Single molecule characterisation of salivary protein aggregates from Parkinson's disease patients – a pilot study

Martin Furlepa*,[1,2] Yu P. Zhang*,[1,3] Evgeniia Lobanova,[1,3] Lakmini Kahanawita,[2] Giorgio Vivacque[4], Caroline H. Williams-Gray,[2] and David Klenerman[1,3]

*MF and YPZ contributed equally to this work.

Abstract

Saliva is a convenient and accessible biofluid that has potential as a future diagnostic tool for Parkinson's disease (PD). Candidate diagnostic tests for PD to date have predominantly focused on measurements of alpha-synuclein in cerebrospinal fluid (CSF), but there is a need for accurate tests utilising more easily accessible sample types. Prior studies utilising saliva have used bulk measurements of salivary α-synuclein to provide diagnostic insight. Aggregate structure may influence the contribution of α-synuclein to disease pathology. Single molecule approaches can characterise the structure of individual aggregates present in the biofluid and may therefore provide greater insight than bulk measurements.

We have employed an antibody-based single-molecule pulldown (SiMPull) assay to quantify salivary α-synuclein and amyloid-β peptide (Aβ) aggregate number and subsequently super-resolved captured aggregates using direct Stochastic Optical Reconstruction Microscopy (dSTORM) to describe their morphological features.

We show that the salivary α-synuclein aggregate/Aβ aggregate ratio is increased almost two-fold in PD patients ($n = 20$) compared to controls ($n = 20$, $p < 0.05$). Morphological information also provides insight with PD saliva containing a greater proportion of larger and more fibrillar Aβ aggregates than control saliva ($p < 0.05$). Furthermore, the combination of count and morphology data provides greater diagnostic value than either measure alone distinguishing between the people with PD ($n = 17$) and controls ($n = 18$) with a high degree of accuracy ($AUC = 0.87$, $p < 0.001$) and a larger dynamic range.

We therefore demonstrate for the first time the application of highly sensitive single molecule imaging techniques to saliva. In addition, we show that aggregates present within saliva retain relevant structural information further expanding the potential utility of saliva-based diagnostic methods.

Author affiliations:

1 Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom.

2 Department of Clinical Neurosciences, University of Cambridge, Cambridge CB2 0PY, United Kingdom.

3 UK Dementia Research Institute at Cambridge, Cambridge CB2 0XY, United Kingdom.

4 Department of Neurology and Psychiatry, Sapienza University, Rome, Italy

Correspondence to: David Klenerman

University of Cambridge, Department of Chemistry, Lensfield Road, Cambridge, CB21EW, UK

E-mail dk10012@cam.ac.uk

Running title: Salivary aggregate structure in PD

Keywords: Parkinson's disease, Diagnosis, Saliva, Single-molecule, Aggregates, alpha-synuclein

Abbreviations: ACE-III = Addenbrooke's Cognitive Examination version 3; Aβ = amyloid-β peptide; αS = α-synuclein; AUC = Area Under the Curve; dSTORM = direct Stochastic Optical Reconstruction Microscopy; IQR = Interquartile Range; LEDD = Levodopa Equivalent Dose; MoCA = Montreal Cognitive Assessment; MDS-UPDRS = Movement Disorder Society's Unified Parkinson's Disease Rating Scale; PD = Parkinson's disease; SAA = Seed Amplification Assay; ROC = Receiver Operator Characteristics; SiMPull = single molecule pulldown; SD = Standard Deviation.

Introduction

Parkinson's disease is the second most common neurodegenerative disorder affecting 1% of those over 60 years of age.[1] Classically Parkinson's disease is characterised by its motor

features of bradykinesia, rigidity, and resting tremor, although non-motor manifestations are now more widely appreciated and incorporated as supportive features in diagnostic criteria.[2] Pathologically the disease is characterised by the accumulation of intracellular Lewy bodies within neurons. α-Synuclein is a key component of Lewy bodies[3,4] and a substantial body of evidence implicates abnormal aggregation of α-synuclein and toxic oligomeric species as a key driver of disease progression.[5] Further, mutations of the gene encoding α-synuclein leads to autosomal dominant forms of Parkinson's disease further implicating its abnormal aggregation in disease progression.[6,7]

Diagnosis of PD primarily relies on clinical assessment which has a diagnostic accuracy of approximately 80% in patients with symptoms onset >5-years,[8] with lower accuracy earlier in the disease course.[9] To date there is no single biomarker which can accurately diagnose PD in clinical practice. Current treatment options focus on relieving PD symptoms by stimulating the dopamine system but do not slow or prevent disease progression. Novel therapies which utilise targeted antibodies to remove α-synuclein aggregates from neuronal tissue have failed to alter the disease trajectory.[10,11] The reliance on clinical diagnosis which typically occurs after motor symptoms develop may impede the development of new therapies as neuronal tissue loss within the substantia nigra precedes motor manifestations.[12] An accurate, easily accessible biomarker which could facilitate early diagnosis and longitudinal sampling could aid the development and utilisation of new therapies.

Outside of the clinical setting, Seed Amplification Assays (SAA) have been shown to accurately diagnose patients based on α-synuclein seeding activity using cerebrospinal fluid (CSF) samples.[13] However, CSF is not easily obtainable and requires the patient to undergo a minor procedure for sample collection. A new method combining SAA and immunoprecipitation has recently reported the detection of α-synuclein seeds within serum and applied this to accurately identify patients with PD[14] raising the possibility that this highly accurate method could be applied to a more easily accessible biofluid. An alternative blood-based approach which isolated CNS α-synuclein from blood exosomes reported a high degree of accuracy when distinguishing between patients with PD, multiple systems atrophy, and healthy controls.[15,16]

Saliva represents a potential alternative biofluid to blood or CSF with several benefits. Sample collection is painless for patients, staff do not require specific training for collection, and there is potential for patients to collect their own samples. Previous studies evaluating saliva for the

diagnosis of PD have had mixed results (reviewed in reference [17]). Studies utilising western blot, ELISA, or Luminex assays to measure total α-synuclein have found reduced levels in PD saliva[18–20] or have been unable to detect any difference.[21–25] Other studies that used ELISA to measure oligomeric α-synuclein levels have shown a greater concentration in PD saliva[19,22,25,26] and that the oligomeric to total α-synuclein ratio is raised in PD providing greater discrimination between groups than either measure alone. Comparisons of salivary α-synuclein have largely been unable to distinguish between PD disease stages although one study has shown an increased concentration of total α-synuclein concentration in later stages of PD[19] and that that oligomeric α-synuclein is higher in de-novo[27] but not in heterogenous PD groups.[19] More recently SAAs have been applied to saliva showing greater seeding activity within the saliva of PD patients in comparison to controls.[28,29] Overall, these studies suggest that α-synuclein can be reliably detected in saliva and that the concentration of different species of α-synuclein aggregates may change between health and disease.

Previous work investigating saliva as a biofluid have used methods which provide bulk measurements of protein concentration but do not provide insight into the size or structure of different species of aggregates present within this heterogenous population. Across a range of neurodegenerative diseases it has been shown that aggregate structure is an key determinant of their toxicity[30] and that in PD, small soluble oligomeric forms of α-synuclein may contribute to disease progression.[5,31] Single molecule approaches can be used to provide a count of the number of individual protein aggregates providing insight into their structure. This can then be combined with super resolution microscopy to visualise individual aggregates to a 20nm spatial resolution, providing detailed morphological information.

It has recently shown that an aptamer based Single-Molecule Pull down assay (SiMPull) can accurately distinguish between PD and healthy control serum samples by measuring an increase in the ratio of α-synuclein/Aβ aggregates in PD patients.[32] This measure, when combined with super resolved morphology data provides superior group discrimination. This confirmed earlier work that utilised immunodepletion in combination with Aptamer DNA Points Accumulation for Imaging in Nanoscale Topography (AD-PAINT),[33] a related single molecule technique, which also showed an increased α-synuclein/Aβ ratio in PD patients. Previous work has identified Aβ co-pathology in patients with PD. Studies have shown that CSF $A\beta_{42}$ decreases with worsening cognition in PD[34] and that this change is detectable several years prior to onset of detectable cognitive decline[35]. In addition, Aβ may act synergistically to increase the aggregation of α-synuclein in PD.[36] Concurrent measurement of both α-synuclein

and Aβ provides a means of adjusting for variation in total aggregate count between participants whilst also capturing additional information related to Aβ co-pathology.

In this study we use SiMPull to capture soluble α-synuclein and Aβ aggregates in saliva of PD patients and controls.[37] We implement this single-molecule imaging technique using paired capture and detection antibodies targeting the same epitope to detect aggregates of interest whilst excluding monomeric forms. We subsequently apply direct Stochastic Optical Reconstruction Microscopy (dSTORM) to super resolve the captured aggregates[38] providing information regarding their size and morphology alongside their single molecule count to investigate if these methods in combination can provide greater diagnostic insight than more conventional methods.

Materials and methods

Participants

Ethical approval for the study was granted by the NRES Committee East of England – Central Cambridge (03/303). All participants provided written consent. The study was conducted in accordance with the Declaration of Helsinki. Patients diagnosed with idiopathic PD by a movement disorder specialist according to the UK Parkinson's Disease Brain Bank criteria[39] were recruited from the Parkinson's Disease Research Clinic at the John Van Geest Centre for Brain Repair, University of Cambridge, UK. Healthy aged-matched controls were recruited from the same centre and were often the partners or relatives of patients attending the clinic. Demographic data, medical history, and medication use was collected from all participants. In addition, PD patients completed standardised assessments of disease severity including the Movement Disorder Society's Unified Parkinson's Disease Rating Scale (MDS-UPDRS) and Hoehn and Yahr scale, and neuropsychological tests including the Montreal Cognitive Assessment (MoCA), Table 1. Levodopa equivalent daily dose (LEDD) was calculated using the conversion factors detailed in the systematic review by Tomlinson et al, 2010.[40]

Saliva collection and processing

Participants were assessed for eligibility to provide a saliva sample. All participants were required to fast for at least one hour prior to sample collection. Exclusion criteria included

patient reported ongoing dental or oral disease, smoking within four hours of sample collection, and consuming alcohol within 12hrs of sample collection. Samples were collected between 10am-12pm using the passive drool method. Participants allowed saliva to pool in their mouth and drooled into a sterile container for 5 minutes, typically 1-4ml of saliva was collected per participant. No stimulation was applied. All samples were collected and processed over ice. After collection, protease inhibitors (Sigma P2714, x10 concentration, 10μL per ml of saliva) and a phosphatase inhibitor (sodium orthovanadate 3μL per ml of saliva) were added to preserve the aggregates' structure. Samples were then centrifuged at 2600xg at 4˚C for 15 minutes. The supernatant was collected and underwent a further 15000xg centrifuge step at 4˚C for 15 minutes. The resulting supernatant was then removed and stored in 100μL aliquots in cryotubes at -80˚C. Received 100μL aliquots were then defrosted and divided into smaller 11μL aliquots before being stored at -80˚C for further analysis. There were no further freeze-thaw cycles.

Materials and antibody preparation

Phosphate buffered solution (PBS, Gibco PBS pH 7.4 1x, cat 1001023) was filtered before use (Whatman Anotop 25/0.02, Cytiva, 6809-2002). Biotinylated azide-free LB509 antibodies (Biolegend, cat 807710) were used to capture α-synuclein aggregates. Biotinylated azide-free 6E10 antibodies (Biolegend, cat 803007) were used as the capture antibody for Aβ containing aggregates. All capture antibodies were diluted to 10nM concentration in PBS.

α-Synuclein aggregates were detected using Alexa 647 labelled LB509 antibodies (Santa Cruz, sc-58480, 647). For the detection of Aβ aggregates, Alexa 647 labelled 6E10 antibodies (Biolegend, cat: 803021) were used. Detection antibodies were diluted to 0.5nM and 5nM concentrations respectively in PBS.

Prior to SiMPull experiments, saliva samples were defrosted and diluted 10-fold in PBS. All sample preparation was completed on ice.

Total aggregate concentrations were measured as duplicates using Invitrogen ELISA kits according to the manufacturer's instructions (Thermo Fisher, α-synuclein #KBH0061, $A\beta_{40}$ #KBH3481, and $A\beta_{42}$ #KBH3441).

Slide preparation

Slide preparation and passivation was completed as previously described using F-127 passivation [Zhang et al, 2023 - manuscript submitted for publication]. In brief, glass coverslips (26x76 mm, thickness #1.5, VWR, cat MENZBC026076AC40) were cleaned for 15 minutes in an argon plasma cleaner. After cleaning a 50 well PDMS chamber gasket (GRACE Bio-Labs CultureWell 50-3mm DIA x 1mm Depth, Cat 103350) was applied to the coverslip. Each well was then filled with 6µL of a 1:1 mixture of Rain-X (Rain-X Rain Repellent) and isopropanol which was allowed to dry with the residue coating the glass surface. The RainX-isopropanol mixture was filtered (Millex, SLGV004SL) prior to use. Each well was then washed twice with PBS, and 10µL of NeutrAvidin 0.1mg/ml (Thermo Scientific, 31000) diluted using PBS was applied and incubated for 10 minutes before removal and a further two washing steps with PBS. Passivation was then completed in each well with the addition of 10µL of F-127 (Invitrogen, P6866, 1% diluted in PBS, filtered 0.22µm) which was incubated for 45 minutes before removal and a further two washing steps with PBS-T (0.05% Tween 20, Fisher BioReagents™ in 1XPBS, Gibco™).

SiMPull assay and dSTORM protocol

10µL of capture antibody was added to each well and incubated for 5 minutes before removal (Fig. 1), each well was then washed twice with PBS-T. 10µL of saliva was added to each well and incubated for 90 minutes before removal and a further two PBS-T washing steps. Sample group was not blinded to the investigator. 10µL of detection antibody was added to each well and allowed to incubate for 30 minutes. Detection antibodies were then removed, and each well was washed twice with PBS-T before being filled with 10µL PBS to prevent the slide from drying out during image acquisition. The same antibody was used for detection and capture.

Diffraction-limited imaging was performed first and then STORM buffer was added for super-resolution imaging. dSTORM buffer was freshly prepared by combining glucose oxidase, catalyse, and MEA (50 mM PBS-Tris, 0.5mM glucose, 1.3µM glucose oxidase, 2.2µM catalase, and 50mM mercaptoethylamine (MEA)). After diffraction-limited imaging, the slide was removed from the microscope and two additional gaskets (GRACE Bio-Labs CultureWell 50-3mm DIA x 1mm Depth, Cat 103350) were stacked on top of the slide to increase the well capacity before STORM buffer was added to each well. The slide was then sealed by applying

a smaller coverslip over the top of the gaskets and applying nail varnish to the sides to produce an airtight seal. The slide was then returned to the microscope for STORM imaging.

Microscope set up

Image acquisition was completed using an in-house TIRF microscope. A 100x 1.49 NA oil-immersion objective (UPLSAPO, 100x, TIRF, Olympus) is mounted on a Nikon Ti2 Eclipse inverted microscope body with a perfect focus system. An excitation laser beam (Oxxius, 638 nm) was circularly polarised by a quarter-wave plate (WPQ05M-405, Thorlabs) and focused onto the back focal plane of the objective. The fluorescent signal from samples was collected via the same objective separated by a dichroic beam splitter (Di01-R405/488/561/635, Semrock). The emission was filtered by a long-pass emitter (BLP01-635R-25, Laser 2000). An air-cooled EMCCD camera (Photometrics Evolve, EVO-512-M-FW-16-AC-110) with frame transfer mode (electron-multiplying Gain of 11.5 e-1/ADU and 250 ADU/photon) was used for image recording. The open-source software Micro-Manager 1.4 was used to operate the imaging system and automate data acquisition. For diffraction-limited imaging a 1.5mW laser was used with a 50ms exposure time to capture 50 frames per field of view (FoV). For dSTORM imaging, a 150mW laser was applied and images were acquired using an exposure time of 15ms to capture 8000 frames. The camera was operated in pre-exposure non-overlapping mode. Continuous illumination with a 405nm laser (LBX-405-50-CIR-PP, Oxxius) was applied at 10mW power. The pixel size of the image was 103.5nm^2. Each FoV contains an area of approximately 2500μm^2. For each patient 16 diffraction-limited and 4 dSTORM images were acquired.

Image processing and statistical analysis

Diffraction limited single-molecule counting was performed using a single-molecule localisation engine in ThunderSTORM.[41] The images were processed using a wavelet filter and the localisations were identified using a hybrid threshold of 0.5*std(Wave.F1)+0.1*mean(Med. F). This arrangement gives a higher resistance to noise by combing the adaptive threshold and the mean-value threshold.

dSTORM data was analysed using published ImageJ[42] plug-ins, with an automation code integrating these independent libraries. The drift correction, image reconstruction, and

morphology analysis were performed by a mean shift algorithm,[43] ThunderSTORM,[41] and morphology library[44] respectively. To avoid blurred images, which may be collected during the microscope focusing stabilisation, the first 200 frames were removed before further analysis. The localisations were identified using ThunderSTORM and drift correction was performed by the mean shift algorithm. The drift corrected localisations were then filtered using localisation merge and a density filter is applied to remove false positive signals, these processed localisations were then used to reconstruct the super-resolved image. Morphology analysis was then performed providing area and circularity measurements.

All field of views were analysed for each participant. Prior to comparisons data was checked for normality using Shapiro-Wilks test and normally distributed data was tested for homogeneity of variance using Levene's test. All data was compared using two tailed tests. Where the variance between the two groups was significant Welch's t-test was applied, otherwise Student's t-test was used. For significant results that were parametric, effect size was measured using Cohen's d, non-parametric effect size (r) was calculated. Correlations were tested using Pearson rank test. Non-normally distributed data was compared using Wilcoxon rank sum test, or for pairwise comparisons Dunn's test was applied. Correction for multiple analysis was made using Bonferroni's method. Non-normal correlations were tested using Kendall Tau test. Categorical variables were compared using Pearson's Chi-Squared test. Optimum cut off values were determined using the value which provided the maximum sum of sensitivity and specificity. For logistic regression p values were calculated using Weld's method, the model's explanatory power was measured using McFadden's pseudo R^2 squared with significance assessed using Chi-Squared test.

Data availability

Code used for image analysis are available at https://github.com/YPZ858/DF-single-molecule-couting (single-molecule counting) and https://github.com/YPZ858/Super-res-code/issues (super-resolution imaging).

Data obtained from image analysis and participant data is available from https://gin.g-node.org/MFurlepa/SalivaAggregates.git

Results

Participants

We recruited two subgroups, each including 10 PD and 10 control participants. The sample size per subgroup was limited by the available space on the imaging slide (40 well coverslip). Subgroups were grouped by time of sample collection. Each subgroup was recruited over a 3-month period and was analysed soon after the last sample was collected. Due to concerns regarding the effect of the duration of sample storage on aggregate number and structure, and due to variation in absolute number of detected aggregates between experimental runs, we initially analysed each subgroup independently before later combining the subgroups and comparing ratio results. Demographic data for each subgroup is shown in Table 1. In subgroup 1, PD participants were younger than controls (PD = 67.04 years, control = 74.15 years), but otherwise the PD cases and controls were well matched across subgroups. Both subgroups included participants with early-stage PD as indicated by disease duration and Hoehn and Yahr score (</-3), but cases in subgroup one had a slightly shorter disease duration than subgroup two (*1.09 vs 2.75 years*). In keeping with previously reported longitudinal data[35] 20% and 40% of PD patients had MoCA scores suggestive of mild cognitive impairment (MCI, score <26) in subgroups one and two respectively,[45] no patients scored in the dementia ranges (score <21).

Subgroup one

Subgroup one - number of aggregates

SIMPull was used to quantify the number of α-synuclein and Aβ aggregates present in saliva. First, we evaluated the sensitivity and specificity of the SIMPull assay for each target (Supplementary Fig. 1-2). Three control conditions were used to ensure that capture was specific to the target protein aggregate, accounting for non-specific protein binding to the coverslip surface (capture control), non-specific detection antibody binding to the coverslip (blank control), and detection/capture antibody interactions (detection control). There was clear signal distinction between the full antibody condition and the control conditions indicating that the assay was specific for the detection of α-synuclein and Aβ containing aggregates. We

propose that by using the same epitope specific antibody for detection and capture, all localisation points are dimers or larger given each monomer only contains a single antibody binding epitope.[46]

The antibody SIMPull assay was then used to compare the total aggregate count (Fig 2, Supplementary Table 3) in PD (*n = 10)* and control groups (*n = 10*). There was a non-significant trend for increased α-synuclein containing aggregates in the saliva of PD patients when compared to controls (median PD = 164.03, control = 62.09, *W = 75, p = 0.063*) whilst there was no clear difference in the number of Aβ containing aggregates (median PD =202.25, control = 214.14, *W = 45, p = 0.74*). Taking the ratio of α-synuclein to Aβ for each participant helps to reduce the effect of differences in total aggregate number in saliva between participants. The α-synuclein/Aβ ratio provides better discrimination between PD and control groups with approximately a 2.2-fold increase in the saliva of participants with PD (median PD = 1.07, control = 0.48, *W = 79, p = 0.029, 95% CI [0.002, 0.890], r = 0.49*; Fig. 2C). Receiver operator characteristics (ROC) was used to evaluate the sensitivity and specificity of the α-synuclein/Aβ aggregate ratio for discriminating between the groups and yielded an *AUC of 0.79*.

Subgroup one - aggregate size and morphology

dSTORM was used to obtain super resolved images of individual protein aggregates with a spatial resolution of approximately 20nm. Using this method, we can provide quantitative measurements of aggregate area and circularity (*PD n = 9, control n = 8*). We have previously shown that our sample preparation allows the dSTORM assay and aggregates to remain stable over 12 hours providing time for image acquisition overnight [Zhang et al, 2023 - manuscript submitted for publication]. Participants were excluded where the number of localisations were insufficient for drift correction. To analyse the morphology of salivary aggregates, we visualised the area and circularity distributions for each aggregate type according to disease status using their cumulative distributions. Across both groups, aggregate area measurements had a large positive skew whilst circularity measurements were normally distributed (Supplementary Table 5). The median area for α-synuclein containing aggregates from PD and control saliva was *0.005*μm^2 with an *interquartile range (IQR) of 0.011*μm^2 and *0.010*μm^2 respectively; the *mean circularity was 0.572* (*standard deviation (SD) = 0.216*) and *0.0578 (SD = 0.217*) respectively. Aβ containing aggregates from PD and control saliva had a *median area of 0.006*μm^2 *with an IQR of 0.013*μm^2 *and 0.011*μm^2 respectively; the *mean circularity was*

0.576 (SD = 0.211) and 0.614 (SD = 0.192). Comparison of mean values gives an indication of the impact of the positive skew on the overall distribution. There was no difference in the mean α-synuclein containing aggregate area between groups (*$0.012\mu m^2$*). In contrast, the mean area of Aβ containing aggregates in PD saliva (*$0.013\mu m^2$*) was higher than in controls (*$0.011\mu m^2$*). This suggests that the overall distribution of aggregate area may differ between the groups with a more significant subpopulation of larger Aβ aggregates in the PD group resulting in a more positively skewed mean area.

To determine if morphology information could distinguish between patients with PD and controls, we utilised a similar workflow to that described by Zhang et al, 2023[32] to isolate subpopulations of aggregates that differed between groups based on area and circularity (Fig. 3). We subtracted the PD cumulative frequency distribution from the control groups distribution to identify the point at which the morphological feature was maximally different. Once identified we then used this value as threshold from which we identified the proportion of aggregates that were larger, or less circular than the identified cut off for each participant. The proportion values were then compared to see if there was a significant difference between the two groups (Supplementary Table 3).

When comparing the area for the α-synuclein containing aggregates we found a small divergence between the two distributions at $0.02\mu m^2$. However, when using this value as a threshold we found no difference in the proportion of α-synuclein containing aggregates larger than $0.02\mu m^2$ between the two groups ($t(16) = -0.709, p = 0.489$). Similarly, when comparing circularity a small difference between distributions was found for aggregates less circular than 0.4 with no significant difference between the two groups in the proportion of aggregates satisfying this threshold ($t(16) = -0.329, p = 0.747$).

For Aβ containing aggregates we found a maximal difference between the two area distributions at $0.03\mu m^2$. Using this as a threshold, we showed a significantly greater proportion of Aβ containing aggregates larger than $0.03\mu m^2$ in the PD group ($t(9.75) = 2.43, p = 0.036, d = 1.15$), applying this area threshold can be used to distinguish between the two groups with an *AUC of 0.76*. Similarly for circularity, the point of maximum difference was *0.4.* There was significantly greater proportion of Aβ aggregates less circular than *0.4* in the PD group ($t(15) = 2.48, p = 0.025, d = 1.23$) and this threshold distinguishes between the two groups with an *AUC of 0.76*. This therefore suggests that Aβ containing aggregates in the PD group are larger

and more fibrillar than those observed in the control group and that morphology data can be used to distinguish between the two groups with a reasonable degree of accuracy.

Subgroup one - combining aggregate count and size data

To determine whether combining diffraction-limited count with super-resolution morphology data might provide a greater discrimination between the two groups, we developed a combined discriminator. First for each participant we found the proportion of aggregates which satisfied both morphology thresholds previously identified (area >0.03μm^2 and circularity <0.4) and then multiplied this proportion to the α-synuclein/Aβ ratio found using diffraction-limited imaging. Using this combined discriminator (Fig. 4), we show a significant difference between the two groups (*t(8.83) = 2.88, p = 0.018, 95% CI [0.013, 0.095], d = 1.36)* with a 4.3-fold increase in the PD saliva. The combined discriminator also provides a greater ability to distinguish between PD and control groups than either count or morphology data alone providing an *AUC of 0.89*. The optimum cut off value identified as *0.039* which provides a sensitivity of 66.6% and a specificity of 100%.

Results - subgroup two

Subgroup two - number of aggregates

Similarly to subgroup one, we found a non-significant trend for increased number of α-synuclein containing aggregates within the saliva from patients with PD (mean PD = 166.25, control = 124.23, *t(18) = 1.44, p = 0.167;* Supplementary Fig. 3A) and no significant difference in the number of Aβ containing aggregates (median PD = 84.50, control = 111.22, *W = 39, p = 0.436,* Supplementary Fig. 3B). Taking the α-synuclein/Aβ aggregate count ratio showed a 1.9-fold increase in the relative number of α-synuclein containing aggregates in the PD group (mean PD = 1.18, control = 1.17, *t(12.68) = 2.42, p = 0.031, 95% CI [0.107, 1.902], d = 1.08,* Fig, 5A), although this difference was less marked than that seen in subgroup 1. Applying ROC gave an *AUC of 0.77* (Fig. 5B).

Subgroup two - aggregate size and morphology

We applied dSTORM imaging to the captured aggregates from the second subgroup. Aggregate morphology data is summarised in Supplementary Table 5 (PD *n = 9*, control *n = 9*).

Participants were excluded where the number of localisations were insufficient for drift correction. The pooled data across PD and controls for each aggregate were marginally larger and rounder than those observed in subgroup 1. Again, a positive skew was seen in the distributions of area values for both aggregate types whilst circularity data was normally distributed. The mean area of Aβ containing aggregates was higher in the PD group.

To determine if the threshold values identified in the first subgroup generalised across participants, we applied the same threshold values for area (*α-synuclein* $>0.02\mu m^2$, *Aβ* $>0.03\mu m^2$) and circularity (<0.4) to the second subgroup (Fig. 5C-F, Supplementary Fig. 4). Using these thresholds, we found no significant difference in the proportion of α-synuclein containing aggregates larger than 0.02μm² ($t(15) = 0.901, p = 0.382$) and no difference in the proportion less circular than 0.4 ($t(15) = 1.157, p = 0.265$). In contrast, for Aβ containing aggregates we again showed a significantly greater proportion of aggregates larger than 0.03μm² ($t(8.365) = 2.852, p = 0.02, d = 1.345$) and when used to separate the two groups this gave an *AUC of 0.77*. Additionally, there was a significantly greater proportion of Aβ containing aggregates with a circularity < 0.4 in the PD group ($t(10.43) = 2.901, p = 0.0152, d = 1.367$) providing an *AUC of 0.81*.

Subgroup two - combining aggregate count and size data

Finally, we combined the combined count and morphology data to produce a combined discriminator. As in subgroup one using this value gave a clear difference between the two groups (4-fold increase in PD, $t(8.669) = 2.713, p = 0.0247$, *95% CI [0.012, 0.133]*, $d = 1.279$; Fig. 5G-H) and allowed them to be accurately distinguished from each other, $AUC = 0.86$. The optimum cut off value was identified as 0.0517 which produced a sensitivity of 66.6% and a specificity of 100%.

Combined subgroups

Discrimination between PD and controls

Currently the sample size of each subgroup is limited by the number of wells available on the imaging slide. We have found that total aggregate counts vary between individual experimental runs, whereas ratio values are more comparable. To maximise our sample size, we combined the two subgroups (Supplementary Table 7). Diffraction limited α-synuclein/Aβ aggregate

ratio (*PD n = 20, control n = 20*) was significantly higher in the PD group (*W = 118, p = 0.026, 95% CI [0.094,1.492], r = 0.351*). There was a 5-fold increase in the combined discriminator for PD saliva compared to controls, the combined discriminator had a high degree of accuracy in distinguishing between people with PD (*n = 17*) and controls (*n = 18*, *W = 41, p < 0.001*, 95% CI [0.0244, 0.0727], r = 0.63, Fig. 6), *AUC = 0.87*. Using a 0.039 combined discriminator cut off gave a sensitivity of 72.2% and a specificity of 94.1%.

In both subgroup one and the combined data, the control group was significantly older than the PD group (Supplementary Table 8). To assess the impact of age on the predictive value of the combined discriminator we constructed two models using logistic regression and analysed the combined subgroup data (Supplementary Table 9). The first used only the combined discriminator to predict disease state, the second combined age and the combined discriminator. We found that age had a non-significant effect on the model's explanatory power. This was also reflected in the measures of model fit; the combined discriminator alone performed well (*pseudo* R^2 *0.376, p=0.2x10*$^{-4}$) and including age provided minimal improvement (*pseudo* R^2 *= 0.396, p=0.1 x10*$^{-4}$) suggesting that the combined discriminator remains predictive of disease state when age is considered.

Correlation between aggregate measures and clinical features

Using the combined data, we investigated whether there was any correlation between the α-synuclein/Aβ ratio (n = 40), Aβ area and circularity threshold proportions (n = 35), and the combined discriminator with demographic data and clinical scores (Table 2).

We found a statistically significant but weak negative correlation between age and the α-synuclein/Aβ ratio (r_τ*=-0.250, p =0.021*) and the combined discriminator (r_τ*=-0.320, p=0.006*). Within the PD group there was no correlation between any of our measures and MDS-UPDRS-III, MDS-UDPDRS-Total, or MoCA scores, which is unsurprising given the small sample size.

Protein concentrations

To determine if our measurements relate to more established neurodegeneration-related proteins, we used commercial ELISA kits to measure the total α-synuclein, $A\beta_{40}$, and $A\beta_{42}$ protein concentration for participants where there was sufficient sample (Supplementary Table

6). As shown in Supplementary Fig 5, there was no difference in the concentration of α-synuclein (PD n = 15, HC n = 16, t(24.174) = 0.446, p = 0.660), $A\beta_{40}$ (PD n = 16, HC n = 17, W = 103, p = 0.242), $A\beta_{42}$ (HC n = 19, PD n = 19, W = 201.5, p = 0.549), or $A\beta_{42}/A\beta_{40}$ ratio (PD n = 16, HC n = 17, W = 171, p = 0.217) between PD and control groups. We also determined if aggregate count taken as a proportion of total protein concentration varied between PD and controls. We showed no difference in α-synuclein count per ng/ml total α-synuclein (W= 78, p = 0.101), $A\beta_{40}$ count per pg/ml (W = 151, p = 0.606), or $A\beta_{42}$ count per pg/ml (W = 190, p = 0.795).

Discussion

Our results show that we can sensitively and specifically detect disease relevant protein aggregates in saliva at a single molecule level. Using an aggregate ratio value allows us to distinguish patients with PD from controls with a high degree of accuracy. By using the same antibody for detection and capture we capture aggregated protein forms; this is also reflected in the size data from our super resolution experiments. As there was no evidence of a difference in the total number of Aβ containing aggregates between the groups, this suggests that there is a greater relative abundance of α-synuclein containing aggregates in saliva from PD patients compared to controls. Using ELISA, we show that there is no difference in the total concentration of α-synuclein, this suggests that the increased aggregate count is due to a shift from monomeric to aggregated forms. Our finding of increased relative levels of α-synuclein aggregates is therefore in agreement with previous studies finding elevated levels of oligomeric α-synuclein in the saliva of patients with PD.[19,20,25] In the pooled data, age is significantly lower in the PD group, and age also has a weak negative correlation with the combined discriminator raising the possibility that the observed difference in the combined discriminator may be confounded by age. Using a regression model, we have however shown that age does not have statistically significant effect on its predictive performance, thus that the combined discriminator is the key variable that predicts group and hence PD disease status.

The majority of previous studies that utilised ELISA based methods to measure salivary alpha-synuclein do not report ROC analysis making comparison difficult.[18,19,21,25] One study that used ELISA to measure total and oligomeric α-synuclein reported that the ratio value provided a sensitivity of 69.77% and specificity of 95.16% but does not report the method used to determine the optimum cut off threshold.[20] Our SIMPull method provides comparable results with both subgroup one and subgroup two reporting a sensitivity 66.6% and a specificity of 100% when using a sum of sensitivity and specificity to identify the optimum cut off value. The sensitivity and specificity from the pooled data across both subgroups are 72.2% and 94.1% respectively. In our study, we do not find any difference in total α-synuclein using ELISA, this suggests that single molecule characteristics, detected using SiMPull, provide greater sensitivity than total protein concentration measures. More recently SAA has been used to compare the seeding activity of α-synuclein present in saliva, and these studies have reported an AUC of between 0.84-0.9.[28,29] For both subgroups, our SIMPull method performs towards the upper end of these values (subgroup one AUC= 0.89, subgroup two AUC= 0.86) whilst having the advantage of allowing easy target customisation as illustrated by quantifying both α-synuclein and Aβ within the same imaging slide.

Our imaging approach has the advantage of allowing the same aggregates captured and counted during diffraction limited SIMPull experiments to be subsequently super resolved with minimal additional processing. This means that we can obtain both single molecule abundance data and morphology data for the same captured aggregates within a single experimental condition. Even though saliva is a protease rich environment, we were able to find measurable differences in aggregate morphology between groups, but unexpectedly this difference was observable in Aβ containing aggregates as opposed to α-synuclein. We propose this may be due to detection of Aβ co-pathology or due to differences in protease resistance between α-synuclein and Aβ.

Changes in Aβ aggregation have been shown to be relevant to PD, particularly in relation to cognition. Longitudinal studies show MCI to be present at diagnosis in approximately 20-30% of PD patients and progression to dementia in 50% after 10-years of follow up.[35,47–51] In keeping with previous studies approximately 30% of PD patients in our subgroups showed evidence of PD-MCI on MoCA testing. In PD, CSF Aβ42 declines with worsening cognition[22,34,35,52], and these changes are predictive of future decline in cognitively normal individuals.[53] No previous studies have described salivary Aβ within a PD population but studies examining the relationship between serum Aβ and cognition have shown variable

results.[54–58] Outside of cognition it has also been suggested that Aβ may correlate with gait disturbance in PD[36] and that Aβ and α-synuclein act synergistically to enhance oligomer formation[59] providing further evidence for the important role of Aβ co-pathology in PD. In our study we used 6E10 to measure Aβ, this antibody is relatively non-specific binding to a range of Aβ isoforms including Aβ40, Aβ42 and APP fragments.[60] This allows us to detect a broad range of aggregates facilitating their separation based on morphology, but limits our ability to relate detected differences to specifically $Aβ_{42}$ which has been implicated in disease. In addition, our ELISA studies are unable to detect differences in the concentration of Aβ42 limiting our ability to comment on the potential composition of the morphologically distinct Aβ subpopulation that we detect.

An alternative explanation for the lack of difference in α-synuclein morphology between groups could be provided by the biofluid studied and the antibodies used. Saliva is a complex biofluid that contains a range of different types of proteases including serine proteases[61,62] which have been shown to have proteolytic effect on Aβ and α-synuclein. We used LB509 to detect α-synuclein which binds to residues 115-122 of the C-terminus, this region is more vulnerable to protease cleavage[63] and hence may reduce antibody binding liming our α-synuclein morphological analysis. This is in contrast to 6E10 which is relatively non-specific and therefore captures a greater variety of aggregates.[60] Aβ aggregates faster than α-synuclein,[64] however the total Aβ concentrations in our samples is low meaning that it is unlikely that aggregation occurring during sample preparation[65,66] explains why we observe a difference in Aβ but not α-synuclein morphology.

The presented work has several limitations. Our low sample size is in part due to the throughput of our methodology which is a limitation of the current study and the future use of our method more generally. To overcome this, we are developing a robotic system using a 96-well microplate which would allow for larger studies. In addition, detection intensities can be used in lieu of dSTORM imaging to indirectly provide a measure of aggregate size but with a far shorter imaging time.[32] Our current super resolution method is restricted to quantifying area and circularity and does not fully describe aggregate structure which will limit our ability to detect differences between groups. Importantly, we detect differences in Aβ using the 6E10 antibody which limits our ability to relate detected differences to disease relevant Aβ isoforms.

Although saliva offers several potential benefits including ease of collection which could allow for self-sampling, the current sample processing protocol is labour intensive. Saliva collection

can also be difficult to collect in the PD patient population, in particular for those with axial or jaw tremors, although this could feasibly be overcome with better design of collection equipment. Future work should aim to assess which factors in collection (e.g. time of day) and processing substantially influence results.

In conclusion, this study demonstrates that disease relevant proteins present in saliva can be detected and characterised using single molecule and super resolution imaging techniques. Our results suggest that structural changes detected may be relevant to disease and may represent a potential new means for PD diagnosis with performance that is superior to ELISA based methods and comparable to SAA methods. Although our results are limited by our small sample size, future work utilising larger automated microplates facilitating greater sample sizes could be used to test this hypothesis.

Acknowledgements

We would like to thank the patients and their families for participating in this research study.

Funding

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain Communications* online.

For the purpose of open access, the authors have applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

References

1. Tysnes OB, Storstein A. Epidemiology of Parkinson's disease. *J Neural Transm*. 2017;124(8):901-905. doi:10.1007/s00702-017-1686-y

2. Postuma RB, Berg D, Adler CH, et al. The new definition and diagnostic criteria of Parkinson's disease. *Lancet Neurol*. 2016;15(6):546-548. doi:10.1016/S1474-4422(16)00116-2

3. Spillantini MG, Schmidt ML, Lee VMY, Trojanowski JQ, Jakes R, Goedert M. α-Synuclein in Lewy bodies. *Nature*. 1997;388(6645):839-840. doi:10.1038/42166

4. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci U S A*. 1998;95(11):6469-6473. doi:10.1073/pnas.95.11.6469

5. Wong YC, Krainc D. α-synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. *Nat Med*. 2017;23(2):1-13. doi:10.1038/nm.4269

6. Singleton AB, Farrer M, Johnson J, et al. α-Synuclein Locus Triplication Causes Parkinson's Disease. *Science (1979)*. 2003;302(5646):841. doi:10.1126/science.1090278

7. Chartier-Harlin MC, Kachergus, Jennifer Roumier C, Mouroux V, et al. α-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet*. 2004;364(9440):1167-1169.

8. Rizzo G, Copetti M, Arcuti S, Martino D, Fontana A, Logroscino G. Accuracy of clinical diagnosis of Parkinson disease. *Neurology*. 2016;86(6):566 LP - 576. doi:10.1212/WNL.0000000000002350

9. Adler CH, Beach TG, Hentz JG, et al. Low clinical diagnostic accuracy of early vs advanced Parkinson disease: clinicopathologic study. *Neurology*. 2014;83(5):406-412. doi:10.1212/WNL.0000000000000641

10. Lang AE, Siderowf AD, Macklin EA, et al. Trial of Cinpanemab in Early Parkinson's Disease. *New England Journal of Medicine*. 2022;387(5):408-420. doi:10.1056/nejmoa2203395

11. Pagano G, Taylor KI, Anzures-Cabrera J, et al. Trial of Prasinezumab in Early-Stage Parkinson's Disease. *New England Journal of Medicine*. 2022;387(5):421-432. doi:10.1056/nejmoa2202867

12. Noyce AJ, Lees AJ, Schrag AE. The prediagnostic phase of Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*. 2016;87(8):871 LP - 878. doi:10.1136/jnnp-2015-311890

13. Siderowf A, Concha-Marambio L, Lafontant DE, et al. Assessment of heterogeneity among participants in the Parkinson's Progression Markers Initiative cohort using α-synuclein seed amplification: a cross-sectional study. *Lancet Neurol*. 2023;22(5):407-417. doi:10.1016/S1474-4422(23)00109-6

14. Okuzumi A, Hatano T, Matsumoto G, et al. Propagative α-synuclein seeds as serum biomarkers for synucleinopathies. *Nat Med*. Published online 2023. doi:10.1038/s41591-023-02358-9

15. Dutta S, Hornung S, Kruayatidee A, et al. α-Synuclein in blood exosomes immunoprecipitated using neuronal and oligodendroglial markers distinguishes Parkinson's disease from multiple system atrophy. *Acta Neuropathol*. 2021;142(3):495-511. doi:10.1007/s00401-021-02324-0

16. Taha HB, Hornung S, Dutta S, et al. Toward a biomarker panel measured in CNS-originating extracellular vesicles for improved differential diagnosis of Parkinson's disease and multiple system atrophy. *Transl Neurodegener*. 2023;12(1):14. doi:10.1186/s40035-023-00346-0

17. Goldoni R, Dolci C, Boccalari E, et al. Salivary biomarkers of neurodegenerative and demyelinating diseases and biosensors for their detection. *Ageing Res Rev*. 2022;76:101587. doi:https://doi.org/10.1016/j.arr.2022.101587

18. Al-Nimer MSM, Mshatat SF, Abdulla HI. Saliva α-synuclein and a high extinction coeffi cient protein: A novel approach in assessment biomarkers of parkinson's disease. *N Am J Med Sci*. 2014;6(12):633-637. doi:10.4103/1947-2714.147980

19. Vivacqua G, Latorre A, Suppa A, et al. Abnormal salivary total and oligomeric alpha-synuclein in Parkinson's disease. *PLoS One*. 2016;11(3):1-12. doi:10.1371/journal.pone.0151156

20. Vivacqua G, Suppa A, Mancinelli R, et al. Salivary alpha-synuclein in the diagnosis of Parkinson's disease and Progressive Supranuclear Palsy. *Parkinsonism Relat Disord*. 2019;63(September 2018):143-148. doi:10.1016/j.parkreldis.2019.02.014

21. Devic I, Hwang H, Edgar JS, et al. Salivary α-synuclein and DJ-1: Potential biomarkers for Parkinson's disease. *Brain*. 2011;134(7). doi:10.1093/brain/awr015

22. Kang JH, Mollenhauer B, Coffey CS, et al. CSF biomarkers associated with disease heterogeneity in early Parkinson's disease: the Parkinson's Progression Markers Initiative study. *Acta Neuropathol*. 2016;131(6):935-949. doi:10.1007/s00401-016-1552-2

23. Goldman JG, Andrews H, Amara A, et al. Cerebrospinal fluid, plasma, and saliva in the BioFIND study: Relationships among biomarkers and Parkinson's disease Features. *Mov Disord*. 2018;33(2):282-288. doi:10.1002/mds.27232

24. Fernández-espejo E, de Fonseca FR, Suárez J, et al. Native α-synuclein, 3-nitrotyrosine proteins, and patterns of nitro-α-synuclein-immunoreactive inclusions in saliva and submandibulary gland in parkinson's disease. *Antioxidants*. 2021;10(5). doi:10.3390/antiox10050715

25. Kang W, Chen W, Yang Q, et al. Salivary total α-synuclein, oligomeric α-synuclein and SNCA variants in Parkinson's disease patients. *Sci Rep*. 2016;6(May):1-8. doi:10.1038/srep28143

26. Cao Z, Wu Y, Liu G, et al. α-Synuclein in salivary extracellular vesicles as a potential biomarker of Parkinson's disease. *Neurosci Lett*. 2019;696(6):114-120. doi:10.1016/j.neulet.2018.12.030

27. De Bartolo MI, Vivacqua G, Belvisi D, et al. A Combined Panel of Salivary Biomarkers in de novo Parkinson's Disease. *Ann Neurol*. 2023;93(3):446-459. doi:https://doi.org/10.1002/ana.26550

28. Luan M, Sun Y, Chen J, et al. Diagnostic Value of Salivary Real-Time Quaking-Induced Conversion in Parkinson's Disease and Multiple System Atrophy. *Movement Disorders*. 2022;37(5):1059-1063. doi:10.1002/mds.28976

29. Vivacqua G, Mason M, De Bartolo MI, et al. Salivary α-Synuclein RT-QuIC Correlates with Disease Severity in de novo Parkinson's Disease. *Movement Disorders*. 2022;(September):1-4. doi:10.1002/mds.29246

30. Bucciantini M, Giannoni E, Chiti F, et al. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*. 2002;416(6880):507-511. doi:10.1038/416507a

31. Winner B, Jappelli R, Maji SK, et al. In vivo demonstration that α-synuclein oligomers are toxic. *Proceedings of the National Academy of Sciences*. 2011;108(10):4194-4199. doi:10.1073/pnas.1100976108

32. Zhang YP, Lobanova E, Emin D, et al. Imaging protein aggregates in Parkinson's disease serum using aptamer-assisted single-molecule pull-down. *Anal Chem*. Published online 2023. doi:10.1021/acs.analchem.3c02515

33. Lobanova E, Whiten D, Ruggeri FS, et al. Imaging protein aggregates in the serum and cerebrospinal fluid in Parkinson's disease. *Brain*. 2022;145(2):632-643. doi:10.1093/brain/awab306

34. Lin CH, Wu RM. Biomarkers of cognitive decline in Parkinson's disease. *Parkinsonism Relat Disord*. 2015;21(5):431-443. doi:10.1016/j.parkreldis.2015.02.010

35. Aarsland D, Creese B, Politis M, et al. Cognitive decline in Parkinson disease. *Nat Rev Neurol*. 2017;13(4):217-231. doi:10.1038/nrneurol.2017.27

36. Lim EW, Aarsland D, Ffytche D, et al. Amyloid-β and Parkinson's disease. *J Neurol*. 2019;266(11):2605-2619. doi:10.1007/s00415-018-9100-8

37. Jain A, Liu R, Ramani B, et al. Probing cellular protein complexes using single-molecule pull-down. *Nature*. 2011;473(7348):484-488. doi:10.1038/nature10016

38. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods*. 2006;3(10):793-796. doi:10.1038/nmeth929

39. Gibb WR, Lees AJ. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry*. 1988;51(6):745-752. doi:10.1136/jnnp.51.6.745

40. Tomlinson CL, Stowe R, Patel S, Rick C, Gray R, Clarke CE. Systematic review of levodopa dose equivalency reporting in Parkinson's disease. *Movement Disorders*. 2010;25(15):2649-2653. doi:10.1002/mds.23429

41. Ovesný M, Křížek P, Borkovec J, Švindrych Z, Hagen GM. ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. *Bioinformatics*. 2014;30(16):2389-2390. doi:10.1093/bioinformatics/btu202

42. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676-682. doi:10.1038/nmeth.2019

43. Fazekas FJ, Shaw TR, Kim S, Bogucki RA, Veatch SL. A mean shift algorithm for drift correction in localization microscopy. *Biophysical Reports*. 2021;1(1):100008. doi:https://doi.org/10.1016/j.bpr.2021.100008

44. Legland D, Arganda-Carreras I, Andrey P. MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics*. 2016;32(22):3532-3534. doi:10.1093/bioinformatics/btw413

45. Litvan I, Goldman JG, Tröster AI, et al. Diagnostic criteria for mild cognitive impairment in Parkinson's disease: Movement Disorder Society Task Force guidelines. *Mov Disord*. 2012;27(3):349-356. doi:10.1002/mds.24893

46. El-Agnaf OMA, Salem SA, Paleologou KE, et al. Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson’s disease. *FASEB J*. 2006;20(3):419-425. doi:10.1096/fj.03-1449com

47. Pigott K, Rick J, Xie SX, et al. Longitudinal study of normal cognition in Parkinson disease. *Neurology*. 2015;85(15):1276-1282. doi:10.1212/WNL.0000000000002001

48. Broeders M, Bie RMA de, Velseboer DC, Speelman JD, Muslimovic D, Schmand B. Evolution of mild cognitive impairment in Parkinson disease. *Neurology*. 2013;81(4):346 LP - 352. doi:10.1212/WNL.0b013e31829c5c86

49. Pedersen KF, Larsen JP, Tysnes OB, Alves G. Natural course of mild cognitive impairment in Parkinson disease: A 5-year population-based study. *Neurology*. 2017;88(8):767-774. doi:10.1212/WNL.0000000000003634

50. Foltynie T, Brayne CEG, Robbins TW, Barker RA. The cognitive ability of an incident cohort of Parkinson’s patients in the UK. The CamPaIGN study. *Brain*. 2004;127(Pt 3):550-560. doi:10.1093/brain/awh067

51. Williams-Gray CH, Mason SL, Evans JR, et al. The CamPaIGN study of Parkinson’s disease: 10-year outlook in an incident population-based cohort. *J Neurol Neurosurg Psychiatry*. 2013;84(11):1258-1264. doi:10.1136/jnnp-2013-305277

52. Irwin DJ, Lee VMY, Trojanowski JQ. Parkinson’s disease dementia: convergence of α-synuclein, tau and amyloid-β pathologies. *Nat Rev Neurosci*. 2013;14(9):626-636. doi:10.1038/nrn3549

53. Siderowf A, Xie SX, Hurtig H, et al. CSF amyloid beta 1-42 predicts cognitive decline in Parkinson disease. *Neurology*. 2010;75(12):1055-1061. doi:10.1212/WNL.0b013e3181f39a78

54. Lin CH, Yang SY, Horng HE, et al. Plasma Biomarkers Differentiate Parkinson's Disease From Atypical Parkinsonism Syndromes. *Front Aging Neurosci*. 2018;10:123. doi:10.3389/fnagi.2018.00123

55. Chen NC, Chen HL, Li SH, et al. Plasma Levels of α-Synuclein, Aβ-40 and T-tau as Biomarkers to Predict Cognitive Impairment in Parkinson's Disease. *Front Aging Neurosci*. 2020;12. doi:10.3389/fnagi.2020.00112

56. Tsai HH, Tsai LK, Lo YL, Lin CH. Amyloid related cerebral microbleed and plasma Aβ40 are associated with cognitive decline in Parkinson's disease. *Sci Rep*. 2021;11(1):1-9. doi:10.1038/s41598-021-86617-0

57. Chung CC, Chan L, Chen JH, Bamodu OA, Chiu HW, Hong CT. Plasma extracellular vesicles tau and β-amyloid as biomarkers of cognitive dysfunction of Parkinson's disease. *The FASEB Journal*. 2021;35(10):e21895. doi:https://doi.org/10.1096/fj.202100787R

58. Chojdak-Łukasiewicz J, Małodobra-Mazur M, Zimny A, Noga L, Paradowski B. Plasma tau protein and Aβ42 level as markers of cognitive impairment in patients with Parkinson's disease. *Adv Clin Exp Med*. 2020;29(1):115-121. doi:10.17219/acem/112058

59. Kim JR. Oligomerization by co-assembly of β-amyloid and α-synuclein. *Front Mol Biosci*. 2023;10(March):1-8. doi:10.3389/fmolb.2023.1153839

60. Grant MKO, Handoko M, Rozga M, et al. Human cerebrospinal fluid 6E10-immunoreactive protein species contain amyloid precursor protein fragments. *PLoS One*. 2019;14(2):e0212815. doi:10.1371/journal.pone.0212815

61. Helmerhorst EJ, Oppenheim FG. Saliva: a Dynamic Proteome. *J Dent Res*. 2007;86(8):680-693. doi:10.1177/154405910708600802

62. Thomadaki K, Helmerhorst EJ, Tian N, et al. Whole-saliva proteolysis and its impact on salivary diagnostics. *J Dent Res*. 2011;90(11):1325-1330. doi:10.1177/0022034511420721

63. Shahnawaz M, Mukherjee A, Pritzkow S, et al. Discriminating α-synuclein strains in Parkinson's disease and multiple system atrophy. *Nature*. 2020;578(7794):273-277. doi:10.1038/s41586-020-1984-7

64. Ojha B, Fukui N, Hongo K, Mizobata T, Kawata Y. Suppression of amyloid fibrils using the GroEL apical domain. *Sci Rep*. 2016;6(August):1-13. doi:10.1038/srep31041

65. Iljina M, Garcia GA, Dear AJ, et al. Quantitative analysis of co-oligomer formation by amyloid-beta peptide isoforms. *Sci Rep*. 2016;6:28658. doi:10.1038/srep28658

66. Novo M, Freire S, Al-Soufi W. Critical aggregation concentration for the formation of early Amyloid-β (1-42) oligomers. *Sci Rep*. 2018;8(1):1783. doi:10.1038/s41598-018-19961-3

Figure Legends

Figure 1 Illustration of the SiMPull method. A – prepared glass coverslip with NeutrAvidin bound to its surface and F-127 passivation. B – application of biotinylated capture antibodies, either LB509 or 6E10 antibodies are used for the detection of α-synuclein or Aβ containing aggregates respectively. C – application of saliva biofluid to the coverslip; Aβ or α-synuclein containing aggregates are specifically captured by the relevant immobilised antibodies on the coverslip surface. D – Alexa-647 labelled LB509 and 6E10 antibodies are applied to the surface

for aggregate detection. E – Representative diffraction-limited image captured using TIRF microscope set up. F – Representative super-resolved aggregates

Figure 2 Analysis of diffraction-limited single-aggregate counting for subgroup one. Each field of view is 2500µm^2 and 16 fields of view were measured for each participant, PD $n = 10$ and control $n = 10$. A - A non-significant increase in the number of α-synuclein containing aggregates was observed in the saliva of people with PD compared to controls ($W = 75$, $p = 0.063$) and there was no difference in the number of Aβ containing aggregates (W = 45, p = 0.74) (B). C - The ratio of the number of α-synuclein to Aβ aggregates is significantly increased 2.2-fold in people with PD compared to controls (W = 79, $p=0.029$, $r = 0.49$). D – ROC analysis of ratio values (*AUC=0.79*)

Figure 3 Morphological analysis of α-synuclein and Aβ containing aggregates from subgroup one using dSTORM super-resolution imaging. For both types of aggregate we compared the cumulative frequency curves for the morphological feature of interest. The two distributions are subtracted from each other to demonstrate how the two curves differ; the point of maximum difference is then used as a threshold to distinguish groups of morphologically distinct aggregates. α-Synuclein A-H. We find no difference in the size (area, A-B, E) or shape (circularity, C-D, G) of α-synuclein containing aggregates (PD n = 9, control n = 9). For Aβ containing aggregates (I-P, PD n = 9, control n = 8) we show that there the area distribution differs between the two groups and that the two groups maximally differ from each other at 0.03µm^2 (I-J, M). Using this value as a cut off, we show that saliva from patient with PD contains a greater proportion of aggregates >0.03µm^2 ($t(9.75) = 2.43$, $p = 0.036$, $d = 1.15$). ROC analysis demonstrates that aggregate size can distinguish between the PD and controls (N, *AUC=0.76*). Shape data analysis shows a visible difference between circularity distributions (K-L, O), we find that the two distributions are maximally different from each other at a circularity value of 0.4 ($t(15) = 2.48$, $p = 0.025$, $d = 1.23$) and ROC analysis shows that shape data can distinguish PD from controls (P, *AUC=0.76*)

Figure 4 Analysis of the combined single-aggregate count and super-resolution morphological data for subgroup one. A combined discriminator is calculated for each participant by multiplying the α-synuclein/Aβ aggregate ratio by the proportion of Aβ containing aggregates satisfying both morphological feature thresholds (area >0.03μm^2 and circularity <0.4, PD n = 9, control n = 8). A – The combined discriminator is significantly higher in the PD group (4.3-fold increase, $t(8.83) = 2.88$, $p = 0.018$, $d = 1.36$), B - applying ROC analysis demonstrates that this metric can accurately distinguish between PD and control participants (*AUC=0.89*)

Figure 5 Analysis of diffraction limited single molecule aggregate counting, super resolution morphological data, and combined discriminator data for subgroup two. For diffraction limited data each field of view is 2500μm^2 and 16 fields of view a captured for each participant, n = 10 PD and 10 control. A - the ratio of the number of α-synuclein to Aβ aggregates is significantly higher in patients with PD (1.9-fold increase, $t(12.68) = 2.42$, $p = 0.031$, $d = 1.08$). B – ROC analysis of diffraction limited ratio values (*AUC=0.77*). For super-resolution data, analysis was completed as described in Fig. 3 (subgroup two PD $n = 9$ and control $n = 9$). C - For Aβ containing aggregates we show that there is a different area distribution between the two groups, applying the threshold values identified in subgroup 1 we show there is significantly more Aβ aggregates larger than 0.03μm^2 in the PD group ($t(8.365) = 2.852$, $p = 0.02$, $d = 1.345$). D - For Aβ shape data, analysis shows a visible difference between circularity distributions, the PD group has more Aβ with a circularity < 0.4 ($t(10.43) = 2.901$, $p=0.015$, $d = 1.367$). Full analysis, including α-synuclein data, is shown in Supplementary Figure 4. A combined discriminator is calculated as previously described. E – The combined discriminator is significantly higher in the PD group (4-fold increase, $t(10.43) = 2.901$, $p = 0.025$, $d = 1.367$), F - applying ROC analysis demonstrate that this metric can accurately distinguish between PD and controls (*AUC=0.86*)

Figure 6 Analysis of combined single molecule aggregate count data and super-resolution morphological data for data combined across subgroups one and two. A – The α-synuclein /Aβ aggregate ratio was significantly higher in when the subgroups were combined. The combined discriminator was calculated as previously described (PD n = 18, control n = 17). A – The combined discriminator was increased 5-fold in the PD group (W = 41, $p <0.001$), B -

applying ROC analysis demonstrated that this metric can accurately distinguish between PD and controls (*AUC=0.86*)