Biophysical characterization of bispecific antibodies developed for enhancement of dual-targeting specificity with switchSENSE®

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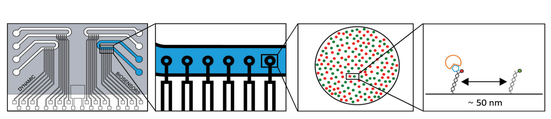
The measurement of kinetic rates and avidity binding in the simultaneous engagement of two antigens is key to optimizing for bispecific antibody target specificity early in the development process. The quantitative analysis provides insight on how to adjust the individual affinities of the bispecific antibody arms, so that the most favorable cooperative action is achieved, specifically maximal on-target and minimal off-target antibody binding. I will describe the application of a novel type of biosensor – **switch**SENSE® – that uses DNA-guided surface functionalization for the precise control over the relative abundance and spatial arrangement of two antigen species. The biosensor emulates the display of two different target antigens on a cancer cell surface and enables dual-color fluorescence detection for the simultaneous single and dual-binding kinetic studies of bispecific antibodies.

switchSENSE® | Electro-switchable DNA nanolevers

DRX² Instrument and Biochip

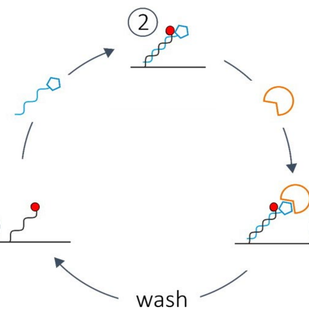
DRX² instrument featuring two light sources and two photon counters optimized for red and green fluorophores for dual binding analysis.

Multi-electrode biochip with 4 separate flow channels, each comprising 6 detection spots.



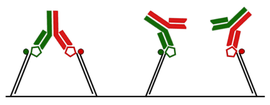
Measurement Cycle

The biosensor surface is functionalized with the target by complementary DNA hybridization. **DNA denaturation enables surface** regeneration and repeated series of hybridizations.



Measuring dual target avidity binding kinetics – immobilization of two different surface antigens

The DNA-based **switch**SENSE® biosurface can be functionalized with two different surface antigens, depending on the experimental design, enabled for the individual nanolevers as well as the dual-color mode for simultaneous and independent kinetics readout.



- Color-coded binding signals
- Measuring cooperativity / avidity

Variable antigen-antigen distance: 7nm / 14 nm

- Immobilization of two atigens at a defined, and tunable, relative distance

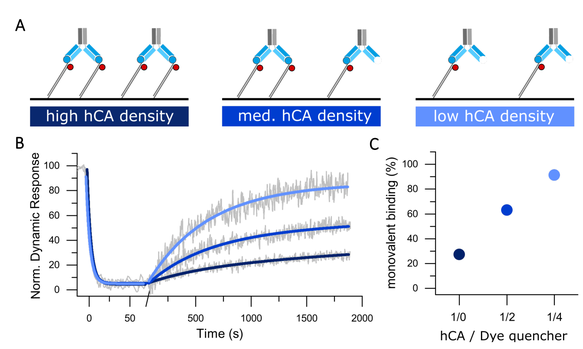
Surface ligand density control | Modulating affinity and avidity binding

Controlling the density of ligand molecules on biosensor surfaces is crucial for experimental success. Tuning the ligand density promotes or suppresses the valency of analyte binding and shifts target engagement between affinity and avidity.

Density Variation using Invisibility Cloaking

Complementary DNA covalently-coupled to a dye quencher (Quencher-DNA) is mixed in to the solution of the Target-DNA conjugate: hybridization of the quencher-DNA to the surface reduces the surface occupancy/density of the target.

Anti-hCA Model System – affinity and avidity as a function of hCA surface density



	k_{ON} (10^7 $M^{-1}s^{-1}$)	k_{OFF} (10^{-5} s^{-1})	K_D (pM)
Affinity	2.1 ± 0.1	187 ± 6	90 ± 6
Avidity		7.4 ± 1.2	4.0 ± 0.6

A) Schematic representation of Anti-hCA surface engagement as a function of the density of surface-immobilized hCA: the inter-hCA distance is larger at lower surface densities, extending beyond the dual-binding reach of the antibody arms, and effectively driving the system to a higher monovalent antibody engagement – affinity binding.

B) Association and dissociation kinetics of the Anti-hCA analyte to surface-immobilized hCA as a function of density. The dissociation curves are fit by a global, double-exponential fit model. The fit amplitudes reflect the contributions of different dissociating species – affinity vs. avidity – to the overall dissociation curve.

C) Ratios of monovalent binding increase as a function of increasing dye quencher, i.e. decreasing amounts of surface-immobilized hCA.

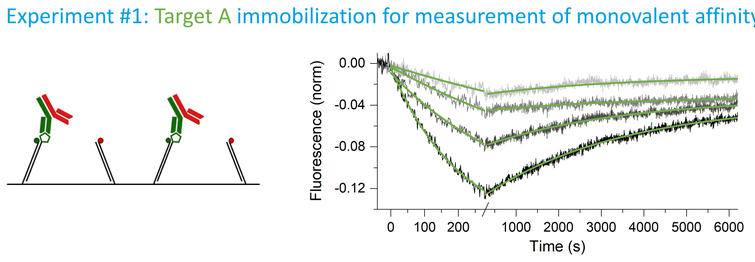
Dual antigen targeting | Bispecific antibody binding kinetics

Antibodies bound via one antigen-binding site (paratope) feature fast dissociation rates (fast $k_{OFF,1}$).
Antibodies bound to via two paratopes feature slow dissociation rates (slow $k_{OFF,2}$).
Comparing the fast and the slow dissociation rates is representative of the avidity advantage: an enhancement of antibody binding affinity upon dual paratopic enagement.

Dual antigen surface functionalization for affinity and avidity kinetic parameter measurements of bispecific antibodies

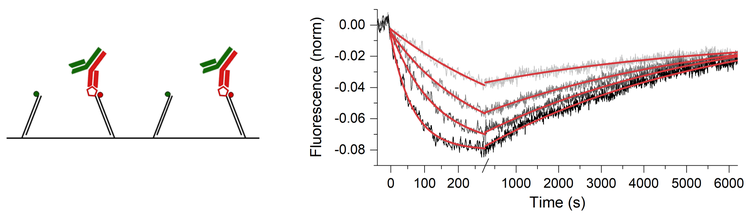
The affinity and avidity binding kinetics parameters are determined using the same assay orientation, where bivalent engagement is distinctly measured as a decrease in the k_{OFF} rate.

Experiment #1: Target A immobilization for measurement of monovalent affinity binding of antibody Paratope A



The surface immobilization with Target A only allows for the affinity binding measurement for the engagement of the antibody via Paratope A.

Experiment #2: Target B immobilization for measurement of monovalent affinity binding of antibody Paratope B

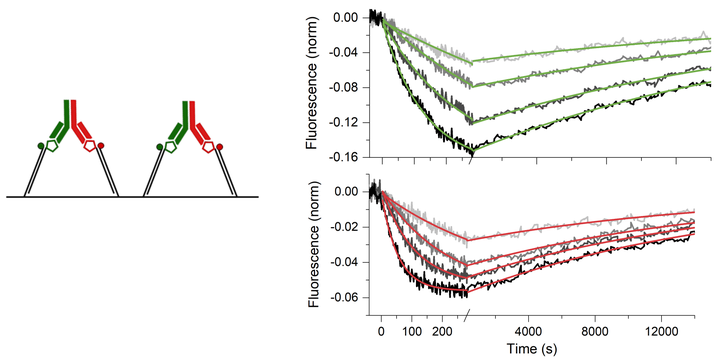


The surface immobilization with Target B only allows for the affinity binding measurement for the engagement of the antibody via Paratope B.

Affinity	k_{ON} (10^6 $M^{-1}s^{-1}$)	k_{OFF} (10^{-4} s^{-1})	K_D (pM)
Target A	0.9 ± 0.1	3.1 ± 0.1	340 ± 40
Target B	3.0 ± 0.1	1.8 ± 0.1	60 ± 4

The outcome of the affinity experiments with single target surface immobilization is the affinity binding kinetics of both of the antibody paratopes to their corresponding targets.

Experiment #3: Target A and Target B immobilization for measurement of bivalent avidity binding of the antibody



Dissociation curves in the green and red channels, measured simultaneously on both detectors. Both colors show the same OFF-rate, reflecting the avidity.

Avidity	k_{ON} (10^6 $M^{-1}s^{-1}$)	k_{OFF} (10^{-4} s^{-1})	K_D (pM)
Target AB	1.5 ± 0.4	0.53 ± 0.04	35 ± 9
Target BA	3.7 ± 0.8	0.63 ± 0.05	17 ± 4

The outcome of the avidity experiments with dual target surface immobilization is the complex binding kinetics of the antibody when engaged via both paratopes: the avidity advantage, measured as a reduction in the k_{OFF} rate in both channels, is measured in addition to the association rates of the individual paratopes.

Avidity Rate Scale Plot for Target A and Target B – individual arm contribution to the combined antibody avidity binding

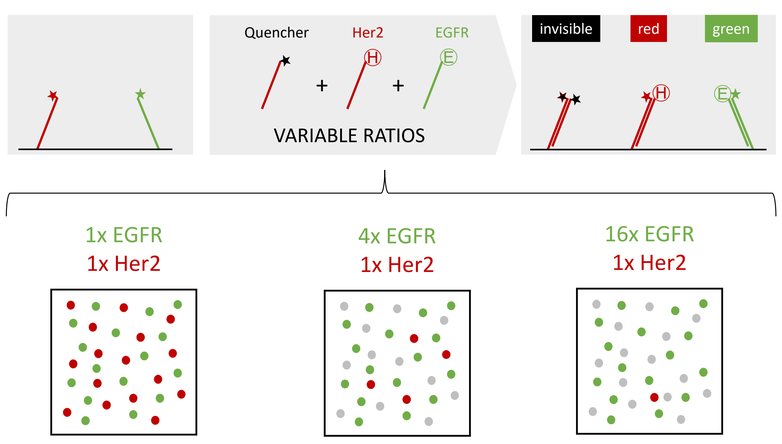
Plotting the kinetic parameters for individual arm paratope engagement, as well as those for the dual-antigen engagement, highlights how the individual arm features factor into the combined binding activity of the bispecific antibody. This effect is unique to each antibody construct, and thus needs to be empirically determined.

Dissimilar antigen abundances | Mimicking cell surface target expression

Target selectivity is a critical parameter for the engineering of therapeutic antibodies, directing the modulation of the antibody affinity to the target antigens to achieve maximal cell target binding, and minimal off-target cell binding. Patterns of cell surface antigen expression, including ratio/abundance of antigen expression, is one manner to distinguish cell types. Here we present an *in vitro* assay for the study of the target selectivity of a bispecific antibody for EGFR and Her2 antigens at dissimilar abundances.

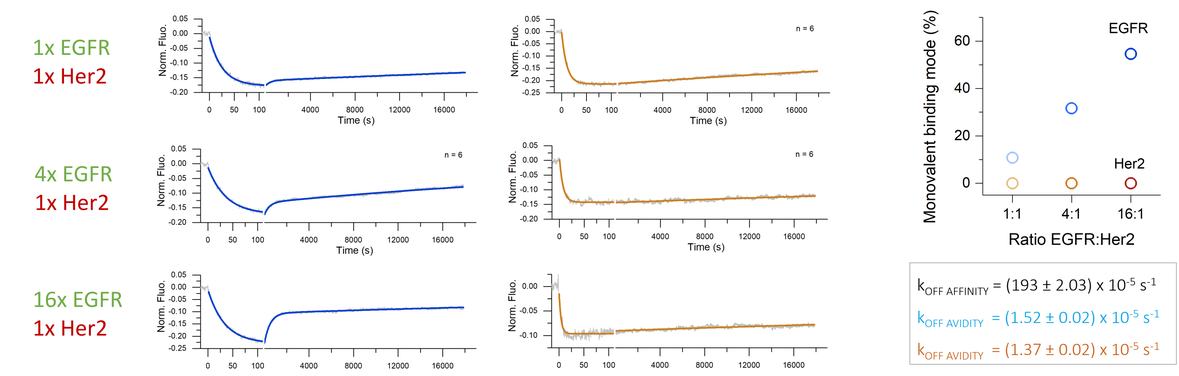
Variable surface ratios of EGFR and Her2 target antigens

The biosurface is functionalized with two color-coded DNA sequences: one sequence carries the red and the second sequence the green fluorophore. Adding complementary DNA conjugated to a black-hole-quencher (Quencher) reduces the ratio of one antigen (color) for a fixed surface density of the other.



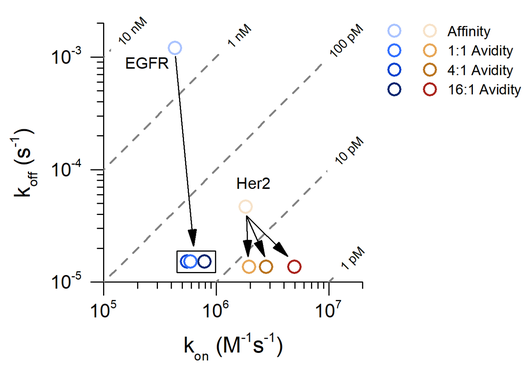
To mimic cell surface target expression, where EGFR and Her2 antigens are presented at different ratios, red-complementary DNA conjugated to a black-hole-quencher (Quencher) selectively reduces the ratio of Her2 antigen for a fixed surface density of EGFR.
Each biosurface type mimics a different cell type, defined by the ratio of the two targets presented on the cellular surfaces.

Dissociation curves for variable surface ratios of EGFR and Her2



Looking at the EGFR signal (left column), we observe an increase in the fraction of monovalently bound antibody, corresponding to the increase in the surface antigen ratio of EGFR. The Her2 signal (right column), as detected in the red channel, demonstrates that the antibody is exclusively bound bivalently – no surface-dependent change in dissociation kinetics. The $k_{OFF\ AVIDITY}$ is slower than the $k_{OFF\ AFFINITY}$ of each of the arms.

Rate Map – k_{OFF} vs k_{ON} : interpreting bispecific target selectivity from variable antigen surface ratio kinetic measurements



Plotting the kinetic parameters for individual arm EGFR and Her2 binding (light circles), as well as those for the dual-antigen engagement at the variable EGFR:Her2 surface ratios. The avidity advantage, as observed in both signals (EGFR in blue, and Her2 in brown), corresponds to a slower dissociation rate – $k_{OFF\ AVIDITY}$ – and is more prominent for the lower affinity EGFR arm.

Interestingly, eventhough Her2 is bound bivalently on all the surfaces, given its presence in lower surface density, its modular ration to EGFR is observed as a change in the k_{ON} of binding to the Her2 antigen: the k_{ON} increases as the ratio of EGFR increases.
Mechanistically, weakening the affinity of EGFR binding, which would predominantly bind the bispecific antibody monovalently on surfaces with limited Her2 antigen expression, drives for the avidity advantage to be predominantly observed on surfaces with increasing amounts of Her2.