# Investigating the Impact of Fragile X Syndrome on Stress Granule Formation

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Submitted to the Faculty of
Worcester Polytechnic Institute

In partial fulfillment of the requirements for the
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# Abstract

The protein FMRP is missing in Fragile X Syndrome (FXS) patients. Stress Granules (SGs) are cytoplasmic bodies where mRNAs are stored during cellular stress to inhibit their translation. FMRP localizes to SGs and regulates mRNA translation; therefore we hypothesized that SGs may differ in FXS and unaffected cells. We used fluorescence microscopy to quantify SG formation in wild type and FXS mouse embryonic fibroblasts and human B lymphocytes. Our results suggest altered stress responses may contribute to the pathophysiology of FXS.

# Acknowledgements

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# Introduction

## Cellular Stress Response

Cellular stress is defined as an introduction to, or a change of, a stimulus that damages the structure and function of macromolecules or the cell itself (Poljšak et al. 2012). These stimuli can lead to damage of proteins, DNA, other molecules, or cell death (Kultz 2005). To combat these stresses, cells have evolved numerous different mechanisms to tackle stressors of various levels called cellular stress response (CSR). One of the mechanisms is the formation of stress granules. These are created so the cell can survive until the stressor subsides.

## Stress Granules

A common method of CSR is the formation of stress granules, which have been found across yeast, protozoa, and metazoa (Anderson and Kedersha 2009). Stress granules are clusters of untranslating messenger ribonucleoproteins (mRNPs) which form from stalled mRNAs (Protter and Parker 2016). Stress granules are most often formed after translational initiation is halted due to stress-induced phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α). During the halted phase, elongating ribosomes are unharmed and simply fall off the stalled polysomes. This results in a circularized, polyadenylated mRNA transcript that is still attached to cellular pre-initiation machinery (Anderson and Kedersha 2009). Figure 1 summarizes the formation and breakdown of stress granules within a cell (Dobra et al. 2018).

Stress granules are believed to sort and degrade mRNA during times of cellular stress and recovery. They can also assist with mRNA regulation and stability (Anderson and Kedersha 2009). In addition, stress granules recruit other molecules which can affect the equilibrium of associated molecules. These molecules can also shift the cellular environment into a different stage. For example, stress granule recruitment of antiviral proteins during an infection enhances innate immune response (Protter and Parker 2016).

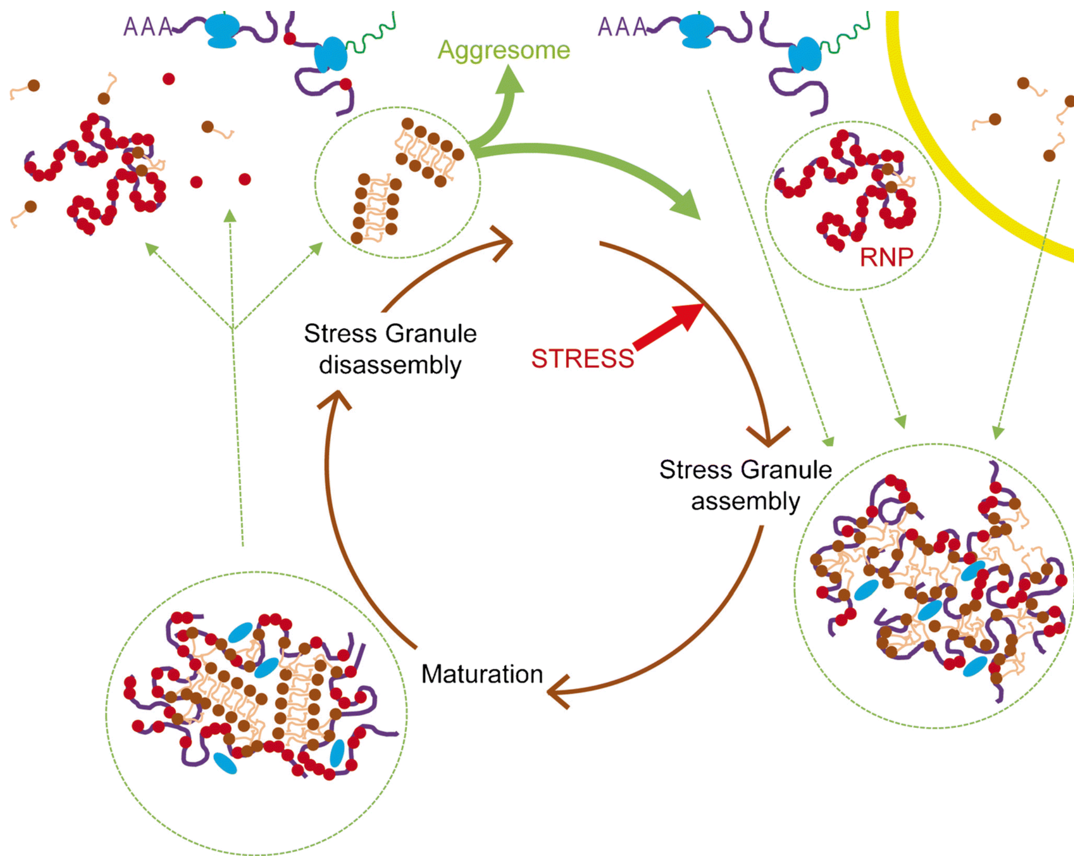


**Figure 1.** Assembly of Stress Granules (Dobra et al. 2018)

Figure 1 displays the formation and breakdown of stress granules in the cytoplasm. The mRNA originates in the nucleus, which is outlined in yellow, and enters the cytoplasm. RNPs bind to mRNAs, which aggregate in the stress granule so the genetic material is preserved until the stress subsides. Some proteins are also present in stress granules. Aggresomes assist in degradation of proteins once the stress granule disassembles (Dobra et al. 2018).

## Bisphenols

Bisphenols are made of two phenols with bridging molecule(s). They are commonly used to manufacture plastics and polycarbonates (Konieczna et al. 2015). Bisphenol A (BPA), as shown in Figure 2, is a public health concern due to its function as an endocrine disrupting molecule, and its analogues are of growing concern as well. BPA is composed of two phenols with a bridging carbon that has two methyl groups attached to the carbon (Chen 2002). Steps have been taken to eliminate BPA from plastic products, but it is often replaced with its analogues, such as bisphenol S or bisphenol F. Despite this removal, BPA is a compound of emerging concern and most people in the developed world are exposed to it on a daily basis.

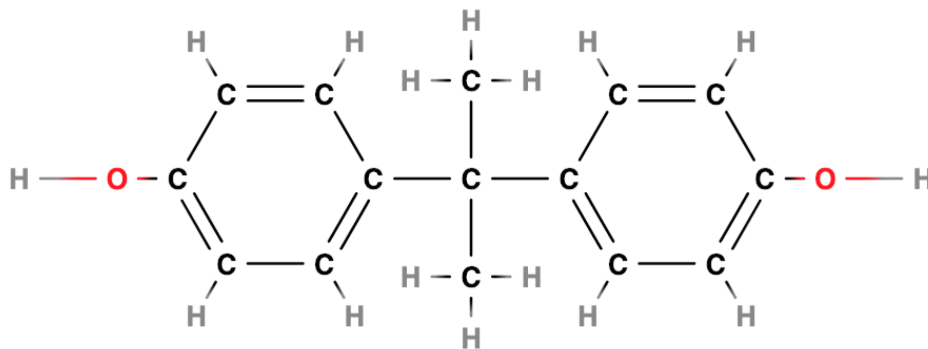

**Figure 2.** Bisphenol A (BPA) molecular structure

Figure 2 shows the bridging carbon between two phenols. There are also two methyl groups attached to the central carbon.

# Bisphenol A (BPA)

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, also known as BPA, is an organic synthetic chemical that is produced worldwide (Konieczna et al. 2015). BPA was first used in 1891 and in the early 1950s it began to appear in industrial and consumer products (Vogel 2009). Since the mid-1970s, BPA has been considered a chemical with high-volume production (Vogel 2009). Over six billion pounds of BPA are produced each year worldwide and an additional 200 tons of the chemical are let out into the atmosphere during its production (Ritter 2011).

Common products that contain BPA are food containers, baby bottles, toys, water pipes, cell phones, laptops, and medical equipment. Humans are exposed to BPA in their everyday lifestyle. In fact, the 2003-2004 National Health and Nutrition Examination Survey discovered 92.6% of the participants had traces of BPA in their urine (Antonia et al. 2008). BPA concentrations in these urine samples ranged from 0.4 µg/L to 149 µg/L and differed between races, household income, gender, and age (Antonia et al. 2008). Frequent exposures to BPA can occur through inhalation, ingestion, and absorption from dermal exposure.

In 1993, BPA's endocrine disruption potential was discovered by endocrinologists at Stanford University. They were searching for an endogenous estrogen in yeast but instead came across BPA from their polycarbonate flask. The endocrinologists' research found that BPA was the chemical competing with estradiol for estrogen receptors and this was not a product of the yeast that they grew in culture. Their published results lead to more research by other scientists on the endocrine disruption potential of BPA (Krishnan 1993).

The human tolerable daily intake, which is defined by the United States Environmental Protection Agency as the maximum amount of a substance a human can be exposed to daily without adverse effects, of BPA is approximately 50 µg/kg/day. However, adverse effects have been detected at lower concentrations of BPA. Low dose effects of BPA have been linked to many diseases including birth defects, neurodevelopmental disorders, cardiovascular disease, some cancers, and autoimmune disease amongst many more (Rochester 2013). BPA activity frequently resulted in genetic damage, epigenetic changes, endocrine disruption, oxidative stress, and/or cell signaling (Rezg et al. 2014).

## Autism Spectrum Disorders and Fragile X Syndrome

Autism spectrum disorders (ASD) are a class of genetic disorders; ASD affect an individual's social skills, communication, and behavior (Lord et al. 2000). People with ASD may react differently to environmental contaminants than people without ASD. One ASD is Fragile X Syndrome (FXS), which is caused by extra CGG repeats in the fragile X mental retardation 1 (*FMR1*) gene promoter region (Hall and Berry-Kravis 2018). An individual with FXS often has varying levels of "intellectual disability, autism, seizures" (Hall and Berry-Kravis 2018). FXS affects more males than females since the *FMR1* gene is located on the X chromosome (Davidovic et al. 2011). Males also have more severe symptoms than females (Hall and Berry-Kravis 2018). The number of repeats present in *FMR1* determines if the individual is unaffected, a carrier, or has FXS (Hall and Berry-Kravis 2018). The CGG repeats in the promoter silence the *FMR1* gene, therefore it cannot translate the fragile X mental retardation protein (FMRP), which participates in protein synthesis at the synapse in neurons (Davidovic et al. 2011; Hall and Berry-Kravis 2018).

FMRP binds within the coding region of translating mRNA, which slows the elongation rate of translating ribosomes (Darnell et al. 2011 ). Therefore, FMRP represses translation. Also, there is evidence that FMRP is present in stress granules and may affect the nucleation of stress granules (Anderson and Kedersha 2008). The relationship between FMRP and stress granules is depicted in Figure 3. Since FMRP is not present in individuals with FXS, they may have increased translation and form stress granules differently than people without FXS.

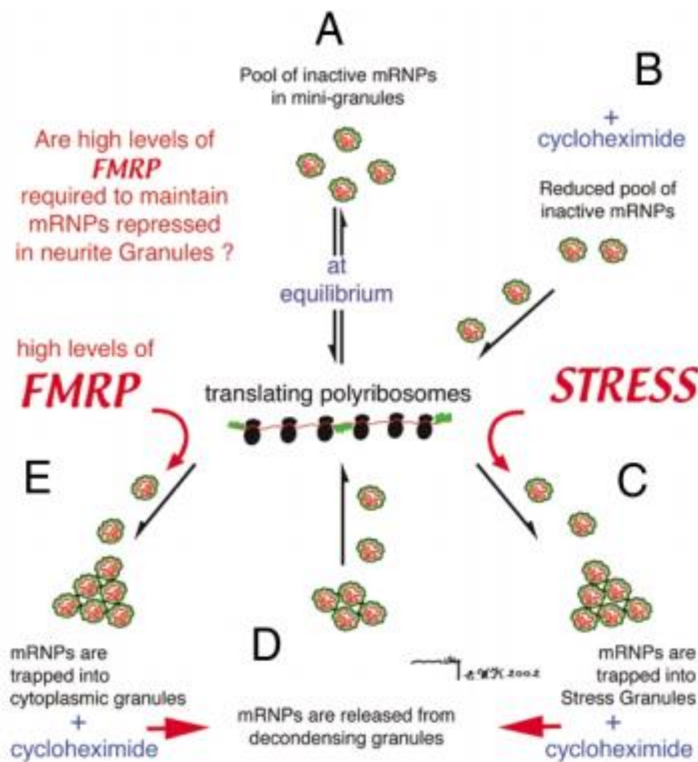


**Figure 3.** The role of FMRP in stress granule formation (Mazroui et al. 2002)

Figure 3 displays the relationship between FMRP and stress granule formation. Both FMRP and stress granules regulate translation. FMRP binds to translating mRNAs, which hinders the movement of ribosomes as they move along the mRNA. Stress granules appear to preserve mRNA during times of stress and no translation occurs in stress granules (Anderson and Kedersha 2008; Mazroui et al. 2002).

In addition, high levels of FMRP might control the repression of mRNPs (Mazroui et al. 2002). To learn more about the connection between FMRP and stress granule formation, Gareau et al. conducted a study to determine FMRP's effect on stress granule formation in *Drosophila* since the localization of FMRP in their stress granules is conserved (2013). The study found that FMRP is a component amongst many other proteins that are involved in stress granule nucleation. Furthermore, Gareau et al. found that FMRP shuttles in and out of stress granules, however, it does not alter nor is necessary in *Drosophila* cells for the formation of stress granules (2013). Therefore, cells that do not produce FMRP can still form stress granules because they have other proteins involved in the nucleation process. Still, the absence of FMRP alters the protein composition of the stress granule (Gareau et al. 2013) and presumably alters the mRNA profile as well. It remains unclear what effects if any these changes in stress granules may have for individuals with FXS.

*FMR1* knockout mice show an increase in protein synthesis of about 20%, which contributes to the effects of FXS on the brain (Udagawa et al. 2013). FMRP binds coding regions of mRNA; in *FMR1* knockout animals their ribosomes translate excessive proteins from the mRNA because they can move freely (Udagawa et al. 2013). In order to make a stress granule, the translating ribosomes must detach or run off from the mRNA so the mRNA can become part of the stress granule. Since ribosomes are able to move with more freedom in *FMR1* knockout cells than in wild type cells, the absence of FMRP may affect the dynamics of stress granule formation.

Experiments addressing concerns about the effects of daily plastic use on the overall population have been conducted, although few have examined the effect on smaller populations, like those individuals with FXS. BPA, a component of many plastic products, is known to cause a stress response in various cell types (Friend et al. 2018). In this study we examined stress granule formation in response to BPA in mouse embryonic fibroblasts (MEF) and human B lymphocytes that were either wild type or affected with FXS. We hypothesized there would be a difference in the dynamics of stress granule formation between the wild type and FXS affected cells when they were exposed to varying concentrations of BPA. Because ribosomes would run off from translating polysomes more quickly in the absence of FMRP, we predicted that mRNAs in FXS cells would be able to move into stress granules in a shorter amount of time or at lower concentrations of environmental stressors. We find that FXS patient lymphoblasts are more sensitive to stress granule formation in response to the stressor sodium arsenite, but we see no significant difference in stress granule formation in response to BPA. The results suggest that, at least in response to some stressors, that FXS-affected cells may be more sensitive to stress, which could contribute to the pathogenesis of this disorder.

# Materials and Methods

## Cell Line Maintenance

Wild type and FXS affected mouse embryonic fibroblasts and human B lymphocytes were a kind gift from Dr. Joel Richter (University of Massachusetts Medical School, Worcester, MA). Double stable GFP-G3BP and RFP-Dcp1 U2OS cells (Kedersha et al. 2008) were a generous gift from Dr. Nancy Kedersha (Brigham and Women's Hospital, Boston, MA). The mouse embryonic fibroblasts and double stable GFP-G3BP and RFP-Dcp1 U2OS cells were maintained in 1x DMEM (Corning Cellgro, Catalog No: 10-013-CV) with 10% fetal bovine serum (Equitech-Bio, Inc., Catalog No: 3FBU3132-0500) and 1% penicillin/streptomycin (Lonza, Catalog No: 17-602E). The human B lymphocytes were maintained in with 1x RPMI (Sigma Life Science, Catalog No: R8758),10% fetal bovine serum and, 1% penicillin/streptomycin.

Adherent cells, mouse embryonic fibroblasts and double stable GFP-G3BP and RFP-Dcp1 U2OS cells, were subcultured approximately every 2 to 3 days. Cell media was removed from the flask and discarded into a waste beaker. A Phosphate-buffered Saline (PBS) rinse was completed and also discarded into the waste beaker. 1.0 mL of Trypsin (Lonza,Catalog No: CC-5012) was added to the flask. The flask was then put in the 37°C incubator for 2-4 minutes to allow the cells time to detach from the flask wall. After the flask was removed from the incubator, the flask was rinsed with new DMEM media. The new cell containing media was split appropriately into the two new flasks; the ratio was between 1:2 and 1:8. More DMEM media was then added to each flask to bring the total volume to 14 mL.

Suspension cells, human B lymphocytes, were also subcultured every 2-3 days. Cell media was split into respective ratio and placed in new flasks. Additional mixture of 1x RPMI media with 10% fetal bovine serum and 1% penicillin/streptomycin was added into each flask to reach a total volume of 14 mL.

## B Lymphocyte Cells Pre-Plating

Coverslips and ultrapure water were autoclaved. The coverslips were then placed one per well on the plates and covered with 0.5 mL of polylysine solution (Sigma Life Science , Catalog No: P4707). The polylysine coating was left on for an hour in a 37°C incubator. After one hour incubation, the polylysine solution was removed and the coverslips were rinsed with 0.5 mL of autoclaved water three times.

## Cell Plating and Pre-Treatment

The cells were plated in 12 well plates containing coverslips at concentrations ranging from 8 x $10^4$ - 1.2 x $10^6$ cells per mL. Depending on the cell type, 1 mL of either DMEM or RPMI based media was placed into each well before incubating the cells at 37°C for approximately 1-2 days. After incubation, 0.5 mL of media was removed from each well and combined with media that contained the same cell type and was receiving the same treatment. A 0.1M stock solution of BPA was diluted in preconditioned media to achieve the final molar concentration indicated in each experimental sample. An untreated sample, the negative control, and an arsenite treated sample, the positive control, were also prepared. Figure 4 shows an example of how the 12 well plates were utilized for plating and treatment. 0.5 mL of the newly mixed media and reagent were added back to each of the appropriate wells and the plate was incubated for one hour at 37°C.

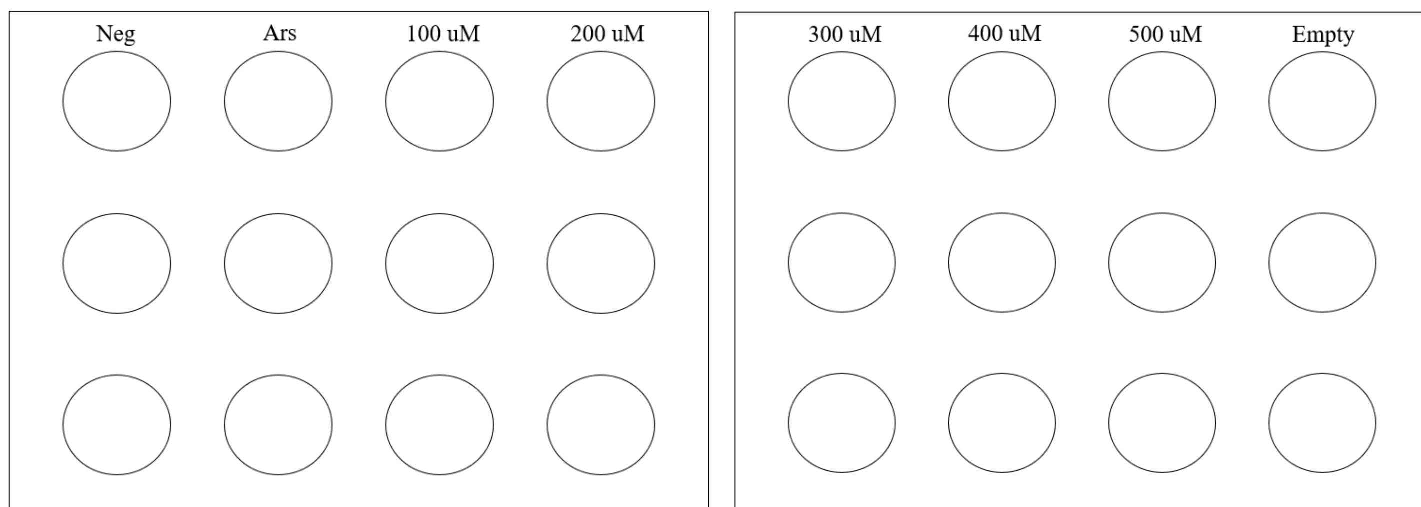


**Figure 4.** Acute Exposure Assay Treatments
The treatments for their respective columns are labelled. Neg is negative control, or untreated. Ars is cells treated with 100uM of arsenite. 100uM-500uM represent the concentrations of BPA added to each well. One of the columns was left empty because only 7 treatments were utilized.

## Cell Fixation

Fixation was done in order to stop any changes the cells were undergoing (reacting to the treatment, dividing, etc). Once the cells were removed from the incubator, and placed in the hood, all media and reagent was removed from each of the wells and placed in the discard beaker. The wells were rinsed twice with PBS, which was removed and placed in the discard beaker. 0.5mL of 4% paraformaldehyde was placed into each well and the plate was placed onto

the shaker for 10 minutes on medium. After 10 minutes, the plate was brought back into the hood and the paraformaldehyde was removed from each well and placed into the hazardous waste bottle. 0.5 mL of 1% triton detergent was put into each well and the plate was placed on the shaker for an additional 10 minutes. After the second 10 minutes on the shaker, the plate was again brought back into the hood, the detergent was removed and placed in the hazardous waste bottle. The wells were rinsed with PBS twice, the PBS was removed and placed in the hazardous waste bottle each time. PBS was then placed in each well a third time, enough to cover the cover slides. The plate was then placed in the refrigerator for storage or brought to the bench for next steps.

## Antibody Staining

The cells were stained in order to see stress granules using fluorescence microscopy. 0.5mL of blocking solution, which consisted of 1% PBS and 5% normal horse serum (NHS), was added to each well being treated and the plates were left on the shaker for approximately 1 hour. The blocking solution was removed and 0.5 mL of a solution containing a ratio of 1uL of the primary mouse monoclonal (Abcam, Catalog No: #181150(EPR3986(B)) antibody anti-G3BP to 1mL of blocking solution was added to each well being treated. The plates were placed on the shaker for approximately 1 hour. The primary antibody solution was removed and 3 1% PBS washes were conducted for 5 minutes each. The plates were put on the shaker during each wash. A solution containing a ratio of 1uL of hoechst (Life Technologies, Catalog No: 333342) to, 1uL of secondary anti-rabbit (red) antibody (Cell Signaling Technology, Catalog No: 8889S), to 1mL of 5% NHS + PBS was prepared. 0.5mL of the solution was added to each well being treated and the plates were wrapped in tinfoil and placed on the shaker for 1 hour. The solution was removed and 3 PBS washes were conducted for 5 minutes each. The plates were put on the shaker during each wash. The coverslips with treated cells were mounted with polyvinol mounting media as described (Fukui et al., 1987) after completing the antibody staining protocol.

## Data Collection and Statistical Analysis

The slides were blinded using tape to cover their respective treatment concentration or control type. The slides were then analyzed using a fluorescent microscope (Zeiss, Vert.A1, AXIO). The microscope lens was set to 20X. Two to three fields were counted, to obtain a total of 250-300 cells. The percent of stressed cells was calculated by dividing the number of cells that contained stress granules by the total number of cells counted on that specific cover slip. The percent of nonstress was calculated by dividing the number of cells that did not have any stress granules by the total number of cells counted on that cover slip. Each coverslip was counted at least two times. The various counts were then averaged.  Experiments were repeated three times unless otherwise indicated. Error bars represent the standard error of the mean. Differences between samples were analyzed using ANOVA and paired t-tests, as indicated in the figure legends. Statistical analyses and data collection were performed using Microsoft Excel.

# Results

## Verification Trials

Verification trials using U20S-DS and human B lymphocyte cell lines were conducted to ensure that the acute exposure assay would yield cells with visible stress granules and that each group member was counting cells, either with or without stress granules, accurately. The cells were treated with varying concentrations of BPA for one hour. The cells treated with arsenite served as a positive control and untreated cells served as the negative control. Cell fixation ensued treatment to allow for scoring of stress granules using a fluorescent microscope.

The first verification trial was performed using U20S-DS cells, which are known to form stress granules when exposed to arsenite and BPA (Friend et al. 2018**).** The data collected in the trial is shown in Figure 5**.** Raw data for the U20S-DS verification trial can be found in Appendix A.

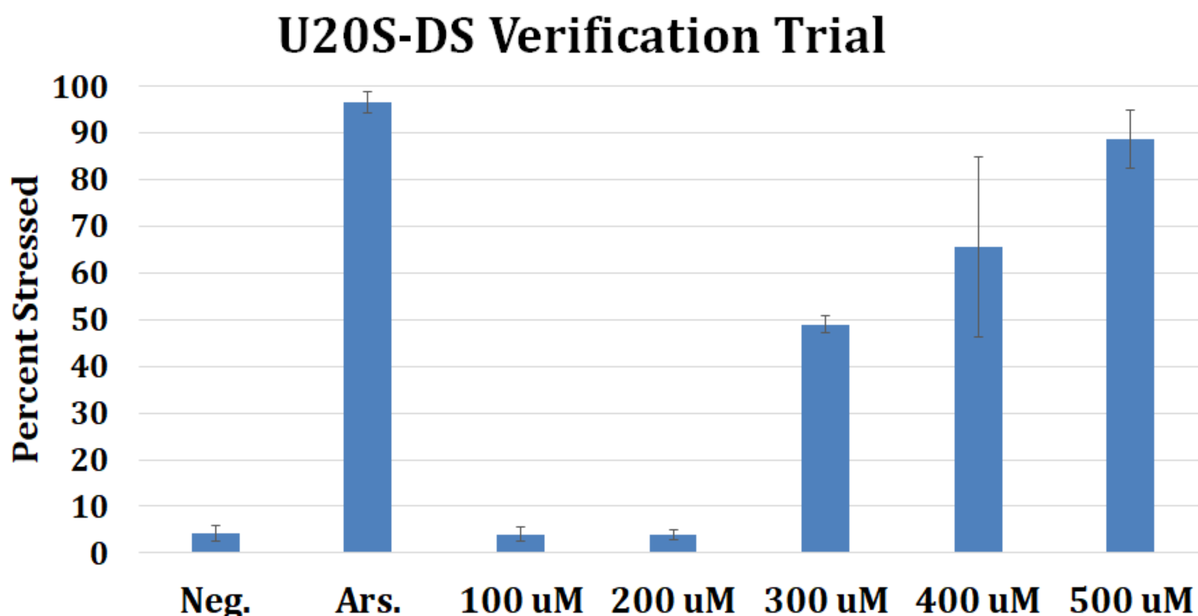


**Figure 5.** The average percentage of U20S-DS cells exhibiting stress. These cells were treated for one hour with varying concentrations of BPA, 500 uM of arsenite, or left untreated as indicated in the figure. One biological replicate was performed. Error bars within the figure represent standard error.

The results were consistent with previous work and showed a dose dependent response as anticipated. One biological replicate was completed and counted by two team members. Standard error was calculated and shows the variance between the two counts. These results provided confidence that the assay would work throughout the experimental period (Friend et al. 2018).

A second verification trial was performed using the human B lymphocytes to ensure that the wild type (unaffected) and FXS affected cell lines would produce a stress response. Again, untreated cells served as a negative control and arsenite treated cells served as a positive control. Figure 6 shows the results from the human B lymphocyte verification trial. Raw data for the human B lymphocyte trial can be found in Appendix B.

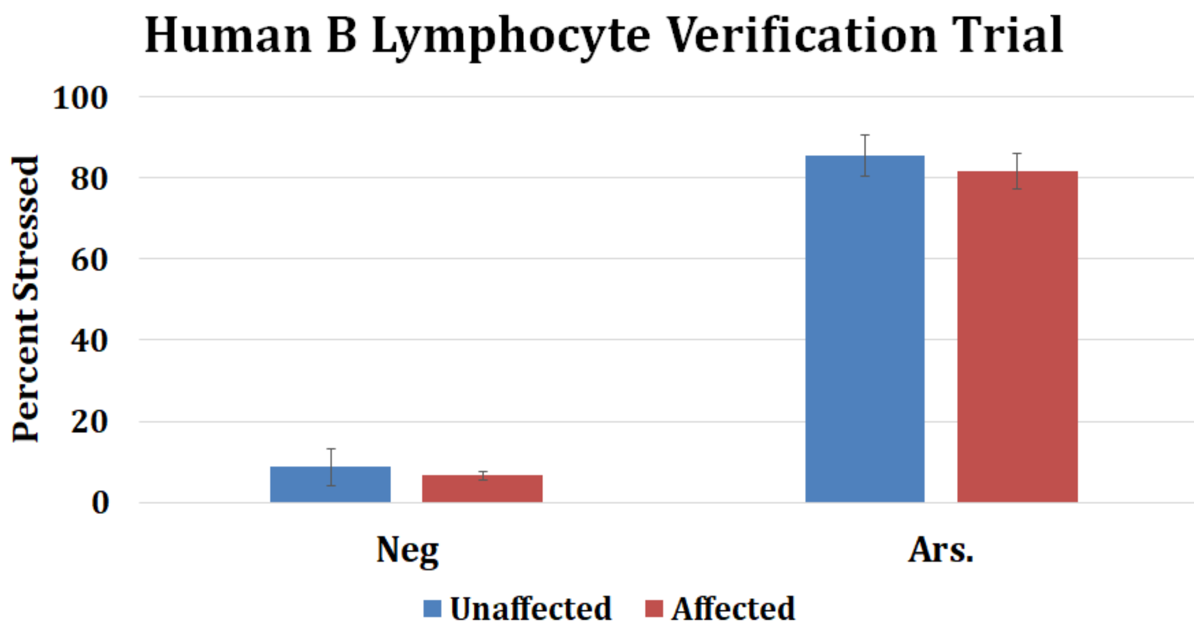


**Figure 6.** The average percentage of human B lymphocytes exhibiting stress. These cells were treated for one hour with 500 uM of arsenite or left untreated as indicated in the figure. One biological replicate was performed. Error bars within the figure represent standard error.

There is a clear difference between the untreated and arsenite treated B lymphocytes, which means these cells are a good candidate for the acute exposure assay. One biological replicate was performed but was counted by all four team members. The standard error shows the variance between the four counts.

## Mouse Embryonic Fibroblasts

In order to determine if there was a difference in stress granule formation between the wild type (WT) and *FMR1* knockout (KO) mouse embryonic fibroblast (MEF) cell lines, an acute exposure assay to arsenite and BPA was performed. Identical to the verification trials, an untreated sample served as the negative control and an arsenite treated sample was the positive control. The fibroblasts were treated with varying concentrations of BPA and the treatment was left on the fibroblasts for one hour. The cells were then fixed, stained with anti-G3BP antibody, and scored for stress granules using fluorescence microscopy. Images taken using the fluorescent microscope can be seen in Figure 7. Panel A shows cells that are not considered to be stressed. Panel B shows cells that contain stress granules (the bright red dots).

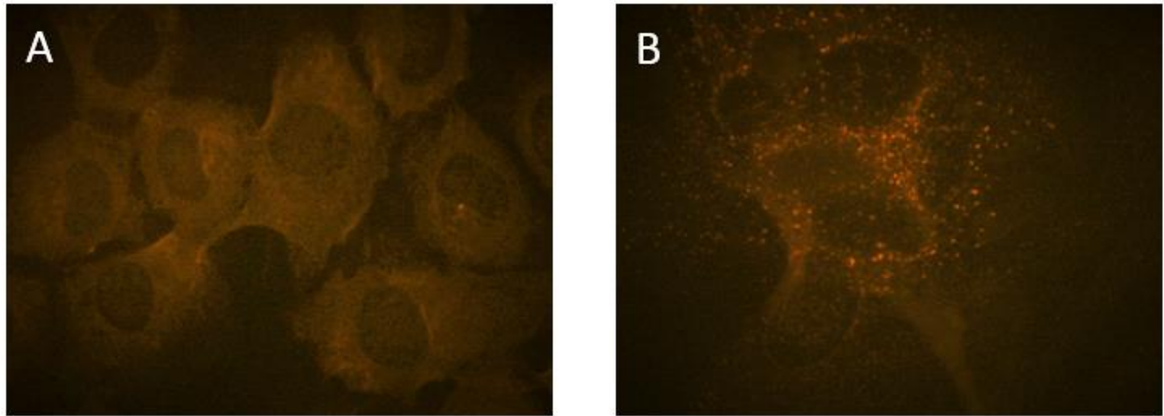


**Figure 7.** Images taken of mouse embryonic fibroblasts using fluorescence microscopy. (A) shows cells that do not have any stress granules and therefore are not considered to be stressed. (B) shows cells that have stress granules and they are considered to be stressed.

The percentage of cells which had stress granules was calculated and depicted in Figure 8. Raw data for the experiment can be found in Appendix C.

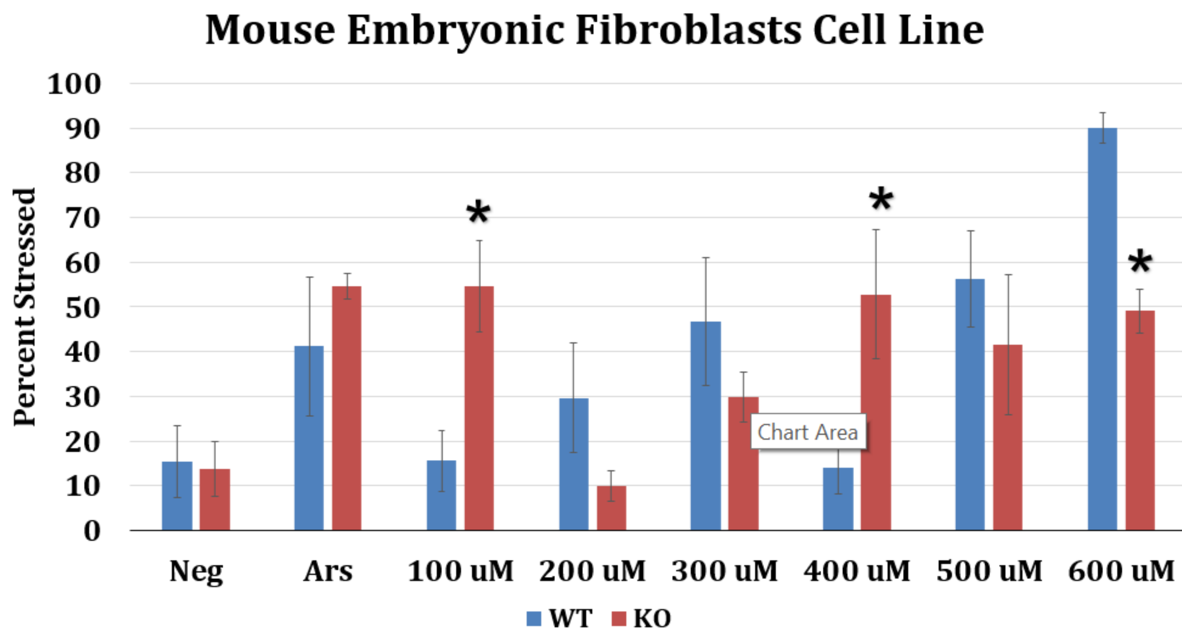


**Figure 8.** The average percentage of MEFs exhibiting stress. These cells were treated for one hour with varying concentrations of BPA, 500 uM of arsenite, or left untreated as indicated in the figure. Three biological replicate were performed. Error bars within the figure represent standard error. ANOVA analysis was completed to find statistical significance and is indicated by an asterisk ($p < 0.05$).

After graphing the average percent of stressed cells, there was no dose dependent response as previously predicted. For WT cells, the greatest percentage of stress was at the 600 uM concentration at 90%. The lowest percentage of stress was at the 400 uM concentration at 14%

stress. For the KO cells, the highest percentage of stress occurred for the positive control and at 100 uM (54%) and the lowest percentage of stress occurred at 200 uM (10%). Three biological replicates were completed and each replicate was counted by two members for a grand total of six counts for each treatment. Standard error shows the variance in biological replicates from experiment to experiment. An ANOVA analysis was conducted to find if data from the WT and KO cell lines were statistically significant at each concentration. The data was found to be statistically significant at the 100 uM, 400 uM, and 600 uM concentration for a p value, $p < 0.05$.

## Human B Lymphocytes

In order to determine if there was a difference in stress granule formation between the WT and FXS affected human B lymphocyte cell lines, an acute exposure assay to arsenite and BPA was performed. An untreated sample served as the negative control and an arsenite treated sample as the positive control. The lymphocytes were treated with varying concentrations of BPA and the treatment was left on the lymphocytes for one hour. The cells were then fixed, antibody stained, and scored for stress granules using a fluorescent microscope. Images of the cells taken by the fluorescent microscope can be seen in Figure 9.

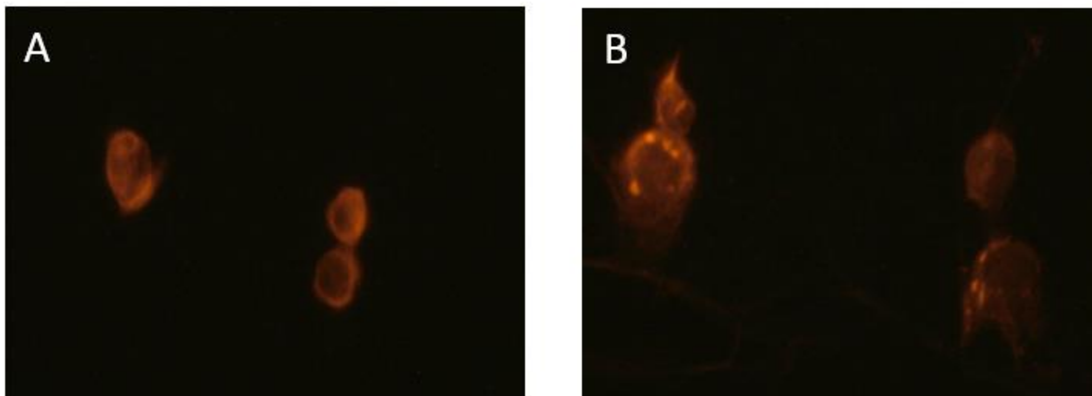


**Figure 9.** Images taken of human B lymphocytes using fluorescence microscopy. (A) shows cells that do not have any stress granules and therefore are not considered to be stressed. (B) shows cells that have stress granules and there are considered to be stressed

The data obtained from this experiment is shown in Figure 10. Raw data for the experiment can be found in Appendix D.

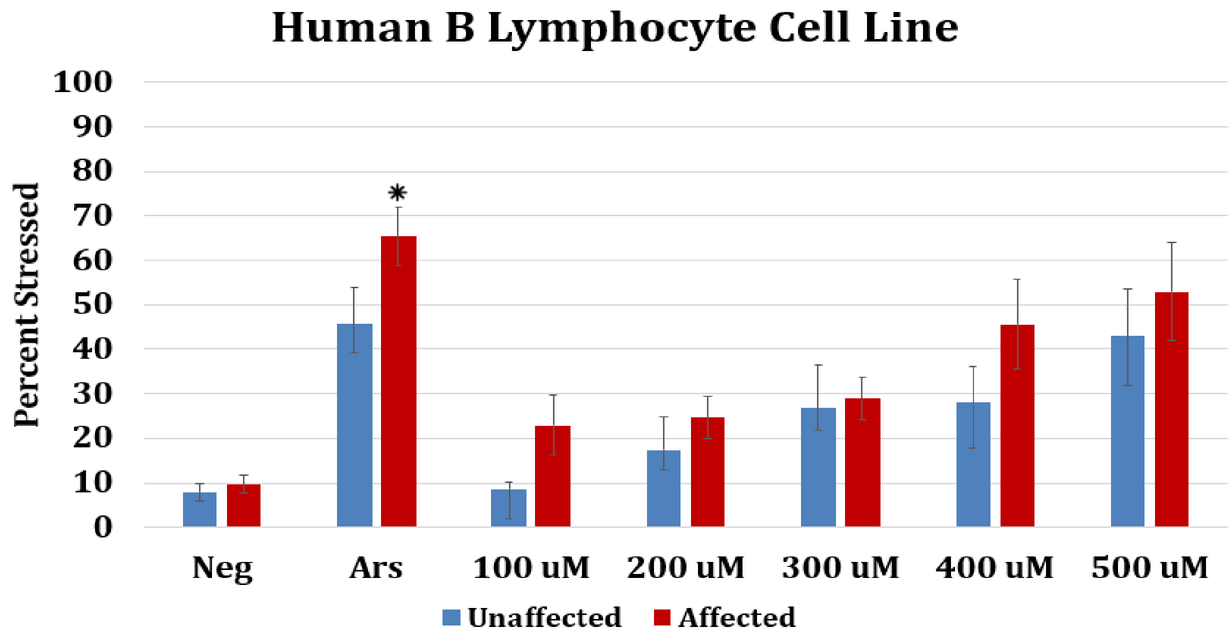


**Figure 10.** The average percentage of human B lymphocytes exhibiting stress. These cells were treated for one hour with varying concentrations of BPA, 500 uM of arsenite, or left untreated as indicated in the figure. Three biological replicate were performed. Error bars within the figure represent standard error. ANOVA analysis was completed to find statistical significance and is indicated by an asterisk ($p < 0.05$).

As seen in Figure 10, the data mimics the dose dependent response as predicted and seen in the U20S-DS cell line. There was an increase in percent of stressed cells as the concentration of BPA exposure increased. Three biological replicates were completed and each replicate was counted by two members for a grand total of six counts for each treatment. Standard error shows the variance in these counts. An ANOVA analysis was conducted to find if data from the unaffected and affected cell lines were statistically significant at each concentration. The data was found to be statistically significant for our positive control, Arsenite, only with a p value where $p < 0.05$.

# Discussion

We noted a significant increase in stress granule formation in FXS affected B lymphocytes in response to arsenite. The FXS affected cells showed increased stress granule formation in response to BPA, however, this difference was not statistically significant. The results from the FXS affected mouse fibroblast cells had some statistical significance, but no conclusive findings. Further research is  necessary to determine if there is a statistical significance in stress granule formation between FXS affected and unaffected cells.

## Reasons for Discrepancies in Data

There were no trends seen in the mouse embryonic fibroblast data, which could be the result of many factors. These cells were the same type but they were not littermate and passage matched: they were not from mice from the same litter, nor were they at the same passage number and therefore they did not follow the same growth trends. These variable growth trends led to the unaffected cells undergoing more passages than the affected cells. This can affect their base level of stress, and therefore, alter the results after the treatment. If one group of cells is more stressed when plating one day over another, this would yield different results. Since we averaged all of the counts we did for each biological replicate, the day-to-day differences could have altered the overall result.

Additionally, some technical errors could have affected the results we obtained. While scoring the embryonic fibroblast cells, the microscope in Goddard Hall broke and required repair, so in order to keep the momentum of the project going, we used a microscope owned by the Biomedical Engineering Department in Salisbury Labs. This scope worked by taking photos of the cells and counting them on a computer screen, rather than looking through the eyepiece of the microscope to count. This change in methods may have altered our results.

Once the cells were mounted on the slides, the team took precautions so the slides would not be exposed to light for an extended amount of time, however, they still may have faded which would cause the fluorescently stained stress granules to appear dimmer, making the counter less likely to see them or count the cell as stressed.

Many of these problems were ameliorated when the team transitioned over to the B lymphocytes. The cells used for the B lymphocyte trials were from brothers: one has Fragile X Syndrome and one does not. This means that these cells were better matched, and they grew at about the same rate so they had about the same number of passages. However, discrepancies are seen between the verification trial and the results with these cells. The level of stress seen in the arsenite treated (positive control) is much higher, and not significantly different between cell types, for the verification trial. We predict that this is because the way this verification trial was set up led to subconscious bias to alter the results. The verification trial was not blinded. Additionally, we knew that the slides were either a positive or negative control, which could cause us to count

cells as stressed when in a blinded count we would evaluate the cell more closely before counting it as stressed or unstressed.

## Future Experimentation

This project grew out of previous experimentation evaluating cellular stress response to various concentrations of BPA. Therefore, we have some suggestions for future experiments with FXS affected cells and BPA. We recommend the following:

1. Repeat the experiment with the mouse fibroblast cells, but remove some of the issues that we came across. Use matched cell lines if they are available, and try to be more consistent with passaging so the cells undergo the same number of passages. Use the same microscope throughout the experiment. Continue with performing biological replications in triplicate and perform at least two technical replicates to account for personal error.
2. Repeat the experiment with B lymphocytes. These cells performed well for this team but assuring that the results are reproducible will add to the validity of the study. If this is the case, treat the cells with BPF or BPA and BPF to see if other bisphenols, or a combination of bisphenols, affects stress response.
3. Another worthwhile change would be to see how affected and wildtype cells react to incubation with arsenite or BPA at different incubation times. Stress granules are dynamic, and by incubating at different times there are more snapshots of the stress response available that would give information about reaction time to the compounds that could vary between affected and unaffected cells.

## Impact on Patients with Fragile X Syndrome

If the results for these experiments are reproducible, they can be used to draw conclusions on how environmental contaminants may affect patients with FXS. For instance, if FXS patients are more sensitive to environmental contaminants, they may form stress granules at lower levels of exposure. If FXS patients' cells have an increased number of stress granules, the cells are not able to synthesize protein because translation is inhibited in stress granules. Therefore, the cell will eventually die, or work improperly, because it is not able to produce proteins. Chronic cell stress can cause ischemia, neurological diseases, and cancer (Reineke and Neilson, 2019).

Once information as to whether or not the differences in cellular stress response translate to differences at the patient level, further research can be done to see if the effects seen in patients with FXS are the same in patients with other ASD and intellectual and developmental disabilities.

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# Appendix

## Appendix A - Raw Data for U20S-DS cell line verification trial

| Treatment | Stressed | Not Stressed | Total | % Stressed |
|---|---|---|---|---|
| Neg | 1 | 258 | 259 | 0.39 |
| | 22 | 252 | 274 | 8.03 |
| | 7 | 258 | 265 | 2.64 |
| | 14 | 255 | 269 | 5.20 |
| Ars | 254 | 1 | 255 | 99.61 |
| | 303 | 0 | 303 | 100.00 |
| | 420 | 44 | 464 | 90.52 |
| | 268 | 1 | 269 | 99.63 |
| 100 uM | 7 | 262 | 269 | 2.60 |
| | 18 | 257 | 275 | 6.55 |
| | 1 | 271 | 272 | 0.37 |
| | 18 | 274 | 292 | 6.16 |
| 200 uM | 7 | 246 | 253 | 2.77 |
| | 16 | 302 | 318 | 5.03 |
| | 3 | 251 | 254 | 1.18 |
| | 16 | 248 | 264 | 6.06 |
| 300 uM | 143 | 133 | 276 | 51.81 |
| | 140 | 137 | 277 | 50.54 |
| | 142 | 144 | 286 | 49.65 |
| | 112 | 146 | 258 | 43.41 |
| 400 uM | 263 | 5 | 268 | 98.13 |
| | 58 | 329 | 387 | 14.99 |

| | | | | |
|---|---|---|---|---|
| | **249** | **10** | **259** | **96.14** |
| | **255** | **89** | **344** | **74.13** |
| **500 uM** | **249** | **4** | **253** | **98.42** |
| | **268** | **5** | **273** | **98.17** |
| | **255** | **14** | **269** | **94.80** |
| | **291** | **113** | **404** | **72.03** |

# Appendix B - Raw Data for Human B Lymphocyte verification trial

| **Unaffected** | | | | | | **Affected** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Stressed** | **Not Stressed** | **Total** | **Percent Stressed** | | **Treatment** | **Stressed** | **Not Stressed** | **Total** | **Percent Stressed** |
| Neg | 4 | 177 | 181 | 2.21 | | Neg | 14 | 191 | 205 | 6.83 |
| | 3 | 104 | 107 | 2.80 | | | 8 | 139 | 147 | 5.44 |
| | 3 | 119 | 122 | 2.46 | | | 11 | 127 | 138 | 7.97 |
| | 44 | 169 | 213 | 20.66 | | | 5 | 168 | 173 | 2.89 |
| Ars | 150 | 13 | 163 | 92.02 | | Ars | 189 | 24 | 213 | 88.73 |
| | 104 | 12 | 116 | 89.66 | | | 78 | 35 | 113 | 69.03 |
| | 113 | 9 | 122 | 92.62 | | | 123 | 22 | 145 | 84.83 |
| | 116 | 47 | 163 | 71.17 | | | 164 | 44 | 208 | 78.85 |

# Appendix C - Raw Data for MEF cell line acute exposure assay

| Wildtype | | | | | | Knockout | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Stressed | Not Stressed | Total | Percent Stressed | | Treatment | Stressed | Not Stressed | Total | Percent Stressed |
| Neg | 1 | 255 | 256 | 0.39 | | Neg | 38 | 96 | 134 | 28.36 |
| | 6 | 232 | 238 | 2.52 | | | 41 | 69 | 110 | 37.27 |
| | 71 | 116 | 187 | 37.97 | | | 7 | 243 | 250 | 2.80 |
| | 89 | 116 | 205 | 43.41 | | | 11 | 247 | 258 | 4.26 |
| | 9 | 250 | 259 | 3.47 | | | 6 | 216 | 222 | 2.70 |
| | 11 | 260 | 271 | 4.06 | | | 16 | 198 | 214 | 7.48 |
| Ars | 20 | 241 | 261 | 7.66 | | Ars | 138 | 80 | 218 | 63.30 |
| | 24 | 235 | 259 | 9.27 | | | 117 | 116 | 233 | 50.21 |
| | 248 | 18 | 266 | 93.23 | | | 89 | 108 | 197 | 45.18 |
| | 17 | 215 | 232 | 7.33 | | | 118 | 77 | 195 | 60.51 |
| | 165 | 126 | 291 | 56.70 | | | 145 | 108 | 253 | 57.31 |
| | 218 | 81 | 299 | 72.91 | | | 130 | 124 | 254 | 51.18 |
| 100 uM | 61 | 155 | 216 | 28.24 | | 100 uM | 60 | 153 | 213 | 28.17 |
| | 62 | 152 | 214 | 28.97 | | | 64 | 131 | 195 | 32.82 |
| | 17 | 245 | 262 | 6.49 | | | 159 | 76 | 235 | 67.66 |
| | 9 | 256 | 265 | 3.40 | | | 140 | 79 | 219 | 63.93 |
| 200 uM | 148 | 133 | 281 | 52.67 | | 200 uM | 20 | 221 | 241 | 8.30 |
| | 163 | 173 | 336 | 48.51 | | | 43 | 170 | 213 | 20.19 |
| | 14 | 245 | 259 | 5.41 | | | 16 | 248 | 264 | 6.06 |
| | 27 | 197 | 224 | 12.05 | | | 11 | 205 | 216 | 5.09 |
| 300 uM | 225 | 29 | 254 | 88.58 | | 300 uM | 30 | 226 | 256 | 11.72 |
| | 221 | 30 | 251 | 88.05 | | | 40 | 223 | 263 | 15.21 |

| | 46 | 204 | 250 | 18.40 | | | 71 | 159 | 230 | 30.87 |
|---|---|---|---|---|---|---|---|---|---|---|
| | 15 | 253 | 268 | 5.60 | | | 112 | 135 | 247 | 45.34 |
| | 109 | 139 | 248 | 43.95 | | | 95 | 137 | 232 | 40.95 |
| | 97 | 177 | 274 | 35.40 | | | 70 | 129 | 199 | 35.18 |
| 400 uM | 105 | 172 | 277 | 37.91 | | 400 uM | 81 | 89 | 170 | 47.65 |
| | 9 | 243 | 252 | 3.57 | | | 114 | 66 | 180 | 63.33 |
| | 40 | 253 | 293 | 13.65 | | | 47 | 195 | 242 | 19.42 |
| | 72 | 218 | 290 | 24.83 | | | 14 | 215 | 229 | 6.11 |
| | 4 | 276 | 280 | 1.43 | | | 199 | 37 | 236 | 84.32 |
| | 7 | 209 | 216 | 3.24 | | | 242 | 10 | 252 | 96.03 |
| 500 uM | 237 | 48 | 285 | 83.16 | | 500 uM | 227 | 39 | 266 | 85.34 |
| | 213 | 47 | 260 | 81.92 | | | 233 | 24 | 257 | 90.66 |
| | 48 | 147 | 195 | 24.62 | | | 51 | 207 | 258 | 19.77 |
| | 47 | 147 | 194 | 24.23 | | | 27 | 257 | 284 | 9.51 |
| | 157 | 102 | 259 | 60.62 | | | 95 | 137 | 232 | 40.95 |
| | 149 | 87 | 236 | 63.14 | | | 6 | 262 | 268 | 2.24 |
| 600 uM | 242 | 37 | 279 | 86.74 | | 600 uM | 144 | 123 | 267 | 53.93 |
| | 236 | 17 | 253 | 93.28 | | | 115 | 145 | 260 | 44.23 |

# Appendix D - Raw Data for Human B Lymphocyte cell line acute exposure assay

| Unaffected | | | | | | Affected | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Stressed | Not Stressed | Total | Percent Stressed | | Treatment | Stressed | Not Stressed | Total | Percent Stressed |
| Neg | 7 | 111 | 118 | 5.93 | | Neg | 7 | 126 | 133 | 5.26 |
| | 24 | 115 | 139 | 17.27 | | | 19 | 139 | 158 | 12.03 |
| | 12 | 166 | 178 | 6.74 | | | 30 | 183 | 213 | 14.08 |
| | 6 | 145 | 151 | 3.97 | | | 3 | 117 | 120 | 2.50 |
| | 12 | 186 | 198 | 6.06 | | | 15 | 198 | 213 | 7.04 |
| | 9 | 88 | 97 | 9.28 | | | 22 | 133 | 155 | 14.19 |
| Ars | 127 | 126 | 253 | 50.20 | | Ars | 165 | 52 | 217 | 76.04 |
| | 81 | 62 | 143 | 56.64 | | | 100 | 78 | 178 | 56.18 |
| | 102 | 156 | 258 | 39.53 | | | 201 | 78 | 279 | 72.04 |
| | 93 | 124 | 217 | 42.86 | | | 142 | 135 | 277 | 51.26 |
| | 108 | 57 | 165 | 65.45 | | | 114 | 87 | 201 | 56.72 |
| | 9 | 91 | 100 | 9.00 | | | 102 | 7 | 109 | 93.58 |
| 100 | 26 | 232 | 258 | 10.08 | | 100 | 97 | 115 | 212 | 45.75 |
| | 14 | 140 | 154 | 9.09 | | | 90 | 158 | 248 | 36.29 |
| | 11 | 116 | 127 | 8.66 | | | 13 | 109 | 122 | 10.66 |
| | 13 | 97 | 110 | 11.82 | | | 7 | 102 | 109 | 6.42 |
| | 21 | 171 | 192 | 10.94 | | | 27 | 208 | 235 | 11.49 |
| | 1 | 154 | 155 | 0.65 | | | 14 | 139 | 153 | 9.15 |
| 200 | 23 | 118 | 141 | 16.31 | | 200 | 31 | 122 | 153 | 20.26 |
| | 26 | 148 | 174 | 14.94 | | | 35 | 112 | 147 | 23.81 |
| | 2 | 258 | 260 | 0.77 | | | 16 | 171 | 187 | 8.56 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 35 | 201 | 236 | 14.83 | | | 34 | 129 | 163 | 20.86 |
| | 63 | 59 | 122 | 51.64 | | | 77 | 121 | 198 | 38.89 |
| | 32 | 69 | 101 | 31.68 | | | 44 | 72 | 116 | 37.93 |
| 300 | 8 | 137 | 145 | 5.52 | | 300 | 37 | 109 | 146 | 25.34 |
| | 0 | 140 | 140 | 0.00 | | | 25 | 165 | 190 | 13.16 |
| | 12 | 204 | 216 | 5.56 | | | 41 | 114 | 155 | 26.45 |
| | 97 | 125 | 222 | 43.69 | | | 39 | 98 | 137 | 28.47 |
| | 129 | 99 | 228 | 56.58 | | | 107 | 109 | 216 | 49.54 |
| | 51 | 109 | 160 | 31.88 | | | 48 | 135 | 183 | 26.23 |
| 400 | 24 | 176 | 200 | 12.00 | | 400 | 149 | 64 | 213 | 69.95 |
| | 11 | 154 | 165 | 6.67 | | | 105 | 75 | 180 | 58.33 |
| | 78 | 196 | 274 | 28.47 | | | 43 | 219 | 262 | 16.41 |
| | 95 | 171 | 266 | 35.71 | | | 20 | 95 | 115 | 17.39 |
| | 41 | 79 | 120 | 34.17 | | | 50 | 63 | 113 | 44.25 |
| | 69 | 40 | 109 | 63.30 | | | 96 | 36 | 132 | 72.73 |
| 500 | 103 | 49 | 152 | 67.76 | | 500 | 98 | 22 | 120 | 81.67 |
| | 72 | 71 | 143 | 50.35 | | | 148 | 46 | 194 | 76.29 |
| | 24 | 67 | 91 | 26.37 | | | 22 | 109 | 131 | 16.79 |
| | 74 | 13 | 87 | 85.06 | | | 31 | 80 | 111 | 27.93 |
| | 47 | 154 | 201 | 23.38 | | | 40 | 74 | 114 | 35.09 |
| | 37 | 119 | 156 | 23.72 | | | 105 | 64 | 169 | 62.13 |