Spring 2019

Cloning the vision related G protein transducin for live cell fluorescence studies

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Course: 3150:497

Date: April 26 2019

Abstract:

G coupled protein receptors (GCPR) are one of the largest families of receptors and mediate a variety of biological responses. Rhodopsin is the largest family and aids in sight, the α-subunit of the GCPR complex in extremely important to the activation and downstream signaling effects of GCPR. The α-subunit contains a small trans-domain portion and in this project the sequence of that portion will be inserted into a vector containing a fluorescent tag. These vectors will then be used to collect fluorescent cross correlation spectroscopy or FCCS data. The unit was cloned using assembly methods that include PCR and purification techniques. The vector containing the transducin α-subunit is then transfected into live Cos-7 cells. Images of the cell can then be taken and FCCS data can also be collected. This will give insight to the dimerization and localization of the subunit in real time live cell experiments. GCPRs are extremely important cell receptors and insights on how molecular interactions between GPCRs and other proteins can lead to new routes for drug discovery.

Abbreviations: GPCR: G protein-coupled receptor; Rho, rhodopsin; GDP, guanine diphosphate; GTP, guanine triphosphate; FCS, Fluorescence Correlation Spectroscopy; PIE FCCS, Pulsed-Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy; GFP, green fluorescent protein;

Introduction:

Guanine nucleotide binding protein (G protein) coupled receptors (GPCR) are not only one of the largest families of receptors found in biological systems but also mediate a large array of biological functions from sight to neurotransmitters.[1] In this study, rhodopsin is examined, the rhodopsin family is the largest of the five GPCR families. The family shares a few conserved sequence motifs that are important for sight and mutations in rhodopsin have been found to contribute to night blindness.[2] Within rod cells there are GPCRs in the discs that form the rod outer segment, with light activation in the transmembrane segment that contains a chromophore that undergoes photoisomerization.[3] The light activation is by a single photon, causing the receptor, Rho, to undergo a conformational change as seen in Figure 1.[4] The conformational change allows for the light signal to be amplified by the heterotrimeric transducin molecule, G_t, and then dissociate.[5] This conformational change is critical to the activation of the receptor and the subsequent signal transduction.

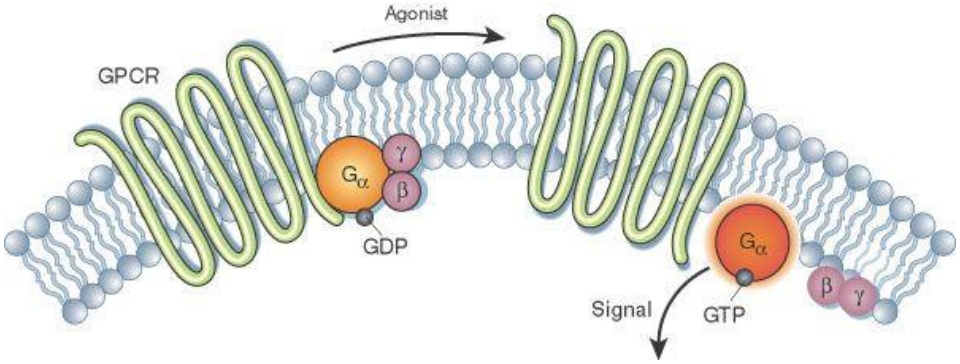

Figure 1: This image shows GPCR on the cell membrane and the α-subunit. This image helps to visualize the activation of the G_α and how the signal transduction works. From Li, J. et al. (2002) Nature 420, 716-717.

The alpha subunit (α-subunit, or G_α) is part of the heterotrimeric G proteins that associate with GCPRs when they are activated. The G_α subunit anchors to the plasma membrane and is important in the binding of GTP or GDP and specifically the amplification of the light signal in rhodopsin. The activation of the rod receptors is related to rhodopsin clustering, which could lead the receptors to localize to particular parts of the membrane effecting the kinetics of the receptor G protein interactions. In a recent study it was speculated that at high levels of illumination would reduce the availability of G_α to activate, this could lead to problems with eye sight.[3]

The G_α subunit is encoded by the gene GNAT1. This portion of the heterotrimeric G protein molecular interacts with the membrane in order to anchor the inactive G_α subunit that is bound to GDP. The molecule uses a N-myristoyl glycine on position 2 as a lipidation site to bind to the membrane.[6] The heterotrimeric transducin domain also contains the binding site for GTP or GDP depending on the activation state of the receptor. As mentioned earlier, light absorption will activate conformational changes in rhodopsin that leads to the binding and activation of transducin to switch out GDP for GTP at the G_α subunit then dissociate from the rest of the

subunits. It has also been found that gene GNAT1 is highly conserved among different species and a missense mutation is often found in congenital stationary night blindness (CSNB).[7] This mutation effects the activation of the receptor and the conformational changes needed to activate the signal. Figure 2 shows the structure for the G_α transducing domain complexed with GTP, this means that the protein is activated and able to dissociate from the membrane.[8]

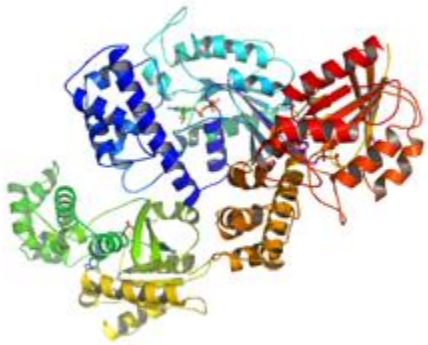

Figure 2: In this figure the structure of the G alpha transducing domain is shown. This figure was created using the mmCIF file found on rcsb.org and software PyMOL. The code for the structure is 1TND.

In this study G_α interaction with rhodopsin will be examined with the technique called fluorescence correlation spectroscopy (FCS) and pulse-interleaved excitation fluorescence cross correlation spectroscopy or PIE FCCS.[9] PIE FCCS uses fluorescently labeled molecules to determine dimerization and activation of membrane bound receptors, this allows for quantification of rhodopsin interaction in situ. This method works by using time-correlated photon counting with fluorescent imaging using custom developed equipment. With PIE FCCS one can determine the protein concentration, mobility, and clustering.[9] This clustering can be analyzed and the order can be determined to be either a monomer, dimer, trimer, or oligomer. This is an extremely powerful and accurate tool since the experiments can be done in live cells without the need to fix the cells. For FCS, a single fluorescent molecule is examined, while with PIE FCCS a two-color fluorescence is evaluated. The spatial organization of the rhodopsin receptor in a dimer or higher order oligomer could affect the transduction of the light signal. The analysis of the data takes into account the different combinations of the labeled proteins that are dimerizing or forming higher order complexes on the membrane.

In this experiment I will create a clone of the transducing Gα domain that will be inserted into a vector containing a GFP tag that will allow for FCS and FCCS data to be taken on the dimerization and activation of rhodopsin. The vector will be created by means of two different methods, one is a subcloning method with an

insertion into the relevant expression vectors while the second method is purchasing a plasmid already containing the GNAT1 gene and a GFP tag. This will allow us to measure the interaction strength and kinetics of G_α and rho.

Materials and Methods:

Constructs:

In order to insert the gene of interest into our florescent vectors, sub-cloning was done with the NEBuilder HiFi DNA Assembly kit. The gene of interest was purchased from AddGene, it was XE101 alpha transducing CS2+ (catalog number 16681). Primers were then designed so that half of the primer would overlap with the gene of interest (GNAT1) and the other half would overlap with the insertion vector. The forward primer being TGAACCGTCAGATCCATGGGGGCTGGGGC and the reverse primer being CGACTGCAGAATTCGTCAGAAGAGGCCACAGTCTTTGAGGTTCTCC with an annealing temperature at 72. The underlined segment of the primer is the base coding for the GNAT1 gene that was in the vector provided by AddGene. The primers where then ordered from GeneWiz. First our empty vectors, pAcGFP1-N1 and pAcmcherry-N1, were digested with Nhel (NEB) and HindIII (NEB) to linearize it for easier amplification during the PCR steps. After each digestion the plasmid was purified to remove any impurities. The vector bought from AddGene containing the gene of interest was also digested with Hind III and Nhel.

After purification, the GNAT1 vector was then amplified with the overlapping primers using PCR. The PCR protocol was as follows: a hot start at 98°C for 30 seconds, 98°C for 10 seconds to denature, 72°C for 20 seconds to allow for annealing, then one minute at 72°C, these three steps are repeated 30 times. Then the mixture is held at 72°C for two minutes and then the PCR product is held at 4°C indefinitely. The product was then ran on a gel (agarose from Bio-Rad)to separate out the product containing the gene from linearized vectors or primer dimers. The NEBuilder HiFi DNA Assembly master mix is then added to a solution containing the PCR product and the pAcGFP1-N1 vector to anneal the DNA back together. This was then purified. The assembly product was then transformed to competent E. coli, DH5α, and plated and allowed to grow for twenty-four hours. Individual colonies were picked and added to LB broth to grow up for twenty-four hours. The vector was then purified using NEB's MiniPrep Kit. The purified DNA was then sequencing to confirm there were no mutations within the gene. The DNA was then transfected into Cos-7 cells with Lipofectamine 2000 (Life Technologies) to check expression. A second construct was ordered from GeneScript that contained the GNAT1 gene in a pcDNA3.1+C-eGFP vector.

Cell Culture:

For the live cell experiments we used Cos 7 cells were obtained from ATCC (CRL-1651). COS-7 cells are primate kidney cancer cells, they are used in this experiment because they are easy to culture and have the correct shape to be used for FCCS experiments. The cells were cultured in a mixture of Dulbecco's modified Eagle's medium, DMEM, (DMEM 1X +GlutaMAX, Life Technologies, Carlsbad, CA), 10% fetal bovine serum, FBS, (Life Technologies, Hyclone, Logan, UT), and 1% antibiotics penicillin/streptomycin (BioReagent, Sigma-Aldrich, St. Louis, MO). The cultures were incubated in a cell only incubator at 37°C with CO_2 at about 5% to 10% in 100% humidity. The cells were grown on 100x20 mm tissue culture plates (Falcon, Corning Inc., Corning, NY). When the cell cultures were split they were at 80% to 90% confluency. The cells were split using trypsin (Life Technologies) warmed to 37°C. The Cos 7 cells were transfected with the construct twenty-four hours before imaging. Transfection was done using Opti-MEM I medium and Lipofectamine 2000 transfection reagent (Life Technologies). Before images and data are collected on the cells, they were washed twice with 1X PBS and the media was be changed to Opti-MEM I media.

Cell Staining:

The cell staining kit (Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit – 134406) comes with two different stains, a red one for the plasma membrane and a blue one for the nucleus. The Alexa Fluor 594 binds selectively to N-acetylglucosamine and N-acetylneuraminic on the plasma membrane. The Hoechst 33342 dye which is a cell-permeant nucleic acid that is selective for DNA. The first step to stain the cells is to prepare a 1.0 mg/mL Alexa Fluor 594 WGS stock solution from the image-IT LIVE Plasma Membrane and Nuclear Labeling kit (134406) from Invitrogen Detection Technologies. The Alexa Fluor stock solution is then diluted 5.0 μg/mL and the Hoechst 33342 stain to a concentration of 1-2 μM. The cells that were previously transfected with the GFP GNAT1 plasmid was washed with PBS twice and 2 mL of imaging media was added to the well. Then a sufficient amount of labeling solution was added to the cells and allowed to incubate for 10 minutes. After the incubation the labeling solution was washed off with PBS and the cells were rinsed twice with PBS.

Live Cell Fluorescence Imaging:

The hOps-mCh / GNAT1-GFP images were taken on a customized Nikon Eclipse Ti inverted microscope. This microscope contained an interleaved excitation and time-correlated single-photon detection to be used for the FCS data collection. After the transfection with the plasmids and a 24-hour incubation period, the DMEM media was aspirated off and the plate was washed twice with PBS. After washing, imaging media was added and the plates where brought to the microscope for imaging. After the microscope was aligned, the

cells were mounted, and the well was searched for cells containing both fluorescence plasmids using a grid pattern. Both plasmids were checked by switching the fluorescence lenses. The images were captured using a Texas Red filter cube with a wavelength of 630 ± 75 nm and an eGFP filter cube with a wavelength of 525 ± 50 nm. The cells were kept on a 37° C on an incubator attached to the microscope. Cell staining images were taken using the Olympus fluorescence microscope in Dr. Paruchuri's laboratory. After the staining protocol was complete the cells were mounted on the microscope and images were taken of the difference fluorophores and then overlapped for comparison.

Results and Discussion:

Expression and Trafficking:

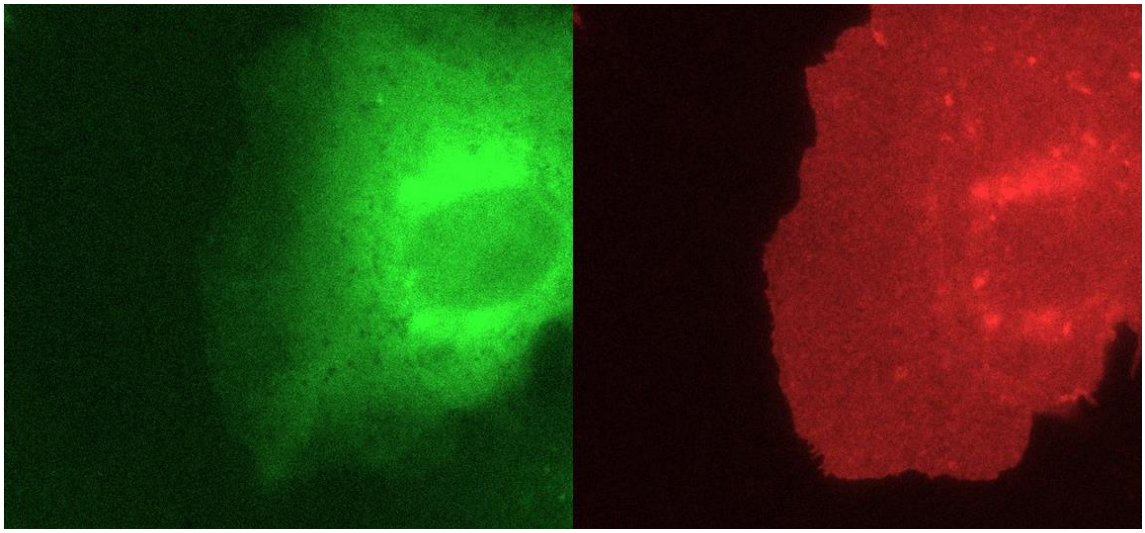

Figure 2: In this figure there are two different colored constructs being expressed in the COS-7 cell. The green GFP construct (GNAT1) is localizing slightly to the membranes but not as well as the red hOps-mCh (mCherry construct).

There was some trouble in creating the first plasmid, after sequencing different colonies of the $G_{\alpha t}$ plasmids there was only one that did not contain a mutation. The mutation was an additional G at the N terminus of the plasmid after the start codon. This could be because there was a sequence of multiple guanines that can lead to multiple issues, like secondary structures and very high annealing temperatures.[10] After a plasmid was successful created, the plasmid was transfected into the COS-7 cells to image. In Figure 4, the COS-7 cells are co-transfected with hOps-mCh and the $G_{\alpha t}$ plasmid. As seen in the Figure, there was very little protein being expressed on the very edges of the membrane. This could be due to multiple reasons, one being that the twenty-four-hour timing of transfection might not be the correct time period to see the protein on the membrane of the cell. Another idea could be that there was just not enough protein localizing on the membrane, to help solve that, one option is to add a lot of plasmid during transfection. The idea being that the more protein being made, the more protein will end up on the membranes of the cell that can be used for data collection.

The COS-7 cells seen in Figure 5, are also a co-transfection of the hOps-mCh plasmid and the GFP-GNAT1 plasmid. In the panel to the left, it is just the $G_{\alpha t}$ plasmid visible, the middle panel contains only the hOps-mCh plasmid visible, and the right panel shows an overlapped image of the two plasmid images. In the overlapped image the yellow color indicated that both the green and the red plasmids are overlapped in that particular location within the cell. This shows that there is some overlap between the plasmids but not enough of the GFP $G_{\alpha t}$ at the edges of the cell to provide accurate imaging data and diffusion data.

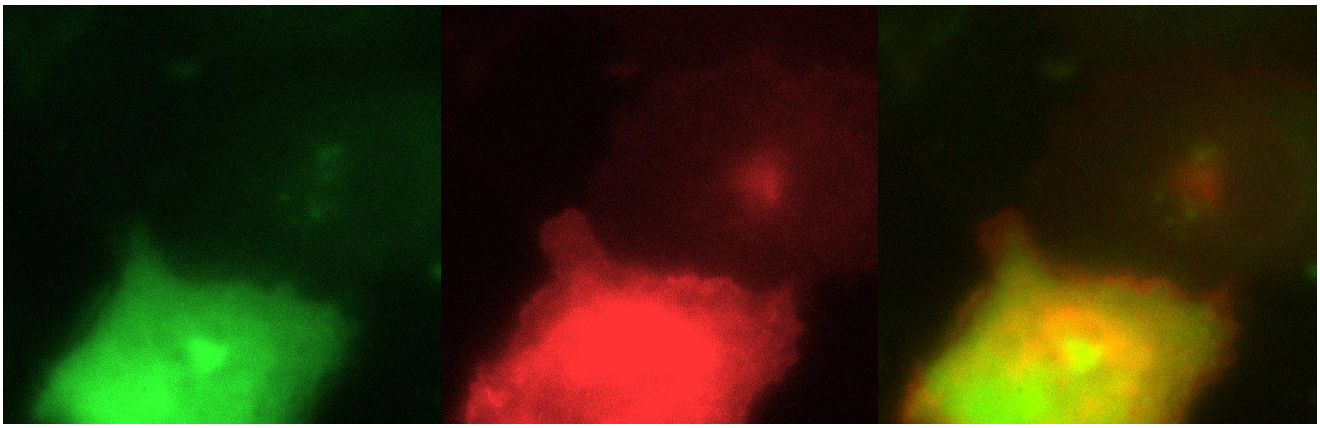

Figure 3: This figure shows three different panels, the one on the most left only shows the GFP GNAT1 plasmid, the middle panel shows the hOps-mCh plasmid, and the right panel shows the overlap of the two plasmids. This shows that the green GFP GNAT1 plasmid is not localizing to the membrane as well as the red hOp-mCh plasmid.

Confocal Staining:

Initial transfections showed that there was some localization of the construct to the membrane but not enough to provide good statistical analysis of the localization and dynamics of the $G_{\alpha t}$ protein. Originally it was thought that perhaps the $G_{\alpha t}$ protein was localizing on the nucleus membrane or stuck on the Golgi apparatus during post-translational modifications. In order to look at where in the cell that the $G_{\alpha t}$ protein might be localizing, a fluorescent dye kit was used to look at the nucleus and membrane. The Alexa Fluor 594 binds selectively to N-acetylglucosamine and N-acetylneuraminic on the plasma membrane. The Hoechst 33342 dye, which is a cell-permeant nucleic acid, is selective for DNA. The images taken of the COS-7 cells are transfected with the GFP $G_{\alpha t}$ plasmid. As seen in Figure 5, the plasma membrane is stained red while the nucleus is bright blue. The image shows that there is some of the $G_{\alpha t}$ plasmid at the membranes of the cells, but a lot of the plasmid is dispersed within the cell but not localized on the edges of the membrane

Since the sequence of the plasmids was confirmed there could be other reasons why the $G_{\alpha t}$ plasmid is not localizing to the edge of the cellular membrane as it should. One way to test this is to transfect the COS-7 cells with more plasmid then the previous calculated concentrations, this could allow for more protein to be sent to the membrane. Another speculation is that the $G_{\alpha t}$ is not being trafficked to the plasma membrane, it could need to be co-transfected with another proteins that traffics the G_{α} subunit to the membrane. A protein of interest to perform a double transfection with is human protein UNC119. This protein binds to the myristoyl moiety found on the N-terminus of proteins and localizes them to the membrane of cells.[11] These two options are the next steps of the project that will be continued by the Smith lab after my graduation.

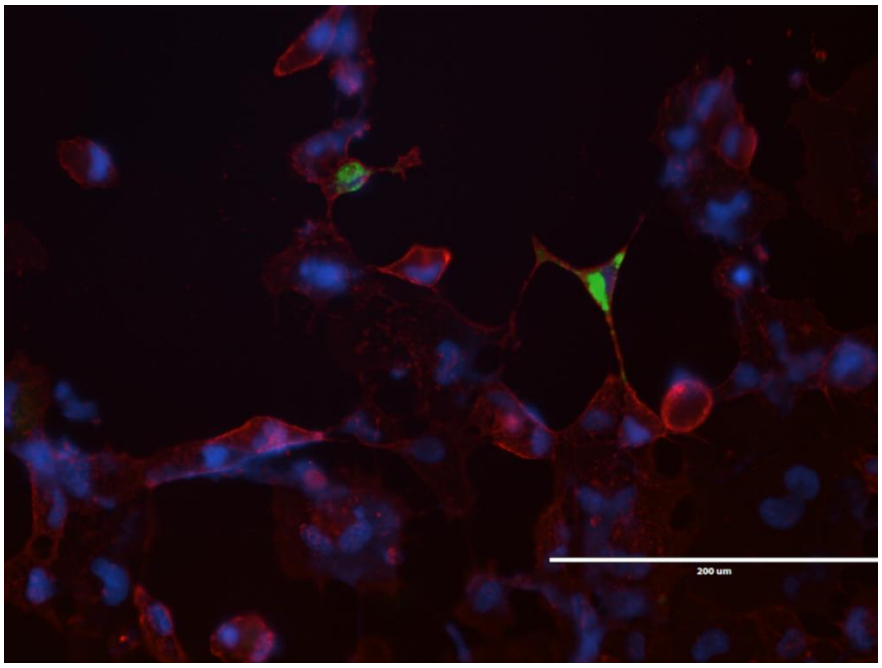

Figure 4: In this figure, the plasma membrane is stained with a fluorescent red color, the nucleus is stained blue, and GNAT1 is visible in the GFP green color. This is to see where the $G_{\alpha t}$ is localizing within the cell.

Conclusion:

The purpose of this project was to create a plasmid that contained the GNAT1 gene that codes for the $G_{\alpha t}$ subunit of rhodopsin. This protein is a point of interest as it plays an important role in the signal transduction of rhodopsin giving sight. Mutations in the rhodopsin GPCR have been implicated in a number of different common sight diseases, like night blindness. A plasmid containing the GNAT1 gene was successfully made but there was a problem with the localization of the $G_{\alpha t}$ protein on the edges of the cell membrane. To look further into this, cell staining was done to determine the localization of the protein within the cell. The cell staining did not lead to any conclusive answers, the next steps are to modify the concentration of plasmid added during transfection to see the effect on the localization of the protein. If additional protein does not help with localization, then a co-transfection with the trafficking protein UNC119 will be attempted. This protein should

help traffic the $G_{\alpha t}$ protein to the edges of the cells. After the protein is successfully trafficked to the membrane a mCherry construct containing the GNAT1 gene will be created. These plasmids with then be used with different constructs that contain different parts of the rhodopsin receptor to see the effects on protein dynamics and dimerization. Mutations may be introduced to the GNAT1 gene to see the effect on the protein. After the creation of all of the plasmids they will be used to collect FCS and FCCS data.

Acknowledgments:

I wish to thank all members of the Smith laboratory where training and materials were provided. Data and processing for all FCS and PIE FCCS experiments and the data figures were done by the University of Akron student D. Paul Mallory. The funding and equipment were provided by Dr. Adam W. Smith of the Chemistry Department at the University of Akron. This research was supported by the National Science Foundation under grant number CHE-1753060 and the National Eye Institute of the National Institutes of Health under award number R15EY024451.

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Appendix 1: Safety Concerns

Laboratory Training: Laboratory equipment and protocol training were provided by the University of Akron graduate students: D. Paul Mallory, Shaun Christie, and Grant Gilmore and graduate William D. Comar. Scientific training included mammalian cell culture, transfection protocol, transformation, DNA purification, and other laboratory molecular biology experiments. This experiment was done under the work of graduate student D. Paul Mallory and he conducted PIE-FCCS and FCS experiments, collected, and analyzed data.

Biohazard Safety:

While working in the lab, gloves were worn at all times with biological substances. Sterile protocol was followed while working in the sterile room with the mammalian cell cultures. All materials that encountered any biological substances, like glassware and pipette tips, were discarded in sperate biohazard waste containers. All liquid biological waste was treated with bleach to prevent any growth and was then disposed. All containers were disposed of by the Department of Environmental and Occupational Health and Safety.