4

Non-linear microscopy

Note that throughout this chapter we assume that the medium is homogeneous and isotropic and as a consequence we can examine vectors and equations on a component-by-component basis.

A linear dielectric medium is characterized by a linear relation between the polarization density P and the electric field E:

$$P = \varepsilon_0 \chi E, \tag{4.1}$$

with ε_0 the dielectric permittivity of empty space ($= 8.854 \cdot 10^{-12} C^2/Nm^2$) and χ a dimensionless quantity called the susceptibility (which is a function of the frequency of the electric field in case of an oscillating field).

From Maxwell's equations (in one dimension), we can derive the inhomogeneous wave equation to describe the effect of the interaction between the electric field and the induced polarization P in the medium.

$$\frac{\partial^2 E}{\partial x^2} - \frac{1}{c^2}\frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2 P}{\partial t^2} \tag{4.2}$$

Combining Eq. 4.2 and 4.1, we have:

$$\frac{\partial^2 E}{\partial x^2} - \frac{1}{c^2}\frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2}{\partial t^2}(\varepsilon_0 \chi E) \tag{4.3}$$

Since $\frac{1}{c^2} = \varepsilon_0 \mu_0$:

$$\frac{\partial^2 E}{\partial x^2} - \frac{(1+\chi)}{c^2}\frac{\partial^2 E}{\partial t^2} = 0, \tag{4.4}$$

which is simply the homogeneous equation with $c \to c/\sqrt{1+\chi} = c/\sqrt{\epsilon/\epsilon_0} = c/n$, with n the (complex) refractive index.

4.1 Second Harmonic Generation microscopy

More generally, P is not proportional to E:

$$P = \varepsilon_0 \left[\chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \ldots\right] \tag{4.5}$$

Usually, $\chi^{(2)} > \chi^{(3)} > \chi^{(4)} > \chi^{(5)} \ldots$. The first term describes linear absorption, scattering and reflection of light, the second term describes second harmonic generation and sum and difference frequency generation and the third term describes two- and three-photon absorption, third harmonic generation and stimulated Raman processes and coherent anti-Stokes Raman scattering (CARS).

For strong enough electric fields, the nonlinear terms cannot be ignored. The wave equation then becomes:

$$\frac{\partial^2 E}{\partial x^2} - \frac{n^2}{c^2}\frac{\partial^2 E}{\partial t^2} = \varepsilon_0\mu_0\chi^{(2)}\frac{\partial^2}{\partial t^2}\left(E^2\right) + \varepsilon_0\mu_0\chi^{(3)}\frac{\partial^2}{\partial t^2}\left(E^3\right) + \ldots \tag{4.6}$$

Assume an oscillating electric field with angular frequency ω:

$$E(t) \propto E_0 \exp(i\omega t) + E_0^* \exp(-i\omega t). \tag{4.7}$$

Then,

$$E(t)^2 \propto E_0^2 \exp(2i\omega t) + 2\left|E_0\right|^2 + E_0^{*2} \exp(-2i\omega t) \tag{4.8}$$

We now have terms that vary at twice the original frequency, second harmonic generation. Since the amplitude of the second-harmonic light is proportional to E^2, also the second-harmonic intensity scales with the square of the intensity of the incident wave. Since the second harmonic emissions are added coherently, the intensity of the second-harmonic wave is also proportional to the square of the length of the interaction volume L.

Therefore, to maximize the SHG efficiency, the incident wave must have the larges possible power. This is typically accomplished by using pulsed lasers which may have a peak power of hundreds of kW (while keeping the average power low enough to limit the amount of photodamage) and focusing the beam with an objective lens.

The constant term in Eq. 4.8 corresponds to small steady contribution to the polarization density, which creates a potential difference across the nonlinear material when the light beam passes.

Energy ($\omega_1 + \omega_2 = \omega_3$) and momentum ($\vec{k}_1 + \vec{k}_2 = \vec{k}_3$) have to be conserved in the process of second harmonic generation. Conservation of energy shows that SHG generates a photon with twice the energy; "two red photons produce one blue photon". Conservation of momentum implies that

$$n(\omega)\frac{\omega}{c} + n(\omega)\frac{\omega}{c} = n(2\omega)\cdot\frac{2\omega}{c}. \tag{4.9}$$

Hence,

$$n(2\omega) = n(\omega) \tag{4.10}$$

This is usually not the case because of dispersion. However, birefringence can be used to satisfy the phase matching requirement. In birefringent materials, the speed at which the wave travels through the medium is different for different polarizations. By choosing the direction at which the waves enters the medium, the birefringence may exactly compensate for dispersion. For perfect phase matching, the SHG emission is 100% forward directed and co-propagates with the laser. This situation holds for SHG from uniaxial crystals (e.g., potassium dihydrogen phosphate (KDP) and β barium borate (BBO)) and from interfaces. The inherent randomness and dispersion in real biological tissues results in a distribution of nonzero Δk values. This imperfect phase matching gives rise to a corresponding distribution of forward and backward emitted components, and as a result SHG in tissues is best described as quasi coherent. Examples of biological materials that produce SHG are collagen and myosin and consequently can be easily imaged with label-free SHG microscopy.

From Eq. 4.5, it is clear that in systems that exhibit inversion symmetry, all even powers of χ disappear. Liquids, gases, amorphous solids (such as glass), and even many crystals display inversion symmetry, these materials cannot produce this type of nonlinear optical interactions. For third-order processes, there is no such condition.

Focusing a laser through an objective lens imposes the Gouy phase shift, which is a phase shift of the coherent light wave upon passing trough the focal point. The Gouy phase shift of the electric field traveling in the z-direction is given by

$$\zeta(z) = \arctan\left(\frac{z}{z_R}\right), \tag{4.11}$$

with

$$z_{\mathrm{R}} = \frac{\pi w_0^2}{\lambda} \tag{4.12}$$

the Rayleight length.

All SHG scatterers inherit and preserve the phase of the illumination wave. Successive scatterers are therefore not in phase along the illumination wave vector but at a certain angle. To estimate this angle, consider two SHG scatterers separated a distance d apart along the z direction. Complete constructive interference of the SHG signal from both scatterers then occurs under the angle θ

$$\cos\theta \approx 1 - \frac{1}{z_R k_\omega}. \tag{4.13}$$

Hence, in order to collect most of the SHG signal, one can show that the NA of the condenser lens NA^C should be at least

$$\mathrm{NA}^C \approx \frac{2\mathrm{NA}^I}{\pi} \approx 0.64\mathrm{NA}^I, \tag{4.14}$$

with NA^I the NA of the objective lens.

Since SHG is most efficiently produced near the maximum intensity of the focused laser beam, SHG offers a better resolution than linear imaging modalities operating at the same wavelength. Alternatively, one can use a longer wavelength, which can penetrate more deeply into the sample and is less damaging. Moreover, the large wavelength difference between the laser beam and the SH signal allows easy filtering of the signal.

The design of an SHG microscope is very similar to a conventional laser-scanning confocal microscope. Typically, a femtosecond pulsed laser beam or relatively long wavelength (800-1100 nm) is focused by an objective lens onto the sample. A set of galvo scan mirrors scans the laser beam over the sample. Since most of the SHG signal is typically produced in "forward" mode, a condenser lens is used to collect the signal an focus is onto a large single-element detector, such as a PMT. A pinhole, and hence descanning, is not needed, since the SHG is intrinsically produced in a small focal volume. A sharp (5 or 10 nm wide) band-pass filter is installed in front of the detector to allow the SHG to pass. The forward SHG is emitted in a dual-lobed pattern, where the angle between these becomes larger at a higher NA. Thus, it is advantageous to use a condenser with somewhat higher NA than the excitation objective,

To penetrate deeply into the sample, what is needed is an objective with a long working distance (e.g., 3 mm for $\times$40, 0.8 NA), while keeping a reasonable NA (0.5–0.9, many higher-NA lenses have insufficient working distances) and optimized for transmission of the near-IR laser excitation.

Due to the coherence, the image formation process cannot be described by a convolution of the object with an intensity PSF. Instead, one has to work with the field H and the object O, and take the squared modulus after integration.

$$i\left(\boldsymbol{x}\right) = \left|\chi^{(2)}\right|^2 \left| \int H^2\left(\boldsymbol{x'} - \boldsymbol{x}\right) O(\boldsymbol{x'}) \mathrm{d}A \right|^2 \tag{4.15}$$

4.1.1 Non-linear effects

This section gives a more detailed description of non-linear effects.

The response of the material to an electric field is described through the density of dipoles, also known as the polarization P. We write the dependency on the electric field as the following power series

$$P(t) = \varepsilon_0 \sum_{n=1}^{+\infty} \chi^{(n)} E^n(t) = \underbrace{\varepsilon_0 \chi^{(1)} E(t)}_{P^{(1)}} + \underbrace{\varepsilon_0 \sum_{n=2}^{+\infty} \chi^{(n)} E^n(t)}_{P^{(NL)}} \tag{4.16}$$

The first term describes the linear response, while higher terms describe the non-linear response. Note that second-order nonlinear optical interactions can occur only in noncentrosymmetric crystals. Namely, only materials that do not display inversion symmetry. Instead, third-order nonlinear optical interactions can occur for both centrosymmetric and non-centrosymmetric media. Higher

order interactions are typically neglected, being extremely inefficient. For simplicity, we now consider only the second-order non-linearity

$$P^{(2)}(t) = \varepsilon_0 \chi^{(2)} E^2(t) \tag{4.17}$$

We now assume an electric field as follows

$$E(t) = E_1 e^{-i\omega_1 t} + E_2 e^{-i\omega_2 t} + \text{c.c.} \tag{4.18}$$

Typically, the two fields are called *pump* and *idler*. The beam generated by the non-linear process is called *signal*. The second-order polarization is then

$$P^{(2)}(t) = \epsilon_0 \chi^{(2)} \left[E_1^2 e^{-2i\omega_1 t} + E_2^2 e^{-2i\omega_2 t}\right] + 2\epsilon_0 \chi^{(2)} \left[E_1 E_2 e^{-i(\omega_1+\omega_2)t}\right] + \tag{4.19}$$

$$+ 2\epsilon_0 \chi^{(2)} \left[E_1 E_2^* e^{-i(\omega_1-\omega_2)t}\right] + \epsilon_0 \chi^{(2)} \left[E_1 E_1^* + E_2 E_2^*\right] + \text{c.c.} \tag{4.20}$$

The following terms describe the second harmonic generation

$$P\left(\pm 2\omega_1\right) = \epsilon_0 \chi^{(2)} E_1^2 \tag{4.21}$$

$$P\left(\pm 2\omega_2\right) = \epsilon_0 \chi^{(2)} E_2^2 \tag{4.22}$$

The following terms describe the sum frequency generation

$$P\left(+\omega_1 + \omega_2\right) = 2\epsilon_0 \chi^{(2)} E_1 E_2 \tag{4.23}$$

$$P\left(-\omega_1 - \omega_2\right) = 2\epsilon_0 \chi^{(2)} E_1^* E_2^* \tag{4.24}$$

The following terms describe the difference frequency generation

$$P(\omega_1 - \omega_2) = 2\epsilon_0 \chi^{(2)} E_1 E_2^* \tag{4.25}$$

$$P(\omega_2 - \omega_1) = 2\epsilon_0 \chi^{(2)} E_1^* E_2 \tag{4.26}$$

The following terms describe the optical rectification

$$P(0) = 2\epsilon_0 \chi^{(2)} \left(E_1 E_1^* + E_2 E_2^*\right) \tag{4.27}$$

If the non-linear terms are not neglectable, the wave equation becomes

$$\nabla^2 E - \frac{n^2}{c^2} \frac{\partial^2 E}{\partial t^2} = \frac{1}{\epsilon_0 c^2} \frac{\partial^2 P^{(NL)}}{\partial t^2} \tag{4.28}$$

Namely, the non-linear polarization acts as a source. Solving the non-linear wave-equation shows that the generated fields have non-neglectable intensity only if the phase matching conditions are respected

$$\Delta \boldsymbol{k} = \boldsymbol{k}_1 + \boldsymbol{k}_2 - \boldsymbol{k}_3 = \boldsymbol{0} \tag{4.29}$$

$$\Delta \omega = \omega_1 + \omega_2 - \omega_3 = 0 \tag{4.30}$$

The first condition describes the *conservation of momentum*, while the second one describes the *conservation of energy*. Using the relation $ck = \omega$ and assuming collinear beams, we can rewrite the first condition as

$$\frac{n_1\omega_1}{c} + \frac{n_2\omega_2}{c} = \frac{n_3\omega_3}{c} \tag{4.31}$$

However, such condition cannot be achieved, because $n(\omega)$ is typically a monotonically increasing function of ω. This limitation is practically circumvented by using birefringent materials. By aligning the polarization of the field with the highest frequency to the crystal axis showing the smallest refractive index, it is possible to achieve anisotropic phase-matching.

4.2 Two-photon fluorescence microscopy

Two-photon fluorescence may look very similar to SHG, since two low-energy photons are 'converted' into one higher-energy photon, but the two processes are quite different. SHG is a second-order scattering process, while two-photon fluorescence is a third-order process that involves actual absorption to an excited state. (In SHG, the destruction and creation of the photons involves virtual transitions in which no energy is absorbed by the specimen. These virtual energy levels are not energy eigenstates of the atom.)

The absorption cross-section σ for two-photon absorption processes is:

$$\sigma = \sigma^{(2)} I, \tag{4.32}$$

where $\sigma^{(2)}$ is the quantity describing the strength of the two-photon absorption process. Under certain assumptions (e.g., the molecular transition rate must be small enough not to alter the population of molecules in the ground state available for excitation), the two-photon absorption cross-section can be expressed as follows:

$$\sigma^{(2)} = \frac{4\pi^2\hbar\omega^2 \operatorname{Im}\chi^{(3)}}{n^2c^2}. \tag{4.33}$$

The transition rate of an absorption process is given by

$$R = \sigma I/\hbar\omega. \tag{4.34}$$

Hence, we find

$$R = \frac{\sigma^{(2)} I^2}{\hbar\omega}. \tag{4.35}$$

Thus, two-photon absorption scales with the square of the excitation intensity. Two-photon absorption was first described by Maria Goeppert-Mayer in 1931. The molecular two-photon absorption cross-section is usually quoted in the units of Goeppert-Mayer (1 GM $= 10^{-50}$ cm^4 s photon^{-1}).

Not that two-photon absorption can also happen with two photons of different energies. This is called non-degenerate two-photon absorption or two-color two-photon excitation.

Compared to single-photon excitation microscopy, two-photon excitation microscopy has several advantages: fewer photointeractions enabling long term imaging of living samples (and photo-damage by UV excitation can be avoided), imaging of thick specimens up to a depth of about 1 mm, simultaneous excitation of different fluorescent molecules reducing 3D colocalization errors.

Similar to SHG, two-photon fluorescence is only produced in the small volume of a focused laser beam.

$$V \approx \frac{33n\lambda^3}{\pi^3(\mathrm{NA})^4}. \tag{4.36}$$

For typical values (NA $= 1.3$, $\lambda = 500$ nm, n $= 1.33$), V is around 0.06 fL.

The two-photon fluorescence intensity collected in a two-photon microscope with objective NA NA, equipped with a laser with power P and wavelength λ is

$$I_f \propto \sigma^{(2)} P^2 \left(\frac{\mathrm{NA}^2}{\lambda}\right)^2. \tag{4.37}$$

The imaging process in a two-photon fluorescence microscope can be described similarly as for one-photon:

$$i(\boldsymbol{x}) \propto [o * h_2](\boldsymbol{x}), \tag{4.38}$$

with o the distribution of emitters in the object plane and h_2 the two-photon PSF.

$$h_2(u,v) = \left| 2\int_0^1 J_o(v\rho)\exp\left(-iu\rho^2/2\right)\rho d\rho \right|^4, \tag{4.39}$$

with $u = k(NA)^2 z$ and $v = k(NA)r$.

Two-photon fluorescence is typically detected in non-descanned detection in backward mode, but also the combination with descanned detection is possible, for example to combine it with ISM.