Quantifying affinity, cooperativity, and kinetic stability in ternary complexes using FCCS

Application note

Developing next-generation biotherapeutics, such as bispecific antibodies, antibody combination cocktails, molecular glues, and PROTACs, requires evaluating multi-protein interaction dynamics that binary assays cannot directly capture. Using the clinically validated dual-HER2 antibody combination (trastuzumab and pertuzumab), this application note demonstrates how Fluorescence Cross-Correlation Spectroscopy (FCCS) provides direct, solution-phase quantification of ternary assembly, cooperativity (α), and kinetic stabilisation (k_{off}) in microplate formats[1]. FCCS measures the cross-correlation between two fluorescent species; in the case of trastuzumab-HER2-pertuzumab, cross-correlation will only occur when HER2 bridges the two antibodies, forming a ternary complex.

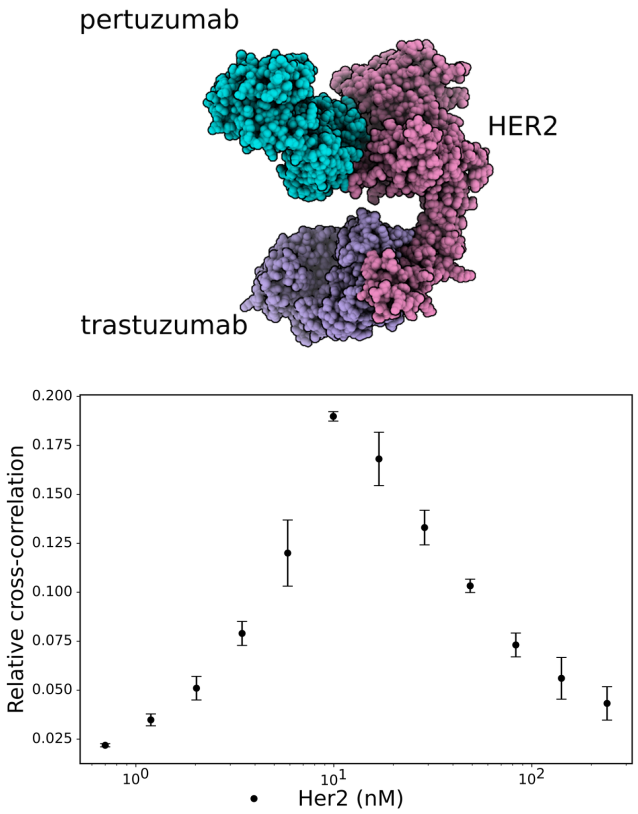

Figure 1 - FCCS directly quantifies dual-HER2 antibody ternary complexes and produces hook plots, determining cooperativity and optimal therapeutic concentration

Overview of this application note:

- FCCS directly quantifies ternary complex formation using cross-correlation, identifying a cooperativity factor of 3.1 for the complex and slower dissociation rates compared to binary species, indicating favourable stabilisation
- Competitive chase FCCS assays confirmed that each of the antibodies binds different epitopes, but dissociation kinetics revealed that pertuzumab may perturb some trastuzumab-HER2 complexes
- FCCS can also be used to derive K_D values for individual antibody-HER2 complexes

Cross-correlation directly identifies ternary complexes, positive cooperativity and complex stabilisation

FCCS assays captured ternary complex formation between labelled antibodies and unlabelled HER2. A titration of HER2 was performed, revealing a characteristic hook plot as HER2 concentration increased (Figure 2a & 2b). Cooperativity values were calculated using the titration data, binary HER2 binding affinities of trastuzumab and pertuzumab, and a thermodynamic equilibrium model, revealing positive cooperativity ($\alpha = 3.1 \pm 0.3$). Importantly, the calculated cooperativity mapped directly onto the hook plot generated from FCCS data, showing that cooperativity can be determined directly from FCCS ternary complex data without requiring data from binary species first.

Dissociation kinetics were also calculated using these binary and ternary data; normalised bound fractions (determined from the cross-correlation signals) were followed over fifty hours and fitted with single-exponential dissociation models (Figure 2d). Here, 1.5-fold (pertuzumab) and 1.9-fold (trastuzumab) lower dissociation rates were observed for antibodies in preformed ternary complexes relative to binary species, suggesting that engagement of HER2 by trastuzumab and pertuzumab kinetically stabilises the assembled complex.

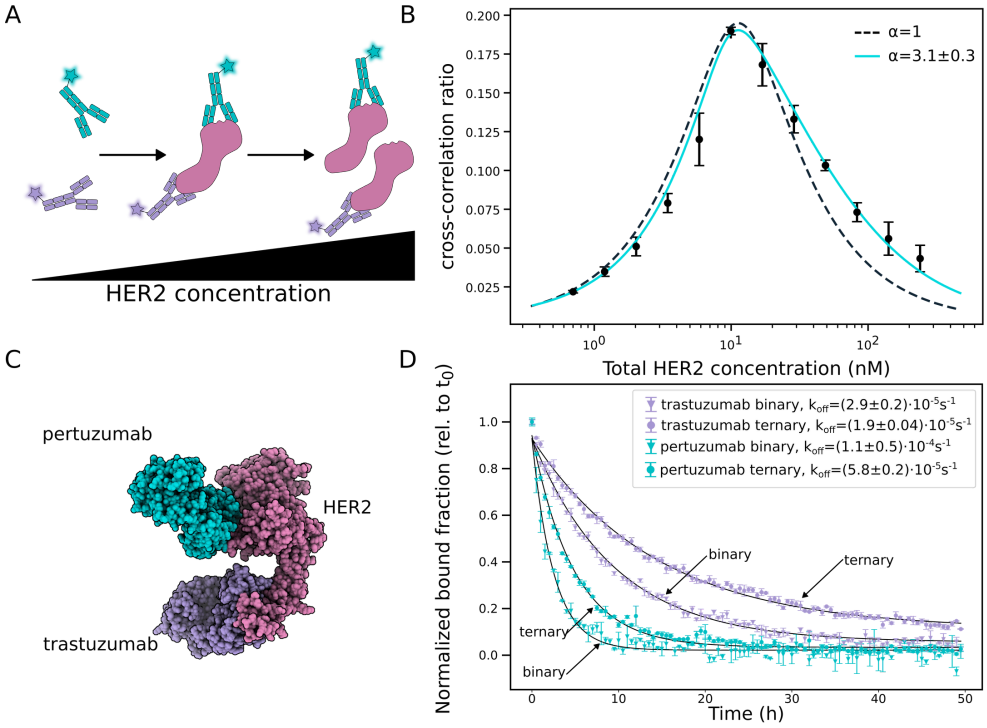

Figure 2 – FCCS directly determines ternary complex formation, cooperativity and dissociation kinetics
A) Schematic of the hook effect with increasing HER2
B) Ternary complex formation of Trastuzumab-HER2-Pertuzumab as a function of unlabelled HER2 concentration; error bars are standard error of the mean, n=3. The dashed line is a numeric fit with the cooperativity parameter α held constant at 1, the cyan line is a numeric fit with α freely fit.
C) The trastuzumab-HER2-pertuzumab ternary complex
D) Dissociation rates of trastuzumab (purple)/ pertuzumab (cyan) from the binary/ternary complexes. Complexes were preformed at saturating/maximal conditions (10 nM of each component).

Competitive-chase FCCS assays confirm trastuzumab and pertuzumab bind distinct HER2 epitopes

Competitive-chase FCCS assays were set up to calculate the dissociation kinetics of the therapeutic antibodies and resolve whether they bind to different epitopes on HER2 (Figure 3a). Dissociation of the labelled antibody would result in a loss of cross-correlation, which was measured over a time course. An orthogonal experiment was also performed; dissociation here would indicate that the two antibodies were competing for the same epitopes on HER2 (Figure 3b).

After 48 hours, cross-correlation for the competition assays had dropped almost to zero, and molecular dissociation rates of $2.9 \pm 0.2 \times 10^{-5} s^{-1}$ for trastuzumab and $1.1 \pm 0.5 \times 10^{-4} s^{-1}$ for pertuzumab were calculated, indicating that trastuzumab dissociates at a slower rate (Figure 2c). Interestingly, in the orthogonal experiments, addition of trastuzumab did not disrupt pertuzumab-HER2 complexes, but the inverse resulted in a reproducible 25 % reduction in trastuzumab-HER2 complexes (Figure 3d). While these data confirm that the two antibodies bind distinct epitopes, pertuzumab binding may perturb the stability of some trastuzumab-HER2 complexes in an allosteric manner.

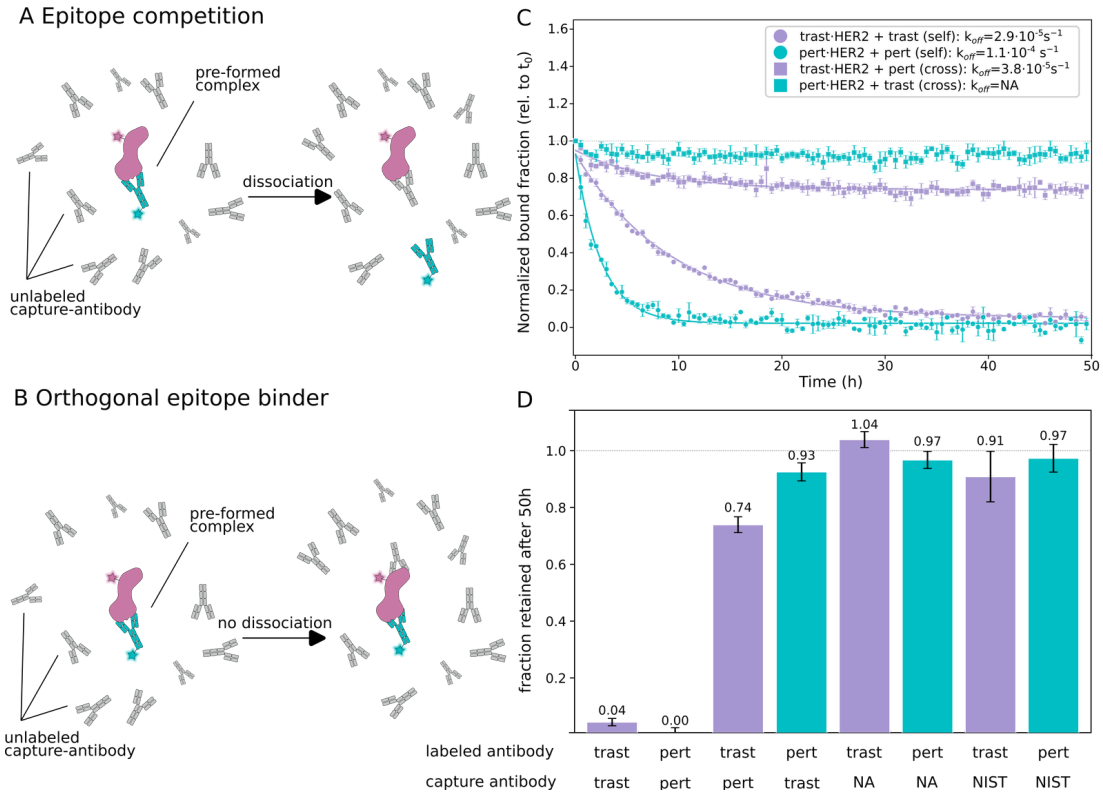

Figure 2 – FCCS epitope binding assays determine distinct binding sites and dissociation kinetics

A&B) Schematic of epitope competition (top)/orthogonal binder (bottom) experiment: Preformed complexes of labelled antibodies and targets were subjected to excess unlabelled competitors to monitor dissociation kinetics.

C) Dissociation of trastuzumab (purple) and pertuzumab (cyan). The controls show trastuzumab competing with unlabeled pertuzumab and vice versa.

D) Normalised cross-correlation fraction retained after 48 h in the presence of capture antibodies: trastuzumab (trast), pertuzumab (pert), None (NA) or non-specific control IgG (NIST).

Cross-correlation data permit the calculation of individual K_D values for antibody-HER2 complexes

Finally, FCCS was used to calculate individual equilibrium dissociation constants (K_D) values for antibody-HER2 complexes. For these experiments, titrations of labelled HER2 were plated with fixed concentrations of labelled antibodies and cross-correlation values were plotted against HER2 concentration to determine K_D values. Dissociation constants for trastuzumab and pertuzumab to HER2 were found to be $K_{D,trast}$ = 1.34 ± 0.14 nM and $K_{D,pert}$ = 1.18 ± 0.10 nM (Figure 4).

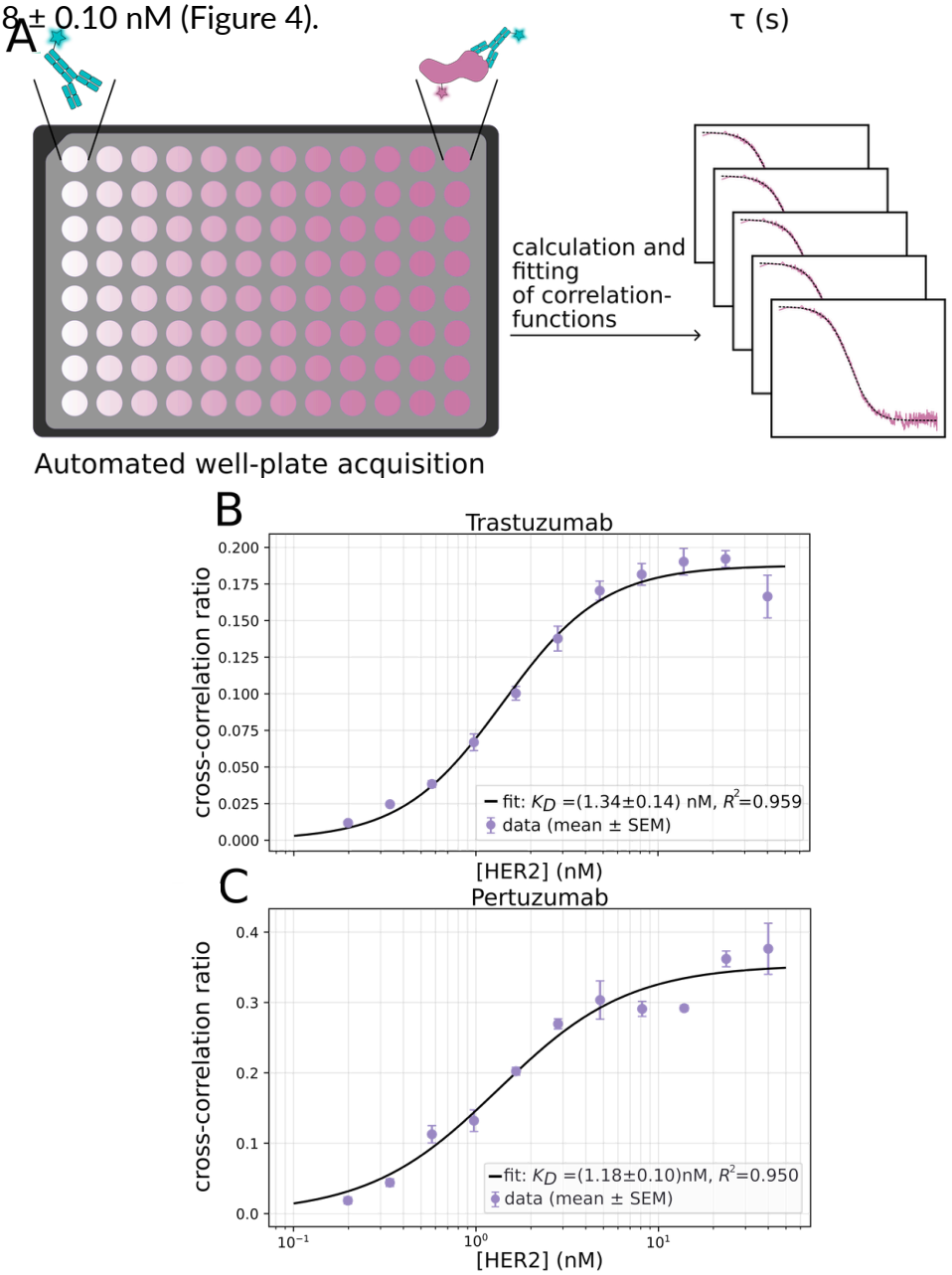

Figure 4 – FCCS analysis of binary complexes determines K_D values
A) Well plate set up showing titration of labelled HER2 against labelled antibody
B) Titration of HER2 to trastuzumab. Error bars depict the standard error of the mean. The data were fit with a quadratic binding model.
D) Titration of HER2 to pertuzumab. Error bars depict the standard error of the mean. The data were fit with a Hill equation

Summary

FCCS was used to characterise the trastuzumab-HER2-pertuzumab ternary complex in several ways: direct quantification of the ternary complex, generating a hook plot that reveals 3-fold positive cooperativity and identifies the optimal concentration of HER2 to be approximately 10 nM for maximising ternary complex formation. Critically, these parameters could be directly determined from the ternary data without requiring analysis of binary species first. In addition, dissociation constants were also calculated, highlighting subtle differences between the antibodies and confirming increased stability of the ternary complex relative to binary species. Lastly, FCCS confirmed that trastuzumab and pertuzumab bind distinct epitopes on HER2 and calculated individual K_D values for these interactions.

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 Pro system on our website.

The EI-FLEX Pro System

The EI-FLEX Pro provides fully integrated, high-throughput workflows for FCCS, with automated sample positioning, focus maintenance and acquisition of 96- and 384-well plate formats. Guided sample acquisition and analysis are performed using dedicated software and bespoke workflows.

The EI-FLEX Pro single-molecule spectrometer

References

1. Mueller, S. H., Mohanan, G., Abdelhamid, M. A. S., Toseland, C. P. & Craggs, T. D. Fluorescence cross-correlation spectroscopy quantifies affinity, cooperativity, and kinetic stability in ternary protein complexes. bioRxiv 2026.07.16.738902 (2026) doi:10.64898/2026.07.16.738902.

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