Developing a FRET Assay to Study Protein Kinase A's Interaction with Smoothened Protein

Advisor: Dr. Rodrigo Maillard
Georgetown University Department of Chemistry

Background

PKA (Protein Kinase A) is an enzyme that regulates cellular processes by phosphorylating target proteins. PKA is composed of two subunits—Regulatory (PKA-R) and Catalytic (PKA-C) subunit. The binding of a signaling molecule cAMP to PKA-R releases PKA-C to phosphorylate.

A Smoothened protein (SMO) is a G-Protein Coupled Receptor which activates the GLI transcription factors during Hedgehog signal transduction. Hedgehog signaling pathway plays crucial role in embryonic development, tissue homeostasis and cell differentiation, including cancer. Previous research in the field has demonstrated that the activation of GLI transcription factors, one of the key steps of Hedgehog signaling pathway, is a result of SMO interacting with the PKA-C to block PKA-C's active site. This interaction would disable PKA-C's ability to phosphorylate or negatively regulate GLI transcription activity and thus activate GLI.

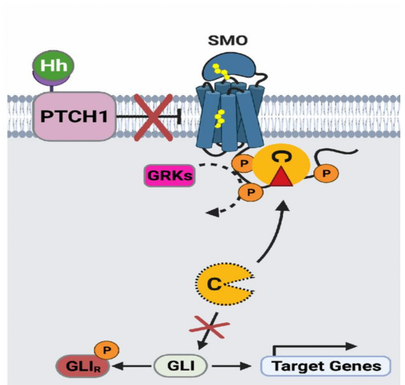

Figure 1. Schematic of the diagram of Hedgehog signal transduction.

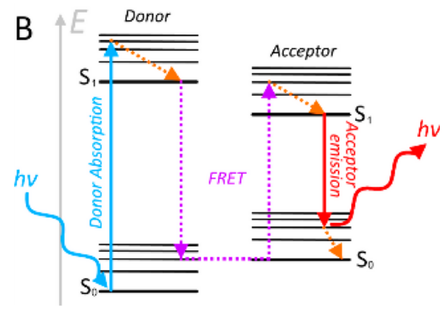

Figure 2. Diagram depicting FRET between the donor and acceptor.

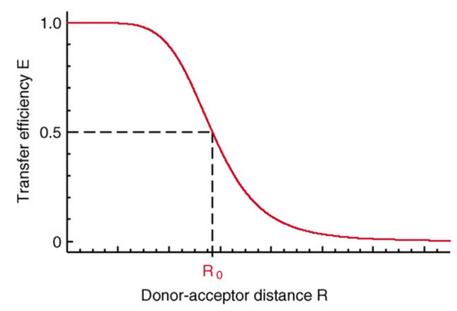

Figure 3. Graph that shows the correlation between FRET efficiency and distance between flourophores.

We developed a FRET (Fluorescence Resonance Energy Transfer) assay that can be used to observe the binding of PKA-C to SMO. FRET utilizes two fluorophores, where the donor excited to a high energy state transfers the energy to the acceptor; the FRET efficiency calculated is indicative of the physical distance between the donor and the acceptor molecules. We used Cy3 and Cy5 flourophores. Efficiency is calculated by dividing the intensity of the Cy5 (acceptor) over the total emission intensity.

Methodology

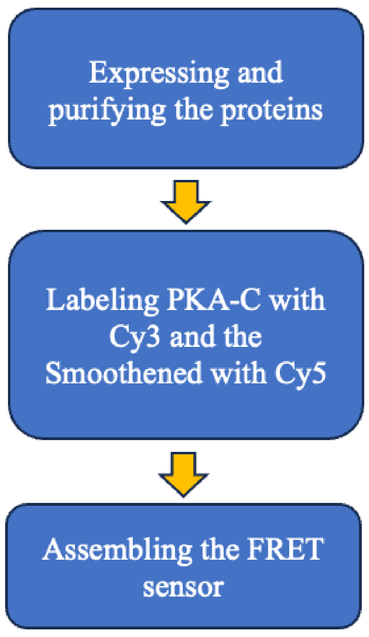

Figure 4. Schematic depicting the preparation process

We collaborated with the Myers lab in University of Utah who provided us with Cy5 labeled SMO. Experiments were conducted to observe an association between PKA-C and SMO using PTI spectrometer. 1 μm of Cy3 labeled PKA-C was titrated with Cy5 labeled smoothened ranging from 0.1 μm to 10 μm. Three trials were performed with the following parameters: λEX = 514 nm, λEM = 540-700 nm.

Results

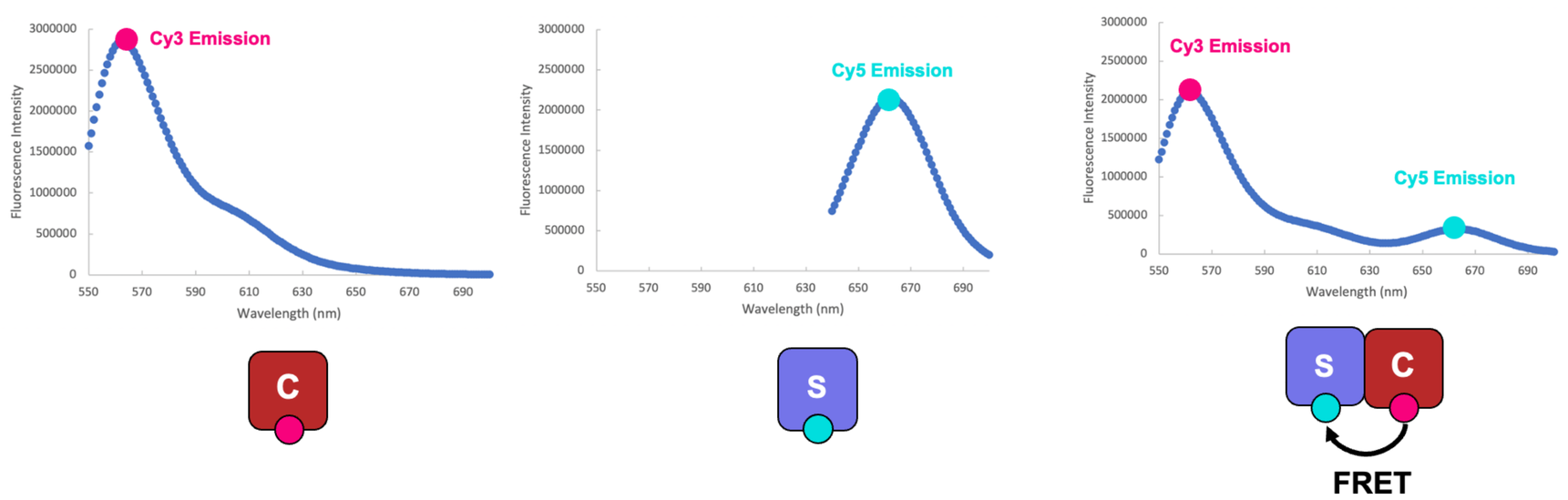

Figure 5. Graphs monitoring the PKA-C and SMO association with FRET. A sequential assay was performed where the intensity of Cy3 and Cy5 were monitored in 1μm PKA-C only (left), 10 μm SMO only (middle), and complex assembly of 10 μm SMO and 1 μm PKA-C (right). The average of three trials were graphed.

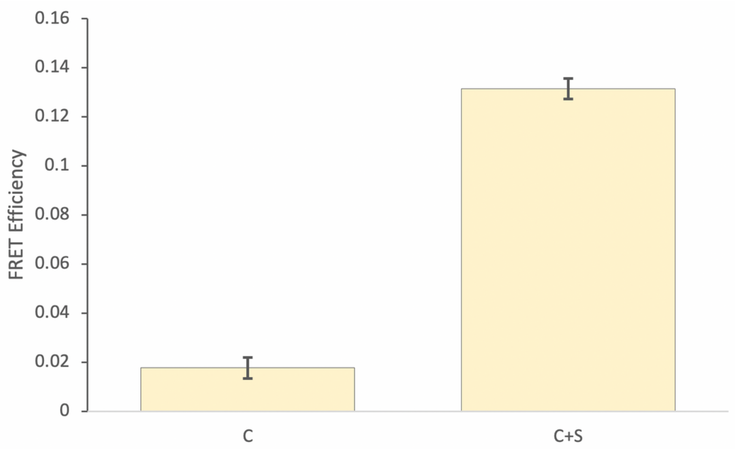

Figure 6. FRET Efficiency of PKA-C and SMO. The FRET efficiency was calculated from the intensity of Cy3 and Cy5 emission peaks with Cy3 being excited at 514 nm. The error bars are SEM and three trials were conducted.

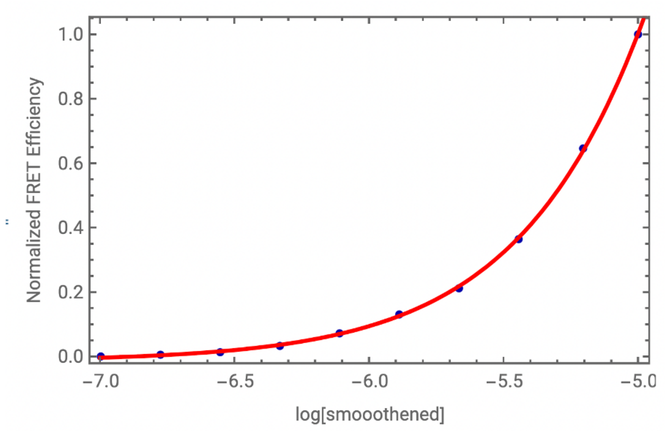

Figure 7. Monitoring the affinity of PKA-C and SMO through FRET. Cy5 labeled SMO was titrated into Cy3 labeled PKA-C and FRET efficiency was recorded for each sample ranging from 0.1 μm to 10 μm of SMO.

Conclusions

For the measurements of apo PKA-C and apo Smoothened, we do not observe a FRET occurring as there is only one peak. However, we observe a FRET occur when the Smoothened is added to PKA, the two peaks indicating that there is a binding affinity. Therefore, we can conclude that this FRET assay supports and can detect the PKA-C and SMO interaction.

For the titration measurements, we observe substantial changes in FRET efficiency as different concentration of the SMO is added. Therefore, we can conclude that this FRET assay can detect the binding process of the two proteins. However, we do see a low binding affinity between PKA-C and SMO, making it hard to detect a sigmoidal curve. Thus, in the future, we can modify this FRET assay to further observe the conformational change of smoothened as it binds to PKA-C by doubling labeling the SMO with Cy3 and Cy5. Understanding the mechanism of this interaction would provide valuable insights into Hedgehog signaling pathways, cancer biology and thus aid in developing future therapeutics.

Acknowledgements

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