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14. ABSTRACT

Purpose: Misfolding and aggregation of amyloid beta (Aß) and tau result in the hallmark pathological plaques and tangles associated with AD while aggregation of the protein alpha-synuclein (a-syn) forms Lewy Bodies, the hallmark feature of Parkinson's disease. While large fibrillar aggregates of each of these proteins are diagnostic features of these diseases, increasing evidence indicates that smaller soluble oligomeric species of these proteins are key neurotoxins involved in the onset and progression of these neurodegenerative diseases. Since Aß, tau and a-syn all also accumulate in the brain following traumatic brain injury (TBI), presence of selected toxic aggregate forms of these proteins can provide valuable early biomarkers for AD and other neurodegenerative diseases and identify specific neuronal damage in soldiers who have incurred traumatic brain injury (TBI). Our lab has developed unique technology that enables us to isolate reagents that bind specific morphologies of a target protein. We have developed various recombinant antibody fragments, or nanobodies, that selectively recognize three different oligomeric Aß species, two different oligomeric a-syn species, and one oligomeric tau species. This pool of six different morphology specific nanobody reagents represent a valuable tool to probe human CSF samples to identify biomarkers for early diagnosis of AD and to identify neuronal damage following TBI.

Scope: Our hypothesis is that characterizing levels of a target set of key toxic biomarkers in CSF will provide a tool to facilitate early diagnosis of AD and other neurodegenerative diseases, and to identify the level of neuronal injury following TBI. The biomarker set will include morphologically distinct toxic aggregate species of Aß, a-syn and tau. Small soluble aggregates of each of these proteins has been indicated as early neurotoxins in different neurodegenerative diseases. Our objective is to develop an assay that can be used to identify the set of CSF biomarkers that are diagnostic for AD and TBI patients. Soldiers suffering TBI who show reactivity to the particular biomarker set have suffered damage similar to that shown in AD brain and are therefore probably much more likely to suffer from AD. We will show that we can rapidly and accurately detect and quantify a selected set of Aß, a-syn or tau species in CSF samples.
Technical Objectives: To achieve our long term goal of identifying biomarkers for AD using our pool of morphology specific nanobodies, we have designed the following specific objectives:
1: Optimize ELISA for testing of multiple biomarkers in CSF using spiked samples.
2: Test CSF samples from TBI, cognitively normal (ND), and AD patients using a subset of morphology specific nanobodies.
Relevance: An early diagnostic indicator for AD, and particularly of increased susceptibility to AD following TBI, is critically important to identify which soldiers have neuronal damage consistent with AD so treatment can begin early and the effectiveness of the treatments can be monitored.

Major Findings: We have essentially finished development of the ELISA protocol for sensitive detection of specific toxic protein variants in human CSF samples. We have determined appropriate concentrations and conditions for immobilizing the primary capture nanobodies onto ELISA plates. We have determined effective secondary detection nanobody configurations which enable low femtomolar or better detection of target antigens. We express the nanobody on a phage particle which enables us to amplify the detection signal by several orders of magnitude. The phage is then biotinylated through carboxyl group chemistry. Bound phage can then be readily detected using a horse-radish peroxidase/aviden complex. For detection either a colorigenic or chemilluminescent substrate can be utilized. The chemilluminescence substrate provides an additional 2-3 order of magnitude improvement in detection signal. Therefore we have a sensitive assay which can detect very low concentrations of toxic compounds specifically associated with AD, TBI or other neurodegenerative diseases. In the next year we will use our nanobodies to characterize CSF samples from control, TBI and AD patients to demonstrate that we can detect the presence of specific toxic protein variants directly in human CSF samples. Successful demonstration in CSF samples can provide a means to detect specific brain damage immediately following TBI and can facilitate presymptomatic diagnosis of AD enabling treatment to begin before extensive neuronal damage has taken place.

15. SUBJECT TERMS beta-amyloid, tau, alpha-synuclein, oligomers, antibody reagents, morphology, Cerebral Spinal Fluid, ELISA					
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Introduction

Background: Misfolding and aggregation of amyloid beta (Aß) and tau result in the hallmark pathological plaques and tangles associated with AD while aggregation of the protein alpha-synuclein (a-syn) forms Lewy Bodies, the hallmark feature of Parkinson's disease. While large fibrillar aggregates of each of these proteins are diagnostic features of these diseases, increasing evidence indicates that smaller soluble oligomeric species of these proteins are key neurotoxins involved in the onset and progression of these neurodegenerative diseases. Since Aß, tau and a-syn all also accumulate in the brain following traumatic brain injury (TBI), presence of selected toxic aggregate forms of these proteins can provide valuable early biomarkers for AD and other neurodegenerative diseases and identify specific neuronal damage in soldiers who have incurred traumatic brain injury (TBI). Our lab has developed unique technology that enables us to isolate reagents that bind specific morphologies of a target protein. We have developed various recombinant antibody fragments, or nanobodies, that selectively recognize three different oligomeric Aß species, two different oligomeric a-syn species, and one oligomeric tau species. This pool of six different morphology specific nanobody reagents represent a valuable tool to probe human CSF samples to identify biomarkers for early diagnosis of AD and to identify neuronal damage following TBI.

Objective/Hypothesis Our hypothesis is that characterizing levels of a target set of key toxic biomarkers in CSF will provide a tool to facilitate early diagnosis of AD and other neurodegenerative diseases, and to identify the level of neuronal injury following TBI. The biomarker set will include morphologically distinct toxic aggregate species of Aß, a-syn and tau. Small soluble aggregates of each of these proteins has been indicated as early neurotoxins in different neurodegenerative diseases. The biomarkers can also be used to assess the effectiveness of a particular therapeutic strategy. Our objective is to develop an assay that can be used to identify the set of CSF biomarkers that are diagnostic for AD and TBI patients. Soldiers suffering TBI who show reactivity to the particular biomarker set have suffered damage similar to that shown in AD brain and are therefore probably much more likely to suffer from AD. We will show that we can rapidly and accurately detect and quantify a selected set of Aß, a-syn or tau species in CSF samples.

Technical Objectives: To achieve our long term goal of identifying biomarkers for AD using our pool of morphology specific nanobodies, we have designed the following specific objectives:

1: Optimize ELISA for testing of multiple biomarkers in CSF using spiked samples.

2: Test CSF samples from TBI, cognitively normal (ND), and AD patients using a subset of morphology specific nanobodies.

Study Design: Objective 1). Optimize ELISA for testing CSF samples. We will utilize a modified sandwich ELISA to detect target antigens in CSF where the capture antibody is one of the morphology specific nanobodies and the detection antibody is a phage displayed version of the capture nanobody. The phage display version increases the detection signal compared to a standard antibody. Using this protocol we have shown we can detect target oligomeric proteins in CSF samples using 1/50 dilutions. 2) Test CSF samples from TBI, cognitively normal (ND), and AD patients using a subset of morphology specific nanobodies. We will quantify levels of a subset of Aß, a-syn or tau aggregate species using the morphology specific nanobodies in combination with the ELISA developed in Aim 1.

Relevance: An early diagnostic indicator for AD, and particularly of increased susceptibility to AD following TBI, is critically important to identify which soldiers have neuronal damage consistent with AD so treatment can begin early and the effectiveness of the treatments can be monitored.

Body:

Progress towards Milestones

Milestone 1

Task 1. Vary Concentration of primary capture nanobody to determine concentration necessary to saturate surface.

The first step in our capture ELISA protocol is to coat the 96-well plates with our different scFvs (also referred to as nanobodies). The scFvs were first produced and then purified. Western blot analysis was then used to confirm the correct molecular weight of the different scFvs. Subsequently, the concentrations of these scFvs were determined using a BCA kit. Once the scFvs were made, we started testing different conditions that would enable saturation of the wells with lowest scFv concentration. Two different methods were used to determine the best concentration at which this saturation would occur. In the first method: we added various concentrations of the different scFvs to an ELISA plate and altered the incubation temperatures and time. The different incubation time and temperature combinations that were tested included 4^{o}C overnight, room temperature for one hour, 37^{o}C for one hour, 37^{o}C for two hours, room temperature for 3.5 hours and 37^{o}C for 3.5 hours. The wells were then washed using our wash protocol for the capture ELISA. Lastly, we determined amount of bound scFv using a BCA.

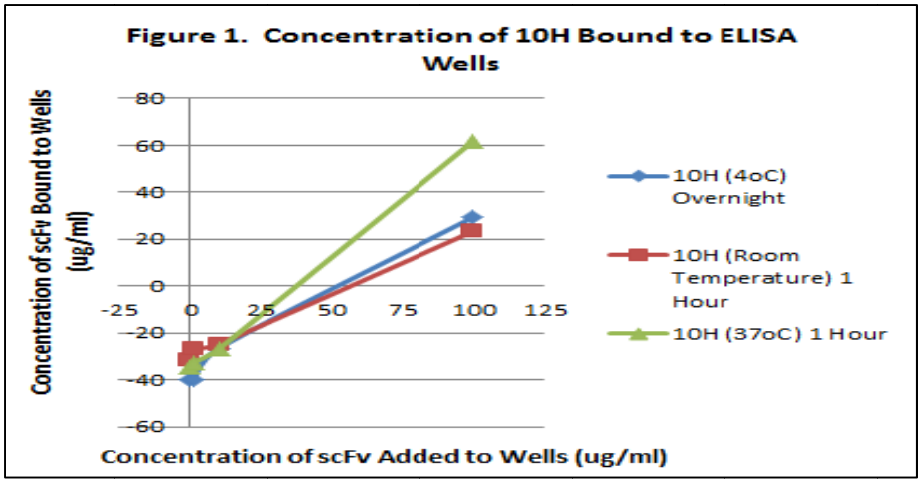
The results of these experiments helped to determine several conditions that are important to our capture ELISA. We have included the results of the scFv 10H (which binds to oligomeric forms of alpha-synuclein) to demonstrate why we chose those conditions. To begin, in one experiment, different concentrations of 10H were incubated at 4^{o}C overnight, room temperature for one hour and 37^{o}C for one hour (Figure 1). The results of this experiment showed that when 100ug/ml of 10H was added to the wells, more than twice the amount of 10H bound after one hour at 37^{o}C

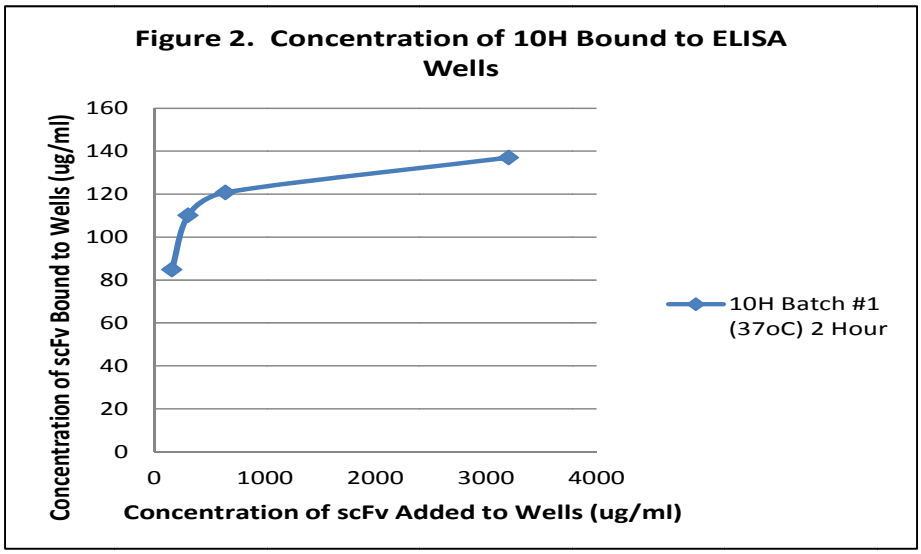
compared to 4^{o}C overnight or room temperature for one hour. Therefore, 37^{o}C was selected for further studies. Looking at figure 1, the lines in the graph are moving in an upward trend rather than leveling off even when 100ug/ml of 10H was added, suggesting that the wells are not saturated. So, in a second experiment, the amount of 10H added started above 100ug/ml and increased up to 3,207ug/ml (the maximum concentration of this scFv that was produced). The 10H was incubated at 37^{o}C for two hours. The results showed that the wells were saturated somewhere between 100-140ug/ml since as the concentration of 10H added increased, the concentration of 10H that remain bound to the wells never went above 140ug/ml (Figure 2). Since ~300ug/ml of 10H allowed for over 110ug/ml of 10H to remain in the wells and ~644ug/ml of 10H only allowed 121ug/ml of 10H to remain in the wells, 300ug/ml of 10H seems like a good concentration to use to saturate the wells. Another batch of 10H that was incubated at 37^{o}C for two hours gave similar results supporting 300ug/ml as the best saturation concentration.

Since, the increase in incubation time from one hour to two hours at 37^{o}C may have also helped with saturating the wells in addition to increasing the 10H concentration, a third experiment was conducted where the incubation time at 37^{o}C was increased to 3.5 hours (Table 1). After 3.5 hours, 200ug/ml of 10H resulted in 84ug/ml of bound 10H. If we look at the two hour incubation at 37^{o}C, ~160ug/ml of 10H allowed 85ug/ml of 10H to bind to the wells. There is no difference between 2 hours and 3.5 hours so 2 hours of incubation with the scFvs seems sufficient to allow saturation of the wells. Comparison of the 3.5 hour room temperature incubation to 3.5 hours at 37^{o}C for 200ug/ml of 10H demonstrated more binding at 37^{o}C, further supporting 37^{o}C as the optimum incubation temperature. Overall, these experiments indicate that we should immobilize scFvs using a concentration of 300ug/ml for 2 hours at 37^{o}C. These same set of experiments above were carried out for other scFvs that will be used in future capture ELISA studies. Their results were similar to that of 10H providing additional support for use of these conditions in further studies.

The second method that was used to support 300ug/ml as the best concentration to saturate the wells was with the use of the 9e10 antibody. Briefly, the ELISA plate was incubated with varying concentrations of the different scFvs and 9e10 was used to detect the scFvs that were bound. Based on the concentration at which the Optical Density started to stabilize this allowed us to validate 300ug/ml as the best concentration.

With the conditions of saturation for the capture scFv determined, the next step will be to ascertain the conditions for biotinylation of the phage.





Task 2. Vary biotinylation conditions to maximize labeling of detection phage without interfering with the antibody binding site.

The next task in the development of our phage capture ELISA protocol was to vary the biotinylation methods to identify the conditions which gave the greatest detection signal without interfering with the antigen-binding site found on the phage-displayed scFv. The phage-displayed scFv utilized here (D10) should bind all forms of the protein of interest including monomers and oligomers, since we intend to use the one phage with multiple capture scFvs that bind different oligomer forms. The phage was first produced and then biotinylated using three different techniques with both a 20 and 40-fold molar excess of biotin. The EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific, USA) was used to achieve amine biotinylated phage and the EZ-Link Pentylamine-Biotinylation kit (Thermo Scientific, USA) produced carboxyl biotinylated phage. The EZ-Link Pentylamine-Biotinylation kit was also employed in the third

biotinylation method except Sulfo-NHS was added to the reaction since this may increase the yield of biotinylated phage. One of the scFvs was also biotinylated using the amine labeling kit to directly compare biotinylated scFv to biotinylated phage.

To determine if biotinylation was successful, we ran titration analyses using different phage or scFv concentrations on an ELISA plate, followed by a blocking step and then detection with avidin-HRP. When examining only the amine biotinylated phages and scFv, both the 20 and 40 mmol biotinylated phages gave higher binding ratios compared to the 40 mmol biotinylated scFv (Fig. 1A). The ratios were determined by dividing the absorbance from wells with phage by that of the avidin-HRP only control. For the 20 and 40 mmol phages, signals could be detected up to the 10^-6 dilution and for the 40 mmol scFv, signals could be detected up to the 10^-4 dilution (when using 1.5 times the avidin-HRP only control as the cut-off). Using these dilutions, we could detect up to femtomolar concentration of the phage and picomolar concentration of the scFv. These results indicate that biotinylated phage is a better choice than similarly prepared scFv for detection of target. Titration analyses on the 20, 40 and 400 mmol carboxyl biotinylated phages and 20 and 40 mmol Sulfo-NHS carboxyl biotinylated phages also revealed that these biotinylation experiments were successful (Fig. 1B). The 10^-4 dilution yielded high signals with all the different biotinylation conditions (Fig. 1A and 1B) and so this dilution was selected for future ELISAs development.

To ensure that the phage-displayed scFvs can still bind to their antigens, the different protein targets were allowed to aggregate over time and used in an indirect ELISA to assess binding abilities. Initially, when we conducted these indirect ELISAs, the background binding in the control wells were extremely high. To remedy this problem, the target antigen was kept at 2ug/ml (a concentration that was within the expected detection range of our phage) and the blocking solutions were varied to see if this could lower the background signals. We first tried 5% milk, 10% milk, 5% BSA and 10% BSA. From fig. 2A, 5% and 10% milk were the only blocking solutions where all four tested phage gave ratios to the no antigen control than were above 1. 5% milk gave slightly better results than 10% milk, however these ratios were still too low and so we continued to test more blocking solutions. Since milk gave better results than BSA, we decided to test additional concentrations of milk and because 5% milk was better than 10% milk we started with concentrations lower than 5%, including 0.5%, 1%, 2% and 4%. The lower milk concentrations blocked non-specific binding better (Fig 2B) since the ratios obtained with lower concentrations were considerably higher than those obtained with higher concentrations (Fig. 2A). A 2% milk solution gave the best results, especially for the 40 mmol carboxyl biotinylated phage and was selected for further ELISA protocols. These experiments facilitated not only the selection of the best blocking solution but also verification that the antigen-binding site was not impeded.

Task 3. **Vary phage concentration in the ELISA assay to obtain highest signal without increasing background**. We altered concentration of phage along with incubation and rinse conditions to determine what assay combination yielded the strongest signal to background ratios. We determined the phage titers and conditions that should give us the highest signals when using CSF or serum samples. Based on the phage titration analyses, 10^-4 was chosen as the phage dilution, however these conditions needed to be verified in an ELISA protocol using the target antigen. Using 2% milk as the blocking solution and 2ug/ml of the antigen, the phage were diluted to concentrations of 10^-5, 10^-6, 10^-7 and 10^-8. Ratios were obtained for all the phage at every dilution that were at or above 1.5 times the no antigen control; many of the ratios approaching 2 times the no antigen control (Fig. 3A). However, because our future intent is to use the phage in a capture ELISA with brain, CSF and serum samples (which may have much lower concentrations of our target antigen as compared to our purified samples), we do not want to limit our detection by using too little of the biotinylated phage and therefore 10^-4 dilution was selected as the best option for further studies.

We still needed to determine which of the six different biotynilated phage preparations to choose for the capture ELISA, so we carried out an indirect ELISA with all of the biotinylated phages at 10^-4 dilution with the target antigen. The carboxyl biotinylated phage all gave ratios above 2 times the no antigen control as compared to the amine biotinylated phages which were below 1.5 times the control (Fig 3B). The increase in sensitivity with the carboxyl biotinylated phage compared to the amino labeled phage was primarily due to lower background with the carboxyl biotinylated phage as opposed to stronger signlas. The Sulfo-NHS kit claims that it should provide higher yields of carboxyl biotinylated phages, however in our assays it did not provide a significantly better outcome than the direct carboxyl labeling (Fig. 3B). Therefore, the 40 mmol carboxyl biotinylated phage was selected for further studies since it gave the best titration results as compared to the other carboxyl biotinylated phages (Fig. 1B) and it also gave the highest ratio with the 2% milk at 10^-4 dilution (Fig. 2B).

Comparison of Amine Biotinylated Phage and scFv and Different Carboxyl Biotinylated Phages

Fig. 1A Titration of Amine Biotinylated Phage and scFv

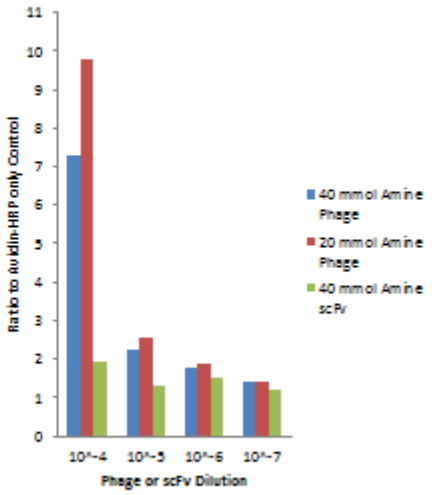


Fig. 1B Varying Biotinylation Conditions

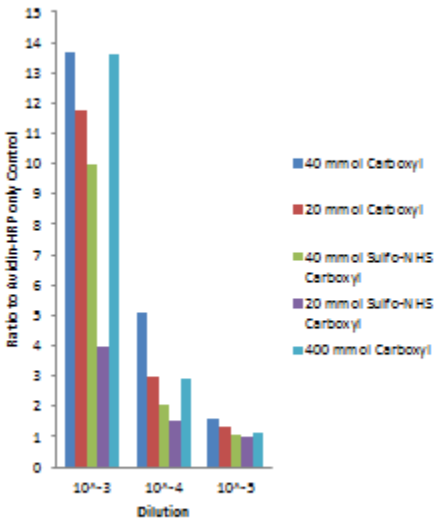


Figure 1. Williams et al. 2013

Ascertain Effective Blocking Solution

Fig. 2A Varying Blocking Solution Concentrations in Indirect ELISA

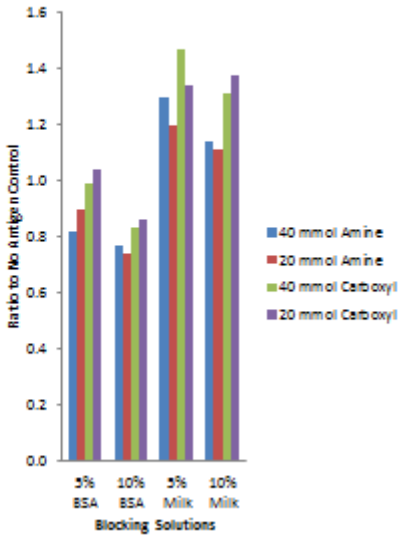


Fig. 2B Varying Blocking Solutions in Indirect ELISA

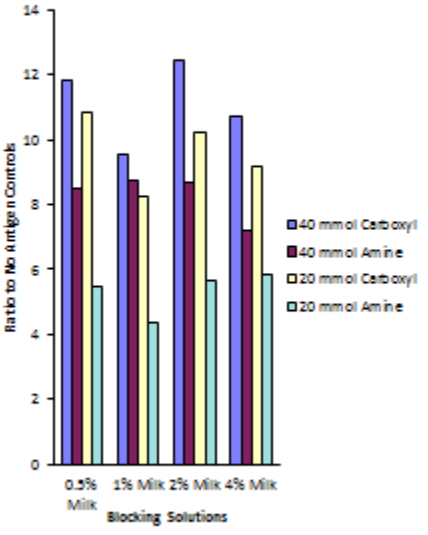


Figure 2. Williams et al. 2013

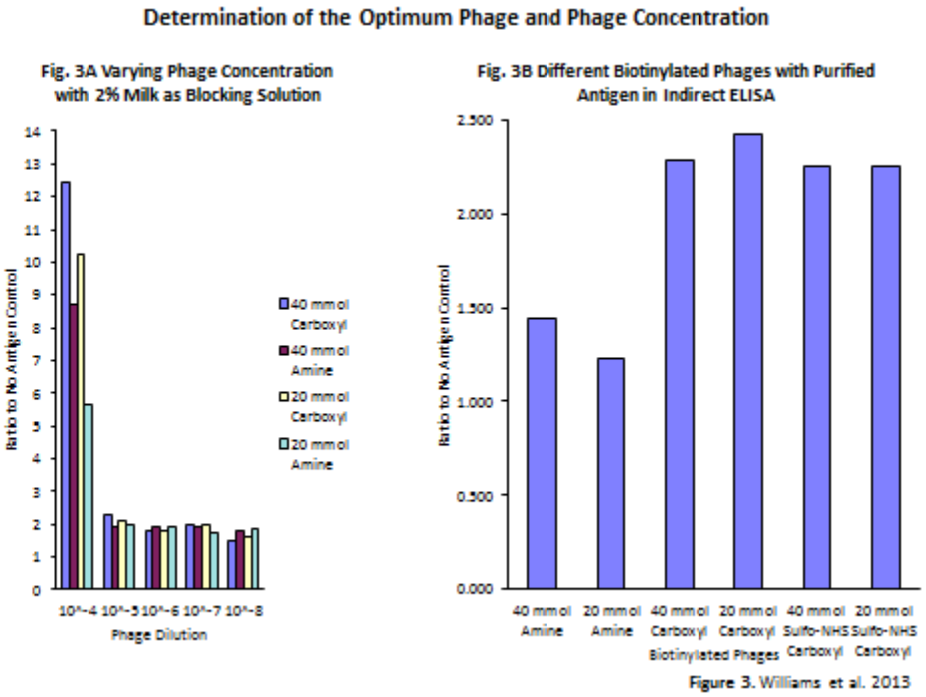


Figure 3. Williams et al. 2013

Task 4. Completion of ELISA protocol.

We previously reported the selected conditions for our primary capture scFv, the optimum blocking procedure and the preferred biotinylation method and working concentration for the detection antibody in our phage capture ELISA system. In our next step, we needed to determine in an indirect ELISA the lowest level of our target detectable by the detection antibody (which binds to all forms of our target including monomers and oligomers). Using various concentrations of aggregated alpha-synuclein, we are able to detect down to the high picomolar range (Figure 1). Ideally, we would like to detect up to femto- molar concentrations of our target and so further optimization was necessary.

To determine the working avidin-horse radish peroxidase (hrp concentration, we ran a complete phage capture ELISA utilizing post-mortem PD human brain tissue we obtained from another project at 1/70 (0.2mg/ml) and 1/140 (0.1mg/ml) dilutions with varying avidin-hrp concentrations. The 1/70 sample dilution gave better signal ratios than the 1/140 dilution at almost every tested avidin-hrp concentration (Figure 2). Based on these results we selected 0.2mg/ml as the antigen concentration and 1/2000 as the avidin-hrp concentration since even at this avidin-hrp concentration the signal was comparable to the 1/500 dilution. To ascertain the optimum incubation conditions for the target antigen we ran another phage capture ELISA using transgenic PD mouse brain tissue due to limited human samples. The PD mouse tissue was also obtained from another project. One of the transgenic PD mouse had been treated with a therapeutic to decrease oligomeric a-syn levels and the other was an untreated PD sample. The samples were incubated overnight at 4^{o}C or for 2 hours at 37^{o}C with 10H (scFv reactive with

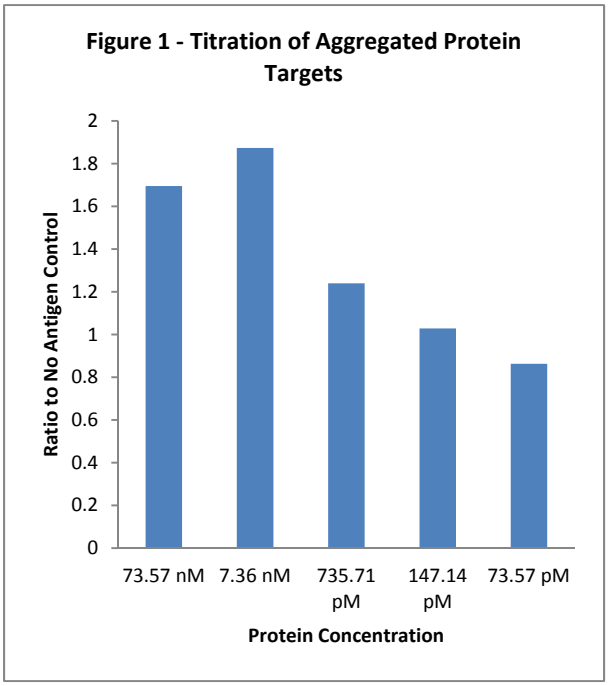
oligomeric alpha-synuclein). The signal ratios were higher for the 37^{o}C incubation compared to 4 C. (Fig 3). Therefore two hours at 37^{o}C was the selected for the sample incubation conditions.

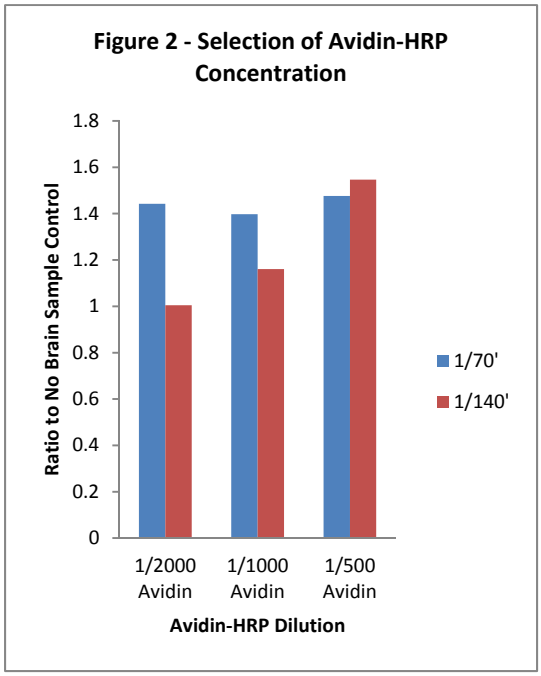
In all our previous ELISAs TMB was used as the substrate to detect bound phage. In order to improve our signals so we could detect antigen at low concentrations expected in CSF or serum samples, we utilized the SuperSignal ELISA Pico Chemilluminescent Substrate kit. In an indirect ELISA carried out in a manner similar to figure 1, we could to detect down to femtomolar concentration of a-syn and lower (Fig. 4). We retested the same mouse brain tissue samples used earlier (Fig. 3) at 0.5mg/ml in a capture ELISA, but this time utilizing the chemilluminescence substrate for detection. The signals obtained with the chemilluminescence substrate were dramatically stronger compared to the TMB substrate (Fig 5). To further improve the signal we tried the SuperSignal ELISA Femto Maximum Sensitivity Substrate for detection. In addition to using the femto kit we varied the biotinylated phage and avidin-hrp using lower concentration of the PD human brain tissue (0.1mg/ml) and used the D5 scFv as the capture antibody (another oligomeric alpha-synuclein reactive scFv). The best signal rations were obtained when using 10^-3 phage and 1/1000 avidin-hrp dilutions and so these parameters were used for the detection and secondary antibodies in further studies (Figure 6).

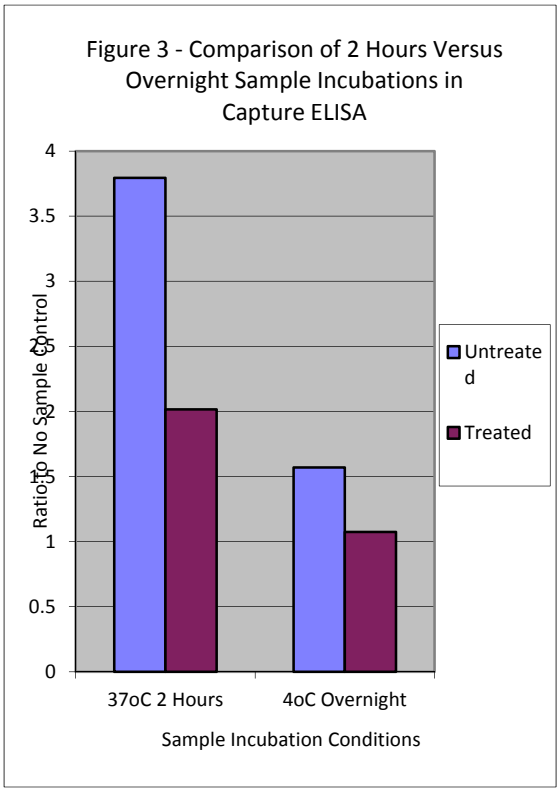
A signal recognition pathway (SRP) that allows for cotranslational translocation of proteins to the periplasm has been shown to help designed ankyrin repeat proteins (DARPins) with the phage display process (Steiner, et al., 2008). We theorized that the SRP signal may increase the sensitivity of our phage detection antibodies by increasing the number of phage that had scFv particles displayed on the tip. We purified phage produced using the SRP signal sequence to express the scFv. Using the same two mice samples again (this time at 0.1mg/ml), we first compared the PelB-phage (original phage) with the SRP-phage. In figure 7A, the ratios to no sample control are higher for the SRP phage and the difference between the untreated and treated mice is also greater. The background signal was also much lower for the SRP phage (figure 7B) and the raw signal with the SRP phage (figure 7C) was higher. Two titration curves using the SRP and Pelb phages were generated using the untreated PD mouse to see the lowest brain sample dilution where a signal could be detected. A higher signal at every dilution was obtained using the SRP produced phage (Fig 8) and a strong signal was obtained even at 1/2000 phage dilutions. These results indicate that using the SRP phage expression system may be further increase the sensitivity of our ELISA assay.

An alternative approach to increase the sensitivity of the detection phage antibody is to increase the number of scFv particles displayed at the tip of the bacteriophage. The M13 hyperphage system can produce phage with multiple copies of the scFv expressed at the tip. Using C6T (an oligomeric Abeta reactive scFv) and a triple transgenic AD mouse model, we ran a titration curve and found the signals using the hyperphage were stronger than using a conventional phage using the PelB signal (Figure 9). We then compared the hyperphage to phage produced using the SRP signal and found that both gave similar signals (results not shown). So using either the SRP or the hyperphage alone seems sufficient to increase our signals.

Overall our ELISA system has improved by several orders of magnitude over the past few months. We are continuing to adjust our protocols to further improve the assays, such as the addition of tween at various steps in the incubation buffers and altering the concentration of the capture scFv needed to saturate the wells of the ELISA plates. However the ELISA protocol is sufficiently sensitive to enable us to detect the presence of oligomeric proteins in CSF samples. We are now waiting to obtain the samples from our collaborators.







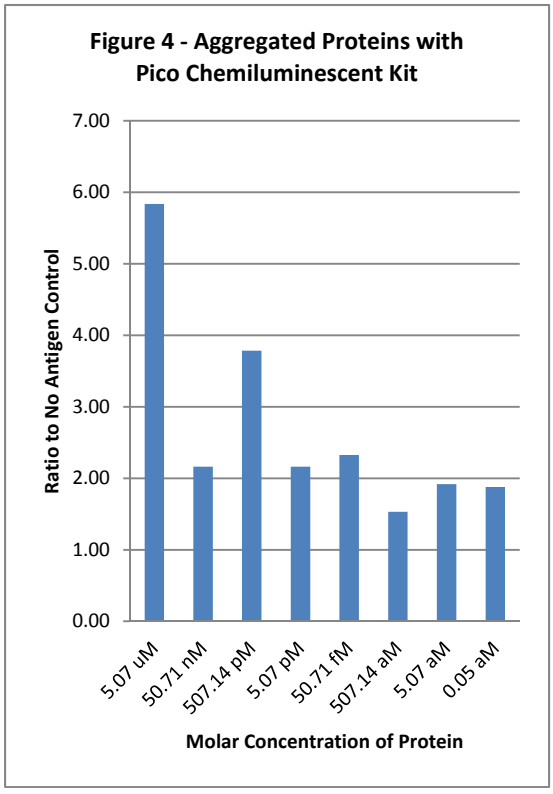


Figure 5 - Capture ELISA using Pico Kit and TMB

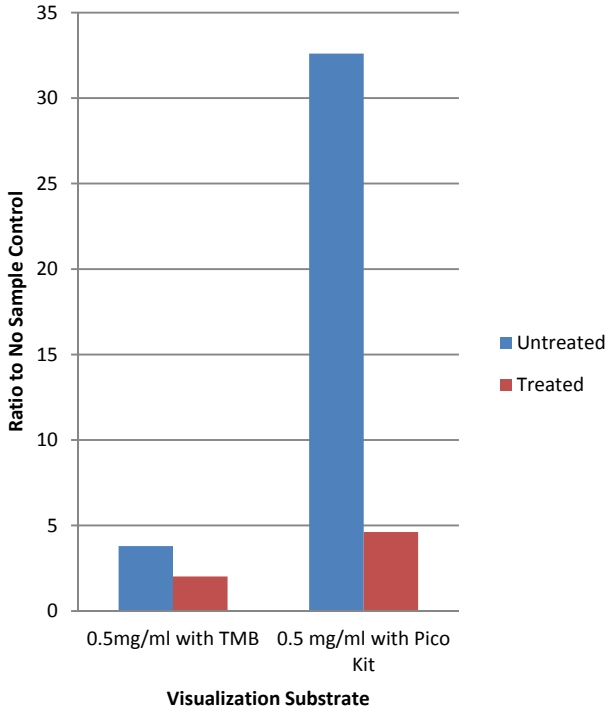


Figure 6 - Binding of D5 to Human Brain Tissue Sample with Femto Kit

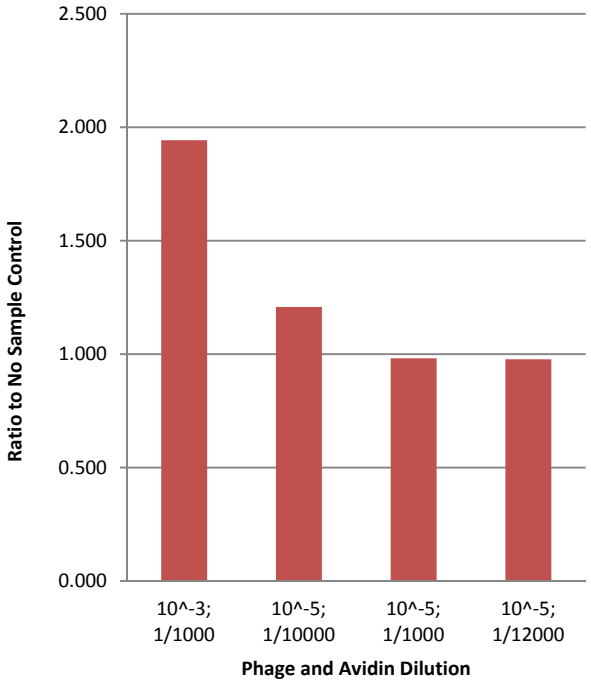


Figure 7A - Comparison of SRP to Pelb using 10H ELISA

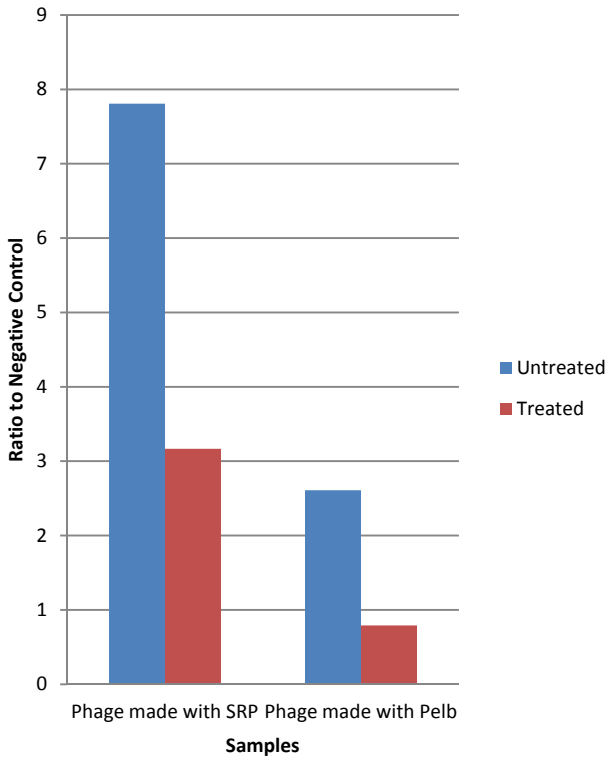
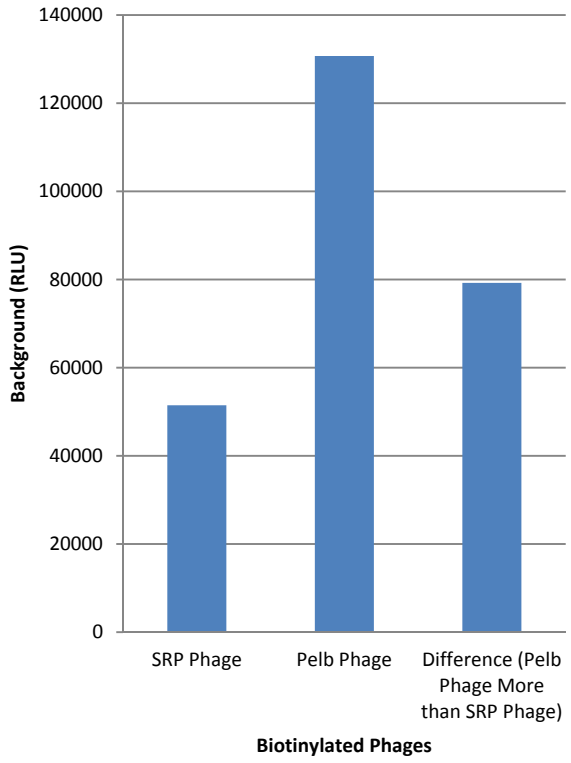


Figure 7B - Comparison of Background Between SRP Phage and Pelb Phage



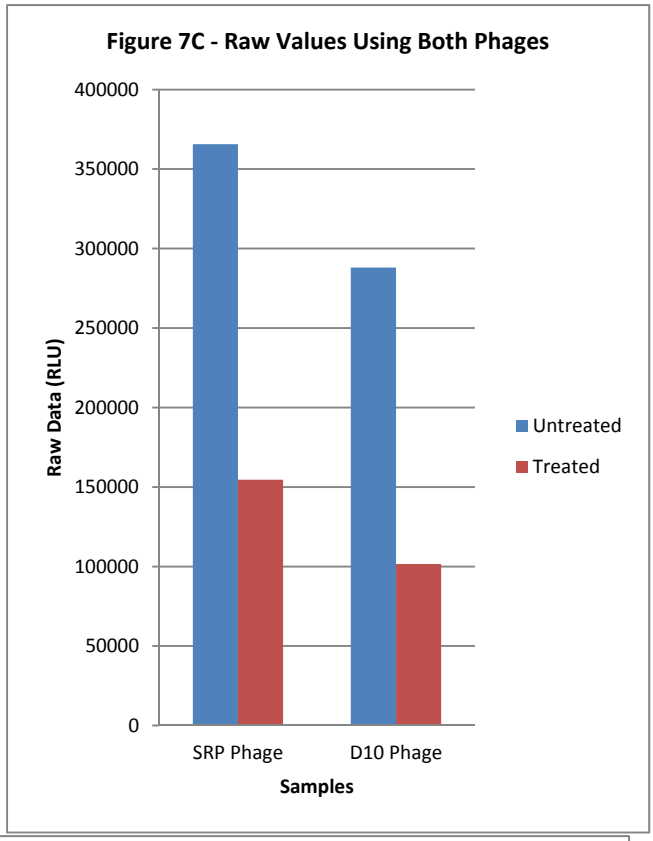


Figure 7C - Raw Values Using Both Phages
400000
350000
300000
250000
200000
150000
100000
50000
0
Raw Data (RLU)
SRP Phage
D10 Phage
Samples
Untreated
Treated

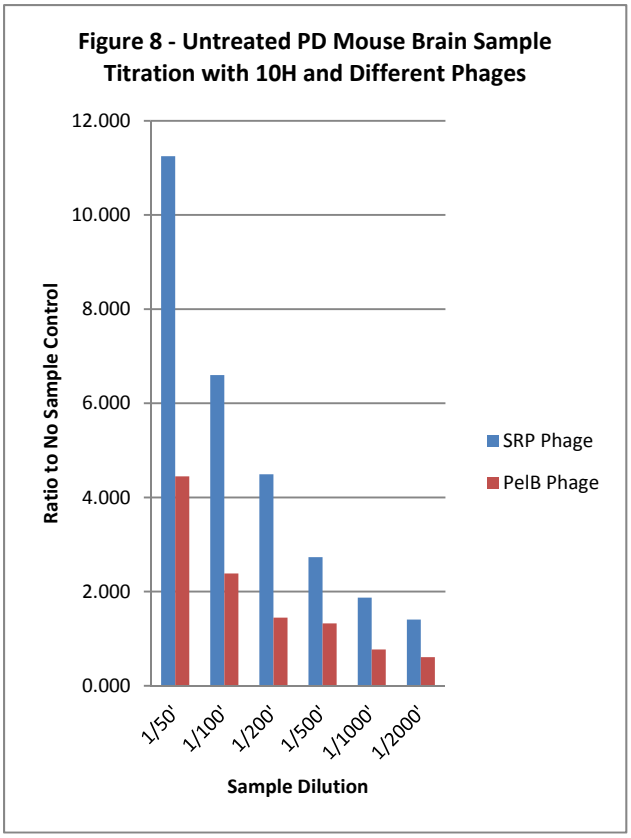


Figure 8 - Untreated PD Mouse Brain Sample Titration with 10H and Different Phages
12.000
10.000
8.000
6.000
4.000
2.000
0.000
Ratio to No Sample Control
1/50'
1/100'
1/200'
1/500'
1/1000'
1/2000'
Sample Dilution
SRP Phage
PelB Phage

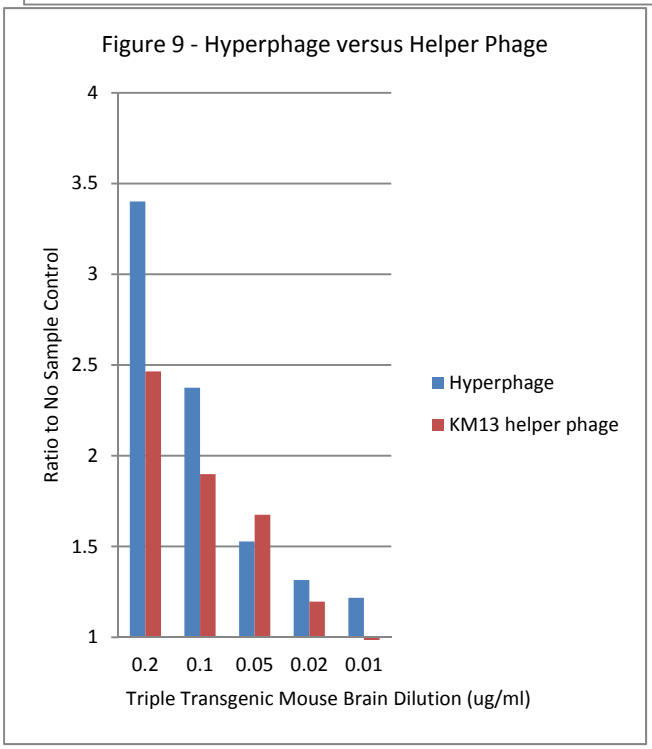


Figure 9 - Hyperphage versus Helper Phage
4
3.5
3
2.5
2
1.5
1
Ratio to No Sample Control
0.2
0.1
0.05
0.02
0.01
Triple Transgenic Mouse Brain Dilution (ug/ml)
Hyperphage
KM13 helper phage

Key Research Accomplishments

- Developed ELISA with femtomolar sensitivity
- ELISA utilizes morphology specific nanobodies against toxic forms of beta-amyloid, tau, and a-synuclein

Reportable Outcomes

We have filed an invention disclosure to utilize the ELISA protocol developed in connection with our morphology specific nanobodies as a method to detect the presence of toxic oligomeric protein species in human CSF and serum samples. Detection should facilitate early diagnosis of Alzheimer's, Parkinson's, traumatic brain injury or other neurodegenerative disease.

Conclusion

We have developed a very sensitive ELISA protocol using our morphology specific nanobodies that can be used to detect the presence of specific toxic protein species that are indicative of various neurodegenerative diseases including Alzheimer's, Traumatic Brain Injury and Parkninson's disease. The ELISA protocol should work effectively on both human CSF and plasma or serum samples. The assay has at least femtomolar sensitivity. We are currently obtaining samples of CSF from our two collaborators, Barrow Neurological Institute (BNI) and Mayo Clinic, Scottsdale. Due to a delay in getting the IRB approvals for the two collaborating institutions, collection of the CSF samples has been delayed and the grant extended for an additional year. The CSF samples from BNI will be both from control samples and from patients that recently suffered TBI. The samples from Mayo will be from patients diagnosed with AD. Once these samples are collected we will analyze them with our panel of morphology specific nanobodies to demonstrate that we can determine the presence of different profiles of toxic protein variants in the control, TBI and AD CSF samples.

References:

Steiner, D., P. Forrer, and A. Pluckthun, *Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display.* J Mol Biol, 2008. 382: p. 1211-27.