The Michael J. Fox Foundation's Efforts to Develop Novel Antibodies for Understanding and Measuring Alpha-Synuclein Modifications in Parkinson's Disease.

Nicole K. Polinski[1], M. Bilal Fares[2], Lara Petricca[2], Salvadora Aguera De Haro[2], Marylin Aebi[2], Hilal A. Lashuel[2,3], Ana Aragon-Gonzalez[4], George K. Tofaris[4], Samuel Strader[5], Ki Woon Sung[5], Andrew B. West[5], Nicholas Pena[6], Laura Volpicelli-Daley[6], Hao Cheng[7], Thomas Kelk[8], Ravindran Kumaran[8], Elisia Clark[1], Gloria Thakuria[1], Jamie Eberling[1]

The Michael J. Fox Foundation for Parkinson's Research[1], ND BioSciences[2], Ecole Polytechnique Fédérale de Lausanne (EPFL)[3], University of Oxford[4], Duke University[5], University of Alabama at Birmingham[6], ABclonal Technology[7], Abcam Ltd[8].

Introduction

Alpha-synuclein misfolding and aggregation play a central role in the pathogenesis of Parkinson's disease (PD). Increasing evidence points to post-translational modifications of alpha-synuclein (aSyn) as important regulators of its aggregation, pathology formation, and pathogenicity. Several types of post-translational modifications have been identified and associated with physiological and aggregated forms of aSyn. However, very little is known about how, where, and when aSyn is modified due to a lack of high-quality and accessible reagents. To address this gap, The Michael J. Fox Foundation (MJFF) has taken an active role in designing, validating, and distributing various tools and models that can be used to investigate PD-related biology, including antibodies to modified forms of aSyn. Here we summarize MJFF-led efforts in partnership with several research teams from academia, industry, and tool manufacturers to develop and characterize antibodies to aSyn truncated at 1-119, aSyn truncated at 1-122, aSyn ubiquitylated at K45/K38/K60, and N-terminal aSyn. We shall provide data on how these antibodies were designed, their sensitivity and selectivity, and their performance in different applications and model systems. In addition, we will include information on how to access these antibodies, an overview of other PD-related tools and models currently in development at MJFF, and a snapshot of other resources MJFF makes available to the scientific community. Ultimately, MJFF's investment in providing the research community with robust, well-characterized tools and models will speed research towards a cure for PD by enabling research, de-risking investment in PD research, and increasing reproducibility by providing the tools to researchers across labs.

N-Terminal aSyn

MJFF embarked on a program to develop antibodies to N-terminal alpha-synuclein (specifically AA40-50) as this is a region not well-represented by alpha-synuclein antibodies currently. Antibodies to this region would be beneficial given that many other antibodies recognize the C-terminus of alpha-synuclein and most post-translational modifications of synuclein that could interfere with antibody binding occur in the NAC domain and C-terminus.

Table 1. Summary of application testing and epitope mapping results for aSyn 40-50 clone 3A4.

Application	Samples	3A4 Clone
Slot Blot	Recombinant Protein	Pass (Figure 1)
Western Blot	Mouse Primary Neurons (WT vs KO)	Pass (Figure 2)
Immunoprecipitation	Recombinant Protein	Pass
Immunocytochemistry	Mouse Primary Neurons (WT vs KO)	Pass (Figure 3)
Immunohistochemistry	Mouse Brain (KO vs Transgenic OE)	Pass (Figure 4)
Immunoassay	Recombinant Protein	Pass (Figure 8)
Epitope Mapping	Epitope is AA46-50 with some binding to AA12-17 due to sequence similarity	

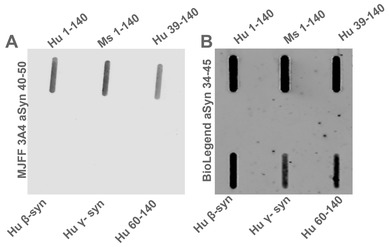

Figure 1. MJFF 3A4 is specific for aSyn. Slot blots using recombinant alpha/beta/gamma-synuclein protein or alpha-synuclein protein fragments. **A)** The 3A4 clone is specific for alpha-synuclein and binds within the expected AA40-50 range. **B)** The BioLegend aSyn 34-45 antibody (clone A15110D) used as a loading control and benchmark binds all synuclein forms.

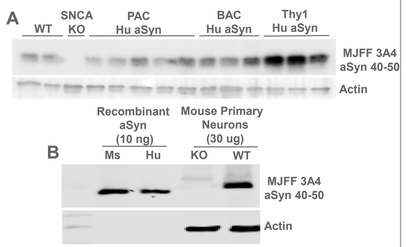

Figure 2. MJFF 3A4 detects aSyn in Western blot. A) Clone 3A4 detects aSyn in the cortex of WT mice and various models overexpressing human aSyn, with signal absent in SNCA KO mice. **B)** Clone 3A4 detects full length recombinant mouse and human aSyn as well as endogenous aSyn in mouse primary hippocampal neuron cultures.

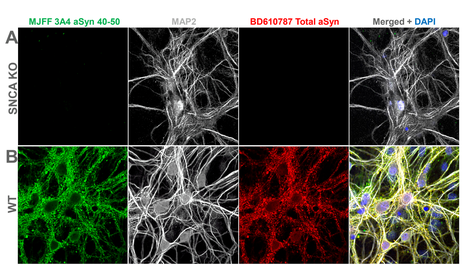

Figure 3. MJFF 3A4 is detects endogenous aSyn in ICC applications. Immunocytochemistry of mouse primary hippocampal neurons. **A)** The 3A4 clone does not cross react with other proteins in SNCA KO cells. **B)** The 3A4 clone detects endogenous levels of aSyn in mouse primary neuron cultures.

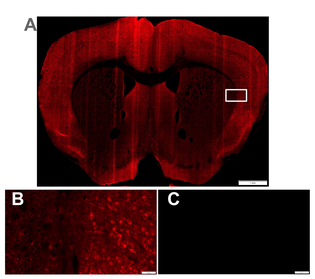

Figure 4. MJFF 3A4 detects aSyn in mouse brain sections. A-B) Clone 3A4 detects aSyn in sections from the Thy1 aSyn (Line 61) mouse with neuronal appearance. **C)** The staining is absent in SNCA KO mouse tissue. White box in A is field of view for images B and C.

Truncated aSyn

MJFF embarked on a program to develop antibodies to two pathogenic truncations of alpha-synuclein that have been previously identified but are not represented by open access antibodies. These truncations are 1-119 and 1-122.

Table 2. Summary of application testing and epitope mapping results.

Application	Samples	aSyn 1-119 Clone 24D2	aSyn 1-122 Clone 10B7
Slot Blot	Recombinant Protein	Pass (Figure 5)	Pass (Figure 5)
Western Blot	Mouse Primary Neurons (WT vs KO) Mouse Primary Neurons (WT +/- PFF) Mouse Brain (WT vs KO vs Transgenic OE)	Fail	Preliminary Pass (Figure 6)
Immunoprecipitation	Recombinant Protein	Fail	Pass (Hu only)
Immunocytochemistry	Mouse Primary Neurons (WT vs KO) Mouse Primary Neurons (WT +/- aSyn PFFs)	Pass (Figure 7)	Fail
Immunohistochemistry	Mouse Brain (KO vs Transgenic OE)	Fail	Fail
Immunoassay	Recombinant Protein	TBD	Pass (Figure 8)
Epitope Mapping	N/A	AA113-120* * = Suspected conformational	AA111-121

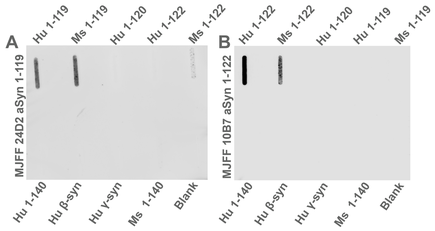

Figure 5. MJFF 24D2 is specific for aSyn 1-119 truncation and 10B7 is specific to aSyn 1-122 truncation. Slot blots using recombinant alpha/beta/gamma-synuclein protein or alpha-synuclein protein fragments. **A)** The 24D2 clone is specific for human and mouse aSyn truncation 1-119. **B)** The 10B7 clone is specific for human and mouse aSyn truncation 1-122. The BioLegend aSyn 34-45 antibody (clone A15110D) was used as a loading control (not shown).

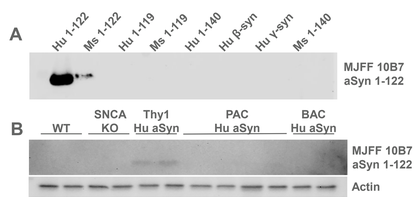

Figure 6. MJFF 10B7 detects truncated 1-122 aSyn in western blot. A) Truncation specificity and species preference for 10B7 indicates it specifically reacts with the 1-122 truncation, with preference to human aSyn vs mouse. **B)** 10B7 detects a faint band in Thy1 Hu aSyn mouse frontal brain around 13-14 kDa in TritonX-100 soluble fractions.

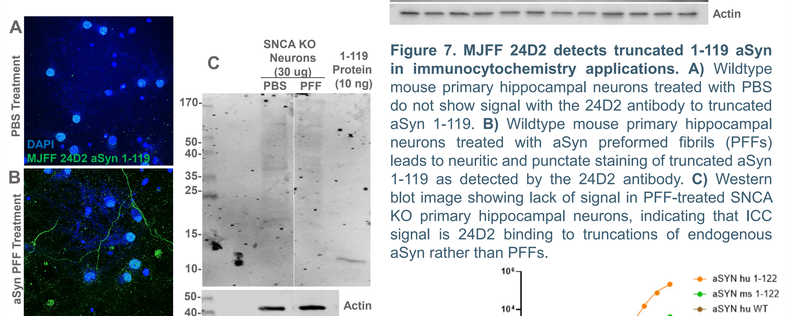

Figure 7. MJFF 24D2 detects truncated 1-119 aSyn in immunocytochemistry applications. A) Wildtype mouse primary hippocampal neurons treated with PBS do not show signal with the 24D2 antibody to truncated aSyn 1-119. **B)** Wildtype mouse primary hippocampal neurons treated with aSyn preformed fibrils (PFFs) leads to neuritic and punctate staining of truncated aSyn 1-119 as detected by the 24D2 antibody. **C)** Western blot image showing lack of signal in PFF-treated SNCA KO primary hippocampal neurons, indicating that ICC signal is 24D2 binding to truncations of endogenous aSyn rather than PFFs.

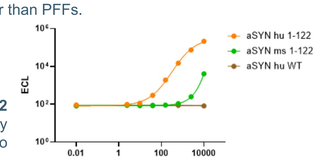

Figure 8. Preliminary results developing assays for the 1-122 truncation. MJFF 3A4 (aSyn 40-50) was used as capture antibody and MJFF 10B7 (aSyn 1-122) was used as detector antibody to measure mouse and human aSyn 1-122 recombinant protein.

Ubiquitylated aSyn

Following a publication from the Tofaris lab in 2023 using a polyclonal antibody that recognizes aSyn ubiquitylated at residues K45, K48, and K60, MJFF began a program to develop open access monoclonal antibodies with similar performance.

Table 3. Summary of application testing for Ubiquitylated aSyn K45/48/60

Application	Samples	Clone 55	Clone 116	Clone 131
Dot Blot	Ubiquitylated aSyn Peptide Ubiquitylated aSyn Protein	Pass (Figure 9, 10)	Pass (Figure 9, 10)	Pass (Figure 9)
Western Blot	Ubiquitylated aSyn Protein SH-SY5Y Cells (WT vs KO)	Pending	Pending	Pending
Immunoprecipitation	SH-SY5Y Cells Mouse Brain (WT vs KO)	Pending	Pending	Pending
Immunocytochemistry	SH-SY5Y Cells (WT vs KO)	Pending	Pending	Pending
Immunohistochemistry	FFPE HEK Cells (WT vs KO)	Fail	Fail	Fail
Immunoassay	Ubiquitylated aSyn Peptide	Pending	Pending	Pending

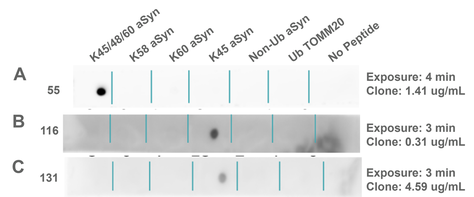

Figure 9. Clones 55, 116, and 131 detect distinct ubiquitylation events on aSyn. Three recombinant clones were tested for sensitivity and specificity to ubiquitylation events of interest on aSyn using recombinant peptides. **A)** Clone 55 detects the triple modified aSyn. **B)** Clone 116 detects ubiquitylation of aSyn at K45 only. **C)** Clone 131 detects ubiquitylation of aSyn at K45 only. No clones bind to non-ubiquitylated aSyn or a different ubiquitylated protein control. Exposure times and antibody concentration are reported.

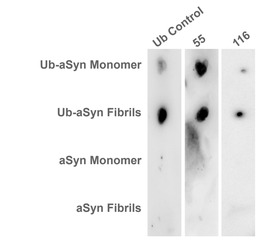

Figure 10. Clones 55 and 116 detect ubiquitylated full length aSyn monomer and fibrils. Full length aSyn monomers and fibrils were ubiquitylated using the in vitro ubiquitylation assay described in Zenko et al Sci Adv 2023. Control antibody is the anti-ubiquitin antibody clone P4D1.

More Information

More information on these and other research tools available and in development can be found at michaeljfox.org/tools-catalog. For questions or suggestions on new tools to develop, email tools@michaeljfox.org. Please visit the pages below to learn more about the various resources MJFF provides the research community:

Patient Biosamples	Patient Datasets	Preclinical Tools/Models	Clinical Trial Resources	Funding	Networking	PD Landscape Reports
17 collections 14 sample types	10 cohorts 8 data types	200 tools 9 tool types	Recruitment Resources Trial Pack	International Non-dilutive Multiple callouts/year	Research Exchange Calls (PDRx) Therapeutics Conference	Targets Biomarkers Therapeutics
michaeljfox.org/biospecimens	michaeljfox.org/data-resources	michaeljfox.org/research-tools	michaeljfox.org/study-recruitment	michaeljfox.org/funding	michaeljfox.org/working-us	zenodo.org/communities/mjff