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The effect of CXCL12 ligand on internalization and dimerization of CXCR4 receptors in live cells

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Honors Research Project

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ABSTRACT

The primary objective of this project was to determine the effect of CXCL12 ligand binding on the CXCR4 receptor, specifically, how it would impact receptor internalization and dimerization. The CXCL12 ligand derives from the stromal cell-derived alpha family [8]. The CXCR4 receptors, known as C-X-C chemokine receptor type 4 play an essential role in controlling cell proliferation. When misregulated, these receptors can drive tumorigenesis and are thus important targets of cancer therapy. These G protein-coupled receptors stimulate a cascade of signaling pathways in specific tissues [1]. These pathways include the positive transcriptional control of CXCR4 via the Nuclear Respiratory Factor-1 (NFR-1) and also include cytokines such as interleukin-2 (IL-2) for CXCR4 expression [2]. From prior studies, it is evident that this receptor has multiple purposes apart from controlling cell proliferation and that is it also controls leukocyte trafficking, and hematopoiesis [5]. My goal was to investigate how the CXCR4 internalization and dimerization is altered by ligand stimulation. I measured CXCR4 levels at the plasma membrane using fluorescent imaging. Dimerization was measured with a time-resolved fluorescence method called PIE-FCCS. This technique allows the study of the CXCR4 membrane organization in living cells at varying densities on the cell surface based on the fluorescence intensity fluctuations [6].

INTRODUCTION

The CXCL12 ligand is a chemokine that binds the CXCR4 receptor, which is a membrane protein highly expressed in multiple cancer cells [3]. The CXCR4 receptor is part of the G-protein family (GPCRs), which will get activated upon the binding of a ligand and initiate a signal transduction pathway. These receptors are extremely diverse in that they regulate actin polymerization, gene transcription, and receptor internalization, cell survival or even apoptosis [3].

The CXCL12 ligand is involved in the inflammatory response. It directs T-cell maturation in the thymus and participates in the T-lymphocyte activation in lymph nodes [3]. It also plays a role in cancer progression during metastasis, which is the spreading of cancer cells away from the primary tumor site to secondary sites and eventually through the rest of the body. Determining the functionality of the ligand itself on the dimerization of the receptor can provide more insight into the function of CXCR4 receptors and their role in tumorigenesis.

My goal was to investigate how the CXCR4 receptor is altered by the addition of ligand. This connects to an ongoing project in the Smith Lab to determine how drugs interact with this membrane receptor, which will aid in cancer research and drug testing in the future. In order to perform the fluorescent imagining experiments, CXCR4 receptors were tagged using the green-fluorescent protein, specifically the Aequorea coerulescens (AcGFP1). COS7 (mammalian kidney cells) were cultured and transfected with fluorescently tagged CXCR4. Time-lapse imaging was performed to observe fluorescent intensity at the plasma membrane post ligand stimulation. The images were taken prior to the addition of ligand and then in 5-minute intervals after the ligand was added for 30 minutes to observe how that has affected the internalization of the CXCR4 receptor. Further experiments were designed to learn more about the oligomer states of the receptor, and this can help build a fundamental understanding of how drugs and ligand affect the composition of the cell membrane and influences cell function and disease progression.

METHODS

COS7 mammalian cells were transfected to express exogenous CXCR4 receptors tagged with green-fluorescent protein (AcGFP1). Lipofectamine 2000 was used as the transfection reagent [7]. The protocol for transfection was followed as described by the *Thermo-Fisher Scientific* vendors. The amount of DNA that was typically used was ~ 1500 ng CXCR4-

AcGFP1, ~ 1500 ng CXCR4-mCherry for FCCS experiment, and ~1500 ng CXCR4-AcGFP1 for fluorescence imaging experiment. For each of the samples, the oligomer-Lipofectamine 2000 complexes were prepared and added to the cell culture. The COS7 cells were stored in an incubator with 5% CO_2 at 37℃ for 24 hours before cell imaging. Separately, the CXCL12 ligand was diluted to 100 nM from a 500 nM stock solution and 10 uL of the prepared solution was pipetted into microcentrifuge tubes and stored in -80℃ freezer. Right before imaging, the ligand was removed from the freezer and stored in a breaker filled with ice.

Single cell fluorescence imaging was performed to measure the levels of CXCR4 receptor at the membrane. Cell imaging was performed on a Nikon Eclipse TI inverted epi-fluorescence microscope. One drop of the immersion oil was added to the objective lens and then the dish was positioned on the sample plate. The objective was then brought up until the cells were visualized with clear membranes and the perfect focus was turned on. Around 10-15 cells were marked with their X and Y position using a programmable motorized sample stage and then the Z positions were recorded for the top and bottom of the cell. The number of cross sections was determined based on the difference between the top and bottom of the cell. Typically, the number of cross-sections would be 10-15. After this process was programmed into the multidimensional acquisition software, the images were recorded. The cells were first imaged at time interval 0. Then using a micropipette, 10 uL of the ligand was added to the dish carefully without moving the dish. Then, in 5-minute intervals, cell imaging was performed for all 10-15 cells up to 30 minutes. This exact procedure was followed for control experiments in which no ligand was added.

We next performed FCCS experiments that more sensitively measures the changes in density compared to imaging alone. The focus was to understand the effect of ligand on

dimerization. PIE-FCCS was used as it directly measures the density instead of using brightness to correlate to density. The FCCS measurements were performed by my postdoctoral mentor. The COS7 preparation is similar to the fluorescent imaging, except this time the COS7 cell lines were transfected with AcGFP1 tagged receptor, mCherry tagged receptor, and both co-expressed as AcGFP1 and mCherry together. This was stored in the incubator with 5% CO_2 at 37°C. The FCCS system was set up with the *Nikon Eclipse* inverted confocal microscope. The fiber-based supercontinuum laser (NKT Photonics) was split up into two beams of wavelength: 488 nm and 561 nm [9]. Each beam was coupled to separate single mode optical fibers with a difference of 15 m in length to introduce approximately 50 ns separation in 488 nm and 561 nm laser pulses [9]. These lasers are AcGFP1 and mCherry which are the two excitation colors. The AcGFP1 represents the green channel and the mCherry represents the red channels. The COS-7 cells were imaged in a microscope stage incubator that has the same temperature as the original incubator (37°C) ensuring that lasers are aligned with proper overlap to one pixel. Then after conducting the alignment, then cells are ready to be imaged. For FCCS specifically, flat cells or cells without any bumps or grains were used.

RESULTS AND ANALYSIS

In this study, imaging was emphasized as this technique allowed us to look closely at how the CXCR4 protein was internalized. All data collected from imaging were analyzed using the *ImageJ software*. Firstly, the cells with proper morphology were shortlisted from the 10-15 cells that were imaged. From that, six cells were selected. A sample cell is shown at each time interval in **Figure 1**. For each of the cells, the brightness and contrast were set to the same minimum and maximum values, so the starting conditions would be comparable for all cells.

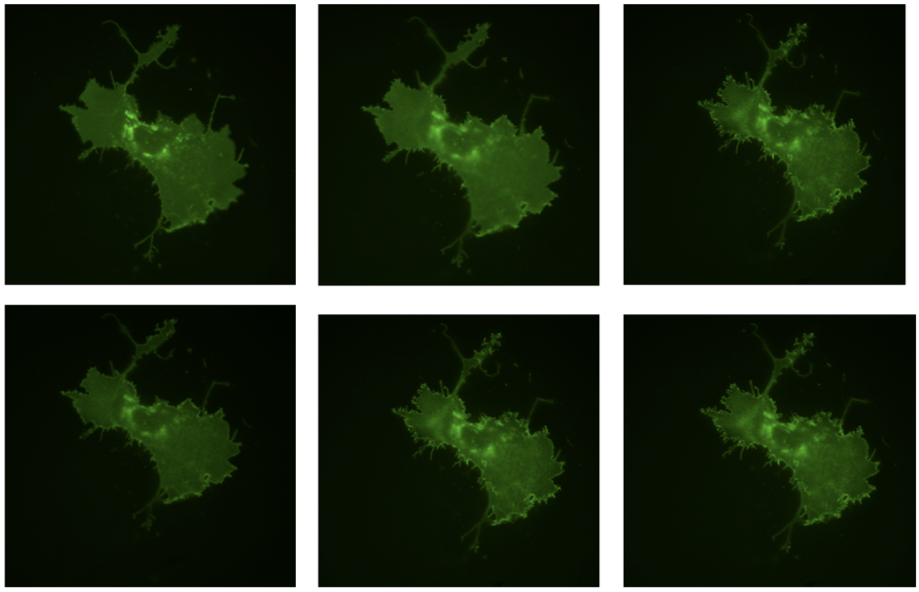

Figure 1. This is one batch of cells from the fluorescent microscopy which observes the images from T=0 to 30 minutes. Time 0 represents prior to the addition of the ligand, and then after adding the ligand, the images were taken in 5-minute intervals. Similarly, multiple cells were imaged, and their densities were recorded over the time period. These images correspond to Cell 3 from Figure 2.

To analyze changes in receptor density in the plasma membrane, each cell was outlined using the freehand tool in Image J. The area and integrated density at each time interval was recorded and output to a spreadsheet. The integrated density was measured using the absolute brightness for each cell outline. This was recorded as the absolute brightness for the cell. The background intensity of the image away from the cell was also recorded in the spreadsheet. The effective brightness can then be calculated from the brightness of the cell and its background brightness for each image. The effectiveness brightness (EB) was calculated from the following equation:

$$EB = [(absolute\ brightness\ of\ cell) - (\frac{absolute\ brightness\ of\ background}{area\ of\ the\ background}) * area\ of\ the\ cell].$$

These values were calculated for the cells at each time interval from 0, 5, 10 up to 30 minutes. The effective brightness of several single cell measurement is shown in **Figure 2**.

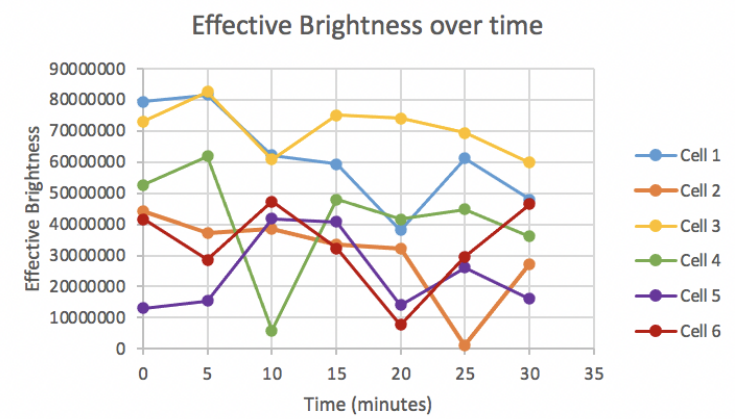

Figure 2. From the fluorescent cell imaging, the integrated cell density was equated to the indirect measure of brightness. This is the effective brightness for each of the cells examined under the CXCL12 ligand. Total of 7 cells were examined for brightness after the ligand was added in a course of 30 minutes in 5-minute intervals.

The normalized brightness was determined for each cell at each interval by dividing the EB of each times series by the EB at the initial time. The normalized brightness values were averaged, and the standard deviations were calculated as shown in the error bars. This was done for the control (- ligand) and experimental (+ ligand) groups, and the results are plotted in **Figure 3**. In the graph, there are two lines, one which represents the experimental sample with the ligand and the other is the control where no ligand was added but the cells were monitored over the same time course to control for extraneous factors. From the results, we conclude that brightness for all cells did not decrease significantly between the treated and untreated groups.

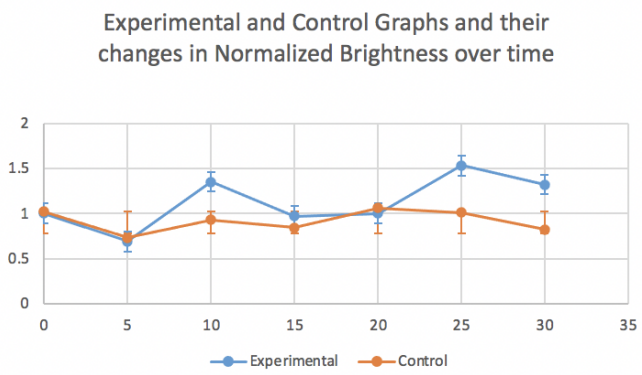

Figure 3. The fluorescent cell imaging, the experimental data and control data for the brightness was normalized and graphed. The error bars denote the standard deviation of the normalized brightness. The CXCL12 ligand was used for the experimental and the for the control, distilled water at the same temperature was used to monitor any extraneous errors.

Because the fluorescent imaging results suggested that the ligand did not significantly decrease the amount of CXCR4 in the plasma membrane, we turned to PIE-FCCS, which is a more sensitive method for quantifying membrane protein density and dimerization. Specifically, the *fc* unit and total density were recorded for each cell at each time interval (T=0,5,10...30). The density is being measured directly using this technique in units of receptor per square micrometer. The total density is the sum of the density of AcGFP1-labeled CXCR4 and the density of mCherry-labeled CXCR4 for every single cell. The f_c unit value is also a key measurement in FCCS. The f_c unit value represents the fraction of the correlated proteins and is a measure of dimerization. The f_c unit for each cell at each time interval was plotted for all nine cells as shown in **Figure 4**. The total density was converted to normalized density for each cell and graphed over the time interval as depicted in **Figure 5**.

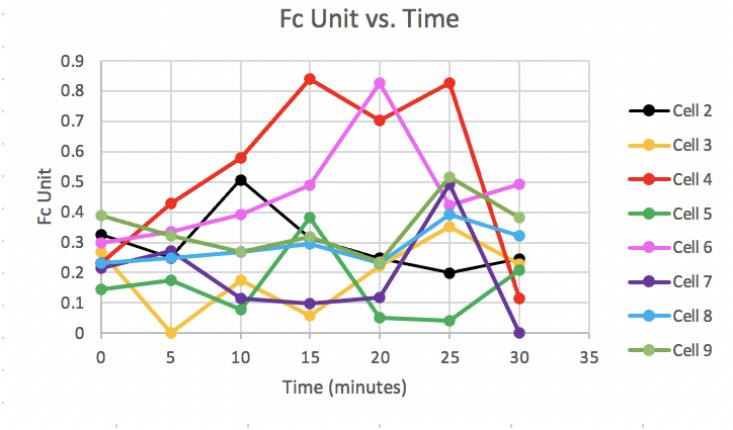

Figure 4. The graph depicts the FCCS data for all 10 cells based on the Fc (fraction of correlated proteins) unit value over time. There are a total of 8 cells that were observed.

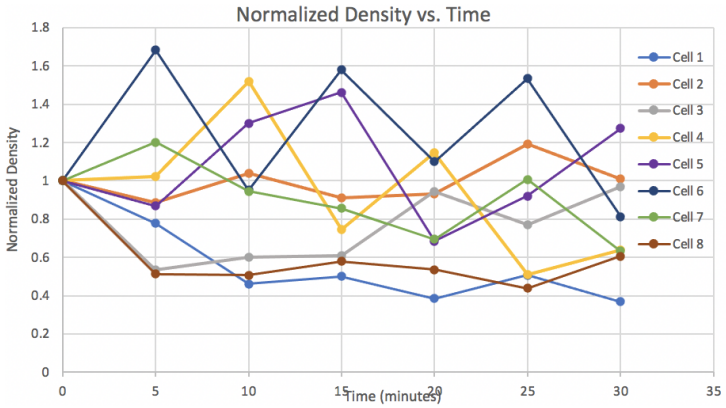

Figure 5. The graph depicts the FCCS data for all 8 cells based on the normalized density which was calculated from the total density of GFP and mCherry each cell over time. There are a total of 8 cells that were observed.

Finally, the total densities were normalized over the time interval for every single cell after the CXCL12 ligand was added. The trend observed was that there was a decline in normalized density of the cells. The standard deviations of each cell's normalized density are indicated with the error bars. The results demonstrate that the CXCL12 ligand changes the

density of CXCR4 at the surface of the cells as the time interval increases (**Figure 6**). It is important to note that these density changes from the PIE-FCCS are small, which is why there weren't detectable in the images.

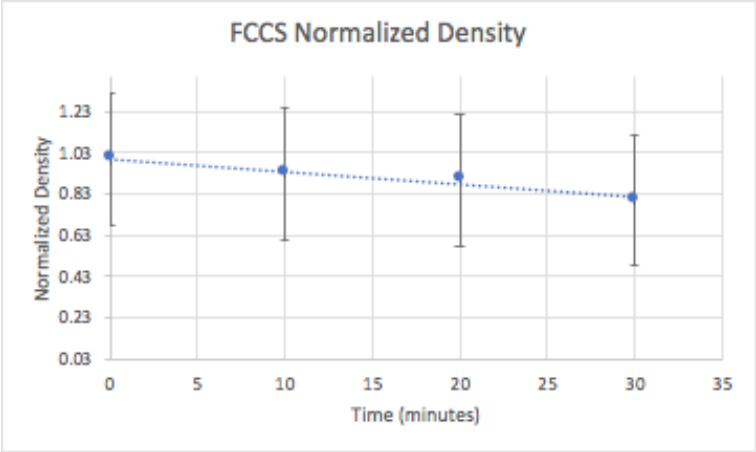

Figure 6. The graph portrays the normalized total density of each of the cells from the FCCS over the time period. It is clear that the ligand CXCL12 created a decrease in cell density over the time interval.

CONCLUSION

The purpose of this experiment was to determine how the CXCL12 ligand affects CXCR4 internalization in living cells. Though the fluorescent cell imaging results were insignificant, the FCCS data affirmed that density is in fact impacted by the ligand. There is a general decrease in the total density of the cell in the GFP and mCherry channels as shown in the results section. It is important to observe that the changes in density is small, hence more experiments must be conducted to confirm these results. The internalization of CXCR4 receptor is thereby affected and that could be an important regulator in how the signal transduction occurs. Overall, I learned the importance of receptor dimerization in lieu of ligand binding as this plays a crucial role in transcriptional activation and efficient DNA binding. Also, dimerization

could be important in the development and screening of drugs that act through supramolecular assemblies of receptors [4].

Specifically, changes in CXCL12 ligand binding could dictate how homo-dimerization of CXCR4 receptors was affected and could lead to more drug testing for advancements in the pharmacological industry. We have found from the results presented in the experiment that ligand binding has caused small changes in the density of the cell as we found that density decreased slightly over the 30-minute interval. The next steps of this should be to analyze exactly what signaling pathways and mechanisms are impacted by this ligand in particular and how that alters the processes of the cell or the expression of the CXCR4 receptor.

For further studies, potentially imaging should be repeated, and more single cell images should be taken for more improved results. We should focus on automated imaging techniques for increased accuracy. We could also add antagonists and observe how that affects internalization and dimerization of CXCR4 membrane receptor. That could also effectively convey more information of how the dimerization of the CXCR4 receptors and the decoupling of the G-proteins would ultimately affect the signal cascade or the expression of proteins within the cell [5]. This could tell us more information regarding the functionality of the receptor and will aid with future drug development.

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