**Dr. Radek Šachl**
**Associate Professor**
**Czech Academy of Sciences**
**J. Heyrovský Institute of Physical Chemistry, v. v. i.**
Dolejškova 2155/3, 182 23 Prague 8, Czech Republic
phone: +420 26605 3505,
e-mail: radek.sachl@jh-inst.cas.cz

# Evaluation report of the doctoral thesis

**Author of the doctoral thesis:** Vandana

**Title of the thesis:** Development of dual-(+1)-Fluorescence Correlation Spectroscopy for Monitoring Protein Oligomerization Leading to Membrane Pore Formation

**Reviewer:** Radek Šachl

The rapid technological advancements of recent decades have paved the way for the development of microscopic fluorescence techniques with the sensitivity to detect single molecules. This breakthrough has facilitated the study of various biological and chemical processes at the single-molecule level. In the context of this PhD thesis, which focuses on membrane pore formation, these single-molecule techniques were naturally applied to investigate the oligomerization of plasma membrane-associated proteins that lead to membrane pore formation.

A widely used approach to study the oligomerization of membrane-associated proteins involves reconstituting fluorescent variants of these proteins into supported phospholipid bilayers and tracking their brightness over time using high-resolution fluorescence techniques. However, the analysis of individual aggregates often yields multimodal and broad distributions of oligomerization states, making it difficult to discern whether these protein aggregates form functional membrane pores or result from nonspecific aggregation due to failed reconstitution of recombinant proteins into the membrane.

Early in her PhD journey, Vandana joined the development of a new method in our laboratory called dual(+1)-FCS. The primary goal was to develop a method enabling monitoring of protein oligomerization at the membrane and membrane pore formation within a single experiment. This technique was designed to directly correlate oligomerization with membrane permeability, thus identifying which oligomeric states are necessary for the formation of functional membrane pores.

As part of her thesis work, Vandana's main task was to complent the development of this method and demonstrate its application on a real protein-membrane system. She successfully accomplished this (Šachl et al Anal. Chem. 2020) and contributed significantly to the further development of dual(+1)-FCS, particularly in detecting changes in oligomerization states over time during membrane pore opening (Vandana et al., Anal.

**Dr. Radek Šachl**
**Associate Professor**
**Czech Academy of Sciences**
**J. Heyrovský Institute of Physical Chemistry, v. v. i.**
Dolejškova 2155/3, 182 23 Prague 8, Czech Republic
phone: +420 26605 3505,
e-mail: radek.sachl@jh-inst.cas.cz

Chem. 2023). In the same work, Vandana applied this method to study FGF2 protein translocation across a biological membrane, a process accompanied by membrane pore formation.

Beyond her main research focus, Vandana also worked on implementing a novel fluorescence cross-correlation spectroscopy (FCCS) binding assay in the lab (Krüger et al., Biophys. J. 2017). She successfully applied this technique to study the binding of cell-penetrating peptides to biological membranes (Nguyen et al., Langmuir 2022). Furthermore, Vandana participated in the development of a new correlative approach that combines the FCCS binding assay with a calcein permeabilization assay, which she employed to investigate the role of apoptotic protein BAX in pore formation under oxidative stress conditions (Mystek et al., Biophys. J.,2024).

In summary, I can only conclude that, in my view, the PhD candidate Vandana has made a solid contribution to advancing our understanding of FGF2 translocation accompanied by pore formation through development of dual(+1)-FCS technique. While there are always areas that could be explored further, her research is solid, well-executed, and offers valuable insights.

I believe that Vandana's thesis meets the requirements for a doctoral dissertation, and I recommend it for defence. I also hope that the discussion during the defence will further highlight the significance of her findings and her ability to contribute to scientific research.

Radek Šachl

In Prague, 23.10. 2024