Genetically Encoded Supramolecular Targeting of Fluorescent Membrane Tension Probes within Live Cells: Precisely Localized Controlled Release by External Chemical Stimulation

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ABSTRACT: To image membrane tension in selected membranes of interest (MOI) inside living systems, the field of mechanobiology requires increasingly elaborated small-molecule chemical tools. We have recently introduced HaloFlipper, i.e., a mechanosensitive flipper probe that can localize in the MOI using HaloTag technology to report local membrane tension changes using fluorescence lifetime imaging microscopy. However, the linker tethering the probe to HaloTag hampers the lateral diffusion of the probe in all the lipid domains of the MOI. For a more global membrane tension measurement in any MOI, we present here a supramolecular chemistry strategy for selective localization and controlled release of flipper into the MOI, using a genetically encoded supramolecular tag. SupraFlipper, functionalized with a desthiobiotin ligand, can selectively accumulate in the organelle having expressed streptavidin. The addition of biotin as a biocompatible external stimulus with a higher affinity for Sav triggers the release of the probe, which spontaneously partitions into the MOI. Freed in the lumen of endoplasmic reticulum (ER), SupraFlipper report the membrane orders along the secretory pathway from the ER over the Golgi apparatus to the plasma membrane. Kinetics of the process are governed by both the probe release and the transport through lipid domains. The concentration of biotin can control the former, while the expression level of a transmembrane protein (Sec12) involved in the stimulation of the vesicular transport from ER to Golgi influences the latter. Finally, the generation of a cell-penetrating and fully functional Sav-flipper complex using cyclic oligochalcogenide (COC) transporters allows us to combine the SupraFlipper strategy and HaloTag technology.

KEYWORDS: fluorescent probes, spatiotemporal control, streptavidin, secretory pathway, mechanochemistry, mechanobiology

INTRODUCTION

The development of chemistry tools to image physical forces in biological systems is an important topic of current concern.1–3 Tension of biomembranes, for instance, significantly impacts cell function, illustrated for example in the activation of signal transduction or the regulation of intraluminal vesicle formation.4,5 Pioneering bioengineered tension reporters operate mostly with mechano-insensitive fluorescence resonance energy transfer (FRET) pairs that respond to distance changes produced by forces applied to protein or DNA constructs in specific model systems.6,7–13 The development of small-molecule fluorescent probes that are mechanosensitive by themselves and do not interfere significantly with biological structure and function could thus contribute toward the practical and general fluorescence imaging of membrane tension at the location of interest inside living cells over time. To address this challenge, we have introduced planarizable push–pull fluorescent probes, known as “flippers”, as membrane tension reporters (Figure 1).14–18 These molecular systems are constructed around an electron-rich and an electron-poor dithienothiophene, which are twisted out of coplanarity due to repulsion between the methyl groups and σ holes on the endocyclic sulfur atoms (Figure 1A–C).19–21 Mechanical planarization imposed by increasingly ordered lipidic membranes turns on the push–pull dipole, supported by the exocyclic cyano acceptor and the ether donor (Figure 1C). Thus, the excitation maximum of flippers is red-shifted in solid-ordered (Sα) and liquid-ordered (Lα) compared to liquid-disordered (Lβ) phases of membranes. In contrast, the

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emission maximum remains basically unchanged because flipper probes report on the order of the MOI, with lifetimes increasing with increasing tension. 

Based on these data, the following guidelines have been developed to interpret the FLIM images of cells stained with flipper probes: (i) At steady state, flipper probes report on the average order of the MOI, with lifetimes increasing with increasing order. (ii) From this steady-state lifetime, changes in membrane tension are reported as increasing lifetimes with increasing membrane tension and vice versa. (iii) Absolute values in lifetimes can vary depending on conditions, whereas lifetime changes in response to changes in tension are almost condition independent. In other words, flipper probes respond to changes in lipid packing caused by changes in lipid composition and membrane tension, and only under conditions where the lipid composition is constant, such as osmotic shock experiments, can one be certain to measure tension.

Extensive data are available on characteristics of flipper probes. Most importantly, flipper probes act as monomers, align along lipid tails of one leaflet, do not affect the structure of the membrane (like, e.g., cholesterol), partition with little preference between differently ordered domains, do not fluoresce in water, and do not respond to membrane tension nor strongly fluoresce when bound to proteins.

The mechanosensitive unit of flipper probes has remained essentially untouched during probe development, maintaining a good compromise between mechanosensitivity, red shift, and stability. On the other hand, the headgroup attached to the first monolayer of the molipid probe causes the disassembly of these microdomains together with bilayer deformations, thus decreasing flipper lifetime. 

Figure 1. (A) Molecular structures of Flipper-TR 1 and HaloFlipper 2. (B) Molecular structure of flipper 6. Release from Sav 5 after the addition of biotin 9, 10 is unfavorable. (C) Structure and molecular mechanism of SupraFlipper 7 and 8. Release from Sav 5 after the addition of biotin 9, 10 is favorable (E). Planarization of the flipper’s in the confining environment turns on the push–pull system, red shifts excitation maxima, and increases fluorescence lifetimes. (D) Design strategy of HaloFlipper, using a covalent tagging system to image localized membrane tension in the MOI around the fusion protein. (E) Design strategy of SupraFlipper. Sav 5 is expressed either in the lumen of the organelle of interest or anchored to the MOI and labeled with SupraFlipper 7 or 8, producing 11. After the addition of biotin 9 or its methyl ester 10, the probe is released from Sav to partition into the MOI, where it is free to laterally diffuse. Schematics of flipper probes in parts D and E are colored according to their lifetime τ (see color bar), which increases with planarization: p, planarized; hp, half planarized.
donor side has been modified extensively to broaden the scope of the probes without loss of function of the mechanophore (Figure 1A–C).55−58 The carboxylic acid terminus in the original Flipper-TR 1 affords selectivity for the plasma membrane (Figure 1A).57 Substitution of the carboxylic acid by empirical tracker motifs provided access to different subcellular organelles such as mitochondria (Mito Flipper-TR), endoplasmic reticulum (ER Flipper-TR), and endolysosomes (Lyso Flipper-TR).58 However, these “trackers” are neither universal nor highly specific.

We have recently introduced HaloFlippers 2 as the optimal structure, to localize the mechanosensitive probe in any membrane of interest (MOI, Figure 1A, D).55 This was achieved by expressing fusion protein 3 composed of the self-labeling enzyme HaloTag combined with a protein in the MOI inside the cell, followed by incubation with the chloroalkylated flipper 2. The probe immediately forms an ester bond to give 4 and directs the fluorophore to the MOI in the vicinity of the protein (Figure 1D).55 HaloFlippers are specific and universal, applicable to organelles out of the scope of empirical trackers, such as the Golgi apparatus and peroxisomes, and capable of reporting the membrane tension changes by changes in fluorescence lifetime. However, a thorough optimization was necessary for the linker tethering the probe to the enzyme, which can also vary depending on the fusion protein.55,55,60,61 Moreover, due to the restricted lateral movement of the tethered probe, it is uncertain whether the local physical properties of the membrane reported by the probe are representative of the MOI or specific to the membrane environment of the fusion protein (Figure 1D). Contributions from the probe interacting with proteins are estimated negligible because protein-bound flippers are insensitive to osmotic stress, i.e., membrane tension, and poorly fluorescent (vide infra).

In the following, we present SupraFlippers as the supramolecular solution to the limitations of covalent targeting with HaloFlippers (Figure 1E). The strategy is based on the precise localization of the probe in the subcellular compartment of choice using a genetically encoded supramolecular tag followed by its controlled release into the MOI with an exogenous chemical stimulus. This strategy adds several advantages to the spatial resolution of HaloFlippers, namely: (1) temporal resolution on the release of the probe, (2) free lateral diffusion of the probe in the MOI and beyond, and (3) no need for tether engineering.

### RESULTS AND DISCUSSION

#### Design of SupraFlippers

Different tools are available to release substrates selectively inside living systems using bioorthogonal external stimuli. Covalent approaches cover photo- and metal-catalyzed cleavable linkers62−66 or “click-to-release” strategies using the trans-cyclooctene/tetrazine pair.67−70 Supramolecular approaches, in contrast, are much less explored and more recent.71,72 We have selected a modified version of the

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**Figure 2.** (A) Schematic representation of model studies using GUVs. After the addition of biotin 9 or 10 to GUVs loaded with 11 or SR (a), SupraFlipper 7 dissociates from Sav and partitions in the membrane of GUVs loaded originally with 11, without diffusing to SR-loaded GUVs over time (b). (B−E) Merged CLSM images of GUVs loaded with SR (1 μM, green channel) and 11 (15 μM, 3 eq of 7, red channel) before (B), 5 min (C), and 1 h (D) after addition of 10 (100 μM). (E) Same image as part D, only in the red channel. Arrows point to the membrane of SR-loaded GUVs, which did not show fluorescence in the red channel, even after time. (F−G) FLIM images of one GUV loaded with 11 (15 μM, 3 equiv of 7) before (F) and (G) 6 min after addition of 10 (100 μM). Scale bars: 10 μm. (H) Excitation spectra of probes 7 (solid) and 8 (dashed, both 100 nM) in DPPC LUVs (75 μM) at 25 °C (red, 5ss) or 55 °C (mint, 5ss) and in buffer at 25 °C (blue).
retention using selective hooks (RUSH) system. \(^\text{73}\) RUSH is based on the expression of streptavidin (Sav) \(^5\) together with a targeting motif and reporter protein linked to a Sav-binding peptide (SBP) motif. Both units remain connected without any stimuli due to the high affinity of SBP to Sav (dissociation constant \(K_d = \text{10}^{-9} \text{ M}\)). However, the addition of biotin (\(K_d = \text{10}^{-14} \text{ M}\)) results in the displacement of SBP and the transport of the reporter protein through the cellular secretory pathway. \(^\text{73,74}\)

In our SupraFlipper strategy, mechanosensitive flipper probes replace or complement the reporter protein–SBP conjugate. The flipper probes are equipped with a biotin derivative to selectively bind Sav inside cells (Figure 1). The biotinylated probe \(^6\) (Figure 1B) has already been reported to validate Sav as a bioorthogonal connector between the mechanosensitive unit and a biotinylated lipid membrane. \(^56\) However, a very strong, quasi-irreversible binding of biotin with Sav impedes any possible selective release, at least in conditions compatible with living cells. Moreover, the biotin residue in probe \(^6\) is very prone to oxidation into the corresponding sulfoxides under ambient conditions, which complicates the functional characterization of this probe (data not shown). \(^56\) To solve these decisive problems, flippers \(^7\) and \(^8\) were designed with a terminal desthiobiocin (Figure 1C). This biosynthetic intermediate of biotin binds less tightly to Sav (\(K_d = \text{10}^{-11} \text{ M}\)) yet strong enough to allow its practical use in biology, down to picomolar concentrations. \(^76-\text{80}\) Thus, the cell-permeable and nontoxic pristine biotin \(^9\) or its methyl ester \(^\text{10}\) can release desthiobiocinylated flipppers \(^7\) or \(^8\) from complex \(^11\) to allow their partitioning in the closest membrane followed by lateral diffusion to report on the global membrane order of the MOI, leaving the inactivated Sav complex \(^12\) behind (Figure 1E).

A high-content and high-throughput cell-penetration assay performed on the HaloFlipper series revealed that probes with an oligo(ethylene)glycol spacer equal or shorter than 8 repeat units display a limited permeability to cellular membranes and poor selectivity for HaloTags. \(^55,56,81,82\) Based on these results, SupraFlippers \(^7\) and \(^8\) were synthesized with a 11-mer and a 23-mer oligo(ethylene)glycol spacer, respectively, between the mechanophore and the desthiobiocin recognition motif (Figure 1C). The probes were synthesized following procedures similar to those of \(^2\) and \(^6\) (Scheme S1). \(^55,56\)

The relative partitioning efficiencies \(D_{\text{rel}}\) of flipppers \(^7\) and \(^8\) were estimated from their fluorescence excitation intensities in large unilamellar vesicles (LUVs) composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) membranes under two temperature-induced phases at two different concentrations (Figures 2H, S1). In comparison, excitation maxima were more blue-shifted and weak in buffer and bound to Sav \(^5\) (Figure 3A).
maximal intensity reached within a few minutes (Figure S2). In GUVs with complex contrast, the membrane of the SR-loaded GUVs did not Simultaneously, the faint surface. Upon chemical stimulation, the membrane of the probe, either in solution and/or in contact with the protein 2.5 ns. This low with KDEL ER retention sequence, i.e., Sav 14. The complex 15 forms spontaneously between one Sav 13 and up to two SBP-GFP-GPI 14. The subsequently added flipper 7 should fill the vacant binding sites to give complex 16 (and, perhaps, partially displace SBP-GFP-GPI 14). The addition of biotin 9 to the resulting complex 16 then should release flipper 7 and SBP-GFP-GPI 14 in the ER. Note, stoichiometries of ligands per Sav presented in complexes 15 and 16 are estimates.

The expression of the fusion proteins was first verified using a cell-impermeable rhodamine-biotin derivative after fixing the cells. The resulting micrographs showed staining of the ER network in the transfected cells, demonstrating efficient binding of biotinylated probes to the expressed Sav 13 (Figure S3).

The transfected HeLa cells were then incubated with flipper probes (100 nM, 1 h), briefly washed, and analyzed using CLSM. Images of such cells with Sav 13, SBP-GFP-GPI 14, and probe 7 showed a bright ER network in the flipper fluorescence channel, with good overlap with the GFP signal from 14 (Figure 3B). Nontransfected cells and regions of transfected cells without expression of the protein, e.g., the nuclei, displayed no or little background signal as expected for poor penetration and retention of the probe along multiple coupled equilibria in the absence of strong binding sites, i.e., a directional driving force (Figure S4, A–D). However, the cell- to-cell heterogeneity of the transfection efficiency, noticeable in the GFP channel, was less evident in the flipper channel, showing a more homogeneous intensity distribution and a generally higher intensity in poorly transfected cells (Figure S4, E–H). This result could be explained by the multivalency of Sav, which keeps the availability of free binding sites high even in poorly transfected cells, as long as the concentration of the probe in the cytoplasm remains below the saturation of the free binding pockets. Some SBP displacement by the added SupraFlipper might also be contributing to this result. For cells transfected with p1, expressing only the luminal Sav 13, the obtained pattern in the flipper channel was similar to that with p2 (Figure S5). The hydrophilic flipper 8, in contrast, displayed very poor fluorescence signals in both sets of transfected cells (Figure S5–S6). Thus, the more lipophilic and brighter probe 7 was chosen in the following experiments.

The addition of biotin 9 (40 μM) to HeLa cells coexpressing Sav 13 and SBP-GFP-GPI 14 after incubation with 7 (100 nM, 1 h) caused the relocation of GFP fluorescence from ER to Golgi and the plasma membrane over time, as anticipated from the role of GPI anchored protein 14 (Figure 3B–F, green channel). The released flipper probes 7 followed the same secretory pathway, staining first the ER and continuing to the Golgi and finally the plasma membrane (Figure 3B–F, red channel). Colocalization with GFP was overall excellent (Figure 3B–F, bottom, yellow). Remarkably, GFP-GPI emission could be observed ahead of time in the secretory pathway. For instance, SBP-GFP-GPI 14 fully transferred to Golgi when flipper emission was still detectable in the ER (Figure 3D, bottom). At dominant colocalization in the Golgi (Figure 3E, yellow), SBP-GFP-GPI 14 could already be seen in the plasma membrane (green), while flippers 7 remained detectable in the ER (red). This difference could be explained by the much lower affinity of SBP to Sav compared to desthiobiotin, which allows more efficient and probably faster displacement by biotin 9. Undetectable GFP translocation to the Golgi already upon flipper addition supported that the displacement of SBP-GFP-GPI 14 by flipper 7 is as unlikely as...
Figure 4. (A–D) FLIM images of HeLa cells transfected with GFP-free p1 after incubation with 7 (100 nM, 1 h) (A) before, (B) 80 min, and (C) 110 min after the addition of 9 (40 μM) and (D) 10 min after addition of sucrose (0.5 M). (E) Schematic representations of images in parts A–D. Stepwise progression of SupraFlipper 7 from intra-ER Sav upon (a) addition of biotin, (b) more time, and (c) hyperosmotic stress. Colors represent the flippers lifetime in each step and organelle (ER, Golgi apparatus, plasma membrane, left to right). (F) Average fluorescence lifetimes of 7 in HeLa cells transfected with p1 before or 120 min after the addition of 9 (40 μM) under isoosmotic and hyperosmotic conditions (sucrose ±, respectively). (G) Kinetics of release and transport of 7 in HeLa cells transfected with p1, based on the increase of the fluorescence lifetime of the probe over time, after addition of 200 (red) and 40 μM (orange) of biotin 9, with solid lines representing the trend curves, solid circles the mean values and bands in light colors the standard deviations; values calculated from >12 cells per condition. (H) Fluorescence lifetimes of 7 in HeLa cells transfected with p1 before the addition of 9 (–) and 50 min after addition of 5, 40, or 200 μM of 9 (+). (I) Violin plot representing the fluorescence lifetime of 7 in HeLa cells transfected with p1 and a nontargeting siRNA (control) and the siRNA for the reduction of expression of Sec12 protein (siSec12) before (–) and 35 min after the addition of 9 (40 μM, +). (F, H, I) Solid blue lines correspond to mean (F, H) or median (I) values, whiskers (F, H) to standard deviation, solid black lines (I) to the quartiles, and solid gray lines match measurements on the same cell (circles). Statistical significance was determined using Student’s t test: *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0005, *****p < 0.0001. Cells where flipper lifetime was below 3.0 ns after 60 min were not considered for the statistical analysis. (I) Solid areas correspond to the probability density for the control (green) and the functional siRNA (blue). Scale bars in A–D: 10 μm.

expected from the availability of vacant binding sites in complex 15.

In cells transfected with p1, SupraFlipper 7 and MitoTracker poorly colocalized both before and after the addition of biotin (Pearson correlation coefficient (PCC) = 0.23 ± 0.04 and 0.4 ± 0.1, respectively, Figure S7). Since mitochondria do not belong to the secretory pathway, this result provided corroborative support that flippers travel between subcellular compartments mainly through secretory vesicles and not through cytosolic media (Figure 4E).

In FLIM images, the lifetime of SupraFlipper 7 in the ER before biotin addition was as low as $\tau_{av} \approx 2.7$ ns (Figures 4A, E and S8A). This value is similar to that of the flipper–Sav conjugate 11 inside of GUVs (Figure 2F) and differed from previous flipper probes targeted to ER.55,56 Moreover, the probe did not respond to a decrease in the membrane tension after the application of hyperosmotic stress (Figure 4F, S8B). Consistent with the previously reported poor fluorescence and mechanosensitivity of flippers interacting with proteins rather than lipid bilayers,55,56 these results demonstrated that the probe 7 within complex 16 stays out of the membranes (Figure 3A).

After the addition of biotin 9, the average fluorescence lifetime of flippers in the whole cell continuously increased to a maximum average lifetime of $\tau_{av} \approx 3.7$ ns in 2 h (Figure 4A–C, F–H). Specific values varied between the different subcellular compartments, from $\tau_{av} \approx 3.1$ ns in the ER network to $\tau_{av} \approx 4.4$ ns in the plasma membrane, together with some defined areas around the nucleus, possibly Golgi, with $\tau_{av} \approx 3.2$ ns (Figure 4C).

The released probe responded to a decrease of membrane tension by decreasing the fluorescence lifetime by $\Delta \tau_{av} \approx 0.5$ ns (Figure 4D–F). The absolute lifetime values of 7 were slightly lower than the ones of previous flipper probes. However, FLIM experiments comparing the lifetimes of Flipper-TR 1 and SupraFlipper 7 in GUVs of different composition supported that the lower $\tau_{av}$ values for the latter are intrinsic to the probe, presumably due to less than perfect...
position of fluorophores in the membranes (Figure S10, Table S2). Absolute lifetime values in general can vary with experimental conditions, whereas general trends between different membranes of, e.g., different organelles were so far always reproducible, also with the new SupraFlipper 7. Most importantly, changes in response to membrane tension were so far fully reproducible independent of conditions and probe.

Decreasing fluorescence lifetimes with decreasing membrane tension was as expected from flipper deplanarization upon tension-induced membrane reorganization, as described in the introduction (vide supra).16 The coinciding loss in fluorescence intensity (Figure 2H) does not influence lifetime measurements except that weak signals can drop below the detection cutoff, as observed, for example, for the plasma membrane staining in Figure 4C, becoming undetectable in Figure 4D. Eventual contributions from surose, added to the medium to vary osmotic pressure, to lower signal intensity in Figure 4D by extracting flippers from the plasma membrane cannot be fully excluded at this point, although they are not supported by extensive previous data.16–18

Kineticks of fluorescence intensity and lifetime increase after the addition of the stimulius was independent of the choice of plasmid, p1 or p2, and the biotin derivative 9 or 10 (Figure S9), but it was clearly dependent on biotin concentration (Figure 4G,H). The addition of 5 μM of 9 only increased the average lifetime modestly by Δτav ≈ 0.1 ns after 50 min of incubation. Increasing the concentration of 9 to 40 and 200 μM boosted the difference in the lifetime to Δτav ≈ 0.4 and 0.7 ns, respectively (Figure 4H).

Modulation of the vesicular trafficking also affected the kinetics of fluorescence lifetime increase (Figures 4I and S8C). Cells were transfected with p1, and an siRNA encoded to lower the expression level of Sec12, a transmembrane protein of ER involved in the stimulation of the budding process and vesicular transport from ER to the Golgi.19 The initial rate of the average lifetime increase upon release of SupraFlipper 7 was clearly lower in these cells compared to cells transfected with a nontargeting siRNA (Figure 5S8C). Cell-by-cell comparison of lifetime changes demonstrates that the population of cells with small lifetime changes upon unleashing of SupraFlipper 7 is larger among cells treated with siRNA against Sec12 compared to those treated with control siRNA (Figure 4I). This result supported that the transport of SupraFlipper 7 occurs significantly through the secretory pathway after external chemical stimulation. Taken together, the access to the study of the membrane order and mechanics along the secretory pathway therefore represents one example for specific advantages of the releasable SupraFlippers compared to the unreleasable HaloFlippers 2 and flippers operating with empirical, covalent ER tracker chemistry,18 which all are retained in the ER within the time frame of interest. Repeated control experiments corroborated that HaloFlippers 2 positioned in the ER as described55 neither relocate nor change lifetime with time (not shown.)

Merging HaloTag Technology and SupraFlippers

The SupraFlipper strategy was further expanded to benefit from the reliable and general targeting of the HaloTag technology. For this purpose, the multifunctional Sav complex 18 was assembled from wild-type Sav 5, SupraFlipper 7, and biotinylated, cell-penetrating chloroalkanes 19 or 20 (Figure
The 2:1 stoichiometry given for complex 18 refers to the equivalents added, which in reality produce a roughly statistical mixture of complexes with the shown 2:1 complex as the main component. Different from the system with Sav described above, the targeted delivery of the wild-type Sav 5 complex is governed by the reaction of the chloroalkane with the HaloTag fusion protein expressed in the MOI (Figure 5A, 1D).

Wild-type Sav 5 does not penetrate cells. To ensure the efficient delivery of 18 to the HaloTagged MOI inside the cell, cyclic oligochalcogenides (COCs) were attached along the trifunctional peptide 19. Not only the best COC oligomers 19 and 20 containing two and three asparagusic acids, respectively, but also their counterparts with diselenolipoic acid instead of asparagusic acid were tested (Figure S11). Delivery of complex 18 to HaloTag 3 in the MOI should then result in covalent capture to yield complex 21, with bound SupraFlipper 7 possibly inserting into the membrane domain containing the fusion protein. Chemical stimulation should then release SupraFlipper 7, while Sav in the resulting complex 22 remains complexed to the fusion protein.

HGM cells, that is a stable cell line expressing GFP-HaloTag in the cytosolic side of mitochondria, were exposed to complex 18 using previously optimized concentration, stoichiometry and incubation time (5 μM of 18, 2 eq of 20, 1 eq of 7, 2 h incubation). In CLSM images before biotin addition, colocalization of GFP and HaloTag probe bound within complex 18 reached the membrane before any release, at least in this particular case (Figure 5D, left). The lifetime values were as high as τav ≈ 4.4 in the plasma membrane and as low as τav ≈ 2.2 for extra and intracellular complex aggregates and τav ≈ 3.6–4.0 ns in subcellular domains. After release, the flipper signal migrated into other membrane networks (Figure S2D, middle). Upon application of hyperosmotic stress, the lifetime values decreased as expected for operational mechanosensitivity (Figure S3D, right). The decrease in intensity coinciding with tension-induced flipper twisting (Figure 2H) reduced detectability. This effect was most pronounced for the plasma membrane as discussed previously (Figure 4D). The bright extracellular aggregates with already very low fluorescence lifetime were not affected by the osmotic shock.

CONCLUSIONS

This study introduces a new strategy to selectively localize fluorescent membrane tension probes in a specific subcellular domain using a genetically encoded supramolecular targeting based on biotin-streptavidin technology. The advantage of supramolecular targeting is the ability to control the release of the probe into the MOI by applying biotin as an external chemical stimulus, which binds better to the protein and thus displaces the mechanophore. These molecular systems, SupraFlippers, thus add temporal resolution to the already gained spatial resolution of our previous HaloFlipper probes.

In artificial model systems, the best SupraFlippers excel with fast and efficient partitioning into the closest membranes upon the addition of biotin, without diffusing into other membranes. In cells expressing KDEL-tagged Sav in the ER lumen, colocalization studies with a protein reporter demonstrate the specificity of the probe. The addition of biotin triggers the synchronized release of the probe to the ER membrane and its transport through membranes following the secretory pathway. Kinetics of the release and diffusion of the probe upon addition of biotin change with concentration of the stimuli and the altered intracellular vesicular transport.
In FLIM, SupraFlipper remains insensitive to membrane order and changes in membrane tension before the addition of biotin. Upon release, the probe becomes operational and reports on the nature of the MOI. For the system used here, the lifetime starts with $\tau_{av} \approx 2.7$ ns of inactive flippers in the ER lumen and evolves up to $\tau_{av} \approx 4.4$ ns in the plasma membrane over time, as a consequence of the insertion and relocation of the active flipper across membranes of a progressively higher order. The observed differences in lifetime for different MOIs are consistent with previously recorded trends\(^5,^8\) and the known differences in lipid composition and packing. Operational mechanosensitivity is also confirmed by decreasing overall lifetime by decreasing tension with hyper-osmotic stress.

Finally, we combine the SupraFlipper strategy with HaloTag technology by taking advantage of efficient thiol-mediated delivery of proteins with COC oligomers. Independent on the nature of the fusion protein, it was possible to transport a functional Sav complex carrying the probe to the cytosol, and localize it in the MOI through Halo-tagging, and finally release the probe with biotin. The migration of the probe into other subcellular domains upon addition of biotin depends on the dynamics of the organelle, mostly absent in mitochondria and pronounced with ER and Golgi. While the strategy is fully operational mechanosensitivity is also confirmed by decreasing overall lifetime by decreasing tension with hyper-osmotic stress.

With conditions taken from the established RUSH system, compatibility and significance with biological systems have been confirmed extensively with regard to release kinetics as well as possible interference, including toxicity.\(^7,^8\) Unrestrained positioning and diffusion within the MOI is one of the key advantages of released SupraFlippers 7 compared to the unreleasable HaloFlipper 2, as exemplified by an increase in fluorescence upon release (Figure 5B) and access to the study of the membrane order and mechanics along the secretory pathway (Figures 3, 4). Negligible trafficking upon controlled release in GUVs (Figure 2) and mitochondria (Figure 5B) demonstrated that contributions from transfer between membranes are not important within the time scale of interest. Altogether, these results validate SupraFlippers as superb transporters, complementing the equally selective but more restricted covalent HaloFlippers. SupraFlippers are already used to unravel the biological implications of membrane tension in the export of proteins through the secretory pathway. Other applications to important biological questions will follow.

**EXPERIMENTAL SECTION**

Detailed experimental procedures for synthesis of 7 and 8, cell cultures, and microscopic imaging can be found in the Supporting Information.

**Controlled Release of SupraFlippers in Live Cells**

**Method 1.** HeLa Kyoto cells were seeded on 35 mm glass bottom dishes, transfected with plasmid p2 as described in the Supporting Information and incubated with 7 (100 nM) in Leibovitz’s medium (1 mL) for 60 min at 37 °C under 5% CO₂. The cells were washed with clean Leibovitz’s medium (2 × 1 mL), incubated for 15 min at 37 °C under 5% CO₂ and finally kept in clean Leibovitz’s medium (1 mL). Right before starting the image acquisition, biotin 9 (80 μM) in Leibovitz’s medium (1 mL) was added to reach a final concentration of 40 μM. The cells were kept at 37 °C during the measurements, and images were acquired over a period of 110 min. The distribution of fluorescent compounds was analyzed using CLSM (Leica SPS) or FLIM (Nikon Eclipse Ti A1R: with a FLIM kit from PicoQuant) without fixing, in different positions of the dish, as described in the Supporting Information.

**Method 2.** Complexes 18 were prepared by adding chloloalkylated transporters 19 (or 20; 0.8 μL, 10 mM in DMSO) and flipper 7 (4 μL, 1 mM in DMSO) to a solution of Sav 5 (0.2 mL, 20 μM in PBS) and shaking the mixtures for 10 min at rt. Then, the mixture was centrifuged (14.0 krpm, 10 min, 4 °C). The supernatant was directly used. HGM cells or HeLa Kyoto transfected with p3 in 8-well plates as described in the Supporting Information were incubated with 18 in Leibovitz’s medium (2–5 μM, 150 μL) for 2 h at 37 °C under 5% CO₂. The cells were washed with Leibovitz’s medium (2 × 200 μL), incubated again for extra 15 min, washed again with Leibovitz’s medium (1 × 200 μL), and imaged. Then, cells were incubated in the presence of biotin 9 (40 μM, 150 μL) for extra 1 h at 37 °C under 5% CO₂. Finally, the cells were washed (1 × 200 μL) and imaged again.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.0c00069.

Detailed experimental procedures (PDF)

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**Author Contributions**

J.L.-A. and K.S. contributed equally to this study.
The authors declare the following competing financial interest(s): The University of Geneva has licensed four Flipper-TR probes to Spirochrome for commercialization.

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