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Detection of Amyloid-β Oligomers in Human Cerebrospinal Fluid by Flow Cytometry and Fluorescence Resonance Energy Transfer

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Abstract. The neuropathology of Alzheimer's disease (AD) has been linked recently to non-fibrillar forms of neurotoxic amyloid-β (Aβ) oligomers of which high levels are observed in the brain of AD patients. This suggests that Aβ oligomers play a key role in the early events of AD, underlining their potential for the early diagnosis of the disease. We have developed an extremely sensitive assay for the detection of oligomeric and fibrillar structures of Aβ that is based on multiparametric analysis of data obtained by flow cytometry and fluorescence resonace energy transfer (Fret). The assay readily detects Aβ oligomers in human cerebrospinal fluid (CSF) as verified by dot blot of the isolated particles. By measuring 174 CSF samples of non-demented control patients with various neurological disorders a high reliability and reproducibility of the method could be demonstrated.

Keywords: Alzheimer's disease, amyloid-β oligomers, cerebrospinal fluid, flow cytometry, fluorescence resonance energy transfer

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative dementia with an average death prognosis of 7 years [7]. Ongoing clinical studies point out promising possibilities for the treatment of this disease [1]. For ideal therapy and timely conservation of essential cognitive functions, however, a diagnostic tool for the early detection of AD is a pre-requisite. Recent studies show that oligomeric assemblies of amyloid-β (Aβ) are neurotoxic in cell culture and *in vivo* as they are able to inhibit long-term potentiation [13,22]. Such low-molecular-weight Aβ oligomers were also shown to induce transient deficits in cognitive function [4]. To further demonstrate the adverse effect of oligomers on nerve cell function, immunotherapy was successfully used to neutralize Aβ oligomers, thereby restoring synaptic plasticity *in vivo* [11]. Further evidence pointing to Aβ oligomers as the neurotoxic species in AD is that the concentration of these structures in human brain were found to be up to 70-fold higher in AD patients than in non-demented controls [9,12,18]. It could thus be demonstrated that the severity of the disease correlates with oligomer concentration rather than with number of plaques [6,14,15] and the presence of globular Aβ oligomers in the brain is suggested to be an early pathological event in AD [2]. Hence, the search for Aβ oligomers was extended to human CSF and recent research studies demonstrated the presence of low amounts of stable Aβ oligomers also in this body fluid [8,17,21,23]. As the concentration of oligomers was consistently higher in CSF of AD patients compared to non-demented age-matched controls, this points toward a correlation between the levels of oligomers and the state of the disease [8], making them a possible

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biomarker and suitable target for the early detection of AD. A sensitive method for the detection and accurate quantification of Aβ oligomers is thus required. One such method is the recently described ultra-sensitive bio-barcode assay for the measurement of amyloid-β-derived diffusible ligands (ADDLs) in CSF [8]. Although this method is very sensitive, the procedure includes a relatively high number of critical steps that may affect the general performance of the assay. We have therefore developed a new assay format that consists of only one step (dilution of the sample in buffer and incubation with fluorescence labeled antibodies) and allows a high throughput of samples. The assay utilizes flow cytometry combined with fluorescence resonance energy transfer (Fret), which is an excellent tool for determining distances and supramolecular organization of biomolecules.

Materials and methods

Chemicals and antibodies

All reagents were purchased from Sigma Aldrich (Hamburg, Germany) unless otherwise specified. The anti-Aβ monoclonal antibodies 4G8 and 6E10 were supplied by Chemicon International. The anti-oligomer antibody A-11 was purchased by Biosource.

Collection of cerebrospinal fluid

The collection of the CSF samples was done according to a vote from the ethics committee of the state of Bavaria. CSF was obtained from 174 neurological patients (mean age 49.3 years; range 8–89) with diagnoses such as multiple sclerosis, neuroboreliosis and inflammation. All samples were obtained by lumbar puncture, frozen within 2 h and stored at $-85\,^{\circ}$C before analysis; repeated freeze/thaw cycles were avoided.

Fluorescence labeling of antibodies

The anti-Aβ antibodies 4G8 and 6E10 were labeled with the fluorescence dyes Alexa Fluor 488 or Alexa Fluor 594 according to the manufacturer's instructions (applying the respective Monoclonal Antibody Labeling Kits; Invitrogen).

Sample preparation for flow cytometry

200 μl of CSF were diluted with 200 μl of 1 $\times$ RIPA buffer (5 $\times$ RIPA buffer contains 250 mM HEPES; 750 mM NaCl, 0.25% SDS, 1.25% Na-deoxycholate, 2.5% NP-40 alternative (Calbiochem) and 1 tablet of protease inhibitor cocktail Complete^{TM} Mini (Roche Applied Science) per 2 ml of 5 $\times$ RIPA buffer). Then the fluorescence labeled antibodies were added to concentrations of 2 nM and 8 nM for donor antibody (4G8, labeled with Alexa-Fluor 488) and acceptor antibody (6E10, labeled with Alexa-Fluor 594), respectively. After incubation (90 minutes at room temperature, protected from light), samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences) as described below.

Flow cytometry and particles sorting

For the detection of Aβ oligomers a FACS Calibur flow cytometer equipped with a 15 mW 488 nm air-cooled argon-ion laser (BD Biosciences) was used. The oligomer particles were gated in logarithmic forward/sideward scatter dot plots (FSC vs. SSC). The green or red fluorescence of the dyes Alexa Fluor 488 and Alexa Fluor 594 was detected by the corresponding FL1 and FL3 (logarithmic scale) photomultipliers through 530/30 or 670LP bandpass filters, respectively. To avoid differences in the measurement due to variation in the CSF samples, all samples were measured in TruCount Tubes (BD Biosciences) and analysis was stopped after 28,000 beads were counted. The number of oligomers/volume of CSF analysed was determined as Fret events. One Fret event corresponded to one particle consisting of Aβ peptide in which several peptides aggregate to form an oligomer. Sorting of the oligomer-specific region was performed on a FACS Vantage cell sorter (BD Biosciences), applying a threshold to the FL1 channel. The population of interest was gated in a dot plot of FL1 vs. FL3 and only events with a Fret signal were sorted.

Western and dot blot analysis

20 μl of CSF samples were applied to 16% Tricine gels (Invitrogen), run for 2 hours, and transferred onto PVDF membranes. Membranes were boiled for 5 min in PBS and blocked for 2 hours in 5% skimmed milk powder in TBS-T (10 mM Tris-HCl, pH 7.6 containing 150 mM NaCl and 0.1% Tween 20). Then the monoclonal anti-Aβ antibody 6E10 was applied

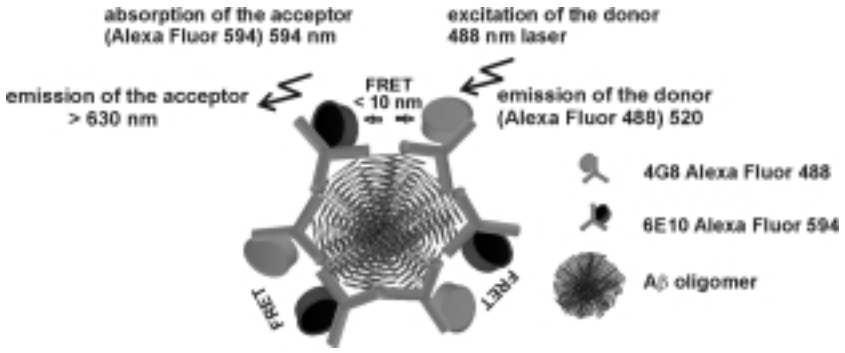

Fig. 1. *Principle of the assay*. Aβ oligomers are detected by two specific monoclonal anti-Aβ antibodies and Fret. The clone 4G8 is used as donor antibody and is labeled with the Alexa Fluor 488 dye, the acceptor antibody 6E10 is labeled with Alexa Fluor 594.

and incubated over night. After washing the membranes in TBS-T, the bound antibody was visualized using HRP-conjugated secondary anti-mouse antibody and ECL detection (SuperSignal West Femto Substrate, Pierce). For dot blot analysis the samples were directly applied to the nitrocellulose membrane and air-dried. The membrane was then processed to determine the presence of Aβ oligomers using the anti-oligomer specific antibody A-11 according to the manufacturer's protocol.

Isolation of Aβ oligomers from brain homogenates

Frontotemporal brain tissue from control persons and AD patients was kindly provided by the German brain bank. Oligomers from control and AD brains were isolated according to Wiltfang et al. [24].

Preparation of seedless Aβ (1–42)

Aβ (1–42) was purchased from Bachem, seedless treated as previously reported [5], further purified by RP-HPLC (column: Source 5RPC 4.6/150 ST (Amersham); solvent A: 0.1% NH_4OH (25%) in H_2O, pH 9.0; solvent B: 60% acetonitrile, 40% solvent A; gradient: 25–56% solvent B in 31 min; flow rate: 1.0 ml/min) and lyophilized.

Generation of in vitro oligomers and fibrils

Oligomers of Aβ were prepared as described by Lambert et al. [13]. For fibrils generation seedless Aβ (1–42) was dissolved in DMSO to 1 mM, diluted to 100 μM into 10 mM HCl and incubated for 24 hours at 37°C [5]. For storage, fibril preparations were diluted to 5 μM into 100 mM Hepes, 2.5 mM DTT, 5 mM EDTA, 250 mM NaCl, 2% glycerol, pH 7.6 and frozen at −85°C.

Statistical analysis

Statistical analysis was performed using the MedCalc software and the Spearman's rank correlation coefficient.

Results

Fluorescence resonance energy transfer (Fret) is a special phenomenon in fluorescence spectroscopy during which energy is transferred from an excited donor molecule to an acceptor molecule under favorable spectral and spatial conditions [10]. In our approach, two monoclonal anti-Aβ antibodies that recognize different epitopes of the Aβ peptide sequence were labeled with the fluorescence dyes Alexa Fluor 488 (mAb 4G8; raised against Aβ 17–24) and Alexa Fluor 594 (mAb 6E10; raised against Aβ 4–13). Thus, the mAb 4G8-Alexa Fluor 488 corresponds to the donor molecule and the mAb 6E10-Alexa Fluor 594 functions as the acceptor molecule (Fig. 1). With this donor/acceptor combination, Aβ monomers are not detectable in our assay, which is due to the low amount of fluorophores that are able to bind to Aβ monomers (a maximum of two antibodies per monomer) and the resulting very low intensity of the emitted fluorescence and Fret signal (data not shown). In contrast, oligomeric structures of Aβ are able to bind sufficient amounts of antibody molecules and give thus rise to a fluorescence signal strong enough to be detected by flow cytometry (Fig. 2A, B). The information content of the fluorescence signal is increased by Fret, as it allows a differentiation of unspecific binding of the mAb 4G8 to other molecules. Hence, signals from the 6E10-Alexa Fluor 594 antibody are only observed if the distance between both antibodies is closer than 10 nm, the Förster dis-

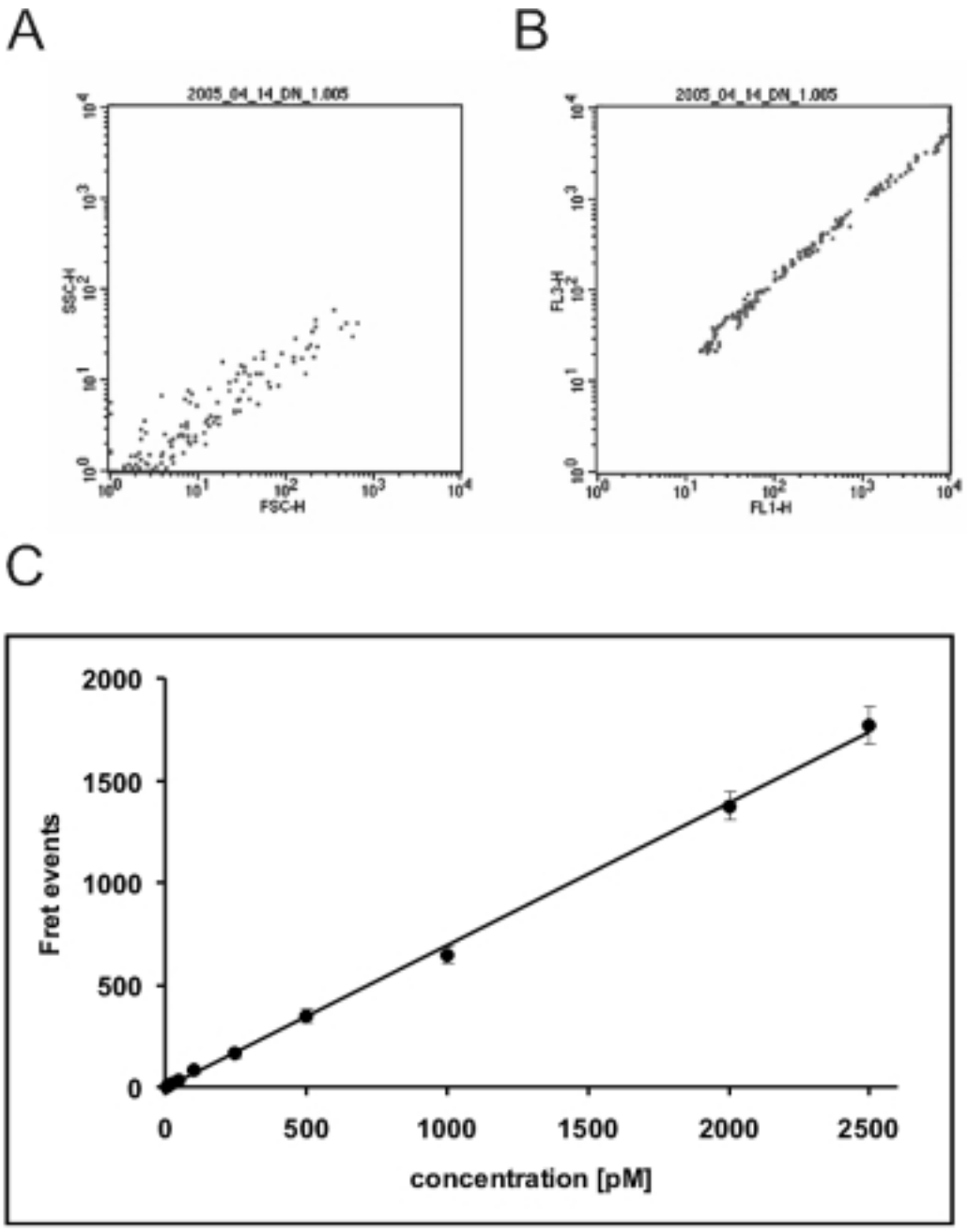

Fig. 2. *Detection of Aβ oligomers prepared in vitro by flow cytometry and sensitivity of the assay.* In contrast to monomers of Aβ the detection of *in vitro* assembled Aβ oligomers can easily be achieved by flow cytometry and Fret (A, B). The sensitivity of the assay was determined by titration of *in vitro* assembled Aβ fibrils. All data points are represented as mean values (± standard deviation) of measurements performed in triplicate. The assay is linear over a wide range of concentrations, ranging from at least 10 pM to 2.5 nM. (C).

tance that allows energy transfer between donor and acceptor fluorophores.

In order to determine the sensitivity of the assay *in vitro* assembled Aβ oligomers and fibrils were analyzed with the procedure described. For the detection of oligomers by flow cytometry ADDLs (Amyloid beta-derived diffusible ligands) were prepared as described by Lambert et al. [13]. Such molecules are claimed to be found in CSF by Georganopoulou et al. [8]. In vitro assembled ADDLs were detected efficiently by

our method. However, our ADDL preparations were not stable (even not at $-85°$C) and transformed to fibrils. As a consequence, the reproducibility of the measurements was poor (data not shown). Therefore we preferred to deal with fibril preparations which were stable. Only differences in the size and Fret-signal intensity were observed between Aβ oligomers and fibrils (smaller for the oligomers). The sensitivity in both cases was the same. The assay is linear over a wide range of concentrations, ranging from at least 10 pM to 2.5 nM (Fig. 2C). Taking into account that the fibril concentration is expressed as concentration of monomers used for fibril assembly and assuming an average fibril size of several hundreds to thousands of monomers, the actual concentration of fibrils is about two to three orders of magnitude lower. The detection limit for the current setup of the assay is therefore in the femtomolar range.

For the determination of the presence of oligomers in human fluids 200 μl of CSF were diluted with 200 μl of 1 $\times$ RIPA buffer, followed by incubation with the Fret antibody mixture. Although we used 200 μl of each CSF sample, this volume is not limiting and can be increased if a higher sensitivity is required. To better visualize the Fret effect, we first incubated human CSF with the antibody 4G8-Alexa Fluor 488 alone. In the subsequent flow cytometric analysis, an oligomer-specific population was detected showing events in the fluorescence 1 channel only (Fig. 3A; FL1-H). Addition of the Fret acceptor antibody 6E10-Alexa Fluor 594 then induced an additional fluorescence signal in the fluorescence 3 channel (Fig. 3B, C; FL3-H). This gain of an Alexa Fluor 594 specific emission demonstrates the binding of both antibodies in close proximity to each other ($<$ 10 nm), allowing energy transfer from the Alexa Fluor 488 donor to the Alexa Fluor 594 acceptor fluorophore. Although the specificity for the detected signal increases upon addition of the acceptor antibody, the overall number of events simultaneously decreases, possibly due to a sterical competition of both antibodies for their respective epitopes as well as a Fret induced fluorescence quenching of the acceptor fluorophores. Since the probability for non-specific binding of both antibodies within a distance closer than 10 nm is extremely low, these data suggest that the detected events are indeed Aβ oligomers. To further validate this assumption, a pool of CSF (6 ml) was prepared and analyzed as described before. The events detected in the region of interest, however, were sorted with a FACS Vantage cell sorter and subsequently analyzed by dot blot, resulting in a positive signal when probed with the oligomer-specific antibody A-11 (Fig. 3D).

It is known that repeated freeze/thaw cycles of CSF lead to a decrease in the concentration of Aβ monomers [19]. The absence of a reliable detection method, however, did render such studies impossible for oligomers. We therefore examined the effect of freeze/thaw cycles using a pool of CSF that was frozen at $-85°$C and thawed consecutively three times. The first value was measured before freezing and was set as 100%. In contrast to the situation observed for Aβ monomers [19], no significant effect was observed for the overall amount of oligomers for all applied freeze/thaw cycles (Fig. 4A). We further investigated the reproducibility of our assay, exemplified for seven different CSF samples in Fig. 4B. The standard deviation for these samples is $\pm$ 2.1% (Fig. 4B) and below 5% for more than 200 measured samples. As can also be seen from this figure, two of the CSF samples were negative for Aβ oligomers, even though all samples contained approximately equal amounts of monomers as detected by Western blot (Fig. 4B, C, lane 3, 5). This demonstrates that there is no correlation between oligomer content and concentration of Aβ monomer as detected by Western blot or ELISA [20] and underlines the advantage of the assay over other methods, i.e. specificity for oligomers, scalable sample volume and thus enhanced sensitivity (Fig. 2C).

In order to test the clinical assay performance, we finally extended our CSF analysis to samples obtained from 174 non-demented individuals with various neurological disorders (Fig. 5). Evaluation of the data shows a large variation in the concentration of the detected oligomers. We found, however, a weak correlation between the age of the individuals and amounts of Aβ oligomers (rho $= 0.22$; $p = 0.0036$).

As flow cytometry is a common method in routine diagnostics, the use of the assay described here could be a low cost alternative to other methods, particularly as it allows a high sample throughput and automation.

Discussion

In the present study we describe a new approach for the detection of Aβ oligomers in cerebrospinal fluid. One of the advantages of the assay is the possibility to discriminate between Aβ monomers, oligomers and fibrils. In addition, the use of a fluid system, i.e. flow cytometry, allows a multiparametric analysis of the detected molecules and thus a better differentiation of the signal. This is not possible with other methods such as ELISA or fluorescence correlation spectroscopy. Stenh

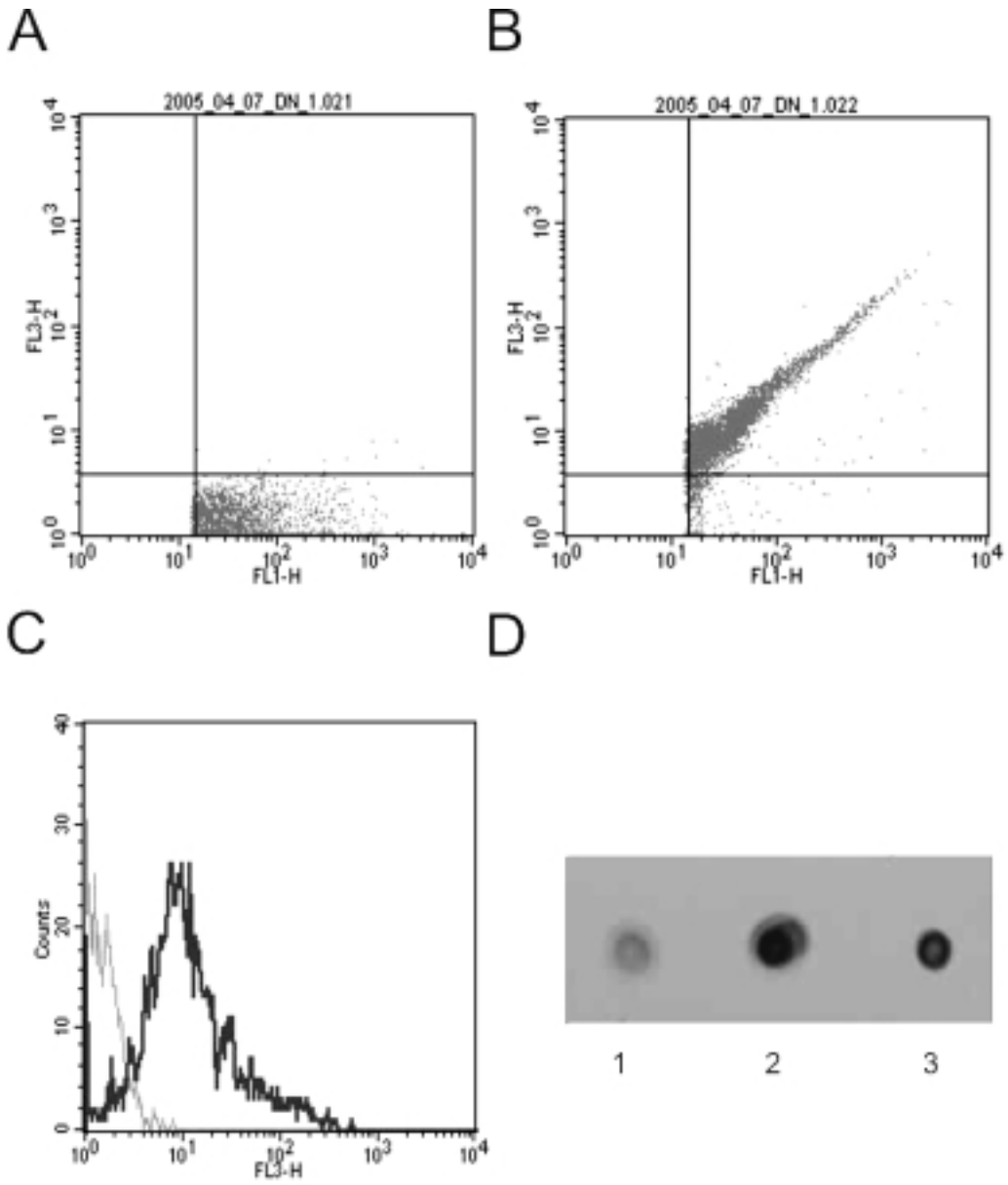

Fig. 3. *Validation of the assay*. Incubation of a CSF sample with donor antibody (4G8-Alexa Fluor 488) alone gives rise to a signal in the FL1 channel only (A). Addition of the acceptor antibody (6E10-Alexa Fluor 594) results in a strong Fret signal, i.e. gain of fluorescence 3 (FL1 and FL3) (B). The histogram analysis of the FL3 channel to visualize the Fret-induced fluorescence shift is shown (C; light grey: only donor antibody; dark grey: donor/acceptor antibody pair). Dot blot analysis of the material isolated from a CSF pool after sorting and probed with the anti-oligomer specific antibody A-11 showed the presence of oligomers in the preparation (D, lane 3). Brain homogenate from an AD patient (D, lane 2) and a non-demented control person (D, lane 1) were used as positive and negative controls, respectively.

et al. [20] showed that the amount of Aβ oligomers determined by ELISA was underestimated due to the suboptimal binding of the antibodies to the oligomers. Pitschke et al. [17] demonstrated the presence of Aβ aggregates in cerebrospinal fluid by fluorescence correlation spectroscopy. Most recently, Georganopoulou et al. [8] showed the presence of A-β-derived diffusible ligands in the same fluid using a bio-barcode assay. Although both assays successfully detected Aβ aggregates in cerebrospinal fluid, flow cytometry possesses the advantages of a widespread availability in all leading biomedical research institutions and the potential

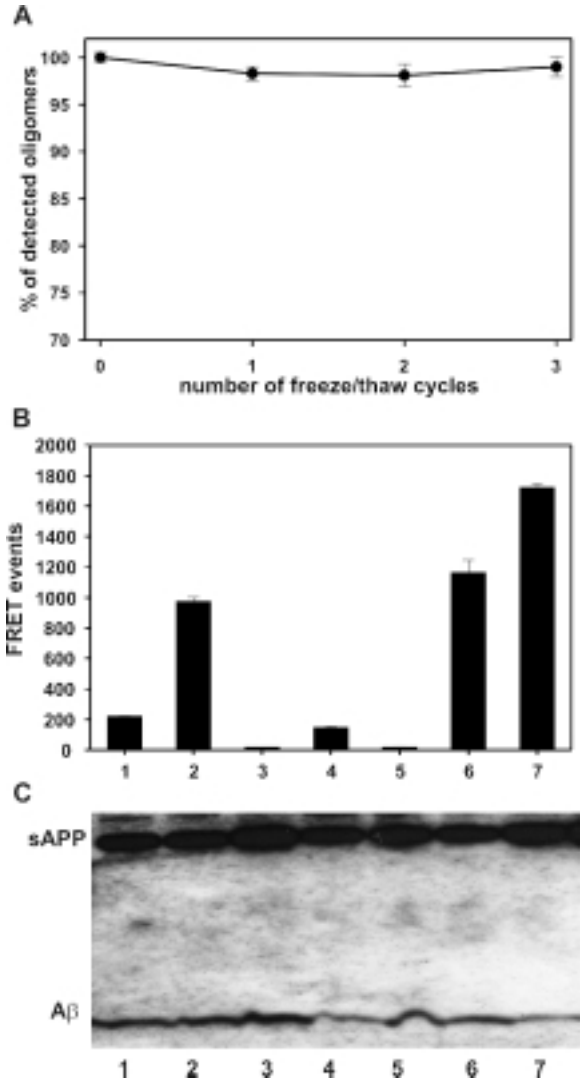

Fig. 4. *Stability of natural Aβ oligomers and reproducibility of the assay.* The effect of freeze/thaw cycles on the detection of natural Aβ oligomers in CSF was tested. The concentration of the Aβ oligomers detected in a CSF pool was not altered by the application of three freeze/thaw cycles (A). The reproducibility of the assay was assessed by measuring seven different CSF samples by flow cytometry. All samples were analyzed in duplicate and values are represented as mean values and standard deviations (B). When a Western blot using 20 μl of CSF samples analyzed in B was carried out no correlation between the amounts of Aβ monomer as detected by Western blot and oligomer content was observed (C). sAPP: soluble amyloid precursor protein.

of a high throughput of samples. In the assay presented here, the combination of flow cytometry and fluorescence resonance energy transfer resulted in an increase in the specificity of the particles detected. Only particles with fluorescence in the FL1 and FL3 channels were counted as a specific signal. Attempts to determine the minimal size of the detected oligomers in CSF by size exclusion chromatography were unsuccessful due to the heterogeneity of the particles. Nevertheless, the size of the detected molecules in CSF and the intensity of the Fret signals corresponded to the size of the *in vitro* assembled ADDLs. The result of the dot blot developed with the anti-oligomer specific A-11 antibody also suggested that the detected molecules were oligomers and not fibrils. Taking all these points together we conclude that the detected particles in CSF are oligomers. Whether Aβ (1–40), Aβ (1–42), or other forms of the peptide (N-terminal or C-terminally truncated) are a constituent of the particles, will be addressed in future studies. The antibodies we used in the present study do not allow distinguishing between Aβ (1–40) and Aβ (1–42) because they bind in the N-terminal and central region of the peptide. Antibodies recognizing epitopes in the C-terminal region could not be used since the C-terminus of the Aβ peptide is highly hydrophobic and thus solvent-inaccessible [16]. Soluble amyloid precursor protein (sAPP) did not interfere with the assay. The donor antibody 4G8 recognized the mid-domain of the Aβ peptide (epitope 17–24) and sAPP lacks this domain. Thus, sAPP could be bound by the acceptor antibody 6E10 but was not exited due to the lack of the donor antibody 4G8 and hence was not detected. Silver gels of the sorted material showed bands not corresponding to multimers of the Aβ peptide, suggesting that other proteins are also constituents of the particles (data not shown). Such proteins could

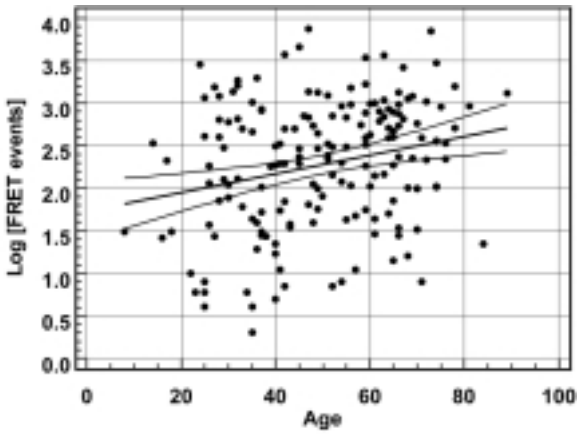

Fig. 5. *Clinical performance of the assay*. The scatter diagram of Aβ oligomers (Fret events) versus age of 174 CSF samples from non-demented control subjects reveals a positive correlation (rho = 0.22; $p = 0.0036$) between age and concentration of Aβ oligomers. All values are mean values of two independent measurements. The linear regression and 95% confidence intervals are represented as solid lines.

be albumin or ApoE which are known to bind to the Aβ peptide [3].

In this study, samples from patients suffering from various neurological disorders other than AD were analysed. A weak correlation between age and the amounts of oligomers was observed. However, the Aβ oligomer concentrations can not actually be contemplated to augment with age. The measurement of samples from patients diagnosed with AD will assess the potential of the assay as a diagnostic tool for AD and the suitability of Aβ oligomers as a biological marker of AD.

Acknowledgement

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