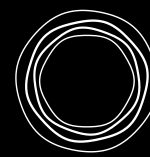

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Antibody-antigen binding affinity screening using FCS

Application note

Produced in collaboration with the Coleman Lab - Lawrence Livermore National Laboratory

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In this application note, we demonstrate how fluorescence correlation spectroscopy (FCS) can assess antibody-antigen binding affinities and generate K_d values for candidate molecules. Nikfarjam et al. used the EI-FLEX to assess the binding of single-chain variable fragments (scFvs) and antibody fragments (Fabs) against the receptor binding domain (RBD) of the SARS-CoV-2 Spike protein[1]. A homebuilt system was used to perform FCS on full-length antibodies.

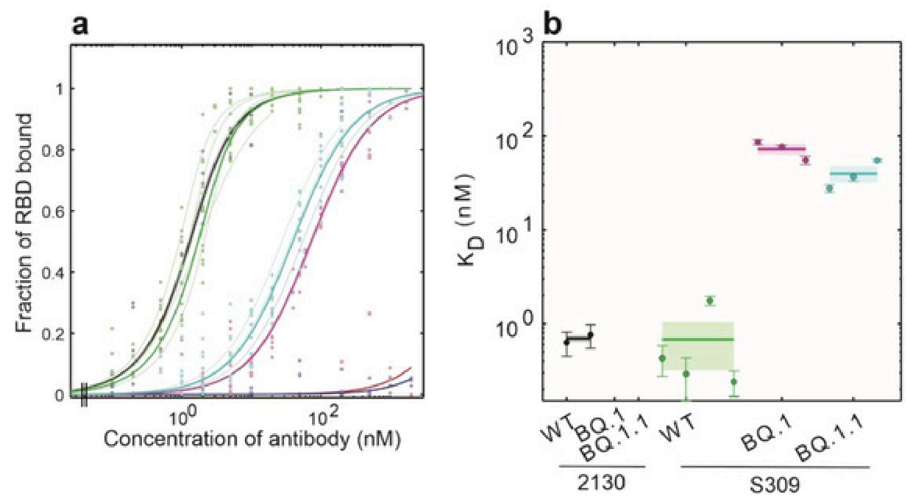

Figure 1 – Comparison of different antibodies and RBD mutants on binding and K_d values

a) Fraction of bound RBD plotted against antibody concentration and b) K_d values for Black: WT RBD with 2130 IgG, Green: WT RBD with S309 IgG, Pink: BQ.1 RBD mutant with S309 IgG, Blue: BQ.1.1 RBD mutant with S309 IgG
Data collected on a homebuilt system.

Overview of this application note:

- FCS can determine K_d values for a range of binders, such as monoclonal antibodies, scFvs and Fabs
- High-throughput data acquisition and analysis permit rapid assessment of binding affinity for molecules produced by different methods, such as in cell-free systems
- Different labels, such as FLAG-tags, can impact antibody-antigen binding

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FCS analysis of antibody-antigen binding affinities

To demonstrate how FCS can be used to determine antibody-antigen binding affinities, Nikfarjam et al. first used a homebuilt system to analyse a well-documented monoclonal IgG (S309 IgG) that is known to bind to a range of RBD variants at nanomolar affinities. Wild-type RBD was kept at a constant concentration of 1 nM, while levels of S309 IgG ranged from 0-50 nM to perform a titration.

Higher concentrations of S309 IgG induced a progressively rightward shift in FCS correlation curves, indicating that RBD binding was occurring; complexes diffuse more slowly through a confocal volume than individual molecules (Figure 2). To calculate K_d values, correlation curves were used to determine diffusion times. The plateauing of diffusion time indicated antibody saturation, and the fraction of bound RBD was calculated using a two-component diffusion model. By plotting the fraction of bound RBD against antibody concentration, the K_d value was derived as 0.240 nM.

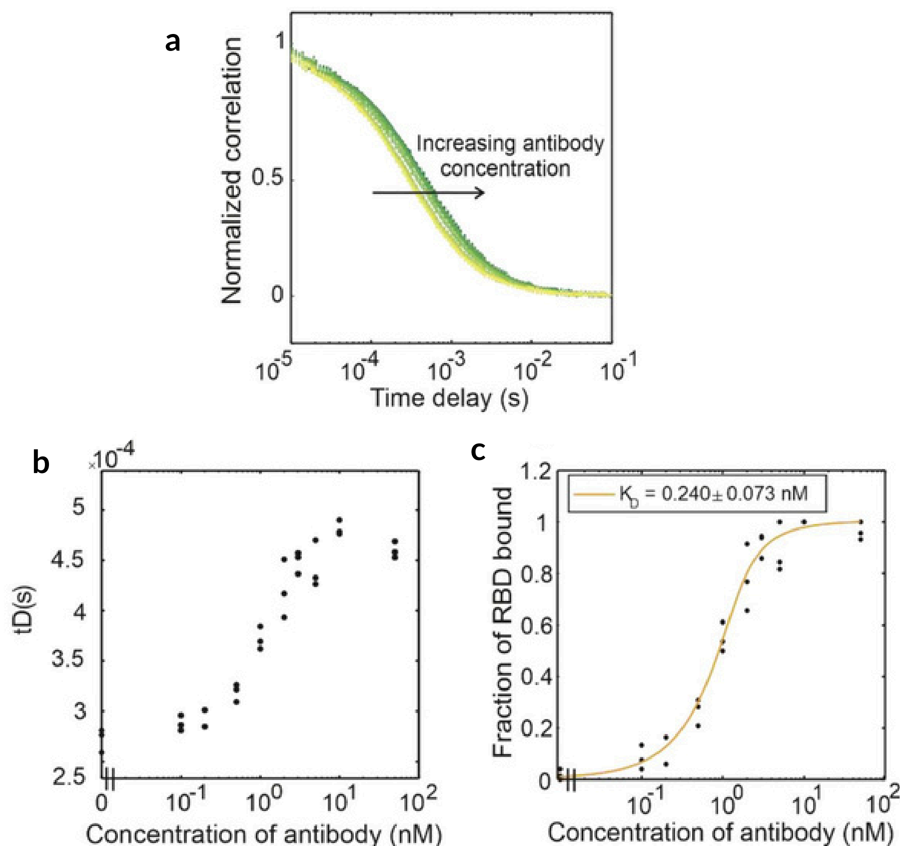

Figure 2 – Calculation of K_d values for antibody-RBD binding from FCS correlation curves and diffusion time
a) FCS correlation curves for various antibody concentrations, indicating slower diffusion time with increasing antibody concentration
b) Diffusion time plotted against antibody concentration
c) Fitting of bound RBD protein against antibody concentration to derive K_d value
Data collected on a homebuilt system.

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High-throughput screening of scFv variants and scalable production methods

Given that IgG production is expensive, Nikfarjam et al. generated scFvs to enable faster and simpler manufacturing of RBD binders without the concern of immunogenic Fc regions. They produced variants using a cell-free protein expression platform with a modified two-stage refolding protocol to maximise yield and ensure scalability. They performed FCS on the EI-FLEX to test the binding affinities of the candidate scFvs, alongside exploring whether their modified protocol also affected the K_d values compared to a more conventional method of scFv production (soluble method). Eight scFv variants were tested, with five candidates showing a range of K_d values on the nanomolar scale (Figure 3). K_d values between scFv variants made by the two-stage refolding protocol and the established soluble method were reported to be comparable, allowing confirmation that these production methods were not affecting overall binding affinity of the same variants.

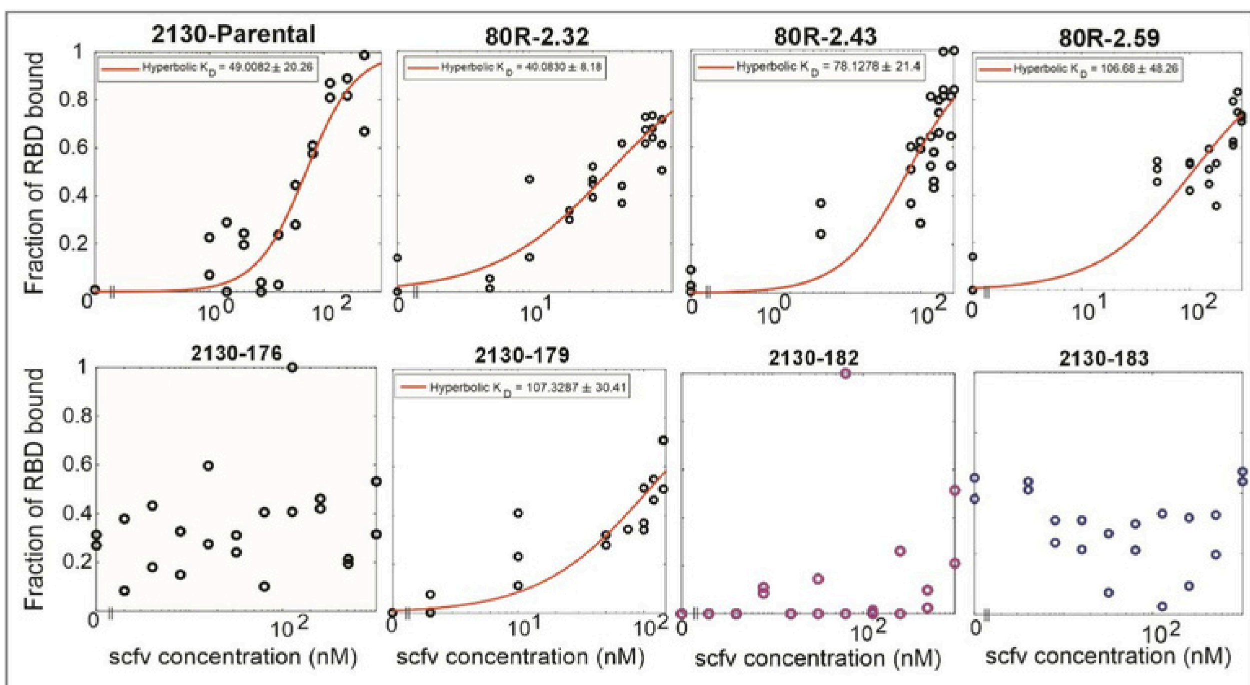

Figure 3 – Screening of eight candidate scFvs for nanomolar K_d values
Fraction of bound RBD plotted against antibody concentration with hyperbolic fitting to derive K_d values.
Data collected on the EI-FLEX.

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Function assessment of Fabs produced by cell-free methods

To take this a step further, the authors used their cell-free production system to generate Fab fragments, which have more similarities to IgG antibodies than scFvs. Binding affinities via FCS were assessed for two Fab fragments, one generated with a His-tag and the other with a His-tag and a FLAG-tag. A measurable binding affinity was achieved with the His-only Fab fragment, around ten-fold weaker than the corresponding full-sized IgG molecule (Figure 4). The Fab fragment that contained a FLAG-tag did not appear to bind to the RBD, indicating that tag interference with epitope availability or conformation may have occurred. Here, FCS was able to rapidly demonstrate that production of functional, cost-effective Fab fragments is feasible, while giving valuable information about how tagging during antibody generation in cell-free systems might affect antigen binding.

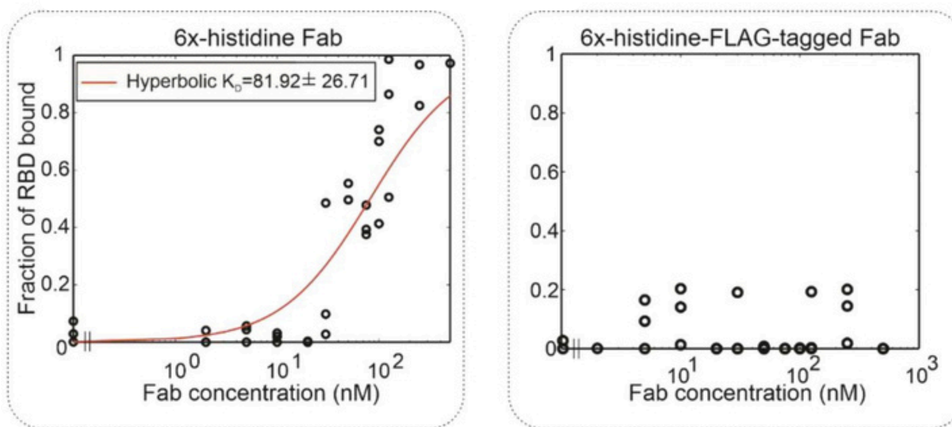

Figure 4 – Impact of his-tag and FLAG-tags on Fab fragment binding affinity to RBD
Left) His-tag only Fab fragment shows binding to RBD, permitting calculation of a K_d value
Right) Presence of a FLAG tag disrupts binding to RBD
Data collected on the EI-FLEX.

In summary, FCS offers a simple but powerful in-solution method for measuring binding affinity and calculation of K_d values. This rapid, high-throughput workflow enables iterative testing of candidate molecules and the methods used to create them, ensuring ease of scalability without compromising accuracy.

For a deeper dive on the techniques used in this application note, we recommend exploring our Resource Library. Discover a range of applications for FCS and the EI-FLEX platform on our website.

References

1. Nikfarjam, S. et al. Rapid Antibody Fragment Production and Binding Analysis Using Cell-Free Protein Synthesis Combined with Fluorescence Correlation Spectroscopy. bioRxiv 2025.08.05.668779 (2025) doi:10.1101/2025.08.05.668779.

All data used in this application note was generated by the authors cited in this publication.
Figures 1 and 2 were generated on a homebuilt system. Figures 3 and 4 were generated on the EI-FLEX.

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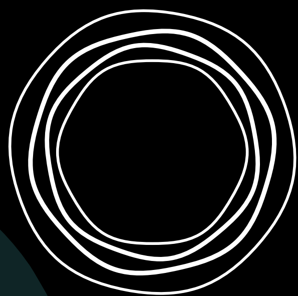

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