ELEC6205
BioNanotechnology

Detection and imaging of molecules and molecular events using optics
Fluorescence

- Excite a molecule with short wavelength light (high energy)
- Electrons jump to higher orbitals, when they return to the ground state they emit light - this is fluorescence.

\[ \Delta E = \frac{hc}{\lambda_0} \]
Optical filters are required to sort out the different absorption and emission spectra of the fluorescent molecules.

Many different fluorescent molecules with different wavelengths are used in biological assays.

Multi fluorescence staining of different proteins in a cell.
Dichroic is a dielectric mirror; reflects excitation wavelength onto sample

Passes the longer (fluorescent) wavelengths back through to detector
Molecular Tagging

- All molecules with aromatic, rigid structures fluoresce, e.g. vitamin B\textsubscript{2} (riboflavin).
- Molecules that don’t naturally fluoresce can be “tagged” with fluorescent molecules.
- Very common fluorescent “tag” is fluorescein.
- Absorbs blue light, emits yellow-green light.
Total Internal Reflection Fluorescence Microscopy  TIRF for single molecule imaging

- Excitation of a surface with light to produce Total Internal Reflection.
- Like light transmission in an optical fibre
- Very little background signal – no light in the bulk (no scatter or fluorescence from molecules in the bulk).
- Only the fluoroprobe near the surface (< 200nm) is imaged.
Penetration depth of the evanescent field depends on the angle of incidence.

Critical angle, point of total internal reflection.
Bioluminescence

Protein Aequorin produces blue light from chemical energy.

This is coupled into a Green Fluorescence Protein (GFP) which emits a blue/green fluorescence.

The GFP is from Jelly fish and the DNA coding for this protein can be “spliced” into other proteins so that cells “manufacture” fluorescent proteins to identify different parts of a cell.

Dinoflagelates – emit light when they are agitated!
Image made from bacteria expressing different variants (colours) of GFP

GFP - Green Fluorescent Protein
ATP and Luciferase

The molecular details of bioluminescence varies from organism to organism, but involves:

- **Luciferin**, a light-emitting substrate catalysed by:
- **Luciferase**, an enzyme that catalyzes the reaction
- **ATP**, the source of energy
- Molecular oxygen, \( O_2 \)

The more ATP - the brighter the light. In the firefly **luciferin** and **luciferase** combine with ATP to produce light.

These compounds are commercially available for measuring the amount of ATP in biological materials – detecting life!

Fireflies use their flashes to attract mates. The pattern differs from species to species. In one species, the females sometimes mimic the pattern used by females of another species. When the males of the second species respond to these "femmes fatales", they are eaten!
Single Molecule Fluorescence – light is QUANTISED

Single Green Fluorescent Protein molecules immobilised on glass

GFP-tagged proteins inside a living cell
Can we image (see) moving molecules?

- Average thermal kinetic energy of any object is $3kT/2$
- Calculate $rms$ velocity of motion of a molecule?

$$\frac{1}{2} m \langle v^2 \rangle = \frac{3kT}{2}$$

$$\Rightarrow rms \text{ velocity} = \left( \langle v^2 \rangle \right)^{\frac{1}{2}} = \left( \frac{3kT}{m} \right)^{\frac{1}{2}}$$

- Single molecule:
- Assume $M_w = 25,000$; Mass $= 25,000/N_A = 4.2 \times 10^{-23}kg$
- If $T = 300K$  $rms$ velocity $= 17.4m/s$ !!
- NO WAY! - this assume molecule is in a vacuum
In Water

• BUT in solution the molecule does not travel anything like this far because it continually collides with water molecules, changing speed and direction.

• For a molecule of Mw = 25,000, radius $a = 2\text{nm}$

$$D = \frac{kT}{6\pi \eta a}$$

Diffusion constant = $1 \times 10^{-10} \text{m}^2\text{s}^{-1}$ (n.b. $\eta = 1 \times 10^{-3}$ for water)

From Einstein, mean free path in 3-D is: $\langle x \rangle = \sqrt{6Dt}$

In 1 second the molecule will travel ON AVERAGE $25\mu\text{m}$

Or in 1 video frame (40msec) $4.5\mu\text{m}$ SO WE CAN SEE IT

Q What is mean free path in 2D (i.e. in x and y dimensions)
• In water the movement is defined by the diffusion constant
• Single molecule movement is stochastic
Surface Plasmon Resonance (SPR) – *label free biosensors*

Water: $n = 1.33$

Metal (Au) film, $d = 40\text{nm}$, R.I. = $n + jk$

Protein layer(s)

Evanescent field

Laser light (p-polarised)

Prism

$\theta$

To detector photodiode
Oscillation of confined electrons at the surface of the metal
Plasmon wave vector has to match incoming light wave vector according to

\[ k_{sp} = k \left( \frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d} \right) = \frac{\omega}{c} \sqrt{\varepsilon_d} \sin \theta \]

Resonance depends on:
- Wavelength of light (\(\omega\));
- Dielectric constant of layer; \(\varepsilon_d\)
- Angle of incidence of light \(\theta\)
SPR

- Resonance occurs when incoming light energy is coupled to excite the free electrons at the metal-dielectric surface.
- The energy ends up as heat, no light is reflected
- Special conditions required to match the wave vector of the incoming light and the plasmon wave vector:
  - Thickness and dielectric constant (must be negative) of metal
  - Correct angle
  - Prism for correct matching conditions.
- Label free – change in resonance condition depends only on the thickness and dielectric constant of the adsorbed layer (protein).
- Can be used in real time to monitor binding kinetics.
Implementation

Prism

Laser light (p-polarised)

Antibody layer

To detector photodiode
Typical experimental biosensor system

Companies: Texas Inst. and Pharmacia (Sweden)
Change in reflected intensity ($R$) with angle of incidence ($\theta$)

Angle of total internal reflection
$$= \sin^{-1}(1/\text{R.I.}) = \sin^{-1}(1/1.51) = 41^\circ$$

Situation for a thick metal film (mirror)

Plasmon resonance – reflected light output goes to zero
When protein binds, the resonance angle shifts

Change is typically $1^\circ$ per 1nm of protein monolayer
Accurate measurement obtained by fitting curve to Maxwell’s equations
Typical experiment

• Immobilise antibody (A) on (gold) surface
• Inject analyte/ligand (L)
• Monitor change in thickness with time until saturation
• Classical kinetics: the rate of association is:

\[
\frac{d[LA]}{dt} = k_{on}[L][A] - k_{off}[LA]K_a
\]

\(k_{on}\) is the association constant, \(k_{off}\) the dissociation constant

After some time steady-state is achieved described by equilibrium constants

\[
K_a = \frac{k_{on}}{k_{off}} \quad K_d = \frac{k_{off}}{k_{on}}
\]
Continuous recording of an assay

Amount of protein on surface

Change in angle

Amplitude of reflection coefficient

Angle of incidence (deg)

Injection of the protein

Rinse with buffer

Rinse with dissociating drug
SPR Microscopy

Small change in angle gives a big change in reflectivity.

Replace photodiode with camera.

Each small segment of the surface is imaged onto a group of pixels – 2-D image of the surface.

Thickness change is obtained from the image.
6 x 6 μm squares of Ag on Au, optical image left, SPR image right
Multiplexed analysis (proteins)

Antibody 1  Antibody 2  Antibody 3

+ Antigen layer

Gold  Glass

Light in  R1  R2  R3

SPR Microscope images – when the protein binds the spot gets brighter. The change in “gray scale” can be related to thickness.
DNA Hybridization Assays

Hybridization to these two spots

Before         After

Probe DNA

Hybridized Targets

SA

Functionalised Gold

Real time analysis

SPR Signal

Hybridization Time (Hours)
Amplification with protein labels in sandwich assay

Amplification with Avidin

SANDWICH Assay

Hybridized Targets

Amplified hybridization

(b) binding target to P1

(c) after regeneration with urea and binding different target to P2
Amplification with gold nano-particles – **Sandwich assay**

Amplification with gold colloid

Hybridized Targets

Functionalised Gold

Sensitivity is $10^9$ oligos/cm²

From He *et al* JACS **122** 9071 (2000)
## Sensitivity Limits

<table>
<thead>
<tr>
<th>Method</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>$10^7$ oligos/cm$^2$</td>
</tr>
<tr>
<td>Unamplified SPR spectroscopy</td>
<td>$10^7$ oligos/cm$^2$</td>
</tr>
<tr>
<td>Unamplified SPR imaging</td>
<td>$10^{12}$ oligos/cm$^2$</td>
</tr>
<tr>
<td>Amplified SPR imaging*</td>
<td>$&lt;10^9$ oligos/cm$^2$</td>
</tr>
</tbody>
</table>
Applications summary:

Cell analysis

Membranes

Carbohydrate analysis

Protein interactions

Nucleic acids (DNA)
BIA Core

• Commercial SPR-based sensing system.
• Now widely used in hospitals and R&D labs
GWC’s SPRimager®II

www.gwctechnologies.com
Spreeta™
TSPR2KXY Biosensor

Spreeta is a multi-channel surface plasmon resonance (SPR) based biosensor for real-time, quantitative measurement of molecular interactions. The device can be used in various applications such as drug discovery, protein-protein interactions, and disease diagnosis.

Refractive Index Range: ........... 1.33 to 1.40
Baseline Noise: ................. 3x10^-7 RIU
Drift:** .......................... <1x10^-6 RIU/min

*Calibrated with 180-300 µg/mL of BSA in PBS at 25°C
**Drift measured with no analyte binding
Plasmonic nano-particles

- Size and composition of nano-particles affects their colour
- Different size and shape particle have different SPR resonances and therefore appear different colours. Particles also scatter the light in different ways.
In the presence of complementary target DNA, oligonucleotide-functionalized gold nanoparticles will aggregate (A), The solution colour changes from red to blue (B) because of change in plasmonic properties of the particles. **This is the basis of a very simple but very sensitive biosensor**
Summary

• Fluorescence – most widely used method for assaying and imaging
• SPR can measure (average) thickness of protein layers in the sub nm range – a label free optical method
• Not as sensitive as fluorescence unless labels (Au particles) used
• Plasmonic nanoparticles – v. simple label free sensing system.
FRET – Fluorescence Resonance Energy Transfer

Molecular ruler

FRET – Measuring molecular interaction

D – orange 580nm
A – red 670nm

Interaction between probes leads to change in emission spectrum

MOLECULAR RULER
Energy coupling varies with $1/r^6$