SibFU 5
February 2012

STRUCTURAL BIOLOGY

Luminol and Quinine are photon calibration standards

Lecture 5. The Structures of Bioluminescence Proteins

The biochemist's articles of faith:
1. The spatial structure of proteins is determined by the **sequence** of amino acid residues comprising the protein polymer.

2. The binding site **environment** modulates the properties of a bound molecule, for ultimate benefit to the organism.

3-Dimensional Structure of Proteins

Determination of the spatial structure of your favorite protein usually will lead to significant advances in understanding its function.

The Protein Data Bank (**PDB**) contains about 55,000 structures most by X-ray crystallography. 10% are determined by Nuclear Magnetic Resonance (NMR).

Crystallography or NMR?

NMR
1. Protein structure is in solution state
2. Needs recombinant protein isotope enriched: ^{13}C, ^{15}N
3. Mass limit < 30 kDa

X-ray
1. Crystal state usually at 77K.
2. Needs a high quality single crystal > 0.1 mm dimension.

Magnetic Nuclei

- The nuclei of these isotopes are magnetic. ^1H ^{13}C ^{15}N ^{31}P
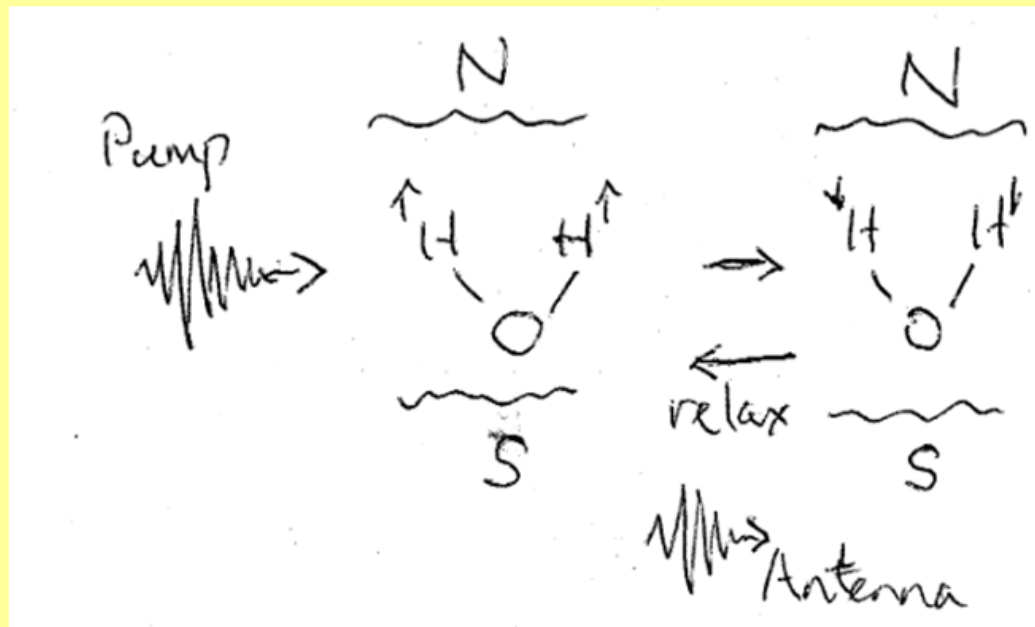
- Molecules will
align in a in a strong field:
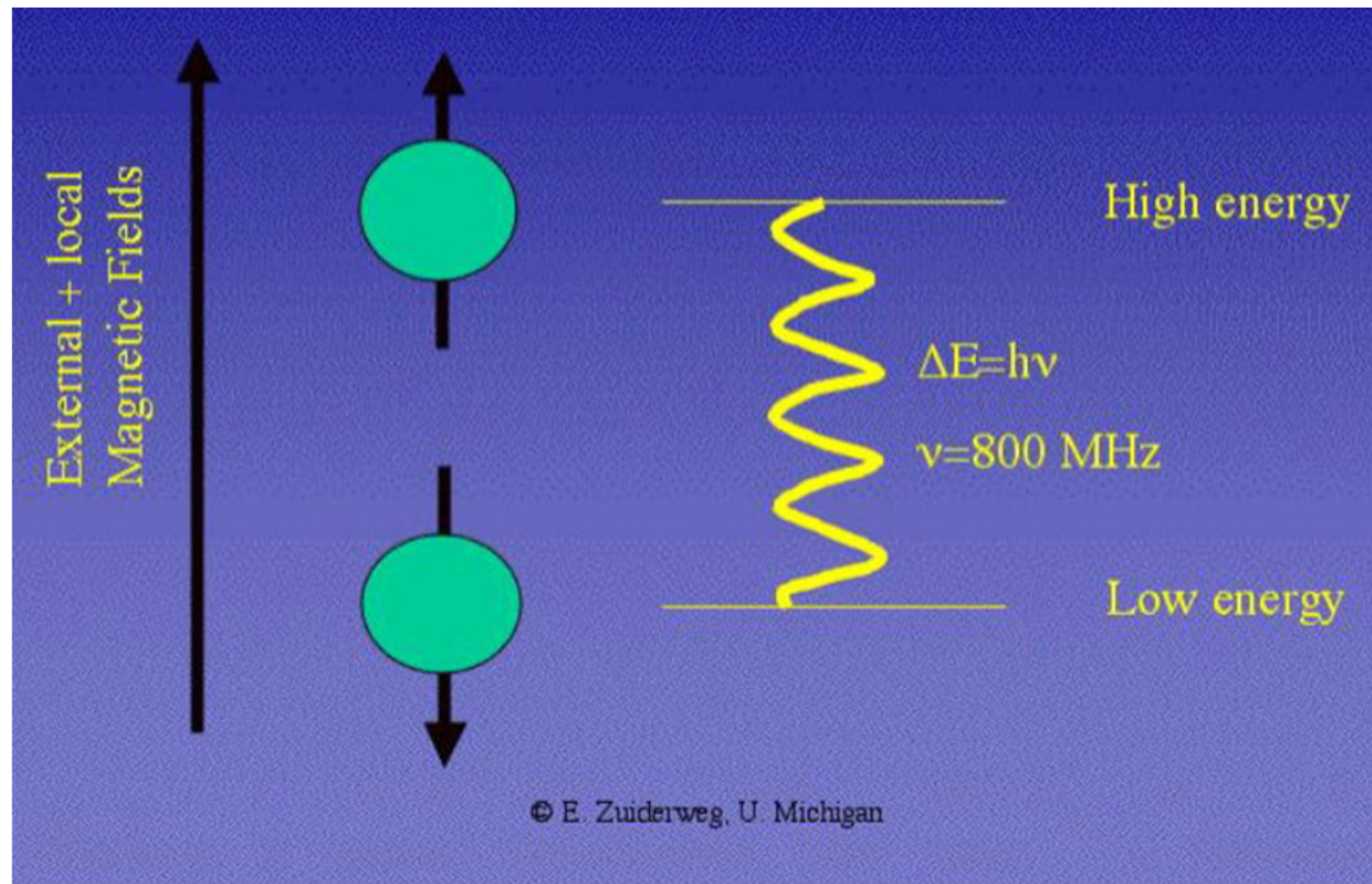
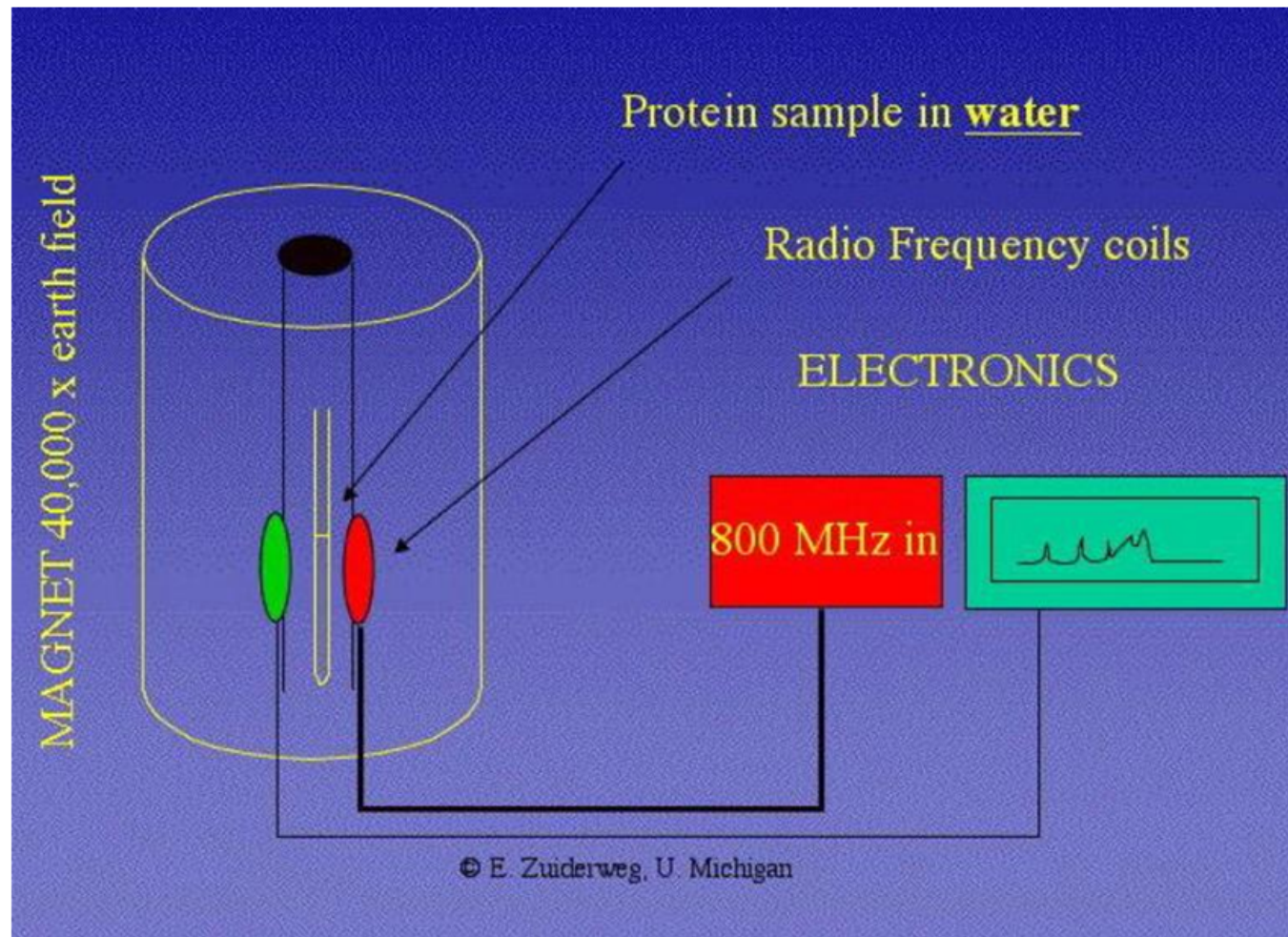# 900 MHz NMR Spectrometer

University of Georgia

Principle of NMR

Shine in a radio-frequency pulse of
exactly the right frequency = **resonance**,
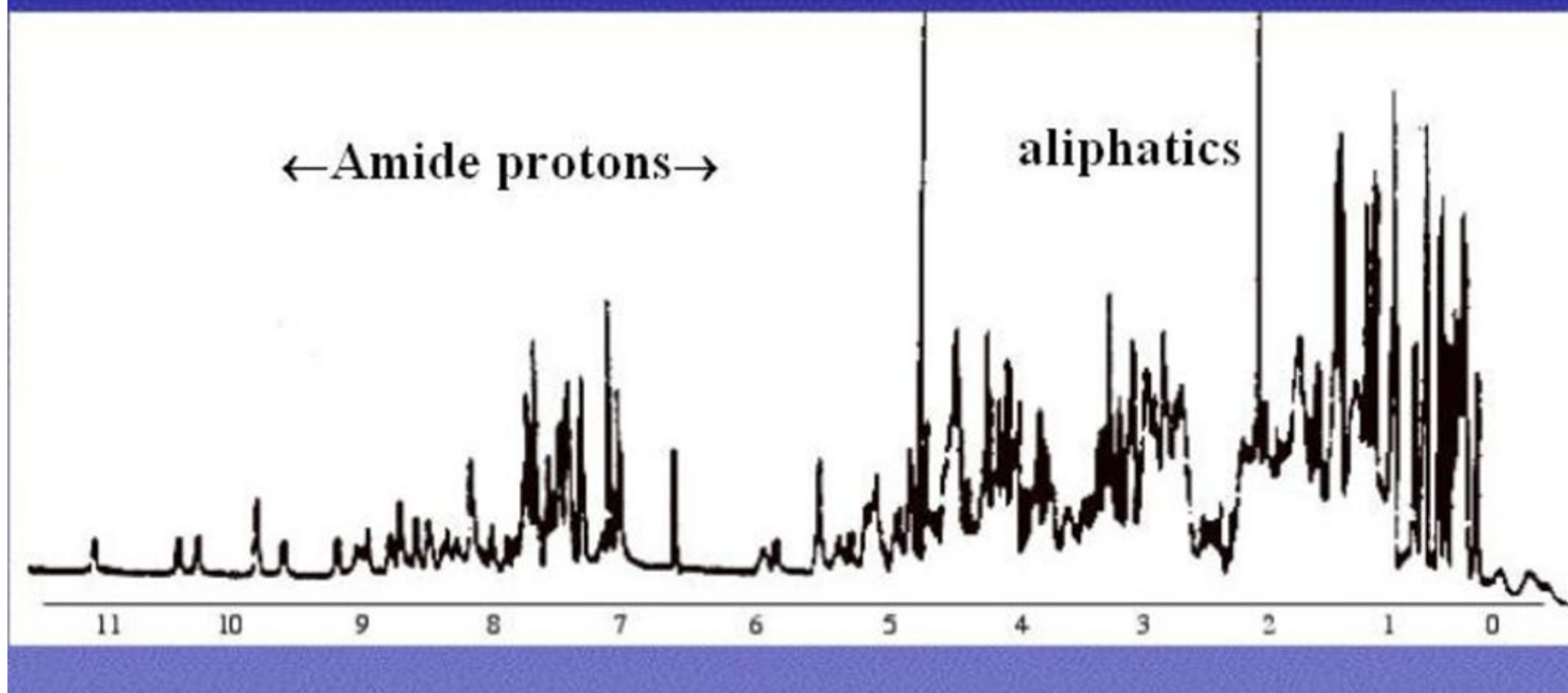will flip the nuclear magnets

Reverse the proton magnet

External + local Magnetic Fields

High energy

$\Delta E = h\nu$

$\nu = 800$ MHz

Low energy

© E. Zuiderweg, U. Michigan

NMR Spectrometer

Protein sample in **water**

Radio Frequency coils

ELECTRONICS

MAGNET 40,000 x earth field

800 MHz in

© E. Zuiderweg, U. Michigan

Every nucleus has its own resonance

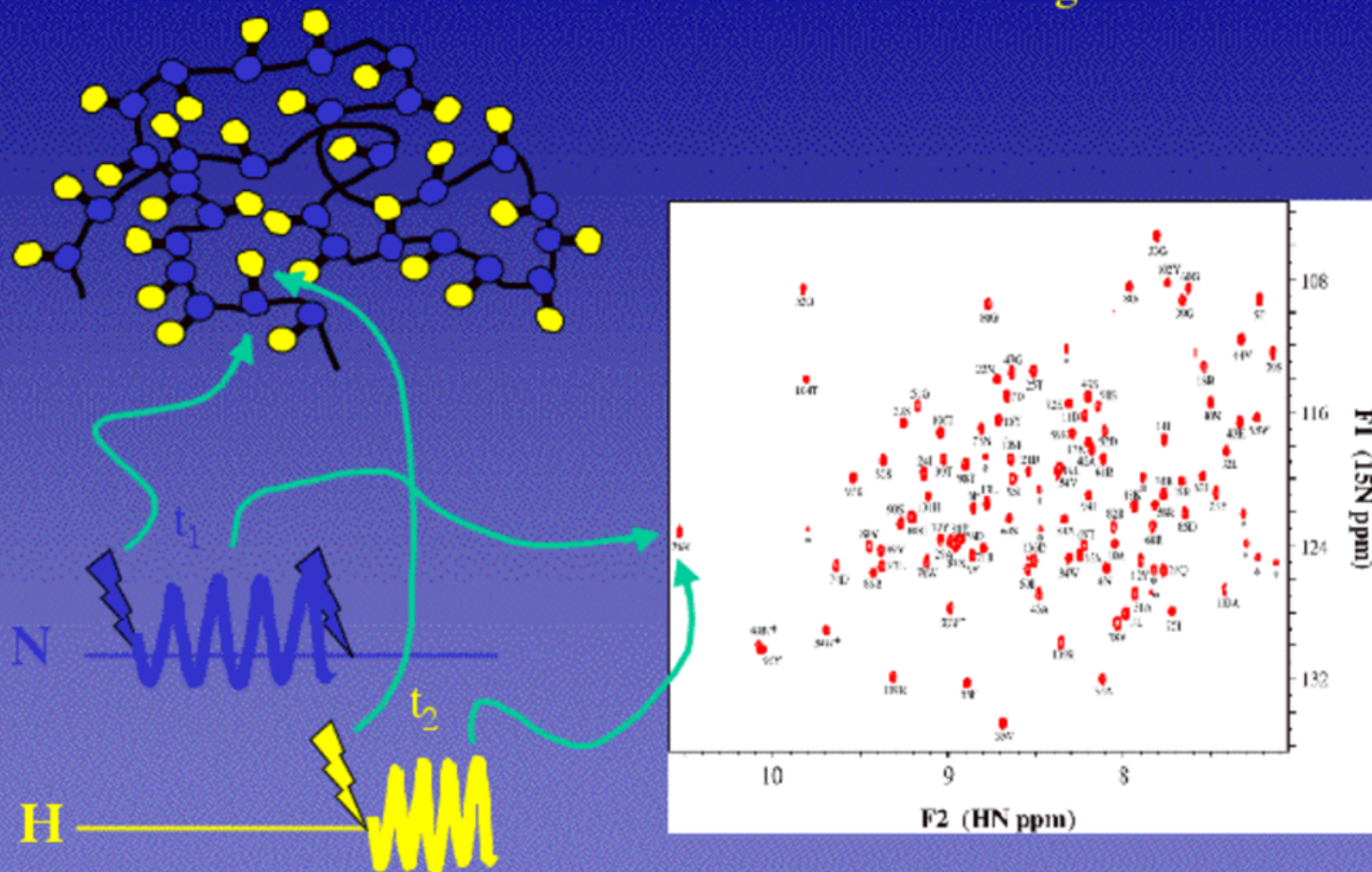←Amide protons→   aliphatics

Parts per million (ppm)

Multinuclear Interactions

• The magnetic property of each nucleus will also influence each other.
• Clever editing of signals called "NMR Experiments" can untangle these.
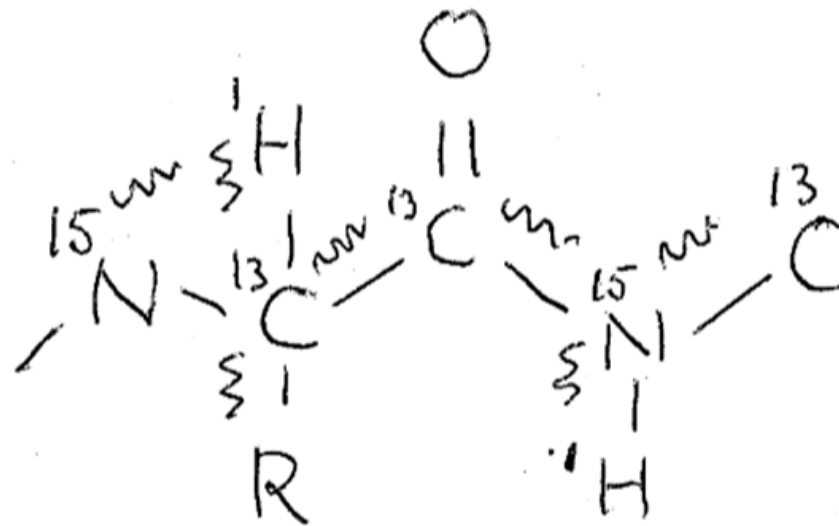
HSQC-NMR GIVES ONE 2D PEAK FOR EVERY NH

And forms the base to modern resonance assignment
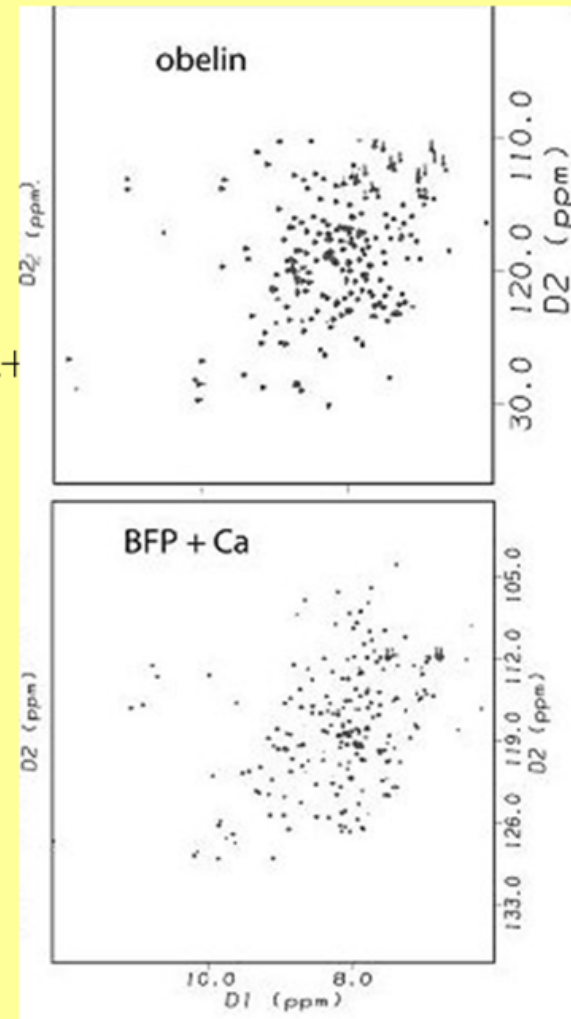

© E. Zuiderweg, U. Michigan

2-Dimensional NMR of Obelin

$^{15}N \Leftrightarrow {}^{1}H$ interactions
          are mapped here

1. Obelin before adding Ca^{2+}

2. After bioluminescence

Ca^{2+}-discharged obelin

Structure of Bioluminescence Proteins

The majority of protein spatial structures have been determined by **X-ray crystallography.**
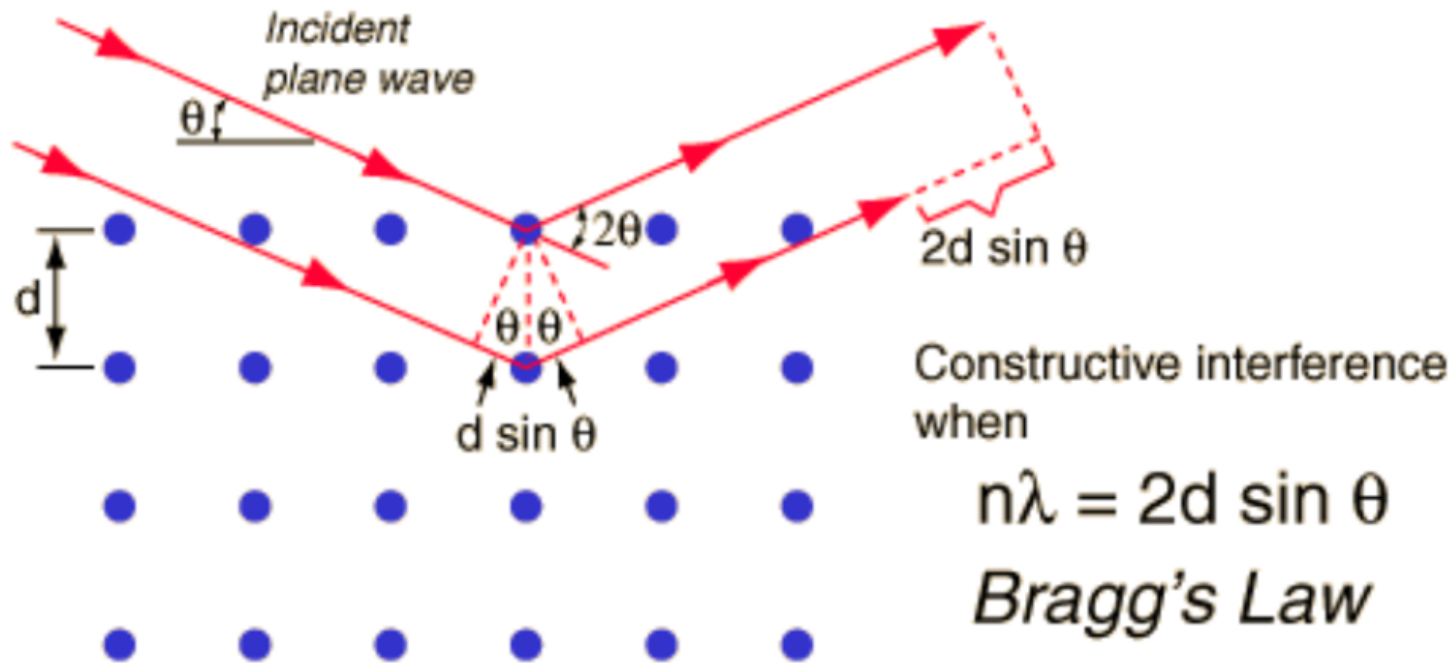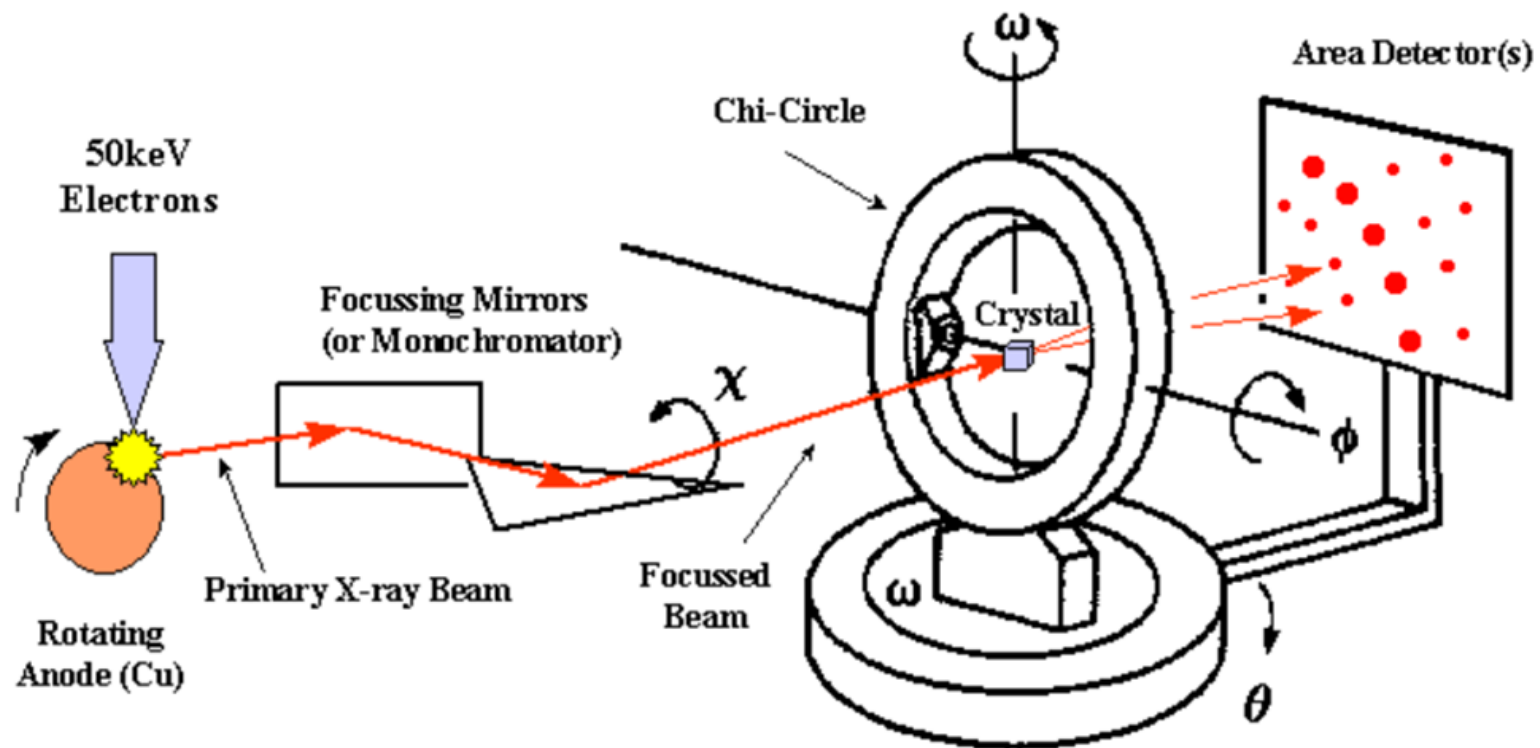
Here is a short tutorial:
http://ruppweb.dyndns.org/

click on "My old Crystallography 101"

Principles of X-Ray Diffraction

Bragg's Law (1913)

Incident plane wave

θ

2θ

$2d \sin \theta$

$\theta \; \theta$

$d \sin \theta$

d

Constructive interference when

$$n\lambda = 2d \sin \theta$$

Bragg's Law

X-Ray Method

50keV Electrons

Chi-Circle

Area Detector(s)

ω

Focussing Mirrors (or Monochromator)

Crystal

χ

ϕ

Primary X-ray Beam

Focussed Beam

Rotating Anode (Cu)

ω

θ

4-Circle Goniometer (Eulerian or Kappa Geometry)

The Crystal

To apply crystallography you only need:

1. Clone and **express** the protein so you can purify amounts > 10 mg.
2. Single crystals > 0.1 mm; usually obtained by methods of "**black magic**".
3. Well equipped X-ray lab at home and access to one of the international (>**$1B**) light sources (Japan, US, UK, Germany).
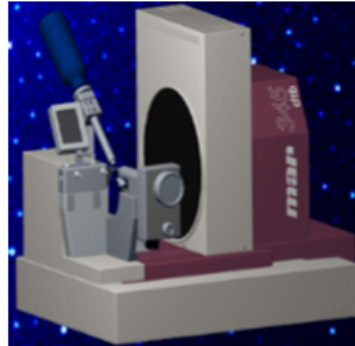
Advanced Photon Source, Argonne
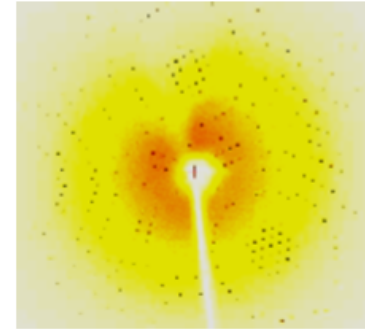
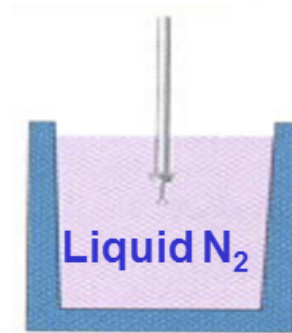# Crystallographic experimental procedure
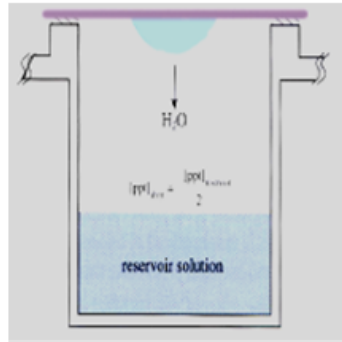
Crystallization
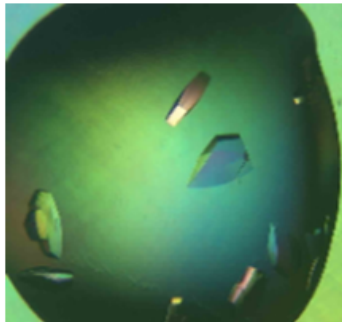
Data collection

Diffraction

Crystals

Liquid N$_2$

Freezing

Computational
Crystallography

OL-obelin Crystal

Obelin from *Obelia longissima*

Space group: $P6_2$
Cell parameters: a = 81.62 Å
c = 86.12 Å
Resolution: 1.73 Å
Dimensions 0.1x0.1x1.0 mm

3-D Bioluminescence

• Available structures of proteins can be viewed and manipulated interactively in the **Protein Data Bank**:
www.wwpdb.org/

• Click RCSB PDB, type keyword or PDB code, then click "View Structure".

Bioluminescence Structures

1995-2011

Bacterial luciferase from *Vibrio*
Firefly luciferases
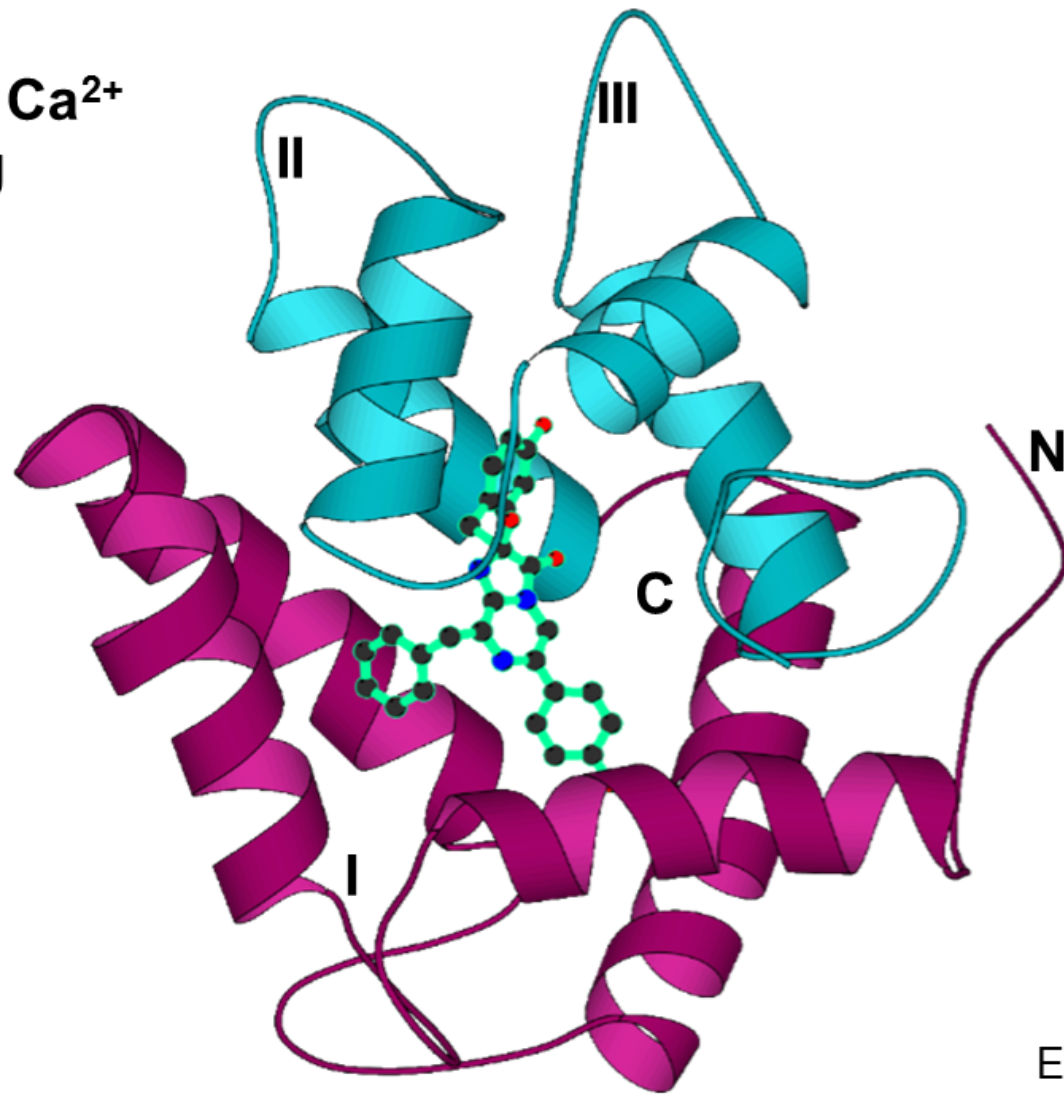Green-fluorescent Proteins (GFP)
Aequorin, obelins, clytin
Lumazine Protein
Dinoflagellate luciferase
Renilla luciferases
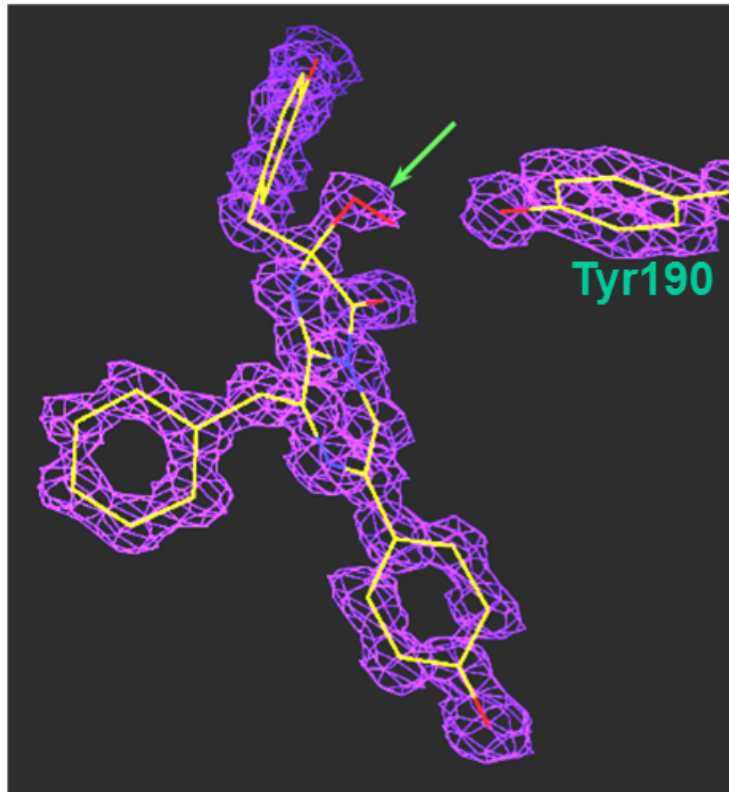*Renilla* coelenterazine binding protein

Structure of obelin at 1.73 Å

I-III are Ca²⁺ binding loops

II

III
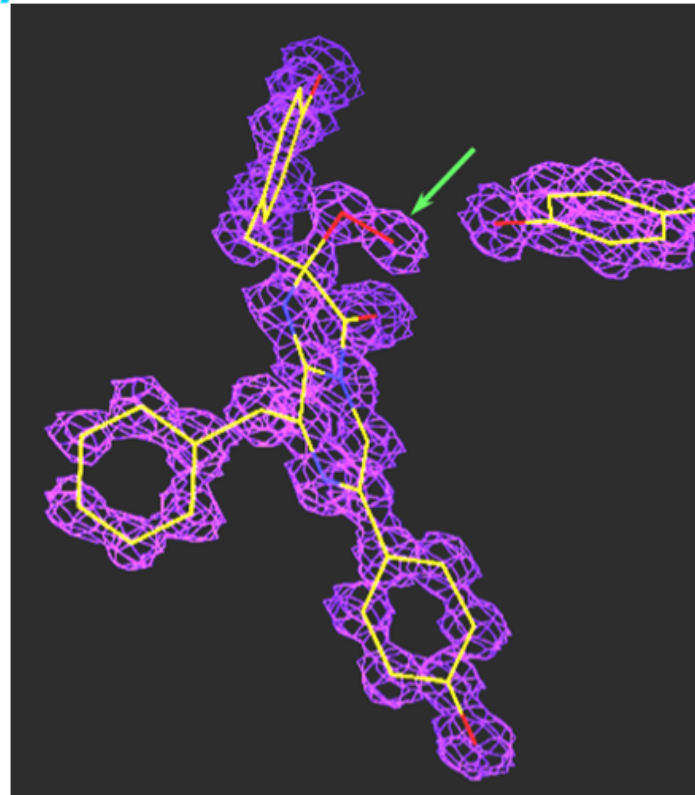
N

C

I

EF-hand motif

Liu et al., Protein science 9, 2000

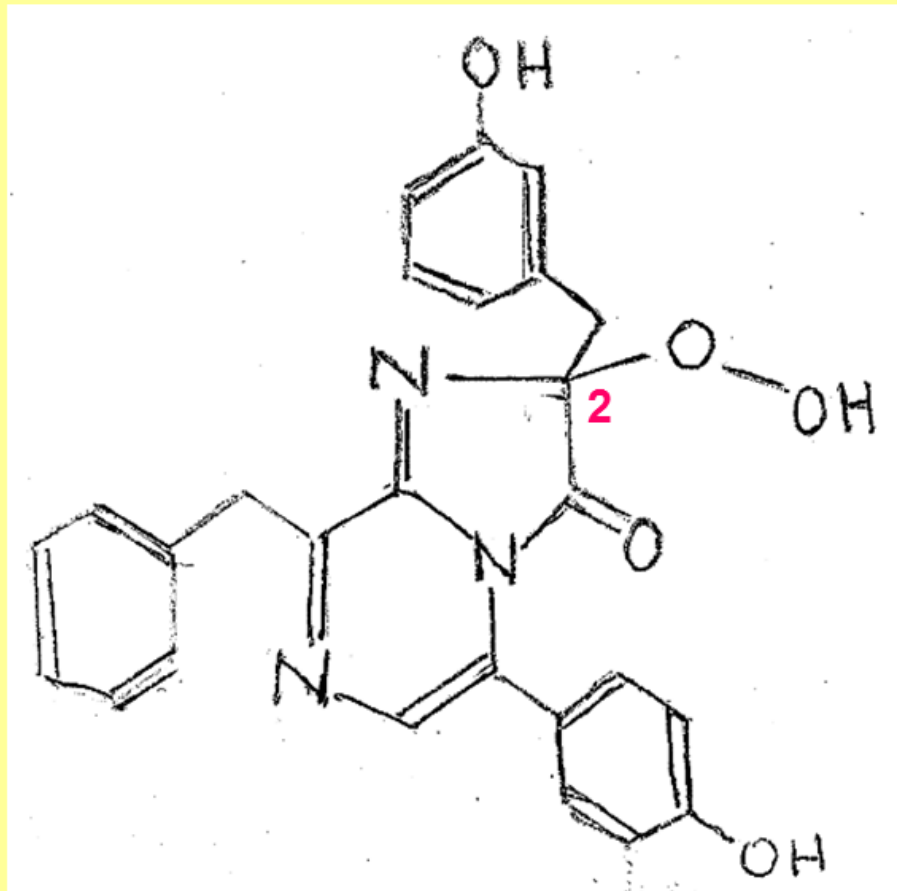# Coelenterazine-oxygen complex covered by electron density map at atomic resolution (1.0 Å)
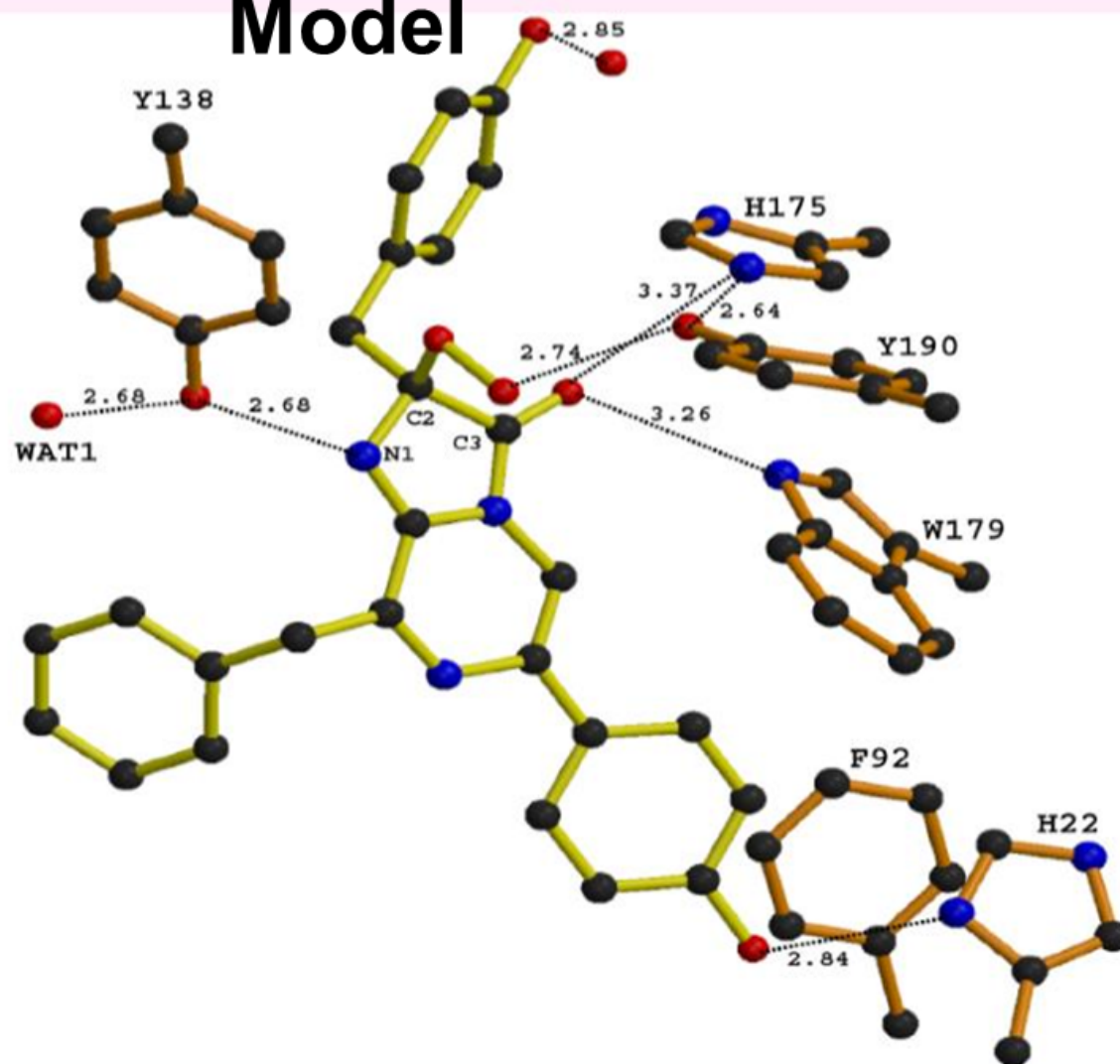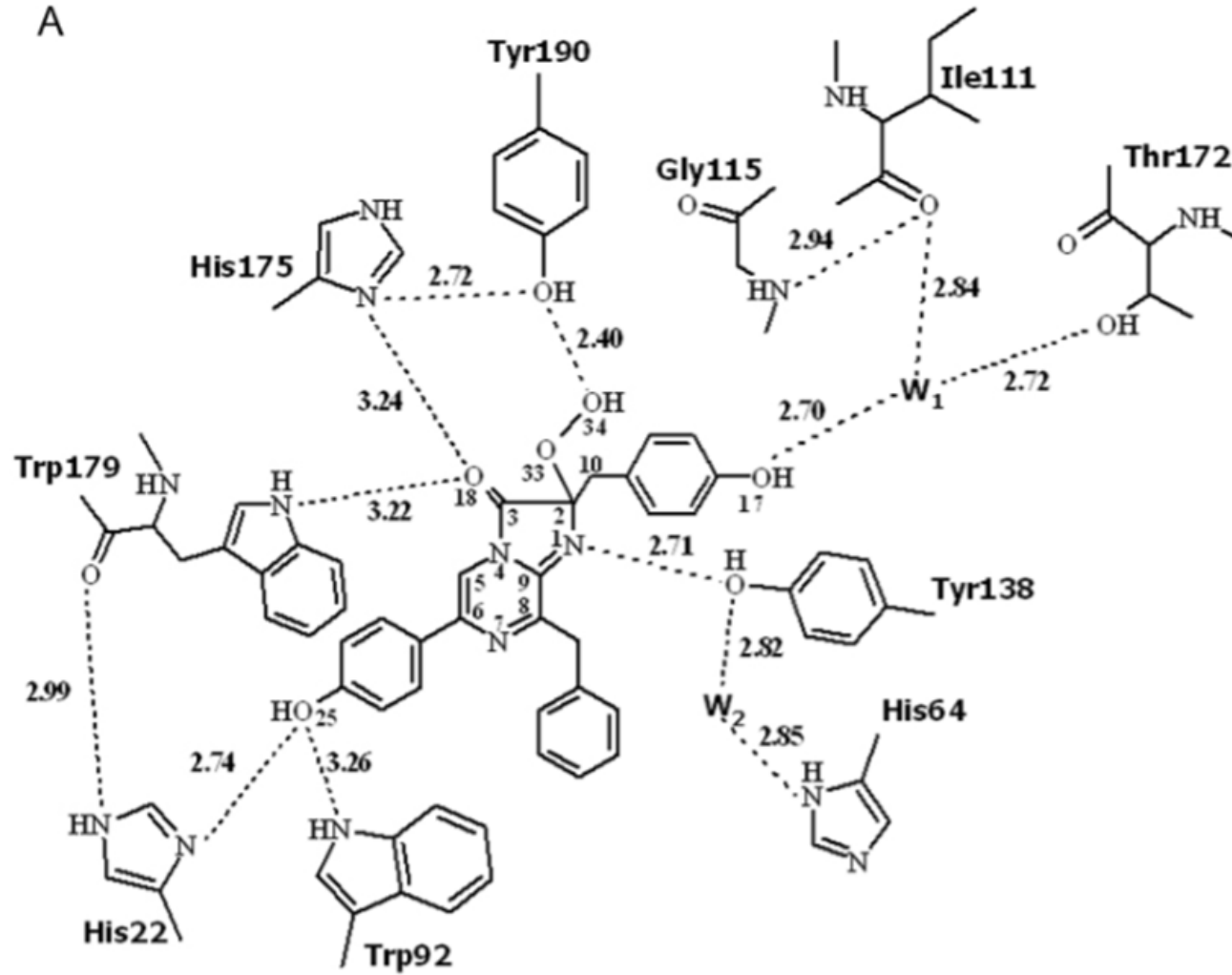

obelin

After binding one calcium

Coelenterazine-OOH

Ball and Stick Model

Hydrogen Bond Network .....

Conclusions

• Coelenterazine lies in a protein cavity •
Electron density nearby its 2-position
shows peroxy substitution.
• Hydrogen bonds between atoms of
coelenterazine side chains stabilize the
peroxy • And also modulate excited state
energy level of the emitter.

GFP Shifts Photoprotein Bioluminescence

Inclusion of clytia GFP in the bioluminescence from the Ca^{2+}-regulated photoprotein clytin, shifts the spectrum to the GFP fluorescence. The mechanism is by <u>Förster Resonance Energy Transfer</u> (**FRET**).
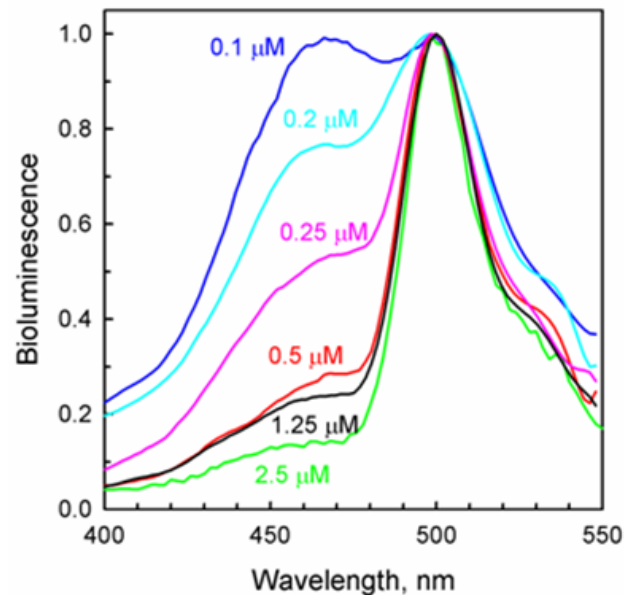
Markova 2010

Spectral Overlap

The large overlap of the blue bioluminescence of clytin with the black GFP absorbance (black), favors the resonance energy transfer.
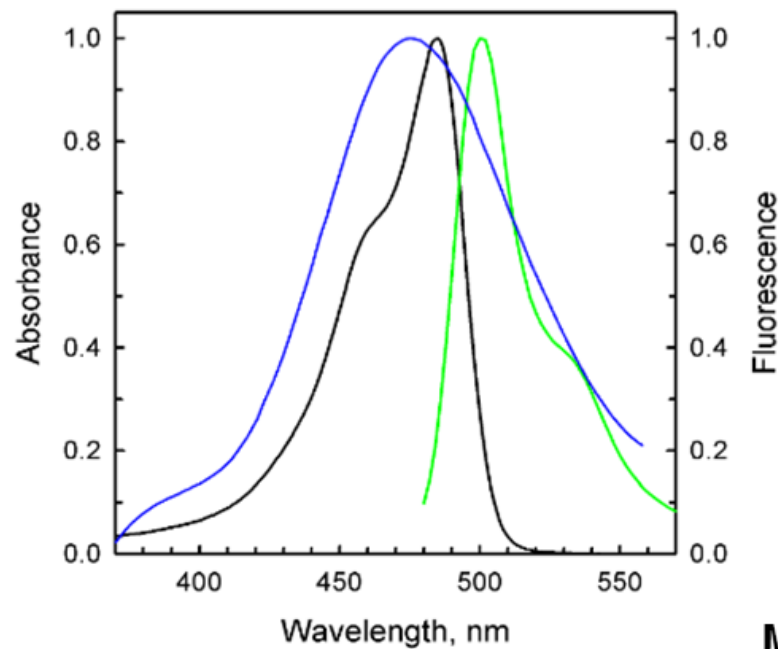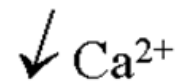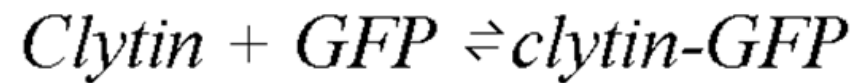

Markova 2010
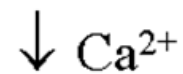
A Clytin-GFP protein-protein complex?

- FRET requires a donor-acceptor separation < ~50 Å
- At 1 μM, free clytin and GFP ~ 1000 Å
- Where is this K_D~1 μM FRET complex?
- **Observed** protein- protein equilibrium

K_D~1 mM!

Hypothesis #1

A complex forms before reaction:

$$Clytin + GFP \rightleftharpoons clytin\text{-}GFP$$

$\downarrow$ Ca²⁺ $\downarrow$ Ca²⁺

blue   green
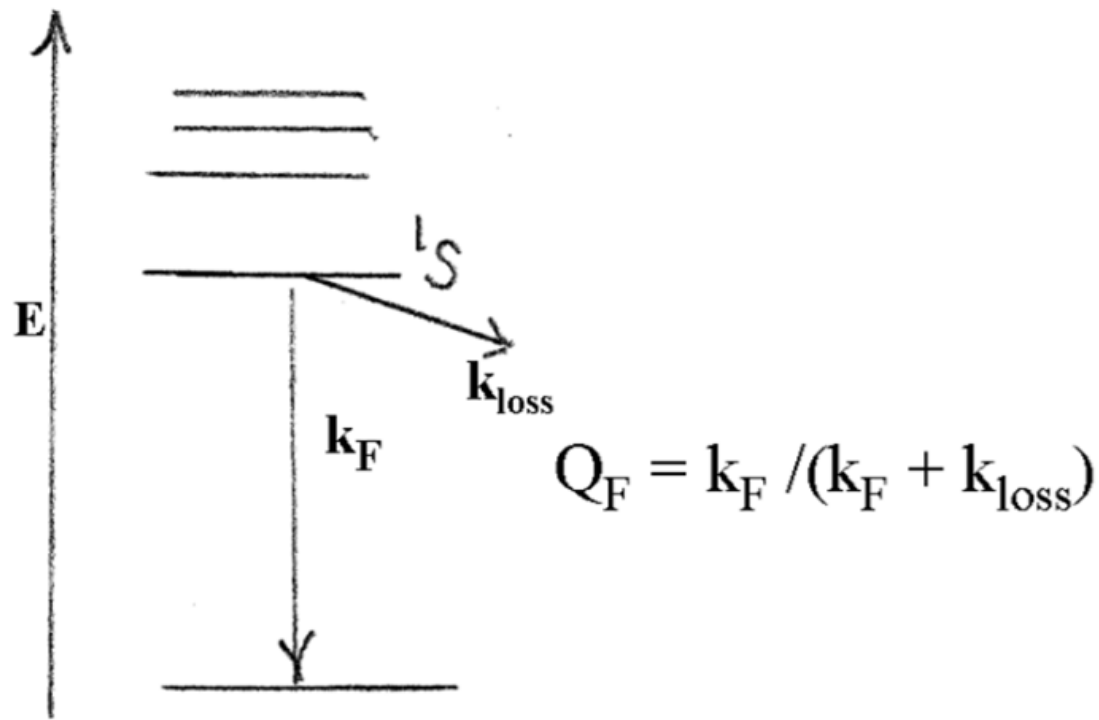
Hypothesis #1 **FAILS**

Hypothesis #2

A complex will be found with the reaction product.

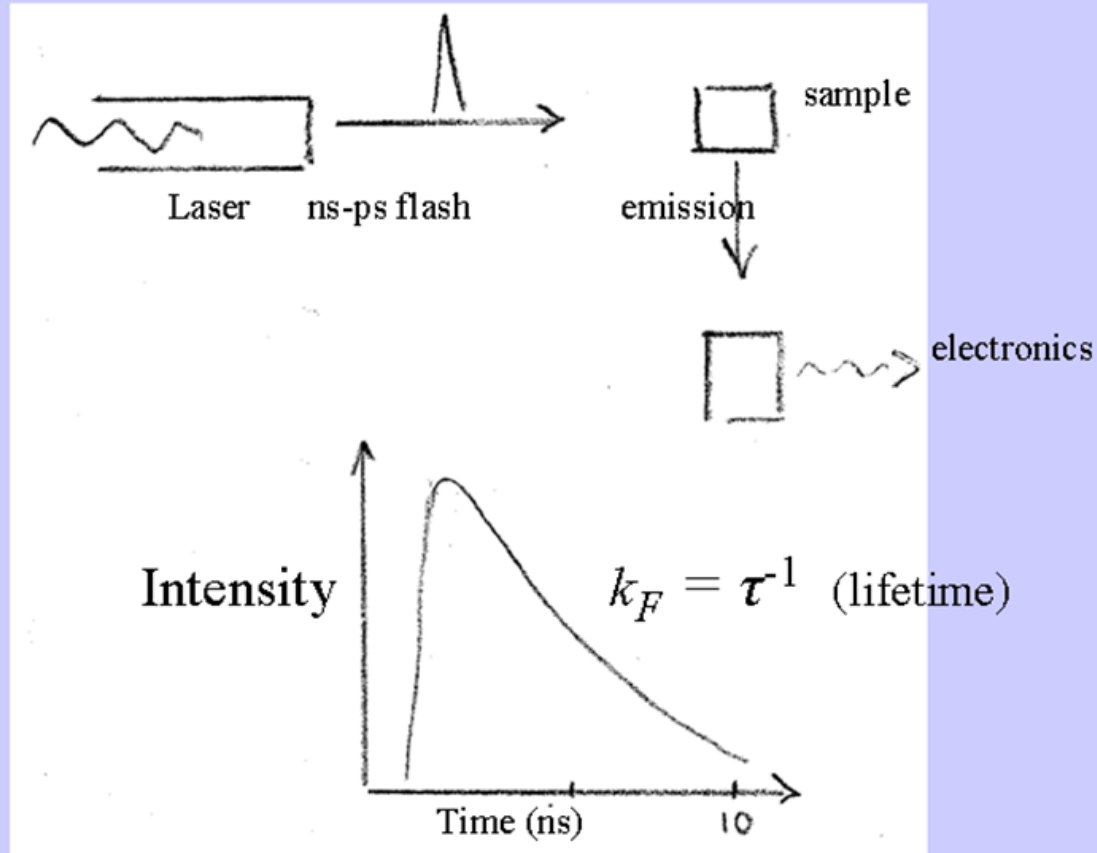Ca^{2+}-discharged clytin + GFP $\rightleftharpoons$ complex

We'll test this hypothesis for complexation using **fluorescence dynamics.**

Fluorescence Dynamics

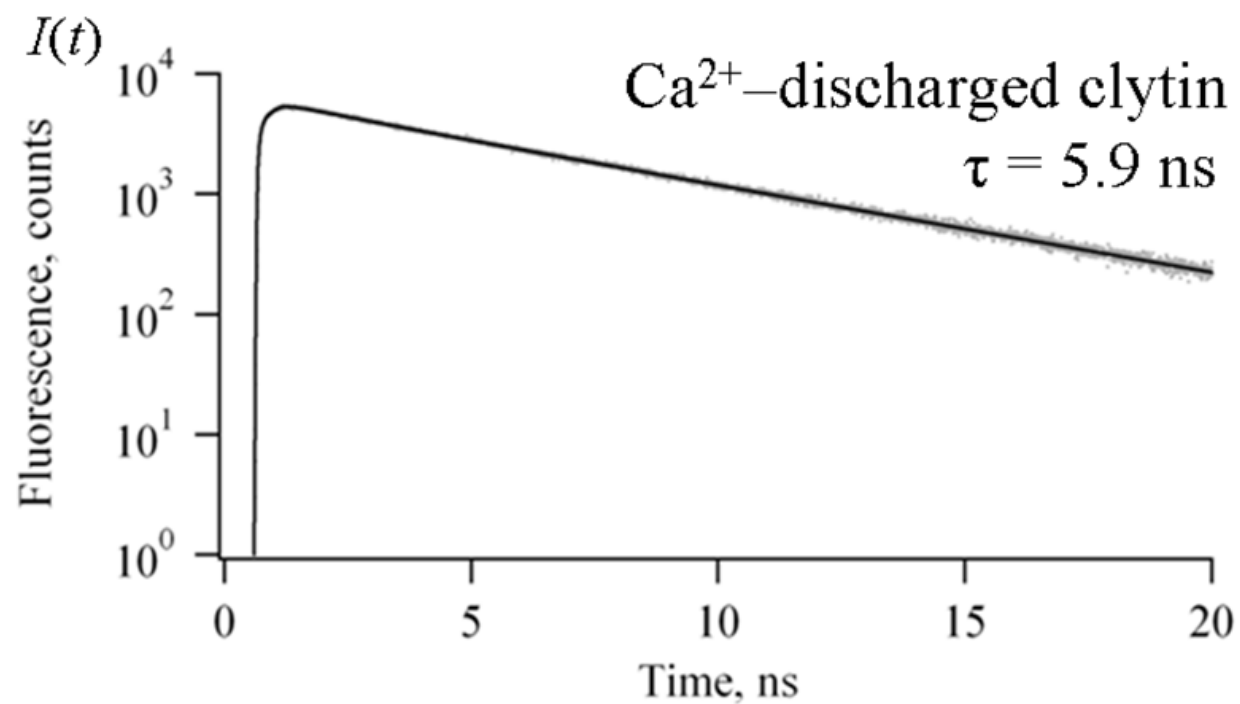Fluorescence quantum yield, Q_F
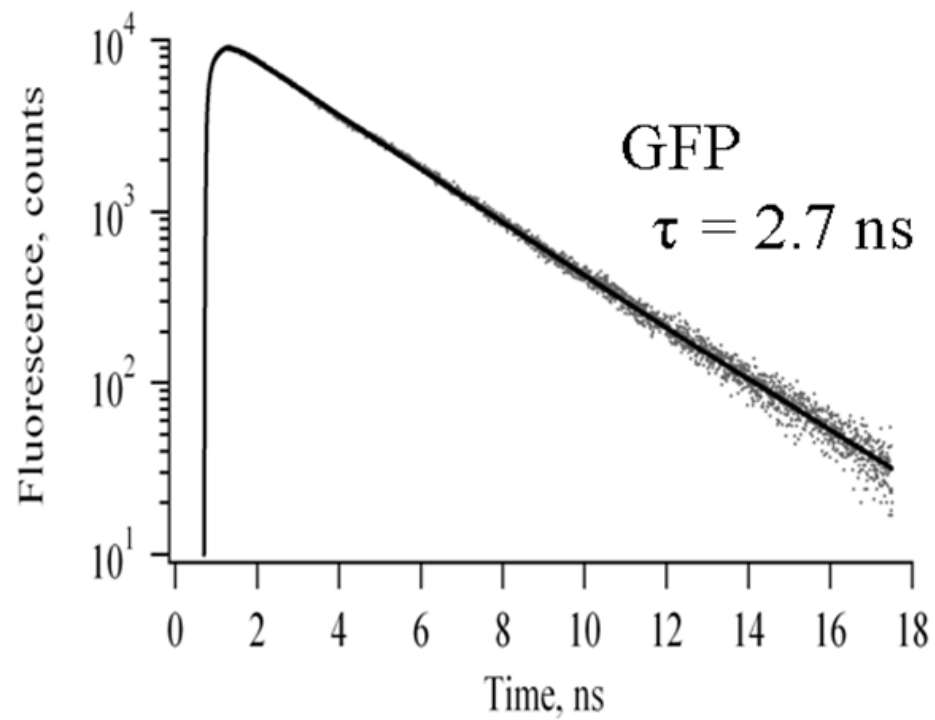
  $\equiv$ probability of photon emission

$$Q_F = k_F /(k_F + k_{loss})$$

Time-resolved Fluorescence

Donor Fluorescence decay

$$I(t) = \exp(-t/\tau)$$

$I(t)$

Ca^{2+}–discharged clytin

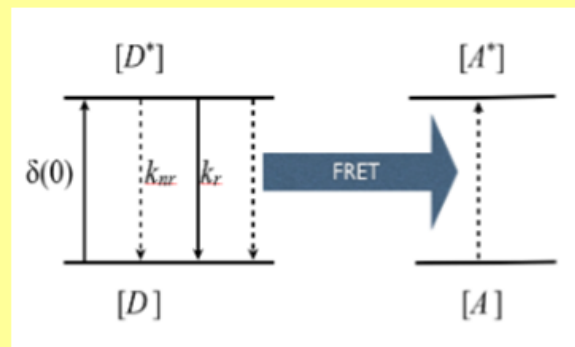$\tau = 5.9$ ns

Malikova 2011

Acceptor fluorescence decay

GFP
$\tau = 2.7$ ns

Malikova 2011

No change in donor lifetime
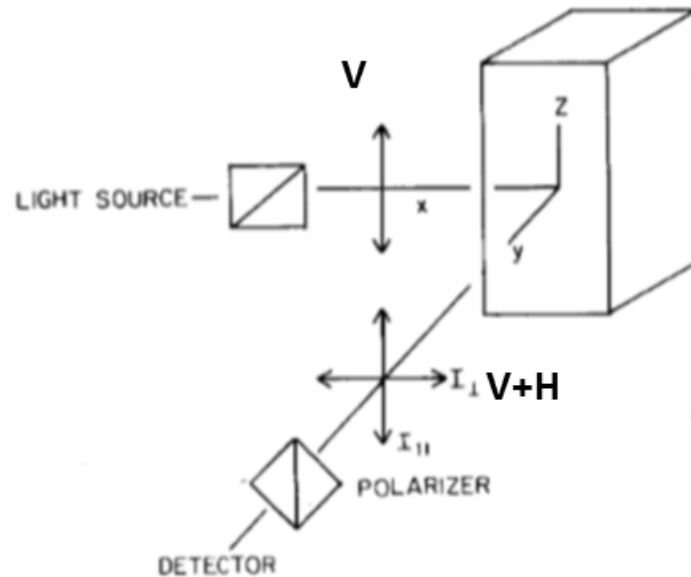

A mixture of Ca^{2+}-discharged clytin and GFP shows the donor decay lifetime the same as donor alone.

$\rightarrow$ No detectable complex!

Fluorescence Polarization

Vertically polarized excitation will result in the fluorescence having both vertical (**V**) and horizontal (**H**) components.

Polarization and Anisotropy

Steady State

$$\text{polarization, } P = \frac{V - H}{V + H}$$
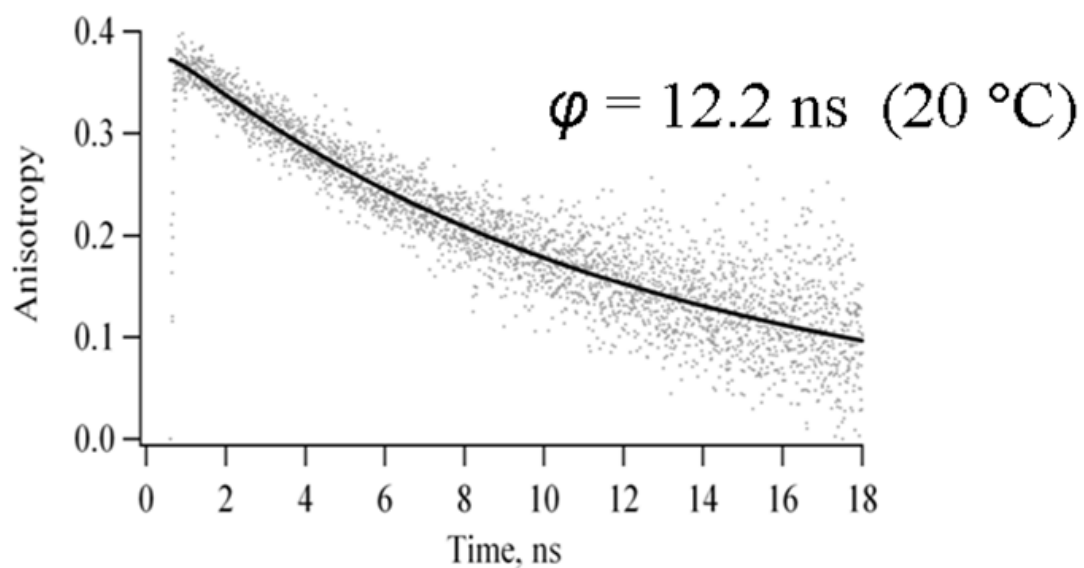
$$\text{anisotropy, } R = \frac{V - H}{V + 2H}$$

Dynamic

$$R(t) = R_0 \exp(-t/\phi)$$

ϕ = rotational correlation time

Fluorescence Anisotropy Decay
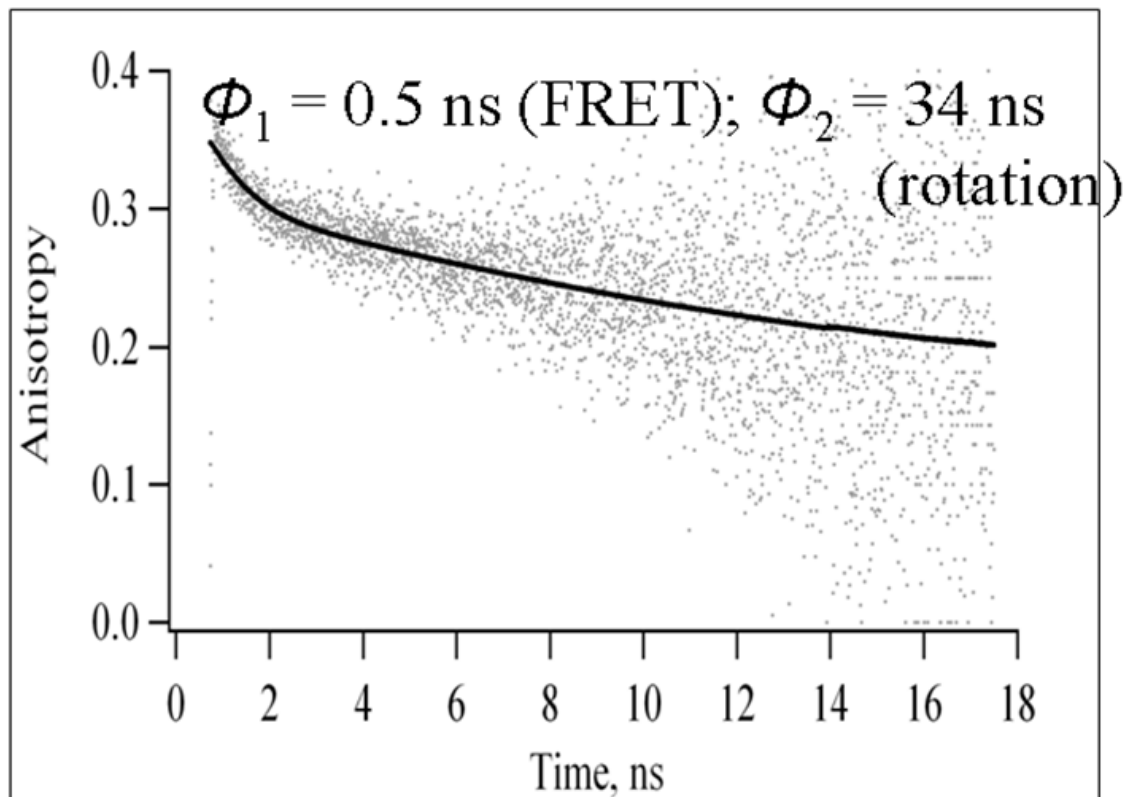
Ca^{2+}–discharged clytin (22 kDa)

$$R(t) = R_0\exp(-t/\varphi)$$

$\varphi = 12.2$ ns (20 °C)

Malikova 2011

Fluorescence Anisotropy Decay

GFP (54 kDa, dimer)

$\Phi_1 = 0.5$ ns (FRET); $\Phi_2 = 34$ ns (rotation)

Malikova 2011

Intra-dimer FRET

Clytia GFP dimer; R(calculated) = 32 Å

$$\varphi_1 = 0.5 \text{ ns}$$
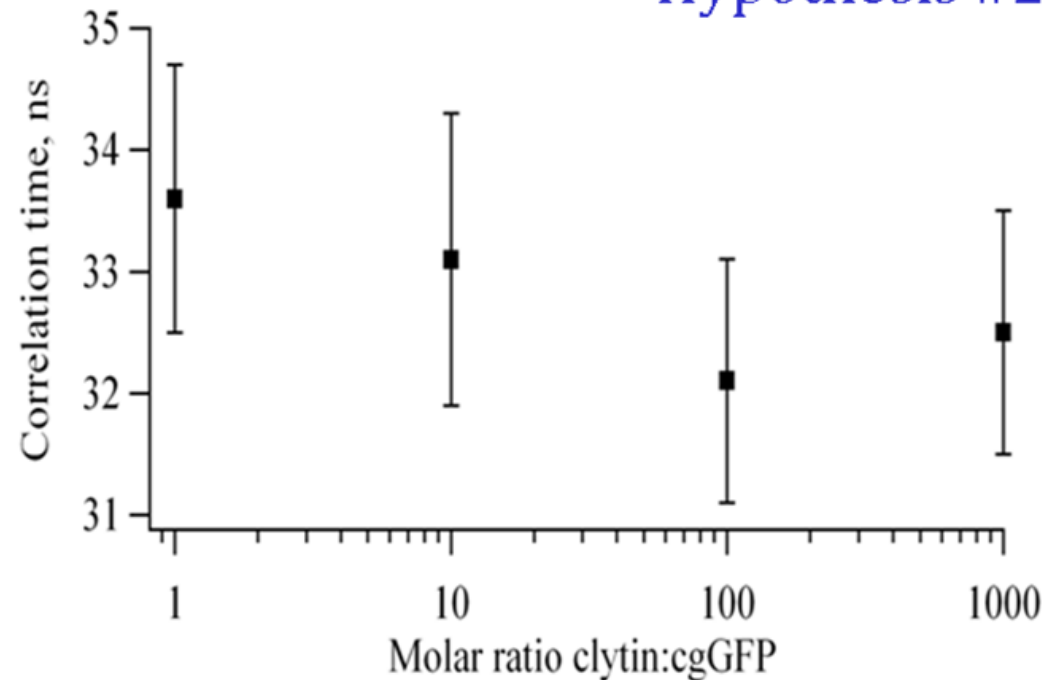

25 Å
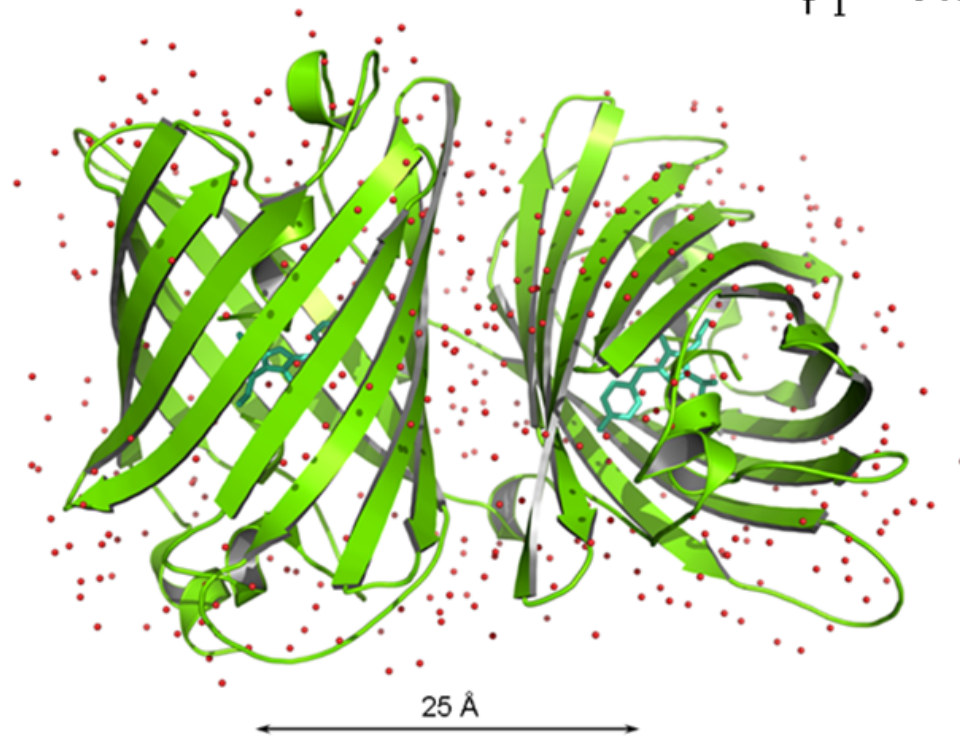
Malikova 2011

Anisotropy Decay Shows No Complex

Ca^{2+}-discharged-clytin + GFP $\rightleftharpoons$?

$\varphi = 12$ $\qquad\qquad$ $\varphi = 34$ $\rightarrow$ $\varphi = 33$ ns

Hypothesis #2 FAILS

Malikova 2011

Hypothesis #3

A transient complex with a reaction intermediate

$$Clytin + Ca^{2+} \rightleftharpoons Ca^{2+}\text{-clytin} \quad (t_{1/2} \sim ms)$$
$$+ GFP \rightleftharpoons Ca^{2+}\text{-clytin-GFP} \rightarrow$$
$$K_D \sim 1 \ \mu M$$

$$\uparrow FRET \downarrow$$
$$\rightarrow Ca^{2+}\text{-discharged clytin*-GFP} \rightarrow \text{Green}$$
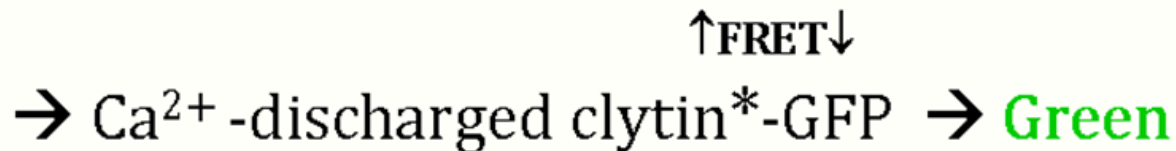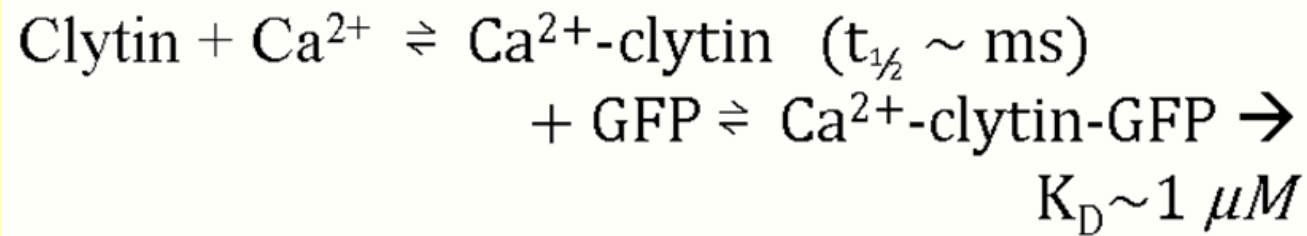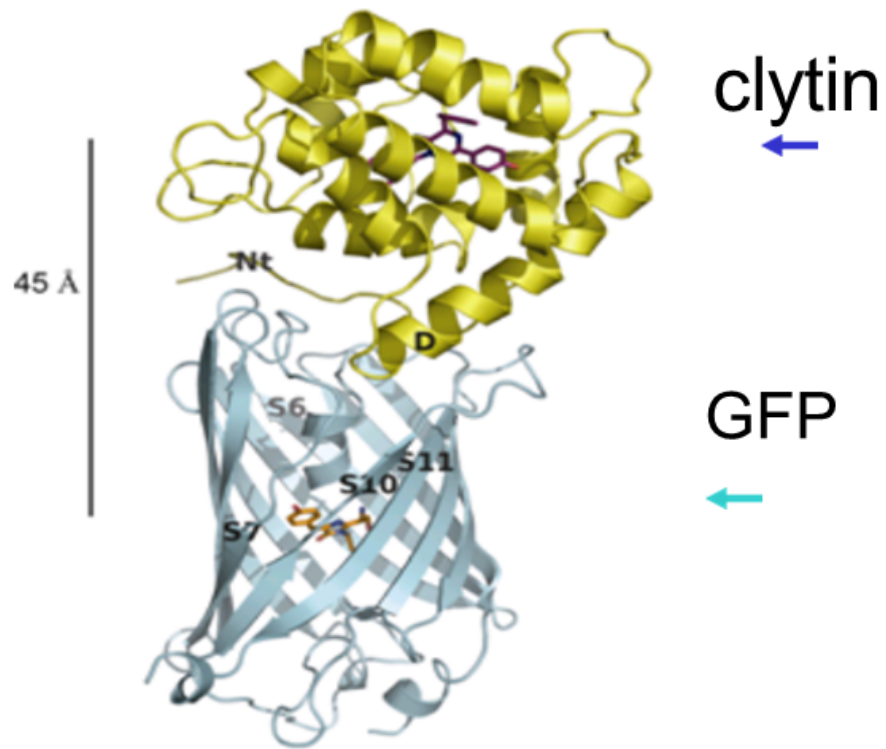
There is analogy to the antenna function of LumP

NMR detection of clytin-GFP complex

The ^{15}N-HSQC resonances from only some amino acid residues are shifted on mixing the two proteins. This locates the weak protein-protein binding site.
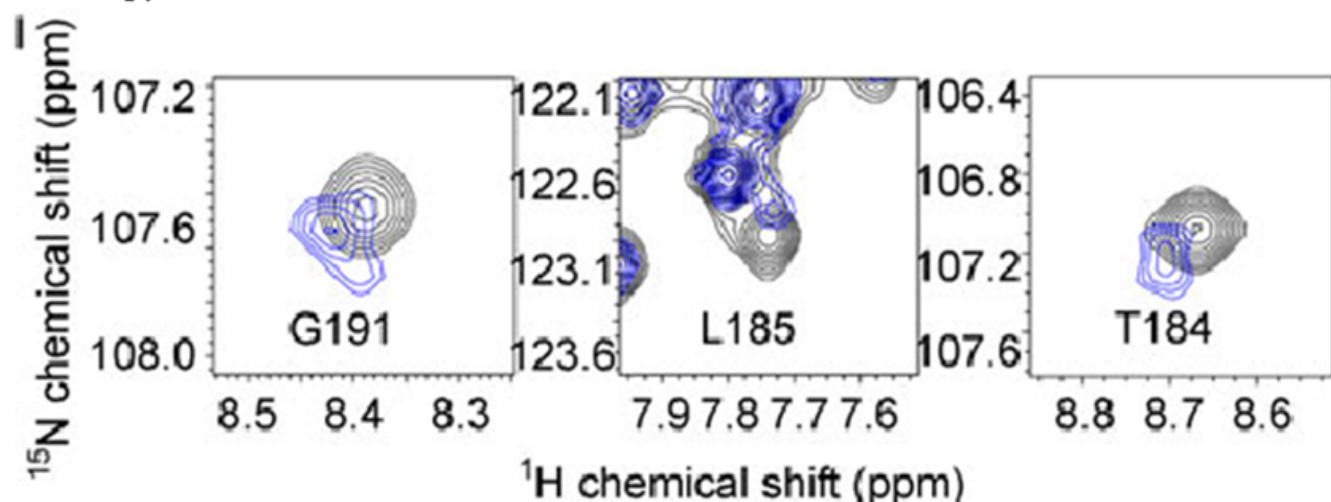
GFP-photoprotein model

$K_D \sim 1$ *mM* (dimer)

clytin

GFP

45 Å

Titushin (2010)

Luciferases from Coleoptera

1. To discover how structure determines beetle bioluminescence colors.
2. Locate the Lase binding site.
3. Are there charged groups positioned to shift the resonance forms?
4. Locate hydrogen bonding interactions of oxy-Luciferin.

The Active Site

• (2006) a team at **Riken** (Japan) confirmed this location of the active site in FF-Lase.

• Determined the structure of FF-Lase (2DIR) bound with a substrate **analog** (DLSA) and a second structure with the **products** FF-L=O and AMP.

• LH$_2$-AMP can't be used because it would react, but it is safe to assume that the analog occupies the same active site.
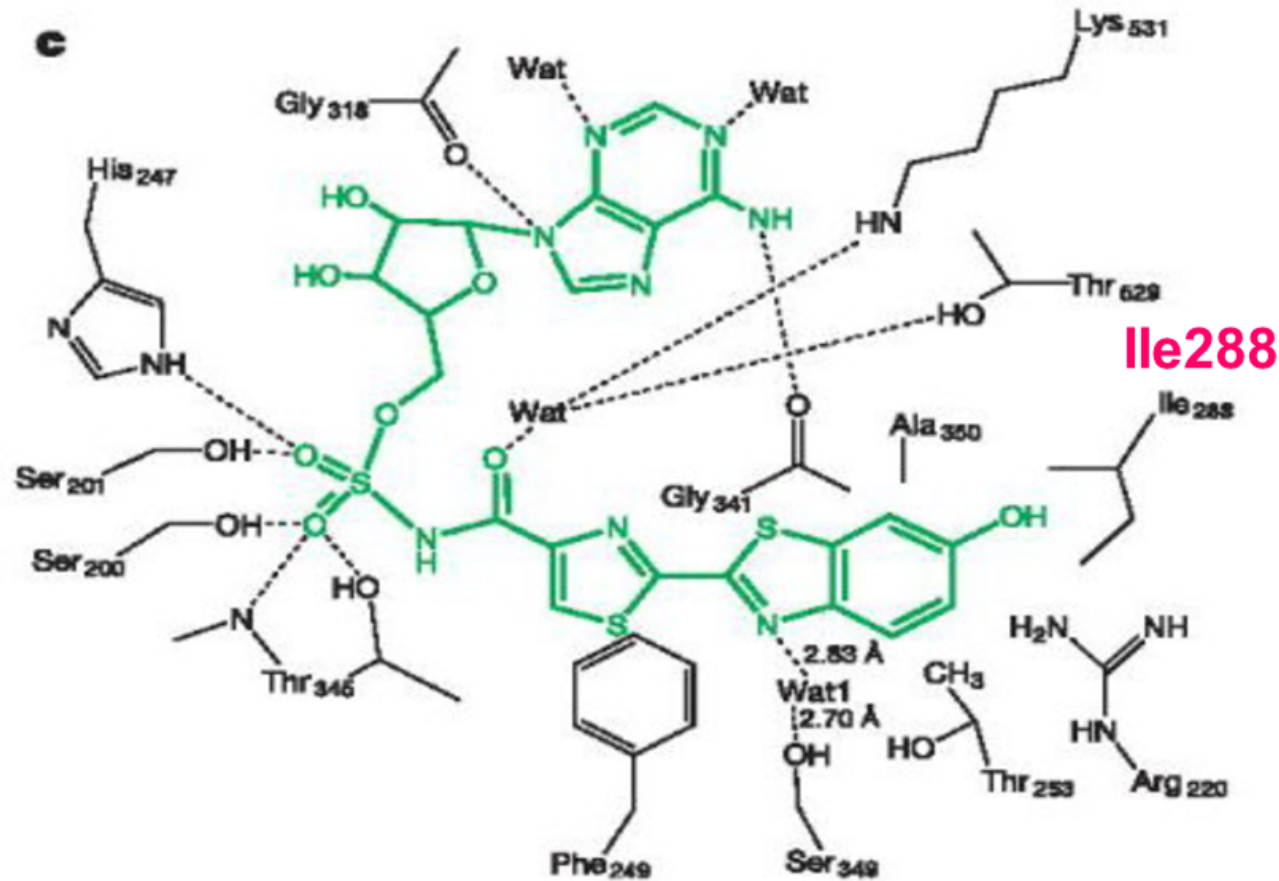
RIKEN Structure

DLSA→
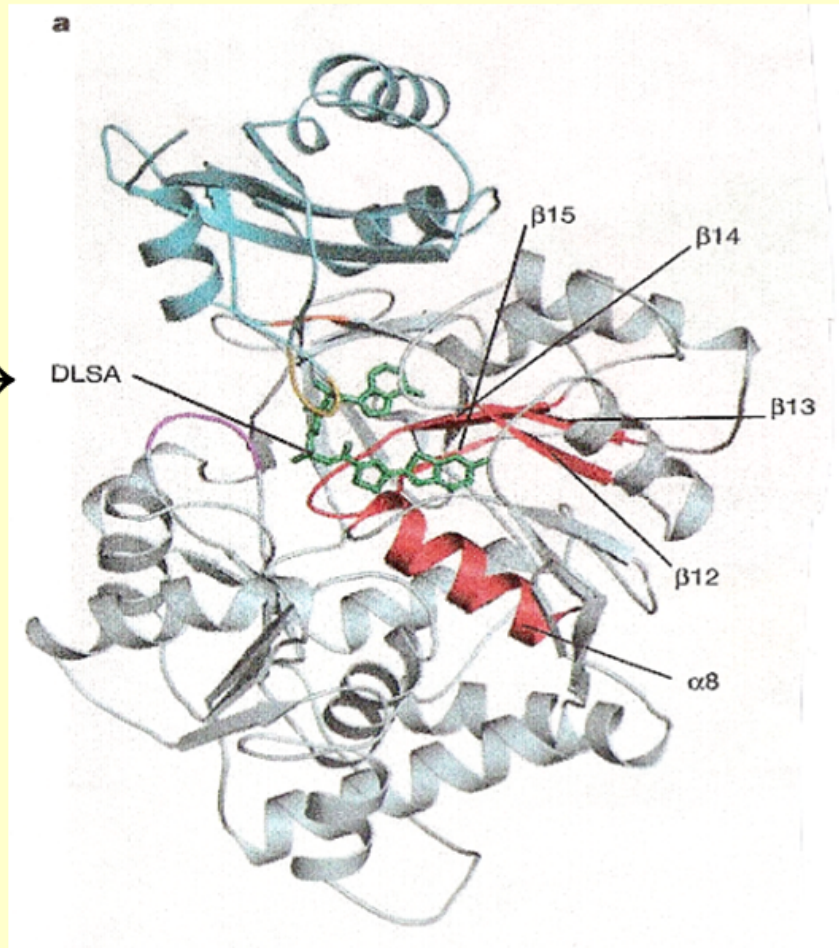
FF-Luciferin Analog

Luciferyl•AMP
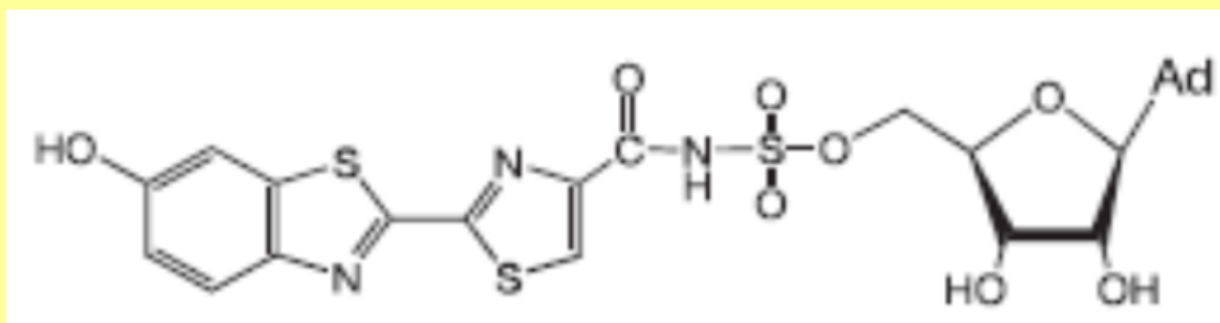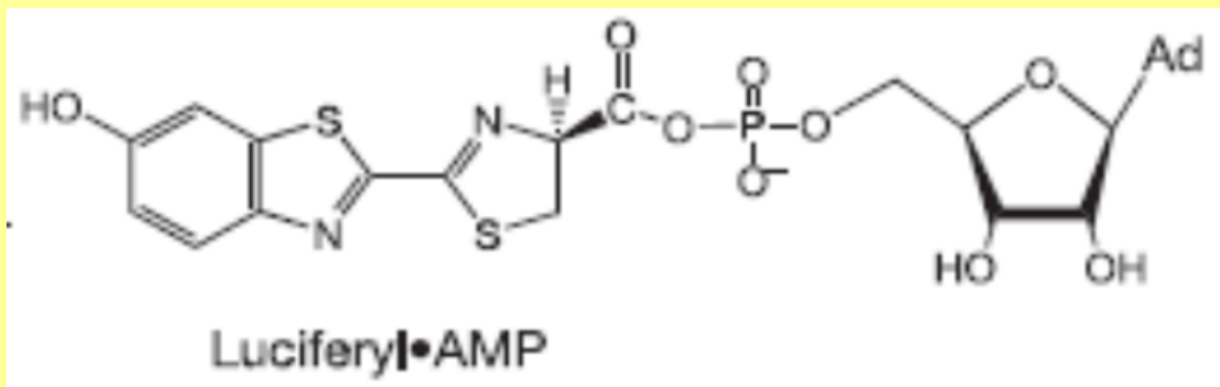
DLSA

Two-D Picture

A 2-D layout of the active site can help visualize interactions.

Conclusion-1

In the FF-Lase active site there are no charged side groups located in suitable positions that would lead to the postulated effect on the change in the resonance forms of L=O.

Conclusion-2

The structure of FF-Lase mutant S286N which gives red BL, was compared to the native form (wild-type, WT) which gives yellow, both containing FF-L=O and AMP. In the WT it was seen that the product is held much more rigidly than in the red mutant.
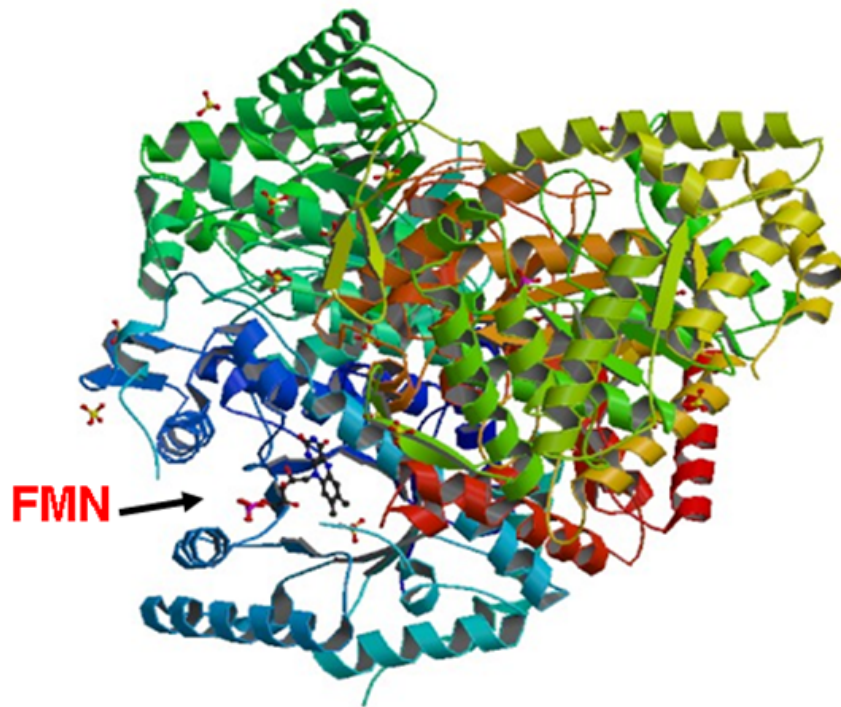A **conformational restriction** of the excited product would explain variations in energy levels for the yellow emission among the types of beetle luciferases.

Sad Conclusion-3

The mechanism of the bioluminescence color shift in the Coleoptera, still remains to be illuminated.
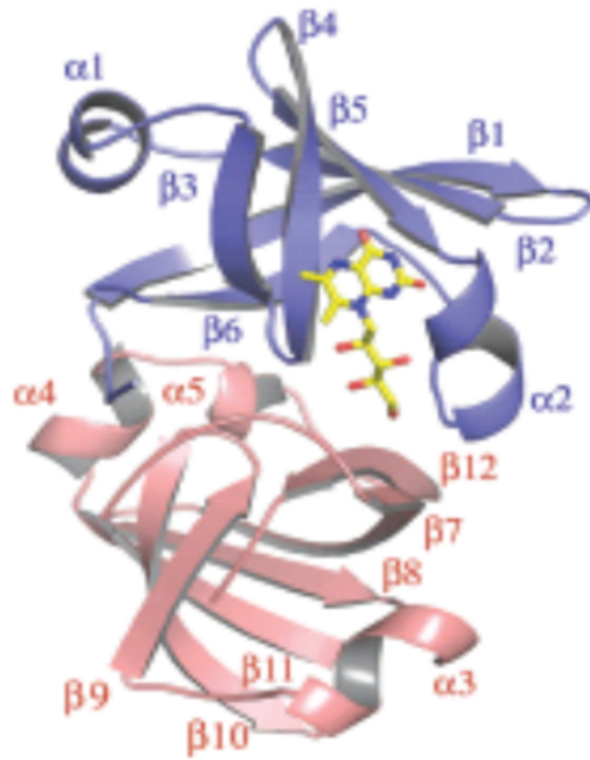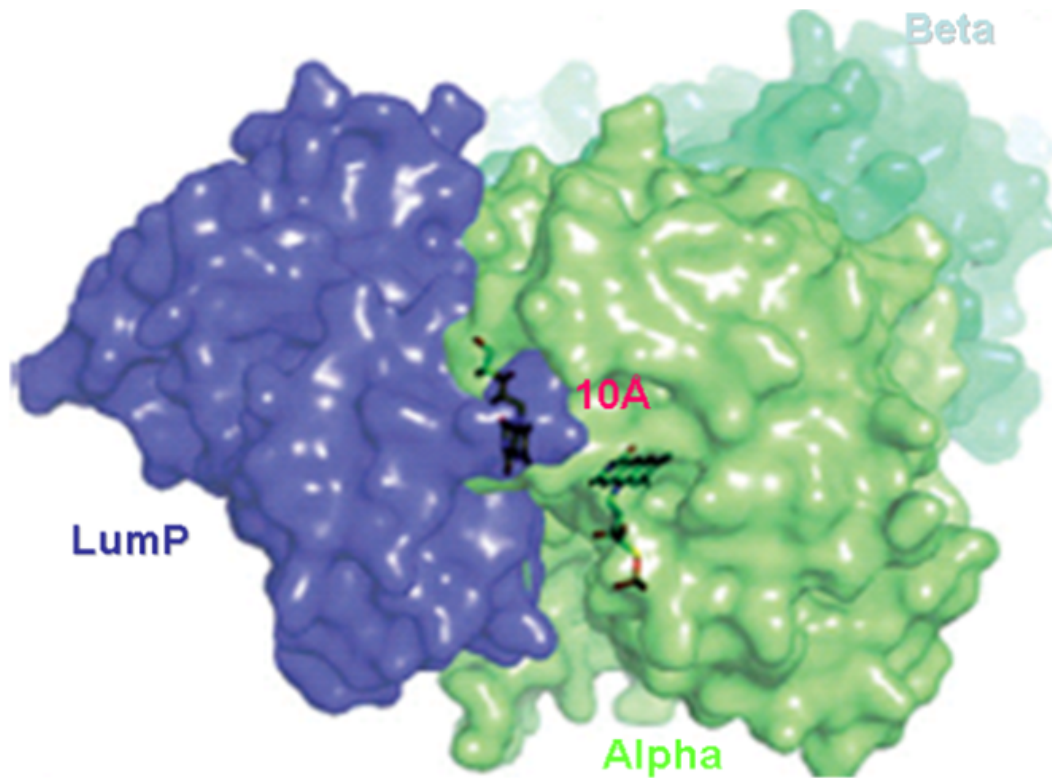
Bacterial Luciferase
77 kDa α-β

FMN →

Campbell (2009) Biochemistry

Lumazine Protein

Sato (2010) J. Bact.

Lumazine Protein-Bacterial Luciferase

Sato (2010) J. Bact.

FRET in bacterial bioluminescence

- Fluorescence dynamics investigation indicated a
separation of < ~20 Å between the B-Lase intermediate and LumP.

- The spatial structure of the complex confirms this.

- Both LumP and the GFP complexes form more tightly in the course of the reaction. This would favor enzyme turnover.

Literature

Lee and Vysotski (2011). Structure and spectra in bioluminescence. On, *Photobiological Sciences Online* (K.C.Smith, Ed.). http://www.photobiology.info/Lee-Vysotski.html

Vysotski and Lee. (2004) Ca^{2+}-regulated photoproteins: Structural insight into the bioluminescence mechanism. Acc. Chem. Res. 37: 405-415.

Literature

Titushin, M., et al. (2011). Protein-protein complexation in bioluminescence, *Protein and Cell* 2: 957.

Nakatsu et al. (2006). Structural basis for the spectral difference in luciferase bioluminescence. Nature 440: 372.