

Resolving ligand-induced conformational heterogeneity using single-molecule FRET

Application note

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In this application note, we demonstrate how single-molecule Förster Resonance Energy Transfer (smFRET) can be performed using the EI-FLEX to resolve the conformational changes of a highly dynamic heterodimeric protein, Rag GTPase, that binds nucleotides and the serine/threonine protein kinase complex mTORC1. smFRET measurements were acquired across a range of conditions to discern how the global structure of Rag GTPase changes and drives downstream signalling in response to cellular amino acid sensing¹.

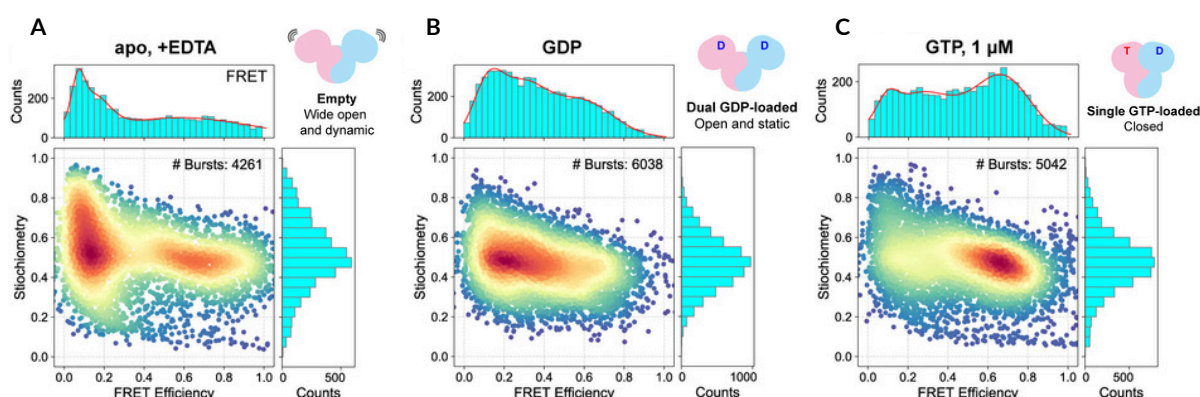


Figure 1 - smFRET detects dynamic conformational changes of Rag GTPase in response to nucleotide binding

Overview of this application note:

- Rag GTPase binding of guanine diphosphate (GDP) and or guanine triphosphate (GTP) drives global conformational changes that differ depending on the combination of bound nucleotides
- Dual binding of GTP induces inter-subunit cross-talk that stabilises Rag GTPase in order for hydrolysis to occur
- Mutations in the nucleotide-binding domain that hyperactivate or inhibit mTORC signalling can be linked to Rag GTPase structural changes, independent of correct nucleotide binding
- Binding of the mTORC subunit Raptor causes Rag GTPase to adopt an open, bound conformation



Glossary of terms used in this application note

FRET Efficiency (E): A measure of how effectively energy is transferred from a donor dye to a nearby acceptor dye. It is determined from the ratio of acceptor fluorescence to total detected fluorescence when only the donor dye is excited by a laser. High FRET efficiency indicates that the labelled sites are closer to each other, low FRET efficiency indicates they are further apart.

Alternating Laser EXcitation (ALEX): A measurement scheme in which a donor and an acceptor laser are switched on and off in rapid sequence, hitting each molecule multiple times with either laser. This allows emission arising from donor and acceptor excitation to be distinguished. This enables the calculation of stoichiometry, which is used to identify and separate doubly labelled molecules from donor-only and acceptor-only species.

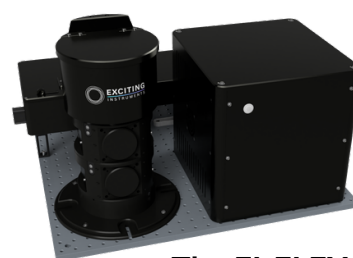
Shot Noise: Photon detection follows Poisson statistics, meaning photons arrive randomly even when the emission rate is constant. In single-molecule FRET, this sets the minimum width of FRET distributions expected for a static molecule due solely to limited photon counts.

Burst Variance Analysis (BVA): A hypothesis test for dynamic conformational changes occurring within bursts. This analysis compares the theoretically expected standard deviation in FRET efficiency with the experimentally observed one. This determines whether the FRET efficiency peak comes from static heterogeneity (multiple distinct, stable species) or dynamic fluctuations (species rapidly transitioning between multiple conformations).

Photon-by-photon Hidden Markov Modelling (H2MM): Analyses the sequence of individual photon arrivals to infer discrete FRET states and the transition rates between them, enabling the reconstruction of the underlying kinetic model with sub-millisecond temporal resolution.

The EI-FLEX brings a biophysics professor into any lab with one simple, confocal benchtop solution that rapidly reveals physiologically-relevant behaviour without immobilising targets or requiring large sample volumes, all at single-molecule precision. With easy-to-use acquisition and analysis protocols and automated, high-throughput options available, high-quality data and publication-ready figures can be generated with ease.

The EI-FLEX System



**The EI-FLEX
single-molecule
spectrometer**

Understanding the effect of GTP and GDP binding on global Rag GTPase conformation

Individual Rag GTPase subunits, RagA and RagC, were labelled with a donor Cy3 fluorophore and acceptor Cy5 fluorophore, respectively. When the Rag GTPase heterodimer was in a closed conformation, the distance between the donor and acceptor dyes was minimised, and FRET efficiency was high. When the heterodimer was in an open conformation, the distance between the dyes increased, resulting in a low FRET efficiency.

To explore the conformational changes upon guanine nucleotide binding to these subunits, Rag GTPase was incubated with EDTA (Figure 2A), GDP (Figure 2B), or GTP and GDP (Figure 2C). smFRET was performed with ALEX to calculate stoichiometries and ensure only doubly labelled molecules were analysed for FRET efficiencies. Burst variance analysis and Hidden Markov modelling were then used to distinguish between dynamic conformational changes and static conformations.

When guanine nucleotides are absent, Rag GTPase occupies an open conformation (low FRET efficiency, Figure 2A). Burst variance analysis shows that the protein's conformation is dynamic, as shown by the increase in variance of observed photon burst frequency compared to the theoretically calculated one. This contrasts with dually GDP-bound Rag GTPase, which remains in a static, open conformation (low FRET efficiency, Figure 1B). When both GDP and GTP are bound, the protein adopts a static closed conformation, generating a high FRET state and no deviation from the theoretical standard deviation of photon bursts (Figure 2C).

Hidden Markov modelling identified a third, intermediate FRET state in the nucleotide-bound complexes that was absent in the unbound condition. smFRET permits the calculation of distances between fluorophores, given that the Förster Radius is known for the particular fluorophore pair (the distance between the fluorophores that generates a FRET efficiency of 50%). The authors calculated the distances for the dyes in the mid and high-FRET states, 61 and 51 Å, respectively, which corroborates with distances determined by crystal structures showing open and closed formations of the nucleotide binding domain.

Understanding the effect of GTP and GDP binding on global Rag GTPase conformation

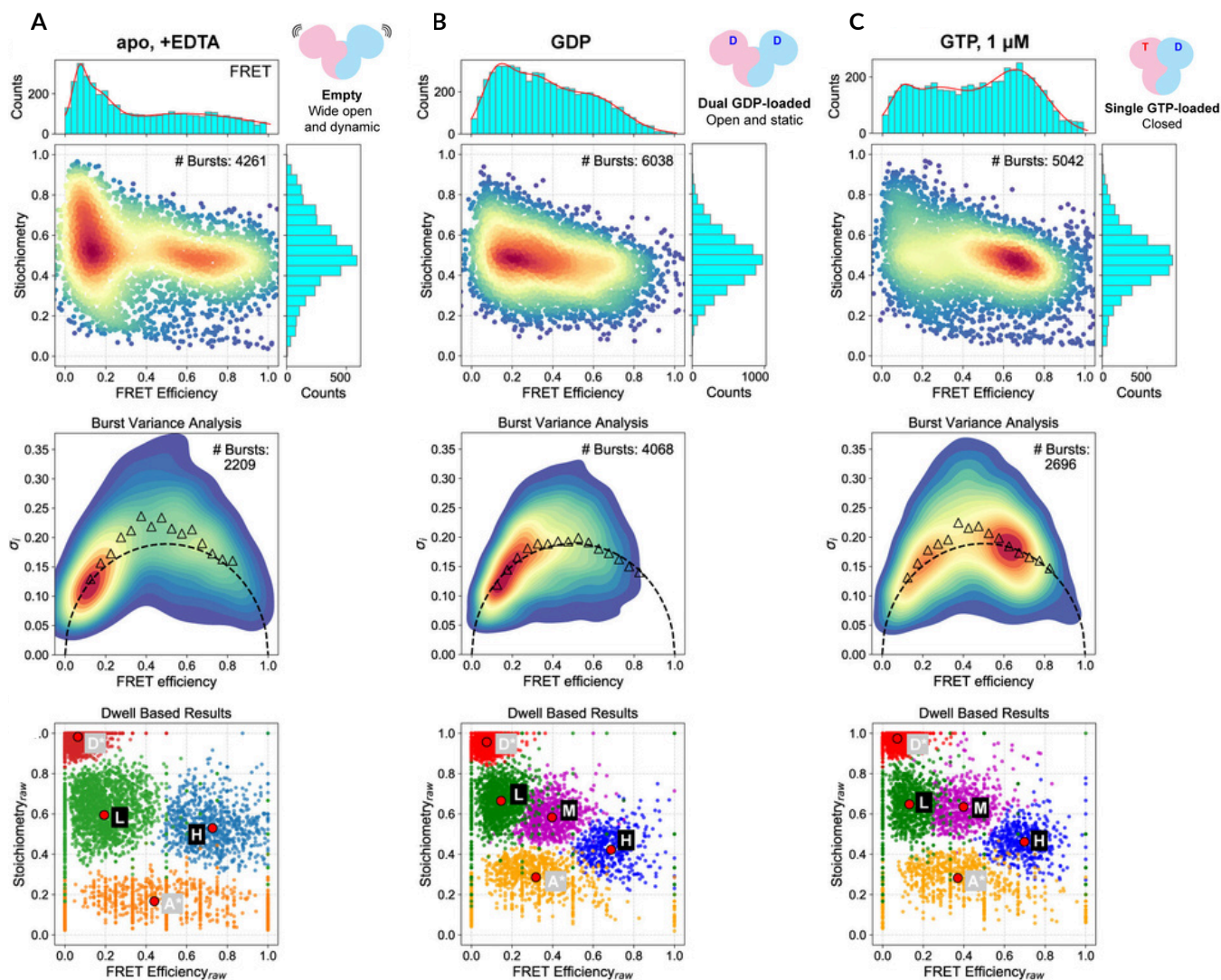


Figure 2 – Binding of GDP alone or with GTP induces global conformational changes in Rag GTPase

Top row: smFRET histograms and FRET efficiency vs stoichiometry plots

Middle row: Burst variance analysis, showing theoretical standard deviation of bursts (dotted line) and experimentally observed standard deviation (triangles)

Bottom row: Hidden Markov modelling data indicating donor only (D), acceptor only (A), low-FRET (L), mid-FRET (M) and high-FRET (H) populations

A) Unbound Rag GTPase (EDTA only)

B) Rag GTPase with GDP only

C) Rag GTPase with GDP and GTP

Understanding the effect of dual GTP binding and hydrolysis on global Rag GTPase conformation

When two GTP molecules bind Rag GTPases, inter-subunit crosstalk ensures that only one of the GTP molecules is hydrolysed, leaving a single bound GTP that is required for correct downstream signalling. To explore the conformational dynamics of this process, the authors performed smFRET, incubating their doubly labelled Rag GTPase with either saturating levels of GTP, GppNHp (a GTP mimetic that is fixed in its ground state) or GDP:AIFX (a GTP mimetic that is fixed in a state that is transitioning towards hydrolysis). Figure 3 shows FRET efficiency histograms and burst variance analysis for these three conditions, whereby dual-bound GTP induces a highly dynamic heterodimer with a reduced high-FRET state and increased mid-FRET state relative to heterodimers with a single-bound GTP (as shown in Figure 2). Additionally, binding of GppNHp also appears to drive RagGTPase out of a stable, closed conformation into one that is highly dynamic with a more pronounced mid-FRET population, indicating that the protein is favouring an open state. By contrast, upon binding of GDP:AIFX, which mimics GTP entering hydrolysis, burst variance analysis data shows Rag GTPase has adopted a static conformation that holds the subunits in their open state, as indicated by a pronounced low FRET population.

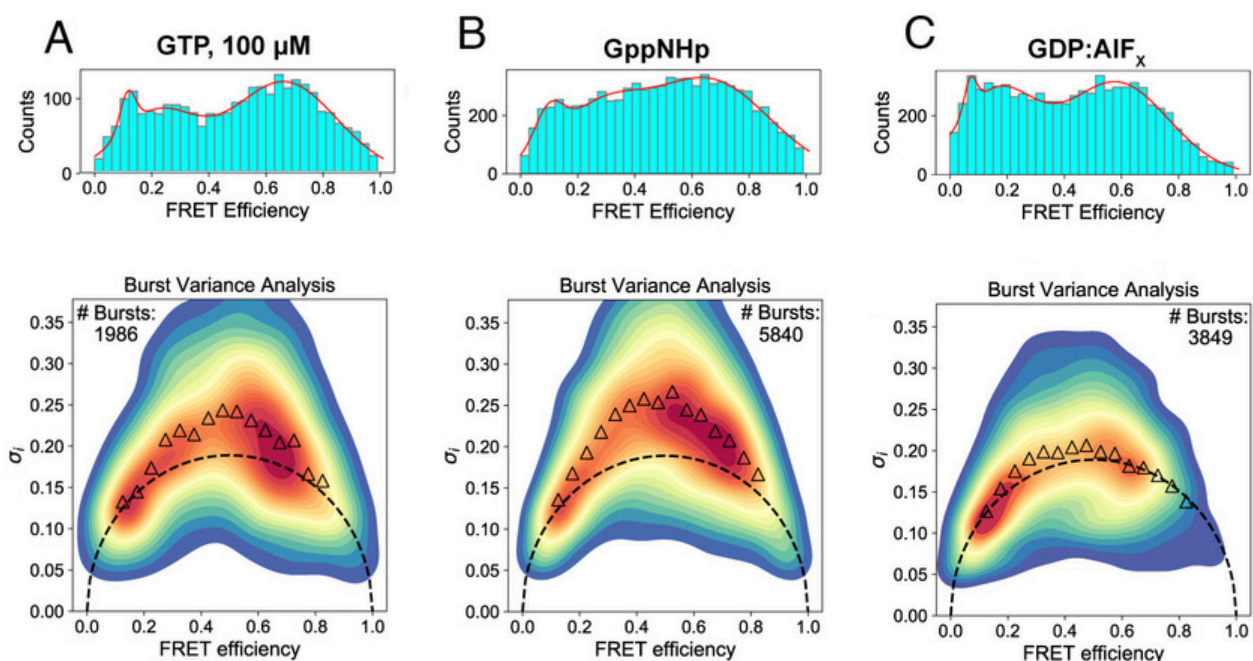


Figure 3 – Effect of dual-GTP binding and initiation of hydrolysis on global Rag GTPase conformation

FRET efficiency histograms and burst variance analysis plots for A) GTP at saturating concentration, B) GppNHp (a ground state mimetic) and GDP:AIFX (a transition mimetic)

Burst variance analysis is presented as theoretical standard deviation of bursts (dotted line) and experimentally observed standard deviation (triangles)

The impact of nucleotide-binding domain mutations on global Rag GTPase conformation

There are two known Rag GTPase mutants that are implicated in disease due to their effect on mTORC1 signalling, RagA-RagC(S75N) and RagA(T21N)-RagC, which hyperactivate or inactivate mTORC1, respectively. smFRET was used to explore the impact of these mutations on the conformational changes of Rag GTPase (Figure 4). Unbound mutant Rag GTPase adopted the open, inactivated state as shown for the wildtype protein, irrespective of which mutation is present. Dual GDP binding of RagA-RagC(S75N) produced a pronounced mid-FRET population, which shifted further to a high-FRET state when GTP was added. This aligns with the observation that RagA-RagC(S75N) mutants drive forward mTORC1 signalling, as Rag GTPase is shifted towards the active conformation even when the incorrect combination of nucleotides is bound. Conversely, the RagA(T21N)-RagC mutant remained in the open, inactive conformation regardless of the combination of nucleotides bound, in agreement with the observation that this mutation inhibits mTORC1.

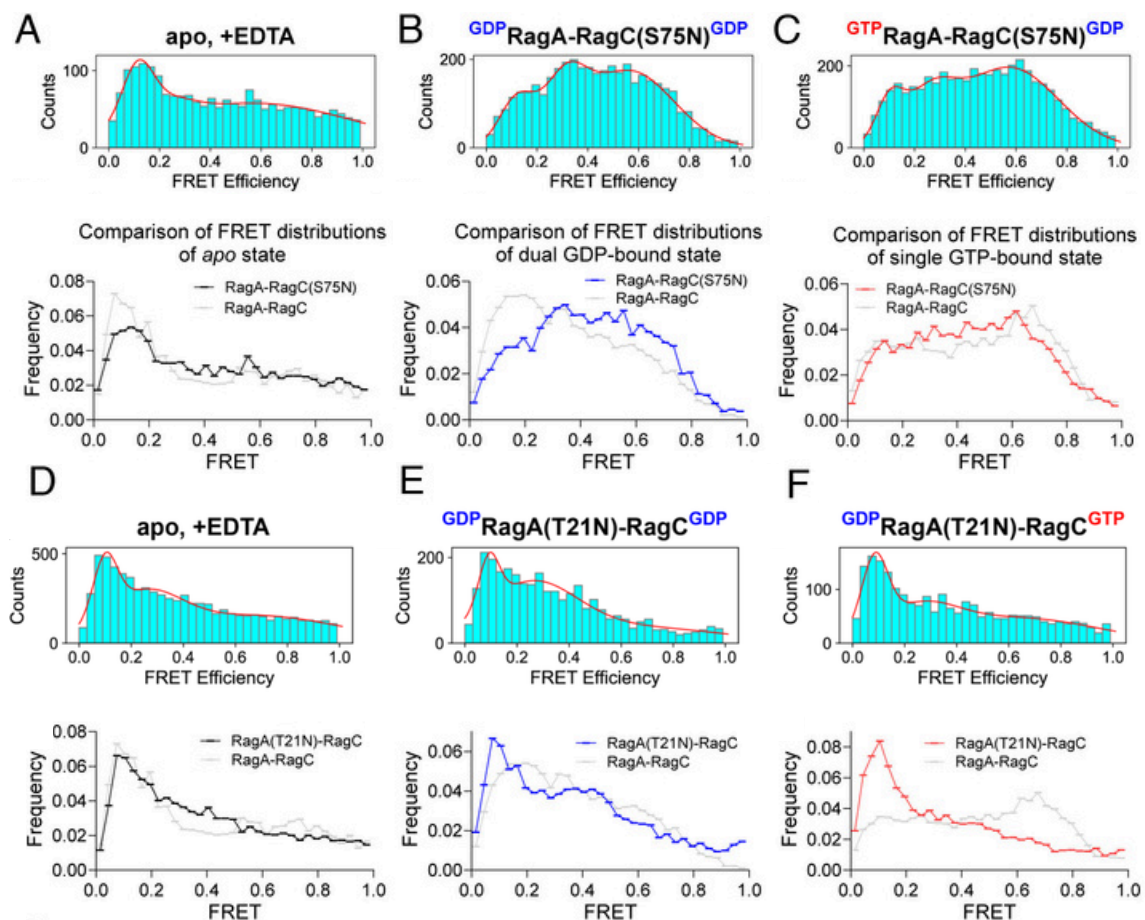


Figure 4 – Impact of nucleotide-binding domain mutations on global Rag GTPase conformations

FRET efficiency histograms for mutated Rag GTPase and overlays between mutant and wildtype for each condition: A) Unbound RagA-RagC(S75N), B) RagA-RagC(S75N) with dual-bound GDP, C) RagA-RagC(S75N) with GDP and GTP, D) Unbound RagA(T21N)-RagC, E) RagA(T21N)-RagC with dual-bound GDP, F) RagA(T21N)-RagC with GDP and GTP

Understanding the impact of Raptor on the global conformation of Rag GTPase

Finally, the authors explored the impact of mTORC1 recruitment in relation to Rag GTPase global conformation, using the Raptor subunit of mTORC1 in their smFRET experiments. Cryo-EM crystal structures show that Rag GTPase is in the open formation when it is bound to both GDP/GTP and Raptor², indicating that a conformational change must occur with this interaction. smFRET and burst variance analysis of the RagA-RagC(S75N) mutant (which favours a closed conformation) show that the binding of Raptor shifts the FRET efficiency to a lower state, indicating opening of the heterodimer, and that this new conformation is static (Figure 5).

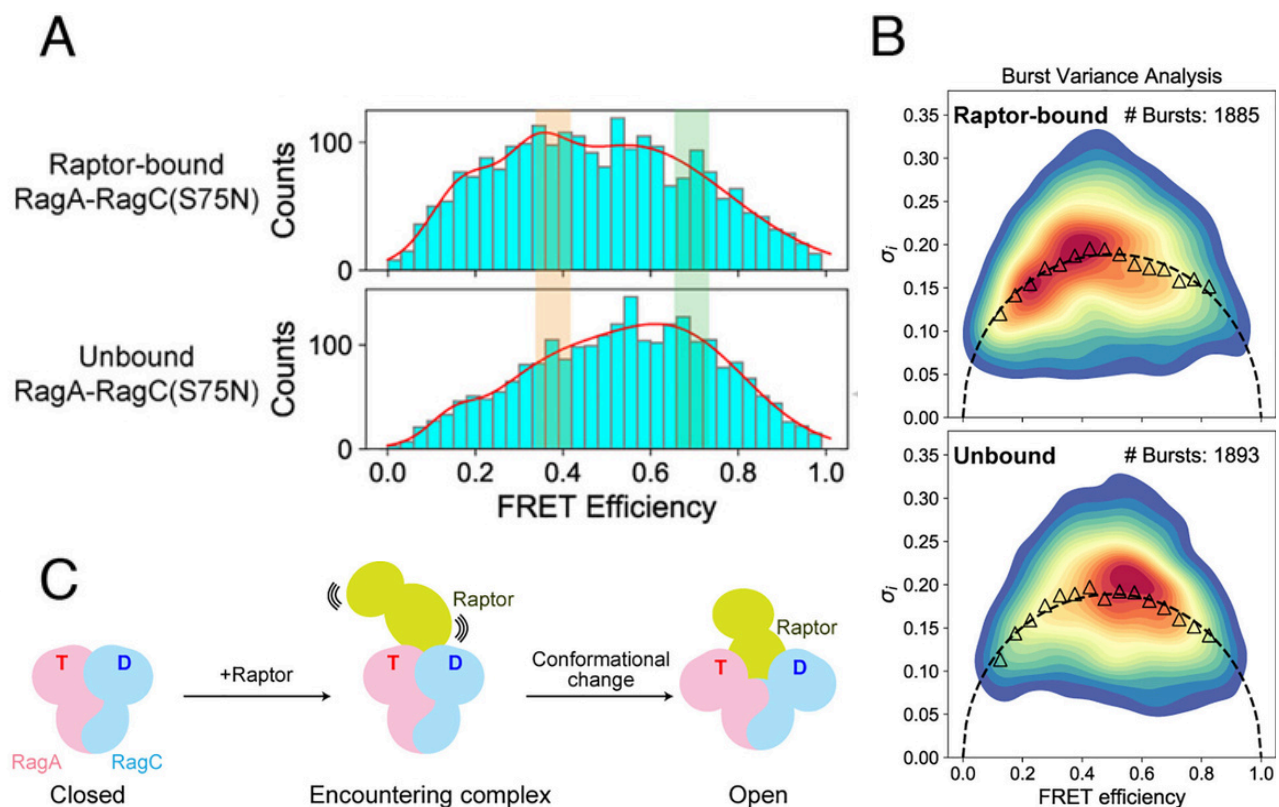


Figure 5 – Conformational changes of Rag GTPase upon Raptor binding

- A) FRET efficiency plots and B) burst variance analysis for unbound and Raptor-bound RagA-RagC(S75N)
C) Proposed structural changes of Rag GTPase upon Raptor binding

Summary

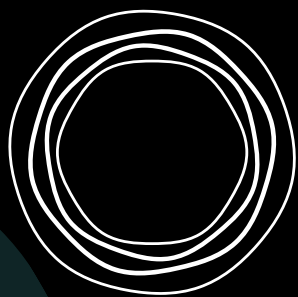
In summary, smFRET on the EI-FLEX system is a robust way to investigate the dynamic conformational changes of Rag GTPase across a range of conditions, including nucleotide binding and hydrolysis, domain mutations, and accessory protein interactions. Burst variance analysis and Hidden Markov modelling permitted the distinction between static and dynamic populations, and quantification of the interconversion rates between them. Data collected with the EI-FLEX system built a clear picture of the conformational landscape of the Rag GTPase heterodimer and how it influences downstream mTORC1 signalling.

For a deeper dive on the techniques used in this application note, we recommend exploring our [Resource Library](#). Discover a range of applications for smFRET and the EI-FLEX system on our website.

References

1. Doxsey, D. D. & Shen, K. Global conformation of the Rag GTPase heterodimer governs eukaryotic amino acid sensing. *Proceedings of the National Academy of Sciences* 122, e2517050122 (2025).*
2. Anandapadamanaban, M. et al. Architecture of human Rag GTPase heterodimers and their complex with mTORC1. *Science* (1979) 366, 203–210 (2019).

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