Optical Fluorescence Microscopy

Advantages

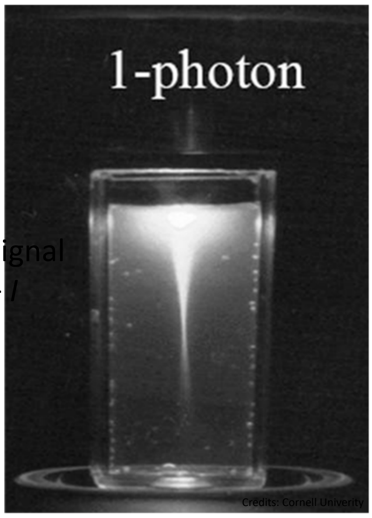
- *high resolution (cellular and subcellular)*
- *no ionizing radiation*
- *specific labelling (*e.g.*, expression of fluorescent proteins)*
- *multiplexing*

Drawbacks

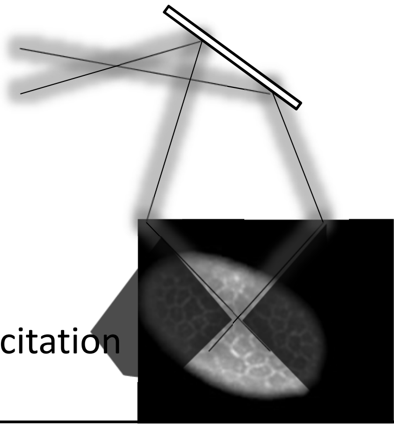
- *limited imaging penetration (scattering, absorption)*
- *signal decrease over time (bleaching)*
- *strong background in tissues*
- *access to axial resolution at expenses of sensitivity*

One-photon excited fluorescence

Fluorescence



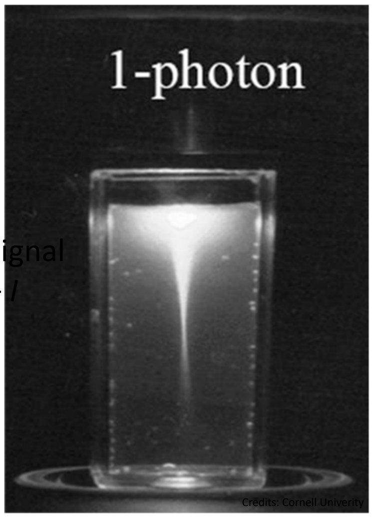
Signal ÷ I



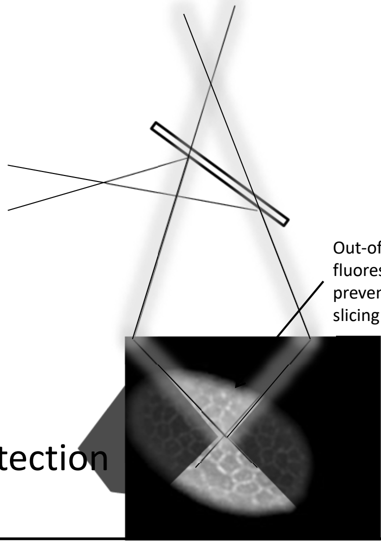
Excitation

One-photon excited fluorescence

Fluorescence



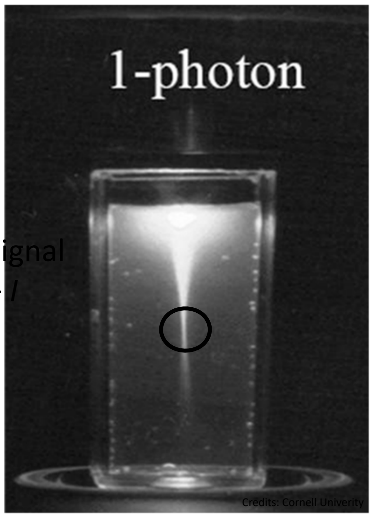
Signal
÷ I



Detection

Fluorescence confocal detection

Fluorescence



Signal
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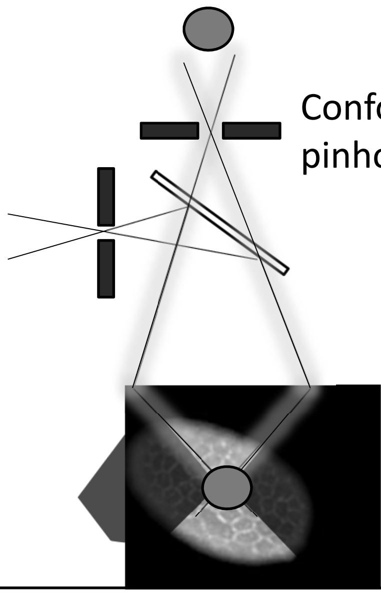
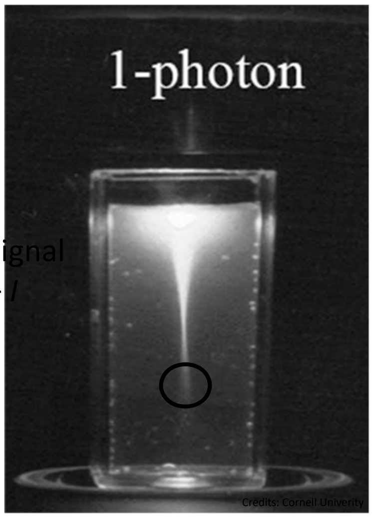


Confocal
pinhole

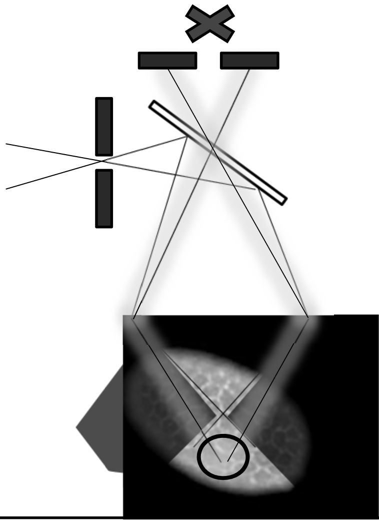
Fluorescence confocal detection

Fluorescence



Signal
÷ *I*

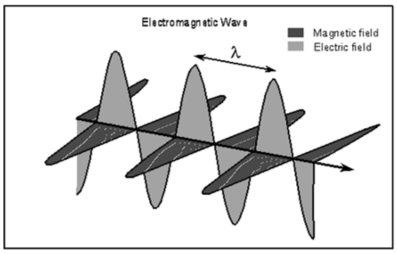
Out-of-focus contributions are blocked by the pinhole



Can we do better?

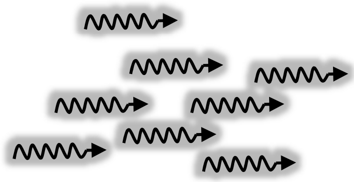
Basic vocabulary

Light as an electromagnetic wave



λ: wavelength

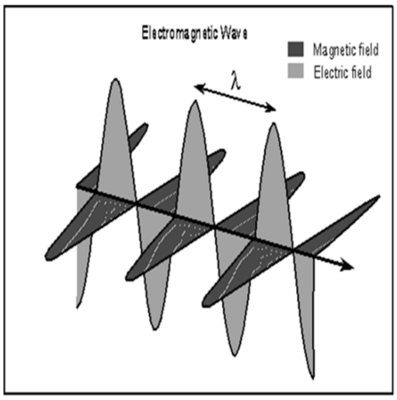
Light as an ensemble of photons



Each photon has energy: hν
ν: frequency (÷ 1/λ)

Basic vocabulary

Light as an electromagnetic wave



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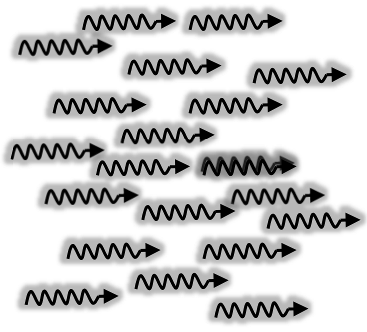

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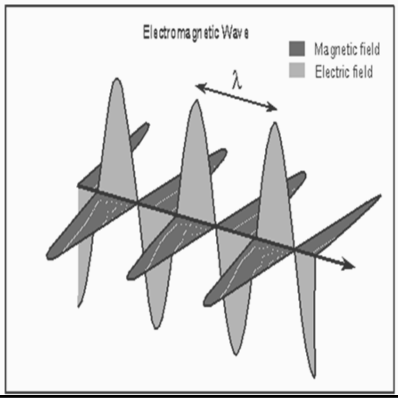
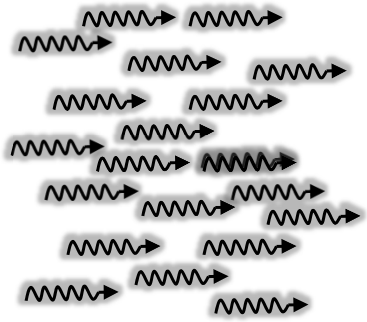


λ: wavelength

Light as an ensemble of photons



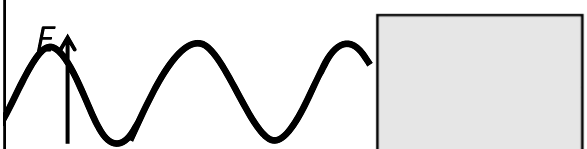
Each photon has energy: $h\nu$

ν; $\omega \div 1/\lambda$

Linear polarization

The oscillating electric field of light interacts with the electrons in an object, determining its polarization:

$$P = \chi(\omega)\, E$$

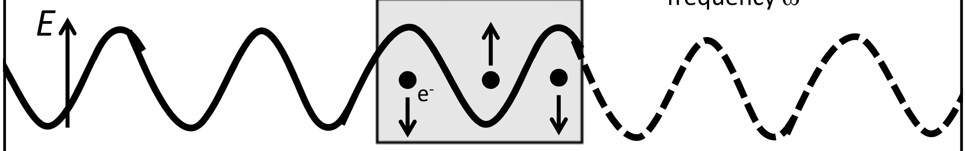


Linear polarization

The oscillating electric field of light interacts with the electrons in an object, determining its polarization:

$$P=\chi(\omega)\,E$$

The oscillating electrons re-emit light at the same frequency ω



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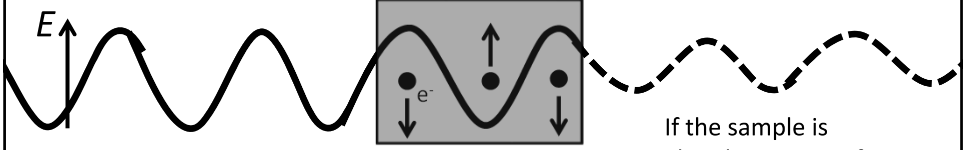
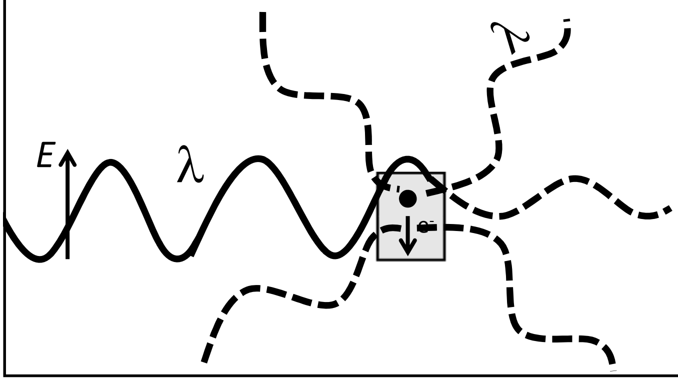


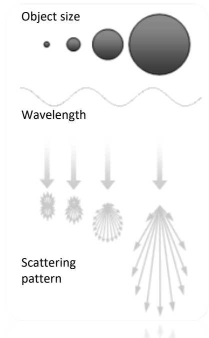
If the sample is absorbing at ω, a fraction of the light remains on the sample

Rayleigh (linear) scattering

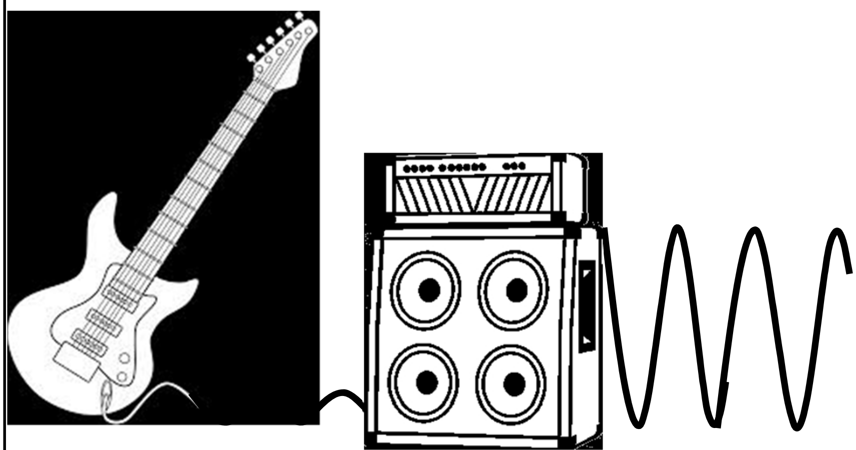
If the object is small compared to λ light is scattered in different directions



Size matters



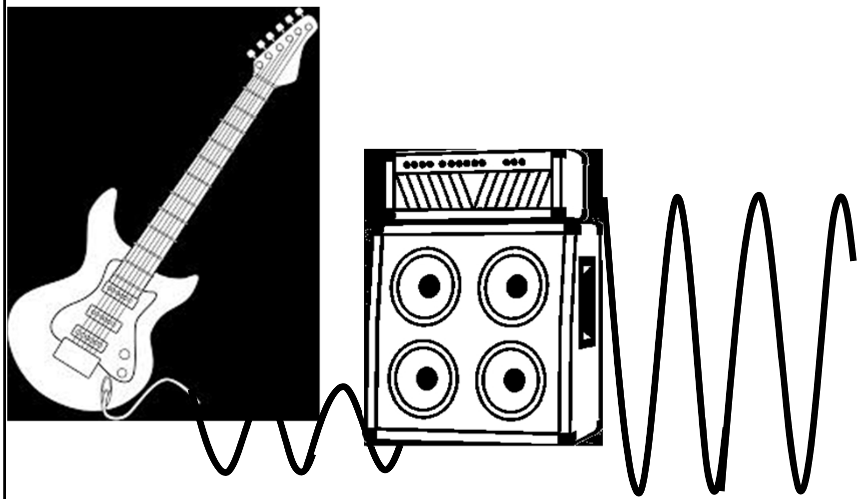
now consider this analogy…



$$I_{OUT} \div I_{IN}$$

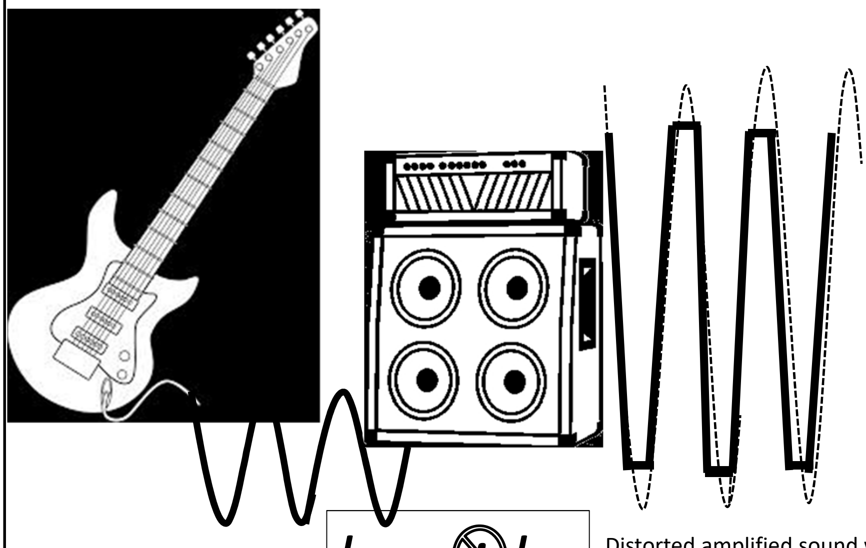
LINEAR RESPONSE

now consider this analogy…



$$I_{OUT} \div I_{IN}$$

now consider this analogy…

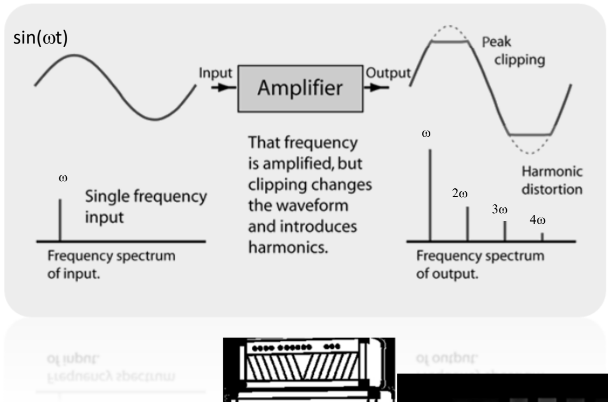


$$I_{OUT} \not\div I_{IN}$$

Distorted amplified sound wave

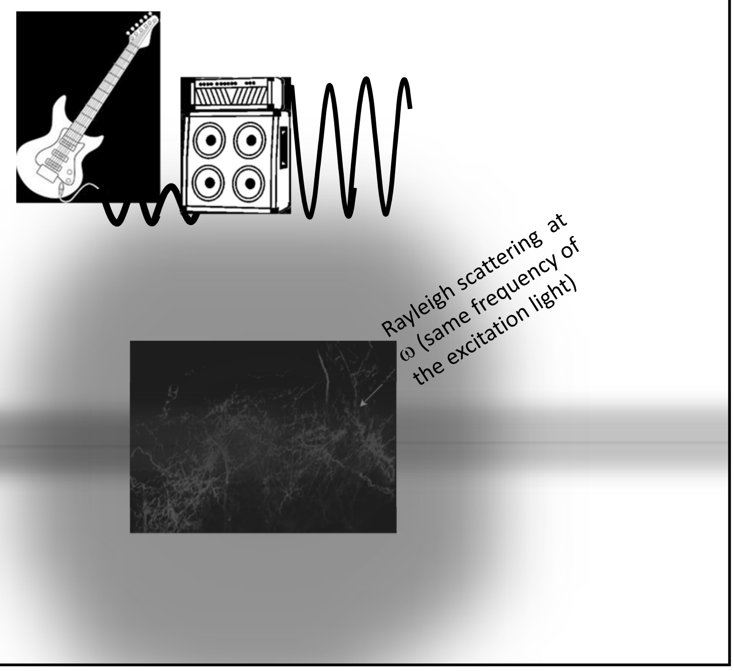
NONLINEAR RESPONSE

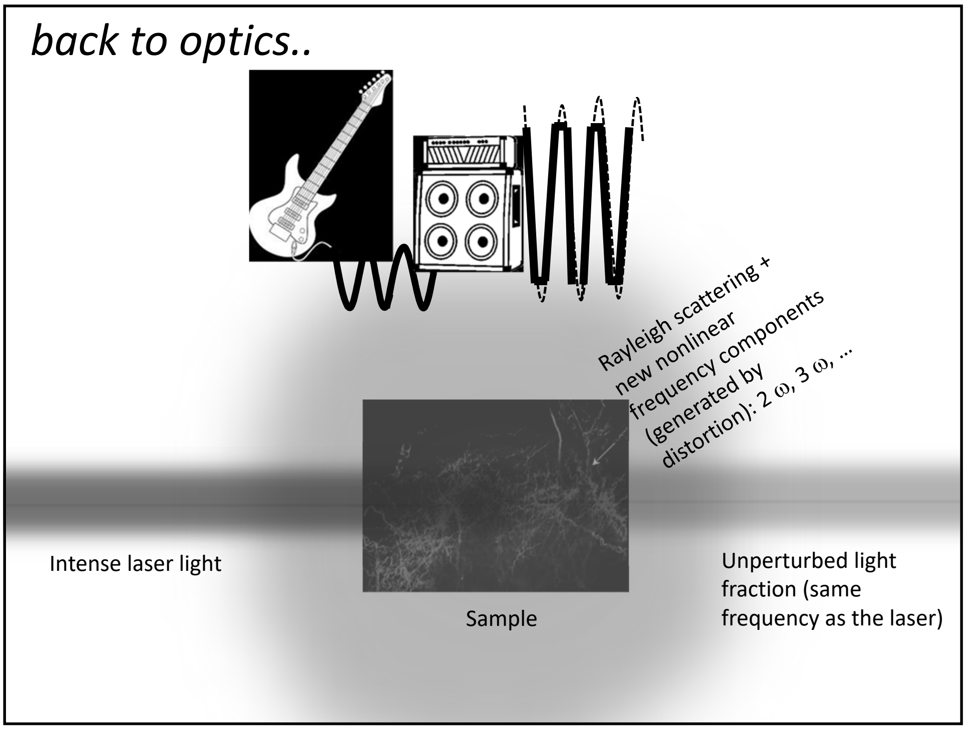
Frequency spectrum of a distorted output

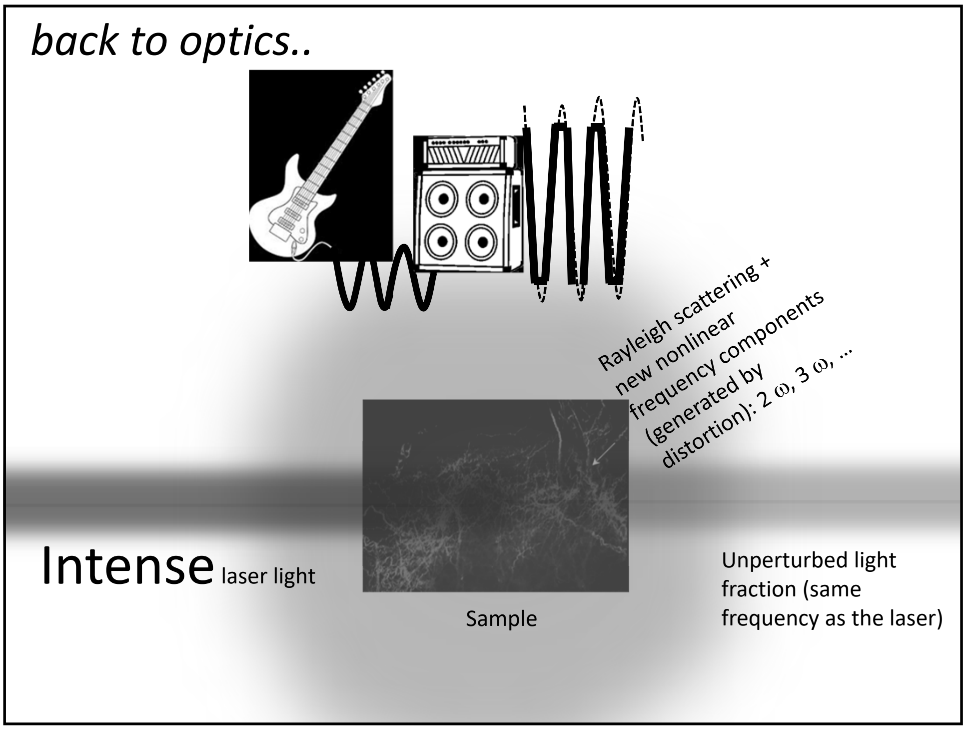


New (harmonic) frequencies will appear at teh output

back to optics..




back to optics..
Rayleigh scattering + new nonlinear frequency components (generated by distortion): 2 ω, 3 ω, ...
Intense laser light
Sample
Unperturbed light fraction (same frequency as the laser)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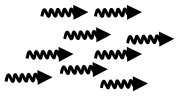
Light intensity, *I*

$$I \div \frac{\#\, photons}{surface \cdot time\ interval} = \frac{power}{surface}\left[\frac{\mathrm{W}}{\mathrm{cm}^2}\right]$$

To increase light intensity we can:



Increase the laser energy
(risk of damaging the sample)

Focus tighter (not always easy/possible…)

Light intensity, *I*

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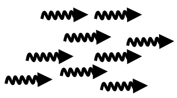

Increase the laser energy
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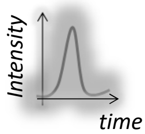
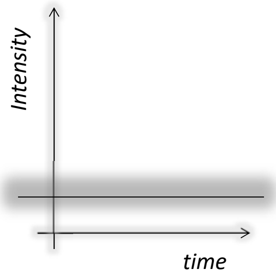
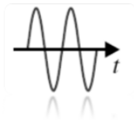


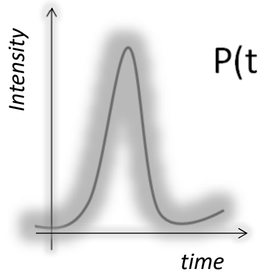
Use a short pulse laser

W. Webb
Cornell University

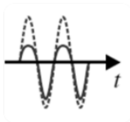


$$P(t) \div \chi(\omega)E(t)$$

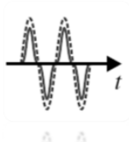




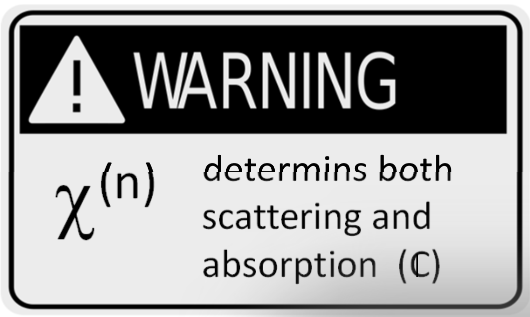
$$P(t) \div \chi(\omega)E(t)+\chi^{(2)}(\omega)E^{2}(t)+\chi^{(3)}(\omega)E^{3}(t)+\ldots$$



Asymmetric distortions

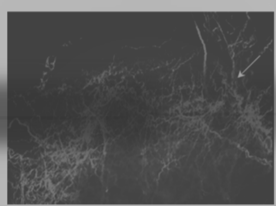


Symmetric distortions

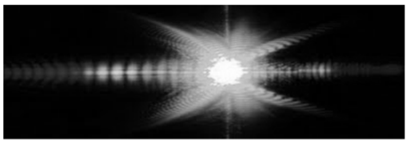


New frequencies can also be absorbed

If the sample was absorbing at ω, under these conditions it can also absorbs at 2 ω, 3 ω, …



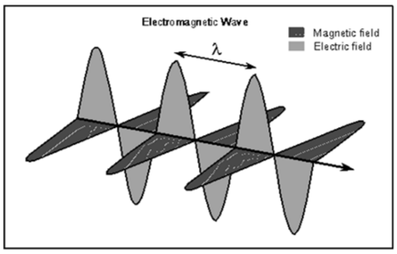
Nonlinear optics phenomena



- Multiphoton absorption
- Harmonic generation (SH, TH,..)
- Frequency mixing
- Spectral broadening
- Nonlinear vibrational techniques
- Optical rectification (THz)
- ...

Basic vocabulary

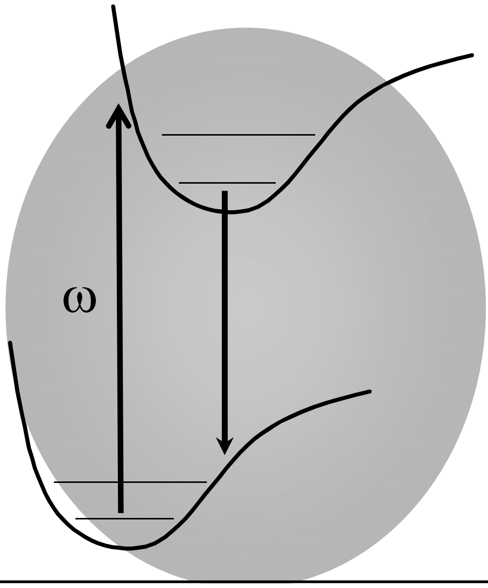
Light as an electromagnetic wave



Light as an ensemble of photons

Each photon has energy: $h\nu$

Two-photon excited fluorescence



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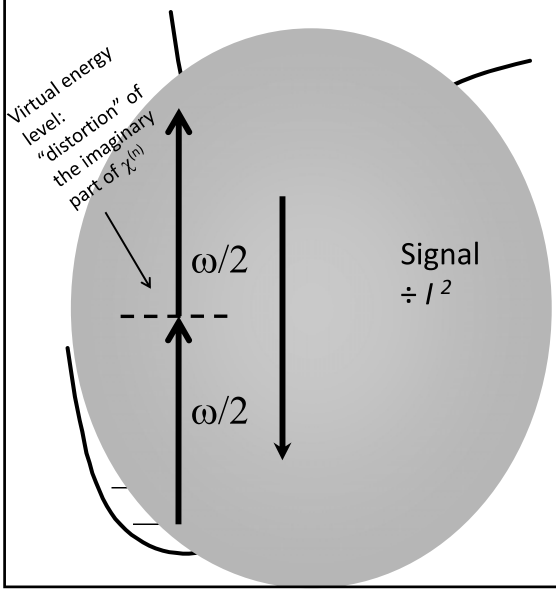
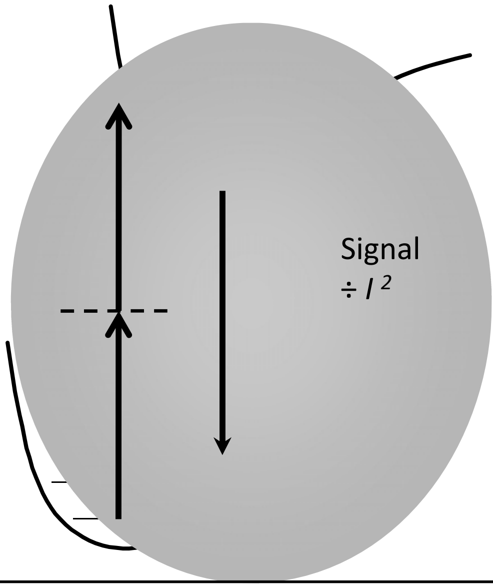


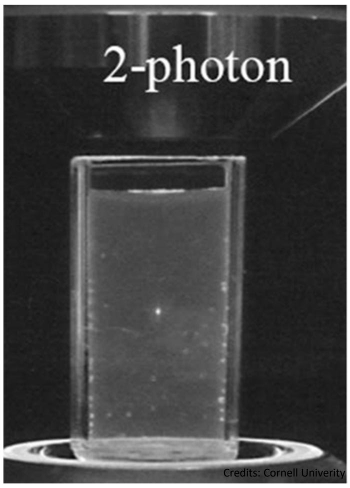
$$I \div \frac{\#\, photons}{surface \cdot time\ interval}$$

Light intensity is high enough for two-photon absorption only in the focal region

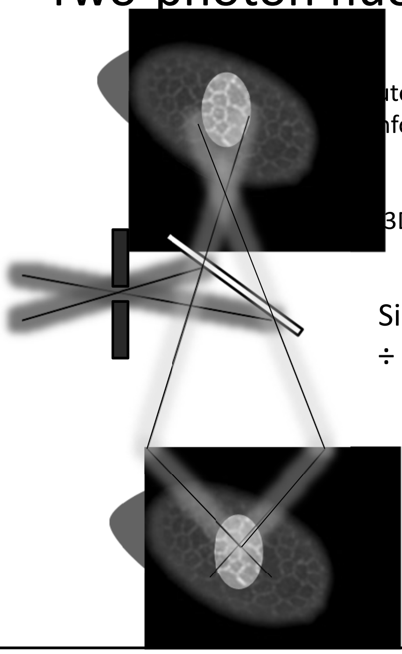
Two-photon excited fluorescence



Signal
÷ I^2



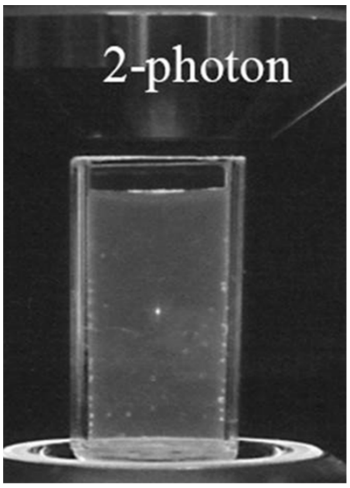
Two-photon fluorescence microscopy



utomatically
nfocal”

3D slicing

Signal
÷ I^2



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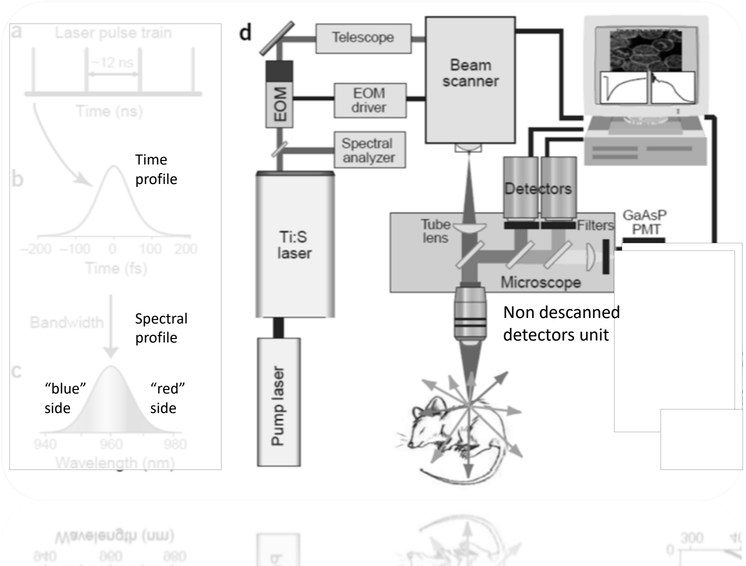
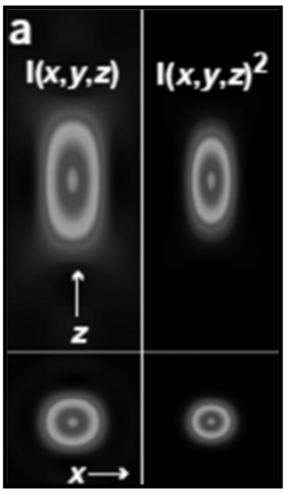


Multi-photon (*effective*) resolution

$$r = \frac{1.22\lambda}{2n\sin\theta} = \frac{0.61\lambda}{\mathrm{NA}}$$

According to Rayleigh criterion the resolution of a multi-photon system should be worst than a linear one

Consider that the Point Spread Function should be taken squared



Zipfel et al., Nature Biotech. 21, 1369 (2003)

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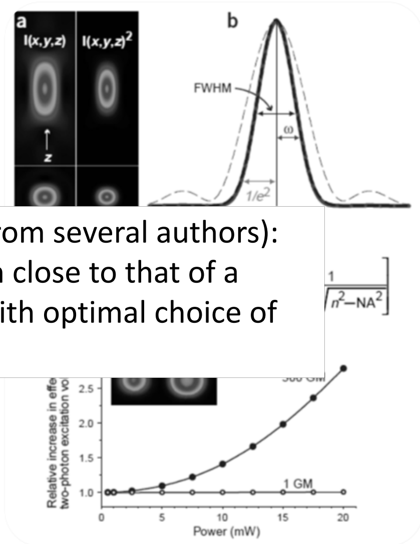
According to Rayleigh criterion the resolution of a multi-photon system should be wors

Take home message (from several authors): multiphoton resolution close to that of a confocal microscope with optimal choice of pinhole aperture size

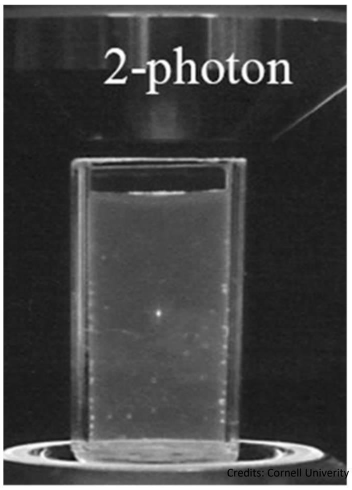
Conside
Spread
taken squared

Other factors intervene: scattering (less than linear), saturation intensity…



Zipfel et al., Nature Biotech. 21, 1369 (2003)

Two-photon fluorescence microscopy



Credits: Cornell Univerity

No need of confocal pinhole

Intrinsically 3D

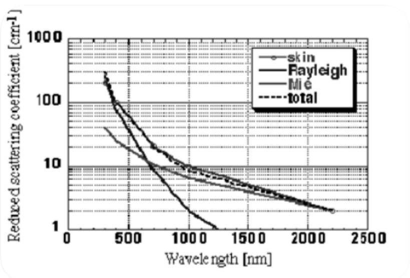
Large excitation/signal spectral separation

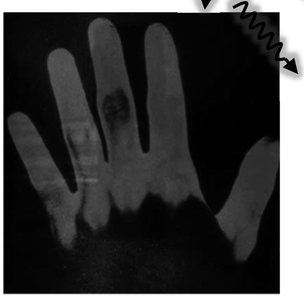
Longer excitation wavelengths

Tissue Scattering

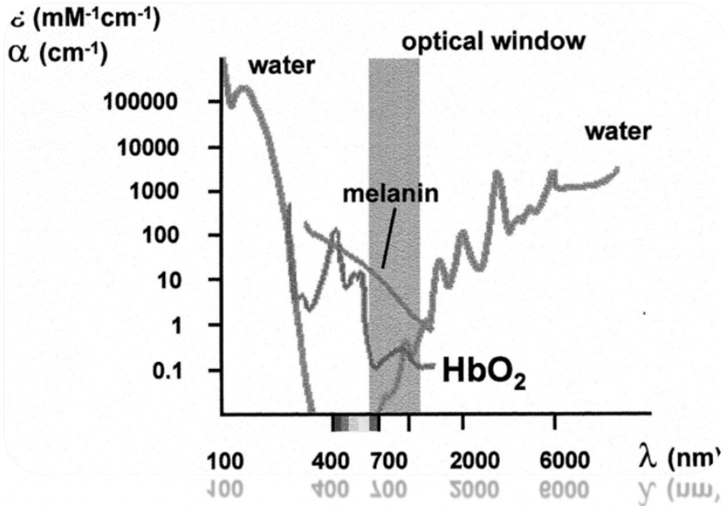
Diffusive and snake photons are lost, not useful for imaging

Tissue Scattering

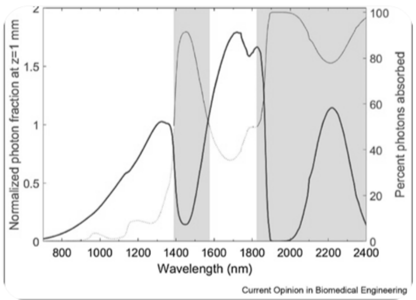




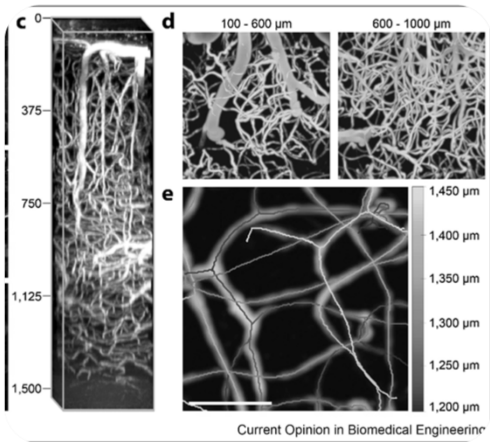
Tissue absorption



New optical windows: NIR-I, II, III

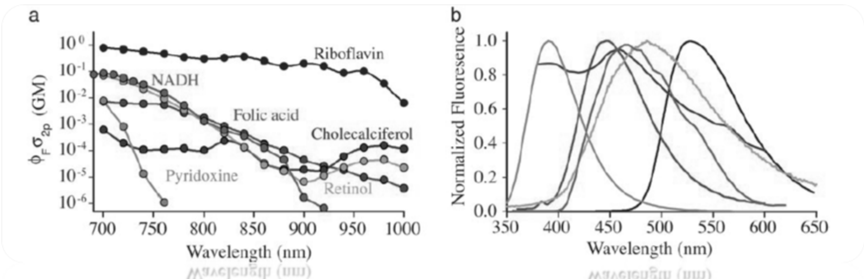


Working in NIR-II allows reaching ~mm penetration in tissue, however the choice of red absorbing probes is still limited



What about selectivity and bleaching in multiphoton fluorescence microscopy?

Endogenous two-photon excited fluorescence

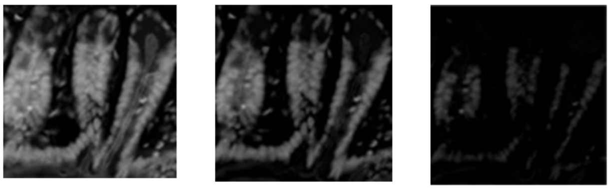


Webb et al, PNAS ***June 10, 2003*** *vol. 100 no. 12* ***7075–7080***

Molecular selectivity remains an issue, as for other fluorescence based images techniques

→ As in linear fluorescence imaging exogenous staining agents can be used / expressed
→ The choice of red absorbing fluorophores is limited

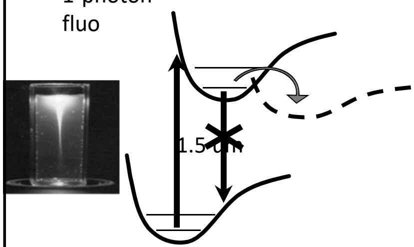
Fluorescence Bleaching



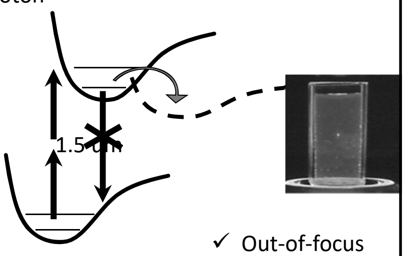
Imaging time

Fluorescence Bleaching

1-photon fluo

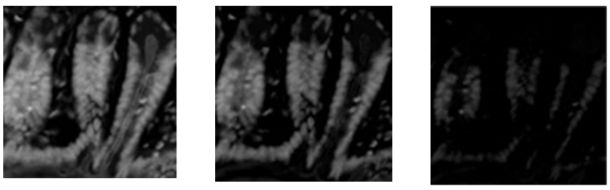


2-photon fluo



Fluorescence

2 photon fluorescence



Imaging time

- ✓ Out-of-focus bleaching: solved

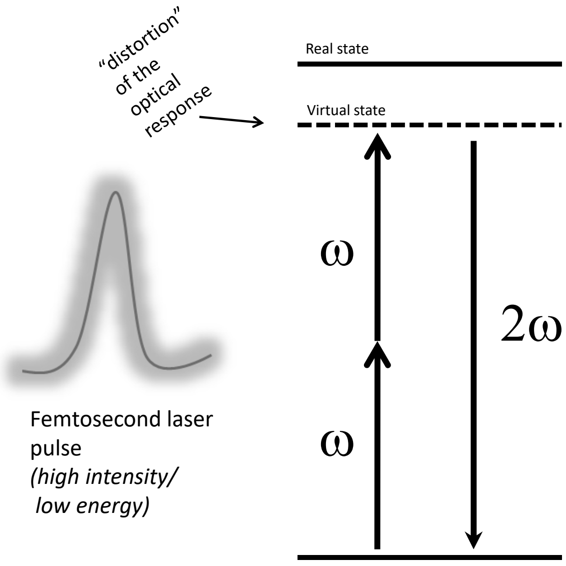
→ Less sample damage

- In-focus bleaching still an issue

- § *Inherently* nonlinear contrast mechanisms?

Possibly not affected by bleaching…

Second Harmonic Generation

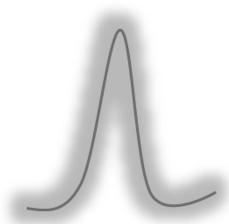


Virtual levels involved:

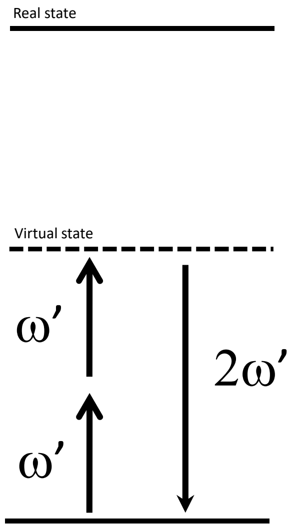
No energy deposited on the sample

$$\omega+\omega=2\omega$$

Second Harmonic Generation



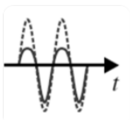
Femtosecond laser pulse
(high intensity/ low energy)



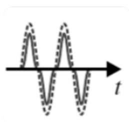
Nonresonant process:
*It works at any wavelength**

* almost true…

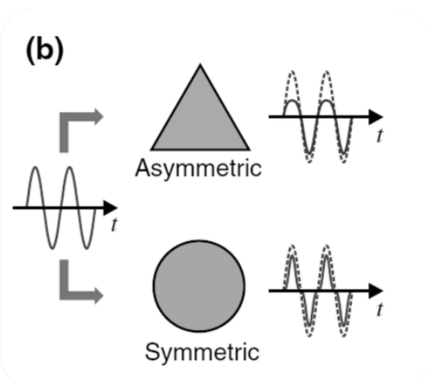
$P(t) \div \chi(\omega)E(t)+\chi^{(2)}(\omega)E^{2}(t)+\chi^{(3)}(\omega)E^{3}(t)+\ldots$



Asymmetric distortions

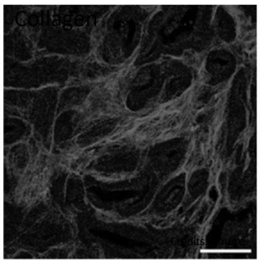


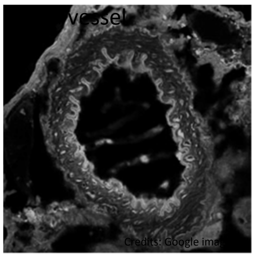
Symmetric distortions

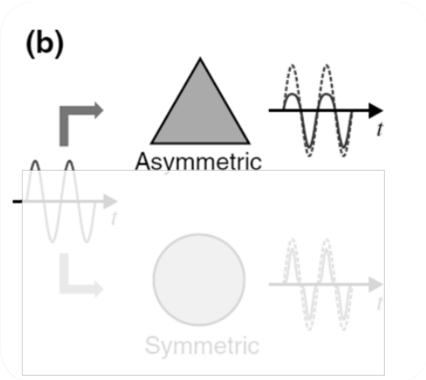


Even harmonics (2ω, 4ω..) appear only for asymmetric distortions

→ Situation when the symmetry of the space is broken
- Fibers
- Membranes
- Surfaces
- …





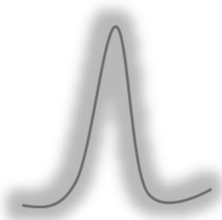


Even harmonics (2ω, 4ω..) appear only for asymmetric distortions

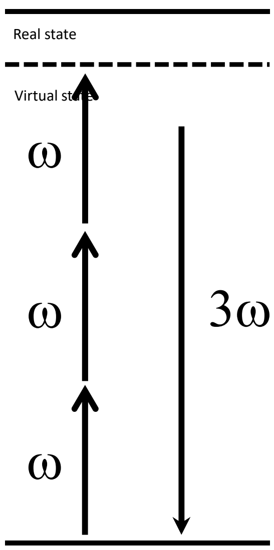
→ Situation when the symmetry of the space is broken

- Fibers
- Membranes
- Surfaces
- …

Third Harmonic Generation

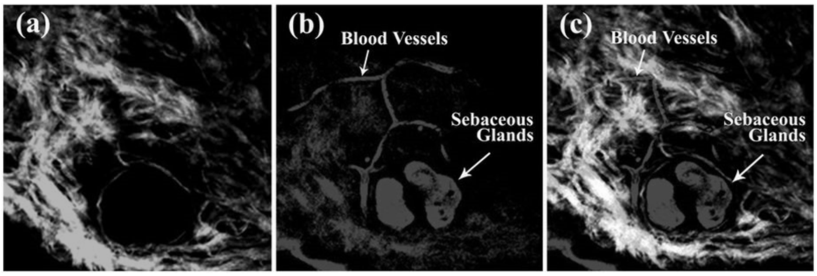


Femtosecond laser pulse
(high intensity/ low energy)



$$\omega+\omega+\omega=3\omega$$

→ THG is particulary strong for lipid droplets



Example of multiphoton harmonic imaging of an unstained tissue:
SHG collagen, THG lipid content

Scientific Reports 5, 8879 (2015)

Third Harmonic Generation

$$\omega+\omega+\omega=3\omega$$

Tunability of the previous generation of lasers for multiphoton microscopy (Ti:sapphire: Mai Tai, Chameleon): 700 -1000 nm

Third harmonic at 333 nm → cut-off of microscope components

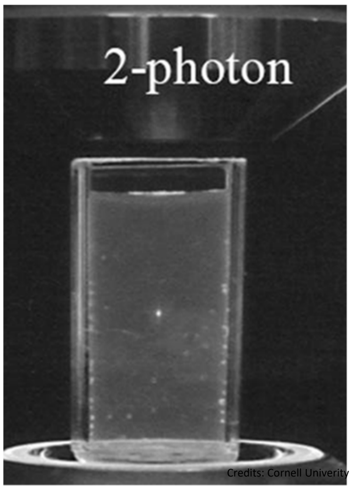
Spectra Physics Insight

Since 2012

Tunability 600-1300 nm

Third harmonic > 400 nm, no transmission problem, at the max. efficiency of GaAsP detectors

To sum up



Intrinsically 3D
High (but not super!!) resolution

Less photobleaching / energy deposition on the sample

Longer excitation wavelengths
→ *Deeper imaging penetration*
→ *Excitation/emission spectral separation*

Simultaneous fluorescence + harmonics

What about multiphoton nanoparticles?

Bioimaging labels: tracking cell populations

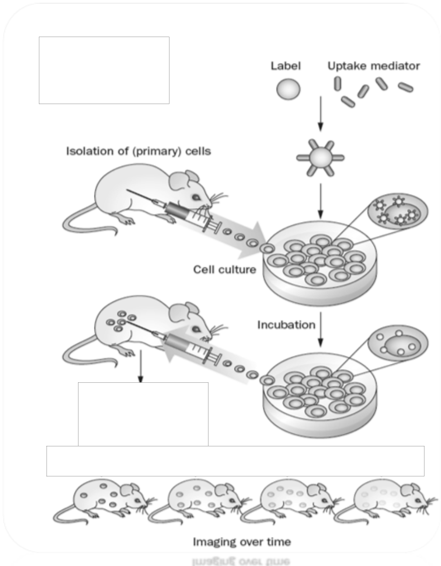


Image adapted from
Kircher, M. F. *et al. Nat. Rev. Clin. Oncol.* 8, 677–688 (2011)

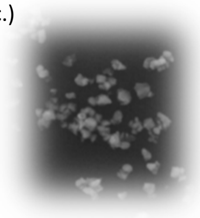
Essential requirements:

Biological
- Good biocompatibility
- Long-term NPs localization in cells (days to weeks)
- NPs uptake with no specific targeting often sufficient

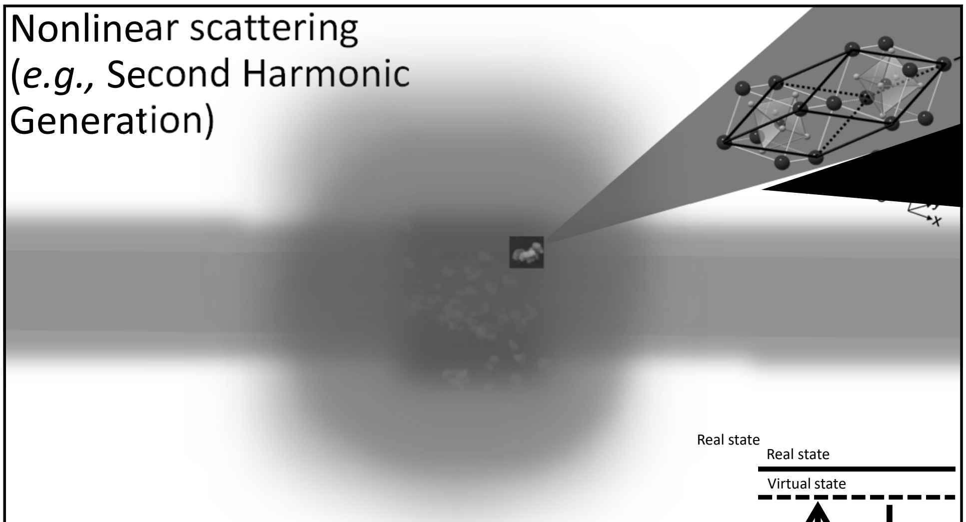
Photonics
- High signal st ver time
- High selecti ainst backgro
- High g depth (> 1 mm)
- C esolution in tissues m)

→ Nonlinear optics

Metal oxide nanomaterials with noncentrosymmetric crystal structure (e.g., *Bismuth Ferrite, Potassium Niobate,* etc.)

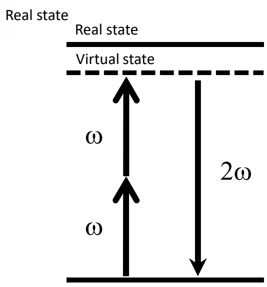


Nonlinear scattering (*e.g.,* Second Harmonic Generation)

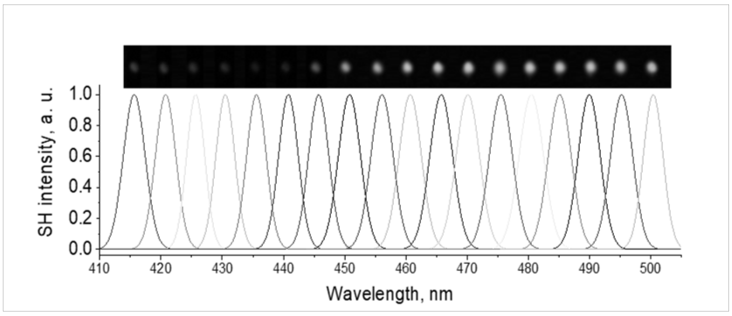


$$P(t) \div \chi(\omega)E(t) + \chi^{(2)}(\omega)E^{2}(t) + \chi^{(3)}(\omega)E^{3}(t) + \ldots$$

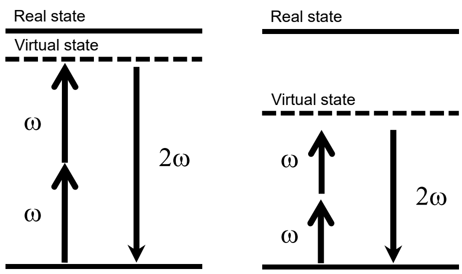
Second Harmonic Generation



Wavelength Flexibility

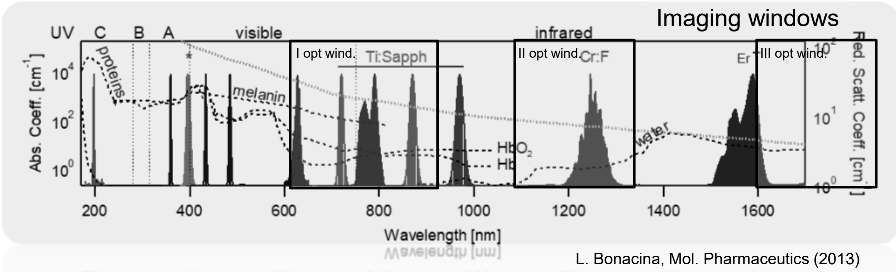


Laser excitation tuned from 820 to 1000 nm

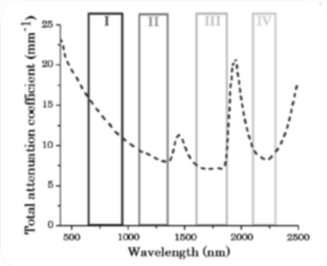


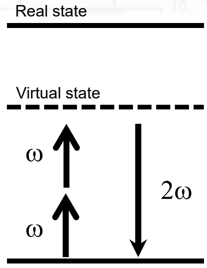
Wavelength Flexibility

New IR transmission windows: Alfano Group CUNY



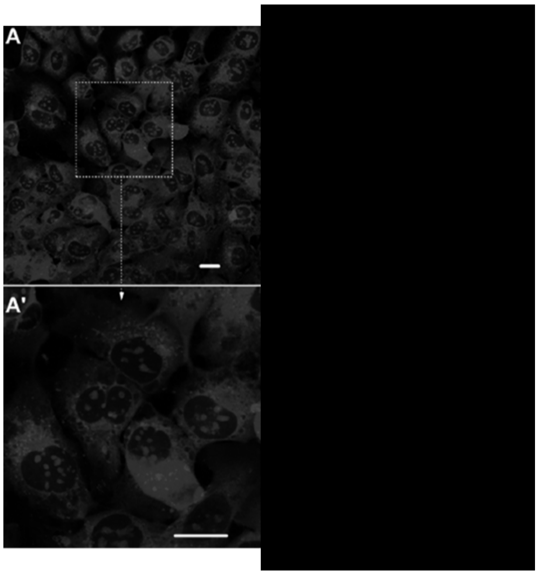
L. Bonacina, Mol. Pharmaceutics (2013)





L. Sordillo Journal of Biomedical Optics 19(5), 056004 (2014)

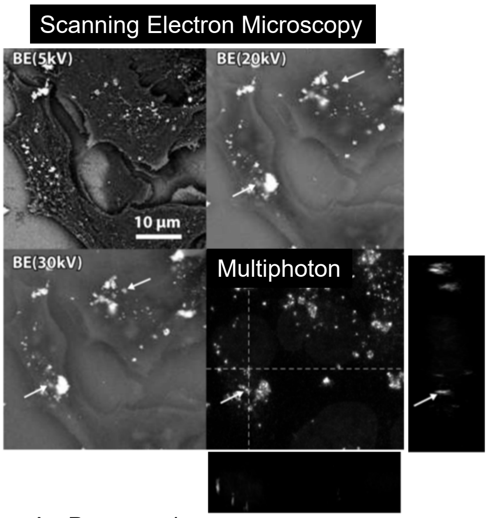
Signal Photo-Stability



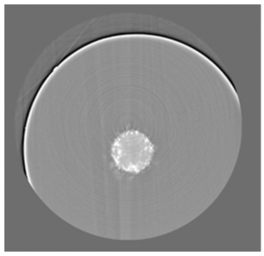
Fluo (membranes)
SHG (HNPs)

D. Staedler ACS Nano (2012)

Multimodal observation: SEM, Multiphoton, and CT scan (Bismuth Ferrite NPs, BFO)



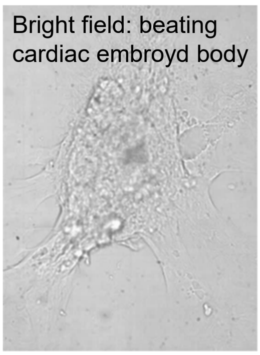
CT scan contrast



Eppendorf containing BFO HNPs labelled cells

A. Rogov *et al.*
ACS Photonics 2015

Cardiac Stem Cells Tracking



Second Harmonic

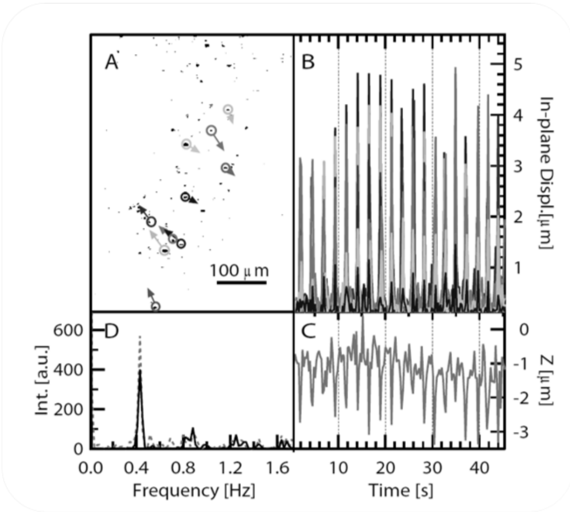
20 frames/s

ROI 1

ROI 2

In collaboration with *M. Jaconi* (UniGE) and *Daniel Ciepielewski* (Nikon Switzerland)

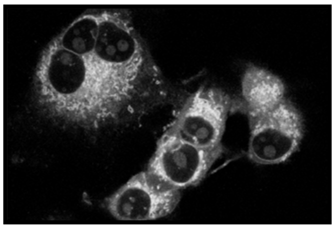
T. Magouroux *et al. SMALL* **8** (17), 2752 (2012)



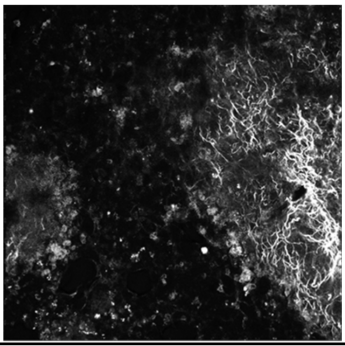
Monitoring at 30 frames/second the 3D movements of a stem cells-derived cardiac cluster

→ → Integration of "regenerative cardiac patches in native tissues*

T. Magouroux *et al. SMALL* **8** (17), 2752 (2012)



From cell cultures to tissue imaging



Tissue imaging

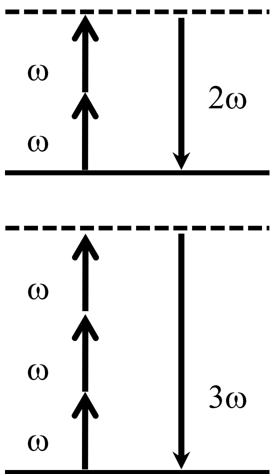
SHG-active nanoparticles against collagen-rich background

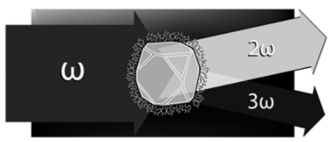
→ no contrast!!!

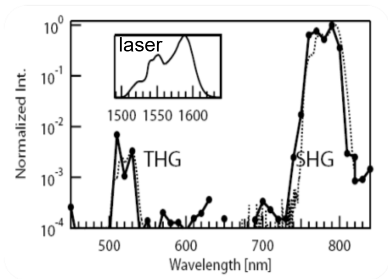
The multi-harmonic approach

For HNPs materials: $|\chi^{(3)}| \div |\chi^{(2)}|^2$

Jap. J. App. Phys. **32**, L905 (1993)







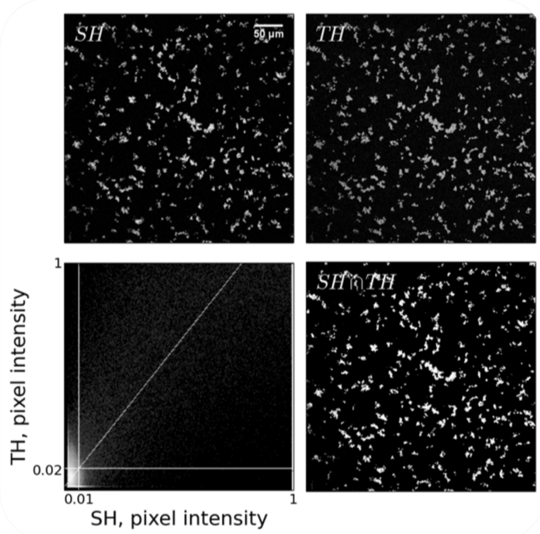
Extermann. et al. Optics Express (2009)

→ Application to microfluidics:
M. Geissbuehler et al., *Nanoletters*, 12, 1668 (2012)

The multi-harmonic approach

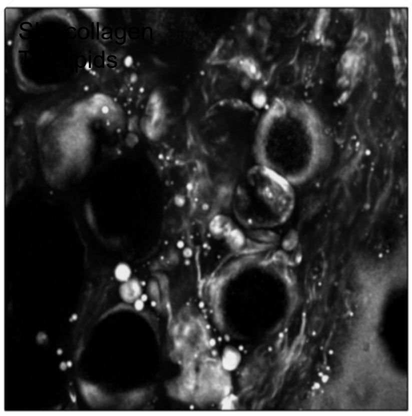
Bare BFO HNPs
on a coverslip

Co-localization
(automatic signal
thresholds)



A. Rogov *et al.*
ACS Photonics 2015

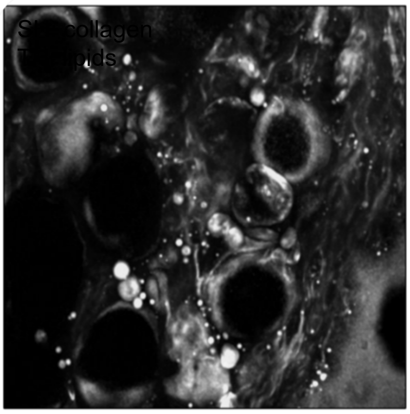
Endogenous sources of Harmonic generation



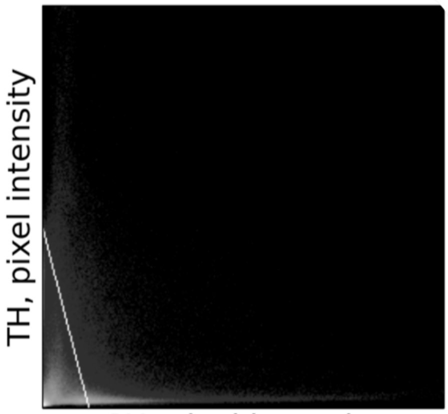
Unlabeled cancer tissue

Laser excitation: 1300 nm

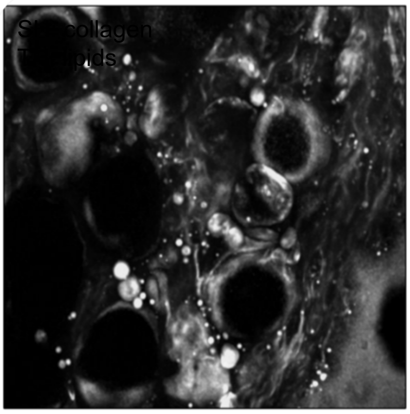
Endogenous sources of Harmonic generation



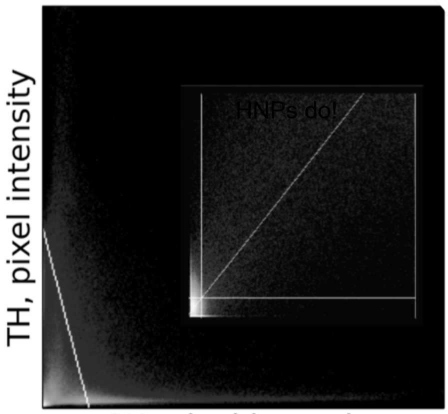
Unlabeled cancer tissue

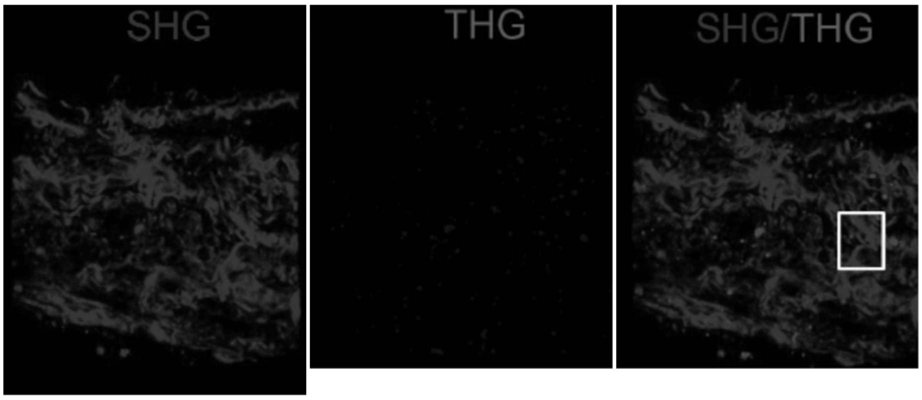


Endogenous sources of Harmonic generation



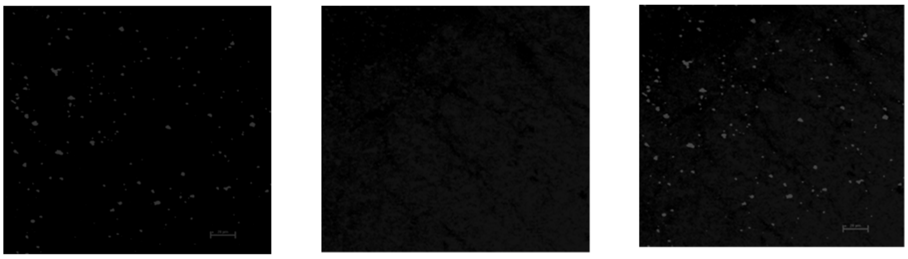
Unlabeled cancer tissue





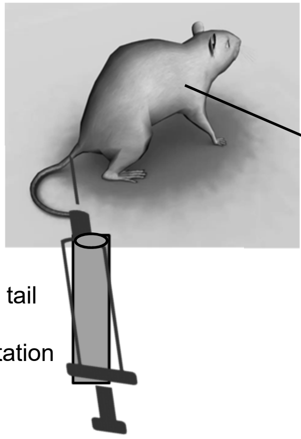
Multiharmonic
nanoparticles against
collagen-rich background

SHG THG SHG/THG



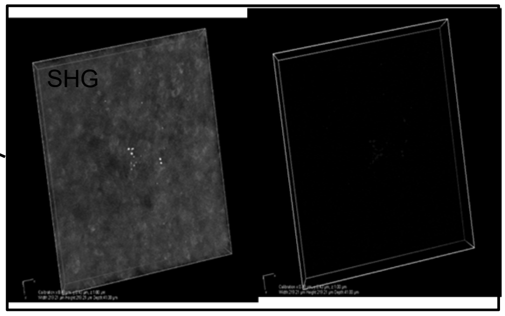
Multiharmonic
nanoparticles against
lipid-rich background

Ex 1: Tracking metastases spreading

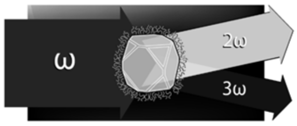


Day 0 tail vein Implatation

BFO-labelled H8N8 human mammary tumor cells

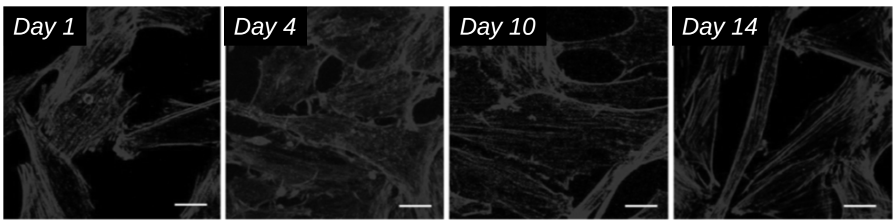


> day 4; *ex vivo* Multi-harmonic imaging in lungs



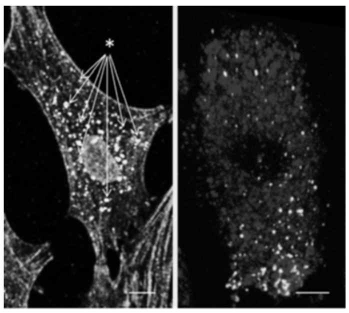
In collaboration with Max Planck for Experimental Medicine, F. Alves' group *Unpublished*

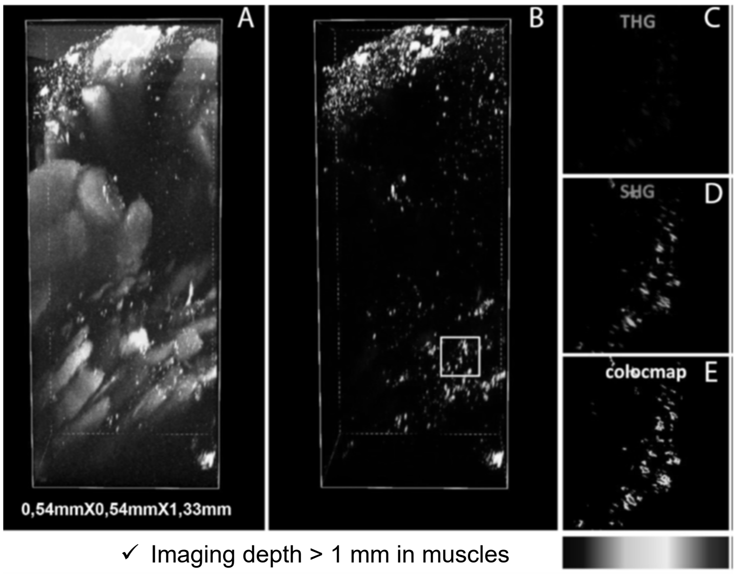
Ex 2: Monitoring cell therapy for Duchenne muscular dystrophy



- ✓ Good labelling and preservation of proliferation and morphological cell properties

HNPs are localized in endosomes and later in lysosomes

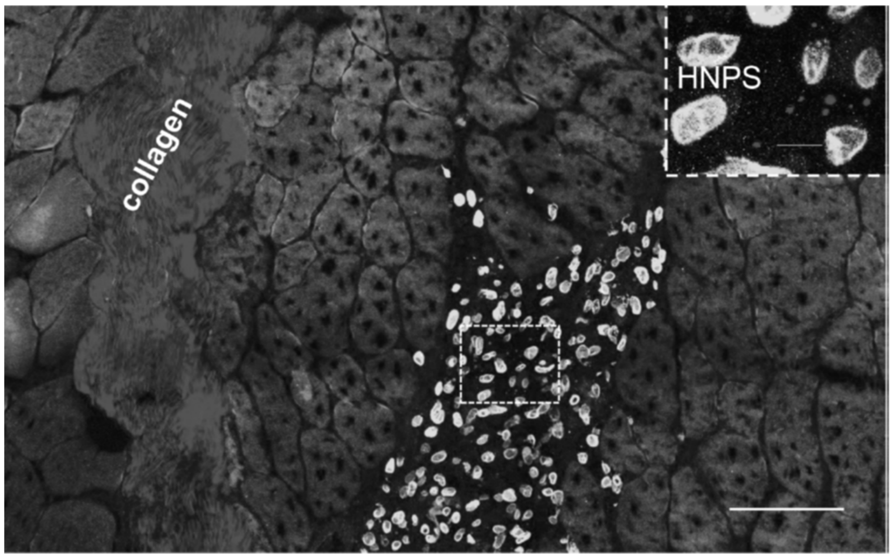




✓ Imaging depth > 1 mm in muscles

Excitation 1300 nm
Spectra Physics, Insight

L. Dubreil *et al.*
ACS Nano 2017



✓ NIR II excited SH and TH co-localization for enhanced selectivity

L. Dubreil *et al.*
ACS Nano 2017

Harmonic Nanoparticles

- Wavelength flexibility → NIR II imaging
- Long term photo-stability
- Multi-harmonic detection for cell tracking in tissue
- Multimodal detection (CT scan, CLEM)