Kavli Synergy Program – Research Proposal

1. **Applicant**

Name Mike Filius, PhD
Affiliation Department of BioNanoScience
Most significant achievements (selected): During my PhD project, I developed a novel high-resolution, fluorescence based protein identification platform (Filius et al, *Nano Letters*, 2021; Filius et al *Nature Nanotechnology*, under second revision) and filed three filed patents. By collaborating with excellent groups in chemistry (Eelkema group, ChemE, TU Delft) and bioinformatics (de Ridder group, Wageningen University) I also co-authored several manuscripts (see CV for full list of publications). Working at the intersection of diverse fields has enhanced my scientific competence and expanded my professional network. This project will serve as a pivotal step in my career development, providing me with the opportunity to delve into the intricacies of biological systems, specifically neuronal cells.

2. **Title of the Project** **High-Resolution Glycosylation Profiling of Synaptic Proteins**
3. **Kavli Host Groups** Dimphna Meijer and Chirlmin Joo
4. **Abstract**

Neurobiology faces the challenges of understanding the complex mechanisms underlying neuronal connectivity. Among various post-translational modifications, glycosylation plays a crucial role in the connections between neuronal cells. However, state of the art technologies for the analysis of protein glycosylation lack the sensitivity and resolution to reveal glycosylation profiles at the molecular level. In light of this gap, my aim for the KIND synergy project is to develop a ground-breaking high-resolution technology that can analyse glycoproteins one molecule at a time. I will apply this novel technology to heavily glycosylated cell adhesion molecules in order to determine how their glycosylation profile dictates synaptic target recognition. My project will shed lights on the significance of protein glycosylation in neurobiology, and broadly human biology. This will lead to better therapeutic strategies and discovery of novel biomarkers for early-stage disease diagnosis of neurological disorders.

5. **Description of the proposed research**

Background and motivation for the research
Recent advances in neurobiology have highlighted the potential role of post-translational modifications, specifically glycosylation, in diversifying the molecular repertoire at the synaptic membrane. For example, glycosylation, as the most diversified type of post-translational modification, plays a crucial role in modulating the specificity of cell adhesion molecules (CAMs). By adding variable glycan chains to these surface-exposed CAM proteins, glycosylation expands the recognition profile of pre- and post-synaptic elements, enabling highly specific matches. Recent studies have identified the presence of specific glycan chains in the binding interfaces of macromolecular complexes at the synapse, further highlighting the significance of glycosylation in synaptic target recognition. Building upon these findings, the Meijer lab is dedicated to investigating how glycosylation extends the diversity of CAMs, providing a code for self-avoidance and synaptic target recognition. Delving deeper into the exploration of glycosylation within the realm of neurobiology is imperative for a comprehensive understanding of its impact on synaptic function and neuronal connectivity, and this is the primary focus of this project.

State-of-the-art
Mass Spectrometry (MS) is currently the gold standard for protein glycosylation analysis. Over the years, several variations of MS have been adopted for the analysis of protein glycosylation. One such approach involves enzymatically removing glycans from proteins, followed by MS analysis. While this approach allows for unravelling all glycans that were present in a particular sample, it lacks information about what protein was glycosylated in which manner. An alternative workflow involves digesting proteins into smaller peptides, with the glycosylation profile determined through the analysis of glycopeptides. However, for proteins with a mixed glycosylation profile (that is variations in type and number of glycans), it is impossible to know how many proteins had which combination of glycans from this glycopeptide analysis. This limitation necessitates advanced glycan analysis tools.

Overall aim
I will respond to this need by developing a technology that can analyse CAMs with high spatial resolution and the ultimate sensitivity—one molecule at a time—and pinpoint where a certain glycan is located within the three-dimensional structure of each individual CAM. This technology will answer outstanding questions such as: “What is the effect of sugar end group on protein-protein interactions?” and "Are glycosylation profiles functional in self-avoidance mechanisms?”

Approach
I will build further on my previously established high resolution protein identification technology. I developed a high-resolution technique that utilizes DNA nanotechnology and single-molecule FRET (a molecule ruler) to localize multiple points of interest in a single nanostructure (e.g. DNA (Filius et al, *Nano Letters*, 2021) or Protein (Filius et al *Nature Nanotechnology*, under second revision)) with sub-nanometre resolution. A key feature of our high-resolution technology is

the repetitive probing a point of interest by exchanging the fluorescent probes. This repetitive probing is achieved when a point of interest is labeled with a short DNA strand (or docking strand) and the complementary fluorescently labeled DNA 'imager' interacts for a short period of time.

During the first part of the KIND Synergy project (4 months), I will develop an approach for the conjugation of docking strands to glycans. As glycans are difficult to label directly due to the presence of many reactive groups, I will utilize naturally occurring enzymes that are capable of specifically modifying *N*-glycan structures. First, I will use an enzyme that is capable of specifically removing one of the main glycan types (Endo-H for Oligo Mannose, Endo-F3 for Hybrid Glycan or Endo-F2 for Complex Glycan) after which another enzyme, B4-GalT, is capable of incorporating a functional group that can be modified with a DNA docking strand. (**Fig. 1**) Next, the glycoproteins (CAMs) will be immobilised using my previously developed universal immobilisation strategy (patent pending) and the fluorescent probes are introduced to determine the location of a glycan within the 3D structure of a protein. This cycle can be repeated for each of the main glycan types and allows us to probe the glycosylation profile of each individual CAM in a mixture, thereby revealing the heterogeneity at the molecular level.

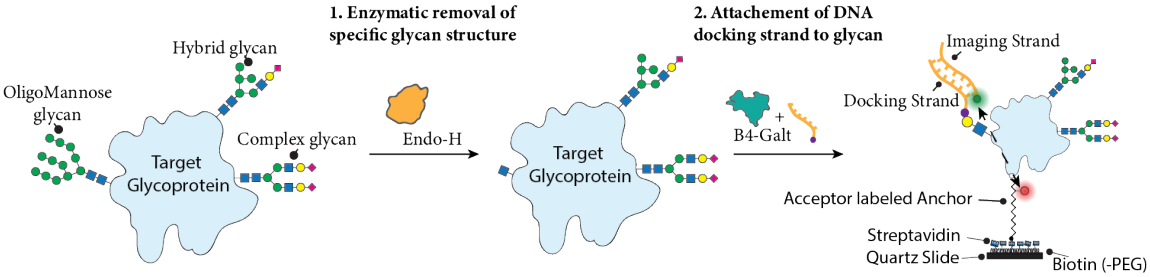


Figure 1 Single-Molecule Glycosylation profiling. 1. One of the three main *N*-glycosylation structures can be enzymatically removed using specific enzymes, Endo-H for Oligo Mannose shown here, leaving a signature (the blue square, middle panel). 2. A second enzyme, B4-GalT is used to incorporate a click handle to the glycosylation signature, which can then be modified using a DNA docking strand. The DNA modified glycoproteins are then immobilised on a quartz slide and the imaging strand is introduced. When the imaging strand interacts with the docking sequence, the distance and location of the glycan can be determined. This can be repeated for any of the main glycosylation types.

During the second part (8 months), I will apply my technology to determine the underlying mechanism of synaptic recognition. Synapses in the central nervous system are highly specialized sites of neuronal adhesion, this process is mediated by many synapse-inducing proteins, including cell adhesion molecules (CAMs). It is speculated that glycan-protein interaction plays an important role in establishing neuronal connections. More specifically, the Meijer group hypothesized that *N*-glycosylation plays a critical role in the fine-tuning of cell-cell recognition by synaptic CAMs. Using my novel glycan analysis technology, I will address this primary question: *what glycan subtypes contribute to synapse formation in primary neurons*? For this I will express and purify CAMs in primary neurons and purify the proteins using a pull down assay. The purified samples will be labeled and analysed using our single-molecule glycan profiling technology. By using different enzymes for the chemoenzymatic labeling approach, we will be able to differentiate which of the main glycan subtypes (oligomannose, hybdrid or complex glycan) are attached to CAMs and are required for synapse formation.

6. Host environment

The combination of a leading group in neurobiology (Meijer lab) and a group that excels in technology developments (Joo lab) will be the key in the success of my project. As from the very beginning I will focus on developing a tool for the relevant biological questions mentioned above, which will allow me to develop my skills for working with complex samples, such as derived from mammalian cell culture. All the necessary access to single molecule microscope setups will be granted by the Joo lab during this project.

7. Benefit to career of the fellow

My long-term aim is to use my pioneering platform for high-resolution glycoproteomics. This project will help me initiate this with neurobiology and consequently expand to other fields such as immunology and therapeutics. This will allow me to become a leading person in this emerging single-molecule field and help me establish my own group. I will also use the results from this project to obtain the Veni grant. Additionally, as this technology can be used for a large number of applications within academic research, but also for discovery and quality control of novel biopharmaceuticals, I am exploring opportunities to create a startup for this technology. I applied for the NWO Take Off grant to validate this business case.

8. Benefit to the synergy of KIND

My project will initiate a new collaboration between the Meijer and the Joo group, that currently do not have any projects running together. Furthermore, single-molecule glycoproteomic will become a new field of research that will be pioneered at KIND. After completing this project at KIND, there will be many opportunities to use this technology in a wide variety of academic groups. For example, we will expand our collaboration with Albert Heck and Geert-Jan Boons (both Utrecht University), who are leading scientists in glycoproteomics. Altogether this will strengthen the bioresearch of KIND.