Optical Biosensors
Concepts and Applications

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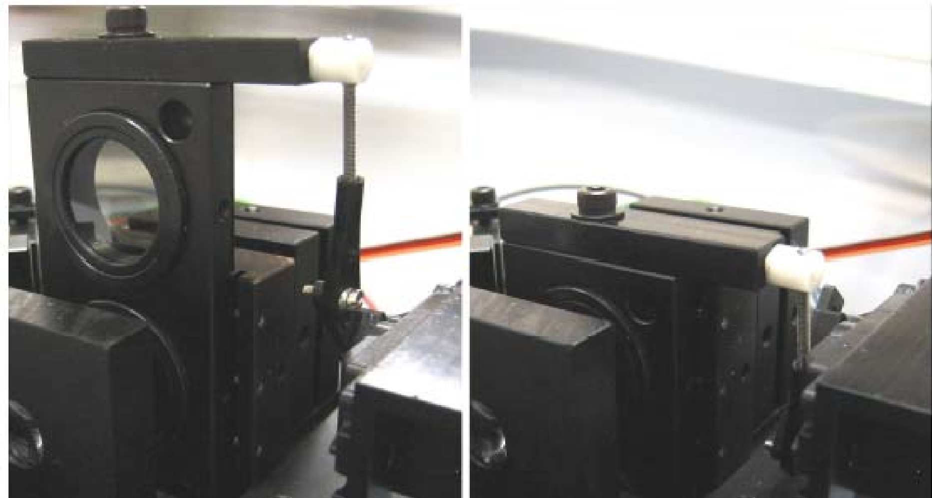


Outline

1. Elements of an Optical Biosensor (very basic principles)
2. Immunosensors: Principles
3. Landscape of optical Readouts
4. **Examaples of some Techniques and Applications**

   - **L**ateral **F**low **A**ssay (**LFA** = Strip-Test)

   - **S**canning **C**onfocal **F**luorescence **S**pectroscopy (**SCFS**)

   - Diffusion Analysis with Fluorescence

     Fluorescence **C**orrelation **S**pectroscopy (**FCS**)

     Fluorescence **P**olarisation **A**nalysis (**FPA**)

   - Integrated Optical Sensing (Labelfree detection)

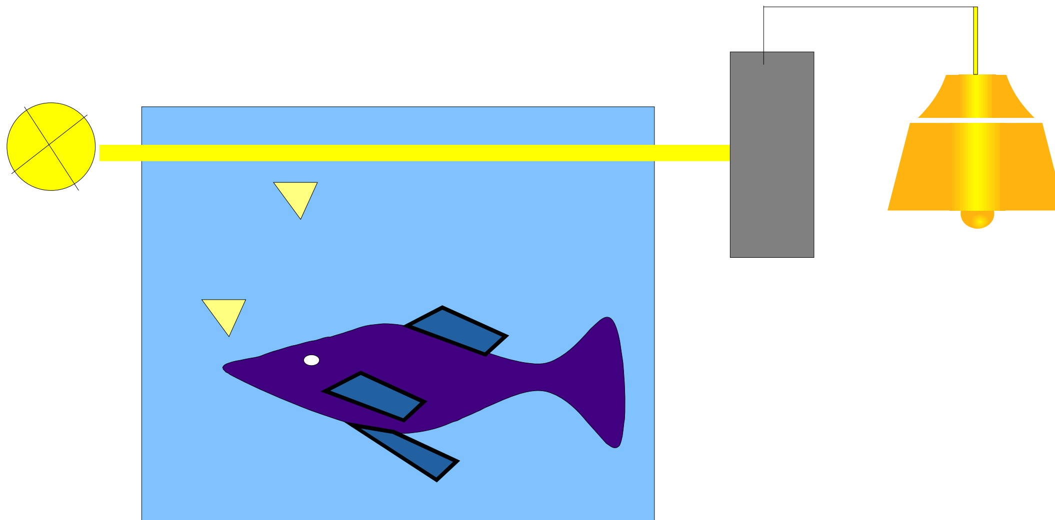
     Different concepts of grating couplers

5. Conclusions

Elements of an Optical Biosensor

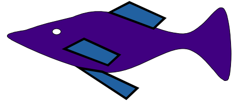
- Biological system (**fish**) reacts to an environmental parameter (**poison**)
- The reaction is detected by an optical set-up (**light barrier**)

  => Light interacts with the biological system (**light barrier is disrupted**)
  => Information about the biological system is stamped on the light
- Light will be analyzed => information is transferred in an electronical form
- Data is analyzed and transfered in a user-friendly format (**bell rings**)



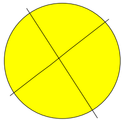
Requirements on the Biological and Optical Systems

The „reaction“ of the biological system to a target should



- occur with only a single „type“ of target => **Specificity**
- be induced by a small stimulus (concentration) => Sensitivity
- easy to be detected => Sensitivity
- have reproducible features (long term stability) => Robustness

The optical system should response to



- small changes in the biological system => **Sensitivity**
- a single parameter of the bio.system (no cross talk) => **Specificity**

Statistical definitions of Sensitivity / Specificity:

pos. detected Samples / # true pos. Samples => **Sensitivity**

neg. detected Samples / # true neg. Samples => **Specificity**

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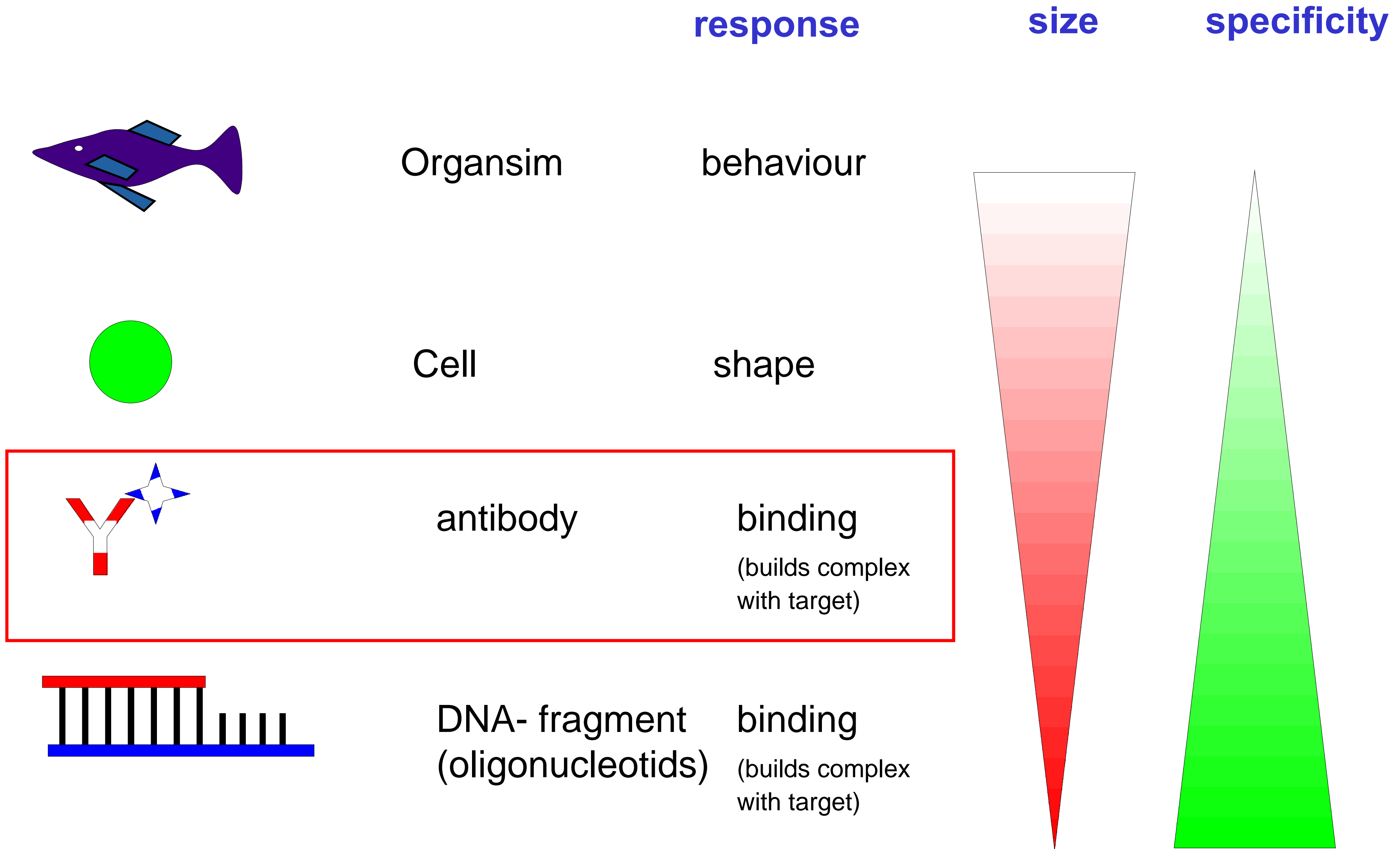
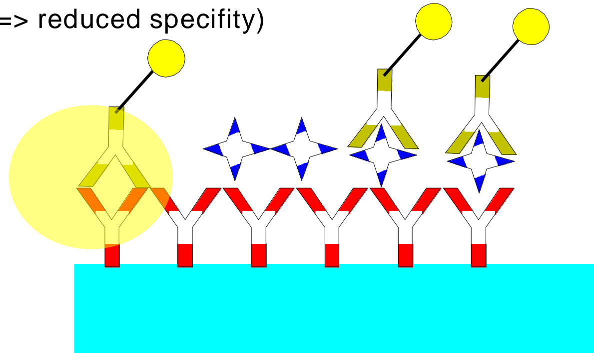
Biological „sensing elements“



Immunosensors: Principles

I. Detection of an Antigen with an immuno-sandwich:

false positive result
(=> reduced specifity)



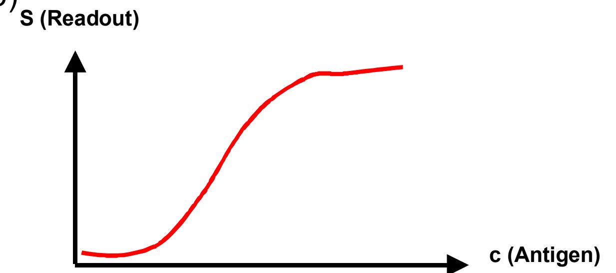
labeling (dye, mass, beads,DNA...)

detection antibody (second Ab)

TARGET: Antigen

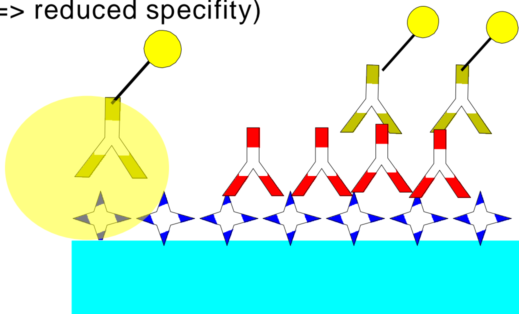
capture antibody

glass, plastic



II. Detection of an Antibody (immuno-response):

false positive result
(=> reduced specifity)



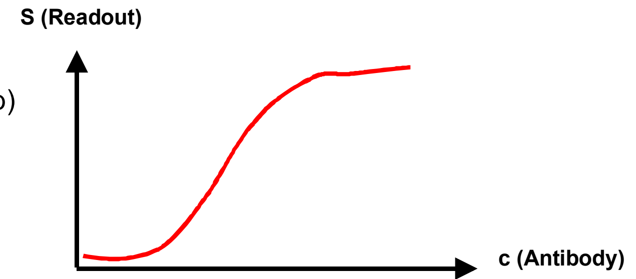
labeling (dye, mass, beads,DNA...)

secondary antibody (against target-Ab)

TARGET: Antibody

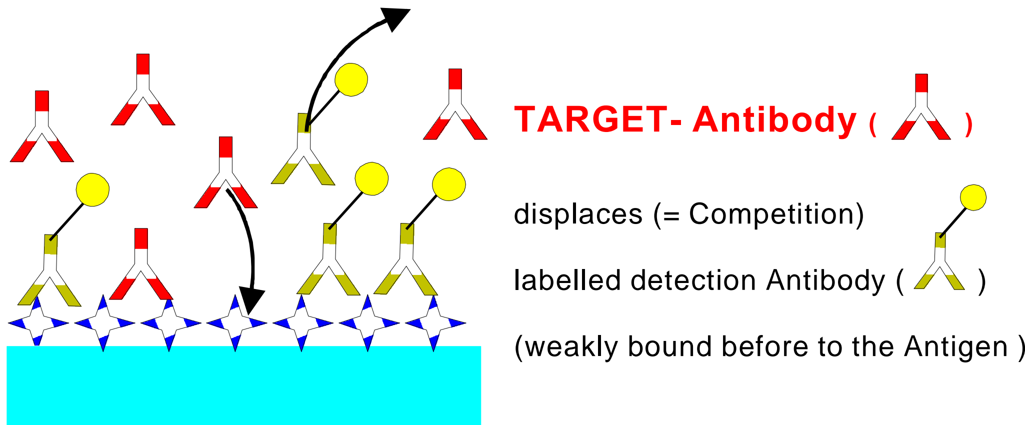
antigen (parts of antigen)

glass, plastic



Immunosensors: Principles

III. Detection of an Antibody (Competition format):

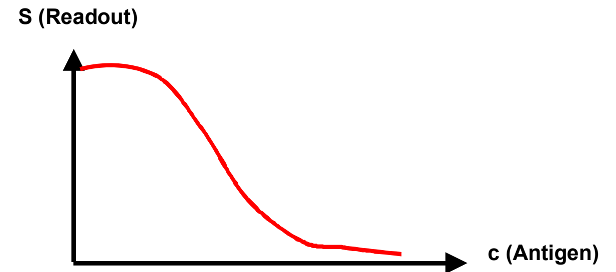


TARGET- Antibody ( )

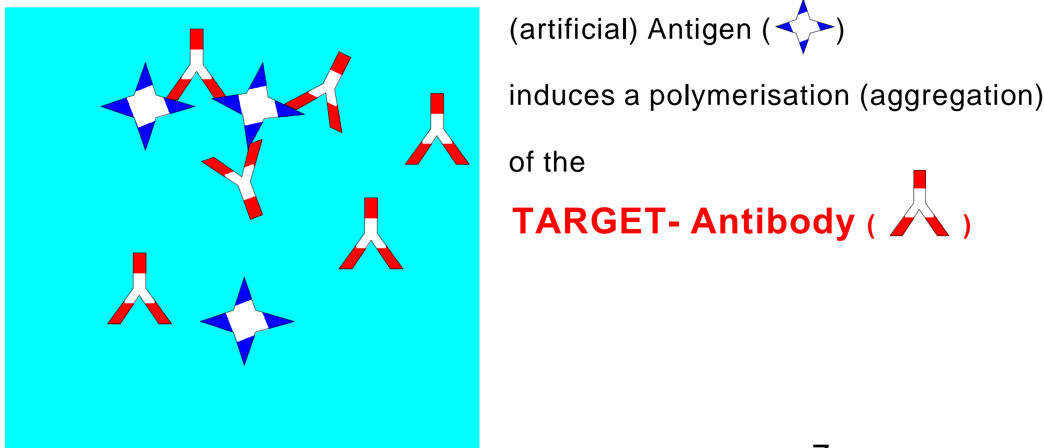
displaces (= Competition)

labelled detection Antibody ( )

(weakly bound before to the Antigen)



IV. Detection of an Antibody (in solution):

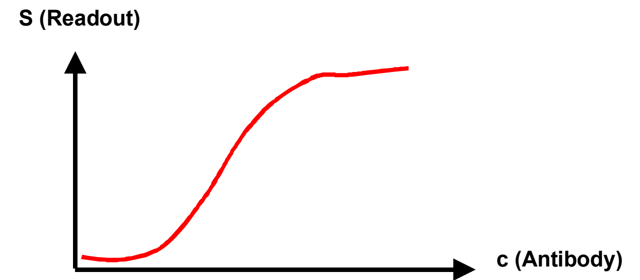


(artificial) Antigen ( )

induces a polymerisation (aggregation)

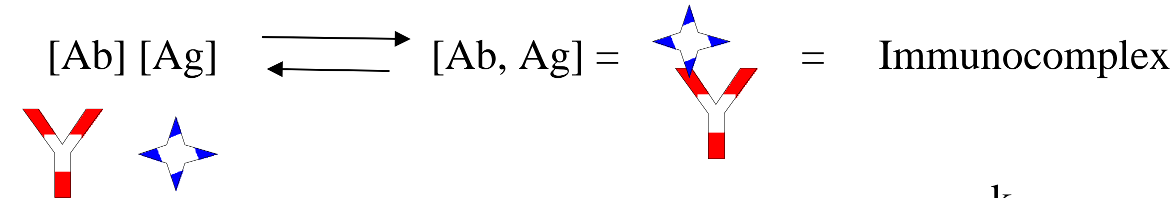
of the

TARGET- Antibody ( )



Kinetics of an immuo reaction (elemantary case):

Equilibrium Reaction: Ab = Antibody; Ag = Antigen

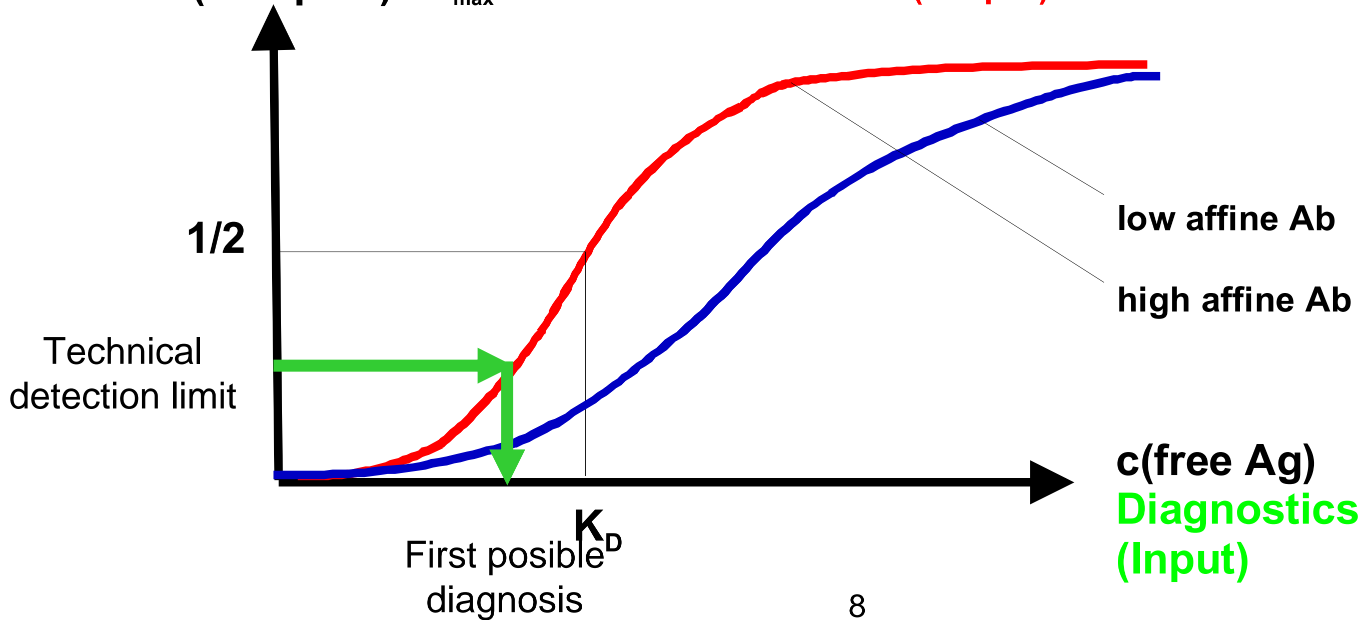


$\frac{d}{dt}[Ab, Ag] = k_{on}\,[Ab]\,[Ag] - k_{off}\,[Ab,Ag]$

0 for equilibrium

$K_D = \frac{k_{off}}{k_{on}}$ characterizes the „strength“ of the Ab-Ag interaction (Affinity)

c(complex) / c_{max} => technical READOUT (Output)



small K_D ⇔ high sensitivity

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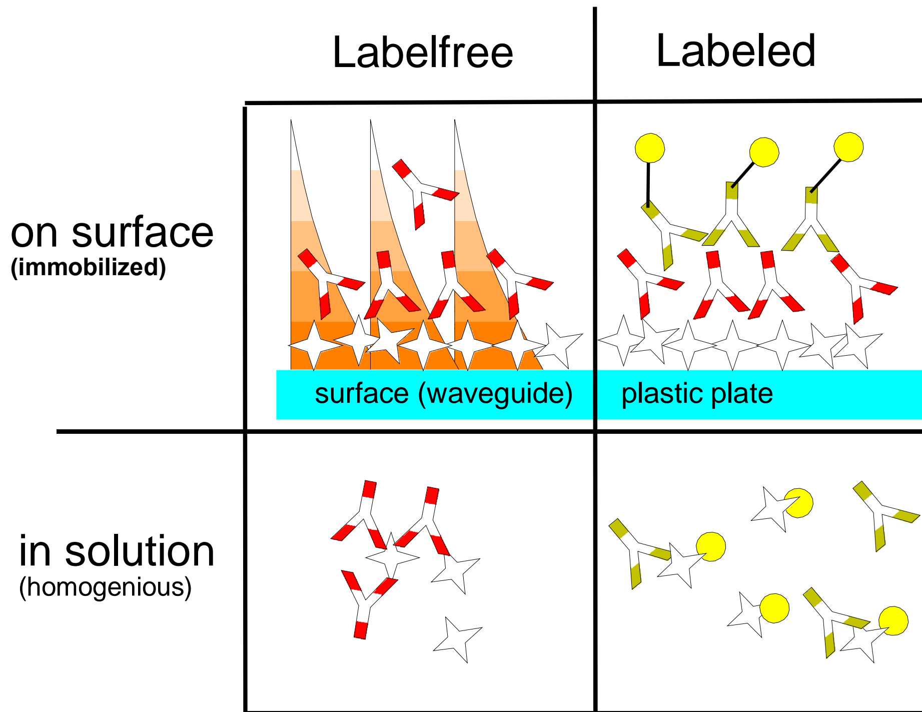
Landscape of Optical Readouts

Illustrated with the detection of Antibodies

Configuration



Optical Read-Outs:

Labelfree	Labeled
Waveguides Plasmon resonance Reflectrometry Spectroscopy	Fluorescence Chem.lumin. FRET Quenching Absorption
Visible by eye Light scattering Absorption	Fluor. Polarisation (FPA) Fluor. Correlation (FCS)

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Principles of optical interactions => Readout-Techniques

1. Absorbance

coloured beads, Example: **Lateral-flow Assay (BSE-Test)**

surface plasmon **resonance** (SPR) Examples: see www.biacore.com

spectroscopy (UV, Infrared) Examples: see www.micro-biolytics.com

2. Fluorescence => intensity, polarisation, quenching, lifetime

dye-labelled „tracer“ (Antibody, peptide, DNA) antigens or DNA

Examples: **Scanning Fluor. Spec. (SFS)**, **Fluor. Corr. Spec. (FCS)**, **Fluor. Polari. Analysis (FPA)**

3. Speed of light => refractive index

integrated optics => waveguides

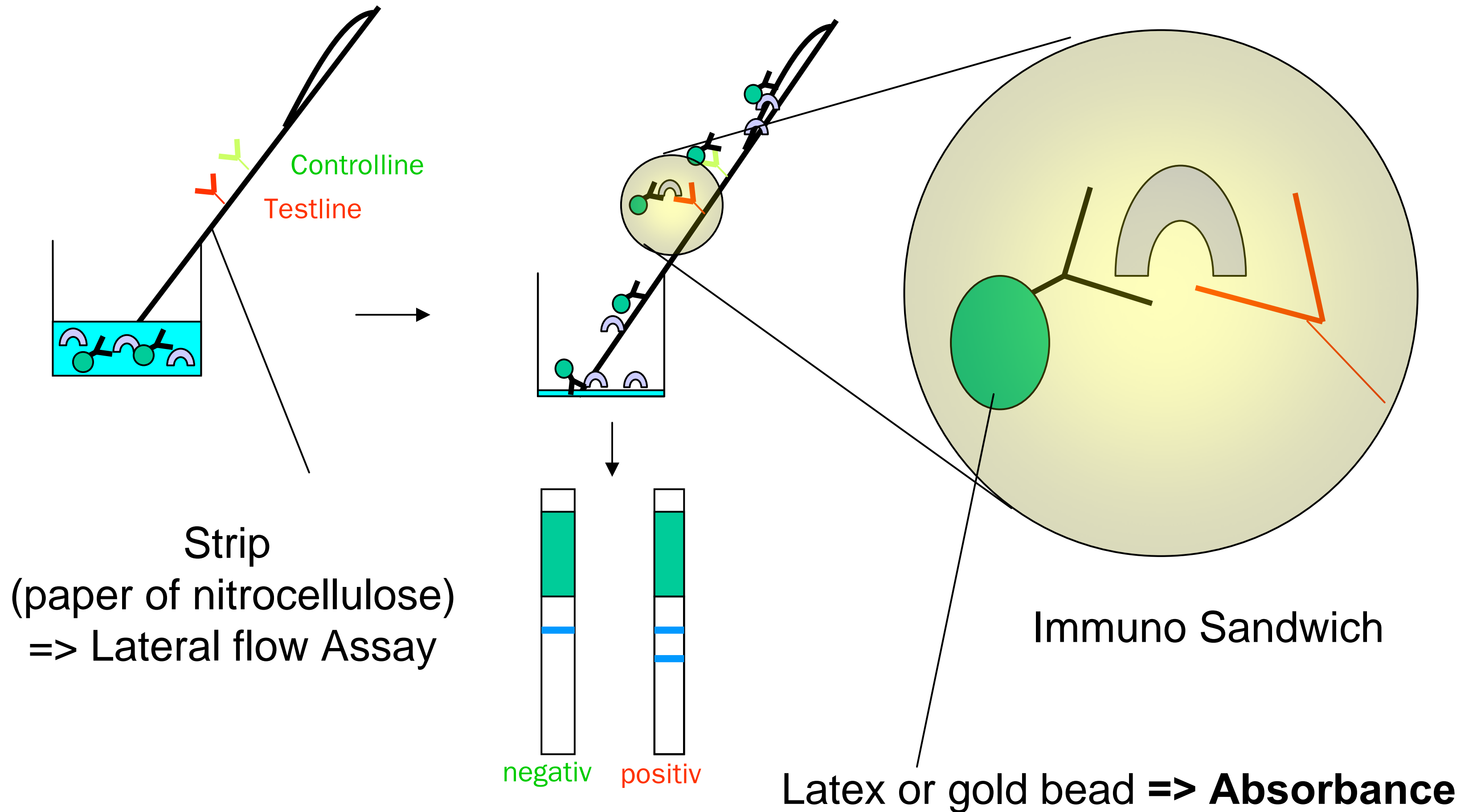
Examples: **Grating couplers**, Interferometers

4. Light scattering => Shape of molecules defines the pattern of scattering

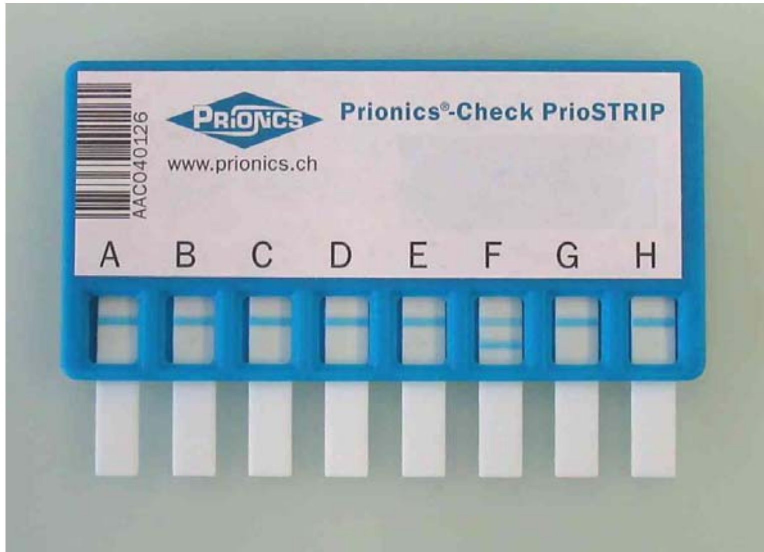
Examples: see www.ap-lab.com

Example of **Absorbance Readout:** Lateral flow Assay

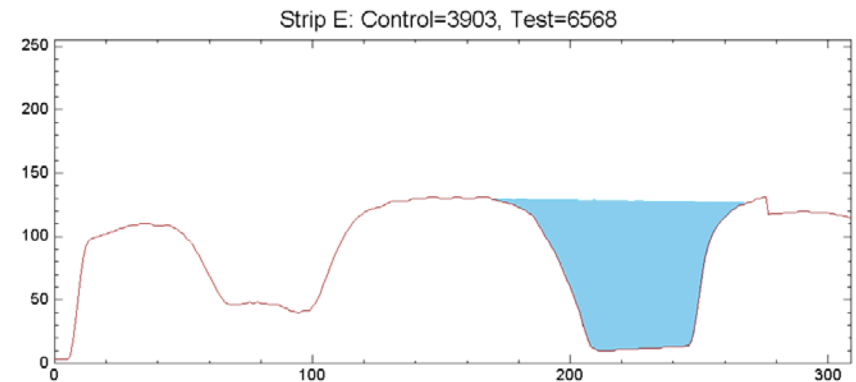
Detection of missfolded Prion Proteins (PrP) (related to BSE = made cow desease)



Human readable signals



← Detection of a BSE case on strip F



The **result** is clear-cut: **one** line means BSE **negative**

two lines means BSE **positive**

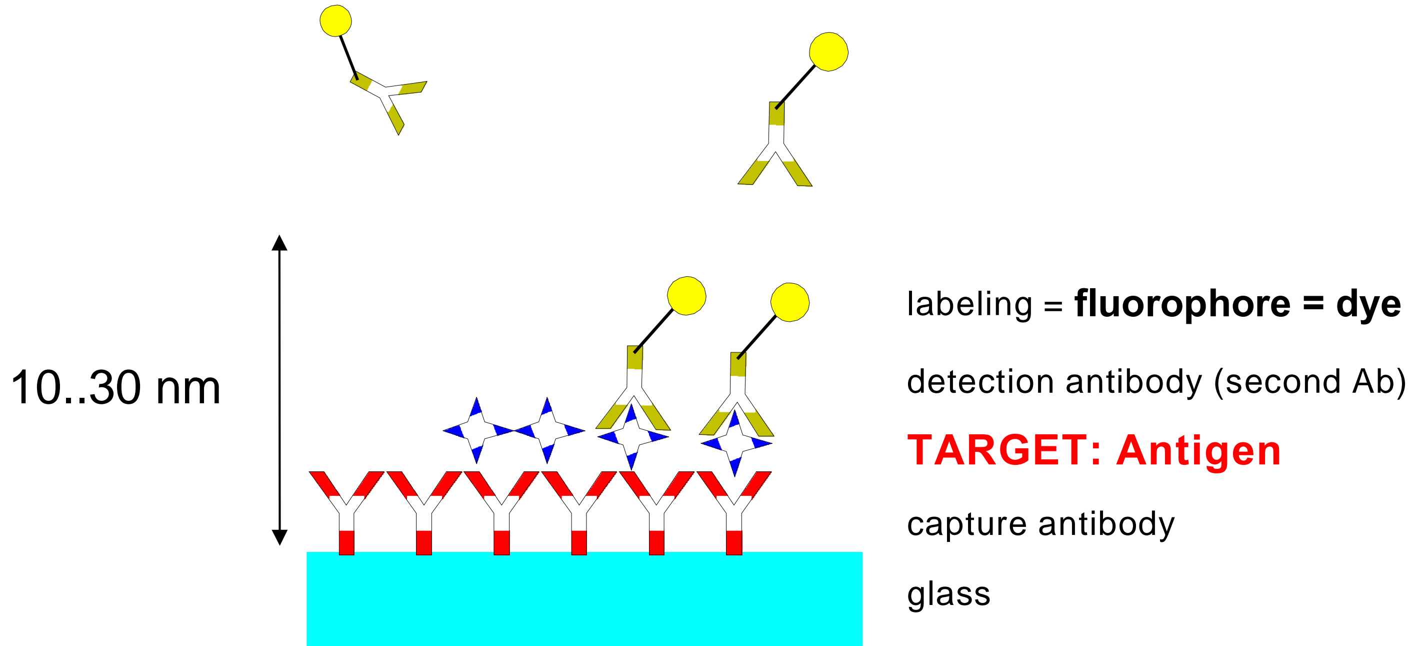
Automated readout for High troughput

1. Assay in 96-well format:

2.Electronic read-out with a Scanner

Comb# / Well#		1 / 1-8	2 / 9-16	3 / 17-24	4 / 25-32	5 / 33-40	6 / 41-48	7 / 49-56	8 / 57-64	9 / 65-72	10 / 73-80	11 / 81-88	12 / 89-96
A	Control	7445	4168	6396	6568	2456	7809	845	8228	7421	7627	925	6699
	Test	0	6222	6111	0	4739	0	4938	0	0	0	7383	0
B	Control	7462	3846	5988	6292	2417	7734	732	7273	7364	7679	930	6348
	Test	0	5979	6189	4423	5062	0	1784	0	0	0	6761	0
C	Control	2513	7768	7275	7039	7512	8265	608	7666	7556	4652	953	6126
	Test	5803	0	0	0	0	0	1805	0	0	5870	6050	0
D	Control	2384	7569	7221	7466	2394	7805	483	7649	7520	3956	196	6378
	Test	265	0	0	0	7408	0	5615	0	0	5613	0	0
E	Control	7893	7351	3415	7179	1319	8031	663	2746	3955	7484	7154	3903
	Test	0	0	6552	0	4925	0	4644	764	1002	0	0	6568
F	Control	7478	7363	3155	7325	4832	7713	761	2644	3957	7231	6865	4093
	Test	0	0	6808	0	0	0	1451	918	0	0	0	6487
G	Control	3242	7390	7079	7383	2444	8275	690	7625	6952	2679	6557	6199
	Test	0	0	427	0	7364	0	2626	0	0	0	3559	0
H	Control	3455	7512	7484	7550	2388	7771	673	7631	6620	2670	6746	6112
	Test	0	0	0	0	101	0	2763	0	0	1616	3172	0

Example of a fluorescence based readout (Intensity)

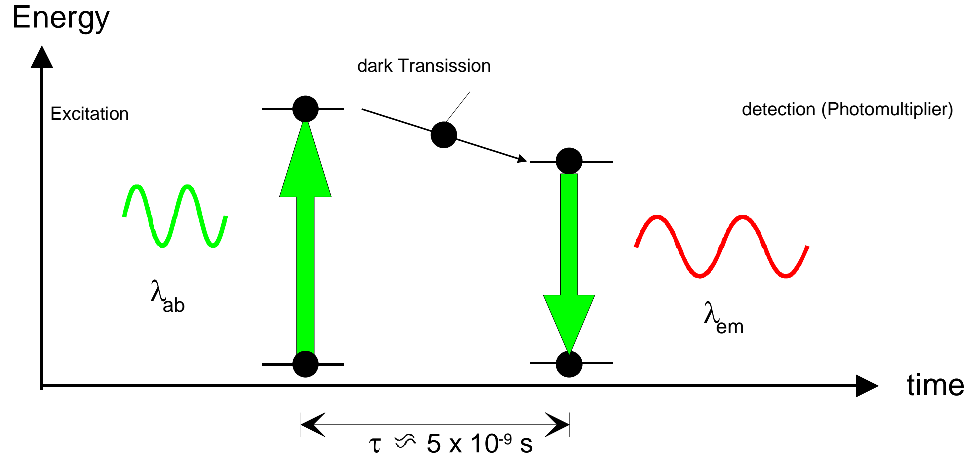


Requirements on an efficient sensitive readout:

Reduction of background signals produced by molecules in solution
Reduction of background signals produced by illuminatation of the target

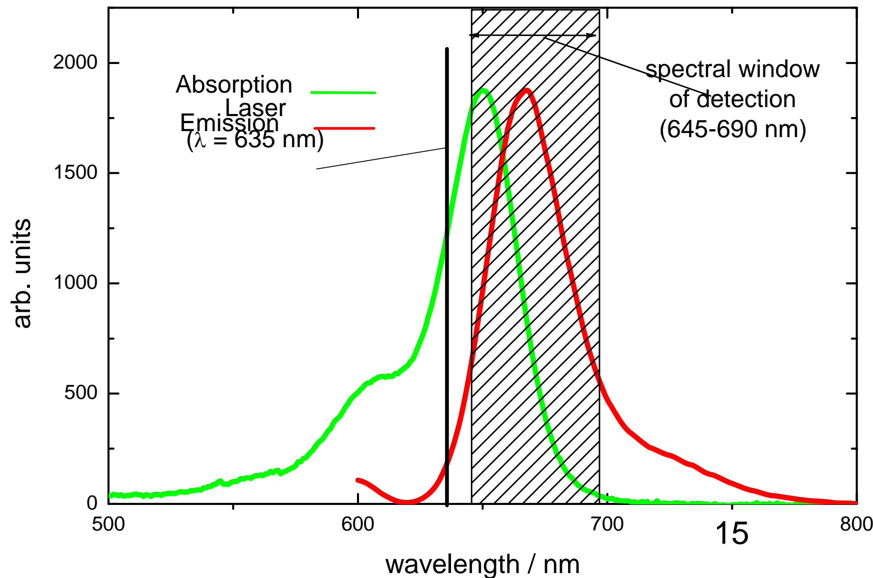
Strategies: Confine the region from where signals are detected.
Separate illumination and emission by different wavelengths

Fluorescence



Principle:

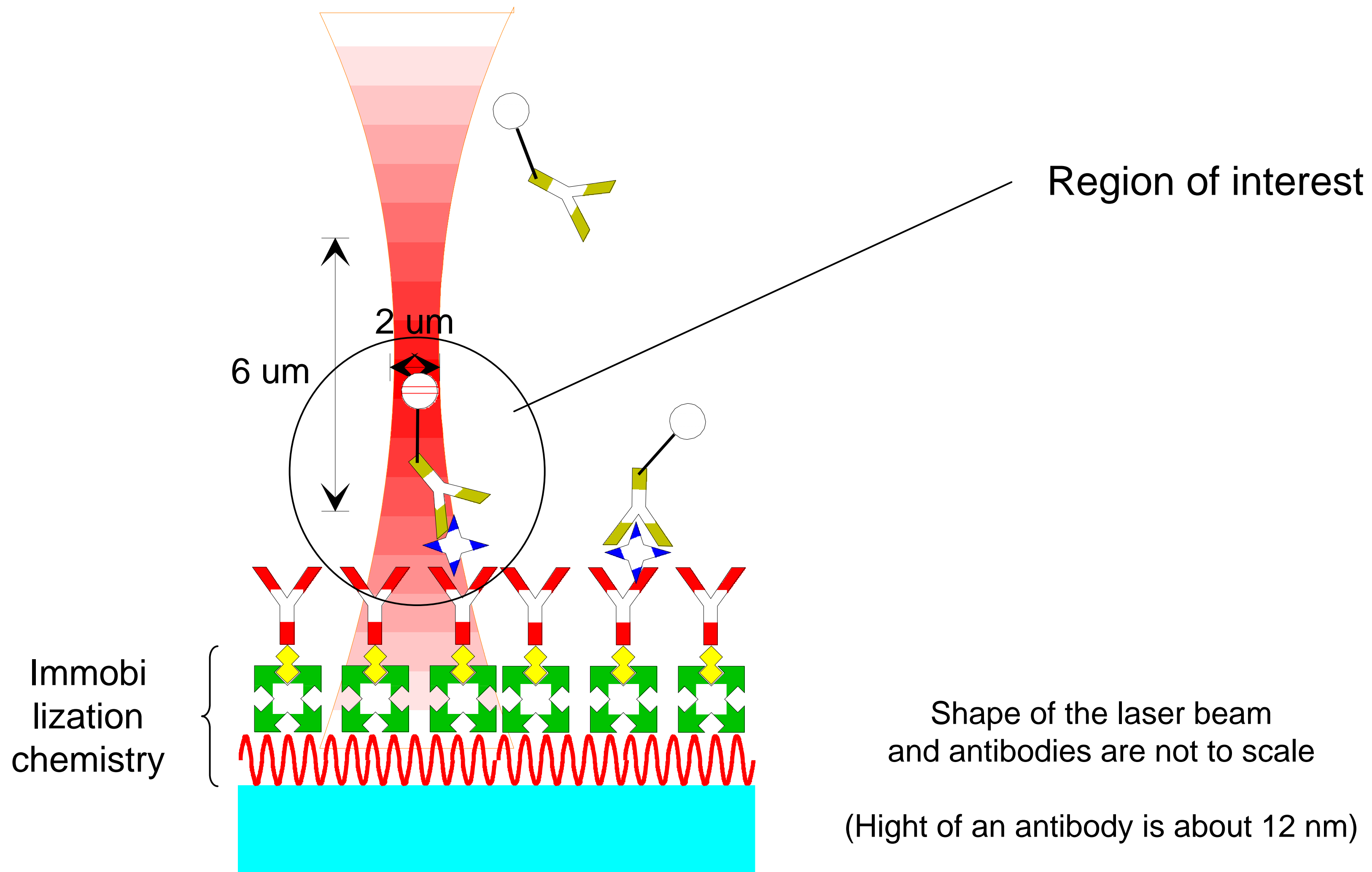
Excitation: at a „short“ wavelength
Emission: at a „longer“ wavelength



Realisation in Practise:

Separation of the light
of excitation and emission

Focusing into the region of interest

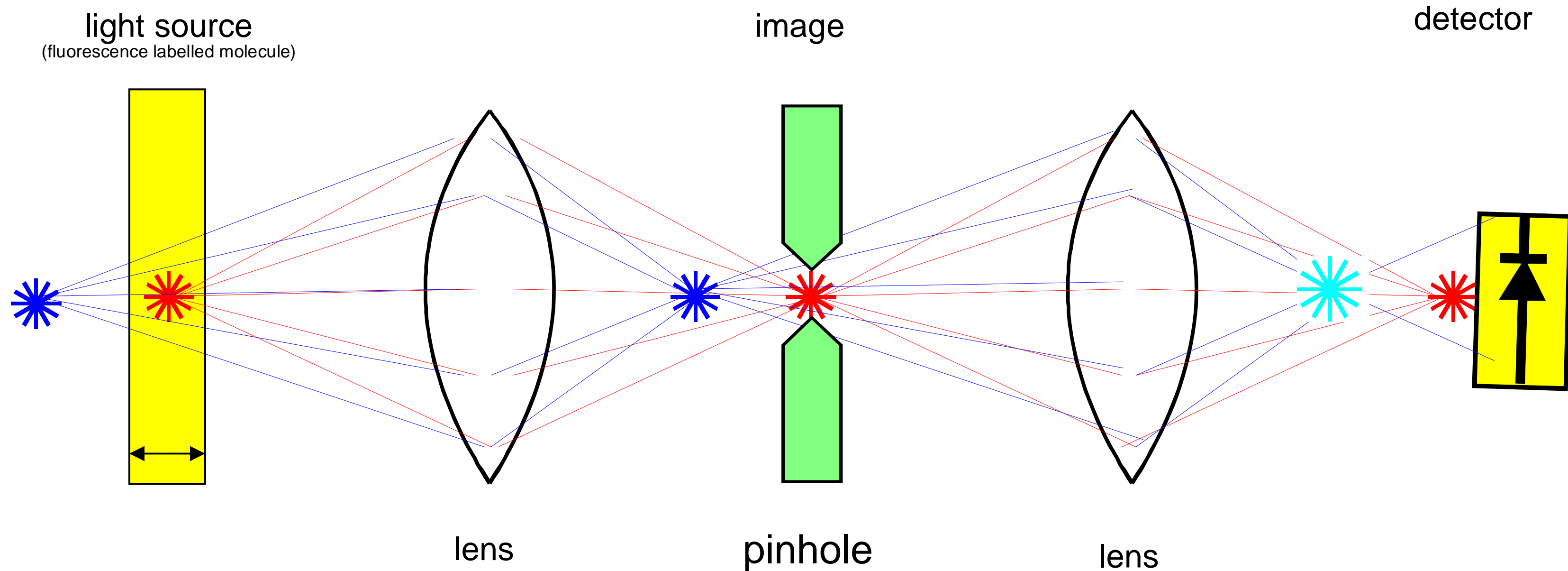


Shape of the laser beam
and antibodies are not to scale

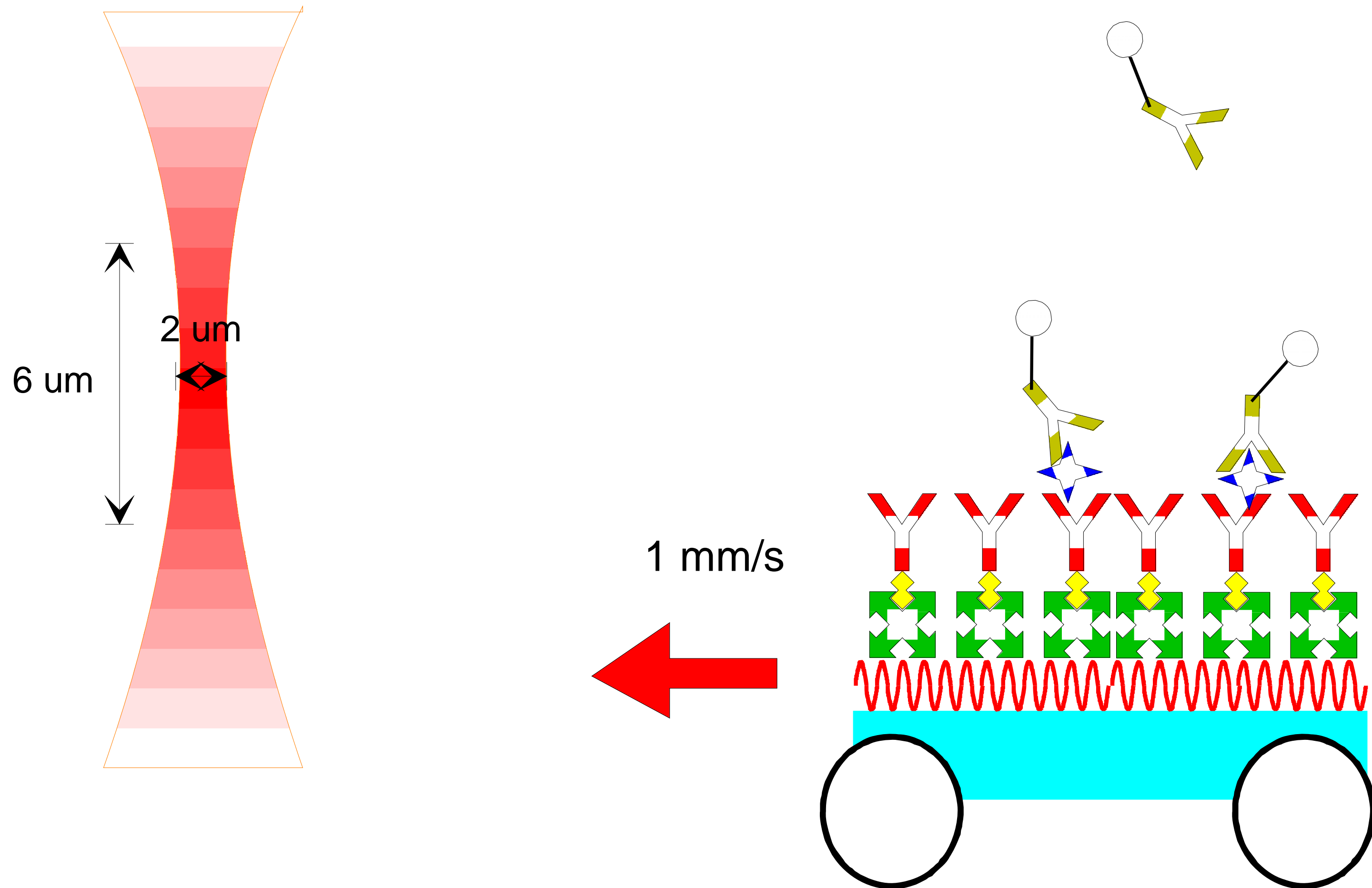
(Hight of an antibody is about 12 nm)

Confocal set-up

signals comming from the region of interest are detected only

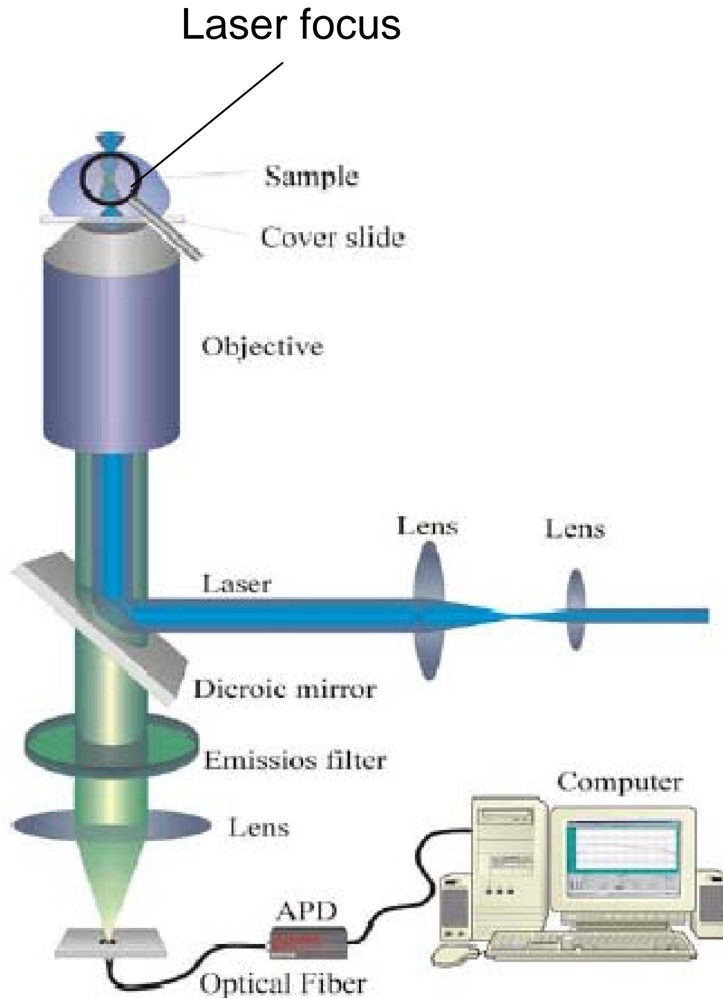


„Oversampling“ by Scanning an area (1000 Samples / mm)

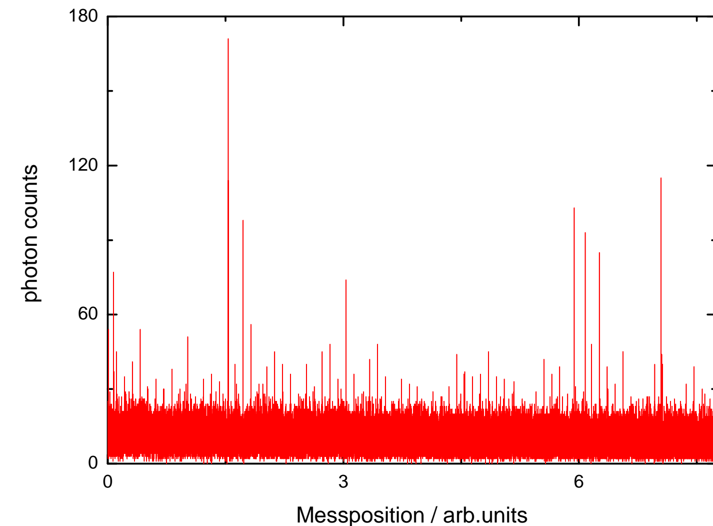


Realisation of the confocal optical set-up

Laser focus



Raw data:

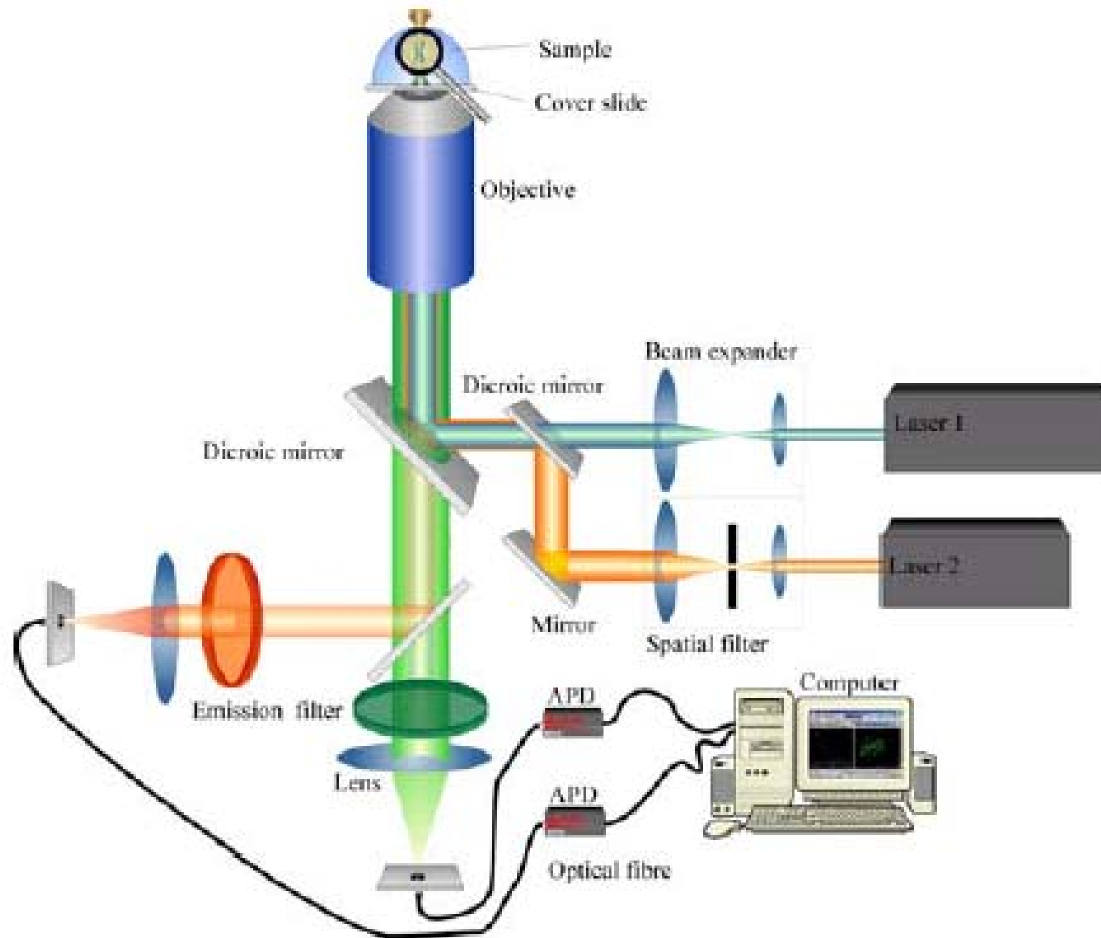


Detection limits

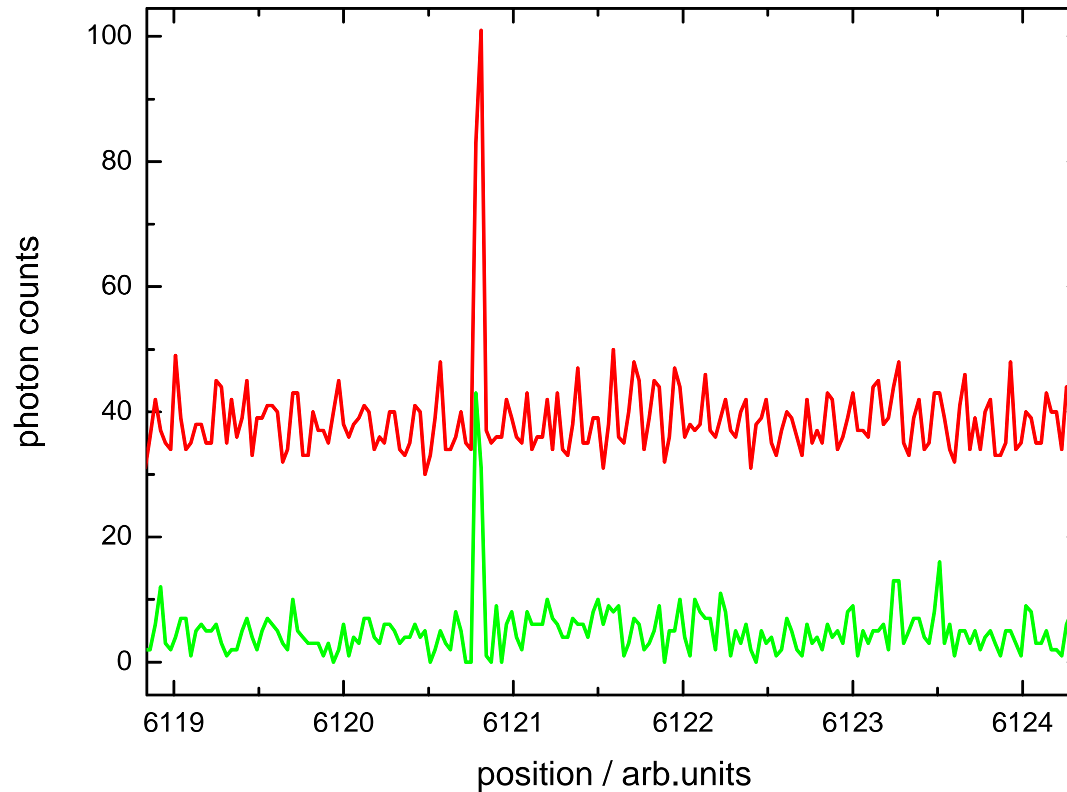
Dye: 10^{-17} M

Antibody 10^{-15} M

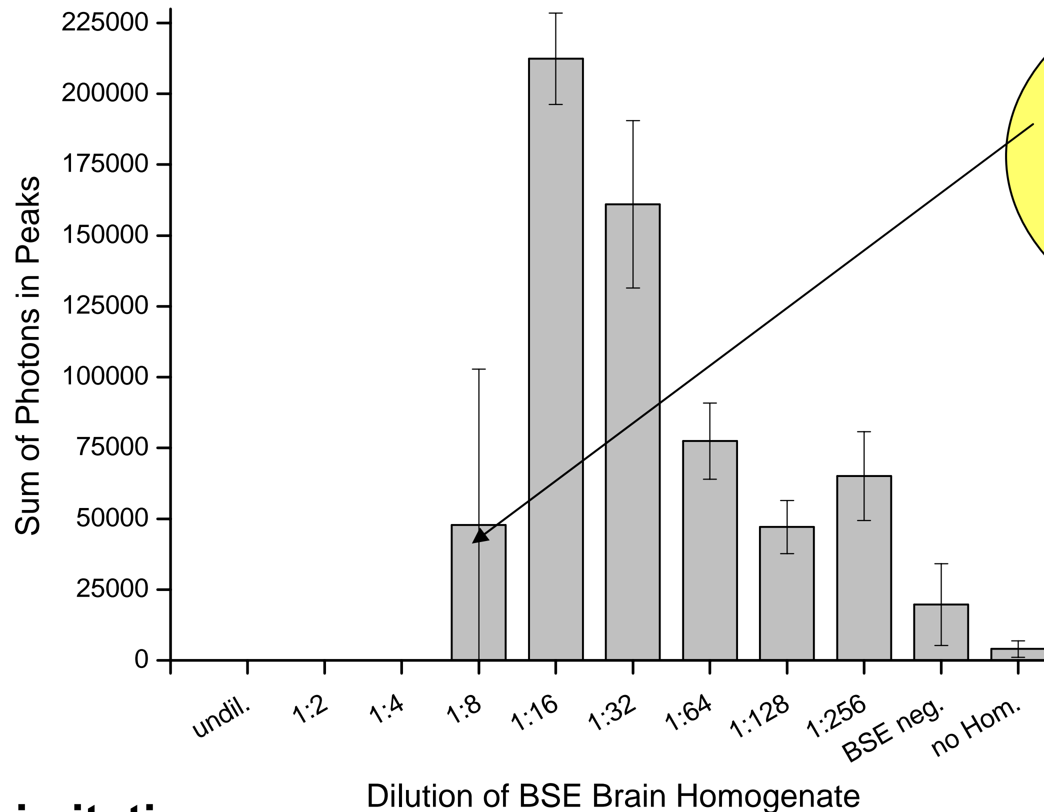
Enhancement to two wavelengths (Correlation)



Detection of an immuno sandwich with correlated signals



Results:



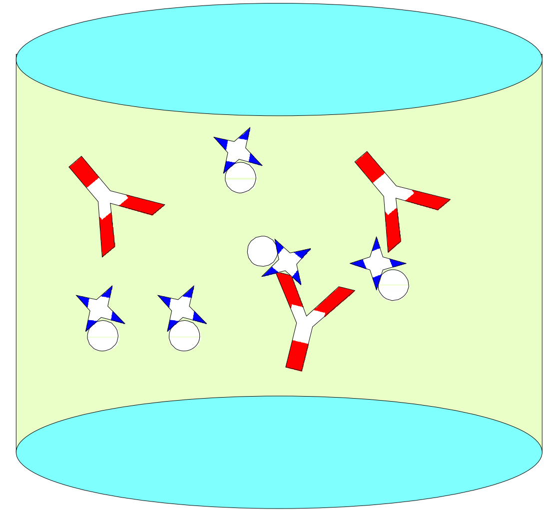
Limitations:

- Reproducibility (inhomogenities of the surface)
- Unspecific Signals (**unspecific binding of the secondary dye-labelled antibody**)

Diffusion Analysis with Fluorescence in Diagnostics

-> **Antigen (fragment) labelled with a fluorphore (dye) = > Tracer**

-> **Binding of the tracer to the antibody (to be detected) occurs in solution (homogenious Assay)**



Read-out:

Analysis of the Brownian motion (diffusion) of the Tracer (small) and the Tracer-Antibody (larger) Immuno Complex

=> Small particles are faster than large particles
=> The free Tracer is faster than the Immuno-Complex (Tracer-Antibody)

How we can measure the speed of motion ?

Translations:
$v = \Delta x / \Delta t$

Rotation:

$\omega = \Delta\phi / \Delta t$

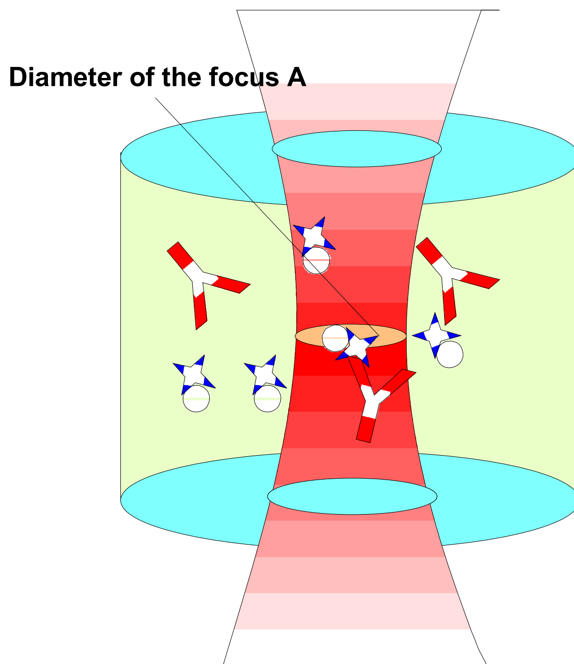
Concepts of Measurements:

Δx is fixed, Δt is measured => **Fluorescence Correlation Spectroycopy (FCS)**

Δt is fixed, $\Delta\phi$ is measuerd => **Fluorescence Polarisation Analysis (FPA)**

Principle of FCS (Correlation Spectroscopy)

Illumination (and detection) of fluorescence-labelled molecules in a very small volume (in a confocal set-up) („Δ**x is given**“).



Diffusiontime τ_D (Δ**t**) through the focus of a laserbeam is **measured.**

(τ_D correspond to the time beeing in the focus)

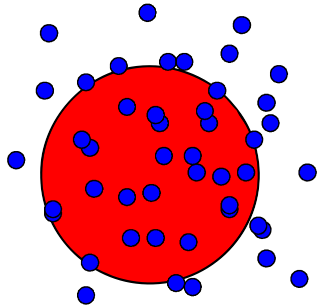
Relation to the geometry of the Tracer / Complex

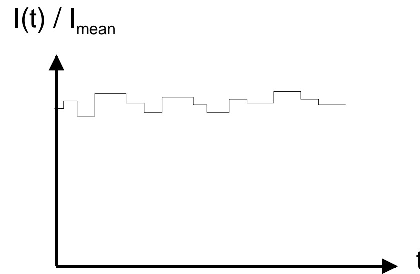
$$\tau_D \propto \frac{A \cdot \eta \cdot r}{T}$$

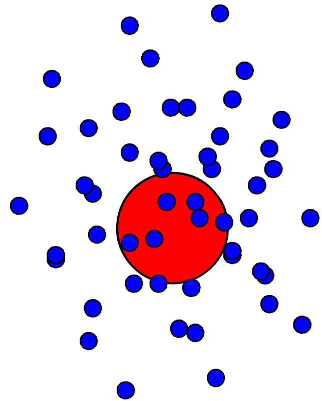
r = radius of Tracer / Immuno-Complex
T = Temperatur [T] = K
A = Diameter of the focus
η = viscosity of the buffer

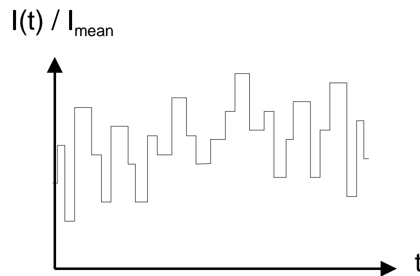
=> Diffusion time is related to the radius of the Immuno-Complex

Measurement of the diffusion time







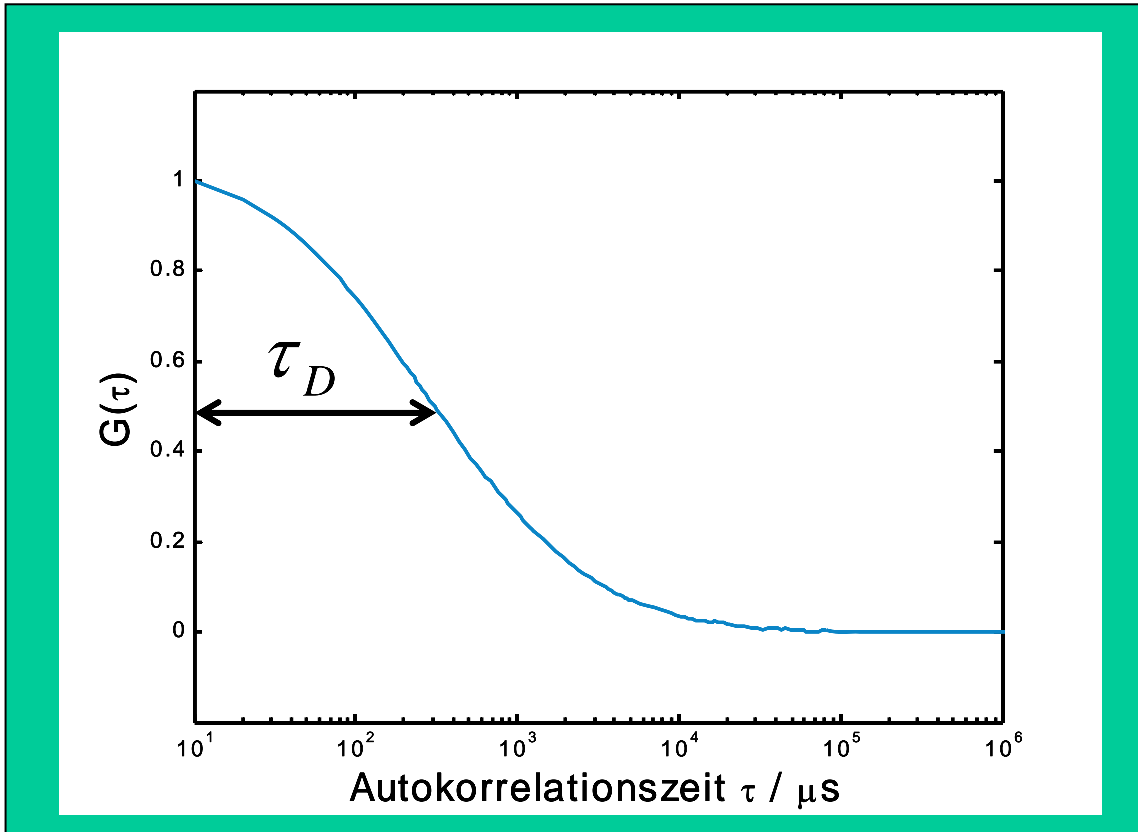


Fluorescene-labelled diffuse in and out of the focus:

=> Noise of the intensity is analyzed
=> small focus volume / low concentrations result in high noise of the **relative** intensity.
(good results for few molecules in the focus (10-50 molecules)
=> Analysis of the noise is done by an Auto-Correlation of the Intensity:

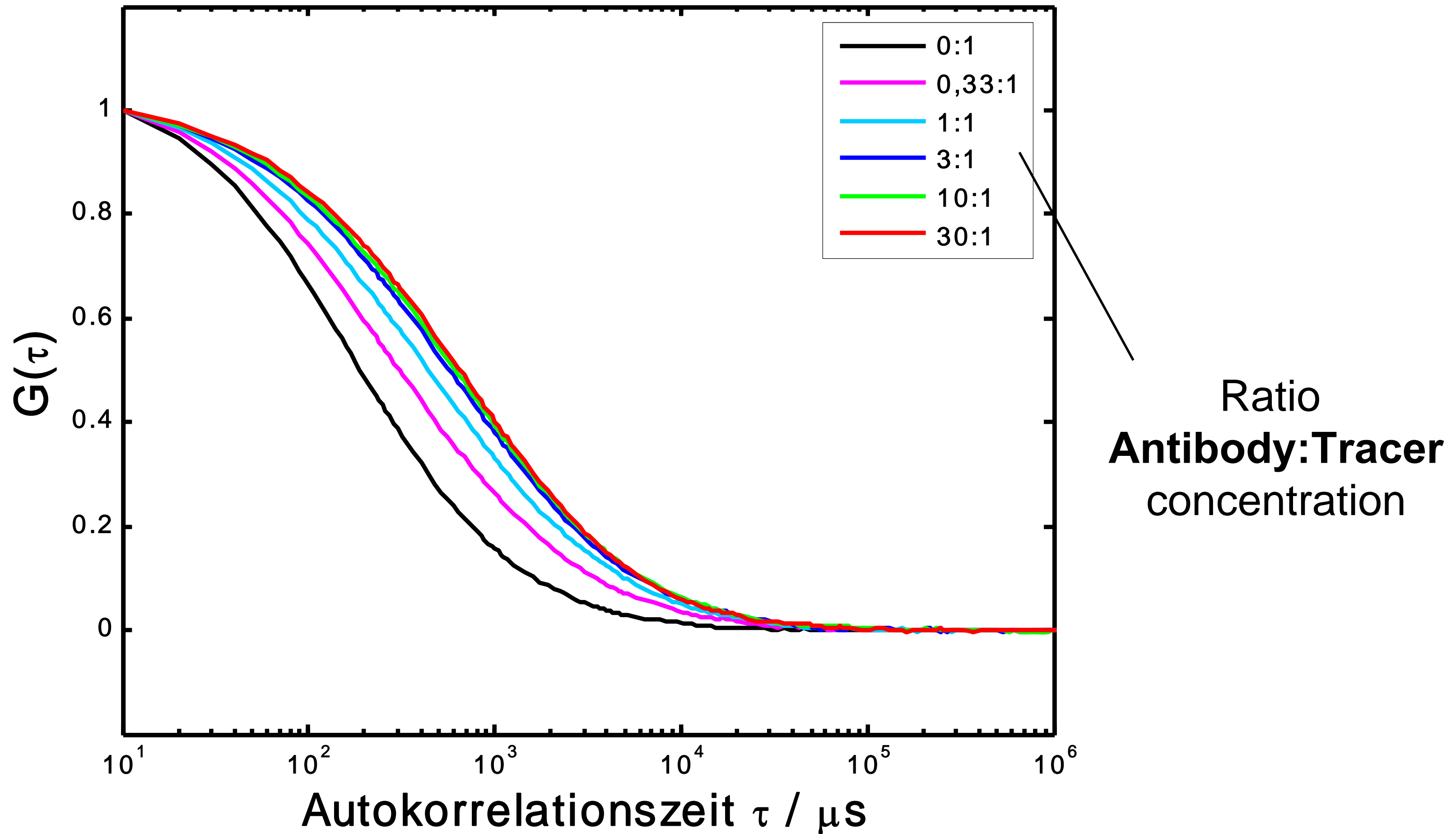
$$G(\tau) = \frac{\int_0^T I(t) \cdot I(t+\tau)dt}{\left(\int_0^T I(t)dt\right)^2} - 1$$

Normalized Autocorrelation of the Fluorescence Intensity I(t)



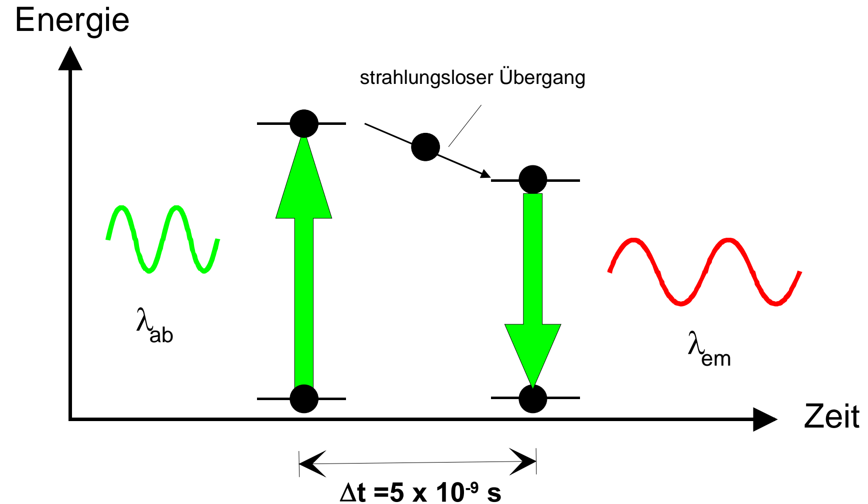
=> Diffusiontime τ_D

Results: Different ratios of Antibody (target) to Tracer

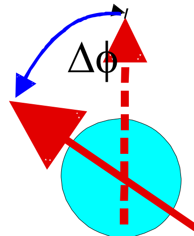


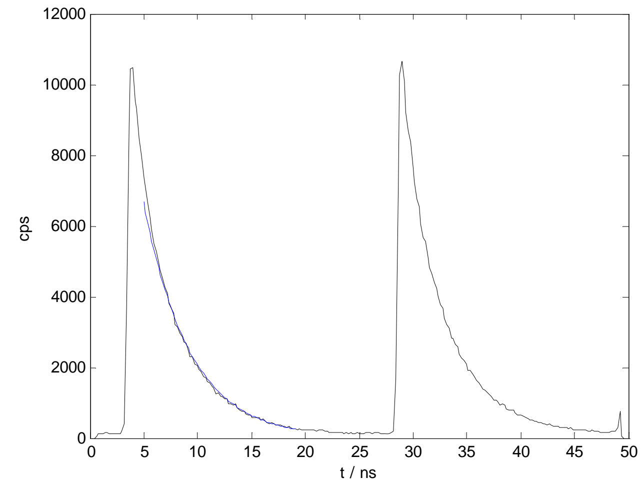
Principle of the Fluorescene Polarization Assay

Intervall of time is given by the lifetime of the excited state of the fluorescene dye (**Δt is given**).



Measured is the angle of rotation (ΔΦ) of the molecule in the given time intervall Δt





Relation to the geometry of the tracer /complex

$$\Delta\phi \propto \frac{\Delta t \cdot T}{\eta \cdot r^3}$$

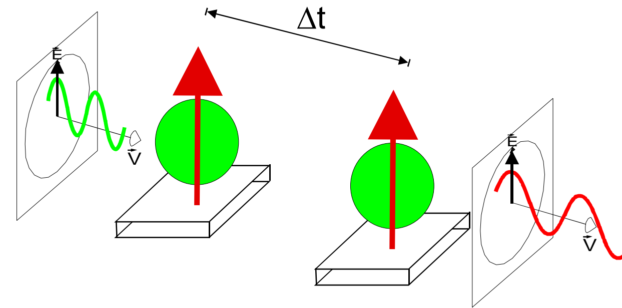
r = radius of the tracers / complex
T = temperature
η = viscosity

Measurement of the rotation angle:

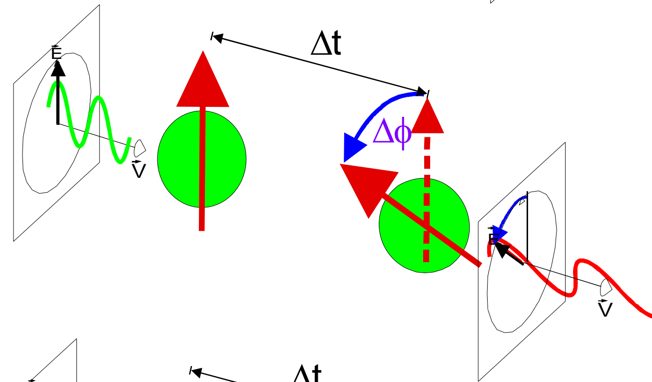
is based on the polarization of the emitted light:

Model experiment:

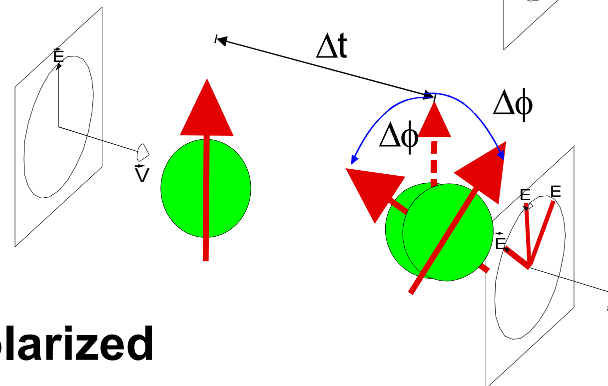
1) Single fixed molecules:



2) Single free molecule (in solution):

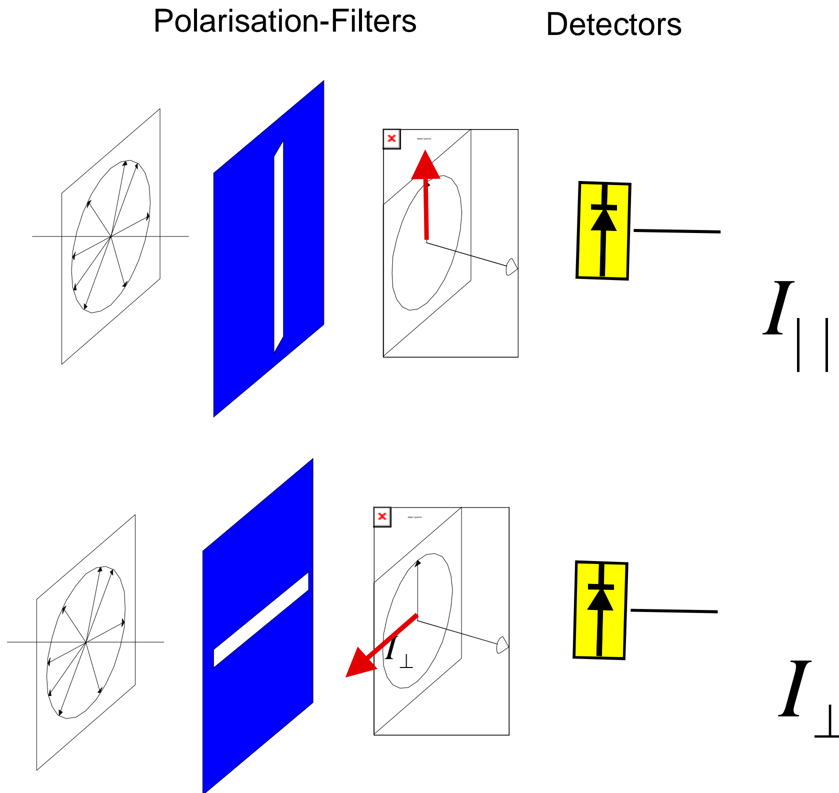


3) **Many** free molecules (in solution):



=> Emitted light is partially depolarized

Measurment of the degree of polarization



- Unpolarized light => $I_{||} = I_{\perp}$
- parallel polarized light=> $I_{\perp} = 0$

Def.of the degree of polarisations

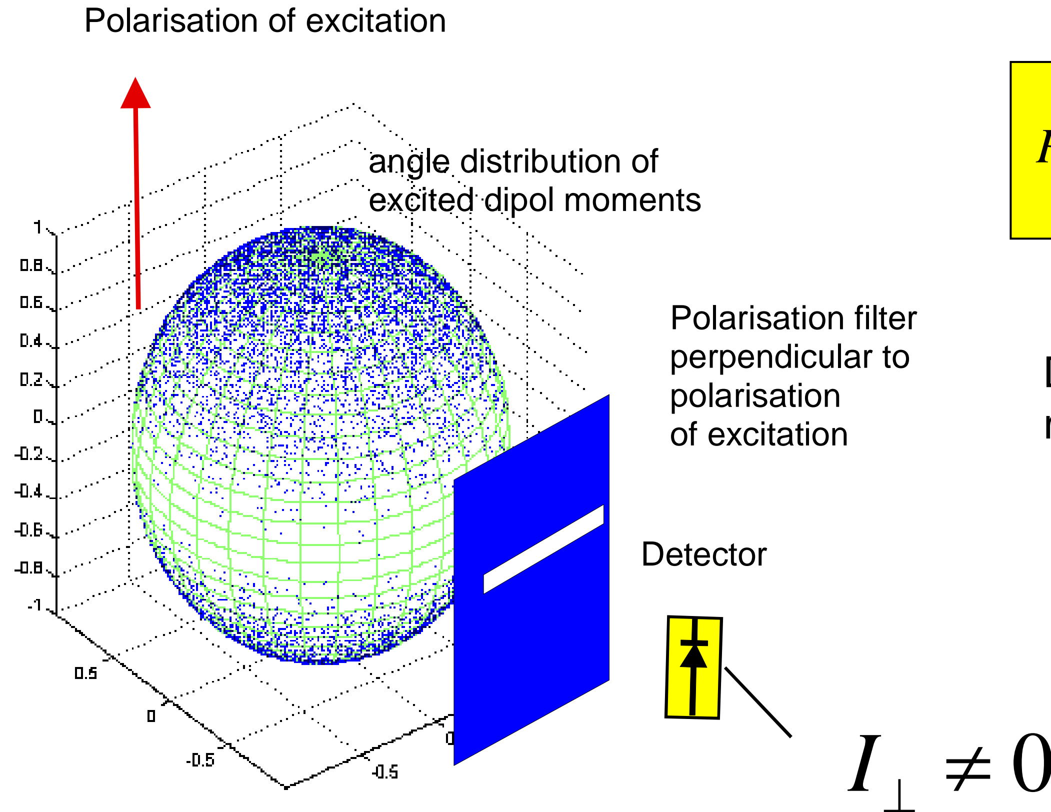
$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

- Unpolarized light => P = 0
- parallel polarized light => P =1

Common units: 1 P = 1000 mP (Millipolarization Units)

Dynamic range of Polarization values

Degree of polarization of stationary but randomly oriented fluorescence dipol moments



$$P_{\max} = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} = 0.5$$

Degree of polarization for free rotating dyes (typically)

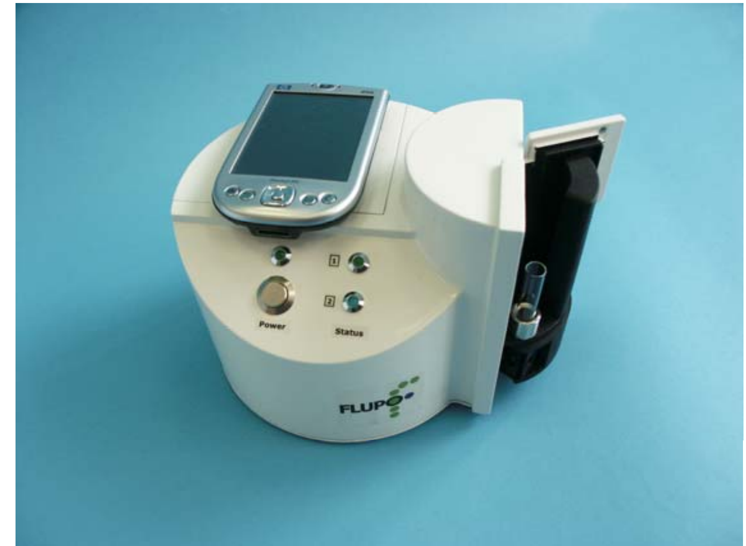
$$P_{\min} = 0.02$$

=> Rather small dynamical range (500 times the resolution of precise FP-Reader)

Instruments:

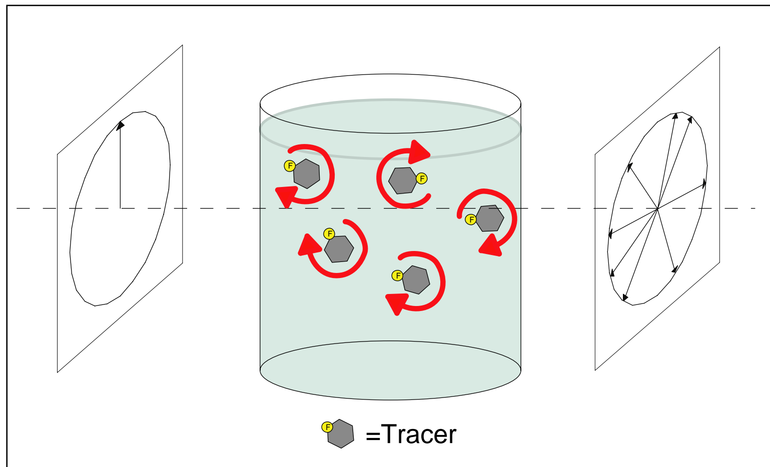
1) 96-well reader (e.g. Tecan GeniosPro):
   (automated injection of buffer and tracer):

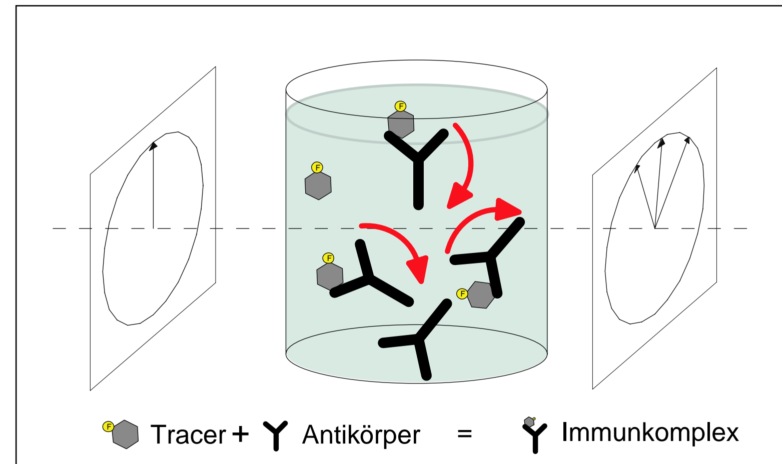
2) Single-well, handheld instrument FLUPO
   (PDA driven, no moving parts) ZHAW-prototype



FPA in diagnostics: e.g. Detection of Antibodies

Artificial antigen (peptide, sugar, part of a bacteria etc) is labelled by a fluorescence dye = Tracer





„light and small" molecule (dye labelled)
=> fast rotation => **unpolarized** light

„big and heavy" molecule (dye labelled)
=> slow rotation => (partially) **polarized** light

Negative Probe

⇒unpolarized light

⇒ 70 mP (typical)

Positive Probe

⇒ (partially) polarized light

⇒ 300 mP (maximal); 130 mP (typical)

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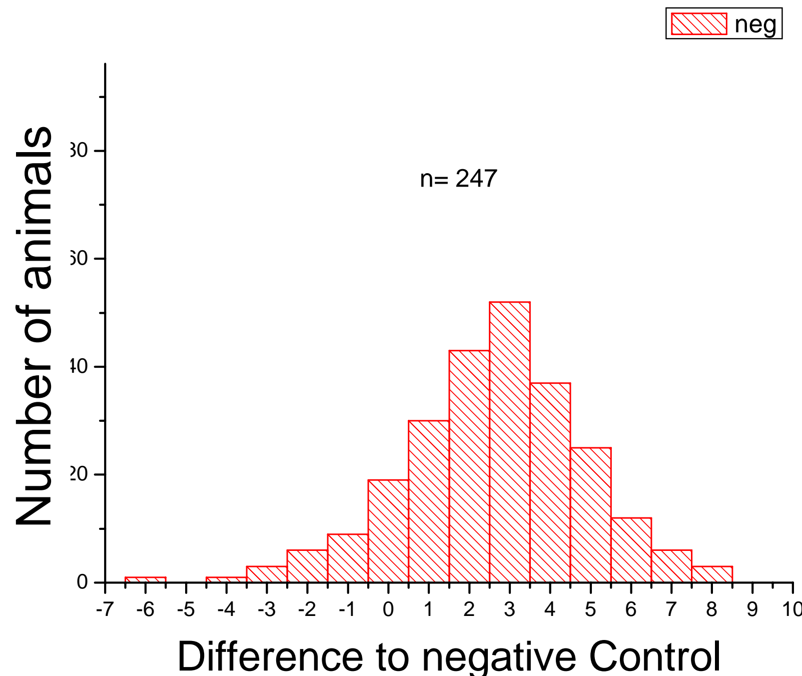
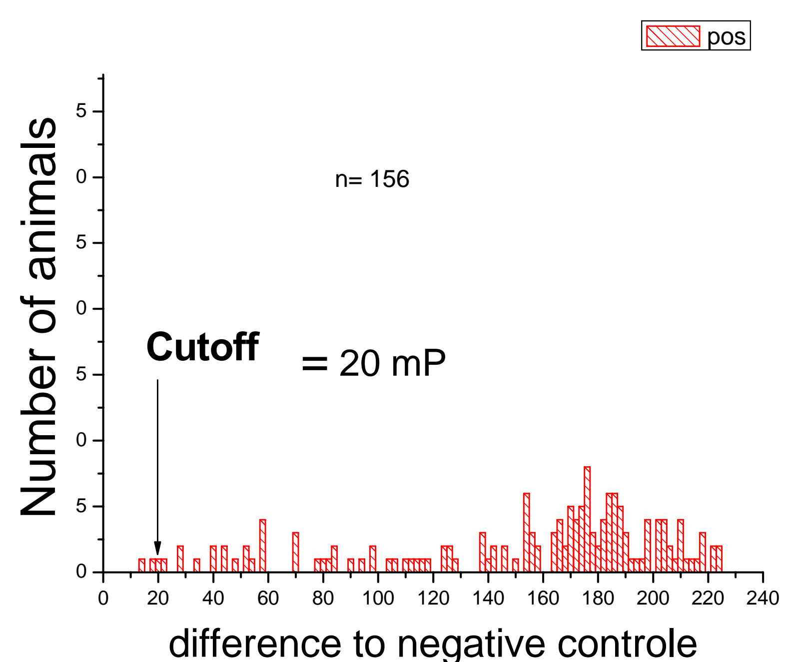
Example 1: Detection of Brucellosis (in bovine sera)

Brucellosis = bacterial desease in cattle, sheep, dogs (human)





100 % Specifity

98% Sensitivity

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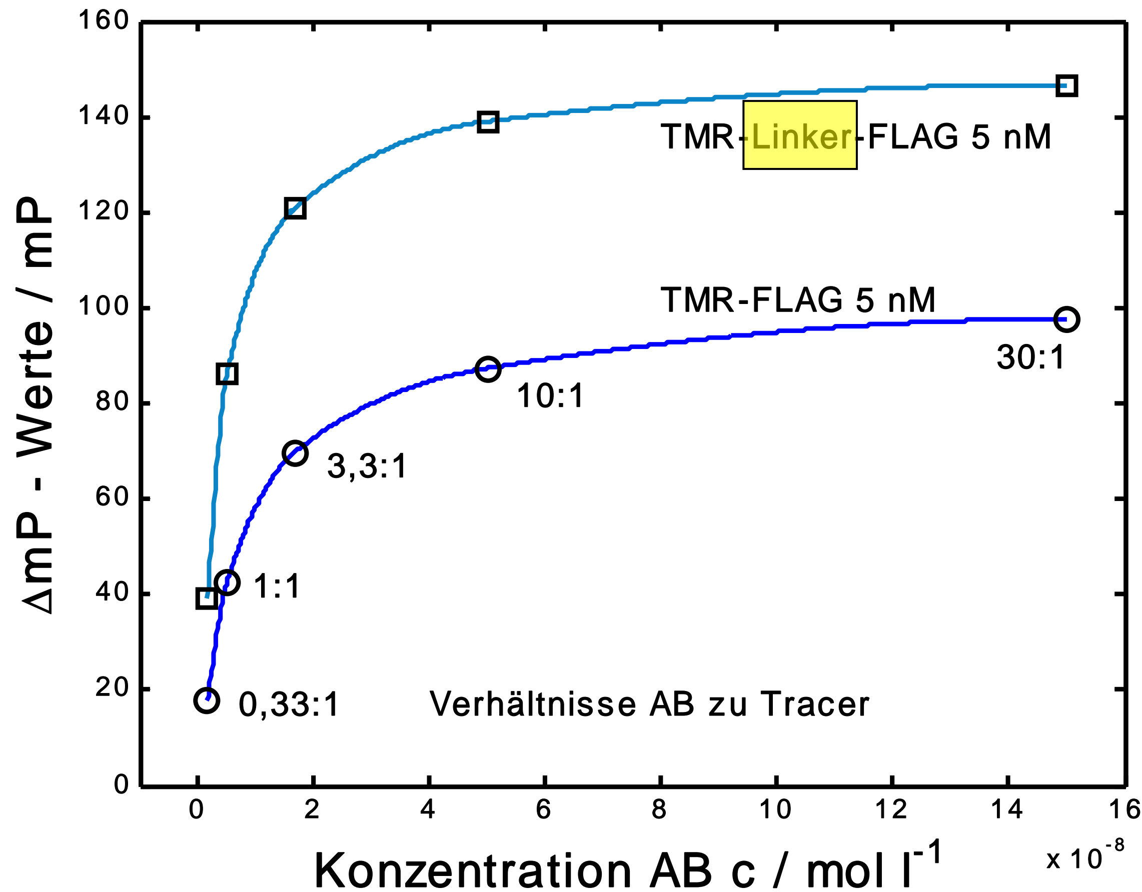
Tracer Development: Influence of the linker (dye labelling):

ΔmP in dependance of the antibody excess



AB = anti-FLAG antibody

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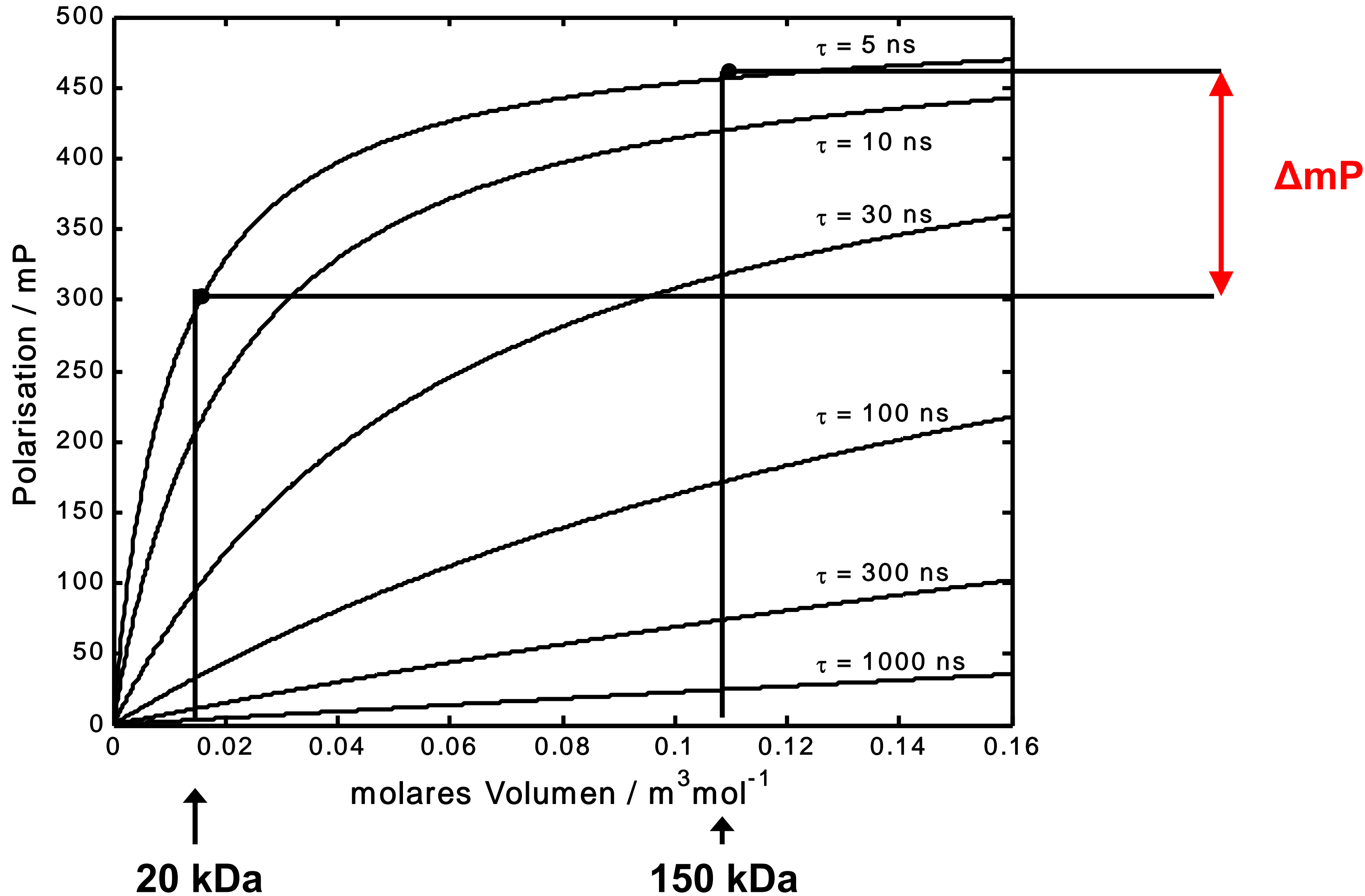
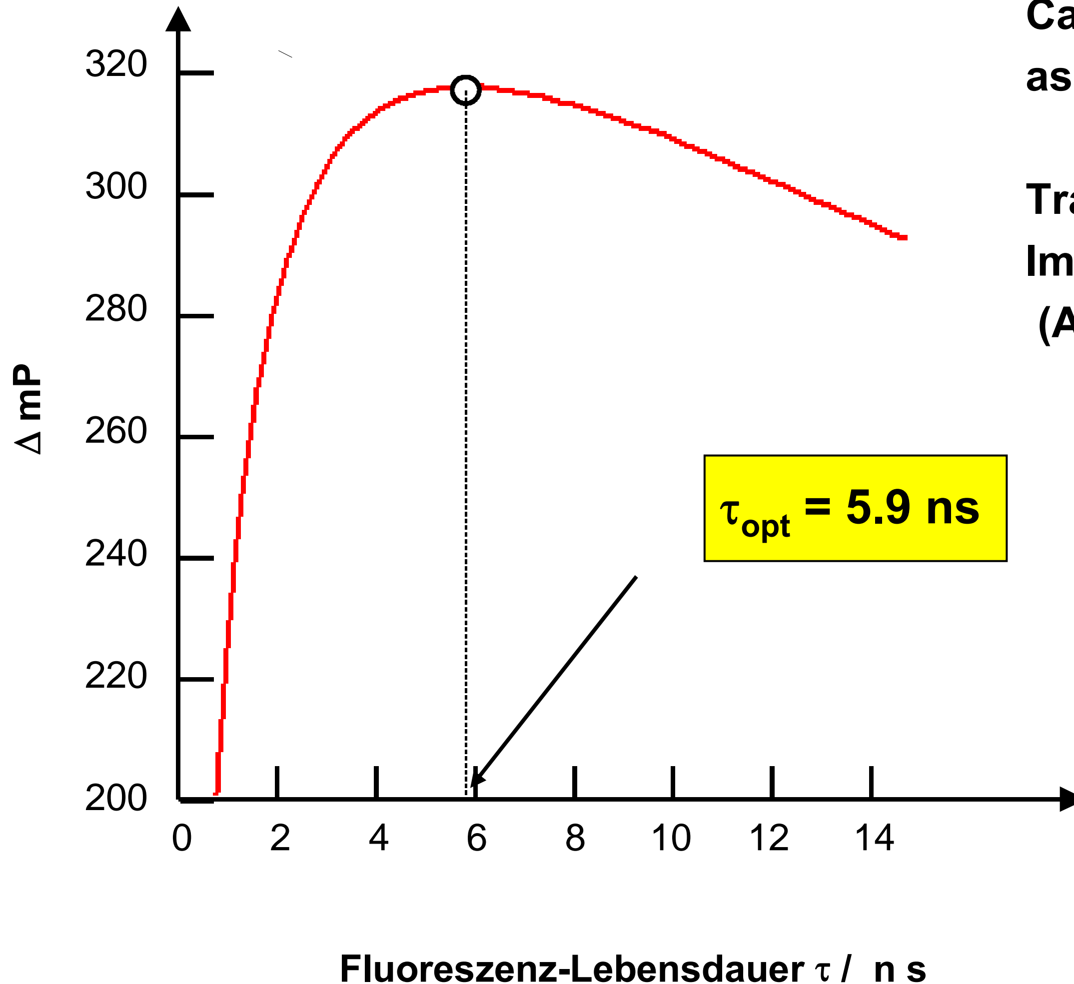
Tracer Development: Influence of the lifetime of the dye



Optimized lifetime of the exicted state τ_{opt}



Calculated for a typical immuno-assay, i.e., detection of antibodies

Tracer m_T **= 2 kDa**
Immunocomplex m_{IK} **= 152 kDa**
(Antibody + Tracer)

$$\sim \sqrt{m_T \cdot m_{IK}}$$

Features of an ideal tracer for FPA (and FCS)

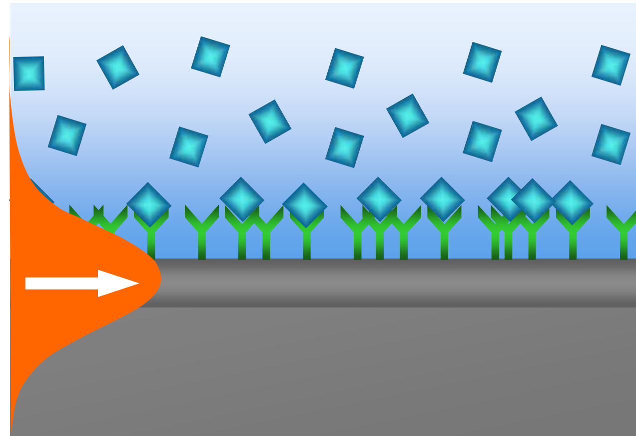
- High affinity to the „target“ (antibody) => **Sensitivity** (K_D determines the detection limit)
- No cross reaction with other molecules in the sample => **Specifity**
- Small mass (relative to „target“) (FP: free tracer should depolarize the light)
- Dye should not distrub the binding process; Binding should not disturb the fluorescence mechanism (quentching!)
- „Good“ fluoerescence features
  - Brightness is essential for working with low tracer concentration => detection limit
  - Optimized lifetime of the excited state is essential for broad dynamic range (**FPA**)
  - Rigid binding of the dye to the specific part of the tracer => no propeller effect (**FPA**)

Development of an FPA (FCS) ⇔ Tracer Development
Tracer Development for FPA is more ambitious than for FCS
Instrumentation for FPA is much simpler than for FCS

Integrated Optical Sensing: Detection Principles

Label-Free / Refractometric Sensors

Measuring surface mass density Γ. $[\Gamma]$ = ng/mm^2



C = Cover (water)

AL = (biol.) Adlayer

F = Film (TaO_5)

S = Substrate

Basic idea:

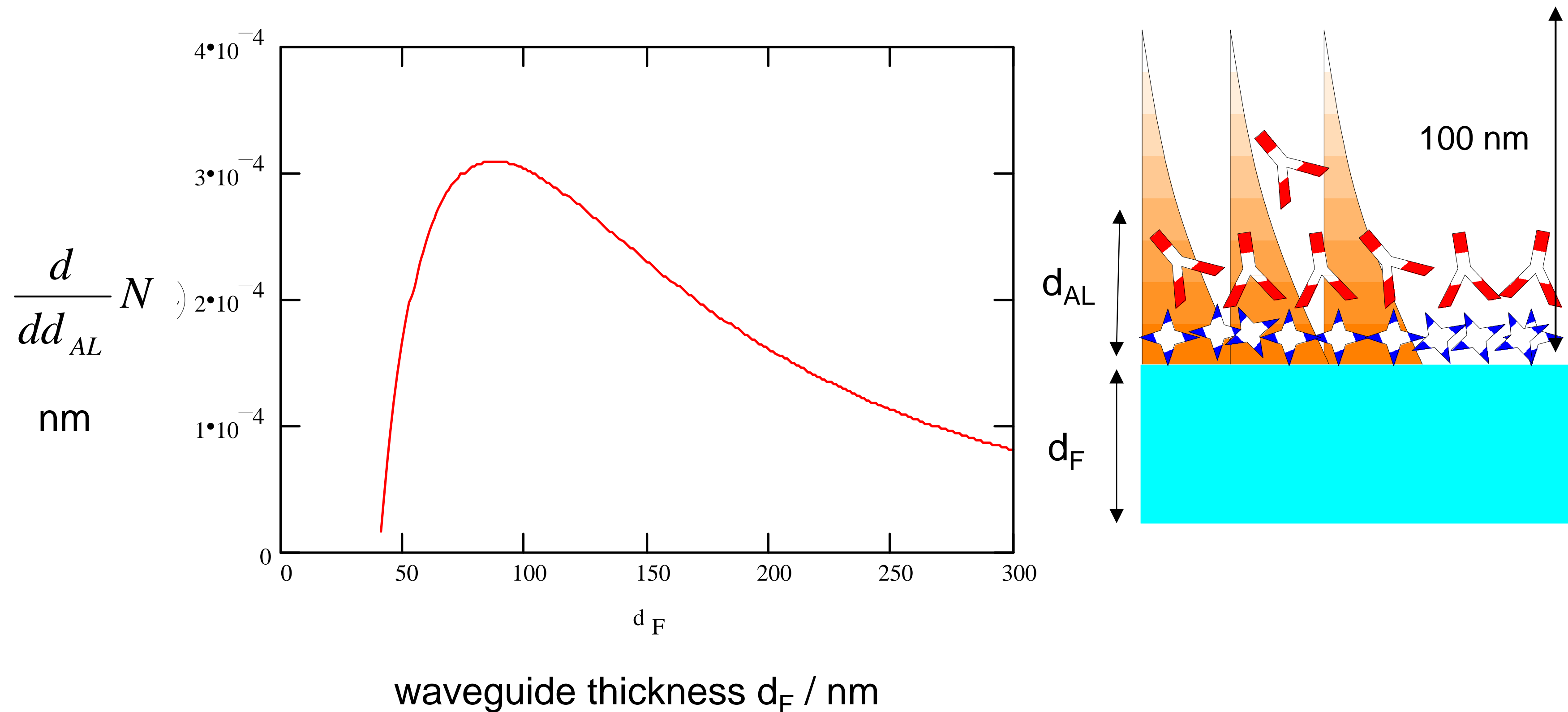
1. The evanescent field of waveguide mode probes the additional layer built by binding molecules (for example antibody-antigen binding)
2. The *effective refractive index N* depends on properties (refractive index, thickness) of the additional layer (adlayer).
3. **Changes** of the *effective refractive index N* has to determined: Instrumentes: **Grating couplers**, Interferometers etc.

Reminder: **Effective refractive Index N** (of a waveguide mode) = c_0 / c_{Mode}

$$N = N(\lambda, n_S, d_F, n_C, n_{AL}, d_{AL}, \text{Polarization})$$

Parameter Optimization

Geometry of the waveguide determines the sensitivity for changes in the adlayer parameter, i.e, the thickness of the adlayer d_{FAL}



waveguide thickness d_F / nm

= > Thickness of the waveguide has to be optimized

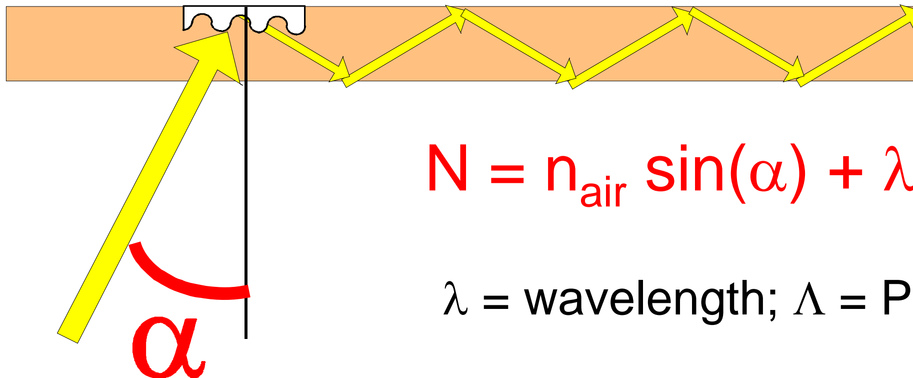
Integrated Optical Grating-coupler

Reminder: **Effective refractive Index** of mode: $N = c_0 / c_{Mode}$

=> Wavevector of the mode = $N k_0$, (k_0 = wavevector in vacuum $= 2\pi / \lambda_0$)

Coupler concept:

1. The incomming light is ***diffracted*** on a surface relief grating: ($k_x' = k_x + 2\pi/\Lambda$)
2. The wavevector of the diffracted light (k_x') **has to** match with the wavevector $k_x' = N k_0$ of the excited mode (sometime called resonance)
3. The wavevector k_x of the incomming light is varried by the coupling angle α (=> resonance angle)

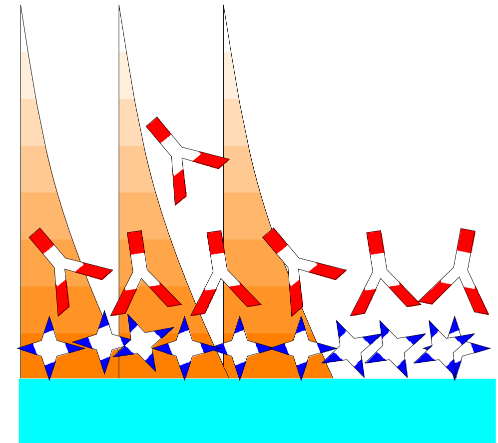


$$N = n_{air} \sin(\alpha) + \lambda / \Lambda$$ (Coupling condition)

λ = wavelength; Λ = Period of the grating

From the „input“ to the „readout“:

Input: **Biological** /chemical interactions =>
change the adlayer (thickness, density etc.)
change the effective refractive index N
change coupling conditions



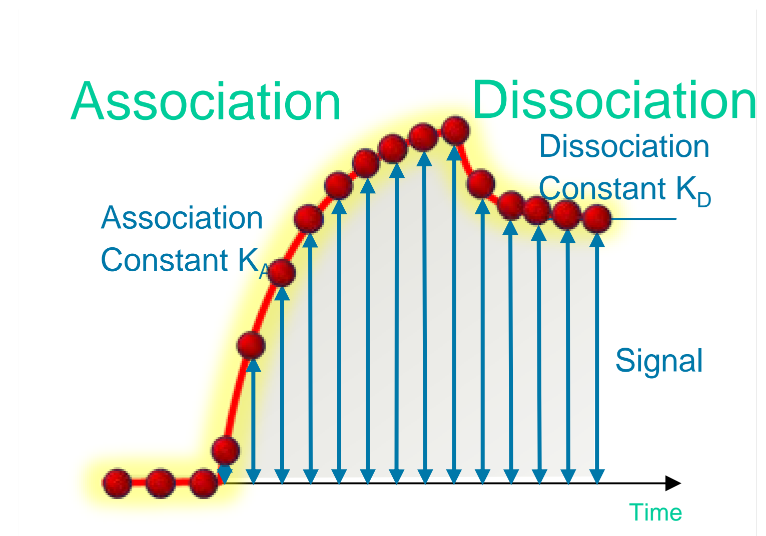
For excitation of the mode coupling parameters (angle, grating periode, wavelength) have to be adjusted

$$N = n_{air} \sin(\alpha) + \lambda / \Lambda$$

(possible) Outputs:

coupling angle <=> mechanical rotation
coupling periode <=> chirped gratings
=> couple position
coupling wavelength <=> tuneable laser

=> Changes on the surface can be monitored in real time



- Kinetic measurement
- Affinity analysis

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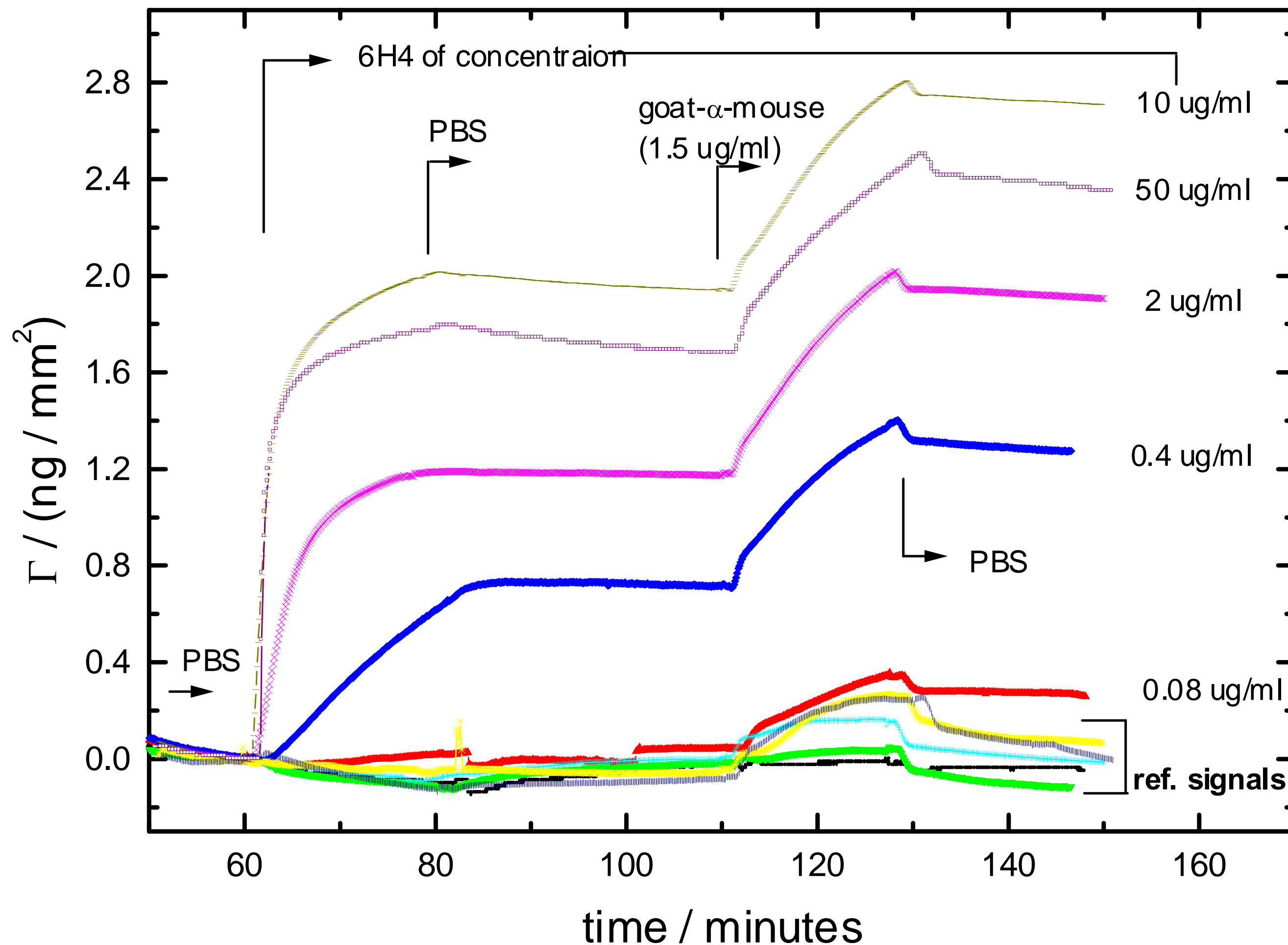
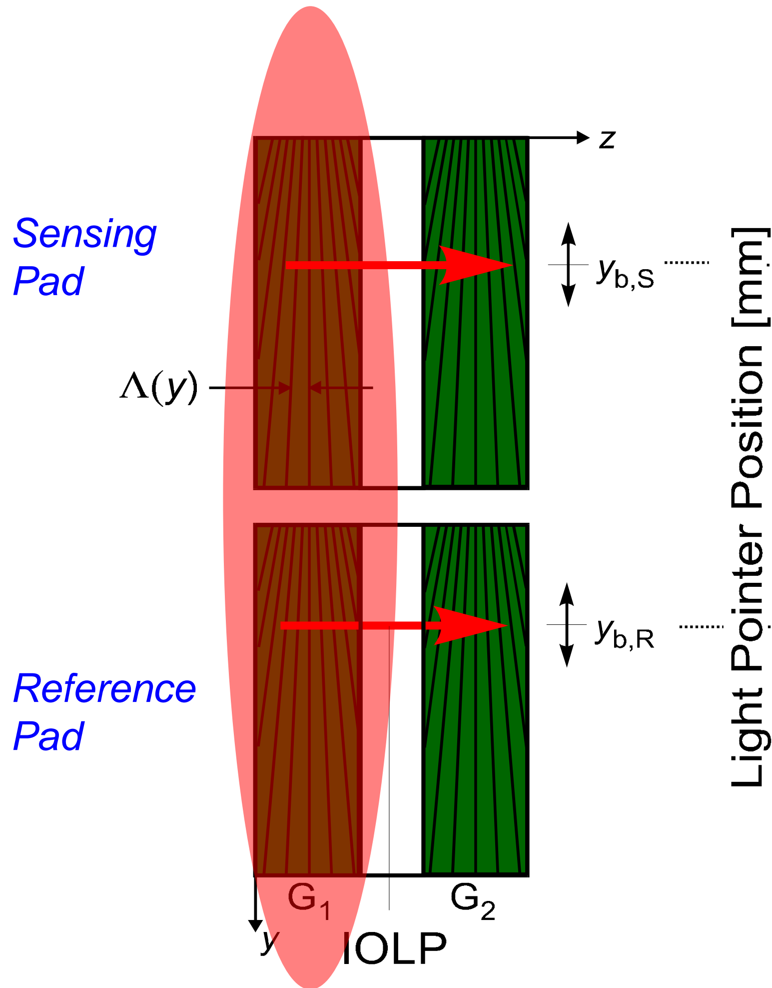
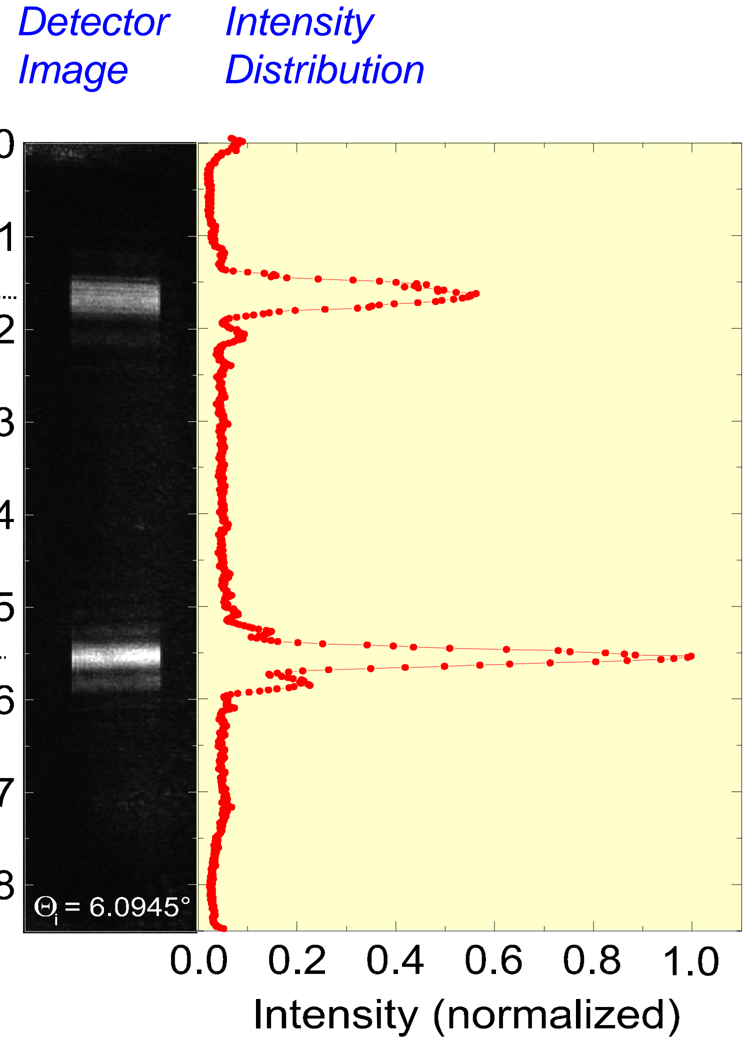
Experimental results:



Working principle: Integrated optical light pointer (chirped grating)

Top View:

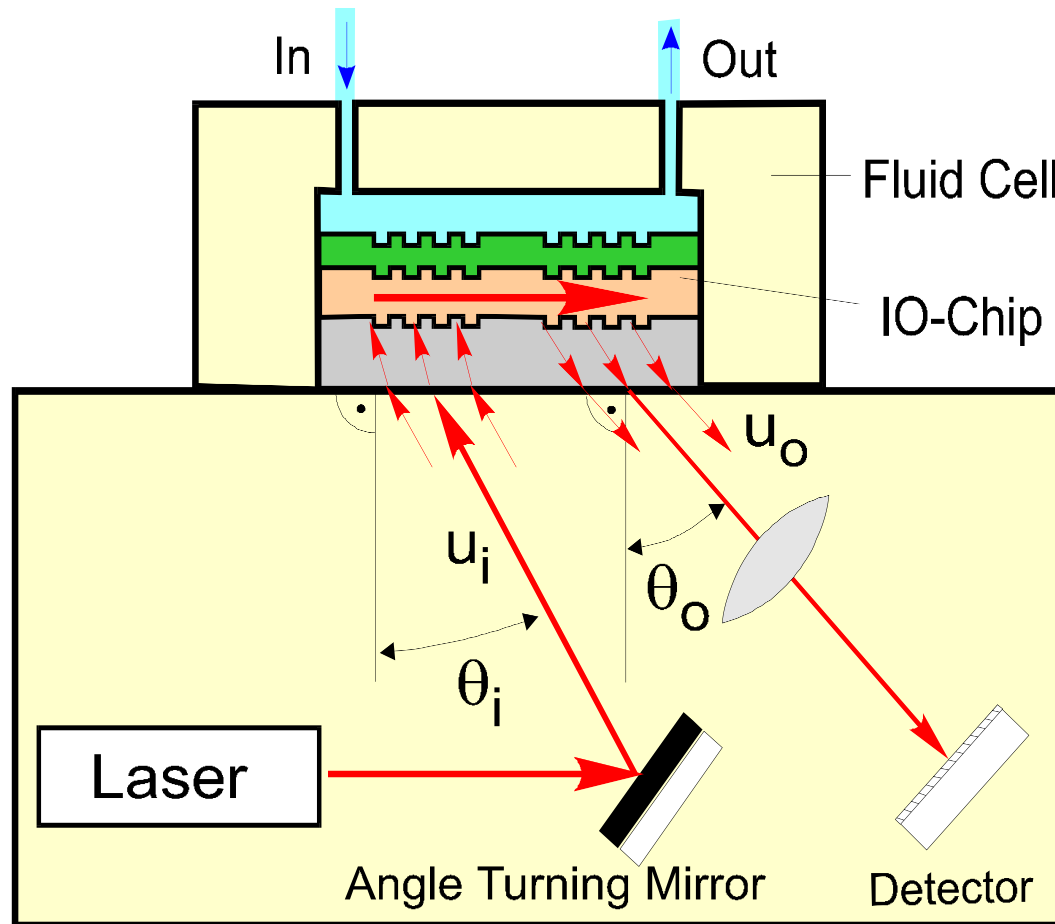




[R. Kunz, CSEM, Neuchatel]

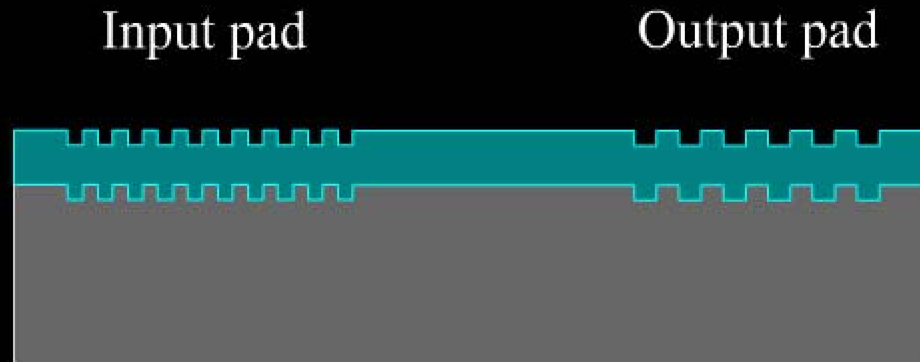
Grating coupler (set-up)

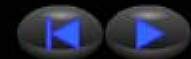
Side View:



[Rino Kunz, CSEM, Neuchatel]

Working principle: wavelength-Interrogated Optical Sensor (WIOS)

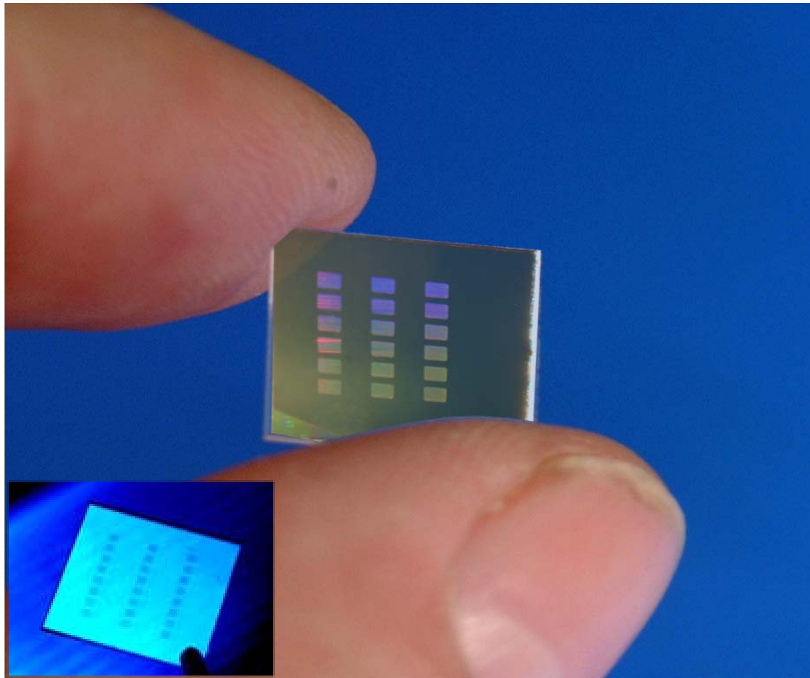




[R.Kunz, CSEM]

Optical Chips: $Ta0_5$ film on a glass substrate

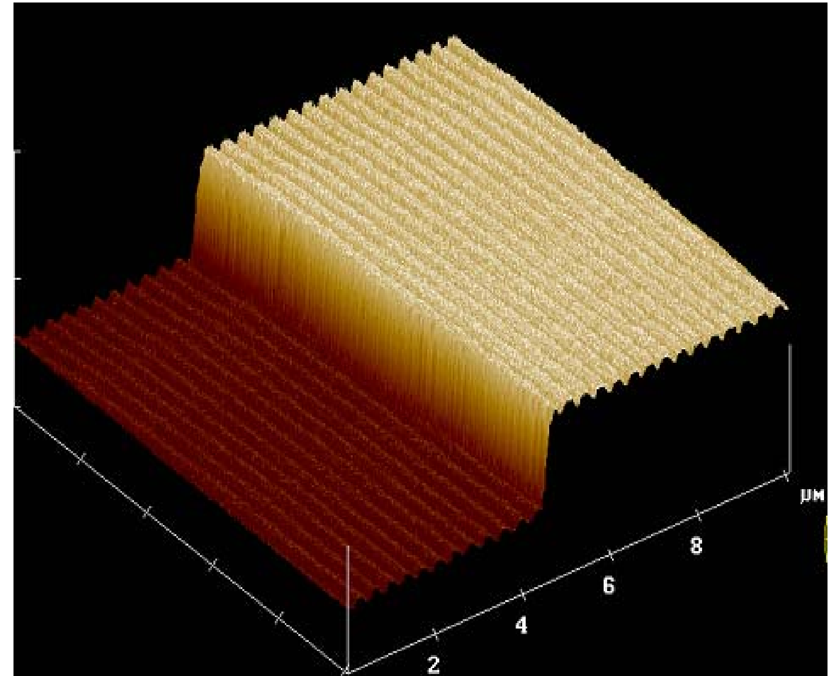
Sensing pad array



Grating nano-structured opto-chips
(period: 360 nm, depth: 12 nm, step: 150nm)

[Max Wiki, Dynetix Biosystems,Landquart]

AFM image



Commercial Instrument:

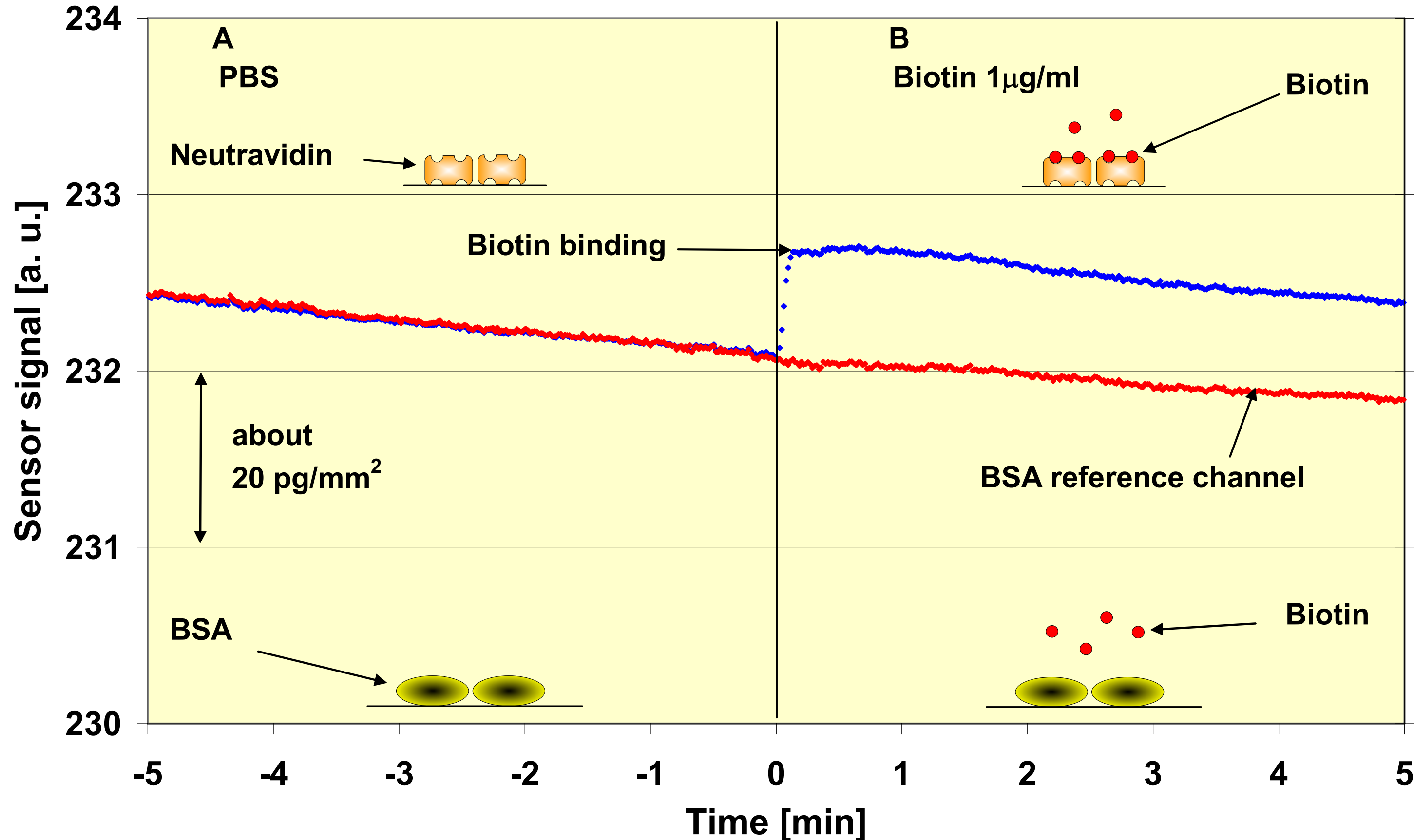
29 cm

8 parallel channels
Sampling rate 10 Hz

27 cm

Details see: www.dynetix.ch

Modell experiments: Binding of Biotin (244 Dalton) to Neutravidin



Std. Dev.: 180 fg/mm² / Detection Limit: 550 fg/mm²

[Rino Kunz, CSEM, Neuchatel]

Conclusions: Fluoresence versus Labelfree detection

Fluoresence

+ Extremely high (physical) sensitivity
+ Information about surface homogenity
+ Multiplexing with high density arrays
- Direct detection needs 2 specific antibodies
- Labeling can disturb biological interactions
- Costly labeling (FPA)
- Possible quentching effects by the sample
- Autofluoescence can mask real signal
- Surface scanning instruments and plates are quite expensive
+ Assays solution (FPA)
  +/- no washing steps (homgenious assay)
  robust and simple detection (point of care)

Labelfree

+ **No labeling, no disturbance of the biological interactions**
+ Direct detection with only one antibody
+ Signal in real-time => kinetic studies
+ No secondary antibody has to be used
+ Insensitive to autofluorescene
- Reasonable sensitivity (compared to Fluors.)
- Sensitive for unspecific signals (any additional mass generates a signal)
- Sensible to the refractive index of buffers
- Fluidhandling can be delicate (bubbles etc.)
- Waveguides are still expensive (Alternative labelfree techniques like reflectometry is cheaper to realize).

However:

=> **Limitation in sensitivity of fluorescence based biosensors are given manly by the biological unspecific interaction/bindung and not by the optical read-out system**