Tau Duplex: A novel assay to detect phosphorylated and total tau protein in neuronal cells using the Alpha technology.

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1 Abstract

Tau continues to be one of the most intensively studied neuronal proteins, and a clear consensus has emerged over the years on the pathological role of aberrant tau in many neurodegenerative conditions, including Alzheimer's disease. There is still much to be learned regarding the mechanisms by which faulty tau can potentiate neurodegeneration. For example, key questions remain as to which phosphorylation sites are critical to pathogenesis. Consequently, improved tools to rapidly and accurately measure endogenous cellular tau levels would be of significant value in this research space.

Here, we introduce a unique cell-based assay where detection of a given phosphorylated tau species (p-tau) can be performed concurrently with detection of total tau in the same sample using a homogenous bead-based technology. Employing a combination of AlphaScreen® and AlphaLISA® Acceptor beads conjugated to various Anti-tau antibodies, we could achieve detection of selected p-tau species concurrently with quantitation of total tau. A tau-specific antibody was conjugated to AlphaLISA Acceptor beads, which was tested in combination with three different p-tau antibodies targeting Thr^{181}, Thr^{205} and Ser^{202} conjugated to AlphaScreen Acceptor beads. A second non-overlapping tau antibody was biotinylated and captured via streptavidin coated Donor beads. Specific AlphaLISA and AlphaScreen signals, generated at 615 and 555 nm respectively, were representative of p-tau and total tau in the samples. Sensitive detection was achieved using human cortical neurons in a disease-relevant model for Alzheimer's disease. Moreover, the assay detected subtle variations in the relative level of each species when various treatments were applied. Using a full-length recombinant tau441, the assay could detect low picogram/ml levels of the protein and the dynamic range spanned more than four log units.

The simplicity of this two-step homogenous assay is a definitive advantage when, for example, conducting siRNA screening campaigns to identify inhibitors of tau phosphorylation. Our results demonstrate the power and versatility of the Alpha technology for studying endogenous cellular targets in a multiplexed mode. The Tau Duplex Assay will be a valuable tool in drug discovery programs for Alzheimer's disease and other tauopathies.

2 Introduction

The oligomerization of the microtubule-associated protein tau into paired helical filaments (PHFs), which give rise to neurofibrillary tangles (NFTs), is one of the defining pathological features of Alzheimer's disease. The molecular mechanism leading to tau oligomerization is thought to involve tau hyperphosphorylation, altered tau conformation and subsequent assembly of tau into PHFs and NFTs. Although the precise toxic tau species remain to be clarified, efforts to block tau phosphorylation and tau aggregation are of great interest as there is no current treatment that can slow the progression of Alzheimer's disease. Recent evidence have challenged the idea that only fibrillar forms of tau (NFT) are toxic to neurons and indicate that other tau species, including soluble forms, might become interesting therapeutic targets.

We have previously reported the combined use of AlphaScreen and AlphaLISA beads to simultaneously monitor substrate phosphorylation and enzyme interaction *in vitro* (Arcand *et al.*, Biochemistry 2010, 49, 3213-3215). Here, we propose a straightforward adaptation of this duplex platform to a cell-based assay where soluble forms of p-tau & total tau can be monitored in a native context.

One of the challenges typically associated with studying cellular proteins lies in the non-homogenous nature of the methods used (ex. Western blot, ELISA); which often preclude any HTS campaigns. The Alpha technology has demonstrated in recent years the ability to successfully address the ever-increasing needs of the drug discovery community for HTS-compatible assays.

3 Materials & methods

- AlphaLISA Acceptor beads (PerkinElmer #6772001) coupled to ThermoFisher Ab #MN1000
- AlphaScreen Acceptor beads (PerkinElmer #6762003) coupled to Ab from: ThermoFisher #MN1020, Abcam #75679, GenScript #A00405
- Streptavidin coated Donor Beads (PerkinElmer #6760002S)
- Biotinylated antibody from Covance #SIG-39416
- AlphaLISA immunoassay buffer (PerkinElmer #AL000C) and lysis buffer (PerkinElmer #AL003C)
- Optiplate-384, white opaque 384 wells microplate (PerkinElmer #6007290)
- EnVision® Multilabel plate reader with Alpha capability (PerkinElmer)
- 555 and 615 nm emission filters (PerkinElmer #2100-5670 & 2100-5090)
- BSA-Biotin Acceptor beads (AlphaLISA & AlphaScreen) (PerkinElmer #AL900D, #6760627D)
- Bulk cell lysates (cortical neurons & astrocytes)

Detection in cortical neuron cell lysates

In a 384-well plate, add:

- 10 µl Ab conjugated AlphaLISA & AlphaScreen Acceptor Beads mix [10 & 20 µg/ml final]
- 5 µl Biotinylated Ab [1 nM final]
- 5 µl lysates from neuronal cells [prepared in AlphaLISA lysis buffer]

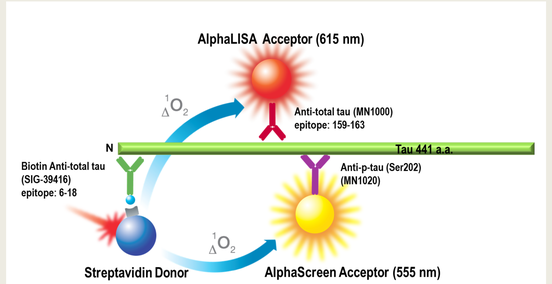
Incubate for 120 minutes at 23ºC

- 5 µl streptavidin-Donor beads [40 µg/ml final]

Incubate for 30 minutes at 23ºC

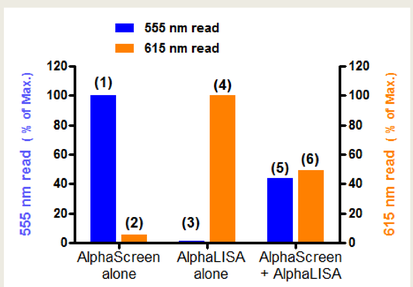
- Read on EnVision®

4 Assay principle



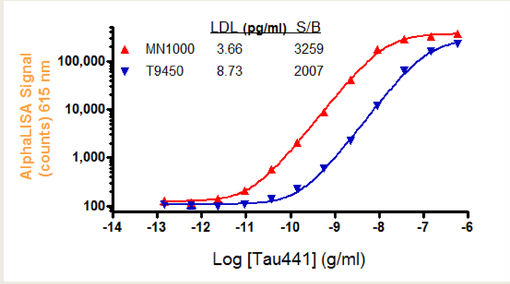
A representation of the Tau Duplex assay. First, a 680 nm laser will excite the Donor beads and singlet oxygen molecules will be released. When Acceptor beads are in close proximity (≤200 nm) a chemiluminescent event occurs resulting in emission of light by the Acceptor beads which is in turn recorded by the instrument. In the Tau Duplex assay, the p-tau detection is first performed (555 nm reading) followed by a second excitation and reading of the plate for total tau signal (615 nm reading). Here the assay set-up involving detection of p-tau(Ser202) is illustrated. Other Ab than those shown in this figure were also successful at detecting total and p-tau. The platform can be used to study over 60 potential phosphorylation sites for tau.

5 Protocol validation



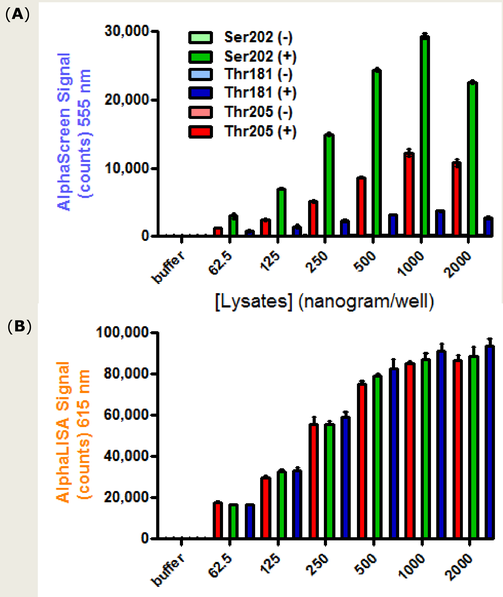
The Tau Duplex assay is made possible by using two custom emission filters for recording of Alpha signals at 555 and 615 nm (AlphaScreen & AlphaLISA beads respectively). BSA-biotin Acceptor beads are used for this protocol validation procedure. Narrow bandwith from the two filters results in minimal signal cross-interference between the two detection channels, as shown with bar graphs (2) and (3). The instrument protocol for this assay can be implemented on any filter-based Envision reader without the need for hardware modification or software upgrade.

6 Recombinant tau441 quantification



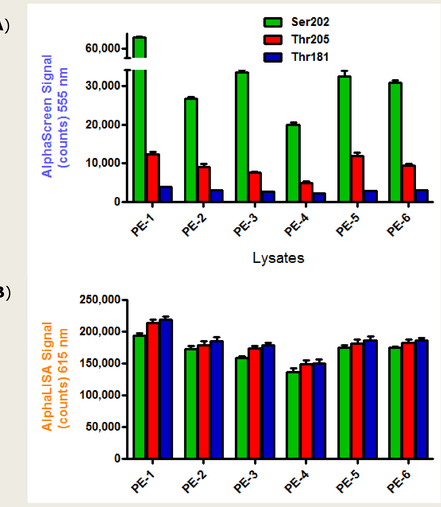
Dose-response curves were performed for recombinant tau441 protein using two different Ab coupled on the AlphaLISA Acceptor beads (total tau detection). The Bio-Ab SIG-39416 was used for both detections. The MN1000 Ab has an epitope located at a.a.159-163 while the T9450 Ab epitope is at a.a. 404-441. A detection range superior to four Logs was obtained for both Ab along with high S/B (max. over min. signal). Lower detection limits (LDL) were defined as 3xSD over background signal. SD errors are shown in the graph (triplicates determination). MN1000 Ab was selected in the final assay configuration (see section 4) in order to allow measurement of shorter tau fragments originating from proteolytic degradation.

7 Assay optimization with lysates



Lysates titration in the Duplex assay for three p-tau species (**A**) and associated Total tau (**B**). The negative control lysate (-) consisted of astrocytes while (+) was cortical neurons lysate. As expected, no p-tau or Total tau was detected in astrocytes. In (**B**) total tau detection was not influenced by the concomitant p-tau detection as all three assay set-up gave similar signal. The optimal amount of lysate to use per well should be located in the linear portion of the signal for both detections (1000 ng/well or less). No treatment were applied to cortical cells therefore p-tau levels found here should represent a basal state.

8 Detection in modulated lysates



Duplex detection for three p-tau species (**A**) and associated Total tau (**B**) in six modulated lysates originating from cortical neurons. All compounds were added one day prior to harvest.

PE-1: 200 nM Okadaic acid (marine toxin with phosphatase inhibition activity)
PE-2: Growth medium alone
PE-3: 1 µM staurosporine (prototypical potent ATP-competitive kinase inhibitor)
PE-4: 10 µM Hesperadin (an inhibitor of human Aurora B kinase)
PE-5: 10 µM PF 431396 (dual FAK and PYK2 kinase inhibitor)
PE-6: DMSO at 0.1% final.

Overall signal obtained with Thr181 was low and no significant modulation was detected, probably indicating that this species was not abundant in our samples. In (**B**) total tau level was not highly modulated following treatments. For each of the three assays, total tau signals were not influenced by the associated p-tau signals, as witnessed by similar counts for a given lysate.

Although results shown here were generated with bulk cell lysates, the assay could be implemented in an All-in-one-well format where cell culture and detection occur in the same well. This in turn allows for interrogation of multiple compounds in an HTS mode.

9 Summary

- In this study, we demonstrated the successful use of the Alpha technology to perform dual detection of a p-tau species along with total tau in lysates of cortical neurons.
- Excellent assay sensitivity was confirmed with a recombinant tau441 protein: as little as 4 pg/ml of purified protein could be detected.
- The specificity and sensitivity of this dual detection assay were demonstrated in a disease-relevant system using only nanogram amount of total cellular proteins.
- Results demonstrated that the assay could detect modulation in the levels of certain p-tau species following cellular treatments.
- This simple and homogenous two-step assay can be carried out in less than 3h and allows a versatile "plug & play" approach for dual interrogation of known p-tau species and Total tau.
- This assay could be helpful in finding promising therapeutics for the treatment of Alzheimer's disease and other tauopathies.

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