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Detection of contactin-2 in cerebrospinal fluid (CSF) of patients with Alzheimer's disease using Fluorescence Correlation Spectroscopy (FCS)

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ABSTRACT

Objectives: Alzheimer's disease (AD) is the most common cause of dementia in the world. As many AD biomarkers occur at rather low abundances in CSF or blood, techniques of very high sensitivity and accuracy are important as diagnostic tools in the clinic. Here, we aimed to provide proof of concept of the use of a single molecule detection technique, Fluorescence Correlation Spectroscopy (FCS) for detection of novel candidate biomarkers for AD.

Design and methods: FCS detects the diffusion times of the antigen-antibody complexes in highly diluted sample solutions, thus eliminating the need of large sample volumes and allows estimating the concentration of the target antigen. We developed a FCS set-up for contactin-2, a neuronal cell adhesion molecule and a ligand of beta-secretase 1 (BACE1) and amyloid precursor protein (APP), the latter proteins being important players in AD. With this method, we investigated whether contactin-2 concentrations are changed after delayed storage and in patients with Alzheimer's disease.

Results: The FCS set-up for measuring contactin-2 in CSF had a lower limit of quantification (LLOQ) of 0.2 ng/ml and intra- and inter-assay coefficients of variation (CVs) of 12.2% and 14.6% respectively. Contactin-2 levels were stable up to one week storage of CSF ($n = 3$) at RT and 4 °C. Further, contactin-2 levels were similar in probable AD patients ($n = 34$, $p = 0.27$) compared to patients with subjective cognitive decline (SCD) ($n = 11$).

Conclusions: FCS is a sensitive tool, which can be used for detecting biomarkers in the clinical setting using very low sample volumes (10 µl) and can measure proteins in their native conformations in the body fluid.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia worldwide. In AD patients, the underlying neurodegeneration is demonstrated by high levels of tau whereas the Aβ plaque pathology is reflected by reduced levels of Aβ42 in the CSF [1]. The third pathological hallmark is loss of synapses [2]. In addition, other disease processes, such as neuro-inflammation too play an important role [3]. As a result, there has been a great focus on characterizing and validating AD biomarkers other than the well-known markers, tau and Aβ [4]. Additional biomarkers are still needed, such as biomarkers reflecting synaptic dysfunction and inflammation or early blood based biomarkers [5]. A major problem is that often such molecules are present at low abundances in the CSF making them hard to be detected by conventional techniques such as ELISA. Moreover, samples such as CSF are not available abundantly. Therefore, there is a growing need for the development of highly sensitive detection methods.

Here, we propose Fluorescence Correlation Spectroscopy (FCS), a highly sensitive single molecule detection technique, and aimed to define a set-up for contactin-2, a soluble synaptic protein present in the CSF, as a proof of concept in the clinical context. FCS is capable of detecting extremely low concentration of biomolecules in solution by use of a confocal detection scheme [6–8]. It requires only very small sample volumes in the range of few microliters and can measure

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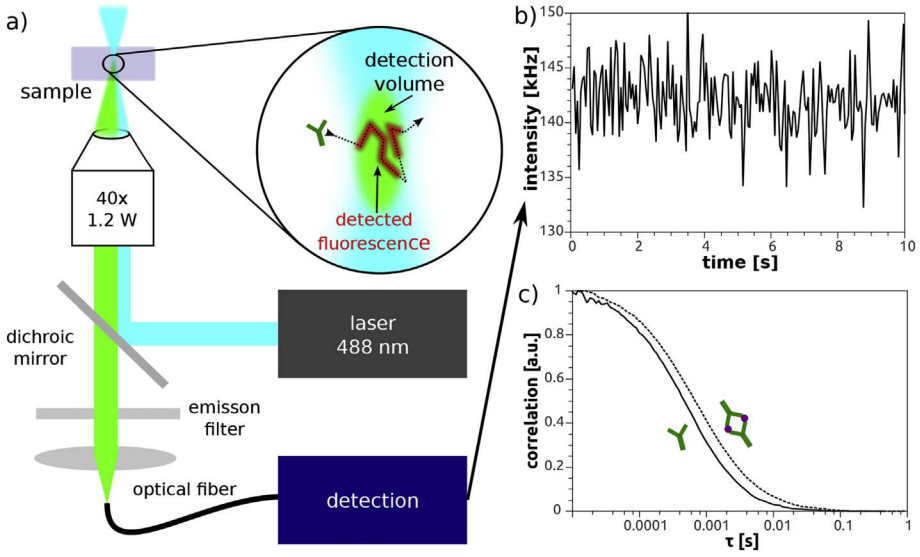

Fig. 1. Sketch of the FCS setup and data analysis. a) Excitation light at 488 nm is emitted by a laser and forms a confocal volume in the liquid sample. Fluctuation in fluorescence intensity is due to Brownian motion occurs (inset). The emitted light is measured by an avalanche photo diode connected via an optical fiber acting as a pinhole. b) Individual timestamps of the arriving photons are collected and visualized as average intensity over time. c) Auto-correlation of timestamps yields correlation curves. Right shift with respect to the reference curve (solid line) indicates higher diffusion time (τ_{diff}) (dotted line).

concentrations in the range of 10 pM (~ 1 ng/ml or below) to 100 nM (~ 15,000 ng/ml). In brief, FCS is based on the principle of detecting single molecules labeled with fluorescent tags as they diffuse freely through a confocal volume. The intensity of the emitted light, fluctuating due to Brownian motion, is recorded, and the fluctuations are evaluated to give the relevant molecules' abundances. Specifically, the temporal photon fluctuations are autocorrelated, resulting in an autocorrelation curve yielding information about the diffusion time(s) of the particles present in the solution and eventually the concentrations of molecules in the solution (Fig. 1).

FCS has some advantages over other available immunological detection methods. Firstly, it uses very small sample volumes and, second, it can detect rather low concentrations in the pico-molar range. Therefore, the samples can be highly diluted. Third, it can simultaneously measure the concentration and diffusion time of molecules, where increases in diffusion time indicate an increase in molecular weight of the tagged molecule brought about by the interaction with other molecules present in the solution. FCS is thus a useful tool for protein-protein interaction studies. Another advantage of FCS is that it can measure samples directly, eliminating any washing step.

In this study, contactin-2 was selected as the candidate biomarker to be detected with FCS in the CSF of patients with AD and patients with subjective cognitive decline (SCD). Contactin-2 is involved in many physiological neuronal processes, such as in the interaction with glial cells [9], axonal guidance [10] and synapse formation [11], which can be altered in AD pathology before clinical symptoms are visible [12]. Contactin-2 is a physiological substrate of BACE1 [13,14]. Also, it was found to co-localize with APP in immuno-stained mice brain sections and it immuno-precipitated together with APP in mice brain lysates [15]. Binding of contactin-2 with APP leads to subsequent release of the APP intra-cellular domain (AICD) which suppressed neurogenesis in cultured neurons [15]. Since contactin-2 interacts with two important molecules involved in the amyloidogenic pathway, we hypothesized that it might be altered in the CSF of AD patients compared with patients with SCD reflecting the ongoing disease process.

2. Materials and methods

2.1. Materials

The N- and C-terminal contactin-2 (SAB42000299, SAB4200251) antibodies were purchased from Sigma Aldrich (St. Louis, USA). The C-terminal antibody was labeled with Alexa fluor 488 following the instructions of the manufacturer using an antibody labeling kit (A10468) from Life Technologies GmbH (Darmstadt, Germany). The labeled antibody was filtered and stored in PBS using Vivacon 500 30000MWCO filters from Sartorius (Göttingen, Germany). Recombinant human Contactin-2 protein (10457-H08H) was purchased from Sino Biological Inc. (Beijing, China). Glass coverslips (BBAD02400600#A) were purchased from Thermo Fisher Scientific Gerhard Menzel B.V. & Co. KG (Braunschweig, Germany). The FCS set-up was calibrated with Alexa dextran 488 (Cat.D22910, Thermo Fisher Scientific, Carlsbad, USA) and fluorescein (Cat.F6377, Sigma Aldrich, St. Louis, USA).

2.2. FCS set-up

In brief, a laser (Cobolt Dual Calypso, 488 and 532 nm) was used for sample excitation. The 532 nm line was blocked and the beam diameter was expanded using a telescope which included a 50 µm pinhole for optical lowpass filtering thereby giving, in a good approximation, a Gaussian beam profile. The expanded beam was then guided through a dichroic beam splitter into the back focal plane of the objective of a Zeiss Axiovert 35 microscope (Fig. 1). For all experiments carried out herein, a Zeiss C-Apochromat 40 × W(1.2 NA) was used as objective. Fluorescent light from the confocal volume (Fig. 1a, inset) was gathered by the same objective. A dichroic mirror, separating the emission from the excitation light, and a subsequent bandpass filter guaranteed that only the emission from alexa-488 and no excitation light (488 nm) could pass. Photon detection was done by a single photon counting module optimized for detection in the green part of the spectrum (Laser Components, Count-10B-FC). Thus, individual photons were detected and assigned a time-stamp in a quTAU device (quTOOLS GmbH) (Fig. 1b). The time stamps were then further processed in a custom python software to obtain autocorrelation curves as described in Section 2.5.

A standard curve was generated using a full length recombinant human contactin-2 protein (135 kDa) as antigen. 10 µL of each protein sample (for final protein concentrations between 0.163 and 41.7 ng/ml in total volume of 60 µL of reaction mix) was incubated overnight at room temperature (RT) with 10 µL of the N-terminal antibody (150 kDa) under gentle shaking. The final concentration of the antibody was 300 nM. Then 10 µL of the C-terminal antibody labeled with Alexa-488 (150 kDa) was added at a final concentration of 10 nM. The mix was kept at 37 °C for 1 h on gentle shaking. Then 30 µL of PBS was added giving a total volume of 60 µL. Patient CSF samples were also measured in the same manner using 10 µL of undiluted CSF replacing the recombinant human contactin-2 protein. 10 µL of PBS was used as a negative control to replace recombinant human contactin-2 protein or