

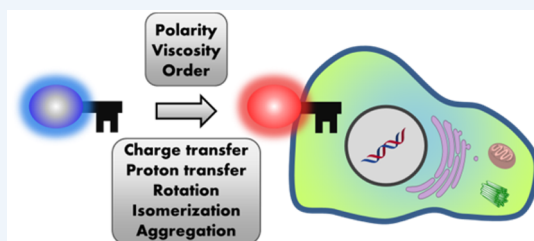
Solvatochromic and Fluorogenic Dyes as Environment-Sensitive Probes: Design and Biological Applications

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CONSPECTUS: Fluorescent environment-sensitive probes are specially designed dyes that change their fluorescence intensity (fluorogenic dyes) or color (e.g., solvatochromic dyes) in response to change in their microenvironment polarity, viscosity, and molecular order. The studies of the past decade, including those of our group, have shown that these molecules become universal tools in fluorescence sensing and imaging. In fact, any biomolecular interaction or change in biomolecular organization results in modification of the local microenvironment, which can be directly monitored by these types of probes. In this Account, the main

examples of environment-sensitive probes are summarized according to their design concepts. Solvatochromic dyes constitute a large class of environment-sensitive probes which change their color in response to polarity. Generally, they are *push–pull dyes undergoing intramolecular charge transfer*. Emission of their highly polarized excited state shifts to the red in more polar solvents. *Excited-state intramolecular proton transfer* is the second key concept to design efficient solvatochromic dyes, which respond to the microenvironment by changing relative intensity of the two emissive tautomeric forms. Due to their sensitivity to polarity and hydration, solvatochromic dyes have been successfully applied to biological membranes for studying lipid domains (rafts), apoptosis and endocytosis. As fluorescent labels, solvatochromic dyes can detect practically any type of biomolecular interactions, involving proteins, nucleic acids and biomembranes, because the binding event excludes local water molecules from the interaction site. On the other hand, fluorogenic probes usually exploit *intramolecular rotation* (conformation change) as a design concept, with *molecular rotors* being main representatives. These probes were particularly efficient for imaging viscosity and lipid order in biomembranes as well as to light up biomolecular targets, such as antibodies, aptamers and receptors. The emerging concepts to achieve fluorogenic response to the microenvironment include *ground-state isomerization*, *aggregation-caused quenching*, and *aggregation-induced emission*. The ground-state isomerization exploits, for instance, polarity-dependent spiro-lactone formation in silica-rhodamines. The aggregation-caused quenching uses disruption of the self-quenched dimers and nanoassemblies of dyes in less polar environments of lipid membranes and biomolecules. The aggregation-induced emission couples target recognition with formation of highly fluorescent dye aggregates. Overall, solvatochromic and fluorogenic probes enable background-free bioimaging in wash-free conditions as well as quantitative analysis when combined with advanced microscopy, such as fluorescence lifetime (FLIM) and ratiometric imaging. Further development of fluorescent environment-sensitive probes should address some remaining problems: (i) improving their optical properties, especially brightness, photostability, and far-red to near-infrared operating range; (ii) minimizing nonspecific interactions of the probes in biological systems; (iii) their adaptation for advanced microscopies, notably for superresolution and in vivo imaging.



1. INTRODUCTION

Fluorescence is one of the most popular among existing biosensing and bioimaging techniques, because it allows investigation of biological samples directly in situ and provides rich information at the biomolecular level with high spatial and temporal resolution. As the biological objects are poorly fluorescent, the current fluorescence techniques rely strongly on fluorescent dyes, proteins and nanoparticles. Fluorescent dyes are of particular importance because of their small size, possibility to finely tune their properties, and ease of chemical modification. Dyes that are specially designed to perform biosensing or bioimaging tasks are called fluorescent molecular probes. Some probes can specifically target biomolecules, cellular organelles, etc., whereas others enable detection and imaging of metal ions, pH, etc. In this Account, we focus on probes that change their emission characteristics in response to fundamental properties of molecular environment: polarity,

viscosity, and molecular order. These probes have become universal tools in fluorescence sensing and imaging, because any biomolecular interaction or change in biomolecular organization results in site-specific modification of these properties. Based on the response type, there are fluorogenic and chromogenic (e.g., solvatochromic) probes, which change their fluorescence intensity and color, respectively. In this Account, the main examples of the environment-sensitive probes and their applications are summarized according to mechanism of their operation (Figure 1). We should note that other types of fluorogenic and chromogenic probes, which respond to ions,¹ pH,² bioconjugation reactions,³ specific cellular organelles,⁴ etc.,⁵ have been well reviewed and they are out of the scope of this Account.

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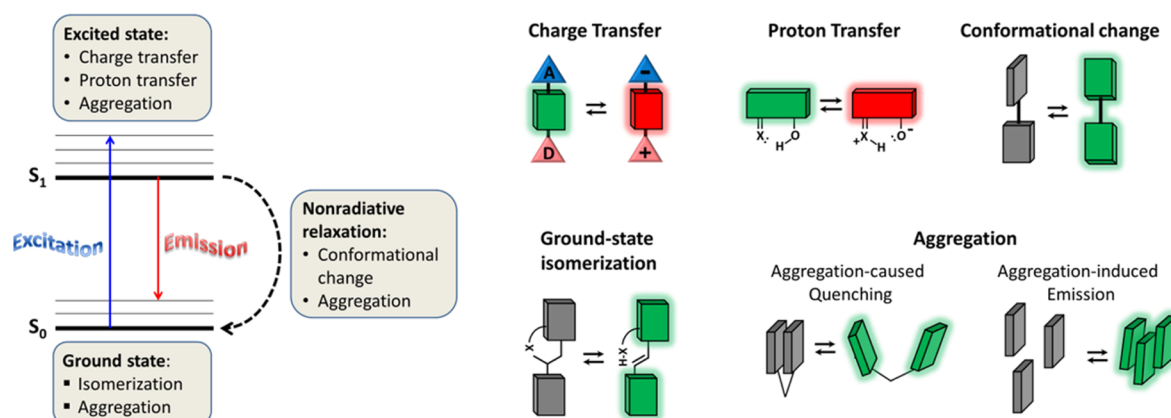


Figure 1. Simplified Jablonski diagram and the key mechanisms involved in the environment-sensitive probes.

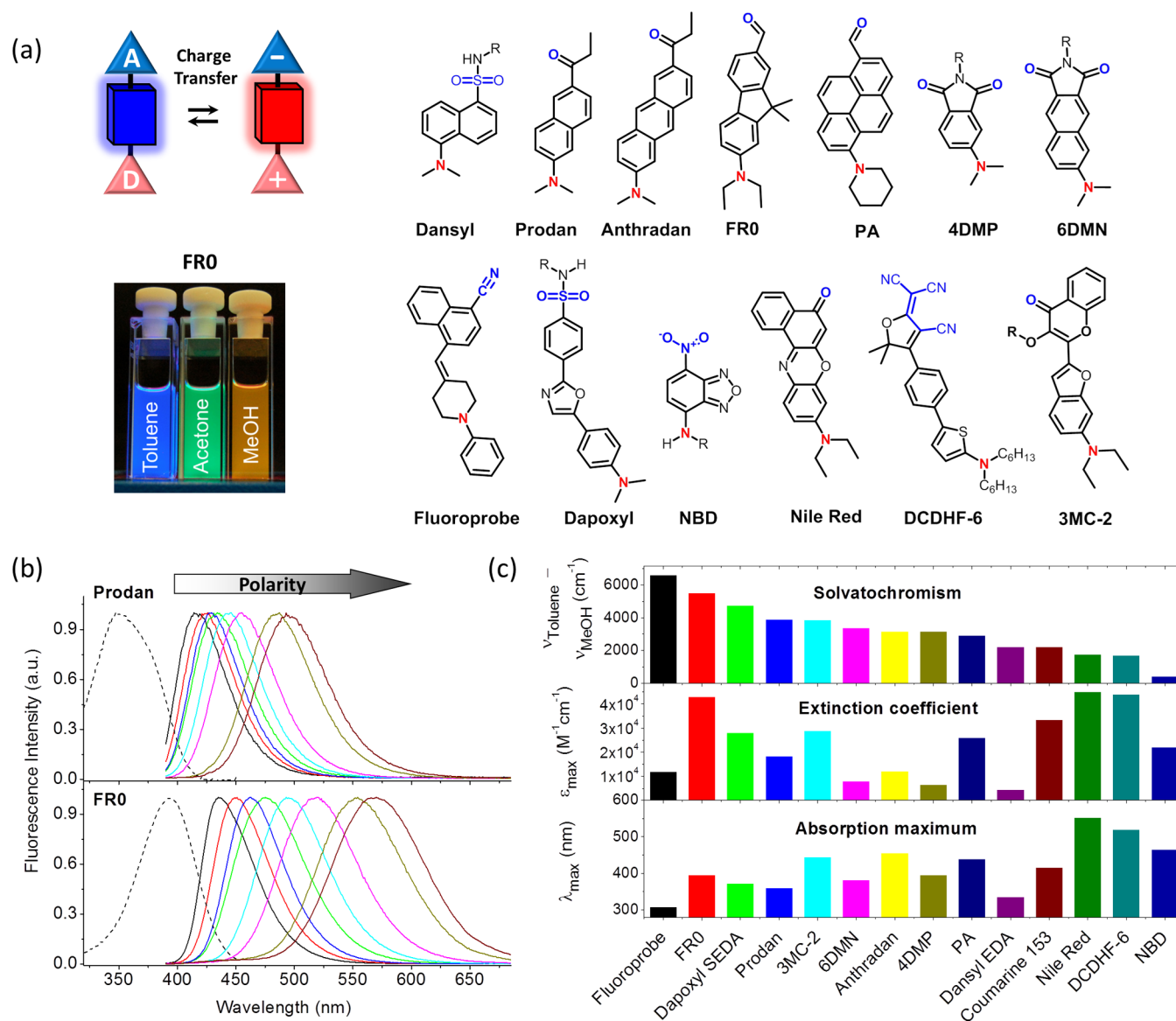


Figure 2. Push–pull solvatochromic dyes and comparison of their spectroscopic properties. (a) Concept and examples of dyes undergoing excited-state charge transfer. Photo of cuvettes of FR0 in solvents of different polarity. (b) Comparison of polarity-dependent band shifts of Prodan and FR0. Reproduced with permission from ref 6. Copyright 2010 American Chemical Society. (c) Comparison of spectroscopic properties of different solvatochromic fluorescent dyes. $\nu_{\text{Toluene}} - \nu_{\text{MeOH}}$ is the shift of the fluorescence maximum from toluene to methanol.

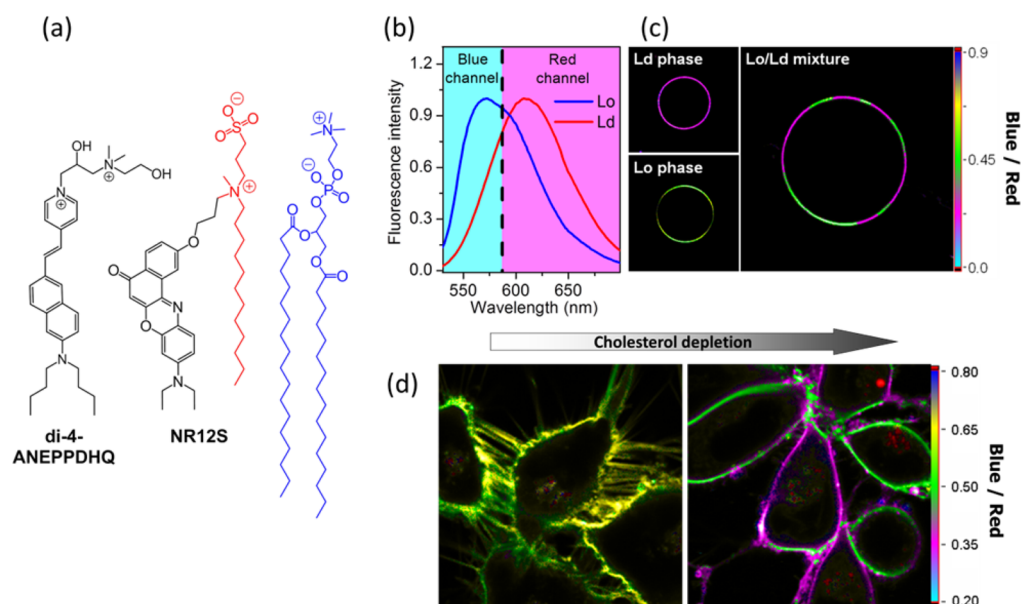


Figure 3. Applications of push–pull probe NR12S for probing lipid order in biomembranes. (a) Examples of leaflet-specific probes for lipid rafts. (b) Fluorescence spectra of NR12S in lipid vesicles presenting Lo and Ld phases. (c) Ratiometric images of giant vesicles presenting Lo and Ld phases and their mixture. (d) Decrease in the lipid order in cell membranes (U87MG human glioblastoma) on cholesterol depletion by methyl- β -cyclodextrin followed by ratiometric imaging with NR12S probe. Adapted with permission from ref 16. Copyright 2010 American Chemical Society.

2. PROBES BASED ON CHARGE TRANSFER

The classical environment-sensitive probes are solvatochromic dyes presenting donor and acceptor groups, so-called push–pull fluorophores. The push–pull structure ensures that after light absorption the charge is transferred from the donor group to the acceptor, which creates a highly dipolar excited state (Figure 2a). The latter relaxes through interaction with the dipoles of solvent and thus its emission shifts to longer wavelengths in more polar solvents (Figure 2a,b). A variety of the push–pull dyes are present in the literature, notably Prodan, Dansyl, 4DMP, NBD, Nile Red, Dapoxyl derivatives, etc. (Figure 2a). Until recently, the dyes with the strongest solvent-dependent red shifts were Fluoroprobe, Dapoxyl, and Prodan, all absorbing in the UV region (Figure 2c). In our previous work, we developed fluorene analogue of Prodan, FR0, featuring extended conjugation (Figure 2a).⁶ In addition to absorption shifted to 400 nm, the new dye showed higher extinction coefficient (43 000 vs 18 400 $\text{M}^{-1} \text{cm}^{-1}$), two-photon absorption cross section (400 vs 75 GM), quantum yield, and photostability. Moreover, FR0 exhibited improved sensitivity to polarity (spectral shift of 5500 cm^{-1} vs 3900 cm^{-1} of Prodan, Figure 1b,c) due to its high transition dipole moment (14 D). The use of pyrene as aromatic moiety also significantly improved properties with respect to Prodan, in particular quantum yield (~ 1.0)⁷ and photostability.⁸ Twieg et al. introduced the DCDHF family (Figure 2a),⁹ which is a rare example of solvatochromic dyes operating in the red region, although the sensitivity of these dyes to polarity remains close to that of Nile Red (Figure 2c). Recent works on push–pull dyes revealed two tendencies. First, photostability of solvatochromic dyes is relatively poor, especially in apolar aprotic solvents, which favor triplet state formation and thus photo-oxidation.^{6,8} Second, most of push–pull dyes are quenched in polar protic solvents, generally due to electron transfer and twisted intramolecular charge transfer (TICT).¹⁰

Due to sensitivity to polarity, push–pull dyes enable monitoring intrinsic biophysical properties of biomembranes,

such as polarity, hydration, electrostatics, etc.¹¹ They are particularly useful for imaging liquid ordered (Lo) and disordered (Ld) phase domains, which, according to hypothesis of lipid rafts,¹² are believed to play important role in organization and functions of biological membranes. Due to their small size and transient nature, lipid rafts are difficult to detect in living cells. Hence, development of advanced fluorescent probes distinguishing Lo and Ld phases is currently in progress.¹³ The Lo phase, tightly packed with saturated lipids and cholesterol, presents much lower hydration and polarity as compared to the loosely packed Ld phase, composed of unsaturated lipids.¹¹ Therefore, the solvatochromic dye Laurdan (a lipophilic analogue of Prodan) showed blue-shifted emission in the Lo phase compared to the Ld phase.¹⁴ In our recent work, a red-shifted analogue of Laurdan, push–pull pyrene PA (Figure 2a), showed similar color response to phase domains, whereas in living cells it revealed a gradual decrease in the lipid order from plasma membrane toward endoplasmic reticulum.⁸ To label specifically the outer plasma membrane leaflet, where the key component of the Lo phase, sphingomyelin, is mainly present, Loew et al. introduced push–pull probe di-4-ANEPPDHQ, bearing both alkyl chains and charged groups (Figure 3a).¹⁵ In our studies, we developed probe NR12S based on Nile Red, bearing dodecyl chain and zwitterion group (Figure 3a).¹⁶ Unlike Nile Red, NR12S binds exclusively the outer membrane leaflet of biomembranes with negligible flip-flop on the time scale of hours. NR12S enabled ratiometric imaging of Lo and Ld phase domains in model membranes (Figure 3b,c) and cholesterol depletion in cell membranes (Figure 3d).¹⁶ In comparison to di-4-ANEPPDHQ, NR12S requires much lower concentration for cell membrane imaging (20–50 nM vs 1–5 μM), although photostability of NR12S is limited.¹⁷ Numerous applications of NR12S include imaging maturation of endosomes,¹⁸ conformational changes of membrane proteins,¹⁹ internalization of nanostructures,²⁰ and detection of apoptosis.¹⁷ We should note that charge transfer dyes, being vertically oriented in biomembranes, can also sense

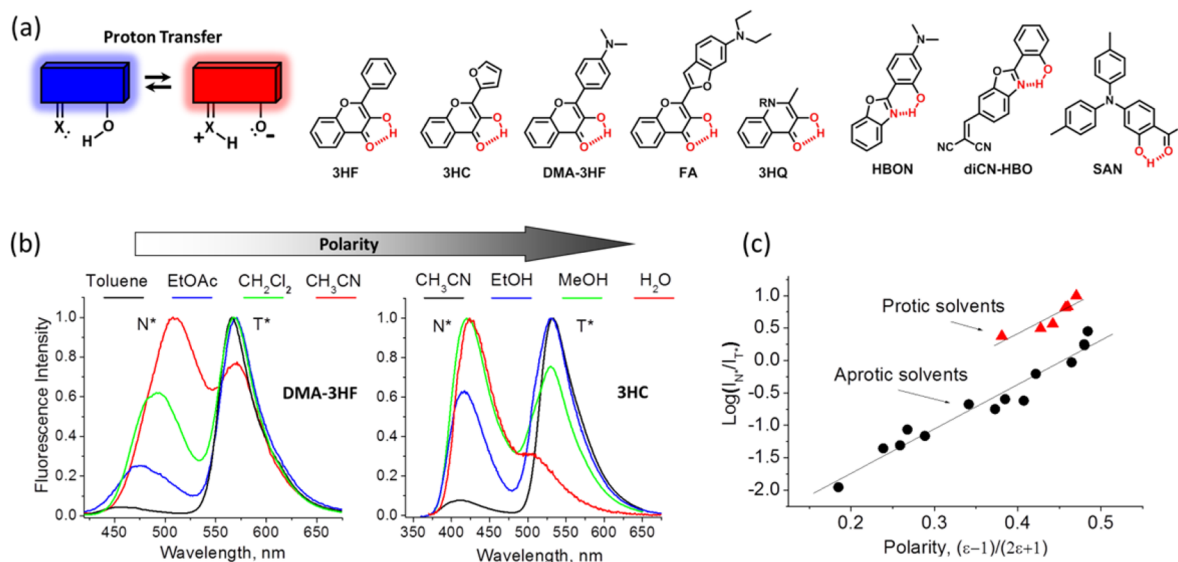


Figure 4. Fluorescent dyes undergoing ESIPT and displaying solvent-dependent dual emission (a). (b) Fluorescence spectra of 3-hydroxychromone dyes in solvents of different polarity. Adapted with permission from ref 22. Copyright 2013 Elsevier. (c) Dependence of dual emission from DMA-3HF on solvent polarity. Adapted with permission from ref 29. Copyright 2003 PCCP Owner Societies.

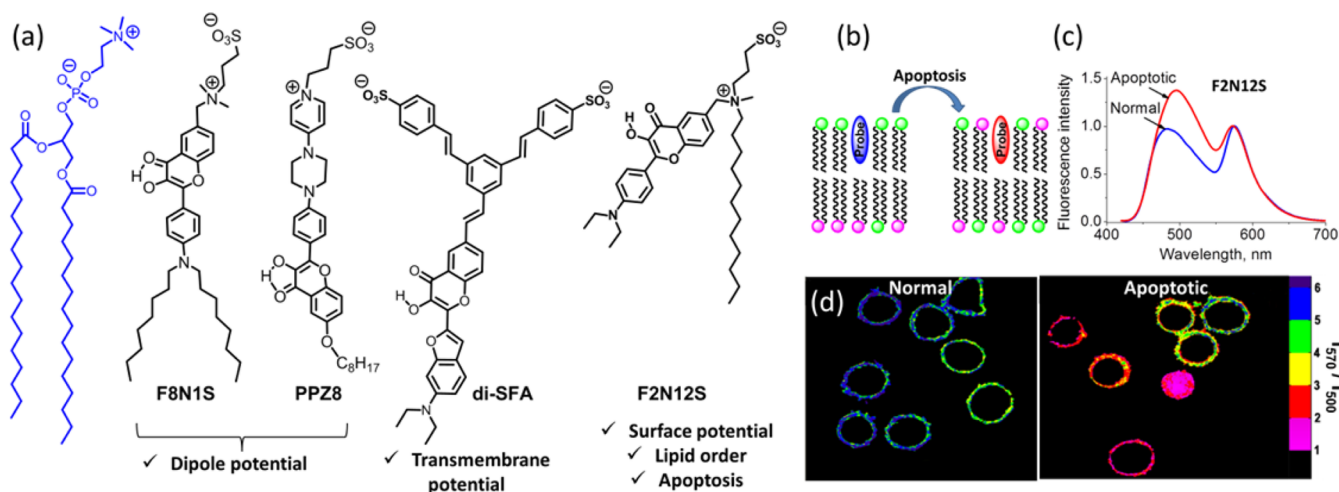


Figure 5. Application of ESIPT dyes based on 3-hydroxychromone for lipid membranes. (a) Membrane probes based on 3-hydroxychromone. (b–d) Application of F2N12S for apoptosis detection. Schematic presentation of probe in plasma membranes during apoptosis (b) and detection of apoptosis by F2N12S using fluorescence spectroscopy (c) and microscopy (d). Adapted with permission from ref 33. Copyright 2007 American Chemical Society.

electric fields, notably transmembrane potential, as shown by Loew et al. for styryl-pyridinium dyes,²¹ analogues of di-4-ANEPPDHQ.

The second important application of solvatochromic probes is detection of biomolecular interactions, as a powerful alternative to traditional methods based on Förster resonance energy transfer (FRET) and fluorescence anisotropy.²² In fact, interaction of biomolecular partners should basically result in the exclusion of the local water molecules, thus decreasing the environment polarity. The early examples of push–pull dyes used to monitor interactions of biomolecules included amino acid analogues of Prodan²³ and 4DMN,²⁴ operating in the ultraviolet–violet region. Using Nile Red conjugated to carbetocin through a polyethylene glycol (PEG) spacer, we obtained a turn-on probe for wash-free detection of G protein-coupled oxytocin receptor operating in the convenient red

spectral region.²⁵ PEG8 linker was important to prevent nonspecific interaction with serum and lipid membranes.

3. PROBES BASED ON INTRAMOLECULAR PROTON TRANSFER

Solvatochromic dyes can operate by excited-state intramolecular proton transfer (ESIPT) mechanism when their intermolecular H-bonding (ESIPT pathway) is weakened by rotational freedom and donor/acceptor substituents.²⁶ The typical examples are HBON, diCN-HBO and SAN, all presenting solvent-dependent dual emission.²⁶ Alternatively, efficient perturbation of H-bonding can be achieved in dyes with 5-membered H-bonding ring, such as 3-hydroxychromone family (3HC; Figure 4)^{11,26} and its aza-analogues, 3-hydroxyquinolones (3HQ).²⁷ Their emission is composed of two well-separated bands of normal (N*) and tautomeric (T*) states (Figure 4b). The ratio of intensities (N*/T*) increases

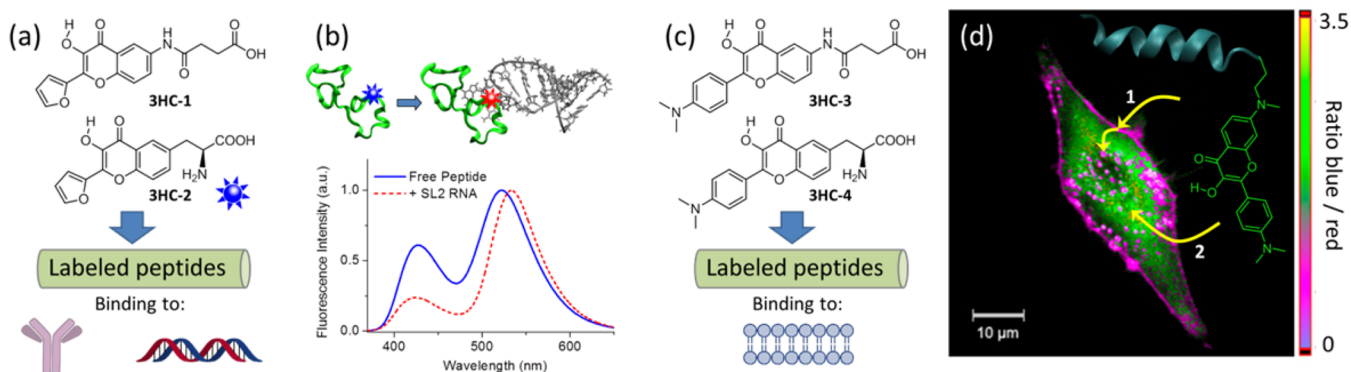


Figure 6. Sensing and imaging biomolecular interactions by ESIPT dyes of 3HC family. (a) Application of 3HCs for sensing interaction of labeled peptide with nucleic acids and antibodies. (b) Example of application of fluorescent amino acid (3HC-2) for detecting interaction of a peptide with a target oligonucleotide. (c) Application of 3HCs as probes for peptide-biomembrane interaction. (d) Ratiometric imaging of internalization of penetratin into HeLa cells using a 3HC label. Adapted with permission from ref 39. Copyright 2014 Royal Society of Chemistry.

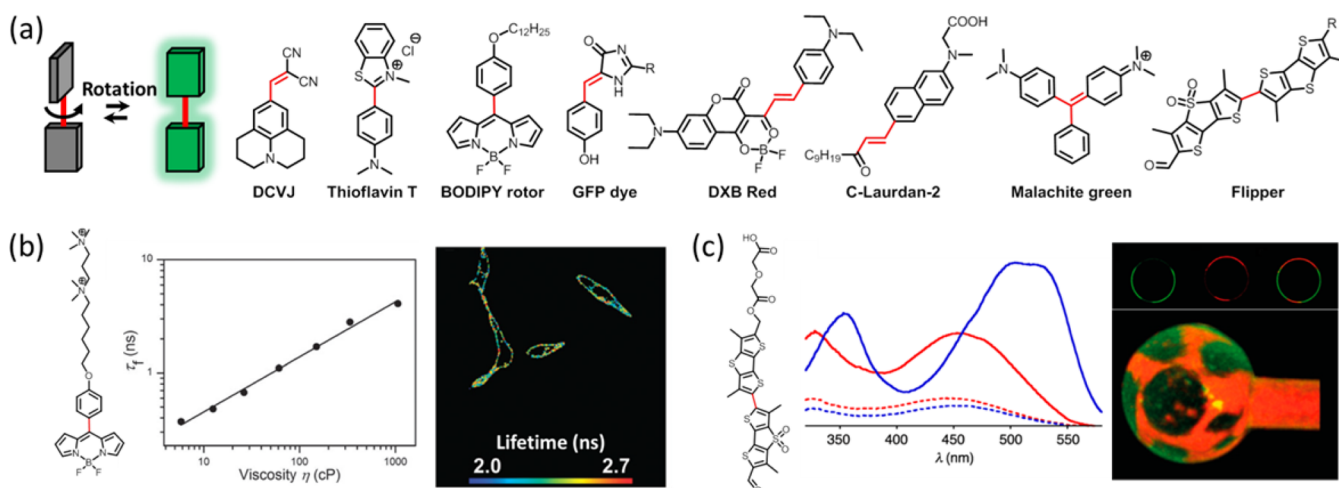


Figure 7. Probes operating by intramolecular rotation and their applications for membrane research. (a) Concept and examples of the probes. (b) Membrane probe based on BODIPY rotor (left), its fluorescence lifetime vs viscosity (middle) and FLIM image of SK-OV-3 cells incubated with this probe (right). Adapted with permission from ref 48. Copyright 2014 Royal Society of Chemistry. (c) Flipper probe (left), its excitation spectra (middle) in dipalmitoylphosphatidylcholine (DPPC) vesicles (solid) and dioleoylphosphocholine (DOPC) vesicles (dotted) at 25 °C (blue) and 55 °C (red). Confocal images of giant vesicles containing both Lo and Ld phases stained with flipper probe obtained by excitation at shorter (green vesicle, $\lambda_{\text{ex}} = 480$ nm) and longer (red vesicle, $\lambda_{\text{ex}} = 560$ nm) wavelength. Below that is a 3D z-stacked image of the same type of vesicle. Adapted with permission from ref 49. Copyright 2015 American Chemical Society.

with increase in the solvent polarity and H-bond donor ability, because polar and especially protic solvents inhibit ESIPT reaction (Figure 4c). Remarkably, polarity range, where solvent-dependent dual emission is observed, depends strongly on the substituents.²⁸ Thus, dyes 3HF and 3HC operate in highly polar environments between water and acetonitrile, whereas DMA-3HF and FA operate in solvents of medium polarity due to strong electron-donor groups enhancing dipole moment of the N* state (Figure 4b).²⁹

3HCs found a variety of applications as membrane probes. Dyes vertically oriented in the lipid bilayer are sensitive to dipole (F8N1S and PPZ8)³⁰ and transmembrane potentials (di-SFA),³¹ while those with tilted orientation are more suitable to sense lipid order and surface potential (Figure 5).^{11,32} In this respect, we should mention F2N12S, which due to specific staining of the outer plasma membrane leaflet, can detect changes in the lipid composition during programmed cell death (apoptosis).³³ The loss of asymmetry between the two leaflets produced by apoptosis increased the negative surface charge

and decreased the lipid order at the outer leaflet, which was detected by F2N12S as change in the dual emission (Figure 5).

Remarkably, 3HCs have become universal probes for almost any type of biomolecular interactions.²² Being grafted to a peptide, these dyes can detect the interactions with nucleic acids,³⁴ proteins,³⁵ and lipid membranes³⁶ (Figure 6). The interaction events in all these cases decreased local polarity, which systematically increased relative intensity of the ESIPT product. 3HC-based fluorescent amino acid (3HC-2) can replace natural ones to probe their insertion into DNA (Figure 6b).³⁴ Nucleotide analogues of 3HCs were applied for detection of DNA hybridization and interaction of chaperon proteins.³⁷ Being attached to α -synuclein, 3HCs detected the early steps of the peptide aggregation, involved in Parkinson's disease.³⁸ Recently, we applied a specially designed ESIPT dye to study a cell-penetrating peptide penetratin. Ratiometric imaging revealed that penetratin at low concentrations can rapidly enter the cytosol by directly crossing the cell plasma membrane, in addition to a classical endocytosis pathway (Figure 6d).³⁹

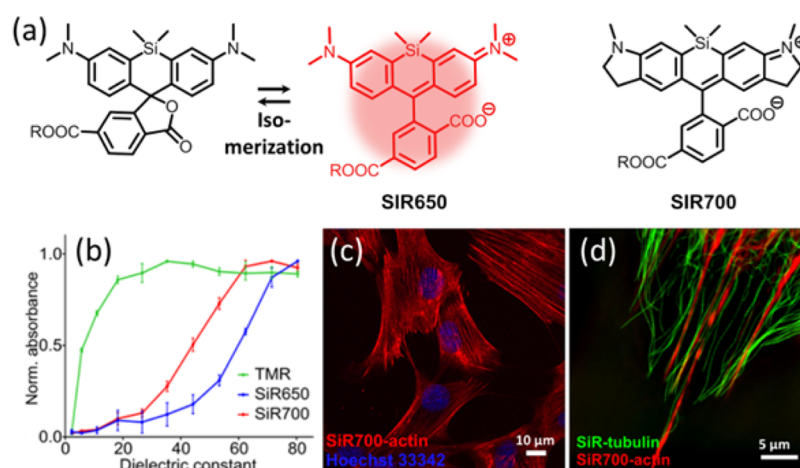


Figure 8. Probes based on intramolecular isomerization. (a) Concept and examples of the probes. (b) Sensitivity of zwitterion–spiro-lactone equilibrium of tetramethyl rhodamine (TMR), silica-rhodamines to the dielectric constant of solvent. (c) Confocal images of human primary fibroblasts stained with SiR700-actin probe (red) and Hoechst 33342 (blue). (d) Two-color SIM of human primary fibroblasts stained with SiR-tubulin (green) and SiR700-actin (red). Adapted with permission from ref 52. Copyright 2016 American Chemical Society.

4. PROBES BASED ON CONFORMATIONAL CHANGES

The internal rotation, disrupting the dye planarity, is a source of nonradiative relaxation (Figure 1), which is used in design of fluorogenic dyes, so-called molecular rotors. In a viscous environment, where the rotations are slowed down, fluorescence intensity and lifetime of these dyes are strongly enhanced. The classical examples are CCVJ,⁴⁰ BODIPY derivative,⁴¹ Thiofavin T,⁴² and green fluorescent protein (GFP) analogues (Figure 7a).^{43,44} Generally, molecular rotors are push–pull dyes, which undergo TICT in polar media, resulting in a poorly fluorescent state.¹⁰ Recently, we introduced a push–pull boron-bridged dye DXB Red,⁴⁵ which is highly emissive in apolar media, but quenched by TICT in polar solvents. In viscous polar solvent glycerol, its emission is restored, which is typical for molecular rotors. Symmetric nonplanar dyes, such as porphyrin dimer⁴⁶ and malachite green (Figure 7a),⁴⁷ can also function as molecular rotors.

Molecular rotors have found a variety of applications, especially for probing lipid membranes. One of the earliest examples is FCVJ, a hydrophobic analogue of DCVJ (Figure 7a) developed by Heidekker and Theodorakis, which was used to visualize shear forces in living cells.⁴⁰ Later on, Kuimova et al. proposed a BODIPY rotor (Figure 7a) for fluorescence lifetime imaging (FLIM) of membrane viscosity in living cells.⁴¹ Its analogue, bearing two cationic groups and long alkyl chain, enabled viscosity imaging specifically in plasma membranes (Figure 7b).⁴⁸ Molecular rotors can distinguish higher local viscosity in Lo phase compared to Ld phase, as shown for C-Laurdan-2 (Figure 7a).⁵⁰ The other application of molecular rotors is background-free imaging of biomolecules. Jaffrey et al. developed RNA aptamers that turn on fluorescence of GFP-derived fluorophore (GFP dye, Figure 7a),⁴³ which enabled intracellular RNA imaging. Bruchez et al. designed single-chain antibodies that turn on fluorescence of malachite green, and proposed them for intracellular protein labeling.⁴⁷ Recently, we developed a conjugate of DXB Red with carbetocin that turns on fluorescence on binding to the target oxytocin receptor, due to transfer of the fluorophore from water into low polar viscous environment. It enabled receptor imaging in wash-free conditions with high signal-to-background ratio (130).⁴⁵

A new class of probes based on conformation changes, so-called “flippers”, was recently introduced by Matile et al.⁴⁹ Due to steric hindrance, flippers present a twisted ground state featuring distorted conjugation between the large aromatic units (Figure 7a). In the rigid environment, such as ordered lipid membranes, the dyes become planar producing the red-shifted absorption. Their excitation spectrum distinguishes well between Lo and Ld phases, while the emission band remains invariant (Figure 7c). Using excitation at two wavelengths, these probes enabled two-color imaging of phase domains in giant vesicles. Sensitivity of flippers to the mechanical forces in the environment makes them different from solvatochromic dyes and molecular rotors.

5. PROBES BASED ON GROUND-STATE ISOMERIZATION

Ground-state isomerization that breaks the dye conjugation is another important mechanism to achieve fluorogenic response. It is particularly important in rhodamine dyes, where nucleophilic carboxylate group in the *ortho*-position of the side phenyl, can attack the electrophilic center of the heterocycle forming spiro-lactone (Figure 8). Importantly, formation of spiro-lactone rhodamines is a reversible process that depends on the solvent polarity, but it takes place only in apolar media. Recently, Johnsson and co-workers proposed to use silica-rhodamines (SiR), which undergo ring closing at much higher polarity (Figure 8).^{51,52} Their conjugates with ligands for tubulin, actin, SNAP tag, etc. displayed open fluorescent form only after binding to the target proteins. This fluorogenic behavior enabled multicolor background-free imaging of target proteins in wash-free conditions using conventional and structured illumination microscopy (SIM, Figure 8).⁵²

6. PROBES BASED ON AGGREGATION

Dyes are flat aromatic structures and, therefore, they have a strong tendency to π -stack into nonfluorescent H-aggregates.⁵³ In water, aggregation-caused quenching (ACQ) is favored by strong hydrophobic interactions, whereas an apolar environment can disassemble the aggregates generating a fluorogenic response. The probes can be based on either intramolecular or

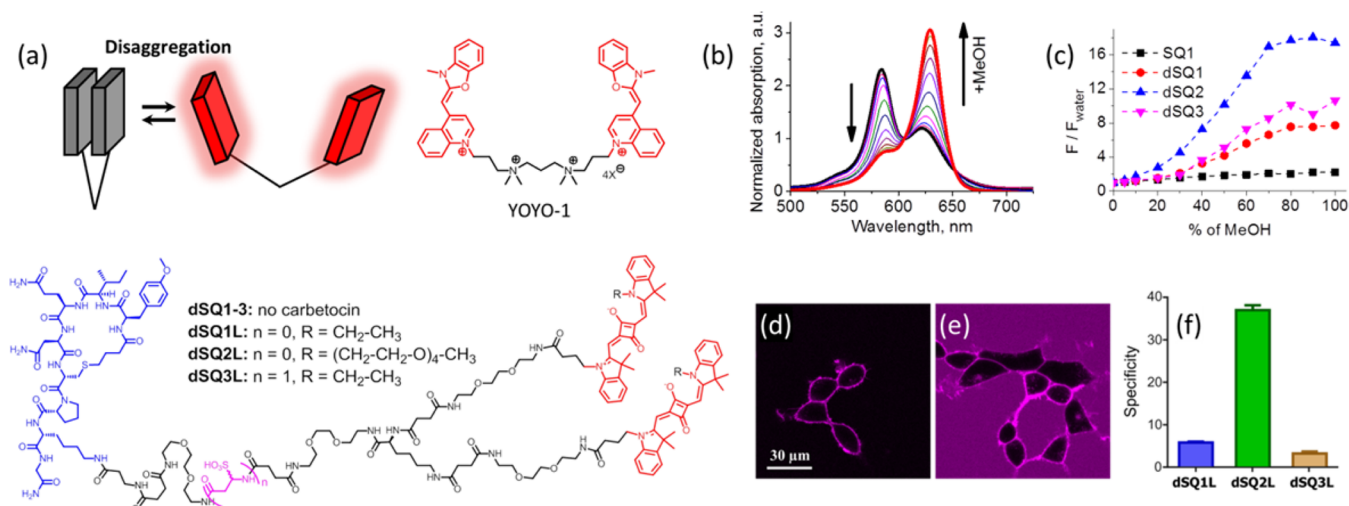


Figure 9. Probes operating by aggregation-caused quenching mechanism. (a) Principle and examples of these probes. (b) Absorption of squaraine dimer dSQ1 in water with addition of methanol (from 0 until 100%). (c) Fluorescence intensities (F/F_{water}) of dyes in H_2O –MeOH mixtures with respect to their intensity in water. (d,e) Confocal images of HEK cells expressing the oxytocin receptor with 100 nM of dSQ2L (d) or rhodamine-carbetocin conjugate (e). (f) Specificity of the probes (10 nM): intensity ratio before and after addition of nonlabeled carbetocin (2 μM). Reprinted with permission from ref 55. Copyright 2015 American Chemical Society.

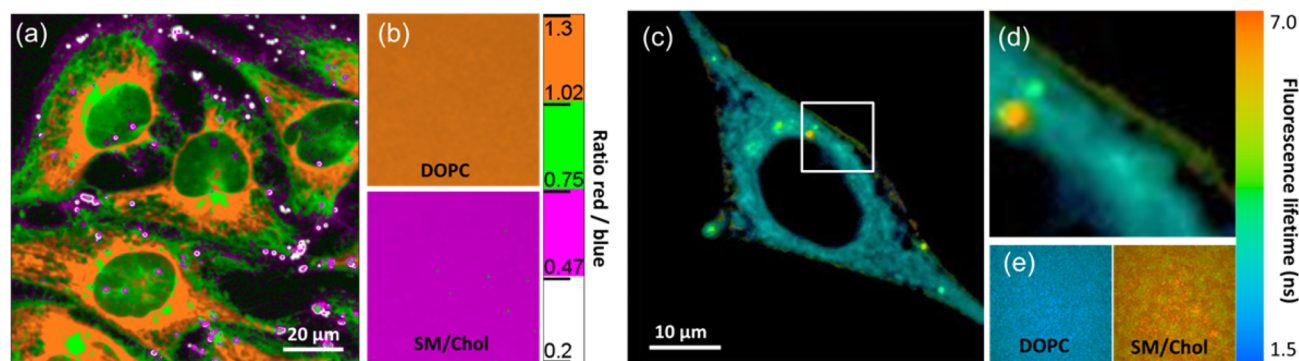


Figure 10. Ratiometric (a,b) and FLIM (c–e) imaging of lipid order in living HeLa cells using solvatochromic probe PA. The pseudocolors represent the ratio of the long- to short-wavelength emission channels (550–700 to 470–550 nm). (b) Calibration of the ratio using suspension of lipid vesicles composed of DOPC (Ld phase) and sphingomyelin/cholesterol (SM/Cho, Lo phase). (c) FLIM image of HeLa cells and (d) zoom on the region of interest. Calibration images of suspensions of DOPC and SM/Chol (e) vesicles recorded with the same instrumental settings. Adapted with permission from ref 8. Copyright 2016 Nature Publishing Group.

intermolecular aggregates. The typical example of the former is commercial probe YOYO-1 (Figure 9a). This dimeric dye is nonfluorescent in water due to H-aggregation, but it opens up on binding to DNA producing fluorescence turn-on. This concept has been applied for sensing interactions with nucleic acids.⁵⁴ Recently, we extended it to polarity sensing. The dimers of squaraine dyes formed poorly fluorescent H-aggregate with characteristic sharp short-wavelength absorption band (Figure 9b).⁵⁵ Decrease in the polarity produced unfolding of the dimer and thus increase in the fluorescence intensity, especially for dimer with additional PEG groups (Figure 9c). This design generated polarity-sensitive fluorogenic dyes with unprecedented brightness due to high extinction coefficient ($\epsilon \sim 700\,000\text{ M}^{-1}\text{ cm}^{-1}$) and quantum yield (~ 0.5). Its conjugate with carbetocin ligand showed turn-on response to the target oxytocin receptor in living cells (Figure 9d), and much lower background in comparison to a nonfluorogenic rhodamine-based ligand (Figure 9e). Moreover, the highest specificity was recorded for the derivative bearing additional PEG groups (Figure 9f). Recently, another group

followed this design concept to further shift the emission to near-infrared for in vivo imaging.⁵⁶

An alternative probe design is to exploit disassembly of intermolecular dyes aggregates in water. Hamachi et al. proposed to aggregate a variety of dyes, such as rhodamine, fluorescein, and BODIPY, using a special hydrophobic linker and targeting ligand.⁵⁷ These aggregates can disassemble on binding to a receptor, producing a fluorogenic response. In our studies, amphiphilic probe NR12S displayed much lower quantum yield in water (0.002) compared to parent Nile Red (0.05) with characteristic blue-shifted absorption, suggesting formation of nonfluorescent micellar H-aggregates.¹⁶ On binding to lipid membranes, these aggregates disassembled into molecular species exhibiting high quantum yield (~ 0.5). Then, we synthesized an analogue of NR12S that was polymerized with cross-linker bearing S–S bond.⁵⁸ The obtained polymerized micelles were nonfluorescent in aqueous media, but disassembled under reductive stimuli producing desired fluorescence turn-on. Thus, we obtained a fluorogenic membrane probe activatable by a reductive stimulus. Later on, we extended the ACQ approach to squaraine-based probe

dSQ12S, displaying >100-fold fluorescence turn-on on binding to lipid membranes.⁵⁹ Due to high brightness ($\epsilon \sim 350\,000\text{ M}^{-1}\text{ cm}^{-1}$ and quantum yield ~ 0.5) and efficient membrane staining, it enabled imaging cell plasma membranes at 1 nM concentration, which is ~ 1000 -fold lower than needed for conventional membrane probe DiD.

The aggregation of dyes does not always lead to ACQ. It can also produce fluorescence enhancement, especially for molecular rotors of propeller-like topology (e.g., tetraphenylethene, hexaphenylsilole, etc.). This phenomenon, so-called aggregation-induced emission (AIE), has been a subject of intensive research in the past decade.⁶⁰ The design of environment-sensitive probes for biosensing based on AIE concept emerged in the last years, with applications to detection of proteins, nucleic acids, polysaccharides, etc.⁶¹

7. QUANTITATIVE AND BACKGROUND-FREE BIOIMAGING WITH ENVIRONMENT-SENSITIVE PROBES

Fluorescence imaging is usually based on analysis of fluorescence intensity, which depends on many factors, such as probe concentration, light source power, sensitivity of the detector, sampling time, etc. Therefore, quantitative fluorescence microscopy uses FLIM and ratiometric imaging, independent of the experimental conditions mentioned above. FLIM and ratiometric imaging require different types of fluorescent probes. Solvatochromic push–pull and ESIPT dyes are perfectly suited to ratiometric imaging, because they change their emission color that can be directly quantified as the ratio at the two channels (Figure 10a,b).⁸ On the other hand, fluorogenic probes, especially molecular rotors, are more appropriate for lifetime imaging.⁴¹ Nevertheless, many solvatochromic dyes can change their lifetime in response to polarity and thus can be used for FLIM. Moreover, as we showed for PA probe, FLIM at the blue edge of the emission band can be used even for solvatochromic dyes that do not change much their lifetime (Figure 10c–e).⁸

The second aspect is the background-free imaging. The fluorogenic dyes can significantly decrease the background signal, especially at high probe concentrations. Our comparative study of turn-on probe with a rhodamine derivative suggested that fluorogenic response becomes important starting from 100 nM ligand concentration (Figure 9d,e).^{25,55} Fluorogenic response is also crucial for specific labeling of biomolecules inside the cells,^{51,52} because the fluorescent label, being used in excess, can be a source of strong background. Moreover, fluorogenic response can improve imaging contrast in different superresolution techniques, including stimulated emission depletion (STED), SIM, and stochastic optical reconstruction microscopy (STORM).⁶² Finally, point accumulation for imaging in nanoscale topography (PAINT) exploits directly fluorogenic and solvatochromic response of dyes (e.g., Nile Red) to obtain superresolution images and decipher the coexistence of different probe environments (e.g., lipid domains).⁶³

8. CONCLUSIONS AND OUTLOOK

Due to sensitivity to fundamental properties of the environment, such as polarity, viscosity, and molecular order, fluorogenic and solvatochromic probes can help understanding properties of lipid membranes, monitoring interactions of biomolecules, imaging target biomolecules directly in living

cells with minimal background. Further development of environment-sensitive probes should address some remaining problems. First, nonspecific interactions should be minimized to prevent false-positive response of these probes, which can be achieved by, for example, PEGylation or the use of highly specific water-soluble ligands.^{25,55} Second, fluorescence brightness and photostability of push–pull, ESIPT, and molecular rotor dyes are generally inferior compared to those of classical dyes, such as cyanines, rhodamines, and BODIPYs. Recent examples, using a large pyrene moiety⁸ and boron-based bridge⁴⁵ in the push–pull dyes, provide some clues to address these issues. Moreover, emerging in vivo applications will require further shifting of the absorption and emission wavelengths of these dyes to NIR region. In the case of ACQ-based dyes, it could be realized using conventional NIR dyes, whereas for others, a dedicated design of fluorophores with extended conjugation will be required. The use of fluorogenic and solvatochromic probes in FLIM and ratiometric imaging can already provide quantitative information. Moreover, the capacity of fluorogenic probes to increase the signal-to-background ratio is a key to enhance rapidly developing superresolution microscopy. Many new biological applications remain to be discovered for the environment-sensitive dyes, and we expect them to become universal tools in the development of next generation of biosensors and probes that can enhance quality and information content of bioimaging.

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The author declares no competing financial interest.

Biography

Andrey Klymchenko was born in Kherson, Ukraine, in 1976. He received his Ph.D. degree in 2003 from Kiev Taras Shevchenko University. After postdoctoral work at the University of Strasbourg and the Catholic University of Leuven, he joined CNRS at the University of Strasbourg in 2006. He got promoted to CNRS Research Director in 2014, and he is an ERC Consolidator fellow since 2015. He is a leader of “Nanochemistry and Bioimaging” group. His research interests include functional fluorescent molecules and nanomaterials for biosensing, imaging and theranostics.

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