LABORATORY DIRECTED RESEARCH & DEVELOPMENT
WHERE INNOVATION BEGINS

SAND20XX-XXXXR
LDRD PROJECT NUMBER: 220608
LDRD PROJECT TITLE: Concurrent evaluation of autophagy induction and *Burkholderia* infection at the single cell level
PROJECT TEAM MEMBERS: PI-Danae Maes (08635), PM-Jerilyn Ann Timlin (08635), Stephen Anthony (08635), Joshua Podlevsky (08631), Colleen Courtney (08621), Steven Branda (08631)

ABSTRACT:

Autophagy is a natural, regulated cellular process that "cleans up" cellular debris by degrading and recycling dysfunctional proteins. There is a high potential impact of exploiting the benefits of autophagy to complement existing treatments, but little has been done to date on bacterial pathogens of defense concern such as *Burkholderia pseudomallei*, a highly virulent Select Agent pathogen that is intrinsically resistant to most classes of antibiotics. Assessment of autophagy in the context of infection typically requires use of multiple technologies in combination (*e.g.*, Western analysis paired with microscopy or flow cytometry) as applied to heterogeneous populations of cells. To address this, we have developed a dual target reporter cell line (RAW264.7 LC3-BFP:mPlum, GFP-RelA) that enables concurrent visualization of infection and autophagy induction. We assessed the effect of clinically approved small molecule inducers of autophagy on infection by *Burkholderia thailendensis*, a closely related but less virulent surrogate for *B. pseudomallei*. The reporter cells were first infected with a *B. thailendensis* strain that constitutively expresses GFP, then treated with one of four known autophagy inducers (rapamycin, niclosamide, bromhexine HCl, or flubendazole) for 4 hours. Confocal fluorescence imaging was used to quantify autophagy stimulation at the single cell level. Autophagy maturation was observed as a decrease in BFP LC3 puncta with a concurrent increase in mPlum LC3 puncta. *B. thailendensis* infection was assessed by monitoring translocation of GFP-RelA (an NFkB subunit) into the nucleus and through quantitating the intracellular bacterial presence in single cells. Preliminary results indicate that bromhexine HCl and niclosamide may hinder *B. thailendensis'* ability to replicate intracellularly and reduce overall bacterial survival.

INTRODUCTION AND EXECUTIVE SUMMARY OF RESULTS:

Autophagy is a natural, regulated cellular process that "cleans up" cellular debris by degrading and recycling dysfunctional proteins. Prior research has displayed autophagy as having potential for improved control of bacterial infections through autolysosomal killing. There is a high potential impact of exploiting the benefits of autophagy to complement existing treatments, but little has been done to date on bacterial pathogens of defense concern such as *Burkholderia*. *Burkholderia*

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pseudomallei, the causative agent for melioidosis, is a Tier 1 HHS & USDA select agent. *Burkholderia* is intrinsically antibiotic resistant and there is also no current vaccine available. In addition, genetic engineering of *Burkholderia* is simple and rapidly evolving.

Current technology relies on separate ensemble measurements such as western blots, plaque assays and single cell analysis (microscopy, flow cytometry) to confirm both autophagy modulation and pathogen infection processes separately. There is no single assay to measure both autophagy modulation and pathogen infection at the single cell level or otherwise. Simultaneous measurement of individual cells is critical to understand how autophagy might be used in combatting pathogen infection because cell-to-cell heterogeneity response is significant.

In this project, we developed a multi-target reporter cell line to concurrently visualize autophagy induction and pathogen infection at the single-cell level. Sandia's prior efforts placed us with unique tools to reduce the time and challenges associated with integrating the multi-target reporters into cells. We leveraged prior work from a 2009 Grand Challenge in which a stable cell line for detecting bacterial infection via microscopy was developed. Combining this with a single cell analysis pipeline for autophagy induction, developed by our team as part of a NMSBA project to improve tuberculosis therapeutics, we assessed the effect of autophagy modulating drugs on *Burkholderia thailandensis* infection in living cells in real time. *B. thailandensis* is a BSL-2 surrogate for the fully virulent *B. pseudomallei.* We first assessed cytotoxicity and overall effects four commercially approved autophagy compounds have on *Burkholderia* infection. We performed a colony formation unit (CFU) assay on a heterozygous population of *Burkholderia* infected mouse macrophage cells. To further assess autophagy induction, western blots were performed to assess LC3 (autophagy protein) quantification. We developed our dual target reporter to visualize *Burkholderia* infection concurrently with autophagy modulation in a mouse macrophage cell line. We performed live cell confocal microscopy followed by 3D, single cell analysis which consisted of quantitating the number, intensity, and volume of LC3 puncta to confirm autophagy induction. We also quantified the number of intracellular bacteria present in the individual macrophage cells.

Our results did not demonstrate that there is a strong statistical connection between autophagy induction and *Burkholderia* clearance. But there are some preliminary indications that niclosamide and bromhexine HCl may lead to reduced bacterial survival and may hinder the *Burkholderia's* ability to replicate intracellularly. This result needs to be validated with further experimentation. We generated several capabilities in this project that will enhance the ability to attract follow-on work, including establishing protocols for *Burkholederia* research in SNL-NM, developing protocols for mammalian fluorescence cell sorting utilizing a FACSAria Fusion sorting flow cytometer, and enhancing our single cell, 3D cell analysis software. Finally, we were able to create a dual target reporter cell line for further studies of synergy between autophagy and other infectious agents and studies of cell-to-cell heterogeneity.

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RESEARCH AND DEVELOPMENT METHODOLOGY:

CellTiter-Glo® Luminescent Cell Viability Assay

RAW 264.7 cells were plated on an opaque cell culture-treated 96-well plate at a density of 8.0 * 10^4 cells/mL with a total volume of 100uL per well. The cells were incubated at 37 degrees Celsius with 5% CO_2 for 24 hours. The CellTiter-Glo® 2.0 reagent was prepared as instructed in the Promega CellTiter-Glo® Protocol and added to each well in a 1:1 ratio of cell culture medium to reagent (for a 96-well plate, add 100uL of CellTiter-Glo® 2.0 Reagent to 100uL of cell culture medium). The 96-well plate was then covered in tin foil to protect from light and placed on an orbital shaker for 2 minutes to promote cell lysis. The 96-well plate was then incubated at room temperature for 20-30 minutes to stabilize the luminescent signal. Following the incubation, the luminesce signal was then read on Synergy H4 Hybrid Multi-Mode Microplate Reader.

Determining effect of small molecules on *Burkholderia* infection (*B. thailendensis* infected RAW 264.7 cells)

RAW 264.7 (mouse macrophage) cells were infected with *B. thailendensis* at MOI25 for 1 hour at 37 degrees and 5% CO_2. Autophagy compounds-Niclosamide 1um, 3uM, and 5um, Bromhexine HCl 10uM, 15uM, and 25uM, Rapamycin 10uM, 15uM, and 25uM, Flubendazol 10uM, 15uM, and 25uM-were then added for 4 hours. The RAW cells were lysed using 0.5% triton and bacterium was collected from this lysate using centrifugation (the lysate was preserved for LC3 Western Blot). The pellet of bacterial cells was washed with PBS and a 1:10 dilution series was created. 100uL of the bacterial suspension was placed in an opaque 96-well plate. The BacTiter-Glo® 2.0 reagent was prepared as instructed in the Promega BacTiter-Glo® Protocol and added to each well in a 1:1 ratio of bacterial suspension to reagent (for a 96-well plate, add 100uL of BacTiter-Glo® 2.0 Reagent to 100uL of bacterial suspension). The 96-well plate was then covered in tin foil to protect from light and placed on an orbital shaker for 1 minute to promote cell lysis. The 96-well plate was then incubated at 37 degrees Celsius for 5 minutes to stabilize the luminescent signal. Following the incubation, the luminesce signal was then read on Synergy H4 Hybrid Multi-Mode Microplate Reader. The 1:10 dilution series was then plated on LB agar plates and grown at 37 degrees for 24-36 hours until colonies formed. The colonies were then manually counted. The Bac-Titer Assay data was an additional matrix to measure the effects of the autophagy compounds on *B. thailendensis.*

Fluorescence-Activated Cell Sorting

A recently acquired FACSAria Fusion sorting flow cytometer from BD Bioscience (San Jose, CA) was used to sort RAW RelA cells expressing GFP with the goal of reducing cell-to-cell expression heterogeneity. A 100-µm nozzle at 20 psi was used during for the sort to reduce shear stress on the

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RAW cells during sorting. In addition, the Neutral Density 2.0 filter was inserted into the beam path to allow simultaneous visualization of single and aggregate RAW cells in the FSC window. Also, BD FACSFlow sheath fluid was replaced with DPBS to remove additional salts and facilitate healthy sorted cell outputs. Subpopulations of the RAW cells were arbitrarily defined by their relative size and GFP fluorescence in the FITC channel. Cells were then sorted into two tubes with a flow rates ≤6000 cells/min, tube 1 contained single cells with high GFP, and tube 2 contained aggregate cells with high GFP. After sorting into sort tubes, cells were gently centrifuged (500xG) for 5 mins, washed, concentrated, and resuspended in DMEM growth media and placed in a tissue culture plates.

Plasmid Constructs

The pLV-Puro-UBC-TagRFP::LC3::TagBFP2 and pLV-Puro-UBC-mPlum::LC3::TagBFP2 were generated by cloning a cassette comprising LC3 flanked by dual-color fluorescent proteins (Sheen et al.) into the pLV third-generation lentivirus (VectorBuilder). The LC3 insert harbors the recognition site for autophagic protease ATG4 cleavage. The dual-color flanking DsRed and eGFP were replaced with either TagRFP or mPlum and TagBFP2. Expression of the TagRFP/mPlum::LC3b::TagBFP2 was driven by the human ubiquitin C promoter.

Development of Dual Target LC3:RelA RAW Transient Cell Line

FACSAria Fusion sorted RAW-RelA cells underwent nucleofection using a an Amaxa 4D-Nucleofector™ X Unit and SF Cell Line 4D-Nuclefector™ X Kit L. 2ug of purified LC3 plasmid DNA (purified using Promega Mini Prep System) was added to RAW RelA cells 24 hours prior to imaging to generate the RAW 264.7 LC3(BFP:mPlum):RelA-GFP dual target reporter cell line.

Live cell fluorescence microscopy of RAW LC3-RelA reporter cell line treated with autophagy inducing compounds

RAW 264.7 LC3(BFP:mPlum):RelA-GFP (dual target reporter cell line) cells were plated on a tissue-culture treated 4-chamber glass bottom dish (40,000 cells per chamber with 0.7mL of DMEM-10/10% FBS) with 400ug/ml G418 antibiotic 24 hours prior to imaging. The cells were cultured at 37 degrees Celsius and 5% CO_2. The cells were infected with *B. thailendensis-GFP* (MOI 25 at OD 1.0) for 1 hour at 37 degrees Celsius and 5% CO_2. The cells were washed with PBS and then treated with each autophagy modulating compound (5uM Niclosamide, 15uM Flubendazol, 25uM Bromhexine HCl, and 25uM Rapamycin), 250ug/mL Kanaymycin, 150ug/mL Gentamicin, and 10ug/mL Impenium. The cells were then incubated at 37 degrees and 5% CO_2. Images of live cells were collected at 4 hours post addition of autophagy modulating compound using a Leica DMi8 DLS confocal fluorescence microscope equipped with a Leica HC PL APO CS2 63x/1.40 oil immersion objective. Three laser diode excitation sources were used (405nm, 488nm, and 552nm) with Leica SP8 LIAchroic low incident angle dichroic beam splitters. Details

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of laser power applied to samples, spectral range detected, and gain applied to signal were as follows – BFP signal: 20% 405nm excitation, detecting 410-490nm, HyD gain= 130, GFP signal: 10% 488nm excitation, detecting 493-560nm, PMT gain = 800, and mPlum signal: 15% 552nm excitation, detecting 625-780nm, HyD gain = 100. Additionally, a transmitted light image was collected using 10% 552nm excitation and a PMT gain of 225. All images were collected using sequential scanning between frames with a resonance scanner at a speed of 8,000Hz (28 fps at 512*512 pixels) at a zoom of 2.00x. Line averaging of 16 was used to improve overall image quality. All images were collected as a 10µm 35-slice z-stack with an axial step size of 0.3µm at an X*Y image size of 186µm*186µm and a pixel size of 363.99nm*363.99nm. LAS X 3.5.5.19976 by Leica Microsystems ©2019 was used as the image acquisition software. 15-20 fields of view were collected at 63x magnification for each treatment condition. This yielded ≥ 100 cells per condition for single cell analysis.

The images were analyzed utilizing Sandia's in-house written software, optimized for these data, to segment the cells and analyze BFP and mPlum puncta in individual cells. Each compound had a minimum of 130 cells analyzed. A flowchart of the analysis process is shown in addendum slide 11. The confocal stack was first merged into a single image to more easily outline individual cells. The images were then processed through an in-house written software, Cell Finder, to segment the individual cells within each image. Cell segmentation was accomplished using a modified marker watershed transformation algorithm, however manual adjustments were made when necessary. Next, the puncta from the coordinates in the 3D image were quantified in another in-house written software, zStackViewer. The identification of each punctum in each slice of the confocal stack was accompanied by an additional procedure to compare the coordinates of the identified puncta in the slices above and below to screen for spatial overlap. If the coordinate indicates the puncta are spatially overlapped, then they are considered a single punctum and for size and intensity purposes their area and intensities will be added. This prevents over-counting or under-counting puncta when they span multiple optical sections. Lastly, the puncta were compared against the coordinates of the cells identified in the cell segmentation and puncta outside the cell boundaries were removed from further analysis.

An excel spreadsheet is then produced that displays the results for the number of puncta, total intensity, average intensity, total puncta volume, and average puncta volume for both BFP and mPlum puncta and the ratio of the two for a total of 15 cell-based metrics. The entire single cell analysis process has been automated to generate these results through the use of Matlab scripts that call the various in house written Matlab functions. To assess the presence of intracellular bacterial infection, we quantified the number of *B. thailendensis* bacteria present in individual cells and measured the GFP fluorescence intensity of the nucleus. We manually counted the bacteria present in the previously segmented cells. We then measured the nuclear GFP fluorescence in each

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of segmented cells. Current analysis is ongoing to determine the correlation between RelA-GFP nuclear fluorescence oscillation and bacterial presence.

Western Blot Analysis

Raw 264.7 macrophage cell lysate was quantitated by BCA protein assay (Pierce) and 7.5 µg total protein was heated at 95°C for 10 min in 1x Laemmli buffer (0.125 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.0025% bromophenol blue), resolved on a 4-12% gradient SDS-polyacrylamide gel, and electro-transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk/1x TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) at 22°C for 5 min followed by the addition of 1:3,500 dilution of anti-Lc3B (L7543) and anti-actin (A2066) rabbit polyclonal primary antibodies (Sigma), incubated for 1 hour, and 3 washes with 1x TBST. The blocking and incubation were repeated at 1:5,000 dilution of anti-rabbit (31462) goat polyclonal secondary HRP-conjugated antibody (Thermo). Following the 3 washes with 1x TBST, the blot was developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo) and imaged on a ChemiDoc system (BioRad).

RESULTS AND DISCUSSION:

The goal of this project was to prove with statistical confidence that inducing a cell's natural clearing process, called autophagy, leads to lower rates of pathogen infection at the single cell level, in *B. thailandensis*, a BSL-2 surrogate for *B. pseudomallei*, the causative agent for melioidosis. To achieve our goal, we conducted the following efforts:

- Performed colony formation unit (CFU) assays to assess the effect of four autophagy compounds (niclosamide, bromhexine HCl, flubendazol, and rapamycin) on *Burkholderia* infection.
- Conducted LC3 Western blots to quantity the level of autophagy protein (LC3II/Actin) in autophagy treated infected RAW 264.7 cells.
- Developed a dual-target reporter to visualize pathogen infection (RelA-GFP) and autophagy induction (LC3 BFP:mPlum) simultaneously.
- Performed 3D, single cell analysis on the dual-target reporter through quantification of LC3 puncta and quantifying the presence of intracellular bacteria.

The results of the clearance assay suggest that flubendazol and bromhexine HCl have a slight effect on the overall survival and viability of intracellular *B. thailendensis.* To validate this finding, we conducted LC3 western blots to assess the level of autophagy induced in the infected macrophage cells. We tested two biological replicates for both flubendazol and bromhexine HCl. For both compounds, one biological replicate displayed a statistical increase in autophagy induction compared to the untreated condition. But the other biological replicate, for each compound,

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displayed no evidence of autophagy induction occurring. We are unsure why the results were inconsistent. We have hypothesized that either the macrophage cells treatment procures lead to inconsistent cellular uptake or perhaps *Burkholderia* is evading the autophagy pathway. Unfortunately, insufficient time remained to identify the source of the variation.

With the development of the dual target cell line, we were able to concurrently visualize autophagy modulation and pathogen infection at the 3D, single cell level. Through live cell confocal imaging and single cell analysis, we were unable to validate a strong statistical connection between autophagy induction and bacterial clearance. Based on the reporter design we expected autophagy induction to be reflected as a decrease in BFP puncta intensity, number, and/or size accompanied by a concurrent increase in mPlum puncta intensity, number, and/or size. Our preliminary data analysis revealed several challenges with our approach. niclosamide and bromhexine HCl displayed a slight increase in the ratio of mPlum intensity to BFP intensity but this was a result of both mPlum and BFP increasing at different amounts instead of the expected decrease in BFP puncta intensity. It was also noted that the signal levels of the BFP fluorophore were weak. Additional analysis is underway to verify if the automated analysis pipeline is extracting reliable signals for this fluorophore. Detailed results can be found in the addendum slides 2-16. Further studies are needed to confirm the BFP fluorophore is being properly cleaved when autophagy is induced.

Based on the combined results of the novel concurrent assay and traditional independent measurements we can conclude that niclosamide and bromhexine HCl does induce autophagy and does influence overall *Burkholderia* clearance, albeit the statistical significance was unable to be proven.

ANTICIPATED OUTCOMES AND IMPACTS:

The goal of this project was to establish technology that can concurrently visualize autophagy induction and pathogen infection at the single cell level. We were successful in the overall creation of a transient dual target macrophage cell line to view both biological processes simultaneously. Although we are not able to validate a statistical connection between autophagy modulation and bacterial clearance, we were able to develop a dual target cell line that allows visualization of synergy between autophagy and bacterial pathogens in living cells in real-time. This capability enables biological hypothesis regarding host-response to be tested that current technology doesn't exist for. We were also able to establish Burkholderia research in SNL-NM, enhance in-house written software to quantify autophagy and pathogen infection in a 3D, single-cell model, and leveraged new FACSS Aria Cell sorter through the establishment of protocols for mammalian cell lines. We are currently preparing two publications that stem from this research project. The first publication originated from a previous NMSBA project but was furthered by this exploratory express project. The second publication is fully attributed to this exploratory express project. We

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are also presenting our new technology at Keystone's virtual conference on autophagy in October 2020. This research has aligned with DOE's mission in Science and Innovation. Follow on efforts would target the Bioscience IAT and/or existing DTRA, NIH, and DHS programs that are pursuing advances in methods to combat emerging infectious disease. There's far reaching applications to utilize CRISPR to regulate autophagy in infectious agents that display high levels of antibiotic resistant and highly virulent agents of biodefense concern. The single cell assay heightens Sandia's standings as a leader in therapeutics for biodefense threats.

CONCLUSION:

There is currently no technology that can concurrently visualize autophagy and pathogen infection, only separate ensemble measurements. The development of a dual reporter cell line allows visualization of synergy between autophagy and bacterial pathogens in living cells in real-time. This assay is an enabling technology for understanding *Burkholderia* and other infectious agents. Although we were unable to establish a statistical connection between autophagy induction and *Burkholderia* clearance, we were able to develop a technology that be leveraged to study cell-to-cell heterogeneity in multiple pathogens of biodefense concern. We also established *Burkholderia* research in SNL-NM as well as leveraging a new FACSS Aria cell sorter for mammalian cell lines thus paving the way for future use of this newly acquired resource. With the establishment of this technology, we can further investigate *Burkholderia* infection and role of autophagy in intracellular bacterial infection.

Acknowledgements: We would like to acknowledge Chuck Smallwood for performing and establishing protocols for mammalian cell sorting. We would also like to thank Anita Sumali for her assistance with image analysis and Steven Bradfute for the LC3 western blot protocols. Finally, we would like to acknowledge Biophagy Inc. for the inspiration of the research idea.

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ADDENDUM:

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Concurrent evaluation of autophagy induction and *Burkholderia* infection at the single cell level

LDRD#: 20-0983

PI: Danae Maes (08635), PM: Jerilyn Ann Timlin (08635)
Team Members: Stephen Anthony (08635), Colleen Courtney (08621), Joshua Podlevsky (08631), Steven Branda (08631)

Program Goal:

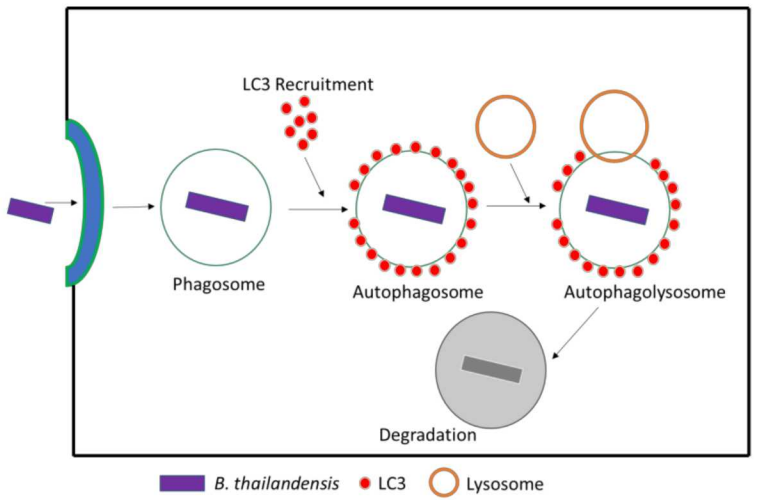
Develop an assay to correlate autophagy induction and pathogen infection at the single cell level, demonstrate this enabling technology on *B. thailandensis*, a surrogate for *B. pseudomallei*.

Current state of the art:

- Autophagy is a natural, regulated cellular process that "cleans up" cell by degrading and recycling dysfunctional proteins. We and others have shown autophagy can provide a "boost" to clear bacterial pathogens.
  - High potential impact on bacterial pathogens of biodefense concern like Burkholderia.
- No assay capable of detecting autophagy AND pathogen infection.
- Ensemble measurements (western blots, plaque assays) and single cell analysis (microscopy, flow cytometry) can confirm each of these biological processes separately
- Recently, our team has developed a single cell analysis pipeline for autophagy induction (NMSBA leverage grant)

Representative Figure

Autophagy and *Burkholderia* Infection: Schematic displaying how autophagy clears *Burkholderia thailendensis* infection in macrophage cells.



Key R&D Results and Significance

Impacts and Outcomes:

- Established *Burkholderia* research in SNL-NM.
- Creation of a dual color reporter to assess autophagy modulation and pathogen infection simultaneously, specifically for *Burkholderia* infection.
  - Integration of LC3 plasmid into RAW RelA-GFP cell line (LDRD Grand Challenge 2009)
- Measured effect of four clinically approved autophagy-inducers have on *B. thailendensis* at the ensemble and single cell level.

Follow-On Plans:

- Generated preliminary results for external sponsors.
  - Will meet with Cathy Branda to discuss external funding opportunities with DTRA, DHS, and NIH to support Sandia's research and technology missions.
- 2 Manuscripts in preparation

Impact of Project:

- Cell line developed can be used to test hypotheses about the relationship between *Burkholderia* and autophagy in future.
- Methods developed are easily translatable to other intracellular bacterial pathogens and viruses.
- Further established Sandia as a leader in biological infectious disease therapies.

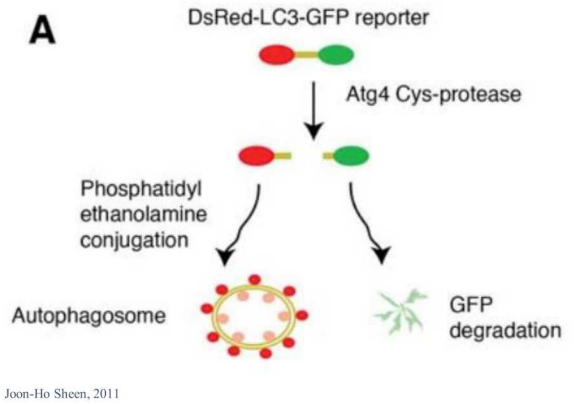
R&D Summary (Methods, Results and Discussions)

Research Question: Can we correlate autophagy induction concurrently with *B. thailandensis* infection in individual cells with a single biological assay?

Clearance assays of 4 commercial autophagy drugs

Quantitatively measured commercial autophagy compound effects on:

- Bulk measurements & single cell
- *B. thailandensis* clearance
- Determine toxicity/effective dosage of drugs



Joon-Ho Sheen, 2011

Development of Dual Reporter Cell Line

Creation, validation, and optimization of stable RAW 264.7 LC3-BFP:RFP, GFP-RelA reporter cell line to evaluate autophagy modulation and infection of *Burkholderia thailandensis.*

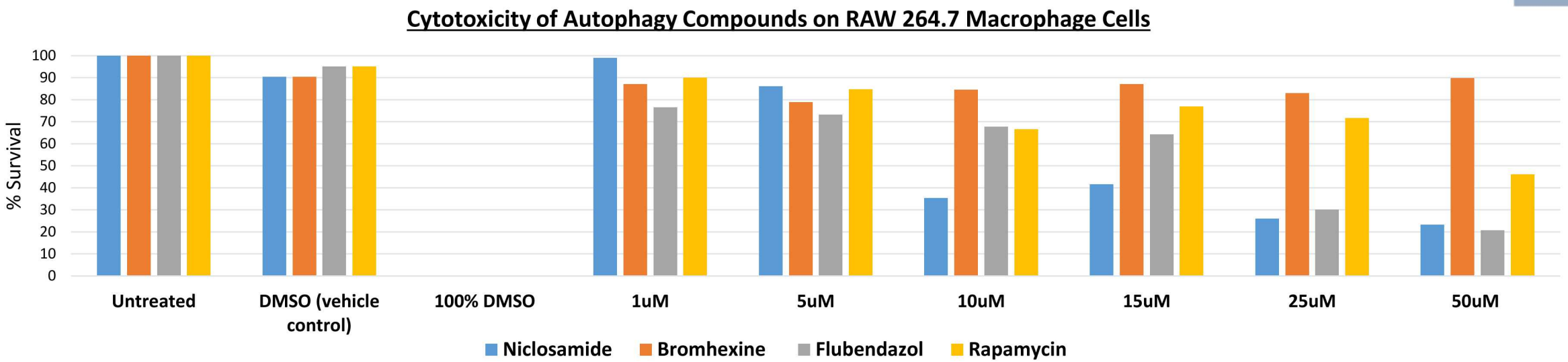
Live cell fluorescence microscopy to assess the effect of autophagy modulating drugs on *B. thailandensis* infection

- Enhancements of Single Cell Analysis of RAW Dual Reporter Cell Line:
  - Generalized code to support arbitrary data (# of channels, # of z steps)
  - Improved automation – background processing of multiple experiments without further user interaction
  - Developed GUI-based visualization allowing a human-in-the-loop to view and validate that the analysis makes sense

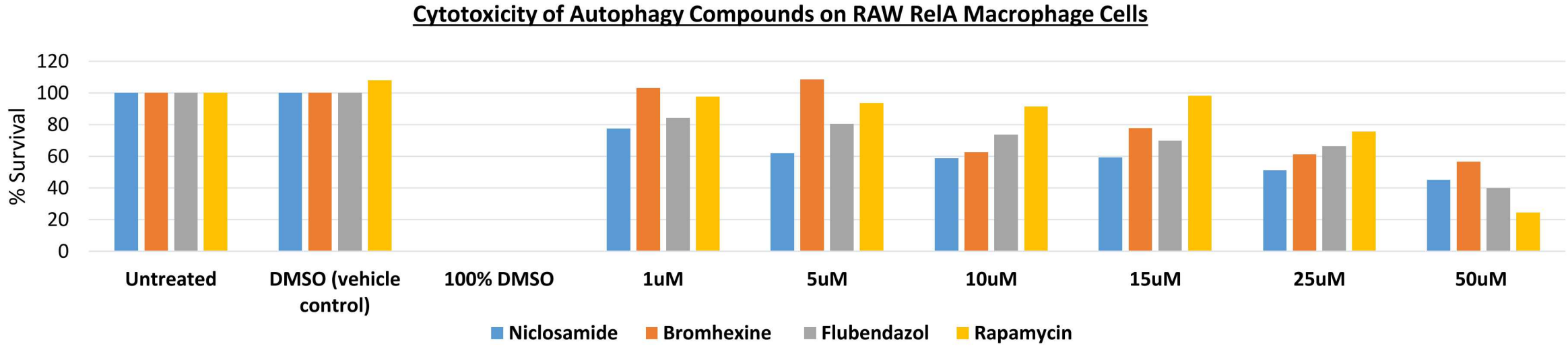
Outcomes:

- Established *Burkholderia* research in SNL-NM.
- Preliminary data suggests that the autophagy compounds affect the *B. thailendensis* ability to propagate and further infect macrophage cells.

Cytotoxicity of Autophagy Compounds on RAW 264.7 & RAW RelA-GFP Macrophage Cells



Cytotoxicity of four autophagy compounds (Niclosamide, Bromhexine HCl, Flubendazol, and Rapamycin) on RAW 264.7 cells at 6hrs. CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was utilized to determine the number of viable cells in culture based upon quantification of ATP present.



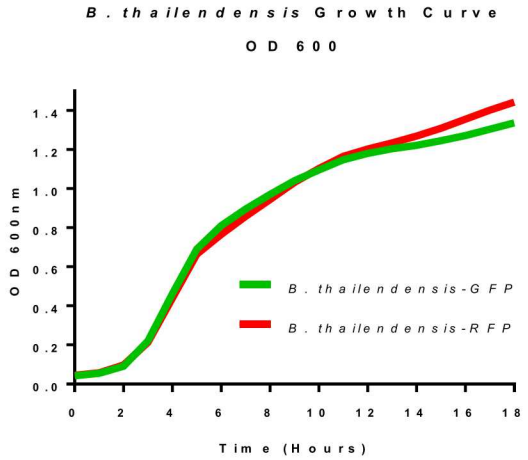
Cytotoxicity of four autophagy compounds (Niclosamide, Bromhexine HCl, Flubendazol, and Rapamycin) on RAW RelA-GFP cells at 4hrs. CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was utilized to determine the number of viable cells in culture based upon quantification of ATP present.

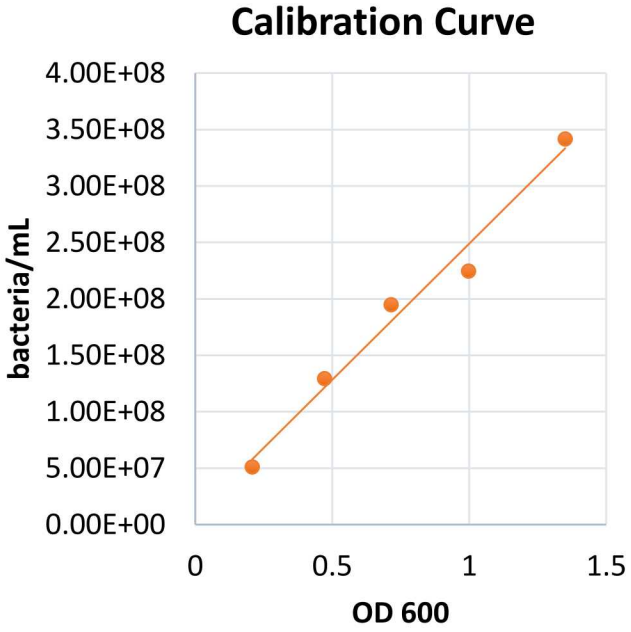
Clearance Assay of *B. thailendensis* on RAW 264.7 Macrophage Cells-Experimental Set-Up

Goal: Quantitatively measure the overall survival of *B. thailendensis* on infected RAW 264.7 macrophage cells following autophagy compound treatment.

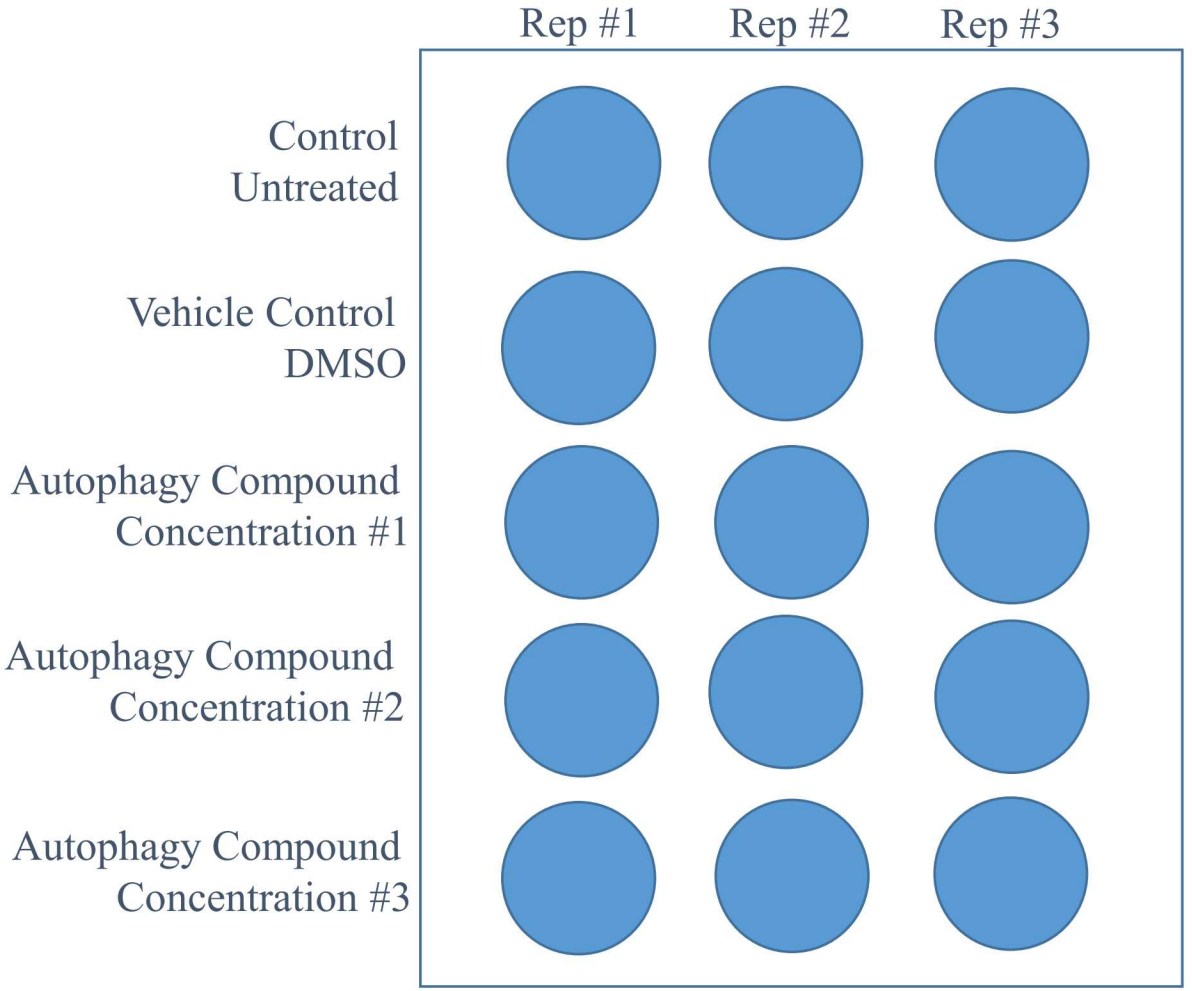
Methods:

1. RAW 264.7 (mouse macrophage) cells were infected with *B. thailendensis* at MOI25 for 1 hour at 37 degrees and 5% CO_2.
2. Autophagy compounds were then added for 4 hours.
3. The RAW cells were lysed using 0.5% triton and bacterium was collected from this lysate using centrifugation (the lysate was preserved for LC3 Western Blot).
4. The pellet of bacterial cells was washed with PBS and a 1:10 dilution series was created and plated onto a 100mm LB agar petri dish.
5. The BacTiter-Glo® Assay was performed to assess bacteria viability.

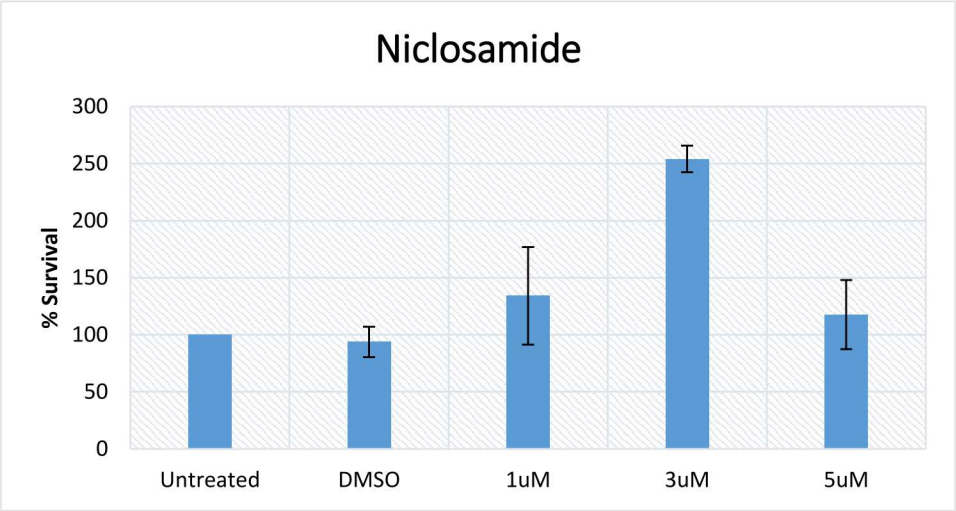


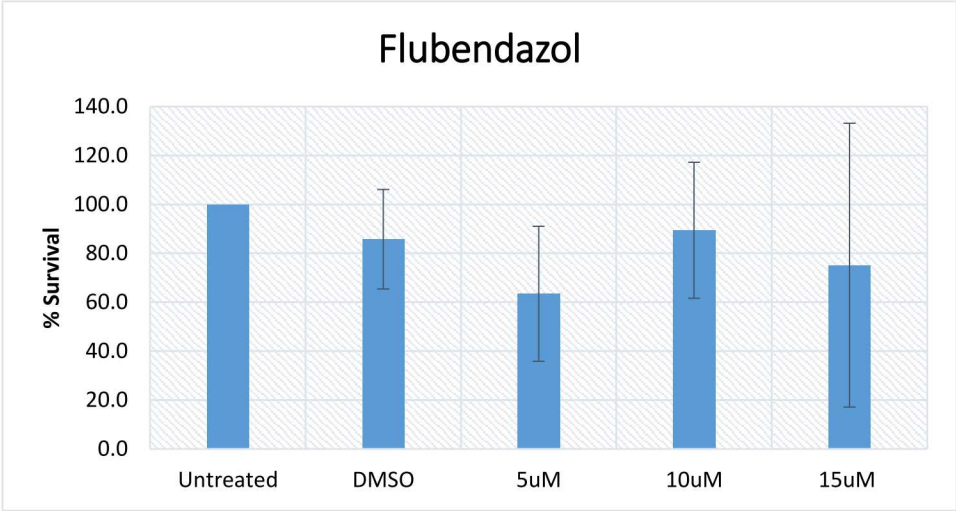


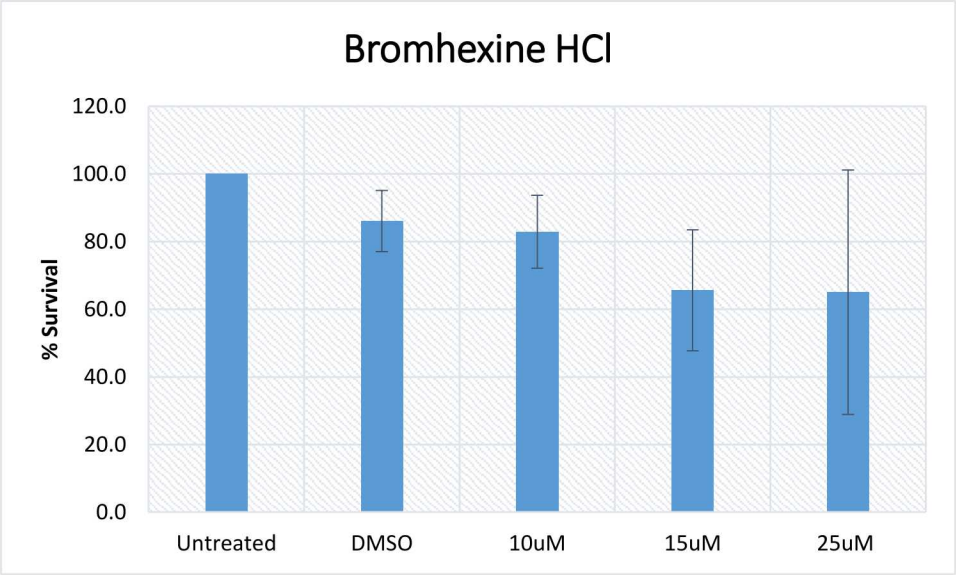
B. thailendensis-GFP growth curve was performed using a multi-plate reader over a 18 hour time period. *B. thailendensis-GFP* calibration curve was determined through a CFU assay plated on 100mm LB agar plates.



Clearance Assay of *B. thailendensis* on RAW 264.7 Macrophage Cells Results







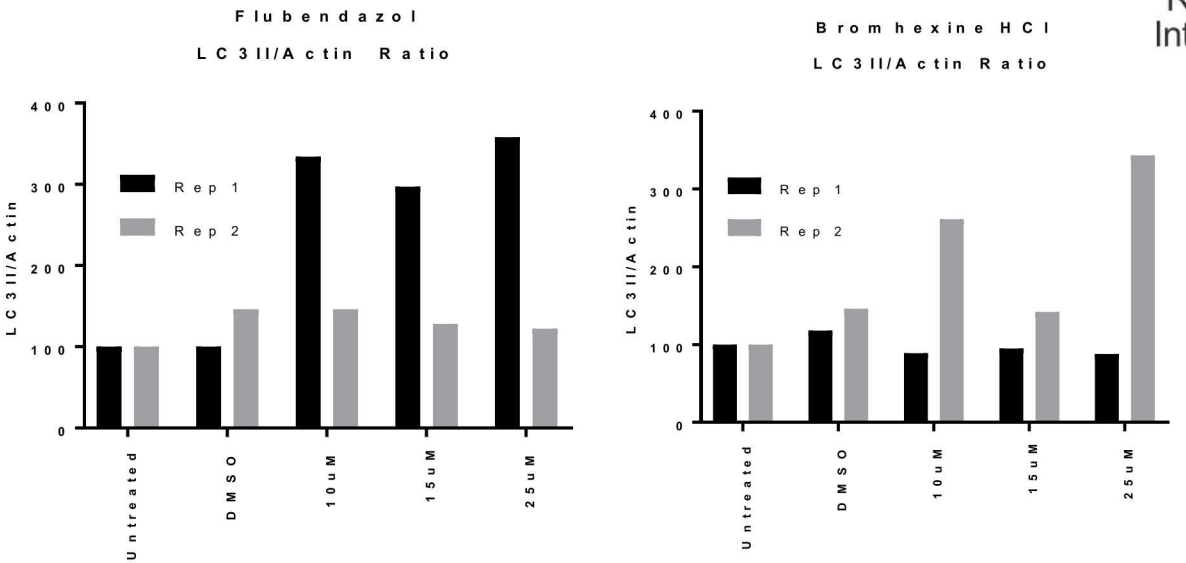
Results of clearance assay to assess overall survival of intracellular *B. thailendensis* in RAW 264.7 (mouse macrophage) cells. *B. thailendensis* was inoculated onto RAW cells for 1 hour. Following incubation, the extracellular bacteria was washed and autophagy compounds were added onto macrophage cells for 4 hours. With the addition of bromhexine HCl and flubendazole, a slight decrease in overall intracellular *B. thailendensis* survival was observed. The addition of niclosamide displayed no evidence of bacterial clearance. Results displayed above are average from three biological replications.

Results from rapamycin were unable to be reported to unexplained experimental results. Further experimentation is needed to understand the abnormalities of the results.

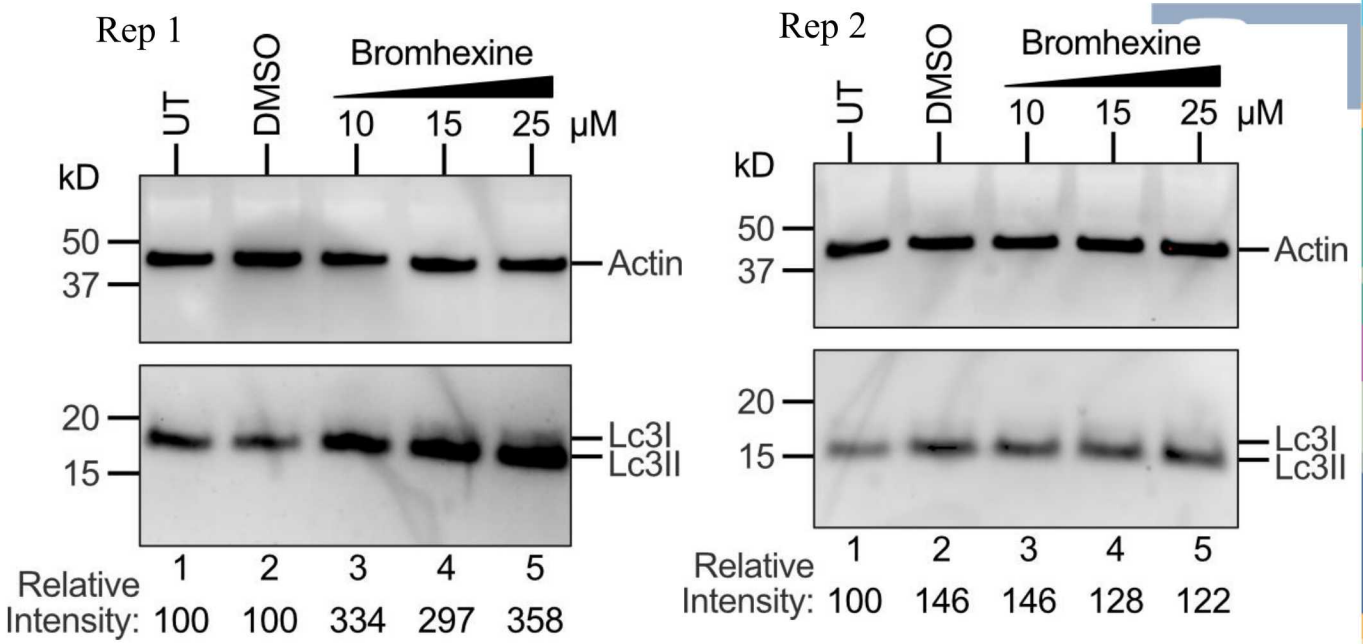
LC3 Western Blots

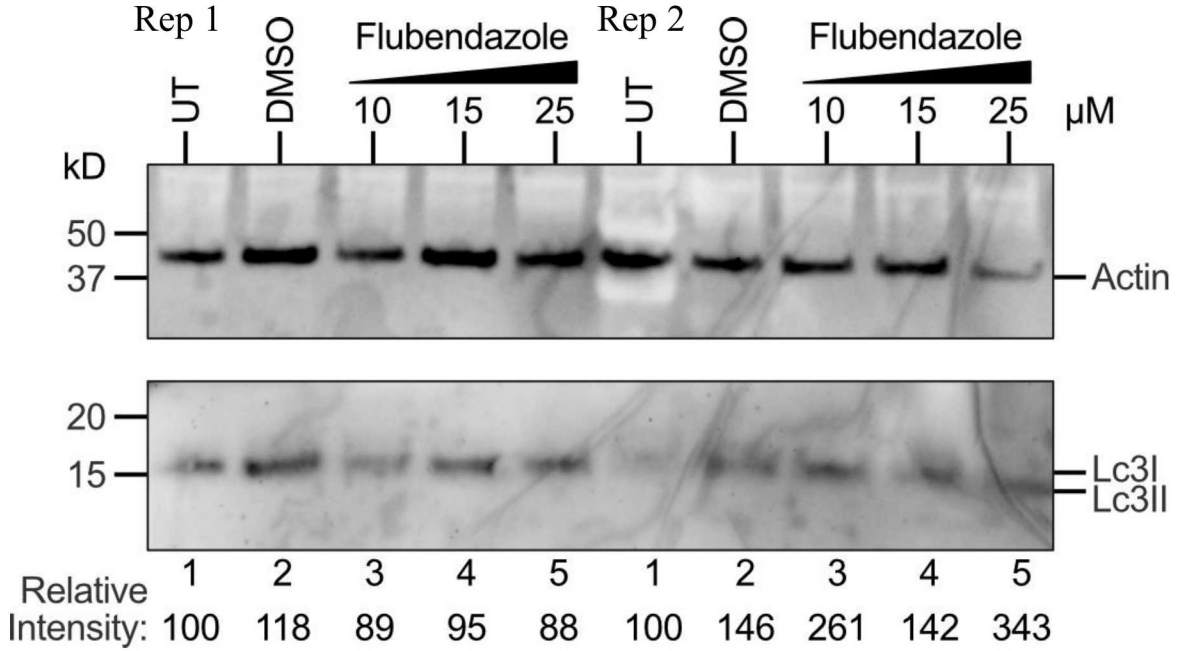
Methods:

1. RAW 264.7 macrophage cell lysate was quantitated by BCA protein assay (Pierce) and 7.5 µg total protein was heated at 95°C for 10 min in 1x Laemmli buffer.
2. Resolved on a 4-12% gradient SDS-polyacrylamide gel and electro-transferred onto a PVDF membrane.
3. The membrane was blocked with 5% nonfat milk/1x TBST at 22°C for 5 min.
4. 1:3,500 dilution of anti-Lc3B and anti-actin rabbit polyclonal primary antibodies were incubated for 1 hour, and 3 washes with 1x TBST.
5. The blocking and incubation was repeated at 1:5,000 dilution of anti-rabbit (31462) goat polyclonal secondary HRP-conjugated antibody.
6. The blot was developed with SuperSignal West Pico PLUS Chemiluminescent Substrate and imaged on a ChemiDoc system.



Western Blot for LC3 was conducted to quantitate the level of LC3 protein (LC3II/Actin) in autophagy treated RAW 264.7 cells. Flubendazol biological replicate one showed no increase in LC3 protein while biological replicate two showed an increase in LC3 protein, demonstrating that autophagy was induced. There was similar trend for Bromhexine HCl where one biological replicate showed an increase in LC3 protein (autophagy induction) while the other biological replicate showed no evidence of increased autophagy induction.



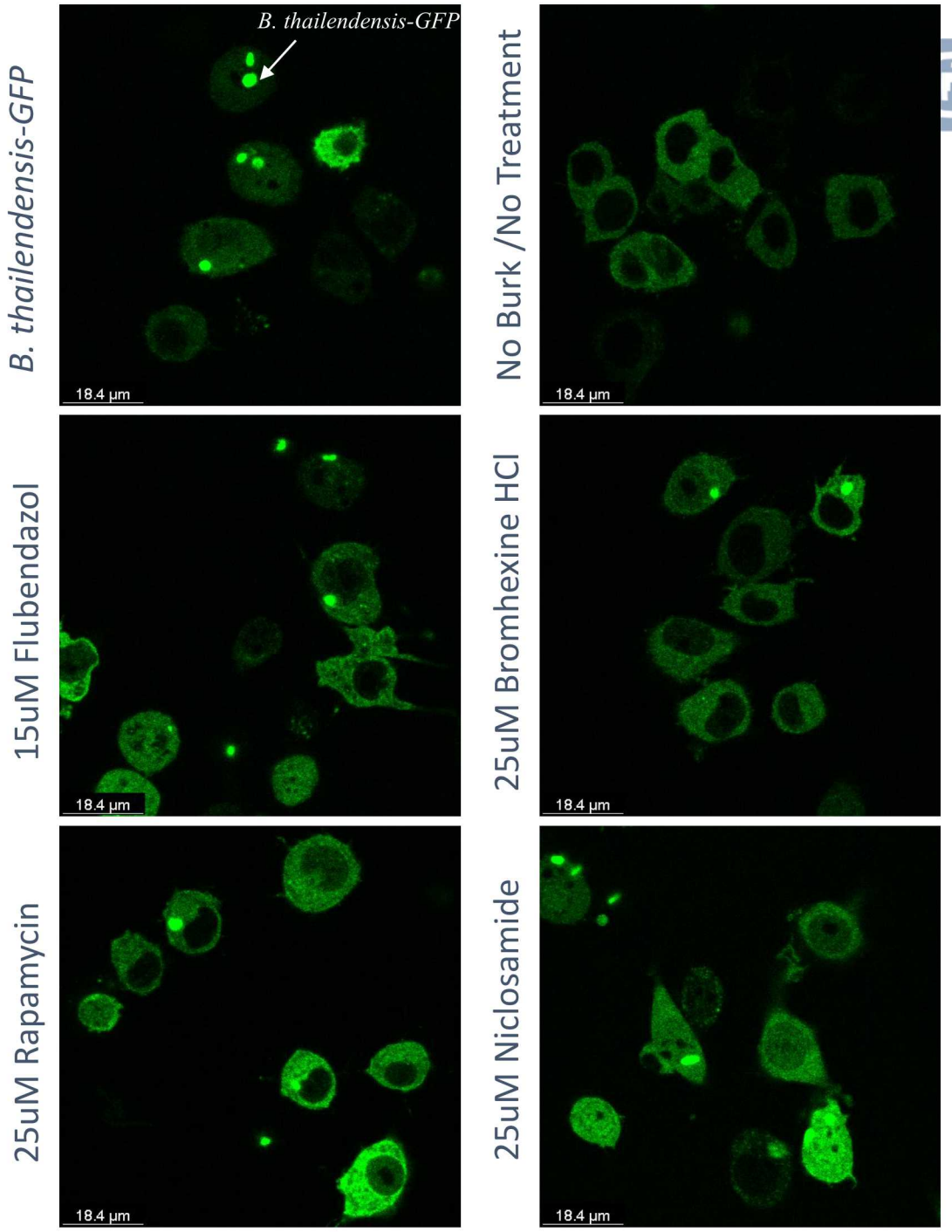


B. thailendensis-GFP on RAW GFP-RelA Cells

Methods:

1. *B. thailendensis-GFP* was inoculated (MOI 25 at OD1.0) onto RAW GFP-RelA macrophage cells for 1 hour.
2. Extracellular *Burkholderia* was removed and autophagy compounds were added (4hrs incubation).
3. Cells were imaged live using a Leica confocal microscope.

Analysis: Quantification of number of bacteria present in individual cells and intensity of GFP-RelA nuclear oscillation.



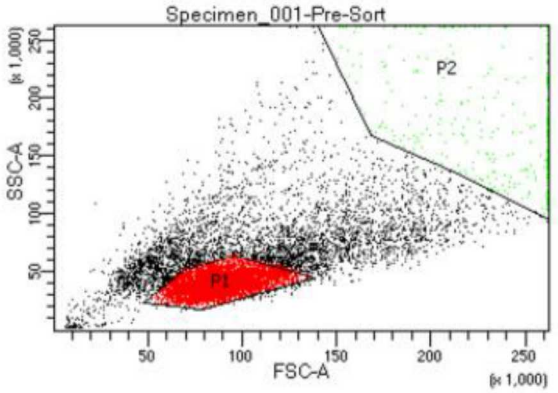
Fluorescence-activated cell sorting on RAW RelA-GFP parental cell line

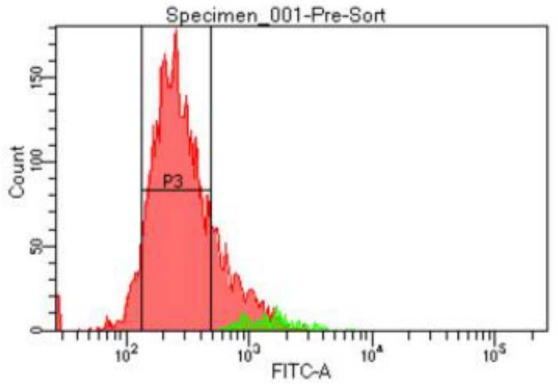
Instrument: FACSAria Fusion sorting flow cytometer from BD Bioscience (San Jose, CA)

Purpose: Sort RAW RelA cells expressing GFP fluorescence

Methods:

1. 100-µm nozzle at 20 psi with a Neutral Density 2.0 filter
2. BD FACSFlow sheath fluid was replaced with DPBS.
3. Subpopulations of the RAW cells were arbitrarily defined by their relative size and GFP fluorescence in the FITC channel.
   - Cells were then sorted into two tubes with a flow rates ≤6000 cells/min.
   - Tube 1 contained single cells with high GFP and tube 2 contained aggregate cells with high GFP.
4. Cells were centrifuged (500xG) for 5 mins, washed, concentrated, and resuspended in DMEM growth media and placed in a tissue culture plates.





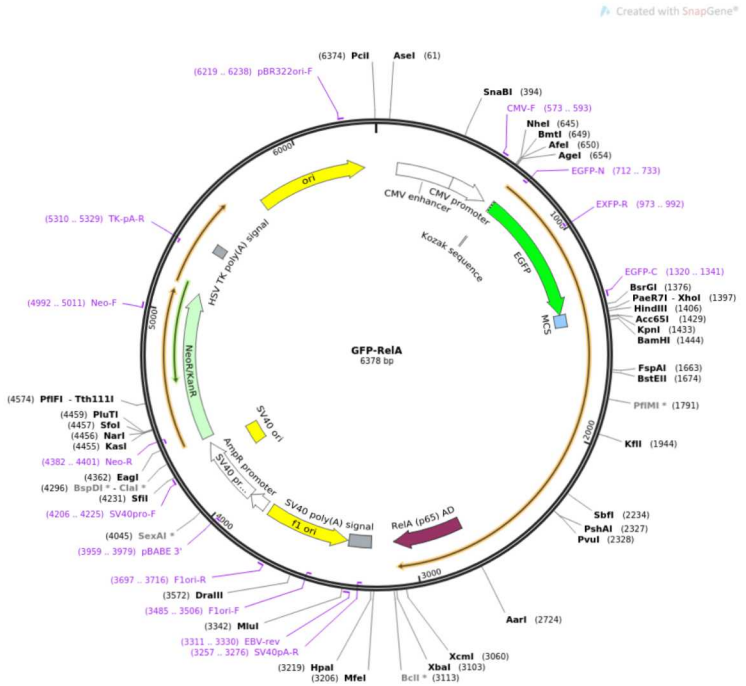
Tube: Pre-Sort

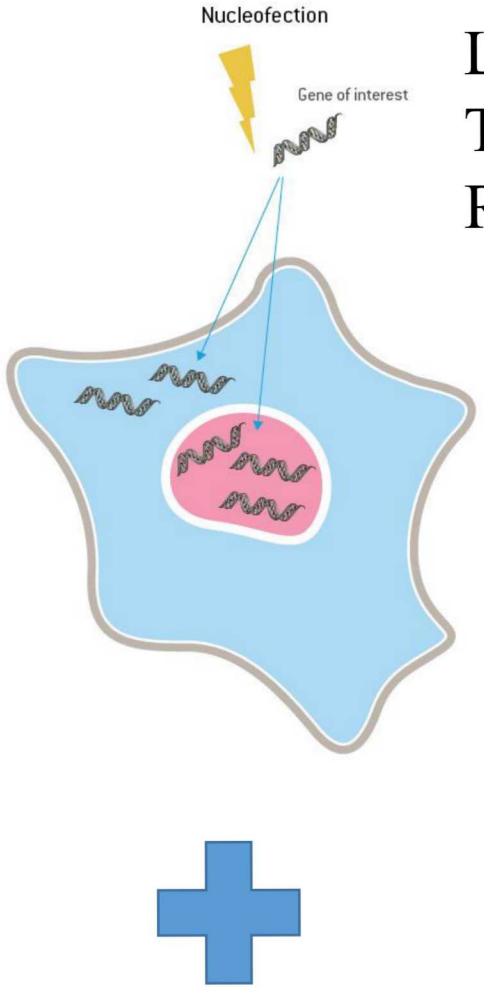
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P2	327	3.3	3.3
P1	5,614	56.1	56.1
P3	6,053	60.5	60.5

RAW RelA-GFP cells were identified from the single-cell population P1 then sorted by strong GFP fluorescence intensity (P3). Target cells were approximately 60.5% of the total population, which yield >95% purity.

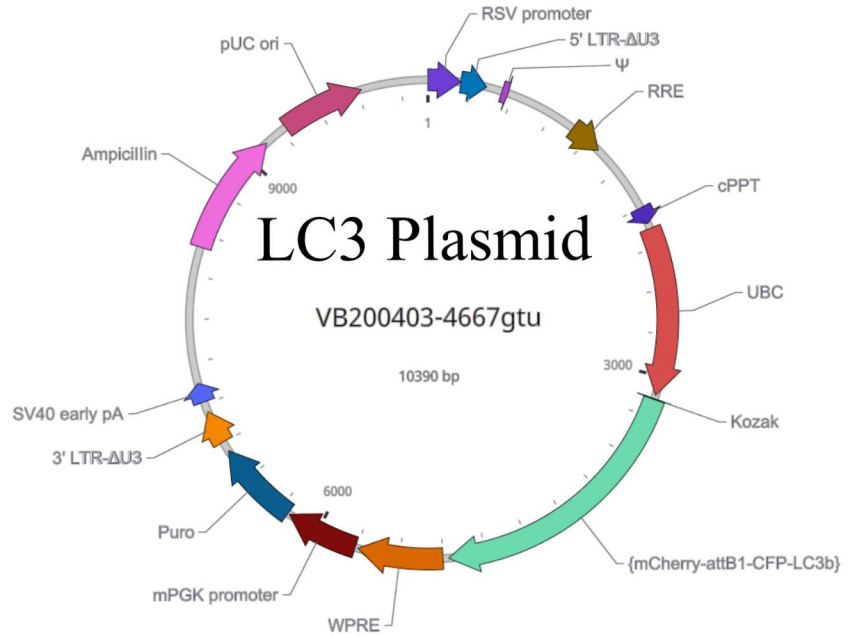
LC3:RelA RAW 264.7 Reporter: Creation of Transient Cell Line

RAW GFP-RelA Cell Line
(Developed in LDRD Grand Challenge 2009)

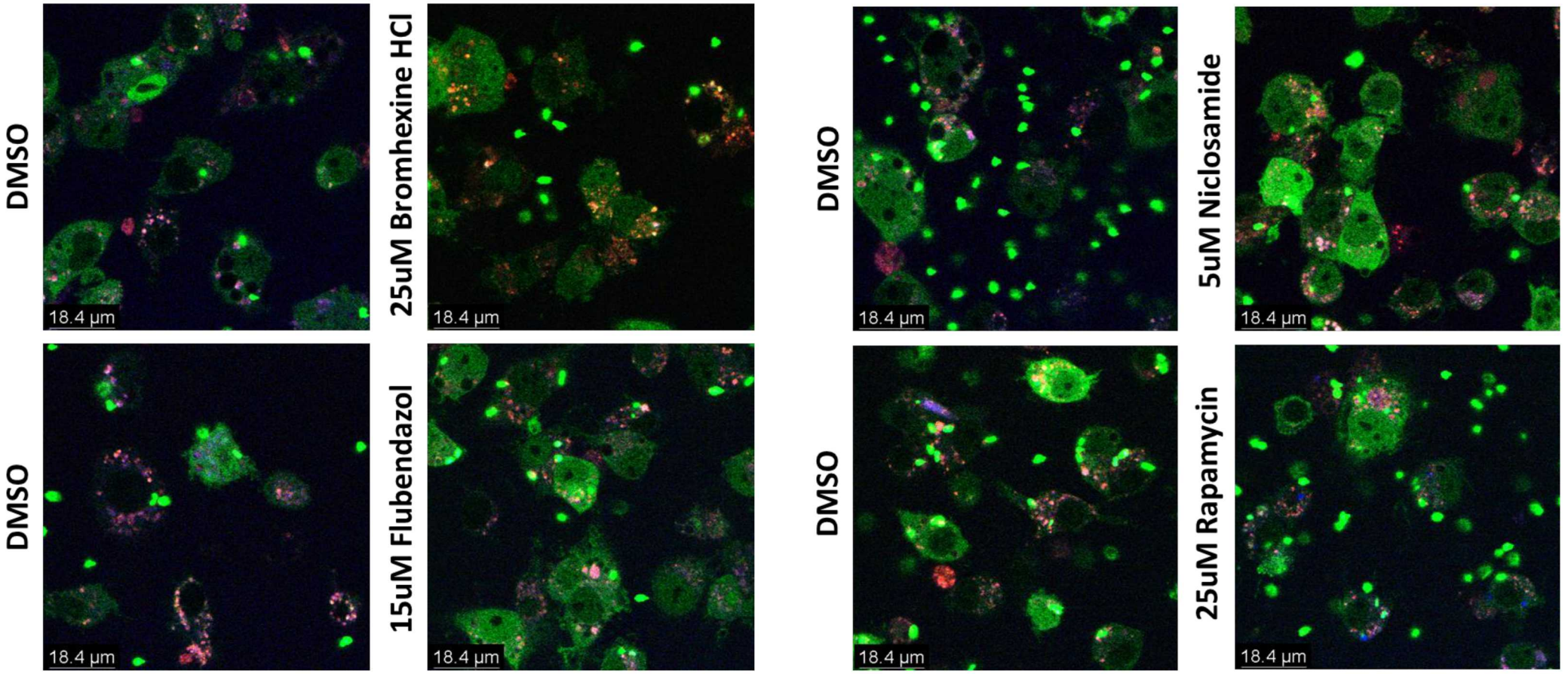




Lonza 4D Nucleofector: Transfection of LC3 Plasmid into RAW GFP-RelA Cell Line



LC3:RelA Reporter Cell Line-Live Cell Confocal Imaging



Methods:

1. Infect macrophage dual target reporter cell line with *B. thailendensis-GFP* for 1 hour.
2. Washed extracellular bacteria with PBS and add autophagy compound on reporter cell line for 4 hours.
3. Imaged cells live using a Leica confocal microscope.
4. Utilize enhanced 3D, single-cell analysis software for analysis
   - Quantify autophagy autophagosome puncta and GFP-RelA oscillation

LC3 Autophagy Puncta Quantification

Single Cell LC3 Autophagy Puncta Quantification

Step 1: Merge and Flatten Tiffs

In-house written software to merge and flatten the 35-stack tiffs to easily identify cells in the image.

Step 2: Segment Image to Identify Individual Cells

Utilize in-house written software, CellFinder, to identify the outline of individual cells in the image.

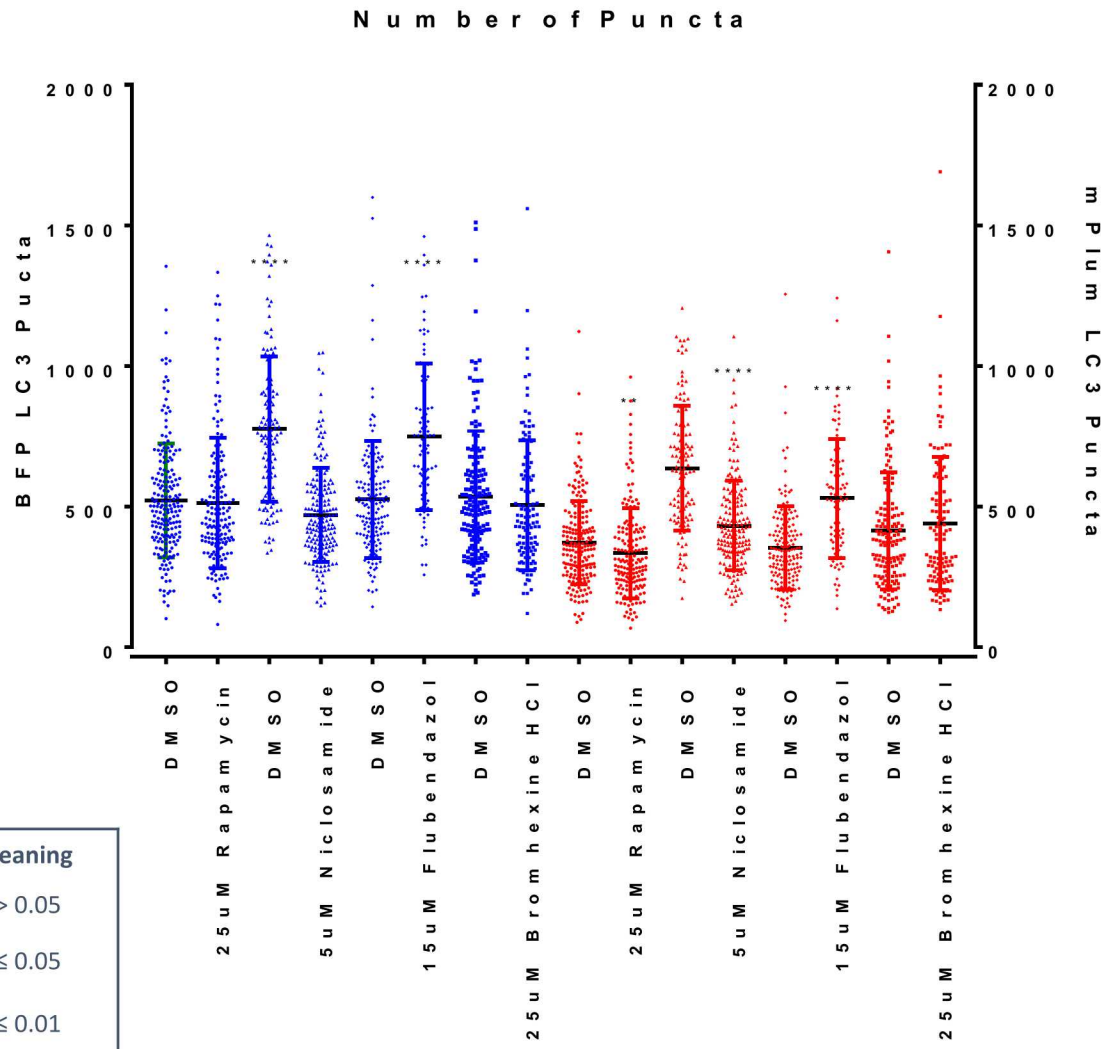
Step 3: Quantification from 3D Projection Coordinates

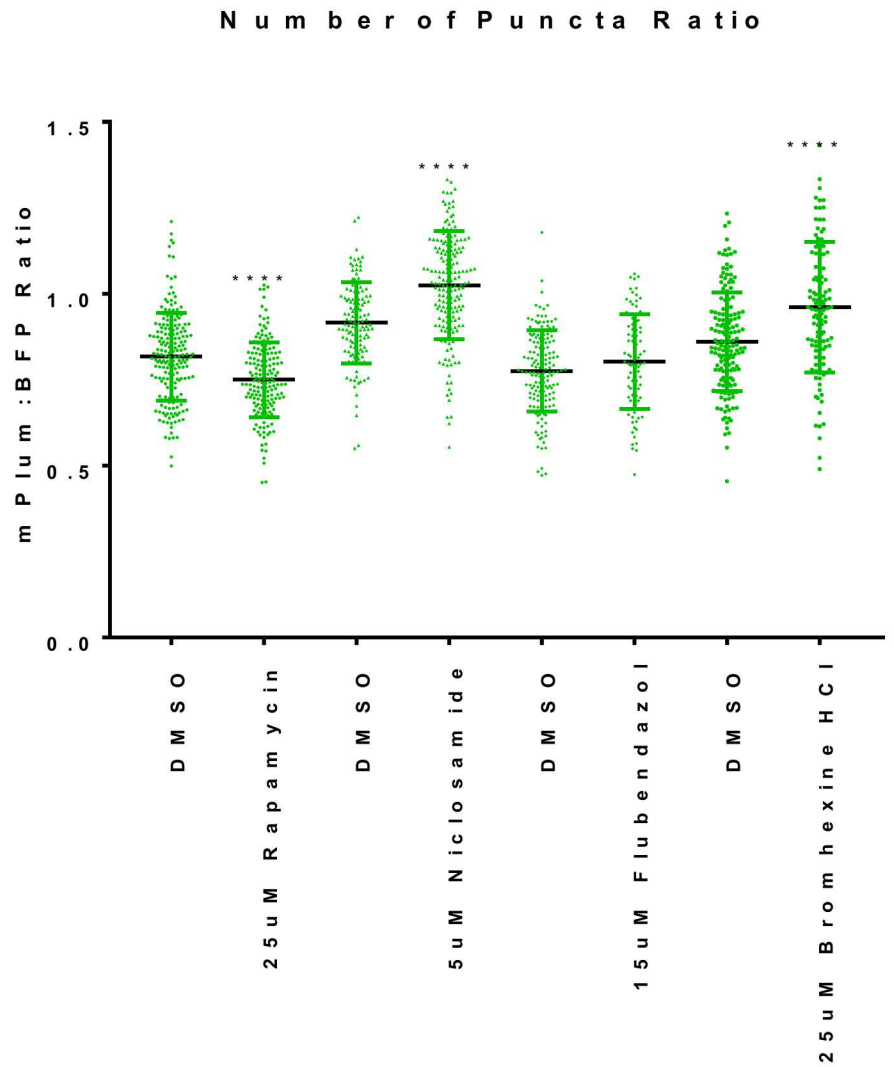
Enhanced in-house written software, zPunctaViewer and zStackViewer, visualization of puncta and quantifies the number, intensity, and volume of the puncta in a 3D cell model.

Single Cell Puncta Code Enhancement's:

- Generalized code to support arbitrary data (# of channels, # of z steps)
- Improved automation – background processing of multiple experiments without further user interaction
- Developed GUI-based visualization allowing a human-in-the-loop to view and validate that the analysis makes sense

Autophagy LC3 Puncta Analysis





Symbol	Meaning
ns	P > 0.05
*	P ≤ 0.05
**	P ≤ 0.01
***	P ≤ 0.001
****	P ≤ 0.0001

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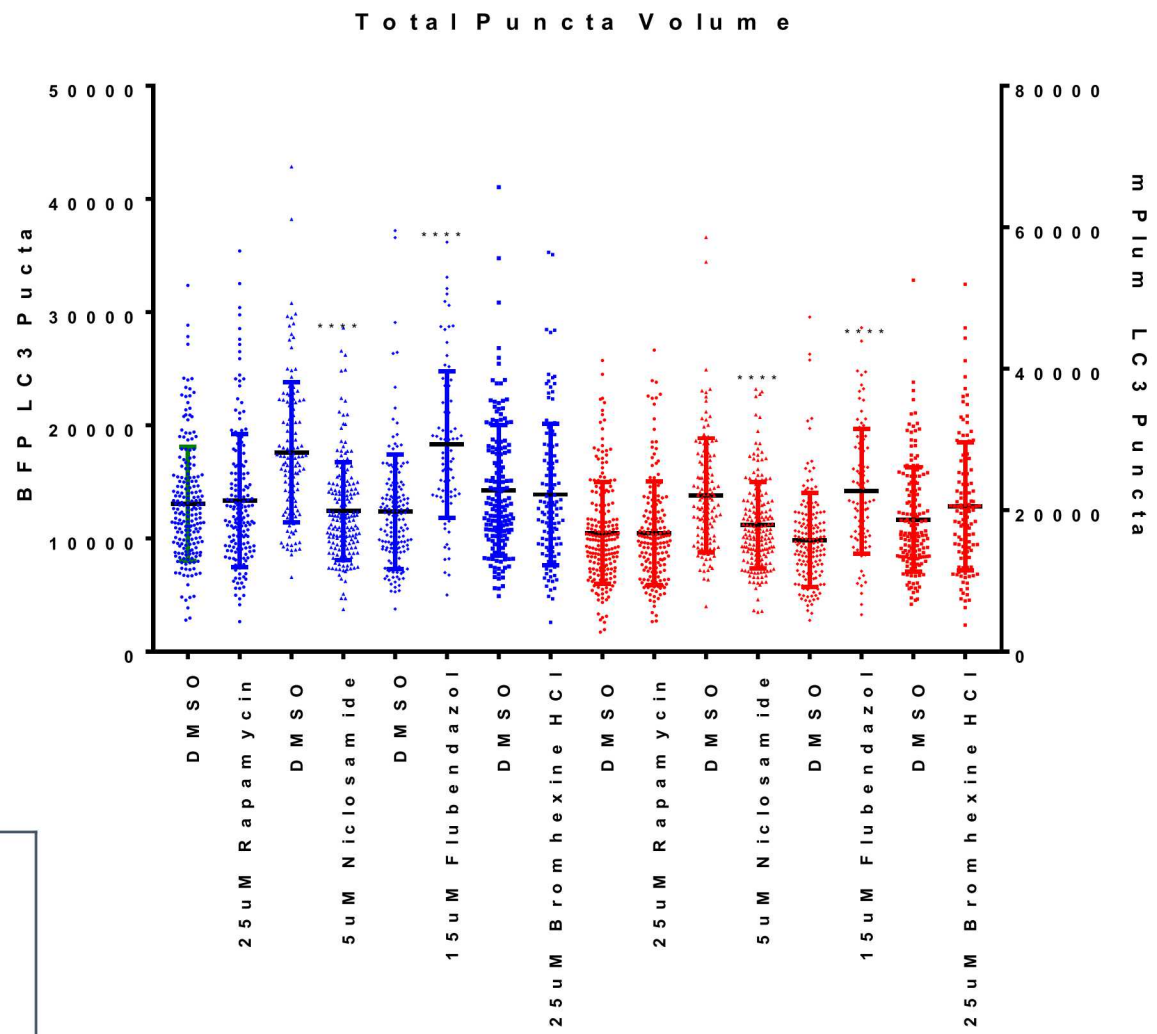

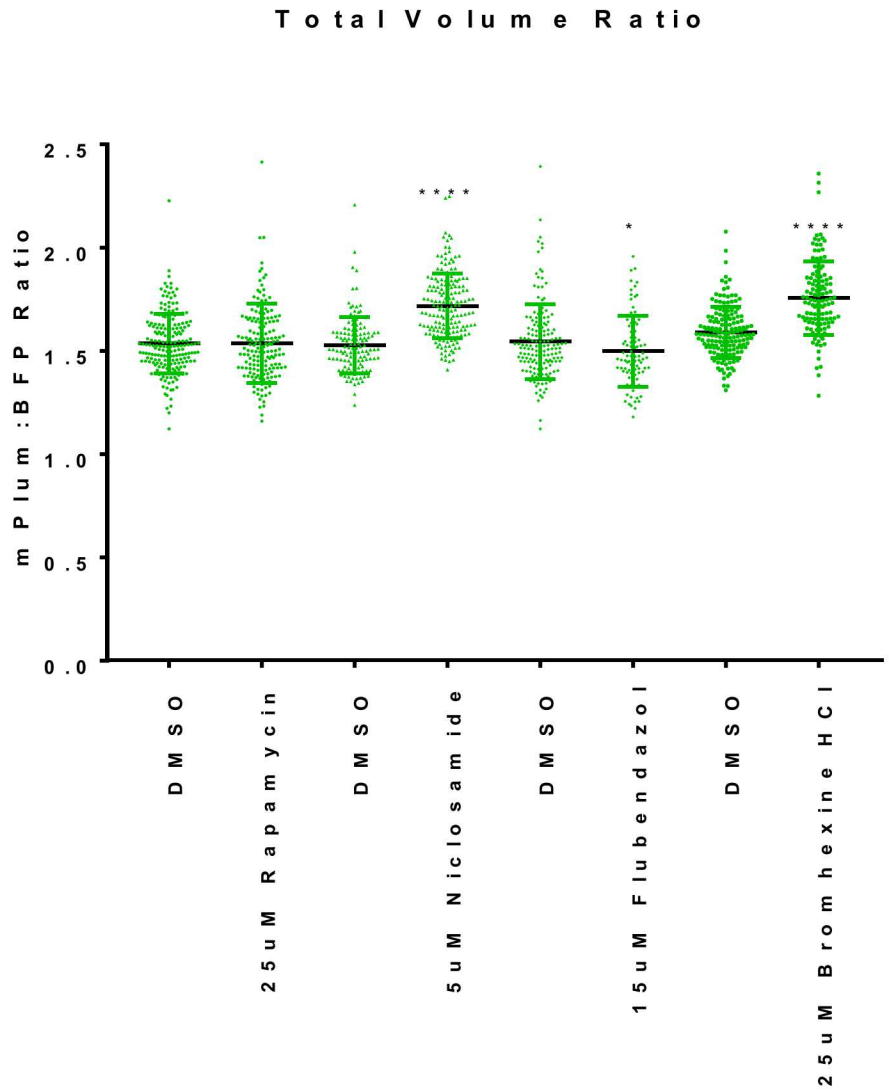

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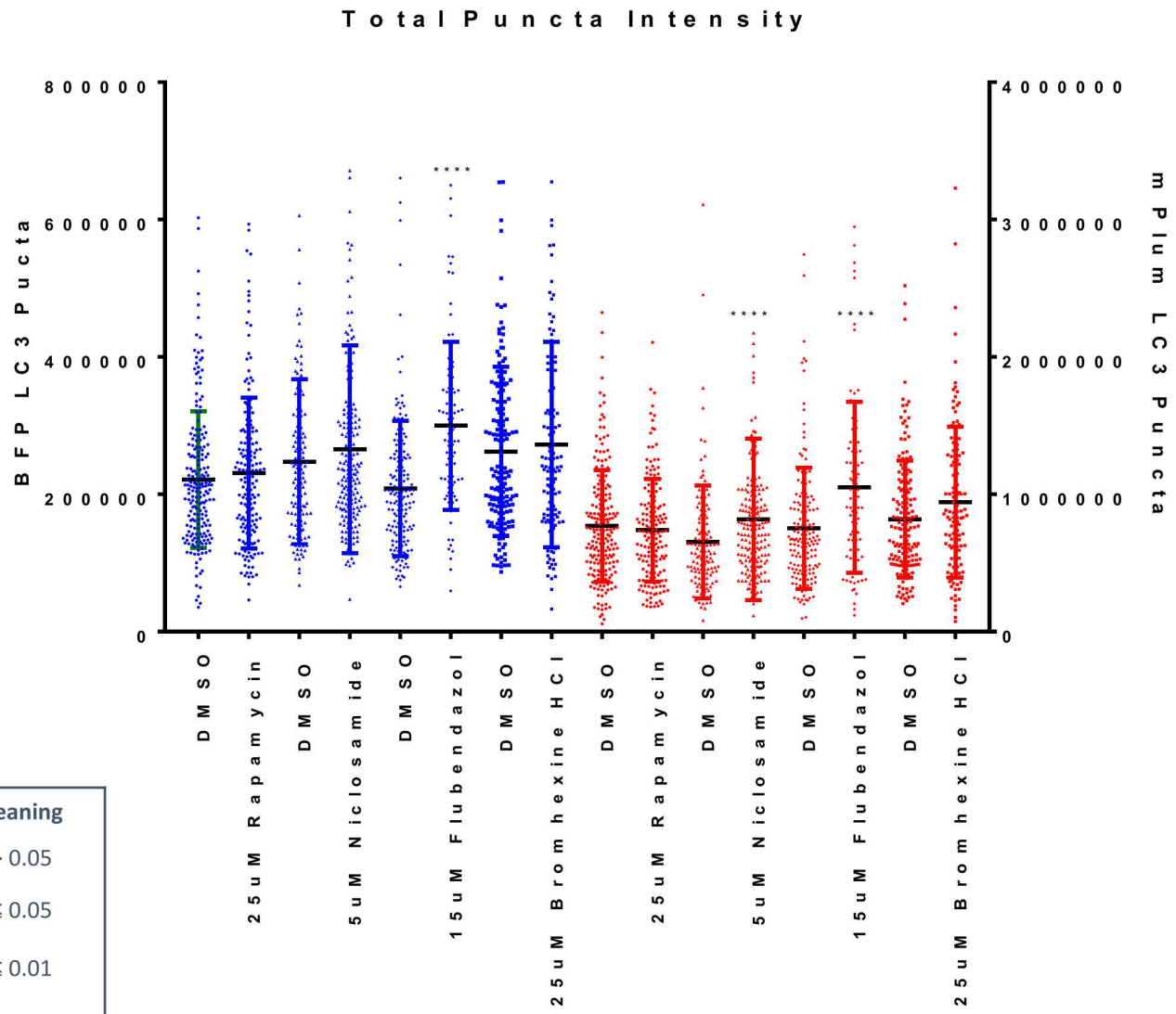

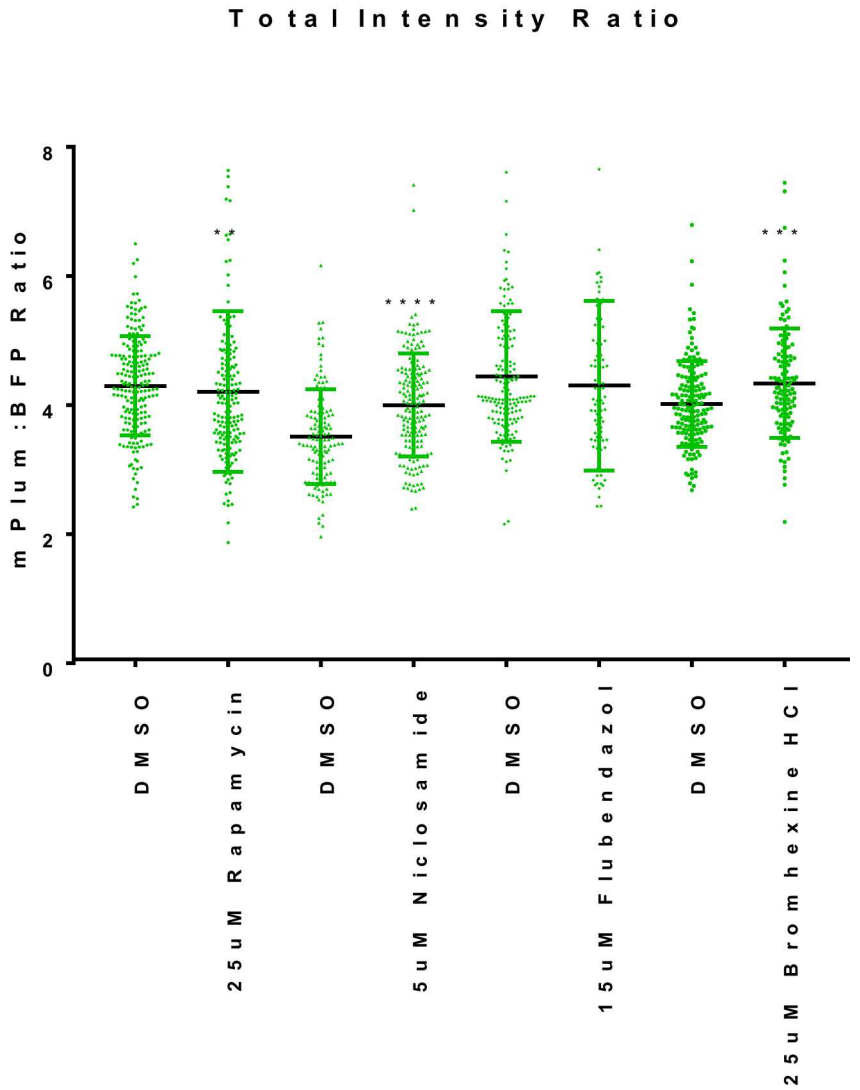

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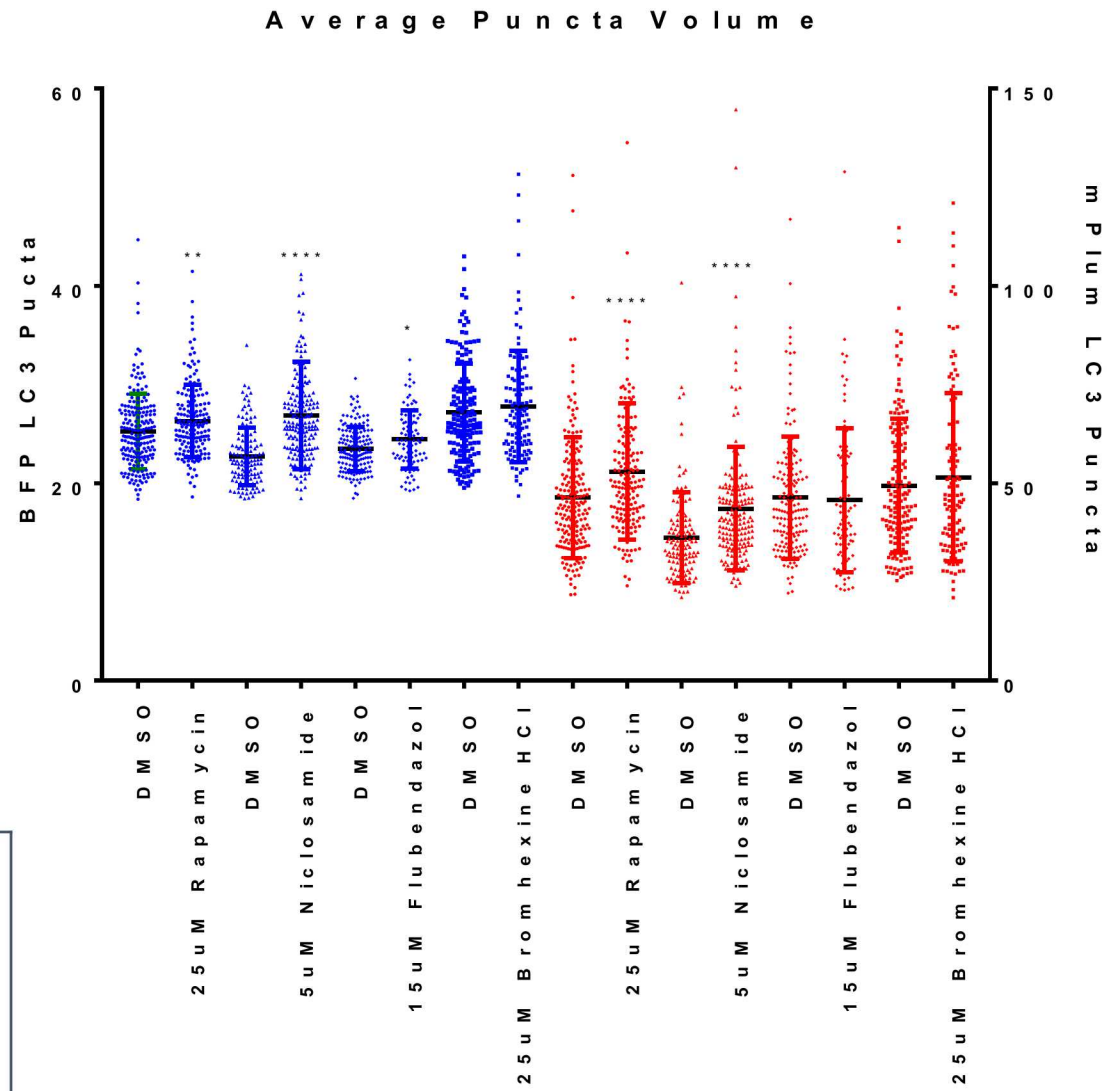

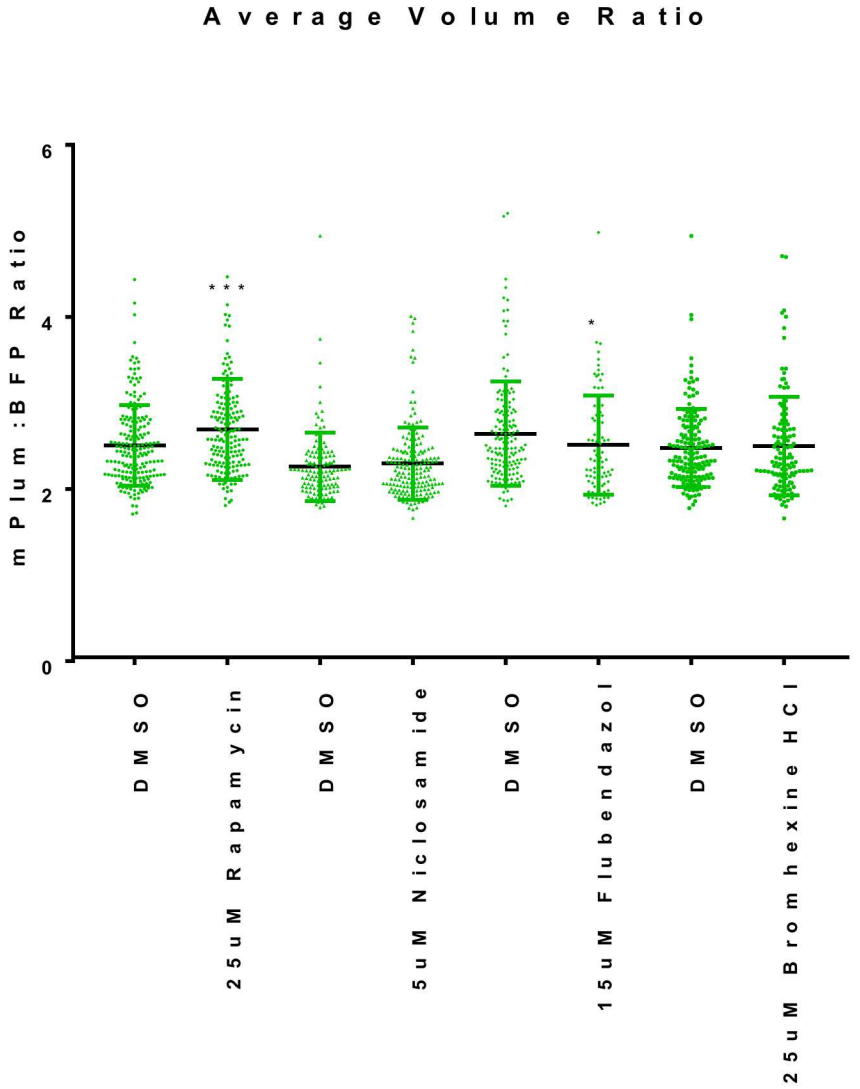

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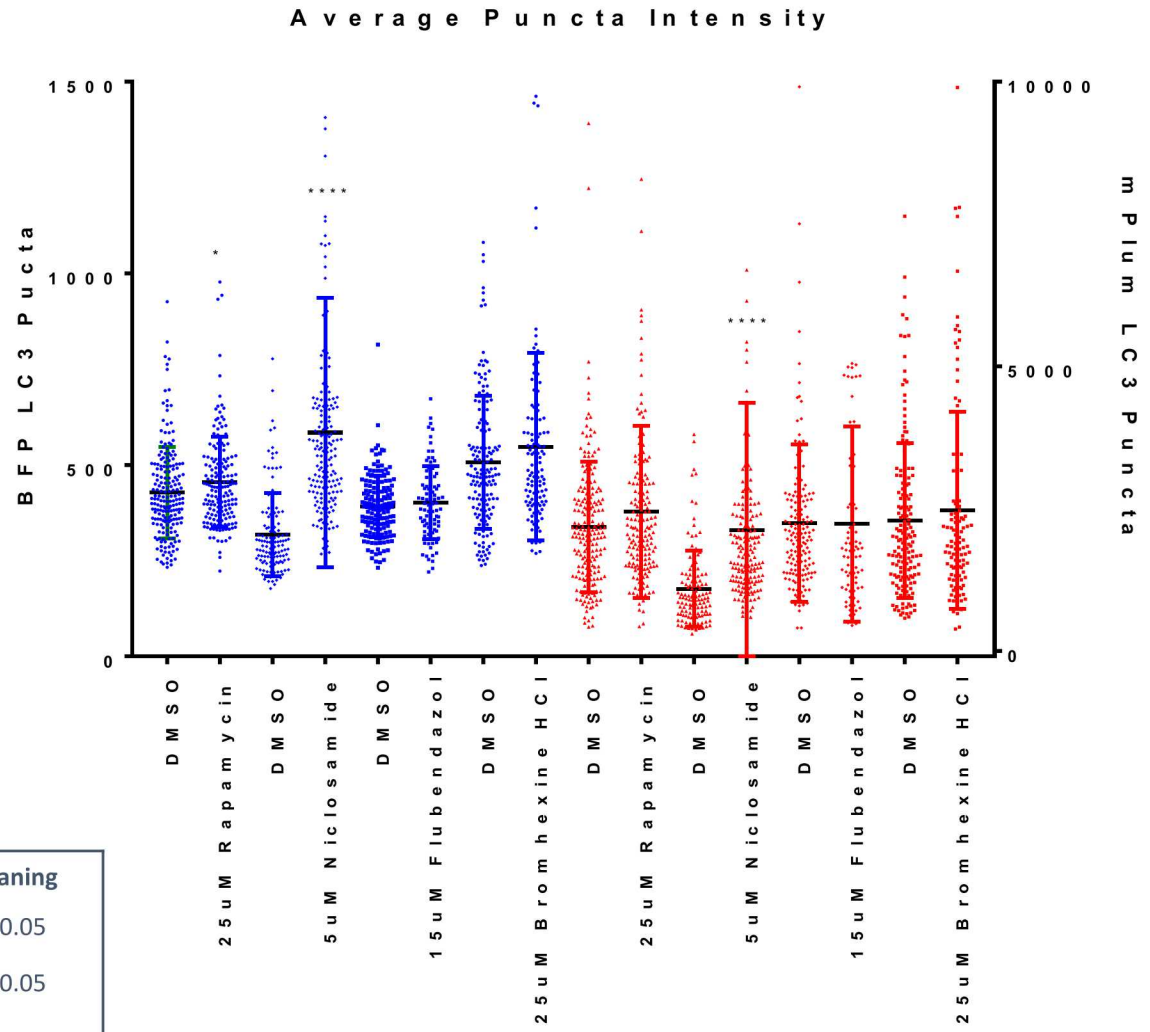

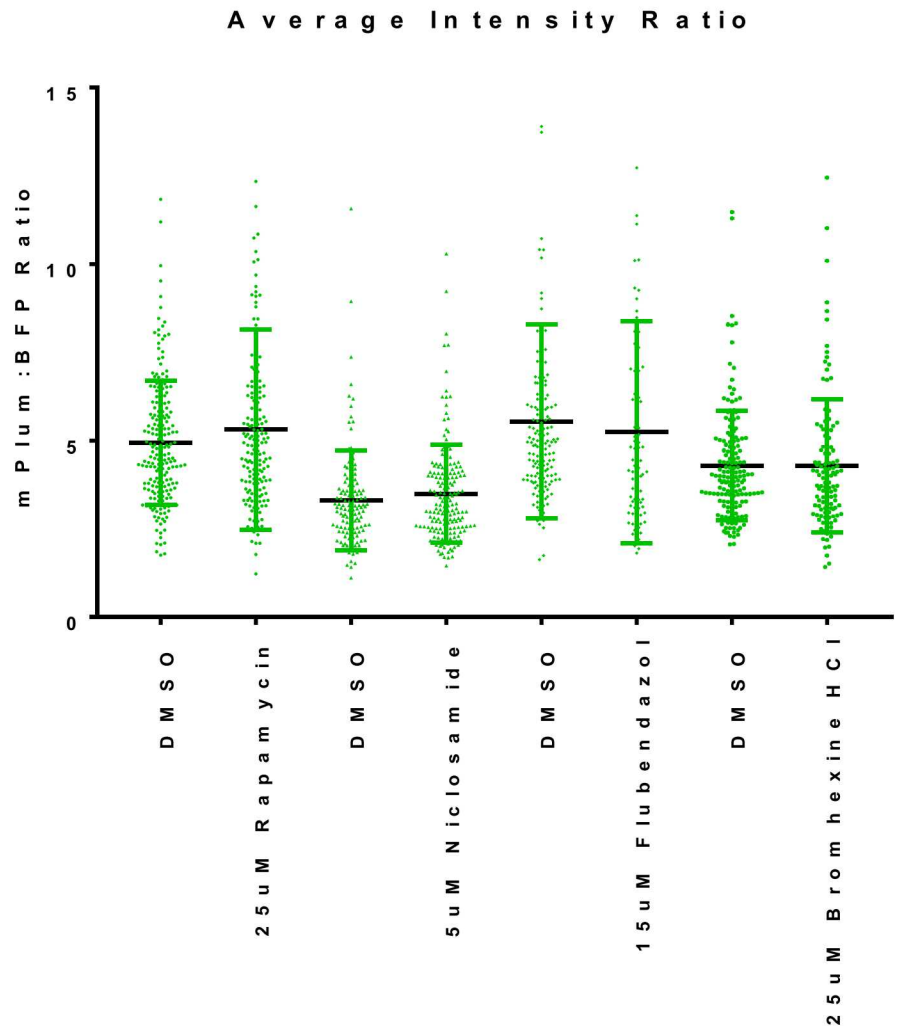

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LDRD Project Metrics

Presentations and Publications

- Virtual Keystone Symposia-Autophagy: Mechanism and Disease, October 5-8, 2020. Presenting "Concurrent evaluation of autophagy induction and *Burkholderia* infection at the single cell level."
- Manuscript's in preparation:
    - Danae Maes, Joshua Podlevsky, Stephen Anthony, Colleen Courtney, Steven Branda, and Jerilyn Ann Timlin, "Concurrent evaluation of autophagy induction and *Burkholderia* infection at the single cell level." Fully attributable to this Ex Ex LDRD
    - Danae Maes, Meghan Barnhart-Dailey, Stephen Anthony, Bryan Carson, Stephen Bradfute, Mary Ortner, and Jerilyn Ann Timlin, "Capacity of Clinically Approved Drugs to Induce Autophagy for the Treatment of Tuberculosis." Originated from prior NMSBA, but furthered under this LDRD with new methods

Tools and Capabilities

- Single biological assay to view autophagy and pathogen infection simultaneously, adaptable for many intracellular bacterial and virus infections.
- Enhancement of in-house written software to quantify autophagy and pathogen infection in a 3D, single-cell model.
- Established *Burkholderia* research in SNL-NM.
- Leveraged new FACSS Aria Cell sorter; Established protocols for mammalian cell lines, prove-in new instrument

Staff Development

- Danae Maes (Graduate Intern)-Project Management, Leadership, Experimental Design
- Colleen Courtney (PostDoc) and Joshua Podlevsky (Early Career Staff)-Mentorship of Danae Maes
- Chuck Smallwood (Early Career Staff) –Development of mammalian cell sorting protocols and experience.

Project Legacy

Key Technical Accomplishment

Development of dual reporter cell line to simultaneously measure autophagy modulation and pathogen infection at the single cell level.

How does this engage Sandia missions?

- Directly aligned with Bioscience Strategies to develop bacterial countermeasures by leveraging SNLs expertise and prior investments in imaging and cellular biology.
- Which customers have you talked to about your results?
  - Bioscience Investment Area
  - Will be discussing with DTRA, DHS, and NIH

Plans for follow-on and partnerships?

- Brief Cathy Branda final results and discuss opportunities for external funding (Oct 2020–PM)
- Discuss results with Biophagy and UNM to gauge interest in partnership (Oct/Nov 2020- PM)

What do you wish you could have done, but didn't?

Due to the rise of mission critical COVID-19 diagnostics lab, our team's efforts were diverted completely (Maes, Timlin, Podlevsky) for about 8 weeks and partially to date. Therefore we were not able to create a stable dual reporter cell line (a cell line that is viable for many years). We instead created a transient cell line that lasts for ~1 week. We have the materials and protocols to recreate the transient cell line as needed for other projects as well as create the stable cell line in the future. Additionally, our planned partnership with UNM Center for Autophagy did not come to fruition because UNM was unable to perform the work due to their own COVID-19 restrictions and responsibilities.