Multiphoton microscopy

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I. 2. c. Multiphoton microscopy

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Abstract

Multiphoton microscopy (MPM) is an optical method adapted for imaging biomedical samples. The practice of MPM is based on an experimental protocol close to standard fluorescence microscopy approaches and delivers information about structure, physiology or pathologies of specimens. The originality of MPM rests on the near infrared range of excitation required for the generation of multiphoton processes. New optical windows of imaging are thus opened, providing images of living samples with a micrometric and subcellular resolution at millimeter depths. Moreover, no labelling is required when biomedical samples contain endogenous fluorophores. This procedure is the exclusive one opening the way to *in vivo*, 3D, label-free and in live imaging. To achieve this ultimate goal, MPM still require many technical development and biological studies in order to identify new optical windows and new biological substances having endogenous fluorescence properties. Besides, new computational strategies for image restoration, based on the more recent and sophisticated mathematical principles have been developed especially for MPM. Based on the estimation of the instrument response function, an optimized image quality can be provided with an upgraded signal to noise ratio resulting in an improved visual quality.

1. Introduction

Multiphoton microscopy (MPM) is an optical method for characterizing biomedical specimens. The routine practice of MPM rests mainly on the detection of fluorescence from samples labeled with fluorescent probes similar to those employed in confocal microscopy (CM). The specificity of MPM concerns the excitation processes requiring the simultaneous spatiotemporal interaction of two photons at least. In MPM and CM,

similar wavelength ranges of emission are detected. This assumption has been experimentally highlighted in the 90's on chromosomes of live cultured pig kidney cells [1], illustrating a theoretical prediction proposed by Maria Göppert-Mayer in 1931 [2].

The interest of MPM in biomedical imaging lies mainly on its excitation principle occurring in the near infrared range (NIR). Compared to a linear excitation in the UV or visible range involved in CM, a NIR of excitation cumulates the advantages to be less energetic and less absorbed by biological compounds of tissues. Such experimental condition is more favorable for deep tissue imaging and *in vivo* or 3D imaging of biological samples. Likewise, in specific experimental conditions, alternative and label-free probing processes can occur with a multiphoton excitation such as harmonic generation [3-5].

In the present chapter, physical principles of MPM will be resumed and typical set-ups detailed. Then, the biomedical relevance and sample preparation will be evoked and illustrated with multiphoton images. A discussion about parameters qualifying image quality and resolution will be proposed. This will open on a discussion about strength and limitations of MPM and hot topics resulting in future developments of MPM.

2. Principles & Set-ups

2.1. Physical Principles

The physical principles of MPM lie on the simultaneous interaction between a minimum of two photons and a biological target – a fluorophore or a structure – leading to a high-order nonlinear light-matter interaction. The resulting optical emission has a quadratic or higher-order dependence on excitation power, delivering to MPM its nonlinear feature. A routine practice of MPM exploits two categories of nonlinear interactions: two-photon fluorescence (TPF) and second harmonic generation (SHG). Alternative

high-order interactions are included more marginally in biomedical MPM such as three-photon fluorescence, third harmonic generation or coherent Raman scatterings. More precisely, a laser beam is associated with an electric field strength E which induces a macroscopic polarization of matter Π, formalized by equation (1), Π_0 being the permanent polarization, ε_0 the vacuum permittivity.

$$\Pi = \Pi_0 + \varepsilon_0\left(\chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \cdots\right) \qquad (1)$$

In equation (1), χ designates the medium susceptibility. $\chi^{(1)}$ corresponds to the linear susceptibility of the matter, $\chi^{(2)}$ and $\chi^{(3)}$ are nonlinear tensors describing nonlinear interactions.

TPF and SHG are two multiphoton probing processes grounded on two distinct physical phenomena. TPF, decomposed in a two-stroke mechanism, involves the third-order susceptibility of the fluorophore. First, the combined energy of two photons matching with the energy gap between ground and excited states of the fluorescent molecule is absorbed and generates a molecular transition to an excited electronic state. For each endogenous or exogenous fluorophore, a specific two-photon absorption (TPA) cross section determines the adapted spectral bandwidth of excitation. Then, a back transition to the electronic ground state gives rise to the emission of a photon of fluorescence. No significant difference is noticeable between the photon of fluorescence emitted with a one- or a two-photon process. As for SHG, an instantaneous nonlinear coherent light scattering phenomenon arises in specific light and matter conditions which constitute the purpose of next chapter (see chapter I.2.d. SHG/THG and chapter I.9.g. Coherent Raman Imaging (CARS & SERS)).

2.2. Typical Set-ups & State-of-the-art

Hardware set-ups

A high-order nonlinear light-matter interaction implicates the merging of four experimental conditions: the (i) simultaneous (ii) spatial interaction of (iii) two photons at least (iv) in the NIR. By definition such a phenomenon is lowly likely. The merging of these specific requirements is the root of several major technical constraints impacting the structuration of multiphoton microscope set-ups. Figure 1 illustrates a typical set-up of MPM.

First, the spatiotemporal confinement of the excitation beam is ensured by a pulsed laser for temporal confinement, in the NIR for matching the energy gap of the fluorophore through a focusing element for spatial confinement. Then, a balance between specimen safety, image signal to noise ratio and standard laser average power arbitrates the density power deposited upon target sample. An optimization of this combination is favored by the use of a laser scanning strategy.

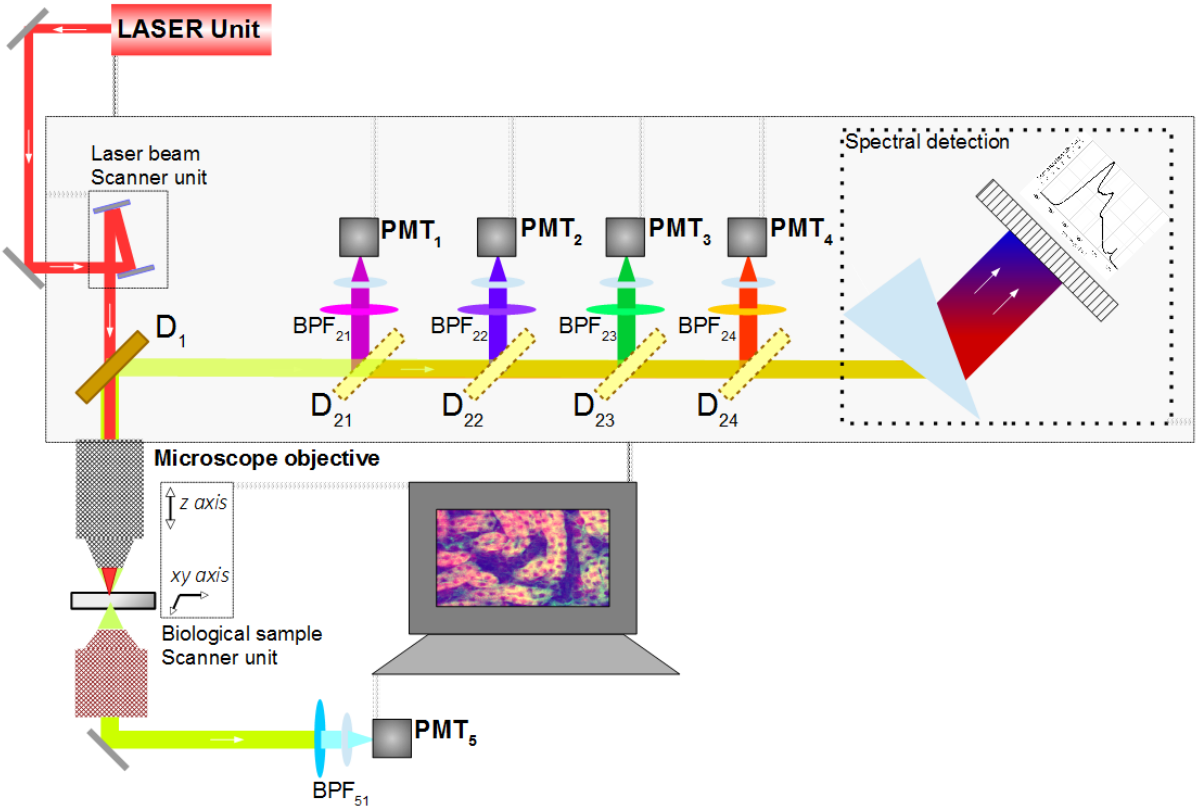

Fig. 1. *Experimental setup of MPM. PMT: Photomultiplier tube, D_{ij}: dichroic mirrors, BPF_{ij}: band pass filters. Typical systems are equipped with two detectors.*

The starting point of typical MPM set-ups is constituted by a titanium-doped sapphire (Ti: Sa) laser. This solid-state mode-locking laser is based on a ion-doped crystal and delivers femtosecond pulse durations in the NIR with a spectral bandwidth of 10 nm at the full-width at half maximum (FWHM), tunable between 650 and 1100 nm. Standard repetition rates are about 80 MHz with an average power of few watts. When the Ti: Sa laser source is coupled with an optical parametric oscillator, the excitation beam with a spectral bandwidth of 10 nm can be tuned until 2.5 µm at best.

Image formation

Excitation beam is focalized on the biological specimen through the microscope objective and gives rise to the signal emission which is detected in backward or in forward position. A backward detection is the more standard situation, mainly due to the impossibility for emission beams to go through the sample in case of tick tissues or *in vivo* imaging. Image formation is thus ensured by a laser scanning strategy which scans point-by-point each pixel region of the sample. The emitted beam is then collected by detectors; photomultiplier tubes (PMTs) are currently one of the more sensitive detection solutions. PMTs must be associated with a dichroic mirror and a band pass filter for spectrally select a wavelength range of emission, depending on the biological target imaged. A spectral detection is an attractive alternative solution. The emission wavelengths are recorded in one shot from the UV to the red wavelengths as long as the emission intensity is enough. Nevertheless, the latter necessitates a longer exposition duration which could be toxic for specimen and generates bigger data, an image being constituted by a spectrum for each pixel. Thus, MPM provides 2D-images reconstructed point-by-point thanks to the laser beam scanner unit synchronized with the detection system. With an axial motion of microscope objective, stacks of 2D images can be generated step by step and results in the realization of 3D images.

Commercial equipment

Commercial multiphoton microscopes are proposed by numerous companies, more or less established and recognized by biologists. Leica, Nikon, Olympus and Zeiss are the four best known companies for microscopy devices which sell turn-key solutions of multiphoton microscopes. The basic structure is composed by a Ti: Sa laser, a galvanometric scanning system, two detectors and a command unit. The cost of a standard solution is about few hundred thousand euros, the main cost item being the Ti: Sa laser (about 150 k€).

3. Biomedical Relevance of multiphoton microscopy

3.1. Application Range & Relevance of multiphoton microscopy (MPM)

Application range of MPM

The development of nonlinear optical microscopy and spectroscopy have historically played an important role for the understanding and advancement of scientific knowledges. The biomedical establishment of MPM is currently in progress, mainly in deep tissues imaging and in the field of neurosciences, cancer detection, structural characterizations, quantification and localization of proteins or metabolic indicators, etc. Figure 2 illustrates visually few of these applications. Concerning preclinical and medical researches, current specific advances in engineering and technical endomicroscopic strategies still on the spotlight drive progressively MPM towards animal or human surgical units (see chapter II.5. Confocal and multiphoton Endomicroscopy).

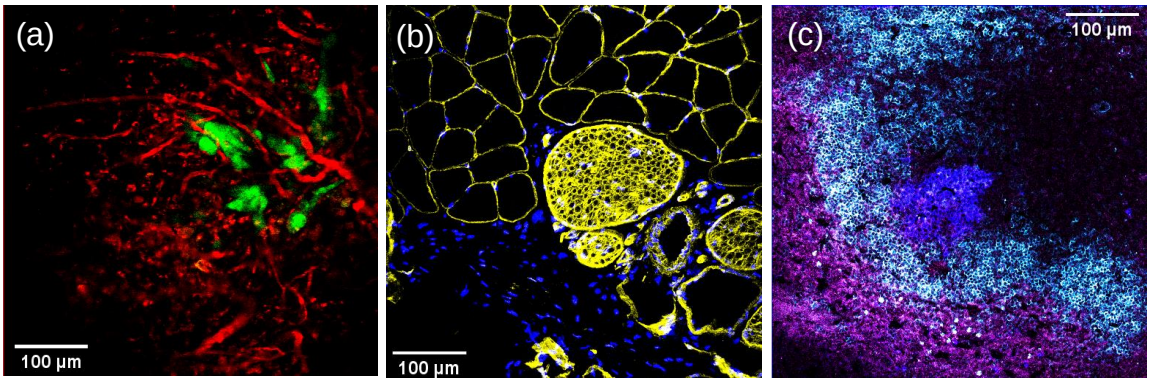

Fig.2. *Multiphoton images generated at XLIM Research Institute with two or three channels. a. Brain imaging:* in vivo *imaging of glioblastoma cells implanted into mouse brain. Green: GFP U87 cells; Red: Rhodamine 6G injected into blood vessels. b. Orthogonal muscle slice from wild mouse: Laminin, (Alexa Fluor 546, yellow) and nucleus (DAPI, blue). c. Localization and quantification of lymphocytes into mouse spleen. Blue: B Cells; Cyan: T Cells; Magenta: D cells.*

Relevance of MPM

More generally, the relevance of MPM is related to the NIR of excitation wavelengths combined with a multiphoton excitation process. Such a strategy opens to (1) additional depths of imaging in biomedical samples with micrometric resolutions, not accessible in CM or alternative imaging strategies, (2) an optical sectioning inherent to the probability of presence of multiphoton interactions and (3) the identification of endogenous fluorescence of biological substances yet unidentified. Therefore, this combination drives MPM towards a label-free 3D microscopic imaging of tick tissues in depths yet unattained. With a NIR of excitation, 3D imaging with 1.6-mm depth has been demonstrated in mouse cortex [6] labeled with a standard fluorophore, thanks to an excitation wavelength centered at 1280 nm. By adjusting correctly the spectral range of excitation, many new windows of transparency of sample imaged can be found on the one hand and new endogenous fluorescence of biological substances can be identified. Moreover, the optical sectioning ability of MPM opens to a 3D vision

of the specimen combined with a sub-micrometer resolution scale, an association with performance not accessible with any other imaging system.

3.2. Sample Preparation

In MPM, similar processes of sample preparation are involved as in confocal or widefield fluorescence microscopy approaches. Multiphoton images are generated from cell cultures, from resected and labeled tissues and also from *in vivo* organs. In MPM and with a noncentrosymmetric structure, SHG can be generated. Third harmonic generation can also be produced when a condition of negative phase mismatch exists, mainly in a non-homogenous medium. These two last methods are detailed in the chapter I. 2. d. SHG/THG. Their interest in biomedical imaging is major, essentially thanks to their label-free generation.

4. Parameters of Image Quality

Multiphoton fluorescence microscopy and CM produce the same kind of photons of fluorescence. Factors affecting image accuracy, precision and sensitivity are similar. More details can be found in Chapter I.1.b Fluorescence & Confocal Microscopy. Concerning harmonic generation, this anisotropic physical phenomenon is optimized by specific properties of light polarization, detailed in Chapter I.2.d. SHG/THG.

More generally and for a routine practice of MPM, two main parameters must be attentively adjusted for an instantaneous optimization of image quality: those concerning the excitation strategy and those affecting the detection sensitivity. For the excitation method, excitation average power, pixel dwell time and scanning speed can impact greatly the levels of fluorescence emitted and also the sample safety. An equilibrium between these two antagonists' properties must be found and is specific to each set of experiment which becomes crucial in case of *in vivo* imaging. Concerning

detection sensitivity, detector voltage, gain and offset play a significant role in the generation of image quality and mainly impact contrast and brightness properties.

When the best image quality is sought, an additional set of experiment can be lead and is detailed in next section: 5. Data processing.

5. Data Processing

In MPM, a data processing strategy is relevant when the best visual image quality is sought. The most elaborated one in optical microscopy, also valuable in MPM, rests on the precise knowledge of the instrument response function also named “point spread function” (PSF). This strategy is based on the consideration that the resulting image generated in MPM is a convolution between the instrument response function and the true image of the sample. With an adapted computational strategy, PSF contribution can be reduced or even erased from the image which can thus be optimally restored. This protocol consists in imaging standardized objects, usually microspheres with sub micrometric dimensions [7]. Identical imaging parameters must be preserved between image generation and PSF characterization: pixel dwell time, detector properties, wavelength ranges of excitation and detection, mounting media, average power, field of view, etc. Then, a deconvolution calculation involving a computational strategy is applied. It results in removing the instrument contribution to the image. This procedure often produces images with a highest visual quality, which sometimes reveals details initially lost into noise and blur of raw image. Several computational strategies exist, the most known being the commercial software Huygens from “Scientific Volume Imaging” which proposes a multiphoton module. More recent approaches are still in research development especially for MPM with the most recent computational and mathematical strategies involving for example robust multivariate Gaussian shaping strategies [8].

6. Conclusions

6.1. Strength & Limitations

Strengths

The main interest of MPM concerns the NIR of excitation presenting two main advantages compared to more standard linear fluorescence technics: being less dangerous for biological targets and on the whole contained in a less absorbing optical window. MPM is thus the exclusive solution able to go toward the generation of deep tissue imaging and 3D images with a micrometer resolution. By evidence, a new outlook of biological samples, structure, constitution, etc. become accessible.

Limitations

Since about ten years, optical platforms dedicated to photonic microscopy for biomedical researches have been equipped with expensive multiphoton microscope systems. Today, after a decade, many of these multiphoton systems are partially or mostly vacant. MPM delivers a new point of view of targets from life sciences but has probably not yet reached an optimal technological maturity for a well-established use in biomedical researches. Several reasons for justifying this fact can be proposed.

First, when a microscopic analysis is necessary, the habits of biomedical researchers often direct them towards usual linear fluorescence strategies with an initial procedure of sample preparation, slicing, labelling and imaging steps, also implemented in MPM. Slices are then scanned, red and the high quantity of data must be analyzed and classified.

Then, light diffraction limit induces a resolution decrease when excitation wavelength increases. Thus, the NIR of excitation induces a less performant resolution than a UV-visible excitation. But this question can finally be lesser than the other questions raised by the realization of 3D images: what field of view dimensions can be observed? A

compromise between field of view and resolution must be chosen; the largest 2D field of view, depending on optical properties of the microscope objective, is often about 0.5 × 0.5 mm. In axial direction, few micrometers of resolution can be hopped at best. Furthermore, tissue scattering and absorption still exist with a NIR of excitation and limit the typical imaging depth to less than a millimeter. Moreover, when a 2D image with two color channels is generated with a standard resolution of 512 pixels × 512 pixels, a 0.5 Mo image is produced. With a 0.5 µm step in axial direction, and for a thickness of 1 mm, an image size of 1 Go is produced. The analysis of data with such a dimension is another challenge reserved to specialists of Big Data management.

6.2. Future Developments

The limitation points raised in the previous paragraph are currently considered for technical progression and numerous novelties are proposed day by day. Other progresses, yet confidentially studied, could also be the subject of scientific efforts highly interesting for future developments of MPM. Indeed, today, an alternative avenue of future development might concern the research of new endogenous fluorophores inside biomedical specimens. Indeed, the structural constitution of the main biological constituents of samples is organic with a structure composed by complex assemblies of proteins, enzymes, genetic material, amino acids, etc. A low quantity of these substances have already been characterized in endogenous fluorescence (tryptophan, tyrosine, NADH and FAD, elastin, collagen or keratin). Considering the organic constitution of biological structures, other biological substances might be endogenously fluorescent. They are often classified as “background noise” in CM. With a linear fluorescence strategy, characterizing endogenous fluorescence looks hard due to the closeness between excitation and emission spectra. A multiphoton excitation strategy might open new characterization

possibilities thanks to the hundreds of nanometer of spectral separation between excitation and emission. Associated with a spectral detection in the UV and visible ranges, a spectral scanning procedure in the NIR of excitation might delivers new information about endogenous fluorescence of biological substances. Such a strategy would require several preliminary steps such as particle separation with a preliminary identification, which covers partially the problematics of flow cytometry.

Such a scientific direction could already be in progress thanks to the implementation of new laser systems in MPM based on optical parametric oscillations [5]. But their expensive cost (about 300 k€) restrains this research field which would rather be favored by the recent apparition of ultrawide band laser systems in MPM, also named "supercontinuum lasers". Based on a simpler and cheaper physical principal of spectral broadening of laser pulses into nonlinear media, a continuum of spectral wavelength is generated and can be selected. Such a strategy is currently in progress and can facilitate this research field highly interesting in readiness for future developments of deep tissue imaging with label-free, 3D, *in vivo* and in live MPM.

7. References & Further Readings: Vancouver Numerical System

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