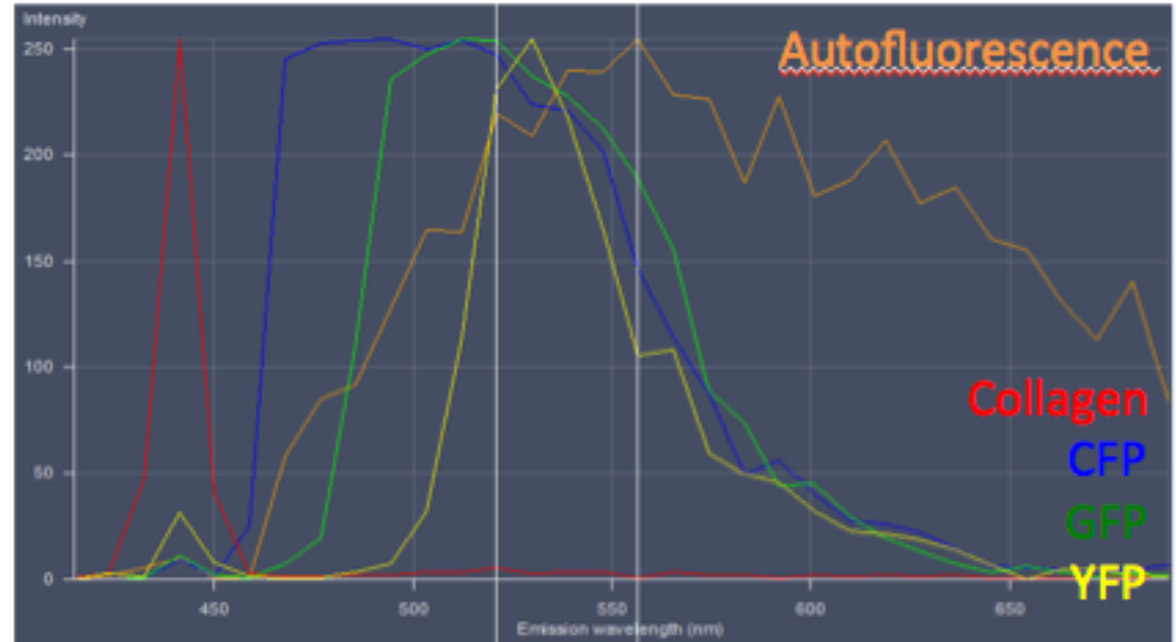
Non-descaned detectors in multi-photon microscopy

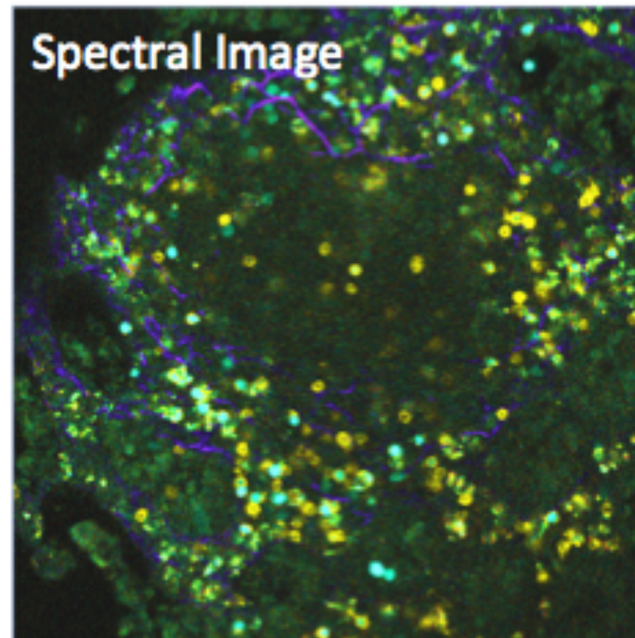


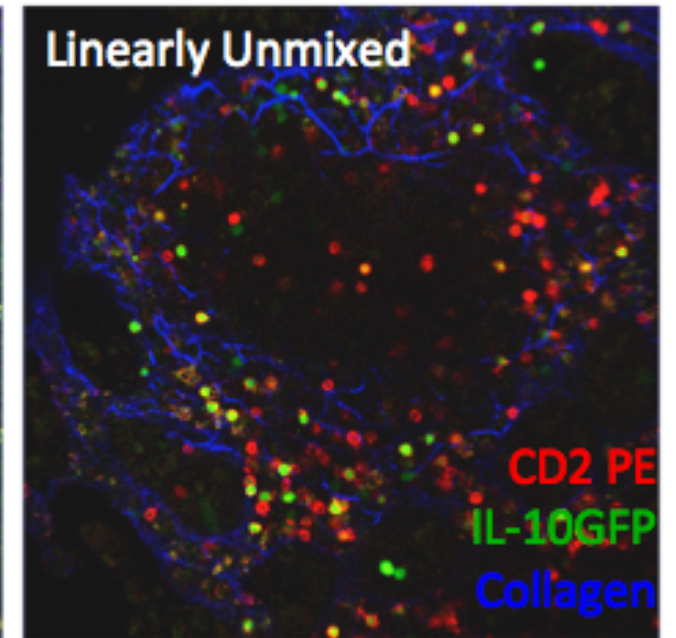
Since we are exciting all the fluorophores simultaneously, it can be difficult to separate the signals

Spectral detection



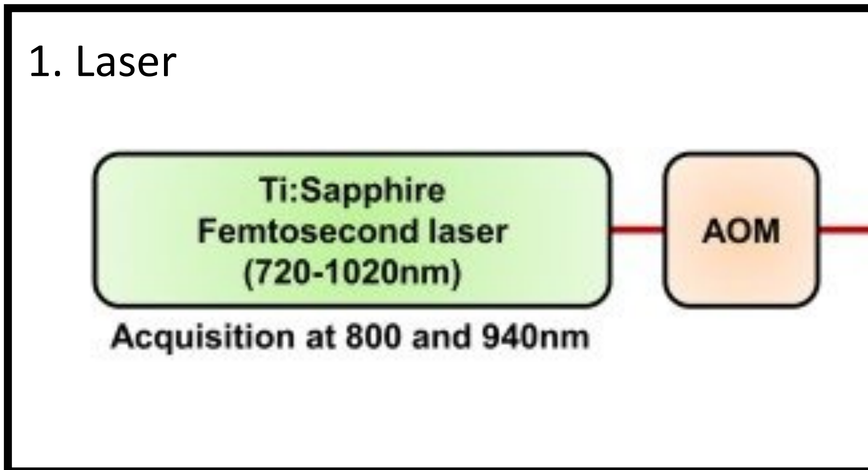


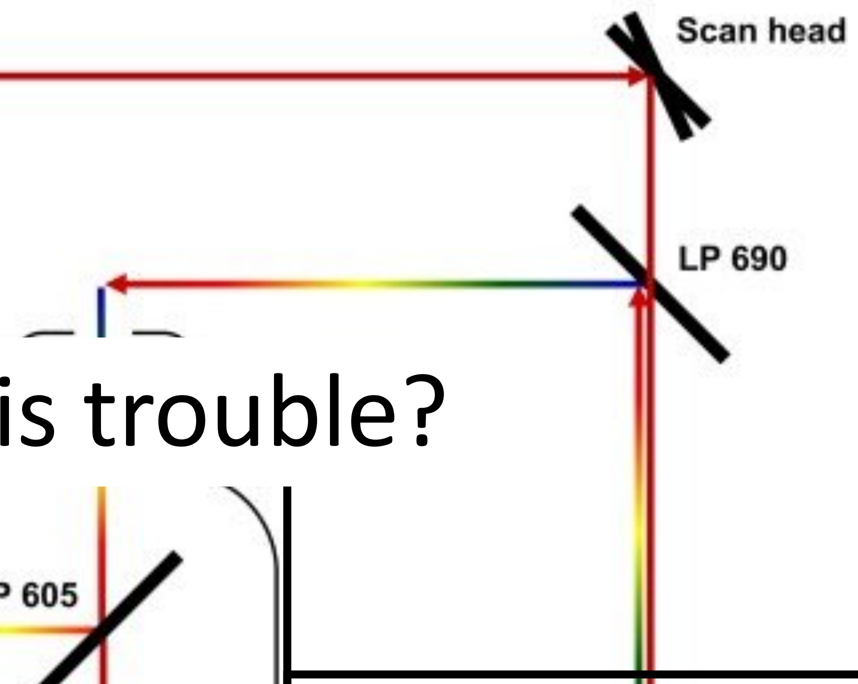




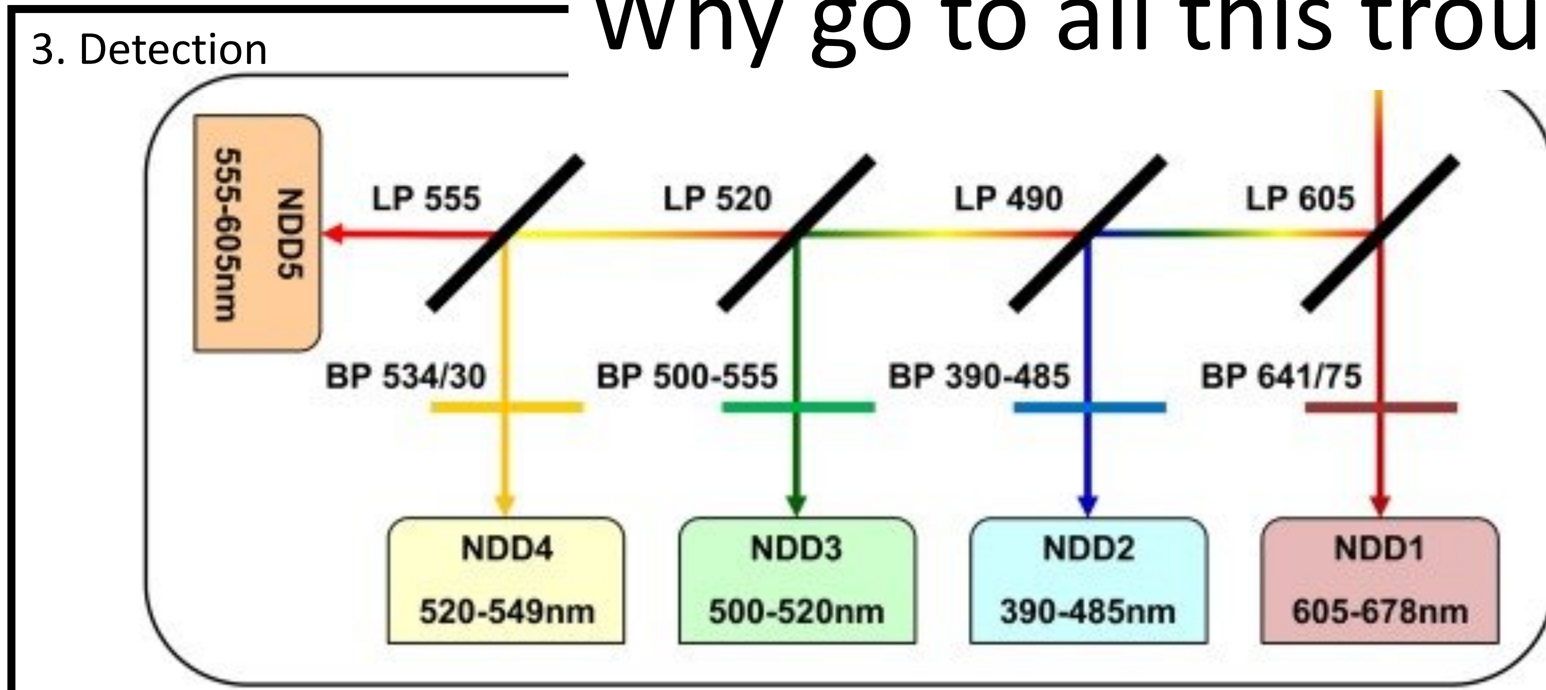
Why go to all this trouble?

1. Laser

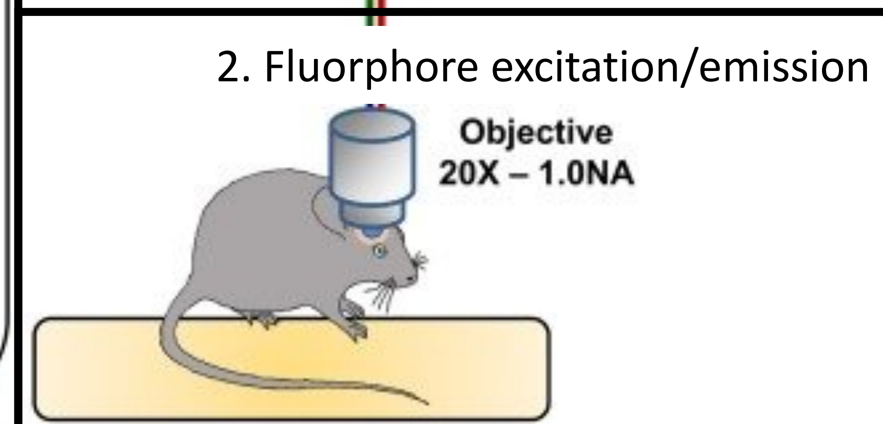




3. Detection



2. Fluorphore excitation/emission



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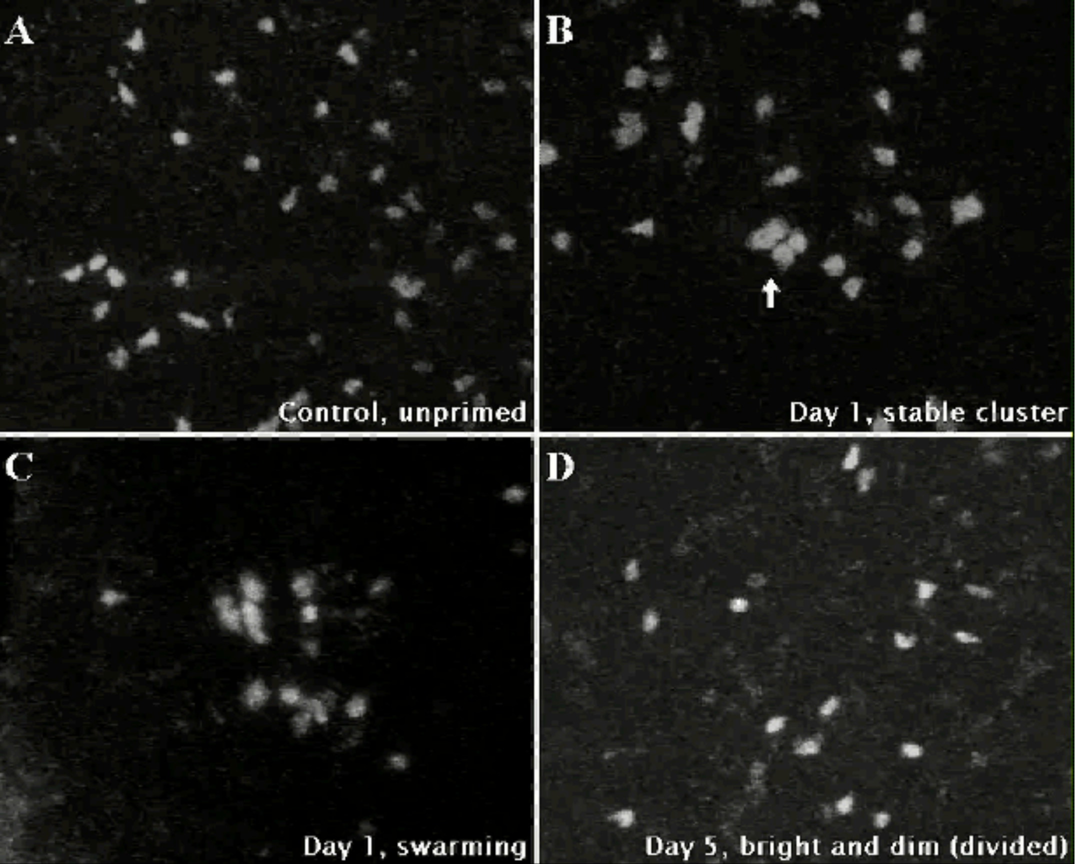
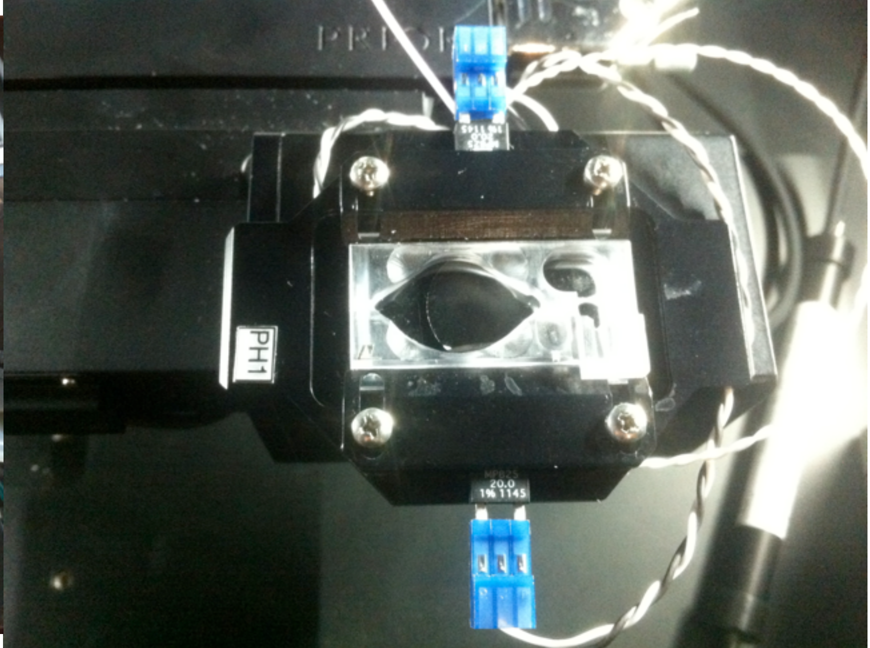
Explant imaging to study T cell behavior in the lymph node



95% Oxygen 5% CO_2

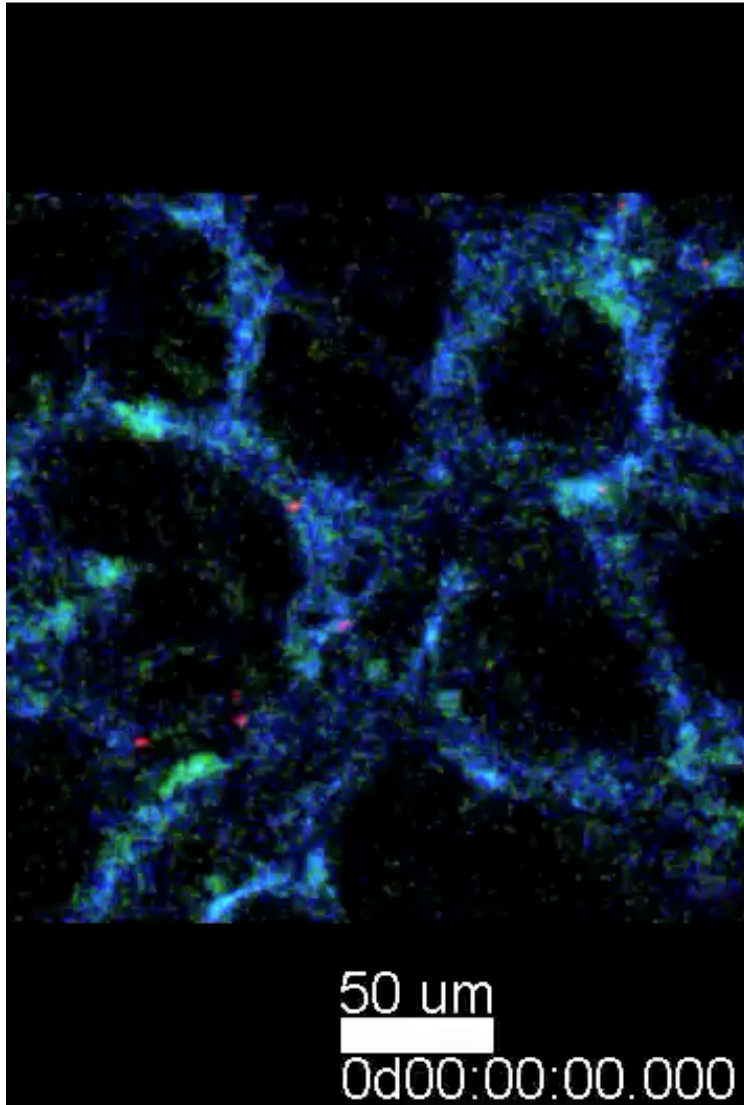


Close to 37 degrees without going over

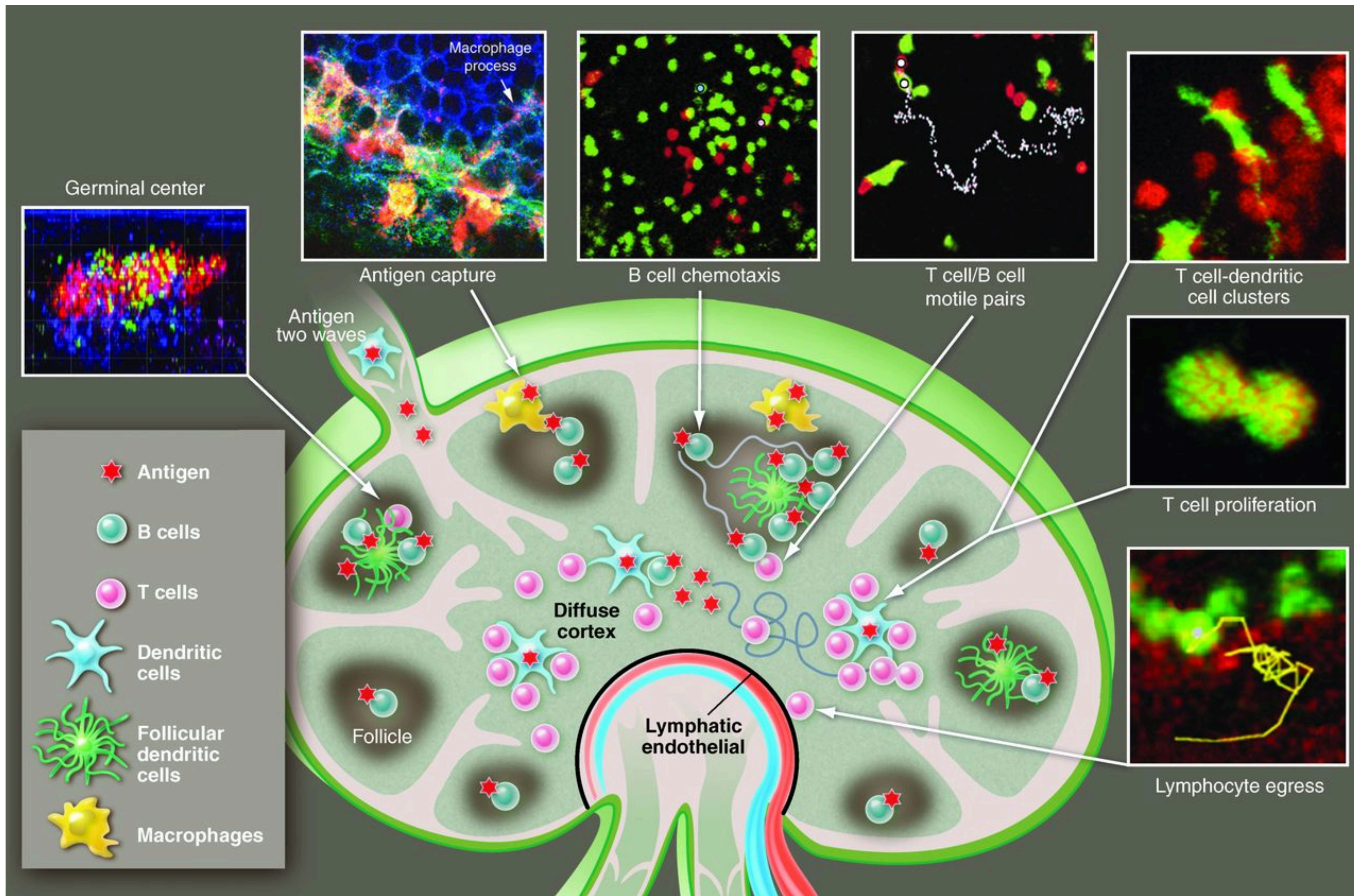
Miller et al. *Science* 2002

Oxygenation and temperature are key

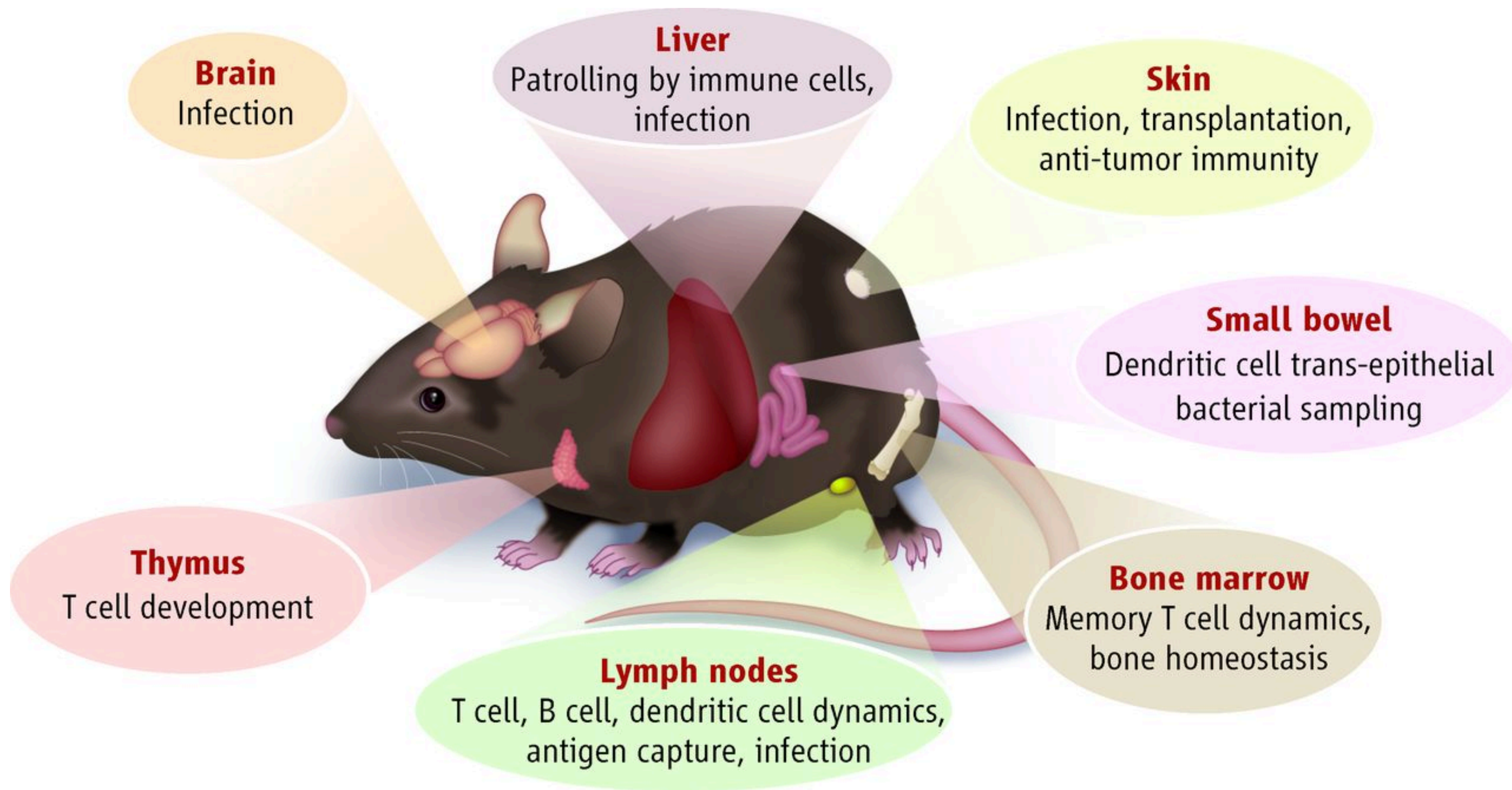
Intravital microscopy to study cell recruitment to the lung



Looney, et al. *Nature Methods* 2011



Germain et al. *Science* 2012



Germain et al. *Science* 2012

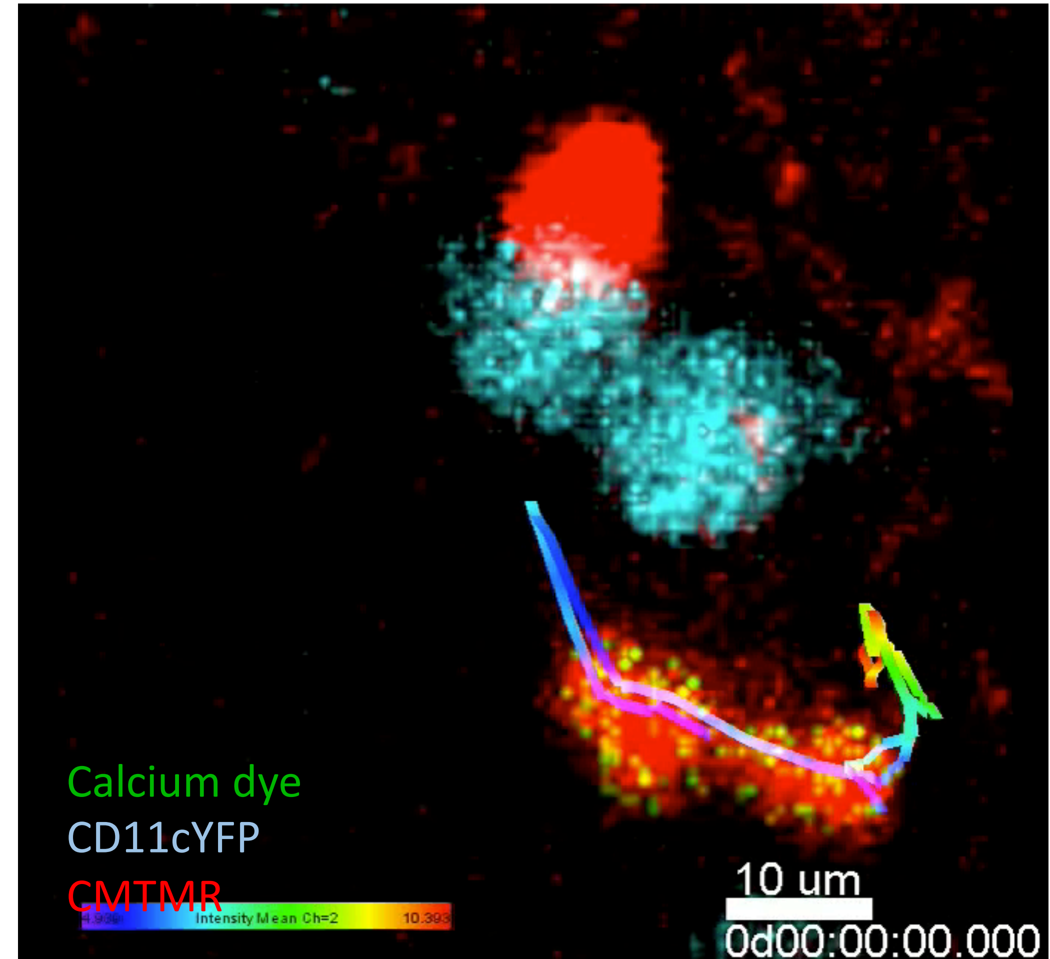
Transferred cells can be labeled

Pros

- Indicator dyes can add information
- Several different populations can be labeled
- No need to breed lots of reporter strains

Cons

- Dyes can be toxic
- Dividing cells dilute out dyes
- Excitation spectra may not work well with other markers



Thornton, et al. *JEM* 2012

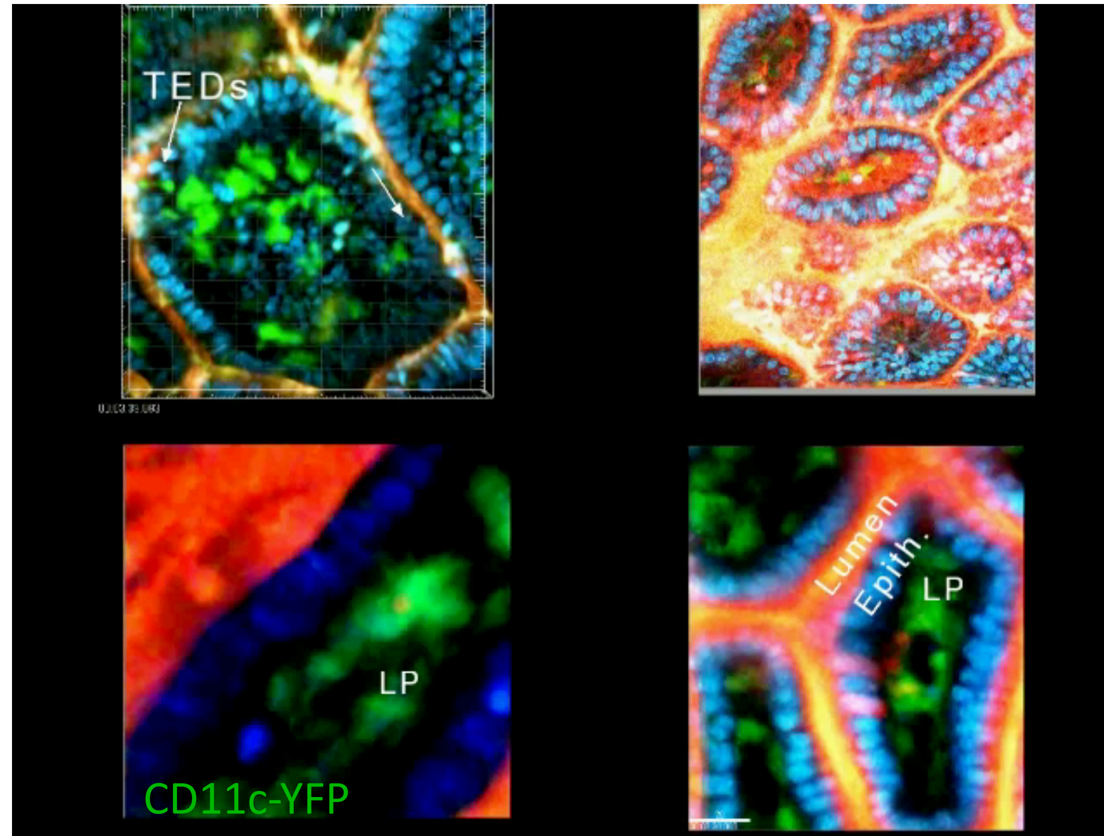
Looking at endogenous populations requires endogenous markers

LETTER

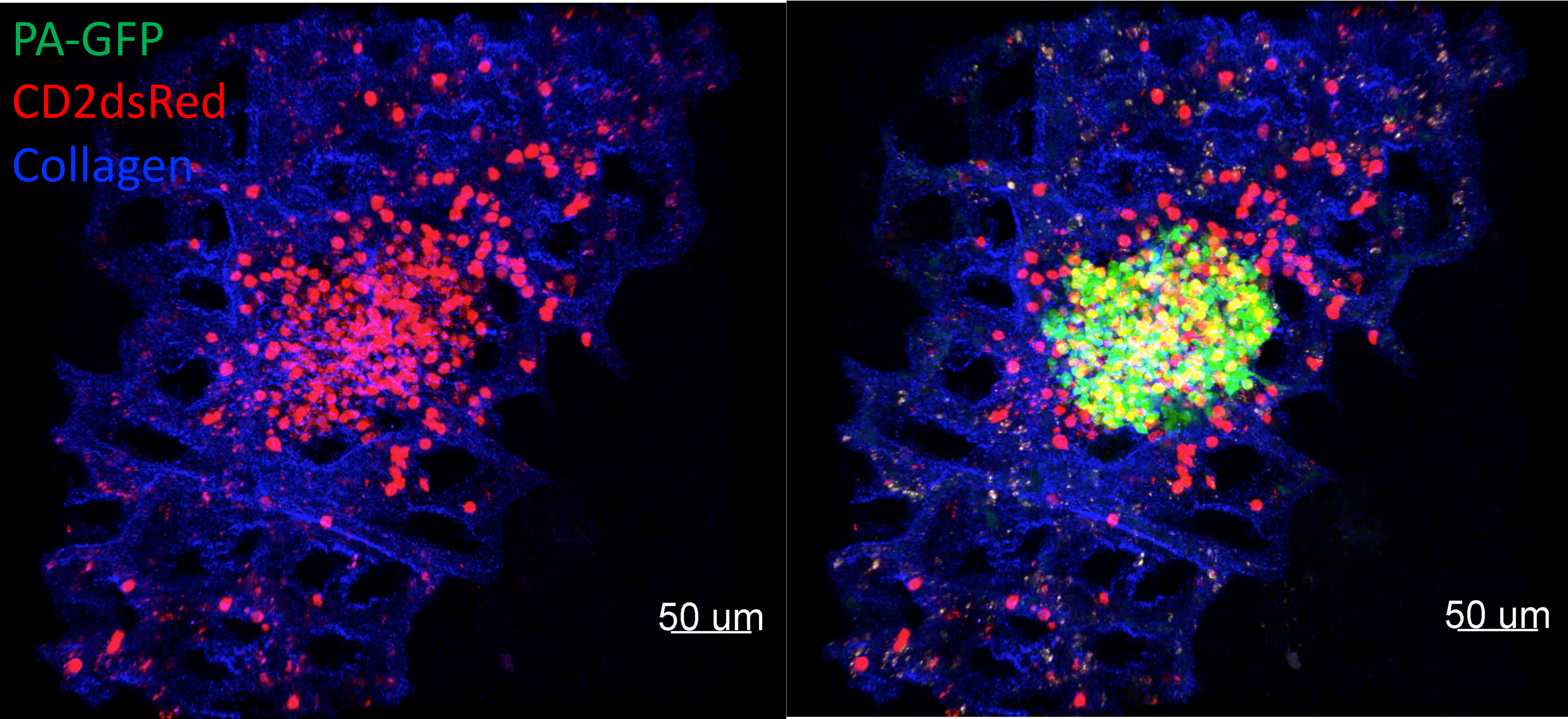
doi:10.1038/nature10863

Goblet cells deliver luminal antigen to CD103$^+$ dendritic cells in the small intestine

Jeremiah R. McDole[1]*, Leroy W. Wheeler[2]*, Keely G. McDonald[2], Baomei Wang[1], Vjollca Konjufca[3], Kathryn A. Knoop[2], Rodney D. Newberry[2] & Mark J. Miller[1]

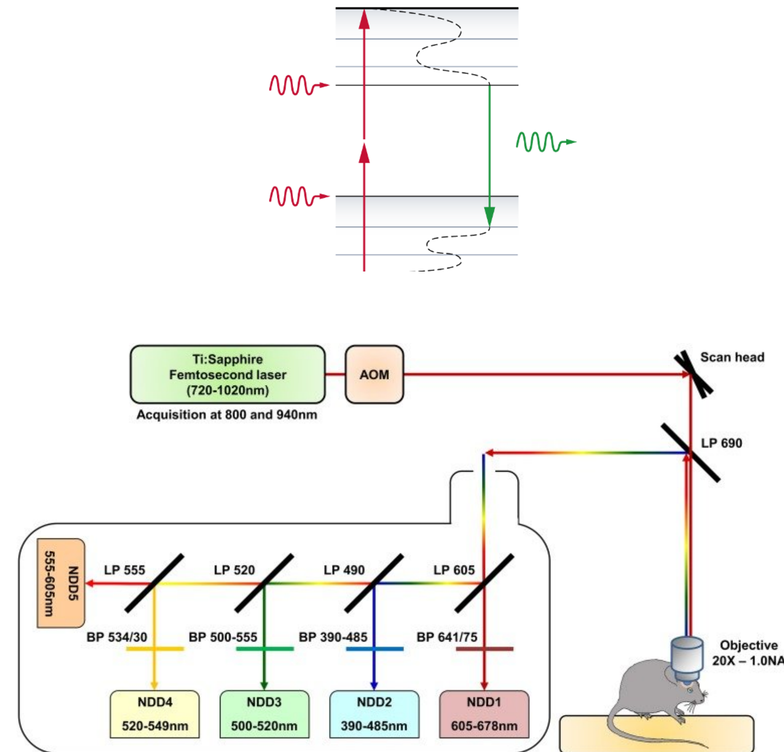


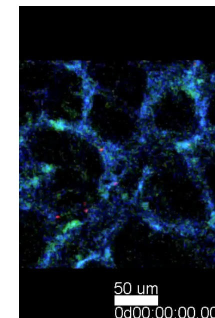
Photoactivation and Photoconversion



Summary

- Two –photon microscopy excitation uses longer wavelengths to penetrate deep into tissue with little damage
- Allow live, deep tissue imaging without cutting or clearing
- Specialized lasers and detectors allow optimal visualization
- Specialists in Oxford are able to image many different tissue types





Questions?