# EVALUATION OF IN VITRO / IN VIVO TAU PHOSPHORYLATION USING THE GYROS IMMUNOASSAY PLATFORM

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## INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that affects millions worldwide. It is the leading cause of dementia, and is most commonly diagnosed in individuals over the age of 65 [1]. Although the initial cause and pathogenesis are not well understood, research has shown that classic hallmarks found in the brains of AD individuals are cholinergic neuron degeneration, extracellular beta-amyloid plaques, and intracellular neurofibrillary tangles (NFT's) composed of hyperphosphorylated Tau protein[1]. Normally, Tau stabilizes microtubules and plays an important role in intracellular transport, but in AD these processes become disrupted[1,2].

The hyperphosphorylated Tau state can be simulated *in vitro* by treating embryonic (E18) rat cortical neuronal cultures with okadaic acid, a phosphatase inhibitor. This primary culture system has been used previously to investigate various Tau phosphorylation pathways[3,4,5,6]. In this study, we used a novel immunoassay system, Gyros, to evaluate the effects of okadaic acid and various kinase inhibitors (AKT inhibitor; MEK inhibitor; staurosporine; MARK inhibitors: PF-05087039, PF-05241320, PF-05120635; and GSK3β inhibitor PF-04802367) on Tau/phospho-Tau.

To investigate Tau phosphorylation *in vivo*, the Tg4510 transgenic mouse model was used. This model carries the P301L mutant human Tau gene (linked to hereditary tauopathies), where expression of the Tau variant leads to development of NFTs, neuronal death, and memory impairment in the mice, which is consistent with the pathology observed in human AD patients[7,8,9,10]. In this study, we used Gyros and traditional Western Blot to evaluate the Tau/phospho-Tau levels in the brain homogenates of rTg4510 mice (wild type -/- male and female, as well as bigenic +/+ male and female; n=10 for each category) at various time points: 3.5, 6.5, and 8 months.

We have used Gyros to develop and optimize several rat and human Tau/phospho-Tau assays, including total Tau, AT270, AT8, AT100, AT180, pSer262, and PHF13. In our studies, we first aimed to establish Gyros as a technology platform to evaluate drug selectivity. We then compared analysis of the rTg4510 brain homogenates using Gyros and Western Blot in order to validate the rTg4510 mouse model on Gyros. Gyros allows for high-throughput analysis of low-volume *in vitro / in vivo* samples with a high level of sensitivity which is comparable to other immunoassay systems, critical parameters for drug discovery.

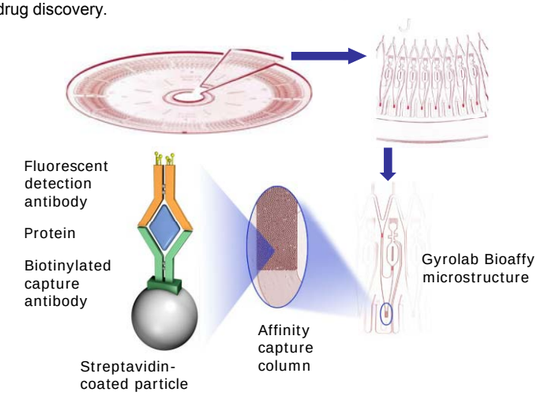



Figure 1: Details of Gyros Technology

The Gyros procedure involves a BioAffy 1000 nL compact disk (CD), which is divided into 12 segments, each with 8 microstructures. At the end of each microstructure is a 15 nL affinity capture column that contains streptavidin-coated particles, which bind a biotinylated capture antibody (specific to protein of interest). The antigen in the sample binds to the capture antibody, and a fluorescently-labeled detection antibody (specific to protein of interest) binds to the antigen. The amount of fluorescence in each affinity column is measured to evaluate the protein of interest.

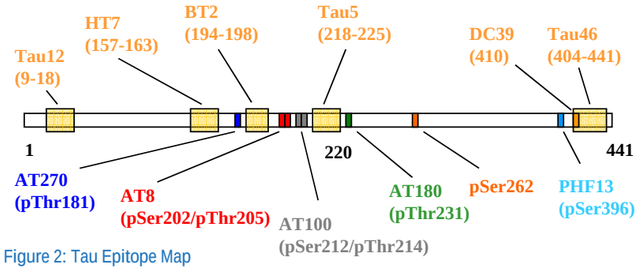



Figure 2: Tau Epitope Map

Tau is a protein composed of 441 amino acids, and depicted are the epitopes for the various assays used in this study. Typically, phospho-Tau antibodies are used for capture, and general Tau antibodies are used for detection.

## MATERIALS AND METHODS

**Rat Cortical Neuron Preparation and Treatment**

1. Rat cortical neurons were harvested from E18 embryos and plated in a 96 well plate, 5000 cells per well. The neurons were cultured for 8 days in Neurobasal Media (Invitrogen) supplemented with B27 (Invitrogen) and Glutamax (Invitrogen), 200 µL volume per well, at 37ºC, 5% $CO_2$.
2. At 8 days *in vitro*, compound solutions were prepared at 2X final concentration (**FC**), 100 µL per well: AKT inhibitor (CalBioChem,10 µM FC); MEK inhibitor (CalBioChem, 10 µM FC); staurosporine (Sigma, 1 µM FC); MARK inhibitors PF-05087039, PF-05241320, and PF-05120635 (all 20 µM FC); GSK3β inhibitor PF-04802367 (20 µM FC).
3. 100 µl of media was removed from each well of the 96 well plate, and 100 µL of the 2X compound solution was added per respective well, followed with 1 hour incubation at 37ºC, 5% $CO_2$.
4. Okadaic acid (OA, Alexis Biochemicals) was then prepared at 5X final concentration (1000 nM OA FC), and DMSO (vehicle, 0 nM OA FC) was prepared for basal levels. 50 µL of either OA or DMSO were added to each respective well of the 96 well plate (250 µL total volume in well), followed with 1 hour incubation at 37ºC, 5% $CO_2$.
5. Subsequently, all media was aspirated off quickly, and Phospho-Safe Lysis Buffer (Novagen) + Protease Inhibitor Cocktail Set III (CalBioChem, 1:1000 in Phospho-Safe) was added 30 µL per well. The plate was tapped gently and then allowed to incubate at room temperature for 5 minutes. The plate was stored at -80ºC till time of assay.

**rTg4510 Mouse Brain Homogenization**

Brains of 120 mice (wild type -/- male and female; bigenic +/+ male and female; n=10 for each category at various time points: 3.5, 6.5, and 8 months) were homogenized using a Polytron or motor-driven glass/Teflon homogenizer. They were then combined with 2mL NP-40 homogenizing buffer, centrifuged at 10,000 rpm for 20 min, supernatant was collected and stored at -80ºC.

**Gyros**

1. All cell lysate samples were thawed prior to assay. All brain homogenate samples were thawed and centrifuged at 12,000 rpm for 20 minutes at 4ºC prior to assay.
2. All cell lysate samples were diluted 1:2 using Gyros Rexxip CCS buffer. All brain homogenate samples were initially diluted 1:50 using NP-40 homogenization buffer. Then, a 1:2 dilution with Gyros Rexxip CCS buffer brought the final dilution of the samples to 1:100.
3. The biotinylated capture antibodies were diluted with PBS-T to a final concentration of 100µg/mL; BT2 (Pierce) for total Tau, AT270 (Pierce), AT8 (Pierce), AT100 (Pierce), AT180 (Pierce), pSer262 (CalBioChem) , Tau46 (Sigma) for PHF13.
4. The fluorescently-labeled detection antibodies were centrifuged at 4ºC at 12,000 rpm for 10 minutes. They were diluted to their respective concentrations (12.5-50 nM) using the Gyros proprietary buffer Rexxip F; antibodies: Tau12 (Covance) for total Tau, AT100; Tau46 (Sigma) for AT270, AT8, AT180, pSer262; PHF13 (Cell Signaling).
5. The samples, antibodies, and wash solution were loaded into 96-well plates according to the experiment lists generated by the Gyros software. The plates were sealed and spun at 4ºC at 3,500 rpm for 5 minutes to eliminate air bubbles.
6. 96-well plates / BioAffy 1000 nL CDs were loaded into the Gyros. Full automation was initiated. See Figure 1 for details. Data is shown as percent control DMSO (vehicle) OR signal:background.

**Western Blot**

1. Protein concentrations were determined using the Pierce BCA kit (Pierce). Samples were prepared under reducing conditions to contain 1.5 µg total protein per µL in E-PAGE loading buffer and heated for 10 minutes at 70ºC.
2. 15 µg of each sample was separated on E-PAGE 8% gels and transferred to nitrocellulose membranes using the Invitrogen i-blot system (gel and blotting systems from Invitrogen). Blots were blocked in Rockland Near Infrared Blocking Buffer for 1 hour at room temperature.
3. Primary antibodies were diluted accordingly in blocking buffer and incubated overnight 1:2000 at room temperature; antibodies: Dako for total Tau, AT270 (Pierce), AT100 (Pierce), AT8 (Pierce), AT180 (Pierce), pSer262 (Calbiochem), PHF13 (Upstate), loading control Tubulin (Covance).
4. After primary incubation, blots were washed 3 x 5 minutes in PBS with 0.1% Tween-20.
5. Species appropriate secondary antibodies were diluted 1:10,000 in blocking buffer (Molecular Probes Alexa Fluor 680 or 800 Invitrogen Corporation) and incubated for 1 hour at room temperature.
6. After another series of washes, blots were imaged on Li-Cor Odyssey infrared imager / quantified using Odyssey software (LI-COR Biotechnology). Integrated intensities of each band were obtained. Data is shown as response units.

## RESULTS

Figure 3: Gyros analysis of *IN VITRO* Tau/phospho-Tau expression in lysates from rat cortical neurons treated with commercially available kinase inhibitors +/- okadaic acid

(A) 1000 nM OA treatment induced significant phosphorylation over basal levels at AT8 and pSer262 sites. (B) At the AT8 site, phosphorylation (post-1000 nM OA treatment) was not reduced with AKT, MEK, or staurosporine kinase inhibitors . (C) At the pSer262 site, phosphorylation (post-1000 nM OA treatment) was reduced with staurosporine.

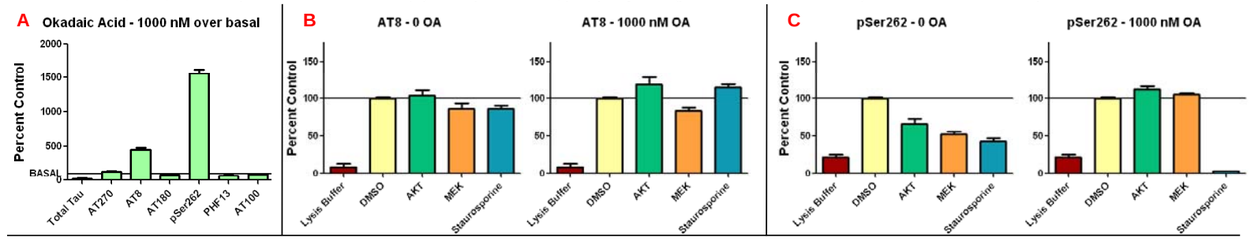



Figure 4: Gyros analysis of *IN VITRO* Tau/phospho-Tau expression in lysates from rat cortical neurons treated with Pfizer developed kinase inhibitors +/- okadaic acid

(A) 1000 nM OA treatment induced significant phosphorylation over basal levels at AT8 and pSer262 sites. (B) At the AT8 site, phosphorylation (post-1000 nM OA treatment) was reduced with MARK kinase inhibitors PF-05241320 and PF-05120635, but not with GSK3β kinase inhibitor PF-04802367. (C) At the pSer262 site, phosphorylation (post-1000 nM OA treatment) was reduced with MARK inhibitors PF-05241320, PF-05120635, and PF-05087039, but was increased with GSK3β inhibitor PF-04802367.

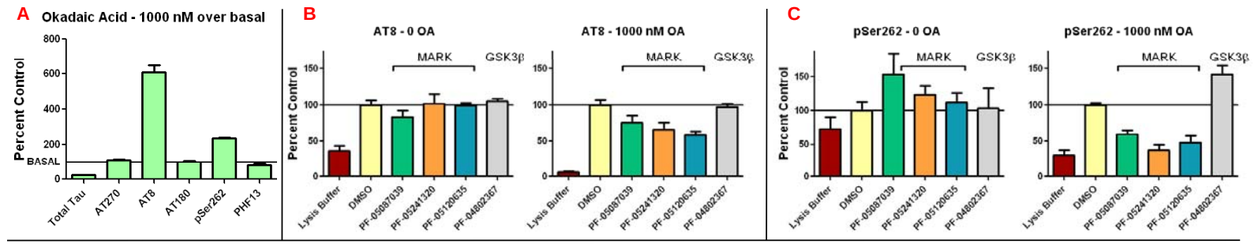
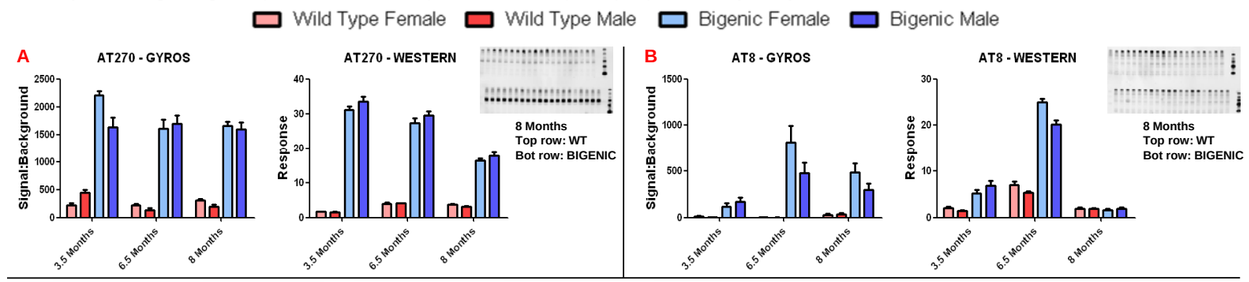



Figure 5: Gyros analysis of *IN VIVO* Tau/phospho-Tau expression in rTg4510 brain homogenates correlates with Western Blot

Tau/phospho-Tau assays on Gyros and Western Blot were performed on samples (WT female, WT male, bigenic female, bigenic male; n=10 each) at various ages (3.5, 6.5, and 8 months) for cross-platform comparison. (A) Gyros and Western AT270 assays showed consistently high bigenic expression at all time points compared to WT. (B) Gyros and Western AT8 assays showed highest bigenic expression at 6.5 months. (DATA NOT SHOWN) Other phospho-Tau assays on Gyros showed correlation with Western.




## CONCLUSIONS

**Gyros technology can be used to evaluate drug selectivity**

- 1000 nM OA treatment induced significant phosphorylation at AT8 and pSer262 sites.
- Phospho-Tau was considerably reduced at AT8 with MARK inhibitors PF-05241320 and PF-05120635, while staurosporine, MARK inhibitors PF-05241320, PF-05120635, and PF-05087039 decreased phosphorylation at pSer262. The GSK3β inhibitor PF-04802367 did not decrease phosphorylation at the AT8 or pSer262 sites.

**Gyros technology can be used to validate transgenic animal models**

- Comparison of the rTg4510 brain homogenates using Gyros and Western Blot indicated bigenic responses were higher than wild type in all Tau/phospho-Tau assays across all time points.
- Result trends from both systems matched up closely with the AT270 and AT8 assays, as well as other phospho-Tau assays (DATA NOT SHOWN) . In some assays, Gyros offered better sensitivity.

**Gyros vs Western Blot**

- Ability to assay large number of samples in 1 experiment:
  Gyros 96 samples in one 1000 nL CD > Western ~50 samples in one blot
  NOTE: Gyros allows you to run 5 CDs in one run, allowing ~500 samples to be run
- Sample volume: Gyros 1000 nL required per column > Western
  NOTE: total sample volume loaded depends on # of assays / # of replicates to be run
- Reagent/sample prep time + experiment time: Gyros 3 hours for one 1000 nL CD > Western 2 days
- Assay development/optimization time: Gyros less than 1 week, given optimal antibody pair is found in screening
  Gyros allows several antibody pairs to be screened in one 1000 nL CD

***Gyros allows for high-throughput analysis of low-volume in vitro / in vivo samples with a high level of sensitivity which is comparable to other immunoassay systems, critical parameters for drug discovery.***

## NEXT STEPS

Perform Gyros Tau/phospho-Tau assays on cell lysates and rTg4510 brain homogenates once again, as well as CSF, this time using newly constructed Tau/phospho-Tau full-length standards to quantify protein levels in the pg/mL range.

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