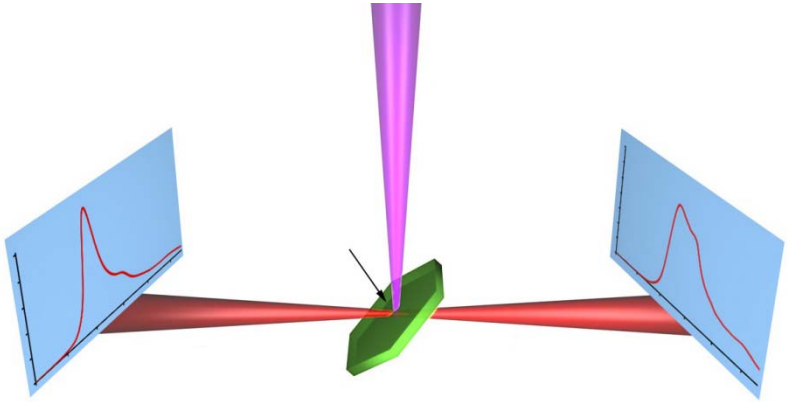


Complementary spectroscopic techniques for protein X-ray crystallography – the new Cryobench

Antoine Royant

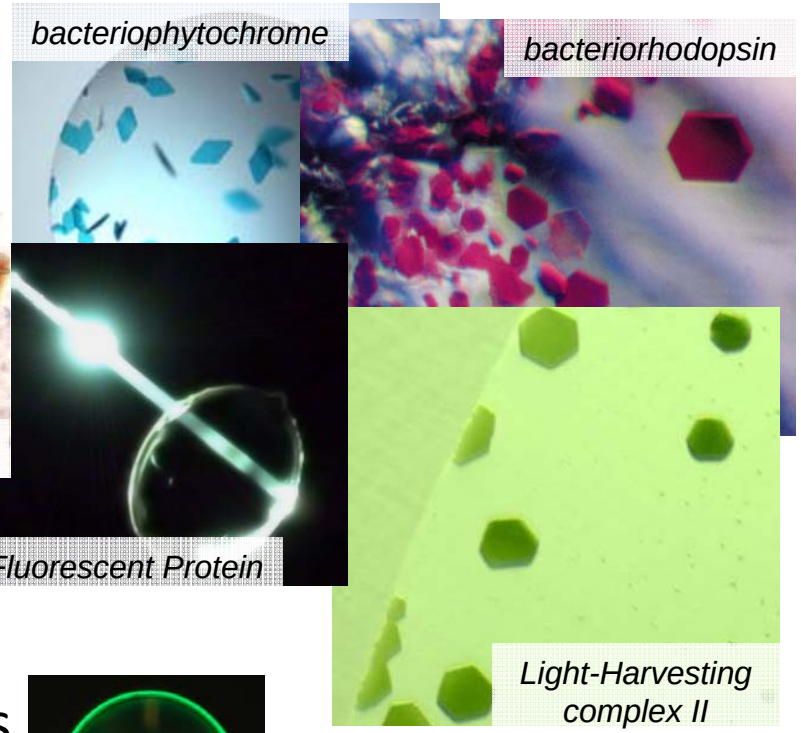
ESRF MX School, February 2010

cnrs cea IBS UNIVERSITE JOSEPH FOURIER SCIENCES.TECHNOLOGIE.SANTÉ

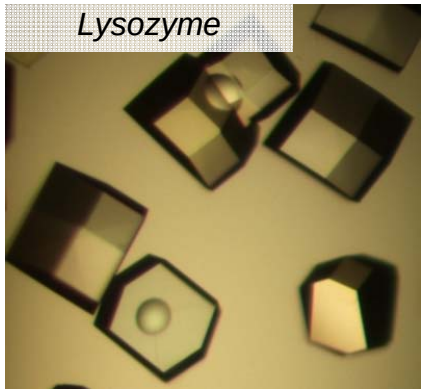
ESRF

What is the Cryobench?
What for?

- Naturally colored/fluorescent proteins
  - redox proteins
  - photoactive proteins
  - photosynthetic proteins
  - fluorescent proteins



- Artificially colored/fluorescent proteins
  - soaking with exogenous fluorophores
  - caged compounds



- Non-colored proteins
  - proteins with specific bonds (S-S, O-Fe, C-Br)

→ **Raman spectroscopy**

The CRYOBENCH lab at the European synchrotron: a historical perspective

1999 - 2006

I

Cryobench I
(in outer ring, next to ID09)

2007 - 2009

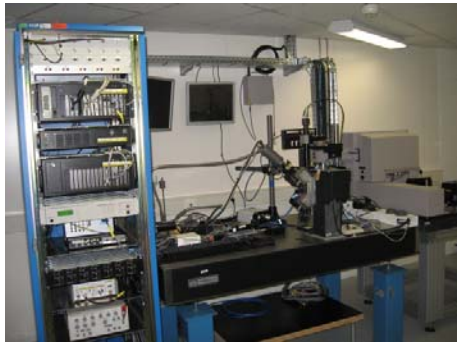
II

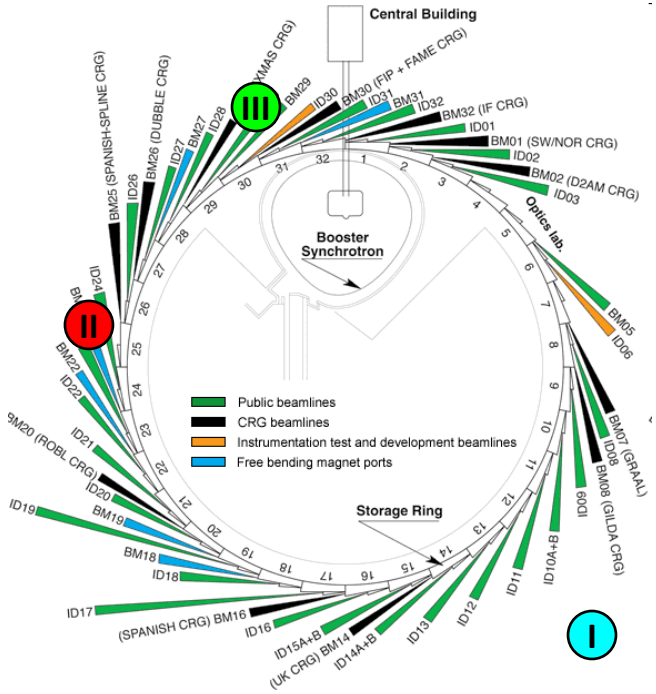
Cryobench II
(on BM23, next to beamline ID23)

2010 -

III

Cryobench III
(on beamline ID29, next to future beamline MASSIF)

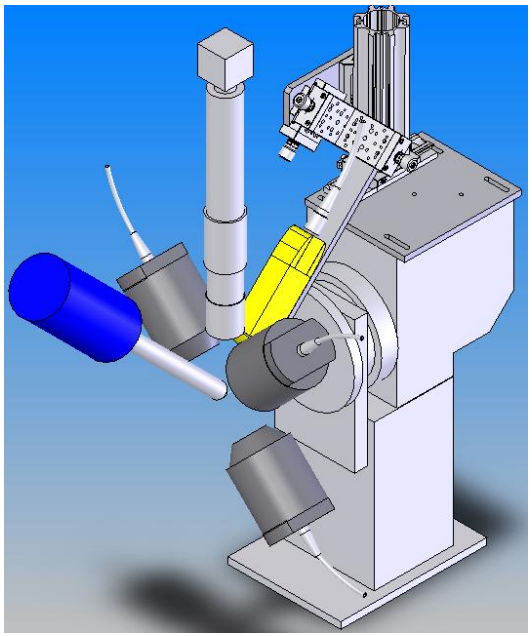


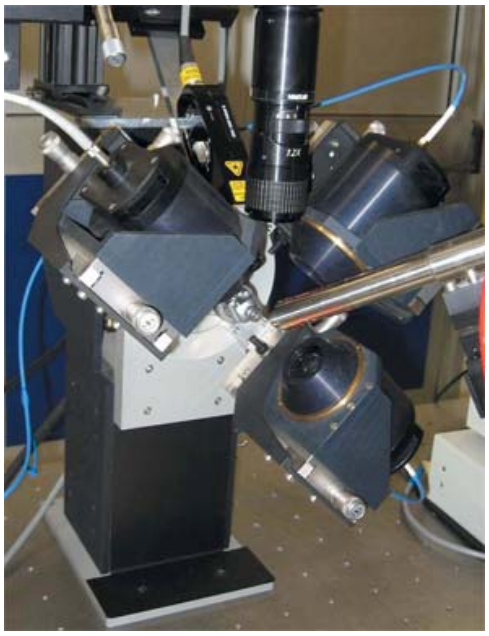


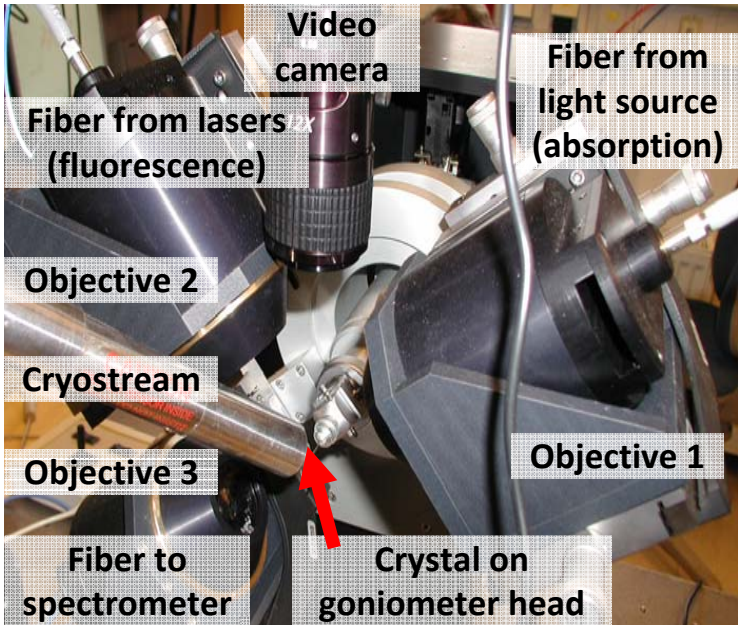
Available setups

Goal: focus and collect light on a ~10-100 µm diameter spot

How: Magnifying objectives, optical fibers, precision translation stages, video camera, lasers







- For protein micro-samples (crystal / 100 nL solution)
- Low- and Room temperature
- Available spectroscopies:

- ***UV-visible light absorption***
- ***Fluorescence (steady-state)***
- ***Fluorescence (lifetime)***
- ***Resonant Raman***
- ***Non-resonant Raman*** → *Non-colored proteins*

<u>Main difficulty:</u> Crystals are extremely concentrated in chromophores

- A simplified version can be mounted on a beamline diffractometer: **on-line microspec**

In crystallo spectroscopy

Why performing spectroscopy on crystals?

- **To check that the crystalline protein is in a comparable situation as in solution (redox state, chromophore state) – to identify functional state**

- **To check the advancement of a reaction with a spectroscopic signature**

Case of photoactive proteins (naturally, artificially) and enzymes

→ intermediate state trapping = **KINETIC CRYSTALLOGRAPHY**

- **To monitor X-ray induced modifications (inherent problem in X-ray crystallography at synchrotrons)**

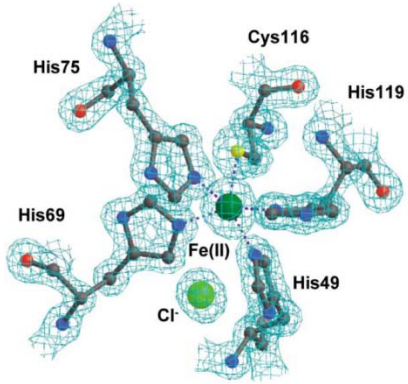
1) Application to radiation damage

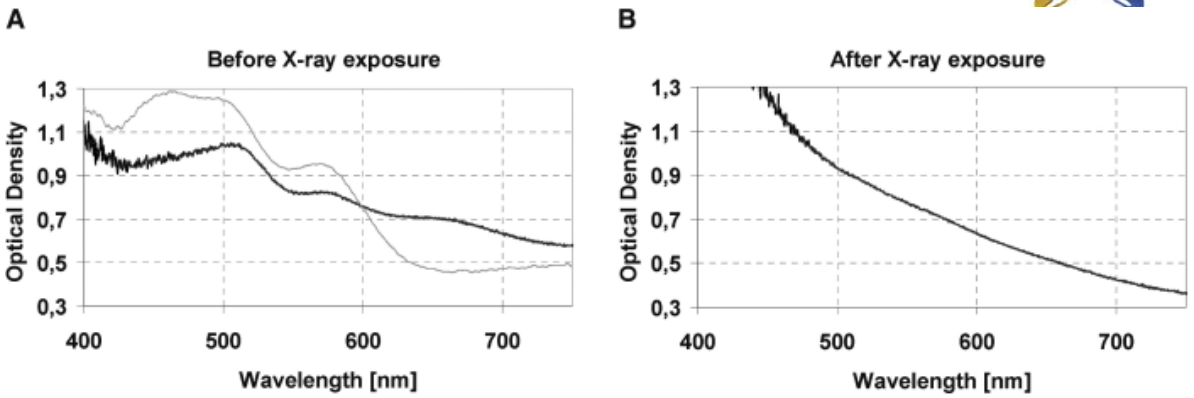
Metal center reduction

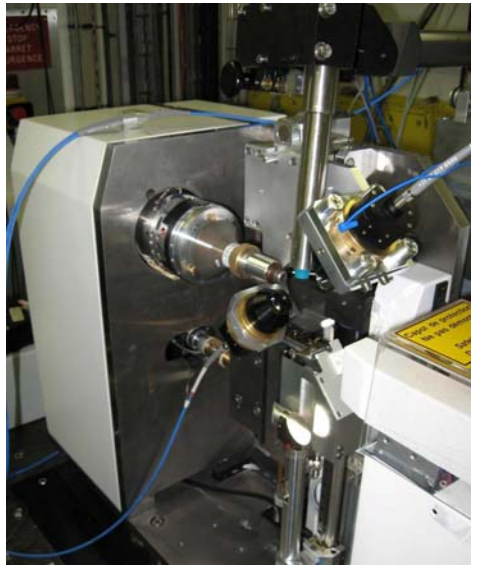
Superoxide Reductase (SOR) from *D. baarsii*

$$O_2^{-\bullet} + 2H^+ + SOR(Fe^{2+}) \rightarrow H_2O_2 + SOR(Fe^{3+})$$

- Found in sulphate-reducing and microaerophilic bacteria
- Similar to SOD, but no release of O_2
- Homodimer, 2 iron centres/ monomer.

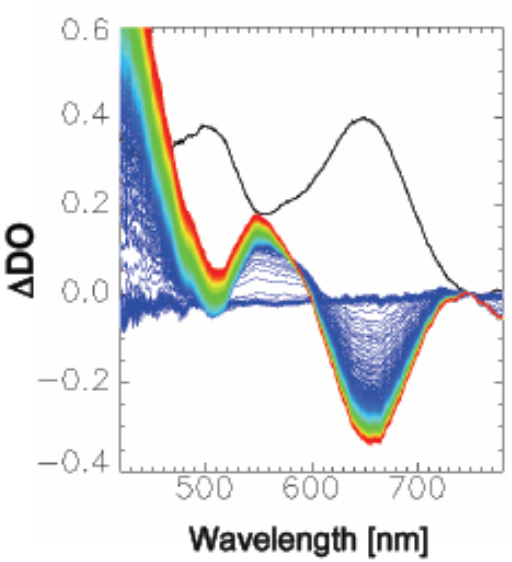


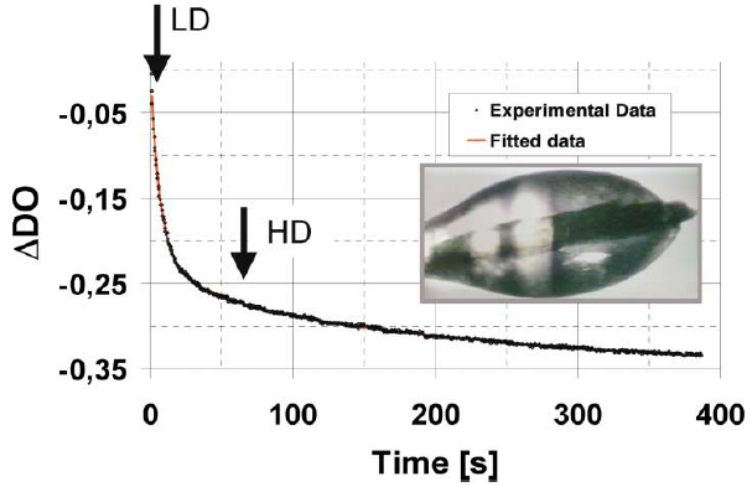


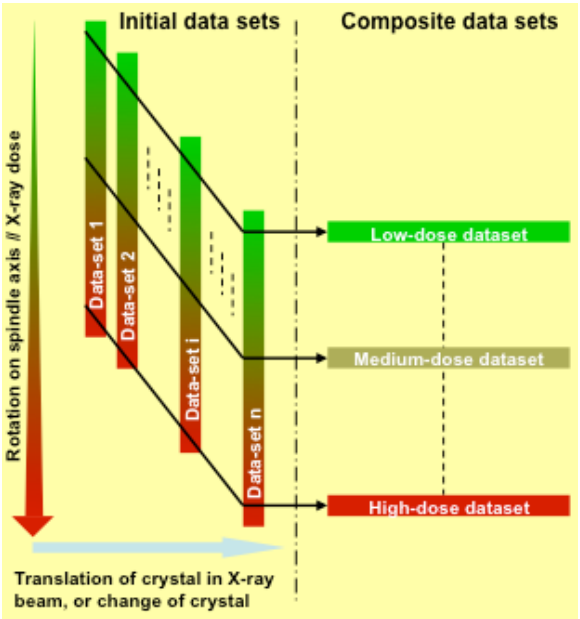


→Online microspec – ID14-eh2/eh4

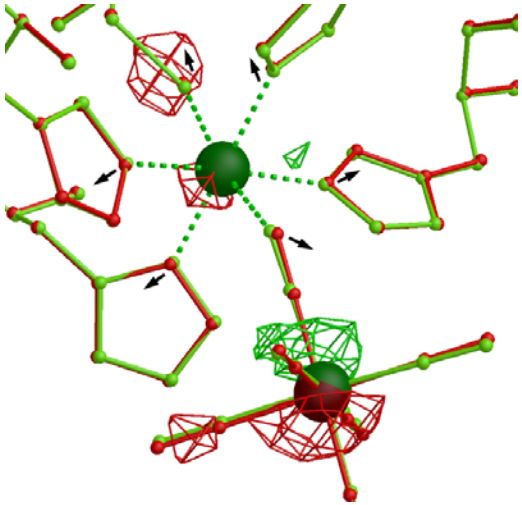
Coupling spectroscopic and structural analyses







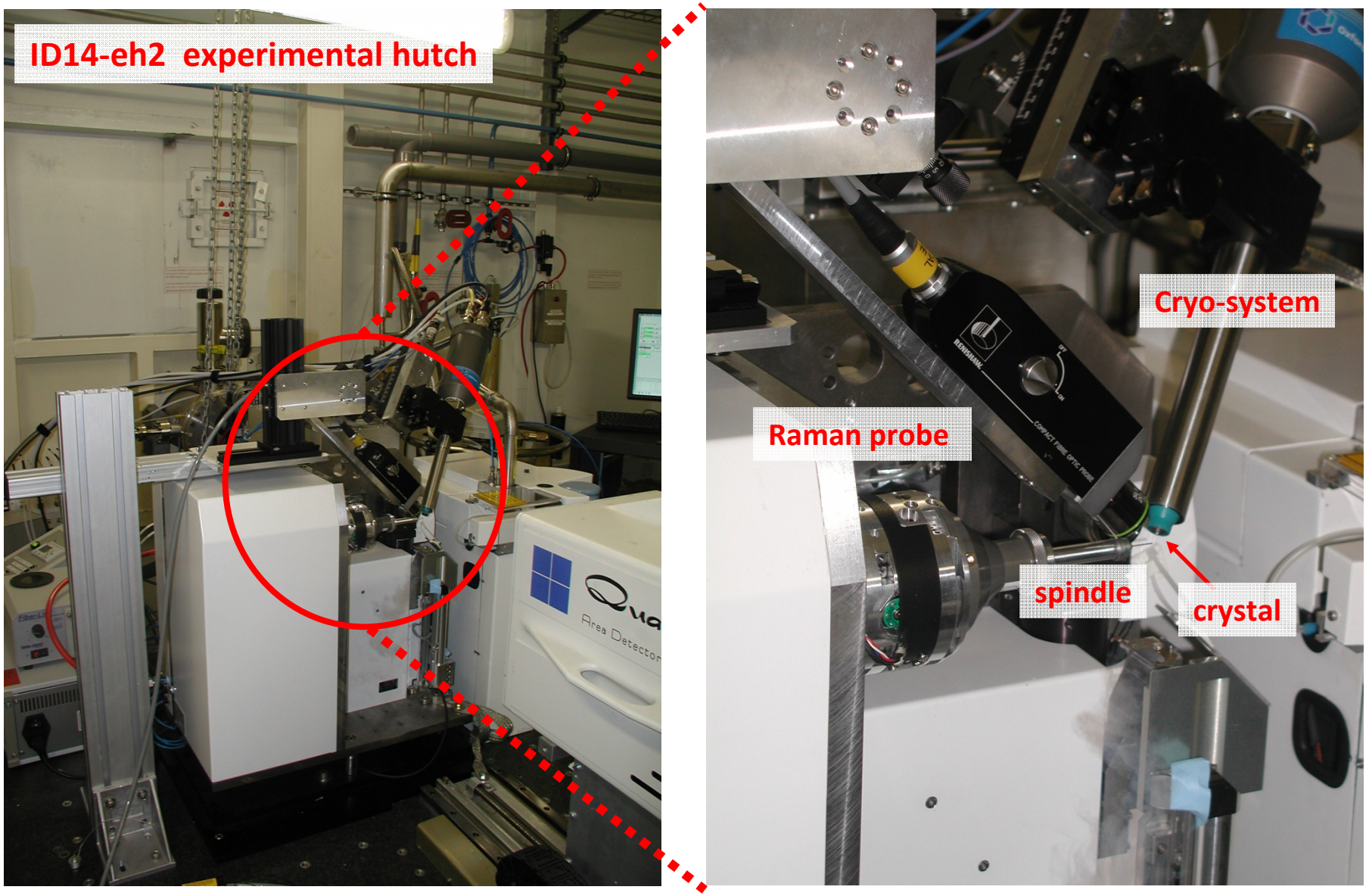
Composite datasets



Subtle active site expansion

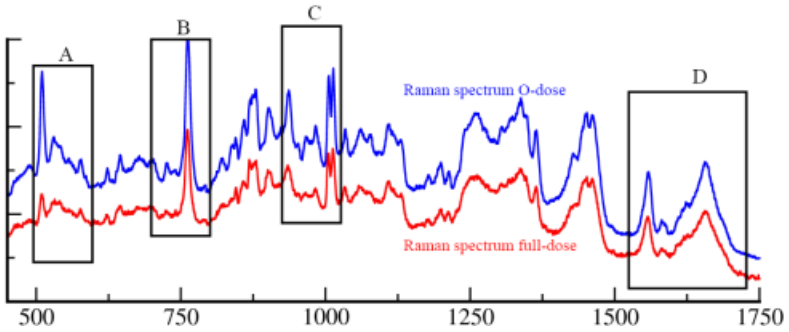
Adam *et al*, *Structure* (2004)

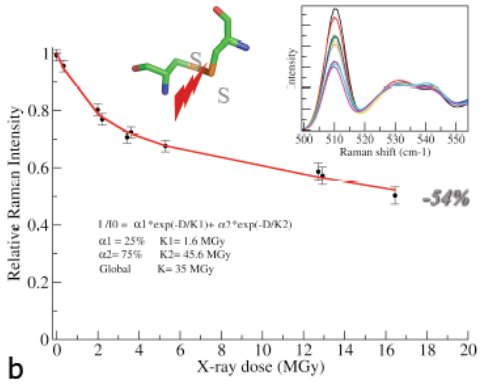
On-line Raman to monitor radiation damage



Monitoring X-ray induced bond breakage

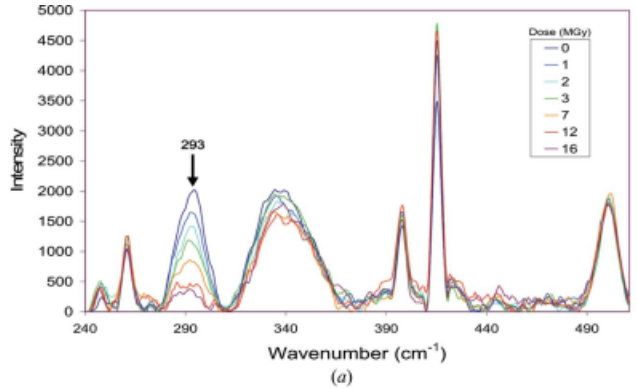
Disulfide bond breakage in lysozyme (S-S bond)

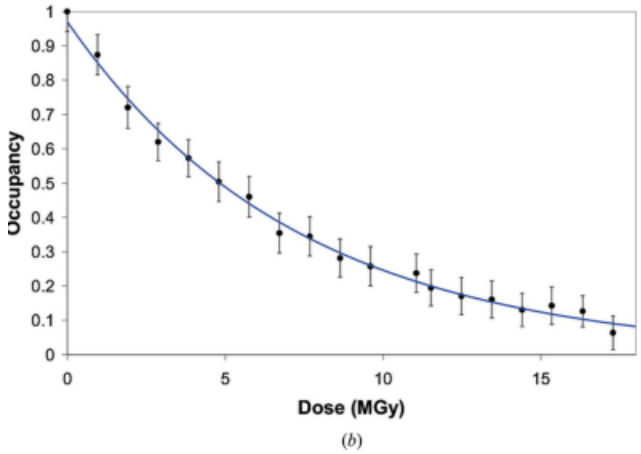




Carpentier *et al.*, *J. Appl. Cryst*. (2007)
Carpentier *et al.*, in preparation

Debromination of brominated DNA (C-Br bond)

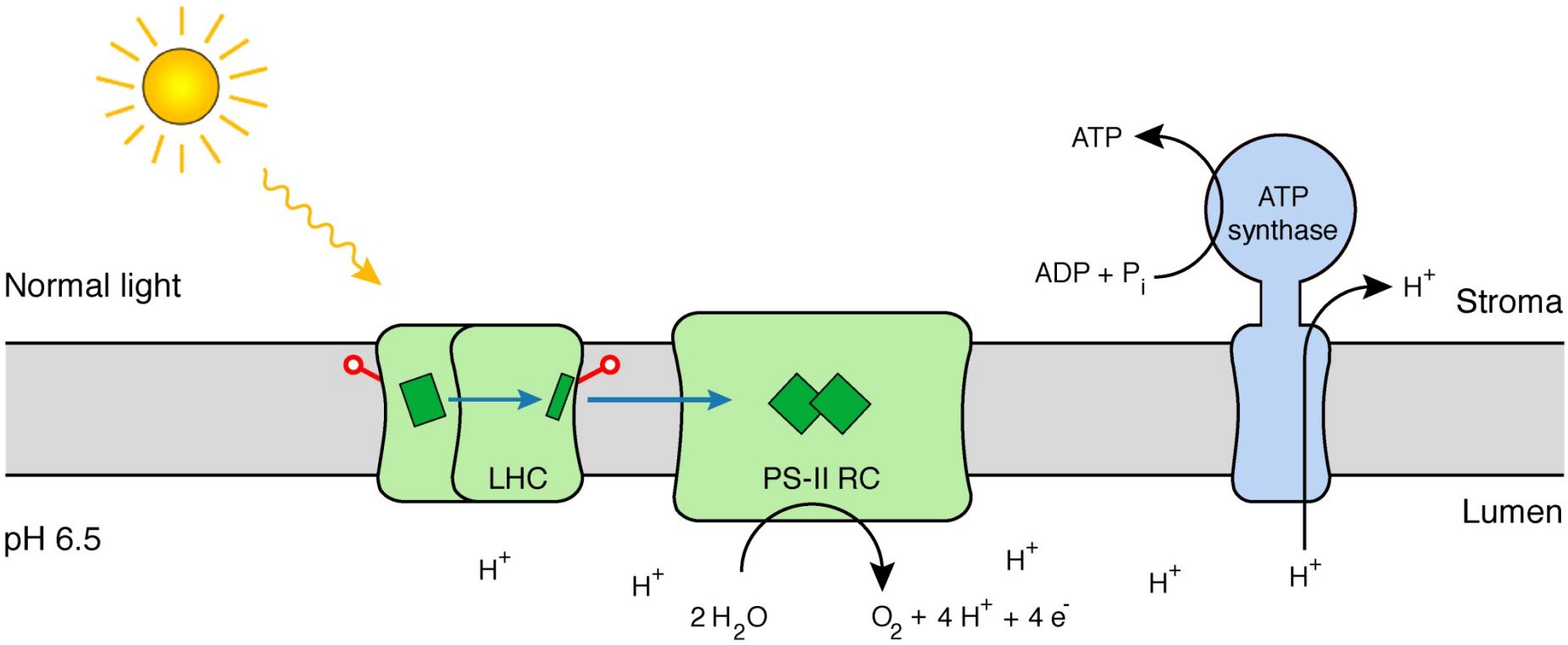




McGeehan *et al.*, *J. Synchrotron Rad.* (2007)

2) Application to functional characterization of proteins

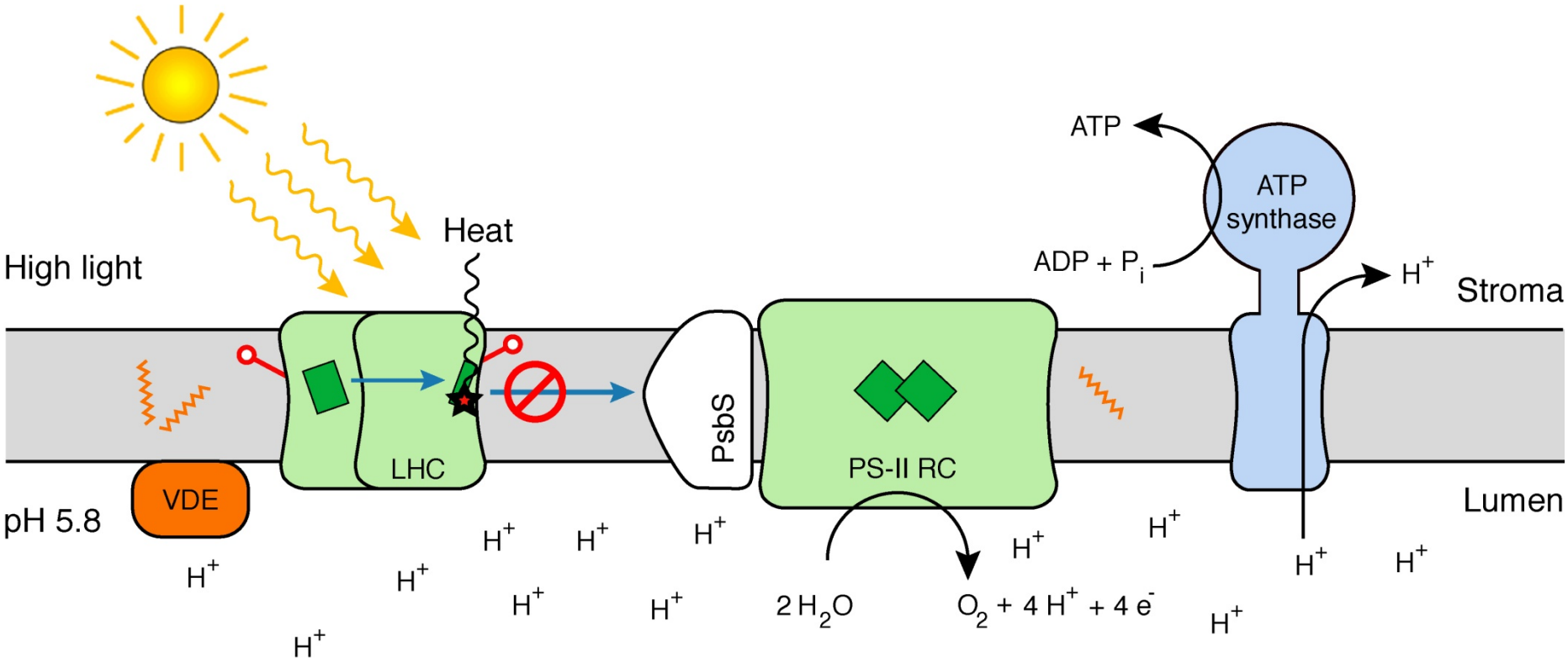
Light-harvesting in plant photosynthesis



LHC-II = Light-harvesting complex of photosystem II

= 30% of all proteins in thylakoid membranes

Light-stress and non-photochemical quenching



Quenching = lowering of fluorescence emission efficiency

LHC-II thought to switch between an active (**energy-transmitting**) and an inactive (**energy-dissipative**) state

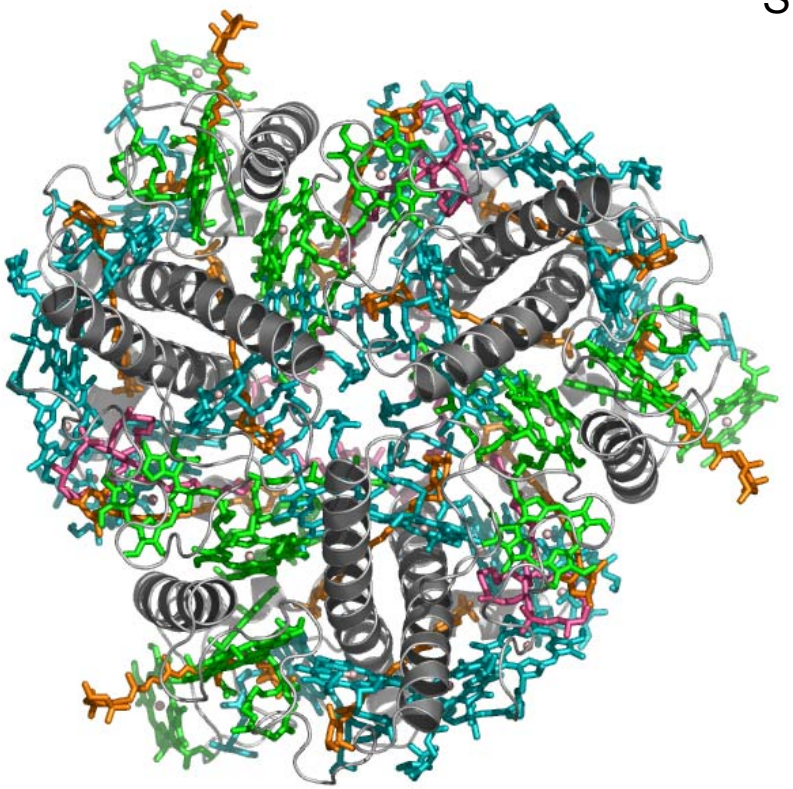
Light-Harvesting Complex II

3 monomers
669 residues

12 carotenoids
6 x lutein
3 x neoxanthin
3 x violaxanthin

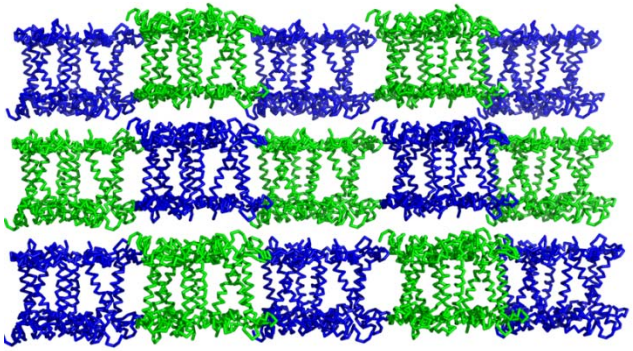
42 chlorophylls
24 x Chl *a*
18 x Chl *b*

6 lipids
3 x PG
3 x DGDG



Standfuss et al., *EMBO J.* (2005)

Pea LHC-II
pH 5.3
2.4 Å
Type I



What is the functional state of the crystalline protein?

First characterization of LHC-II crystals

- Pascal *et al.*, *Nature*, 2005

Fluorescence of crystals measured in a regular bench fluorimeter

Active state (unquenched)

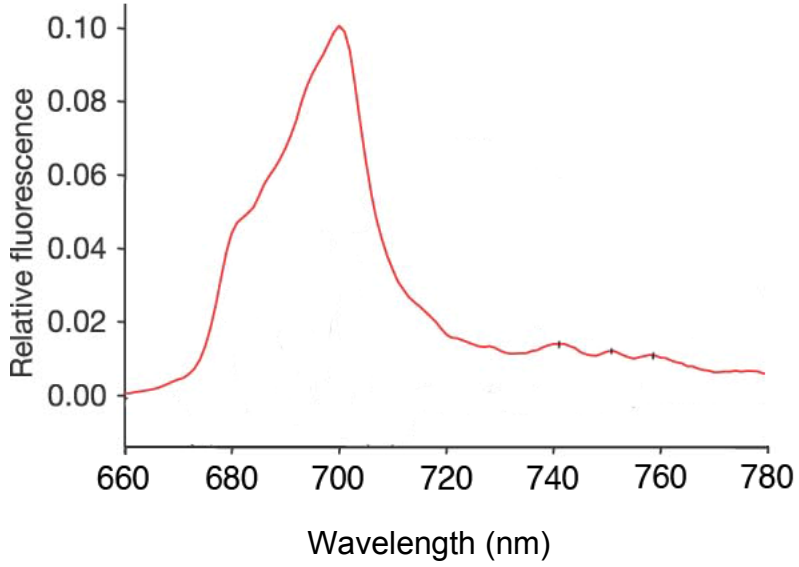
Long fluorescence lifetime (ns)

680 nm

Inactive state (quenched)

Short fluorescence lifetime (ps)

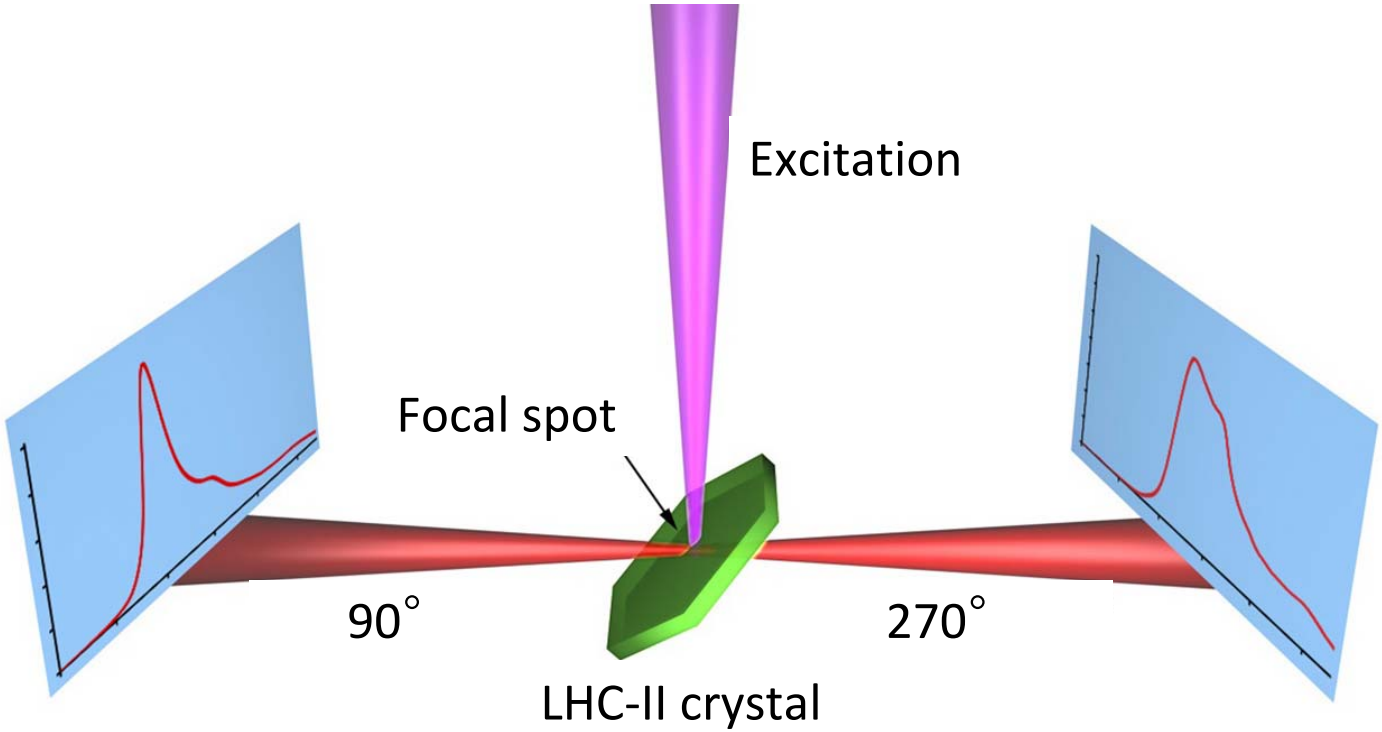
700 nm



→ **LHCII inactive (quenched) in crystals**

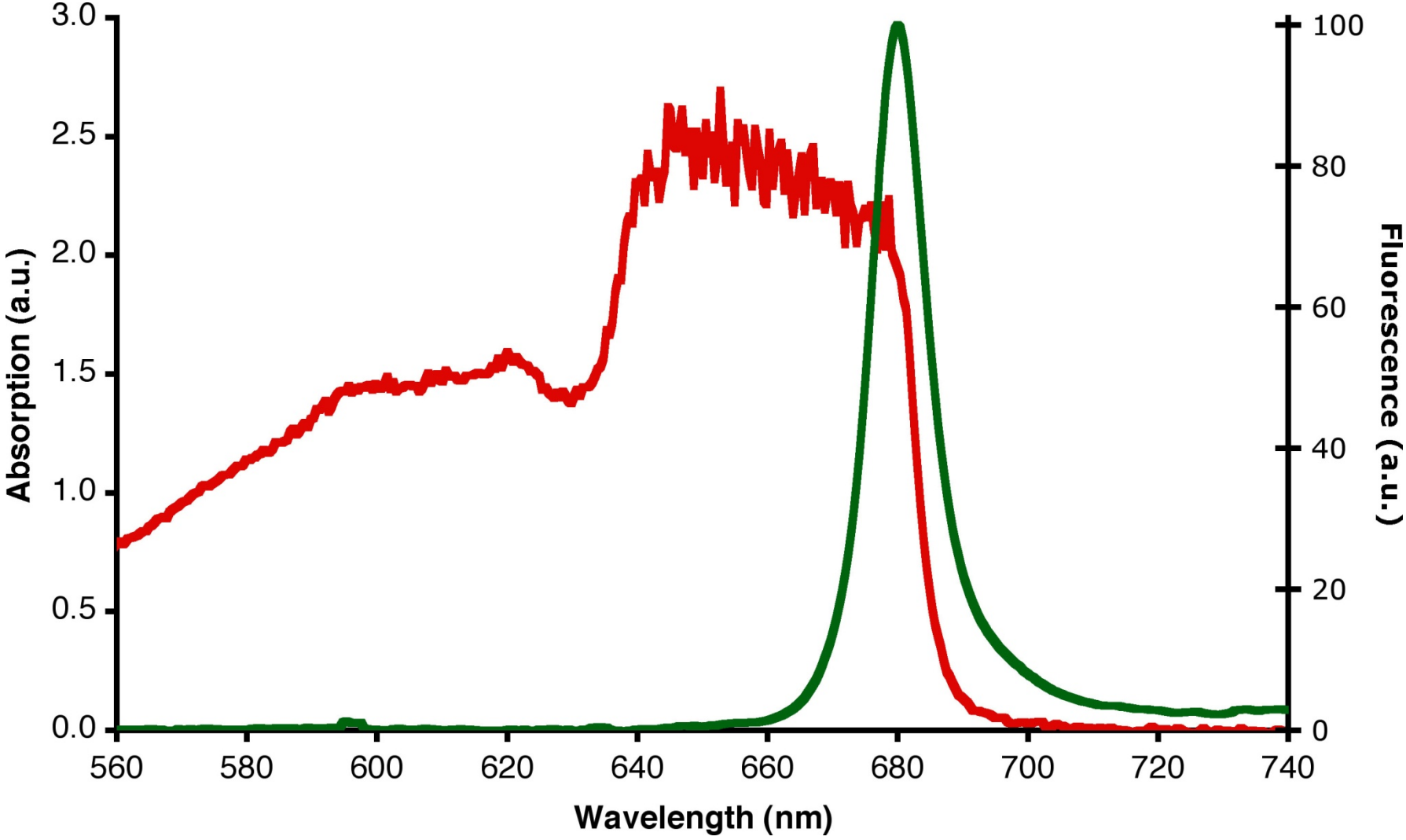
Functional properties of LHC-II in single-crystals

Single-crystal spectroscopy



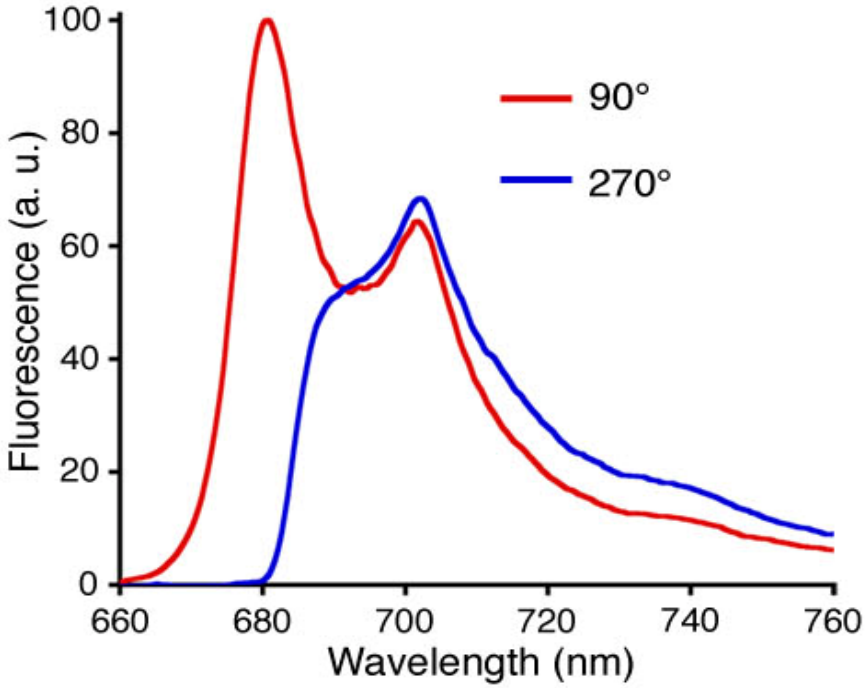
Barros *et al.*, *EMBO J.*, 2009

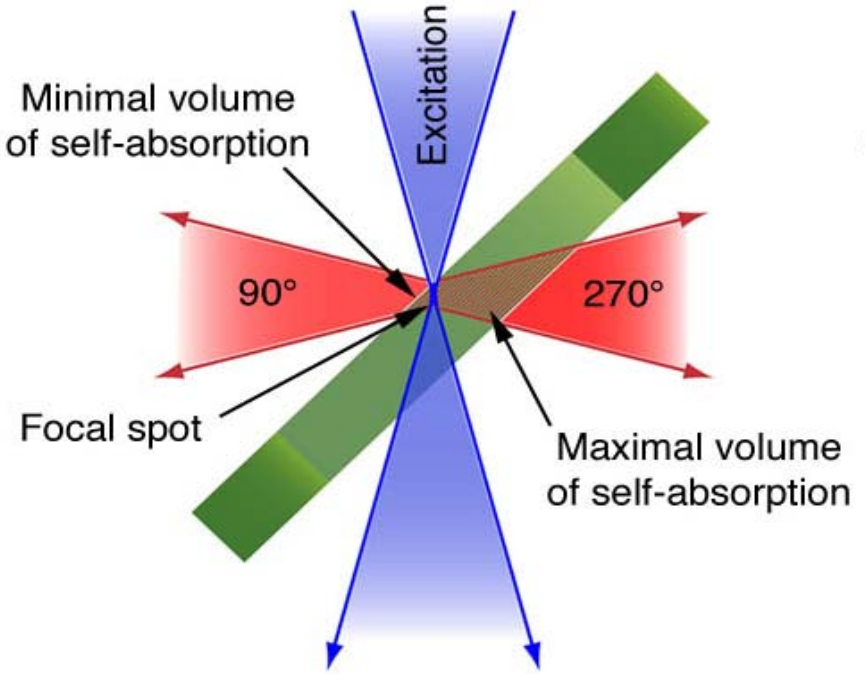
Absorption and emission spectra of a pea LHC-II crystal



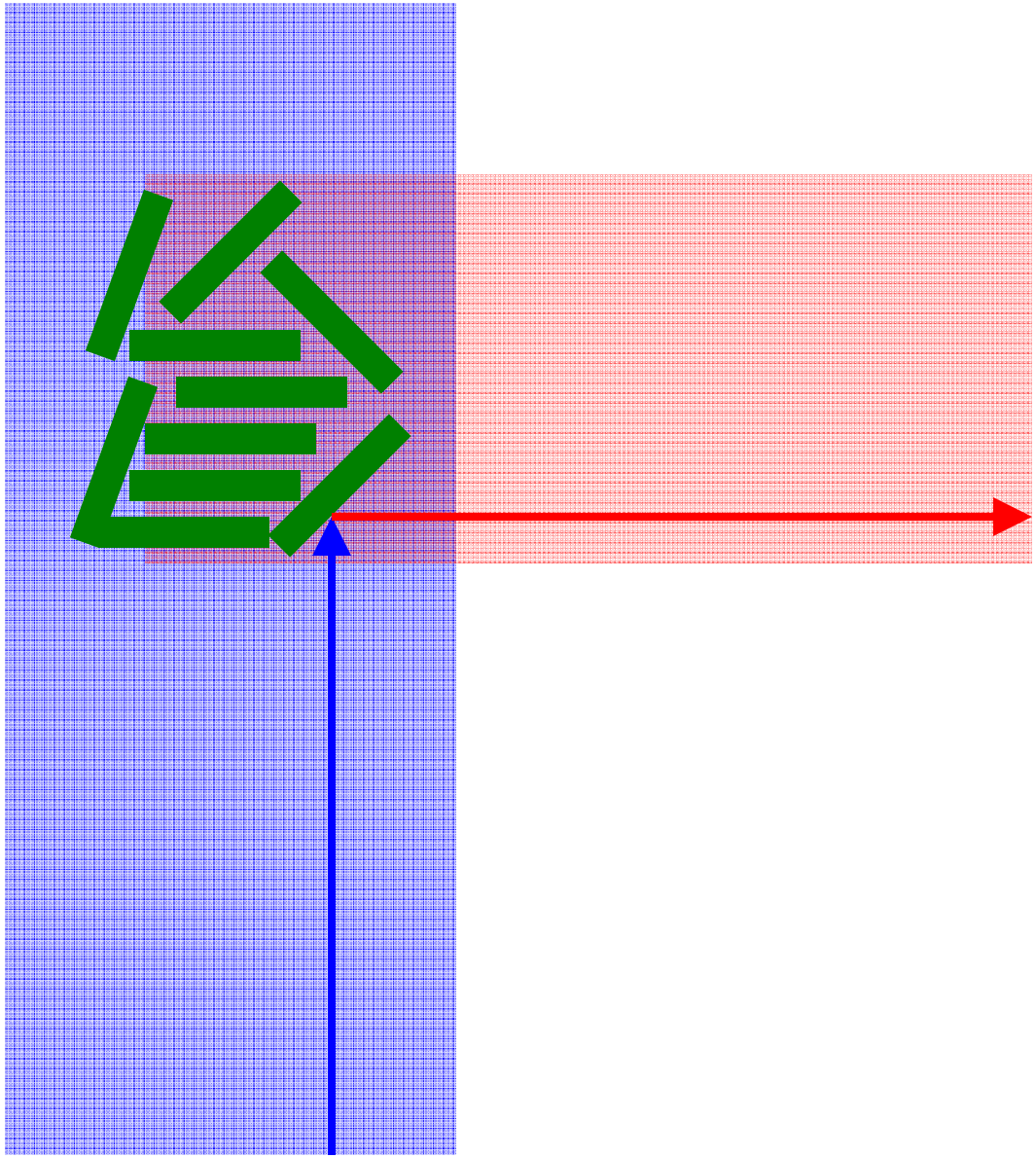
Functional properties of LHC-II in single-crystals

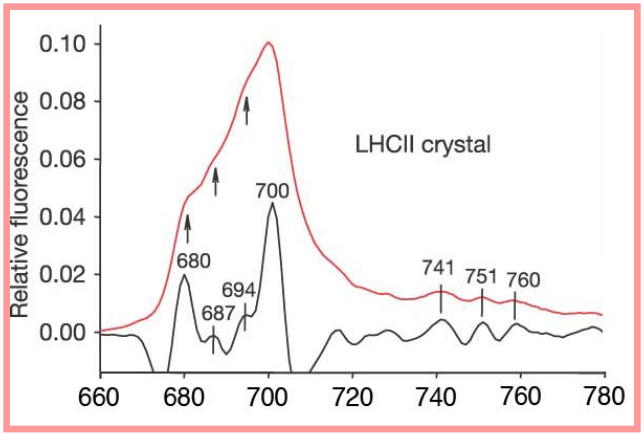
Self-absorption of LHC-II fluorescence

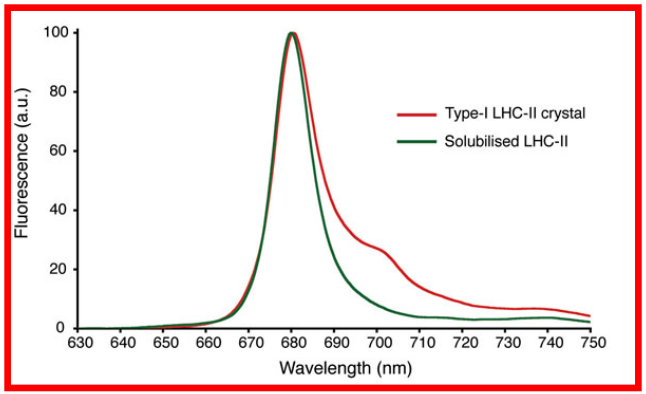




Single crystal *vs.* fluorimeter

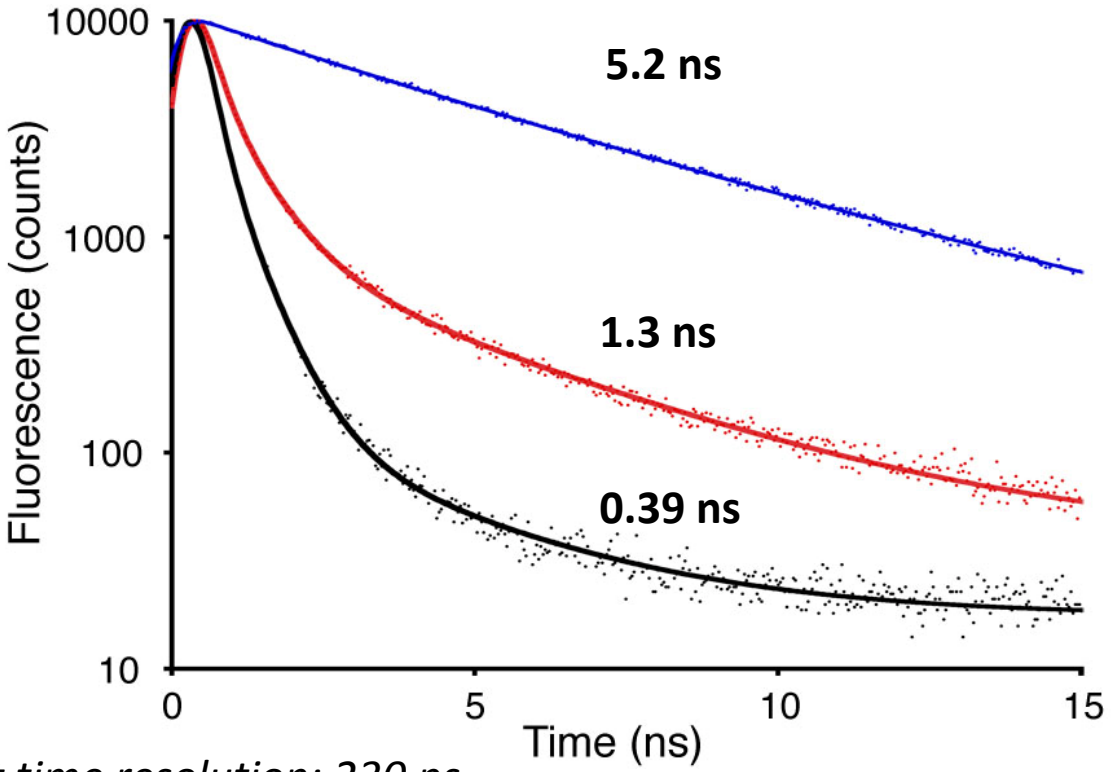






Functional properties of LHC-II in single-crystals

Fluorescence lifetimes (low temperature)



mg (Chl) /mL

Solution **0.28**

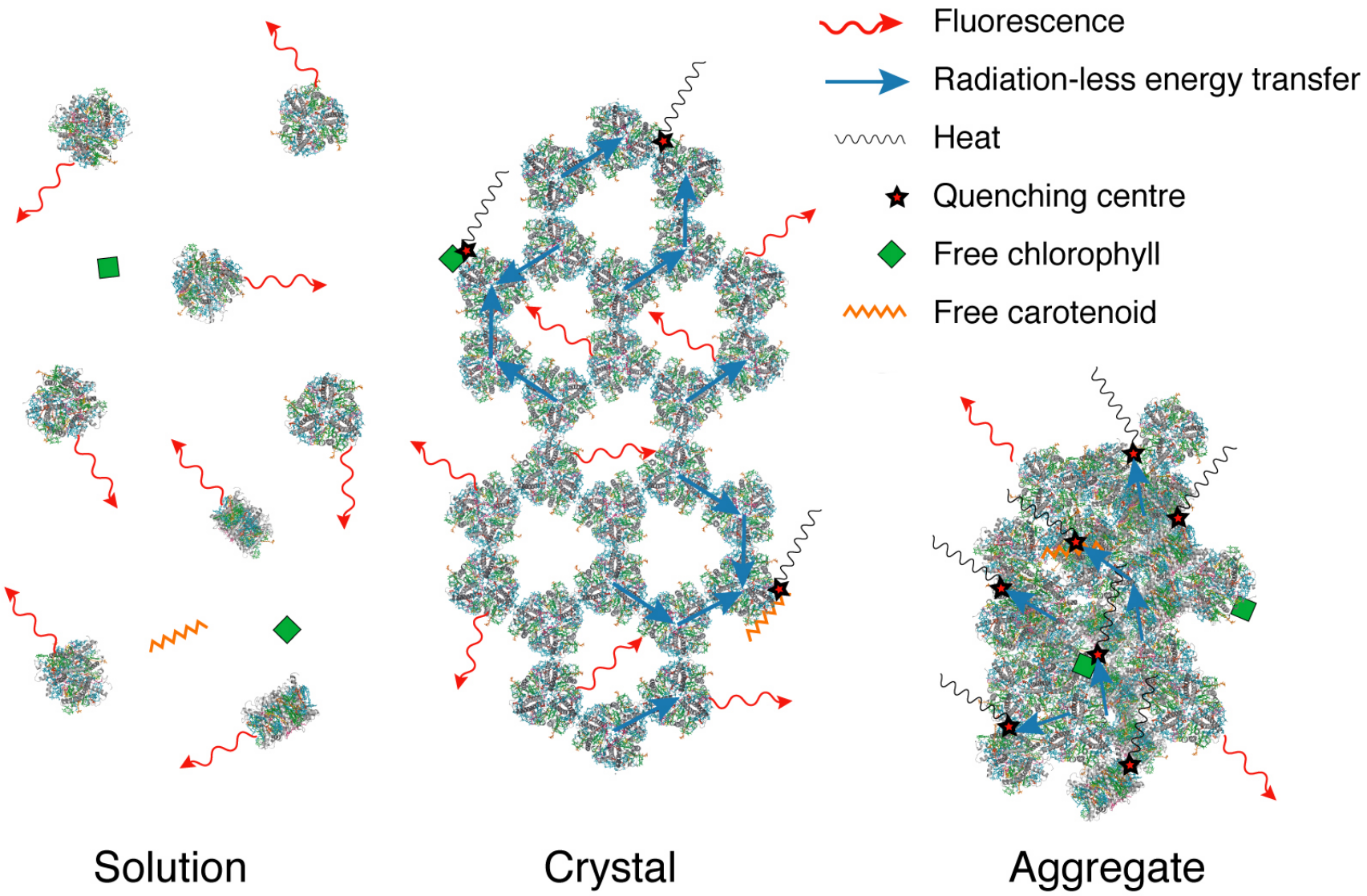
Single crystal **~ 150**

Aggregates

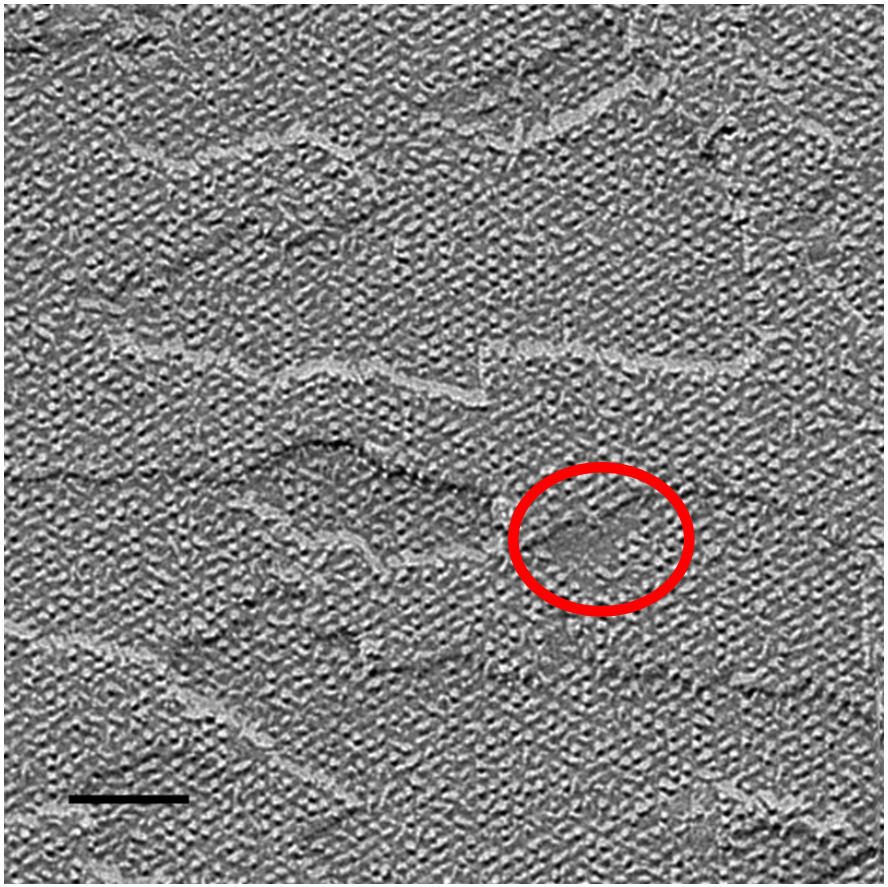
Instrument time resolution: 230 ps

Fluorescence in crystals is quenched, but cannot originate from a conformational change

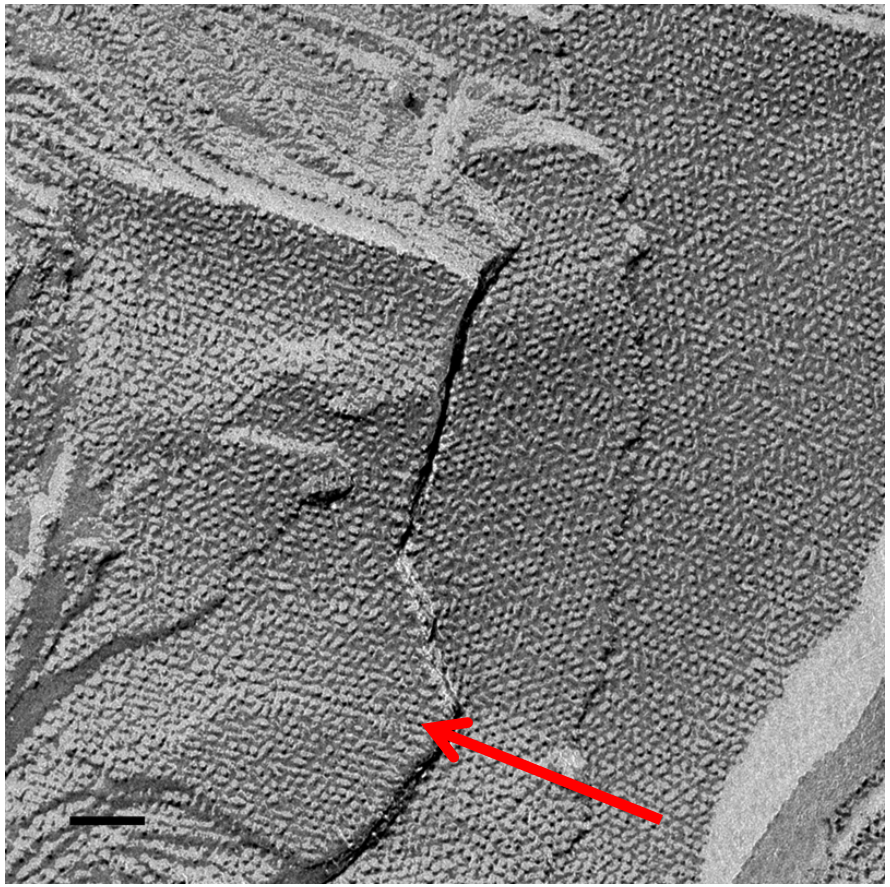
Energy transfer and dissipation *in vitro*



Defects in the type-I LHC-II crystal lattice



Gap in the crystal lattice



Layers out of register

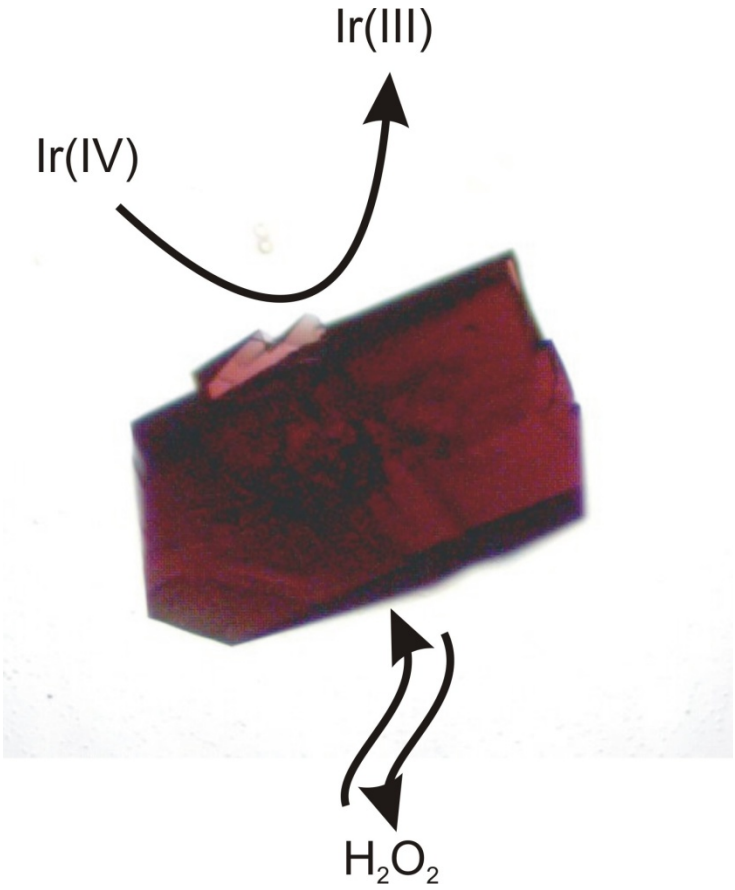
Barros & Kühlbrandt , *BBA*, 2009

3) Application to kinetic crystallography

Trapping peroxide intermediates in SOR

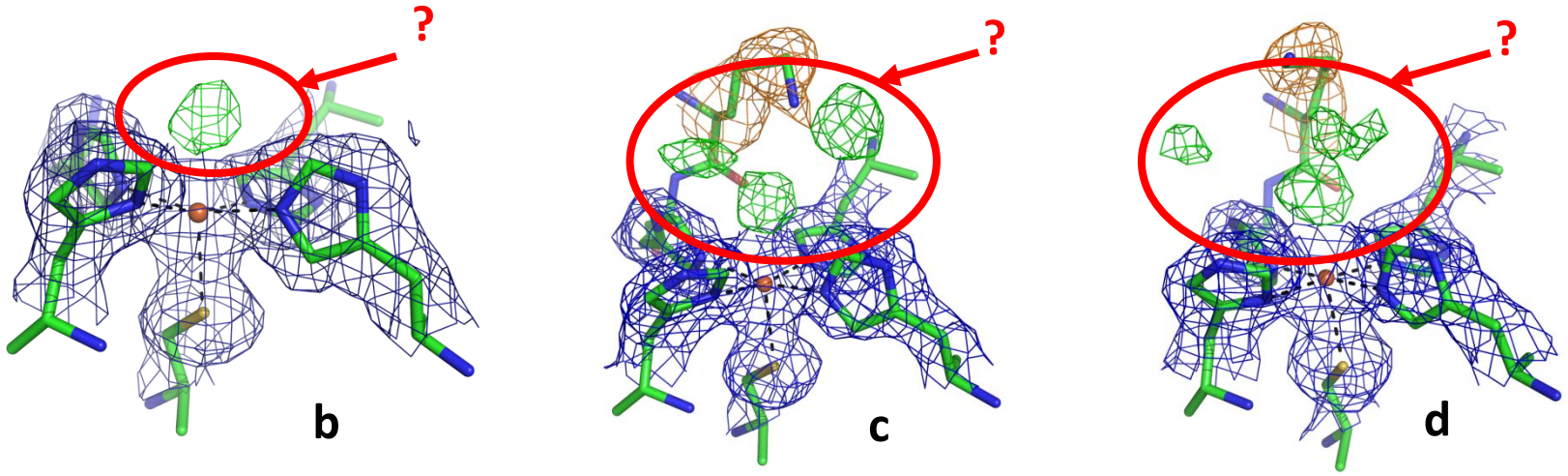
$$O_2^{-\bullet} + 2H^+ + SOR(Fe^{2+}) \rightarrow H_2O_2 + SOR(Fe^{3+})$$

- **Using the E114A mutant**
  - **Stabilizes peroxide intermediate**
- **Driving the reaction backwards at pH 9.0**
- **Soak & flash-cool**
  - **First oxidize enzyme with 8 mM $[IrCl_6]^{2-}$**
  - **Then 10 mM H_2O_2**



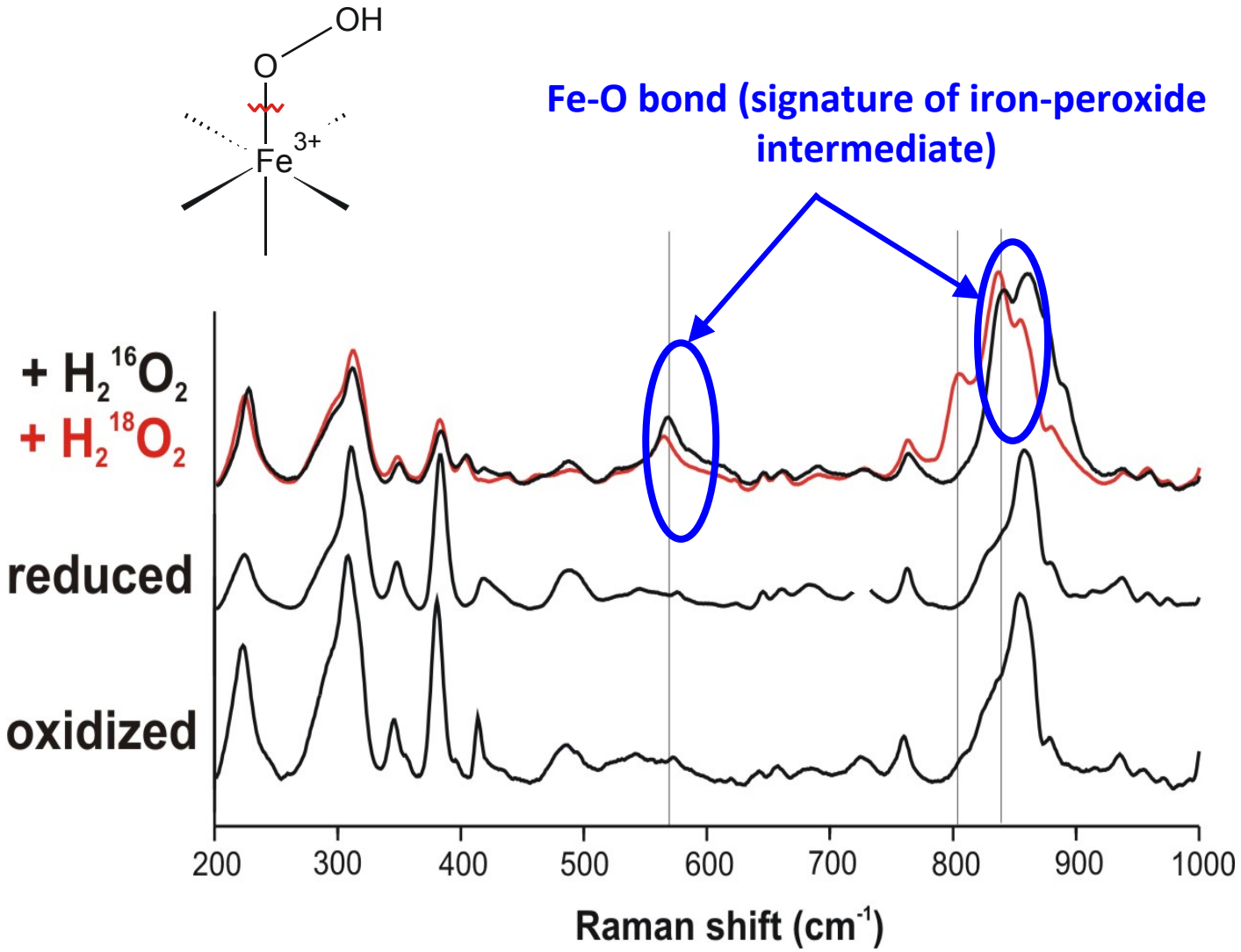
Katona *et al*, *Science* (2007)

Puzzling crystallographic data

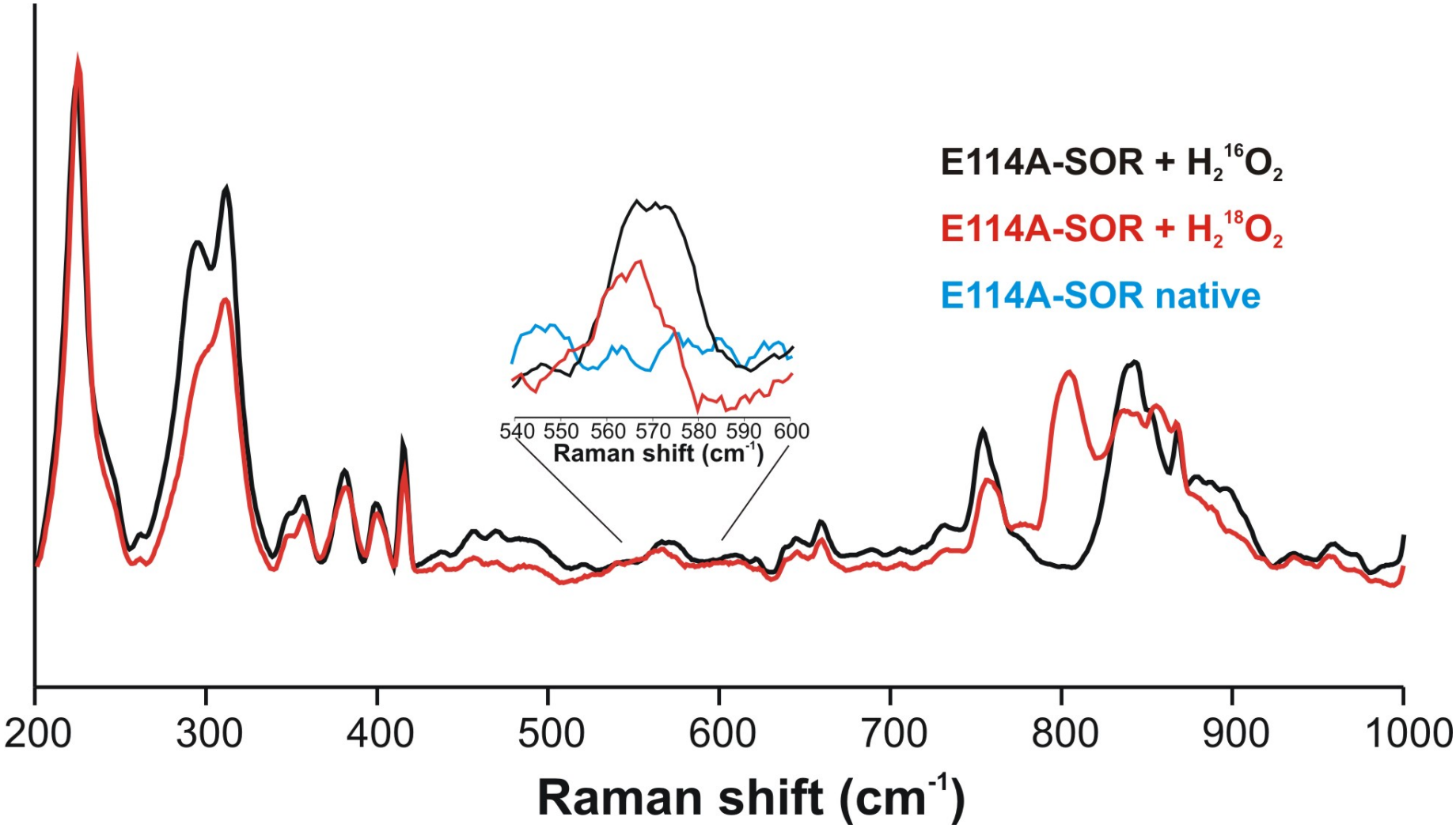


- Limited crystallographic resolution (1.95 Å)
- Multiple observations in the asymmetric unit
- Can these be relevant intermediate species?

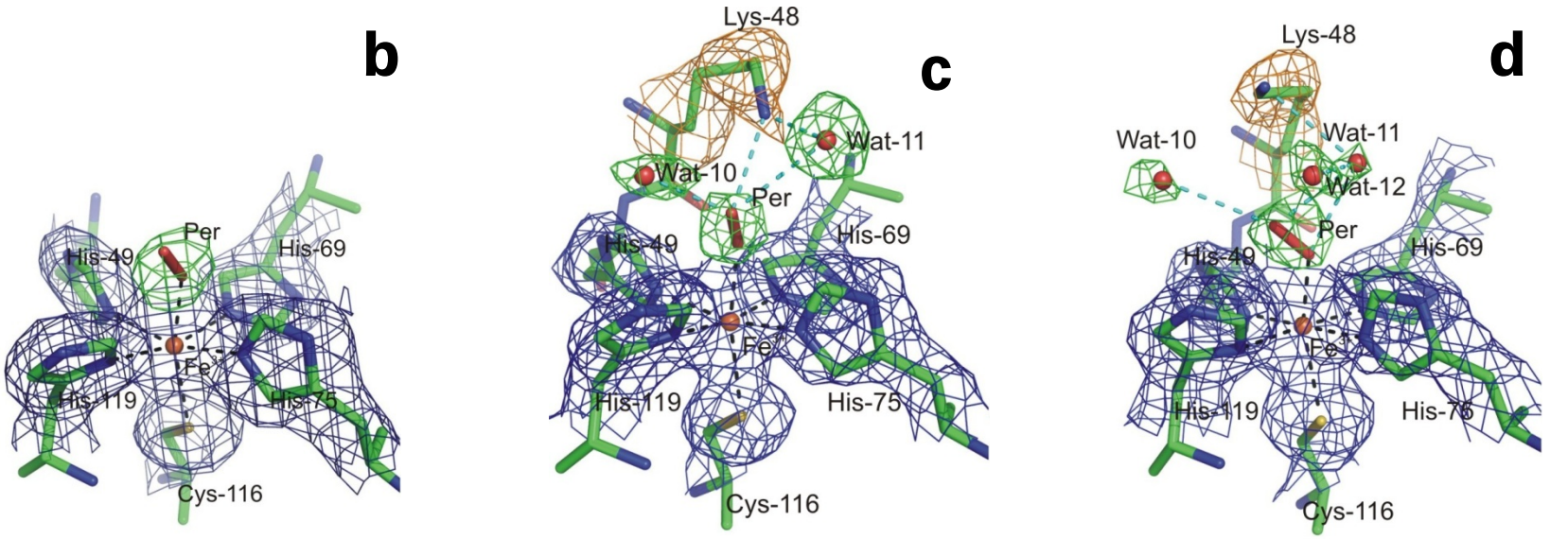
In crystallo Raman Spectroscopy of SOR

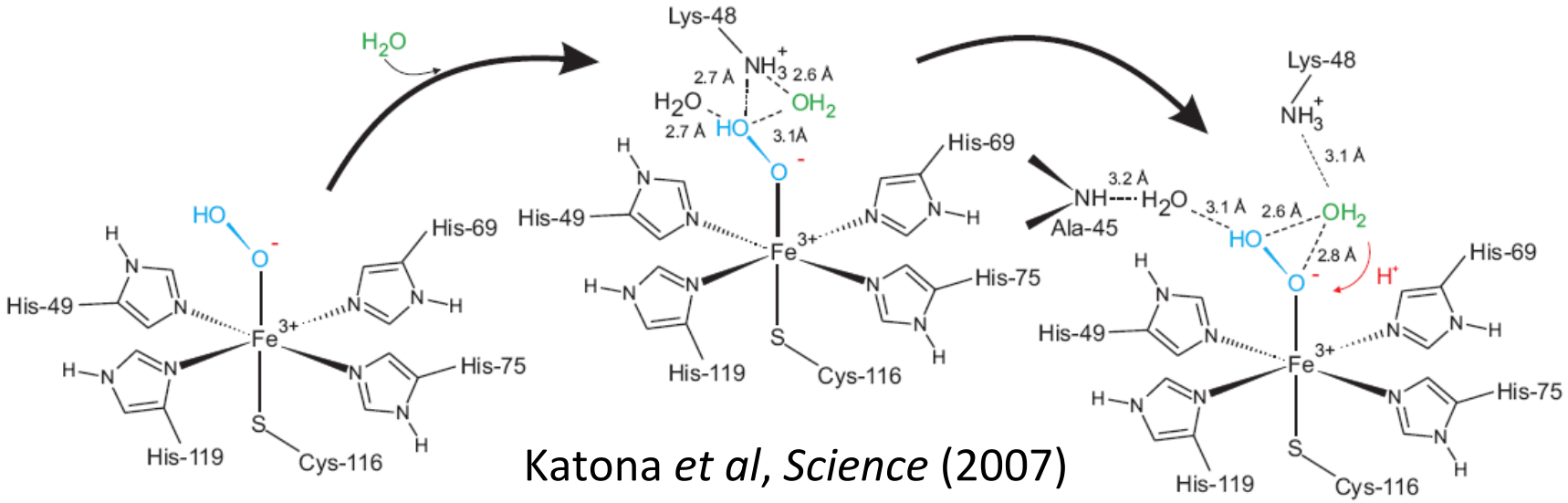


Comparison with solution state



Proposed mechanism

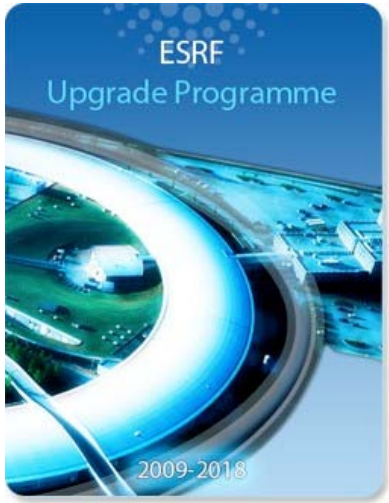




Katona *et al*, *Science* (2007)

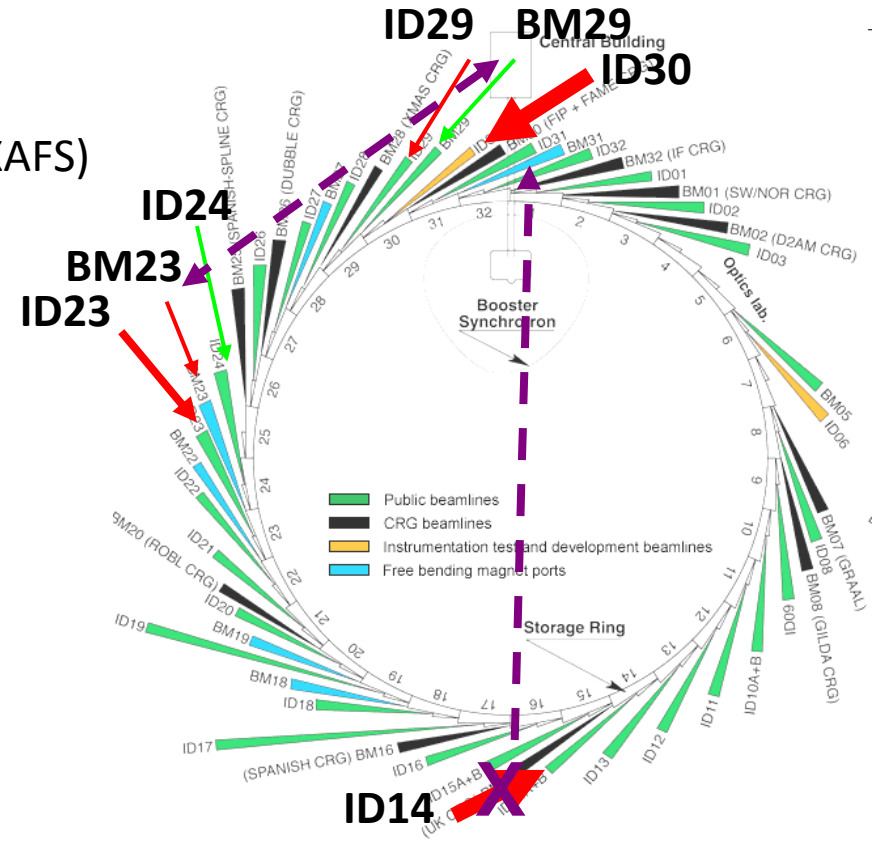
The new Cryobench

Context of Crybench moving

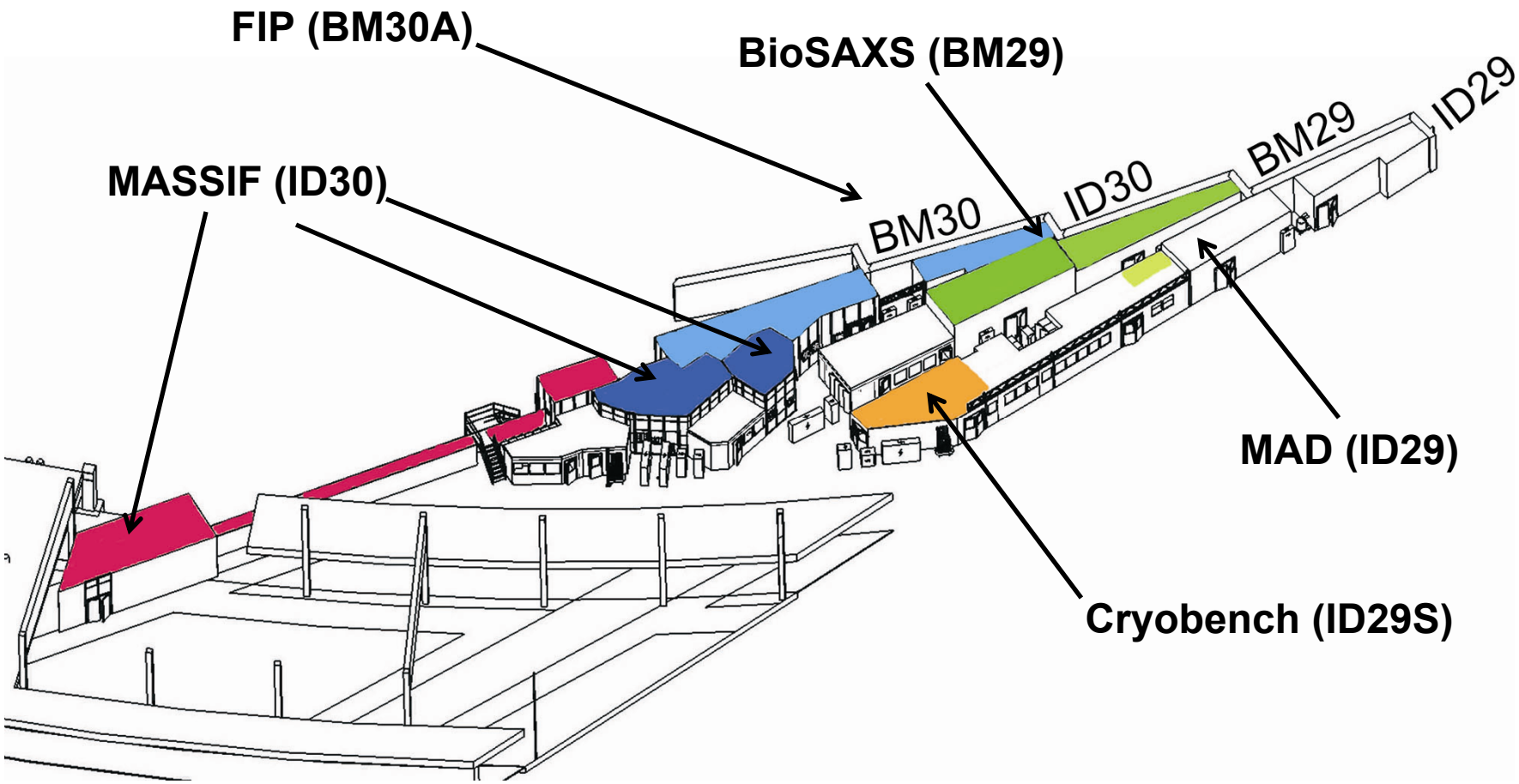


- ESRF upgrade program → reorganization of beamlines, search for synergies (regrouping of BLs)
- Exchange **BM23** (Cryobench, '*Structural Biology*' group) with **BM29** (X-ray Absorption Spectroscopy, '*Electronic Structure and Magnetism*' group)
- New **BM23** grouped with **ID24** (Dispersive EXAFS)
- Cryobench moved on ID29

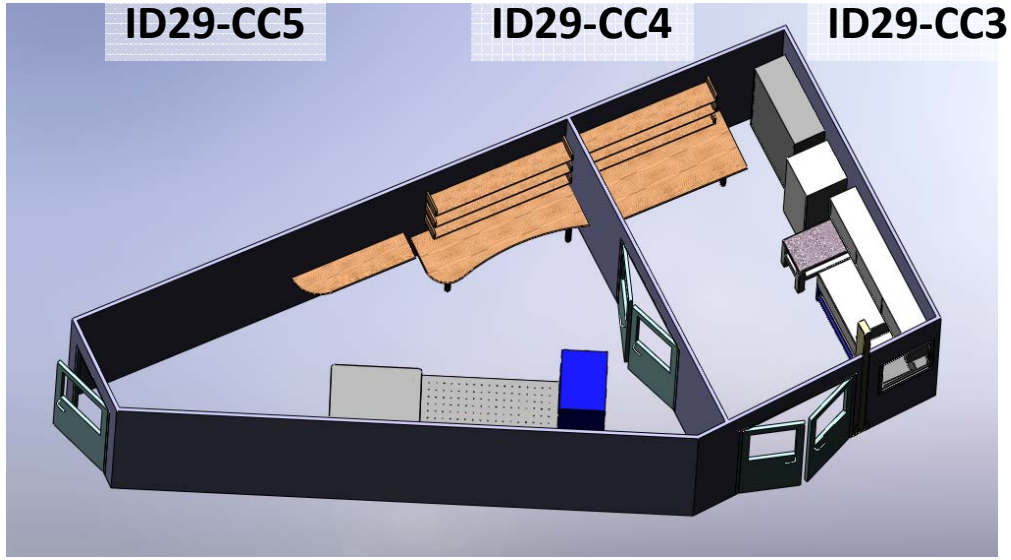
  → ID29S, grouped with [**ID29** - future **BM29** (BioSAXS) - future **ID30** (MASSIF)]

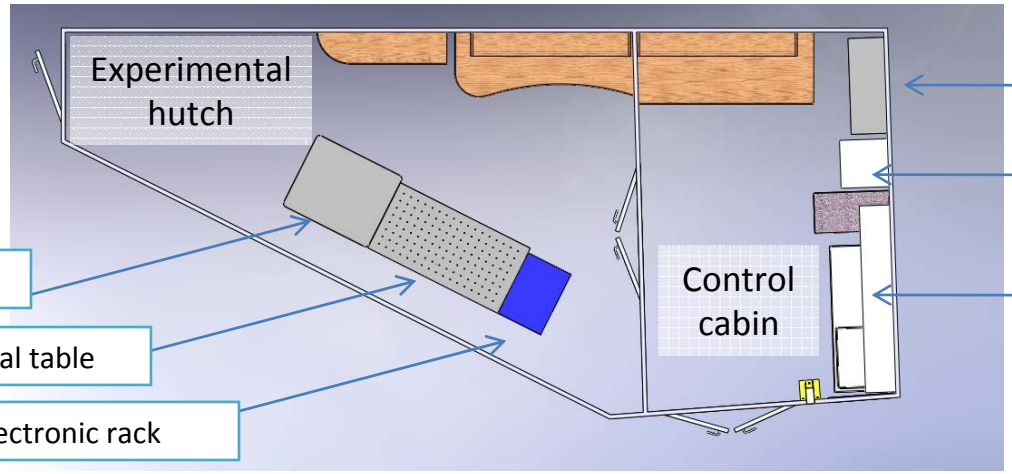


UPBL10 project (MASSIF)



Cryobench v3.0





Storage cupboard

Fridge/freezer

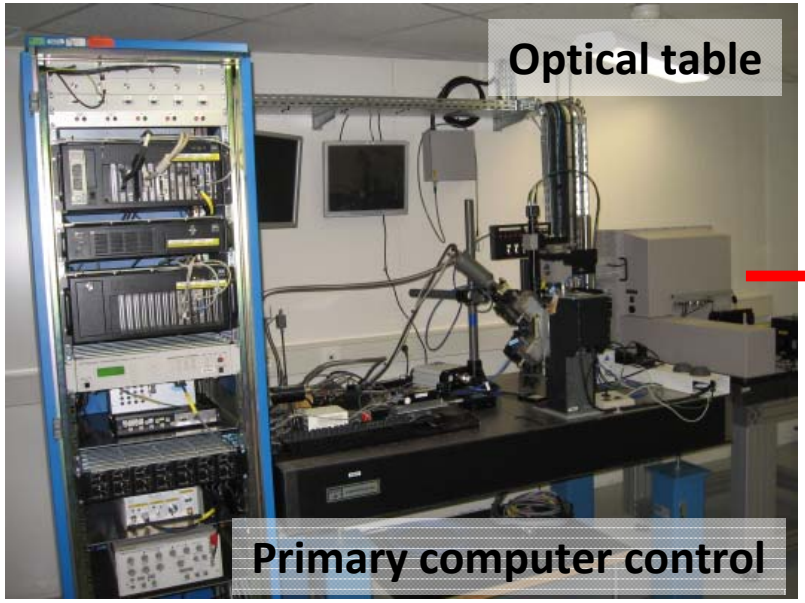
Wet bench

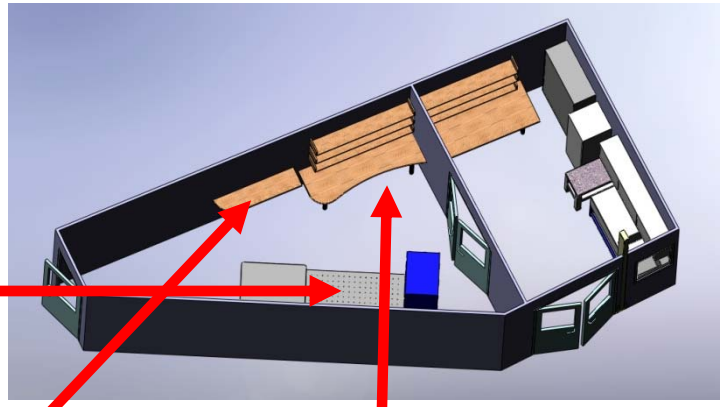
Raman spectrometer

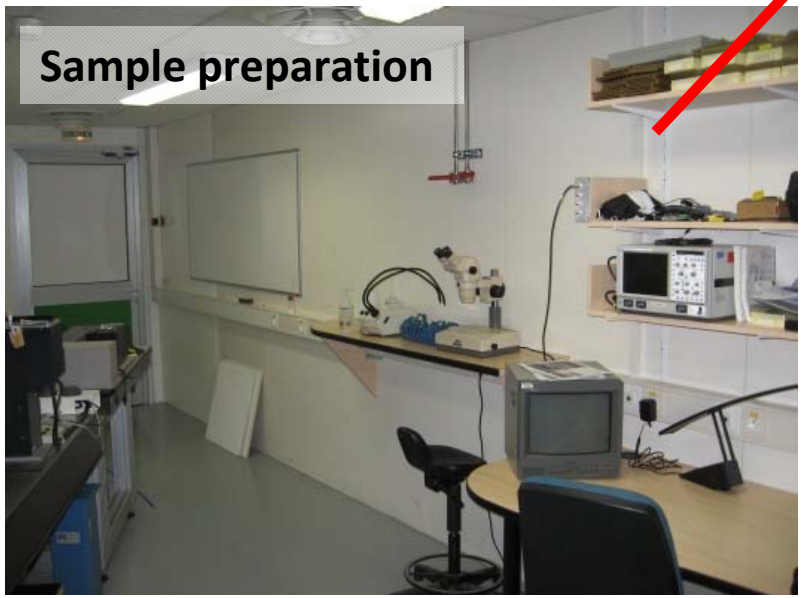
Main optical table

Electronic rack

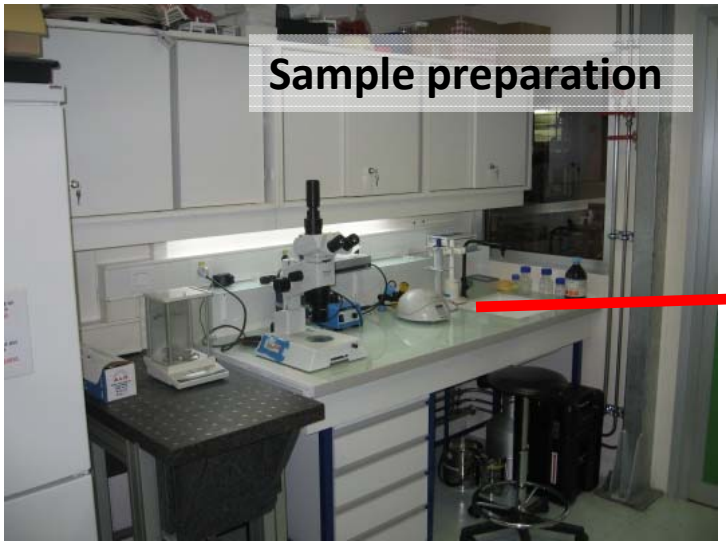
Experimental hutch

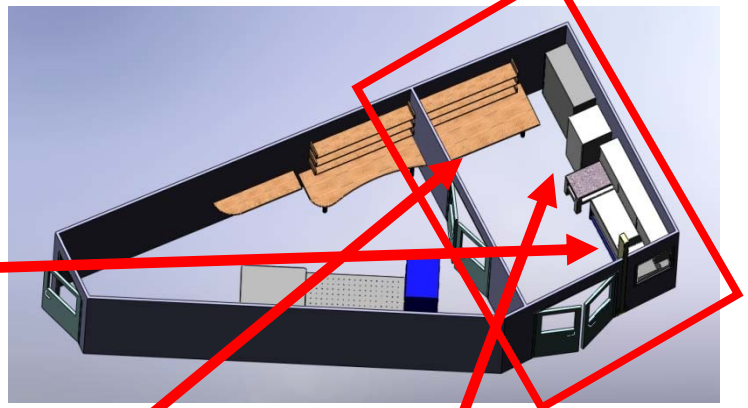


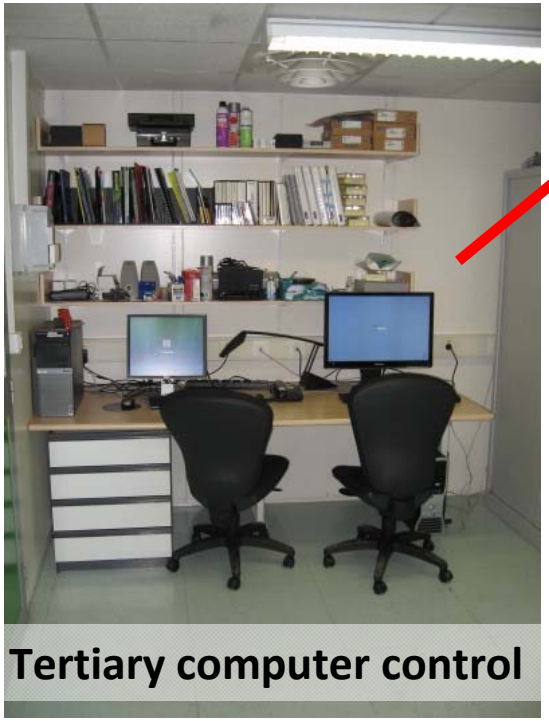




Control cabin





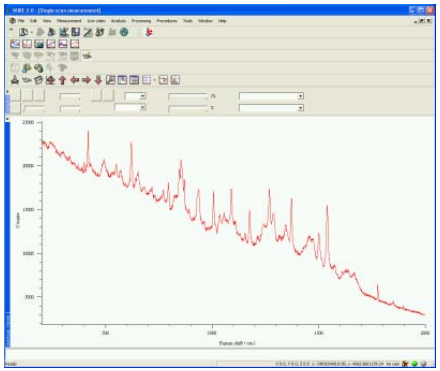


Computing environment

- Proprietary software

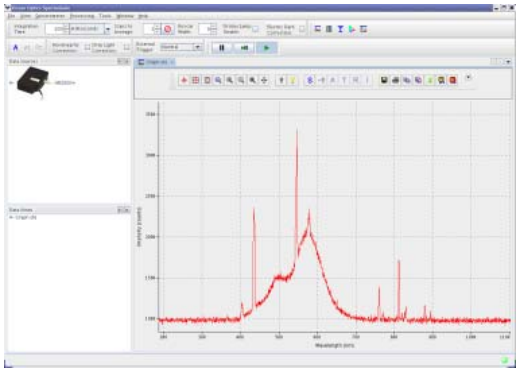
Raman

Wire2 (Win XP)



UV-Visible absorption, fluorescence

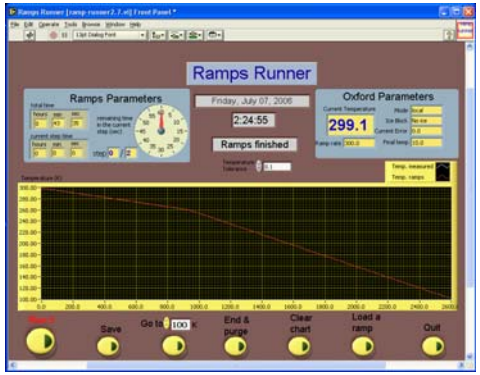
SpectraSuite (Linux)



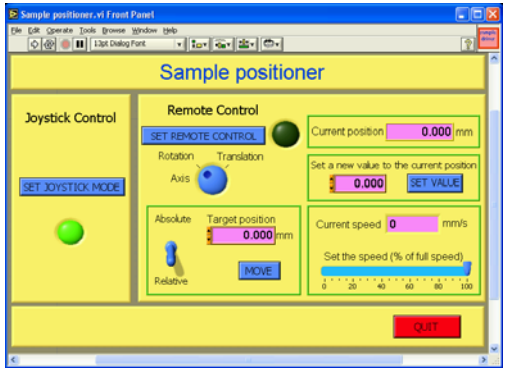
- Instrument environment: LabVIEW modules

TTL signal generation for laser triggering

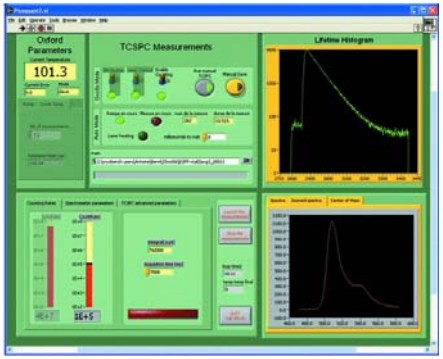
Oxford cryosystem temperature control and monitoring



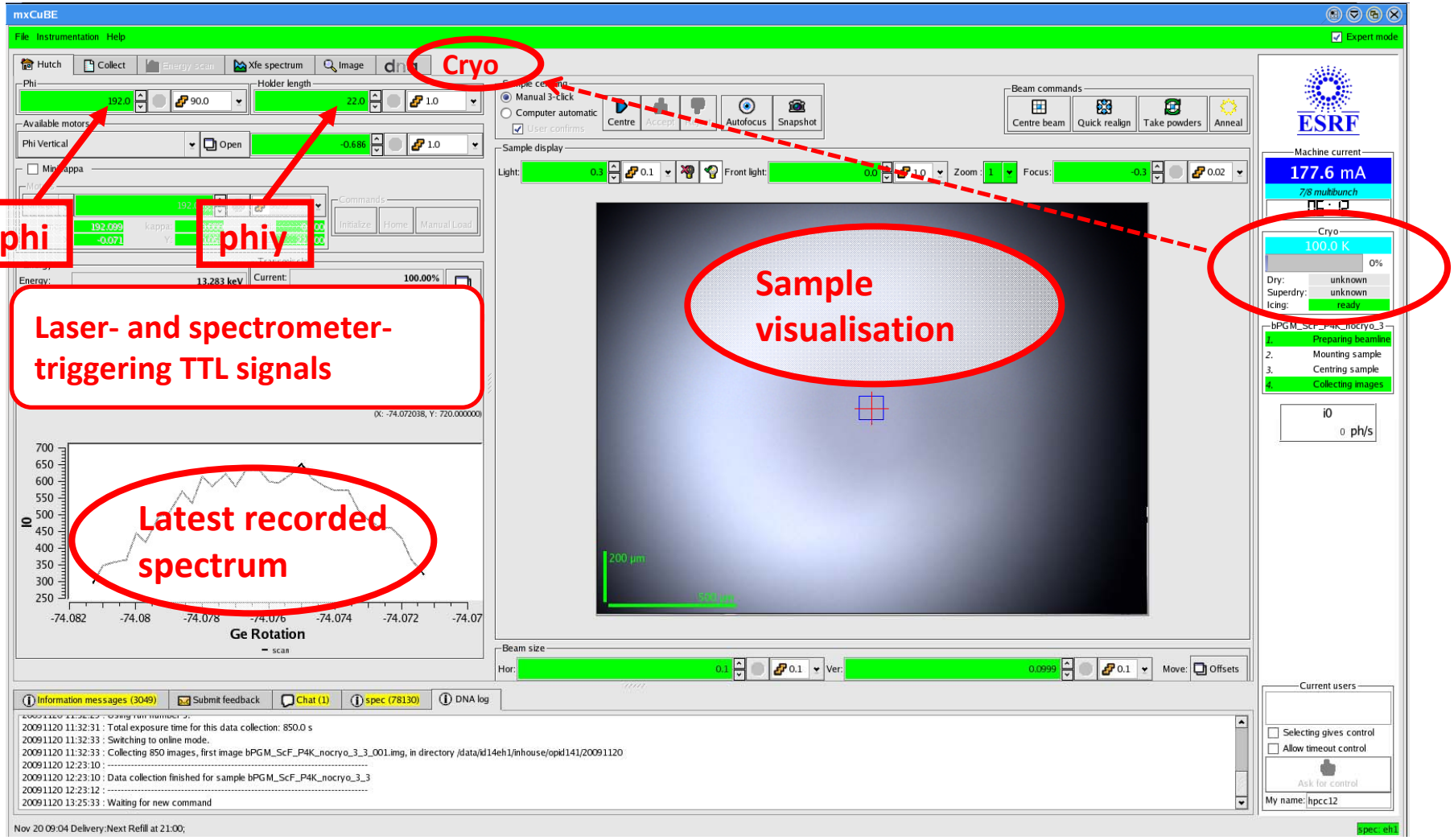
Computer control of goniometer



Fluorescence lifetime / emission



Outlook of speCuBE



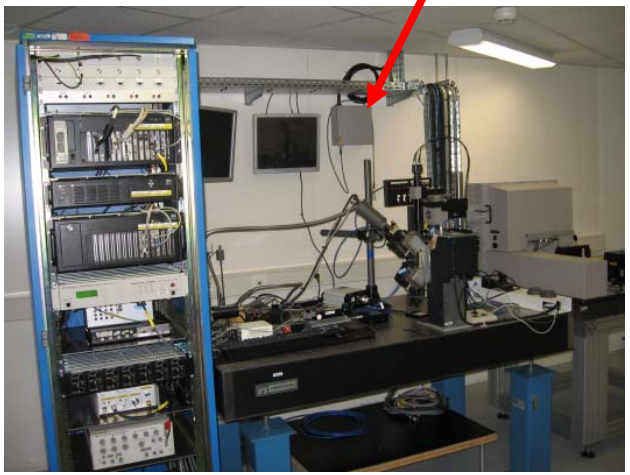
Data storage on Nice `/data/id29s/external/...`
`/data/id29s/inhouse/...`

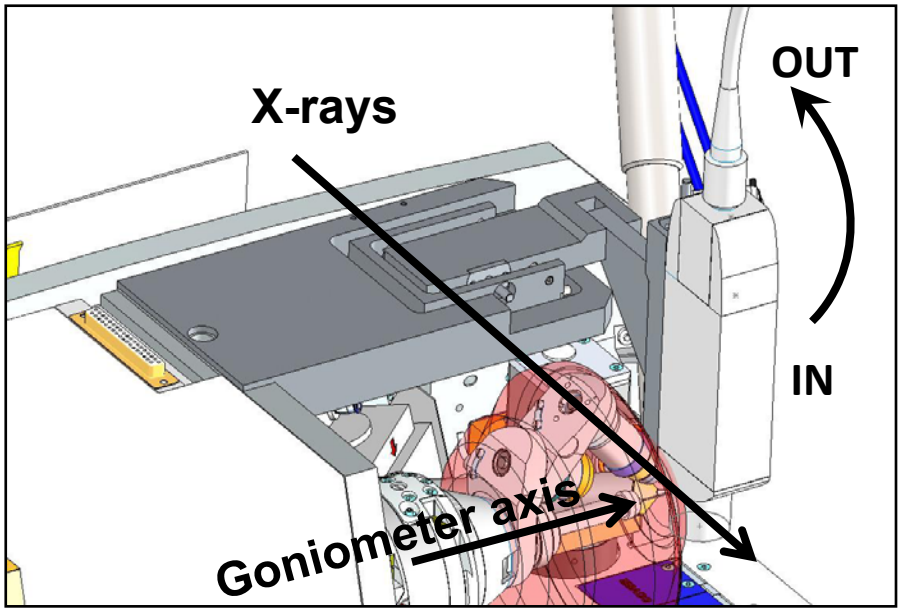
On-line Raman

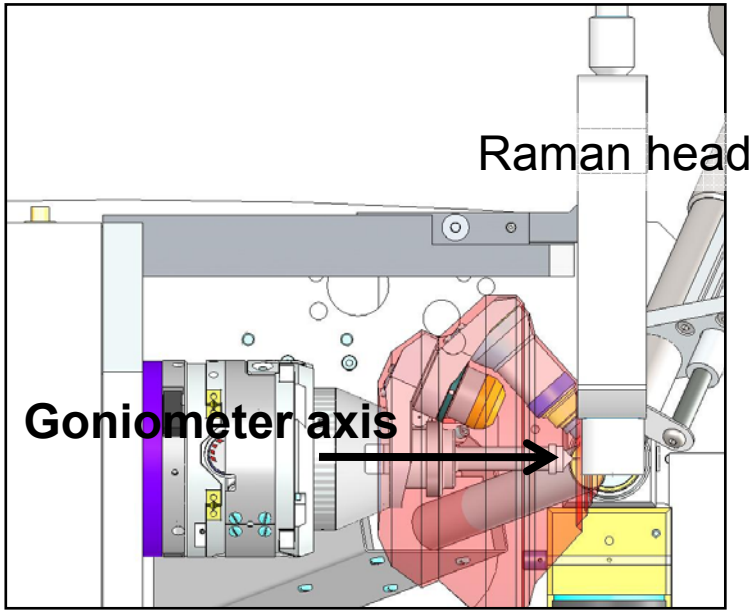
- Goal: getting the Raman head on-axis with X-rays on ID29 goniometer (microdiff MD2)

  → easier centering

  → use of x50 objective



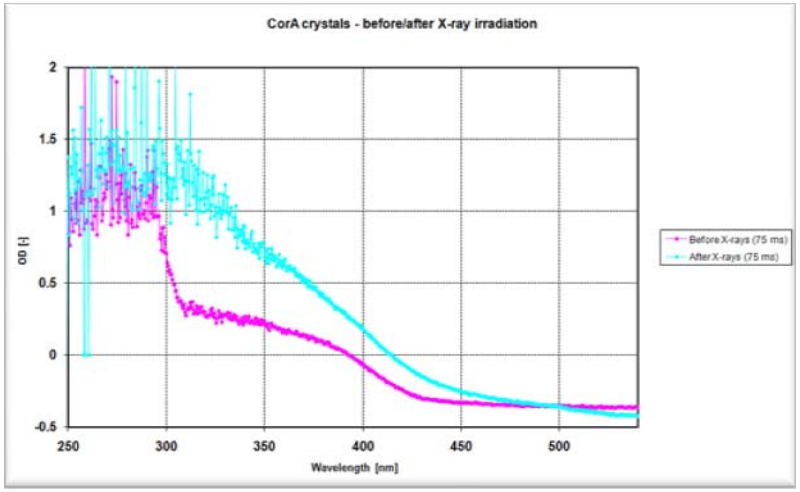




First spectra recorded on ID29S

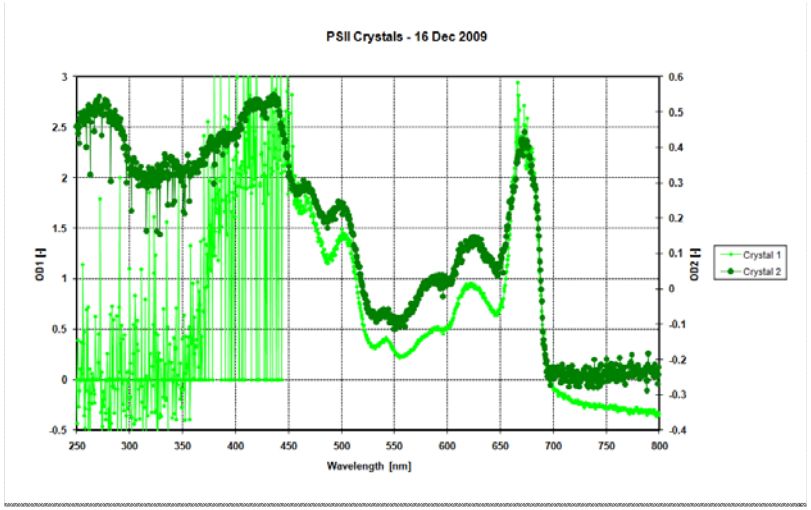
- **10/12/09 - Ronny Helland (NorStruct, MX853)**

  Crystals of CorA



- **16/12/09 - Dario Piano (ESRF Structural Biology group)**

  Crystals of photosystem II



Cryobench beamtime allocation

- **BAG users**

  $\rightarrow$ request through BAG (XX days per year, scheduled as beamline beamtime)

- **Non-BAG users**

  $\rightarrow$ request through standard protein crystallography rolling applications (requesting ID29S as beamline)

- **1st-time users**

  $\rightarrow$ access through in-house beamtime

Acknowledgements

- **The whole ESRF Structural Biology group**
- **Technical help** Thierry Giraud

Fabien Dobias

- **On-line microspec** Martin Weik

- **On-line Raman** Daniele de Sanctis

- **Computing support** Franc Sever