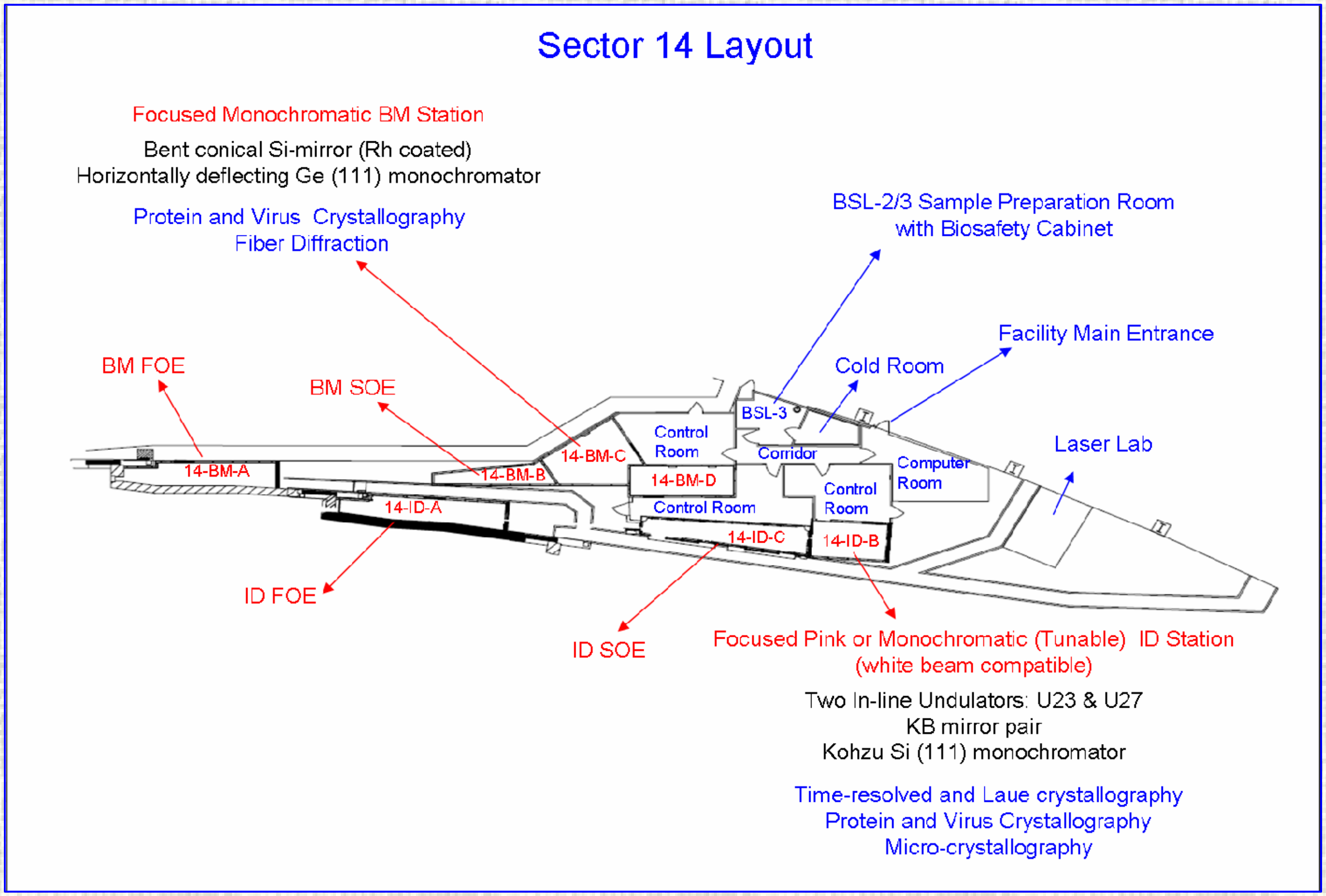
Time-resolved macromolecular crystallography: past, present and future

Vukica Srajer

BioCARS, University of Chicago
Sector 14, Advanced Photon Source, Argonne National Laboratory

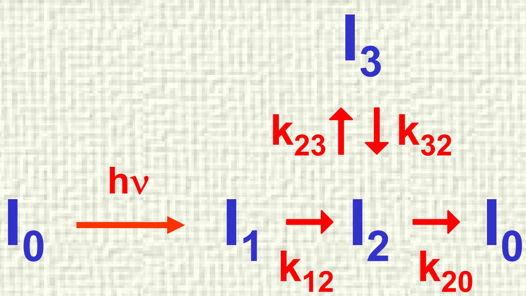


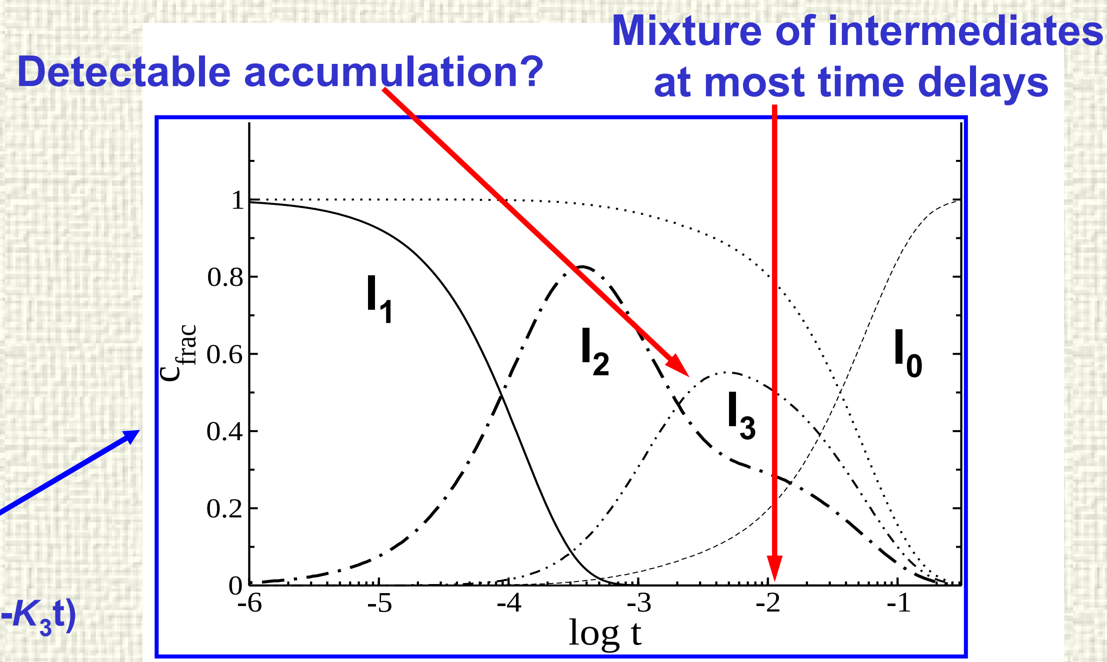
Time-resolved X-ray Macromolecular Crystallography

Why? Static structures of many biological macromolecules are available,
but
the detailed mechanism by which they function often remains elusive.

➡ **Need to capture molecules in action.**

Ultimate goal: From recorded movies of electron density maps, $\Delta\rho(t)$
➡ structures of intermediates and the reaction mechanism





Concentrations of intermediates:
$C_i(t) = C_{1i}\exp(-K_1 t) + C_{2i}\exp(-K_2 t) + C_{3i}\exp(-K_3 t)$

How to Capture Structural Intermediates?

Extend the lifetime of intermediates: physical or chemical trapping

- Low temperature
- Trigger-freeze – trap by freezing
- pH change or other solvent modification
- Chemical modification

or

Real-time snap-shots of evolving structural changes: no trapping

- Probe fast structural changes at ambient temperature
- Requires
  - rapid reaction initiation (short laser pulses)
  - rapid data collection (short X-ray pulses, Laue technique)

For sub-sec time resolution Laue diffraction is needed: high-flux pink X-ray beam & stationary crystal

Brief History of Fast (sub-sec) Laue Time-resolved Studies

Early days
(end of 1980-ies and early 1990-ies)

Proof of principle:
CHESS/Cornell U undulator runs

J. Appl. Cryst. (1992). **25**, 414–423

Quantitative Analysis of Laue Diffraction Patterns Recorded with a 120 ps Exposure from an X-ray Undulator

By Doletha M. E. Szebenyi
Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA

Donald H. Bilderback
Cornell High Energy Synchrotron Source and School of Applied and Engineering Physics, Cornell University, Ithaca, NY 14853, USA

and Alan LeGrand,* Keith Moffat,*† Wilfried Schildkamp,* Brenda Smith Temple‡ and Tsu-yi Teng*
Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA

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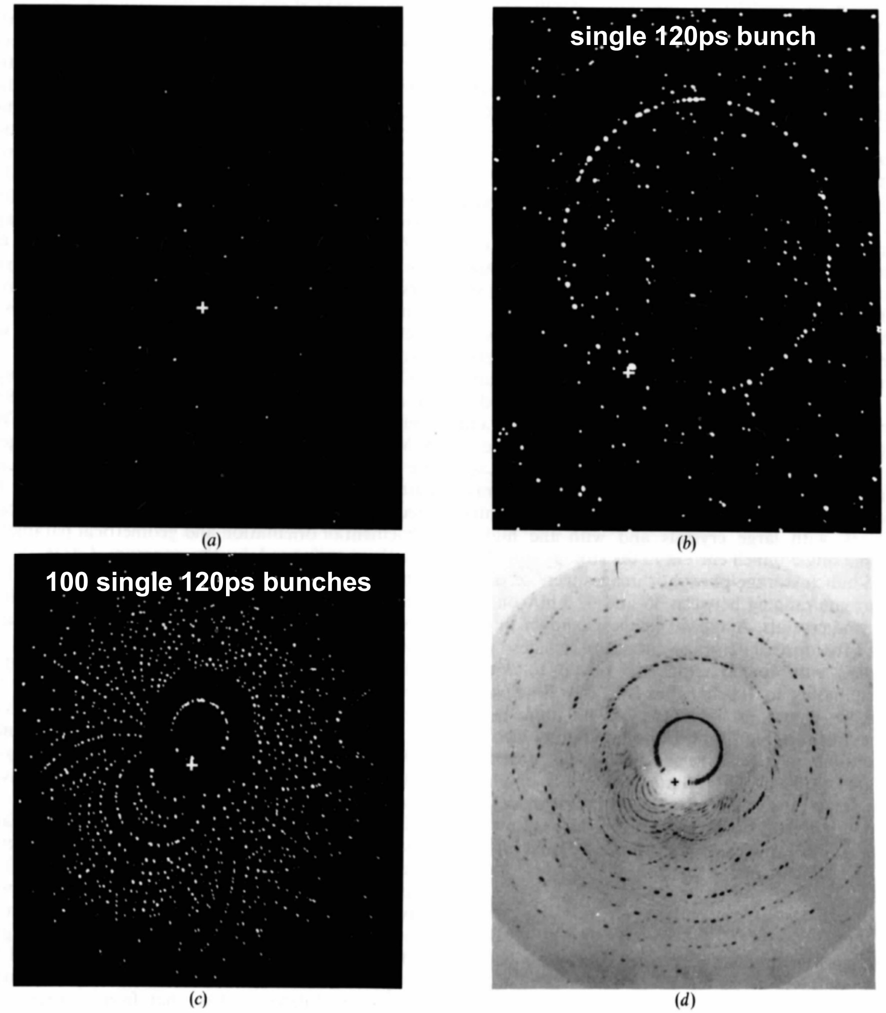


Fig. 2. Laue diffraction patterns. (*a*) 120 ps single-bunch exposure from a crystal of GA381 recorded on Polaroid film. (*b*) 120 ps single-bunch exposure from a crystal of lysozyme, recorded on a storage phosphor. (*c*) 2.56 μs, 100-bunch exposure from a crystal of lysozyme, recorded on a storage phosphor. (*d*) 600 s exposure from the same crystal as in (*b*), using a sealed-tube laboratory X-ray source and recorded on photographic film. See also Fig. 2 of Szebenyi *et al.* (1988).

Feasibility studies (1994-2001)

• ms time-resolved studies of photoactive yellow protein (PYP) at X26C/NSLS

Genick, U.K., G.E.O. Borgstahl, K. Ng, Z. Ren, C. Pradervand, P.M. Burke, V. Srajer, T.Y. Teng, W. Schildkamp, D.E. McRee, K. Moffat, E.D. Getzoff "Structure of a protein photocycle intermediate by millisecond time-resolved crystallography.", Science (Wash.) 275 1471-1475 (1997).

• ns time-resolved studies of Mb and PYP at ID09/ESRF:

- **small structural changes (0.2-0.3Å) can be detected**
- **low- occupancy docking sites of small diatomic ligands (10-20%) can be detected**

Bourgeois, D., T. Ursby, M. Wulff, C. Pradervand, A. LeGrand, W. Schildkamp, S. Laboure, V. Srajer, T.Y. Teng, M. Roth, K. Moffat "Feasibility and realization of single pulse Laue diffraction on macromolecular crystals at ESRF.", J. Synchrotron Rad. 3 (2) 65-74 (1996).

Srajer, V., T.Y. Teng, T. Ursby, C. Pradervand, Z. Ren, S. Adachi, W. Schildkamp, D. Bourgeois, M. Wulff, K. Moffat "Photolysis of the carbon monoxide complex of myoglobin: nanosecond time-resolved crystallography", *Science (Wash.)* 274 1726-1729 (1996).

Perman, B., V. Srajer, Z. Ren, T.Y. Teng, C. Pradervand, F. Schotte, D. Bourgeois, T. Ursby, M. Wulff, R. Kort, K. Hellingwerf, K. Moffat "Energy Transduction on the Nanosecond Time Scale: Early Structural Events in a Xanthopsin Photocycle", *Science (Wash.)* 279 (5358) 1946-1950 (1998).

Ren, Z., B. Perman, V. Srajer, Z. Ren, T. -Y Teng, C. Pradervand, D. Bourgeois, F. Schotte, T. Ursby, M. Wulff, R. Kort, K. Moffat K "Molecular movie at 1.8 Å resolution displays the photocycle of photoactive yellow protein, a eubacterial blue-light receptor, from nanoseconds to seconds", *Biochemistry (Wash.)* 40 (46) 13788-13801 (2001).

Srajer, V., Z. Ren, T. -Y Teng, M. Schmidt, T. Ursby, D. Bourgeois, C. Pradervand, W. Schildkamp, M. Wulff, K. Moffat "Protein conformation relaxation and ligand migration in myoglobin: a ns-ms molecular movie from time-resolved Laue X-ray diffraction studies", *Biochemistry (Wash.)* 40 (46) 13802-13815 (2001).

• critical software developments: Laue data processing

- **summarized in:** Ren, Z., D. Bourgeois, J.R. Helliwell, K. Moffat, V. Srajer, B.L. Stoddard "Laue crystallography: coming of age", *J. Synchrotron Rad.* 6 (4) 891-917 (1999)

Coming of age (2001- present)

• time-resolution extended to 100ps at ID09/ESRF; now implemented also at 14ID/APS and NW14/AR-PF, Japan

Schotte F, Lim M, Jackson TA, Smirnov AV, Soman J, Olson JS, Phillips GN Jr, Wulff M, Anfinrud PA: Watching a protein as it functions with 150-ps time-resolved x-ray crystallography. Science 2003, 300:1944-1947.

• comprehensive studies of structures of intermediates in PYP and structural relaxation and ligand migration in Mb at 14ID/APS and ID09/ESRF

Bourgeois D, Vallone B, Schotte F, Arcovito A, Miele AE, Sciara G, Wulff M, Anfinrud P, Brunori M: Complex landscape of protein structural dynamics unveiled by nanosecond Laue crystallography. Proc Natl Acad Sci USA 2003, 100:8704-8709.

Schotte F, Soman J, Olson JS, Wulff M, Anfinrud PA: Picosecond time-resolved X-ray crystallography: probing protein function in real time. J Struct Biol 2004, 147:235-246.

Hummer G, Schotte F, Anfinrud PA: Unveiling functional protein motions with picosecond x-ray crystallography and molecular dynamics simulations. Proc Natl Acad Sci USA 2004, 101:15330-15334.

Schmidt M, Nienhaus K, Pahl R, Krasselt A, Anderson S, Parak F, Nienhaus UG, Srajer V: Ligand migration pathway and protein dynamics in myoglobin: A time-resolved crystallographic study on L29W MbCO. PNAS 2005, 102 (33): 11704-11709.

Bourgeois D, Vallone B, Arcovito A, Sciara G, Schotte F, Anfinrud PA, Brunori M: Extended subnanosecond structural dynamics of myoglobin revealed by Laue crystallography. PNAS 2006, 103 (13): 4924-4929.

Anderson S, Srajer V, Pahl R, Rajagopal S, Schotte F, Anfinrud P, Wulff M, Moffat K: Chromophore conformation and the evolution of tertiary structural changes in photoactive yellow protein. Structure 2004, 12:1039-1045.

Schmidt M, Pahl R, Srajer V, Anderson S, Ren Z, Ihee H, Rajagopal S, Moffat K: Protein kinetics: structures of intermediates and reaction mechanism from time-resolved X-ray data. Proc Natl Acad Sci USA 2004, 101:4799-4804.

Rajagopal S, Anderson S, Srajer V, Schmidt M, Pahl R, Moffat K: A structural pathway for signaling in the E46Q mutant of photoactive yellow protein. Structure 2005, 13:55-63.

Ihee H, Rajagopal S, Srajer V, Pahl R, Anderson S, Schmidt M, Schotte F, Anfinrud PA, Wulff M, Moffat K: Visualizing reaction pathways in photoactive yellow protein from nanoseconds to seconds. Proc Natl Acad Sci USA 2005, 102:7145-7150.

• software for analysis of time-resolved data (SVD)

Schmidt M, Rajagopal S, Ren Z, Moffat K: Application of singular value decomposition to the analysis of timeresolved macromolecular X-ray data. Biophys J 2003, 84:2112-2129.

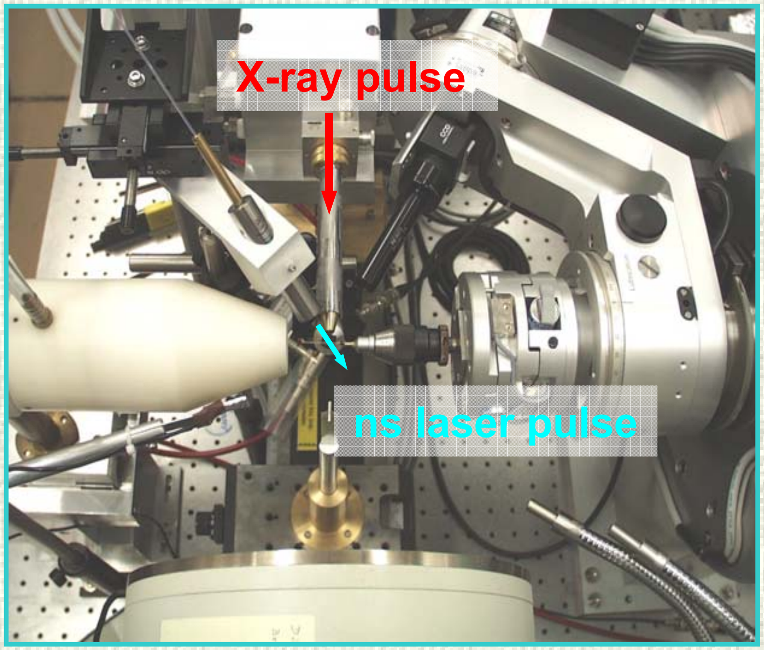
Rajagopal S, Schmidt M, Anderson S, Ihee H, Moffat K: Analysis of experimental time-resolved crystallographic data by singular value decomposition. Acta Crystallogr D Biol Crystallogr 2004, 60:860-871.

Time-resolved Experiments Today

Pump-probe

Pump: laser pulses (100fs-10ns), μs flash lamps

Probe: 100ps X-ray pulse or longer pulse trains



Data collection strategy

- Slow variable: crystal angular setting
  Fast variable: pump-probe delay time, Δt
  ➡For each crystal orientation collect:
  no laser, Δt_1, Δt_2, Δt_3… Laue frames

- Repetition rate depends on: sample (lifetime of intermediates)
  heat dissipation (laser-induced heating)
  ➡1-3 Hz typical

- 40-60 images per data set
  2-3° angular increment with undulator sources (few % bandwidth)

X-ray Source Requirements for Time-resolved Macromolecular Crystallography

- Pulse duration: structural changes to be probed span sub-ps to sec and min
  - 100ps available presently at synchrotron sources
  - longer pulse trains quite suitable for slow reactions
  - sub-100ps desirable to probe very fast structural changes:
    - short-lived intermediates
    - fast protein relaxation
    - rapid ligand migration

- X-ray flux: > 10^{10} photons/pulse needed for single pulse image acquisition
  - at present, at TR PX beamlines (APS 14-ID, ESRF ID09, PF-AR NW14) >10-100 single X-ray pulses needed
  - the aim of the 14-ID/APS upgrade: single 100ps X-ray pulse acquisition in hybrid and 24-bunch mode (dual, in-line undulators; KB mirror pair – focused beam size 90 (h) X 35 (v) μm^2)
  - single-pulse acquisition will allow study of fast, irreversible processes
  - higher pump laser pulse energies can be used (crystal motion not a problem)

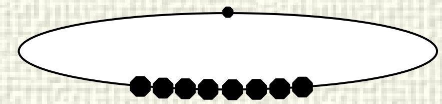
- **X-ray energy: few % bandwidth at 12-15keV**
  - **softer X-rays increase radiation damage**
  - **harder X-rays diffract less strongly and are detected less efficiently**
  - **undulators better sources than wigglers:**
    - **high peak intensity**
    - **low polychromatic background**
    - **reduced spatial and harmonic overlap in Laue patterns**
    - **data processing software can handle wavelength normalization in the presence of sharp spectral features**
      (Srajer et al., J. Sync. Rad 7: 236, 2000)
    - **better data quality (as judged by R_{merge}, completeness, map quality)**
      (Bourgeois et al., Acta Cryst. D 56: 973, 2000)

- **X-ray focal spot:**
  - in principle should match the sample size
  - investigating small crystals requires small beam but at full flux (short exposures)
  - small vertical beamsize needed for isolating single X-ray pulses by a chopper
  - use of relatively low pulse energy fs-ps lasers requires small laser beam to be matched by a small X-ray beam

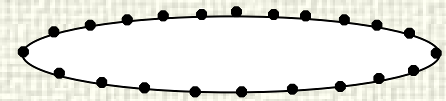
50-100 μm focal spot

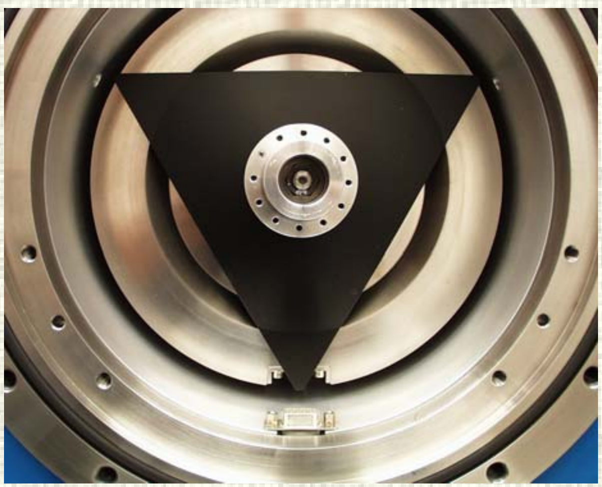
- **Storage ring mode and beamtime availability:**
  - need to isolate single X-ray pulse in pink mode
  - old Sector 14 ultra-fast chopper could isolate single 100ps pulse only in the APS hybrid mode
  - upgraded chopper can isolate single pulses in the 24 bunch mode
  - technically challenging experiments – unlike standard PX data collection require significant beamtime ➡ standard rather than special operating mode

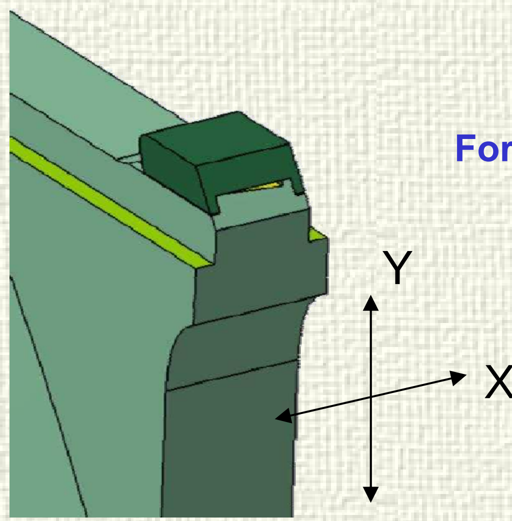
Upgraded BioCARS ultra-fast chopper



Geometry	**TIMETAL (Ti6Al4V)**
	triangle, 166 mm side
	multiple aperture size
	vacuum (< 10^{-3} Torr)
Rep. Rate	**60,320 rpm (994.7 Hz)**
	T = 1.005 ms
Pulse Width	**t_{open}= 190 – 420 ns, 0.46 – 11.5 µs, >ms**







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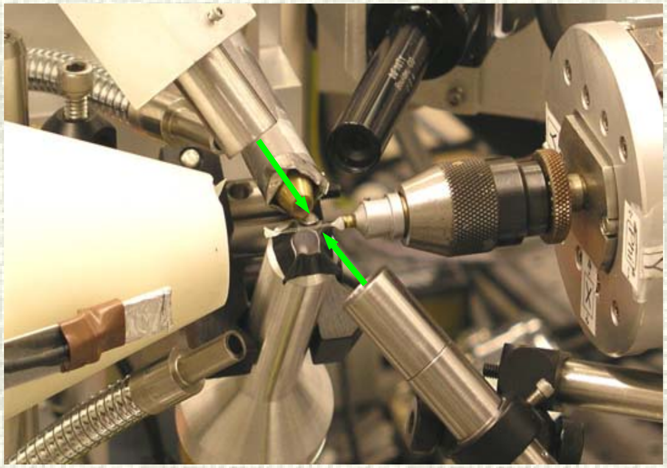
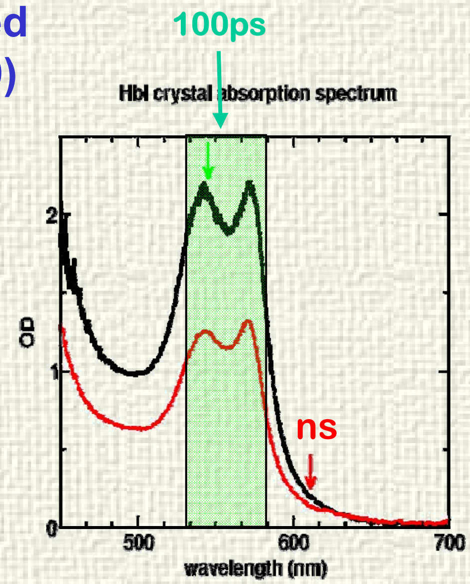
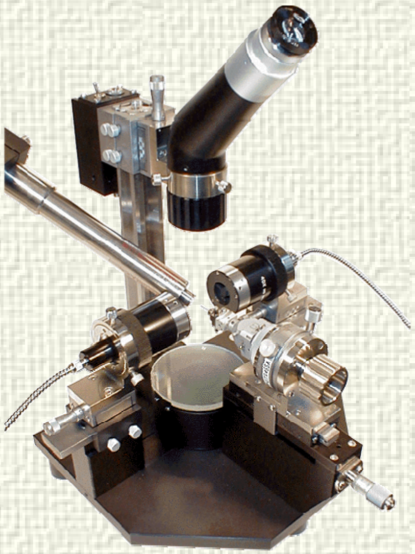
Reaction Photo-initiation in Crystals

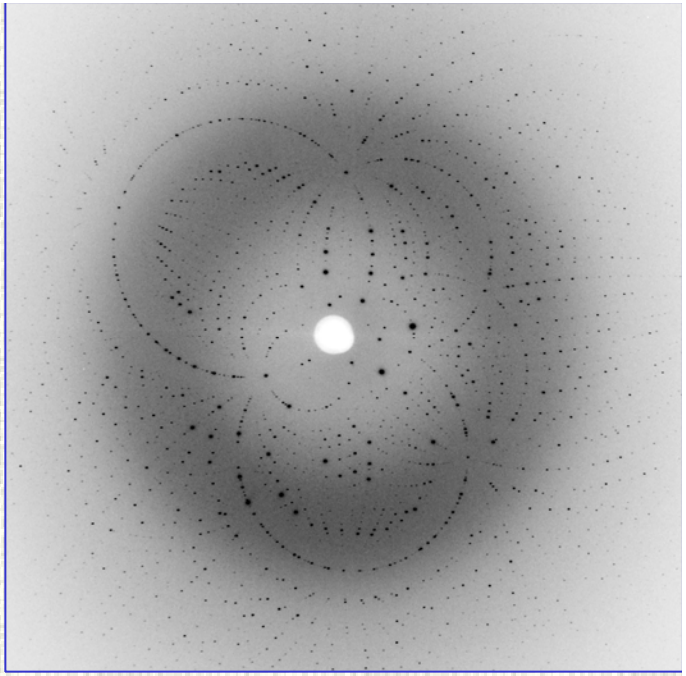
- Short laser pulses: 100fs-10ns duration
- Large number of molecules in crystals: $10^{13} – 10^{14}$
  - ▶Need >10-100 μJ/pulse
  - ▶100ps studies: >40μJ / 100-200μm beam dia
    ns studies: >1-2mJ / 600-800μm beam dia
- Uniform and efficient photo-initiation
  - ▶tunable wavelength
  - ▶double-sided illumination (BioCARS)
  - ▶optically thin crystals (OD < 1) or
  - ▶probing by X-rays only laser-illuminated surface layer of the crystal (ESRF ID09)
- Avoiding crystal damage
  - ▶maximum laser pulse energies?
  - ▶100fs pulses typically stretched to 10-100ps to avoid sample damage
- Extent of photo-initiation?
  - ▶monitoring absorption changes by a micro-spectrophotometer







Laue data processing software: critical component of the TR success story !



- **Mature stage:**
  - problems of wavelength normalization, spatial and harmonic spot overlap resolved in mid 1990-ies
  - automation ongoing – minimal user input and intervention
    ➡ online data processing

- **Very good software packages:**
  - Precognition (Renz Research Inc)
  - LaueView (Ren and Moffat, J. Appl. Cryst. 28: 461,1995)
  - TReX (Schotte & Anfinrud)
  - PrOW (Bourgeois, Acta Cryst. D55: 1733, 1999)
  - Daresbury Laboratory Laue Software Suite
    (Campbell J.W., J. Appl. Cryst. 28:228, 1994; Arzt et al., J Appl. Cryst. 32: 554, 1999)
  - Leap
    (Wakatsuki, S. Data Collection and Processing, Sawyer, N. W. Isaacs & S. Bailey, Eds., Daresbury Laboratory, Warrington, UK, p.71, 1993)

Quality of Laue data and electron density maps derived from Laue data are comparable to monochromatic data.

Time-resolved Data Analysis

Step 1: Difference electron density maps

From Laue structure factor amplitudes: $|F(t)|_{hkl}$

➡time-dependent difference electron density maps $\Delta\rho(t)$: $|F(t)| - |F(t=0)|$, $\varphi_{t=0}$

Step 2: Time-resolved data analysis challenge:

Time-dependent series of difference electron density maps, $\Delta\rho(t)$, each possibly a mixture of states

→

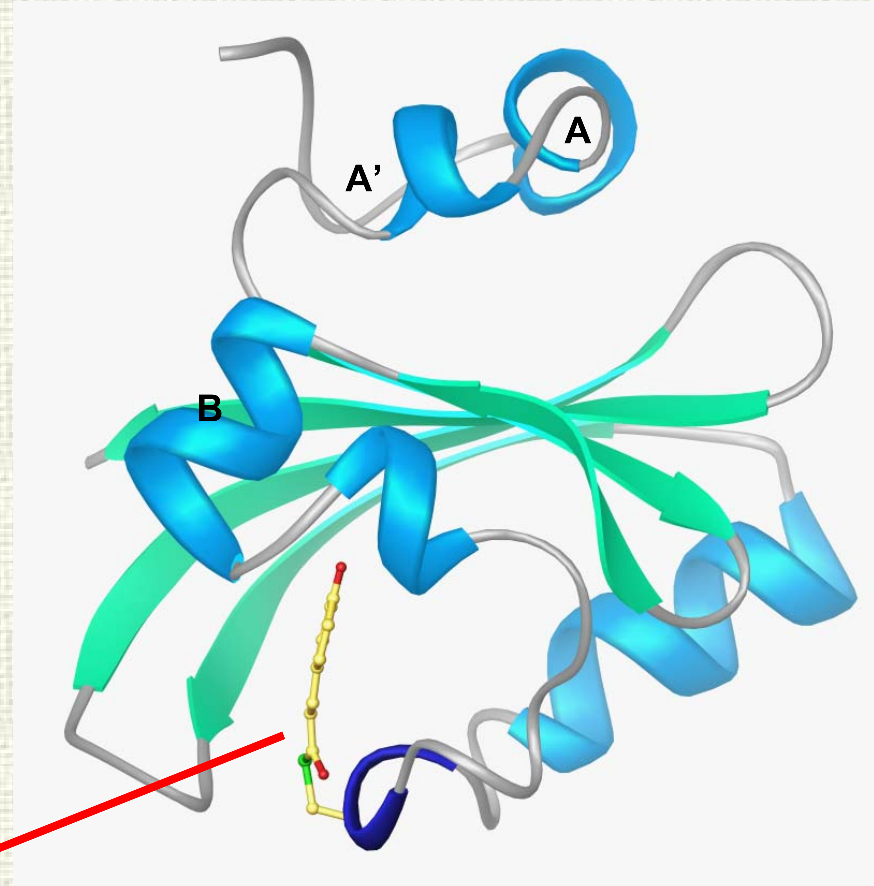
- Number of intermediates
- Maps for pure intermediates, $\Delta\rho(I_i)$ (time-independent)
- Structures of intermediates I_i
- Reaction mechanism

Most promising method: Singular Value Decomposition

Ihee et al., PNAS 102, 7145 7150 (2005)
Rajagopal et al., *Structure* 13, 55-63 (2005)
Rajagopal et al., *Acta Cryst. D*60, 860-871 (2004)
Schmidt et al., *PNAS* 101, 4799-4804 (2004)
Schmidt et al., *Biophys. J*. 84, 2112-2129 (2003)

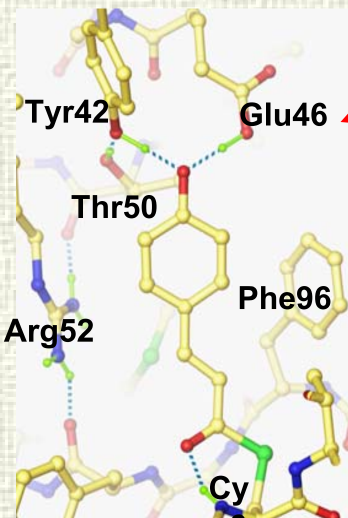
Photoactive Yellow Protein:
an example of
complete TR data analysis

- **Blue light photoreceptor from the purple eubacterium *Ectothiorhodospira halophila***
- **Involved in negative phototactic response of *E. halophila* to blue light**
- **PYP exhibits a photocycle: several intermediates spanning time-scales from <ps to seconds**



Coumaric Acid Chromophore

light-induced *Trans* to *Cis* isomerization

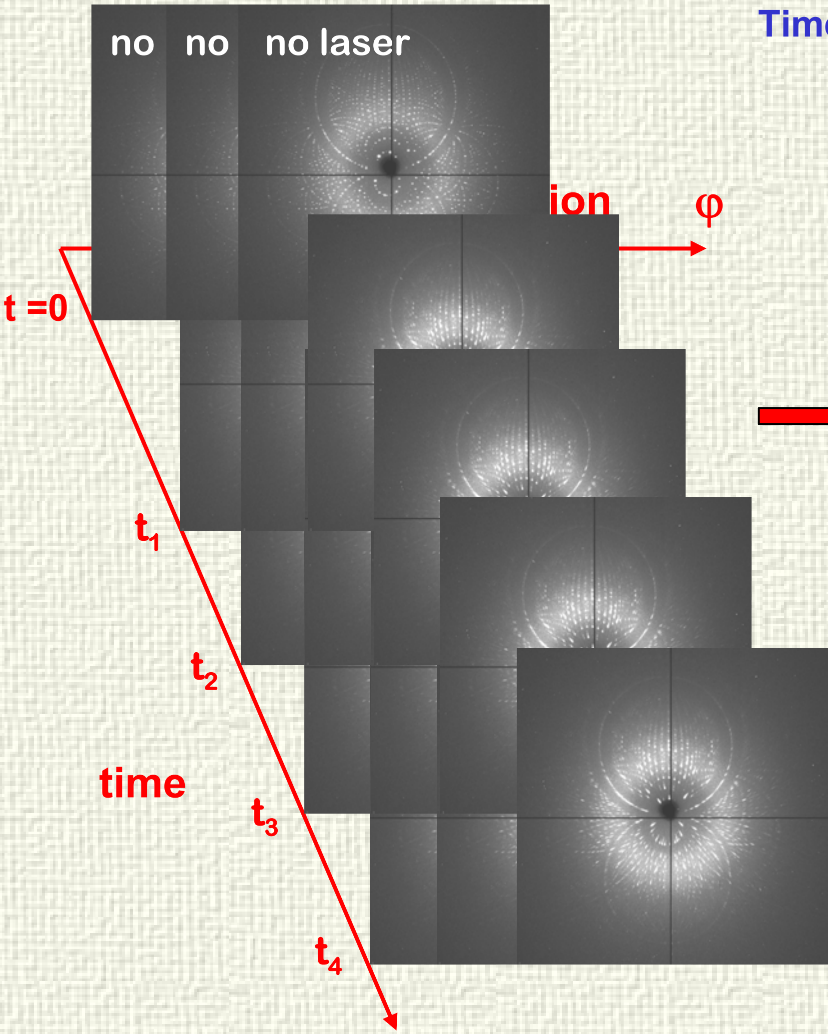


BioCARS 14-ID, APS

Spencer Anderson, Sudarshan Rajagopal, Harry Ihee, Marius Schmidt, Keith Moffat
University of Chicago

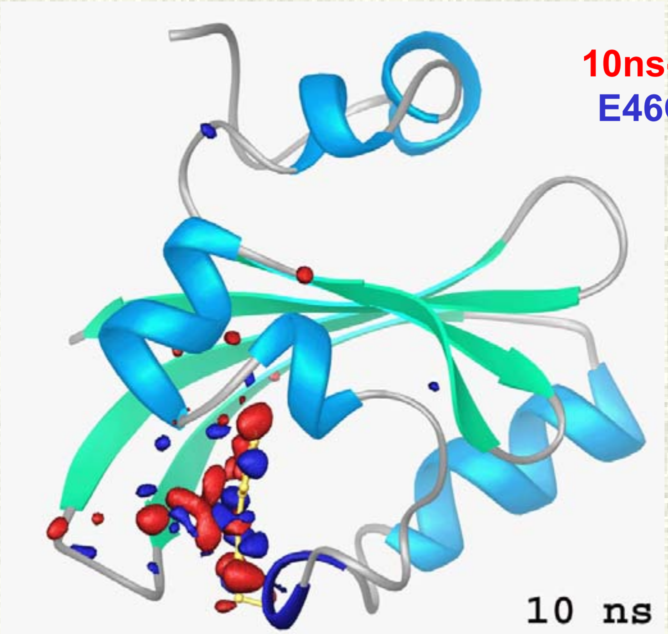
Vukica Srajer, Reinhard Pahl, BioCARS

Ihee et al. PNAS 102, 7145 (2005)
Rajagopal et al., Structure 13, 55 (2005)
Anderson et al., Structure 12,1039 (2004)
Rajagopal et al., Acta Cryst. D60, 860 (2004)



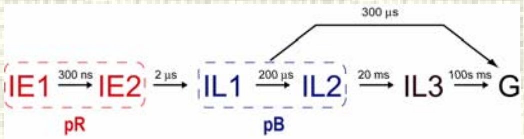
TR data collection

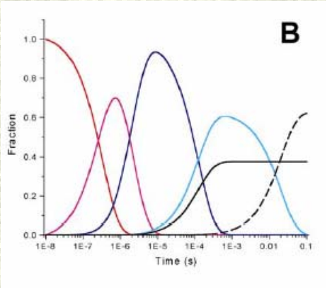
Time-dependent difference electron density map movie, $\Delta\rho$ (t)

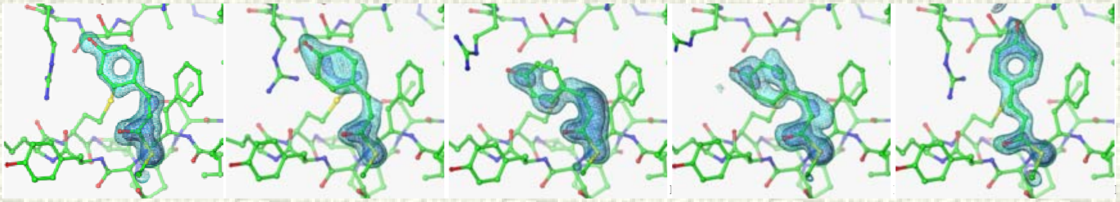


10ns-100ms
E46Q PYP

SVD/post-SVD analysis: mechanism & structures of intermediates







300ns 2µs 200-300µs 20ms >100ms t

Time-resolved Crystallography: Conclusions and Future Outlook

- Mature phase of the technique: demonstrated ability to detect small structural changes even at relatively low levels of reaction initiation (15-40%)

- Development of essential methods for global time-resolved data analysis, such as SVD, is well under way

- Challenges:
  - Application of the technique to other systems of biological interest, photosensitive and beyond
  - Reaction initiation: system-specific efforts to determine a suitable reaction initiation method
  - Irreversible processes and smaller crystals: need more intense X-ray sources and faster read-out detectors
  - Further improvements in time resolution: sub-100ps X-ray sources?
  - Combining experimental results from time-resolved crystallography with computational and theoretical approaches to describe reaction pathways completely, including the transition states

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bioCARS

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