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Confidential report on the thesis "Quantitative fluorescence microscopy techniques to study three-dimensional organisation of T-cell signalling molecules" by Mgr. Tomáš Chum

General remarks: The thesis by Tomáš Chum is a truly comprehensive body of work. He first approached the question, how the sorting and the plasma membrane localization of transmembrane adaptor proteins (TRAPs) is linked to their particular transmembrane domain length, amino acid sequence and palmitoylation state. Mr. Chum used quantitative confocal microscopy to study the distribution of genetically modified TRAPs and to provide insights into underlying mechanisms. He then focused on the protein LAT (linker for activation of T cells) - one key player during early T cell signaling- which turned out to undergo a different sorting control compared to other TRAPs. Together with co-workers he found that central proline residues within the transmembrane part of LAT destabilized the helix by inducing a kink.
Finally, Mr. Chum developed a novel substrate for artifact-free adhesion and subsequent superresolution imaging of non-activated T cells. A glycine coating of glass coverslips together with thoroughly cleaning procedures preserved nanoscopic structures on the T cell plasma membrane. Using a modified 3D superresolution approach developed by co-workers he studied the localization of CD4 and CD45 – two receptors involved in T cell signaling –with nanometer resolution in all three dimensions.

In general, the thesis is well written (with some minor typos, though), the text is easy to follow, but never at the expense of a scientific precision. Having studied many different proteins labeled with numerous fluorescent markers in various cell types the reader was still able to keep the track. Several sketches were provided that are very helpful for understanding the experimental settings and research questions. A few minor but important aspects were not absolutely clear to the reader and will be listed as *comments* in the detailed report.

Detailed report: The thesis contains seven chapters: A General introduction (Chapter 1), Aims of the work (Chapter 2), Materials and Methods (Chapter 3), Results (Chapter 4), General conclusions and discussions (Chapter 5) and References and Appendices (Chapters 6 &7).

In the introduction, Mr. Chum starts with an overview over the building blocks of biological cells, with a focus on the class of proteins that were studied in his work. He introduces relevant post-translational modifications of membrane proteins and how these proteins are currently be thought to be sorted to finally reach the plasma membrane. *Comment: sorting in multicellular organisms rather takes place via (ATP-driven) directed motion than diffusion. The latter would take way too long to transport molecules over micrometer-distances (Chapter 1.4, page 24).*
Two sections are devoted to the plasma membrane of T cells. He then introduced fluorescence microscopy concepts, with a focus on confocal microscopy and superresolution microscopy – both techniques have been deployed to study protein sorting and plasma membrane localization in his work. *Comment: Mr. Chum provides the single molecule localization accuracy (Chapter 1.9.3.3, page 53, first formular), however, beside the "2", which should be a superscript, the provided formular (I guess it was taken from Thompson et al. 2002) is not correct and should be replaced by the one given by Mortensen et al. 2010. Mr. Chum cites both papers so I am sure he is aware of this fact.*
Finally, he briefly discusses sample preparation and closes the introductory chapter with a really good overview over all proteins involved in his study.

In the aims chapter, Mr. Chum lists biological aims together with the chosen experimental approach, as well as methodological problems with their solution.

The Materials and Methods chapter contains a detailed overview about sample preparation and the techniques and analysis methods used for each project. Importantly, Mr. Chums briefly explains the improved 3D superresolution analysis methodology dTRABI, which was applied by him and coworkers for one study.

The Results chapter is divided into four projects. (1) Mr. Chum applied quantitative 2-color confocal microscopy to study sorting and plasma membrane localization of transmembrane adaptor proteins (TRAPs) and TRAP-like proteins. As representative TRAPs he chose PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains), NTAL (non-T cell activation linker) and LAT (linker for activation of T cells) – the latter being a key protein in early T cell signaling. In summary he found, that sorting and plasma membrane targeting is linked to the particular transmembrane domain length, amino acid sequence and palmitoylation state. Interestingly, non-palmitoylated LAT was found to be trapped on its way to the plasma membrane – in contrast to the other studied TRAPs. This part of his thesis was published in 2016 in the Journal of Cell Science, with Mr. Chum as the first author. (2) In the second project, Mr. Chum together with co-workers studied the role of the kink-forming amino acids proline and glycine in the transmembrane domain of LAT. Mr. Chum's contribution was to perform quantitative confocal microscopy to determine the localization of various LAT mutants in living cells. The non-peer-reviewed work was posted on a pre-print server in September 2020. (3) Here, he applied an improved 3D superresolution method termed dTRABI to study the surface distribution of CD4 and CD45 – two important proteins on the plasma membrane of T cells. Together with co-workers he showed, that CD4 is accumulated on the tips of microvilli of non-activated T cells, while CD45 is spatially segregated. This finding is highly important to understand the molecular orchestration during early T cell activation. Importantly, these findings were only possible due to the development of a minimally-disruptive surface for the adherence of T cells. This non-peer-reviewed work was posted on a pre-print server in July 2020 with Mr. Chum as first author. (4) The last project deals with more details about the surface coating which was developed to study T cells in project 3.

In the next chapter, Mr. Chum discusses in great detail his results gathered in the previously mentioned four projects. After the references, he lists three important protocols which were used to prepare the novel surface for the adhesion of cells and to perform superresolution microscopy.

Concluding remarks: Mr. Chum's thesis is a comprehensive work focusing on the sorting and localization of transmembrane adaptor proteins as well as transmembrane proteins relevant in T cell signaling. By using quantitative microscopy, he was able to show highly convincing arguments about the role of the transmembrane part in protein sorting and the nanoscopic localization of proteins, respectively. The thesis is well written and structured. Finally, I appreciated the detailed discussion, especially in Chapter 5, which also pointed out some disadvantages of applied methodologies.

In conclusion, Mgr. Tomáš Chum's thesis exhibits independent scientific work and I highly recommend him for being awarded with the academic degree doctor.

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