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DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY

UTILIZATION OF ALFA-TAG SYSTEM FOR STUDYING ZIKA VIRUS LIFE CYCLE IN INFECTED HOST CELLS

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ABSTRACT

Orthoflaviviruses, such as Zika virus (ZIKV) and Dengue virus (DENV), are single-stranded RNA viruses that can cause diseases like congenital Zika syndrome and dengue fever, respectively. Their viral life cycle in infected cells, especially the fate of viral mRNA and the dynamics of transcription and replication, is currently not well understood. In this study, we employed the ALFA-Tag system and fluorescence nanobodies against the ALFA-Tag to investigate the early events of ZIKV infection in human cells using confocal fluorescence microscopy. We genetically engineered recombinant ZIKV cDNA clones expressing the ALFA-Tag and created nanobody (NbALFA-RFP-His) expression constructs under the control of CMV and inducible Tet promoters, respectively. Our findings suggest that the ALFA-Tag system is a viable tool for visualizing early ZIKV infection events. However, the introduction of ALFA-Tag on ZIKV affected virus formation in host cells. We also observed leaky expression of the nanobody in the Tet-inducible system. Future studies will focus on reintroducing the ALFA-Tag on different locations of the ZIKV genome to avoid disruption of virion formation, optimizing nanobody expression control in the Tet-inducible system, and optimizing co-transfections with ZIKV ALFA-Tag clones and NbALFA-RFP-His to better understand the viral life cycle.

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Chapter 1

INTRODUCTION

Origin & History of the Zika Virus

The Zika virus is a mosquito-borne flavivirus that was first isolated in 1947 in Uganda. Although only a few cases were sporadically found in the local areas, the virus began gaining attention in 2007 following an outbreak on Yap Island with 49 confirmed cases and the islands of French Polynesia in 2013, with 30,000 cases. There were large-scale outbreaks of Zika virus in Brazil and South American countries later in 2015, causing multiple fatalities and cases of congenital Zika syndrome and microcephaly[1,2,3].

Zika Virion & Genome

Zika Virus (ZIKV) belongs to the Flaviviridae family, a group of viruses that home similar RNA viruses such as Dengue Virus (DENV) and Yellow Fever Virus (YF). The Zika Virus genome is a positive single-strand RNA with a single large open reading frame (ORF) and untranslated regions (UTRs) at both 3' and 5' ends. The ORF codes both the structural proteins and nonstructural proteins1. The C, prM, and E proteins comprise the Zika Virus's structural proteins. The capsid protein (C) is responsible for packaging the positive strand genome of the Zika virus. The prM protein is the precursor protein for M and pr proteins; the prM is usually cleaved by the host furin protease to generate the M protein. The M protein and the membrane-anchored envelope (E) protein form heterodimers and dimerize to form a raft-like arrangement

covering the virus surface. The E protein is responsible for viral attachment and fusion into host cells3.

The ORF also encodes seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS1 protein is responsible for aiding in regulating RNA processing, viral genome replication and helping the virus evade immune detection. The N2B-NS3 protein complex is a serine protease and host proteases responsible for cleaving the polyprotein following viral translation, creating both structural and nonstructural proteins. The NS5 is involved in viral replication as it encodes the RNA-dependent RNA polymerase, which is responsible for viral replication and capping the mRNA[2].

Replication Cycle of Zika Virus

Zika virus first enters the host cell by endocytosis through clathrin-coated vesicles. These vesicles later fuse with endosomes. Upon acidification of these endosomes, the acidic environment changes the conformation of the E protein. This conformational change causes the E protein to turn into a fusion-active trimer state, allowing the fusion of the viral and host membrane and releasing the RNA genome into the cytoplasm. The RNA genome, once released, will undergo translation as a single polyprotein by using ribosomes located on the endoplasmic reticulum (ER) membrane. The polyprotein is co-translationally translocated onto the ER membrane, and the polyprotein is cleaved by viral and cellular proteases into three structural and seven non-structural proteins. The nonstructural proteins and the viral RNA form the replication complex and replicate the positive sense genomic RNA through a double-stranded RNA

intermediate. During this stage, the RNA genome will be synthesized using a complementary (minus strand) copy of the positive RNA strand in specialized membrane compartments formed by ER modification. This allows the RNA-dependent RNA polymerase to create more copies of the RNA genome. Once the viral genome has been replicated, it could either be translated to produce more viral proteins, be used as a template for RNA replication to generate more viral RNA or be packaged by capsid protein into nucleocapsid cored that bud into the ER lumen forming enveloped immature virus particles with a lipid bilayer embedded with prM and E protein heterodimers arranged as trimeric spikes. The immature virus is later glycosylated and travels through the trans-Golgi Network (TGN), where the virion begins to mature, and the prM, or the precursor membrane protein, will be cleaved by a host furin protease to pr and M proteins forming the smooth mature particles. Subsequently, the mature virion is released from the host cell through the exocytosis pathway[4].

Transmission of Zika Virus

Zika is an arthropod-borne virus, meaning that it is transmitted by an arthropod vector, specifically the *Aedes aegypti* mosquitos. This mosquito population can breed in stagnant water pools, many of which can be found in ongoing construction sites and urban areas. Human transmission happens when an infected mosquito bites an individual[4]. However, this virus can be transmitted in multiple ways, such as through sexual intercourse and across the placental barrier, causing fetuses to be infected by this virus. The transmission of this virus onto the fetus during pregnancy can leads to congenital Zika syndrome, microcephaly, and fetal loss[2].

Clinical Features of Zika Virus

Zika has been documented to have symptoms similar to other mosquito-borne viruses, including arthralgia, conjunctivitis, headache, fatigue, and rash. In severe cases, multi-organ failure, meningitis, and encephalitis have also been documented. The Zika virus can also cause Guilian-Barre syndrome, a condition where the patient begins to lose muscle control, which leads to paralysis. Another prominent disease caused by Zika is the microcephaly congenital ZIKV syndrome (CZS), a syndrome where the virus can pass from the mother onto the fetus; a fetus suffering from this syndrome will experience microcephaly, or size reduction of the head, as well as learning and neurological disabilities[4,5]. There are currently no specific treatments or vaccines available against Zika infections. Treatment usually includes bed rest and supportive care; patients are recommended to drink fluids to prevent dehydration. Apart from that, it is recommended to take acetaminophen to alleviate both fever and pain[6].

ALFA-Tag System

The ALFA-Tag system includes a peptide tag (ALFA-Tag) and high-affinity nanobodies that recognize and bind to an ALFA-Tag sequence in proteins of interest. The ALFA-tag is a 15 amino acid sequence with an α-helical structure. This epitope tag is both hydrophilic and uncharged. This particular tag can be placed within several parts of a protein of interest, including both the N- and C-terminus, as well as between two different folded protein domains. Nanobodies (Nb), also known as camelid single-domain antibodies, are small and monomeric as they are 15 kDa in size. Specifically, high-affinity nanobodies (NbALFA) have been proven to be good models for binding to the ALFA-Tag. When modified NbALFA tagged with fluorescent

protein was co-expressed with ALFA-tagged proteins, the ALFA-tagged proteins were successfully identified using immunofluorescence and fluorescence confocal microscopy[7].

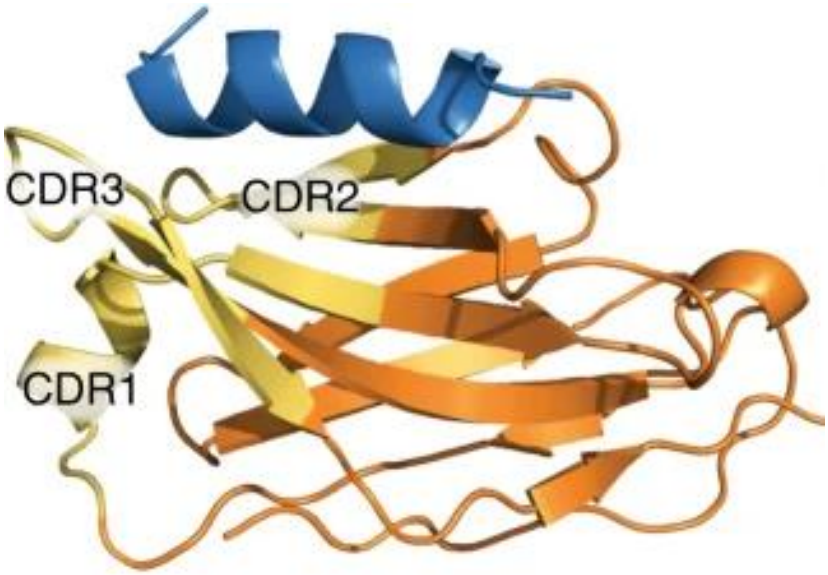

Figure 1: NbALFA (Orange) with CDRs 1-3 (Yellow) Interacting with ALFA-TAG (blue).

Experimental Design

ALFA tag system has proven effective for resolution imaging and detection of any proteins tagged with this ALFA-Tag sequence[7]. Therefore, the purpose of this thesis study is to implement this ALFA-Tag system into the Zika virus model for high-resolution imaging of ZIKV genome translation, and assembly in infected host cells.

We genetically engineered the Zika virus cDNA to introduce the Alfa Tag attached to capsid and NS2A proteins by site-directed mutagenesis (Fig 2). We obtained plasmid encoding ALFA tag nanobody NbALFA-RFP-His under a CMV promoter from Addgene (#159991). We subcloned the ORF coding for NbALFA-RFP-His into a mammalian expression vector for

expression under an inducible Tet promoter. Mammalian cells were transfected with the plasmids for ALFA tag-based fluorescence microscopy of ZIKV-infected mammalian cells.

One of the objectives of this study is to characterize the formation of RNA viral proteins, replication, and packaging into the virions. Another objective of this study is to standardize the ALFA-tag system to help visualize the infection and growth of viruses studied in the Jose laboratory. This would help establish a system within the lab to help with future studies, including protein-protein interaction by pull-down studies. This would help advance the understanding of the viral mechanisms and help advance the progress for future therapies/drugs to benefit patients infected with this virus.

Chapter 2

MATERIALS AND METHODS

Site-directed mutagenesis (SDM) and Sequence Analysis

Site- directed mutagenesis (SDM) was employed to create the ZIKV cDNA with ALFA-Tag clones under the CMV promoter clone. SDM was performed using the Phusion DNA Polymerase (Thermo Scientific, #3091494) and oligonucleotide primers, as in Table 1. PCR Samples were later treated with *DpnI* enzyme, transformed into NEB Stable *E. coli* cells, and selected in media plates of Lysogeny broth (LB) agar plates containing 100 ug/mL ampicillin (Amp). Two colonies from the transformation plate were inoculated for overnight growth in Lysogeny Broth (LB). Plasmid DNA was purified/extracted from bacterial cultures following EconoSpin spin column miniprep kit (Epoch Life Science, #1920-250). Purified plasmid samples were quantified using NanoDrop spectrophotometry, and to confirm the location of ZIKV ALFA-TAG and NbALFA-RFP-His-CMV promoter, samples were sent for Sanger Sequencing at the Pennsylvania State University Genomics Core.

Transformation and overnight cultures

A 3 uL sample of DNA was incubated with ~25 uL of NEB-stable *E. coli* cells for 30 minutes. Cells were heat shocked at 42 ℃ for 60 seconds, followed by a 5-minute incubation on ice. 250 uL of LB broth were added to the cell mixtures; cell mixtures were incubated in the shaker at 25℃ at 180 RPM. Cells were finally plated on 100 ug/mL of Ampicillin LB plates and incubated

overnight at 37 ℃. 2 Inoculations from each plated sample were performed using 6 mL of AMP-LB broth and were later incubated in the shaker at 32℃ at 180 RPM overnight.

Minipreps/ Plasmid Extraction

Minipreps/Plasmid Extraction was performed following the EconoSpin spin column miniprep kit (Epoch Life Science, #1920-250). Cultures following overnight inoculation were centrifuged for 10 minutes. Supernatants were removed. Pellets were each resuspended in 250 uL of P1 buffer, following 3 minutes of lysis with 250 uL of P2 buffer. Subsequently, 350 uL of N3 buffer was added to neutralize each solution. Reactions were later transferred to clean Eppendorf tubes, which were centrifuged for 15 minutes at 13,000 RPM. Supernatants were then transferred to columns that were later centrifuged for 1 minute at 13,000 RPM. The flowthroughs were discarded, and 500 uL of PB binding buffer was added to the columns, which were later spun for 1 minute at 8,000 RPM. Flow-throughs were discarded, and 750 uL PE wash buffer was added to each column. Reactions were later centrifuged at 13,000 RPM for 1 minute. Flowthroughs were discarded, and samples were centrifuged for 2 minutes at 13,000 RPM. Columns were placed onto clean Eppendorf tubes, and plasmids were eluted using 50 uL of EB elution buffer. The buffer was placed in the column for 1 minute before it was centrifuged at 10,000 RPM for 1 minute. Samples were later run under agarose gel electrophoresis to detect desired plasmids. Plasmid concentrations were quantified using a Nanodrop spectrophotometer.

Agarose Gel Electrophoresis/ Gel Extraction

PCR and purified plasmid samples were run using agarose gel electrophoresis. 2 uL of DNA samples combined with 2 uL of gel loading dye (New England Biolabs, #18251015) and 2.5 uL of 1kb DNA ladder (New England Biolabs, #N3233) were run in low-melting 0.8% agarose gel. The gel was run at 90V for 20 minutes and was later analyzed using a UV transilluminator.

For gel extraction, the desired band was cut from the gel and melted at 50℃ for 10, periodically mixing using a gel-dissolving buffer. The melted gel was inserted into a Monarch spin collection tube and spun for 1 min at 13,000 RPM. Flow through was discarded. The sample was then washed with 200 microliters of wash buffer and spun for 1 min at 13,000 RPM. This step was repeated twice. The column was transferred to a clean Eppendorf tube, and 20 uL of elution buffer was added and incubated for 1 minute. DNA was later eluted after 1 minute of spinning at 13,000 RPM.

Ligation-independent cloning into Tet-on vector

ALFA-NB-RFP-His sequence was amplified by Polymerase chain reaction (PCR) using primers as given in Table 1 from Addgene #159991. The sample was run on an agarose gel, and the band of interest was extracted using a gel elution procedure using the Monarch Spin DNA Gel Extraction Kit (New England Biolabs, #10258290). The purified insert was treated with T4DNA polymerase and dCTP to create 5' and 3' overhangs. The T4-treated insert was added to the T4-treated pCW vector and transformed into E.coli NEB stable cells. Samples were then transformed into NEB Stable *E. coli* cells and plated in 100 ug/ml amp-combined LB agar plates.

Overnight cultures of bacterial colonies were prepared for plasmid purification. Plasmids were digested with 0.5 uL of the *NheI* and *BamHI* enzymes to confirm the presence of the insert. Finally, the samples were analyzed for whole plasmid sequencing at the Pennsylvania State University Genomics Core Facility.

Cell-Lines & Transfection of ZIKV-ALFA TAG and NbALFA-RFP-His Clones

Human embryonic kidney (HEK-293T) was used to transfect ZIKV cDNA clones with ALFA-TAG insert to create virus stocks. Plasmid DNA with the ZIKV cDNA with ALFA tag insertions on the capsid and NS2A proteins were transfected into human embryonic kidney (HEK-293T) cells grown on 24-well culture plates using Polyethyleneimine Hydrochloride (Polyscience, 24765 (1)) combined with Opti-MEM (Gibco, 51985034). Following 18-20 hours of incubation at 37℃, OPTI-MEM was replaced with cell culture media (DMEM + 10% FBS). After 96 hours following transfection, cells were visualized using fluorescence confocal microscopy. Cell culture supernatant was harvested and stored in a -80℃ freezer.

For co-transfection experiments, plasmids encoding NbALFA-RFP-His with CMV promoter (for constitutive expression) and Tet-on promoter (for induced expression) were co-transfected with ZIKV cDNA clones with ALFA TAG into HEK-293T cells grown on imaging glass-bottomed plates using Polyethyleneimine Hydrochloride. Cell grown on 24-well plates were transfected with the plasmids using PEI max reagent. Following 18-20 hours of incubation at 37℃, the transfection mixture was replaced with cell media (DMEM + 10% FBS). Cells were later treated with 1 ug/mL of anhydrotetracycline, which was added to the cell cultures to induce

expression of the NbALFA-RFP-His. After 48 hours following induction, cells were visualized by fluorescence confocal microscopy and imaged using Nikon NIS elements software.

Plaque Assays

VeroE6 cells were grown to confluence in a 24-well plate. P0 stocks of ZIKV with ALFA TAG inserts were serially diluted using MEM media containing 1% FBS. 125 uL of each serial dilution was added to each well of VERO E6 cells, and the plate was incubated at room temperature in a rocker for 1 hour. Following incubation, overlay media (an equal mixture of 2X DMEM + 2% Cellulose) was added to the wells and incubated at 37℃. After 4 days of incubation, cells were fixed with 500 uL of 3.7% formalin for 1 hour at room temperature, followed by a 1% PBS wash. Cells were later stained with 250 uL of crystal violet dye for 15 minutes, followed by washing with water.

Chapter 3

RESULTS AND DISCUSSION

Generation of ALFA-tagged ZIKV cDNA clones

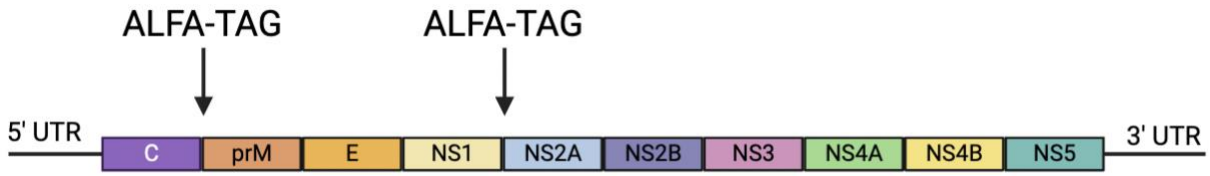

Figure 2: Locations of ALFA-TAG on ZIKV Genome. Figure was made on BioRender.

To create these ZIKV cDNAs with the ALFA-TAG insertions (Fig. 2), we performed site-directed mutagenesis on the plasmid encoding ZIKV cDNA using primers listed in Table 1. These amplified products were treated with *DpnI* and transformed into NEB-stable *E. coli* cells. Plasmids were isolated as described in the material and methods section. The recombinant plasmids were sequenced to confirm the location of the ALFA-Tag in the ZIKV genome. We confirmed the location of both ALFA-Tag locations on the ZIV genome from sequencing data. We successfully obtained two different ALFA-tag clones. For clone 1 (#2586), the ALFA tag was inserted at the C terminus of the capsid protein, which already has an Emerald on NS2A, and for clone 2 (#2760), the tag was inserted on the N-terminus of NS2A, which already has an Emerald on C protein (Figure 3).

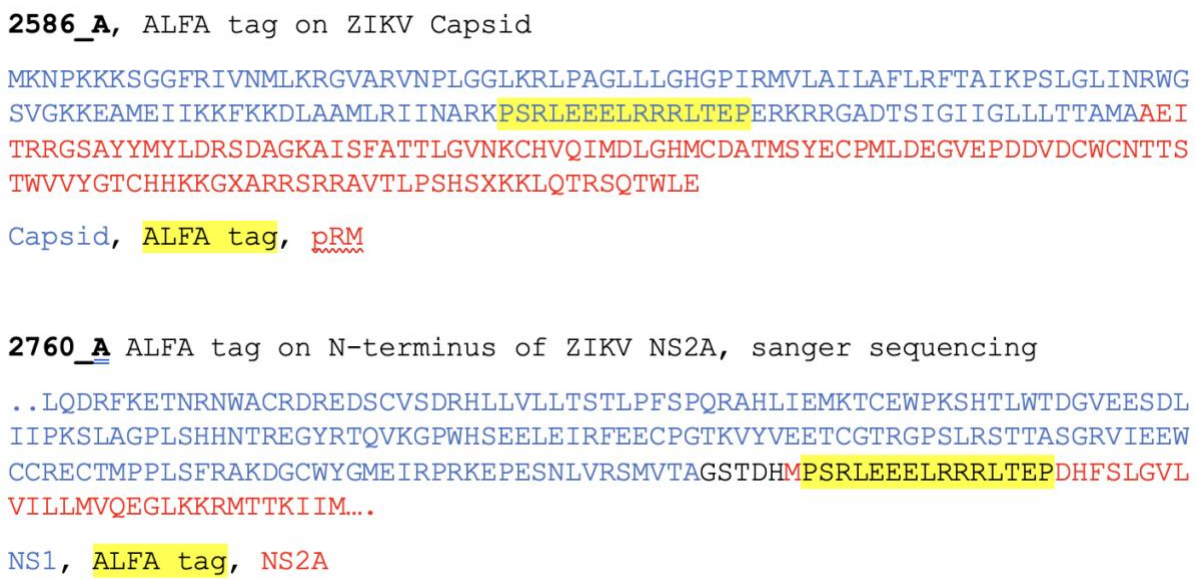

Figure 3: Sequence results of ZIKV cDNA ALFA-TAG inserts on ZIKV Capsid and N-terminus of ZIKV NS2A

(Fig 3). Sequencing results of ZIKV cDNA clones with AFLA-Tag inserts on ZIV. 2586_A clone contains the AFLA tag (yellow) on ZIKV capsid (blue). 2760_A clone contains the ALFA tag (yellow) in the N-terminal domain of NS2A (red). Both plasmids were sequenced using Sanger sequencing.

Creation of NbALFA-RFP-His clones under Tet-on promoter

For this study, we created a recombinant plasmid that encoded ALFA-Tag nanobody NbALFA-RFP-His under a CMV promoter. We also subcloned the ORF coding for NbALFA-RFP-His into a mammalian expression vector for expression under an inducible Tet promoter. To create the NbALFA-RFP-His into the mammalian expression vector, the pCW, ALFA-NB-FRP-His sequence was first amplified using Polymerase chain reaction (PCR). Samples were run under agarose gel electrophoresis, and bands were extracted using the gel elution procedure as listed in the material and methods procedure. Ligation-independent cloning was employed to

clone the ORF into the pCW vector. The sequence of the clone was confirmed by whole plasmid sequencing at the Pennsylvania State University Genomics Core Facility

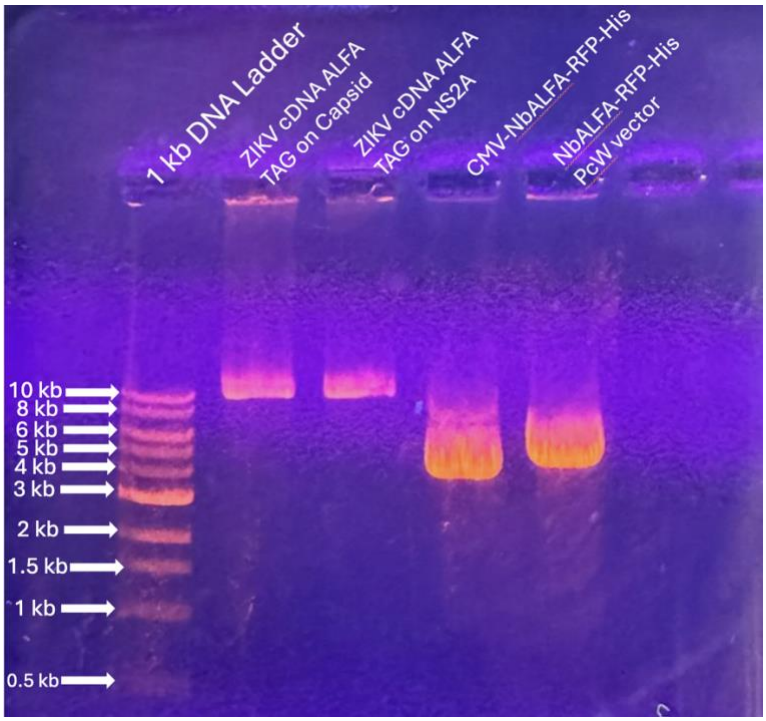

Figure 4: Agarose Gel electrophoresis of Recombinant Plasmid DNA Samples

(Fig 4). Agarose gel electrophoresis of samples. 1 kb DNA ladder was loaded in lane 1; ZIKV cDNA ALFA TAG on Capsid was loaded in lane 2; ZIKV cDNA ALFA TAG on NS2A was loaded in lane 3; CMV-NbALFA-RFP-His clone was loaded in lane 4; NbALFA-RFP-His PcW vector clone was loaded in lane 5.

Transfection of ALFA-tagged ZIKV clones on HEK-293T cells

To test the effect of adding the ALFA tag on the ZIKV Capsid or NS2A proteins, we transfected the plasmids into Human embryonic kidney (HEK-293T) cells and determined transfection efficiency and virus titers in the P0 supernatants. Cells grown in 24-well plates were transfected with the plasmids using PEI max reagent, and transfection efficiency was determined after 4 days of incubation using fluorescence microscopy. We observed green, fluorescent cells

in both experiments, which suggested successful transfection. P0 stocks were harvested and stored at –80°C for determining virus titers.

The P0 stocks collected from Clone #2586 (ALFA tag on Capsid) were added to fresh VeroE6 cells and observed on a fluorescence microscope after 4 days of incubation. We did not observe green fluorescence in these cells, suggesting the ALFA tag on the C terminus of the capsid did interfere with the virus assembly process. Next, we tested the clone with the ALFA tag on NS2A protein for its effect on virus assembly. Successful transfection was confirmed by fluorescence microscopy. The P0 stocks were collected from another batch of fresh VeroE6 cells and were observed using a confocal microscope after 4 days of incubation. We did not observe fluorescence in these cells, suggesting that the ALFA tag on the NS2A protein also interferes with virus assembly.

Co-transfection of ALFA-tagged ZIKV clones with the CMV-Nb-RFP-His

We assessed the binding between ALFA-tag nanobody to ALFA-Tag capsid or NS2A proteins of ZIKV by confocal microscopy. We co-transfected the first HEK 293T cell plate with the CMV-Nb-RFP-His clone combined with the ZIKV with ALFA-Tag and emerald fluorescent protein on NS2A protein (Fig 3). The second plate was co-transfected with the same CMV-Nb-RFP-His clone and with ZIKV ALFA-Tag clone and emerald fluorescent protein on capsid protein (Fig 4). When we observed the plates, we detected colocalization of red (Nb on ALFA-tag) and green (ZIKV Alfa-Tag clone) fluorescence, resulting in yellow fluorescence in the cells, indicating nanobody binding to the ALFA-Tag region on NS2A. However, further experiments should be performed to assess how effectively the nanobodies bind to the ALFTA-Tag region of

Zika Virus. We did not observe colocalization of red and green fluorescence (Fig 4), indicating that the ALFA tag on the capsid protein was not accessible to the nanobody. Further experiments must be conducted to understand why the ALFA tag was not accessible for the nanobody to bind to.

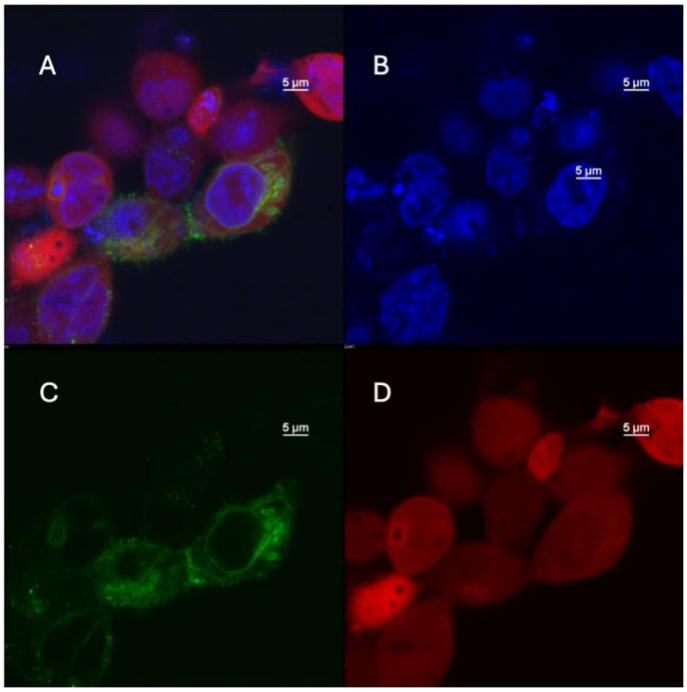

Figure 5: Co-expression of CMV-Nb-RFP-His with ZIKV ALFA-TAG on Capsid in HEK-293T cells

A). Merged micrograph indicating fluorescence in infected HEK293T cells with the CMV-NB-RFP-His (Red) with ZIKV-ALFA TAG on Capsid protein(green) . B). Blue fluorescence shows the cells' nucleus stained with Hoechst dye. C). The micrograph of green channel D). and red channel. Cells were visualized using fluorescence confocal microscopy at 60X magnification on a Nikon A1R microscope

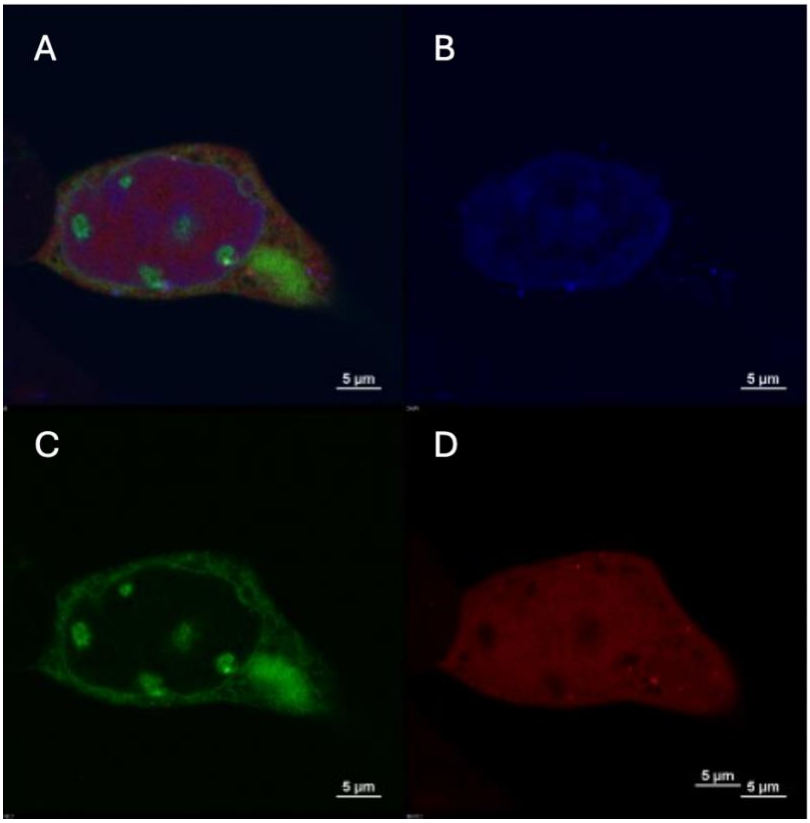

Figure 6: Transfection of HEK293T cells with CMV-Nb-RFP-His with ZIKV ALFA-TAG on NS2A Protein

A). Merged micrograph shows fluorescence in infected HEK293T cells with the CMV-NB-RFP-His (red) with ZIKV-ALFA TAG –Emerald on NS2A (green). B). Blue fluorescence shows cells' nucleus stained with Hoechst dye. C). green channel and D) red channel. Cells were visualized using fluorescence confocal microscopy at 60X magnification.

Transfection of NbALFA-RFP-His Tet On Vector

The NbALFA-RFP-His under the Tet-inducible system was tested for inducible expression of the nanobody. Human embryonic kidney (HEK-293T) cells were transfected with the NbALF-RFP-His with Tet-On Vector clones. Following transfection, we added 1ug/mL of anhydrotetracycline as the inductor to induce the gene expression. We took images of the cells before (Fig 7 A) and after induction (Fig 7B) using confocal fluorescence microscopy.

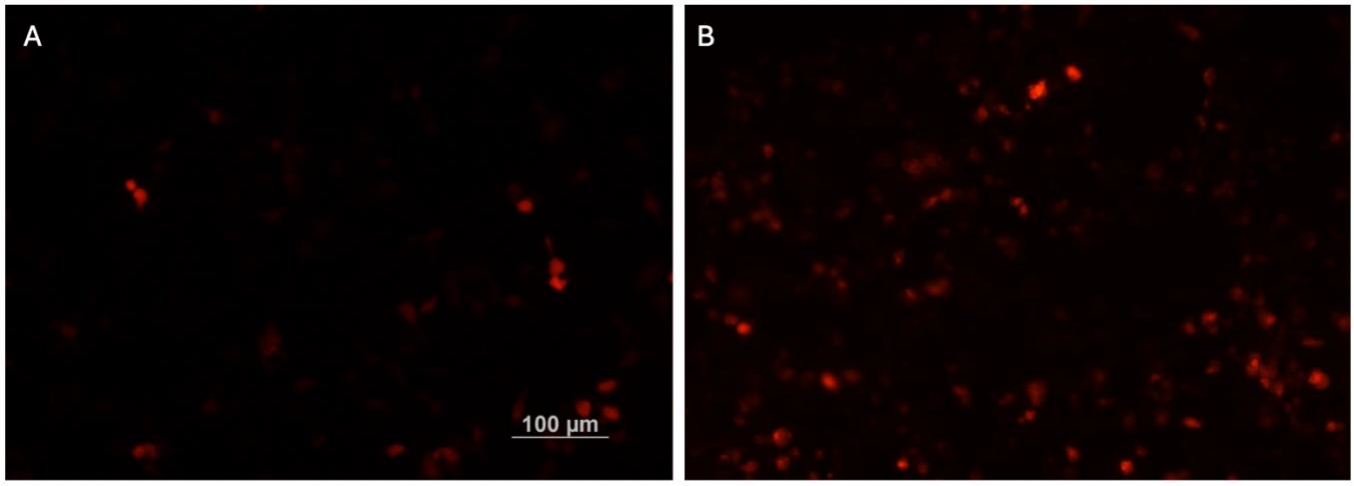

Figure 7: Transfection of NbALFA-RFP-His with Tet-On Vector in HEK293T cells before induction

A). Micrograph of transfections NbALFA-RFP-His with Tet-on Vector (Red) before induction. Red fluorescence indicates leaky expression of NbALFA-RFP-His in the Tet-On Vector. B). Micrograph of transfections NbALFA-RFP-His with Tet-on Vector (Red) after induction. Red fluorescence indicates expression of NbALFA-RFP-His

Our results show a baseline leaky expression of the nanobody in uninduced cells (Fig 5A). However, a substantial increase in the expression of Nanobody was observed upon induction of expression (Fig 5B). Further experiments should be conducted to help maximize the expression of Nanobodies under the Tet-inducible vector upon induction and minimize any leaky expression to improve the efficiency of this system.

Plaque Assay of ALFA-TAG ZIKV cDNA clones on Vero E6 Cells

Plaque assays of Alfa-Tag ZIKV clones were performed to assess if the ALFA tag interferes with virus assembly. Using the virus supernatant obtained from the P0 from each ZIKV ALFA-Tag clone after transfection, we tested for the virus by performing plaque assays on Vero E6 cells. No visible plaques were observed on the plaque assay for serial dilutions using either the ZIKV clones or control (Fig 7). Our data suggest that adding the ALFA-Tag on the

capsid and NS2A viral proteins could interfere with the viral assembly process. The only visible gap on the 51:1 is primarily due to a pipetting error when adding the P0 stock of the 51:1 ZIKV sample. However, since the control did not produce any plaques, the data obtained from these experiments is not valid, and therefore, further experiments should be conducted to assess this hypothesis.

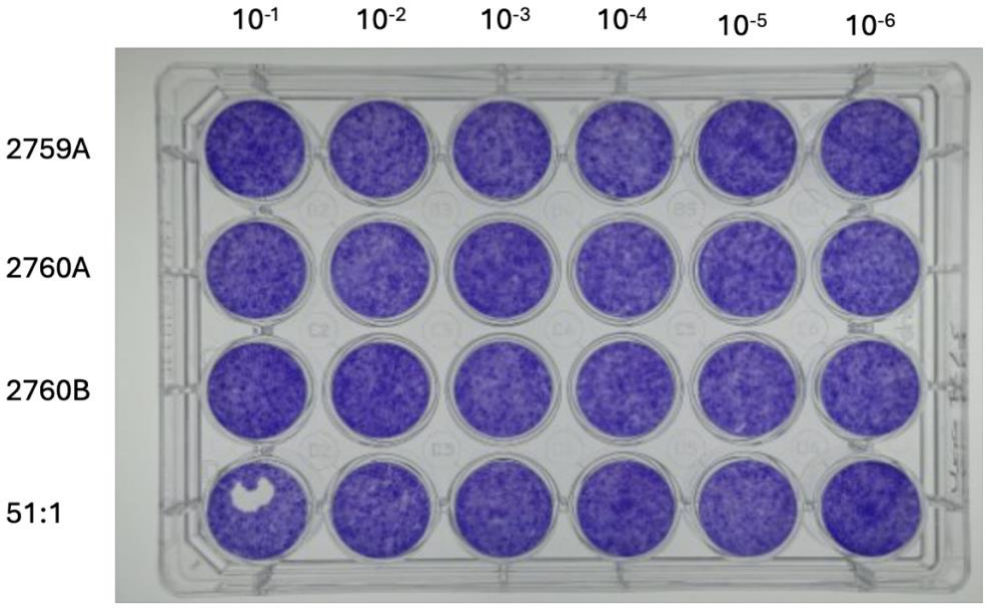

Figure 8: Plaque Assay of ZIKV ALFA-Tag Clones on Vero E6 cells.

P0 stocks from each virus stock were used to create Plaque assays on Vero E6 cells. Serial dilutions of each stock were performed to transfect the cells; Cells were incubated for 4 days, followed by fixation, 1% PBS wash, staining with Crystal violet, and a wash with water.

Chapter 4

CONCLUSION

Sequencing data revealed the successful introduction of ALFA-Tag in both the NS2A protein and Capsid protein of the Zika virus. The transfection of these AFLA-Tag ZIKV cDNA clones was successful due to the appearance of green, fluorescent proteins under the fluorescence confocal microscope. However, when the plaque assay was conducted on VeroE6 cells using serial dilutions from the P0 stocks from each viral sample, we were not able to observe any plaques. This indicates that the ALFA-Tag attached to the capsid and NS2A proteins could inhibit or disrupt virion formation in infected host cells. Further studies and research should be conducted to identify new areas in the ZIKV genome to attach the ALFA-Tag sequence and stabilize virion formation. Another potential future direction would be to use the ALFA-Tag sequence on a different virus family.

Based on our sequencing data, we successfully subcloned the nanobody sequence into two different vector systems for expression: one under the CMV promoter and another under a Tet-inducible promoter. Using induction assays, we were able to confirm the successful expression of NbALFA-RFP-His under the Tet-inducible promoter following induction with anhydrotetracycline. However, leaky expression of NbALFA-RFP-His was observed due to the appearance of red, fluorescent cells before induction. Future studies and experiments should focus on optimizing this system to have a more efficient expression of nanobodies upon induction.

We also tested the binding between the ALFA-Tag nanobody and the ALFA-tag on ZIKV Capsid and NS2A proteins. Through co-transfections of each ZIKV cDNA ALFA-Tag

clone with the CMV-Nb-RFP-His clone. Colocalization of ALFA-Tag region on NS2A and CMB-Nb-RFP His clone was observed due to the appearance of yellow fluorescence because of the overlap of both green and red fluorescence. This indicates successful binding of the nanobody to the ALFA-Tag region on NS2A protein. However, no colocalization was observed in the co-transfection of the ALFA-Tag region on the Capsid and CMB-NB-RFP-His clone, indicating that the nanobody was not able to access the ALFA-Tag region on the capsid protein. Further experiments must be conducted to understand why the ALFA tag was not accessible for the nanobody to bind to, as well as to assess how effective binding between nanobody and ALFA-Tag region on NS2A was.

In conclusion, although certain aspects of the ALFA-Tag system need optimization. The ALFA-Tag system could potentially serve as a new method for visualizing Zika virus assembly inside host cells. However, the system needs to be optimized in multiple ways, such as investigating new areas to insert the ALFA-Tag in the ZIKV genome to allow virion formation and nanobody binding to the ALFA-Tag. Further experimentation should also be focused on improving the Tet-inducible system to promote more effective expression of nanobodies. However, perfecting this system and using it in conjunction with future studies, such as protein-protein interaction by pulled own studies, could bring new insights into the viral mechanisms employed by flaviviruses. Using new, versatile biotechnological tools, like the ALFA-Tag system, could help progress the research on future therapies and drugs to help aid patients/populations infected or affected by the spread of Zika Virus and other flaviviruses.

Chapter 5

APPENDIX

Table 1: Molecular Clones for Project

Clone #	Purpose	Forward (F) and Reverse (R) sequences (5' – 3')
2759/2760	ALFA Tag on NS1-NS2A ZIKV	F)# 4608 AGGAACTGCGTCGTCGCCTGACCGAACCAGACCACTTC TCTCTTGGAGTG (R)# 4609 CGACGACGCAGTTCCTCTTCCAGGCGAGATGGCATATG ATCGGTTGACCC
2586	SDM for adding ALFA-Tag to C term of ZIKV Capsid	(F)# 4275 AGAGGAACTGCGTCGTCGCCTGACCGAACCAGAGAGGA AGAGACGTGGCGC (R)# 4276 ACGCAGTTCCTCTTCCAGGCGAGATGGTTTCCTAGCATT GATTATT
2602	Lic primers for amplifying ALFA-NB mCherry His into PCW Lic Vector 1662-A tet vector	(F) #4269 CACACCATCTCACGGCCACCATGGAAAAGGAGTCTGGG GAAGTAC (R)# 4270 CTCACATTCCACGCTAGTGGTGATGGTGATGGTGATG
2302	Adding ALFA-Tag NB as CT mCherry-His Under CMV promoters	(F) #3720 TACTTCCAATCCAATGCCACCATGGAAAAGGAGTCTGG GGAAGTACAATTG (R) #3721 CTCCCACTACCAATGCCACAATCGTCATCGTCATCATCG GAGG

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Academic Vita | Juan-Rodrigo Solares

EDUCATION

The Pennsylvania State University **University Park, PA**
Bachelor of Science in Biochemistry & Molecular Biology 2021 - 2025
Schreyer Honors Scholar Dean's List: 2021-2024

RESEARCH EXPERIENCE

Jose Lab – *Undergraduate Researcher* – Penn State University August 2023 – Present
- Leading independent honors thesis work on visualization of Zika Virus using nanobodies and fluorescence microscopy
- Perform daily site-directed mutagenesis, PCR, gel electrophoresis, transformation, and DNA extraction experiments
- Design DNA primers for site-directed mutagenesis
- Conducted plaque, transfection, and infection assays
- Presented research findings at Penn State Undergraduate Exhibition 2025

Oliver Lab – *Undergraduate Researcher Intern* – Johns Hopkins Medicine May **-** July 2024
- Lead independent project on understanding the development of chronic spontaneous urticaria
- Performed daily cell culture harvests and stimulation experiments using wide array of stimulants (IL-4, TNF-α, PGD2)
- Performed flow cytometry experiments on stimulated cells
- Isolated, purified and stimulated neutrophils and basophils
- Presented research findings at Pulmonary and Critical Care Medicine (PCCM) Symposium,
- Obtained over 50 hours of shadowing in an immunology clinic at Johns Hopkins Medicine
- Presented research findings at 2024 Eberly College of Science Undergraduate Poster Exhibition, SACNAS 2025 Research Conference, and ABRCAMS 2025 Research Conference
- Won "Outstanding Presenter" Award at SACNAS 2025 Conference among 1,000+ other student presenters

Goncalves Lab – *Undergraduate Researcher Intern* – Weill Cornell Medicine June – August 2023
- Lead an independent project on how fructose stimulation affects zonation patterns along intestinal villi.
- Performed immunohistochemistry using 3 antibodies: Lat2 (*Slc7a8),* GLUT5 (*Slc2a5)*, and Lysozyme
- Operated brightfield microscope and imaged and scanned over 2,000 pictures using ImageJ.
- Presented research at the Leadership Alliance National Symposium and the Annual Gateways Symposium
- Completed 30 hours of shadowing physicians in endocrinology, surgery, and internal medicine at New York-Presbyterian hospitals.
- Presented research findings at St. Jude Graduate School National Symposium for Predoctoral Research

Showalter Lab - *Undergraduate Researcher* – Penn State University January 2022 – May 2023
- Researched Pancreatic Duodenal Homeobox Domain 1 (PDX1), a transcription factor that helps regulate insulin levels in the pancreas/
- Constructed DNA and PDX1 binding assay through protein binding experiments using fluorescence anisotropy and presented findings at the 2022 Eberly College of Science Undergraduate Poster Exhibition

- Created binding assay graphs using python on the Spyder Platform
- Performed rounds of protein purification using size-exclusion and ion-exclusion columns
- Ran daily PCR, mutagenesis, and gel electrophoresis experiments.

LEADERSHIP EXPERIENCE

Intro to Immunology (MICRB/VBSC 410) - *Learning Assistant* – Penn State August 2024 – December 2024
- Hosted weekly office hours to help students engage with material
- Proctored monthly mid-terms and makeup exams in a class section of over 100 students
- Helped students navigate and think critically of the material
- Worked alongside the professor to create material for students to work on during class
- Grade and revised 100 monthly exams in class section

Lion's Pantry - *President* - Penn State April 2024 - Present
- Lead a team of 11 executive members, 12 interns, and 10 pantry managers
- Manage a budget of over $400,000 used for pantry operations
- Delegate tasks and conduct productive meetings with executive members
- Host and lead executive team leadership retreat
- Structure and attend weekly 1-hour exec meetings
- Design and coordinate 2 major fundraising events
- Serve as board member on 3 boards: Student Round Leadership Table, Student Wellness and Health advisory board, and Student Sustainability board

Lion's Pantry - *Vice President* - Penn State May 2023 – May 2024
- Co-lead a team of 13 executive members, 12 interns, and 10 pantry managers
- Manage a budget of over $130,000 used for pantry operations
- Delegate mandatory officer trainings at the beginning of each school year
- Organize new member orientation for incoming pantry managers and volunteers
- Structure and attend weekly 1-hour exec meetings
- Led 23 food drives to help stock the main pantry facility
- Hosted 2 major service collaborations

Lion's Pantry - *Cub Pantry Coordinator & Pantry Manager* August 2021 – May 2023
- Coordinated, managed, and communicated with over 12 different pantries on-campus
- Set up an online ordering system to help restock the on-campus pantries
- Organized 2 shifts at the main pantry with over 12 to 20 volunteers
- Raised awareness of food insecurity with promotion and participation in on-campus food drives

Lion Ambassador - *Diversity, Equity, & Inclusion Chair & Member – Penn State* June 2023 – May 2024
- Coordinated 8 different on topics related to DEI throughout the school year
- Arranged several external events with different multicultural groups
- Engaged and created a strategic plan for the organization
- Planed and lead school spirit events
- Participating in multiple service events across campus and State College
- Gave private tour to families and/or influential alumni

Lion Ambassador - *Touring Education Chair* November 2023 – March 2024
- Created teaching material on touring for new members
- Led a 3-hour teaching session for new members on touring information and procedures
- Evaluated 40 new members on touring
- Collaborated with different multicultural offices to include more resources on touring material

HONORS

Millennium Scholars Program Recipient: The Penn State Millennium Scholars Program is a merit-based scholarship designed for high-achieving Science, Technology, Engineering and Math (STEM) students who will become leaders in their chosen fields and who are committed to pursuing STEM-related discipline. Millennium Scholars are students with both the passion and the ability to advance the frontiers of knowledge and change the world.

Penn State Presidential Leadership Academy: A selective program that fosters leadership fundamentals necessary for solving complex issues. The program promotes the exploration of different viewpoints and inductive and deductive thinking through civil discourse during class taught by Dr. Neeli Bendapudi, the President of The Pennsylvania State University.

The Braddock Scholarship Recipient: A $10,000 awarded to high school seniors interested in studying biological sciences at Penn State's University Park campus. This award is given to high achieving students with overall outstanding applications consisting of "strong essay responses, high school performance, extra-curricular achievements, and letters of recommendation".