Serial protein crystallography

Sample preparation, model building and biological data interpretation

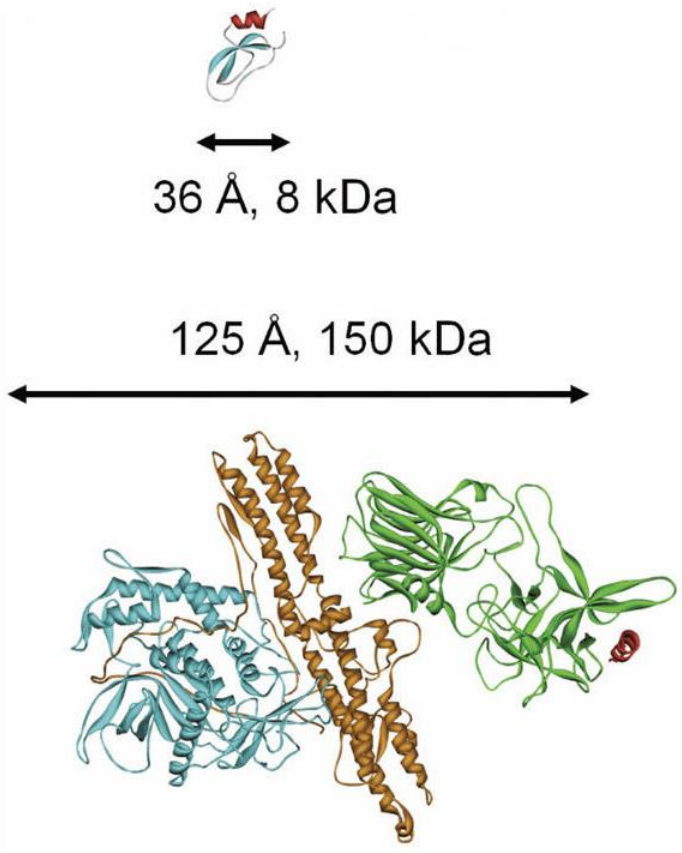
Lars Redecke

Structural Infection Biology applying new Radiation Sources (SIAS)

Joint Laboratory for Structural Biology of Infection and Inflammation
of the Universities Hamburg and Lübeck

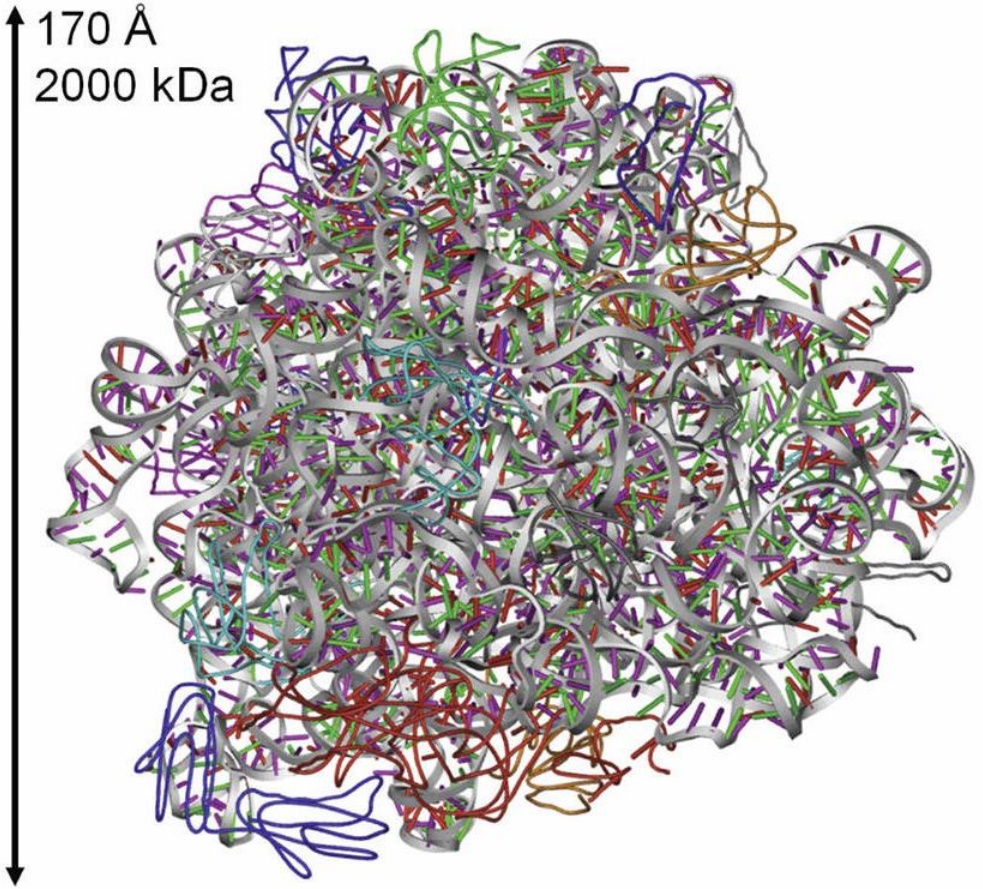
Structure of Biomolecules

Trypsin inhibitor (58 aa)



Botulinumtoxin (1,300 aa)

Ribosome, 50S subunit



PDBs: 1bpi, 3bta, 1ffk

Conventional Crystallization of Macromolecules

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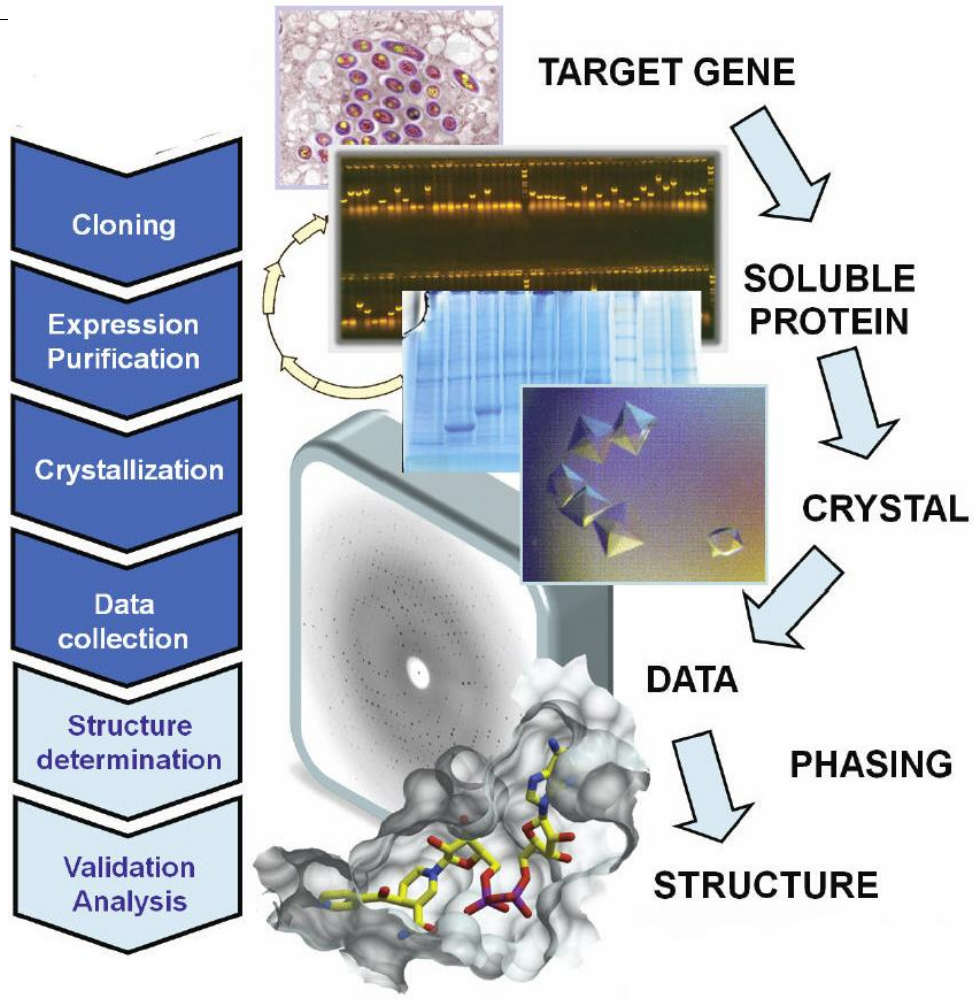
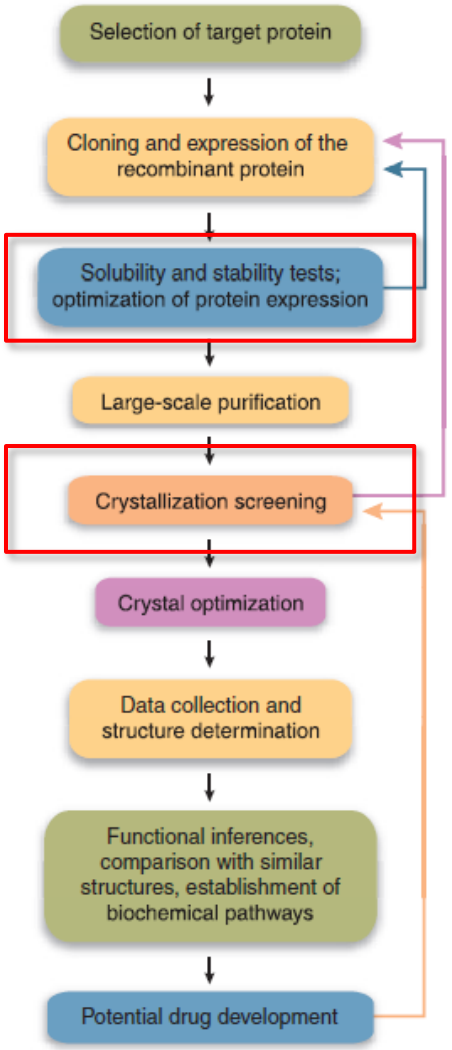
X-ray Crystallography





Rupp, Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology (2009)
Chayen & Saridakis, *Nature Methods* **5**, 147-153 (2008)

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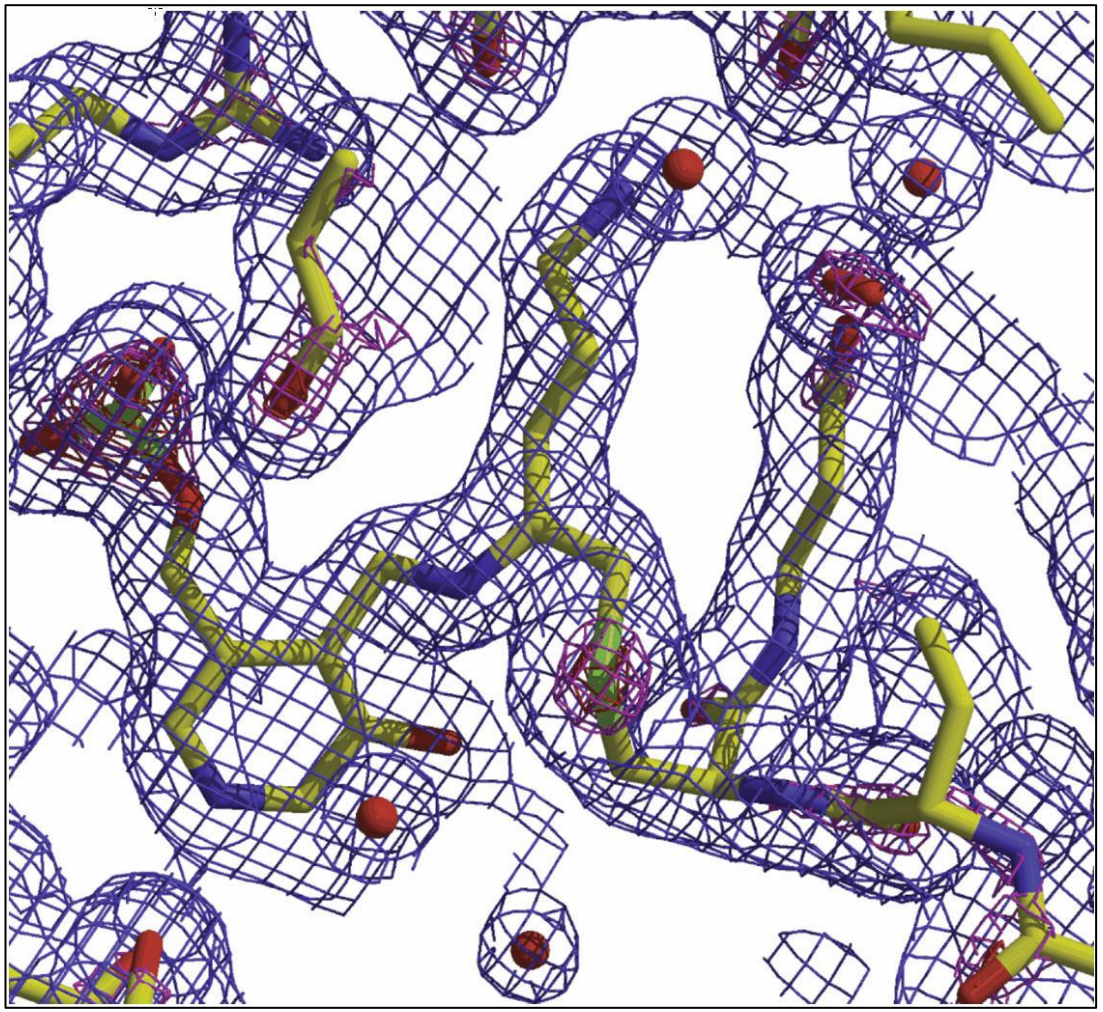
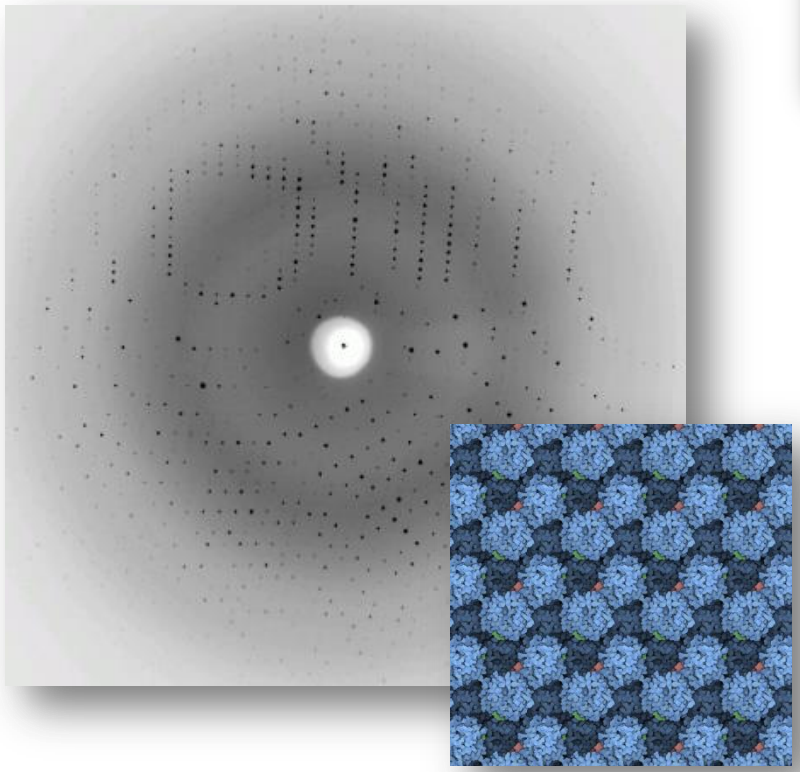
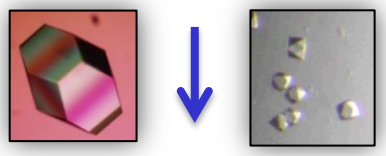
Diffraction, Resolution and Structural Details



1 Å = 10^{-10} m

Diffraction, Resolution and Crystal Size

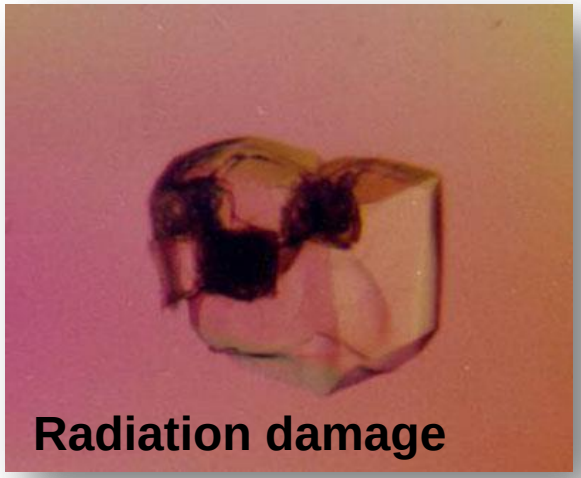




Crystal size

X-ray intensity

Increased number of crystals



N9 neuraminidase from avian influenza, following 100K data collection at the ESRF *(www.bioch.ox.ac.uk/garmangroup)*

Signal of single molecules is very low, crystals are required for strong reflections

Serial X-ray crystallography

Crystallization - Principles

Basic principle of crystallization:

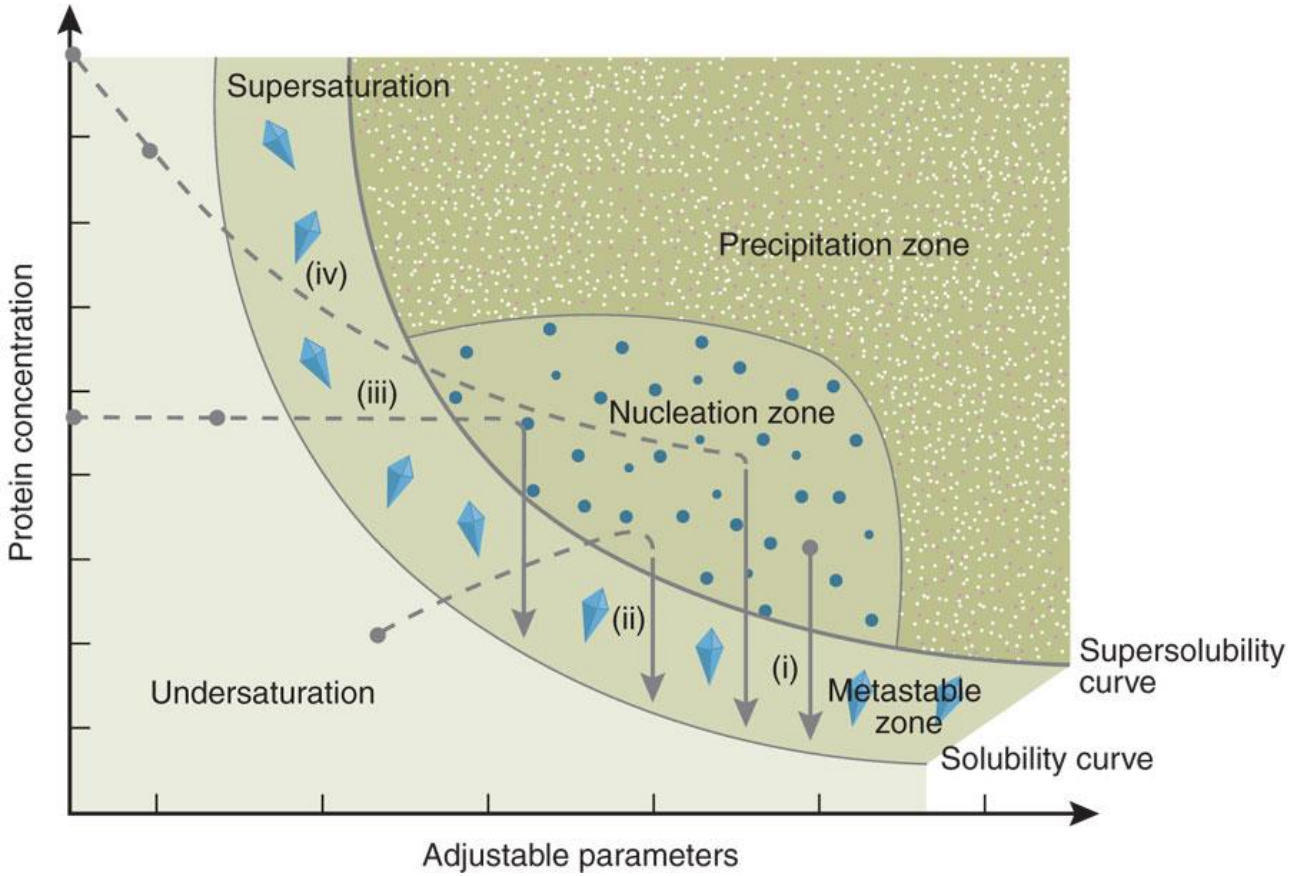
Obtaining a supersaturated state of the protein solution, from which the equilibrium is re-established by formation of a solid state, optimally crystals.

Number and size of the crystals is determined by the rate of nuclei formation, which depends on:

- Concentration of the protein and the precipitant in solution
- Degree of supersaturation of the protein solution
- Presence of other particles (contaminants)
- Size and material of the crystallization well

Crystallization - Principles

There are **not any obvious correlations** between **crystallization conditions** and **protein structure** or family, nor are there any set rules or ‘magic bullets’ that will guarantee the production of good crystals!



Chayen & Saridakis, *Nature Methods* **5**, 147-153 (2008)

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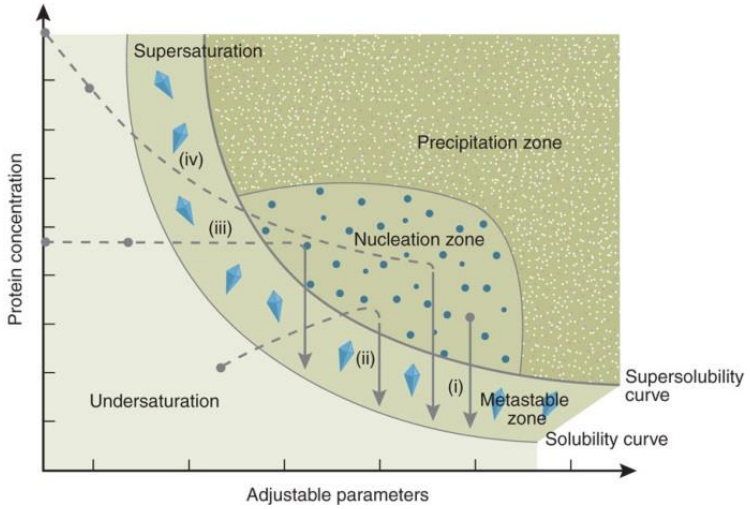
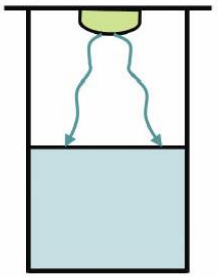
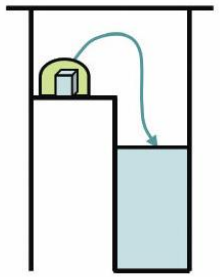
Crystallization Techniques



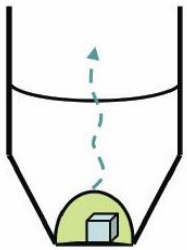


The classic: hanging-drop vapor diffusion

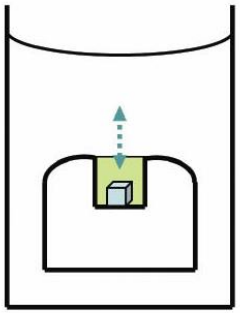


The variant: sitting-drop vapor diffusion

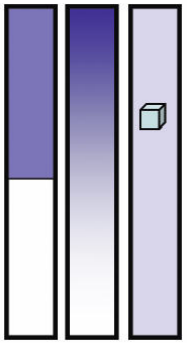
Vapor diffusion



Micro-batch under oil

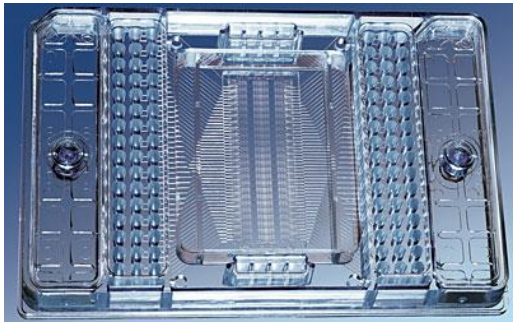


Micro-dialysis



Free-interface diffusion

+ Microfuidics…



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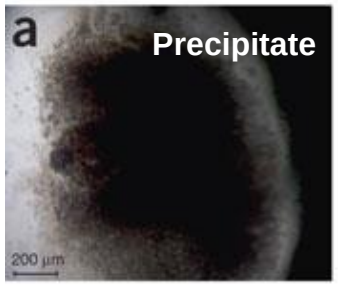
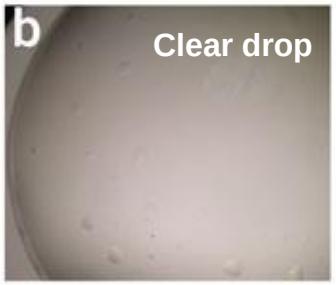
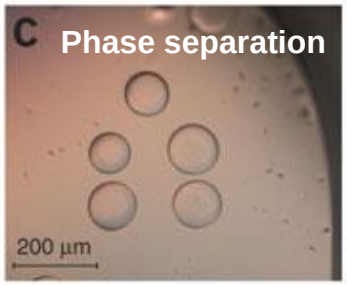
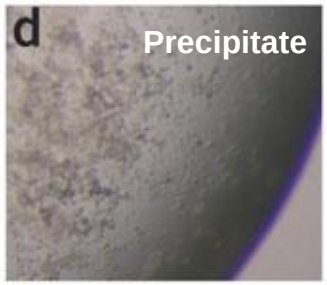
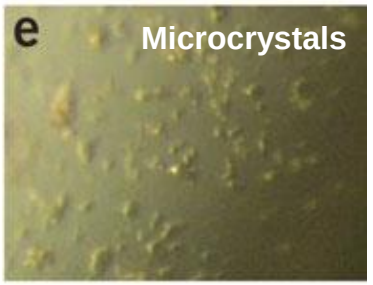
Crystallization Screening

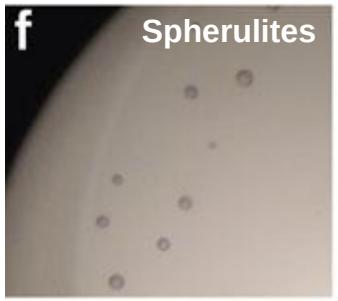


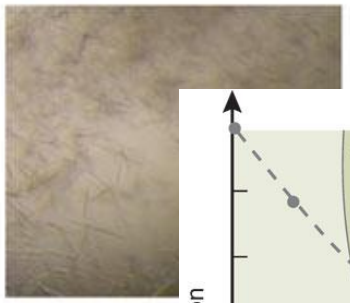


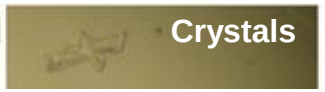


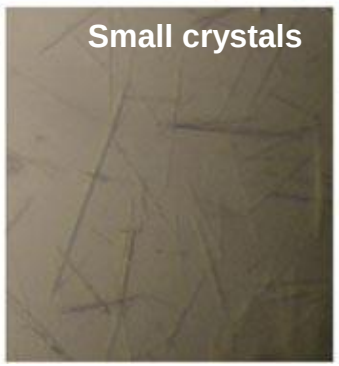


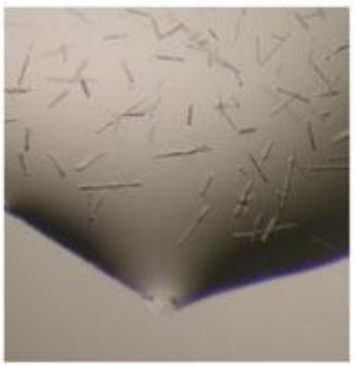


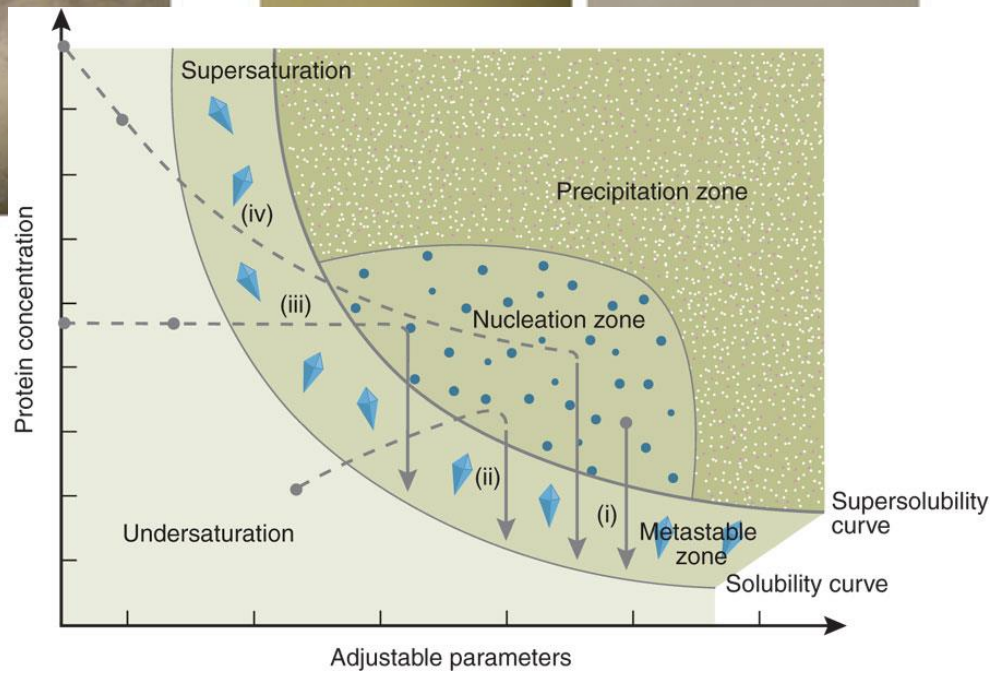












Benvenuti & Mangani, *Nature Protocols* **2**, 1633 - 1651 (2007)

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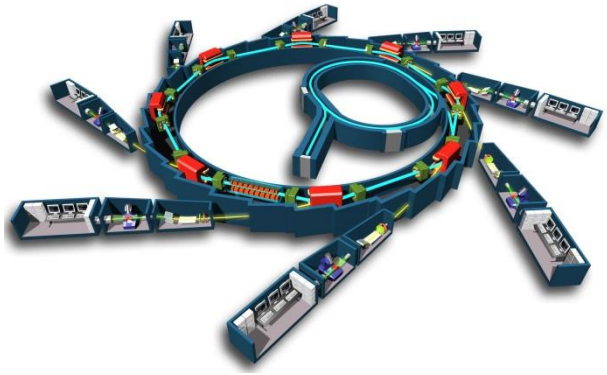
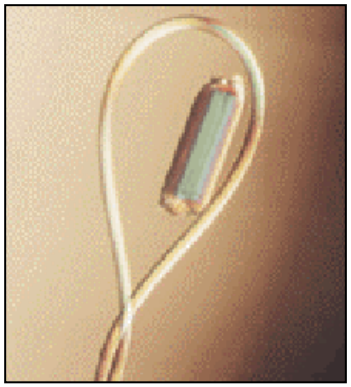
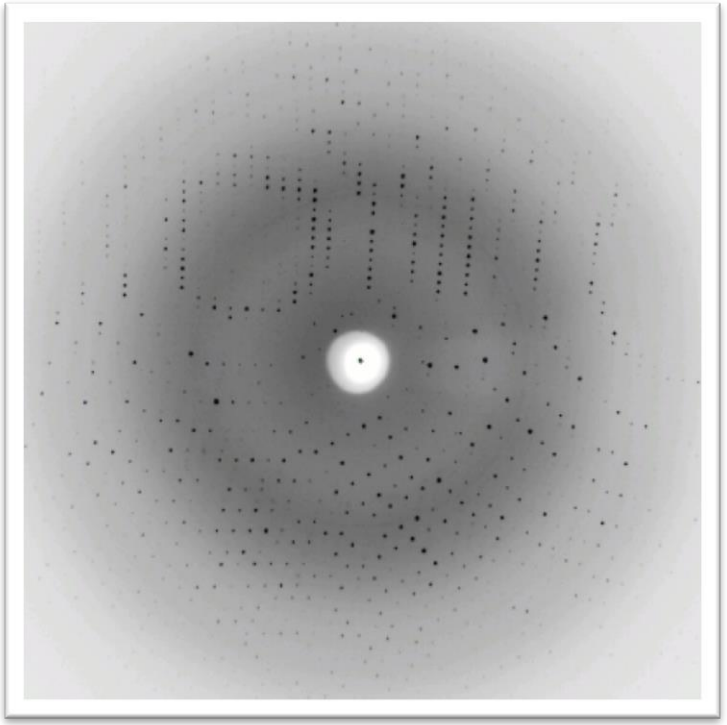
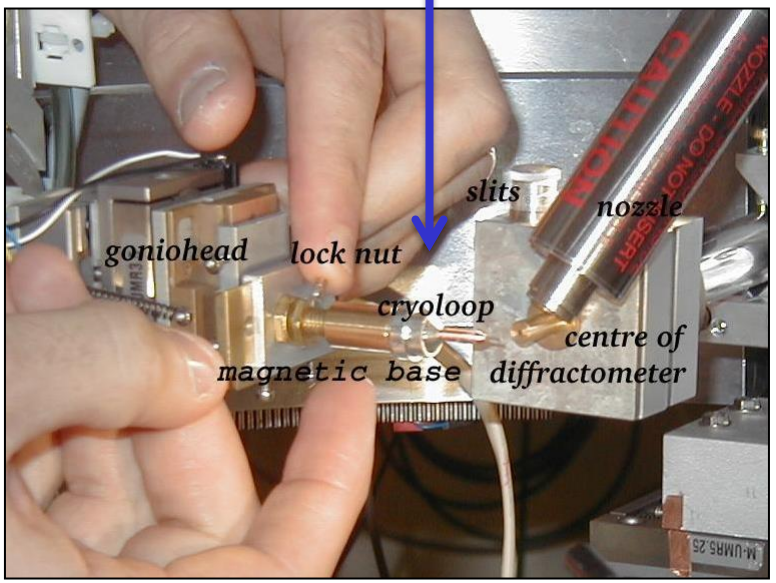
Diffraction Data Collection









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Protein crystallization - more information:

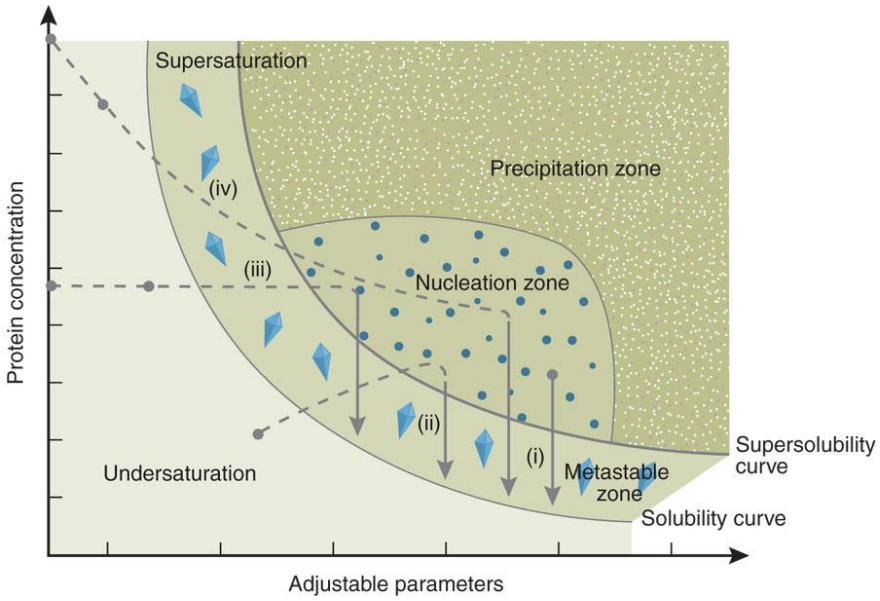
- Chayen NE & Saridakis E (2008) Protein crystallization: from purified protein to diffraction-quality crystal. *Nat. Methods* **5**, 147

- McPherson A & Gavira JA (2014) Introduction to protein crystallization. *Acta Cryst* **F70**, 2-20

How to grow large amounts of small crystals?

Formation of Protein Micro- and Nanocrystals

… undesired in past decades: new approaches have to be investigated!



Batch method

-> volume = amount of crystals!

Example 1: Lysozyme

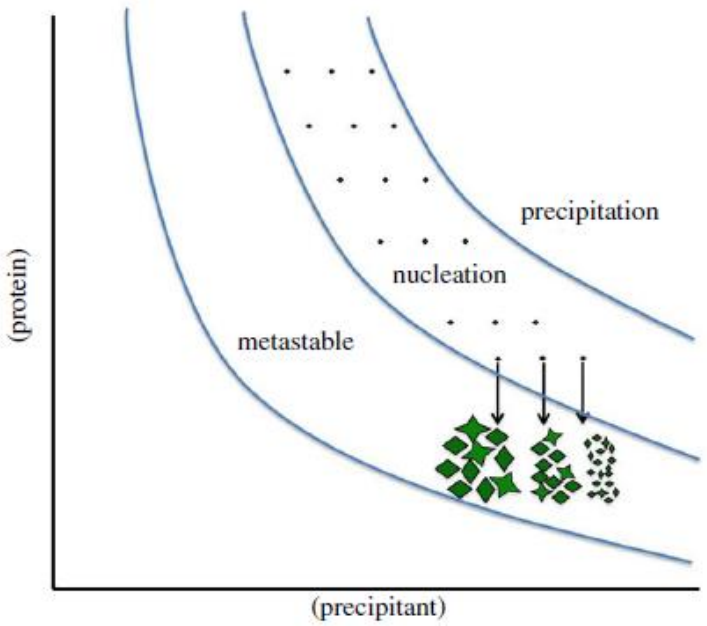
- Mix precipitant [14.7 % (w/v) NaCl, 22 % (w/v) PEG 8,000 in 500 mM NaAc pH 3] with protein solution (100 mg/ml)
- Stirring for 2 min
- Incubation for 12 hrs at RT:

5 x 10^7 microcrystals /ml

F. Stellato *et al*. *IUCrJ* 1 (2014)

Formation of Protein Micro- and Nanocrystals

Example 2: Photosystem II (PSII)

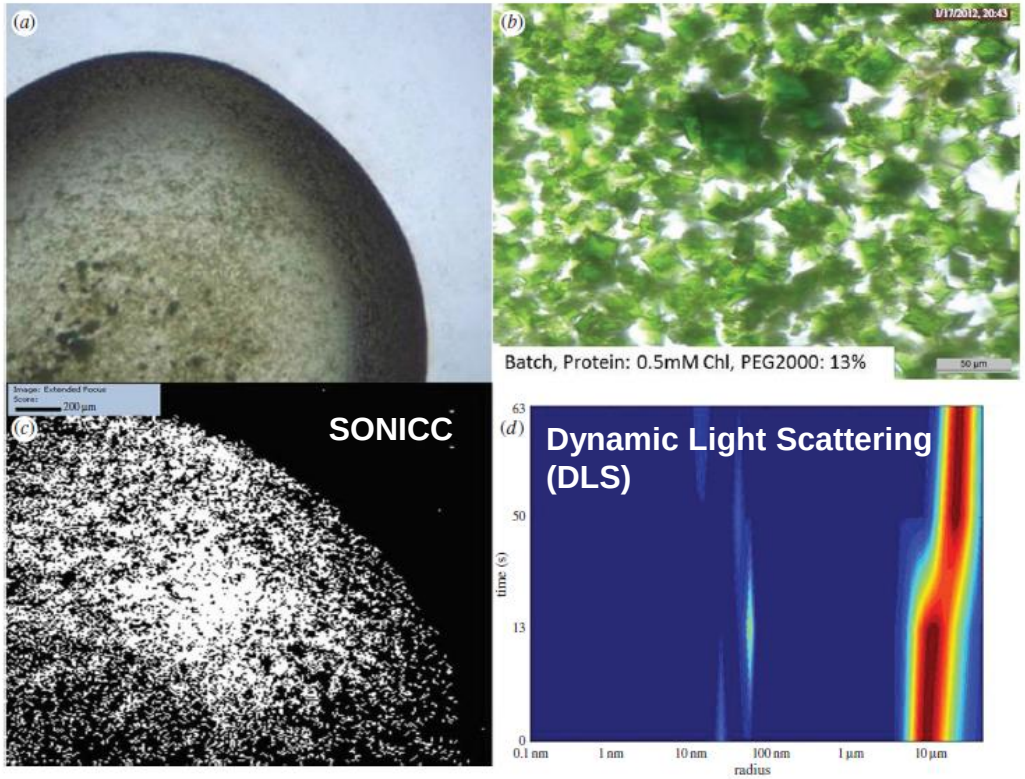


Phase diagram should be known for batch crystallization!

Seeding with nano-crystals increases the nucleation rate

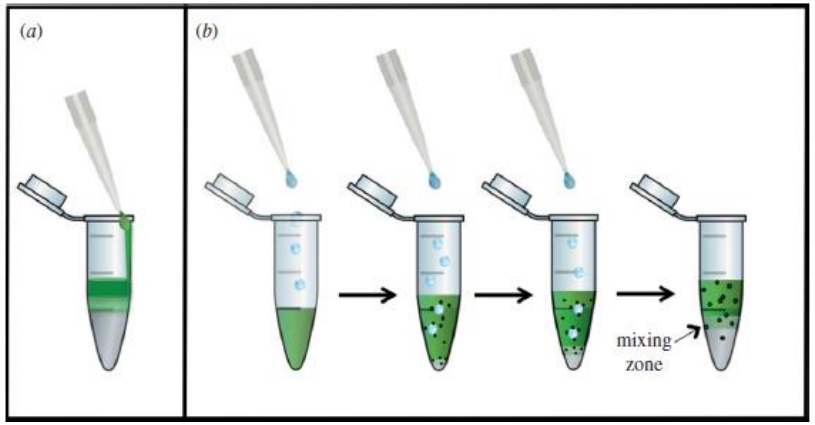
Broad size distribution, fast crystallization: small crystals grow fast into larger ones!

Batch method



C. Kupitz *et al*. *Phil Trans R Soc B* 369 (2014)

Formation of Protein Micro- and Nanocrystals



For nano-crystal growth:
maximize the surface / volume ratio!

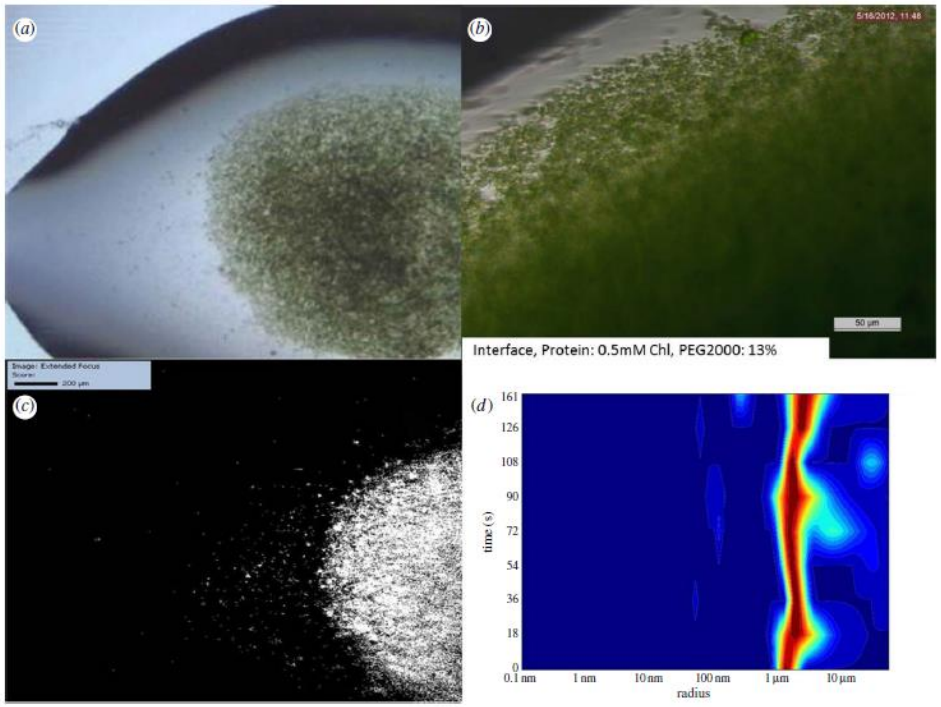
Setup in 1.5 ml reaction vessels

Precipitant is slowly dropped through the protein layer at a rate of 20 µl / min – large transient interface!

Small crystals with 1-2 µm radius, much less polycrystallinity!

Example 2: Photosystem II (PSII)

Free interface diffusion

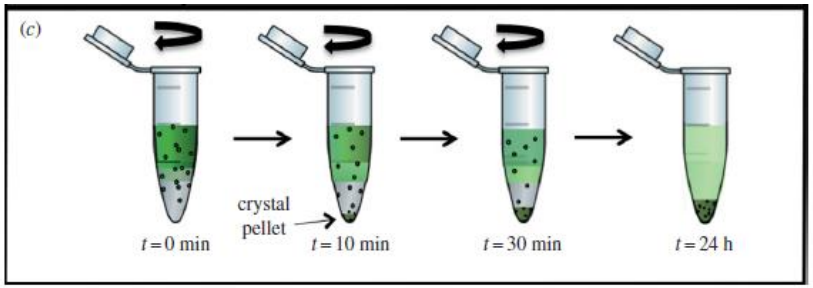


C. Kupitz *et al*. *Phil Trans R Soc B* 369 (2014)

Formation of Protein Micro- and Nanocrystals

Example 2: Photosystem II (PSII)

200 – 500 g

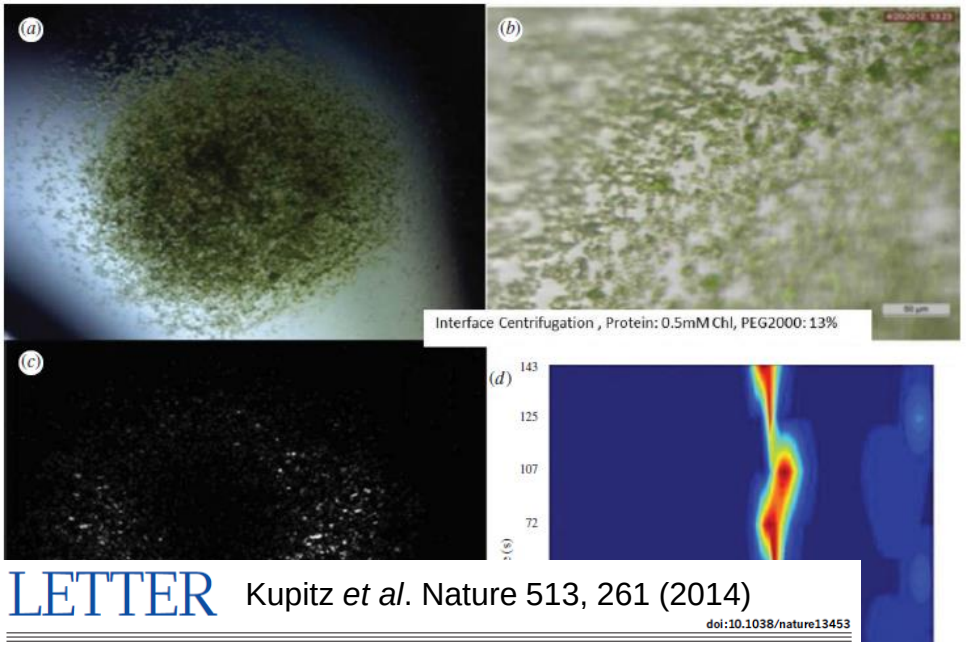


Modification of FID approach:
Formation of two phase system is followed by centrifugation

- Nano-crystals sediment into precipitant after reaching a specific size
- Growth of crystals stops as soon as they enter precipitant layer
- Majority of crystals formed after 30 min
- Upscaling to 6 ml protein plus 6 ml precipitant in 15 ml Falcon tubes possible

Very small crystals with uniform size distribution with radius of around 500 nm!

Free interface diffusion centrifugation



LETTER Kupitz *et al*. Nature 513, 261 (2014)
doi:10.1038/nature13453

Serial time-resolved crystallography of photosystem II using a femtosecond X-ray laser

C. Kupitz *et al*. *Phil Trans R Soc B* 369 (2014)

Formation of Protein Micro- and Nanocrystals

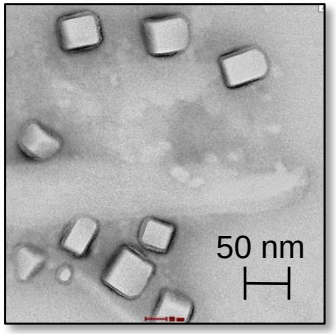
Parameters influencing the growth of crystals by FID

Crystal size depends on:

- protein concentration
- precipitant concentration
- Viscosity of precipitant solution

But not significantly on:

- centrifugation speed (within 200 to 500 g range!)



Crystal size is limited to below 2 µm by FID

Quenching of crystal growth to avoid large crystal formation…
… by addition of high concentration precipitation buffer

Control of crystal size: DLS, optical imaging (EM), visualization by SONICC

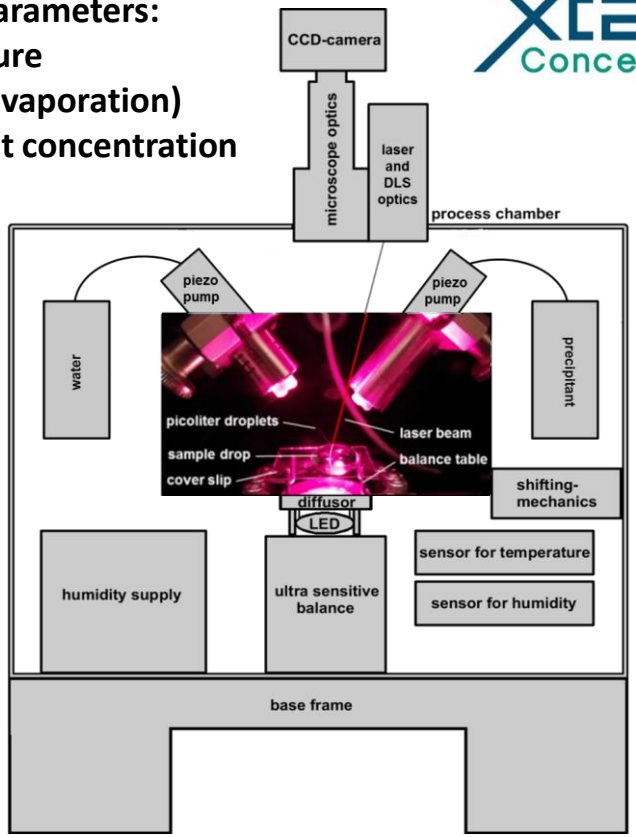
C. Kupitz *et al*. *Phil Trans R Soc B* 369 (2014)

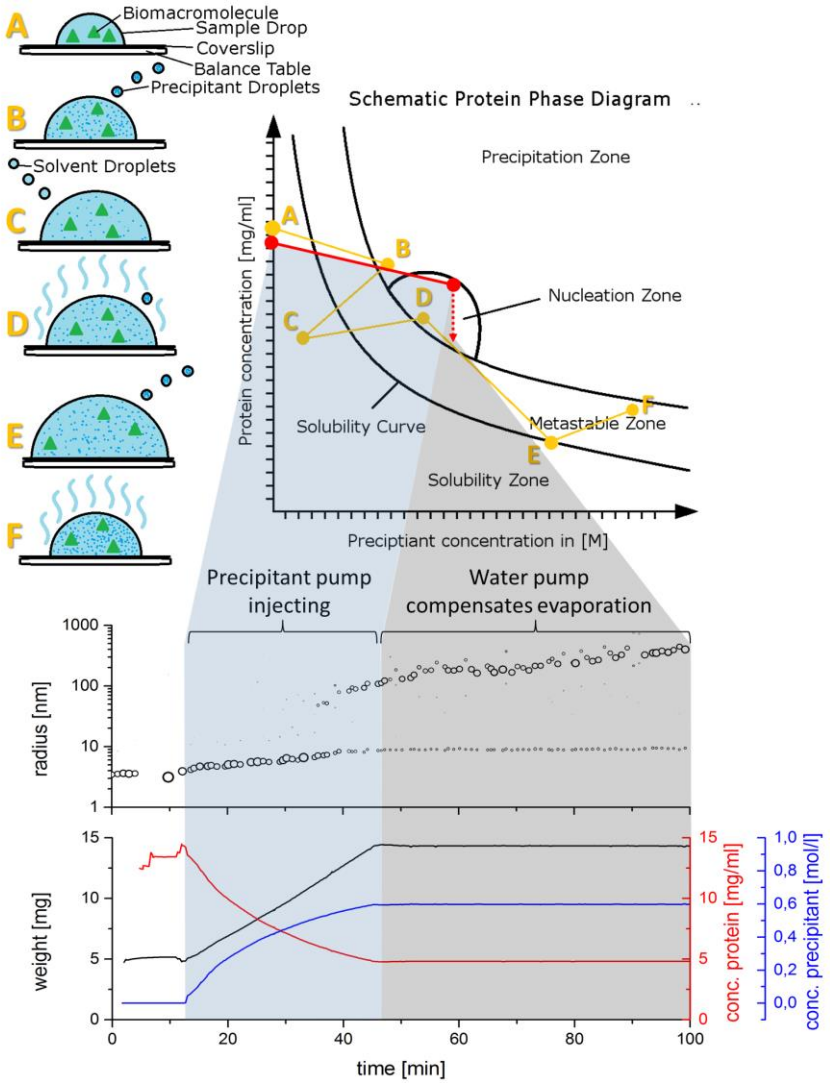
Xtal Controller – Designed Crystal Growth

Setup based on DLS:

- **Determination of hydrodynamic radius**

- **Variable parameters:**
- **Temperature**
- **Volume (evaporation)**
- **Precipitant concentration**





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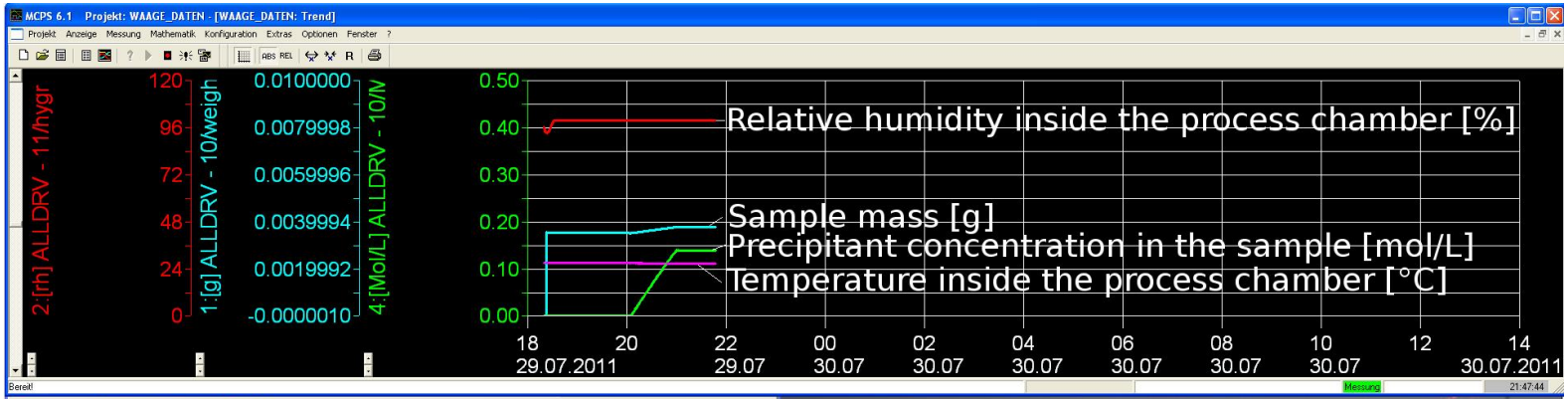
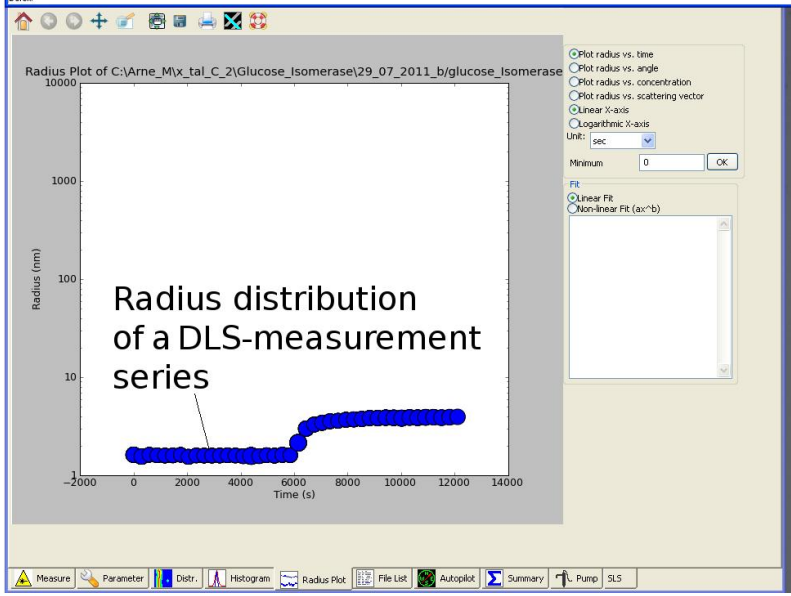
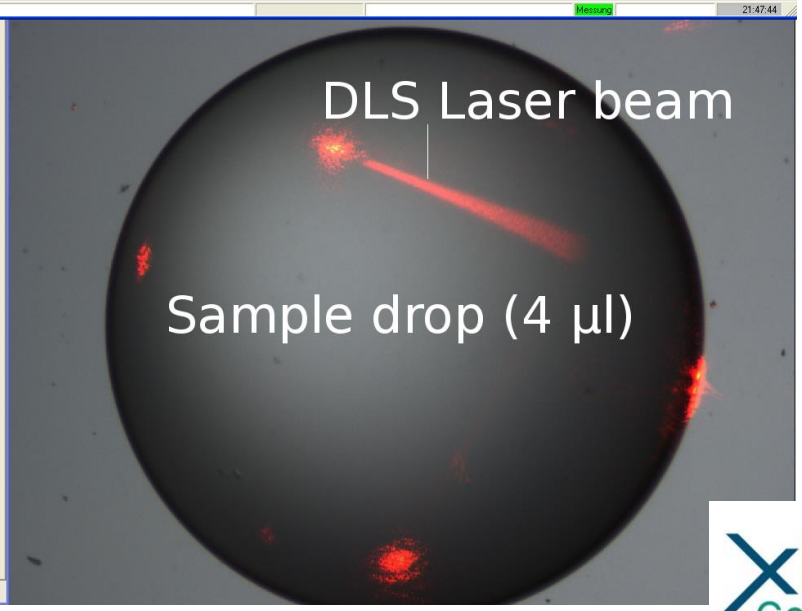
Xtal Controller – Designed Crystal Growth





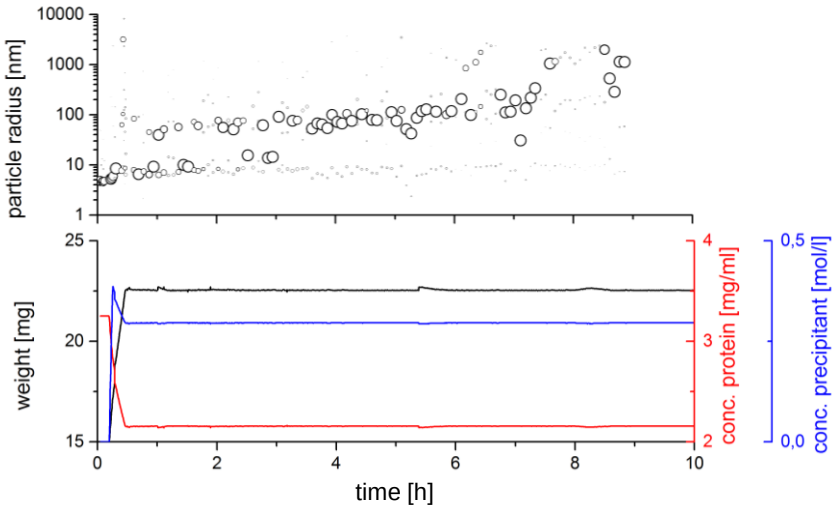


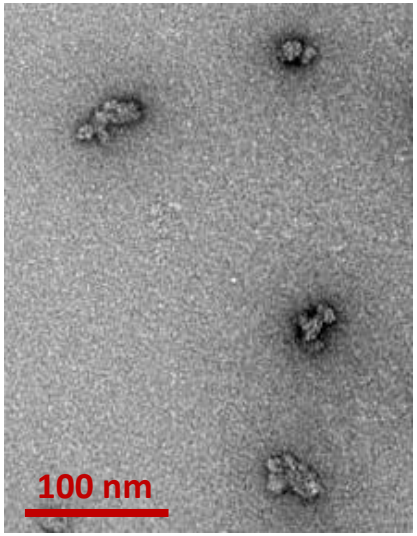
Xtal Concepts

Xtal Controller – Designed Crystal Growth

Aggregates

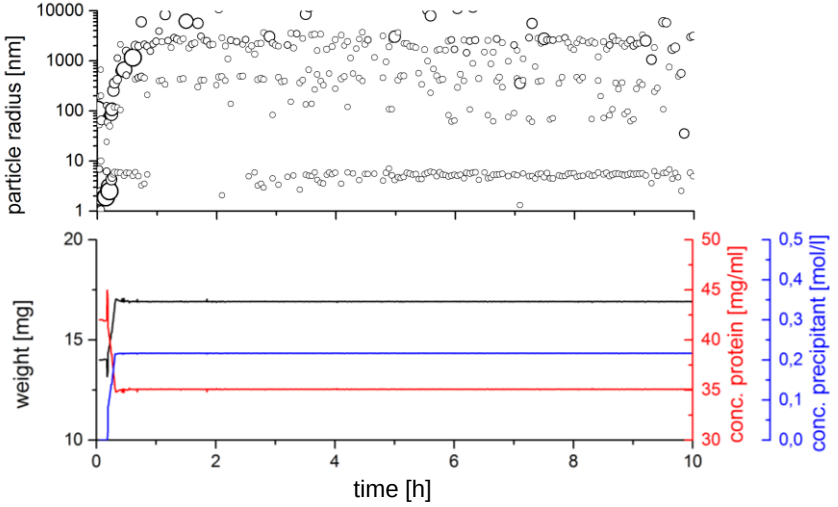
- Mistletoe lectin (*viscum album*)
- crosslinking with glutaraldehyde
- negativ stain

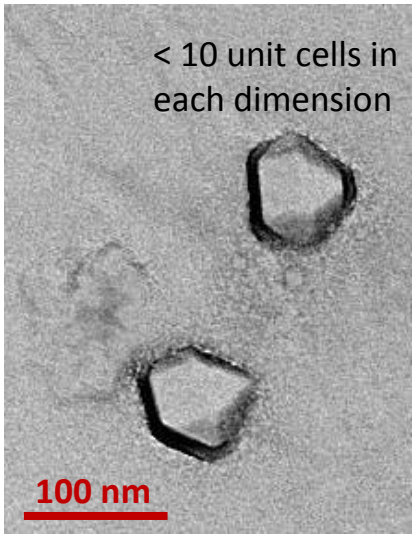




Crystals

- Thaumatin 1 (*thaumatococcus daniellii*)
- crosslinking with glutaraldehyde
- negativ stain





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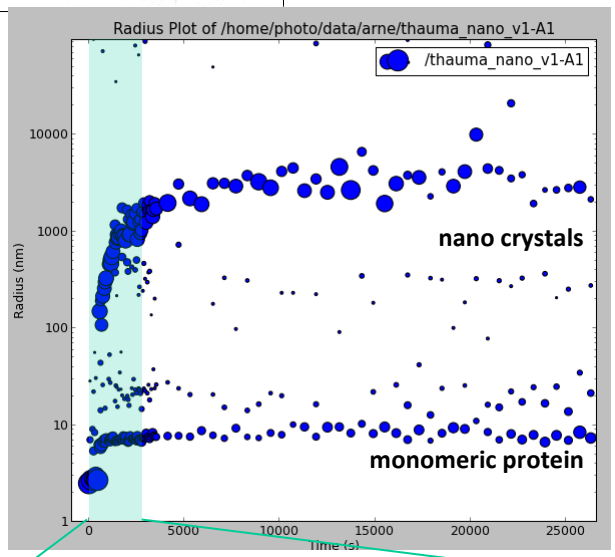
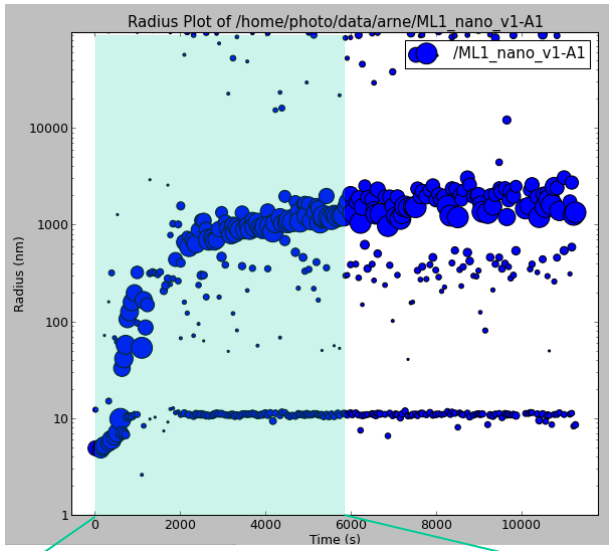
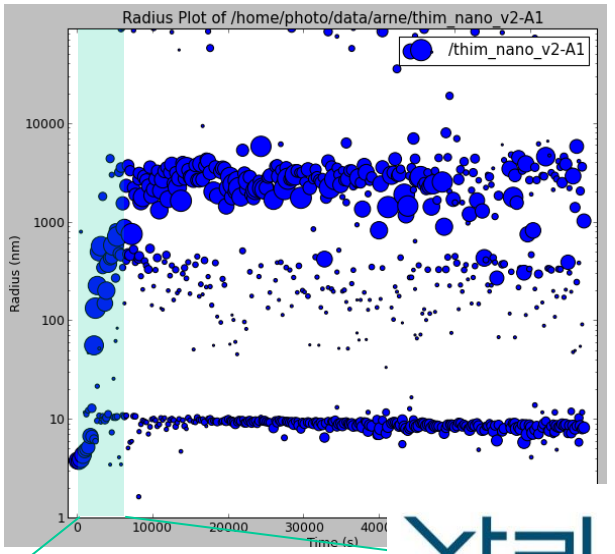
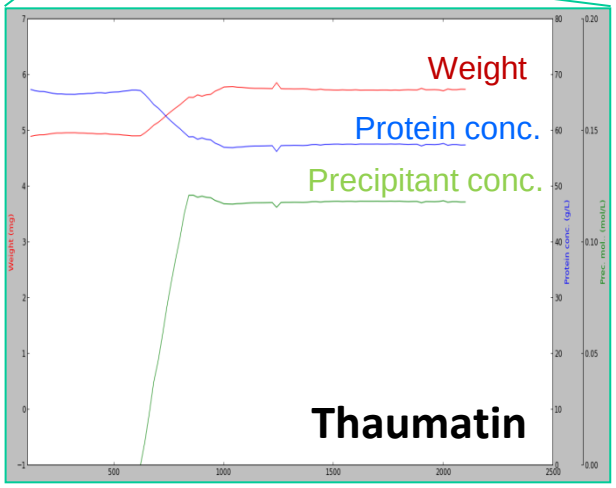
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen

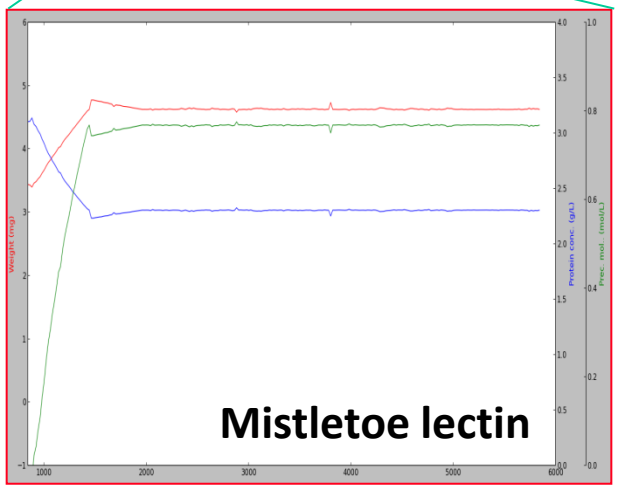
Xtal Controller – Designed Crystal Growth

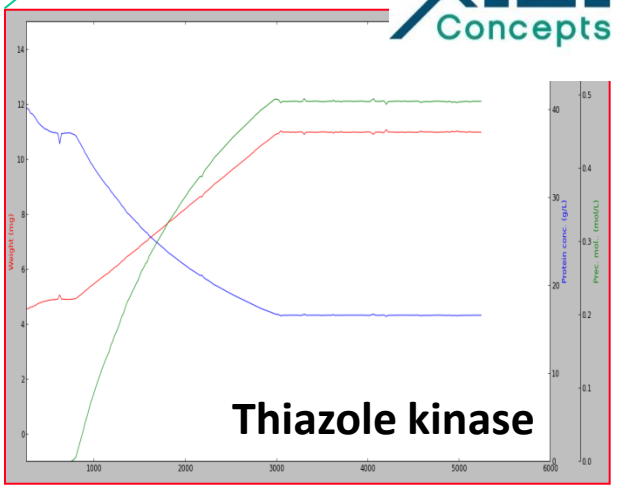










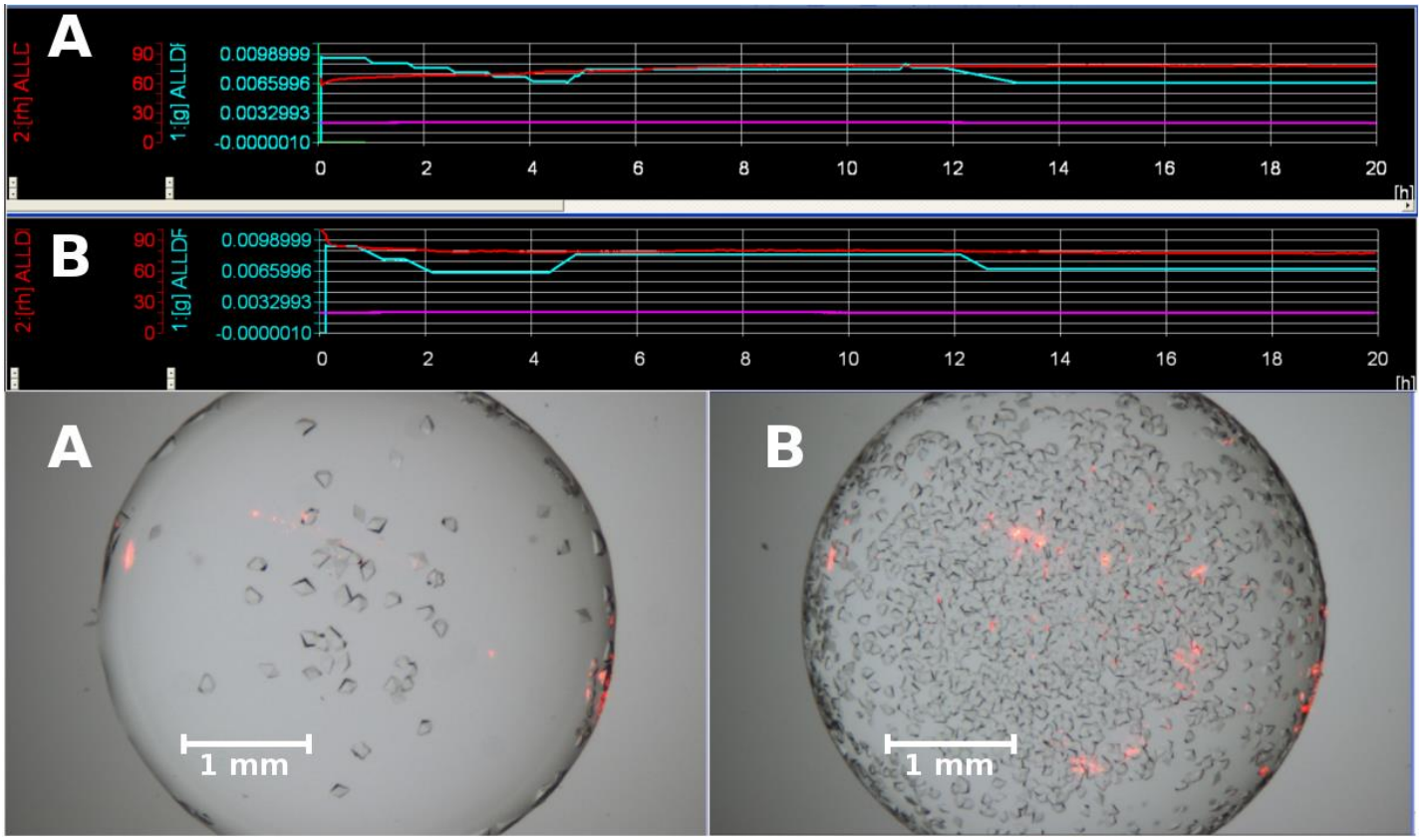


Xtal Concepts

Robin Schubert, University of Hamburg

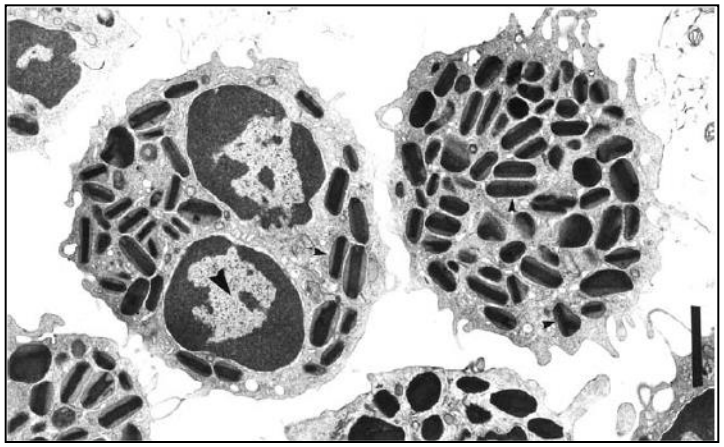
Xtal Controller – Designed Crystal Growth

Experiments A and B under identical conditions. Only difference is the time within the supersaturated region (S).

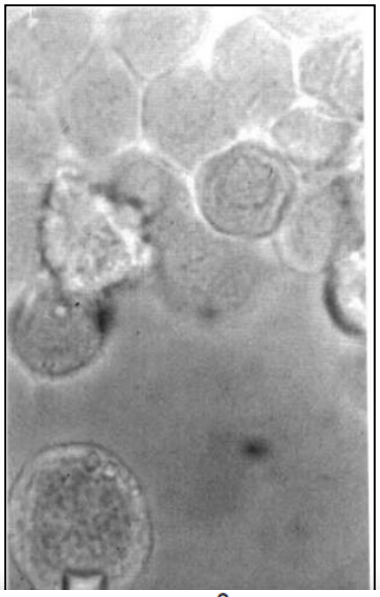


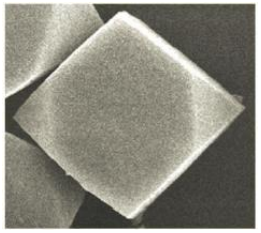
Crystallization of Biomolecules – A native process?

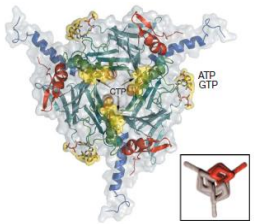
Native Protein Crystallization *in vivo*



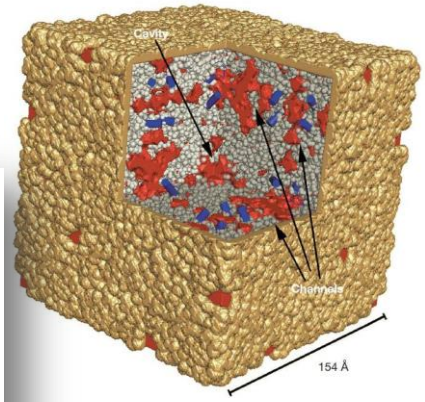
Eosinophils containing rectangular crystals of eosinophil major basic protein. (1999)







- Cypovirus polyhedra
- 5 – 12 µm crystals
- 2 Å resolution

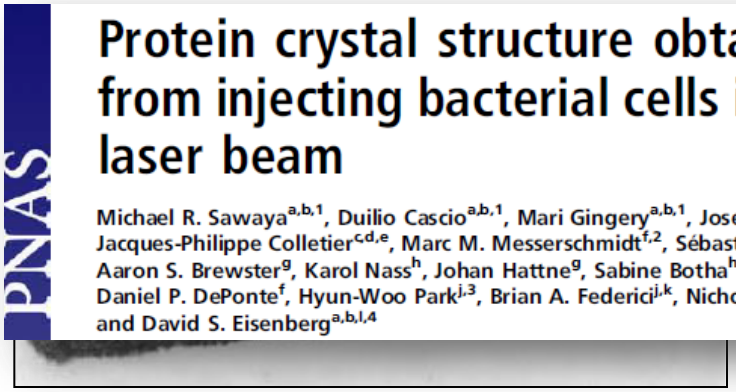


Protein crystal structure obtained at 2.9 Å resolution from injecting bacterial cells into an X-ray free-electron laser beam

PNAS

PNAS 111, 12769 (2014)

Michael R. Sawaya[a,b,1], Duilio Cascio[a,b,1], Mari Gingery[a,b,1], Jose Rodriguez[a,b], Lukasz Goldschmidt[a,b], Jacques-Philippe Colletier[c,d,e], Marc M. Messerschmidt[f,2], Sébastien Boutet[f], Jason E. Koglin[f], Garth J. Williams[f], Aaron S. Brewster[g], Karol Nass[h], Johan Hattne[g], Sabine Botha[h], R. Bruce Doak[h,i], Robert L. Shoeman[h], Daniel P. DePonte[f], Hyun-Woo Park[j,3], Brian A. Federici[j,k], Nicholas K. Sauter[g], Ilme Schlichting[h], and David S. Eisenberg[a,b,l,4]



Protein toxin crystal within *Bacillus thuringiensis*. (1995)

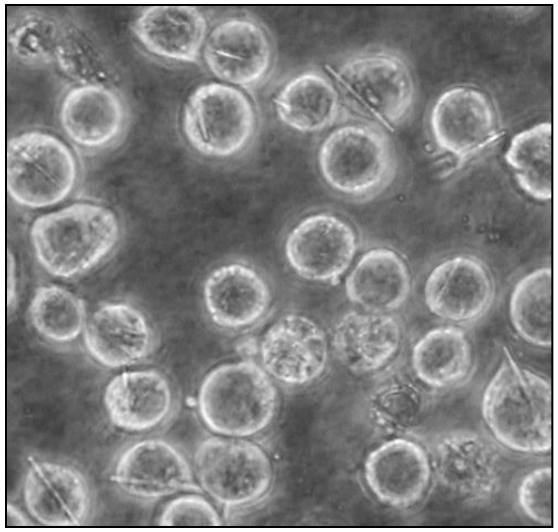
) - no structural data

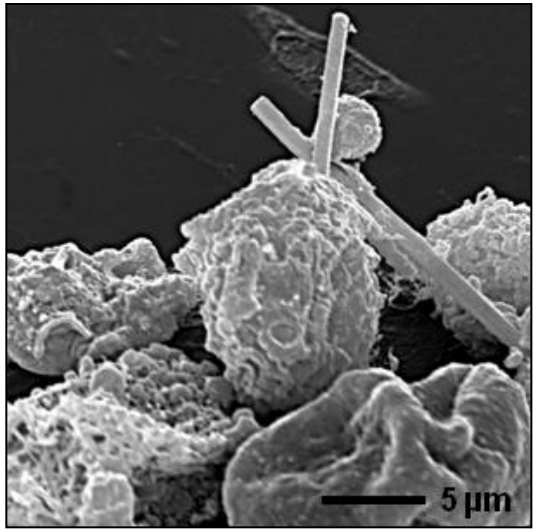
Coulibaly *et al*., *Nature* 446, 97-101 (2007)

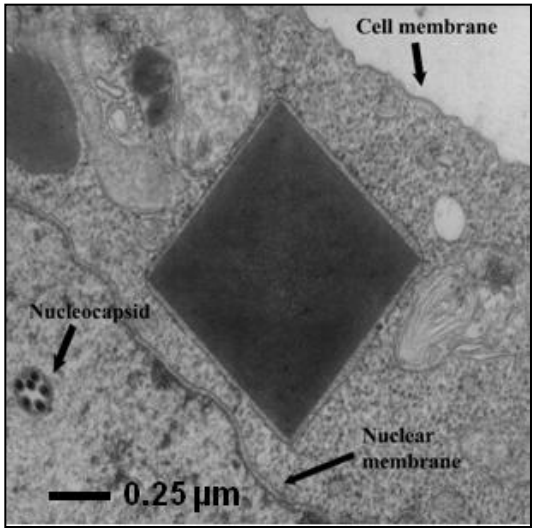
Fan *et al*., *Micros. Res. Tech.* 34, 77-86 (1996)

Doje & Poon, *Curr. Opin. Colloid Inferf. Sci.* 11, 40-46 (2006)

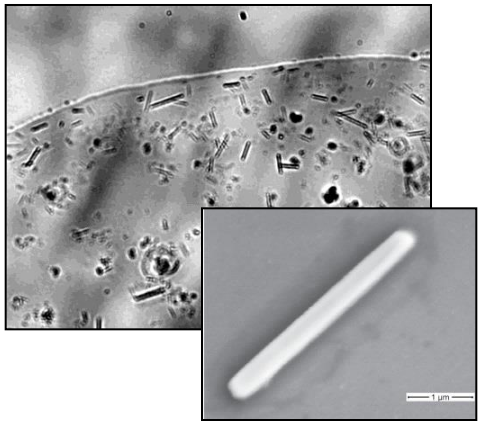
In vivo Crystallization of Recombinant Proteins







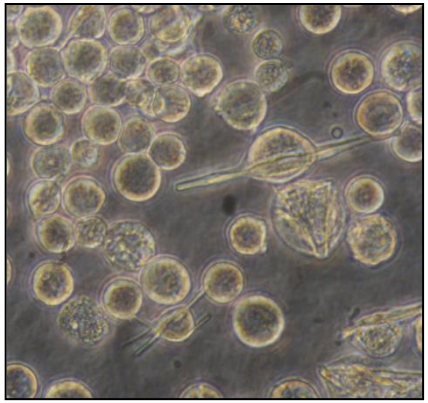
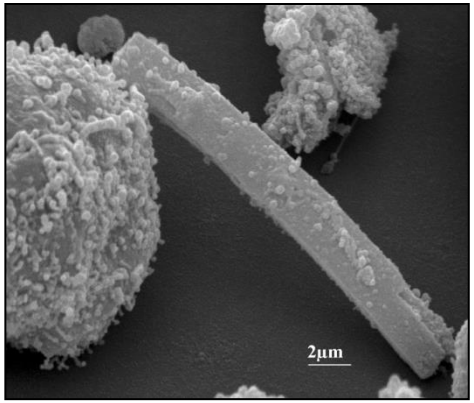
- **pre-pro-cathepsin B** from parasite ***Trypanosoma brucei*** (*Tb*CatB)
- recombinant expression in **SF9 insect cells** using **baculovirus expression system**
- crystals surrounded by **membranes** decorated with ribosomes – **origin** of crystallization in the **ER**?

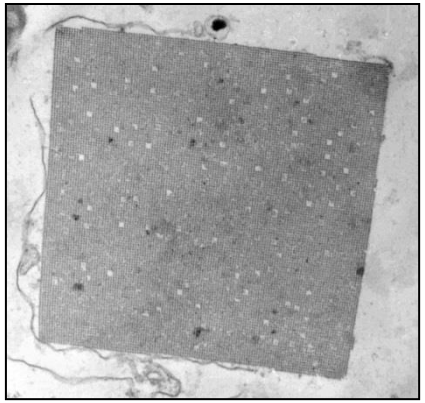
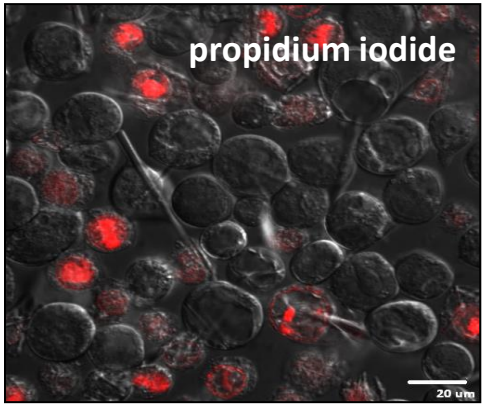


Koopmann*, Kupelli*, Redecke* *et al*., *Nat. Methods* 9, 259-262 (2012)

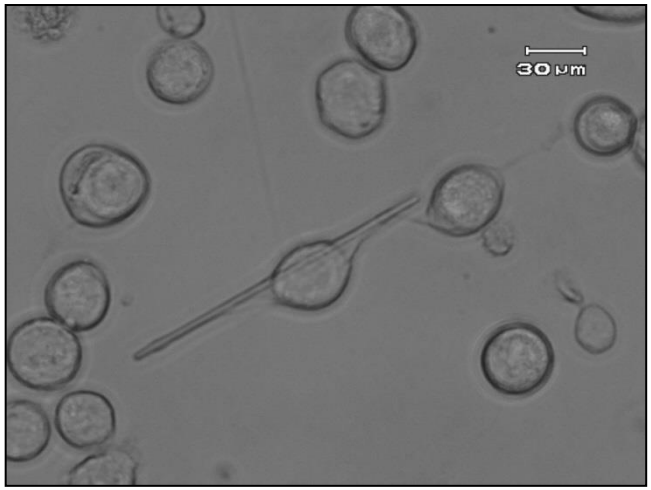
SIAS
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen

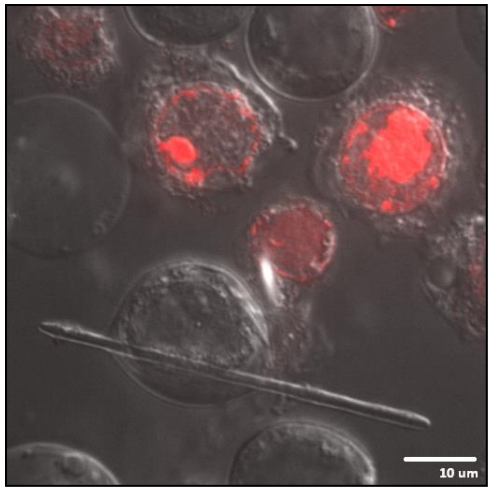
In vivo Crystallization of Recombinant Proteins

Inosine monophosphate dehydrogenase from parasite ***Trypanosoma brucei*** (*Tb*IMPDH)

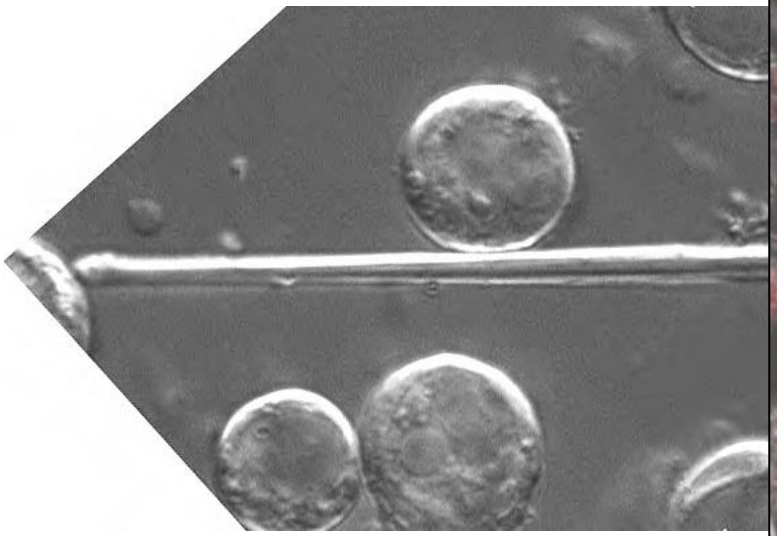




Firefly luciferase

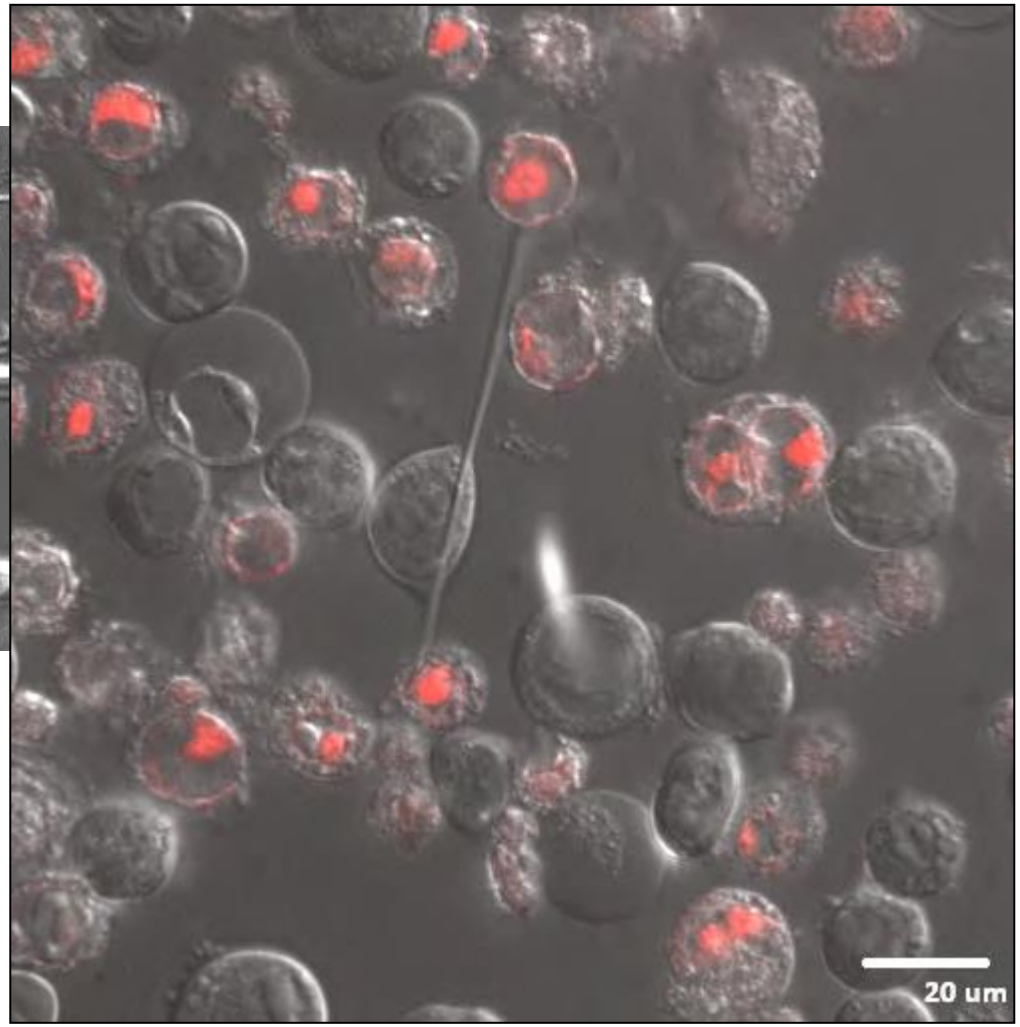
L. Redecke*, K. Nass* *et al*. (2014), to be submitted
R. Schönherr*, M. Klinge*, … , and L. Redecke (2014), to be submitted

In vivo Crystallization of Recombinant Proteins



120 µm in length!

Firefly luciferase



Schönherr *et al.* and Redecke (2014), to be published

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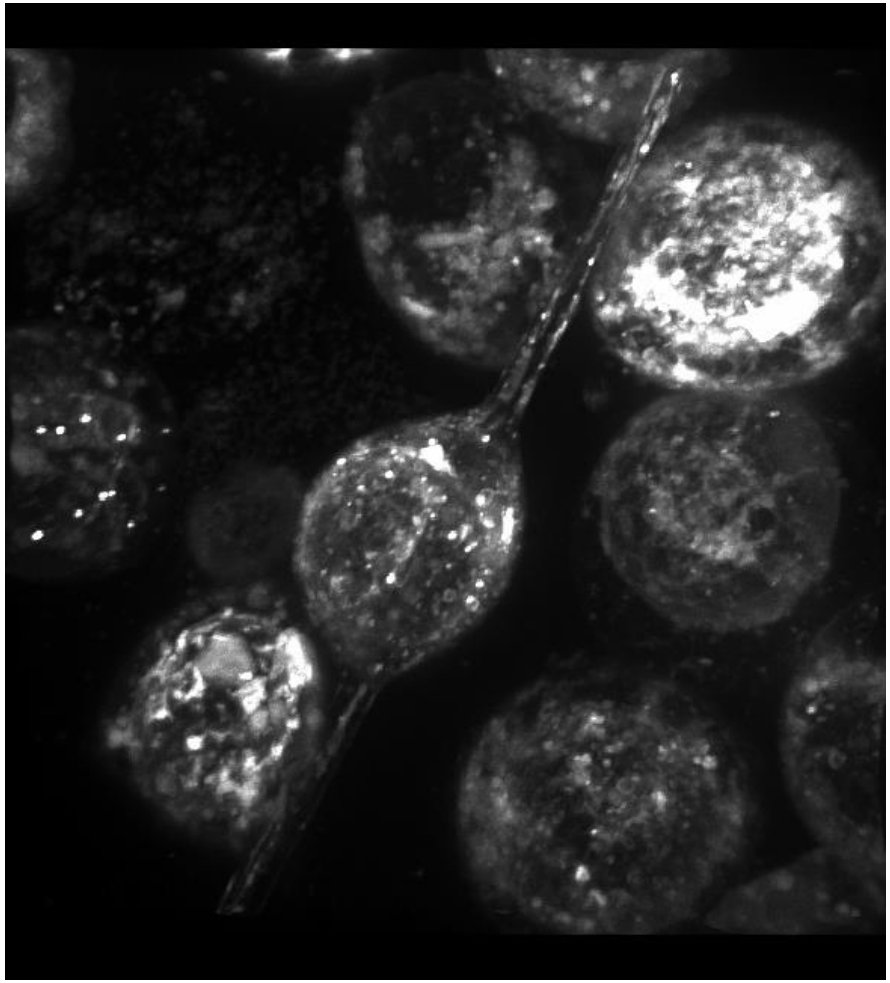
In vivo Crystallization of Recombinant Proteins



Firefly luciferase

Membrane stain
by Bodipy 558

Rainer Duden
Robert Schönherr
Institute of Biology
University of Lübeck

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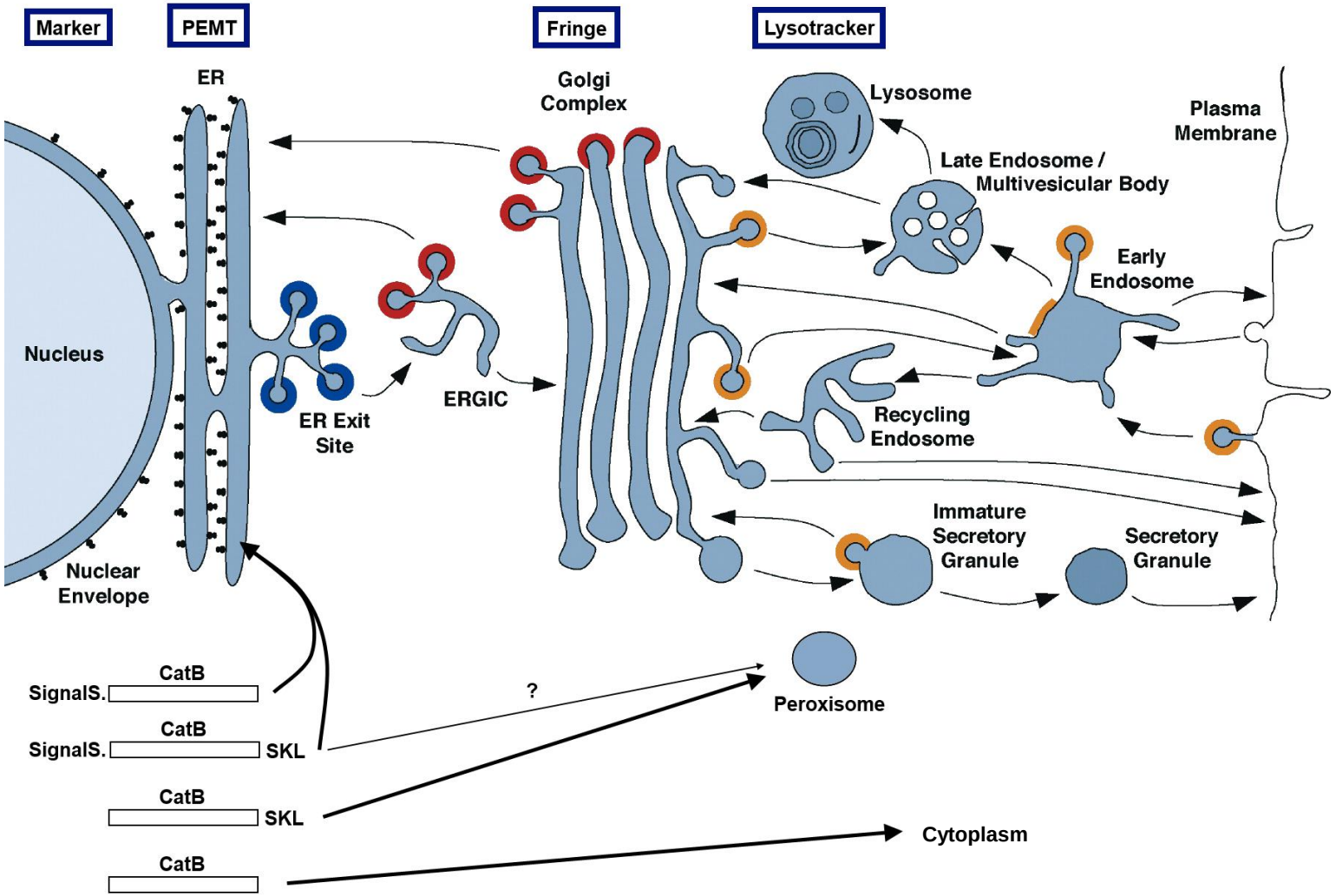
Cellular Compartments for Protein Crystallization



Modified from: Bonifacino *et al.*, *Cell* 116, 153-166 (2004)

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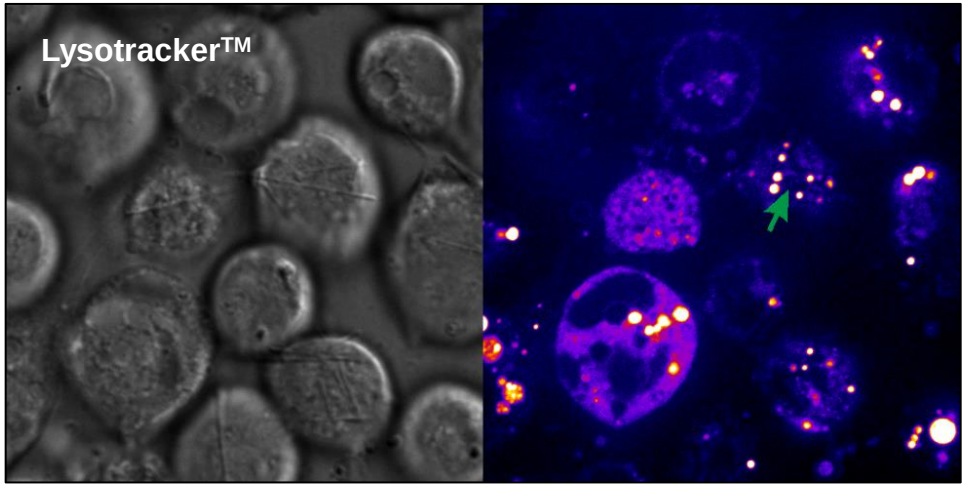
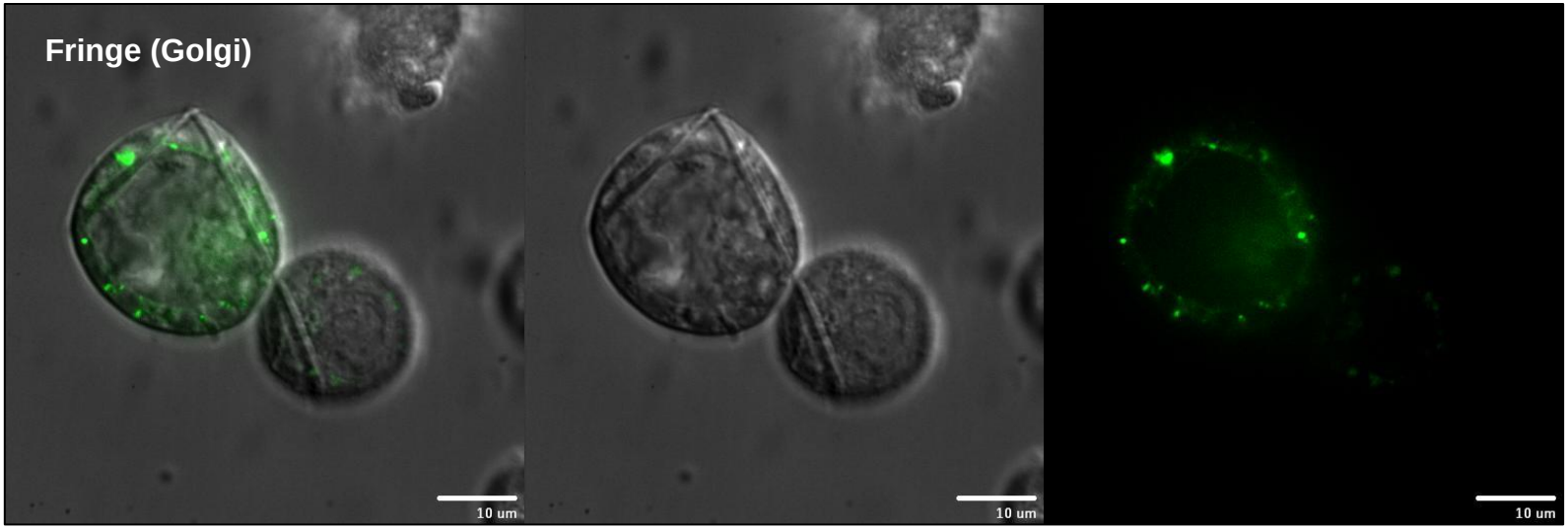
In vivo crystallization of cathepsin B

Cellular compartment?





Klinge*, Schönherr* *et al.* and Redecke (2014), to be published

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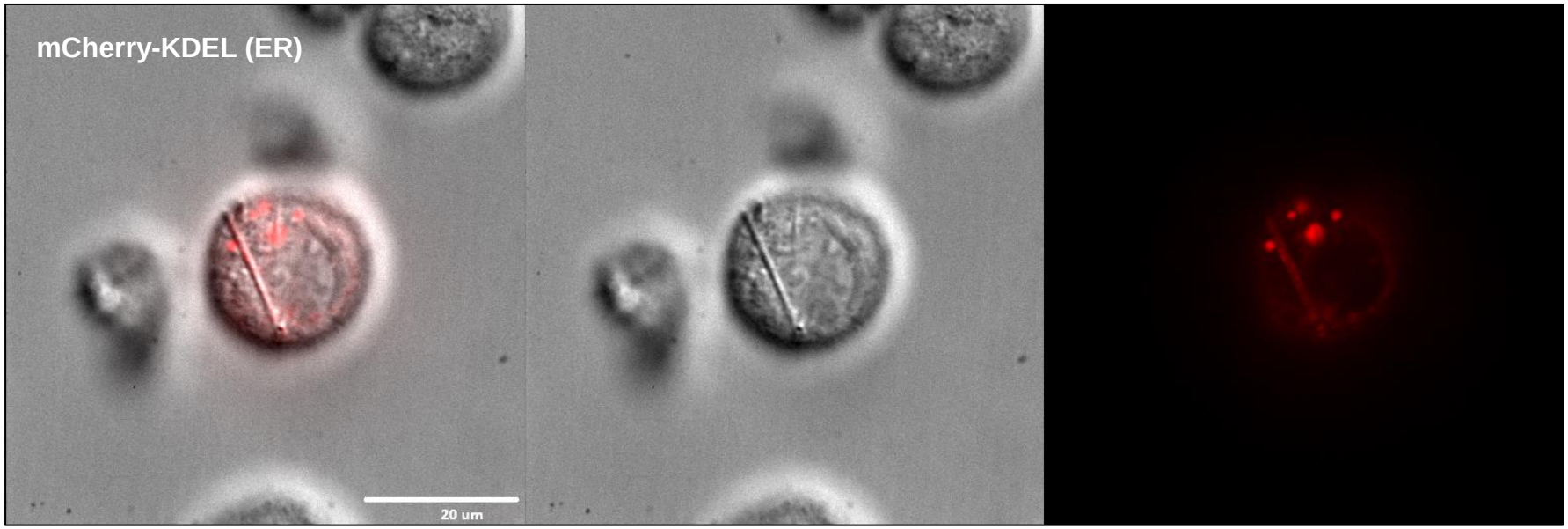
In vivo crystallization of cathepsin B

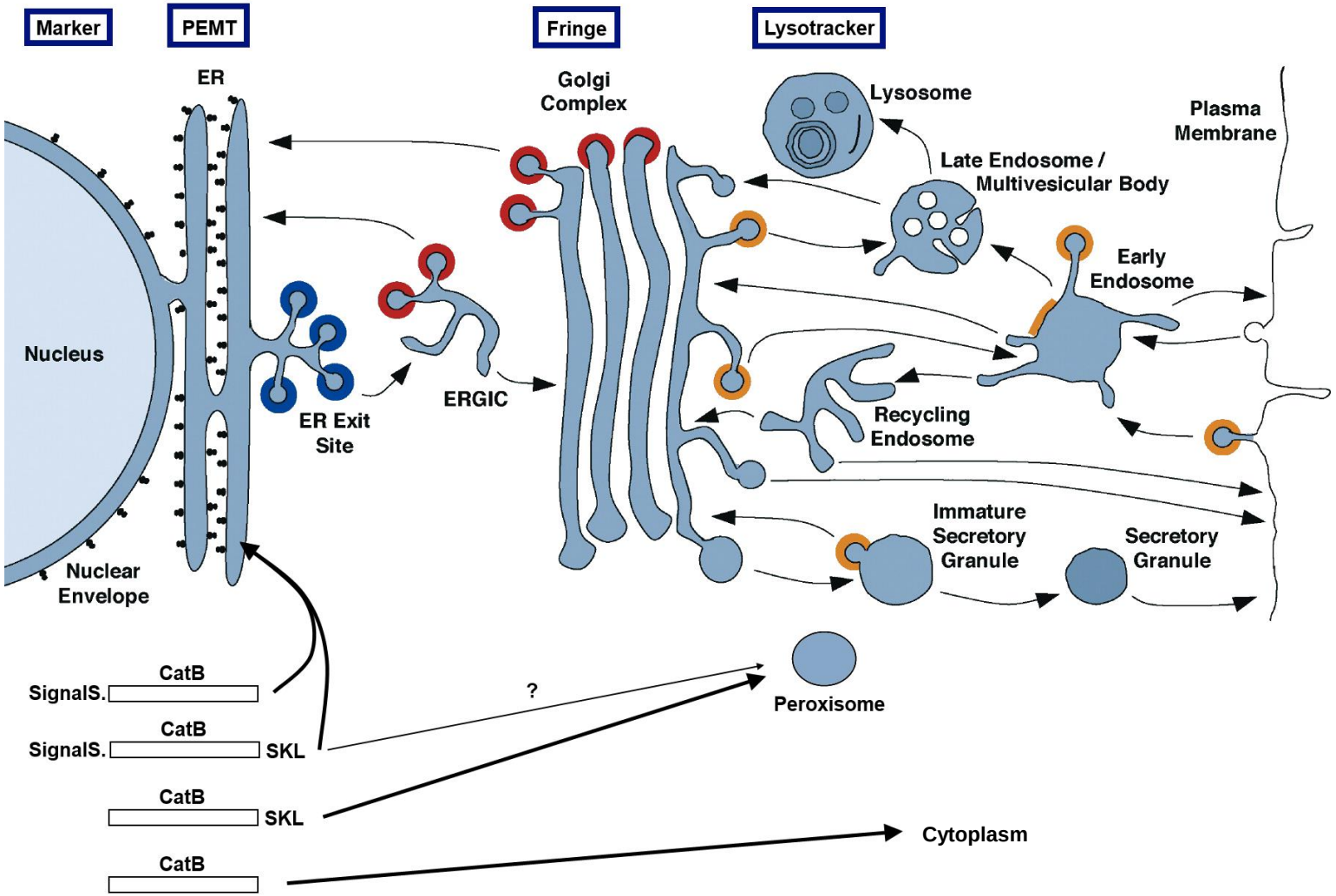
Cellular compartment – Endoplasmatic reticulum (ER)!



Klinge*, Schönherr* *et al.* and Redecke (2014), to be published

SIAS
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen

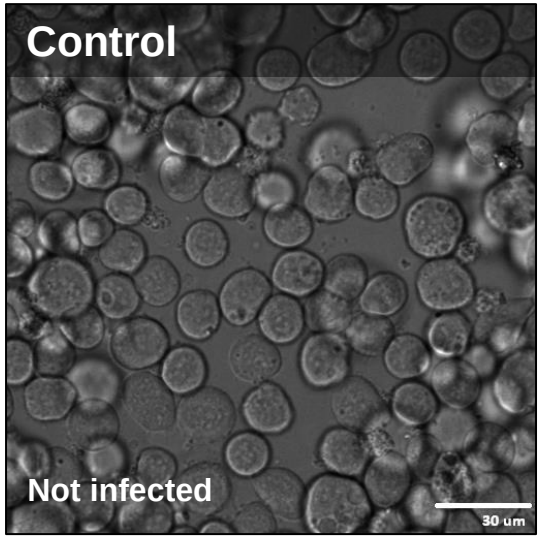
Cellular Compartments for Protein Crystallization

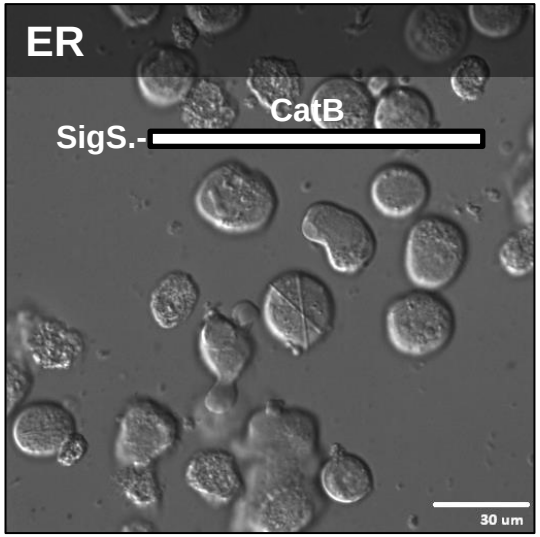


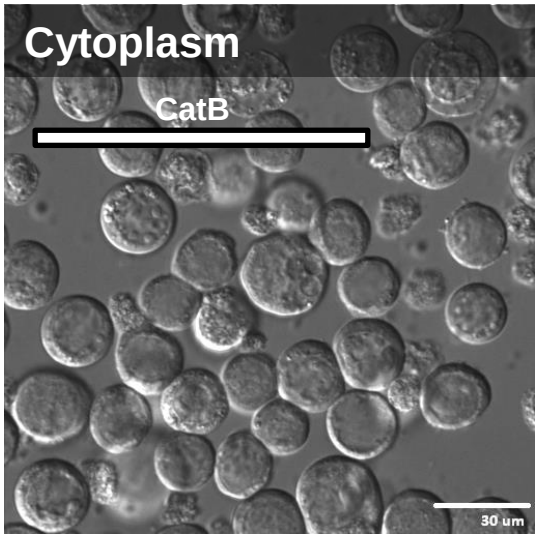
Modified from: Bonifacino *et al.*, *Cell* 116, 153-166 (2004)

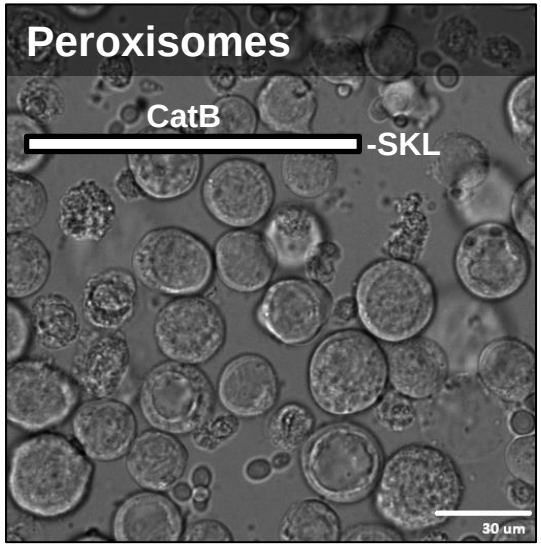
SIAS
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen

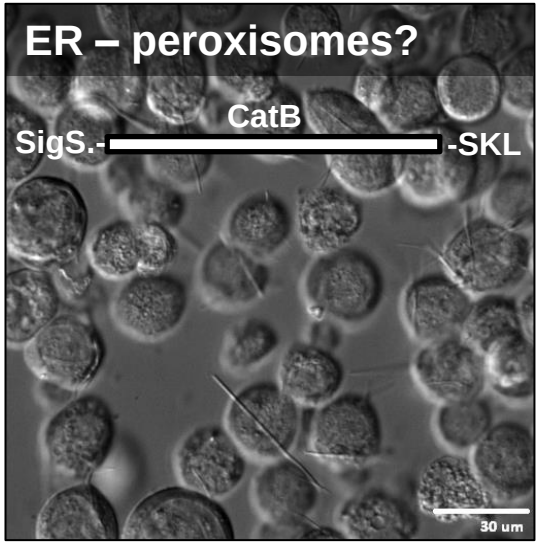
In vivo crystallization of cathepsin B







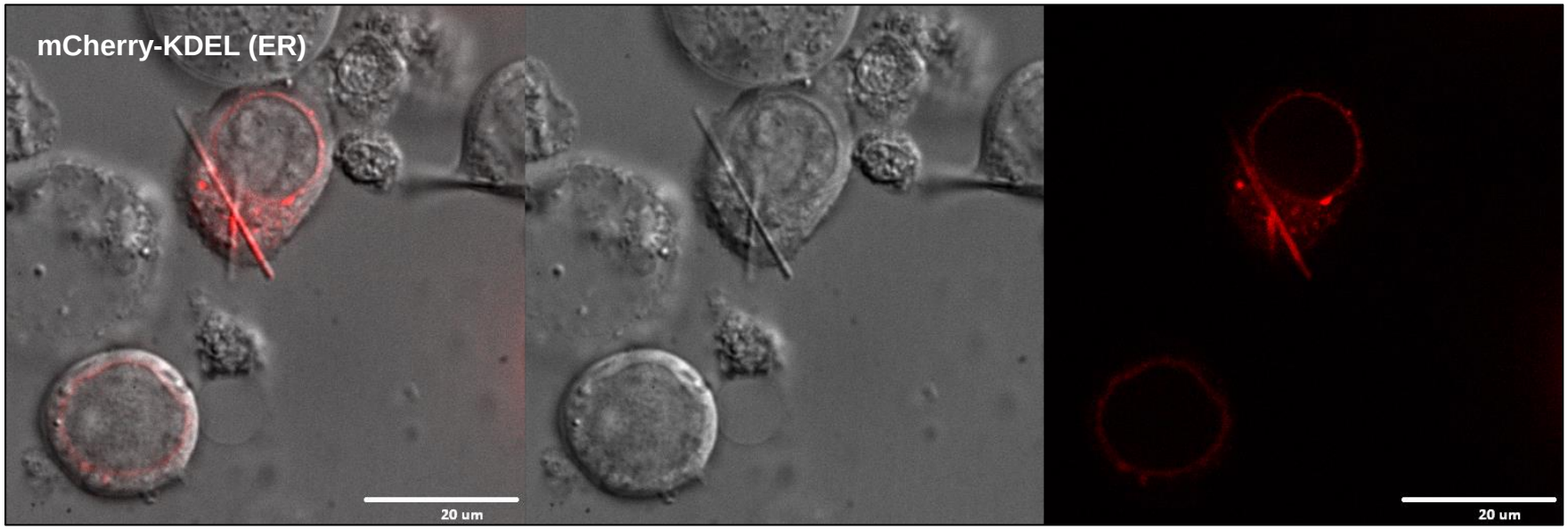




Klinge*, Schönherr* *et al.* and Redecke (2014), to be published

In vivo crystallization of cathepsin B

SKL effect – transport or crystallization?



SigS.-CatB-SKL	large Xtals
SigS.-CatB-SEL	wt Xtals
SigS.-CatB-SKLG	large Xtals
SigS.-CatB-SKI	large Xtals

Klinge*, Schönherr* *et al.* and Redecke (2014), to be published

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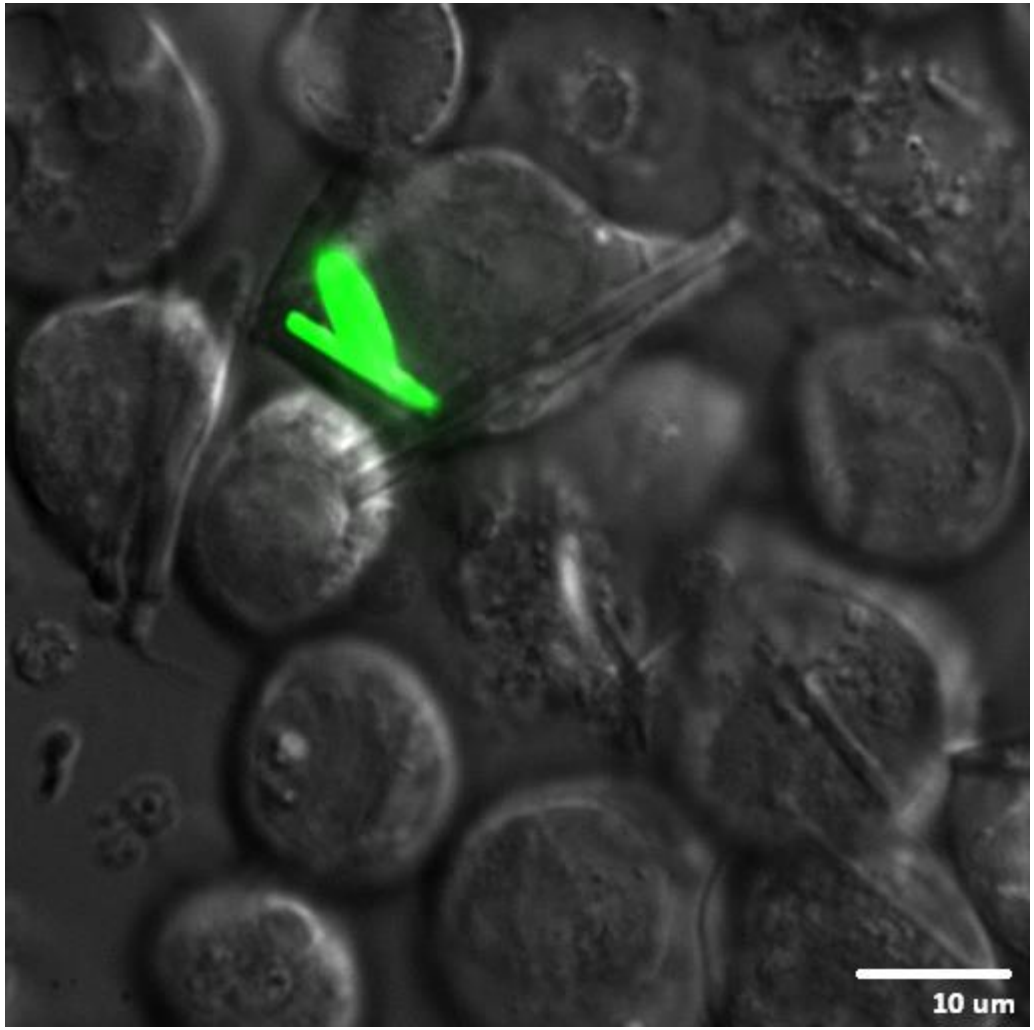
“Co-crystallization” *in vivo*? – IMPDH and GFP-µNS!



Rainer Duden
Robert Schönherr
Institute of Biology
University of Lübeck

Serial X-ray Crystallography Approaches

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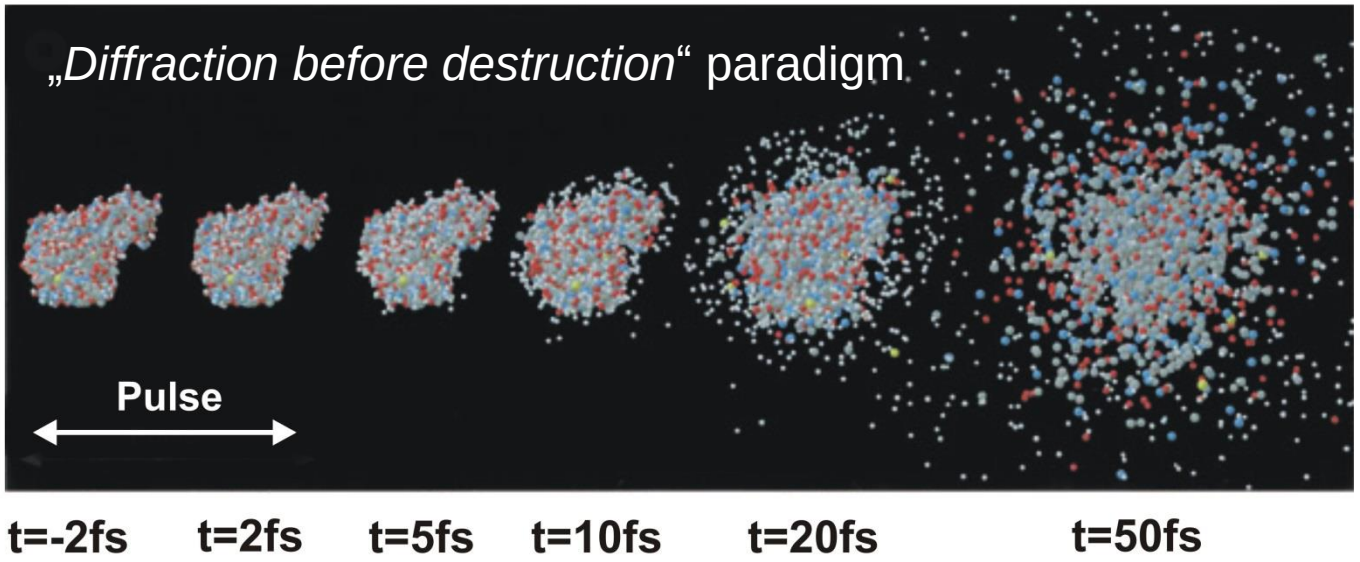
Radiation damage

Sample damage by x-rays limits the resolution of structural studies



Theorie:

A **single diffraction pattern** from a macromolecule may be recorded from a **ultrashort** and **extremely bright** coherent **X-ray pulse** before the sample explodes.

Neutze, R. *et al*., *Nature* 406, 752-757 (2000)

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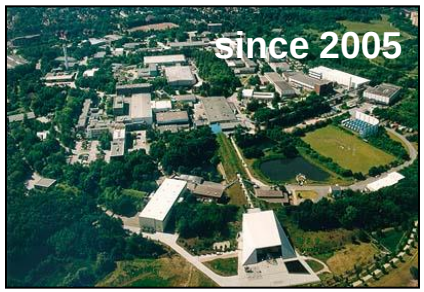
Free-Electron Laser (FEL)

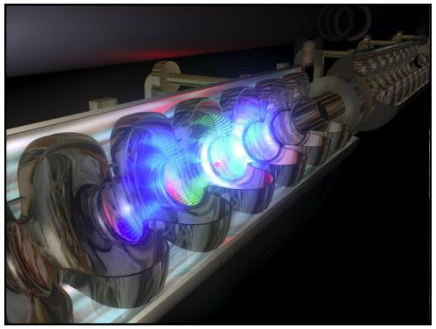


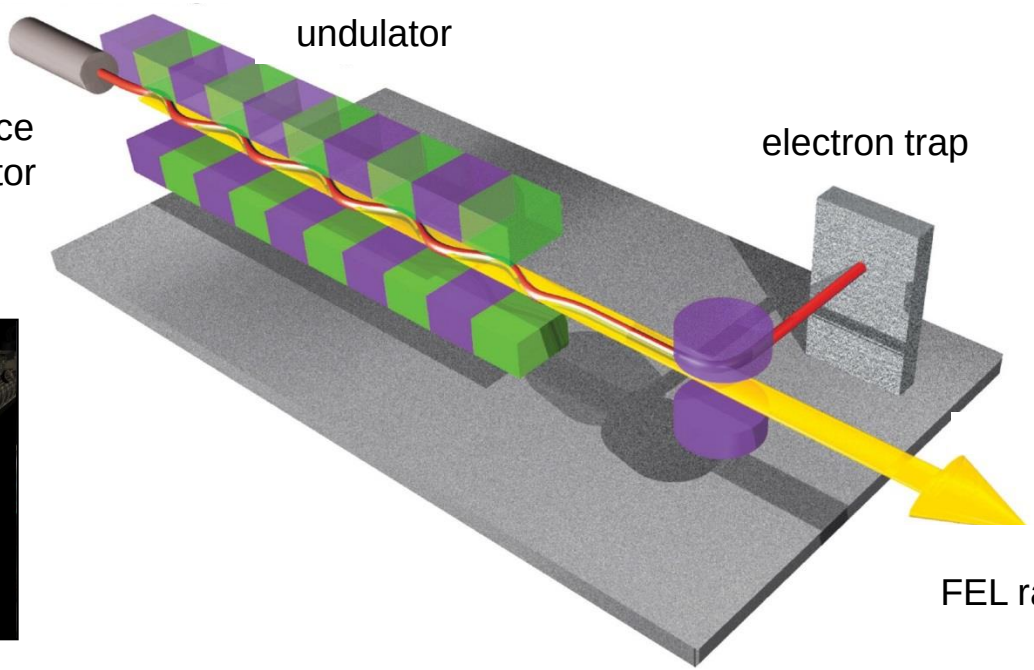
FLASH (DESY/GER)

LCLS (SLAC/USA)

SACLA (RIKEN8/JAP)

EU XFEL (DESY/GER)





Serial Femtosecond Crystallography (SFX)

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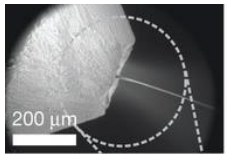
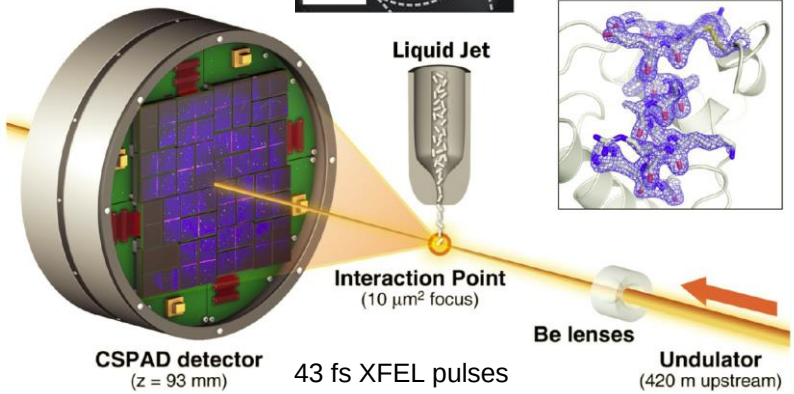
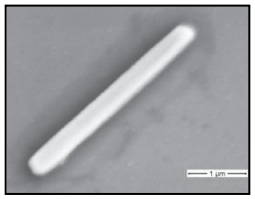
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen



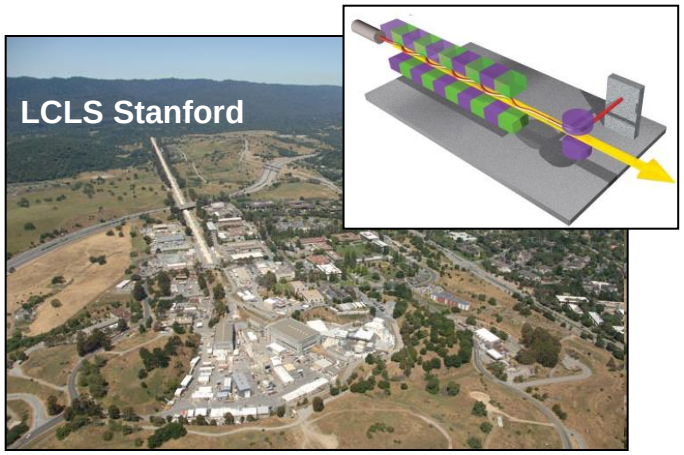


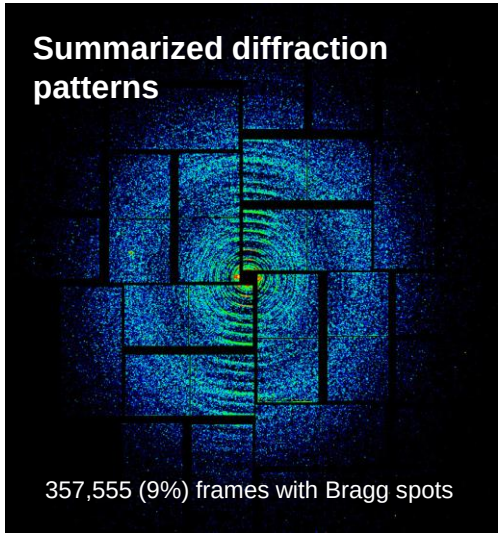
43 fs XFEL pulses
31 MGy sample dose
RT

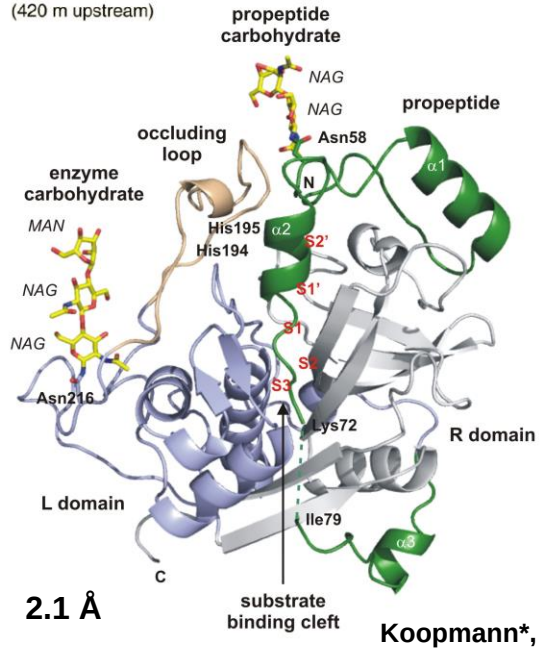
Isolated *in vivo* crystals of cathepsin B (*T. brucei*)



Average crystal:
0.9 x 0.9 x 11 µm
8.9 µm³







- **First new biological structure** solved applying SFX techniques **at a free-electron laser**
- ***In vivo*** grown **crystals** are particularly **suitable for SFX**
- **Is collection of suitable diffraction data also possible using synchrotron radiation?**

L. Redecke*, K. Nass* *et al*. *Science* 339, 227-230 (2013)
Koopmann*, Kupelli*, Redecke* *et al*., *Nat. Methods* 9, 259-262 (2012)

Femtosecond Crystallography Using Large Crystals

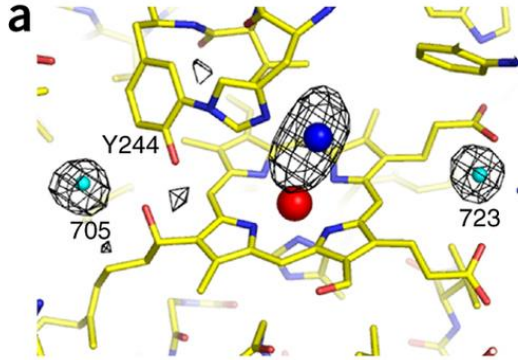
SIAS
Strukturelle Infektionsbiologie unter Anwendung neuartiger Strahlungsquellen

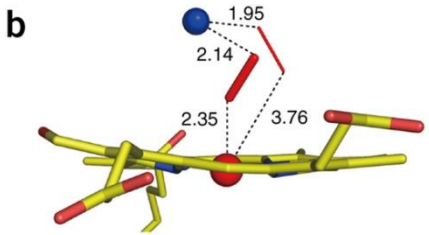
BRIEF COMMUNICATIONS

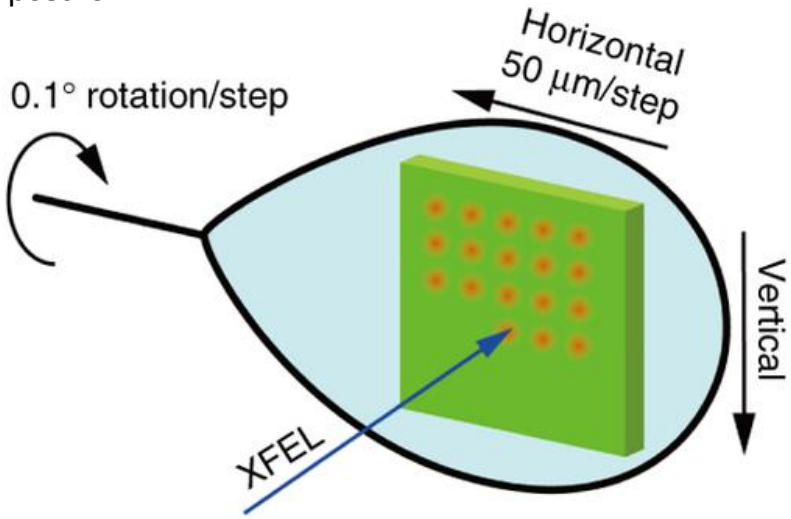
Determination of damage-free crystal structure of an X-ray–sensitive protein using an XFEL

- 1.8 x 1.2 μm² beam size
- 3.5 x 10^{10} ph/s at 10.0 keV
- 10 fs pulse duration
- 76 crystals at 100 K
- 9.9 MGy per exposure

SACLA (RIKEN8/JAP)





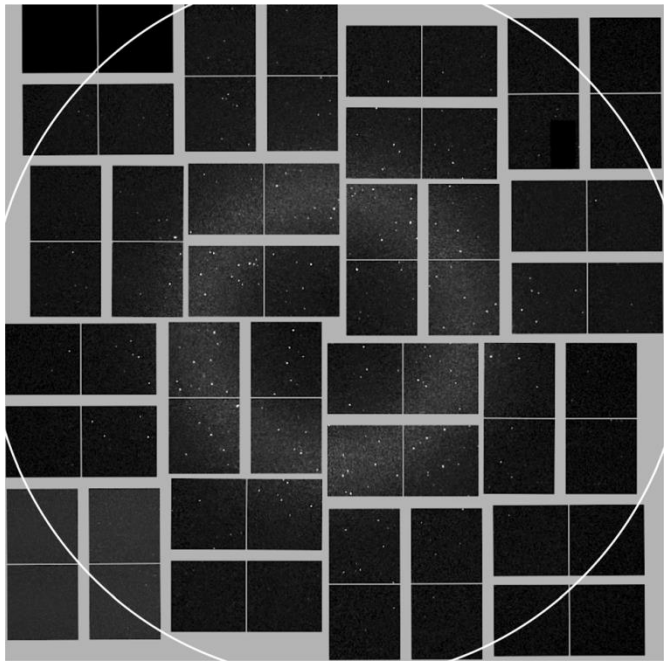


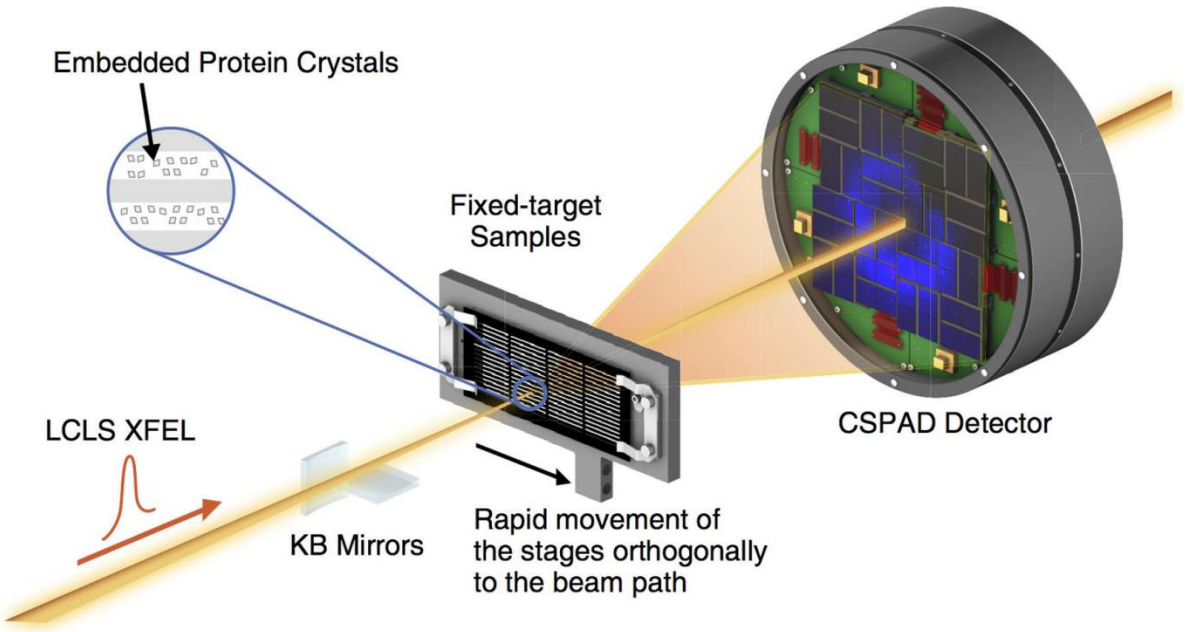
Bovine cytochrome c oxidase

- Data collection from large crystals (100 x 500 μm²)
- Highly sensitive membrane protein
- 1.9 Å radiation damage-free structure, could not be obtained at microfocus synchrotron beamline (Spring-8)

K. Hirata *et al*. *Nat Methods* 11, 734 (2014)

Femtosecond Crystallography with Fixed Crystals



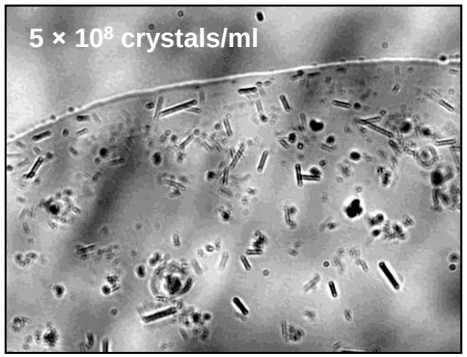


REP24 crystals embedded in paratone N measured *in vacuo* diffracted FEL pulses up to 2.5 Å

MS Hunter *et al*. *Scientific Reports* 4, 6026 (2014)

Serial Synchrotron Crystallography at 100 K

Isolated *in vivo* crystals
of cathepsin B (*T. brucei*)

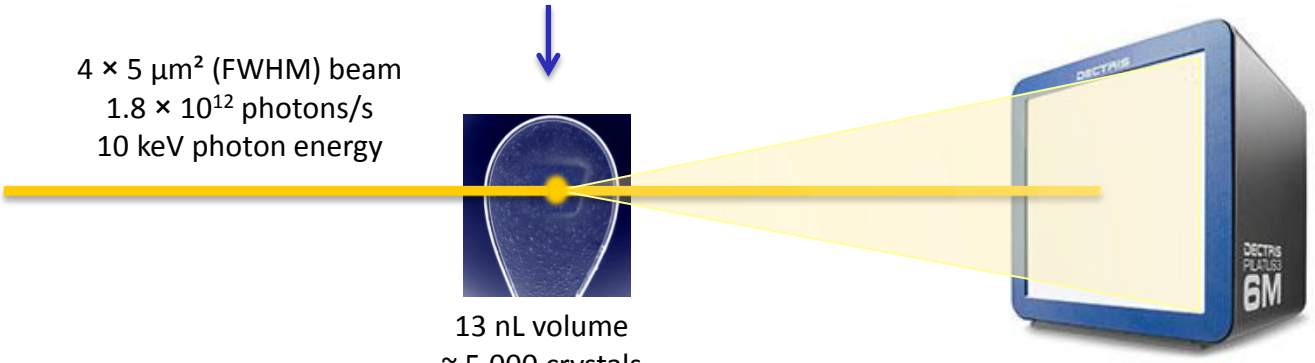


Average crystal:

0.9 x 0.9 x 11 µm = 8.9 µm³

P14 microfocus beamline
@ PETRA III

4 × 5 µm² (FWHM) beam
1.8 × 10¹² photons/s
10 keV photon energy

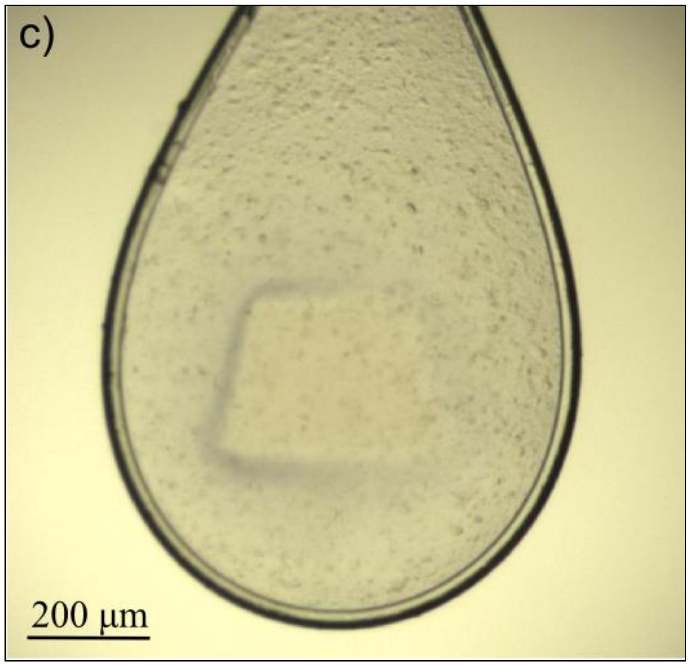


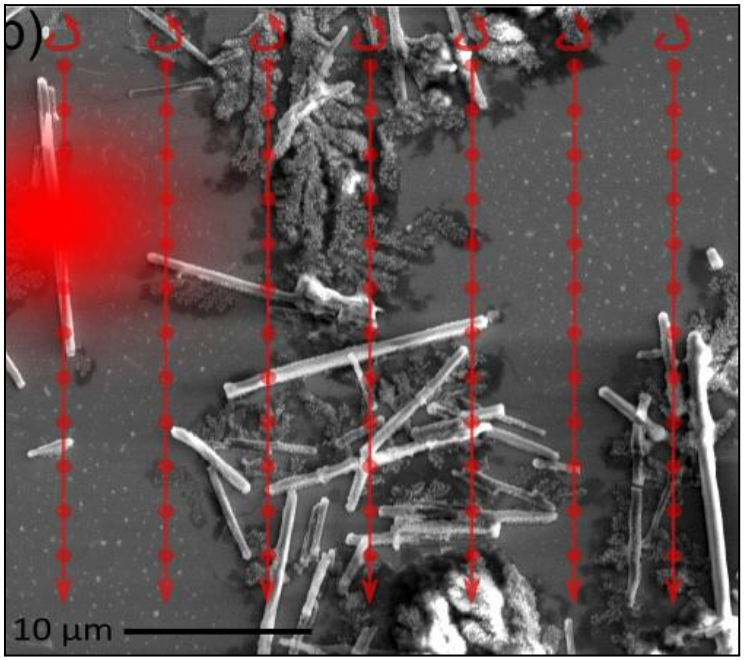
13 nL volume
~ 5,000 crystals

MK3 mini-kappa goniometer head
MD2 microdiffractometer

C. Gati*, G. Bourenkov*,…, and L. Redecke, *IUCrJ* 1 (2014)

Serial Synchrotron Crystallography at 100 K





- **120 parallel helical scans spaced 5 µm** apart
- Goniostat **rotation** from **Ω = -45° to Ω = +45°** and **600 µm translation** during each scan
- **240 exposures for 1 s** -> rotation of 0.375° and translation of 2.5 µm for individual frame
- **Radiation dose of 50 to 60 MGy** for each crystal
- **28,800 detector frames** during 8 hrs

C. Gati*, G. Bourenkov*,…, and L. Redecke, *IUCrJ* 1 (2014)

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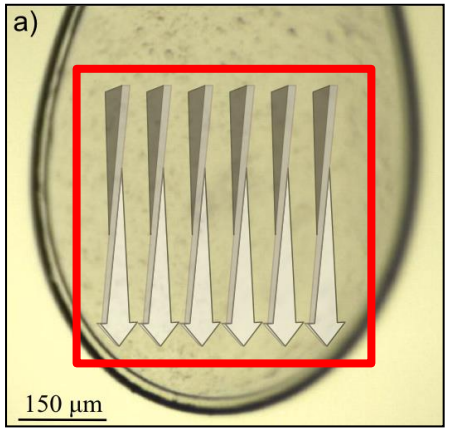
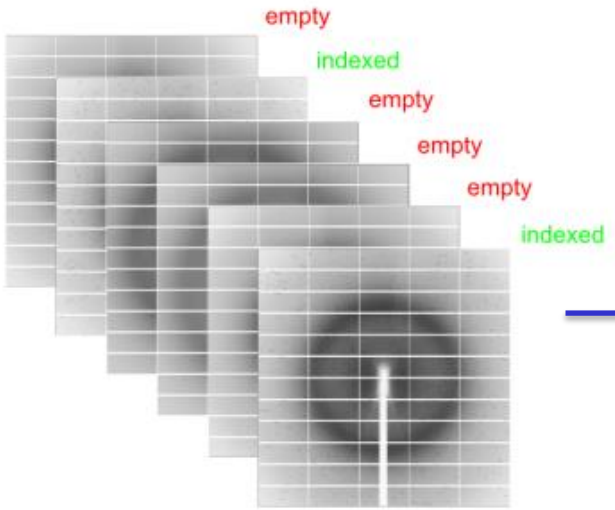
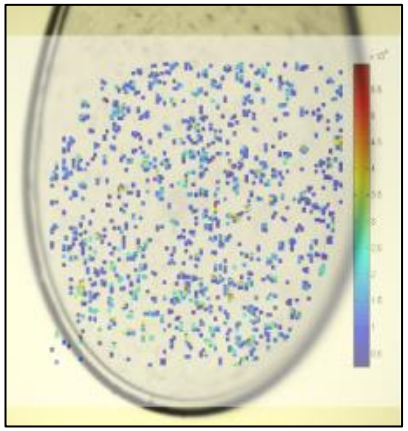
Serial Synchrotron Crystallography at 100 K







Serial helical line scans of randomly orientated crystals

Detector frames

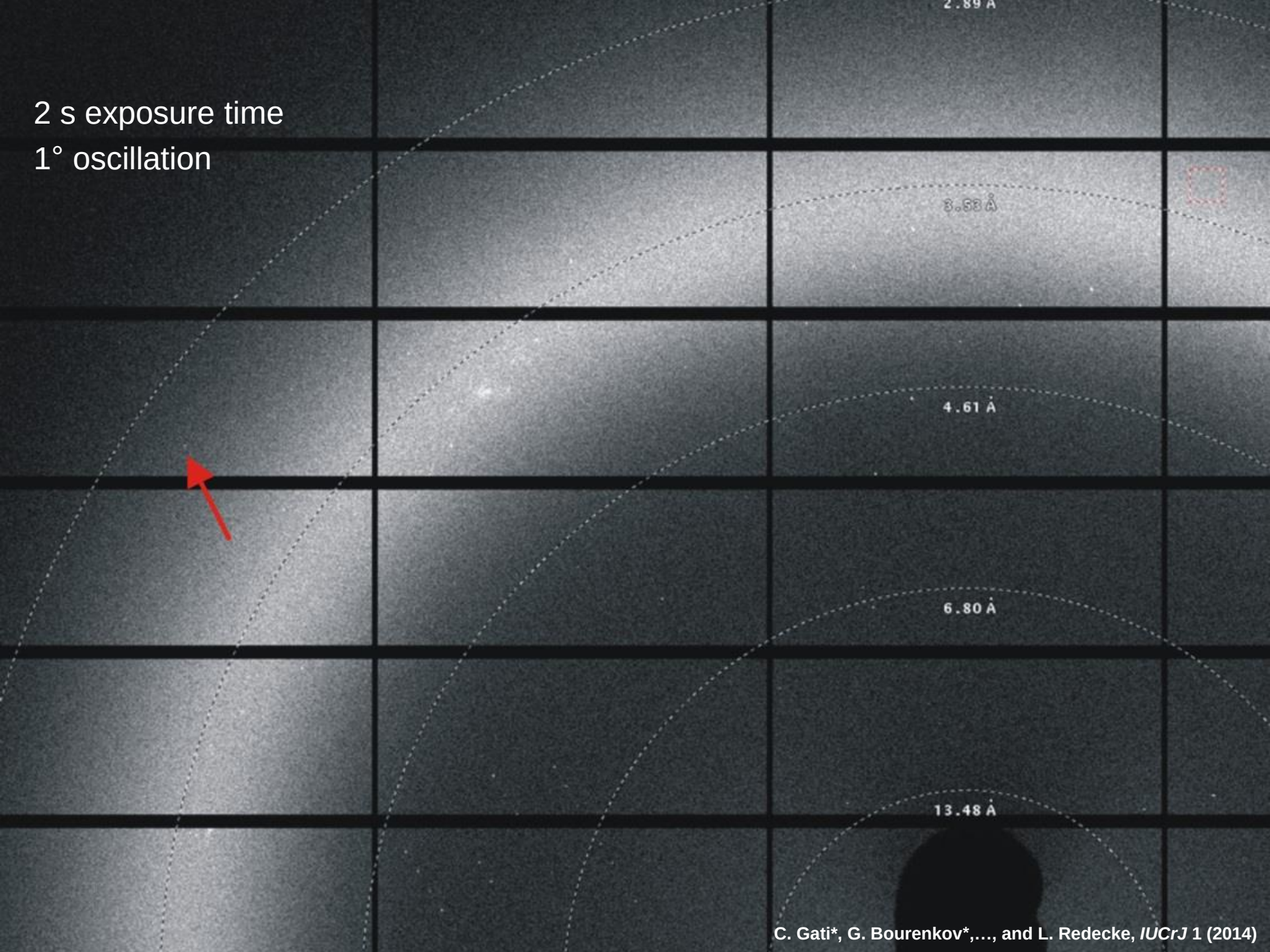
Identification of idexable diffraction images using

- **2,233** out of 28,800 **patterns were indexable** by CrystFEL
- **595 groups of adjacent diffraction images** containing 2 to 10 frames defined -> **regular rotation data (XDS)**
- Final dataset: **109,661 reflection intensities** (88 - 3.0 Å) from **426 diffraction patters** from only **80 crystals**

CrystFEL

T. A. White *et al*. J. Appl. Cryst. 45, 335-341 (2012)

C. Gati*, G. Bourenkov*,…, and L. Redecke, *IUCrJ* 1 (2014)



C. Gati*, G. Bourenkov*,…, and L. Redecke, *IUCrJ* 1 (2014)

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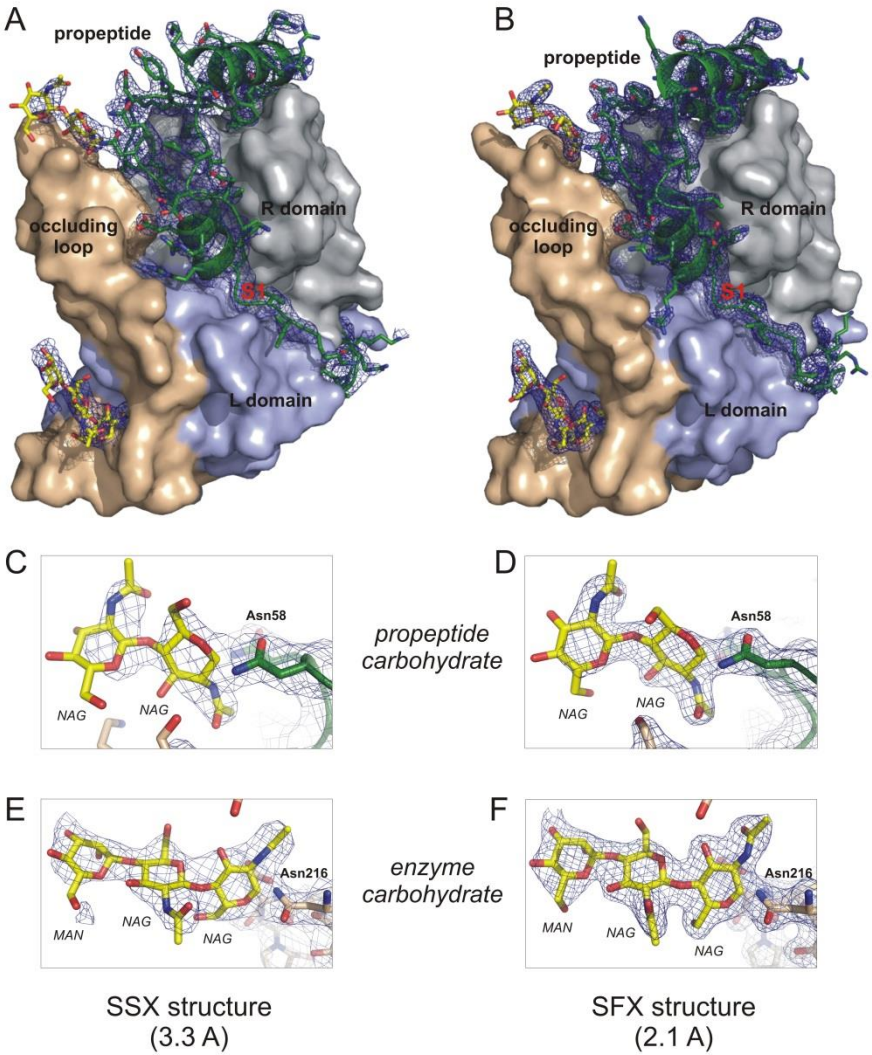
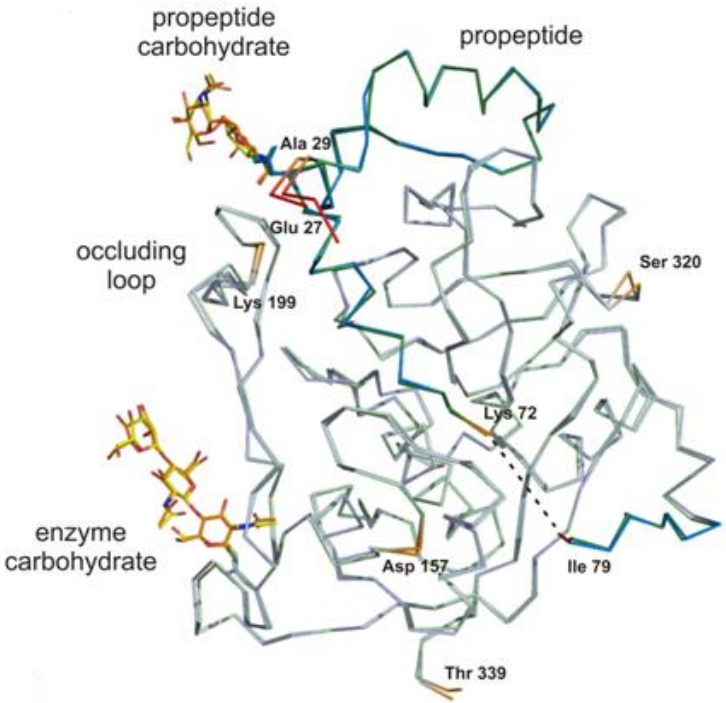
Serial Synchrotron Crystallography at 100 K

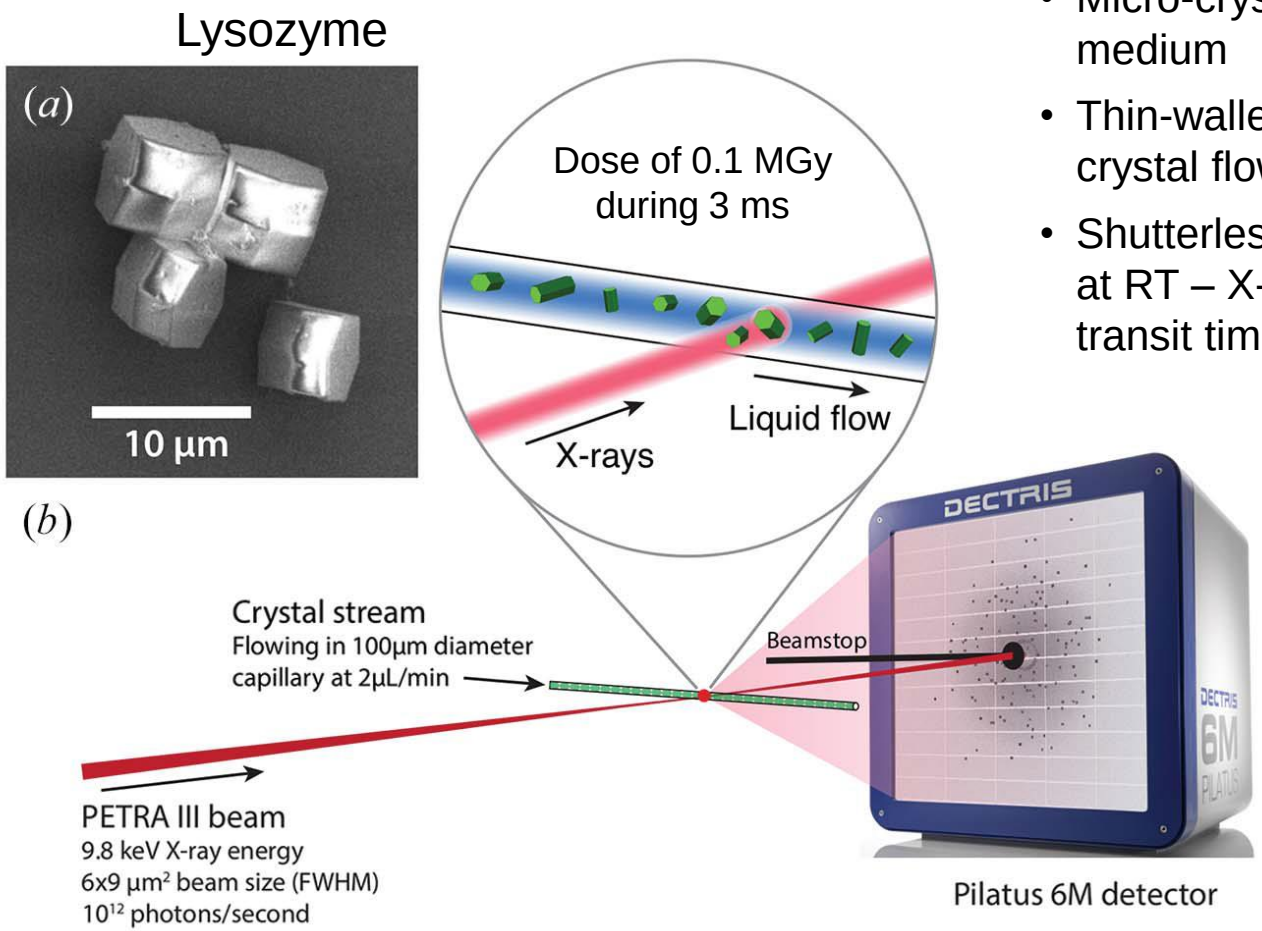




- model superimposable to 2.1 Å structure solved by SFX at an FEL
- no radiation damage!

C. Gati*, G. Bourenkov*,…, and L. Redecke, *IUCrJ* 1 (2014)

Serial Synchrotron Crystallography at RT



- Micro-crystals suspended in growth medium
- Thin-walled capillary with continuous crystal flow
- Shutterless diffraction data collection at RT – X-ray dose depends on transit time through the X-ray focus

Problem:

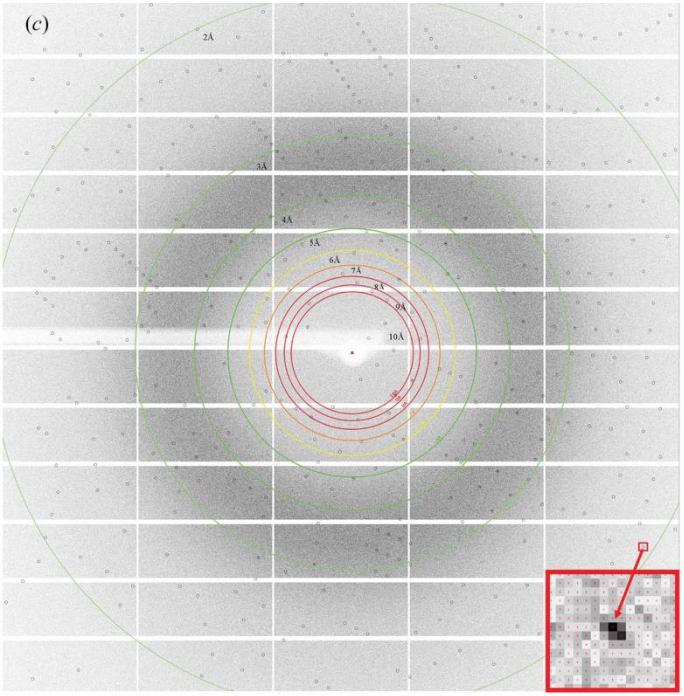
Crystal settling in the sample reservoir

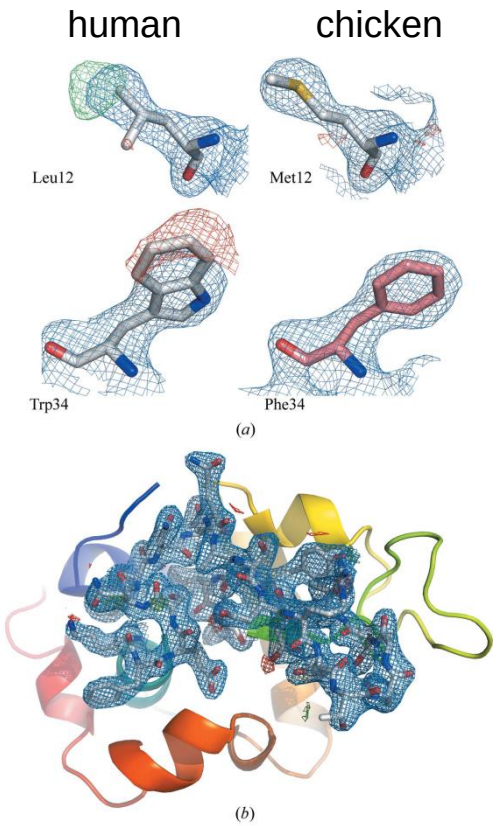
-> increase viscosity!

Crystal flow by syringe pump

F. Stellato *et al*. *IUCrJ* 1 (2014)

Serial Synchrotron Crystallography at RT



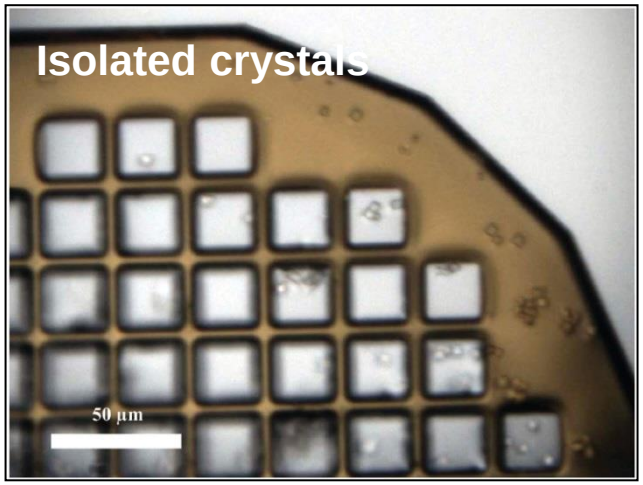


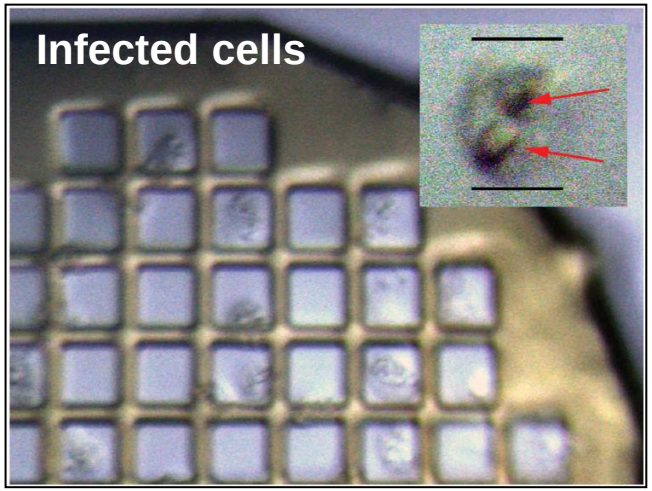
Lysozyme @ 2.1 Å

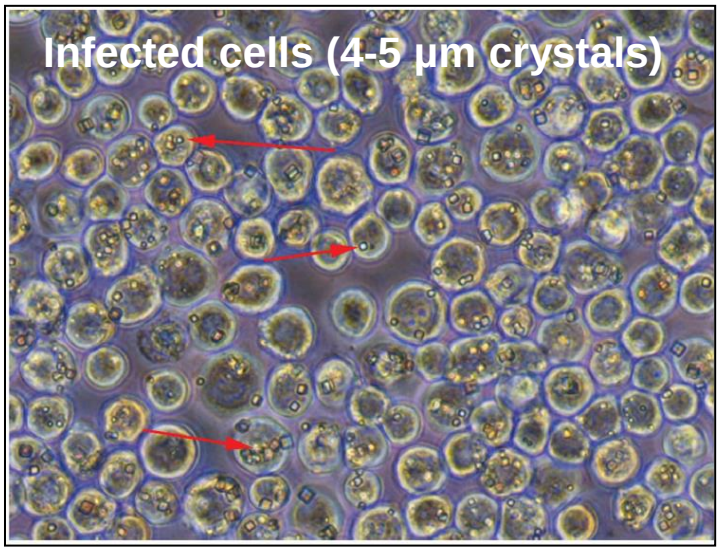
- 40.233 indexed individual diffraction patters (from > 1 million recorded patterns) during 17h data collection time
- Sample consumption of 2.5 ml crystal suspension (250 mg protein)
- Processing of diffraction frames using CrystFEL

F. Stellato *et al*. *IUCrJ* 1 (2014)

In cellulo Structure Determination by Serial Synchrotron Crystallography







- Insect cells infected with cytoplasmatic polyhedrosis virus (CPV) form spontaneous polyhedrin crystals *in vivo*
- Frozen live cells directly mounted in the X-ray beam, cryo-protectant: ethylene glycol
- Crystals are maintained in a biologically relevant environment!

D. Axford *et al*. *Acta Cryst* D70, 1435 (2014)

In cellulo Structure Determination by Serial Synchrotron Crystallography

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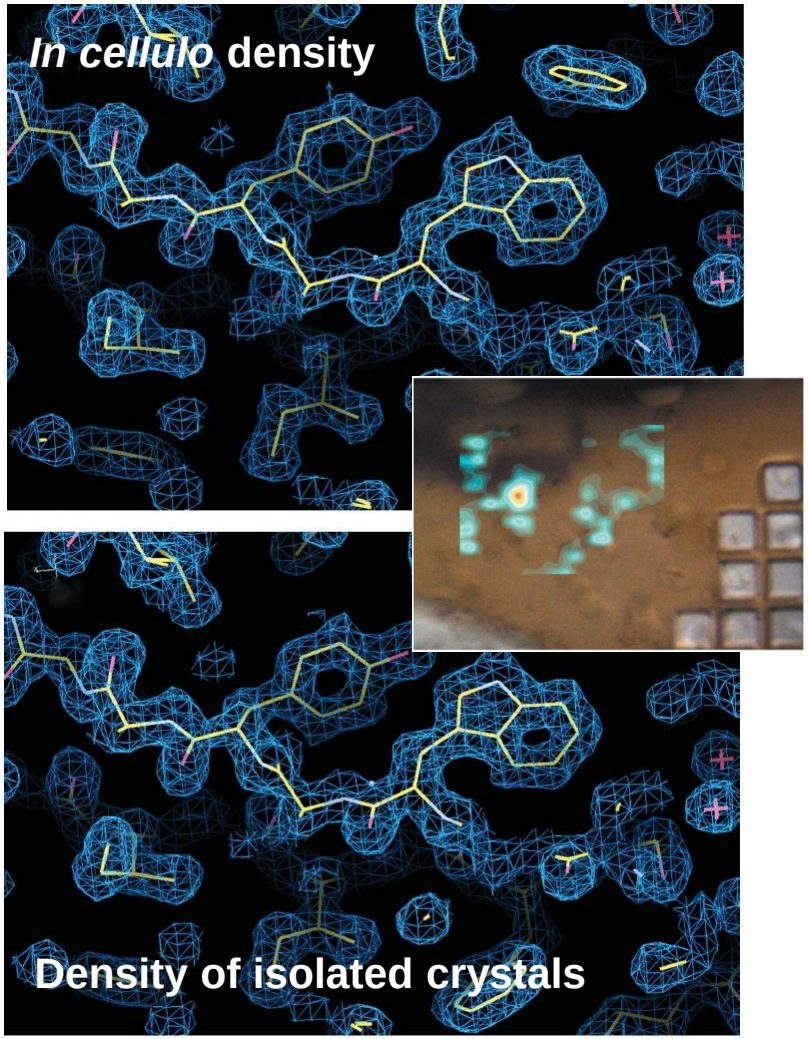
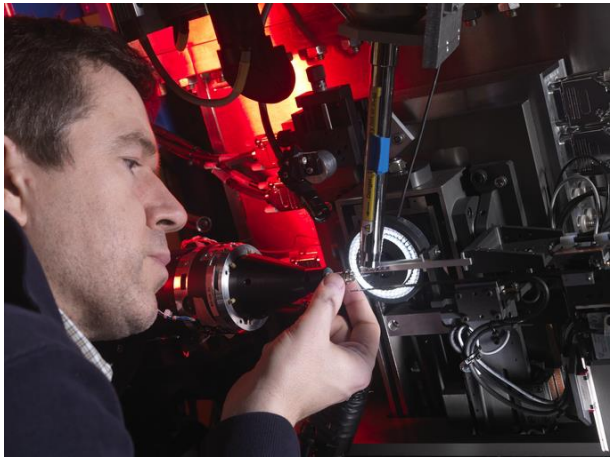


Beamline I24
@DIAMOND

- **6 x 6 µm² beam**
- **2 x 10^{11} ph/s at 12.8 keV**
- **1.4 MGy/s at each crystal**



- Crystal location by raster scan of sample loop
- 12 data collection points *in cellulo*
- 40 images of 2° of data with 0.5 s exposure recorded at each point
- Isolated crystals: 26 partial data sets consisting of 30 images of 3°, exposure 0.25 s

Similar datasets for *in cellulo* measurements and isolated crystals

D. Axford *et al*. *Acta Cryst* D70, 1435 (2014)