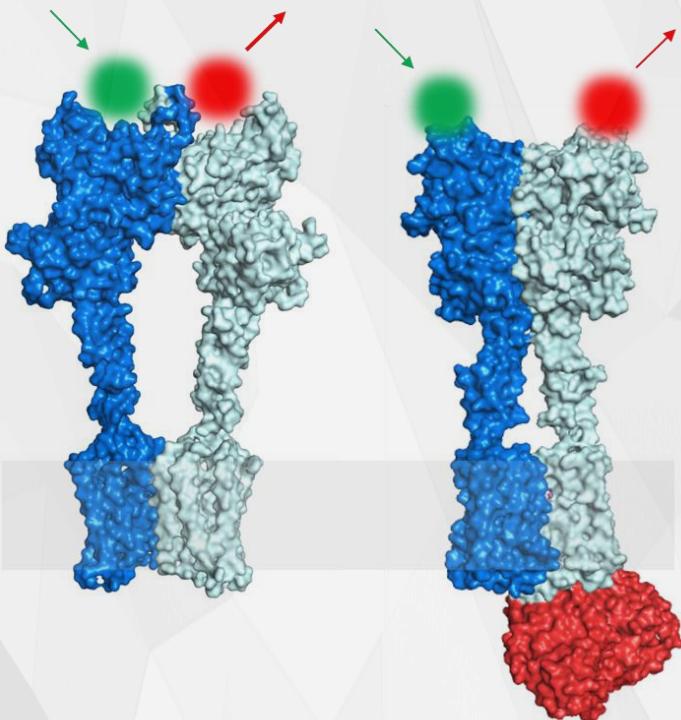


INTRODUCTION TO SINGLE MOLECULE FRET



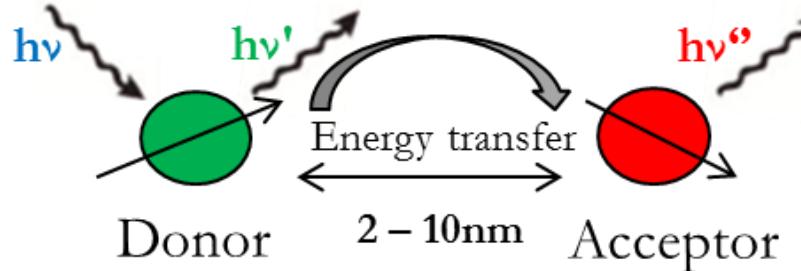
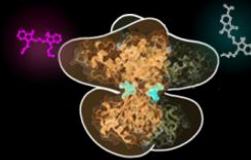
Emmanuel MARGEAT

Team Integrative Biophysics of Membranes

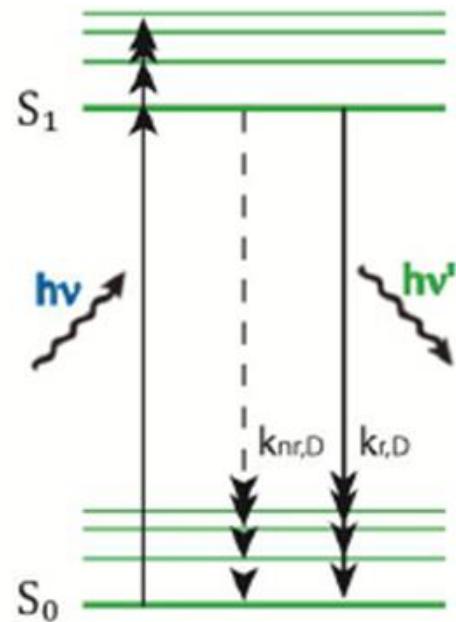
Centre de Biochimie
Structurale
Montpellier



FRET: Förster Resonance Energy Transfer



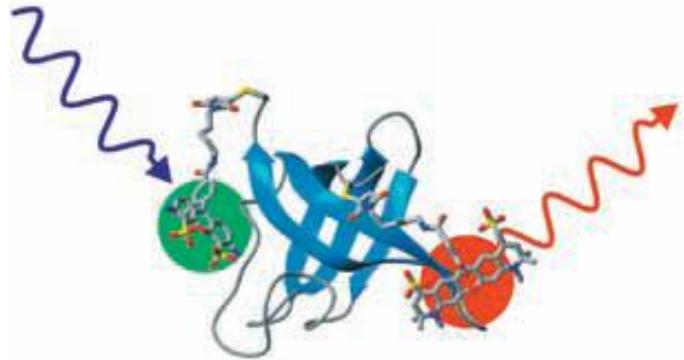
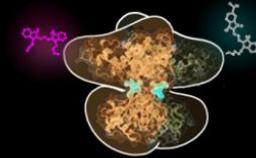
$$FRET(PR) = \frac{\text{Red signal}}{\text{Green signal} + \text{Red signal}}$$



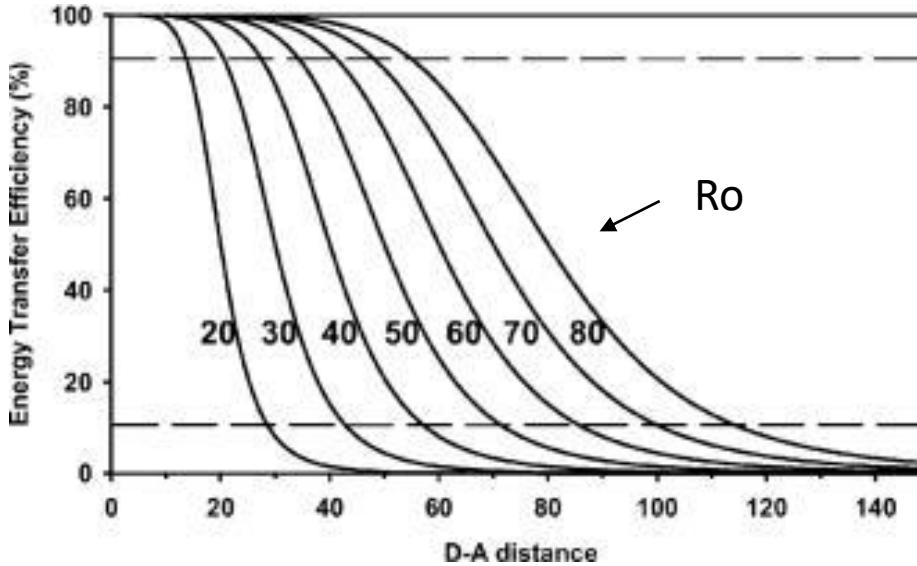
$$\tau_D = \frac{1}{k_{nr} + k_r}$$

- ✓ Informs on the distance separating two fluorescent dyes
- ✓ Ratiometric FRET efficiency (PR)
- ✓ $\tau_{D(A)}$ decreases when FRET increases

FRET: Förster Resonance Energy Transfer

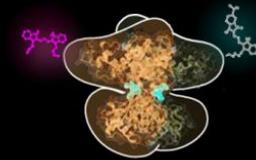


$$FRET = \frac{R_0^6}{R_0^6 + R^6}$$

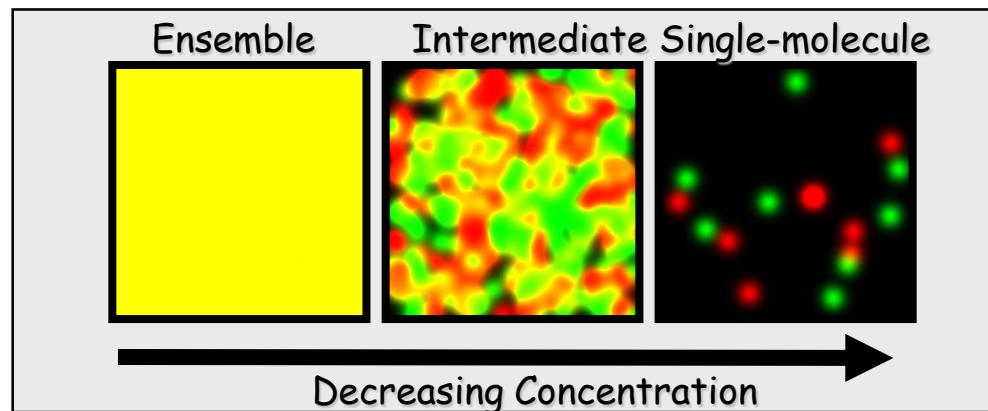


- ✓ FRET can be used as a ruler
- ✓ Very sensitive to distance changes around R_0
- ✓ An accurate FRET measurement can report on distance change, down to the Angstroms and nanosecond timescales

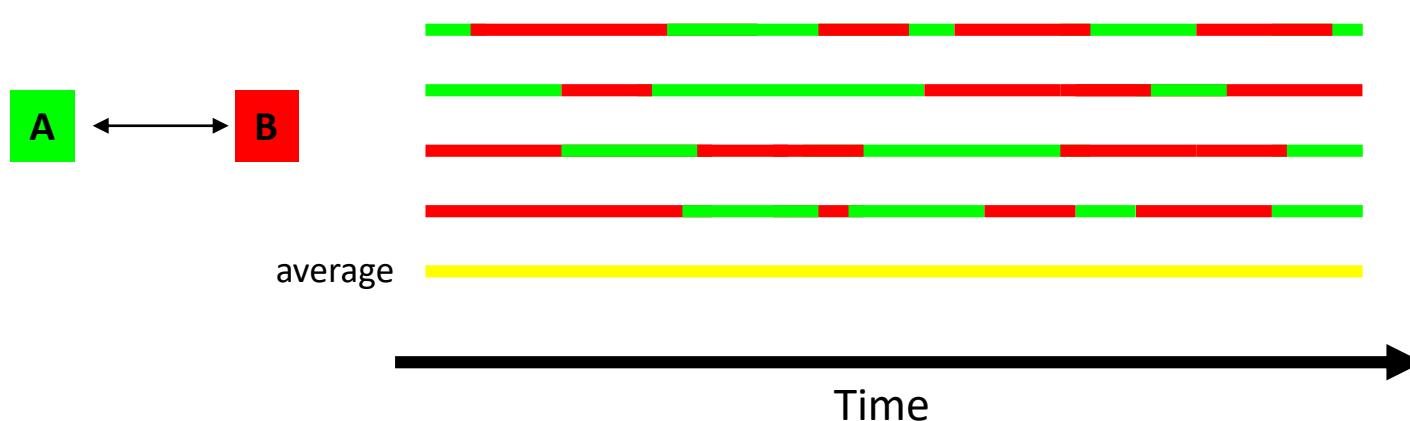
Why do we study single molecules ?



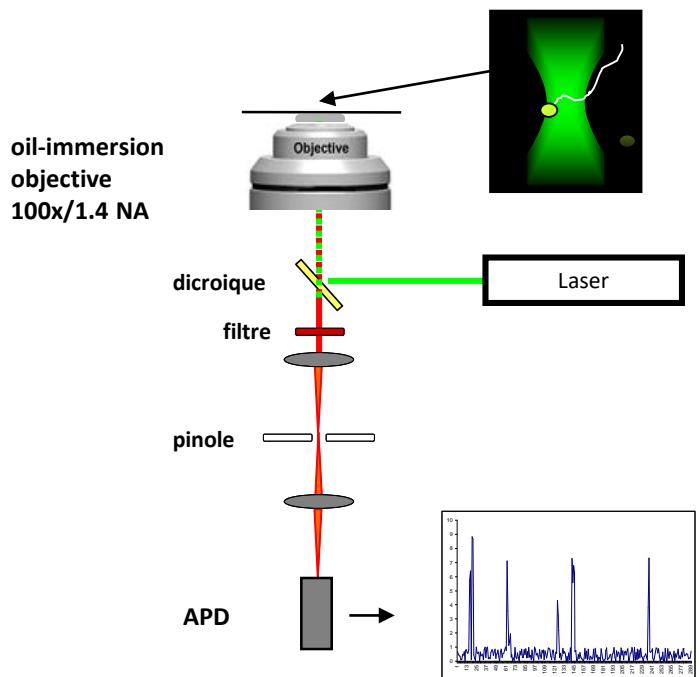
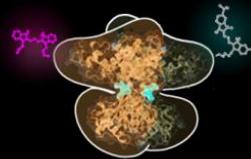
- Looking at ensemble of molecules generally leads to average values
- In particular, you cannot see **static heterogeneities**, i.e. the presence of subpopulations :



- Moreover, ensemble measurement do not allow to see the dynamics of unsynchronized molecules (**dynamic heterogeneities**)

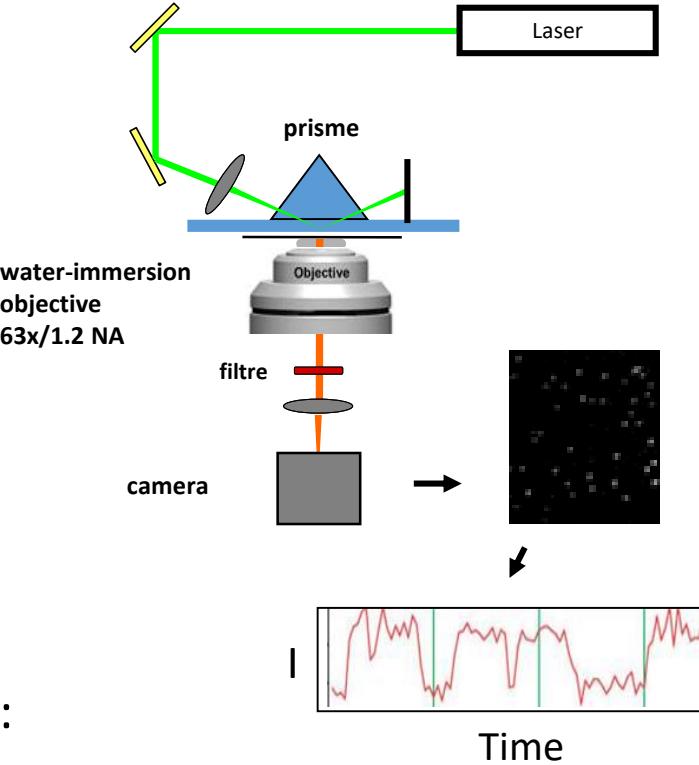


Confocal and TIRF microscopies



Confocal :

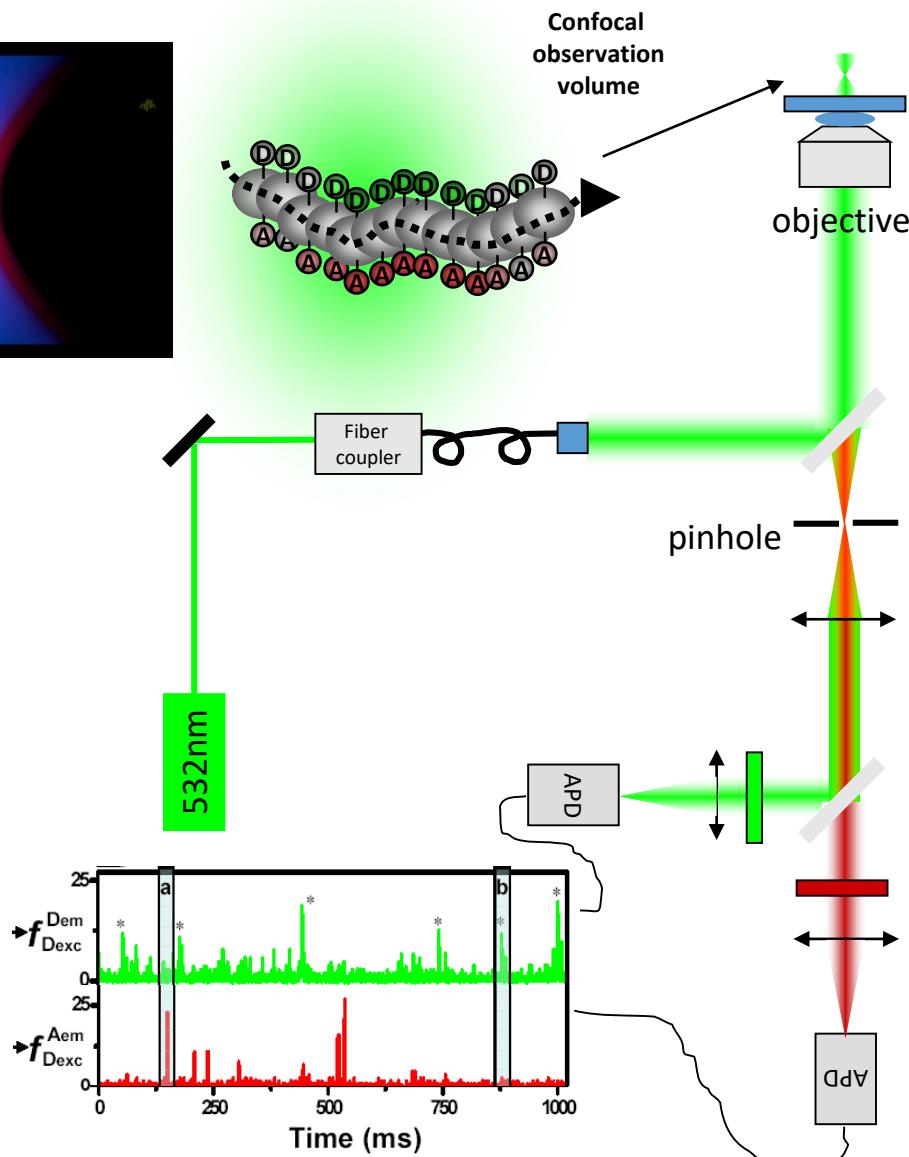
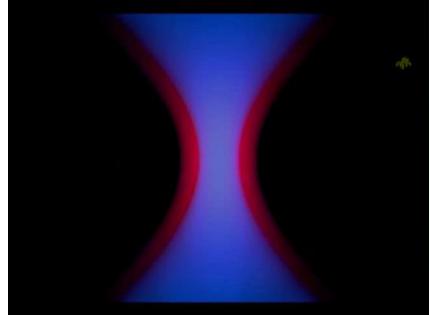
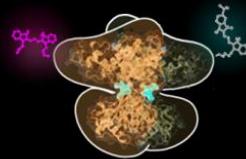
- immobilized or diffusing molecules in 3D
- 1 molecule at the time
- detection sub- populations
- High temporal resolution ($\mu\text{s-ns}$)



TIRF :

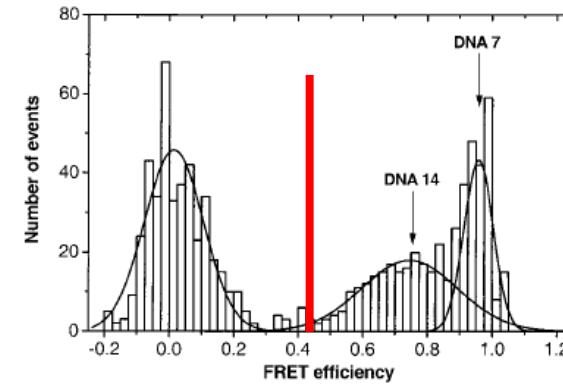
- immobilized or diffusing molecules in 2/3D
- dozens of molecules
- follow each molecules vs.time
- lower temporal resolution (ms)

smFRET on diffusing molecules



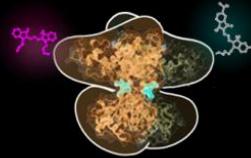
For each molecule :

$$E_{PR} = \frac{F_{Aem}}{F_{Dem} + F_{Aem}}$$



→ resolve subpopulations

Single laser smFRET



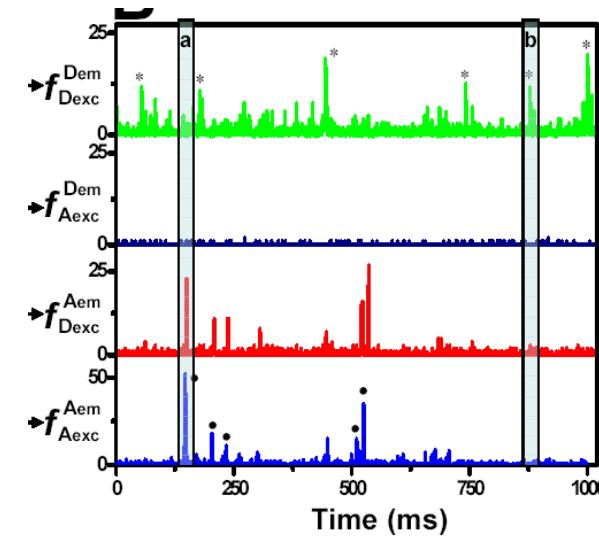
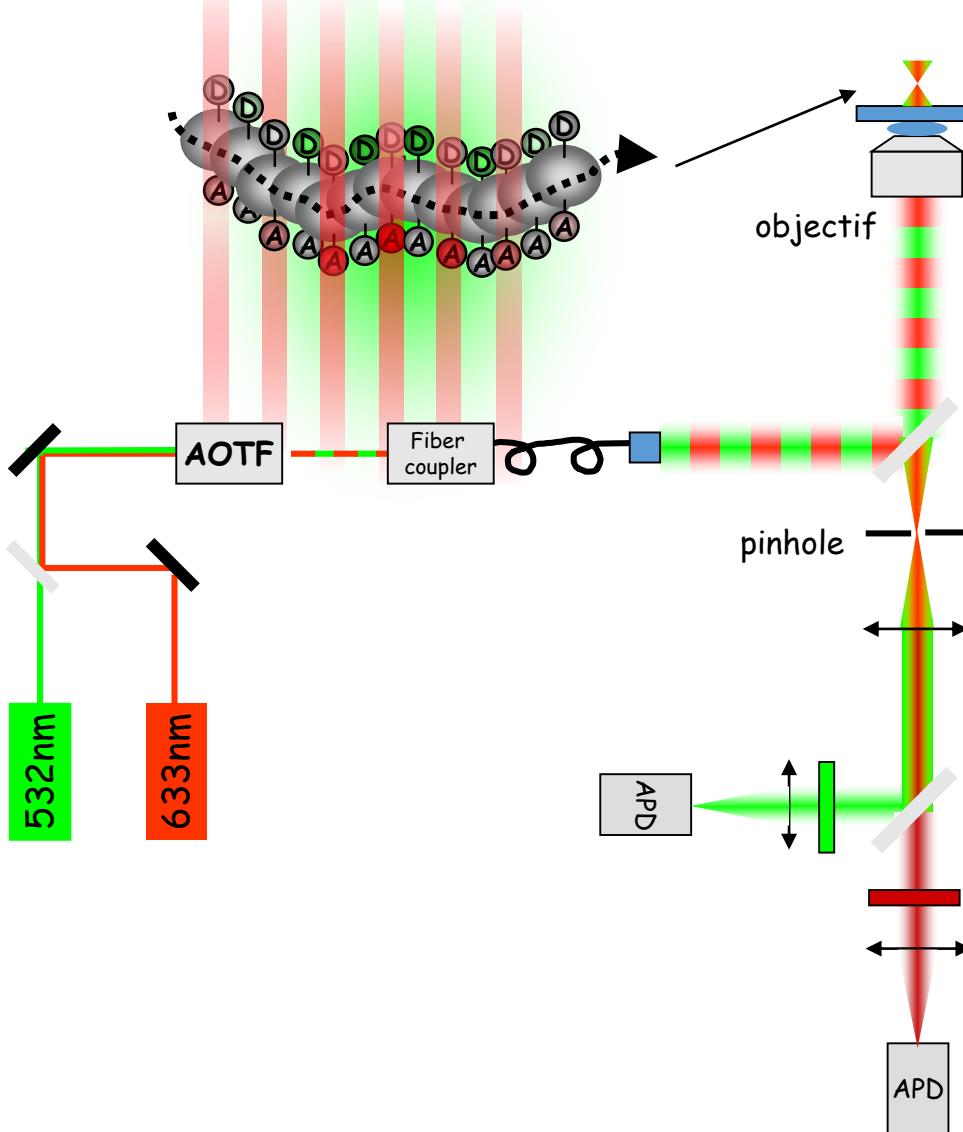
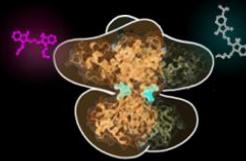
- + Not necessary to have a high yield of labeling, or to know it exactly
- + Ability to recover subpopulations
- + On immobilized molecules, spFRET traces = $f(\text{time})$

- Acceptor photophysics : blinking, bleaching : D-only species
- Sensitive to fluorescent contamination, appears as D-only
- Unable to recover the correction factors needed to extract quantitative FRET efficiency, and thus distances

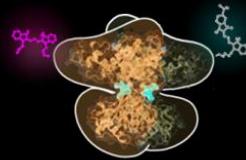
→ Addition of a second laser to directly probe the emission of the acceptor

Alternating Laser EXcitation

Alternating laser excitation (ALEX) - smFRET



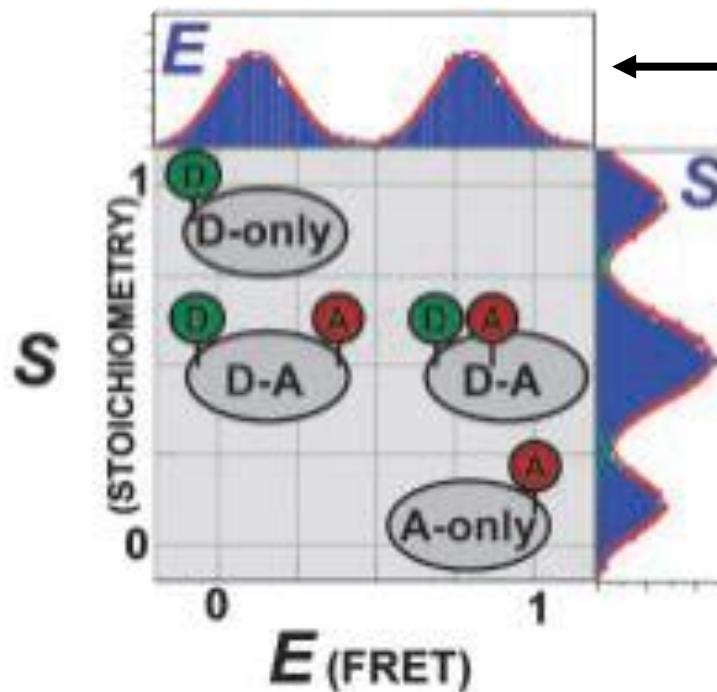
Identifying the species with ALEX



For each molécule

$$E_{PR} = \frac{F_{Dex, Aem}}{F_{Dex, Dex} + F_{Dex, Aem}}$$

$$S = \frac{F_{Dex}}{F_{Dex} + F_{Aem}}$$



E_{PR} : distance between fluorophores

S : Presence of each fluorophores
→ Interaction
If D and A are on different molecules

Application example



ARTICLE

<https://doi.org/10.1038/s41467-021-25620-5>

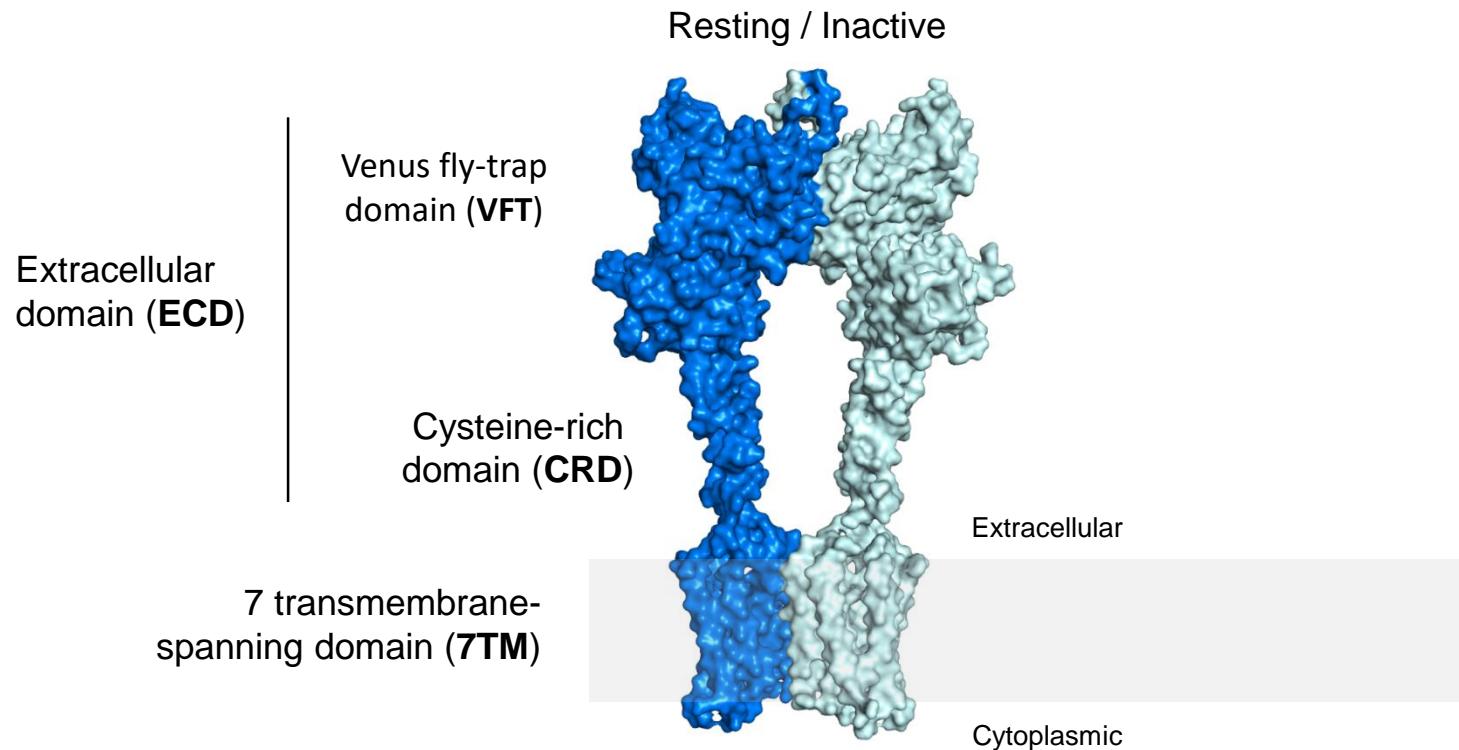
OPEN

 Check for updates

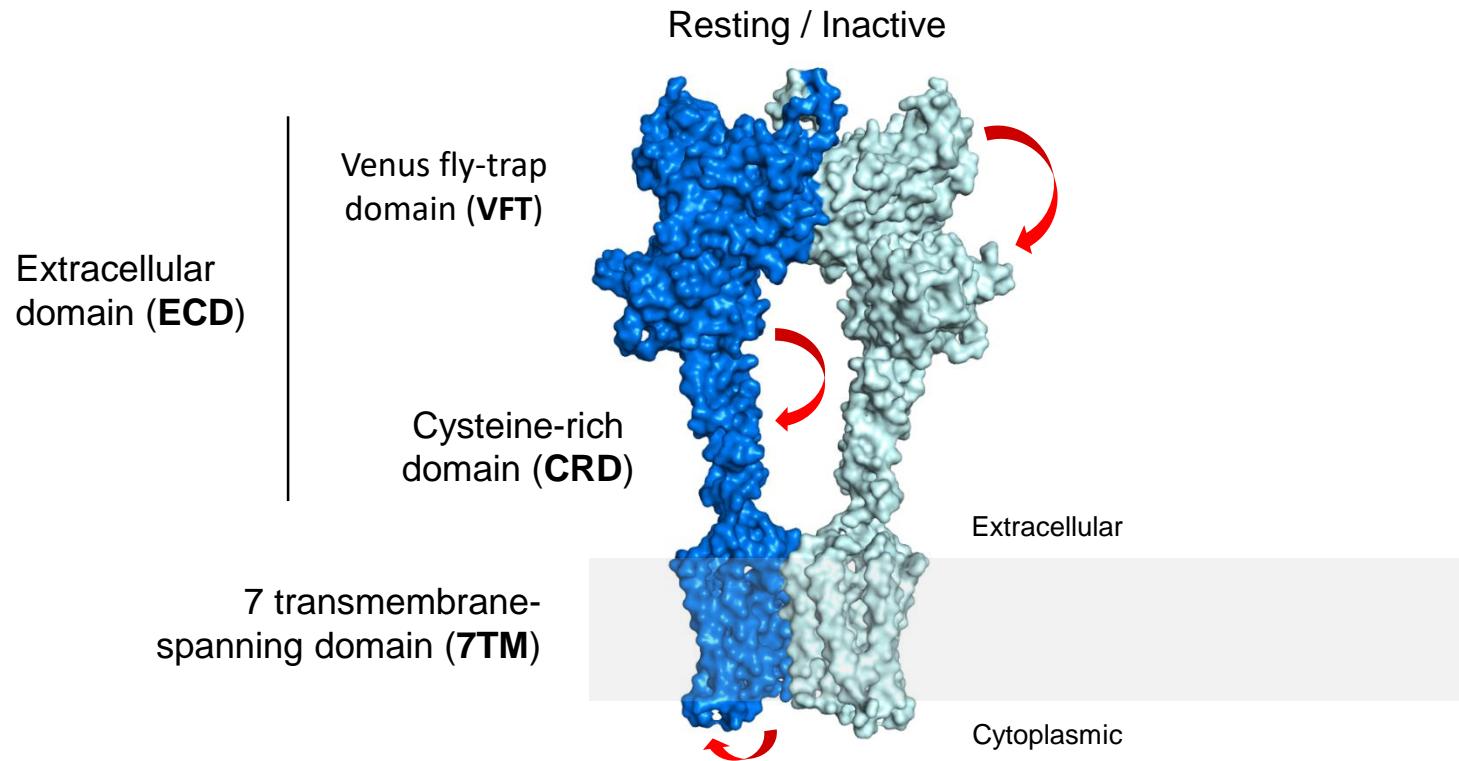
Allosteric modulators enhance agonist efficacy by increasing the residence time of a GPCR in the active state

Anne-Marinette Cao  ^{1,4,5}, Robert B. Quast  ^{1,5}, Fataneh Fatemi^{1,2}, Philippe Rondard  ³, Jean-Philippe Pin  ³ & Emmanuel Margeat  ¹

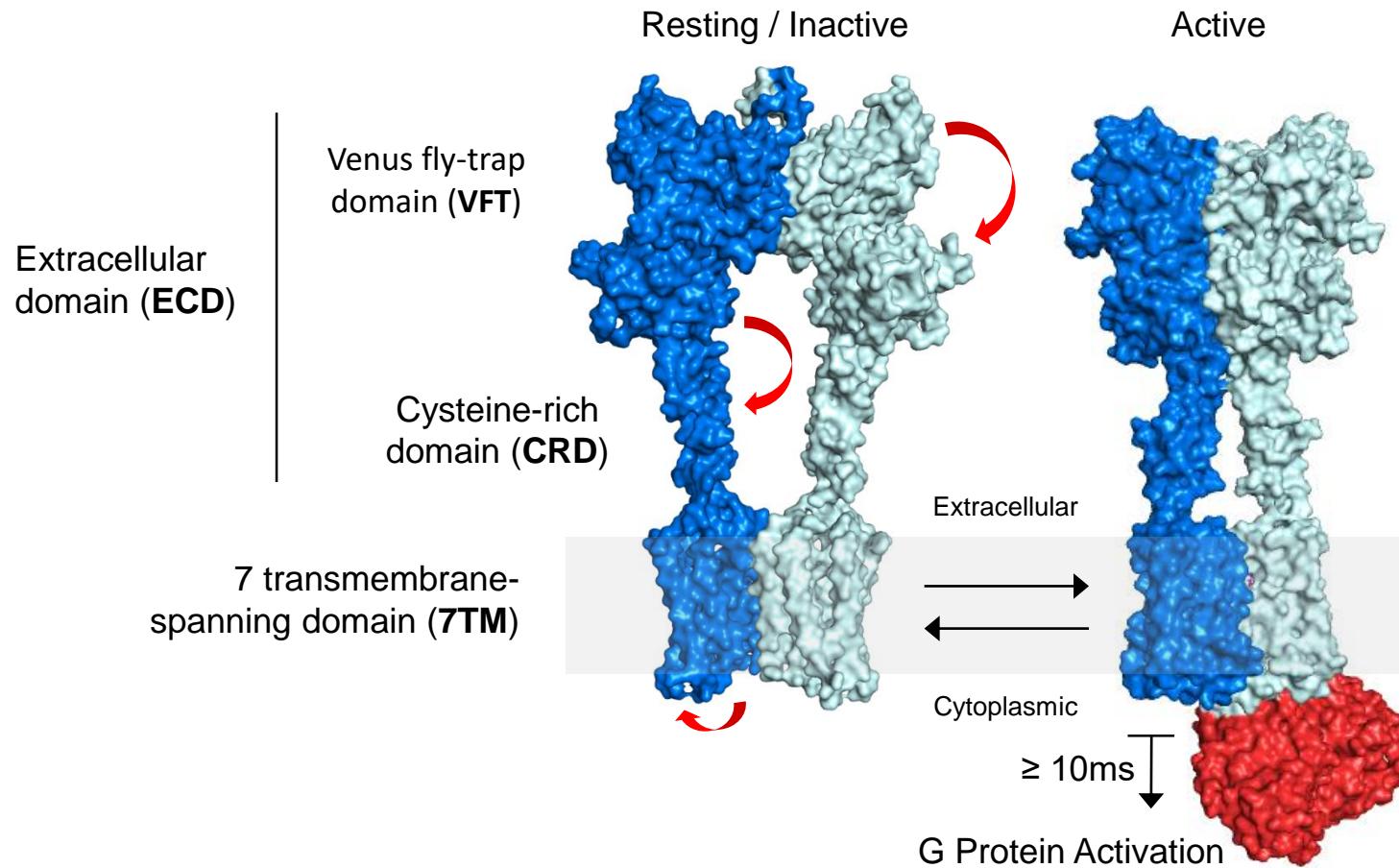
Structure and Activation



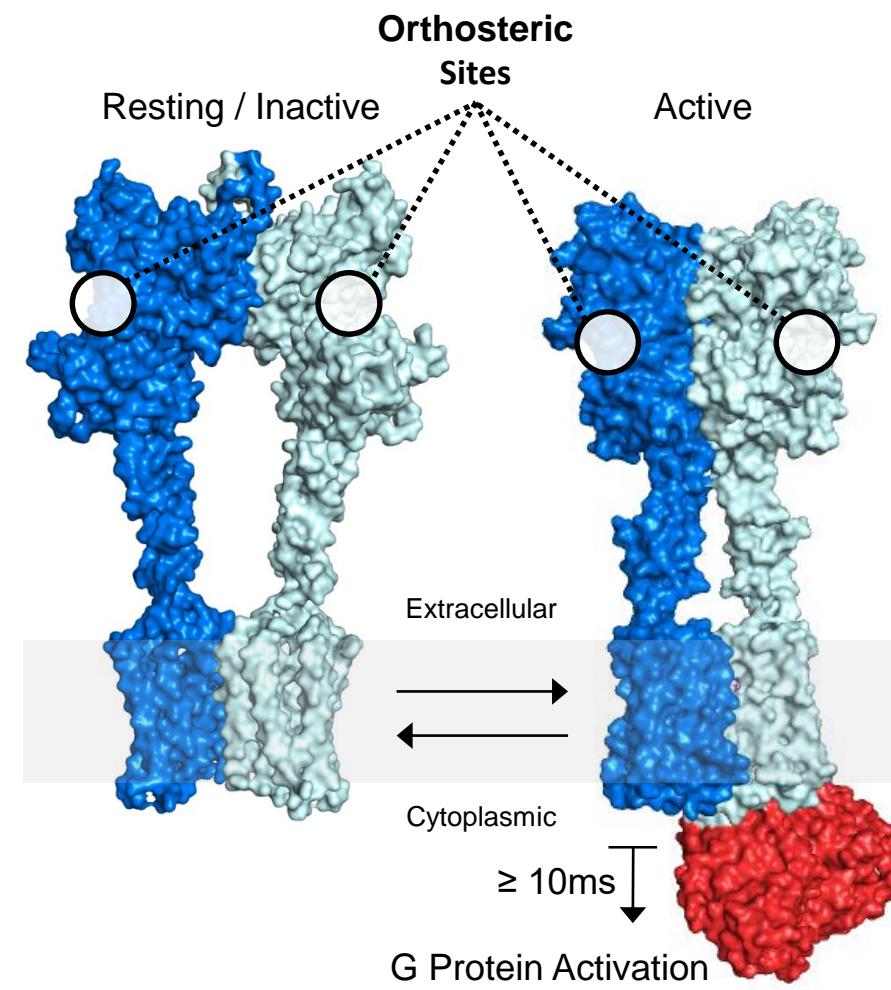
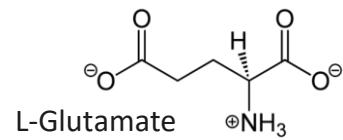
Structure and Activation



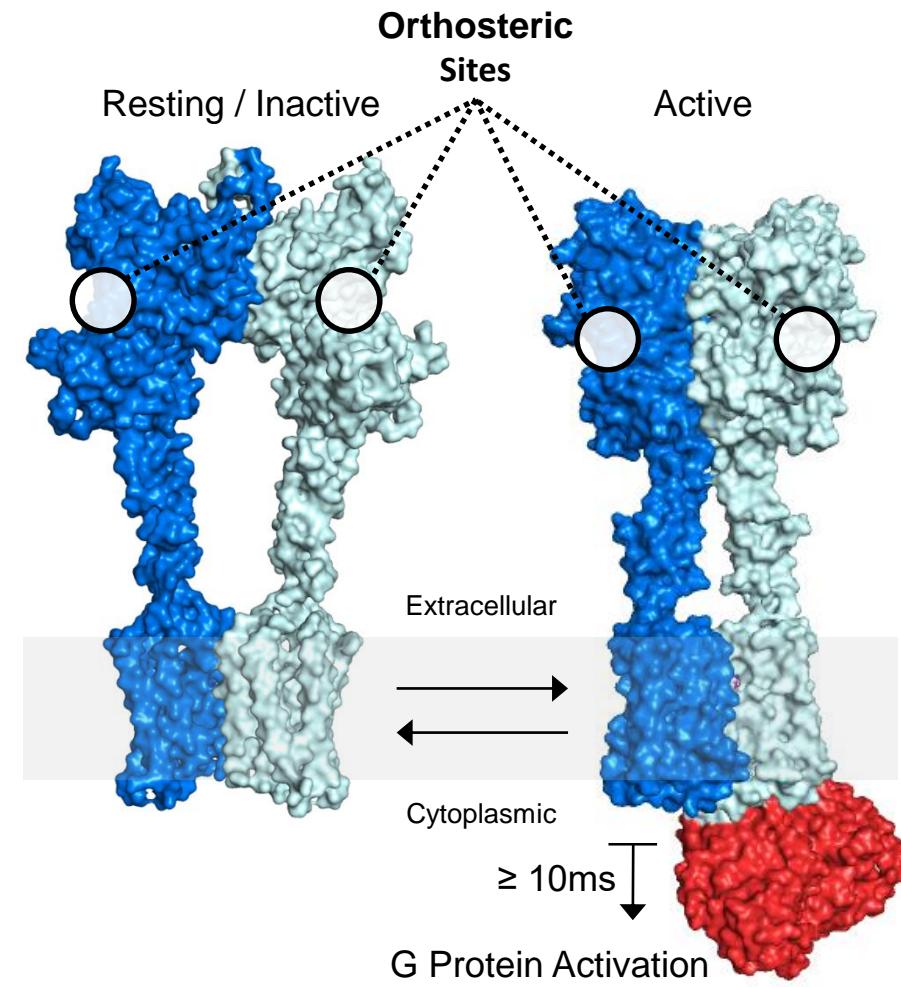
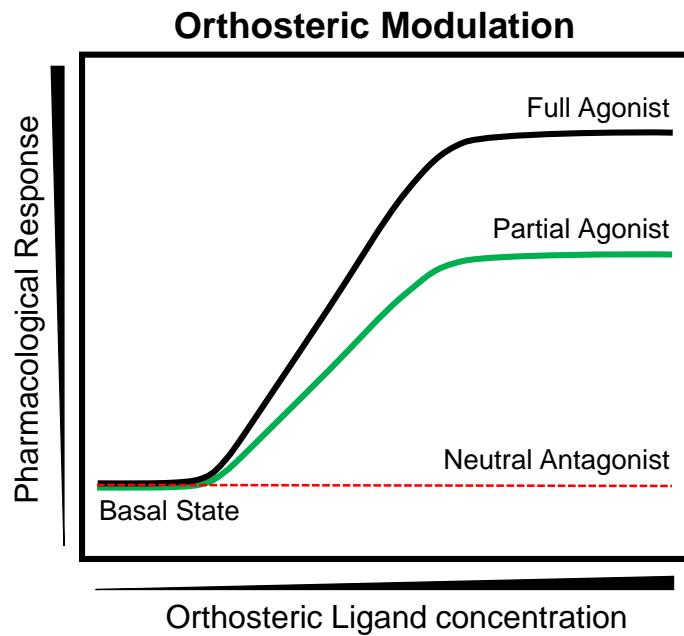
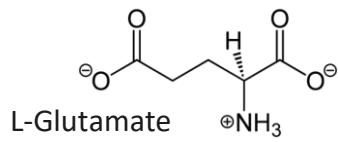
Structure and Activation



Orthosteric Ligands



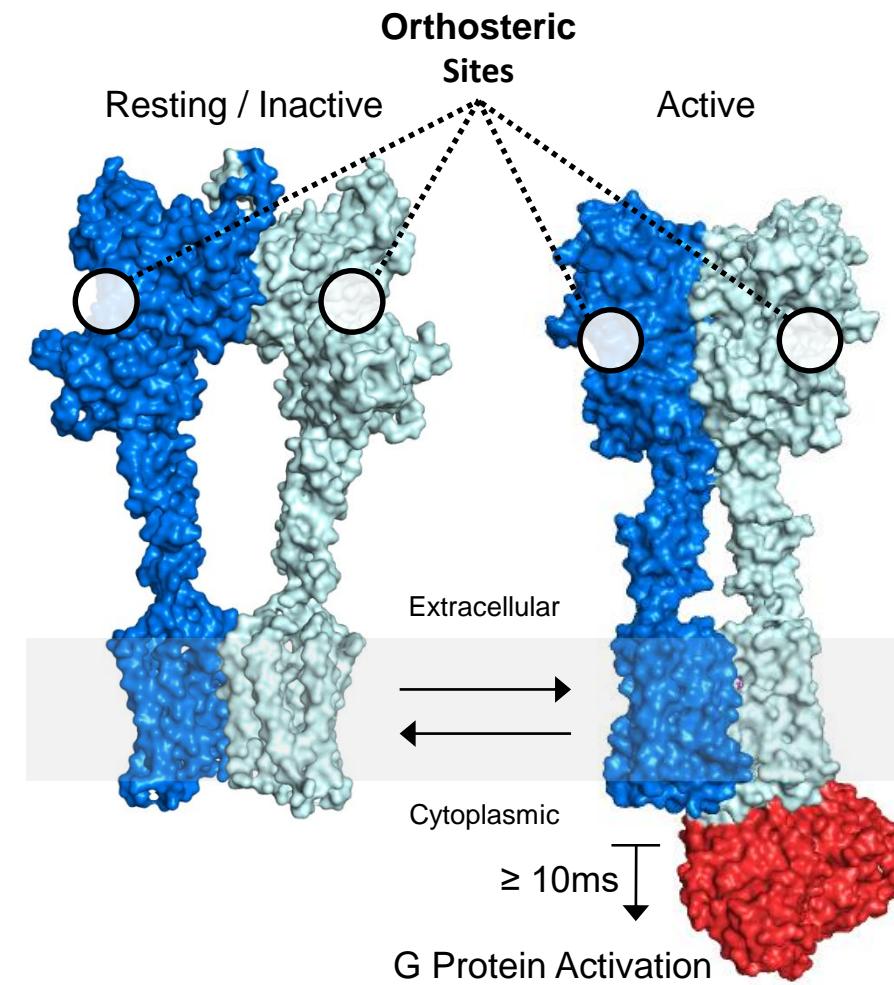
Orthosteric Ligands



Allosteric Modulators

Positive Allosteric Modulator - PAM

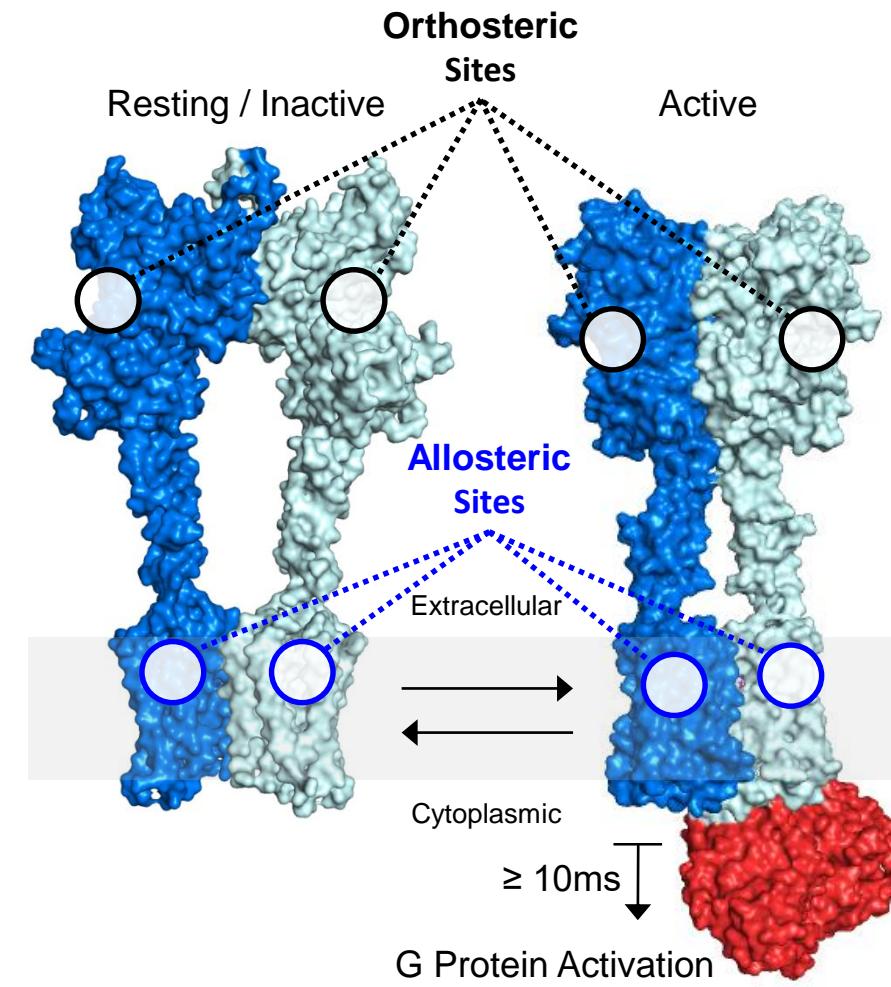
Negative Allosteric Modulator NAM



Allosteric Modulators

Positive Allosteric Modulator - PAM

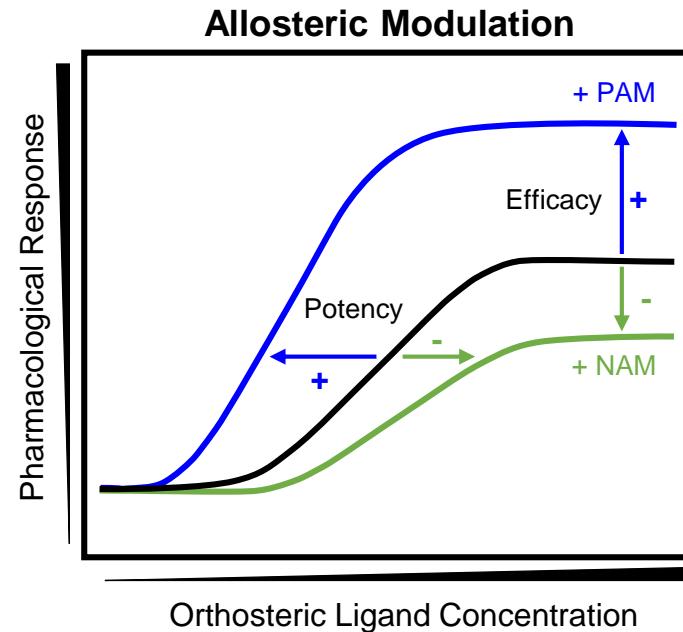
Negative Allosteric Modulator NAM



Allosteric Modulators

Positive Allosteric Modulator - PAM

Negative Allosteric Modulator NAM



Orthosteric
Sites

Resting / Inactive

Active

Allosteric
Sites

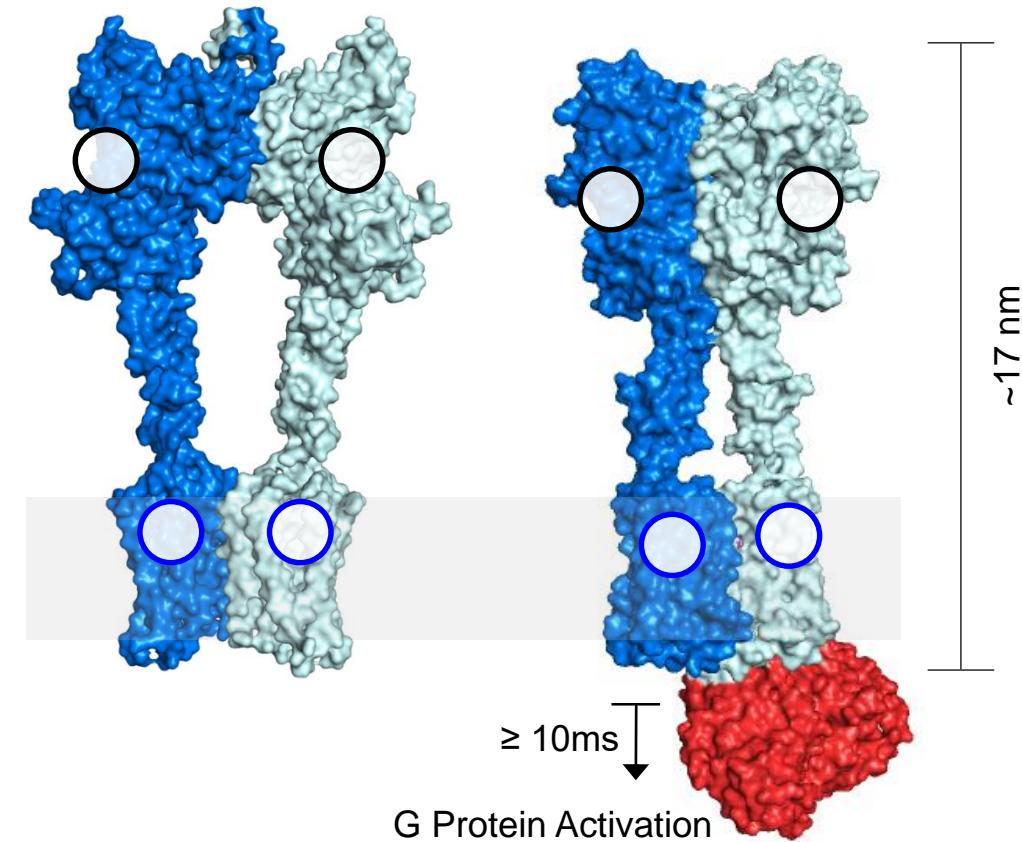
Extracellular

Cytoplasmic

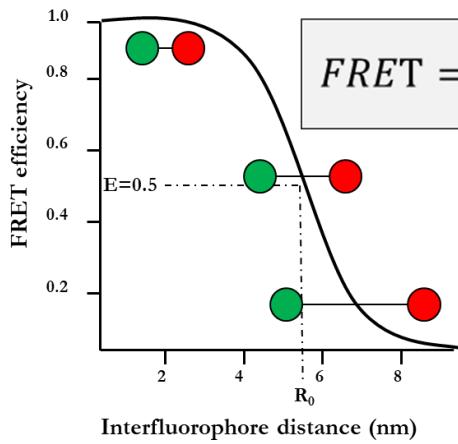
$\geq 10\text{ms}$

G Protein Activation

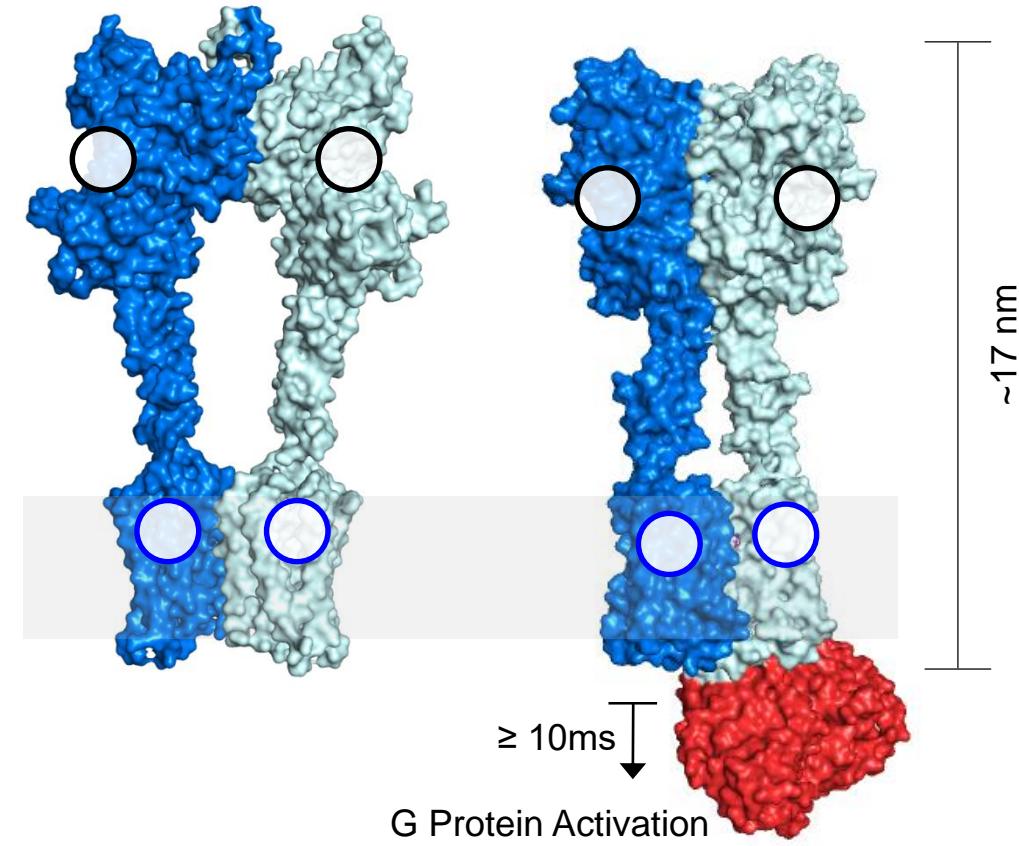
How do allosteric modulators modulate agonist action?



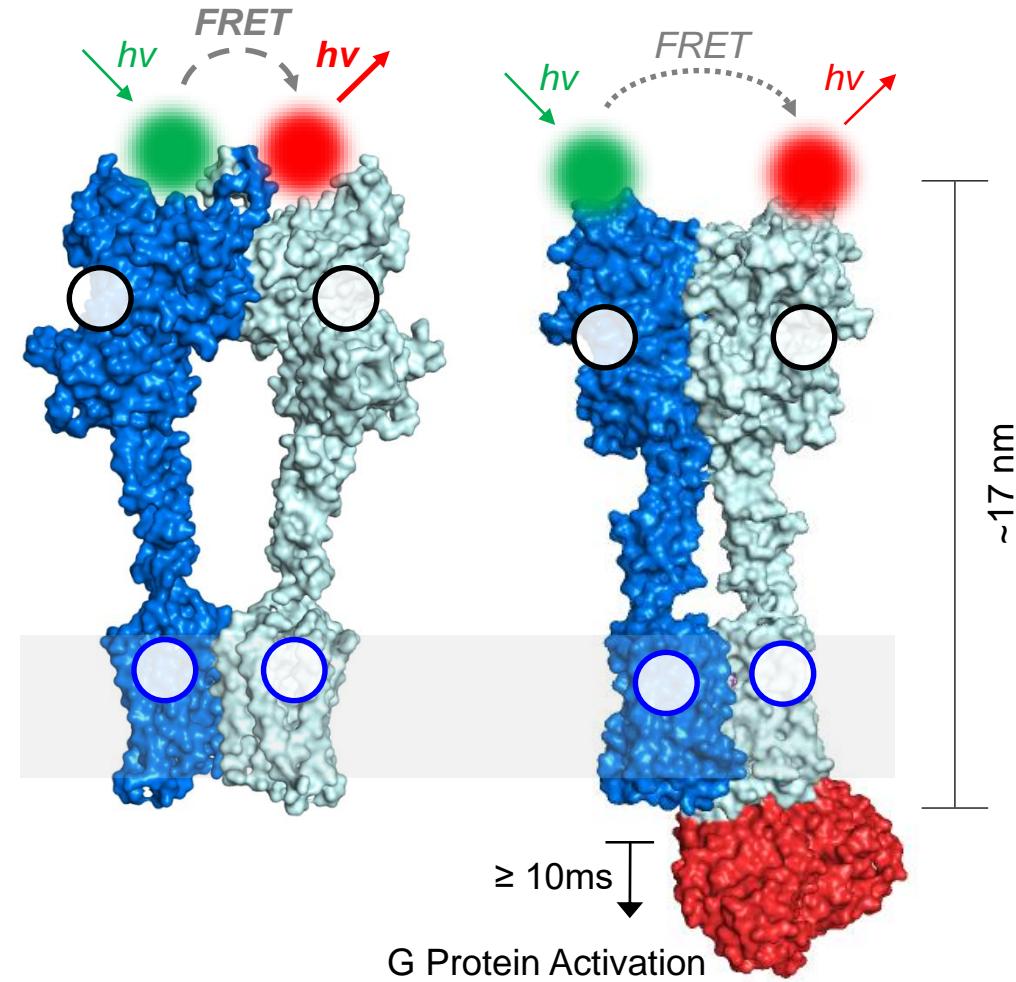
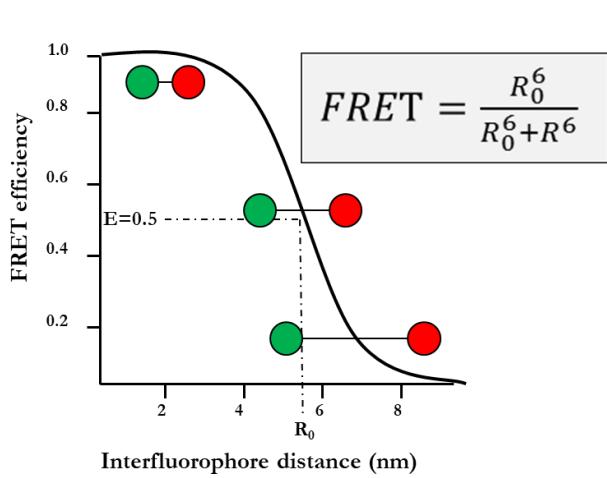
How do allosteric modulators modulate agonist action?



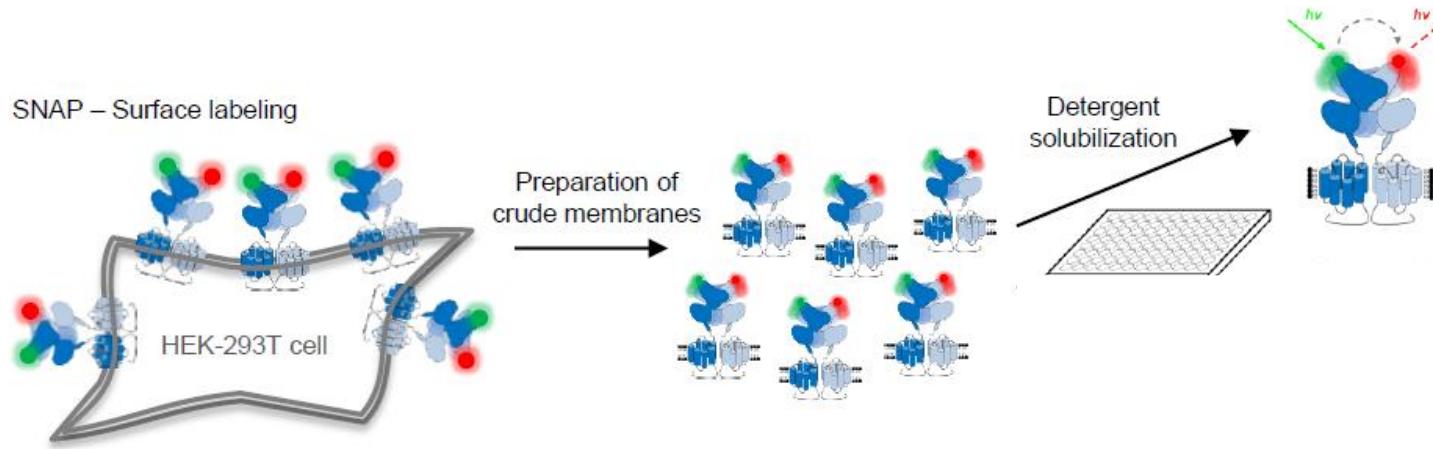
$$FRET = \frac{R_0^6}{R_0^6 + R^6}$$



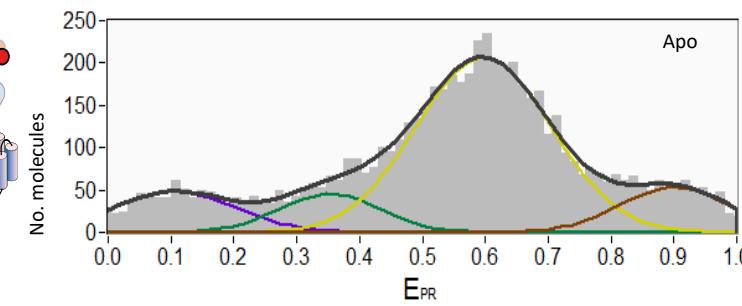
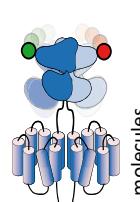
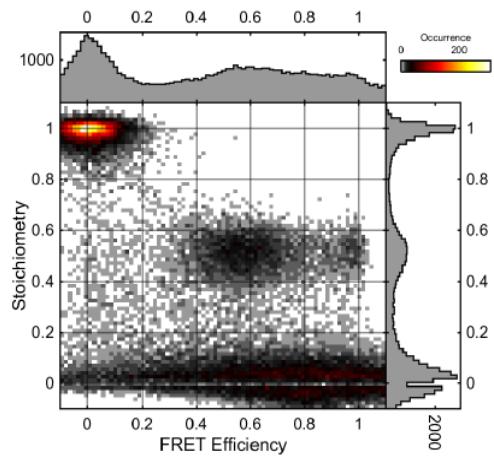
How do allosteric modulators modulate agonist action?



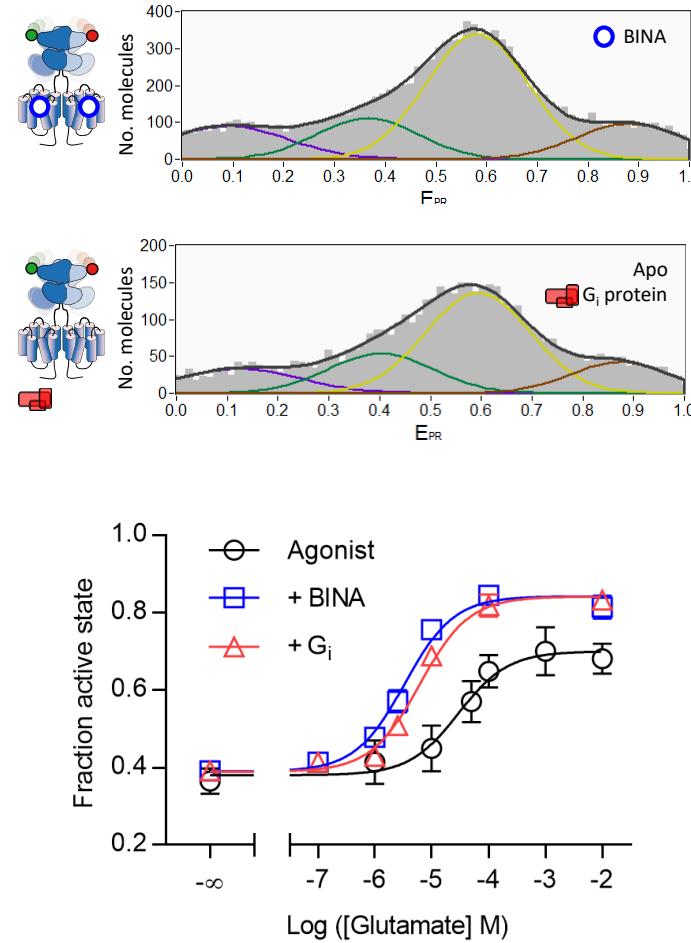
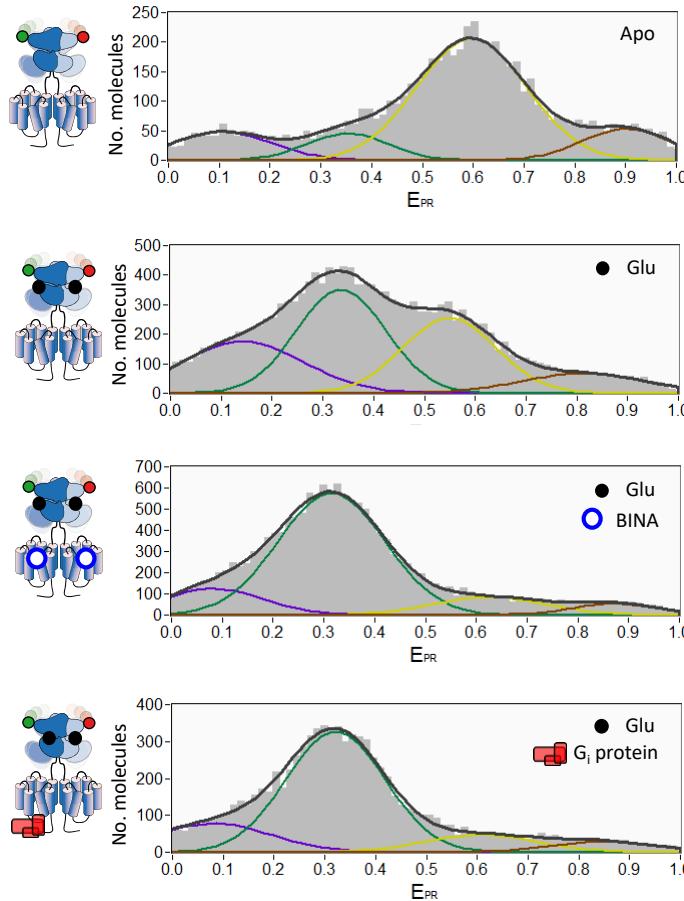
How do allosteric modulators modulate agonist action?



a Apo

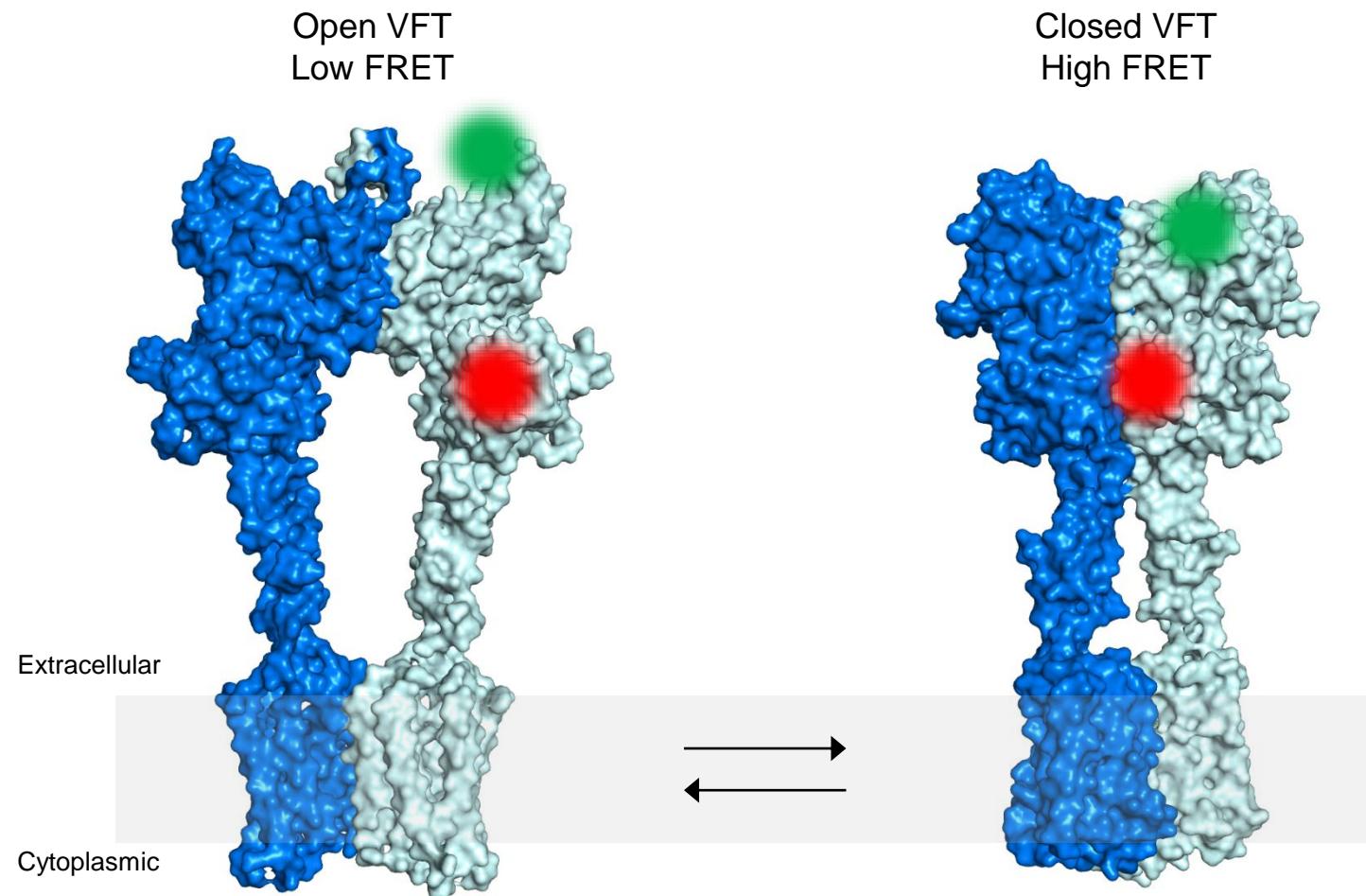


Allosteric modulators are required to stabilize the active state

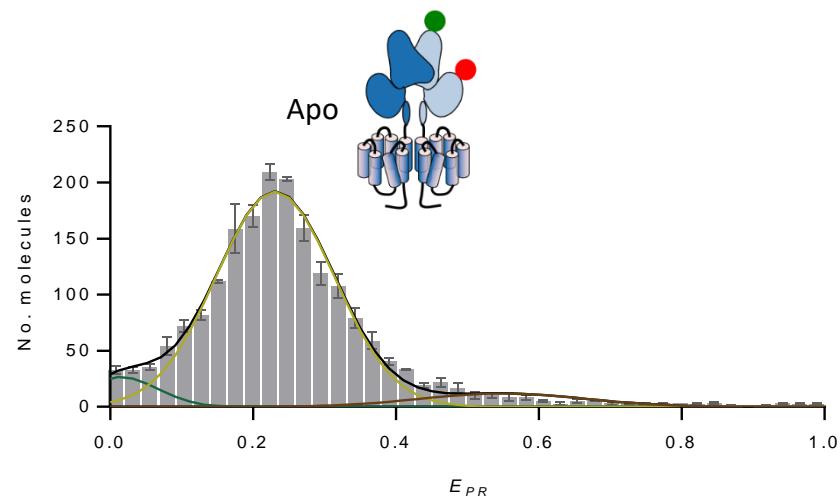


→ PAM or G_i push the receptor toward its active state

VFT Closure – Intrasubunit Sensor



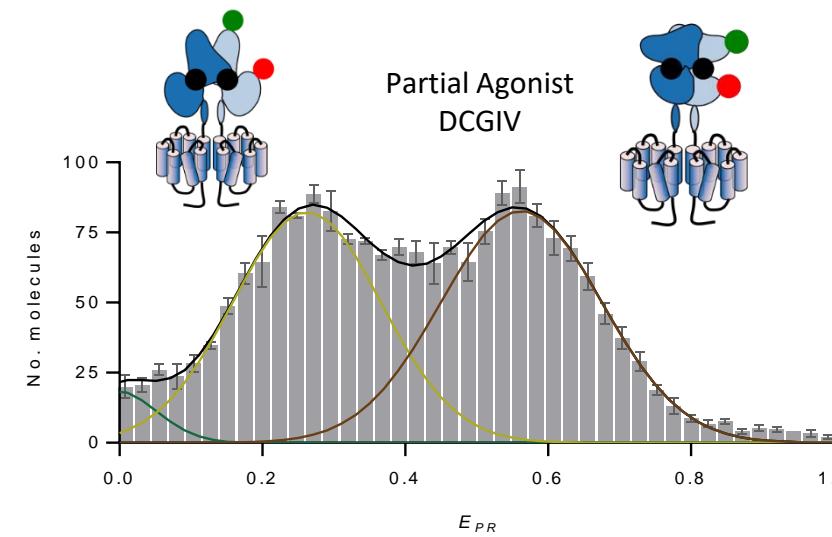
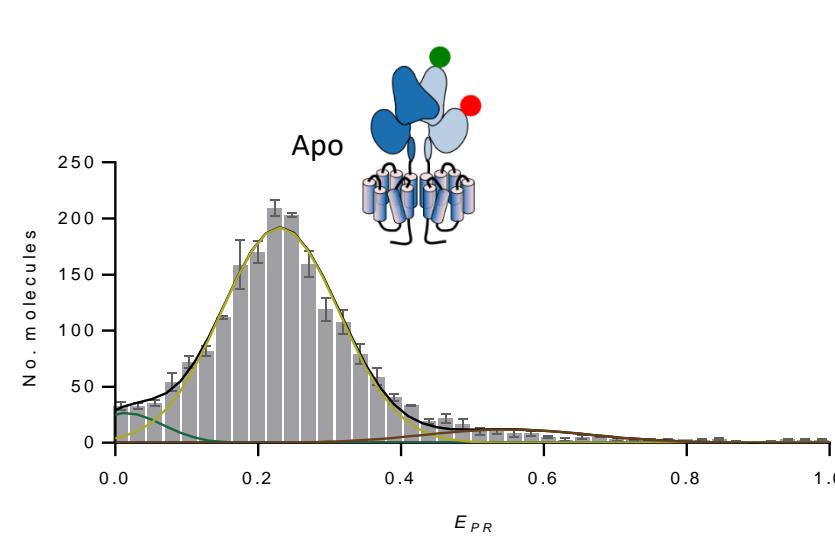
Agonist-dependend Allosteric Effect on VFT Closure



Unpublished data

Quast et al., in preparation

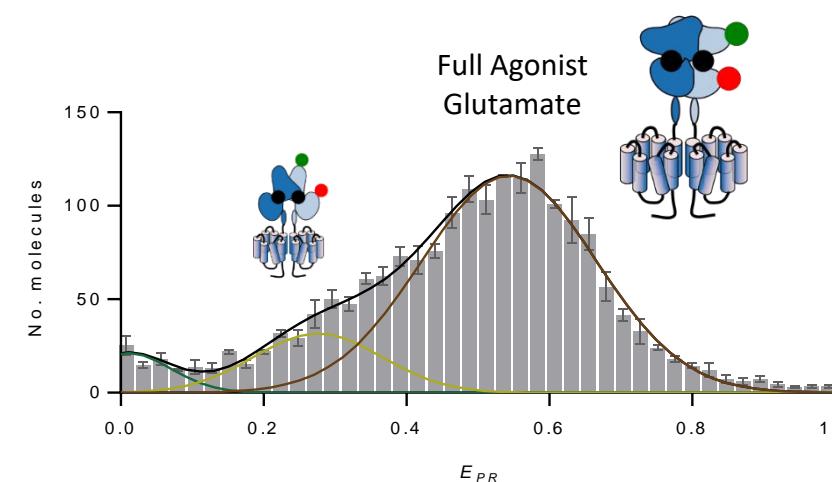
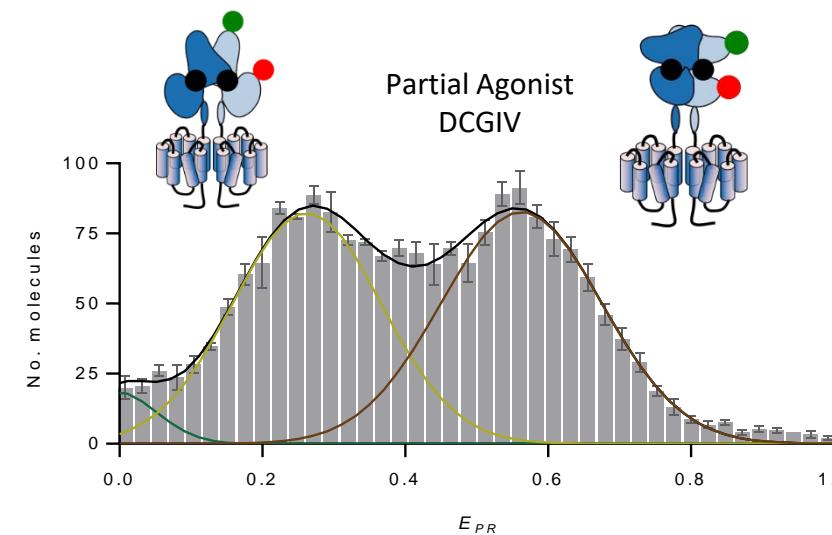
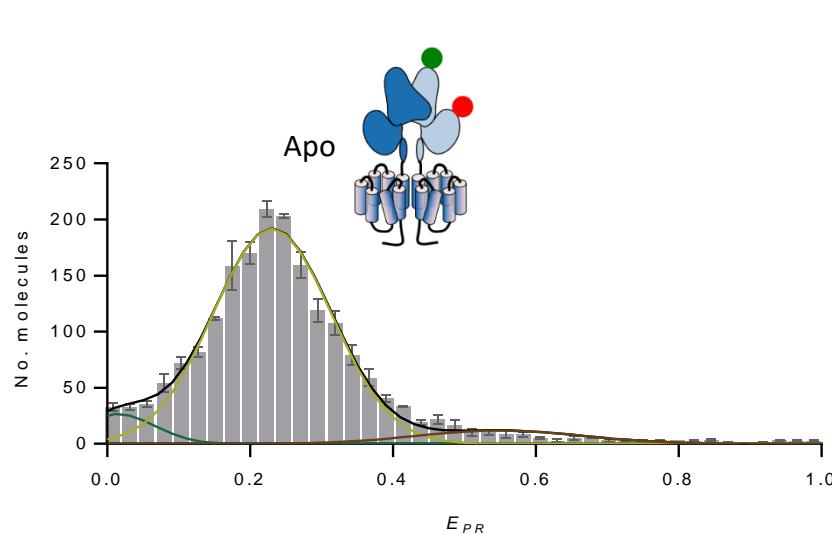
Agonist-dependend Allosteric Effect on VFT Closure



Unpublished data

Quast et al., in preparation

Agonist-dependend Allosteric Effect on VFT Closure



Unpublished data

Quast et al., in preparation

Summary & Conclusions

