I. Review of Steady State Kinetics

If any of this is at all unfamiliar, review a basic biochemistry text like Voet & Voet

\[ E + S \rightarrow E \cdot S \rightarrow E + P \]

E = enzyme, S = substrate, P = product

Graph of product produced over time:

- Steady State assumption: \([E \cdot S]\) is constant, \(\frac{d[E \cdot S]}{dt} = 0\)

Steady state rate equation:

\[ v = \frac{k_{\text{cat}} ([E]_o[S])}{(K_m + [S])} \]

\[ K_m = \frac{(k_1 + k_{\text{cat}})}{k_1} \] (definition)

Note that as \([S] \rightarrow \infty\), \(v_{\text{max}} = k_{\text{cat}} [E]_o\)

Also, when \(K_m = [S]\), \(v = \frac{1}{2} v_{\text{max}}\)

Example: how to measure \(K_m\) & \(k_{\text{cat}}\) for RS\text{Ile}:

\[ \text{Ile} + \text{ATP} + \text{RS}_{\text{Ile}} \rightarrow \text{RS}_{\text{Ile}} \cdot \text{Ile-AMP} \rightarrow \text{RS}_{\text{Ile}} + \text{tRNA-Ile} + \text{AMP} \]

There are 3 substrates (Ile, ATP, and tRNA), therefore, there are 3 \(K_m\)'s. To measure \(K_m\) for Ile, provide excess of tRNA and ATP (saturate) and titrate in various amounts of Ile. Plot the amount of product produced over time, and calculate the reaction velocity from the linear region (slope = reaction velocity). Then plot reaction rate against substrate concentration- \(K_m\) & \(k_{\text{cat}}\) can be easily taken from this graph.
II. FRET (fluorescence resonance energy transfer)
A way to estimate distances between two fluorophores
-done when emission $\lambda$ (em) of 1 fluorophore (donor) overlaps with excitation $\lambda$ (ex) of another (acceptor)
-NOT a reabsorption (no intermediate photon) – dyes are coupled by a dipole-dipole interaction- the excited states of 2 fluorophores
NOT radiative

Example: Fluorescein and Rhodamine
Fluorescein ex= 494nm, em = 514nm (donor)
Rhodamine ex= 528nm, em = 551nm (acceptor)

Attach both fluorophores to a protein
Emission spectrum when the two fluorophores are excited at 494nm shows two peaks.
Separate the fluorophores by digesting the protein (trypsin), or eliminate the acceptor by photobleaching
Now see only one peak, corresponding to the donor, intensity (I) increased since energy no longer transferred to acceptor

FRET efficiency $E= 1 - \frac{I_{\text{donor before bleach}}}{I_{\text{donor after bleach}}}$

High FRET= big increase after bleach= small fraction= $E$ near 1.0
Low FRET= small increase after bleach = big fraction = $E$ near 0

Can also get $E$ from lifetime measurements
$E= 1 - \frac{\tau_{\text{donor before bleach}}}{\tau_{\text{donor after bleach}}}$

To get distances, use FRET theory $E= \frac{R_o^6}{(R_o^6 + r^6)}$
where $r$ = distance between donor and acceptor
$R_o$ = “Forster radius” - distance at which $E=0.5$

A plot of $E$ vs $r$ shows that you can get the most info about distance at around $R_o$ (30-60 angstroms) for most dyes- conveniently about the diameter of an average protein
$R_o$ is specific to the fluorophores used
III. GFP - green fluorescent protein
Why? – to label proteins for optical imaging with perfect specificity
features: from jellyfish (GFP= FRET acceptor for auquorin-->conveys blue light to green)
238 amino acids
11 strand beta-barrel (anti-parallel)
Ser65-Tyr66-Gly67 form chromophore
(ex=395nm, em=505nm; anionic form ex=475nm, em=505nm)
maturation time after expression (1-4 hrs)
slow step: oxidation
imidazolinones spontaneously auto-oxidize in air
No fluorophore develops in anaerobic conditions

RFP= red fluorescent protein
features-from coral
1st becomes green, then red ~12 hrs later
mass spec shows red protein 2 mass units smaller than green intermediate
obligate tetramer

other rationally designed colors: yellow(YFP), blue(BFP), cyan(CFP)
EGFP=enhanced GFP, has only one ex peak, @ 475nm (S65T mutant) --> destroys H
bond with Glu222, forcing it to be protonated--> stabilizes anion form of Tyr 66

advantage- GFPS are genetically encoded (can attach to desired protein with perfect
specificity)
disadvantage- REALLY big

IV. Example of use of FRET- Ras activation

Nature (2001) 411, 1065

\[
\text{Ras} \cdot \text{GTP} \quad \text{Ras} \cdot \text{GDP}
\]

\[
\text{GAP} \quad \text{GEF}
\]

\[
\text{Ras} \cdot \text{GTP} \quad \text{“on”} \quad \text{Ras} \cdot \text{GDP} \quad \text{“off”}
\]

Ras is a GTPase that acts as a molecular switch in many signaling cascades. When “on,” Ras•GTP interacts with many downstream effectors, including Raf. Raf binds only to “on” Ras.

Approach:
- make DNA for intramolecular complex of CFP(cyan)-Raf-linker-Ras-YFP(yellow)
- introduce into cells
- express protein
- image

See figure 1a in Nature (2001) 411, 1065 for a cartoon image of the protein construct in the GTP and GDP bound state.

To image: excite CFP, collect YFP emission
- excite CFP, collect CFP em
- divide YFPem/CFPem -> ratio image

In the figures, red = high YFP/CFP ratio -> high FRET
blue = low YFP/CFP ratio -> low FRET

Compare 2 reporters: Ras activation reporter “Raichu-Ras”
- Rap1 reporter “Raichu-Rap”

Rap1 is another GTPase
- Goal- use FRET to compare in vivo spatial and temporal activation of these GTPases
- Experiment: add EGF (epidermal growth factor) -> Ras or Rap1 construct -> cell growth
- observations: Ras activated @ plasma membrane 1st
- Rap1 activated @ perinuclear membrane 1st

Ras activated @ free edges of cell

Conclusions: Ras and Rap1 act by different mechanisms
small subpopulation of each are activated by EGF, most stays initially inactive again, in Nature (2001) 411, 1065

**Figure 1b** shows emission spectra of Ras and Rap1, red line= GTP bound (high FRET), blue= GDP bound (low FRET), green = protein digest control

**Figure 1a** compares cells expressing Ras and Rap1- notice that Ras is first activated at free edges of cell (as indicated by red high FRET), and Rap1 is activated at perinuclear membrane.

**Figure 2c** compares location of red high FRET areas (from Ras) to border of cell, notice activation at free cell edge