Two-Photon Imaging

Giuseppe Sancataldo[1], Olga Barrera[2,3], Valeria Vetri[1]

[1] Department of Physics and Chemistry – E. Segrè, University of Palermo, Palermo, Italy
[2] Oxford Brookes University, Oxford, UK
[3] University of Oxford, Oxford, UK

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What you will learn in this chapter

This chapter will provide an overview of two-photon microscopy from elements of the theory underpinning fluorescence phenomena to functioning principles of a two-photon microscope including step-by-step practical advice on how to conduct an experiment using a two-photon microscope. In this context multiphoton excitation is also taken in consideration.

By reading this chapter, you will have a synopsis of the basic principles of two-photon excitation, optical sectioning and 3D microscopy. Furthermore, fundamentals of promising advanced methods for tissue imaging available for two-photon imaging as Second Harmonic Generation (SHG) and Fluorescence Lifetime Imaging Microscopy (FLIM) are briefly described together with classical applications on deep tissue imaging and functional brain imaging.

Keywords: Two-Photon Excitation Process, Second Harmonic Generation (SHG), Fluorescence Lifetime Imaging Microscopy (FLIM), Phasor approach, 3D tissue imaging

Introduction

Vision is one of our primary senses and it is highly informative. This is the reason why the idea to boost up it, increasing the possibilities to see objects magnifying them to better distinguish shapes, distances, colors and details has been prioritized in many fields of experimental sciences. Lenses were already described by Seneca about 2000 years ago and first attempts of compound microscopes are reported in XVI century. Optical microscopes are made of a light source, an objective lens and an eyepiece allowing the observer to see a magnified image with respect to the one accessible to the naked eyes. These three elements are now technologically advanced, but they still constitute the foundations of these magnificent tools. Since the construction of the first microscope, there have been numerous advances in solving questions in life sciences. Scientists became able to see what was previously hidden to their eyes as cells and, with further developments, subcellular organelles. Introducing fluorescence as contrast method has been an incredible step towards measuring biological and chemical reactions in real time. Fluorescence is an exquisitely quantistic phenomenon, resulting in the emission of light by a molecule after the absorption of electromagnetic energy. In the last decades, fluorescence microscopy has become a key tool in several biomedical research fields, allowing researchers to localize specific molecules and to characterize cell and tissue morphology at sub-micrometer resolution. The characteristics of the emitted light are critically dependent on the properties of the molecules including their structural, dynamic and environmental evolution. Therefore, fluorescence signals are the fingerprint of the molecules and of their environment. Fluorescence microscopy images are maps of fluorescent molecules, which can be endogenous in the sample or extrinsic markers and their analysis provide spatial and functional information (Diaspro, 2011; Pawley, 2006). Fluorescent molecules can be introduced as a probe into the specimens allowing mapping or tracking them, reporting the properties of the molecules they are interacting with and visualizing physico-chemical processes with high specificity. Due to the availability of measuring multiple observables (intensity, spectral features, lifetime), also as a function of time, the amount of information that can extracted from a single fluorescence experiment dramatically increases. Advances in digital imaging and analysis allow quantitatively monitoring, with high temporal resolution, specific signals from specimens of different nature ranging from proteins, polymeric systems, living cells, living animals (Konig, 2000), or solid state samples like solar panels or synthetic ceramic superconductors (Barnard et al., 2013; Gaury and Haney, 2016).

Image resolution using optical microscopy classically remains within submicron micron scale (hundreds nanometers), which is still limited with respect to other methods like electron or atomic force microscopy. However, the possibility of investigating living cells/organisms with minimal perturbation and biological structures close to physiological conditions remains a great advantage of fluorescence methods. It is worth mentioning that recently huge effort has been made in order to push fluorescence microscopy to the nanoscopic level by overcoming resolution limits imposed by diffraction (Diaspro, 2016). However, these methods are not discussed in this chapter.

In this scenario, two (multi)-photon fluorescence microscopy has been established as a powerful tool for the imaging and the analysis of three-dimensional (3D) samples providing high spatial and temporal resolution ideal for in vivo experiments. The development of this method is a clear sign of the continuous evolution and efforts that are devoted in continuously improving optical and more specifically fluorescence microscopy possibility. Two-photon microscopy is a non-linear method, this means that it uses "higher order" light-matter interactions, involving multiple photons, to generate contrast in images. The features of this non-linear interaction produce several advantages with respect to standard methods that will be described in this chapter.

In conventional fluorescence microscopy, fluorescence is stimulated by the absorption of a single photon by a molecule raising the molecule to an excited energy state. When the molecule returns to its ground state emits a less energetic photon. In contrast, two (multi)-photon fluorescence microscopy is based upon the simultaneous absorption of two (multiple) low-energy photons by a molecule. Since the excitation depends on the simultaneous absorption of two photons, the probability of triggering a two-photon process is extremely rare to occur at a low photon density. To enhance the probability of simultaneously absorbing two photons, a two-photon microscope concentrates a high dose of light in a small spot within the sample which generates fluorescence only from this confined region. This feature makes two-photon microscopy the most suitable tool for imaging highly scattering samples. It also provides the possibility of three dimensional (3D) optical microscopy in a non-invasive way of thick samples (mm scale penetration depth). Moreover, compared to single-photon fluorescence microscopy, two-photon microscopy results in an improved image quality with highly reduced background signal, minimized photobleaching and photodamages as lower energy is delivered to the sample (Konig, 2000). For its properties this method is gaining high popularity for instance in analysising biological tissues that typically strongly scatter light, making high-resolution deep imaging highly challenging.

In the following, we will describe fundamentals of fluorescence microscopy focusing on the rationale of its application in tissue imaging and giving an introduction of functional brain imaging using two-photon microscopy. Furthermore, in this chapter, Second Harmonic Generation (SHG) microscopy and Fluorescence Lifetime Imaging Microscopy (FLIM) methods will be described that can be conveniently coupled with two-photon microscopy to increase the level of information accessible in a single experiment.

1. Two-Photon Excitation Process

1.1 Historical perspectives

Two-photon excitation process is a relatively old concept in quantum physics. The theoretical basis of two-photon absorption was predicted by Maria Goeppert-Mayer (the first Nobel Prize in theoretical physics awarded to a woman) in her doctoral dissertation in 1931 (Göppert-Mayer, 1931). Since two-photon absorption is an uncommon event at normal light intensities, the experimental evidence of this phenomenon was achieved only in 1960s mainly due to the advent of lasers source capable of delivering a high photon density. The first spectroscopic report on two-photon fluorescence of CaF2:Eu2C was in 1963 by Kaiser & Garret (Kaiser and Garrett, 1961). Only years later, in 1971, two-photon fluorescence of organic dyes was demonstrated. The first applications of two-photon

fluorescence in microscopy were presented at the beginning of the 1990s by Denk and colleagues, who demonstrated the potential of imaging two-photon excited fluorescence in a raster scanning microscope coupled to an ultrafast pulsed laser (Denk et al., 1990). The development of commercially available mode-locked lasers, with high peak-power, femtosecond pulses and repetition rates around 100 MHz was then the trigger for a fast uptake of the multi-photon method in biology (Hoover and Squier, 2013). Nowadays two-photon microscopy is a widespread technique that, combined to specific labeling technology of biological structures, provides a sensitive means to study a plethora of phenomena in biomedical research (Pawley, 2002).

1.2 Principles of two-photon excitation

Fluorescence emission commonly takes place when a single photon of the appropriate energy is absorbed by a fluorophore. The single photon excitation process is schematically illustrated in Figure 1a by means of the Jablonski diagram. This diagram illustrates the electronic states of a fluorescent molecule and the transitions between these states. In Figure 1a the singlet ground (S_0) and first electronic state (S_1) together with the vibrational energy levels in which they may exist are shown. The electronic states are arranged vertically by the energy level and vertical arrows indicate the transition between levels. The absorption of a photon (occurring in 10^{-15} s time scale) causes the transition of the fluorophore from the ground electronic state to the excited state. A fluorophore is usually excited to high vibrational levels of the excited electronic state so that a rapid relaxation process, named internal conversion (10^{-12} s time scale), occurs to the lowest vibrational level of the excited state. The fluorophore then returns to its ground state by emitting a new photon (10^{-9} s time scale). All of these processes result in a loss of energy, therefore the emitted photon has less energy and a larger wavelength than the exciting photon. The difference between the exciting and emitted wavelengths, known as the Stokes shift, is an important feature to be considered when choosing fluorescent dyes for microscopy experiments. One-photon excitation typically requires photons with energies in the ultraviolet (UV) or visible spectral range. In order to generate fluorescence by a two-photon process, two different photons must be absorbed simultaneously (within a temporal interval of 10^{-16} s as consequence of the Heisenberg uncertainty principle) by the same fluorophore. The sum of the energy of the photons required in a two-photon absorption event must be equal to the energy needed for the single-photon absorption. This phenomenon may also occur with multiple photon absorption e.g. three-photon excitation needs the absorption of three photons and so on. An example of a two-photon excitation process is schematically illustrated in Figure 1b, where the transition to the excited state is induced by two photons of lower energy with respect to the energy gap between the two electronic levels.

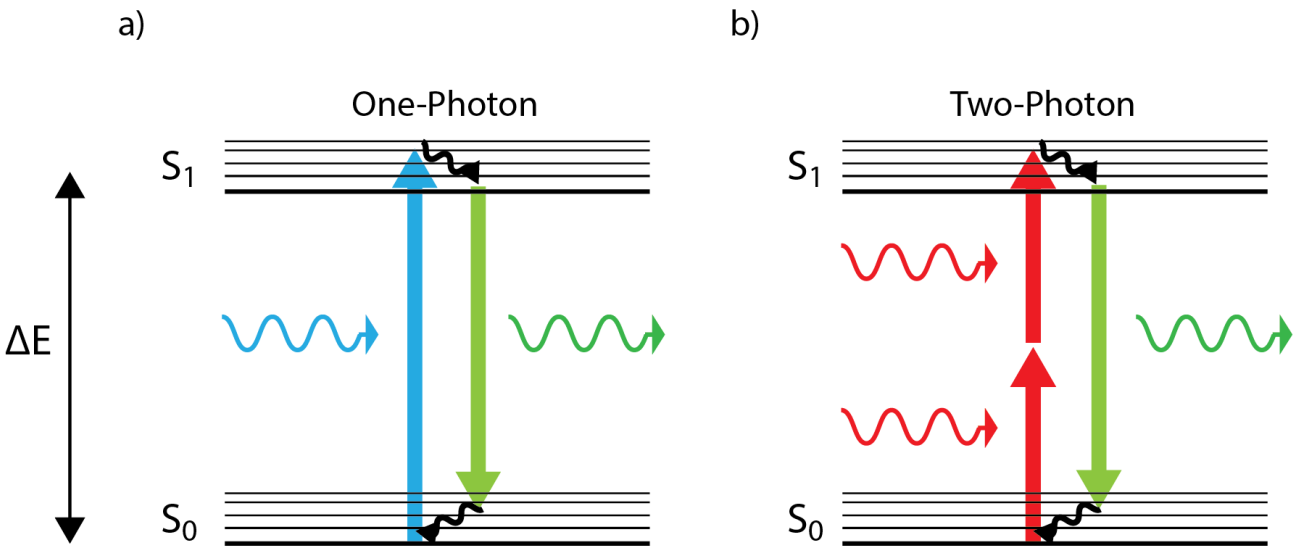

Figure 1 *Jablonski scheme for one-photon and two-photon excitation. a) one-photon excitation results from the absorption of single photon (blue arrow). B) Two-photon excitation results from the simultaneous absorption of two low energy photons by a fluorophore. Green arrows in a) and b) indicates fluorescence emission.*

For practical reasons, due to laser excitation, the two photons are usually at the same energy about one-half of the energy that is necessary to excite the molecule (two times longer than the wavelength required by a single-photon excitation), more in general the following relation should apply:

$$\lambda_{1P} = \left(\frac{1}{\lambda_1} + \frac{1}{\lambda_2}\right)^{-1}$$

Where λ_{1P} is the wavelength corresponding to the energy for one-photon excitation and λ_1 and λ_2 are the wavelength of the two-photon excitation respectively. For this reason, two-photon absorption process typically requires photons with lower energy and, in the larger part of the experiments, the excitation occurs with high power laser in the infrared spectral range.

In general, one-photon and two-photon selection rules are different thus, it is possible that electronic transitions forbidden for one of them is allowed for the other one (Birge, 1986; Birge and Pierce, 1979; Friedrich, 1982; Loudon and von Foerster, 1974). However, a rule of thumb exists for experiments suggesting that, if one-photon excitation occurs at a wavelength of specific excitation, two-photon will occur at about the double of this wavelength. Usually the two-photon absorption spectra are found to be wider. It is important to note that, independently of the way in which the molecule is excited, the emitted light will have the same properties in terms of spectral shape and lifetime since fluorescence occurs from the same excited state.

The probability that a two-photon excitation phenomenon occurs is critically lower than the single photon one. Qualitatively this can be understood by considering that Rodhamine B, which is an common fluorescent dye, absorbs one photon each second in the day light (roughly) but a two-photon event may occur once every 10 million years. For this reason, two-photon excitation requires a high concentration of photons in time and, in order to increase the probability of the event, photons should be also highly focused in small spatial regions (Denk and Svoboda, 1997). This can be obtained with high-power continuous wave lasers or using pulsed laser sources with high-energy short pulses. Two-photon excitation in optical microscopy is usually made possible by laser scanning and titanium-sapphire lasers; these are tunable lasers which generate ultrashort pulses in the Near Infrared range. By using an ultra-short pulsed laser sources higher peak power are reached significantly increasing the probability of two-photon excitation maintaining low incident power (Denk et al., 2006). The use of short pulses at high frequency allows image acquisition without irradiating the sample at high power levels.

Therefore two-photon excitation becomes a peerless tool for biological imaging as electromagnetic radiation at longer wavelength is delivered to the sample. This facilitates measurements on many endogenous fluorophores in cells like NAD(P)H, flavins, collagen and lipopigments (Aubin, 1979). The use of important exogenous markers like the DAPI, Hoechst 3342 gold standards for nuclear staining as well as the membrane dye Laurdan is facilitated as they require UV or blue excitation wavelengths that may induce serious damages to specimen both in terms of photobleaching and phototoxicity (Cunningham et al., 1985; Tyrrell and Keyse, 1990).

Non-linear light-matter interaction gives rise to other important advantages with respect conventional light microscopy such as the restriction of the excitation to a tiny volume which allows sample scanning and 3D measurements, higher penetration depth in turbid samples and improved image quality.

1.3 Optical Sectioning and 3D Microscopy

The capability to image biological specimens in three dimensions represents one of the major achievements' optical microscopy. In the past years, using conventional instruments, destructive sectioning procedures had to be practiced for thick samples (larger than > circa 30 μm). Indeed, out-of-focus signal can completely obscure the in-focus information and greatly reduces the image's contrast. Sample sectioning in thin slices, besides being a complex and time-consuming procedure, is highly undesirable as it might modify what is observed inducing perturbation or breakage of specimens' structural organization, thus possibly inducing artifacts.
Two-photon microscopes exploiting nonlinear nature of light-matter interaction give the possibility of producing 3D images of thick specimens preserving the structure and functionality. 3D reconstruction is performed collecting and recording a series of two-dimensional images acquired at different planes throughout the specimen. Optical sectioning, namely the ability of the microscope to reject out-of-focus fluorescence background, covers a key role in the process. The elimination of unwanted light provides greater contrast and allows correct three-dimensional (3D) reconstructions.
Optical sectioning is achieved thanks to the high localization of the excitation intrinsically provided by two-photon excitation. Indeed, as reported in the previous section, in order to obtain a significant number of two-photon absorption events, the photon density must be remarkably higher than what is

required to generate the same number of one-photon absorptions. Two-photon excitation relies on the simultaneous absorption of two photons so that the number of photons absorbed in the time unit (and thus fluorescence) is proportional to the squared intensity of the excitation light.
In microscopy experiments, the light of the beam is focused (by the objective) on a point of the sample, this decreases its size and increases the intensity. As a result, for two-photon excitation, the amount of light absorbed across the sample is not constant, it is actually weaker in out of focus points. Fluorescence is only excited in a diffraction limited region centered at the focus point. In two-photon experiments, using high power laser beams, focused through high Numerical Aperture (NA) objectives, multiphoton absorption is spatially confined to a tiny femptoliter scale volume. This is significantly different from other microscopy methods which exploit one photon excitation as confocal fluorescence microscopy which requires the physical screening of unwanted signal using a pinhole. In order to achieve one-photon excitation, laser beam focusing does not change the total amount of light passing through a plane due to linear dependence of absorbed light from excitation light intensity. In figure 2 the comparison between the two conditions (one-photon and two-photon) is sketched considering a cone shaped (focused beam) illuminated region as usually created by objectives.

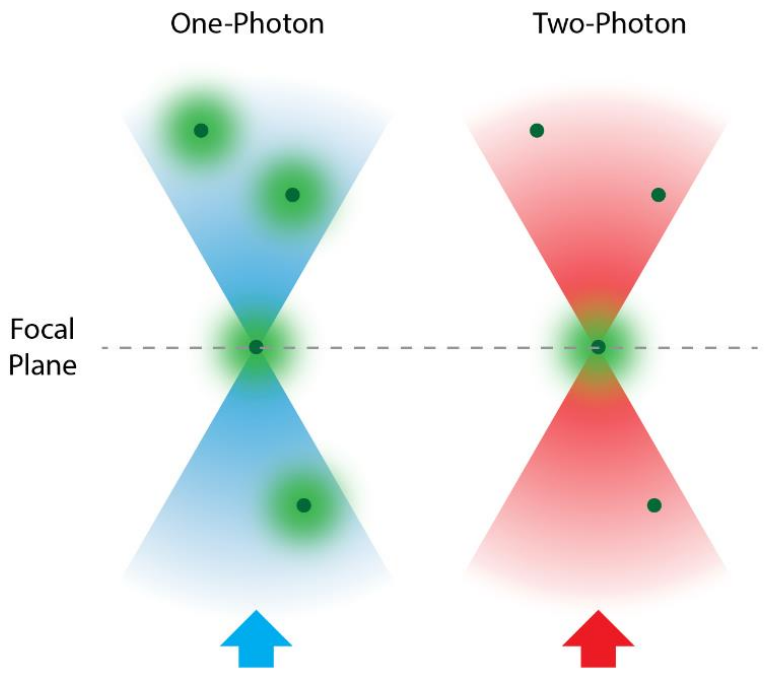

Figure 2 *A schematic representation of the localisation of one-photon and two-photon excitation. In one-photon excitation, a continuous wave ultraviolet or visible light laser excites fluorophores throughout the volume. Out-of-focus fluorophores are exited and emit. In two-photon microscopy, an infrared laser provides pulsed illumination such that the density of photons sufficient for simultaneous absorption of two photons by fluorophores only occurs at the focal point. Arrows indicate the excitation direction.*

In one-photon excitation conditions, fluorescence signal is generated in molecules in the whole cone, whilst in two-photon excitation conditions only molecules localized in a small volume at the focus emit light. Summarizing, in two-photon microscopes fluorescence signal comes from molecules localized in a tiny region at the focus. As the focus position moves in the x-y plane, light is delivered

to sequential points of the sample and the emission signal reconstructs the distribution of fluorescent objects with high resolution. A 3D reconstruction of the distribution of fluorescent molecules is then obtained through volume-rendering procedures preserving the structure and the functionality of the system. This procedure is sketched in figure 3.

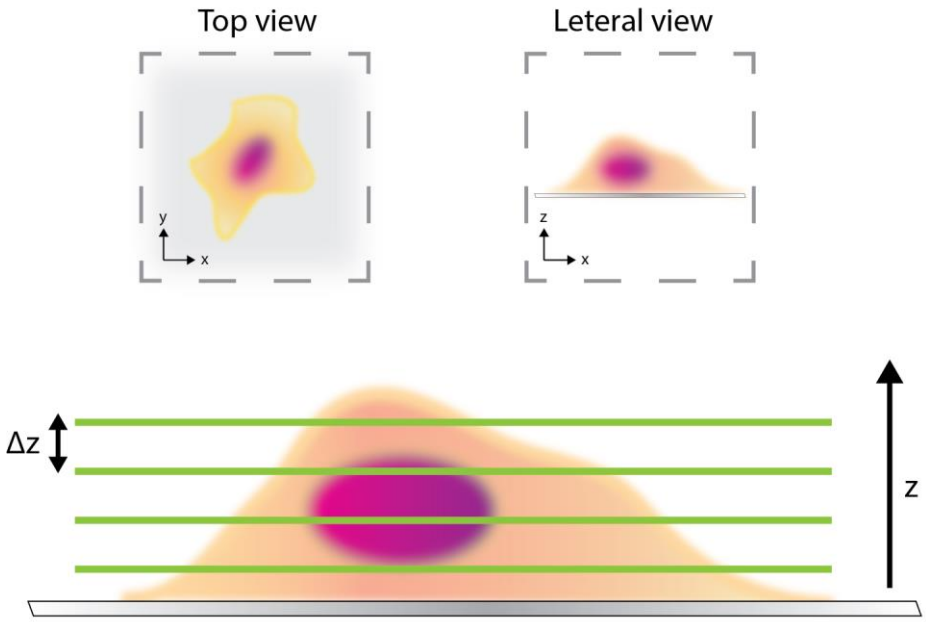

***Figure 3**. Schematic representation of 3D acquisition by means optical sectioning. 2D images are acquired in the x,y plane at defined Δz steps changing focus plane position.*

1.4 Scattering and photobleaching reduction due to fluorescence excitation in the NIR window

Generally, fluorescent molecules undergo multiphoton excitation when incident light is in the near infrared (NIR) spectral region (700-1000 nm). This long-wavelength illumination light presents advantages also in the achievable penetration depth. Shifting the excitation towards the infrared region of the electromagnetic spectrum allows deeper penetration in high scattering samples like biological tissues as a result of the combination of two different features: i) minimization of scattering effects ii) reduction of losses in the excitation light due to sample absorption. These two properties permit a significant decreasing in image degradation, especially when imaging is performed in deep regions of scattering samples. First, the NIR excitation reduces scattering effects which results from the deflection of light from different regions of the specimen due to the inhomogeneity in refraction index. Scattering occurs in most of the samples and may cause artifacts or high reduction of detected signal. As the number of photons which are not scattered reaches the objective, the focus exponentially decreases with depth. In highly turbid sample, the intensity of fluorescence generated at the focal plane can be dramatically reduced by this effect. Scattering intensity is reduced at higher

wavelengths which is, in first approximation, inversely proportional to the fourth power of incident light (this is strictly valid in Rayleigh scattering conditions) (Pecora, 2000). Moreover, the analysis of biological tissues takes advantage of the spectral range between 650-1350 nm of the excitation, which is often referred as NIR window (Xu et al., 1996b). In this range, biological tissues generally do not contain endogenous molecules which absorb light (water, hemoglobin, melanin etc.); consequently, the light penetration is maximized. The reduced excitation volume also implies the reduction of unsought background signal which balances the poorer spatial resolution compared to one-photon measurements (which require shorter excitation wavelengths). Another important advantage of non-linear excitation of fluorescence to consider is the lack of out-of-focus excitation which reduces photodamage during 3D imaging. Photobleaching occurs only at the focal plane, while along the excitation light path absorption is prevented, thus the integrity of the sample at molecular level is also prevented. This makes non-linear excitation of fluorescence extremely suitable for imaging and analyzing biological structures.

1.5 Two-photon microscope

Two-photon microscopy is typically realized in laser-scanning microscopes and the main setup shares with confocal microscopes many aspects. The main difference is the light source, two-photon excitation in optical microscopy is usually made possible by titanium-sapphire lasers. These are tunable lasers which generate ultrashort pulses in NIR range. The use of short pulses at high frequency allows image acquisition without irradiating the sample at high power levels. The pulsed laser is focused to a diffraction-limited spot and scanned in a raster over the sample. When the tiny spot overlaps with fluorophores of the sample, fluorescence signal is generated selectively within the small spot. An image is formed as a well-ordered 2D sequence of points by collecting the fluorescence by a single detector. The temporal signal of the detector is mapped to the corresponding raster-scanned point by the data acquisition computer. Figure 4 shows the optical scheme of a typical two-photon microscope.

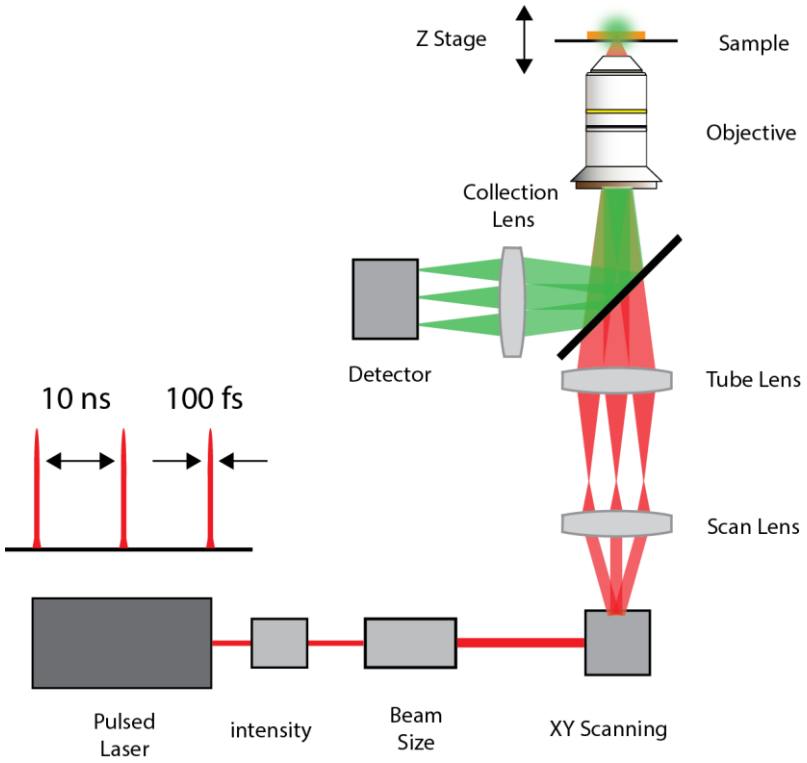

Figure 4. *A schematic of typical components in a two-photon scanning microscope. The system contains of a high-peak-power pulsed laser, a scanning microscope and high-sensitivity detector.*

The light beam from the laser unit is optimized in size and intensity by means of a telescope and by an intensity modulator (intensity and Beam size boxes in figure 4) and directed to the scanning system. This is a fundamental part, composed of two rotating mirrors, that is used to raster the excitation beam in the focal plane of the objective. The objective is generally used both for exciting the sample and for collecting emitted fluorescence. Fluorescence signal is separated from the excitation beam on the path using a wavelength-sensitive dichroic mirror. Finally, a single-element detector such as a photomultiplier tube (PMT) or an avalanche photodiode (APD) collects the signal.

2. Advanced analysis and applications

2.1 Second Harmonic Generation (SHG)

When the energy density at the focal spot of a microscope is sufficiently high, other interesting non-linear optical effects can be observed. These optical phenomena can be used as complementary data in an optical microscope to study biological samples. Among these phenomena, Second Harmonic Generation (SHG) is one of the widespread forms of biological nonlinear microscopy. SHG is a second order coherent process in which two lower energy photons are up-converted to exactly twice the incident frequency of an excitation laser. It results from phase matching and sum of light fields induced in ordered non-centrosymmetric structures. This means that a scattered beam is produced at half the wavelength of the illumination one in phase with the input. SHG is essentially an

instantaneous process, which makes it distinguishable from other fluorescence phenomena occurring in the nanosecond timescale. SHG imaging was implemented first time to biological imaging in 1986 by Freund (Freund et al., 1986) and can be performed simultaneously with multiphoton excitation measurements using the same incident light. Since SHG signal does not require the absorption phenomenon, these measurements present reduced photobleaching and phototoxicity effects with respect to fluorescence methods. Given that the signal is generated using same laser sources as the ones of two-photon microscopy, the same advantages discussed for two-photon microscopy hold. In general, molecules that are strongly and directionally affected by electric field generates SHG signal. In biological specimens many types of spatially ordered structures such as muscle myosin lattices or microtubules, polysaccharides as cellulose and starch, are able to generate sufficient amount of SHG and can be identified without performing any sample staining (Campagnola et al., 1999; Chen et al., 2012; Cox et al., 2005; Gauderon et al., 2000; Moreaux et al., 2000). Most studies in biosciences carried out by SHG microscopy is focused on the visualization of collagen fibers in the extracellular matrix and in different kinds of organs and connecting tissues. The signal is highly sensitive to collagen structural organization and it is reported to change in diseased tissues (Campagnola, 2011). In the last years, SHG has risen back to prominence as a powerful contrast mechanism for label-free imaging of biological specimens in physiological as well as in disease state enhancing basic research possibilities in biology and medicine and providing quantitative tool for diagnosing a wide range of diseases (Campagnola and Loew, 2003; Friedl et al., 2007).

2.2 Fluorescence Lifetime Imaging Microscopy (FLIM)

The advent of two-photon microscopy has provided an alternative way to obtain improved 3D images due to its optical sectioning capability and its intrinsic enhanced contrast. In most common applications, fluorescence intensity from a chromophore is measured in a specific spectral window. However, the fluorescence is not only characterized by the steady-state emission spectrum, it has also a characteristic lifetime. The fluorescence lifetime is defined as the time the fluorophore remains in the excited state. For a fluorophore generally the lifetime (τ) is given by:

$$\tau = \frac{1}{\Gamma + k_{nr}}$$

Where Γ and k_{nr} are the rates of radiative and non-radiative processes from the excited state. In a real measure, as fluorescence is a random process, the fluorescence intensity presents the following exponential time dependence:

$$I(t) = I(0)e^{-t/\tau}$$

Where I(0) is the intensity at time zero (upon excitation). The fluorescence lifetime τ can vary from picosecond to nanosecond range and it is a peculiar characteristic of the molecule constituting a fingerprint for every dye and its environment.

Two-photon microscopes, as other fluorescence microscopes, allow equipment upgrades that make the measure of fluorescence as a function of time (in a sub nanosecond timescale) at each pixel of the image, thus allowing Fluorescence Lifetime Imaging Microscopy (FLIM) measurements. FLIM

is a fluorescence imaging technique where the contrast is based on the fluorescent lifetime of chromophores in the sample. Two photon setups take advantage from short pulsed laser sources, to perform fluorescence lifetime measurements recording the time decay of the signal after each short excitation pulse. For example, an 80 MHz pulsed laser, commonly used in two-photon microscopy, provides an excitation pulse every 12.5 ns making accessible measurement of fluorescence decays in this timescale. To this aim, Time-Correlated Single-Photon Counting (TCSPC) is commonly used (Becker et al., 2004; Gratton, 2003) and fluorescence decays can be measured at discrete locations during raster scan making accessible this valuable observable to imaging. Figure 5 is a schematic example of using lifetime measurements to discriminate between spectrally similar fluorophores.

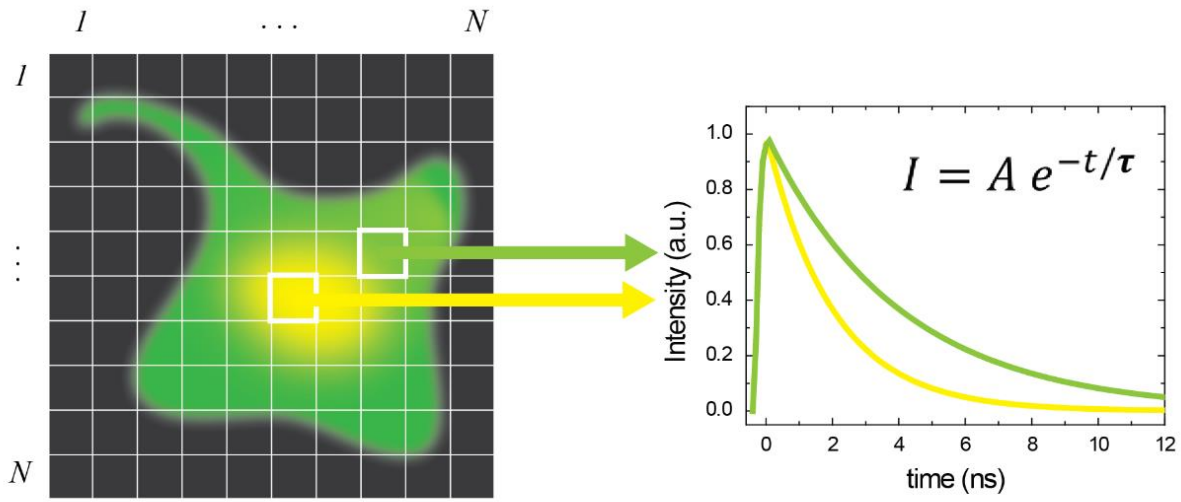

Figure 5. *Fluorescence Lifetime Imaging Microscopy (FLIM). Contrast is given by different lifetimes measured at each pixel of the image, this is independent on the fluorescence intensity, number of fluorescence molecule of intensity of excitation light.*

FLIM measurements present several advantages when dealing with biological samples which are dynamical and highly heterogeneous. These advantages clearly overcome the apparent difficulty in data analysis and interpretation and the need of sophisticate hardware. A feature that limits the quantitative interpretation of intensity-based fluorescence images is the lack of knowledge of fluorophore concentration at different locations. A fluorescent dye, for instance, can inhomogeneously accumulate in different regions of a cell due to the intrinsic heterogeneous physicochemical features of the environment. As the measured fluorescence intensity linearly depends both on the quantum yield (i.e. the number of emitted photons relative to the number of absorbed photons) and on the number of molecules, it is not always easy to separate the two effects. This means that experiments involving the use of fluorescent dyes which report environmental properties for example pH (Fluorescein and carboxyfluorescein) (Robertson et al., 2013) or calcium concentration (Fluo-3, Calcium green) (Agronskaia et al., 2004) via variation in fluorescence emission intensity are non-trivial in cellular environments. The most important advantage of FLIM over fluorescence intensity imaging is that fluorescence lifetimes are independent of fluorophore concentration and laser excitation intensity. Since the fluorescence lifetime of a fluorophore is sensitive to the local environment (pH, charge, presence of quenchers, refractive index, temperature and so forth), their measurements under a microscope offer the important advantage of contrast by spatial variations of lifetimes. Fluorescence lifetime measurements can then investigate

photophysical events that are difficult or impossible to observe by fluorescence intensity imaging. Furthermore, as fluorescence lifetime provide "absolute" measurements, FLIM is certainly less susceptible to artefacts arising from chromophore inhomogeneity, photobleaching, uneven refraction index and so on. Importantly, scattering or SHG signals can be easily discriminated from fluorescence as they are instantaneous phenomena. Importantly, FRET (Foster Resonance Energy Transfer) is largely employed to evaluate the molecular mechanisms governing diverse cellular processes become simpler as FRET events are marked by the reduction of donor fluorescence lifetime (Wallrabe and Periasamy, 2005).

It cannot be neglected that, although the decay law is based on first order kinetics, in real experiments often fluorescence decays are more complex and present multi exponential behavior. This behavior of fluorescence decays may be due to various reason depending for instance on molecular structures, inhomogeneous environments, quenching processes, presence of multiple species among other factors. The interpretation of these data traditionally required specific expertise, complex fitting procedures requiring a specific model of system under studies. However, this has been overcome by recent development of FLIM analysis, introduced by M. Digman and E. Gratton at the beginning of this century (Digman et al., 2008). The so called "phasor approach" allows fit-free analysis of FLIM data and a rapid extraction of fluorescence lifetime information without the need of prior lifetime knowledge (Sancataldo et al., 2020; Stringari et al., 2011a). This method provides simple and fast mapping of fluorescence lifetime distribution in the image and, as proved by application in many fields from its implementation in commercial instrumentation and analysis software, phasor approach makes FLIM data analysis accessible to non-expert audience rapidly becoming mainstream. A representation of phasor plot analysis is reported in Figure 6.

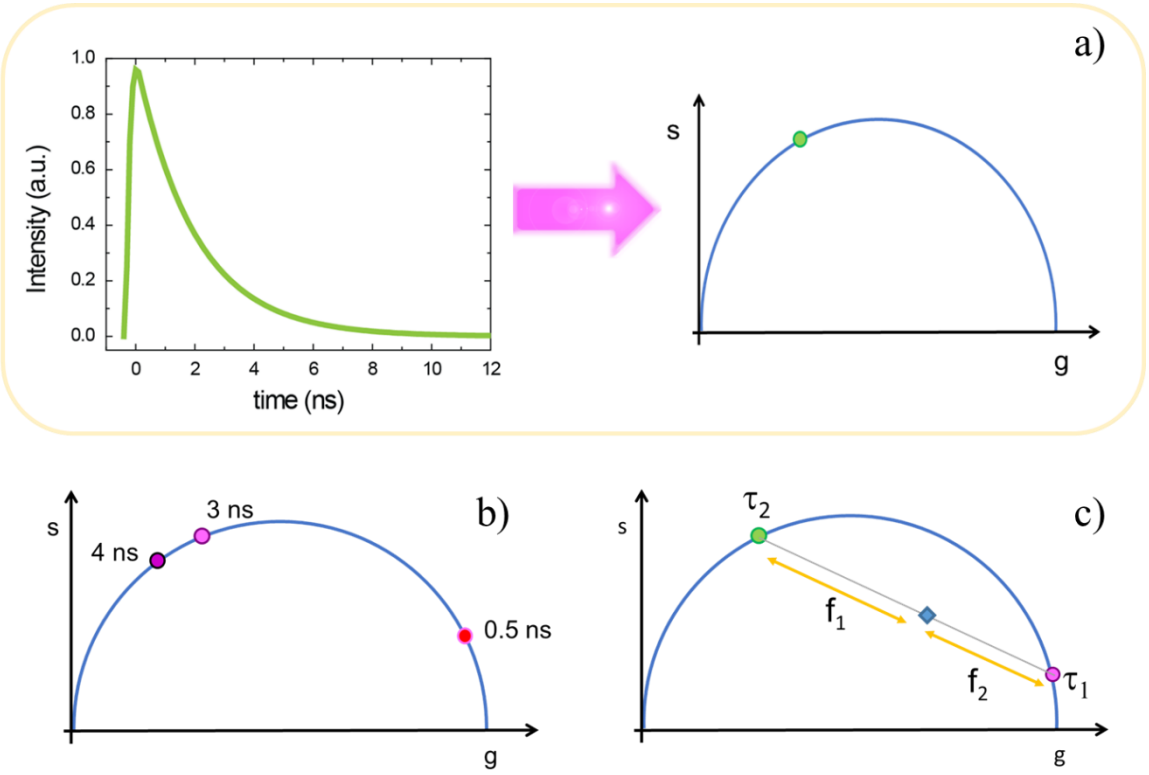

Figure 6 *Phasor analysis fundamentals: a) the fluorescence intensity decays are Fourier transformed in a point in the phasor plot which represents the fluorescence lifetime of the fluorophore (called phasor). b) Single exponential decays give rise to phasors lying on the universal circle which becomes a lifetime ruler. The ruler scale can be easily experimentally calibrated from known lifetime of well-known fluorophores (e.g. fluorescein presents a single exponential. c) Complex decays are located inside the universal circle as phasors follow vector algebra. For example the phasor corresponding to a double exponential decay* $I(t)=f_1e(-t/\tau_1)+f_2e(-t/\tau_2)$ *is located along the line connecting individual species* (τ_1) *and* (τ_2) *phasors. The distance between each phasor and the single exponential phasors on the universal circle* (f_1 *and* f_2) *represents the fraction of each component.*

For each pixel of the image, the phasor analysis transforms fluorescence decay traces to a set of phasor coordinates in a polar plot corresponding to the real and imaginary portions of the Fourier transformed decay. When using phasor analysis each pixel of the image is mapped to a point in the polar plot corresponding to the measured fluorescence lifetime. Single exponential lifetimes lie on the so called "universal circle"; long lifetimes are located near the origin (0 on the x axis), while short lifetimes are shifted on the circumference toward the bottom right intersection with the x axis (1 on the x axis) (Digman et al., 2008; Ranjit et al., 2018). In real measurements, data in the phasor plot appear as clouds of points representing the fluorescence lifetime distributions. Points in the phasor plot can be selected using a colored cursor and the corresponding pixels are mapped back with selected color to the image pixels. The phasors in the plot follow vector algebra, so that the combination of two single exponential decays components (that lie on the universal circle) will lie on a straight line joining the phasors of the two fixed components (Figure 6c). Importantly, each molecular species is represented in a specific location in the phasor plot becoming essentially the fingerprint of the molecule in a selected environment. This is particularly useful when attempting the identification of specific molecular components and their interactions within biological samples using their intrinsic fluorescence signal. Applications of FLIM coupled with two photon spectroscopy in biosciences are uncountable, they range from selective visualization of fluorescent molecules with overlapping spectral features, to biosensing applications and tracking of molecular interactions in living tissues (Chen and Periasamy, 2004; Stringari et al., 2017; Yellen and Mongeon, 2015).

2.3 Tissue imaging

Features of two-photon excitation microscopy described in previous sections made this technique a valuable and increasing widespread method for the analysis of biological tissue both in vivo and ex vivo. The most common application is the topological analysis in 3D of tissues architecture (Helmchen and Denk, 2005; Mulligan and MacVicar, 2007; Wu and van Zandvoort, 2014). Moreover, it is possible to extract additional quantitative information physicochemical features of the tissue component and on functional reaction by coupling the visualization of fluorescence observables with spectroscopic analysis (Chen and Periasamy, 2004; Meleshina et al., 2016; Rubart, 2004).

Biological tissues can be analyzed in 3D and with different levels of accuracy by means of the use of multiple fluorescent markers. Fluorescent dye exists for a wide range for applications to label biomolecules, mark organelles or specific targets and to monitor physicochemical properties of the sample allowing dynamic monitoring different important parameter such as membrane potential,

membrane order, reactive oxygen species (ROS) or metal ion presence, mitochondrial membrane potential, pH and so on. Most of these dyes are accessible to multiphoton microscopy. However, two-photon microscopy has become an incomparable tool for specific applications as it opens the way to the analysis of intrinsic fluorescence of endogenous molecules in biological samples (autofluorescence) that still recently was considered an annoying background when analyzing stained samples. Instead, it is becoming increasingly evident, also owing to FLIM availability, that a large number of information can be extracted from the analysis of these signals. Indeed, as it is possible to infer, changes occurring during physiological processes, variations of the spatial distribution of intrinsic fluorophores, their concentration, chemical modifications or change in their local environment may occur. These variations result in changes of the detected fluorescence signal. Autofluorescence measurements require minimal or no specimen treatment thus allowing reduction of artifacts induced by exogenous molecule presence.

Endogenous chromophores are often the main players of molecular reactions of great interest for biological functions/dysfunctions and their fluorescence can be excited by means of IR laser beam and acquired in a broad region in the near-UV-visible range also allowing multicolor experiments. For reference, few examples of endogenous fluorophores in tissues are listed in table 1 together with suitable single photon excitation wavelengths which fall in the UV side of the electromagnetic spectrum.

Fluorophore	λ_{exc}	Ref.
Aromatic aminoacids	240-300 nm	(Lakowicz, 2006)
Collagen	330-340 nm	(Fujimoto et al., 1977)
Elastin	350-420 nm	(Blomfield and Farrar, 1969)
Vitamin A	350-380 nm	(Sobotka et al., 1943)
Flavins	350-450 nm	(Berg, 2004)
NAD(P)H	330-380 nm	(Schneckenburger, 1992)
Folic acid	< 400 nm	(Baibarac et al., 2019)

Table 1 *Non-exhaustive list of fluorescent endogenous molecules in biological tissues, that can be excited using two photon excitation using wavelengths between 700 and 850 nm. As a note aromatic aminoacids cannot be detected in fluorescence microscopy without the use of dedicated optics.*

The dominant contribution in tissues autofluorescence is given by extracellular matrices collagen and elastin, which are massively present, and they are often organized in micron scale supramolecular arrangements. The molecular origin of their signal is still puzzling possibly being attributed to cross-links (Deyl et al., 1980; Fujimoto et al., 1977) their fluorescence has been reported in different conditions and in different spectral range both for excitation and emission. Two-photon microscopy can be used to monitor chemical modification in tissues due oxidation or AGE effects, lipopigments for example can be excited in a range between 340–400 nm and to have an emission spectrum with

two main peaks centered at 450 nm and 600 nm respectively (Sohal, 1984). Tyrosine oxidation induced by UV can be excited at about 350 nm, these and other oxidation or AGE products contribute the autofluorescence of biological systems in the visible range. Other interesting fluorescent molecules with relevant application are NAD(P)H and FAD which can be used to determine the redox state of the cell (Agati et al., 2003; Richards-Kortum and Sevick-Muraca, 1996; Schomacker et al., 1992). Interestingly, in a recent studies a combination of two-photon imaging, FLIM and phasor approach was used to image cellular metabolism quantifying variations in NADH lifetime distribution (Gómez et al., 2018).

The use of FLIM methodology is particularly valuable and will certainly soon bring to numerous advances overcoming the main issue of autofluorescence analysis. Indeed, interpretation of data based on autofluorescence is not always straightforward since most of the endogenous molecules are excited in superimposed spectral range in the UV and emit in the far UV or in the green region of the visible spectrum (Zipfel et al., 2003a). In this instance and depending on the sample, the molecular fingerprint provided by FLIM and phasor analysis can be a valuable additional information.

In Figure 7, two-photon cross sections (a) and fluorescence spectra of some endogenous fluorophores (b) in biological specimens are reported. These underly the challenge in the spectral separation, when single species and their variations need to be identified. A valid support in solving this issue is provided by phasor analysis.

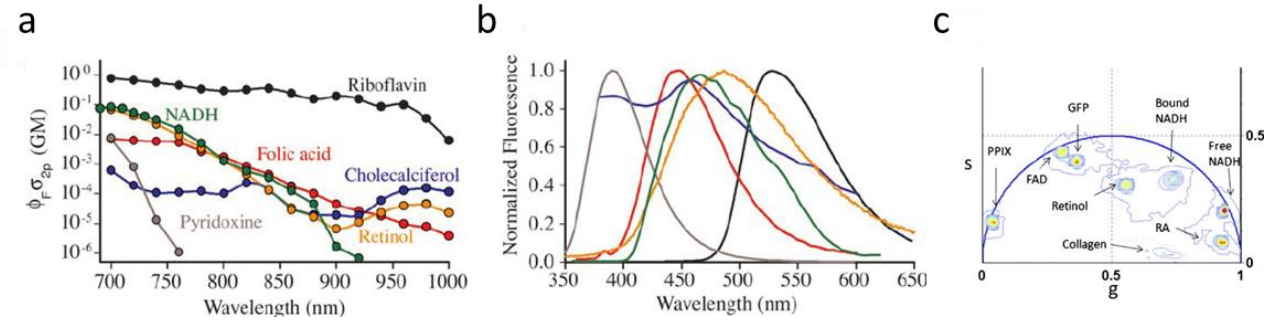

Figure 7 *Spectral properties of endogenous fluorophores in biological tissues. (a) two-photon cross sections (b) fluorescence spectra and (c) lifetime distributions of endogenous fluorophores occupy a specific position in the phasor plot. Adapted from* (Zipfel et al., 2003a) *and* (Stringari et al., 2011b)

The most common use of two-photon fluorescence microscopy is deep tissue imaging performed in combination with SHG that "comes for free" when performing measurements. Two-photon fluorescence microscopy combined with SHG readily provide accurate microscale 3D reconstruction of collagen and elastin fibrils architecture and orientation. This approach was successfully applied revealing the structure of bones, tendons, cardiovascular tissue, and cartilage orientation of elastin and collagen bundles (Andrews et al., 2014; Berezin and Achilefu, 2010; Brockbank et al., 2008; Brown et al., 2014; Chen et al., 2012; Coluccino et al., 2017; Georgiadis et al., 2016; Pang et al., 2017; Schenke-Layland, 2008; Xu et al., 1996a; Zipfel et al., 2003b, 2003a; Zoumi et al., 2004). Usually elastin is mapped by means of the fluorescence signal which is more "red shifted" whilst SHG is used for efficient collagen detection. Collagen as its triple-helix structure is not centro-

symmetric, and it is very effective in generating SHG. The high directionality of SHG provides also information on fibrils.

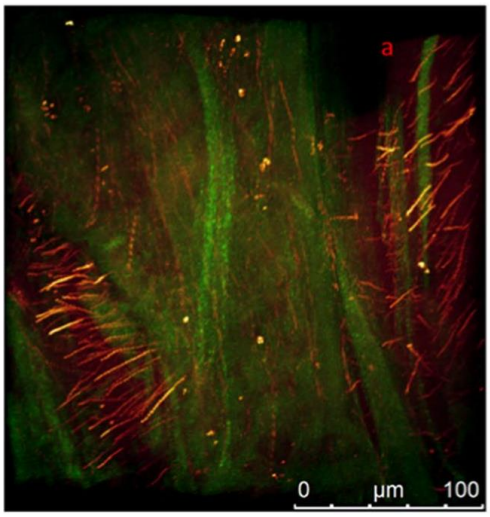

Figure 8 *two-photon analysis of the radial section of the central portion of the lateral meniscus. 3D reconstruction of a 60 um depth measurement. The overlap of SHG (green channel) mainly due to collagen fibrils and autofluorescence (red channel) is reported, as can be seen elastin fibers are easily distinguishable for their morphology and stronger intensity. Adapted from* (Vetri et al., 2019)

In Figure 8, as an example, measurements acquired on lateral meniscus tissue. Representative 3D reconstruction is shown. SHG signal (green) is acquired under excitation at 880 nm and acquired at about half of incident wavelength. Fluorescence signal (red) is collected in the range 485 nm–650 nm. Fluorescence signal is not highly specific in this case as it can be attributed to multiple contributions of endogenous molecules, however elastin fibrils are clearly distinguishable in their beautiful arrangement for their fibrillar morphology and their higher intensity of fluorescence signal. Collagen sheets, characterized by widths between 5 and 10 µm, run along the images. The 3D reconstruction makes possible to appreciate a group of collagen bundles arranged in straight and wavy patterns which are enclosed by elastin fibers oriented along the perpendicular direction.

In tissue studies, two-photon measurements can be acquired at different depths and over different space scale (from micron to nano) to quantify orientation and periodicity of the fibrils and bundles. These measurements are important to relate the architecture of the tissues to its mechanical properties, or to specific pathological conditions.

2.4 Functional brain imaging

The biological function occurs in such a complex tissue environment that ultimately has to be studied in intact samples (Fee, 2000; Svoboda et al., 1997; Venkatachalam et al., 1999). Optical recordings are the only means by which the intact brain can be studied with micrometer-spatial resolution. Due to the strong scattering of visible light in tissue, imaging of the deeper layers of cortex had to await the introduction of two-photon excitation fluorescence microscopy into neurobiology (Denk and Svoboda, 1997). Brain function emerges from the coordinated activity, over time, of large neuronal populations placed in different brain regions. Understanding the relationships of these specific areas

and disentangling the contributions of individual neurons to the overall function remain a central goal for neuroscience. In this scenario, two-photon microscopy has been proved to be the suitable tool of choice for the in vivo recording of brain activity (Figure 9).

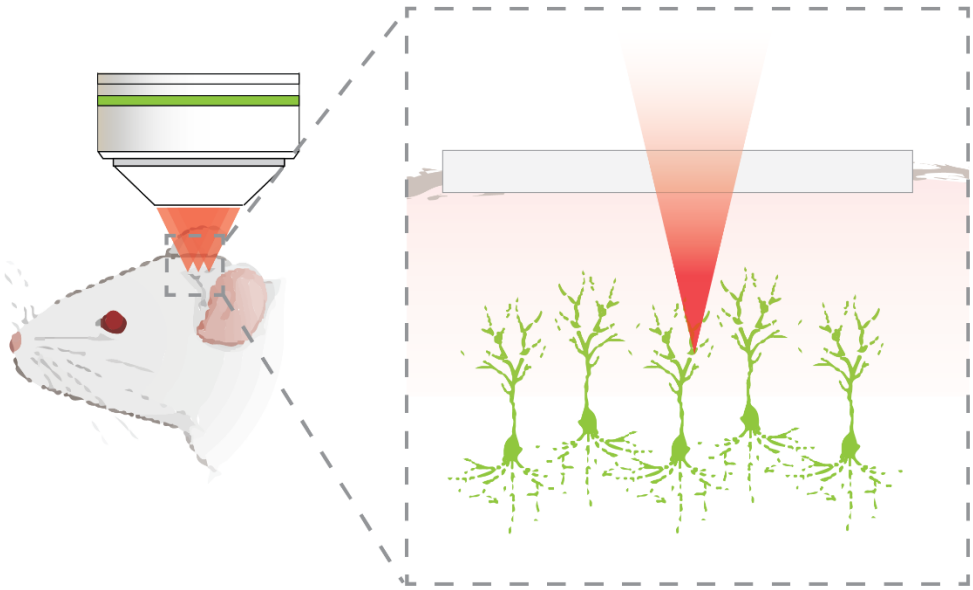

Figure 9. *In vivo brain imaging. Schematic of two-photon microscopy trough the skull of an awake mouse. Fluorescence from different neurons can be acquired in deep tissue up hundreds of micrometers.*

Optical advances combined with genetically encoded indicators allow, nowadays, a large flexibility in terms of spatial-temporal resolution and field-of-view, while keeping invasiveness in living animals to a minimum (Sancataldo et al., 2019). Calcium imaging with fluorescent indicators provides an optical approach to monitor action potentials and is being used systematically to measure in vivo neuronal activity. Recently, much effort has been devoted to the development of new optical architectures for advanced two-photon microscopy. Nowadays, optical architecture that allow imaging of large region (Large Field of View) of model animal brain can be achieved by custom made (Bumstead, 2018; Tsai et al., 2015) or commercial (Sofroniew et al., 2016) microscope. Emergent optical tools opened the way to fast beam scanning that boosted imaging recording (Grewe et al., 2011; Reddy and Saggau, 2005; Yang et al., 2016). The optical in vivo optical strategies enabled to tackle complementary aspects of neuronal dynamics, encompassing small networks to whole brain. The functionality of deep neurons with subcellular resolution in alive and awake animals can now be achieved which seemed unattainable only a decade ago.

3. Step-by-step protocol

Microscopes are often thought as instrument easy to manage and quickly suitable for everybody. These beautiful machines open continuously new possibilities pushing forward the number information that can be obtained and the number of questions that can be approached due to the high sensitivity and specificity of fluorescence. However, knowing the fundamentals of the methods, and details of instrumental setup is of utmost important to avoid error during image acquisition that may

lead to wrong results. In this context, it has also be taken in account the importance of sample preparation, as manipulations and staining procedure may misrepresent the results (North, 2006). Thus, the specimen is the most important part of the experiment and its properties should match both the characteristics to answer the scientific question and the instrumentation features.
Designing a two-photon imaging experiment is most often an iterative process. Image acquisition parameters and analysis tools usually need to be set and tested several times until a reproducible standard protocol is validated. In the following a starting and general protocol for imaging samples by means of a two-photon microscope.

■ Setting up the microscope

Thanks to the development of commercial turnkey pulsed laser systems, multiphoton microscopy is now available for everyone to use without extreme complexity. Turn on the laser and the microscopy hardware according to the manufactures. Ideally, the laser or any illumination source should be warmed up for a minimum of a couple of hours before imaging to avoid fluctuation of the light intensity.

■ Selecting an objective lens

The choice of the suitable objective for two-photon imaging of the sample is determined by three main considerations: numerical aperture (NA), working distance and magnification. The NA must be large enough to provide enough spatial resolution and to adequately collect fluorescence from the specimen. The working distance of the objective must be great enough to reach the desired plane of focus within the specimen. Magnification must be chosen in order to image an ample portion of the sample. Choice of immersion medium of the objective is a compromise between accessible resolution and the working distance, depending on the sample, one immersion medium can be more advantageous than others as mismatch between specimen and immersion media refraction index may induce the reduction of collected signal and image aberrations. Usually the ideal objective for typical two-photon imaging is the lowest magnification lens with the highest available NA.

■ Configuring and optimizing fluorescence channels

The photophysical properties of fluorescent probes are critical for two-photon experimental design. It is not so simple to predict two-photon excitation spectra from the one-photon spectra since they follow fundamentally different quantum-mechanical processes and obey very different selection rule. The two-photon spectra of several useful fluorescent molecules have been measured. Compared to one-photon spectra, two-photon spectra tend to be broader and shifted toward the blue.

The choice of excitation wavelength is generally determined by the fluorophore(s) to be excited. As a rule, select a wavelength that is about two times longer than the wavelength required by a single-photon excitation. Select the appropriate emission range for the fluorophore(s). Commercial microscopes usually allow to select band pass filter or a specific spectral range. In case of multicolor detection, avoid overlaps among bandwidths to reduce crosstalk among different channels.

■ Setting up the specimen

Mount the sample on the microscope stage and focus on it using eyepiece in bright-field or epifluorescence mode. A motor in steps of hundreds of nanometers usually controls the microscope focus. Once the sample is in focus it is important to look at two-photon fluorescence. To avoid photobleaching or photodamage keep the laser power to its minimum intensity. Once the region of interest has been found optimize laser intensity till a suitable signal to noise ratio.

■ Optimizing spatiotemporal parameters

Commercial two-photon microscopes offer many different acquisition modes. Volumetric imaging and time-lapse modes are most common modalities and allow to image the specimen over time. Set the frame rate by independently adjusting frame size, scan speed and number of sections. In case of advanced fluorescence microscopy technique (i.e. spectral detection and FLIM) set the appropriate parameters. Be prepared to start it over several times, the best practice indeed, requires that comparison between samples is made using the same experimental parameters.

■ Data acquisition and visualization

It is important to look for compromise between acquisition parameters that allow the faster possible measurements with higher signal to noise ratio. Avoid (if possible) pixel in the image where intensity is out of scale (saturation) in these conditions light arriving to the detector above this limit will not be taken in account certainly affecting image quantification. Image with saturated pixels omit a number of spatial features and they are not pretty! Imaging of samples could span a large range of timescales. In case of long acquisition, check image quality, sample defocusing and photobleaching.

Once data are collected, use appropriate software for visualizing and analysis.

Table 1. **Glossary**

Dichroic mirror	Mirror that selectively reflects one or more parts of the light spectrum while transmitting the rest of the spectrum. In a two-photon microscope it is commonly used to filter the fluorescence from the excitation light.
Field of view (FOV)	The area that can be imaged by the microscope. It depends on the magnification of the optics and on the raster scanning.
Fluorophore	Molecule in which the absorption of a photon triggers the emission of a photon with a longer wavelength, making it visible in a fluorescence microscope. Biological tissues are commonly labelled with fluorophores through immunostaining or genetic expressed fluorescent proteins
Numerical aperture (NA)	Dimensionless number that characterizes the range of angles over which the system can accept or emit light. It is defined as the product of the medium's refractive index n and the sine of the half angle of the maximum cone of light that can enter or exit the lens.

Penetration depth	Depth to which a satisfying image quality can be achieved; depends on the transparency of the sample and the imaging modality used.
Photo-bleaching	Process whereby a molecule is rendered non-fluorescent. Almost all fluorophores fade during light exposure.
Raster imaging	Process in which the final image is built up piece by piece through the sequential illumination (and detection) of small areas (point or lines); required in microscopes that image only a fraction of the image at a time, such as confocal and multi-photon microscopes.
Resolution	Smallest distance between two points in a specimen that can still be distinguished as two separate entities. Theoretically only dependent on the NA and the wavelength of light used to image the object. In practice, the achievable resolution also depends on additional parameters such as contrast, noise and sampling. In single-lens microscopy, the axial resolution along the axis of the detection objective is always worse than the lateral resolution.
Scan mirrors	Rapidly moving mirrors required in point or line scanning microscopes to sequentially illuminate the whole area of interest.

TAKE HOME MESSAGE

- Fluorescence microscopy images are maps of fluorescent molecules, which can be endogenous in the sample or extrinsic markers and their analysis provide spatial and functional information. Fluorescence signals are the fingerprint of the molecules and of their environment.
- Two (multi)-photon fluorescence microscopy is a powerful tool for imaging and analyzing three-dimensional (3D) samples providing high spatial and temporal resolution ideal for in vivo experiments.
- The development of Advanced methods for tissue imaging available for two-photon imaging such as Second Harmonic Generation (SHG) and Fluorescence Lifetime Imaging Microscopy (FLIM) increase the level of information accessible in a single experiment.
- Image resolution using two (multi)-photon fluorescence microscopy remains within submicron micron scale (still below with respect to other methods like electron or atomic force microscopy). However, the possibility of investigating living cells/organisms close to their physiological conditions remains a great advantage of fluorescence methods.

4. References and further reading

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