The Amyloid-β Oligomer Count in Cerebrospinal Fluid is a Biomarker for Alzheimer's Disease

Lei Wang-Dietrich[a,1], Susanne Aileen Funke[a,1], Katja Kühbach[a], Kun Wang[a], Astrid Besmehn[b], Sabine Willbold[b], Yeliz Cinar[a], Oliver Bannach[c], Eva Birkmann[a,c] and Dieter Willbold[a,c,*]

[a]*Institute of Complex Systems, ICS-6: Structural Biochemistry, Forschungszentrum Jülich, Jülich, Germany*
[b]*ZCH, Forschungszentrum Jülich, Jülich, Germany*
[c]*Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany*

Abstract. Recent studies indicate that small amyloid-β peptide (Aβ) oligomers are the major toxic species responsible for development and progression of Alzheimer's disease (AD). Therefore, we suggest that the number of Aβ oligomers in body fluids is the most direct and relevant biomarker for AD. Determination of the Aβ oligomer content of cerebrospinal fluid (CSF) samples from 14 AD patients and 12 age-matched controls revealed a clear distinction between both groups. All samples of the control group showed homogenously low numbers of Aβ oligomers, while the samples of the AD group exhibited significantly higher levels of Aβ oligomers. The Aβ oligomer numbers correlated with the patients' Mini-Mental State Examination scores. This indicates that the quantity of Aβ oligomers in CSF reflects the severity of the disease and that Aβ oligomers play a crucial role in AD pathology and in turn can be used as a diagnostic biomarker.

Keywords: Alzheimer disease, amyloid-β peptide, early diagnosis, sFIDA, surface based fluorescence intensity distribution analysis

INTRODUCTION

Alzheimer's disease (AD) is a fatal neurodegenerative and progressive disorder and the most common form of dementia, which will become a threat to our health care systems, if prevention and treatment efforts continue to fail. The sensitivity and specificity for clinical diagnosis of AD is 70 to 90% in specialized centers, but considerably lower for patients with early AD or in primary care settings [1–3]. Early diagnosis will aid treatment decisions substantially, as the majority of scientists currently agrees that AD treatment will be most effective in preclinical stages of the disease [4]. Therefore, a reliable biomarker and a reliable method for its quantification are urgently needed to allow trial inclusion of humans at very early, pre-symptomatic stages of the disease. Reducing the level of the very same biomarker and the delayed or avoided appearance of clinical symptoms could then be clearly defined treatment objectives.

Aggregation of neurotoxic amyloid-β (Aβ) is believed to play a major role in the development of AD. Aβ is derived from the amyloid-β protein precursor (AβPP) by sequential activities of the β- and γ-secretases [5–7], and is the major component of amyloid plaques. The most abundant Aβ species consist of 38 to 43 amino acid residues of which Aβ$_{42}$ and especially pyro-glutamated species are prone to aggregation and undergo formation from monomers to oligomers, protofibrils, insoluble fibrils, and plaques [8, 9]. As originally suggested by the amyloid cascade hypothesis [10, 11], Aβ aggregates initiate cellular events leading to the pathologic effects of AD. Recent studies support that, in particular, the small diffusible

---

Aβ oligomers are the major toxic species responsible for disease development and progression [12]. Although the monomeric form of Aβ is not the main responsible isoform for neurotoxicity and neurodegeneration, a variety of studies have demonstrated that reduced levels of $Aβ_{1-42}$ monomers in CSF, as determined by ELISA or related methods, implicate a high predictive value for the identification of prodromal AD even in cases of mild cognitive impairment (MCI). Evidence was found that combined biochemical analysis of tau and phosphorylated tau in CSF, both of which are increased in CSF of AD patients in comparison to controls, and detection of Aβ levels can improve the diagnostic value predicting AD with sensitivity and specificity values of 80 to 95% [13, 14]. However, because of substantial readout overlap between individuals of different groups, no definite diagnosis for all individual patients is currently possible. Regardless of the potential role of hyperphosphorylated tau protein and its aggregates, aggregation of Aβ is regarded as a very early event in the disease. Thus, the appearance of Aβ aggregates in body fluids, especially in CSF, may be one of the earliest events to be detectable during disease development and progression, and the ability to quantify them could not only enhance AD diagnosis, but also aid in the investigation of the contribution of Aβ aggregates to AD pathology. The quantification of Aβ aggregates in CSF basically requires the solution of two major technical problems: first, full discrimination between Aβ monomers and Aβ aggregates is needed, and second, maximum sensitivity is desired, preferably at the single aggregate level.

Starting in 2005, a number of reports have been published describing techniques for the measurement of Aβ aggregate contents in body fluids. Many of them are based on ELISA, but to date, only a few methods were suggested that have the potential for single Aβ aggregate detection sensitivity in body fluids [15, 16]. In 2006, Birkmann et al. reported on the detection of single prion protein particles by fluorescence correlation spectroscopy (FCS) in solution after labeling them by two different fluorescence-labeled anti-prion protein antibodies [17]. In 2007, the method was refined by including an immobilization step for the aggregates to a glass surface using prion protein-specific capture antibodies, and subsequent detection of the fluorescent probe decorated prion protein aggregates by the laser focus of a FCS device while it was moved across the glass surface. This procedure allowed counting fluorescence events originating from each prion protein particle in CSF of bovine spongiform encephalopathy (BSE) infected cattle. The assay

was denominated Surface-FIDA (surface fluorescence intensity distribution analysis) or sFIDA [18]. Application of sFIDA even allowed the distinction of Scrapie infected sheep from healthy sheep by quantifying prion protein particles in blood plasma samples [19]. In recent years, it was shown that sFIDA is principally applicable to count Aβ aggregates in human CSF samples. In addition, it was possible to replace the FCS device by a laser scanning microscope (LSM) [20–22].

Based on the sFIDA principle, we optimized the assay into an ultrasensitive Aβ aggregate detection system using anti-Aβ-antibodies as capture and detection probes. We describe the experimental features of the redesigned sFIDA assay and report on the results that we obtained from a challenge of the assay with 26 human CSF samples that were obtained from AD-affected subjects and age-matched non-AD controls.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Munich, Germany) with analytical grade or chemically pure unless noted otherwise. Ultrapure water (Milli-Q, water resistivity = 18.2 MΩ·cm, Millipore GmbH, Schwalbach/Ts., Germany) was used in all experiments.

Preparation of synthetic Aβ monomers and oligomers using size exclusion chromatography (SEC)

Lyophilized, synthetic $Aβ_{42}$ peptide was purchased (JPT Technologies, Berlin, Germany). To dissolve pre-existing aggregation seeds, the Aβ peptide was dissolved in 1,1,1,3,3,3- hexafluoro-2-propanol (HFIP) to a concentration of 400 μM and incubated overnight at room temperature. After HFIP was evaporated, Aβ was dissolved in running buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.4 with 0.6% Tween-20) to a final concentration of 500 μM. The sample was centrifuged for 5 min at $16,100 \times g$. 100 μl of supernatant were loaded onto a SEC column (Superdex 75 10/300 GL, GE Healthcare, Uppsala, Sweden). The samples were eluted at room temperature, at a flow rate of 0.5 ml/min, with running buffer and detected at 214 nm, according to the protocol of Johansson et al. [23]. The concentrations of Aβ oligomer and monomer fractions were determined using the Micro BCA[TM] Protein Assay

Kit (Thermo Scientific, Rockford, USA) following the instructions of the manufacturer. The Aβ-containing fractions were incubated for 30 min in 3 M Urea at 60°C to disassemble Aβ oligomers. The solutions were mixed with micro BCA reagents and incubated further for 30 min at 60°C. Each sample was tested in duplicate in a 96-well microplate (Greiner Bio-one, Frickenhausen, Germany) and the peptides were detected with a wavelength of 570 nm in a plate reader POLARstar OPTIMA (BMG Labtech, Offenburg, Germany). For Aβ species detection using sFIDA, Aβ monomers and oligomers were further diluted in PBS to the desired concentrations.

Fluorescence labeling of antibodies

6E10-Alexa-Fluor 488 was purchased (Covance, Dedham, MA, USA). For labeling of Nab228 (Sigma-Aldrich, Munich, Germany), the fluorescence monoclonal antibody labeling kit Alexa-Fluor 647 (Molecular Probes, Karlsruhe, Germany) was used according to the manufacturer's instructions. The purified fluorescence-labeled antibodies were stored in PBS and 2 mM sodium azide, at 4°C in the dark.

Design of the sFIDA assay

Glass surface preparation and covalent immobilization of Aβ capture antibodies

384-Well SenoPlate[TM] Plus microplates (Greiner bio-one, Frickenhausen, Germany, borosilicate glass bottom with a well surface of 10 mm^2) were used to perform sFIDA. Because the polystyrene frame is chemically not inert, a gentle protocol was applied to activate the glass surface for subsequent amination using ethanolamine, partly according to Janissen et al. [24]. After cleaning the glass surface with cleaner solution in an ultrasonic bath, the surface was incubated with 5 M NaOH for 3 h, rinsed with water and ethanol and dried afterwards. The glass was subsequently immersed in DMSO containing 5 M ethanolamine overnight [25], followed by washing three times with DMSO and three times with ultrapure water. For the reduction of unspecific fluorescent background caused by the binding of fluorescent probes or sample material to the surface, glass-shielding carboxymethyldextran (CMD) was immobilized to the glass surface activated with amino groups. CMD was dissolved in water to a concentration of 10 mg/ml and mixed with 200 mM *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC) and

50 mM N-Hydroxysuccinimide (NHS). After 10 min pre-incubation the solution was incubated on the amino group activated glass surface for 2 h at room temperature, leading to covalent linking of CMD to the glass surface via ester bonds. Finally the glass surface was rinsed with water.

A second activation step with EDC/NHS (5 min, 200/50 mM, respectively) was applied to the plates prepared as described above. The capture antibodies (Nab228) were added and incubated for 2 h at 4°C for their covalent immobilization on the CMD activated glass surface of the microtiter plate wells. To deactivate remaining carboxylate end groups of the CMD spacer, 1 M ethanolamine in DMSO was applied for 15 min. The wells were rinsed with PBS three times before the samples were added.

Sample application to sFIDA and data evaluation

Samples were either synthetic Aβ monomers or oligomers in PBS, or human CSF. None of the samples was blinded prior to analysis. Samples were added to the capture antibody coupled glass microtiterplate wells and incubated for 1 h to immobilize Aβ present in the sample at the bottom of the glass well. The wells were rinsed twice with TBST (0.1% (w/w) Tween-20, 50 mM Tris-HCl; 0.15 M NaCl, pH 7.4). Next, fluorescence-labeled Aβ detection antibodies 6E10-Alexa-Fluor 488 and Nab228-Alexa-Fluor 647 were added. After 1 h incubation at room temperature and consecutive washing steps, the measurements were performed on a laser scanning microscope LSM710 (Carl Zeiss, Jena, Germany). The microscope was equipped with an argon ion laser (λex = 488 nm) and a helium-neon laser (λex = 633 nm).

The glass surface was scanned by the argon (λex = 488 nm) and helium-neon laser (λex = 633 nm). In total, 50 images (213 μm × 213 μm per image) containing 1024 × 1024 pixels were scanned for each sample in the tile scan mode, 25 images per tile scan. The area for each tile scan was randomly chosen, but the edges of the well were avoided. The totally scanned area covered about 20% of each well.

The image data were analyzed using the open source image processing software ImageJ (http://rsbweb.nih.gov/ij/). The images obtained from channel 1 (6E10-Alexa-Fluor 488) and channel 2 (Nab228-Alexa-Fluor 647) were merged and the number of crosscorrelated pixels that were above the threshold value in both channels was counted for every of the 50 images per sample. Intensity cut-off values of two for the green channel and 1 for the red

Table 1
Summary of sample specifications

Specification	Subject ID No.	Age	Gender	MMSE	sFIDA readout
Control	7090076	69	M	30	10387
Control	7090084	72	M	30	22474
Control	7807	74	F	30	5016
Control	7808	66	M	29	4494
Control	7827	73	F	28	8124
Control	7828	70	M	30	11814
Control	7830	80	F	30	6169
Control	7837	73	F	30	24413
Control	7840	76	F	29	10898
Control	7844	75	M	30	8682
Control	7846	70	M	30	8255
Control	7852	81	F	30	7530
Mean value		73		30	6169
AD	308	93	M	2	22524
AD	312	86	F	24	445840
AD	322	94	F	9	350160
AD	8001	83	M	15	329032
AD	8005	80	M	14	450637
AD	8026	78	F	15	308302
AD	8029	74	M	14	274639
AD	8040	87	M	19	161557
AD	8057	77	F	15	152676
AD	8058	72	F	11	149527
AD	8060	79	M	17	150300
AD	8061	82	F	16	170509
AD	8077	77	F	15	139320
AD	8106	71	F	15	138045
Mean value		83		15	231647.71
p-value of sFIDA readout means between AD and control groups					<0.001

MMSE, Mini–Mental State Examination score.

channel excluded background with low fluorescence intensities from the analysis.

Human CSF samples

CSF samples of elderly controls and subjects diagnosed with AD were purchased from PrecisionMed, Inc. (San Diego, CA) and stored at $-80°C$ until use. The sample specifications can be seen in Table 1.

RESULTS

Design of the sFIDA assay

Starting from the basic principle of the "sFIDA" assay [17, 18], which has been developed to detect and quantify prion protein aggregates from scrapie-infected hamster as well as BSE-infected cattle, and an earlier version of this assay adapted to detect Aβ aggregates [20, 21], we optimized the assay to an even more sensitive and highly specific Aβ oligomer detection and quantification assay. The details are described

in the methods section and a general scheme of the procedure is shown in Fig. 1. Differences between the earlier sFIDA version and the assay described here are summarized in Table 2.

Briefly, in order to avoid unspecific binding of Aβ aggregates to the glass surface of microtiter plate wells, we covalently covered the glass surface with CMD. An anti-Aβ-antibody (Nab228) was covalently linked to the CMD treated surface as a capture for Aβ species eventually contained in the samples. After application of the sample and thorough washing, fluorescence-labeled anti-Aβ-antibodies (6E10-Alexa-Fluor-488 and Nab228-Alexa-Fluor-647) were applied to decorate potentially present Aβ oligomers in the sample. The use of capture and detection antibodies with overlapping epitopes avoided fluorescence-labeling of capture-bound Aβ monomers. After final washing, the presence of fluorescent probes on the surface indicated the presence of Aβ oligomers. The use of two different anti-Aβ-antibodies, each one labeled with another fluorescent dye, further increased the specificity of the detection procedure using LSM by counting only fluorescence events that show fluorescence
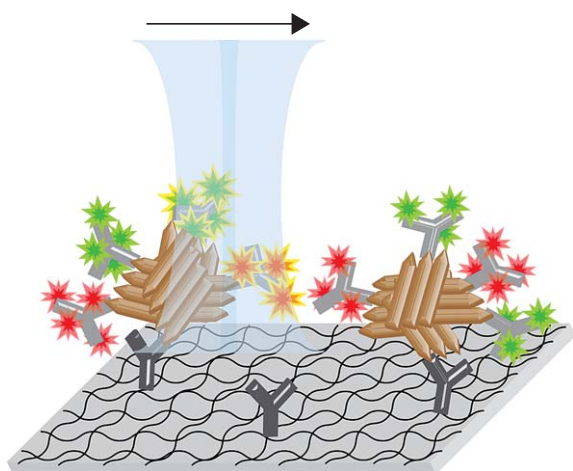
Fig. 1. Scheme of the sFIDA assay. Aβ aggregates or oligomers are captured and concentrated onto a two-dimensional surface by immobilizing them on a glass surface (grey bottom plane) with covalently linked Aβ capture antibodies (dark grey Y-letters). Aβ aggregates (brownish pale bundles) are detected by adding at least two fluorescence-labeled anti-Aβ antibodies (light grey Y-letters). Laser beams with wavelengths suitable for excitation of the employed fluorescent dyes are focused on the surface of the glass chip and the fluorescence light which is emitted by the fluorescence-labeled antibodies is detected, allowing single aggregate detection sensitivity. Either a fluorescence correlation spectroscopy (FCS) device or a laser scanning microscope (LSM) can be used.

intensities above background thresholds in both emission channels.

Validation of sFIDA with synthetic Aβ oligomers

We tested the newly designed sFIDA with known amounts of synthetic Aβ oligomers prepared by SEC. SEC purified Aβ monomer samples with the same total Aβ content and samples without any Aβ were used as negative controls. Various amounts of Aβ monomers and oligomers in PBS buffer were applied to the sFIDA assay. Images from the Aβ oligomer containing samples as well as of the control samples (Fig. 2A–C) showed that the Aβ oligomers were spread homogenously on the surface. In both negative

controls, virtually no fluorescence was detectable. The sFIDA readout is shown in Fig. 2D. Application of Aβ oligomers resulted in considerably high signals, whereas the readout for Aβ monomers was as low as for the control without any Aβ. To evaluate the linearity of the assay, Aβ oligomers were diluted serially in PBS and applied to the assay. The correlation between Aβ concentration and readout was linear over a wide range of Aβ amounts, even down to the low picogram range (Fig. 2E).

Application of human CSF samples from AD patients and age-matched controls to sFIDA

To challenge the sFIDA assay with real samples, we applied CSF samples in 12.5 µl volume from AD patients and age-matched non-demented controls. The exact specifications of the samples as well as the respective sFIDA readouts are shown in Table 1. As can be seen in Fig. 3, the mean value of the sFIDA readout of the AD group was significantly higher than that of the age-matched control group, allowing a clear and highly significant distinction between both groups. The AD group showed high variations among samples, whereas the control group showed homogenously low levels of Aβ aggregate count. All but one AD samples resulted in significantly elevated sFIDA readouts as compared to the control samples. For the small number of samples tested, this yields a sensitivity of 93% and a specificity of 100%.

Interestingly, the sFIDA readout correlated with the Mini-Mental State Examination (MMSE) scores of the CSF donors (Fig. 4). We found a significant negative correlation with the MMSE scores ($r^2 = 0.35$; $p = 0.0016$). Thus, the sFIDA readout seems to reflect the severity of the disease. The correlation between sFIDA readout and age of the patients was less clear ($p = 0.0116$, $r^2 = 0.2373$), but a trend toward more oligomers with age could be observed, which might be affected by the fact that the AD patients were on average 10 years older (mean 83, range 71 to 94) than the controls (mean 73, range 76–84).

Table 2
Summary of specifications for the earlier and novel sFIDA version

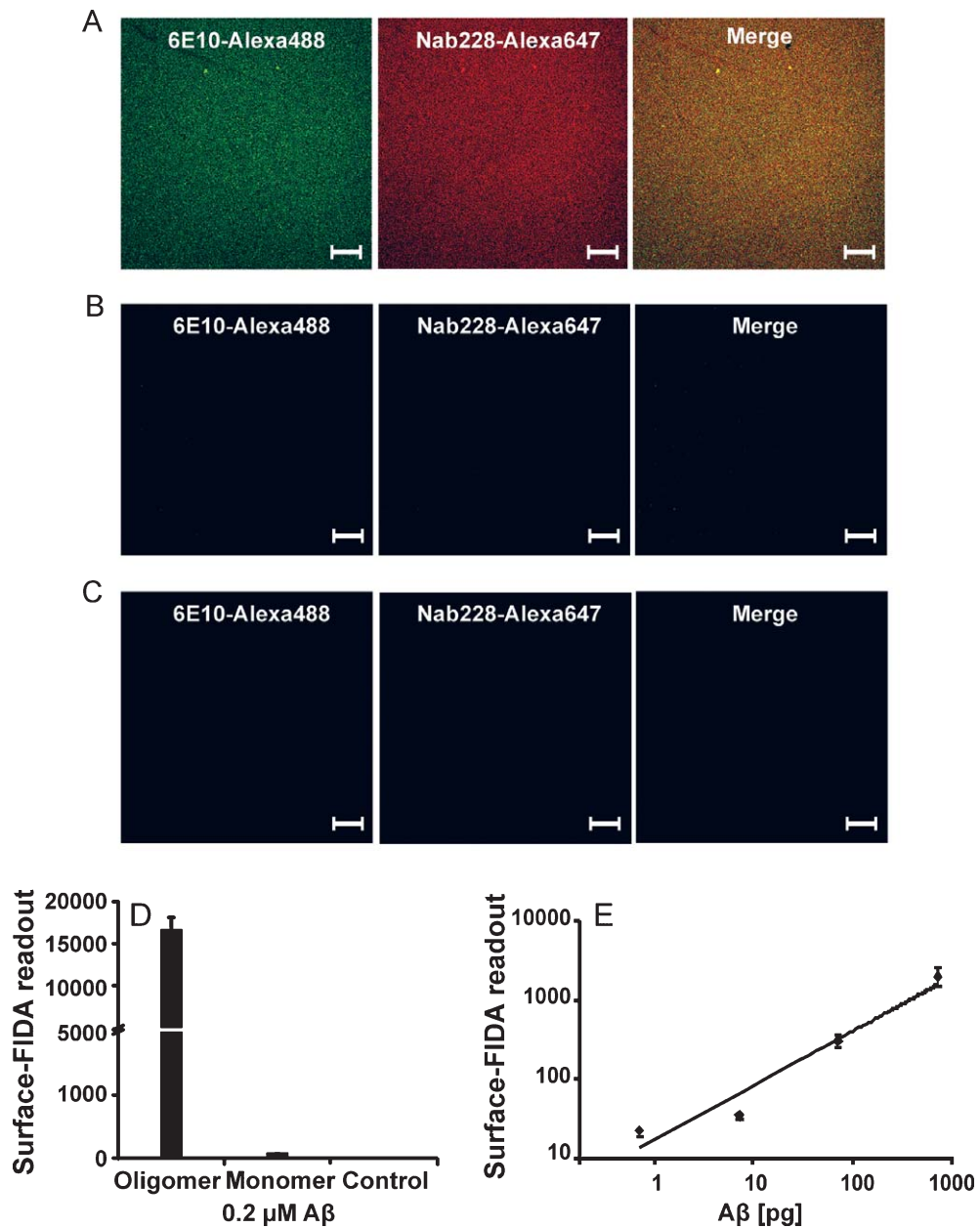| Specification	Version 2007 [20]	Recent version
Capture antibody immobilization	Unspecific adsorption, poly-D-lysine	Covalent, carboxymethyldextran
Background fluorescence	Comparably high due to unspecific binding of fluorescent probes and sample proteins to glass surface	Significantly reduced
Principle of detection	Fluorescence correlation spectroscopy	Laser scanning microscopy
Data output	Fluorescence intensities over time	Images, Pixel
Sensitivity as judged by detection of synthetic Aβ oligomers	nM scale	pM scale

Fig. 2. sFIDA is insensitive to Aβ monomers. Aβ oligomers and monomers were prepared using size exclusion chromatography (SEC) according to the protocol of Johansson et al. [23]. The Aβ species were immobilized on the CMD glass surface via capture antibody Nab228. As detection antibodies Nab228-Alexa-Fluor 647 and 6E10-Alexa-Fluor 488 were applied. PBS without Aβ was used as control. LSM images of 0.2 μM Aβ oligomers (A), 0.2 μM monomers (B), and PBS control without Aβ (C). The sFIDA readouts of all these samples are given in D. The standard deviation was calculated from six tile scan measurements. The sensitivity of the assay was determined by dilution of the oligomers in PBS buffer (E).

DISCUSSION

Aβ oligomer formation and toxicity is reported to be a key event in AD progression [12, 26]. The development of techniques for the specific detection of Aβ oligomers is technically challenging. Aβ oligomers may be present in CSF only in trace amounts [27] and are highly diverse in respect of size and structure. A variety of different oligomer conformers, ranging from dimers to 56 mers and higher order
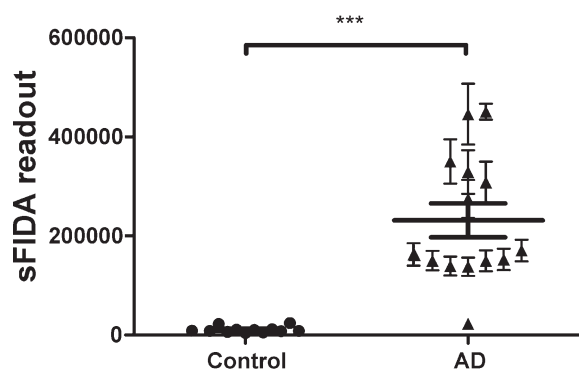
Fig. 3. Results of sFIDA from AD and control CSF samples. Of each CSF sample, 12.5 μl were directly applied to capture antibody covered wells. In total, 50 images (213 μm × 213 μm per image) of 1024 × 1024 pixels were recorded for each sample. The total number of crosscorrelated pixels above a threshold of one for the red channel and a threshold of two for the green channel was calculated for each of the 50 images, and the mean value is given for each sample. Error bars indicate the standard error of the mean (SEM). The significance was calculated using unpaired student's *T*-test. The horizontal bars represent the mean value of all data points within one group, the corresponding SEM is indicated. ***$p < 0.001$.

Fig. 4. A) Correlation between sFIDA readout and MMSE scores ($p = 0.0016$; $r^2 = 0.35$). Each point represents a single case. The best-fit linear regression is shown by a solid line and the 95% confidence interval by interrupted lines. B) Correlation between sFIDA readout and age of the patients, independent of diagnosis. ($p = 0.0116$, $r^2 = 0.2373$). Each point represents a single case. The best-fit linear regression is shown by a solid line and the 95% confidence interval by interrupted lines.

oligomers, have been described *in vitro* and *in vivo* [8, 12, 26, 28–32]. The expected low abundance of oligomers demands for methods with intrinsically high sensitivity.

We hereby present an updated sFIDA assay with improvements to render it fully applicable as a tool to quantify Aβ oligomers with single particle sensitivity in human CSF samples (also see Table 2). Compared to other assays and principles, sFIDA has some major advantages. i) The assay is completely insensitive to Aβ monomers. ii) It does not require any pretreatment of the CSF samples and thus avoids artificial changes in the aggregation state of Aβ species contained in the samples. iii) By using conformation-unspecific anti-Aβ antibodies, the assay is not restricted to certain Aβ aggregate species. In the present study, we used anti-Aβ antibodies that are directed against linear epitopes located at the amino-terminal end of Aβ, which seems to be exposed in all Aβ aggregate conformers [33]. If desired, it is straightforward to use conformation specific capture and/or detection antibodies to allow for example quantification of certain sub-species of Aβ oligomers. iv) Although not shown in the present work, it may be straightforward to correlate the detected fluorescence signal intensities with the sizes of the respective aggregates, because the larger the aggregates, the more epitopes are present, the more detection antibodies will bind. This is a property clearly superior to sandwich ELISA protocols, which
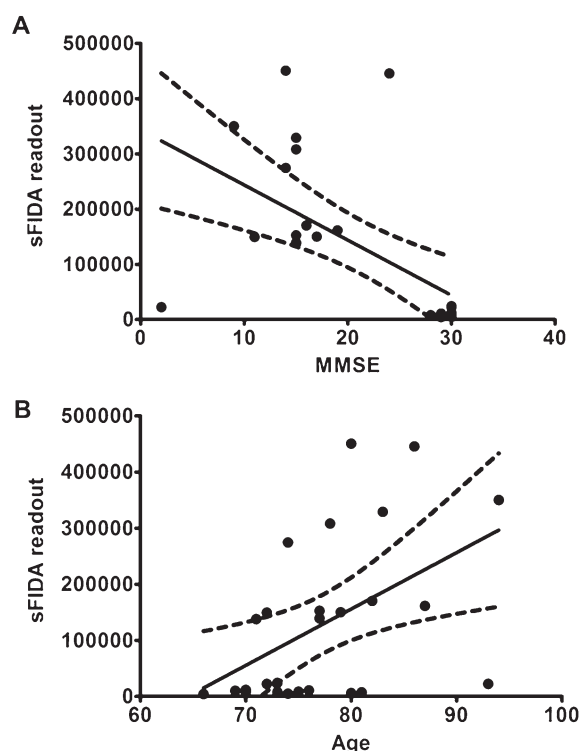
cannot differentiate a large number of small oligomers from a small number of large aggregates. (v) The most important advantage of sFIDA in comparison to ELISA approaches is its sensitivity. Each CSF sample applied to an ELISA yields exactly one readout value. Thus, the presence of only a few oligomers will possibly not lead to a readout value clearly above background. Each CSF sample applied to sFIDA, however, yields millions of readout values, dependent on the pixel size and the number of the images taken. Each image pixel is able to generate one readout value for each observed emission channel. We have developed several options to combine this wealth of information into one or more readout values per well. In the current mode of the sFIDA data evaluation, we counted the number of pixels that show fluorescence intensities above background in both emission channels that were used for detection. This number may correlate with the number of Aβ aggregate particles. (vi) The principle of sFIDA can easily be adapted to any other protein aggregates

to be detected. (vii) It is straightforward to include more detection probes with additional fluorescence colors in order to obtain even higher specificity and/or more information on the detected aggregates. (viii) We used a LSM for data collection. Other methods like total internal reflection fluorescence microscopy are working comparatively well (data not shown). If desired, the data evaluation may be carried out with any super-resolution imaging method, e.g., stimulated emission depletion or stochastic optical reconstruction microscopy [34], in order to yield highly resolved information about size and shape of the aggregates.

We challenged the assay with 26 human CSF samples that were obtained from AD-affected subjects and age-matched non-AD controls. Only 12.5 μl of each sample were sufficient and adequate to investigate the Aβ oligomer readout of each sample. The Aβ oligomer count allowed a clear distinction between both groups. All but one AD samples resulted in a clearly increased sFIDA readout compared to the control samples. We do not want to over-stress this singular result, but interestingly the one CSF sample of the AD group with very low sFIDA readout was obtained from a donor with an MMSE of only 2. Possibly the brain of this donor was already damaged to an extent that interfered with the production of Aβ. Another potential explanation is more general: if the current gold standard of AD diagnosis has a specificity of 90%, then any diagnostic tool with 100% specificity needs to disagree with the current gold standard in 10% of the cases. Thus, the outlayer of the AD CSF sample group may have been misdiagnosed and might have been affected by any other form of dementia. Unfortunately, a follow-up study of this CSF donor was not possible.

Our study emphasizes the usefulness of Aβ oligomers or higher molecular weight aggregates as a biomarker for AD. Together with other reports on various approaches to quantify Aβ aggregates, we also find that the amount of Aβ aggregates was higher in CSF samples from AD patients as compared to CSF samples from non-demented controls [16, 20, 27, 35–40]. Similar to our study, Fukomoto et al. [36] also found a correlation between the amount of Aβ aggregates in CSF and the MMSE score of the respective donors. These results further strengthen the view that the number of Aβ oligomers may reflect disease severity and progress. The finding that the concentration of Aβ oligomers is increased in CSF of AD patients in comparison to healthy controls seems to contradict ELISA studies revealing that the total or monomer Aβ amount decreases with disease progression [41–44]. Englund et al., however, found evidence that the lowering of monomeric $Aβ_{42}$ might be caused by its oligomerization and deposition in plaques [35]. Indeed, the decrease of $Aβ_{42}$ concentration in CSF is strongly and inversely correlated with Aβ deposition in the brain, as measured by amyloid imaging studies using positron emission tomography [45]. Thus, the reported decrease of monomeric Aβ might well be in accordance with the observed increase of aggregated Aβ with disease progression.

In summary, our results demonstrate that Aβ oligomers as quantified by sFIDA have a high potential to be used as a biomarker to clearly discriminate CSF from AD patients and healthy age-matched controls. The correlation between sFIDA readout from the CSF samples and the MMSE score of the respective patients supports the role of Aβ aggregates for disease development and progression. Thus, Aβ aggregate quantification by sFIDA may allow early diagnosis of pre-symptomatic AD cases. This in turn may render the assay as a useful tool to include pre-symptomatic cases into clinical trials for therapy development. The Aβ oligomer count may become useful for therapy monitoring and its reduction may even become a potential therapy objective. Currently, a variety of compounds for treatment of AD are developed [46], and reliable biomarkers will be necessary to prove that the drug of interest has disease modifying effects and changes the pathophysiology of the disease [41].

For potential use of sFIDA detected Aβ oligomers as biomarker in clinics, robustness of the method will have to be proven in longitudinal studies of large cohorts. Although lumbar puncture is moderately invasive and has low incidence of complications [47], the detection of Aβ oligomers in blood plasma would be of great value for wider diagnostic use and for therapy monitoring. Consequently, sFIDA may become a method for reliable detection and quantitation of Aβ oligomers in any biological sample (e.g., CSF, blood, organs) in order to investigate their role in AD pathology and progression. It may be sensitive enough to follow the "prion-like" propagation of Aβ aggregates *in vitro* and *in vivo* to assess the role of "prion-like" Aβ aggregates for transmission of the disease from one animal to another.

ACKNOWLEDGMENTS

Diseases (JPND) and the Kompetenznetz Degenerative Demenzen (KNDD) of the German Federal Ministry of Education and Research (BMBF). CSF samples were kindly provided by EISAI Co., Ltd.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1619).

REFERENCES

[1] Knopman DS, DeKosky ST, Cummings JL, Chui H, Corey-Bloom J, Relkin N, Small GW, Miller B, Stevens JC (2001) Practice parameter: Diagnosis of dementia (an evidence-based review). Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* **56**, 1143-1153.

[2] Visser PJ, Scheltens P, Verhey FR (2005) Do MCI criteria in drug trials accurately identify subjects with predementia Alzheimer's disease? *J Neurol Neurosurg Psychiatry* **76**, 1348-1354.

[3] Ganguli M, Rodriguez E, Mulsant B, Richards S, Pandav R, Bilt JV, Dodge HH, Stoehr GP, Saxton J, Morycz RK, Rubin RT, Farkas B, DeKosky ST (2004) Detection and management of cognitive impairment in primary care: The Steel Valley Seniors Survey. *J Am Geriatr Soc* **52**, 1668-1675.

[4] Golde TE, Schneider LS, Koo EH (2011) Anti-abeta therapeutics in Alzheimer's disease: The need for a paradigm shift. *Neuron* **69**, 203-213.

[5] Haass C, Selkoe DJ (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell* **75**, 1039-1042.

[6] Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733-736.

[7] Weidemann A, Konig G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115-126.

[8] Finder VH, Glockshuber R (2007) Amyloid-beta aggregation. *Neurodegener Dis* **4**, 13-27.

[9] Schlenzig D, Manhart S, Cinar Y, Kleinschmidt M, Hause G, Willbold D, Funke SA, Schilling S, Demuth HU (2009) Pyroglutamate formation influences solubility and amyloidogenicity of amyloid peptides. *Biochemistry* **40**, 7072-7078.

[10] Hardy JA, Higgins GA (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256**, 184-185.

[11] Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* **6**, 487-498.

[12] Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol* **8**, 101-112.

[13] Bibl M, Esselmann H, Wiltfang J (2012) Neurochemical biomarkers in Alzheimer's disease and related disorders. *Ther Adv Neurol Disord* **5**, 335-348.

[14] Zetterberg H, Blennow K (2008) Biological CSF markers of Alzheimer's disease. *Handb Clin Neurol* **89**, 261-268.

[15] Funke SA, Willbold D (2011) Quantitation of Amyloid-β oligomers in human body fluids for Alzheimer's disease early diagnosis or therapy monitoring? In *Alzheimer's Diagnosis*, Ronson CE, ed. Nova Science Publishers, Inc., Hauppage, NY.

[16] Funke SA, Birkmann E, Willbold D (2009) Detection of Amyloid-beta aggregates in body fluids: A suitable method for early diagnosis of Alzheimer's disease? *Curr Alzheimer Res* **6**, 285-289.

[17] Birkmann E, Schafer O, Weinmann N, Dumpitak C, Beekes M, Jackman R, Thorne L, Riesner D (2006) Detection of prion particles in samples of BSE and scrapie by fluorescence correlation spectroscopy without proteinase K digestion. *Biol Chem* **387**, 95-102.

[18] Birkmann E, Henke F, Weinmann N, Dumpitak C, Groschup M, Funke A, Willbold D, Riesner D (2007) Counting of single prion particles bound to a capture-antibody surface (surface-FIDA). *Vet Microbiol* **123**, 294-304.

[19] Bannach O, Birkmann E, Reinartz E, Jaeger KE, Langeveld JP, Rohwer RG, Gregori L, Terry LA, Willbold D, Riesner D (2012) Detection of prion protein particles in blood plasma of scrapie infected sheep. *PloS One* **7**, e36620.

[20] Funke SA, Birkmann E, Henke F, Gortz P, Lange-Asschenfeldt C, Riesner D, Willbold D (2007) Single particle detection of Abeta aggregates associated with Alzheimer's disease. *Biochem Biophys Res Commun* **364**, 902-907.

[21] Funke SA, Birkmann E, Henke F, Gortz P, Lange-Asschenfeldt C, Riesner D, Willbold D (2008) An ultrasensitive assay for diagnosis of Alzheimer's disease. *Rejuvenation Res* **11**, 315-318.

[22] Funke SA, Wang L, Birkmann E, Willbold D (2010) Single-particle detection system for Abeta aggregates: Adaptation of surface-fluorescence intensity distribution analysis to laser scanning microscopy. *Rejuvenation Res* **13**, 206-209.

[23] Johansson AS, Berglind-Dehlin F, Karlsson G, Edwards K, Gellerfors P, Lannfelt L (2006) Physiochemical characterization of the Alzheimer's disease-related peptides A beta 1-42Arctic and A beta 1-42wt. *FEBS J* **273**, 2618-2630.

[24] Janissen R, Oberbarnscheidt L, Oesterhelt F (2009) Optimized straight forward procedure for covalent surface immobilization of different biomolecules for single molecule applications. *Colloids Surf B Biointerfaces* **71**, 200-207.

[25] Ebner A, Hinterdorfer P, Gruber HJ (2007) Comparison of different aminofunctionalization strategies for attachment of single antibodies to AFM cantilevers. *Ultramicroscopy* **107**, 922-927.

[26] Kawahara M (2010) Neurotoxicity of beta-amyloid protein: Oligomerization, channel formation, and calcium dyshomeostasis. *Curr Pharm Des* **16**, 2779-2789.

[27] Georganopoulou DG, Chang L, Nam JM, Thaxton CS, Mufson EJ, Klein WL, Mirkin CA (2005) Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease. *Proc Natl Acad Sci U S A* **102**, 2273-2276.

[28] Barghorn S, Nimmrich V, Striebinger A, Krantz C, Keller P, Janson B, Bahr M, Schmidt M, Bitner RS, Harlan J, Barlow E, Ebert U, Hillen H (2005) Globular amyloid beta-peptide oligomer - a homogenous and stable neuropathological protein in Alzheimer's disease. *J Neurochem* **95**, 834-847.

[29] Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A* **95**, 6448-6453.

[30] Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* **440**, 352-357.

[31] Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL (2007) Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci* **27**, 2866-2875.

[32] Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ (2008) Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* **14**, 837-842.

[33] Tycko R (2006) Molecular structure of amyloid fibrils: Insights from solid-state NMR. *Q Rev Biophys* **39**, 1-55.

[34] Tonnesen J, Nagerl UV (2012) Superresolution imaging for neuroscience. *Exp Neurol*, in press. doi: 10.1016/j.expneurol.2012.10.004

[35] Englund H, Degerman Gunnarsson M, Brundin RM, Hedlund M, Kilander L, Lannfelt L, Pettersson FE (2009) Oligomerization partially explains the lowering of Abeta42 in Alzheimer's disease cerebrospinal fluid. *Neurodegener Dis* **6**, 139-147.

[36] Fukumoto H, Tokuda T, Kasai T, Ishigami N, Hidaka H, Kondo M, Allsop D, Nakagawa M (2010) High-molecular-weight beta-amyloid oligomers are elevated in cerebrospinal fluid of Alzheimer patients. *FASEB J* **24**, 2716-2726.

[37] Haes AJ, Chang L, Klein WL, Van Duyne RP (2005) Detection of a biomarker for Alzheimer's disease from synthetic and clinical samples using a nanoscale optical biosensor. *J Am Chem Soc* **127**, 2264-2271.

[38] Pitschke M, Prior R, Haupt M, Riesner D (1998) Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy. *Nat Med* **4**, 832-834.

[39] Englund H, Sehlin D, Johansson AS, Nilsson LN, Gellerfors P, Paulie S, Lannfelt L, Pettersson FE (2007) Sensitive ELISA detection of amyloid-beta protofibrils in biological samples. *J Neurochem* **103**, 334-345.

[40] Gao CM, Yam AY, Wang X, Magdangal E, Salisbury C, Peretz D, Zuckermann RN, Connolly MD, Hansson O, Minthon L, Zetterberg H, Blennow K, Fedynyshyn JP, Allauzen S (2010) Abeta40 oligomers identified as a potential biomarker for the diagnosis of Alzheimer's disease. *PLoS One* **5**, e15725.

[41] Blennow K (2010) Biomarkers in Alzheimer's disease drug development. *Nature Med* **16**, 1218-1222.

[42] Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat Rev Neurol* **6**, 131-144.

[43] Fagan AM, Holtzman DM (2010) Cerebrospinal fluid biomarkers of Alzheimer's disease. *Biomark Med* **4**, 51-63.

[44] Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: A follow-up study. *Lancet Neurol* **5**, 228-234.

[45] Fagan AM, Mintun MA, Mach RH, Lee SY, Dence CS, Shah AR, LaRossa GN, Spinner ML, Klunk WE, Mathis CA, DeKosky ST, Morris JC, Holtzman DM (2006) Inverse relation between *in vivo* amyloid imaging load and cerebrospinal fluid Abeta42 in humans. *Ann Neurol* **59**, 512-519.

[46] Jakob-Roetne R, Jacobsen H (2009) Alzheimer's disease: From pathology to therapeutic approaches. *Angew Chem Int Ed Engl* **48**, 3030-3059.

[47] Andreasen N, Minthon L, Davidsson P, Vanmechelen E, Vanderstichele H, Winblad B, Blennow K (2001) Evaluation of CSF-tau and CSF-Abeta42 as diagnostic markers for Alzheimer disease in clinical practice. *Arch Neurol* **58**, 373-379.