Engineered HaloTag variants for fluorescence lifetime multiplexing

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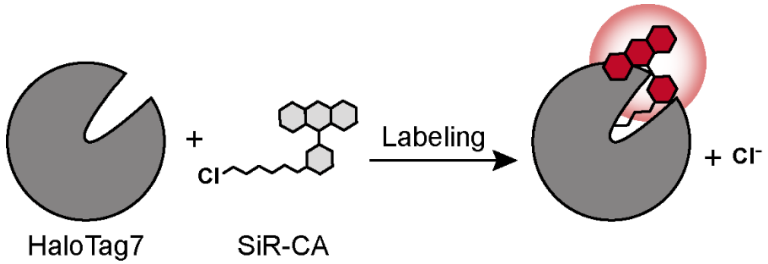
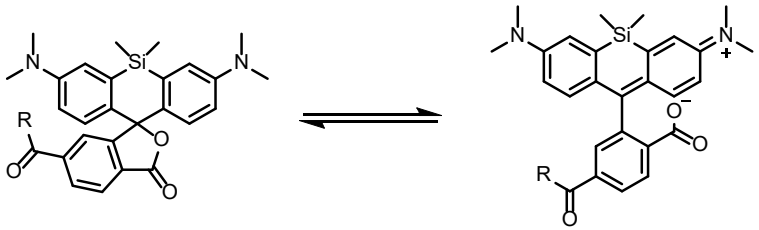
Introduction

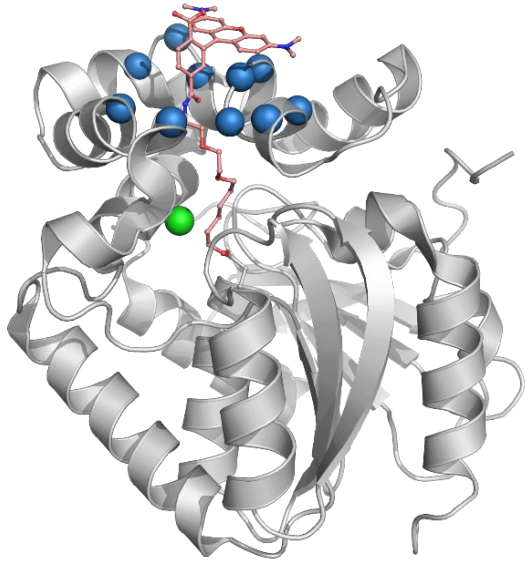
The self-labeling protein tag HaloTag7 (HT7) shows a fluorogenic response with rhodamine based fluorophores due to their open-close equilibrium.



The position of the equilibrium is determined by the local environment and therefore the protein surface.



Can we engineer the protein surface to influence the fluorophore's properties?

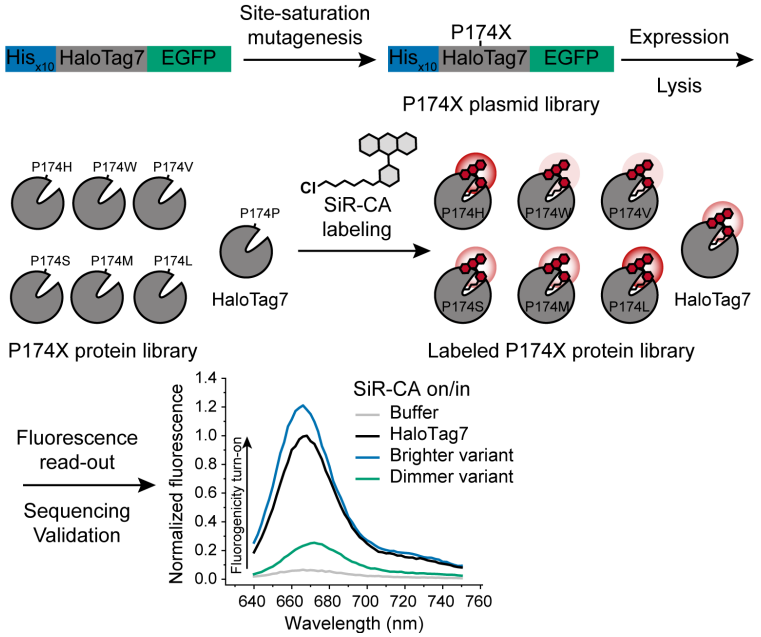


HaloTag7-TMR
Protein Data Bank ID: 6Y7A

Results

Structure guided protein engineering lead to the identification of both brighter* and dimmer# HaloTag variants.

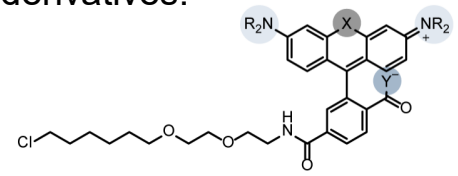
- **HaloTag9*** (HT9): HT7-Q165H-P174R
- **HaloTag10#** (HT10): HT7-Q165W
- **HaloTag11#** (HT11): HT7-M175W

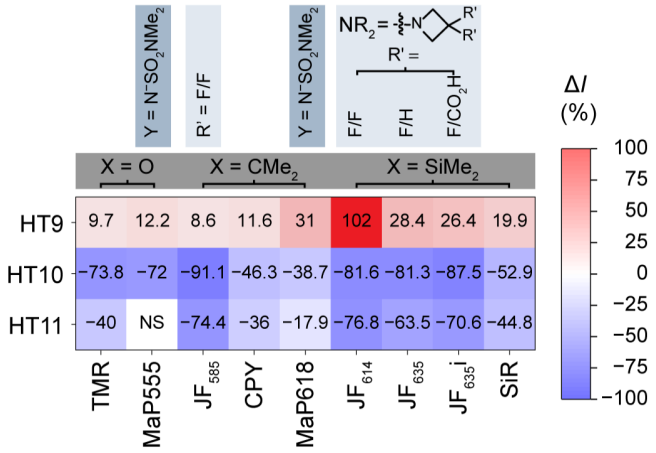


Characterization of HT9, HT10 and HT11

The brightness of the new HaloTags was characterized with a panel of 46 different fluorophores in comparison to HT7.

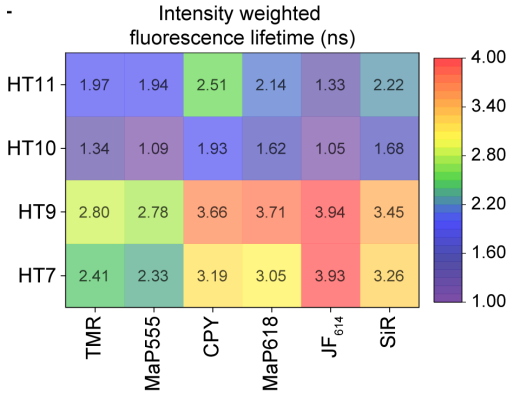
- HT9 rendered primarily fluorogenic fluorophores brighter.
- HT10 and HT11 decreased the brightness of most rhodamine derivatives.





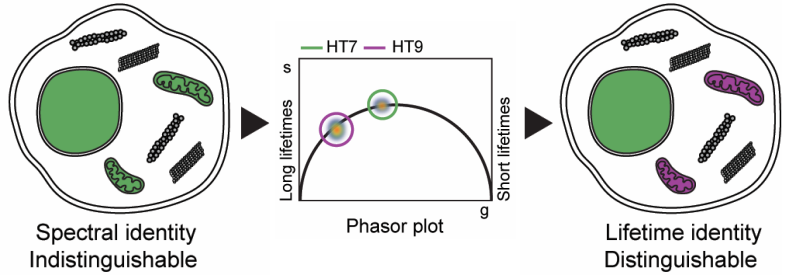
Comparison of photophysical parameters such as quantum yield, extinction coefficient, or fluorescence lifetime in combination with X-ray crystal structure analysis, allowed us to get a better picture of the molecular mechanisms inducing the increases and decreases in fluorophore brightness.

The fluorescence lifetime of several fluorophores bound to HT7, HT9, HT10, or HT11 were spanning a range of up to 3 ns. In the case of MaP555 and MaP618[1] they were gradually distributed, making these fluorophores ideally suited for fluorescence lifetime multiplexing.



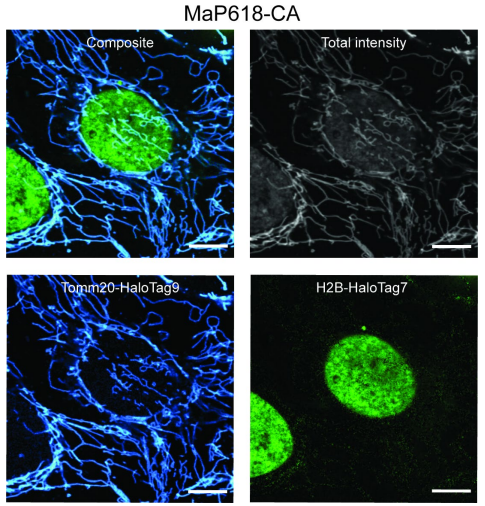
[1] Wang, L. *et al. Nat. Chem.* (2020).

Fluorescence lifetime multiplexing

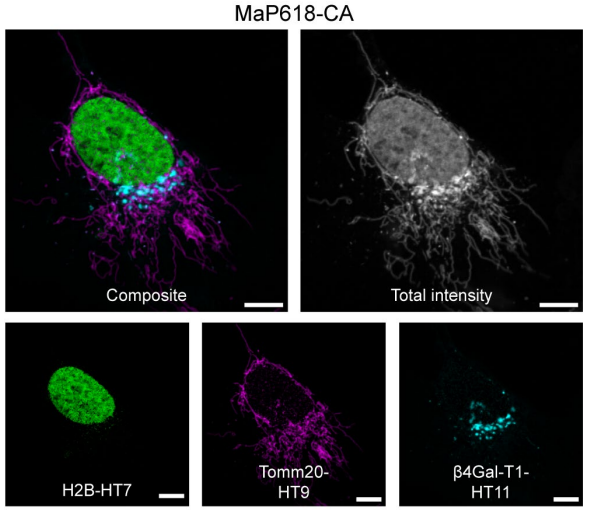


Fluorophores cannot only be separated based on their spectral information but also using fluorescence lifetime information acquired via fluorescence lifetime imaging microscopy (FLIM). Separation can be achieved using Pattern Matching[2] of decay curves or in phasor space[3]. Using HT7, HT9, HT10, and HT11 we were able to separate up to three components in one spectral channel.

Two components



Three components

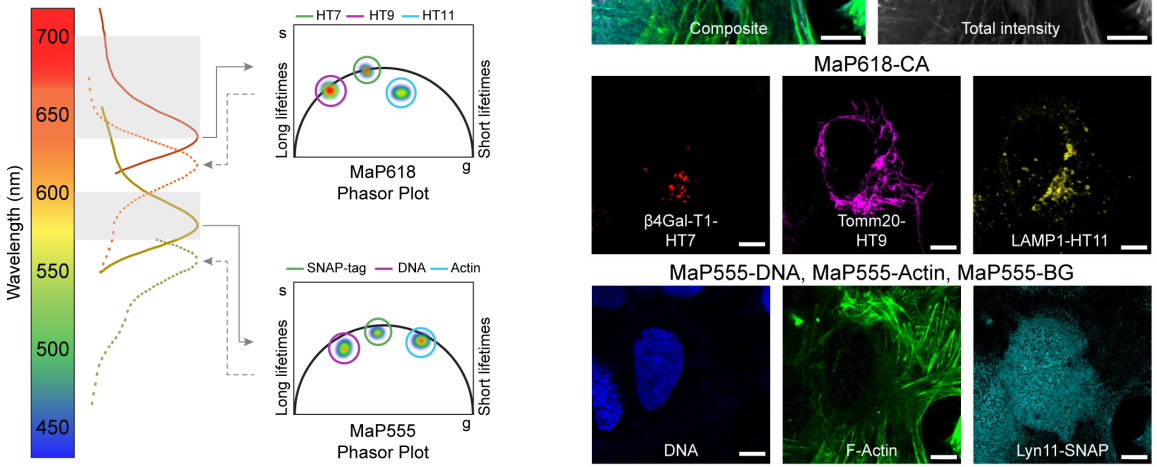


Living U-2 OS cells expressing Tomm20-HT9, H2B-HT7 (left), and β4Gal-T1-HT11 (right) labeled with MaP618-CA. Scale bars, 10 µm.

[2] Niehörster, T. *et al. Nat. Methods* (2016). [3] Digman, M. A., *et al. Biophys. J.* (2008).

Six component imaging

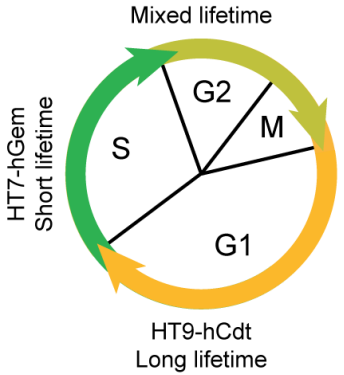
Through the combination of two distinct spectral channels (MaP555 and MaP618) it became possible to image six components at the same time.



Living U-2 OS cells expressing β4Gal-T1-HT7, Tomm20-HT9, LAMP1-HT11, and Lyn11-SNAP-tag were labeled with MaP618-CA, MaP555-BG, MaP555-DNA, and MaP555-Actin. Scale bars, 10 µm.

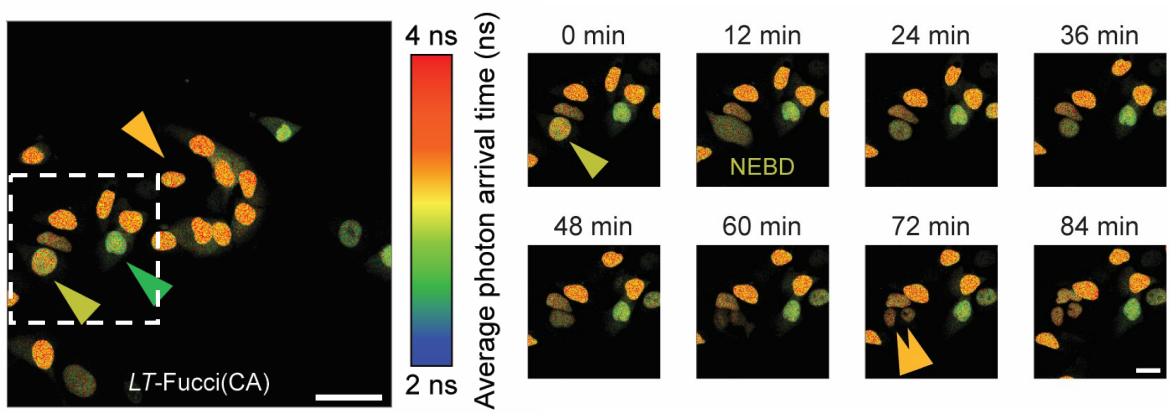
Fluorescence lifetime-based Fucci biosensors

Through the adaption of Fucci biosensors used to indicate the cell cycle stage[4], we created a fluorescence lifetime-based Fucci sensor. It only occupied one (variable) spectral channel instead of the green and red channel and can therefore be multiplexed more easily.



[4] Sakaue-Sawano, A. *et al. Mol. Cell* (2017).

Fluorescence lifetime-based Fucci biosensors



FastFLIM image of U-2 OS cells expressing *LT*-Fucci(CA) labeled with MaP618-CA. Scale bars, 50 µm and 25 µm.

Conclusion

Modulation of the HaloTag surface can indeed influence the photophysical properties of bound rhodamine based fluorophores. Our screening based approach has not only led to the identification of the brighter HaloTag9 but to a series of HaloTag variants that can be combined for fluorescence lifetime multiplexing.

Frei, M. S. *et al. bioRxiv* (2021).

Acknowledgment

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