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# Fluorescent probes for lipid membranes: from cell surface to organelles

Andrey S. Klymchenko\*

Laboratoire de Bioimagerie et Pathologies, UMR 7021 CNRS, Faculté de Pharmacie, Université de Strasbourg, 67401 Illkirch, France

## CONSPECTUS

Biomembranes are ubiquitous lipid structures that delimit cell surface and organelles and operate as platforms for multitude of biomolecular processes. Development of chemical tools for sensing and imaging of biomembranes, fluorescent probes, is a rapidly growing research direction, stimulated by a high demand from cell biologists and biophysicists. This Account focuses on advances in these smart molecules, providing a voyage from the cell frontier – plasma membranes (PM), towards intracellular membrane compartments – organelles. General classification of the membrane probes can be based on *targeting principles*, *sensing profile* and *optical response*. Probes for PM and organelle membranes are designed based on multiple targeting principles: conjugation with *natural lipids* or *synthetic targeting ligands*, and *in situ cell labelling by bio-orthogonal chemistry*, *conjugation to protein tags* and *receptor-ligand interactions*. Thus, to obtain membrane probes targeting PM with selectivity to one leaflet, we designed *membrane anchor* ligands based on a charged group and an alkyl chain. According to the sensing profile, we define *basic membrane markers* with constant emission and *probes for biophysical and chemical sensing*. The markers are built from *classical fluorophores*, exemplified by a series of bright cyanines and BODIPY dyes bearing the PM anchors (MemBright). Membrane probes for the biophysical sensing are based on *environment-sensitive fluorophores*: (1) polarity-sensitive *solvatochromic dyes*; (2) viscosity-sensitive *fluorescent molecular rotors*; (3) mechano-sensitive *fluorescent flippers*; (4) voltage-sensitive *electrochromic dyes*. Our solvatochromic probes based on Nile Red (NR12S, NR12A, NR4A), Laurdan (Pro12A), and 3-hydroxyflavone (F2N12S) through polarity-sensing can visualize liquid ordered and disordered phases of lipid membranes, sense lipid order and its heterogeneity in cell PM, detect apoptosis, etc. *Chemically sensitive probes*, combining a dye, membrane targeting ligand and molecular recognition unit, enable detection of pH, ions, redox species, lipids and proteins at the biomembrane surface. In terms of the optical response profile, we can identify (1) *fluorogenic (turn-on) probes*, allowing background-free imaging; (2) *ratiometric probes*, e.g. solvatochromic probes, which enable ratiometric imaging by changing their emission/excitation color; (3) *fluorescence lifetime-responsive probes*, e.g. fluorescence molecular rotors and flippers, suitable for fluorescence lifetime imaging (FLIM); (4) *switchable probes*, important for single-molecule localization microscopy. We showed that combining solvatochromic probes with on-off switching through a reversible binding specifically to cell PM enables mapping their biophysical properties with superior resolution. While the majority of efforts have been focused on PM, the probes for cellular organelles, such as endoplasmic reticulum, mitochondria, Golgi Apparatus, etc, emerge rapidly. Thus, non-targeted solvatochromic probes can distinguish organelles by the emission color. Targeted solvatochromic probes based on Nile Red revealed unique signatures of polarity and lipid order of individual organelles and their different sensitivity to oxidative or mechanical stress. Lipid droplets, which are membraneless lipidic structures, constitute another interesting organelle target for probing the cell stress. Currently, we stand in front of long and broad way with big challenges ahead, in particular (1) to achieve superior organelle specificity; (2) to label specific biomembrane leaflet, notably the inner leaflet of PM; (3) to detect lipid organization in a proximity of specific proteins; (4) to probe biomembranes in tissues and animals.

## KEY REFERENCES

- Danylchuk, D. I.; Jouard, P. H.; Klymchenko, A. S. Targeted Solvatochromic Fluorescent Probes for Imaging Lipid Order in Organelles under Oxidative and Mechanical Stress. *J. Am. Chem. Soc.* **2021**, *143*, 912-924.<sup>1</sup>

*A series of organelle-targeting fluorescent probes based on solvatochromic Nile Red dye were synthesized. They enabled ratiometric imaging of local polarity and lipid order in specific subcellular compartments under varied cholesterol, oxidative and mechanical (osmotic) stress.*

- Danylchuk, D. I.; Moon, S.; Xu, K.; Klymchenko, A. S. Switchable Solvatochromic Probes for Live-Cell Super-resolution Imaging of PM Organization. *Angew. Chem. Int. Ed. Engl.* **2019**, *58*, 14920-14924.<sup>2</sup>  
*Anionic membrane-targeting anchors were proposed for designing solvatochromic probes with controlled membrane binding/unbinding-induced on/off switching for the super-resolution microscopy. The probes enabled nanoscale cartography of the lipid organization at the cell plasma membranes.*

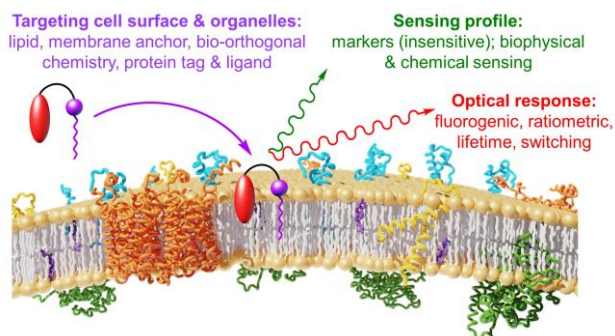
- Collot, M.; Ashokkumar, P.; Anton, H.; Boutant, E.; Faklaris, O.; Galli, T.; Mély, Y.; Danglot, L.; Klymchenko, A. S. MemBright: A Family of Fluorescent Membrane Probes for Advanced Cellular Imaging and Neuroscience. *Cell. Chem. Biol.* **2019**, *26*, 600-614.e607.<sup>3</sup>

*A family of bright fluorescent probes for cell plasma membranes (MemBright) was designed based on cyanine dyes with two zwitterionic anchors. The probes enabled imaging cancer cell lines, primary neurons and tissues using conventional, two-photon excitation and super-resolution microscopy.*

- Ashokkumar, P.; Ashoka, A. H.; Collot, M.; Das, A.; Klymchenko, A. S. A fluorogenic BODIPY molecular rotor as an apoptosis marker. *Chem. Commun.* **2019**, *55*, 6902.<sup>4</sup>

*A fluorescent BODIPY molecular rotor was combined with a targeting ligand for specific recognition of phosphatidylserine lipid. This design yielded the fluorogenic (turn-on) probe for detection of apoptosis on the cell surface.*

## TOC GRAPHIC



## 1. INTRODUCTION

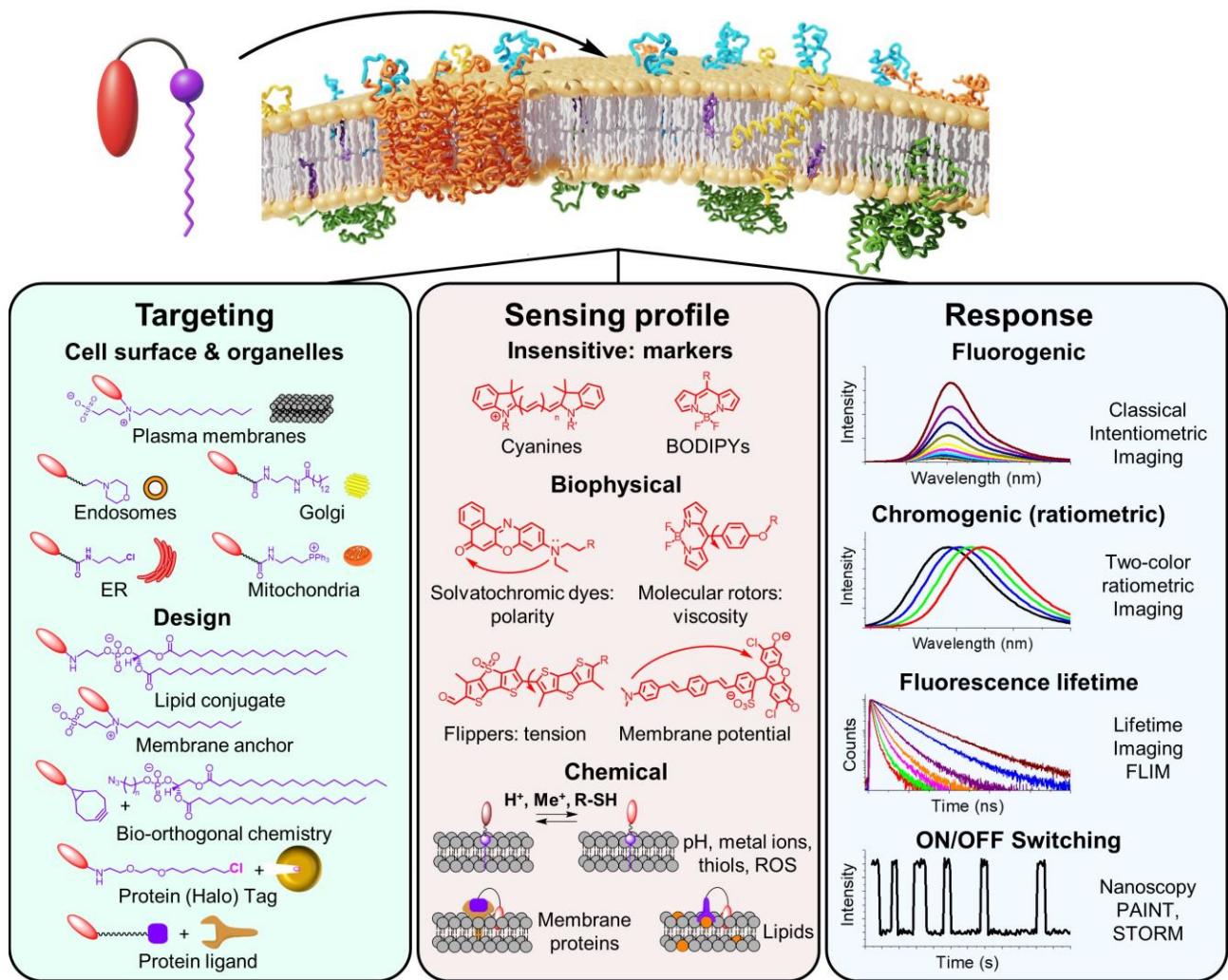
Biomembranes are ubiquitous lipid structures in living cells. They constitute cell plasma membranes (PM) and they delimit majority of cell organelles. Sensing and imaging of biomembranes reveal remarkable heterogeneity in their lipid organization. Thus, majority of sphingomyelin and cholesterol are present at the PM, where they preferentially localize at the outer leaflet and constitute microdomains responsible for the plethora of cell functions.<sup>5,6</sup> Development of chemical tools for biomembranes is an important research direction, stimulated by a high demand from cell biologists and biophysicists. In particular, it concerns fluorescent probes, because of high sensitivity of fluorescence techniques, boosted by remarkable developments in fluorescence microscopy.

The great multitude of developed fluorescent membrane probes urged us to classify and further organize them in this Account based on their *targeting principles*, *sensing profile* and *optical response* (Figure 1). In terms of *the targeting*, there are probes for PM and organelles. The cell PM is probably the most explored target, whereas the intracellular membranes of organelles, e.g. endoplasmic reticulum (ER), Golgi Apparatus, mitochondria, etc are emerging targets.<sup>7</sup> As biomembranes are lipid bilayers with different inner or outer membranes leaflets, the leaflet-specific targeting should be considered.<sup>8</sup> Lipid droplets (LDs) stand apart, because they are membraneless lipid-protein structures with core-shell organization. The design principles of probes for biomembranes are based on their interaction or reaction with lipids or membrane proteins (Figure 1). To target the lipid part, fluorescent dyes are conjugated to natural lipids or synthetic membrane targeting ligands (anchors). Other methods include *in situ* bio-orthogonal chemistry, grafting to protein Tags (e.g. HaloTag) or non-covalent receptor-ligand interaction. Within the lipid tools, we introduced a membrane anchor based on dodecyl chain and zwitterion (Figure 1), which was found to be universal for localizing different fluorophores at the PM: 3-hydroxychromones,<sup>9</sup> Nile Red,<sup>10</sup> squaraine,<sup>11</sup> cyanines,<sup>3</sup> BODIPY, etc.<sup>12</sup> Then, we replaced the zwitterionic group with an anionic sulfonate, affording Nile Red<sup>2</sup> and Prodan<sup>13</sup> derivatives characterized with highly efficient membrane staining. These anchors ensure specific staining of the outer membrane leaflet.<sup>10,13</sup>

Regarding *sensing profile*, we identify basic membrane markers with constant emission and probes for biophysical and chemical sensing (Figure 1). The basic membrane markers are built from classical fluorophores insensitive to the environment and used exclusively for imaging of biomembranes. Membrane probes for the biophysical sensing are based on environment-sensitive fluorophores, which change their properties, such as emission color, fluorescence intensity or fluorescence lifetime, as a function of environment polarity, viscosity, etc. Our group contributed to solvatochromic membrane probes, which detect local polarity and lipid order.<sup>14</sup> Other groups developed molecular rotors for monitoring local viscosity<sup>15,16</sup> and flippers to address the lipid order and membrane tension.<sup>17,18</sup> The chemically sensitive probes detect at the biomembranes local chemical species, such as protons (pH), ions, redox species, as well as biomolecules, like lipids and proteins.

Finally, the membrane probes can be classified by their *response profile*. Fluorogenic probes, which light up their fluorescence, are used with conventional microscopy techniques, allowing background-free bioimaging. Ratiometric probes respond by changing their emission/excitation color, with typical example of solvatochromic probes, extensively explored by us. Under microscope, their emission is split into blue and red parts and further analyzed as a ratio or as a “generalized polarization” (GP) parameter. Then, fluorescence lifetime-responsive probes, such as fluorescence molecular rotors and flippers, are compatible with fluorescence lifetime imaging (FLIM). Finally, probes undergoing fluorescence blinking are suitable for single-molecule localization microscopy (SMLM). One should identify SMLM probes operating by reversible binding to biomembranes (PAINT) and those that undergo photo-switching (STORM).

In this Account, we focus on our advances in the fluorescent probes for biomembranes and key examples from other groups, where we propose a voyage from the cell frontier – plasma membrane, towards intracellular membrane compartments – organelles.

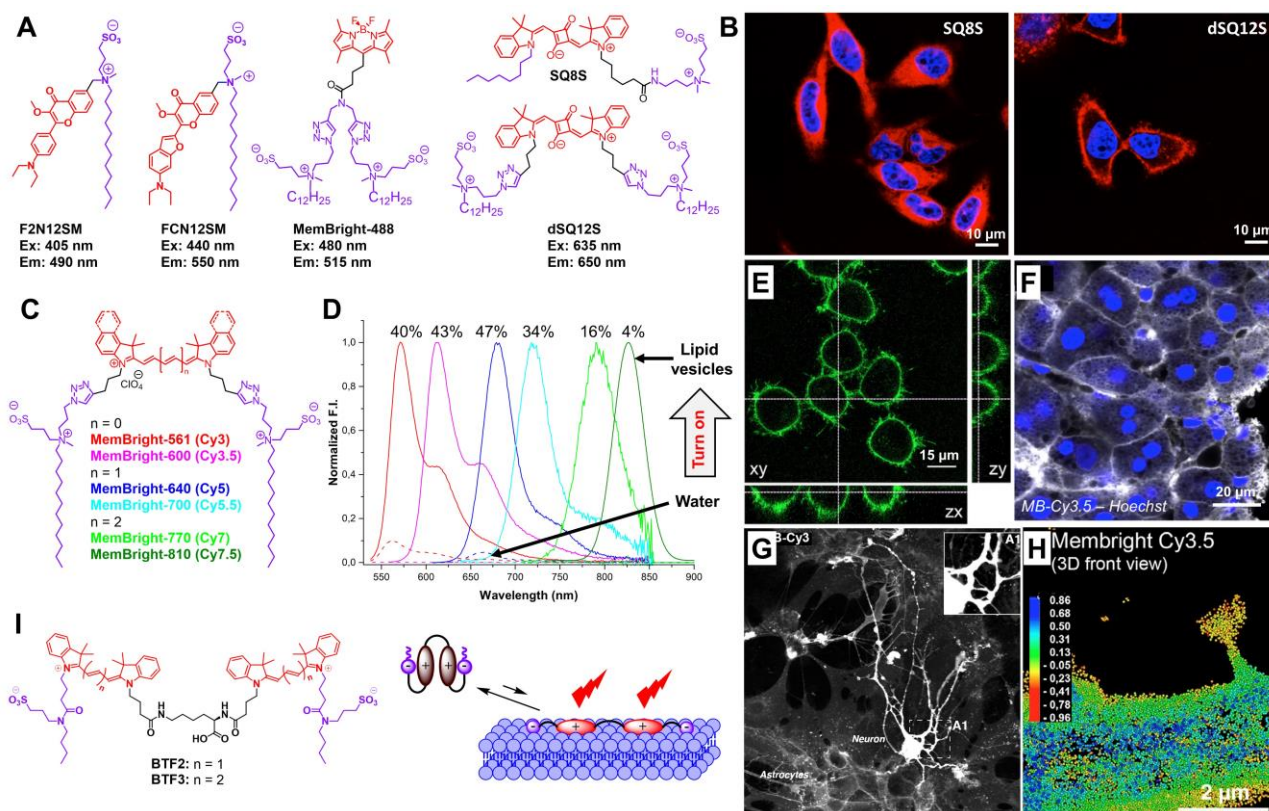


**Figure 1.** Generic scheme of membrane probes classification.

## 2. BRIGHT PM MARKERS

Frequently, cell biologists require robust tools to simply label cell PM. A traditional tool is fluorescently labelled wheat germ agglutinin (WGA), which targets membrane glycoproteins. However, its large size does not always allow efficient staining of cells and tissues. Early examples of small-molecule probes are lipophilic cyanine derivatives, like DiI, DiD, PKH26, etc. They generally suffer from poor water solubility and, therefore, require excessive concentrations ( $\sim 5 \mu\text{M}$  vs  $50 \text{ nM}$  of WGA) and provide heterogeneous labelling. To address these problems, we exploited our zwitterionic anchor concept, originally developed for solvatochromic probe F2N12S,<sup>9</sup> for different bright fluorophores. First, we synthesized a methylated analogue of F2N12S, F2N12SM (Figure 2A), excitable with a common violet light source and emitting with high quantum yield and Stokes shift.<sup>19</sup> It showed much brighter membrane staining than F2N12S, allowing imaging at  $20\text{-}50 \text{ nM}$  concentration.<sup>19</sup> F2N12SM was found compatible with green and red fluorescent proteins, thus occupying the available blue channel, like DAPI for the nucleus imaging. Next, we selected a far-red squaraine dye, characterized by high extinction coefficient and quantum yield. Surprisingly, squaraine probe SQ8S bearing a zwitterionic group on one end and an alkyl chain on the other (Figure 2A) displayed effective internalization with ER labelling (Figure 2B).<sup>11</sup> In contrast, probe diSQ12S bearing two zwitterionic anchors on each end of the squaraine showed excellent membrane staining (Figure 2B), superior to the analogue with the single anchor.<sup>11</sup> diSQ12S displayed high-quality staining at concentrations as low as  $1 \text{ nM}$ , while commercial marker

Vybrant™ DiD even at 2  $\mu\text{M}$  and CellVue® Claret at 20 nM showed heterogeneous labelling. We further explored this membrane targeting strategy using green dye BODIPY, which was functionalized with one, two and three zwitterionic membrane anchors.<sup>12</sup> The BODIPY derivative with two anchors (MemBright-488, Figure 2A) showed the brightest PM labelling, in line with the squaraine work. To access other colors, we applied the design concept with two zwitterionic anchors to cyanine dyes (Figure 2C). The obtained probes showed a fluorescence light up on binding to lipid membranes and high selectivity to PM, allowing high-quality imaging at 20 nM concentrations (Figure 2D,E). They were compatible with multiple imaging modalities, including two-photon excitation imaging of tissues (Figure 2F). In primary neuronal culture, they showed some selectivity to neurons vs astrocytes (Figure 2G), allowing long-term neuron imaging. Membright-Cy3.5 displayed on/off switching behavior, which enabled SMLM super-resolution imaging of synapses (Figure 2H). Thus, we developed a pallet of bright PM markers operating from blue to near-infrared region. The BODIPY and cyanine analogues are named MemBright and nowadays commercialized. Recently, we reported fluorogenic cyanine dimers bearing two anionic membrane anchors (“butterfly” BTF probes), which are non-fluorescent in water due to intramolecular H-aggregation, but undergo ON/OFF switching on reversible PM binding (Figure 2I).<sup>20</sup> Their high brightness and slow lateral diffusion enhanced performance of 3D PAINT imaging of PM in live cells. Works of other groups on bright PM markers include dyes undergoing aggregation-induced emission,<sup>21,22</sup> and fluorescent chitosan functionalized with cholesterol and biotin.<sup>23</sup>

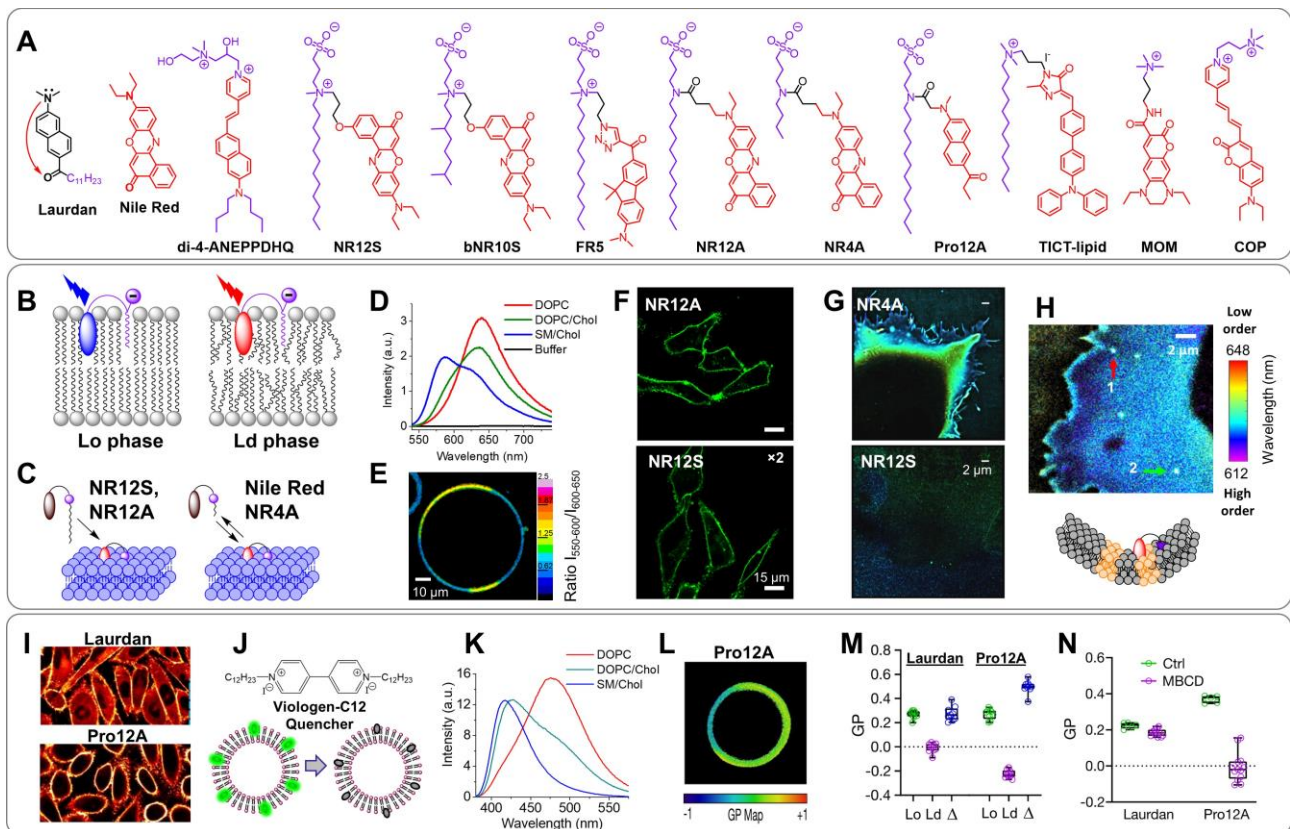


**Figure 2.** Bright PM markers based on zwitterionic membrane anchor. (A,C) Examples of bright membrane markers. Excitation and emission wavelengths are indicated in (A) panel. (B) Confocal microscopy images of HeLa cells incubated with 1 nM of SQ8S and dSQ12S (red). Nucleus was stained with Hoechst (blue). Adapted with permission from ref <sup>11</sup>. Copyright 2015 Royal Society of Chemistry. (C) MemBright probes and (D) their emission spectra in water or with lipid vesicles. Fluorescence quantum yields are shown in %. (E) Confocal microscopy of live KB cells stained with MemBright-Cy7 (20 nM) with orthogonal projections obtained from z stacks. (F) Two-photon imaging of liver tissue slice, stained with Membright-Cy3.5 (white). Nucleus was stained with Hoechst (blue). (G) Confocal microscopy of hippocampal primary neurons and astrocytes labeled with MemBright-Cy3. (H) 3D-STORM microscopy of hippocampal neurons using MemBright-Cy3.5. Adapted with permission from ref <sup>3</sup>. Copyright 2019 Elsevier. (I) Fluorogenic dimer probes for PAINT imaging and

principle of their ON/OFF switching on membrane binding. Adapted with permission from ref <sup>20</sup>. Copyright 2022 American Chemical Society.

### 3. PM PROBES SENSITIVE TO POLARITY

Polarity is a fundamental parameter measured in lipid membranes, which describes an extent of dipole-dipole interactions, H-bonding with water and solvent relaxation. As it was shown in the seminal works on solvatochromic dye Laurdan (Figure 3A), sensitivity to polarity and hydration enables imaging the lipid order, which describes organization of lipid microdomains.<sup>24</sup> Indeed, liquid ordered (Lo) phase enriched in cholesterol and saturated lipids<sup>6</sup> is characterized by decreased polarity and hydration and observed as a blue shift in emission of a solvatochromic dye (Figure 3B), because tightly packed lipids exclude water from the bilayer and slow down dipolar relaxation around the dye.<sup>25</sup> Another early example is di-4-ANEPPDHQ (Figure 3A), which distinguishes the two phases by the emission color.<sup>26</sup> While it was designed to target the outer PM leaflet, its microinjection inside the cells allowed the inner leaflet staining and monitoring transmembrane asymmetry.<sup>8</sup> However, both Laurdan and di-4-ANEPPDHQ require relatively high concentrations (>1  $\mu\text{M}$ ) and the selectivity of the former to the outer/inner leaflet was not clear. To address these issues, we developed NR12S probe from solvatochromic dye Nile Red and the zwitterionic anchor group (Figure 3A).<sup>10</sup> The probe displayed emission color response to the lipid order and bright PM staining at low dye concentrations (~100 nM). Owing to its capacity to switch off in the presence of sodium dithionite, we showed that NR12S binds the outer membrane leaflet and undergoes very slow flip-flop (only 9% after 4 h at 37 °C). NR12S found numerous applications in cell membrane biology (nowadays commercialized). Although NR12S enabled monitoring the lipid order in PM and its loss on cholesterol extraction,<sup>10</sup> it showed rather homogeneous emission color on the cell surface,<sup>10</sup> probably because hypothetical lipid microdomains were too small and dynamic. To understand better phase-heterogeneity in the PM, we designed NR12S analogue bNR10S bearing a bulky alkyl chain (Figure 3A), which was excluded from the Lo phase (Ld-phase staining), and black whole quencher-2 BHQ10S with the same bulky alkyl chain that quenched NR12S in the Ld phase (Lo-phase staining).<sup>27</sup> In cells, bNR10S showed signature of Ld phase, while combination of NR12S with BHQ10S showed signal from the Lo phase, which suggested co-existence of both Lo and Ld phases in cell PM.<sup>27</sup>



**Figure 3.** Polarity-sensitive PM probes. (A) Chemical structures of solvatochromic membrane probes and (B) principle of their color response to lipid organization. (C) Membrane probes that irreversibly (left) or reversibly (right) bind to biomembranes. (D) Fluorescence spectra of NR12A in an aqueous buffer and in liposomes of different lipid composition. (E) Ratiometric confocal microscopy of giant vesicles composed of DOPC/SM/Chol, 1/1/0.7, stained with NR12A. (F) Confocal microscopy images of HeLa cells stained with NR12A and NR12S (20 nM). (G) 3D PAINT super-resolution images of COS-7 cells with NR4A (10 nM) and NR12S (20 nM). (H) Nanoscopic mapping of lipid order in cell membranes with NR4A. Color represents single molecule spectral mean (color scale bar). (C–H) Adapted with permission from ref <sup>2</sup>. Copyright 2019 Wiley-VCH GmbH. (I) Confocal microscopy images of CHO cells stained with Laurdan (top) and Pro12A (bottom); (J) Viologen-C12 and its use for quenching of the probes located at the outer bilayer leaflet. (K) Fluorescence spectra of Pro12A in LUVs of different compositions. (L) Response of Laurdan and Pro12A to lipid packing in phase separated GPMVs. (M) Quantification of GP for the Lo and Ld phases obtained from GPMVs and for CHO under cholesterol extraction by methyl- $\beta$ -cyclodextrin (MBCD). (I–N) Adapted with permission from ref <sup>28</sup>. Copyright 2020 American Chemical Society.

An attractive approach to assess membrane lipid organization by solvatochromic dyes is super-resolution PAINT microscopy, which relies on reversible binding-unbinding processes.<sup>29</sup> However, Nile Red, commonly used in PAINT, rapidly distributes between the two membrane leaflets and enters the cells. Therefore, we designed two Nile Red probes with membrane anchors composed of an anionic sulfonate and an alkyl chain of varied lengths (Figure 3A): NR12A with dodecyl chain for high-affinity membrane staining and NR4A with butyl chain for low-affinity (reversible) staining (Figure 3C).<sup>2</sup> The new probes preserved their sensitivity to the lipid order in model membranes (Figure 3D,E), while NR12A showed improved performance in classical confocal microscopy compared to NR12S (Figure 3F). Thus, the anionic sulfonate anchor of NR12A was more efficient PM targeting ligand than the zwitterionic one of NR12S. The low-affinity probe NR4A, in contrast to NR12S and NR12A, exhibited efficient single-molecule blinking. It allowed PAINT imaging of cell surface with spectral resolution, which revealed the presence of vesicular and fiber like protrusions and invaginations characterized by lower lipid order (Figure 3G,H). Thus, the co-existence of ordered and disordered phases in



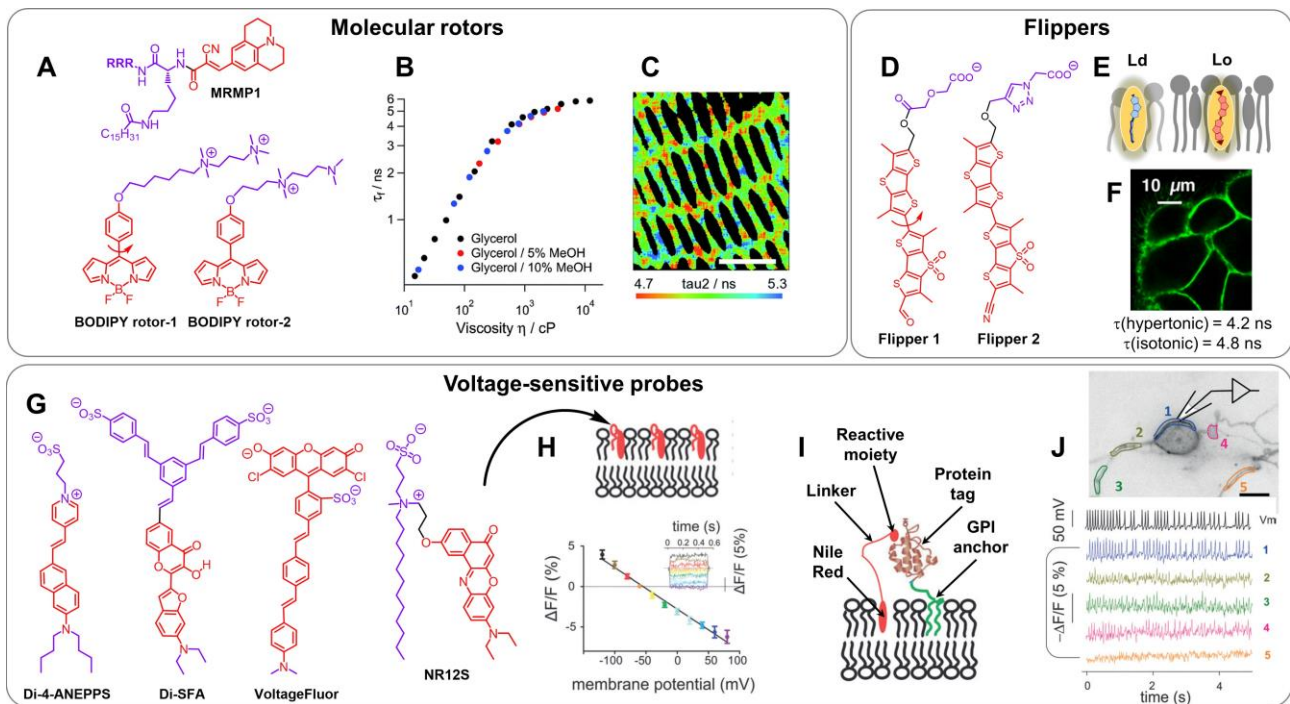
the PM can be at least in part linked with membrane geometry, where the cell surface dominated by Lo-like phase contains Ld-like structures in form of cell protrusions or invaginations.

To assess other wavelengths, we applied our concept of membrane anchor to other solvatochromic dyes: push-pull fluorene (FR5)<sup>30</sup> and Laurdan (Pro12A)<sup>28</sup> (Figure 3A). Compared to Laurdan, Pro12A displayed much higher specificity to PM (Figure 3I) and selectivity to the outer PM leaflet, confirmed by specially designed viologen quencher Viologen-C12 with expected leaflet-specificity due to two positively charged anchor groups (Figure 3J). Pro12A also showed stronger color change distinguishing the Lo and Ld phases in model membranes and giant PM vesicles (GPMVs) (Figure 3K-M), as well as stronger response to cholesterol extraction in live cells (Figure 3N), owing to more precise membrane localization. Finally, its blue-green emission operating range makes it compatible with common yellow and red fluorescent proteins.

One should note that applications of the polarity-sensitive PM probes go well beyond imaging of the lipid order. Previously, we designed solvatochromic probe F2N12S based on 3-hydroxyflavone, which revealed drastic increase in the local polarity at the outer PM leaflet after apoptosis, due to the loss of transmembrane lipid asymmetry.<sup>9</sup> Later on, we found that NR12S can also detect apoptosis featuring red-shifted emission<sup>31</sup> and allows monitoring formation of exocytotic blebs characterized by dramatically increased local polarity.<sup>27,32</sup> Recently, Feng and co-workers developed polarity-sensitive fluorogenic probes based on coumarin MOM<sup>33</sup> and COP<sup>34</sup>, which light up at the PM of tumor cells, because tumor cells could exhibit lower local polarity. Finally, using NR12A,<sup>2</sup> we found that a hyperosmotic mechanical stress generates clear heterogeneities of the PM local polarity,<sup>1</sup> probably because of local membrane bending. An oxidative stress produced slight increase in the local polarity of PM imaged by NR12A,<sup>1</sup> which is probably linked to a lipid peroxidation. These results open exciting applications for solvatochromic probes.

#### **4. PM PROBES SENSITIVE TO VISCOSITY, TENSION AND ELECTRIC POTENTIAL**

PM viscosity is another fundamental biophysical parameter, which directly controls membrane dynamics. Fluorescent molecular rotors (Figure 4A) can detect viscosity increase by inhibition of their intramolecular rotation in the excited state, which increases their emission lifetime. Haidekker and co-workers originally introduced 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) derivatives in form of lipid conjugates.<sup>35</sup> Recently, CCVJ conjugate with a charged peptide and a long alkyl chain (MRMP1, Figure 4A) was proposed for imaging PM of living cells and exosomes.<sup>36</sup> Kuimova and co-workers pioneered PM probes based on BODIPY molecular rotor functionalized with two cationic groups,<sup>16,37</sup> displaying strong dependence of the emission lifetime on the viscosity (Figure 4A,B). These probes revealed changes in the membrane viscosity in highly organized tissues of porcine eye lens (Figure 4C)<sup>37</sup> and in neurons under oxidative stress and neuroprotection.<sup>38</sup>



**Figure 4.** PM probes sensitive to viscosity (molecular rotors), membrane tension (flippers) and membrane potential. (A) Examples of molecular rotor probes. (B) Fluorescence lifetime calibration vs. viscosity obtained for BODIPY rotor-2 in methanol/glycerol mixtures at various temperatures. (C) FLIM images of lifetime ( $\tau_f$ ) distributions recorded at 20 °C. Scale bar: 10  $\mu$ m. (B,C) Adapted with permission from ref <sup>37</sup>. Copyright 2015 the Royal Society of Chemistry. (D) Flipper PM probes and (E) principle of their operation due to planarization in ordered lipid environment (red form in Lo phase vs blue form in Ld phase). (F) Confocal images of HeLa Kyoto cells stained with Flipper 2. Lifetime values for isotonic and hypertonic (mechanical stress) conditions are shown. (E,F) Adapted with permission from ref <sup>39</sup>. Copyright 2019 American Chemical Society. (I) Voltage-sensitive membrane probes. (J) Mean response of NR12S to changes in transmembrane potential in patch-clamped HEK293T cells. The graph inset shows fluorescence responses in a typical NR12S-labeled HEK293T cell subjected to square voltage steps of various magnitudes. (L) Genetic targeting of Nile Red-based voltage indicator. Illustration of STeV11-Nile Red derivative tethered to a protein tag by a molecular linker. (L) Tracking action potentials in neurons. (Top) Wide-field image of dorsal root ganglia culture cell expressing ACPGPI and labeled with CoA-PEG11-NR. Scale bar is 10  $\mu$ m. (Bottom) Membrane potential (top trace, black) was recorded from the cell body under current clamp mode. Epifluorescence from single-trial optical recordings for the color-matched somatic and axon areas (colored traces) highlighted with colored ROIs in the image (top). (J-L) Adapted with permission from ref <sup>40</sup>. Copyright 2019 Wiley-VCH GmbH.

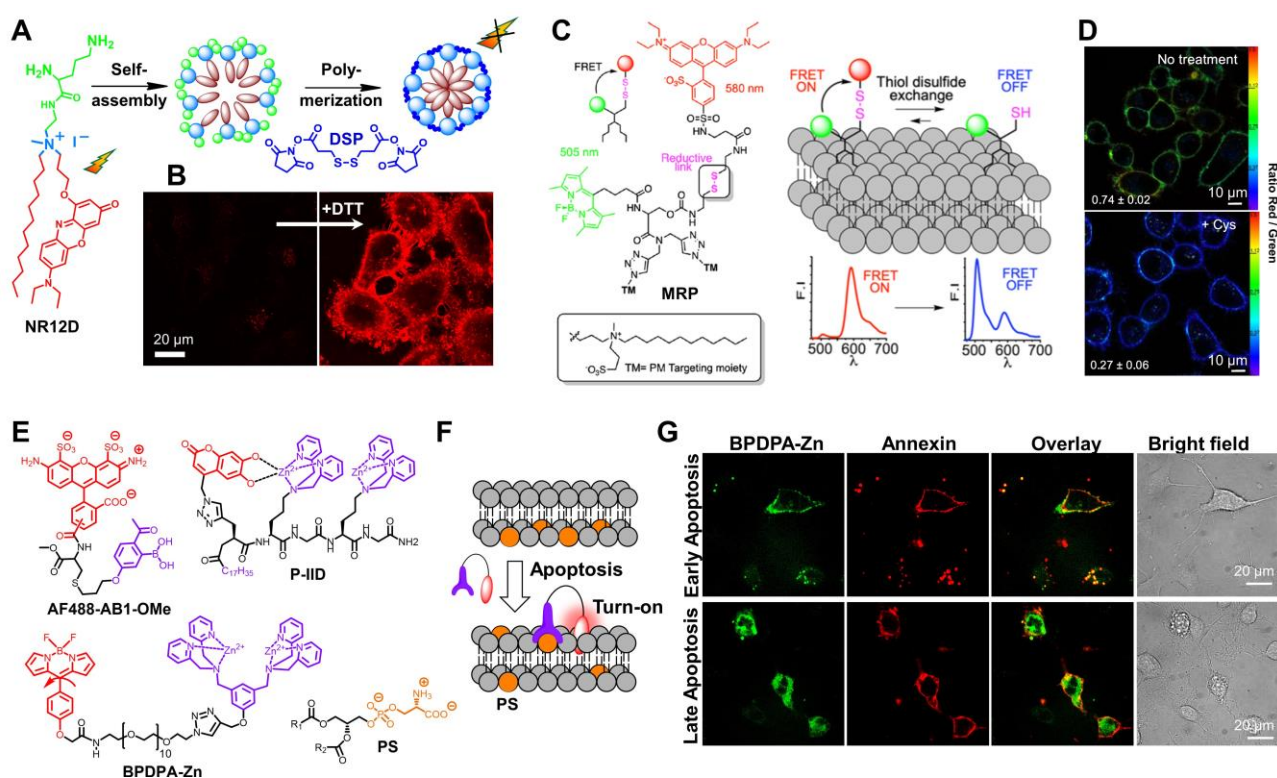
A remarkable class of membrane probes are flippers, introduced by Matile and co-workers.<sup>18</sup> These dyes change their ground state conformation, where the Lo phase drives their planarization (Figure 4D,E), leading to increase in their emission lifetime.<sup>17</sup> Carboxylate derivatives flippers 1 and 2 can label specifically PM (Figure 4D,F),<sup>18</sup> while their mechanosensitive nature enabled monitoring membrane tension in cells under a mechanical stress, affecting their fluorescence lifetime (Figure 4F).<sup>17,39</sup>

Significant efforts have been done to design probes for electric potential in PM, in order to wirelessly monitor activity of neurons. The early seminal works by Loew and co-workers introduced styrylpyridinium probes di-4-ANEPPS (Figure 4G) and di-8-ANEPPS, operating by electrochromism.<sup>41</sup> We selected 3-hydroxychromone derivative, undergoing excited state intramolecular proton transfer (ESIPT) sensitive electric fields,<sup>42</sup> and grafted two “legs” bearing sulfonate groups in order to impose its vertical orientation and deep insertion into

biomembranes. The obtained probe di-SFA showed ~15% ratiometric response of its dual emission to 100 mV voltage change in cells.<sup>43</sup> Moreover, NR12S, due to vertical orientation of its push-pull fluorophore in biomembranes, also displayed good sensitivity to transmembrane potential (Figure 4H).<sup>40</sup> Its analogue labelled to tagged GPI protein (Figure 4I) allowed detection of action potential in neurons (Figure 4J).<sup>40</sup> A remarkable concept was proposed by Miller and co-workers,<sup>44</sup> which combines fluorophore with electron donor unit undergoing voltage-sensitive photoinduced electron transfer (PET).<sup>45</sup> It featured higher sensitivity compared to di-4-ANEPPS (up to 20-60 % vs 6-10% per 100 mV)<sup>44</sup> and allowed designing probes of different color using fluorescein, rhodamine, BODIPY, etc.<sup>44</sup>

## 5. PM PROBES FOR CHEMICAL SENSING

PM probes bearing molecular recognition units enable sensing biologically important chemical species at the cell surface, which include protons (pH), metal ions, redox species and biomolecules, like lipids and proteins. Thus, Zhang and co-workers combined membrane targeting unit composed of lipid-DNA conjugate with pH-sensitive fluorescein and pH-insensitive rhodamine,<sup>46</sup> which enabled ratiometric imaging of pH at the cell surface.<sup>46</sup> To achieve near-membrane detection of metal ions, such as  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ , the corresponding probes for ions were functionalized with long alkyl chains.<sup>47</sup> To sense thiols, we made a derivative of Nile Red, bearing a membrane anchor and a lysine residue, which formed non-fluorescent micelles polymerized by dithiol cross-linker (Figure 5A).<sup>48</sup> The thiols induced micelle disassembly that lighted up the cell surface (Figure 5B).<sup>48</sup> Recently, we designed a FRET-based probe named MRP composed of BODIPY donor, coupled to a membrane anchor and to a rhodamine acceptor through a thiol-sensitive S-S bond (Figure 5C).<sup>49</sup> The probe enabled ratiometric imaging of thiols at PM (Figure 5D) and revealed that cancer cell lines presented a reduced thiol disulfide exchange activity compared to noncancer cells.<sup>49</sup>



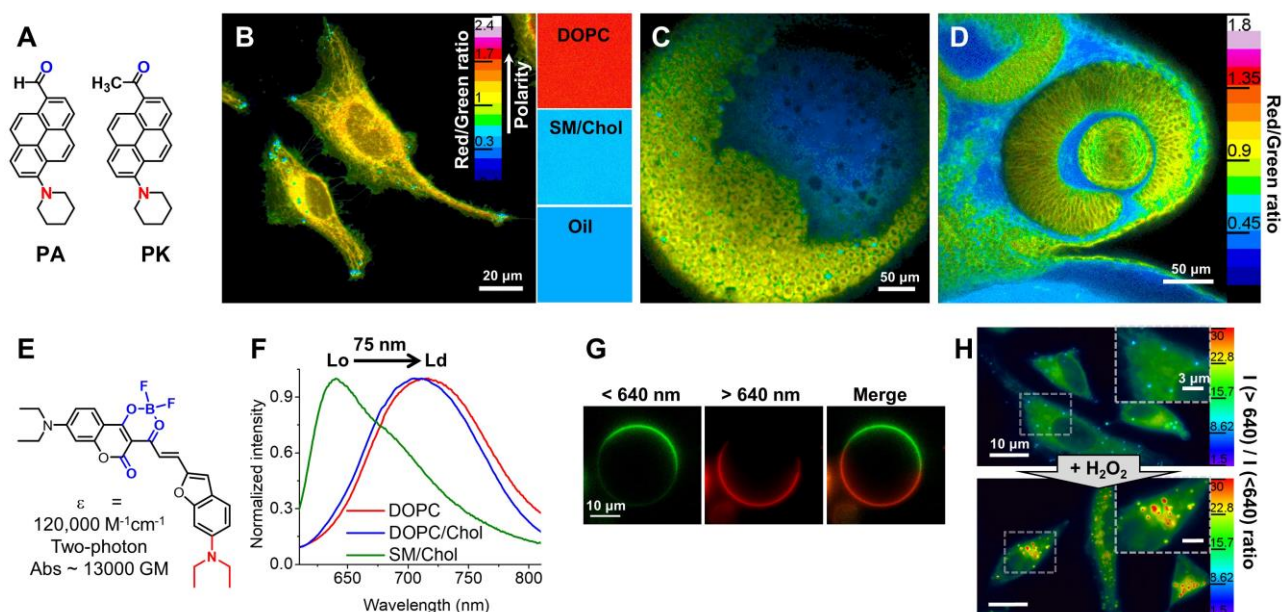
**Figure 5.** (A) Presentation of the concept of disassembly-driven fluorescence turn-on probe for thiols. (B) Fluorescence confocal images of HeLa cells incubated with the nanoprobe before and after addition of thiol (DTT). (A-B) Adapted with permission from ref <sup>48</sup>. Copyright 2014 Wiley-VCH GmbH. (C) Principle of FRET probe MRP for thiol detection. Adapted with permission from ref <sup>49</sup>. Copyright 2020 American Chemical Society. (D) Ratiometric imaging of KB cells stained with MRP in normal conditions (top) and with cysteine

(bottom, Cys). (E-G) Fluorescent probes for detection of lipids on the cell membranes. (E) Examples of fluorescent probes for amino-lipids (AF488-AB1-OMe) and PS (P-IID and BPDPA-Zn). (F) Design concept of turn on probe for detection of lipids in biomembranes. (G) Fluorescence images of early and late apoptotic HeLa cells induced by 1 and 3  $\mu\text{M}$  staurosporine, respectively, for 2 h at 37°C, which were stained with BPDPA-Zn (green) and Annexin V Alexa Fluor 568. Adapted with permission from ref <sup>4</sup>. Copyright 2015 the Royal Society of Chemistry.

A special class is PM-selective probes that recognize lipids. Gao and co-workers reported AF488-AB1-OMe probe bearing 2-acetylphenylboronic acid (Figure 5E), which specifically reacts with amino-containing lipids through a dynamic iminoboronate bond and enables detection of bacteria presenting phosphatidylethanolamine at their surface.<sup>50</sup> Another elegant example is fluorogenic probe P-IID (Figure 5E) bearing dipicolylamine-zinc (DPA-Zn), which recognizes specifically phosphatidylserine (PS), allowing detection of apoptosis.<sup>51</sup> Recently, we developed a fluorogenic probe BPDPA-Zn for PS by conjugating the BODIPY molecular rotor with DPA-Zn through a PEG linker (Figure 5E,F).<sup>4</sup> The probe showed light up on binding to PS due to increase in the local viscosity, which allowed wash-free detection of apoptosis in cancer cells (Figure 5G). Similar approach was used by us to detect membrane proteins – G-protein coupled receptors, where environment-sensitive dyes coupled with a receptor ligand light up on protein binding, because the dye is inserted in a low-polar lipid environment.<sup>52,53</sup> In case of solvatochromic reporter Nile Red, this approach enabled sensing polarity of PM lipid surrounding of GPCR.<sup>53,54</sup>

## 6. LIPIDIC PROBES FOR ORGANELLES

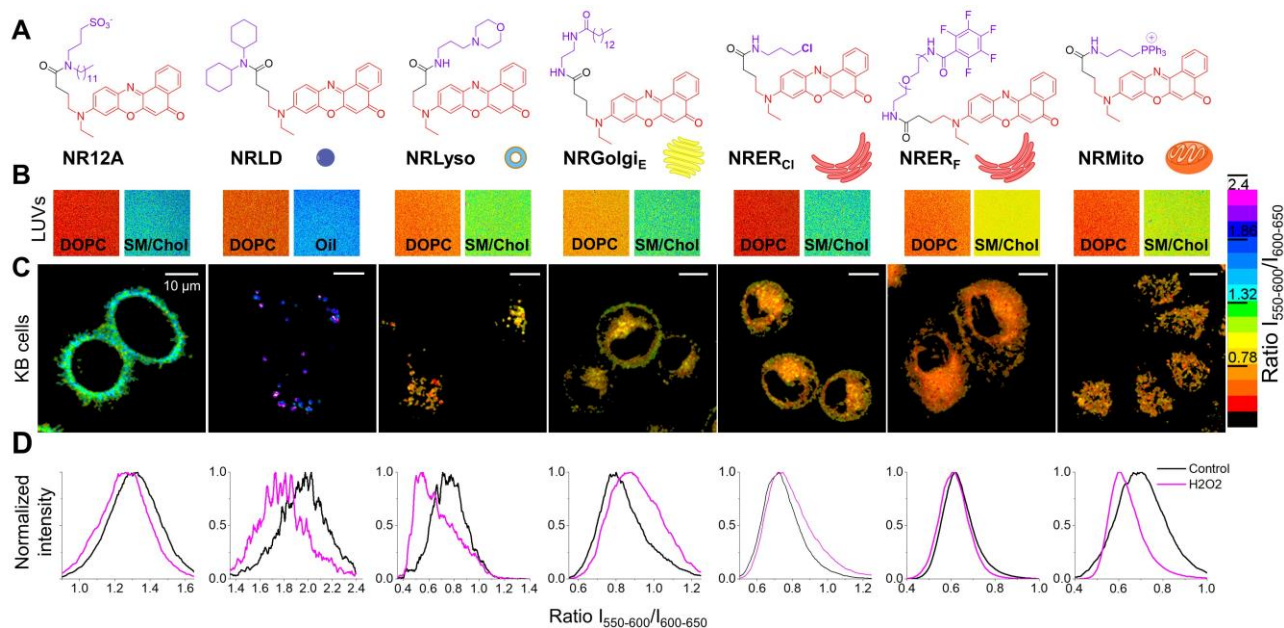
The cell organelles are delimited by lipid membranes with the exception of LDs. Each organelle carries essential function in cells, e.g. lysosomes digest molecules entering the cells, ER takes care of synthesis of biomolecules, mitochondria produce energy, Golgi apparatus is responsible for protein and lipid trafficking, etc. To monitor the lipid organization within organelle membranes, the direct approach is to use a cell-permeable lipophilic solvatochromic dye, as we showed for push-pull pyrene dye PA<sup>55</sup> and its improved analogue PK<sup>28</sup> (Figure 6A). Ratiometric two-color imaging of the cells stained with these probes revealed a polarity gradient from the most apolar and highly ordered PM to more polar intracellular membranes (Figure 6B),<sup>28,55</sup> in line with preferential localization of sphingomyelin and cholesterol in the PM. This polarity gradient was also found in cells of live zebrafish embryo stained with PK (Figure 6C,D).<sup>28</sup> Besides, LDs were systematically identified by different solvatochromic dyes<sup>28,55,56</sup> as the most apolar structures, owing to their oil core. This low polarity makes LDs a good target for highly hydrophobic fluorescent dyes,<sup>57</sup> including our ultrabright dioxaborine dyes StatoMerocyanines.<sup>58</sup> Moreover, using near-infrared emitting solvatochromic dioxaborine dye DXB-NIR, showing outstanding sensitivity to the lipid order (Figure 6E-G), we monitored changes in polarity of subcellular lipid structures in response to external stimuli, such as cholesterol extraction, oxidative stress and starvation, which generally led to increase in the local polarity (Figure 6H).<sup>59</sup> Particularly strong polarity increase was in LDs (Figure 6H), which probably accumulated the products of lipid peroxidation. Overall, non-targeted solvatochromic probes, despite their non-specific distribution in organelle membranes, can distinguish sub-cellular structures by emission color, which was recently used for cartography of multiple organelles at high resolution.<sup>60</sup>



**Figure 6.** Polarity-sensitive probes for whole-cell imaging of lipid membrane organization. (A) Solvatochromic dyes PA and PK. (B) Red/green ratio image with a pseudocolor scale reflecting probe environmental polarity. (Right) Calibration ratiometric images for PK probe in environments of different polarity: DOPC and SM/Chol liposomes, Labrafac oil. (C-D) Two-color ratiometric confocal imaging of zebrafish embryo at different development stages: 6 h (C) and 32 h (D) postfertilization, stained with 500 nM of PK probe. (D) Eye part is shown. (B-D) Adapted with permission from ref <sup>28</sup>. Copyright 2020 American Chemical Society. (E) Solvatochromic dye DXB-NIR. (F) Normalized emission spectra of DXB-NIR in liposomes of various composition. (G) Two-color microscopy imaging of giant vesicles (DOPC/SM/Chol, 1/1/0.5: Lo/Ld mixture) stained with DXB-NIR: far red (<640 nm) and NIR (>640 nm) channels, and the merge. (H) Two-color ratiometric imaging of HeLa cells using DXB-NIR (200 nM) under oxidative stress: 2 mM H<sub>2</sub>O<sub>2</sub> for 1 h at 37 °C. (F-H) Adapted with permission from ref <sup>59</sup>. Copyright 2019 American Chemical Society.

To achieve specific organelle targeting, a purely chemical approach is to functionalize fluorescent dyes with targeting ligands. In particular, probes bearing PM anchors can also label endosomes, because endocytosis recruits PM inside endosomes together with the probes. Although NR12S provides selective labelling of cell surface for at least 4h at 4 °C and for at least 30 min at room temperature, after 30 min at 37 °C a significant fraction of the probe labels endosomes.<sup>61</sup> The endosomal accumulation depends on the probe charge because for zwitterionic NR12S it is much slower than for cationic FM4-64.<sup>61</sup> Endosomal targeting by NR12S was used to monitor maturation of endosomes:<sup>61</sup> the observed red shift after 2h incubation at 37 °C suggested that, after its transfer to the inner leaflet of endosomes, NR12S detected decrease in cholesterol and sphingomyelin content and thus loss of the lipid order. Recently, using a dimeric zwitterionic anchor with pH-sensitive chromenoquinoline fluorophore, we showed a gradual change in the endosomal pH.<sup>62</sup> Krishnan and co-workers designed a DNA-based probe that combined a voltage-sensitive PET dye with a reference dye and exploited receptor-mediated endocytosis for ratiometric detection of membrane potential in specific endocytic compartments.<sup>63</sup> A selective strategy to label endosomes/lysosomes is to use protonatable chemical ligands, such as N-substituted morpholine, having pKa close to pH in lysosomes.<sup>7,39</sup> The ligand approach was successfully applied for targeting other organelles. Thus, cationic alkyl-triphenyl phosphonium was used to target different dyes to mitochondria because of a high transmembrane potential of its inner membrane.<sup>7</sup> Propyl chloride and pentafluorobenzoyl amide were used for targeting ER, hypothetically due binding to its chlorine pump<sup>7</sup> and reacting with thiols in ER-membrane proteins,<sup>39</sup> respectively. Myristic acid was used for targeting Golgi apparatus,<sup>64</sup> because one of its functions is to process lipids. A systematic approach to address biophysical properties of organelle membranes was proposed by Matile and co-workers using flippers coupled with mentioned ligands.<sup>39</sup> External stimuli, in particular mechanical stress, produced significant organelle-dependent variation in the lipid order. We adapted the chemical ligand strategy to

solvatochromic Nile Red and obtained probes for endosomes, ER, Golgi, mitochondria and LDs (Figure 7A).<sup>1</sup> By calibrating each probe directly under the microscope and analyzing green/red fluorescence intensity ratios ( $I_{550-600}/I_{600-650}$ , Figure 7B), we could directly image and compare the lipid order of different organelles (Figure 7C,D), which decreased in the following sequence: PM  $\gg$  mitochondria  $\sim$  ER > lysosomes > Golgi apparatus > ER (with shallow probe NRER<sub>F</sub>). LDs, which were targeted by bulky lipophilic dicyclohexylamino group, appeared again as the most apolar lipid structures in the cells (the highest  $I_{550-600}/I_{600-650}$  value, Figure 7). This toolkit of solvatochromic probes revealed that organelles are differently sensitive the external stress. LDs, mitochondria and lysosomes were the most sensitive to oxidative stress, where a significant polarity increase (i.e. lower values of the  $I_{550-600}/I_{600-650}$  ratio, Figure 7D) was observed, probably due to the lipid oxidation, whereas lysosomes, ER (NRER<sub>F</sub>), and mitochondria showed the strongest response to hyperosmotic conditions (mechanical stress).<sup>1</sup>



**Figure 7.** Solvatochromic probes for organelles, based on chemical ligand strategy. (A) Chemical structures of Nile Red-based probes for organelles. (B) Ratiometric confocal imaging of liposomes presenting Lo (DOPC) and Ld (SM/Chol) phases for calibration of the probes (in case of NRLD, sesame oil was used). (C) Ratiometric confocal imaging of KB cells by the organelle probes. (D) Distribution histograms of the pixel-to-pixel green/red fluorescence intensity ratios ( $I_{550-600}/I_{600-650}$ ) for corresponding probes in KB cells before and after oxidative stress (2 mM H<sub>2</sub>O<sub>2</sub> for 1 h at 37 °C). The total intensity for each ratio  $I_{550-600}/I_{600-650}$  is calculated for all pixels of the corresponding image. (B-D) Adapted with permission from ref <sup>1</sup>. Copyright 2021 American Chemical Society.

An alternative organelle-targeting strategy is a bio-orthogonal reaction inside the cells between a targeting ligand and a fluorophore.<sup>65,66</sup> For example, ceramide derivative Ser-TCO can localize in Golgi or ER depending on temperature and then react with tetrazine derivative of spontaneously blinking Si-rhodamine dye HMSiR-Tz, allowing their super-resolution imaging.<sup>67</sup> Recently, we explored dynamic covalent chemistry to couple Nile Red ketone derivative with hydrazide ligands located in mitochondria, PM or lipid droplets,<sup>68</sup> which allowed localizing the same dye in organelles of interest. Specific targeting of mitochondria and PM led to significantly higher photo-toxicity compared to LDs or non-targeted dye, showing that the organelle targeting can modulate the biological activity.

Another effective approach is to label organelles chemo-genetically by protein tags. Kuimova and co-workers reported molecular rotor BODIPY functionalized with a ligand for targeting HaloTag of proteins localized in cytosol, nucleus, ER and mitochondria.<sup>15</sup> Using FLIM, a characteristic low viscosity profile was found in mitochondria, revealing its heterogeneity and response to a mechanical stress and a variation in membrane

potential.<sup>15</sup> Matile and co-workers used this approach to target flippers bearing HaloTag ligand (HaloFlippers) through a linker of optimized length.<sup>69</sup> Overall, in the chemo-genetic strategy, a care should be taken about exact localization of protein-grafted fluorophore with respect to a lipid membrane.

## 7. CONCLUSIONS AND OUTLOOK

Ubiquitous nature of lipid membranes and their essential role in cell life make them an important research subject, which stimulates development of new chemical tools – fluorescent probes. Here, we present our advances and those of other groups in the field of membrane probes by organizing them based on targeting principles, sensing profile and optical response. Among membrane targeting strategies, we highlight our synthetic membrane anchors that yielded a large family of PM probes. Sensing biophysical properties of biomembranes is achieved by design of environment-sensitive probes, while grafting recognition units to the probe allows chemical sensing at the biomembrane. Although the main efforts have been focused on PM, the probes for organelles emerge rapidly. Both non-targeted and targeted probes, including solvatochromic probes developed by us, revealed unique signatures of organelle membranes and their response to cell stress.

In this fruitful research direction, there is a number of big challenges ahead. First, superior organelle specificity should be achieved, which requires design of new ligands exploiting profound knowledge of biochemistry of organelles and lipids. In case of PM, localization of probes specifically at the inner leaflet remains challenging, with key questions on specific targeting of this leaflet and preventing the probe spreading into the cytoplasm. The other challenge is long-term imaging of PM, which requires probes with minimized endocytosis. Sensing lipids and their organization should be done with relation to specific membrane proteins, by designing, for example, bio-functional probes able to target proteins and sense their lipid environment. Rapid development of super-resolution techniques generates strong demand in appropriate membrane probes. Here, we consider promising to design probes implementing both sensing and on/off switching functions. Ultimately, the biomembrane probes should go beyond cells and allow imaging tissues and animals, which would require their specific delivery, proper biodistribution and low toxicity as well as compatibility with intravital and whole animal near-infrared imaging.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: andrey.klymchenko@unistra.fr.

### ORCID

Andrey S. Klymchenko: 0000-0002-2423-830X

### Notes

Competing financial interest: multiple fluorescent membrane probes described here were commercialized by SATT Conectus/University of Strasbourg: F2N12S (Thermo Fisher Scientific), NR12S, NR12A, NR4A and MemBright family (Cytoskeleton Inc.).

### 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 was an ERC Consolidator fellow in 2015-2020. He is a leader of

“Nanochemistry and Bioimaging” group and co-founder of BrightSens Diagnostics SAS. His research interests include functional fluorescent molecules and nanomaterials for biosensing, imaging and theranostics.

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