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Short Report

# Internalization of tau antibody and pathological tau protein detected with a flow cytometry multiplexing approach

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**Abstract**

**Introduction:** Tau immunotherapy has emerged as a promising approach to clear tau aggregates from the brain. Our previous findings suggest that tau antibodies may act outside and within neurons to promote such clearance.

**Methods:** We have developed an approach using flow cytometry, a human neuroblastoma cell model overexpressing tau with the P301L mutation, and paired helical filament (PHF)–enriched pathologic tau to effectively screen uptake and retention of tau antibodies in conjunction with PHF.

**Results:** The flow cytometry approach correlates well with Western blot analysis to detect internalized antibodies in naïve and transfected SH-SY5Y cells ($r^2$ = 0.958, and $r^2$ = 0.968, $P$ = .021 and $P$ = .016, respectively). In transfected cells, more antibodies are taken up/retained as pathologic tau load increases, both under co-treated conditions and when the cells are pretreated with PHF before antibody administration ($r^2$ = 0.999 and $r^2$ = 0.999, $P$ = .013 and $P$ = .011, respectively).

**Discussion:** This approach allows rapid in vitro screening of antibody uptake and retention in conjunction with pathologic tau protein before more detailed studies in animals or other more complex model systems.

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## 1. Introduction

Antibody immunotherapies are a major focus of treatment approaches for Alzheimer's disease and other neurodegenerative diseases [1–3]. Our laboratory has pioneered targeting pathologic tau proteins for clearance with active and passive immunotherapies for Alzheimer's disease and other tauopathies [4,5], which have been confirmed and extended by multiple groups over the last several years [6–33]. With numerous potential tau epitopes to target, as well as various antibody isotypes and effector mechanisms to consider, it has become a daunting task to prescreen potential candidate antibodies to test in animal models.

We have developed an approach using flow cytometry and a human neuroblastoma cell model to detect internalization of tau antibodies and paired helical filament (PHF)–enriched pathologic tau protein. Our findings show that this approach correlates very well with Western blot detection of tau antibody uptake and confirms such uptake observed in different model systems [4,13,14,21,28,34]. This rapid and highly quantitative approach is an effective means to screen antibody internalization with or without pathologic tau, which can be detected in a similar manner. Tau antibodies can in

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theory lead to tau clearance extracellularly and/or intracellularly. To target both pools of the pathologic protein simultaneously is likely more efficacious. Hence, it is important to be able to quickly and quantitatively screen antibody's ability to enter cells, which varies and likely depends primarily on its charge [1].

Overall, such a multiplexing approach to detect antibodies and pathologic targets in cells has great potential to be used with conventional techniques to better understand the mechanism of action of prospective immunotherapies for neurodegenerative diseases.

## 2. Methods

### 2.1. PHF protein preparation

Human brain slices were homogenized and prepared in buffer (pH 6.5; 0.75-M NaCl, 1-mM EGTA, 0.5-mM $MgSO_4$, and 100-mM 2-(*N*-morpholino) ethanesulfonic acid) along with protease inhibitor cocktail (Roche) and centrifuged at 11,000 $\times$ *g* for 20 min at 4°C. Supernatant was subsequently centrifuged in an ultracentrifuge at 100,000 $\times$ *g* for 60 min at 4°C. The pellet was then resuspended in paired helical filament (PHF) extraction buffer containing sucrose (10-mM Tris; 10% sucrose; 0.85-M NaCl; and 1-mM EGTA, pH 7.4) and spun at 15,000 $\times$ *g* for 20 min at 4°C. The pellet was re-extracted in the sucrose buffer at the same low-speed centrifugation. The supernatants from both sucrose extractions were pooled and subjected to 1% sarkosyl solubilization by stirring at ambient temperature and then centrifuged at 100,000 $\times$ *g* for 60 min at 4°C in a Beckman 60 Ti rotor (Beckman Coulter; Fullerton, CA). The resulting pellet was re-suspended in 50-mM Tris-HCl (pH 7.4), using 0.5 μL of buffer for each milligram of initial weight of brain sample protein. It was then dialyzed in PBS overnight at 4°C, using a 3500 MW cassette, and designated the PHF enriched preparation.

### 2.2. Tau antibody and fluorescence labeling

In this study, we used the tau antibody, 6B2G12 (6B2), which detects Ser396/404 tau epitope and has previously been characterized by our laboratory [14,35]. The antibody and the PHF-enriched brain fraction were tagged with Alexa Fluor 488 and Alexa Fluor 647, respectively, using protein labeling kits, as detailed in the manufacturer's instructions (Invitrogen).

### 2.3. Cell culture

SH-SY5Y human neuroblastoma cells obtained from ATCC were transfected using Lipofectamine 2000 (Invitrogen) and pcDNA3.1(+) P301L hTau 4R0N with gentomycin (G418) selection. Naïve and transfected cells were cultured in complete media (Dulbecco's Modified Eagle Medium [DMEM] with glutamax (Invitrogen), 10% heat inactivated fetal bovine serum (FBS), 10,000-units/mL penicillin, and 10,000-μg/mL streptomycin). Transfected cells media also contained 200-μg/mL gentomycin. Cells were plated at 4 $\times$ $10^2$ cells/$mm^2$, allowed to recover for 3 days before each experiment and grown in an incubator with 5% $CO_2$ at 37°C. For dose response experiments, naïve and transfected cells were treated with increasing doses of 6B2 antibody (0–60 μg/mL) for 24 hours. For cotreatments and pretreatments, transfected cells were either pre-treated with increasing levels of tagged PHF (0.1–10 μg/mL) for 24 hours, then washed several times to remove remaining extracellular PHF, and subsequently incubated with tagged 6B2 tau antibody (5 μg/mL) for another 24 hours or co-treated with both for 24 hours.

### 2.4. Flow cytometry

All cells analyzed with flow cytometry were incubated with tagged 6B2 antibody and/or tagged PHF. Before measurements, cells were washed with PBS, trypsinized, resuspended in ice-cold complete media and then spun down and pelleted at 200 $\times$ g for 5 minutes. Supernatant was discarded, and pelleted cells were then resuspended in ice-cold PBS and put through a 0.2-micron filter (PARTEC). To quench any surface bound fluoresence, trypan blue was added to each sample at a final concentration of 0.02% (w/v; Sigma Aldrich), and the sample was placed on ice. Samples were analyzed using flow cytometery (LSRII, Becton Dickinson) to a count of 10,000 cells per sample and were gated for viable and singlet cell populations. Cells were further analyzed using FlowJo for either Alexa Fluor 488 and/or 647 positive cells. Median fluoresence intensity (MFI) values were obtained for both fluorescent signals.

### 2.5. Western blot

All cells analyzed using western blot were incubated with nontagged 6B2 antibodies and/or PHF. All samples were homogenized in RIPA buffer and prepared as described previously [14]. Samples were boiled and loaded on 10% SDS-PAGE gels, electrophoresed, and then transferred to nitrocellulose membranes, which were blocked in 5% milk with 0.1% TBS-T. Blots were then probed for total tau (Dako polyclonal antibody), phospho-tau (PHF-1 monoclonal antibody), or GAPDH (Abcam polyclonal antibody) primary antibodies overnight at 4°C, washed and then probed with anti-horseradish peroxidase (HRP) conjugated rabbit or mouse secondary antibody (Pierce) for 1 hour. PHF characterization was conducted in the same manner with CP27 monoclonal antibody. For antibody uptake detection, membranes were incubated with an anti-mouse IgG1 HRP-conjugated secondary antibody with specificity against the heavy chain (Bethyl Laboratories), and signal was detected with an ECL substrate (Thermo Scientific). Images of immunoreactive bands were then acquired and quantified using the Fuji LAS-4000 imaging system.

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