Following Abeta fibrillization/aggregation in real-time using a FLUOstar® Omega microplate reader

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- Long-term shaking capability of the microplate reader used to monitor Aβ aggregation
- Lag times derived from signal curves proved to be a useful measure for the fibrillation process

Introduction

Aggregation of the amyloid-β (Aβ) peptide is a fundamental hallmark for Alzheimer’s disease. The formation of extracellular senile plaques will lead to synaptic and neuronal damages in clinical demented patients. The aggregation process of Aβ peptide is seen as seed driven. These seeds consist of small stable aggregates of Aβ. It is thought that these aggregates are already present in early stages of Alzheimer’s even before a patient experiences any symptoms. If this is true, determination of these early aggregates (aggregation seeds) would be an excellent diagnostic tool.

Here we present a cell-free assay (FRANK-Assay = Fibrilization of recombinant Aβ nucleation kinetics) that allows determination of the amount of aggregation seeds from brain tissue homogenates. The assay is run over 2-3 days using the FLUOstar® Omega microplate reader from BMG LABTECH.

Materials & Methods

- FLUOstar Omega, BMG LABTECH, Germany
- black, clear bottom 96-well plates, Greiner
- Sealing films, non-sterile, Excel Scientific

Donor brain tissue and extract preparation

APP23 transgenic [tg] mice were used as seed donors for all studies. This transgenic mouse model is characterized by an overexpression of mutant human APP. Transgenic APP (amyloid precursor protein) is subject to proteolytic cleavage and gives rise to β-amyloid peptide which in turn is aggregation prone and results in amyloid plaques consisting of β-amyloid deposits.

Donor brain tissues were obtained from APP23 tg mice as well as from non tg wild type (WT) mice. After removal the brain was divided into hemispheres. One hemisphere was immediately fresh-frozen (but not fixed) in dry ice while the other hemisphere was immersion-fixed in formaldehyde solution. After finishing the fixation process this hemisphere was frozen on dry ice and stored at -80°C until use (Fig. 2).

Assay Principle

The assay uses Thioflavin T to follow the amyloid formation (Fig. 1). Thioflavin T is a benzothiazole salt that is known to show increased fluorescence when bound to beta sheet-rich structures, such as in amyloid fibrils of Aβ.

![Fig. 1: Fibrillation process followed over time.](image)

Before aggregation or fibrillation can start a critical amount of initial aggregation seeds need to be present or spontaneously formed. This is a thermodynamically unfavoured process even in the presence of excess monomeric Aβ, kinetically slow and results in a delay in time before measurable aggregation starts. Once enough seeds have formed a massive and steep increase of ThT fluorescence due to incorporation into newly formed fibrils.

![Fig. 2: Method to obtain comparable fixed and fresh-frozen brain samples from either APP23 mice or wild type mice.](image)

Prior to use fixed and fresh-frozen tissues were homogenized, centrifuged and the supernatant aliquoted. For all experiments, a 10 % (w/v) extract was used.

Thioflavin T aggregation assay

1 μl of brain extract, protease inhibitor cocktail (Complete, Roche), 20 μM Thioflavin T, 25 μM Aβ1-40 were incubated in aggregation buffer (50 mM phosphate and 150 mM NaCl) at 37 °C. Each brain homogenate was present in 8 replicate wells. The fluorescence increase was

Keywords: aggregation, Alzheimer’s, bottom reading, fibril formation, seeding activity, Thioflavin T
Results & Discussion

A typical result for signal curves over time is shown in figure 3:

![Signal curves for samples containing either fixed or fresh-frozen wild type or APP23 brain homogenates. Error bars represent deviation of replicate wells within one plate from mean.](image)

All signal curves show a clear increase in fluorescence after a certain time. This increase illustrates the incorporation of Thioflavin T into the newly formed Aβ fibrils. After some time a plateau is reached that is considered as endpoint of amyloid formation and Thioflavin T incorporation process. The time until the signal starts to increase is the lag time. The MARS data analysis software offers the possibility to create 4-parameter fits of the signal curves from which the lag times are calculated (lag times correspond to the EC20 value of the fit). Initial fibril seeds are formed until the lag time is reached. Considering this, the lag time can be used as a measure to compare different brain homogenates (Fig. 4).

![Lag times created from signal curves shown in figure 3. Fixed and fresh frozen tg and WT mice are compared. As a control thioflavin T only (ThT) was measured on the same microplate (n=1). Error bars refer to 3 biological replicates.](image)

From figure 4 it can be followed that the lag times of the wild type are bigger compared to the lag times obtained for the tg mice. Further a difference can be seen between fixed and fresh-frozen APP23 samples. As expected the fresh samples induce Aβ deposition much faster. Nonetheless, the fixed APP23 samples show compared to the WT a significantly lower lag time indicating that fixation in formaldehyde is not sufficient to prevent Aβ aggregation.

Conclusion

With the help of the FLUOstar Omega microplate reader it is possible to prove that the in vitro assay is reliable to detect seeding activity in brain samples. In addition it allows quantitative comparison of seeding activity which with only little effort can be statistically validated. The method is also open for bigger sample cohorts which would easily burst the limited capacity of the in vivo approach. Since data can be obtained much easier (no animal surgery and animal permission needed) and earlier (2-3 days vs. 4-6 months) compared to the in vivo assay it is meant to replace the latter for qualitative and quantitative seed determination measurements in the future.