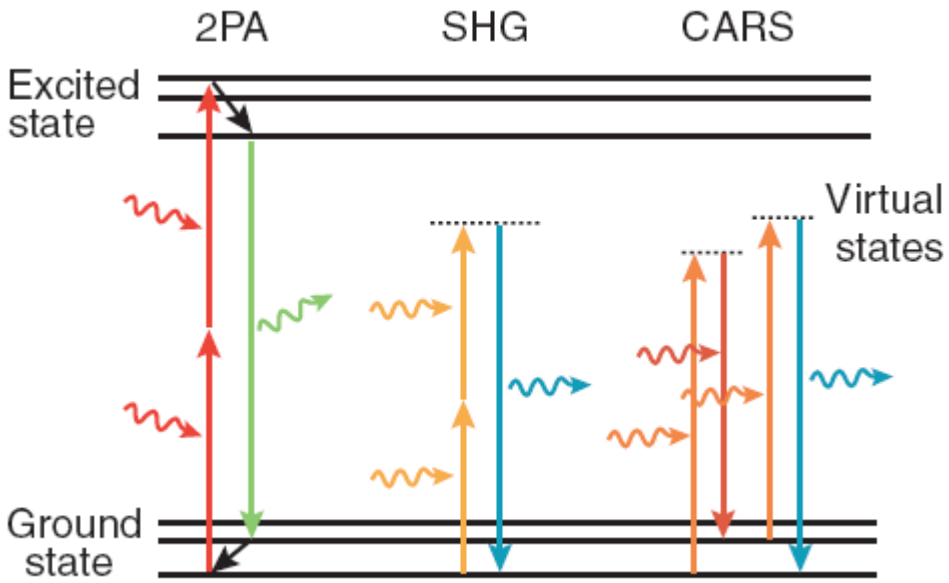
# Multiphoton Microscopy

# Jablonski Diagram: Nonlinear Optical Microscopy

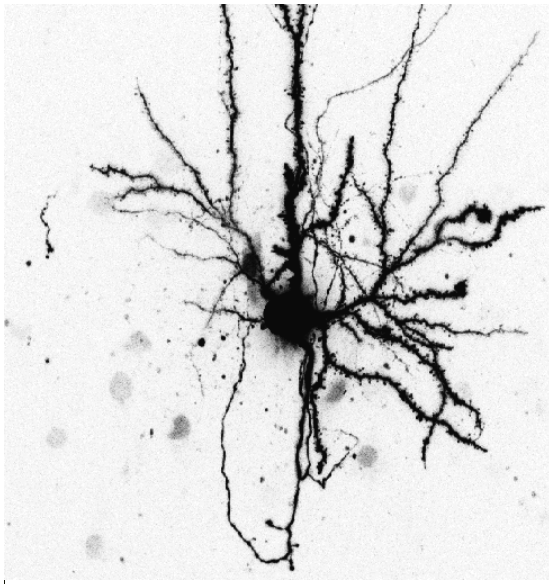





F.- Helmchen, W. Denk, Deep tissue two-photon microscopy, *Nat. Methods* 2, 932-940

# Typical Samples – Small Dimensions & Highly Scattering





T. Nevian
Institute of Physiology
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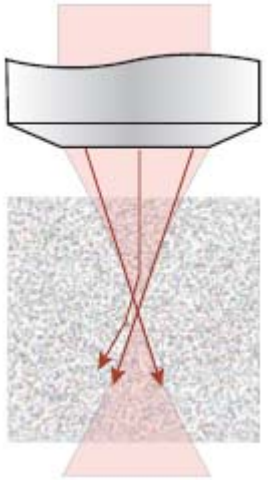
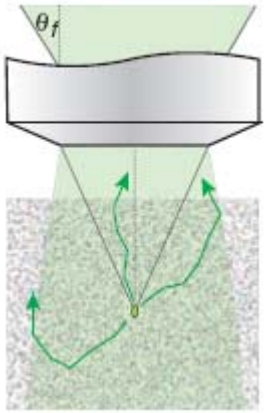





$l_s$ ~ 50-100 µm (@ 630 nm)

$l_s$ ~ 200 µm (@ 800 nm)

F.- Helmchen, W. Denk
Deep tissue two-photon microscopy.
*Nat. Methods* 2, 932-940

# Why Multiphoton microscopy?

- **Today main challenge:**

→ **To go deeper into samples for improved studies of cells, organs or tissues, live animals**

**Less photodamage, i.e. less bleaching and phototoxicity**

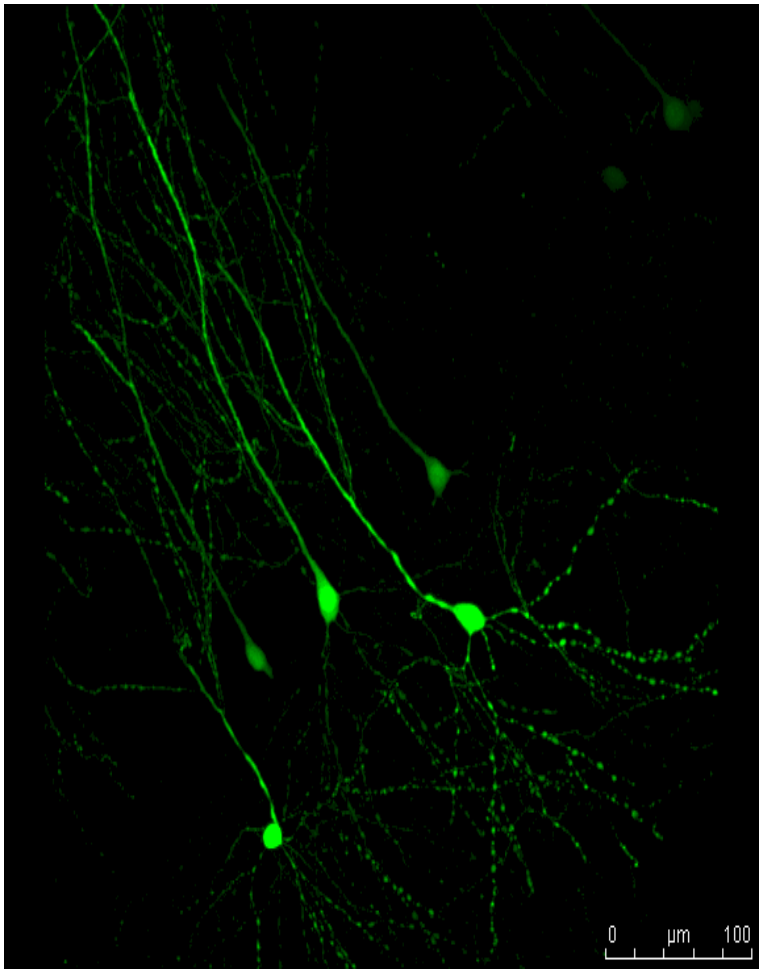
- **Why is it possible?**

→ **Due to the reduced absorption and scattering of the excitation light**

# The depth limit

- Achievable depth: ~ 300 – 600 µm

- Maximum imaging depth depends on:
  - Available laser power
  - Scattering mean-free-path
  - Tissue properties
    - Density properties
    - Microvasculature organization
    - Cell-body arrangement
    - Collagen / myelin content
  - Specimen age
  - Collection efficiency



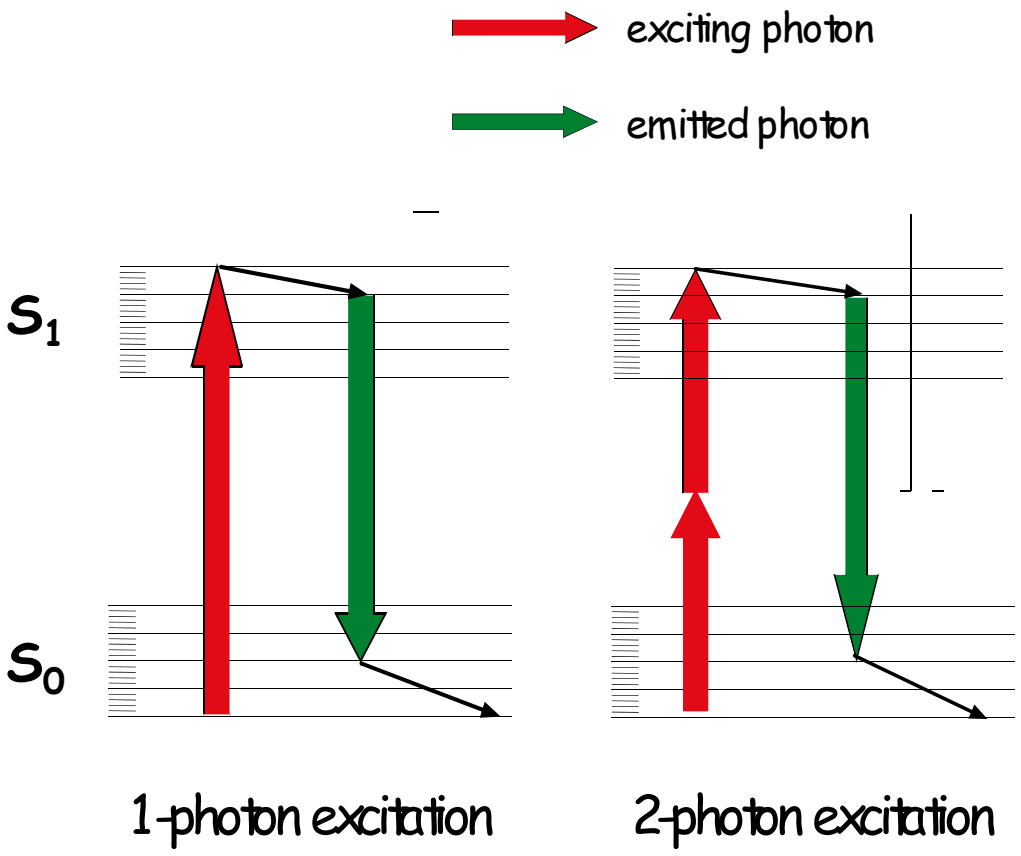

**Acute mouse brain sections containing YFP neurons,maximum projection, Z stack: 233 µm**

Courtesy: Dr Feng Zhang, Deisseroth laboratory, Stanford University, USA

# What is Two-Photon Microscopy?



A 3-dimensional imaging technique in which 2 photons are used to excite fluorescence emission




Simultaneous absorption of 2 longer wavelength photons to excite a fluorochrome, emission as with 1-photon

# 2-photon: excitation probability - importance of high NA



$$n_a \propto \delta \left( \frac{P_{avg}^2}{\tau f^2} \right) \left( \pi \frac{NA^2}{hc\lambda} \right)^2$$

**$n_a$: probability of excitation**

**$\delta$: excitation cross section**

**$P_{avg}$: average power incident light (peak power)**

**$\tau$: pulsewidth**

**$f$: repetition rate**

***NA*: Numerical aperture**

***h*: Planck's constant**

***c*: Speed of light**

**$\lambda$: Wavelength**

**MP excitation is favoured when we have:**

- **Molecules with large cross-section**
- **High peak power**
- **High-NA objective lenses**

# Two photon microscopy
# fluorescence yield – non-linear process



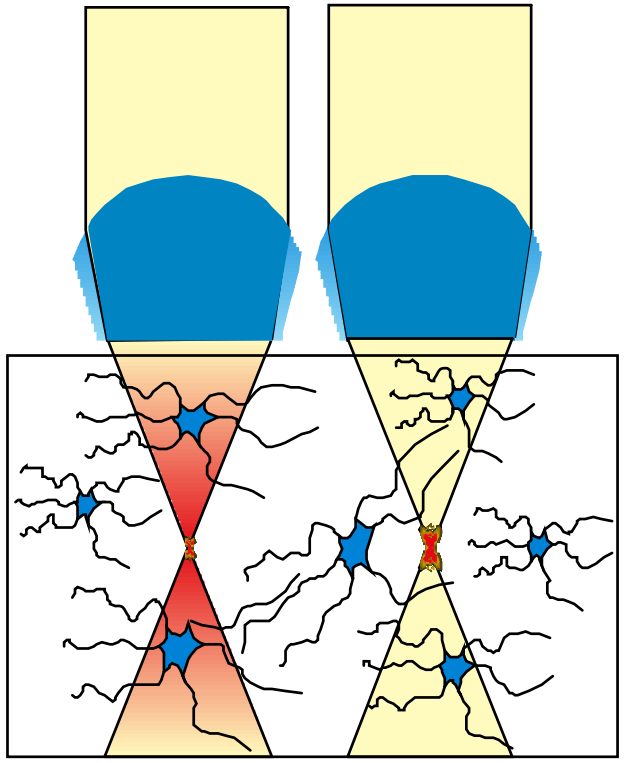
$$Fl \cong \frac{\langle P_{avg} \rangle^2}{\tau \cdot f}$$

Fl = fluorescence photons/sec
P = average laser power
$\tau$ = pulse width → fs/psec
f = laser repetition rate

**Efficiency of excitation increases with the square of the laser power**

# What is Two-Photon Microscopy ?

**1-Photon 2-Photon** ⟶



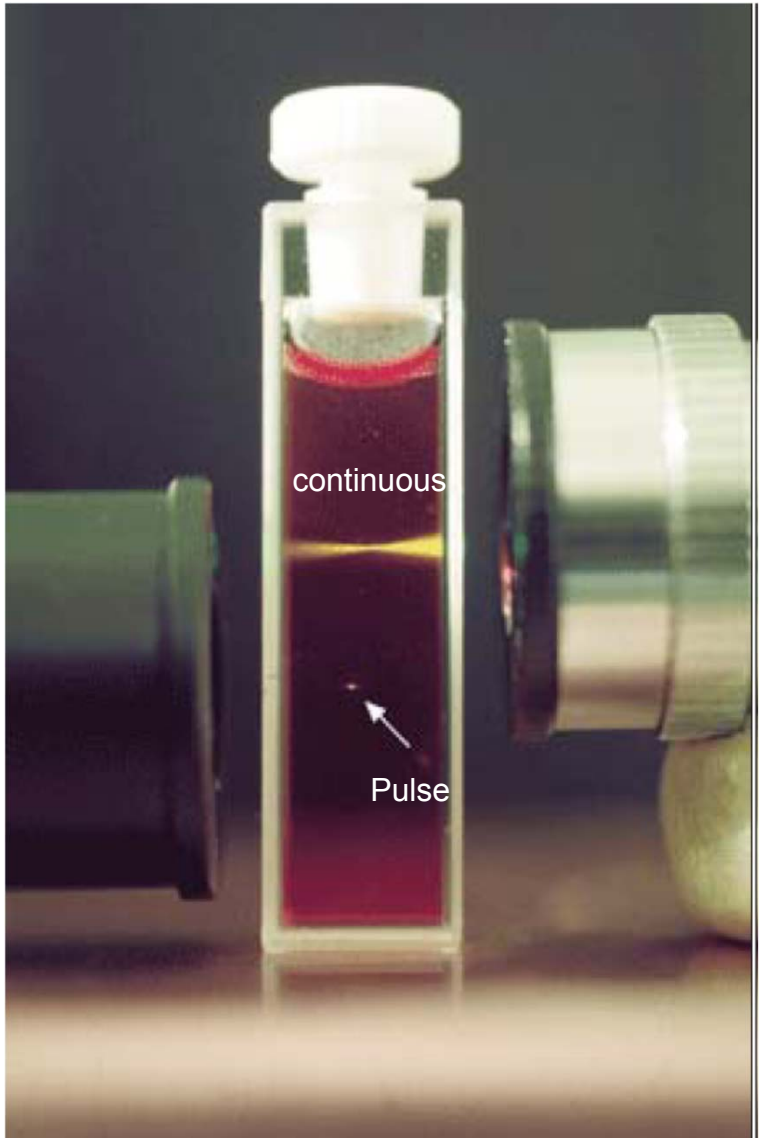
label is excited only at the focus of the beam where probability of 2P event is highest

No out-of-focus-fluorescence:

- No need of confocal aperture

- Dye bleaching and photo toxicity limited to the plane of focus

# Confocal vs. Multiphoton microscopy




Two-photon optical probe interacts with the sample *only* in the focus region.

2-photone and 1-photon excitation
at the same time
In dye solution

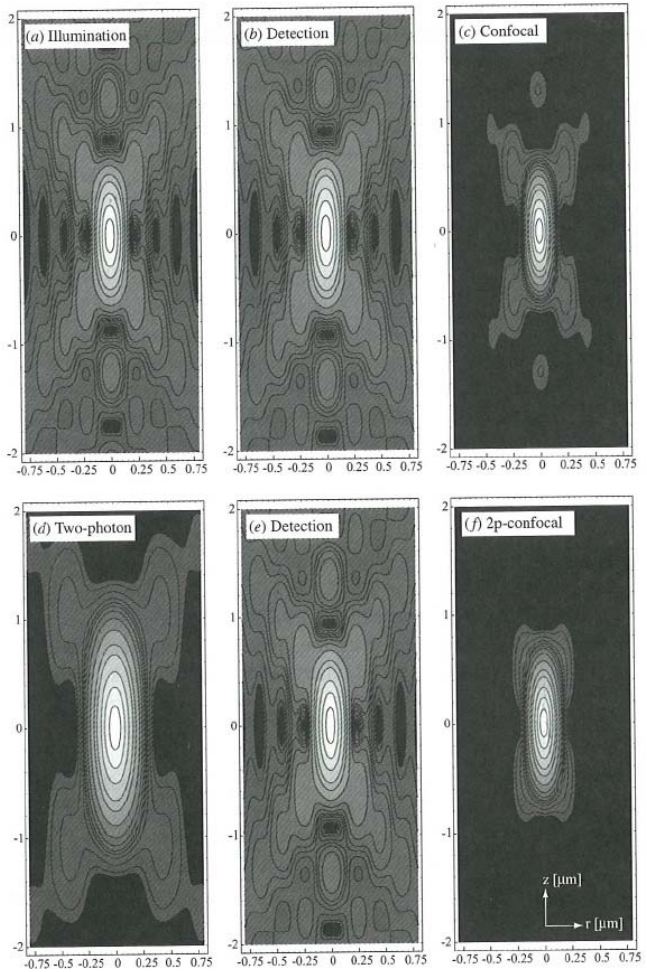
# Optical resolution: PSF - Confocal vs. 2-Photon

**Resolution in 2-Photon Microscopy is ~ 2x worse compared to Confocal Microscopy**

| NA=1,4<br>n= 1,51 | Wavelength<br>$\lambda$ (nm) | Lateral resolution<br>(x-y, µm) | Axial resolution<br>(x-z, µm) |
|---|---|---|---|
| Confocal | 488 | 0,16 | 0,52 |
| 2-photon | 900 | 0,29 | 0,97 |

Confocal

2-Photon

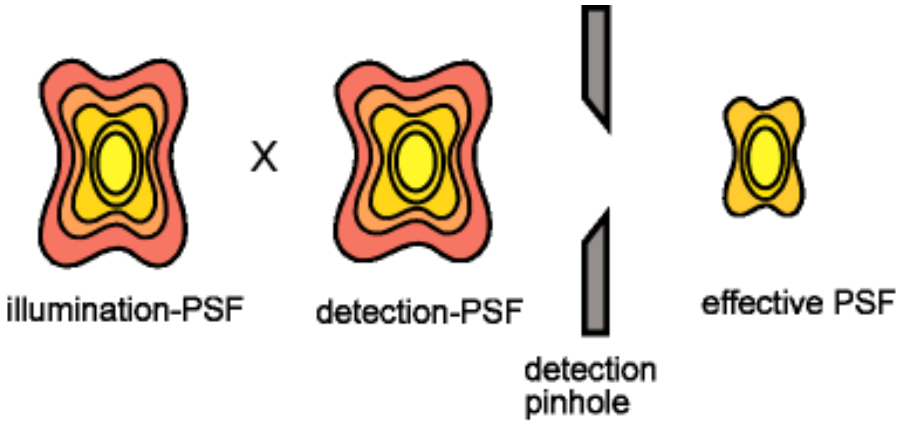



**Calculated PSFs**

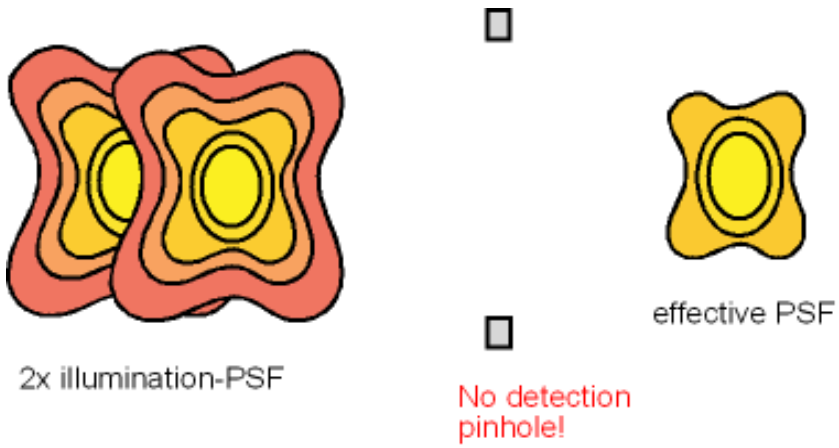
**taken from „Confocal and Two Photon Microscopy", ed. Alberto Diaspro, 2002**

# Optical resolution of Two Photons imaging

## Geometry of MP illumination spot




confocal microscopy




2P microscopy

# Optical resolution: Conventional vs. Confocal

## Conventional Microscopy

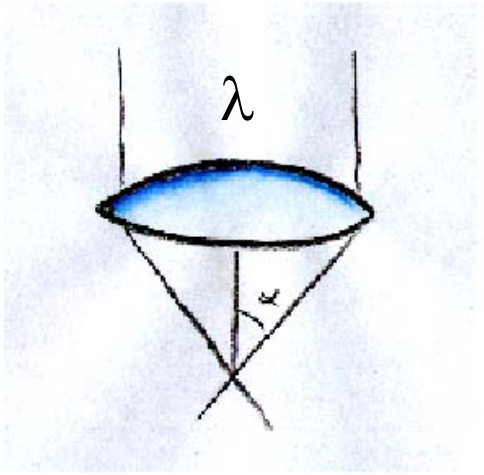
- Wavelength of light
- Numerical aperture

$$r_{xy} = \frac{0{,}61 \cdot \lambda}{NA}$$

## Confocal Microscopy

- Pinhole size
- Geometry of the probing beam spot

$$r_{xy} = \frac{0{,}46\,\lambda}{NA} \qquad r_z = \frac{1{,}4\ n\lambda}{NA^2}$$




$NA = n\sin\alpha$

$n = 1$ for air
$n = 1.518$ for oil immersion

# Optical resolution: Confocal vs. Two photons

**Confocal Microscopy:**

$$r_{xy} = \frac{0{,}46\lambda}{NA} \qquad r_z = \frac{1{,}4\ n\lambda}{NA^2}$$

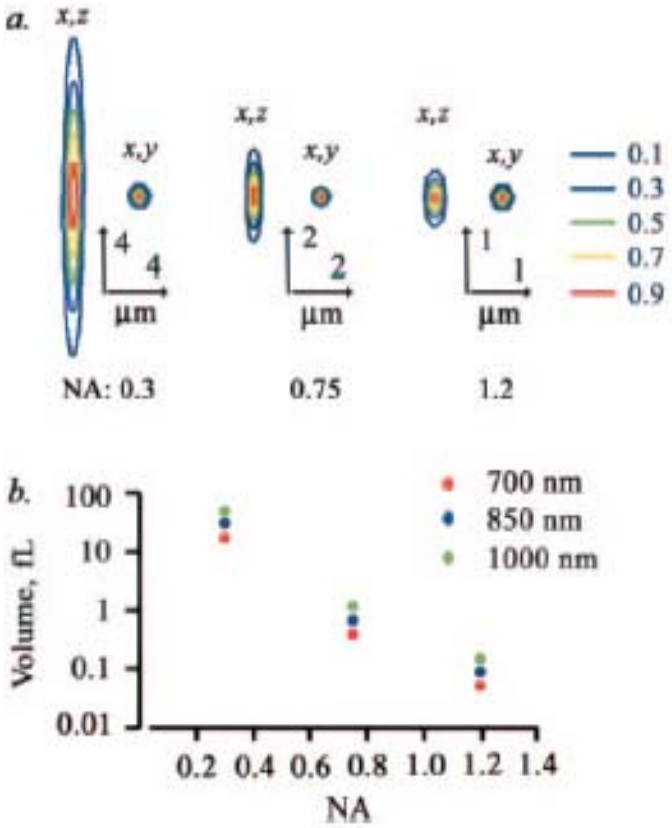
**Example:**

| NA=1,4<br>n= 1,51 | Wavelength $\lambda$ *(nm)* | Lateral resolution (x-y, µm) | Axial resolution (x-z, µm) |
|---|---|---|---|
| Confocal | 488 | 0,16 | 0,52 |
| 2-photon | 900 | 0,29 | 0,97 |

**In two photon microscopy, lower resolution due to longer wavelength**

# TPE volumes: wavelength & NA dependence

Living up to Life



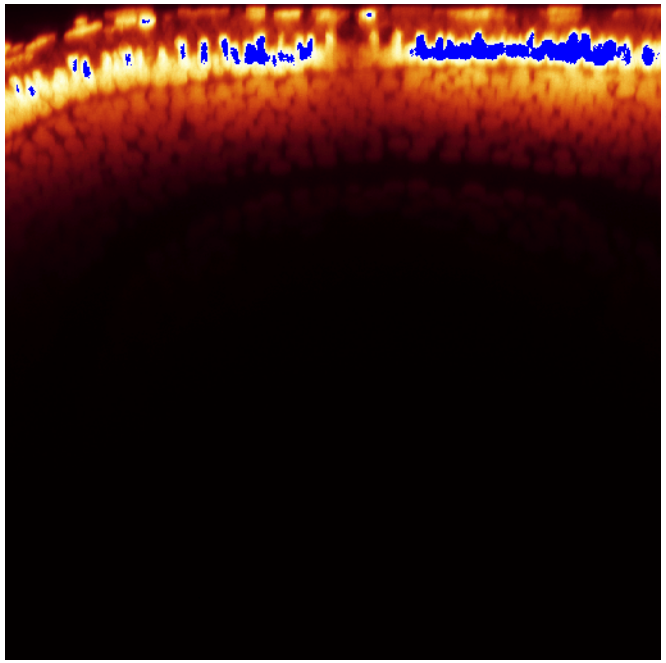

Rubart , M., Two-Photon Microscopy of Cells and Tissue, *Circ. Res.* 2004;95;1154-1166

# Comparison of penetration: UV – IR (internal detectors)



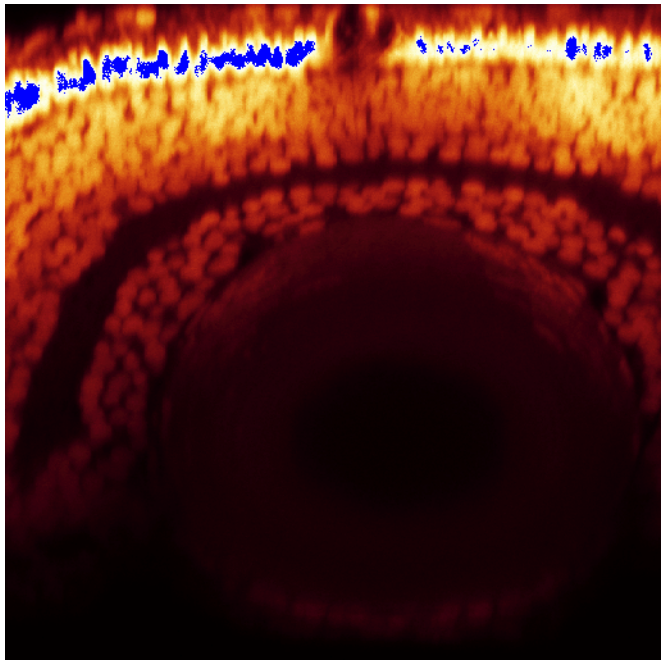
**Eye of zebrafish larvae (stained with DAPI)**

Image size (xz): 125 µm x 125 µm - Objective: 63x 1.2 Water - Detection range: 400nm – 500nm



◀ **Exc.:** UV
**PMT:** 300V

**Exc.:** IR
**PMT:** 360V
▶



# 2-Photon excitation probability

**2-Photon excitation is a very very rare event!**

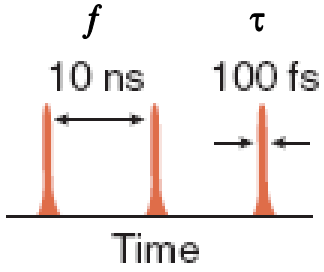
In bright day light a good one- or two-photon absorber absorbs
in a 1-photon process: once a second
in a 2-photon process: every 10 million years

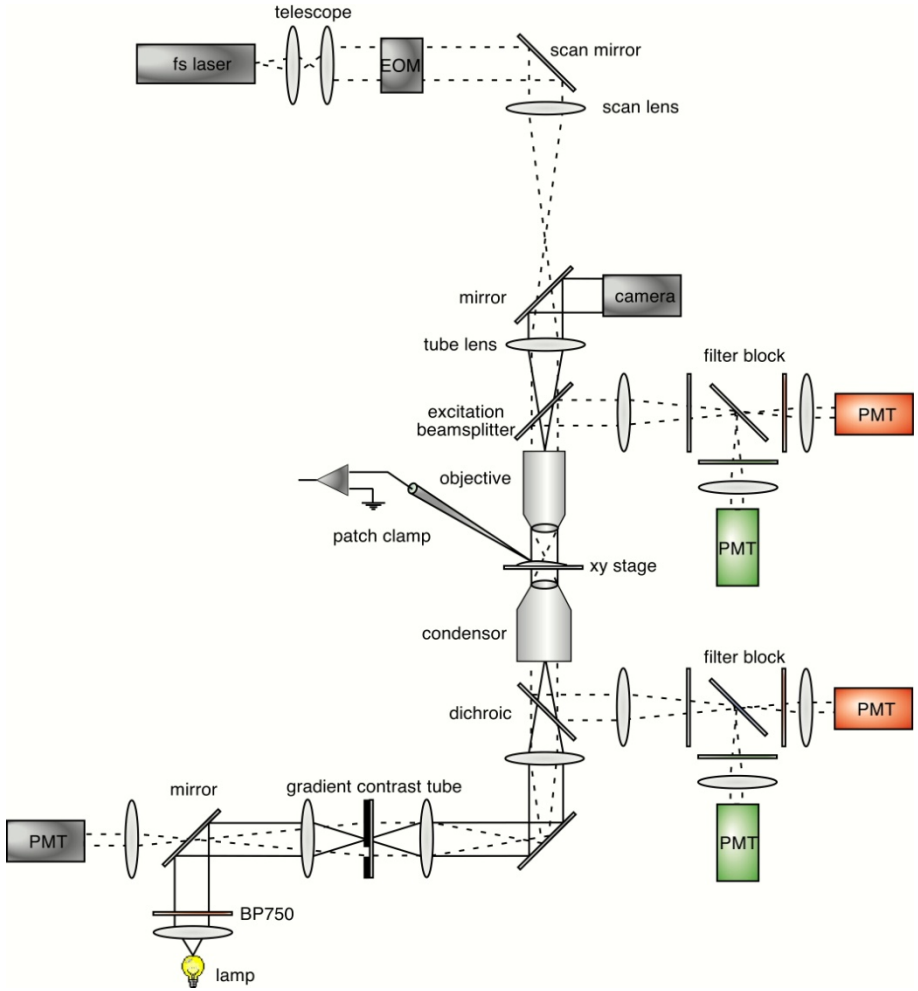
Solution:

Use of
laser sources –
focused beam

The probability of a molecule to absorb 2 photons simultaneously is expressed as the 2-photon cross section

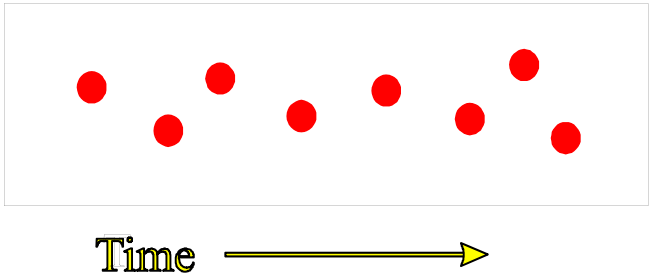
# Multiphoton Microscope setup

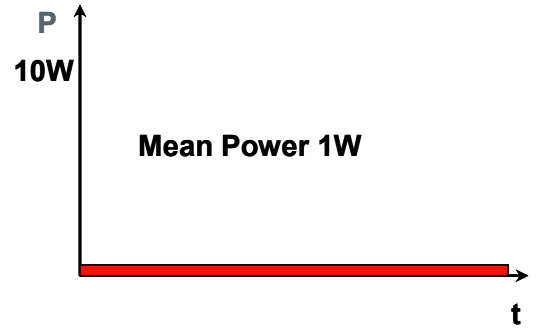


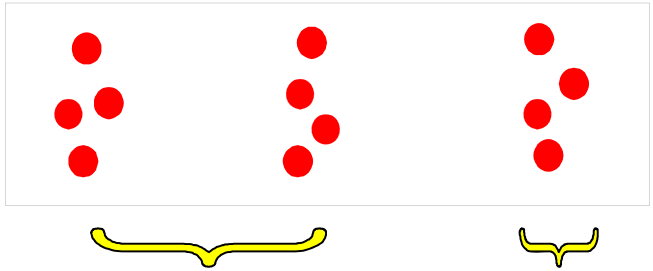
# CW and Pulsed Lasers

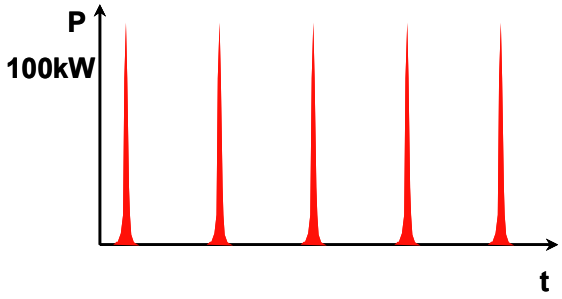
CW



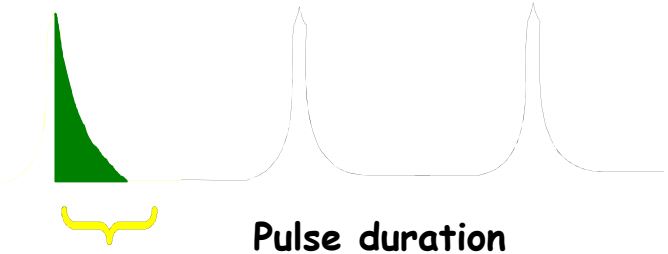




Pulsed




**Pulse repetition rate f ( 80 MHz) in range of fluorescence life time $10^{-8}$ s**
**few nsec → 80MHz**



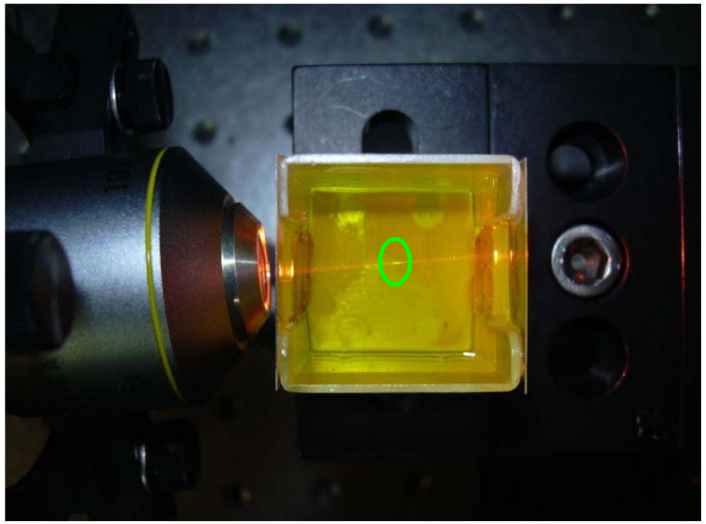

**Pulse duration (width) τ**
**$10^{-13}$ s (fs/ps)**

Short Pulse Advantage

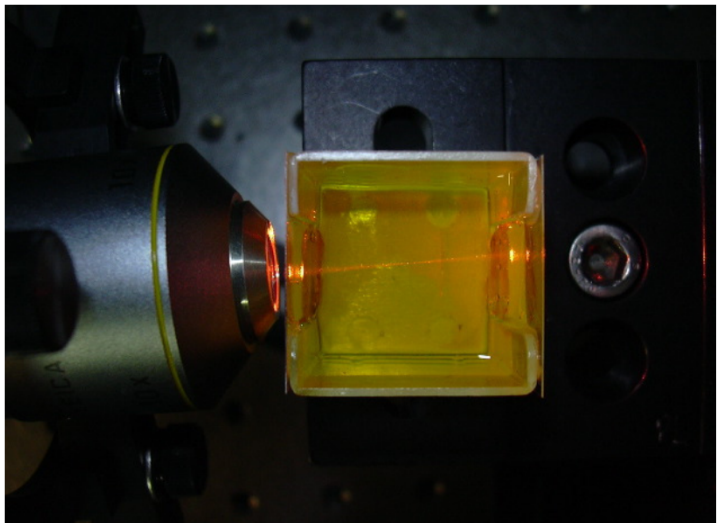
Fluorescence proportional to 1/pulse width x repetition rate

# Continuous lasers vs. pulsed lasers

**Pulsed mode**



**Continuous mode**



Two photon excitation in Lucifer yellow at 750nm wavelength (same power)

*Courtesy: Magendie institute, PICIN, P. Legros, Bordeaux, France*

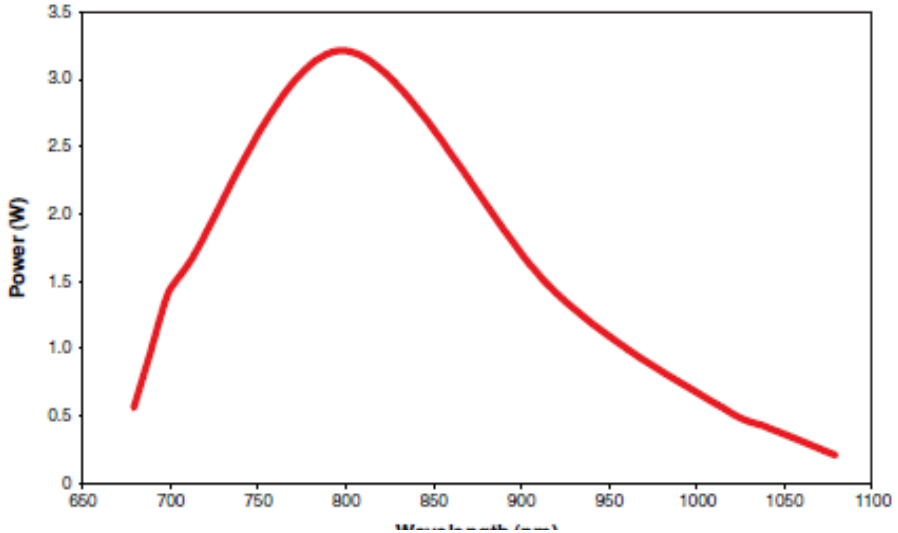
## 3 conditions necessary for 2P excitation

- High intensity ➔ Hence in focal point
- High cross section ➔ Not always double 1P
- Short pulses: ➔ Pico or Femto second laser

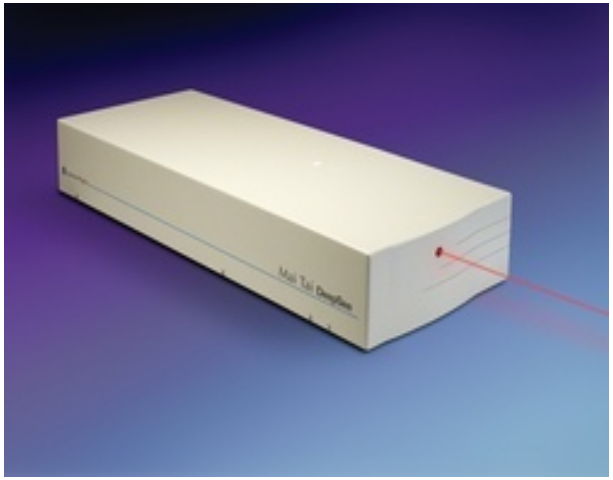
# Typical Tuning Curve IR Laser

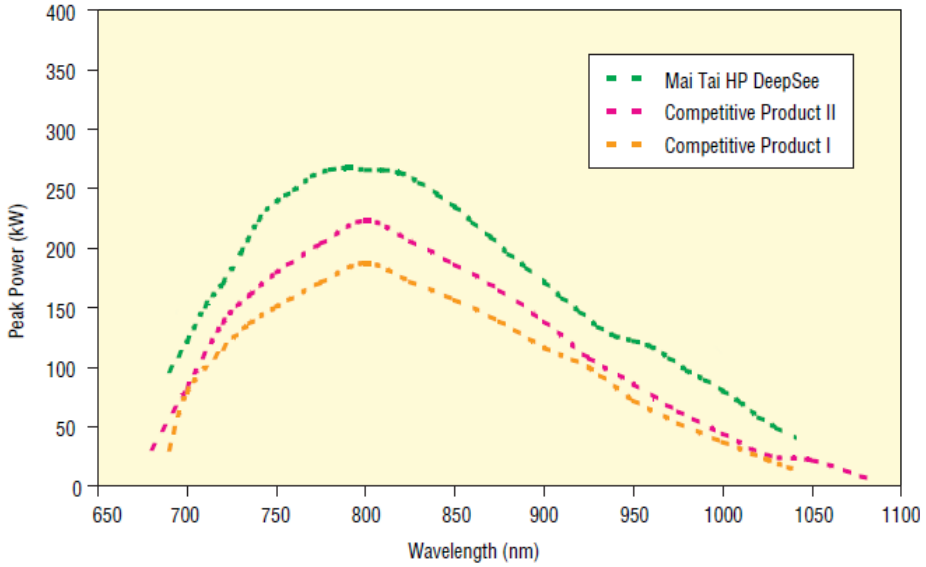
Living up to Life

## Coherent
## Chameleon Vision II




## Spectra Physics
## MaiTai DeepSee

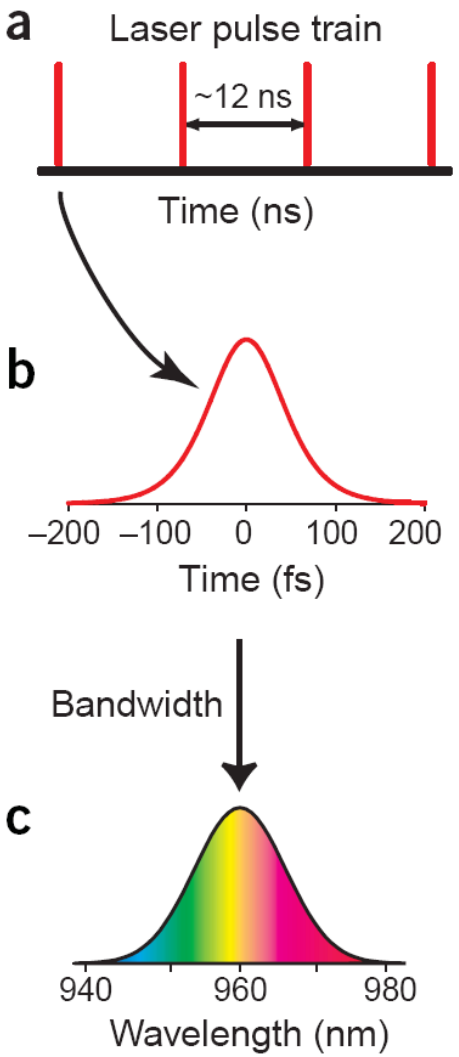





*calculated using specified values for average power and pulse width for each laser with dispersion compensation

# Ultra Short Pulses






**80 MHz repetition rate**

**FWHM 100 fs – time**

**FWHM 10 – 15 nm**

Short pulses of monochromatic light have colors! - Spectral components

Nat. Biotech. Vol 21 No 11, 2003, Zipfel et al. Nonlinear magic: multiphoton microscopy in the biosciences

# Continuous or pulsed laser

## Notion of fluorescence emission rate and cross section

One photon of fluorescence is generated by 2 incident photons

**With continuous laser the fluorescence emission rate (f) is proportional to the square of intensity**

$$f = \tfrac{1}{2} \sigma I^2$$

f : photons/seconde
$I^2$ : Intensity of laser
$\sigma$: cross section in GM (Göppert-Mayer)
1 GM= $10^{-50}$ $cm^4$/photons

**The two photon cross section is the probability of a molecule to absorb 2 photons simultaneously**

$\sigma$ (cross section) is dependent on the wavelength and in general between 1 and 100 GM
GFP = 10 GM
Qdots = $10^4$ – $10^5$ GM

# Continuous or pulsed laser

**With pulsed laser, the fluorescence emision rate is described as follows:**

$$f_m = \tfrac{1}{2}\,\sigma\,(t.F)^{-1}\,I_m^{\,2}$$

t : Pulse duration (s)
F : Frequency (Hz)
$f_m$ : Average fluorescence emission rate
$I_m^{\,2}$ : Average intensity
t. I : Intensity per pulse (peak power)
$\sigma$: cross section in GM (Göppert-Mayer)

Because $I_m = t.F.\ I$

**Examples**

$t = 10^{-13}$ s
$F = 10^{8}$ Hz ➔ $(t.F)^{-1} = 10^{5}$
To compare $(t.F)^{-1} = 1$ for continuous laser

So for equal power we have $10^5$ time more excitation of fluorophore with pulsed laser

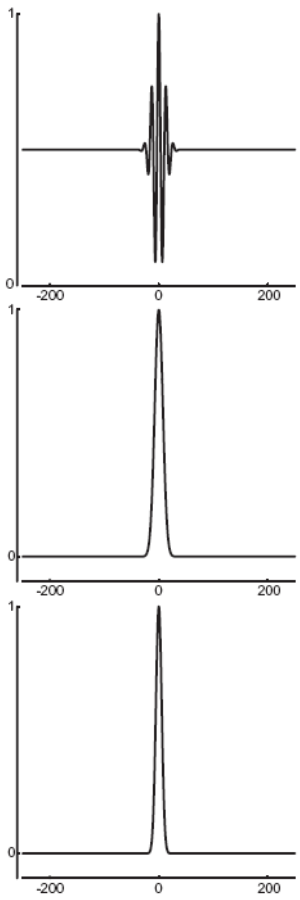
# Group Delay Dispersion (GDD)

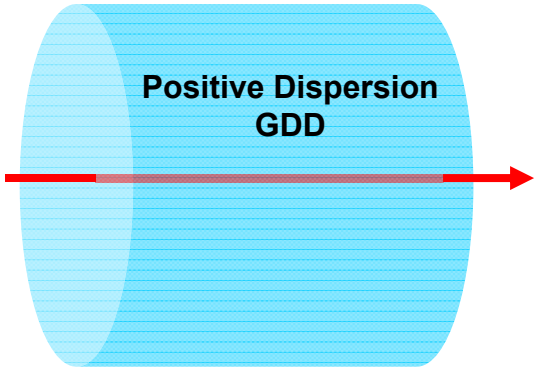
**Electrical field**

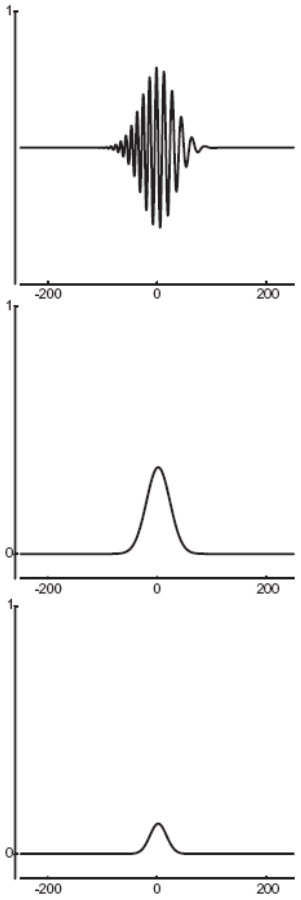
**Intensity**

**(Intensity)$^2$**

**= 2PE efficiency**




$\tau in = 100\ fs$

**@ 690nm ~700 fs**

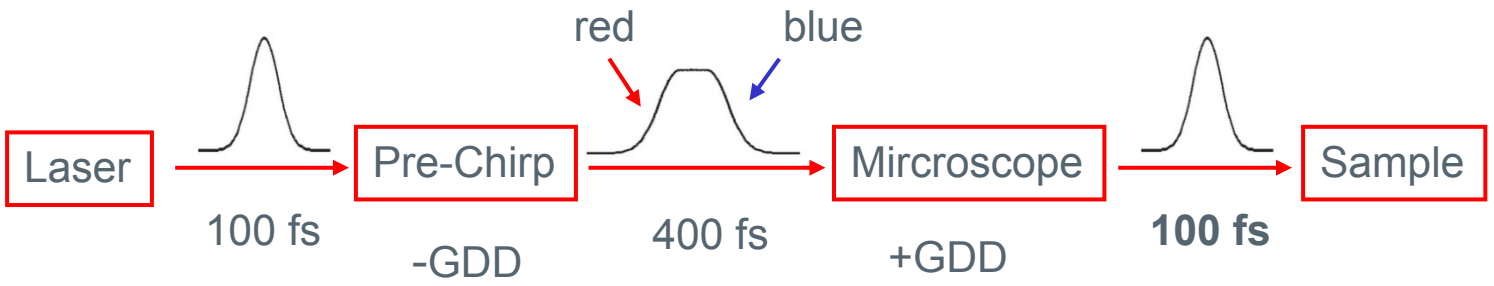
**@ 800nm ~500 fs**

**@ 1020nm ~300 fs**

Amended, from Pawley, Chapter 28 Denk et al.,
Multi-Photon Excitation in Laser Scanning Microscopy

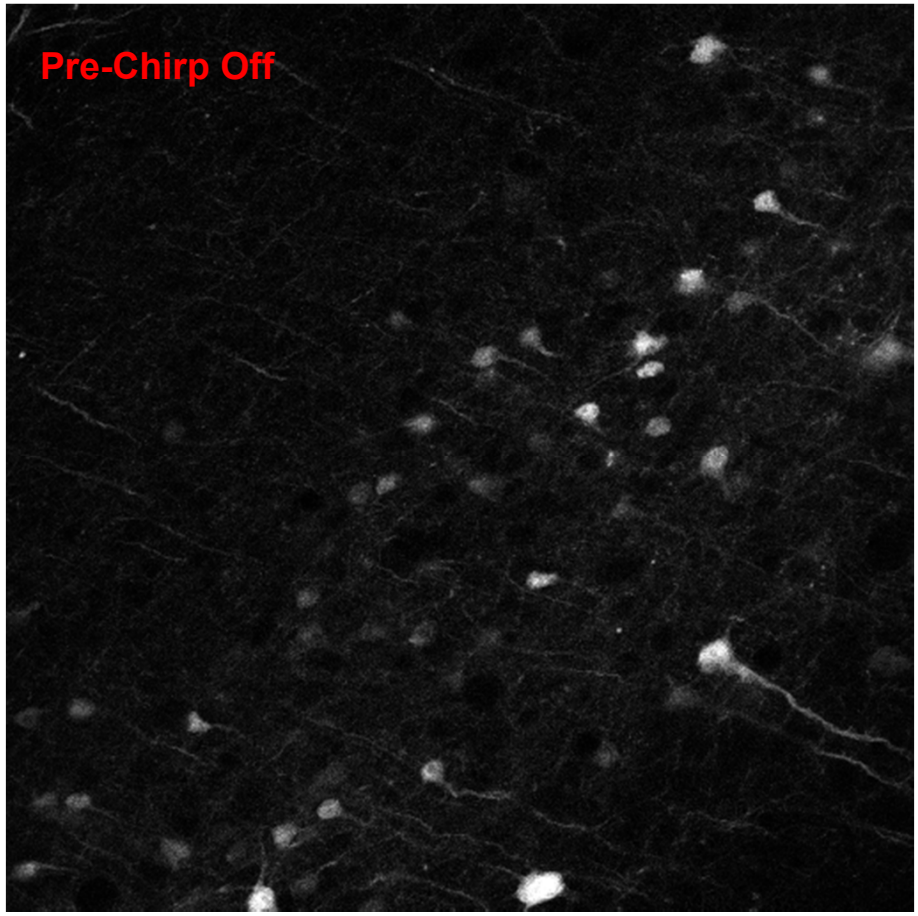
# Principle of Precompensation

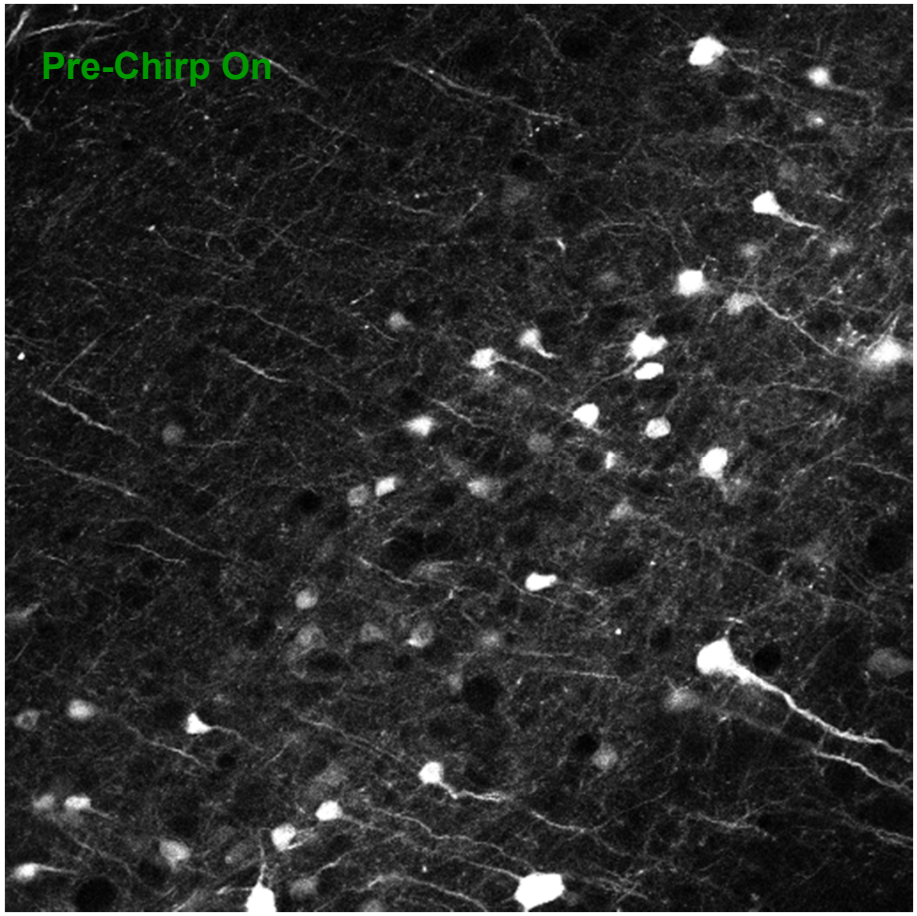

Group Delay Dispersion: GDD



# Performance comparison
# Dispersion Compensation – On / Off



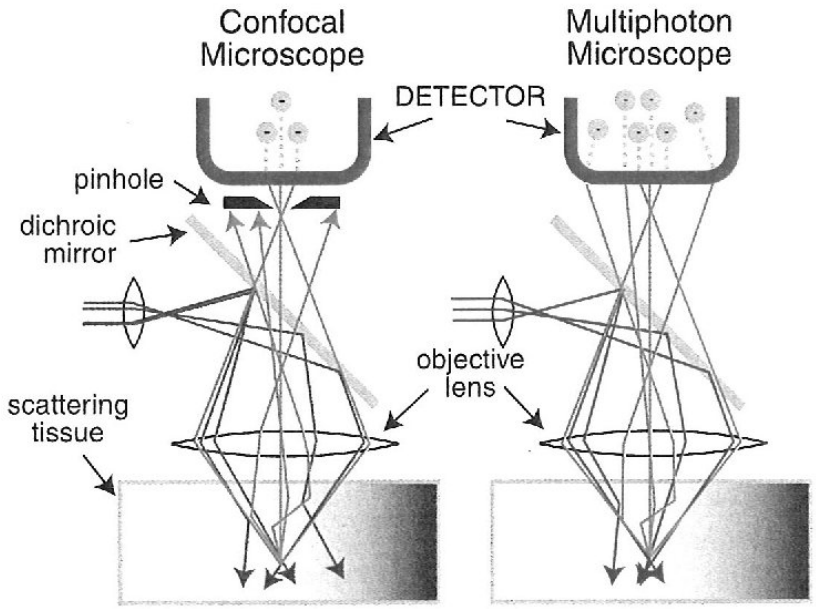



Objective lens: 20 x 1,0
Sample: Brain Slice, GFP @ 920 nm
Chameleon Vision

- **Mean intensity approx. 2,5 x higher**

# MP-imaging for highly scattered tissue




**Confocal Microscopy:**

- **pinhole aperture rejects the out of focus fluorescence but also scattered light**
  **→ difficult to image highly light scattering tissue like thick brain slices**

**Multi-photon Microscopy:**

- **no confocal pinhole required because all fluorescent light originates from the focal spot;**
  **→ detectors can be placed as close as possible to the specimen (NDDs)**
- **enables also scattered photons to be collected**
- **much higher photon collection efficiency compared to confocal microscopy**

**→ Large area detector**

# Detection Path

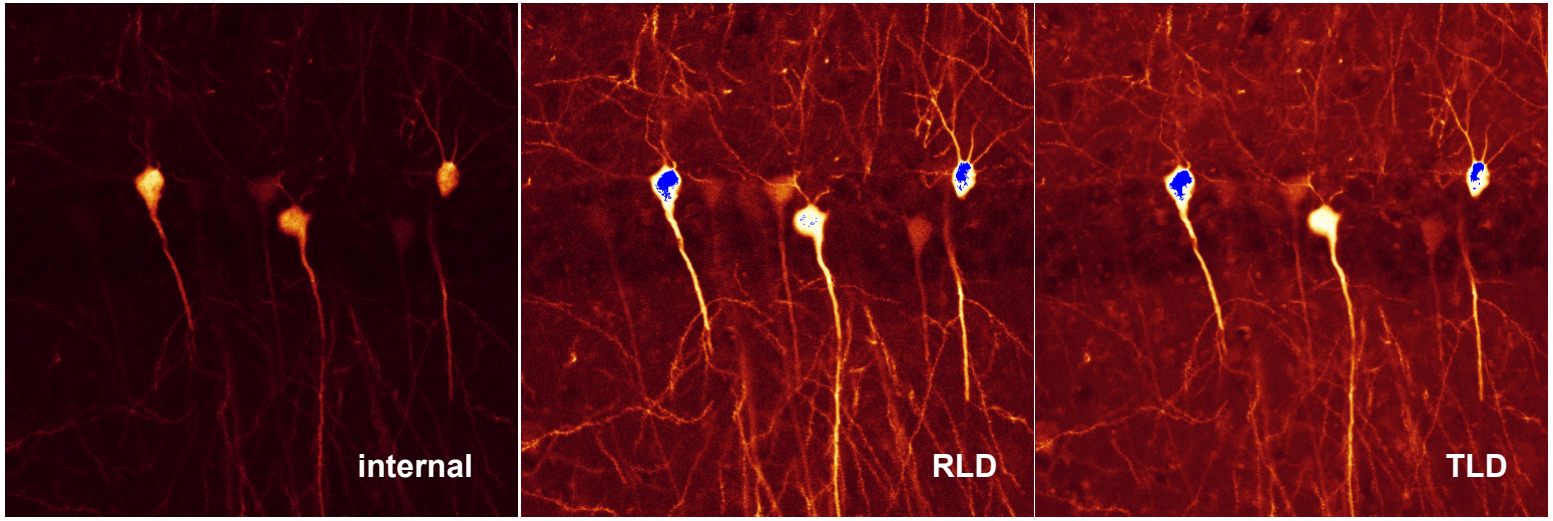
- **Descanned pathway can be used – but clipping at pinhole**
- **Strategy: collect as many photons as possible**

  **⟶ i.e. if descanned detection: open pinhole completely!**

- **Non-Descanned-Detection:**
  - **Large-area detectors (predominantly PMTs)**
  - ***epi*-detection**
  - ***trans*-collection – high-NA Condensor, prefferably oil (!)**

# Photon Collection Efficiency - Internal vs. NDDs

**Mouse brain slice:** ~ 20 µm (center plane)
**Detection range:** 500 – 550 nm
**PMT:** 950 V
**Objective:** 20 x 1.0 W




| | | | |
|---|---|---|---|
| **Mean intensity:** | **20** | **52** | **58** |

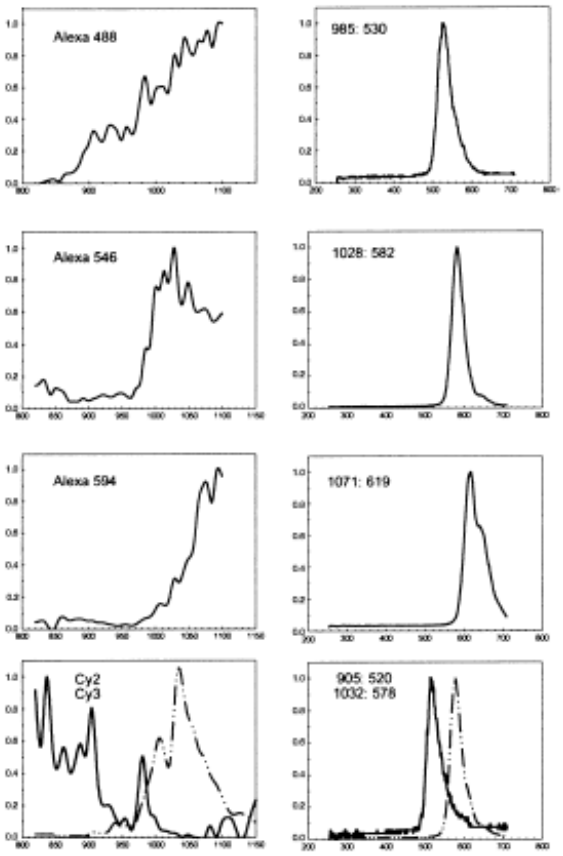
# Fluorochromes: TPE - Overview 1

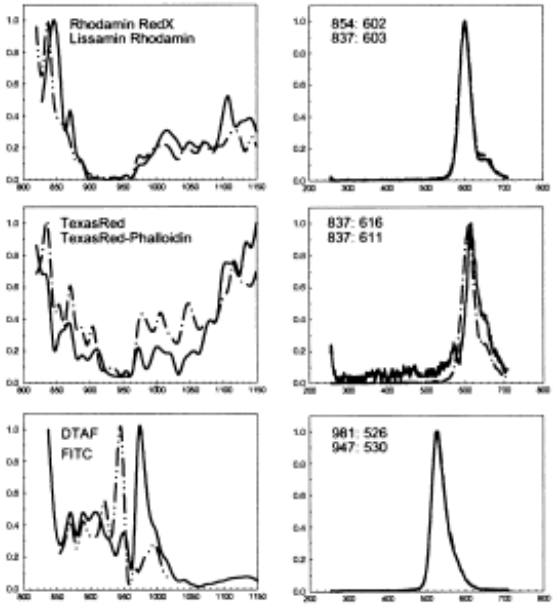
| Fluorochrome | TPE absorption maximum (nm) | Absorption shift TPE vs. 2 × OPE (nm) | TPE emission maximum (at excitation) (nm) | Emission shift TPE vs. OPE (nm) |
|---|---|---|---|---|
| Dyes | | | | |
| Acridine Orange | 837 > 882 >> 981 | – | 548 (837) | – |
| aminomethylcoumarin methylcoumarin | 703 < 722 >> 863 | +4 | 458 (820) | + 11 |
| Bodipy FL[a] | 920 < < 972 | –38 | 526 (972) | +15 |
| Bodipy TRoad | 1032 < < 1108 | –70 | 684 (1032) | +61 |
| DAPI | 685 > 697 | –31 | 467 (685) | +6 |
| DCF2 ($H_2$DCFDA)[a] | 1065 | +65 | 532 (1065) | ±0 |
| Hoechst 33342 | 660 > 715 | –40 | 497 (660) | +10 |
| lissamine rhodamine | 837 >> 1116 | –24 | 600 (837) | +5 |
| PhenGreen-FL | 1074 | +90 | 435 << 522 (1074) | +5 |
| PhenGreen-FL + Fe | 1074 | | 440 (1074) | |
| propidium iodide | 989 > 1015 >> 1099 | –13 | 612 << 657 (981) | –5, + 40 |
| quinacrine | 678 (697) | –168 | 517 (837) | +14 |
| rhodamine 123 | 913 (1090) | –101 | 536 (947) | +7 |
| SNARF-1 | < 837 | –200 | 622 < < 660 (837) | +10 |
| Organelle trackers | | | | |
| ER-Tracker white/blue | 728 | –20 | 586 (728) | +11 |
| LysoTracker Yellow | 972 | –104 | 435 << 552 (981) | ±0 |
| LysoTracker Red | 1010 < 1100 | – | 605 (1032) | +15 |
| MitoTracker Red | 1133 | –22 | 444 (1065) << 608 (1133) | +9 |
| Antibody-conjugated | | | | |
| Alexa Fluor 488 | 985 | +3 | 530 (985) | +15 |
| Alexa Fluor 546 | 1028 | – 78 | 582 (1028) | +13 |
| Alexa Fluor 594 | 1074 | –114 | 619 (1074) | +9 |
| Cy2-IgG | 837 < 905 > 981 | –73 | 520 (905) | +14 |
| Cy3-IgG | 1032 | –84 | 578 (1032) | +5 |
| F-DTAF-IgG | < 820; 820 > 837 > 972 | –8 | 527 (981) | +7 |
| FITC-IgG | 947 | –39 | 530 (947) | +5 |
| RedX-IgG | 845 >> 1108 | – | 602 (854) | ±0 |
| TexasRed-IgG | 1108 < (> 1150) | – | 616 (837) | ±0 |
| TexasRed-Phalloidin | > 1150 | – | 611 (837) | +9 |

[a]Dissolved in DMSO.

*Bestvater et al.*
*Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging*
*Journal of Microscopy, Vol. 208, Pt 2 November 2002, pp. 108–115*

# TPE cross-sections of various fluorochromes




*Bestvater et el.*
*Two-photon fluorescence absorption and emission spectra of*
*dyes relevant for cell imaging*
*Journal of Microscopy, Vol. 208, Pt 2*
*November 2002, pp. 108–115*

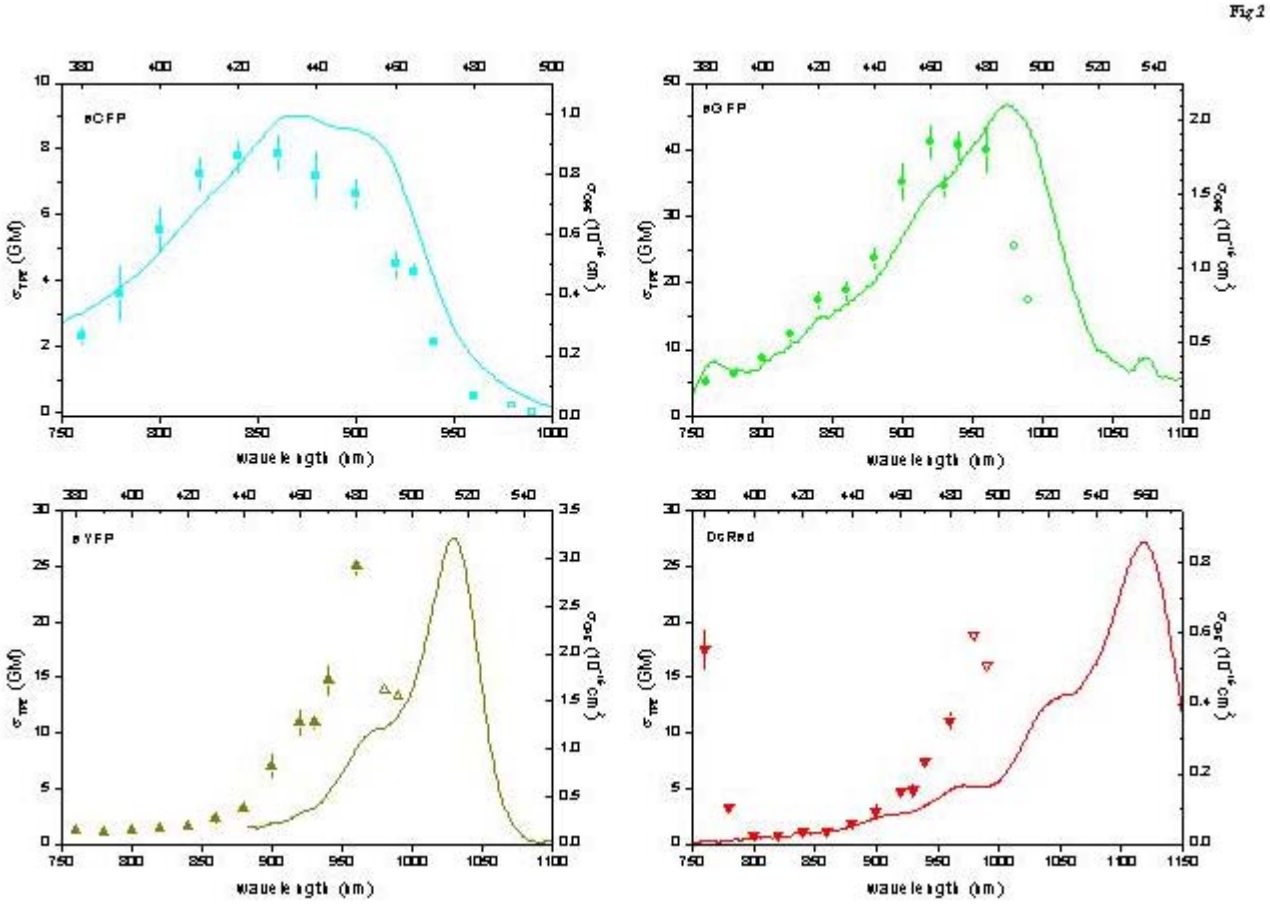
# Fluorochromes: TPE - Overview 2

Table 1. Fluorophores and Chromophores for Two-Photon Excitation

| Fluorophores/Choromophores | Φ[a] (GM) | 2PE[b] (nm) | Em. (nm) | Note | References |
|---|---|---|---|---|---|
| **Calcium indicators** | | | | | |
| Fluo -3, -4, -5F, 4FF et al. | | 810[c] | 520–530 | | (Yasuda et al., 2004) |
| Oregon Green BAPTA -1, -2 et al. | | 810[c] | 520 | | (Yasuda et al., 2004) |
| Calcium green-1 + $Ca^{2+}$; Calcium green-1 – $Ca^{2+}$ | 30, 2 | 820 | 530 | | (Xu and Webb, 1996; Xu et al., 1996) |
| Fura-2 + $Ca^{2+}$; Fura-2 – $Ca^{2+}$ | 6, 0.2 | 800 | 505 | | (Wokosin et al., 2004) |
| Indo-1 + $Ca^{2+}$; Indo-1 – $Ca^{2+}$ | 3.5, 1.5 | 700 | 400 | | (Xu and Webb, 1996; Xu et al., 1996) |
| **Quantum dots** | | | | | |
| Quantum dots | up to 47,000 | broad | variable | | (Larson et al., 2003) |
| **Fluorescent proteins** | | | | | |
| eCFP | 100–200 | 800–900 | 505 | | (Zipfel et al., 2003) |
| eGFP | 100–200 | 900–1000 | 510 | | (Zipfel et al., 2003) |
| eYFP | 100–200 | 930–1000 | 530 | | (Zipfel et al., 2003) |
| mRFP, mCherry | | 1030[c] | 610 | Ytterbium-doped laser | (Campbell et al., 2002; Shaner et al., 2004) |
| **Photoswitchable fluorescent proteins (see also Lukyanov et al., 2005)** | | | | | |
| paGFP | | 750[g] | 515 | | (Patterson and Lippincott-Schwartz, 2002; Schneider et al., 2005) |
| Kaede | | 730[d] | 520→580 | green to red; tetramer | (Ando et al., 2002) |
| KFP1 | | 1120[d] | 600 | tetramer | (Chudakov et al., 2003) |
| Dronpa | | 780[d,e], 1010[d,f] | 520 | reversible | (Ando et al., 2004; Habuchi et al., 2005) |
| psCFP | | 800[d] | 470→510 | cyan to green | (Chudakov et al., 2004) |
| PA-mRFP | | 760[d] | 605 | | (Verkhusha and Sorkin, 2005) |
| KikGR | | 760[c] | 520→590 | green to red; tetramer | (Tsutsui et al., 2005) |
| Dendra | | 960[d] | 505→575 | green to red | (Gurskaya et al., 2006) |
| mEosFP | | 780[d] | 520→580 | green to red | (Wiedenmann et al., 2004) |
| **Caged glutamate** | | | | | |
| MNI-glutamate | 0.06 | 730 | | | (Matsuzaki et al., 2001) |
| **Caged calcium** | | | | | |
| DM-nitrophen | 0.013 | 730 | | $K_d$: 2 nM[h], 1.5 mM[i] | (Brown et al., 1999; Momotake et al., 2006) |
| Azid-1 | 1.4 | 700 | | $K_d$: 230 nM[h], 0.12 mM[i] | (Brown et al., 1999; Momotake et al., 2006) |
| NDBF-EGTA | 0.6 | 710 | | $K_d$: 14 nM[h], 1 mM[i] | (Momotake et al., 2006) |

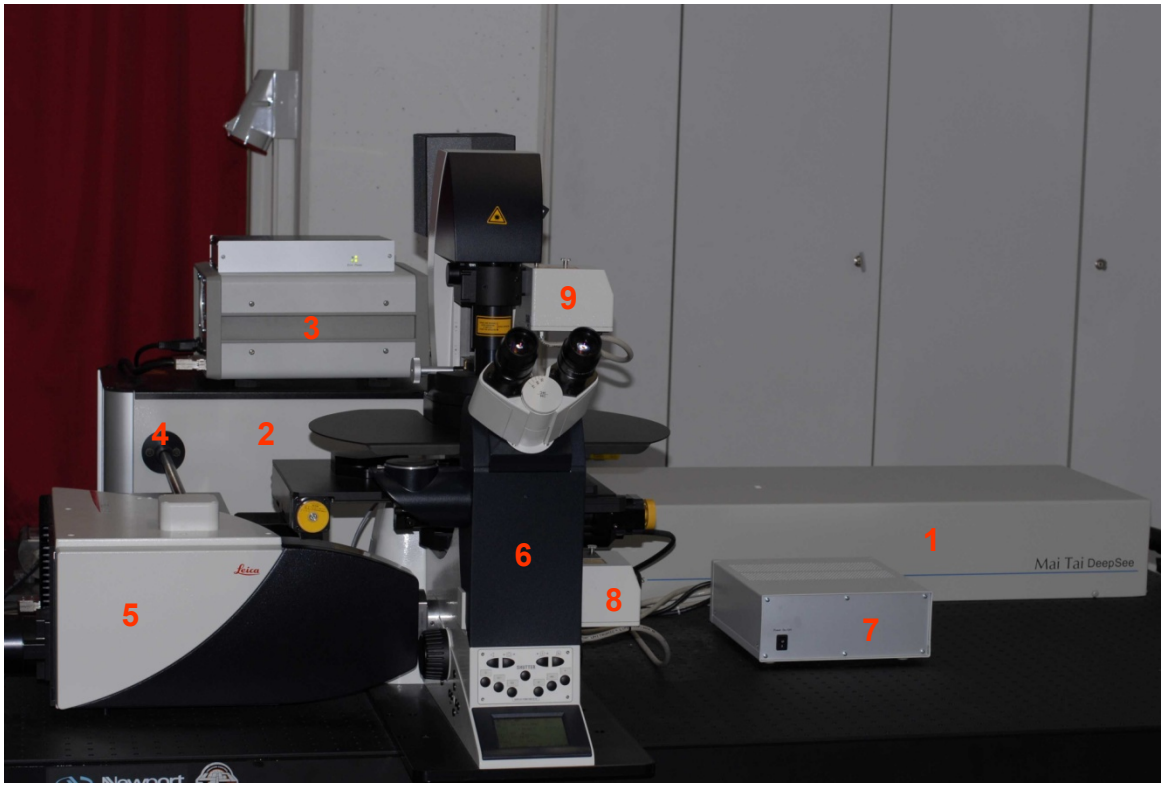
*Svoboda & Ryohei*
*Principles of Two-Photon Excitation Microscopy and its Applications to Neuroscience*
*Neuron 50, 823 – 839, June 15, 2006*

# TP cross-section of standard FPs



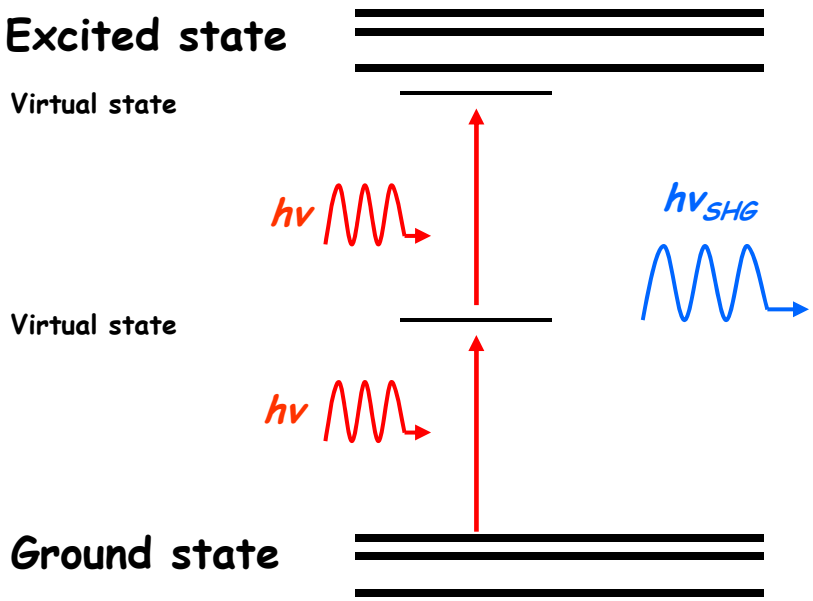

*Blab et al., 2001*
*Two-Photon Excitation Cross-Sections of the Autofluorescent Proteins. Chemical Physics Letters 350: 71-7*

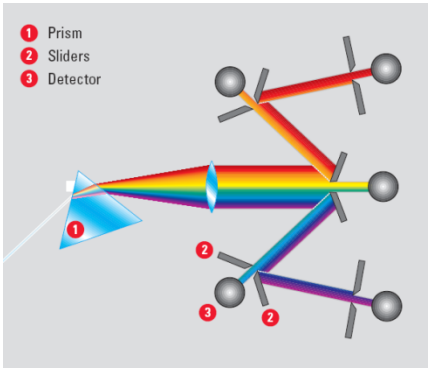
# TCS SP 5 MP: Typical Setup - inverted




1) **IR Laser (Mai Tai DeepSee)**
2) **Safety Box**
3) **EOM (Driver)**
4) **Beam Routing (direct coupling)**
5) **Scanhead**
6) **DMI 6000 Microscope Stand**
7) **NDD Detection Unit**
8) **NDD: RLD**
9) **NDD: TLD**

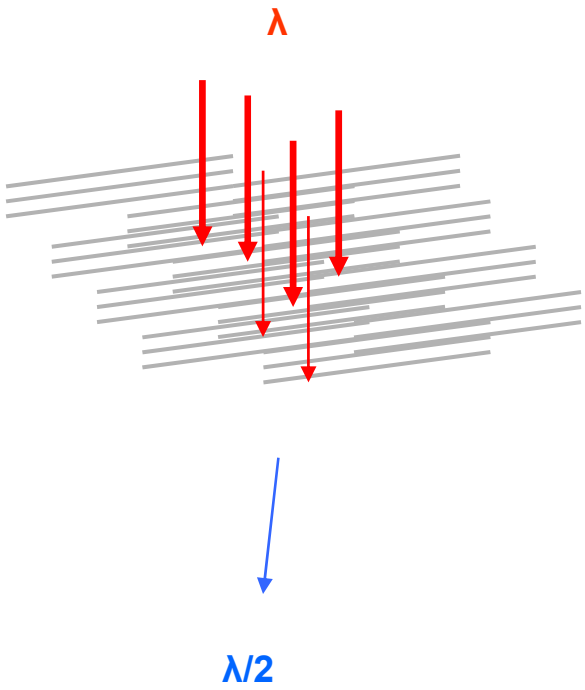
# Second Harmonic Generation (SHG)




- **SHG is a nonlinear scattering process that conserves energy and results in the SHG λ exactly half of the illumination λ'**
- **involves virtual transitions in which no energy is absorbed**
- **2 photons „simultaneously" scattered, resulting in „frequency doubling"**
- **In contrast 2PE involves absorption (real transition) and excitation of molecules**
- **SHG = $\lambda_{incident}/2$**
- **Investigate with spectrophoto-meter-PMT or NDD**

# SHG: how it works – lite

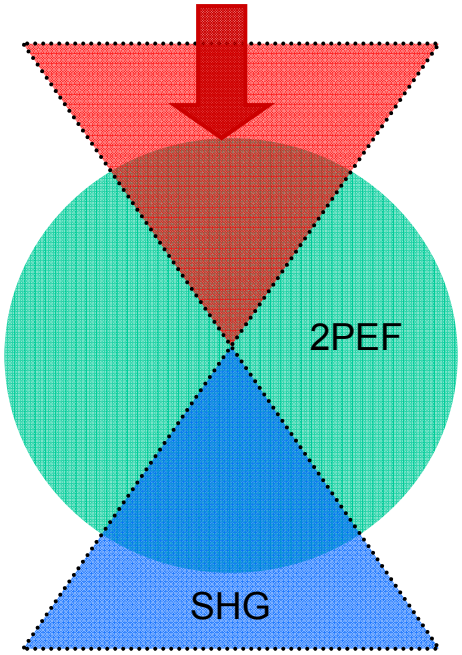



**Incident beam polarizes illuminated matter**

**non-centrosymmetric organization oscillating dipoles**

**Simultaneous scattering**

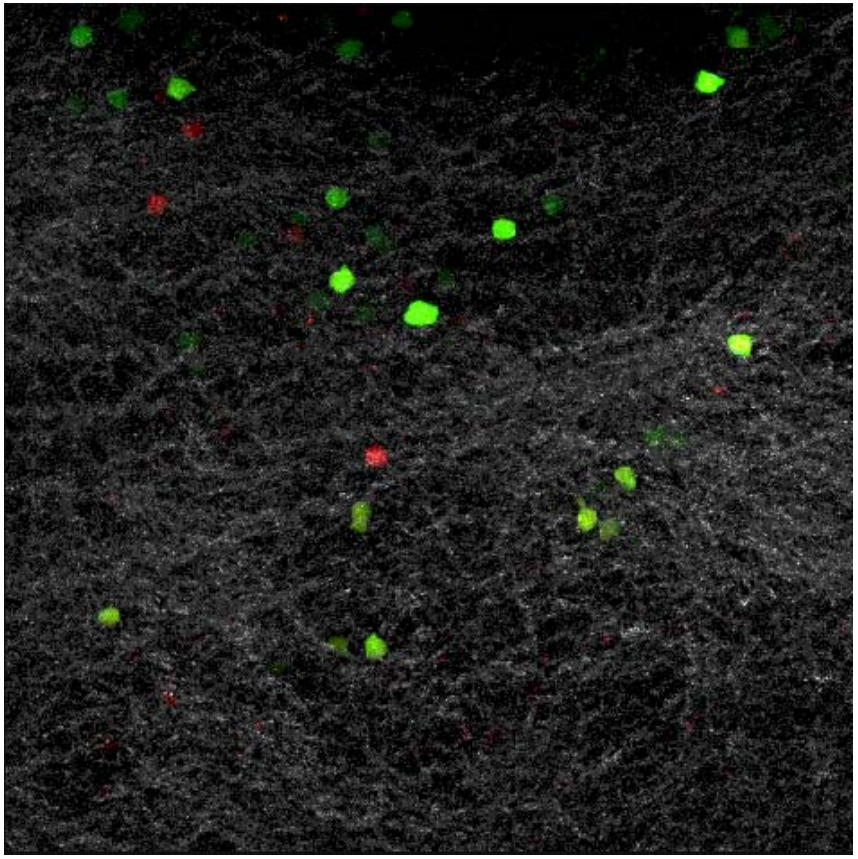
# SHG – direction & structures



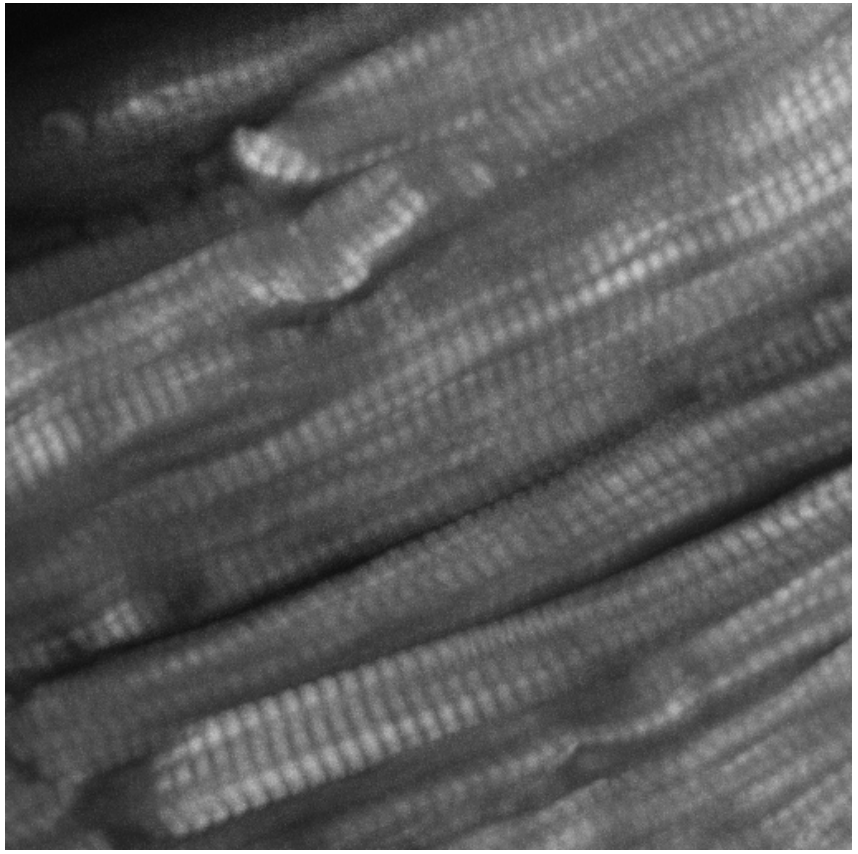

- **Predominantly forward-directed emission**
  - **i.e. *trans*-detection**
    - **High NA condensor (1,4 oil)**
    - **2nd objective (on DMI)**
  - **backscattered possible**

- **Visualize well-ordered structures:**
  - **Collagen fibers**
  - **Microtubules**
  - **Muscle myosin**
  - **Membrane potential via dyes**

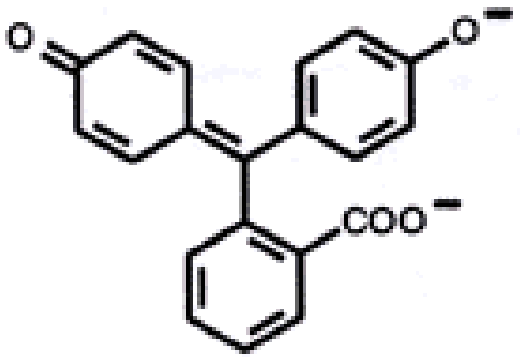
# SHG – images



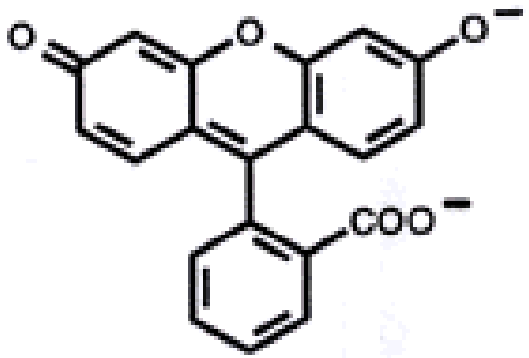
SHG combined with fluorescence:
Collagen fibrils (SHG, grey), Macrophages (Fluorescence, green & red)



Striation pattern of murine heart



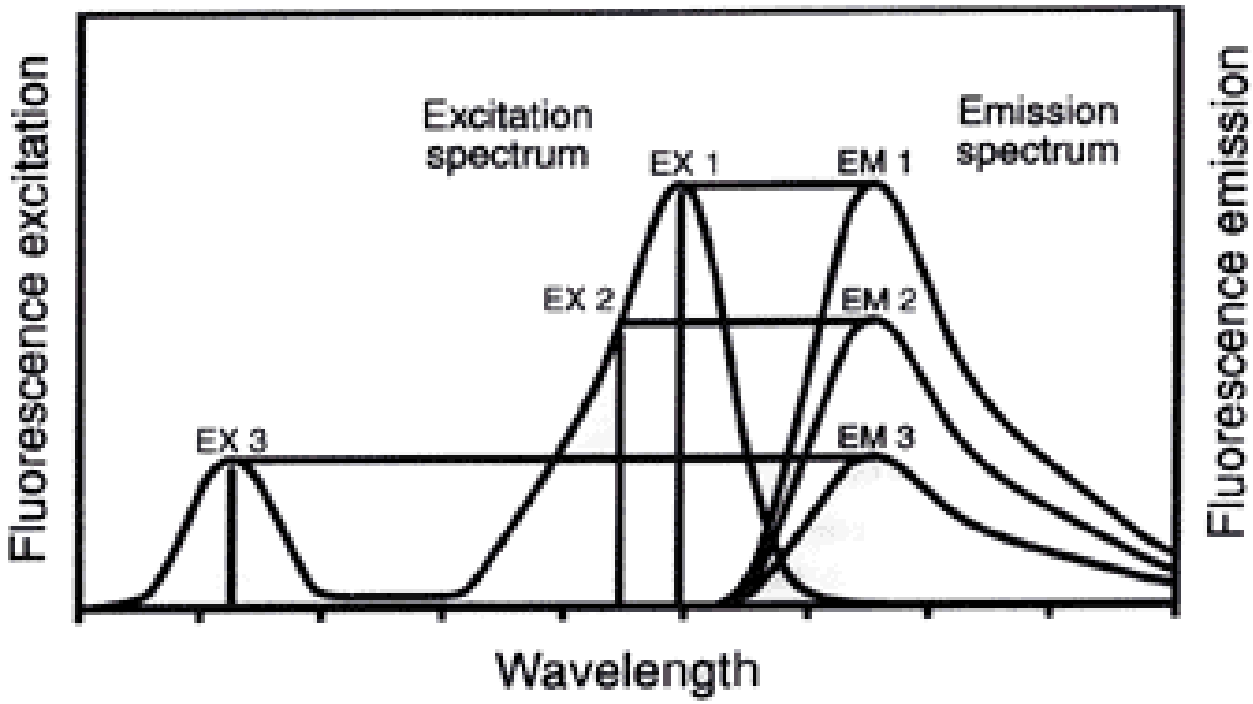
Phenolphthalein
Flexible
Nonfluorescent



Fluorescein
Rigid
Fluorescent

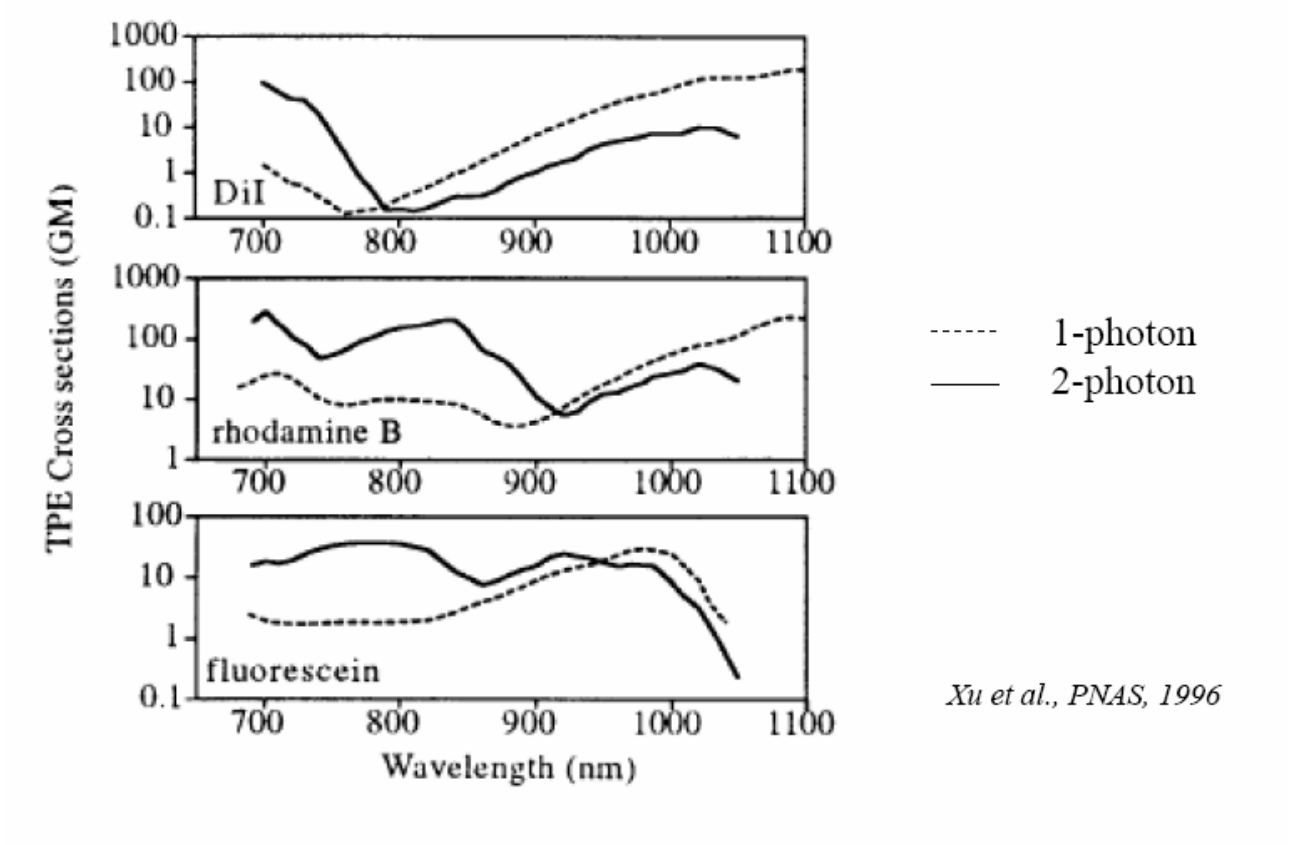
| Fluorophore | ε ($cm^{-1}$ $M^{-1}$) |
|---|---|
| Oregon Green®488 | 87,000 |
| BODIPY FL | 91,000 |
| FAM | 79,000 |
| JOE | 71,000 |
| TAMRA | 103,000 |
| ROX | 82,000 |
| Texas Red | 139,000 |

| Fluorophore | QY |
|---|---|
| Oregon Green®488 | 0.9 |
| BODIPY FL | 0.9 |
| FAM | 0.9 |
| JOE | 0.6 |
| TAMRA | 0.2 |
| ROX | 0.7 |
| Texas Red | 0.9 |


Fluorescence excitation
Excitation spectrum
EX 1
EM 1
Emission spectrum
EX 2
EM 2
EX 3
EM 3
Fluorescence emission
Wavelength

# Examples of 2P excitation cross sections

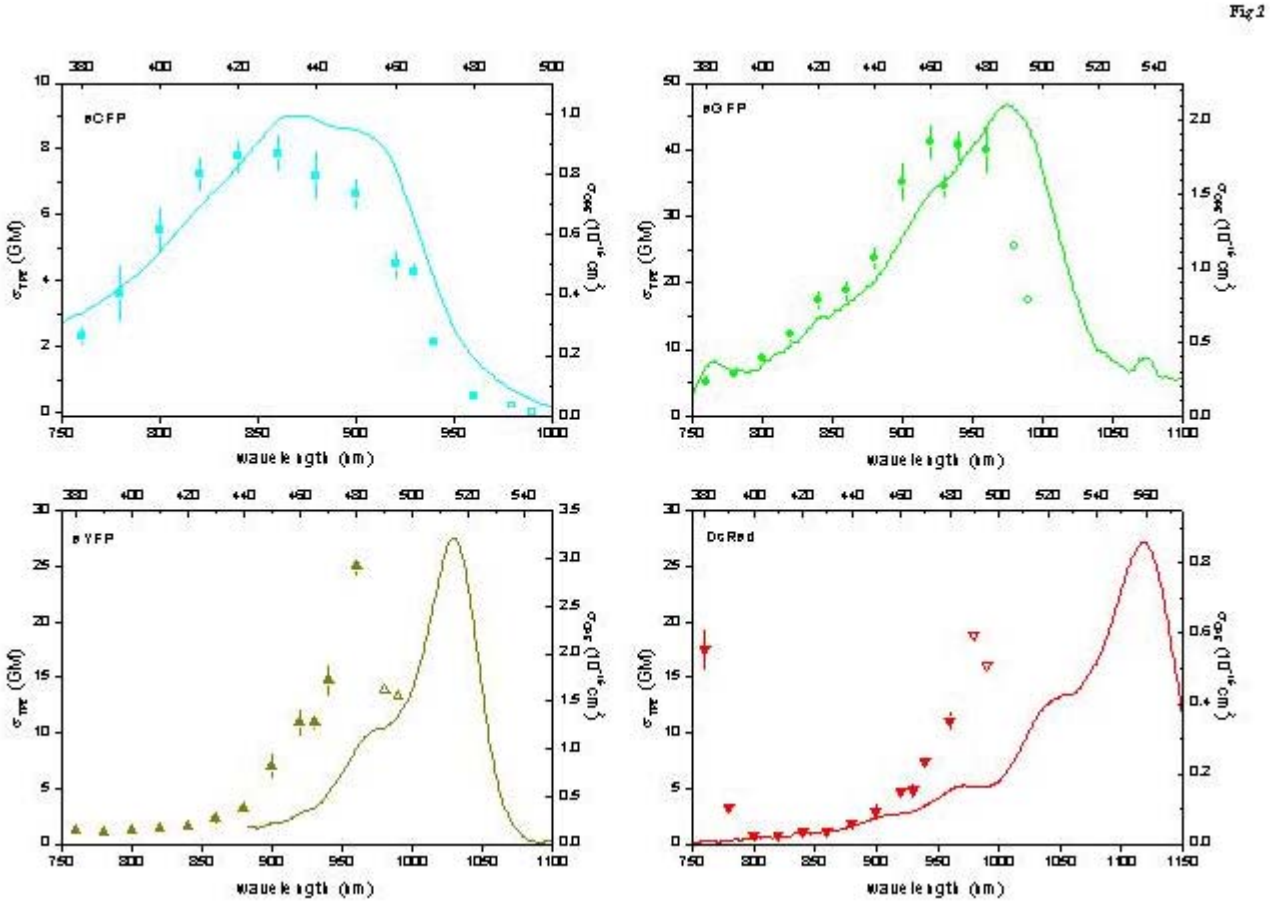





*Xu et al., PNAS, 1996*

**The best excitation is not always double 1P excitation**

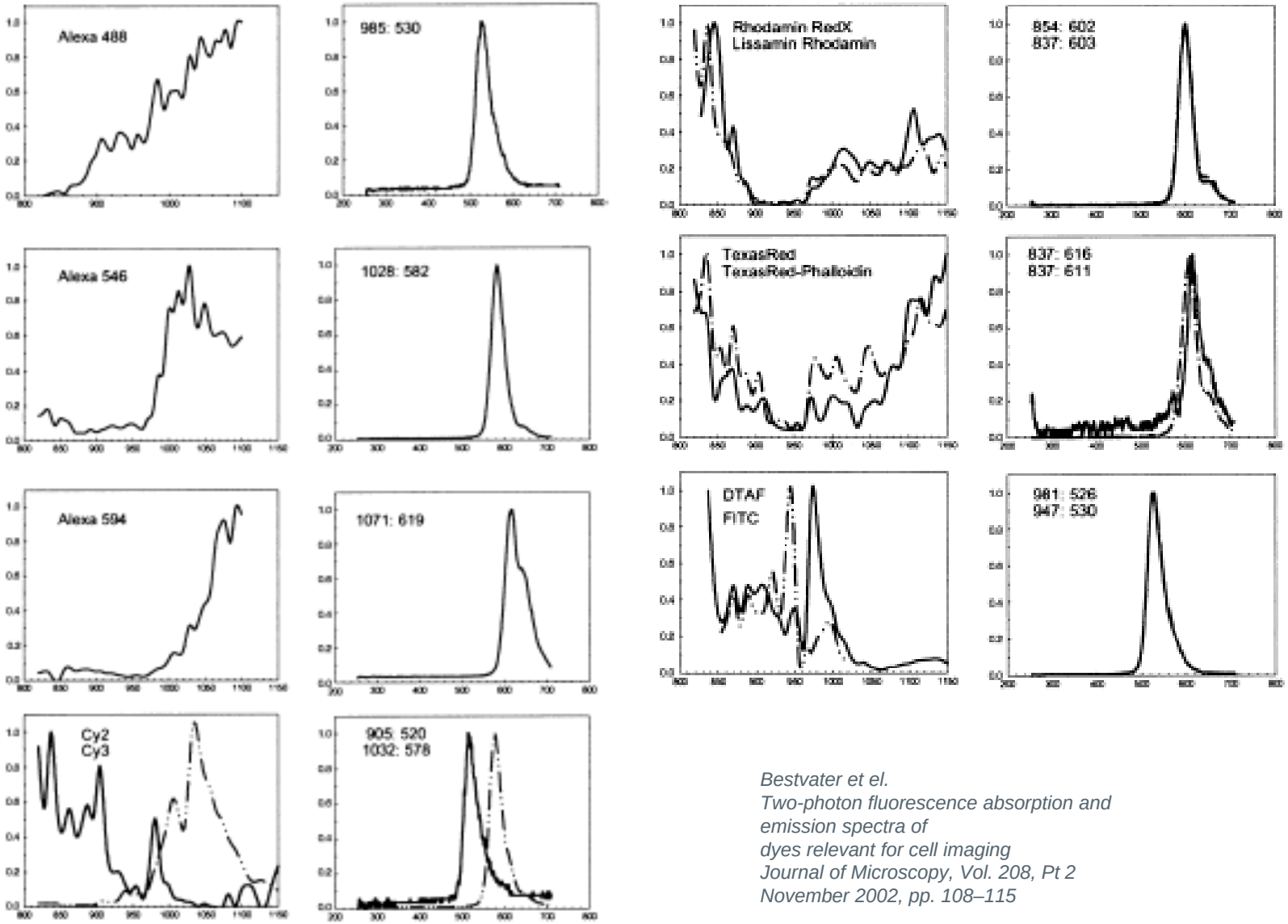
# TP cross-section of standard FPs




*Blab et al., 2001*
*Two-Photon Excitation Cross-Sections of the Autofluorescent Proteins. Chemical Physics Letters 350: 71-7*

# Examples of cross section in 2P excitation






*Bestvater et el.*
*Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging*
*Journal of Microscopy, Vol. 208, Pt 2 November 2002, pp. 108–115*

# Multiphoton excitation of selected dyes



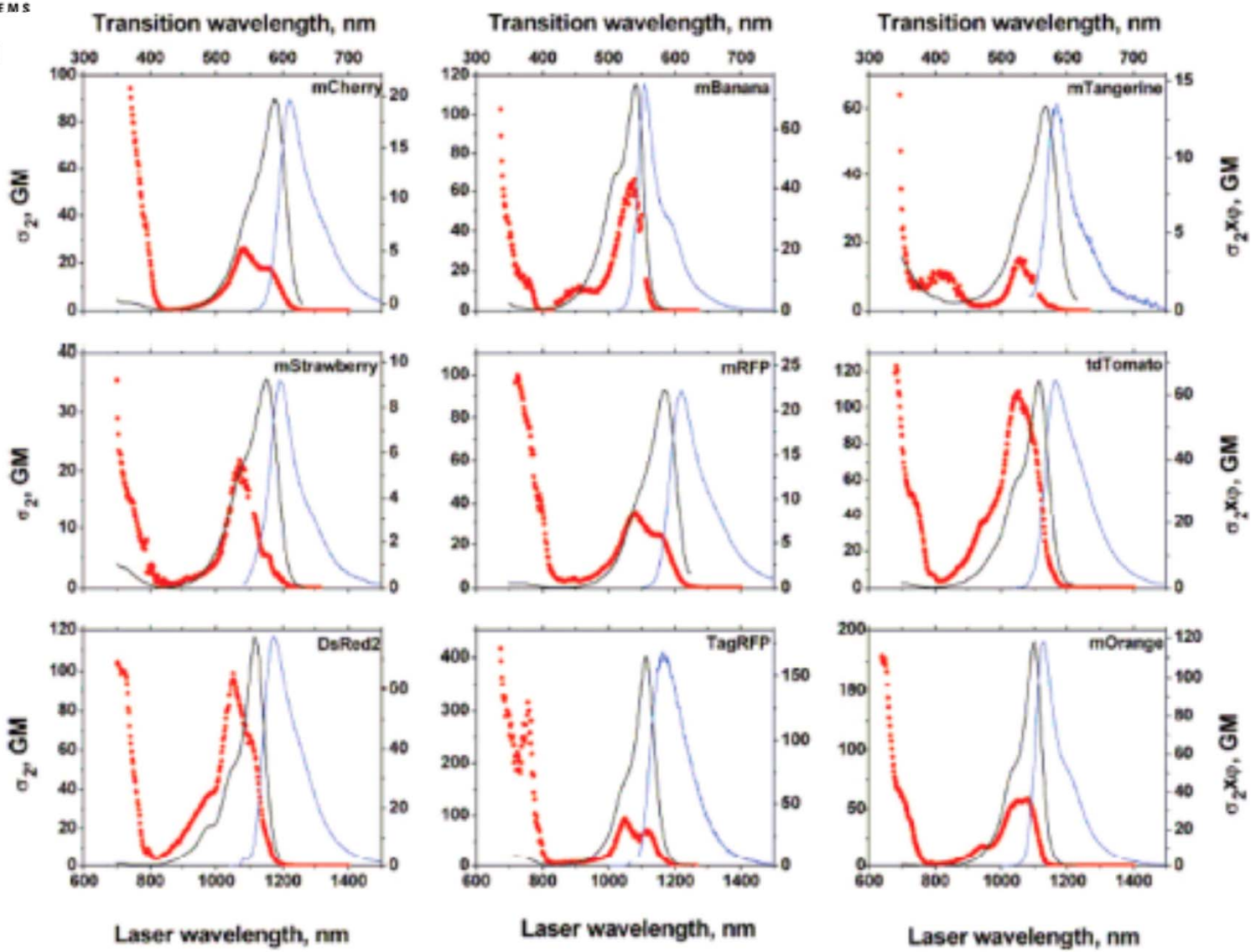
| | 780 nm | | 820 nm | | 1064 nm | |
|---|---|---|---|---|---|---|
| Cell Wall Stain | Calcofluor White | 440/500-520 | Calcofluor White | 440/500-520 | | |
| Nucleic Acid Stains | DAPI, Hoechst | 350/470 350/460 | DAPI (885,970 3P), Hoechst | 350/470 350/460 | | |
| | | | Ethidium Bromide | 518/605 | Propidium Iodide | 530-615 |
| | Feulgen | 480/560 | Feulgen | 480/560 | Feulgen | 480/560 |
| Cell Viability | Fluorescein Di Acetate | 495/520 | Fluorescein Di Acetate | 495/520 | | |
| Calcium | Indo 1 (720, 885 3P) | 340-365 /400-480 | | | | |
| | Fura 2 (720) | 340-380/512 | | | | |
| | Calcium Green/Texas Red (770) | 488/530, 596/620 | | | | |
| | Calcium Green | 488/530 | | | | |
| Protein Conjugates | AMCA | 431/498 | | | | |
| | FITC | 490/525 | FITC | 490/525 | | |
| | CY2 (760 nm) | 489/506 | CY2 | 489/506 | BodipyR6G | 528/547 |
| | CY3 (760 nm) | 550/570 | CY5 | 649/670 | Oregon Green 514 | 506/526 |
| | CY5 (760 nm) | 649/670 | TRITC | 541/572 | TRITC | 541/572 |
| | Texas Red | 596/620 | | | Texas Red | 596/620 |
| | | | | | CY3 | 550/570 |
| Gene Expression | GFPuv | 395/509 | S65T (860) | 488/507 | S65T | 488/507 |
| Mito Tracers | Rhodamin 123 | 507/529 | Rhodamin 123 | 507/529 | Rhodamin 123 | 507/529 |
| | | | | | Rosamin | 550/574 |
| | | | | | Rhodamin 6G | 530/590 |
| | | | | | JC1 | 514/529 |
| Vacuolar Tracer | | | | | FM4-64 | 515/614 |
| Lipid Tracer | | | | | Nile Red | 485-530 /526-605 |
| | | | | | DiI (12, 16) | 549/565 |
| Neuronal Tracer | DID (760-780) | | Lucifer Yellow | 430/535 | Evans Blue | 550/610 |

# Fluorescent Proteins –Covering the entire visible spectra

| | Excitation Maximum (nm) | Emission Maximum (nm) | Molar Extinction Coefficient | Quantum Yield | in vivo Structure | Relative Brightness (% of EGFP) |
|---|---|---|---|---|---|---|
| **Orange Fluorescent proteins** | | | | | | |
| Kusabira Orange | 548 | 559 | 51,600 | 0.6 | Monomer | 92 |
| mOrange | 548 | 562 | 71,000 | 0.69 | Monomer | 146 |
| dTomato | 554 | 581 | 69,000 | 0.69 | Dimer | 142 |
| dTomato-Tandem | 554 | 581 | 138,000 | 0.69 | Monomer | 283 |
| DsRed | 558 | 583 | 75,000 | 0.79 | Tetramer | 176 |
| DsRed2 | 563 | 582 | 43,800 | 0.55 | Tetramer | 72 |
| DsRed-Express (T1) | 555 | 584 | 38,000 | 0.51 | Tetramer | 58 |
| DsRed-Monomer | 556 | 586 | 35,000 | 0.1 | Monomer | 10 |
| mTangerine | 568 | 585 | 38,000 | 0.3 | Monomer | 34 |
| mStrawberry | 574 | 596 | 90,000 | 0.29 | Monomer | 78 |
| AsRed2 | 576 | 592 | 56,200 | 0.05 | Tetramer | 8 |
| mRFP1 | 584 | 607 | 50,000 | 0.25 | Monomer | 37 |
| JRed | 584 | 610 | 44,000 | 0.2 | Dimer | 26 |
| mCherry | 587 | 610 | 72,000 | 0.22 | Monomer | 47 |
| HcRed1 | 588 | 618 | 20,000 | 0.015 | Dimer | 1 |
| mRaspberry | 598 | 625 | 86,000 | 0.15 | Monomer | 38 |
| HcRed-Tandem | 590 | 637 | 160,000 | 0.04 | Monomer | 19 |
| mPlum | 590 | 649 | 41,000 | 0.1 | Monomer | 12 |
| AQ143 | 595 | 655 | 90,000 | 0.04 | Tetramer | 11 |

# Two Photon Excitatation Spectra of Fluorescent Proteins

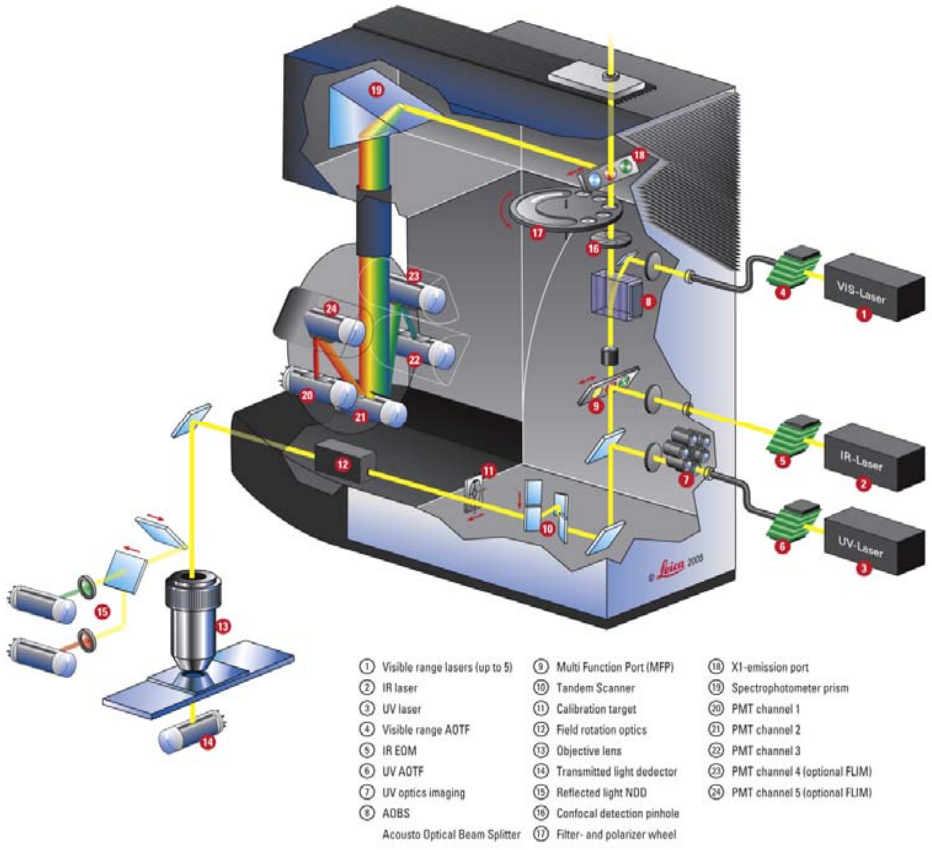
Living up to Life




igure 1. 2PA spectra of orange and red FPs (symbols) shown along with fluorescence emission (blue line) and one-photon fluorescence excitati
lack line) spectra. The left vertical scale shows the 2PA cross section. The scale on the right represents two-photon brightness. One-phot

M. Drobizhev, S. Tillo, N. S. Makarov, T. E. Hughes,and A. Rebane, Absolute Two-Photon Absorption Spectra and Two-Photon Brightness of Orange and Red Fluorescent Proteins, J. Phys. Chem. B, 2009,

# TCS SP5 MP: requirement



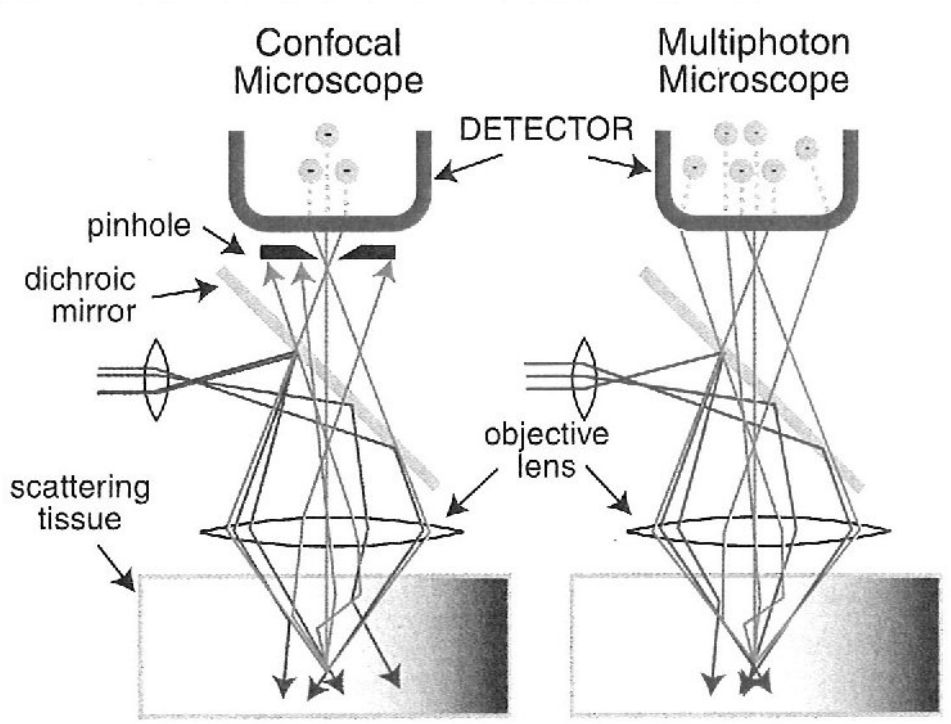

- Dedicated Multifunction Port (MFP)
- Direct coupling
- IR lasers: Tuneable over a wide range (690-1040nm)
- Attenuation
  - Half wave plate
  - EOM (Electro Optical Modulator)
    - Fine tuning of attenuation of excitation light
    - 0% - 100%
    - ROI scan
      - Uncaging
      - photoactivation experiments
      - FRAP
- Detection
  - Descanned detectors (PMT / APD)

## Advantage of Multifphoton vs Confocal for imaging scattering tissue

- No confocal pinhole necessary
- detectors as close as possible to the specimen

enables scattered photons to be collected

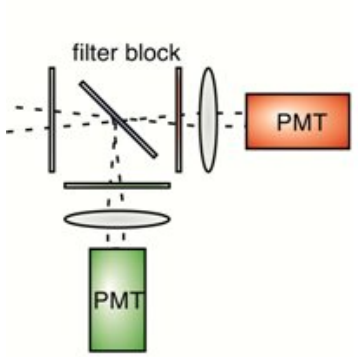
→ much higher photon collection efficiency compared to confocal microscopy




## 2 NDDs architecture

- different dichroics available

to separate pairs of fluorochromes



# TCS SP5 MP: NDDs



## Highest photon collection efficiency

Detectors directly behind Objective, RLD

Detectors directly behind Condensor, TLD

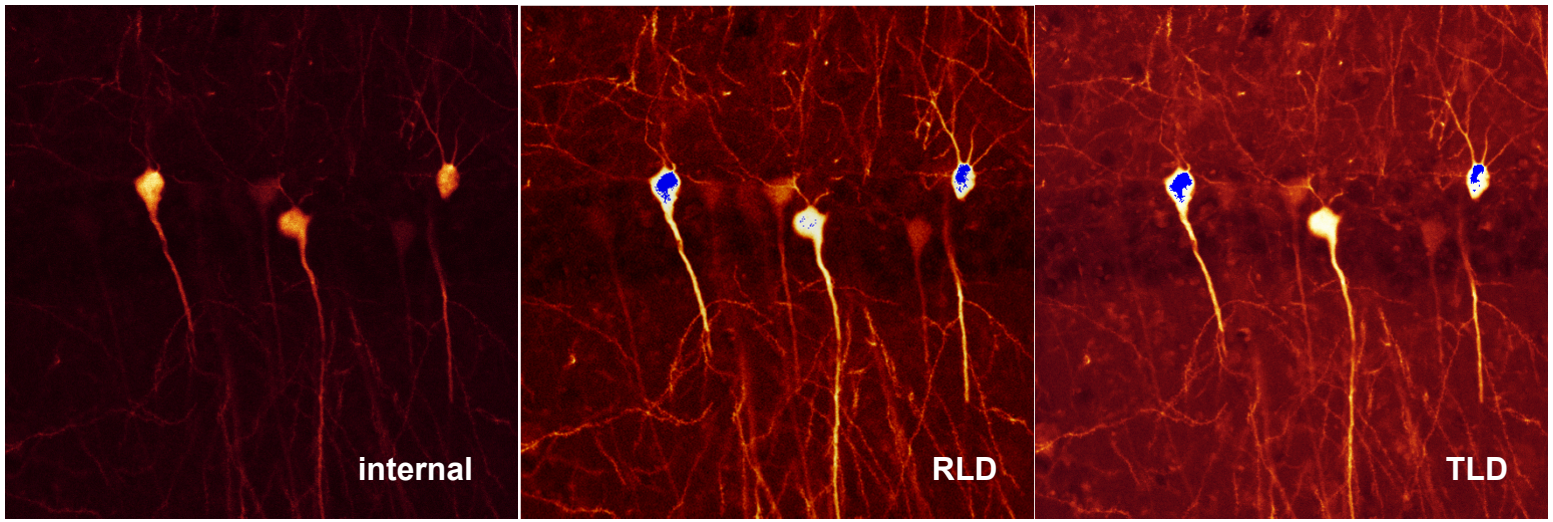
## Advantage:

- Scattered fluorescent photons can also be collected
- Special dichroic allows simultaneous acquisition of fluorescence and IR-SGC
- Protected by Leica patent US 6,831,780 B2

# Photon Collection Efficiency - Internal vs. NDDs

Living up to Life

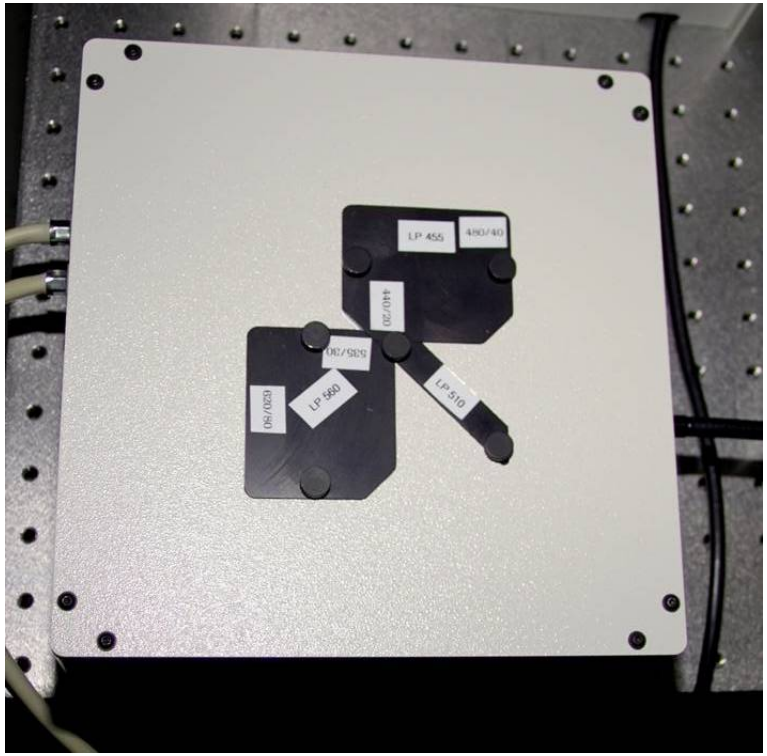
Mouse brain slice: ~ 20 µm (center plane)
Detection range: 500 – 550 nm
PMT: 950 V
Objective: 20 x 1.0 W
Excitation: 920 nm, power level identical




Mean intensity image: 20 52 58

# TCS SP5 MP: 4 NDDs solution



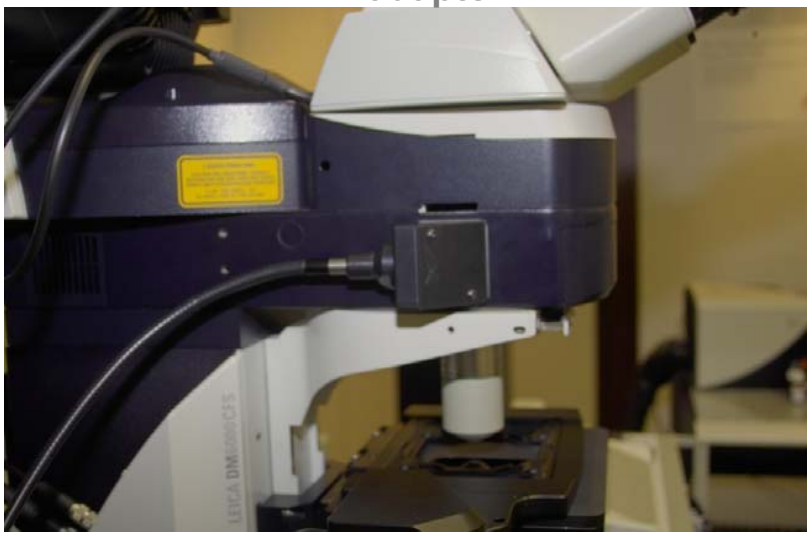



## 4 NDDs solution

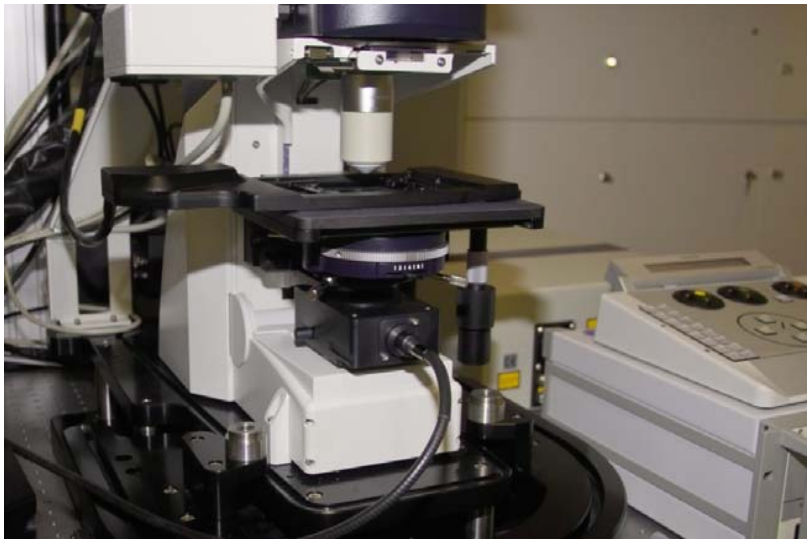
- Simultaneous acquisition of 4 colors
- Solution: adaptor + liquid light guide + 4NDDs module
- Photon transmission via liquid light guide
- Adaptor directly behind Objective, RLD
- Adaptor directly behind Condensor, TLD

# TCS SP5 MP: 4 NDDs solution

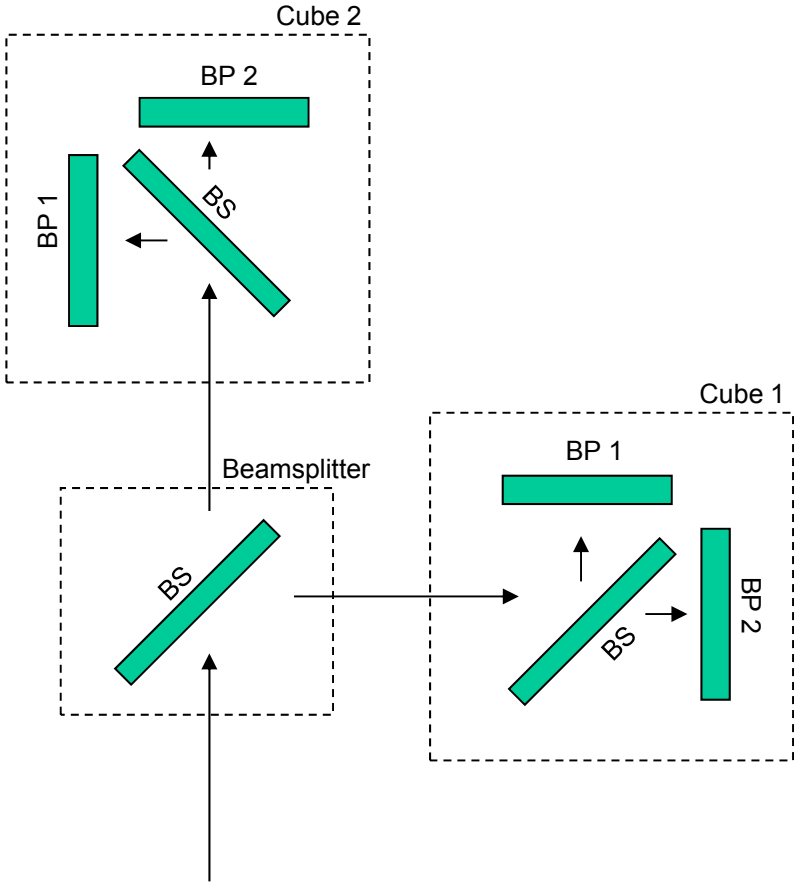
RLD adaptor



TLD adaptor



Architecture of the module containing the filtercubes

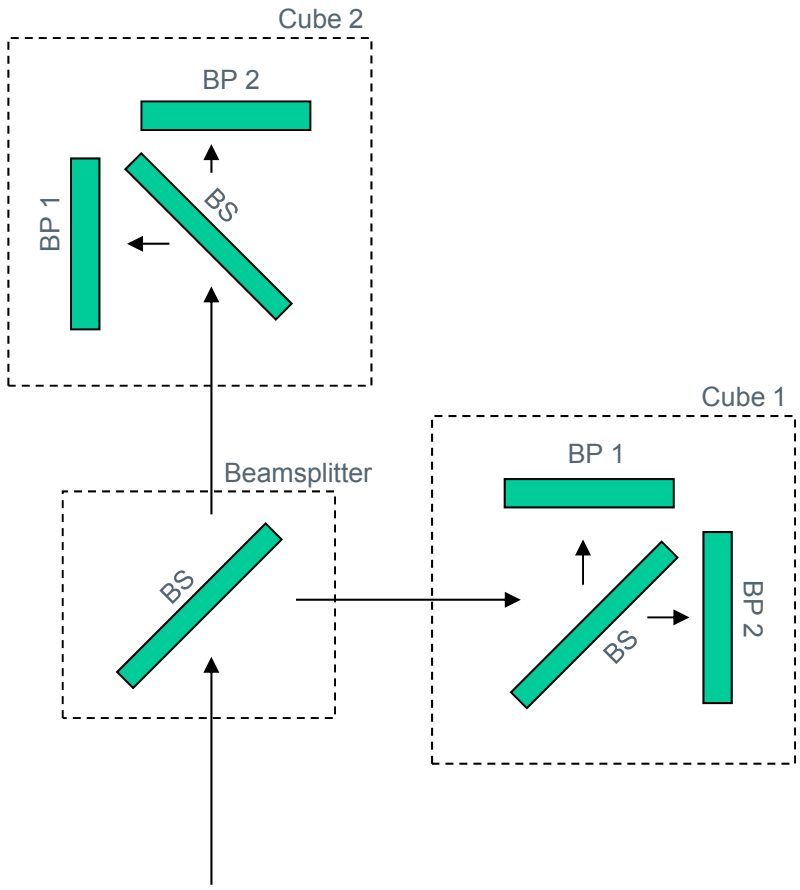


# TCS SP5 MP: Filtersets for 4 NDDs

Living up to Life

| Set 1 156504245<br>DAPI / FITC / TRITC / ALEXA633 | | |
|---|---|---|
| Cube1: DAPI / FITC | BP1 457/50 | BS RSP495 |
| | BP2 525/50 | |
| Cube2: TRITC / Alexa633 | BP1 585/40 | BS RSP620 |
| | BP2 650/50 | |
| Beamsplitter | | BS RSP560 |

| Set 2 156504246<br>SHG440 / CFP / YFP / DSRED | | |
|---|---|---|
| Cube1: SHG440 / CFP | BP1 440/20 | BS RSP455 |
| | BP2 483/32 | |
| Cube2: YFP / DSRED | BP1 535/30 | BS RSP560 |
| | BP2 585/40 | |
| Beamsplitter | | BS RSP505 |



# MP-imaging: 4 Dyes with the 4 channel NDDs

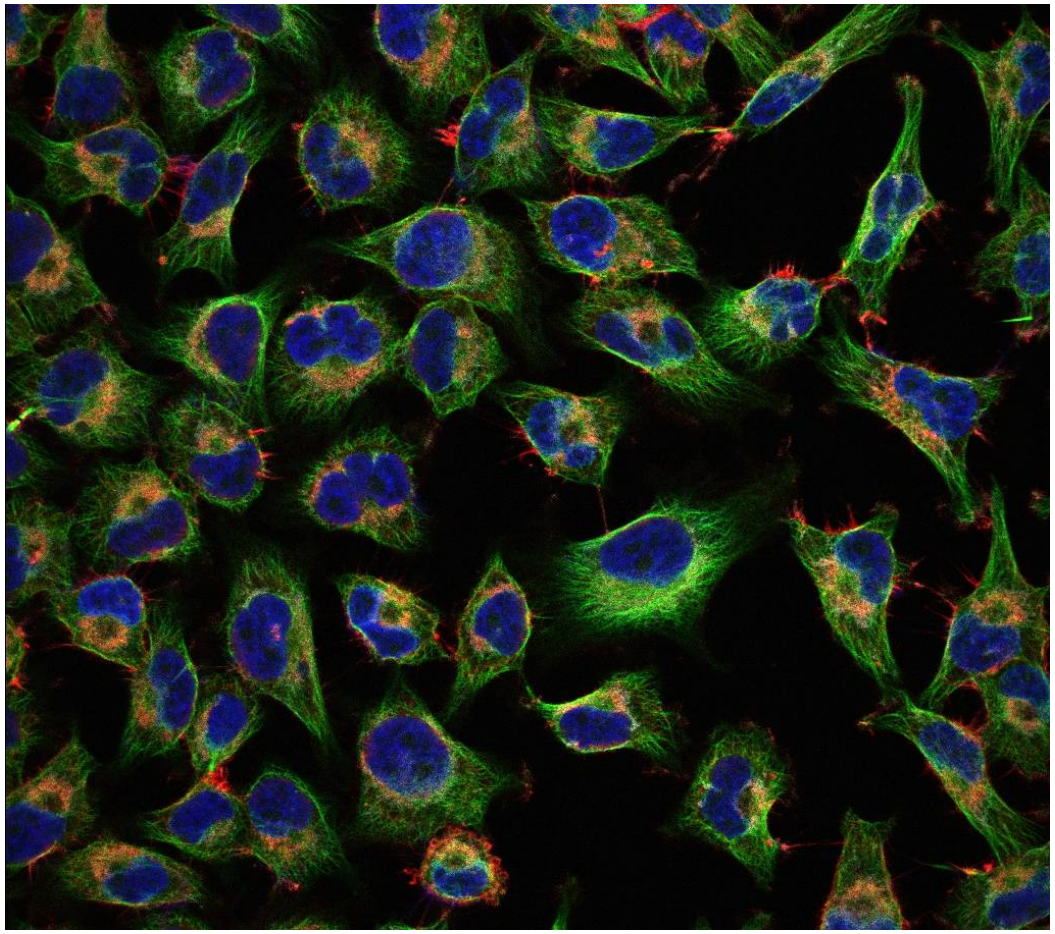


**Sample: HELA-cells**

**MP Sequential Scanning**

• Sequence 1:
Excitation 800nm
Blue: Nuclei – DAPI
Red: Actin - Phalloidin-TRITC

• Sequence 2:
Excitation 920nm
Green: Tubulin - Alexa 488
Grey: Mitochondria - Mito-Tracker 599nm



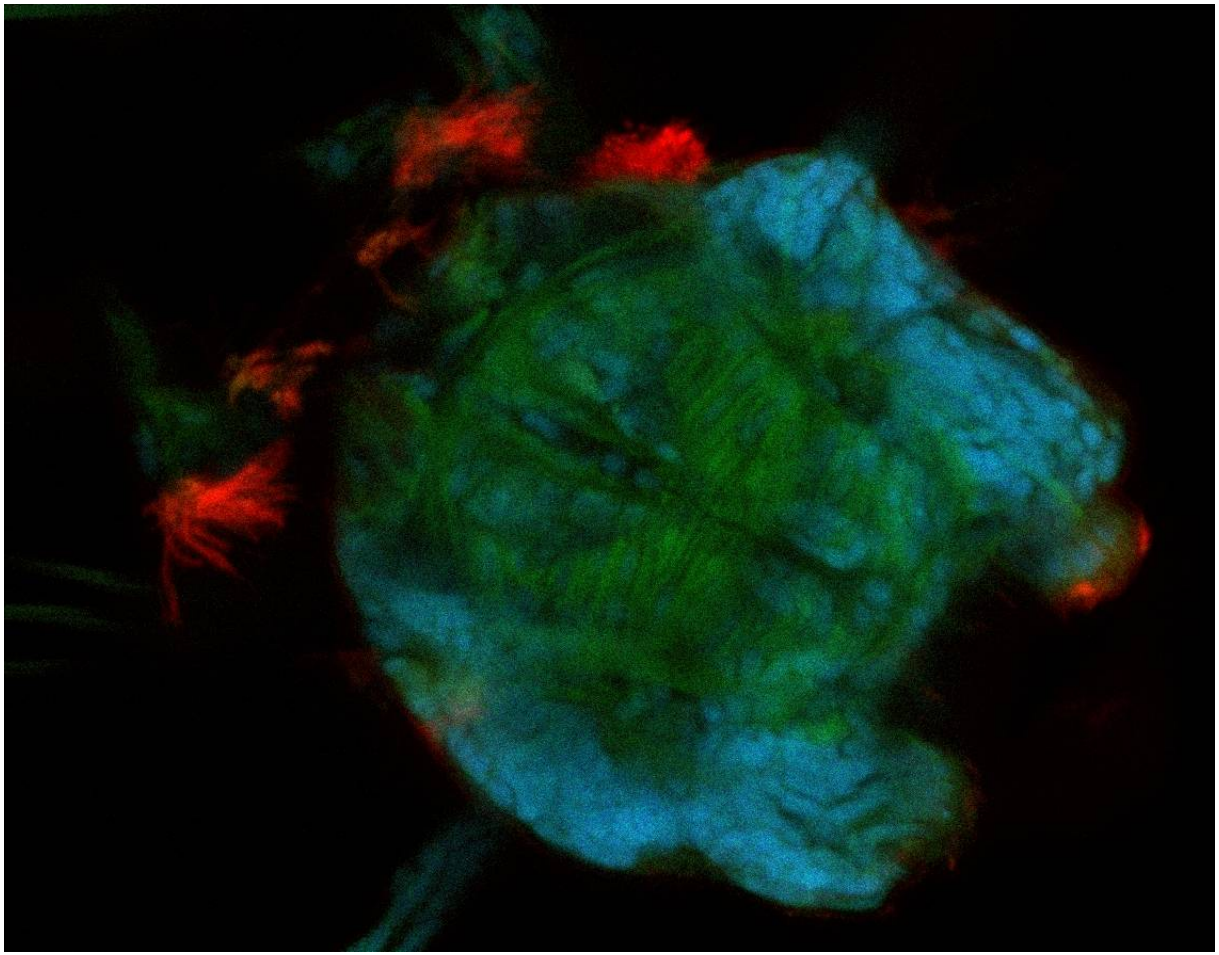
**Sample is a courtesy of Dr. G. Giese and Annemarie Scherbarth, MPI Heidelberg, Germany**

# MP-imaging: 3 dyes with the 4 channel NDDs



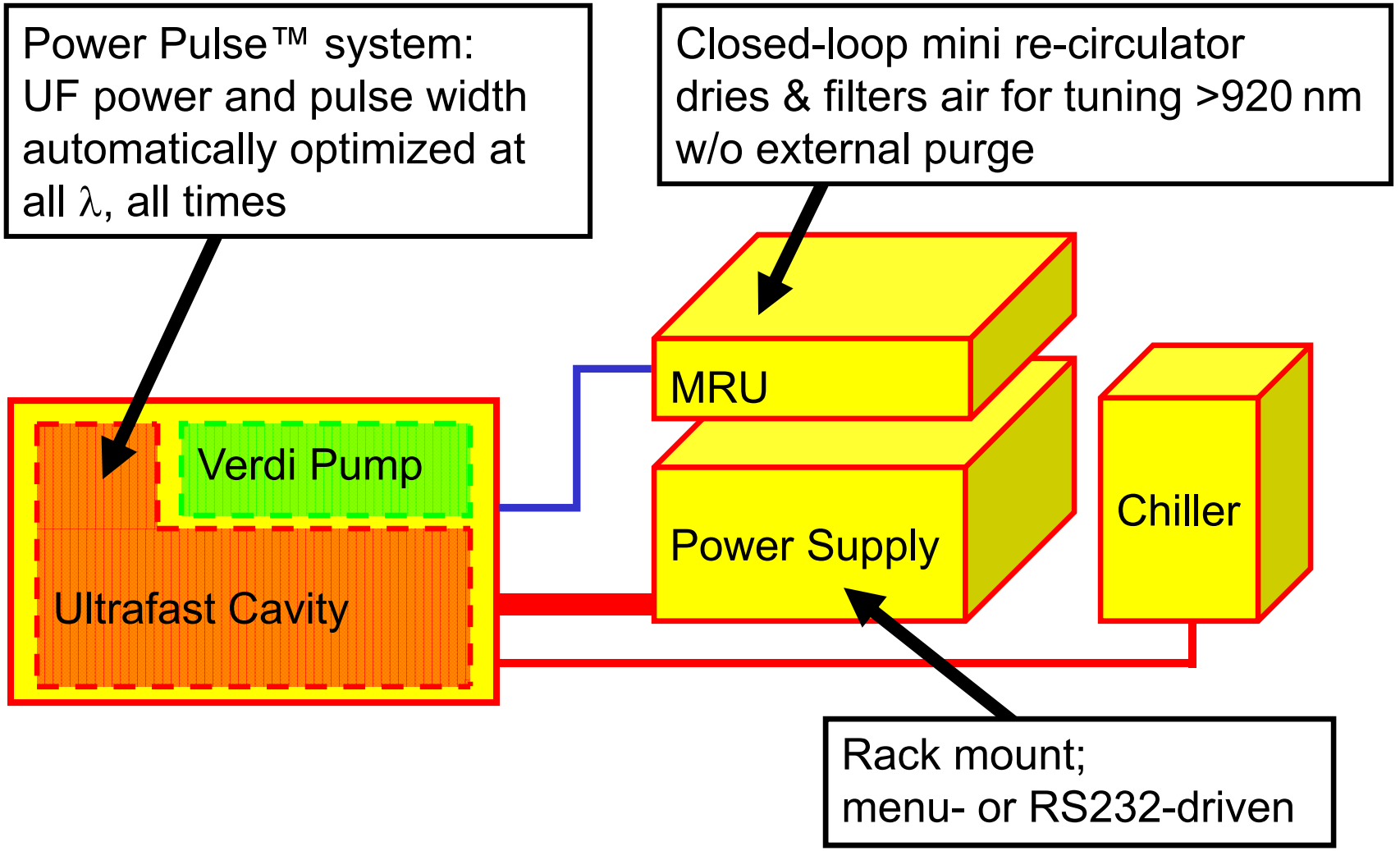
**Platynereis**
Two-photon excitation: 860 nm

- Blue: Nuclei - DAPI
- Green: Actin - Alexa 568
- Red: Tubulin - Alexa 633



Sample is a courtesy of Dr. Leonid Nezlin, RSA, Moscow, Russia

# Coherant laser


Power Pulse™ system:
UF power and pulse width automatically optimized at all λ, all times
Closed-loop mini re-circulator dries & filters air for tuning >920 nm w/o external purge
MRU
Verdi Pump
Ultrafast Cavity
Power Supply
Chiller
Rack mount;
menu- or RS232-driven