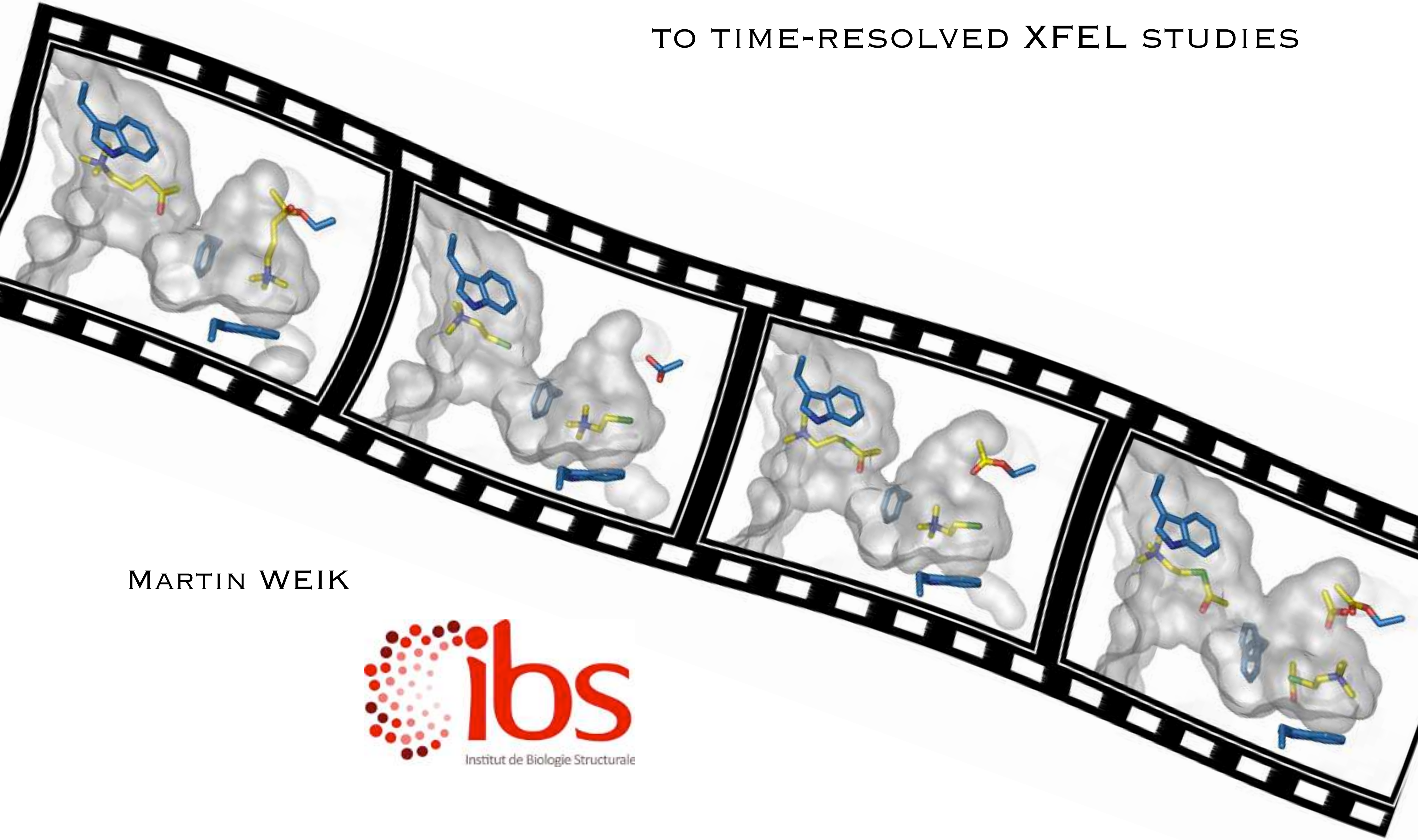
KINETIC CRYSTALLOGRAPHY: FROM CRYO-TRAPPING TO TIME-RESOLVED XFEL STUDIES

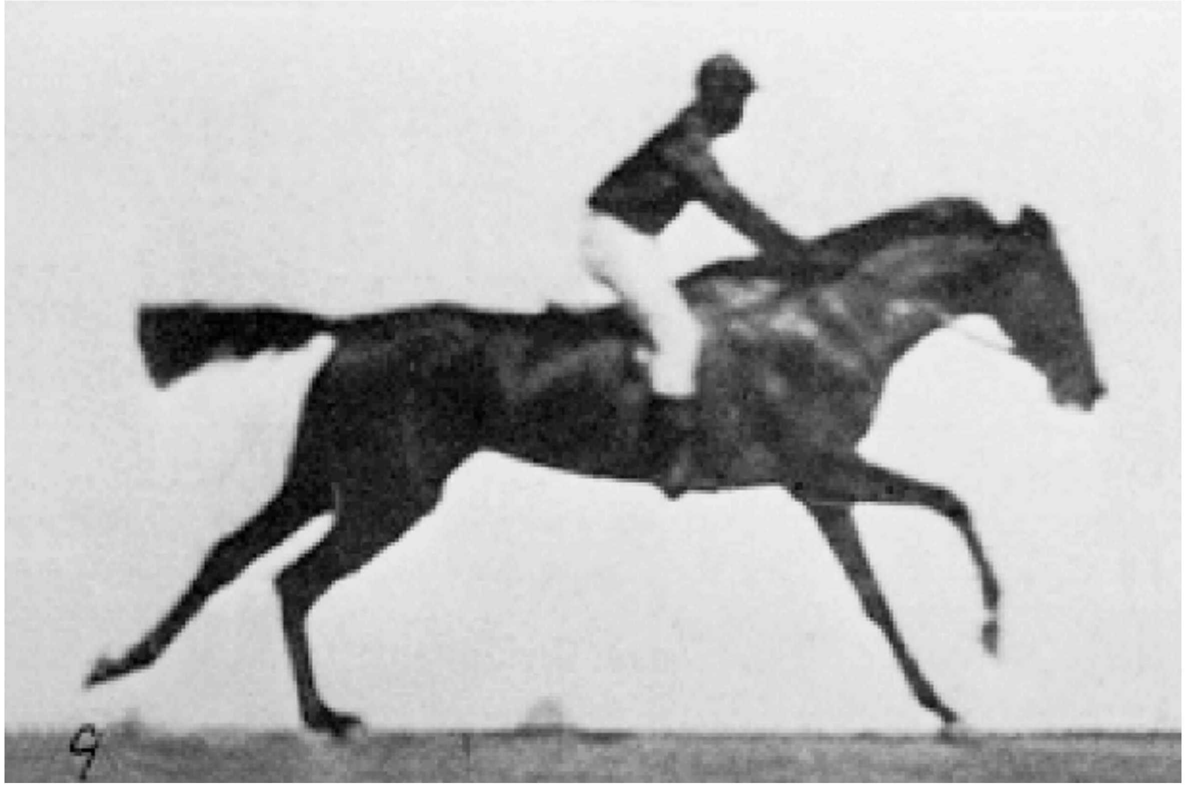


Martin WEIK

Institut de Biologie Structurale

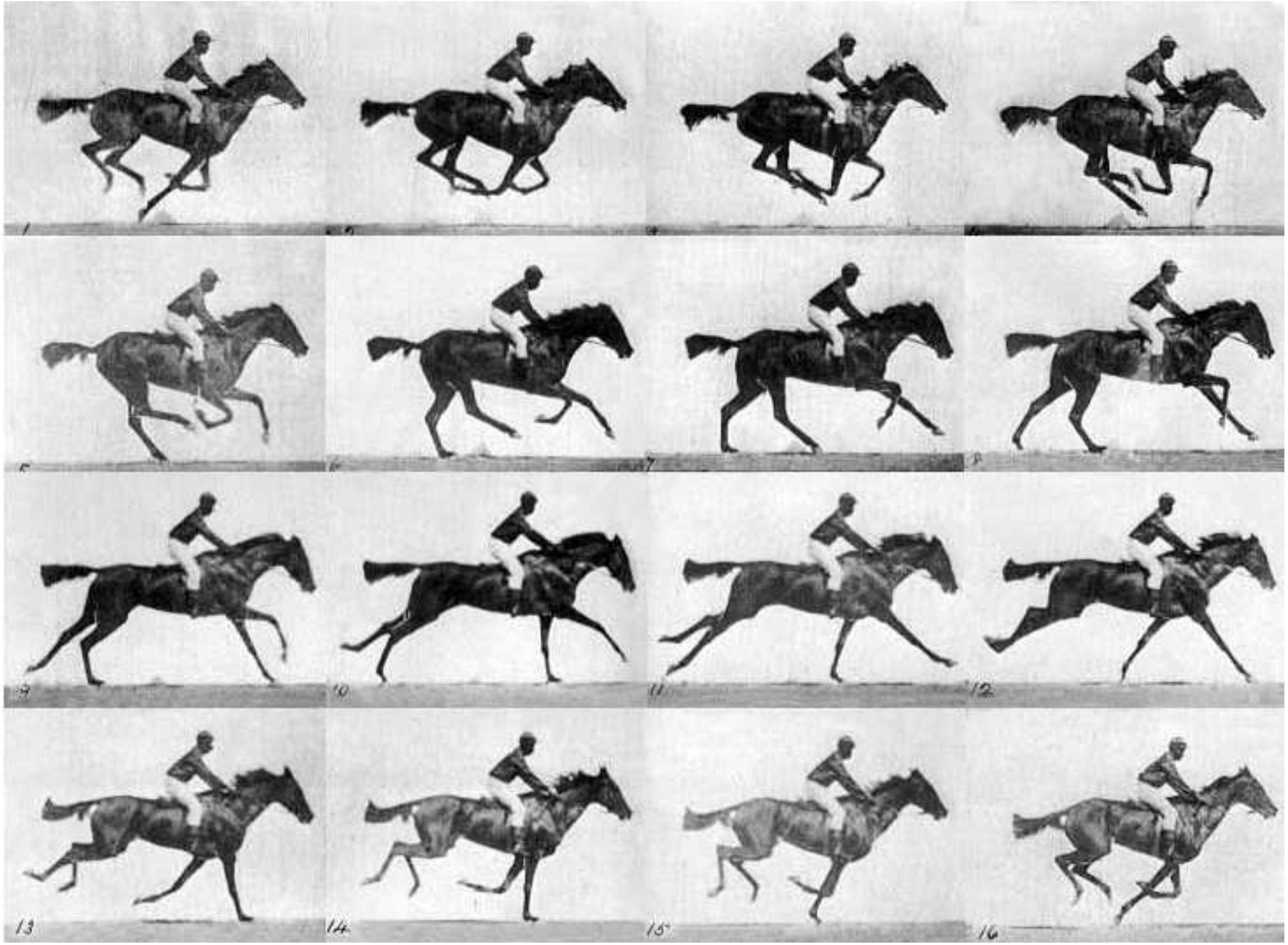
Challenge in 1878:

Structure and dynamics of a horse in motion



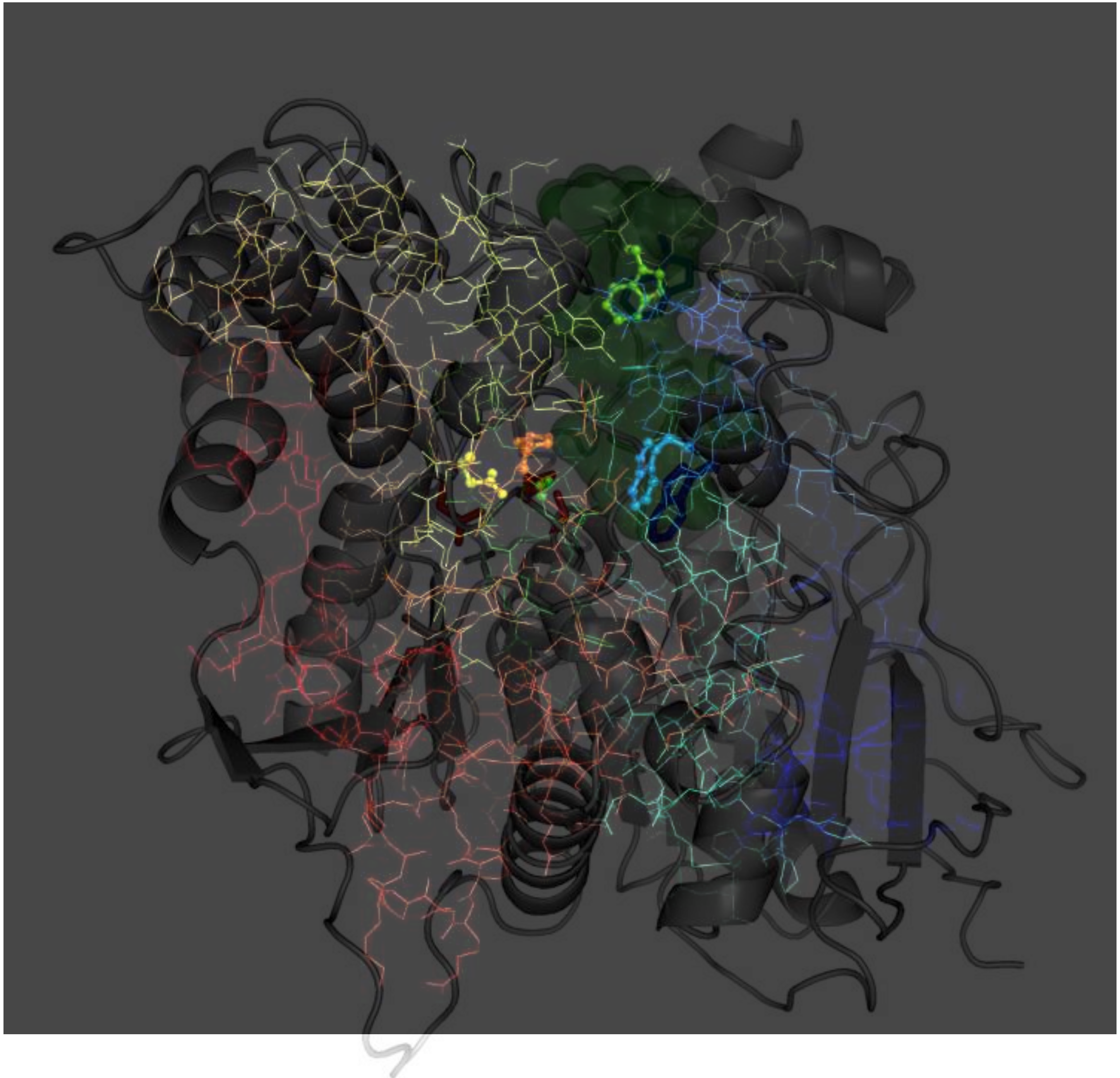
Are all four of a horse’s hooves off the ground at the same time ?

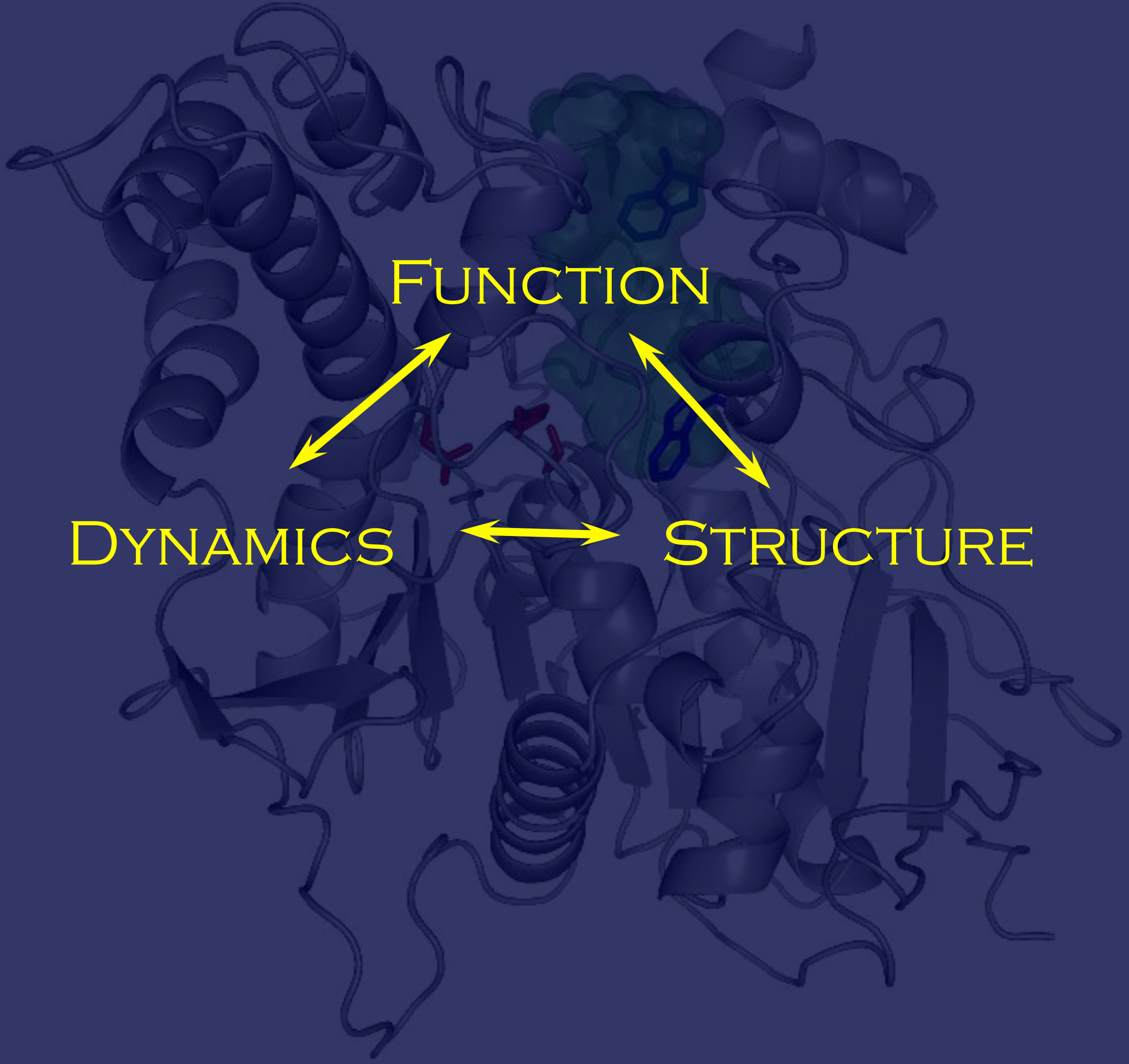
Eadweard Muybridge, Sacramento, California


1
5
6
7
8
9
10
11
12
13
14
15
16

Eadweard Muybridge, *The Horse in Motion*

Sussman et al. (1991) *Science* 253, 872



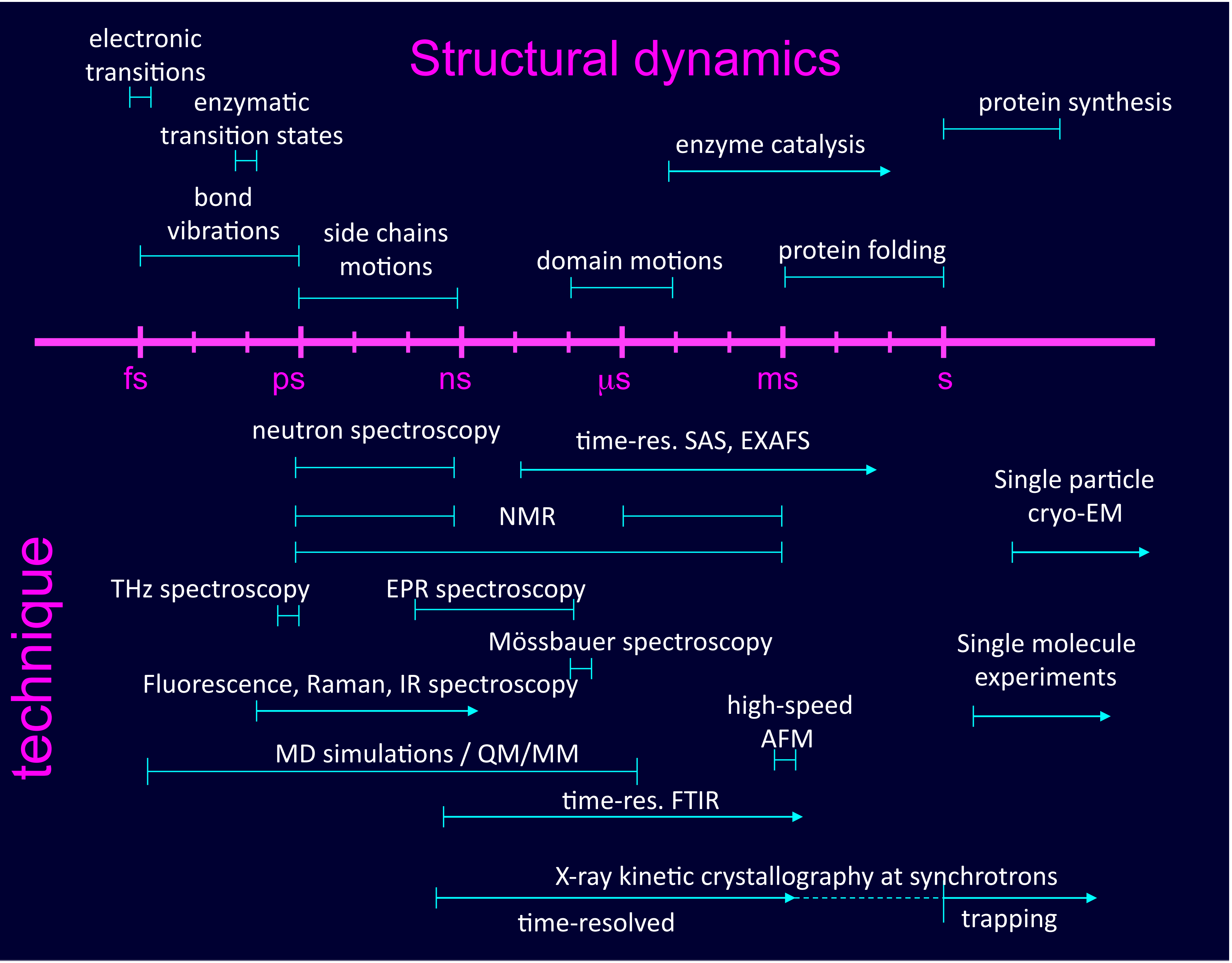


Function

Dynamics

Structure

Structural dynamics



electronic transitions

enzymatic transition states

bond vibrations

side chains motions

domain motions

protein folding

enzyme catalysis

protein synthesis

fs

ps

ns

µs

ms

s

technique

neutron spectroscopy

time-res. SAS, EXAFS

Single particle cryo-EM

NMR

THz spectroscopy

EPR spectroscopy

Mössbauer spectroscopy

Single molecule experiments

Fluorescence, Raman, IR spectroscopy

high-speed AFM

MD simulations / QM/MM

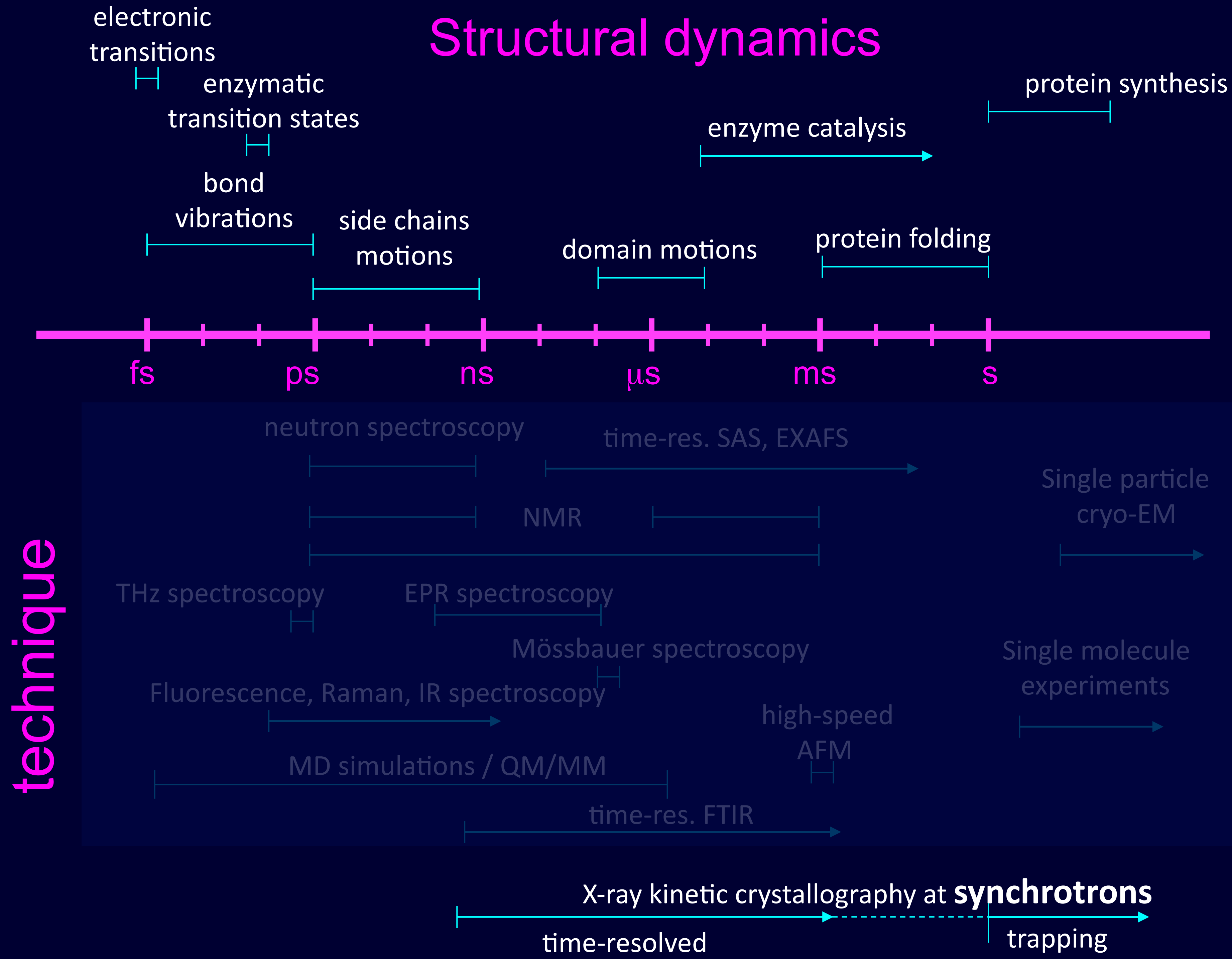
time-res. FTIR

X-ray kinetic crystallography at synchrotrons

time-resolved

trapping

Structural dynamics



electronic transitions

enzymatic transition states

bond vibrations

side chains motions

domain motions

protein folding

enzyme catalysis

protein synthesis

technique

neutron spectroscopy

time-res. SAS, EXAFS

NMR

Single particle cryo-EM

THz spectroscopy

EPR spectroscopy

Mössbauer spectroscopy

Single molecule experiments

Fluorescence, Raman, IR spectroscopy

high-speed AFM

MD simulations / QM/MM

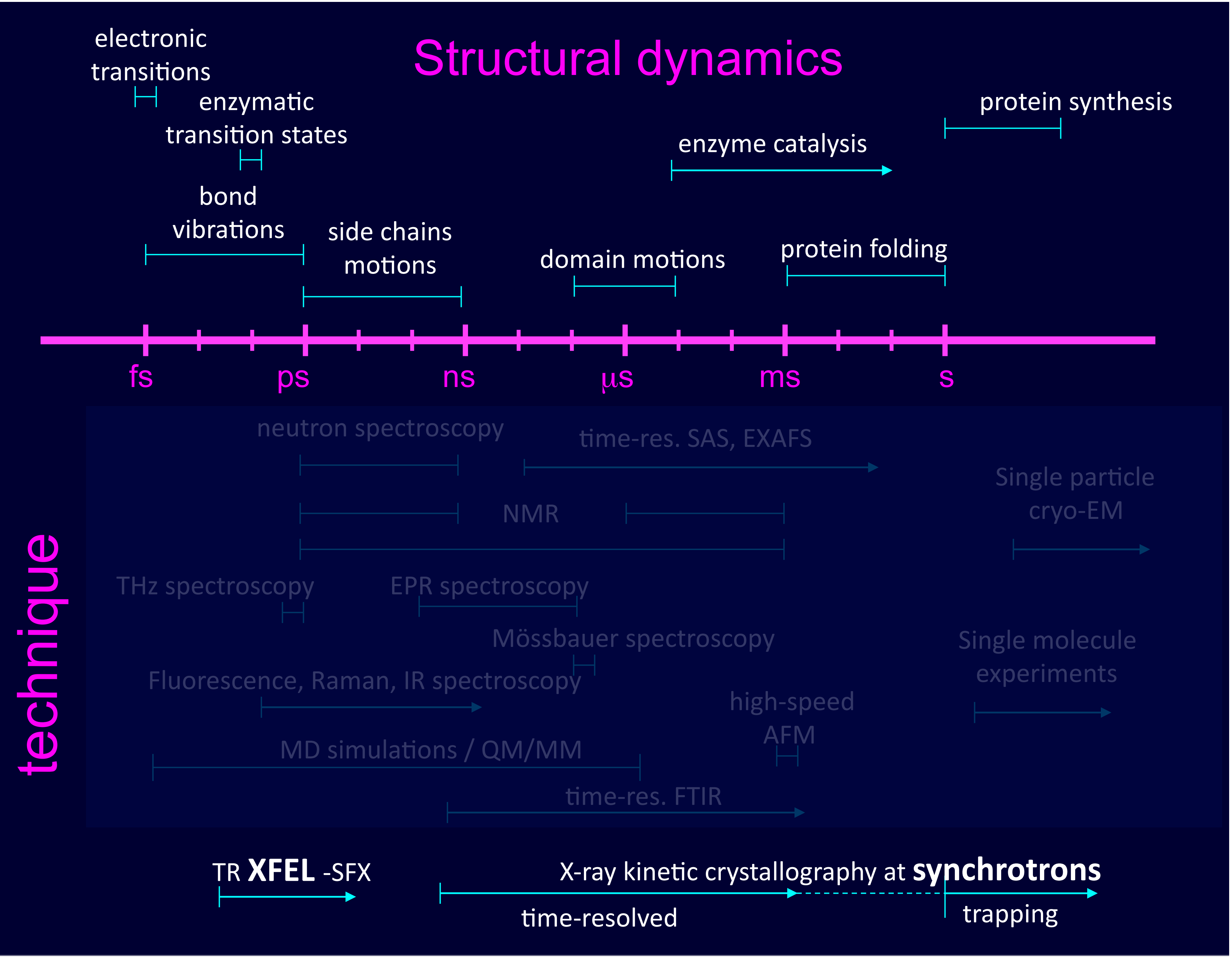
time-res. FTIR

X-ray kinetic crystallography at **synchrotrons**

time-resolved

trapping

Structural dynamics



electronic transitions

enzymatic transition states

bond vibrations

side chains motions

domain motions

protein folding

enzyme catalysis

protein synthesis

fs ps ns µs ms s

technique

neutron spectroscopy

time-res. SAS, EXAFS

Single particle cryo-EM

NMR

THz spectroscopy

EPR spectroscopy

Mössbauer spectroscopy

Single molecule experiments

Fluorescence, Raman, IR spectroscopy

high-speed AFM

MD simulations / QM/MM

time-res. FTIR

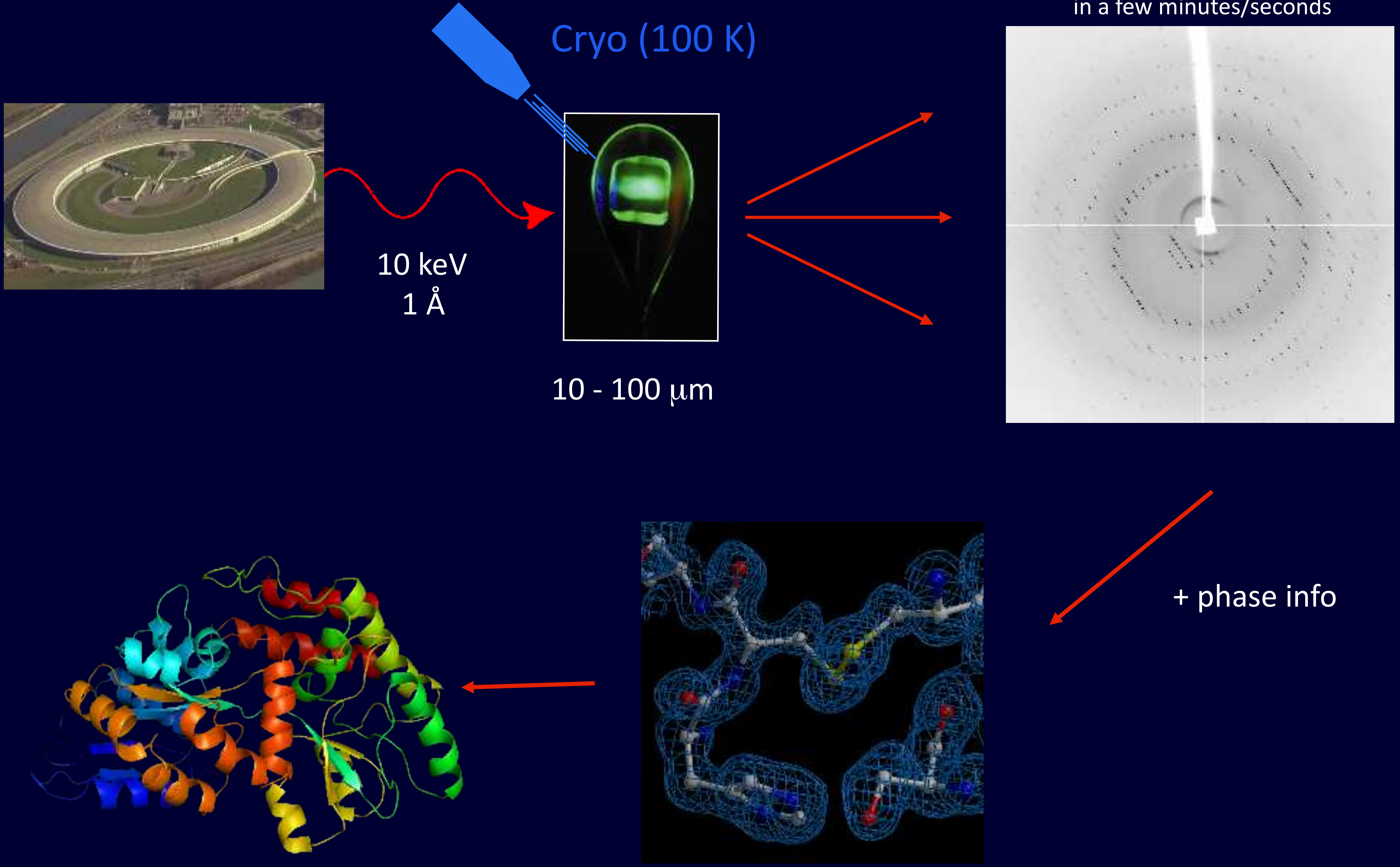
TR **XFEL** -SFX

X-ray kinetic crystallography at **synchrotrons**

time-resolved

trapping

CONVENTIONAL MACROMOLECULAR X-RAY CRYSTALLOGRAPHY



Cryo (100 K)

10 keV
1 Å

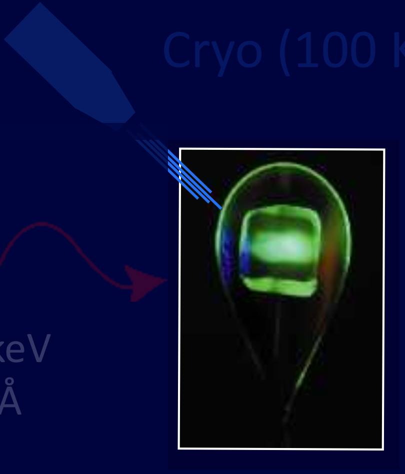
10 - 100 μm

Collect 100 diffraction images
on a single crystal
in a few minutes/seconds

+ phase info

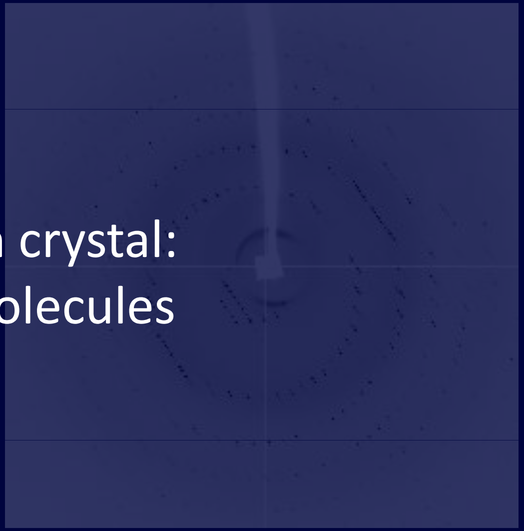
… CONVENTIONAL X-RAY CRYSTALLOGRAPHY

10 keV
1 Å



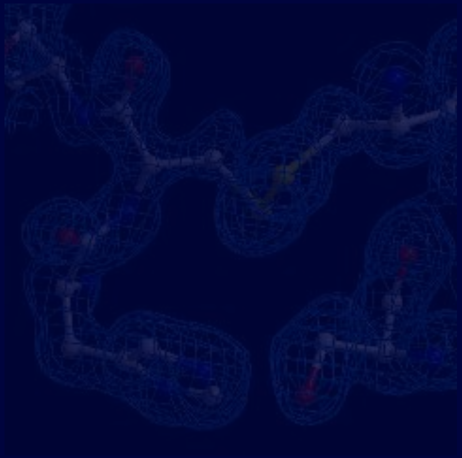
Cryo (100 K)

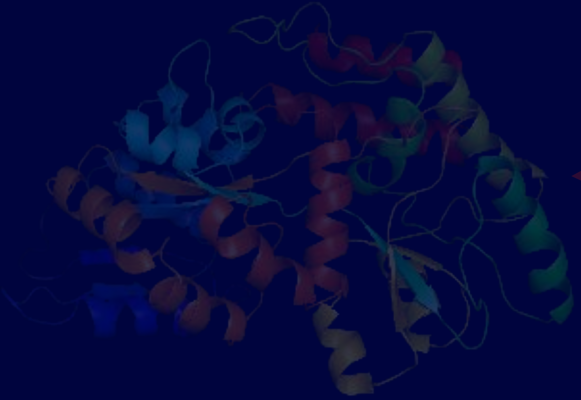
100 μm

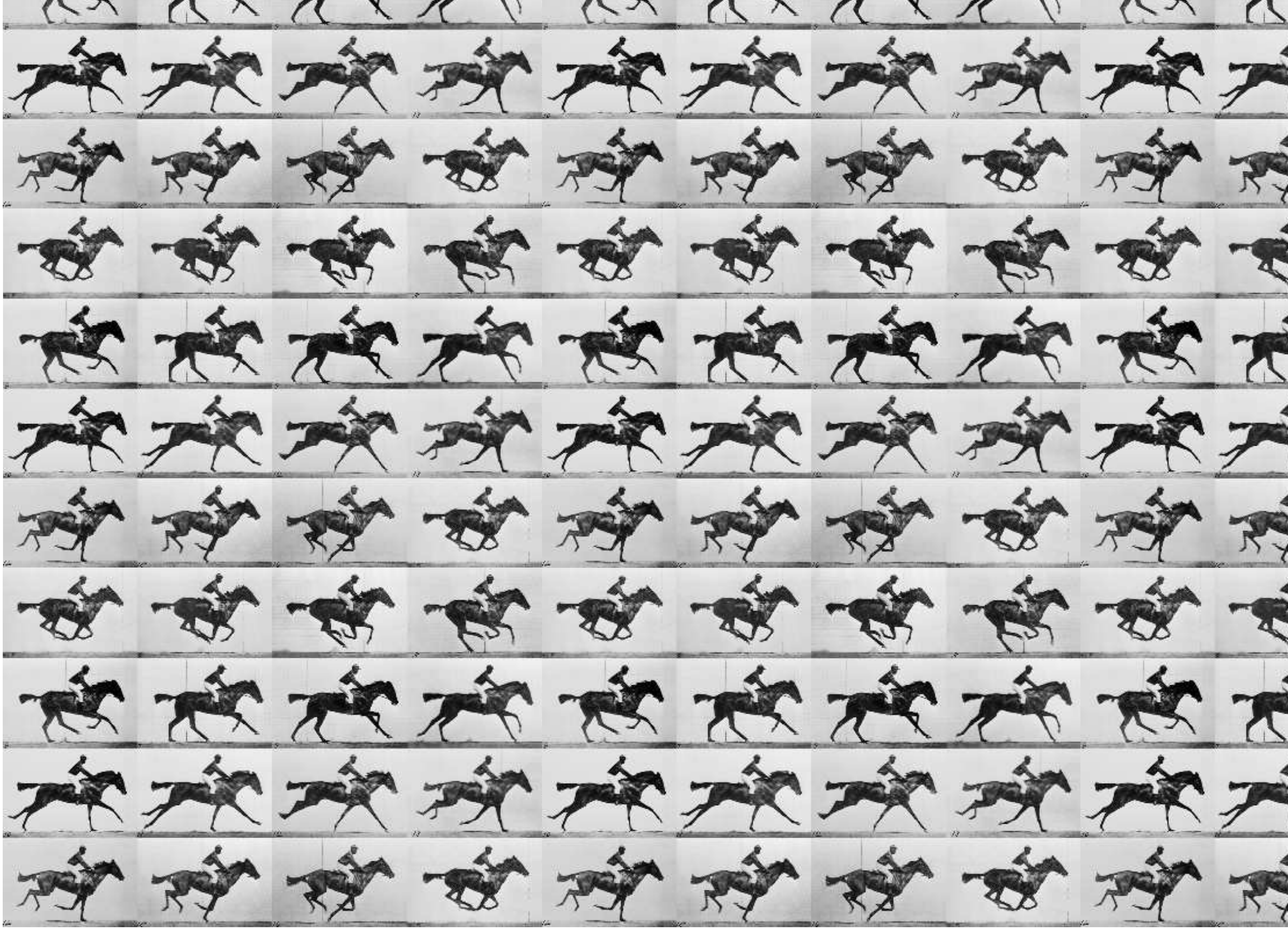


Protein crystal:
10^{15} molecules

+ phase info







refined model: average structure

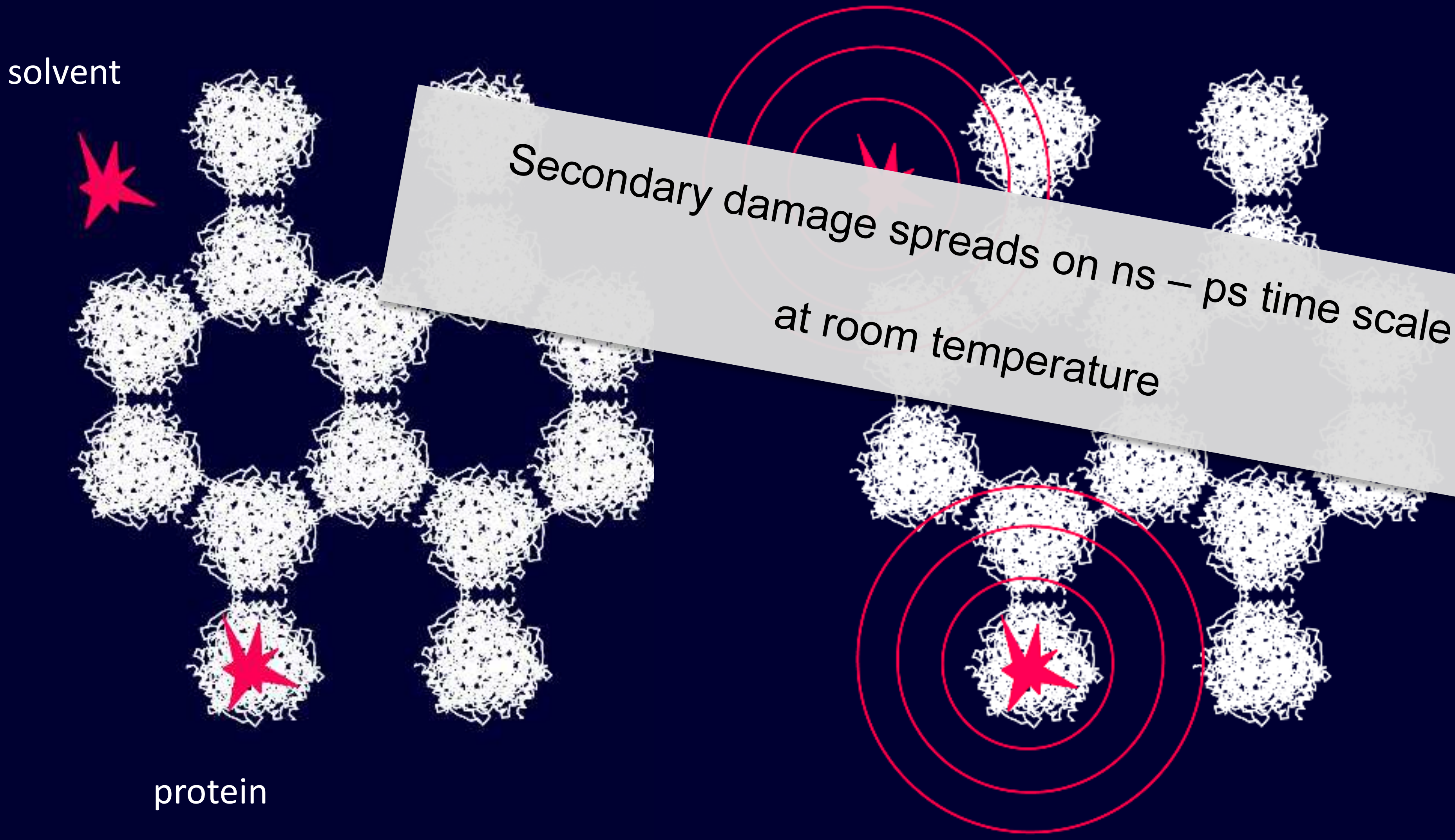
Courtesy James Holton

Problem: X-rays produce radiation damage

Courtesy James Holton

X-ray radiation damage

Primary and secondary damage


Primary damage

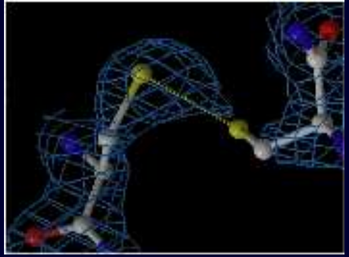
Secondary damage

Secondary damage spreads on ns – ps time scale

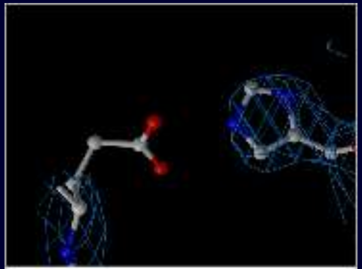
at room temperature

Secondary radicals produce specific radiation damage

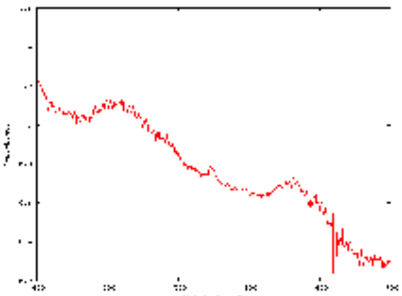
Disulfide bond cleavage



Decarboxylation



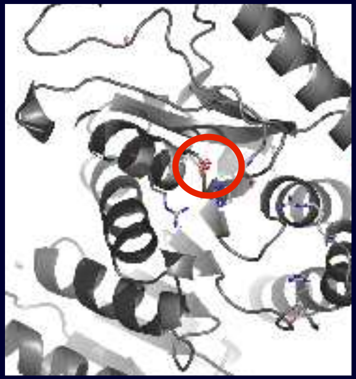
Metal center reduction …



… within seconds,

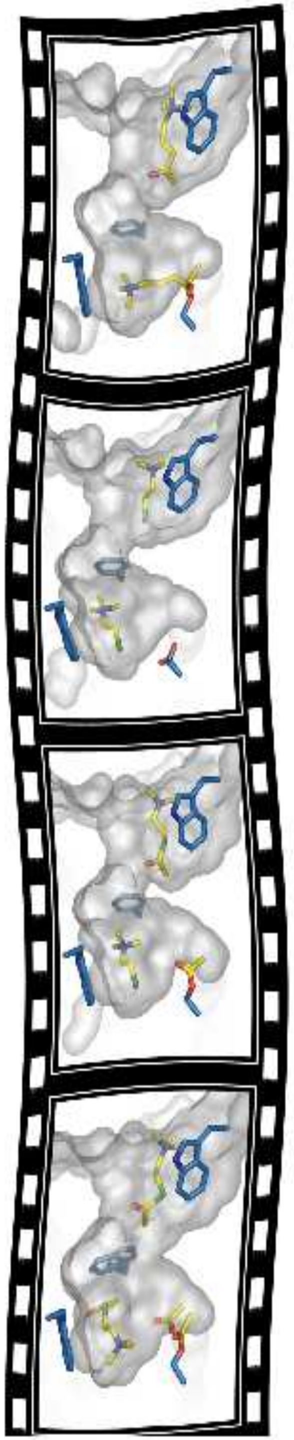
before full data set is collected

Active site damage



in PDB: many protein structures
with reduced redox centers

Protein crystals are 100 x more radiation sensitive at room temperature than at 100 K



Kinetic crystallography

Aim: obtain structural snapshots of proteins *at work*
(e.g. enzymatic intermediate states)

cryo-trapping

of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography

to follow reactions in real time
at room temperature

at synchrotrons

- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

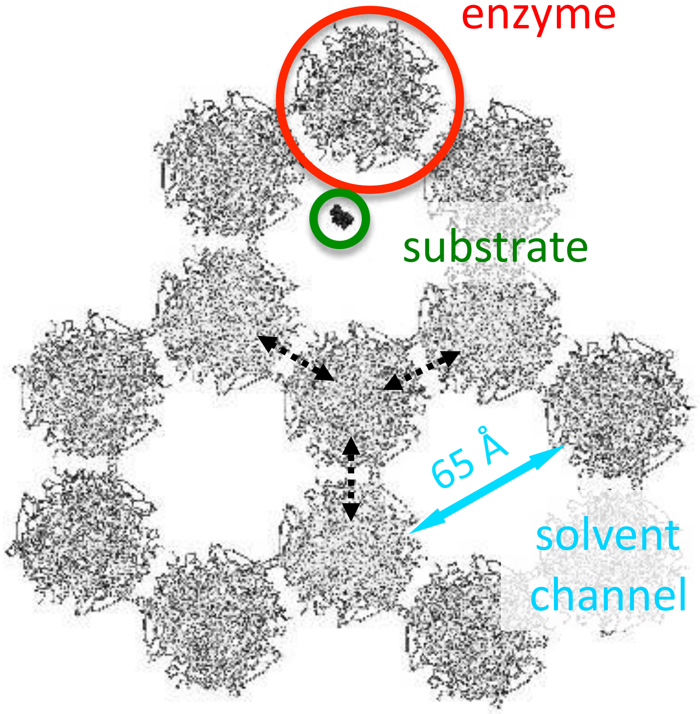
at XFELs

- microcrystals
- ‘monochromatic’ X-rays
- time resolution: 10 fs

Kinetic crystallography ‘works’ because most proteins are active in the crystalline state

Protein crystal:

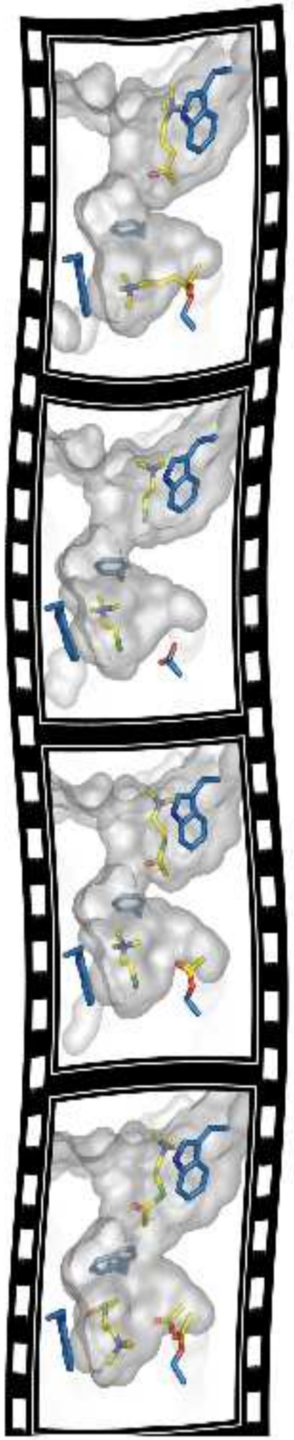
- 50% solvent in a crystal
- only few crystal contacts



- ligands diffuse through solvent channels
- proteins remain flexible and thus active
- but reaction rate most often slowed down

Kinetic crystallography requires synchronization
($k_{trigg} >> k_{cat}$)

Courtesy James Holton



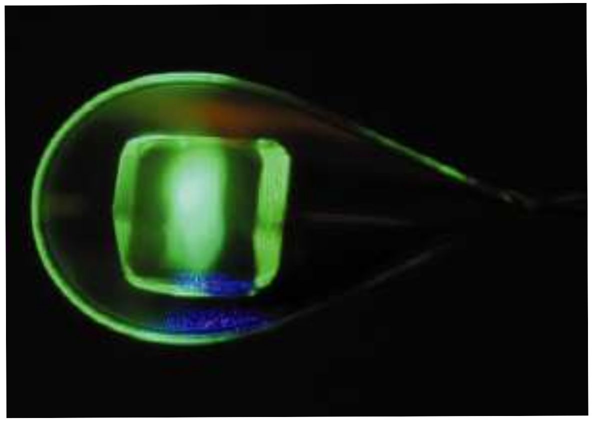
Reaction triggering methods

- UV-vis light illumination

  (inherent light sensitivity or caged compounds)
- diffusion of small molecules
- pH jump
- X-irradiation
- THz irradiation
- electric field

Monitoring reaction progression by *in crystallo* spectroscopy …

…. offline or online on Cryobench platform at ESRF (von Stetten, …., Royant (2015) ActaD 71, 15)



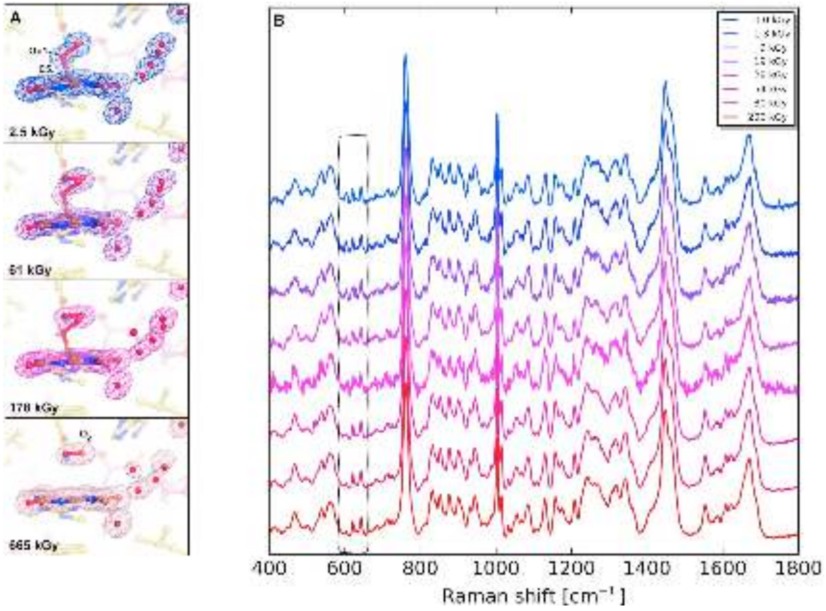
Various optical spectroscopies can be applied to protein crystals :

- **UV-Visible absorption**
- **Fluorescence emission**
- **Raman**

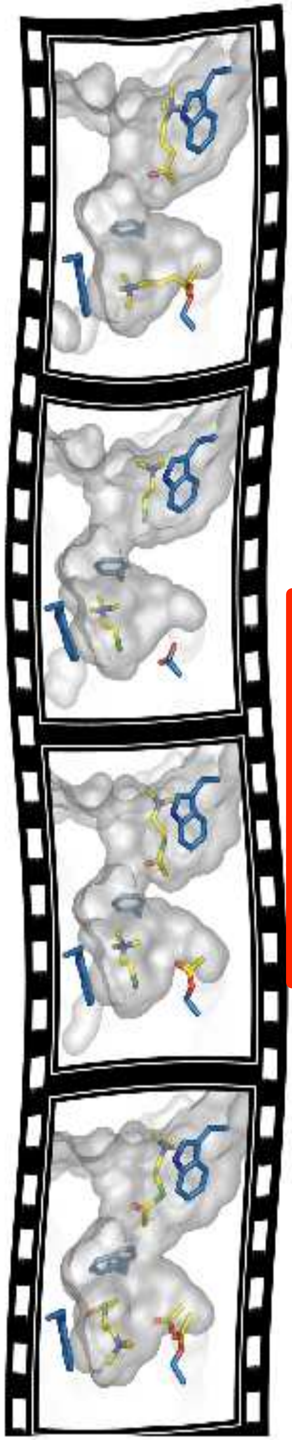
Example: online Raman spectroscopy

von Stetten, …., Royant (2017) J Struct Biol 200, 124
Bui et al (2014) Angew Chem 53, 13710

Peroxide intermediate state in the mechanism of urate oxidase shown to be extremely sensitive to X-rays

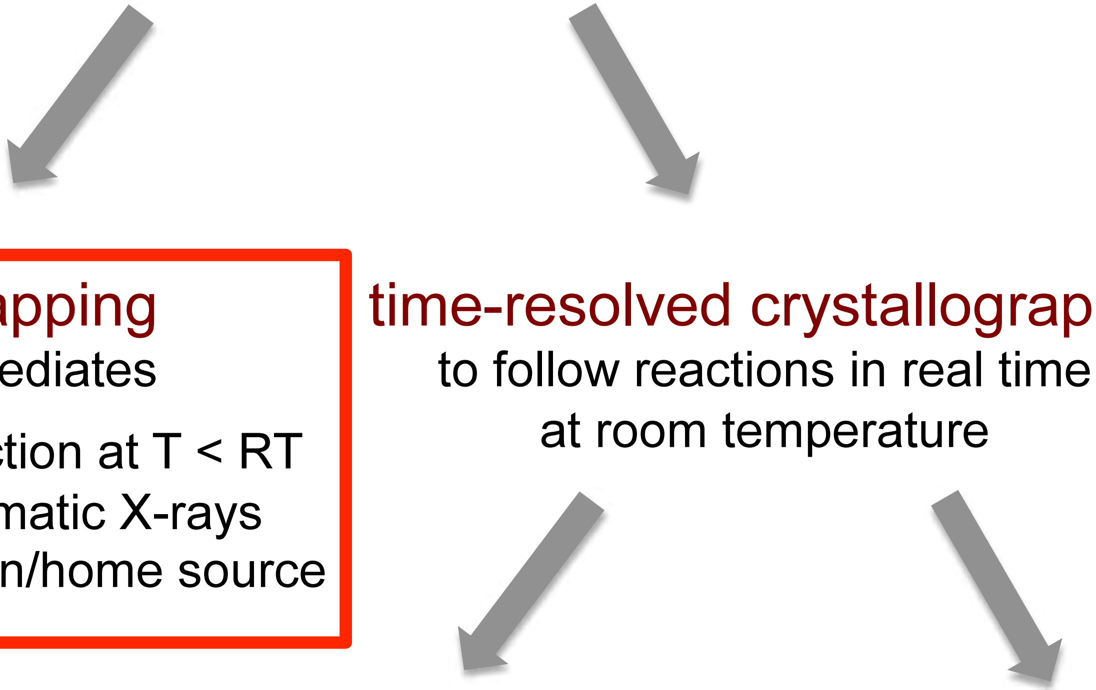


Courtesy Antoine Royant



Kinetic crystallography

Aim: obtain structural snapshots of proteins *at work*
(e.g. enzymatic intermediate states)



cryo-trapping

of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography

to follow reactions in real time
at room temperature

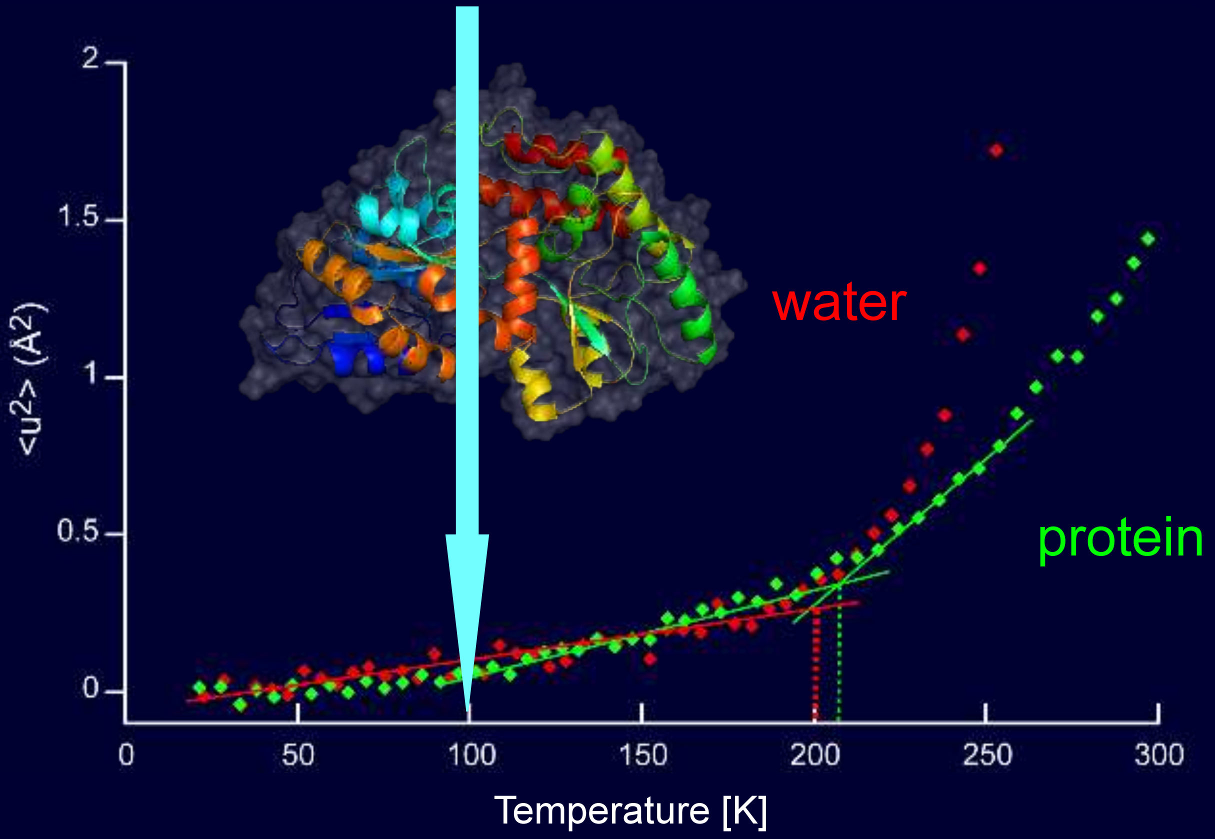
at synchrotrons

- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

at XFELs

- microcrystals
- ‘monochromatic’ X-rays
- time resolution: 10 fs

Temperature-dependent side-chain flexibility from neutron scattering



Cryo X-ray data collection

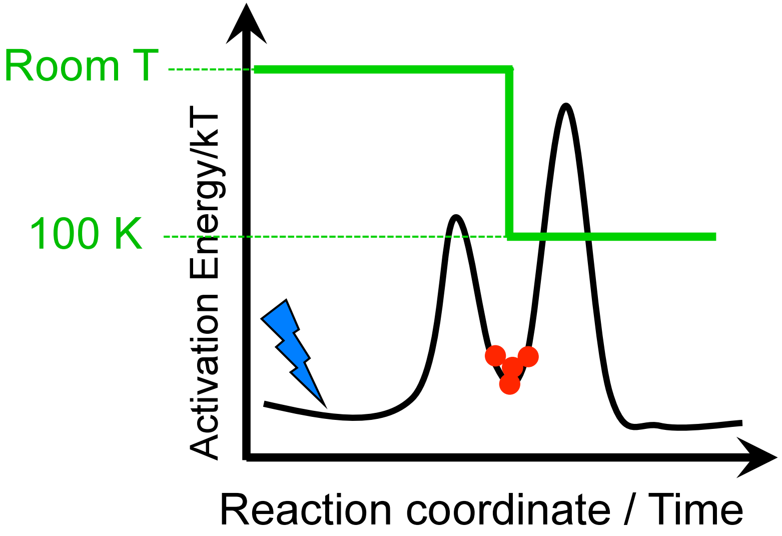
Wood et al (2008) JACS 130, 4586

Cryo-cooling at 500 K / s : protein conformational changes quenched at 200 K

Halle (2004) PNAS 2004, 4793

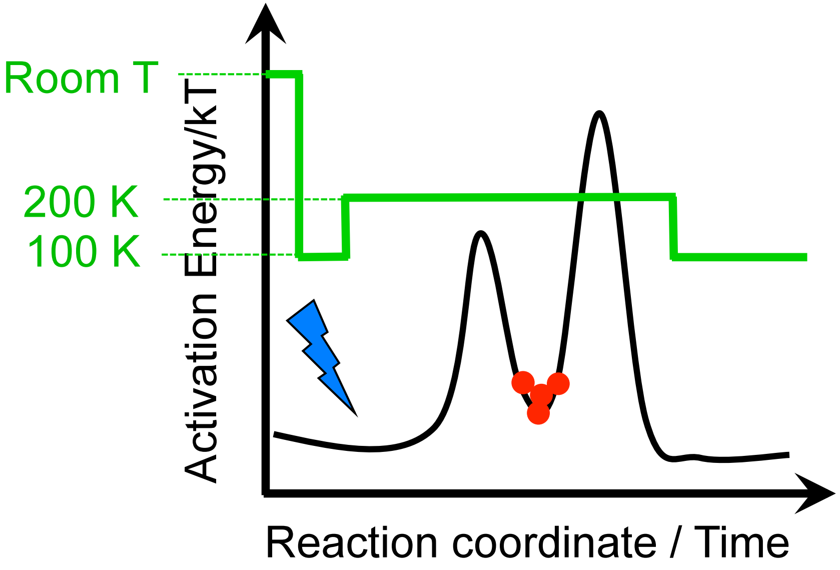
Cryo-trapping of intermediates

Trigger - Freeze



Limitation:
- only processes slower than cooling speed (500 K/s) can be trapped

Freeze - Trigger



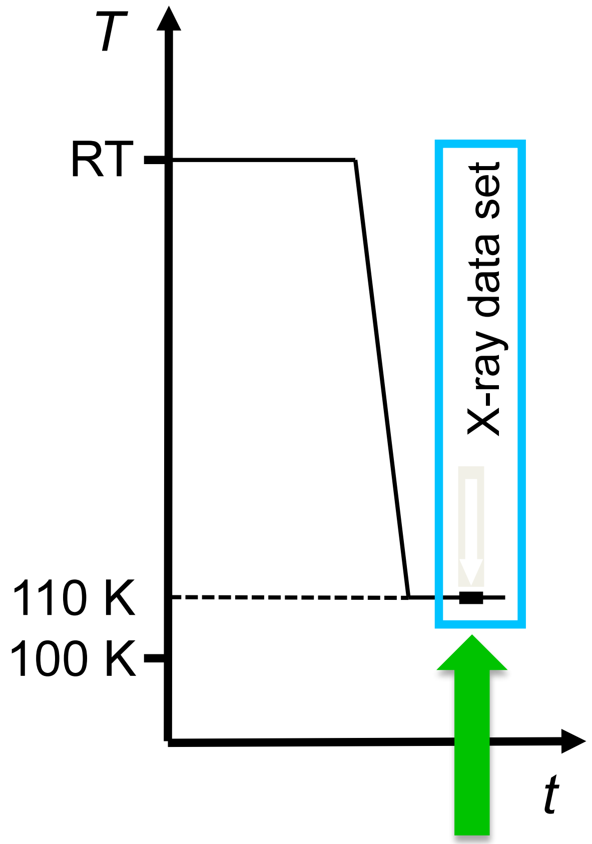
Limitations:
- energy landscape might be different at 100 K and at RT
- only intermediates with low barriers are accessible

Bourgeois & Weik (2009) Cryst. Rev. 15, 87

Example:

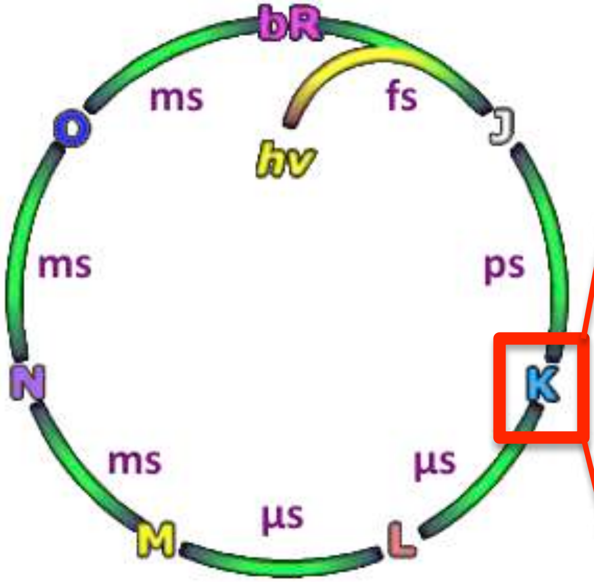
Early photo-intermediate in the light-driven proton pump bacteriorhodopsin

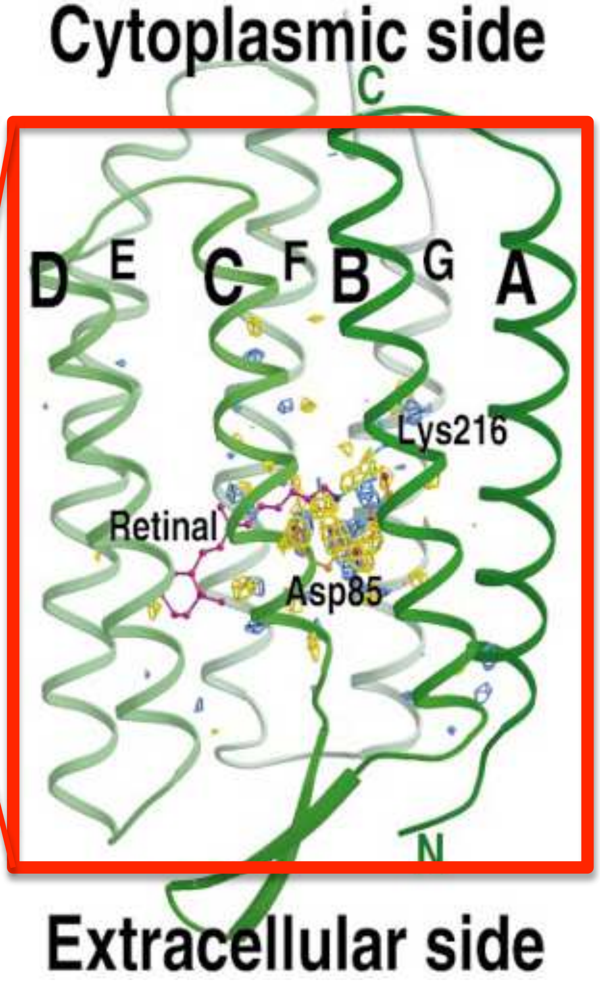
Edman, Nollert, Royant, Belrhali, Pebey-Peyroula, Hadju, Neutze, Landau (1999) Nature 401, 822



Trigger: continuous illumination at 527 nm

Photocycle

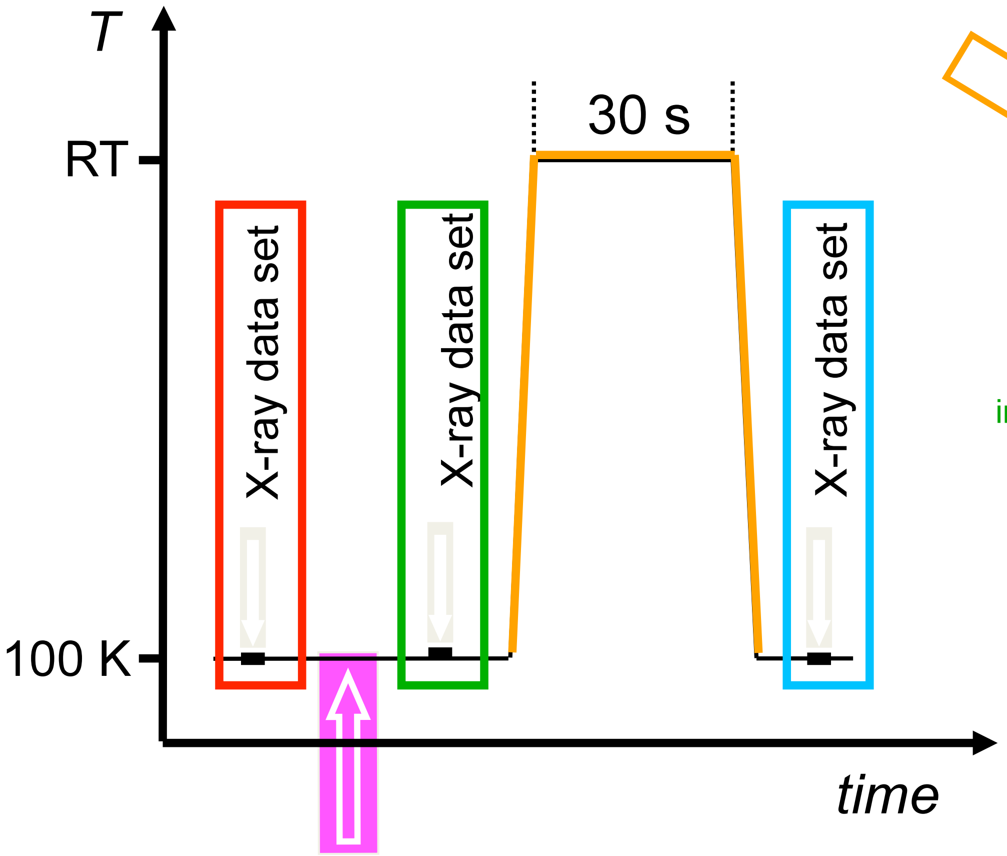


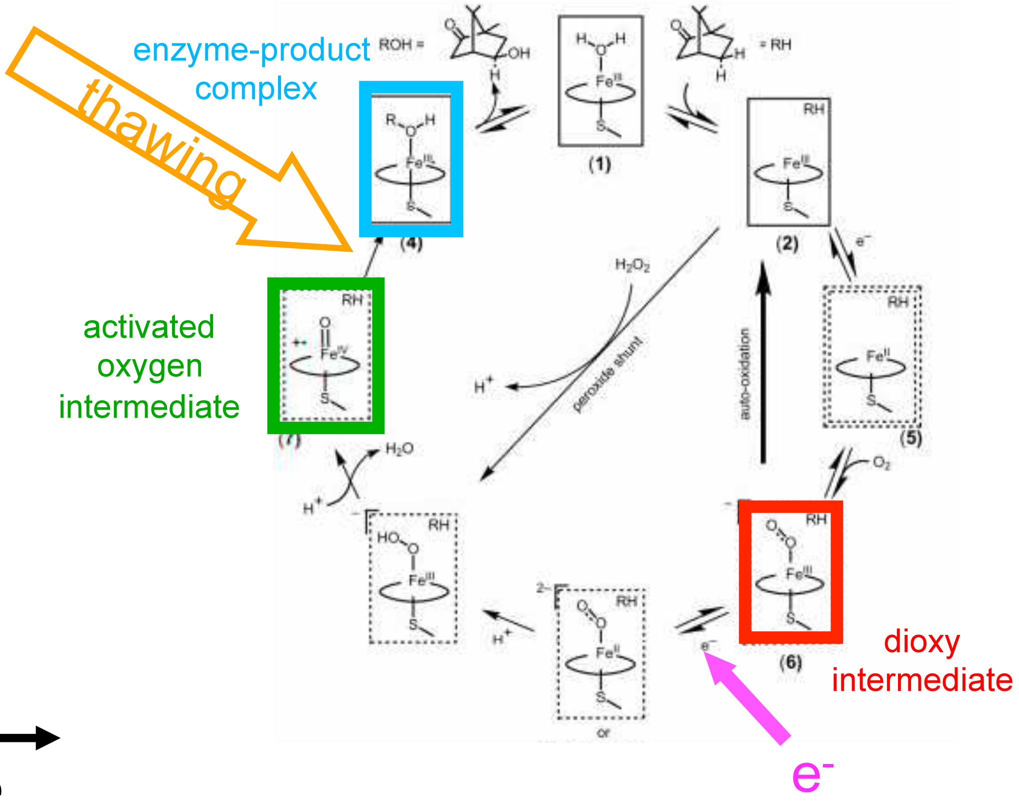


Review: Wickstrand et al (2015) BBA 1850, 536

Example: Metastable species on the P450cam reaction pathway

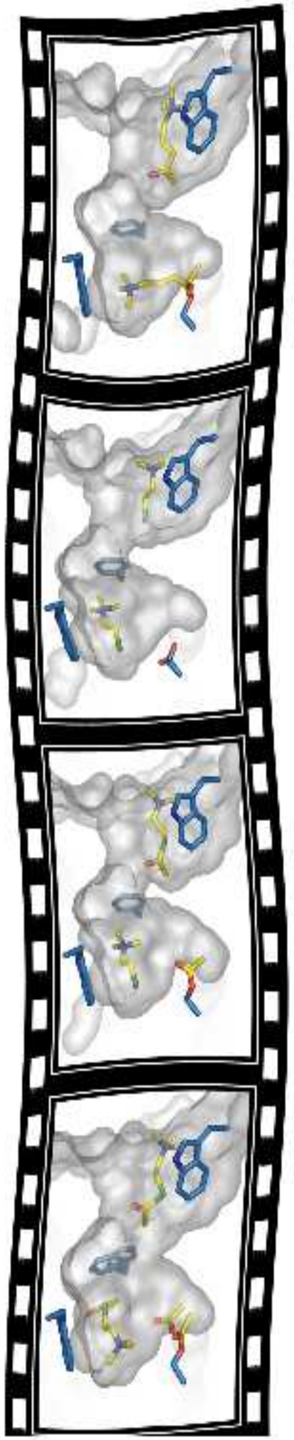
Schlichting, Berendzen, Chu, Stock, Maves, Benson, Sweet, Ringe, Petsko & Sligar (2000) Science 287, 1615





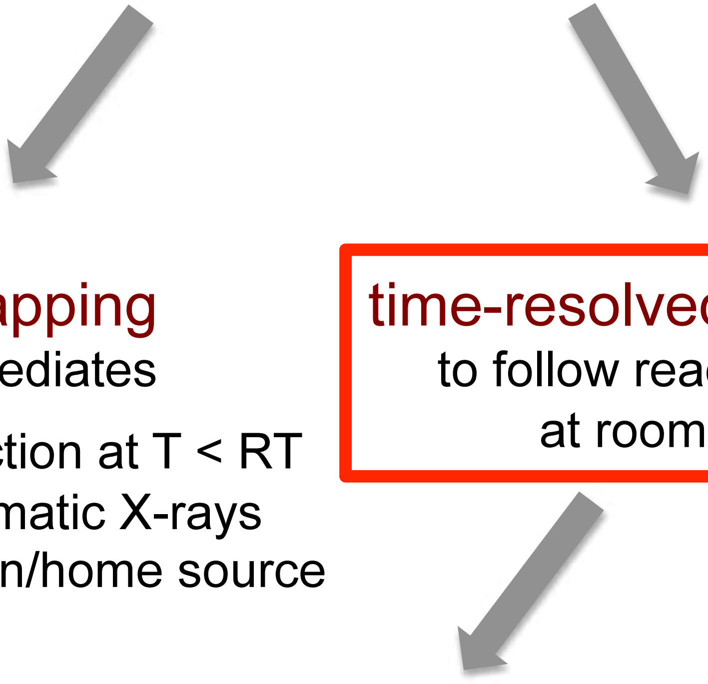
Electron generation by X-irradiation (1.5 Å) for 3h

Thawing: increasing protein and substrate flexibility, backbone flip



Kinetic crystallography

Aim: obtain structural snapshots of proteins *at work*
(e.g. enzymatic intermediate states)



cryo-trapping
of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography
to follow reactions in real time
at room temperature

at synchrotrons

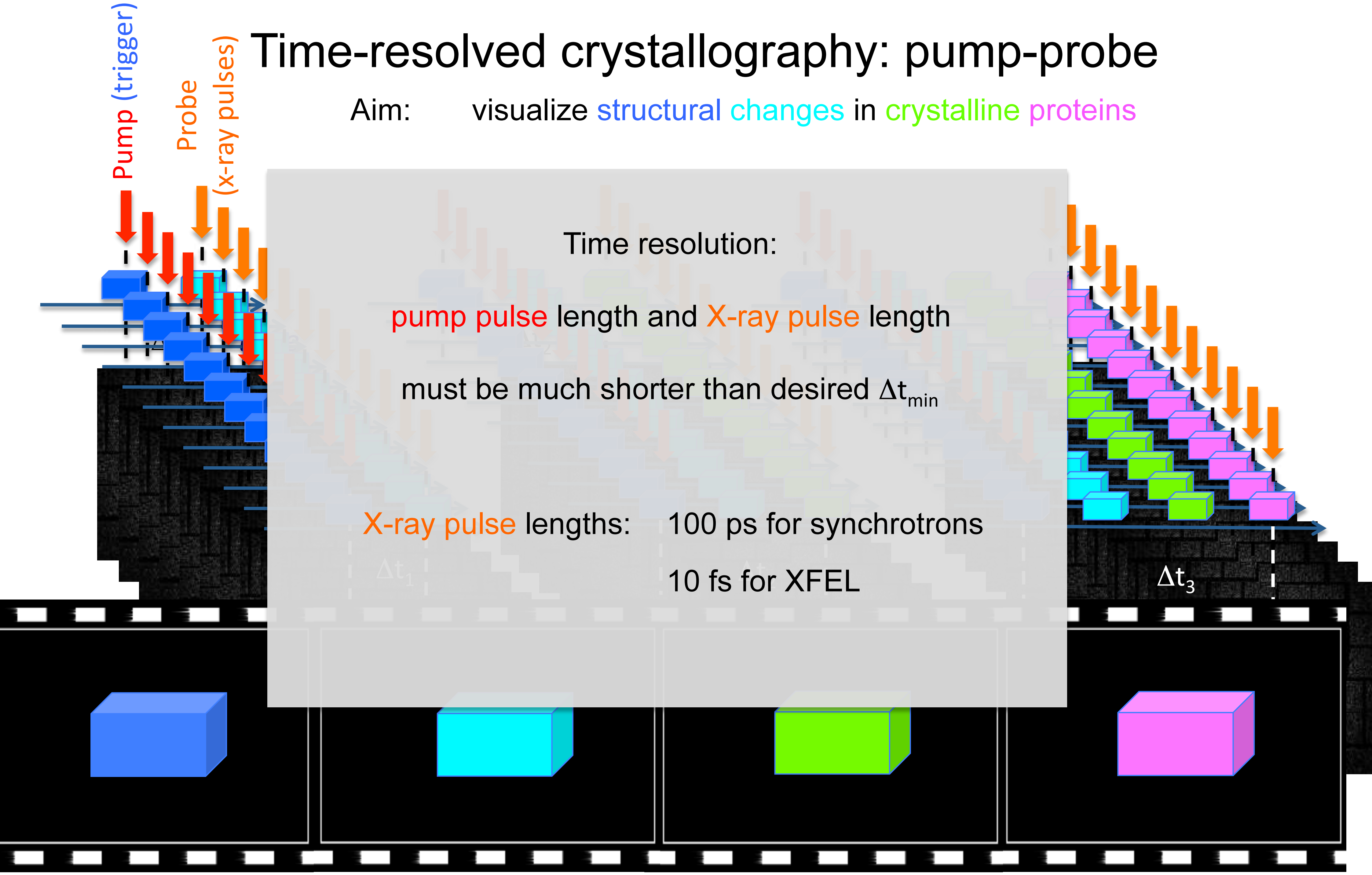
- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

at XFELs

- microcrystals
- 'monochromatic' X-rays
- time resolution: 10 fs

Time-resolved crystallography: pump-probe

Aim: visualize structural changes in crystalline proteins



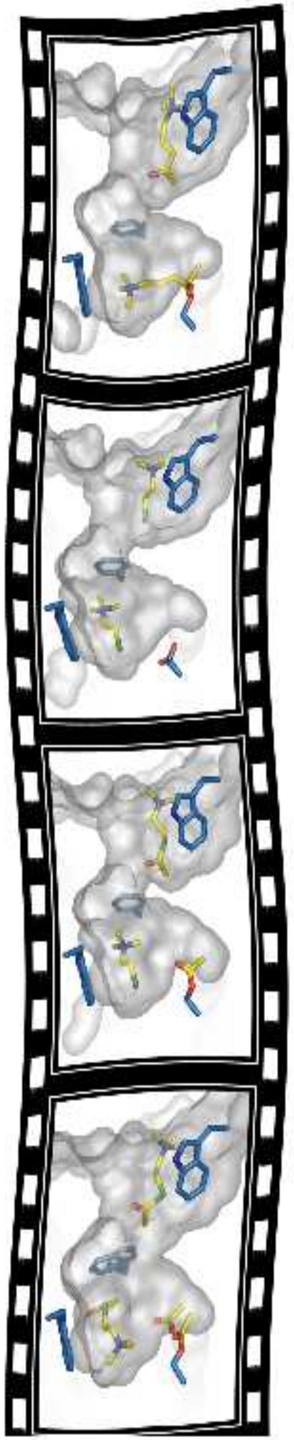
Time resolution:

pump pulse length and X-ray pulse length

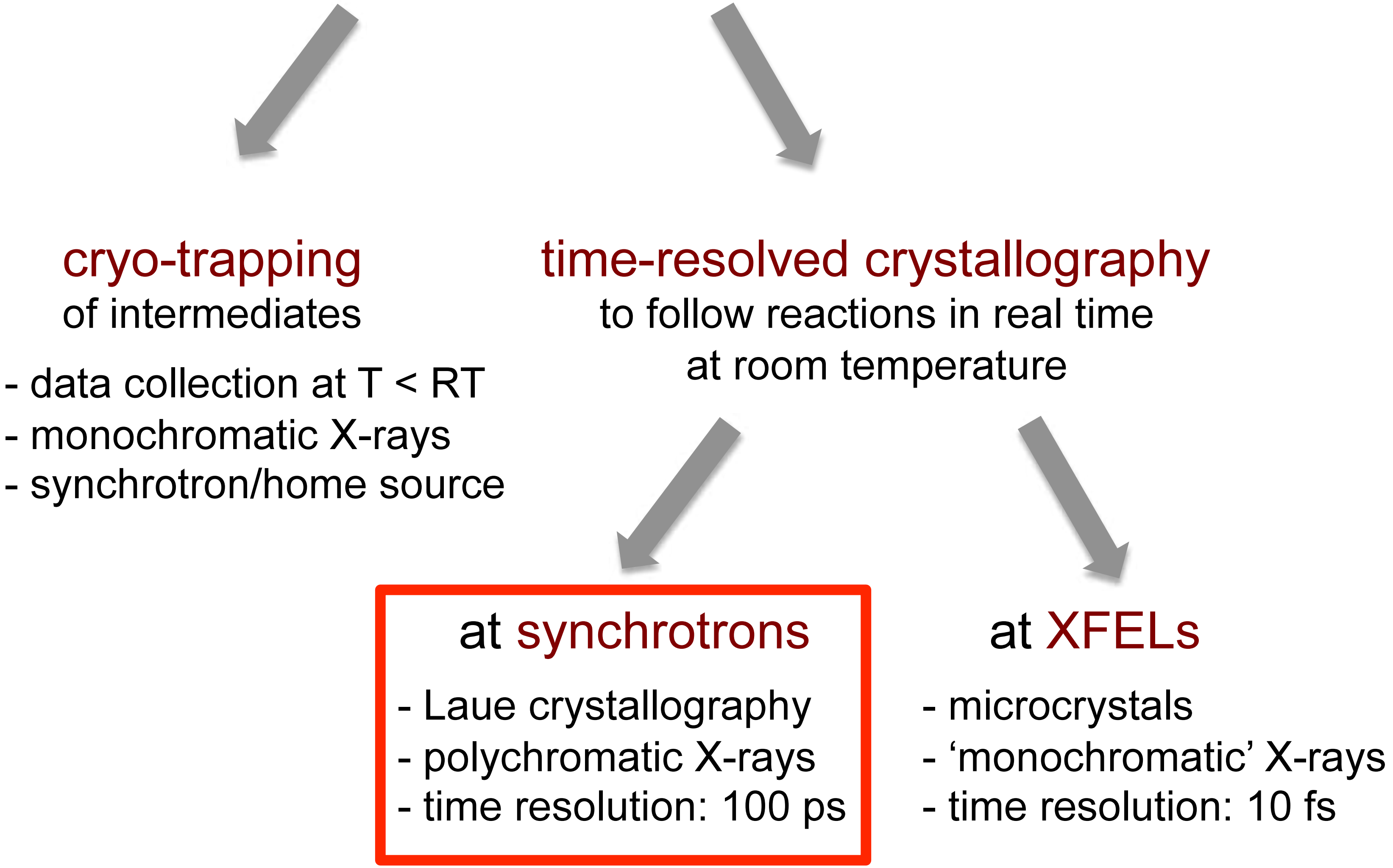
must be much shorter than desired Δt_{min}

X-ray pulse lengths: 100 ps for synchrotrons

10 fs for XFEL



Kinetic crystallography



Aim: obtain structural snapshots of proteins *at work*
(e.g. enzymatic intermediate states)

cryo-trapping
of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography
to follow reactions in real time
at room temperature

at synchrotrons

- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

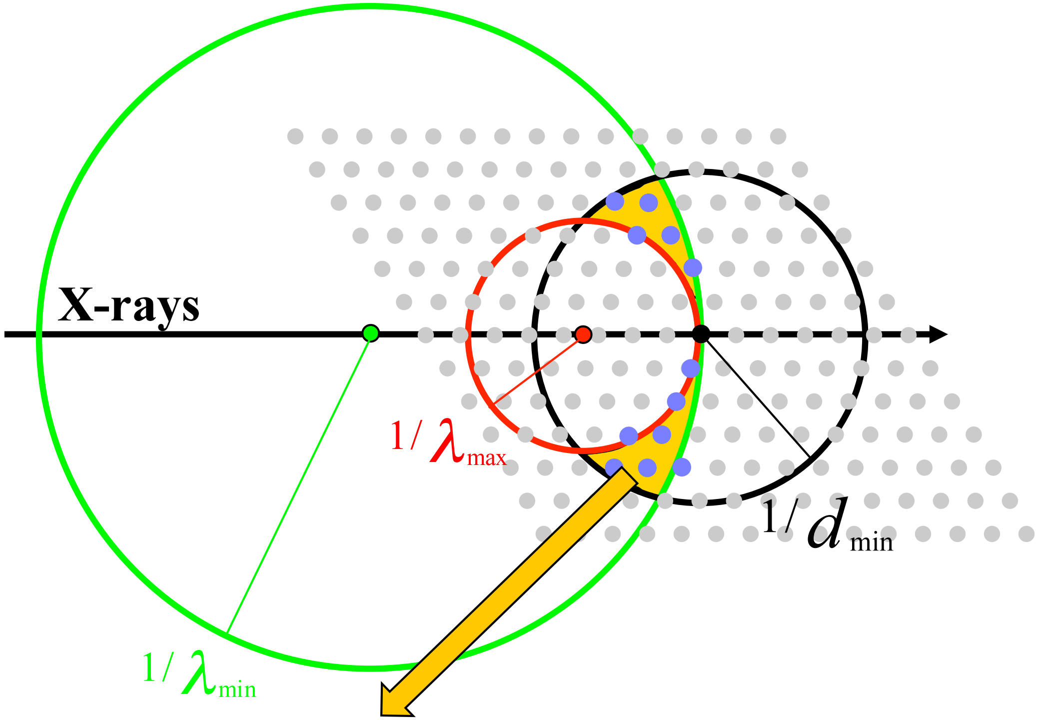
at XFELs

- microcrystals
- ‘monochromatic’ X-rays
- time resolution: 10 fs

Laue crystallography :

Expose the static crystal to a wide band pass X-ray beam

Ewald sphere construction for Laue geometry



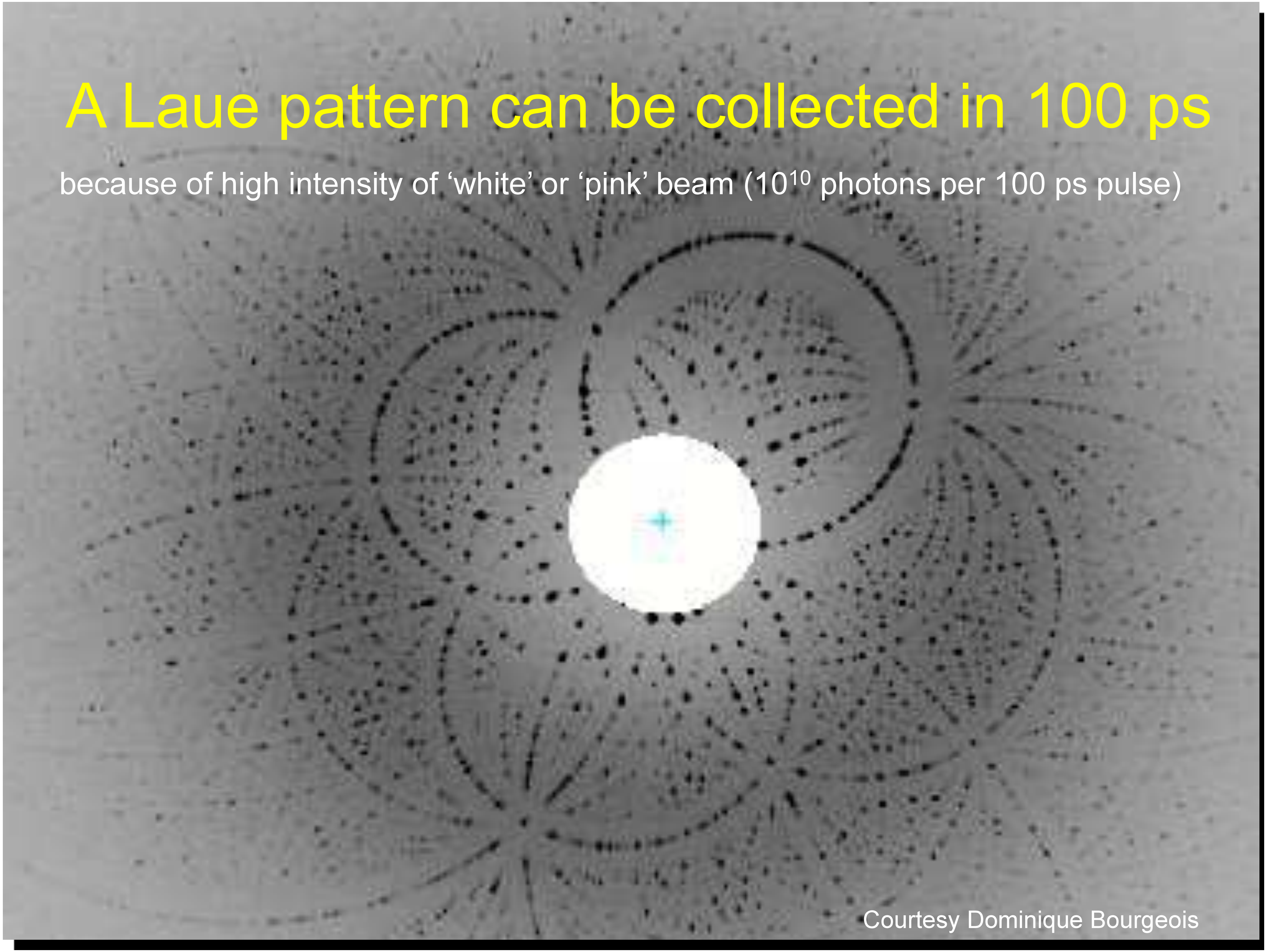
You get more Bragg peaks per diffraction pattern than with monochromatic X-rays:

Fewer images are needed for a full data set

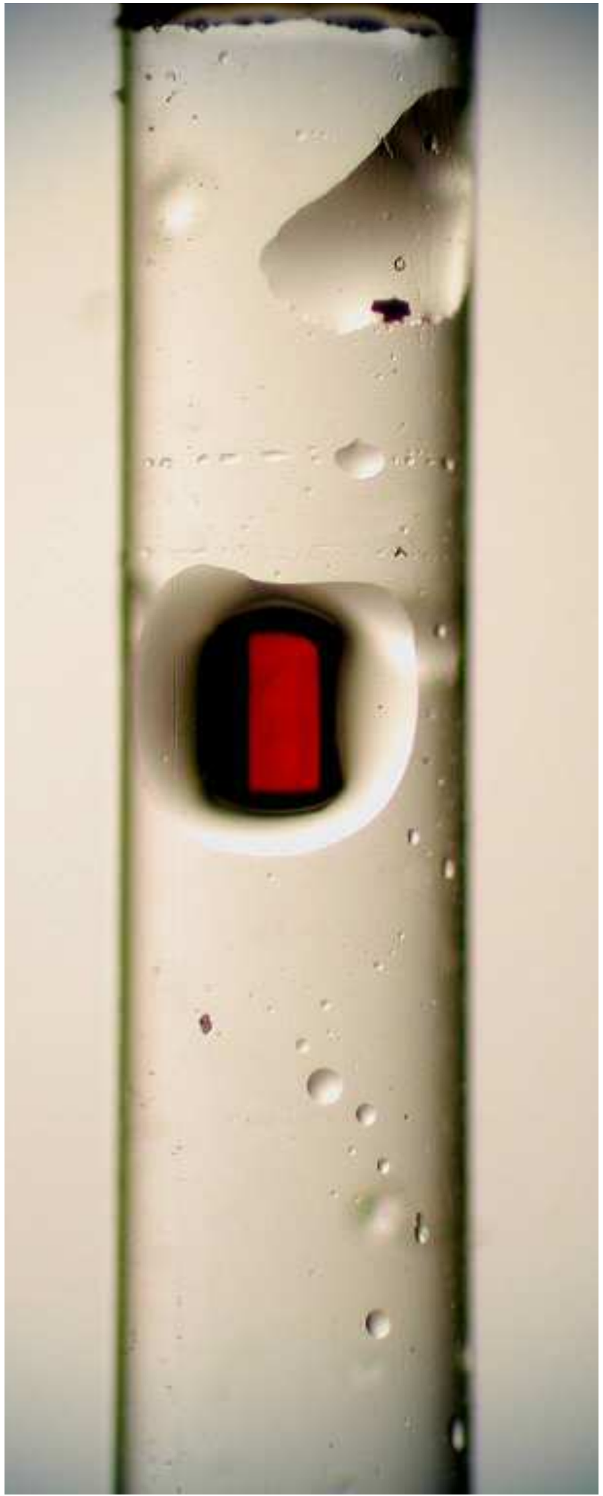
Courtesy Dominique Bourgeois

A Laue pattern can be collected in 100 ps

because of high intensity of ‘white’ or ‘pink’ beam (10^{10} photons per 100 ps pulse)



Courtesy Dominique Bourgeois



Time-resolved pump-probe Laue crystallography at synchrotrons:

- collect several pump-probe sequences on same part of crystal:
  only cyclic reactions can (in practice) be studied

- radiation damage:
  radicals spread on time-scale of X-ray pulse length (100 ps)

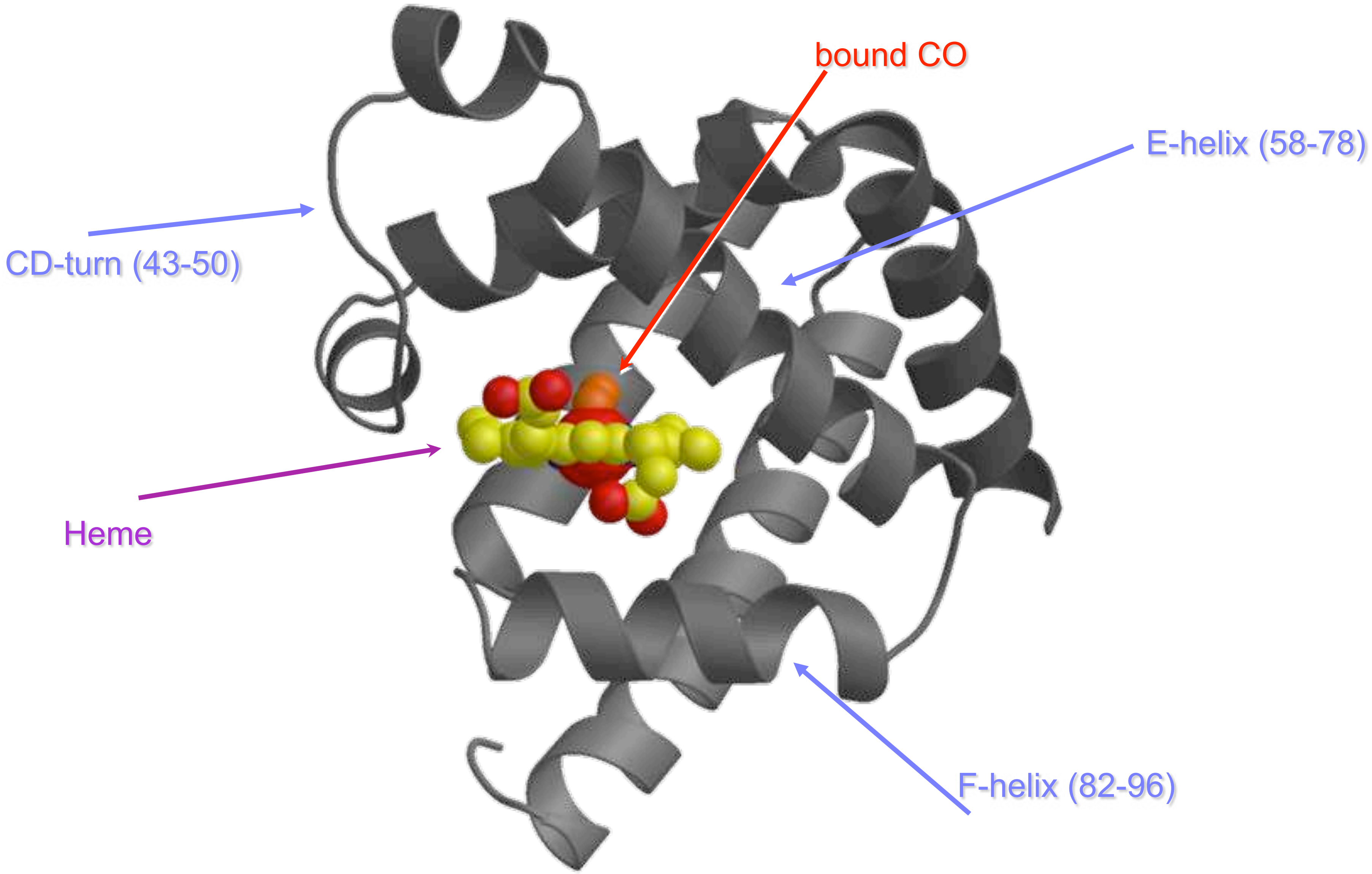
- requires crystals that:
  - are radiation resistant
  - have low mosaicity (overlap of reflections)
  - small unit cells (overlap of reflections)

- very useful, but limited to a few model systems
  (myoglobin, photo active yellow protein, GTPase Ras, photosynthetic reaction center)

Courtesy Dominique Bourgeois

Example:

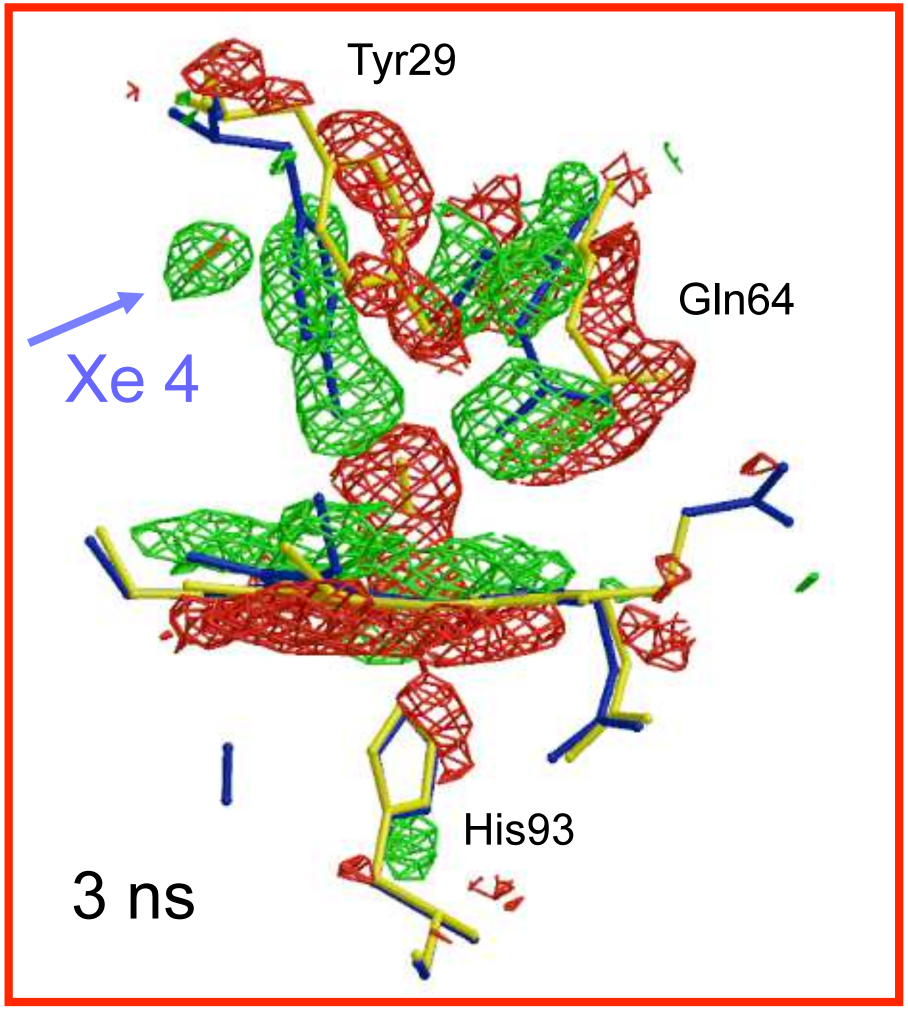
Myoglobin: model protein to study conformational dynamics

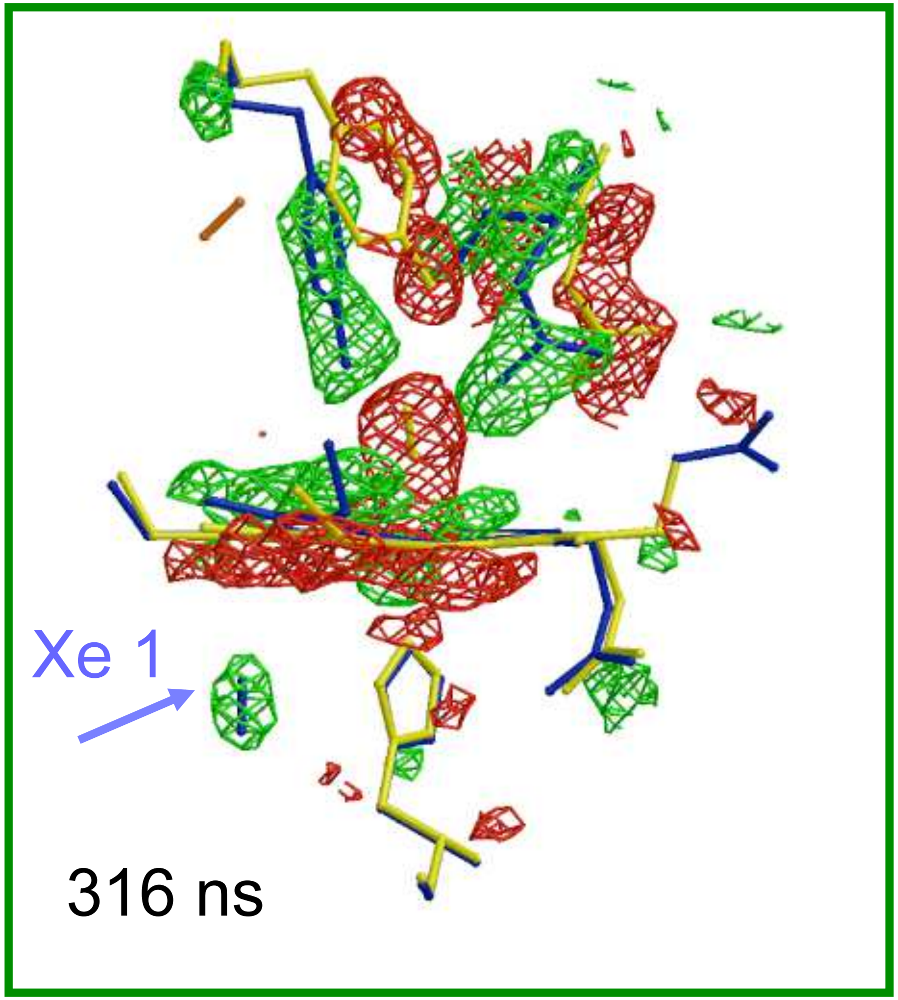


First protein structure ever solved, J. Kendrew, M. Perutz, 1959

Courtesy Dominique Bourgeois

Motions in the heme vicinity …



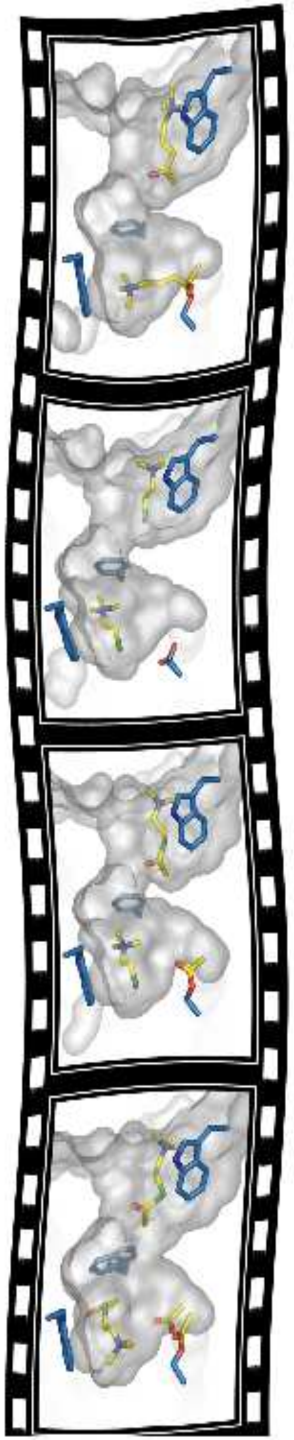


Bourgeois, Vallone *et al*, (2003) PNAS 100, 8704-9

Yellow CO Mb
Blue : Mb*

Green +3.0 σ
Red -3.0σ

Courtesy Dominique Bourgeois



Kinetic crystallography

Aim: obtain structural snapshots of proteins *at work*
(e.g. enzymatic intermediate states)

cryo-trapping

of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography

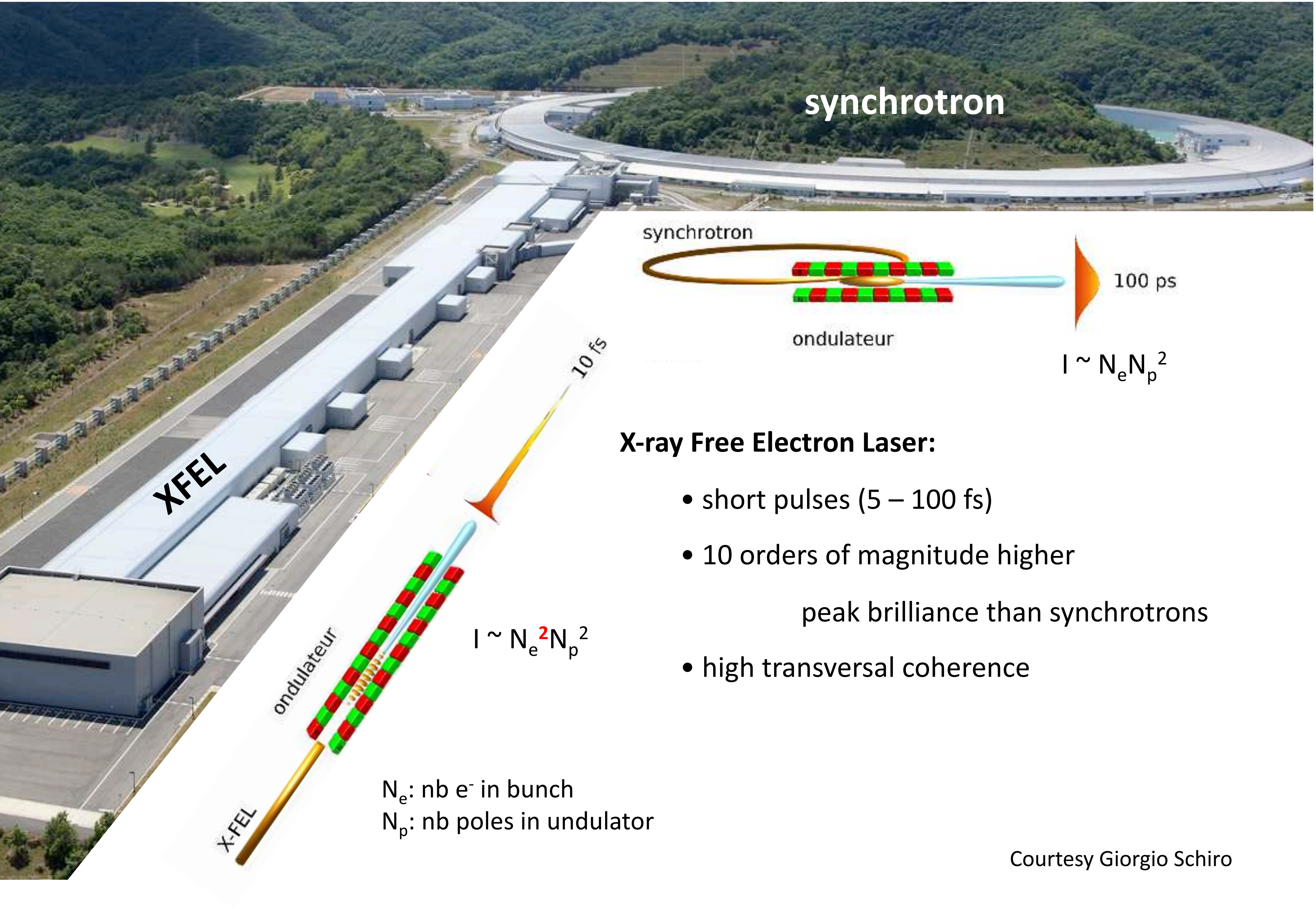
to follow reactions in real time
at room temperature

at synchrotrons

- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

at XFELs

- microcrystals
- ‘monochromatic’ X-rays
- time resolution: 10 fs



$I \sim N_e N_p^2$

X-ray Free Electron Laser:

- short pulses (5 – 100 fs)
- 10 orders of magnitude higher peak brilliance than synchrotrons
- high transversal coherence

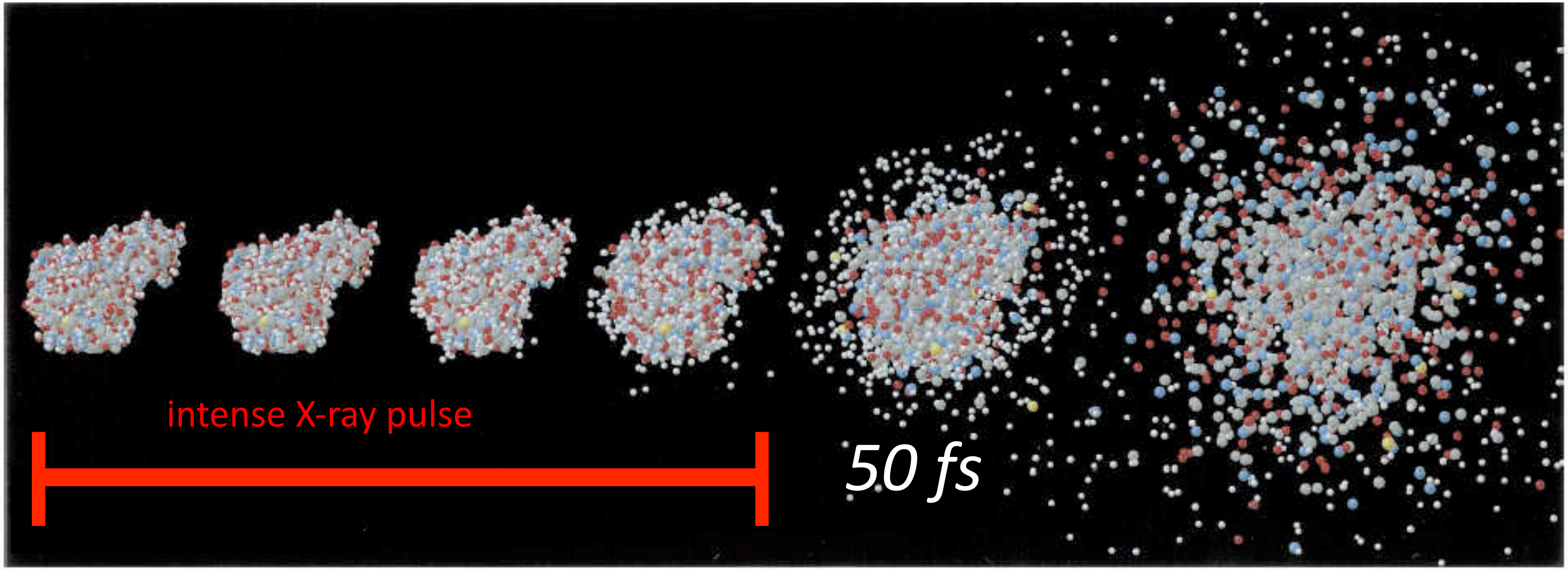
$I \sim N_e^2 N_p^2$

N_e: nb e^- in bunch
N_p: nb poles in undulator

Courtesy Giorgio Schiro

Diffraction-before-Destruction Imaging

Idea: Neutze, ..., Hajdu (2000) Nature 406, 7524

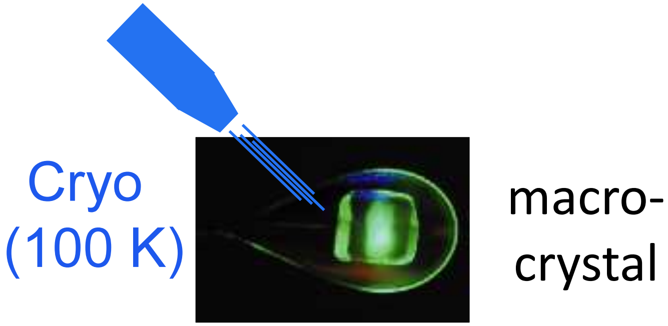


Collect data before the sample has time to respond (explode)

Remember: radials spread on ps – ns time scale, i.e. longer than pulse length

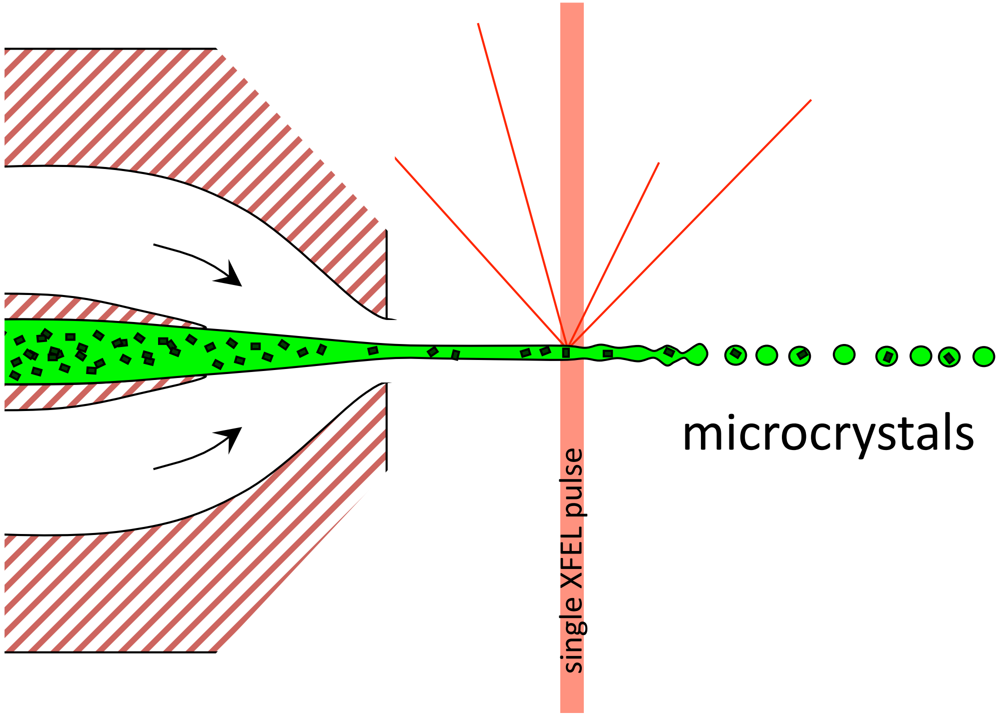
Serial femtosecond crystallography (SFX) at XFELs

Conventional protein crystallography at synchrotrons



Serial femtosecond crystallography

Chapman, …, & Spence (2011) Nature 470, 73

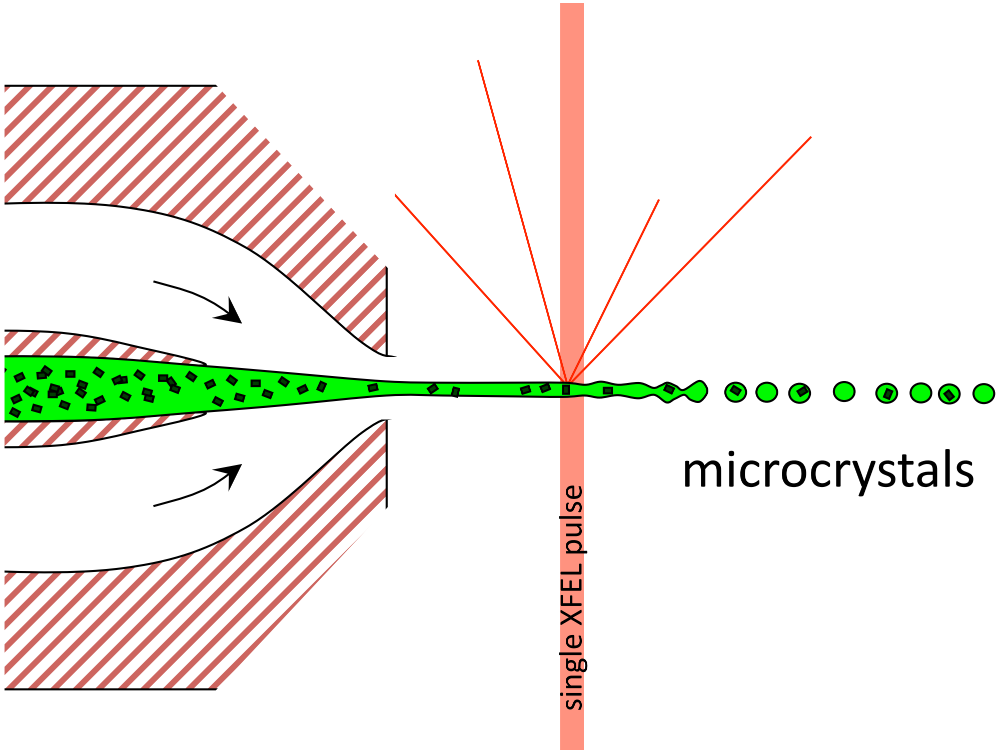


- Each crystal is immediately destroyed
- No *a priori* control over orientation
- Crystals effectively stand still during a 50 fs pulse
- Only part of reflection intersects Ewald sphere (“partials”, no “fullies”)
- Collect 100 000 – 1 000 000 images

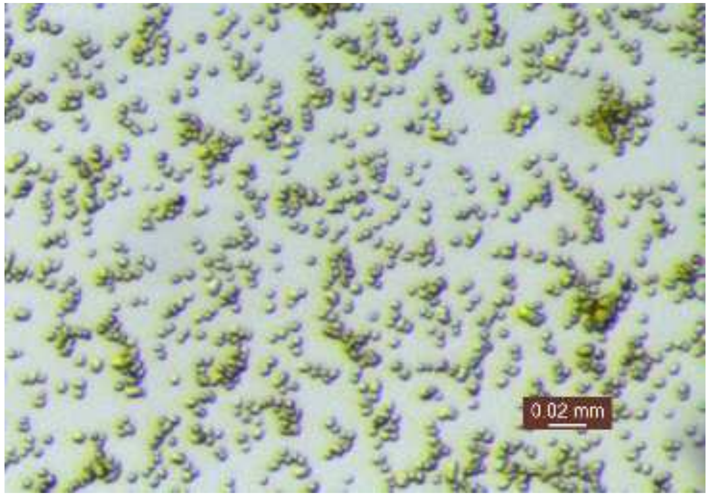
Serial femtosecond crystallography (SFX) at XFELs

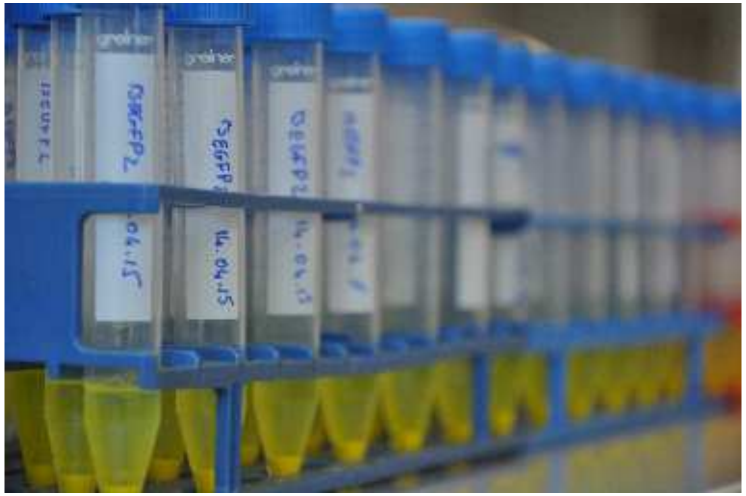
Practical implications:

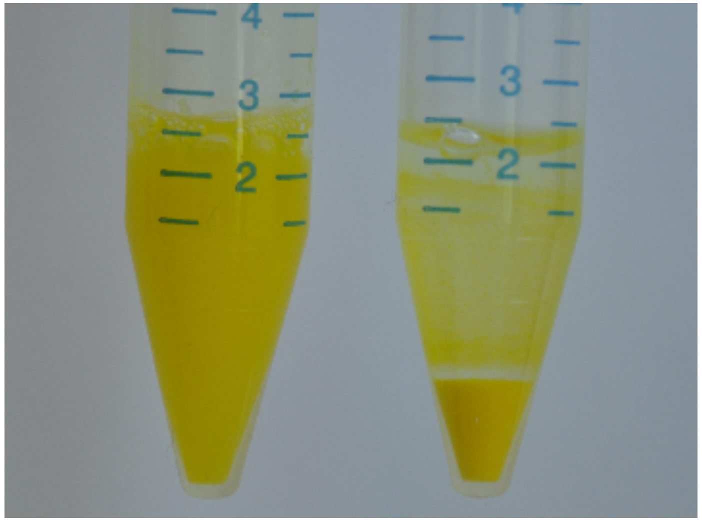
- samples
- sample injection
- ~~SFX data processing~~
- ~~phasing~~



SFX needs lots of microcrystals



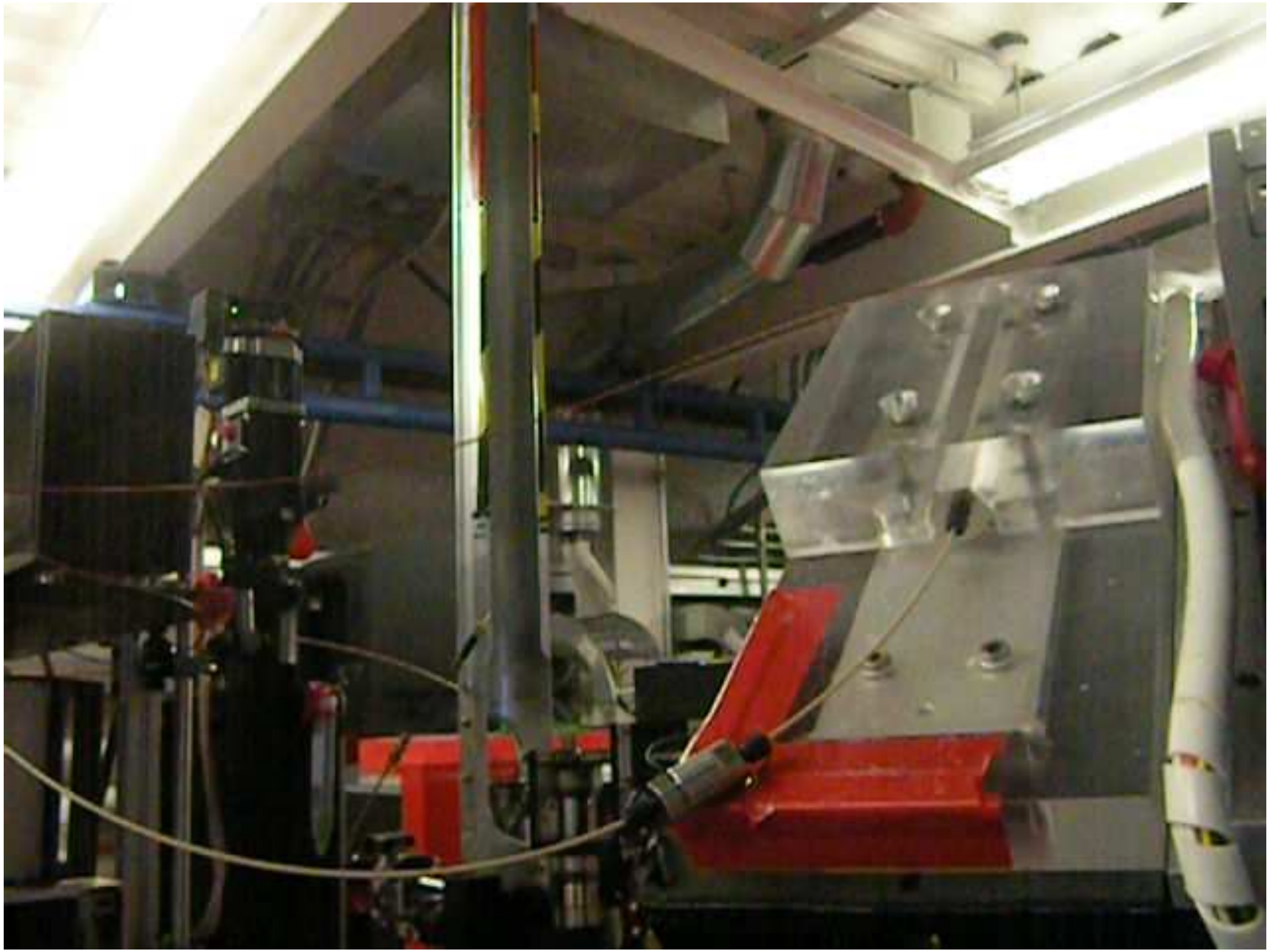




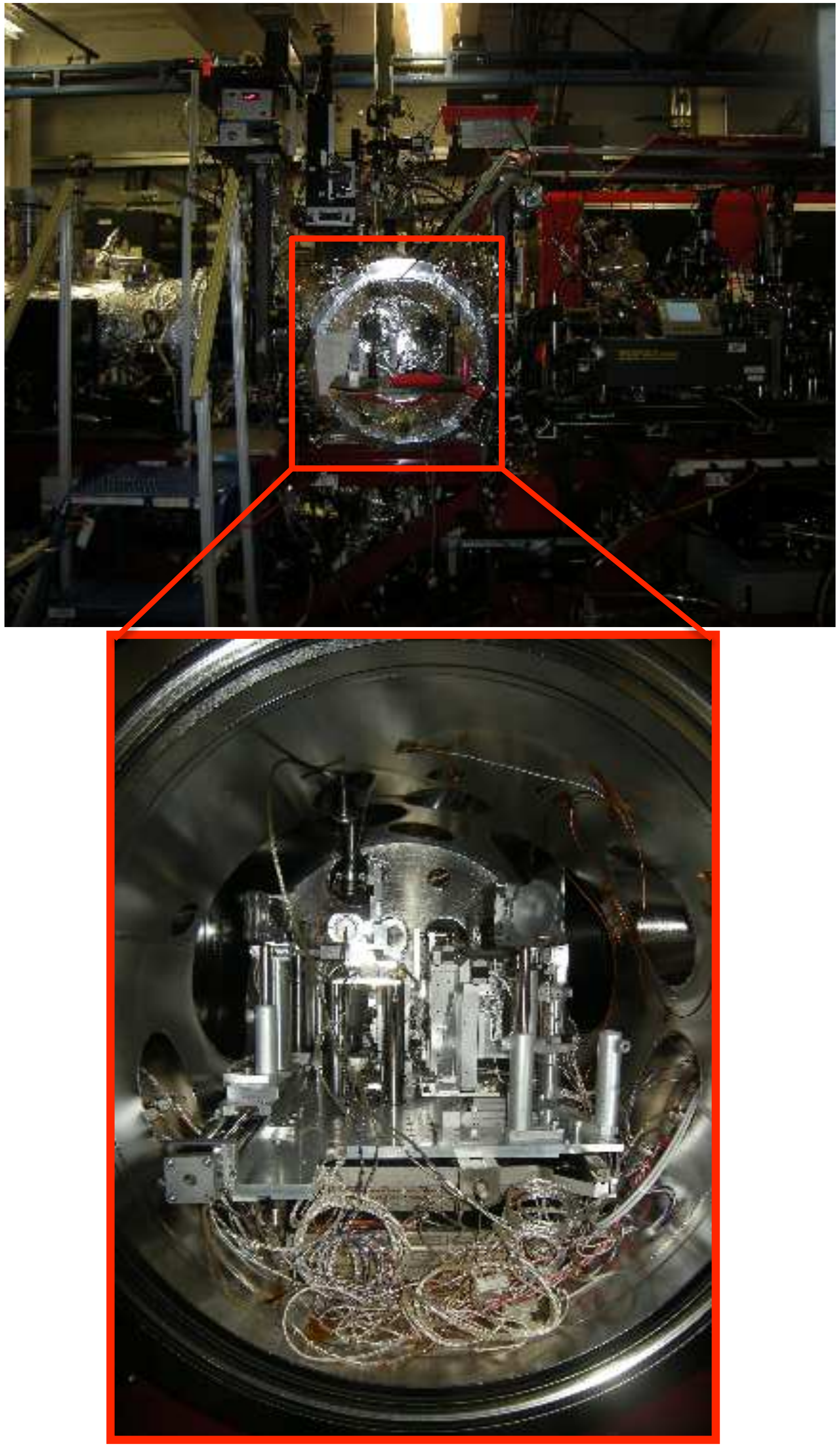
Concentration: 1/3 of settled crystals

Anti-settling device

Lomb *et al* (2012) J Appl Cryst **45**:674

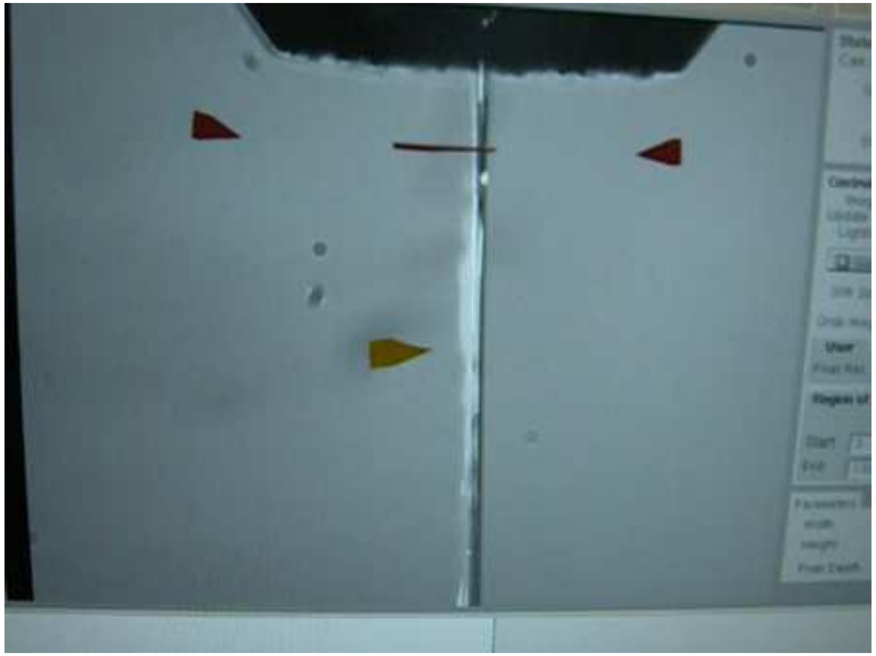


CXI beamline at LCLS



Microcrystal injection

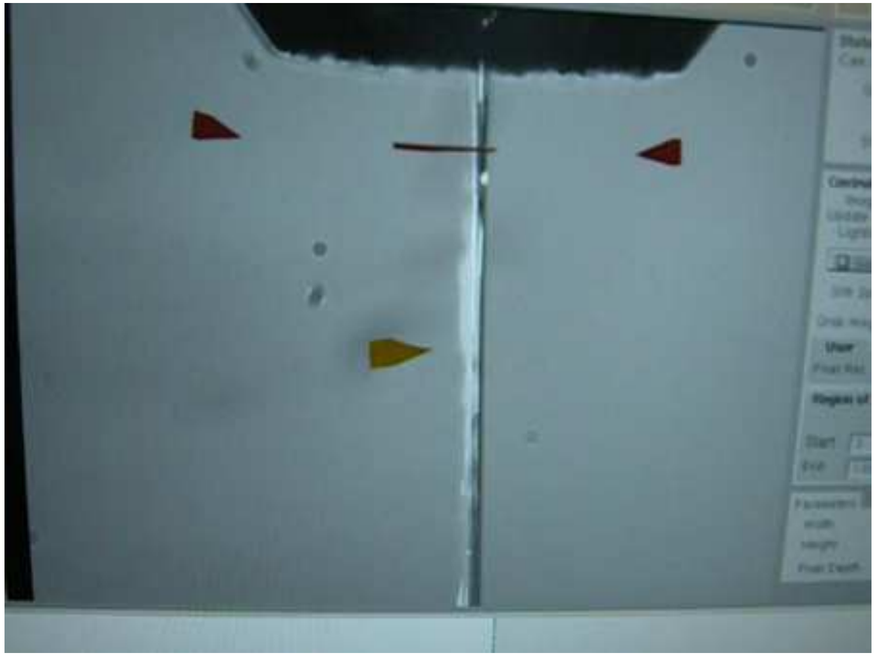
Liquid jet (GDVN)



- 5 μm diameter: low background
- transparent: good for pump laser excitation
- high speed (10 m/s):
  - high sample consumption
  - only 1 out of 500 000 xtals hit by X-ray beam
  - 100 mg – 1 g per data set

Microcrystal injection

Liquid jet (GDVN)

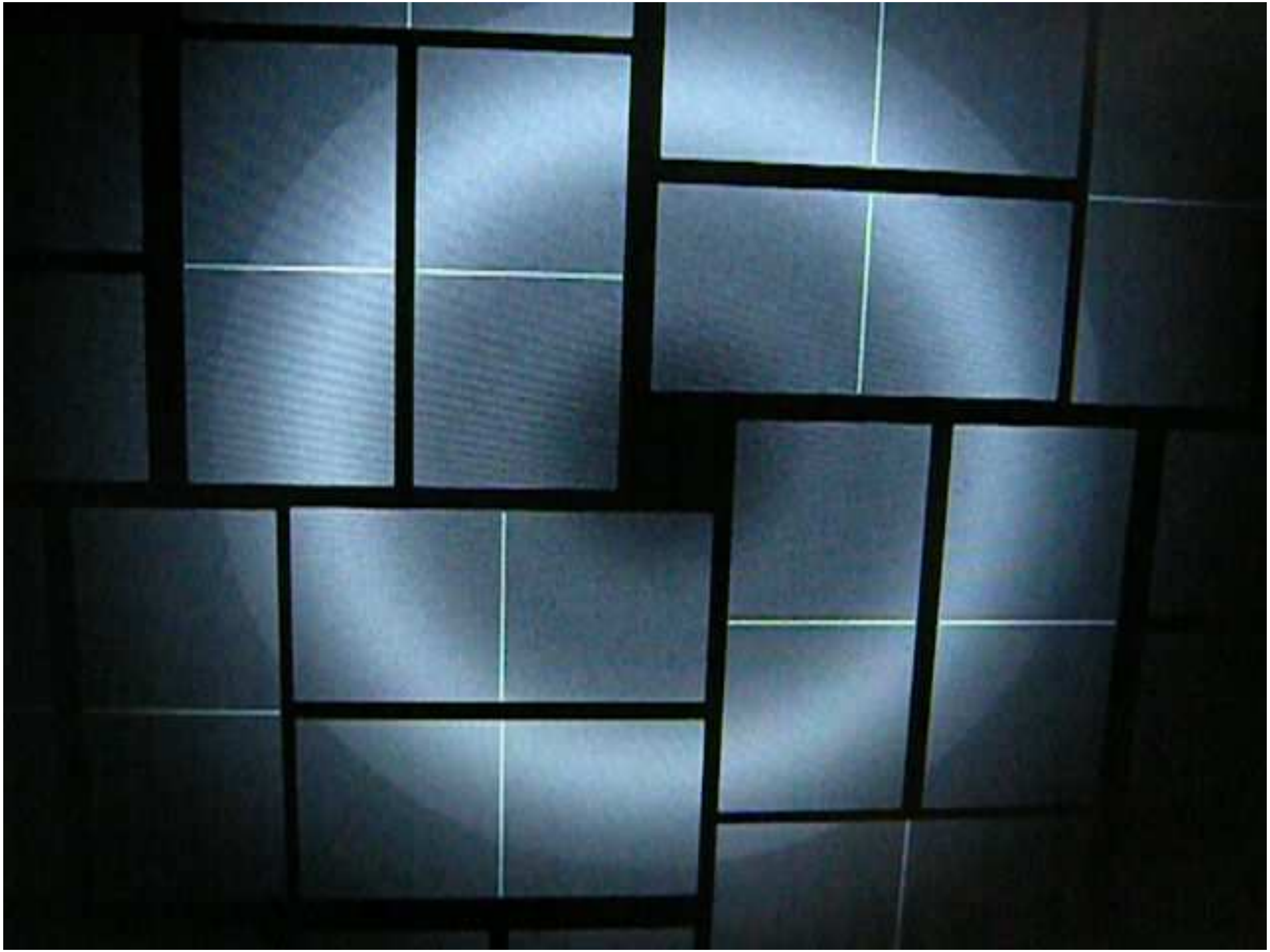


- 5 μm diameter: low background
- transparent: good for pump laser excitation
- high speed (10 m/s):
  - high sample consumption
  - only 1 out of 500 000 xtals hit by X-ray beam
  - 100 mg – 1 g per data set

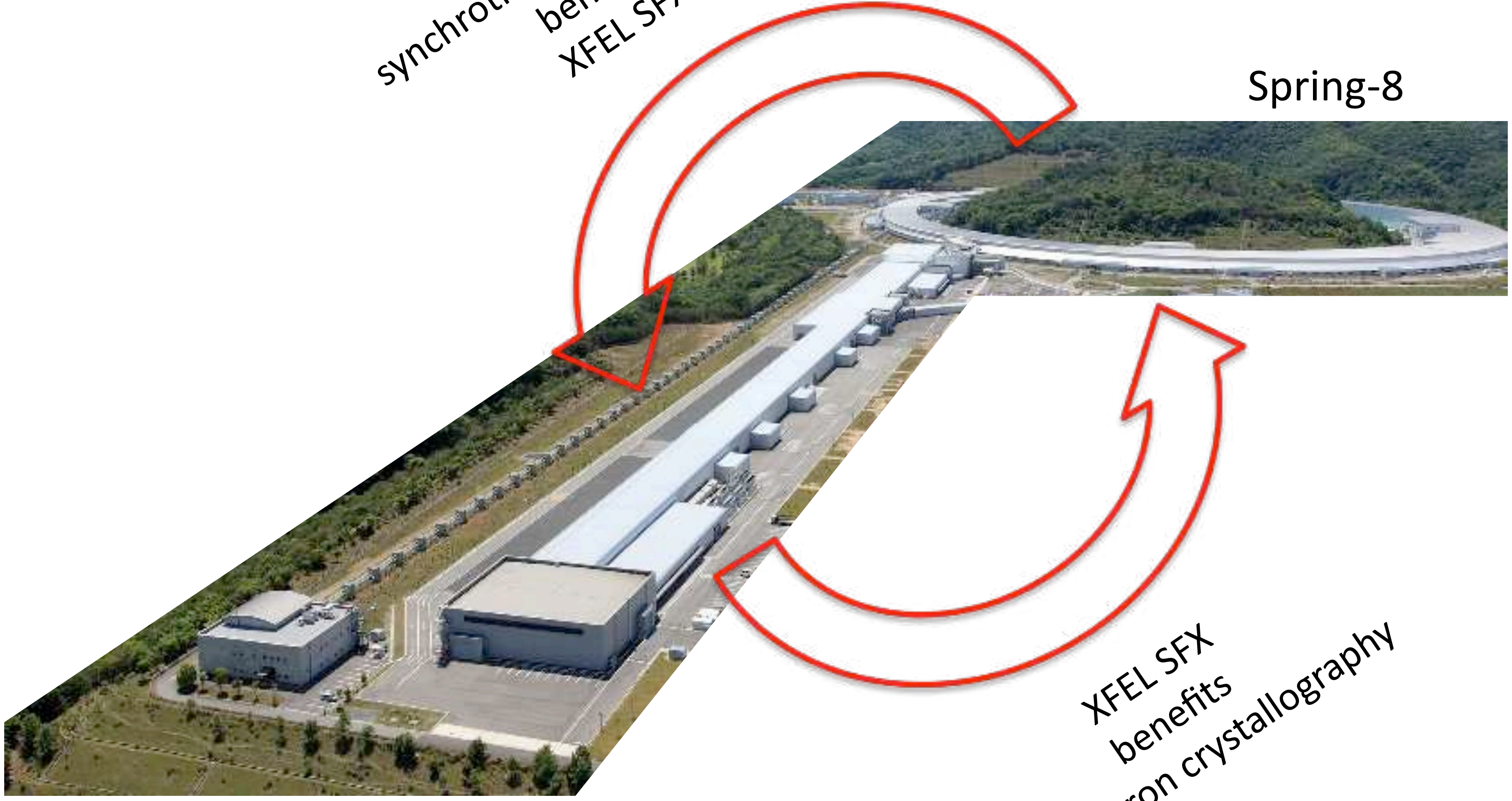
High viscosity injector

- crystals in viscous matrix (LCP, grease, …)
- 100 μm diameter: high background
- reduced transparency
- low speed (mm/s):
  - low sample consumption
  - 1 mg per data set

Alternatively: microcrystals on solid supports + raster scanning



XFEL SFX does not replace, but complements synchrotron crystallography

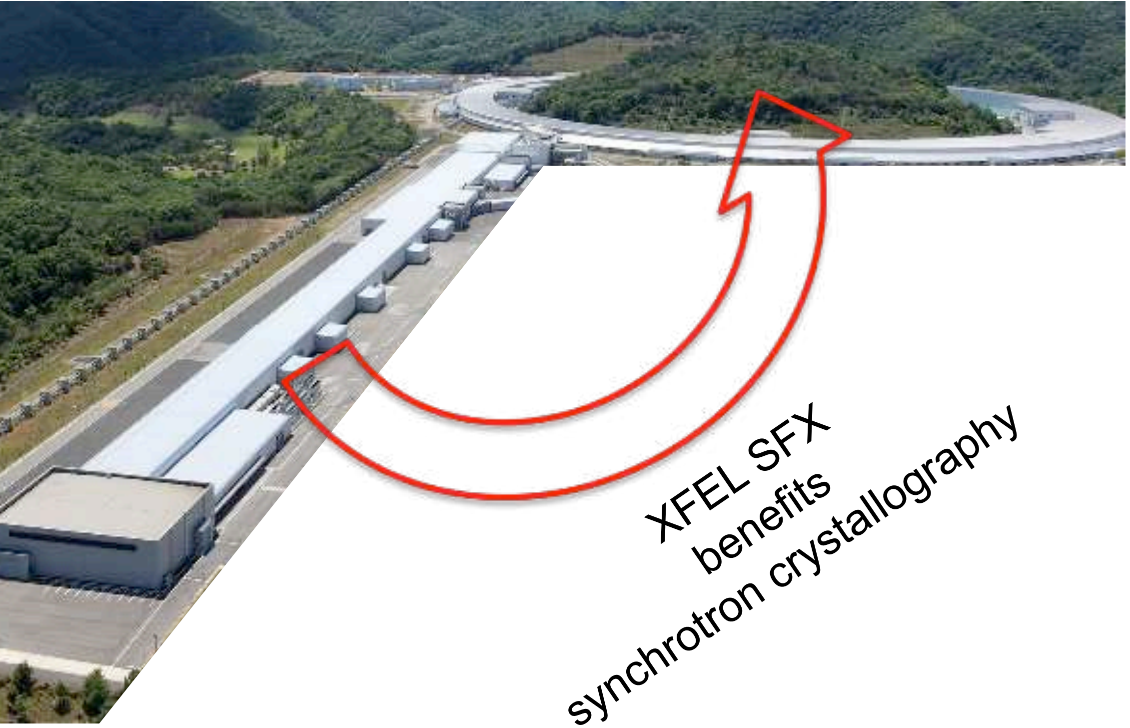


synchrotron crystallography
benefits
XFEL SFX

Spring-8

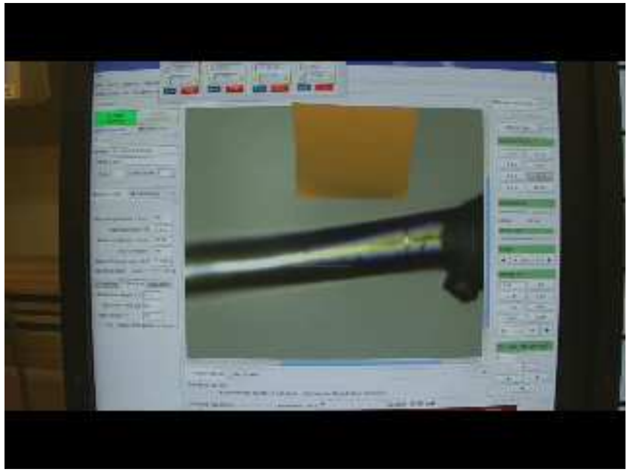
XFEL SFX
benefits
synchrotron crystallography

SACLA



XFEL SFX
benefits
synchrotron crystallography

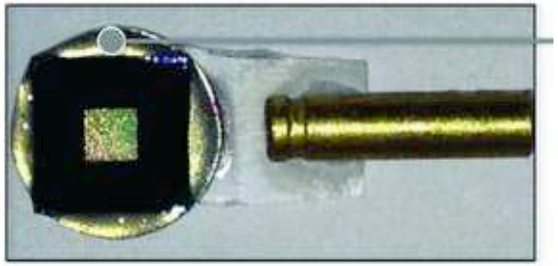
Serial synchrotron crystallography



Slow extrusion injector at SLS

Botha et al. (2015) ActaD71, 387

Gati *et al.* (2014) IUCrJ 1, 78
Stellato *et al.* (2014) IUCrJ 1, 204
Heyman *et al.* (2014) IUCrJ 1, 349
Nogly *et al.* (2015) IUCrJ 2
Botha *et al.* (2015) Acta D71, 387
Coquelle *et al.* (2015) Acta D71, 1184
Roedig *et al.* (2016) J Appl Cryst 40, 968
Huang *et al.* (2016) Acta D72, 93
Jaeger *et al.* (2016) Adv Exp Med Biol 922, 137
Owen *et al.* (2017) Acta D73, 272
Martin-Garcia *et al.* (2017) IUCrJ 4
Beyerlein *et al.* (2017) IUCrJ 4
Weinert *et al.* (2017 Nature Commun 8, 542
Meents *et al.* (2017) Nature Commun 8, 1281



Solid support at ESRF

Coquelle et al. (2015) ActaD71, 1184

Five applications of XFEL SFX that can’t (easily) be done at a synchrotron

- Microcrystals of fragile proteins (membrane proteins, protein complexes, …)
- *in vivo* crystallography
- Room temperature crystallography (preserves conformational heterogeneity)
- Damage-free structures of radiation sensitive proteins (metalloproteins, chromophore-containing proteins, …)
- Time-resolved experiments on ps-fs time scales

Five applications of XFEL SFX
that can't (easily) be done at a synchrotron

- Microcrystals of fragile proteins
- *in vivo* crystallography
- Room temperature crystallography
- Damage-free structures
- Time-resolved experiments

G protein coupled receptors (GPCR) are targeted by 30% of known drugs

Microcrystals of GPCR (5-HT2B) too small for synchrotron crystallography

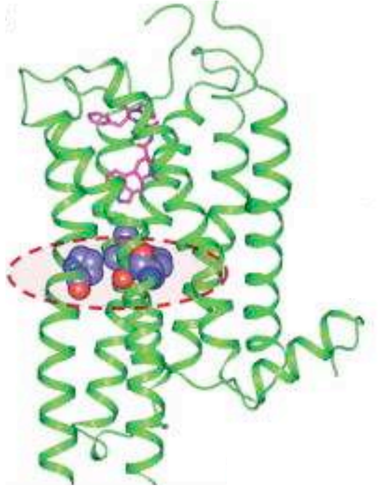
Structure solved by **XFEL SFX**

Liu et al. (2013) Science 342, 1521

6 y ars

(V. Cl ezov)

Structure of GPCR (5-HT2B) solved from **macrocrystals** by **synchrotron** crystallography



Wacker et al. (2013) Science 340, 615

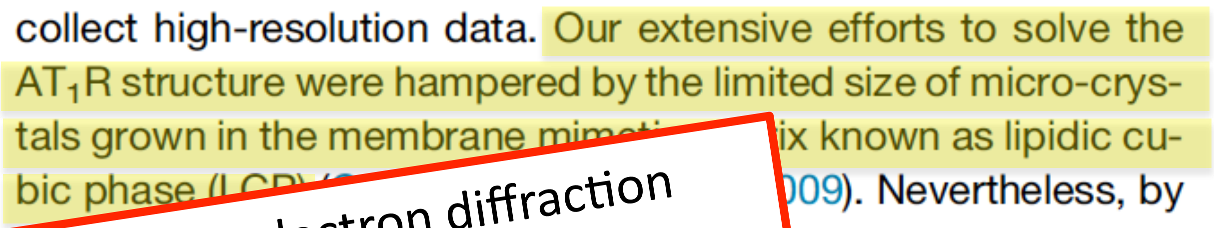

Article

Cell

Structure of the Angiotensin Receptor Revealed by Serial Femtosecond Crystallography

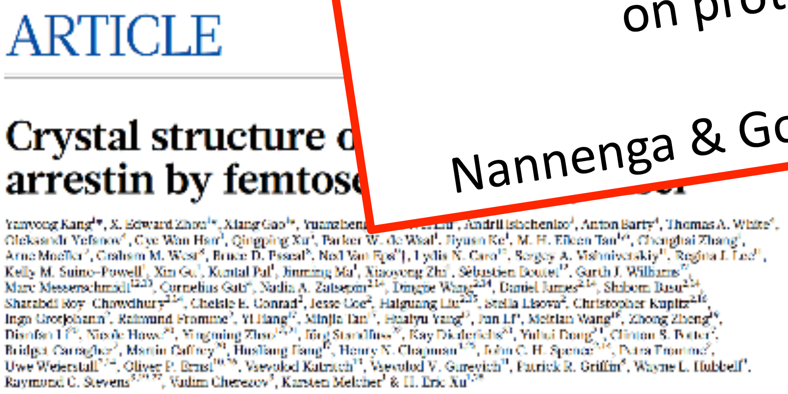
Haitao Zhang,[1] Hamiyet Unal,[2] Cornelius Gati,[3] Gye Won Han,[4] Wei Liu,[5] Nadia A. Zatsepin,[6] Daniel James,[6]
Dingjie Wang,[6] Garrett Nelson,[6] Uwe Weierstall,[6] Michael R. Sawaya,[7] Qingping Xu,[8] Marc Messerschmidt,[9]
Garth J. Williams,[10] Sébastien Boutet,[10] Oleksandr M. Yefanov,[3] Thomas A. White,[3] Chong Wang,[11] Andrii Ishchenko,[4]
Kalyan C. Tirupula,[2] Russell Desnoyer,[2] Jesse Coe,[5] Chelsie E. Conrad,[5] Petra Fromme,[5] Raymond C. Stevens,[1,4,12]
Vsevolod Katritch,[1] Sadashiva S. Karnik,[2] and Vadim Cherezov[4,*]

Zhang *et al.* (2015) Cell 161, 833


collect high-resolution data. Our extensive efforts to solve the AT$_1$R structure were hampered by the limited size of micro-crystals grown in the membrane mimeti ix known as lipidic cubic phase (LCP) (C 009). Nevertheless, by

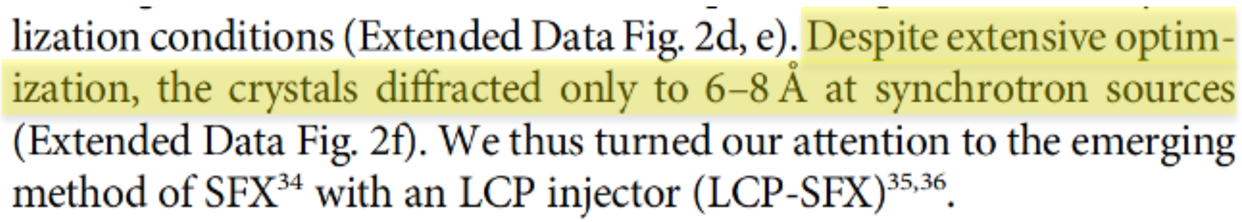
Overlap with micro-electron diffraction
on protein nanocrystals

Nannenga & Gonen (2016) COSB 40, 128


ARTICLE

Crystal structure o arrestin by femtose

Kang *et al.* (2015) Nature 523, 561


lization conditions (Extended Data Fig. 2d, e). Despite extensive optimization, the crystals diffracted only to 6–8 Å at synchrotron sources (Extended Data Fig. 2f). We thus turned our attention to the emerging method of SFX[34] with an LCP injector (LCP-SFX)[35,36].

Five applications of XFEL SFX that can't (easily) be done at a synchrotron

- Microcrystals of fragile proteins
- *in vivo* crystallography
- Room temperature crystallography
- Damage-free structures
- Time-resolved experiments

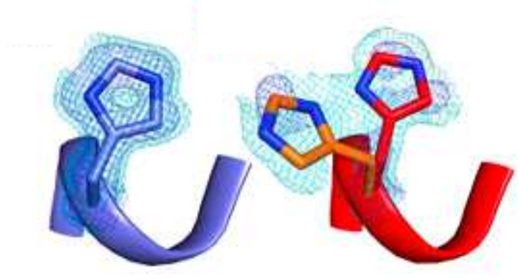
Naturally occurring nanocrystals *in vivo*

Colletier et al. (2016) Nature 539, 43

Five applications of XFEL SFX
that can’t (easily) be done at a synchrotron

- Microcrystals of fragile proteins
- *in vivo* crystallography
- Room temperature crystallography
- Damage-free structures
- Time-resolved experiments

Room temperature crystallography preserves physiological conformational heterogeneity



100 K

Room T

Fraser et al. (2011) PNAS 108, 16247

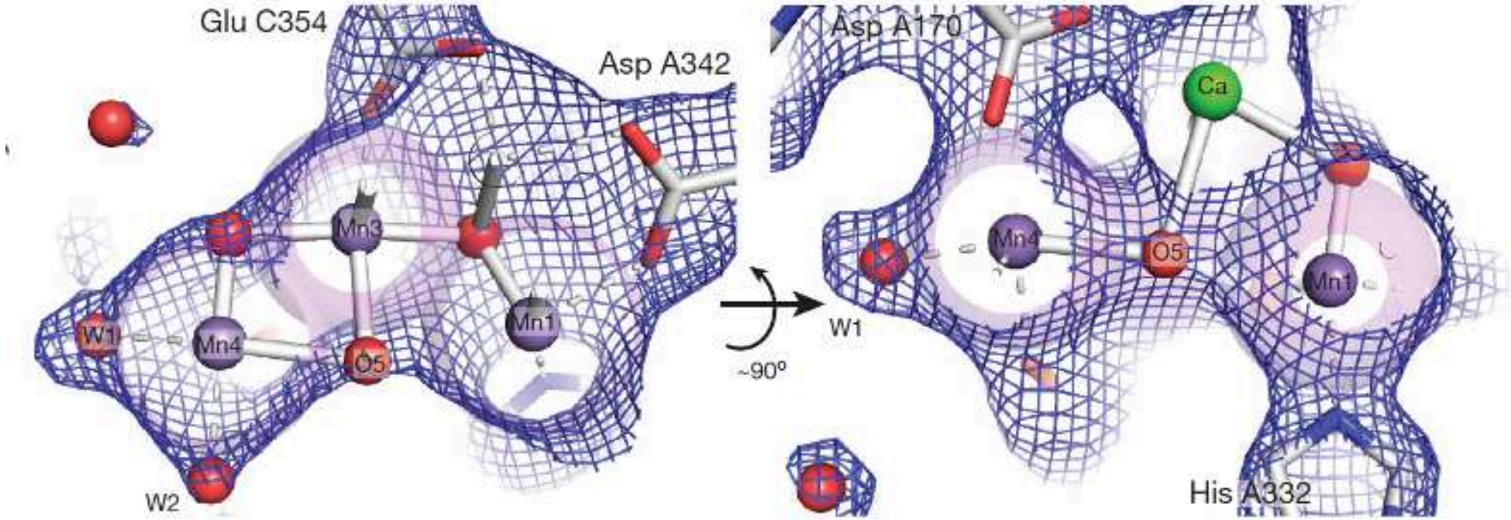
Five applications of XFEL SFX that can't (easily) be done at a synchrotron

- Microcrystals of fragile proteins
- *in vivo* crystallography
- Room temperature crystallography
- Damage-free structures
- Time-resolved experiments

- short XFEL pulse even outruns electronic damage
- good news for radiation sensitive proteins

(metalloproteins, chromophore containing, …)

Mn_4CaO_5 cluster of crystalline photosystem II retains intact electronic structure



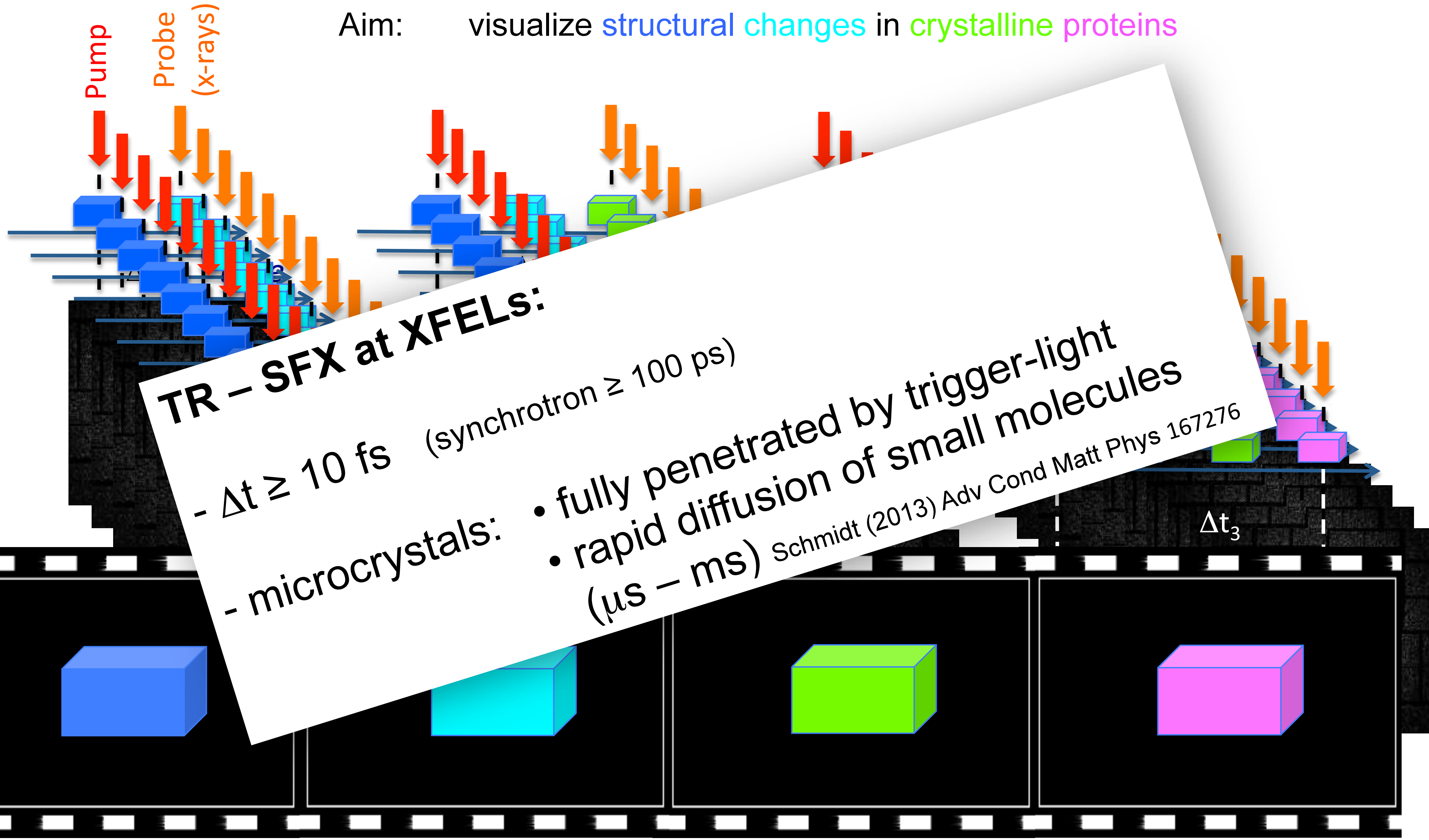
Young et al. (2016) Nature

Five applications of XFEL SFX that can’t (easily) be done at a synchrotron

- Microcrystals of fragile proteins
- *in vivo* crystallography
- Room temperature crystallography
- Damage-free structures
- Time-resolved experiments

Time-resolved crystallography: pump-probe

Aim: visualize structural changes in crystalline proteins

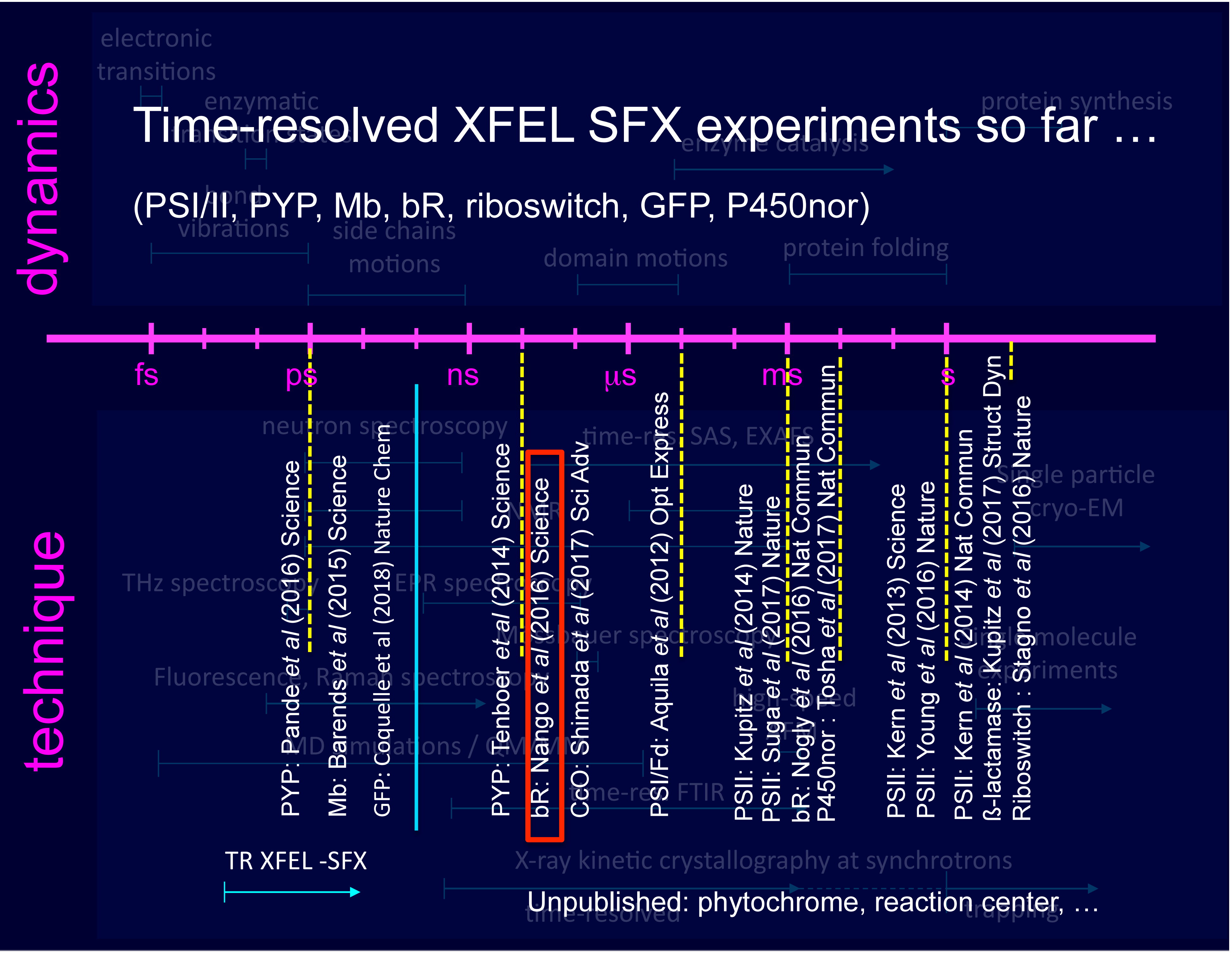


TR – SFX at XFELs:

- Δt ≥ 10 fs (synchrotron ≥ 100 ps)
- microcrystals:
  - fully penetrated by trigger-light
  - rapid diffusion of small molecules (μs – ms) Schmidt (2013) Adv Cond Matt Phys 167276

Time-resolved XFEL SFX experiments so far …

(PSI/II, PYP, Mb, bR, riboswitch, GFP, P450nor)



PYP: Pande *et al* (2016) Science

Mb: Barends *et al* (2015) Science

GFP: Coquelle et al (2018) Nature Chem

PYP: Tenboer *et al* (2014) Science

bR: Nango *et al* (2016) Science

CcO: Shimada *et al* (2017) Sci Adv

PSI/Fd: Aquila *et al* (2012) Opt Express

PSII: Kupitz *et al* (2014) Nature

PSII: Suga *et al* (2017) Nature

bR: Nogly *et al* (2016) Nat Commun

P450nor : Tosha *et al* (2017) Nat Commun

PSII: Kern *et al* (2013) Science

PSII: Young *et al* (2016) Nature

PSII: Kern *et al* (2014) Nat Commun

ß-lactamase: Kupitz *et al* (2017) Struct Dyn

Riboswitch : Stagno *et al* (2016) Nature

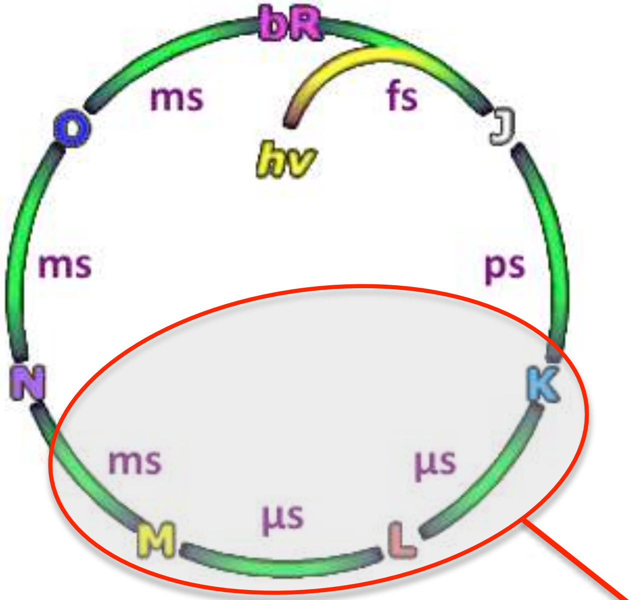
Unpublished: phytochrome, reaction center, …

A three-dimensional movie of structural changes in bacteriorhodopsin

Nango, Royant, Kubo, …., Neutze, Iwata (2016) *Science* **354**, 1552

- Bacteriorhodopsin = proton pump
- Diffraction data recorded at the XFEL SACLA in Japan, with LCP injector (1 mg sample / data set)

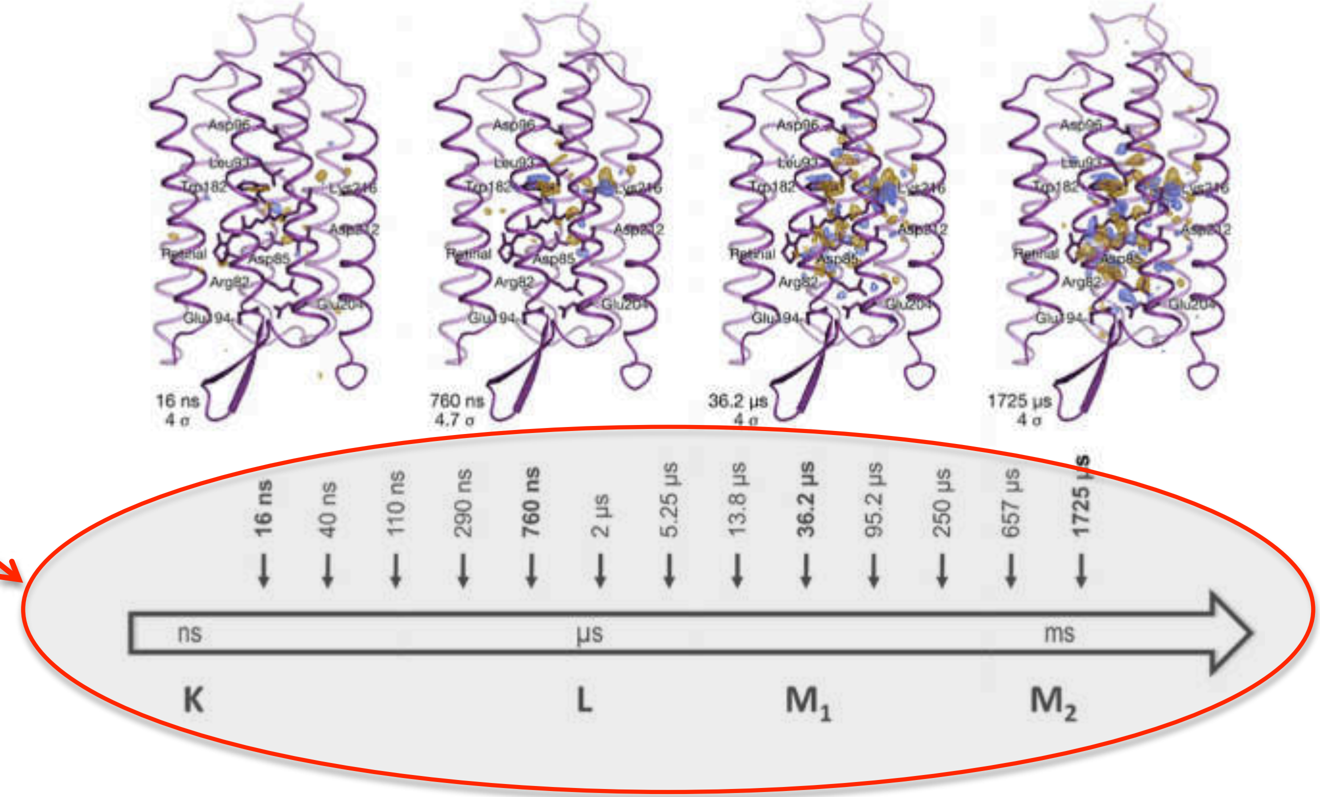
The photocycle



Courtesy Antoine Royant

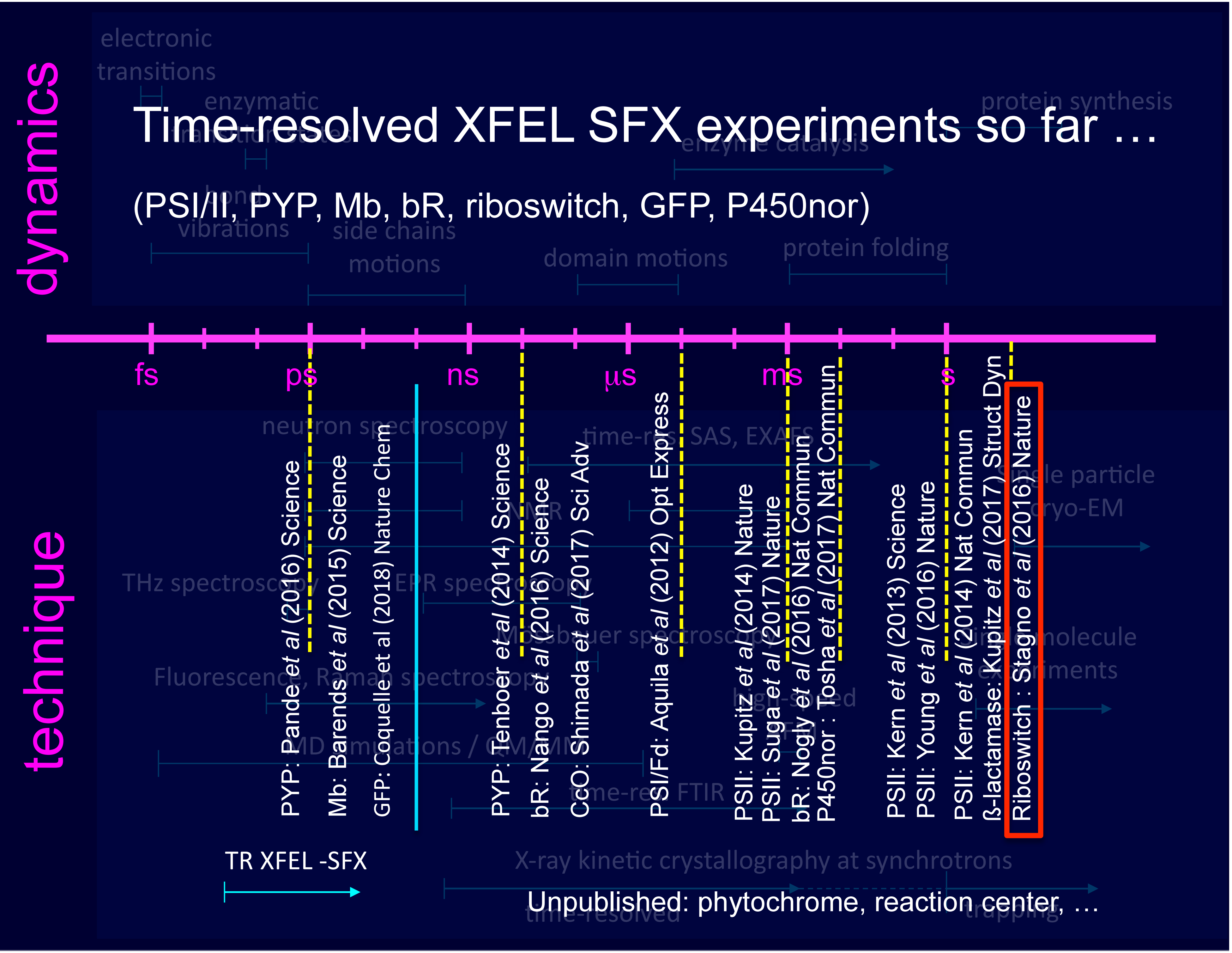
Diffraction data at 2.1 Å resolution on 13 time points

16 ns -> 1.7 ms (5 orders of time magnitude)



Time-resolved XFEL SFX experiments so far …

(PSI/II, PYP, Mb, bR, riboswitch, GFP, P450nor)



PYP: Pande *et al* (2016) Science

Mb: Barends *et al* (2015) Science

GFP: Coquelle et al (2018) Nature Chem

PYP: Tenboer *et al* (2014) Science

bR: Nango *et al* (2016) Science

CcO: Shimada *et al* (2017) Sci Adv

PSI/Fd: Aquila *et al* (2012) Opt Express

PSII: Kupitz *et al* (2014) Nature

PSII: Suga *et al* (2017) Nature

bR: Nogly *et al* (2016) Nat Commun

P450nor : Tosha *et al* (2017) Nat Commun

PSII: Kern *et al* (2013) Science

PSII: Young *et al* (2016) Nature

PSII: Kern *et al* (2014) Nat Commun

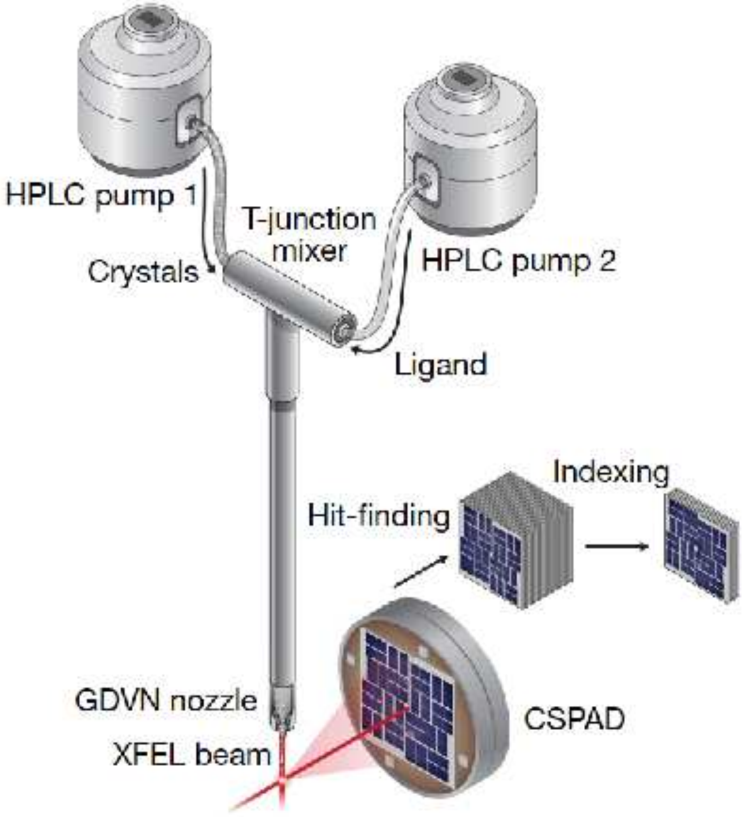
ß-lactamase: Kupitz *et al* (2017) Struct Dyn

Riboswitch : Stagno *et al* (2016) Nature

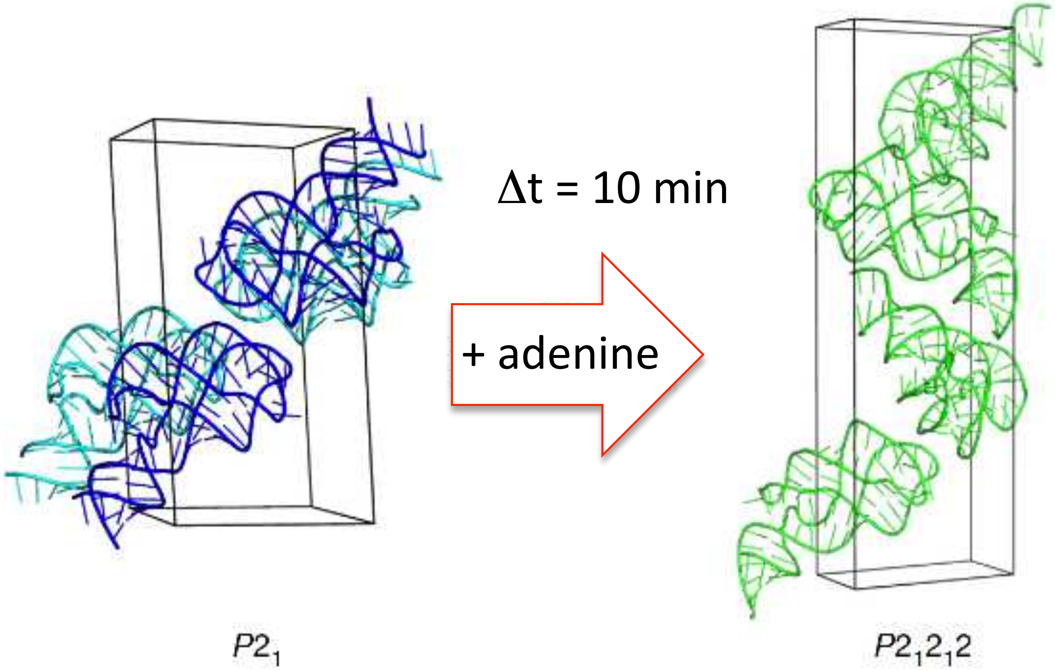
Unpublished: phytochrome, reaction center, …

Structures of riboswitch RNA reaction states by mix-and-inject XFEL serial crystallography

J. R. Stagno[1], Y. Liu[1], Y. R. Bhandari[1], C. E. Conrad[2,3], S. Panja[4], M. Swain[1], L. Fan[5], G. Nelson[6], C. Li[6], D. R. Wendel[1],
T. A. White[7], J. D. Coe[2,3], M. O. Wiedorn[7,8], J. Knoska[7,8], D. Oberthuer[7], R. A. Tuckey[1], P. Yu[1], M. Dyba[1], S. G. Tarasov[1],
U. Weierstall[3,6], T. D. Grant[9], C. D. Schwieters[10], J. Zhang[11], A. R. Ferré-D'Amaré[12], P. Fromme[2,3], D. E. Draper[13], M. Liang[14],
M. S. Hunter[14], S. Boutet[14], K. Tan[15], X. Zuo[16], X. Ji[17], A. Barty[7], N. A. Zatsepin[3,6], H. N. Chapman[7,8], J. C. H. Spence[3,6],
S. A. Woodson[4] & Y.-X. Wang[1]



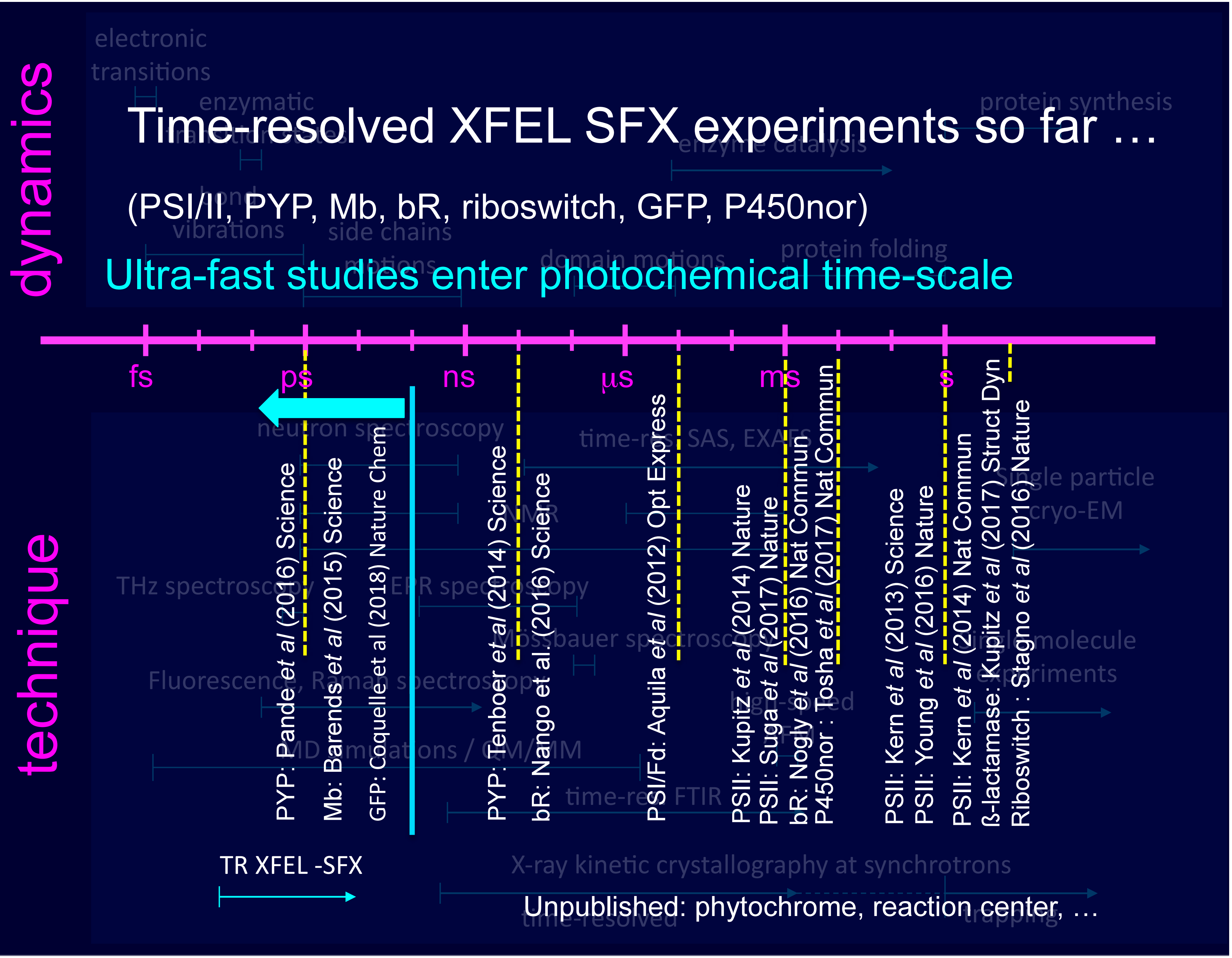
Large conformational changes tolerated by microcrystals (large S/V ratio, small V)



Time-resolved XFEL SFX experiments so far …

(PSI/II, PYP, Mb, bR, riboswitch, GFP, P450nor)

Ultra-fast studies enter photochemical time-scale



PYP: Pande *et al* (2016) Science

Mb: Barends *et al* (2015) Science

GFP: Coquelle et al (2018) Nature Chem

PYP: Tenboer *et al* (2014) Science

bR: Nango et al. (2016) Science

PSI/Fd: Aquila *et al* (2012) Opt Express

PSII: Kupitz *et al* (2014) Nature

PSII: Suga *et al* (2017) Nature

bR: Nogly *et al* (2016) Nat Commun

P450nor : Tosha *et al* (2017) Nat Commun

PSII: Kern *et al* (2013) Science

PSII: Young *et al* (2016) Nature

PSII: Kern *et al* (2014) Nat Commun

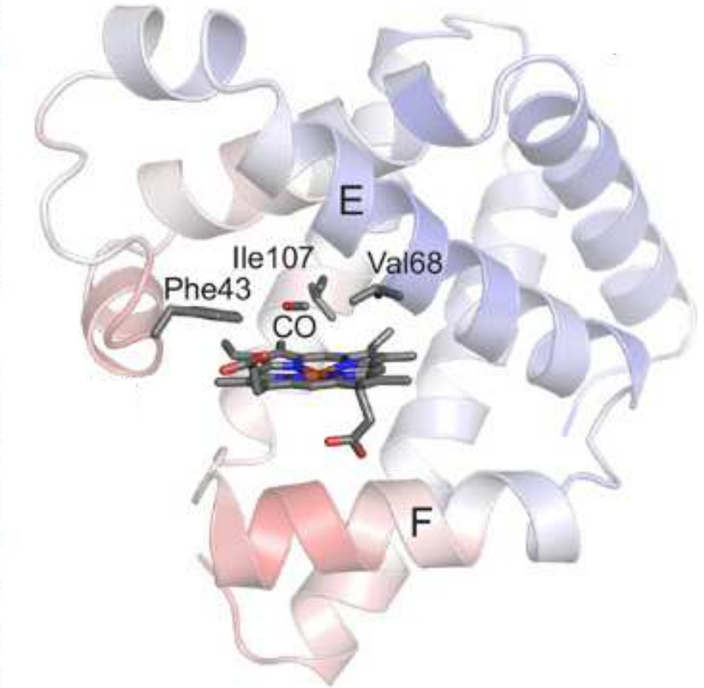
ß-lactamase: Kupitz *et al* (2017) Struct Dyn

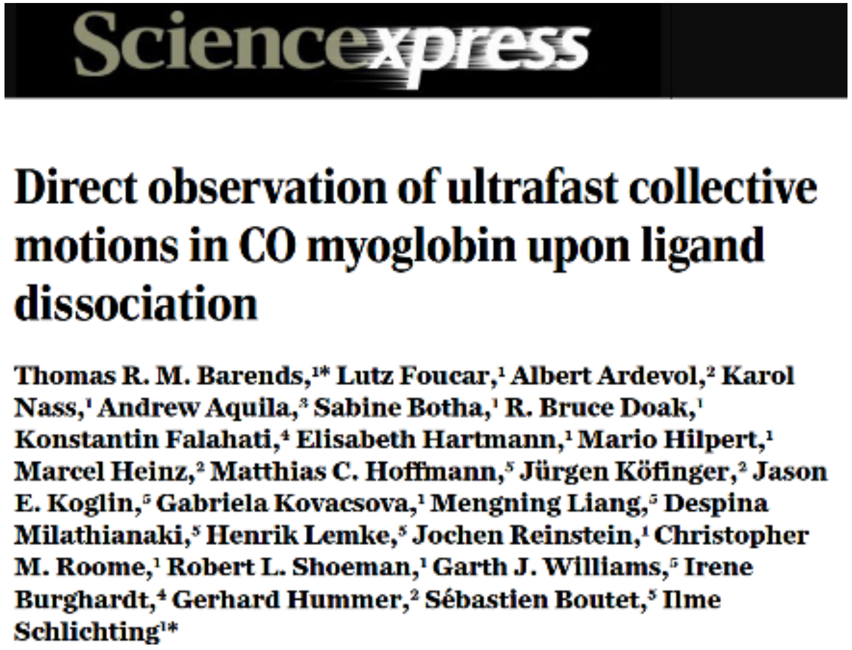
Riboswitch : Stagno *et al* (2016) Nature

Unpublished: phytochrome, reaction center, …

youtube

First sub-ps TR-SFX experiment



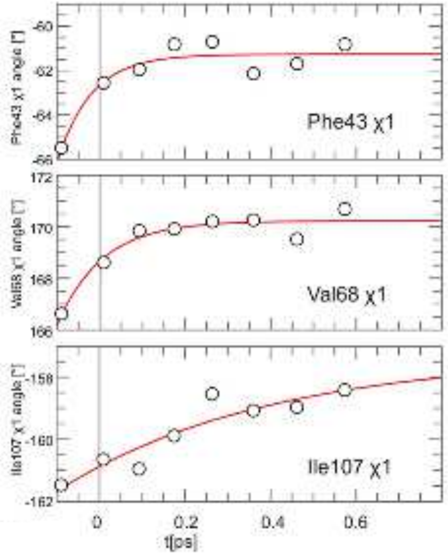


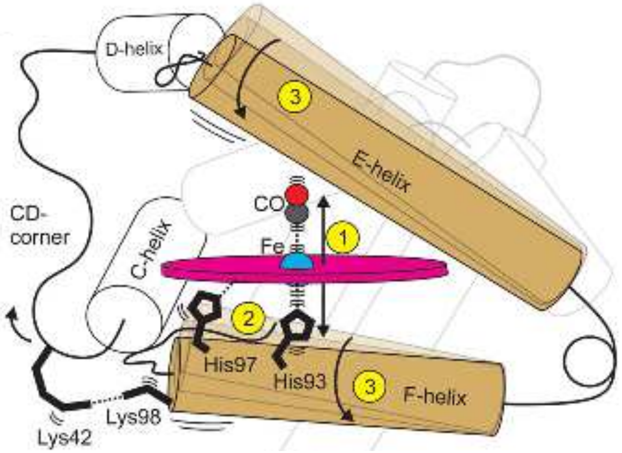
Sciencexpress

Direct observation of ultrafast collective motions in CO myoglobin upon ligand dissociation

Thomas R. M. Barends,[1]* Lutz Foucar,[1] Albert Ardevol,[2] Karol Nass,[1] Andrew Aquila,[3] Sabine Botha,[1] R. Bruce Doak,[1] Konstantin Falahati,[4] Elisabeth Hartmann,[1] Mario Hilpert,[1] Marcel Heinz,[2] Matthias C. Hoffmann,[5] Jürgen Köfinger,[2] Jason E. Koglin,[5] Gabriela Kovacsova,[1] Mengning Liang,[5] Despina Milathianaki,[5] Henrik Lemke,[5] Jochen Reinstein,[1] Christopher M. Roome,[1] Robert L. Shoeman,[1] Garth J. Williams,[5] Irene Burghardt,[4] Gerhard Hummer,[2] Sébastien Boutet,[5] Ilme Schlichting[1]*

Ultrafast local motions …………… couple to ……………… slower, global motions

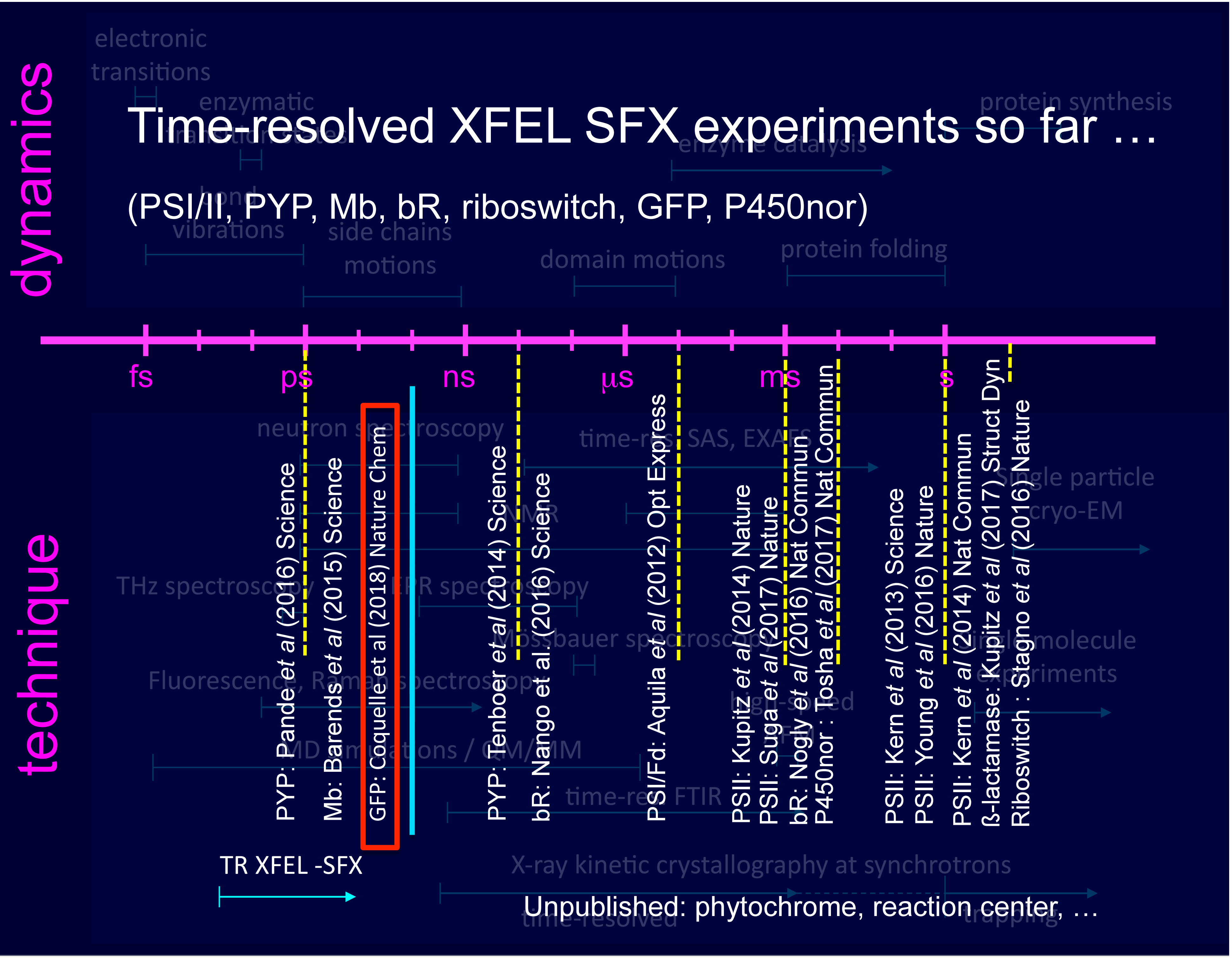




Barends *et al* (2015) Science 350, 445

Time-resolved XFEL SFX experiments so far …

(PSI/II, PYP, Mb, bR, riboswitch, GFP, P450nor)



PYP: Pande *et al* (2016) Science

Mb: Barends *et al* (2015) Science

GFP: Coquelle et al (2018) Nature Chem

PYP: Tenboer *et al* (2014) Science

bR: Nango et al. (2016) Science

PSI/Fd: Aquila *et al* (2012) Opt Express

PSII: Kupitz *et al* (2014) Nature

PSII: Suga *et al* (2017) Nature

bR: Nogly *et al* (2016) Nat Commun

P450nor : Tosha *et al* (2017) Nat Commun

PSII: Kern *et al* (2013) Science

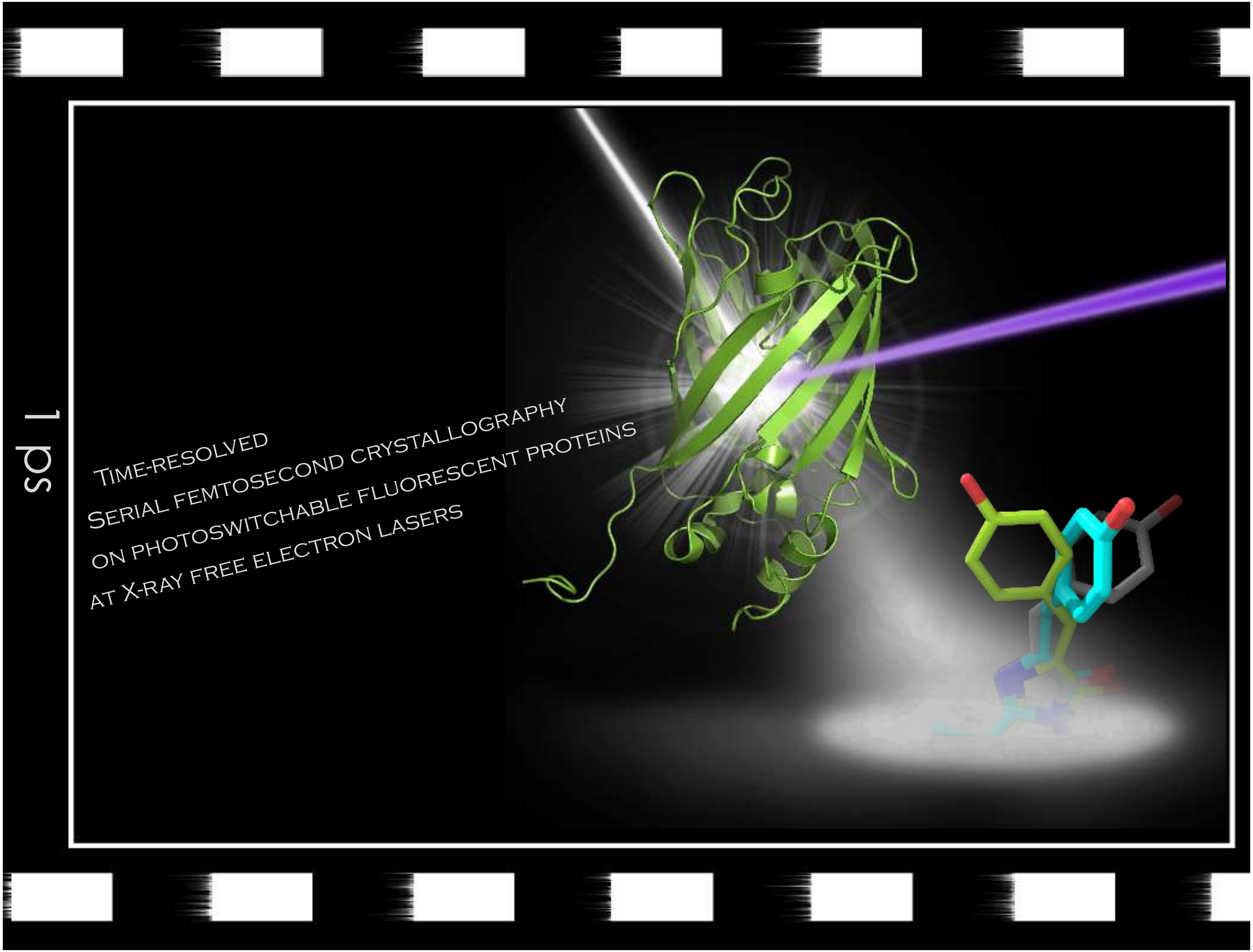
PSII: Young *et al* (2016) Nature

PSII: Kern *et al* (2014) Nat Commun

ß-lactamase: Kupitz *et al* (2017) Struct Dyn

Riboswitch : Stagno *et al* (2016) Nature

Unpublished: phytochrome, reaction center, …



Time-resolved Serial Femtosecond crystallography on photoswitchable fluorescent proteins at X-ray free electron lasers

IBS Grenoble
France

Virgile ADAM
Dominique BOURGEOIS
Martin BYRDIN
Jacques-Philippe COLLETIER
Nicolas COQUELLE
Mikolaj FELIX
Martin FIELD
Franck FIESCHI
Virginia GUILLON
Pauline MACHEBOEUF
Eugenio de la MORA
Hugues NURY
Giorgio SCHIRO
Michel THEPAUT
Martin WEIK
Joyce WOODHOUSE

Rennes University
France

Marco CAMMARATA

U Paris Sud
France

Isabelle DEMACHY
Bernard LEVY
Jacqueline RIDARD

LASIR Lille
France

Michel SLIWA

SLAC

Andy AQUILA
Sébastien_BOUTET
Sergio CARBAJO
Mark HUNTER
Jason KOGLIN
Thomas LANE
Mengning LIANG
Joseph ROBINSON
Matthew SEABERG

MPI Heidelberg
Germany

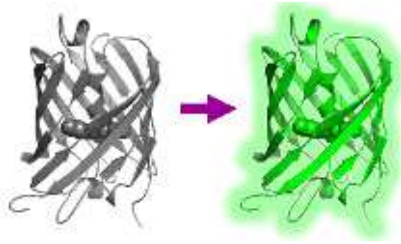
Thomas BARENDS
Bruce DOAK
Lutz FOUCAR
Mario HILPERT
Gabriela KOVACSOVA
Karol NASS
Christopher ROOME
Ilme SCHLICHTING
Robert SHOEMAN

MPI Göttingen
Germany

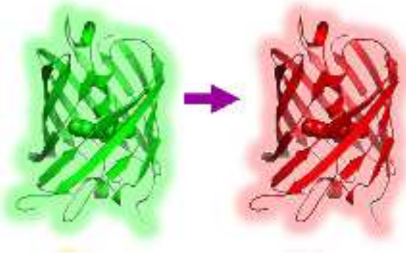
Stefan JAKOBS

Phototransformable
fluorescent proteins (FP)

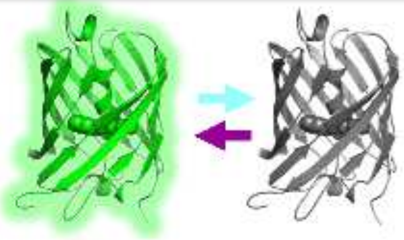
Photoactivatable FP
eg PAGFP



Photoconvertibe FP
eg Kaede, Dendra2



Reversibly switchable FP
eg DRONPA, rsEGFP2, IrisFP



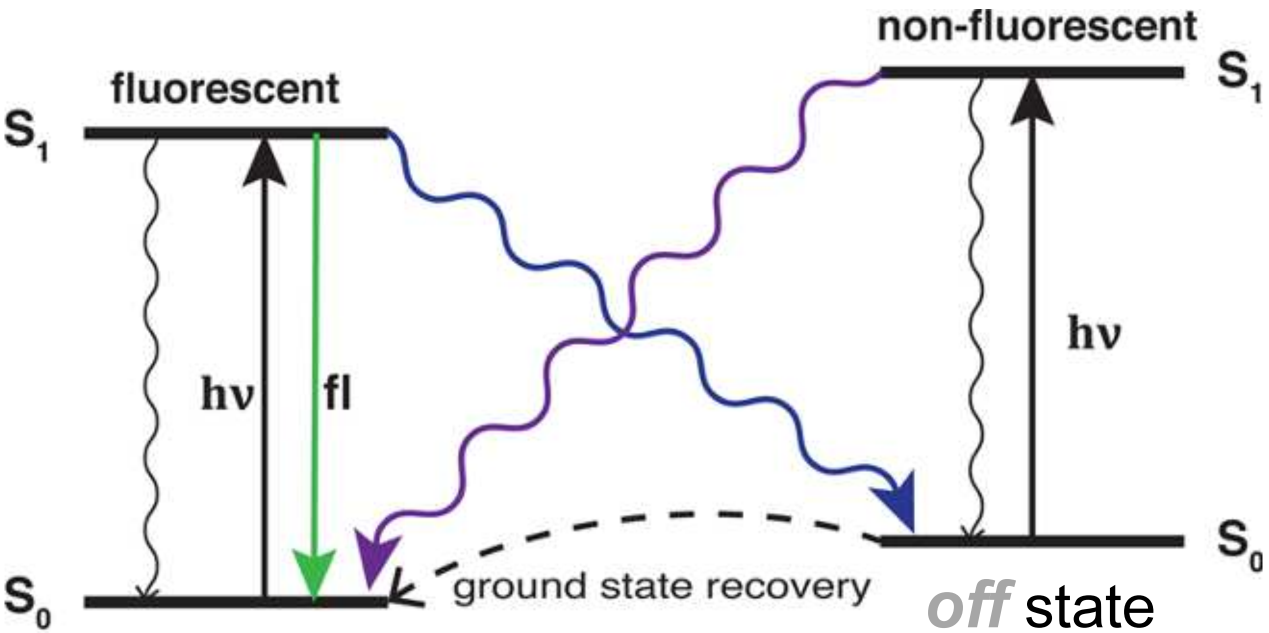
V. Adam

M. Byrdin

D. Bourgeois

Adam (2014) Histochem Cell Biol 142, 19

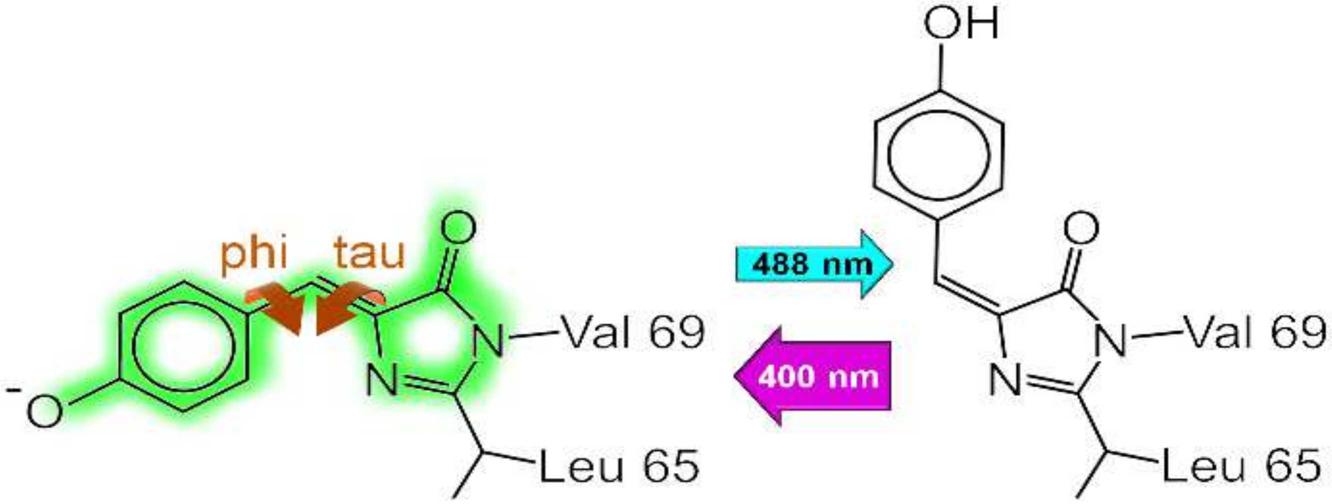
Fluorescent and non-fluorescent forms interconvert *via* excited-state processes



on state
cis anionic

off state
trans neutral

Acharya et al (2017)
Chem Rev 117, 758

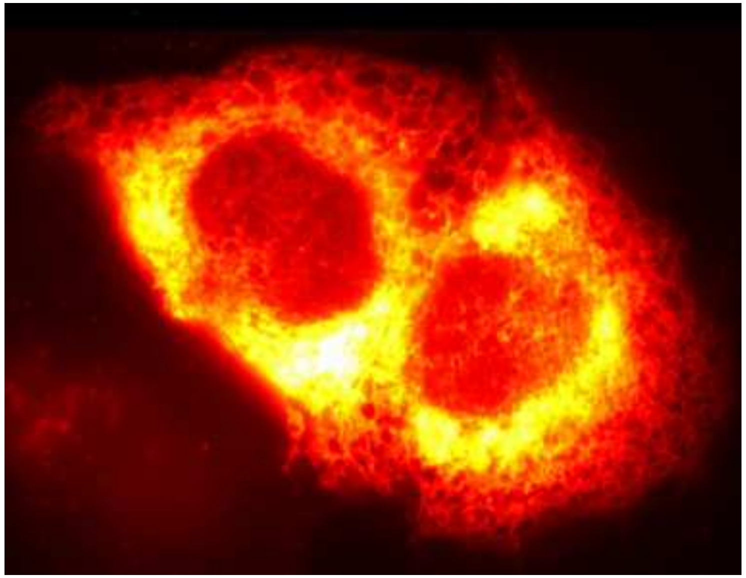


rsEGFP2
Grotjohann et al (2012)
eLife 1:e00248

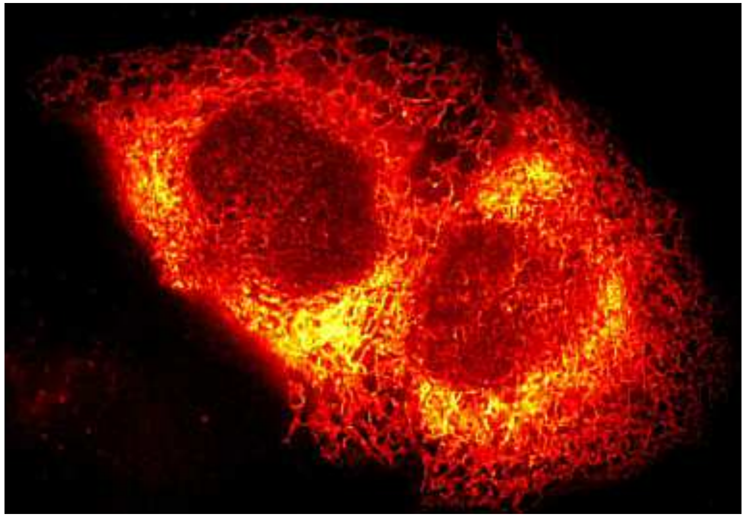
Super-resolution fluorescence microscopy

(2014 Chemistry Nobel Prize to Hell, Betzig, Moerner)

HeLa cell expressing KDEL-rsEGFP2 for visualization of the endoplasmic reticulum



Widefield microscopy
Diffraction limited ($\lambda/2$ = 200 nm)

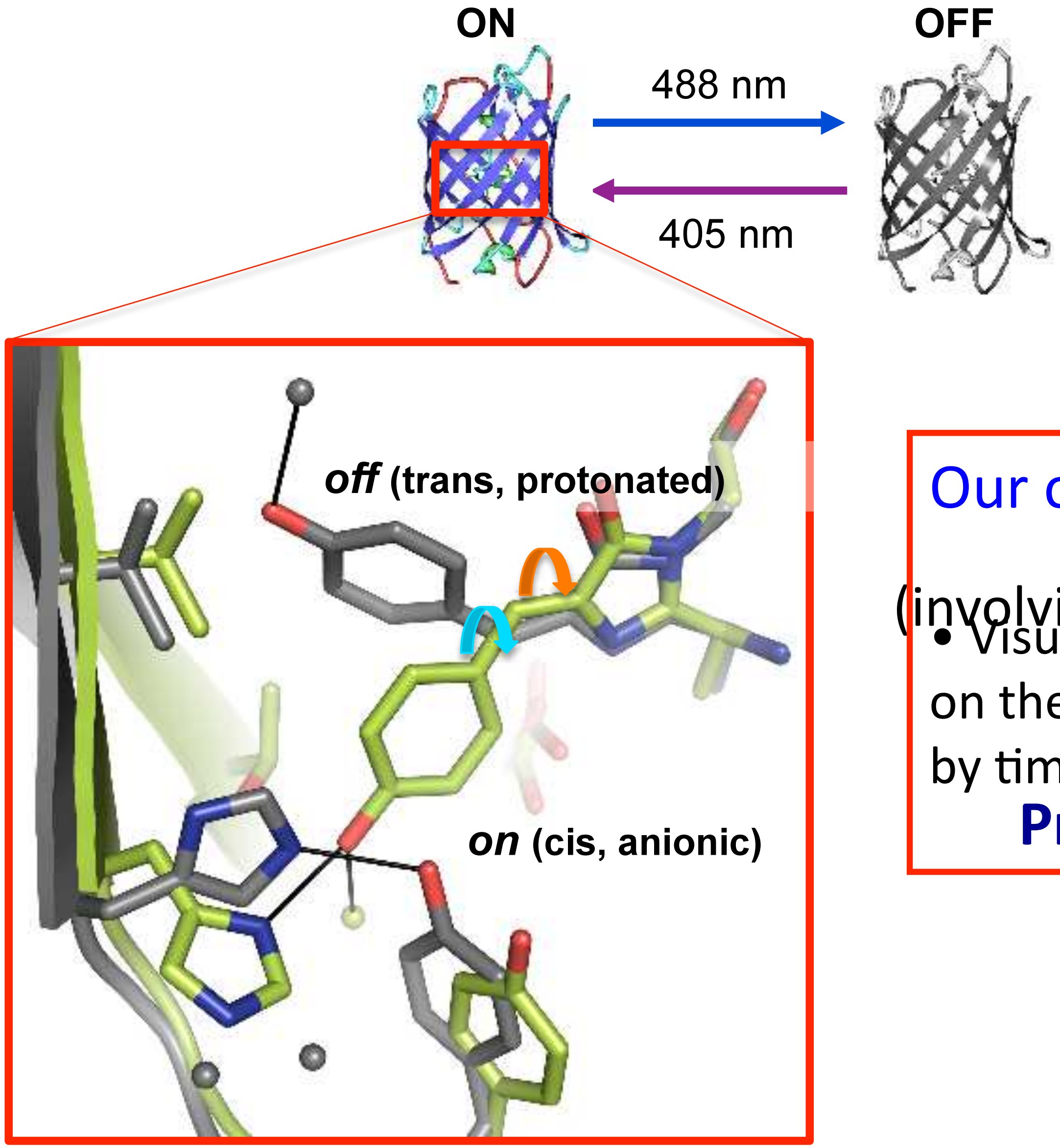


RESOLFT (super-resolution) microscopy
Resolution: 80 nm

Abberior Instruments

Reversibly switchable fluorescent protein rsEGFP2 – molecular marker in RESOLFT nanoscopy

Grotjohann, …, Jakobs (2012) eLife 1:e00248



El-Khatib, Martins, Bourgeois, Colletier, Adam (2016) Scientific Rep 6: 18459

Photoswitching =

Isomerization

(involving changes in *tau* and *phi*)

+

Protonation changes

(of phenol group)

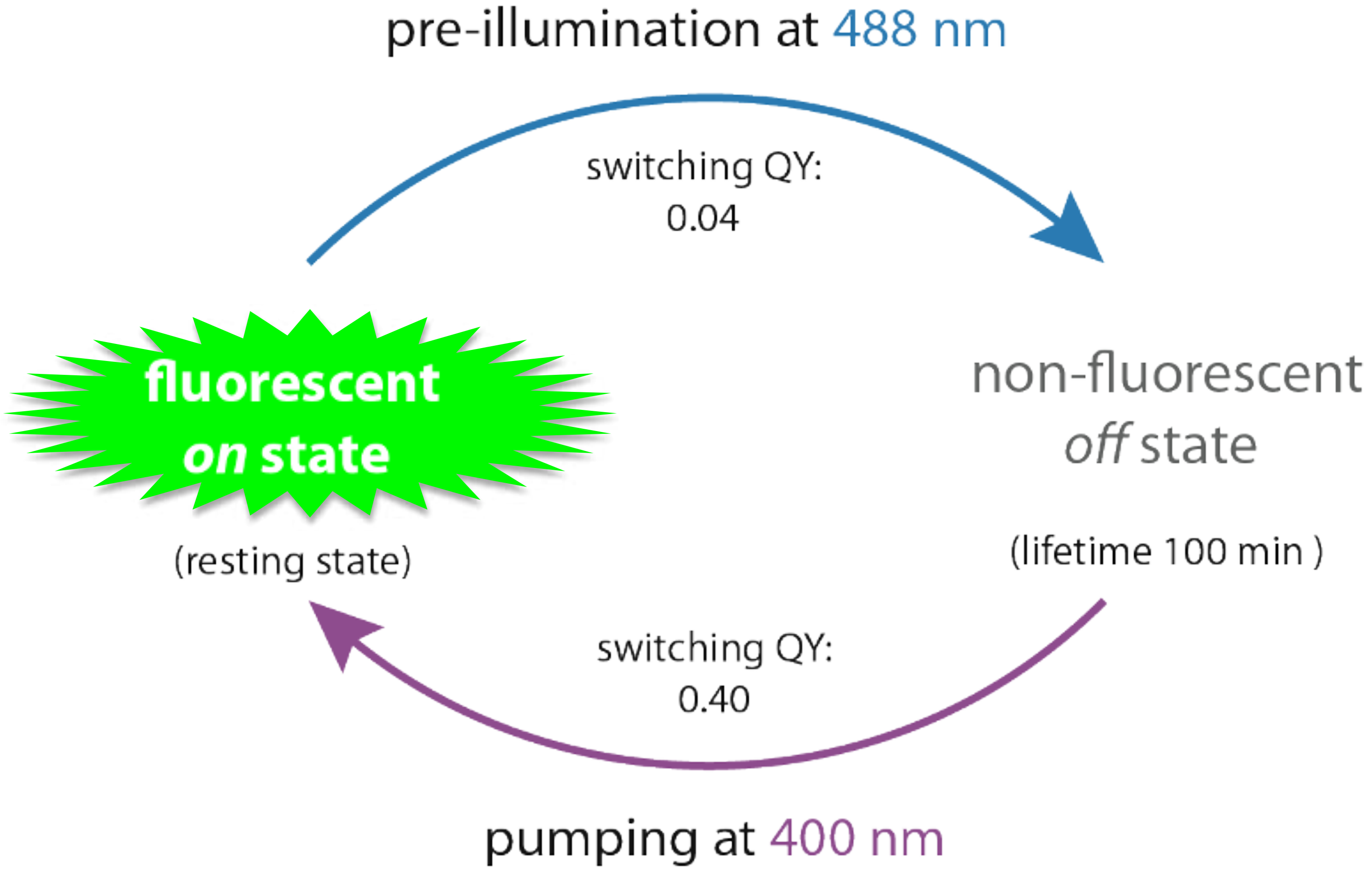
Our objective:

- Visualize photointermediates

on the fs - µs time scale

by time-resolved SFX

In particular: *off* to *on* switching because of high QY



pre-illumination at 488 nm

switching QY:
0.04

fluorescent
***on* state**

(resting state)

non-fluorescent
off state

(lifetime 100 min)

switching QY:
0.40

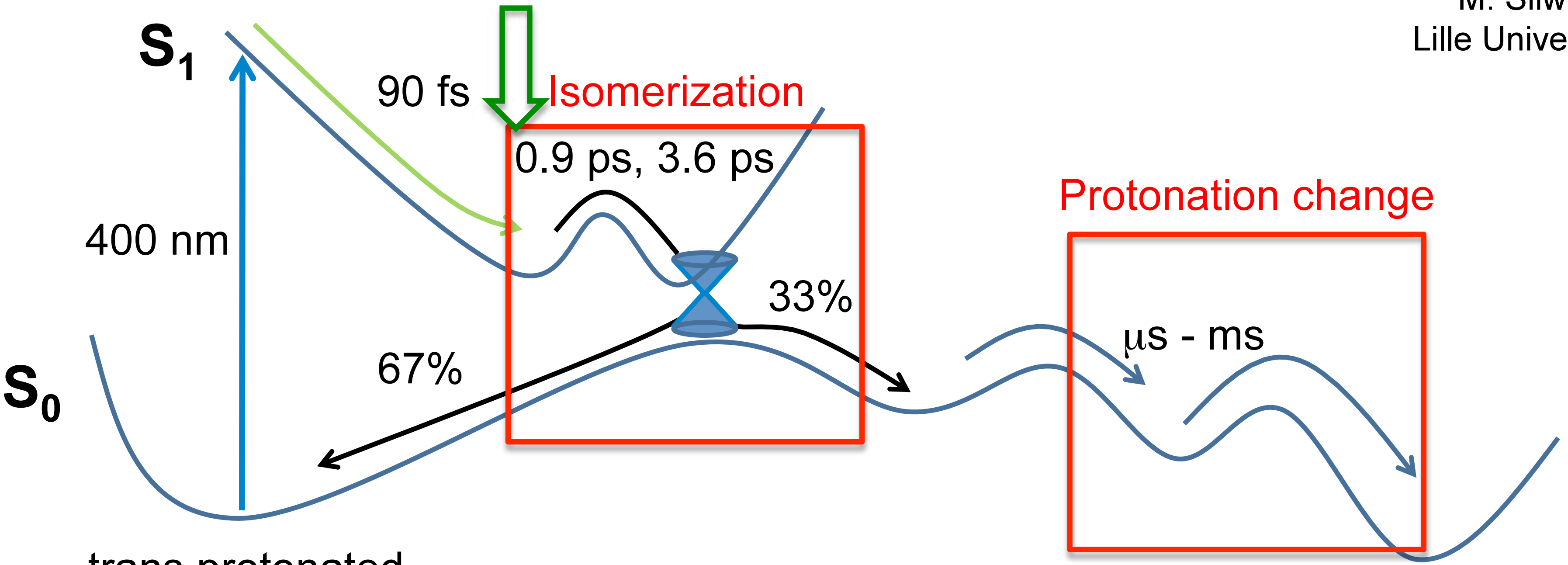
pumping at 400 nm

Time-scale of photoswitching intermediates from time-resolved absorption spectroscopy in **solution**

M. Sliwa
Lille University

Pump-probe delay: 1 ps

Isomerization intermediate ?



trans protonated
off state

cis anionic
on state

LCLS XFEL

May 2015

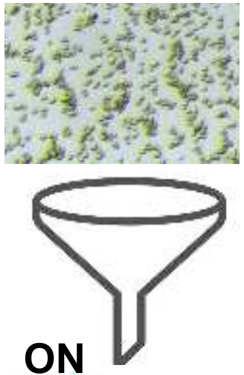
Injection and nozzles


B. Shoeman


G. Kovacsova


B. Doak

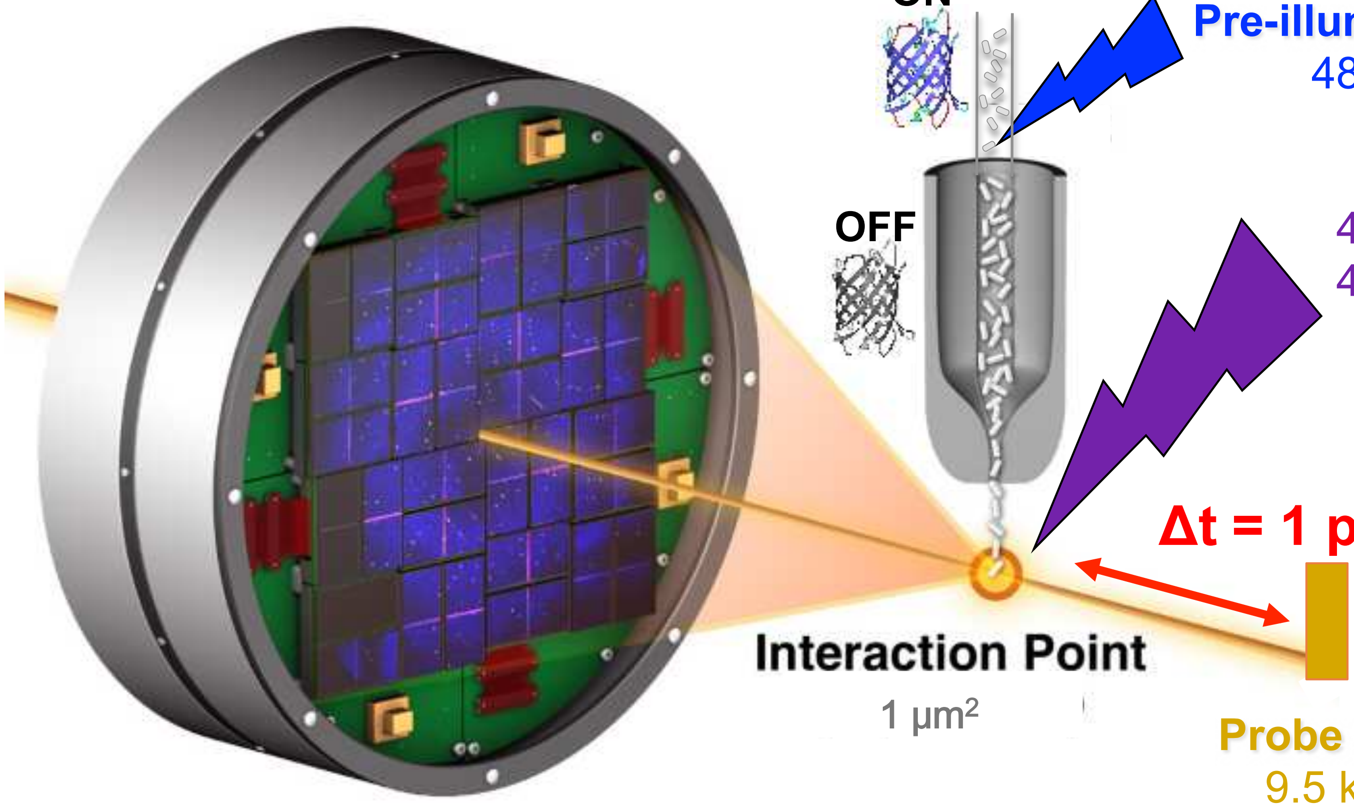
Protein microcrystals




J. Woodhouse


I. Schlichting


G. Schiro
B. Shoeman



Pre-illumination laser
488 nm cw

Pump laser
400 nm, 230 fs,
40 μm x 40 μm;
1.5 μJ/pulse
400 GW cm^{-2}


M. Cammarata

Δt = 1 ps

Interaction Point
1 μm^2

Probe laser
9.5 keV

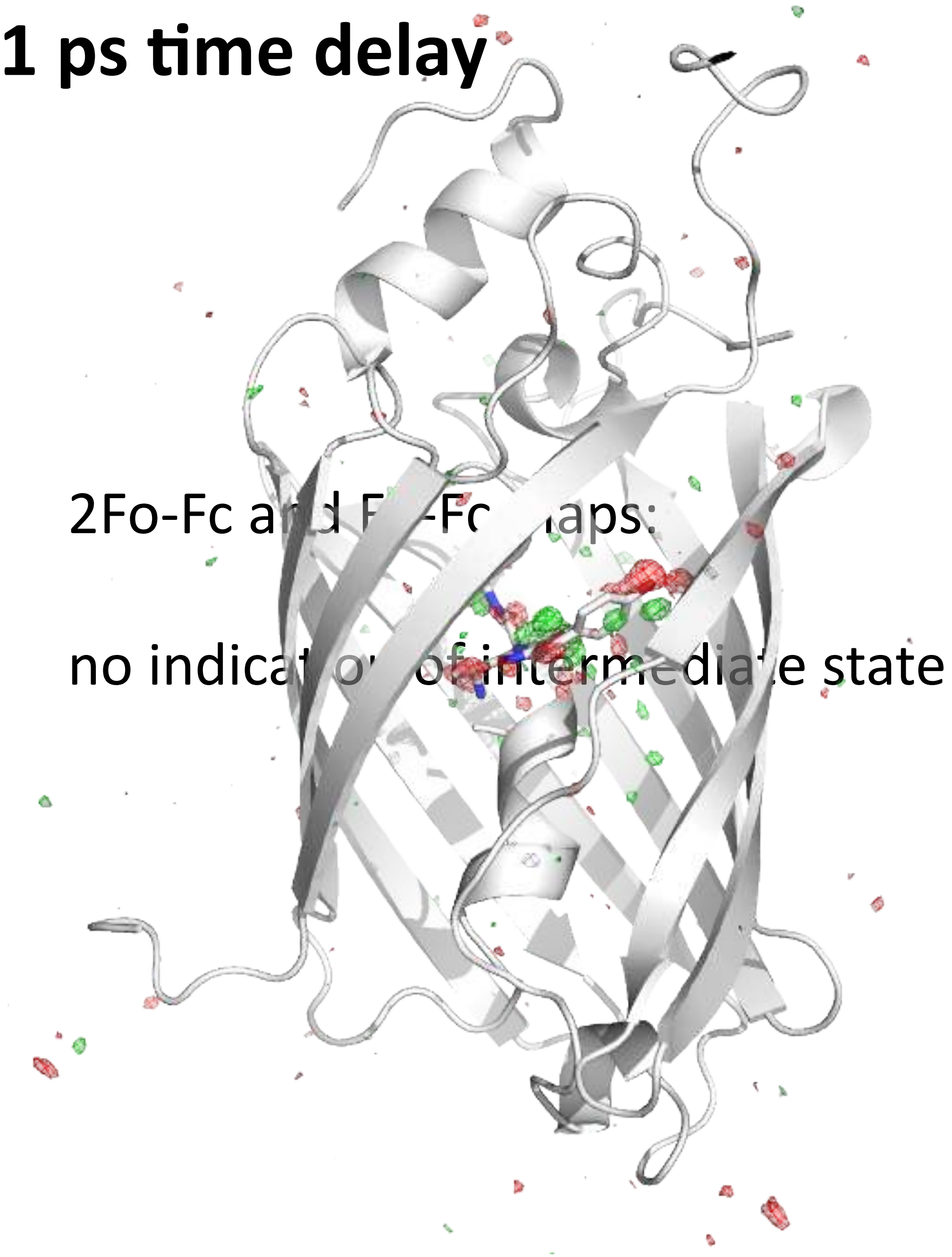
CSPAD detector


S. Boutet


A. Aquila

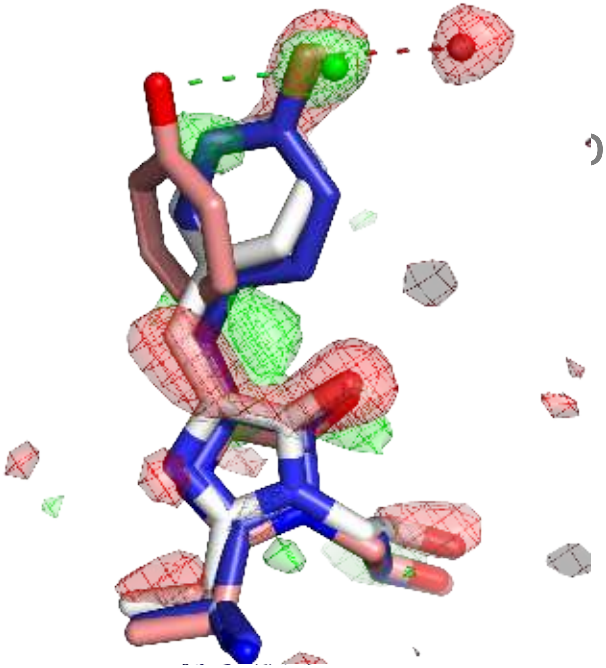
adapted from Boutet, … Schlichting Science **337**:362 (2012)

1 ps time delay



2Fo-Fc a d F -Fc aps:

no indicat o of intermediate state

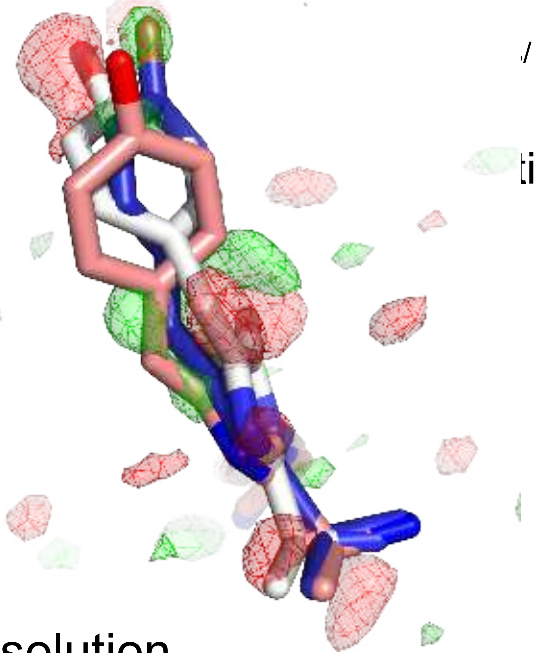


otonated

ns/10% cis

/10% cis (±3 σ)

tion



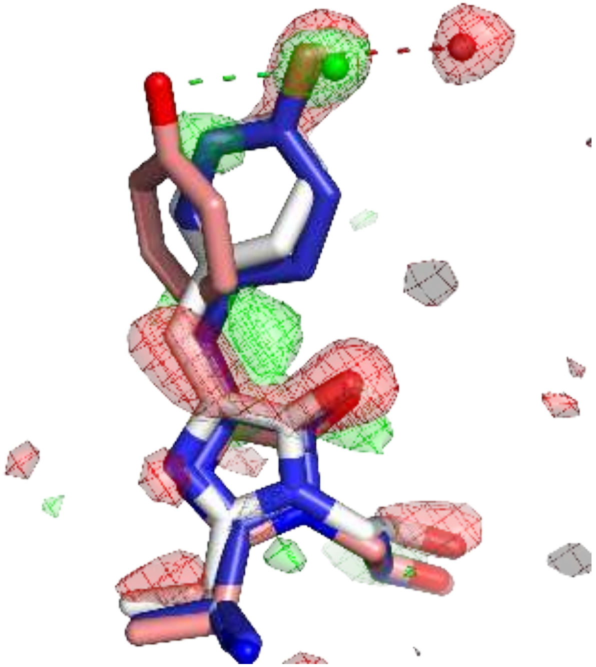
$F_o^{1\ ps} – F_o^{dark}$ (± 3.5 σ), 1.6 Å resolution

1 ps time delay


J.-P. Colletier


D. Bourgeois


T. Barends



Difference refinement:

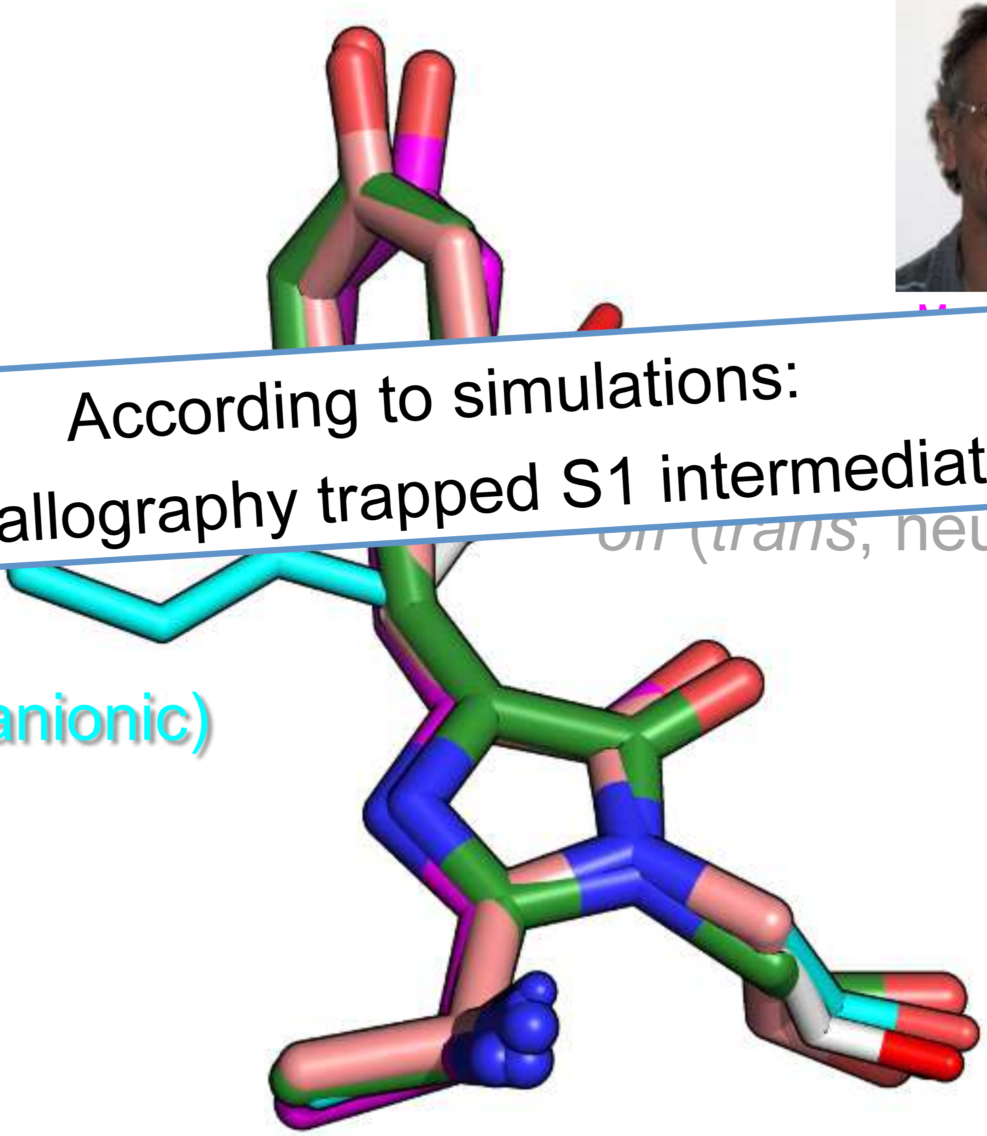
$$F_{extrapolated}^{laser\text{-}on\text{-}\Delta 1ps} = \alpha \; * \; Q/\langle Q\rangle \; * \left(F_{obs}^{laser\text{-}on\text{-}\Delta 1ps} - F_{obs}^{laser\text{-}off}\right) + F_{obs}^{laser\text{-}off}$$

Model 1 and **Model 2** occupied to a total of 7%

Issue: low occupancy of intermediates makes modeling and refinement difficult

1 ps after photoexcitation, chromophore is fully twisted (two rings are perpendicular)

Excited-state chromophore conformations by TR-SFX, excited-state QM/MM and excited–state MD simulations




ield


Isabelle Demachy

According to simulations:
crystallography trapped S1 intermediate

Issue: pump-laser power density was high (400 GW / cm^2; nominally > 1 abs photon/molecule)

Twisted chromophore …

Higher-order excited state ?

Probably not: QM/MM simulated S_2 and S_3 states have no twisted chromophore

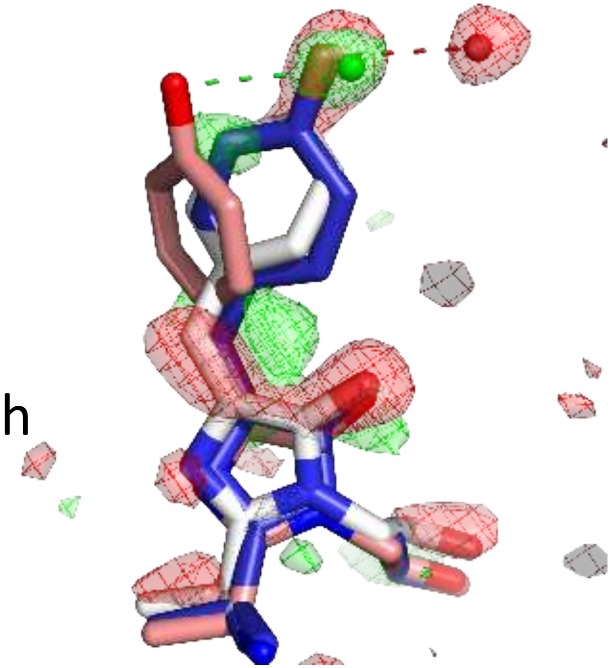
Permanently photobleached state ?

1 ps time delay

Difference Fourier refinement:

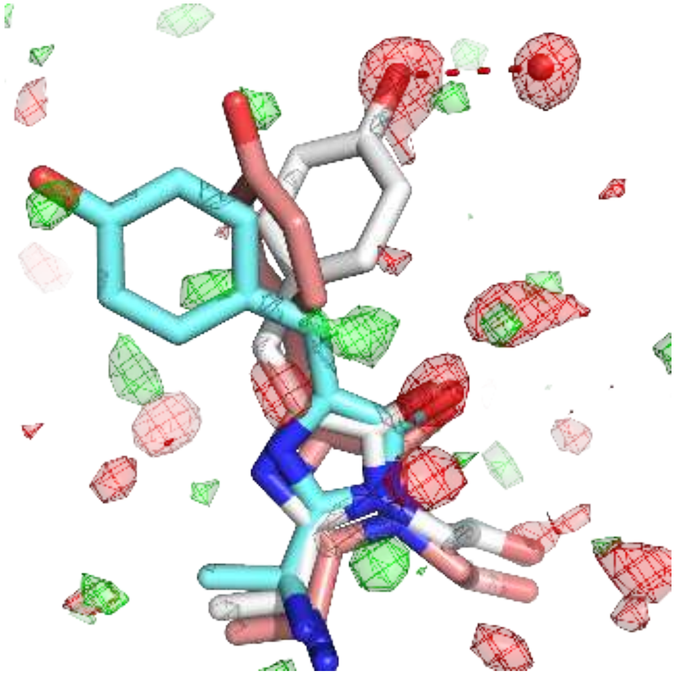
Model 1 and **Model 2** occupied at 50% each

(absolute total occupancy 7 %)



3 ps time delay

Model 2 less occupied, *cis* isomer appears



Issue: pump-laser power density was high (400 GW / cm^2; nominally > 1 abs photon/molecule)

Twisted chromophore …

Higher-order excited state ?

Probably not: QM/MM simulated S_2 and S_3 states have no twisted chromophore

Permanently photobleached state ? Probably not, because :

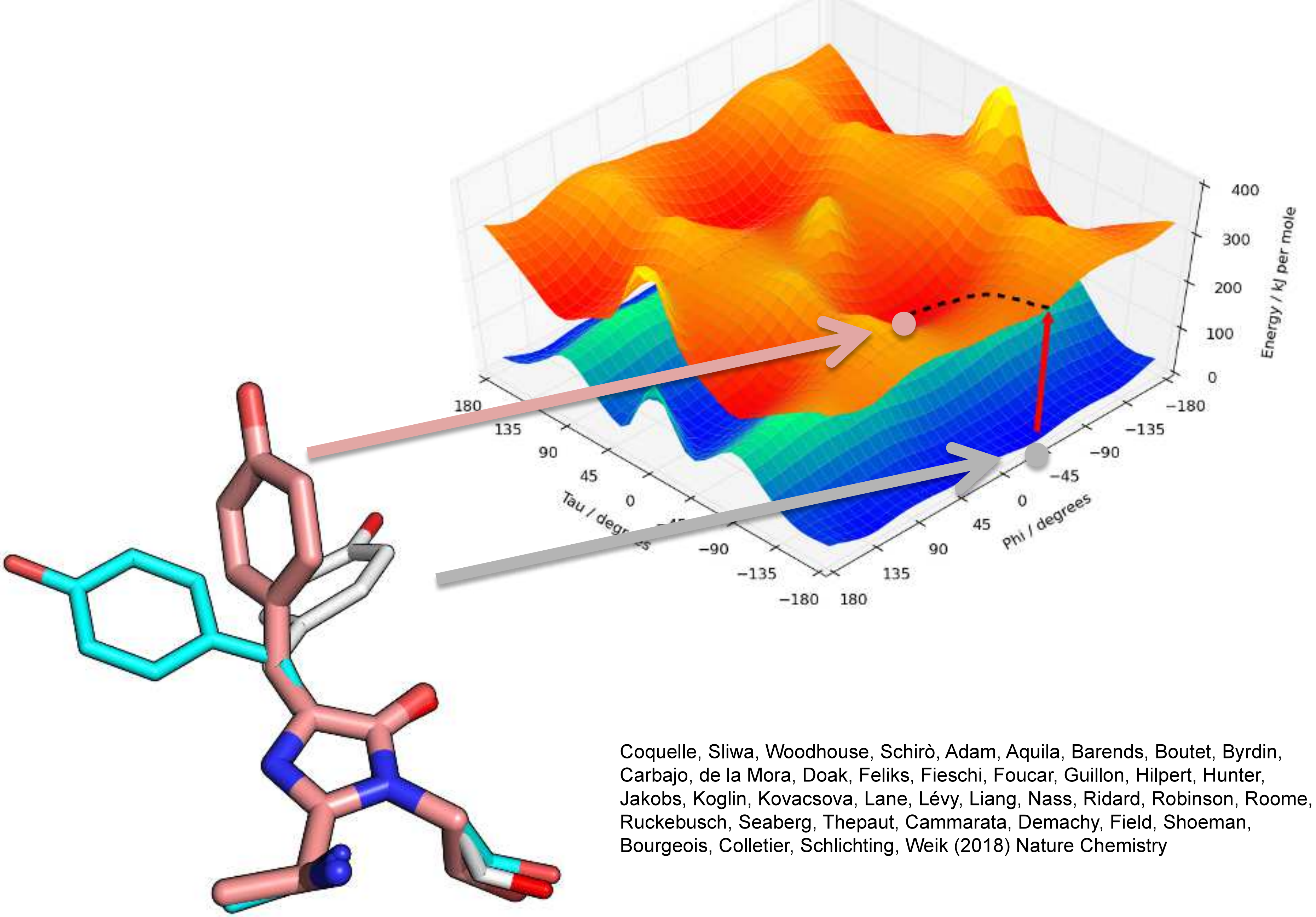
- isomerization proceeds to *cis* on 3 ps time scale
- steady state absorption spectra of rsEGFP2 in solution (M. Cammarata) after fs laser excitation at 400 GW / cm^2 shows switching is reversible

(inspired by Hutchinson et al. (2016) Chem Phys Lett 654, 63)

Radical ?

Probably not: cationic radical forms indeed ≥ 60 GW / cm^2 but is stable up to ns

… most probably S1 excited state isomerization intermediate at or close to conical intersection

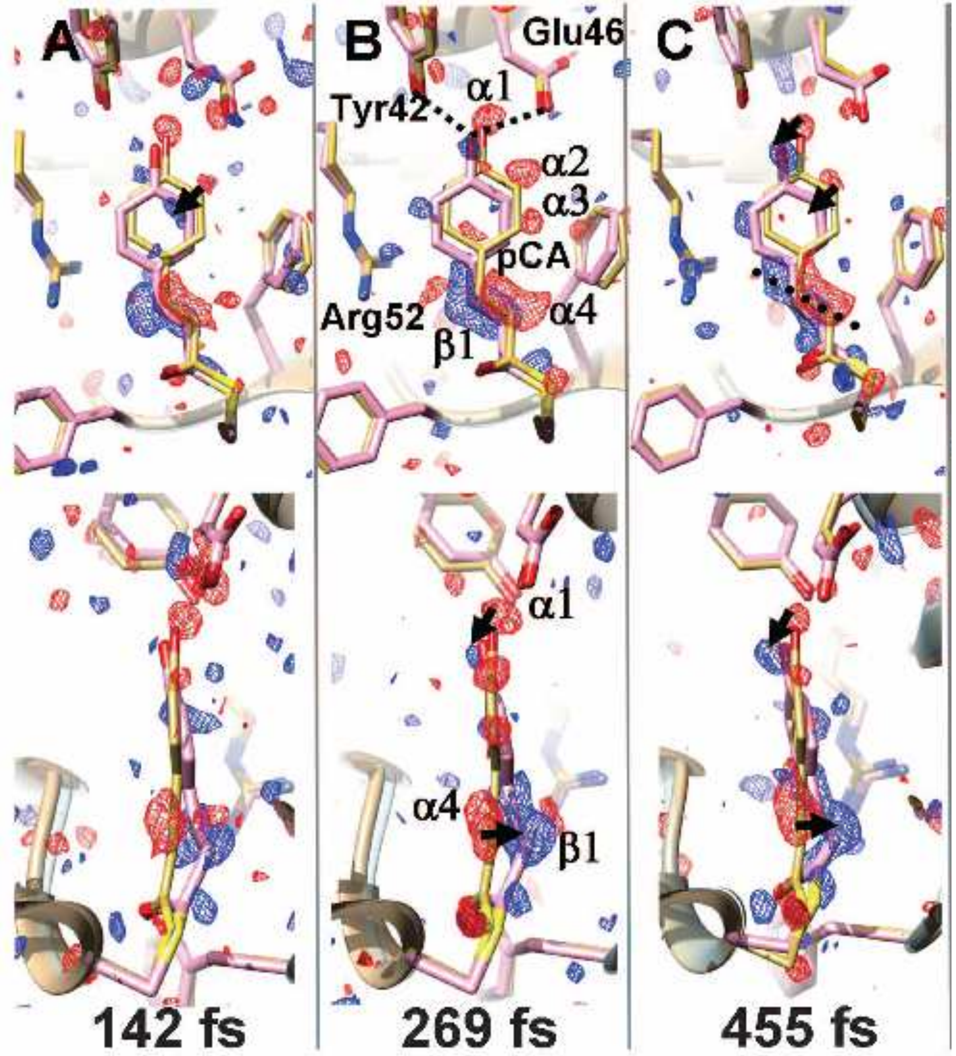


Coquelle, Sliwa, Woodhouse, Schirò, Adam, Aquila, Barends, Boutet, Byrdin, Carbajo, de la Mora, Doak, Feliks, Fieschi, Foucar, Guillon, Hilpert, Hunter, Jakobs, Koglin, Kovacsova, Lane, Lévy, Liang, Nass, Ridard, Robinson, Roome, Ruckebusch, Seaberg, Thepaut, Cammarata, Demachy, Field, Shoeman, Bourgeois, Colletier, Schlichting, Weik (2018) Nature Chemistry

Twisted chromophore: S_1 excited state isomerization intermediate between *trans* and *cis* at conical intersection

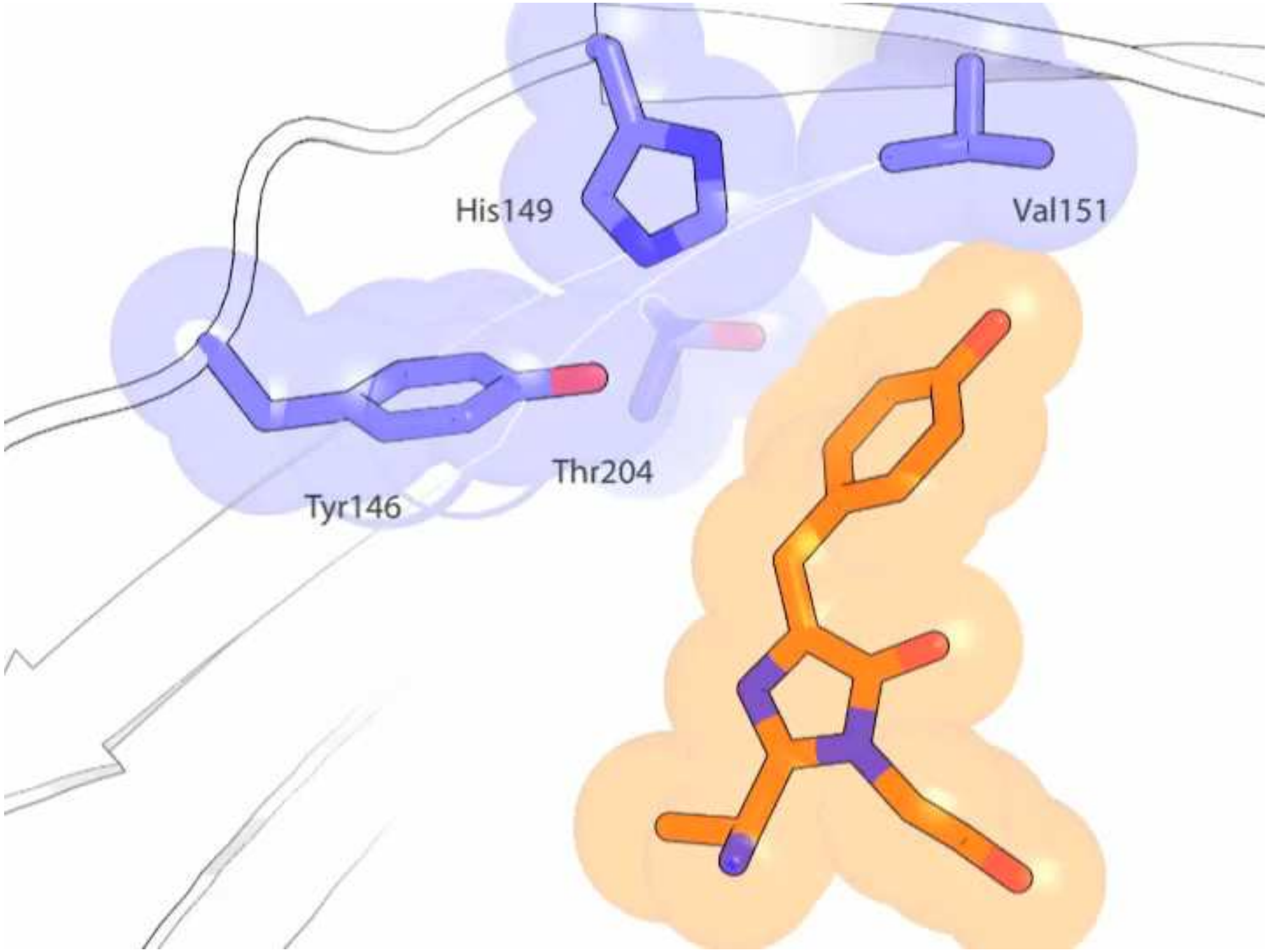
Similar situation in PYP: **Distorted chromophore in excited-state**

Pande, …, van Thor, Schmidt (2016) Science 352, 725



Rational improvement of rsEGFP2 for super-resolution imaging based on excited-state structures ?

Rational improvement of rsEGFP2 for super-resolution imaging based on excited-state structures ?

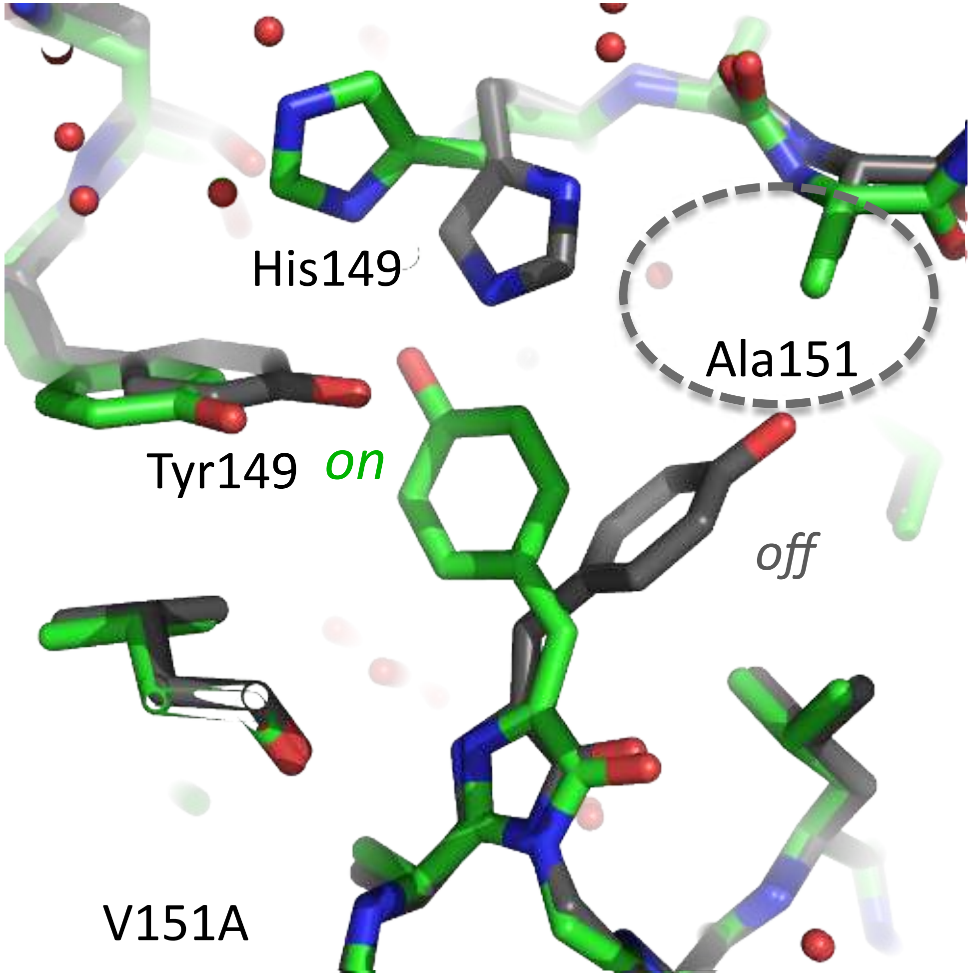


Morph between off- and 1 ps excited-state structure

Coquelle et al (2018) Nature Chemistry 10, 31

Rational improvement of rsEGFP2 for super-resolution imaging based on excited-state structures ?

Photoswitching in rsEGFP2 V151A mutant is facilitated



	WT	**V151A**
on > *off* switching QY:	0.04	0.06
off > *on* switching QY:	0.40	**0.77**

Coquelle et al (2018) Nature Chemistry 10, 31

IBS Grenoble France

Virgile ADAM
Dominique BOURGEOIS
Martin BYRDIN
Jacques-Philippe COLLETIER
Nicolas COQUELLE
Mikolaj FELIX
Martin FIELD
Franck FIESCHI
Virginia GUILLON
Pauline MACHEBOEUF
Eugenio de la MORA
Hugues NURY
Giorgio SCHIRO
Michel THEPAUT
Martin WEIK
Joyce WOODHOUSE

Rennes University France

Marco CAMMARATA

U Paris Sud France

Isabelle DEMACHY
Bernard LEVY
Jacqueline RIDARD

LASIR Lille France

Michel SLIWA

SLAC

Andy AQUILA
Sébastien_BOUTET
Sergio CARBAJO
Mark HUNTER
Jason KOGLIN
Thomas LANE
Mengning LIANG
Joseph ROBINSON
Matthew SEABERG

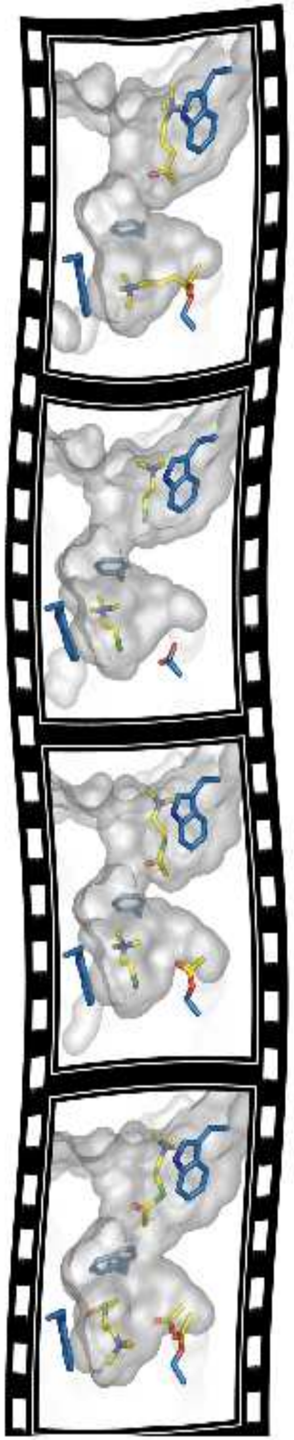
MPI Heidelberg Germany

Thomas BARENDS
Bruce DOAK
Lutz FOUCAR
Mario HILPERT
Gabriela KOVACSOVA
Karol NASS
Christopher ROOME
Ilme SCHLICHTING
Robert SHOEMAN

MPI Göttingen Germany

Stefan JAKOBS

Kinetic crystallography



Aim: obtain structural snapshots of proteins *at work*

cryo-trapping
of intermediates

- data collection at T < RT
- monochromatic X-rays
- synchrotron/home source

time-resolved crystallography
to follow reactions in real time
at room temperature

at synchrotrons

- Laue crystallography
- polychromatic X-rays
- time resolution: 100 ps

at XFELs

- microcrystals
- ‘monochromatic’ X-rays
- time resolution: 10 fs

Near Future: 4th generation synchrotrons

- 100 – 1000 x higher flux
- monochromatic X-rays
- serial or multi-crystal crystallography
- time-resolution: μs - ms