# Small is beautiful

# Protein micro-crystallography at SLS

**MX Frontiers at the One Micron Scale**

**NSLS II at BNL, USA, July, 23rd 2009**

**Clemens Schulze-Briese, SLS at PSI**

# Outline

- Introduction to SLS and its MX beamlines
- Instrumentation
- Ultralente Insulin
- Crystal Structure of Cypovirus Polyhedrin
- Challenges in 1 micron protein crystallography

# The SLS

2.4 GeV, 288 m circumference , 12 straight sections

| | |
|---|---|
| low emmittance | 5.5 (7.3) nm·rad |
| high current | 400 mA |
| long straight sections | 4, 7, 11 m |
| low energy undulator radiation | 8 eV |
| high energy undulator radiation | 20 (35) keV |
| booster in storage ring tunnel | 270 m, 9 nm·rad |

top-up injection

variable polarisation

high beam stability

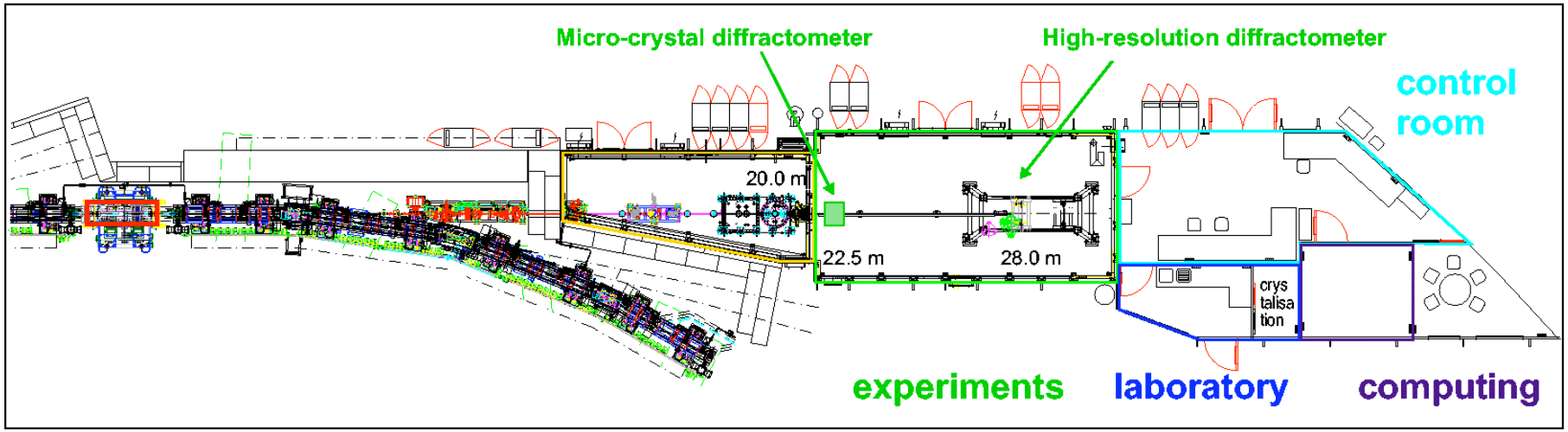
Femto second pulses by bunch slicing

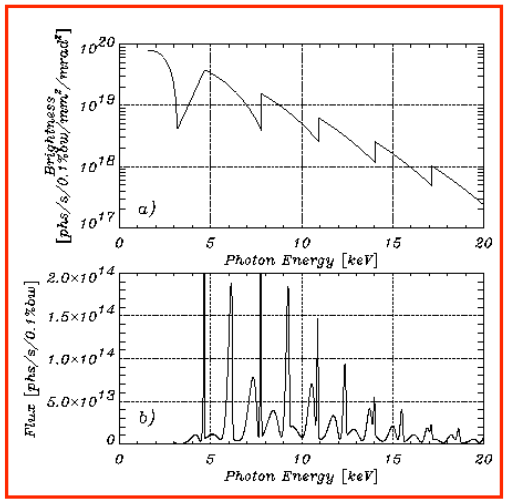
# o Introduction

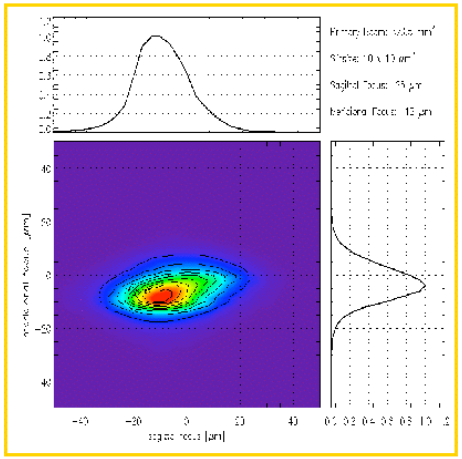
**High brightness in-vacuum undulator radiation**

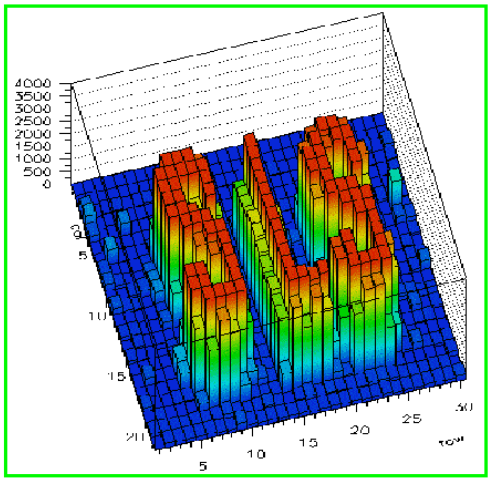
**Decoupled, dynamically focussing optics**

**Fast read-out, large area Pixel Detector**

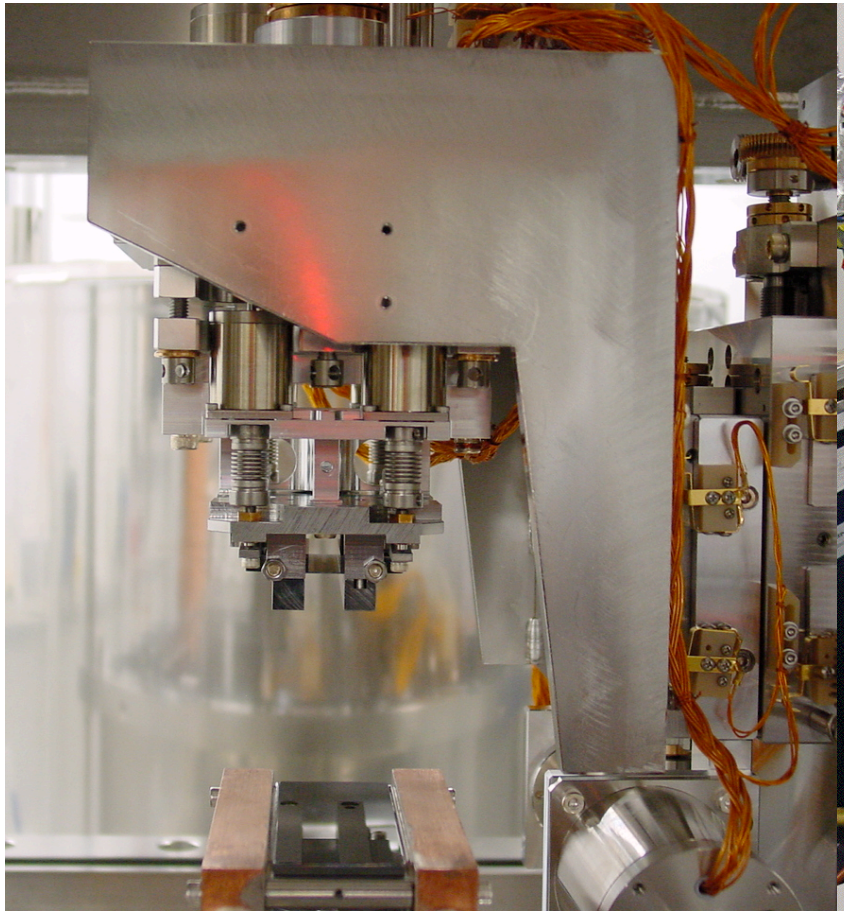


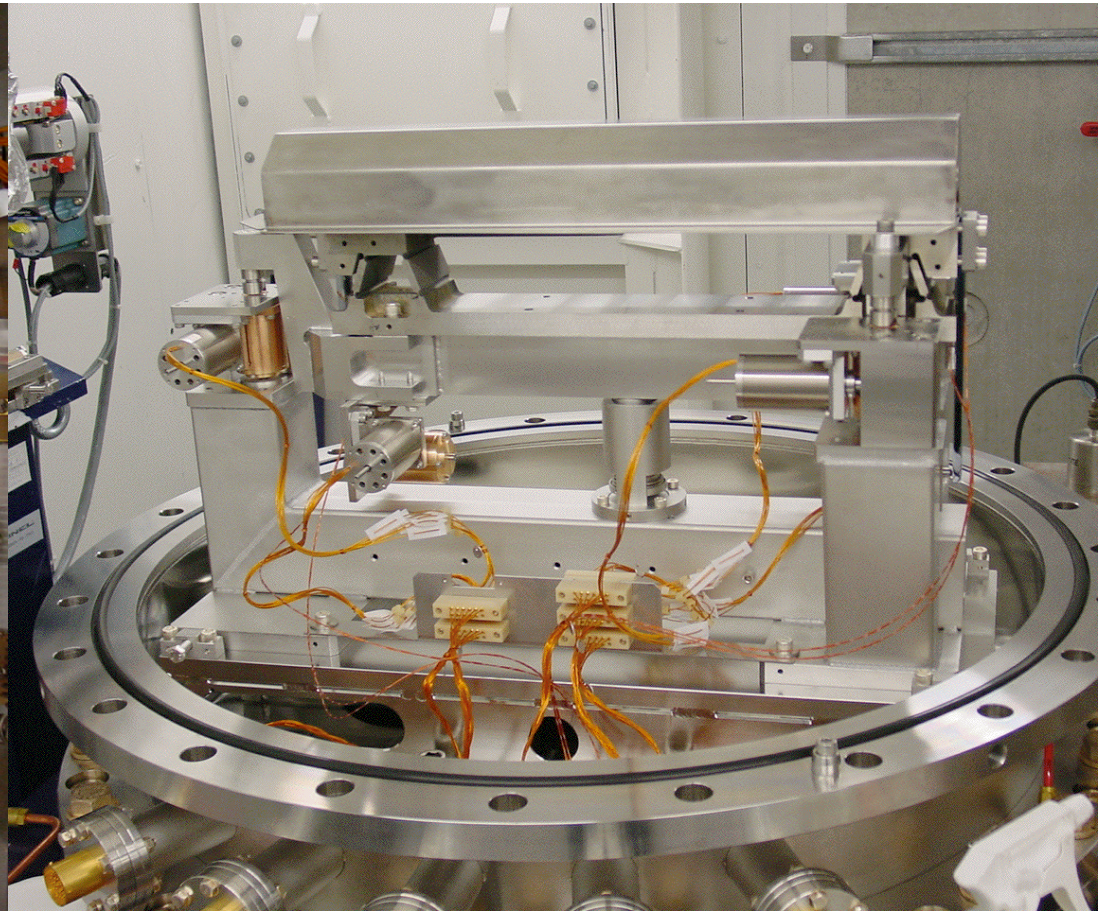





# Dynamically focussing optics





DCM with Sagittal Bender

Meridionally focussing bent mirror

# Emittance conserving optics

| | | Horizontal | | Vertical | |
|---|---|---|---|---|---|
| | | Focus [μm] | Emmit. [nm ·rad] | Focus* [μm] | Emit. [pm · rad] |
| Pre-chicane | X06SA | 82.5 | 4.93 | 5.4 | 38.8 |
| | X10SA | 49.1 | 5.03 | 4.3 | 90.2 |
| Post-chicane | X06SA | 87.0 | 5.48 | 13.2 | 242 |
| Modulator | X10SA | 52.2 | 5.72 | 6.8 | 225 |
| OPEN | X10SA | | | 2.1 | 20.7 |
| Modulator | X06SA | | | | |
| CLOSED | X10SA | 59.1 | 7.3 | 9.9 | 477 |

# High Resolution Diffractometer with PILATUS 6M

- 2527 x 2463 pixel
- 172 µm pixel size
- 20 bit counter
- 1 MHz / pixel
- 5 ms read out time
- 12.5 Hz frame rate
- 8.4 % dead area

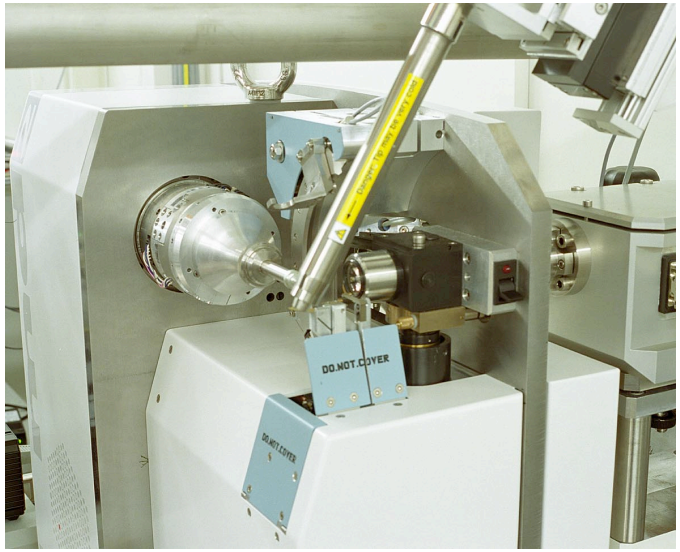
# High Resolution Diffractometer with PILATUS 6M

*AChBP as Template for nAChR binding Pocket*

acceptable merging statistics was obtained to 3.3 Å resolution. The crystal belongs to space group $P3_121$ and has the following cell dimensions: $a = 76.81$ Å, $b = 76.81$ Å, $c = 725.52$ Å, $\alpha = 90.0°$, $\beta = 90.0°$, $\gamma = 120.0°$. The structure was solved by molecular replacement using MOLREP and the open C-loop structure of Ac-AChBP as the search model (PDB accession code 2c9t). The initial model was refined with REFMAC[39] using NCS and TLS[40] restraints. A second data set with diffraction data beyond 2.8 Å resolution was collected at beamline X06SA at the Swiss Light Source, Villigen Switzerland. Several technical measures were required to minimize spot overlaps along the 725 Å axis, including alignment of the crystal with the long cell axis along the rotation axis, reduction of the beam size, and focusing of the beam onto the detector. Data were processed with EVAL15[41] to correct for residual spot overlaps. The model was further refined with REFMAC to $R_{work} = 23.7\%$ and $R_{free} = 26.7\%$.

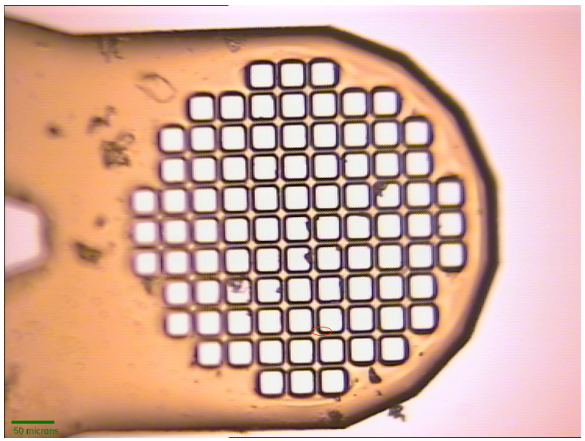
C. Ulens et al., J. Med. Chem., 2009

# Micro-diffractometer setup at X06SA




- 6 keV – 17.3 keV
- Flux (12.66 keV): 1.05 $\cdot 10^{12}$ pht/s
- Energy resolution: 0.02 %
- Spot size: 25 (15) µm x 5 µm
- Detector: mar225

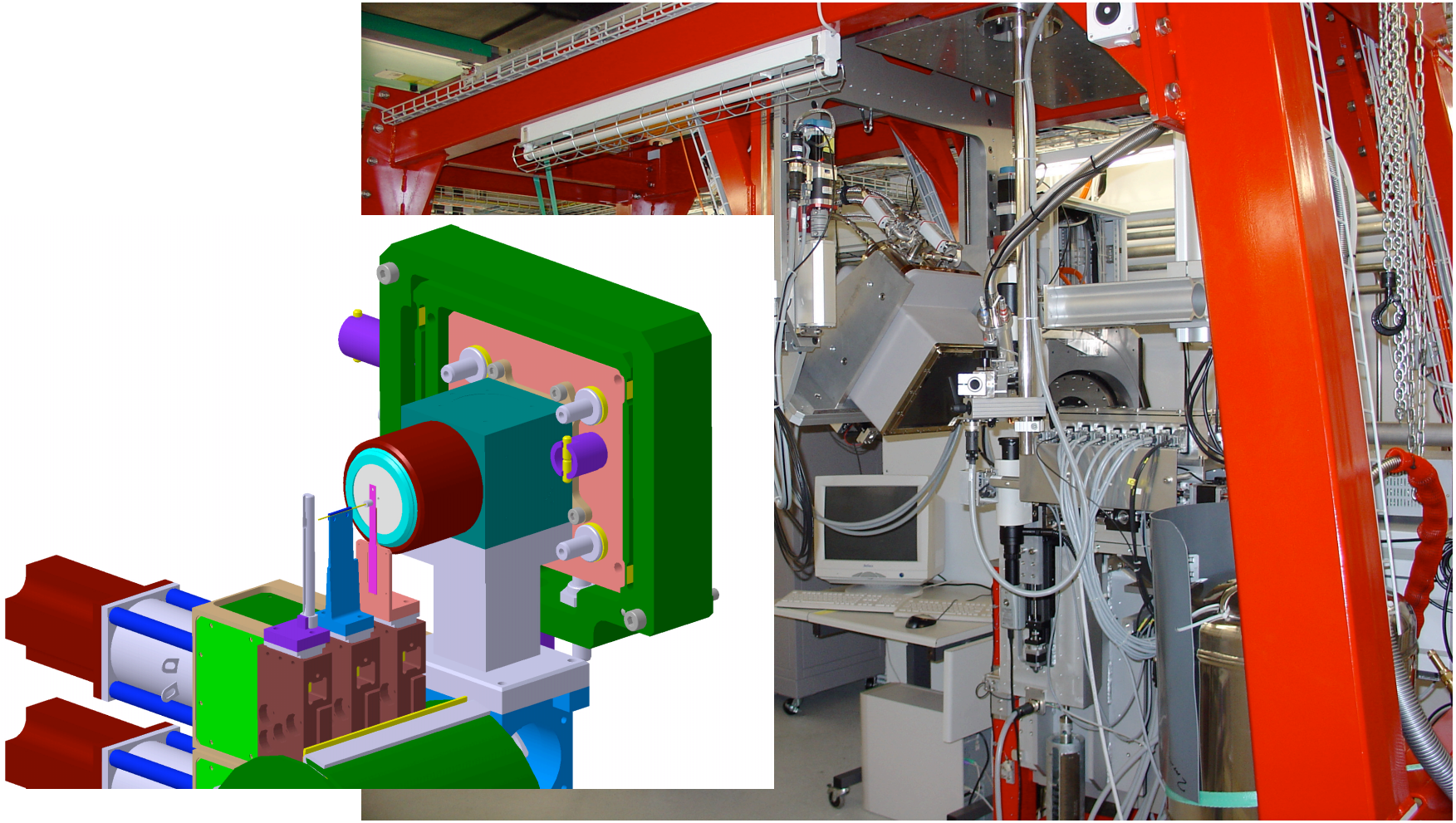
## Mounting crystals



- Change between Diffractometers:

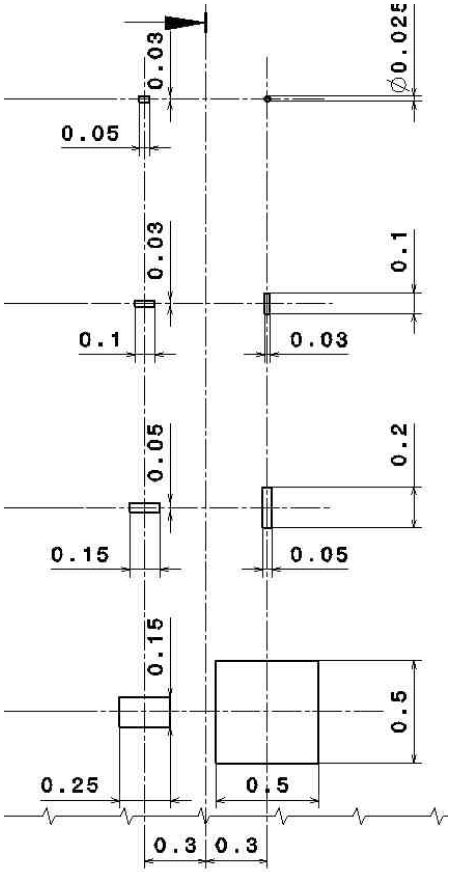
30 - 45 min

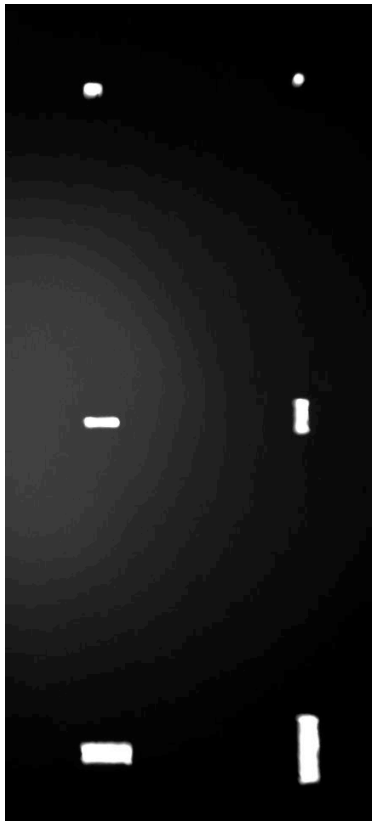
# SLS on-axis system at X10SA



# Apertures for beam shaping

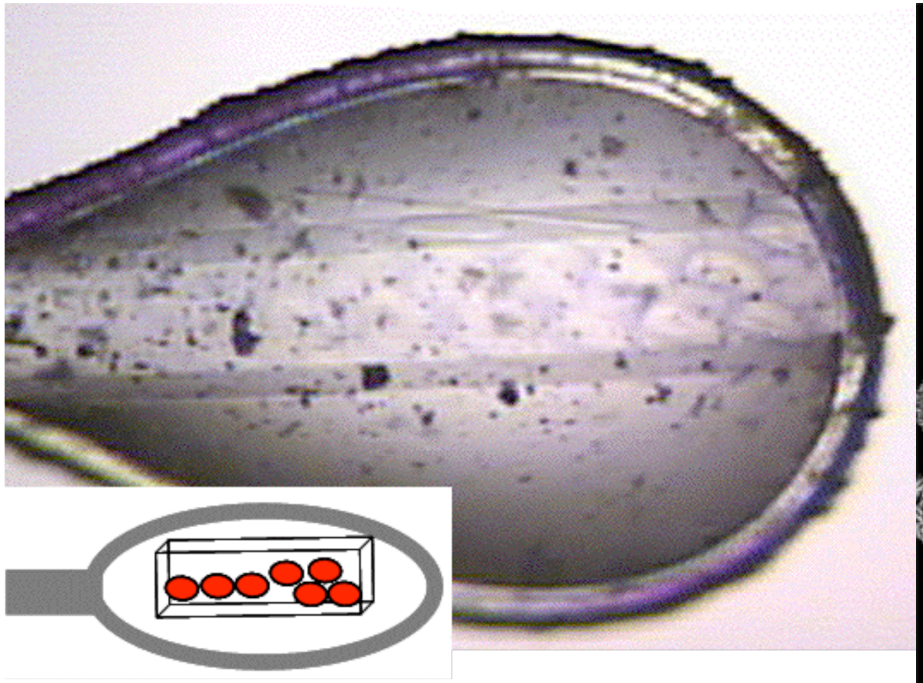
| Nr. | h (µm) | v (µm) |
|---|---|---|
| 1 | 50 | 30 |
| 2 | 25 | 25 |
| 3 | 100 | 30 |
| 4 | 30 | 100 |
| 5 | 150 | 50 |
| 6 | 50 | 200 |
| 7 | 250 | 150 |
| 8 | 500 | 500 |



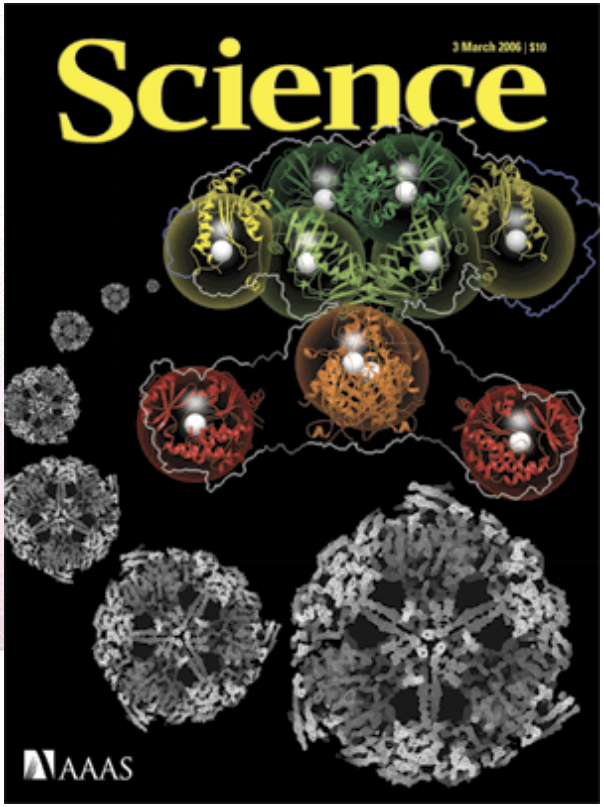



## o Examples

All crystallographic data were measured at the PX beamline X06SA at the Swiss Light Source (SLS), Switzerland, using the microdiffractometer in the case of the needle-like crystal form (15 x 15 x 150 $\mu m^3$ ) ,



after 18 translations at SLS MD2



# Ultralente

- Introduction in 1953
- Suspension of rhombohedrally shaped human insulin microcrystals
- Long duration of action
- Effect starts 4 hours after sub-cutanous injection
- Maximum between 8 and 24 hours
- Stops after 28 hours

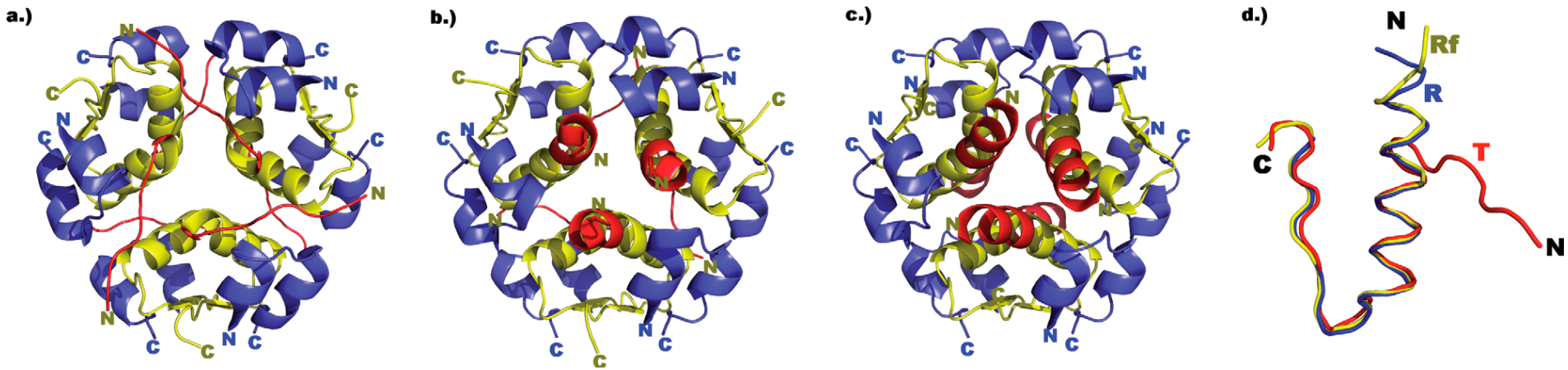
- *A. Wagner et al., Proteins, 2009*

# Ultralente

- Pharmacokinetics to large extend determined by dissociation of Zinc containing hexamer to biologically active insulin monomer.

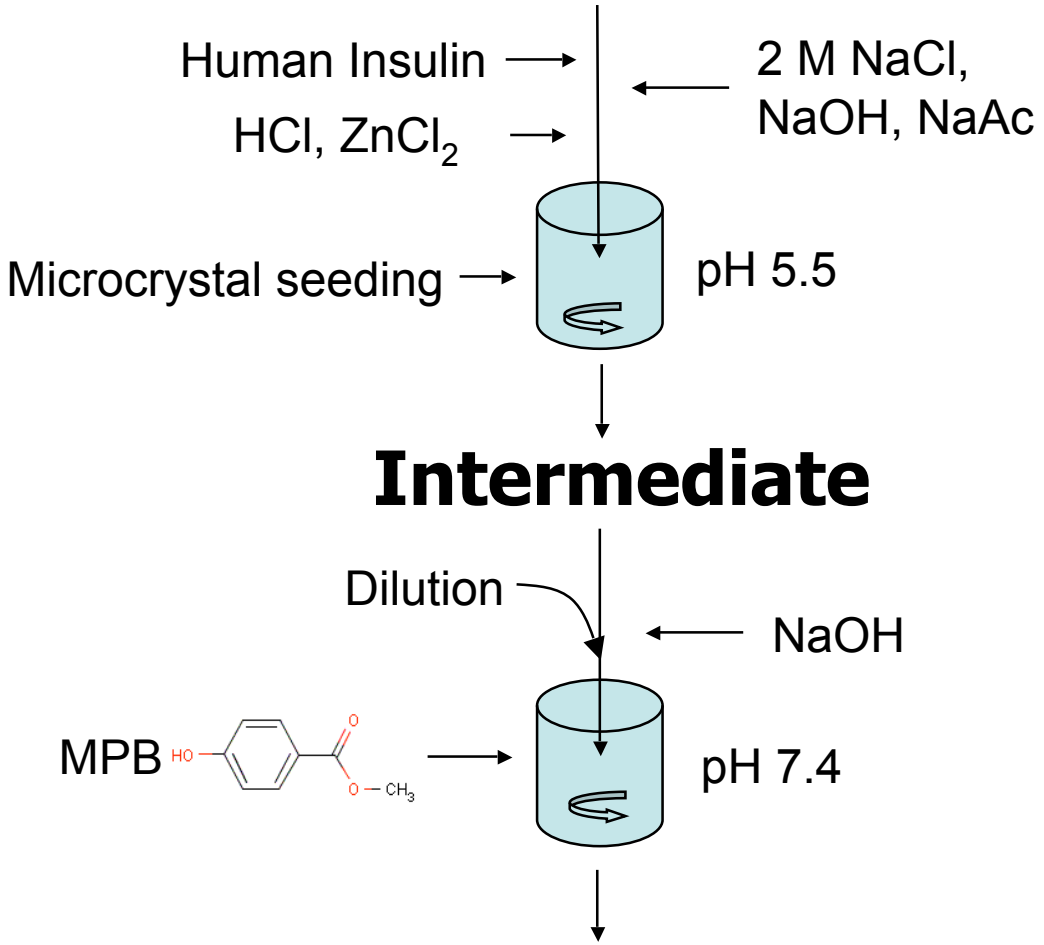
- Cell constants determined by powder diffraction.
- Hexameric arrangement of insulin dimers investigated by AFM.
- Because of the small size of the crystals (25 x 25 x 5 $\mu m^3$) structure characterization has been elusive so far.

# The three different conformational states of the insulin hexamer



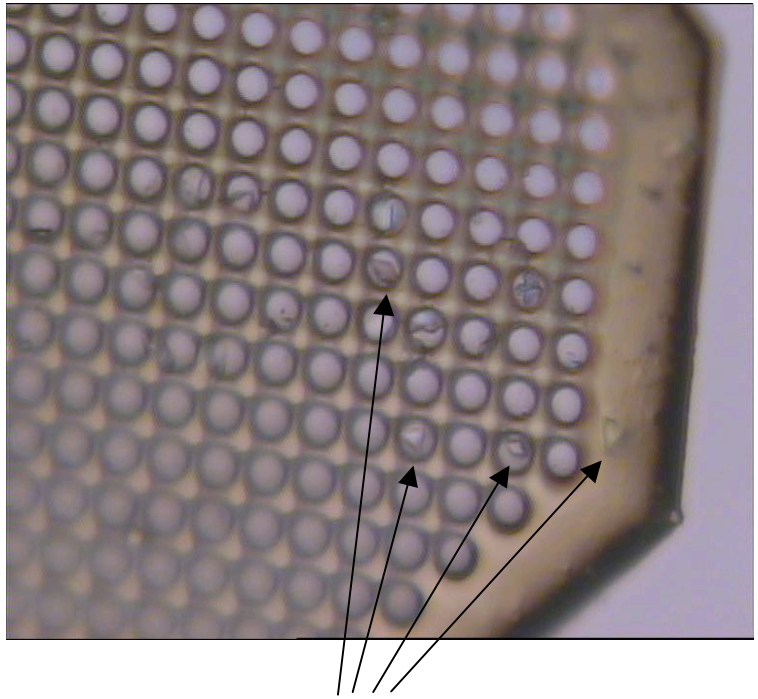

$T_6$ $T_3R_3^f$ $R_6$

# Industrial Ultralente Production

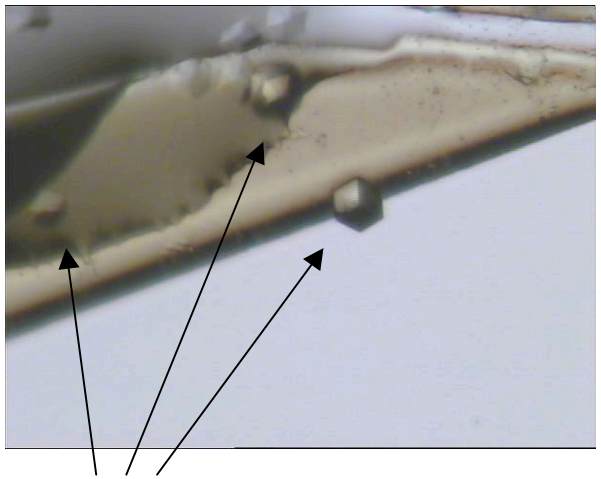



Flow chart (simplified)

# Microcrystals



Precursor crystals
(8 x 8 x 3 $\mu m^3$)



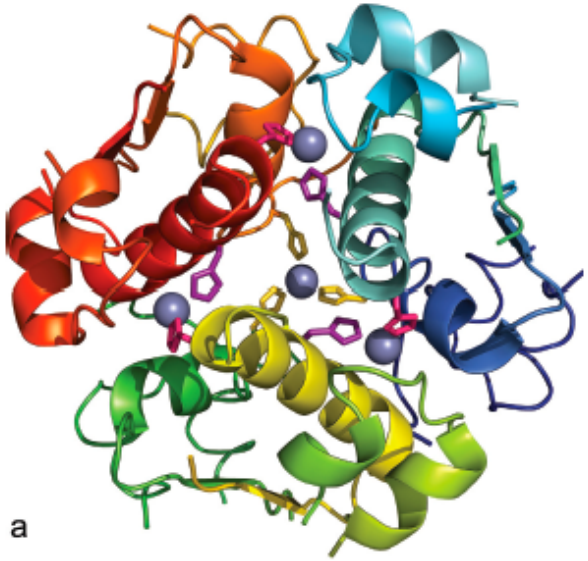
Ultralente
(25 x 25 x 5 $\mu m^3$)

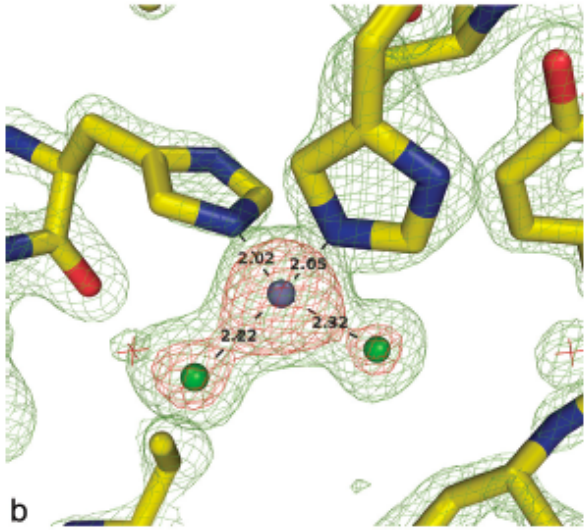
# Processing and refinement

| | Ultralente | Precursor |
|---|---|---|
| Fold | $T_6$ | $T_3R_3^f$ |
| Crystal size ($\mu m^3$) | 25 × 25 × 5 | 8 × 8 × 3 |
| Number of crystals | 1 | 3 |
| Beam transmission | 30% | 23% (2 crystals), 100% (1 crystal) |
| Wavelength (Å) | 0.9786 | 0.9183 |
| Space group | R3 | R3 |
| Cell parameters (*a*; *c*) (Å) | 81.03; 33.90 | 79.82; 36.71 |
| Resolution range (Å) | 41.0–2.2 (2.3–2.2) | 33–1.8 (1.9–1.8) |
| Total number of reflections | 13231 (1707) | 33133 (2368) |
| Number of unique reflections | 3958 (537) | 7433 (1035) |
| *I*/sigma (*I*) | 11.8 (5.7) | 9.5 (1.8) |
| Completeness (%) | 98.8 (98.7) | 96.8 (84.4) |
| $R_{merge}$[a] (%) | 7.2 (20.9) | 10.5 (53.7) |
| Refinement statistics | | |
| Reflections used in refinement | | |
| Resolution range | 40.5–2.2 | 32.4–1.8 |
| No. of used reflections | 3958 | 7433 |
| Selected for $R_{free}$ | 209 | 391 |
| $R_{cryst}$[b]/$R_{free}$[b] (%) | 24.0/28.2 | 17.3/23.9 |

# Main Results - Precursor

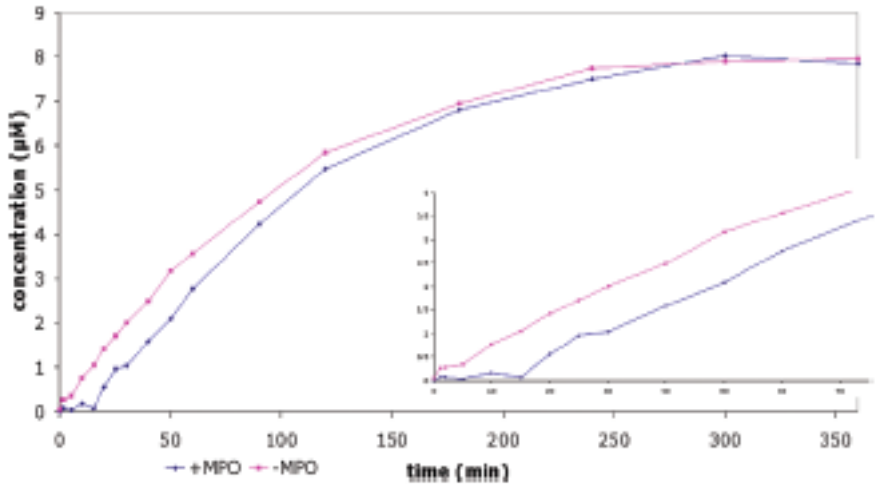
- 4 Zn cations per hexamer in precursor with off-axis binding site coordinated by His B10 and His B5 and 2 chloride anions
- $T_3R_3^f$ to $T_6$ transition in microcrystals (c-axis change from 36.7 to 33.9 Å) yields crack-free crystals

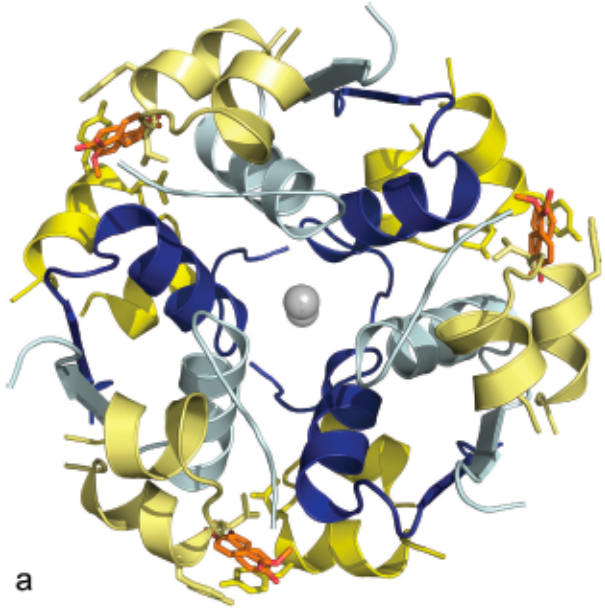


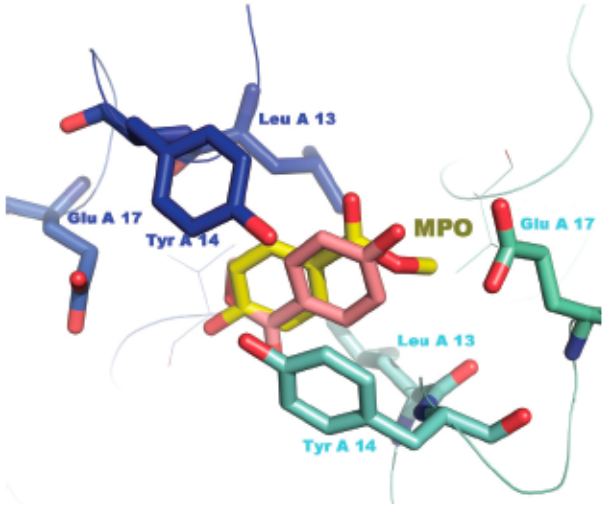



# Main Results - Ultralente

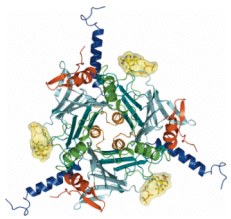
- Methylparaben (MPO) bound to Ultralente at dimer-dimer interface
- Bound MPO delays dissolution



# Viral Polyhedra



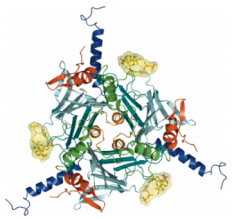
- Virus-containing protein crystals produced by ubiquitous insect pathogens
  - Polyhedra in Baculoviruses, Cypoviruses
  - Spheroids in Entomopoxviruses

- Puzzle for virologists…
  - Identified in the early 1900s
  - Visible by light microscopy
  - Mostly protein
  - Remain infectious for years like bacterial spores

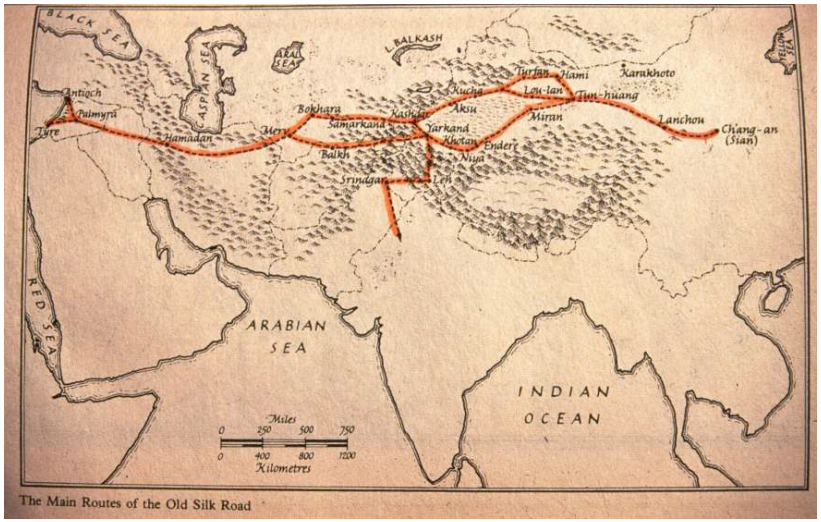
- and biochemists alike
  - Up to 50% of the cell
  - Crystals forming *in vivo*
  - Remarkable robustness
  - Structure?

Gulam Manji,
University of Wisconsin
(Manji & Friesen, J. Biol. Chem., 2001)

# Cytoplasmic & Nuclear Polyhedrosis Virus (CPV & NPV)

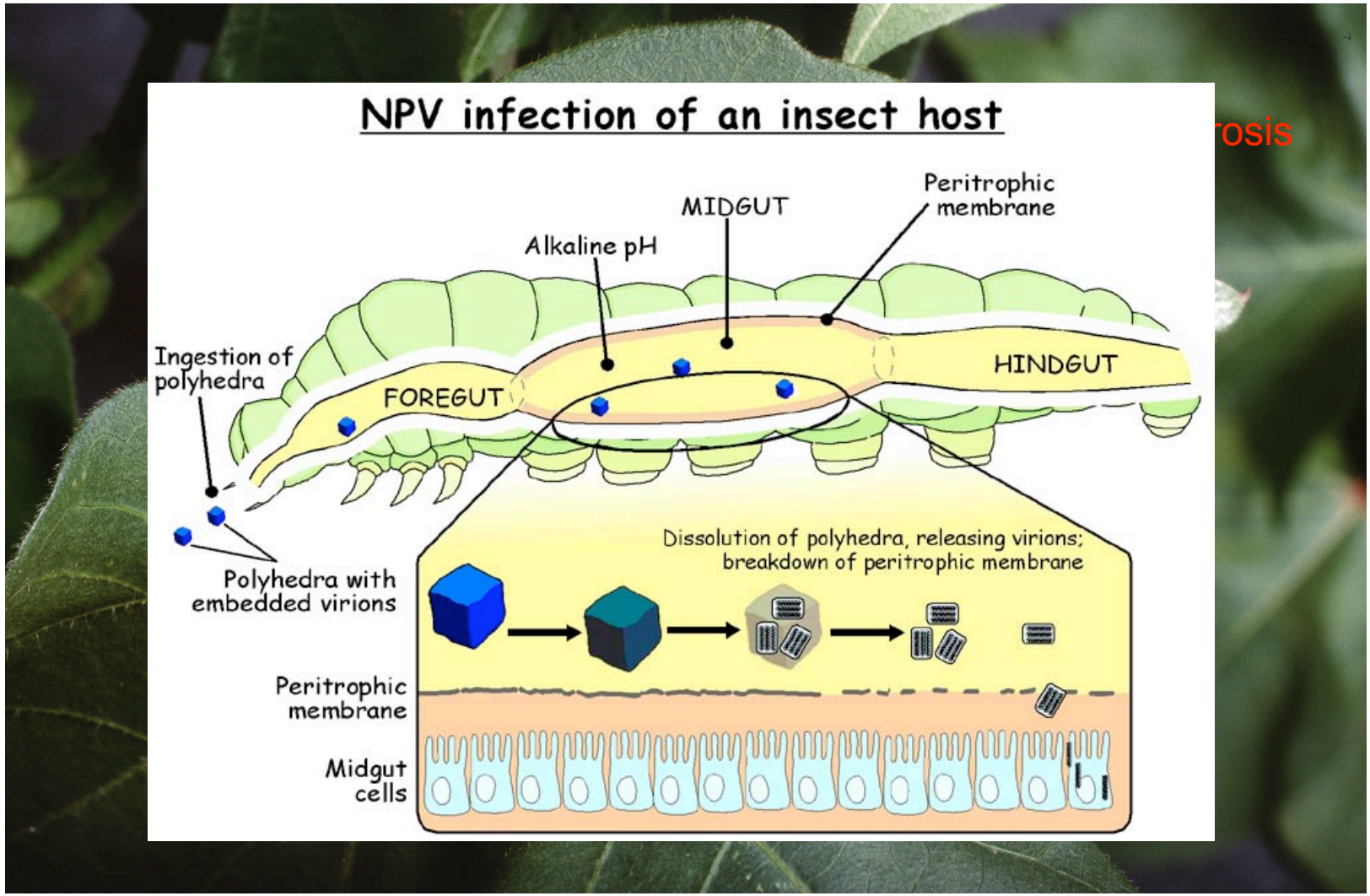


- Ubiquitous viruses that infect over 600 species of insects
  - significant losses to the industry of silk
  - Cause natural epizootics limiting insect populations
  - bioinsecticides



The Main Routes of the Old Silk Road

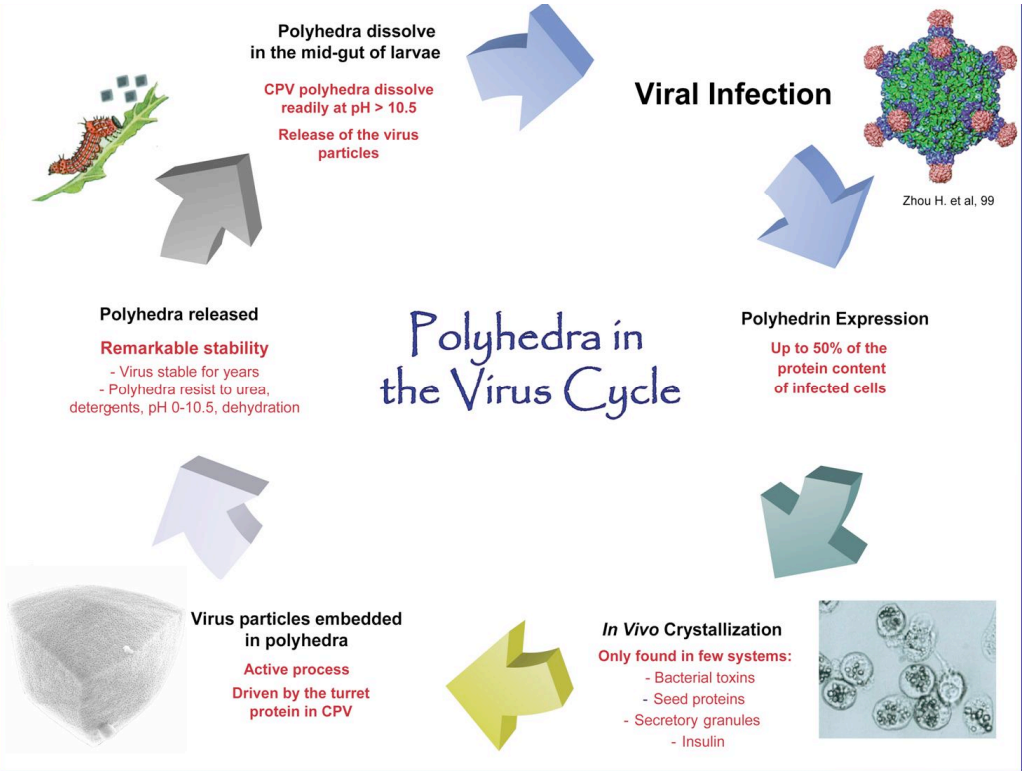
# Introduction PH




Beet armyworm - *Spodoptera exigua*

Source: www.ars.usda.gov

## Polyhedra in the viral cycle




## Some facts...

- Cypovirus (CPV) causes polyhedrosis.
- Cypoviruses are packed into micron-sized protein crystals called polyhedra.
- The protein that forms polyhedra is called polyhedrin.
- Polyhedrin is composed of 248 amino acids.
- Polyhedra are very stable, but dissolve at basic pH (midgut of insect larvae).
- The structure of polyhedrin and the molecular organization of polyhedra are unknown.

## Aim of the project:

To solve the structure of polyhedrin in order to gain insights into the molecular organization of polyhedra (*in vivo* crystals).

## Team:

Swiss Light Source

MX Group

Altana Pharma AG

Peter Haebel

Kyoto Institute of Technology

Keiko Ikeda

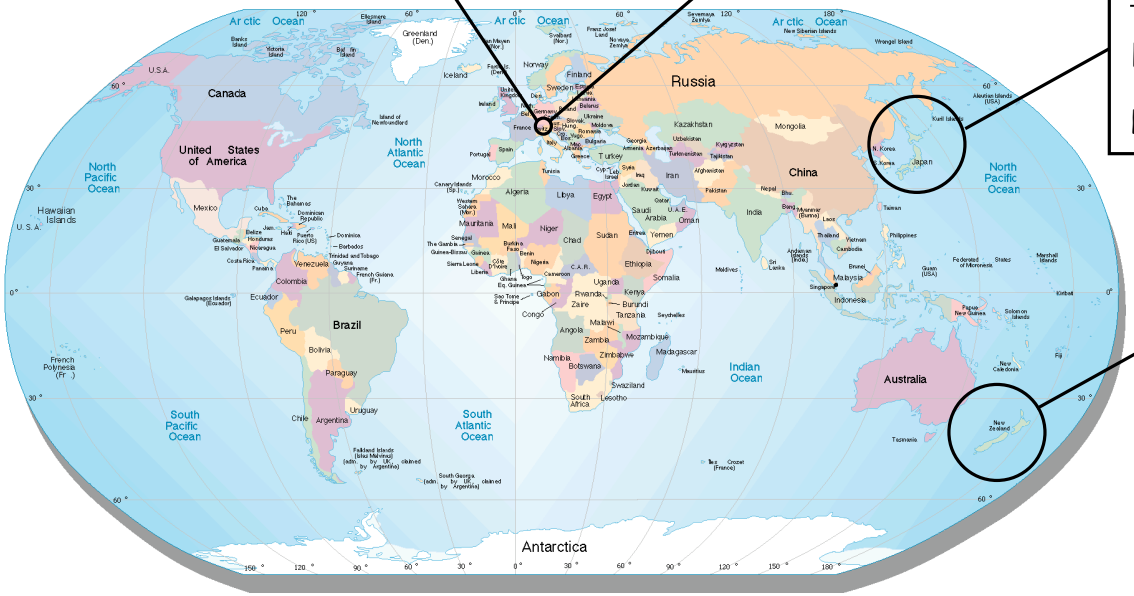
Hajime Mori

University of Auckland

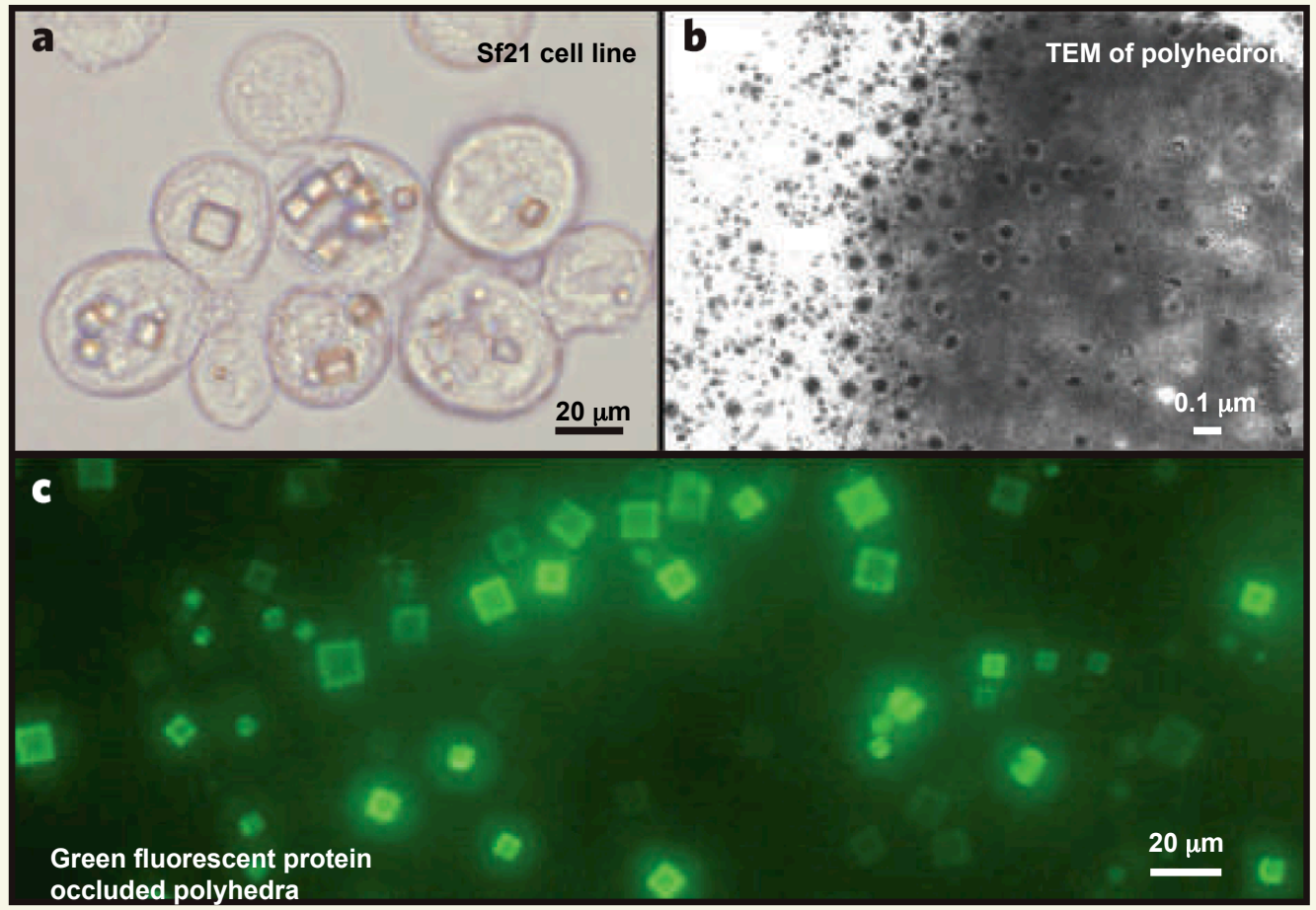
Elaine Chiu

Fasséli Coulibaly

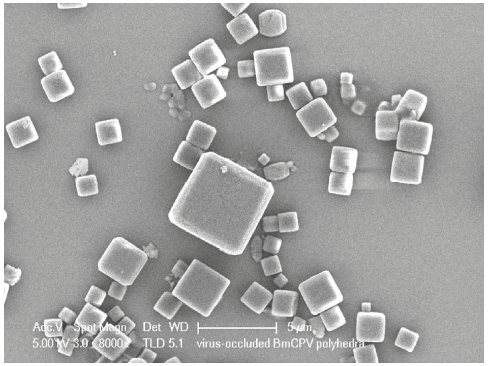
Peter Metcalf



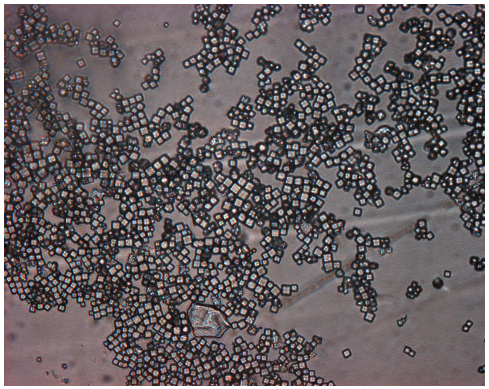
# Getting the crystals




Source: Nature 446, 35 – 37 (1 March 2007), Photos courtesy of H. Mori, Protein Crystals Corp., Kyoto Institute Technol., Japan)



Scanning electron micrographs of infectious polyhedra (Elaine Chiu)



Light microscope image of infectious polyhedra (Elaine Chiu)

# Structure of polyhedrin

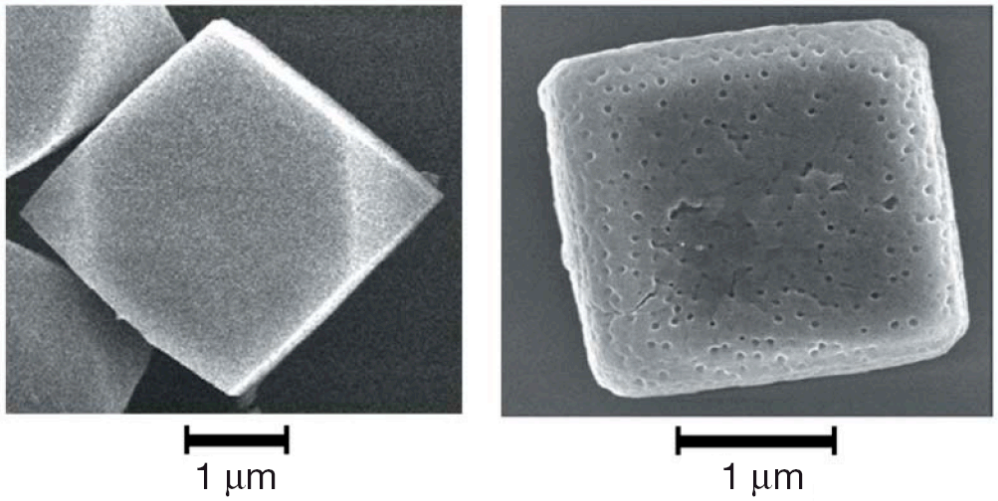
## Data collection statistics (Multiple isomorphous replacement)

| | Native | $KAuCN_2$ | $KI/I_2$ | $AgNO_3$ | Se-Met |
|---|---|---|---|---|---|
| **Data collection** | | | | | |
| Cell dimensions $a = b = c$ (Å) | 102.96 | 103.11 | 102.36 | 102.92 | 103.00 |
| Resolution (Å) | 14.5–2.1 (2.17–2.10) | 18.8–2.8 (2.9–2.8) | 24.1–2.8 (2.9–2.8) | 24.3–2.6 (2.69–2.60) | 18.8–2.6 (2.69–2.6) |
| $R_{sym}$ or $R_{merge}$ | 0.13 (0.43) | 0.13 (0.26) | 0.13 (0.28) | 0.14 (0.30) | 0.15 (0.32) |
| $I/\sigma I$ | 7.6 (2.6) | 7.5 (4.0) | 12.7 (6.7) | 12.1 (4.7) | 13.1 (6.9) |
| Completeness (%) | 97.9 (98.2) | 96.9 (96.6) | 95.4 (98.2) | 98.7 (97.1) | 97.8 (99.6) |
| Redundancy | 3.6 (2.6) | 2.6 (2.6) | 5.8 (5.6) | 5.3 (3.4) | 7.3 (7.0) |

Space group: I23. Number of crystals merged to obtain a full data set: Native: 2, $KAuCN_2$: 2, $KI/I_2$: 3, $AgNO_3$: 4, Se-Met: 2

| | Infectious | Kinase-containing |
|---|---|---|
| **Data collection** | | |
| Cell dimensions $a = b = c$ (Å) | 102.78 | 102.74 |
| Resolution (Å) | 20–1.98 (2.05–1.98) | 20–2.45 (2.54–2.45) |
| $R_{sym}$ or $R_{merge}$ | 0.14 (0.50) | 0.13 (0.35) |
| $I/\sigma I$ | 13.9 (4.3) | 12.6 (3.7) |
| Completeness (%) | 99.0 (100) | 99.9 (99.4) |
| Redundancy | 7.2 (7.3) | 8.2 (3.5) |
| **Refinement** | | |
| Resolution (Å) | 18.76–1.98 | 18.8–2.45 |
| Number of reflections | 11,348 | 6,092 |
| $R_{work}/R_{free}$ | 0.093/0.154 | 0.112/0.221 |
| Number of atoms | | |
| Protein | 2,056 | 2007 |
| Ligand/ion | 92/3 | 92/3 |
| Water | 286 | 137 |
| *B*-factors | | |
| Protein | 11.4 | 20.6 |
| Ligand/ion | 41.5/27.1 | 54.7/45.5 |
| Water | 26.3 | 21.5 |
| r.m.s. deviations | | |
| Bond lengths (Å) | 0.013 | 0.016 |
| Bond angles (°) | 1.490 | 1.680 |

The space group for all data sets is *I*23. The number of crystals merged to obtain a complete data set is indicated in parentheses for the following data sets: infectious (3) and kinase-containing (5). The highest-resolution shell is shown in parentheses.



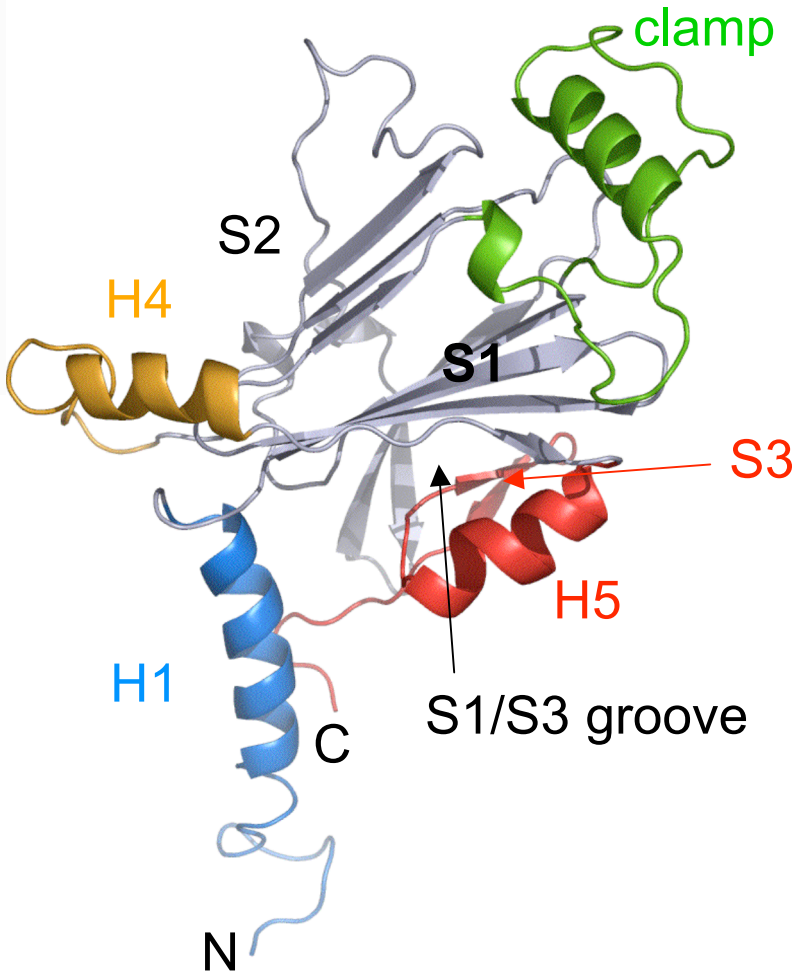

Scanning electron micrographs of recombinant (left) infectious (right) polyhedra (Elaine Chiu)

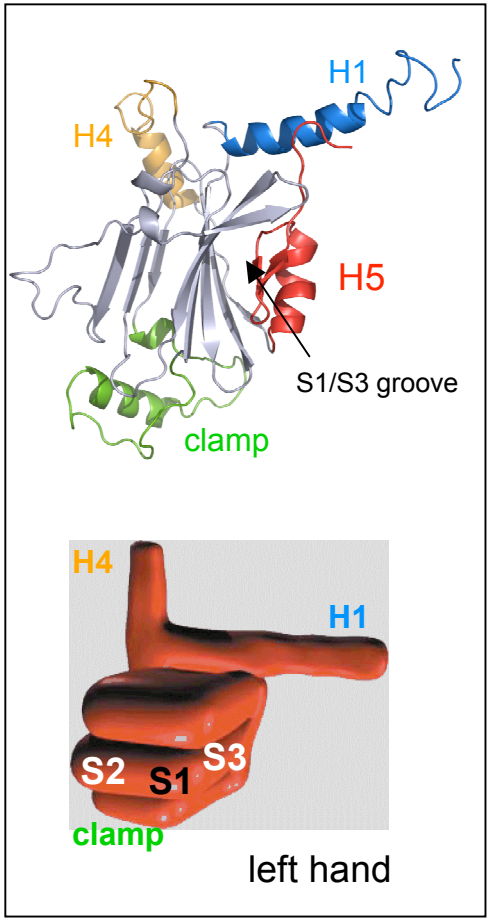
# Mechanical and chemical stability of polyhedra

| | Incubation time | Diffraction |
|---|---|---|
| Chaotropic agents | | |
| 8 M urea | 2 days | Yes |
| Detergents | | |
| 10% SDS | 2 days | Yes |
| Acids | | |
| 20% acetic acid, pH 1.9 | 2 days | Yes |
| 1 M HCl | 2 days | No |
| Bases | | |
| 20 mM $NaH_2CO_3$, pH 10.5 | <1 min | Dissolved |
| 10 mM PBS, pH 11.0 | <1 min | Dissolved |
| Solvent | | |
| 100% ethanol | 30 min | Yes |
| Physical stress | | |
| Dehydration* | 24 h | Yes |
| Freeze–thaw | - | Yes |
| 90 °C | 16 h | No |

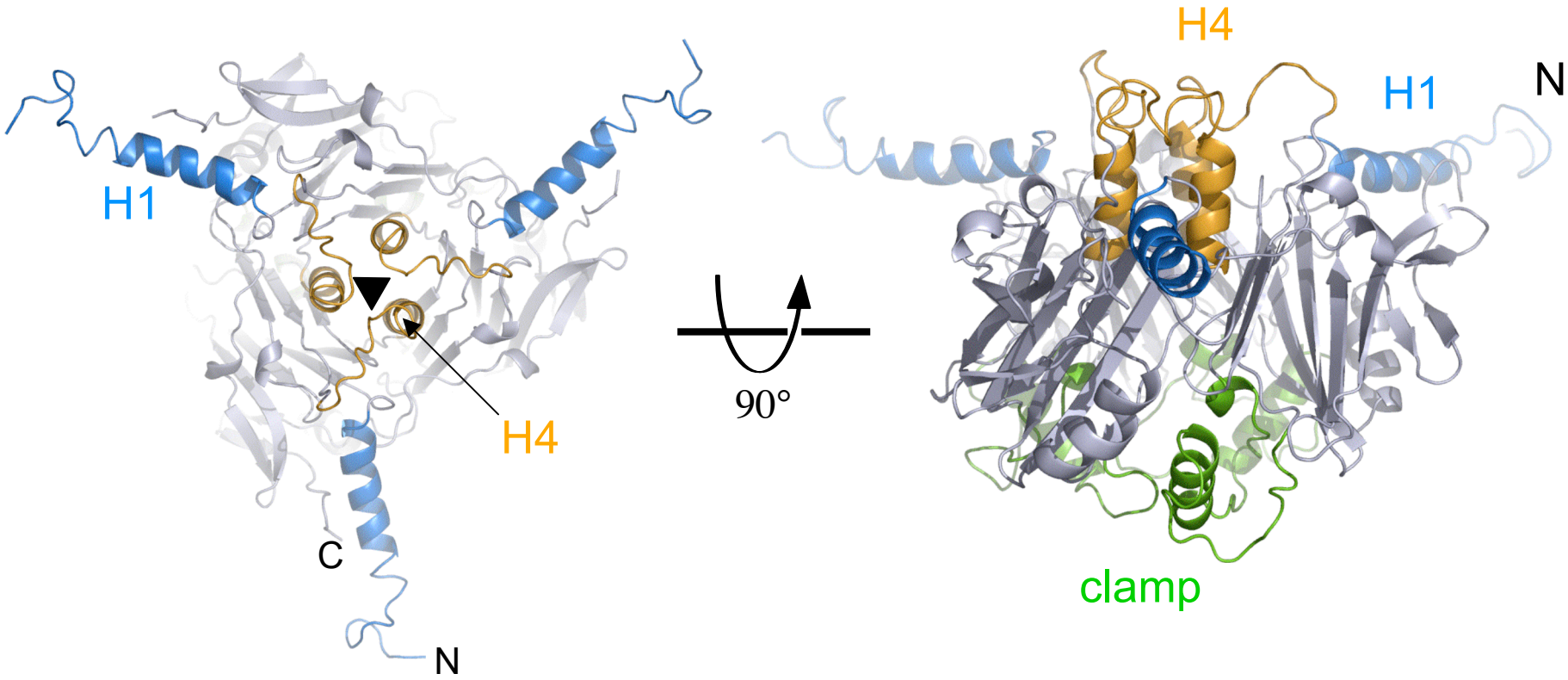
* Polyhedra in water were left to dry on a micromesh mount at relative humidity of 50% and frozen in liquid nitrogen without rehydration or cryoprotectant.

# o Structure of BmCPV polyhedrin



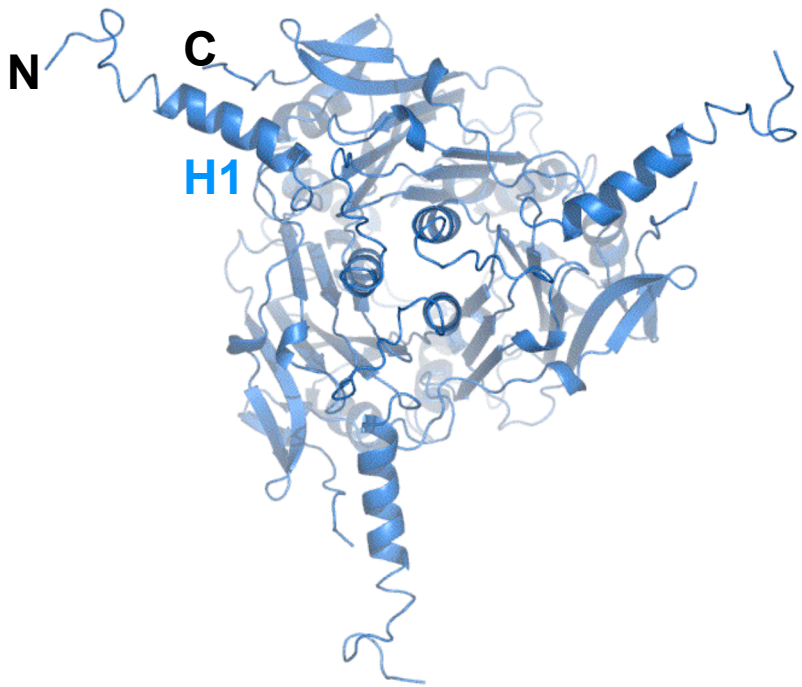



# Trimers of polyhedrin

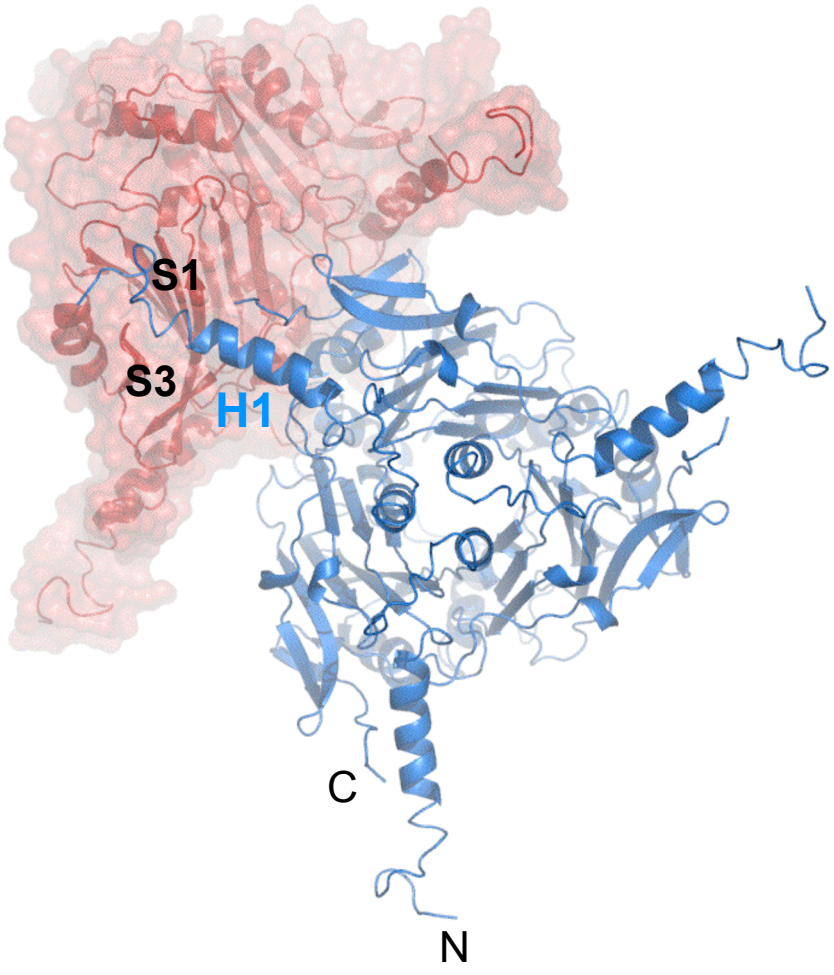


# o Molecular organization of cypovirus polyhedra

## Tetrahedral cluster of polyhedrin trimers

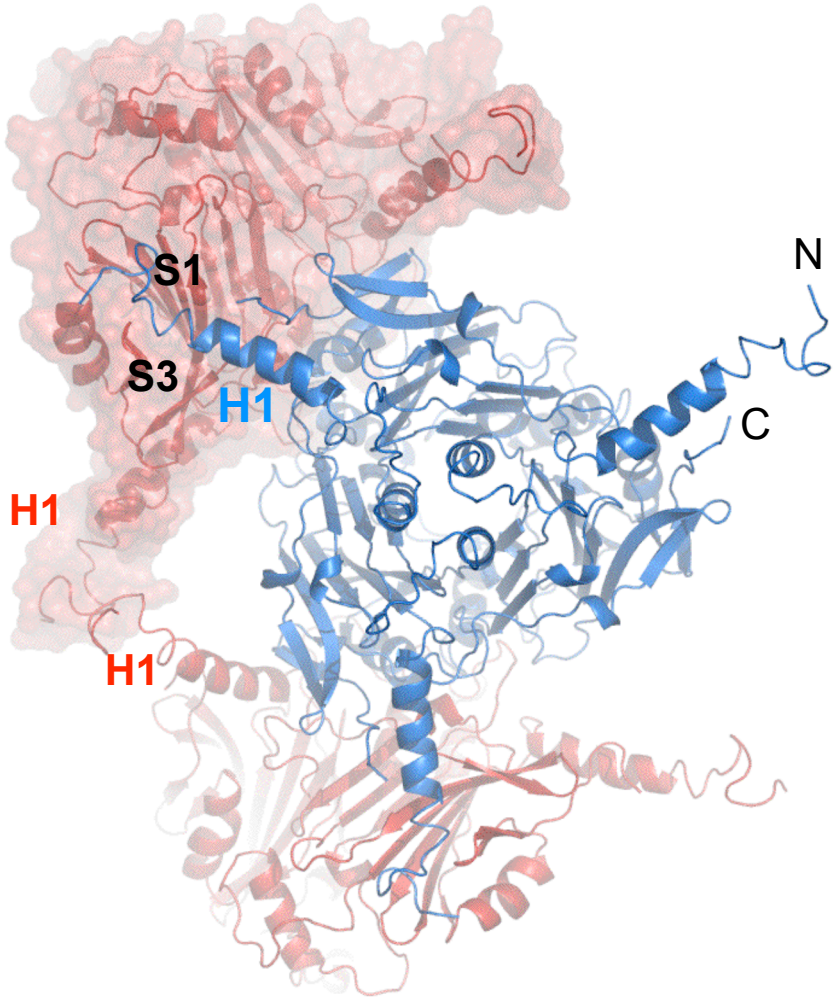


# o Molecular organization of cypovirus polyhedra



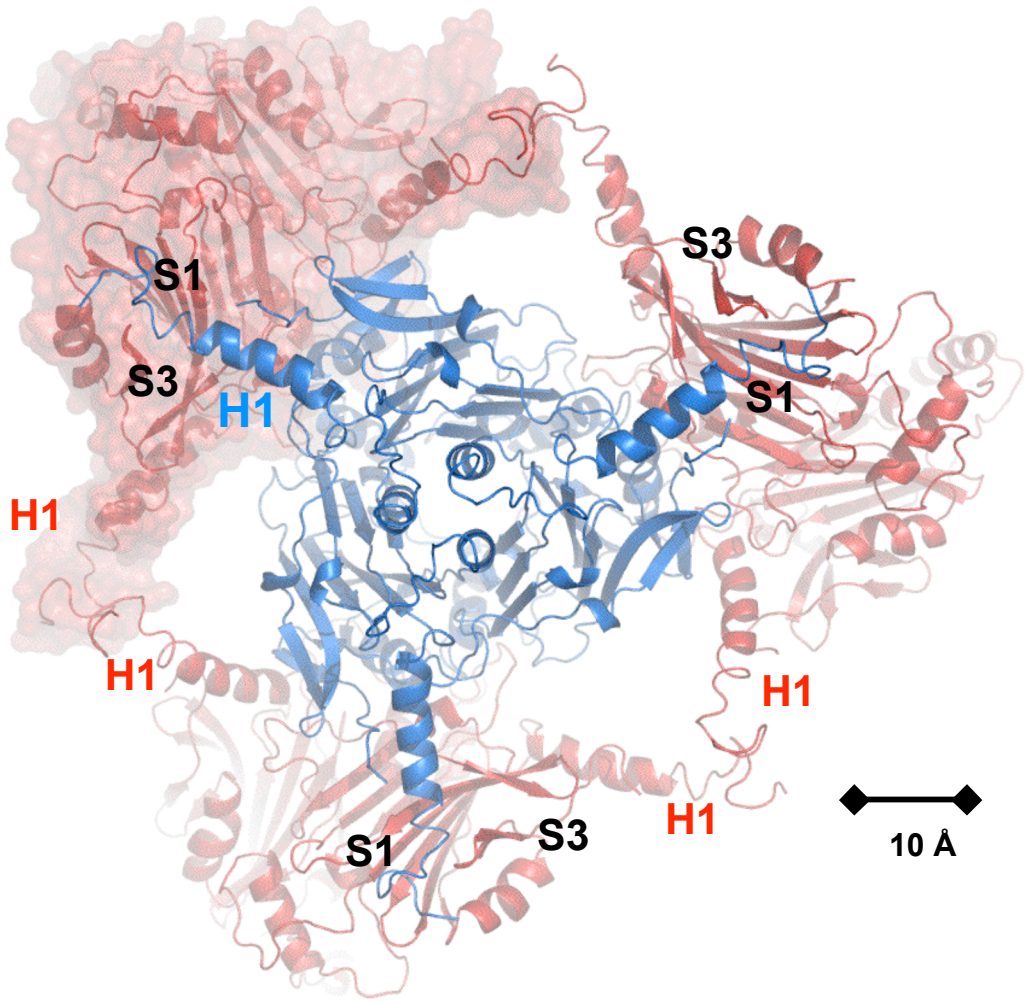

Tetrahedral cluster of polyhedrin trimers

# o Molecular organization of cypovirus polyhedra



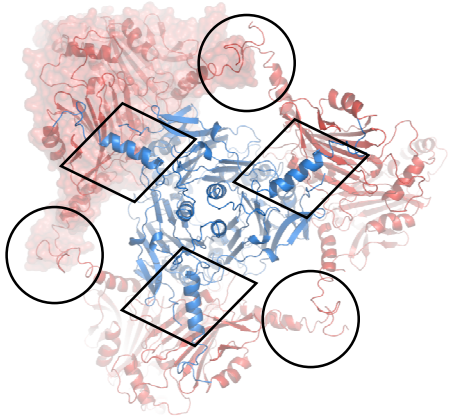

Tetrahedral cluster of polyhedrin trimers

# Molecular organization of cypovirus polyhedra




Two tetrahedral clusters constitute the unit cell of the crystal

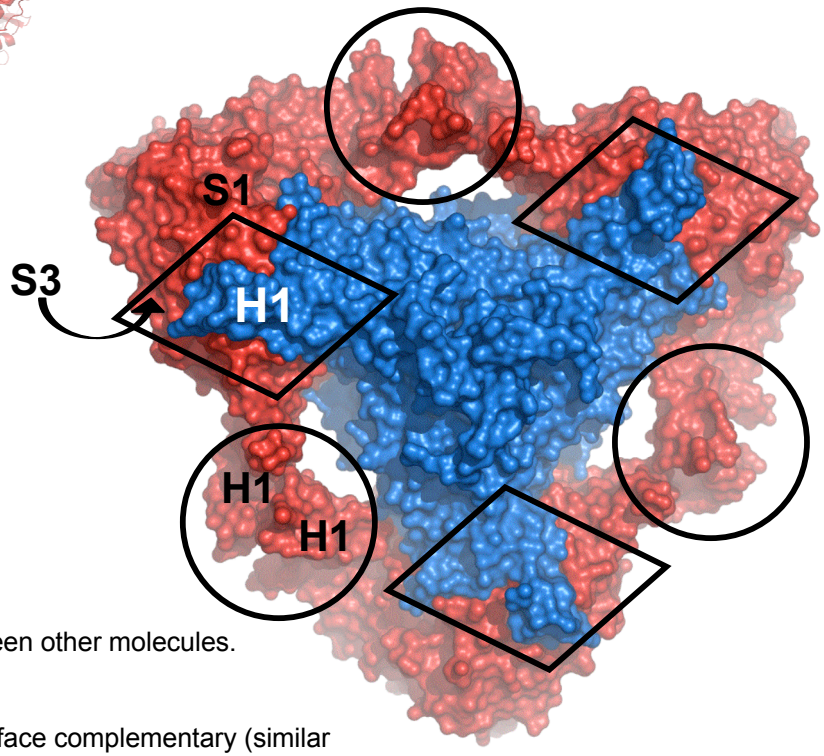
# o Molecular organization of cypovirus polyhedra



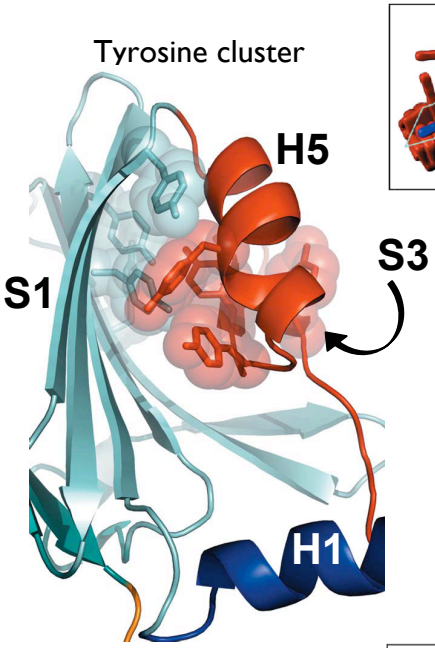
Non-covalent trimer interactions

finger-to-fist

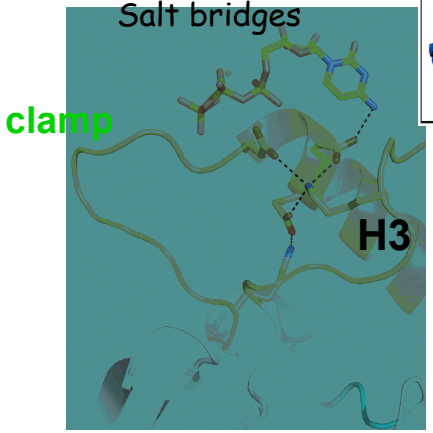
finger-to-finger




Tyrosine cluster



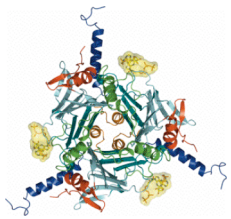
Salt bridges

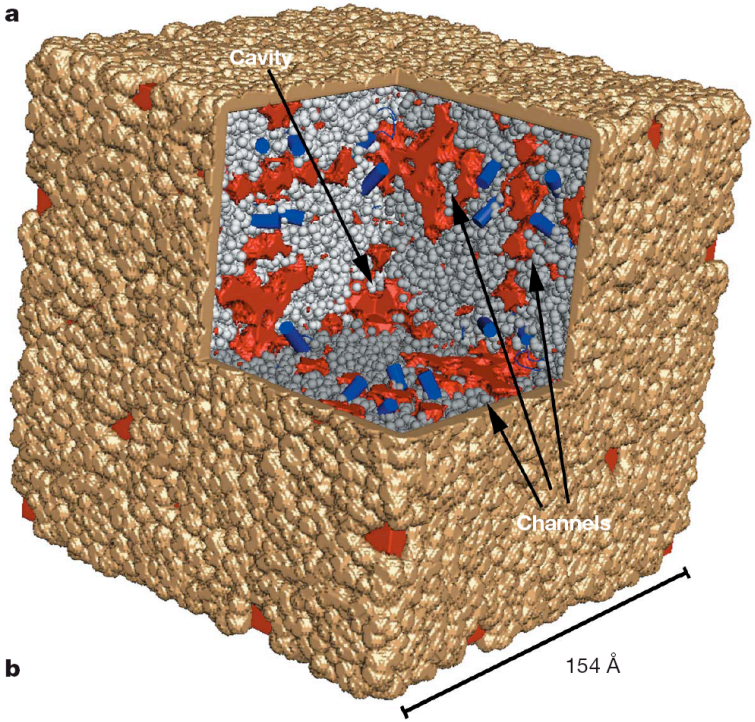


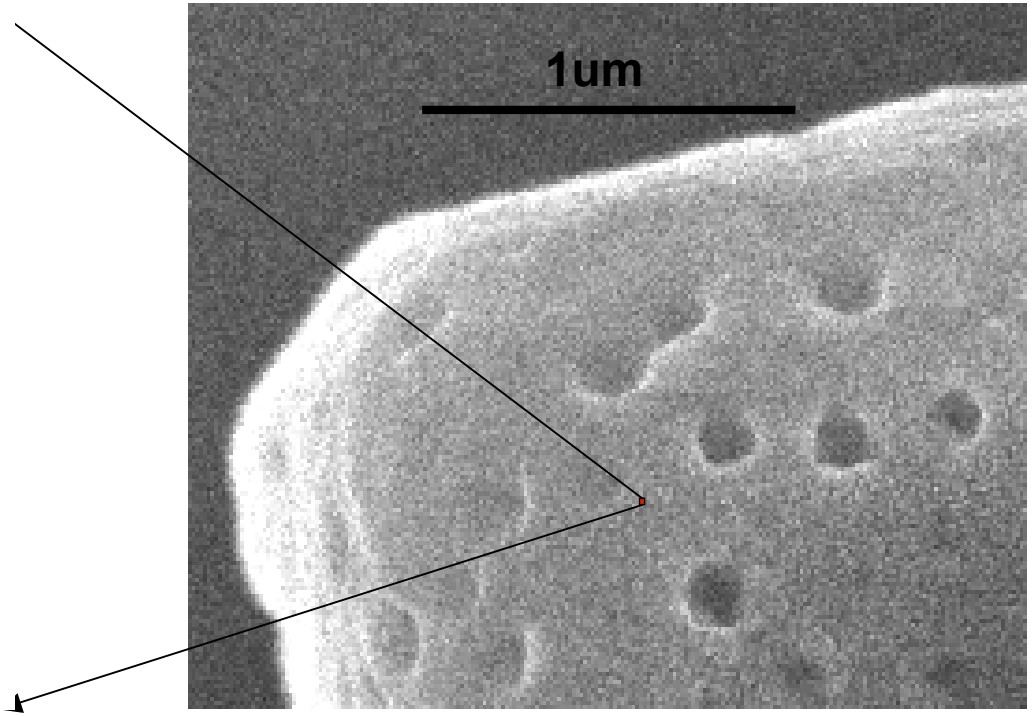
Stabilty of polyhedra:

- ❖ Each polyhedrin contacts eighteen other molecules.
- ❖ Low solvent content (19 %).
- ❖ Packing surfaces have high surface complementary (similar to antibody-antigen interactions).
- ❖ Over 25 % of the total polyhedrin surface is buried in trimer interfaces.

# A sealed viral carapace



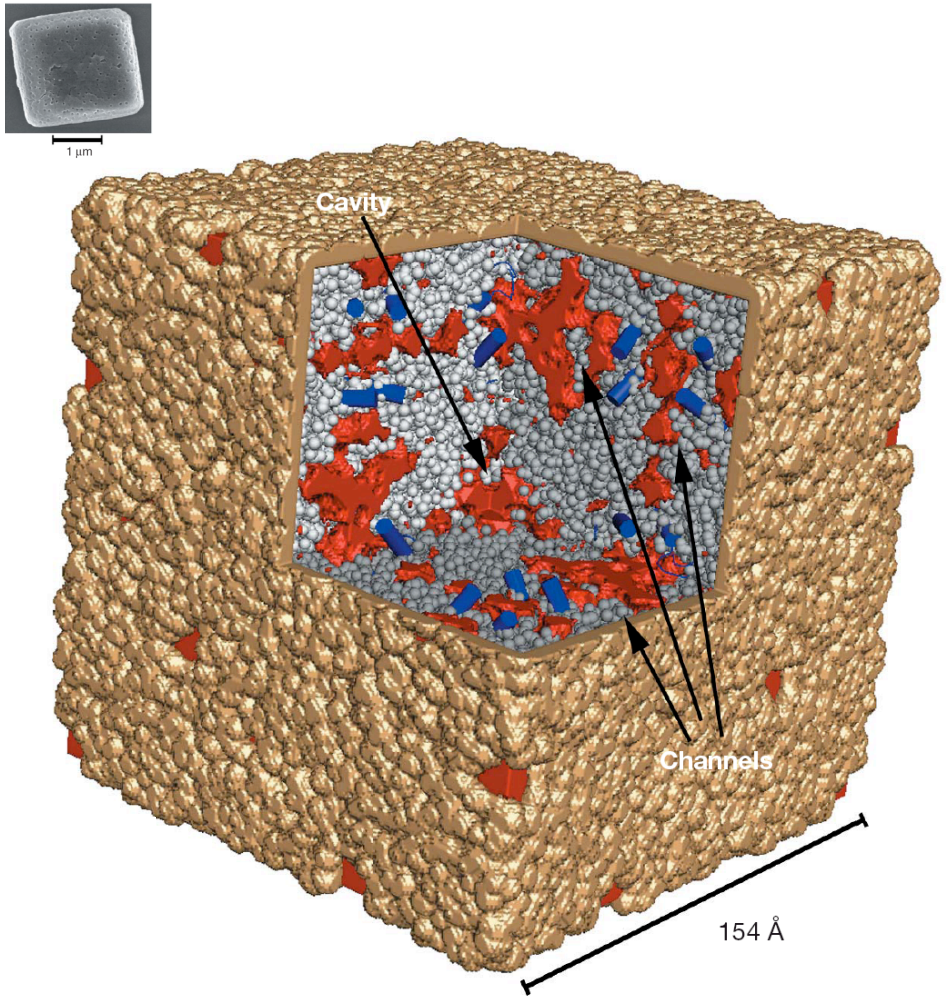


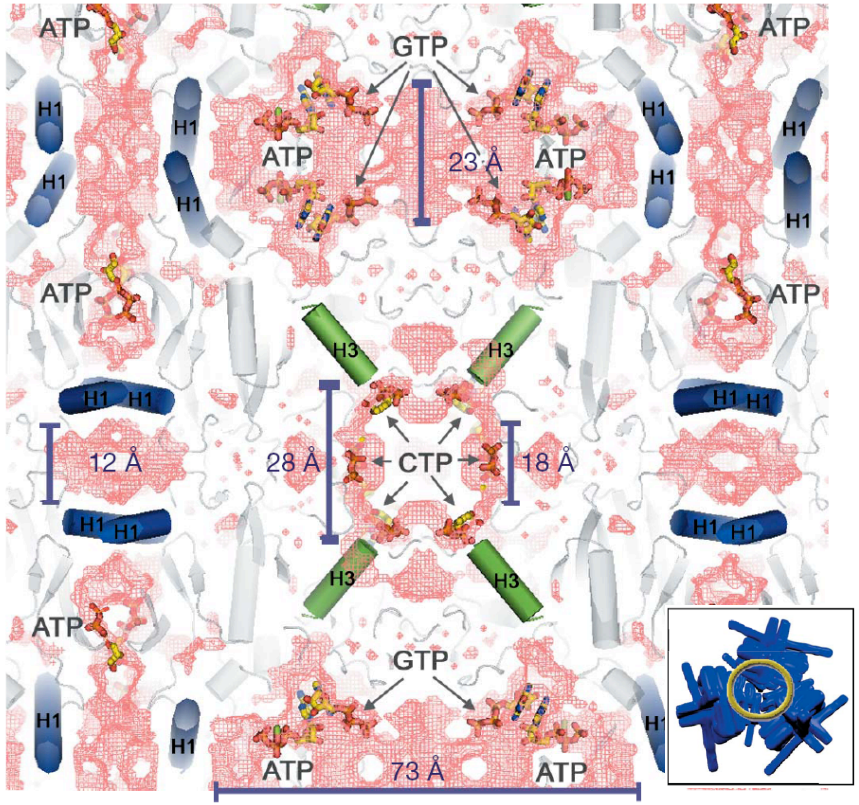




- Helices H1 block the small solvent channels
- Helices H4 close the cavities
- Limit diffusion in and out of the crystal and protect virus particles

# o Molecular organization of cypovirus polyhedra

Biologically active form of polyhedrin is the polyhedra (=crystal)!



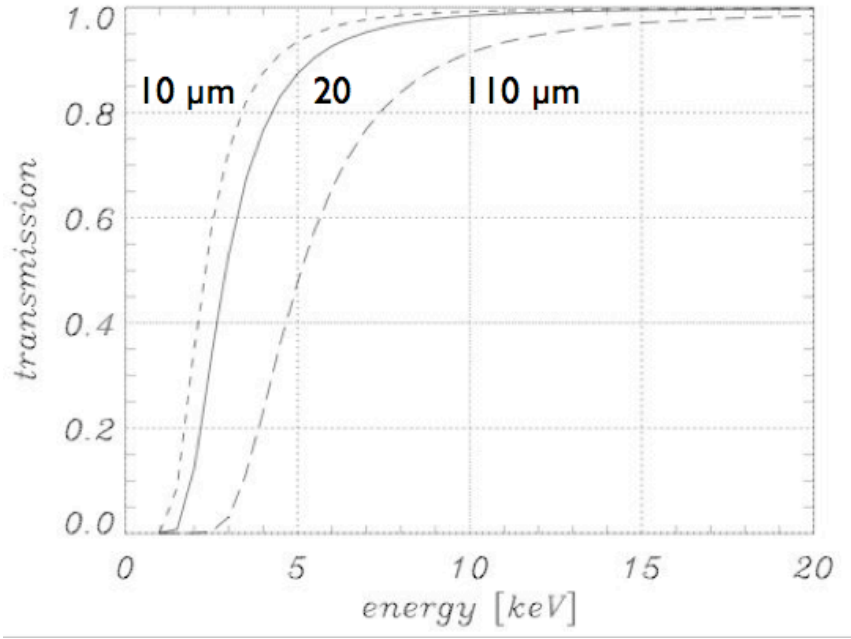
# Beam stability

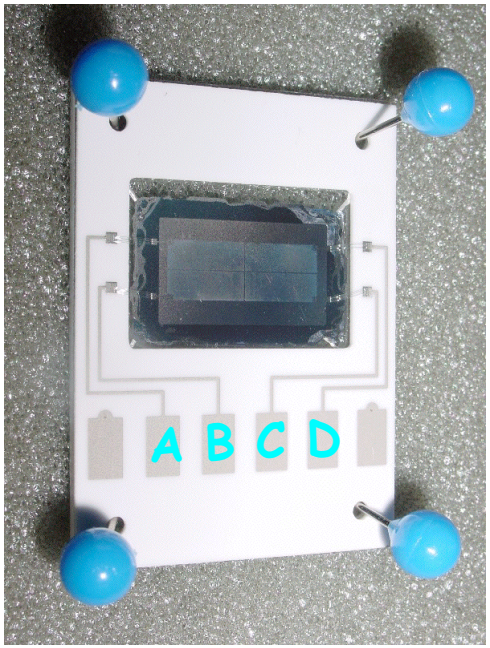
Beam should be kept stable to within 10% beam dimension

=> 0.1 µm at up to 100 Hz

SLS MX Beamlines:

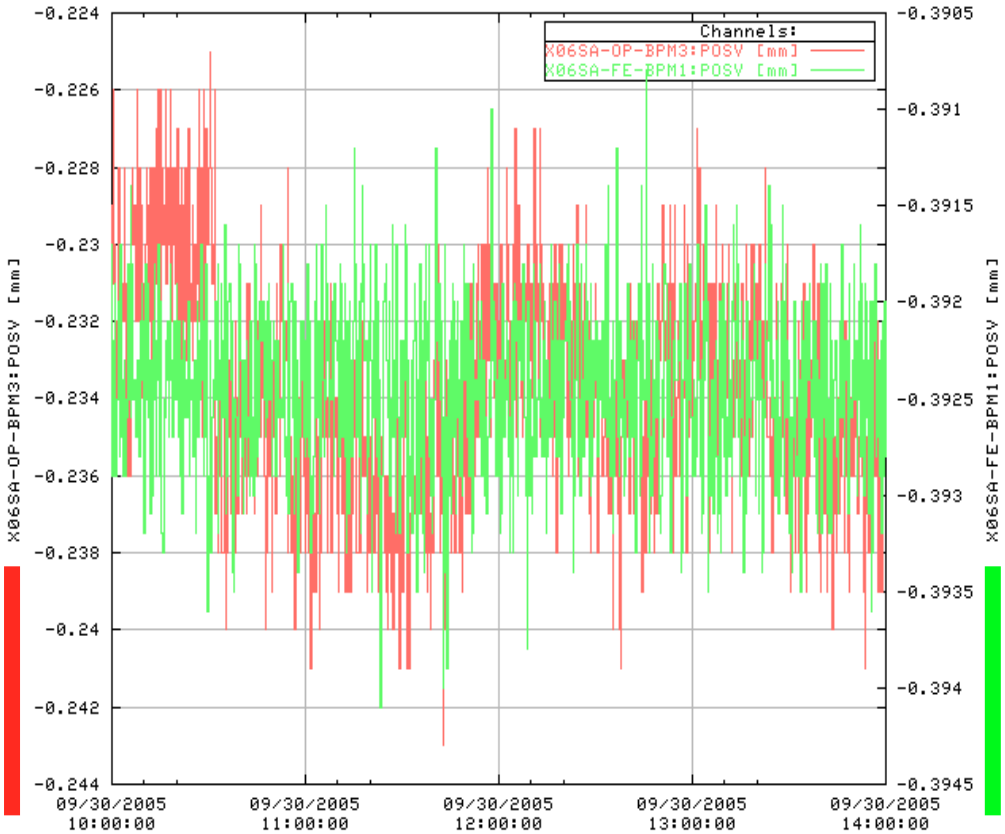
CVD Diamond Quadrant XBPMs



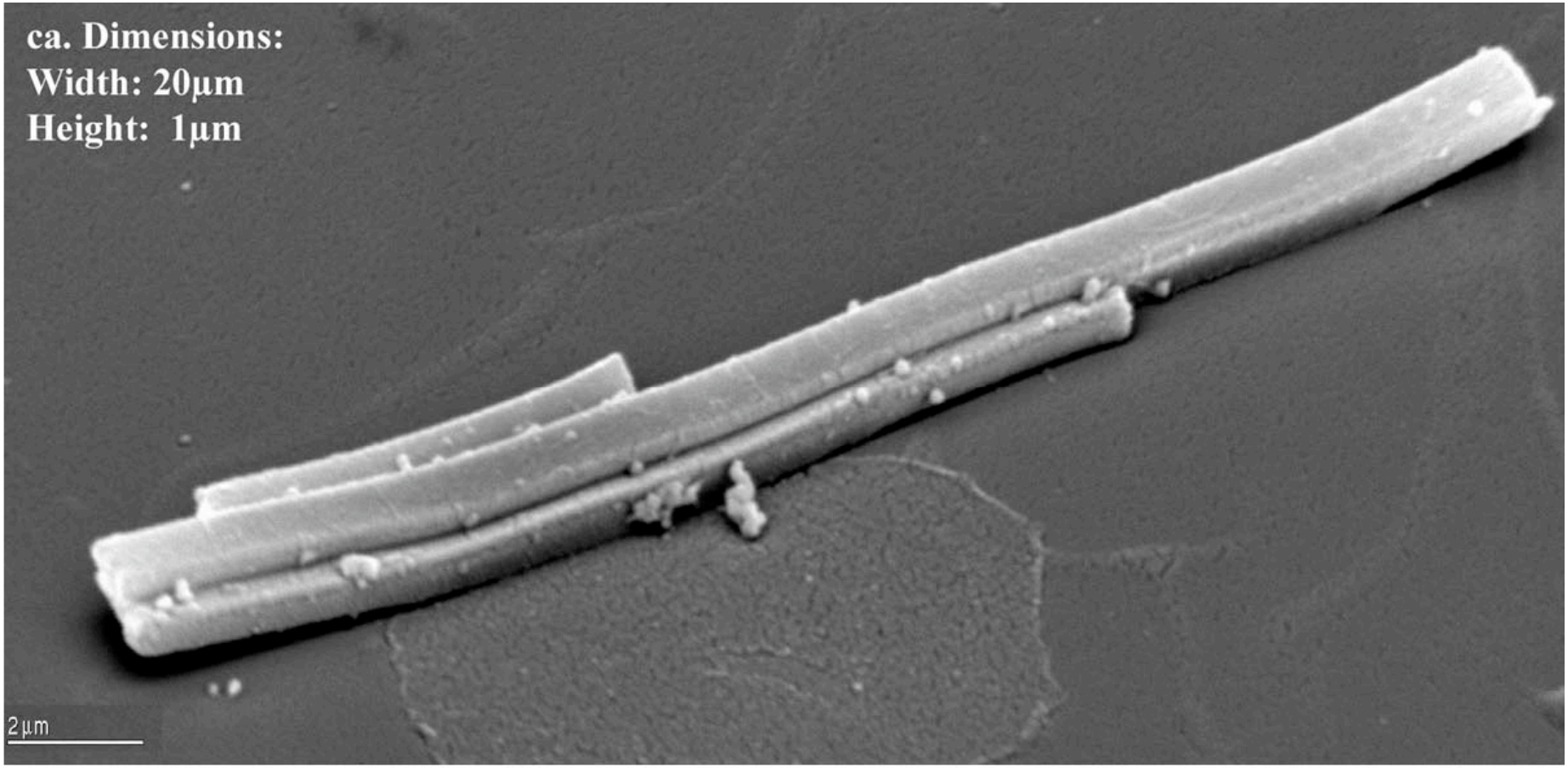



# Slow Feedback

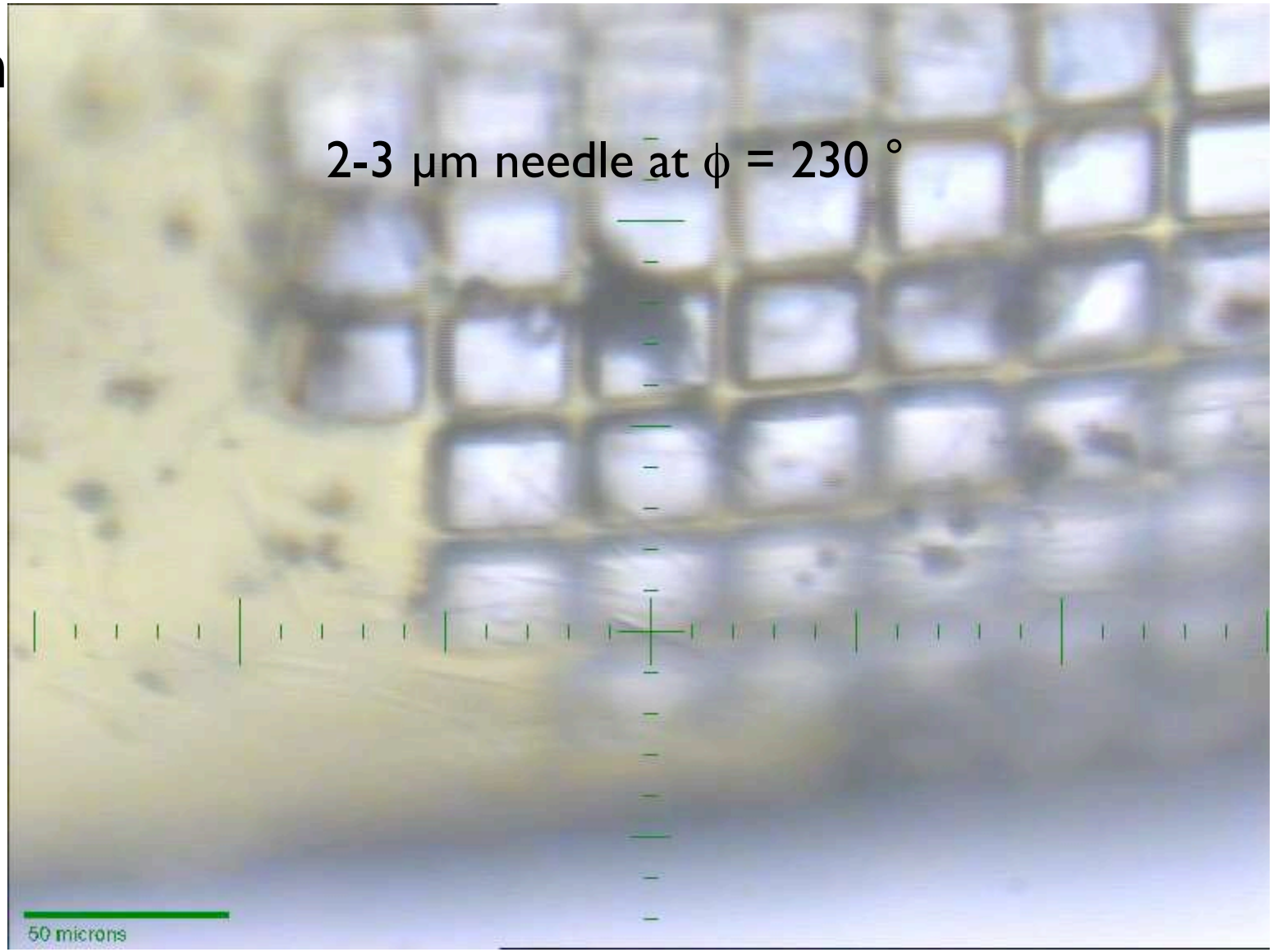
- Exposure box Q-XBPMs feedback on mirror and sagittal bender
- Data acquisition: 10 Hz
- Frequency: 0.1 - 1 Hz



# Sample Visualisation



# Sam

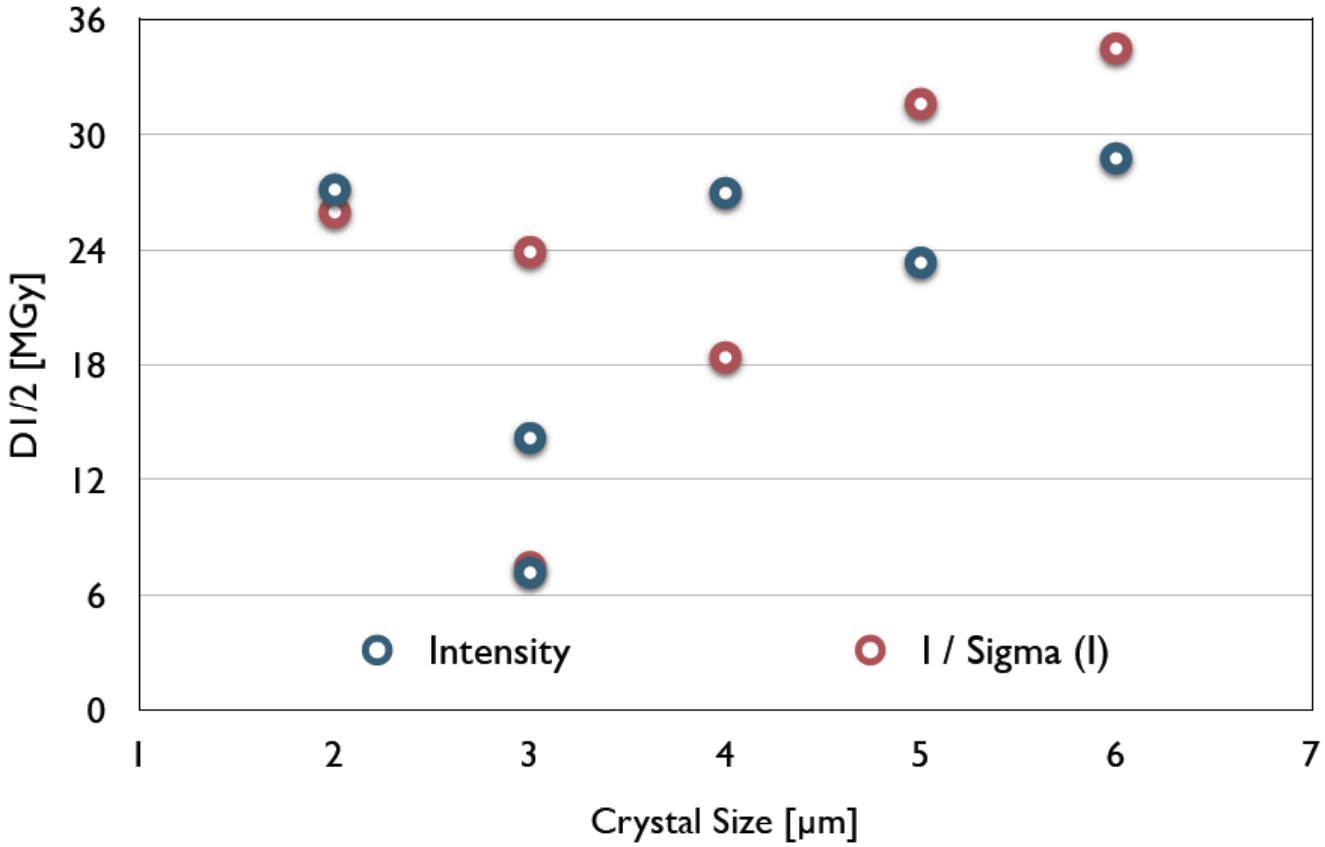


# Radiation damage

- Minimal useful crystal size set by maximal tolerable dose
- Test system BmCPV crystals of 2 to 6 µm size
  - Wavelength 1 Å
  - Beamsize 15 x 5 $\mu m^2$
  - Flux (100%) $3 \cdot 10^{11}$ phs/sec

- Linear fit of Intensity Decay and I/sigma(I) decay between 30 and 3 Å

# Radiation damage - Minimal dose




One 0.8 µm CPV crystal should provide 1 frame

# Many thanks to

**Fasseli Coulibaly, Elaine Chiu, Chitra Rajendran, Joachim Diez, Sascha Gutmann, Peter Haebel, Peter Metcalf, Hajime Mori**

**Gerd Schluckebier, Armin Wagner**

**Takashi Tomizaki, Claude Pradervand, Roman Schneider, Alke Meents, Pirmin Bühler and the entire SLS MX-Group**

**our Users**