Purification-free smFRET: Utilising a universal protein ladder directly in mammalian cell lysates

Application note

This application note was produced in collaboration with the Twelvetrees lab at the University of Sheffield.

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The FRET community has long required a universal standard that can be used as a positive control to decouple troubleshooting novel biology from the labelling process and instrument readout, while also bridging the gap between *in vitro* and in-cell measurements. Recently, Smith et al. addressed the lack of a universal standard to harmonise FRET data across instruments, platforms, and labelling methods by producing a protein ladder for FRET[1]. Notably, their method is suitable for in-cell labelling and direct analysis of mammalian cell lysates, as well as for purified proteins.

In this application note, we demonstrate how this universal protein ladder can be integrated into smFRET analysis using two distinct in-cell labelling methods and performed directly on cell lysates, without the need to purify proteins of interest[1]. We also showcase a recent example of how in-cell labelling and direct smFRET analysis using cell lysates was performed on mammalian Kinesin-1 proteins[2].

Key takeaways:

- A universal protein ladder for FRET analysis provides a stable, modular benchmark to harmonise FRET measurements and a predictable calibration curve
- The protein ladder is formed of CLIP and SNAP tags flanking increasing TPR repeats
- smFRET can be performed directly on mammalian cell lysates, using proteins that have been labelled using in-cell methods via CLIP and SNAP tags, or site-directed mutagenesis and incorporation of non-canonical amino acids

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.

Self-labelling enzyme: Self-labelling enzymes are protein tags (typically 20-30 kDa) derived from modified enzymes that have been engineered to form a permanent, covalent bond with a specific synthetic ligand attached to a fluorophore. These enzymes label themselves by reacting specifically with their substrate.

Click Chemistry: A class of biocompatible, high-yield chemical reactions that ‘click’ two molecules together rapidly and reliably under mild conditions (such as room temperature and aqueous buffers). In protein labelling, it typically involves a reaction between an azide and an alkyne group. One part of the pair (e.g., an azide) is incorporated into the protein, often via a non-canonical amino acid, while the other part (e.g., a DBCO-alkyne) is attached to the fluorophore.

Non-canonical amino acid (ncAA): An unnatural amino acid that is not part of the standard 20 amino acids. They can be coded into a protein by encoding a stop codon and then using a specialised pair of an engineered tRNA and an aminoacyl-tRNA synthetase. This provides a highly targeted site for fluorescent labelling.

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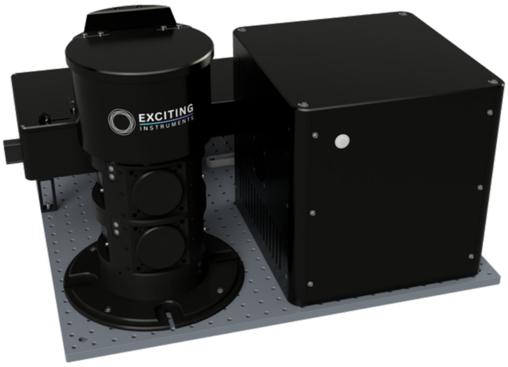

The EI-FLEX single-molecule spectrometer

A universal protein ladder for smFRET

Smith et al. recently addressed the lack of a universal standard to harmonise FRET data by developing a CLIP-SNAP fusion protein that can be extended using tetratricopeptide repeat (TPR) motifs. This functions as a protein ladder, providing a standardised reduction in FRET efficiency as more motifs are incorporated (Figure 1). This protein ladder can be characterised by direct analysis of mammalian cell lysates or purified proteins. It is also compatible with both self-labelling enzymes and non-canonical amino acid (ncAA) incorporation, as demonstrated by the authors.

In smFRET, where data is often used to resolve distinct structural states, the ladder offers a predictable calibration curve. By using a protein-based standard, which is more stable in intracellular environments than DNA-based alternatives, the ladder also enables consistent interpolation between diverse modalities, such as confocal smFRET, flow cytometry-based FRET, and Fluorescence Lifetime Imaging Microscopy.

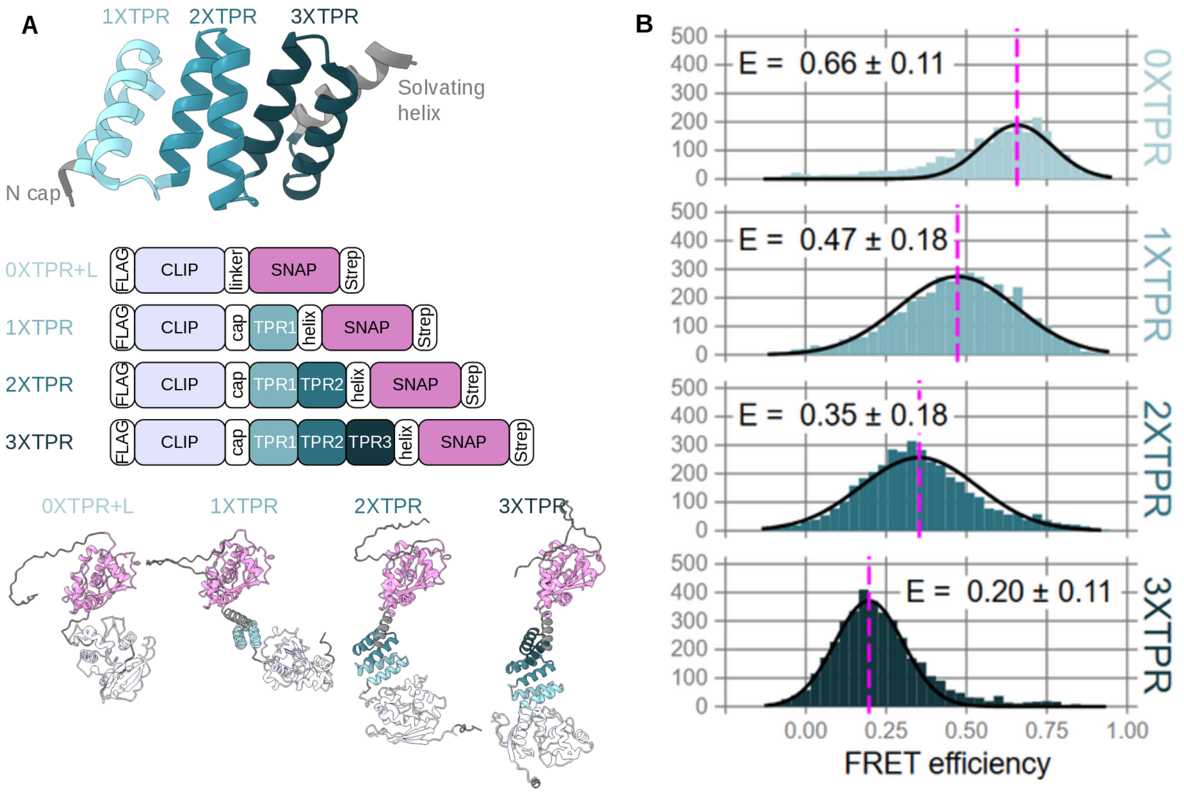

Figure 1 – A universal protein ladder for FRET

A) Schematic and protein structure of the protein ladder showing CLIP and SNAP tags flanking increasing TPR repeats

B) FRET efficiency histograms for ladder proteins with 0, 1, 2 or 3 TPR repeats; mean FRET efficiency ± standard deviation is indicated

Modified figure from publication by Smith et al.[1]

In-cell labelling for direct smFRET analysis of cell lysates

In-cell labelling of proteins for smFRET provides several benefits over labelling of purified proteins *in vitro*. These include the preservation of native folding and post-translational modifications, alongside the retention of endogenous binding partners, while avoiding artefacts associated with purification.

There are two main methods used for in-cell protein labelling for smFRET that enable direct analysis of diluted cell lysates: self-labelling enzymes, such as SNAP and CLIP tags, ncAA labelling. Both of these methods were used to generate data with the universal protein ladder.

Self-labelling enzymes

Cell lines such as HEK293 cells are transfected with a plasmid encoding a CLIP-SNAP fusion protein. These proteins are joined by a non-flexible linker, creating a distance between the two tags within the range for smFRET detection. Once transfected, the cells can then be incubated with dyes such as TMR-Star and 647 SiR ligands to label the CLIP and SNAP tags, respectively, and the unbound ligand washed away. In the example shown in Figure 2, the authors optimised ligand concentration, incubation time and washout time to ensure that minimal background was present from free dye[2]. This enabled smFRET to be performed directly on diluted cell lysates, with high reproducibility between biological repeats.

Non-canonical amino acid (ncAA) labelling

Cells are transfected with a plasmid encoding a CLIP-SNAP fusion protein with a mutated amino acid; in Figure 2, the glutamine residue at position 149 was replaced with an amber stop codon[1]. Two additional plasmids are also required, encoding the orthogonal aminoacyl-tRNA synthetase/tRNA pair (tR-NAPyl/NESPylRSAF) and an early release factor (eRF1-(E55D)), alongside the ncAA of choice (the authors used TCO*A). The ncAA is incorporated at the mutated position, which can then be labelled via copper-free click chemistry using tetrazine as the ligand. smFRET was performed directly on diluted cell lysates and purified protein, demonstrating highly reproducible FRET efficiencies between the two methods (0.58 ± 0.14 and 0.62 ± 0.12, respectively).

In-cell labelling for direct smFRET analysis of cell lysates

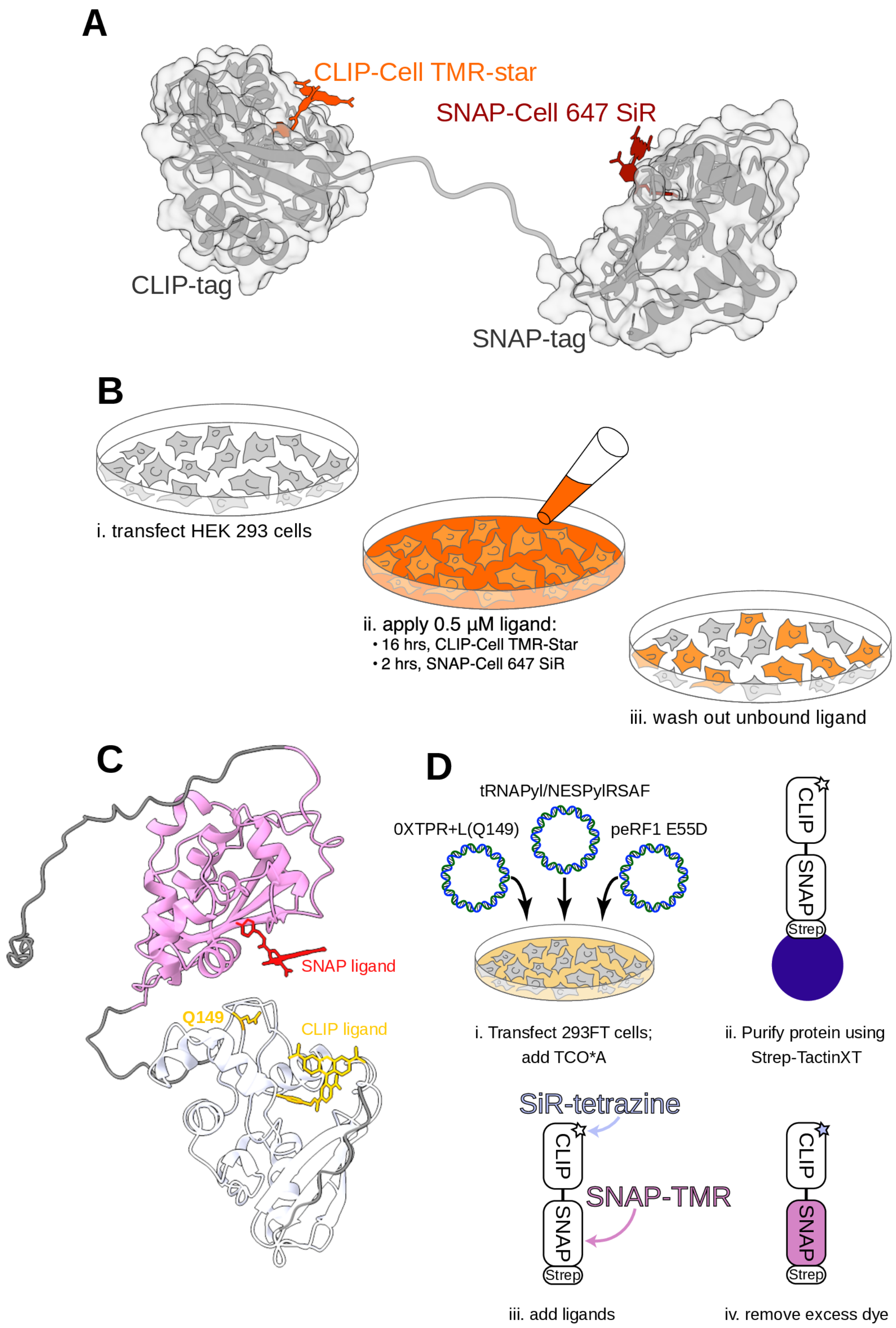

Figure 2 - In-cell labelling for smFRET using self-labelling enzymes

A schematic of the CLIP-SNAP fusion protein, detailing the sites that will be labelled by TMR-Star and 647 SiR, respectively, B) The workflow for in-cell labelling of HEK293 cells using self-labelling enzymes. C) Schematic of CLIP-SNAP fusion protein, detailing the sites for fluorescent labelling and the glutamine residue at position 149 for site-directed mutagenesis, D) The workflow for ncAA labelling of 293T cells, Modified figure from publications by Smith et al.[1,2]

A real-life example: Mapping the conformational dynamics of Kinesin-1 directly from cell lysates

The neuron-specific molecular motor KIF5A was inserted between CLIP and SNAP tags to create the fusion protein shown in Figure 3[2]. smFRET was performed directly on diluted cell lysates in the presence of NaCl or unlabelled microtubules to observe their effect on motor protein conformational heterogeneity and autoinhibition. In brief, the addition of 150 mM NaCl increased the chance of KIF5A autoinhibition, resulting in a more closed conformation with a high FRET efficiency. Equally, the addition of microtubules also increased this high FRET signal compared to KIF5A alone, indicating that transient autoinhibition of Kinesin-1 is more likely in the presence of microtubules when cargo is absent. Both populations are highly heterogeneous, highlighting the benefits of using smFRET to capture this diversity.

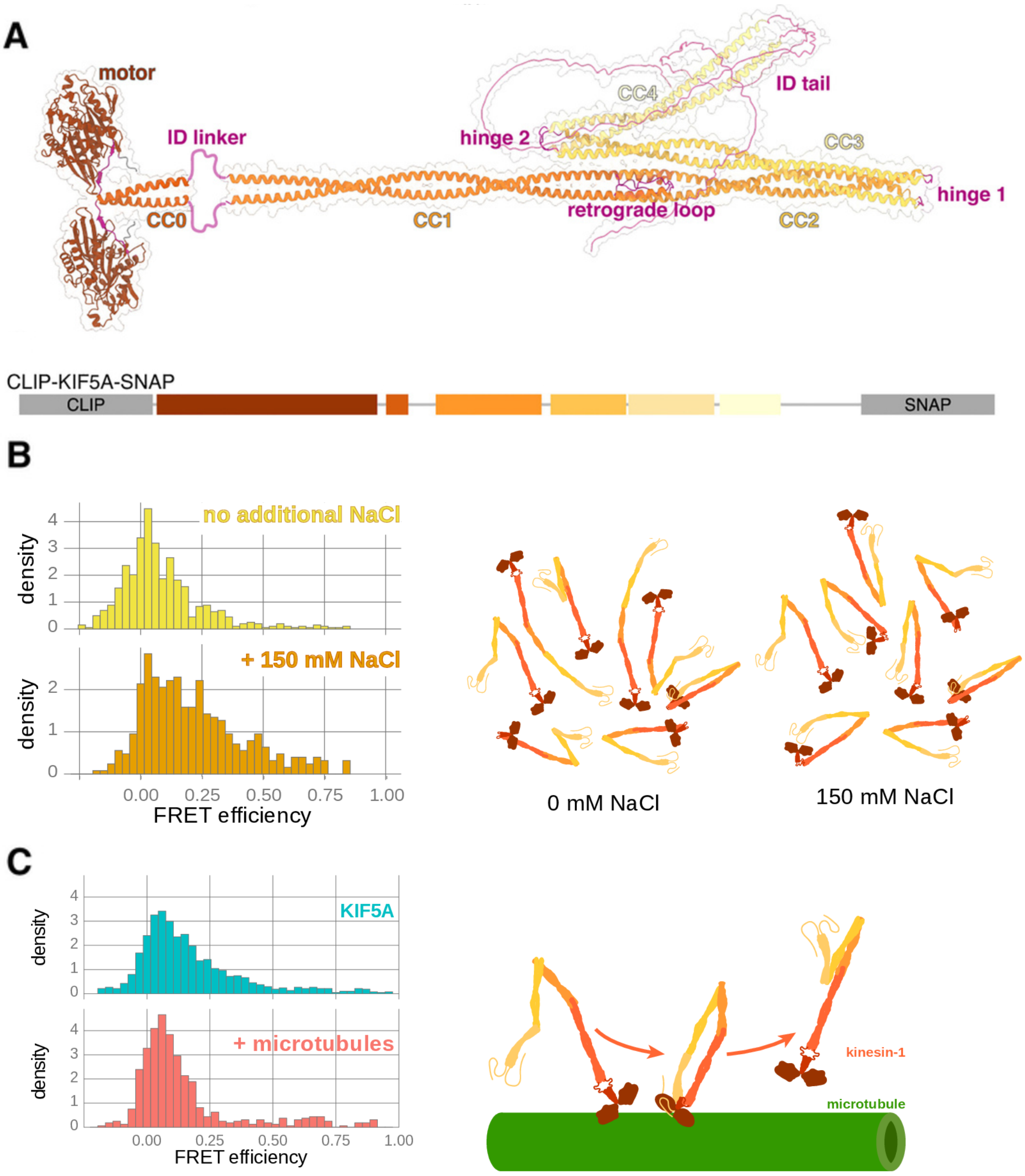

Figure 3 – Investigating the dynamic heterogeneity of Kinesin-1

A) Structure of mouse KIF5A and schematic of the positions for the CLIP and SNAP tags B) FRET efficiency histograms for labelled KIF5A with 0 or 150 mM NaCl (left), and a schematic showing predicted heterogeneous populations (right) C) FRET efficiency histograms for labelled KIF5A with or without unlabelled microtubules (left), and a schematic of predicted structures during microtubule interaction (right)

Modified figure from publication by Smith et al.[2]

Summary

smFRET can be performed directly in diluted mammalian cell lysates or on purified proteins, following fluorescent labelling by self-labelling enzymes such as CLIP and SNAP, or by site-directed mutagenesis and incorporation of non-canonical amino acids. The use of a universal protein ladder for FRET provides a positive control and a method for normalising results across different instruments and techniques.

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. Smith, E. R. et al. A universal protein ladder for standardisation of diverse FRET assays. bioRxiv 2026.03.05.709886 (2026) doi:10.64898/2026.03.05.709886.*
2. Smith, E. R., Turner, E. D., Abdelhamid, M. A. S., Craggs, T. D. & Twelvetrees, A. E. Kinesin-1 is highly flexible and adopts an open conformation in the absence of cargo. iScience https://doi.org/10.1016/j.isci.2026.114875 (2026) doi:10.1016/j.isci.2026.114875.*

*The figures used in this application note were derived from images and data published by the authors in these publications.

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