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Marie-Geneviève Blanchin

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# Non-linear photonics for intravital microscopy in health sciences: application to detection of nanoparticles in organs

**M.G. Blanchin**

Institut lumière matière, UMR5306 Université Claude Bernard Lyon1-CNRS,  
Université de Lyon 69622 Villeurbanne cedex, France.

E-mail: Marie-Genevieve.Blanchin@univ-lyon1.fr

**Abstract.** Intravital microscopy (IM) for health sciences can be achieved by means of Laser Scanning Microscopy (LSM) in two Photon (2P) excitation conditions using Infrared (IR) illumination which has good penetration in all tissues and organs. Imaging is then performed using 2P emission of fluorescence (2PE) and second harmonic generation (SHG) modes which provide high contrast and good spatial resolution. These modes do reveal the different parts of organs due to differential fluorescence and SHG. In the case of atherosclerotic aorta, for instance, the formation of the atherosclerotic plaque can be visualised and located with respect of aorta's structure: an important application is the diagnosis about instability or stability of the plaque. Also the spatial resolution of LSM allows revealing the heterogeneous biodistribution of iron oxide core (USPIO) nanoparticles for diagnosis of the atherosclerotic plaque, from the aorta's scale down to the subcellular level: detection of aggregates of USPIOs at the subcellular level supports the model of USPIOs phagocytised by macrophages which target the plaque.

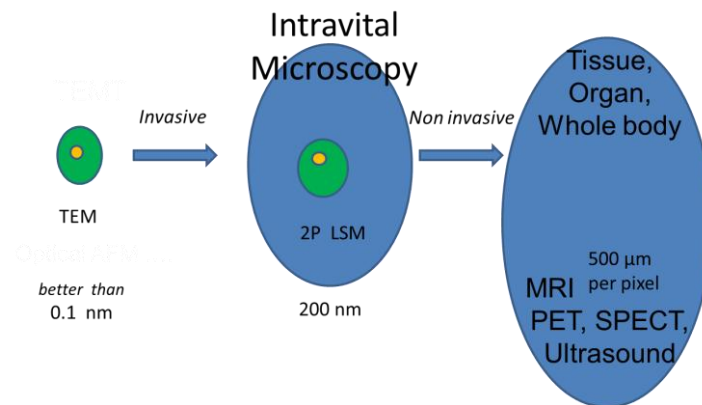
## 1. Introduction

The interest for nanoparticles (NPs) in biomedical applications is strongly growing during the last decade [1]. They are often designed as a multimodal platform for combined therapy and diagnosis (theranostics) applications [2]. Their core may contain a drug that is protected from the biological environment by a shell structure [3] like e.g. silica. The imaging moieties at the shell surface or in the core may be radioactive, paramagnetic or fluorescent for positron emission tomography (PET/SPECT) [4], magnetic resonance imaging (MRI) [5] and intravital microscopy (IM.) [6] respectively. MRI as one of the most widely used non-invasive imaging technique, permits analysis going from the whole body distribution to the microscopic biodistribution in organs and tissues, but its spatial resolution is limited at 500  $\mu\text{m}$  per pixel (Figure 1). On the other hand studies of structure and biolocalisation of NPs requires techniques having spatial resolution better than a few 0.1 nm, like transmission electron microscopy (TEM). Figure1 sketches the resolution gap between MRI and TEM, for instance, which can be filled by Intravital Microscopy achieved at a resolution of a few hundred nm by means of Laser Scanning Microscopy (LSM).

## 2. Intravital microscopy (IM)

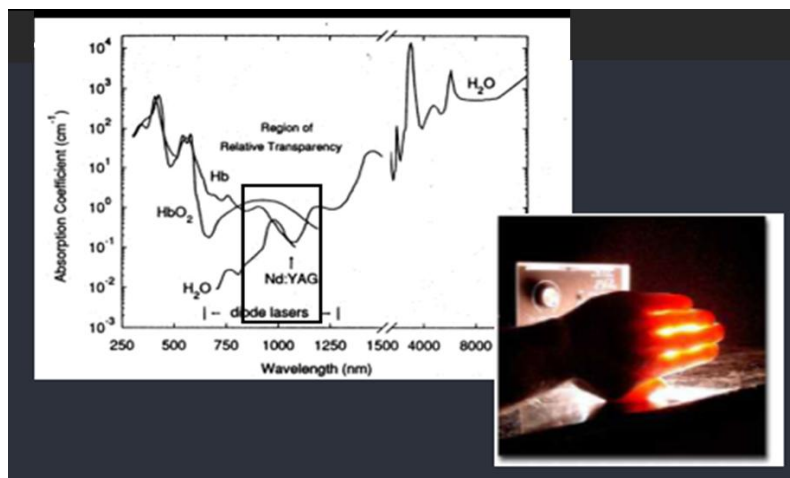
IM relies on the transparency of the human body in the infrared region of light spectrum (Figure 2). Major components (hemoglobin, water) of tissues and organs exhibit a minimum of absorption





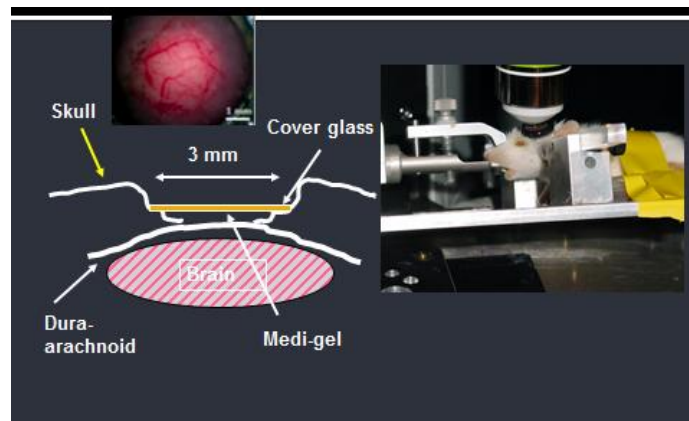
**Figure 1:** Two photon (2P) laser scanning microscopy (LSM) fills the resolution gap between MRI and TEM

coefficient in that region, as depicted in Figure 2. This induces a transparency window when human body parts are illuminated in the infrared range: the photon penetration can then reach several millimeters to several centimeters, depending on the organ considered.



**Figure 2:** Variation of absorption coefficients of hemoglobin, oxyhemoglobin and water versus radiation wavelength, showing window of relative transparency in infrared (IR) range (left); example of IR radiation penetration in human hand (right)

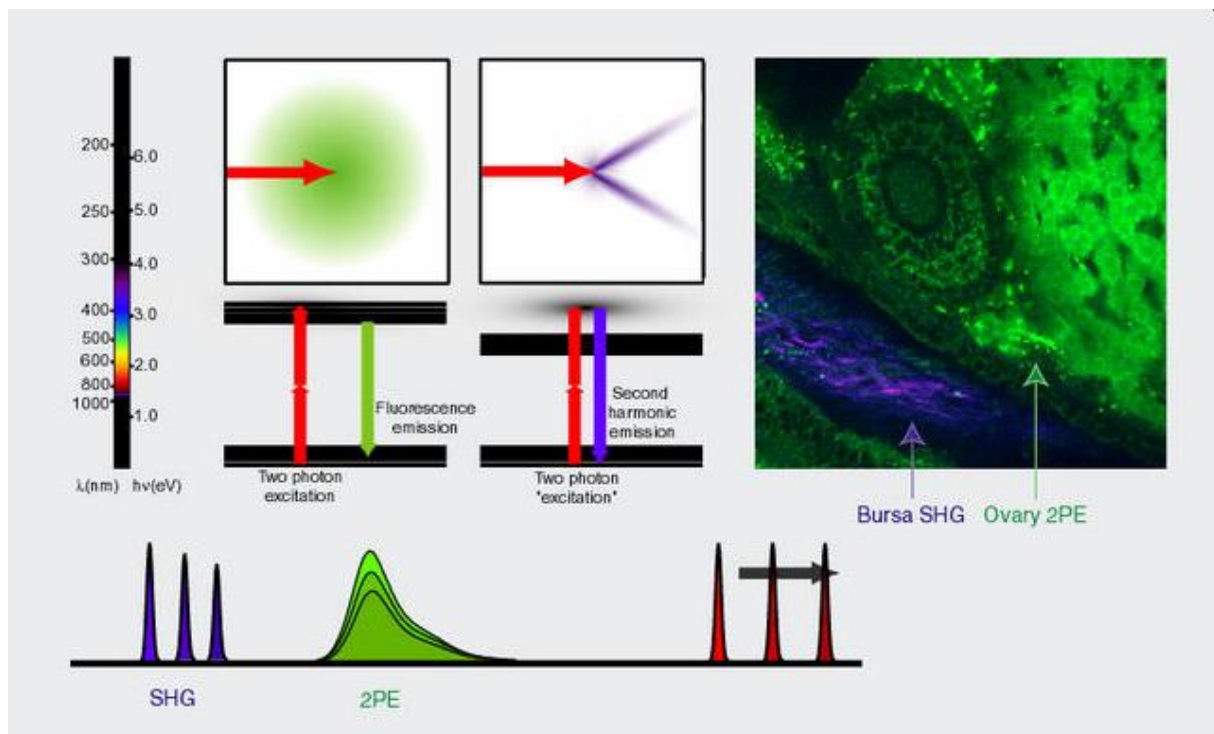
Figure 3 illustrates an example of brain exploration achieved by IM through a skull micro window in an anesthetized mouse.



**Figure 3:** Exploration of mouse's brain by Intravital Microscopy

### 3. Non linear photonics for I.M.

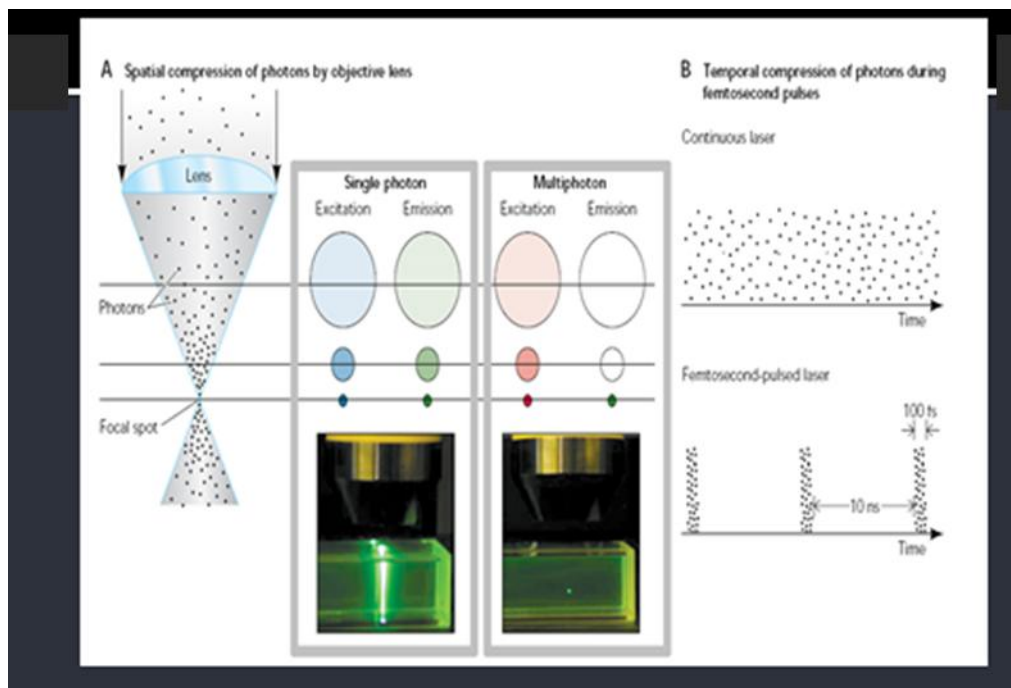
Latest developments in IM thus involve non linear photonic imaging with illumination in infrared range and use of two photon fluorescence emission (2PE) and Second Harmonic Generation (SHG) modes for imaging [7,8]. Generation of these modes and corresponding energy levels are sketched on Figure 4.



**Figure 4:** Two photon (2P) excitation from IR radiation giving rise to emission of fluorescence (2PE) and Second Harmonic Generation (SHG), with example of image from different organ components exciting preferentially 2PE or SHG mode.

Excitation of 2PE and SHG modes require spatial compression of photons (Figure 5) by the objective lens of a laser scanning microscope (LSM) and temporal compression during the femtosecond pulses of a femtosecond illuminating laser (Figure 5), typically a mode-locked Titanium-Sapphire laser, which produces femtosecond pulses.

The high photon density and flux required for two photon excitation typically correspond to a pulse width of approximately 100 femtoseconds and a repetition rate of about 80 MHz. The excitation wavelength can be varied between 700 and 1000 nm, a region of the specimen being illuminated by raster scanning of x-y mirrors by means of a galvanometer-driven scanner. A custom made dichroic mirror is used to maximize the reflection of the infrared radiation and to transmit the radiation in the blue-green region of the spectrum. The fluorescent radiation emitted from the sample is collected by the objective lens and transmitted to the dichroic mirror for the detection system: the most commonly used photodetectors are photomultiplier tubes due to their good sensitivity, large active areas, low price.



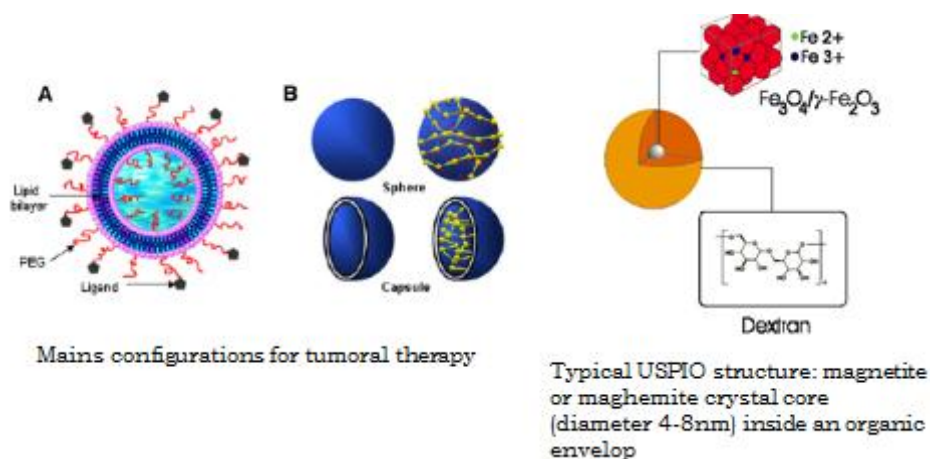
**Figure 5:** Temporal and spatial compression in a laser scanning microscopy (LSM) for 2P excitation

Since only ballistic photons do contribute to the image, only the specimen region corresponding to the objective focal plane is imaged and 3D imaging is obtained by 3 D reconstruction from images acquired from specimen slices successively illuminated (see section 4) [9]

#### 4. Nanoparticles for diagnosis and /or therapy

During the past years, nanocapsules for drug targeting have been obtained from different biocompatible polymers [10 to 13]. Nanovectors ( nanospheres or nanocapsules) can also be loaded with radioactive compounds for radionuclide intratumoral therapy [14] (Figure 6). Nanoparticulate systems made of biopolymers encapsulating magnetic nanoparticles have been intensively studied in the last decade due to the interesting combination of their structural specifications with their magnetic properties. This combination gives rise to applications for diagnosis like Magnetic Resonance Imaging (MRI) or ultrasonic imaging, and for therapy: drug targeting, hyperthermia. In MRI studies especially, maghemite or magnetite iron oxide loaded nanosystems (Figure 6) are extensively used as contrast agents. The advantage of iron oxide nanoparticles, compared with other metallic compounds (for example gadolinium), is their relatively low toxicity [15]. The latest studies [16] show that such nanovectors can be used as specific contrast agents for targeted organs.

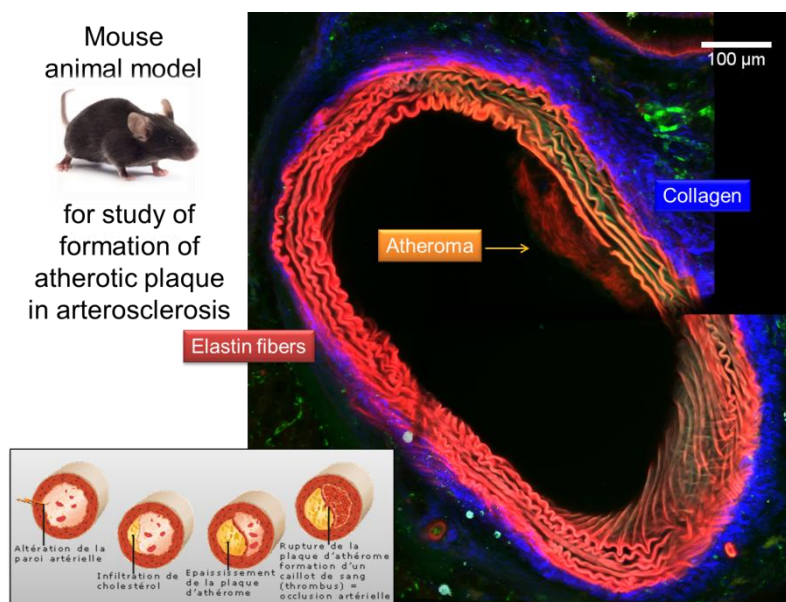




**Figure 6:** Structure of nanoparticles (NP) for diagnosis or/and therapy: examples

### 5. IM. and detection of nanoparticles in organs: validation of medical imaging

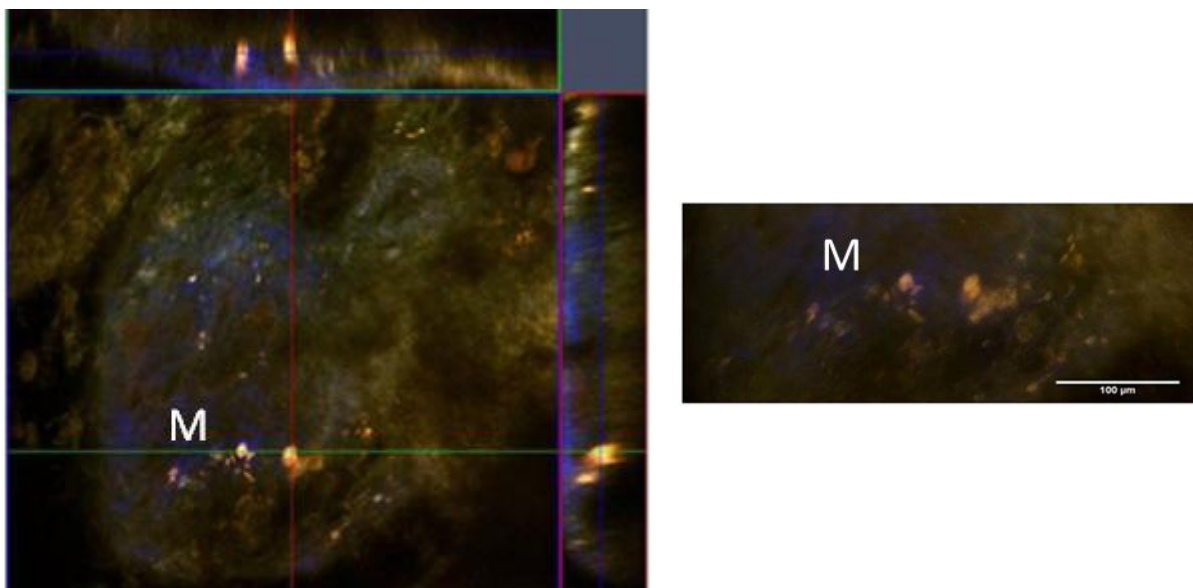
The use of ultra small superparamagnetic iron oxide (USPIO) NPs ( having magnetic iron oxide core) in biomedical applications like MRI has increased significantly in the last decade, but it is not completely understood how these NP reach their target, for instance the atherotic plaque or atheroma, in diagnosis of arterosclerosis [17]. USPIOs designed as MRI contrast agents were grafted (GUERBET Labs, Paris) at the coverage surface with a dye (Rhodamine family) having emission wavelength close to 600 nm [17] and injected into atherosclerotic ApoE<sup>-</sup> model mice [17] rapidly developing atherotic plaque under high-fat diet. At 32 weeks of age when the aortic plaque was well developed, the animals were administered with the same dose of 1000  $\mu\text{mol Fe/kg wt.}$ , as for MRI studies. Thin rings of the targeted atherosclerotic aorta were taken from the mice sacrificed after deep anesthesia (see *Ethics* alinea herafter).



**Figure 7:** Overview of an atherotic aorta's ring imaged by 2PLSM, showing the aortic wall structure and the presence of atheroma (formed as sketched in left inset) in the aorta's lumen

Such aortic rings from the atherosclerotic aortas were imaged using a 2PM LSM equipment of the type described in section 3, an example of ring's overview being presented in Figure 7. The whole fluorescence emitted in the visible range 347 – 660 nm was detected, to construct such color images.

The images were acquired slice by slice in the sample depth (z-axis), depth steps of 3 to 5  $\mu\text{m}$  being used to form depth stacks of several tens of slices. For a given stack, the z-projection images were obtained by averaging the pixel intensities over the depth of the stack. Orthogonal views along a given line in a given image were also recorded: they correspond to the distribution of the intensity in a slice perpendicular to the initial image plane and are displayed parallel to the chosen line with respect to the initial image. Figure 7 shows that the 2P modes of LSM does reveal the different components of the aortic wall due to fluorescence for elastin fibres and SHG blue colour for collagen fibers [17]. Also the presence of atherotic plaque is clearly visualised and located with respect of aorta's structure : an important application is the diagnosis about unstability or stability of the plaque [17]. At higher magnification the spatial resolution of LSM allows revealing the heterogeneous biodistribution of the USPIOs seen as yellow spots in the atherotic plaque ( Figure 8, left). Figure 8 displays aggregates of USPIOs like in M: size and scaling of such aggregates seen at higher magnification (Figure 8, right) support the model of USPIOs phagocytised by macrophages which target the plaque.



**Figure 8:** Biodistribution of USPIOs in atherotic plaque: plan view and orthogonal images showing USPIO's aggregates , like in M (left); high magnification of M region, consistent with USPIOs phagocytised in macrophages, bar is 100 $\mu\text{m}$  (right)

After such ex vivo samples were analysed by 2PLSM, specimens for TEM studies could be prepared from USPIO rich regions. Such TEM studies allowed to follow the biostructural transformations undergone by the USPIO NPs at the atomic scale [18,19]. Hence could be completed a multiscale approach from the organ's scale down to the atomic subcellular level.

## 6. Conclusion and perspectives

I.M. using 2PLSM permits differential imaging of organ components and tissues together with detection of NPs at the subcellular level. This allows to bridge the gap towards TEM investigations for atomic resolution. Thus 2PLSM naturally takes place in multimodal imaging platforms and supports the development of endoscopic techniques for medical imaging.

*Ethics:* all the animal experimentations mentioned in the present paper were conducted according to European and French ethical regulations



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