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Abstract

A variety of protein tags are available for genetically encoded protein labelling, which allow their precise localization and tracking inside the cells. A new dimension in protein imaging can be offered by combining protein tags with polarity-sensitive fluorescent probes, which provide information about local nanoscale environment of target protein within the subcellular compartments (organelles). Here, we designed three fluorescent probes based on a solvatochromic Nile Red dye, conjugated to a HaloTag reactive targeting group through PEG linkers of varying length. The probe with medium linker length, NR12-Halo, was found to label specifically a large variety of proteins localized in defined cell compartments, such as plasma membranes (outer and inner leaflets), endoplasmic reticulum (ER), Golgi Apparatus, cytosol, microtubules, actin and chromatin. Owing to polarity-sensitive fluorophore, the probe clearly distinguished the proteins localized within apolar lipid membranes from other proteins. Moreover, it revealed dramatic changes in the environment during the life cycle of proteins from biosynthesis to their expected localization and finally, to recycling inside lysosomes. Heterogeneity in the local polarity of some membrane proteins also suggested a formation of low-polar protein aggregates, for example within cell-cell contacts. The approach also showed that mechanical stress (cell shrinking by osmotic shock) induced general polarity decrease in membrane proteins, probably due to condensation of biomolecules. Finally, the nano-environment of some membrane proteins was affected by polyunsaturated fatty acid (PUFA) diet, which provided the bridge between organization of lipids and proteins. The developed solvatochromic HaloTag probe constitutes a promising tool for probing nanoscale environment of proteins and their interactions within subcellular structures.

Introduction

Specific targeting and imaging of proteins of interest directly inside the cells is the key for studying protein functions in their native environment.[1-3] For such purpose, optical imaging using fluorescent reporters is undoubtedly the most widely used imaging modality.[4-7] The gold standard of genetically encoded protein visualization via fluorescence involves the use of expressed protein of interest fused with the green fluorescent protein (GFP).[8-10] Beyond basic fluorescent labelling, this approach enabled monitoring protein function in form of biosensors that responds to an analyte, enzymatic reaction or/and protein conformational change inside the cells.[6,11] However, the fluorophore of the fluorescent proteins is caged inside the protein beta-barrel scaffold, which renders them poorly sensitive to the molecular environment. Even though, some fluorescent proteins exhibit sensitivity to local pH[12,13] and redox potential,[14] fluorescent proteins are generally not adapted for monitoring the protein surrounding (nano-environment). New possibilities appeared in the last two decades by the introduction of self-labeling protein tags, such as SnapTag,[15,16] HaloTag[17] and ClipTag,[18] which covalently attach specific substrates.[3] These chemogenetic tags enable protein labelling with a wide range of small fluorescent probes,[5,19] which makes the labelling with small molecules highly versatile and adaptable to different types of sensing and imaging techniques. One of the most used protein tags of that kind is probably the dehalogenase-based HaloTag[17] that enables effective ligation with its corresponding ligand, the chloroalkane scaffold. Indeed, in addition of being a robust and efficient targeting system, the chloroalkane motif is chemically simple, small and rather bioorthogonal in eukaryotic cells.[20]

Nowadays, a wide range of HaloTag fluorescent ligands are commercially available and more have been reported in literature, covering almost all the UV to NIR spectra with various spectroscopic properties.[5,21] They are generally based on the most standard fluorescent scaffolds used in bioimaging, which include different dye families based on e.g. fluoresceins, rhodamines, cyanines, BODIPYs, allowing tracking proteins down to single-molecule level with nanoscale resolution.[5,19]

To go further in protein imaging, smart fluorescent dyes can be designed. Thus, fluorogenic light-up probes enable protein imaging in cells with minimal background.[16,22-24] A particularly promising direction is protein labelling with a fluorescent probe that provides information about the protein close neighborhood, because intracellular environment is complex, presenting multitude of subcellular structures, such as organelles. Thus, genetically encoded protein targeting enabled monitoring pH[25] and redox potential[26] in different subcellular compartments. New possibilities are offered by using environment-sensitive probes,[22] which change their emission color or intensity in response to local molecular surrounding properties, such as polarity, viscosity and molecular order. Indeed, molecular rotors, which change their intensity and fluorescence lifetime in response to viscosity, were successfully used to label proteins using HaloTag and then mapping microviscosity dynamics in cellular organelles and the effects external mechanical stress.[27] Fluorescent flippers, sensitive to molecular order,[28] were successfully used to visualize lipid surrounding of HaloTagged proteins, in particular membrane tension in different orgenelles.[29] Solvatochromic dyes change emission color in response to surrounding polarity,[22] which is a fundamental property of environment related to dipolar interactions and hydrogen-bonding interactions driven by hydration. Solvatochromic dyes have been particularly useful to monitor protein-protein interactions,[30] and protein aggregation in cells[31] as well as lipid organization of biomembranes.[22] Among them, Nile Red dye and its derivatives has been particularly popular, allowing super-resolution imaging of biomembranes,[32-34] polarity mapping of cell organelles[35] and monitoring ligand-protein interactions.[36] Recently, it has also been applied for probing lipid surrounding of receptors in the cell plasma membrane using SnapTag-based labeling[37,38] or specific ligands.[39] Genetically encoded tagging of Nile Red also helped to monitor protein aggregation in cells.[40] An attractive direction that has not been well explored

to date is to use solvatochromic dyes for chemogenetic labeling proteins in different organelles for polarity mapping of their local surrounding, as it has been done for molecular rotors and fluorescent flippers.

In the present work, we developed an environment-sensitive HaloTag probe based on Nile Red for the chemogenetic labeling of proteins expressed in different organelles of live cells. We found that length of the polar PEG linker between the dye and the chloroalkane reactive group was crucial to ensure specific protein targeting. The probe revealed characteristic signatures of environment polarity for proteins in different subcellular locations, with the following order of polarity decrease free protein > protein complexes > membrane proteins. Moreover, the approach enabled monitoring changes in the protein nano-environment during protein recycling, mechanical stress and lipid diet, showing that it depends drastically on protein location.

Experimental section

General Methods and Materials. All the reagents were purchased from Sigma-Aldrich, Alfa Aesar, or TCI and used as received. Milli-Q-water (Millipore) was used in all experiments. NMR spectra were recorded at 20 °C on a Bruker Avance III 400 spectrometer. Mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer. Absorption spectra were recorded on a Cary 5000-UV-Vis-NIR spectrophotometer (Agilent), and emission spectra were recorded on a FS5 (Edinburgh Instruments) spectrofluorometer. NR probes titrations with liposomes have been done using a TECAN Spark fluorescence plate reader in black flat-bottomed PS 96-wells plates without lid. **NR12A** was synthesized according to the previously published procedure.

Cell Lines, Culture Conditions, and Treatment. KB (ATCC CCL-17) cells were grown in Dulbecco's modified eagle medium (DMEM, Gibco Invitrogen) and supplemented with 10% fetal bovine serum (FBS, Lonza), 1% L-glutamine (Sigma-Aldrich), 1% non-essential amino acid solution (Gibco-Invitrogen), and 1% MEM vitamin solution (Gibco-Invitrogen) at 37 °C in a humidified 5% CO2 atmosphere. Cells were seeded onto a chambered coverglass (IBIDI) at a density of 5×10^4 cells/well. Cells were seeded 24 h before the microscopy measurement for **NR12A** probe. For Halo-NR, cells have been imaged 24 h after transfection. For microscopy imaging with **NR12A**, the attached cells in IBIDI dishes were washed once with warm Hanks' balanced salt solution (HBSS, Gibco-Invitrogen); after that, 1 mL of a corresponding dye solution in warm HBSS was added and the cells were incubated for 10 min at room temperature. For HaloTag probes, cells in IBIDI dishes were washed once with warm phosphate buffer saline (PBS, Gibco-Invitrogen), after that, 1 mL of a corresponding dye solution in warm Opti-MEM was added and the cells were incubated for 30 min at 37 °C. Then, the staining solution was washed and attached cells were incubated in complete medium for 30 min at 37 °C under 5% CO_2 atmosphere to remove unreacted dye. Before imaging, attached cells were always washed with Opti-MEM once and microscopy experiments were run in Opti-MEM.

Unsaturated lipid diet. For **NR12A** imaging, 16 h to 24 h after been seeded into Ibidi chambers, KB cells have been incubated in growing media supplemented with docosahexaenoic acid (DHA) (20 μM). After 24 h of diet, KB cells have been washed with HBSS and incubated in HBSS with **NR12A** (1 μM) for 10 min. Then, cells were washed with warmed HBSS twice and imaged in Opti-MEM. For **NR12-Halo**, 16 h after transfection, KB cells have been incubated in growing media supplemented with docosahexaenoic acid (DHA) (20 μM). After 24 h of diet, KB cells have been labeled as previously described.

Results and discussion

Design of the probes

The concept of nano-environment polarity sensing of proteins exploits solvatochromic dye grafted to a protein of interest through an appropriate linker. The free protein is expected to expose its solvatochromic label to highly polar aqueous medium. On the other hand, interactions of proteins with nucleic acids, other proteins and especially lipid membranes are expected to decrease the local polarity, thus leading to blue shifts in the emission of the solvatochromic dye (Figure 1A). To address the nano-environment of proteins within organelles, the solvatochromic probe should be grafted to a protein of interest in situ inside live cells (Figure 1B). For this purpose, we designed three solvatochromic probes based on Nile Red bearing HaloTag targeting group through PEG linkers of different length: **NR2-Halo**, **NR12-Halo** and **NR26-Halo** (Figure 1C). The linker length is expected to affect two properties: (1) minimize non-specific interactions with hydrophobic sites of the cells; (2) optimize cell membrane permeability; (3) ensure sufficient freedom for Nile Red moiety to interact with environment of the protein of interest (e.g. proximal lipid membrane). To synthesize the probes, we used Nile Red derivative, NR-COOH (Figure S1), which was recently used in conjugation with different ligands for chemical targeting of different cell organelles.[32,35] We have chosen the derivatization of Nile Red at the amino-end because it was shown to ensure good photostability of the fluorophore.[32] In case of the probe with the shortest linker containing two ethylene glycol units (**NR2-Halo**), NR-COOH was coupled directly to corresponding amine **1a**. The other two probes containing additional 10 and 24 ethylene glycol units, **NR12-Halo** and **NR26-Halo**, were obtained by coupling NR-COOH with the corresponding t-butyl protected PEG amino acids, followed by removal of the t-butyl and finally coupling to the amine **1a** (Figure S1). The identity of the new compounds was confirmed by NMR and mass spectrometry (Figures S2-S4).

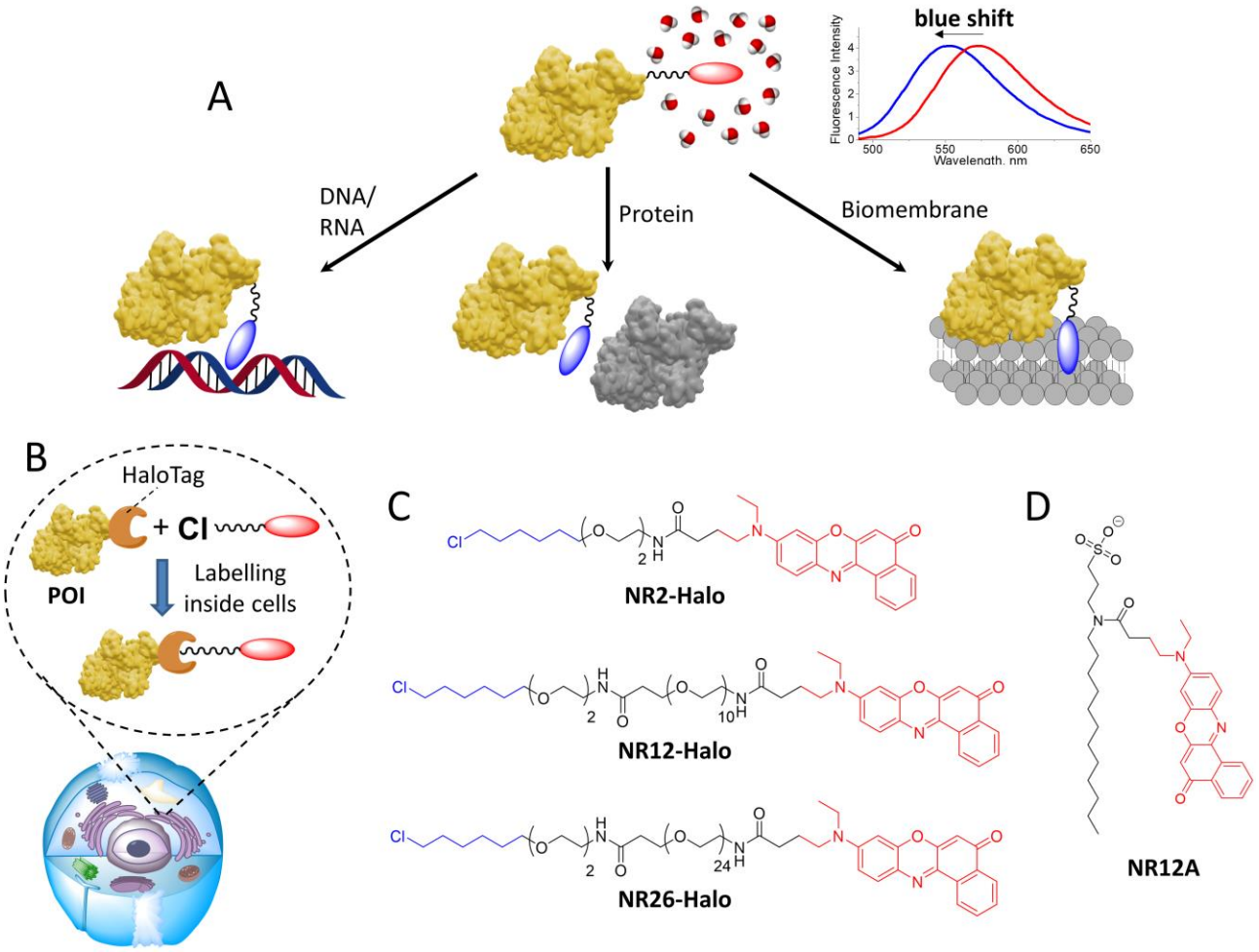

Figure 1. Principe of the genetically encoded polarity sensing. (A) Sensing of nano-environment of a protein and its interactions with partners by a solvatochromic dye. (B) Protein-HaloTag labeling by a solvatochromic dye inside the cells. (C) Chemical structures of the synthetized fluorescent probes: **NR2-Halo**, **NR12-Halo**, and **NR26-Halo**. (D) Structure of NR12A.

Absorption and fluorescence spectroscopy in various solvents showed that **NR2-Halo**, **NR12-Halo** and **NR26-Halo** exhibited no significant difference from Nile Red (NR) in terms of spectroscopic properties and solvatochromism (Figures 2 and S5). Indeed, all NR derivatives showed similar bathochromic shifts in absorption and emission spectra on increase in solvent polarity, indicating that these Halo-derivatives preserved the solvatochromism of their Nile Red fluorophore.

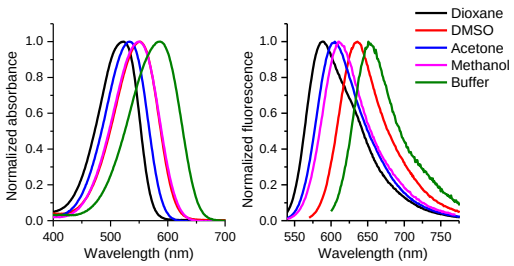

Figure 2. Normalized absorption and fluorescent spectra of studied **NR12-Halo** in various solvents. Excitation wavelength are respectively 520 nm, 550 nm, 530 nm, 550 nm, 590 nm in 1,4-dioxane, DMSO, acetone, methanol and in phosphate buffer 20 mM at pH = 7.4.

Then, in order to estimate affinity of NR-Halo derivatives to lipid membranes, they were titrated with suspensions of large unilamellar vesicles (LUVs) composed of unsaturated lipid DOPC (Figure 3). **NR2-Halo** exhibited significant increase in the fluorescence intensity with addition of DOPC LUVs, which was similar to that of NR. Thus, **NR2-Halo** remains relatively hydrophobic and, thus, similarly to NR binds non-specifically to lipid membranes. In sharp contrast, **NR12-Halo** and **NR26-Halo** exhibited negligible changes in the fluorescence intensity in the tested concentrations up to 500 µM (Figure 3), indicating poor affinity to lipid membranes. This effect could be due to hydrophilic PEG moiety that is long enough for **NR12-Halo** and **NR26-Halo** to prevent the lipophilic NR core to bind to lipid bilayer. We can conclude that **NR12-Halo** and **NR26-Halo** are advantageous compared to **NR2-Halo** because they are expected to exhibit lower off-target fluorescent signal in live cells imaging caused by non-specific binding to biomembranes.

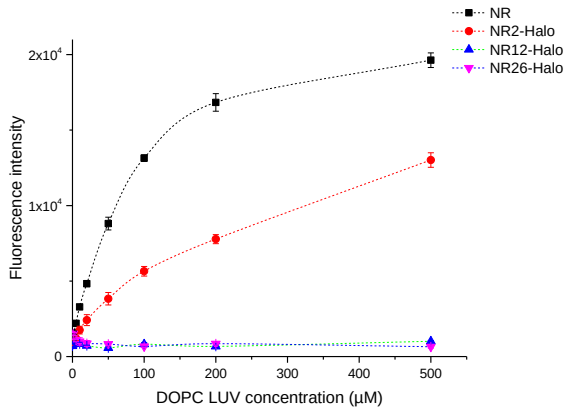

Figure 3. Titration of studied NR derivatives in PB 20 mM at pH 7.4 with DOPC LUVs. Excitation wavelength 540 nm, emission wavelength 640 nm. The indicated concentration corresponds to the lipid concentration.

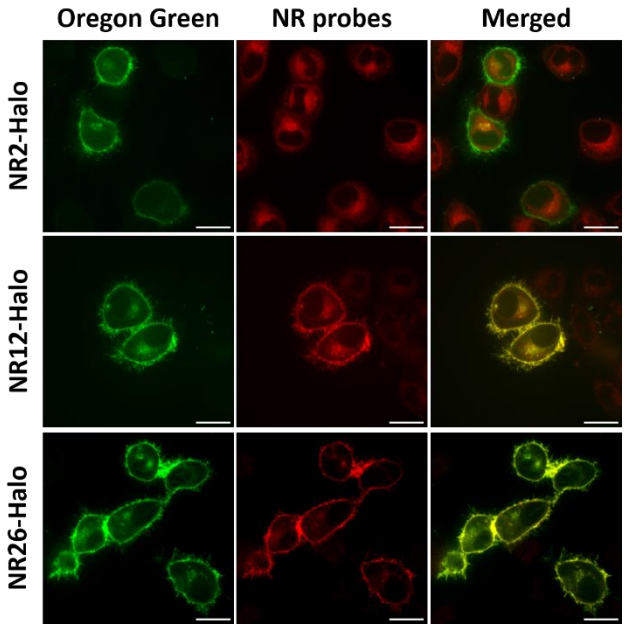

Figure 4. Evaluation of NR Halo probes in cells expressing HaloTag together with a membrane protein. Confocal spinning disk microscopy of **NR2-Halo**, **NR12-Halo** or **NR26-Halo** with Oregon Green HaloTag in KB cells transfecfted with HaloTag fused to cell surface protein (cell surface 1). Transfected cells were incubated in Opti-MEM supplemented with NR Halo probe (500 nM) and Oregon Green HaloTag (500 nM) for 30 min at 37 °C. Afterwards, cells were incubated in complete medium for 30 min at 37 °C and finally washed and imaged in Opti-MEM. Scale bars: 20 µm.

Protein labelling in cells

Next, the ability of NR-Halo probes to target HaloTagged fused proteins in different subcellular structures have been explored in transfected live cells. To this end, we fused HaloTag to (i) the platelet derived growth factor receptor (PDGFR) transmembrane domain for plasma membrane outer leaflet labeling, (ii) an endoplasmic reticulum (ER) targeting sequence for ER labeling, (iii) the N-terminal 81 amino acids of the human beta-1,4-galactosyltransferase for Golgi apparatus labeling, (iv) the zebrafish H2B for chromatin labeling, (v) the microtubule-associated protein (MAP) 4 for microtubule labeling, and (vi) the actin-binding peptide LifeAct for actin labeling.[41] Localizations of the HaloTagged proteins were validated by fluorescence microscopy using reference probe TMR-HaloTag (Figure S6). One should note that here we selected proteins with different types of environments: free proteins, assembled proteins and those located close to biological membranes. The latter are particularly important, as we expect to see significant decrease in polarity close to lipid membranes compared to free proteins. The cells were transfected with corresponding proteins and then labelled with NR-Halo probes and a commercial probe (Oregon green HaloTag), in order to study their colocalization by fluorescence microscopy. First, we compared three NR Halo probes in cells expressing HaloTag fused to plasma membrane protein (outer leaflet). It was clear, that **NR2-Halo** showed fluorescence in all cells, while Oregon green HaloTag labelled specifically cell surface of only some cells (Figure 4). Moreover, in cells identified by Oregon green HaloTag, the staining of **NR2-Halo** was all over the cells without clear membrane profile. Non-transfected control cells as well as cells expressing HaloTag in other cell compartments (cytosol and Golgi apparatus) showed the same non-specific fluorescence (Figure S7). Thus, in line with experiments in liposomes, **NR2-Halo** showed strong non-specific interactions with lipid membranes all over the cells and thus cannot target specifically HaloTag in live cells. In sharp contrast, **NR12-Halo** and **NR26-Halo** showed good colocalization with Oregon green HaloTag, as showed in Figure 4 for cells transfected with HaloTag at the cell surface. Non-transfected control cells showed negligible signal for both **NR12-Halo** (Figure 5) and **NR26-Halo** probes (Figure S8), confirming their low non-specific interactions with lipid membranes. In cells transfected with HaloTag in different subcellular compartments, such as cell surface 2 outer leaflet, Golgi, actin and chromatin (Figure 5) as well as cytosol, microtubules, ER and inner leaflet of plasma membrane (Figure S8), both probes showed generally good colocalization with the reference marker Oregon Green HaloTag (Table S2). However, **NR26-Halo** usually gave significantly lower signal and, in some cases (endoplasmic reticulum and cytosol HaloTag), lower colocalization coefficients (Table S2). We can conclude that the **NR12-Halo** contains the linker of optimal size: it is long enough to prevent non-specific binding to biomembranes (in contrast to **NR2-Halo**) and at the same time short enough to ensure good chemical reactivity with HaloTag protein and probably better cell penetration, compared to **NR26-Halo**. These results corroborate well with the earlier studies based on Halo-Flipper probes, where intermediate-sized PEG linker was also necessary to achieve effective labelling of proteins inside the cells.[29] For those reasons, the further experiments where focused on **NR12-Halo**, which was clearly the most promising probe.

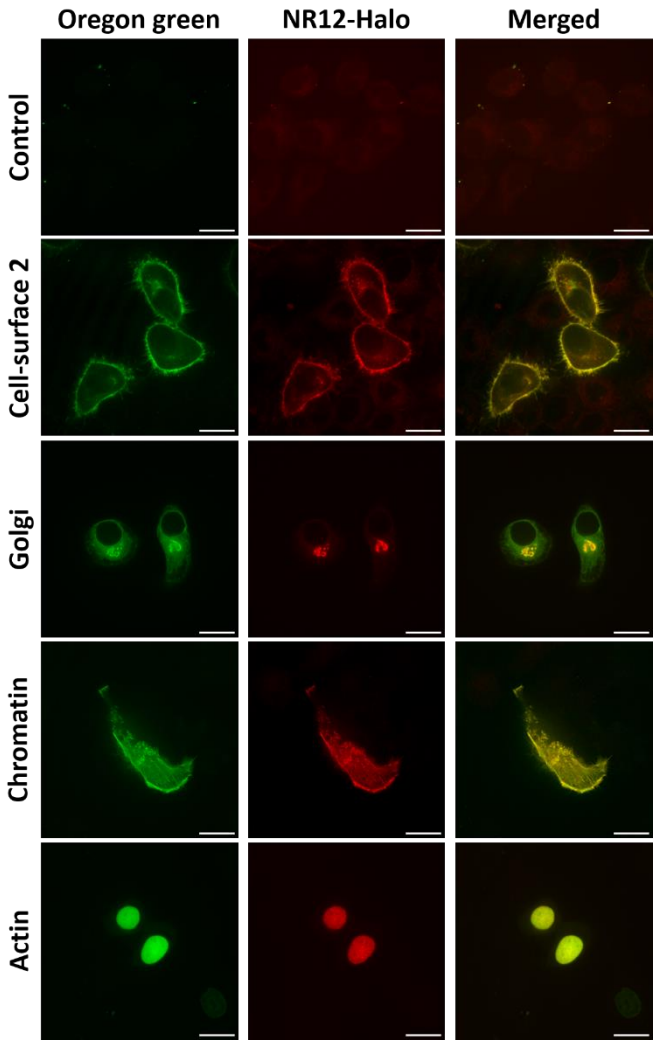

Figure 5. Examples of fluorescence images of NR12-Halo applied in cells transfected with HaloTag in different cell compartments. Colocalization of NR12-Halo with Oregon Green HaloTag in transfected KB cells imaged by confocal spinning disk microscopy. Transfected cells were incubated in Opti-MEM supplemented with NR12-Halo (500 nM) and Oregon Green HaloTag (500 nM) for 30 min at 37 °C. Afterward, cells were incubated in complete medium for 30 min at 37 °C and finally changed for Opti-MEM before imaging. Scale bars: 20 μm.

Sensing local polarity of proteins

The next steps of our study were dedicated to exploring solvatochromism of NR fluorophore to sense the nano-environment of targeted proteins in different sub-cellular environments. To this end, ratiometric confocal microscopy on transfected KB cells were performed, where we recorded a blue-shifted region (550-600 nm) and the red-shifted one (600-650 nm) with respect to NR emission maximum in lipid membranes[32,35] and then built the polarity index. As the ratio changed to a broad range, simple ratio was not convenient because, which nonlinear function provided non-even presentation for low and high values of the ratio (Figure S9-S10). Therefore, we propose to use arctan function of the long-wavelength (600-650 nm) to short-wavelength (550-600 nm) ratio, which provides is a more convent function which changes smoothly in the whole range of polarity from 0 to 1 (Figures S11-S13). The details on the polarity index analysis are given in the supplementary information. In the obtained polarity index, the higher values correspond to higher contribution of the red-shifted channel vs blue shifted one, indicating higher polarity. Strikingly, the pseudo-color in the ratiometric images showed strong dependence on the localization of the HaloTag in the cells (Figure 6). Indeed, for HaloTag fused to membrane proteins, e.g. those collocated in plasma membrane (both

leaflets), ER and Golgi, the polarity index values were generally low, close to those of solvent acetone (Figure 6). On the other hand, HaloTag in cytosol or in complex with actin or inside chromatin showed high polarity values, close to that of ethanol (Figure 6). Thus, our polarity-sensing approach can distinguish proteins located close to biological membranes from those located in cytosol. First, for all these proteins, the local polarity is significantly higher than that of membrane probe **NR12A** (Figures 1D, 6). Thus, the NR fluorophore being linked though PEG linker to a protein is much less deeply imbedded into lipid membranes compared to that in the lipid membrane probe. One should note the intermediate polarity values for HaloTag with microtubules, probably because their environment is less exposed to water compared to free proteins in cytosol (Figure 6). Thus, our results provide evidence that Halo-Tag labelling of proteins with solvatochromic dye Nile Red allows sensing local environment of protein of interest (Figure 1). The approach enables clear distinction of protein neighborhood, particular presence of low polar lipid membranes, and to lower extent protein assembly.

Careful look into the local polarity of membrane proteins yield additional interesting observations. Comparison within membrane proteins shows that polarity increases in the following order PM inner leaflet < Golgi < cell surface 2 < cell surface 1 < ER (Figure 6). These observations are unexpected, because one should expect the outer leaflet to be less polar than the inner one, because of high concentration of sphingomyelin lipid in the former. Importantly, the bleaching of NR by sodium dithionite[42] confirmed that the leaflet-specific labelling by **NR12-Halo**, with strong bleaching for the outer leaflet labelling, and no bleaching effect for the inner leaflet labelling (Figure S14). Thus, the unexpected difference in polarity shows that the response of the probe depends on the labelled membrane protein, probably defined by the distance between the fused HaloTag and the lipid membrane surface. This assumption can explain also the fact that local polarity of Halo Tag in the cell surface is even higher than that in Golgi, which does not correspond to the observations made by organelle-specific lipid binding NR probes.[35] A dedicated structural studies on different membrane proteins fused with HaloTag would be needed to understand better the correlation with the probe response.

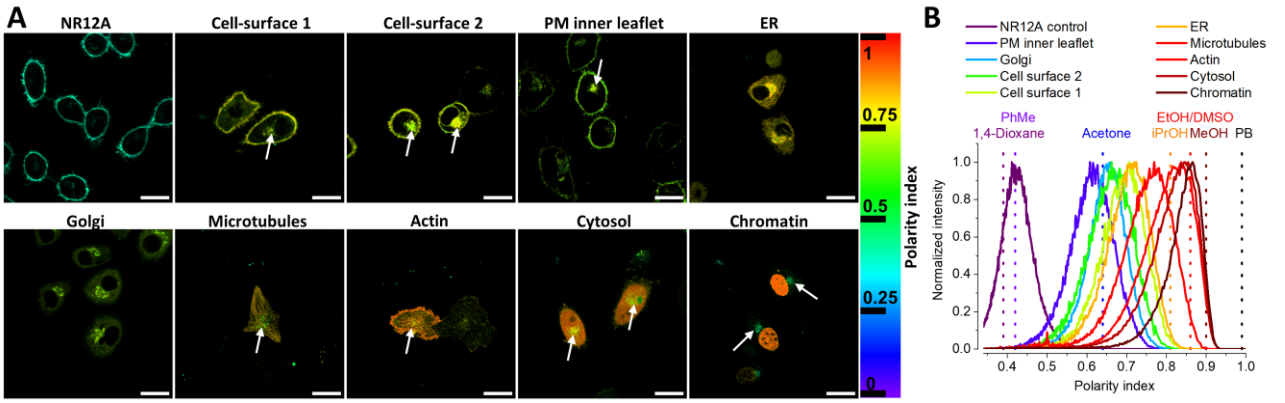

Figure 6. (A) Polarity imaging of KB cells expressing HaloTagged proteins at various localization in CLSM. **NR12-Halo** at 1 µM in Opti-MEM used as fluorescent probe. Scale bars: 20 µm. White arrows point at inner cells structures. (B) Distribution curves of the polarity index normalized at their maximum.

Monitoring protein trafficking

In addition to the signal from expected sub-cellular location, we also observed some fluorescence from perinuclear region of the cells, characterized by different local polarity values (Figure 6, white arrows). To understand better the origin of this additional labelling, transfected cells were stained with **NR12-Halo** and then washed to remove the excess of dye and prevent further labeling. Then, cells were incubated in the complete medium for 30 min or 5 h to observe the trafficking of the labelled proteins at two different times

after the labelling. For this experiment, membrane protein labeling at the PM outer and inner leaflets was chosen. To identify the localization of proteins inside the cells, they were co-stained with ER Tracker green and Lyso tracker deep red before imaging (Figure S15). At 30 min incubation, **NR12-Halo** labelling for outer leaflet showed emission mainly at the plasma membrane with additional weak emission inside the cells. The latter co-localized to some extend with ER tacker, but not with Lyso Tracker. After 5h incubation, the labelling significantly changed, showing lower emission at the plasma membrane and strong emission in the perinuclear region (Figure S15). The latter colocalized well with Lyso Tracker, but not with ER Tracker, which is the opposite to that observed for a short incubation time. The labelling profile for the inner leaflet labelling was somewhat different, but the tendency in the co-localization over time was similar (Figure S15): at the early time, the intracellular fluorescence of **NR12-Halo** was closer to that of ER tracker, while after 5h incubation it was very similar to that of Lyso Tracker. We expect that at the early times after the **NR12-Halo** labelling, intracellular fluorescence is probably related to freshly synthesized protein within ER compartments, in line with the observed co-localization with the ER tracker. However, after the long incubation, the protein recycling leads to accumulation of the labelled proteins inside the lysosomes, which explains the observed co-localization with the Lyso Tracker. Thus, we can conclude that the observed emission close to nucleus are proteins being synthesized at the ER or trapped in the lysosomes. This could explain much lower local polarity for those structures in case of proteins destinated for the microtubules, actin, cytosol and chromatin. Indeed, when the protein is synthesized inside ER or trapped in the lysosomes it is probably trapped close to lipid membranes, which may force decrease in the local polarity. The situation is more complex for membrane proteins, because they are expected to be bound to lipid membranes during their life cycle starting from synthesis up to their recycling.

To reveal the evolution of nano-environment polarity of a membrane protein during its life cycle, the time-dependent study was performed using ratiometric microscopy imaging at 30 min and 6 h after **NR12-Halo** washing with transfected cells addressing HaloTagged protein on outer leaflet 2 (Figure 7). At the early incubation time (30 min), the intracellular fluorescence, which could be at least in part assigned to newly synthesized proteins in ER showed significantly lower polarity compared to that on the plasma membrane. After 6h incubation, the intracellular fluorescence appeared in form of dots, which according to the data above should correspond to recycled protein inside lysosomes. The dominating green pseudo-color of these dots suggest that in these compartments the local polarity is lower than that at the plasma membrane. Thus, after recycling into the lysosomes, the local polarity of proteins also decreased compared to the plasma membranes. Another remarkable observation is that over time the local polarity of proteins at the plasma membrane increased, and this was associated with the decrease in the local concentration of the protein on the surface (Figure 7), as also observed in the co-localization studies (Figure S15). It was also found that zones with high intensity showed tendency to present lower values of local polarity (Figure S16), suggesting that higher local concentration of the protein could explain in part the decrease in the local polarity. Assuming that the protein structure and insertion inside the membrane does not change over time, we hypothesized that the color variations of the protein in the membrane could be related to protein-protein association at the level of membrane, which should directly affect the local polarity by water exclusion from the site of interaction. Indeed, at the short incubation time, the concentration of the protein at PM was much higher, which favored dye-dye aggregation, thus decreasing the local polarity. This hypothesis can be directly supported by the significantly decreased local polarity in the cell-cell contacts (see green emission between the two cells in Figure 7), where protein-protein association is favored. The protein aggregation is also in line with the fact that the cells surface for both short and long incubation times is heterogenous in terms of emission color, where relatively large green and orange zone could be identified (Figure 7). It is well established that the cells surface is highly heterogenous, where proteins and lipids form microdomains with highly varied local concentrations and properties.[43,44] Moreover, platelet derived growth factor receptor used

in this study, is prone to dimerization and multimerization, which support the hypothesis.[45] On the other hand, the protein dimerization or aggregation can also explain lower polarity observed within ER at the early labelling time (30 min) and inside lysosomes after the long incubation time (6h). Indeed, we could speculate that after the transfection, its overexpression leads to high local concentration at the ER during synthesis and further accumulation inside lysosomes, both favor protein-protein interactions and thus decrease in the local polarity. We need to stress, this protein dimerization/aggregation is our preliminary explanation of these significant variations in polarity of the membrane protein, and a dedicated biological study would be required to understand the exact mechanism of the observed phenomena.

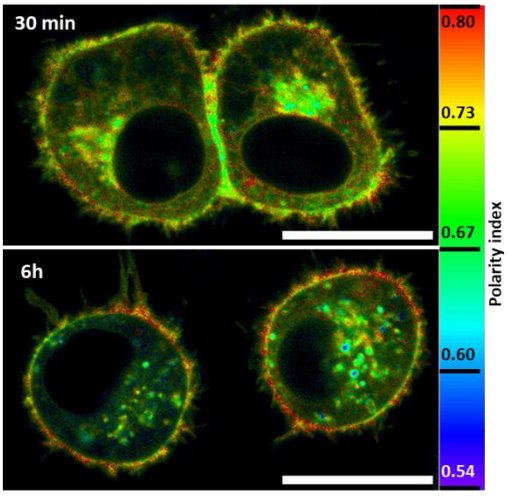

Figure 7. Polarity imaging of transfected KB cells expressing HaloTagged proteins cell surface 2 in CLSM. **NR12-Halo** at 1 µM in OptiMEM was used as fluorescent probe. Scale bars: 20 µm.

Monitoring mechanical stress and diet

To explore the ability of **NR12-Halo** to sense dynamically its local environment, transfected KB cells labelled at the corresponding subcellular compartment with **NR12-Halo** were subjected to mechanical stress applied using osmotic shock (Figure 8). The latter is commonly used to induce mechanical effects on biological membranes, monitored by fluorescent flippers,[29,46] molecular rotors[27] and solvatochromic dyes.[35] Hypertonic condition, using concentrated sucrose solutions, is expected to compress cells, while hypotonic condition, using desalted water, is expected to swell them. After applying a mechanical stress, the cells were immediately imaged by ratiometric confocal microscopy and the results were compared to isotonic conditions. For this study we used only membrane associated proteins located in different cell compartments. In case of plasma membrane proteins, we observed a decrease in the local polarity upon hypertonic conditions (Figure 8). It should be noted that the opposite was observed for the reference membrane probe **NR12A**, in line with the earlier study.[35] This opposite behavior is probably related to different processes in response to the mechanical stress: **NR12A** detects changes at the lipid bilayer, while labelled membrane proteins sense changes in their insertion and/or aggregation state in the lipid membrane. We could speculate that cell shrinking contracts the cell membranes which brings fluorescent probe closer to plasma membranes, thus decreasing the local polarity. Moreover, cell shrinking could also bring membrane proteins closer to each other leading to their aggregation, that could also contribute to the blue shifts in the probe emission. Upon hypotonic conditions, the effects were either negligible or small in the direction of higher polarity (Figure 8). Again, the reference plasma membrane probe **NR12A** showed the opposite effect: decrease in the local polarity, highlighting different sensing profiles protein labelled and lipid membrane labelled NR dye. Importantly, we could see that pseudo-color of membranes depended slightly

on the orientation of the plasma membrane with respect to the polarization of the excitation light. The latter is usually observed in giant vesicles because of selective excitation effects,[42] and thus clearly indicates that Nile Red fluorophore is inserted into lipid membrane, while being covalently grafted to the membrane proteins. The polarization effect is only observed in flat membranes of swelled cells dye to the hypotonic conditions but not in normal ones, presenting highly rough plasma membranes. The fluorophore insertion into the membrane is in line with significantly lower polarity observed for all membrane proteins compared to other labelled proteins.

In case of ER, hypertonic conditions showed decrease in the local polarity. Contraction of the cells could also produce compaction of ER and deeper immersion of the labelled protein (and thus the probe) into lipid membrane. This dramatic polarity drop is in line with our earlier data for Nile Red ER-targeting probe, which was poorly inserted into lipid membrane,[35] as well as with reported increase in viscosity, reported for ER-targeted molecular rotor. Hypotonic conditions, however, produced changes in ER morphology, which could be explained by possible damage at the level of ER, making interpretation of color changes more difficult. In case of Golgi, the small decrease in local polarity was observed in hypertonic conditions, while hypotonic conditions produced again significant changes in the morphology of this organelle.

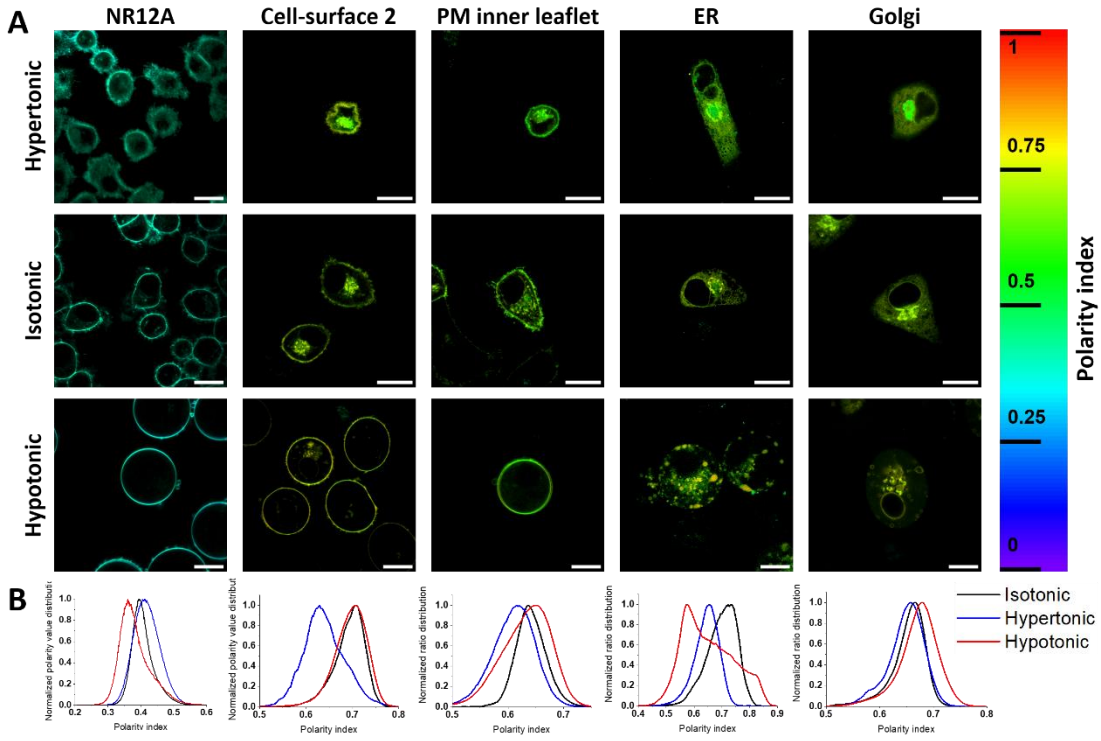

Figure 8. (A) Polarity imaging of transfected KB cells expressing HaloTagged proteins at various localization in CLSM under condition of osmotic stress. **NR12-Halo** at 1 µM in Opti-MEM was used as fluorescent probe. Scale bars: 20 µm. (B) Distribution curves of the polarity index normalized at their maximum.

To explore further the sensitivity of **NR12-Halo** to its environment, KB cells were submitted to a fatty acid diet using a growing media supplemented in docosahexaenoic acid (DHA) (Figure S17). DHA was selected because it was reported to induce lipidome remodeling in mammalian cells via a lipidic homeostasis mechanism.[47]

First, the membrane probe **NR12A**, was used to control the effect of DHA diet on KB cells. The DHA diet provided higher polarity values, which is consistent with the expected increase concentration of unsaturated lipids in the plasma membrane. Similar effect was observed for KB cells expressing HaloTagged proteins on

PM outer leaflet. The lower sensitivity for the labelled proteins can be explained by **NR12-Halo** being inserted in the membrane than **NR12A**. By comparison, KB cells expressing HaloTagged proteins addressed to the PM inner leaflet displayed no polarity changes, probably because the inner leaflet naturally contains more unsaturated lipids compared to the outer leaflet.[43] Concerning the internal organelles (ER and Golgi), an increase in the reported polarity was observed. Overall, these results confirm that being labelled to membrane proteins, NR dye can detect changes in their lipid environment, probably because of partial insertion of the dye into lipid membrane.

Conclusion

In the present study, a solvatochromic probe for HaloTag targeting have been developed based on the Nile Red scaffold. The exploration of several linker lengths allowed us to optimize the unspecific binding by balancing the lipophilicity of the NR moiety with the hydrophilicity of the PEG linker. The resulting optimized probe **NR12-Halo** showed an ability to access and specifically target HaloTagged expressed proteins in corresponding subcellular structures, such as plasma membranes, organelles, or cytosol. Because of its solvatochromic properties, **NR12-Halo** provided not only the localization of the protein of interest but also information about its close neighborhood. Owing to sensitivity of the dye to polarity, it clearly distinguished proteins localized within apolar lipid membranes from other proteins. It revealed dramatic changes in the environment during the life cycle of proteins from synthesis in ER to their expected localization and finally, to recycling inside lysosomes. The effects were particularly strong for non-membrane proteins with naturally high polarity in the cytosol and much lower polarity at the early and late stage of their life cycle, being close to lipid membranes. Heterogeneity in the local polarity of some membrane proteins also suggested formation of low-polar protein aggregates, for example within cell-cell contacts. However, a dedicated study is needed do understand the hypothetic aggregation, because the finding may have a large range of applications. The mechanical stress (cell shrinking by osmotic shock) showed general polarity decrease in membrane proteins, probably due to condensation of biomolecules with exclusion of highly polar water molecules. The phenomenon was opposite to that observed for reference solvatochromic membrane probe **NR12A**, suggesting that the same solvatochromic dye targeted to proteins and lipids detects different processes during the mechanical stress. Finally, nano-environment of some membrane proteins was affected by polyunsaturated fatty acid (PUFA) diet, which provided the bridge between organization of lipids and proteins. Overall, in comparison to traditional HaloTag labels and organelle tracers, the new probes provide information about polarity of the local nanoscale environment of proteins in cells, which corelates with their localization with respect to biomembranes, folding, aggregation and interactions with other biomolecules. The approach could be extended to variety of proteins and protein tags, and it could be explored for other types of polarity-sensitive dyes. It is expected to help cell biologists to study proteins in cells by monitoring their local environment polarity, in order to better understand their life cycle, aggregation, interaction with lipids and other biomolecules, implication in biomolecular condensates and monitoring their response to external cell stress.

Conflicts of interest

There are no conflicts to declare.

Supporting information

Additional experimental details, synthesis protocols, MS spectra of synthetized probes, additional spectroscopy and microscopy data and detailed methodology concerning the polarity index.

Acknowledgements

This work was supported by Agence Nationale de la Recherche (ANR) AmpliSens ANR-21-CE42-0019-01. R.P. acknowledges support from SATT Conectus Alsace (Maturation grant LUNA). D.I.D. was supported by a fellowship from the Ministère de la Recherche (France).

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