# Towards improved descriptions of FRET dye movements

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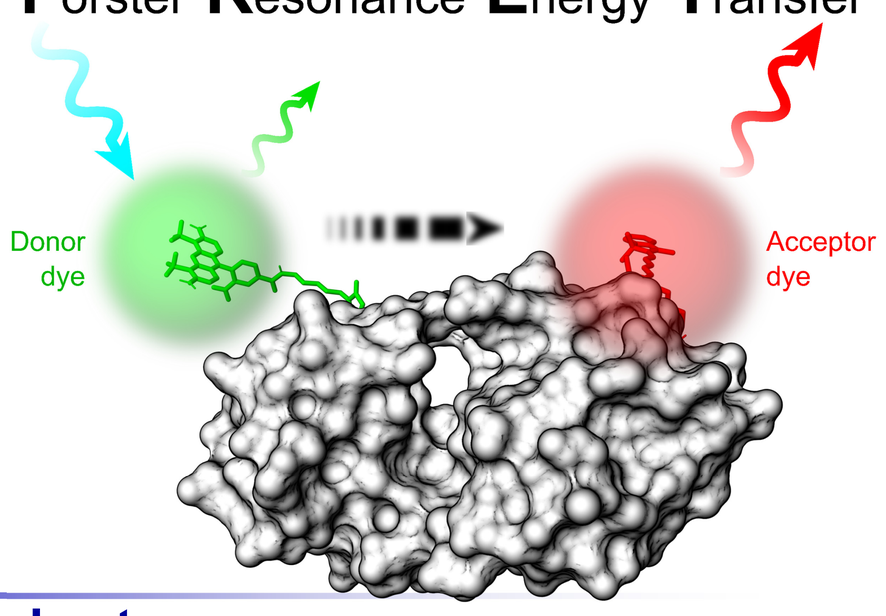
## Introduction

Being able to analyse biomolecular dynamics is a central element in understanding a biomolecule's function and mechanism. The Förster Resonance Energy Transfer (FRET) [1] describes the energy transfer between two fluorescent molecules (dyes). As FRET provides insights into inter-dye distances and also covers dynamic processes on biologically relevant timescales, it is often referred to as a "spectroscopic ruler" [2].

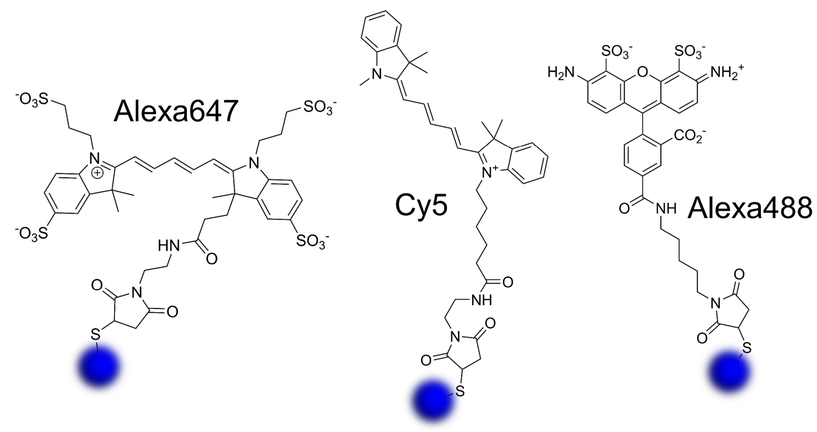




## Experimental setup

We performed all-atom molecular dynamics (MD) simulations with a commonly used hydrophobic dye (**Cy5**) or hydrophilic dyes (**Alexa488, Alexa647**) attached to five distinct positions of T4-Lysozyme, resulting in a cumulative simulation time of over 350 µs. To ensure the free movement of the dyes, all dyes contain long flexible linkers that are covalently bound to cysteine variants (I150C, D127C, N132C, R119C, and P86C) of T4L.

The FRET effect depends on the distance between the two dyes and the relative orientation factor $\kappa^2$ of their electronic transition dipole vectors. However, both values are approximated for interpreting experiments, and thus, can increase the uncertainty of the underlying measurement.

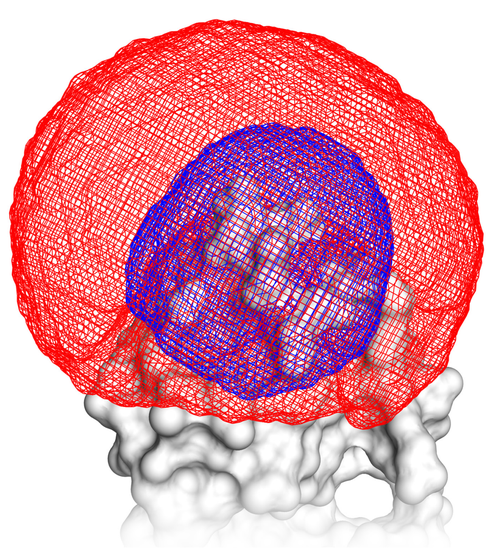
To tackle this issue, we are developing a general method for the accurate and efficient prediction of the dye movement and orientation.



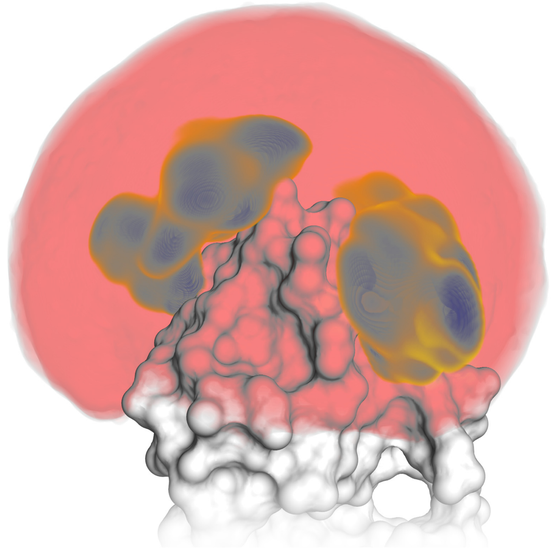

## Commonly used approximations in FRET measurements

To interpret FRET measurements, the position and orientation of the dyes have to be approximated, as both values cannot be measured directly. One commonly used approximation is the available volume of the dye. The available volume is the theoretical space a dye can occupy during the FRET experiment and depends on the linker length, the used dye, and chosen attachment site. Every position of the dye within the available volume has the same propensity. A second commonly used approximation is the weighted Available Volume, which considers the tendency for dyes to be near the protein surface. It consists of a defined sub-volume, which covers only the region near the protein surface. The sub-volume is the region where dyes are most likely localized during FRET experiments.

Weighted available volume



Density map



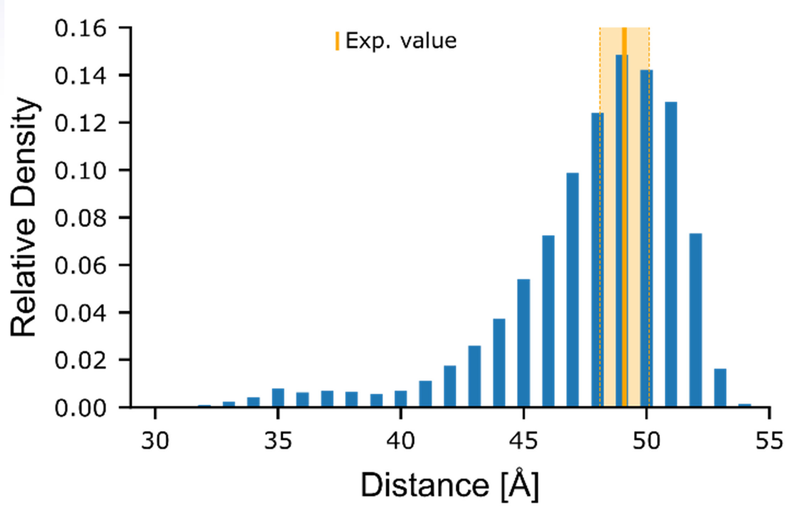
## Spatial probability density maps derived from MD simulations

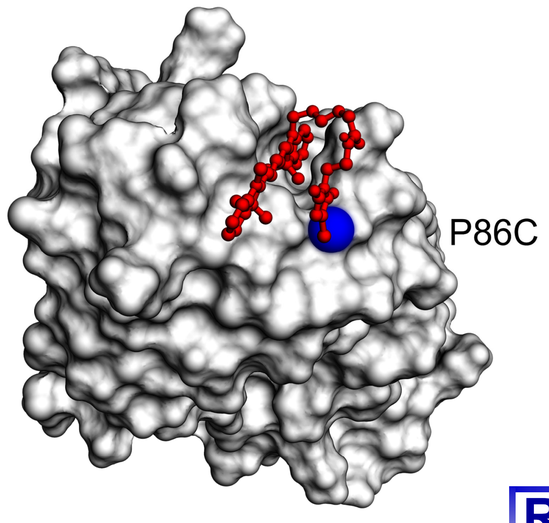
From the MD trajectories we calculated spatial probability density maps for every combination of dye and attachment site. The density maps are divided into three regions: RED covers 100% of the space occupied by the dye during the simulations and corresponds to the AV; YELLOW is the region where the probability to detect the dye is ~33% of the maximum; BLUE consists of the space with the maximum probability for the dye to be during the simulations. Single small blue regions point to sticking events, while multiple or large blue regions indicate different favored conformations of the dye.

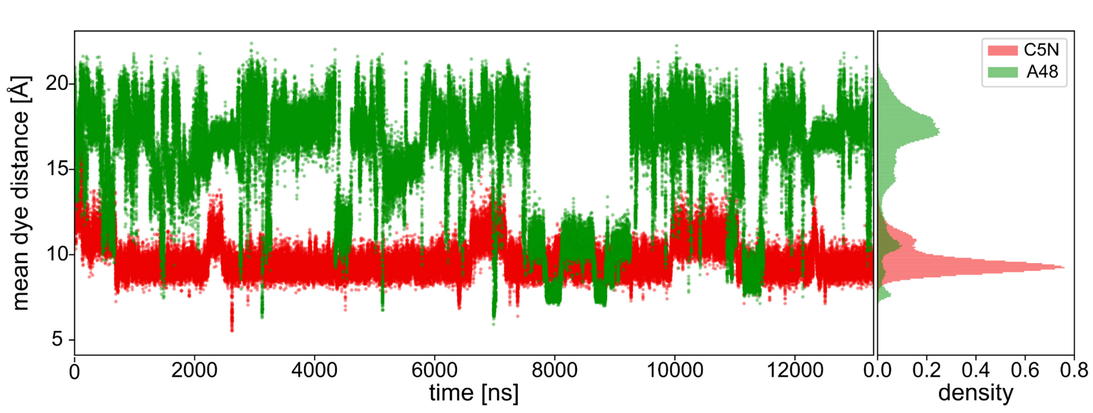
## Results

Similarly to FRET experiments, the distance of two simultaneously attached dyes in MD simulations is in good agreement to the distance estimated by FRET measurements [3].

In contrast to commonly used approximations, density maps show specific regions with a higher tendency for the dye to be found during FRET experiments. Notable, these regions are not equally distributed over the protein surface or in a specific region, as implicated by the the weighted available volume. These regions can have a large impact on the FRET efficiency and their interpretation, due to a shift of the mean dye position between available volumes and density maps in the range of 5.5 to 17.2 Å. Furthermore, sticking of the dye on the protein surface for an elongated period of time is also not considered by the approximations. Our MD simulations show that sticking occurs in 5 out of 10 cases for the hydrophobic dye, and in 2 out of 10 cases for the hydrophilic dyes. Based on our results, we identify those attachment sites at which dye sticking can occur and thus need to be excluded from subsequent FRET measurements. Additionally, the approximation of $\kappa^2 = 2/3$ is not fulfilled in 18 out of 20 cases, regardless of a free moving or sticking dye.




## Summary

In summary, our data questions widely used approximations as to isotropic or semi-isotropic density distributions of commonly used fluorescent dyes around attachment sites on proteins. They also provide the starting point for developing more accurate descriptions for dye movements. Ultimately, being able to predict dye movements and potential sticking will allow to rationally select suitable dyes and attachment sites on the surface of a protein.

## References

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