Frequency domain fluorescence microspectrometry: Application to cellular uptake and drug distribution

Petr Praus^{a,*}, Eva Kočíšová^a, Peter Mojzeš^a, Josef Štěpánek^a, Franck Sureau^b and Pierre-Yves Turpin^b

^a Charles University in Prague, Faculty of Mathematics and Physics, Prague, Czech Republic

^b Acides Nucleiques and Biophotonique, Evry Cedex, France

Abstract. Time-resolved confocal microspectrofluorometry and fluorescence microimaging were used to monitor how the model antisense oligonucleotide is transported into 3T3 living cells and distributed inside them. Phosphorothioate analog of 15-mer oligothymidylate labeled by ATTO 425 was complexed with Zn(II) 5,10,15,20-tetrakis(4-N-methylpyridyl) porphyrin as an uptake-mediating agent. Homodyne phase-resolved technique based on a high frequency analog modulation of both exciting diode laser and detector image intensifier was used for time-resolved measurements. Decay-time data obtained within a broad range spectral region have provided unique information about the fate of both fluorophores inside the cell.

Keywords: Fluorescence lifetime, oligonucleotide, porphyrin, microspectrofluorometry, cellular uptake

1. Introduction

Methods of fluorescence microscopy are widely used in cellular studies employing natural intracellular chromophores (NAD(H), flavin, porphyrin), synthetic fluorescent dyes (ionic probes, organelle and structure markers), or endogenous fluorescent fusion proteins (GFP, YFP, CFP, etc.) [2,6,12,13]. These applications include also monitoring of the cellular uptake and intracellular interaction of exogenous fluorescent or fluorescently labeled compounds such as antitumoral or photosensitizing drugs [3,4,8,9]. Standard microscopic imaging based on the fluorescence intensity measurement at certain wavelength may not, however, be suitable to distinguish between different fluorophores whose emission spectra are highly overlapped. Determination of fluorescence lifetimes over the proper spectral region may help not only to distinguish individual components in fluorescence emission of a fluorescent mixture, but also to provide useful information about interactions in intracellular environment.

In order to gain lifetime characteristics from multifluorophore molecular systems in living cells, we have build up a homodyne time-resolved microfluorometer with a fully modulated short wavelength excitation and a broad spectral window. It enables simultaneous spectral and lifetime analysis either from a single subcellular spot in a confocal mode or from a wide field area selected inside the single cell.

*Corresponding author: Petr Praus, Charles University in Prague, Faculty of Mathematics and Physics, Ke Karlovu 3, Prague 2, CZ-121 16, Czech Republic. Tel.: +420 2219 11474; Fax: +420 2492 2797; E-mail: praus@karlov.mff.cuni.cz.

2. Experimental set-up

Our fluorescence lifetime microspectrofluorometer (see Fig. 1) is based on a phase modulation principle [11]. Two synchronized frequency synthesizers generate the same modulation frequency that is used to modulate the excitation light and the optical detector gain (homodyne method). A variable phase adjustment of the slave synthesizer is used to change the phase shift between the light source modulation and the image detector. Broad spectral detection window (375 nm wide) is chosen to cover both the excitation wavelength, detected via elastic scattering, and the fluorescence emission. From at least three spectral images acquired at different phase shifts, the phase difference of the fluorescence signal from excitation and consequently the fluorescence lifetime can be determined for all emission wavelengths. To resolve multiple components of the fluorescence lifetime, the measurements have to be performed several times using different modulation frequencies. Spectrum acquisition time is within an interval of several minutes at each frequency to get datasets enabling lifetime resolution of ± 0.1 ns.

Laser Diode Module (Omicron LDM 442.50.A350) with 50 mW output is used for excitation at 442 nm wavelength. The most severe limit for the excitation power is a danger of the sample photo-bleaching [1,5,7]. Hence, the 50 mW output power of the laser is attenuated by neutral density filters to the level from 1 to tens μW at the sample. Analog intensity modulation of the laser is obtained by direct coupling of the output sine wave of the Master synthesizer (model 2025 from IFR) to the laser diode excitation module. A laser-beam clean-up filter (LD01-439/8, Semrock, NY, USA) is used to suppress the green fluorescence emission of the laser diode.

Fig. 1. Block diagram of the experimental set-up of homodyne time-resolved microfluorometer with a broad spectral window.

Confocal epi-fluorescence upright microscope (Zeiss UMSP-80) is used with a high-numerical-aperture water-immersion $63\times$ objective (Zeiss Neofluar, NA 1.2). Fluorescence signal collected from the pinhole is focused on the entrance slit of the Jobin-Yvon HR640 spectrograph equipped with a 100 line/mm grating, which enables to direct whole 375 nm spectral region on a 25 mm diameter photocathode of the image intensifier. This high frequency gain modulated (5–200 MHz) image intensifier (Lambert Instrument, Netherlands) is optically coupled with CCD detector (1024×1024 pixels). The CCD controller is connected to the control computer via USB communication interface. The control computer also directly drives both high frequency synthesizers (modulation frequency, the phase and the amplitude of the output signal) via RS232C interface.

The LIFLIM software (Lambert Instruments) is used for the setup control and data acquisition. A special data conversion program (PHR) has been written to calculate the phase shift and the demodulation spectral dependence of the fluorescence signal from the acquired data. Subsequent lifetime determination and spectral decomposition is performed by using a global fitting procedure.

3. Sample preparation

The modified oligonucleotide, phosphorothioate oligothymidylate (15-mer), labeled by ATTO 425 at the 3'-end (ATTO-dT₁₅) was synthesized by Biomers, Germany. Chloride salt of Zn(II) 5,10,15,20-tetrakis(4-N-methylpyridyl) porphyrin (ZnTMPyP) was acquired from Frontier Scientific, USA. Living adhesive cell line (3T3 mouse fibroblasts) was cultured as monolayers in 25 cm² flask in a humidified 5% CO₂ atmosphere at 37°C, in Dulbecco's Modified Eagle's Medium (DMEM). DMEM was supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (0.1 mg/ml) and penicillin (100 U/ml), all purchased from Biomedix, France.

Cells were carried to Petri dishes (35 mm in diameter) and subcultured there for 48 h before microspectrofluorometric experiments. Prior to the experiments, the cells were overnight treated with added solution of oligonucleotide/porphyrin complex. This solution was prepared by gently mixing 60 μ l of ZnTMPyP stock solution (2.8×10^{-4} M) with 30 μ l oligonucleotide stock solution (3.3×10^{-4} M) for 15 min.

4. Experimental results

As shown in [10], the combination of fluorescence imaging and time-resolved microspectrofluorometry represents promising tool for reliable monitoring of the penetration, distribution and post-delivery fate of the labeled oligonucleotides inside the living cells. Fluorescence imaging can readily confirm successful penetration and shows subsequent distribution of the fluorophore in cellular compartments. On the other hand, the frequency domain lifetime analysis can be used as convenient method to verify integrity of the labeled molecules, stability of their complexes with delivery agents as well as to study their interaction with subcellular structures [11].

In the present study, cellular uptake of ATTO-dT₁₅ oligonucleotides was mediated by fluorescent ZnTMPyP. The fluorescence microimaging proved presence of labeled oligonucleotide and ZnTMPyP in cellular nucleus (figure not shown). Afterwards time-resolved microfluorescence spectra were collected from the cellular nucleus. Fluorescence signal of the two fluorophores was excited by 442 nm laser line that is close to their excitation maxima (both 436 nm). Obtained two-fluorophore experimental

Fig. 2. Original lifetime-resolved spectrum taken from the cellular nucleus of the 3T3 mouse fibroblast treated with complex of ATTO labeled oligonucleotide and cationic porphyrin (ATTO-dT₁₅/ZnTMPyP).

spectrum is shown in Fig. 2, ATTO-dT₁₅ and ZnTMPyP fluorescence contributions are clearly separated spectrally (~485 and ~640/680 nm, respectively). Elastic scattering peak serves as a zero lifetime reference. As one can see, labeled oligonucleotide and ZnTMPyP contributions are clearly separated. Global analysis has provided two lifetimes: ATTO-dT₁₅ fluorescence can be characterized by a single lifetime of 3.3 ns and porphyrin fluorescence possesses also a single lifetime but shorter – nearly 1.1 ns. The lifetime of ATTO-dT₁₅ in the cellular nucleus is shortened in comparison with solution (3.6 ns). The porphyrin lifetime also exhibits some shortening after the delivery process (1.7 ns when ATTO-dT₁₅/ZnTMPyP complex is in solution). Single lifetime of ZnTMPyP in the nucleus indicates that all porphyrin molecules remain in the complexes with oligonucleotide, in contrast to our previous experiments with complexes of rhodamine-labeled oligonucleotides and free-base H₂TMPyP [10], when two distinct lifetimes of H₂TMPyP indicated its partial dissociation from the oligonucleotide inside the nucleus [10]. Lifetime shortenings of both fluorophores in ATTO-dT₁₅/ZnTMPyP complex probably indicate non-covalent interactions in nuclear environment.

5. Conclusion

The build-up homodyne time-resolved microspectrofluorometer with broad spectral window was demonstrated as an efficient tool for monitoring multifluorophore molecular systems inside living cells. Complex of fluorescently labeled phosphorothioate homooligonucleotide with cationic porphyrin ATTO-dT₁₅/ZnTMPyP was found to penetrate successfully into living cells and to be accumulated in the cellular nucleus without dissociation.

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