Two-photon (2P) microscopy

Principles & Applications in Life Sciences

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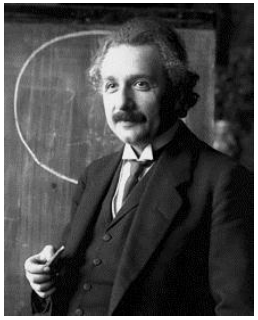
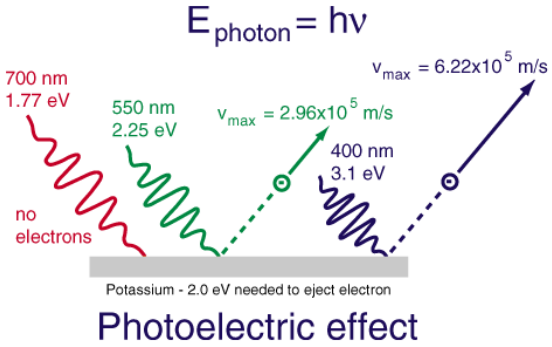
Background

1905



Light consists of bundles of energy, called **photons**.
The energy **E** of a **single** photon is given by **E = hν**.



1931

Theory of **two-photon absorption** first described by **Maria Göppert-Mayer** in **1931**.

1990

W. Denk

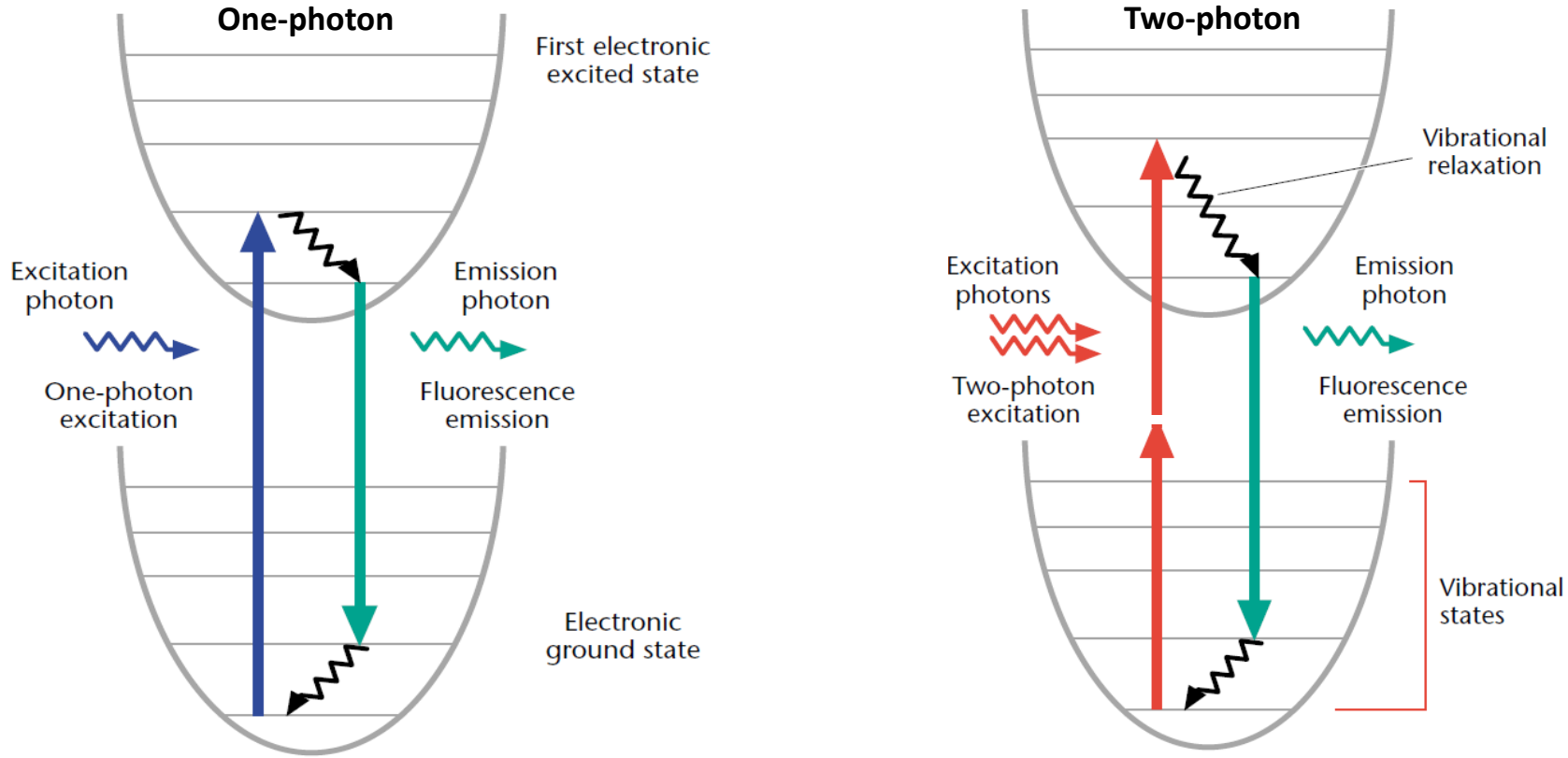
W. Webb

The first **2P microscope** was developed by **Winfried Denk** in the lab of **Watt Webb.**

Denk combined the idea of ***2P absorption*** with the use of a ***laser scanner***.

The fluorescence principle

Jablonski diagram



The simultaneous absorption of two low energy photons results in the emission of a single fluorescence photon.

Peter TC So (2002) Encyclopedia of Life Sciences

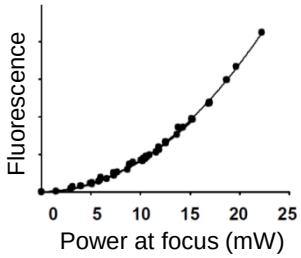
How likely is 2P absorption ?

$$\Delta x \Delta p \geq \frac{h}{4\pi}$$

Heisenberg's Uncertainty

A molecule should be capable of absorbing two photons in the same quantum event within 10^{-16} – 10^{-17} s.

$$F \propto I^2$$



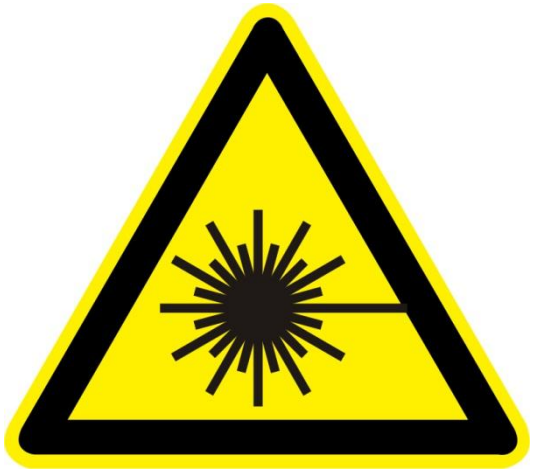
Probability for 2P Absorption

There is a quadratic relation between 2PA probability and light intensity = **nonlinear** process**.**

Exposed to **sunlight**, **a single molecule of Rhodamine** absorbs about **one photon per second**.

Here, **2P absorption** happens every **10^9 years.**

How 2P microscopy is achieved ?!



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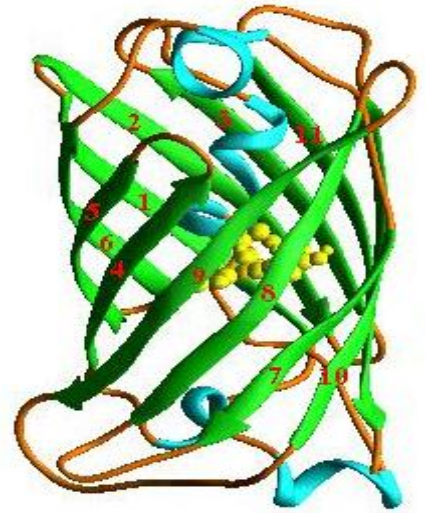


Ultrafast near-infrared light source

Focusing elements with high IR transmission

2PE compliant fluorophore

CONCENTRATING excitation PHOTONS in TIME and SPACE

$F \propto I^2$

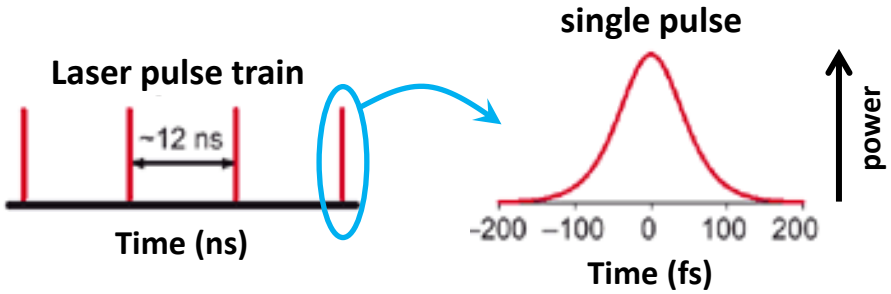
INCREASING the RATE of FLUORESCENCE emission

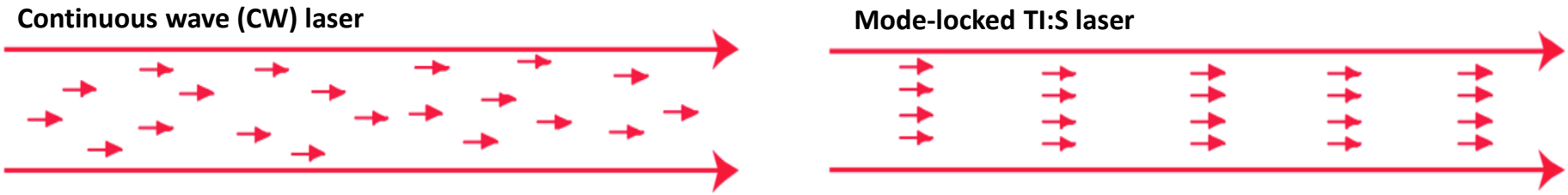
How 2P microscopy is achieved ?!

1. Ultrafast and near-infrared light source

Use of **titanium–sapphire laser** (Ti-S):

- femtosecond **pulse** width
- **high repetition** rate (100 MHz)
- tunable from **700-1000 nm**
- high peak power





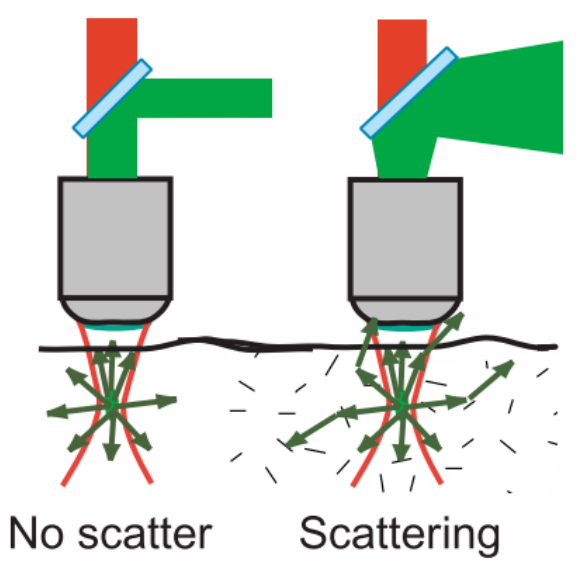
✓ **Pulsed lasers allow to concentrate photons in time**
<u>within a same average power</u>

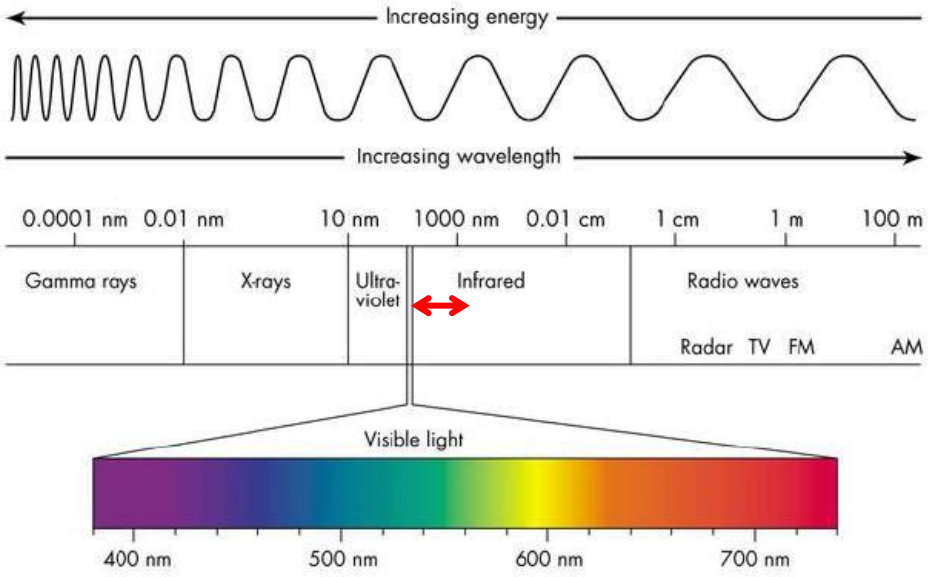
⬇

INCREASE THE PROBABILITE OF 2PE by ~10^5

How 2P microscopy is achieved ?!

1. Ultrafast and near-infrared light source





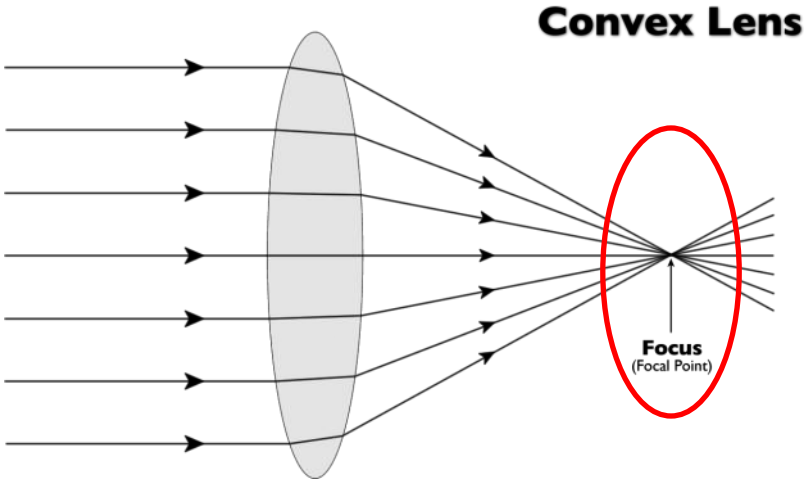
Rayleigh scattering: $I_s \sim \lambda^{-4}$

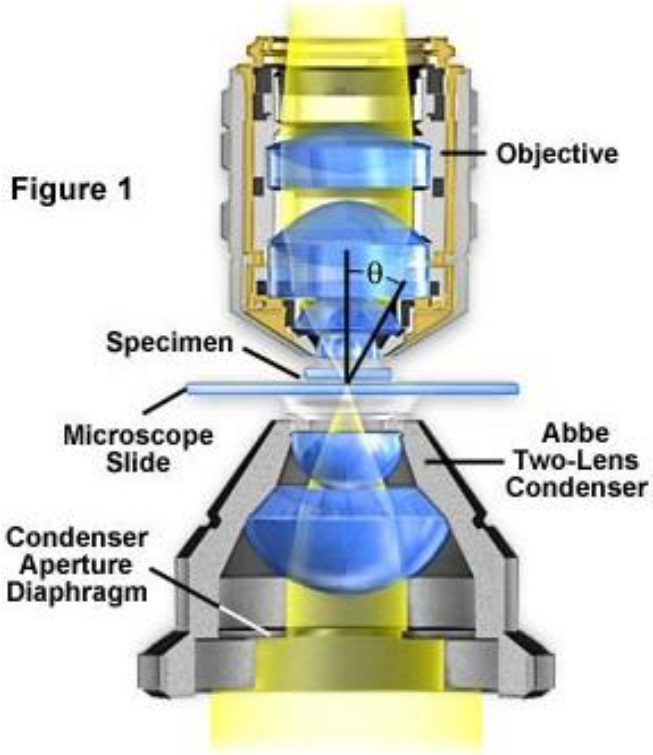
- ✓ longer excitation wavelengths penetrate deeper
- ✓ decreased energy = less photodamaged to living tissue

How 2P microscopy is achieved ?!

1. **Ultrafast and near-infrared light source**
2. **Focusing elements with high IR transmission**

- $P_{2PE} \propto I^2$
- I = number of photons passing through a **unit area** per unit time ($cm^{-2}\ s^{-1}$)





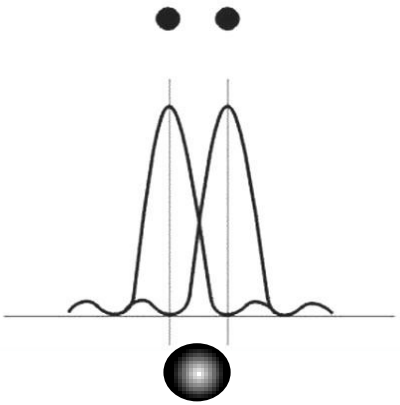
2PE probability increases by ~10^7 in the focal plane

How 2P microscopy is achieved ?!

1. Ultrafast and near-infrared light source
2. Focusing elements with high IR transmission
3. A 2PE compliant fluorophore

*The **2PE probability** per laser pulse and per **fluorophore** is:*

$$1 - \exp(-\alpha\sigma_{2P} \cdot P^2 \cdot IPSF^2_{(x,y,z)}/(R^2\,\tau))$$



P: laser power
R: number of laser pulse per sec
τ: full-width half-maximum (FWHM) of the pulse
α: conversion constant

IPSF = Intensity Point Spread Function

How 2P microscopy is achieved ?!

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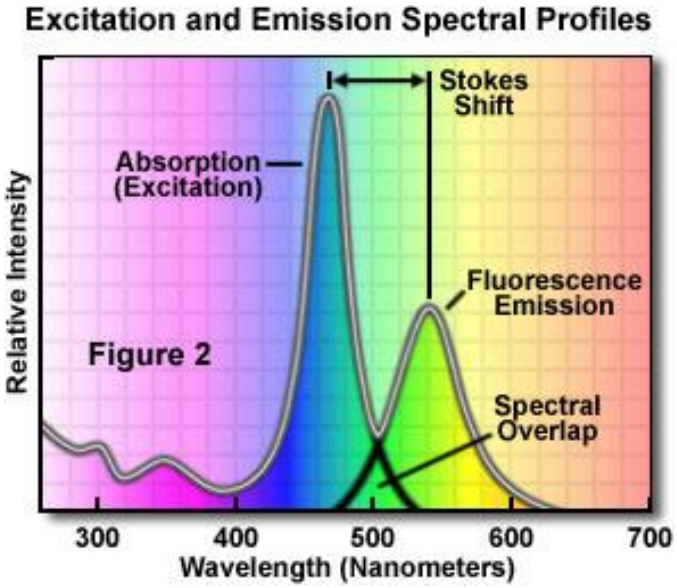
α: conversion constant

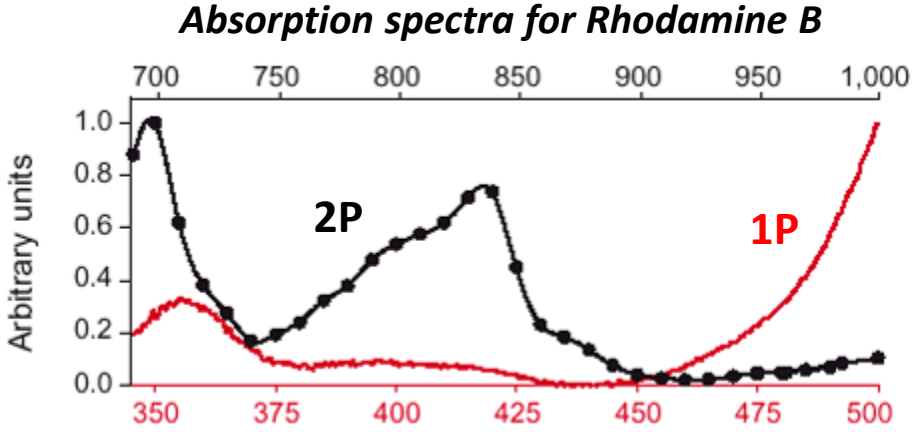
$$IPSF = \overbrace{\sigma_{2P}}^{\text{absolute PSF}} \times \phi F$$

ϕF : fluorescence quantum yield
(ratio of photons absorbed to photons emitted through fluorescence).

How 2P microscopy is achieved ?!

1. Ultrafast and near-infrared light source
2. Focusing elements with high IR transmission
3. A 2PE compliant **fluorophore**

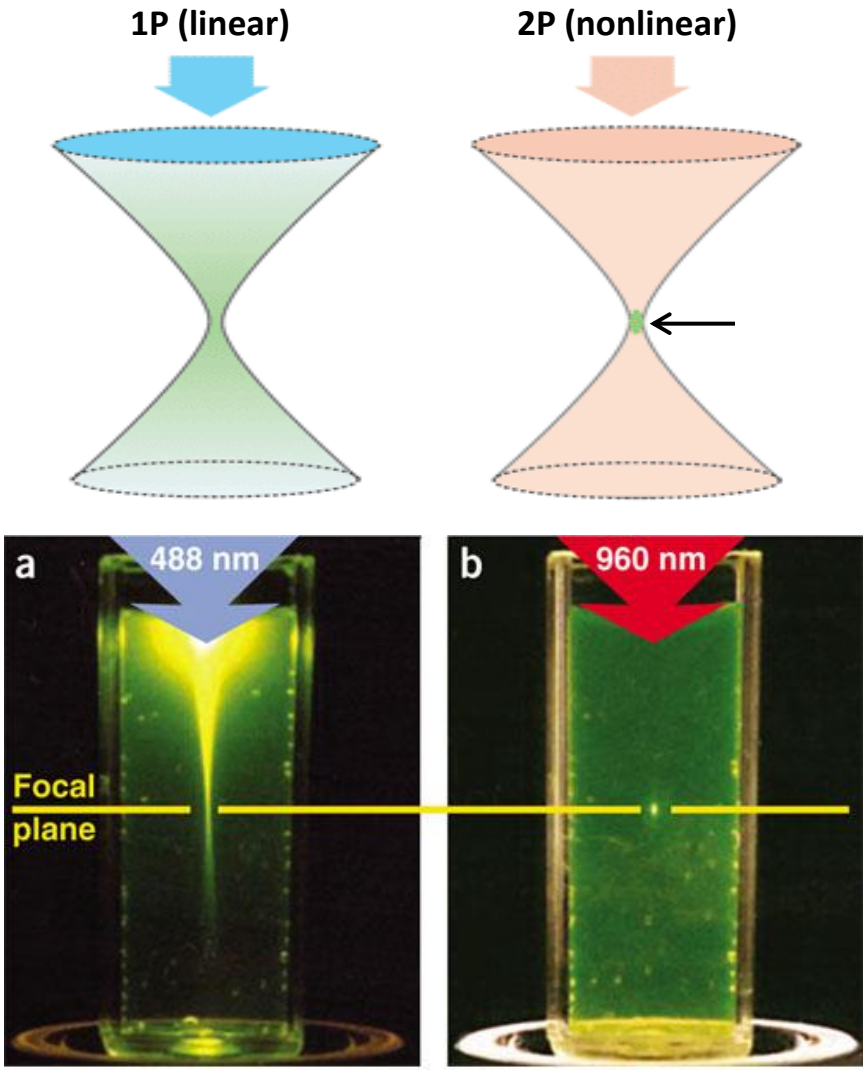




$2 \times \lambda \neq (2\lambda)$

2P spectra of many molecules can be significantly different from their scaled 1P equivalent

1P versus 2P excitation (1)



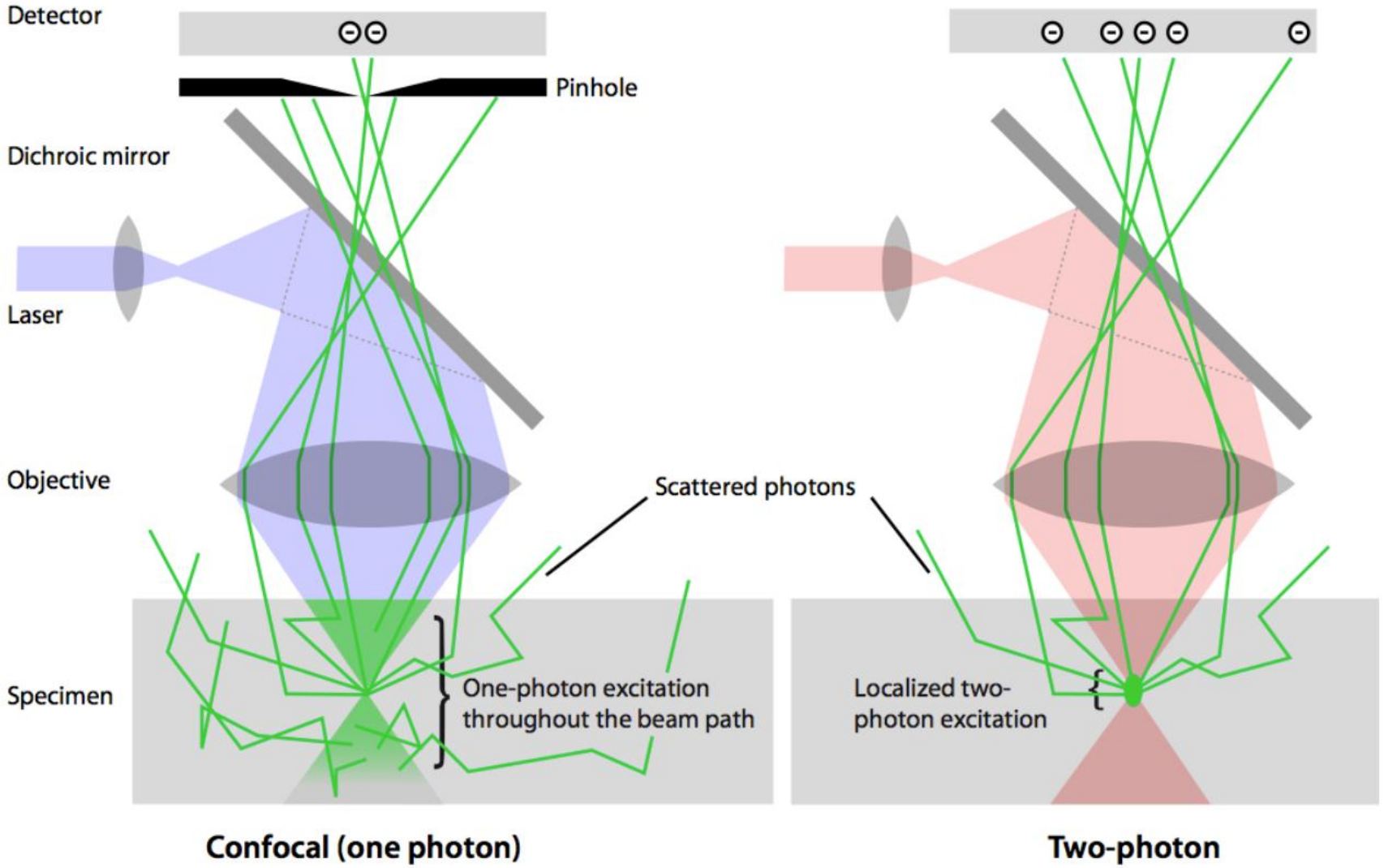
Fluorescein cuvette

Probability of 2PE is very low away from the focal plane,

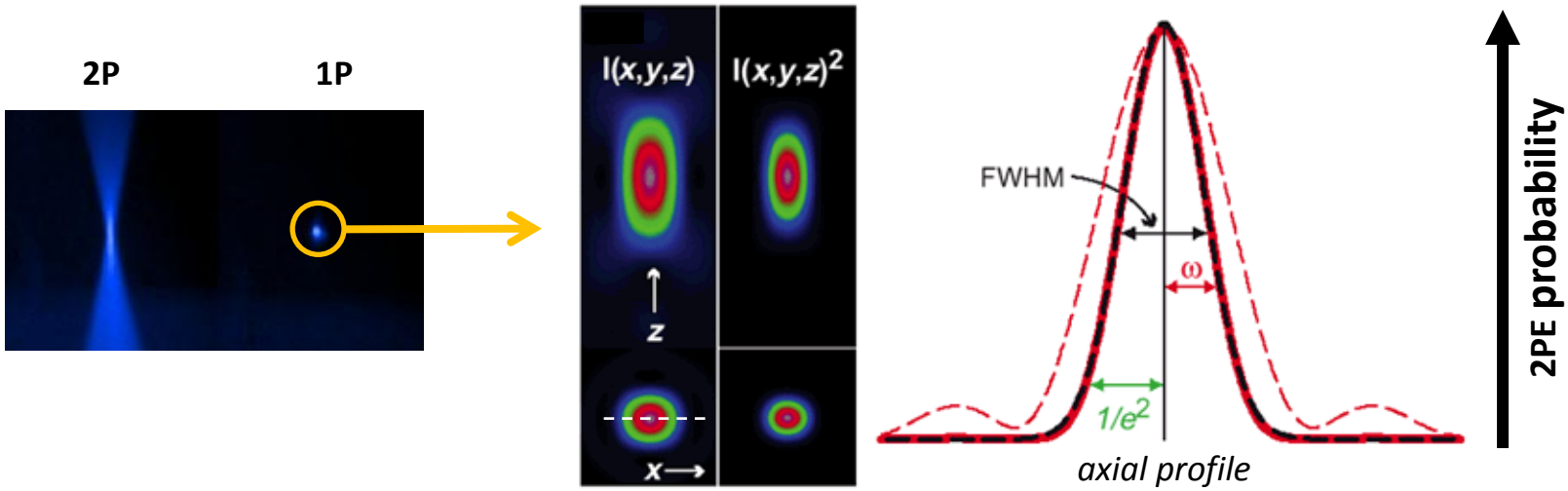
Three-dimensionally localized excitation

Zipfel et al. (2003) Nature Biotechnology, 21.

1P versus 2P excitation (2)



Resolution of 2P microscopy (IPSF)



IPSF describes intensity everywhere in space near the focus

$$d = \frac{0.61\lambda}{NA}$$

The optical resolution increases with:

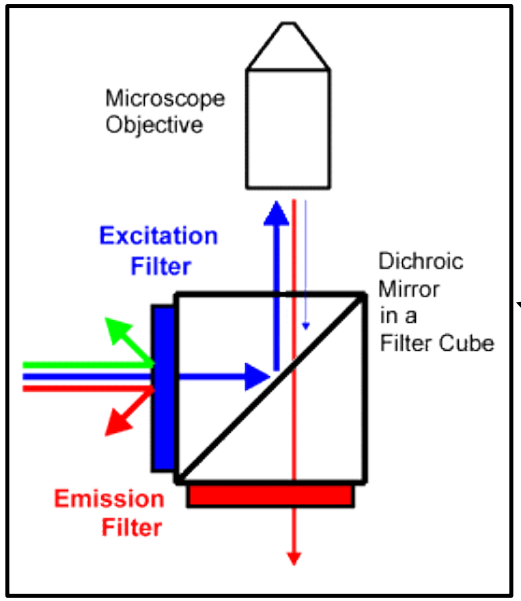
- **smaller λ**
- **higher NA (numerical aperture)**

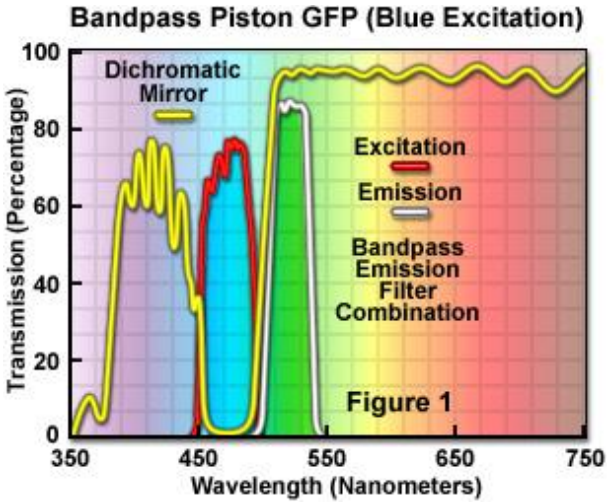
d_{2PE} = 0.3 mm x 0.9 mm

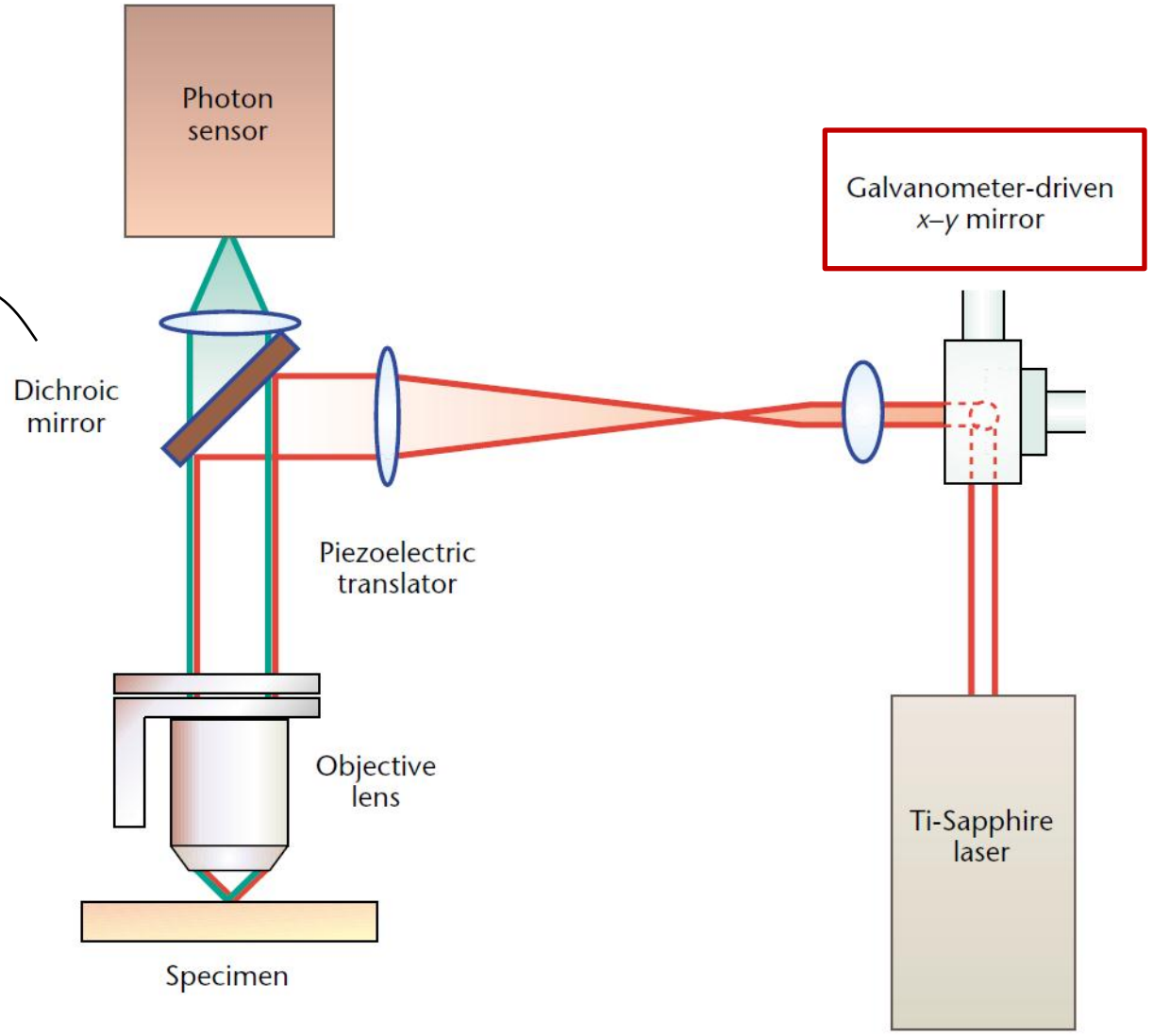
FWHM: Full-width half-maximum value

Zipfel et al. (2003) Nature Biotechnology, 21.

2P microscopy setup





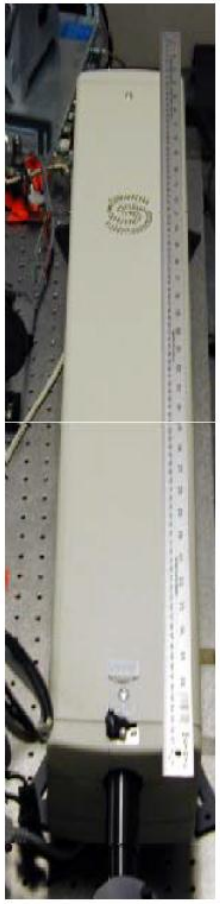


Peter TC So (2002) Encyclopedia of Life Sciences

Evolution of 2P lasers

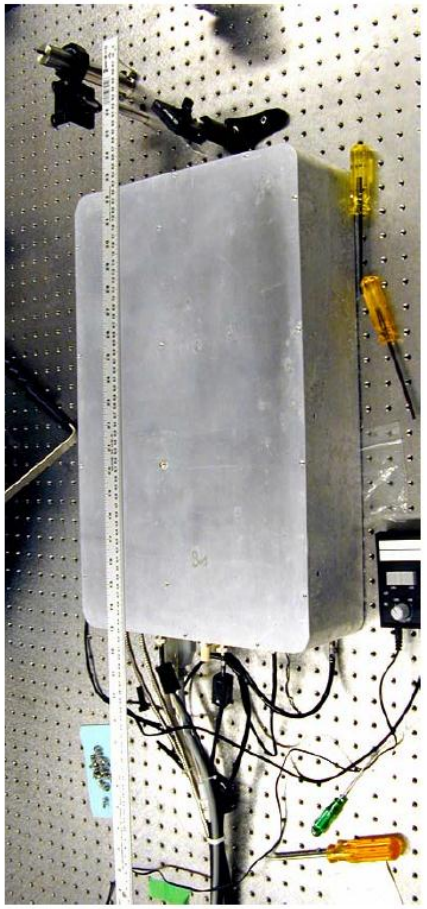
1991: Argon pump and Ti:S

1997: Diode pumped Nd-vanadate 5 W pump



1998: Diode pumped Nd-vanadate 10 W pump

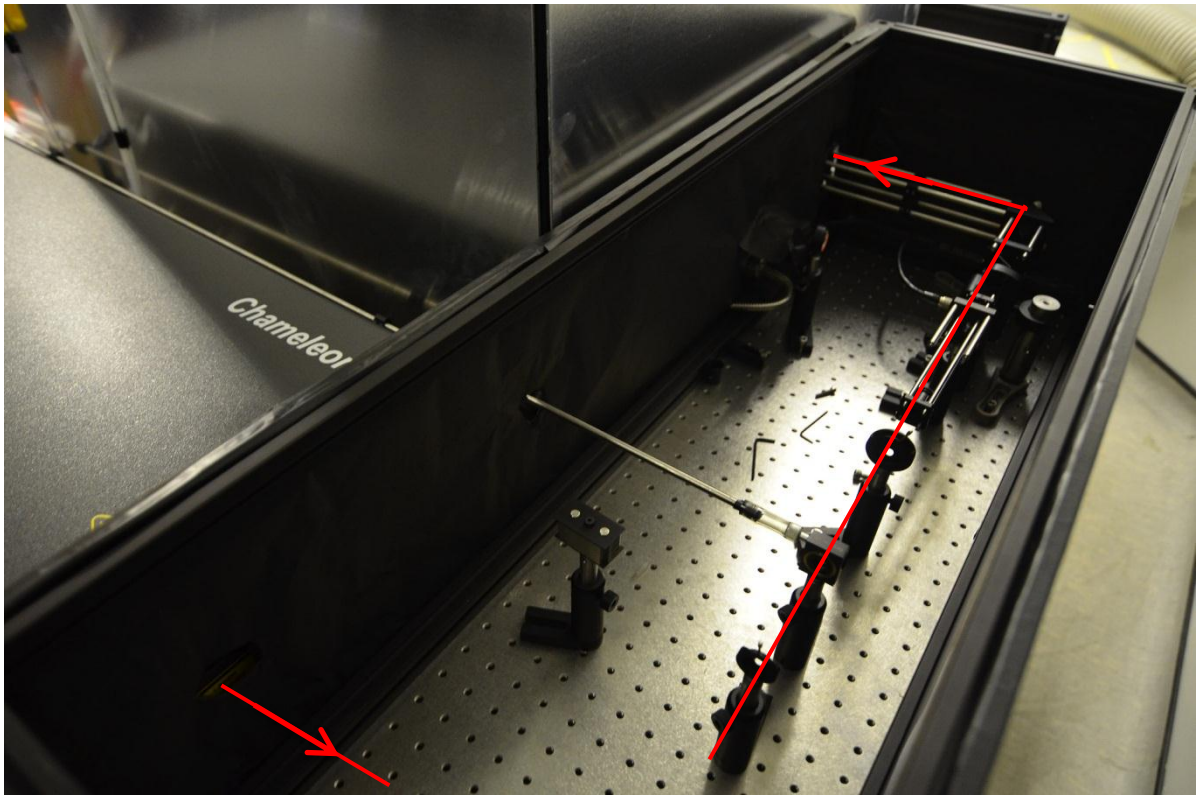
1999: Diode Pump and Ti:S resonator in a single case.



From Alex Kwan (Cornel University)

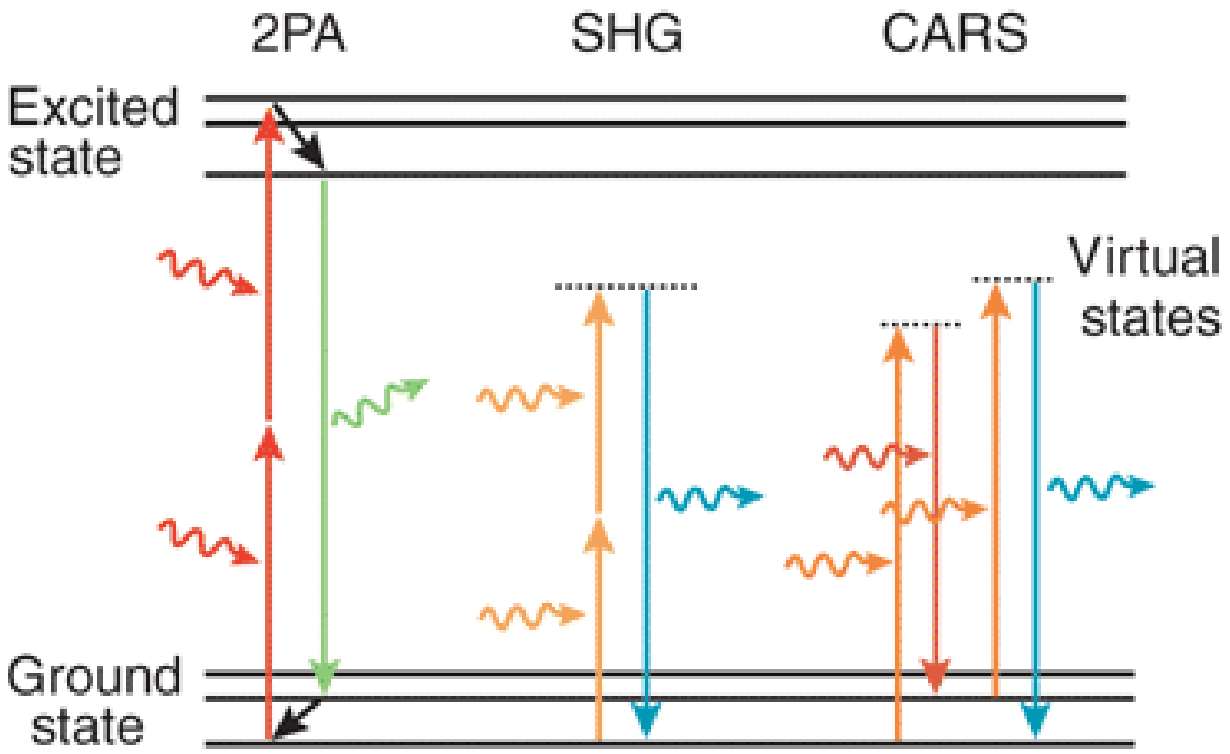
A 2P microscope setup in 2013

A 2P microscope setup in 2013



customized 2P microscope with laser-intensity controler

Other type of nonlinear optical microscopy



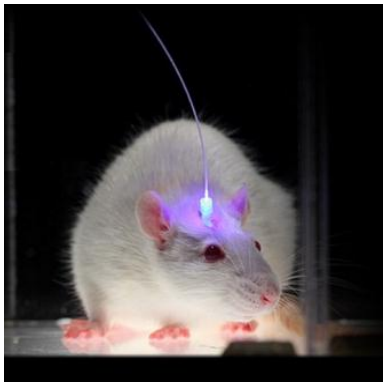
SHG: Second Harmonic Generation
CARS: coherent anti-Stokes Raman scattering

Helmchen & Denk (2005) Nature Method, 2.

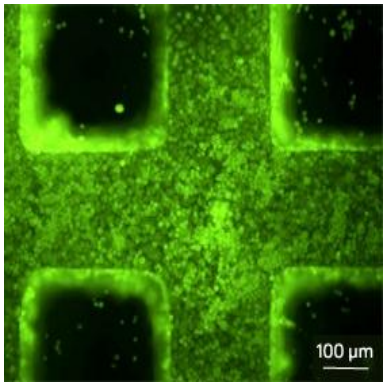
Applications

Main applications

RESEARCH

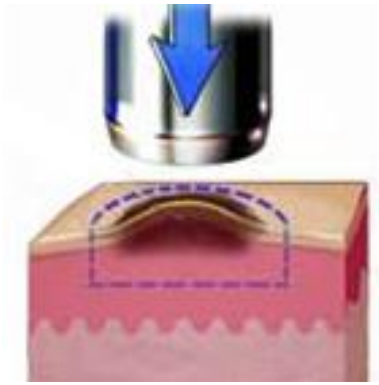


life sciences



tissue engineering & biomaterials

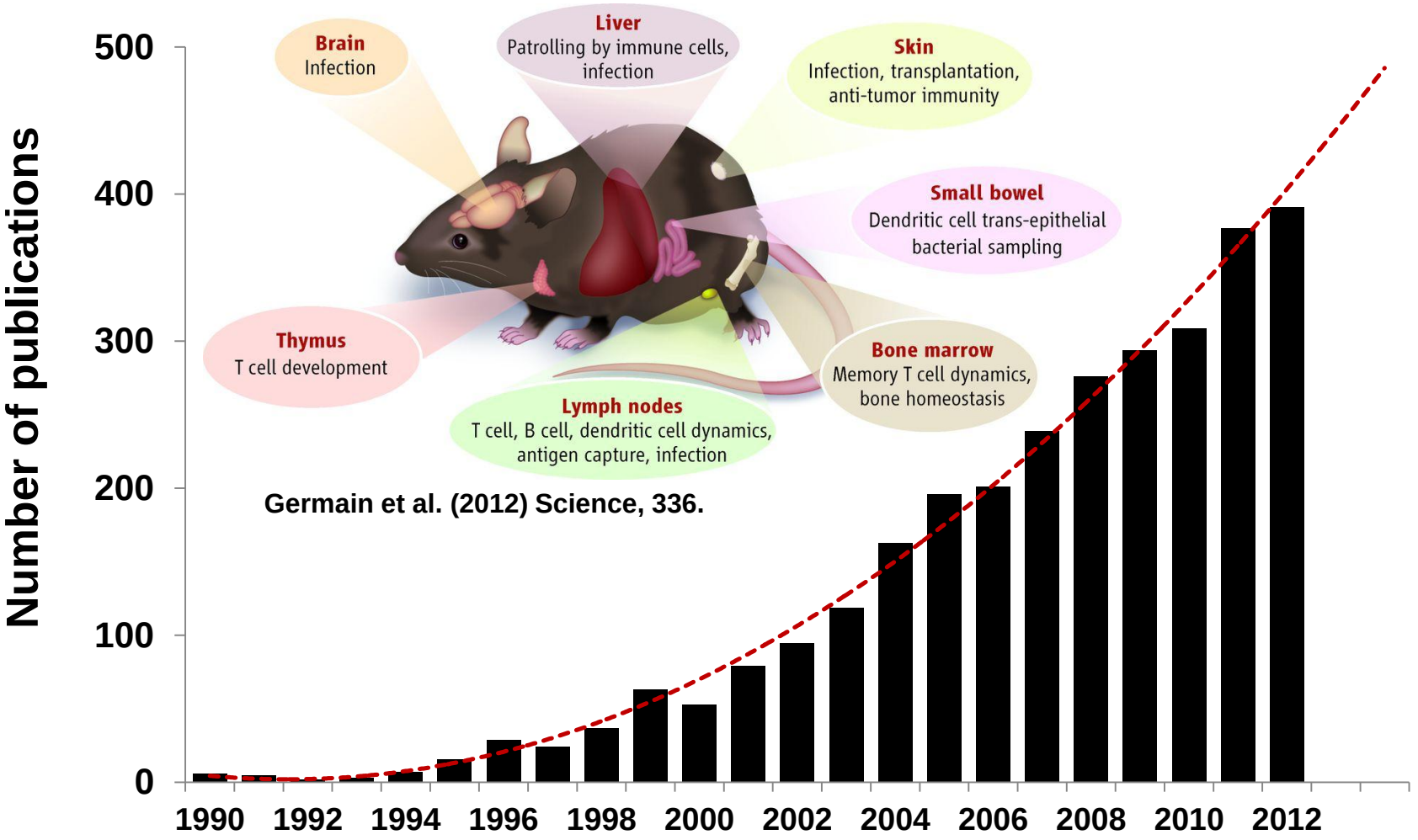
CLINICAL



non invasive biopsy

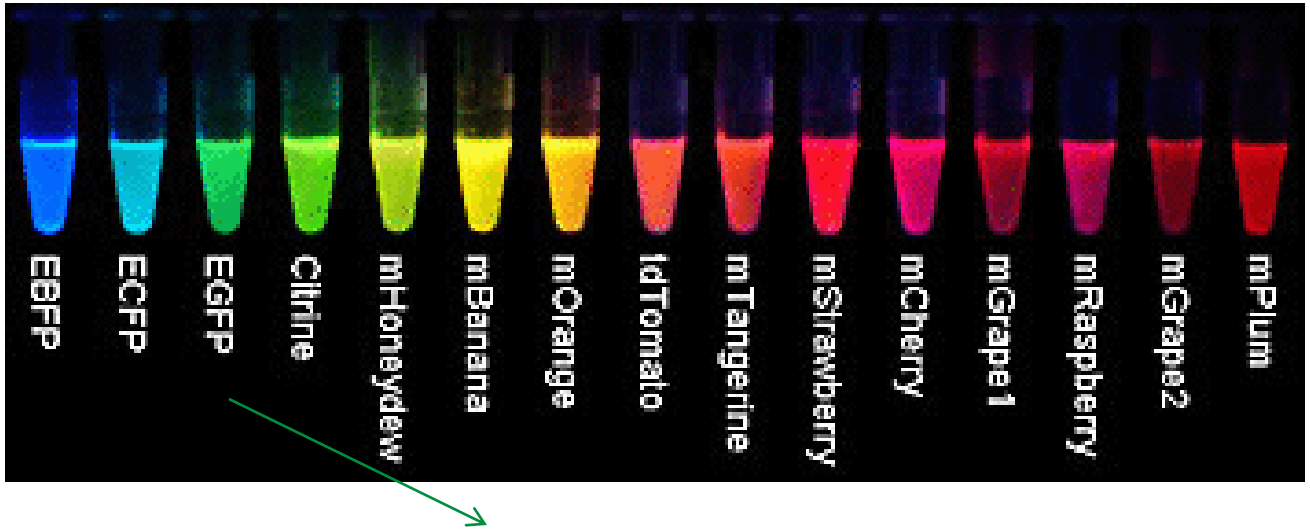
skin imaging

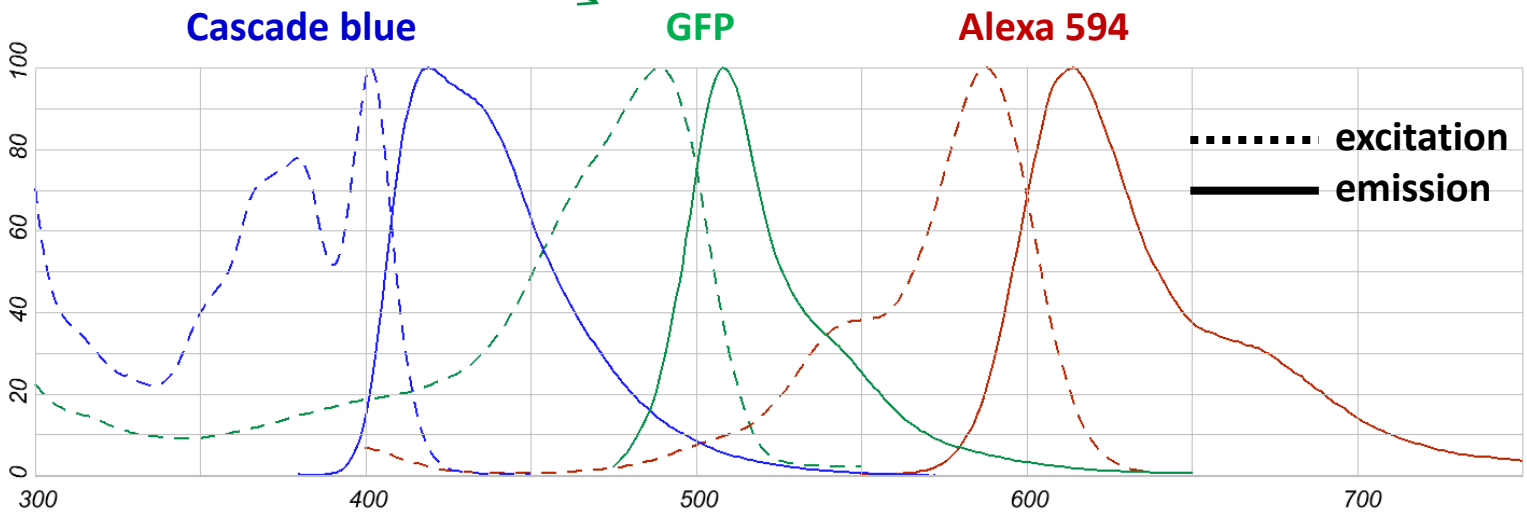
2P microscopy and life sciences



Search for "two-photon microscopy" in Pubmed database

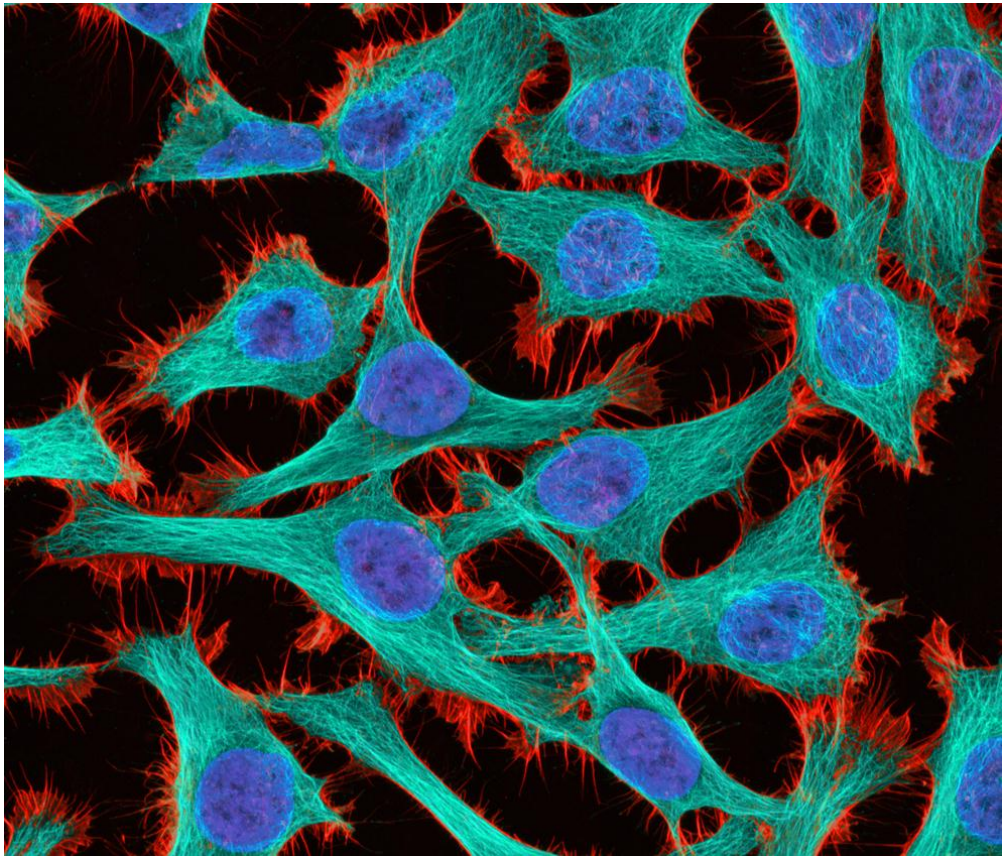
Multicolor 2P imaging





Multicolor 2P imaging

HeLa (cancer) cells at 300x magnification



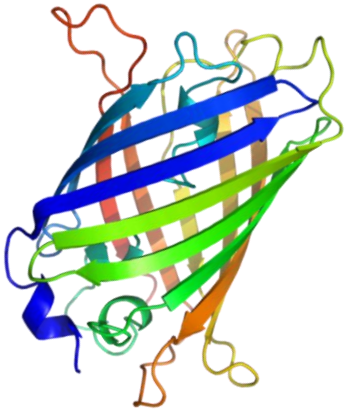
simultaneous excitation of fluophores emitting at widely diverging wavelengths

Thomas Deerinck
National Center for Microscopy & Imaging Research
La Jolla, USA

The GFP revolution

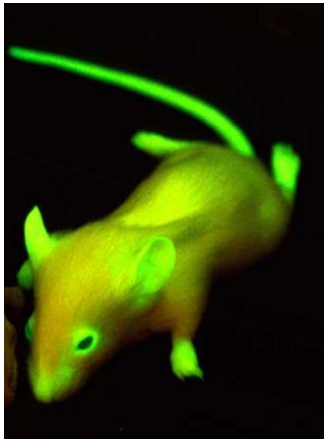
Aequorea victoria

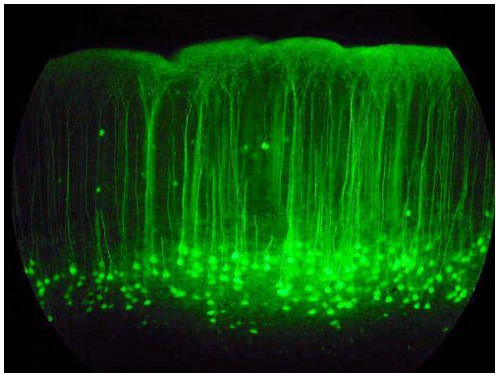
extraction



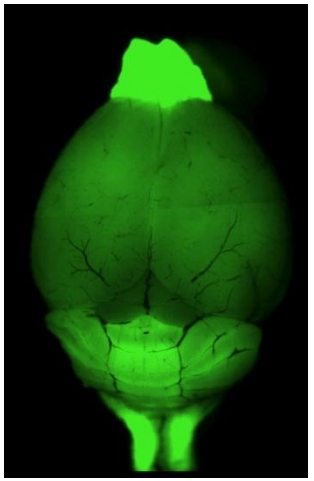
genetic targeting

Roger Tsien
Nobel Prize in 2008

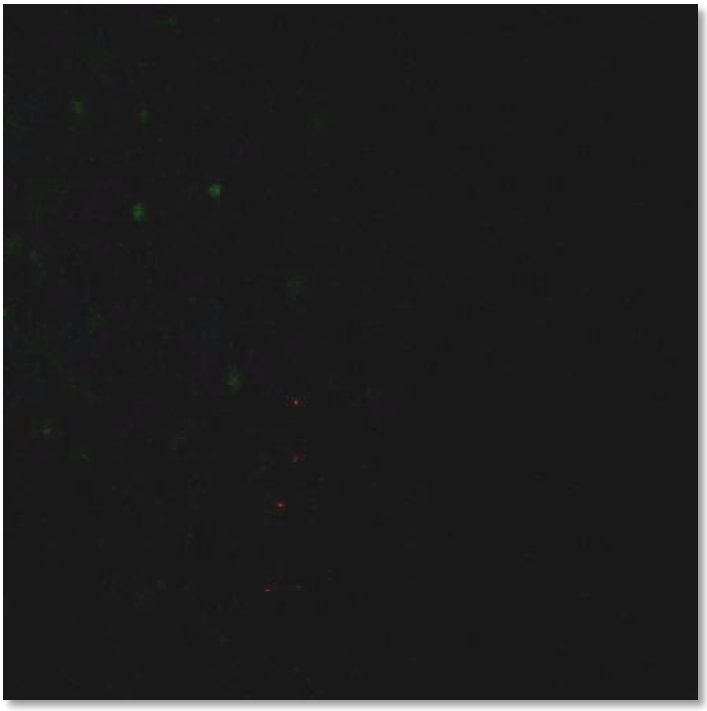




2P imaging

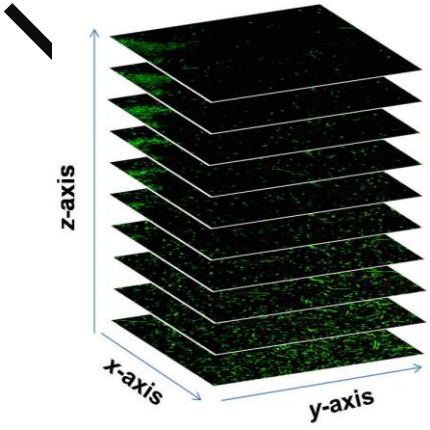


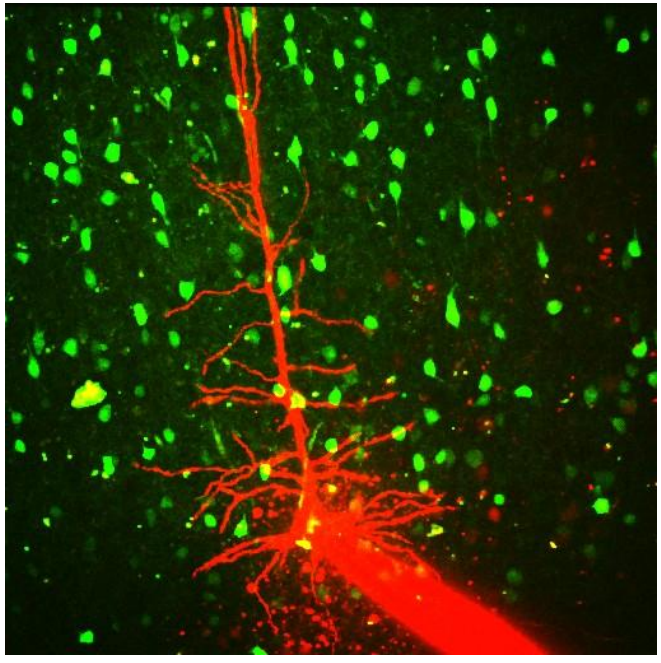
Imaging the brain in depth *ex vivo* (2D)



300 µm thick mouse brain slice (visual cortex)

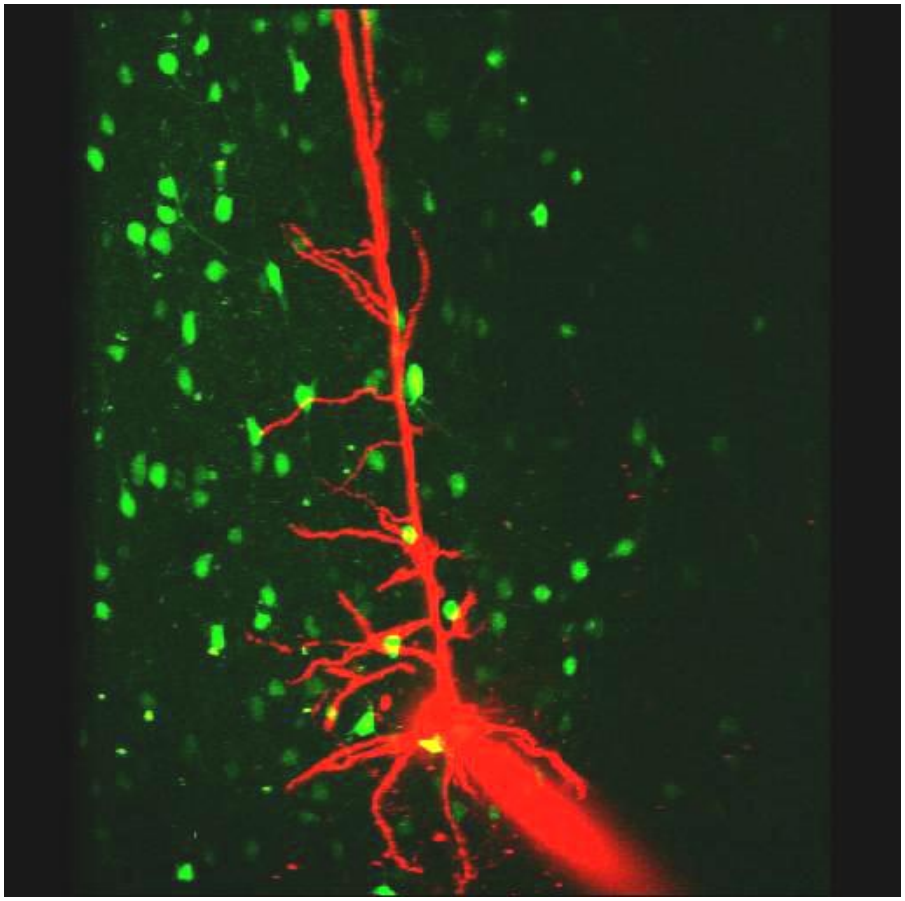
56 x 1 µm optical sections through the z-axis



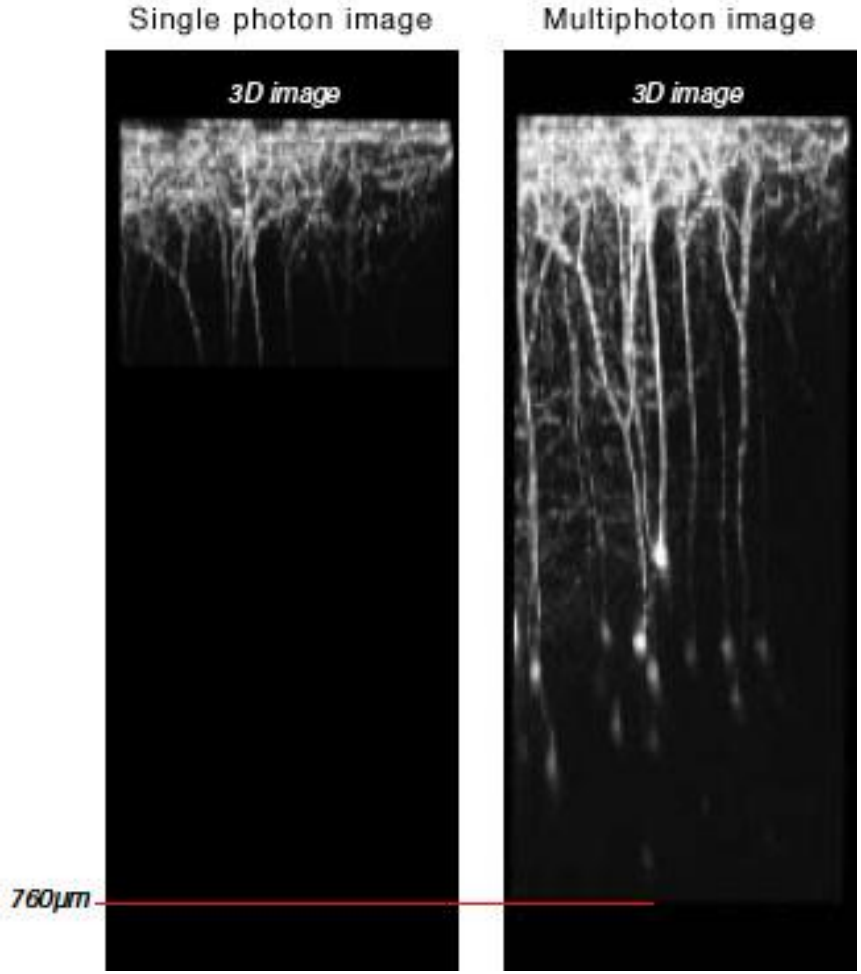


Green = Inhibitory neurons (GFP)
Red = Excitatory pyramidal neuron (Alexa 594)

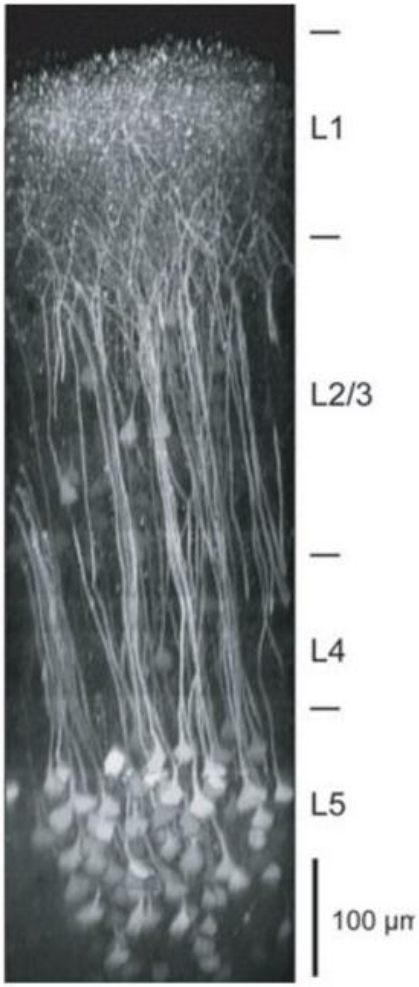
Imaging the brain in depth *ex vivo* (3D)



Imaging the brain in depth *in vivo*

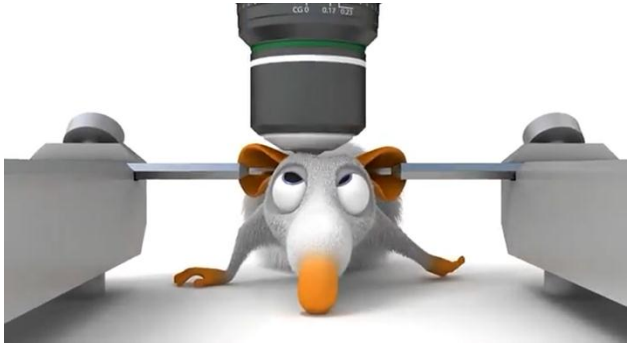


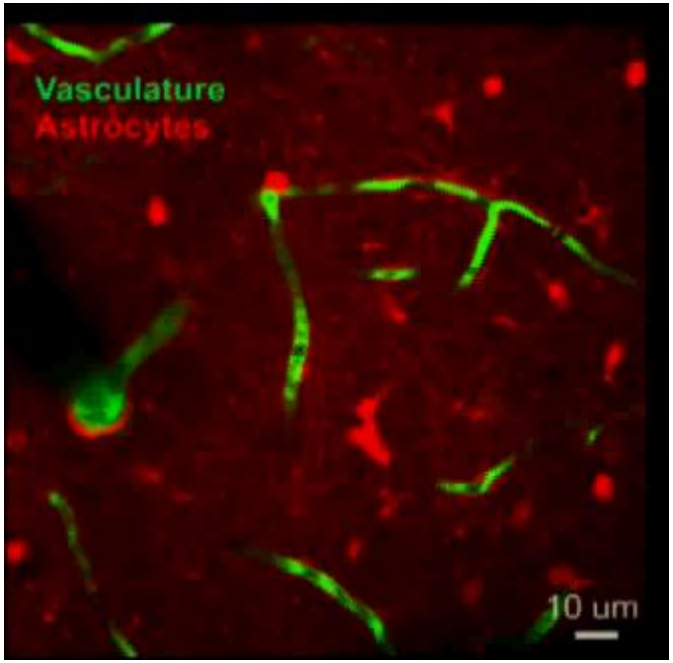
Svoboda & Yasuda (2006) Neuron, 50(6).

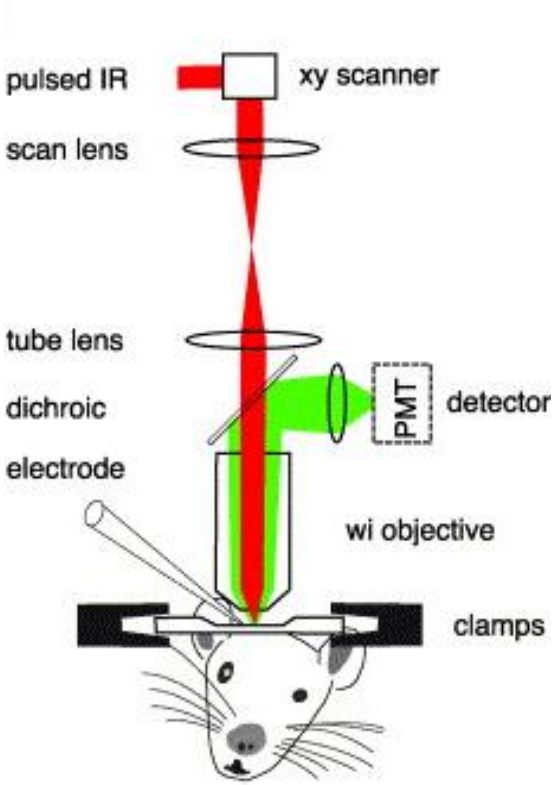


Helmchen & Denk (2005) Nature Method, 2.

Watching inside the brain *in vivo*



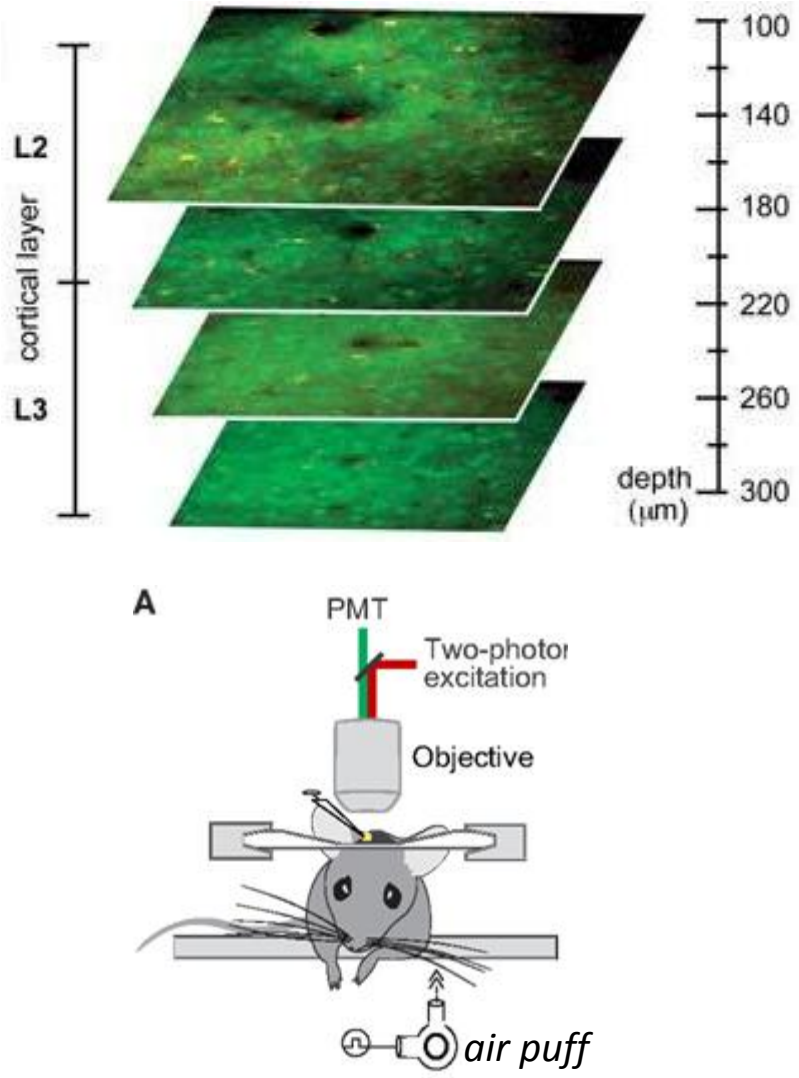


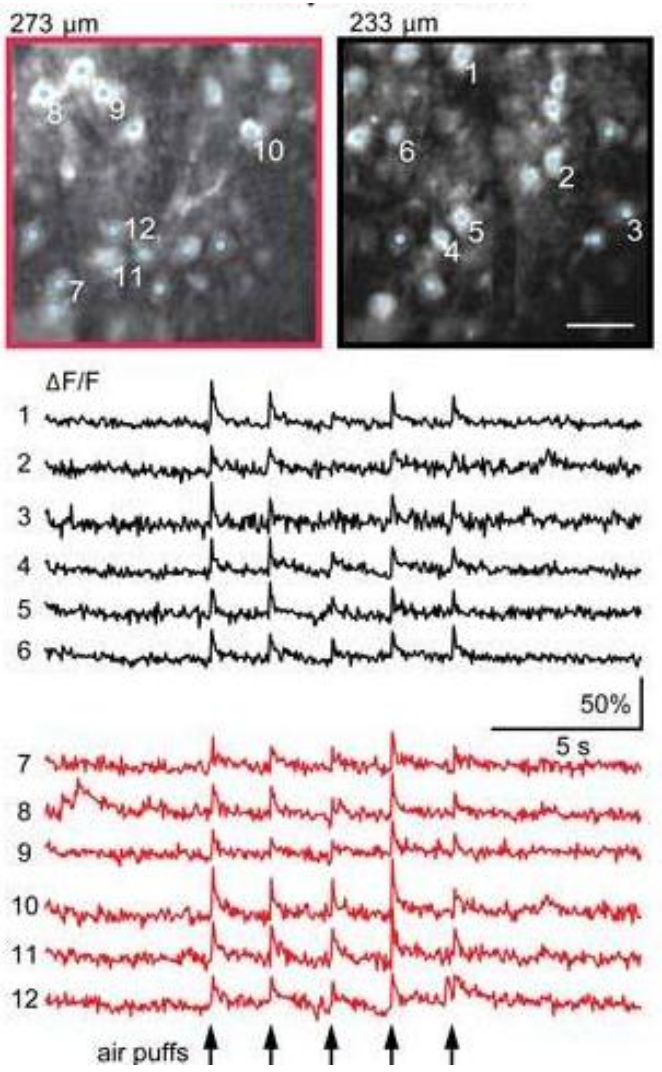


Blood flow in anesthetized rat brain capillaries

Neurotar Ltd

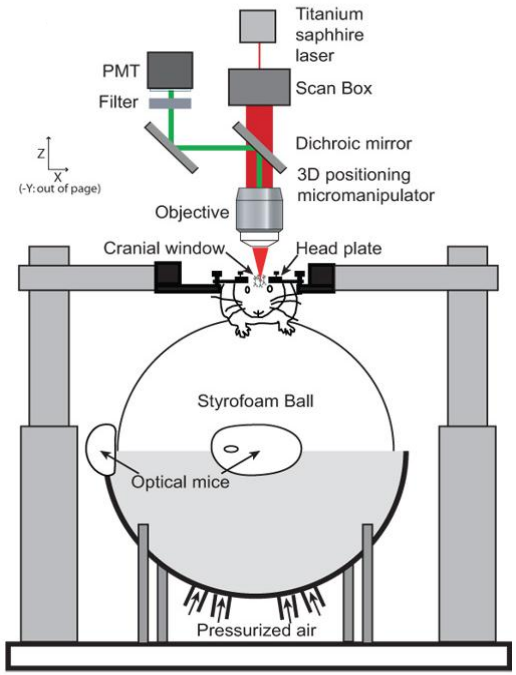
Population imaging *in vivo*

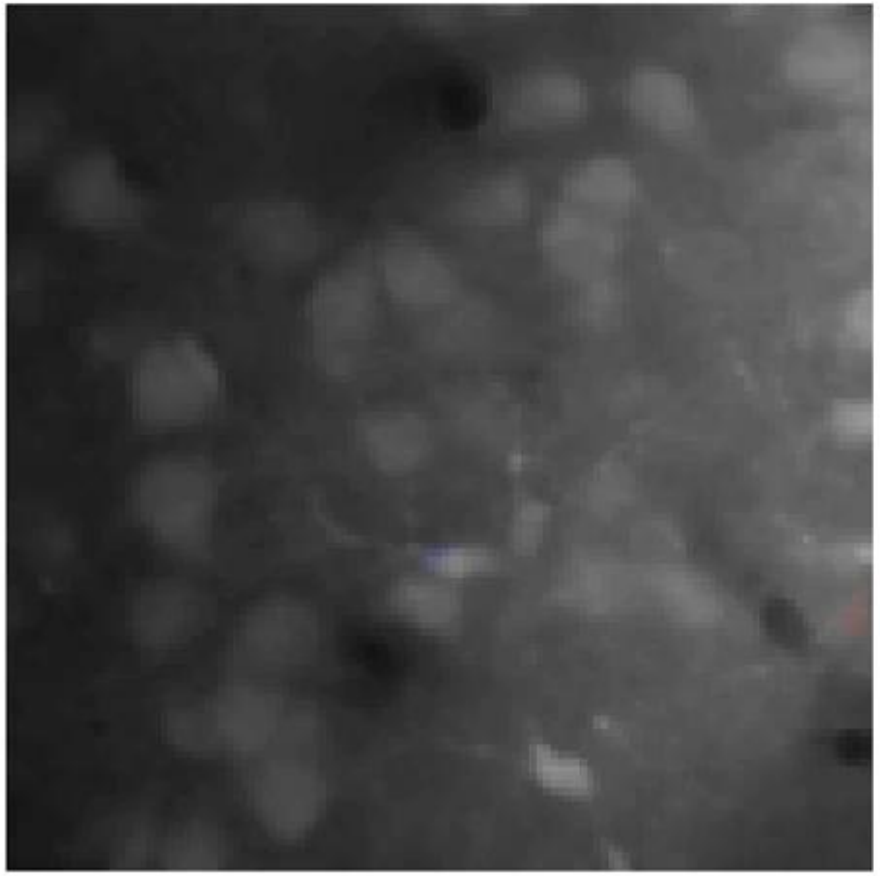




Johannssen & Helmchen (2012) Exp Neurol.

Population imaging *in vivo*

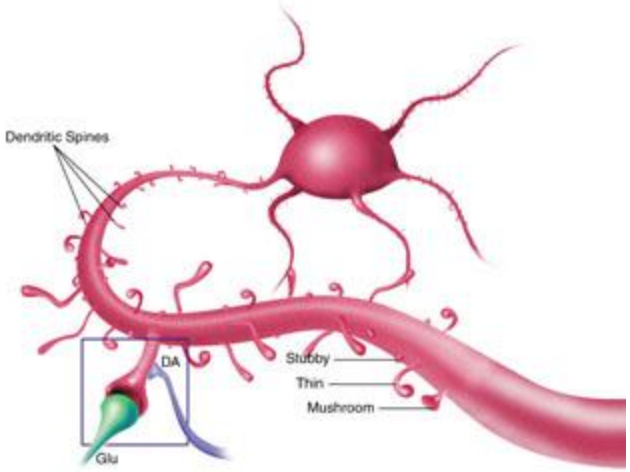




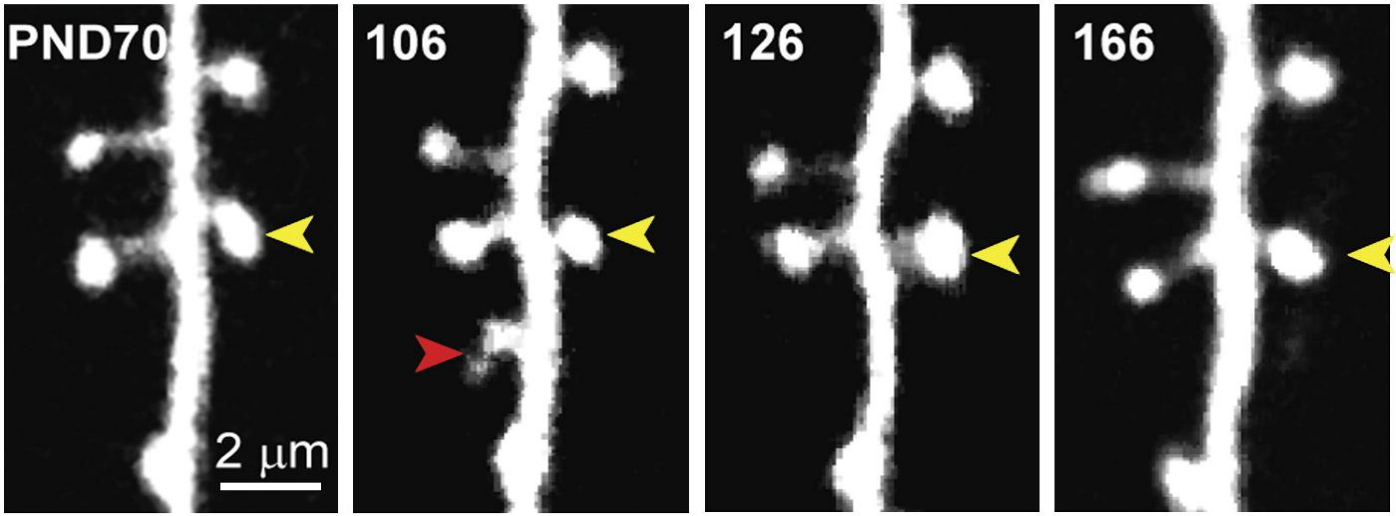
Calcium-imaging in the awake behaving mouse (sensory cortex)

Dombeck et al. (2007) Neuron, 56(1).

Long-term *in vivo* imaging



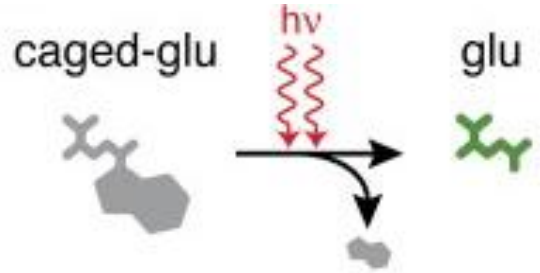
Long-term in vivo imaging of dendrites and spines expressing GFP

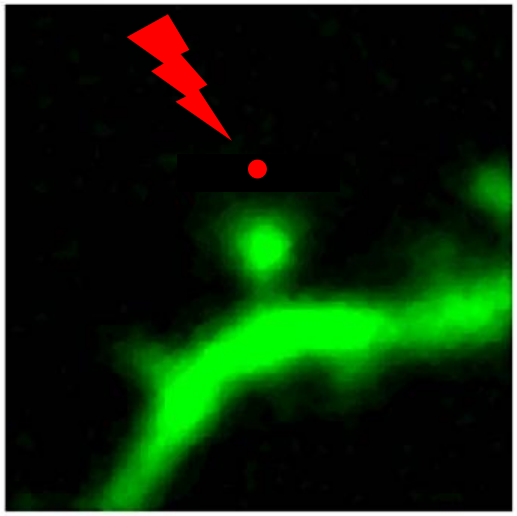


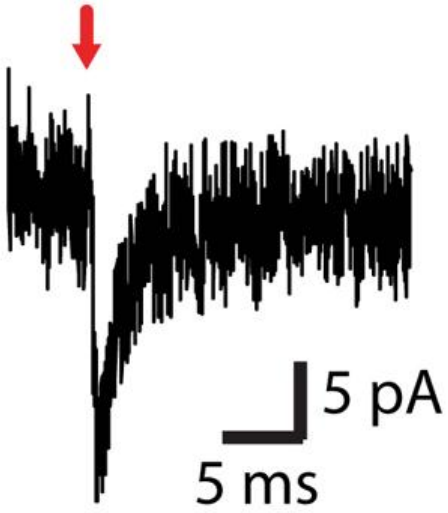
Svoboda & Yasuda (2006) Neuron, 50(6).

Local activation of neurons inside the brain

Use of two 2P lasers : **imaging (IR)** and **stimulating (UV)**







Activation of synaptic glutamate receptors by 2P glutamate uncaging

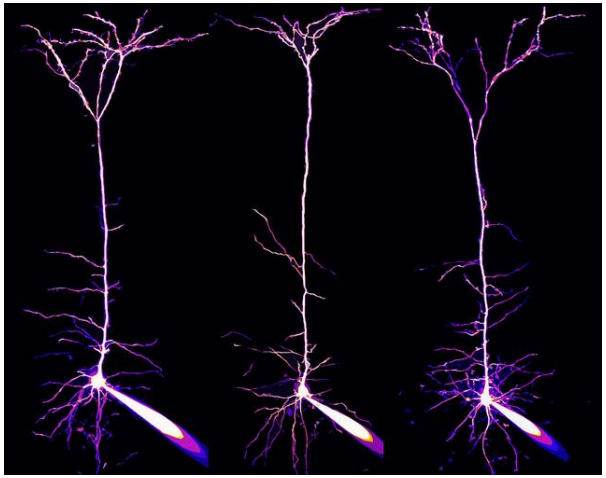
Judkewitz et al. (2006) Neuron, 50(2)

Conclusion

Advantages	Inconvenient
+ working with **living** and **highly scattering** tissues	- **Cost** (laser ~50-100 k£)
+ imaging up to very **high depth**	
+ reduced **photo-bleaching**	
+ **3D** imaging	
+ **temporal resolution**	

Want to learn more about 2P microscopy?

- **"Imaging in Neuroscience and Development"** from R. Yuste and A. Konnerth
- **http://www.microscopyu.com/**
- **a.moreau@ucl.ac.uk**



THANK YOU