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The cytoplasm of a cell contains a high concentration of proteins, mRNAs, sugars, ions, and small molecules. The high density of macromolecules makes the cytoplasm crowded and hence the intracellular environment is markedly different from bulk solution. It was recently shown that the high macromolecular crowding in the intracellular environment can be quantified with Förster resonance energy transfer (FRET)-based sensors. However, to better understand the crowding effect in vivo, there are some questions that need to be answered: 1) The sensing mechanism of the sensors; i.e. how does crowding influence the sensors? 2) The artifacts that influence the read-out of the sensors; i.e. how does the maturation of fluorescent protein influence the read-out of the sensors? 3) The effect of crowding in the cell under different growth conditions; i.e. how does crowding change during adaptation to hyperosmotic shock?

In order to answer these questions, in this thesis, I systematically changed the linker of the sensors, identified the factors that influenced the fluorescent protein maturation efficiency, and tracked the change in crowding during hyperosmotic stress in bacterial cells.

In chapter 1, I present an overview on the complex composition of the cytoplasm, the effects of the crowded interior on the macromolecular diffusion, as well as the conformational changes of macromolecules due to crowding, and methods to quantify crowding in cells. The recent developments in crowding sensing will be discussed in detail, including the mechanism, applications, and (dis)advantages of the available sensors.

To understand the sensing mechanism, in chapter 2, I present a novel set of FRET-based crowding-sensitive probes and investigate the role of the linker design. We investigate the sensors in vitro and in vivo and by molecular dynamics simulations. We find that in vitro all the probes can be compressed by crowding, with a magnitude that increases with the probe size, the crowder concentration, and the crowder size. We capture the role of the linker in a heuristic scaling model, and we find that compression is a function of size of the probe and volume fraction of the crowder. The FRET changes observed in the cell are more complicated, where FRET-increases and scaling behavior are observed solely with probes that contain the helices in the linker. The probe with the highest sensitivity to crowding in vivo yields the same macromolecular volume fractions as previously obtained from cell dry weight. The collection of new probes provides more detailed readouts on the macromolecular crowding than a single sensor.

To quantify the influence of fluorescent protein maturation on the read-out of the sensors, in chapter 3, we show that variation of both
the protein expression conditions and the fluorescent proteins influence FRET. Our findings show that artifacts from slow maturing fluorescent proteins can be significant with increasing inducer concentration, but can be minimized by expression with stable protein levels (without inducer). We built a model to quantify the influence of the maturation efficiency on the measured FRET efficiency. The model indicates that the ratiometric FRET relates to the maturation of the fluorescent proteins and depends mostly on the maturation of mCitrine (acceptor), while the maturation of mCerulean3 (donor) influences the ratiometric FRET at very low level of maturation. These results demonstrate that the maturation efficiency has a significant effect on the measured FRET efficiency under expression with inducer. Similar outcomes should apply to other fluorescent protein-based FRET sensors described in the literature.

To investigate the change in crowding during adaptation to hyperosmotic shock, in chapter 4, we tracked the crowding changes in *Escherichia coli* with previously developed macromolecular crowding sensors (crGE, crE6, and crG18). The results demonstrate that macromolecular crowding increases immediately after osmotic upshift, then decreases to a lower level over 2–5 h, where it remains. The crowding initially follows cell volume, but upon adaptation arrives at a lower value. With these results, in combination with literature observations, we hypothesize that the decrease could be due to an increase in self-association of the macromolecules in the cell upon adaptation. The self-association creates a heterogeneous distribution in the cytoplasm, resulting in some regions experiencing less crowding. This interpretation would be fundamental to the organization of the crowded cellular cytoplasm, and would apply to other species as well.

Finally, to detect the ionic strength *in vivo*, in Chapter 5, we present the first sensors to determine the ionic strength in living cells, by designing protein probes based on FRET. These probes allow observation of spatiotemporal changes in the ionic strength on the single-cell level.

In summary, in this thesis, we developed and characterized a set of FRET-based sensors for the crowding and ionic strength in the cytoplasm. With these sensors, we can observe the crowding and ionic strength of the intracellular environment spatiotemporally. By characterization of these sensors, we started to clarify the mechanism of the effect of crowding in cells. However, more work is needed for deeper understanding of macromolecular crowding in cells, as detailed below.

We cannot predict how the crowding affects different macromolecules *in vivo*, due to the complex nonspecific chemical interactions in the intercellular environment. The steric crowding effect is often overshadowed by these other effects. Hence, we suggest to design more sensors, which are larger or have specific chemical interactions with the intracellular environment, to map the effect of crowding *in vivo*. 


We hypothesized that macromolecules increasingly self-associate after adaption. A consequence of this hypothesis, is that the crowding would different in different subcellular locations. Hence, we suggest to locate the sensor to different subcellular location to verify this. This also helps us to understand the physical chemical parameters of intracellular environment.

Additionally, the maturation of the fluorescent proteins influences the accurate quantification of the change in FRET ratio. The maturation of fluorescent protein depends on the protein itself and the expression conditions (e.g. with/without inducer). Hence, the development of a new fluorescent protein, which rapidly matures and is not affected by its environment, will allow the quantification of macromolecular crowding under various conditions.

In conclusion, the macromolecular crowding can be quantified by a set of crowding FRET-based sensors, depending on crowder volume fractions, crowder size, sensor size, and additional factors that need elucidation. With these sensors, one can determine the crowding in subcellular locations and track the change in crowding when exposing the cells to different environments. As a result, we may link many biological processes to the change in crowding, which may help us understanding these biological processes a physical view.