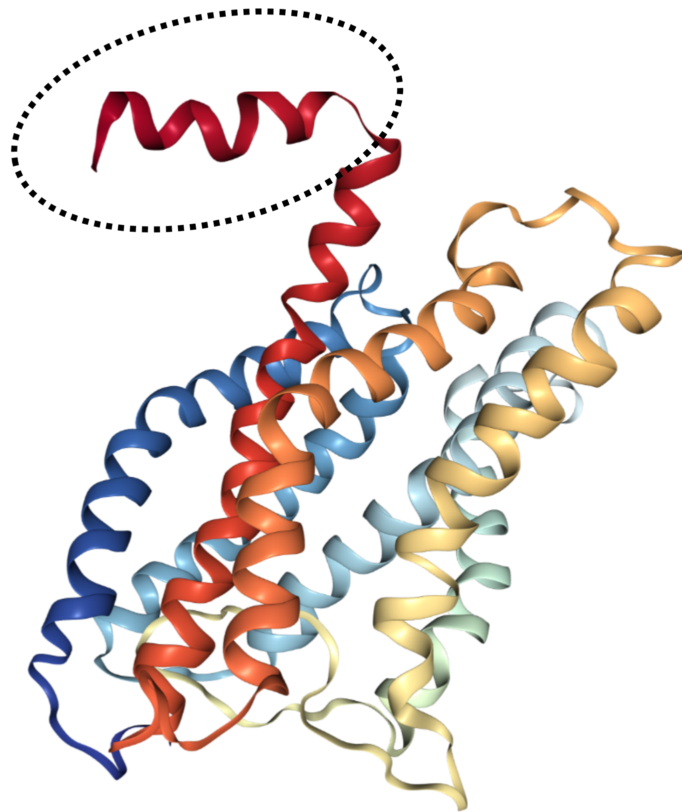
Gliadin Cuff

The Gliadin Cuff Understanding the human receptor of Gliadin was the first step for this cuff as we needed to see what we could use as a 'cuff' to sequester the Gliadin. The first thought was to utilize nano-bodies however the library I had sought out from U-Lethbridge didn't have one that I could use. There was a full-length antibody however since E. Coli can't create full antibodies so we had to explore other avenues. Further research revealed that a receptor called **CXCR3** interacted with Gliadin and played a part in the autoimmune response in celiac patients. CXCR3 is a G-protein coupled receptor (GPCR) or a 7-transmembrane receptor which mean that there could be some problems incorporating those transmembrane domains into a gram-negative *E. coli.* Since E. coli is often used as an expression vector for GPCR crystallization experiments we understood that CXCR3 was easily expressed by *E. coli*. However, there were a few solutions to the transmembrane domain problem as we consulted our advisors and they suggested using solely the extra cellular domain as the receptor as we don't require the signal transduction part which includes much of the receptor. Now that we decided the methods we were going sequester gliadin we now had to find a way to anchor the receptor on the surface so it could do its job.



Circled portion is Extracellular Domain

Source: pdb.org

Figure 1: CXCR3

The research led to many ways to anchor and express this receptor in significant enough quantity so have an effect at gut concentrations of gliadin. Designs **0.0.1, 0.0.7, 0.1.1, and 0.1.7** use the truncated CXCR3 and all other designs utilizes the full-length receptor. The first seven designs utilized FLAG or His-Tags since they are cheap, small and have a high specificity. Designs **0.1.1, and 0.1.7** also were the designs that were decided to be the most sound, easiest to test, most cost effective and were therefore our primary designs.

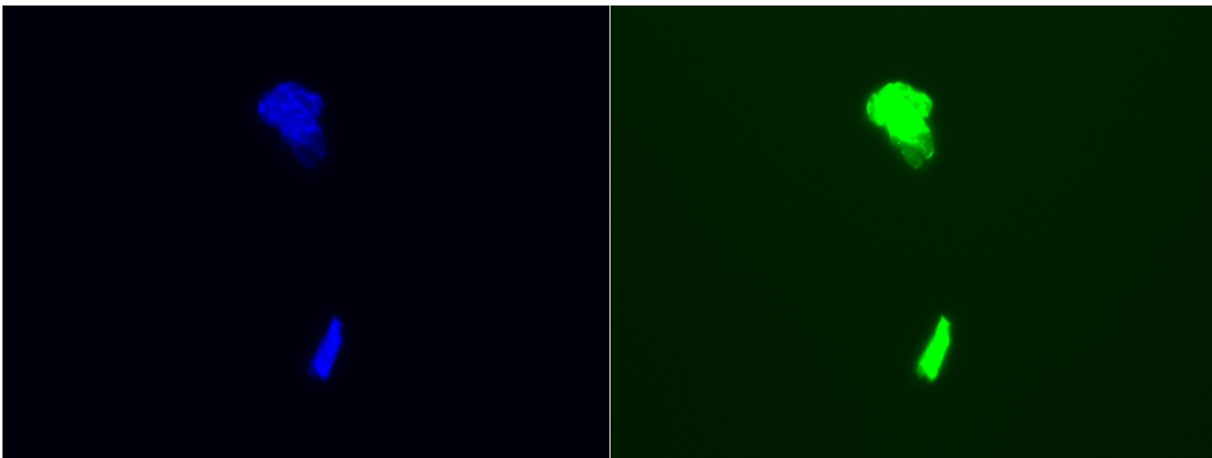
0.0.1 *(OMPa - Trunc. CXCR3 - 3xFLAG)* utilizes a protein called OMPa which is a beta barrel structure that can transport fused proteins to the surface of an *E. Coli*. OMPa is also from a past iGEM team as well and we have improved the part by giving it the ability to bind to gliadin by fusing it to CXCR3. OMPa has shown to be effective with sfGFP and we are only using the extracellular domain of the receptor in this design since its anchored to the cell and a FLAG tag is used as well. **0.0.2** *(MBP – Linker – CXCR3 – 6xHis)* follows a design detailed in a paper that expressed GPCRs on the surface of *E. coli*. Maltose Binding Protein (MBP) acts as a solubilization agent to avoid inclusion bodies and the linker was to aid with folding of the receptor. However, this design was much larger than the others and the mechanism was not well documented therefore this was one of the risker designs. **0.0.3** *(PelB – Linker – CXCR3 – 6xHis)* the PelB leader sequence was pulled from the iGEM registry and has been shown to localize fused protein to the periplasm however it has not been shown to integrate receptors. The reason this was proposed was because it followed the same build as 0.0.2 which also has a protein that aids in periplasm localization (MBP) and this also faced the same problems as 0.0.2. **0.0.4** *(OMPa Signal seq – CXCR3 – 6xHis)*, **0.0.5** *(DsbA Signal seq – CXCR3 – 6xHis),* **0.0.6** *(PhoA Signal seq – CXCR3 – 6xHis)* all follow the same build with varying signal sequences that help localize the construct to the periplasm. Each of these signals originate from their respective protein that is typically localized to the periplasm in E. coli. These designs stemmed from 0.0.2 due to similar mechanisms as well. **0.0.7** (*BclA - Trunc. CXCR3 - 3xFLAG*) consists of a glycoprotein anchor, BclA, which presents the truncated receptor to the media. This would be test by utilizing the FLAG tag.

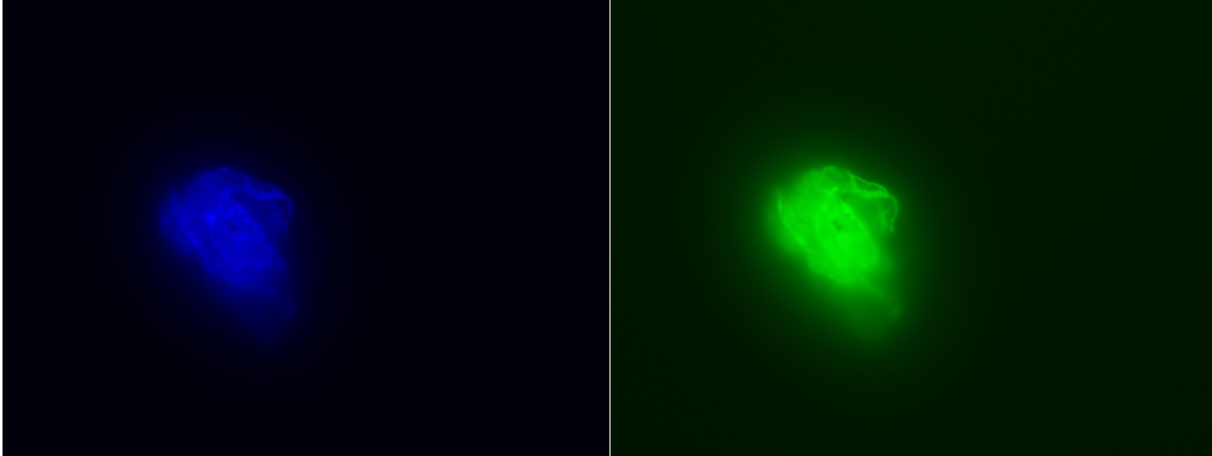
Our finalized designs utilized a florescent protein called mNeonGreen for some testing protocols. The circled designs were the ordered constructs but that does not mean it was tested and data was collected. These designs stem from the original designs and therefore have the same mechanisms albeit with different testing methods. The main reason to use a FP was that we could better visualize where the receptor localized by using florescent microscopy and were still able to measure binding efficiency by Bradford assay. **0.1.1** *(OMPa – Trunc. CXCR3 – TEV Site – mNeonGreen)* utilizes OMPa as the anchoring motif and the same truncated receptor to bind to the excess gliadin. mNeonGreen is a monomeric green-yellow FP which performs very well as a fusion protein. The TEV site was added as a contingency if the mNeonGreen adversely affected the binding capability of the trunc. receptor due to its much larger size. **0.1.2** *(MalE (aka MBP) – GS Linker – CXCR3 – mNeonGreen)* This design was not pursued further due to its large size due to the fusion of mNeonGreen. This design stemmed from a paper that detailed how they were able to express and embed functional GPCRs into the E. coli outer membrane. **0.1.7** *(BclA – Trunc. CXCR3 – TEV Site – mNeonGreen)* Design 0.0.7 was improved by utilizing a FP instead of a FLAG tag. The mechanisms in this design are the same but the main difference being the FP and testing parameters.

Gliadin Cuff Performance

<u>Confirmation of Gliadin Cuff Expression</u>

The gliadin cuff was designed with an mNeonGreen protein attached so that expression could be confirmed microscopically. This does not confirm that the Gliadin Cuff is attaching to the outer membrane, but does confirm that our bacteria expresses the coding sequence inserted.



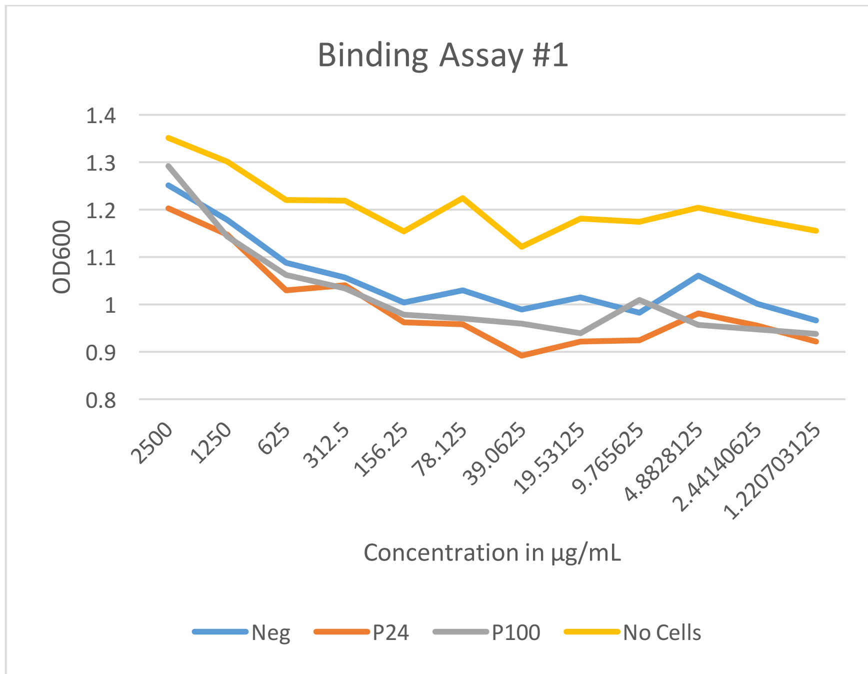


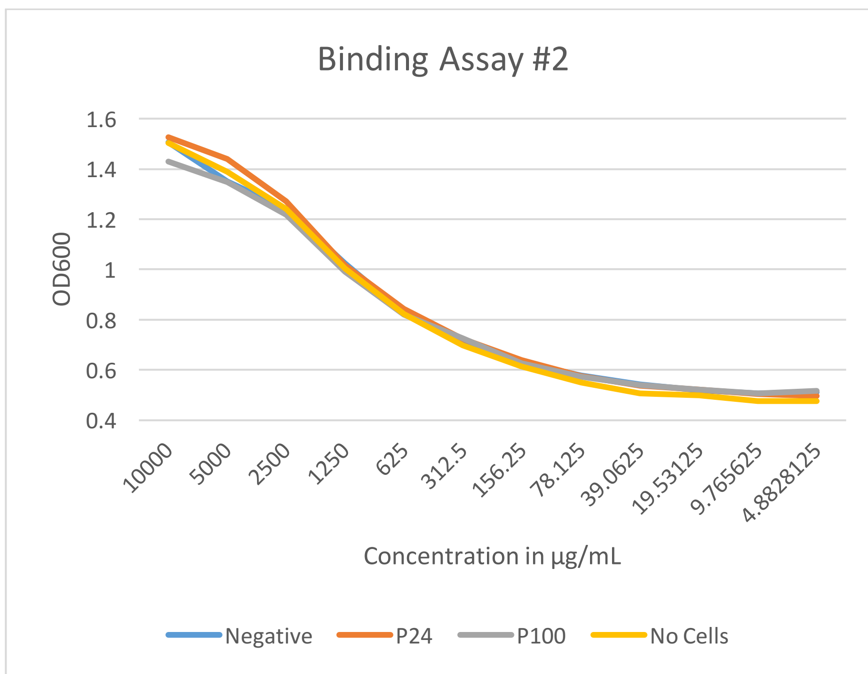
The above images show bacteria with a DAPI stain (in blue) and the same bacteria expressing green fluorescence. This confirms our cells are expressing the inserted coding sequences.

Gliadin Cuff Binding Assay

Gliadin is not water soluble and so stock solutions of gliadin in DMSO at 25 mg/mL and 100 mg/mL were made. Initially, multiple assays were conducted using PBS as a buffer. However, gliadin still has limited solubility in PBS and so different buffers were tested that could solubilize more gliadin while still not causing adverse reactions with the Bradford. Eventually, a PBS solution with 0.01% Triton X-100 was settled on as it appeared to solubilize the most gliadin and was still compatible with the Bradford reagent.

For each assay 2 plates were run and the values of each row of a device was averaged together to form the following graphs.





The first assay suggests that gliadin is binding nonspecifically. Meaning there was no significant different between OD values in a row that had cells with the gliadin cuff versus cells without the gliadin cuff. The second assay suggests no difference between rows with cells and without. Both assays suggest that the cells are not binding gliadin as expected. This could be caused by the cuff not being inserted onto the outer membrane or it could be that the protein is on the outer membrane but is misfolded. We are awaiting confocal microscopy to confirm whether the cuff is inserted onto the outer membrane.