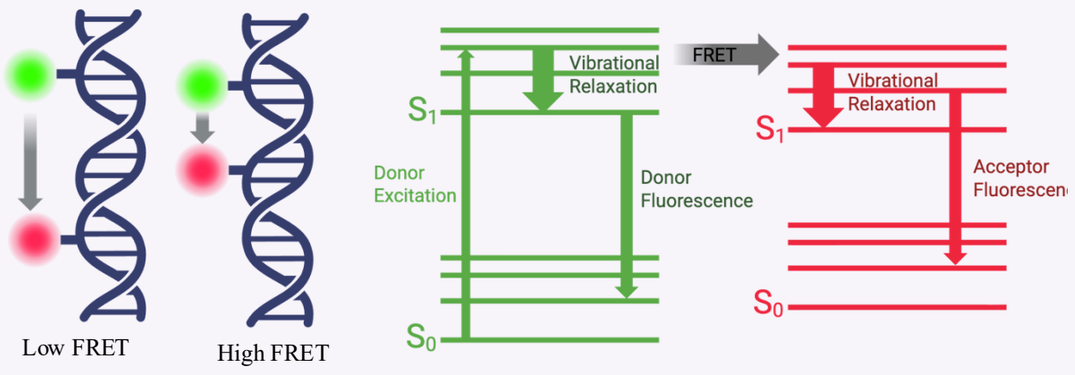
Expanding the Reach of FRET: Quantitative Quenching for probing DNA Allostery and Sub-3 nm Distance Determination

Sophie E Fountain[1], Benjamin Ambrose[1], Victoria E Hill[1], Gabriel Rosenblum[3], Rebecca Mächtel[2], Thorben Cordes[2], Hagen Hofmann[3], Timothy D Craggs[1]

[1]Department of Chemistry, University of Sheffield, UK, [2]Ludwig-Maximilians Universitat Muchen, Munich, Germany, [3]Structural Biology, Weizmann Institute, Rehovot, Israel

1. Fluorescence Resonance Energy Transfer (FRET)



- Fluorescence Resonance Energy Transfer (FRET) is the non-radiative transfer of excitation energy from a donor fluorophore to an acceptor fluorophore.
- Upon excitation, the donor transfers energy to the acceptor when they are in close proximity.
- The acceptor subsequently returns to its ground state by emitting a photon.
- FRET efficiency is distance-dependent.

FRET Efficiency

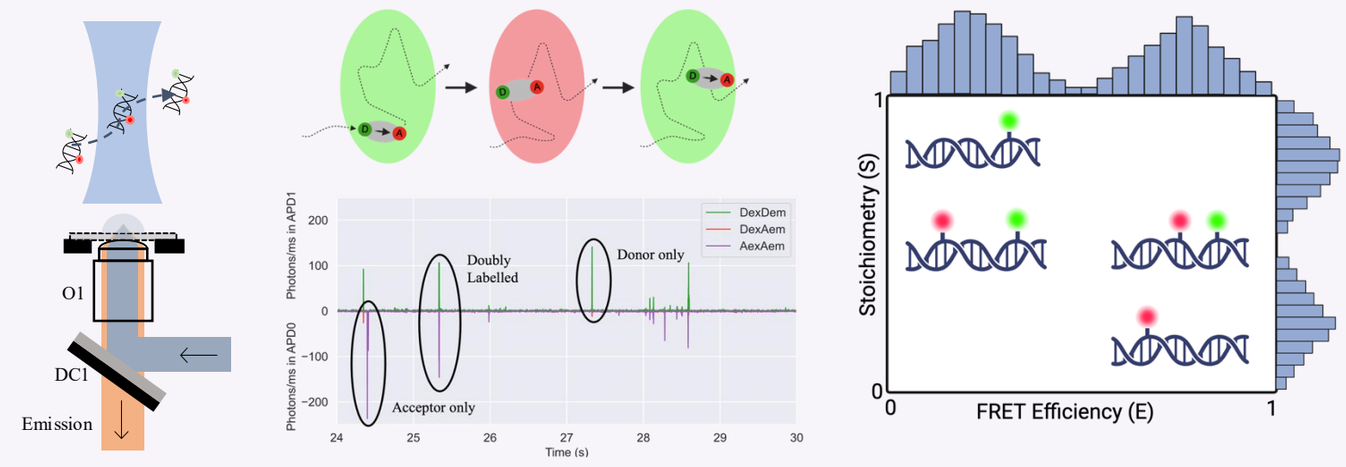
$$E = \frac{1}{1 + (\frac{R}{R_0})^6}$$

$$E^* = \frac{Acceptor}{Donor + Acceptor}$$

R = Donor-Acceptor distance
R_0 = Donor-Acceptor distance where E = 50%

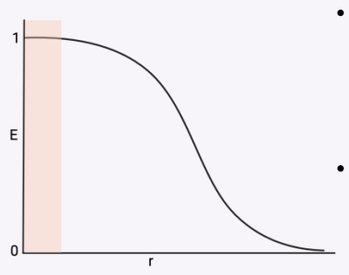
2. smFRET

Alternating-laser excitation (ALEX) uses two alternating lasers to directly excite the donor and acceptor fluorophores on single molecules.



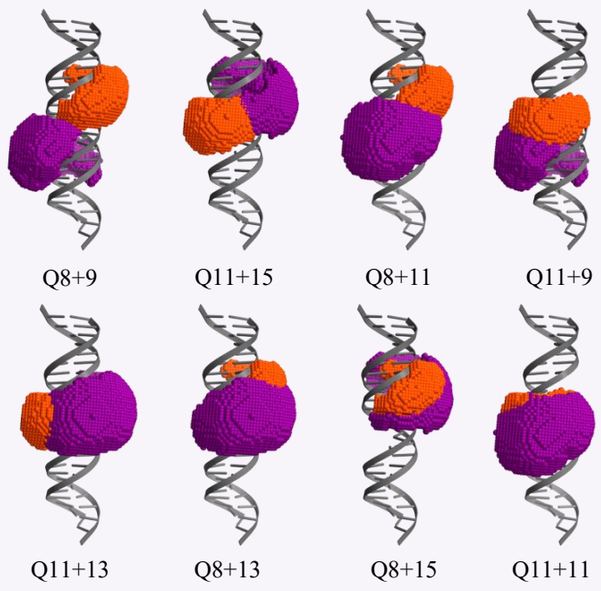
DNA concentration is kept low so that only one molecule is present in the detection volume at a time, producing distinct photon bursts.
By identifying donor-acceptor bursts and excluding donor-only or acceptor-only signals, the FRET efficiency of each DNA molecule can be determined.

3. Quenching Constructs and AV Overlap

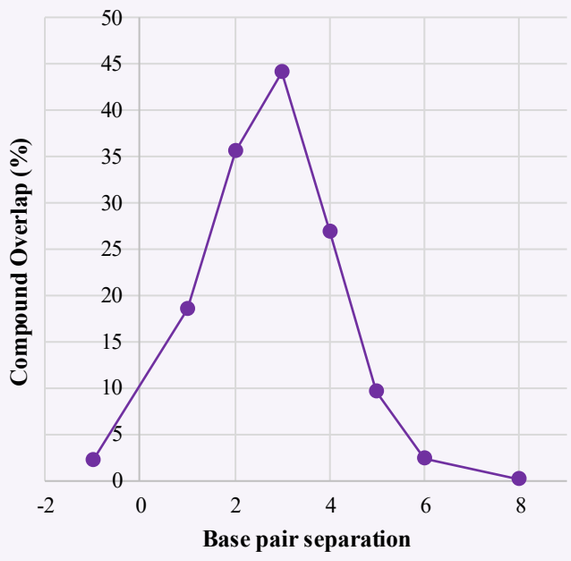


Contact Quenching

- Traditional smFRET loses sensitivity below ~3 nm due to the Förster radius (R_0).
- At these short distances, contact quenching reduces fluorescence through non-radiative energy transfer.
- Rather than treating quenching as a limitation, qqFRET uses it as an additional signal to extract short-range distance and structural information.

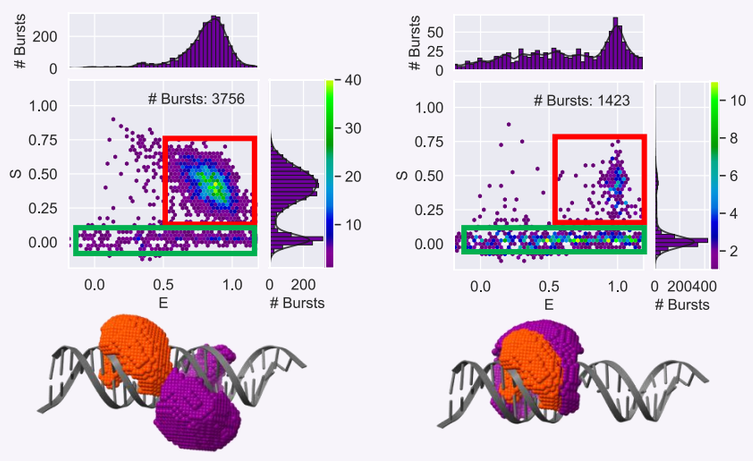


- DNA constructs with 2 donor and 3 acceptor positions were designed, yielding 8 constructs with separations from -1 to 8 bp.
- Accessible volume (AV) clouds were generated from the experimental sequence, and overlap was calculated by snapping one AV onto another on a 0-0.2 Å grid.
- Overlap peaked at 3 bp separation.



- The accessible volume (AV) model describes the range of positions a dye can occupy due to linker flexibility.
- AV calculations were performed with Olga software for Cy3B (donor) and ATTO647N (acceptor).
- The overlap between their AVs estimates the likelihood of direct dye contact and resulting quenching.

4. Quantitative qFRET



AV Overlap: 0.02%
Quenching Ratio: 24.8%

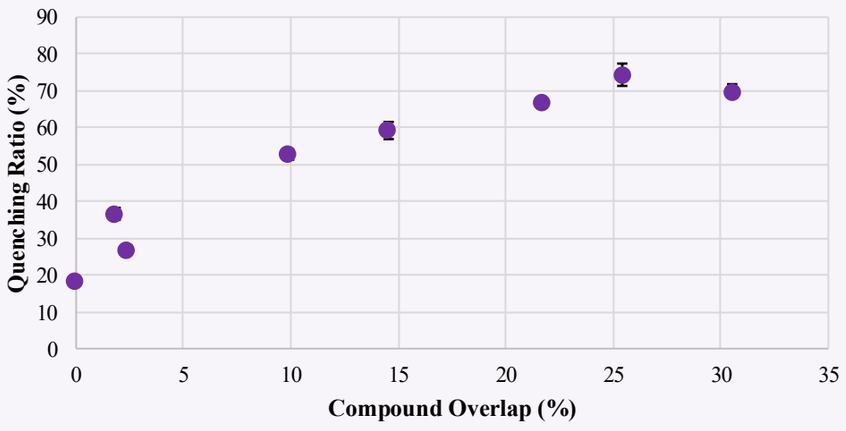
AV Overlap: 44.1%
Quenching Ratio: 71.6%

The quenching ratio (QR) is defined as:

$$QR = \frac{AA}{AA + FRET}$$

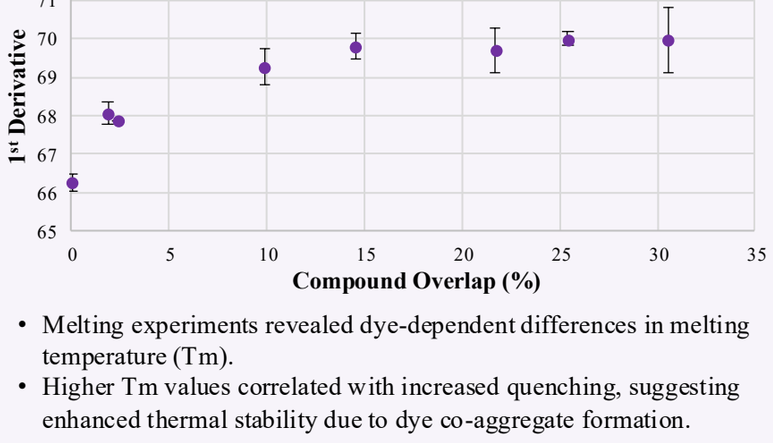
where AA is the acceptor-only population and FRET is the number of FRET events.

- Eight dsDNA constructs with varying Cy3B-ATTO647N separations were analysed to investigate quenching.
- Quenching increased with accessible volume (AV) overlap, being strongest at short distances (2-4 bp) and minimal at longer separations (6-8 bp).
- The results show that qqFRET can detect distance changes in the 10-25 Å range, extending sensitivity beyond the ~30 Å limit of standard FRET.



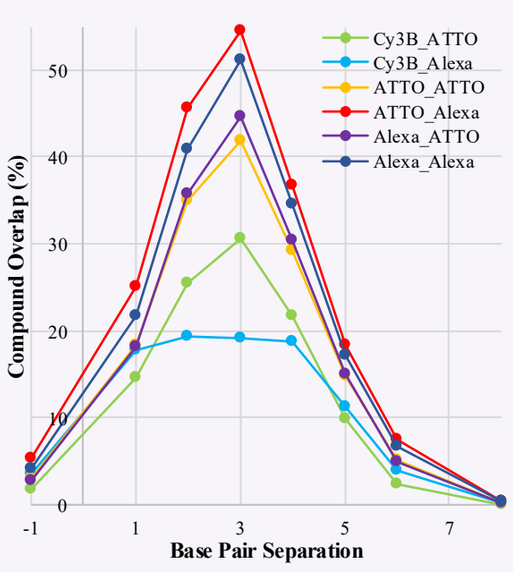
5. Melting Experiments

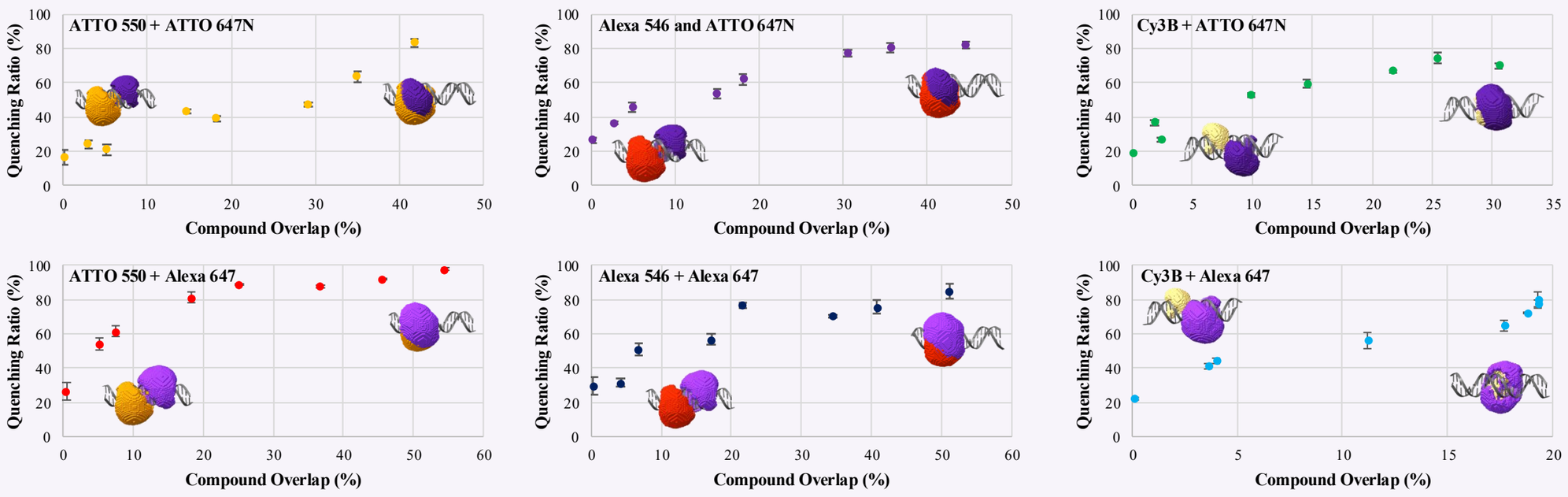
Construct	Compound Overlap	Quenching Ratio	1st derivative	Sd of 1st derivative
Q8+9	0.02	20.5	66.3	0.32
Q11+15	1.86	41	68.1	0.12
Q8+11	2.38	31.6	67.9	0.28
Q11+9	9.89	72.1	69.3	0.12
Q11+13	14.56	65.6	69.8	0.35
Q8+13	21.71	72.9	69.7	0
Q8+15	25.42	81.6	70	0
Q11+11	30.54	54.4	69.9	0.25



- Melting experiments revealed dye-dependent differences in melting temperature (Tm).
- Higher Tm values correlated with increased quenching, suggesting enhanced thermal stability due to dye co-aggregate formation.

6. Different dyes





7. ComK

ComK binds cooperatively to promoter DNA, inducing structural changes associated with competence.

Labelled within spacer region at 1 and 2 base pair separation.

1 Base Pair Separation (1 bp)

	Unbound (%)	Bound (%)	Percentage Change (%)
Overlap Prediction from Model	10.80	15.60	44.4
Quenching Ratio from measurement	59.50	90.08	51.4

2 Base Pair Separation (2 bp)

	Unbound (%)	Bound (%)	Percentage Change (%)
Overlap Prediction from Model	11.60	27.04	133.1
Quenching Ratio from measurement	43.44	95.85	120.6

- qqFRET measurements showed increased quenching upon ComK binding, in close agreement with accessible volume overlap predictions.
- This strong correlation validates qqFRET as a sensitive method for detecting DNA conformational changes and quantifying protein-DNA interactions beyond the resolution of traditional FRET.

8. MalE

Maltose-binding protein is part of the maltose transport system and undergoes a conformational change from an open to a closed state upon maltose binding.

Compound Overlap: 1.3%
Quenching Ratio: 37.7%

Compound Overlap: 8.7%
Quenching Ratio: 57.1%

- qqFRET measurements on labelled MalE showed high sensitivity to protein binding, enabling both qualitative and quantitative analysis with a Kd of ~1–2 µM.
- These results demonstrate the potential of qqFRET for studying protein interactions and characterising biologically relevant targets.

Acknowledgements

Thank you to BBRSC White Rose for funding my PhD Project.
Thank you to Craggs Lab and Exciting Instruments for support with the project.

References

1. T. Cordes, Y. Santoso, A. I. Tomescu, K. Gryte, L. C. Hwang, B. Camará, S. Wigneshweraraj and A. N. Kapanidis, **Biochemistry**, 2010, *49*, 9171–9180, DOI: 10.1021/bi101184g.
2. J. Hohlbein, T. D. Craggs and T. Cordes, **Chem. Soc. Rev.**, 2014, *43*, 1156–1171, DOI: 10.1039/c3cs60233h.
3. G. Rosenblum, N. Elad, H. Rozenberg *et al.*, **Nat. Commun.**, 2021, *12*, 2967, DOI: 10.1038/s41467-021-23148-2.
4. R. Mächtel, A. Narducci, D. A. Griffith, T. Cordes and C. Orelle, **Res. Microbiol.**, 2019, *170*, 321–337, DOI: 10.1016/j.resmic.2019.09.004.
5. G. Agam, C. Gebhardt, M. Popara *et al.*, **Nat. Methods**, 2023, *20*, 523–535, DOI: 10.1038/s41592-023-01807-0.