Focal Molography: From Fundamentals to DNA-Encoded Library Screening and Membrane Protein Target Characterization

Miltenyi Biotec

lino Biotech AG
a Miltenyi Biotec Company

SPS Meeting 11.9.2024

The people behind focal Molography - lino Biotech AG

- Founded in 2020 as ETH/Roche spinoff
- Acquired by Miltenyi Biotec in Feb. 2023
- located in Adliswil

12 People (9 PhDs, 3 MA):

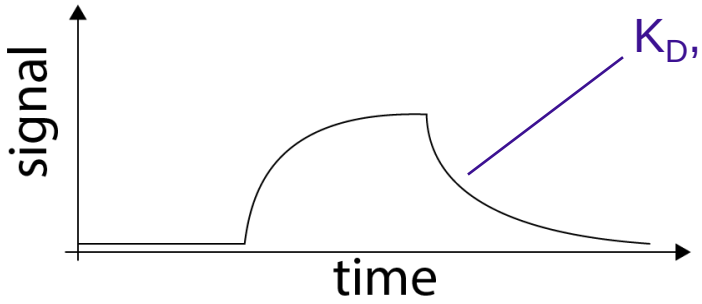
- 3 Physicist
- 2 Biologists
- 4 Chemists
- 2 Material scientists
- 1 Mech. Engineer

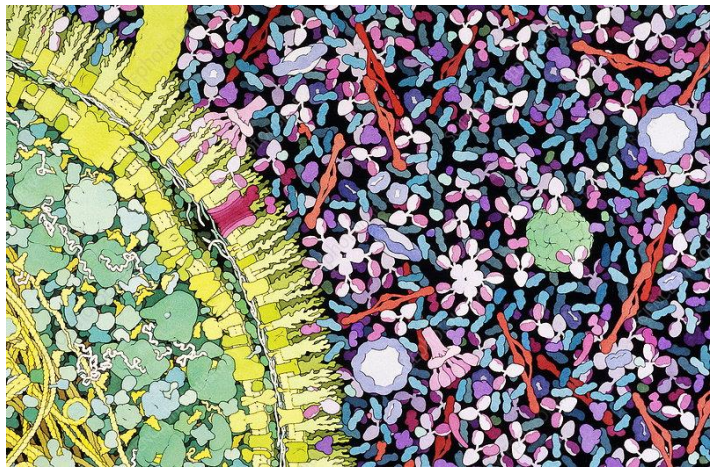
What is focal Mology raphy?

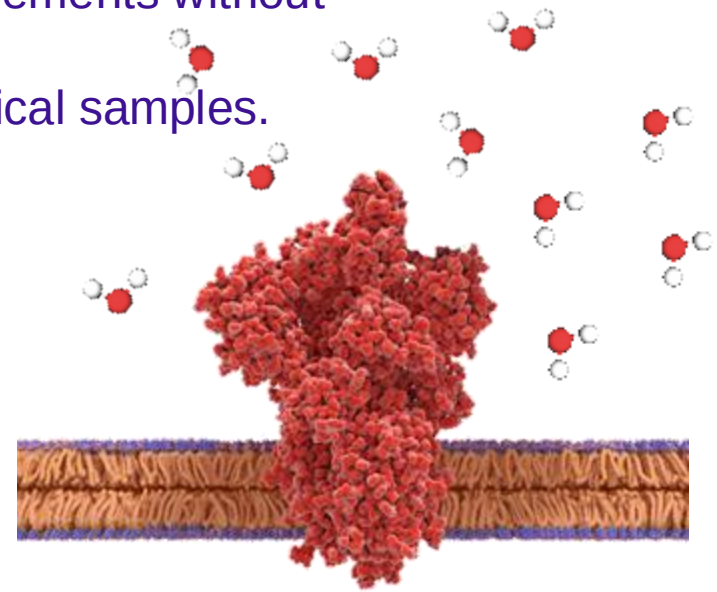
- focal Mology raphy is a surface based real-time label-free biosensor

- This allows two measurement principles:
  - affinity and kinetic measurements
  - concentration measurements

- Key feature: It can do such measurements without any stabilization (temperature) and in nonpurified complex biological samples.







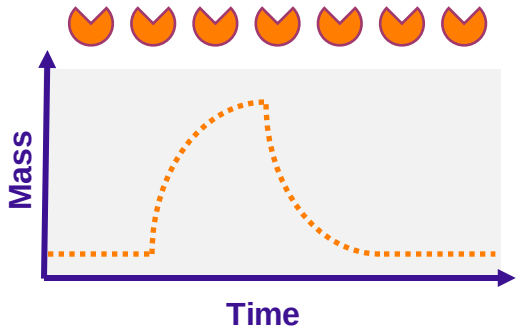
How are biomolecular Interactions measured today?

Biomolecular interaction analysis (BIA)

Miltenyi Biotec

Detection principle

in label-free detection

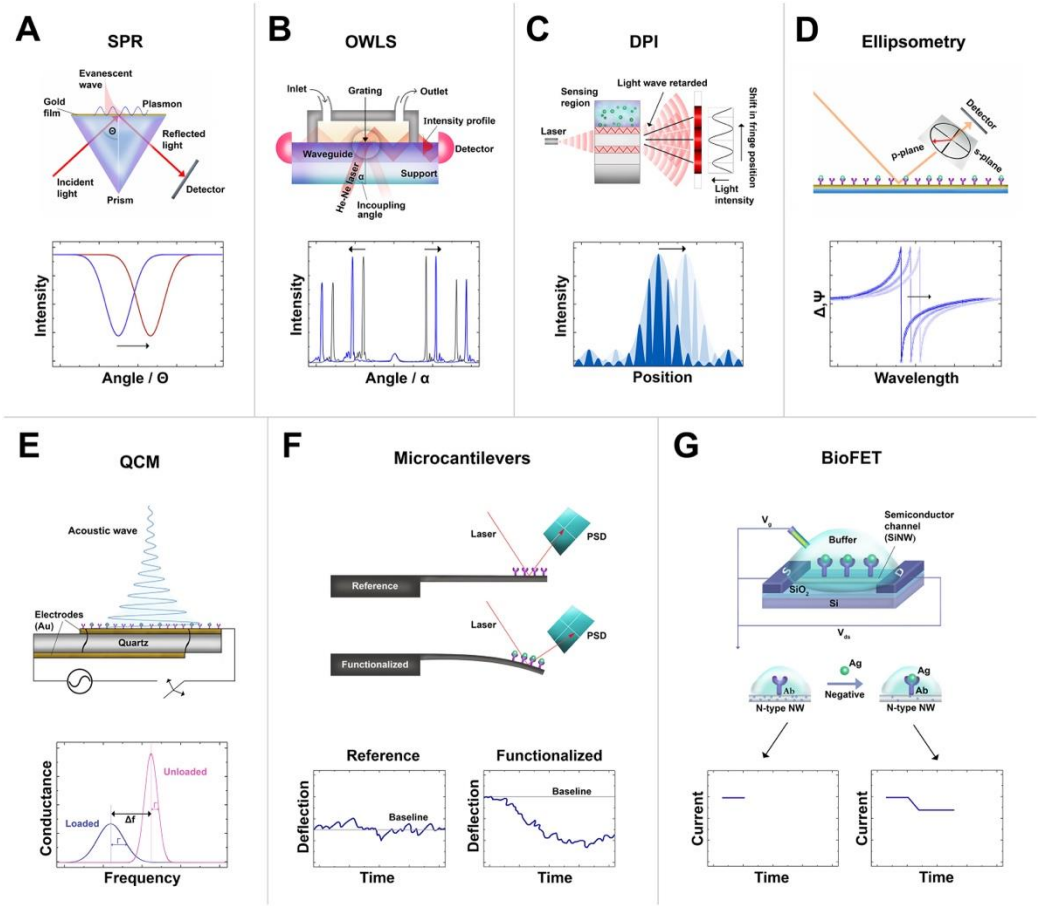


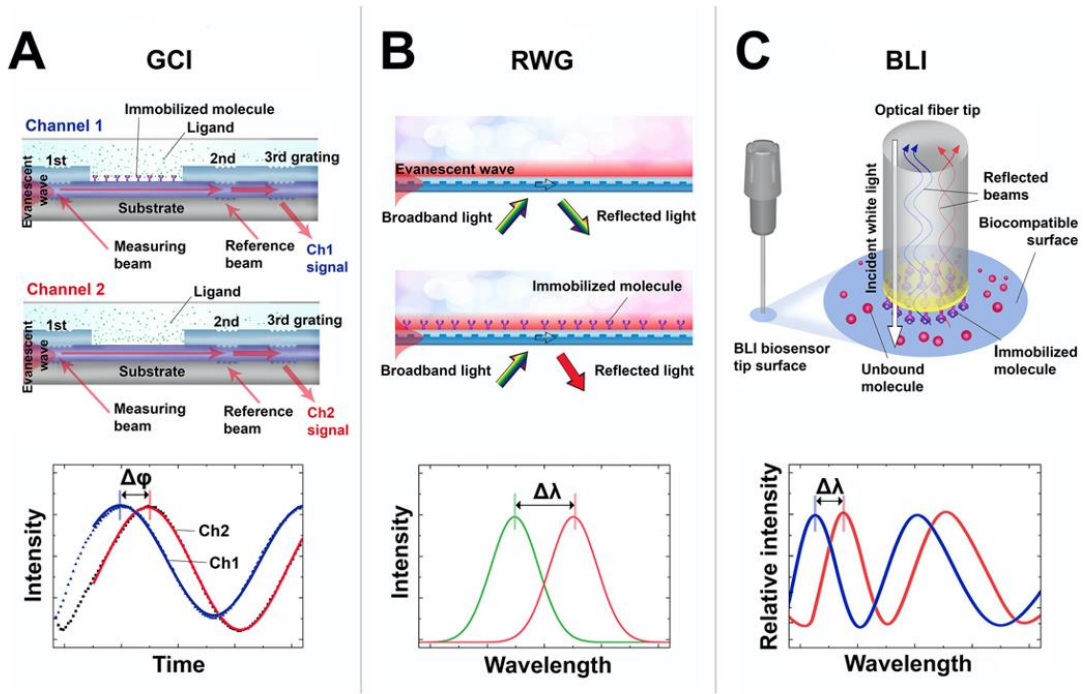
Kinetics and thermodynamic parameters in clean media which are important to in-detail understand biological interactions.
Results: Conc., K_D, off-rate, on-rate, enthalpy (ΔH) and entropy (ΔS)

Very established field

by different technologies and companies

How do these sensors work?

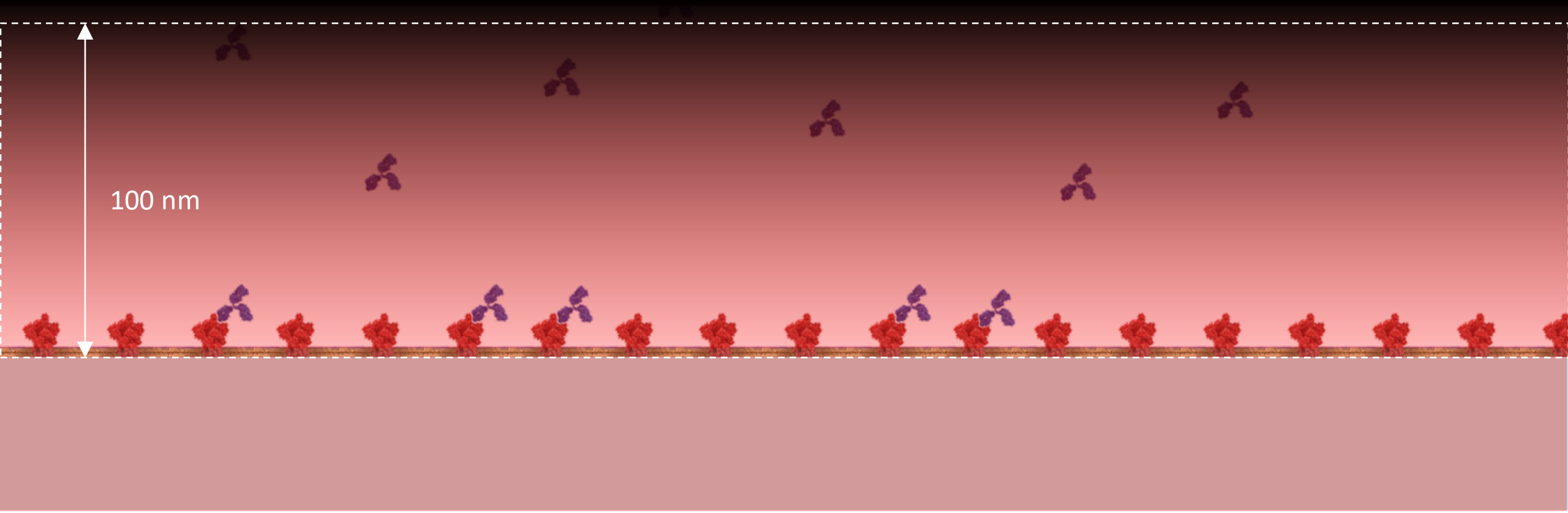




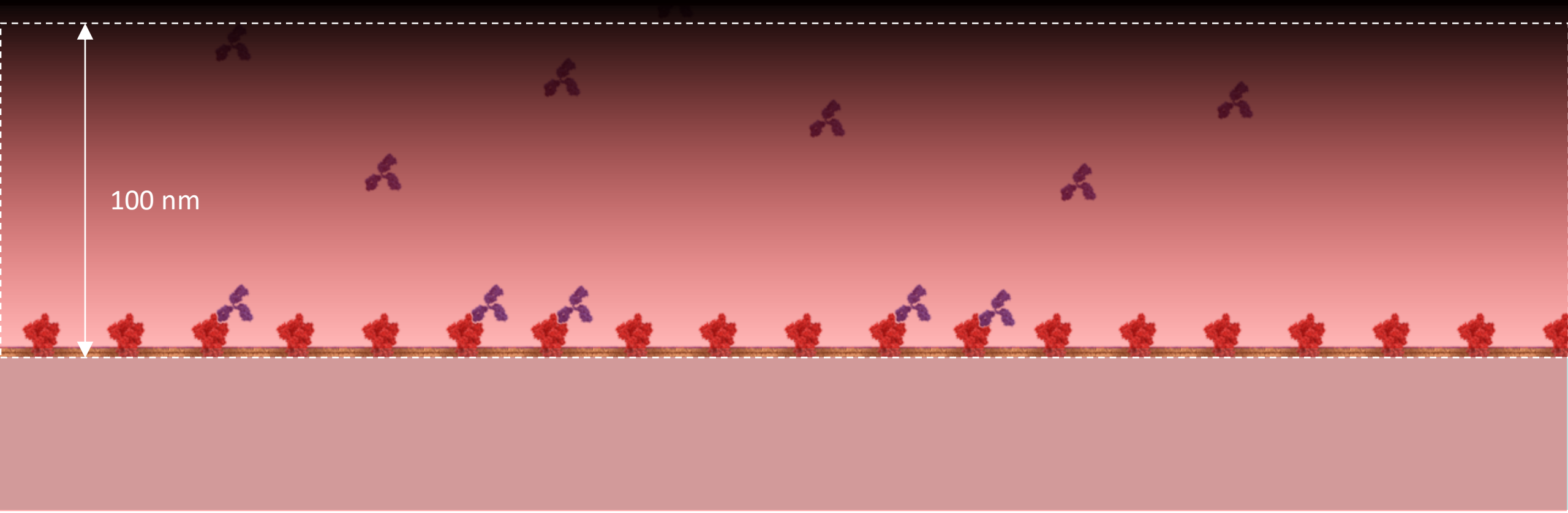
- Known as refractometric sensors
- In principle: Very sensitive techniques

A. Saftics, S. Kurunczi, B. Peter, I. Szekacs, J. J. Ramsden, and R. Horvath, *Data Evaluation for Surface-Sensitive Label-Free Methods to Obtain Real-Time Kinetic and Structural Information of Thin Films: A Practical Review with Related Software Packages*, Adv. Colloid Interface Sci. **294**, 102431 (2021).

Sensing principle: Correlation of the refractive index change within a tiny measurement volume defined by the evanescent field to a change in the number of molecules



Lets put one of these sensors in **a biological environment**



Lets put one of these sensors in a biological environment

In a biological sample,
molecules of interest represent only a **small fraction of the biomolecular mass** in the sensing volume, despite the affinity difference.

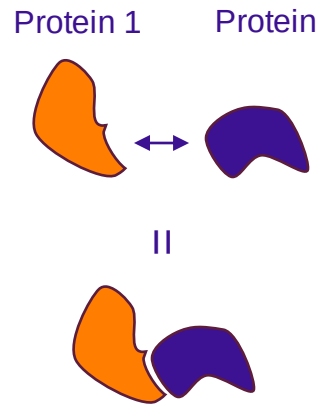
In addition, temperature, concentration and buffer changes all affect the refractive index.

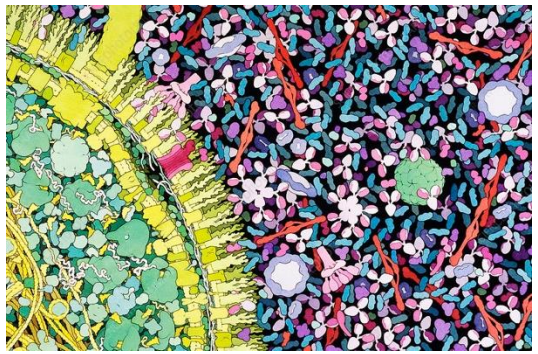
State of the art molecular sensors are cross-sensitive and can only operate in controlled conditions.

Why characterization in complex media (blood, living cells etc)?

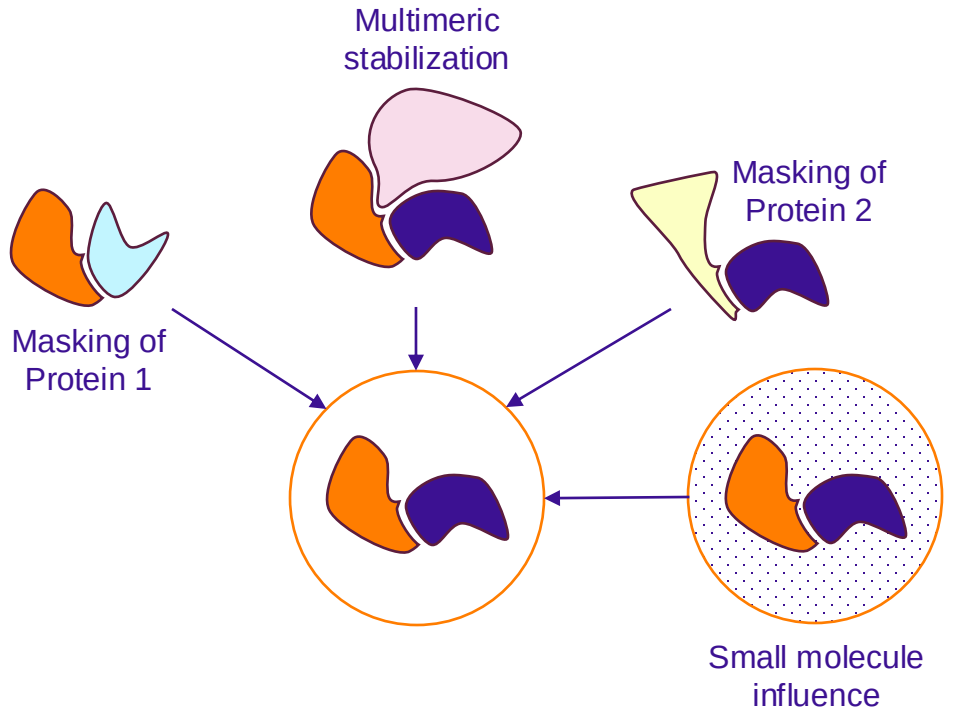
Miltenyi Biotec

Typical simplified interaction experiment





This is how the reality looks like



To measure interactions in a complex media we need

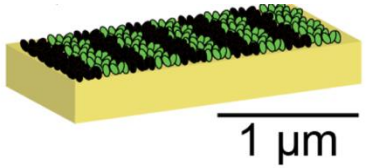
Miltenyi Biotec

Robust and sensitive biosensors

I. PILLAR
Darkfield illumination
By evanescent waves

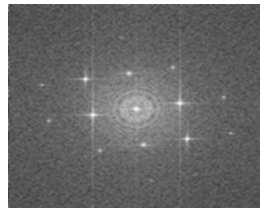
Only illuminate where needed

II. PILLAR
Submicron Modulation



Make the analyte bind in a pattern

III. PILLAR
Detection in Fourier space



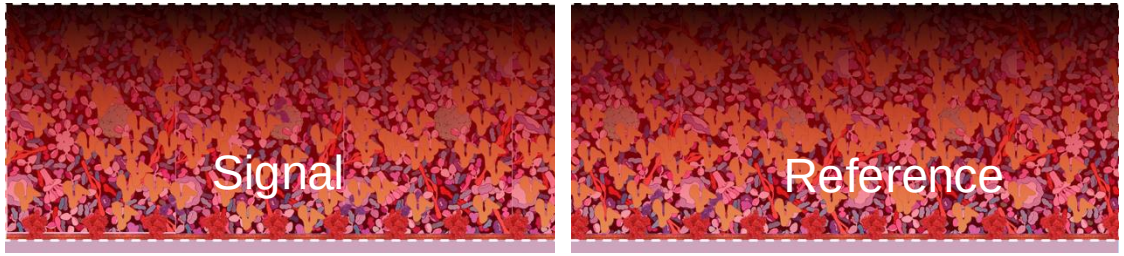
Read the pattern by diffraction

A. Frutiger, C. Fattinger, and J. Vörös, Ultra-Stable Molecular Sensors by Sub-Micron Referencing and Why They Should Be Interrogated by Optical Diffraction—Part I. The Concept of a Spatial Affinity Lock-in Amplifier, Sensors 21, 469 (2021).
A. Frutiger, K. Gatterdam, Y. Blickenstorfer, A. M. Reichmuth, C. Fattinger, and J. Vörös, Ultra Stable Molecular Sensors bySubmicron Referencing and Why They Should Be Interrogated by Optical Diffraction—Part II. Experimental Demonstration, Sensors 21, 9 (2020).

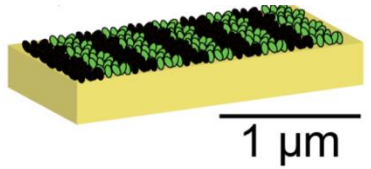
Why is a macroscopic reference inferior to a distributed reference on the molecular length scale?

Miltenyi Biotec

Macroscopic reference:



II. PILLAR
Submicron Modulation



Make the analyte bind in a pattern

To understand it we need to look at the **length scales of environmental noise**...

Most environmental noise is situated at low spatial frequences (longer length scales)...

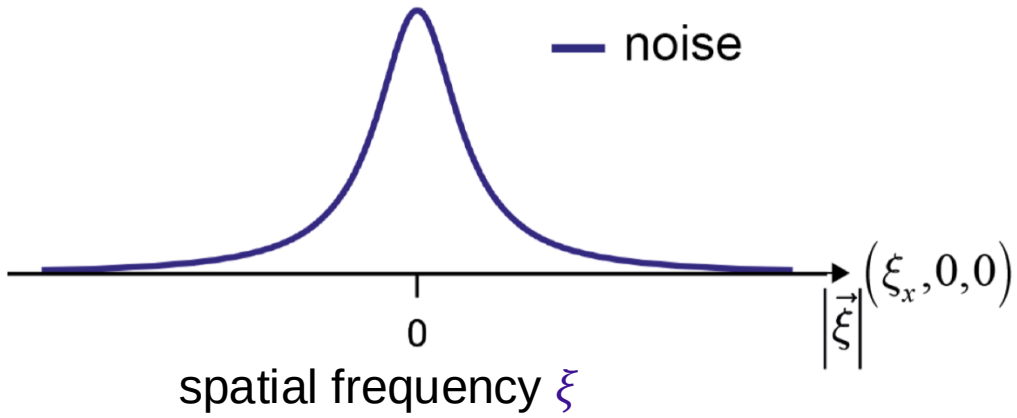
Miltenyi Biotec

...because it is governed by the advection-diffusion equation.

$$\frac{\partial T(\vec{r},t)}{\partial t} = -D_T \nabla^2 T(\vec{r},t) + \frac{q(\vec{r},t)}{\rho c_p},$$

Intuitive explanation: Speed of diffusion is finite
→ **gradients in concentration,**
temperature relax faster on nm than mm scales

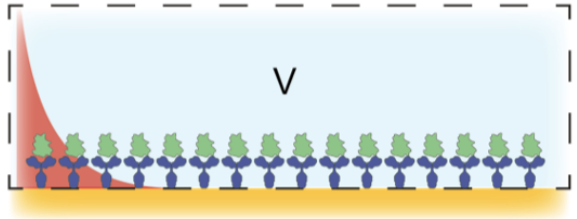
Power spectrum is $1/\xi$ distributed:

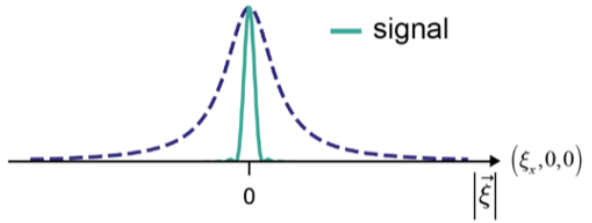


A. Frutiger, C. Fattinger, and J. Vörös, *Ultra-Stable Molecular Sensors by Sub-Micron Referencing and Why They Should Be Interrogated by Optical Diffraction—Part I. The Concept of a Spatial Affinity Lock-in Amplifier*, Sensors **21**, 469 (2021).

The binding signal can be shifted to a high spatial frequency and separated from the noise by modulation

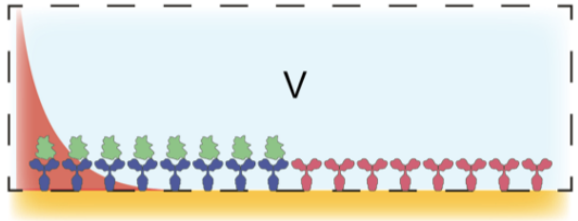
Sensor signal **unreferenced**

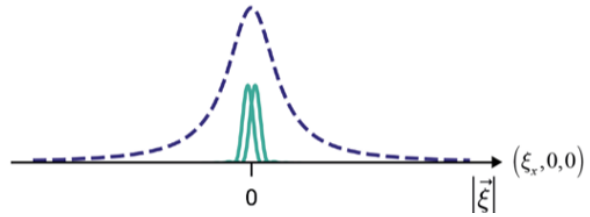




Noise and signal **overlap**

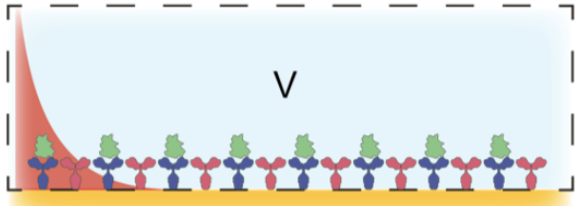
Sensor signal **referenced**

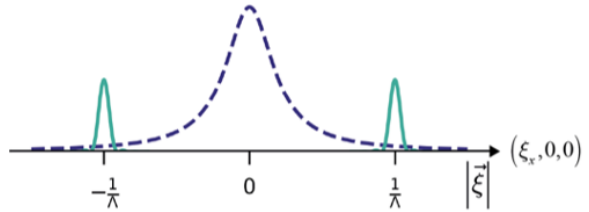




Noise and signal **still overlap**

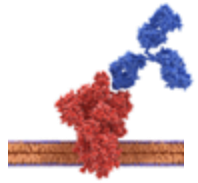
Sensor signal **sub-micron referenced**





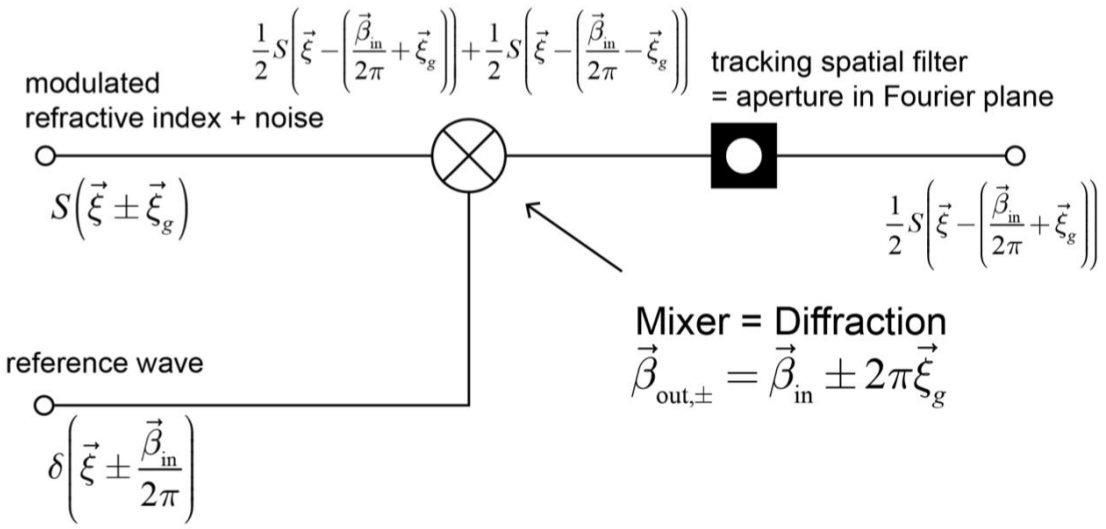
Noise and signal **separated**

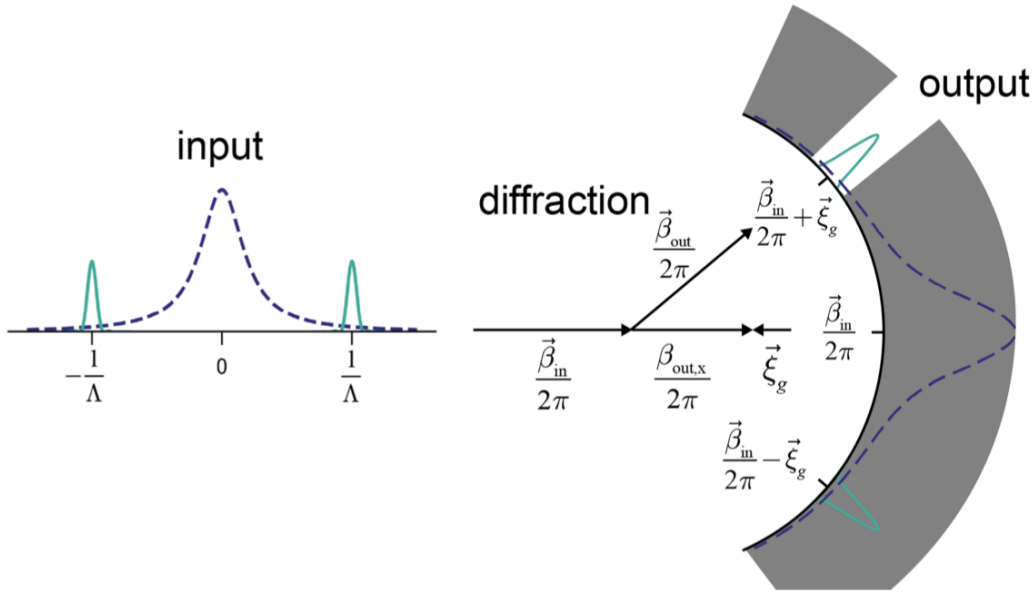
Modulation frequency should be as close to the inverse molecular length scale as possible.



5 nm

Mixing and filtering can be achieved via diffraction of waves and Fourier plane filtering

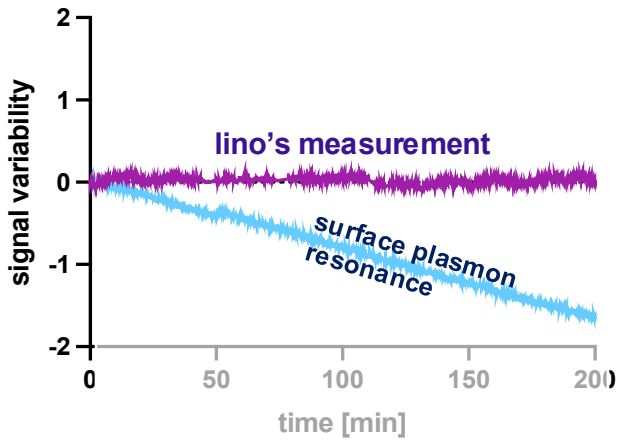




Tracking of the signal (lock-in) can be achieved by a pinhole or an array detector in a Fourier plane.

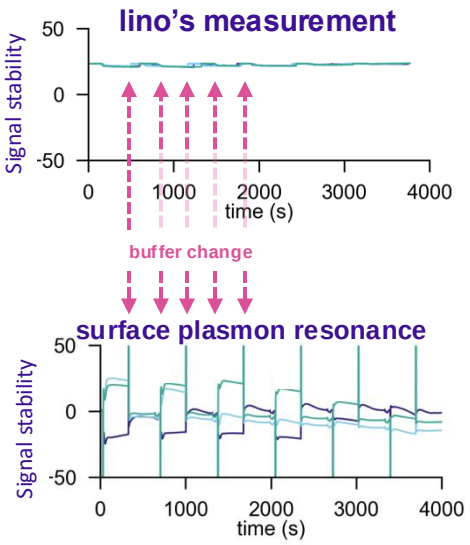
Spatially locked in sensors have unique properties

Long-term signal stability



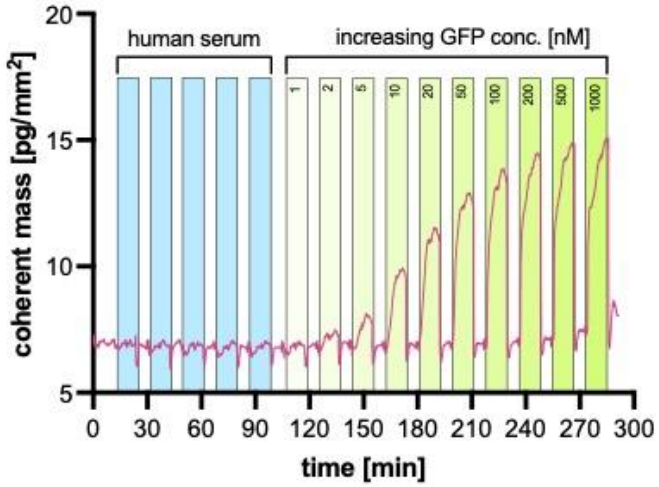
The technology is essentially drift-free. Here, the signal against the latest versions of the Biacore 8K is shown over multiple hour. *[Experiment was the signal stability under running buffer flow over multiple hours; no binding signal change is expected]*

Low medium influence



Molography has no artefacts from buffer changes during the measurement. Competitors (SPR) cannot tolerate buffer changes. *[Experiment was the injection of different glycerol concentrations; no binding signal is expected]*

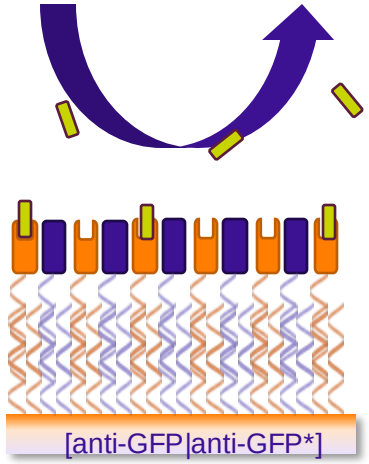
Excellent signal to noise ratio in complex media

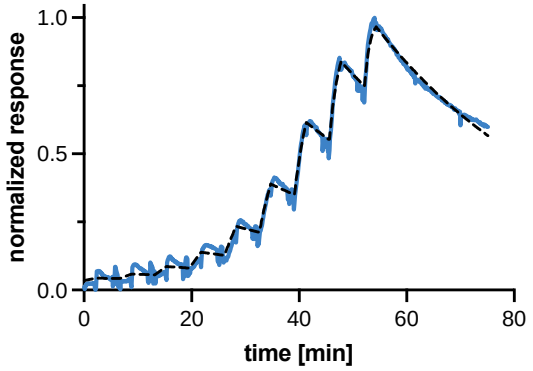


Molography works in crude samples like competitors can only do in buffer. Non-specific binding of serum proteins hardly generates a response. *[Experiment detects spiked GFP in 50% human serum down to 2 nM concentrations]*

Kinetic characterization
PBS-T versus human serum

Spiked PBS-T buffer



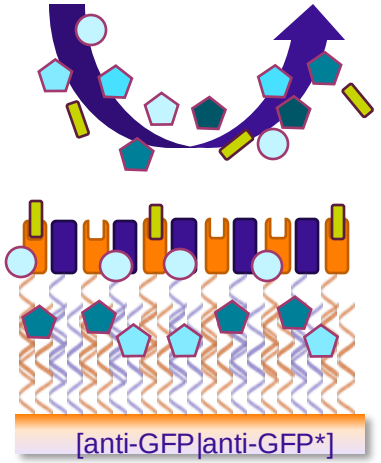


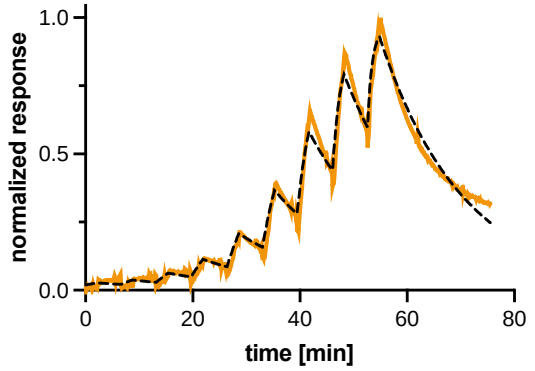
The assay format was first established in a classical PBS-T based interaction system. Therefore, increasing GFP concentrations were injected onto sensor surface and evaluated by a 1:! Langmuir model. The median sensorgram is shown exemplary.

Mean kinetic values with SD (N=54)

k_{on}	4.4±8.5 x10^{5} $M^{-1}s^{-1}$
k_{off}	4.5±0.8 x10^{-4} s^{-1}
K_D	1.2±1.2 x10^{-9} M

Spiked human serum





In a second step the assay was performed in 50% human serum. The medium was spiked with identical increasing concentrations of GFP followed by the identical evaluation. The median sensorgram is shown exemplary.

Mean kinetic values with SD (N=51)

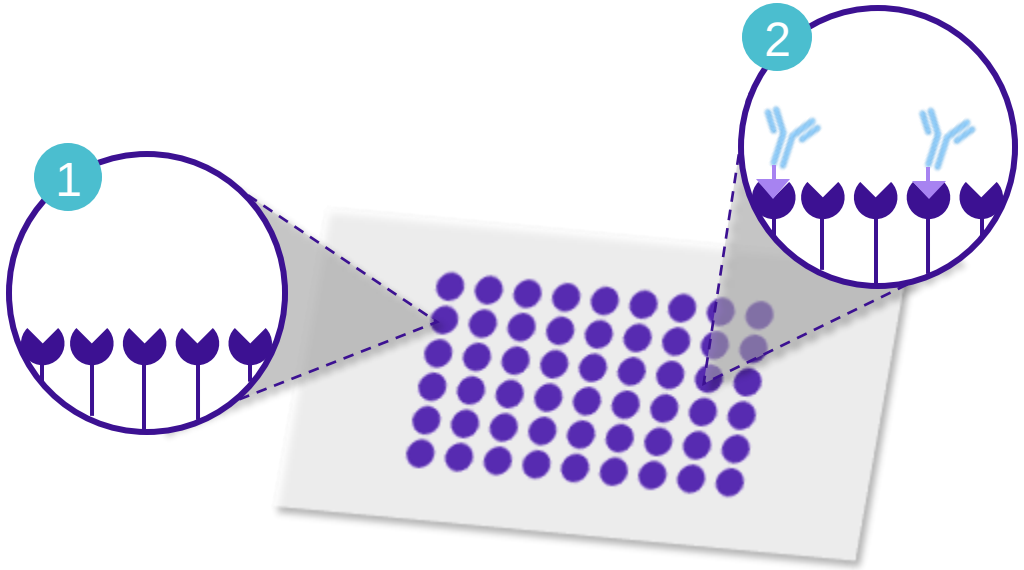
k_{on}	6.1±3.5 x10^{5} $M^{-1}s^{-1}$
k_{off}	1.3±1.0 x10^{-3} s^{-1}
K_D	2.6±3.5 x10^{-9} M

General surface functionalities for sensor chips

Miltenyi Biotec

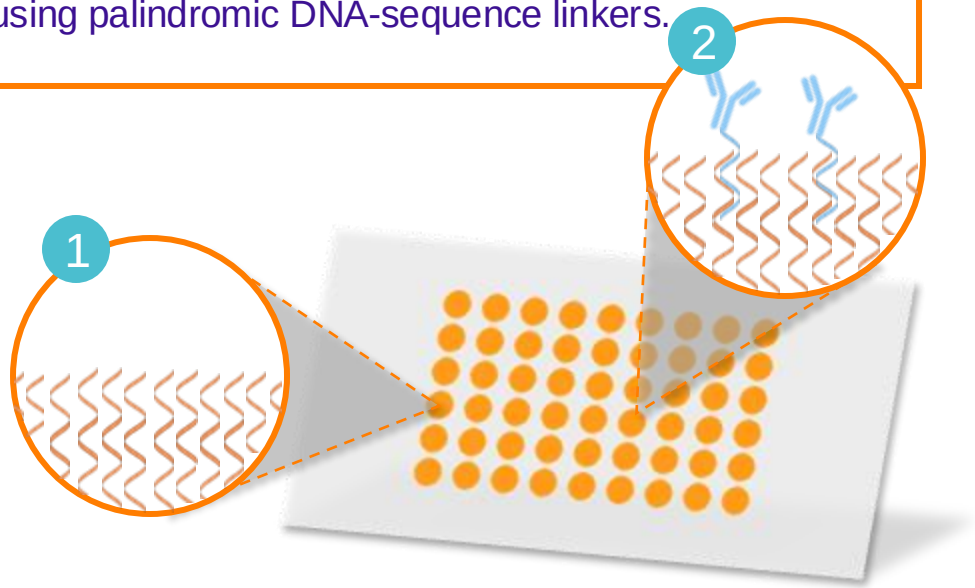
Click Chemistry

Biomolecules can be immobilized in a covalent fashion to the sensor surface via an optimized Click Chemistry strategy.
The underlaying biocompatible polymer is a 2D PEG layer equipped with the molographic template structure.



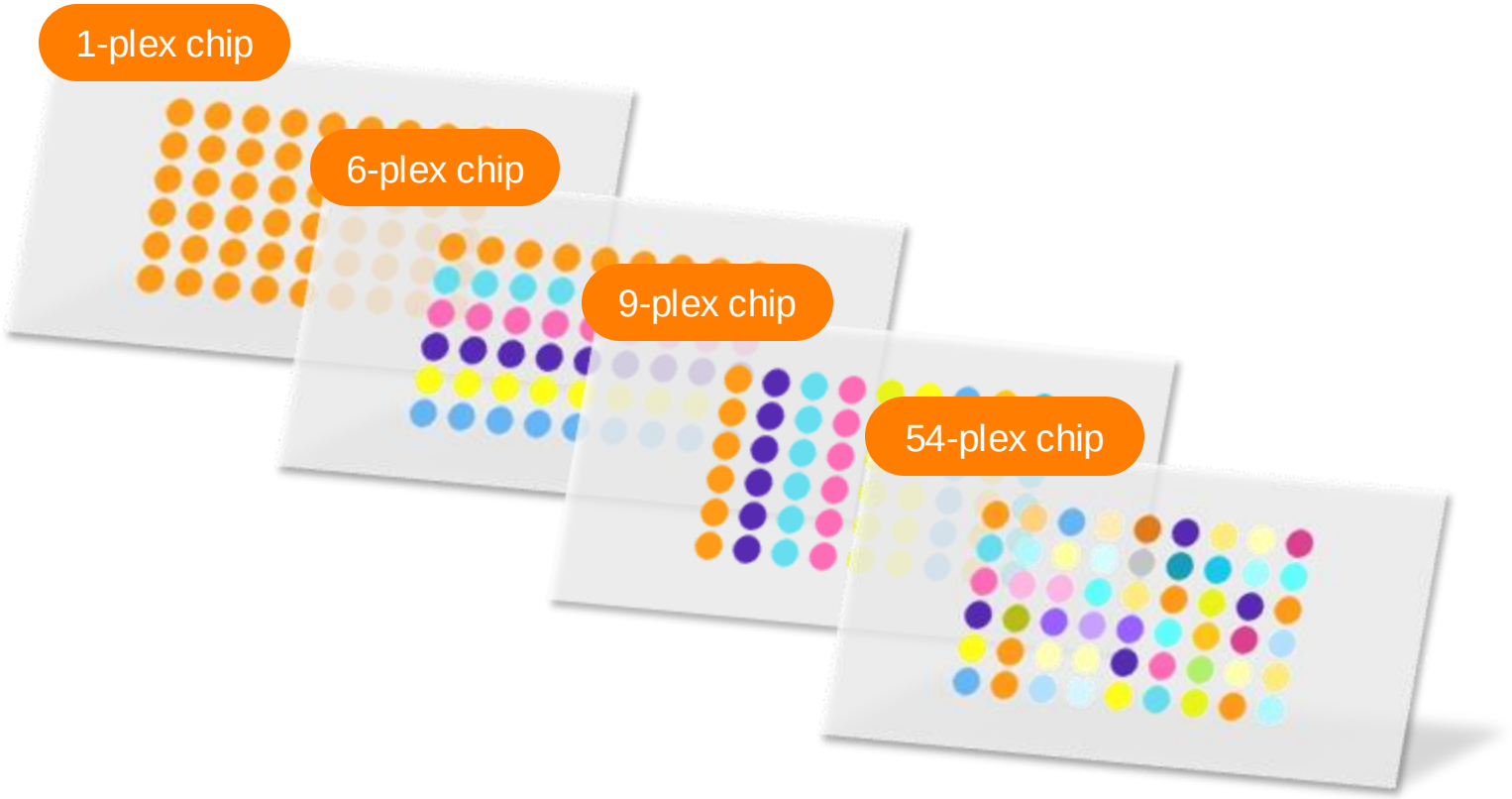
DNA directed

Sensor chips are fabricated with a well-defined DNA oligonucleotide pattern for individual Mologrames. The different DNA sequences are attached to the Mologram by established micro-spotting techniques. For biological functionalization, different target-types can be linked to individual Mologrames using palindromic DNA-sequence linkers.



DNA directed immobilization is the basis for multiplexed chips

Miltenyi Biotec



Sensor Chip

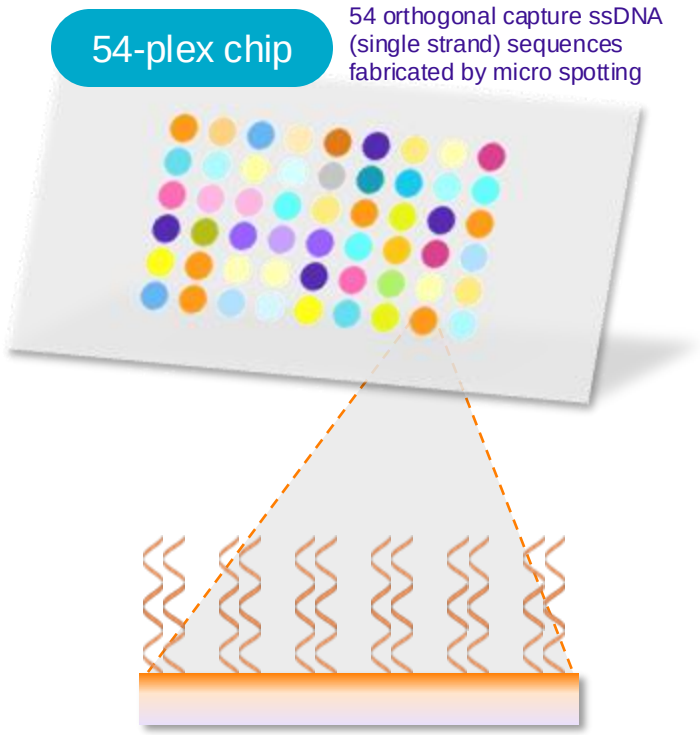
Sensor chips are spotted with a pre-functionalized pattern for various multiplexed applications.

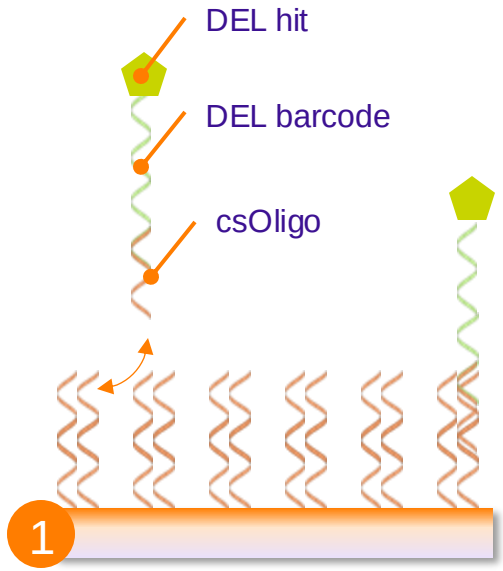
In combination with a disposable fluidic cassette a broad set of sensor chips can be easily realized

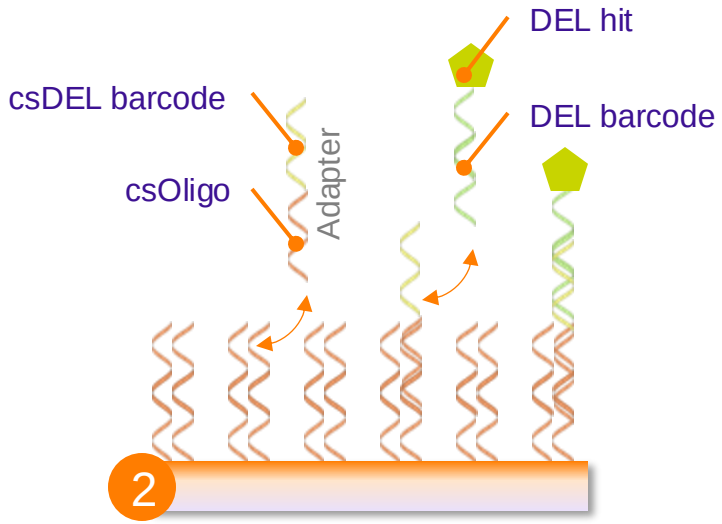
Miltenyi Biotec

DEL (DNA encoded library) hit validation

DEL hit screening with Mologaphy
Working principle





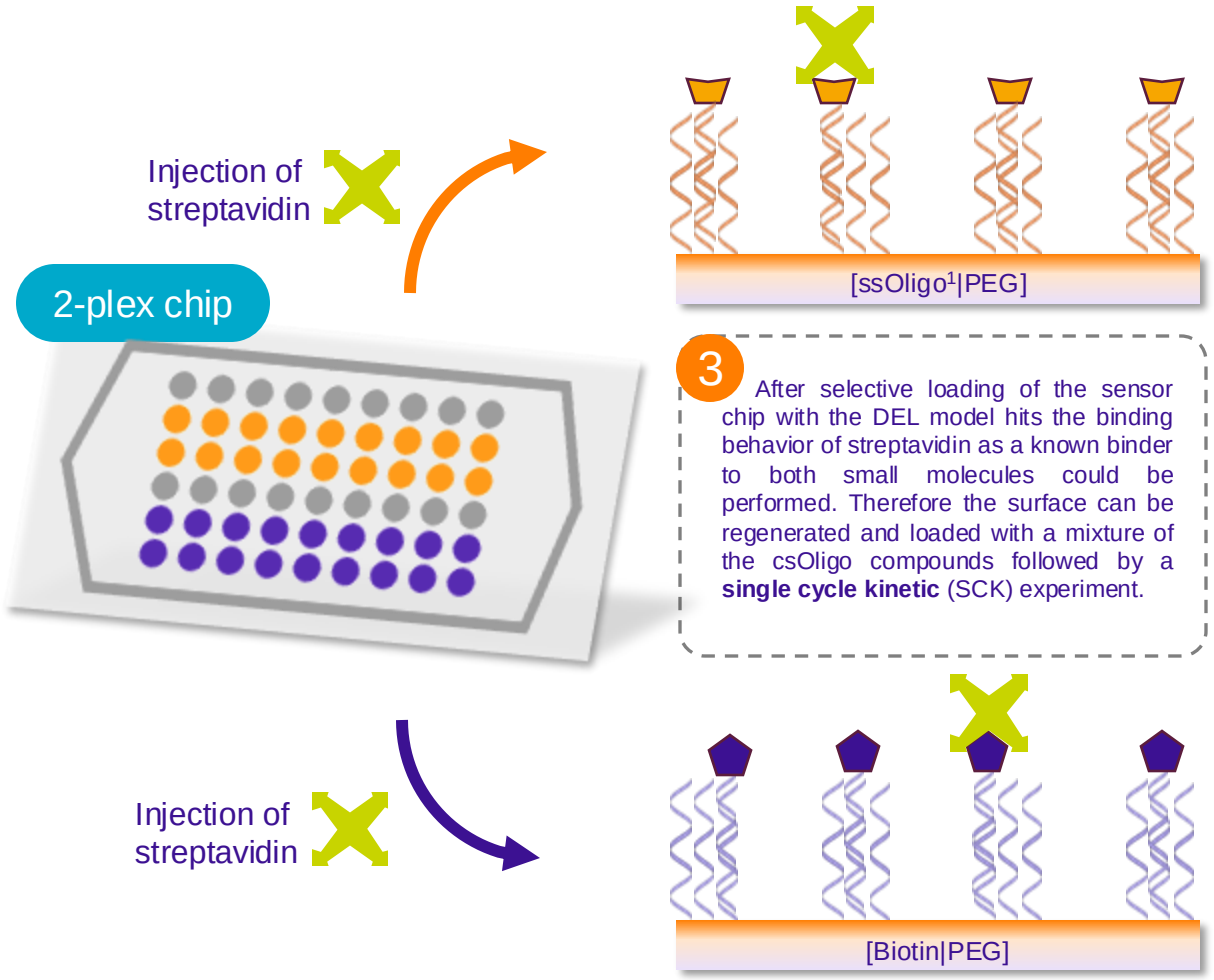


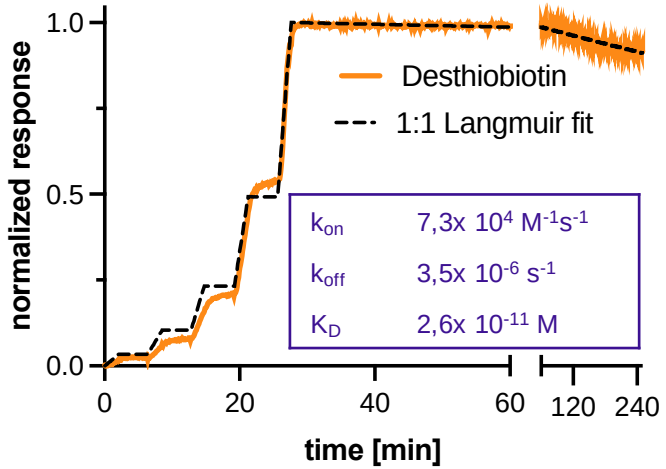
A **pre-functionalized sensor chip** with 54 mologramms modified with different **ssOligos** (surface strain) is used for further kinetic characterization of DEL hits. The DNA based DEL barcode attached to the small molecule compound is used for a convenient immobilization on the ready to use multiplexed oligonucleotide chip. Currently two working modes are possible.

If the customer is able to elongate the DNA barcode of the DEL hits a **csOligo** (complementary strain) can be added accordingly to the sequences on the sensor chip. A single injection of 54 DEL hits with unique csOligo tags can be automatically injected by the autosampler resulting in **DNA directed immobilization (DDI)** of the ligands to the 54 mologramms.

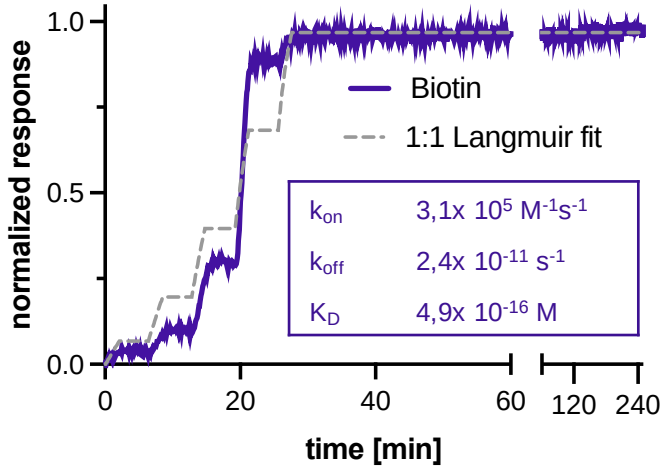
If the DEL barcode can not be modified an adapter oligonucleotide can be provided consisting of a **csOligo** and **csDEL barcode**. This bridging oligo can be used for DDI guided functionalization of the 54 mologramms. The kinetic characterization of the target protein can be performed by a single cycle kinetics approach fully guided by the instruments software.

DEL hit screening with Molography
Direct kinetic characterization





It is known in literature that Biotin/streptavidin interaction is nearly covalent while the desthiobiotin/streptavidin interaction can be broken by specific condition. The weaker interaction is also visible by the existing off-rate in the long dissociation phase of the experiment.



The plotted graphs are again a single sensorgram of a representative mologram of the whole 18 mologram subarray. The used 1:1 Langmuir fit of the SCK shows small deviations for the biotin interaction. This could be due to depletion effect. In general such a high affinity interaction is also borderline to what can be measured

Injection of ligand (at 50 µl/min)

DEL screen of Carbonic Anhydrase IX on 3 plex chip

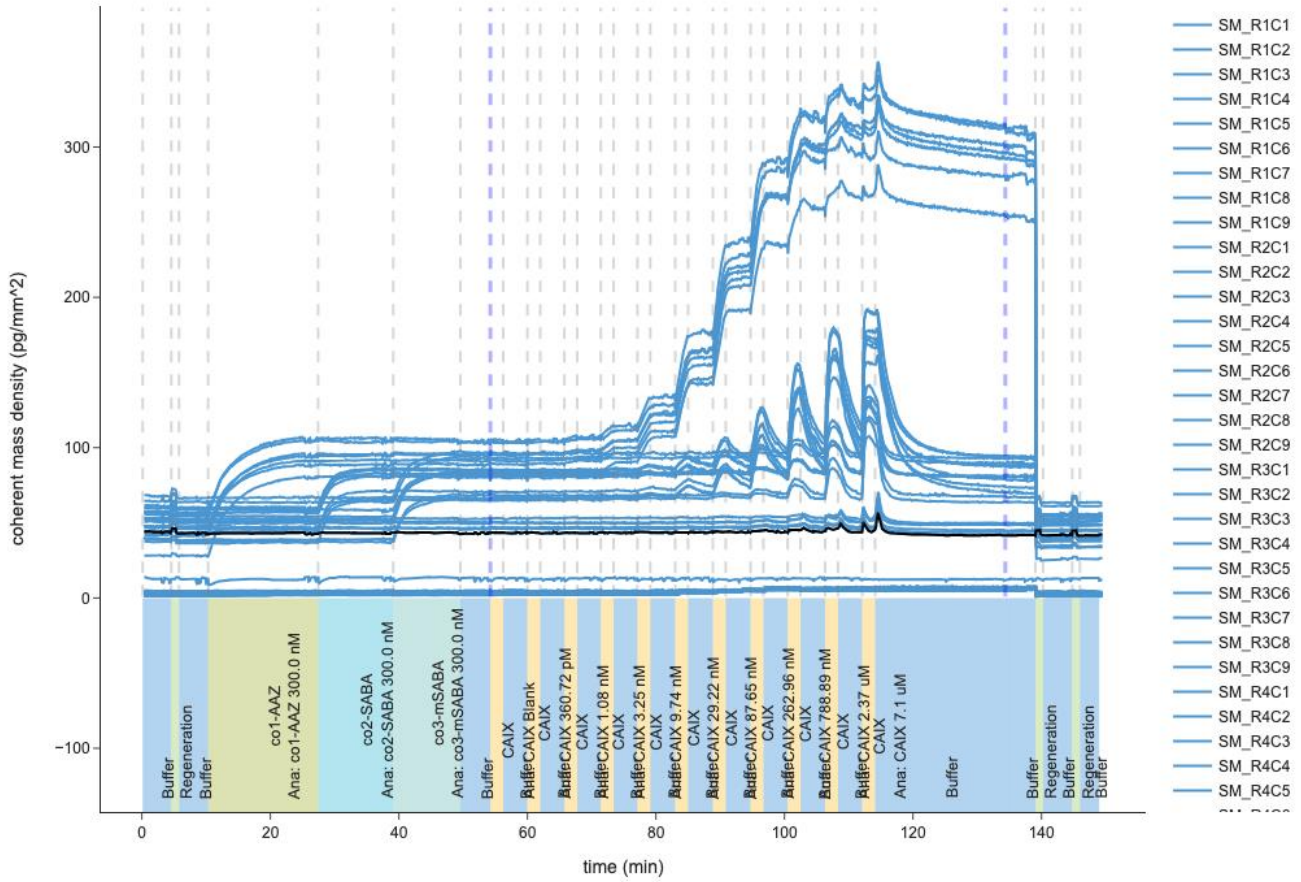
Miltenyi Biotec

Compound Immobilization

Kinetic Screen

Compounds:

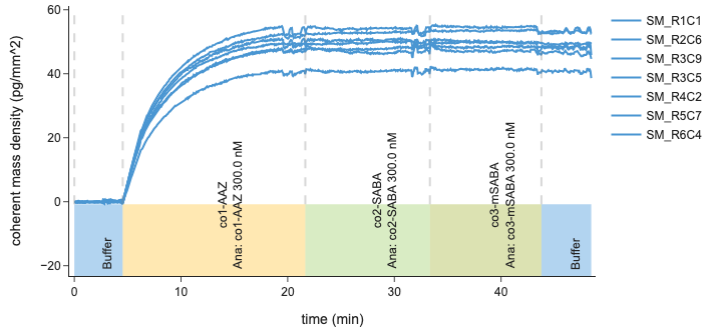
- AAZ (Acetazolamide)
- SABA (Sulfamoylbenzoic Acid)
- mSABA (meta-Sulfamoylbenzoic Acid)

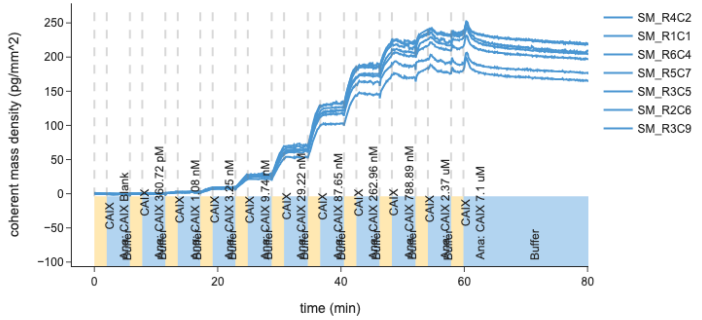


Compound Immobilization

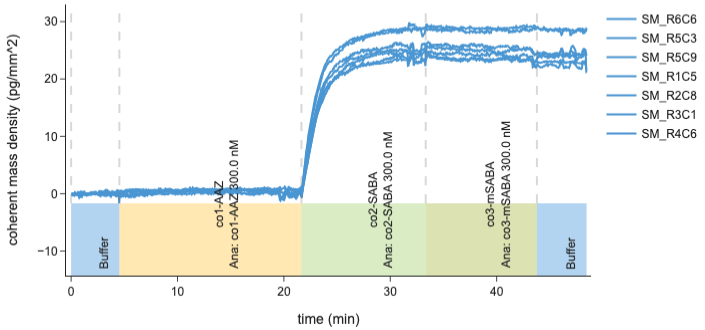
Kinetic Screening

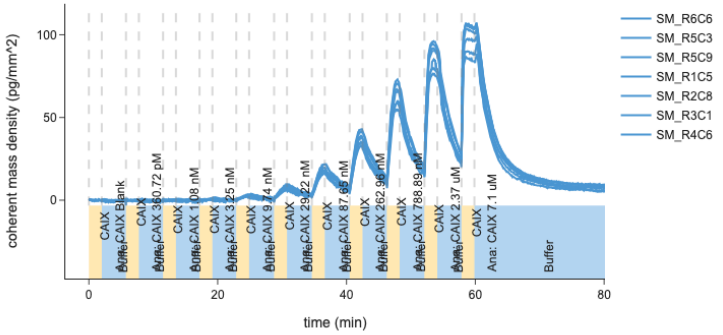
AAZ



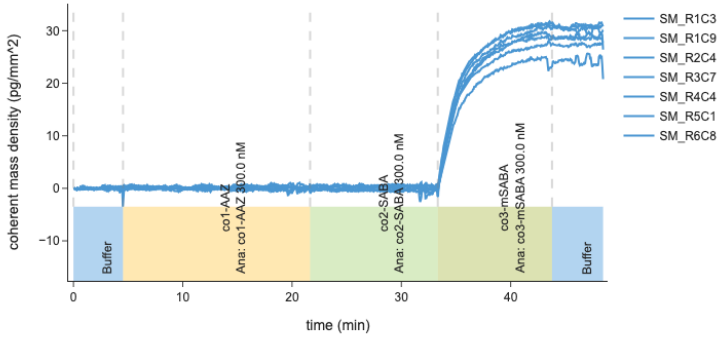


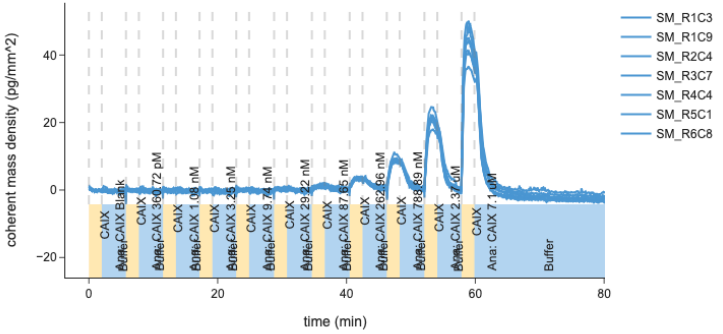
SABA





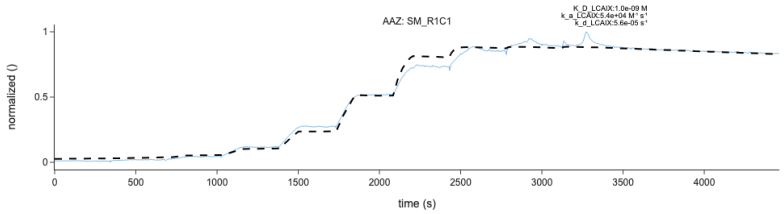
mSABA



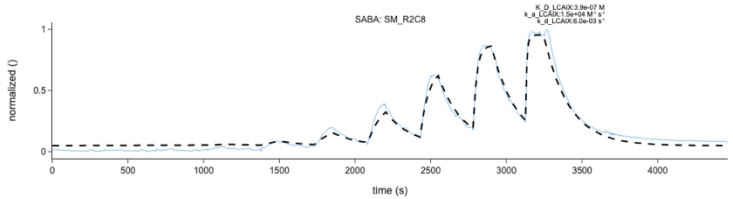


Kinetic parameters Carbonic Anhydrase IX

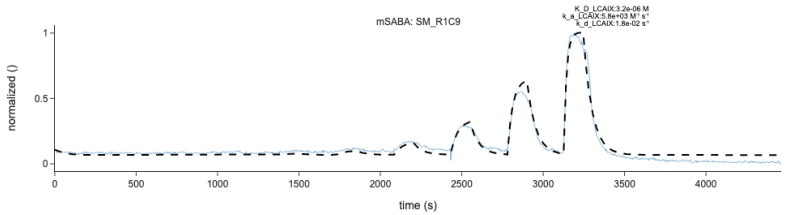
AAZ



SABA

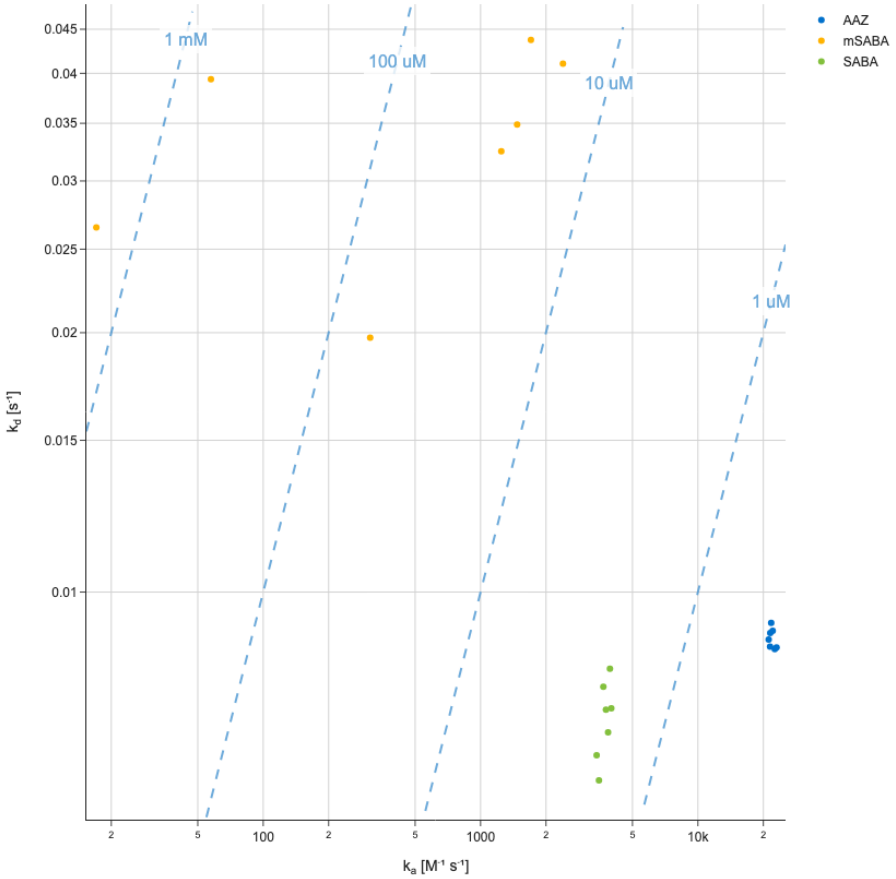


mSABA

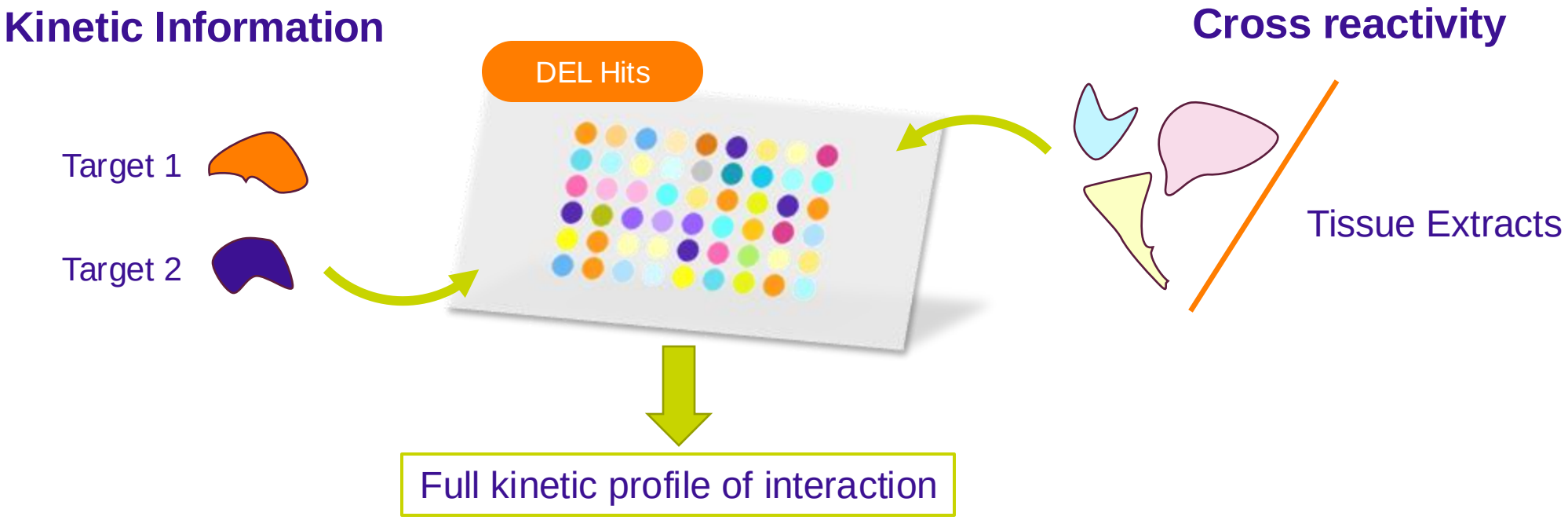


Compound	K_D	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]
AAZ	790 pM	5.75e04	4.6e-05
SABA	326 nM	2e04	6.5e-03
mSABA	2 uM	7.5e03	1.46e-02

Isoaffinity map



DEL hit validation with Mology raphy
Customer benefits



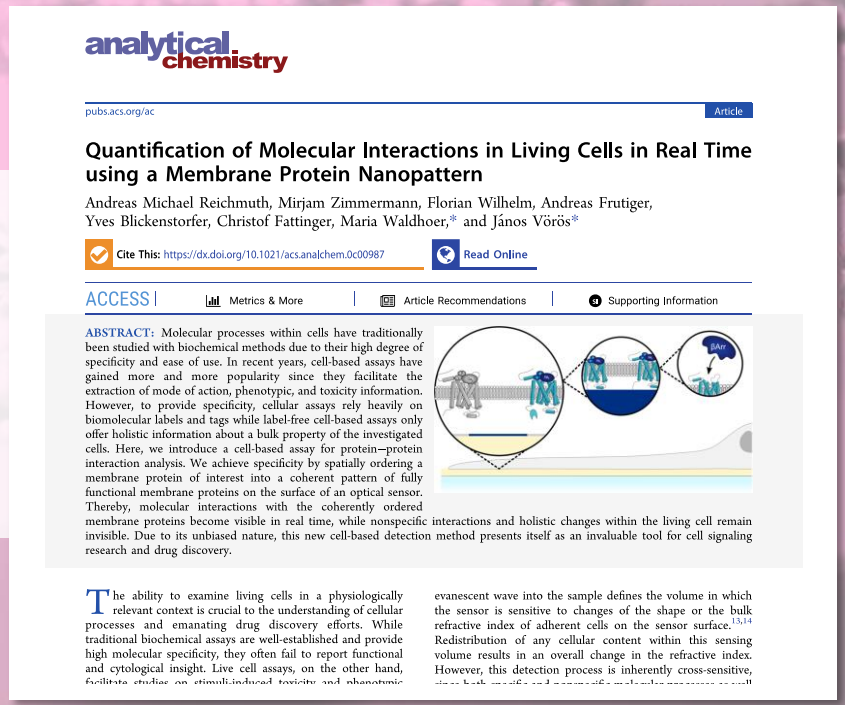
Going crude: An additional unique benefit of focal Mology raphy would be to test DEL hits against the biological matrix where the drug would be finally delivered. Thereby potential interfering biological interactions (drug masking) could be evaluated.

Application example

Miltenyi Biotec

Cell mediated interactions

Membrane receptor Characterization



analytical chemistry

pubs.acs.org/ac Article

Quantification of Molecular Interactions in Living Cells in Real Time using a Membrane Protein Nanopattern

Andreas Michael Reichmuth, Mirjam Zimmermann, Florian Wilhelm, Andreas Frutiger, Yves Blickenstorfer, Christof Fattinger, Maria Waldhoer,* and János Vörös*

Cite This: https://dx.doi.org/10.1021/acs.analchem.0c00987 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

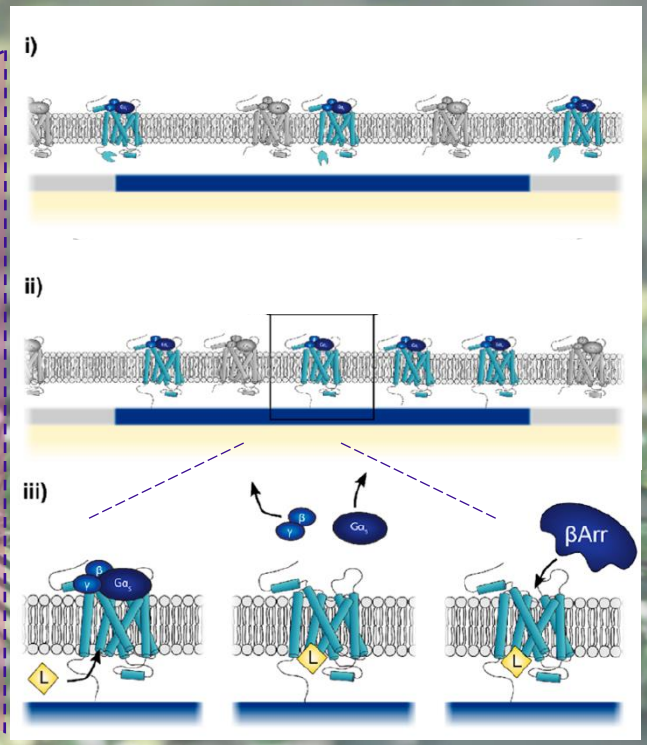
ABSTRACT: Molecular processes within cells have traditionally been studied with biochemical methods due to their high degree of specificity and ease of use. In recent years, cell-based assays have gained more and more popularity since they facilitate the extraction of mode of action, phenotypic, and toxicity information. However, to provide specificity, cellular assays rely heavily on biomolecular labels and tags while label-free cell-based assays only offer holistic information about a bulk property of the investigated cells. Here, we introduce a cell-based assay for protein−protein interaction analysis. We achieve specificity by spatially ordering a membrane protein of interest into a coherent pattern of fully functional membrane proteins on the surface of an optical sensor. Thereby, molecular interactions with the coherently ordered membrane proteins become visible in real time, while nonspecific interactions and holistic changes within the living cell remain invisible. Due to its unbiased nature, this new cell-based detection method presents itself as an invaluable tool for cell signaling research and drug discovery.

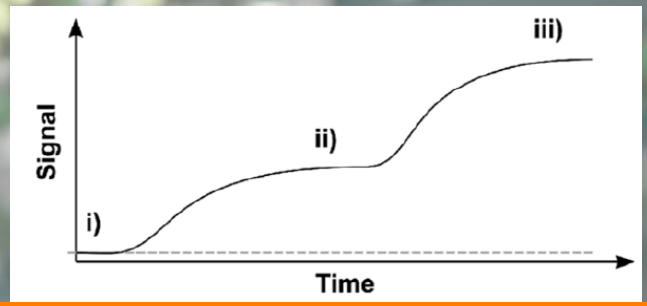
The ability to examine living cells in a physiologically relevant context is crucial to the understanding of cellular processes and emanating drug discovery efforts. While traditional biochemical assays are well-established and provide high molecular specificity, they often fail to report functional and cytological insight. Live cell assays, on the other hand, facilitate studies on stimuli-induced toxicity and phenotypic

evanescent wave into the sample defines the volume in which the sensor is sensitive to changes of the shape or the bulk refractive index of adherent cells on the sensor surface.[13,14] Redistribution of any cellular content within this sensing volume results in an overall change in the refractive index. However, this detection process is inherently cross-sensitive,

Drug screening: Ruling out off-target binding and accelerating drug development

Miltenyi Biotec





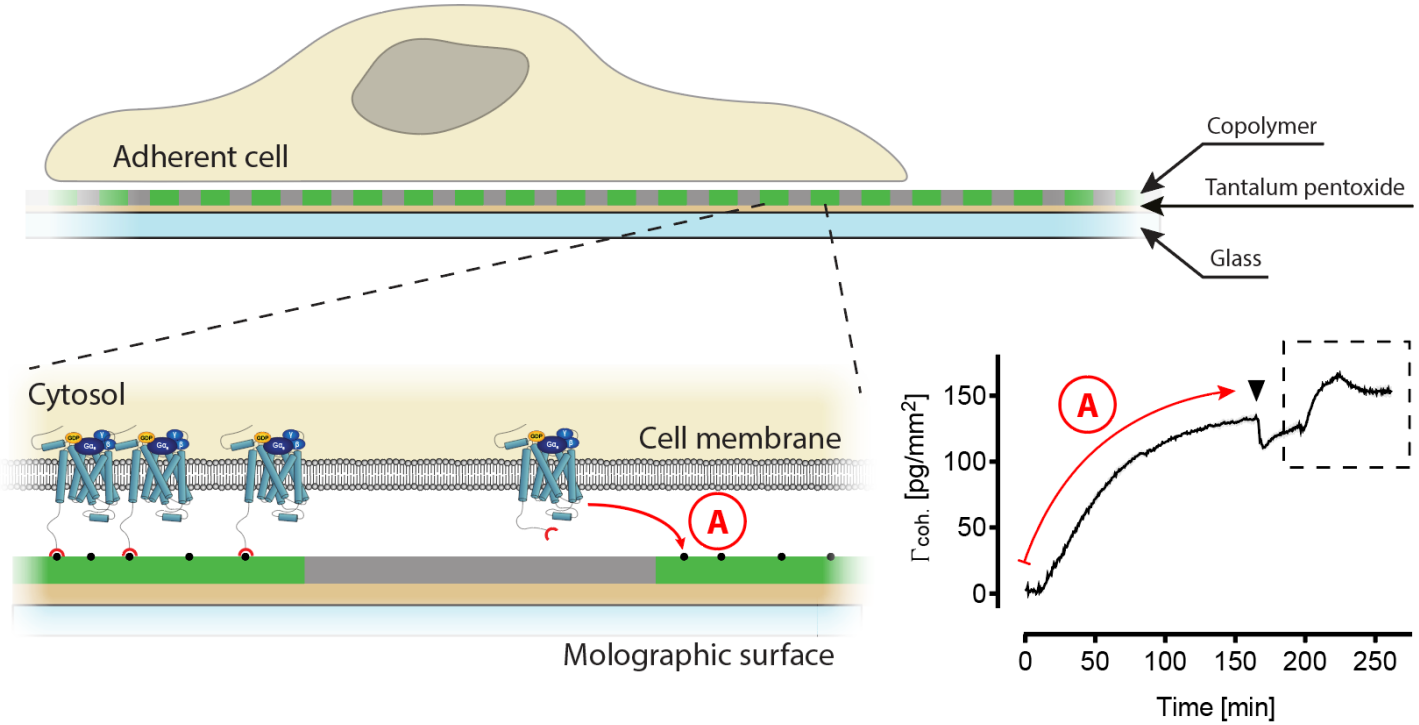
Membrane proteins can be directly investigated in their native environment

Detection of G-protein coupled receptors (β2AR) in living cells

Miltenyi Biotec

Formation of a functional SNAP-β2AR transmembrane mologram in HEK293 cells.

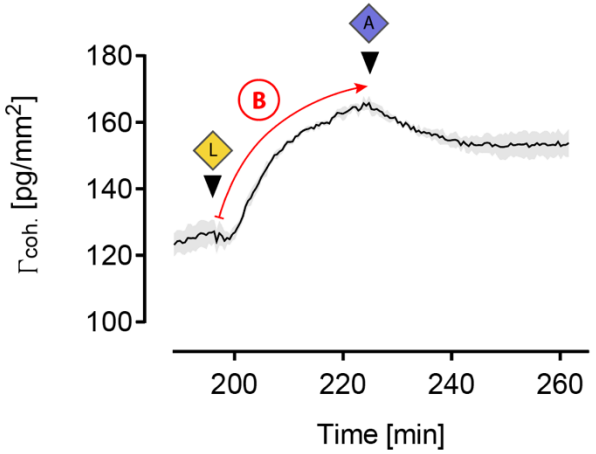
Cells are plated on a sensor chip with previously employed template mologram. The GPCRs arrange within the cell membrane over the course of about 150 min shown by the molographic signal (red arrow). Medium is exchanged for the assay (black triangle).



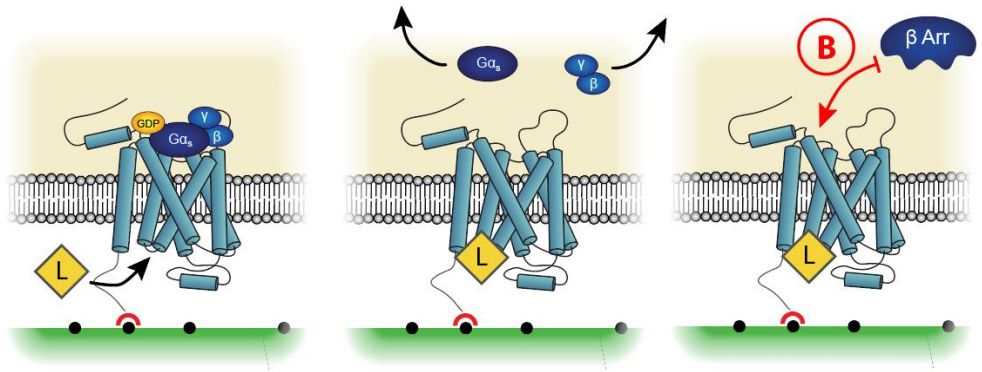
Additional Data
✓ Unmodified HEK293 cells → no signal increase

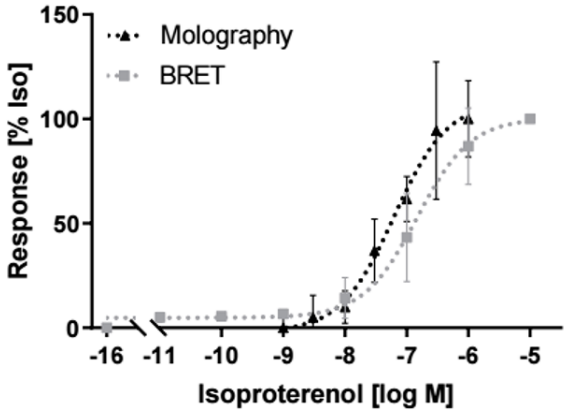
Source: Reichmuth et al. 2020. "Quantification of Molecular Interactions in Living Cells in Real Time Using a Membrane Protein Nanopattern." *Analytical Chemistry* 92 (13): 8983–91.

Detection of G-protein coupled receptors (β2AR) in living cells



Stimulation of the SNAP-β2ARs with 1µM isoproterenol (first triangle) shows an increase in the molographic signal, which is partially reversed by the addition of a 10µM amount of the competitive antagonist ICI 118,551 (second triangle).





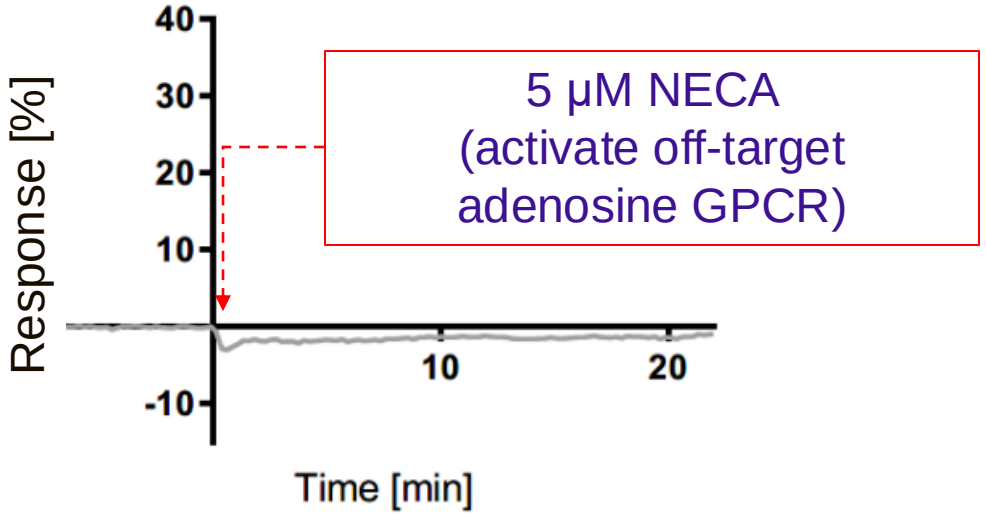
Cell-based molography compared to classical BRET arrestin recruitment assay.
For the molographic assay, cells were treated with increasing concentration of isoproterenol (left). The molographic signal was normalized to 1 µM isoproterenol (=100%).

- ✓ Quantification and identification of binding partner
- ✓ Determination of receptor occupancy
- ✓ Concentration-response curves
- ✓ Receptor deorphanization

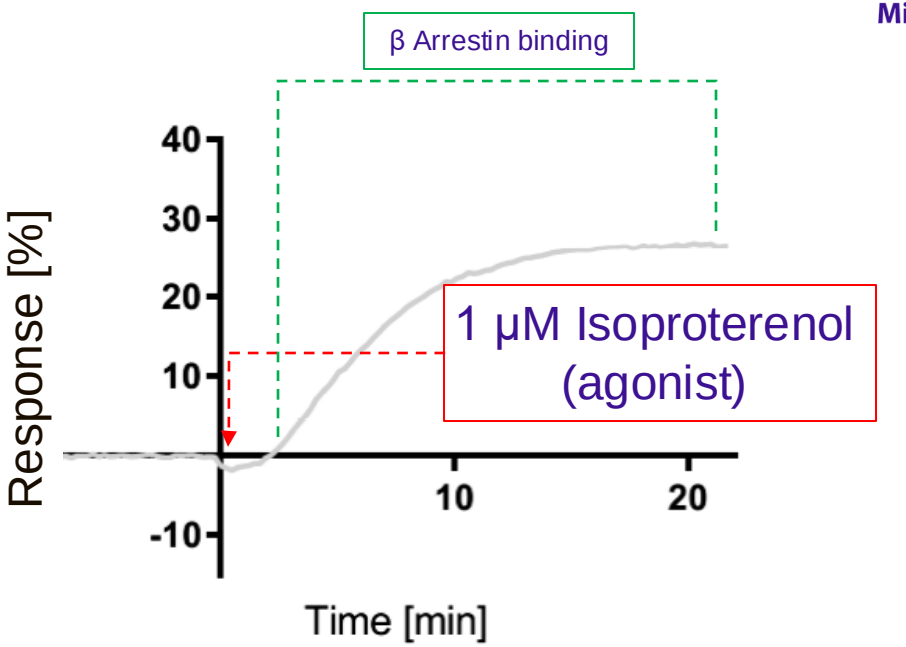
Compatibility of target specific characterization with living cells

Source: Reichmuth et al. 2020. "Quantification of Molecular Interactions in Living Cells in Real Time Using a Membrane Protein Nanopattern." *Analytical Chemistry* 92 (13): 8983–91.

Detection of G-protein coupled receptors (β2AR) in living cells → *off-target controls*



- ✓ Effect of injection of dummy ligand
- ✓ Effect on off-target GPCR signaling

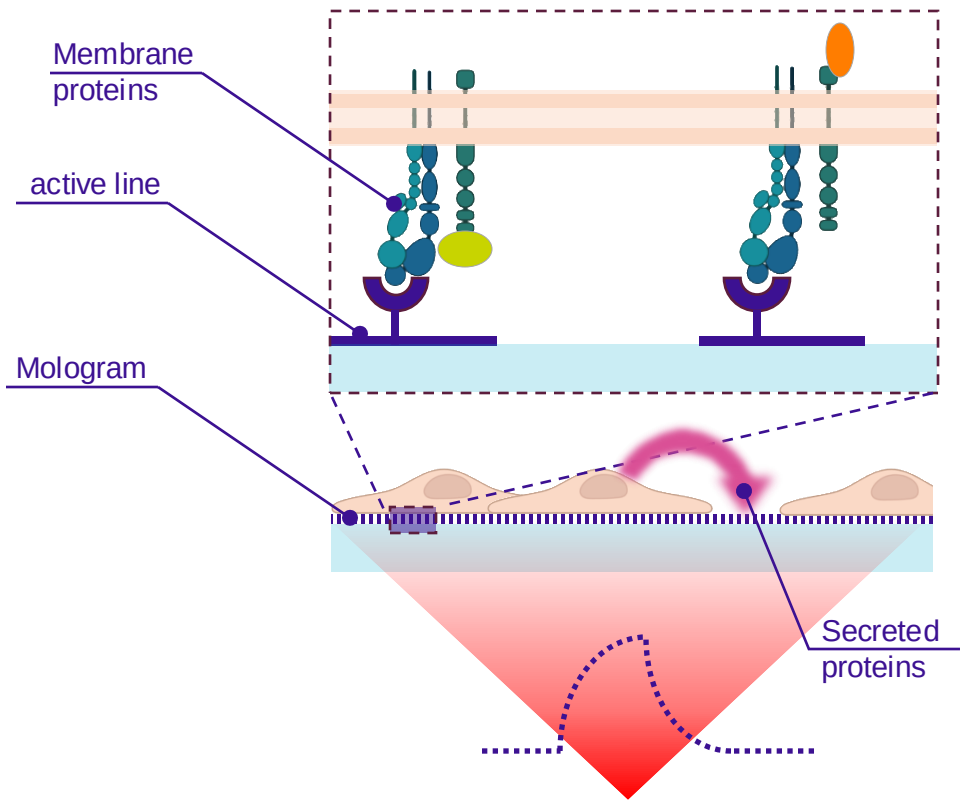


- ✓ Positive control for target GPCR

Additional Data
- ✓ β Arrestin knock-out → no signal increase
- ✓ β Arrestin knock-out + β Arrestin plasmid → signal increase

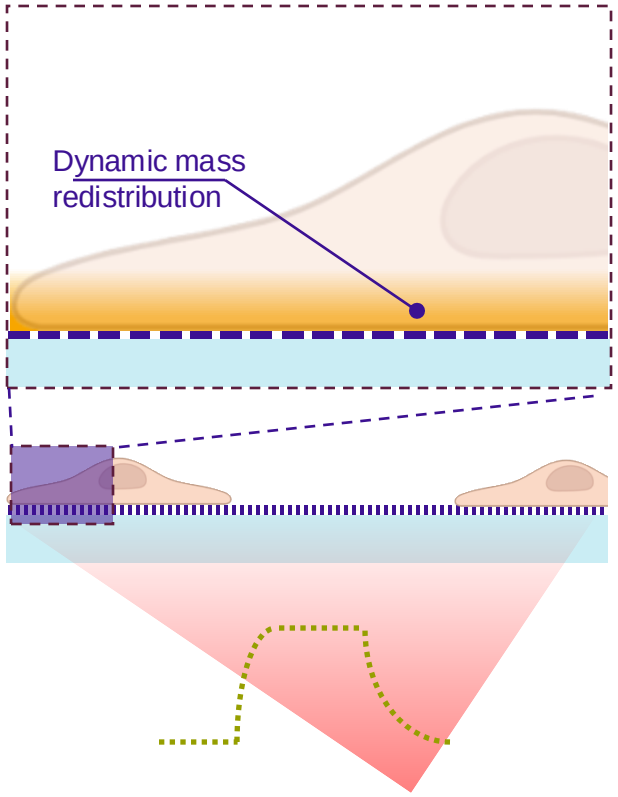
Data generation for cell mediated interaction

Diffractometric Data
- Focal Molography -



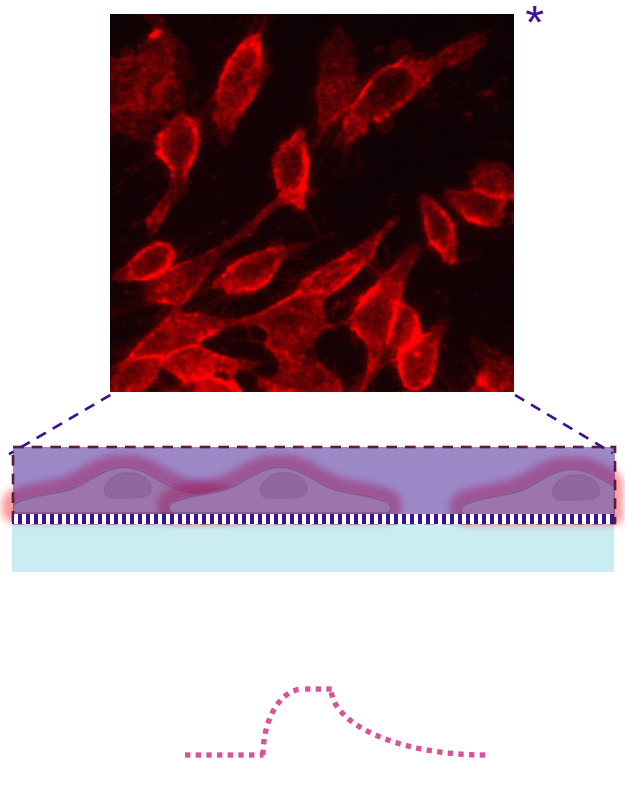
Spot intensity

Refractometric Data
- SPR like -



Spot movement

Fluorescence Data
- Microscopy like -



Fluorescence intensity

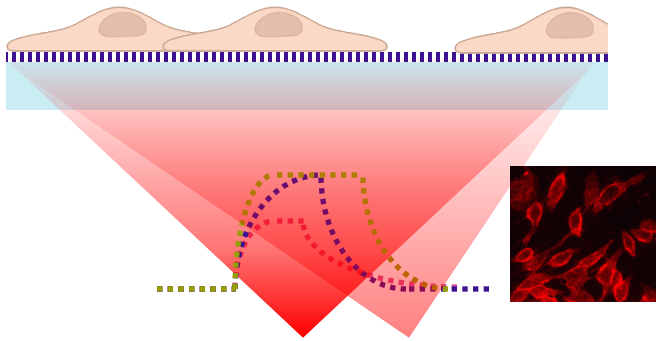
* Example image from aatbio.com; recorded with third-party microscope

Data generation for cell mediated interaction

Diffractometric Data
- Focal Mology raphy -

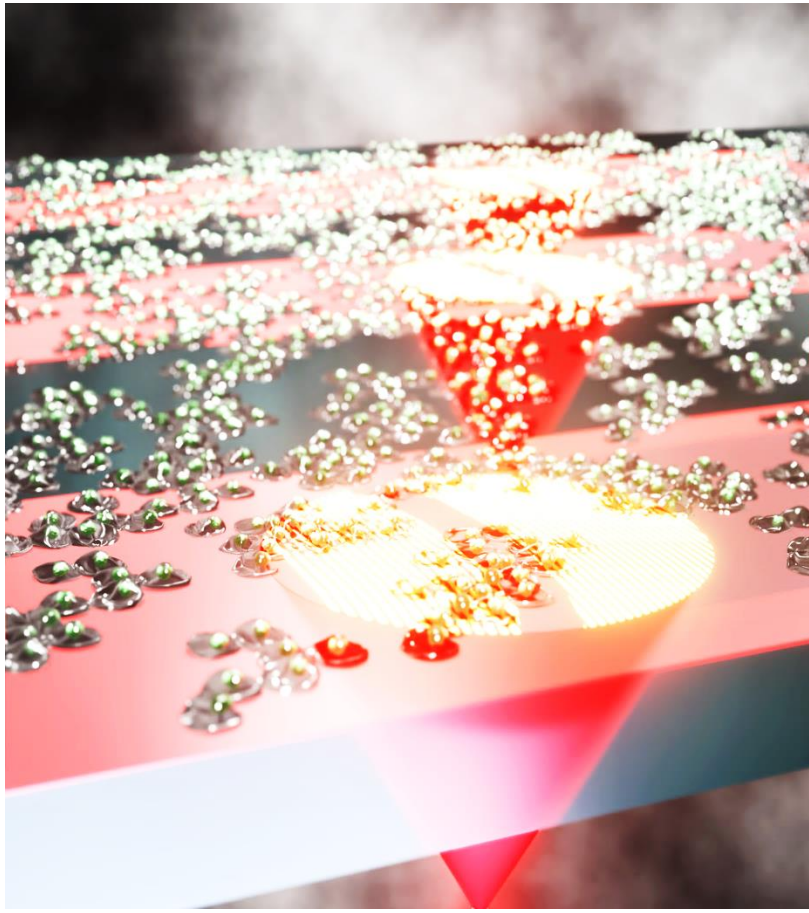
Refractometric Data
- SPR like -

Fluorescence Data
- Microscopy like -



All combined in one benchtop instrument

Summary



Miltenyi Biotec
Thank you for your attention!

Dr. Andreas Frutiger
VP R&D

E-mail andreasfru@miltenyi.com
Web: www.lino-biotech.com