CONFOCAL AND WIDE-FIELD DECONVOLUTION MICROSCOPY: INTEREST OF MULTI-PHOTON EXCITATION IN OPTICAL BIOENGINEERING

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ABSTRACT

A variety of optical techniques have been developed over the years for experimental study into living specimens. The increase in lateral and spatial resolutions is one of the major targets of research and development in the field of optical miscroscopies applied to living tissue. The optical geometry of Confocal Laser Scanning Microscopy (CLSM) demonstrates its undeniable advantage on conventional fluorescence microscopy by segregating the planes outside the focussing plane. The methodological and technological advances of the last five years have been fast evolving, especially with regard to the optimisation of CLSM and deconvolution process. The limited analysis in thick tissue have given rise to the development of other techniques, MultiPhoton excitation Microscopy in particular.

In this paper, we have applied these techniques on major biological applications in bioengineering (skin, endothelial cell, chondrocyte) and discussed the technical limitations and perspectives.

1. INTRODUCTION

Molecular emission spectroscopic techniques (fluorescence, phosphorescence, chemo- and bio-luminescence), which correspond to excitation deactivation processes, are now widely used to study variably complex media such as cells or tissues. Among the physical methods available to investigate biological media, fluorescence, with its high analytical sensitivity and resolutions (spectral, spatial, temporal, order) offers interesting possibilities for cell or tissue biological analysis. But it was mainly in the past decade that it became possible to shift from whole-specimen analysis to smaller and smaller volumes, through microscopy techniques (far and near-field microscopies, fluorescence correlation, etc), down to almost single-molecule exploration (1). Fluorescence microscopy is exempt from a number of constraints normally attached to standard fluorometry (probe concentration, low cellular density, exploration and display at the cellular scale). Furthermore, it is the only methods of investigation currently available with a high enough resolution to specify the distribution of the fluorescent probe, because it collects fluorescence signals emitted at the probe incorporation site.

Multiphoton imaging with near IR laser pulses significantly reduces tissue damage due to laser beam absorption and scattering of short wavelength visible or near UV-excitation sources. This technique allows living cells to be probed in real time.

Fluorescence can be used in different ways, especially with regard to the excitation mode. Also, when combined with adequate optical instruments, fluorescence measurements can be useful in terms of spectral microscopy, optical microscopy, or a combination of both (time-resolved fluorescence microscopy). The capabilities of these techniques can be significantly enhanced by choosing the appropriate fluorescent markers, by making proper use of their emission characteristics (wavelength, *i.e.*, spectral resolution; excited state lifetime, *i.e.*, temporal resolution; fluorescence quantum yield, etc.) and by changes in the immediate surroundings of fluorescent probes (phase transition temperature, polarity, viscosity, pH, pressure, temperature, presence of inhibitors, etc.).

1. OPTICAL SCANNING MICROSCOPY

One of the major limitations of fluorescence microscopy, whether applied conventionally (Fig.1.A) or confocally (Fig.1.B), is the progressive bleaching of the fluorochrome during prolonged exposure. Unfortunately, when focusing at a particular depth within a transparent fluorescently labelled 3D specimen, fluorochromes molecules throughout the whole of its thickness are excited. The diffraction light disrupts the image which reduces contrast and spatial resolution, allowing few strategies to overcome these limitations:

2.1 Confocal Laser Scanning Microscope

The optical geometry of Confocal Laser Scanning Microscopy (CLSM)

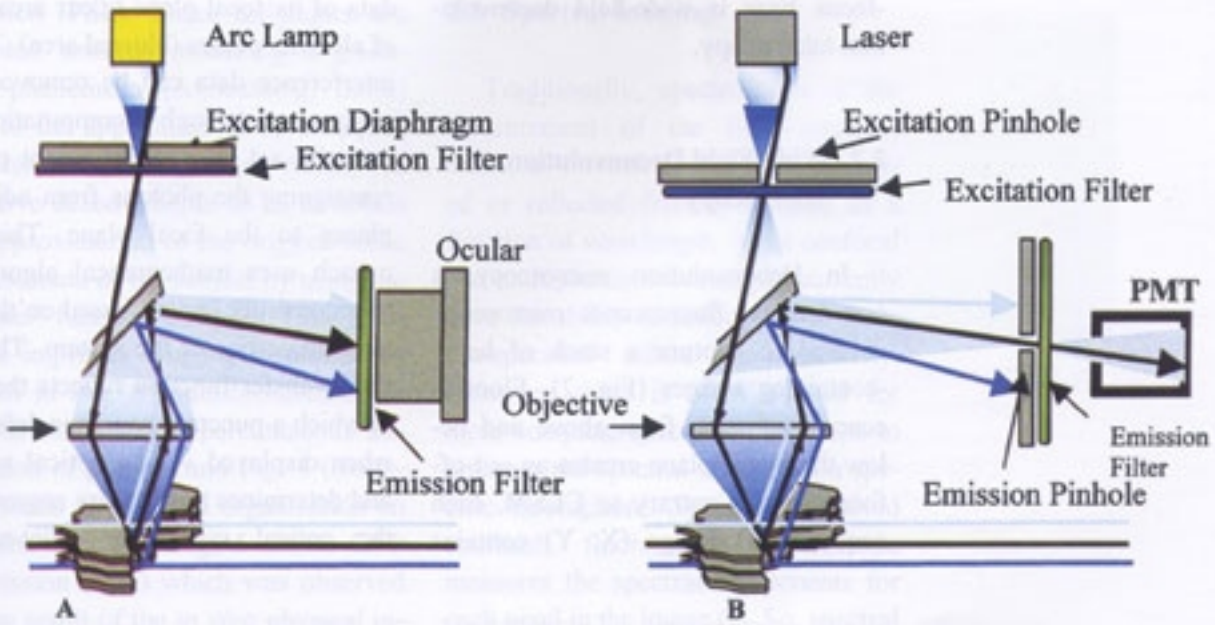

Fig. 1 – A. In conventionnal epi-illumination light microscopy, the simultaneous illumination of the entire field of view of a specimen will excite fluorescence emissions or reflections throughout the whole depth of the specimen, rather than just at the focal plane. Much of the light collected by the objective lens to form the image will thus come from regions of the specimen above and below the selected focal plane, contributing as out-of-focus blur to the final image, and seriously degrading it by reducing contrast and sharpness. In confocal Microscopy (**B**), a laser beam is focused to a small waist within the specimen, then reimaged onto a detector through a confocal aperture (pinhole) which serves to block fluorescence from all other z positions along the beam path (from Robinson).

demonstrates its undeniable advantage on conventional fluorescence microscopy by rejection out-of the focus plane. In a confocal imaging system, a single point of laser light is scanned across the specimen and the light passing through the emission pinhole (Fig. 1.B) is detected by a photomultiplier.

The benefits of confocal microscopy are 1) Increased effective resolution 2) Improved signal to noise ratio 3) Clear examination of thick specimens 4) Depth perception in Z-sectioned images 5) Magnification can be adjusted electronically 6) Reduced blurring of the image from light scattering.

The improvements were essentially aimed at offering solutions to the problems posed by CLSM 1) by multiple marking in fluorescence (cross-talking) 2) the too low scanning rate to catch rapid biological events, 3) the fast photodegradation of the fluorescent probe as well as cytotoxicity (under UV light mainly) 4) the low quantum efficiency of detector 5) the fluorescence emission restricted by optical configuration (pinhole).

Moreover, CLSM posed certain problems linked to the use of living cells due to the high density of incident light (laser source) focussed on a small volume (femtoliter).

To obviate these limitations, an alternative method for removing out-of-

-focus haze is wide-field deconvolution microscopy.

2.2 Wide-Field Deconvolution Microscopy

In Deconvolution microscopy, a conventional fluorescence microscope is used to capture a stack of haze--containing images (Fig. 2). Fluorescence emanating from above and below the image plane creates an out-of-focus haze. Contrary to CLSM, each acquired 2D image (X, Y) contains data of its focal plane (clear area) an of all other planes (blurred area). Thes interference data can be removed an images run through a computationall intensive set of algorithms that permi reassigning the photons from adjacen planes to the focal plane. This ap proach uses mathematical algorithm to reconstruct images based on the op tical properties of the system. The op tical transfer function reflects the wa in which a punctual source is deforme when displayed via an optical syste and determines the impulse response o the optical system or point-sprea

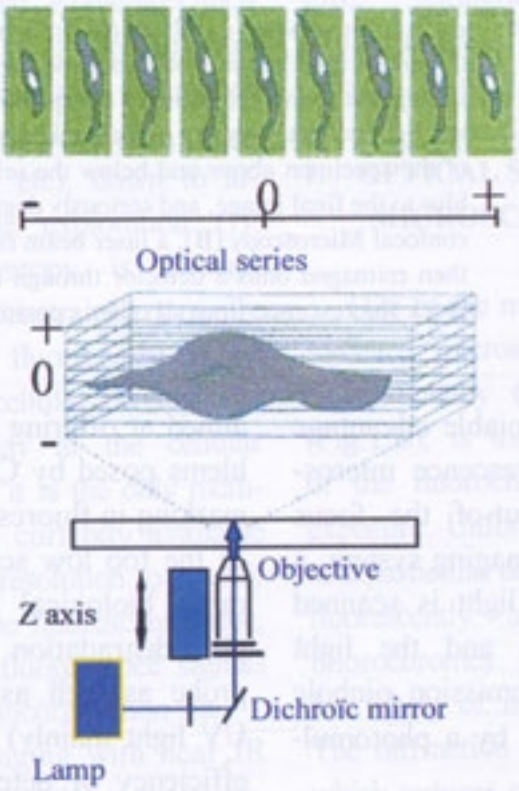

Fig. 2 – Optical sections were obtained with an Olympus IX-70 epifluorescence inverted micro scope equipped with the CellScan™ optical sectioning acquisition system (Scanalytics Billerica, Massachusetts) and a 60xPSF/1.2-NA water immersion apochromatic objectiv (Olympus, France). The scanning along the optical axis was performed by a piezoelectri z-axis focus device (at a z spacing of 0.25 μm). Images of biological samples were col lected on a cooled 12 bit Charge-Coupled Device (CCD) camera (Princeton Instrument Inc, USA). The intensity of the excitation source throughout one measurement (0.1 s) di not induce any photobleaching. A filter set (green WU cube, red WNG cube) selected th fluorescence excitation (330-365 nm or 560-590 nm) and the integral part of the monome emission spectrum (390-420 nm or 615-650 nm). An image intensity kit (InSpeck, Mo lecular Probes, Eugene City, OR., USA) was used for the calibration.

function (PSF). These techniques are oriented towards modelling degradation phenomena (defocussing, noise) and to the application of reverse procedures (mathematical reversion) or iterative deconvolution so as to obtain an approximation of the original scene (an estimate of the subject by using the transfer function image). This technique improves imaging resolution as shown in Fig. 3 (autofluorescence of the rat skin and the percutaneous absorption of pyrene) and Fig. 4 (oxidative stress and F-actin organization on endothelial cells). Fig. 5 shows GFP expression (spot) which was observed as the result of the *in vivo* physical introduction in rats cartilage cells of a plasmid DNA construct.

2.3 Spectral Imaging

Traditionally, spectroscopy is the measurement of the light intensity which is emitted, transmitted, scattered or reflected from a sample, as a function of wavelength. Most confocal and multiphoton microscopes currently have the ability to collect two or three pre-specified colors simultaneously. However, there is often a need for more complete spectral information to optimize the spectral window of a specific fluorophore. Spectrally resolved confocal fluorescence microscope measures the spectral components for each pixel in the image (7). So, spectral bio-imaging can be used to identify and map several fluorophores, simulta-

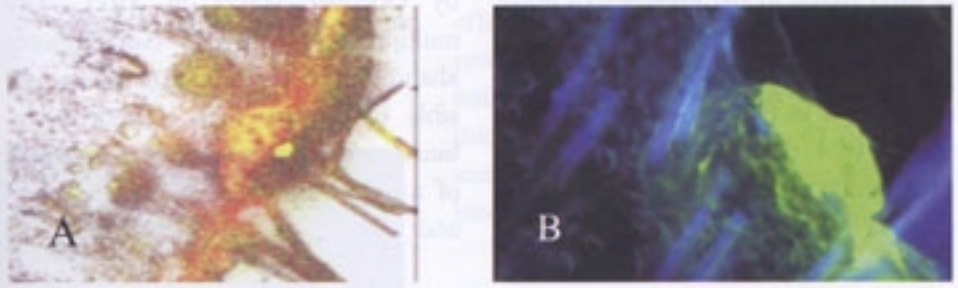

Fig. 3 – Distribution of Pyrene (**A**) in Hairly rat skin and autofluorescence (green-red) of stratum corneum and deep areas (**B**).

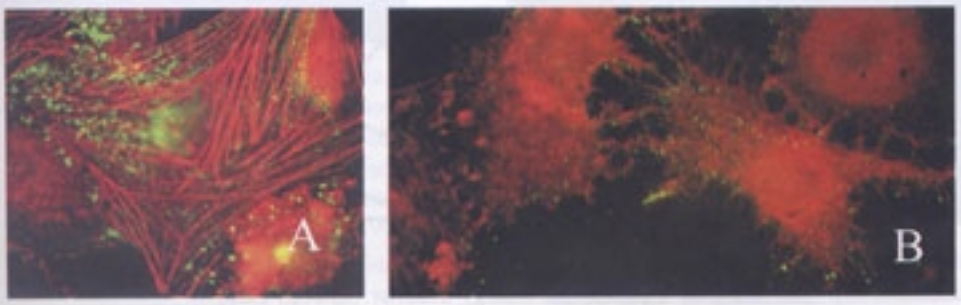

Fig. 4 – Effect of Ciprofloxacine (10^{-5} M) on organisation of F-actin filament (red) and distribution of Icam-1 (green) in human endothelial cells monolayer before (**A**) and after (**B**) irradiation (20 J/cm^2).

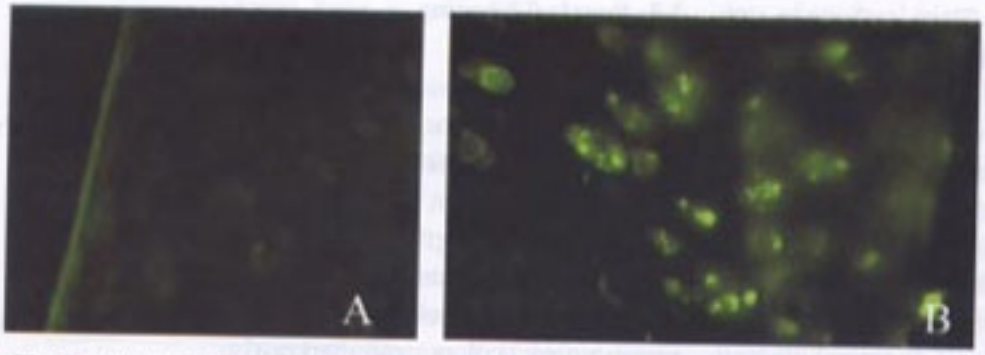

Fig. 5 – Rats were submitted to electro-permeabilization-mediated gene transfer to achieve gene expression into chondrocytes *in vivo*. (**A**). No GFP spots were observed in the experiment with plasmid DNA intra-articular injection without electric pulses administration or with electric stimulation alone. (**B**) Cell expression GFP was located in definite areas (superficial layer, middle and deep areas) indicating that the target tissue was transduced in its whole depth.

neously, in one measurement. Benefits of spectral imaging are 1) optimization and discrimination of spectral windows for multiple labels 2) collection of the desired signal in the presence of noise (detection noise, fluorescence background, etc...). Fig. 6.A illustrates the endocytosis in Human Vascular Endothelial Cell (Huvec) of Low-density lipoproteins (LDL) loaded with carbocyanine dye (diI).

2.4 Multiphoton Microscopy

The technique is based upon the two-photon principle first described by Goeppert-Mayer in 1931. This multiphoton process (simultaneous absorption of 2 photons) is made possible by a very high intensity combined with time-related concentration of a very brief (pico or femtosecond) and very high-frequency (about 80

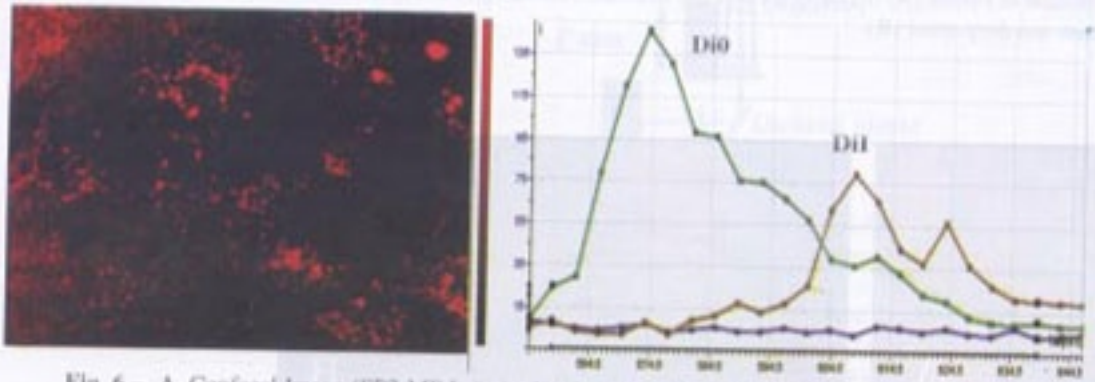

Fig. 6 – A. Confocal Image (SP2-MP Leica) of endocytosis of Lipoprotein combined to DiI or in Human Endothelial Cell (HUVEC). **B**. Fluorescence Energy Transfer between LDL-DiO and LDL-DiI in HUVEC. Excitation at 488 nm. Step: 5nm.

MHz) laser flash (2). Because in multi-photon mode spatial resolution is the result of absorption (and excitation) confinement to the focal event (smaller of one femtoliter) (3), the photobleaching patterns and photodegradation outside the focal plane are reduced (4). According to the pulse duration/peak power ratio, two red photons can excite a fluorophore whose excitation spectrum is in UV for an emission spectrum in the blue. Although the excitation properties of a fluorescent molecule are different for single photon and multi-photon events, the emission spectrum remains unchanged, regardless of how the electronic transition occurred. Fig. 7 shows endothelial cells labelled with three fluorophores (emission in blue, green and red) simultaneously excited by 2P-excitation using 820 nm laser light.

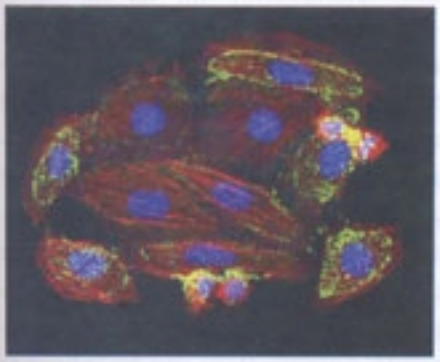

Fig. 7 – Simultaneous illumination by 2-Photon absorption light at 820 nm of endothelial cell: F-actine in red (rhodamine-phalloïdine), nucleus in blue (Hoescht), and Icam-1 in green (Alexa 488™-Mab). Laser femtoseconde MIRA 900.

Since the red or near infrared illuminating light used for 2-P excitation has approximatively twice the wavelength of that employed for 1-P excitation, scattering effects at the excitation wavelengths are greatly reduced allowing deeper penetration into tissues than with visible or UV excitation (Fig. 8). This experiment illustrates the need for deeper penetration on rat cartilage head cap to study the chondrocyte-matrix interaction (5). As shown in Fig. 9, depth penetration in thick specimen (rat articular cartilage) is greatly enhanced (from 64 µm to 219 µm). The effects of the heat produced after high-frequency pulse illumination in a restricted volume (approx. 1 μm^3) and the optical aberrations are thought to be negligible and similar to those observed with single-photon confocal microscopy (6).

The benefits of Multiphoton Excitation microscopy are: 1) Increased effective resolution (deeper spatial resolution/1P) 2) Improved signal to noise ratio 3) Clear examination of thick and difficult specimens 4) Z-axis scanning (penetration enhanced into biological or material specimens) 5)

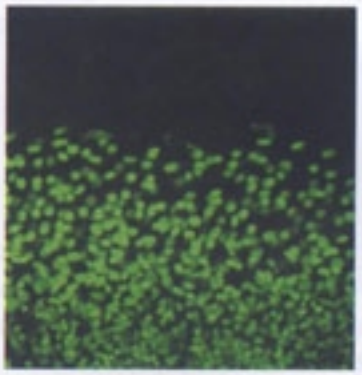

Fig. 8 – Illumination by 2-Photon absorption light at 780 nm (Laser femtoseconde MIRA 900) of chondrocyte encapsulated in alginate bead (diameter 2 mm) – Nucleus, Hoescht – (depth illumination: ***1.6 mm***).

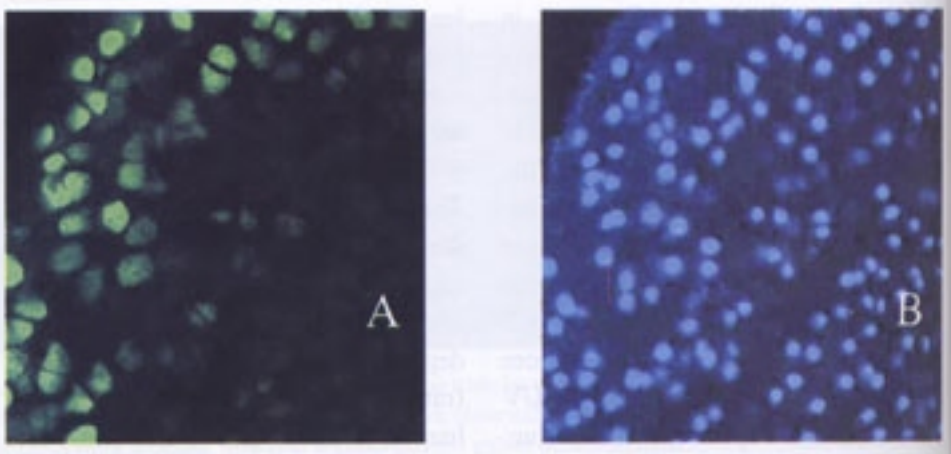

Fig. 9 – **A.** Illumination by 1-Photon absorption light at 488 nm (Laser Argon) of chondrocyte in rat cartilage head cap (F-actine. Oregon Green- Phalloidine; depth illumination: ***64 µm***). **B.** Illumination by 2-Photon absorption light at 780 nm (Laser femtoseconde MIRA 900) of chondrocyte in rat cartilage head cap (Nucleus, Hoescht; depth illumination: ***219 µm***).

Phototoxicity due to the long wavelength negligible/1P 6) Reduced bleaching 7) Reduced blurring of the image from light scattering.

At this time, the main disadvantages of Multiphoton Microscopy are the cost of femtosecond laser sources, the incomplete data on the two-photon absorption and fluorescence properties of commonly used fluorophores, the possible thermal damage, and the lack of affordable optical fiber coupling.

2.5 Lifetime Imaging

Because the excitation mode conditions the nature of data available, users can have access to two main types of measurements :

- Fluorescence intensity values with continuous excitation, the steady-state conditions give a balance between excited state production and deactivation.
- Fluorescence temporal decay values with pulsed mode or modulated mode (kinetic conditions; decay statistic of acquiring (single photon counting)/streak camera, time window (Boxcar type); phase modulation (phase fluorometry).

Measurements based on fluorescence intensity generally require rather simple instrumentation, essentially an excitation source (lamp or laser beam) and a detector (photomultiplier or Charge-Coupled Device camera). These components lack stability with time and fluorescence intensity determination can be subject to fluctuations, among other shortcomings. Measuring source correction and intensity can partly solve that problem, but sometimes in a non-satisfactory manner. Furthermore, this method does not take into account the changes of fluorescent marker concentration resulting from photobleaching, which induces a significant

amount of fading. In biological specimens, either *ex vivo* or *in vitro*, under physiological conditions (pH, temperature, culture medium...) fluorescence quantitation at the sub-cellular level with a fluorescence microscope suffers from the same limiting factors.

Two obviate these difficulties, (excitation source stability, photo bleaching, fluctuating marker concentrations...) another method is available, which consists in dealing with the lifetimes of excited states (fluorescence decay). The fluorescence lifetime of a substance represents an average amount of time the molecule remains in the excited state prior to its return to the ground state, while emitting fluorescence. The advantages and disadvantages of measuring the fluorescence signal in terms of intensity or lifetime have often been discussed (7). The precise nature of the fluorescence decay can reveal more details about the interactions of the fluorophore with its close surrounding: this parameter can reveal the frequency of collisional encounters with quenching agents, the rate of excited state reactions. Multiple decay constants can be the reflection of several distinct surroundings of a fluorophore or of the presence of several conformational states of a molecule. Factors such as ionic strength, hydrophobicity, oxygen concentration, binding to macromolecules can all modify the lifetime of a fluorophore, considered as an indicator of these parameters.

The shift from fluorescence measurement in a stationary state to time-resolved fluorescence involves all fluorescence-based techniques (flow cytometry, spectrometry and microscopy). So, Fluorescence Lifetime Imaging Microscopy (FLIM) combines the advantages of lifetime spectroscopy with fluorescence microscopy by revealing the spatial distribution of a fluorescent molecule together with information about its microenvironment.

In spectral and lifetime imaging used to discriminate among multiple fluorophores on the basis of spectra as well as lifetime (Fig. 10), the Fluorescent Resonant Energy Transfer (FRET) is a powerful technique for measuring intermolecular distances, the proximity of molecules that can deplete the excited state by resonance energy transfer, as shown in Fig. 6.B between a donor (DiO) and a acceptor (DiI) loaded to LDL (8).

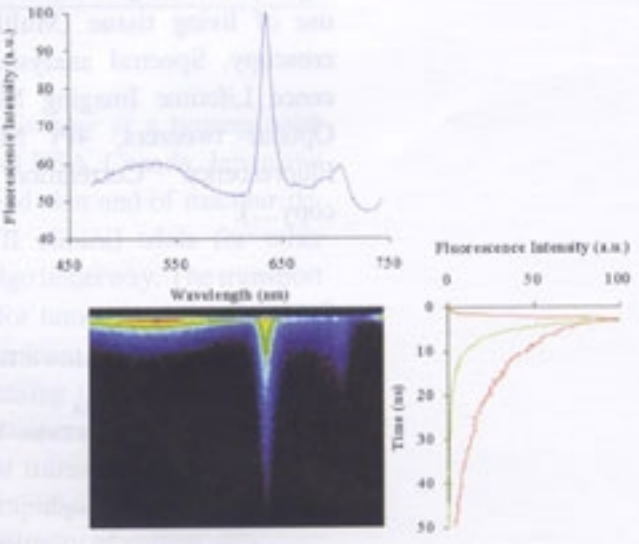

Fig. 10 – Streak image where the horizontal and vertical axis are respectively the wavelength and the decay of fluorescence (lifetime) of intrinsically fluorescent molecules (NAD/FAD) which emit at 520 nm (green), shorter than those of the extrinsic fluorophore (porphyrin derivative) which emits at 630 nm (red).

3. CONCLUSION

Even if the deconvolution techniques require considerable computing power capacities and extended computation time, they prove very useful in situations of low intensity levels or restricted use of confocal microscopy to improve spatial resolution in muliphoton microscopy. At this time, Multiphoton Imaging has established itself as an important method for optical microscopy particularly for subcellular events within organized tissue environment. With the elimination of UV excitation, more imaging scans are permitted, and time-sequence studies on sample kept at physiological conditions are permitted. Moreover, a number of variants have been developed allowing optimised imaging for high speed or high resolution with the use of living tissue (Multifocal Microscopy, Spectral analysis, Fluorescence Lifetime Imaging Microscopy, Optical tweezers, 4Pi Microscopy, Fluorescence Correlation Microscopy...).

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