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Effect of an Oncogenic HER2 Mutant on EGFR Activity

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Honors Project Report

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Introduction

Every year, about 1.9 million people are diagnosed with cancer. Out of that number, it is estimated that around 287,850 women and 2,710 men will be diagnosed with breast cancer.[1] These statistics make breast cancer the most common type of cancer. Of every five breast cancer cases, one is caused by having high concentrations of human epidermal growth factor receptor 2, HER2.[1] HER2 proteins control how fast cells in breast tissue grow and divide. When breast tissue contains extra HER2 receptors, the protein is overexpressed which causes the cells to divide uncontrollably.[1] HER2 is the only member of the HER family that does not require a ligand in order to dimerize.[2] Therefore, it is always ready to dimerize. The dimerization process causes phosphorylation, the addition of a phosphate group to the intracellular portion of the receptors.[2] As receptors are dimerized, signaling molecules are allowed to bind to docking sites on the receptors. Pathways such as the PI3K and MAPK pathways can be activated.[2] The PI3K pathway may prevent apoptosis of cancer cells while the MAPK pathway can lead to an increase in the proliferation of cancer cells.[2] Another reason for breast cancer cells to rapidly divide is because of a mutation. The HER2 S310F mutation is the most common mutation in this protein. This missense mutation increases HER2 activation receptors.[3] Regardless of the cause of HER2-positive breast cancer, it responds well to targeted therapies.

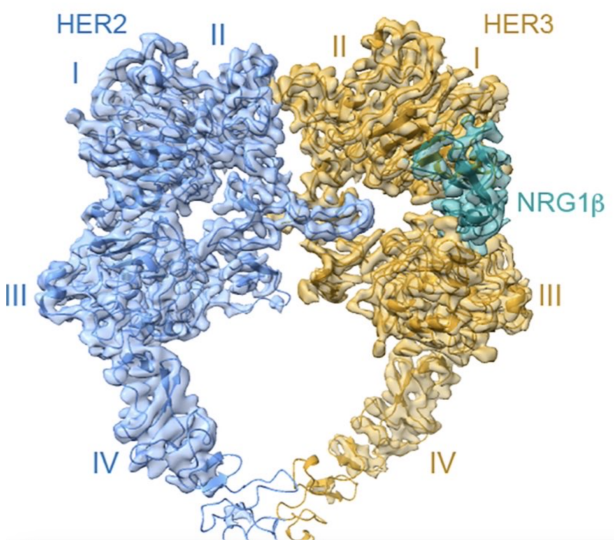

Figure 1. Structure of HER2-HER3 dimerization with NRG1β ligand binding.[4]

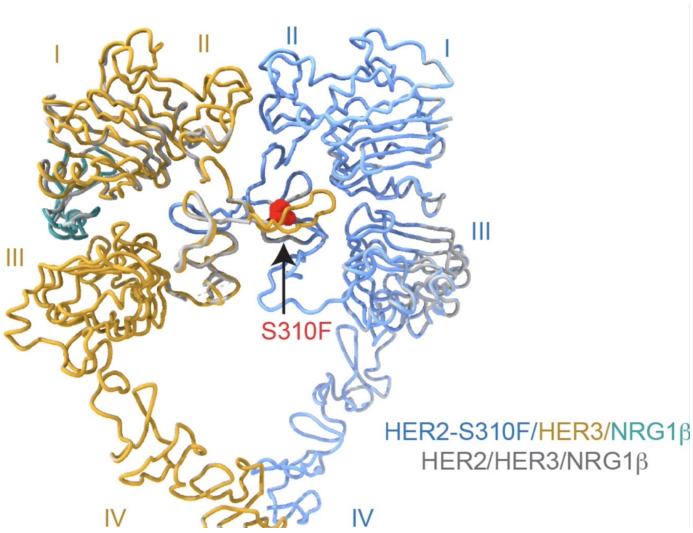

Figure 2. Structure of HER2-HER3 dimerization with the HER2 S310F mutation.[4]

Targeted therapy is a type of cancer treatment that is aimed to interrupt molecular abnormalities that are responsible for the growth of cancer.[5] Trastuzumab is a type of targeted therapy that is specific to HER2-positive breast cancer. It is a monoclonal antibody that binds to HER2.[6] By doing so, it could assist the immune system to fight and kill cancer cells. According to Diwanji et al, it was found that trastuzumab did not interfere with HER2-HER3 heterodimerization. The antibody altered its shape and formed complexes with the receptor dimers, as seen in Figure 3.

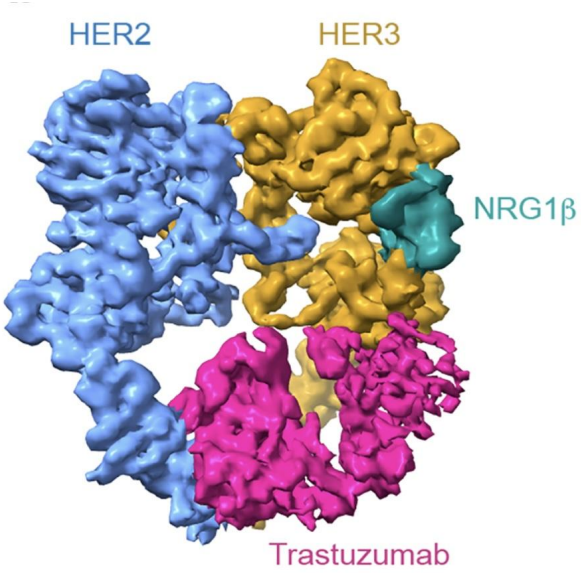

Figure 3. HER2-HER3 dimerization with NRG1β ligand and Trastuzumab antibody binding.[4]

One way to diagnose HER2 status is immunohistochemistry (IHC). IHC uses a variety of anatomical, immunological and biochemical techniques to capture images in tissues.[7] This is done by using a specific antibody that binds to antigens.[7] When diagnosing breast cancer, IHC measures the amount of HER2 proteins found in breast cells. In this Honors Research Project, immunohistochemistry is used to capture the amount of antibody binding by treating HER2 WT

and its S310F mutation with the Recombinant Alexa Fluor 488 Anti-ErbB2/HER2 (phospho Y877) antibody. Images of the HER2 wild type (WT) and S310F mutation were taken from a confocal microscope and analyzed through the software Fiji.

The goal of this honors project was to successfully collect images of the cos-7 cells before and after an antibody treatment. By doing this, the effects of the antibody on the receptor tyrosine-kinase HER2 and its mutant protein S310F can be observed. This study was motivated around the possibility of adding onto a recently discovered therapeutic approach that can be used in treating cancers, specifically for HER2-positive breast cancer. Despite difficulties in the lab moving to Texas, a first round of results were obtained and analyzed.

Materials and Methods

Materials:

A COS-7 cell line was obtained from the Knight Chemical Laboratory 205(KNCL205) Cell Bank (ATCC CCL-70). Dublecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM) was obtained from ThermoFischer Scientific. 0.25% Trypsin-EDTA, phenol red, was obtained from ThermoFischer Scientific. Opti-MEM, Reduced Serum Medium, was obtained from ThermoFischer Scientific. Lipofectamine 2000 (Lipo) was purchased from ThermoFischer Scientific (California, USA). Imaging was done by a Nikon Eclipse T*i* confocal microscope. Images were captured through the Nikon Elements program. The immersion oil was obtained from Nikon (Japan). Phosphate-buffered saline was obtained from ThermoFisher Scientific. 4% Paraformaldehyde (formaldehyde solution in PBS) was obtained from Electron Microscopy Sciences (Hatfield, PA). 0.5% Triton X-100 in PBS was obtained premade from KNCL 205. 3% bovine serum albumin in PBS (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). The

Recombinant Alexa Fluor 488 Anti-ErbB2/HER2 (phospho Y877) antibody was purchased from Abcam. The EGF samples were obtained from the -80°C freezer in KNCL 205.

Growing Cells from Stock:

A COS-7 cell line was obtained from the KNCL205 Cell Bank and thawed. A 10 cm cell culture plate was obtained and labeled with the COS-7 cell line, passage number (p#), the date, and lab student's name. 10 mL of DMEM full was pipetted into the plate. 1 mL of the COS-7 cell stock was added to the plate and incubated at 37°C for four to 5 days.

Splitting Cells:

The cells were observed under a microscope for 70-80% confluency in order to split. A new 10 cm plate was obtained and labeled along with adding 10 mL of DMEM. The old media was aspirated off of the old plate. 2 mL of Trypsin was added to suspend the cells. The plate was placed back in the incubator to sit for 3 minutes. To stop the enzyme, 2 mL of DMEM was added. Using a P1000 pipette and angling the plate, the solution was pipetted up and down to rinse cells off the plate until a majority of the cells were turbid. This solution was pipetted into a 15 mL falcon tube. 135 µL of the solution from the falcon tube was added to the new plate for a growth period of 3 days. After the appropriate growth time, the cells were split again. Following the same procedure as above, the cells were split into six 2 cm glass bottom plates. 2 mL of DMEM and 135 µL of the cell solution were added to each plate. The plates were placed in the incubator to grow for 3 days.

Transfection:

Two different single transfections were performed in this experiment. Two plates were transfected with HER2 WT m.Ch, two plates with the S310F mutant, and two plates were untransfected as a control group. Each plate was labeled with the cell line, passage number,

transfection DNA, date, and the student's name. A sample of HER2 WT m.Ch and S310F m.CH was obtained from the -80°C freezer in KNCL205 and thawed. The concentration of the HER2 WT was used in a programmed Excel sheet to calculate the amount of HER2 and Opti-MEM needed to have a total of 2.5 μg of DNA for each plate. A microcentrifuge tube was used to combine the necessary amounts of HER2 and Opti-MEM. Another microcentrifuge tube was used to combine 2.5 μL of Lipo and 25 μL of Opti-MEM. Each individual microcentrifuge tube was let to sit for 5 minutes at room temperature to gently mix. After 5 minutes, the Lipo and Opti-MEM microcentrifuge tube was pipetted into the HER2 and Opti-MEM microcentrifuge tube and let sit for 25 minutes at room temperature. The combined microcentrifuge tube was then pipetted into the center of one of the six small imaging plates and placed into the incubator for about 19 hours. The previous transfection procedure was repeated for the S310F m.Ch mutant. The plates that were untransfected received 2 mL of Opti-MEM and were placed in the incubator with the transfected plates.

Imaging:

After 19 hours, the transfected plates were ready for the first round of imaging. The plates were removed from the incubator. The old media was aspirated and replaced with 2 mL of fresh Opti-MEM. Each individual plate was imaged using a confocal microscope. A drop of immersion oil was placed on the lens of the confocal microscope to avoid possible damage. Images of the cells were captured through the 'Texas Red' fluorescence filter. Images in the 'GFP' fluorescence filter were also captured to ensure there was no contamination. The exposure time used for all HER2 m.Ch plates was 90 ms. Light filters ND8 and ND4 were used and adjusted to focus on the cells. About 10-15 images were taken for each plate. Upon the completion of imaging, the plates were returned back to the incubator to sit overnight.

Fixation, Permeabilization, and Blocking:[8]

The cells were removed from the incubator and the media was aspirated off. 1 mL of 4% Paraformaldehyde was added to each plate and then incubated for 15 minutes at room temperature. The fixative solution was removed and each plate was washed by pipetting 1 mL of PBS. The solution was swirled around and then removed. The washing step was repeated 3 times. 1 mL of 0.5% Triton X-100 was added to each plate and then incubated for 15 minutes at room temperature. The permeabilization solution was removed and each plate was washed 3 times with 1 mL of PBS. 2 mL of 3% BSA was added to each plate and was incubated for at least 60 minutes at room temperature. The plates were left to incubate with the blocking solution overnight at room temperature. The cells are now locked in place and are prepared for immunolabeling.

Immunofluorescence:[9]

The blocking solution was removed from each plate. 1 mL of a 1:1000 dilution of the Recombinant Alexa Fluor 488 Anti-ErbB2/HER2 (phospho Y877) antibody was added to each plate. The plates were incubated for 1 hr covered at room temperature. The antibody staining solution was aspirated off the plates and washed 3 times with PBS. 2 mL of Opti-MEM was added to the HER2 WT, S310F, and untransfected plates that were not receiving EGF treatment. 3 samples of EGF were thawed and prepared for the HER2 WT, S310F, and untransfected plates that were receiving treatment. 1.5 mL of Opti-MEM was added to each plate while 500μL of Opti-MEM was added to each EGF sample. The EGF-Opti-MEM solution was then added to each plate and incubated for 4 minutes at room temperature. The solution was aspirated off the plates and 2 mL of new Opti-MEM was added. The plates were then ready to be imaged. The

previous imaging protocol was followed. Upon the completion of imaging, all plates were discarded in the biohazard waste bin.

Results and Discussion

Preliminary data obtained by Yasmine Elshoweikh, a Ph.D. student at the University of Akron, suggests how strongly the proteins dimerize with each other. Using an advanced fluorescence spectroscopy system, called PIE-FCCS, previous work in the lab showed that the fraction of correlation of WT EGFR-HER2 heterodimerization is close to zero without EGF. When EGF is added, there is stronger heterodimerization. The S310F-EGFR heterodimerization without EGF is stronger compared to the WT. This suggests that the mutant heterodimerization with EGFR can lead to unregulated cell growth and be an oncogenic pathway. When EGF is added to the S310F-EGFR heterodimerization, the strength of dimerization becomes stronger. This data is the motivating data for this experiment. [10] Our hypothesis is that there will be more EGFR phosphorylation when mutant HER2 is present, even in the absence of a ligand.

The data analysis of this experiment consisted of measuring the intensity of the fluorescence from the images. This was done by using the software Fiji, a software based on imageJ.[11] For each image, three ROIs and a blank were taken and measured by the software. The three ROIs on the cell were averaged and recorded before and after the antibody treatment. Following the preliminary data, the intensity of the fluorescence of HER2 and S310F, with and without EGF, were compared before and after the antibody treatment. This was analyzed in hope of seeing similar results that phosphorylation is higher with the S310F with EGF.

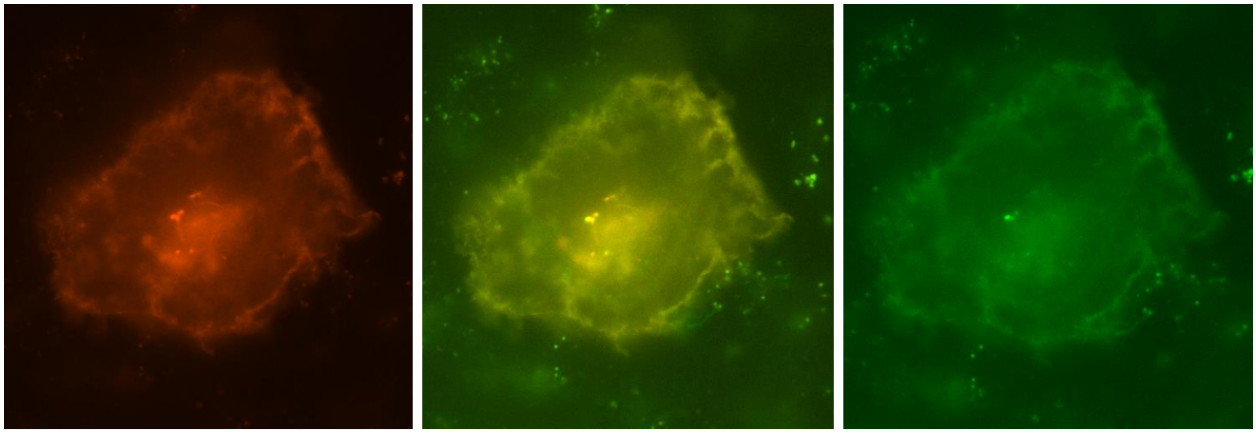

Figure 4. Images of HER2 WT with no ligand before the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filters and merged (middle) into one channel.

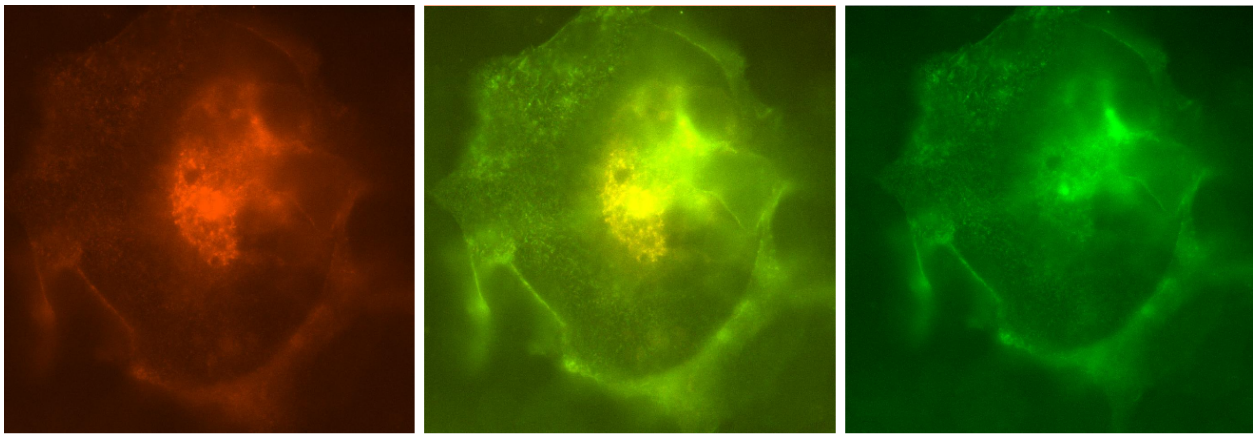

Figure 5. Images of HER2 WT with no ligand after the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filters and merged (middle) into one channel.

Channel	Before	After
Texas Red	9783.4	12269.8
GFP	9387.5	14720.2

Table 1. This table reports the average intensities of HER2 WT with no ligand measured from three ROIs by using the Fiji software.

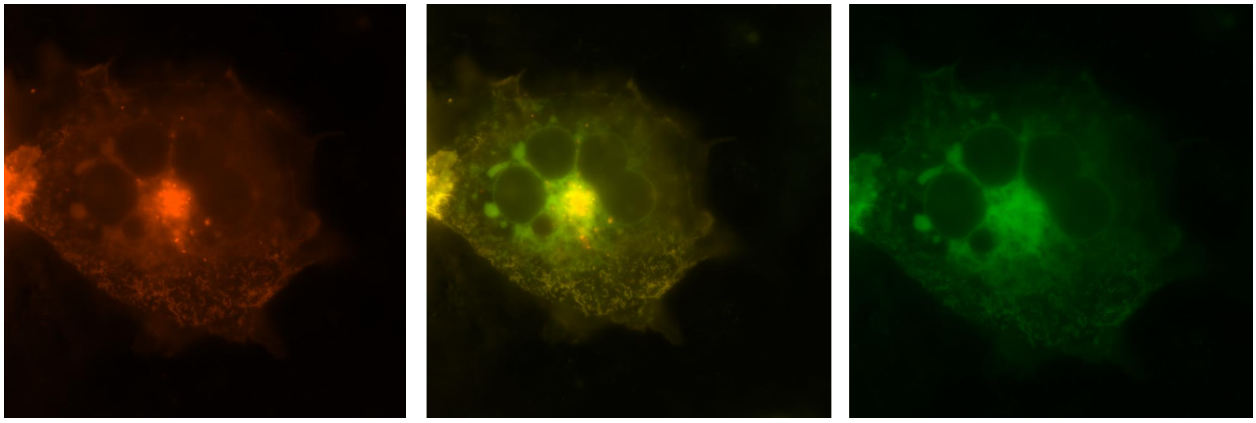

Figure 6. Images of HER2 WT with EGF before the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filters and merged (middle) into one channel.

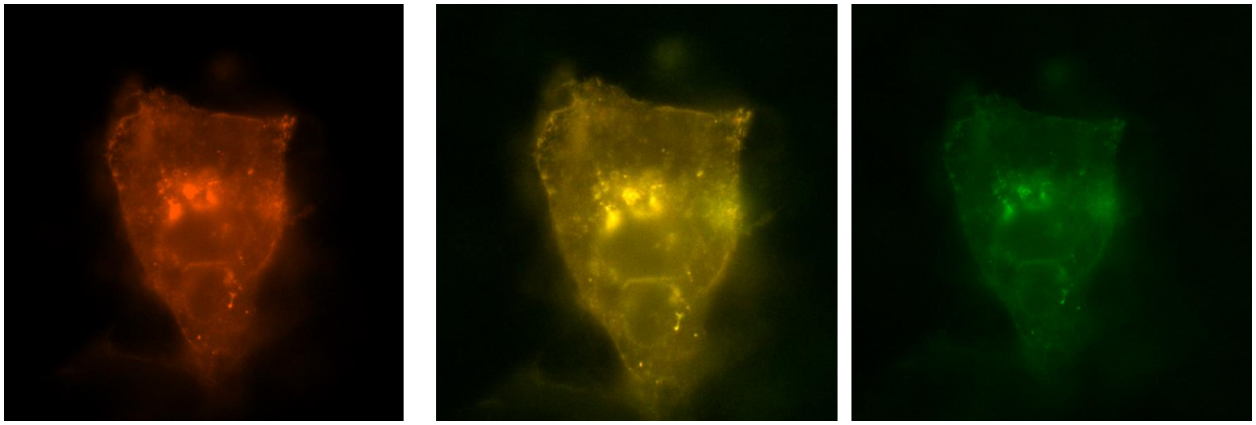

Figure 7. Images of HER2 WT with EGF after the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filters and merged (middle) into one channel.

Channel	Before	After
Texas Red	7051.8	19182.2
GFP	14313.4	14904.9

Table 2. This table reports the average intensities of HER2 WT with EGF measured from three ROIs by using the Fiji software.

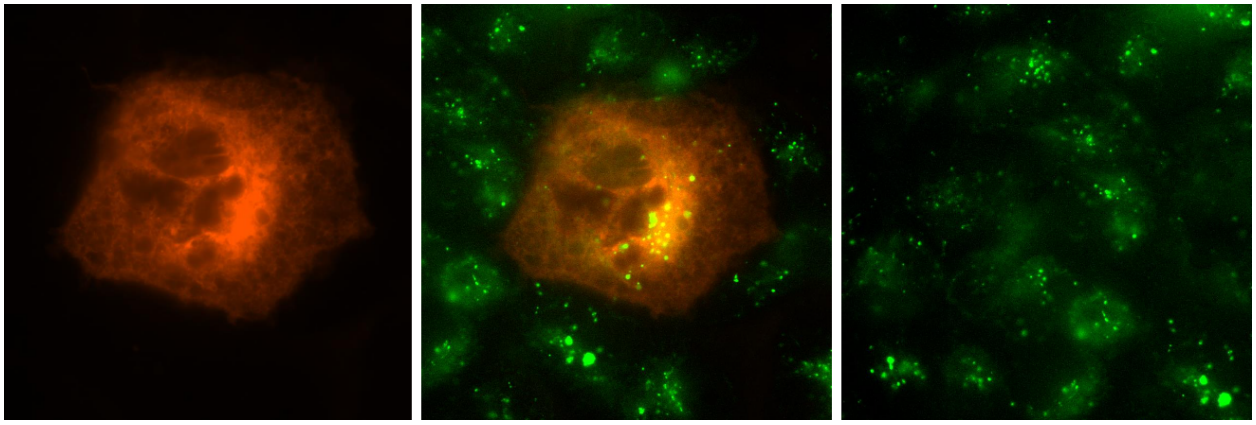

Figure 8. Images of S310F with no ligand before the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filter and merged (middle) into one channel.

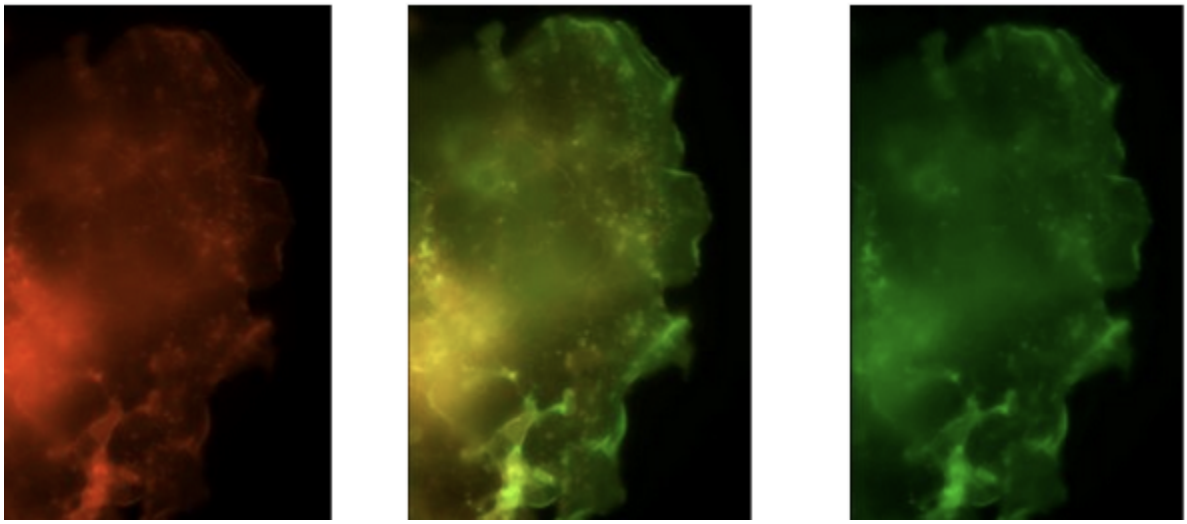

Figure 9. Images of S310F with no ligand after the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filter and merged (middle) into one channel.

Channel	Before	After
Texas Red	13764.9	15129.3
GFP	1378.2	11434.9

Table 3. This table reports the average intensities of S310F with no ligand measured from three ROIs by using the Fiji software.

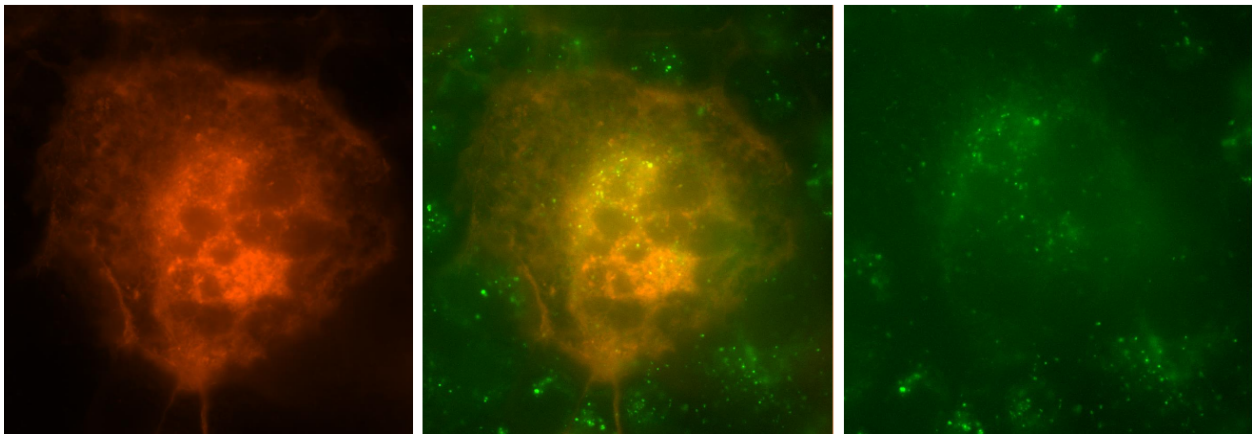

Figure 10. Images of S310F with EGF before the antibody treatment. The images were taken in the Texas Red (left) and GFP (right) filter and merged (middle) into one channel.

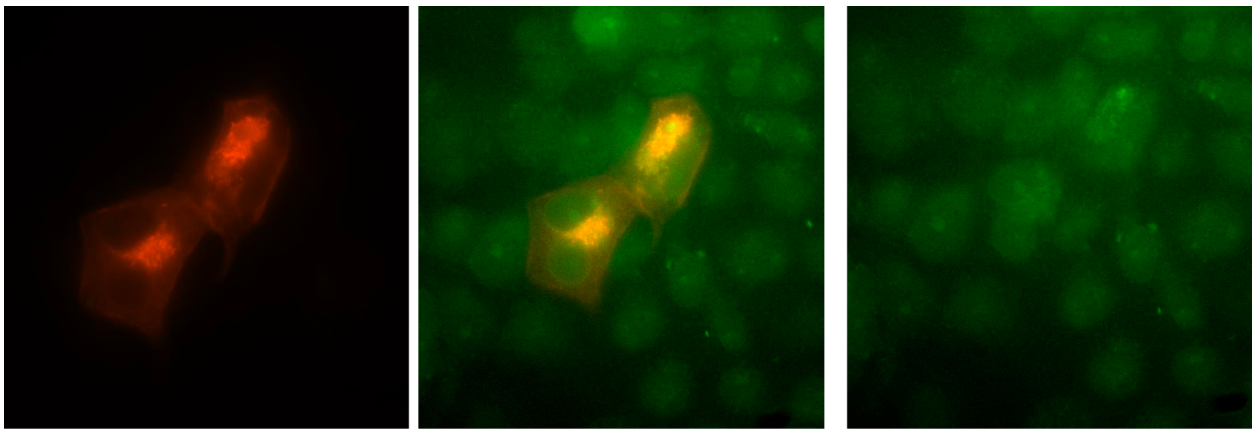

Figure 11. Images of S310F with EGF after the antibody treatment. Images were taken in the Texas Red (left) and GFP (right) filter and merged (middle) into one channel.

Channel	Before	After
Texas Red	9986.6	14344.9
GFP	1924.7	N/A

Table 4. This table reports the average intensities of S310F with EGF measured from three ROIs by using the Fiji software.

The data obtained from Table 1 and Table 2 suggest that there is an increase in phosphorylation for HER2 WT when EGF is added compared to when no EGF is added. The intensity increased after antibody binding for both with and without EGF.

The data obtained from Table 3 and Table 4 does not provide sufficient evidence that the phosphorylation with the S310F mutant is higher with or without EGF. This is due to problems arising while imaging. Along with not capturing sufficient images, there is believed to have been some contamination when transfecting the cells with the S310F mutant. As of right now, it can't be said whether or not the phosphorylation is higher with the mutant with or without EGF.

Considering the preliminary data, it was expected that the phosphorylation of the S310F mutant would be the highest with EGF, but right now we do not see any evidence of this. After the first round of experiments, the lab moved to Texas, so no other results were obtained. The next step to achieve successful results would be to pass along the experiment to a new graduate student to continue. New HER2 m.Cherry WT and S310F samples must be prepared via miniprep in order to avoid contamination. By using fresh samples during transfection, better images should be produced and captured via the confocal microscope.

Conclusion

This study was conducted in hopes of adding more knowledge to a recently discovered therapeutic approach in treating HER2 positive breast cancer. Our hypothesis was that there would be more EGFR phosphorylation when the mutant S310F is present, even in the absence of a ligand. Cos7 cells were grown and transfected with wild-type HER2 and its S310F mutant, the most common mutation found in the HER2 domain. After fixation, permeabilization, and blocking, the cells were treated with a recombinant antibody and imaged using a confocal

microscope. The data analysis concluded that there was an increase in phosphorylation for HER2 WT when the EGF ligand was bound compared to when it was not bound. There was not enough sufficient evidence collected to conclude that the phosphorylation with the S310F mutant is higher with or without EGF. Considering these results, it was expected that the highest phosphorylation would be for the S310F mutant with the EGF ligand, but there is currently no evidence of this. The hypothesis can be further tested with more experimental rounds in the future to obtain better results. More studies and replicate experiments are needed to determine with confidence the effect of the antibody treatment. As the results from the first round of data is mostly inconclusive, there is hope for researchers to continue this experiment to further develop therapeutic treatments for HER2 positive breast cancer.

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x-perm-block.html#:~:text=Fix%2C%20Perm%2C%20and%20Block%20Protocol%20This%20protocol%20provides,antibodies%20to%20access%20the%20interior%20of%20the%20cell.

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Appendix 1

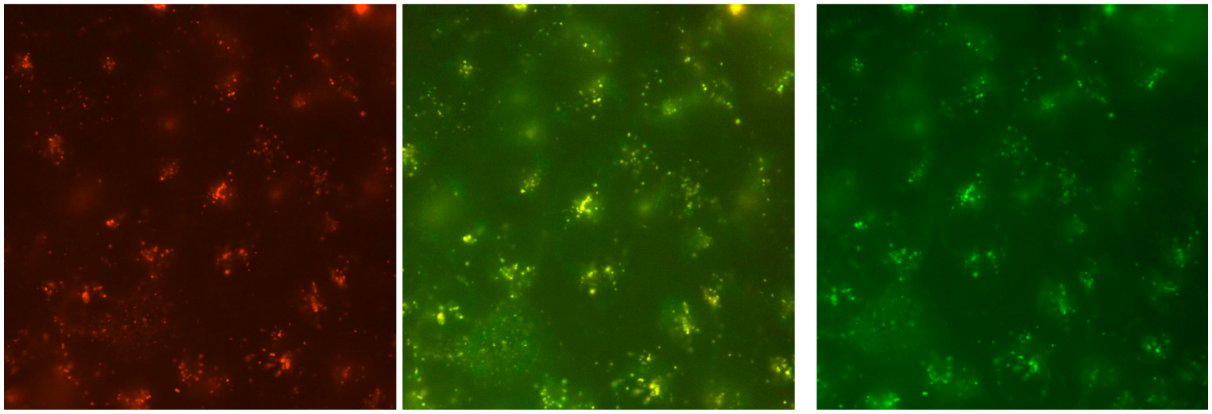

Figure 12. Images of untransfected cells with no EGF before the antibody treatment.

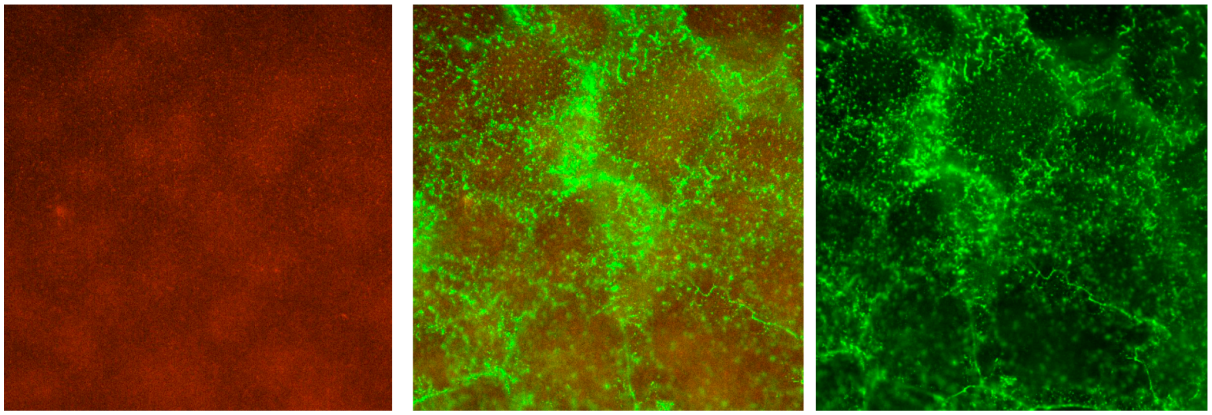

Figure 13. Images of untransfected cells with no EGF after the antibody treatment.

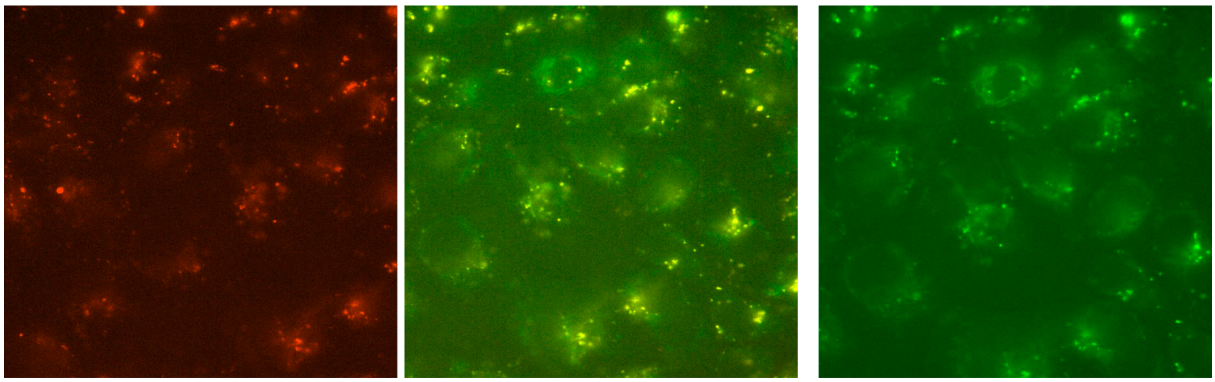

Figure 14. Images of untransfected cells with EGF before the antibody treatment.

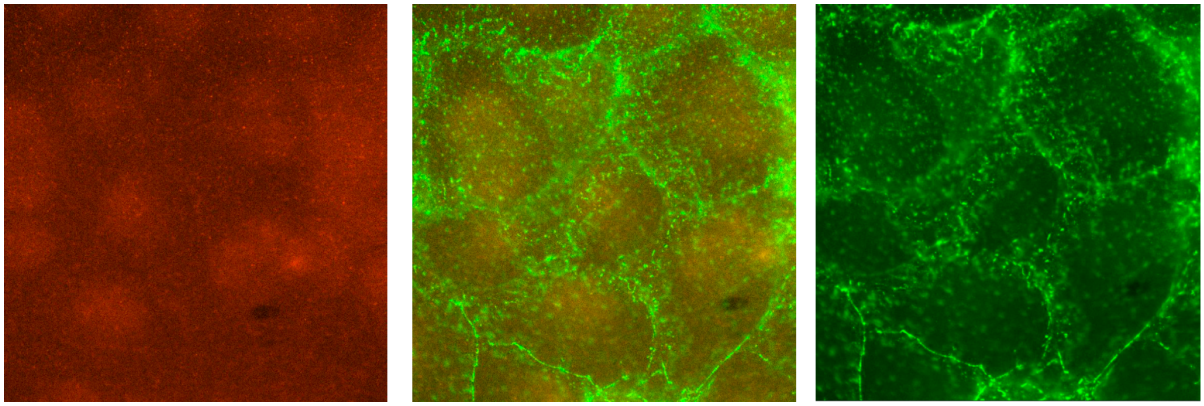

Figure 15. Images of untransfected cells with EGF after the antibody treatment.

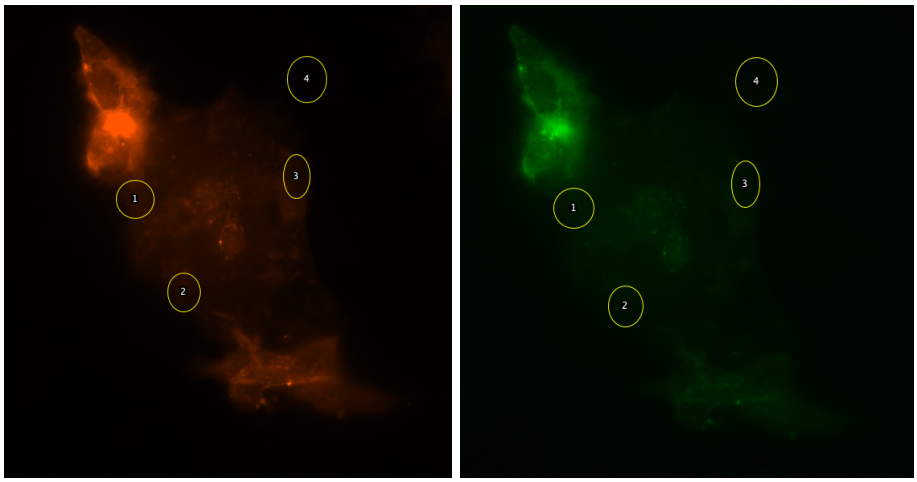

Figure 16. Example of ROI measurements using the FIJI software.