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ORIGINAL ARTICLE

Intracellular Monitoring of AS1411 Aptamer by Time-Resolved Microspectrofluorimetry and Fluorescence Imaging

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Abstract Time-resolved microspectrofluorimetry and fluorescence microscopy imaging—two complementary fluorescence techniques—provide important information about the intracellular distribution, level of uptake and binding/interactions inside living cell of the labeled molecule of interest. They were employed to monitor the "fate" of AS1411 aptamer labeled by ATTO 425 in human living cells. Confocal microspectrofluorimeter adapted for time-resolved intracellular fluorescence measurements by using a phase-modulation principle with homodyne data acquisition was employed to obtain emission spectra and to determine fluorescence lifetimes in U-87 MG tumor brain cells and Hs68 non–tumor foreskin cells. Acquired spectra from both the intracellular space and the reference solutions were treated to observe the aptamer localization and its interaction with biological structures inside the living cell. The emission spectra and the maximum emission wavelengths coming from the cells are practically identical, however significant lifetime lengthening was observed for tumor cell line in comparison to non–tumor one.

Keywords Aptamer · AS1411 aptamer · Intracellular fluorescence · Fluorescence microscopy imaging · Phase-modulation lifetime measurement

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Introduction

Different oligonucleotide strategies (e.g., antigen, antisense and aptamer strategy) that employ the sequences of synthesized deoxyribo– or ribonucleotides to inhibit and/or regulate cellular gene expression have been extensively studied in recent decades [1]. As they foresee considerable therapeutic promise in the treatment of various hereditary and non–hereditary diseases, with the emphasis on strain of different types of the cancer recently, unflagging interest in this research has become important. Aptamer strategy emerged at the end of the 1980s and aptamers as such became a group of potential therapeutics because they possess several biophysical and biological properties making them very attractive in pharmacological applications. Aptamers (coming from the Latin word "*aptus*" that means fit) are generally small oligonucleotides (12–30 bases usually) or peptides that form, on appropriate conditions, a specific three–dimensional structure. After its binding to the key targeted protein, often with a high affinity, and following cellular uptake, demanded biological response in the cellular environment is elicited [2, 3].

The molecule of AS1411 is considered to be an advanced therapeutic aptamer with potential in anticancer treatment [4]. It has been previously found that AS1411 is able to inhibit the proliferation of cells and to induce the cell death in many types of cancer cell lines. Moreover, it was demonstrated that its effect on normal cells is negligible [2, 4]. AS1411 is a guanine–rich 26mer sequence (5′-GGTGGTGGTGGTTGTGGT GGTGGTGG) with a non–modified, i.e., natural (phosphodiester), backbone. In the presence of K^+, this specific sequence adopts a stable dimeric quadruplex structure with surprising resistance to cellular and serum nucleases. This stability enables effective interaction with its primary target—nucleolin, a protein involved in different cellular processes that is present on the surface of many cells as well as in the cellular

environment [4, 5]. Aptamer/nucleolin complex is able to cross the membrane, to distribute in the intracellular environment and to activate processes leading to cell death. Mode of the penetration inside the cell is not yet completely elucidated. However different internalization pathways were observed for different cell types: uptake of AS1411 in DU145 prostate cancer cells occurred by macropinocytosis, while in the case of Hs27 non–malignant human foreskin fibroblasts non–macropinocytosis pathway was noticed [5].

Advanced microfluorescence techniques provide the possibilities to monitor the distribution of molecules or structures of interest and to follow their "fate" (findings about target, its environment and related interactions) via a convenient fluorescent label. Classic or confocal fluorescence microscopy imaging, a standard way for intracellular distribution studies, when completed with time-resolved microspectrofluorimetry represents a strong tool to fulfill this task [6]. In this paper we introduce both mentioned techniques primarily focused on the U-87 MG tumor cell line incubated with AS1411 aptamer labeled by ATTO 425. We show the aptamer distribution inside the cells and discuss its interaction in connection with measured fluorescence lifetimes in the reference solutions and the cellular environment as well. Differences in lifetimes obtained from interior of the cells are subject of interest mostly when making a comparison between the tumor and non–tumor cells. It seems that fluorescence lifetime is very sensitive feature that can reflect changes linked with tumor environment of the cell.

Materials and Methods

The ATTO 425 fluorescence dye (Fig. 1) is a product of the Atto–Tec company (www.atto-tec.com). This dye is based on the coumarin structure and is characterized by λ_{abs}=436 nm, λ_{em}=484 nm and $\varepsilon_{max}=4.5\times10^4$ l/mol.cm. Synthetic 29mer oligonucleotide with sequence 5′-d(TTTG GTGGTGGTGGTTGTGGTGGTGGTGG) based on a 26mer AS1411 (5′-GGTGGTGGTGGTTGTGGTGGTG GTGG) with three thymine bases added at the 5′end and labeled by ATTO 425 was synthesized and purified (HPLC) by Biomers, Germany. In the labeling procedure the NHS-version of the ATTO 425 is used, an amino linked oligonucleotide (amino C5 at 5′) is attached to the label. Thymine bases were inserted as a spacer to avoid fluorophore quenching. Moreover, to insure the stability of the structure, the first four linkages (from 5′ end) were modified: the phosphodiester linkage was replaced by phosphorothioate.

The aptamer sample was dissolved in a PBS buffer with K^+ at concentration 150 mM to induce the formation of a quadruplex structure [7]. Final concentration of the aptamer quadruplex stock solution was 0.25 mM. Liposome

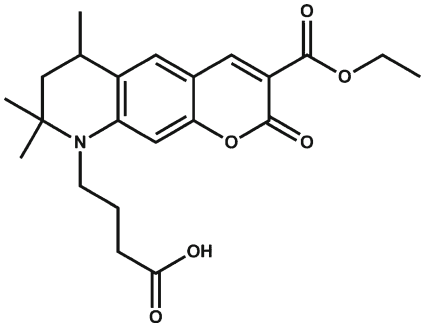

Fig. 1 Structure of ATTO 425 fluorescence label

suspension from 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC, Avanti Polar lipids, Inc.) was formed through 100 nm pore size polycarbonate membrane using a small–volume LiposoFast™ apparatus (Avestin Europe, GmbH, Germany) according to a standard protocol [8]. This suspension was mixed with the aptamer solution with final concentration of 1.25 mg/ml and 1.25 μM for lipid and the aptamer respectively.

Tumor cell line U–87 MG (human glioblastoma cells) and non–tumor cell line Hs68 (human foreskin cells) were used in the experiment. Cells were cultured by standard method in 25 cm^2 flasks at temperature 37 °C in a humidified 5 % CO_2 atmosphere. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % calf fetal serum, 2 mM L–glutamine, streptomycin (0.1 mg/ml) and penicillin (100 U/ml) were purchased from Sigma–Aldrich and Biomedia, France. Cells were subcultured in Petri dishes (μ–Dish 35 mm high glass bottom, ibidi, Germany) 48 h before experiments. Incubation with the aptamer solution for about 2–3 h before the measurement was found to be sufficient. The aptamer concentration in Petri dishes was about 2 μM.

Our microspectrofluorimeter has been built on a phase-modulation principle by using a homodyne data acquisition method [6, 9, 10]. Its block diagram can be seen in Fig. 2. The fluorescence lifetime can be determined for all emission wavelengths by acquiring several spectral images (regularly 6) by changing the phase shift of the modulated excitation laser beam (master synthesizer) in relation to fixed detector gain modulation phase shift (slave synthesizer) [11]. In order to resolve multiple lifetime components of the fluorescence, the spectra acquisition was performed for each of usually 8 individual modulation frequencies covering evenly 10 to 180 MHz interval. Analog modulated Laser Diode Module (Omicron LDM 442.50.A350, Germany) with 50 mW peak output (attenuated to 1 to tens of μW at the sample level) was used for the excitation at 445 nm wavelength. It is driven by the master synthesizer (IFR Aeroflex 2025 signal generator, USA). This excitation wavelength matches free ATTO 425 label excitation band (436 nm at maximum value) quite well.

Confocal epifluorescence upright microscope (Zeiss UMSP–80, Germany) is used with a high–numerical aperture

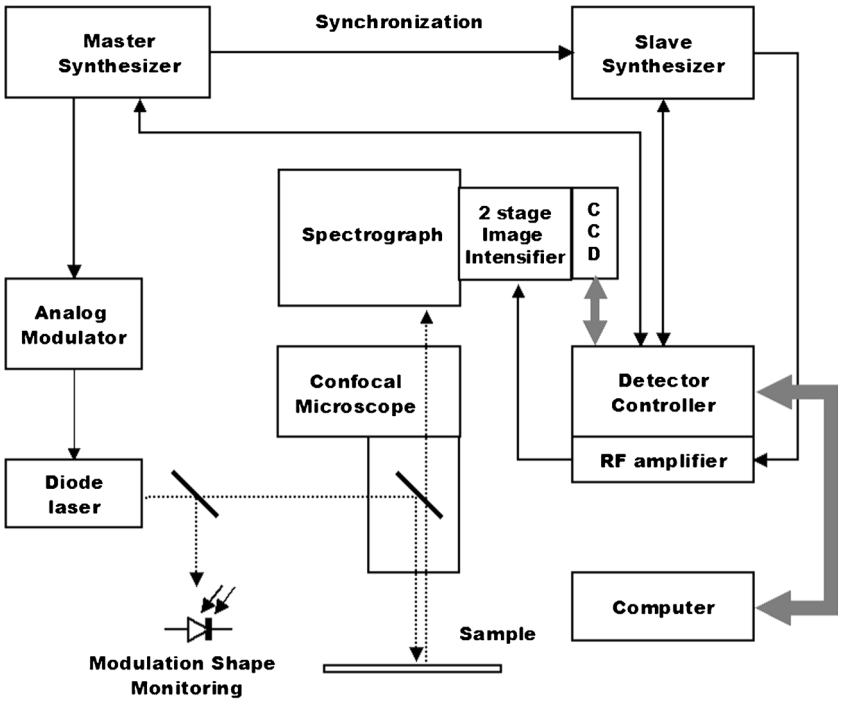

Fig. 2 Block diagram of the time-resolved phase modulation confocal microspectrofluorimeter with homodyne data acquisition principle

water–immersion 63× objective (Zeiss Neofluar, NA 1.2). Fluorescence signal collected from the microscope pinhole is focused on the entrance slit of the Jobin–Yvon HR640 spectrograph equipped with a 100 line/mm grating. A spectral detection window (375 nm wide) is covering both the excitation wavelength (elastic scattering, used as a lifetime reference) and the fluorescence emission of the fluorophore (see Fig. 3). The detection part consists of a gain modulated image intensifier driven by amplified output of the slave synthesizer (IFR Aeroflex 2025 signal generator, USA) working at precise synchronism with the master one. The image intensifier is optically coupled with TE–cooled CCD detector (1024× 1024 pixels). The data transfer from the CCD detector readout and control unit to the data acquisition computer uses a USB interface. Both high frequency digital synthesizers (modulation frequency, its phase and the output level) are directly controlled by the computer via RS232C serial interface.

The spectrometer setup control and data acquisition were done by the LIFLIM software (Lambert Instruments, Belgium). To calculate the phase shift and the demodulation spectral dependence of the fluorescence signal from the acquired data, application specific software called "PHR" has been written in the Institute of Physics. For each frequency, the output data of LIFLIM are values of intensity, phase and modulation for 512 or 1024 equidistant wavelength points. In our program, the elastic scattering peak (taken as a reference) is fitted by asymmetric Lorentzian curve to find exact position of the maximum. Then, reference values of phase (P_{ref}) and modulation (M_{ref}) at this position are evaluated from the smoothed phase and modulation curves (see Fig. 2). Specific wavelengths (usually 3 to 8 distinctive values for each examined interval) are selected in the fluorescence emission spectrum window. Corresponding values of phase ($P_{\lambda em}$) and modulation ($M_{\lambda em}$) are evaluated from the smoothed data curves. Calculated $P_{\lambda em}$ - P_{ref} differences and $M_{\lambda em}$/M_{ref} ratios are used as an input table for GLOBAL fitting program (Laboratory for Fluorescence Dynamics, Irvine, USA) to calculate single fluorescence lifetime components and their percentage representation.

White light and fluorescence images of the cells were obtained with inverted fluorescence microscope (Nikon Eclipse Ti, Japan) by using an oil–immersion objective 100×/1.3 (Nikon Plan Fluor OFN25 Ph3 DLL). For fluorescence images the excitation (482/35 nm) and the emission (536/40 nm) filters were used. This setting enabled us to observe intracellular distribution of labeled AS1411 aptamer very well.

Results and Discussion

Fluorescence microscopy imaging is typically used as a highly informative technique providing the overall view about the distribution of the labeled molecule of interest throughout the population of cells in the cell line. It indisputably demonstrates the general state of the staining, the presence or absence of the labeled molecule on the surface of the cells and/or its localization inside the cell and in the specific subcellular structures. Typical white light image from the tumor cells of human glioblastoma line U-87 MG without any fluorescence agent

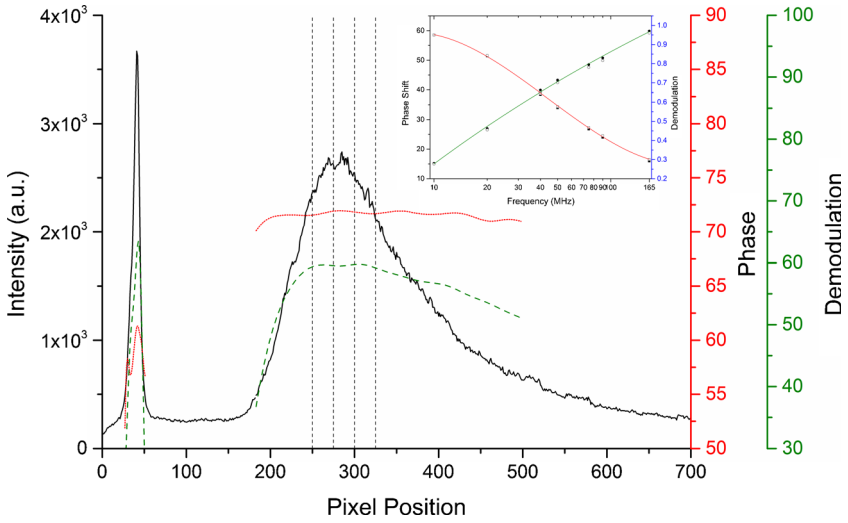

Fig. 3 Experimental emission spectrum and corresponding phase and demodulation values (displayed in the part of the elastic scattering and the fluorescence band as well) depending on pixel position of the detector. *Vertical dashed lines* demonstrate positions at which the reading of phase shift and demodulation was done

and fluorescence image incubated with AS1411 aptamer labeled by ATTO 425 after three hours of incubation are shown in Fig. 4. Fluorescence image (Fig. 4 right) clearly reveals the aptamer presence throughout the cells with prevalent occurrence in the cytoplasm. Thus, our labeled aptamer is clearly able to cross the cellular membrane and to spread out in the cell, although the cell nuclei rest practically unstained. From the non–homogenous character of the staining we can conclude that uptake process is more likely mediated rather than simply diffusive. As already known from the published results [4], tumor cells possess increased levels of the cytoplasmic and plasma membrane nucleolin in comparison to normal healthy cells. Moreover, nucleolin localized in the plasma membrane is considered to be a very effective shuttle and it enables the aptamer uptake [4]. The fact that no additional 'delivery system' is necessary to support the aptamer transport through the cellular membrane is a real advantage of such molecules. In addition, uptake of labeled AS1411 aptamer into U-87 MG cells is obtained after a shorter time (1–3 h) and doesn't need a long overnight incubation.

Time-resolved microfluorescence spectra were obtained from a set of U-87 MG cells and from reference aptamer aqueous solutions (or suspensions) as well. The solution measurements are commonly done in order to imitate and simplify in parts the complex cellular environment with the aim to recognize possible molecular targets. In this way, the outcome lead to easier understanding and interpretation of the data obtained from the cellular environment.

First reference measurement was done on solution of AS1411 aptamer labeled by ATTO 425 in PBS buffer with K^+ ions needed to form a quadruplex structure. Measured spectra were processed with our PHR program and the lifetime analysis was then performed by GLOBAL fitting procedure to distinguish single lifetime components. Fluorescence emission spectrum obtained from the labeled aptamer in solution with fitted phase shift and demodulation values is shown in Fig. 5. Specific lifetime was assessed from the grayish area of the fluorescence band. Single lifetime value of 4.4 ns has been calculated for several measurements of the aptamer concentration in μM scale.

In view of the fact that we are interested in the transport of AS1411 aptamer through the cellular membrane, its behavior in the presence of the lipid environment was considered. The lipid membrane model system, DPPC liposome, was formed in this prospect. DPPC is convenient lipid molecule very often found in high amount in the animal and plant cellular

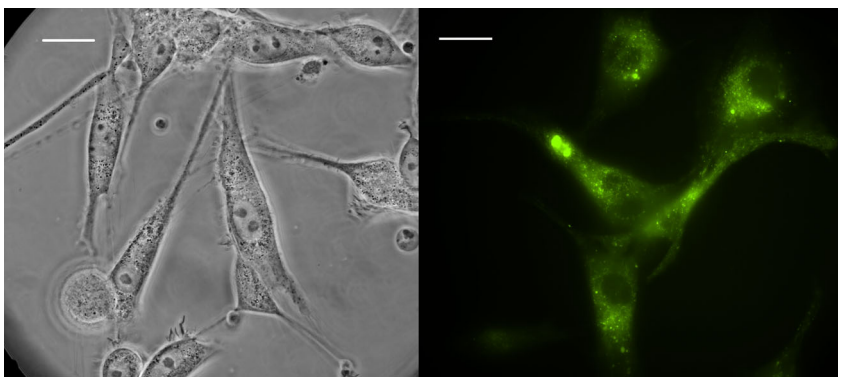

Fig. 4 White light image (*left*) from the cells of U–87 MG cell line (tumor human glioblastoma cells) and fluorescence image (*right*) of the cells incubated with AS1411 aptamer labeled by ATTO 425. Time of incubation was approximately 3 h. Bar= 20 μm

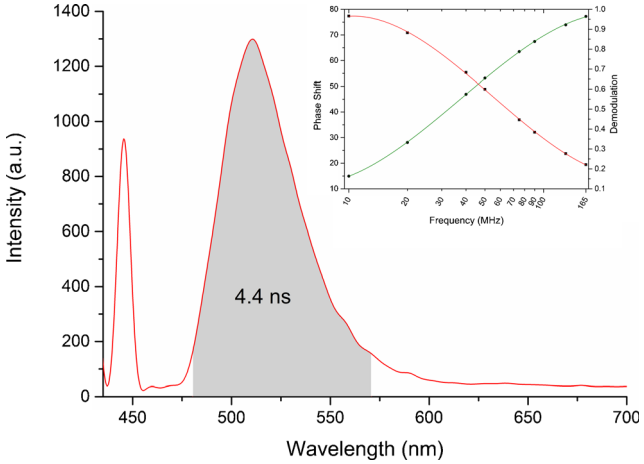

Fig. 5 Emission spectrum of AS1411 aptamer labeled by ATTO 425 in PBS buffer solution containing K^+ ions. Fluorescence lifetime was calculated from the grayish part of the fluorescence band. Inset: Fitted phase shift and demodulation data curves indicating single component fluorescence lifetime

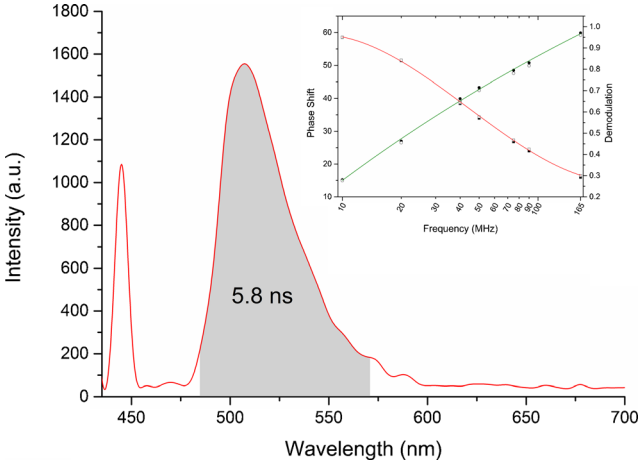

Fig. 6 Typical intracellular fluorescence emission spectrum and corresponding lifetime obtained from U-87 MG tumor cells after 3 h incubation with 2 μM of AS1411 aptamer labelled with ATTO 425. The narrow band observed on the left corresponds to the elastic scattered light of the excitation (442 nm). Inset: Fitted phase shift and demodulation data curves indicating single component fluorescence lifetime

membranes. It undergoes the phase transition at 41 °C, a temperature that is higher than the one at which the cells are cultured. Moreover, all our measurements were done at a temperature lower than 41 °C. Thus DPPC membranes of liposome are in the same ordered state, i.e., potential problems caused by the phase changes due to the temperature can be excluded. The mixture of AS1411 aptamer with the liposome suspension (incubated for several tens of minutes up to 3 h) shows single lifetime component 3.7 ns. This value slightly differs from the one in the aqueous buffer solution and it reveals that the environment composed of amphiphilic lipid molecules possessing polar heads and hydrophobic chains leads to the lifetime shortening.

As for intracellular time-resolved measurements on U-87 MG and Hs68 cell lines, several independent incubations in the presence of the labeled AS1411 aptamer were accomplished. Obtained dataset accumulated from incubation medium and from the intracellular environment of several different uptaken cells was processed in the same way as those obtained from solutions or suspensions. Treatment of spectra coming from the incubation medium where the cells with labeled AS1411 aptamer are cultured shows single fluorescence lifetime component of 3.5 ns. Typical fluorescence emission spectrum observed in U-87 MG cells consists of ATTO 425 labeled aptamer emission band and of the lifetime reference elastic scattering peak (see Fig. 6). The same spectral shape is obtained in Hs68 cells (data not shown). Corresponding curves of the fitted phase shift and the demodulation data clearly, indicating a single lifetime component, can be seen in Fig. 6 inset. The value of the corresponding calculated fluorescence lifetime is 5.80 ns (with the calculation error within±0.15 ns). As one may note the intracellular lifetime of the labeled AS1411 aptamer in U-87 MG tumor cells is markedly lengthened when compared to those obtained for aptamer in K^+ buffer solution, mixed with liposome suspension or present in the incubation medium (see Table 1). This result indicates that intracellular space in tumor cell line represents a distinct microenvironment for the aptamer localization and the fluorescence lifetime reflects its intracellular binding. As for Hs68 cell line, the incubation time of AS1411 aptamer and its distribution inside the cells with a dominant presence in the cytoplasm are similar to U-87 MG cell line (images not shown in this paper). However calculated fluorescence lifetime shows single 4.30 ns lifetime component in this case. This lifetime value resembles the one obtained for the labeled aptamer solution measurement but it is definitely distinct from the lifetime value in tumor U-87 MG cell line. Thus, a significant lifetime lengthening was observed for the tumor cell line in comparison to the non-tumor one.

As for emission spectra in Figs. 5 and 6, the maximum emission wavelength observed in the spectrum of AS1411 aptamer labeled by ATTO 425 in PBS buffer solution (Fig. 5) and in the spectrum of tumor U-87 MG cells with AS1411 aptamer (Fig. 6) are practically identical.

Table 1 Fluorescence lifetime values of ATTO 425 labeled AS1411 aptamer determined in different environments

ATTO 425 labeled AS1411 aptamer	Fluorescence lifetime (nsec)
In 150 mM K^+ PBS buffer	4.40±0.10
In DPPC liposome suspension	3.70±0.10
In Cell culture medium	3.50±0.10
In Hs68 cells cytoplasm	4.30±0.15
In U-87 MG cells cytoplasm	5.80±0.15

This characteristic is therefore not sufficient to distinguish between them.

Thus, as obtained results demonstrate, the fluorescence lifetime is important and very sensitive characteristic which is able to reflect differences that are not noticeable only by making a comparison between fluorescence spectra and their maxima. Fluorescence lifetime measurement is a stronger tool that can distinguish tumor and non-tumor cellular environment.

Conclusion

The classic fluorescence microscopy imaging and time-resolved microspectrofluorimetry techniques were employed to study the AS1411 aptamer tranport inside tumor and non–tumor cell lines. Fluorescence imaging reveals the aptamer presence throughout the cells with dominant occurrence in the cytoplasm. The aptamer labeled by ATTO 425 and dissolved in PBS buffer with K^+ ions gives a single fluorescence lifetime of 4.40 ns. In the lipid environment, its lifetime is shortened to 3.70 ns. As for the intracellular measurements in tumor and non–tumor cell lines, the emission spectra and the maximum emission wavelengths are not significantly modified; however some difference was found in their respective lifetimes. Intracellular lifetimes obtained inside the tumor cells and non–tumor cells are 5.80 ns and 4.30 ns, respectively. Hence, lifetime of ATTO 425 labeled AS1411 aptamer in U-87 MG tumor cell line is markedly lengthened when compared to those obtained in the solution while the lifetime value of non–tumor Hs68 cell line does not change considerably. Thus, our study suggests that ATTO 425 labeled aptamer could be a powerful tool to differentiate between tumor and non–tumor cells based on its fluorescence lifetime.

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