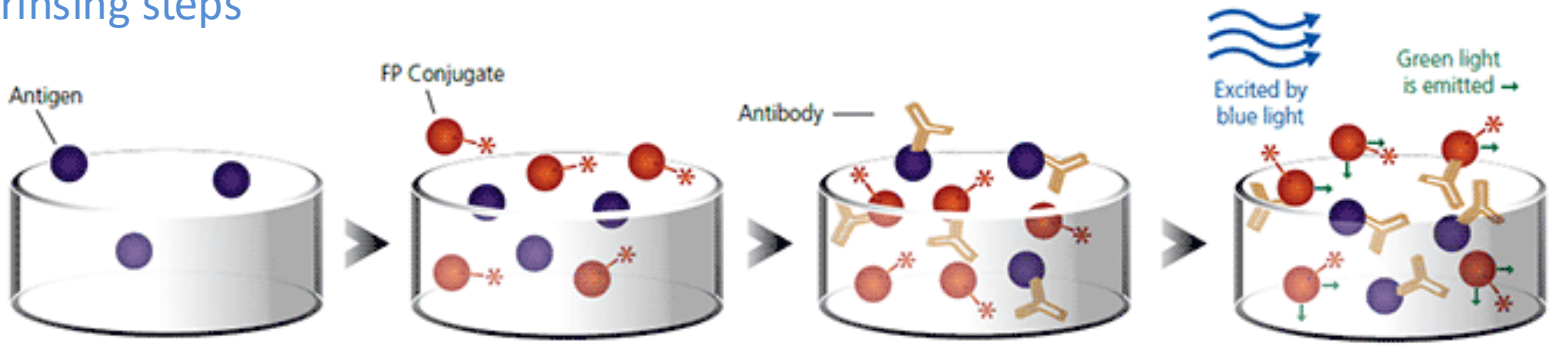
(4) ASSAY CONFIGURATIONS, MATRIX-DEPENDENT PERFORMANCE

Outline

- Classifications and configurations of molecular assays
  - Homogenous and heterogeneous
  - Direct, sandwich, competition, inhibition assays
- Main issues encountered in surface sensing

Homogeneous and heterogeneous assays

Homogeneous assays. Binding occurs in solution,
Systems with typically low throughput (e.g. FPIA: Fluorescence Polarization Immunoassay).
No rinsing steps



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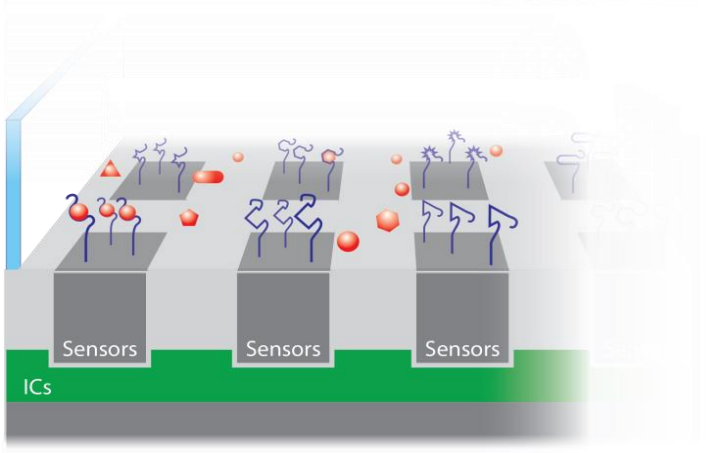
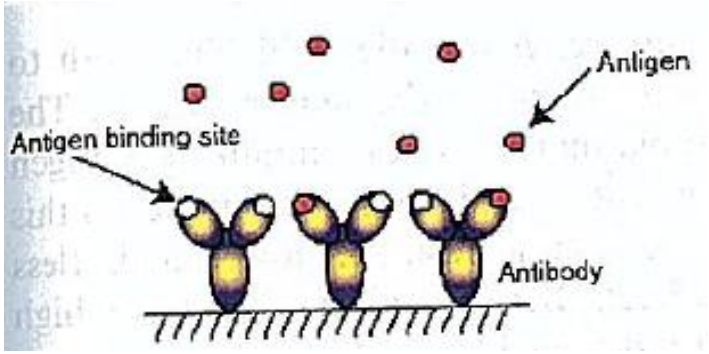
Heterogeneous assays. Binding occurs on the sensor surface.
Typically high throughput (e.g.: microarrays).
Rinsing steps required (ELISA)



Direct assay

Antigen = target
Antibody = probe/ligand



-Can be used when the signal provided directly by the target is large enough or when the target can be combined with a label.

- Essential for real-time observation of binding

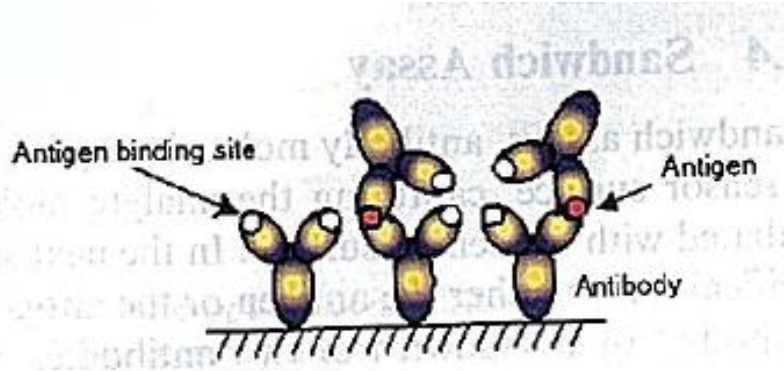
- Sensor surface preparation: Probes are immobilized on the sensor surface.
- Detection:

Sample solution containing the target is incubated with the sensors surface. Target might be conjugated with a label.

- Signal-measurand relationship:

The signal-increase correlates with the amount of target in the sample. Readout is *"SIGNAL-ON".*

Sandwich Assay



To be selected for relatively high molecular weight antigens and when high affinity antibodies are available.

-Selected when the direct detection cannot be applied. In this case, the secondary probe provides signal or carries a label.

- Can be used when the target can bind two antigens at the same time.

- More reagent-demanding

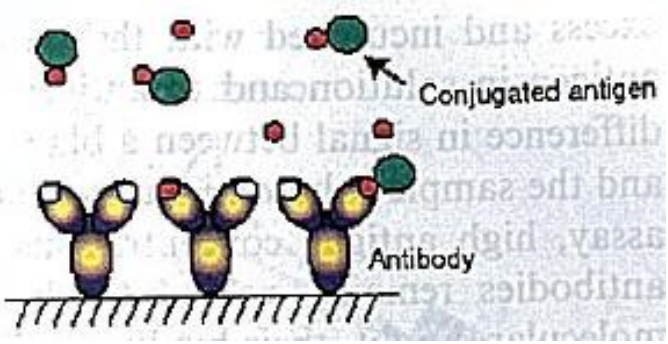
- Sensor surface preparation: Probes are immobilized on the surface
- Detection:

Sample solution containing the target is incubated with the sensors surface. In a second step, a secondary probe binds specifically with the target that has been previously captured on the surface.

- Signal-measurand relationship:

The increase in signal is proportional to the amount of target in the sample. The high molecular weight of the secondary probe can provide a signal sufficient to monitor the detect the event. Conjugated probes can be used as well for signal amplification. Readout is "*SIGNAL-ON*".

Competition Assay



Employed when neither direct or sandwich assay can be applied, in particular in case of small molecules.

Uses only one antibody.

- Sensor surface preparation: Probes are immobilized on the surface
- Detection:

Sample solution that contains the target of unknown concentration is mixed with a known concentration of conjugated targets (Mixture S).

- Signal-measurand relationship:

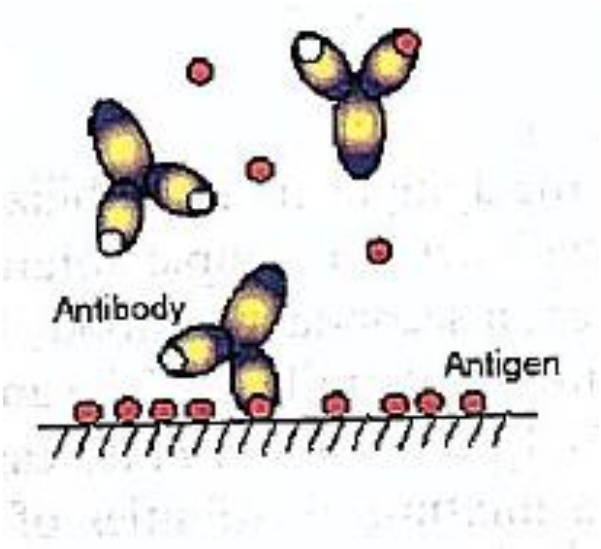
The **output** is the the difference in the measured binding events on the surface between:

- a reference sample containing only conjugated-target

- and the *Mixture S.*

High target concentration in the original sample result in low **output**. Readout is *"SIGNAL-OFF".*

Inhibition Assay (or "antigen-down")



Employed when neither direct or sandwich assay can be applied and conjugation of the target molecule with a label is difficult or impossible.

Uses only one antibody.

- Sensor surface preparation: A known density of antigens is previously immobilized on the sensor surface (this is not the sample antigen).
- Detection:

Sample solution that contains the target is mixed with specific probes in excess (*Mixture SH)*. Probes bind both to the target in solution and to the target bound previously on the sensor surface.

- Signal-measurand relationship:

The output is the the difference in the measured binding events on the surface between:

- a reference sample containing only the probes

- and the *Mixture SH.* High target concentration result in low signals. Probes have high molecular weight and can be directly detected. Readout is "*SIGNAL-OFF*".

Outline

- Classifications and configurations of molecular assays
  - Homogenous and heterogeneous
  - Direct, sandwich, competition, inhibition assays
- Main issues encountered in surface sensing
  - Availability of reagents
  - Mass-transport phenomena
  - Interference/Sensing in matrices
  - Design, stability, accessibility of the ligand. Regeneration/Reuse of the surface

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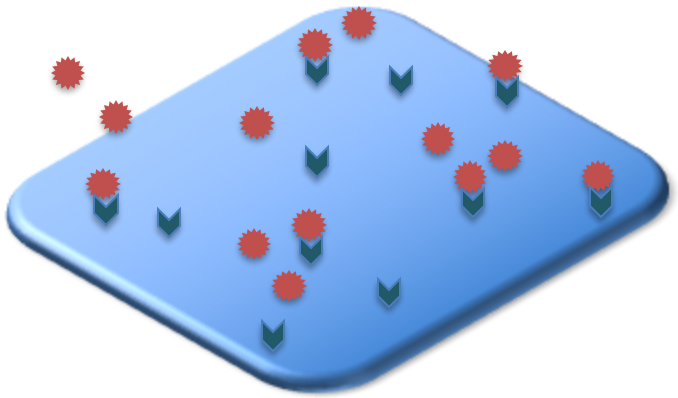
Molecular quantification (determination of the Concentration of analytes)

Upper and lower limit of measurable concentrations (dynamic range).

Upper limit: conc. of the targets should be lower than the one leading to saturation coverage (thus, we should remain in a regime of approx linear dependence of concentration and density at equilibrium). I.e., we want to avoid "excess reagent" conditions.

Lower limit: The lower limit on concentration is not only determined by the concentration that translates in the smallest density of targets on surface that the sensor can detect (e.g.: limit of detection), but also to another phenomenon: the condition of "Limited reagent". In fact, in a limited volume, the amount of molecules available in solution might be insufficient to keep the volumetric concentration constant upon binding (amount of available molecules is too small vs the number of molecules that will bind at equilibrium).

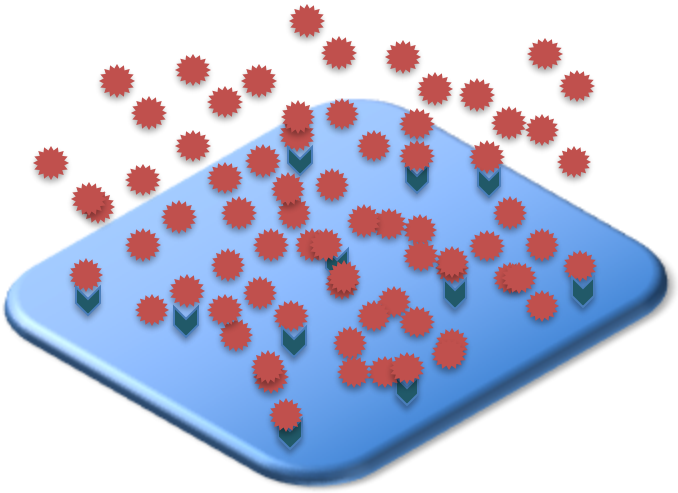


Limited reagent. Langmuir equation is no longer valid: C decreases due to the molecules adsorbed on the surface.

Excess reagent conditions

However, excess reagent conditions works well for **molecular detection** (not for quantification). When the aim of the sensor is simply to detect the presence of a molecule, without quantifying the concentration, the only condition on the concentration is that it should be large enough to provide a signal transduced from the molecules captured on the surface .

Excess of target molecules (concentration leading to the saturation coverage)

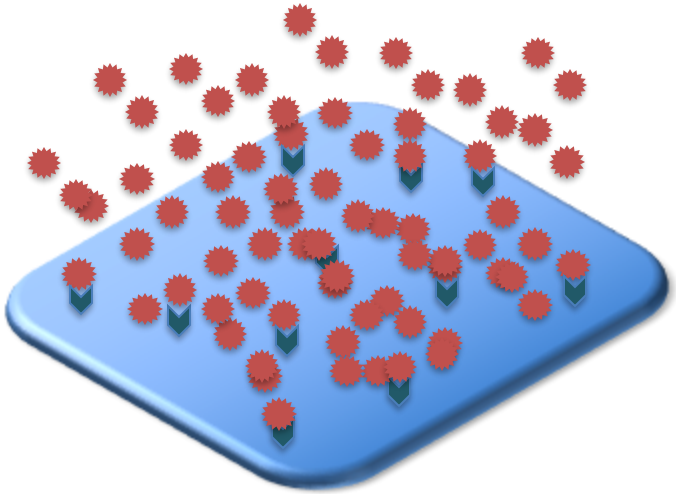


Excess reagent (*target molecules*)

Target molecules (reagent) availability changes with the surface size (1/2).

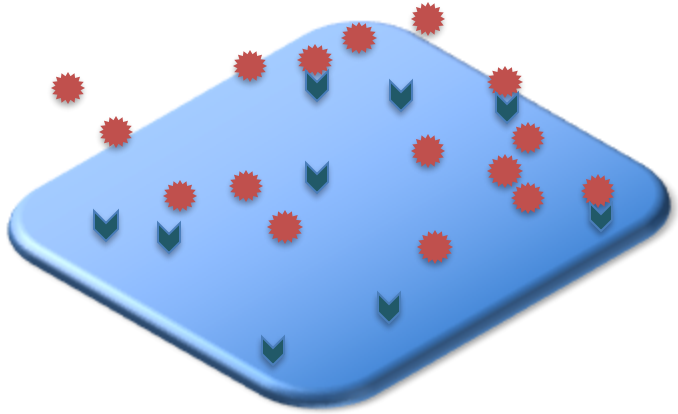
For a given surface size, the availability depends on the concentration

Excess reagent



High concentrations

Target limited



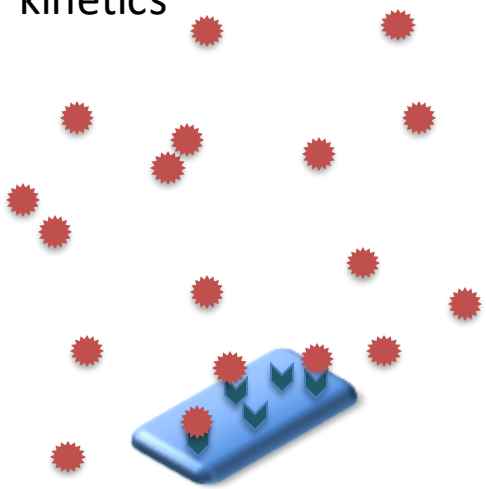
Low concentrations (and low overall number of analytes available in the sample volume)

Target molecules (reagent) availability conditions depends on the surface size (2/2).

Nevertheless, for a given concentration, availability depends on surface size.

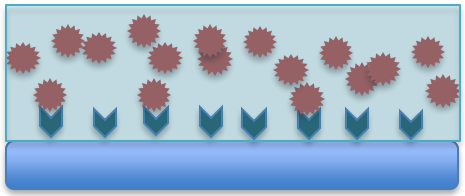
Non Reagent-limited

Even in case of small concentration!
In fact, with small spots in large volumes, C is kept constant.
The equilibrium can be reached according to the Langmuir kinetics



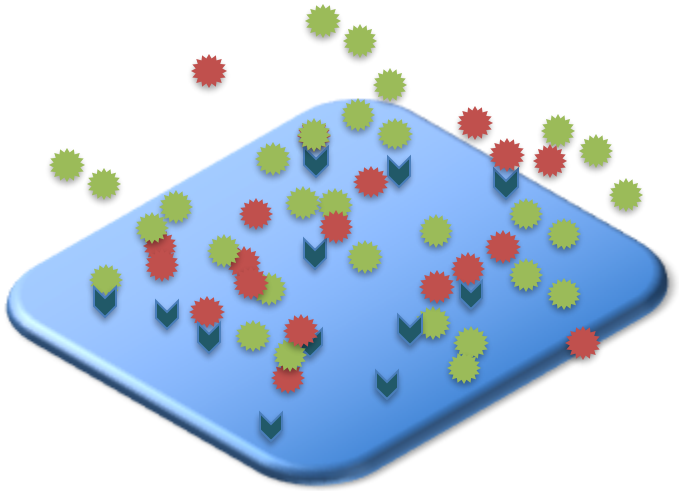
Reagent limited

Even in case of relatively large concentrations!
In fact, when large sensing surfaces are incubated in small volume chambers, the concentration can be easily depauperated. eg: microarrays in hybridization chambers.



Molecular quantification (determination of the Concentration). Other limitations.

Finally, an excess of non-specific molecules can interfere with the surface binding. In complex matrixes, non-specific molecules might be several orders of magnitude more concentrated than the target molecules.



Interference-limited. Excess of non specific molecules. Non specific bindings interfere with specific bindings

- Main issues encountered in surface sensing
  - Availability of reagents
  - Mass-transport phenomena
  - Interference/Sensing in matrices
  - Design, stability, accessibility of the ligand. Regeneration/Reuse of the surface

Mass-transport limited binding

Surface sensing: Binding occurs between the analyte in bulk solution and the receptor on the sensor surface.

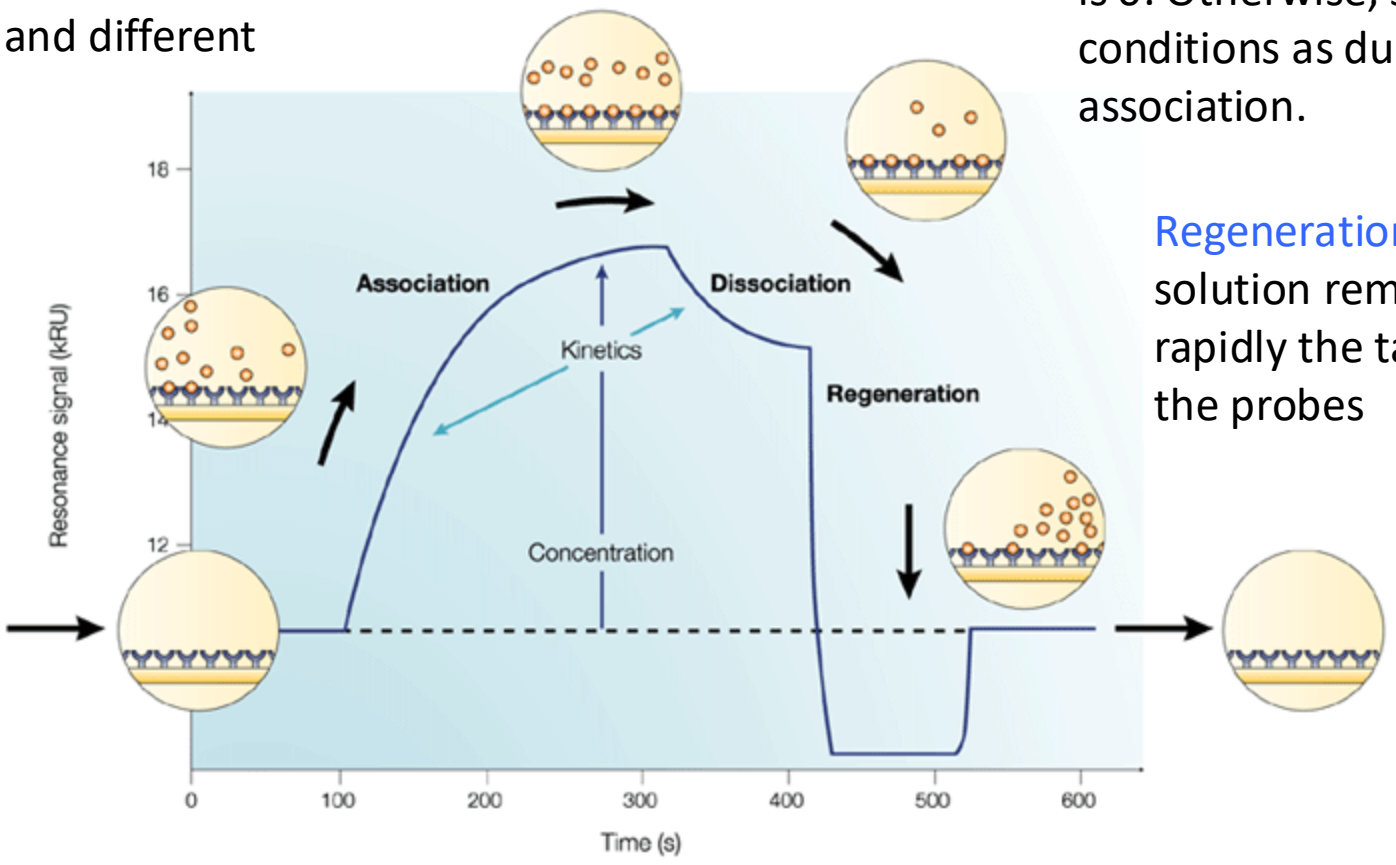
- Mass-transport limited binding occurs when the association of the analyte to the receptor is faster than the diffusion of the analyte.
- The phenomenon results in increased re-binding of the analyte in the dissociation phase, as the released analyte can re-bind to free receptor before it diffuses into the bulk solution. The effect is most pronounced with very large analytes (which have low diffusion rates), and with analytes that have very fast association rates (comparable to the diffusion rate). Dissociation rate is evaluated with a lower value than it has in reality.
- To minimize the effect, very low density of receptors are immobilized and high flow rates are used, the latter have the effect of reducing the depth of the surface-associated 'depletion region' (unstirred layer).

Binding and dissociation kinetics on surface.

Association: Concentration of targets molecules in bulk is constant and different from 0

Dissociation: Concentration of targets molecules in bulk is 0. Otherwise, same conditions as during association.

Regeneration: Harsh solution removes rapidly the targets from the probes



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- Main issues encountered in surface sensing
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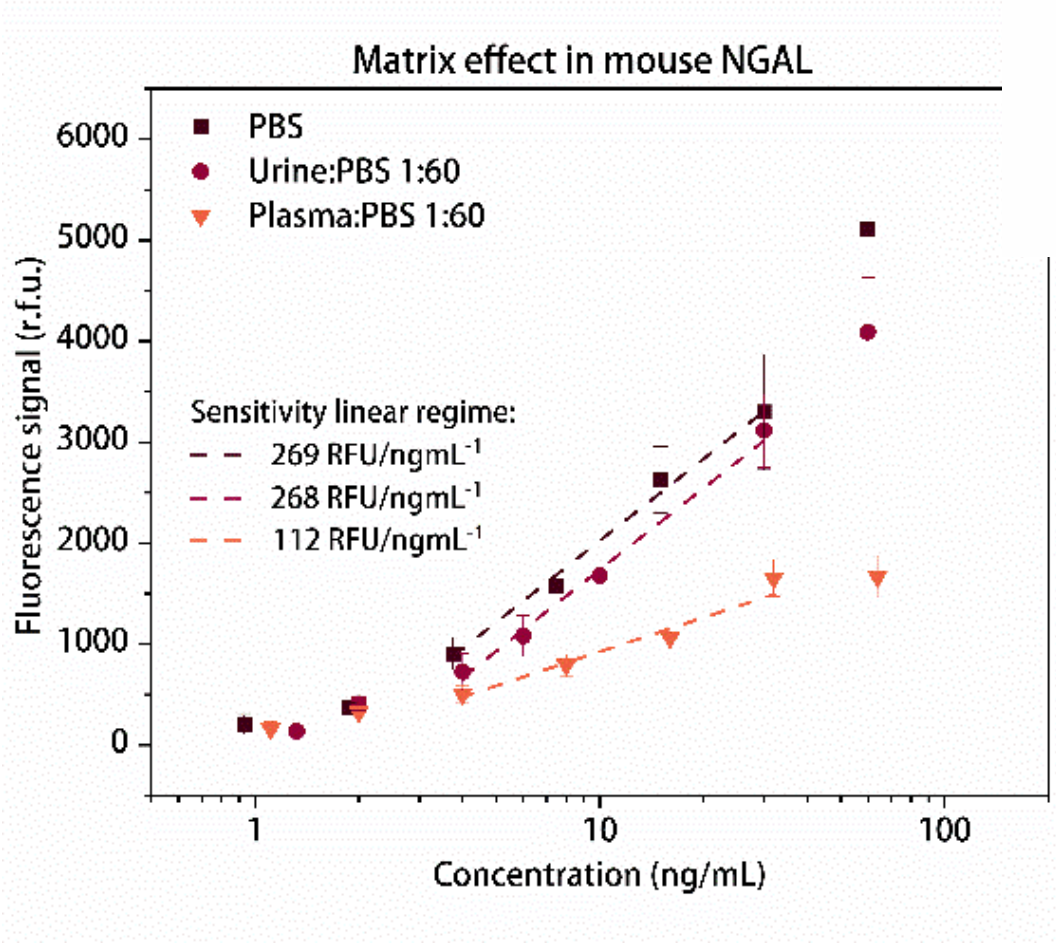
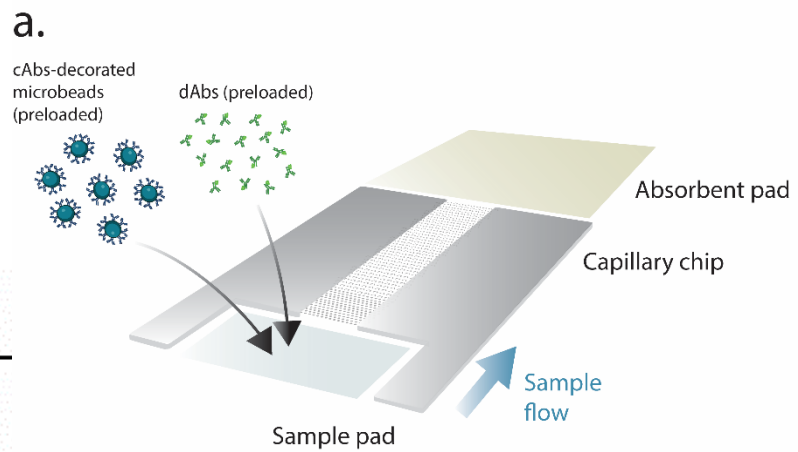
Matrix effect

- The presence of a complex matrix vs. a simple buffer influences the binding of the analyte molecules on the surface and the output signal overall.
- Translational effects do not depend on the concentration of the analyte or on the binding taking place, while rotational effects do.
- Translational effects can be subtracted with a negative control (reference sensor or reference surface).
- Rotational effects depend on the concentration of the analyte molecules.

Rotational matrix effect



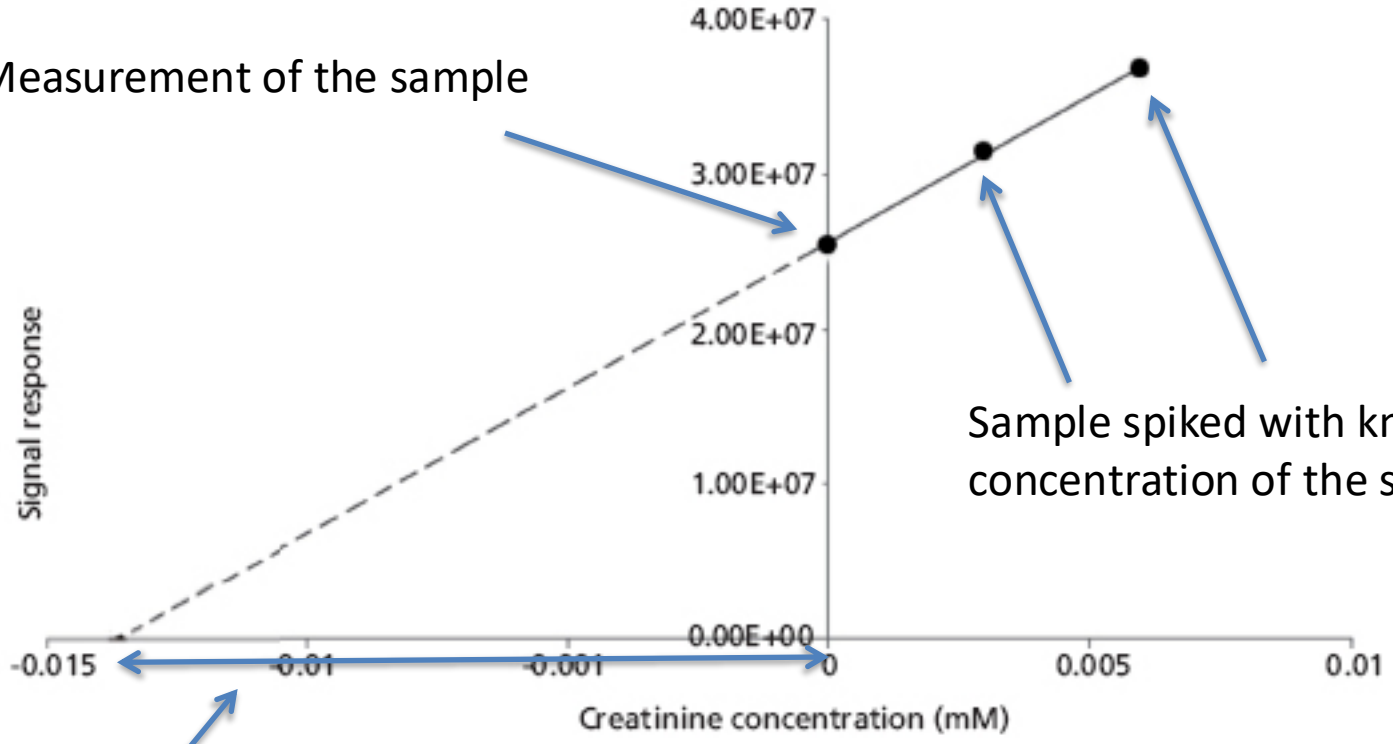


credits: Gloria Porro, Guiducci Lab

Overcome rotational matrix effects

Standard additions method

Measurement of the sample



Sample spiked with known concentration of the same analyte

Reveals the concentration of the unknown sample (absolute value)

→ *Standard additions: myth and reality*
Stephen L. R. Ellison and Michael Thompson, Analyst, 2008, 133, 992–997

Main issues in surface-based assays

Issues and artifacts

Related to phenomena involved in binding of probe-target (including affinity and diffusion)?

Bulk effects and time-dependent artifacts such as drift (translational effects).	No
Matrix effects: translational	No
Matrix effects: rotational	Yes
Various artifacts: ▪ Issues related to the interaction of labels with the surface. ▪ Steric hindrance and repulsive forces	Yes
Mass-transport limited binding (to be discussed in details when considering systems in flow)	Yes

- Main issues encountered in surface sensing
  - Availability of reagents
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Sensing surface

- Surface properties are imparted by
  - Physical attributes (roughness, elastic modulus)
  - Presence and arrangement of "biologically active" molecules which are meant to
    - provide specific interactions
    - suppress unwanted non specific interactions

Sensing surface (cont.)

- Interaction of analytes with ligands on the surface depends on
  - Orientation of the receptor
  - Its separation from the surface
  - Its surroundings on a molecular length scale and on the homogeneity on various length scales
  - Spatial organization in 2D and 3D

Nature of surfaces and substrates

Substrates:

- Oxides, metals, polymers
- Flat substrates or complex 3D structures

The ligand (probe) can undergo:

- Direct binding or adsorption on surfaces
- Mediated attachment (mediating or precursor layer).
  A precursor layer serves in two ways:
  - Barrier function: passivates the underlying substrate
  - Active function: provides attachment sites for further chemical attachment and bioconjugation

Mediating or precursor layer

- Modification of metal and oxide surfaces: Self Assembled Monolayers assembled by solution or from gas phase
- Thin-polymeric films deposited by spin or dip coating or by plasma polymerization

SAM

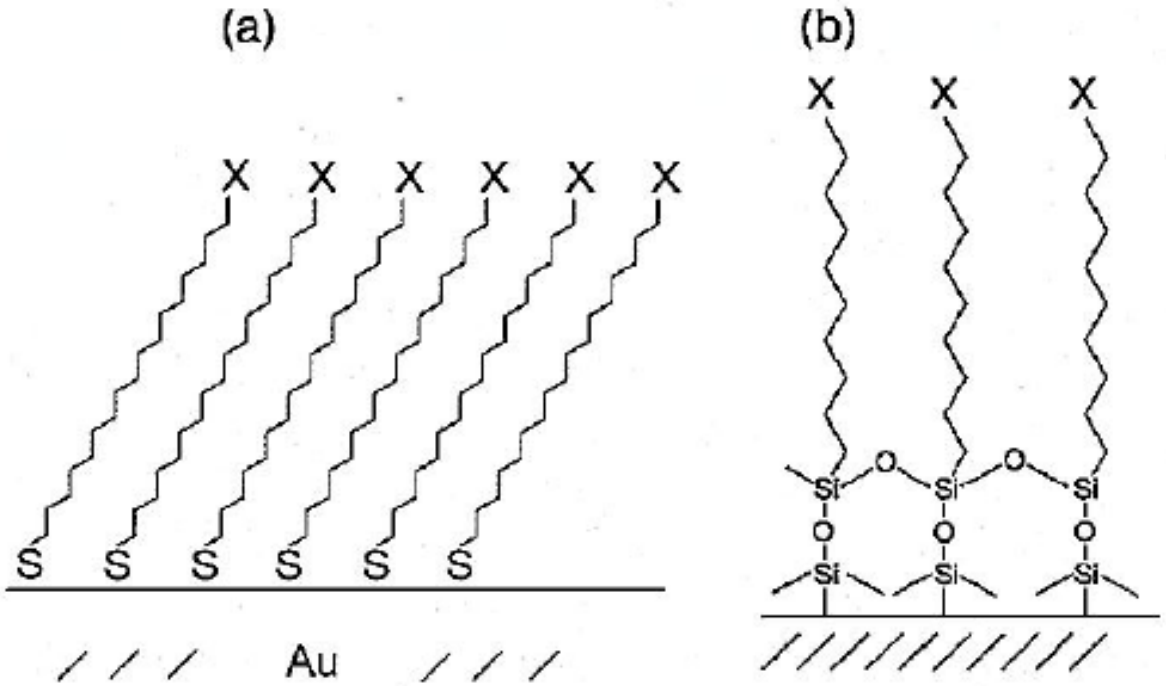
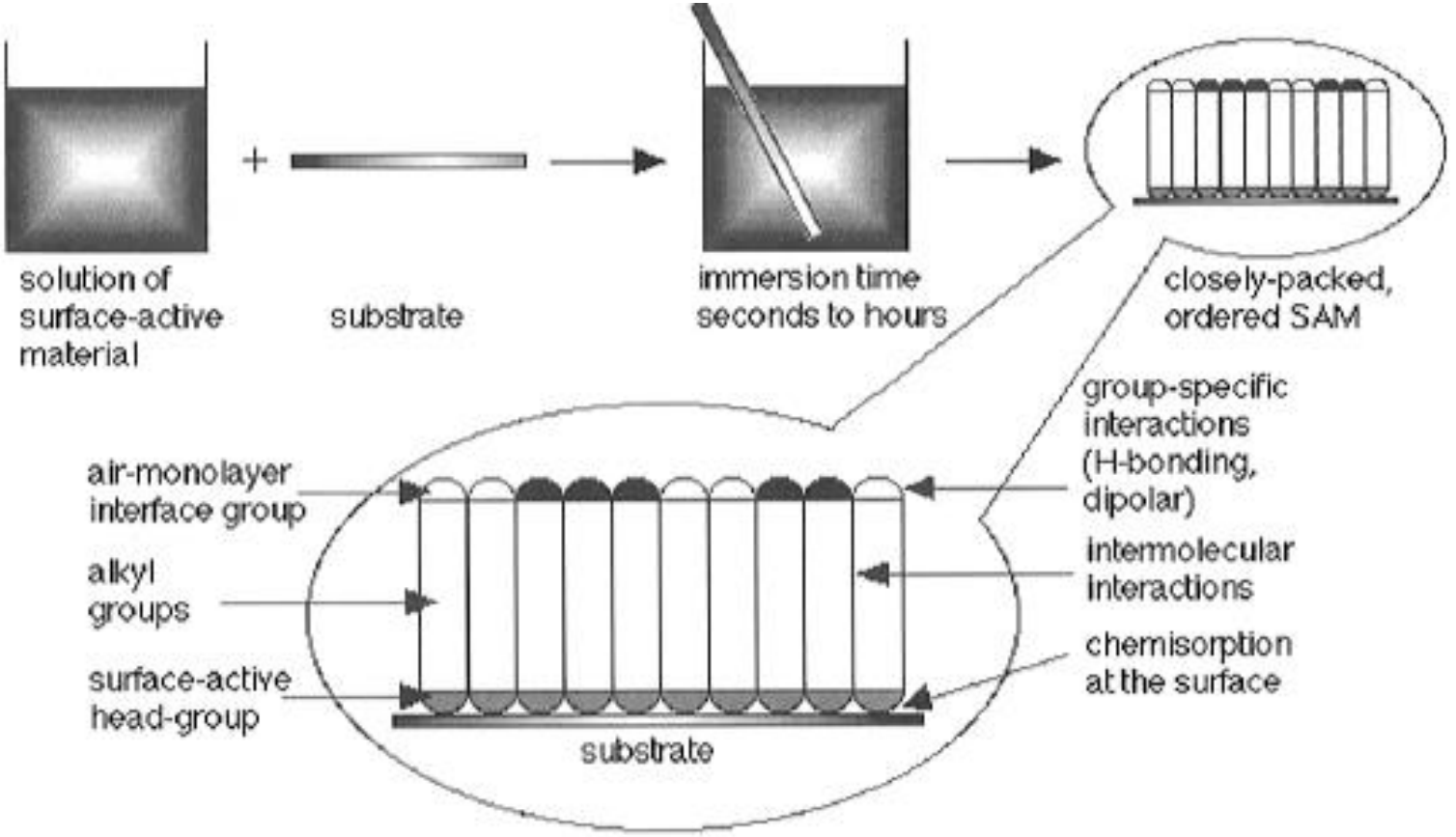


Figure 2 (a) Schematic structure of self-assembled monolayer of end-functionalized alkane thiols and disulfides on gold. The range of functional groups X that can be introduced is almost unlimited due to the rare crossreactivity of the functional groups typically employed and the thiol or disulfide chain end [9, 11]. (b) Schematic of silane-based SAM. SAMs on gold or silicon may suffer from degradation under oxidative conditions (ozone or oxygen and UV light) and condition of extreme pH, respectively. In addition, SAMs may possess intrinsic molecular scale defects, such as pinholes.

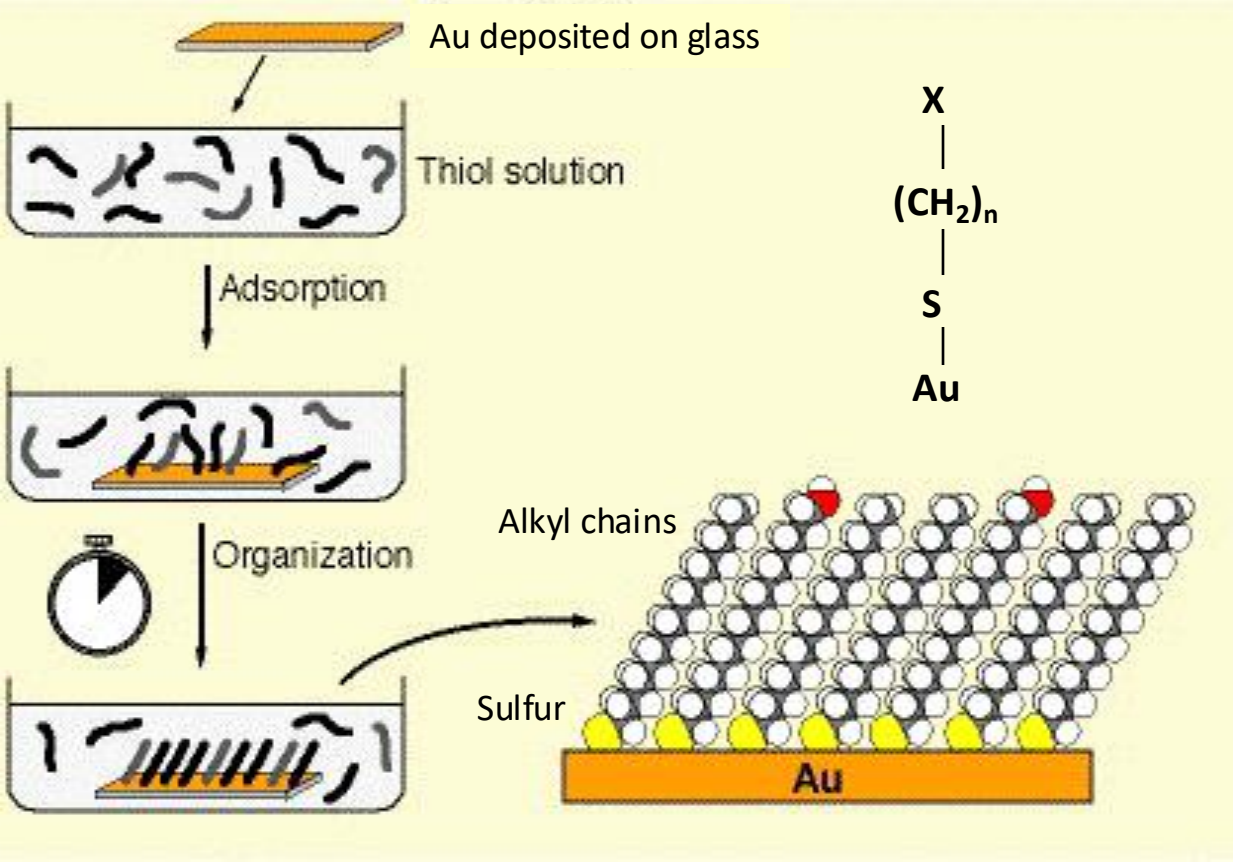
SAMs formation

Self-assembled monolayers are formed by simply immersing a substrate into a solution of the surface-active material. The driving force for the spontaneous formation of the 2D assembly includes chemical bond formation of molecules with the surface and intermolecular interactions.



SAM on gold

Self-assembly of thiols on gold films



X = $-CH_3$, $-OH$, $-COOH$, $-SO_3^-$, $-PO_4H_2$, $N^+(CH_3)_3$, $-(OCH_2CH_2)_nOH$, ...

SAMs on gold

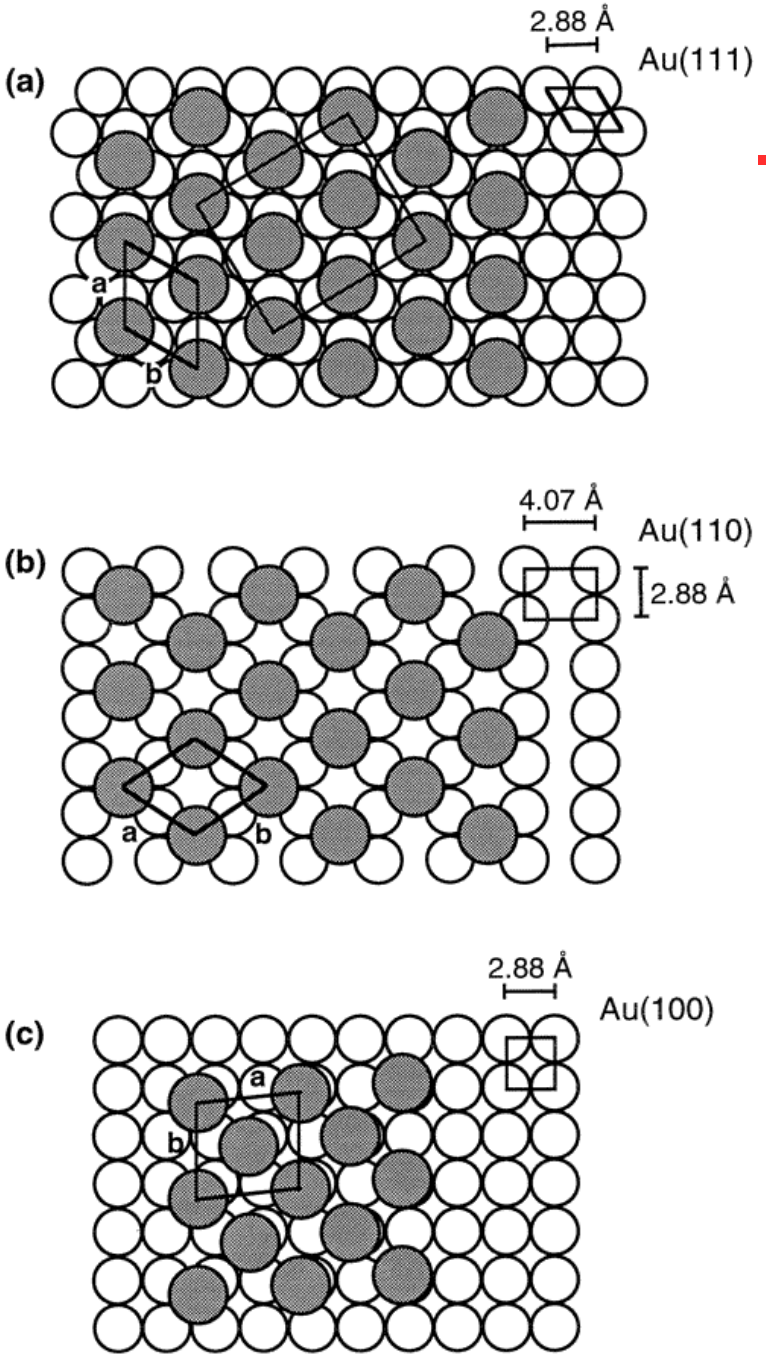
Alkanethiols on single crystals of gold. Open circles represent gold atoms, and shaded circles represent alkyl chains.

(a) Au(111). The smaller rhombus shows the Au(111) lattice. The larger rhombus shows a unit mesh with $a = b = 4.97$ Å and $\alpha = 120$ (angle between a and b).

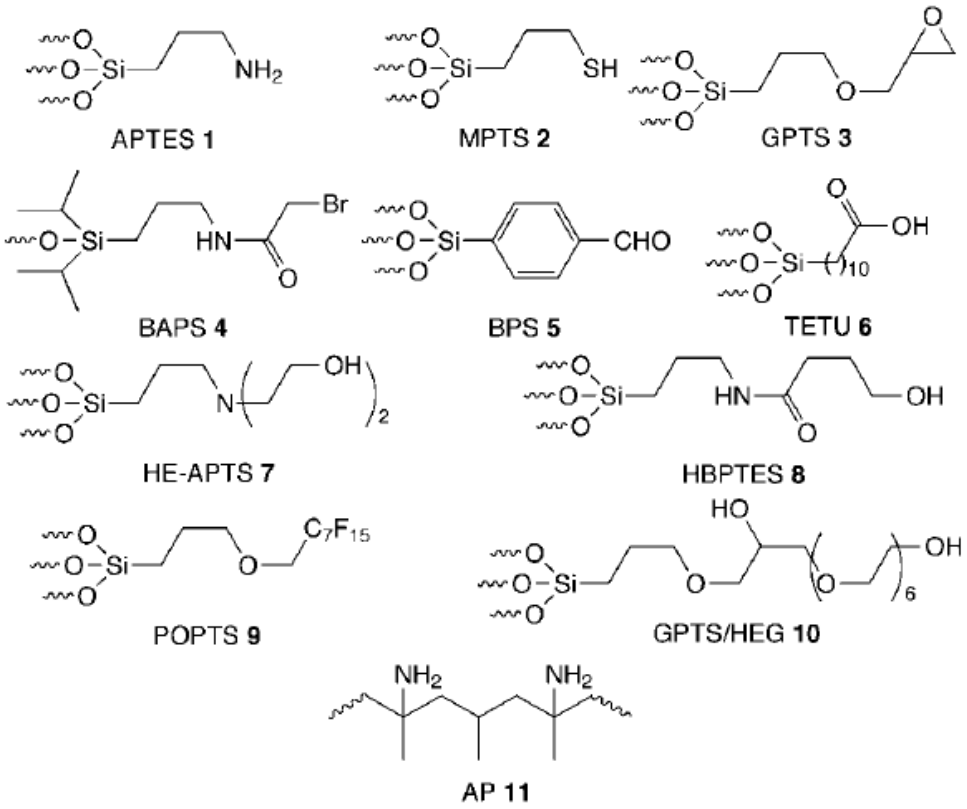
(b) Au(110). Also shown is a unit mesh with $a = b = 4.99$ Å and $\alpha = 109.5$.

(c) Au(100). Also shown is an oblique mesh with $a = b = 5.97$ Å and $\alpha = 95$.

Camillone et al, J. Chem. Phys., 1993. Hou, Stroeve et al., Langmuir 1998.



Silane chemistry

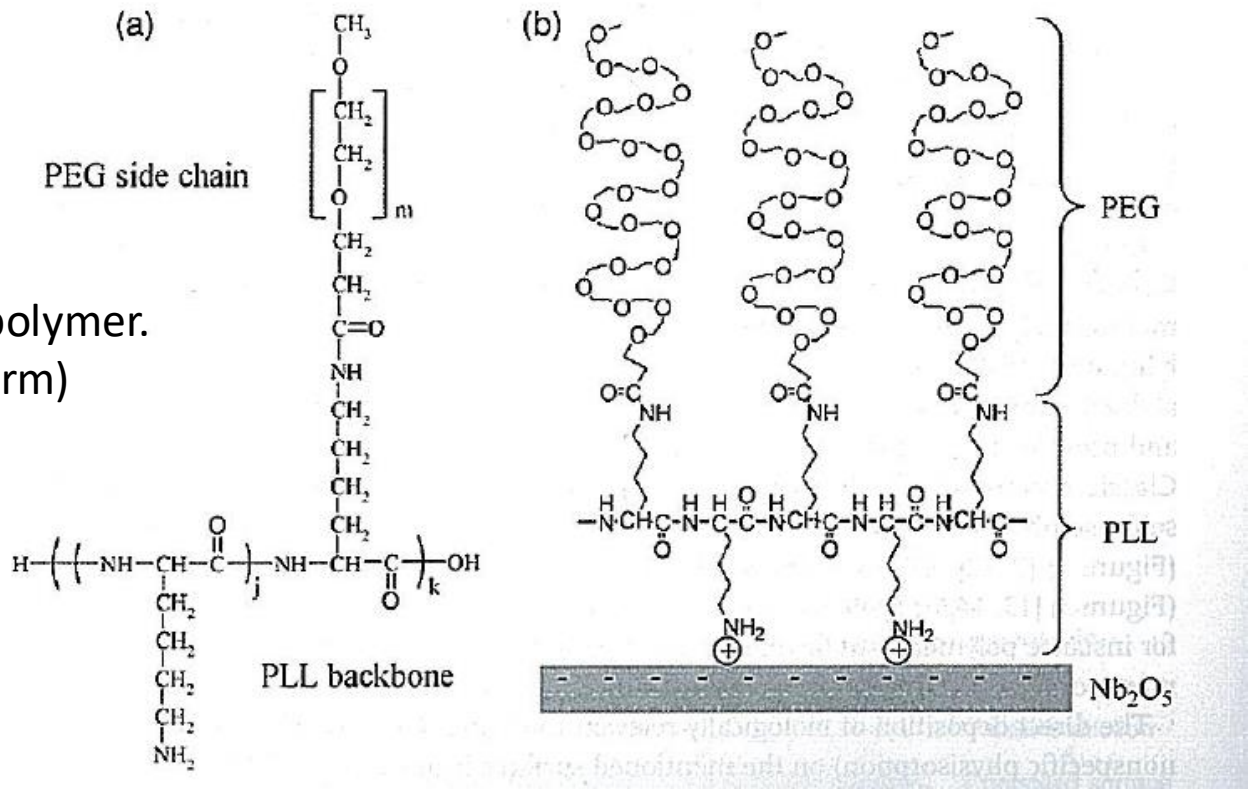


Examples of reagents for the silane-based chemistry for the derivatization of surfaces.

- APTES aminopropyltriethoxysilane
- MPTS 3-mercaptopropyltrimethoxysilane
- GPTS glycidoxypropyltrimethoxysilane
- HE-APTS bis(hydroxyethyl)aminopropyltriethoxysilane
- HBPTES hydroxybutyramide propyltriethoxy silane
- POPTS (perfluorooctyloxy)propyltriethoxysilane

Polymeric film

PLL-g-PEG copolymer.
(uncharged form)



Interfacial structure of a monolayer of PLL-g-PEG adsorbed on a metal-oxide substrate via electrostatic interactions between the negatively charged metal oxide surface and positively charged amino-terminated PLL side chains

Active biomolecule and mediating layer

1. Direct adsorption of the active biomolecule (ligand) on a mediating layer
   - Non suitable for molecules that require control on position, coverage and orientation. In general suitable for DNA

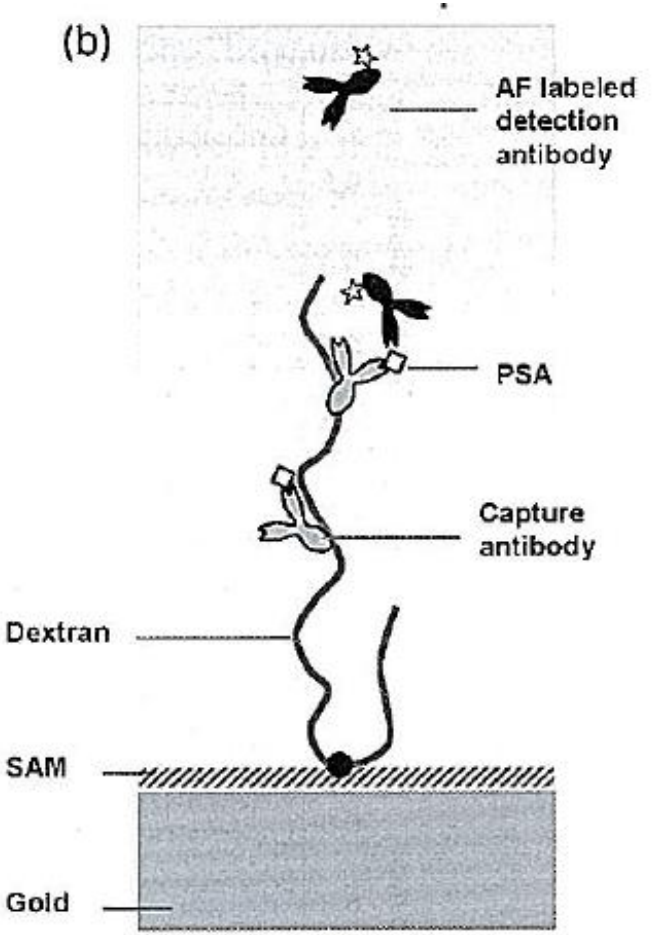
2. More sophisticated approaches relying on surface chemical reactions starting from the mediating layer. E.g.: Heterobifunctional spacers. To link a given functional group exposed by the mediating layer with an active molecule

3D structures vs. SAM

- 3D surface structures are used to increase surface coverage and release the constraint of ordered SAM.
- These are based on dendrimers, thin polymeric films, or structured surfaces obtained by micromachining

Examples of 3D structures

3D molecular layer



3D-patterned solid surface

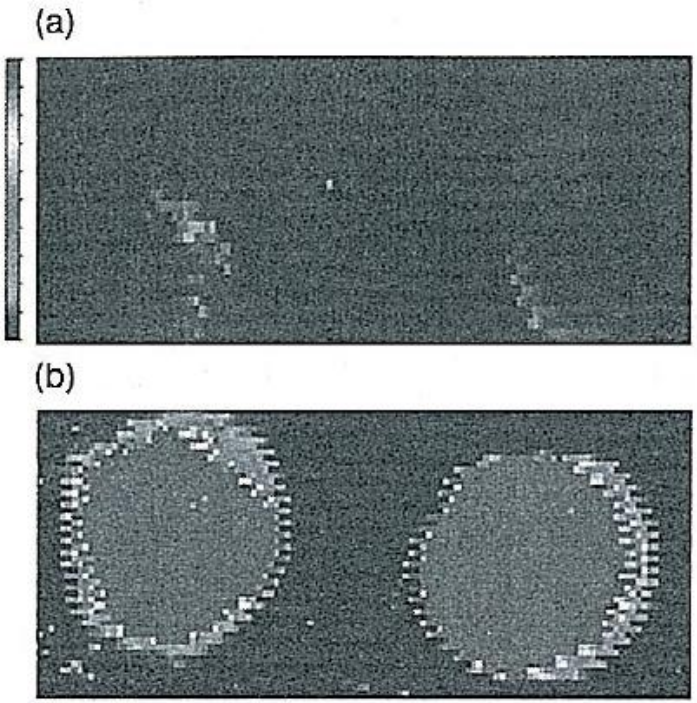


Figure 5 13.2 mm × 3.06 mm fluorescence images of two-element oligonucleotide arrays on (a) planar PMMA and (b) nanopillared PMMA (R = 343) surfaces. Scale is 0 to 10 000 counts. Pixel size is 101.6 µm. (Reproduced with permission from reference [22]; G. Chen, R. L. McCarley, S. A. Soper, C. Situma, J. G. Bolivar, Chem. Mater. 2007, 19, 3855, Copyright 2007 by American Chemical Society).

Prominent problems related to surface molecular recognition

1. Susceptibility to contaminations
2. Limited mobility of immobilized functional groups and spacers
   - Translation is impossible: reduction in the degree of freedom.
   - active biomolecules cannot freely diffuse in solution
3. Hindered access of reactants to reactive sites in organized assemblies
4. The presence of surface forces

1. Susceptibility to contaminations

- Number of reactive surface-bound functional groups is limited
- Largest density : SAM on gold.

Footprint of one molecule: 20-25 $Å^2$

Coverage: 4-5 × 10^{14} molecules/cm^2.

Few tens-hundreds of nanograms on 1 cm^2.

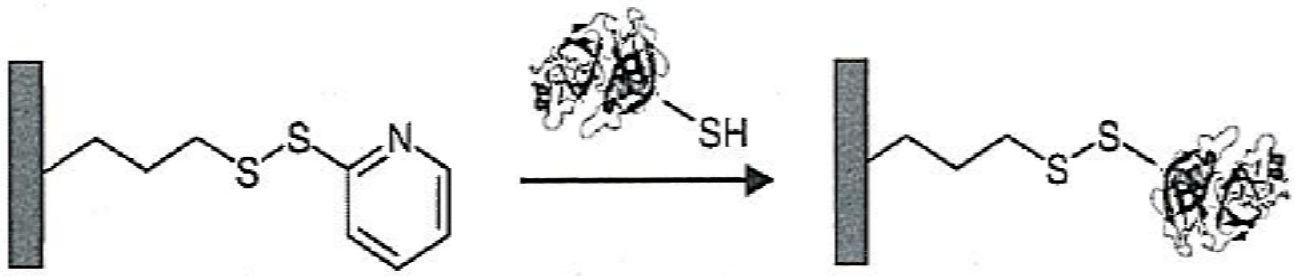
The presence of nanograms of contaminants can consistently reduce the available reaction surface and lead to non heterogeneous surfaces

Covalent attachment reactions

Non covalent attachment reactions

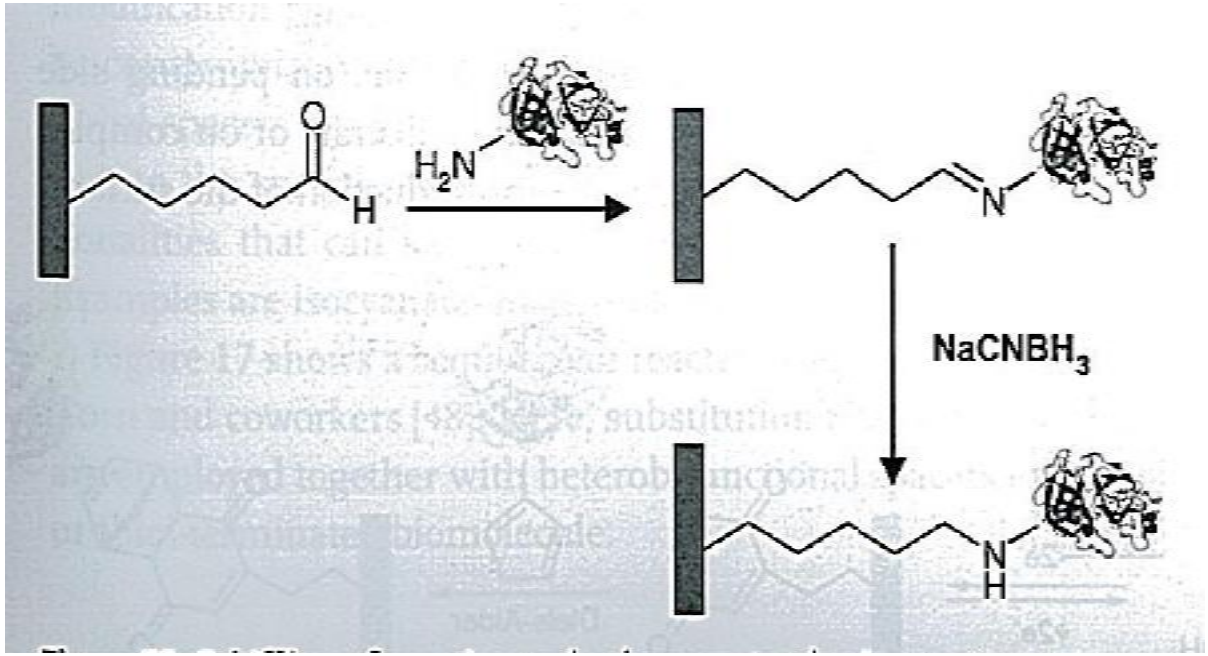
COUPLING CHEMISTRIES

Covalent attach.: Substitution reactions.



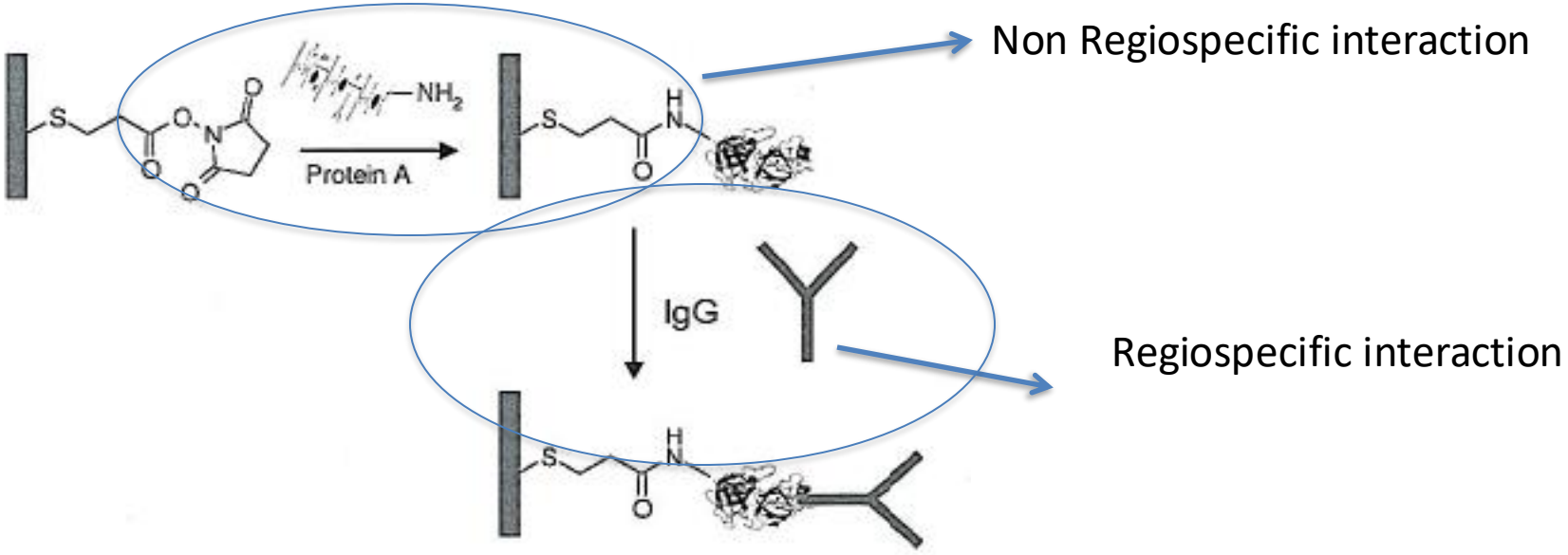
A disulphide exchange group reaction can be used to immobilize proteins via the thiol group of a pending cysteine residue or via a synthesized thiol-ends of DNA.

Covalent attachment: Addition reactions



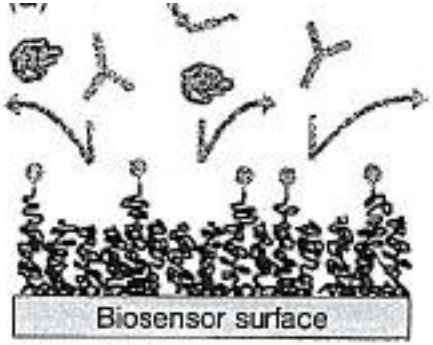
Schiff base formation and subsequent reductive treatment using sodium cyanoborohydrid.
The imin reaction product is in dynamic equilibrium with free reactants and can be exploited to regenerate the surface.

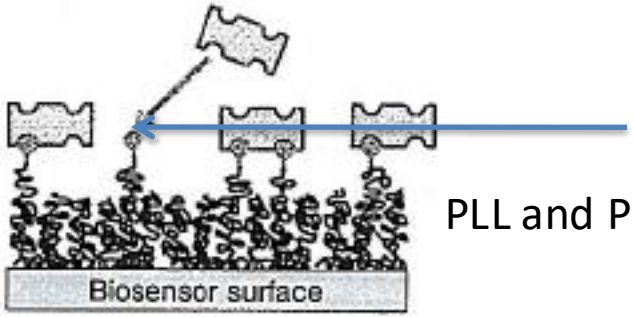
Oriented antibody attachment.



Protein A has a Fc receptor that bonds to the Fc portion of an IgG

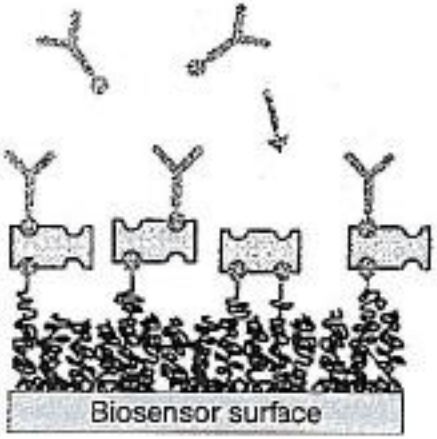
Bio-conjugation

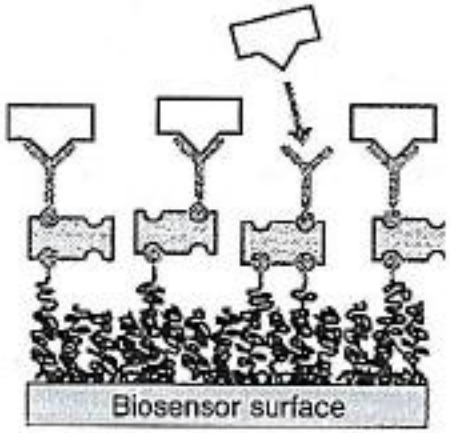




biotin

PLL and PEG brushes





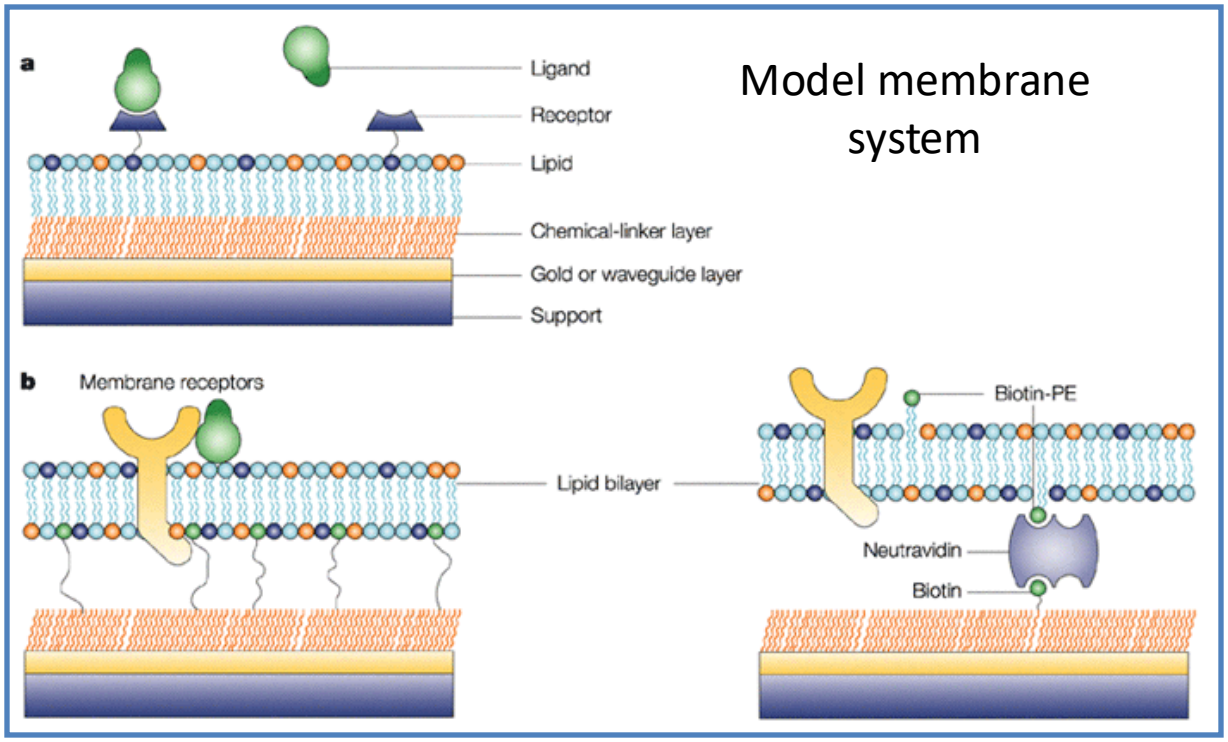
- Charged lysine residues adhere electrostatically to the biosensor surface
- PEG brushes prevent non specific adsorption or proteins
- Biotin is used to immobilize tetrafunctional streptavidin.
- The remaining streptavidin binding sites serve as attachment site for biotin-modified antibodies.

Non covalent attach.: bioconjugation

- Biotin-streptavidin. 10^{15} M^{-1} K_A
- Biotin-avidin. 10^{13} M^{-1} K_A
- Optimum surface coverage in SAM for Biotin is 0.1 per alkyl chain. This provides sufficient lateral dilution and to avoid the effect of surface reactions in highly organized assemblies.

LIPID BILAYERS

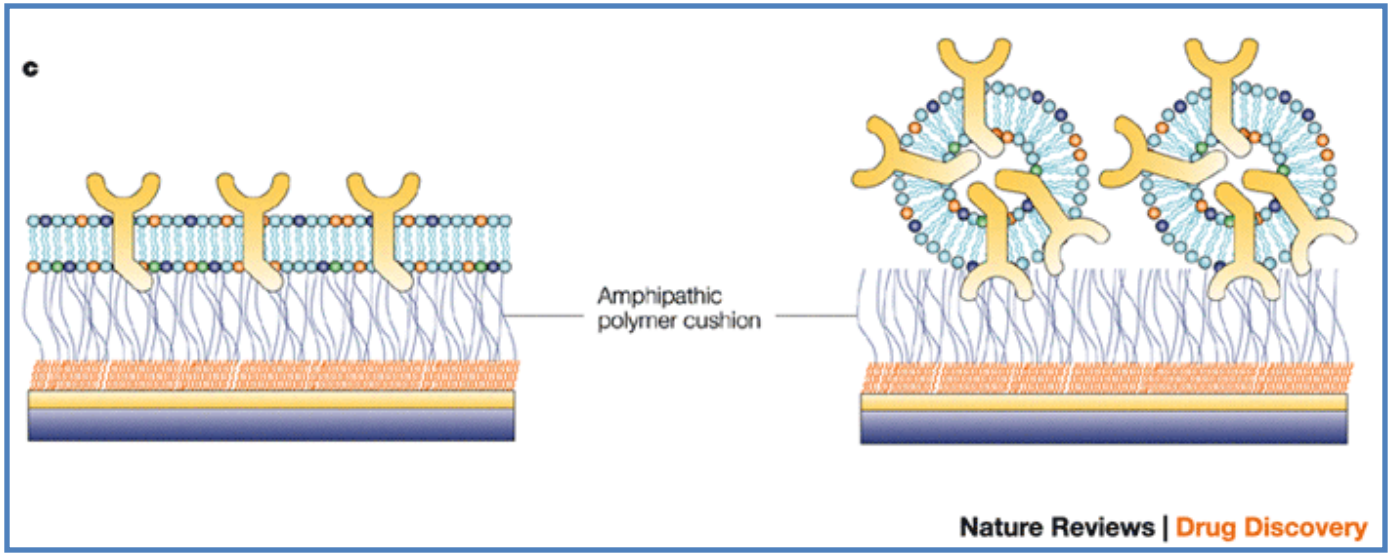
Case study: Screening against membrane receptors



a | A supported lipid monolayer that has been formed on top of a hydrophobic, self-assembled monolayer.

b | Two examples of tethered lipid bilayers that contain an integral (transmembrane) receptor. The bilayer is either captured on the surface using synthetic phospholipids that are tethered to the support by **flexible, hydrophilic linkers** (left), or through immobilized neutravidin in conjunction with biotinylated lipids or a biotinylated receptor. PE, biotinyl-phosphoethanolamine-*N*-(biotinyl).

Case study: Screening against membrane receptors



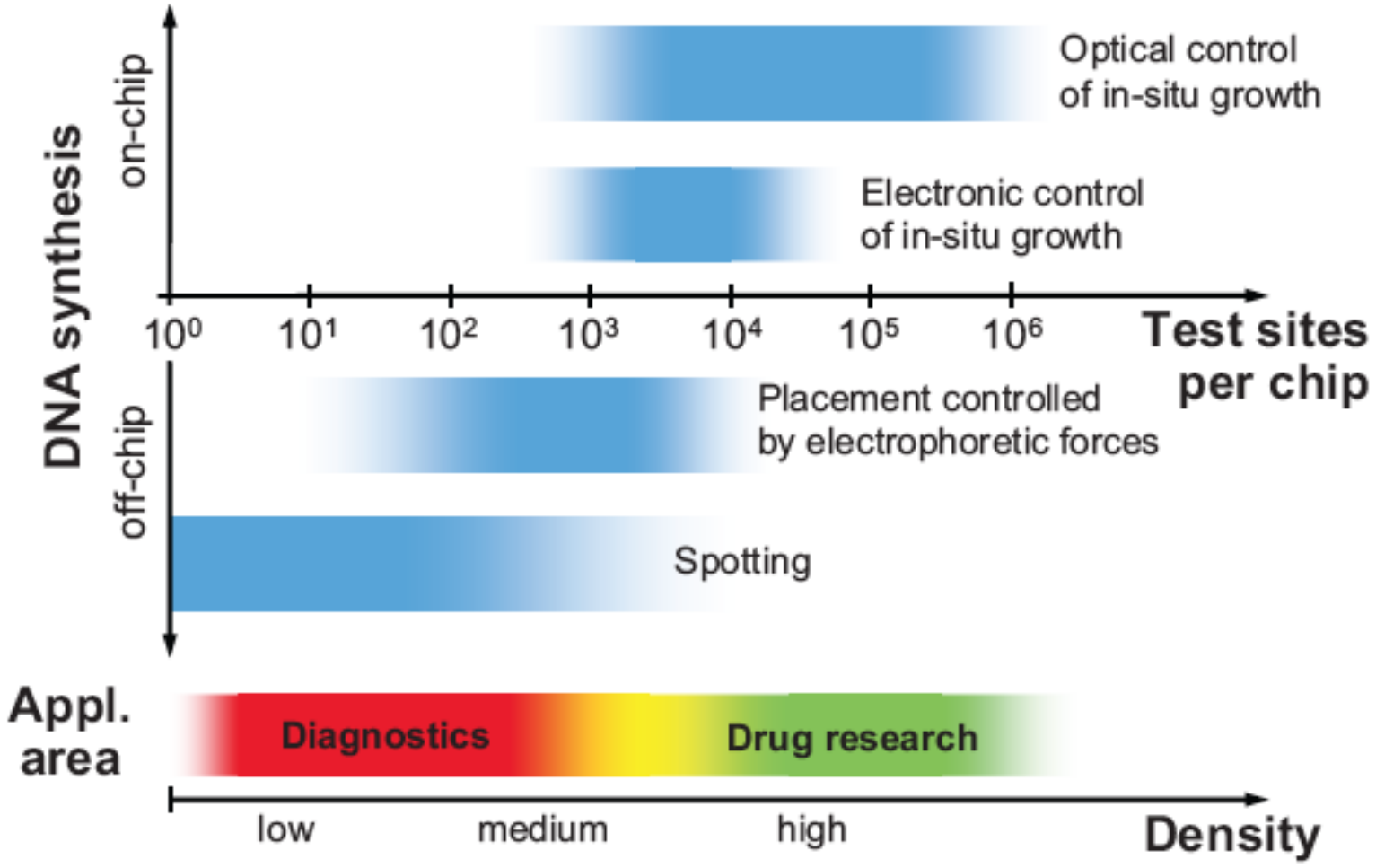
c | Flexible, amphipathic polymer cushions support membranes as either supported lipid bilayers or captured proteoliposome layers.

In this manner, the polymer cushion resembles the cytoskeleton that anchors the plasma membrane of a cell

Definition of different probe sites

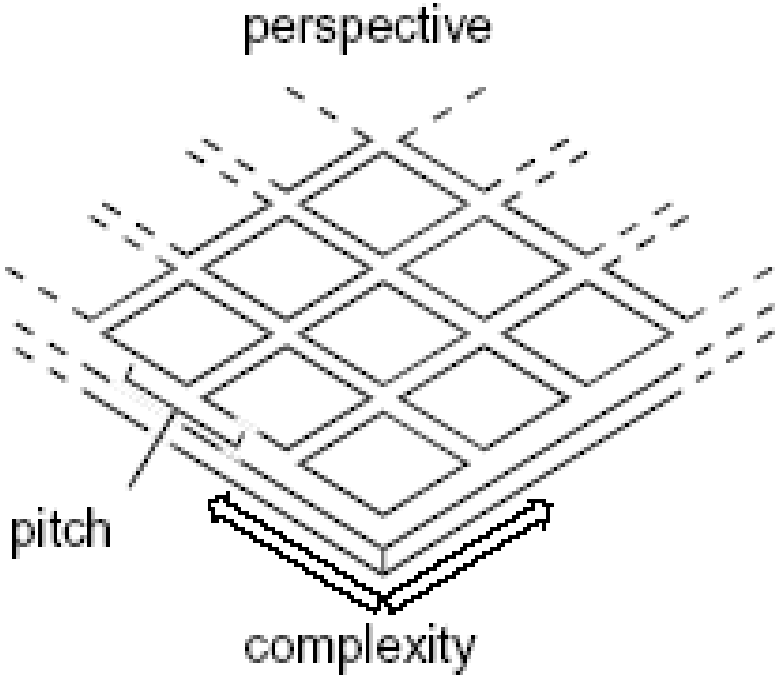
TECHNOLOGIES FOR SURFACE PATTERNING

DNA microarray functionalization techniques and application areas

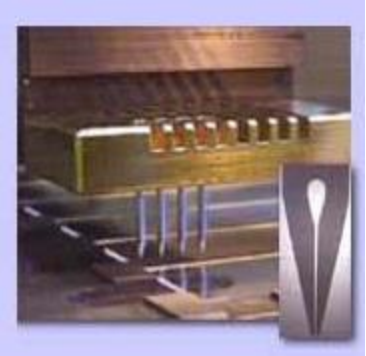


Micro-Technology for patterning of probes/ligands/receptors features

- Direct printing
  - contact printing
  - non contact printing
- Photolitography. In-situ synthesis of probes

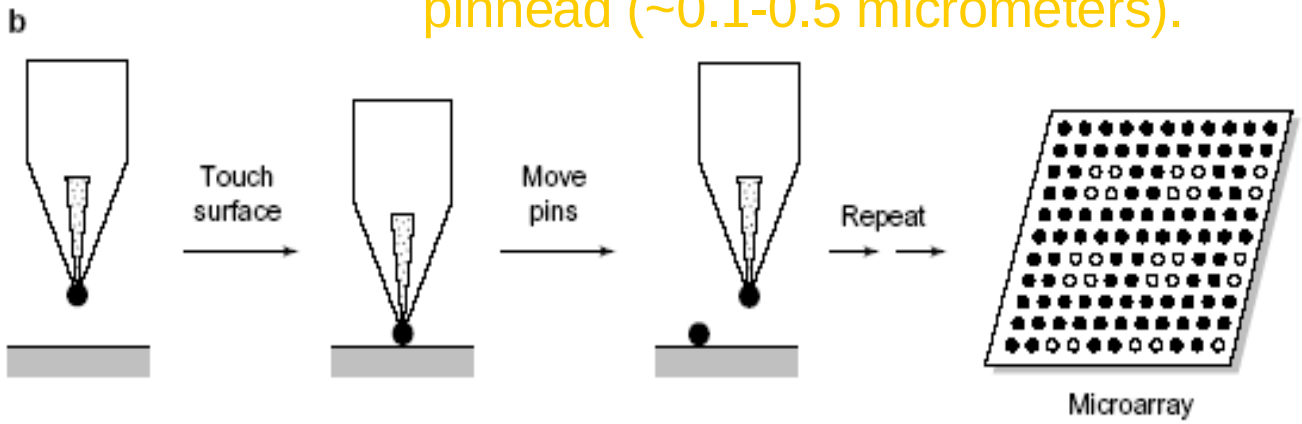


Direct printing. Pin-contact printing



- Up 40,000 different probes on a microscope slide
- Serial/parallel Approach

pinhead (~0.1-0.5 micrometers).



Direct printing. Pin-contact printing

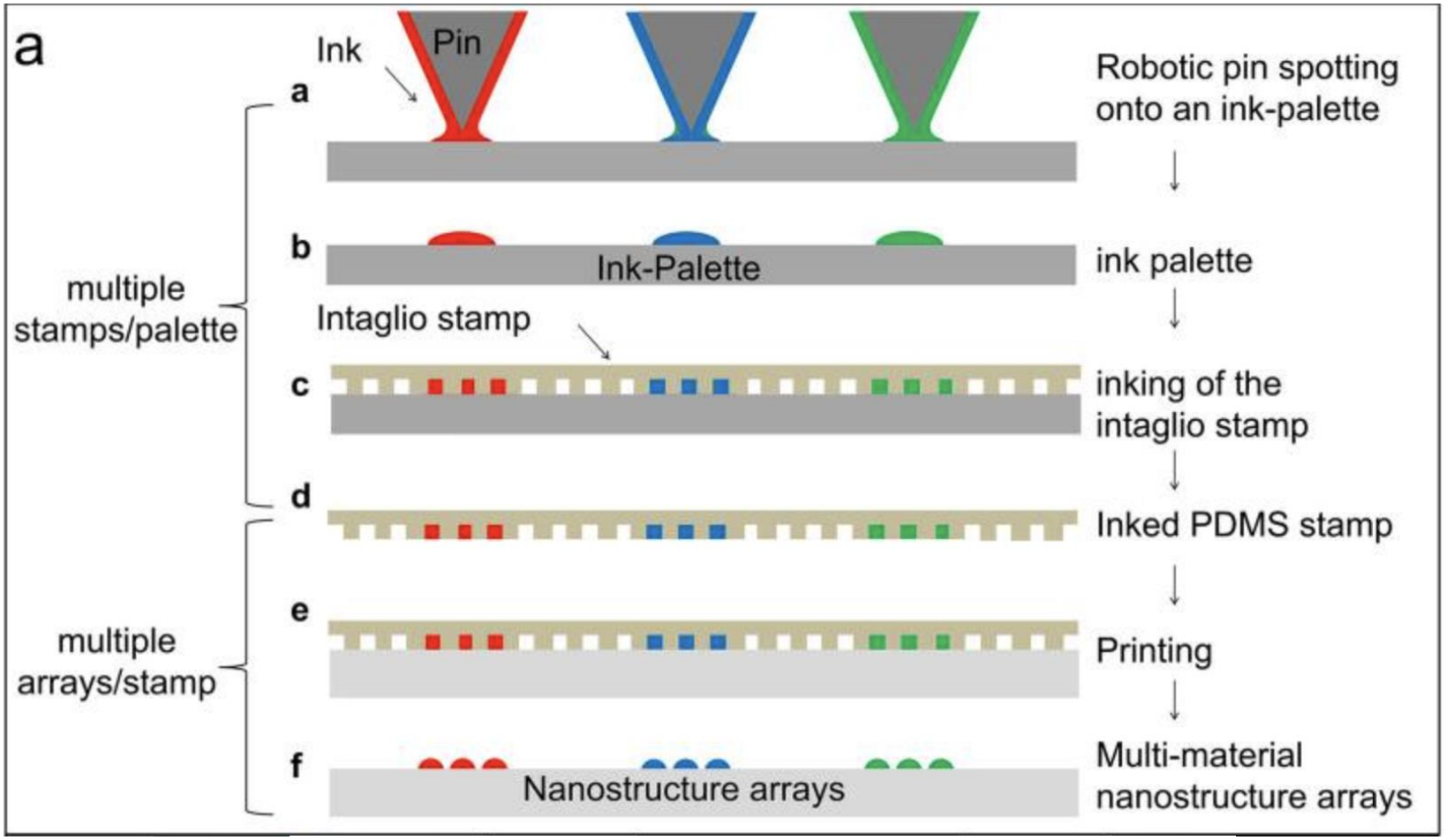
Pin spotting, also known as pin contact printing, is a simple and widely used technique for microarray fabrication. It works by using capillary forces to deposit small droplets of biomolecules onto a substrate, such as a glass slide. The amount of reagent dispensed depends on the pin's structure, the solution's viscosity, and the surface's hydrophilicity. Various pin designs (solid, quill, or split) made from materials like stainless steel or titanium are used to control the fluid's flow and spot size.

Several factors affect spot morphology, including pin configuration, solution viscosity, and contact time. Additives like DMSO or glycerol are often used to prevent evaporation and maintain consistent spot size. Typical micro-spots range from 50–300 µm in diameter, with smaller nano-spots (as low as 15 nm) achievable through techniques like atomic force microscopy.

Despite its ease of use, pin spotting has some limitations. It struggles with inks of high viscosity or large particles, which can clog the pin. Additionally, contact between the pin and substrate risks damaging cells or biomolecules. It is, however, effective for printing biomolecules like DNA, proteins, and antibodies, as well as cell growth media.

The method can also be applied to 3D substrates like nitrocellulose membranes. However, inconsistencies in reagent deposition and the risk of cross-contamination when printing multiple antibodies remain challenges.

Direct printing. Micro/nano-contact printing



Scalable lipid droplet microarray fabrication, validation, and screening

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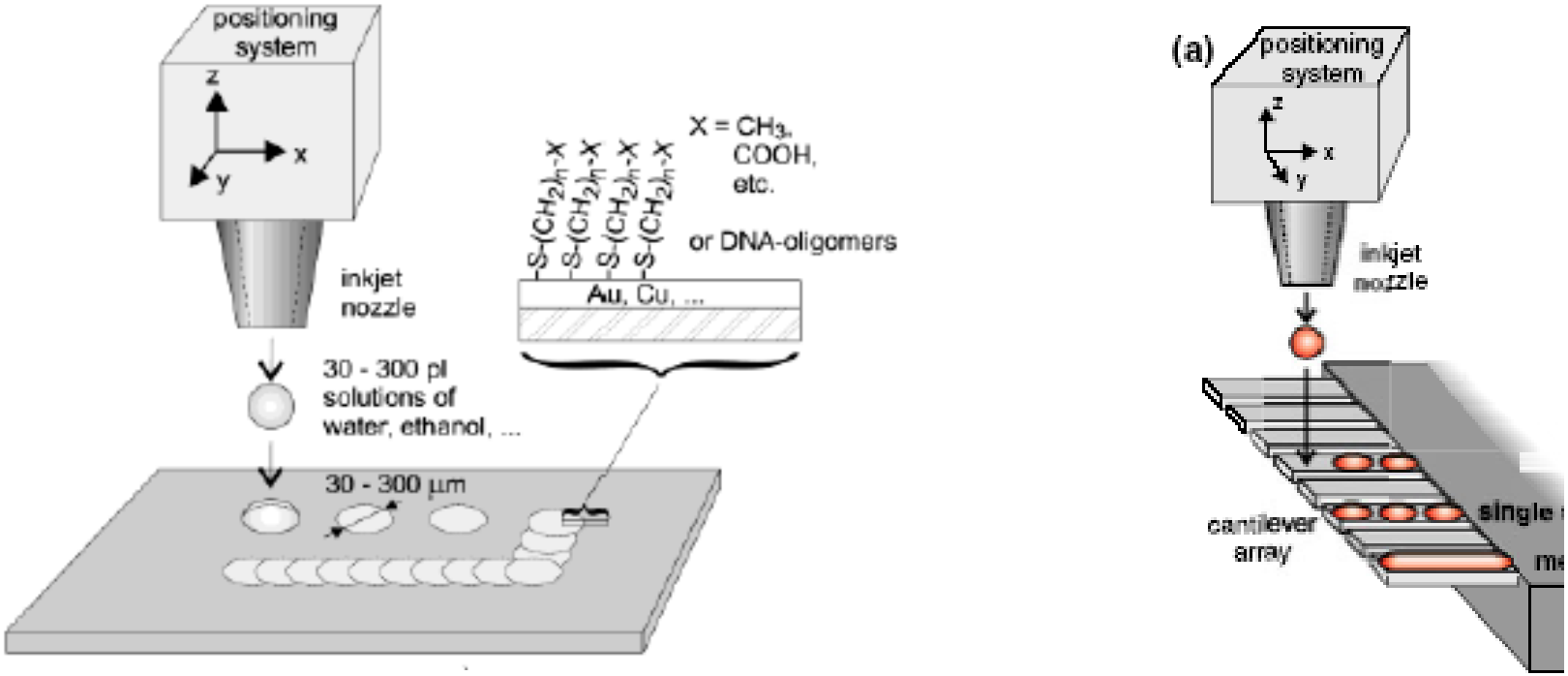
Direct printing. Micro/nano-contact printing.

Micro and nano contact printing utilize specially molded **PDMS (Polydimethylsiloxane)** stamps to transfer molecules from low-energy surfaces like PDMS to higher-energy surfaces such as cleaned glass. PDMS, an elastic silicone-based polymer, enables nanoscale resolution and is created through soft lithography. This method is effective for printing features at both the micro and nano scales, such as extracellular matrixes and proteins. However, it is less flexible than pin printing for addressing individual spots, as the same bioink is used across the entire stamp.

An enhanced method combines **solid pin printing and PDMS stamp printing** to develop scalable lipid microarrays. These microarrays are used for high-throughput studies on lipophilic drug functions and cell responses. The process involves printing different drug solutions into arrays on a 384-well plate, with each spot around 200 µm in diameter.

Direct printing. Contact-free printing



Contact-free printing, also known as **non-contact printing**, is a method of printing microarrays without direct contact between the printer and the surface. This reduces contamination risks and prevents damage to sensitive biomolecules and cells while delivering consistent reagent volumes. Two main techniques under this category are **inkjet printing** and **drop-impact printing**.

Direct printing. Contact-free printing

In **inkjet microarray printing**, droplets of bioink are ejected from a nozzle when a voltage signal is triggered. The droplets are generated through mechanical or pressure-based actuators, with **piezoelectric** and **acoustophoretic** actuators being common options. Piezoelectric inkjet printers are effective for small spot sizes (<100 µm) but struggle with high-viscosity inks. In contrast, acoustophoretic inkjet printers can handle viscosities up to 25,000 cP (mPa·s). Inkjet printing can print a wide range of biomolecules, including proteins, DNA, and cells, while preserving their functionality.

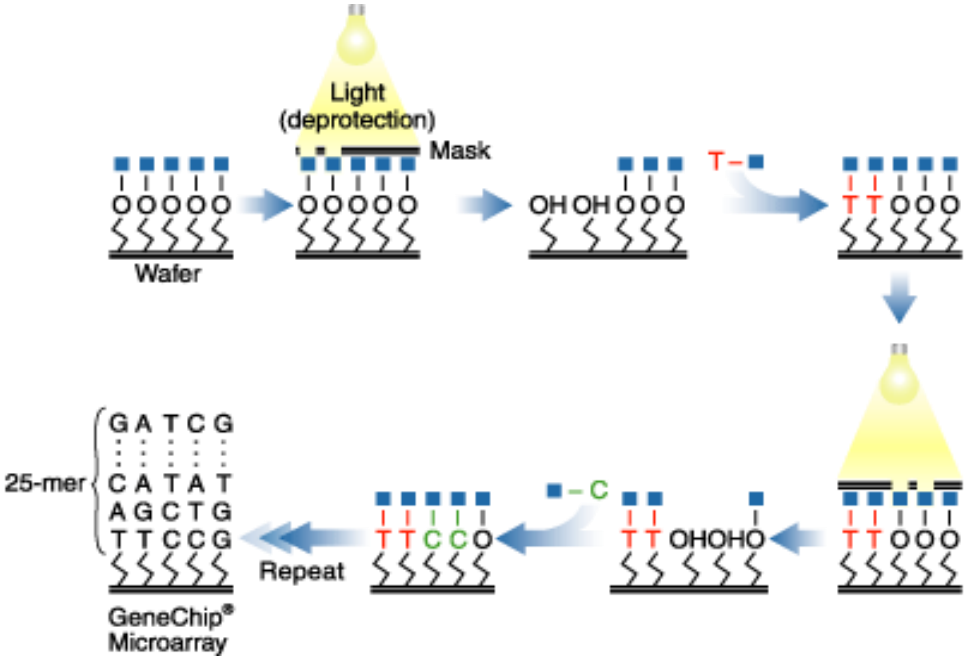
This technology enables sensitive detection with low reagent volumes and short incubation times, making it suitable for field use. The method allows for consistent spot sizes and reliable printing of various inks.

Drop impact printing involves using a syringe to dispense droplets onto a **superhydrophobic sieve**, where the mother droplet breaks into smaller daughter droplets. The size of these droplets depends on the sieve's properties and the ink's characteristics. Drop impact printing can handle more viscous inks than inkjet printing and reduces the risk of clogging, especially when printing cells. However, the process is prone to **satellite droplet formation**, which can cause inconsistencies.

The method has been demonstrated for single-cell printing, where red blood cells were successfully printed without affecting cell viability. However, drop impact printing generally results in uniform spots from the same drop, limiting its use for microarrays with diverse probes.

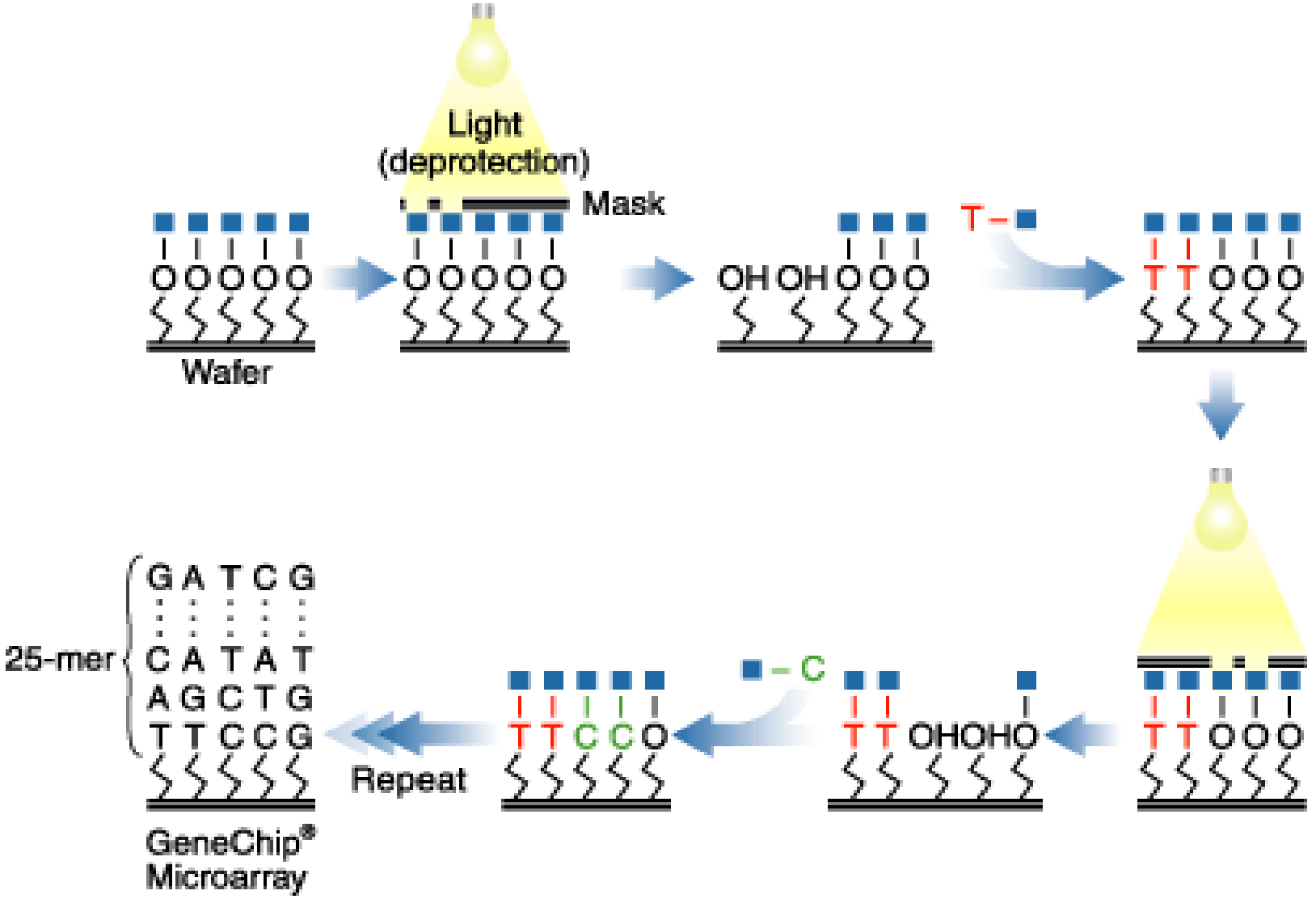
Synthesised in situ. Photolitography

- Each feature hosts thousands of identical DNA molecules
- Maximum number of masks 25*4=100
- A few micrometers pitch



Affymetrix, GeneChip®

Each array contains up to 900,000 different oligos and each oligo is present in millions of copies in each location.



Photolithography is a technique that uses light to create specific patterns on a surface. It involves a **photomask**, which is an opaque material with a pre-designed pattern, and a **photoresist**, a light-sensitive material. The mask blocks light in certain areas while exposing others. When light hits the exposed regions of the photoresist, the pattern is imprinted onto the surface. Depending on the photoresist's properties, the pattern can be either a **positive or negative imprint**.

Photolithography typically achieves a resolution of about **100 nm**, and with some methods, even **sub-50 nm** resolution is possible. The quality of the mask, photoresist, and light used influences the resolution. A significant advantage of photolithography is its ability to enable **large-scale production**. However, any change in the pattern requires creating a new mask, which may involve using techniques like **electron-beam (e-beam) lithography**.

Photolithography is commonly used for creating **protein and DNA microarrays**. In **DNA microarrays** (e.g., Affymetrix GeneChips), single nucleotides are added in a cyclic process of unblocking and coupling, where a **photosensitive blocker** is used to prevent further nucleotides from being added in specific areas. The blocker is removed with photolithography, allowing the next nucleotide to be added. Since the process is carried out nucleotide by nucleotide, this technique requires numerous masks, each unique to the cycle.

D.D. Dalma-Weiszhausz, J. Warrington, E.Y. Tanimoto, C.G. Miyada, The Affymetrix GeneChip® platform: an overview, Methods Enzymol. 410 (2006) 3–28, https://doi.org/10.1016/S0076-6879(06)10001-4.