Blue fluorogenic probes for cell plasma membranes fill the gap in multicolour imaging†

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Blue fluorescent probes for cellular imaging are poorly developed and rarely used because of strong cell auto-fluorescence at these wavelengths. However, multi-colour imaging needs blue probes, such as ubiquitous nucleus markers DAPI and Hoechst, because they can be readily combined with common green and red markers based on dyes and fluorescent proteins. Cell plasma membrane is an important target for imaging, but membrane probes that absorb and emit light between 400 and 500 nm are missing. Here, using 3-methoxychromone dyes we designed two blue membrane probes exhibiting >100-fold fluorescence turn-on (fluorogenic response) on membrane binding, large Stokes shift (70–90 nm) as well as high brightness and photostability. These unique properties enabled cellular imaging at low probe concentrations (20–50 nM) with minimal background from cell auto-fluorescence and from free probe. RGB multicolour imaging was successfully realized using these probes in combination with common green and red markers. As the new probes enable high-quality imaging of cell plasma membranes in the poorly explored blue spectral region, they may become popular tools that fill the gap in multi-colour microscopy.

1. Introduction

Recent years have seen a tremendous expansion of fluorescence techniques and tools for cellular research. In addition to genetically encoded fluorescence proteins, a number of molecular probes for monitoring cellular life have been developed.1–4 Notably, membrane probes underwent rapid development during the last years.5–8 The first reason is that plasma membrane plays a key role in cell functions and it is the first barrier that molecules and ions need to cross to enter the cells. Secondly, fluorescent probes able to concentrate within the confined space of lipid plasma membrane permit not only to clearly delimit the cell surface, but also to characterize its biophysical properties and monitor cell internalization of molecules.5,9–12 Finally, the hypothesis of lipid rafts formed by sphingomyelin/cholesterol rich domains13 stimulated chemists to develop appropriate imaging tools.6,15–17 Nevertheless, the performance of membrane probes is limited, so that biologists generally use membrane proteins bearing fluorescent protein tags or fluoroscently labelled membrane binding-proteins, such as wheat germ agglutinin (WGA).18 Among the existing molecular membrane probes, two large families, namely fluorescently labelled lipids and specially designed probes, should be mentioned. Labelled lipids are ideal for model membranes, but their use for living cells requires special lipid delivery systems, such as cyclodextrins.19 The second class is based on specially designed fluorescent probes, which can spontaneously stain lipid membranes without using delivery agents.5,7,11–15,35 To achieve specific staining of cell plasma membranes with minimal internalization, fluorescent dyes are usually modified with a polar head group, as it was done for FM4-64,20 TMA-DPH,21 di-4-ANEPPDHQ,22 C-Laurdan,23 Mem-SQAC,24 push–pull glycoconjugates,25 and oligothiophene amphiphiles.11 We should also mention F2N12S25 and NR12S,18 bearing an amphiphilic anchor group that enables specific staining of the outer membrane leaflet with minimal internalization and flip-flop between the leaflets.11 However, the membrane probes developed so far, being highly useful for studying biomembranes, overlap with the 500 to 650 nm spectral window of common molecular probes and fluorescent proteins,26 which limit their applications for multi-colour imaging. Exceptions are TMA-DPH and C-Laurdan and their analogues, but these probes require ultraviolet excitation (≈360 nm), which is harmful for the cells. Therefore, there is a strong need for powerful blue probes for cell plasma membranes, which, similarly to DAPI and Hoechst, the blue stains of nucleus, could become ubiquitous cell imaging tools. These blue probes should be excitable by violet light (≈400 nm),

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1. Introduction

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emit efficiently around 450–500 nm and exhibit large Stokes shift in order to minimize the contribution of cell autofluorescence, commonly observed in the blue spectral region. Moreover, they should be fluorogenic, i.e. non-fluorescent in water but highly fluorescent in lipid membranes, which would enable imaging without background noise from the non-bound probes.\textsuperscript{27,28} As prospective building blocks for preparation of blue membrane probes, we considered 3-methoxychromones, because they were recently shown to be bright and photostable, and to exhibit large Stokes shift and fluorogenic response to solvent polarity.\textsuperscript{29}

In the present work using 3-methoxychromone dyes, we developed the first high-performance blue membrane probes, characterized by high brightness and photostability as well as by suitable absorption and emission wavelengths, allowing their combination with green and red markers. We showed that these probes could be used at concentrations as low as 20–50 nM and that their high brightness, large Stokes shift and efficient membrane binding with fluorogenic response provided excellent signal-to-noise ratio in cellular imaging.

2. Experimental

2.1. Synthesis of probes

All chemicals and solvents for synthesis were from Sigma-Aldrich. Synthesis of F2N12S was described elsewhere.\textsuperscript{25} 2-(6-(Diethylamino)benzofuran-2-yl)-3-hydroxy-4-methoxymethyl)-4H-chromen-4-one (1). 6-(Diethylamino)benzofuran-2-carbaldehyde\textsuperscript{30} (1 eq., 1 g) and 5-chloromethyl-2-hydroxyacetophenone\textsuperscript{31} (1 eq., 0.85 g) were dissolved in 10 mL of cold DMF (ice bath) and then treated with 0.5 g sodium methoxide (4 eq., 1 g). The obtained mixture was stirred for 24 h to give dark red chalcone, which was used in the next step without isolation. This mixture was then diluted with 40 mL of ethanol followed by addition of sodium methoxide (12 eq., 1.5 g) and hydrogen peroxide (10 eq., 2.3 mL). The mixture was shaken intensively and heated gently to reflux. Within 5 min, the dark red solution turned orange. Then, the solution was rapidly cooled to room temperature (RT) and poured into water. The product was then extracted with dichloromethane. The solvent was removed under vacuum and the crude product was purified by column chromatography with dichloromethane/MeOH (95/5) as an eluent. Yellow powder, yield 19%.\textsuperscript{1}H NMR (400 MHz, chloroform-d) δ 8.20 (s, 1H), 7.75–7.63 (m, 3H), 7.50 (d, J = 8.8 Hz, 1H), 6.89 (d, J = 2.2 Hz, 1H), 6.78 (dd, J = 8.8, 2.2 Hz, 1H), 4.39 (s, 1H), 3.53–3.43 (m, 7H), 1.26 (t, J = 7.0 Hz, 6H). HR-LCMS: (m/z) C23H23NO5 calc. 393.1576, found 393.1592.

6-(Bromomethyl)-2-(6-(diethylamino)benzofuran-2-yl)-3-hydroxy-4H-chromen-4-one (2). 2-(6-(Diethylamino)benzofuran-2-yl)-3-hydroxy-4-methoxymethyl)-4H-chromen-4-one (1) (1 eq., 260 mg) was dissolved in a hydrobromic acid solution (48%) (5 mL) and the mixture was heated for 10 min at 100 °C. The reaction was then cooled down to RT and neutralized with a solution of NaOH (50%) (2 mL). The obtained brown orange precipitate was filtrated and washed with 15 mL of heptane. The product was dried and used directly without further purification. Brown red powder, yield 85%. \textsuperscript{1}H NMR (400 MHz, chloroform-d) δ 8.27 (d, J = 2.3 Hz, 1H), 7.78–7.73 (m, 1H), 7.67 (d, J = 11.2 Hz, 2H), 7.50 (d, J = 8.7 Hz, 1H), 6.88 (s, 1H), 6.81–6.76 (m, 1H), 4.63 (s, 2H), 3.48 (q, J = 7.0 Hz, 4H), 1.26 (t, J = 7.0 Hz, 6H). HR-LCMS: (m/z) C22H13BrNO4 calc. 441.0576, found 441.0582.

2-(6-(Diethylamino)benzofuran-2-yl)-6-((dodecyl(methyl)amino)-3-hydroxy-4H-chromen-4-one (3). 6-(Bromomethyl)-2-(6-(diethylamino)benzofuran-2-yl)-3-hydroxy-4H-chromen-4-one (2) (1 eq., 150 mg) dissolved in THF (10 mL) and then N-methyl-dodecylamine (2.5 eq., 210 μL) was added upon stirring. After 2 h of stirring, the mixture was analysed by TLC, showing full conversion. The crude product was then dried under vacuum and purified by column chromatography with CH2Cl2/MeOH (95/5) has eluent. Brown orange oil, yield 25%. \textsuperscript{1}H NMR (400 MHz, chloroform-d) δ 8.03 (dd, J = 2.0 Hz, 1H), 7.67 (dd, J = 8.4, 2.0 Hz, 1H), 7.55 (t, J = 4.4 Hz, 2H), 7.40 (d, J = 8.7 Hz, 1H), 6.79 (s, 1H), 6.68 (dd, J = 8.7, 2.2 Hz, 1H), 3.51 (s, 3H), 3.38 (q, J = 7.0 Hz, 5H), 2.32 (t, J = 8.0 Hz, 2H), 2.13 (s, 3H), 1.45 (p, J = 7.7, 6.9 Hz, 2H), 1.17 (q, J = 6.7 Hz, 34H), 0.84–0.73 (m, 6H). HR-LCMS: (m/z) C35H48N2O4 calc. 560.3614, found 560.3632.

3-(((2-(6-(Diethylamino)benzofuran-2-yl)-3-hydroxy-4-oxo-4H-chromen-6-yl)methyl)(dodecyl)-(methyl)ammonio)propane-1-sulfonate (4). 2-(6-(Diethylamino)benzofuran-2-yl)-6-((dodecyl(methyl)amino)methyl)-3-hydroxy-4H-chromen-4-one (3) (1 eq., 50 mg) was dissolved in CH2CN (5 mL) and then 1,3-propanesultone (3 eq., 23 μL) was added. The mixture was heated to reflux for 24 h. The solvent was then removed under vacuum and the crude product was solved in CH2Cl2 and washed with a brine solution 3 times. It was used without further purification directly into the next step. Dark orange oil, yield 33%.

3-(((2-(6-(Diethylamino)benzofuran-2-yl)-3-methoxy-4-oxo-4H-chromen-6-yl)methyl)(dodecyl)(methyl)ammonio)propane-1-sulfonate (FC12SM). 3-(((2-(6-(Diethylamino)benzofuran-2-yl)-3-hydroxy-4-oxo-4H-chromen-6-yl)methyl)(dodecyl)(methyl)ammonio)propane-1-sulfonate (4) (1 eq.; 15 mg) was firstly dissolved into MeOH (5 mL), then K2CO3 (4 eq., 12 mg) and CH2I2 (10 eq., 31.92 mg) were added to the mixture. The reaction was stirred overnight at RT. After one night, 5 eq. of K2CO3 (15 mg) and a large excess of CH3I (1.14 g) were added to the mixture. After a few hours, solvent was removed under vacuum and the crude product was purified by preparative TLC using CH2Cl2/MeOH (8/2) as eluent. Yellow orange powder, yield 85%. \textsuperscript{1}H NMR (400 MHz, methanol-d4) δ 8.26 (d, J = 2.1 Hz, 1H), 7.86 (dd, J = 8.6, 2.2 Hz, 1H), 7.74 (d, J = 8.7 Hz, 1H), 7.65 (s, 1H), 7.44 (d, J = 8.6 Hz, 1H), 6.76 (d, J = 9.5 Hz, 2H), 4.61 (s, 2H), 3.94 (s, 3H), 3.47–3.37 (m, 8H), 2.93 (s, 3H), 2.81 (t, J = 6.7 Hz, 2H), 1.46–1.37 (m, 2H), 1.34–1.11 (m, 20H), 0.87–0.76 (m, 9H). HR-LCMS: (M + H) C35H48N2O7S calc. 696.3808, found 696.3793. Extinction coefficient in ethanol is 30 000 ± 3000 M⁻¹ cm⁻¹ at 457 nm.

3-(((2-(4-(Diethylamino)phenyl)-3-methoxy-4-oxo-4H-chromen-6-yl)methyl)(dodecyl)(methyl)ammonio)propane-1-sulfonate (F2N12SM). F2N12S (1 eq., 15 mg) was first dissolved into MeOH (5 mL), and then K2CO3 (2 eq., 10 mg) and CH2I2 (10 eq., 15 μL) were added to the mixture. The reaction was stirred for two days at RT. The solvent was then removed under vacuum and the crude product was purified by preparative TLC using CH2Cl2/MeOH (9/1) as eluent. Yellow orange powder, yield 80%.

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2.2. Lipid vesicles

Dioleoylphosphatidylcholine (DOPC) and cholesterol were purchased from Sigma-Aldrich. Bovine brain sphingomyelin (SM) was from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (LUVs) were obtained by the extrusion method as previously described. Brieﬂy, a suspension of multilamellar vesicles, prepared from the hydrated lipid film of lipids, was extruded with the Lipex Biomembranes extruder (Vancouver, Canada). The size of the filters was ﬁrst 0.2 μm (7 passages) and thereafter 0.1 μm (10 passages). This generates monodisperse LUVs with a mean diameter of 0.11 μm as measured with a Malvern Zetasizer 3000 (Malvern, U.K.). LUVs were labelled by adding aliquots (generally 2 μL) of probe stock solutions in dimethyl sulfoxide to 1 mL solutions of vesicles. Since the probe binding kinetics is very rapid, the ﬂuorescence experiments were performed a few minutes after addition of the aliquot. 20 mM phosphate buffer, pH 7.4, was used in these experiments. Concentrations of the probes and lipids were generally 0.4 and 200 μM, respectively, unless indicated.

2.3. Cell lines, culture conditions, and treatment

HeLa cells (ATCC) were cultured in Dulbecco’s modiﬁed Eagle medium (DMEM, Low glucose, Gibco-Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Lonza), and 1% antibiotic solution (penicillin-streptomycin, Gibco-Invitrogen) in a humidified atmosphere with 5% CO2 atmo-

sphere at 37 °C. Cells cultures passages were realized every 2–3 days.

In ﬂuorescence spectroscopy experiments, HeLa cells were detached by trypsinization. DMEM medium was ﬁrst removed from the culture dish, and cells were washed two times with DPBS. Trypsin 1× (LONZA) solution in DPBS was added to the cells and the cells were incubated at 37 °C for 4 min. The solution of trypsinized cells was then diluted by DPBS, transferred to Falcon tubes and centrifuged for 5 min. The washing procedure was repeated one more time with HBSS solution. To stain the cell suspension with the probes, an appropriate aliquot of their stock solution in DMSO was added to 0.5 mL of HBSS buffer, and after vortexing, the solution was immediately added to 0.5 mL of the cell suspension to obtain a ﬁnal probe concentration of 40 nM (<0.25% DMSO) and a cell concentration of 5 × 10^5 to 10^6 cells per mL. It should be noted that only freshly prepared solutions of the probes in HBSS should be used (<1 min) for cell staining, because of the slow aggregation of the probe in water. Before measurements, the cell suspension with the probe was incubated for 7 min at RT in the dark.

For microscopy studies with the probes, attached HeLa cells were washed two times by gentle rinsing with HBSS. Then, a freshly prepared solution of F2N125, F2N12SM or FC12SM in Opti-MEM (or HBSS) was added to the cells to a ﬁnal probe concentration of 50 nM (<0.25% DMSO volume) and incubated for 7 min in the dark at RT. The obtained samples were imaged directly without washing.

For tri-colour ﬂuorescence imaging, HeLa cells were transfected by a plasmid coding mCherry. 3 × 10^5 HeLa cells were seeded in 35 mm glass coverslips (µ-Dish IBIDI, Germany) in DMEM supplemented with 10% FBS and antibiotics (penicillin 100 UI mL^-1, streptomycin 100 UI mL^-1) and kept at 37°C in a 5% CO2 atmosphere. Transfection was performed at 24 hours post-seeding with 1 µg of pCMV3-mCherry plasmid using jetPEI™ (PolyPlus transfection, France) according to supplier’s recommendations. All observations were done between 16–24 hours post DNA transfection. To stain lysosomes in green, LysoTracker® Green DND-26 (Life technologies) was added to the cells at 50 nM ﬁnal concentration and incubated for 30 min at 37 °C. Then F2N12SM was added as described above.

2.4. Fluorescence spectroscopy and microscopy

Absorption spectra were recorded on a Cary 4 spectrophotometer (Varian) and ﬂuorescence spectra on a Fluorolog (Jobin Yvon, Horiba) spectrophotometer. Fluorescence emission spectra were systematically recorded at 410 nm excitation wavelength at room temperature, unless indicated. All spectra were corrected for the baseline (suspension of cells or lipid vesicles without the probe) and wavelength-dependent sensitivity of the detector. Fluorescence quantum yield were measured using 4′-(dimethylamino)-3-hydroxylavone in methanol as a reference (27%).

Confocal microscopy experiments were performed by using a Leica TCS SPE-II microscope with HXC PL APO 63×/1.40 OIL CS objective. The excitation was provided by 405 and 488 nm laser and the images were processed with the Image J software.

3. Results and discussion

3.1. Design and synthesis

F2N12SM is an analogue of F2N12S, where the 3-hydroxy group is methylated (Fig. 1). It was prepared by direct methylation of F2N12S with methyl iodide in acetonitrile (Fig. S1 in ESI†). To prepare FC12SM (Fig. 1), 6-(diethylamino)benzofuran-2-carbaldehyde was condensed with 5-chloromethyl-2-hydroxyacetophenone in basic conditions and then oxidized in the presence of hydrogen peroxide into the corresponding 3-hydroxychromone derivative (Fig. S2 in ESI†). It was then reacted with N-methyl-dodecylamine and the obtained tertiary amine was quaternized with 1,3-propanesulfonate. The 3-hydroxy group of the chromone was then methylated to obtain the FC12SM probe. The final probes were puriﬁed by thin layer chromatography and their structure was conﬁrmed by NMR and mass spectrometry.
3.2. Fluorescence spectroscopy in model membranes

**F2N12SM** and **FC12SM** showed very poor fluorescence intensity in aqueous media (QY < 0.5% for both probes), but they were highly emissive in organic solvents (Table 1). On addition of large unilamellar vesicles (LUVs), the fluorescence intensity of both probes grew rapidly with lipid concentration and reached stable values at lipid/probe ratios ≥ 125 (Fig. 2), indicating that above this probe/lipid ratio nearly all dye molecules were bound to lipids. These data indicate an efficient binding of the probes to lipid membranes. Remarkably, the fluorescence intensity increased >200-fold and the emission band shifted from 50 to 65 nm to the blue for **F2N12SM** and **FC12SM**, respectively (Fig. 2). The position of the emission band also stabilized at lipid/probe ratios ≥ 125, confirming that optimal binding was achieved in these conditions. The obtained quantum yields in DOPC vesicles were 33 and 37%, for **F2N12SM** and **FC12SM**, respectively, which confirmed their efficient binding to the lipid vesicles. The fluorogenic response of **F2N12SM** and **FC12SM** to membrane binding can have two mechanisms. On one hand, their 3-methoxychromone moieties, owing to the charge transfer binding can have two mechanisms. On one hand, their 3-methoxychromone moieties, owing to the charge transfer

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their hypothetic location with respect to a lipid (black) of the membrane bilayer.

![Fig. 1 Chemical structures of F2N12S and the new membrane probes, F2N12SM and FC12SM, and their hypothetic location with respect to a lipid (black) of the membrane bilayer.](image1)

| Table 1 Spectroscopic properties of F2N12SM and FC12SM in different media* |
|-----------------|-------|-------|-------|
| Probe | Medium | $\lambda_{abs}$ nm | $\lambda_{max}$ nm | QY, % |
| F2N12SM | Water | 412 | 537 | <0.5 |
|  | Acetone | 398 | 501 | 61 |
|  | Dioxane | 400 | 471 | 56 |
|  | DOPC LUVs | 402 | 480 | 33 |
| FC12SM | Water | 445 | 621 | <0.5 |
|  | Acetone | 445 | 578 | 69 |
|  | Dioxane | 449 | 536 | 51 |
|  | DOPC LUVs | 450 | 544 | 37 |

* $\lambda_{abs}$ and $\lambda_{max}$ are positions of the absorption and fluorescence maxima, respectively (nm); QY is the fluorescence quantum yield (%).

![Fig. 2 Fluorescence intensity (open symbols) and emission maximum (filled symbols) of F2N12SM (squares) and FC12SM (triangles) in lipid vesicles (composed of DOPC) at different concentrations of lipids. Probe concentration was 0.4 μM.](image2)

![Fig. 3 Normalized absorption and fluorescence spectra of F2N12SM and FC12SM in lipid vesicles composed of DOPC. Probe and lipid concentrations were 0.4 and 200 μM, respectively.](image3)
the commercially available blue dyes of the coumarin (aminomethylcoumarin acetate, AMCA) or pyrene (Cascade Blue) families, our probes exhibit significantly larger Stokes shifts, which are of key importance for minimizing contribution of the auto-fluorescence in microscopy applications. These large Stokes shifts originate from the dipolar nature of these chromone derivatives, which ensures significant solvent relaxation of the fluorophores.27 As these dyes are solvatochromic, we checked their sensitivity to changes in the lipid composition. Surprisingly, the new dyes showed relatively small variation of their emission maximum for different lipid compositions corresponding to liquid crystalline (DOPC), liquid disordered (DOPC/cholesterol) and liquid ordered (sphingomyelin/cholesterol) phases (Fig. 4). This poor sensitivity to lipid composition is in clear contrast with cholesterol) phases (Fig. 4). This poor sensitivity to lipid composition is in clear contrast with F2N12S or other solvatochromic membrane probes based on Nile Red13 and Lardan.25,26 Nevertheless, this property is of interest for standard imaging and FRET applications, which require stable position of the emission maximum.

Then, we evaluated the photostability of the new probes bound to lipid membranes in comparison to their parent analogue F2N12S. After 1 h of illumination (light flux of ~1 mW cm⁻²), the fluorescence decreased only by 16% and 14% for F2N12SM and FC12SM, respectively, whereas for F2N12S the corresponding fluorescence loss was as high as 88% (Fig. 5). This strongly improved photostability is in agreement with our previous report on 3-methoxycromones in organic solvents29 and is explained by the absence in these dyes of the excited state intramolecular proton transfer generating the less photostable tautomeric form.

3.3. Application for cellular imaging

After addition to suspensions of HeLa cells, the new probes exhibited a single emission band similar to that observed in model membranes composed of DOPC/cholesterol mixture (Fig. 4). The fluorescence intensity and the band shape stabilized within <5 min. The new probes were then added to adherent HeLa cells and studied by confocal fluorescence microscopy. A clear membrane staining was observed for both new probes. They exhibited excellent fluorescence contrast even at low probe concentrations (50 nM), with a signal-to-background ratio of 15–20, while for our reference membrane probe F2N12S it was only 3–4 (Fig. 6B–D). Fluorescence imaging at different concentrations of probes revealed that already at 20 nM of F2N12SM we obtained high-quality images, in contrast to F2N12S that needed concentrations >100 nM (Fig. S4†), in line with our earlier studies of F2N12S.25 To the best of our knowledge, these are the first blue dyes that can be used at such low concentrations for cell membrane staining. Despite these low concentrations, the cell auto-fluorescence remained negligible, which is due to the efficient binding of the probes to cell plasma membranes as well as their high brightness (extinction coefficient × quantum yield ~ 30 000 × 0.4) and large Stokes shift allowing detection far from the excitation wavelength. Importantly, the blue emission region is very convenient, as biologists use extensively green and red emission channels with fluorescent proteins and organic dyes. Particularly interesting in this respect is F2N12SM, because its absorption maximum matches perfectly with the 405 nm laser excitation, while its emission does not overlap with the green channel. We evaluated the compatibility of this probe with markers representative of the most commonly used colours. The first one is LysoTracker® Green DND-26, which labels lysosomes and uses the same instrumental settings as fluorescein, AlexaFluor 488, and eGFP. The second one is mCherry, which stains the cytoplasm and corresponds spectrally to common Rhodamine and Cyanine 3 dyes. From the obtained multi-colour images (Fig. 6E–H), it can be seen that F2N12SM clearly stains the plasma membranes in the presence of the other markers (Fig. 6E). Moreover, its fluorescence is not detectable in the green (Fig. 6F) and red (Fig. 6G)
channels, indicating the absence of cross-talk between the channels. Thus, F2N12SM is perfectly compatible with both LysoTracker® Green DND-26 and mCherry, allowing RGB imaging. Therefore, this new probe could be used to stain the cellular contour with a complementary blue colour, similarly to Hoechst or DAPI used for staining nucleus.

In conclusion, we describe fluorescent membrane probes with convenient absorption in a blue region allowing their combination with common green and red cellular markers for multicolor imaging. To overcome the problem of autofluorescence common for blue dyes, our probes were developed with several key features. Firstly, these probes are fluorescent, so that they turn-on their fluorescence >100-fold after nearly quantitative binding to the lipid membranes. Secondly, they exhibit high brightness and large Stokes shift, which enable their use at low concentrations (20 nM), applying a simple staining protocol. We expect that these new probes may become as common for multicolour cellular staining as blue nuclear staining dye Hoechst or DAPI.

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