I. Fluorescence

The fluorescence, $F = 2.3 I_0 \Phi_f f(\theta)g(\lambda) \varepsilon l c$ where $I_0$ is the intensity of the incident light; $f(\theta)$ is the fluorescence that reaches the detector; $g(\lambda)$ is the efficiency of the detector; $\varepsilon$ is the molar extinction coefficient; $l$ is the pathlength; $c$ is the molar concentration; $\Phi_f$ is the quantum yield.

**Quantum yield** = \( \frac{\text{Number of photons } F}{\text{Number of photons } A} \) where $0 \leq \Phi_f \leq 1$

The quantum yield is essentially the emission efficiency of a given fluorochrome.

Once the fluorophore, $M$, has absorbed a photon and goes to the excited state $M^*$, it can decay in a number of ways. The rate constants for the different processes are:

- $k_t$: thermal inactivation
- $k_p$: photochemistry (photobleaching)
- $k_f$: fluorescence
- $k_0$: quenching
M + hν → M*

\[
\begin{align*}
\text{N} & \quad \text{kp} \\
\text{kt} & \quad \text{kr} \\
\text{M} & \quad \text{F} = k_d [M^*]
\end{align*}
\]

And as a result, M* → M + hν

\[
F \propto \frac{d(M^*)}{dt} = k_d [M^*]
\]

\[k_d = kr + kt + kp + [Q]k_0\]

\[
F \propto \frac{d(M^*)}{dt} = kr [M^*] + kt [M^*] + kp [M^*] + k_0 [M^*][Q]
\]

M* + Quencher → M + Q*

Q* → Q + Heat

\[
\Phi_f = \frac{k_f[M^*]}{k_d[M^*]} = \frac{k_f}{k_d} = \frac{\tau}{\tau_0}
\]

\[
\ln [M^*] = -k_d t + \ln [M^*_0]
\]

\[F \propto [M^*]\]

\[\ln F = -k_d t + \text{constant}\]

Two amino acid side chains have substantial fluorescence:

- tryptophan and tyrosine.

A polar environment next to a fluorescent group results in \(\Phi_f \downarrow\) and a non-polar environment results in \(\Phi_f \uparrow\). Therefore the fluorescence of the side chains give information about the environment. Conformational changes that change the environment around a fluorescent group can be detected as a change in fluorescence.

Amino acid side chains with reactive groups such as –SH, –NH₂, or COOH can be chemically labeled with a fluorophore and used to detect the changes in conformation, binding to ligands, etc..

Some co-factors such as NADH are naturally fluorescent.

\[
\begin{align*}
\text{NADH} & \quad \leftrightarrow \quad \text{NAD}^+\n\end{align*}
\]
Oxidation to NAD+ results in loss of fluorescence. Thus one can follow these changes in intact cells by measuring the fluorescence.

II. Examples of the use of fluorescence: Lateral Motion and Vesicle Fusion

The motion of proteins, lipids, etc. inside a cell or in a cell membrane can be detected using fluorescent molecules. The protein or lipid is tagged with a fluorophore either chemically or with a fluorescently labeled antibody. Processes such as endocytosis can be monitored as a function of time. Lateral diffusion in a membrane can be measured by photobleaching an area and measuring the rate of recovery of the fluorescence.

**Figure 2**: Photobleaching experiment where a component in the plasma membrane is tagged with a fluorescent dye. Then a small area is bleached with a laser beam of intensity strong enough to destroy the fluorescent dye by weak enough to not destroy the cellular structures. The fluorescence of the bleached patch recovers as tagged molecules diffuse into the patch from the rest of the membrane. The larger molecules move slowly than the smaller ones (Picture courtesy of NIH)

Another experiment that can be used to monitor vesicle fusion. Three different experimental protocols are illustrated below. (a) the outer leaflets of the vesicles are differentially labeled with two different fluorophores. (b) same but the inner leaflets are labeled (c) the vesicle contents are labeled. The mixing of the two different fluorescent molecules is used to show mixing of the different compartments. Alternatively, 1 fluorescent molecule and quencher can be used. Mixing would diminish the fluorescence. Below is the membrane fusion from Kason et al. (PNAS P.11916—P.11921, 2006) that utilized two different fluorescent molecules.
III. Examples of the use of fluorescence: Membrane Potential

The potential difference between inside and outside of the cells can be measured by using a fluorescent molecule that is amphipathic and has either a dipole or an asymmetric net charge allowing it to respond to the electric field. Thus the electric field favors the spanned state. In this state the environment around the fluorophore changes resulting in changes in fluorescence. Which form would you expect to be more fluorescent?

*Figure 3:* The membrane potential can cause the charged fluorophore to change location.
Based on figure 3 and graph 1, Adsorbed (F↓) \(\xrightarrow{\Delta \psi}\) Span (F↑). This process can be understood quantitatively:

\[
F \propto \frac{[\text{Spanned}]}{[\text{Adsorbed}]} = e^{-\frac{(\Delta G - \vec{B} \cdot \vec{E})}{RT}}
\]

where \(\vec{B}\) is dipole moment and \(\vec{E}\) is the electric field.

**IV. Examples of the use of fluorescence: Vesicle Permeation**
To be able to see, **self-quenching** is used.

Vesicles are filled either with (a) a high concentration of fluorophore so that there is strong self-quenching or (b) with a combination of fluorophore and a quenching agent. Thus release of the vesicle contents will increase the fluorescence either by (a) elimination of self-quenching by dilution or (b) elimination of quenching by the quenching agent by dilution.

**V. Quantitation of Quenching**

\[ \Phi_{f}^{0} = \frac{k_{f}}{k_{d}} \]

This is the quantum yield without the quencher. Here \( k_{d} \) includes all the rate constants EXCEPT \( k_{Q} \).

\[ \Phi_{f} = \frac{k_{f}}{k_{d} + k_{Q}[Q]} \]

\[ \frac{\Phi_{f}^{0}}{\Phi_{f}} = 1 + \frac{k_{Q}[Q]}{k_{d}} \]

And therefore Stern Volmer equation can be derived as:

\[ \frac{F_{0}}{F} \propto \Phi_{f}^{0} - 1 = k_{Q} \tau_{0}[Q] \]

\( F_{0} \) is the fluorescence without the quencher. \( \tau_{0} \) is the time constant of the fluorescence decay without the quencher. A plot of \( F_{0}/F \) vs \([Q]\) yields the \( k_{Q} \). With this constant one can now understand/predict how the fluorescence will change as \([Q]\) changes.