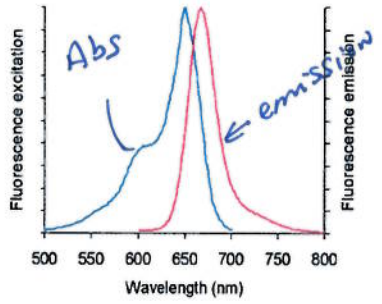
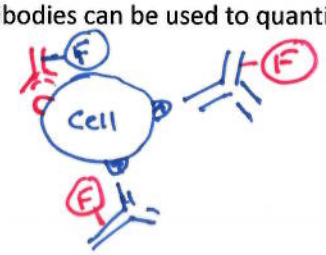
Lecture 15 - Immunoassays II

Fluorescence-Activated Cell Sorter

a) The bound fluorescence on the surface of cells can be detected.
b) Fluorescent cells can be separated from non-fluorescent cells
c) Using different antibodies with different fluorescent molecules allows the separation by different cell surface markers. For example, anti-CD4 and CD8 antibodies can be used to quantify and separate T_H from T_C cells.





Fluorescent $\propto$ [antigen]

Steps:

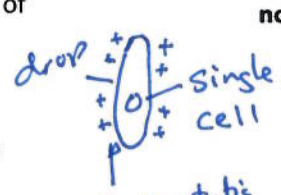
i) Cells are labeled with fluorescent antibody, or multiple antibodies, each of which has a different fluorescent emission spectrum.

ii) Small droplets are made, each containing a **single** cell.

iii) The droplets are charged so they can be deflected by electric fields.

iv) The fluorescence of the drop is measured by the laser. Multi-color detection is feasible, as is quantification of the amount of fluorescence, which is proportional to the number of bound antibodies, which is proportional to the amount of epitope. If two labels are used, each cell generates a point in a 2D plot.

iv) The fluorescence properties of each cell is used to alter the trajectory of the drop. "Gates" are set which define regions of the 2D plot that are to be separated into different samples.



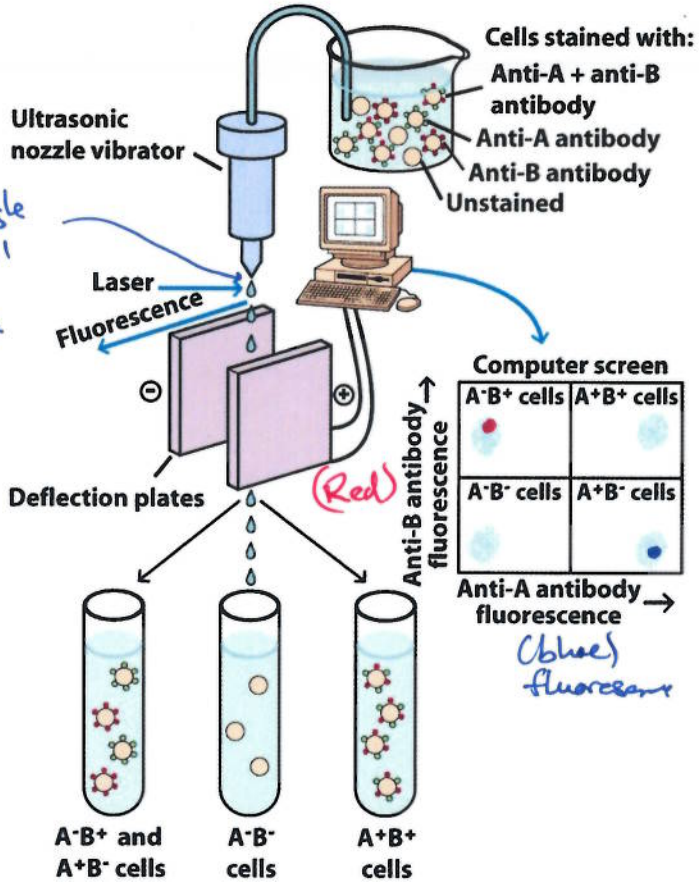


Figure 6-15
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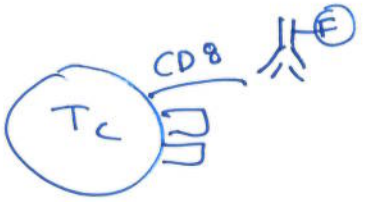
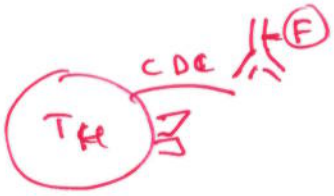
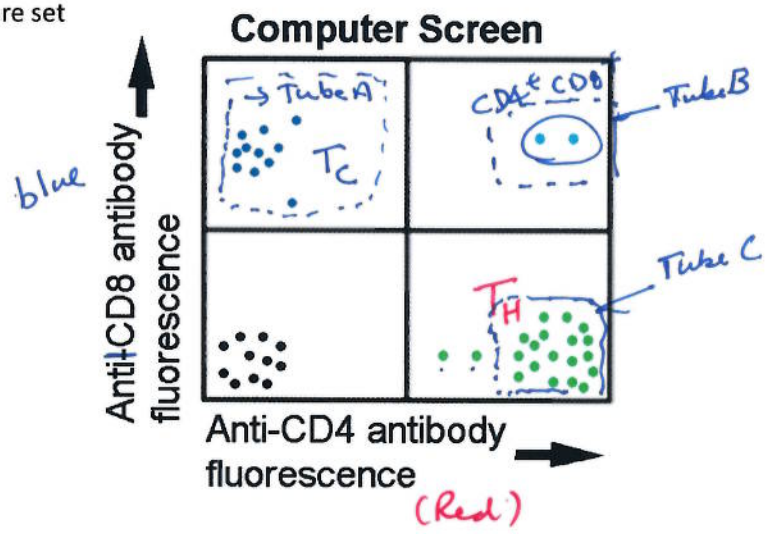




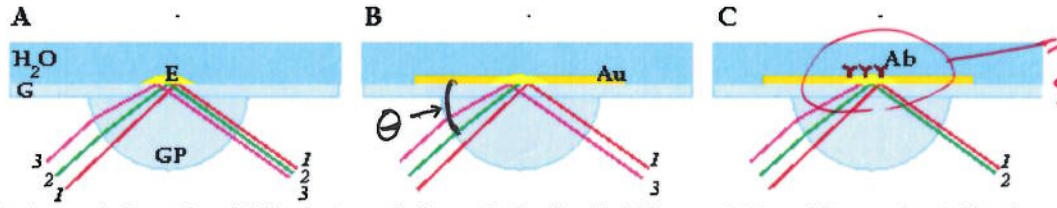


Surface Plasmon Resonance (SPR)

- Measure the binding affinity
- Kinetic on- and off-rates

$$M + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ML$$

$K_D = \frac{(M)(L)}{(ML)}$, $K_A = 1/K_D$

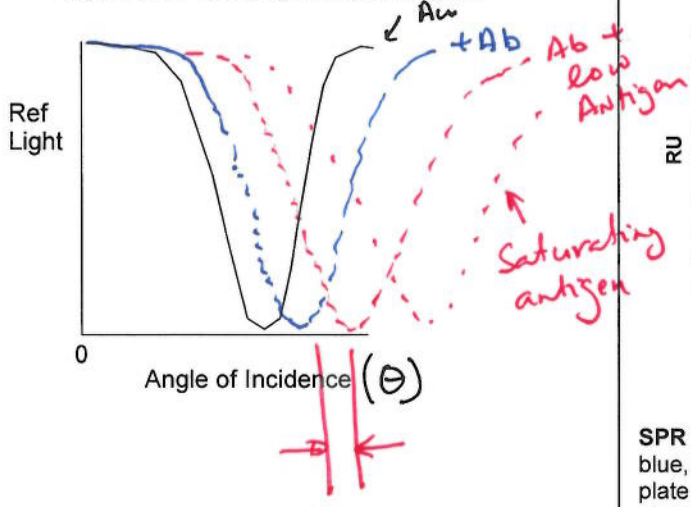


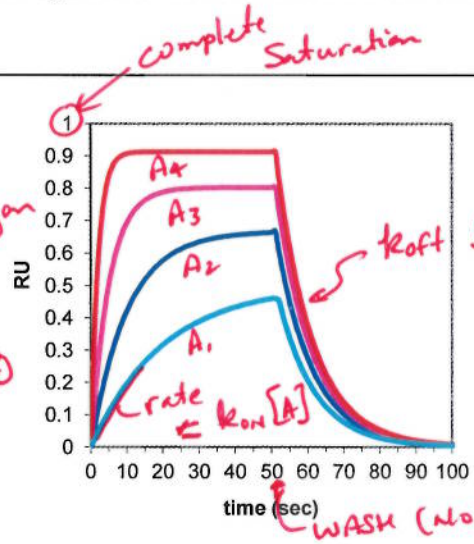
Surface properties affect Ab. of light

SPR Instrumentation. Panel A illustrates only the optical path. Light beams 1, 2, and 3 are refracted by the circular glass (GP) prism and show total internal reflection at the glass (G) water interface, generating an electrical field (E, yellow) that extends several hundred angstroms into the aqueous compartment. Note that the three light beams strike the glass plate at different angles due to the curvature of the prism. Although three light beams are shown, in practice a continuous spread of light is used. In an SPR sample cell (B) a thin layer of gold (Au) is coated on the glass slide. Coupling between the light and the electrons will excite plasmon waves in the metal, leading to absorption of the light. The strength of the coupling depends on the angle of incidence and the refractive index of the aqueous compartment. In this example light beam 2 is absorbed by the gold film. Panel C shows the effect of increasing the mass at the gold surface by the attachment of antibody molecules (Ab). The change in refractive index alters the incidence angle that stimulates plasmon waves. In this example, light beam 3 is now selectively absorbed by the gold after the attachment of antibodies. The binding of ligand to the antibody (not illustrated) would lead to a further change in the angle of incidence that stimulates plasmon waves in the gold. The change in the incidence angle required to stimulate plasmon waves is measured by the instrument.

The angle of incidence where the light is absorbed depends on the refractive index.

- The refractive index depends on the mass loaded on the surface.
- The angular change that occurs due to changes in mass at the surface is reported by the instrument, in arbitrary units (response units, RU).
- One of the binding partners (e.g. antibody) is immobilized on a surface within the sample cell.
- Antigen is flowed over the immobilized protein.
- By varying the antigen concentration it is possible to determine the dissociation constant, K_D, from the measured RU at equilibrium.
- The initial rate of the RU change after addition of the ligand can be used to measure the kinetic on-rate: $k_{on}^{obs} = k_{on}[L]$.
- The off-rate can be obtained by measuring the signal after washing out the antigen.





SPR Data. Increasing amounts of antigen (cyan, blue, magenta, red) were introduced at time=0 to a plate containing antibody. As ligand absorbs to the plate the response increases. At t=50 the ligand was removed. The height of the response after equilibrium is reached is proportional to the fractional saturation, Y. The rate of signal build-up at the different ligand concentrations can be used to estimate the on-rate. The decay of the signal after ligand is removed provides the off-rate.