Linking single crystal spectroscopy to structure

Workshop on time-resolved macromolecular crystallography
November 2008

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Four spectroscopic techniques are currently combined with macromolecular X-ray crystallography

- UV / visible absorption.
- Fluorescence.
- Raman.
- X-ray absorption.
- These can be done either “off-line” in the laboratory, or “on-line” during X-ray data collection.

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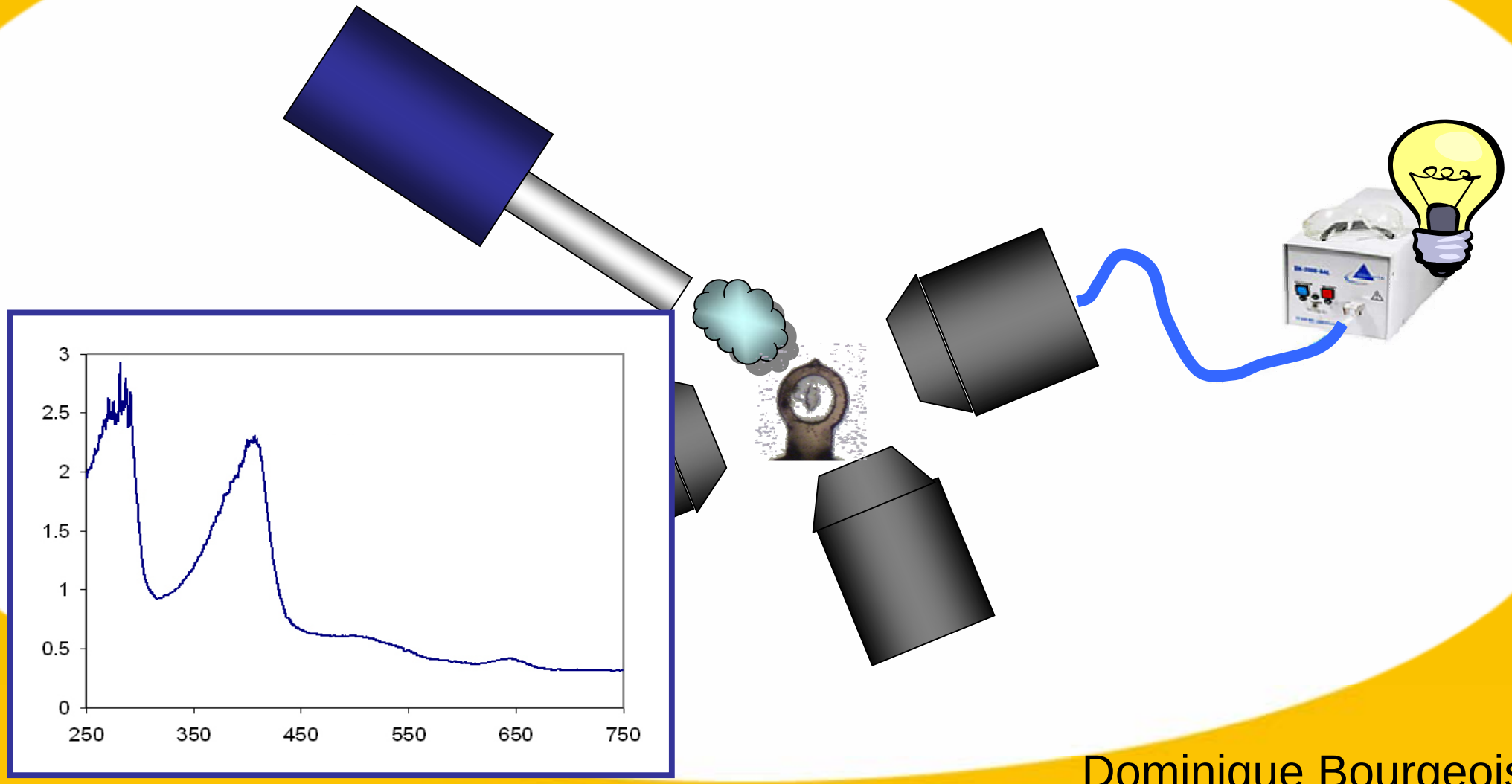
Absorption spectrum measurement



Dominique Bourgeois

Single crystal UV/vis microspectrophotometry

- **Know what you have in your crystal:** If a chromophore is present, *e.g.* flavin / heme, then the the UV/vis spectrum of the crystal enables the structure to be directly linked to a particular chromophore chemical / redox state.
- **Track unintended photoreduction:** X-rays oxidize solvent water to produce photoelectrons. These can reduce redox active cofactors, disulfide bonds *etc.*
- **Kinetic crystallography:** Changes in UV/vis absorbance that occur during chemical reactions can be followed in the crystal. This information can be used to trap reaction intermediates.

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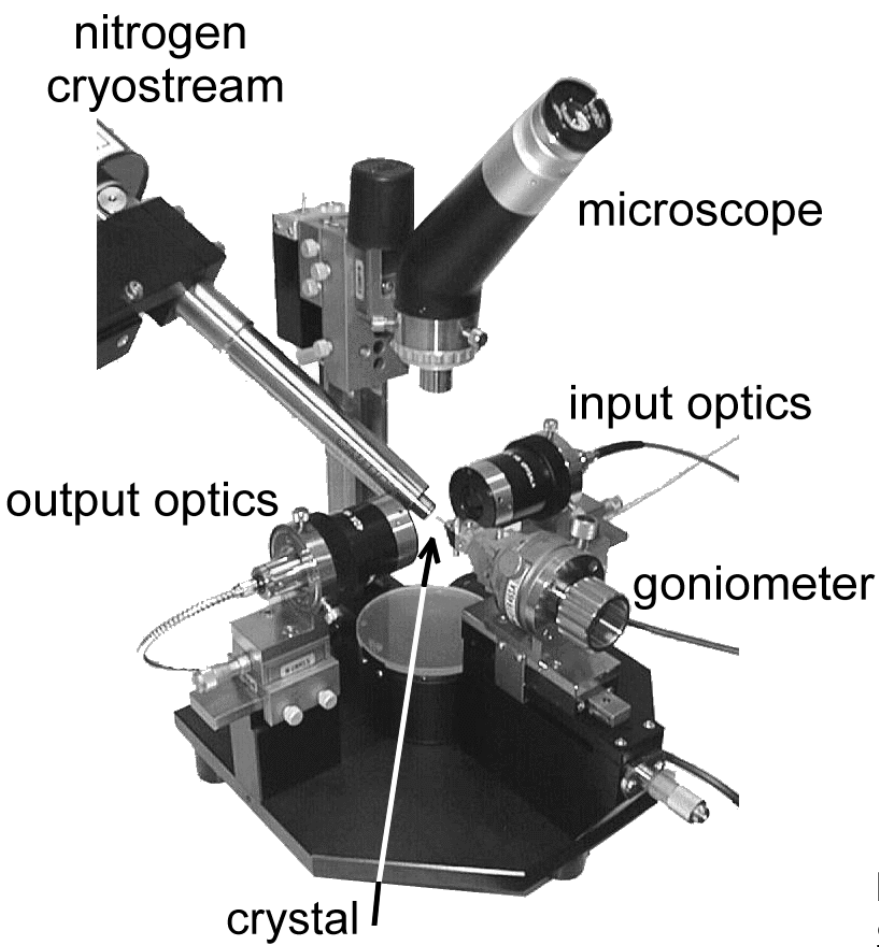
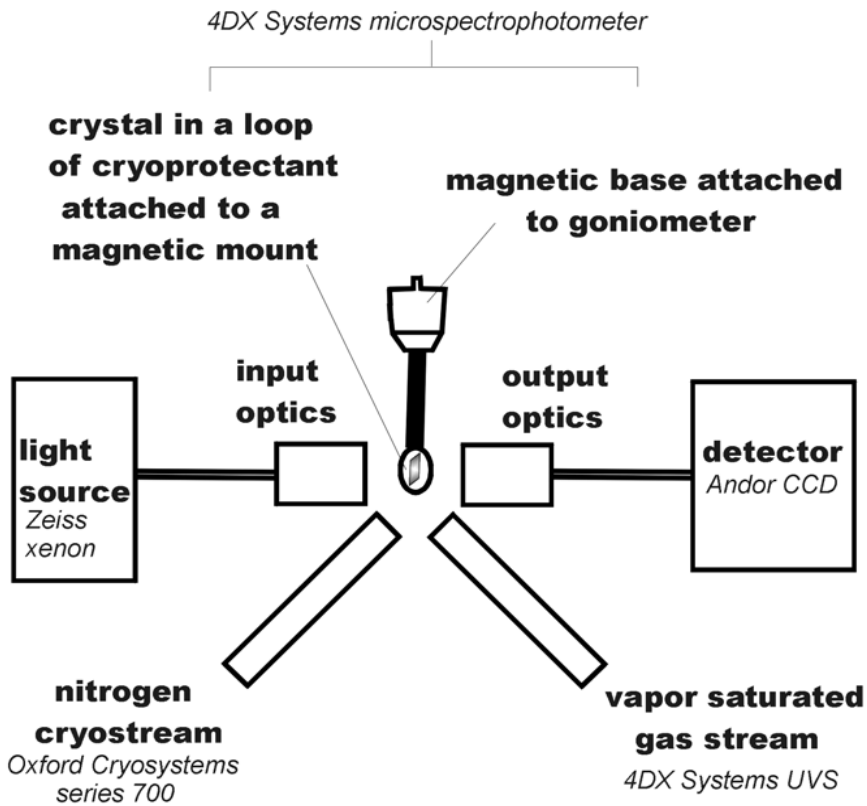
Single crystal UV/vis microspectrophotometer





Hadfield, A. & Hajdu, J. (1993) *J. Appl. Cryst*. 26: 839-42.
Sjögren, T. *et al.* (2002) *J. Appl. Cryst*. 35: 113-6.

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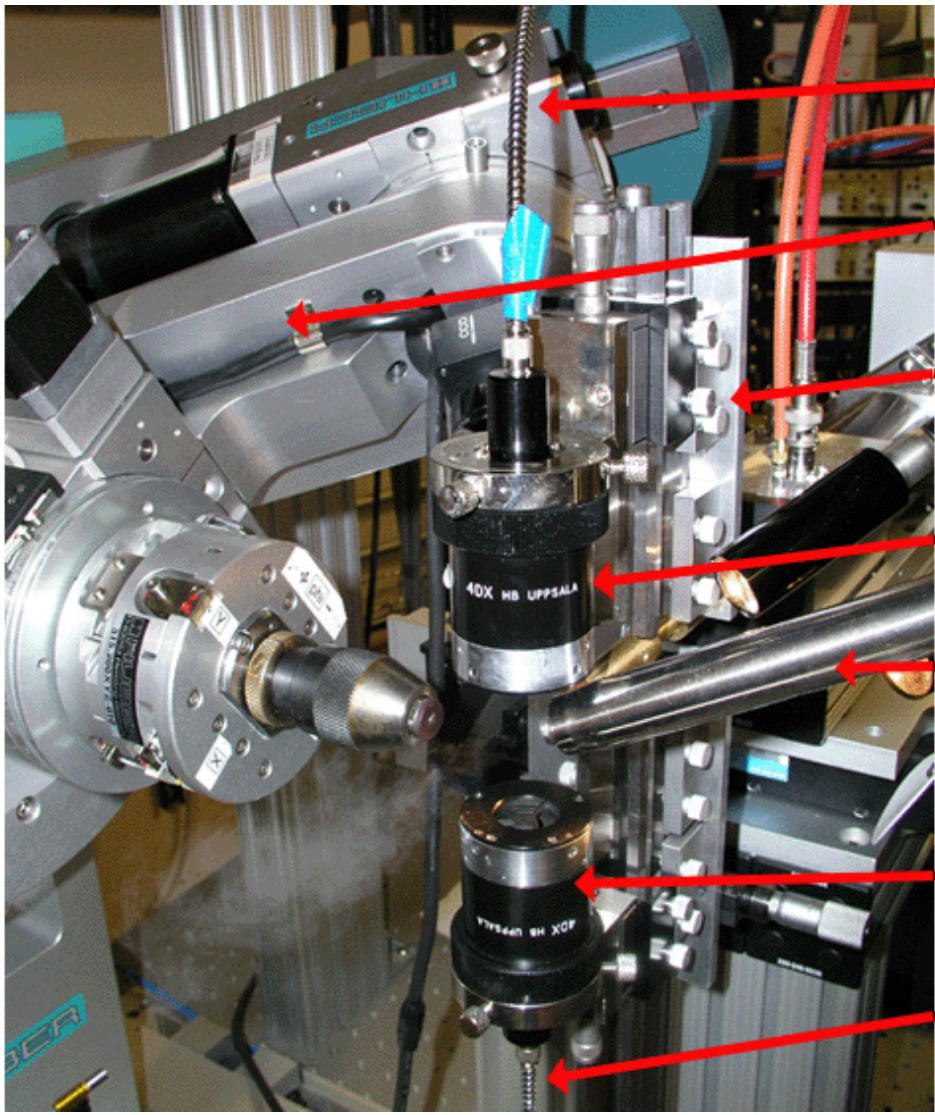
On-line microspectrophotometer mount



Fibre optic light guide

κ-diffractometer

Custom mount
for 4DX optics

4DX focusing optic

Cryo-stream

4DX focusing optic

Fiber-optic light guide

BioCARS
14-BM-C

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Single crystal UV/vis microspectrophotometry

- The spectra from single crystals are anisotropic.
- Therefore, the isotropic solution spectrum cannot be emulated with a single crystal orientation.
- Chromophores with extended planar π electron systems, such as heme, exhibit the strongest orientation dependent anisotropy.
- Crystal prism effects also distort the spectrum.
- Path length (crystal size) varies.
- Absorbance changes in crystals are qualitative, not quantitative.
- Despite this, a crystal orientation can normally be found that has the features of the solution spectrum, and ***relative*** changes can be tracked.

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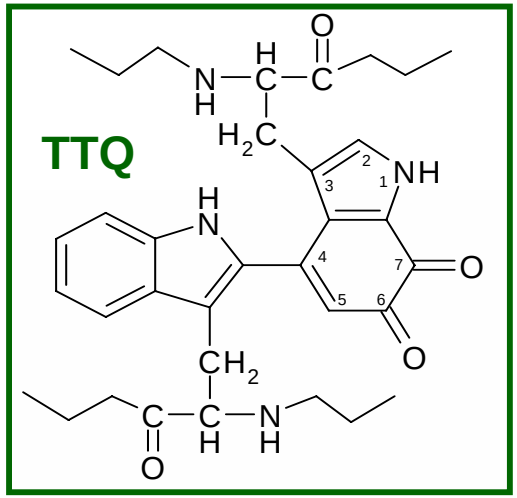
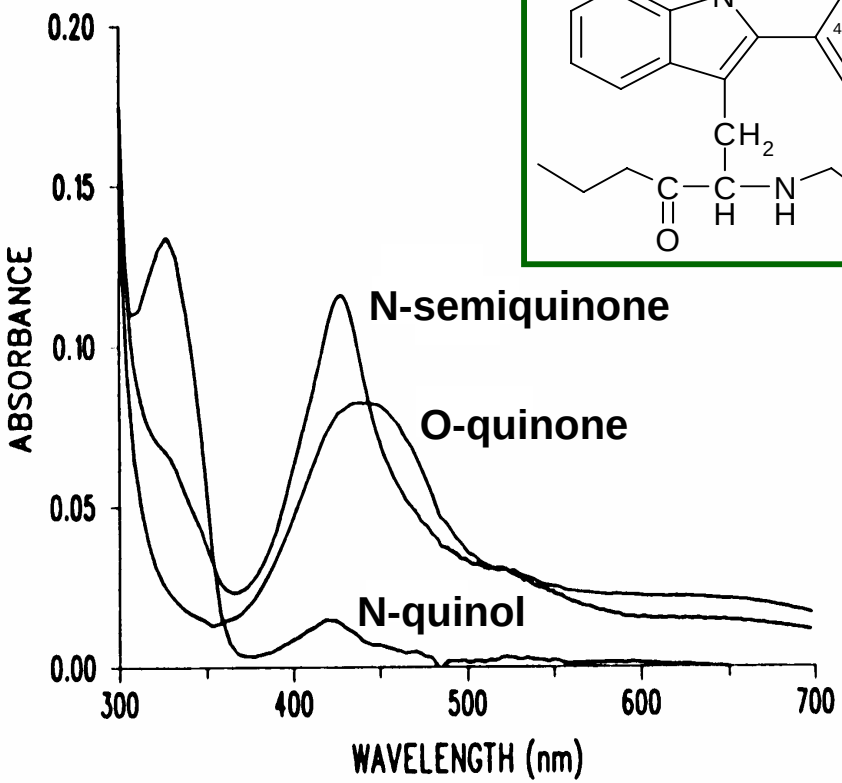
UV/vis of methylamine dehydrogenase

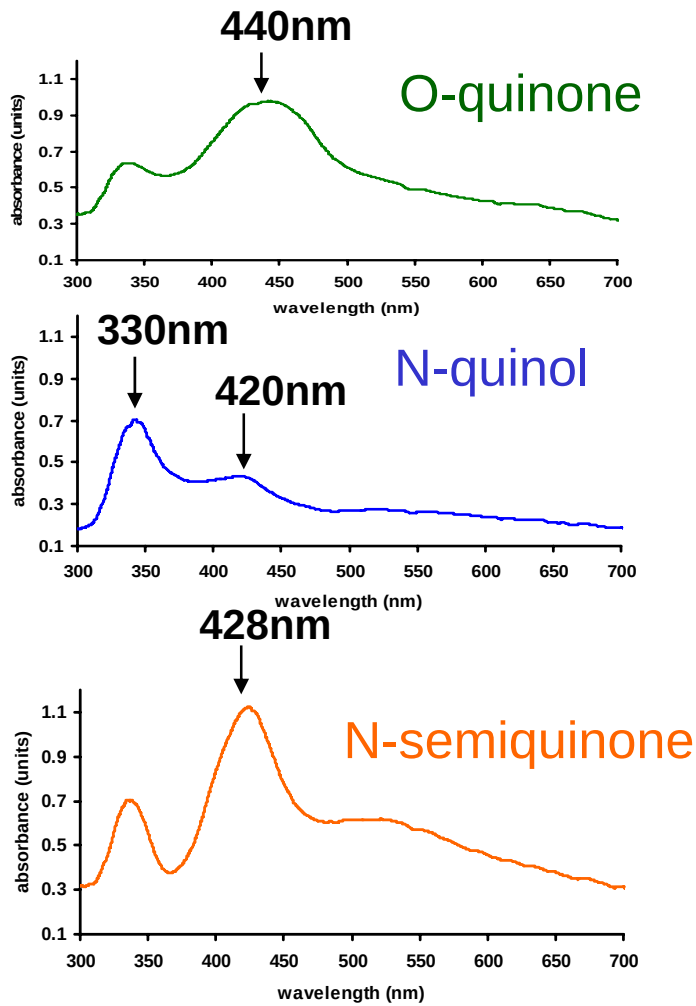
Solution





Pearson, A.R. & Wilmot, C.M. (2003) *Biochim. Biophys. Acta*, 1647: 381-9.

Single crystal



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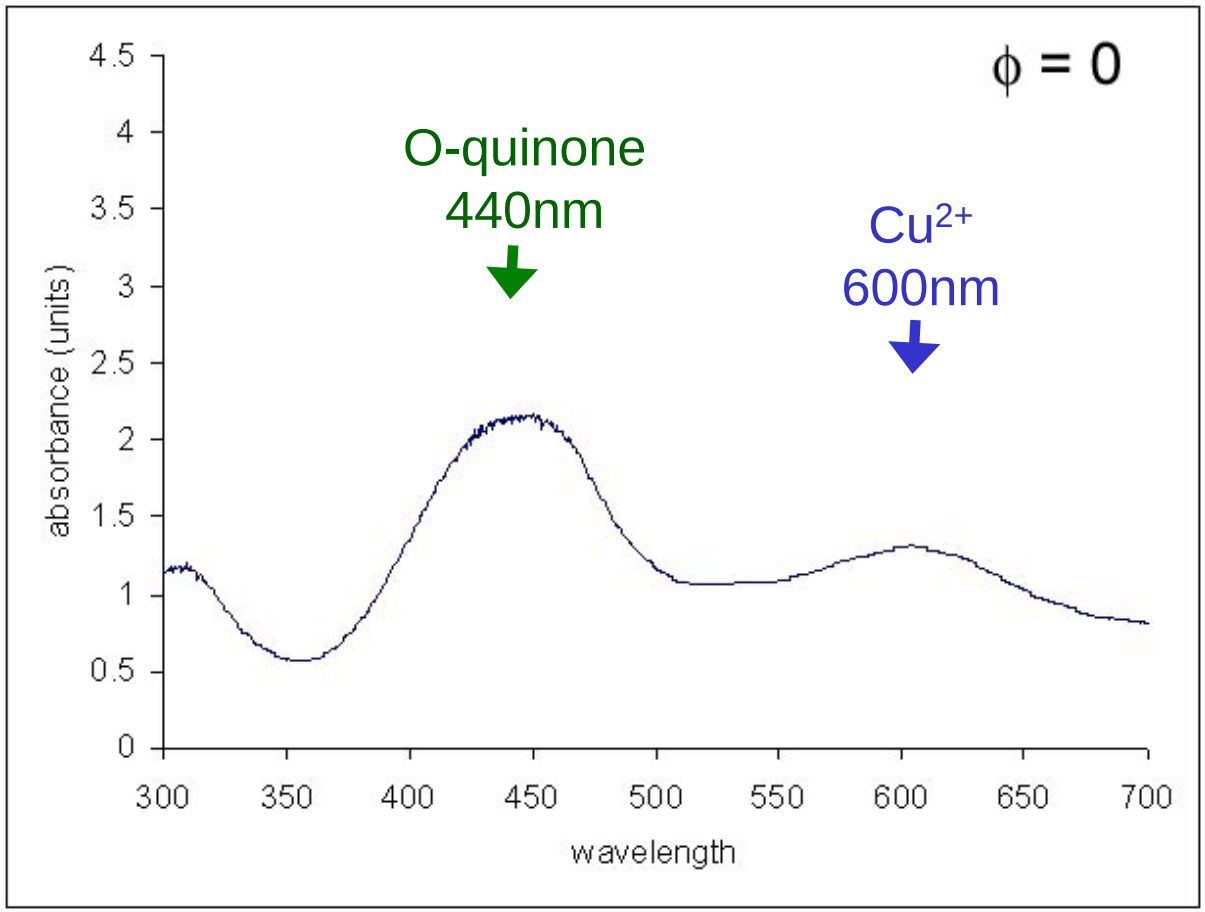
UV/vis of methylamine dehydrogenase in complex with amicyanin

Rotation of O-quinone / Cu^{2+} containing crystal



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Reduction of redox centers by photoelectrons

- X-rays oxidize solvent water to produce photoelectrons, and these can reduce oxidized redox centers.
- The kinetics and dose dependence of reduction at redox centers can be determined.
- Composite datasets can then be constructed from multiple crystals where only the early time-points during X-ray exposure are used. This enables the structure of the oxidized species to be determined.
- Experimental tip: If possible only use data where loss of the oxidized species is less than 20%.

Sjögren T & Hajdu J (2001) *J. Biol. Chem.* 276: 13072-6. Berglund *et al.* (2002) *Nature* 417:463-8. Pearson AR *et al.* (2007) *J. Synch. Rad.* 14: 92-98

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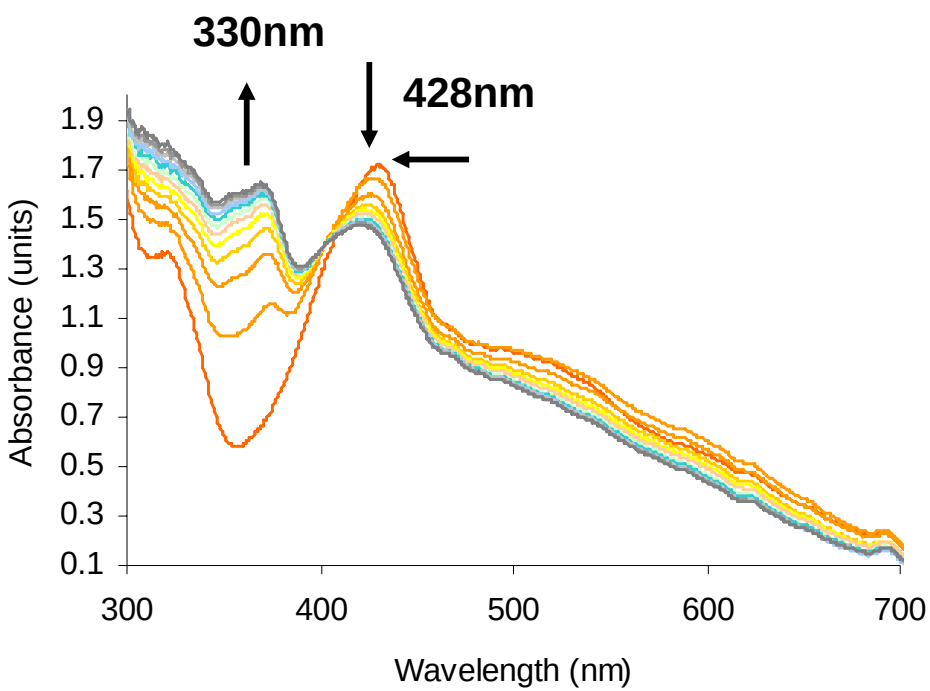
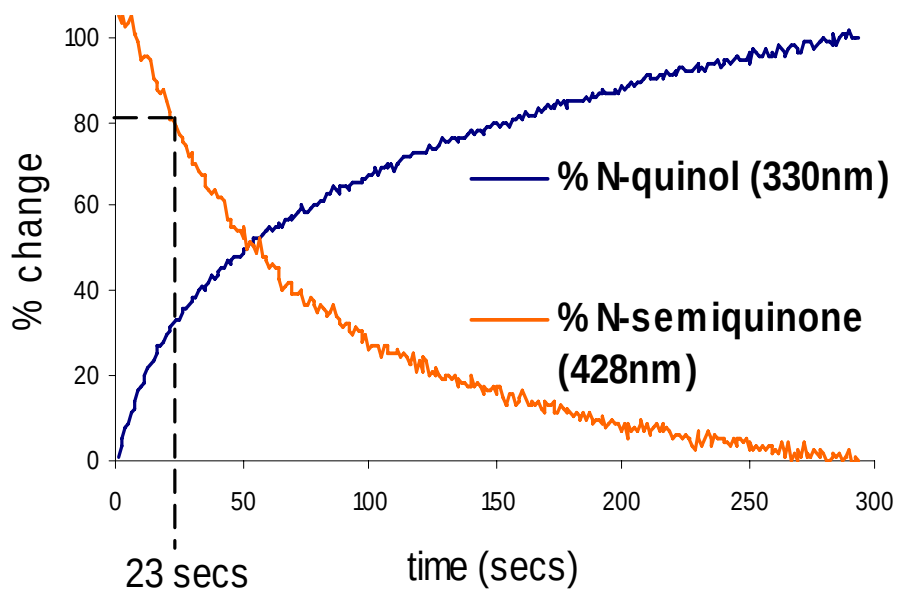
Reduction of redox centers by X-rays

Photoreduction of N-semiquinone→N-quinol in holo-binary MADH / amicyanin crystals.
Copper is already reduced to Cu(I)





For N-semiquinone containing intermediate only 20s of X-ray data were used per crystal

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Enzymology in crystals

- [protein] in crystals ≈ [protein] in the cell.
- The crystal acts likes a porous cage that enables molecules, such as substrates, to diffuse through the solvent channels.
- Many enzymes retain catalytic activity in the crystal.
- If there are no large conformational changes during catalysis, many proteins remain crystalline during turnover.
- Need the majority of the protein molecules in a crystal to be in the same state to "see" that state in the structure.
  - desired intermediate must accumulate
  - must remain stable during X-ray data collection
- Depending on the system, spectroscopy can track the reaction in the crystalline protein.

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Enzymology in crystals

- **Chemical trapping:** Intermediates can be built up within the crystal using knowledge of the reaction mechanism.
- For example, (1) an intermediate prior to an acid catalyzed step may be captured by initiating the reaction at high pH. (2) A reaction may be halted in an ordered or ping-pong mechanism by addition of only the 1st substrate. (3) A “slow” substrate can be used.
- **Kinetic crystallography:** The constraints of the crystal lattice can dramatically slow reaction steps that involve conformational change. The reaction can be tracked by spectroscopy. When the build-up of an intermediate reaches a maximum, the crystal can be frozen to “pause” the reaction. In this way “snapshots” along the reaction pathway can be generated and assembled into a “movie of catalysis” at the molecular level.

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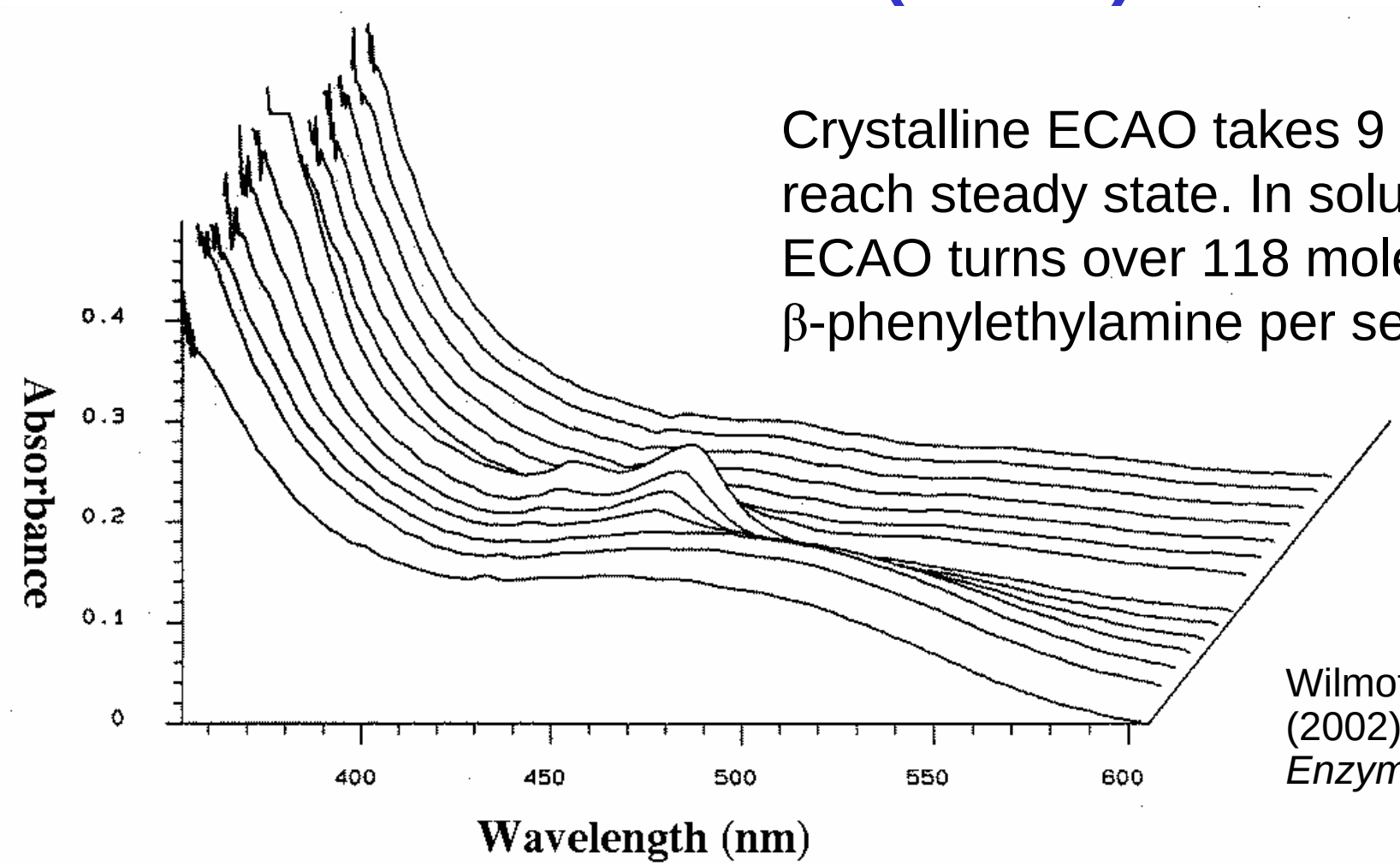
Single crystal kinetics in *E. coli* amine oxidase (ECAO)



Crystalline ECAO takes 9 minutes to reach steady state. In solution ECAO turns over 118 molecules of β-phenylethylamine per second.

Wilmot CM *et al.* (2002) *Methods in Enzym.* 353:301-18.

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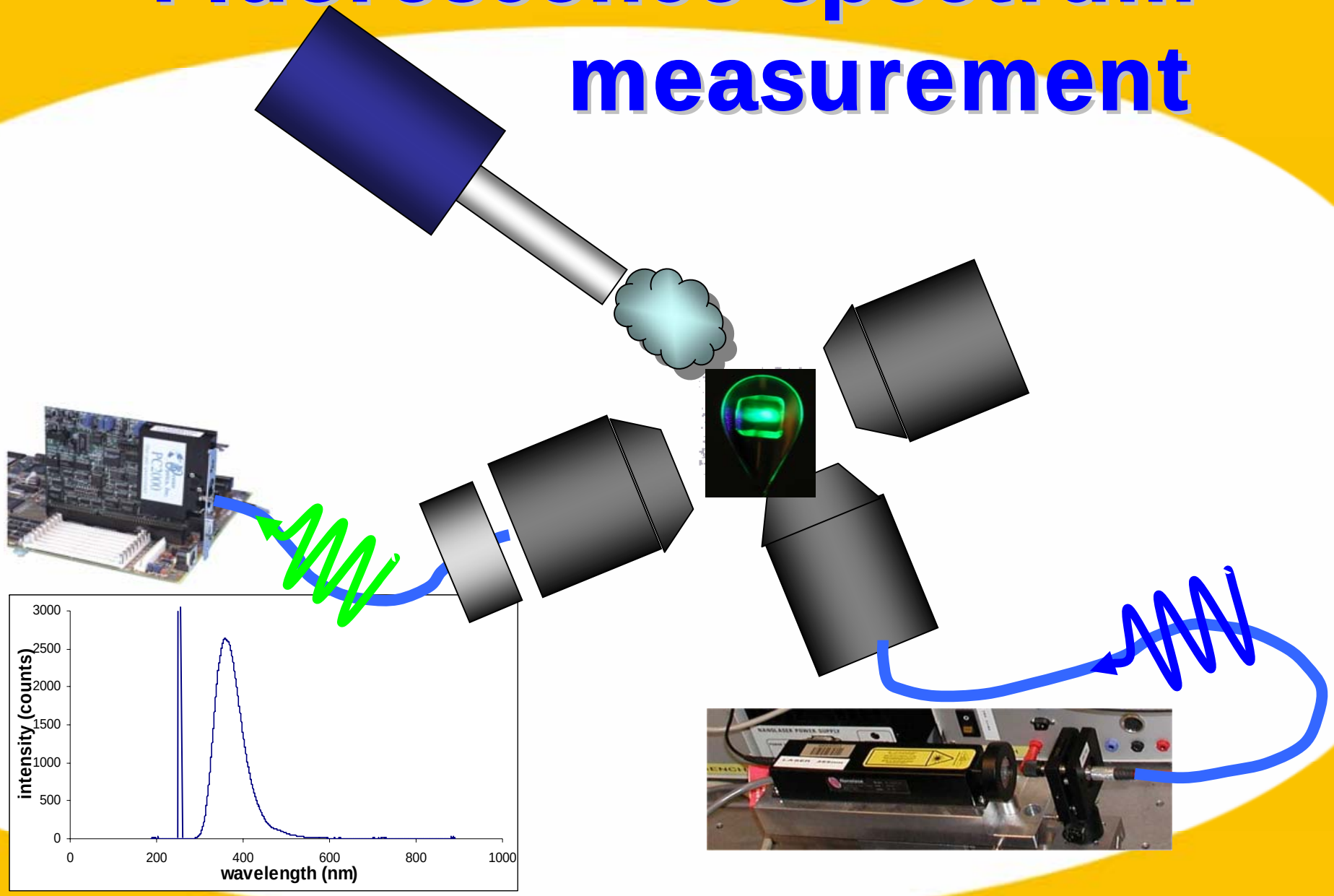
Fluorescence spectrum measurement



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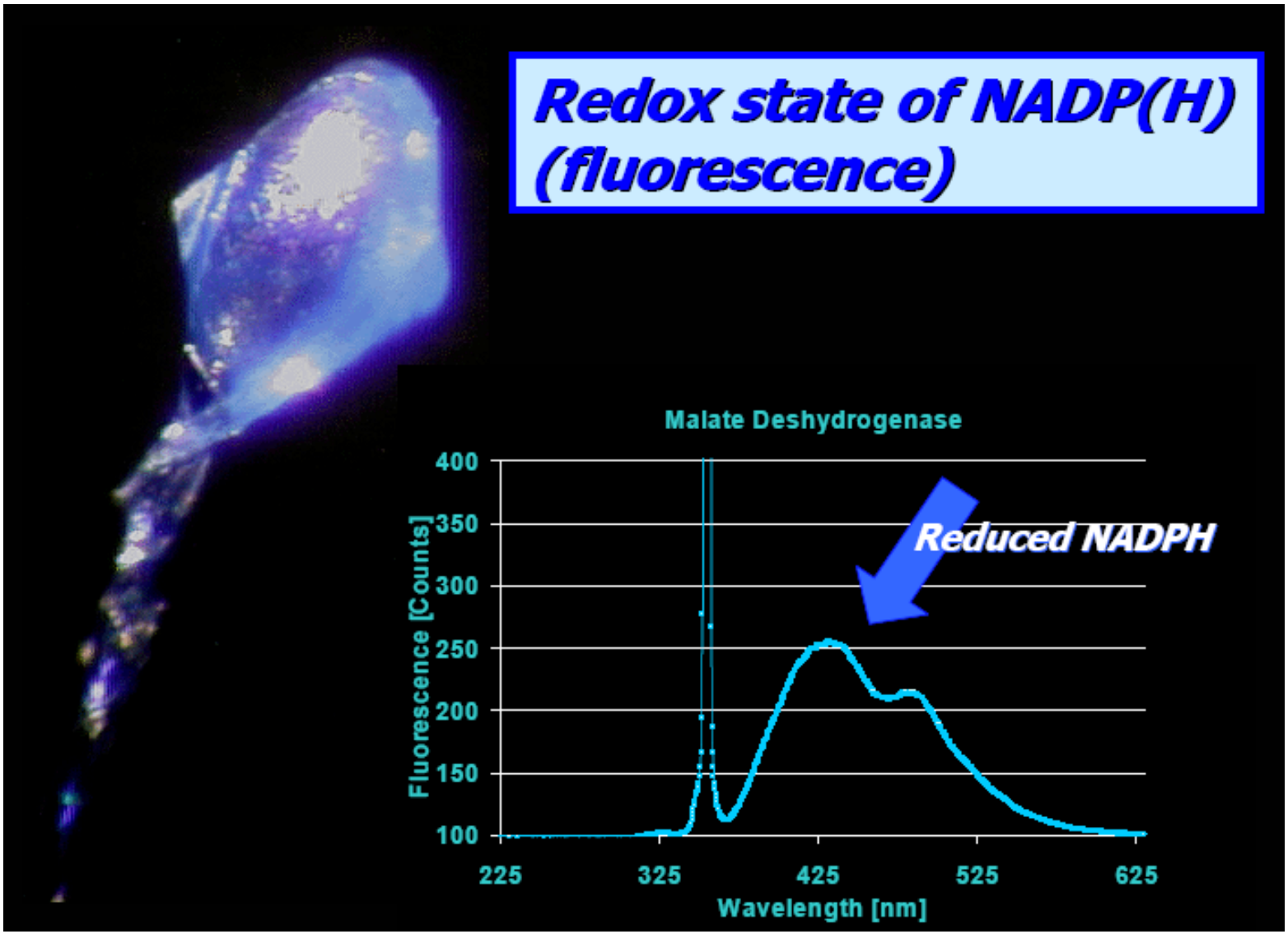
Single crystal fluorescence



Can also be used for visualizing protein crystal in a loop.

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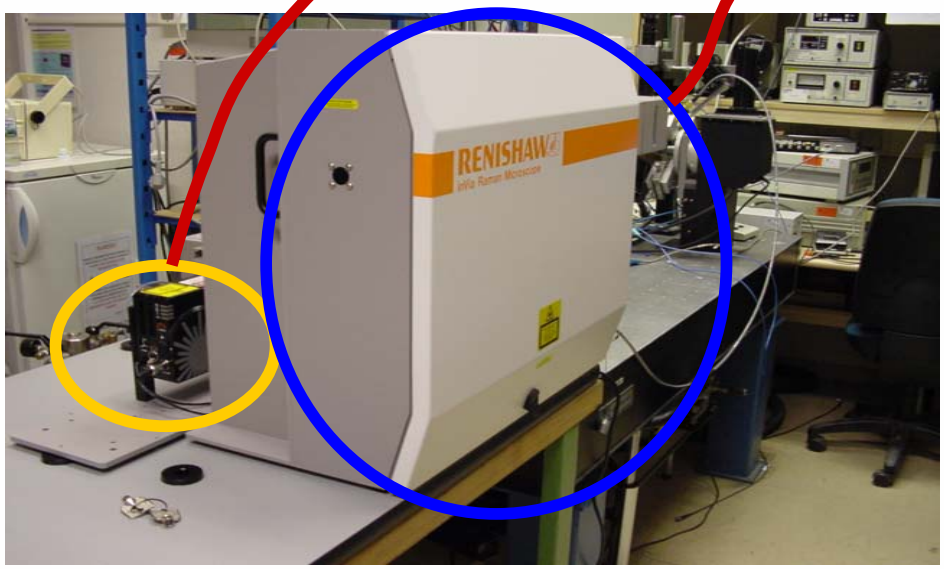
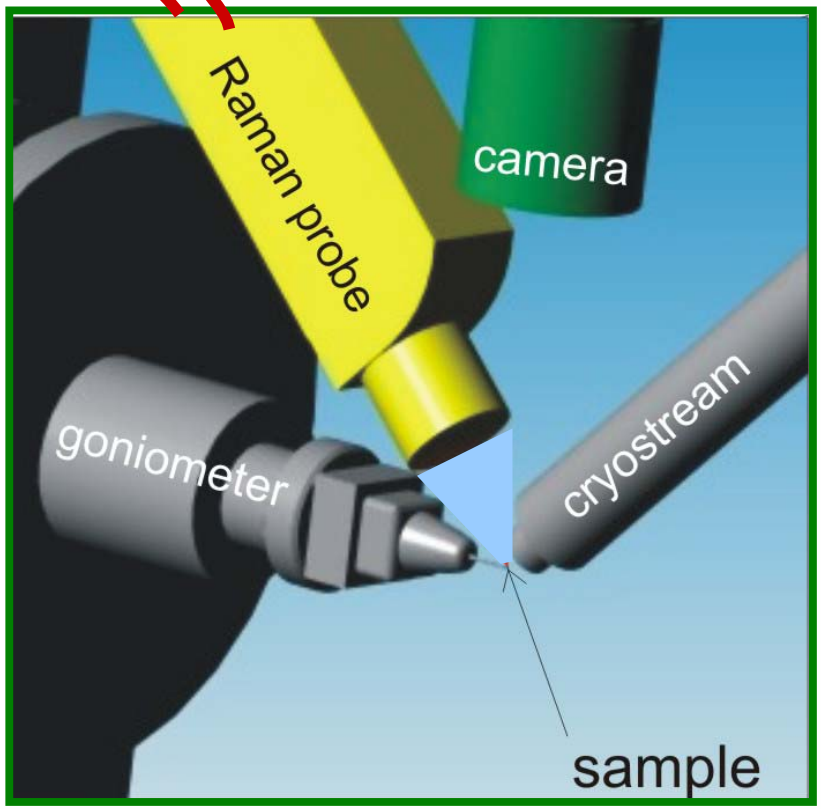
Raman spectrum measurement

785 nm





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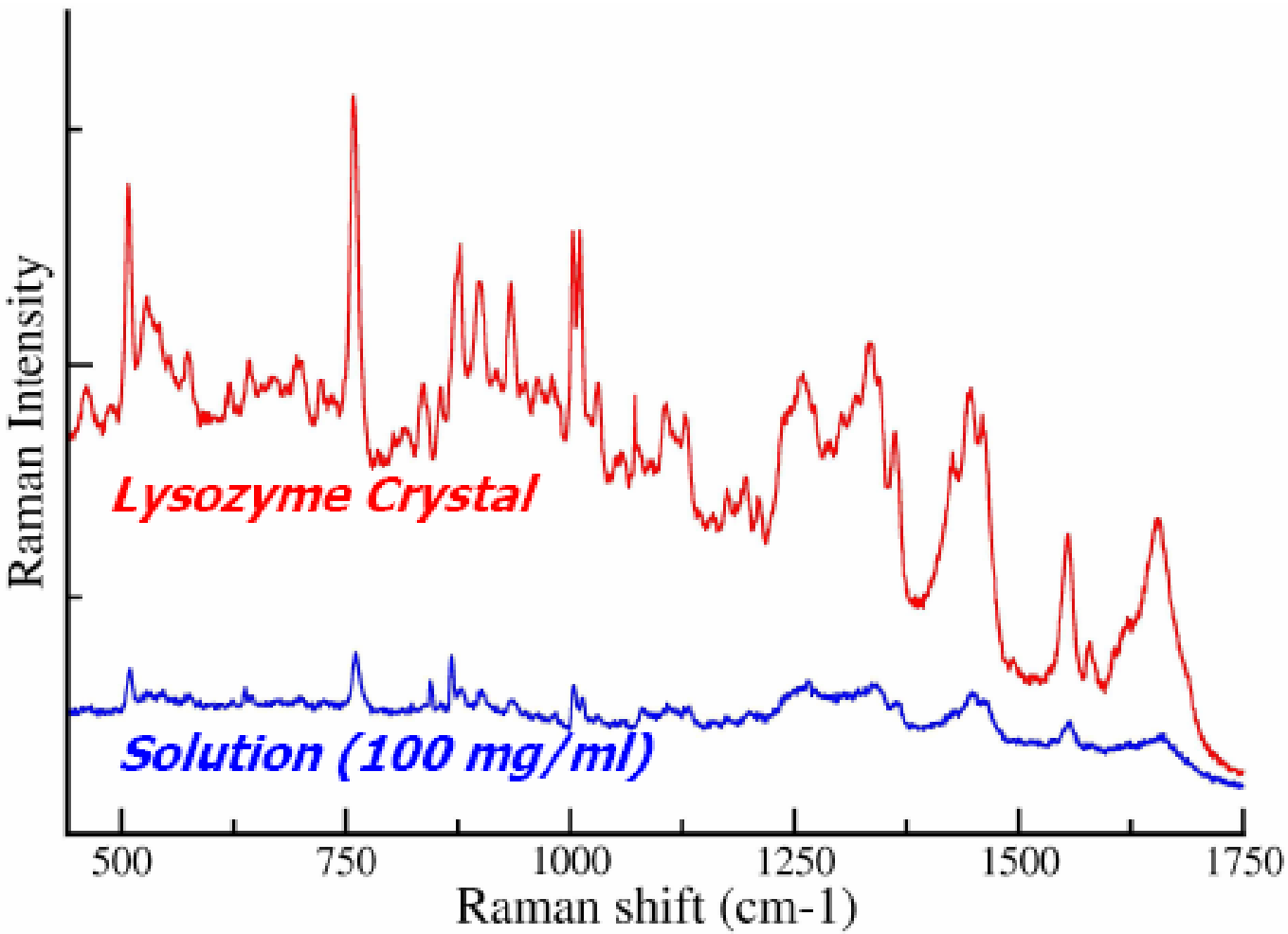
Single crystal non-resonant Raman



- [protein] in crystals much greater than in solution.
- Therefore more protein signal, and less solvent contribution.
- The crystal restricts motions, which leads to sharper features in the Raman.

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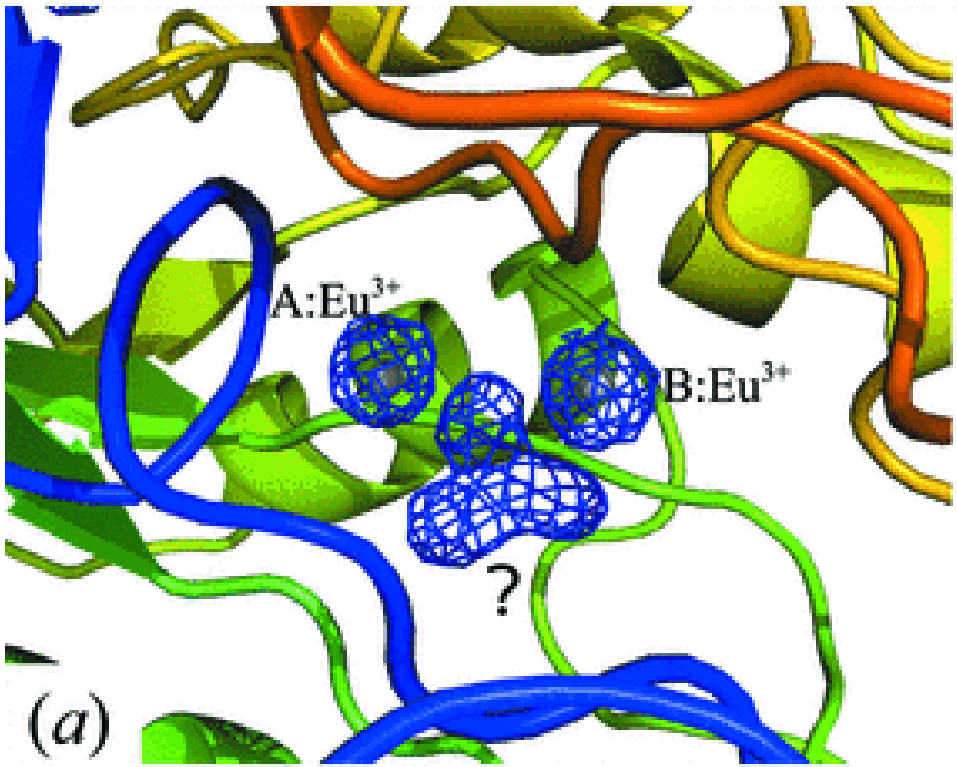
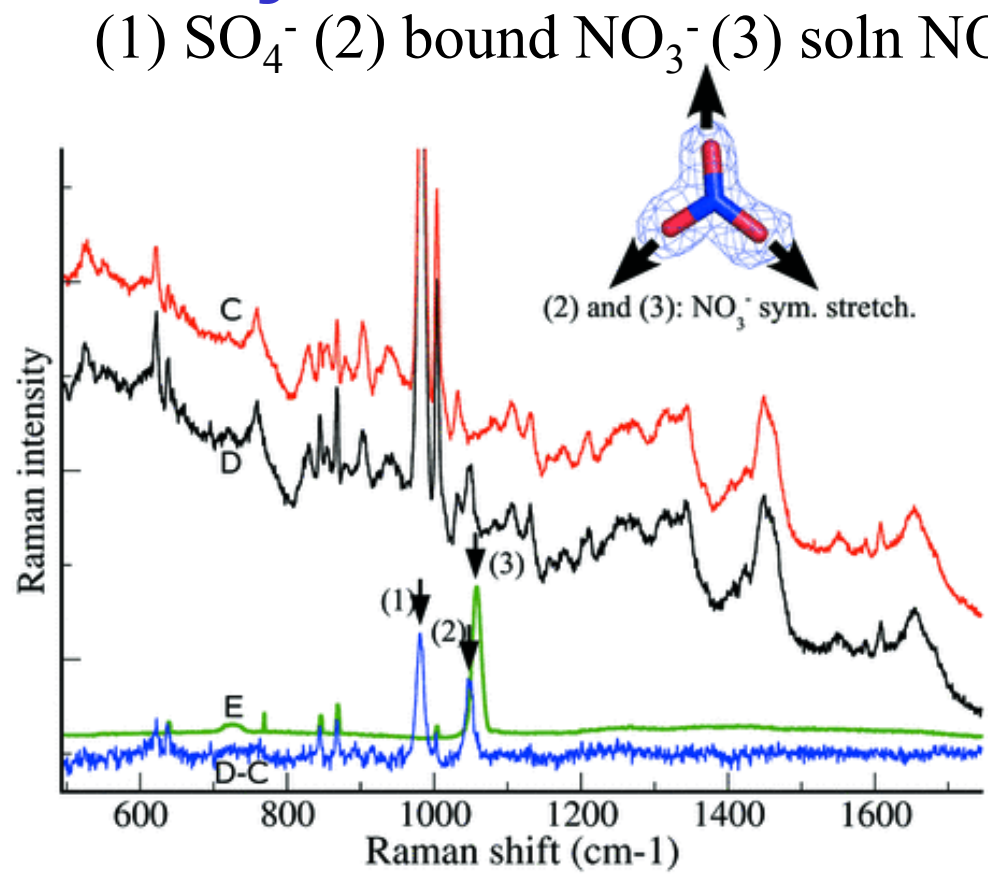
Ligand identification in xylose isomerase





Crystal: C: Native; D: Eu derivative, ($EuNO_3^-$); D-C: difference

Solution: E: 1M $NaNO_3$

Carpentier P *et al.* (2007) *J. Appl. Cryst.* 40:1113-22

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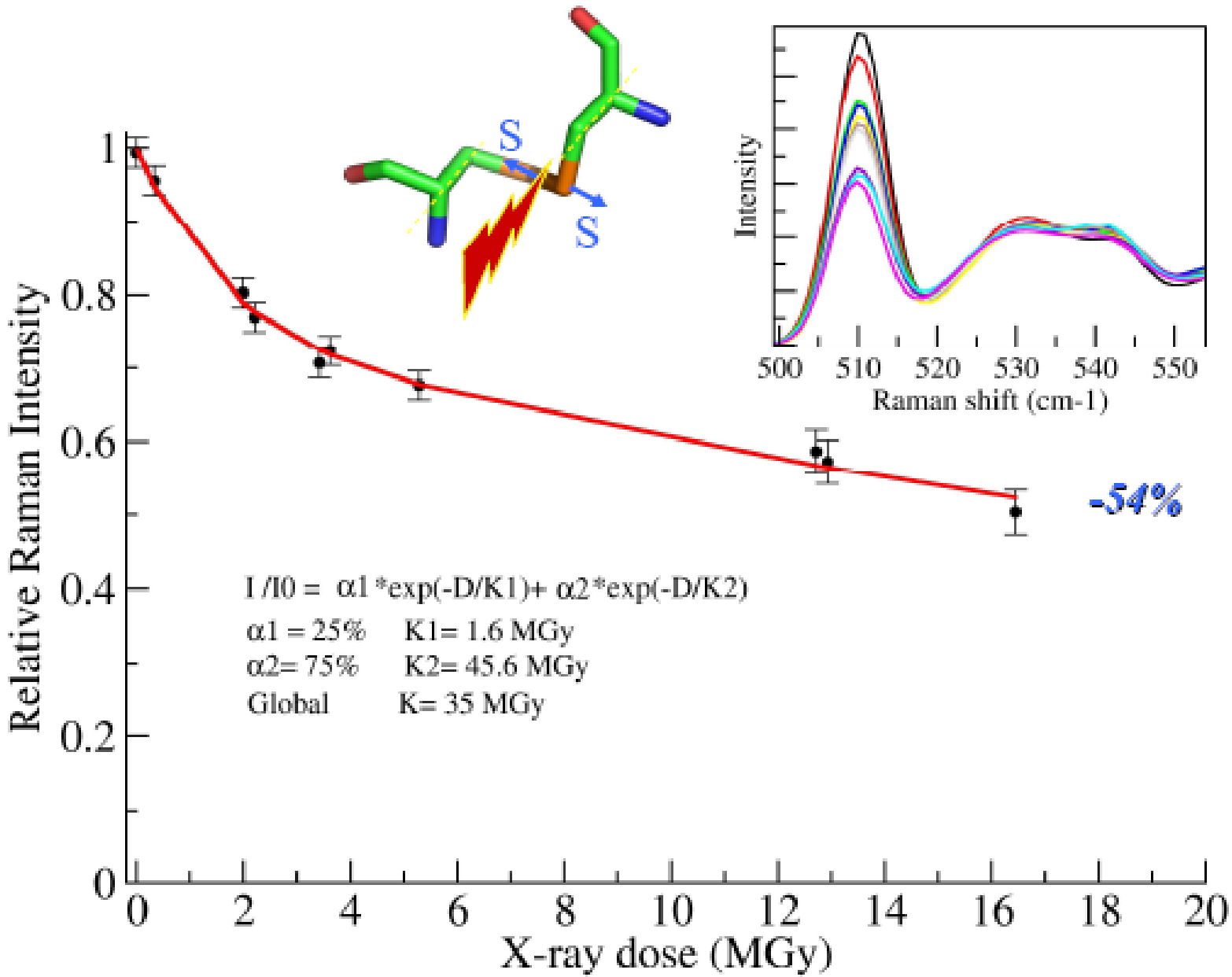
Breakage of disulfide bonds



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X-ray absorption spectroscopy

- Genuine XAS / PX combinations are still rare. SSRL BL9-3 has been developed to support these types of experiments.
- Polarized XAS on crystals of photosystem II (PS-II) has been used to shed light on the structure of the oxygen evolving complex, Mn_4Ca.
- The Ca was replaced with Sr, which still gives a functional complex.
- Different intermediate states were probed with XAS.

Latimer, MJ *et al.* (2005) *J. Synch. Rad.* 12: 23-7; Pushkar, Y *et al.* (2008) *PNAS*, 105: 1879-84; Yano J *et al*. (2006) *Science* 314: 821-5.

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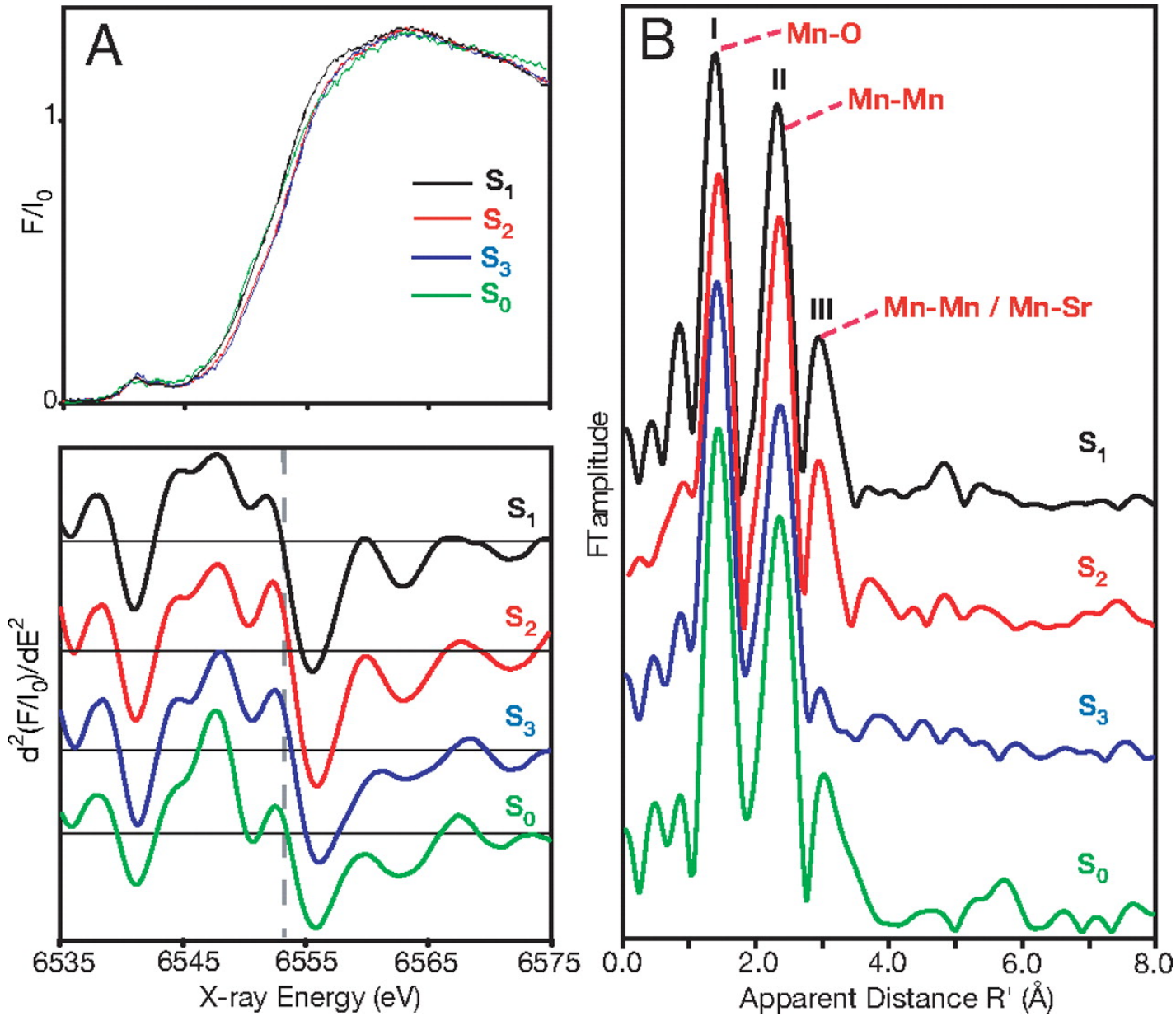
Polarized XAS on OEC of PS-II



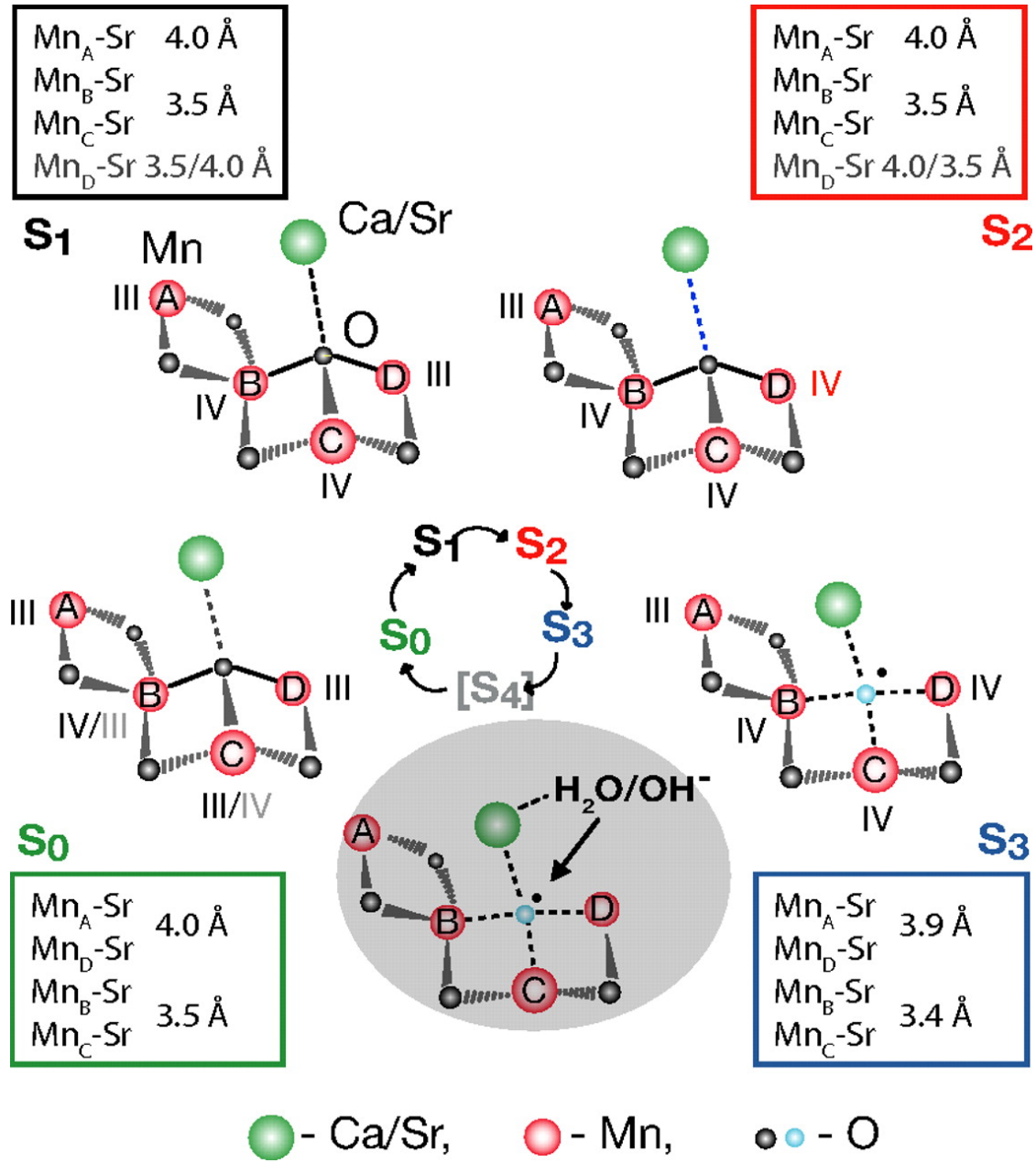
A) Mn *K*-edge XANES. Lower panel 2nd derivative.

B) FT of Mn EXAFS.

Pushkar, Y *et al.* (2008) *PNAS*, 105: 1879-84.

Polarized XAS on OEC of PS-II

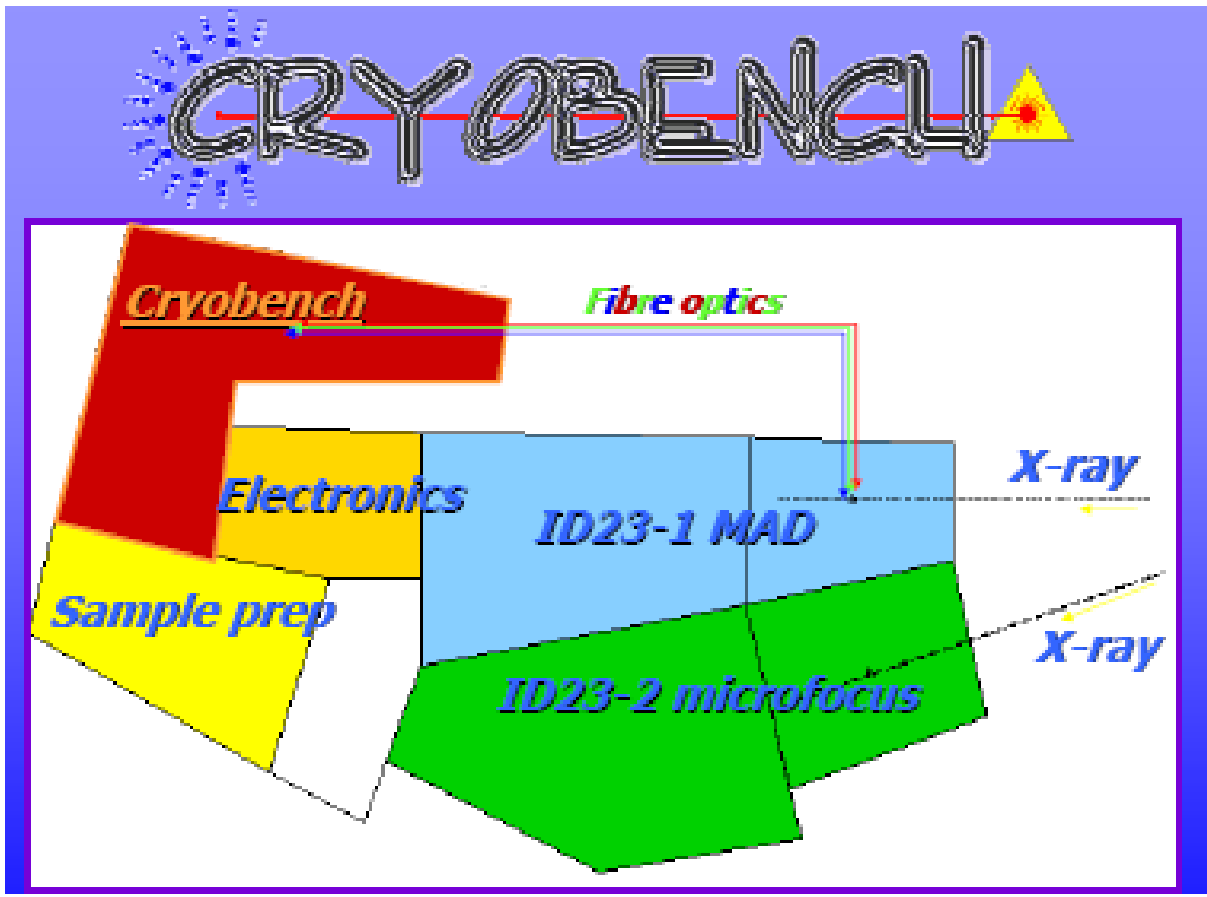
- The data indicated substantial changes to the cluster during OEC turnover.



Pushkar, Y *et al.* (2008) *PNAS*, 105: 1879-84.

Single crystal spectroscopy / PX facilities

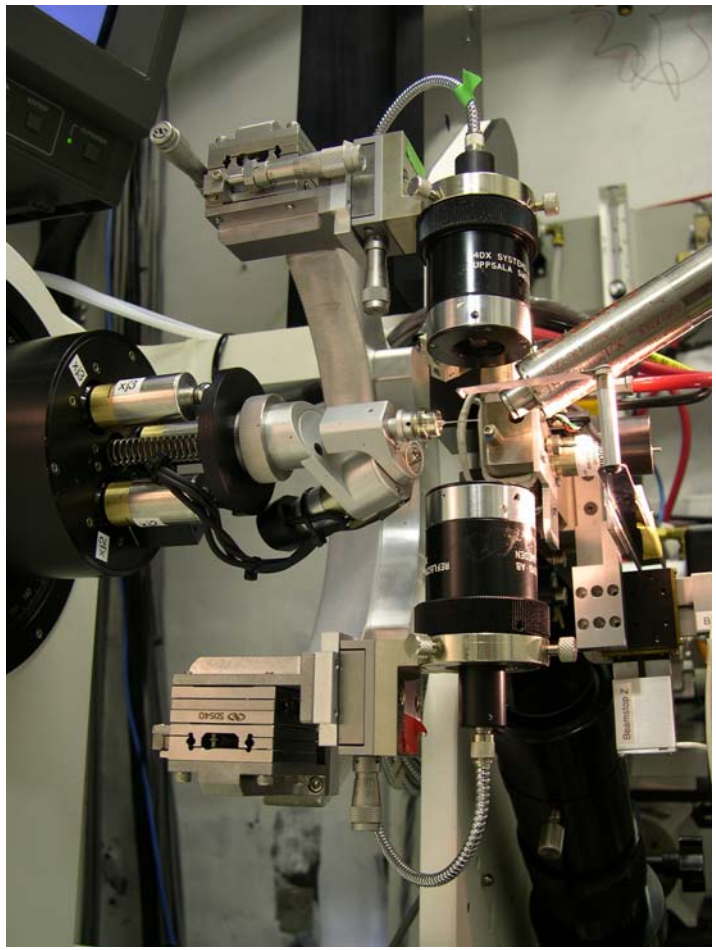
- Single UV/visible spectroscopy is becoming widely available at synchrotrons, *e.g.* BioCARS at APS.
- Currently, one of the best set up labs for combined single crystal spectroscopy / PX is sector 23 at ESRF.



http://www.esrf.eu/UsersAndScience/Experiments/MX/Cryobench/

Single crystal spectroscopy / PX facilities

- A similar facility to cryobench is being built at NSLS-II, Brookhaven National Laboratory, but will also include X-ray absorption spectroscopy.



http://www.px.nsls.bnl.gov/x26c/x26c_info.html

More info……

Reviews on single crystal spectroscopies:

- Pearson, A.R., Mozzarelli, A. & Rossi, G.L. Microspectrophotometry for structural enzymology. *Current opinion in structural biology* **14**, 656-662 (2004).
- Bourgeois, D. & Royant, A. Advances in kinetic protein crystallography. *Current opinion in structural biology* **15**, 538-547 (2005).
- De la Mora-Rey, T. & Wilmot, C.M. Synergy within structural biology of single crystal optical spectroscopy and X-ray crystallography. *Current opinion in structural biology* **17**, 580-586 (2007).
- Bourgeois, D., de Rosay, E. & Katona, G. Kinetic crystallography: a tool for filming proteins in action. *Biofutur*, 48-51 (2007).

More info……

UV/visible (kinetics):

- Hajdu, J., *et al.* Analyzing protein functions in four dimensions. *Nature structural biology* **7**, 1006-1012 (2000).
- Wilmot, C.M., Sjogren, T., Carlsson, G.H., Berglund, G.I. & Hajdu, J. Defining redox state of X-ray crystal structures by single-crystal ultraviolet-visible microspectrophotometry. *Methods in enzymology* **353**, 301-318 (2002).
- Berglund, G.I., *et al.* The catalytic pathway of horseradish peroxidase at high resolution. *Nature* **417**, 463-468 (2002).

More info……

Photoreduction:

- Pearson, A.R., Pahl, R., Kovaleva, E.G., Davidson, V.L. & Wilmot, C.M. Tracking X-ray-derived redox changes in crystals of a methylamine dehydrogenase/amicyanin complex using single-crystal UV/Vis microspectrophotometry. *Journal of synchrotron radiation* **14**, 92-98 (2007).

Fluoresence:

- Royant, A., *et al.* Advances in spectroscopic methods for biological crystals. 1. Fluorescence lifetime measurements. *Journal of Applied Crystallography* **40**, 1105-1112 (2007).

More info……

Raman:

- Carey, P.R. Raman crystallography and other biochemical applications of Raman microscopy. *Annual Review of Physical Chemistry* **57**, 527-554 (2006).
- Carpentier, P., Royant, A., Ohana, J. & Bourgeois, D. Advances in spectroscopic methods for biological crystals. 2. Raman spectroscopy. *Journal of Applied Crystallography* **40**, 1113-1122 (2007).
- Carey, P.R. & Dong, J. Following ligand binding and ligand reactions in proteins via Raman crystallography. *Biochemistry* **43**, 8885-8893 (2004).