High sensitive detection and quantification of biomarkers for Parkinson's disease

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- **Erisyon introduces the platform technology, fluorosequencing, for single molecule peptide sequencing.**
- **Alpha-synuclein is a protein biomarker implicated in parkinson's disease. The observation of phosphorylation on serine-129 is correlated to disease states. Cerebrospinal fluids and bloods are sources for identifying this diseased form of the protein.**
- **Antibody assays and mass spectrometry have difficulty in accurate quantification of the protein's modification state. This is either due use of different calibration standards (in antibody assays) or different detection modalities used for identifying modified and unmodified proteins (in mass spectrometry).**
- **By fluorosequencing of the enriched protein, the positions and proportional counts of the phosphorylated residues to the unmodified forms can be mapped and accurately quantified.**

Overview

Parkinson's disease (PD) is a neurodegenerative disorder that is typically diagnosed when 60% of a brain region's dopaminergic neurons have already degenerated[1]. With an aging population and lack of effective therapies, massive efforts are underway to develop molecular biomarkers and diagnose the condition decades' earlier (michaeljfox.org). Sensitive and accurate diagnostic tests would offer the best bet for early detection, monitoring the progression of the disease, design clinical trials and establish better therapeutic approaches to treating the disease. Fluorosequencing, a parallelized single molecule peptide sequencing technology[2], is an alternate platform technology for biomarker detection owing to its intrinsic features of high sensitive detection and absolute quantification.

Alpha-synuclein modifications as a biomarker

One of the proteins implicated for PD is alpha-synuclein. Its neuronal function is speculated to be in maintaining synaptic vesicle density and regulating dopamine release. It is clinically important protein since its elevated expression leads to an insoluble aggregated fibrillar form (called Lewy bodies) and are found in the brain tissue of deceased parkinson's patients. While this protein in PD patients is present in lower levels in their cerebrospinal fluid (CSF) when compared to age matched controls, the patient specific level and its relationship to disease progression is unclear. In addition, alpha-synuclein is also expressed in other cells such as red blood cells making its quantification in blood for PD biomarker challenging. Therefore, the use of total levels of alpha-synuclein as a molecular biomarker for PD has been controversial[3].

Modified forms of alpha-synuclein, such as oligomers, C-terminal truncation mutants or site-specific post-translational modifications such as phosphorylation and ubiquitination are seen as better predictors of the disease and some significantly correlate with disease severity[4]. As an example, in human brain samples, Ser129 phosphorylation (pSer129) represents more than 90% of the total alpha-synuclein while only 4% in age matched controls. Its presence in CSF has been shown to increase with disease progression 4. It has also been observed that a number of modifications

forms of alpha-synuclein are immunoassays and mass spectrometry. Briefly, immunoassays use the binding affinity of antibodies, that are specific to different regions (epitopes) or modifications of the alpha synuclein protein, to detect and quantify the amount of the specific alpha-synuclein modification. Luminex's xMAP, Perkin Elmer's AlphaLISA assays or assays with TR-FRET have enabled identifying alpha-synuclein and its modifications (pSer129) with small sample amounts and in many bodily fluids such as saliva, tear glands etc. with high sensitivity[3]. Protein or peptide

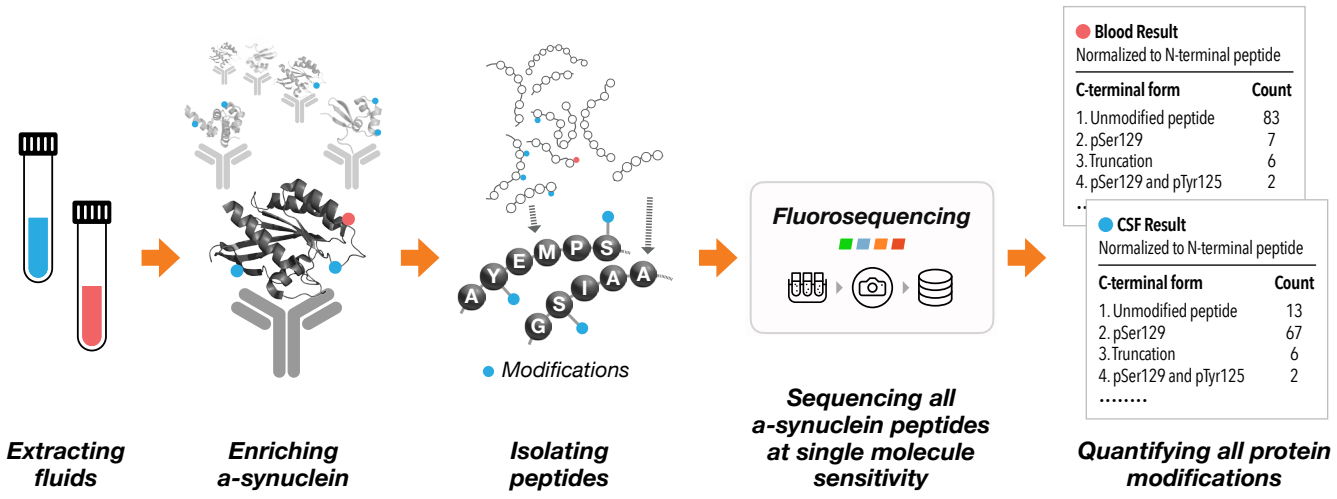

Figure 1. Workflow of fluorosequencing technology to detect and quantify all protein forms of alpha-synuclein – The protein alpha-synuclein is enriched from the bodily fluids such as CSF, blood etc. using well characterized antibody binding to the N-terminal epitope of the protein. After digestion and isolation of peptides, they are labeled and sequenced by fluorosequencing. Since all peptides are analyzed simultaneously, all the sites of phosphorylation, C-terminal truncation and other variants can be quantified and normalized to the N-terminal peptide observations. An example of the output data is shown.

(such as pSer129, pTyr125 and ubiquitination or C-terminal truncation) can co-occur in the same protein sample[5]. Due to this a number of assays are being researched and clinically tested to detect multiple modified forms of alpha-synuclein in a number of bodily fluids.

Current challenges in detecting and quantifying the modified alpha-synuclein

Currently, the major technologies for detecting the different

mass spectrometry has been used to identify intact and modified alpha-synuclein proteins (C-terminal truncation, pSer129) or their peptides by their intact masses and/or the fragmentation patterns of their digested peptides[6].

While each of these techniques have their merits and have enabled a better understanding of PD, they suffer from limitations. Specifically the identification and accurate quantification of all modifications on alpha-synuclein proteins in the same, low abundant sample. For example,

after more than 20 different studies, it is still unclear whether there is any difference in the levels of total alpha-synuclein in CSF observed in PD patients. Inaccurate calibration standards have been pointed out for immunoassays detecting phosphorylated residues[5]. Additionally, for a given sample, both multiplexed immunoassays and top down proteomics do not always identify all the modifications present in the same experiment. This can be attributed to limitations of protein material or the separate detection methods used for quantifying modified and unmodified protein forms. Hence both immunoassays and mass spectrometry fail as an accurate diagnostic platform to probe all the alpha-synuclein protein forms.

Fluorosequencing as an alternate platform technology

By sequencing peptides at a single molecule level and accurately quantifying each of the modifications on the digested peptide fragments, fluorosequencing can not only perform the basic tasks of immunoassays and mass spectrometry with higher sensitivity, but also provide a line of information that was otherwise inaccessible. After the enrichment of alpha-synuclein with an antibody binding to the N-terminus of protein, peptide fragments are generated, labeled and sequenced by the fluorosequencing technology. By probing and sequencing all the peptide fragments, the counts of the epitope bound N-terminal fragment can act as an internal calibration standard. The counts for all the different modified forms of the C-terminal fragment of alpha-synuclein can be normalized to the reference counts. Apart from providing information of all the different modifications of alpha-synuclein in one experiment at very high sensitivity, the technology potentially provides information of the diversity of alpha-synuclein in different bodily fluids that was previously inconceivable.

A basic experimental workflow for using fluorosequencing technology and the expected result is illustrated in **figure 1**. This pipeline inherits the high sensitivity and specificity of antibody binding and is agnostic to the C-terminal modifications on the proteins. This single molecule peptide sequencing technology is similarly translatable for probing the protein forms of many other biomarkers such as LRRK2, RIPK or Parkin associated with neurodegenerative diseases.

References

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