Journal of Fluorescence, Vol. 12, No. 2, June 2002 (© 2002)

Visualization of Intracellular CA^{2+} Dynamics with Simultaneous 2-Photon Excited Fluorescence and Third Harmonic Generation Microscope

S. Rivet,[2] L. Canioni,[2] L. Sarger,[2] R. Barille,[1,4] Pierre Vacher,[3] and Thomas Ducret[3]

Received September 27, 2001

We show that the simultaneous coupling of two laser scanning non-linear microscopies (2-photon excited fluorescence and third harmonic generation microscopy) give functional and morphological information for improving our understanding on intracellular calcium flows.

KEY WORDS: Intracellular CA^{2+} dynamics; 2-photon excited fluorescence; third harmonic generation microscopy.

The 2-photon excited fluorescence (TPEF) [1] microscopy, now a laboratory standard, has allowed a great extension of the capabilities of the powerful confocal imaging technique. Recently, a new non invasive microscopy technique has been developed that takes advantage of the discontinuity of the third-order nonlinear susceptibility (χ^3) at the material interfaces. At the focusing point of the laser beam at wavelength λ, this discontinuity gives rise to a new coherent wave in the forward direction at the third harmonic $\lambda/3$ (THG process) [2]. The validity of this technique has been demonstrated and extended to biological samples [3].

These two techniques use femtosecond laser pulses because their high peak power and high repetition rate are the keys for cellular biology imaging. In another hand, specific mechanisms of communication between cells, which are done by transcription of extracellular signals into cellular response, remain elusive, specifically in neuroscience, where calcium plays an important role. We have recently demonstrated that optical properties, particularly either χ^3 or local refractive indices, are correlated to calcium flows inside cells.

Two-photon excited fluorescence is ideally suited for observing the location of given ions inside cells and can be detected with high sensitivity and selectivity. On the other hand, THG can be used to interpret the location of all the possible interface phenomena involved, particularly at the cell surface [4]. Thus, a coupling of these two techniques can bring skeletal as well as functional insight of the cellular mechanism.

The experimental setup (Fig. 1) uses as laser sources the various outputs of a synchronously pumped OPO system, i.e, 130 fs at $\lambda = 1.5$ μm (for THG) and 100 fs at $\lambda = 810$ nm (for TPEF).

The two laser beams are focused into the sample by the same microscope objective (LOMO Apochromat $90\times/1.25$ oil). Two prisms are inserted in the beam path for compensating the GDD introduced by the different optical glasses and to adjust the pulse temporal width. The third harmonic light (in the forward direction at a wavelength of 0.5 μM) is collected through a condenser filtered from the fundamental wavelength using an inter-

[1] Laboratoire des Propriétés Optiques des matériaux et applications/ CNRS UMR 6136, 4, Bd Lavoisier, BP 2018,49016 Angers Cedex, France.
[2] Centre de Physique Moléculaire et ondes Hertziennes/CNRS UMR 5798 351, Crs de la libération, 33405 Talence Cedex, France.
[3] Laboratoire de Physiologie et Physiopathologie de la Signalisation Cellulaire/CNRS UMR 5543 146, rue Léo Saignat, 33076 Bordeaux Cedex, France.
[4] To whom correspondence should be addressed. Tel.: (33) 5 56 84 28 82. Fax: (33) 5 56 84 69 70. E-mail: regis.barille@univ-angers.fr

1053-0509/02/0600-0197/0

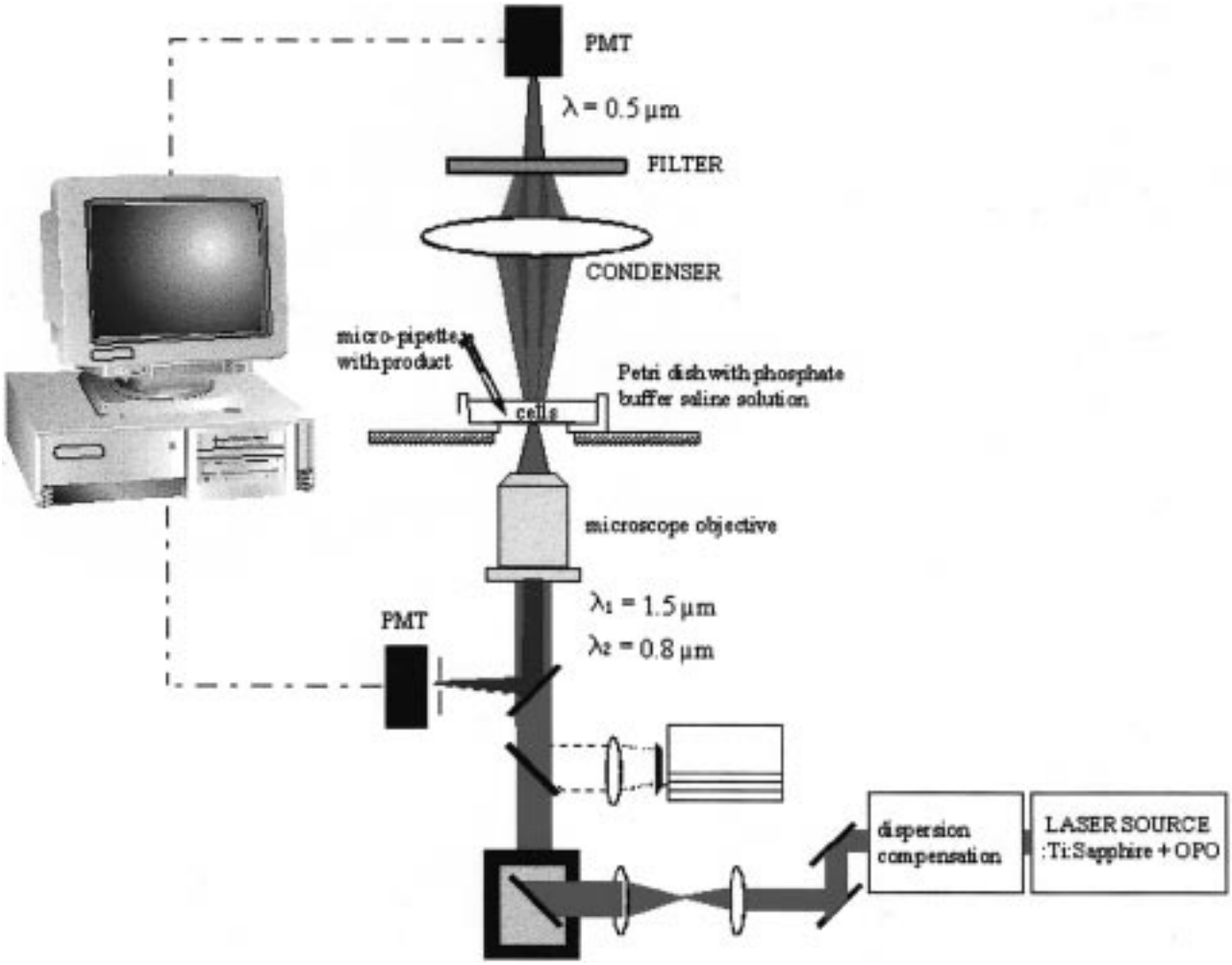

Fig. 1. Details of the set-up for coupling TPEF and THG microscopy.

ference filter (λ_o = 500 nm, $\Delta\lambda$ = 40 n*M*) to increase the signal-to-noise ratio and measured by a photomultiplier tube (PMT). The 2-photon excited fluorescence is collected in the backward direction through a bandpass filter, discriminated with a cold mirror associated with a bandpass filter centered at λ = 400 nm and detected by another PMT. The transmitted power through the objective corresponds to 62% of the input beam (average power at the sample level is $\sim$ 8.7 m*W* for λ = 810 nm and 15.5 m*W* at λ = 1.5 μ*M*) with power at the geometric focal point estimated to be approximately 74.8 GW and 32.7 GW, respectively. The resolution of our microscope is R $\approx$ 254 nm and 412 nm for 1.5 μ*M* and 810 and 810 nm respectively.

The cell material consists of the human astrocytoma cell line, U-87 MG. They serve as scaffolding for the actual nerve cells and having a major influence on the formation and reformation of synapses; they may play a crucial role in learning [5]. Researchers attempt to exploit them to develop therapies for illnesses such as Alzheimer's disease [6].

The cells are prepared with 10 μ*M* of dye Fura-2 AM (molecular probes) and stimulated with EGF (epidermal growth factor), which is responsible for cellular multiplication and whose the action on $[Ca^{2+}]_i$ has been demonstrated in several cell types. Upon Ca^{2+} binding, the dye excitation spectrum with TPEF is shifted, while the emission spectra remain unchanged in such a way that an increase in $[Ca^{2+}]_i$ results in an increase in the emitted fluorescence intensity measured at 505 nm for λ_{exc} = 345 nm (and a concomitant decrease for λ_{exc} = 380 nm)-close to our TPEF operating conditions at 810 nm.

Several images have been acquired without stimulation in TPEF and THG to ensure the validity of the technique. During the whole experiment no cell damage was observed with the average power used at the sample

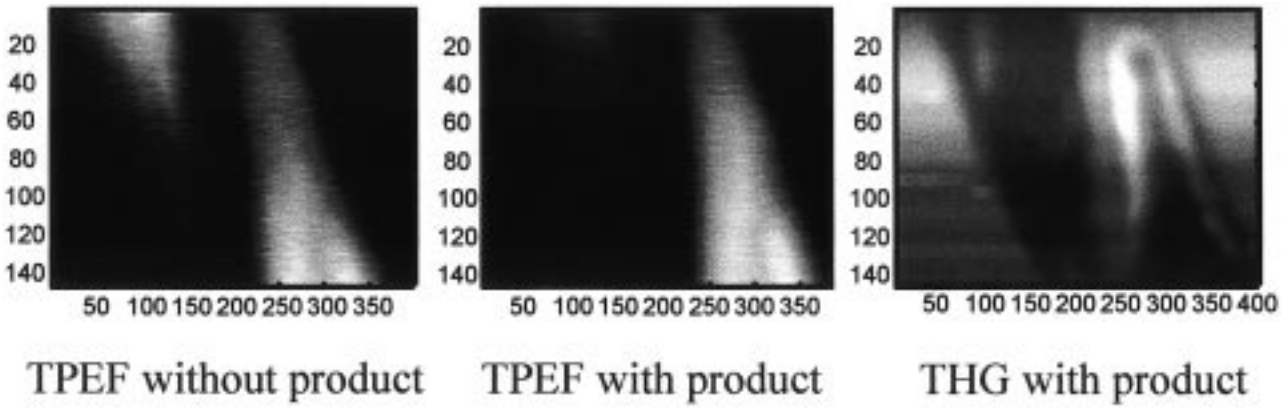

Fig. 2. Comparison between images with TPEF microscopy before and after Thapsigargine injection in the sample and THG microscopy of a cell's part (cells: U 87 MG).

for both excitation wavelengths. The possible damage for THG microscopy can be a locally heat increase which leads to calcium release. We have verified that the generation of heat by IR absorption at $\lambda = 1500$ nm is not a mechanism that induces damages. For Fura-2, the dependance of the fluorescence emission intensity as a function of the beam power was controlled and found, as expected, to depend on the square of the laser power. The focal plane for TPEF imaging has been chosen to give the maximum of dynamic on the THG signal. The calcium release acts in the entire cells volume and can be detected at different focal planes with only difference in the maximum intensity. Moreover, the microscope objective is achromatic but the possible chromatic aberrations for IR wavelength domain are less than the confocal parameter for TPEF and there is no loss of information. After EGF injection, the free calcium inside the cell implies a decrease on the TPEF intensity revealing the different location of the calcium flow. Here, no morphological informations could be obtained on the plasma membrane whereas images by THG microscopy show a strong sensitivity on the location of the cell's membrane (Fig. 2). Moreover the axial THG sectioning capability reveal the rounded form of the cell.

For extensive comparison between these two volumetric imaging techniques, different images of one cell's part without stimulation has been done as function of depth. Two scans were performed with $\Delta z = 500$ nm. A cross-section along the different images allows comparing the variation of the intensity measured by TPEF and THG. In TPEF there is no further information brought by the depth variation and the contrast on the intensity is not improved, whereas THG imaging gives more information, revealing a higher sensitivity on the membrane contrast. These results come from the order of process, one is a second-order process depending of the quadratic intensity, whereas the other one is a third-order process depending of the cubic intensity.

We show that these two powerful techniques using femtosecond pulses when they are coupled optimize the measurement of intracellular calcium flow and give a better efficiency for the localization of the different ion flows (Ca^{2+} flow) crossing the plasma membrane. Two types of complementary informations can be revealed and, whereas the TPEF microscopy brings functional informations on the cell, the THG microscopy confirms these informations giving further details on the cell's morphology. These coupling microscopy techniques optimize the confocal microscope by concurrently acquiring information in transmission with THG and in reflection with TPEF.

Future studies might be extended to completely define the full characteristics of THG microscopy in view of discrimation of the various physiological and chemical aspects of the biochemical pathways involved in calcium dynamics without fluorescent markers. In this case the TPEF microscopy can serve to validate these results.

REFERENCES

1. W. Denk, J. H. Strickler, and W. W. Webb (1990) *Science* **248**, 73–76.
2. D. Yelin and Y. Silberberg, (1990) *Opt. Exp.* **5**(8), 169.
3. A. C. Millard, P. W. Wiseman, D. N. Fittinghoff, K. R. Wilson, J. A. Squier, and M. Müller (1999) *Appl. Opt.* **38**(36), 7393.
4. L. Canioni, S. Rivet, L. Sarger, R. Barille, P. Vacher, and P. Voisin (2001) *Opt. Lett.* **26**(8), 515–517.
5. J. W. Deitmer, A. J. Verkhratsky, and C. Lohr (1998) **24**(5/6), 405–416.
6. O. Thibault, N. M. Porter, K. C. Chen, E. M. Blalock, P. G. Kaminker, G. V. Clodfelter, L. D. Brewer, and P. W. Landfield (1998) *Cell calcium* **24**(5/6), 417–433.