Opponent's opinion on Vandana Singh's dissertation

Vandana Singh prepared her dissertation "Development of dual-(+1)-Fluorescence Correlation Spectroscopy for Monitoring Protein Oligomerization Leading to Membrane Pore Formation" under the guidance of supervisor Assoc. Prof. Radek Šachl.

The author's main goal was to develop a way to examine membrane-bound complexes of FGF2 protein, and at the same time to find out whether or not they are functional oligomers. The procedure that was developed and presented in the work is very clever and probably unique. The work is based on two publications in peer-reviewed journal of Analytical Chemistry (IF 6.8), where Vandana is the first author (Singh et al. 2020) or co-author (Šachl et al. 2023).

The submitted thesis contains all the necessary details, such as an abstract, a literature review, an explanation of the goals of the work, the research objective explaining the main results, conclusions, bibliography, publications of the author, declaration of Vandana’s contribution to the manuscripts.

The introductory chapters prepare the reader well for the content of the work. The chapter is divided into two parts. The first deals more with experimental approaches to the study of membranes and membrane proteins. The second then targets the FGF2 protein. This section also provides a clear rationale for the importance of studying the pore-forming proteins. Some of the opening paragraphs might deserve certain expansion, however. In the paragraph "Artificial membrane systems", for example, I miss a little mention of other low molecular weight components of membranes, the functions of membrane proteins or the role of membrane microdomains.

The objectives of the work (p. 30) are very clearly defined and explain what the author was dealing with and why:

Part I: Development of a functional assay to correlate protein oligomerization states with membrane pore formation.

Part II: Determining the functional oligomeric state of membrane-associated FGF2 oligomers forming membrane pores on giant lipid vesicles.

The results of the work are analyzed and discussed in detail on many pages (pp. 30-54). As a separate, very brief chapter, the thesis contains Supplementary Information, more precisely Material and methods (pp. 58-59). For more details of the methods used, there is a need to consult the attached publications, however.

I have some reservations about the graphic side of the work. The work contains several images taken from literature, either by the author herself or by other authors. However, some unattributed images contain certain inaccuracies. Small unilamellar vesicles (SUVs in Fig. 2) contain apparently "small" phospholipids which does not correspond to reality. The confocal microscope is not clearly depicted (Fig. 9A), the objective is missing in the diagram and it is not clear in which direction the light beam travels. It is not obvious, for example, what is the role of pinhole in the confocal imaging.

The work contains a large number of references. Individual papers are usually well cited. The exception is the citations in the figure legends, where the citations are non-standard and inconsistent. Other minor inaccuracies also appear in the text itself, an overview of which I attach in the last part of the review.

However, the aforementioned shortcomings are not fundamental and do not significantly reduce the quality of the work.

Questions for discussion:

1) Is it possible to change the size and shape of the monitored volume during the FCS measurement? Which instrumental parameters of the microscope have an effect on this? What could a targeted confocal volume change be useful for?

2) What is the experimentally observed diffusion coefficient (D) of FGF2-GFP and DOPE-Atto-633 in the membrane? Are these results consistent with theory? (D value for Rhodamine given in the thesis is wrong by the orders of magnitude, p. 35).

3) The fluorescence channel of FGF2-GFP is very intense (p. 32, Fig. 13). Are the GUVs labeled in this way usable for the FCS experiments? Does the GFP signal in the solution interfere with the experiments?

4) Is the FGF2 protein fully functional after GFP attachment *in vivo*? Where exactly was the GFP genetically attached to FGF2 in the experiments? "GFP and FGF2-GFP" are sometimes confused in the text, while free GFP could of course also be used for calibration purposes.

5) Is the triplet state of the fluorophore significant for the investigated protein?

6) Do you expect that any pore in GUV membrane that is leaky for Alexa Fluor dye can also serve for translocation of FGF2 protein?

In conclusion:

Mgr. Vandana Singh contributed fundamentally to the development of a innovative method of *dual-(+1)-FCS*. The method which can be generally used to detect *functional* oligomers of membrane pore-forming proteins against the background of other *non-functional* aggregates. Thanks to her work, the long-standing problem of many scientists dealing with oligomeric membrane proteins seems to be solved. All presented experiments were properly carried out and evaluated, as evidenced by their successful publication in a journal with a high impact factor.

Dissertation thesis of Mgr. Vandana Singh meets the requirements placed on it. In her work, the author proved that she is a capable experimenter with a well-founded theoretical and methodological background. The author's publications publication in a high-quality journals show the high experimental level.

I recommend accepting the dissertation as a basis for further proceedings for the award of a doctoral degree.

In Prague 13/11/2024 Doc. RNDr. Radovan Fišer, PhD.

Additional questions:

(The questions below do not need to be answered during the defense)

- For work with FGF2, carboxylmethylphenylalanine was included as an unnatural amino acid. With what intention was the point mutation Y91pCMF (p. 58) introduced into the protein? What role does this swap play in the activity of the protein? For example, have you also considered mutations at positions C77 and C95?

- In what organisms can FGF2 homologues be found?

- Does the FGF2 protein show oligomers also in solution? Does this state of the protein change during storage?

- Does the Poisson statistics manifest itself in the detection of oligomers on GUVs? Is it possible to change the amount of oligomers by different setup of the experiment?

- To study oligomers of pore-forming proteins, monitoring the concentration dependence of membrane activity can be used in a double logarithmic plot. The data is then fitted with a power function. What is this simplified model based on?

- Which fluorescent probes exert "self-quenching" (p. 9) and what is the reason for that?

- Is there an advantage to using calcein over carboxyfluorescein in LUV or GUV?

- How is a sample with GUV monitored at different wavelengths, regarding the polarization of the used excitation laser and the sensed polarized fluorescence? Is the source polarization (and its spatial alignment) important for DOPE and GFP when observing in XZ mode?

- How does fluorescence bleaching manifest itself in the analysis of autocorrelation functions?

- The membrane composition of the GUV suggests that it was a rather complicated mixture of phospholipids (p. 58). What is the composition of the mixture based on?

- What exactly does the quantity "oligomeric state" mean? Why does this value differ for individual GUVs (Fig. 20)? How many individual oligomers have been observed in the membrane of a single GUV and how do they vary in number of subunits?

Additional comments:

- Equation 7 is written incorrectly (probably a typographical error, p. 20).

- The reference 65 (p. 14) is not related to STORM or PALM but to the Bax oligomers.

- Some more extensive introductory passages are poorly supported by the cited literature (p. 15-16). The text is a paraphrase of a paragraph from the review article ref 55. This work is not properly cited on that page. Similarly, page 25 apparently draws from a paper that is not properly cited (doi: 10.3390/pharmaceutics12060508).

- Some informations are communicated with certain errors. E.g. the amount of heparin that binds to the investigated protein is 10 times different in the literature (p. 25).

- List of abbreviations - used abbreviations are listed (p. 81), but some did not even need to be introduced (EM, FCCS, SMLM, YFP, CFP, ...), as they are used only once (or not at all). Some abbreviations are incorrectly defined (MLKL - Pore Forming Proteins).

- The following text is a bit inaccurate (p. 21):
  Consequently, the probability of detecting photon pairs from a single molecule within a very short lag time between them approaches zero as the lag time itself approaches zero. This characteristic drop in probability is determined by the fluorescence lifetime of the molecule.
  … The probability of capturing any event always decreases as the integration time (or lag time) decreases. And it doesn't have much to do with antibunching or fluorescence lifetime.

- What is "membrane katsinpotential" (p. 28)?

- Chapter 3.5 contains longer texts without citations (pp. 39-40). It is probably a summary of the author's observations and experiences. Nevertheless, specific values are given here, when it is not clear how the author arrived at them.

- I don't like the use of the term "*time-resolved measurement*", since fluorescence lifetimes are not determined in the experiment (p. 36, 37, Fig. 17).

- Latin names of bacteria should be given in italics.

- Fig. 15 shows what the FCS record (and corresponding autocorrelation curve) would look like when the protein "oligomer", "monomer" and DOPE-Atto-633 were present in the sample (p. 35). But the example is rather unfortunate. The intensity curves over time are identical, they differ only in the intensity, and this corresponds to the situation when the autocorrelation functions have the same shape (inflection point at the same time). It is also not clear how the analysis was realistically performed when different protein forms were present simultaneously in one sample.

- Statement on p. 39:
  As the main limitation of dual-(+1)-FCS can be considered the fact that it yields the average oligomer size per single GUV, i.e. it does not provide a distribution of single oligomer species as the true single-molecule techniques do.
  Q: Is it not possible to determine the true distribution of the number of protein oligomer subunits for a set of GUVs? Is it necessary to calculate the average value?

- Statement on p. 41:
  ...we studied the translocation of FGF2 across the plasma membrane … the presence of membrane pores indicates that the protein is functional and capable of being translocated across the plasma membrane...
  Q: Do you expect that any pore in GUV membrane that is leaky for Alexa Fluor day could also serve for translocation of FGF2 protein?