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SMASH: Single Molecule Antibody Screening with High-throughput imaging system

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INTRODUCTION

Larger, more diverse antibody libraries are being screened in recent years. Despite enabling the high-throughput screening of extensive libraries, the process for identification and characterization of antibodies remains laborious and time-intensive.

Here we report a high-throughput antibody affinity measurement platform utilizing single-molecule protein interaction detection (SPID) technology that not only compiles hit finding and validation processes, but also provides high-resolution binding affinity (K_D) as well as rank order among the library being screened.

We streamlined the antibody library construction and affinity screening via microscale transient expression in HEK293T cells with designed antibody sequence and direct quantification of antibody-antigen binding affinity which reduced the timeline from multiple weeks to a single week.

METHODS

We constructed single-chain Fv and Fab libraries by ligating duplex DNA-encoded variants into the CDRs of interest to investigate affinity changes. This sequence-to-affinity matching approach enables us to bypass traditional sequencing once hits have been identified through antibody screening **(Fig. 1)**.

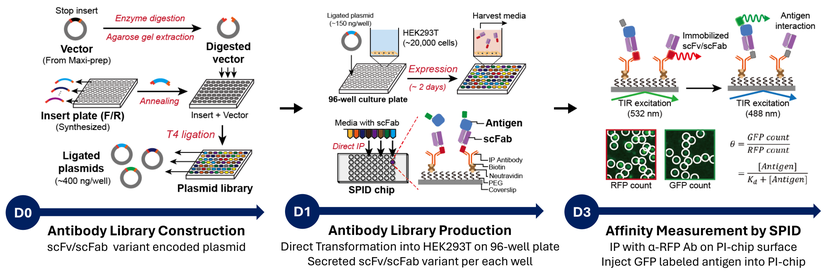

Fig. 1 Schematic illustrating the workflow of the SMASH.

The Single-molecule resolution of SPID technology provides ultra-sensitivity **(Fig. 2)**, with the SMASH platform determining binding affinity (KD) using just 10 pg of crude antibody, contrasting with techniques like SPR and BLI that typically necessitate DNA amplification to produce µg of antibody for analysis. The SMASH platform consumes 10^6 times less antibody.

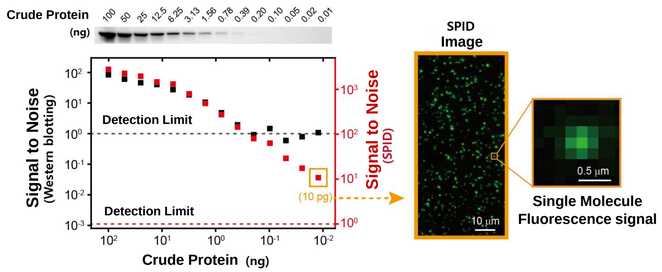

Fig. 2 Ultra-sensitivity of SPID enabling the analysis of crude protein types.

USE CASE 1. AFFINITY MATURATION

In this study, we produced 8 FDA-approved Fab antibodies and 2 targeting the Coronavirus spike protein. Their binding affinity (K_D) was assessed using SPID and compared with SPR, revealing a correlation coefficient of 0.98. Single-point measurements at equilibrium provide reliable K_D determination, consistent with binding curve analysis **(Fig. 3)**.

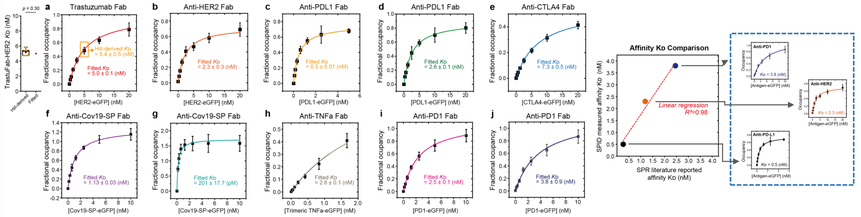

Fig. 3 Binding affinity K_D of 10 Fab antibodies against 6 therapeutic antigens was measured using SPID (a-j), with SPR comparison (right).

As a proof-of-concept, we generated a site-saturated mutagenesis library comprising 720 Trastuzumab scFab variants, with 9% exhibiting higher affinity than the wild-type (WT scFab, K_D 5 nM) **(Fig. 4)**. Subsequently, key residues were selected to design a 200-variant combinatorial library, where 50% demonstrated higher affinity than the WT, resulting in sequences with 2-5 times higher affinity compared to the WT.

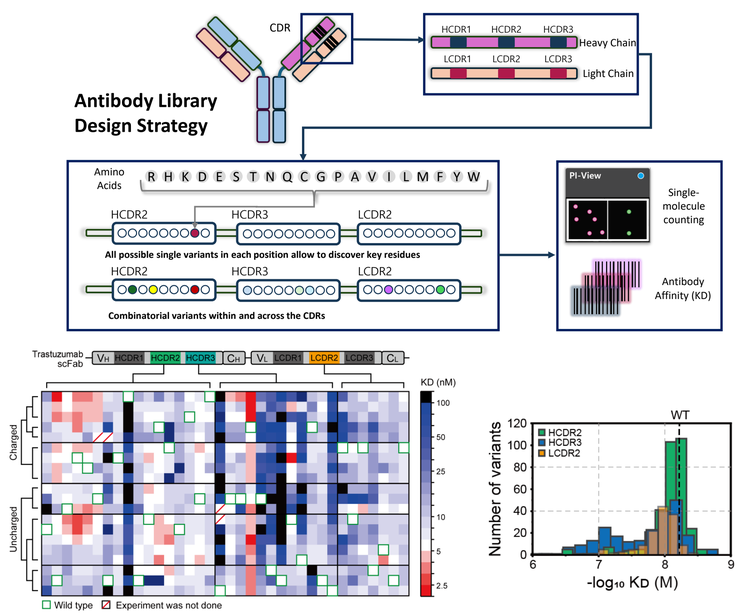

Fig. 4 Antibody library design strategy (top) and K_D distribution heatmap of 720 Trastuzumab scFab variants from site-saturated mutagenesis library (bottom).

USE CASE 2. CAR-T BINDER TUNING

The unmet needs in CAR-T therapy involve selecting CAR binders with moderated affinity and fast dissociation rates (k_{off}) to enhance both the efficacy and safety **(Fig. 5.)**.

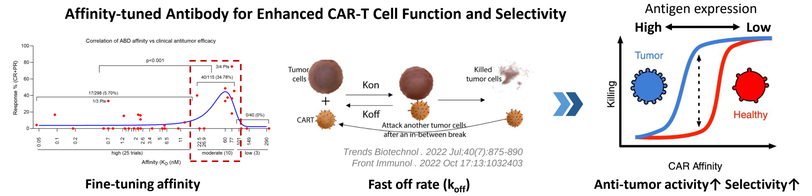

Fig. 5 Unmet needs in CAR-T: CAR binders with affinity-tuned and fast off-rate.

We utilized SPID technology to measure association rates (k_{on}) via real-time single-molecule imaging, quantifying new binding events at equilibrium **(Fig. 6.)**.

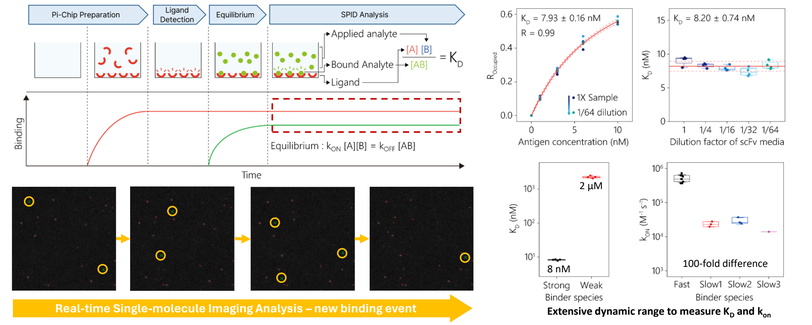

Fig. 6 Technical capabilities of SPID applicable for CAR-T binder tuning.

We selected mFMC63 as our model system and used the SMASH platform to generate 760 scFv variants. From this, we identified 6 novel key residues that were not previously reported in the literature, which modulate binding **(Fig. 7.)**. Among these variants, 114 exhibited the desired affinity range (100 – 1000 nM K_D).

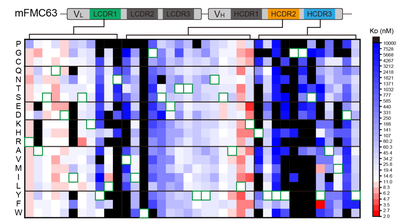

Fig. 7 K_D distribution heatmap of 760 mFMC63 scFv variants from site-saturated mutagenesis library.

CONCLUSION

We have demonstrated that the SMASH platform is an efficient approach for screening antibody affinities, whether in scFab or scFv format, making it directly applicable in antibody discovery, affinity maturation, and CAR-T cell therapy. Complementing this, PROTEINA's core technology SPID enables a faster turnaround time of 2 weeks from antibody library design to production, along with binding affinity measurement and comprehensive analysis.

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