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Aggregation-Induced Emission

Mapping Live Cell Viscosity with an Aggregation-Induced Emission Fluorogen by Means of Two-Photon Fluorescence Lifetime Imaging

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Abstract: Intracellular viscosity is a crucial parameter that indicates the functioning of cells. In this work, we demonstrate the utility of TPE-Cy, a cell-permeable dye with aggregation-induced emission (AIE) property, in mapping the viscosity inside live cells. Owing to the AIE characteristics, both the fluorescence intensity and lifetime of this dye are increased along with an increase in viscosity. Fluorescence lifetime imaging of live cells stained with TPE-Cy reveals that the lifetime in lipid droplets is much shorter than that from the general cytoplasmic region. The loose packing of the lipids in a lipid droplet results in low viscosity and thus shorter lifetime of TPE-Cy in this region. It demonstrates that the AIE dye could provide good resolution in intracellular viscosity sensing. This is also the first work in which AIE molecules are applied in fluorescence lifetime imaging and intracellular viscosity sensing.

Introduction

Intracellular viscosity is a crucial parameter that determines the rate of the diffusion-controlled reactions in many biological processes.[1] It controls the speed of biomass transportation as well as signal transduction in the living system.[2] The abnormality of intracellular viscosity has been regarded as a vital contributor to, or indicator of, a large variety of diseases such as atherosclerosis, diabetes, and Alzheimer's disease.[3] Drugs that influence the intracellular viscosity can be used to manipulate the cell activity. There are certain anesthetics that belong to these types of drugs. They can insert into the neuron membrane, reduce the membrane fluidity (the viscosity of the lipid bilayer membrane), regulate the net flux of neurotransmitter receptors, and thereby decrease the nerve activity.[4] Given its important roles in cell functioning and pathogenic conditions, the sensing and imaging of the intracellular viscosity is of great significance and has attracted a great deal of attention.

Although many methods for bulk viscosity measurement have been developed, determination of the viscosity in microenvironments still remains elusive.[5] In particular, the fluorescence recovery after photobleaching (FRAP) technique is frequently employed by biologists to elucidate the mobility of the fluorophore. The intracellular viscosity in a local area can thus be deduced.[6] In this method, a high-power laser is applied to bleach a small defined area inside the cell; the recovery rate of the fluorescence from unbleached fluorophores in the neighboring region can be correlated to the local viscosity. Intrinsically, FRAP is for macroscopic measurements, which can barely provide information down to submicron resolution. Early in the 1970s, viscosity-dependent fluorescence depolarization was reported for the measurement of viscosity in lipids.[7] However, this method is indirect and less commonly used. More recently, a group of molecular rotors was developed as microviscosity sensors.[8] The fluorescence intensity or lifetime signals of these dyes are responsive to the viscosity

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changes. However, there is still room for improvement in terms of sensitivity to resolve the viscosity variation inside a cell.

A series of dyes was observed to exhibit an unusual property called aggregation-induced emission (AIE).[9] These dyes are non-emissive or weakly emissive when molecularly dissolved, but become fluorescent once aggregated or hindered in a confined environment. Mechanistic studies suggest that restriction of intramolecular motions (RIM) plays a major role in this turn-on process of the AIE phenomenon.[9b–f] On the basis of this understanding, it is envisaged that the AIE dyes are sensitive to viscosity. In a more viscous environment, the intramolecular motions—that consequently populate the radiative decay and therefore enhance the fluorescent intensity—would be impeded. Our previous observation suggests that the fluorescence lifetime of the AIE dyes is prolonged with an increase in solvent viscosity.[9a] We thus expect that the lifetime signals of the AIE molecules could be applied to intracellular viscosity sensing and mapping. Compared with traditional molecular rotors, the AIE dyes contain more rotatable parts, which might make them more sensitive to the change in viscosity in the microenvironment.

To demonstrate the feasibility of this, for this work, we selected TPE-Cy, an AIE-active hemicyanine dye for microviscosity sensing, because of its cell permeability and excellent biocompatibility.[10] TPE-Cy exhibits longer fluorescence lifetime in solution with higher viscosity as well as in a membrane environment with more tightly packed lipids. Upon two-photon excitation, the lifetime image of living cells stained with TPE-Cy is collected and analyzed, from which lipid droplets with lower local viscosity can be easily identified. The lifetime value of TPE-Cy varies from a few hundred picoseconds to more than 1.5 ns inside the cells, thereby providing much higher resolution than most of the reported systems.

Results and Discussion

TPE-Cy is a pH-sensitive dye that can change its emission color from red to blue with an increase in the pH.[10a] Under basic conditions, the OH^- anion could serve as a nucleophile and transform TPE-Cy to TPE-Cy-OH (Scheme 1). With the breakage of the conjugation, TPE-Cy-OH exhibits blue emission. Interacting with the lipid membrane promotes this reaction and shifts its color-transition point from pH 10 to neutral pH.[10b] In the cell-staining process, when the TPE-Cy molecules diffuse into the cells through the cell membrane, they stain the majority of the cytoplasmic region, especially the membrane-bound organelles with blue emission. To mimic the intracellular conditions, TPE-Cy was first chemically converted to TPE-Cy-OH, and its fluorescence behaviors in response to viscosity change were investigated prior to its application in live cells.

To afford solvents with varying viscosity but constant polarity, ethylene glycol and glycerol mixtures with different glycerol volume fractions were prepared. As shown in Figure 1A, TPE-Cy-OH is weakly emissive in pure ethylene glycol. When the glycerol fraction was increased, the solvent became more viscous and the blue emission from TPE-Cy-OH was enhanced gradually with the increase in viscosity (Figure 1B and Figure S1 in the Supporting Information). According to the AIE mechanism, the free rotation of the peripheral phenyl rings of the TPE serves as the nonradiative channel to decay excited species, whereas in a viscous medium, such intramolecular motions become difficult and thus inhibit the nonradiative decay and populate the radiative decay species. As a result, the fluorescence intensity is enhanced about 18-fold in solution with 99.8 vol% glycerol.

On top of the enhanced fluorescence intensity, the fluorescence lifetime is also gradually prolonged with the increase in solvent viscosity (Figure 1C). From pure ethylene glycol to 99.8% glycerol, the lifetime of TPE-Cy-OH is lengthened from

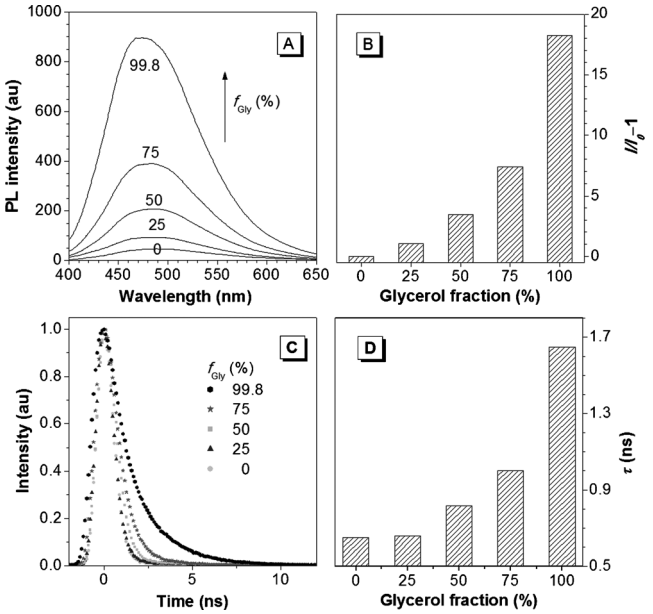
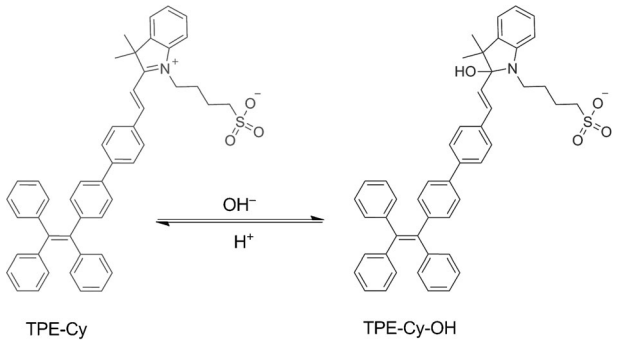


Figure 1. Viscochromism and viscosity-dependent fluorescence lifetime of TPE-Cy-OH in solvent mixtures. A) Emission spectra of TPE-Cy-OH in mixtures of ethylene glycol and glycerol with different glycerol fraction (f_{Gly}). B) Plot of I/I_0-1 versus glycerol fraction in which I and I_0 represent the peak intensity with a specific f_{Gly} and in pure ethylene glycol, respectively. C) Fluorescence decay traces recorded for TPE-Cy-OH in ethylene glycol and glycerol mixtures with different f_{Gly}. D) Plot of fluorescence lifetime versus f_{Gly}; $\lambda_{ex}=375$ nm, $\lambda_{em}=480$ nm.



Scheme 1. The structure of TPE-Cy and its transformation to the base form, TPE-Cy-OH.

0.65 to 1.65 ns (Figure 1D). The observed longer lifetime is consistent with the increase in the excited population decaying through the radiative channel as well as the enhanced emission intensity in the solution with high viscosity. Hence, along with fluorescence intensity, the fluorescence lifetime is another parameter of TPE-Cy-OH to indicate the environmental viscosity.

To further verify this hypothesis, we examined the change in fluorescence lifetime of TPE-Cy in the membrane environment with different fluidity. Membrane fluidity refers to the viscosity of the lipid bilayer of a cell membrane, which can be regulated by the composition of phospholipids with different structures.[11] We prepared artificial lipid vesicles with different lipid compositions and TPE-Cy was used as a probe to sense the difference in fluidity of these lipid vesicles. Here, we did not change TPE-Cy to TPE-Cy-OH, because such a conversion can take place spontaneously in neutral pH in the presence of a phosphocholine lipid membrane.[10b]

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol were the four model membrane lipid components used in this study (Figure 2). As shown in Figure 3, the lifetime of TPE-Cy was indeed different in the lipid composition with the shortest lifetime (0.71 ns) in the presence of the unsaturated lipid DOPC and the longest (1.36 ns) in the presence of its saturated form DSPC. When cholesterol was added into the DOPC lipid vesicles, the lifetime value became longer than in pure DOPC. In the presence of DHPC, a saturated lipid with shorter aliphatic chains, TPE-Cy exhibited a lifetime between those in DSPC and in DOPC/cholesterol vesicles.

This result is in agreement with our above observation that the lifetime of TPE-Cy-OH is shortened with the decrease in viscosity, or in other words, the increase in fluidity. The proposed

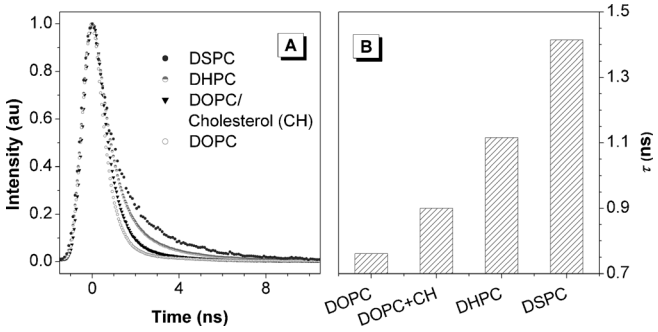


Figure 3. A) Recorded fluorescence decay traces, and B) plot of fluorescence lifetime of TPE-Cy stained lipids in PBS; $\lambda_{ex} = 375$ nm, $\lambda_{em} = 490$ nm.

packing modes of lipid vesicles with different compositions are shown in Figure 2. DOPC with unsaturated C=C bonds could not pack very tightly, and the resultant vesicles were less viscous. The insertion of cholesterol filled up the gap between these unsaturated tails and thus decreased the fluidity of the DOPC vesicles. DSPC, in contrast, has long saturated alkyl chains, which allowed the maximum interaction of fatty-acid tails. The tight packing of DSPC molecules afforded the vesicles with the highest viscosity and thus the lowest membrane fluidity. DHPC, though with saturated chains, possesses much shorter alkyl chains, which reduced the tendency of the hydrophobic tails to interact with each other. The viscosity of these four model systems can thus be ordered as DOPC < DOPC/cholesterol < DHPC < DSPC, in accordance with the lifetime values of TPE-Cy. When the membrane environment was more rigid with higher viscosity, the lifetime of TPE-Cy in the blue channel became longer. However, in the membrane with higher fluidity, the phenyl rings of TPE-Cy could still undergo free rotation, which increased the non-radiative decay population and led to a shorter lifetime of the dye. These results further prove the feasibility of using TPE-Cy for sensing the viscosity/fluidity in a membrane environment.

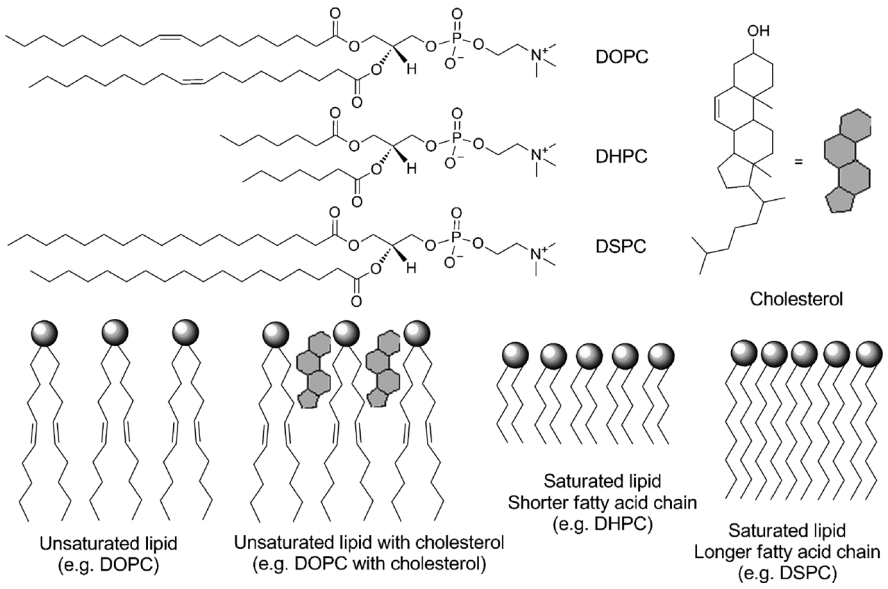


Figure 2. The chemical structures of DOPC, DHPC, DSPC, and cholesterol, and the illustrated representation of the proposed lipid packing modes. DOPC is a model of unsaturated lipids; DHPC and DSPC are models of saturated lipids with shorter and longer fatty-acid chains, respectively.

The results in model lipid vesicle systems encouraged us to further explore the application of TPE-Cy in sensing intracellular viscosity. Co-localization experiments suggested that the blue signals of TPE-Cy inside the cells were mainly located in the membrane-bound organelles such as mitochondria and lipid droplets (Figure 4). The stained tubular structures are mainly mitochondria, as verified by the overlay signals from the mitochondria-specific dye, rhodamine 123 (Figure S2A–D in the Supporting Information). However, Nile red is widely known to stain cytoplasmic lipid droplets,[12] a fat storage organelle

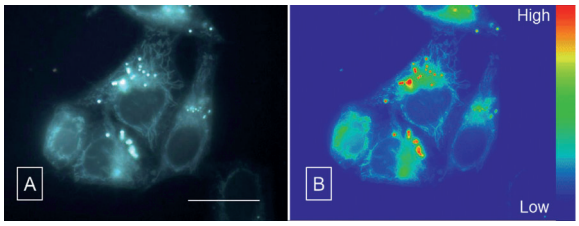


Figure 4. A) Fluorescence image and B) the corresponding surface intensity plot of TPE-Cy stained HeLa cells. [TPE-Cy] = 10 μM. Scale bar: 35 μm, λ_{ex} = 330–380 nm.

surrounded by a monolayer of phospholipids. The co-staining experiment indicates that the dots in the cytoplasm with intense blue signals (Figure 4) are mainly lipid droplets, as they can be satisfactorily colocalized with signals from Nile red (Figure S2E in the Supporting Information). From the structural point of view, the chromophoric part of TPE-Cy is basically lipophilic, which encourages the dye molecules to accumulate in the hydrophobic regions inside the cells such as the membrane-bound and the lipid-rich organelles.

Certain organelles, however, can produce intrinsic fluorescence upon photoexcitation. Such autofluorescence might interfere with the fluorescence signals from external probes. To avoid the interference from autofluorescence, two-photon excitation was employed in this study. By using two-photon excitation at 600 nm, signals from intracellular TPE-Cy that peaked at around 490 nm could be satisfactorily separated from the autofluorescence signals at around 350 nm, which originate from proteins that bear tryptophan residues inside the cells (Figure 5A).[13] At the same time, we employed fluorescence lifetime imaging microscopy (FLIM) for the detection of intracellular viscosity because the lifetime of a fluorophore depends on its molecular environment but is less dependent on other variables such as dye concentration or the power of the excitation light source, which can affect fluorescence intensity significantly.[14]

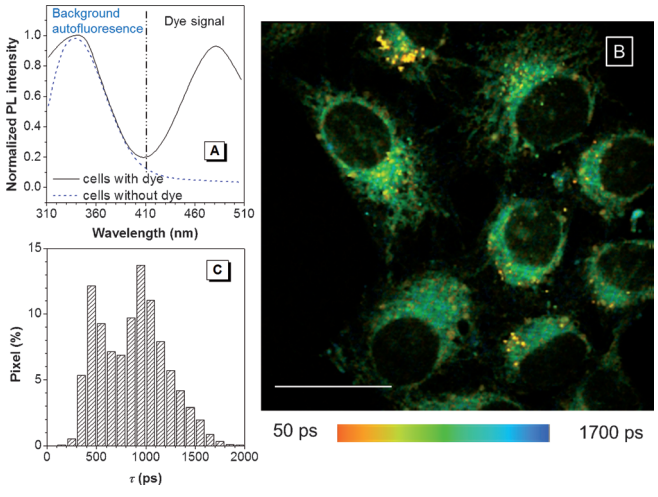


Figure 5. A) Comparison of PL signals from cell autofluorescence and cells stained with TPE-Cy upon two-photon excitation. B) FLIM images and C) fluorescence lifetime distribution histogram of HeLa cells stained with TPE-Cy. [TPE-Cy] = 10 μM. Scale bar: 30 μm. Two-photon excitation wavelength: 600 nm.

Fluorescence lifetime imaging of the TPE-Cy-stained live HeLa cells was carried out by means of a homemade time-resolved spectroscopic imaging system. The FLIM image obtained by two-photon excitation at 600 nm is shown in Figure 5B with detailed analysis in Figure S3 of the Supporting Information. In the cellular environment, the lifetime of TPE-Cy was distributed over a broad range from 300 to 1500 ps. In particular, in the lipid droplets, the lifetime (≈500 ps) was much shorter than that of the other stained regions such as the tubular mitochondria (≈1 ns). The two populations with distinct lifetimes can be easily differentiated from the two peaks in the fluorescence lifetime histogram (Figure 5C).

The broad distribution of the lifetime of TPE-Cy reflects the different fluidity of the microenvironments inside the cell. Unlike the phospholipid bilayer, such as the mitochondrial membrane, lipid droplets are surrounded by a lipid monolayer membrane with lipids stored inside. The interior of the lipid droplets possesses high fluidity with lipid molecules loosely packed because of their unique structure.[15] The hydrophobic TPE-Cy molecules thoroughly "dissolved" in this lipid reservoir could still undergo active intramolecular motions, thus leading to the shorter fluorescence lifetime. This result is in line with the results obtained by other research groups using molecular rotors.[16]

Conventional fluorescence-based microviscosity probes are mainly based on molecular rotors. Molecular motors are defined as a group of fluorescent molecules with twisted intramolecular charge-transfer (TICT) properties.[17] A TICT molecule consists of electron-donor (D) and -acceptor (A) units that can rotate against each other. In the TICT state, the charge separation of D and A results in a redshift and decreased emission of the molecule.[18] In a viscous environment or nonpolar solvent, the TICT rate is reduced and the emission of the molecular rotor is thus enhanced.[8] However, D–A units are not required in the AIE systems.[9] The AIE fluorophores can turn on the emission with prolonged fluorescence lifetime once the intramolecular motions are restricted. The restriction of intramolecular motions might result from the aggregation of the dye molecules (like the first time when the AIE phenomenon was observed and named) and also the increase in the local viscosity.[9] The different mechanisms of the molecular rotor and AIE will both facilitate the development of viscosity-sensitive probes. In this sense, by introducing the AIE molecules, the library of fluorescent probes that can be used for microviscosity sensing would be greatly enlarged and more alternatives with better performance might be discovered.

Conclusion

In this paper, an AIE active dye, TPE-Cy, was utilized as a probe for extracellular and intracellular viscosity sensing. With the increase in viscosity, the fluorescence intensity is enhanced and the lifetime becomes longer, which has been demonstrated by using solvent mixtures of ethylene glycol and glycerol with different glycerol fractions and lipid vesicles comprised of differ-

ent phospholipid derivatives. Depending on the structures of the alkyl chains, the lipid molecules in the membrane can pack either tightly or loosely, thereby resulting in the difference in the fluorescence lifetime of the dye.

Thanks to its two-photon absorption at 600 nm, the fluorescence signals of TPE-Cy can be thoroughly separated from the cell autofluorescence, thereby eliminating the intrinsic interference during FLIM imaging. Once entering the cells, the dye exhibits a wide range of fluorescence lifetime distribution, which is indicative of the heterogeneity of the intracellular viscosity and the complexity of the intracellular environment. The long lifetime is mostly distributed in membrane-bound organelles such as mitochondria: The ordered packing of the lipid bilayers of most organelles results in high viscosity/low fluidity and thus the long lifetime of the dye. The shortest lifetime is observed in the lipid droplets. The loose packing of the lipid molecules in lipid droplets offers a low-viscosity environment that allows the free intramolecular motions of the dye and thus results in the short lifetime. Because of the hydrophobicity of the lipid droplets, the AIE dyes are prone to accumulation in these organelles, thus leading to high dye concentration and intense fluorescence. The lifetime, independent of the dye concentration, can overcome such interference and reveal the true feature of the low viscosity in lipid droplets. This method could be applicable in other biological systems as well as in screening drugs that can alter the intracellular viscosity.

Experimental Section

Materials and instruments

Minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Acros. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids, Inc. All other reagents used in this study were purchased from Sigma–Aldrich. Photoluminescence (PL) spectra of the solutions were recorded using a Perkin–Elmer LS 55 spectrofluorometer. Single-photon lifetime measurements were carried out using an Edinburgh FLSP920 spectrophotometer equipped with a 375 nm laser. TPE-Cy-OH was generated by adding TPE-Cy in DMSO (10 mM stock) (1:9 v/v) into a NaOH (1 M) solution.

Lipid vesicle preparation

Lipids (DOPC/DHPC/DSPC) were dissolved in chloroform to afford 10 mg mL^{-1} stock solution. Solvent was removed by vacuum evaporation. Lipids were re-suspended in phosphate-buffered saline (PBS; pH 7.4) to a final concentration of 2 mM. The suspension was then shaken and sonicated. The lipid vesicles with a diameter of about 200 nm were prepared by extrusion and analyzed by using a ZetaPlus Potential Analyzer (Brookhaven Instruments Corporation, USA). TPE-Cy was added into the solutions to a final dye concentration of 10 μM. All the samples were placed in room temperature overnight before measurement.

Cell culture

HeLa cells were cultured in MEM in a humidity incubator at 37 °C with 5% CO_2. The medium contained 10% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin.

Cell staining

HeLa cells were seeded in a 35 mm petri dish with a glass cover slide. After culturing overnight, cells were stained with 10 μM TPE-Cy for approximately 2 h in a humidity incubator at 37 °C with 5% CO_2. Before imaging, the cells were washed three times with PBS solution (pH 7.4). The staining solutions for cell staining were prepared by adding an aliquot of DMSO stock solution of TPE-Cy (10 mM) into the culture medium.

Cell imaging

The fluorescence images of the stained cells were recorded by using a fluorescence microscope (Olympus BX41). UV excitation: excitation wavelength = 330–380 nm, dichroic mirror = 400 nm, long-pass emission filter. Blue excitation: excitation wavelength = 460–490 nm, dichroic mirror = 505 nm, long-pass emission filter.

Two-photon lifetime imaging

A homemade time-resolved spectroscopic imaging system was used for this study. The excitation sources in the visible wavelength of 600 nm were generated from the ultrafast supercontinuum generation in a photonic crystal fiber, pumped by a tunable femtosecond Ti:sapphire laser. The supercontinuum was generated from a photonic crystal fiber (PCF, NL-1.4-775, Crystal Fiber). The femtosecond Ti:sapphire laser (Mira 900, Coherent) of wavelength tuned at 750 nm was used as the pump source. The supercontinuum beam in the visible band was purified by means of interference band-pass filters at (600 ± 20) nm (D600/40M, Chroma). The excitation power was less than 10 mW on the sample. A water immersion objective (40 × 1.15 NA, Olympus) was used to focus the excitation beam(s) into the examined samples and collect the backscattered two-photon excited fluorescence (TPEF) signals. A dichroic mirror (FF510, Semrock) was placed above the objective to reflect the TPEF signals to the detection system and a short-pass filter (500 sp, Edmund Optics) was used to reject the reflected excitation light. The TPEF signals were relayed by a telescope to a fiber bundle that conducted the TPEF signals to a spectrograph, coupled with a linear array of photomultiplier tubes (PMT) connected with a time-correlated single-photon counting (TCSPC) module (PML-16-C-0 and SPC-150, Becker & Hickl). The system recorded time-resolved fluorescence signals in 16 consecutive spectral bands from 300 to 500 nm at 13 nm intervals and provided the capability to simultaneously detect the TPEF signal in time and wavelength domains. The microscope imaging system produced lateral and axial resolutions of about 0.4 and 1.5 μm, respectively. The spectrum and image acquisition time was set to 8 s. An incomplete dual-exponential fitting model, $I = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + A_1\exp[-(t+\Delta\tau)/\tau_1] + A_2\exp[-(t+\Delta\tau)/\tau_2]$, for fluorescence lifetime analysis was utilized to analyze the decay curve for each image pixel after deconvolution, with system response by commercial software (SPC Image, Becker & Hickle GmbH). A_1 and A_2 denote the amplitudes of two decay terms, whereas τ_1 and τ_2 are the lifetime components, respectively. The lifetime detecting range of the TCSPC module for each excitation pulse $\Delta\tau$ was 12.5 ns, determined by the pulse repetition rate. The average fluorescence lifetime was calculated using the equation: $\tau_{avg} = A_1\tau_1 + A_2\tau_2$.

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