MULTIPHOTON MICROSCOPY

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. Uppsala University . Platform . Biological Visualization .

BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

Information

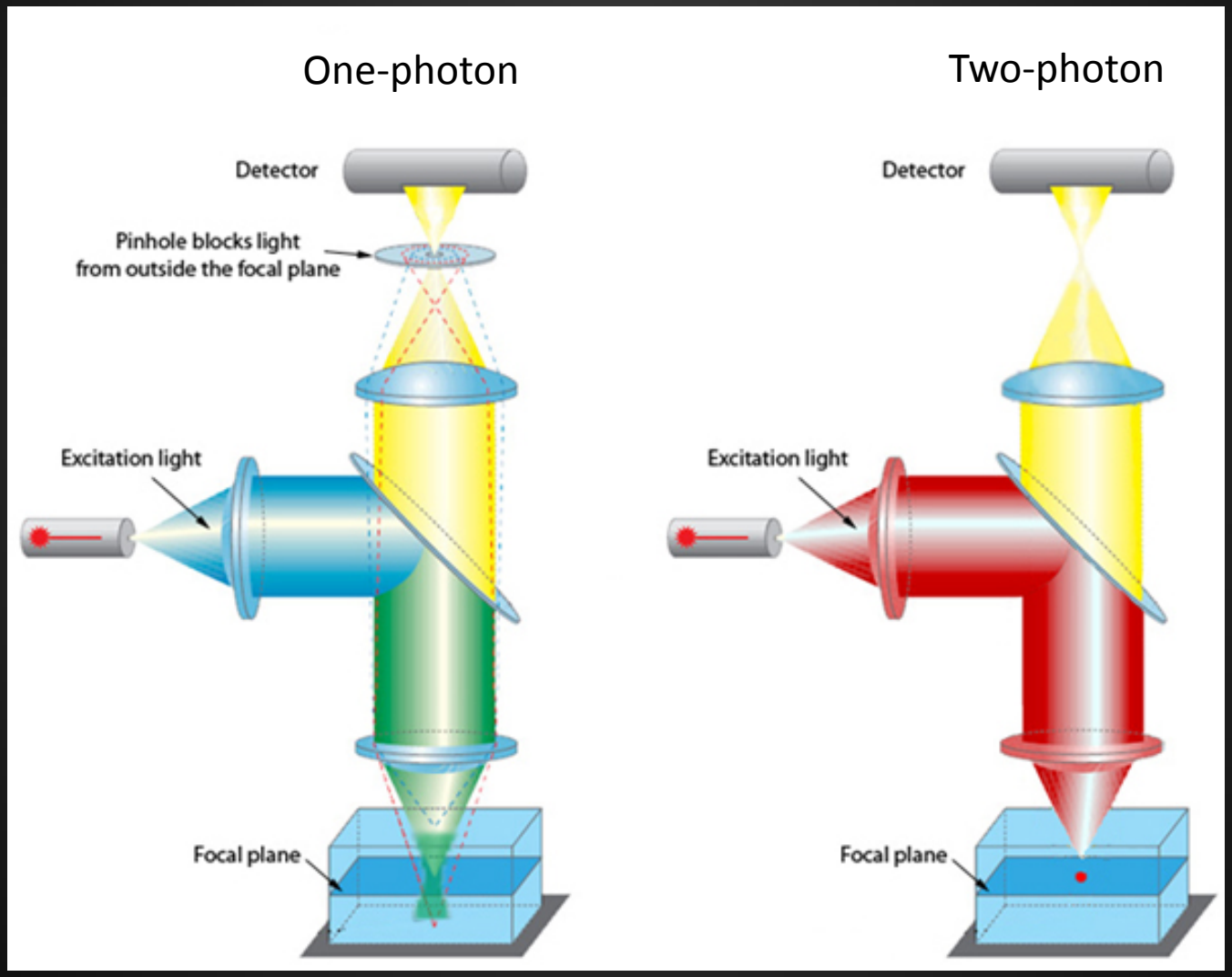
Information given here about 2 Photon microscopy were mainly taken from these sources:

Background information on 2-Photon microscopy:
http://micro.magnet.fsu.edu/primer/techniques/fluorescence/multiphoton/multiphotonintro.html

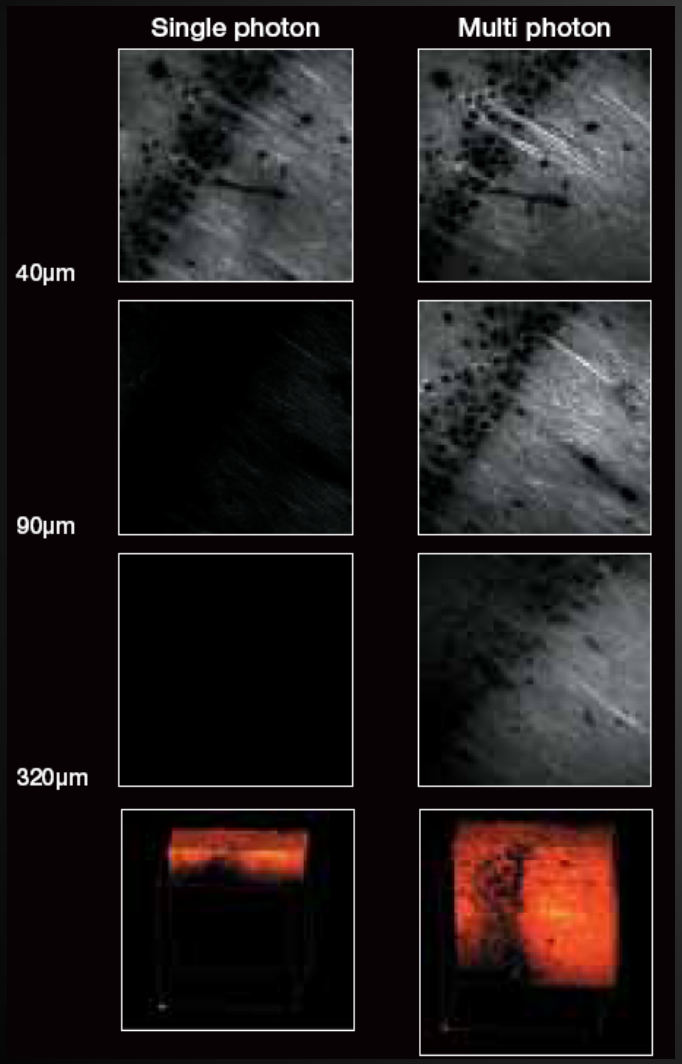
The microscopes:
Zeiss LSM 710 NLO; http://www.zeiss.com
Olympus Fluoview 1000 MPE, http://www.olympusamerica.com

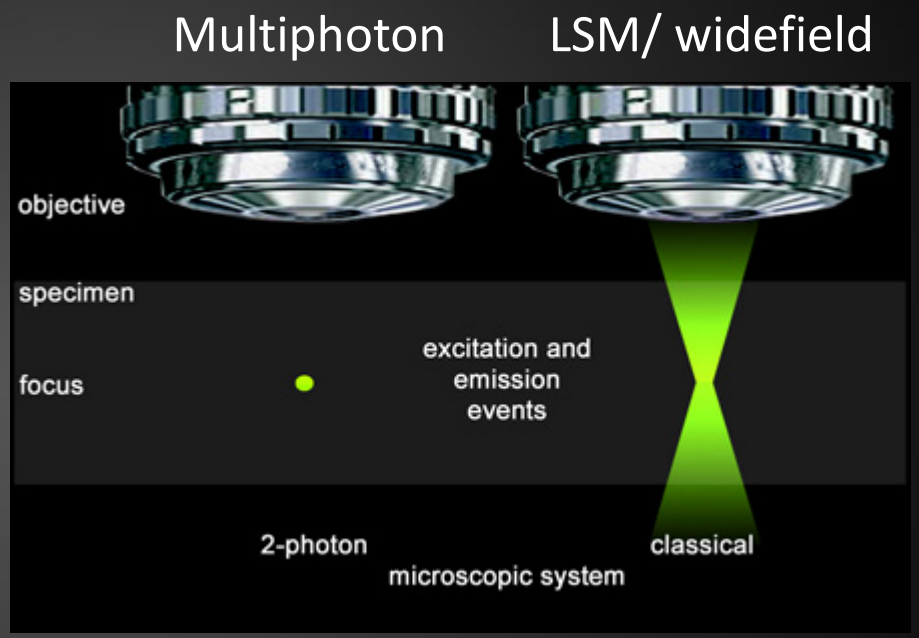
Spectra-Physics Laser:
http://www.newport.com/store/selectcountry.aspx?newpurl=/Lasers/361887/1033/catalog.aspx

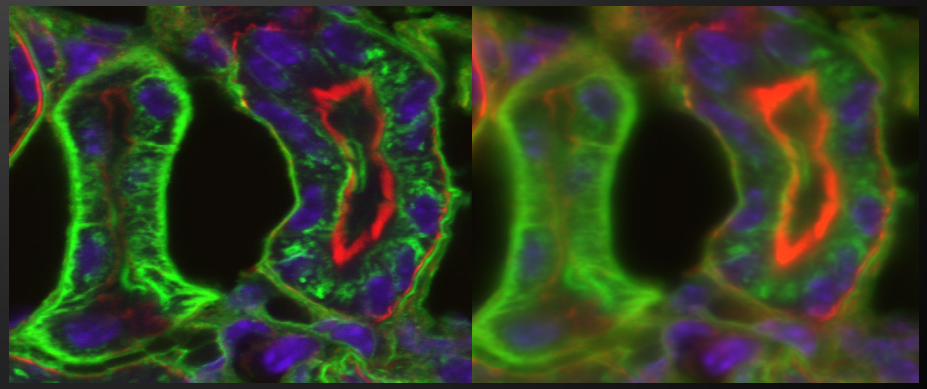
Schematic drawing of LSM



Why use 2-Photon microscopy ?







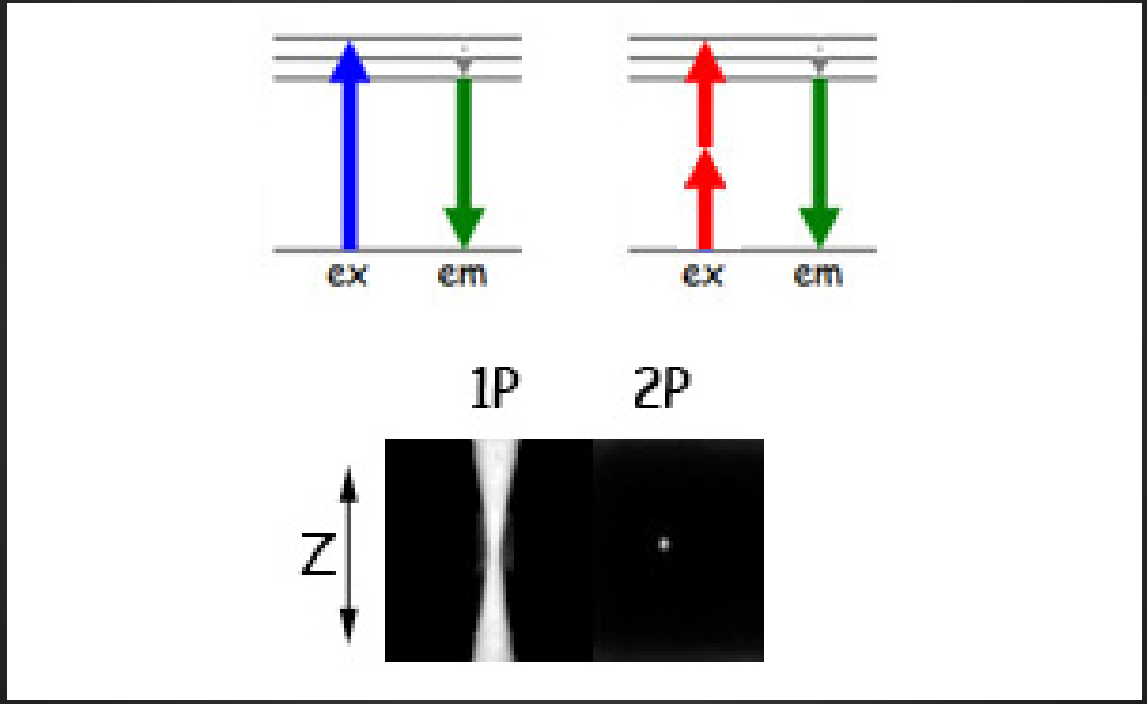
The message to keep in mind

A multiphoton microscope gives you the opportunity to get images from deep (e..g. 500 µm) within (living) tissue, whilst photodamaging only the imaged volume.

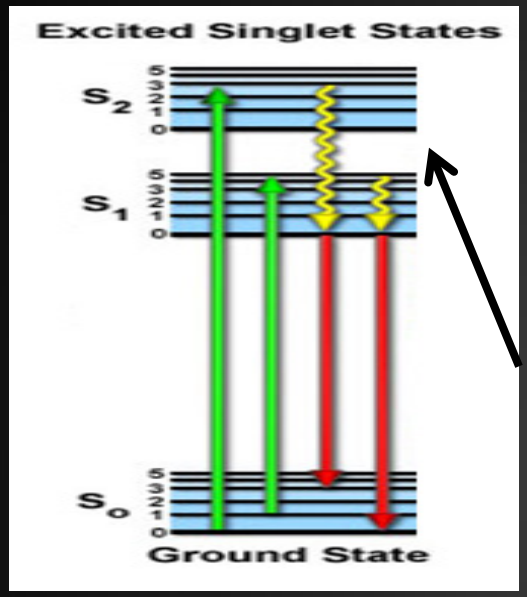
A Multiphoton microscope is a point scanning system which excites fluorophores within the Focus volume only. Therefore you collect emission light from this volume only, enabling you to acquire optical slices, without the use of confocal pinholes.

Beside this, one is able to photomanipulate tissue/cells within a very small volume.

THE THEORY OF 2PM



Theory for 2PM : The 1Photon Excitation



Stokes shift

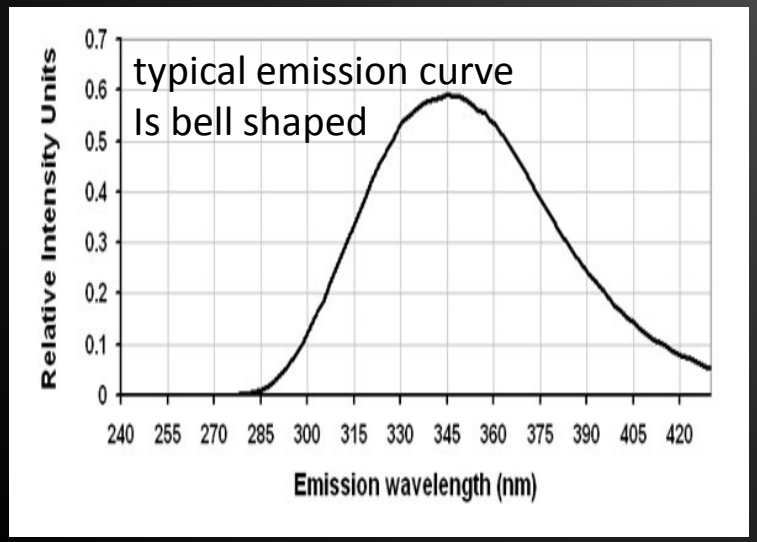
- Illuminate a fluorophore with appropriate λ of light
- 1 (excitation) photon absorbed gives 1 emission photon

BUT

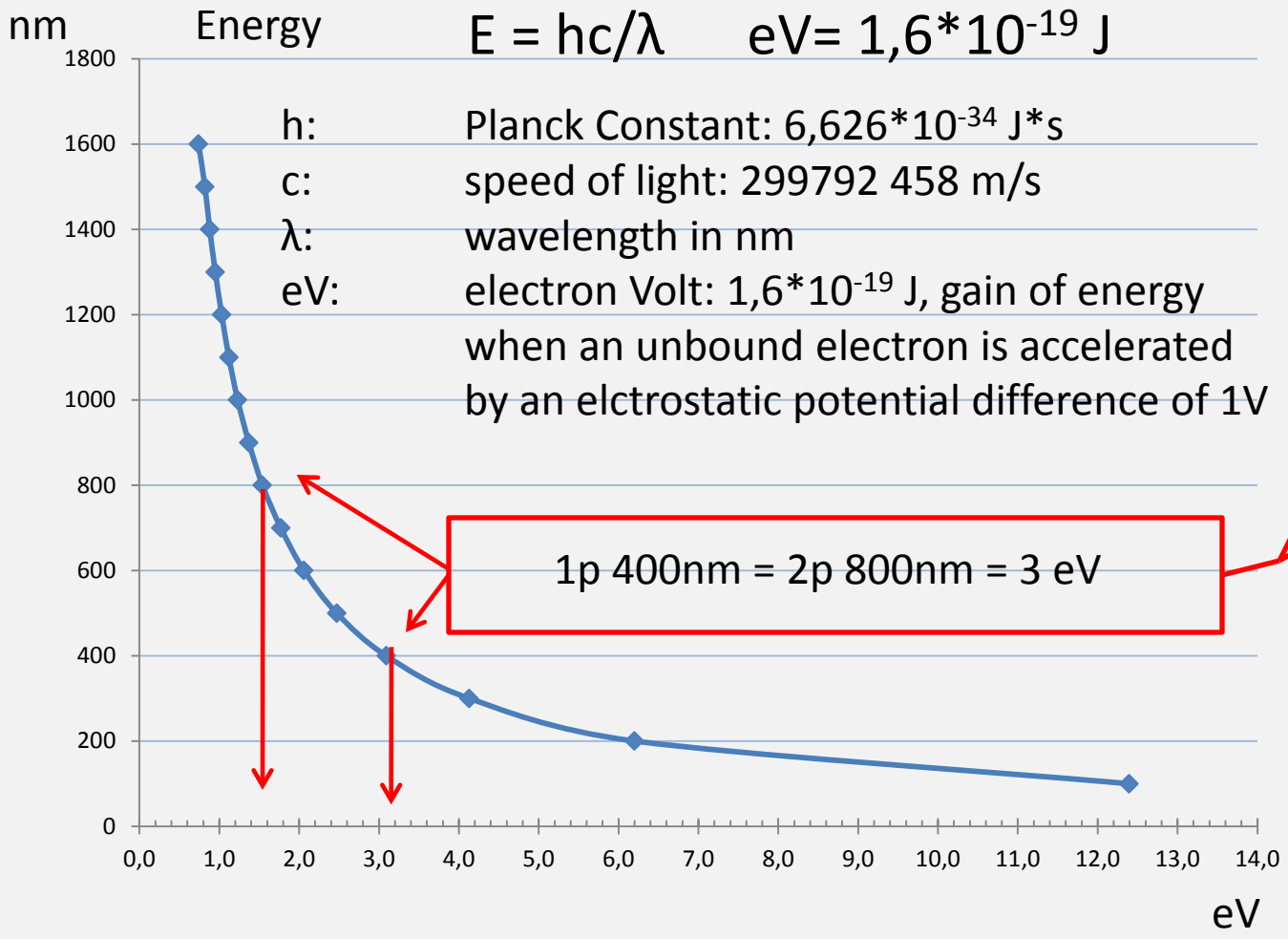
- emission photon will have less energy i.e. longer λ than excitation photon

AND

- it´s λ and energy vary due to which S_0 level (0,1,2,3) the fluorophore relaxes
- Fluorescence - photons with different λ emission curve is bell shaped



Theory for 2PM : λ ~E - The Energy of a Photon



$E = hc/\lambda$ $eV = 1{,}6*10^{-19}$ J

h: Planck Constant: $6{,}626*10^{-34}$ J*s

c: speed of light: 299792 458 m/s

λ: wavelength in nm

eV: electron Volt: $1{,}6*10^{-19}$ J, gain of energy when an unbound electron is accelerated by an elctrostatic potential difference of 1V

1p 400nm = 2p 800nm = 3 eV

eV	nm
12,39	100
6,20	200
4,13	300
3,09	400
2,47	500
2,06	600
1,77	700
1,54	800
1,37	900
1,23	1000
1,12	1100
1,03	1200
0,95	1300
0,88	1400
0,82	1500
0,74	1600

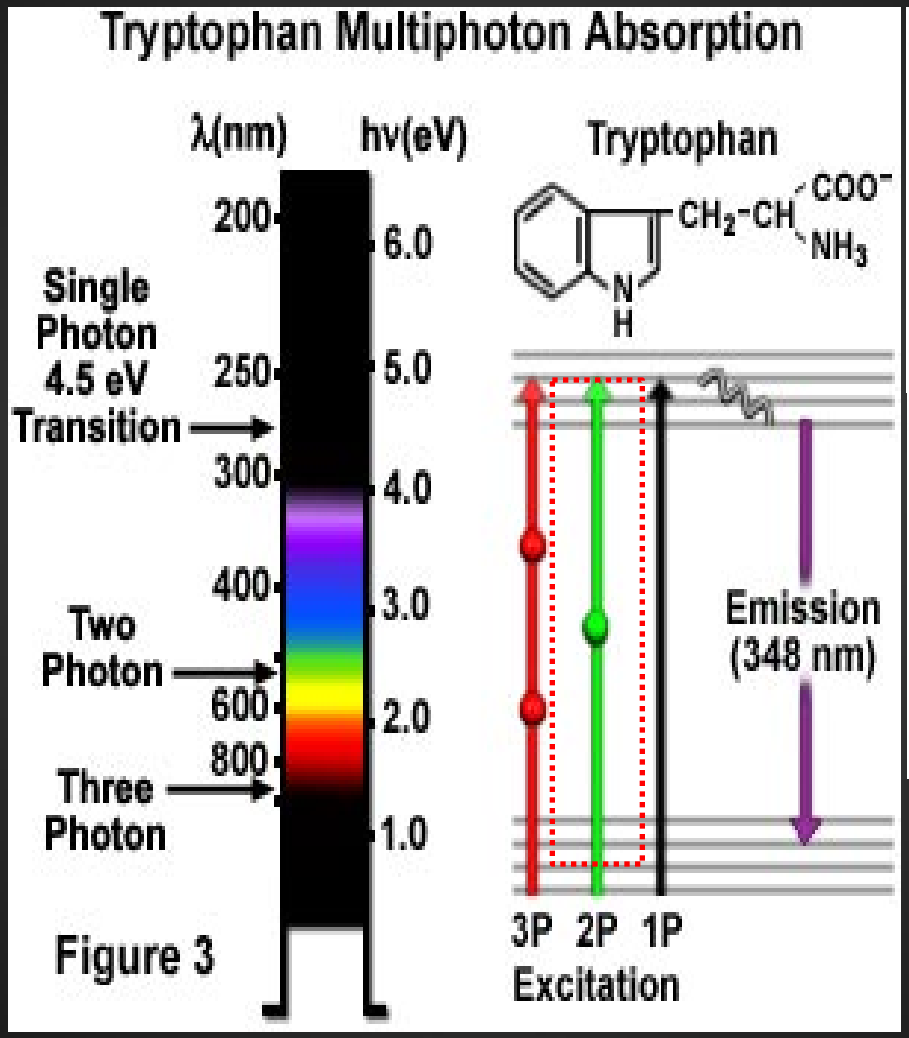
Theory for 2PM : How to excite (Tryptophan)

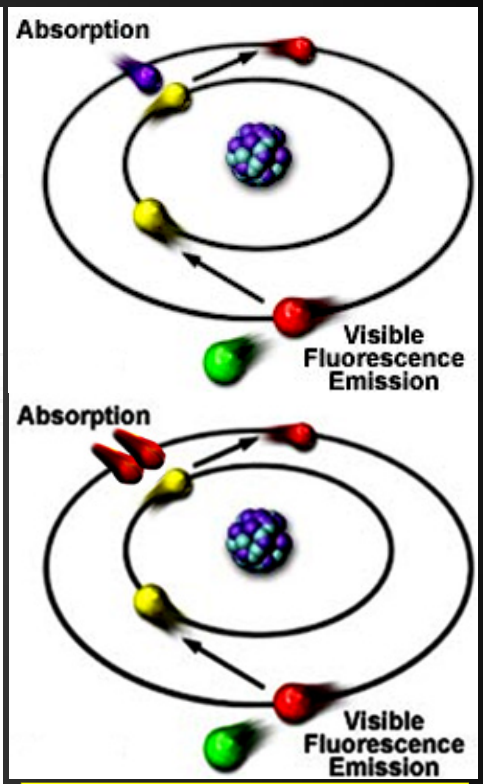
Single-photon
1 photon, 280 nm
4.5 eV
No laser for this...

$$\frac{A}{B} = \frac{8\pi h \nu^3}{c^3}$$

Two-photon
2 photon, 580 nm
2.13 eV x2
4.26 eV

Three-photon
3 photon, 840 nm
1.47 eV x3
4.41 eV



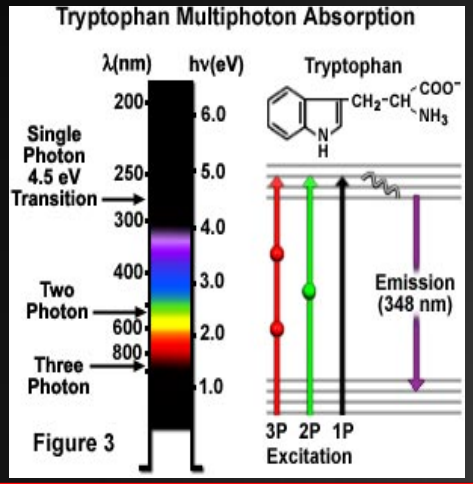


virtual state
VERY short
0.01 fsec
(10^{-17} sec)

2-PM hypothesis introduced by
Maria Göppert-Mayer, doctoral thesis 1931

Notes

Theory for 2PM : λ ~E - The Energy of a Photon



Observe: range of overlap of potential Excitation
760nm : excite A488 & A633 *

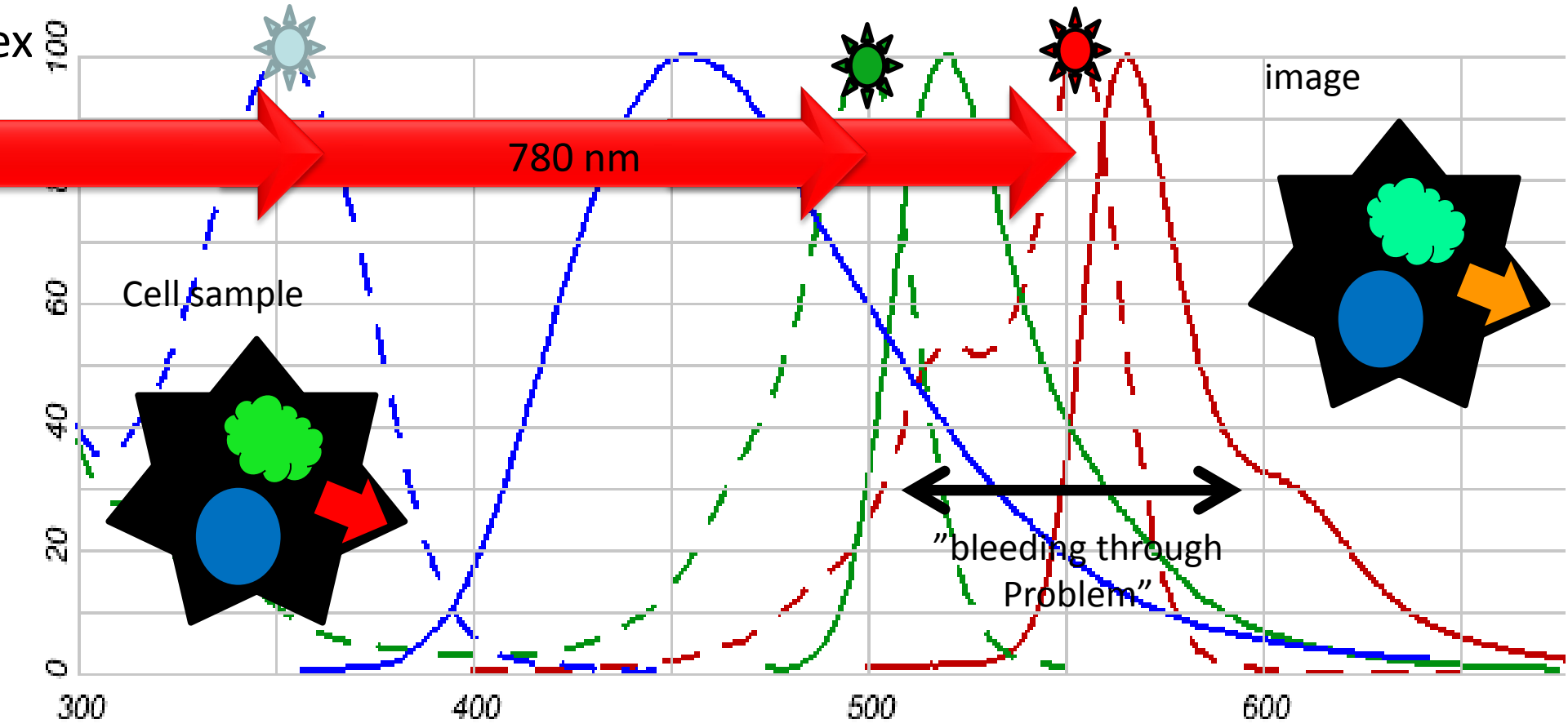
For multicolor 2PM choose fluorophores so that they do overlap in excitation
BUT NOT emission

* has to be checked on microscope

Fluorochrome	Absorption	Emission
Alexa Fluor 350	720-800	440
Alexa Fluor 488	720-800	515
Alexa Fluor 546	720-840	569
Alexa Fluor 568	720-840	596
Alexa Fluor 594	720-850	610
Alexa Fluor 633	720-900	647
AMCA	780-800	444
bis-MSB	680-750	420
Bodipy	900-950	512
Calcium Crimson	900	615
Calcium green	780-850	531
Cascade Blue	750-800	420
Coumarin 307	780-800	530
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
Dansyl Hydrazine	700-750	440

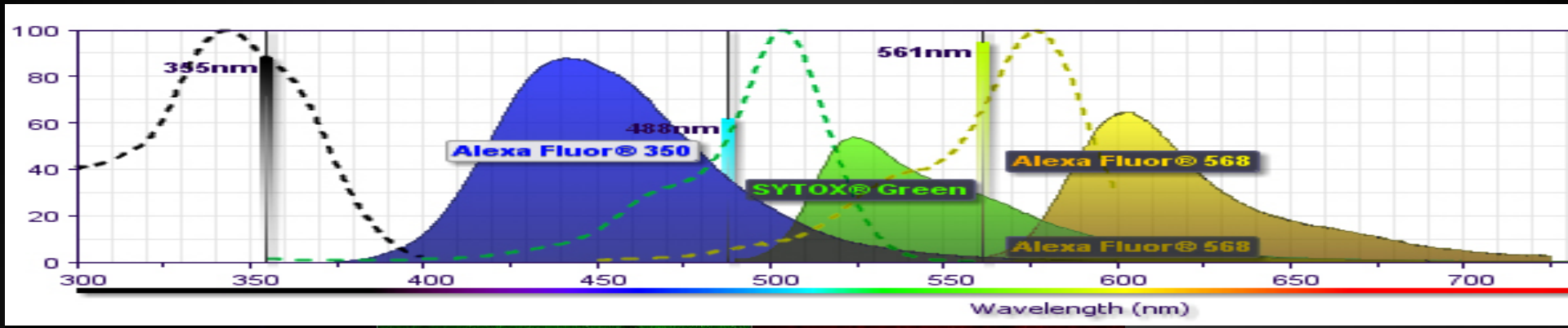
Near Infra Red

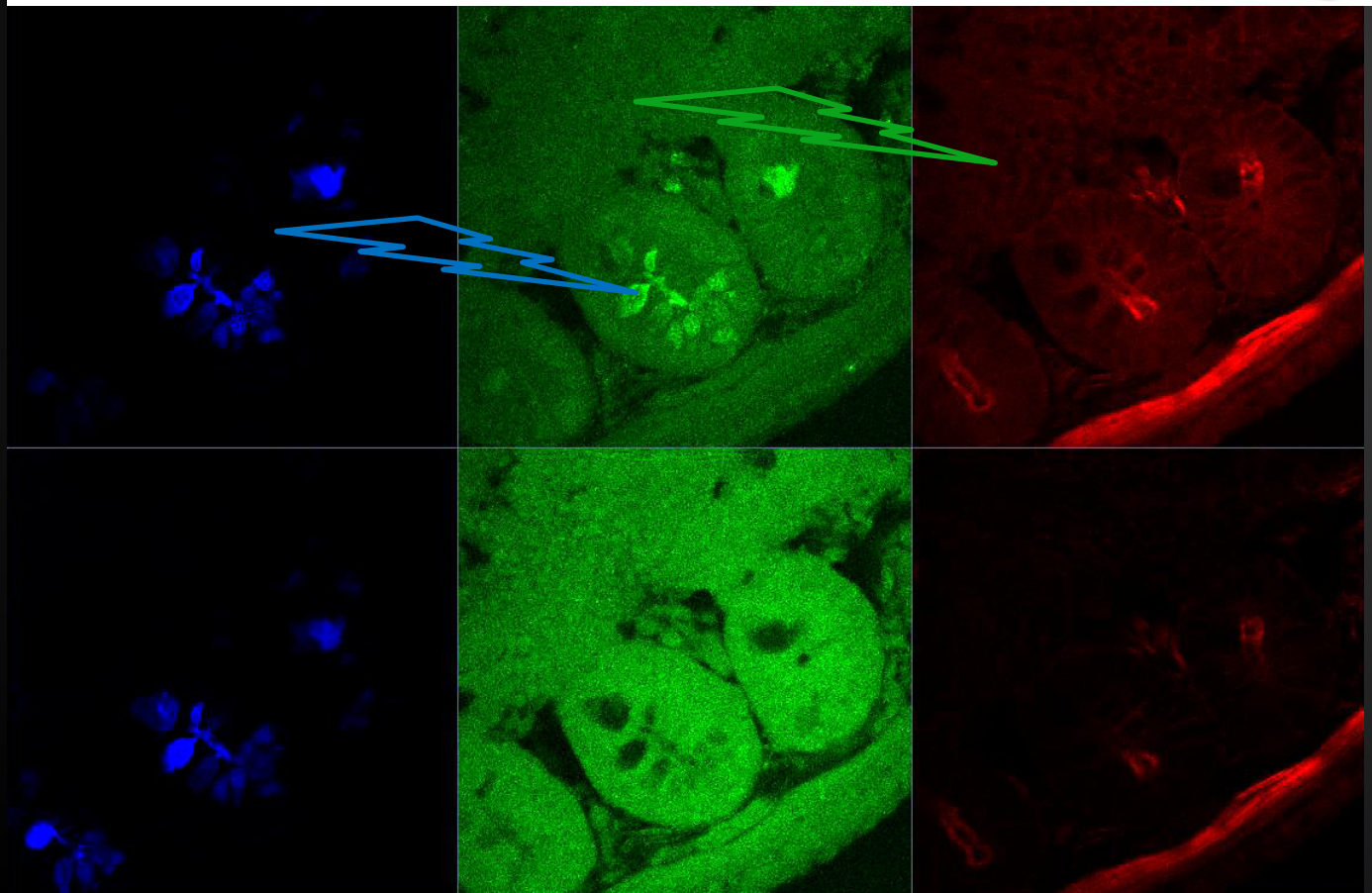
Dealing with fluorescence in 2P



The 780nm NIR Laser might/will excite all three fluorophores, the Instrument has to unmix the mixture of Blue/Green/Red, or we have to use better fluorophore combination

Reminder – simultaneous vs sequential scanning



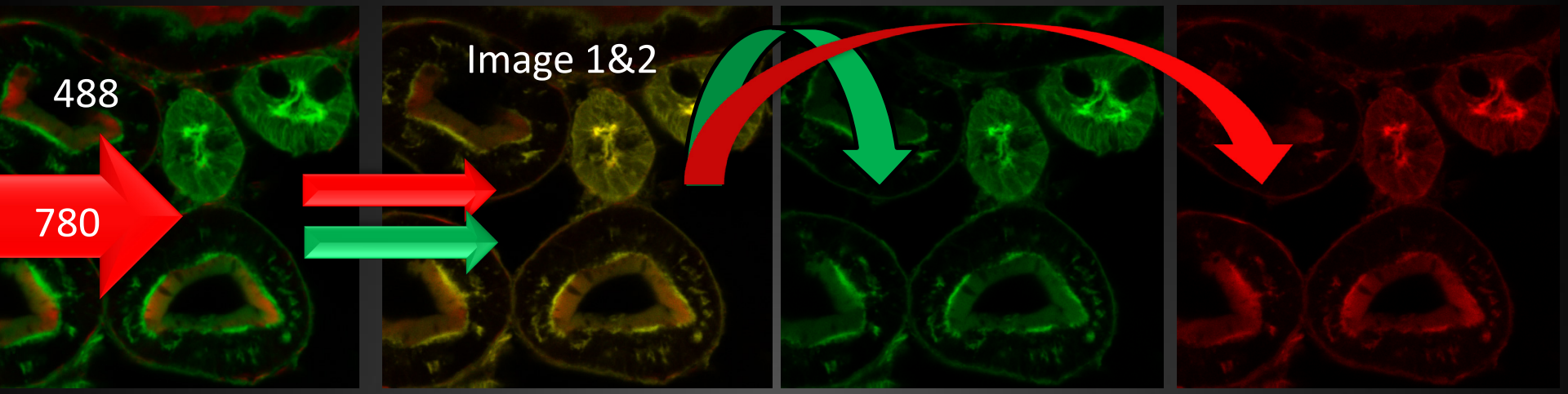


Simultaneous Excitation resulted in artifact due to bleeding through on ”green” image,where the ”blue” appears; and on the red image where the ”green appears”

Sequential scanning does not show such artifacts, therefore in THIS sample the excitation are far apart.

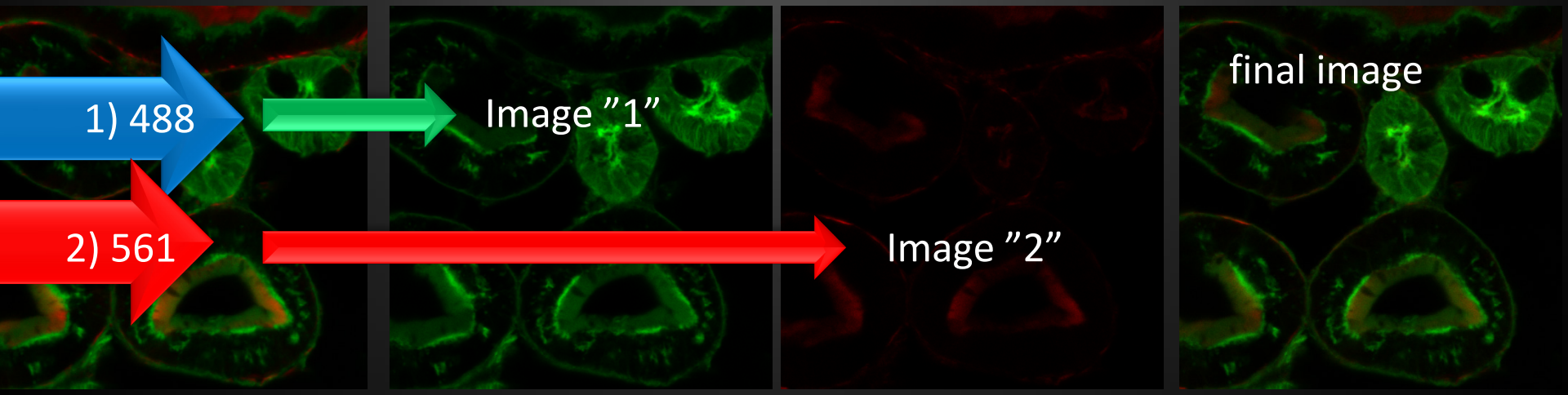
Notes

Multicolor imaging in 2P



Simultaneous scan excites several fluorophore at once, emission is guided by filter and beamsplitter to PMTs. If FL-green bleed over into PMT of FL-red it will be seen here (in red). Sequential scan excites and collects one fluorophore at a time.

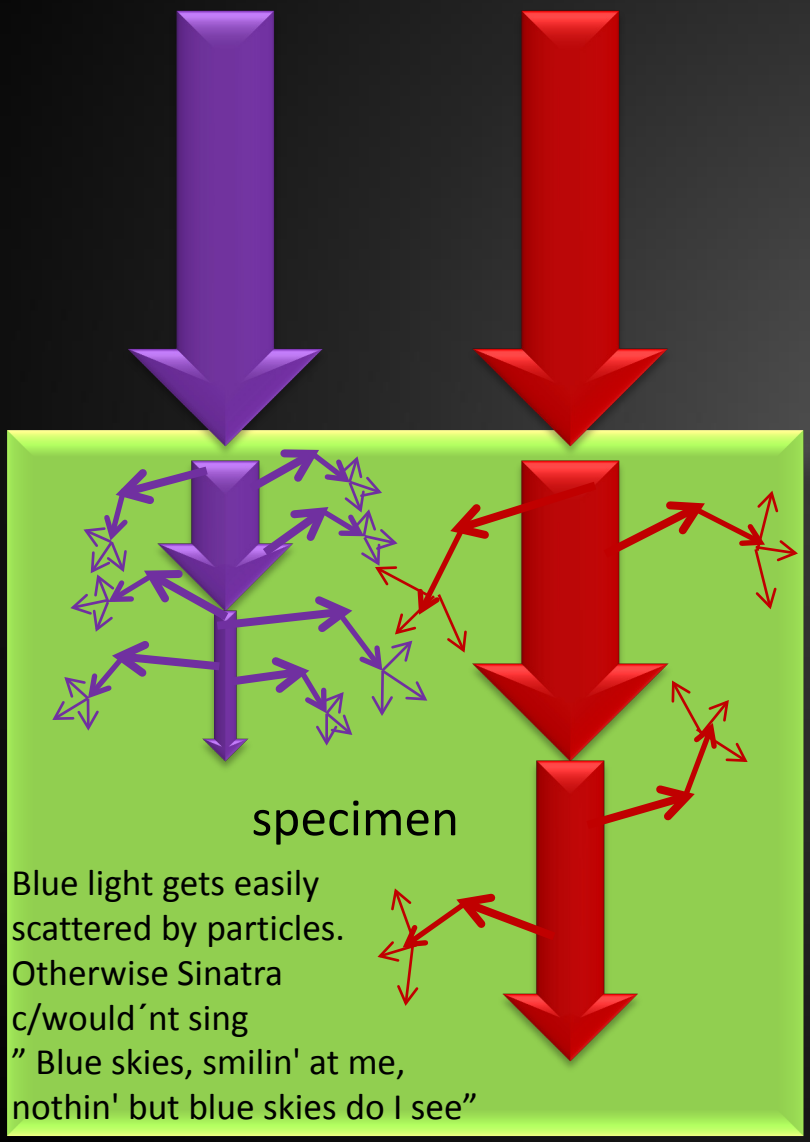
! Be sure that 488 does result in emission of FL red in the ”green range”... Test that...



WHY USE 2P?
- to see deeper

Nikon instruments

See deeper – scattering problem



NIR light : 700-1100nm travelling through Specimen to focal plane will not scatter and disperse* as much as light of shorter λ (350-633 nm for FL microscopy)

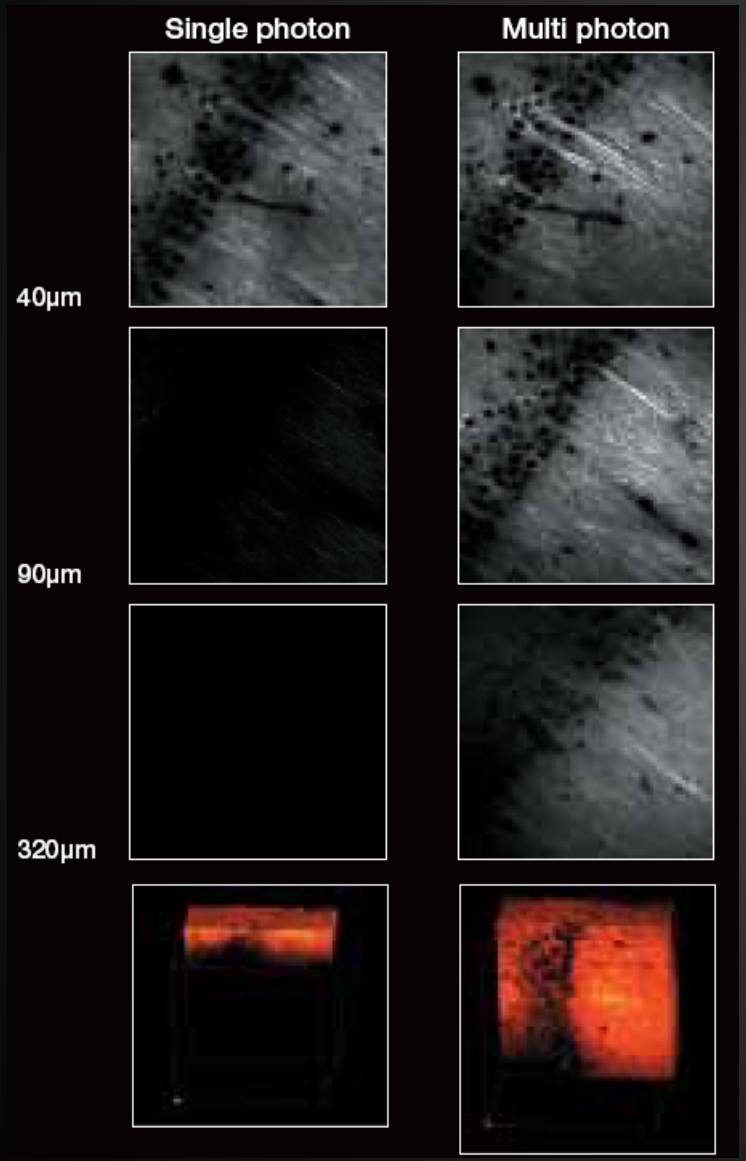
- We can excite deeper fluorophores

Problem: different fluorophores need its own NIR Laser?

Solution: Laser can be tuned from e.g. 690 to1040 nm, fluorophores have wide excitation range in 2PM

*(due to different refractive indices of the various components in specimen)
See also :
Optical Clearing

See deeper



XYZ images of mouse brain sections expressing GFP, comparing single-photon 488 nm excitation and two-photon 910 nm excitation.

With single photon excitation, tissue can be observed only to a depth of about 90 µm, but with two photons, observation to a depth of about 320 µm is possible (FOR THIS SAMPLE!).

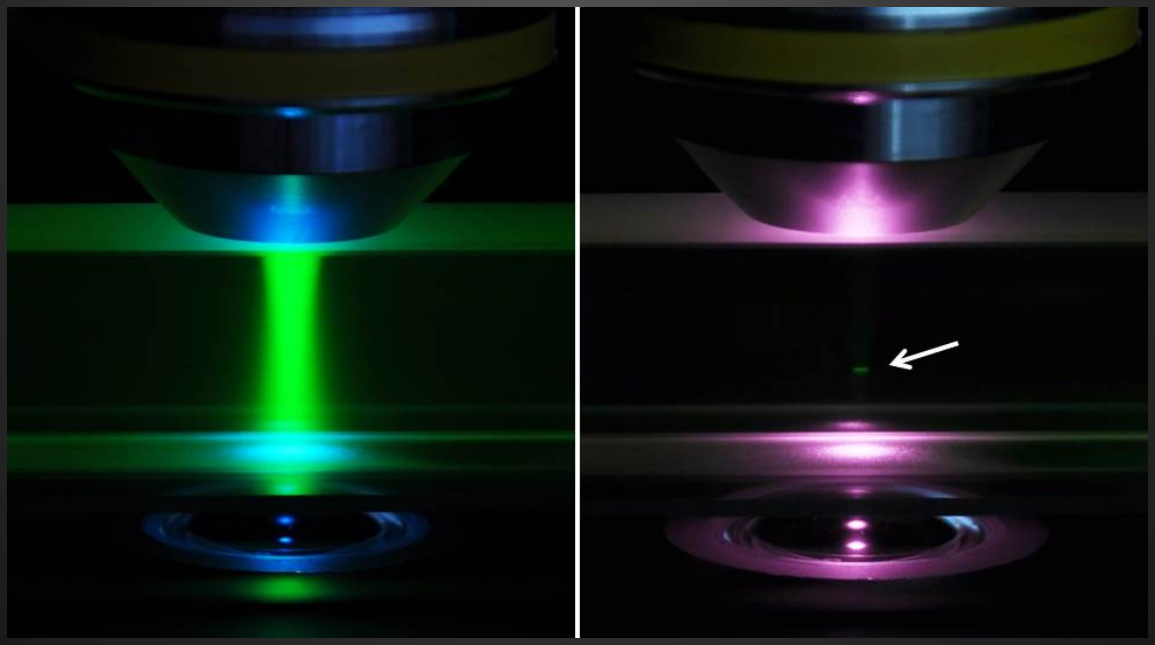
Items displayed in color are vertical cross sections of 3-dimensionally constructed images.

Specimens provided by:
Kimihiko Kameyama, Tomoyo Ochiishi, Kazuyuki Kiyosue, Tatsuhiko Ebihara
Molecular Neurobiology Group, Neuroscience Research Institute,
National Institute of Advanced Industrial Science and Technology, Japan

Brochure, OLYMPUS, FV1000MPE

WHY USE 2P?

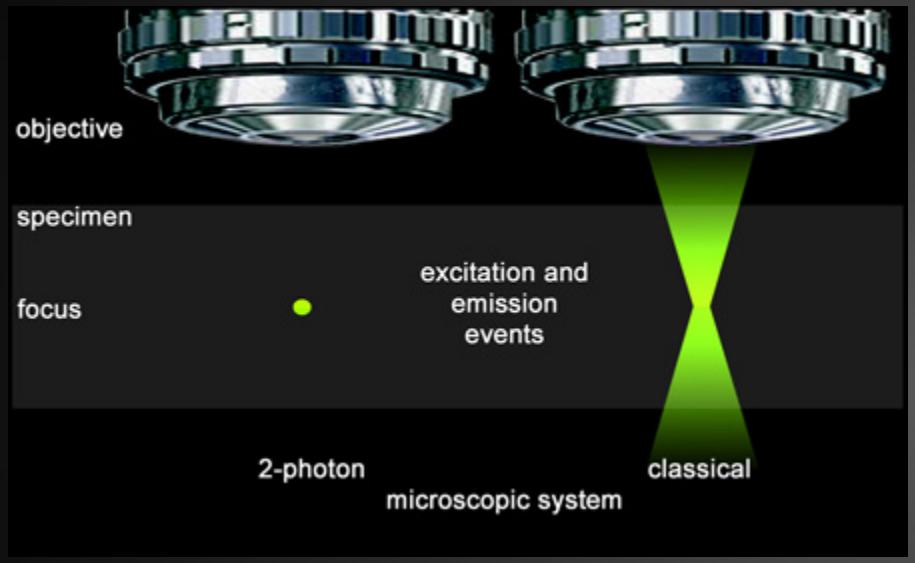
- *small excitation volume, no pinhole*



Matyas Molnar

Small focus spot

Multiphoton LSM



$Ex \sim (P_{avg}/A)^2 = I^2$ $Ex \sim P_{avg}$

That´s why Multiphoton is also named Nonlinear.
Chance for 2PM event drops drastically with distance to focus

Two-Photon event occurs only in focus volume

- All emission light is directly from focus

Resolution is similar (or worse) to LSM

- 0.3x1µm ellipsoid (high NA objective)

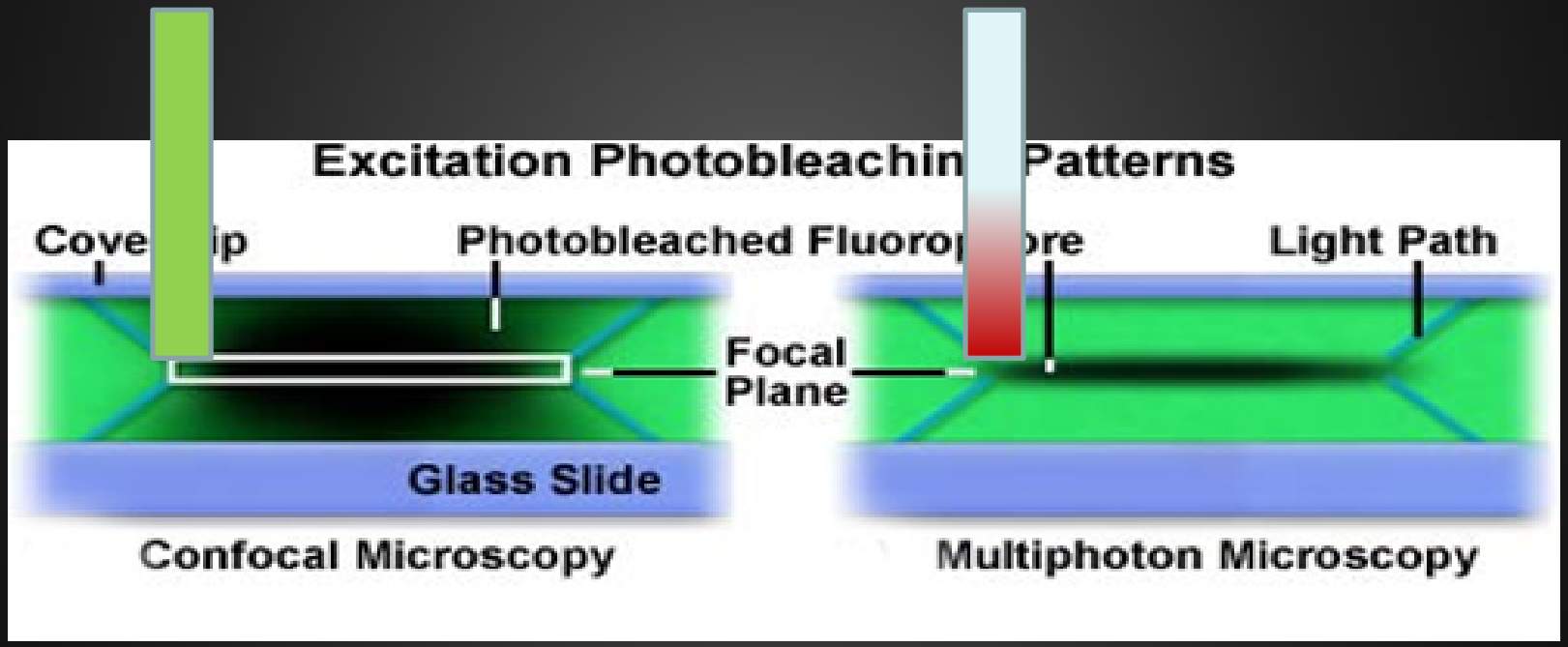
Penetration depth depending on specimen and optical parameter but might be up to nearly 1mm

These features will be important for various live cell imaging techniques, like bleaching, photodamaging, uncaging ...

Small focus spot

Laser of LSM scans through specimen

Laser of 2PM scans through specimen



excitation/emission and photodamage/heat

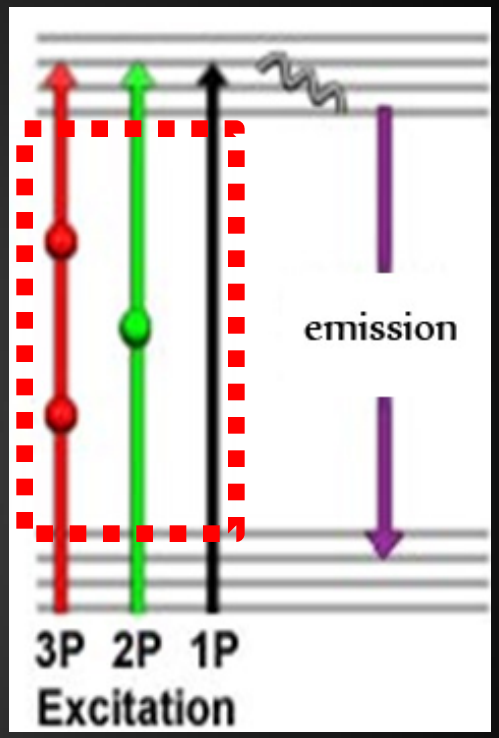
occurs within specimen
also outside the focal plane

occurs within specimen
only in the focal plane

Two-photon excitation's probability

What is the chance that 2 photons hit the same fluorophore at almost the same time?

- a matter of time and area
- **The probability of observing a two-photon absorption event on a bright sunny day is 1 per 10,000,000 years, whereas the one-photon absorption takes place every second**



Time → the virtual state

→ Δt of intermediate virtual state = 10 attosec (10^{-17} s)

→1 attosecond (10^{-18} s) is the time window

→ light travels 3 hydrogenatoms within 1 attosec

Area → the fluorophore

→quite small target

Problem: Light can not travel faster than speed of light

Solution: More photons are needed (high density of photons)

We need a million times more photons than in single photon fluorescence and "good" objectives.

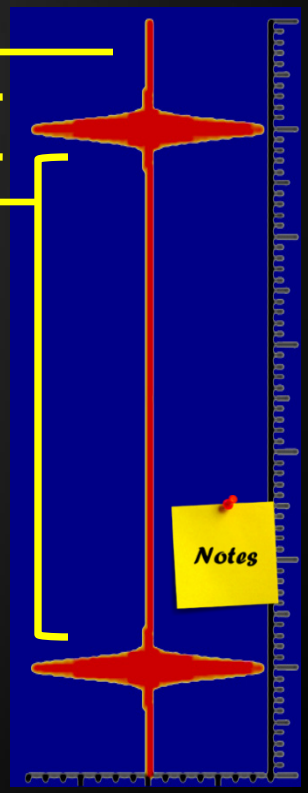
More photons please

Problem:
1 million times more photons? Very strong laser...
There is no continuous wave laser to achieve this.

Solution: A moderate Laser with high photon intensity pulses

- low average power (0.3 - 2.5 W)
- high peak power (30-300 kW) pulses 50-100 fs wide
- pulse frequency 80 Mhz (1pulse/ 12,5ns)

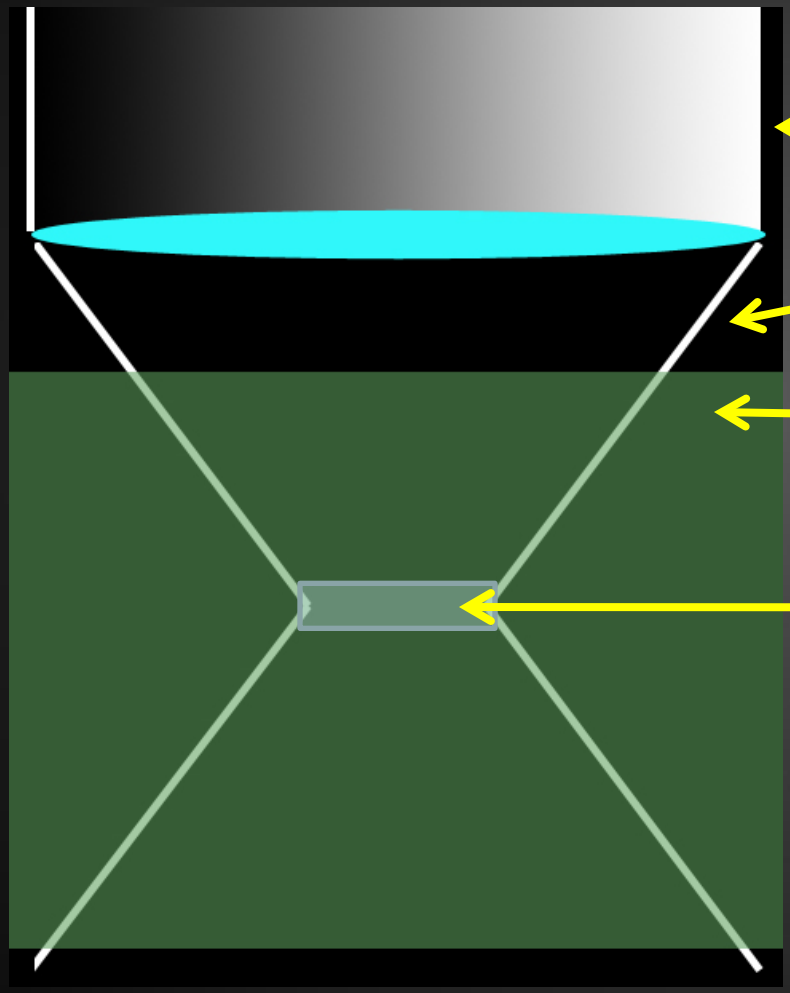
This laser is dangerous when used (Class 4)!



Problem: Many fluorophores but one Laser
Solution: To excite a wide range of fluorophores the laser is tuneable for e.g. 700-1040 nm

Pulsed NIR Laser is tuneable for excitation wavelength twice the 1Photon-excitation wavelength

Principle of 2P excitation



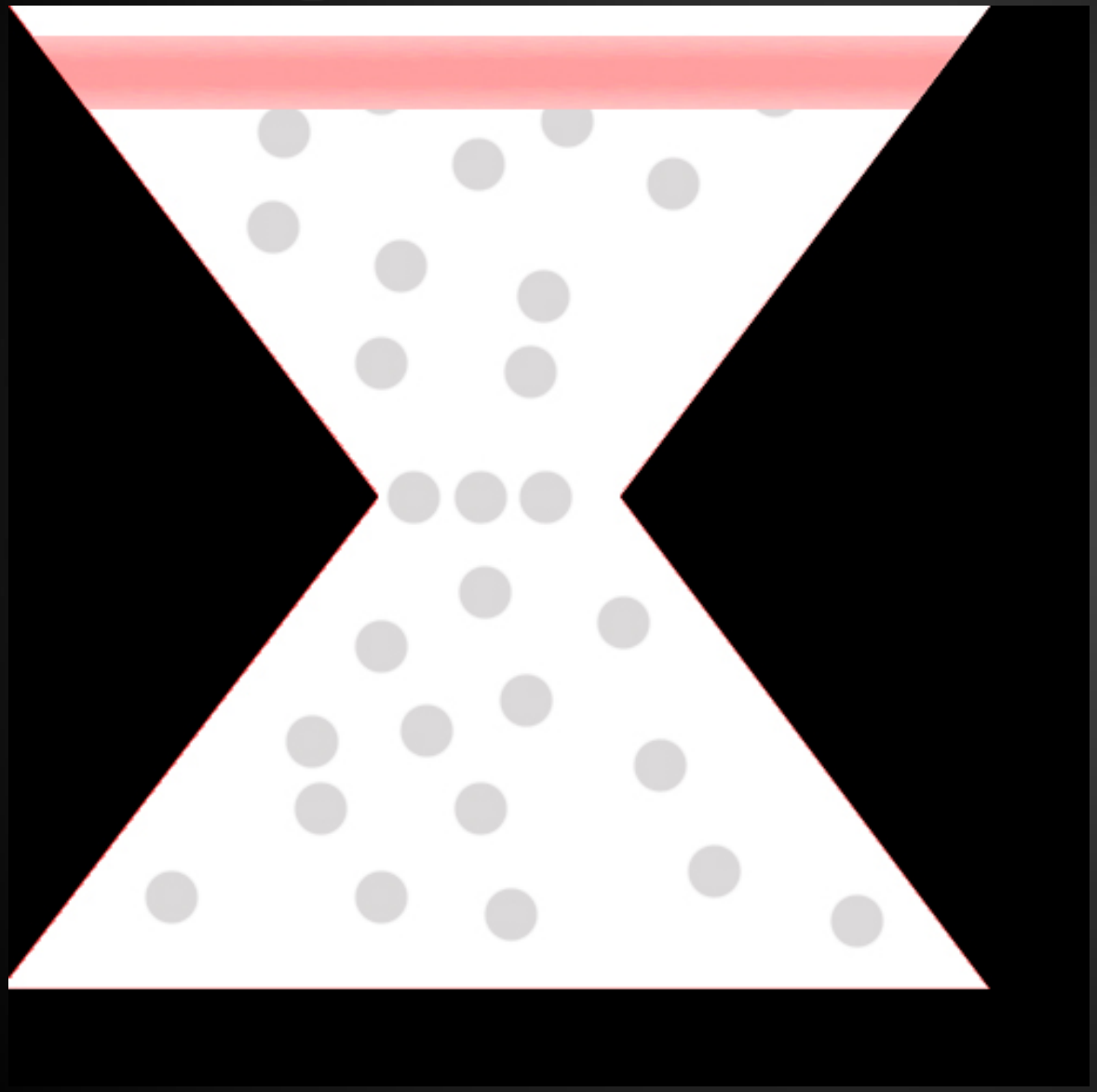
objective

aperture of objective

specimen

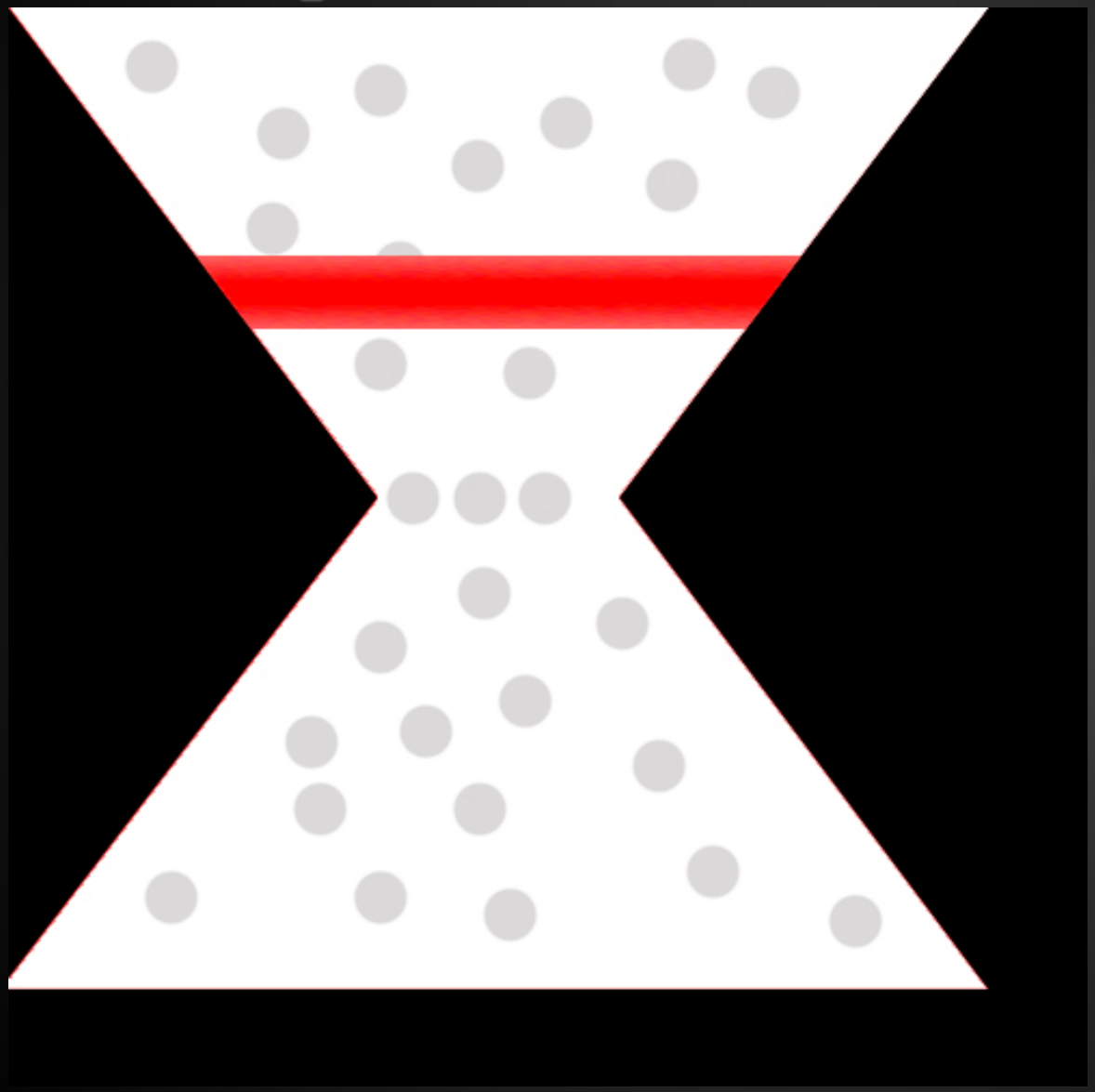
focal plane of objective
(depth of focus),
light is focused here

Principle of 2P excitation



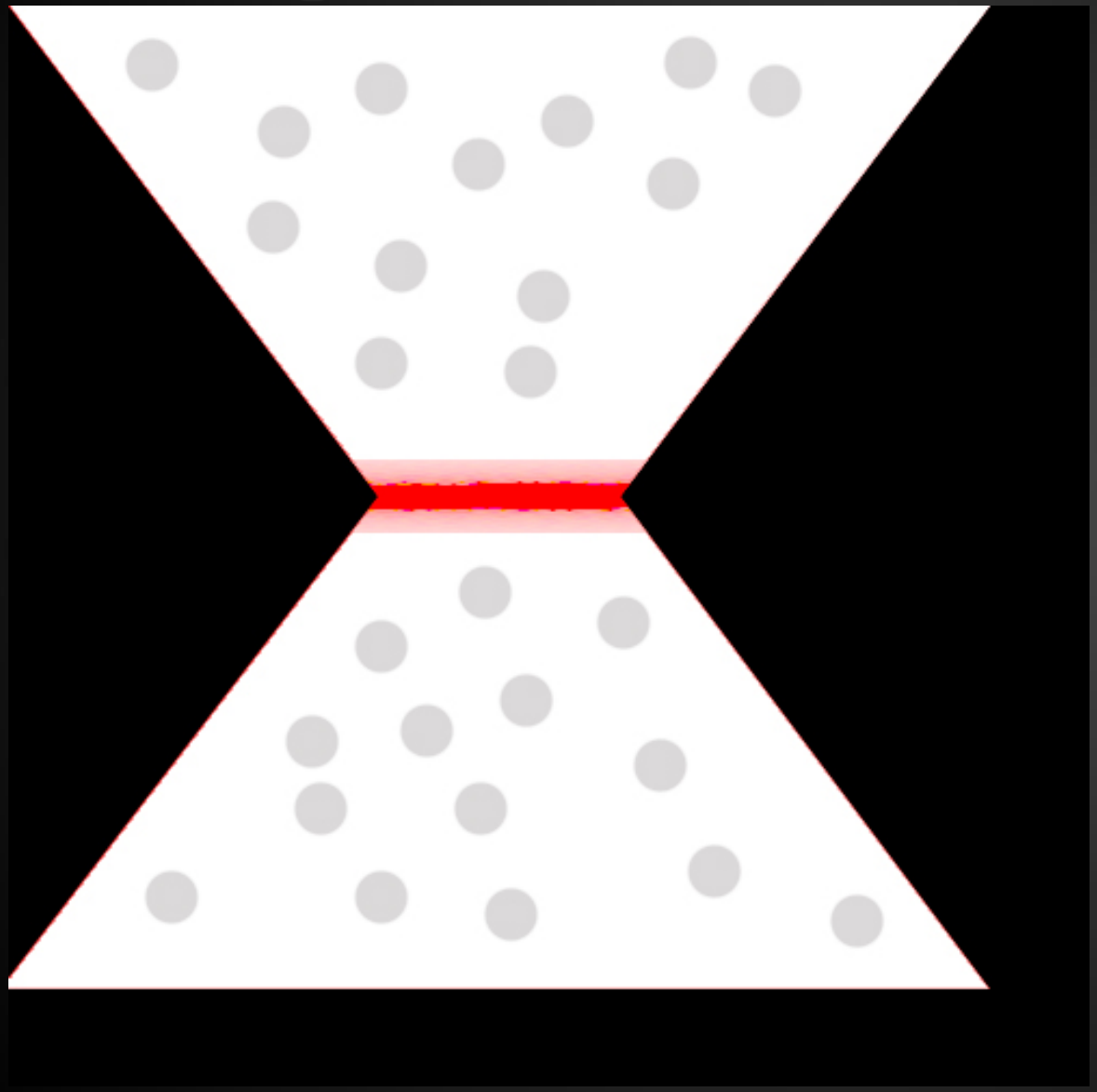
Laser pulse is far from focal plane, photon density is low, no chance for two photons to hit a fluorophore in one time

Principle of 2P excitation



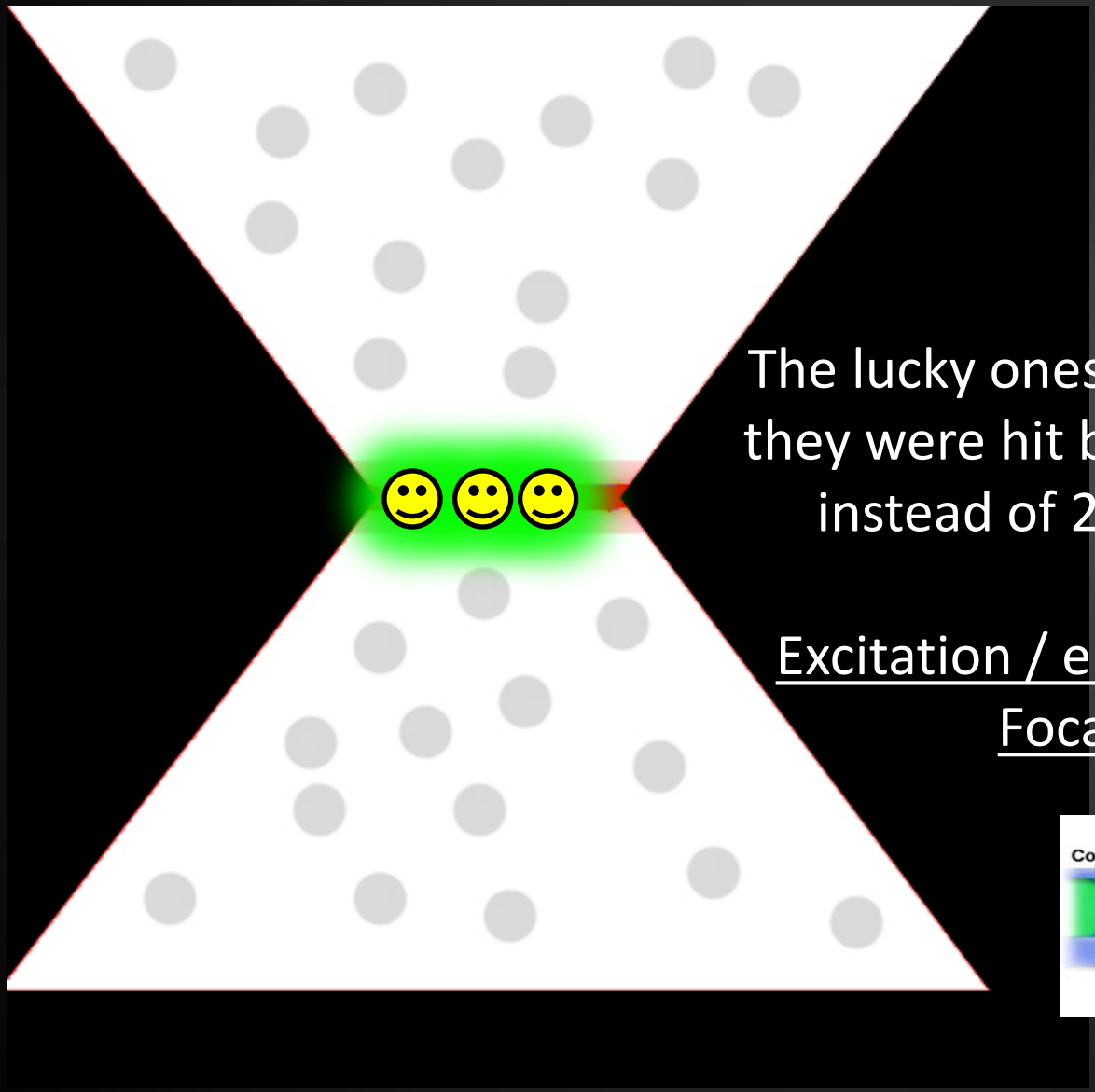
Laser pulse is closer to focal plane, photon density is more concentrated but still low, no chance for two photons to hit a fluorophore in one time

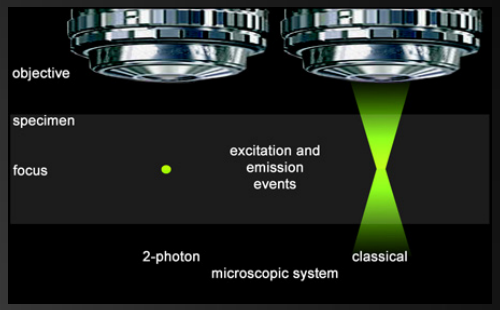
Principle of 2P excitation



Laser pulse reached the focal plane, photon density is high, high probability for 2 photons to hit one fluorophore within 10 attosec

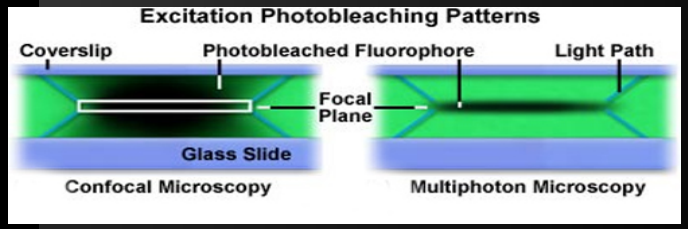
Principle of 2P excitation



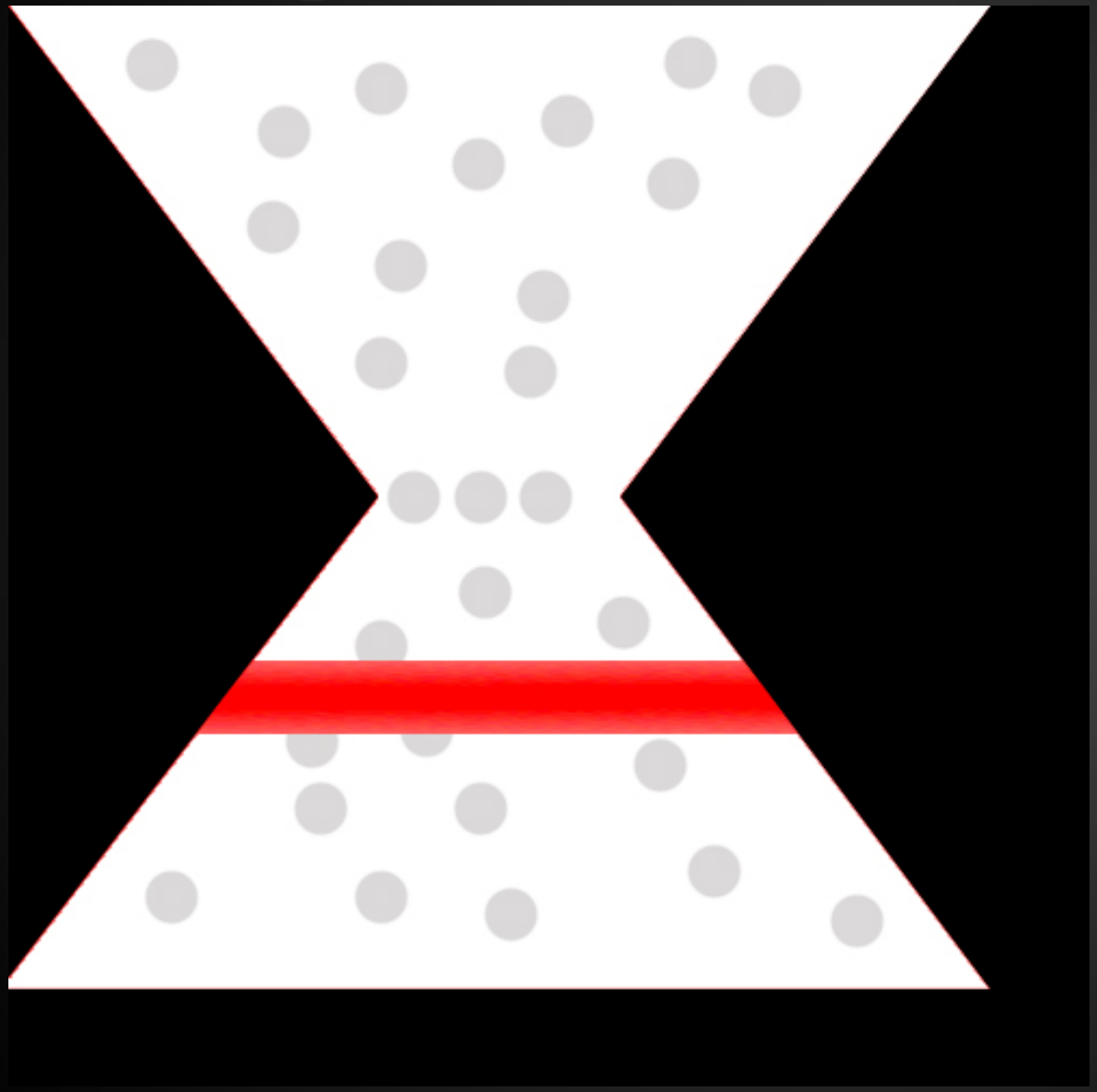


The lucky ones emit fluorescence like they were hit by 1 high energy photon instead of 2 low energy photons

Excitation / emission occurs only in Focal plane /spot

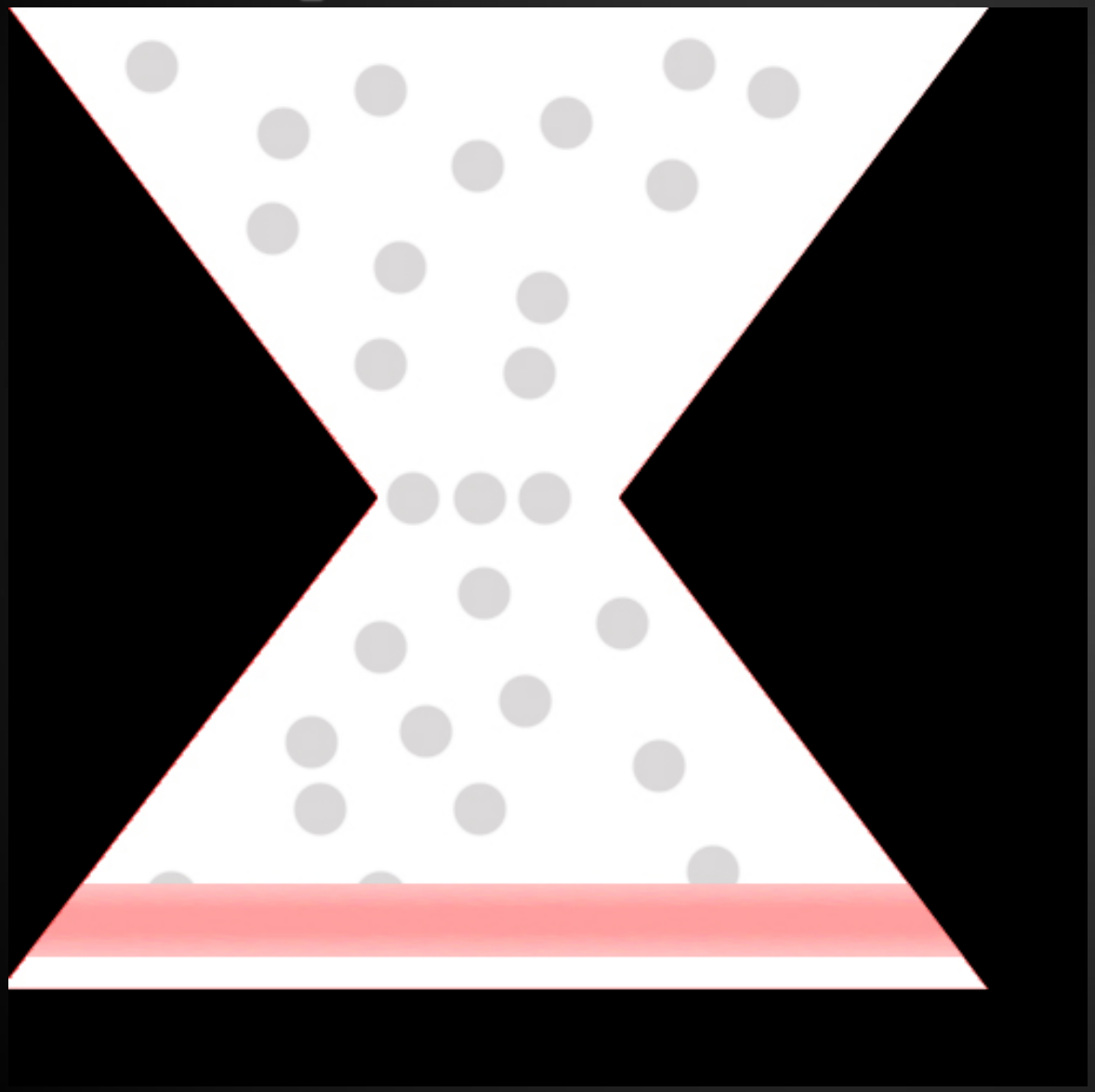


Principle of 2P excitation



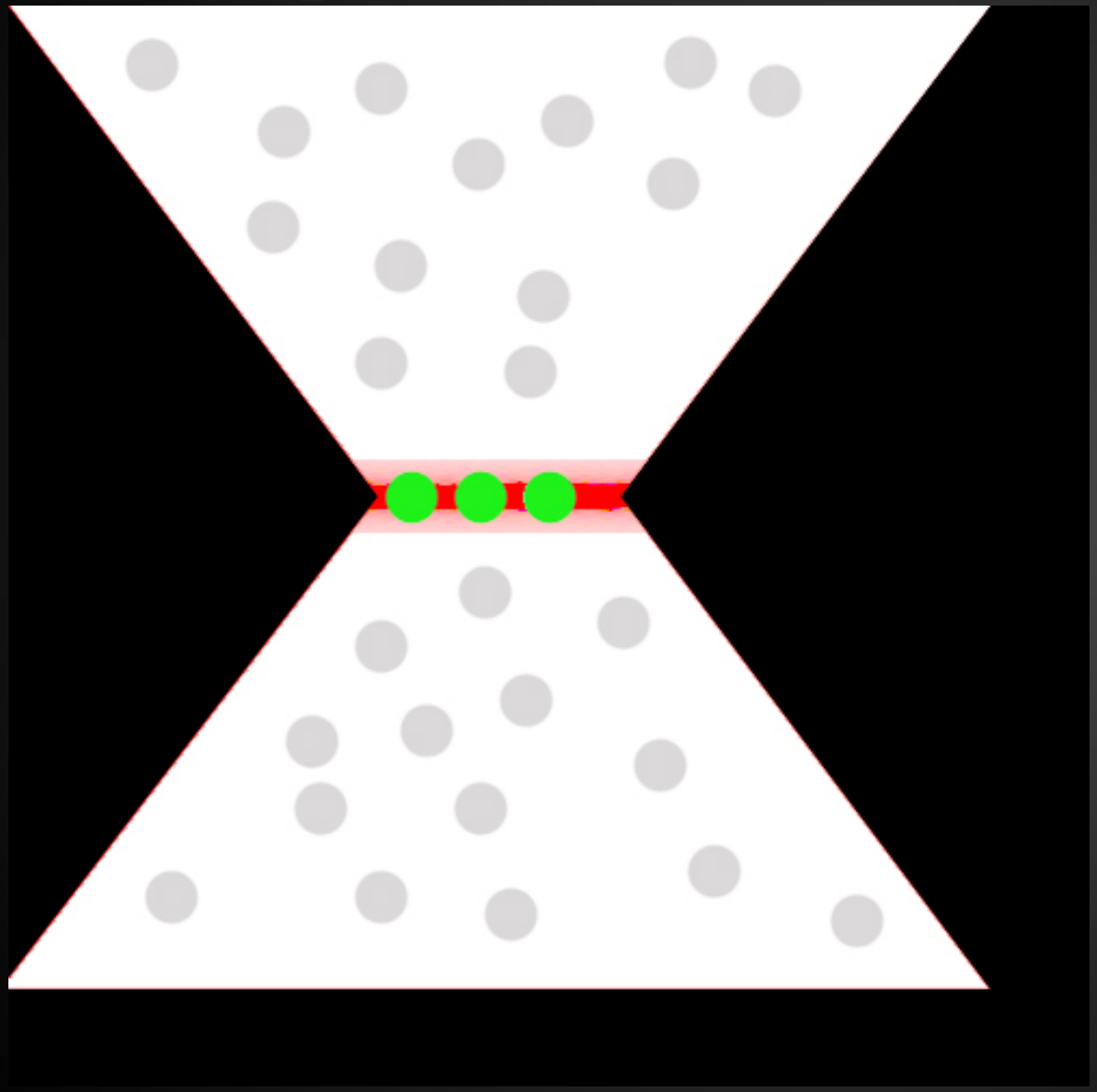
Laser pulse leaves focal plane,
NO incident of two photons hitting one fluorophore

Principle of 2P excitation



Laser pulse disperses in tissue, NO incident of two photons hitting one fluorophore

Principle of 2P excitation



REMEMBER

The probability for two-photon excitation is extremely low.

Excitation / emission occurs only in focal plane /spot, where the photon density is very high. This is a confocal system without a pinhole.

Repeat again

Recapitulate:

- NIR Laser to reach deep
- Excitation of ”normal” fluorophores via 2P effect
- NIR is tuneable over range e.g. 690 nm – 1040 nm
- 2P is only happening in focal volume
-Ex/Em/photodamage only at focal volume and bleaching is limited due to the low energy of NIR

<u>Applications:</u>

Living animals

Manipulation of ”precise” small volumes

Non-linear effects

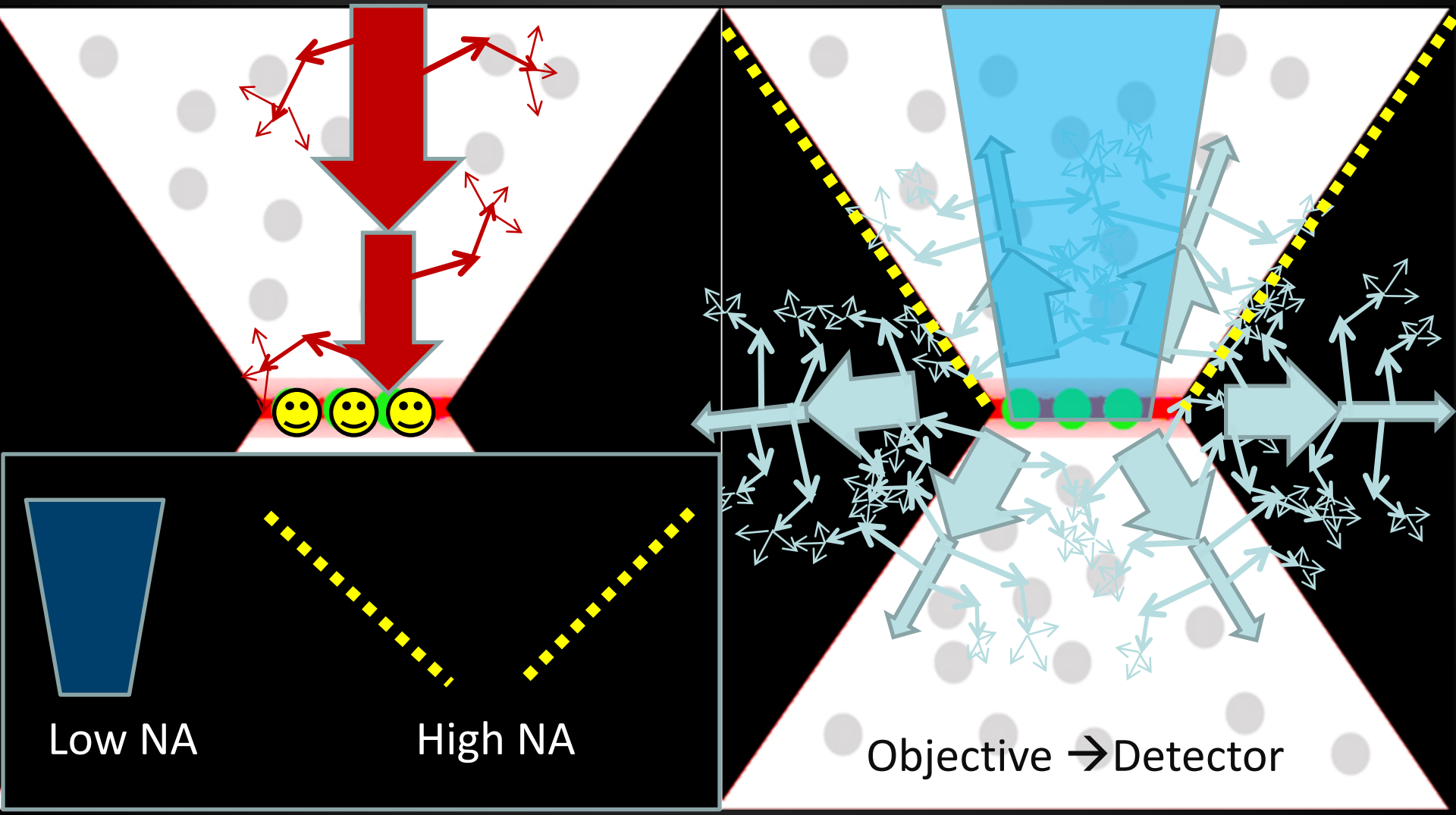
Multiphoton microscopy Objectives and Detectors

Light must come in to depth
Light must get collected from the depth

Bring back home the photons

Laser → Objective → Excitation

Emission → Objective → Detector



Multiphoton objectives

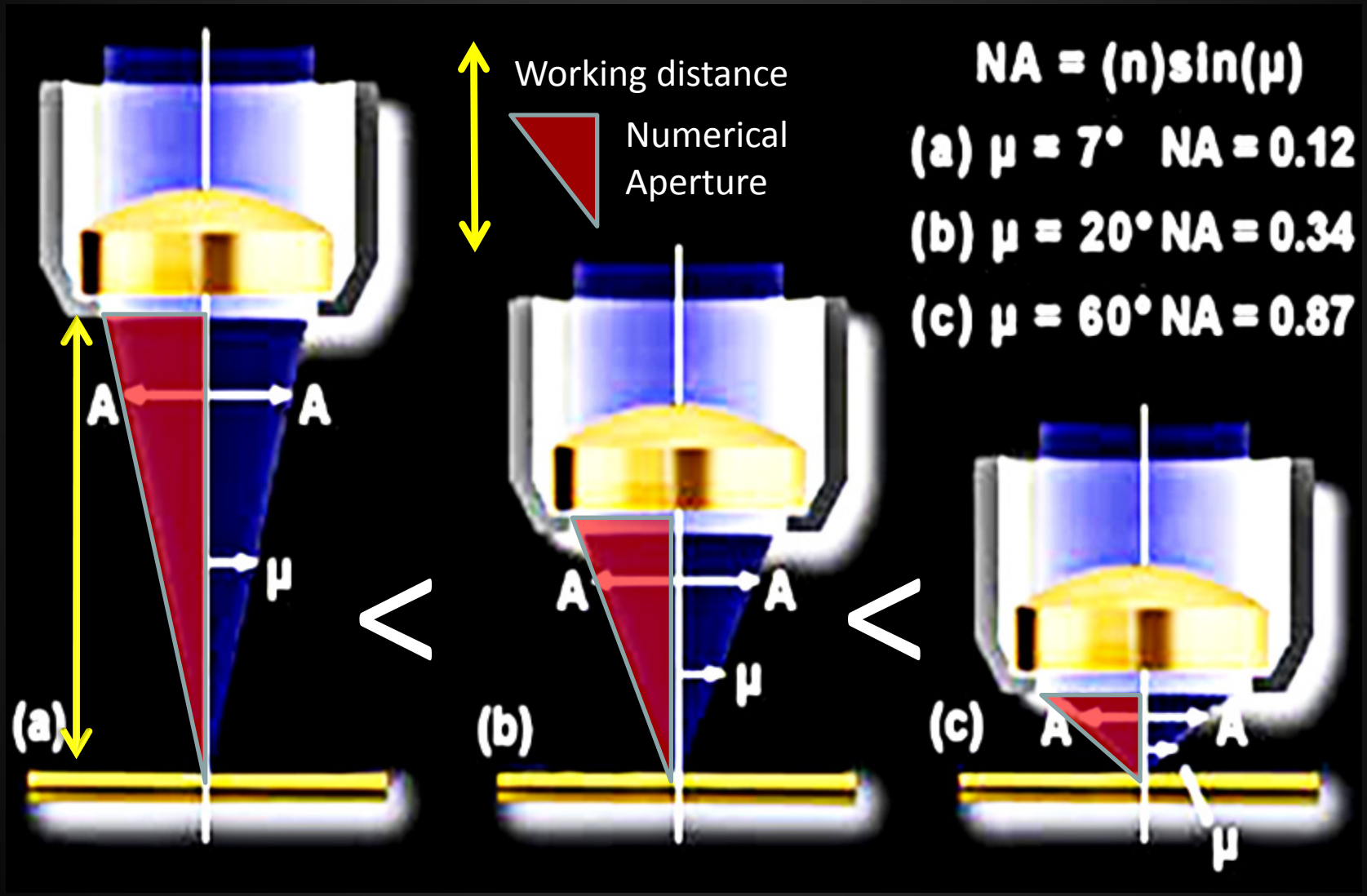
The Olympus
XLPlan N
25x, NA 1.05

Long Working distance (2mm) including (!)
High Numerical Aperture
(good resolution/focus, narrow depth of focus)

- all photons to the focus for high chance of 2P-Ex

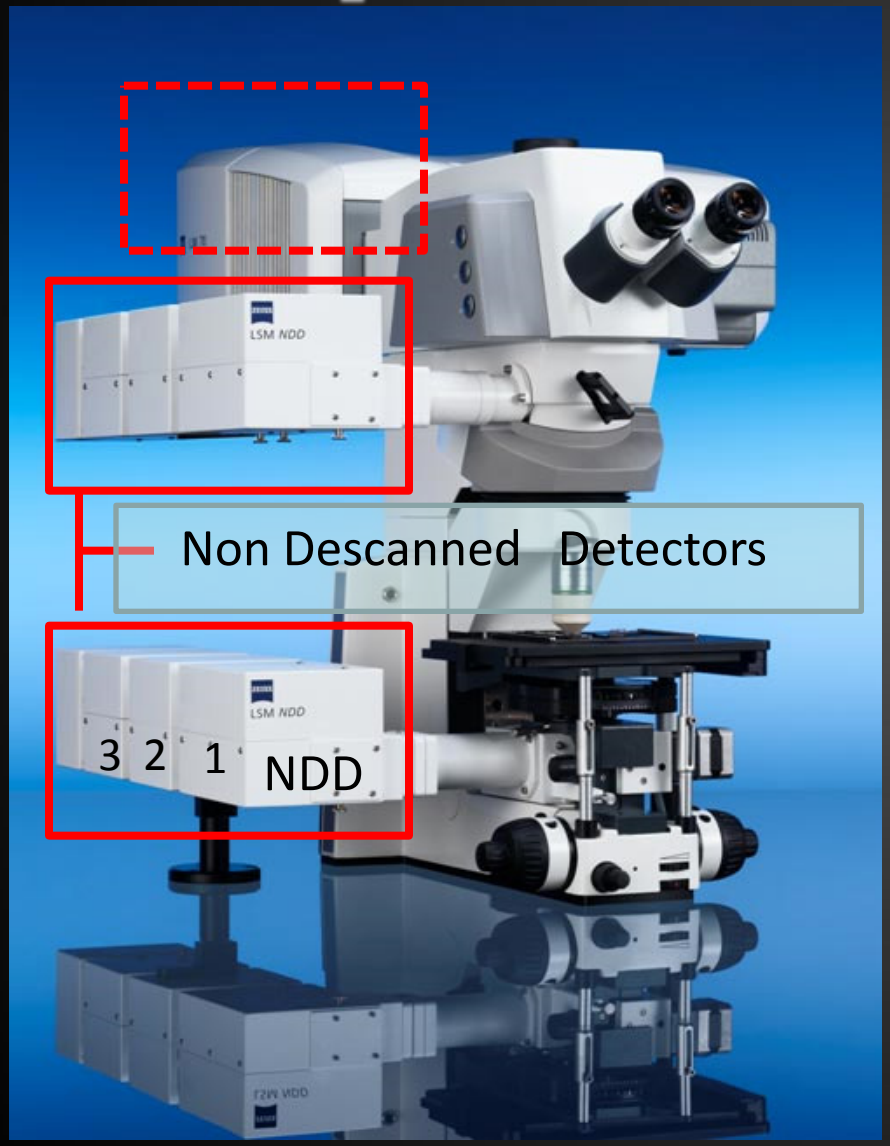
- High transmittance and correction for broad range of e.g. 400 nm to 1000 nm
- Water dipping (remember *in vivo* imaging) / cover slip
- Correction collar (!) to compensate for different refractive indices (water 1.3, specimen 1.34-1.4)
- 34 degree angle at lense top for better accessibility to specimen for manipulation

Multiphoton objectives



High NA + Long WD = expensive objective

Multiphoton detectors - NDD



Confocal detector (LSMD)

Using the ”long way” gives more flexibility, the confocal filterfree scanhead can be finetuned what range of light shall be collected, BUT

- the way is long (equals 32 cm glas!) and hence light is lost...

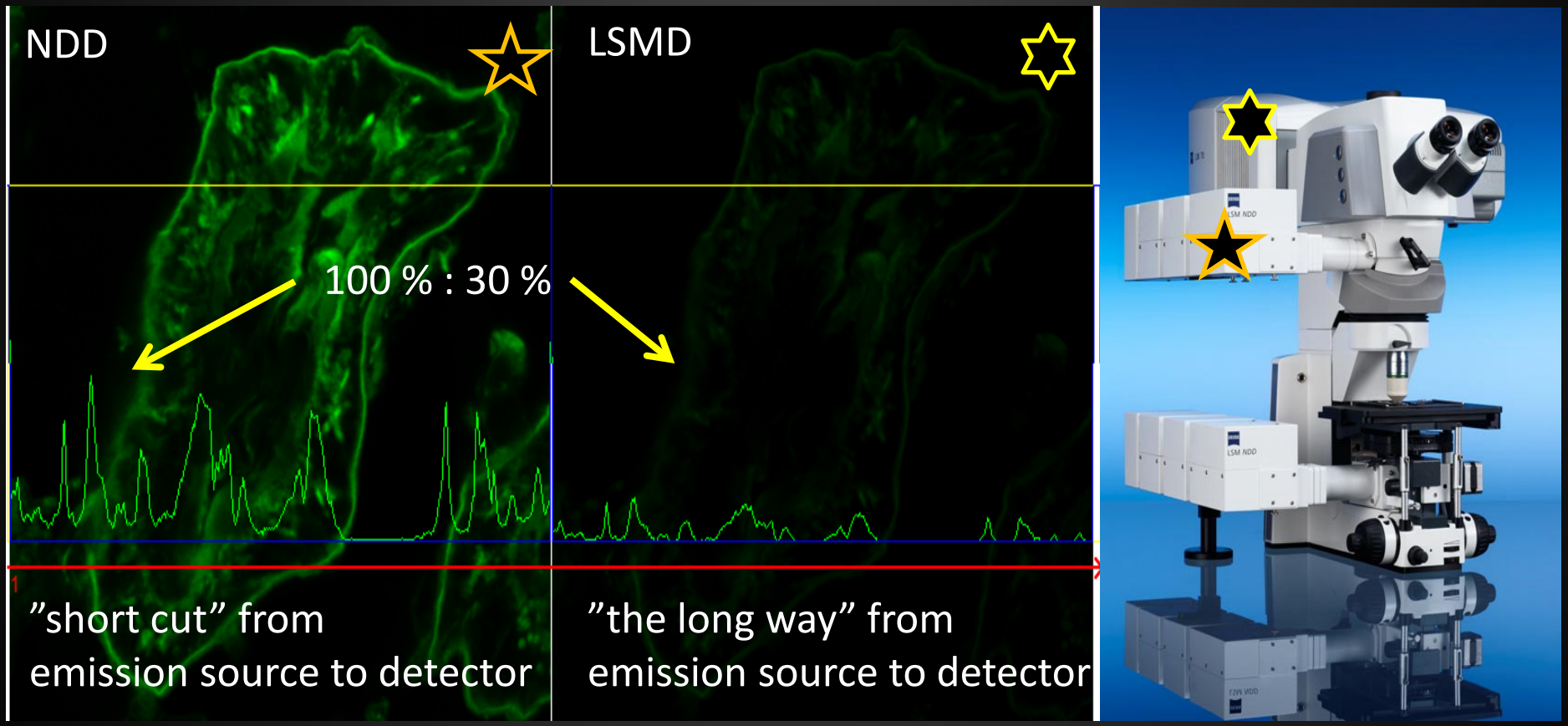
Non descanned detector (NDD)

Using the NDDs as ”short cuts” avoids loss of light. NDDs filter light via ”old days” filtercubes and therefore lack in flexibility.

- 2 sets: Epi- and transmitted directions

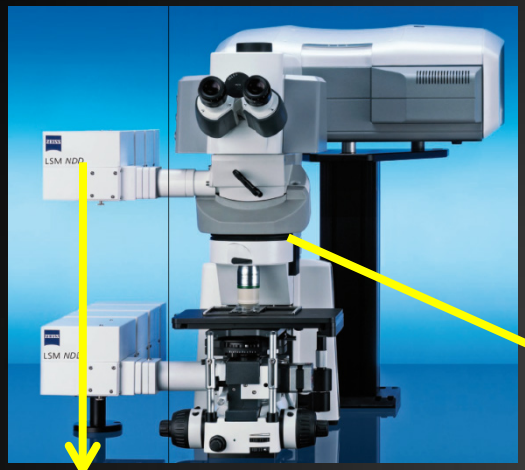
Multiphoton detectors - NDD

Loss of emission light: NDD vs LSMD I



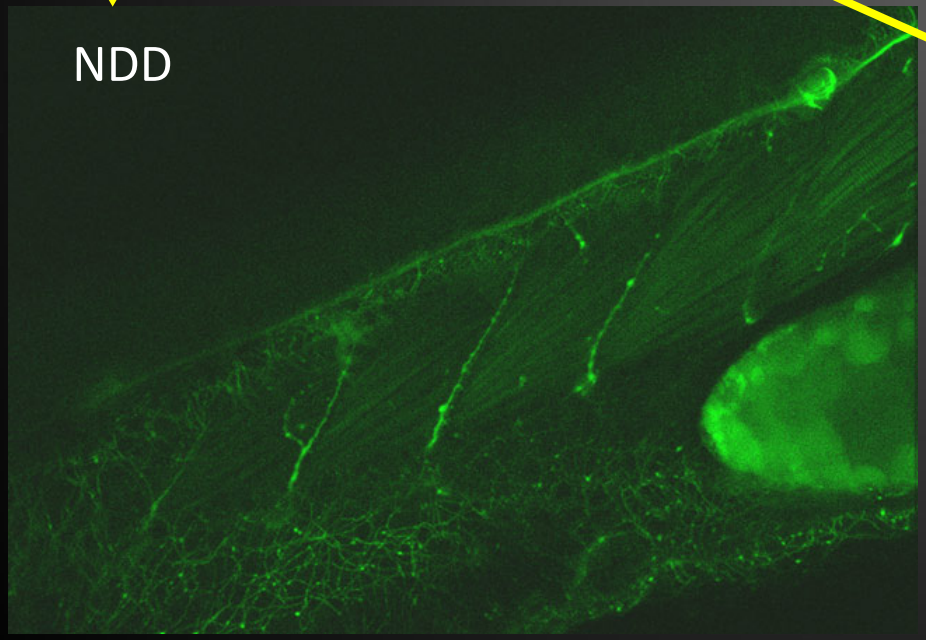
Alexa 488, MaiTai 780nm, 5% (quite high), spectral range emission 500-550nm, no/open pinhole, digital gain etc for NDD (no over/under exposure)

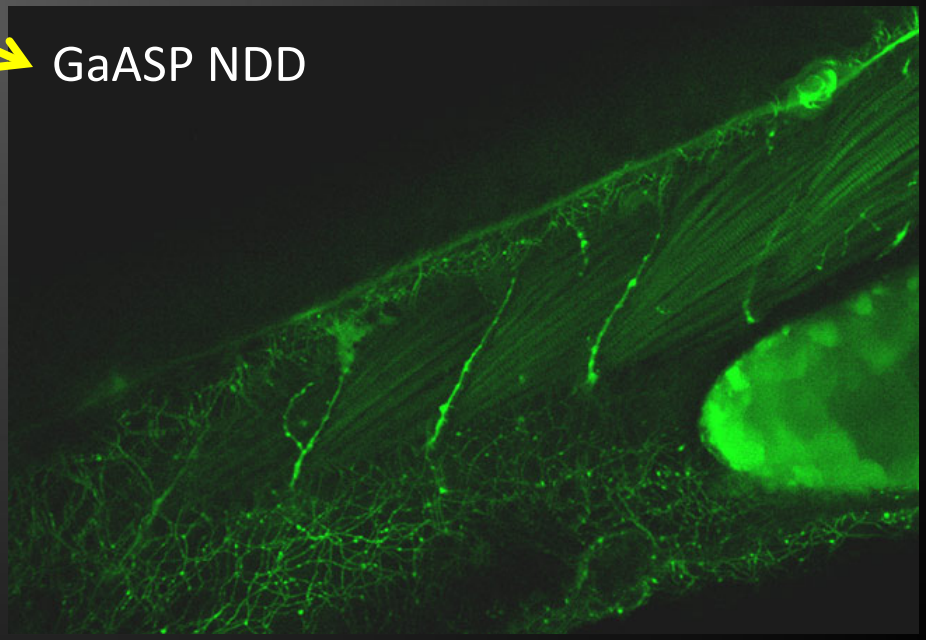
Multiphoton detectors - GaAsP



With the very sensitive GaAsP detector right behind the objective we are able to collect more light from weakly fluorescent specimen (higher signal to noise ratio)

- one detector with no filter
- no distinction between different fluorophores...
- Efficience 40 % for 400 -700 nm



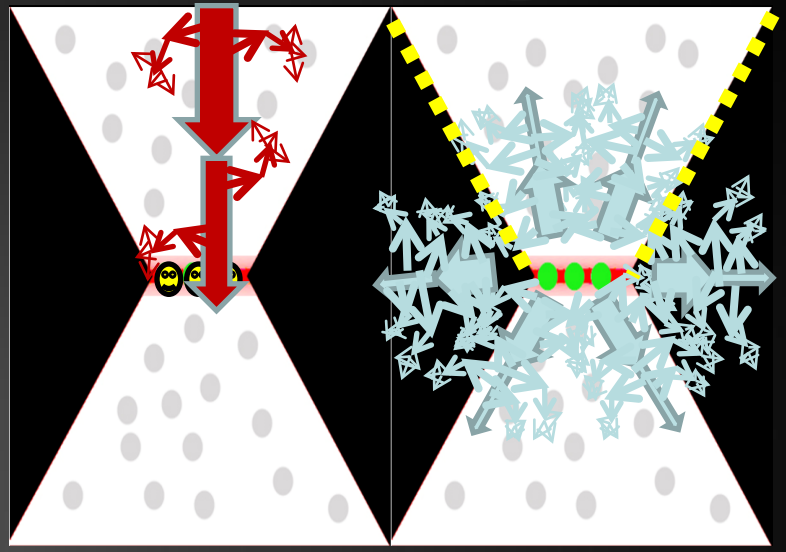


Loss of emission light: NDD vs GaAsP

Bring back home the photons - summary

FL emission is shorter in λ and get more scattered and dispersed than NIR Ex light

- Loss of emission light i.e. signal light
- light gets lost via the optical pathways



To compensate this loss
Detectors should have

- better sensitivity
- proximity to specimen
- more

Keep in mind...

A multiphoton microscope gives you the opportunity to get images from deep (e..g. 500 µm) within (living) tissue, whilst photodamaging only the imaged volume.

A Multiphoton microscope is a point scanning system which excites fluorophores within the Focus volume only. Therefore you collect emission light from this volume only, enabling you to acquire optical slices, without the use of confocal pinholes.

Beside this, one is able to photomanipulate tissue/cells within a very small volume.

Comparison of CLSM and 2P

	LSM	Multiphoton
light source	laser UV to VIS	tuneable 50-100fs pulsed IR laser
depth of visualization	up to 100 µm depending on tissue/sample	up to 1000 µm depending on tissue/sample
XYZ resolution	via focal plane of objective, pinhole and wavelength	Similar (or worse) as LSM, no pinhole needed
volume of exitation	throughout the Illuminated tissue	only the focal plane
sensibility	Loss of signals via optics ➢ Descanned detectors	Enhance signal by use of ➢ Non-descanned detectors ➢ GaAsP or Hybrid/avalanche

THANKS FOR YOUR ATTENTION!