UNIVERSITY OF CALIFORNIA
RIVERSIDE

Intracellular Detection of Viral Replication of Dengue Virus Serotype 1 Using a Molecular Beacon Fluorescent Probe

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Microbiology

by

Katie Elouise Curnyn

December 2016

Thesis Committee:
Dr. Marylynn V. Yates, Chairperson
Dr. Sharon Walker
Dr. Quan (Jason) Cheng

The Thesis of Katie Elouise Curnyn is approved:

University of California, Riverside

Acknowledgements

I thank my Lord for providing me with the resources, support, love, peace and strength I needed to successfully complete my degree.

Dr. Marylynn Yates, thank you for giving me my first exposure to laboratory research and continuing your support into and throughout graduate school. You have allowed me to pursue my interests and to become an independent researcher and educator. Thank you to my committee members Dr. Sharon Walker and Dr. Jason Cheng, who have been encouraging and enthusiastic during my entire research project. Thank you to all the professors who have served as both excellent educators and mentors. Thank you Dane Reano for training me in laboratory techniques and research fundamentals. Daniel Wasik, thank you for your collaborative spirit and companionship, our research conversations have been irreplaceable.

Thank you to my family and friends for your incredible emotional and financial support. Particularly my parents Gary and Patricia Brodersen, and my Uncle and Aunt Ray and Sherri Gosselin. Thank you for remaining strong and believing in me, especially in the moments when I was not. Thank you to Ryan Foltz, you taught me more advanced statistical analysis methods and how to write code to analyze my data and for that I will forever be indebted to you. Even more importantly, your love has liberated me into the person I am today and inspires me to continue to pursue my greatest potential.

Thank you to my funding sources; Water Sense IGERT NSF fellowship, UCR Graduate Division Dean's Fellowship, and USDA Hatch funding.

ABSTRACT OF THE THESIS

Intracellular Detection of Viral Replication of Dengue Virus Serotype 1 using a Molecular Beacon Fluorescent Probe

by

Katie Elouise Curnyn

Master of Science, Graduate Program in Microbiology
University of California, Riverside, December 2016
Dr. Marylynn V. Yates, Chairperson

Dengue virus (DENV) is currently the most prevalent arbovirus, causing an estimated 390 million new infections and 500,000 deaths each year in tropical and subtropical regions. The economic burden of medical care, missed work and school days, and mortality has been approximated to be $950 million per year, although recent research indicates this number is higher as a significant number of patients have begun to report prolonged fatigue following infection. The development of rapid, sensitive, and accurate methods for its detection are greatly needed to aid in the treatment and prevention of the disease, such as the development of antiviral drugs, vaccines, and vector control. This work has resulted in the development of two different molecular beacons (MB) fluorescent probes specific to conserved regions of the DENV1 genome. For both MBs, results show a significance increase in fluorescence intensity in the presence of the target viral genome. MBs have a wide range of applications and can be a powerful tool for the detection of viral replication, with high sensitivity and specificity, in a shorter amount of time than traditional methods.

Table of Contents

Table of Figures

1.0 Introduction to the Dengue Virus

1.1 Virus Replication

Dengue virus (DENV) belongs to the family *Flaviviridae*, the same virus family as Yellow Fever and West Nile viruses. DENV is an enveloped virus with a single-stranded positive sense RNA (+ssRNA) genome of 10.7kb with 5' and 3'untranslated regions. The genome is translated into a single polyprotein and post-translationally cleaved by viral and host proteases into ten proteins. Three of these proteins are structural; the other seven are non-structural and have a variety of functions in the host cell (Knipe & Howley, 2013). A virion adsorbs to and enters a cell via clathrin-dependent receptor-mediated endocytosis; this forms an endosome containing the entire intact virion. Endosome formation recruits lysosomes, which are vesicles containing reactive oxygen species as one of the cell's defense mechanisms to kill and destroy pathogens. The lysosome fuses with the endosome, causing a rapid change in pH, forming the endolysosome. The virion undergoes a conformational change upon this acidification. This conformational change results in fusion of the virion envelope to the endolysosome membrane, which then releases the viral genome into the cytosol (Welsch, et al., 2009). Electron microscopy shows endosomes fuse with lysosomes at 24 hours post infection (hpi); this stage of digestion is found up to 72hpi (Barth, 1992). Following the genome release into the cytosol, the +ssRNA locates to the rough endoplasmic reticulum (ER) and induces invagination, forming a vesicle with a pore opening to the cytosol. Once formed, the vesicles are the sites of both translation and replication (Welsch, et al., 2009). Maturation and release occur in the Golgi apparatus.

1.2 Epidemiology and Pathology

Dengue virus (DENV) is currently the most prevalent arbovirus, causing an estimated 390 million new infections and 500,000 deaths each year, and these numbers continue to increase exponentially each decade (Bhatt, et al., 2013) (Guzman, 2010). **Figure 1** illustrates this increase in the distribution and prevalence of disease. The economic burden of medical care, missed work days, and deaths has been approximated to be $950 million per year, although recent research indicates this number will be higher as a significant number of patients have begun to report prolonged fatigue following infection (Shepard, et al., 2013).

All four serotypes of dengue virus (DENV1-4) are capable of causing a range of disease from asymptomatic to severe dengue classically called dengue hemorrhagic fever (DHF) (Knipe & Howley, 2013). Patients with the febrile form of dengue fever typically experience a high fever (up to 105 F); a maculopapular rash typically located on the torso, headache, joint and bone pain. The pain can be quite severe and debilitating, hence the common name "break-bone fever." Approximately only 3% of patients infected with DENV for the first time will develop the severe form of disease, DHF, about 3-5 days after appearance of the initial symptoms (CDC, 2014). DHF is characterized by three criteria: vascular permeability resulting in plasma leakage, abnormalities in hemostatic responses including thrombocytopenia (low blood platelets), and a bleeding diathesis (unusual susceptibility to hemorrhage and slowness to heal) (Rothman, 2004). If significant plasma loss occurs, hypotension and circulatory collapse (shock) will occur, usually resulting in death. DHF mortality rate can be greatly reduced to 2-5% with

supportive therapy (Guzman, 2010). Secondary infections (SI) occur when a patient becomes infected with a second serotype. SIs greatly increase a patient's risk of developing DHF. In some outbreaks, all individuals with DHF were a result of a SI, yet, not all patients who experience a SI will develop DHF (Guzman, et al., 2013).

There are many proposed mechanisms for DHF, all of which trigger an excessive inflammatory immune response. The most widely accepted mechanism is antibody dependent enhancement (ADE). ADE mainly occurs upon a SI. The antibodies that the patient has from the primary infection bind to the virus of the SI, forming an immune complex that is recognized by FcyRI and FcyRII (Fc gamma receptors on certain immune cells bind to the Fc portion of antibodies). This binding facilitates the uptake of the virus complex into the host cell (Green, et al., 2014). Usually, these cells would function to provide immunity against the antibody-trapped pathogen, however, DENV evades these mechanisms once inside the immune cells and infects them. Antibodies have also been documented in the activation of the complement pathway, which has been shown to cause plasma leakage. However, since a very small percentage of patients experience DHF upon primary infection, ADE cannot be the only responsible mechanism (Rothman, 2011). The precise mechanisms that lead to DHF remain unknown, however, it appears there are many complex interactions that take place between the virus and the host that lead to the activation of many pathways with pro-inflammatory responses. Factors such as age, geographic location and serotype order also play major roles. All of these immunological phenomena require a vaccine that protects against all four serotypes with high efficacy; anything less would place individuals at greater risk for developing DHF.

1.3 Vector and Distribution

DENV and its mosquito vector are endemic to over 100 countries in tropical and subtropical regions, including Southeast Asia, Africa, the eastern Mediterranean, the Western Pacific, and much of Latin America. *Aedes aegypti* and *Aedes albopictus* are the mosquito vectors for DENV, and continue to spread geographically due to climate change, world globalization (rapid/frequent transportation), crowding, rapid and unplanned urbanization, and poor water management (Harrington, et al., 2005). **Figure 2** displays the current global distribution of the virus as well as the regions where the vector is present. Everywhere that the vector is found are areas with potential for epidemics. Repeated epidemics place a region at high risk of becoming hyperendemic (co-circulation of multiple serotypes). *A. aegypti* and *A. albopictus* mosquitoes are found throughout the southern U.S., however, DENV is not endemic to the continental United States. There have been outbreaks of DENV in Texas and Florida, though these outbreaks were due to travelers and immigrants whose infections were acquired outside of the country (CDC, 2014). With the exception of Florida in 2010, where an outbreak of 66 cases in Key West was locally acquired, no Dengue cases have been reported since 2010 (Florida Department of Health, 2016).

The *A. aegypti* mosquito is also the vector for yellow fever (YF), however, this is not a large concern anymore since the discovery of an effective yellow fever vaccine. Prior to the YF vaccine, in the 1950's and 1960's a large and fairly effective mosquito eradication program targeted at *A. aegypti* eliminated the mosquito from 23 countries in Central and South America. However, once the program was stopped in the 1970's due to

concerns over the use of DDT, the mosquitos were able to immediately repopulate the region. Simultaneously, rapid urban growth was occurring, and epidemics of all four DENV serotypes from Asia occurred from 1977-1994, each becoming endemic thereafter (Gubler, 2011). It is during this period of hyperendemicity that epidemics of DHF began to emerge in the Americas. A similar pattern was observed in Pacific Islands, where DENV was not endemic, and DHF epidemics were not observed prior to hyperendemicity. After just a few decades of epidemics from Southeast Asia, the Pacific islands became hyperendemic with all four serotypes, and severe and fatal dengue is the leading cause of death in children to the present day (Gubler, 2011). The first reported cases of DHF epidemics were in countries of Southeast Asia following hyperendemicity due to World War II, and postwar urbanization in the 1950's (Gubler, 2011). DENV continues to be a major public health problem as there are no antiviral medications, no available vaccines, and DENV is increasing both geographically and in virulence (Guzman & Harris, 2015).

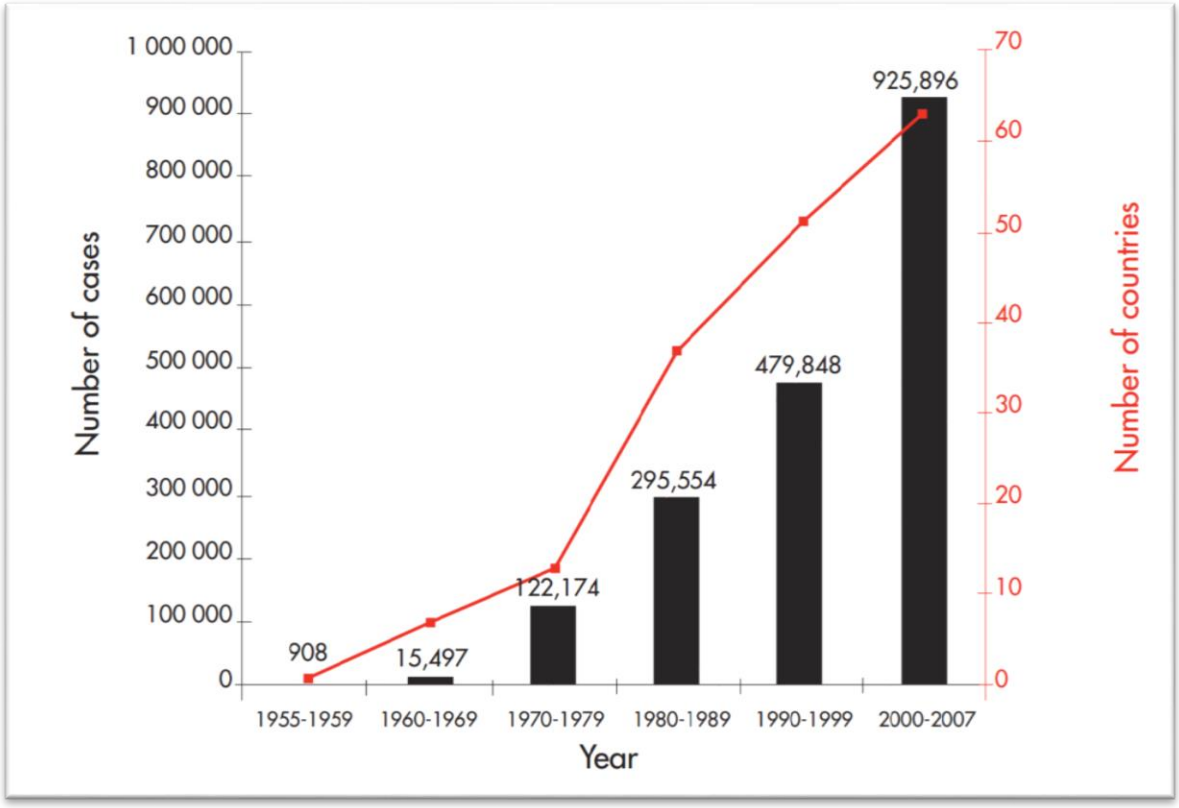


Figure 1Average Annual Number of DF and DHF Cases Reported to WHO, and of Countries Reporting Dengue, 1955–2007. Dengue: Guidelines for diagnosis, treatment, prevention and control. (2009). Geneva: World Health Organization

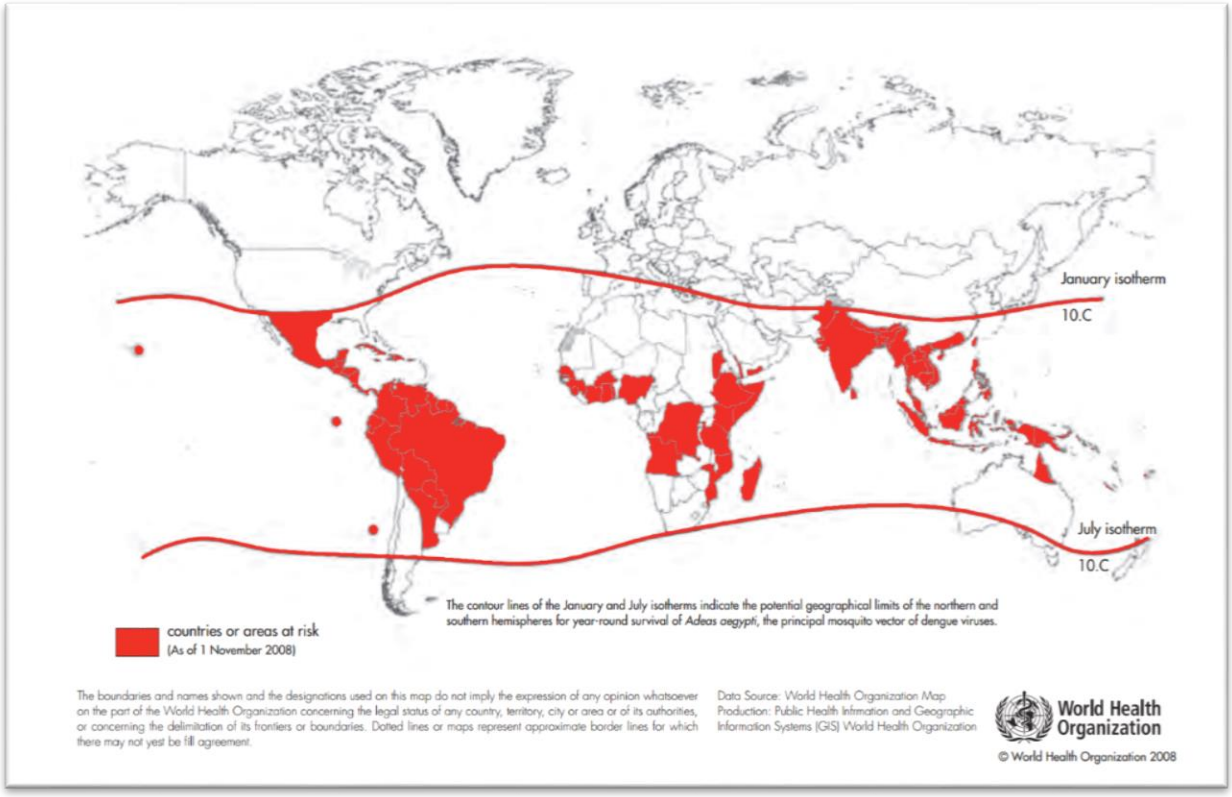


Figure 2 Countries/Areas at Risk of Dengue Transmission, 2008. Dengue: Guidelines for diagnosis, treatment, prevention and control. (2009). Geneva: World

2.0 Molecular Beacon Structure and Function

Current methods to detect viral infections include nucleic acid detection, virus culture, antigen detection, and serological methods. Plaque assays are beneficial because they allow one to determine viability and concentration of virus, however, they are highly technical and can take up to two weeks or more to perform, depending on the virus of interest. Another limitation of the plaque assay is that not all viruses form plaques, for example DENV1. Virus culture is limited still further, as some viruses do not show visible signs of infection, called cytopathic effects (CPE). DENV1 does form CPE, however, it requires 5-7 days to appear. Therefore, for these viruses that do not form plaques nor produce CPE, it would be impossible to differentiate between infected and non-infected cells under regular microscopy. Nucleic acid detection, mainly via PCR, is also highly technical and labor intensive. Although PCR is more rapid than virus culture, it cannot distinguish between infective and non-infective virus. Serological methods are less complex and much more rapid, however, they are also less sensitive and less specific than PCR and culture methods (Storch, 2000). **Figure 3** depicts the relationship between ease of the assay and its specificity.

A molecular beacon (MB) is a synthetic single-stranded oligonucleotide with a fluorophore on one end and a quencher on the other. It is designed such that it will fold into a hairpin shape with a loop and stem region as shown in **Figure 4**. The hairpin shape brings the quencher and fluorophore into close proximity, subsequently quenching the fluorescence by FRET (fluorescence resonance energy transfer) and releasing the light energy as heat. The target sequence, located on the loop portion of the MB, must be

designed such that the hybrid structure of the target and probe is energetically favorable over that of the hairpin loop structure. When the loop sequence comes into contact with the target viral genome sequence, it will hybridize, causing the separation of the quencher and fluorophore. Therefore, fluorescence is an indication of the presence of viral genome.

MBs have been used for many applications, such as RT-PCR and detection of intracellular replication of viruses (Yeh, et al., 2008) (Galil, et al., 2004). A unique strength of MBs is the ability to use them for *in situ* studies. When the MB is introduced inside of cells, if virus replication is occurring it can be detected, even before the signs of CPE occur. The MB can be functionalized with a cell-penetrating peptide (CPP), such as the TAT protein, which provides nearly 100% intracellular delivery of the MB without membrane electroporation or the use of harsh chemicals (Nitin, 2004). CPP-MBs allow for real-time intracellular detection of viral replication in living cells. One potential problem with using MBs to detect virus replication *in situ* is the stability of the beacon. For real time *in situ* imaging, a beacon would be enzymatically degraded by host endonucleases in a short period of time. A modification to the sugar-phosphate backbone structure of the beacon will change the target site of the endonuclease and prevent degradation, allowing it to remain intact for at least 12 hours (Yeh, et al., 2008). However, if fixation and permeabilization of cells is the method of choice, then no modifications to the MB are necessary.

In addition to detecting intracellular viral replication, another benefit of MBs is their high specificity. MBs are documented to have single nucleotide specificity; neither a single nucleotide change nor a single nucleotide deletion on an otherwise perfect

complementary strand results in hybridization (Tyagi, 1996). Although MBs are not particularly bright, they have a remarkable difference in fluorescence between the background and the MB-target hybrid: as much as a 100-fold increase has been documented (Marras, et al., 2005).

Another benefit of MBs is that one can design the target sequence to be highly specific to one strain or serotype, or, to be more general to a group or family of viruses that share a conserved sequence. The ability to distinguish between serotypes is particularly useful with viruses like DENV, where it is vital to monitor the serotype distribution.

The objectives of this research are to design two MBs specific to DENV1, to test the MBs *in situ* and to create a means to analyze the fluorescent images for total fluorescence. The total fluorescence will be used to determine whether or not the MBs can detect the presence of the target, and to test if the MBs can be used in an assay to detect virus replication.

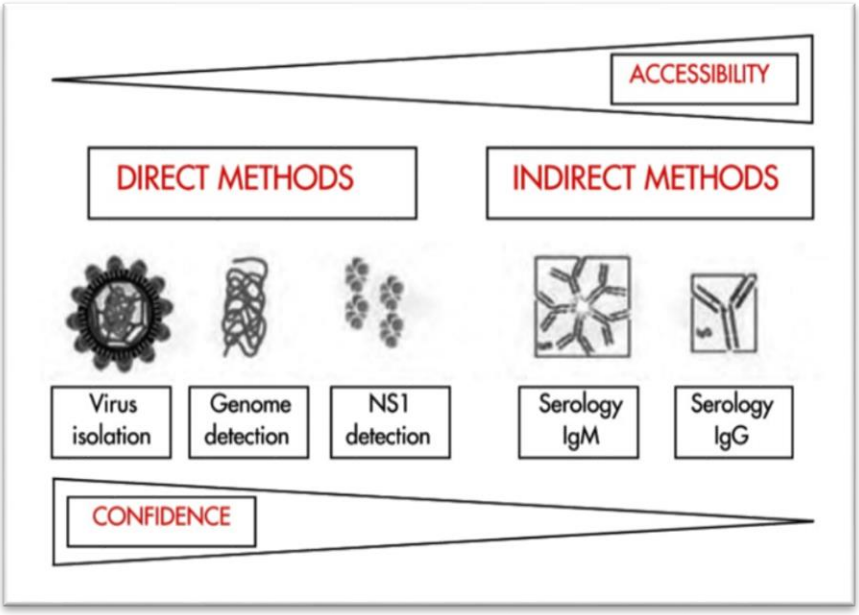


Figure 3 Comparison of Diagnostic Tests. Dengue Guidelines for Diagnosis, Treatment, Prevention and Control: Geneva: World Health Organization; 2009

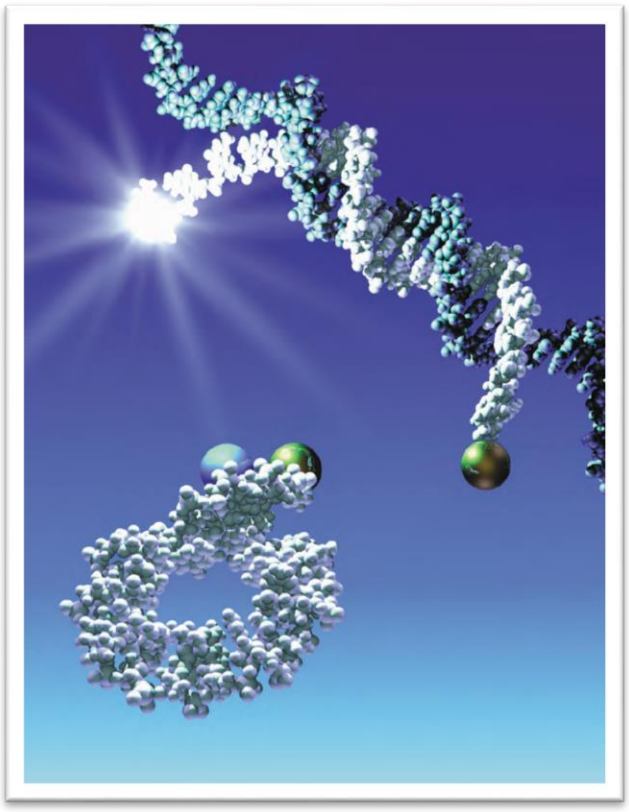


Figure 4 Molecular Beacon Structure and Function. A chemical model of a MB folded into a hairpin loop with a fluorophore and quencher in close proximity to each other resulting in no fluorescence (bottom) and hybridization of the loop sequence to the target sequence on a strand of genetic material, causing separation of the quencher from the fluorophore, resulting in fluorescence (top). Image permission from Public Health Research Institute Rutgers, State University of New Jersey.

3.0 Materials and Methods

3.1 Beacon Design

Reference sequences for all four serotypes of dengue virus were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), and a sequence alignment was performed on these four sequences using Clustal Omega (http://www.clustal.org/). Only sequence regions that were unique to DENV1 were used as candidate MB sequences. To insure these regions were conserved in DENV1 strains and not found in other bloodborne pathogens, the sequences were run through NCBI's basic local alignment search tool (BLAST). Predictions of the folding and thermodynamics of the different sequences were obtained using mfold (http://unafold.rna.albany.edu) and IDTfold (https://www.idtdna.com/UNAFold) webservers, specifically, to see if they form the stem-loop structure and no secondary structures under the precise experimental molar NaCl and MgCl2 conditions. Mfold and IDTfold were also used to predict the melting temperature of the hairpin loop. Oligocalc (http://biotools.nubic.northwestern.edu/) was used to measure the melting temperature of the hybridized loop sequence to the target RNA. Three sequences were found to meet all these criteria, and two were chosen for study. The fluorophore 6-FAM and the quencher Dabcyl-Q were used for both MBs.

MB-1 sequence is 5' [6FAM]CCATACATTGTTAAGTTTCTTTTCGTATGG-[Dabcyl-Q] 3' where the underlined regions are the arm sequences, and the middle is loop sequence which is the reverse compliment to DENV1 nucleotides 5074-5091, the nonstructural protein NS3 protein coding region. MB-2 sequence is 5' [6FAM] -

CAATATCGCAGTTGTTCCATGTTCTGATATTG[Dabcyl-Q] 3' where the loop sequence is the reverse compliment to DENV1 nucleotides 1402-1420 the envelope protein E coding region.

3.2 Cell Culture

Cell culture of Vero cells, purchased from American Type Culture Collection (ATCC) CCL-81, was performed in accordance with ATCC recommendations. Briefly, cells were grown in media made from minimal essential media (MEM) powder with glutamate (Corning Cellgro, Manassas VA), supplemented with 10% fetal bovine serum FBS (Hyclone, Logan UT), 1M HEPES pH 7.4, 1% penicillin 100 units/mL (Hyclone, Logan UT), 1% streptomycin 100 units/mL (MP, Solon OH), and sodium bicarbonate per manufacturer's directions (Fisher Scientific, Fairlawn NJ). The medium was adjusted to pH 7.3 and then filter sterilized and stored at -20°C. Cells were grown to 90% confluence in monolayers in T-75 cell culture flasks (Corning Cellgro, Manassas VA) incubated at 37°C with 5% CO_2 and humidity, rinsed with 1x PBS to remove cell debris prior to splitting with 0.25% trypsin in 0.02% EDTA (VWR, Radnor PA). Equal parts of media were used to neutralize the trypsin. Cell stocks of passage numbers below 10 were stored at -80°C. Low passage numbers (10-40) were used for all work.

3.3 Virus Propagation and Stock Preparation

DENV1 VR-1586 Thailand and Philippines strain was purchased from ATCC. A T-75 culture flask of 90% confluent monolayer Vero cells was infected with DENV1for five days. The supernatant was harvested at 5 days post-infection and clarified by low speed centrifugation (10 minutes, 4,000 × G at 4°C). The cell debris pellet was discarded,

and the supernatant was further concentrated to create higher titer samples by ultracentrifugation in a 20% sucrose cushion in ultrapure H_2O for three and half hours at a speed of 100,715 x G at 4°C to pellet the virus. The pellet was suspended in PBS, aliquoted, and stored at -80°C. Viral stock titer was approximately 5.8 x 10^5 PFU/mL, as determined by TCID50 with Vero cells in a 96-well plate.

Human Poliovirus-1 was purchased from ATCC VR-1562. A monolayer of Vero cells at 90% confluence was infected overnight, and harvested by three cycles of freeze-thaw (-20°C for 30 minutes, 37°C for 20 minutes) for cell lysis. The cell solution and equal parts chloroform were added to 50-mL centrifuge tubes and vortexed on high for two minutes, followed by centrifugation at 2200 X G at 4°C for 15 minutes. The supernatant was homogenized and filter sterilized with a 0.45 PVDF syringe filter, and 250-µL aliquots were stored at -80°C. Viral titer was approximately 1.9 x 10^9 PFU/mL as determined by plaque assay in 6-well plates (Corning Cellgro, Manassas VA) using Vero Cells.

3.4 Reconstitution of Molecular Beacons and Oligonucleotides

Both MBs and complimentary oligonucleotides of the target sequence were ordered from Eurofins Operon (Louisville, KY) and were shipped salt-free and lyophilized. Each vial was centrifuged (table top for 2 minutes) and then reconstituted in 1x TE buffer with 1mM $MgCl_2$ pH 8.0 to make a100mM solution. Aliquots of 50.0µL were placed in black cryogenic tube and frozen at -20°C.

3.5 Molecular Beacon and Complimentary Oligonucleotide Control Test

8-well tissue culture-treated bottoms Lab-Tek II CC2 chamber slides (Fisher Scientific, NJ) were seeded with 400µL of 2.0x10^5/mL Vero cells in growth media and incubated overnight at 37℃ with 5% CO_2 and humidity. Cells were aspirated and washed twice with Tris buffered saline solution (TBSS). Cells were fixed with 300µL ice cold 100% methanol (Sigma-Aldrich, St. Louis MO) for 5 minutes, after which the methanol was aspirated. The methanol addition and aspiration was repeated once. Cells were rehydrated with 350µL TBSS for 5 minutes at room temperature, aspirated and then rinsed once with TBSS. Cells were permeabilized with 300µL 0.1% Tween in TBSS for 5 minutes at 4℃. Cells were aspirated and washed three times with 350µL TBSS. Cells were incubated with 100µL of 2µM MB and 2µM oligo in 1:1 ratio, for 1 hour at room temperature in the dark prior to imaging. All MB and oligo dilutions were made in 1:1 ratio of 2x Leibovitz (L-15) media without phenol red (Lonza, Walkersville MD) and Tris HCL with 1mM $MgCl_2$ at pH 8. The test was performed once, in triplicate. The 8-well microscope slide included one negative control well consisting of cells not treated with molecular beacon, nor virus.

3.6 Poliovirus and Molecular Beacon Confirmation of Specificity Test

8-well chamber microscope slides were seeded with 400µL of 2.0 $x10^5$ cells/mL in media made from MEM powder with glutamate, supplemented with 10 % FBS, 1M HEPES pH 7.3, 1% penicillin 100 units/mL, 1% streptomycin 100 units/mL, and 2.2g/L sodium bicarbonate per manufacturer's directions. The pH was adjusted to 7.3 and then the medium was vacuum filter sterilized and stored at -20℃. Slides were incubated overnight at 37℃ with 5% CO_2 and humidity.

All wells were aspirated by hand pipetting to avoid cell detachment throughout the procedure. After incubation, cells were washed with 450µL TBSS. Three wells were infected with 100µL of 1.9 $x10^3$ PFU/mL and another three with 100µL of 1.9 $x10^2$ PFU/mL, for one hour at 37℃, with rocking every fifteen minutes. Cells were aspirated and filled with 400µL MEM with 5% FBS, and incubated overnight at 37℃ with 5% CO_2 and humidity.

Wells were aspirated and cells washed twice with TBSS. Cells were fixed with 300µL ice cold 100% methanol for five minutes at room temperature, twice. Then 350µL TBSS was added for five minutes at room temperature, aspirated and cells washed once with 300µL TBSS. Cells were then permeabilized with 300µL of 0.1% Tween 20 in TBSS for five minutes at 4℃. Cells were aspirated and washed three times with 350µL TBSS. Cells were incubated for one hour at room temperature in the dark (covered in foil) with 100µL of 2µM MB. All MB dilutions were made in 1:1 ratio of 2x Leibovitz (L-15) media without phenol red and Tris HCL with 1mM $MgCl_2$ at pH 8. The poliovirus confirmation of specificity test was performed once, in triplicate. The 8-well microscope plate included one negative control well consisting of cells not treated with molecular beacon, nor virus; and one control well of cells treated with MB but not virus, from which the background fluorescence of the beacon was measured and is hereafter referred to as the control.

3.7 Dengue and Molecular Beacon Image Assay

8-well chamber microscope slides were seeded with 400µL of 2.0 $x10^5$ cells/mL in media made from MEM powder with glutamate 9.53g/L, supplemented with 10 %

FBS, 1M HEPES pH 7.3, 1% penicillin 100 units/mL, 1% streptomycin 100 units/mL, and 2.2g/L sodium bicarbonate per manufacturer's directions. The pH was adjusted to 7.3 and then the medium was vacuum filter sterilized and stored at -20℃. Slides were incubated overnight at 37℃ with 5% CO_2 and humidity.

Wells were aspirated and washed with 450µL TBSS (Tris buffered saline solution). Three wells were infected with 100µL of 5.0 x 10^3 PFU/mL and another three were infected with 5.0 x 10^2 PFU/mL virus for one hour at 37℃, with rocking every fifteen minutes. Cells were aspirated and filled with 400µL MEM with 5% FBS, and incubated for 72 hours at 37℃ with 5% CO_2 and humidity.

Wells were aspirated and cells washed twice with TBSS. Cells were fixed with 300µL ice cold 100% methanol for five minutes at room temperature, twice. Then 350µL TBSS was added for five minutes at room temperature, aspirated and cells washed once with 300µL TBSS. Cells were then permeabilized with 300µL of 0.1% Tween 20 in TBSS for five minutes at 4℃. Cells were aspirated and washed three times with 350µL TBSS. Cells were incubated for one hour at room temperature in the dark (covered in foil) with 100µL of 2µM MB. All MB dilutions were made in 1:1 ratio of 2x Leibovitz (L-15) media without phenol red, and Tris HCL with 1mM $MgCl_2$ at pH 8. All DENV experiments were performed in triplicate and repeated three times. Each 8-well microscope plate included one negative control well consisting of cells not treated with molecular beacon nor virus, and one control well of cells treated with MB but not virus, from which the background fluorescence of the beacon was measured for each trial and is hereafter referred to as the control.

3.8 Image Processing and Analysis

The Olympus IX71 inverted microscope equipped with a reflected fluorescence system (Olympus America, Inc., San Diego, CA) was used to take fluorescence and phase-contrast images. The 6-FAM-labeled fluorescent probe was detected by the filter set consisting of a D480/30 nm exciter and a D535/40 nm emitter (Chroma Technology, Bellows Falls, VT). All images were obtained by a ProgRes MF^{scan} monochrome CCD camera (Jenoptik, Rochester, NY). Fluorescence images and phase-contrast images were analyzed by the Image-Pro PLUS analysis software (Media Cybernetics, Inc., Bethesda, MD). All images were taken with the 20x objective. Exposure times are documented for each and are 250ms, 400ms, or 500ms.

To analyze the relative fluorescence of each image, and each set of images, a macro was written in Image-Pro Plus to output the bit map data for each image into a plain text file. The bit map contains each individual pixel value for the image. Pixel intensity is on a scale from 0-255, where 0 is black, and 255 is white light. Each image contains 1041 pixels. A Python code was written to import the plain text files and calculate the relative average fluorescence intensity, the standard deviation, perform a standard t-test, and to perform Bayesian Estimation statistical analysis. The full code and steps of the statistical analysis are given in detail in the appendix.

4.0 Results

4.1 Summary of Results

As expected, no fluorescence was detected for the wells consisting of cells only. All Dengue-infected cells have a significant difference (increase) in fluorescence compared to the control (cells with MB only). The complimentary oligo tests for MB-1 and MB-2 produced an 8.4-fold and 11.8-fold increase in fluorescence, respectively. Lastly, there is no difference in fluorescence for poliovirus-infected cells compared to the control.

4.2 MB-1 Dengue Trials

Low levels of fluorescence were detected in non-virus-infected cells treated with MB-1 only. High levels of fluorescence were detected with MB-1 in virus-infected cells. **Figures 5, 6, & 7** display the data collected for all three trials at 500ms exposure time; the individual points are individual images taken within the control well (no virus) and the three virus-infected wells. Trial-1 wells have a mean intensity of 62 +/- 10 and the control well has a mean intensity of 19 +/- 6. Trial-2 wells have a mean intensity of 36 +/- 8 and the control well has a mean intensity of 6 +/- 5. Trial-3 wells have a mean intensity of 127 +/- 12 and the control well has a mean intensity of 67 +/- 20. For all three trials, the virus-infected cells are significantly brighter than the control well with T-test $p<<0.01$. **Figure 8** illustrates the mean of all the images taken in a trial at a given exposure, with the mean of the control well subtracted, thus removing the background noise level of fluorescence from unbound MB and showing only the total fluorescence of the bound MB. **Figure 9** is a color composite image for Trial 1 to visually demonstrate the difference in fluorescence between the virus-infected cells and the control.

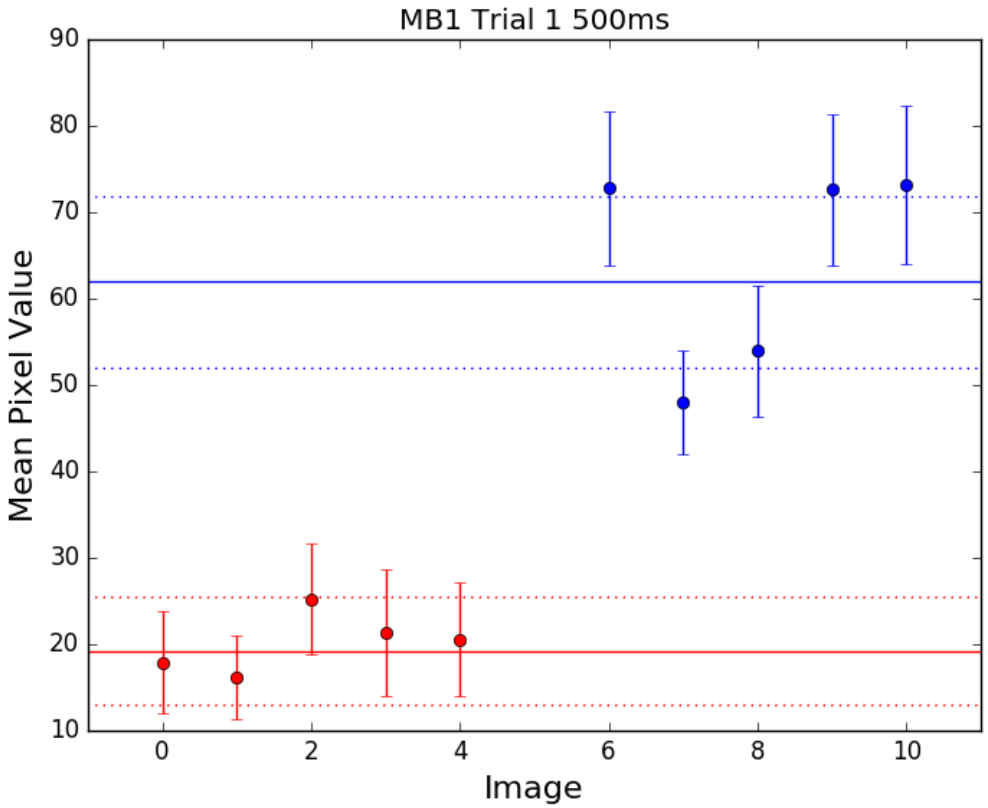


Figure 5 MB-1 DENV1 Trial 1 at 500ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 19, and the solid blue line is the mean of all images in the trial, which is 62.

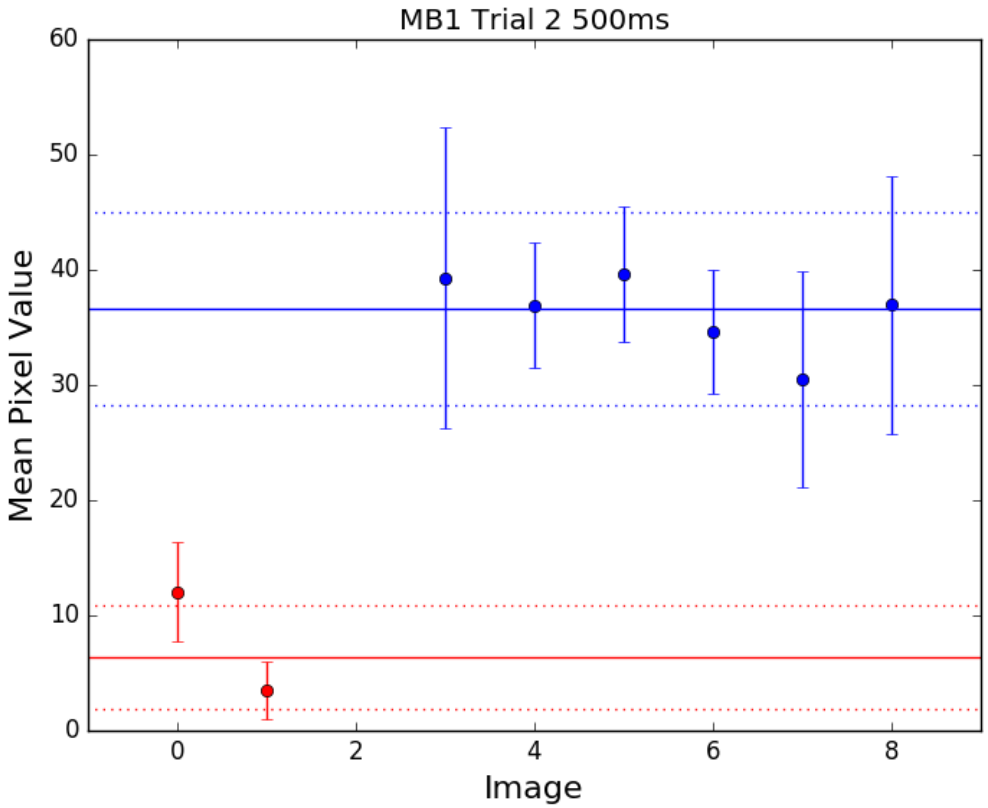


Figure 6 MB-1 DENV1 Trial 2 at 500ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 6, and the solid blue line is the mean of all images in the trial, which is 36.

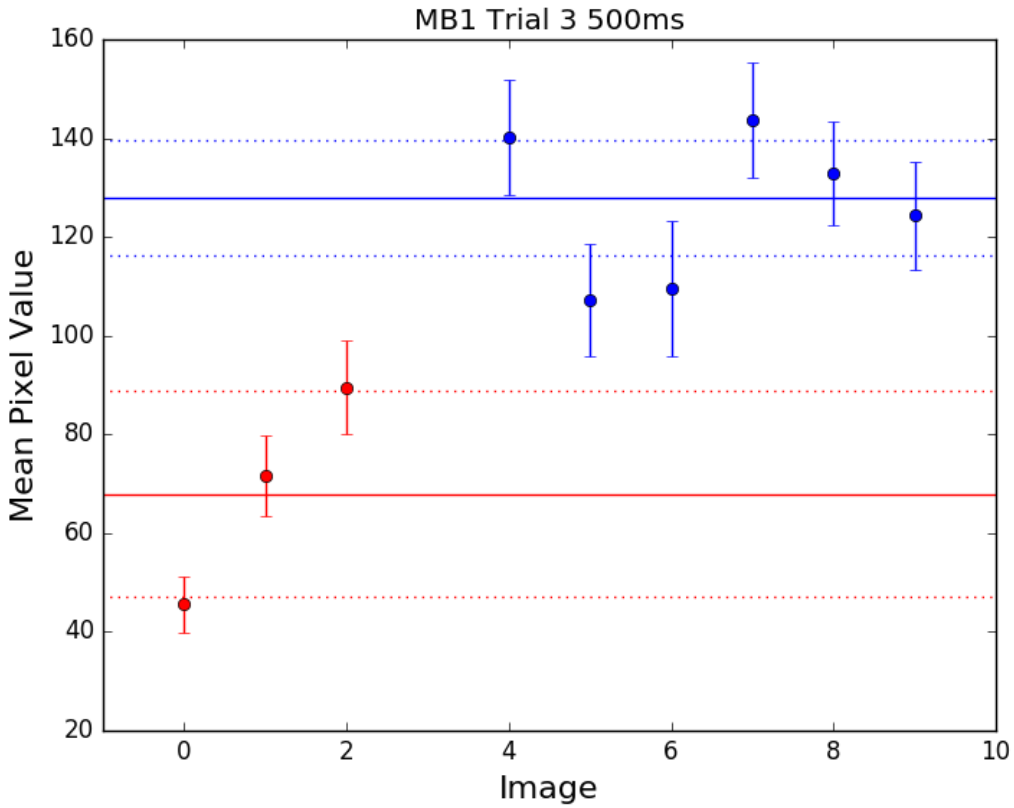


Figure 7 MB-1 DENV1 Trial 3 at 500ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 67, and the solid blue line is the mean of all images in the trial, which is 127.

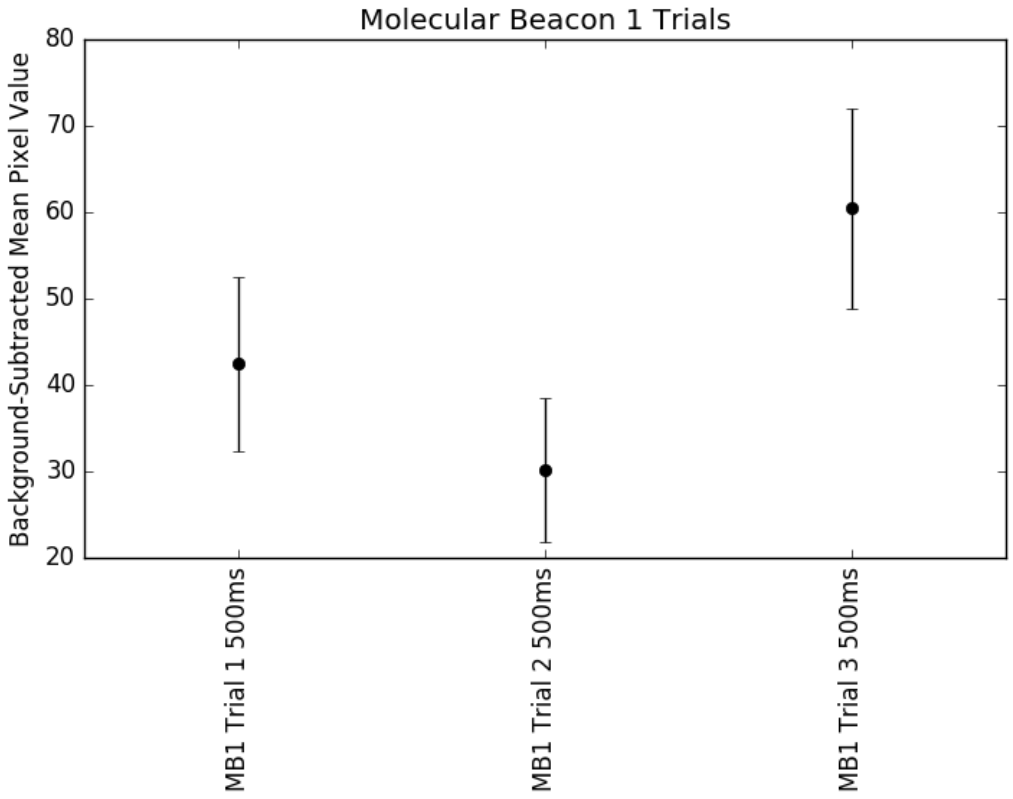


Figure 8 MB-1 Dengue Trials Total Fluorescence. Each point is the mean of all the images taken in a trail at the given exposure, with the mean of all the images of the control subtracted, thus removing the background noise and showing only the total fluorescence of the viral genome bound MB.

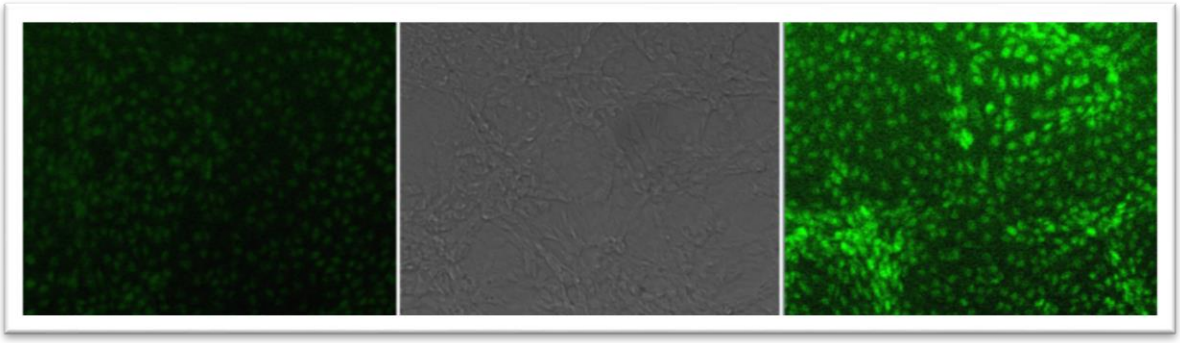


Figure 9 MB-1 Trial 1 Color Composite Image Cells treated with MB-1 only (Left). Cells not infected and not treated with MB under phase contrast microscopy (Middle). Cells infected with DENV1 and treated with MB-1 (Right).

4.3 MB-2 Dengue Trials

Low levels of fluorescence were detected in non-virus-infected cells treated with MB-2 only. High levels of fluorescence were detected in virus-infected cells treated with MB-2. **Figures 10 & 11** display the data collected for Trial 1 and Trial 2 at 500ms exposure time, and **Figure 12** displays the data collected for Trial 3 at 250ms. The individual points are individual images taken within the control well (no virus) and the three virus-infected wells. Trial-1 wells at 500ms exposure time have a mean intensity of 127 +/- 22 and the control well has a mean intensity of 87 +/- 8, T-test $p<0.05$. Trial-2 wells at 500ms have a mean intensity of 290 +/- 32 and the control well has a mean intensity of 196 +/- 23, T-test $p<<0.01$. Trial-3 wells at 250ms have a mean intensity of 125 +/- 11 and the control well has a mean intensity of 81 +/- 6, $p<<0.01$. **Figure 13** illustrates the mean of all the images taken in a trial at a given exposure, with the mean of all the images of the control well subtracted, thus removing the background noise level of fluorescence from unbound MB and showing only the total fluorescence of the bound MB. **Figure 14** is a color composite image for Trial 1 to visually demonstrate the difference in fluorescence between the virus-infected cells and the control.

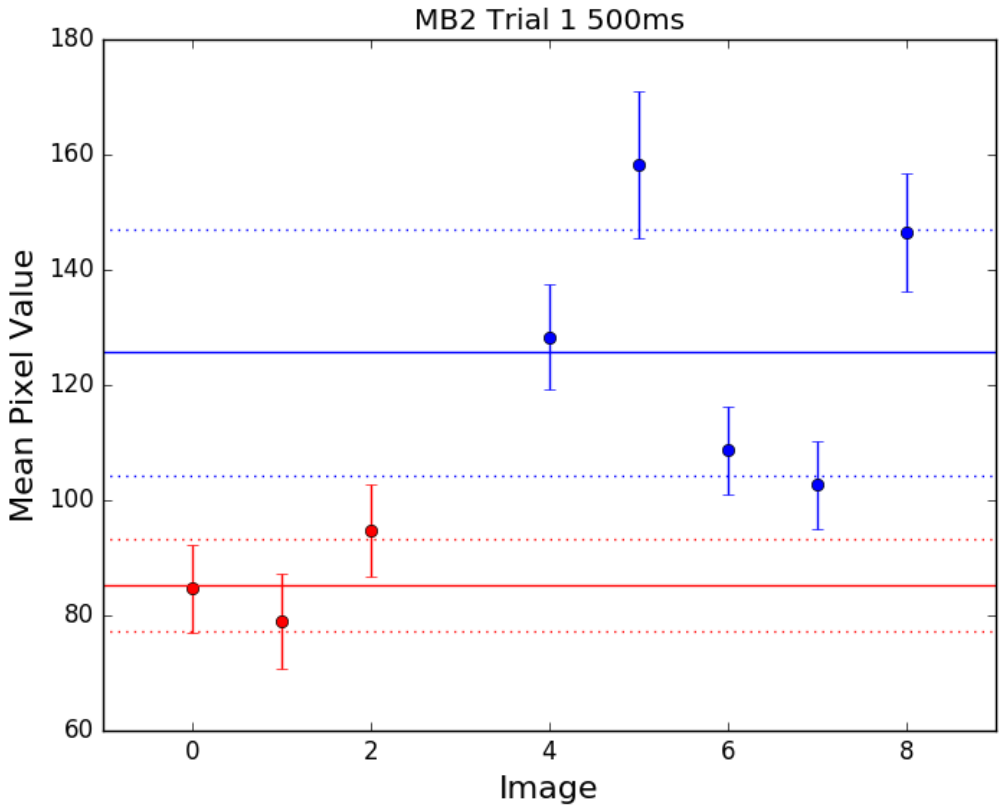


Figure 10 MB-2 DENV1 Trial 1 at 500ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus-infected cells. The solid red line is the mean of all images in the control well, which is 85 +/- 8, and the solid blue line is the mean of all images in the trial, which is 126 +/- 21.

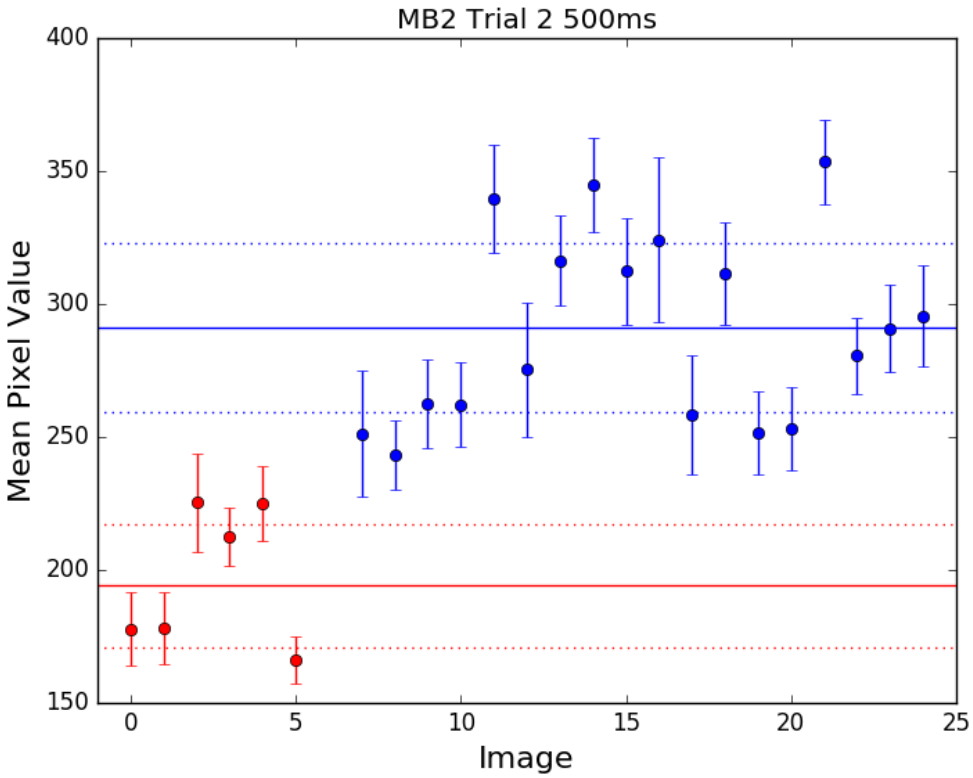


Figure 11 MB-2 DENV1 Trial 2 at 500ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 194 +/- 23, and the solid blue line is the mean of all images in the trial, which is 291 +/- 32.

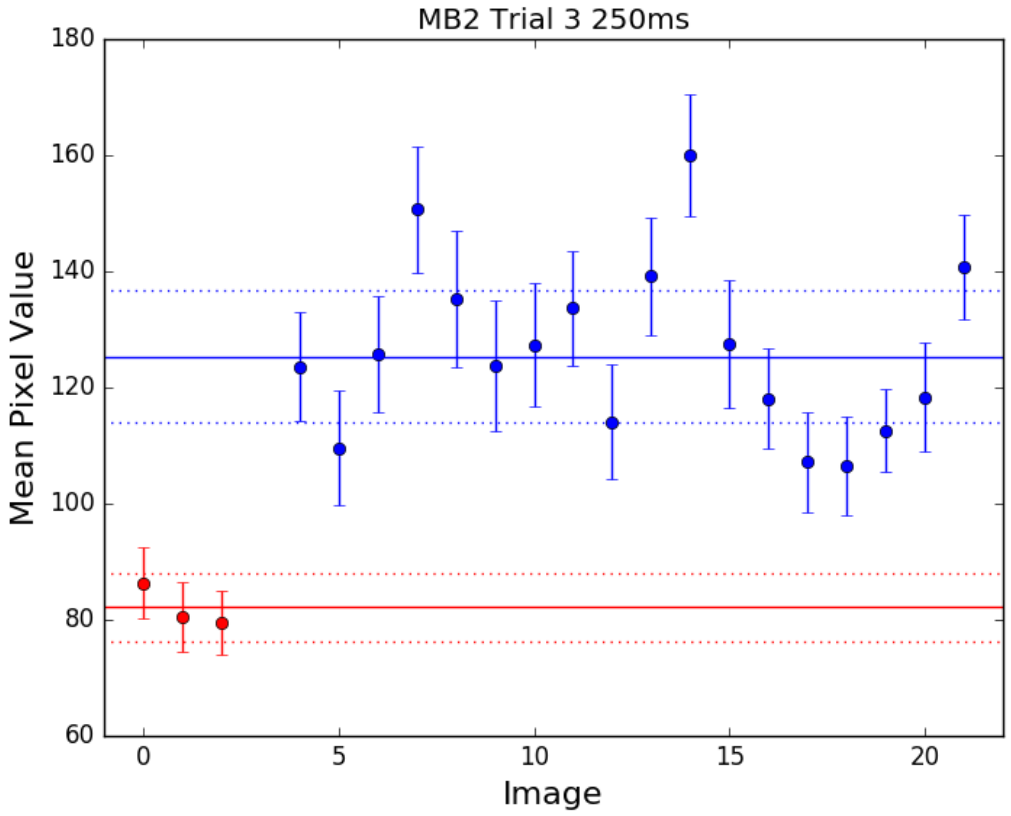


Figure 12 MB-2 DENV1 Trial 3 at 250ms Exposure Time. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 82 +/- 6, and the solid blue line is the mean of all images in the trial, which is 125 +/- 11.

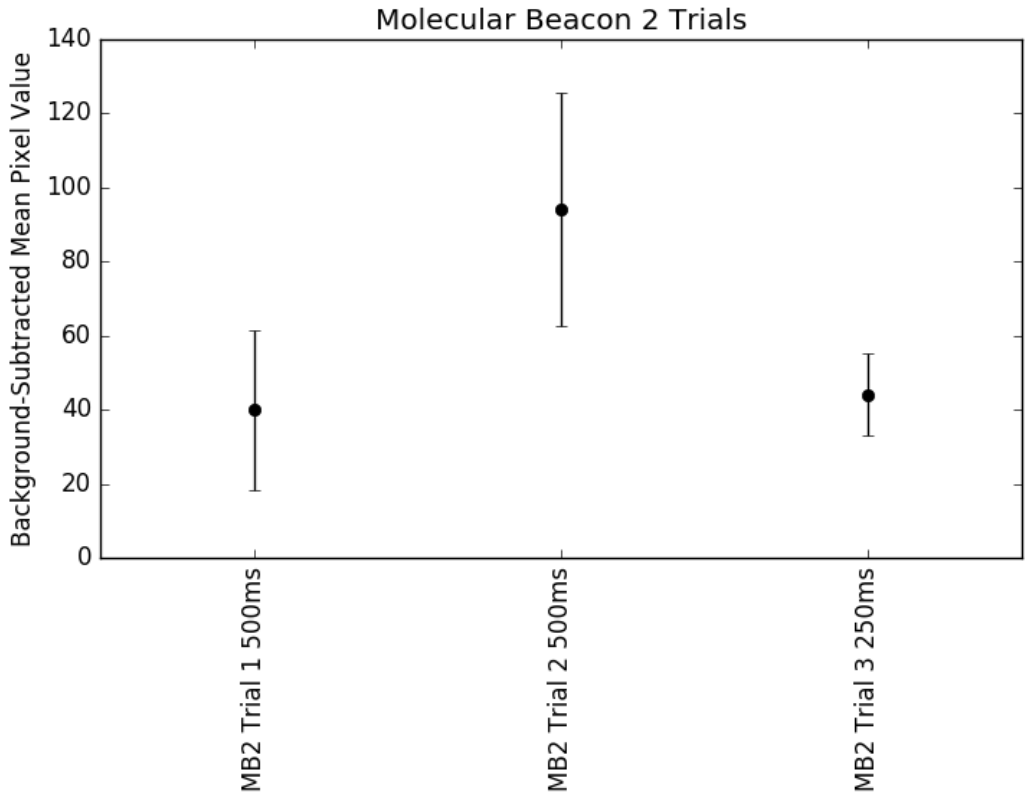


Figure 13 MB-2 DENV1 Trials Total Mean Fluorescence. Each point is the mean of all the images taken in the given trail at the given exposure. The mean of all the images of the control have been subtracted, thus removing the background noise and showing only the total fluorescence of the viral genome bound MB.

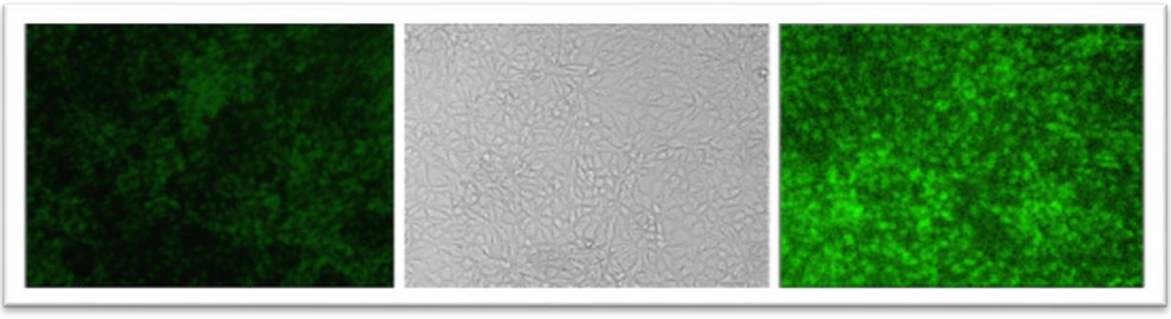


Figure 14 MB-2 Trial 1 Color Composite Image Cells treated with MB-2 only (Left). Cells not infected and not treated with MB under phase contrast microscopy (Middle). Cells infected with DENV1 and treated with MB-2 (Right).

4.4 Complimentary Oligonucleotide and MB-1 Test

An 8.4-fold increase in fluorescence was detected for MB-1 oligonucleotide-bound wells compared to the control well (MB only) as seen in **Figure 15.** Each point is an individual image; red points are the images taken within the control well and blue points are the images of all three wells treated with MB-1 and the complimentary oligonucleotide. All images were taken at 500ms exposure time. The mean intensity of the oligo-MB wells is 420 +/- 86 and the mean intensity of the control well is 50 +/- 23. T-test $p<<0.01$.

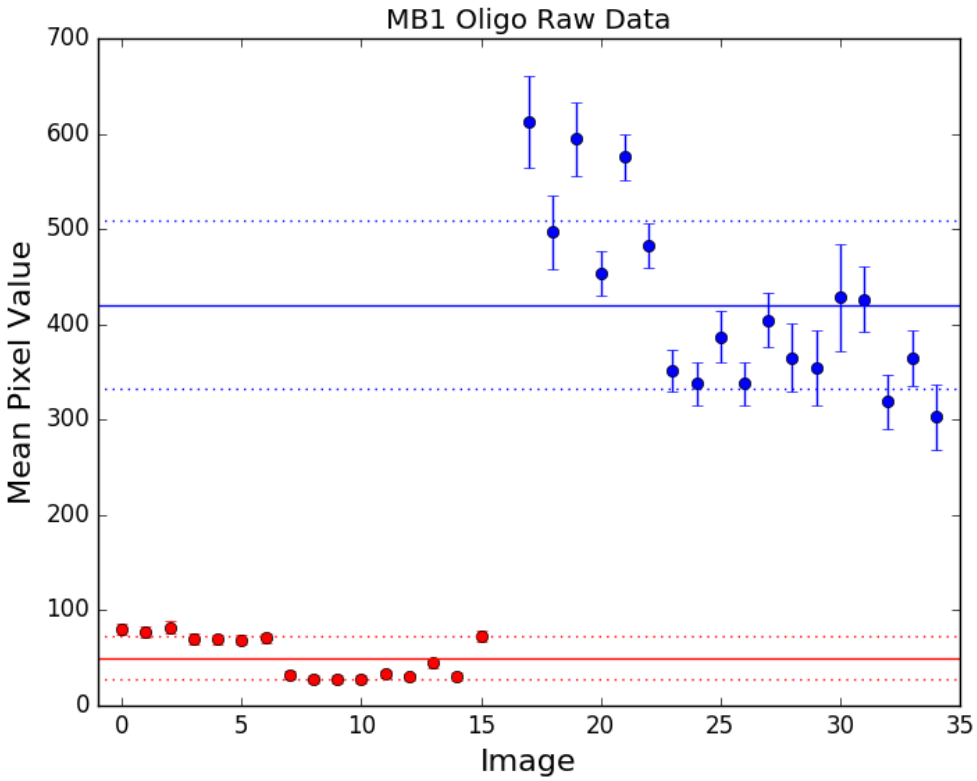


Figure 15 MB-1 and Complimentary Oligonucleotide Test. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no oligonucleotide) and blue points are the cells treated with MB-1 and the complimentary oligonucleotide. The solid red line is the mean of all images in the control well, which is 50 +/- 23, and the solid blue line is the mean of all images of all three wells in the trial, which is 420 +/- 86.

4.5 Complimentary Oligonucleotide and MB-2 Test

An 11.8-fold increase was detected between cells treated with complimentary oligonucleotide and MB-2, compared to cells treated with only MB-2. **Figure 16** displays the data, including the mean intensity of the control well is 91 +/- 14, and the mean intensity of the hybrid (MB-oligo) is 1073 +/- 106 (T-test $p<<0.01$).

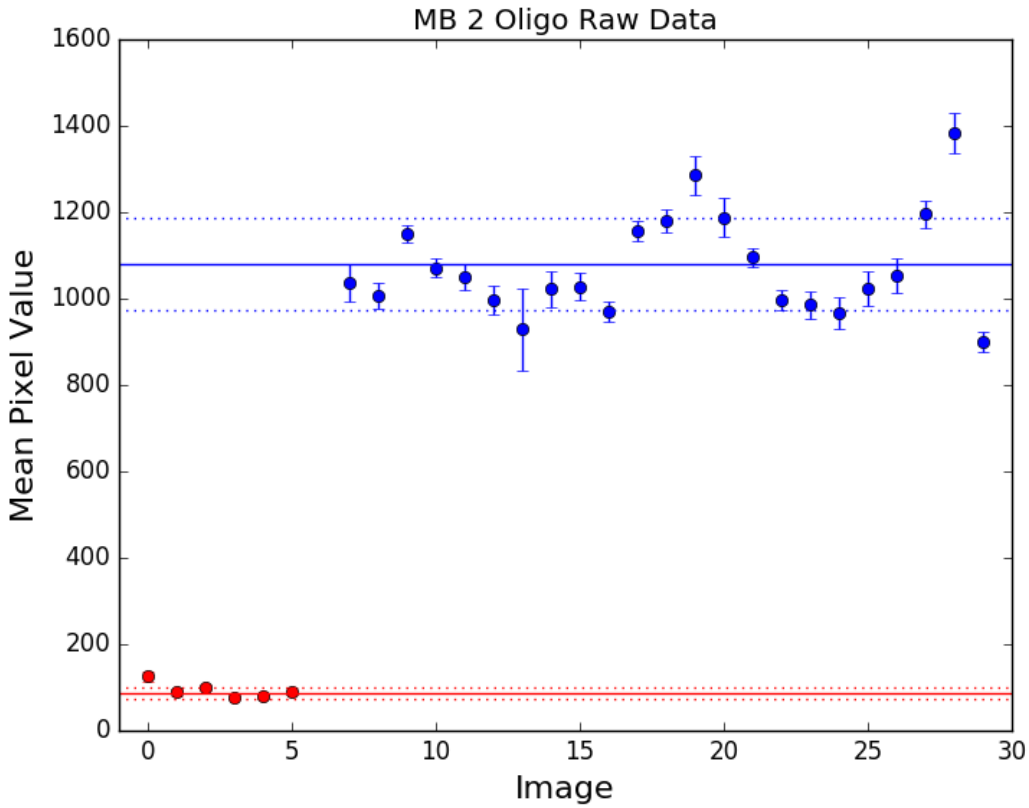


Figure 16 MB-2 and Complimentary Oligonucleotide Test. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no oligonucleotide) and blue points are the cells treated with MB-2 and the complimentary oligonucleotide. The solid red line is the mean of all images in the control well, which is 91 +/- 14 , and the solid blue line is the mean of all images of all three wells in the trial, which is 1073 +/- 106.

4.6 Poliovirus MB-1 Test

Human poliovirus-1 infected cells and non-infected cells were imaged at 500ms exposure time, as shown in **Figure 17**. The control images have a mean intensity of 78 +/- 51 and the trial wells have a mean intensity of 90 +/- 31. The T-test p>>0.05, thus the null hypothesis cannot be rejected, therefore, the distributions between polio-infected cells and non-infected cells are not significantly different.

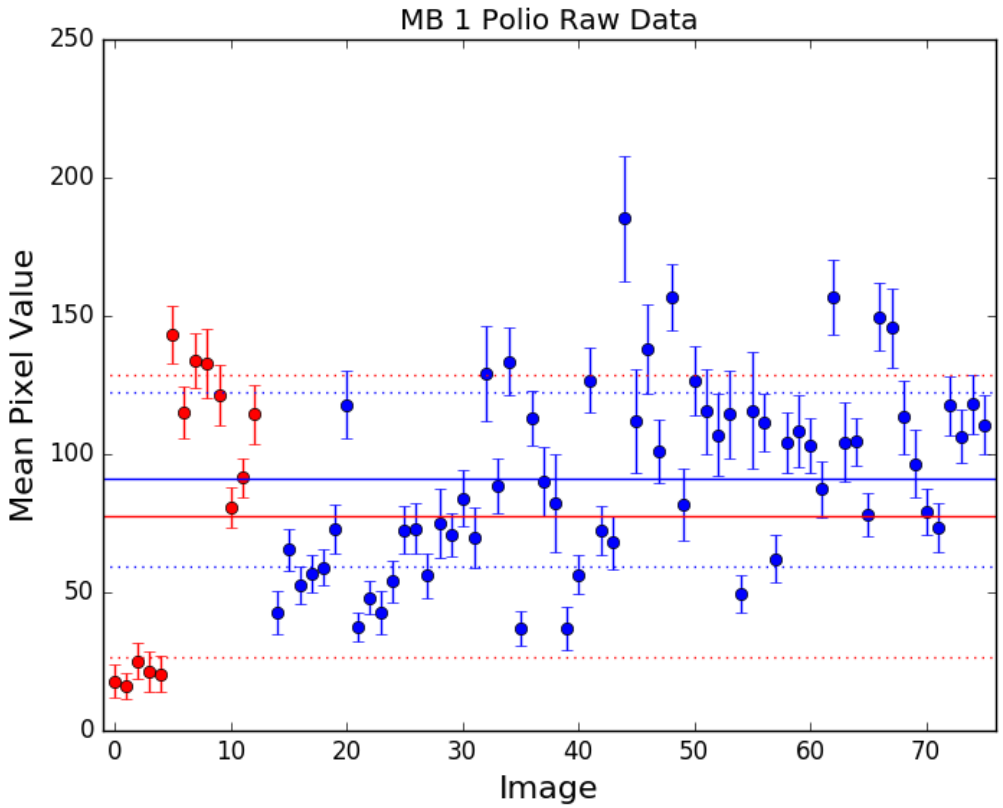


Figure 17 Poliovirus and MB-1 Confirmation of Specificity Test. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 78+/- 51, and the solid blue line is the mean of all images in the trial, which is 90 +/- 31.

4.7 Poliovirus MB-2 Test

Human poliovirus-1 infected cells and non-infected cells were imaged at 500ms exposure time, as shown in **Figure 18**. The control images have a mean intensity of 138 +/-35 and the trial wells have a mean intensity of 115 +/- 31. The T-test $p>0.05$, thus the null hypothesis cannot be rejected and therefore there is no statistical difference in the distributions between the poliovirus-infected cells and the non-infected cells.

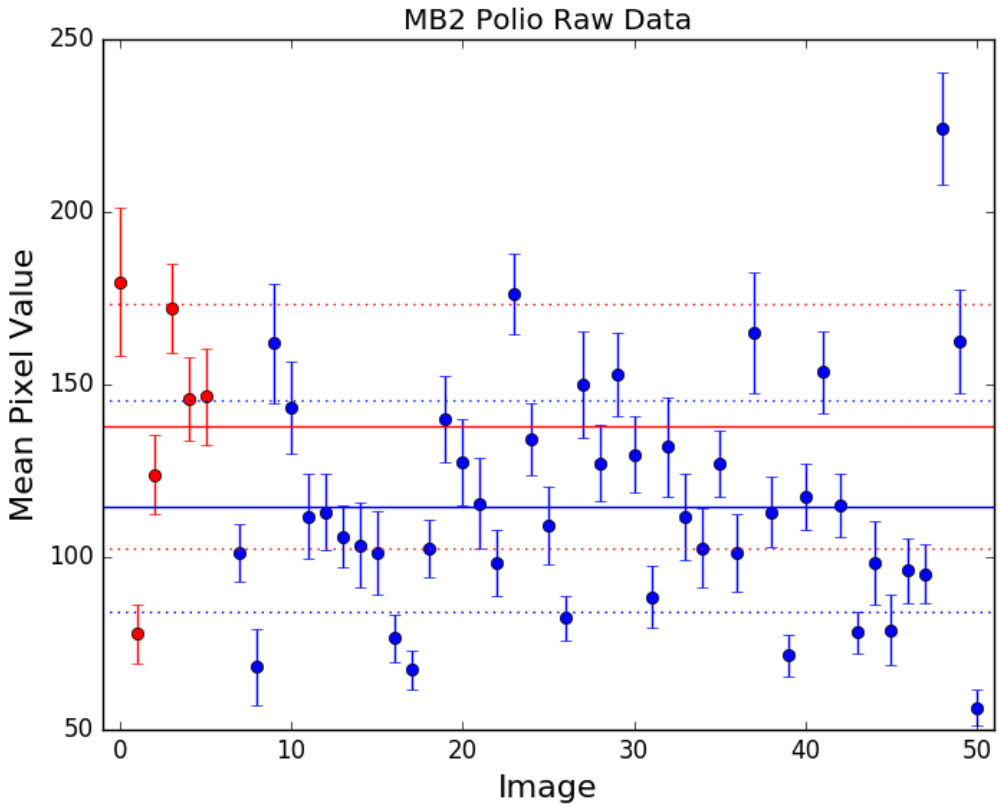


Figure 18 Poliovirus and MB-2 Confirmation of Specificity Test. Each point is the mean value of all pixels of a single image. Red points are images taken from different locations within the control well (no virus) and blue points are the virus infected cells. The solid red line is the mean of all images in the control well, which is 138 +/- 35, and the solid blue line is the mean of all images in the trial, which is 115 +/- 31.

5.0 Discussion

Dengue virus is a significant cause of worldwide morbidity and mortality. As there is yet no widely accepted vaccine, the development of rapid, sensitive, and accurate methods for its detection are needed. This project has resulted in the development of two different molecular beacon probes capable of detecting DENV1.

For both probes, there is a significant increase in fluorescence intensity with the presence of the DENV viral genome. The poliovirus test confirms the specificity of the beacon to the target viral genome. The complimentary oligonucleotide test served as a positive control for detecting the target, and had a much more intense signal than that of the DENV1 trials. This is to be expected, however, because for the DENV1 trials the MB has to diffuse into the cell, locate to the ER vesicles and bind to the viral genome target, resulting in a loss of fluorescent signal. For the oligonucleotide test, the oligonucleotides and MB were mixed together prior to being added to the cells, thereby maximizing the potential for binding.

No difference was able to be measured between viral concentrations 10^3 PFU/mL and 10^2 PFU/mL. It is unlikely that the beacon was saturated as seen by the difference in florescence using the oligonucleotide. It is possible that the amount of viral genome present (or accessible) in the cells was about equal, given the incubation time of 72 hours. The 72-hour incubation period was chosen because of reports of endosomes still present at 72 hours. The difference in fluorescence between the oligonucleotide test and the DENV1 test is therefore likely a limitation of the MB localizing to the viral target inside the cell. Whereas for the oligonucleotide test, the oligonucleotides and MB are mixed

together and then added to cells, and thus their binding together is not dependent on the MB diffusing into the cell and accessing the target.

One important limitation of the assay is that there are no units for intensity as a measure of pixel values, which are unitless; intensity is based on the specifications of the camera/software that were used on the microscope/computer. This means that it would be difficult to make any direct comparisons in data between different laboratories, and no standard values can be made for particular beacon sequences. Rather, each time a beacon is used, the investigator would have to measure for a significant difference between the control and the test.

6.0 Future Directions

Optimization of the MB to detect viral concentration is still needed. A possible strategy would be to try the viral concentrations 10^1 PFU/mL, 10^2 PFU/mL, and 10^3PFU/mL and test at 24hpi and 48hpi. This would help determine if the viral genome is accessible at the shorter infection times, if viral concentration is distinguishable, and would establish the detection limit. This study used 100µM MB to detect as few as 10PFU/mL. The next step would be to use varying concentrations of MB to find the lowest optimal concentration of MB needed to detect as few as 1PFU/mL.

There are many possible applications of MBs. The MBs in this thesis are unique to DENV1, and can be a tool for future experiments to differentiate between the serotypes. MBs unique to the other three serotypes could be designed, each with a different fluorescent molecule so that they can be distinguished from one another.

Another possible application of MBs would be a drug screen. Drug screens require one to work with living cells to test toxicity of the chemical to living cells, and to test the efficacy of the chemical on the viral reproduction cycle. Although virus research has produced an array of antiviral drugs for HIV in the past few decades, antiviral compounds remain in high demand for the treatment of other diseases such as hemorrhagic, respiratory and diarrheal diseases.

7.0 Conclusion

Molecular beacons are an effective way to detect intracellular replication of viruses. Both MB-1 and MB-2 are unique to DENV1 and have been tested against DENV1, poliovirus, and their respective complimentary oligonucleotide. Significant differences in fluorescence were detected for both the complimentary oligonucleotide and the DENV1-infected cells. No significant difference in fluorescence was detected for the poliovirus-infected cells, confirming the specificity of the beacons toward the target viral genome, DENV1. MBs have a wide range of applications and can be a powerful tool for the detection of viral replication, in a shorter amount of time than required for the development of CPE or plaques.

8.0 References

Aguirre, S. et al., 2012. DENV Inhibits Type I IFN Production in Infected Cells by Cleaving Human STING. *PLOS Pathogens,* 4 October.

Anon., 2009. *Dengue Fever.* [Online]
Available at:
http://www.niaid.nih.gov/topics/DengueFever/Research/BasicResearch/pages/howdenguematures.aspx
[Accessed April 2015].

Anon., 2014. *Dengue.* [Online]
Available at: http://www.who.int/topics/dengue/en/
[Accessed April 2015].

Barth, O. M., 1992. Replication of Dengue Viruses in Mosquito Cell Cultures-A Model from Ultrastructural Observations. *Mem. Inst. Oswaldo Cruz,* Oct/Dec, Volume 87 (4), pp. 565-574.

Bhatt, S. et al., 2013. The global distribution and burden of dengue. *Nature,* 25 April.Volume 496.

CDC, 2014. *Dengue Epidemiology.* [Online]
Available at: http://www.cdc.gov/dengue/epidemiology/
[Accessed April 2015].

Cherrier, M. et al., 2009. Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. *European Molecular Biology Organization,* 27 August.28(20).

Clyde, K., Kyle, J. & Harris, E., 2006. Recent Advances in Deciphering Viral and Host Determinants of Dengue Virus Replication and Pathogenesis. *Journal of Virology* , December.80(23).

Florida Department of Health, 2016. *Dengue Fever.* [Online]
Available at: http://www.floridahealth.gov/%5C/diseases-and-conditions/dengue/index.html

Galil, K. et al., 2004. Combined Immunomagnetic Separation-Molecular Beacon-Reverse Transcription-PCR Assay for Detection of Hepatitis A Virus from Environmental Samples. *Applied and Environmental Microbiology,* July.

Green, A., Beatty, R. & Alexandros Hadjilaou, E. H., 2014. Innate Immunity to Dengue Virus Infection and Subversion of Antiviral Responses. *Journal of Molecular Biology,* 20 March.

Gubler, D., 2011. Dengue, Urbanization and Globalization: The Unholy Trinity of the 21st Century. *Tropical Medicine and Health,* 25 August.Volume 39.

Gulley, M., Zhang, X. & Michel, K., 2013. The roles of serpins in mosquito immunology and physiology. Volume 59.

Guzman, M., Alvarez, M. & Halstead, S., 2013. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection. *Archives of Virology,* March.

Guzman, M. G. a., 2010. Dengue: A continuing global threat. *Nature Reviews Microbiology.*

Guzman, M. & Harris, E., 2015. Dengue Seminar. *The Lancet,* 31 January .Volume 385.

Harrington, L. et al., 2005. Dispersal of the Dengue Vector Aedes aegypti Within and Between Rural Communities. 72(2).

Hess, A. et al., 2011. Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense. *BMC Microbiology.*

Jain, B., Chaturvedi, U. & Jain, A., 2014. Role of intracellular events in the pathogenesis of dengue; an overview. *Microbial Pathogenesis,* 20 March.

Kearney, M. et al., 2009. Integrating biophysical models and evolutionary theory to predict climatic impacts on species' ranges: the dengue mosquito Aedes aegypti in Australia. Volume 23.

Knipe, D. M. & Howley, P., 2013. *Fields Virology Sixth Edition.* s.l.:Wolters Kluwer Health.

Kwissa, M., Nakaya, H. & Onlamoon, N., 2014. Dengue Virus Infection Induces Expansion of a CD14+CD16+ Monocyte Population that Stimulates Plasmablast Differentiation. *Cell Host & Microbe,* 9 July.

Kyle, J. L. & Harris, E., 2008. Global Spread and Persistence of Dengue. *Annual Review of Microbiology,* October.Volume 62.

Mackenzie, J. S., Gubler, D. J. & Petersen, L. R., 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses.

Marras, S., Tyagi, S. & Kramer, F., 2005. Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes. *Clinica Chemica Acta,* 18 August .

Moreira, L. et al., 2009. A Wolbachia Symbiont in Aedes aegypti Limits nfection with Dengue, Chikungunya, and Plasmodium. *Cell*, 24 December.

Murdock, C. C. et al., 2012. Complex effects of temperature on mosquito immune function.

Nitin, N., 2004. Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. April.32(6).

Pan, X. et al., 2012. Wolbachia induces reactive oxygen species (ROS)-dependent activation of Toll pathway to control dengue virus in the mosquito Aedes aegypti. *PNAS*, 3 January .Volume 109.

Pena, J. & Harris, E., 2011. Dengue virus modulates the unfolded protein response in a time-dependent manner. *Journal of Biological Chemistry*, 22 Aprill.Volume 286.

Pena, J. & Harris, E., 2012. Early Dengue Virus Protein Synthesis Induces Extensive Rearrangement of the Endoplasmic Reticulum Independent of the UPR and SREBP-2 Pathway. 4 June.

Richardson, J., Molina-Cruz, A., Salazar, M. & Black, W., 2006. Quantitative analysis of dengue-2 virus RNA during the extrinsic incubation period in individual Aedes aegypti. January.74(1).

Rico-Hesse, R., 2009. Dengue virus markers of virulence and pathogenicity. *Future Viology*, November.4(6).

Rothman, A. L., 2004. Dengue: defining protective versus pathologic immunity. *The Journal of Clinical Investigation* , April.113(7).

Rothman, A. L., 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. 15 July.Volume 11.

Sanchez-Vargas, I. et al., 2009. Dengue Virus Type 2 Infections of Aedes aegypti are Modulated by the Mosquito's RNA Interference Pathway. 5(2).

Schnettler, E. et al., 2012. Noncoding Flavivirus RNA Displays RNA Interference Suppressor Activity in Insect and Mammalian Cells. *Journal of Virology* , December .86(24).

Shepard, D. S., Undurraga, E. A. & Halasa, Y. A., 2013. Economic and Disease Burden of Dengue in Southeast. *PLOS Neglected Tropical Diseases*, 21 Feburary.7(2).

Sirot, L. et al., 2008. Identity and transfer of male reproductive gland proteins of the dengue vector mosquito, Aedes aegypti: Potential tools for control of female feeding and reproduction. Volume 38.

Storch, G. A., 2000. Diagnostic Virology. *Clin Infect Dis*, Volume 31 (3), pp. 739-751.

Tun-Lin, W., Burkot, T. R. & Kay, B. H., 2000. Effects of temperature and larval diet on development rates and survival of the dengue vector Aedes aegypti in North Queensland, Australia. Volume 14.

Tyagi, S., 1996. Molecular Beacons: Probes that Fluoresce upon Hybridization. Volume 14.

Vasilakis, N. et al., 2011. Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. July .

Welsch, S. et al., 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host & Microbe,* 23 April.5(4).

Xi, Z., Ramirez, J. & Dimopoulos, G., 2008. The Aedes aegypti Toll Pathway Controls Dengue Virus Infection. 4(7).

Yeh, H.-Y.et al., 2008. Detection of Hepatitis A Virus by Using a Combined Cell Culture-Molecular Beacon Assay. *Applied and Environmental Microbiology,* February.

Yeh, H.-Y., Yates, M., Mulchandani, A. & Chen, W., 2008. Visualizing the dynamics of viral replication in living cells via Tat peptide delivery of nuclease-resistant molecular beacon. 105(45).

9.0 Appendix

9.1 Image Pro-Plus Macro

The following code was used for the Macro in Image Pro-plus software to make a plain

text file for each image's pixel information:

```
Option Explicit
Attribute VB_Name = "Module1"

' Global directory starting string, for saving where we last processed
Dim gDirStart As String

Sub ProcessOpenDocs()
' Obtain and sort document ID's. This is so that images are processed
' in order of opening, rather than the most recent front-to-back display order.
    ' ret = IpMacroStop("starting", MS_MODAL)
    Dim docIDs() As Integer
    Dim numDocs As Integer
    Dim tempDoc As Integer
    Dim i As Integer, j As Integer

    ' Get the document list
    ret = IpDocGet(GETNUMDOC, 0, numDocs)
    ReDim docIDs(0 To numDocs-1)
    ret = IpDocGet(GETDOCLST, numDocs, docIDs(0))

    ' Clear for output
    debugclear
    ret = IpOutputClear()
    ' Sort (selection sort)
    ' This is not terribly efficient, but is quite easy to code and
    ' understand.
    For i=0 To numDocs-1
        For j=i+1 To numDocs-1
            If docIds(i) > docIds(j) Then
                tempDoc = docIds(j)
                docIds(j) = docIds(i)
                docIds(i) = tempDoc
            End If
        Next j
    Next i
```

```
    For i=0 To numDocs-1
        ret = IpAppSelectDoc(docIDs(i))

        ' Your processing here ***
        ' DoStuff docIDs(i), Format$(docIDs(i))
        DoStuff docIDs(i)

        ' Debug.Print i; docIDs(i)
    Next i

   ' Let the user know that we've finished
    ret = IpMacroStop("All open images processed.", MS_MODAL)
End Sub

Private Sub DoStuff(source As Integer)
    Dim fullName As String*255
    Dim fileName As String*255
    Dim path As String*255
    Dim extension As String, baseName As String
    Dim delim As Integer

    ret = IpDocGetStr(INF_FILENAME, source, fullName)
    delim = InStrRev(fullName, "\")
    fileName = Right(fullname, Len(fullName) - delim)
    delim = InStrRev(fileName, ".")
    baseName = Left(fileName, delim - 1)

    path = "C:\Users\Katie\Desktop\data_output\" + baseName + ".dat"
    'path = "C:\Users\Katie\Desktop\" + baseName

    'ret = IpMacroStop(path, MS_MODAL)
    ret = IpBitShow(1)
    ret = IpBitSaveData(path, S_LEGEND)
End Sub

Sub ProcessDirectory()
' Scan through and process all files in a directory
    Dim IName As String*255
    Dim fName As String
    Dim workStr As String
    Dim docID As Integer

    ' Make certain we have a reasonable starting point. This code is
```

```
 ' only executed the first run after macro compilation. If you have
 ' a desired standard starting point, initialize the directory
 ' search here.
 If gDirStart = "" Then
     gDirStart = "C:\"
 End If

 ' Get a file name in the desired directory
 workStr = GetFilePath("", "*", gDirStart, _
   "Select a file in the desired directory", 0)

 ' Check to make certain the user did not cancel
If workStr = "" Then
 Exit Sub
End If

' Close all open images prior to processing (this can be removed if desired)
 ret = IpAppCloseAll()

 ' Extract the directory name from the full file name
gDirStart = Left(workStr, InStrRev(workStr, "\"))

 ' Clear the output for work purposes
debugclear
ret = IpOutputClear()

 ' Call a setup routine ***
 ' Insert your setup call here

 ' Look for standard files, no directories or system files.
' See the 'Dir' command help for details
fName = Dir(gDirStart + "*.*", 32)

While fName <> ""
 ' Print out the file name and its attributes
    Debug.Print GetAttr(gDirStart + fName); " "; fName

    ' Load the image
    docID = IpWsLoad(gDirStart + fName, "")

    ' Don't process if there is a failure loading
    If docID >= 0 Then
     ' Call processing routine here ***
     ' The document ID and file name are sent here
```

```
        ' in case the processing routine needs them.
           DoStuff docID

           ' Close the initial image
           ret = IpAppSelectDoc(docID)
           ret = IpDocClose()
        Else
            Debug.Print "Error loading "; gDirStart + fName
        End If

       ' Get the next file name
       fName = Dir()
   Wend

    ' Call a finish routine ***
    ' Insert your code here.

   ' Let the user know that we've finished
    ret = IpMacroStop("All images in directory processed.", MS_MODAL)
End Sub

Sub ProcessSubDir()
' Scan through and process all files in a directory
' recursively. Each sub-directory will be processed in
' turn, as well as the files in the current directory.
'
' Note that 'IpStSearchDir( )' and a counter are used, rather
' than the IpBasic 'Dir'; the 'Dir' command uses a global
' position counter, and thus cannot be called recursively.

    Dim workStr As String

    ' Make certain we have a reasonable starting point. This code is
    ' only executed the first run after macro compilation. If you have
    ' a desired standard starting point, initialize the directory
    ' search here.
    If gDirStart = "" Then
        gDirStart = "C:\"
    End If

    ' Get a file name in the desired directory
    workStr = GetFilePath("", "*", gDirStart, _
      "Select a file in the desired directory", 0)
```

```
    ' Check to make certain the user did not cancel
    If workStr = "" Then
     Exit Sub
    End If

    ' Extract the directory name from the full file name
    gDirStart = Left(workStr, InStrRev(workStr, "\"))

    ' Clear the output for work purposes
    debugclear
    ret = IpOutputClear()
    ret = IpOutputShow(1)

    ' Perform any setup, such as tuning Count/Size ***
    Call SetupRecursive()

    ' Process each file and subdirectory
     Call RecursiveSub(gDirStart)

    ' Clean up, show results, etc ***
    Call FinishRecursive()

End Sub
Sub RecursiveSub(startStr As String)
    Dim X As Integer
    Dim DStat As Integer
    Dim Iname As String * 255
    Dim shortName As String
    Dim docID As Integer

    ' Search through all files in the directory
    X = 0
    DStat = IpStSearchDir(startStr, "*.*", X, Iname)

    Do While DStat > 0
        X = X + 1

        Select Case DStat
        Case 1
            ' File, such as an image file. Attempt to
            ' load it...
            docID = IpWsLoad(Iname, "TIF")
            If docID >= 0 Then
                Debug.Print "Processing: "; Iname
```

```
            ' Processing code goes here ***
            DoStuff docID

            ' Clean up...
            ret = IpAppSelectDoc(docID)
            ret = IpDocClose()
        End If

    Case 2
        ' Directory. Ignore "\." and "\..", as they
        ' have already been visited.
        shortName = Right(IpTrim(Iname), 2)
        If shortName <> "\." And shortName <> ".." Then
            ' Dive down a level and process this one
            RecursiveSub(IpTrim(Iname) + "\")
        End If
    End Select
    ' Next file
    DStat = IpStSearchDir(startStr, "*.*", X, Iname)
  Loop
End Sub
Private Sub SetupRecursive()
  Debug.Print "Setup for the recursive routine here"
End Sub
Private Sub FinishRecursive()
  Debug.Print "Clean up after the recursive routine here"
End Sub
```

9.2 Python Code

The following is the code that was used to process all the plain text files to calculate the intensity values, the mean intensity values, the standard deviations, perform the standard T-test, perform the Bayesian Estimation analysis, produce the figures that are in the results section, and produce the distribution plots that are in Appendix 9.4. The code is broken into two files; the first file is the process code and the second is the fit code (the full Bayesian Statistical analysis code).

Process code

```
# -*- coding: utf-8 -*-
"""Statistical measurements of molecular beacon data."""
import numpy as np
import matplotlib.pyplot as plt
from scipy.stats import ttest_ind

import datetime
import time
import os

# the bayesian fitting code
import evo.fit

def run_all(main_directory='/katie/data'):
  """Run the analysis code for all folders contained within a
  master data folder.
  This automates the processing and logging of all data sets.

  main directory (string): path to directory containing sub-directories
  """
  filelist = os.listdir(main_directory)
  start_time = time.time()
  for folder in filelist:
    path = os.path.join(main_directory, folder)
    if not os.path.isdir(path):
      continue
    print path
    process_trial(path)
```

```
    print ''
  print 'time to process all data:'
  print '--- {} seconds ---'.format(time.time() - start_time)

def process_trial(path):
  """Read in images contained in the folder at path, and perform
  a bayesian statistical comparison between the control and trial images.
  path (string): path to folder
  """
  control_path = os.path.join(path, 'control')

  # perform photometry on all dat files in the folder
  x, e = average_directory(path)
  cx, ce = average_directory(control_path)

  # perform bayesian analysis and plot prior probability distributions
  image_path = os.path.join('output', path.split('/')[-1] + ' posterior.png')
  mu, sig, cmu, csig, p = evo.fit.compare(x, e, cx, ce, plotfile=image_path,
plotTitle=path.split('/')[-1])

 tval = ttest_ind(x, cx)

  # if measurement error overwhelms intrinsic scatter, we are limited by
  # measurement error and should use its average value instead.
  if sig < np.mean(e):
    sig = np.mean(e)
  if csig < np.mean(ce):
    csig = np.mean(ce)

  # plot the trial data
  image_path = os.path.join('output', path.split('/')[-1] + '.png')
  fig = plot_trial(x, e, cx, ce, mu, sig, cmu, csig, path.split('/')[-1])
  fig.savefig(image_path)
  plt.close(fig)

  # log the trial in the output directory
  log_trial(path, x, e, cx, ce, mu, sig, cmu, csig, p, tval=tval)
  print 'RESULTS:'
  print 'trial: {:.3f} +/- {:.3f}'.format(mu, sig)
  print 'control: {:.3f} +/- {:.3f}'.format(cmu, csig)
  print 'probability that the distributions differ: {:.2f}'.format(p)
  print 't statistic: {}\tt-test pval: {}'.format(tval[0], tval[1])

def average_directory(path, silent=True):
```

```
  """For a given folder path, read all the images in the folder and
  measure their average intensities and standard deviations.
  Return arrays containing the averages and sigmas.
  """
  averages = []
  sigmas = []
  for filename in list_data_files(path):
    ave, std = imave(os.path.join(path, filename))
    if not silent:
      print '{}: {} +/- {}'.format(filename, ave, std)
    averages.append(ave)
    sigmas.append(std)
  return np.array(averages), np.array(sigmas)

def list_data_files(path):
  """List valid data files in a given directory."""
  data_files = []
  for data in os.listdir(path):
    if data == '.DS_Store':
      continue
    if os.path.isdir(os.path.join(path, data)):
      continue
    data_files.append(data)
  return data_files

def imave(filename, plot=False):
  """Read in an ImagePro exported raw data file and measure its
  average intensity and standard deviation.
  filename (string): path to ImagePro exported raw image data
  """
  data = np.loadtxt(filename, skiprows=1)
  image = np.array([row[1:] for row in data[:]])
  if plot:
    f, axarr = plt.subplots(2)
    axarr[0].imshow(image)
    axarr[1].hist(image.flatten())
    f.suptitle(filename)
    plt.show(f)
  return np.mean(image.flatten()), np.std(image.flatten())

# plotting and logging functions
def plot_trial(ave, sig, cave, csig, mu_trial, scatter_trial, mu_control, scatter_control,
title):
  """Visualize the intensity values for the individual images of a trial."""
```

```
  def hline(y, color='b', linestyle='-'):
    """Quick and dirty plotting code to create a horizontal line at height y."""
    plt.plot((-100, 100), (y, y), color=color, linestyle=linestyle)
  fig = plt.figure()
  x = np.arange(len(cave))
  plt.errorbar(x, cave, yerr=csig, fmt='o', color='r')
  x = np.arange(len(ave)) + len(cave) + 1
  plt.errorbar(x, ave, yerr=sig, fmt='o', color='b')
  hline(mu_trial, color='b')
  hline(mu_trial+scatter_trial, color='b', linestyle=':')
  hline(mu_trial-scatter_trial, color='b', linestyle=':')
  hline(mu_control, color='r')
  hline(mu_control+scatter_control, color='r', linestyle=':')
  hline(mu_control-scatter_control, color='r', linestyle=':')
  plt.xlim(-1, len(cave) + len(ave) + 1)
  plt.xlabel('Image', fontsize=16)
  plt.ylabel('Mean Pixel Value', fontsize=16)
  plt.title(title)
  return fig
def log_trial(path, x, e, cx, ce, mu, sig, cmu, csig, p, tval=None):
  """Given the trial directory and output data, write everything
  to a log file in the output folder.
  """
  dirname = path.split('/')[-1]
  with open(os.path.join('output', dirname + ' log.txt'), 'w') as f:
    f.write(dirname)
    f.write('{:%Y-%m-%d %H:%M:%S}\r\n'.format(datetime.datetime.now()))
    f.write('mean trial: {:.3f} +/- {:.3f}\r\n'.format(mu, sig))
    f.write('mean control: {:.3f} +/- {:.3f}\r\n'.format(cmu, csig))
    f.write('probability that the distributions differ: {:.2f}\r\n'.format(p))

    if tval:
      f.write('t statistic: {}\tt-test pval: {}\r\n'.format(tval[0], tval[1]))
    f.write('----------------------\r\n')
    f.write('\r\n')
    trial_dat = list_data_files(path)
    control_dat = list_data_files(os.path.join(path, 'control'))
    f.write('----- TRIAL DATA ------\r\n')
    for filename, a, s in zip(trial_dat, x, e):
      f.write('{}: {:.3f} +/- {:.3f}\r\n'.format(filename, a, s))
    f.write('MEAN: {:.3f} +/- {:.3f}\r\n'.format(mu, sig))
    f.write('\r\n')
    f.write('----- CONTROL DATA ------\r\n')
    for filename, a, s in zip(control_dat, cx, ce):
```

```
      f.write('{}: {:.3f} +/- {:.3f}\r\n'.format(filename, a, s))
    f.write('MEAN: {:.3f} +/- {:.3f}\r\n'.format(cmu, csig))

# summary plot code
def beacon_plots():
  """Create summary plots for both sets of beacon data.
  These plots show the background-subtracted average intensity values
  for each data set for both trials.
  """
  trials = ['MB1 Trial 1 400ms', 'MB1 Trial 1 500ms', 'MB1 Trial 2 500ms', 'MB1 Trial 3
250ms', 'MB1 Trial 3 500ms']
  title = 'Molecular Beacon 1 Trials'
  full_plot(trials, title)
  trials = ['MB2 Trial 1 250ms', 'MB2 Trial 1 500ms', 'MB2 Trial 2 500ms', 'MB2 Trial 3
250ms']
  title = 'Molecular Beacon 2 Trials'
  full_plot(trials, title)
def full_plot(trials, title, main_directory='/katie/data'):
  """Plot the average values minus control for a series of trials."""
  aves = []
  sigs = []
  fig = plt.figure()
  ax = fig.add_axes([0.1, 0.3, 0.8, 0.6])
  for trial in trials:
    print trial
    path = os.path.join(main_directory, trial)
    control_path = os.path.join(path, 'control')
    x, e = average_directory(path)
    cx, ce = average_directory(control_path)
    mu, sig, cmu, _, _ = evo.fit.compare(x, e, cx, ce)
    if sig < np.mean(e):
      sig = np.mean(e)
    aves.append(mu - cmu)
    sigs.append(sig)
  x = range(len(trials))
  ax.errorbar(x, aves, yerr=sigs, fmt='o', color='k')
  plt.xticks(x, trials, rotation='vertical')
  plt.xlim([-0.5, len(x)-0.5])
  plt.ylabel('Background-Subtracted Mean Pixel Value')
  plt.title(title)
  plt.show(fig)
```

Fit Code

```
# -*- coding: utf-8 -*-
"""Bayesian fitting of intrinsic scatter.
"""
from sklearn import preprocessing
import matplotlib.pyplot as plt
import numpy as np
# import ipdb
from scipy.interpolate import interp1d
from scipy import stats
from scipy.special import erf
from scipy.special import erfc


def dual_exp(sig, mu, x, e):
  return np.log(1 / (np.sqrt(sig**2 + e**2) * np.sqrt(6.28)) * np.exp(-((x-
mu)**2)/(2*(e**2 + sig**2))))
vec_exp = np.vectorize(dual_exp, excluded=[0, 1])


def fit_scatter(dist, error, plot=False):
  """Find the intrinsic scatter parameter given a set of distances
  from ideal value and their error bars.
  """
  cut = np.isfinite(dist)
  dist = dist[cut]
  error = error[cut]
  dist = dist - np.mean(dist)
  sigs = np.linspace(0, 10, 1000) + 0.01
  ls = np.empty(len(sigs))
  for i, sig in enumerate(sigs):
    ls[i] = 0
    for x, e in zip(dist, error):
      ls[i] += np.log(1 / (np.sqrt(sig**2 + e**2) * np.sqrt(6.28)) * np.exp(-(x**2)/(2*(e**2
+ sig**2))))
  ls = np.exp(ls - max(ls))
  if plot:
    plt.plot(sigs, ls)
    plt.show()
    X = np.arange(len(dist))
    y1 = np.empty(len(dist))
    y1.fill(sigs[ls == max(ls)][0])
    plt.errorbar(X, dist, yerr=error, fmt='.')
```

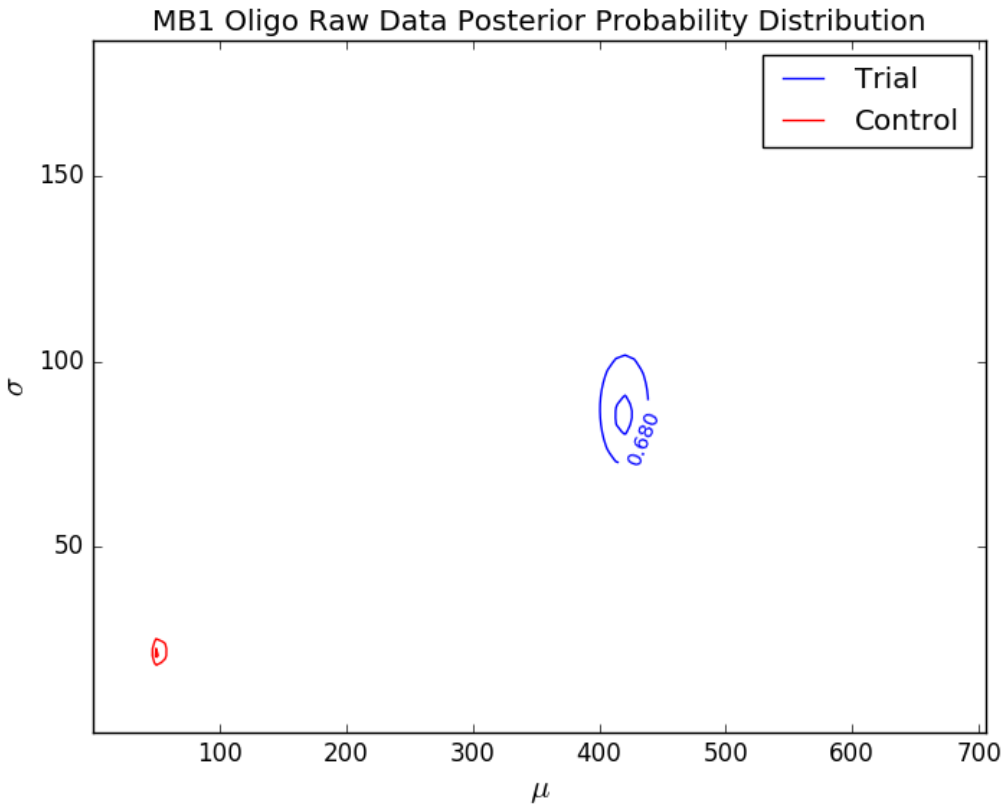
```
    plt.plot(X, dist, 'ro')
    plt.plot(X, y1, linestyle='--', color='black')
    plt.plot(X, -1* y1, linestyle='--', color='black')
    plt.show()
  return sigs[ls == max(ls)][0]

def fit_dist(dist, error, plot=False, siglim=None, mulim=None, gridN=100,
plotTitle=None):
  """Find the intrinsic scatter of a sample given a set of values
  with error bars.
  We use a Bayesian robust estimation to directly calculate the probability
  that a given (sigma, mu) fit the data, for a grid of sigma and mu.
  returns: the grid sigma, mu and probability matrix.
  """
  if siglim == None:
    siglim = [0, np.mean(error)*3]
  if mulim == None:
    mulim = [0, max(dist) + siglim[1]]

  cut = np.isfinite(dist)
  dist = dist[cut]
  error = error[cut]

  sigs = np.linspace(siglim[0], siglim[1], gridN) + 0.01
  mus = np.linspace(mulim[0], mulim[1], gridN) + 0.01
  ls = np.empty([len(sigs), len(mus)])
  for sig_index, sig in enumerate(sigs):
    for mu_index, mu in enumerate(mus):
      ls[sig_index, mu_index] = 0
      ls[sig_index, mu_index] = sum(vec_exp(sig, mu, dist, error))
  ls = np.exp(ls - max(ls[ls != float('-inf')]))
  if plot:
    plot_prior(sigs, mus, ls, plotTitle=plotTitle)
  return sigs, mus, ls

def compare(x1, e1, x2, e2, plotfile=None, plotTitle=None):
  """A robust Bayesian equivalent of a t-test.
  Find the confidence interval that a given set of values have a
  different average.
  """
  siglim = [0, max(np.std(x1), np.std(x2))*2]
  mulim = [0, max(max(x1), max(x2)) + siglim[1]/2]

  sigs, mus, ls1 = fit_dist(x1, e1, siglim=siglim, mulim=mulim)
```

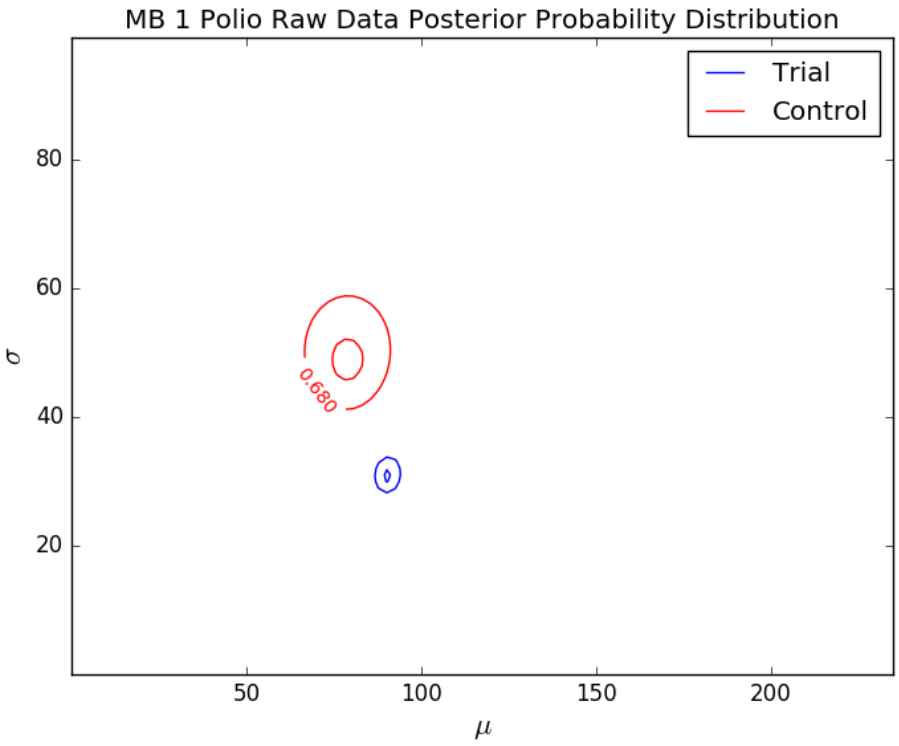
```
  sigs2, mus2, ls2 = fit_dist(x2, e2, siglim=siglim, mulim=mulim)

  mm1 = np.sum(ls1, axis=0) * (sigs[1]-sigs[0])
  ms1 = np.sum(ls1, axis=1) * (mus[1]-mus[0])
  mm2 = np.sum(ls2, axis=0) * (sigs[1]-sigs[0])
  ms2 = np.sum(ls2, axis=1) * (mus[1]-mus[0])
  mu1 = mus[mm1 == max(mm1)][0]
  sig1 = sigs[ms1 == max(ms1)][0]
  mu2 = mus2[mm2 == max(mm2)][0]
  sig2 = sigs2[ms2 == max(ms2)][0]
  if plotfile != None:
    fig = plt.figure()
    plot_prior(sigs, mus, ls1, fig=fig, label='Trial', color='Blue', plotTitle=plotTitle)
    plot_prior(sigs2, mus2, ls2, fig=fig, label='Control', color='Red', plotTitle=plotTitle)
    plt.legend()
    fig.savefig(plotfile)
    plt.close(fig)
  return mu1, sig1, mu2, sig2, prob_differ(mu1, sig1, mu2, sig2)

def prob_delt(x, p1, p2, dlim=None, gridN = 100):
  """Given p1(x) and p2(x), directly calculate the probability distribution
  p(p1 - p2).
  """
  f1 = interp1d(x, p1, fill_value='extrapolate')
  f2 = interp1d(x, p2, fill_value='extrapolate')
  if dlim==None:
    dlim = [0, 1]
  delts = np.linspace(dlim[0], dlim[1], gridN) + 0.01
  prob = np.empty(len(delts))
  for i, d in enumerate(delts):
    prob[i] = 0
    for xx in x:
      prob[i] += f1(xx) * (f2(xx + d) + f2(xx - d))
    prob[i] = prob[i] / len(x)
  return delts, prob / sum(prob)


def conf(x, p, plot=False):
  """For a general probability distribution function p(x), find the
  coverage probability for 1 sigma.

  returns: (x_low, x_mean, x_high) where x_low and x_high are the
  68% confidence interval over x.
  """
```

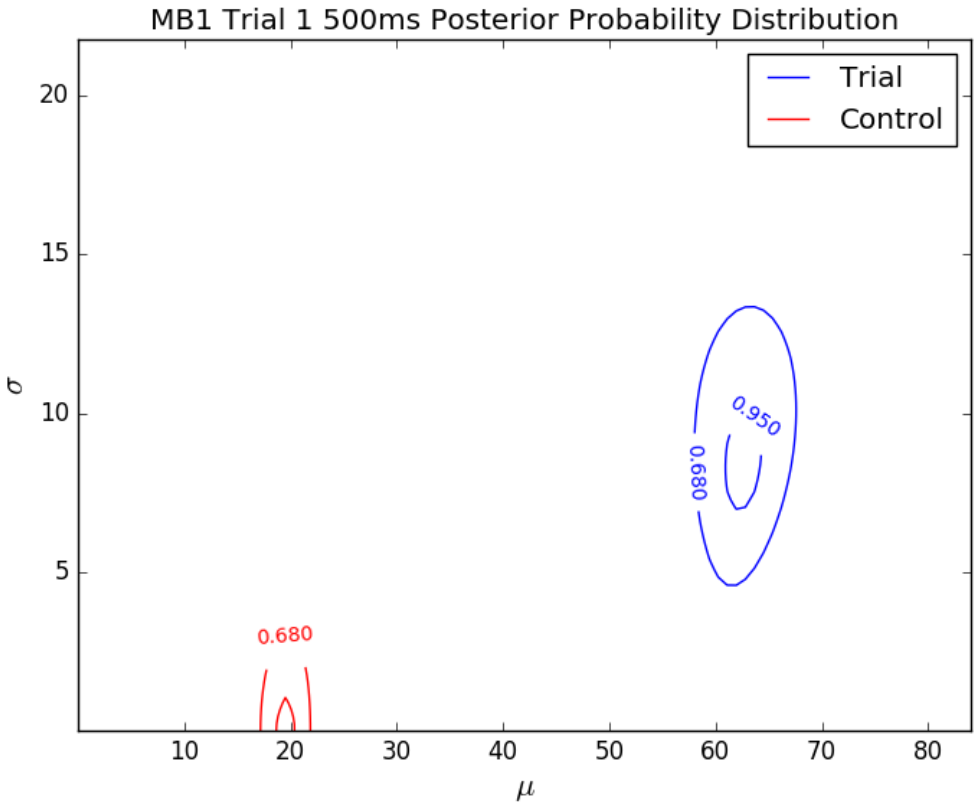
```
  center = np.arange(len(p))[p == max(p)][0]
  csum = 0
  low = center
  high = center
  while csum < 0.68 * sum(p):
    high += 1
    low -= 1
    if low < 0:
      low = 0
    if high > len(p)-1:
      high = len(p)-1
      break
    csum = sum(p[low: high])
  if plot:
    plt.plot(x, p)
    plt.axvline(x=x[low], color='r')
    plt.axvline(x=x[high], color='r')
    plt.axvline(x=x[center])
    plt.show()
  return x[low], x[center], x[high]

def sig_diff(x, p):
  """For a given probability distribution function p(x), find the
  largest coverage probability that excludes 0.

  returns the coverage probability.
  """
  center = np.arange(len(p))[p == max(p)][0]
  csum = 0
  low = center
  high = center
  while low > 0:
    high += 1
    low -= 1
    if high > len(p)-1:
      high = len(p)-1
    csum = sum(p[low: high])
  return csum

def plot_prior(sigs, mus, ls, fig=None, plotTitle=None, label=None, color=None):
  """NOTE: really should be called 'plot_posterior'.
  Plotting code that shows the 68% and 95% confidence interval over the
  posterior distribution in sigma, mu.
  """
```

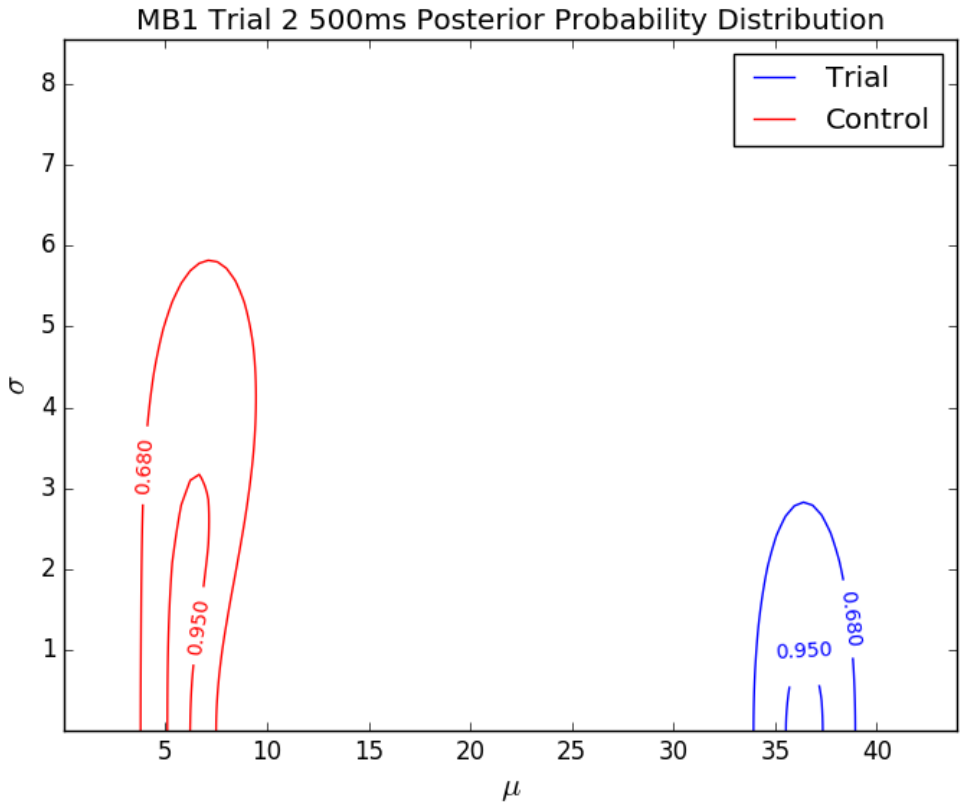
```
  suppress = True
  if fig == None:
    fig = plt.figure()
    suppress = False
  CS = plt.contour(mus, sigs, ls, levels=[0.68, 0.95], colors=color, label=label)
  plt.ylabel('$\sigma$', fontsize=16)
  plt.xlabel('$\mu$', fontsize=16)
  plt.clabel(CS, inline=1, fontsize=10)
  plt.title(plotTitle+' Posterior Probability Distribution')
  if label != None:
    CS.collections[0].set_label(label)
  if not suppress:
    plt.show()
def prob_differ(mu1, sig1, mu2, sig2):
  """For two normal distributions given by (mu1, sigma1) and (mu2, sigma2)
  The probability function of the difference of their random variables
  is another normal distribution.
  This function returns the largest coverage probability of the difference
  distribution that excludes 0, and thus the probability that
  the two normal distributions differ.
  """
  joint_mu = mu1 - mu2
  joint_sig = np.sqrt(sig1**2 + sig2**2)
  return erf(abs(joint_mu) / (np.sqrt(2) * joint_sig))


def simulate(scatter, escale=0.1, n=100, plot=False, ave=0):
  """Simulate a data set with intrinsic scatter."""
  err = np.random.normal(scale=escale, size=n)
  x = []
  for e in err:
    x.append(np.random.normal(scale=(np.sqrt(scatter**2 + e**2)))+ave)
  x = np.array(x)
  if plot:
    X = np.arange(len(x))
    y1 = np.empty(len(x))
    y1.fill(scatter)
    plt.errorbar(X, x, yerr=err, fmt='.')
    plt.plot(X, x, 'ro')
    plt.plot(X, y1, linestyle='--', color='black')
    plt.plot(X, -1* y1, linestyle='--', color='black')
    plt.show()
  return x, err
```

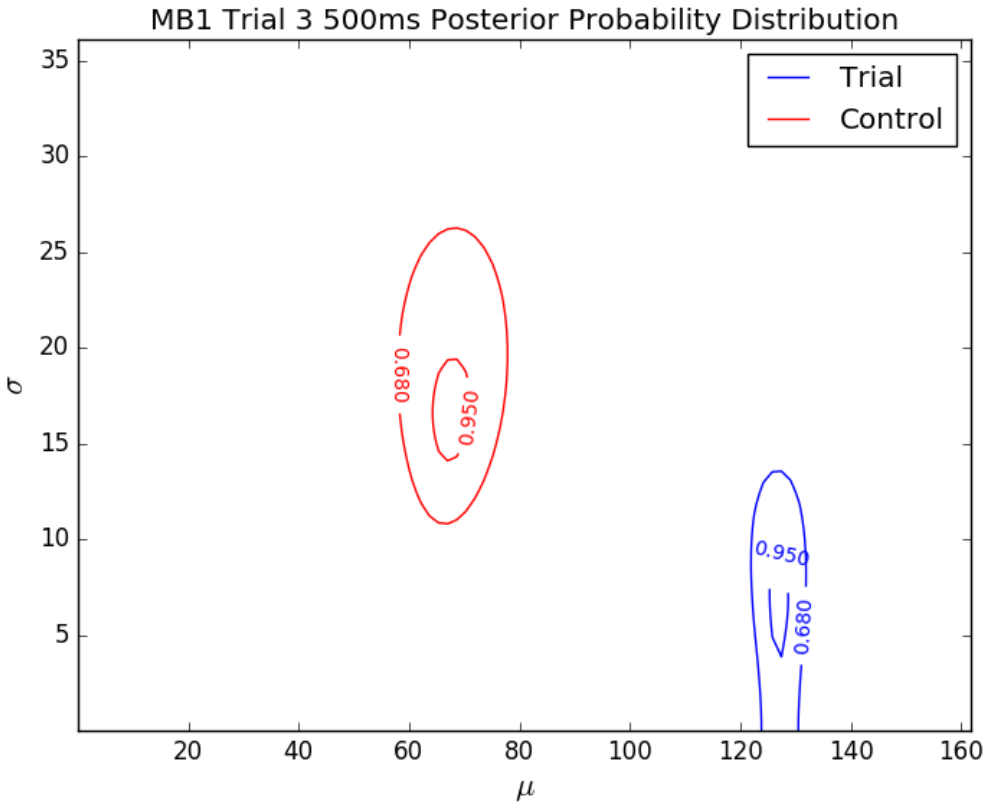
9.3 Distribution Plots from the Bayesian Estimation Statistical Analysis

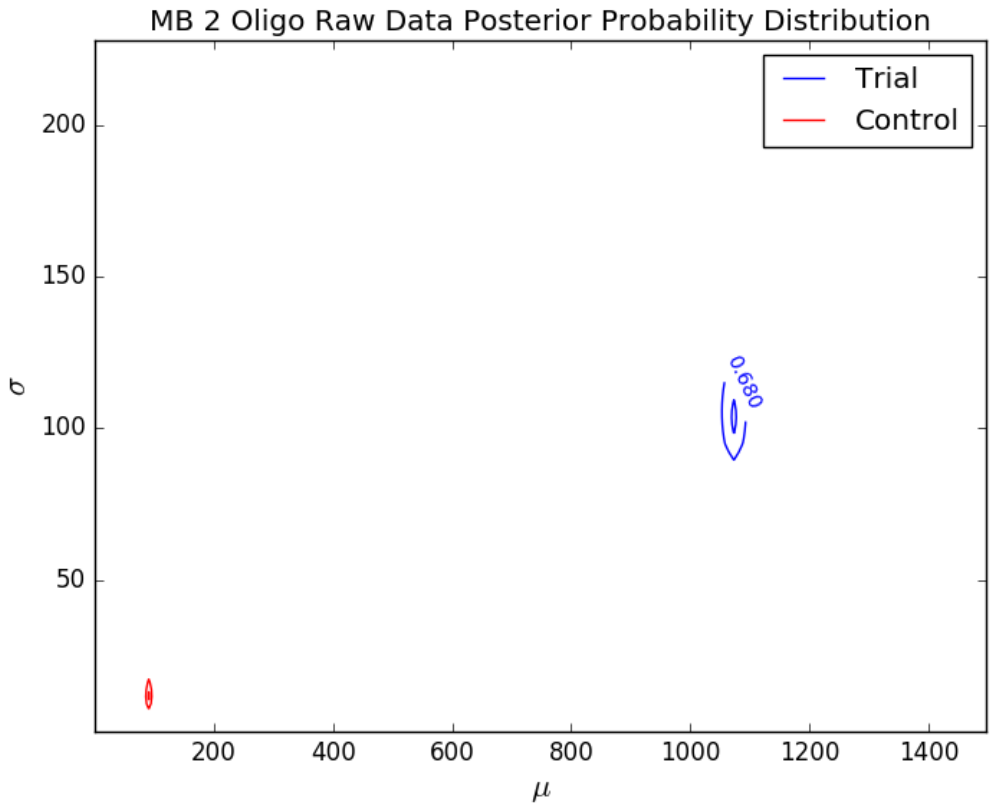


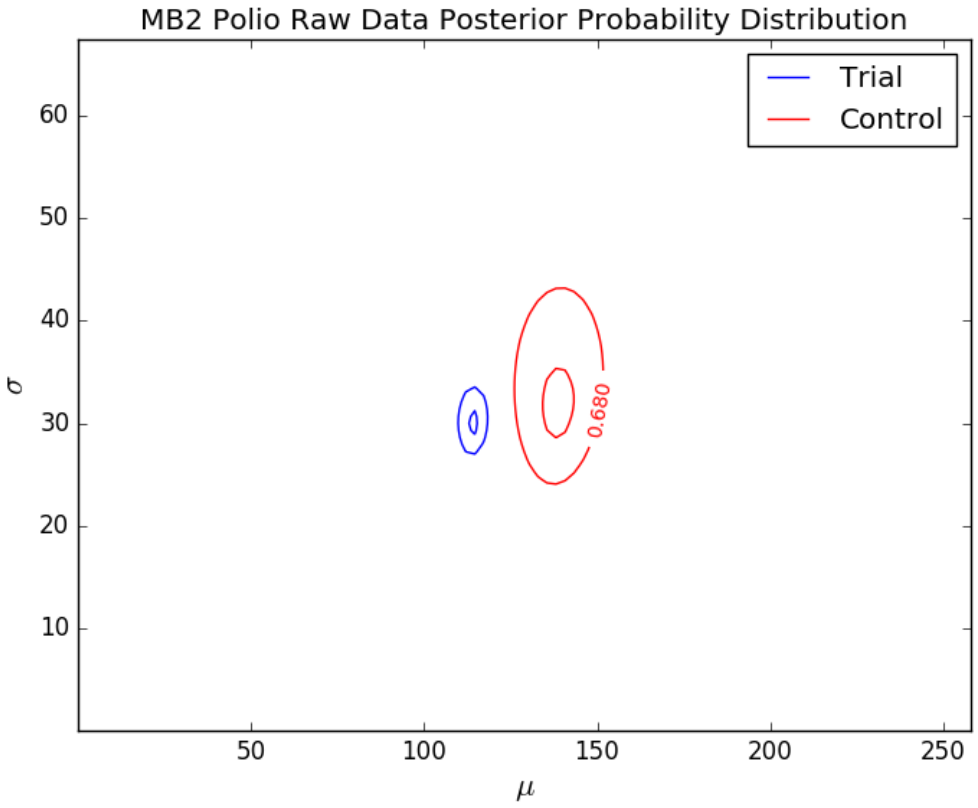

MB 1 Polio Raw Data Posterior Probability Distribution
Trial
Control
80
60
40
20
σ
0.680
50
100
150
200
μ

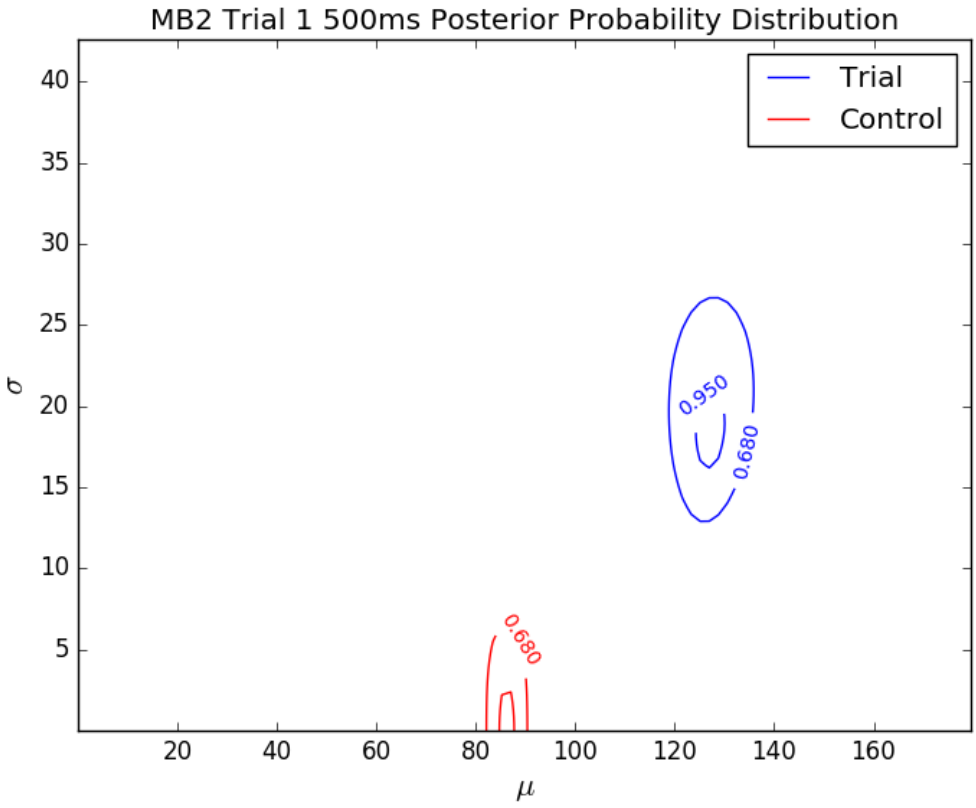

MB1 Trial 1 500ms Posterior Probability Distribution
Trial
Control
0.950
0.680
0.680
σ
μ
20
15
10
5
10
20
30
40
50
60
70
80

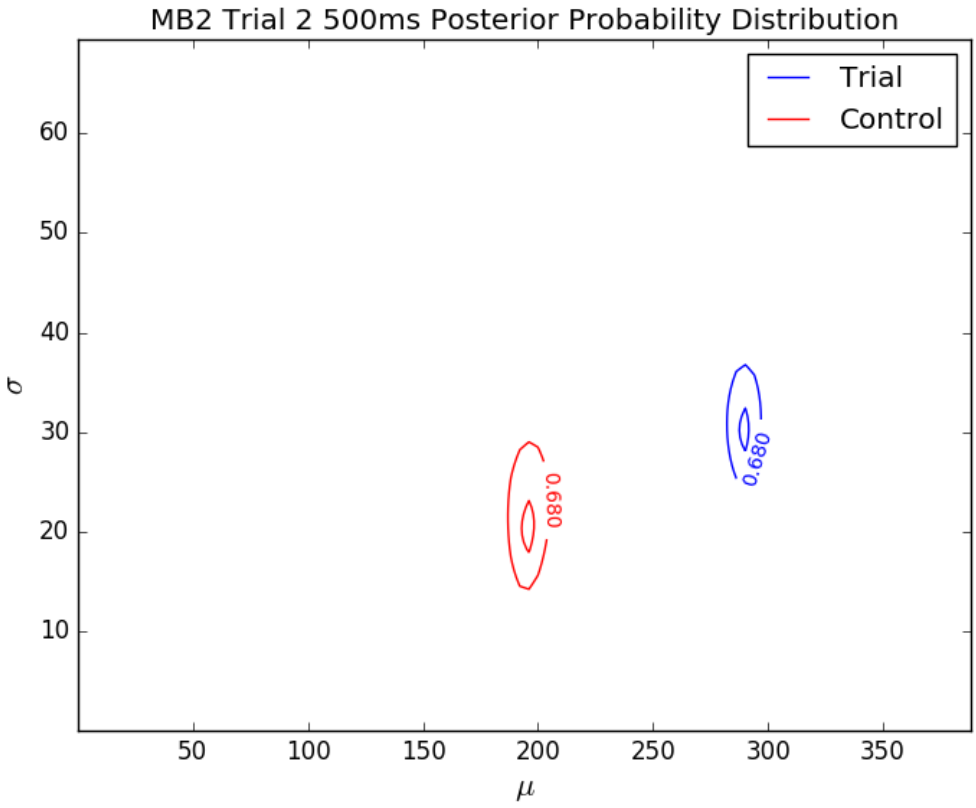

MB1 Trial 2 500ms Posterior Probability Distribution
Trial
Control
0.680
0.950
0.950
0.680
σ
μ
1
2
3
4
5
6
7
8
5
10
15
20
25
30
35
40

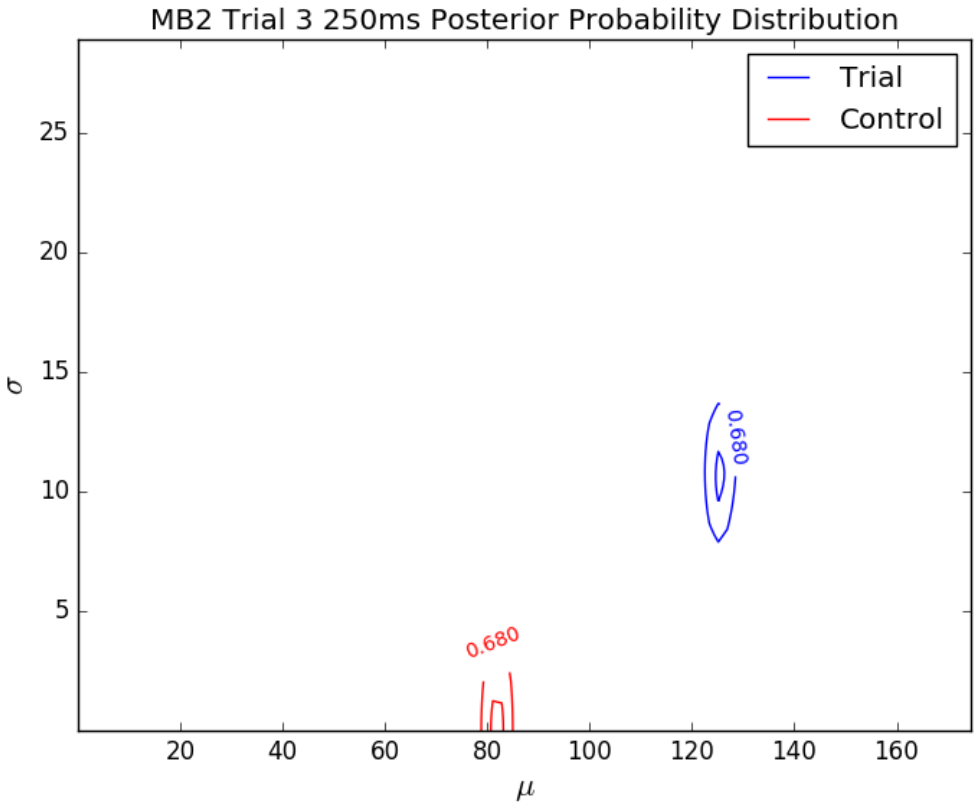

MB1 Trial 3 500ms Posterior Probability Distribution
Trial
Control
0.680
0.950
0.950
0.680
35
30
25
20
15
10
5
20
40
60
80
100
120
140
160
σ
μ


MB 2 Oligo Raw Data Posterior Probability Distribution
Trial
Control
0.680
200
150
100
50
σ
200
400
600
800
1000
1200
1400
μ


MB2 Polio Raw Data Posterior Probability Distribution
Trial
Control
0.680
60
50
40
30
20
10
50
100
150
200
250
σ
μ


MB2 Trial 1 500ms Posterior Probability Distribution
Trial
Control
0.950
0.680
0.680
40
35
30
25
20
15
10
5
20
40
60
80
100
120
140
160
σ
μ


MB2 Trial 2 500ms Posterior Probability Distribution
Trial
Control
60
50
40
30
20
10
σ
50
100
150
200
250
300
350
μ
0.680
0.680


MB2 Trial 3 250ms Posterior Probability Distribution
Trial
Control
0.680
0.680
σ
μ
25
20
15
10
5
20
40
60
80
100
120
140
160