CH-413 Nanobiotechnology

Optical Biosensing

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March 20, 2025

You may already be using biosensors

Measuring heart rate using light

- Measuring heart rate using light = photoplethysmography
- The device measures the **change in concentration of red blood cells** as the **blood vessels expand and contract**
- Expanded blood vessels absorb more green light; contracted blood vessels absorb less green light.
- The detector measures the reflected light and a software algorithm converts the changes in light intensity into your pulse rate.

https://edu.rsc.org/feature/the-science-of-smartwatches/4013008.article
10.1056/NEJMoa1901183

Biosensors in the news

TechRadar
https://www.techradar.com › ... › Fitness Trackers

The Apple Watch could soon track how much you sweat

21 Feb 2024 — There are sweat patches and **biosensors** that can already track your sweat, passing information to, among other things, the **Apple Watch**. That ...

Sports Business Journal
https://www.sportsbusinessjournal.com › 2024/02/22

Apple secures hydration tracking patent for Apple Watch

22 Feb 2024 — The U.S. Patent and Trademark Office recently issued **Apple** a new patent for the measurement of sweat through a wearable sensor.

Fierce Biotech
https://www.fiercebiotech.com › medtech › apples-long...

Apple Watch creeps closer to adding glucose tracking

23 Feb 2023 — For much of the last decade, rumors have suggested that **Apple** is aiming to one day bring completely noninvasive glucose tracking to the ...

ScienceDaily
https://www.sciencedaily.com › releases › 2017/03

Wearable biosensors can tell you when to see the doctor

10 Mar 2017 — Researchers supported by the National Institutes of Health have revealed the ability of wearable **biosensors**, similar to the **Apple Watch** or ...

The next Apple Watch will reportedly add 3 new health sensors for diabetes, blood pressure, and sleep apnea

Apple continues exploring the potential of health monitoring tools.

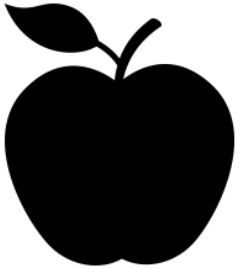
Biosensors are widely used

Medical diagnostics

Glucose meters
Breathalyzer
Ovulation test
Pregnancy tests
Home INR test for blood clotting

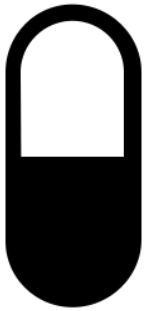
Environmental monitoring

Air quality
Water quality
Soil moisture
Noise level
Radiation level
Greenhouse gases
UV radiation
Weather



Food safety

Temperature, gas, pH, moisture, metal detection
Pathogen detection
Optical sensors for sorting produce
Pesticide, herbicide detection



Drug development

ELISA, SPR
Fluorescence
Cell-based
Microfluidic
Affinity
Arrays and chips
High-throughput

Personal health and fitness

Heart rate monitor
Wearable fitness trackers
Blood glucose
Blood pressure
Oximeter
Sleep trackers
Skin temperature
Smart clothing

Biotechnology

Enzyme-based
DNA-based
Cell-based
Immunosensors
Microbial sensors
Lab-on-a-chip
Wearable

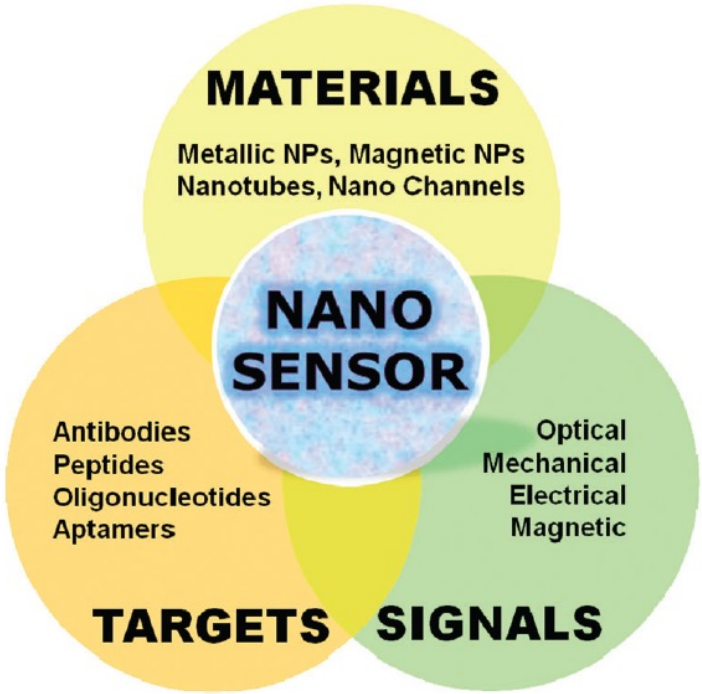
Nano(bio)sensors

Motivation

- Early detection of disease biomarkers (pM sensitivity)
- Cheap & reliable
- Point-of-care testing

Nanotechnological solutions promising

Components of a nanosensor



Swierczewska et al., Chem Soc Rev 2011

Biosensing – Plan for the next lectures

Sensing modalities

- Optical sensors
- Electric sensors
- Nanopores and next-generation sequencing

Integration

- Microfluidics and lab-on-a-chip devices

Learning objectives

1. Understanding Key Concepts and Technologies

Define and describe the principles of Lateral Flow Assays, ELISA, and Surface Plasmon Resonance (SPR), differentiating between label-dependent and label-independent biosensing methods.

2. Application in Scientific Contexts

Analyze data from various biosensors and evaluate their applications in scientific case studies, particularly in areas like medical diagnostics and biotechnology.

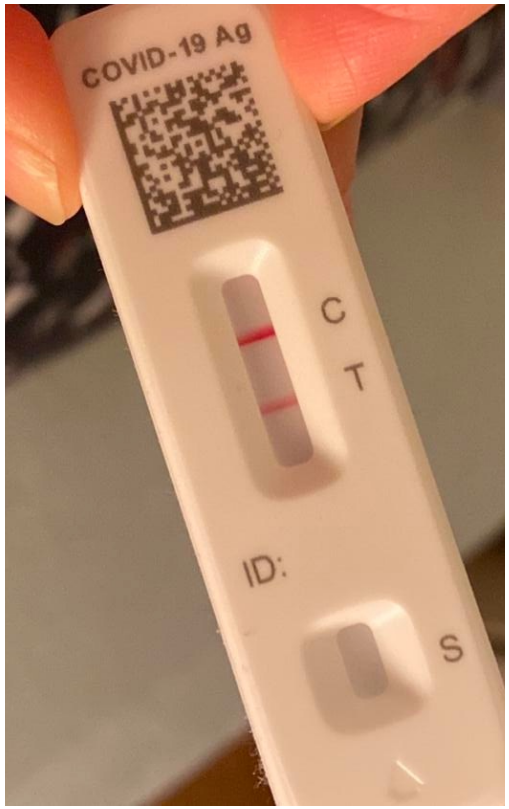
3. Planning and Analyzing SPR Experiments

Outline the planning of SPR experiments and the analysis of SPR sensorgrams, including understanding the technical setup, data interpretation, and extraction of key information like association/dissociation rates and binding affinities.

4. Designing Research Questions

Formulate research questions and conceptualize how biosensing technologies can be applied or adapted to address these scientific inquiries.

Testing positive...

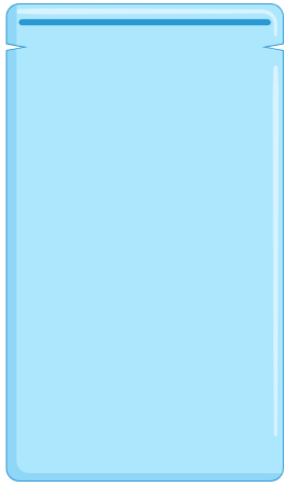


Optical biosensors convert the **biological interaction** (e.g., antigen-antibody binding, enzyme-substrate reaction) into an **optical signal.**

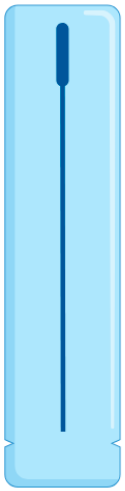
LFA test kit

Budd et al. Nat Rev Bioeng 2023

Extraction tube holder



Waste bag

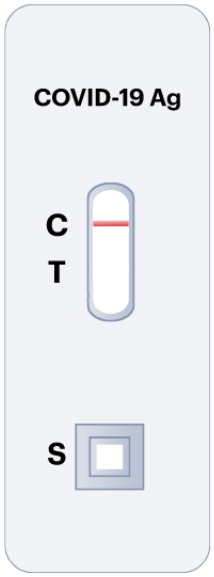


Swab inside sealed wrapper

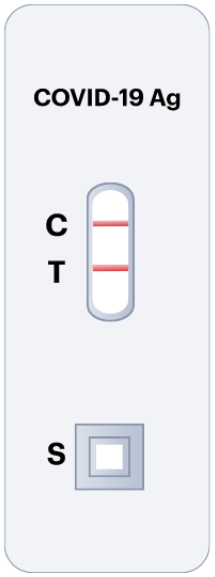
LFT in sealed packaging

Extraction buffer sachet

Extraction tube



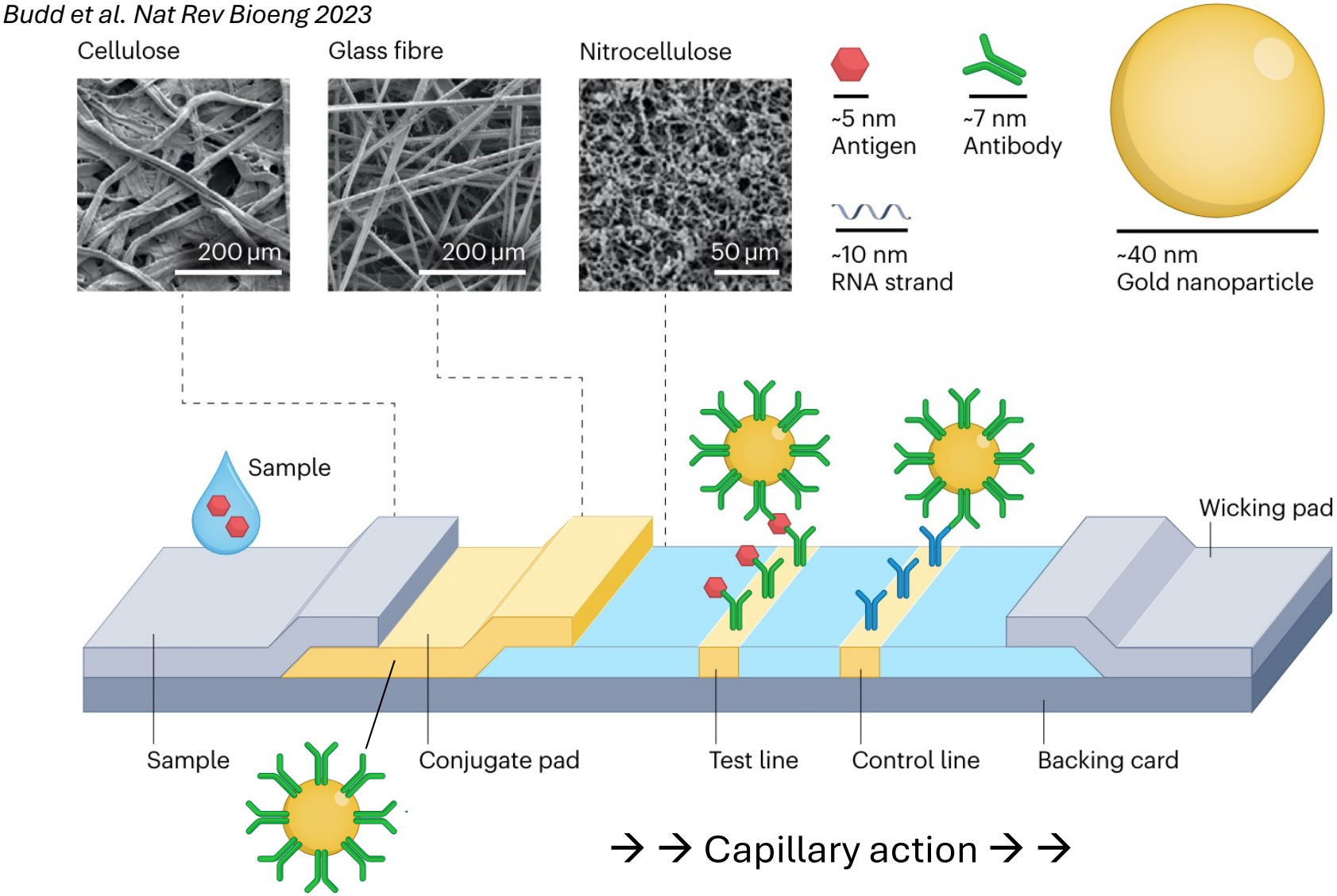
Negative



Positive

Lateral flow assay (LFA) components

Budd et al. Nat Rev Bioeng 2023



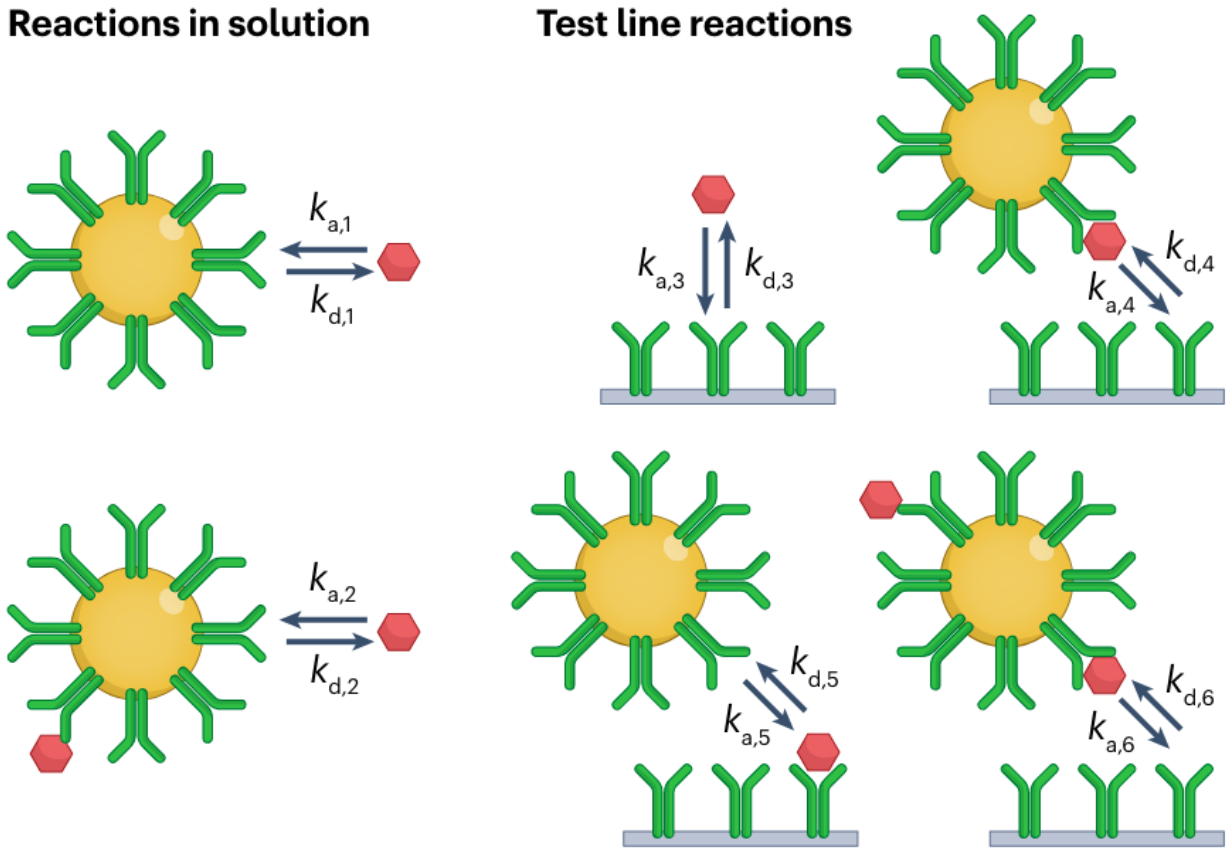
Lateral flow assay (LFA) components

Budd et al. Nat Rev Bioeng 2023

- **Cellulose sample pad**: absorbs the sample
- **Glass fibre conjugate pad**: stores dried nanoparticle–receptor conjugates
- **Nitrocellulose membrane**:
  - With a test line of immobilized capture receptors
  - With a control line functionalized with antibodies that bind to the antibodies on the nanoparticles
- **Wicking pad**: absorbs the sample on the other end of the test

LFA reactions

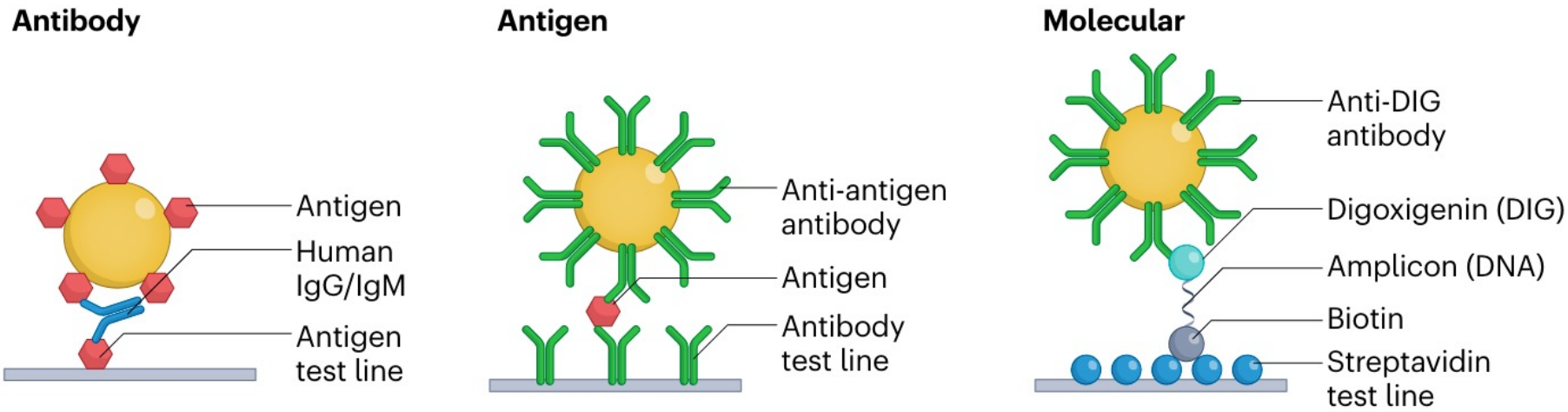
Budd et al. Nat Rev Bioeng 2023



- Initial analyte capture by nanoparticle-antibody (NP-Ab) should be rapid and stable enough for transport. (**High $k_{a,1}$ and $k_{a,2}$**)
- Test-line antibody capture of NP-Ab-analyte complexes must have the highest affinity to ensure stable and robust detection signals. (**High K_D for reactions 4 and 6**)
- Detection Ab (on NP) and capture Ab (on test line) are usually not the same.

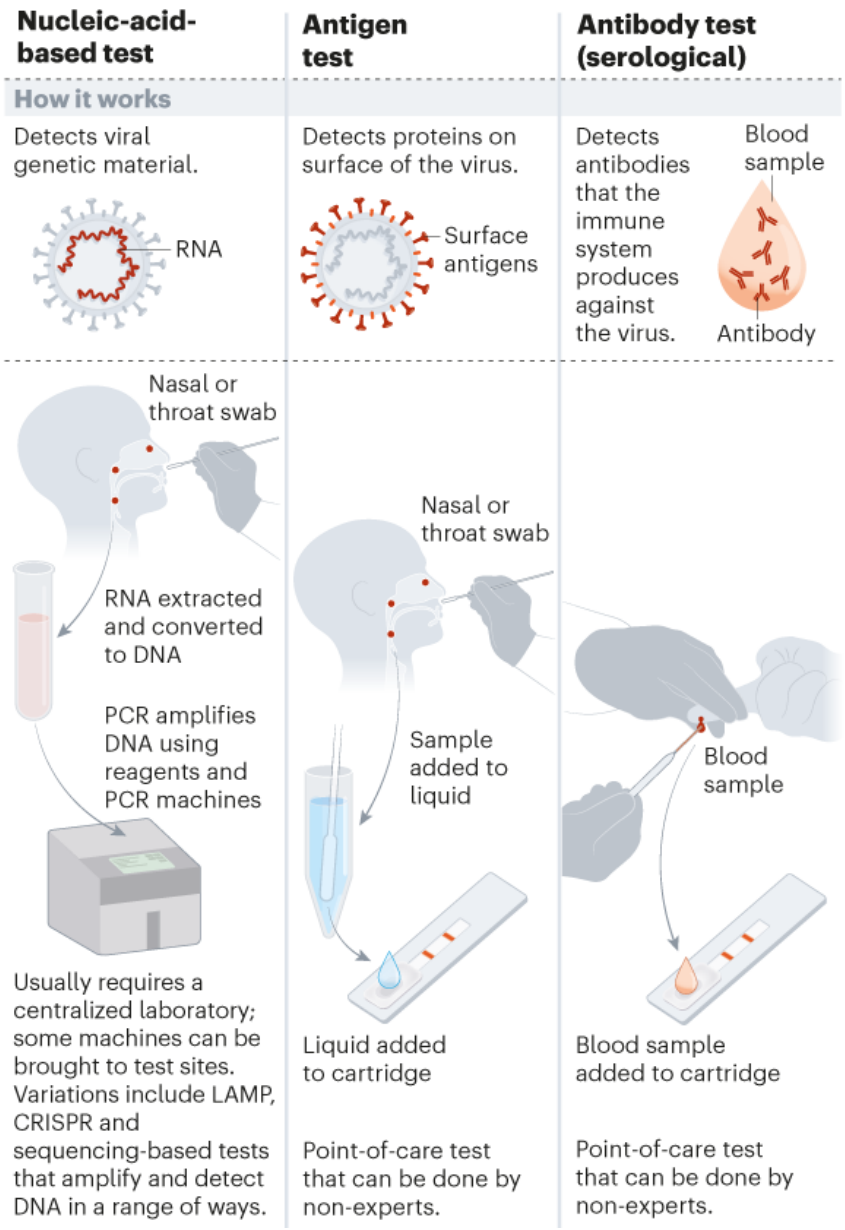
LFA detection complexes in different assay designs

Budd et al. Nat Rev Bioeng 2023



- **Sandwich formation in all cases:**
  - Mixed analyte-nanoparticle complex binds to test line in a sandwich-type complex

Rapid testing for COVID-19



Nucleic-acid-based test	Antigen test	Antibody test (serological)
What a test tells you		
Whether any viral genetic material is present, even at low levels.	Whether the virus is present in high concentrations. (Whether you are likely to be infectious.)	Whether you are likely to have had the virus. It does not detect an active infection.
Time and cost		
Hours/days $$$	Minutes $	Minutes $
General reliability*		
Very sensitive and specific.	Misses infections with low virus levels.	Variable, but some tests are very specific.

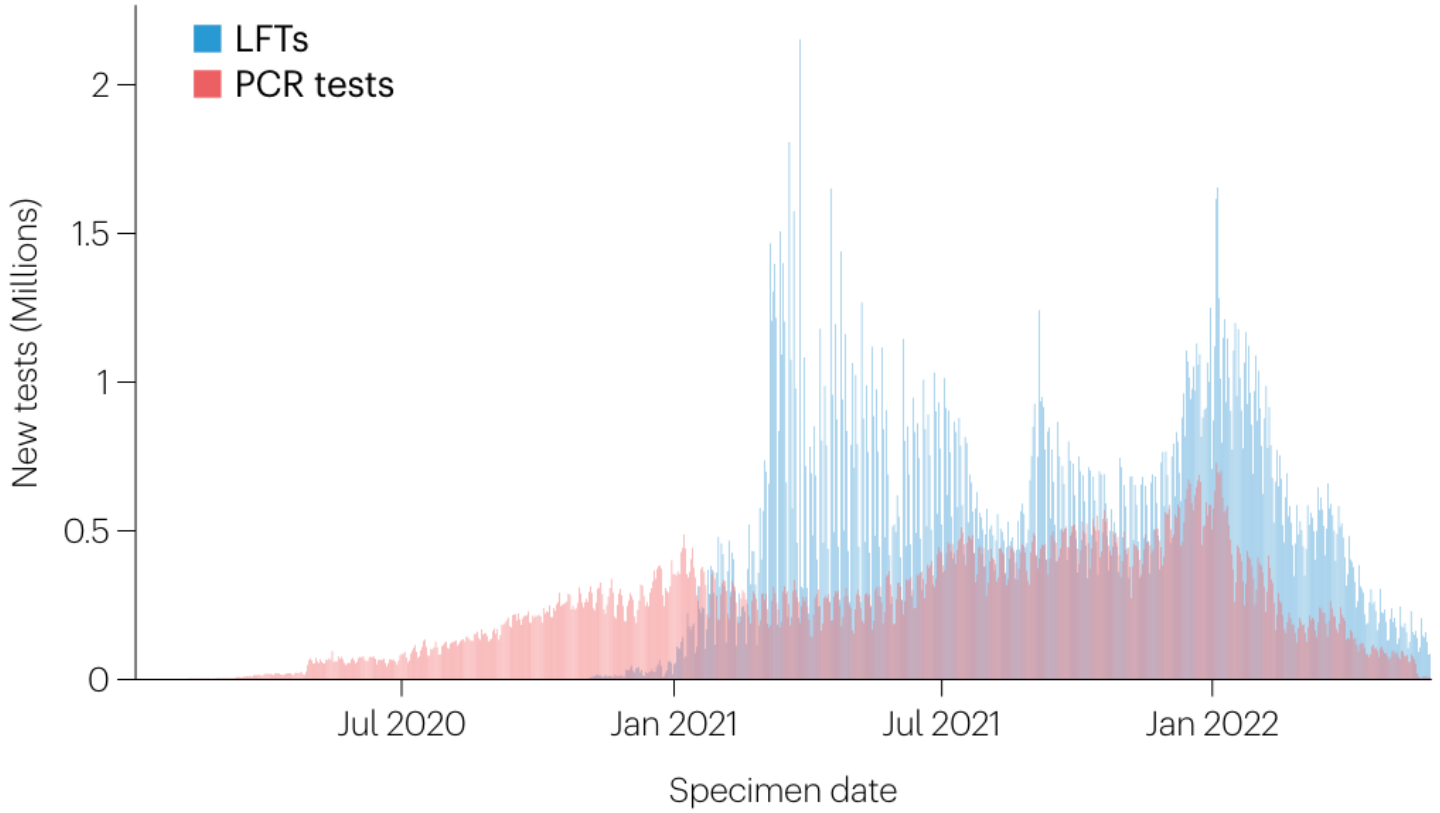
*The chance that a test result is a true positive or a true negative depends not only on a test's own reliability, but also on background rates of infection, and on whether a person shows symptoms.

©nature

https://www.nature.com/articles/d41586-021-00332-4

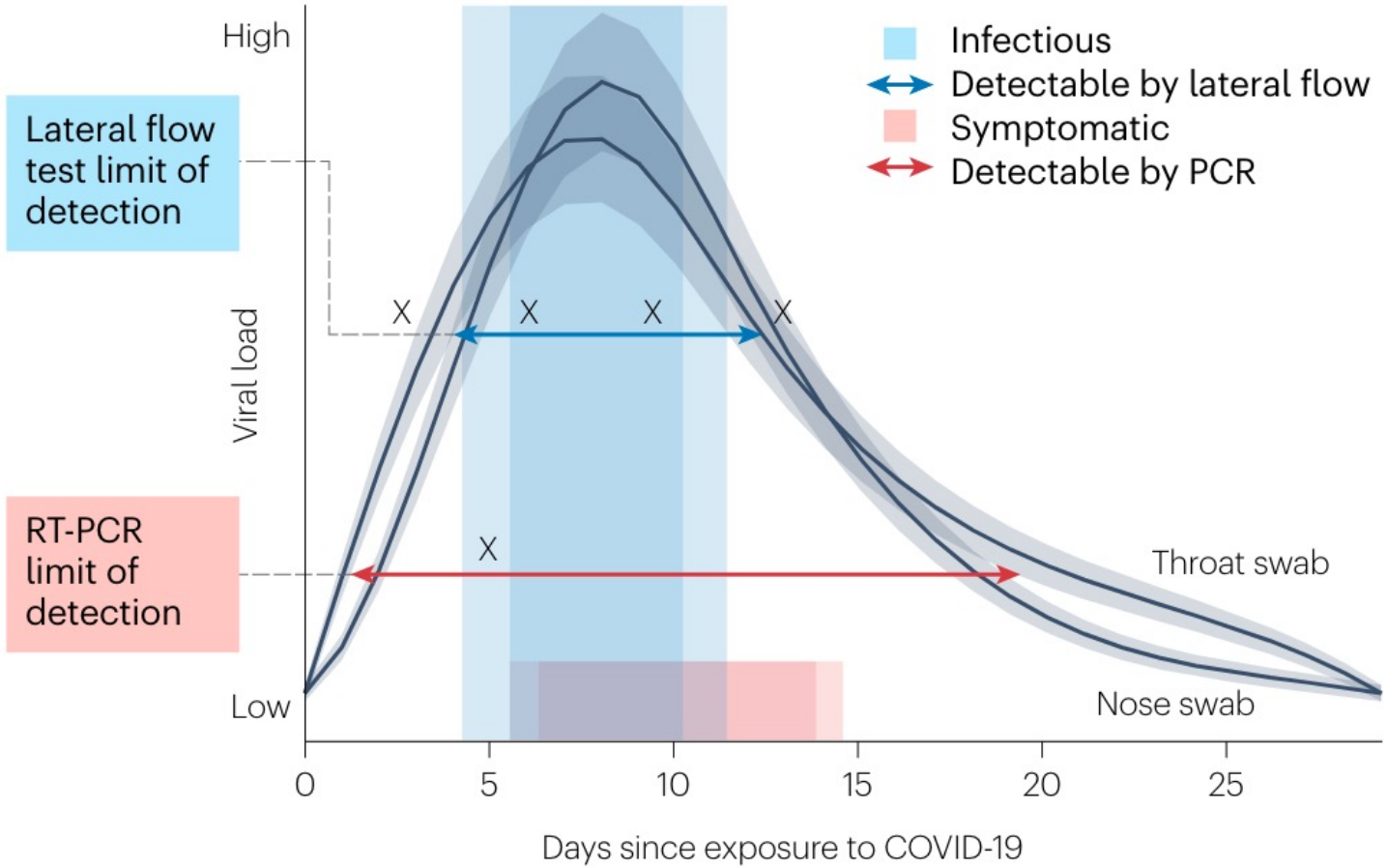
COVID tests in England over time

Budd et al. Nat Rev Bioeng 2023



Sensitivity: LFA vs. RT-PCR

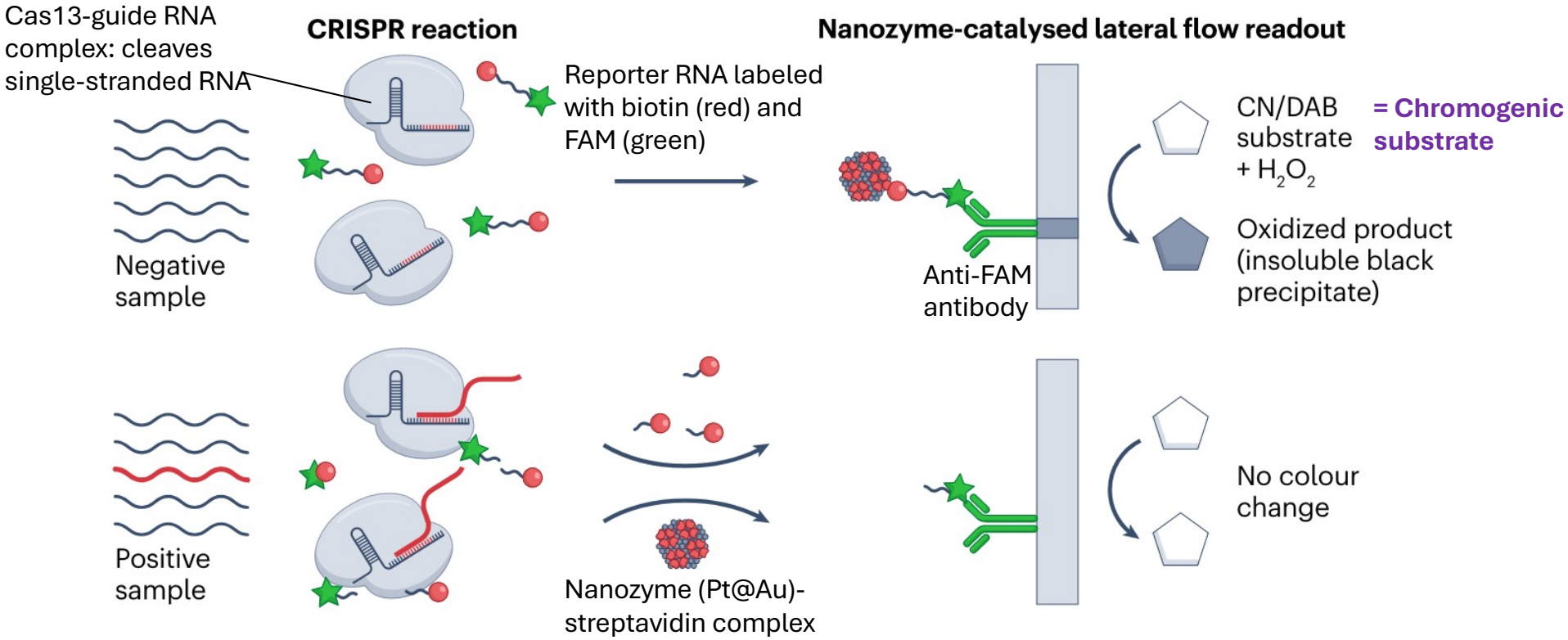
Budd et al. Nat Rev Bioeng 2023



Activity: Next-generation LFTs

Nanozyme-amplified lateral flow test

Broto et al. Nat Nanotech. 2022



Discuss with another student: 1) How does this assay work? Describe the figure. 2) Why is a nanozyme-catalyzed readout necessary? For a positive test result, what's the expected readout without adding **a chromogenic substrate** vs. after adding it?

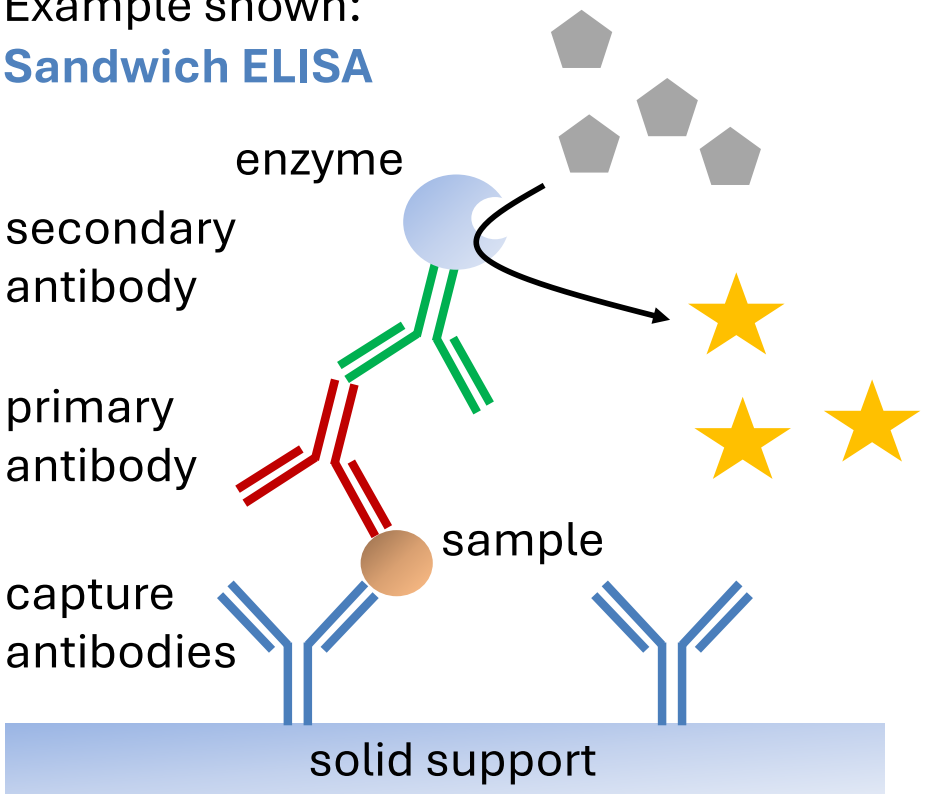
How are LFAs made?

- https://www.youtube.com/watch?v=o4iJgz9ugy4&ab_channel=DCNDiagnostics

Established methods:
ELISA-type biosensing

ELISA: Enzyme-linked immunosorbent assay

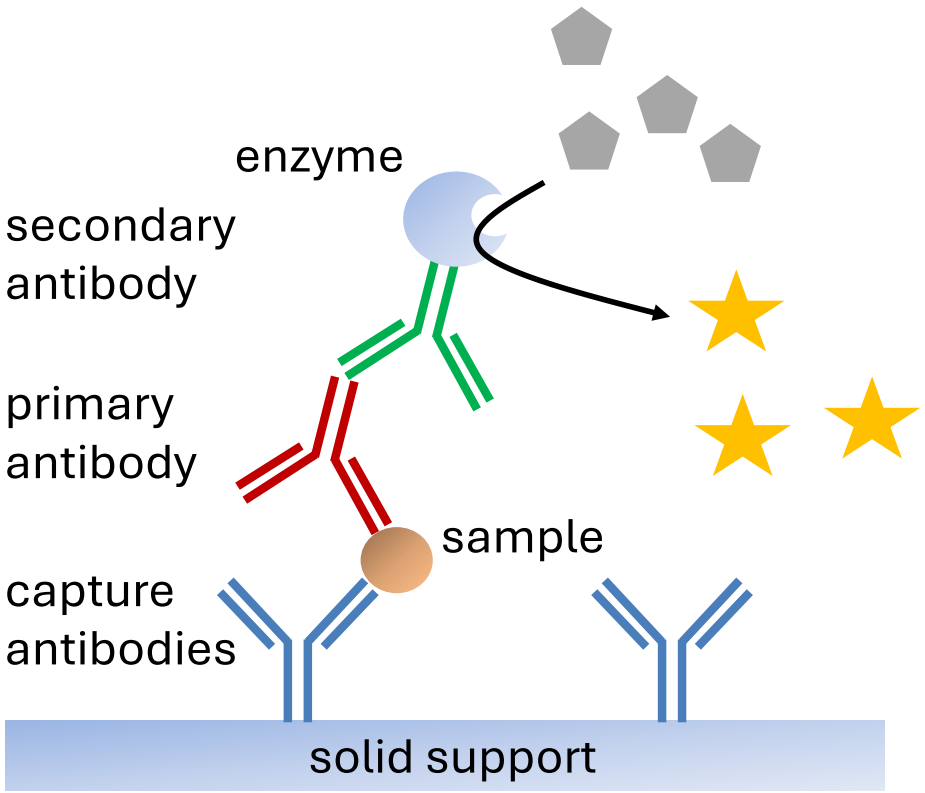
Example shown:
Sandwich ELISA



1. Sample is flowed over immobilized capture antibody, specific for target molecule (protein)
2. Solution containing specific primary antibody (=detection antibody) is bound (**sandwich assay**)
3. Enzyme-linked secondary antibody is added
4. Enzyme substrate solution is added. An enzyme-catalyzed reaction converts substate into colored compound. (→ Signal enhancement)
5. Optical detection

Established methods: ELISA-type biosensing

ELISA: Enzyme-linked immunosorbent assay



Labels:

Fluorophores, radioactive labeling, enzymes, antibodies

Advantage:

Specificity, sensitivity

Disadvantages:

Chemistry involved / might not be possible (patient sample)

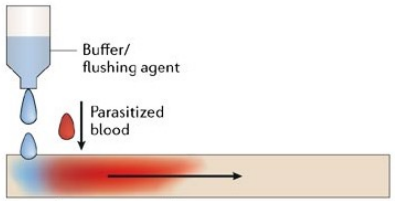
Labels might disturb interaction

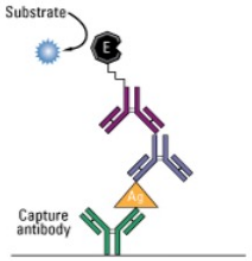
How to do an ELISA

- https://www.google.com/search?sca_esv=7e140dff066c04a6&sca_upv=1&hl=en&sxsrf=ACQVn08DvN3HcQsO9X32SHwlFOjiV2Xc9Q:1710796791157&q=elisa+assay&tbm=vid&source=lnms&sa=X&ved=2ahUKEwia55PY3v6EAxX7gv0HHZuQBEkQ0pQJegQIExAB&biw=1532&bih=814&dpr=2#fpstate=ive&vld=cid:d24af6ef,vid:alQT_soh_V0,st:0

Summary: Comparison of LFA and ELISA

	Sensitivity	Measurement time
LFA	0.1 µM	minutes
ELISA	1 pM	hours





LFA: Ideal point-of-care characteristics, but often not sensitive enough (disease biomarkers)

ELISA: complex and slow, but high sensitivity due to large amplification

Goals:
- fast analysis time (< 1 min)
- very high concentration sensitivity (< 1 pM)
- quantitative readout
- minimal sample manipulation

Surface plasmon resonance (SPR)

SPR is a gold standard technique used in the biotechnology industry to measure **binding kinetics** and **molecular interactions**.

DOI: 10.1007/978-3-319-76556-3_9

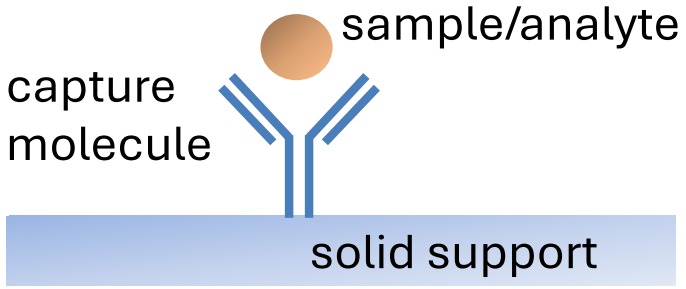
Label-free biosensing:
Surface plasmon resonance (SPR)

Label-free sensor:

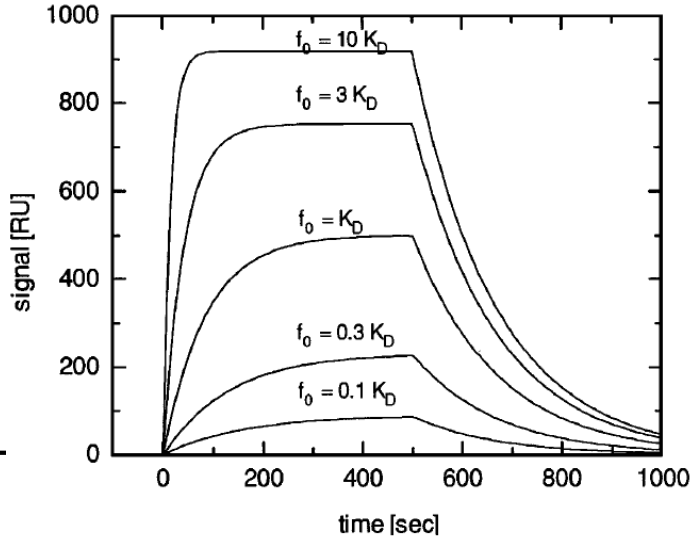
The signal is generated only due to the presence of the target molecule

Surface plasmon resonance:

- Measures biomolecular, protein-protein or protein-ligand (small molecule, nucleic acid, lipid, etc.) interactions quantitatively at a **surface**.
- Provides equilibrium and kinetic information
- High sensitivity, down to single-molecule level
- Scaleable method → can be miniaturized / integrated into a microfluidic chip

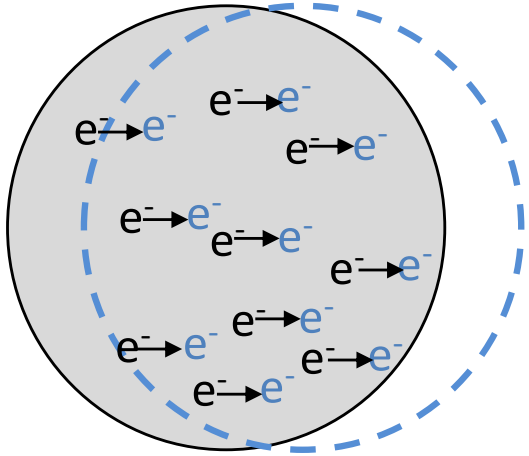


Measurement: **sensorgram**

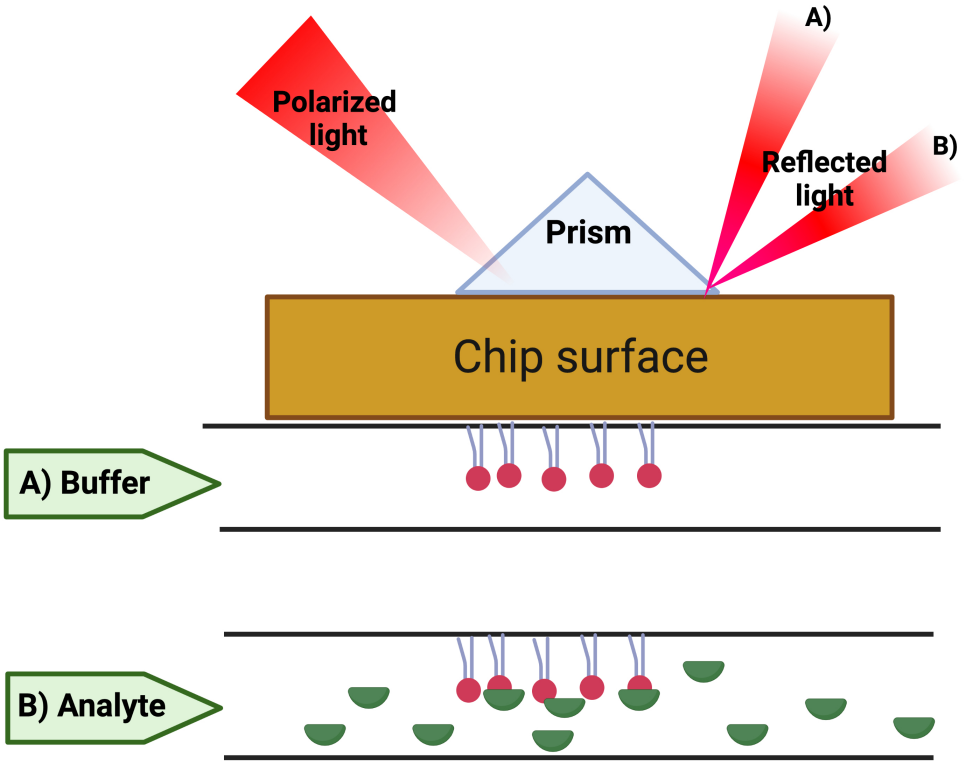


Terminology: plasmons in metal

- **Metallic bonding:** Lattice of positively charged metal nuclei surrounded by a 'sea' of delocalized, mobile electrons
- **Plasmons:** collective oscillations of the free electron gas (=electron plasma)
- **Generation of dipoles:** free electrons oscillate relative to fixed positive lattice, they create oscillating dipole moments.
- **Interaction with light**: when light is shined on the metal surface, it can interact with the electron plasma, inducing plasmon oscillations.
- **Frequency dependence**: ability to induce oscillations depends on factor like **light frequency**, **metal type**, **nanostructure size**. There is a specific frequency (**plasmon frequency**) where the resonance is strongest.



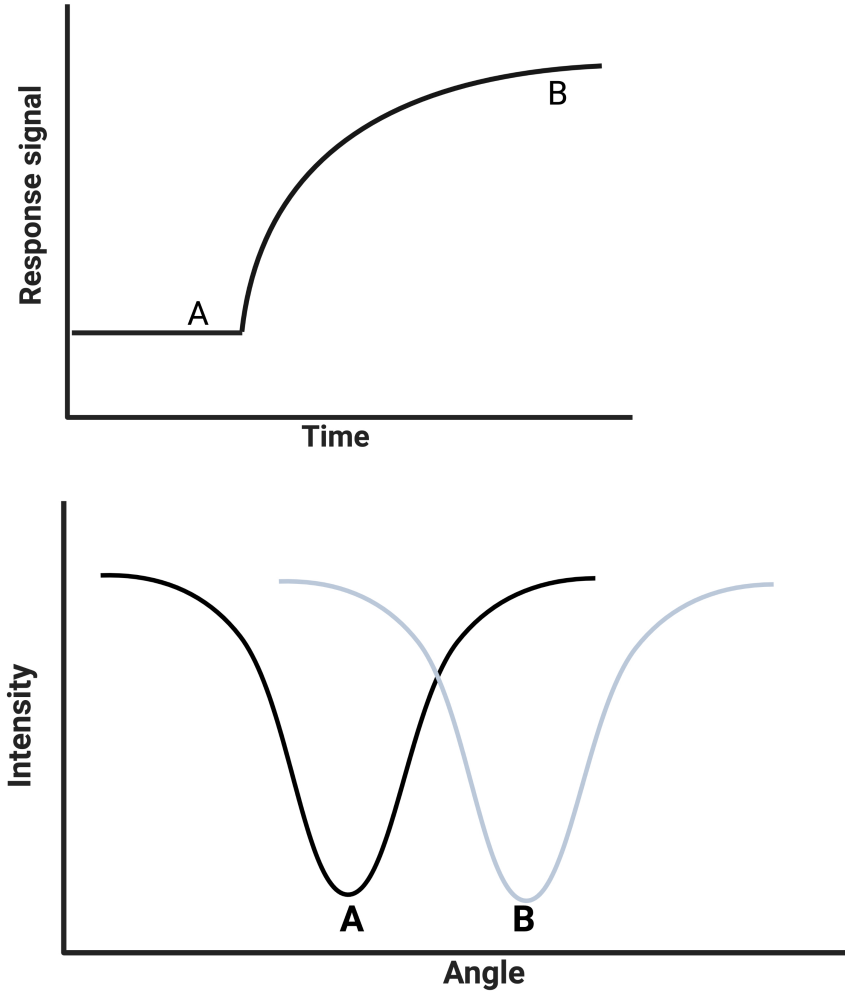
Label-free biosensing:
Surface plasmon resonance (SPR)



- Measures **change in refractive index at metal-dielectric interface** (most commonly gold)
- Incident polarized light is introduced at a precise, **resonant angle** through a prism
- This excites collective electron oscillations called **surface plasmons**
- This interaction creates an **evanescent electromagnetic field at the metal surface**, which is highly sensitive to changes in refractive index caused by biomolecule binding

Motsa et al. Biochem (Lond) (2023) 45 (1): 18–22.

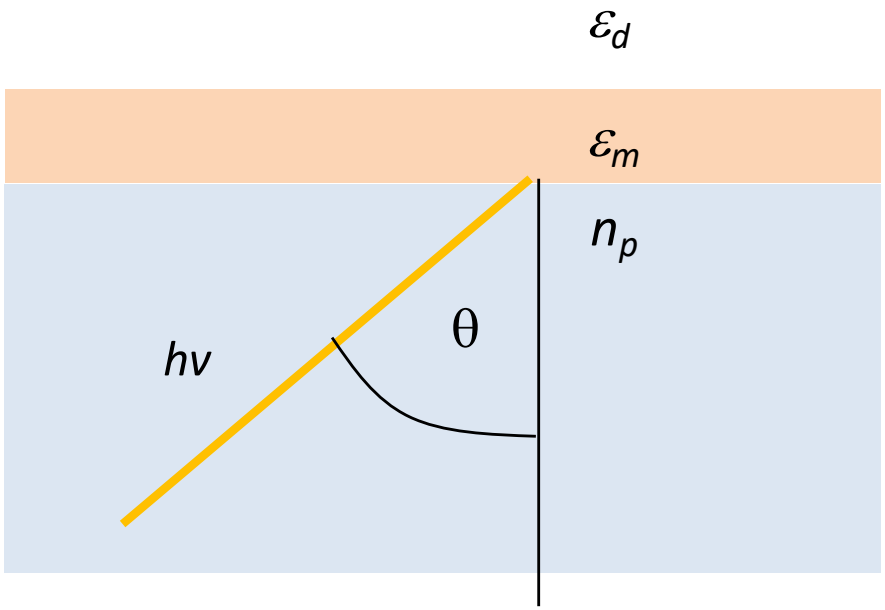
Label-free biosensing:
Surface plasmon resonance (SPR)



Motsa et al. Biochem (Lond) (2023) 45 (1): 18–22.

- Binding increases the local refractive index, **shifting the resonant angle** and altering the reflected light intensity
- Resonance angle dip occurs because **incoming photons are being absorbed to excite surface plasmons** rather than being reflected
- Monitoring intensity shift in real-time: **kinetic parameters and affinity constants of biomolecular interactions**

Angular dependence of resonance



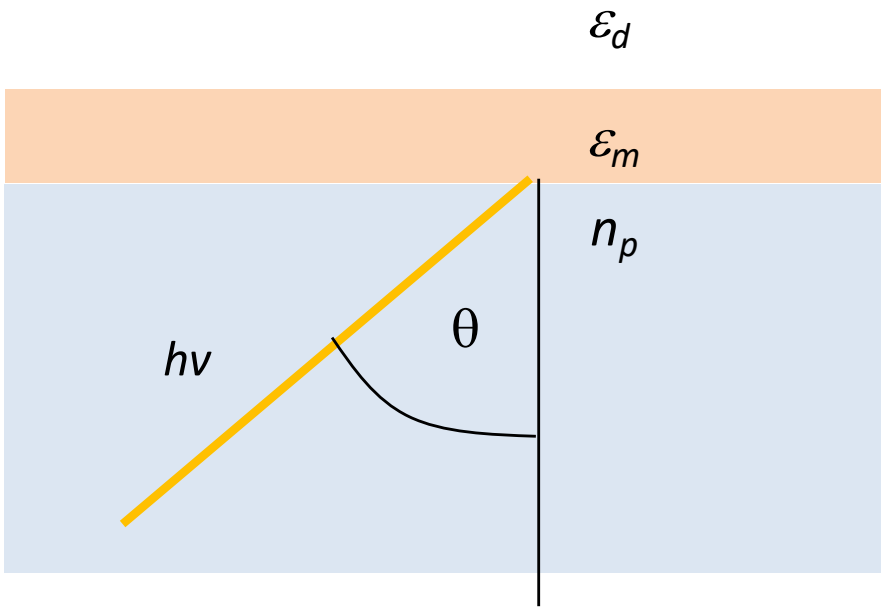
3) **Refractive index (n_p) and material properties**: The coupling efficiency is also influenced by the refractive index of the medium through which light passes (usually a prism is used) and the material properties of the metal and dielectric medium (ε_m *and* ε_d*)*.

Parameters that influence SP coupling efficiency:

1) Matching wavelength (*hv*): Incident light must have a wavelength that resonates with electrons in the metal. Resonance ensures effective energy transfer from the incident light to electrons at the metal interface.

2) Incident angle (θ): The angle at which light strikes the surface determines how effectively the incident beam's momentum matches the momentum required to excite electrons at the metal interface. Only specific angles enable optimal interaction and energy transfer to create a strong electromagnetic field at the metal surface.

Equation of resonance condition



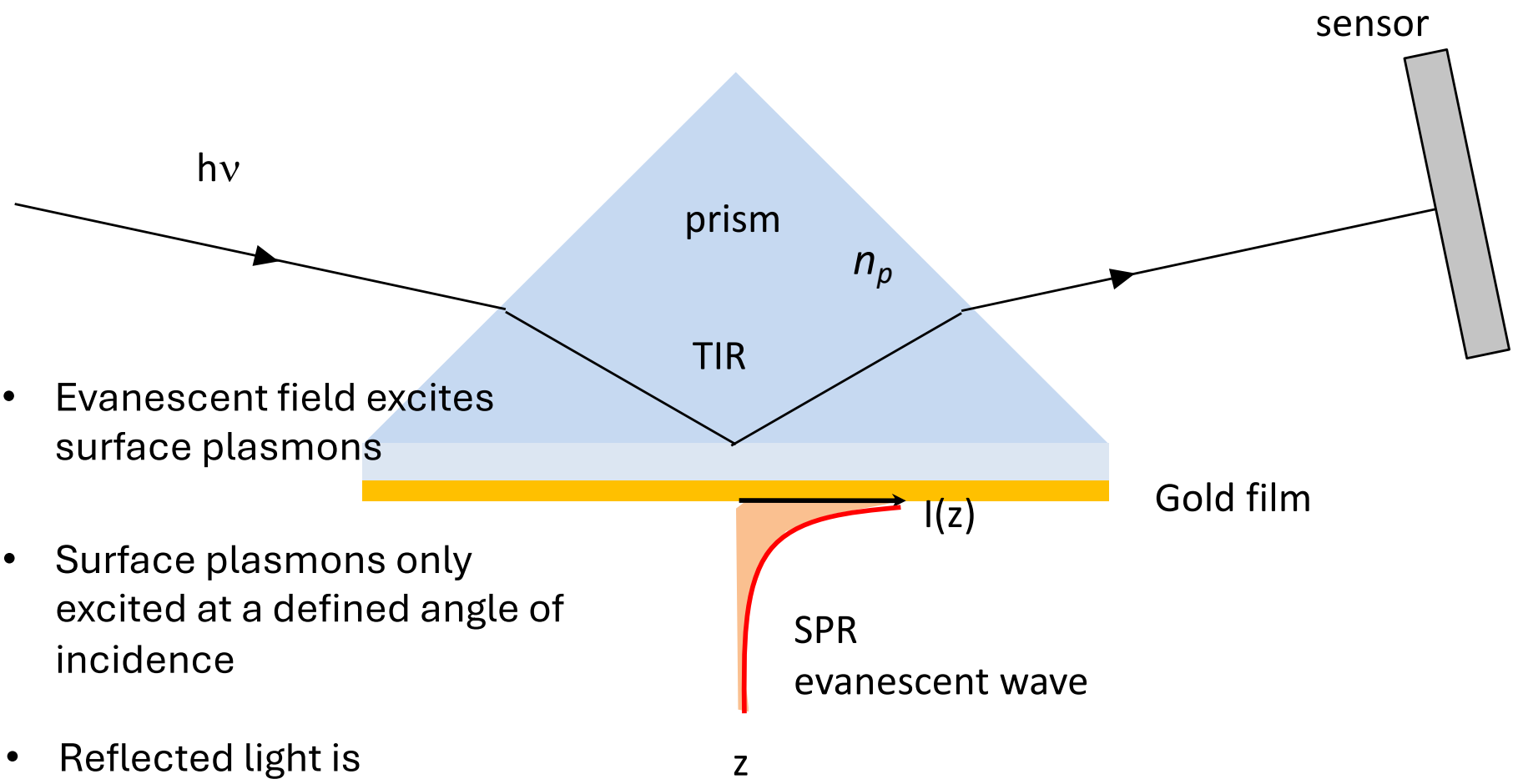
$$\left(\frac{2\pi}{\lambda}\right) n \sin\theta_i = \frac{2\pi}{\lambda_{spp}} \sqrt{\frac{\epsilon_d \epsilon_m}{\epsilon_d + \epsilon_m}}$$

The **resonance condition** can be described by an equation that relates the properties of the incident light (wavelength λ, angle θ) to the properties of the metal (surface plasmon wavelength λ_{spp}, permittivities of the metal and dielectric medium, defined by ε_m and ε_d).

Permittivity: a measure of the material's ability to "*permit*" the presence of an electric field. The permittivity is a complex value in metals due to their high conductivity and is denoted by the symbol ε. The relationship between the dielectric constant (ε) and the refractive index (n) of a non-magnetic material in optics is given by:

$$\boldsymbol{\varepsilon = n^2}$$

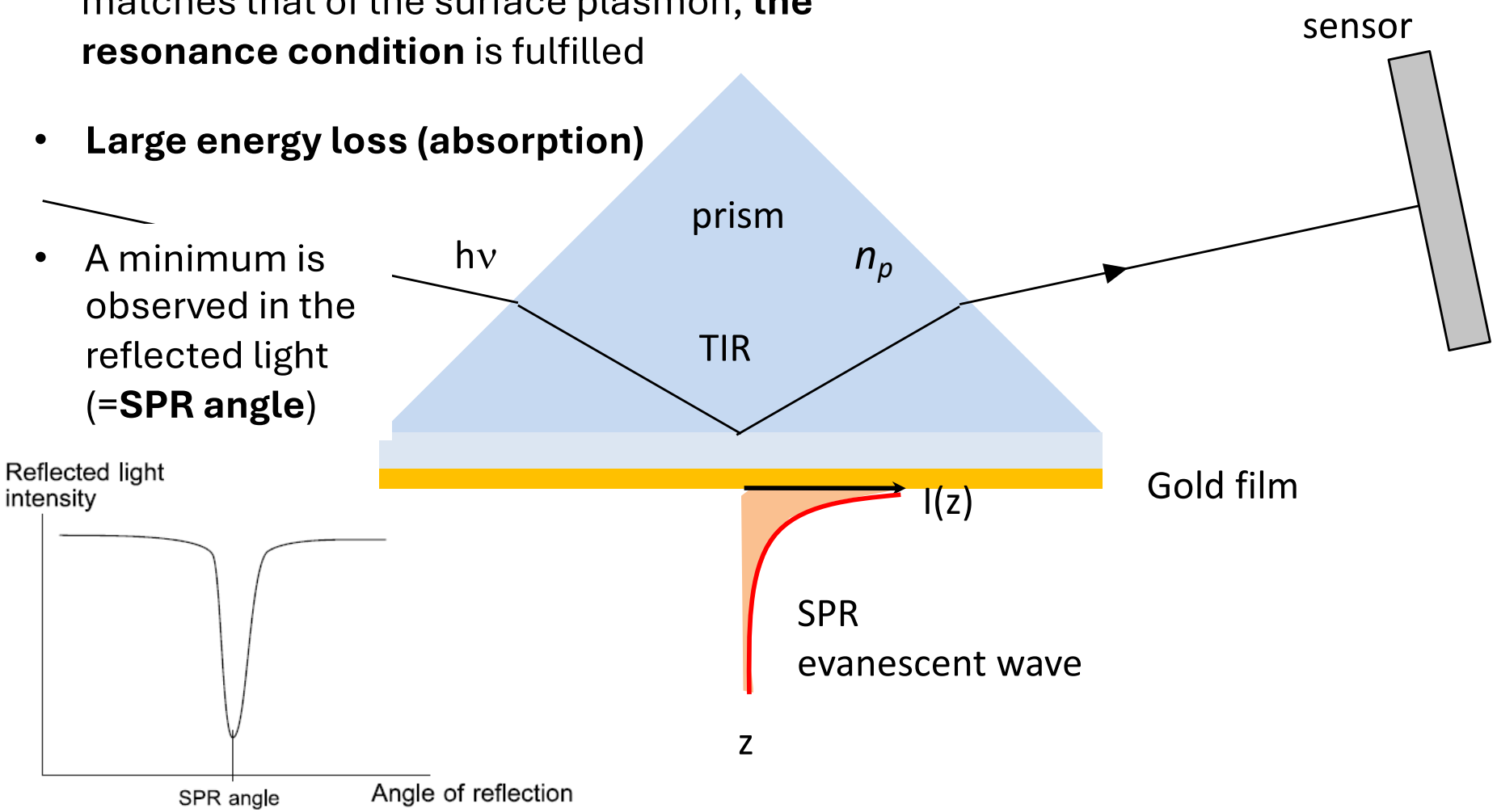
Total internal reflection condition at metal surface (Kretschmann configuration)



- Evanescent field excites surface plasmons
- Surface plasmons only excited at a defined angle of incidence
- Reflected light is monitored in a sensor

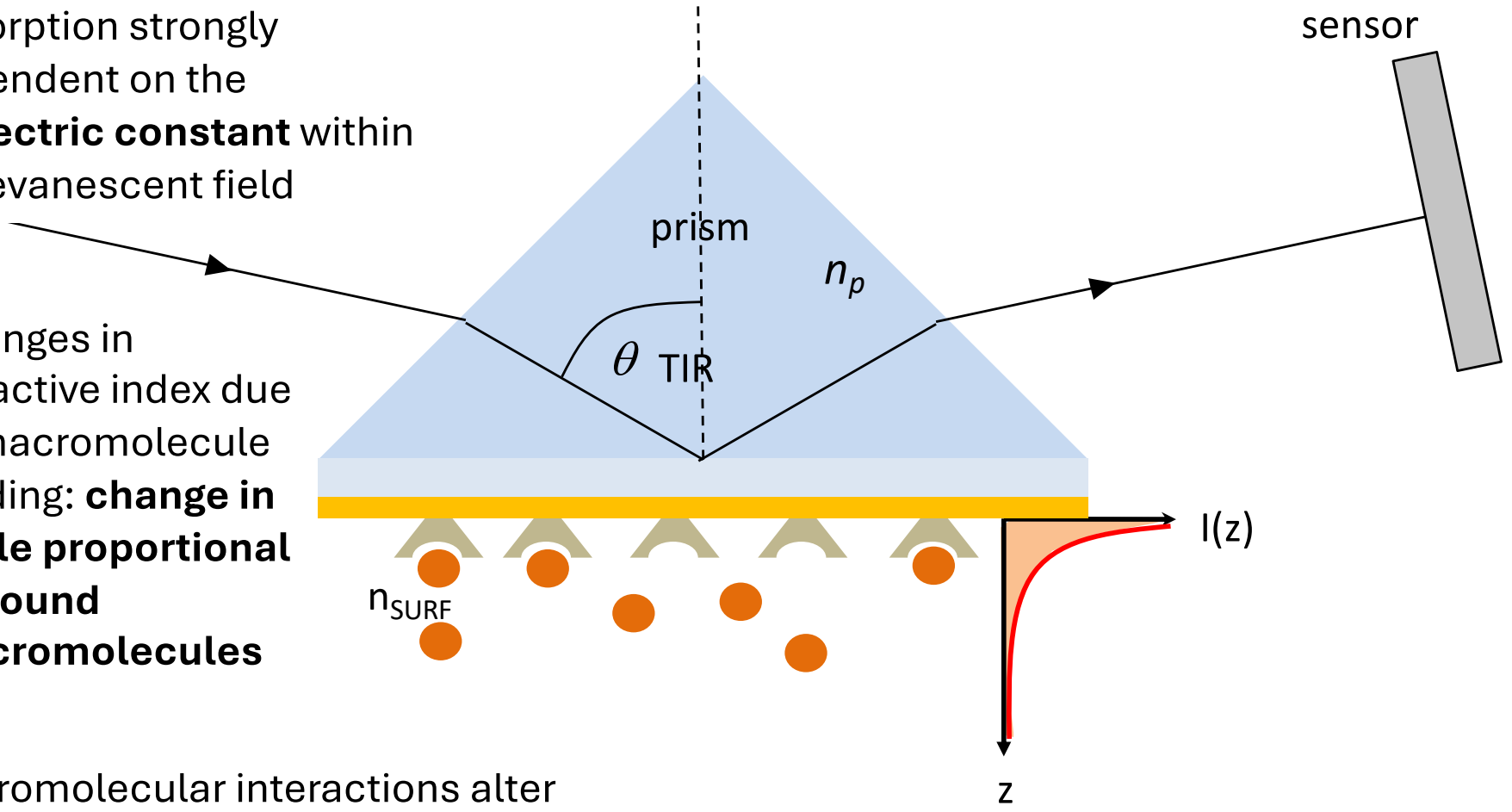
SPR: Kretschmann configuration

- If the wave vector of the light in the plane matches that of the surface plasmon, **the resonance condition** is fulfilled
- **Large energy loss (absorption)**
- A minimum is observed in the reflected light (=**SPR angle**)

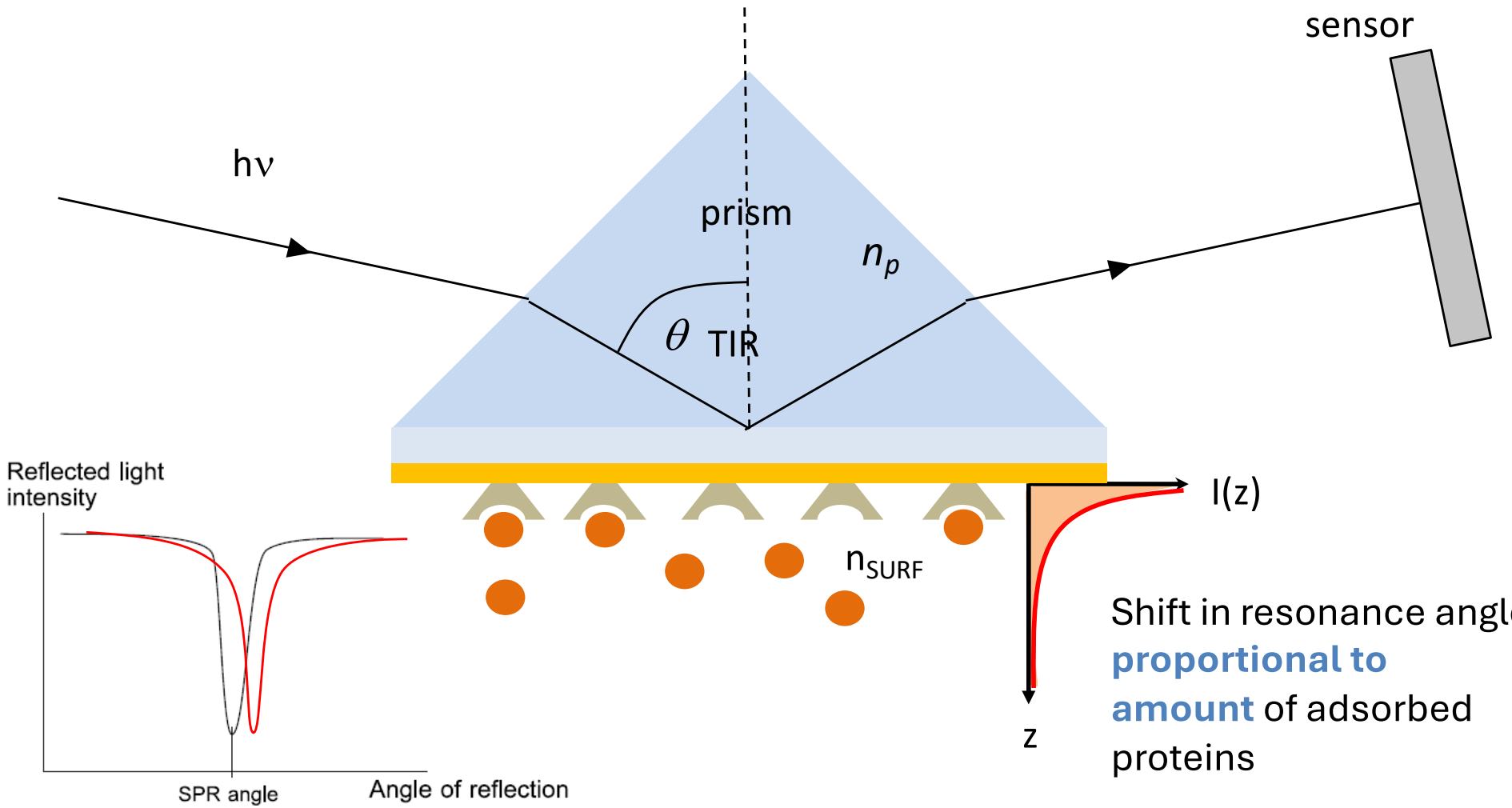


SPR: Kretschmann configuration

- Angle of maximal absorption strongly dependent on the **dielectric constant** within the evanescent field
- Changes in refractive index due to macromolecule binding: **change in angle proportional to bound macromolecules**
- Macromolecular interactions alter refractive index at dielectric side



SPR: Kretschmann configuration



Resonance condition: Analogy to the macroscopic world

Image source: ChatGPT

The **child on a swing** has a natural swinging rhythm (natural frequency).

You must **match your pushes (energy input)** exactly to the child's swinging rhythm to effectively transfer energy and make the child swing higher (**resonance**).

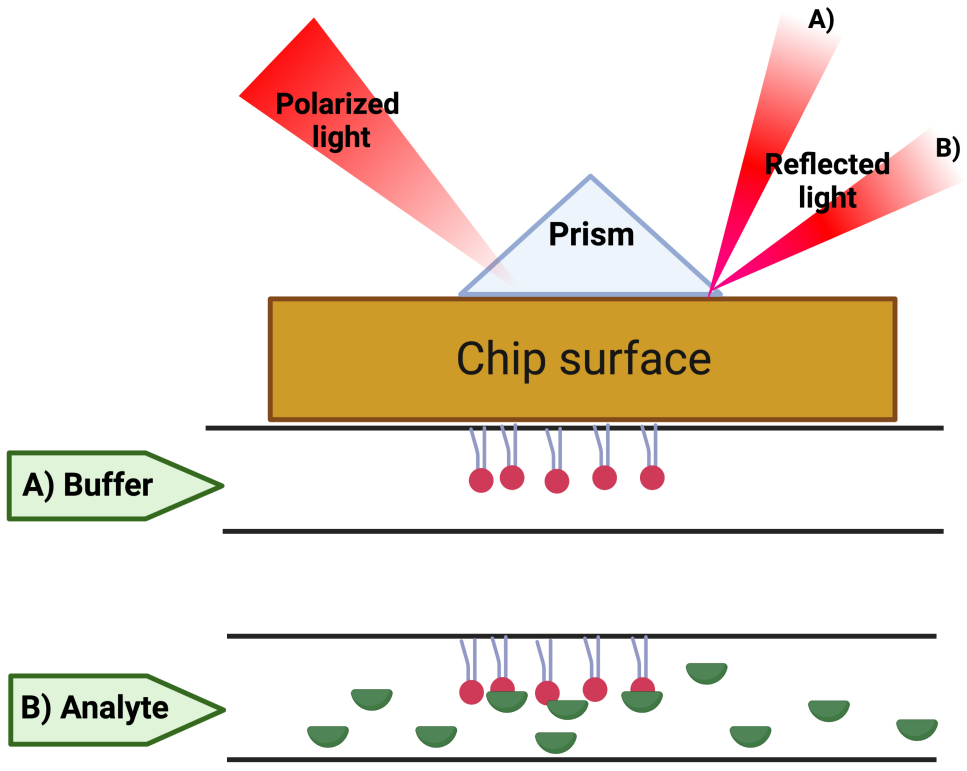
Resonance condition: Analogy to the macroscopic world

Image source: ChatGPT

Child swings through water

- **Air to water medium change** → **Change in refractive index** at the metal interface.
- **Slightly altered swing rhythm** → **Resonance angle shifting** in SPR.
- **Adjusting your push timing** → Adjusting incident angle of incoming photons to re-establish resonance conditions.

Activity: what is wrong in this picture?



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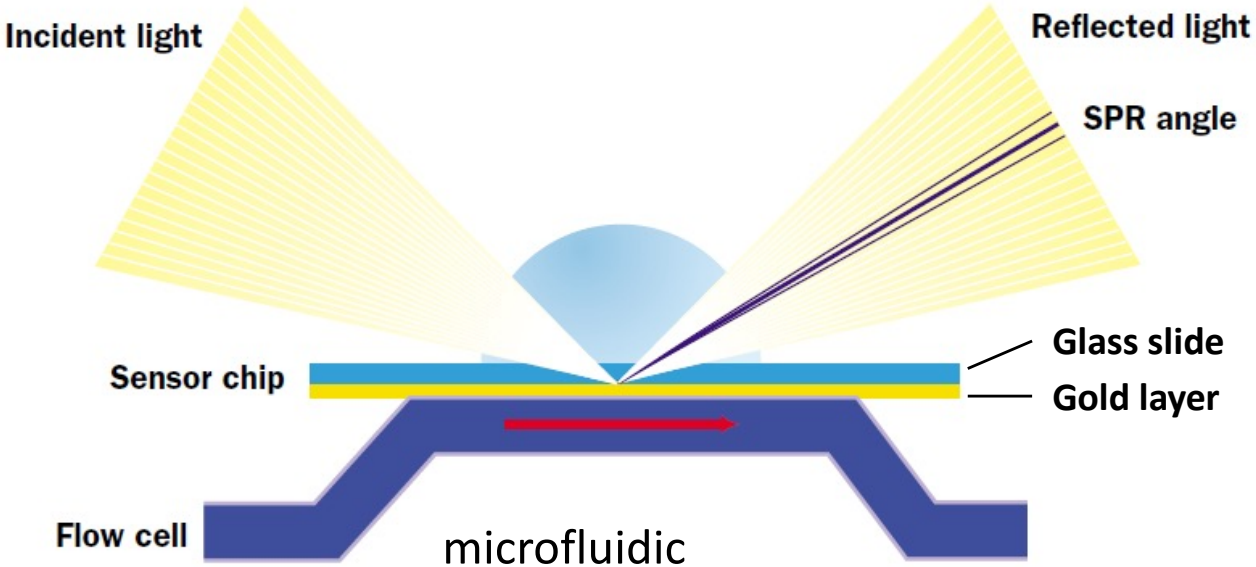
Motsa et al. Biochem (Lond) (2023) 45 (1): 18–22.

BIAcore biosensor

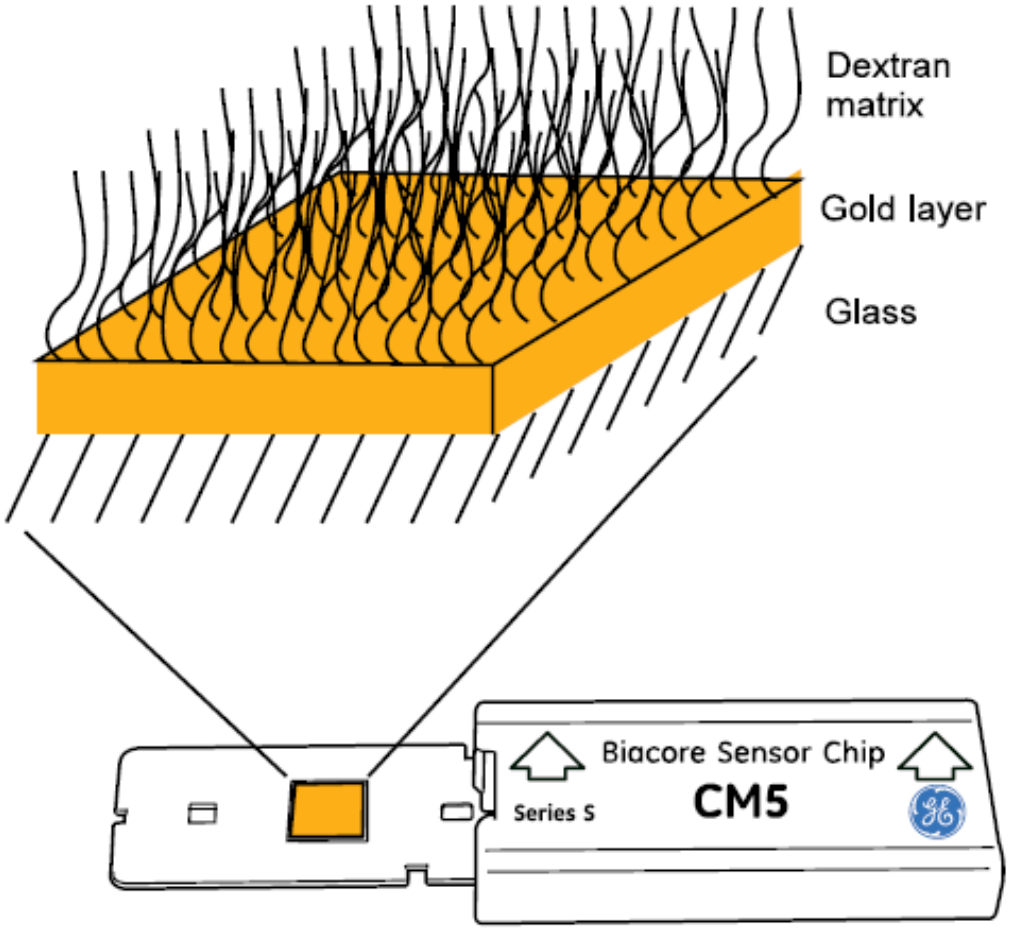


microfluidic
sample flow cell

BIAcore Tech Brochure

- Measurements require very exact measurements of the angle
- Commercial solution: all integrated
- Commercially available chips, contain all the necessary microfluidics to perform the assay

BIAcore chip



Surface attachment chemistry:

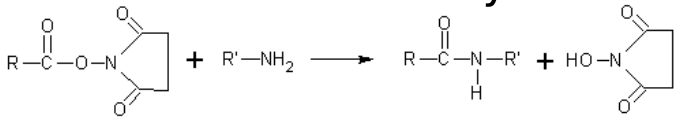
Non-covalent

Ni:NTA

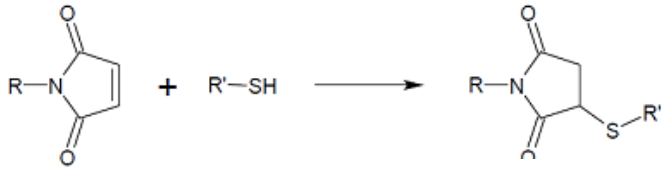
Streptavidin-biotin

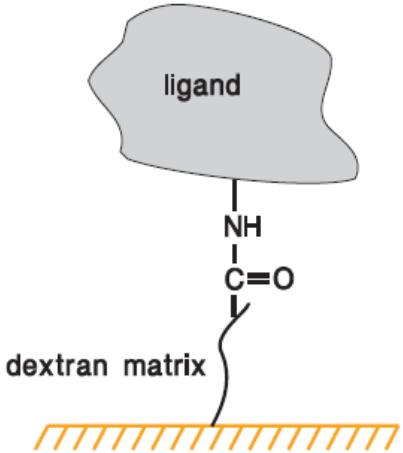
Covalent

Succinimide chemistry → amines

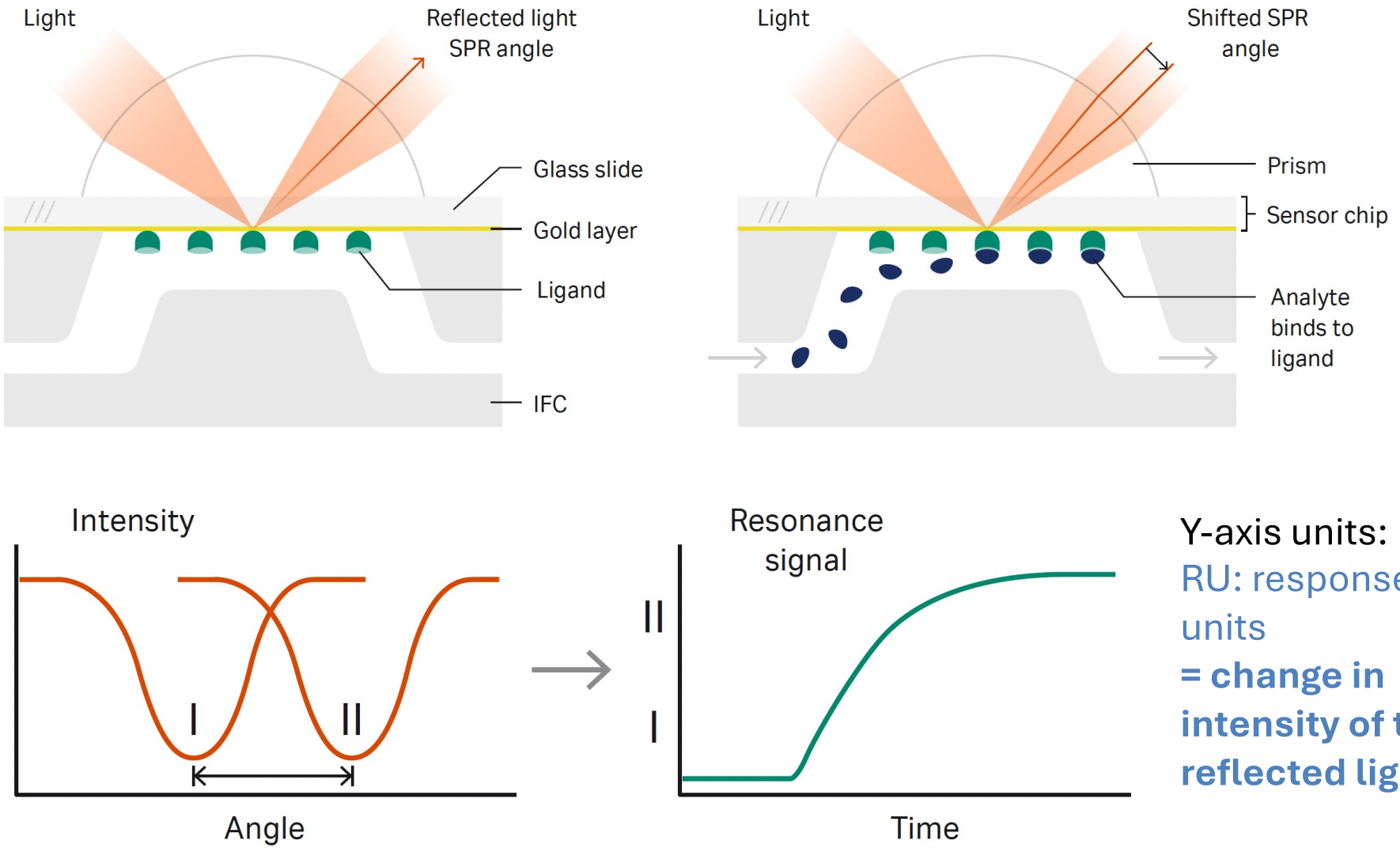


Maleimide chemistry → thiols





SPR detection principle

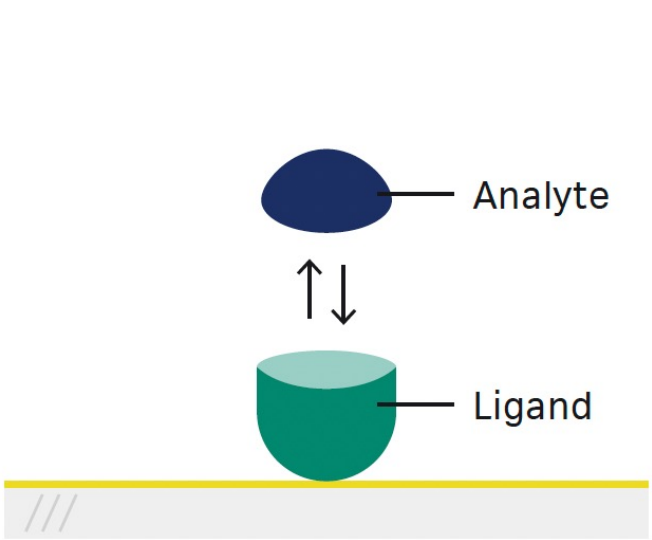


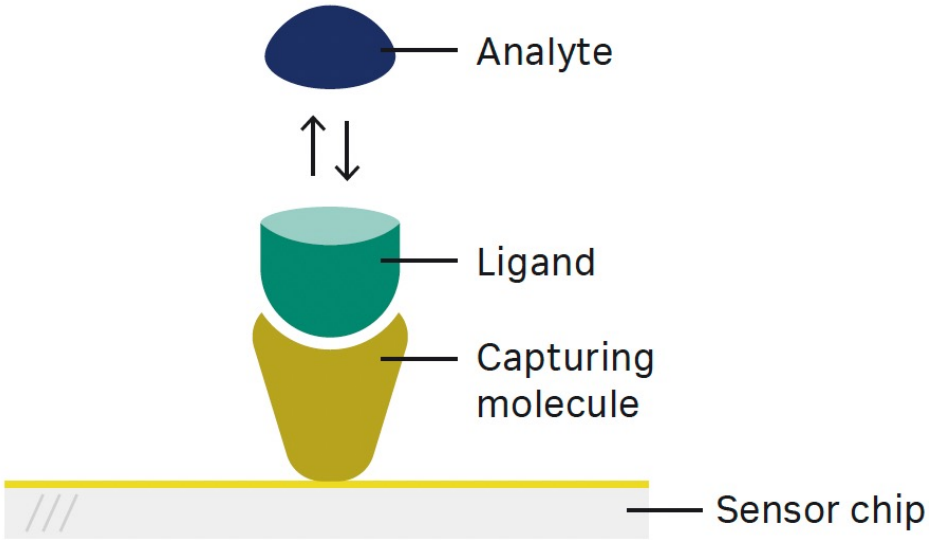
Y-axis units:

RU: response units

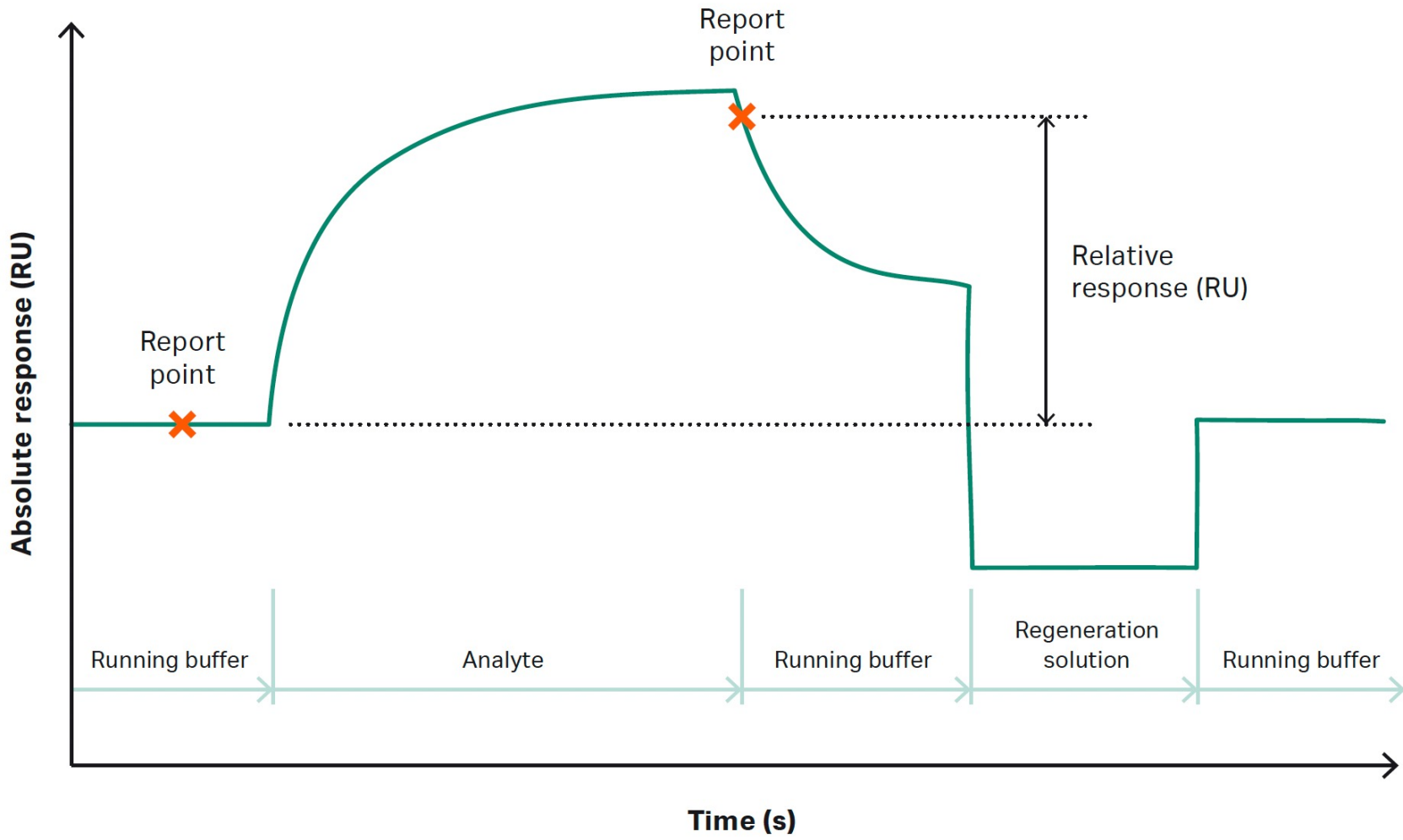
= change in intensity of the reflected light

Ligand, analyte, and capturing molecule in relation to the sensor surface

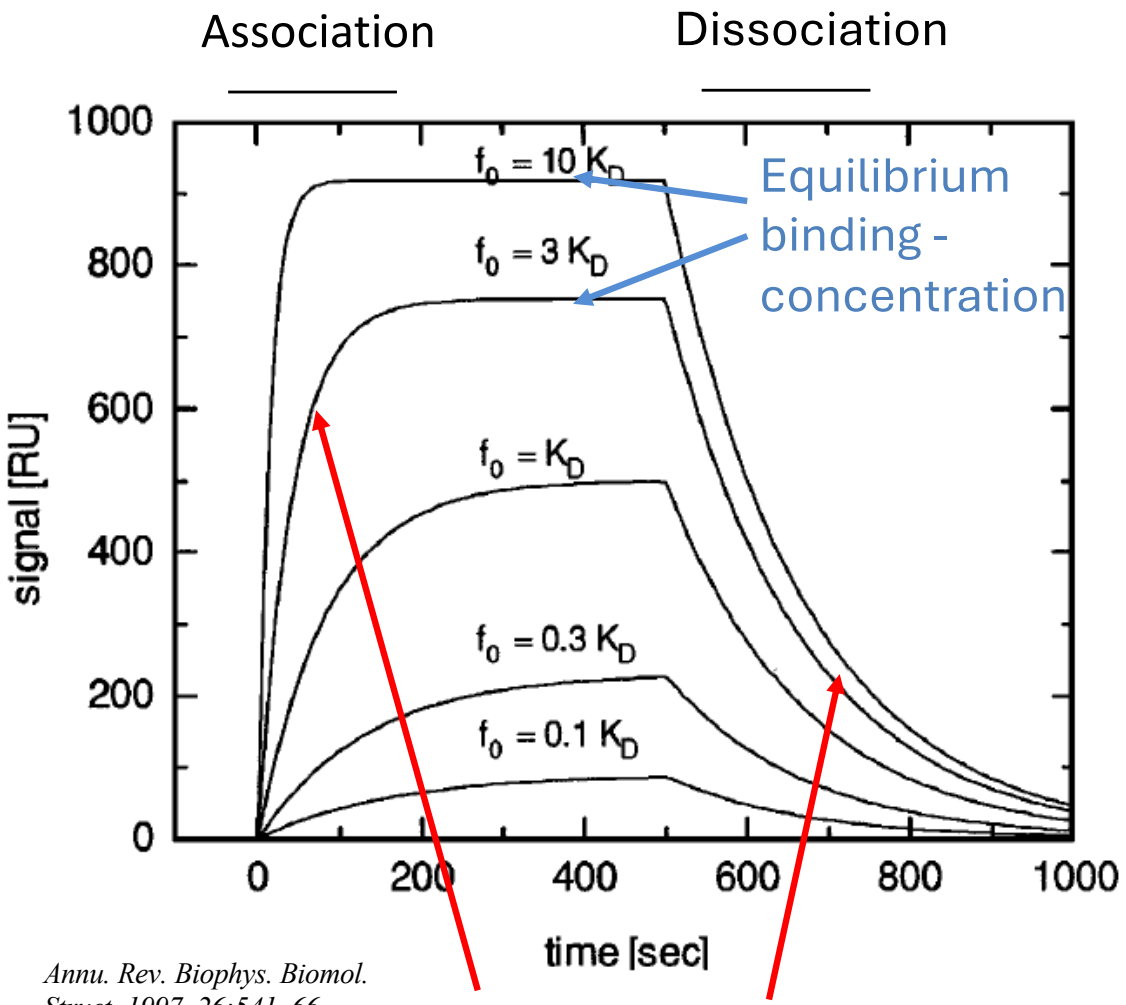




Sensorgram


Report point
Report point
Relative response (RU)
Absolute response (RU)
Running buffer
Analyte
Running buffer
Regeneration solution
Running buffer
Time (s)

The sensorgram



Annu. Rev. Biophys. Biomol. Struct. 1997. 26:541–66

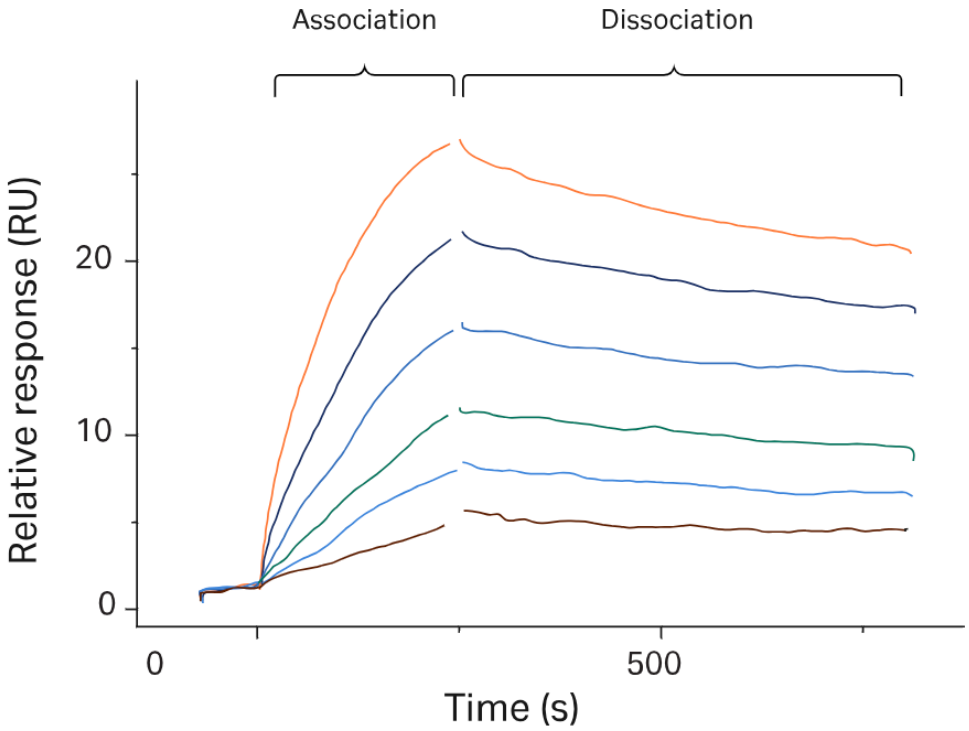
RU: response units
= change in intensity of the reflected light

1 RU = 1pg protein mm^{-2}

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} LR$$

$$K_D = \frac{k_{on}}{k_{off}} = \frac{[LR]}{[L][R]}$$

What is kinetics?



$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

Kinetics is a measure of association and dissociation of a complex within a given time span. It tells you how fast the interactions occur.

Association (k_a) refers to how fast molecules bind and form a complex. (on-rate, units: in M^{-1} s^{-1})

Dissociation (k_d) refers to how fast a complex falls apart. (off-rate, units: s^{-1})

Kinetics are determined from the change in response as a function of time, as represented in this sensorgram.

From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course

What is affinity?

Affinity is quantified using the equilibrium dissociation constant (K_D) is the ratio of the on-rate and off-rate constants ($\frac{k_{on}}{k_{off}}$). It describes how much complex is formed at equilibrium and tells you the strength of a complex. (Units: M)

Lower K_D = higher affinity:
A small K_D means high affinity (tight binding).
A higher K_D means lower affinity (weak binding).

K_D value (typical)	Affinity description
1 pM – 1 nM	Very high affinity
1 nM – 1 µM	Moderate affinity
1 µM – 1 mM	Lower affinity (weak)
> 1 µM	Weak affinity

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} LR$$

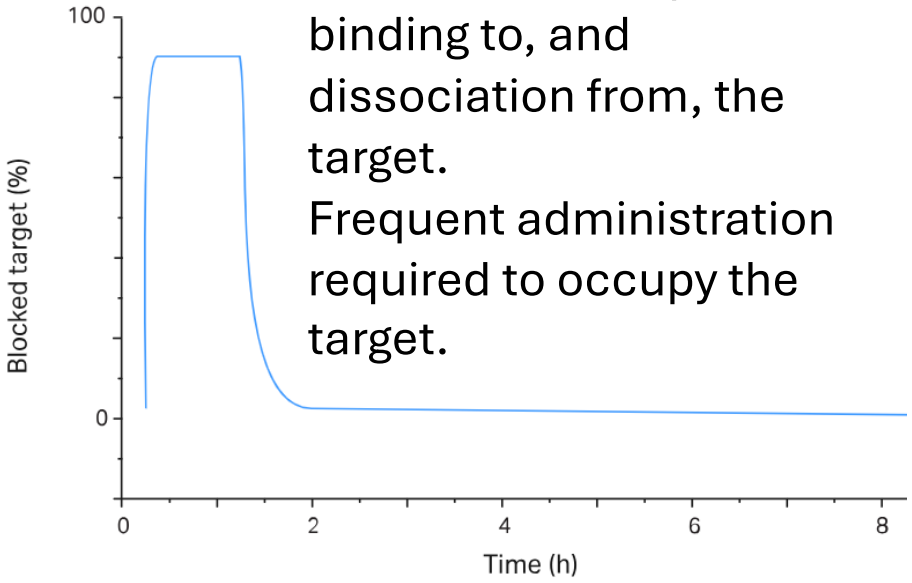
$$K_D = \frac{k_{on}}{k_{off}} = \frac{[LR]}{[L][R]}$$

From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course

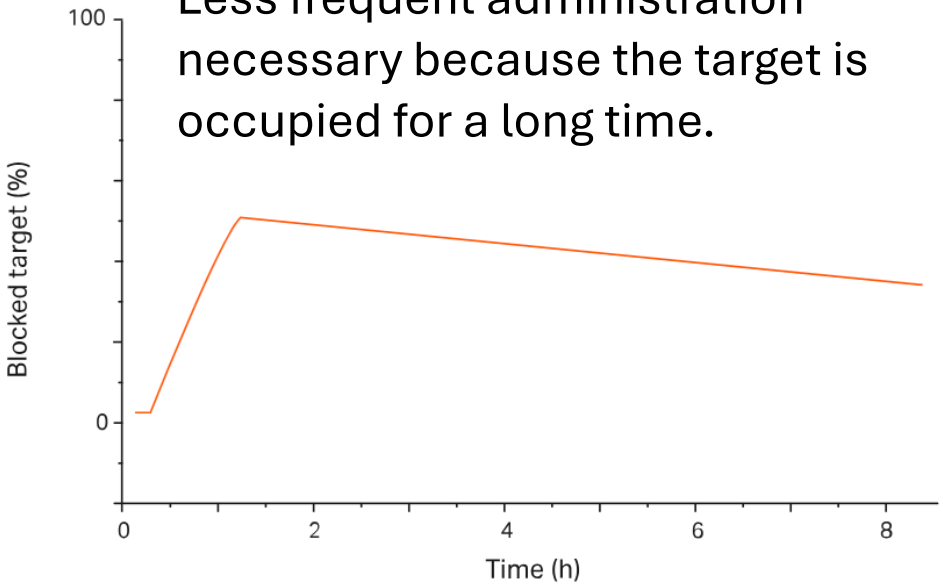
Why study kinetics and not just affinity?

We study kinetics because it gives us more information than affinity alone. Interactions with the same affinity can have different kinetics, which could have an impact on biological significance and drug function, such as pharmacokinetics and dosing.

Fast kinetics: rapid binding to, and dissociation from, the target.
Frequent administration required to occupy the target.



Slow kinetics: slow binding to, and dissociation from, the target.
Less frequent administration necessary because the target is occupied for a long time.



From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course≈

Mass transport considerations

- Mass transport is needed to supply analyte to surface during sample injection and to remove analyte during dissociation.
- Observed rate of analyte binding is the net result of mass transport and interaction rate.

- Three scenarios:
  1. **Fast mass transport, slow interaction rates**: observed binding limited by interaction rate. Rate constants relevant for interaction.
  2. **Slow mass transport, fast interaction rates**: observed binding limited by mass transport. Rate constants will reflect mass transport processes, not relevant to interaction. This should be avoided.
  3. **Mass transport and interaction rates similar**: observed binding represents both processes. Evaluation possible if interaction model takes mass transport processes into account.

From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course≈

Minimizing the effect of mass transport limitation

- **High flow rate for analyses, low ligand level:**

Ensures that absolute rate of analyte binding is low, less likely to be limited by mass transport.

- **Check:**

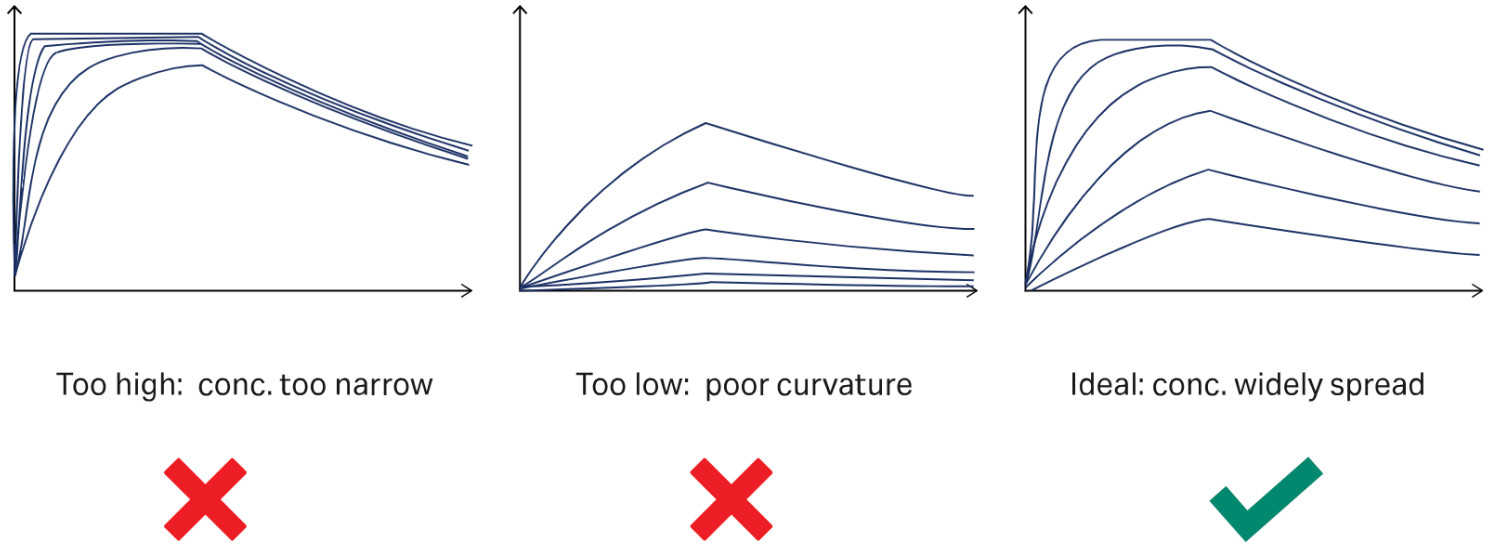
Run assay at multiple flow rates and check how this affects the obtained rate constants.

1. Kinetic constants remain unaffected by flow changes: interaction probably not mass transport-limited
2. Faster kinetics are obtained at higher flow rate: interaction is limited with respect to mass transport.

From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course≈

Analyte concentration

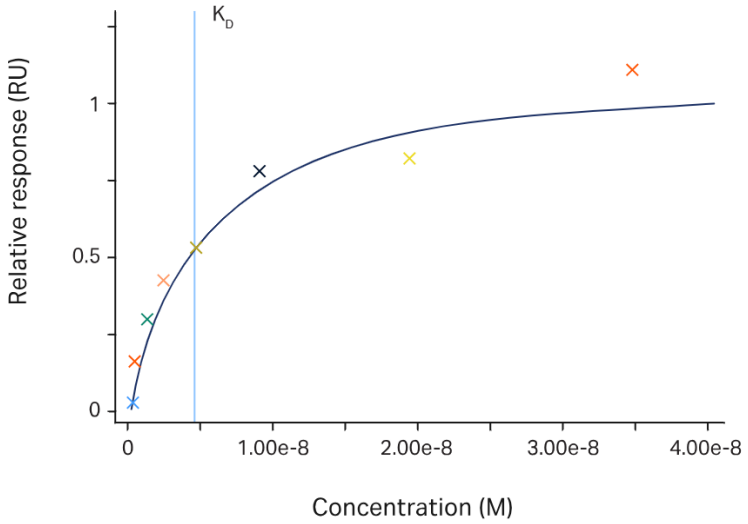
- Knowing your analyte concentration is key because it directly affects kinetics.
- Analyte concentration **between 0.1 and 10 times the expected K_D**.
- Highest concentration should reach steady state, if possible.
- Unknown K_D: pilot experiments with wide range: 2-1500 nM, adjust and narrow if necessary.

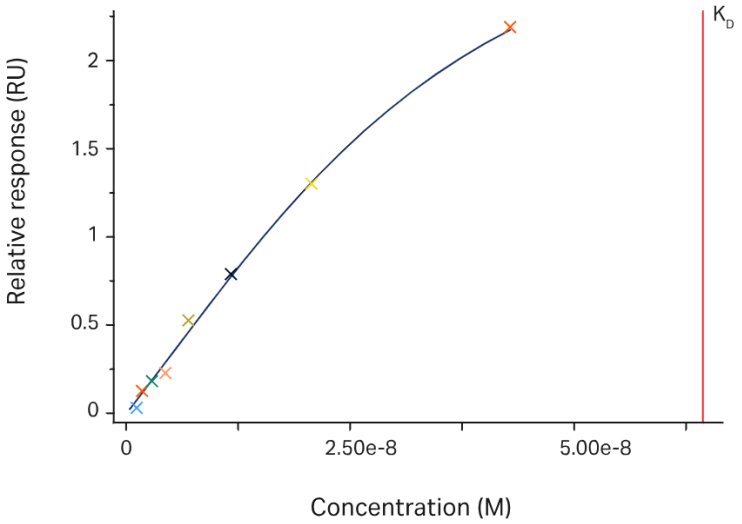


From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course≈

Analyte concentration for affinity

- On the right: concentrations are too low, K_D is outside concentration range.



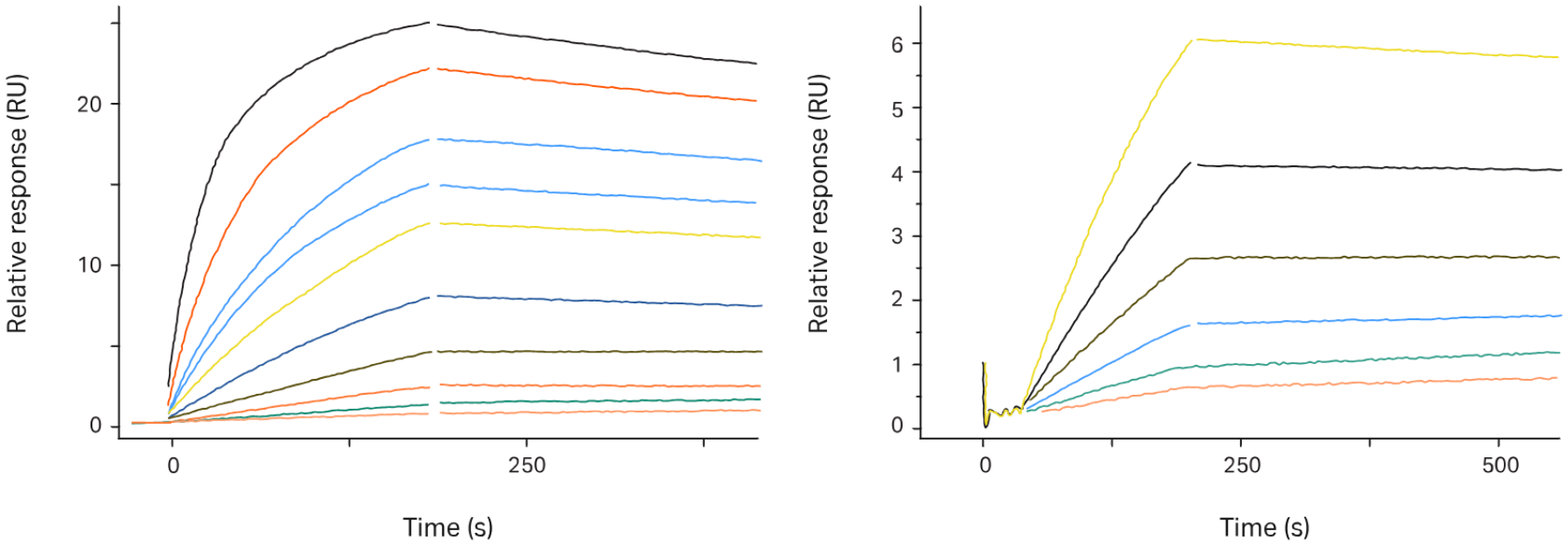


From: Cytiva, *Get to know SPR kinetics and affinity measurements*, Online course≈

Analyte association time for kinetics

- Association time should be long enough to allow for sufficient curvature of the sensorgrams for at least some of the injections.

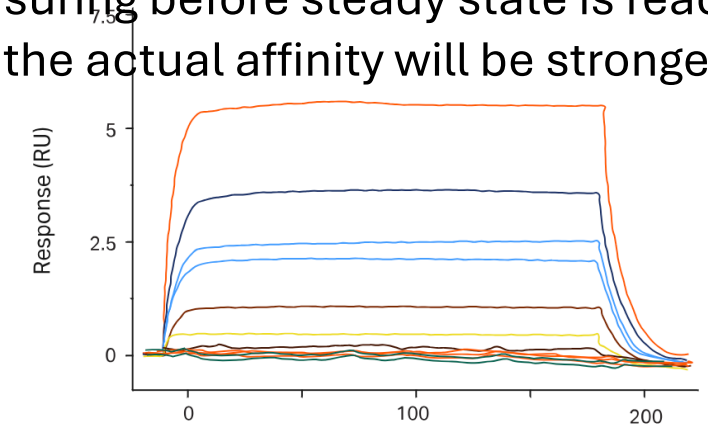
Examples of both good (left) and insufficient (right) curvature:



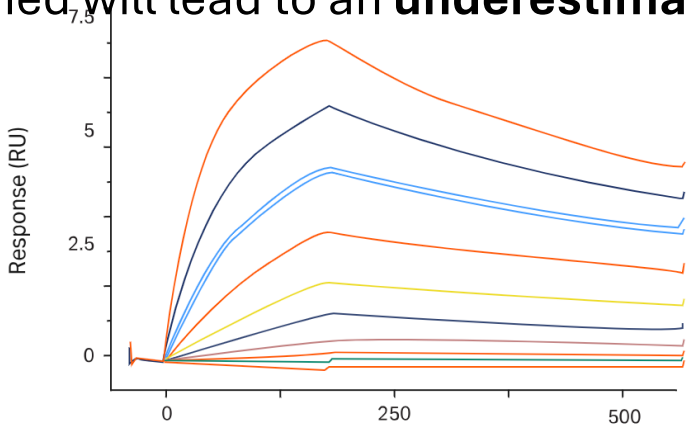
Analyte association time for affinity

Association time for **affinity** assay should be **long enough to allow the injections to approach steady state.**

- Time to reach steady state is related to **analyte concentration and dissociation rate**.
- If some concentrations but not all reach steady state, the value will be close but not exact.
- If none of the concentrations reaches steady state, **the value does not represent K_D.**
- Measuring before steady state is reached will lead to an **underestimation of K_D** and the actual affinity will be stronger.



Steady state



No steady state

Activity: SPR quiz

Question 1:

You want to assess the affinity of a protein-protein interaction using steady-state affinity analysis. The estimated affinity of the interaction is 20 µM. What would be a suitable concentration range for your analyte?

A. 2-200 µM in two-fold dilution steps (eight concentrations)
B. 15-25 µM (three concentrations)
C. 1-20 µM in two-fold dilution steps (six concentrations)

Activity: SPR quiz

Question 2:

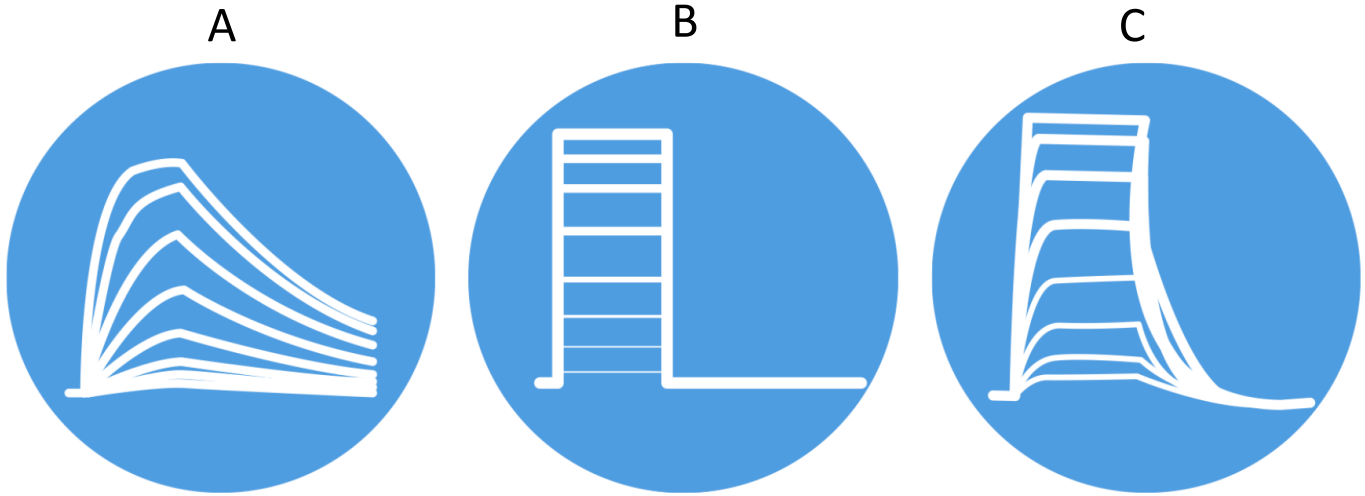
You are setting up a kinetics experiment. What do you need to take into account for highest data quality?

A. Ligand level is not critical if you use a high flow rate.
B. Run the experiment with high ligand levels and at a low flow rate.
C. Run the experiment with low ligand levels and at a high flow rate.

Activity: SPR quiz

Question 3:

Determination of binding kinetics and steady state affinity place different demands on experimental data. It is usually not possible to meet both these requirements in the same experiment. However, on some occasions it is possible. Below, you see three sets of binding curves. From which curve set is it possible to extract both kinetics and steady state affinity?



More resources

- https://www.youtube.com/watch?v=OE-NxmsULps
- https://www.sprpages.nl/sensorgrams/quiz
- https://www.youtube.com/watch?v=BZFFPB5zGxg&ab_channel=Carterra
- https://portlandpress.com/biochemist/article/45/1/18/232564/A-beginner-s-guide-to-surface-plasmon-resonance

Applications of BIAcore biosensor

- **Widely used** to characterize binding reactions of biomolecules (ligand-receptor, protein-protein, DNA etc.)
- Quantitative and label-free sensing Determination of kinetics* and equilibria
- Inhibitor screening
- Biomolecule self-assembly on a surface

* Can be difficult to interpret due to diffusion, multivalency, avidity

You are stuck.

1. In your research on membrane protein dynamics, you have observed through standard fluorescence microscopy that a certain transmembrane receptor undergoes clustering upon ligand binding. However, due to the high background signal from the cell interior, the precise dynamics at the membrane are unclear.
2. Your project involves understanding how mechanical forces influence the folding and stability of a certain enzyme. Biochemical assays have shown changes in enzyme activity under different physical conditions, but you need direct measurements of the forces acting on the enzyme.
3. In your investigation of a new therapeutic antibody's binding affinity to its target receptor, traditional binding assays have provided initial qualitative results. However, you need a more thorough understanding of the binding kinetics and affinity to guide the optimization of the antibody for therapeutic use.
4. In studying intracellular signaling pathways, you've used fluorescence-tagged proteins to observe that two signaling molecules co-localize in the same cellular compartment upon stimulation. You need to determine whether these molecules directly interact – a crucial piece of your signaling model.
5. Your work on neuronal development has revealed interesting patterns of protein distribution in dendrites using wide-field fluorescence microscopy. However, the intricate three-dimensional structure of dendrites is lost in these images.
6. In your research on membrane protein dynamics, you have observed through standard fluorescence microscopy that a certain transmembrane receptor undergoes clustering upon ligand binding. However, due to the high background signal from the cell interior, the precise dynamics at the membrane are unclear.
7. Your project involves understanding how mechanical forces influence the folding and stability of a certain enzyme. Biochemical assays have shown changes in enzyme activity under different physical conditions, but you need direct measurements of the forces acting on the enzyme.
8. In your diagnostic research for a specific viral infection, you've identified a unique viral protein present in saliva during early infection stages. You need to develop a rapid and user-friendly diagnostic tool.

What method do you choose to approach these research questions?

Connections

- https://connections.swellgarfo.com/game/-NtNaldpLBmWXRgPx1zO

A note about connections: Refractive index changes

SPR and Refractive Index Changes:

- In SPR, a change in the refractive index at the surface of a sensor chip (typically a thin metal film) is crucial. SPR measures changes in the refractive index near the sensor surface that occur due to molecular interactions (such as the binding of a biomolecule to the sensor surface). These changes alter the resonance condition of surface plasmons, which is detected as a change in the intensity or angle of reflected light.

TIRF and Refractive Index Changes:

- TIRF, on the other hand, doesn't rely on changes in the refractive index for its primary function. Instead, it utilizes the phenomenon of total internal reflection to generate an evanescent wave at the interface between two media (like a glass slide and a sample). The refractive index difference between these two media is important to achieve total internal reflection, but it’s the evanescent wave, rather than refractive index changes per se, that is key in TIRF. This wave penetrates only a short distance into the sample and is used to excite fluorescent molecules near the surface.