607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2024/25, 1st semester

Part1:

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Lecture 5

607SM - TECNICHE AVANZATE DI INDAGINE MICROSCOPICA - AA 2024/25

1. Optical Microscopy

1.1. Basics (Image formation, magnification, resolution, image quality) NO → Thalhammer

1.2. Digital camera (image acquisition, formats, properties) NO → Thalhammer

1.3. Phase imaging (qualitative and quantitative)

1.4. Dark field and Polarization microscopy

Lecture 1

1.5. Super resolution microscopy – STED, PALM/STORM, SIM, MINFLUX , DNA PAINT, RISE ....

Lecture 2

1.6. Photobleaching-based Techniques for Assessing Cellular Dynamics

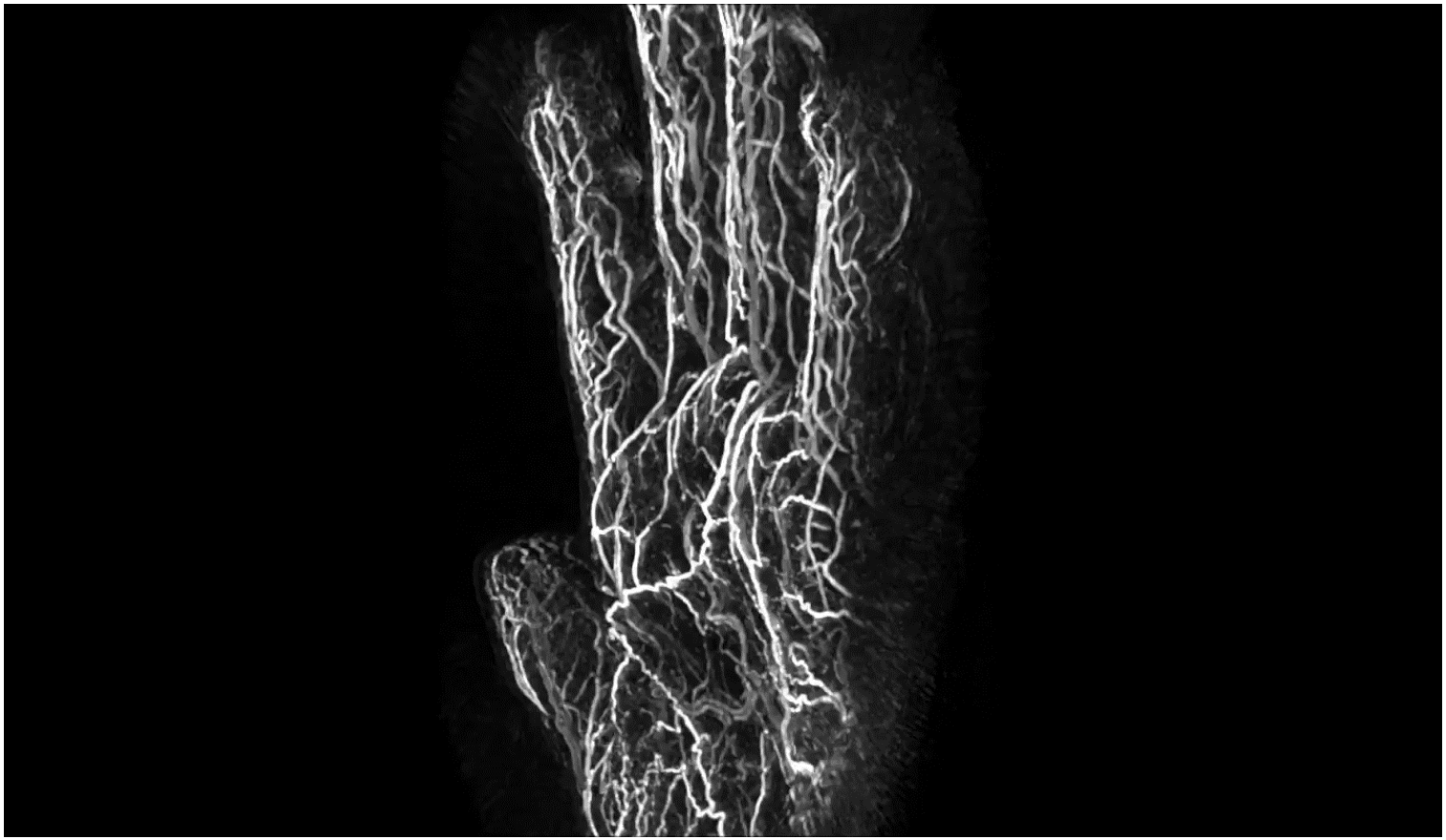
1.7. PhotoAcoustic Microscopy PAM

Lecture 3-4

1.8. Non Linear Optical Microscpy - Lecture 5

!!! The order of the last three topics is slightly changed from the initial plan

Remember last topic discussed PhotoAcoustic Microscopy PAM



Matsumoto et al. (2018) *Scientific Reports*

PAM (technique younger than 20 y) has the following notable features:

(1) PAM breaks through the optical diffusion limit, with highly scalable spatial resolution and maximum imaging depth in both the optical and acoustic domains.

(2) PAM images optical absorption contrast with 100% sensitivity i.e., a given percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic amplitude, and provides images without speckle artifacts.

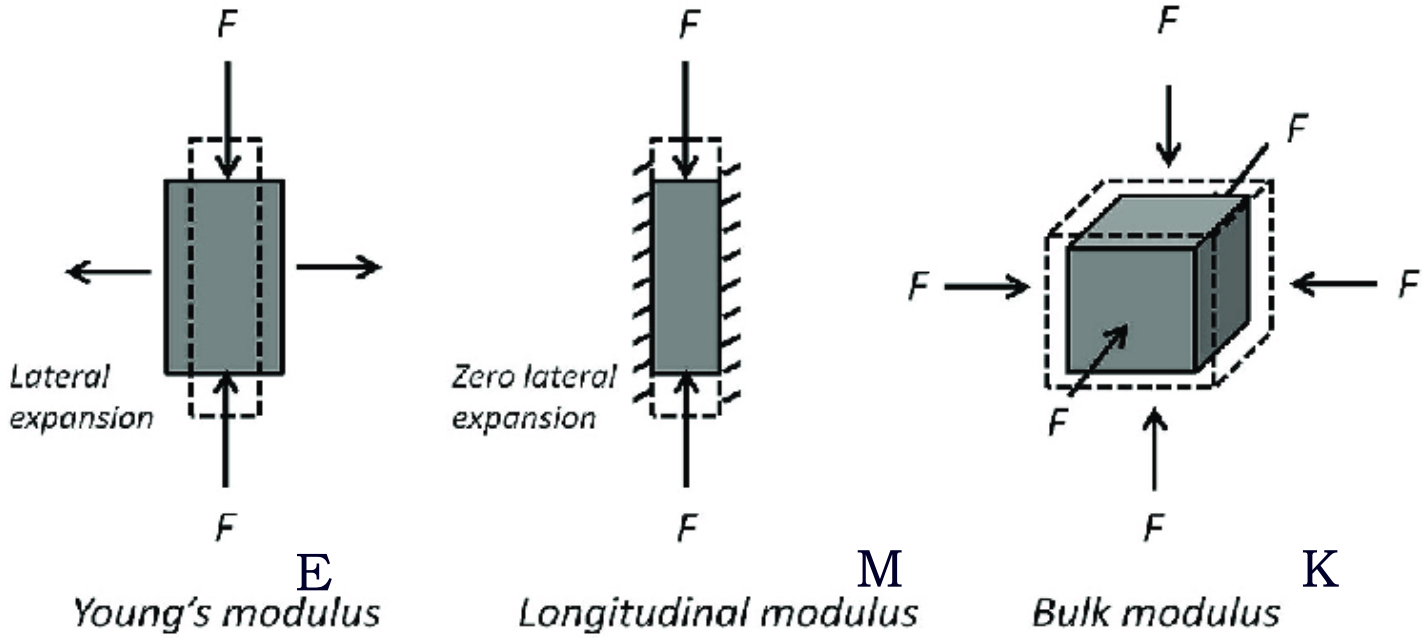
(3) PAM can essentially image all molecules at their absorbing wavelengths.

(4) PAM is capable of functional and metabolic imaging in absolute units using endogenous contrast agents.

PAM is expected to find new applications in both fundamental life science and clinical practice, which include but are not limited to tumor angiogenesis, lymphatic dynamics, neural activity, brain metabolism, cancer detection, drug delivery and intraoperative monitoring.

Brillouin Microscopy

Brillouin microscopy provides an alternative and complimentary assessment of material elasticity and viscosity through measurement of the longitudinal modulus in the GHz frequency range. What is longitudinal modulus?



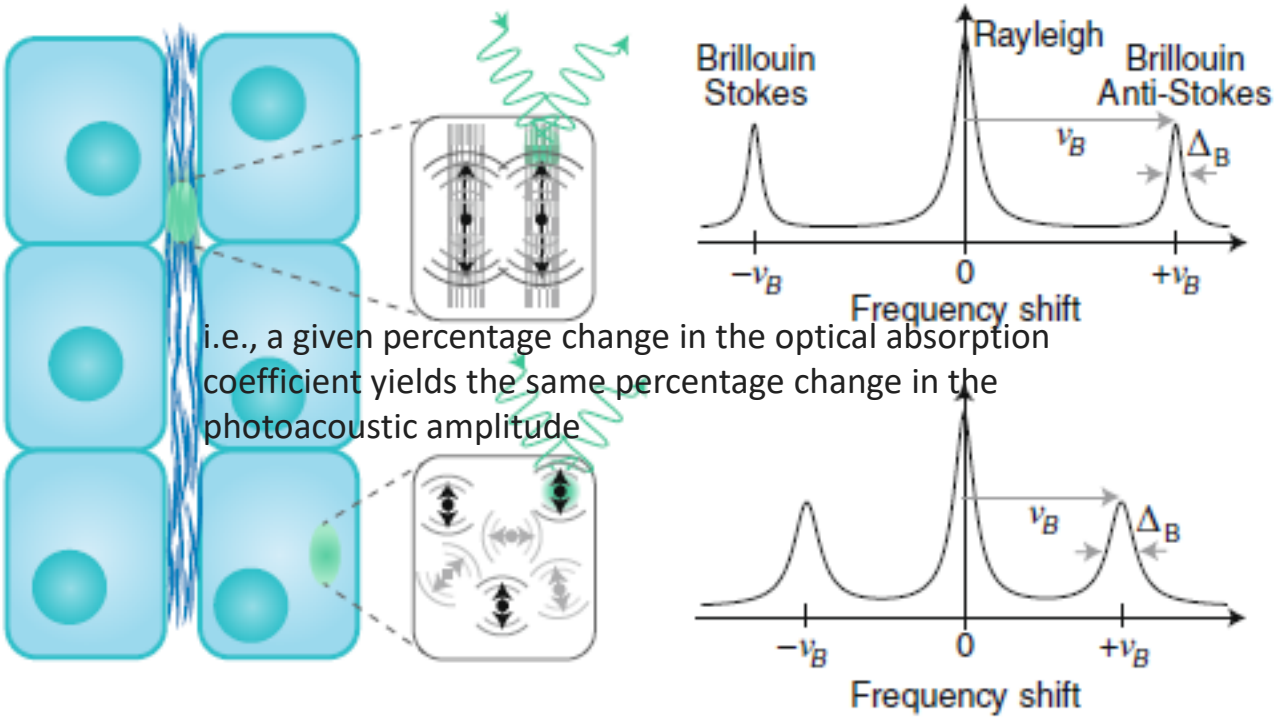
$$K = E/3(1 - 2\nu)$$

$$M = E(1 - \nu)/(1 + \nu)(1 - 2\nu)$$

ν Poisson ratio:
ratio between strain in the transverse direction to strain in the axial direction

Brillouin scattering in heterogeneous biological samples

Brillouin light scattering is an inelastic process arising from the interaction of light with spontaneous, thermally induced periodic density fluctuations (phonons) which can be described as a population of microscopic acoustic waves often, called phonons.



i.e., a given percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic amplitude

Monochromatic laser light is inelastically scattered by phonons propagating in the longitudinal (axial) directions. Light scattered off solid components (for example, collagen fibers; top) experiences a high Brillouin frequency shift. In contrast, the spectrum from the liquid-like cytosol (bottom) results in lower shift (indicating a less rigid material) and larger linewidth (indicating a more viscous medium).

Prevedel R. et al Nature Methods 2019

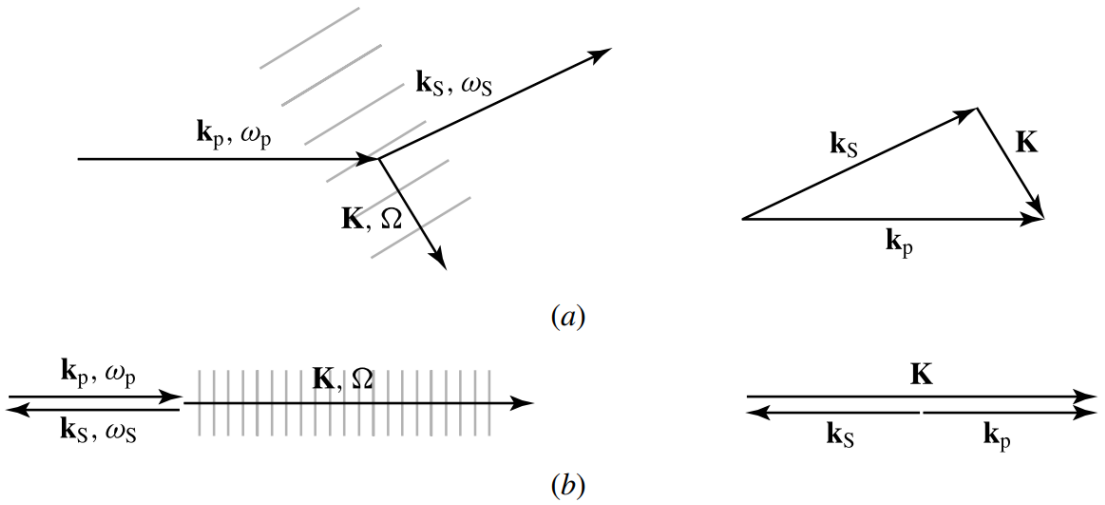
Raman vs Brillouin scattering

In Raman scattering photons are scattered by the effect of the light interaction with the vibrational and rotational transitions in the bonds between first-order neighboring atoms, while Brillouin scattering results from the scattering of photons caused by large scale, low-frequency phonons.

Both are inelastic, **non-linear optical processes** (characterized by the imaginary part of a complex 3rd order nonlinear susceptibility χ). Both cause a shift of the optical frequency of the probing beam (laser),

Raman or Brillouin Stokes process can be viewed as an interaction among a pump wave, a Stokes wave, and a material excitation wave. The material excitation wave is characterized by a frequency Ω and a wavevector K. The Stokes interaction is governed by the following conditions:

$$\Omega_s=\omega_p-\Omega;\ k_s = k_p - K$$



phase matching among the pump wave, the Stokes wave, and the material excitation

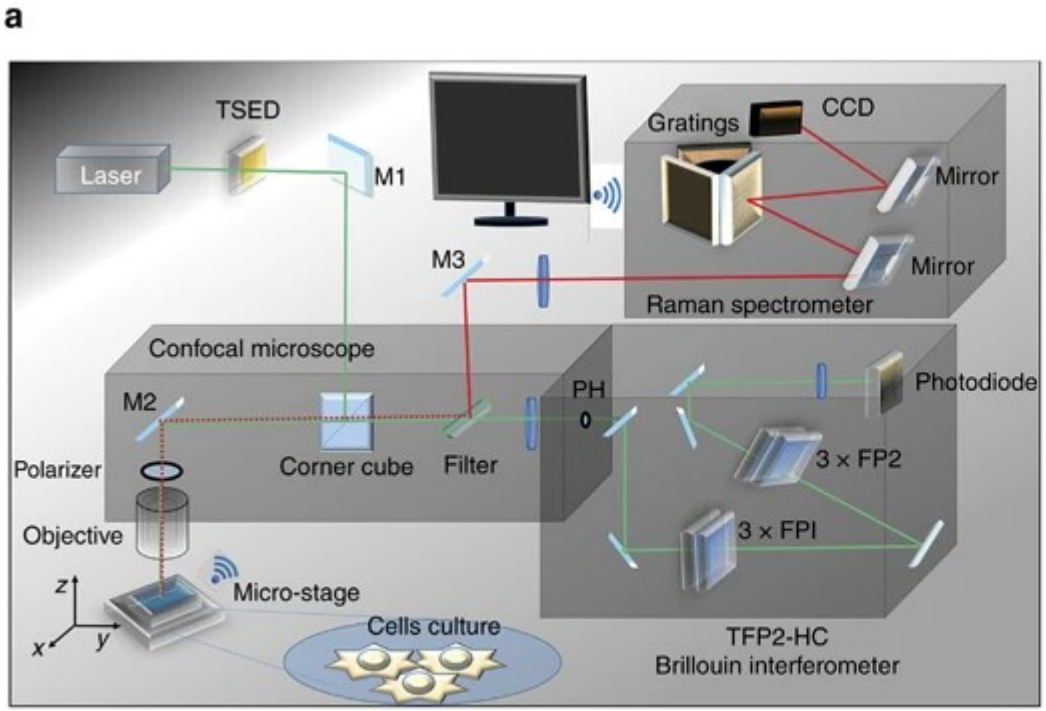
RAMAN scattering provides information on CHEMICAL composition,

BRILLOUIN scattering provides information on mechanical properties (elasticity, viscosity)

Raman vs Brillouin scattering

Tutorial on Brillouin scattering Wolff C. et al, J Opt Soc of America B (2021)

They can be performed on the same platform of a confocal microscope + interferometer + spectrometer



Mattana S. et all Light :Science & applications (2018)

Non contact mechanical and chemical analysis of single living cells by microspectroscopic techniques

Complex setup to buld and to use

Low efficiency (low number of photons from the incident light interact with sample for Raman and Brillouin):

Elastic Rayleigh scattering 10^{-4}

Inelastic Raman scattering 10^{-8}

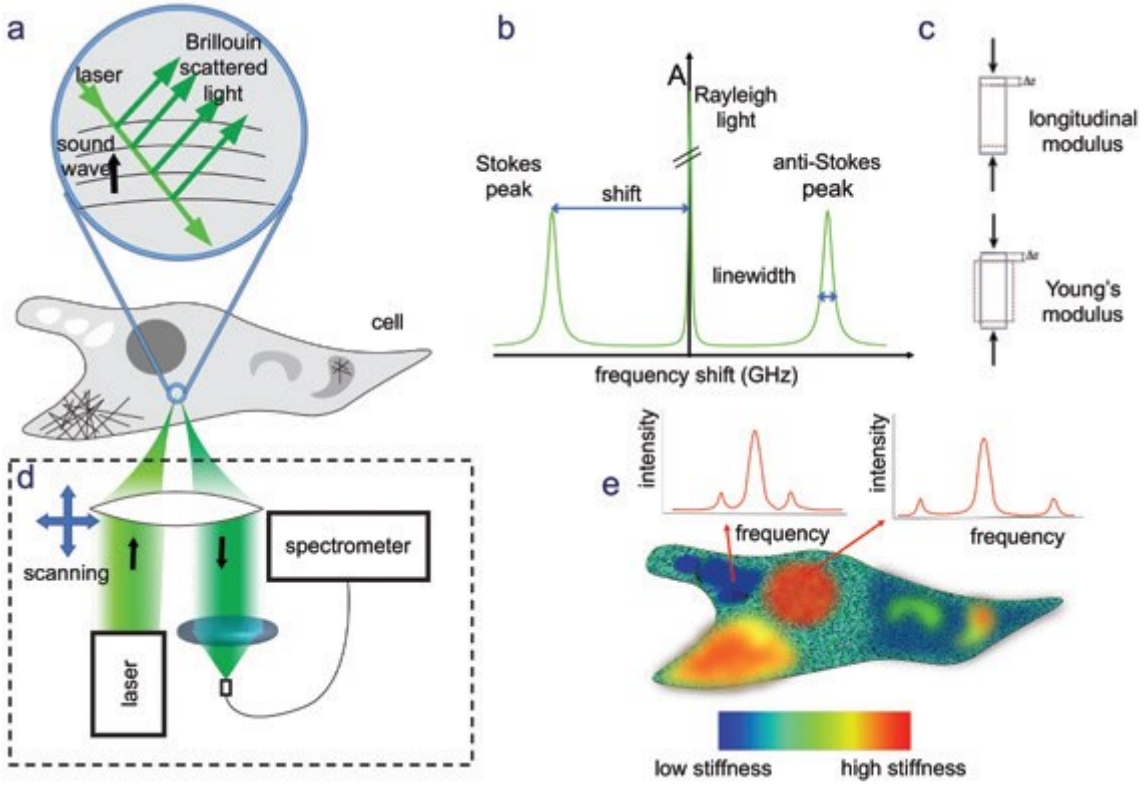
Brillouin scattering 10^{-12}

Brillouin microscope

The speed of the soundwave induced by the laser beam and its lifetime determine the frequency shift and the linewidth of Brillouin scattered light, typically in the GHz regime.

Confocal implementation of Brillouin microscopy: the laser is focused on the sample by an objective lens and Brillouin scattered light is collected by the same lens. Subsequently, a single mode fiber acts as a confocal pinhole and delivers the light to the spectrometer.

A stiffness map can be constructed by scanning the focal point across the sample and plotting the frequency shift at each position.



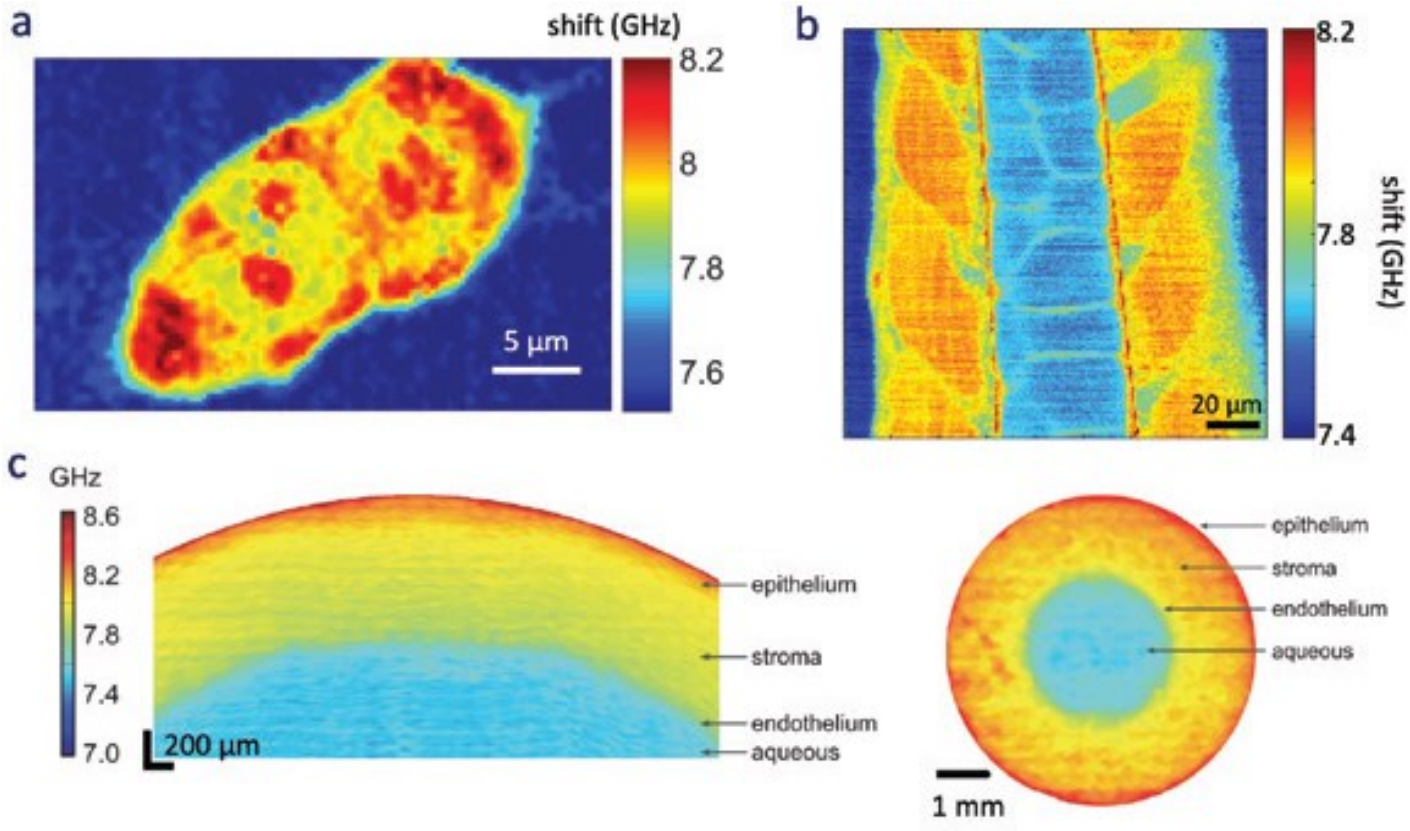
Bevilacqua et al InFocus Heidelberg 2019

Brillouin images - biological samples examples

a) A mouse embryonic stem cell, showing sub-cellular scale stiffness differences.

b) Tail of a zebrafish larva where anatomical structures (muscles, sheath cells, notochord) can be clearly distinguished based on their stiffness properties.

c) Left: Sections of a bovine cornea showing the heterogeneity of mechanical properties. Left: Cross section. Right: Optical section from a deep layer.



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1.8. Non Linear Optical Microscpy

Invention of laser in 1960 – is considered the most important event in the history of modern optics (intense coherent light) becaused it allowed the development of new technologies with applications in all the domains of science and life

LASER : Light Amplification by Stimulated Emission of Radiation

Two important properties:

- Coherence:

!A light field is called coherent when there is a fixed phase relationship between the electric field values at different locations or at different times.

- Collimation:

the laser beam propagates almost without diverging

With the discovery of the LASERof Optical Physics was open:

NONLINEAR OPTICS

deals mainly with various new optical effects and novel phenomena arising from interactions of intense coherent optical radiation with matter.

Linear Optics

To interpret the refraction, reflection, dispersion, scattering, as well as birefringence of light propagation in a medium, one should consider the electric polarization $\vec{P}$ induced in the medium by an electric (optical) field $\vec{E}$.

In Linear Optics the electric polarization is (linerally) proportional to the electric field strength

$$\vec{P} = \varepsilon_0 \chi \vec{E}$$

ε_0 is the electric permittivity in vacuum; χ is the electrical susceptibility (material constant)

$\alpha = \varepsilon_0 \chi$ - polarizability measures the ability of a material to become transiently polarized

Relationship between electrical susceptibility and refractive index

The refractive index n describes how matter affects light speed through electric permittivity ε and magnetic permeability μ ($\mu \approx \mu_0$ for a dielectric material)

$$n = \sqrt{\frac{\varepsilon}{\varepsilon_0}\frac{\mu}{\mu_0}} = \frac{c}{v}$$

The electric polarization can be also written as:

$$\vec{P} = (\varepsilon - \varepsilon_0)\vec{E} = \varepsilon_0 \chi \vec{E}$$

The refractive index n is a function of electrical susceptibility χ:

$$n^2 = \frac{\varepsilon}{\varepsilon_0} = 1 + \chi$$

Some properties of **Linear Optics**

1) In **linear optics** there is no coupling between different light beams or between different monochromatic components when they pass through a medium i.e. if there are several monochromatic optical waves of different frequencies passing through a medium simultaneously, no coherent radiation at any new frequency will be generated.

2) In **linear optics** the attenuation of the intensity of an optical beam propagating in an absorptive medium is **linear**, i.e. the decrease of the beam intensity in a unit propagation length is linearly proportional to the local intensity itself.

$$\frac{dI(z)}{dz} = -\alpha I(z) \quad \Longleftrightarrow \quad I(z) = I(0) \cdot e^{-\alpha z}$$

α - absorption coefficient of the medium

Demonstration of the attenuation relation in the following slides

In **NON - linear optics ,** a second-harmonic frequency wave can be generated and the attenuation of the intensity of an optical beam propagating in an absorptive medium can be quadratic or higher power electric field strength

One of the important features of the parameters described above (electrical susceptibility, refractive index, polarizability) is that they are frequency dependent

The wave equation (in a dielectric) :

$$\frac{\partial^2}{\partial z^2}E = \left(\frac{1}{v^2}\right)\frac{\partial^2 E}{\partial t^2} = \left(\frac{n^2}{c^2}\right)\frac{\partial^2 E}{\partial t^2}\Bigg)_{\substack{chem \\ material}}$$

states that the variation of the electric field with respect to the distance travelled is proportional to its variation with respect to time). The solution is of the form:

$$E(z,t) = E_{max}\cos(\omega t - kz + \phi_o) \quad \text{where:}$$

ω is the frequency, k the wave vector $k = \omega/v = \omega\, n / c = 2\pi\, n/\lambda$, and Φ_O the initial phase

To understand the frequency dependence, one may analyze the electric susceptibility by describing the chemical material as through it were a collection of N electrons bound to N atomic nuclei per volume. Since the charges are bound, they have a natural resonant frequency, designated as ω_0. Assuming a linear response to incident electric fields, and that oscillations fade with time after their source is turned off, the system acts as a damped harmonic oscillator.

Solving this system reveals that χ is given by:

$$\chi = \frac{Ne^2}{m_e\varepsilon_0}\frac{1}{\left(\omega_0^2 - \omega^2\right) + 2i\gamma\omega}$$

where the charge of an electron is e, the mass of the electron is m_e and the damping coefficient is γ (which is the inverse of the lifetime of the oscillator)

The electrical susceptibility χ is a complex quantity and a function of frequency:

$$\chi = \chi_{real} + i\chi_{imag}$$

$$\chi = \frac{Ne^2}{m_e\varepsilon_0}\frac{1}{\left(\omega_0^2 - \omega^2\right) + 2i\gamma\omega}$$

The physical meaning of this result: the photon (light) irradiation induces two independent physical response in the material:

- dispersion : propagation of the light through materials is wavelength dependent
- absorption : energy transfer to the structure of the material is wavelength dependent

Reference _0 Light Propagation in a Dielectric:

www1.udel.edu/chem/sneal/sln_tchng/CHEM620/CHEM620/Chi_3._Light-Matter_Interactions.html

For the Non-Linear Microscopy part there are also other 2 references
see material loaded on moodle: Reference_1 and _2

What about the refractive index n ? Since n is related to χ : $n^2 = 1 + \chi$

it means that also the refractive index is a complex quantity: $n^2 = \underbrace{1 + \chi_{real}}_{} + \underbrace{\chi_{imag}}_{}$

$n_{real} = \sqrt{1 + \chi_{real}}$

$n_{imag} = \sqrt{\chi_{imag}}$

$n = n_{real} + i \cdot n_{imag}$ ⟵ $n^2 = n_{real}^2 + i \cdot n_{imag}^2$

$$E(z,t) = E_{max} \cos(\omega t - kz + \phi_o)$$

Remember the solution of the wave equation ?

where the wave vector k is defined as : $k = \frac{2\pi}{\lambda} n$

Since n is complex, we have to re-write the solution of the wave equation using complex quantities:

$$\boldsymbol{E(z,t)} =$$

$$= E_{max} \cdot e^{i\omega t} \cdot e^{-i\frac{2\pi}{\lambda} n \cdot z} \cdot e^{\varphi_0}$$

$$= E_{max} \cdot e^{i\omega t} \cdot e^{-i\frac{2\pi}{\lambda} n_{real} \cdot z} \cdot e^{-\frac{2\pi}{\lambda} n_{imag} \cdot z} \cdot e^{\varphi_0}$$

The electric / optical field (scalar complex representation) :

$$\boldsymbol{E(z,t)} = E_{max} \cdot e^{i\omega t} \cdot \boldsymbol{e^{-i\frac{2\pi}{\lambda}n_{real}\cdot z}} \cdot \boldsymbol{e^{-\frac{2\pi}{\lambda}n_{imag}\cdot z}} \cdot e^{\varphi_0}$$

The intensity of the electrical / optical field:

$$\boldsymbol{I(z,t)} = |\boldsymbol{E(z,t)}|^2 = \boldsymbol{E(z,t)} \cdot \boldsymbol{E(z,t)}^* = E_{max} \cdot \boldsymbol{e^{-\frac{2\pi}{\lambda}n_{imag}\cdot 2\cdot z}} \cdot e^{2\cdot\varphi_0}$$

Modulus squared

$$I(z) = I(0) \cdot e^{-\alpha z}$$

$$\alpha = \frac{4\pi}{\lambda} n_{imag}$$

The intensity I(z) at the propagation distance z in the material/medium as a function of the initial intensity *I(0)* and the attenuation / absorption constant α

In linear optics the attenuation of the intensity of an optical beam propagating in an absorptive medium is linear

$$\frac{dI(z)}{dz} = -\alpha I(z)$$

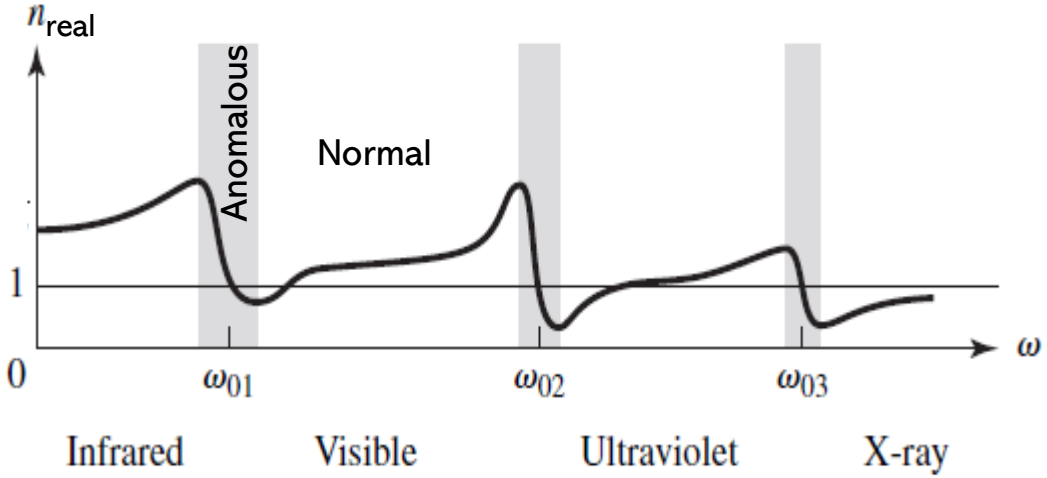
Remember: both n_{real} and n_{imag} are wavelength dependent !

Both n_{real} and n_{imag} are wavelength dependent !

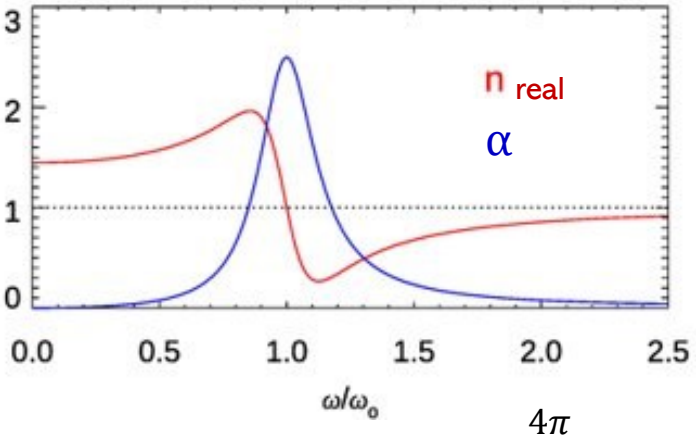
n_{real} – is related to dispersion

n_{imag} – is related to absorption

Dispersion vs light frequency



Dispersion - absorption around a resonance frequency



$$\alpha = \frac{4\pi}{\lambda} n_{imag}$$

OPTICS

the focus is off – resonances where n_{imag} is negligible and n_{real} is used (as n)

e.g. to control beam direction through refraction

SPECTROSCOPY

the focus is on resonances where n_{imag} is large and changes in n_{real} are small

In **linear optics** the attenuation of the intensity of an optical beam propagating in an absorptive medium is **linear:**

$$\frac{dI(z)}{dz} = -\alpha I(z) \quad \Longleftrightarrow \quad I(z) = I(0) \cdot e^{-\alpha z}$$

Non Linear Optics: for **high intensities** of the light, the attenuation constant α becomes a variable that depends on the incident intensity and the relation above does not hold

→ In a one-photon absorptive process / material, a saturable absorption effect takes place i.e. the intensity of light reaches a saturation threshold

→ In a two-photon absorption process / material:

$$\frac{dI}{dz} = -\alpha I - \beta I^2$$

where

β - is the two-photon absorption coefficient of the medium

Linear Optics (materials)

Electric Polarization $\vec{P}$

induced in a medium by an electric field $\vec{E}$

$$\vec{P} = \varepsilon_0 \chi \vec{E}$$

ε_0 is the electric permittivity in vacuum; χ is the electrical susceptibility (material constant)

Polarizability $\alpha = \varepsilon_0 \chi$ measures the ability of a material to become transiently polarized

Non Linear Optics

$$\vec{P} = \varepsilon_0 \left[\chi^{(1)} \vec{E} + \chi^{(2)} \vec{E}^2 + \chi^{(3)} \vec{E}^3 + \ldots \right]$$

$$= \vec{P}_{Linear} + \vec{P}_{non-linear}$$

In a non-linear optical medium (e.g. some crystals) the electric polarization P is a nonlinear function of E, i.e. P depends not only of E but also of E^2, E^3, with correspondent proportional constants (susceptibility) $\chi^{(1)} \chi^{(2)} \chi^{(3)}$

This generalization allowed to explain / model the experimental results obtained with the discovery of lasers and use of high power / intensity beams

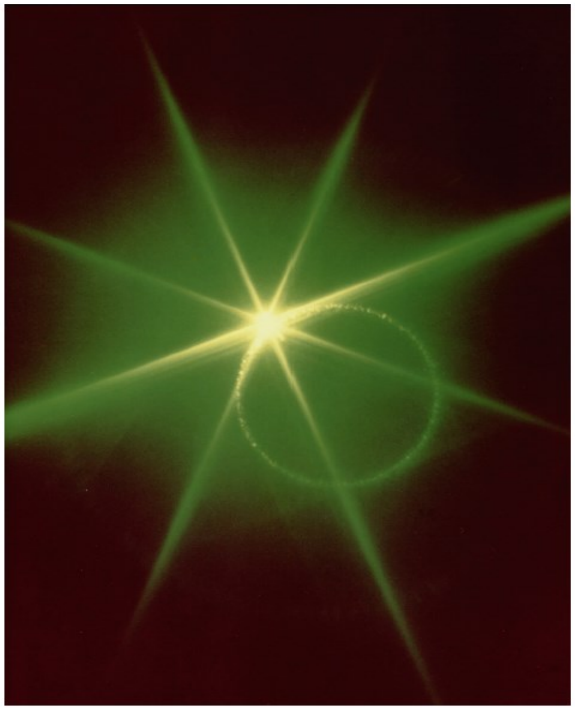
The nonlinearity can lead to the generation of new frequency components (explained by the Maxwell equations in non linear medium)

Nonlinear Optics can produce many exotic effects

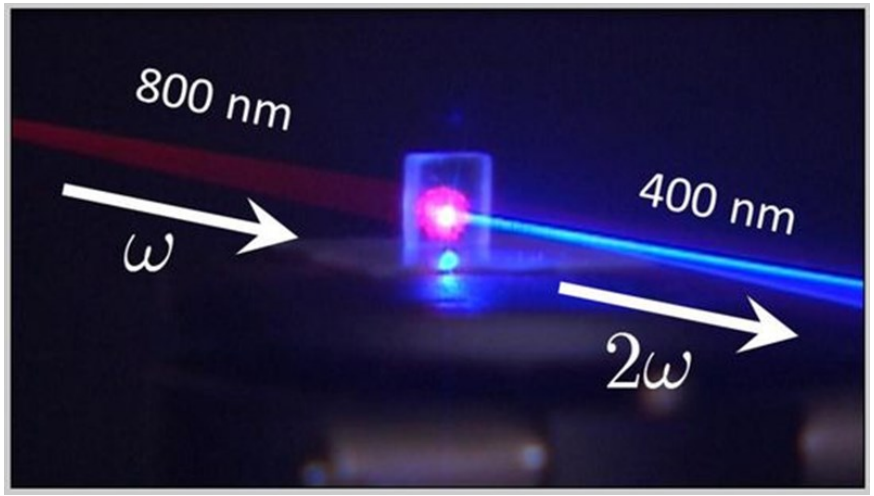
Sending infrared light into a crystal yielded this display of green light:

Nonlinear optics allows us to change the color of a light beam, to change its shape in space and time, and to create the shortest events ever made by humans.

Nonlinear optical phenomena are the basis of many components of optical communications systems, optical sensing, and materials research.



Second Harmonic Generation SHG (generating a light with double frequency of that of the incident beam)



Nonlinear Optical Microscopy NLO

NLO exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths

NLO microscopy offers:

- deep tissue penetration (>500 um) since the infrared excitation wavelengths inherently provide a reduced light scattering and absorption
- overcoming the use of staining, allowing one to obtain rich morphological /structural /molecular information from a sample which shows nonlinear properties and/or distinctive chemical composition

The most relevant NLO microscopy techniques for biological investigations are:

- **two-photon excited fluorescence (TPEF)**
- **second and third harmonic generation (SHG and THG)**
- **coherent Raman scattering (CRS)**

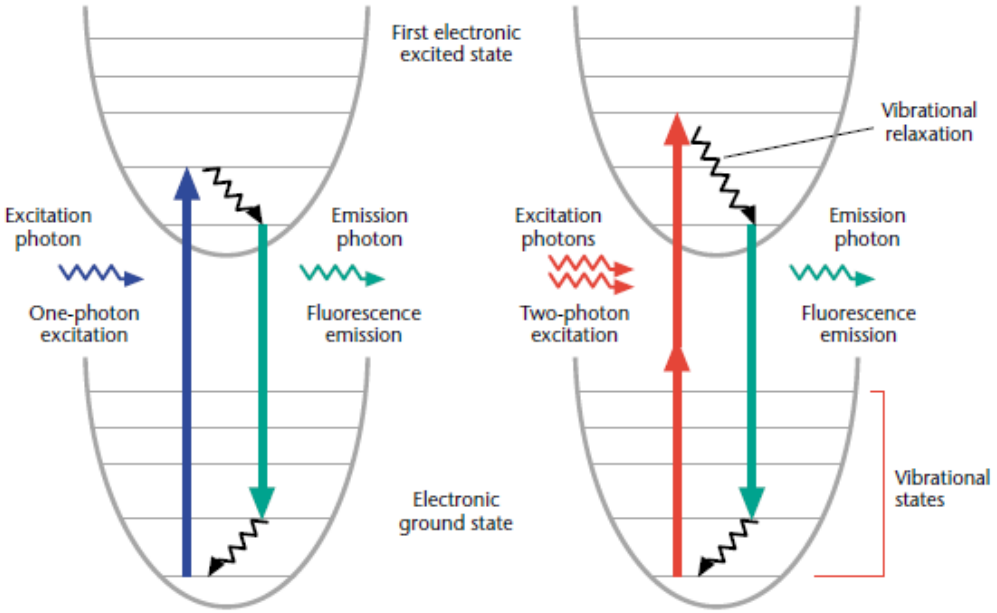
Multiphoton microscopy MM is regarded as the method of choice for imaging of living, intact biological tissues on length scales from the molecular level through the whole organism.

MM is suited to perform experimental measurements with minimal invasion over long periods of time, thereby providing exquisite detail of inherently dynamic biological processes having time scales from microseconds to days or weeks.

Two Photon Process - principle

Jablonski diagram of one photon and two-photon excitation

1 Photon



2 Photon

Two-photon excitation is a fluorescence process in which a fluorophore is excited by the simultaneous absorption of two photons.

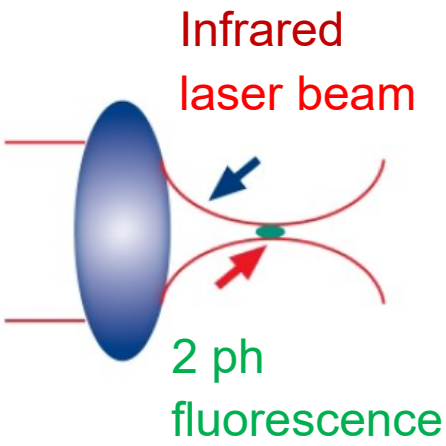
The probability, *p* of 2 photon absorption is proportional to the intensity, the intensity of the beam squared I^2 :

$$p \sim I^2$$

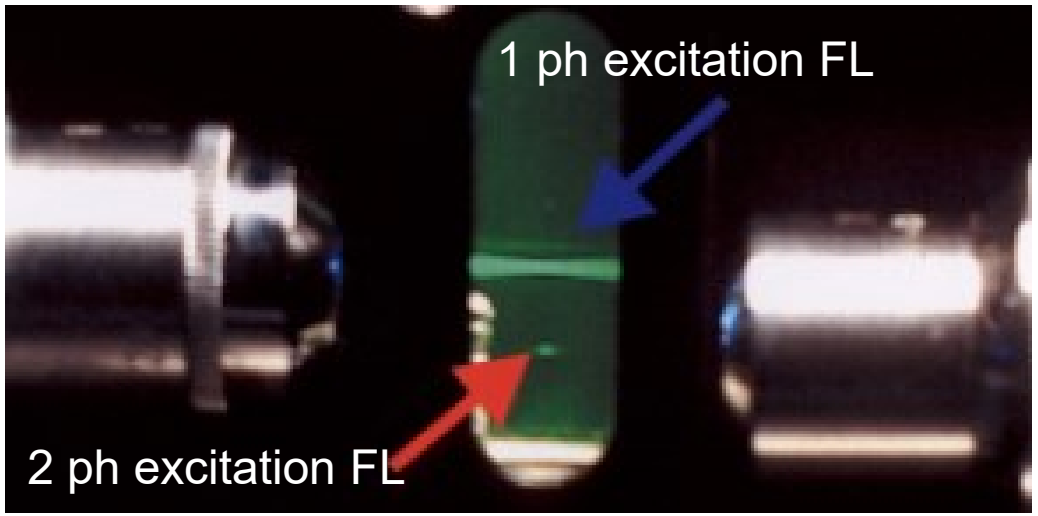
Two Photon is a 3rd order NLO process discovered in 1931 by Maria Göppert-Mayer !!! doctoral thesis !!!

$$P = \varepsilon_0 \left[\chi^{(1)} E + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \ldots \right]$$
$$= P_{Linear} + P_{non-linear}$$

The localization of excitation in 2 ph



1 ph vs 2 ph excitation



The excitation in 2 ph is localized only in the focus of the beam.

However, the size of the excited volume (voxel) is given by the lateral (radial) and axial resolution (spot) of the IR beam, which are still diffraction limited !

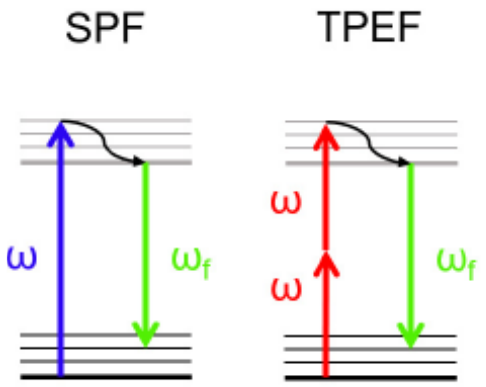
2 ph excitation is somehow similar to TIRF but it can be moved inside the sample, while in TIRF is limited to the coverslip interface.

The big advantage of 2 ph is the use of IR light which allows to go and excite dyes deeper in tissues (~ 500 um).

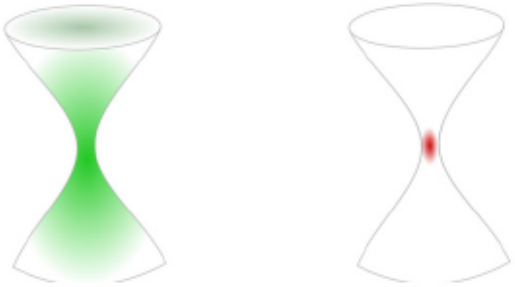
Beside imaging, 2ph is very useful to produce localized chemical reactions.

Single Photon Fluorescence (SPG) vs Two Photon (Excited) Fluorescence (TPEF)

Jablonski diagrams

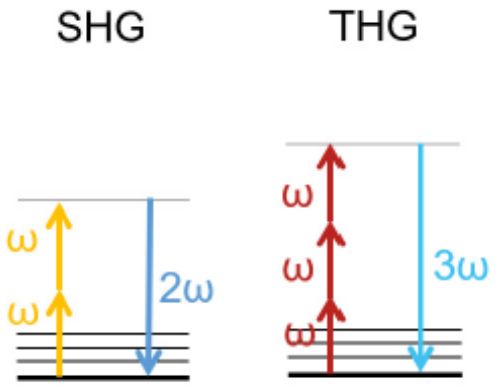


Size of the excited volume



TPEF is a 3rd order NLO process discovered in 1931 by Maria Göppert-Mayer !!! doctoral thesis !!!

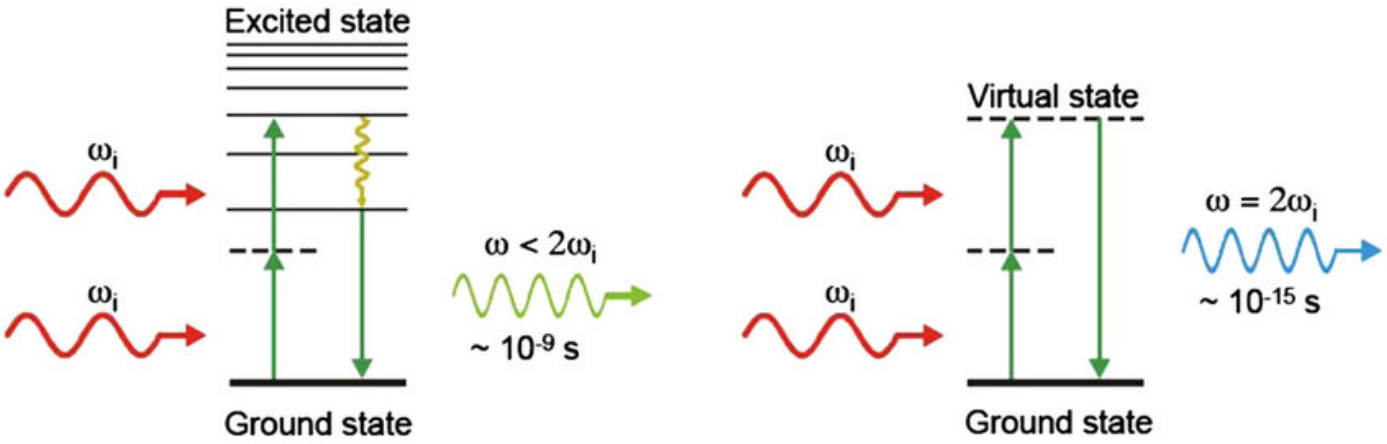
Second and Third Harmonic Generation SHG THG



$$P = \varepsilon_0 \left[\chi^{(1)} E + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \ldots \right]$$
$$= P_{Linear} + P_{non-linear}$$

SHG and THG are 2nd and 3rd order NLO process

The difference between TPEF and SHG



Two-Photon Excited Fluorescence

Involves real transition
Energy is partially lost
Nanosecond response time
Frequency lower than SHG

Second Harmonic Generation

Involves virtual transition
Energy is conserved
Femtosecond response time
Frequency exactly doubled

Summary of the main properties of TPEF , SHG and THG microscopy techniques

Principle	Contrast mechanism	Biological target	Advantages	Limits
Two low energy photons simultaneously absorbed to excite a fluorescent species	Fluorescence	Endogenous fluorescent proteins and molecules, cell autofluorescence	• High penetration depth (>500 μm) • High resolution below the diffraction limit of light • High sensitivity, down to the single molecule • Intrinsic 3D scanning capability • Applied *in vitro*, *in vivo* (intravital) and in fixed samples • Can be coupled with FLIM, FRET and FRAP techniques	• Risk of photodamage
Two photons at the same frequency simultaneously interact with nonlinear species to generate a photon with the double frequency	Second-order nonlinear polarization of non-centrosymmetric structures	Non-centrosymmetric biological structures such as: collagen fibrils I/II, myofibrils, myosin filaments, microtubules	• Intrinsic 3D scanning capability • Fibrillar orientation dependence • Usually coupled with TPEF • Applied *in vitro*, *in vivo* (intravital) and in fixed samples • Low risk of photodamage • Easy interpretability	• Backscattering signal is less intense than forward one. • Fixation did not affect signal generation except in microtubular imaging • Low intensity signal
Three photons simultaneously interact with a medium with refractive index mismatch to generate a photon with the triple frequency	Refractive index mismatch within inhomogeneous medium	Interfaces between lipid and water, cell membranes and vesicles	• Intrinsic 3D scanning capability • Completely label-free • Applied *in vitro*, *in vivo* (intravital) and in fixed samples • Low risk of photodamage	• High infrared wavelength (>1200 nm) • Depend on distribution of signal eliciting-structures and incoming light polarization

TPEF

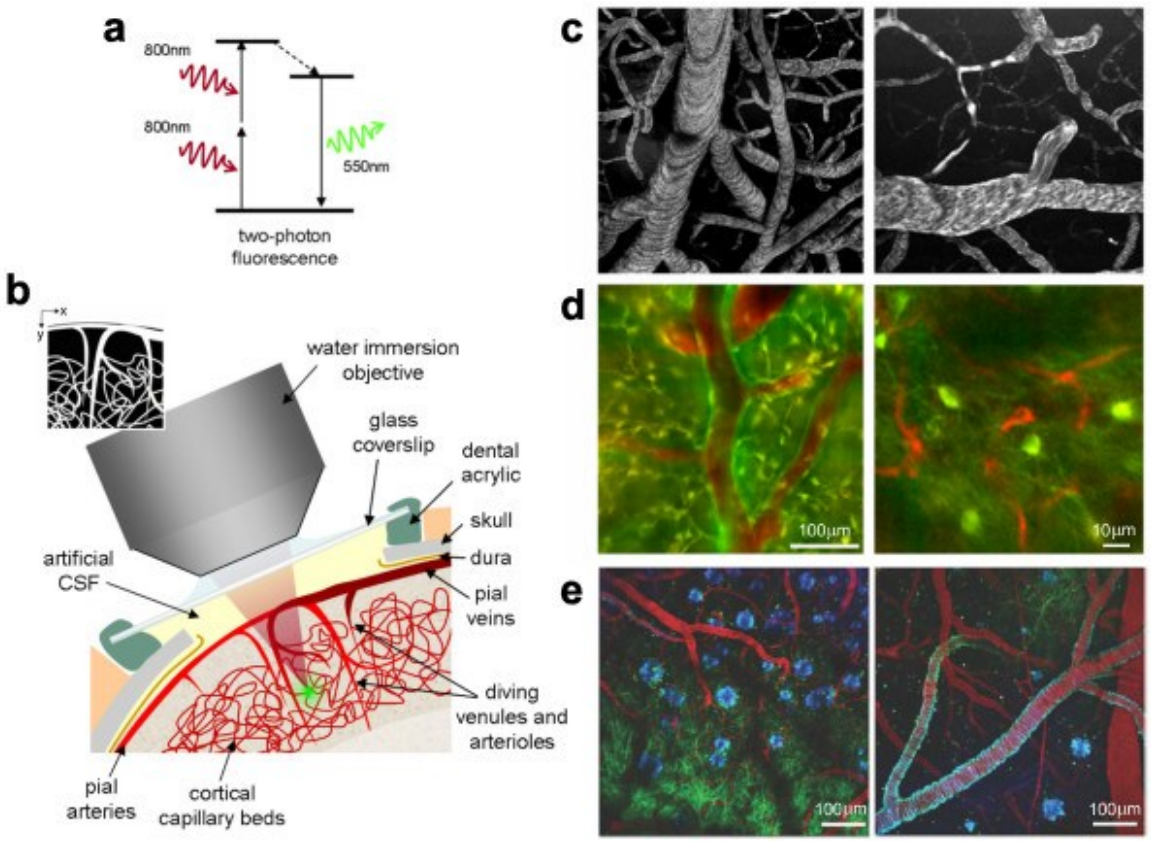
allows the visualization of both exogenous (dye molecules, quantum dots, and fluorescent proteins such as GFP, RFP, and YFP) and endogenous fluorophores (such as nicotinamide adenine dinucleotide phosphate-NAD(P) H-, flavin adenine dinucleotide - FAD-, flavoprotein -FP-), and it is often exploited in fluorescence lifetime imaging (FLIM) studies, resulting suitable for metabolic and hybrid investigations.

SHG

THG

REVIEW : Parodi V, et al (2020) Nonlinear Optical Microscopy: From Fundamentals to Applications in Live Bioimaging. Front. Bioeng. Biotechnol. 8:585363. doi: 10.3389/fbioe.2020.585363

Two-photon microscopy of in vivo brain function.



(b) Schematic of surgical preparation of exposed cortex, with sealed glass window and microscope objective positioning. Green dot shows location of two-photon fluorescence.

(c) Examples of two-photon maps of the vasculature following intravenous injection of dextran-conjugated fluorescein. Black dots and stripes show red blood cell motion.

(d) Dual-channel imaging of neuronal (green) and vascular (red) signals:

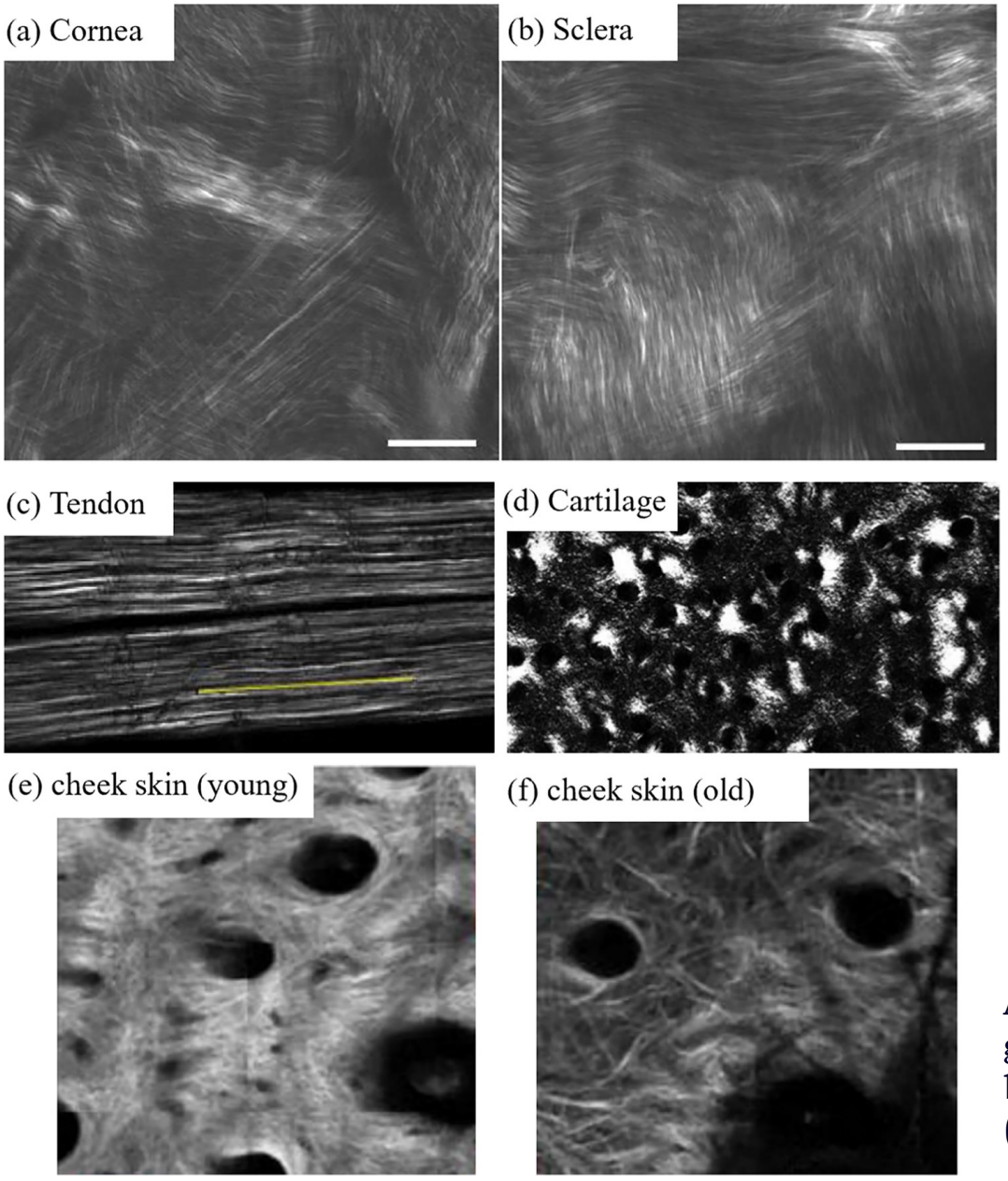
(left) Oregon Green 488 BAPTA-1 AM calcium sensitive dye stained neurons and

(right) transgenic mouse expressing green fluorescent protein (GFP) in a subpopulation of neurons Texas dextran red is the intravascular tracer in both cases.

(e) Three channel imaging of Tg2576 APP Alzheimer's disease mouse model with amyloid-targeting dye (blue), GFP expressing neurons and dendrites (green) and vasculature (red).

Hillman EM: Optical brain imaging in vivo: techniques and applications from animal to man.
J Biomed Opt 2007,12(5):051402

SHG microscopy of different samples



a) cornea and (b) sclera, the scale bar is 20 µm. Collagen fibrils type I in two different components: lamellar configuration in cornea, randomly packed and highly scattering

c) Tendon (500 × 150 µm)

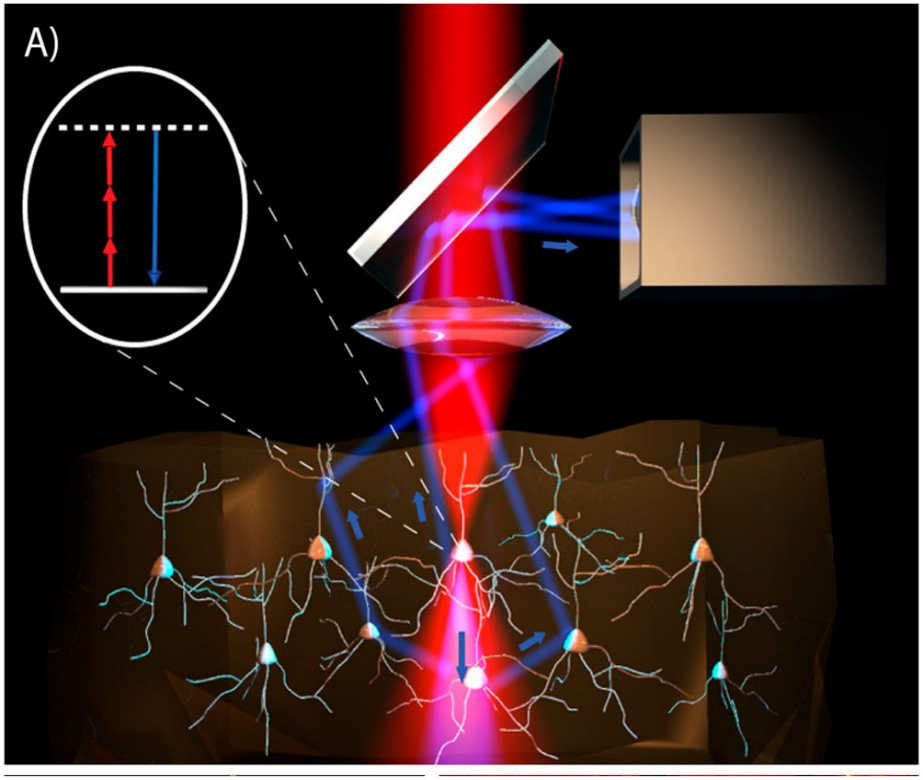
d) Cartilage (350 × 200 µm)

e-f) SHG image of skin in young vs. old age (1.6 × 1.6 mm)

Aghigh, A., et al. Second harmonic generation microscopy: a powerful tool for bio-imaging. Biophys Rev 15, 43–70 (2023). Doi:10.1007/s12551-022-01041-6

Label-free live brain imaging with THM microscopy

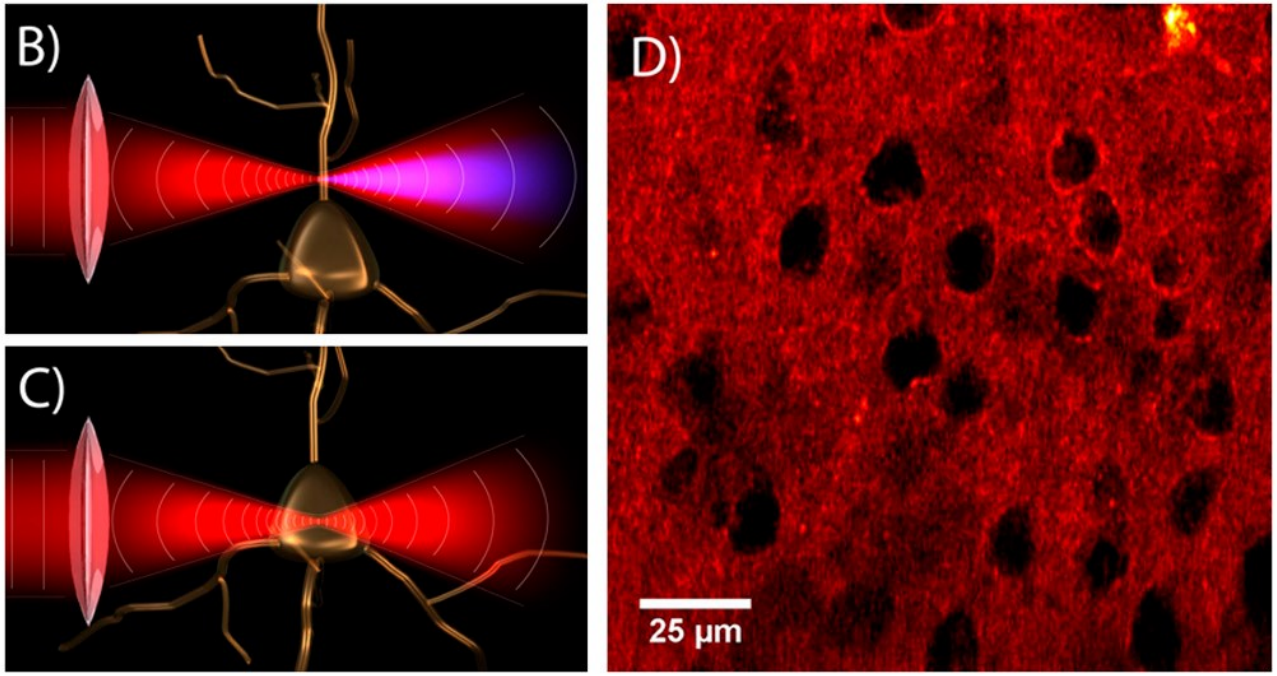
High-contrast imaging of live brain tissue at cellular resolution, without the need for fluorescent probes, using THG. The specific geometry and lipid content of brain tissue is exploited at the cellular level to achieve THG, providing an alternative contrast mechanism to fluorescence.
THG brain imaging allows rapid, noninvasive label free imaging of neurons, white-matter structures, and blood vessels simultaneously.



Schematic of THG microscopy on brain tissue in an epidetection geometry.

S. Witte et al Label-free live brain imaging and targeted patching with third-harmonic generation microscopy, Proc. Natl. Acad. Sci. U.S.A. 108 (15) 5970-5975, https://doi.org/10.1073/pnas.1018743108 (2011).

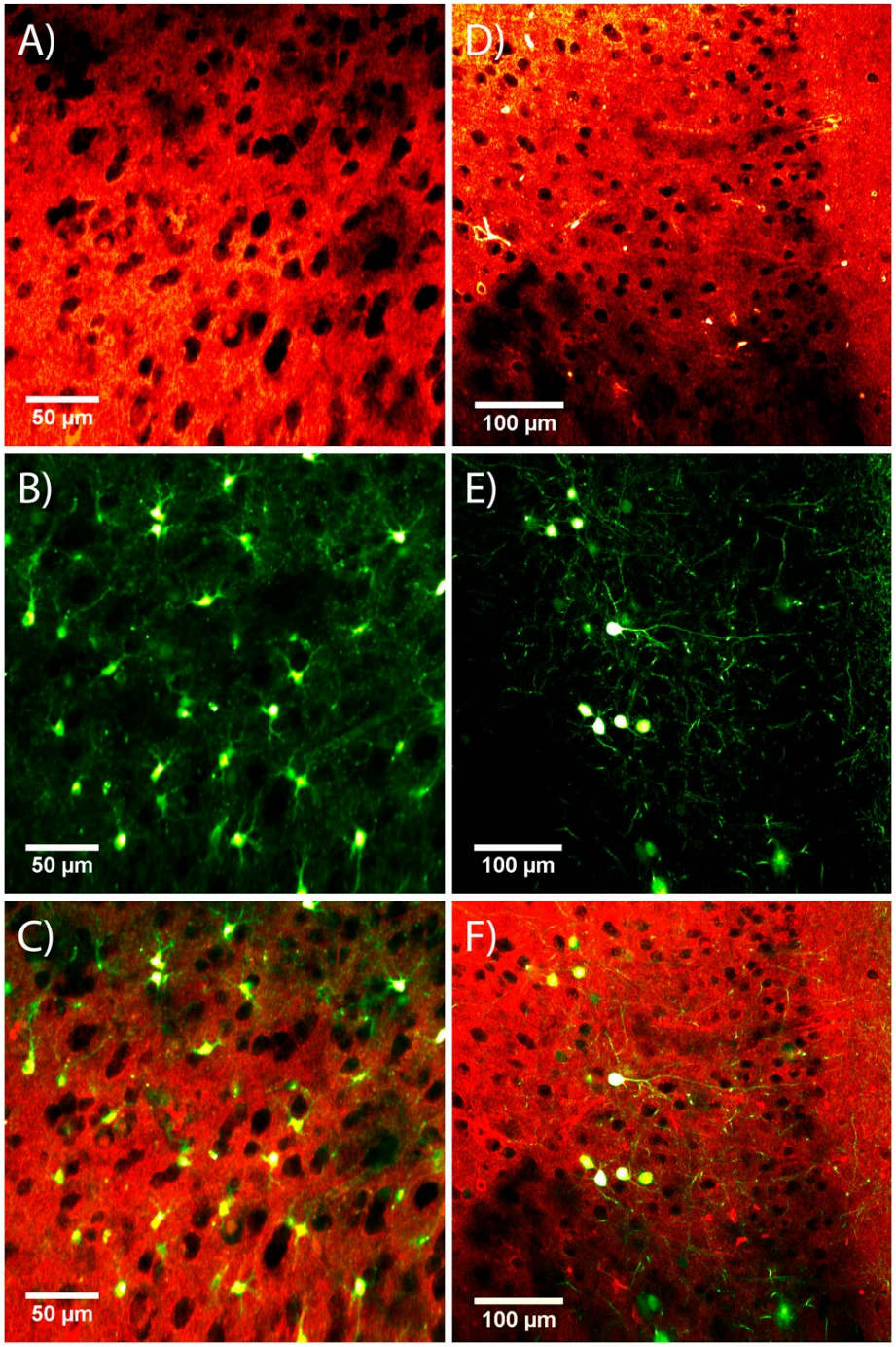
Label-free live brain imaging with THM microscopy



(B) Focused laser beam at a dendrite. By setting the laser focal volume several times larger than the dendrite diameter, partial phase matching is achieved, and a significant THG signal is produced.

(C) Focused laser beam in the cell body. Due to the poor structural phase-matching conditions, no THG is produced.

(D) THG microscopy image of living neurons in mouse brain tissue. The somata appear as dark shadows.



Combined THG and 2PLSM imaging.

(A) THG image of mouse prefrontal cortex.

(B) 2PH fluo image of astrocytes labeled with SR-101.

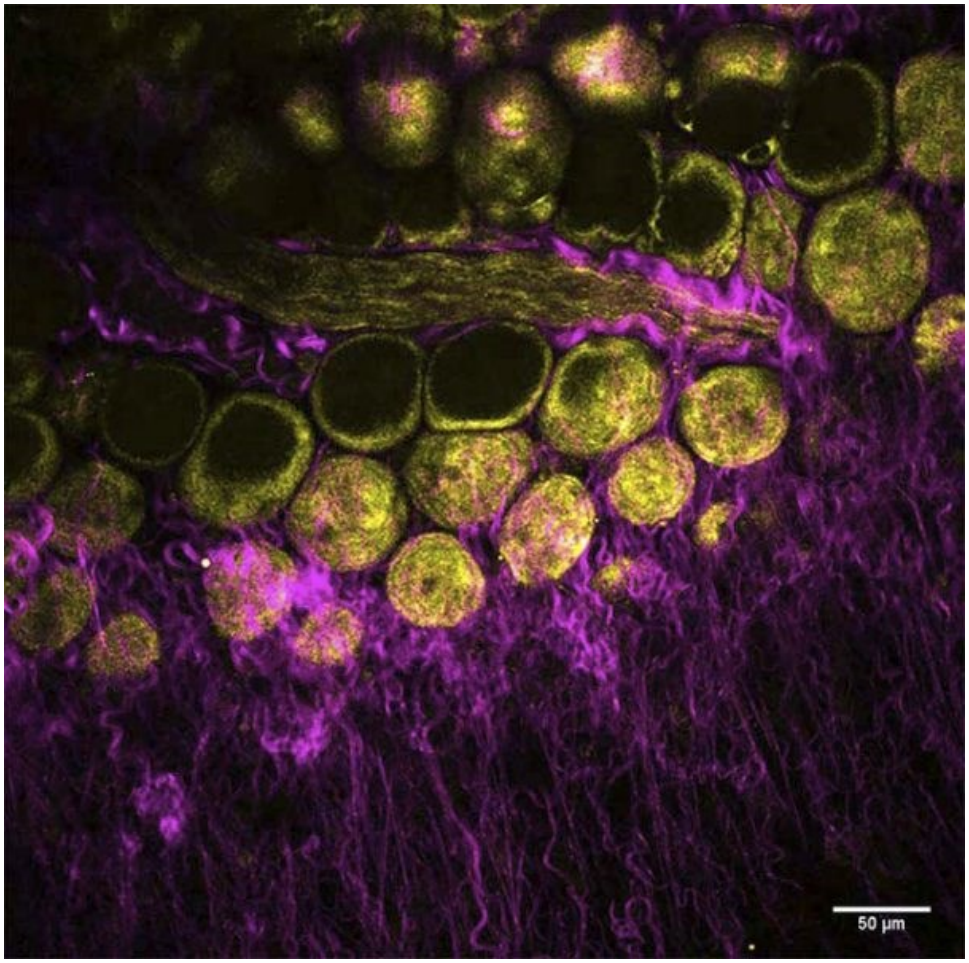
(C) Overlay of A and B, providing information on the distribution of astrocytes within the neuronal network.

(D) THG image of mouse prefrontal cortex.

(E) 2PH fluo image of GFP-labeled somatostatin-positive neurons.

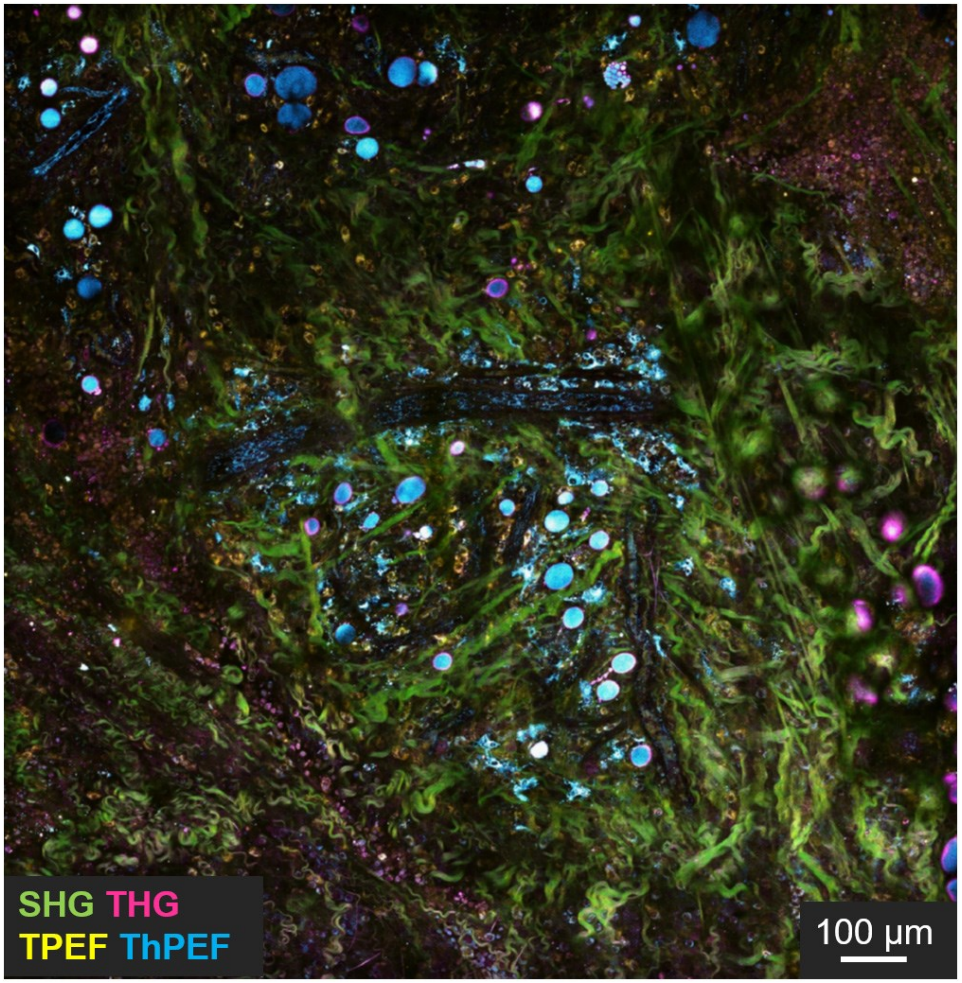
(F) Image overlay of D and E, showing the distribution of somatostatin-positive cells in the structure of the PFC.

Combined SHG and THG imaging



Mouse mammary gland, label free image of
collagen (SHG, magenta) and adipocytes (THG, yellow),

Combined TPEF, SHG and THG imaging



Intravital imaging of mouse tumor microenvironment showing

SHG (green), THG (magenta), TPEF (yellow), and three photon fluorescence (cyan) signals.

In evidence the green signal from collagen fibers, cyan from NADH, yellow from FAD and magenta from interfaces. Scale bar = 100 um.

Nicotinamide Adenine Dinucleotide Phosphate
Flavin Adenine Dinucleotide

You et al. 2018 Nat. Comm

Spontaneous Raman Scattering and Coherent Raman Scattering Microscopies

Spontaneous Raman Scattering (SRS) microscopy

Raman scattering is a powerful technique for label-free identification of a molecule/material based on the characteristic vibrational spectrum.

In SR microscopy, a monochromatic laser at frequency ωp ("pump") excites the molecules to a virtual state, which then relax to the ground state scattering photons with lower frequency ωs ("Stokes").

The inelastic frequency shifts:

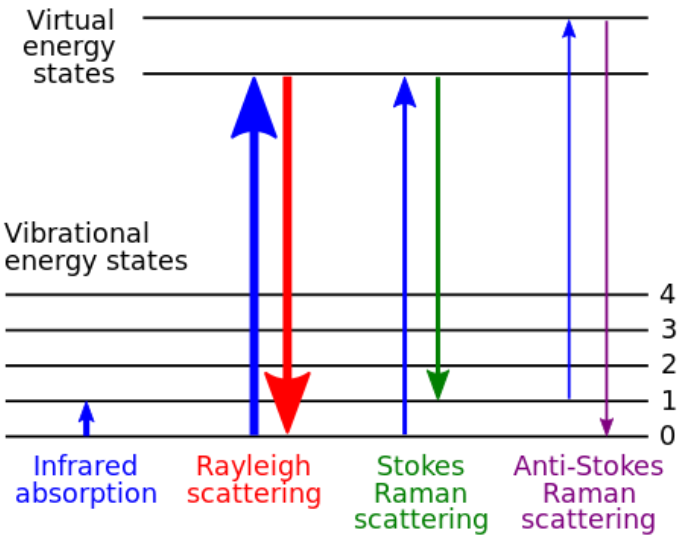
$$\Omega = \omega p - \omega s$$

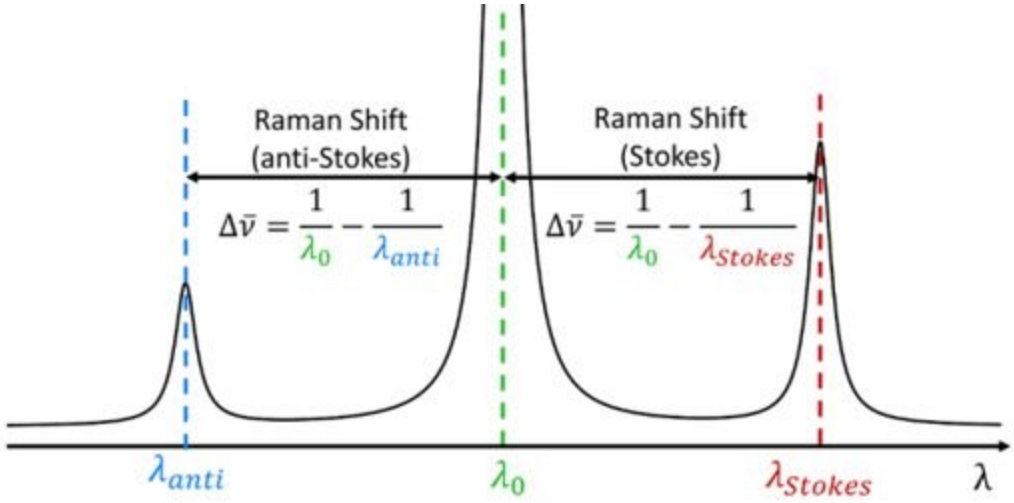
match the molecular vibrations, which in turn reflect the molecular structure.

The resulting SR spectrum provides a detailed picture of the biochemical composition of the measured cells/tissues

Drawback: very weak scattering cross section, > 10^{10} order of magnitude lower than fluo.

Energy-level diagram showing the states involved in Raman spectra





Raman shifts are typically reported in wavenumbers, which have units of inverse length. In order to convert between spectral wavelength and wavenumbers of shift in the Raman spectrum, the following formula can be used:

$$\Delta\tilde{\nu} = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_1}\right),$$

where $\Delta\tilde{\nu}$ is the Raman shift expressed in wavenumber, λ_0 is the excitation wavelength, and λ_1 is the Raman spectrum wavelength.

$$\Delta\tilde{\nu}(\mathrm{cm}^{-1}) = \left(\frac{1}{\lambda_0(\mathrm{nm})} - \frac{1}{\lambda_1(\mathrm{nm})}\right) \times \frac{(10^7\,\mathrm{nm})}{(\mathrm{cm})}.$$

Coherent Raman Scattering (CRS) microscopy

overcomes Simultaneous Raman Scattering SRS limitation by generating the Raman signal from a coherent superposition of the molecules in the sample, illuminated by

two synchronized ultrashort laser pulses of different color:

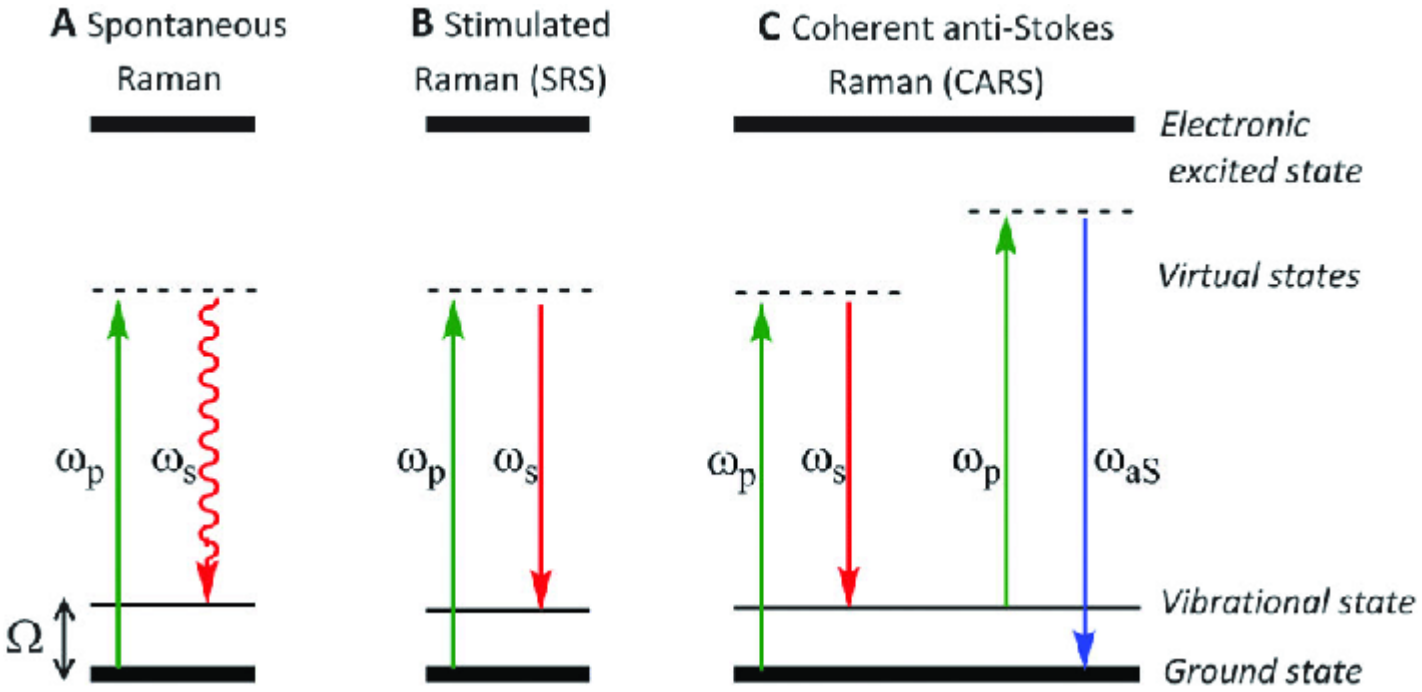
the pump (at frequency ωp) and the Stokes (at frequency ωs).

When the difference between pump and Stokes frequencies matches the vibrational frequency Ω, i.e. $\Omega = \omega p - \omega s$

then all the molecules in the focal volume are resonantly excited and vibrate in phase.

This vibrational coherence enhances the Raman response by many orders of magnitude with respect to the incoherent SRS process, decreasing the acquisition times from seconds to microseconds per pixel.

The two most widely employed **CRS** techniques are **C**oherent **A**nti-Stokes **R**aman **S**cattering (**CARS**) and **St**imulated **R**aman **S**cattering (**StRS**)

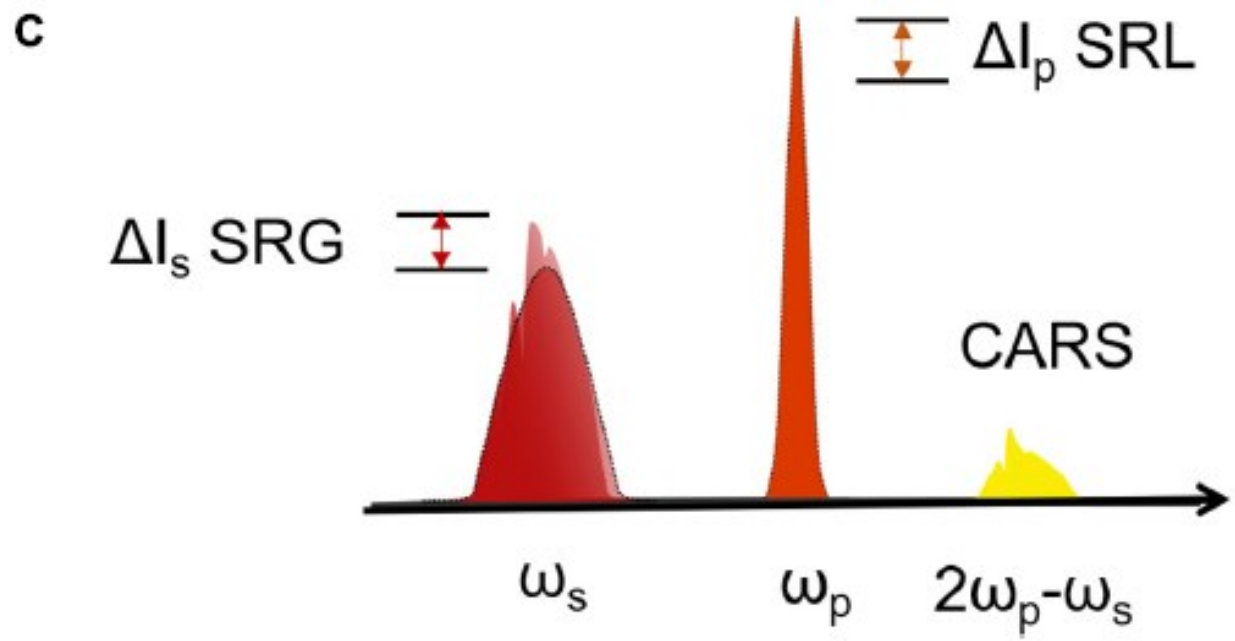


CARS and StRS are third order nonlinear phenomenons.

In CARS the vibrational coherence is read by a further interaction with the pump beam, generating a coherent radiation at the anti-Stokes frequency $\omega_{as} = \Omega + \omega_p = 2\omega_p - \omega_s$

In StRS the coherent interaction with the sample induces stimulated emission from a virtual state of the sample to the investigated vibrational state, resulting in a Stokes field amplification (Stimulated Raman Gain, SRG) and in a simultaneous pump-field attenuation (Stimulated Raman Loss, SRL).

The two most widely employed **CRS** techniques are
Coherent **A**nti-Stokes **R**aman **S**cattering (**CARS**) and **St**imulated **R**aman **S**cattering (**StRS**)



Schematic representation of the excitation and emission frequencies involved in StRS and CARS: in evidence the StRS signal in terms of gain (SRG) on the Stokes pulse or the loss (SRL) on the pump pulse.

CARS benefits from being a background-free process, since the emitted signal has a frequency ω_{aS} differing from those of pump and Stokes

CARS suffers from the so-called non-resonant background (NRB) generated both by the molecular species under study and by the surrounding medium, according to a four-wave mixing scheme. The NRB does not carry any chemically specific information and, when the concentrations of the target molecules are low, can distort and even overwhelm the resonant signal of interest.

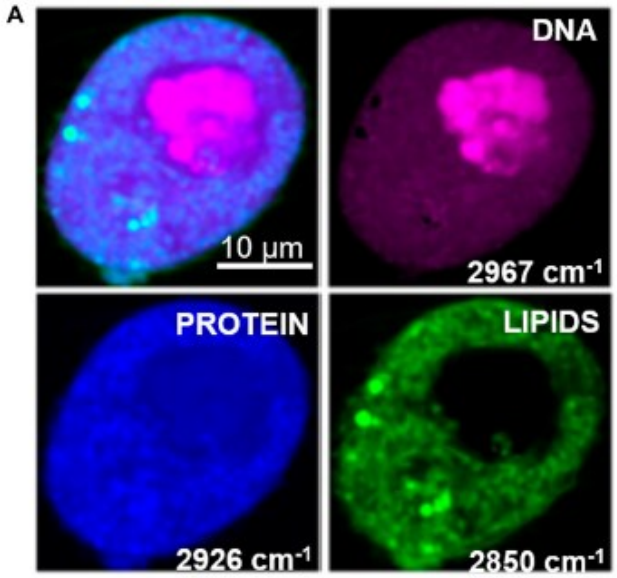
SRS signal is proportional to the imaginary part of the third-order susceptibility tensor $\chi 3$
Since the NRB is a real quantity, SRS is inherently free from NRB.

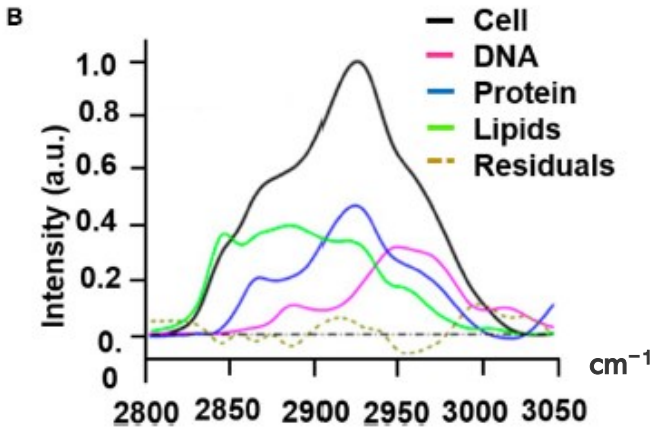
CARS microscopy can be preferentially performed on high molecular concentration specimens, while SRS with high-sensitive and high-speed acquisition systems can be performed on less chemically dense samples

Technically SRS is more challenging than CARS

Summary of the main properties of CARS and SRS microscopy techniques

The difference in frequency between the pump and Stokes pulses match the vibrational resonance of the chemical bond emitting an anti-Stokes photon	Anti-Stokes frequency match the resonance frequency of the target molecular bond **CARS**	Chemical bond resonant frequency from fingerprint region (nuclei acids and proteins) to the C-H stretch region (lipids and proteins) of the Raman shift	• Chemically selective technique • Intrinsic 3D scanning capability • Completely label-free • Applied *in vitro*, *in vivo* (intravital) and in fixed samples • Multicolor imaging	• Non-resonant background contribution • Phase mismatch • Electronic contribution from background molecules • Four-wave mixing process • Risk of photodamage • Fixation process may perturb the chemical stability of target chemical bonds
The difference in frequency between the pump and Stokes pulses match the vibrational resonance of the chemical bond enhancing the vibrational transition emitting a photon	SRG or SRL on the Stokes or on the pump pulse, respectively, match the resonant frequency of the target molecular bond **SRS**	Chemical bond resonant frequency from fingerprint region (nuclei acids and proteins) to the C-H stretch region (lipids and proteins) of the Raman shift	• Chemically selective technique • Intrinsic 3D scanning capability • Completely label-free • Same spectral profile of spontaneous Raman scattering • Fast acquisition process enables real time monitoring of biological processes • Low risk of photodamage • Multicolor imaging • No background fluorescence • Non-resonant background free	• Difficult signal isolation from incoming fields • Fixation process may perturb the chemical stability of target chemical bonds





Example of multi-color SRS microscopy on living tumor cells during prophase:

(A) DNA (magenta) at 2967 cm^{-1}, proteins (blue) at 2926 cm^{-1} and lipids (green) at 2850 cm^{-1}

(B) Raman spectrum extracted from the cell pellet showing the signatures of the different species.

Lu et al., (2015) PNAS

$$\Delta\tilde{\nu}(\mathrm{cm}^{-1}) = \left(\frac{1}{\lambda_0(\mathrm{nm})} - \frac{1}{\lambda_1(\mathrm{nm})}\right) \times \frac{(10^7\,\mathrm{nm})}{(\mathrm{cm})}.$$

Biological Applications :

See examples from reference mentioned in the review:

Reference 2

Parodi V, et al (2020)

Nonlinear Optical Microscopy: From Fundamentals to Applications in Live Bioimaging.

Front. Bioeng. Biotechnol. 8:585363. doi: 10.3389/fbioe.2020.585363

Non Linear Optical Microscopy - non staining -

Exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths : Two Photon Excitation Fluorescence (TPEF),

Non linear effects:

Second Harmonic Generation (SGH), Coherent Raman Scattering (CRS)

Optical Microscopy - CONCLUSIONS

- The optical microscope offers the unique advantage to study living cells
- The image contrast is formed by the changes introduced by the sample to the amplitude, phase, polarization of the intensity of the electrical field (non-stained samples)
- Quantitative phase imaging / digital holographic microscopy provides height and refractive quantitative information
- The resolution in a classical optical microscope is limited to about $\lambda/2$ (200 nm) lateral and 400 nm axial. This limitation is due to the diffraction of light and it is common to all imaging techniques with electromagnetic waves.
- To overcome this limit, different super-resolution techniques as STED and PALM have been recently proposed. They are based on the possibility to switch ON/OFF the state of the dye molecule ! There are also other techniques as BALM based on the nuceic acid stains binding on DNA strand. Spatial resolution can thus be pushed to 1 nm !and less using visible light microscopy !!!
- Photobleaching-based Techniques for Assessing Cellular Dynamics (FRAP, FLIP, FLAP, PA)
- Photoacoustic Microscopy – no staining, deeper penetration, essentially image all molecules at their absorbing wavelengths

Optical Microscopy - CONCLUSIONS

- Brillouin Microscopy – nonlinear non-staining, probes elasticity and viscosity
- Non Linear Optical Microscopy : Exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths;

Two Photon Excitation Fluorescence, Second Harmonic Generation, Third Harmonic Generation – extend the depth of focus, reduce photodamage, no need of staining, specific components imaging

Spontaneous Raman Scattering and Coherent Raman Scattering Microscopies – chemical composition, CARS and StRS enhance efficiency and specificity

Optical Microscopy is Beautiful and Useful ! ☺

What is Nonlinear Optics ? (Some notions recalling the microscopic approach)

the branch of optics that studies the properties and the interaction of light in a medium where the polarization density interacts nonlinearly with the electric field of light.

The Electric Polarization / Polarization Density

describes the formation of electric dipoles within a material, consisting of the electric charge possessed by the atoms and molecules of which it is composed, following the application of an electric field.

Electric dipole

deals with the separation of the positive and negative electric charges found in any EM system.

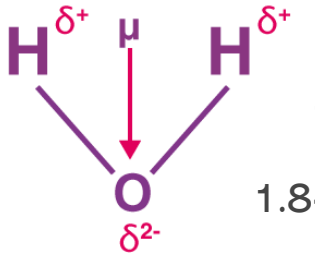
Dipole moment

measures the polarity of a chemical bond between two atoms in a molecule

Dipole Moment has a **Magnitude** and a **Direction**

$\mu = \delta . d$

SI Unit Debye : [D] = [C · m]



μ =

1.5 D - OH bond

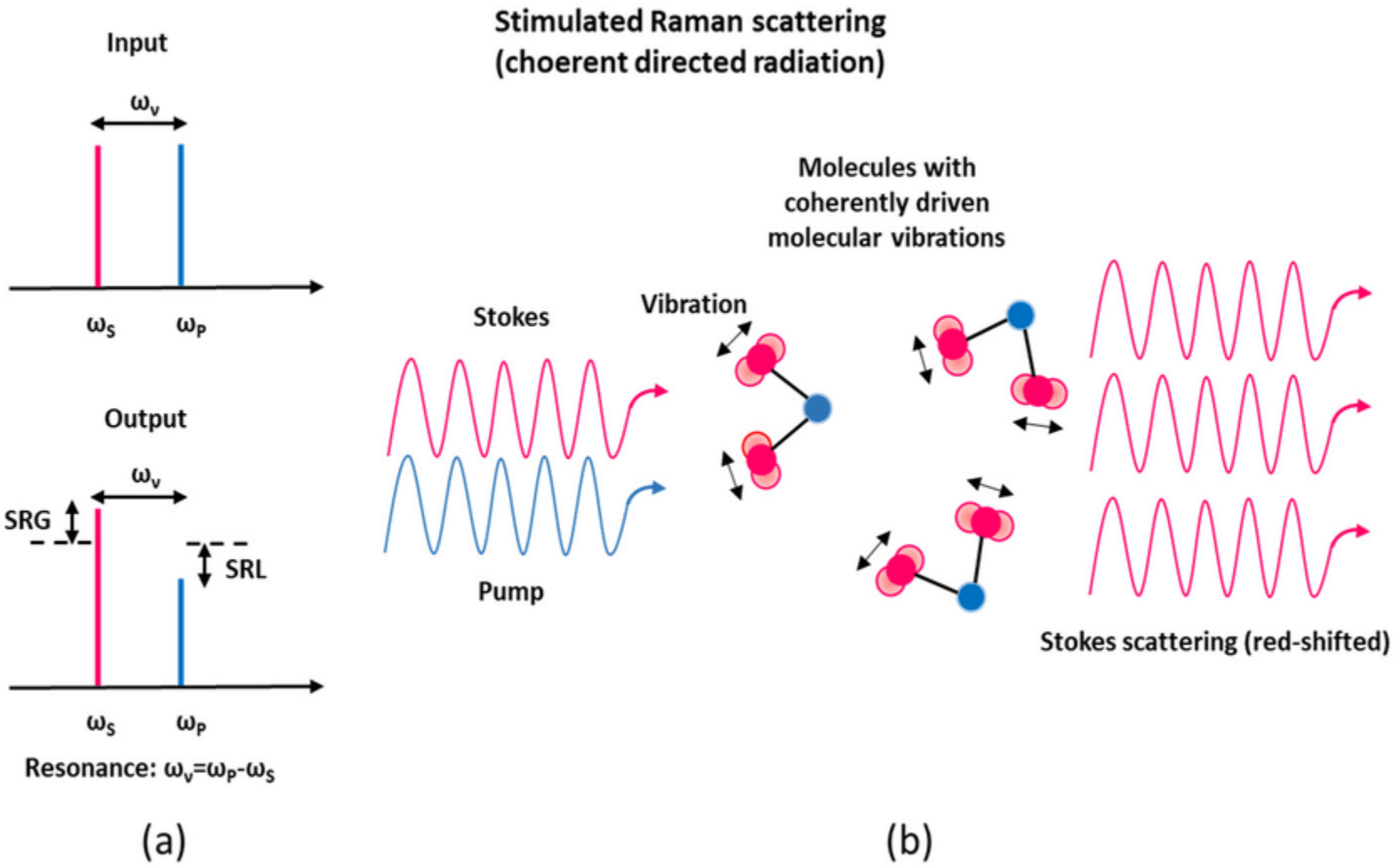
1.84 D - H20 molecule

Polarization density is a function of the intensity of the electric field and the susceptibility of the material

$$\vec{P} = \varepsilon_0 \chi \vec{E}$$

Polarization density is represented by a vector ***P***. In SI units [C / m^2] or [D/m^3]

Stimulated Raman Scattering (StRS)



Stimulated Raman-Scattering (StRS) principle. (a) Pump-probe modalities associated with the SRS process are pointed out: SRG, stimulated Raman gain; SRL, stimulated Raman loss. (b) Stimulated Raman scattering occurs through inelastic scattering of probe photons off from vibrationally excited molecules that interfere coherently.