Mechanical forces play a critical role in the regulation of biochemical events at the cellular level, and the field of mechanobiology is accordingly attracting growing interest. Despite this, many of the complex molecular mechanisms underlying cellular responses to mechanical stimuli remain unknown, and their understanding will require novel specific tools capable of dissecting mechanobiology at high spatiotemporal resolution. Fluorescence microscopy provides an ideal modality to monitor dynamic processes in living cells, but in contrast to the large number of available fluorescent probes for small molecules and ions, the development of molecular sensors suited for mechanobiology has lagged behind. In this issue of ACS Central Science, Matile and coauthors report an intracellular membrane tension probe designed by combining a synthetic fluorescent sensor with a genetically encoded self-labeling tag. This hybrid probe can be localized to specific membranes of interest inside living cells, and thus provides a promising tool to investigate mechanobiology at the subcellular level using fluorescence microscopy.

Probing mechanical forces in biological membranes is a complex task. Indeed, forces can typically only be assessed indirectly through downstream physical and morphological parameters such as membrane rigidity, curvature, or tension. Most of the current approaches rely on physical methods such as optical tweezers, micropipette aspiration, and atomic force microscopy to extract quantitative information, with only a limited number of reporters compatible with fluorescence imaging in living cells. For the past few years, researchers in the Matile group have been addressing this need, by developing fluorescent mechanosensitive small molecules able to report on membrane states within living cells. Recently, they reported Flipper-TR, a fluorescent probe tailored for membrane tension imaging (Figure 1a). Flipper-TR is a push−pull small molecule, built around a bis(dithienothiophene) core separating the donor and acceptor. When delivered onto cells, this compound can readily insert into the plasma membrane. In relaxed membranes, it adopts preferentially a twisted conformation emitting fluorescence with blue-shifted excitation. As membrane tension increases, local reorganization of lipids causes the probe to adopt a planarized conformation leading to a red-shift in excitation and an increase in fluorescence lifetime. Therefore, this compound can quantitatively report on membrane tension changes by fluorescence lifetime imaging microscopy (FLIM), but its use is restricted to the external membrane of cells.

Changes in membrane state, however, are not confined to the plasma membrane. Intracellular organelles are also...
delimited by lipid membrane bilayers subjected to mechanical forces.\textsuperscript{5} To extend the application of Flipper to intracellular membranes, previous work introduced organelle-specific targeting groups in the sensor scaffold.\textsuperscript{6} These targeting groups are small synthetic motifs that exploit the intrinsic physicochemical properties of certain organelles to direct localization of the probe. For instance, the cationic triphenylphosphonium group is preferentially sequestered in the mitochondria due to the negative potential of the mitochondrial inner membrane. When applied to Flipper, this strategy enabled visualization of membrane tension changes in the endoplasmic reticulum (ER), mitochondria, and lysosomes, but it remains limited to the subset of organelles for which targeting groups have been reported. In addition, organelle targeting groups can also show some affinity, albeit lower, to other subcellular compartments, which can compromise localization and result in unwanted fluorescence signal.

In the present work, the authors implement a broadly applicable strategy to target their sensor to any desired intracellular membrane with superior specificity. For this purpose, they use the self-labeling HaloTag, a protein engineered from a bacterial enzyme that binds specifically and irreversibly to a synthetic chloroalkane ligand. Appending the HaloTag ligand to the Flipper scaffold yielded HaloFlipper (Figure 1a), which labels with high specificity membranes expressing HaloTag fusion protein. The resulting hybrid probe retains the mechanosensitivity of the parent Flipper and can report on changes in membrane tension at the subcellular level (Figure 1b).

The strength of the approach lies in that HaloFlipper can in principle be adapted to any membrane of interest. Using appropriate HaloTag fusion proteins, the authors demonstrate the versatility of the probe by selective labeling of the ER, mitochondria, peroxisomes, lysosomes, and the Golgi apparatus. Targeted to the ER membrane, HaloFlipper gave a comparable fluorescence lifetime response to osmotic shocks as the previously reported ER-specific Flipper,\textsuperscript{6} which confirms that mechanosensitivity is unaffected by HaloTag tethering (Figure 1c). As Flipper probes are also membrane composition sensitive, an absolute comparison of membrane tension across organelles is not possible. Nevertheless, the authors report similar tension changes in various internal membranes, with hypertonic treatment resulting in shorter fluorescence lifetimes, which is characteristic of a decrease in membrane tension. Overall, these results demonstrate that HaloFlipper is applicable for imaging membrane tension changes in intracellular compartments which were previously inaccessible using organelle-targeting motifs.

The strength of the approach lies in that HaloFlipper can in principle be adapted to any membrane of interest.
advanced molecular probes. This powerful approach, however, presents a molecular design challenge, as attachment of the ligand or interaction with the protein often compromises performance of the probe. With the added complexity of working within membranes, the development of HaloFlipper certainly illustrates this challenge. Careful molecular engineering of the linker between the Flipper and the HaloTag ligand was critical to ensure robust labeling without affecting mechanosensitivity, and the authors found that the optimal linker length is around 6 nm. While a longer linker improved cell-permeability, it also resulted in poor membrane insertion. In contrast, derivatives with shorter linkers showed insufficient cellular uptake. Although certain future applications might require further linker engineering, depending on the protein used as the HaloTag fusion partner, the current iteration of HaloFlipper already shows applicability in diverse intracellular membranes, thus, emerging as a promising addition to the existing mechanobiology toolbox.

From a molecular engineering perspective, this work further establishes the value of hybrid synthetic—genetic strategies for generating advanced molecular probes.

While physical methods to probe membrane tension can typically only be applied to the external surface of cells, the versatility and easy use of HaloFlipper make it a promising tool for the investigation of intracellular mechanobiology with minimal perturbation. Looking forward, fluorescent mechanosensors such as HaloFlipper hold the potential to be used in combination with other existing probes to provide functional insights correlating mechanical and biochemical events in living cells. We can expect that this tool will be adopted by the biology community and enable functional imaging studies previously out of reach, therefore increasing our understanding of the complex molecular mechanisms underlying the mechanobiology of cells and tissues.

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