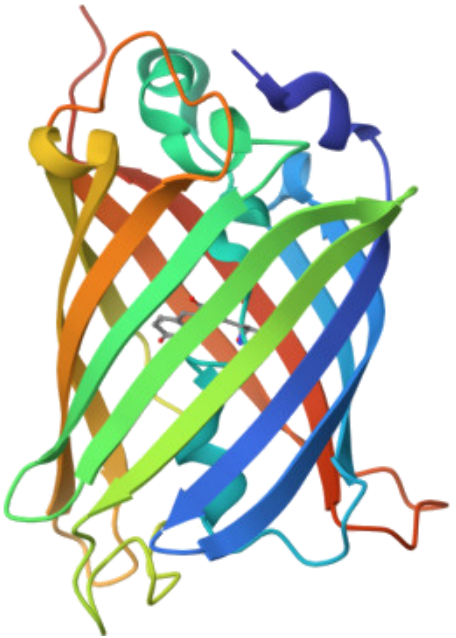
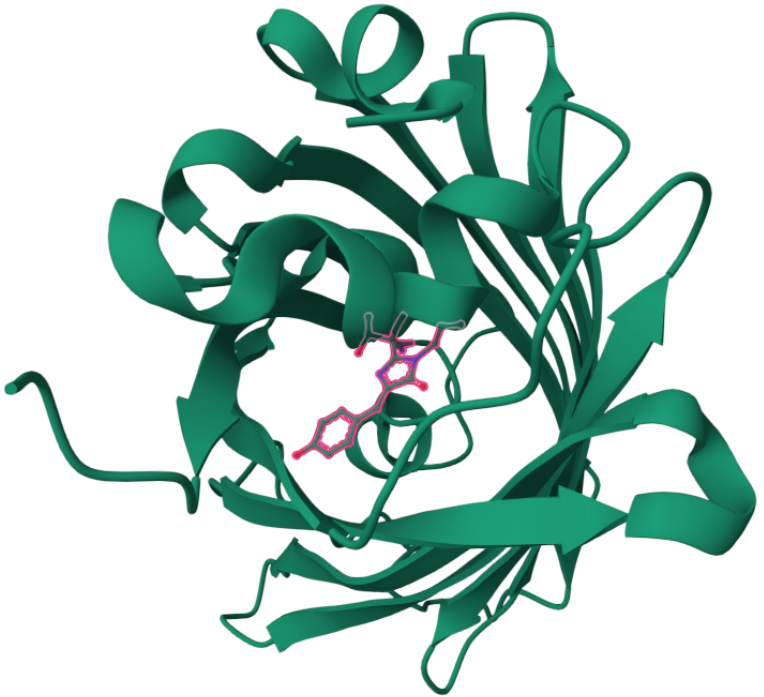
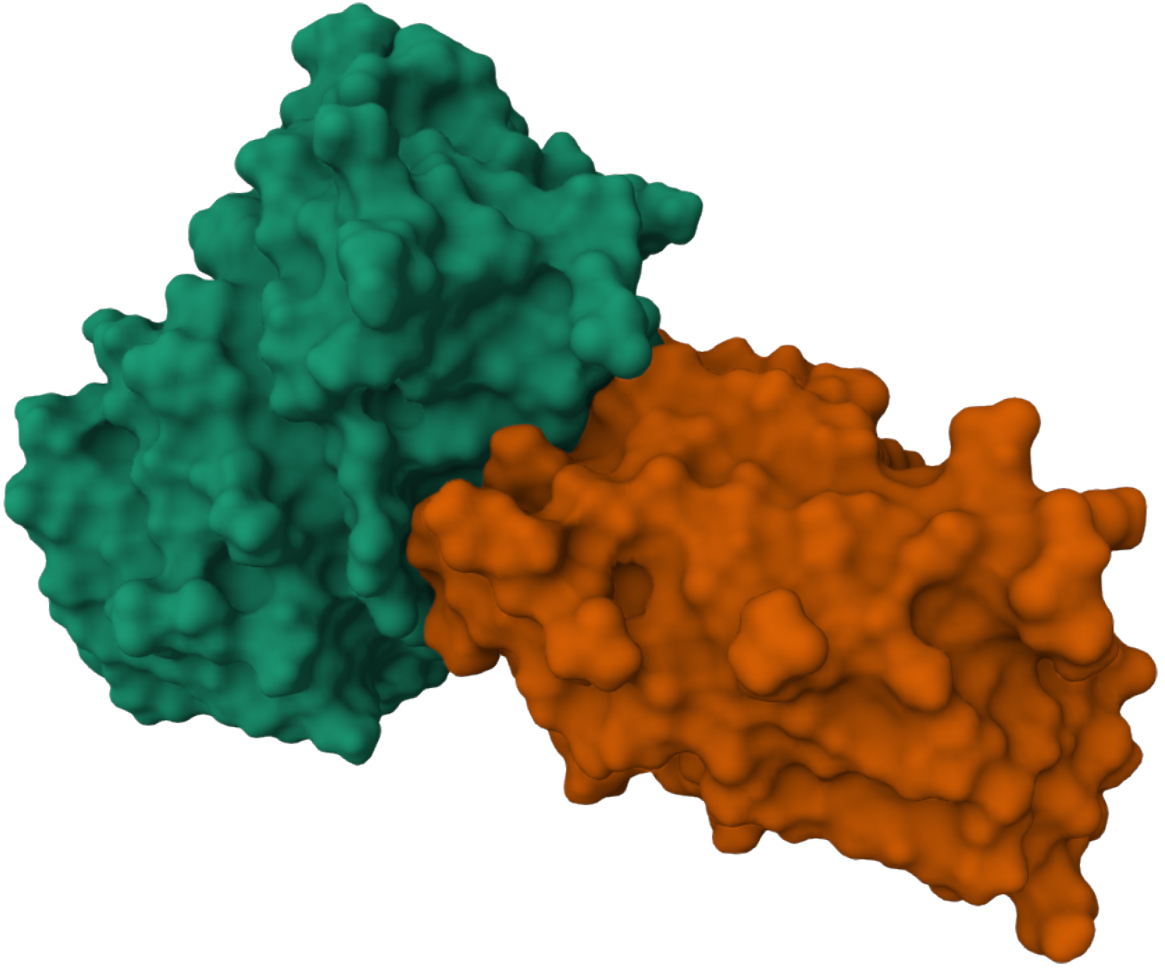
Superfolder Green Fluorescent Protein (sfGFP)





Orange
Nanobody Enhancer

Green:
Superfolder Green
Fluorescent Protein (sfGFP)



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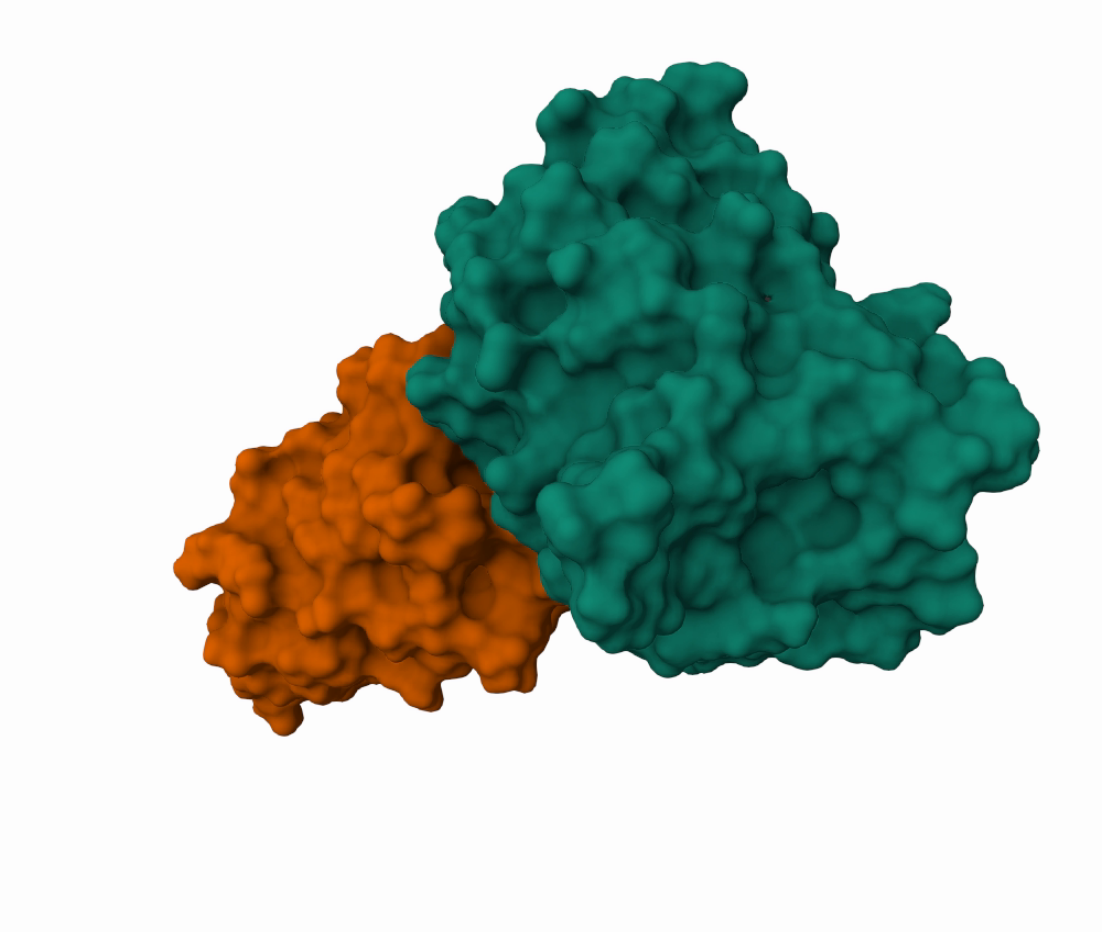

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

1) What do we need to learn?
2) Do we have individual reliable absorbance spectra?
3) What concentrations do we need to measure?
4) How do we get reliable molar extinction coefficients?
5) Are there any hypochromic/hyperchromic shifts upon binding?
6) What wavelengths need to be acquired?
7) How fast and long do we need to spin?
8) How do you interpret the molar mass of complexes? Need to know the molar masses of the individual species and their vbar values

How to design a reliable multi-wavelength AUC experiment:

Start by asking the right questions:

What do we need to learn?

- **What do our controls look like by themselves in the AUC?**
- **What are their molar extinction coefficients?**
- **What are their binding strengths (k_D)?**
- **What are their binding stoichiometries?**
- **What are the molar masses of each species? (expected: free and complexed reactants)**

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

Do we have individual, reliable absorbance spectra?

- Measure dilution series for each protein individually
- Determine the intrinsic molar extinction spectrum
- Overlay the two spectra to find regions of overlap and difference

DEMO

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

What concentrations do we need to measure?

- Make sure to stay within the dynamic range of the detector (0.1-1.2 OD)
- Decide if you are in the range of the k_D or much above or below
- This decides what type of experiment you can perform
- Use orthogonal information from ITC and MST to get ballpark k_D values
- Make sure you have enough protein
- Consider a titration experiment measuring multiple MW-AUC experiments

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

1) **How do we get reliable molar extinction coefficients?**
   - Rely on a protein that has a single, well known chromophore, with a calibrated molar extinction coefficient at one wavelength (e.g., aromatic amino acid side chains, a heme, or a fluorescent chromophore, and use that value to normalize the absorbance curve.
   - Calibrate the unknown molecule by forming a complex and measuring the concentration and mass of the complex to decide on stoichiometry

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

Are there any hypochromic/hyperchromic shifts upon binding?

- These are regions that change the shape or amplitude of a wavelength range upon binding
- Perform a titration experiment and normalize for concentration
- Overlay plots and visually inspect the sameness/difference for different ratios of ligands

DEMO

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

What wavelengths need to be acquired?

- Try to deconvolute a mixture of the two proteins to find the regions
- Find regions without hypochromic/hyperchromic shifts in the spectrum
- If there are shifts, determine their magnitude and determine in
- Minimize the wavelengths needed
- Use the most distinct wavelength in spectrum
- Maximize the angle between the spectral vectors using this formula:

$$\theta = \cos^{-1}\left[\frac{u \cdot v}{\|u\| \cdot \|v\|}\right]$$

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

How fast and long do we need to spin?

- Optimize number of scans for each wavelength against resolution
- Faster sedimentation gives better resolution, but reduces # of scans before pelleting
- Simulate with the UltraScan software to get best combination
- Molar mass determination also requires good diffusion signal, which is enhanced in slower speed.
- Decide on the best compromise

How to design a reliable Multi-wavelength AUC experiment:

Start by asking the right questions:

How do you interpret the molar mass of complexes? Need to know the molar masses of the individual species and their vbar values.

- Calculate the weight average partial specific volume of the proteins for a range of stoichiometries (using the solution tool in UltraScan)
- Plot the expected molar masses from s and D values for the complex when using the expected molar ratios vbar values
- Which molar mass/stoichiometry gives a matching molar mass when adding the individual molar masses? This is the key.