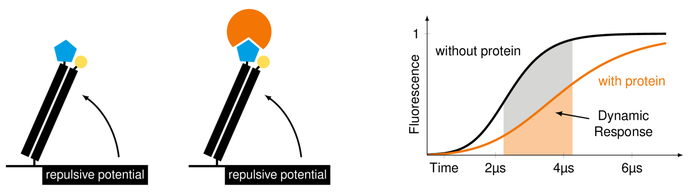
Advanced Biophysical Analysis of Mono- and Bispecific Antibody Formats with the switchSENSE® Biosensor Platform

dynamic BIOSENSORS

Johannes Reusch and Ulrich Rant; Dynamic Biosensors GmbH, Martinsried, Germany

High-affinity and bispecific antibody formats are challenging analytes for interaction analysis systems. The apparent binding kinetics crucially depend on how the target molecules are presented on the sensor surface. In order to emulate the presentation of heterogeneous antigens on a cell surface with a biosensor platform, it is necessary to functionalize the sensor with different antigens at a defined stoichiometry. Further it is crucial to control the spatial arrangement of these antigens relative to each other, which has not been feasible up to now.
Here, we introduce the DNA-templated assembly of different ligands on a switchSENSE sensor and demonstrate the quantitative measurement of binding cooperativity, i.e., avidity effects. The influence of different ligand arrangements on the binding kinetics, in particular the off-rate, is discussed for antibody formats. We show how the ligand-to-ligand distance can be controlled with sub-nanometer precision using bifunctional DNA scaffolds, i.e. nanolevers with adjustable arm-lengths. We believe the introduced workflows will be highly instrumental in the discovery and selection of bispecific biotherapeutics.

The switchSENSE® Principle | Electro-Switchable DNA Nanolevers

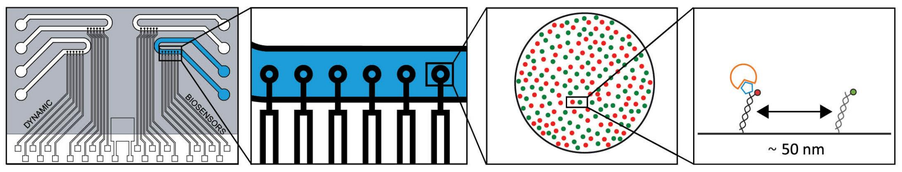


DNA nanolevers are electrically actuated at high-frequency on microelectrodes, while their orientation is monitored by time-resolved single photon counting. The binding of analyte molecules slows the switching dynamics in a characteristic way, providing an unprecedented level of information about the target.

Nature Commun. 4:2099 (2013) | Bioanal. Rev. 4 (2) 97-114 (2012) | JACS 134, 15225 (2012) PNAS 107, 1397 (2010) | JACS 132, 7935 (2010)

switchSENSE® DRX / DRX²

switchSENSE® Biochip



Biochip with 4 flow channels, each with multiple microelectrodes in series | 6 microelectrodes in 1 flow channel | Each detection spot, a mixture of red & green nanolevers | 2 nanolevers, each with a different sequence & fluorescent dye

Interaction and Biophysical Analysis

High Sensitivity
Measurement of analyte concentrations from fM to mM
From ultra-fast to ultra-slow kinetics.
LOD = 10 fM

Kinetics and Affinity
Determination of binding rate constants
k_{ON}, k_{OFF} and dissociation constants K_D in real-time.

Size and Conformation
Analysis of protein diameters on chip with 0.1 nm accuracy,
and monitoring of conformational changes.

Cooperativity and Avidity
Identification of multiple binding sites in a single measurement
using variable capture molecule densities.

Thermodynamics
Characterization of melting transitions, thermal stability or thermodynamic analyses. 8° - 75°C

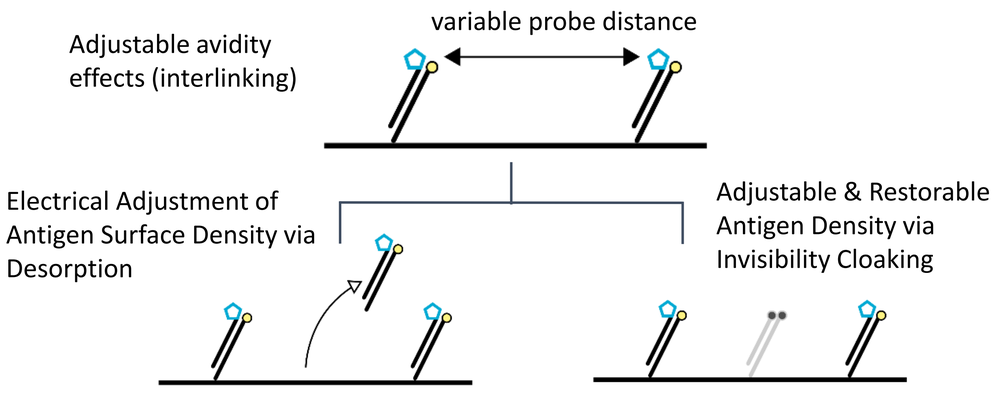
Control over Steric Configurations

Prerequisites for reliable kinetics measurements

Phase	Potential meas. artifact	Cause	Consequence
Association	Mass-transport limitation	• Ligand density too high • Flow rate too low	→ Apparent k_{ON} < real k_{ON}
Dissociation	Rebinding	• Ligand density too high • Flow rate too low	→ Apparent k_{OFF} < real k_{OFF}

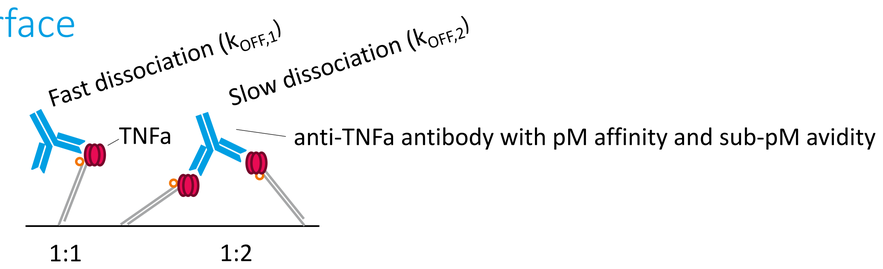
Adjust surface density of the ligand as low as possible & flow rate as high as possible

Biochip Surface Density Variation



switchSENSE provides the control over steric configurations of antigens and a homogenous surface density

Density Variation | IgG binding to an TNFα-modified surface

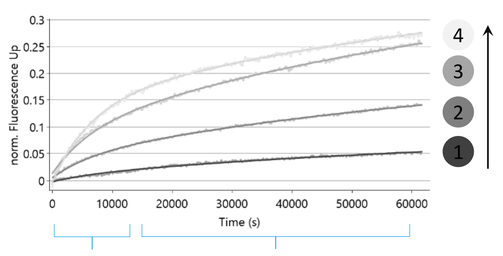


Complex binding situations → bi-phasic dissociation:
- Antibodies bound via one antigen binding site to ligand feature fast dissociation (fast $k_{OFF,1}$)
- Antibodies bound to two ligand feature slow dissociation rate (slow $k_{OFF,2}$)

→ By interlinking of both effects: slow and fast dissociation exists in parallel

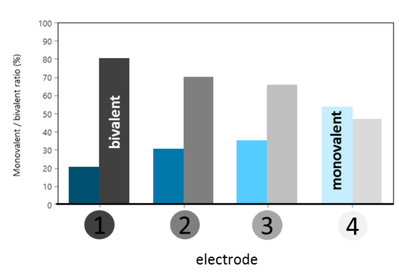
Monovalent vs. Bivalent Binding – Affinity vs. Avidity

Adjusting the nanolever density → distinction between monovalent and bivalent binding (Affinity vs. Avidity)
Amplitudes reflect respective contributions of different dissociating species to the overall dissociation curve



monovalent phase $k_{OFF,1}$ = 1.55 E-4 s^{-1}
bivalent phase $k_{OFF,2}$ = 0.12 E-4 s^{-1}

Dissociation curves fitted with a global, double-exponential model:



Amplitudes of bi-phasic fit analysis reflect respective contributions

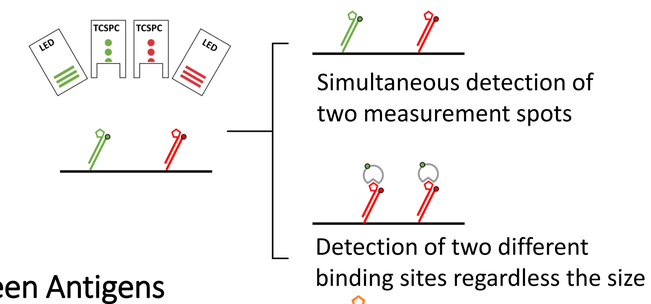
The thinner the ligand density the more dominant becomes the monovalent phase.

Bispecific Functionalization of Sensor Surface

Immobilization of Two Different Antigens with DRX² instrument

On-spot reference
Green and red fluorophore signal

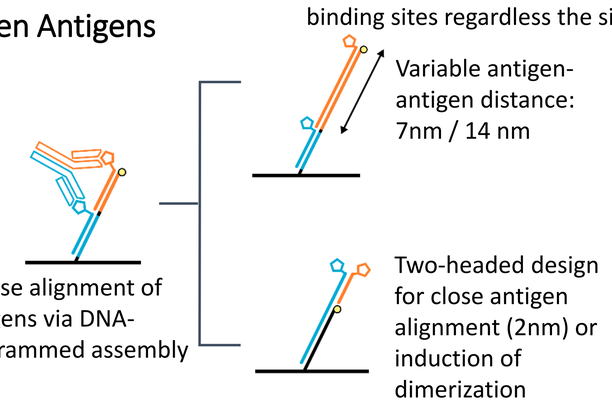
More advanced assay
Detection of color-coded binding signals from individual antigens → analyzing bispecific binder



Bi-functional Nanolevers: Defined Distances between Antigens

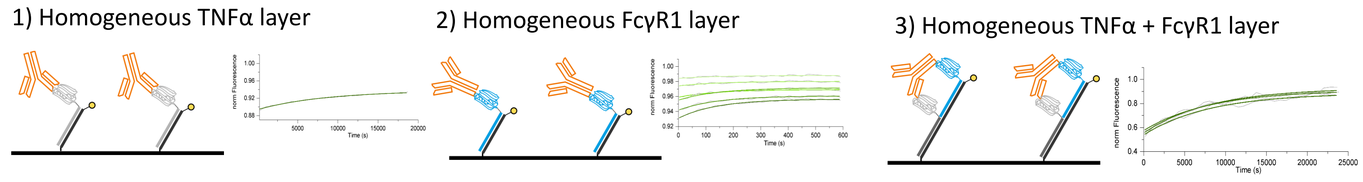
Bispecific functionalization of the sensor surface
- Immobilization of **two antigens**
- Assembly of antigens at **defined distance** and **known position**
- **Control** over **surface density**

→ switchSENSE offers ultimate solution for understanding bivalent / bispecific antibody design & characterization of challenging bispecific antibody formats (femto-molar range)



Bivalent Interaction | anti-TNFα – FcγR1

1) Homogeneous TNFα layer | 2) Homogeneous FcγR1 layer | 3) Homogeneous TNFα + FcγR1 layer



Use of bifunctional nanolevers allows to study the contribution of a weak and strong binding site to the overall affinity.

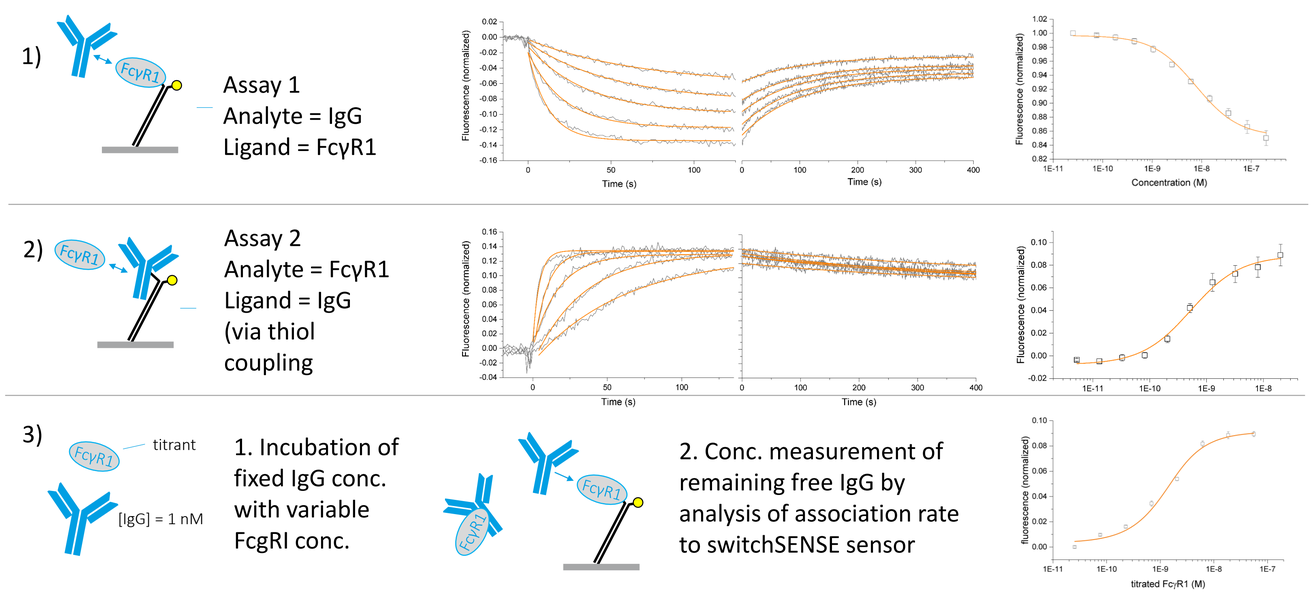
Immobilized binding partner (Ligand)	Kinetics k_{ON} (10^6 $M^{-1}s^{-1}$)	k_{OFF} (10^{-4} s^{-1})	$K_D = k_{OFF}/k_{ON}$
1) TNFα layer	23.7 ± 0.4	1.7 ± 0.1	6.9 ± 0.2 pM
2) FcγR1	1.4 ± 0.1	71 ± 4	4900 ± 400 pM
3) TNFα + FcγR1 layer	20.0 ± 0.2	0.61 ± 0.09	3.0 ± 0.5 pM

Assay Orientation & Solution Titration | FcγRI – IgG binding

1) Assay 1
Analyte = IgG
Ligand = FcγR1

2) Assay 2
Analyte = FcγR1
Ligand = IgG (via thiol coupling

3) 1. Incubation of fixed IgG conc. with variable FcgRI conc.
2. Conc. measurement of remaining free IgG by analysis of association rate to switchSENSE sensor



FcγR1 - IgG interaction supposed to follow 1:1 stoichiometry

	Kinetics k_{ON} (10^6 $M^{-1}s^{-1}$)	k_{OFF} (10^{-3} s^{-1})	$K_D = k_{OFF}/k_{ON}$ (nM)	Equilibrium Titration (Langmuir) K_D (nM)
1	1.21 ± 0.02	10.3 ± 0.1	8.50 ± 0.20	7.60 ± 1.00
2	3.69 ± 0.51	2.59 ± 0.17	0.70 ± .11	0.53 ± 0.09
3		n.a.		0.80 ± 0.17

- Affinity influenced by **assay orientation** and **coupling strategy**
- **10 fold weaker** affinity of immobilized FcγR1 (overcome by in-solution competition assay)
- Complex formation of IgG and FcγR1 in solution: surface-bound ligands determine free fraction of IgGs yielding „**true**" dissociation constant - a factor 10 lower then expected affinity of FcγR1 → matching K_D from kinetic measurements of immobilized IgG

www.dynamic-biosensors.com | info@dynamic-biosensors.com