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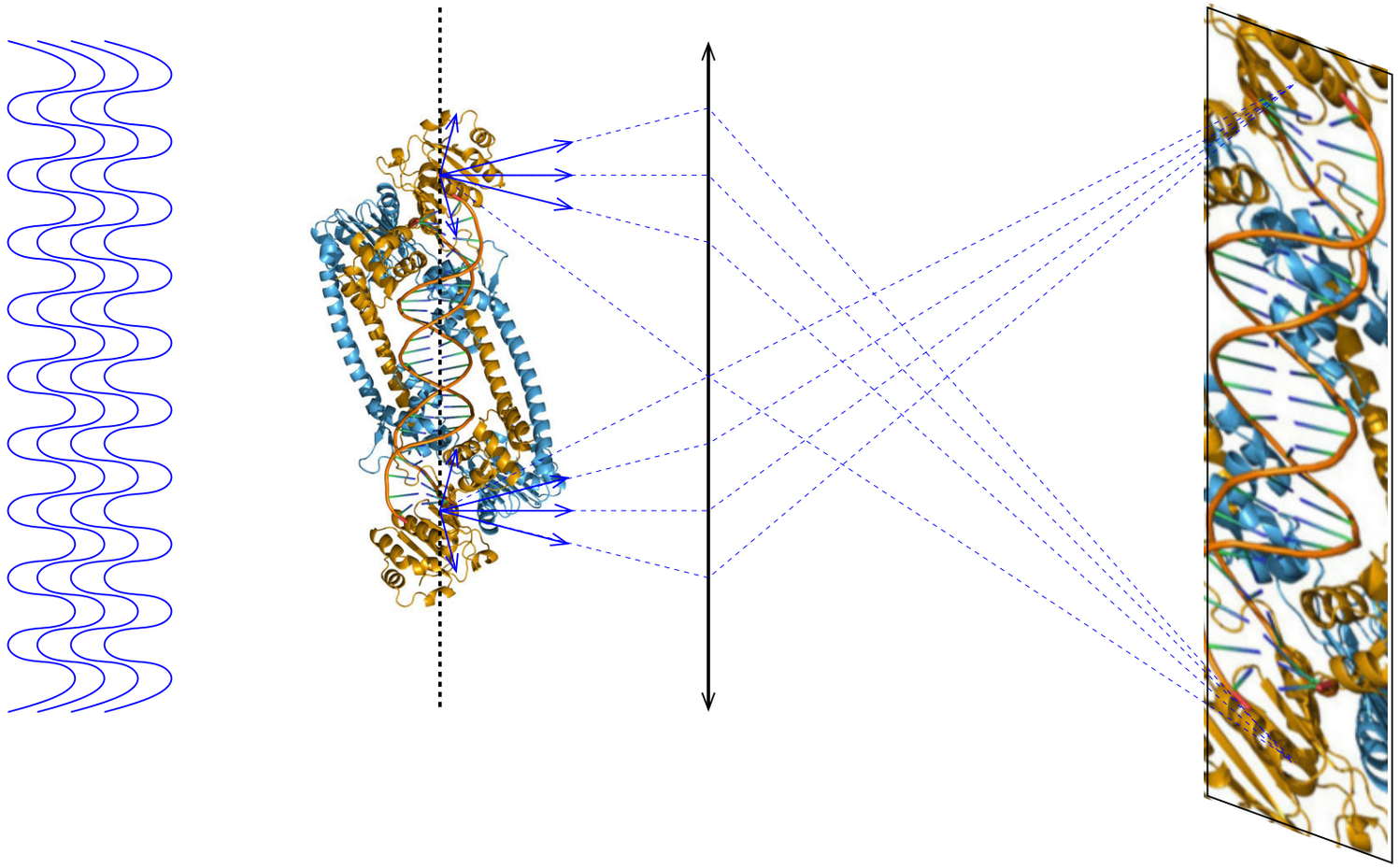
Seeing the Small with Electron Crystallography

Center for Chemistry and Biomedicine Innsbruck

31st May 2017

1 - Seeing the small

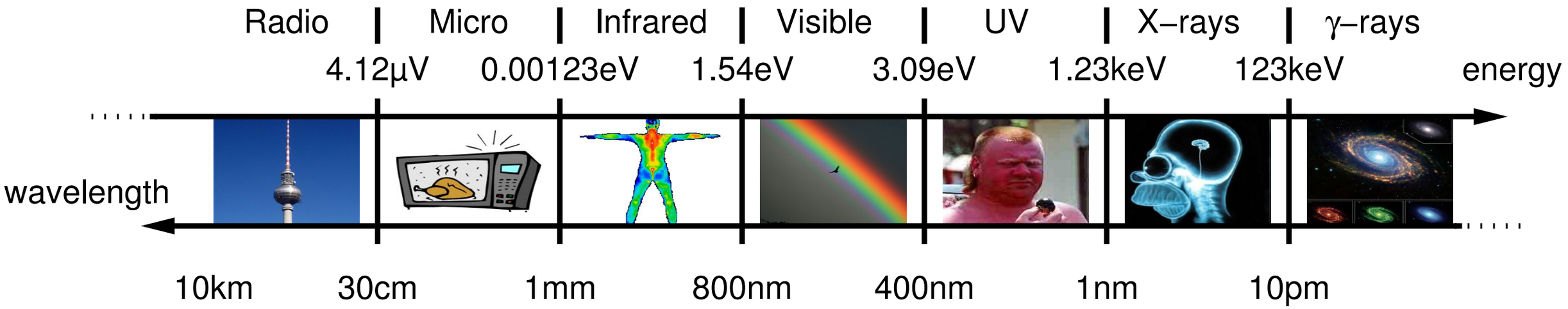
Microscopy



Light Object Lense Image Plane (Detector)

Enlarged image from object

Microscopes



- Light has a wavelength: 800nm = **red** . . . 400nm = **blue**
- Resolution (possibility to distinguish between two neighbouring points) $\approx \lambda$
- Size of interest for molecules: $\approx 1\text{Å} = 0.1nm$
- **No lenses = no microscopes** available for such short wavelengths!

Workaround I: Electron Microscopy

- Light / Electromagnetic waves: no microscopes

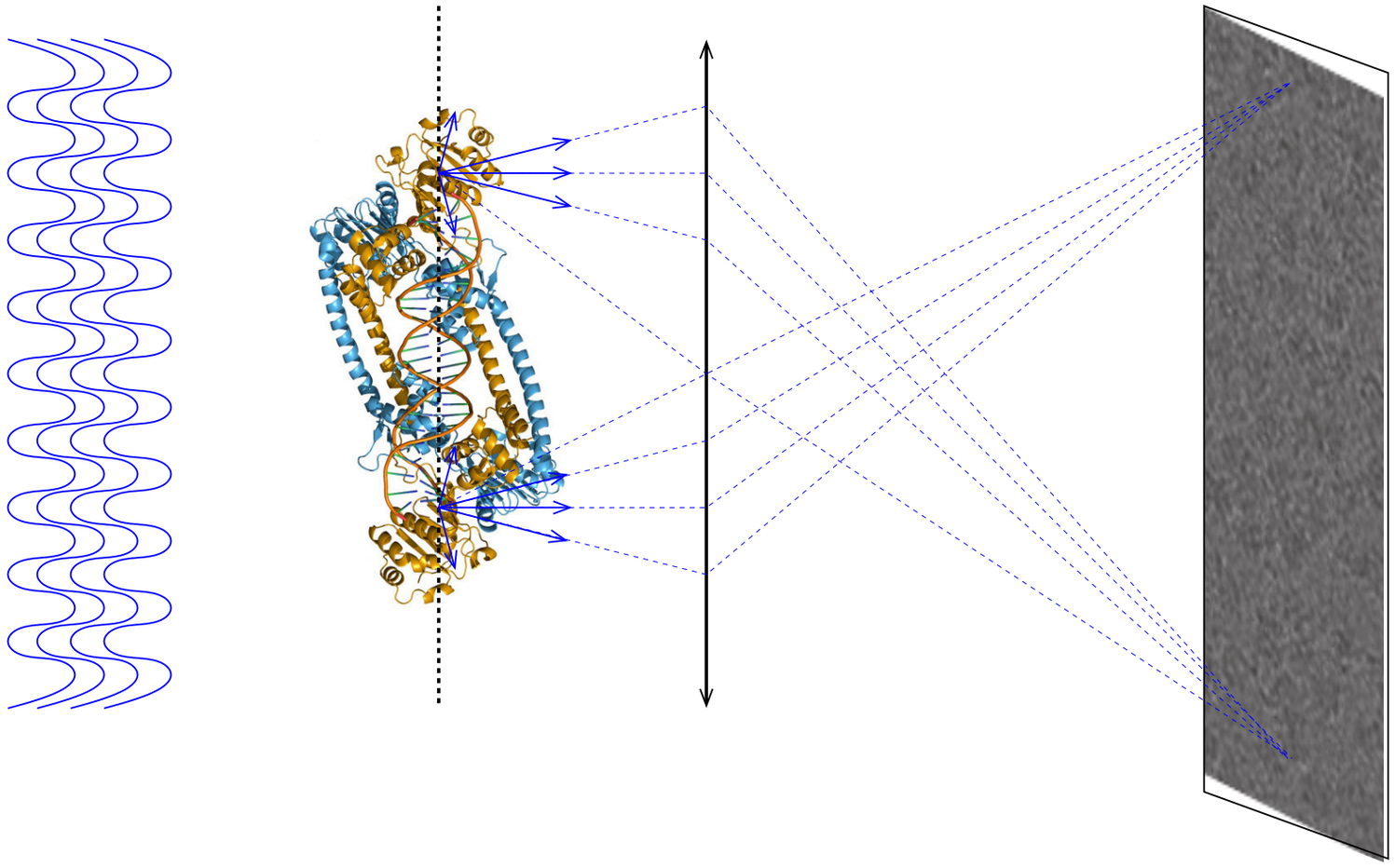
- Use electrons as wave and use **magnetic** lenses: even at very short wavelength

- Since quantum mechanics: electrons are waves, de Broglie wavelength

$$\frac{m_e v}{\sqrt{1-(v/c)^2}} = \frac{h}{\lambda}$$

- Commonly used: Electron energies 200–300keV, wavelength $\lambda = 0.025 - 0.017$Å.

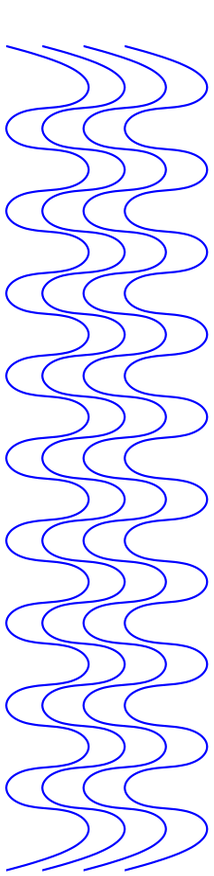
Electron Microscope: Imaging Mode



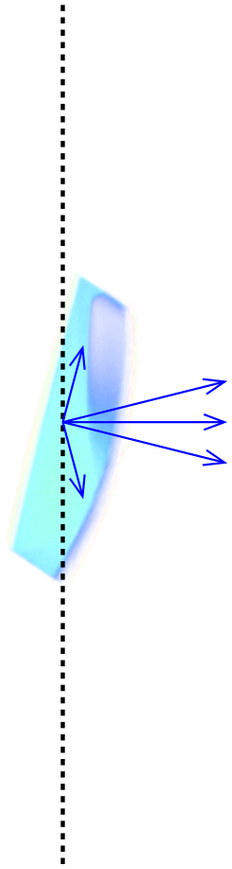
Detector noise and radiation senstivity require low contrast images

Martinez-Rucobo *et al.* Molecular Cell (2015) 58, 1079–1089

Workaround II: Crystallography

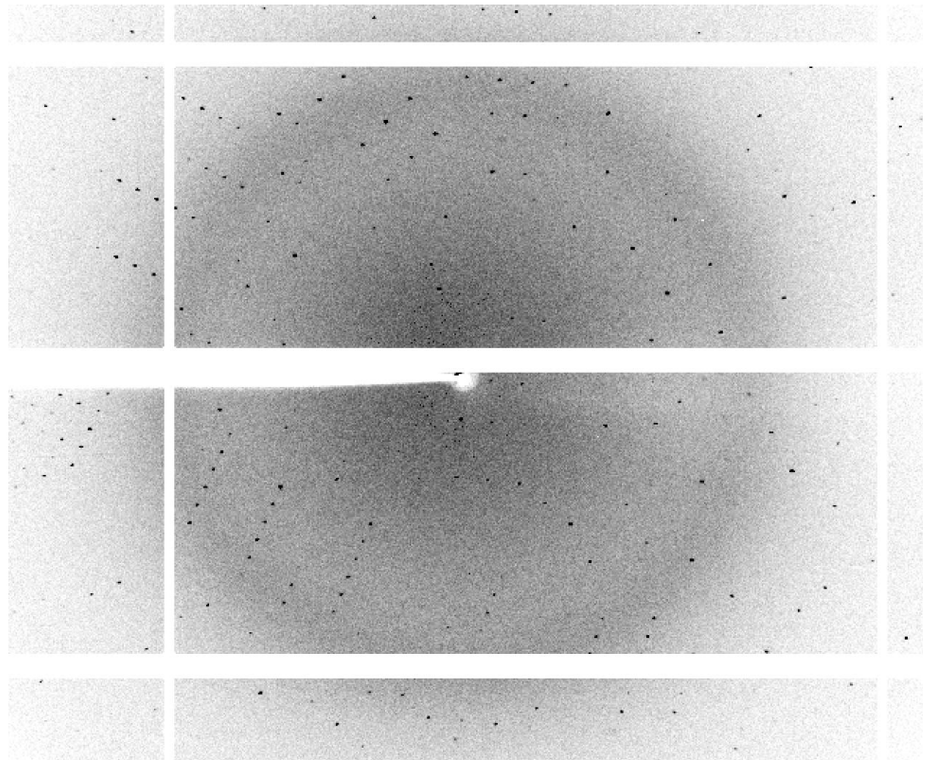


Wave



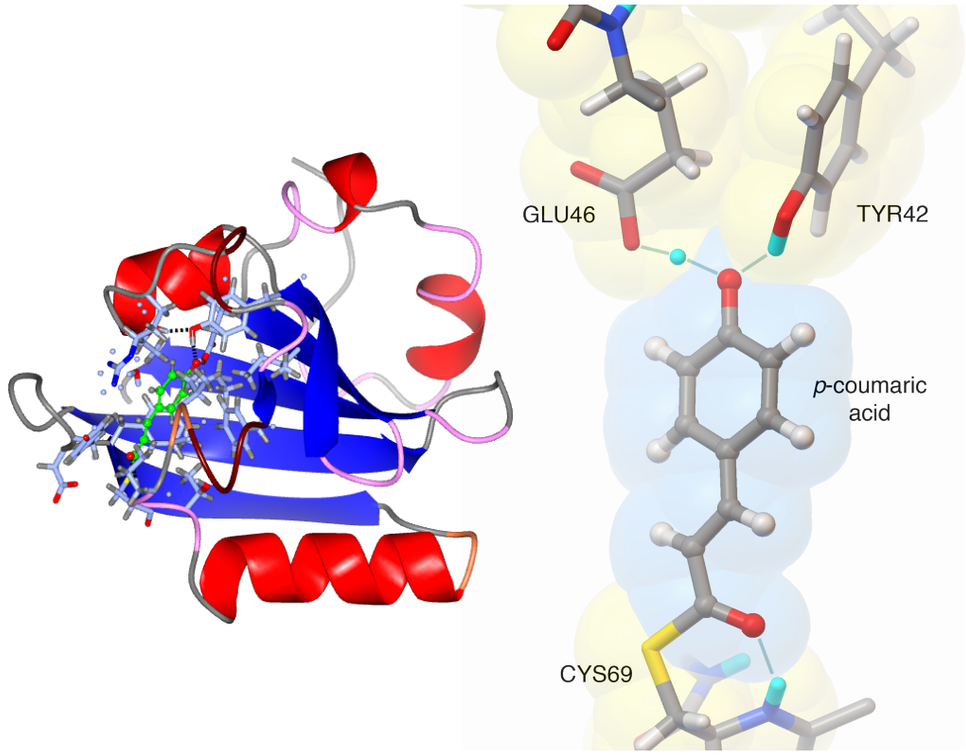
Crystal

No lens!



- Crystal requires no lens.
- Crystal **concentrates** its signal on individual spots *aka* **reflections**

Crystallography in Brief

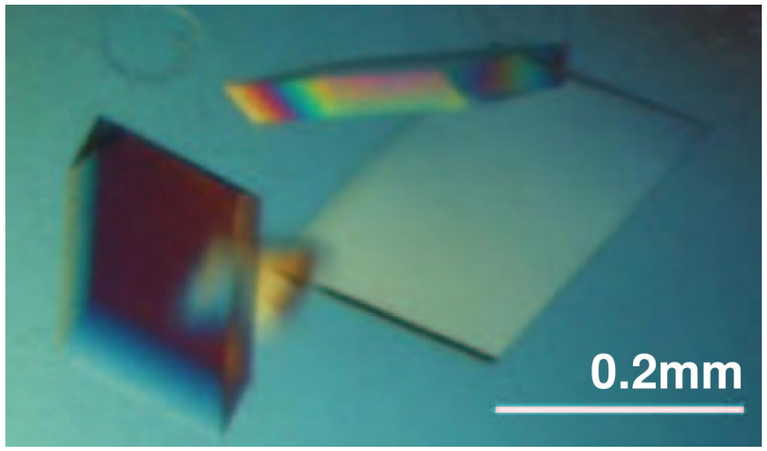


- Crystals amplify the signals from atoms so that the signal can be detected.
- Data are spot positions and spot intensities
- Data **are not** atoms, some calculations are required
- Crystal structures provide high level of detail insight

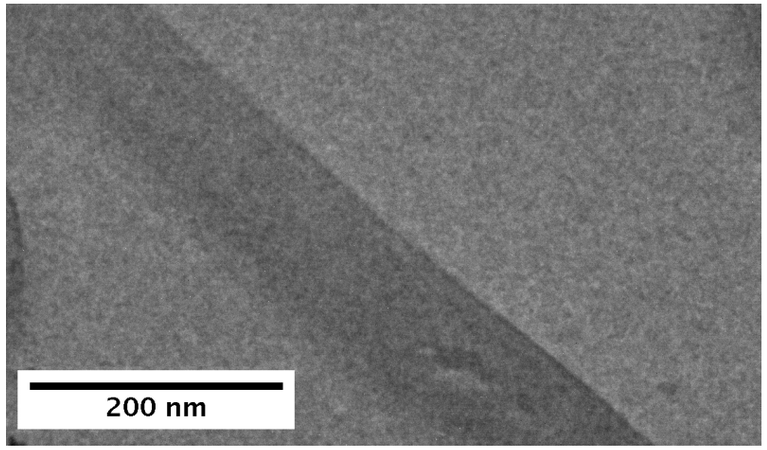
Photoactive Yellow Protein (2ZOI): Turns light into molecular movement. PDB Molecule of the Month March 2017

Electron Crystallography

Combining Electron Radiation (and Microscopes) with Crystallography

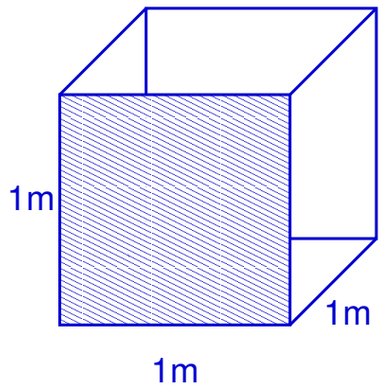


typical protein crystal size for X-rays (0.2mm = $200\mu m$)



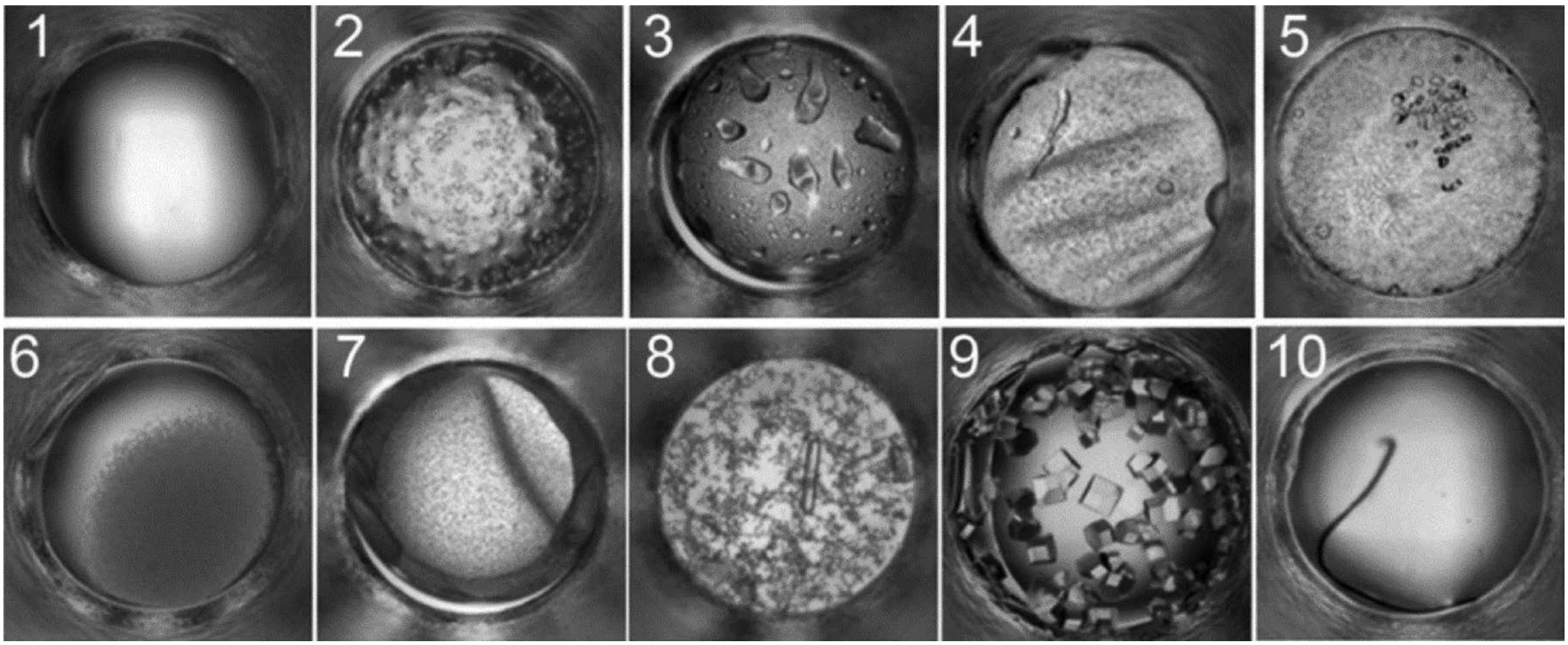
typical protein crystal size for electrons, $0.1 \times 0.14 \times 1.7\mu m^3$

The combination of **electron radiation** (as in EM) with **crystallography** permits crystallographic data collection from tiny crystals.



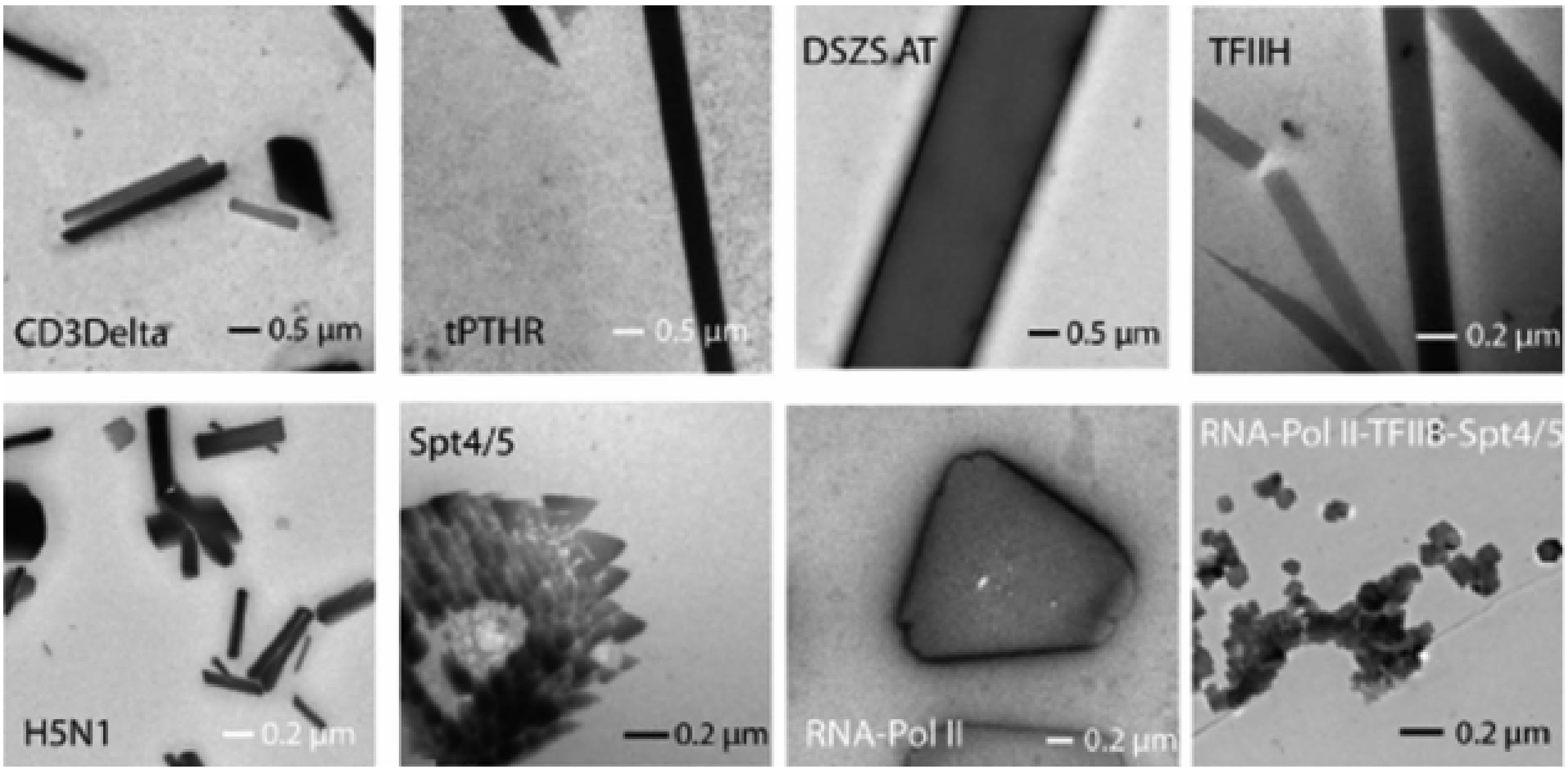
volumes compare like 6 bath tubs of water *vs.* a $10\mu l$ drop from a pipette

Typical Protein Crystallisation Trials



Luft, Wolfley, Snell, Crystal Growth& Design (2011), 11, 651–663

Drops viewed through TEM



Stevenson,. . . , Calero, PNAS (2014) 111, 8470–8475 / Calero, . . . , Snell, Acta Cryst (2014) F70, 993–1008

Drug Research: The Novartis Library

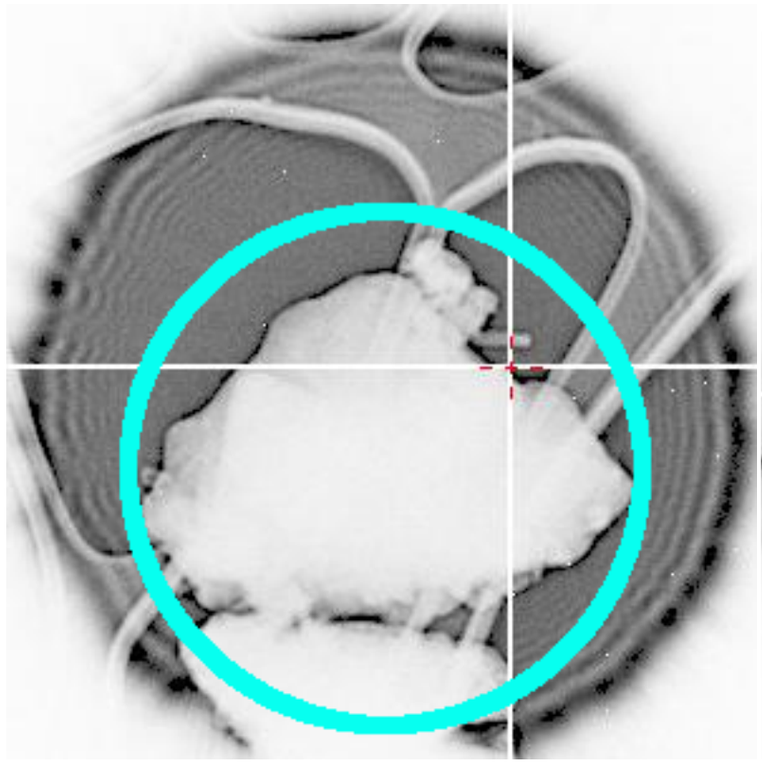
- 2,000,000 compounds of potential drug targets

- 30-40% suitable for X–ray powder analysis

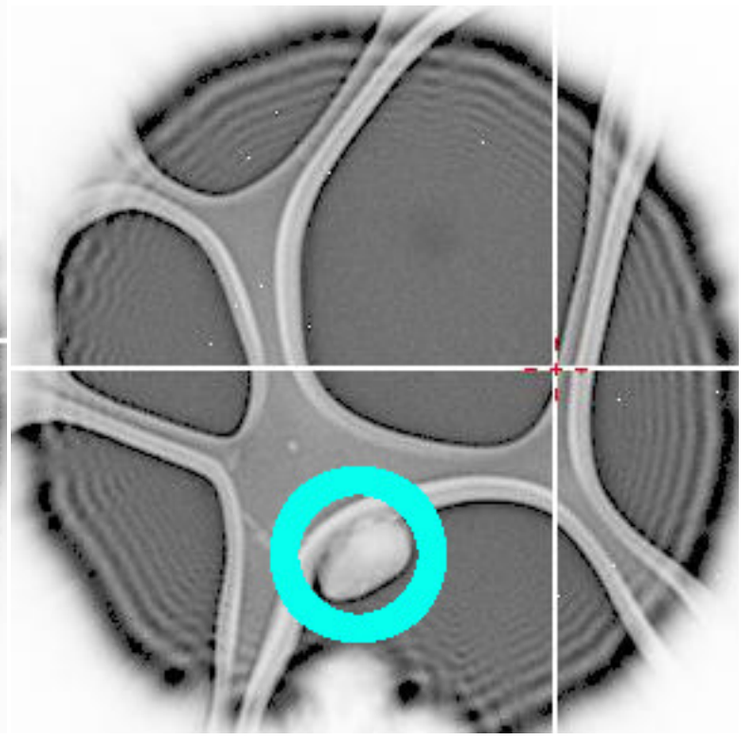
- 10% suitable for single crystal X–ray analysis

Dr. Trixie Wagner (2012)

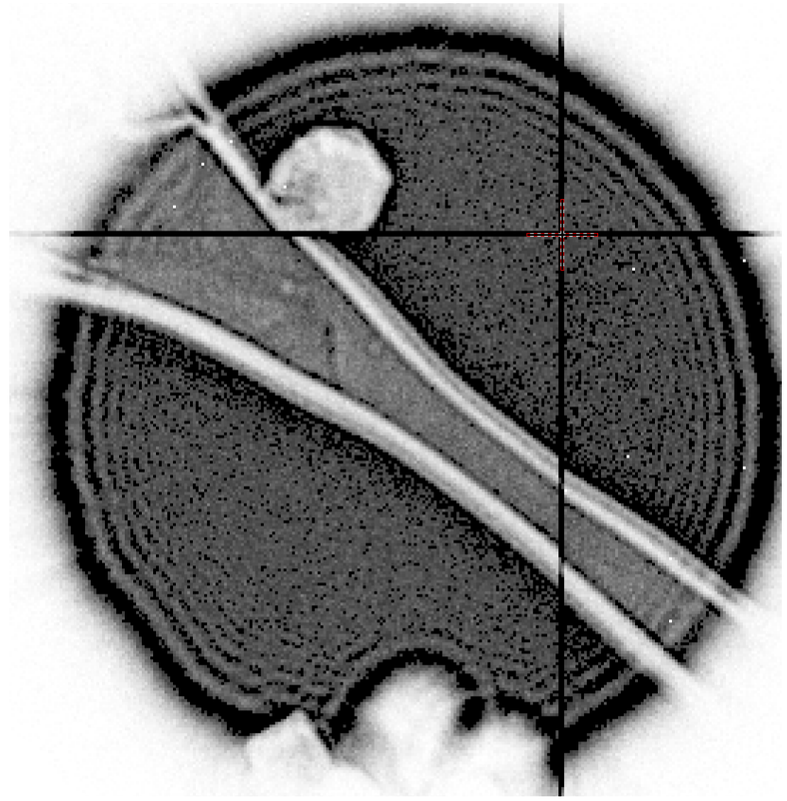
Powders contains Single Crystals



Novartis IRELOH:
$\varnothing = 1,700nm = 1.7\mu m$



Novartis EPICZA:
$\varnothing = 500nm = 0.5\mu m$



Zeolite (Prof. Bokhoven):
$\varnothing = 300nm = 0.3\mu m$

2 - Structures

Macromolecular Structures

- Protein Data Bank (PDB, www.pdb.org) contains about 13 protein structures from 3D electron diffraction

- Started 2013 (PDB ID 2013)

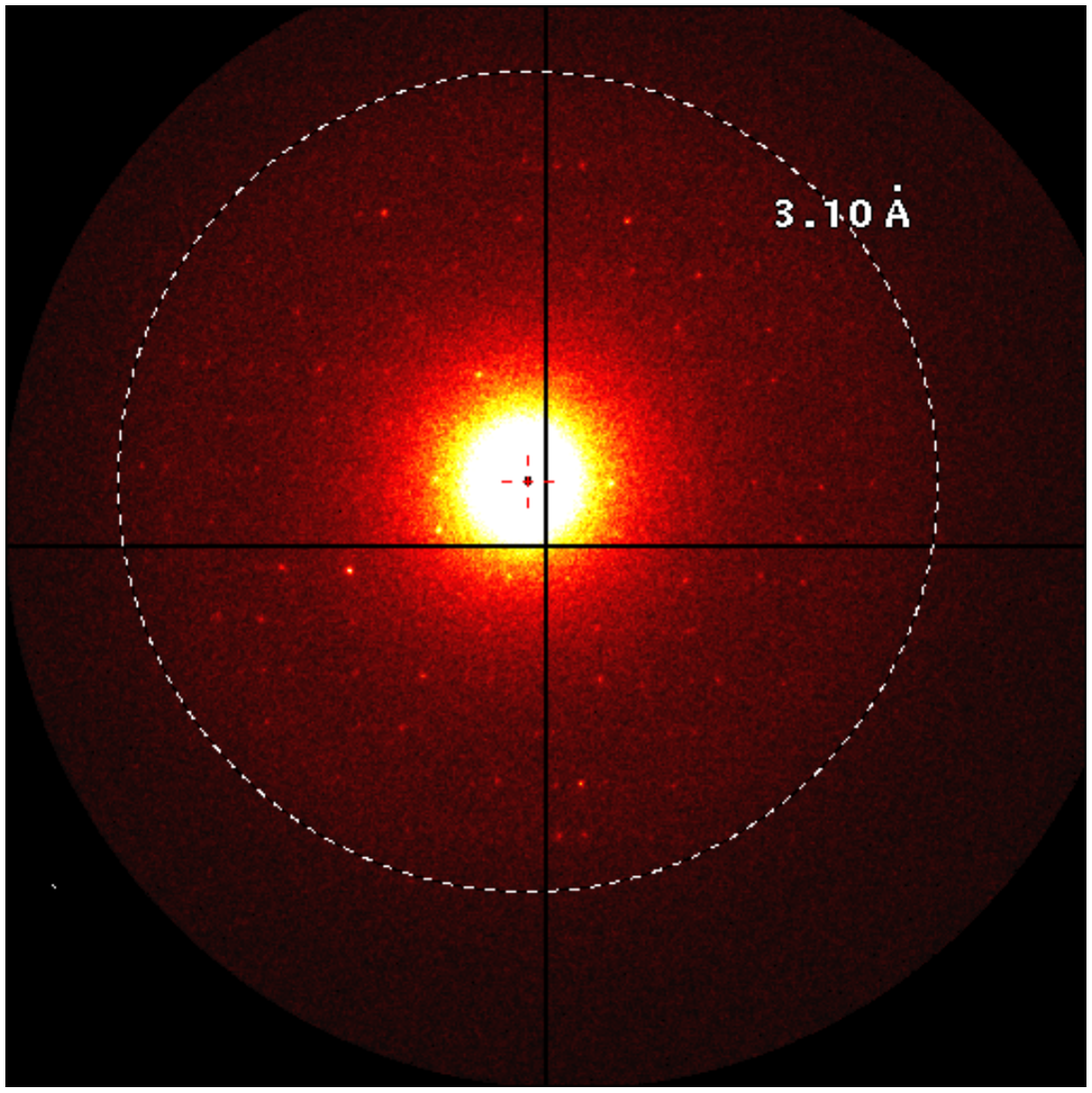
- (Mostly) commonly known protein — not a new structure to date.

Structures from Test Proteins

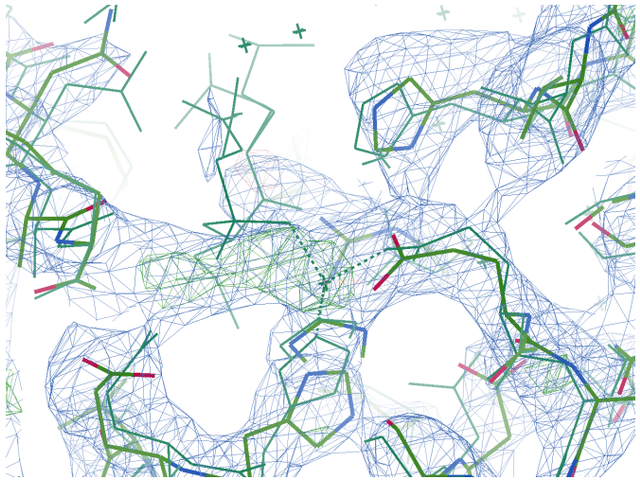
Sample (PDB id; EMDB id)	Tau peptide (5k7n; EMD-8216)	Lysozyme (5k7o; EMD-8217)	TGF-βm:TβRII (5ty4; EMD-8472)	Xylanase (5k7p; EMD-8218)	Thaumatin (5k7q; EMD-8219)	Trypsin (5k7r; EMD-8220)	Proteinase K (5k7s; EMD-8221)	Thermolysin (5k7t; EMD-8222)
	Data collection							
Resolution (Å)	14.70–1.10	30.58–1.50	26.64–2.90	25.55–1.90	27.73–2.11	27.63–1.50	20.75–1.30	30.14–1.60
# crystals	2	7	3	4	3	10	6	4
$\langle T_{exposure} \rangle$ (s)	159.9	127.7	140.8	172.7	179.7	155.8	122.2	187.6
Molecular weight (kDa)	0.7	14.4	19.1	21.0	22.2	23.4	28.9	34.6
	Data processing							
Resolution[1] (Å)	14.70–1.10 (1.23–1.10)	30.58–1.80 (1.84–1.80)	26.64–2.90 (3.07–2.90)	25.55–2.30 (2.38–2.30)	27.73–2.51 (2.61–2.51)	27.63–1.70 (1.73–1.70)	20.75–1.60 (1.63–1.60)	30.14–2.50 (2.61–2.50)
Space group	*C*121	$P4_32_12$	$P2_12_12_1$	$P2_12_12_1$	$P4_12_12$	$P2_12_12_1$	$P4_32_12$	$P6_122$
Unit cell								
a, b, c (Å)	29.42, 4.99, 37.17	76.23, 76.23, 37.14	41.53, 71.33, 79.51	48.16, 59.75, 69.81	58.12, 58.12, 150.31	53.18, 56.43, 64.67	67.06, 67.06, 100.71	92.07, 92.07, 128.50

Cruz et al., “Atomic-resolution structures from fragmented protein crystals with the cryoEM method MicroED”, Nature Methods(2017), 14, 399–405

Thermolysin (sample courtesy Ilme Schlichting)



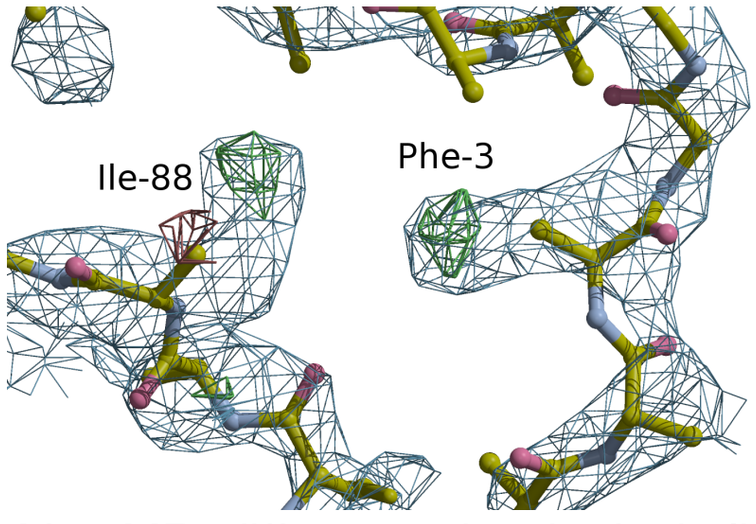
- Spacegroup $P6_122$
- Unit Cell 94.3 94.3 130.4 90° 90° 120°
- $d_{\text{min}} = 3.5$Å
- 72.4% completeness
- MR with 3DNZ poly Alanine: TFZ=26.4, LLG=433
- Buccaneer: side chain extension 315/316
- Refmac5: R1/“Rfree” = 28.0% / 29.9% (4N5P w/o water)



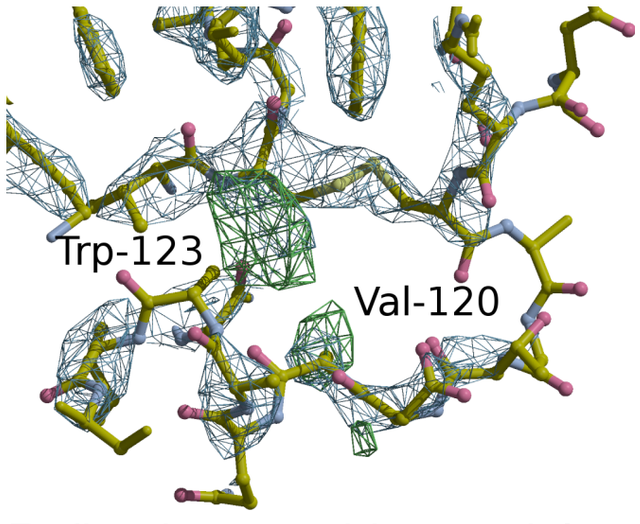
Lysozyme

	Single crystal	Merged data
Data integration		
Space group	$P2_12_12$	
Unit cell dimensions		
a, b, c (Å)	104.56, 68.05, 32.05	
α, β, γ (°)	90.0, 90.0, 90.0	
Number of crystals	1	6
Resolution (Å)	32.05-2.50 (2.57-2.50)	57.04-2.50 (2.57-2.50)
R_{merge} (%)	31.7 (107.3)	35.7 (113.2)
I/σI	2.92 (1.10)	2.87 (1.10)
Completeness (%)	41.0 (40.5)	69.0 (51.3)
Reflections	9518 (817)	25148 (1373)
Unique reflections	3445 (236)	5808 (299)
Redundancy	2.76 (3.46)	4.33 (4.59)
Refinement		
R1 (%)	25.90	23.54
$R_{complete}$ (%) [4]	32.49	27.21
<B> (Å^2)	33.08	36.49
RmsZ bonds	0.779	0.765
RmsZ angles	0.974	0.911

1. MR (Phaser) from poly Ala **monomer** determines space group $P2_12_12$ (TFZ=19.8, LLG=335.3)
2. Side chain completion with Buccaneer all except 27 atoms
3. Refinement with refmac5



After MR: difference density for bulky side chains



Refined map guides model completion

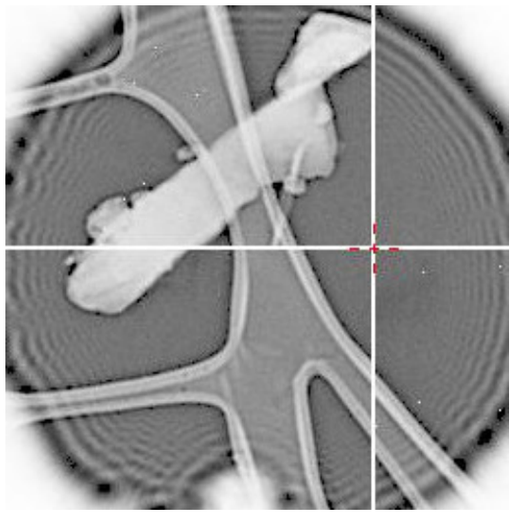
Organic Structures

- Pioneers: ZG Pinsker, BK Vainshtein (1940s +; 1990s)
- D Dorset (1995: Textbook Electron Crystallography)
- U Kolb (recording of 3D diffraction patterns, ADT, 1997+)
- X Zou, S Hovmóller (recording of 3D diffraction patterns with beam precession, RED, 2008+)

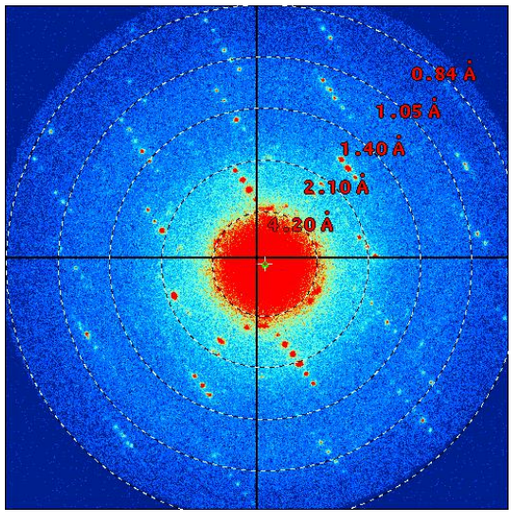
Pharmaceutical I: Visualisation of Hydrogen Atoms

H–atom positions can be refined against electron diffraction data
CCDC: IRELOH, Dai et al., Eur. J. Org. Chem (2010), 6928-6937

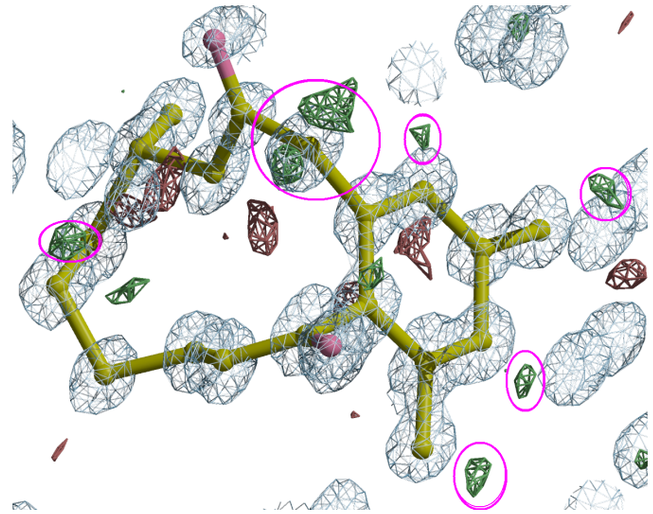
Sample courtesy Novartis



- Field of view: $3\mu m$
- Crystal: $1.6\mu m \times 400nm$



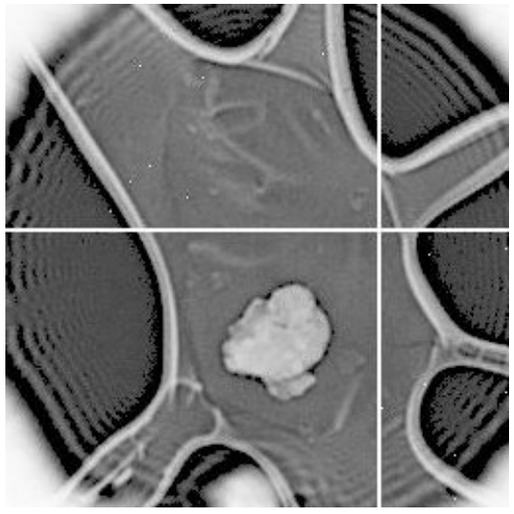
- $d_{\text{min}} < 0.8$Å
- $P2_12_12_1$: 85% completeness with 3 crystals
- a=8.06Å b=10.00Å c=17.73Å



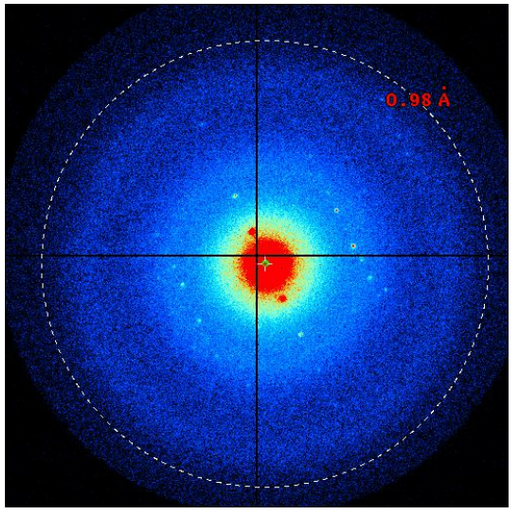
- **Hydrogen atoms** in difference map even with poor model
- 1334 reflections, 195 parameters, 156 restraints (RIGU)
- $R1 = 15.5\%, R_{\text{complete}} = 18.5\%$

Pharmaceutical II: Differentiation of Atom Types

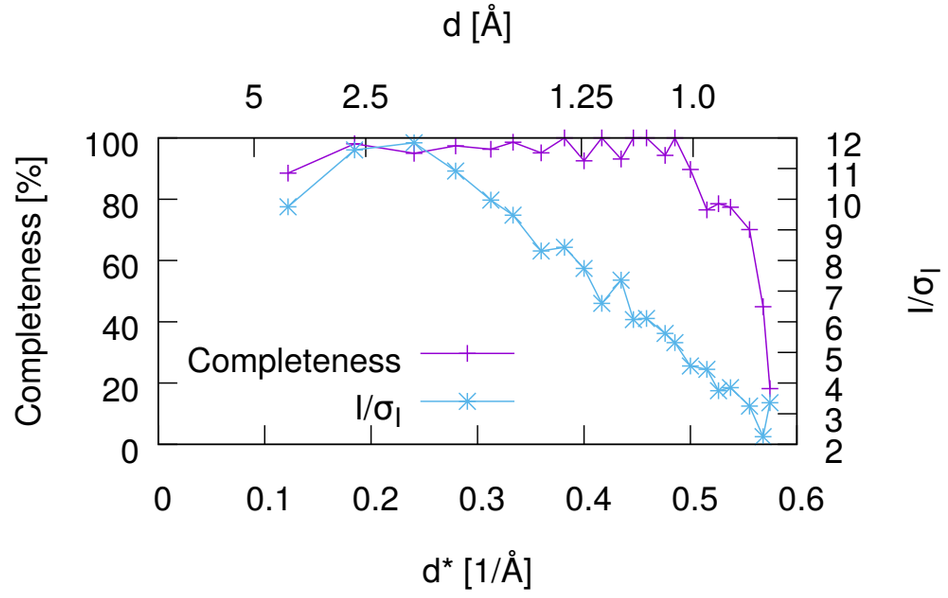
Data quality: recognition of atom types, C *vs.* O *vs.* N *etc.* (CCDC: EPICZA)



- Field of view: $3\mu m$
- Crystal: $400nm$ diameter

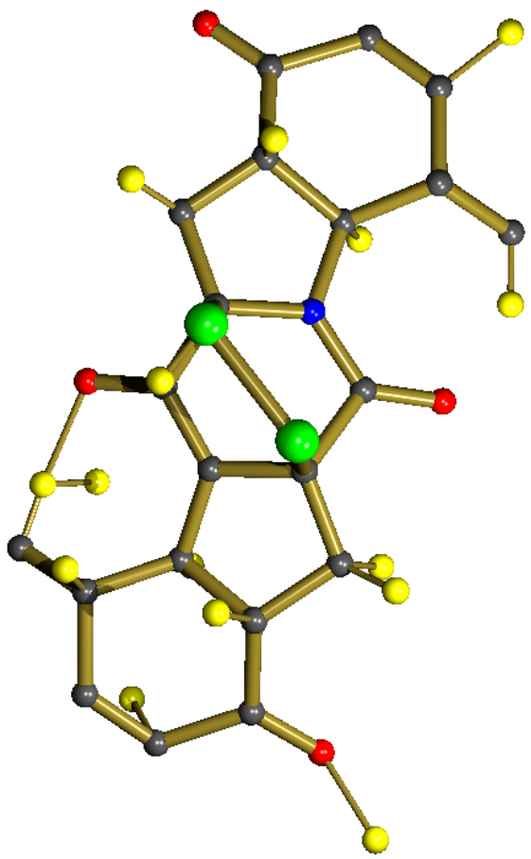


- $d_{\text{min}} = 0.87$Å
- a=11.35Å, b=12.7Å, c=13.0Å
- $P2_12_12_1$: completeness with 4 crystals: 86%

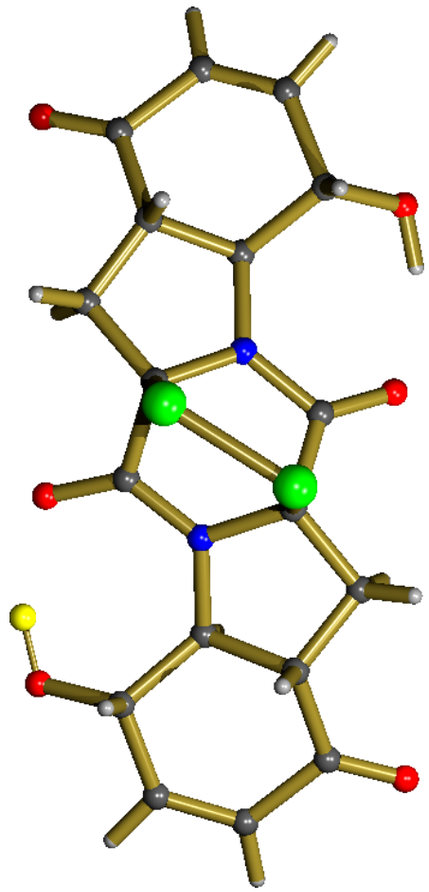


- 2545 refl., 258 param., 267 restraints (RIGU)
- all data: $R1 = 15.9\%, R_{\text{complete}} = 19.1\%$
- $R1 = 14.7\%, R_{\text{complete}} = 18.0\%$

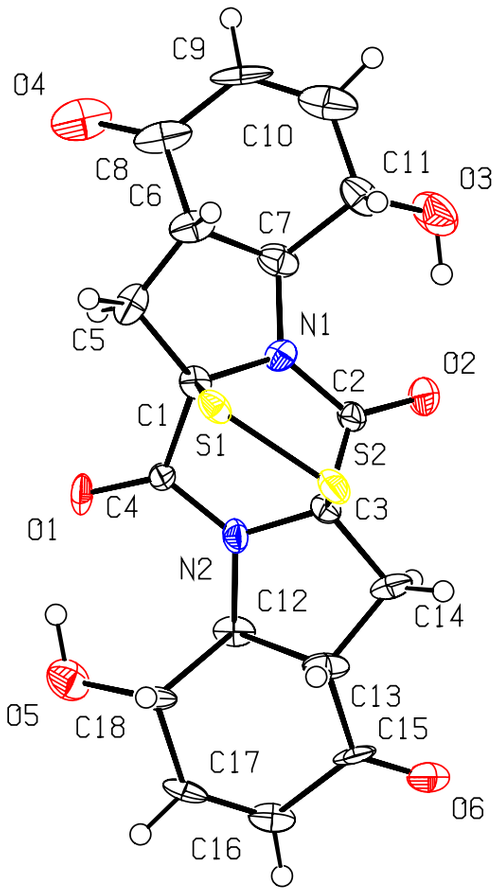
Pharmaceutical II (EPICZA): Structure Solution Process



Direct methods reveal H atoms
=data quality



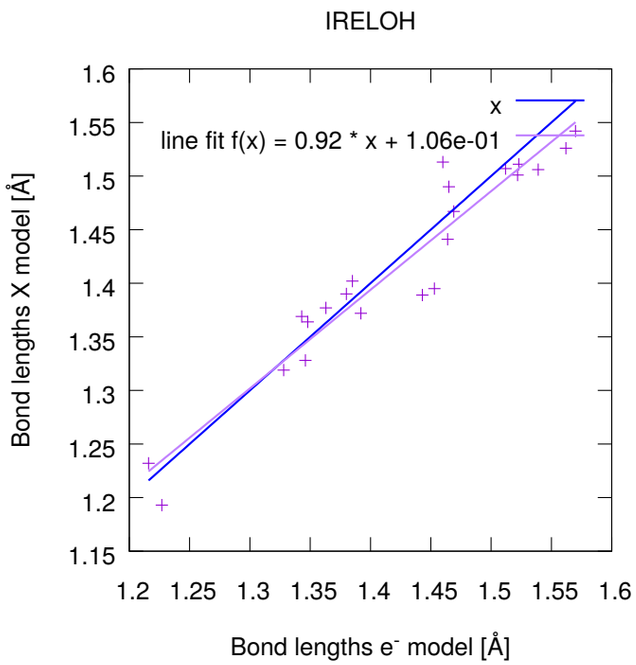
HFIX: all except 1 H
=model quality

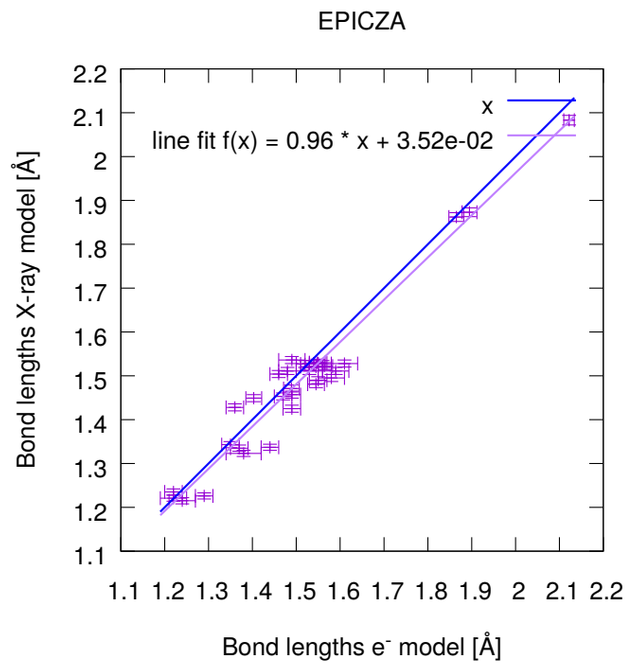


Final Structure

Summary: Electron Diffraction of Organic Compounds

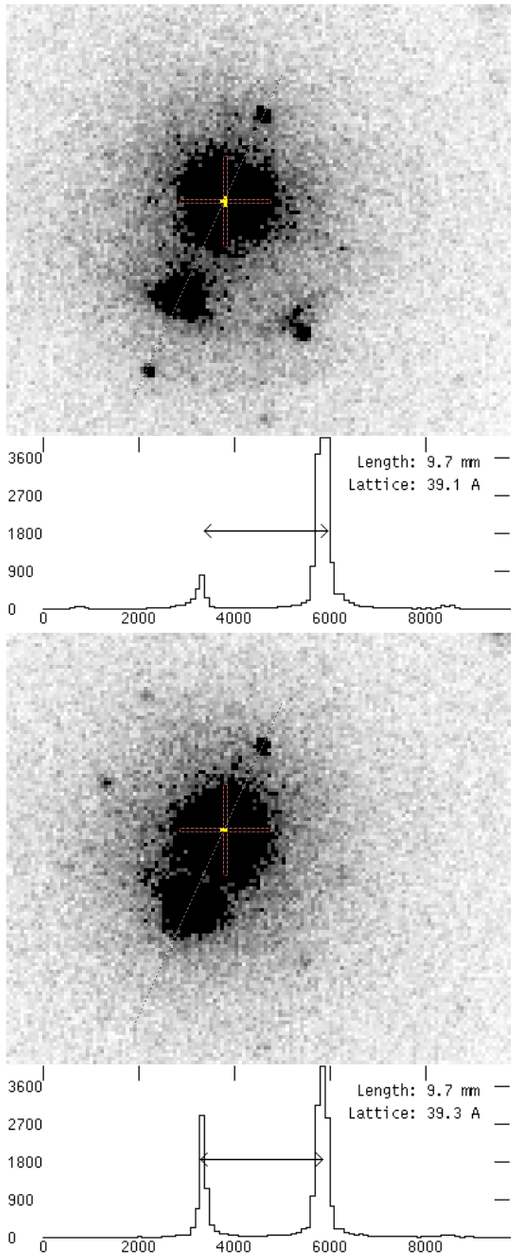
- Structures can be solved with X–ray knowledge and methods.
- Radiation damage present, but not (always) limiting
- Kinematic approximation sufficient for high quality structures





3 - Technical Aspects

Dynamic Scattering

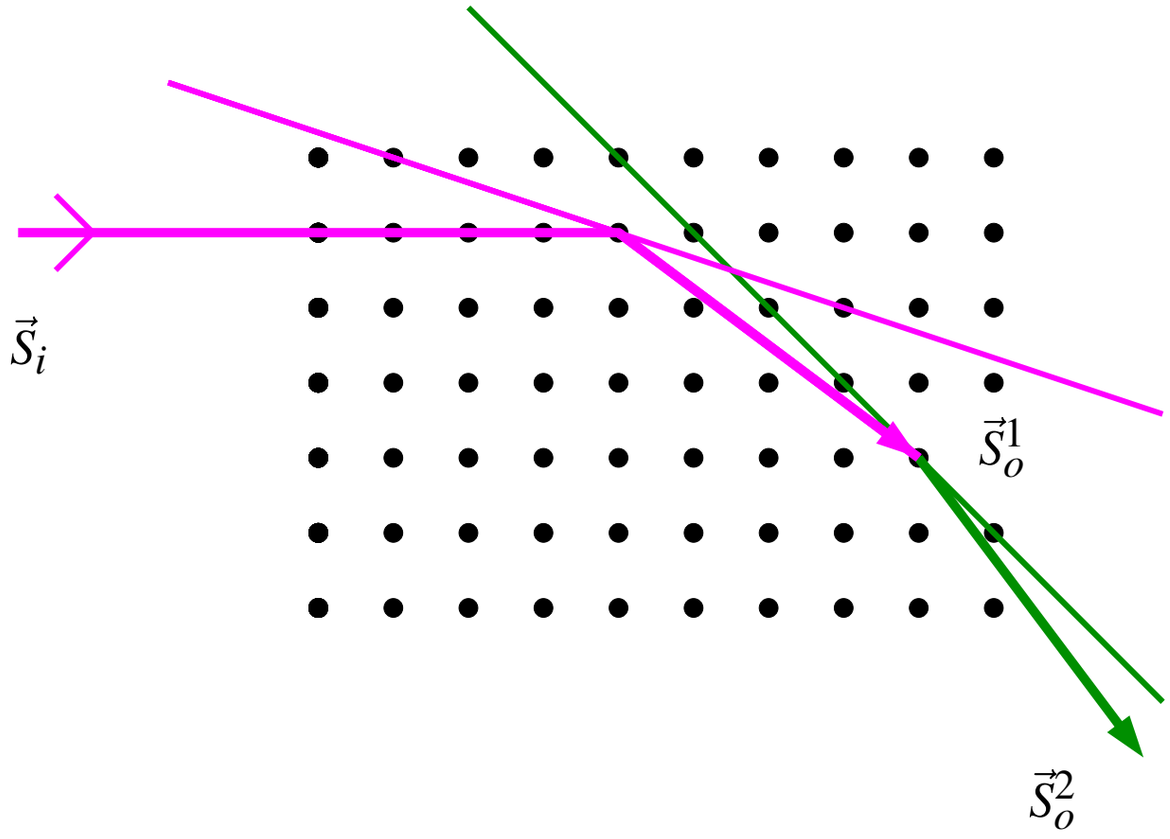


Data from SAPO–34: $I(-2,-1,1) > I_{\text{direct beam}}$ (Eiger chip, 256x256 px)

Kinemtic (X–ray) and Dynamic (e^-) Scattering

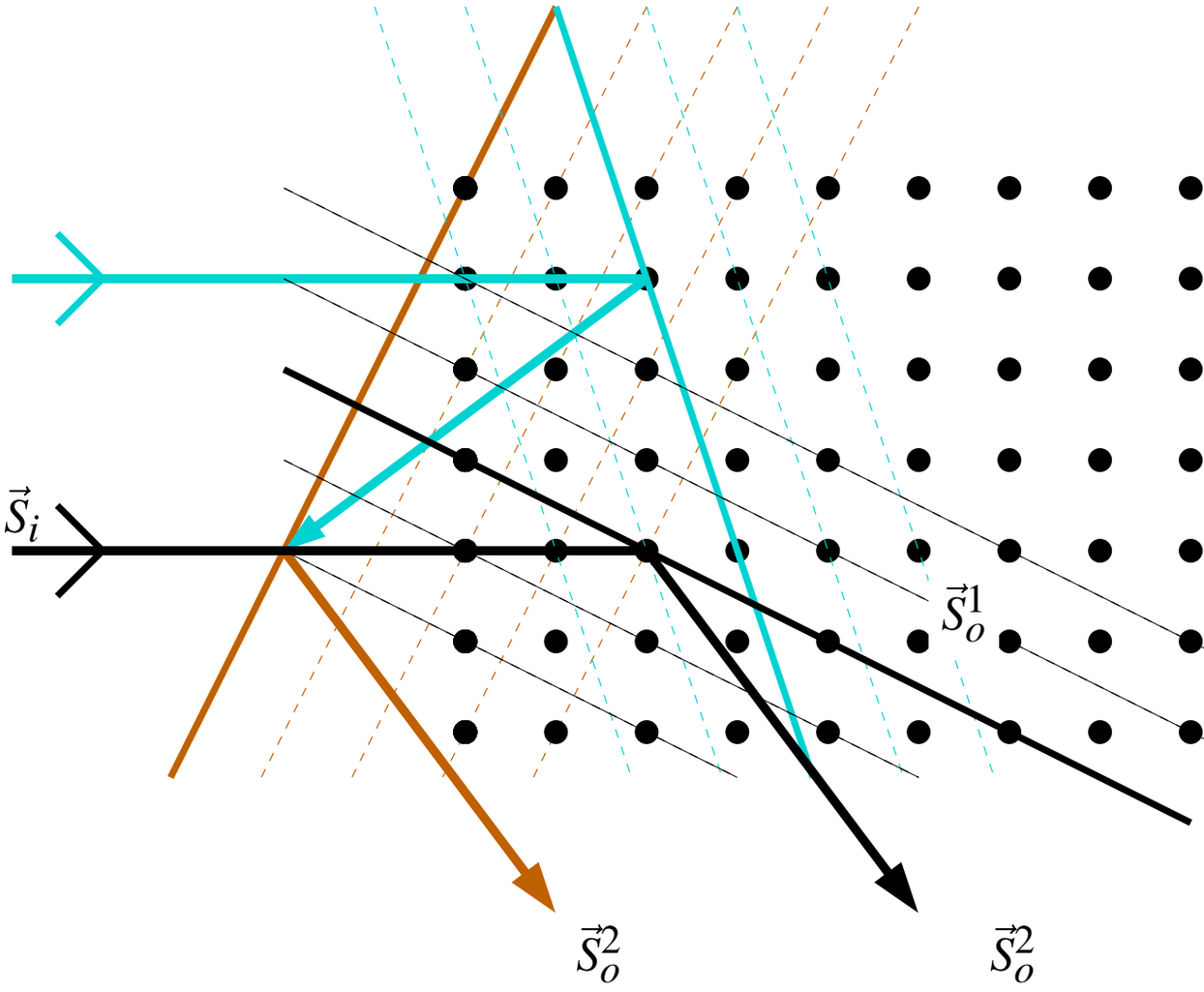
- Kinematic Theory of Diffraction: Every photon / electron / neutron scatters once in the crystal
- $|F_{\text{ideal}}(hkl)| \propto \sqrt{I_{\text{exp}}(hkl)}$
- Dynamic Scattering: Multiple Scattering events occur
- Electron Diffraction: Multiple Scattering occurs even with nanocrystals
- For data from proteins: Currently no satisfactory treatment

Multiple (Dual) Scattering



- Outgoing ray $\vec{S}_o^1$ acts as incoming ray for reflection $\vec{S}_o^2$.
- Probability of re–reflection thickness dependent

Multiple (Dual) Scattering



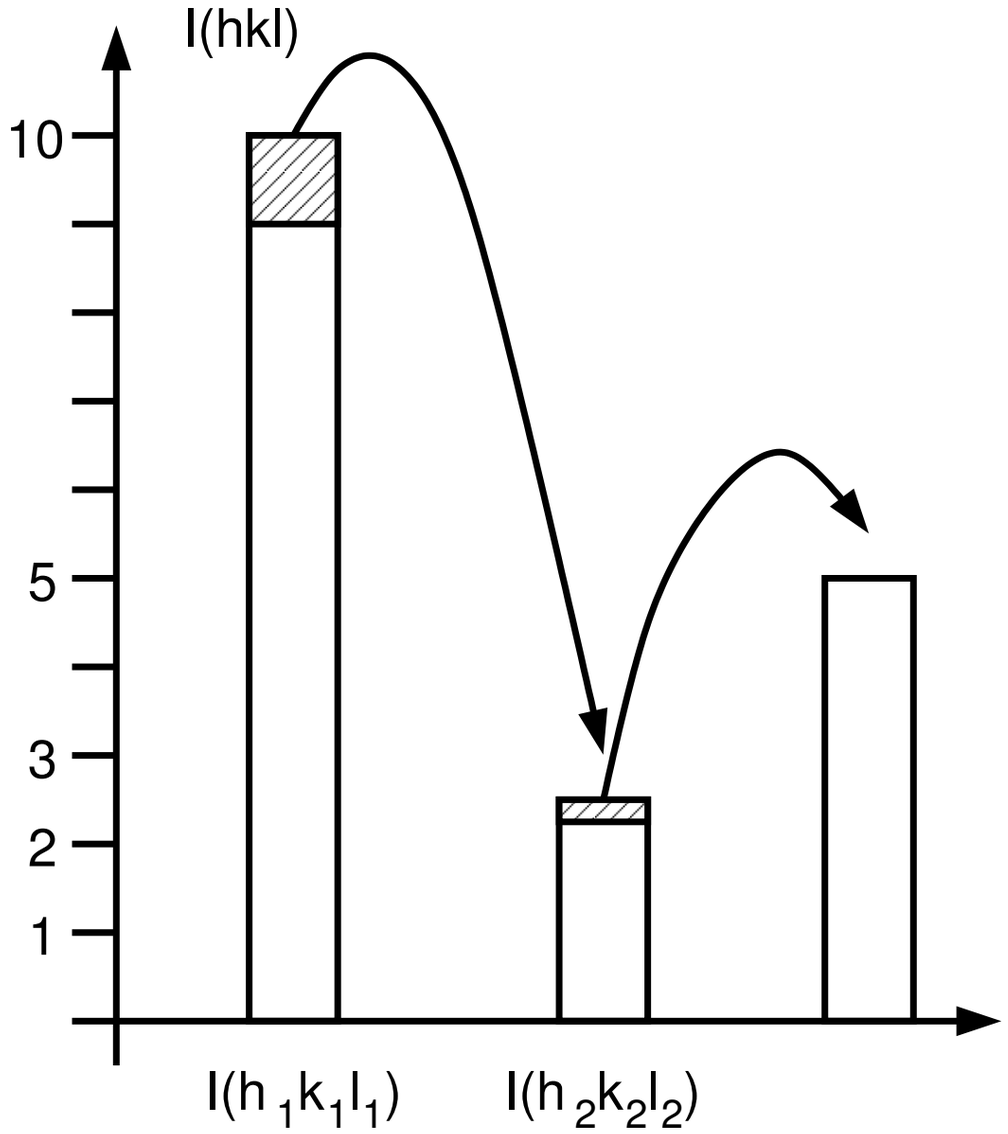
Laue Conditions (accordingly $\vec{b}$ and $\vec{c}$):

$$(\vec{S}_o^1 - \vec{S}_i) \cdot \vec{a} = h_1$$
$$(\vec{S}_o^2 - \vec{S}_o^1) \cdot \vec{a} = h'$$
$$(\vec{S}_o^2 - \vec{S}_i) \cdot \vec{a} = h_1 + h' = h_2$$

Requirement for detrimental effect on $I(h_2, k_2, l_2)$

- $I(h_1 k_1 l_1)$ must be strong
- $I(h', k', l')$ must be strong
- $I(h_2 k_2 l_2)$ must be weak
- $I(h_1 k_1 l_1)$ and $I(h_2 k_2 l_2)$ on same frame

Multiple (Dual) Scattering

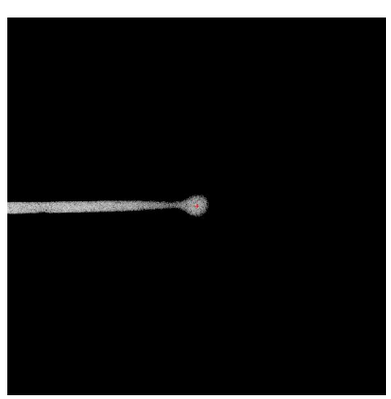


- Re-reflection more likely for thicker crystal(path)
- Percentage similar for all reflections on frame ($2\theta \approx 0$)
- 10% of strong reflection affects weak reflection

$\Rightarrow$ Measured intensities “shifted” from strong to weak

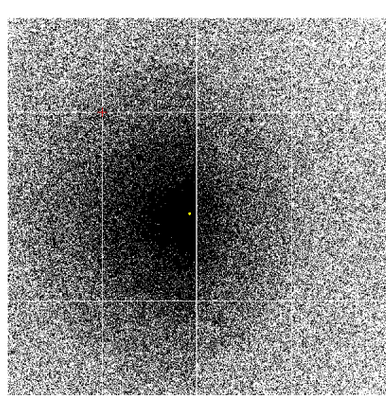
$\Rightarrow$ Low resolution reflection under–, high resolution reflections overestimated

Electron Detectors for Diffraction



TVIPS CMOS

$20 \leq I \leq 21$ cts



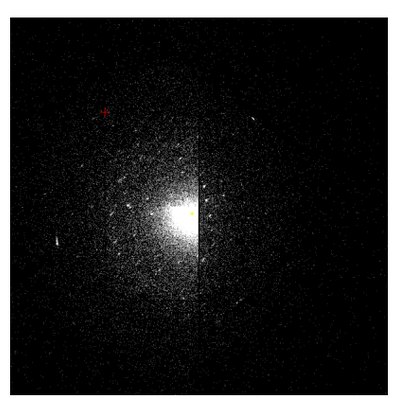
1024x1024 Timepix

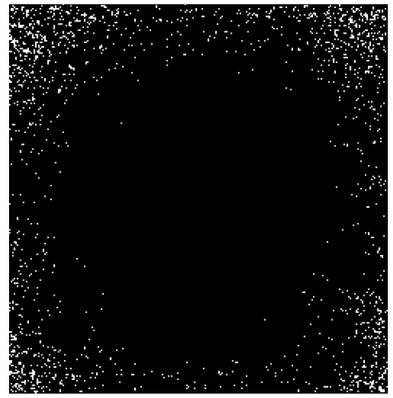
$0 \leq I \leq 1$ ct Lysozyme (inv[d])

$\approx 1kHz, 50\mu\text{m} \times 50\mu\text{m}$

cut–off: 11809

dead time $\approx 0.01s$





256x256 Eiger (PSI)

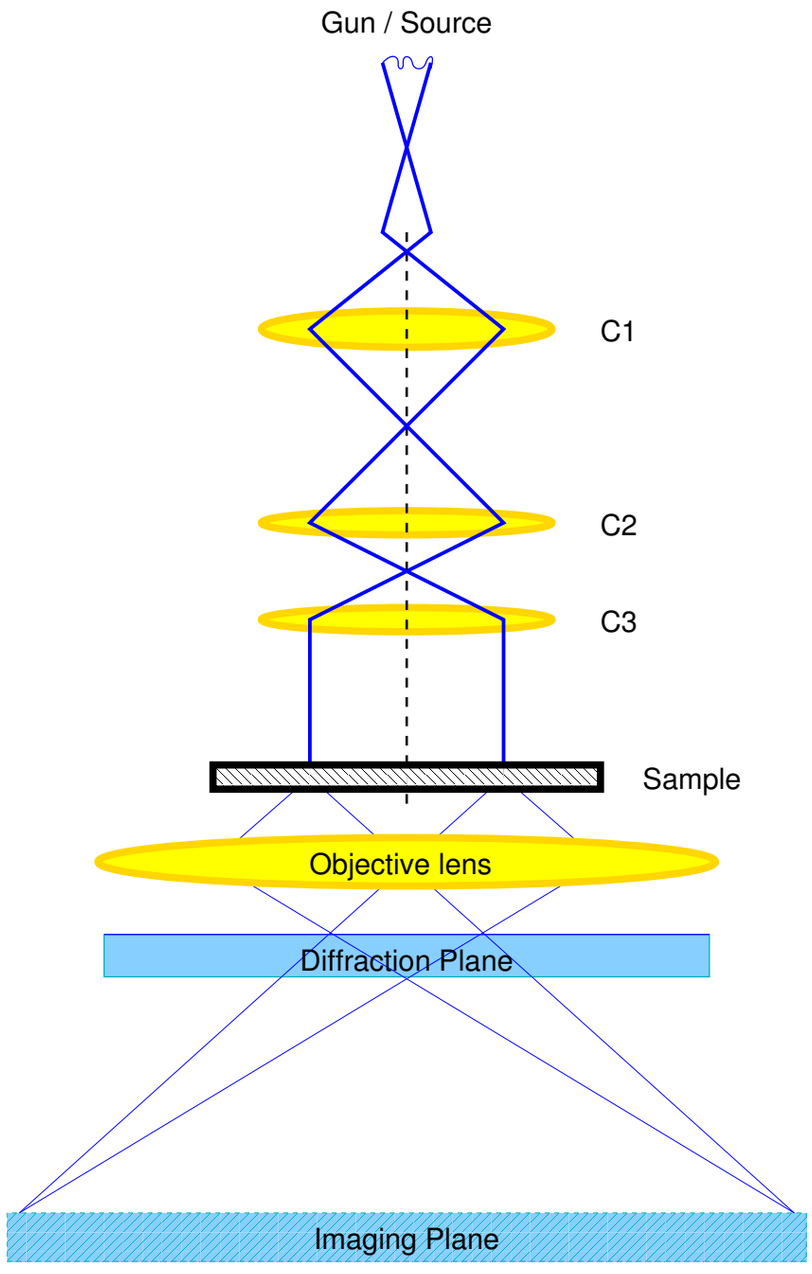
$0 \leq I \leq 1$ ct SAPO–34 crystal

$\leq 23kHz, 75\mu\text{m} \times 75\mu\text{m}$

cut–off: 16, 64, or 4096 (4, 8, 12 bit)

dead time 3μs

The Lens System



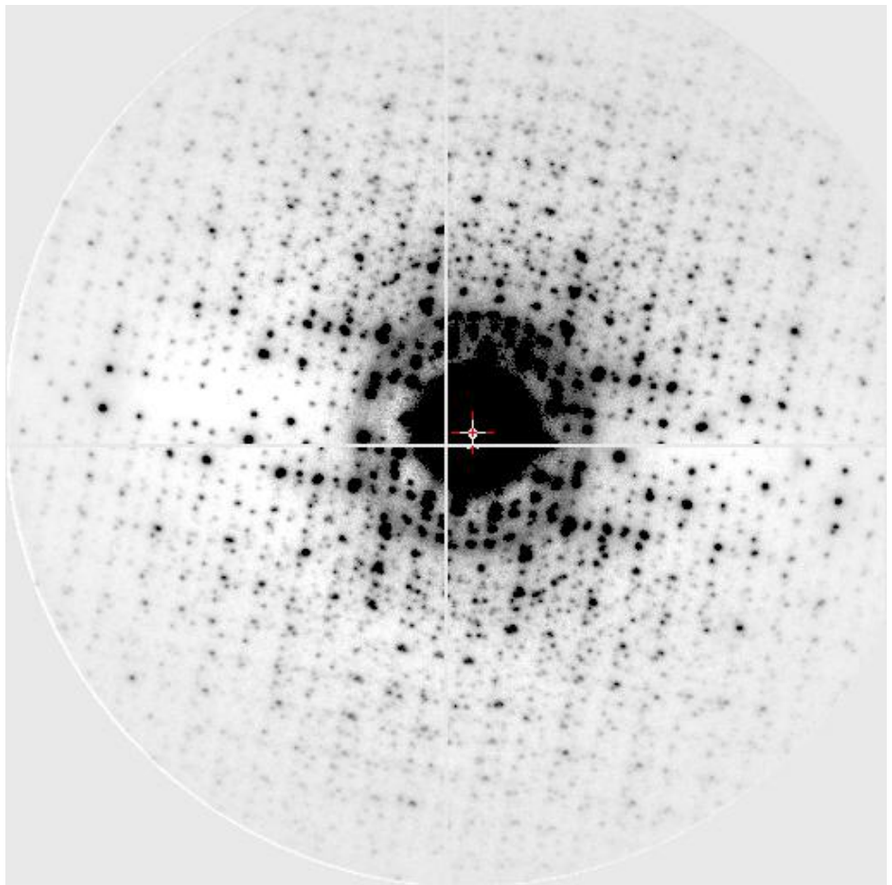
- Lenses C1–C3 shape beam
- Crystallography: Parallel beam
- Objective lens: sets effective detector distance to back-focal plane = diffraction mode
- C3 not present in all microscopes

Lenses cause distortions.

Garnet Andradite

- The garnet Andradite, $Ca_3Fe_2{}^{3+}(SiO_4)_3$, radiation hard
- 2 grids courtesy Xiaodong Zou (Stockholm)
- Space group $Ia\bar{3}d$, $a = 12.06314(1)$Å (ICSD No. 187908)

(Wikipedia)



- Summed images from Garnet (200keV)
- 66.8° rotation
- good coverage of detector surface

Spatial Correction for the Detector Surface

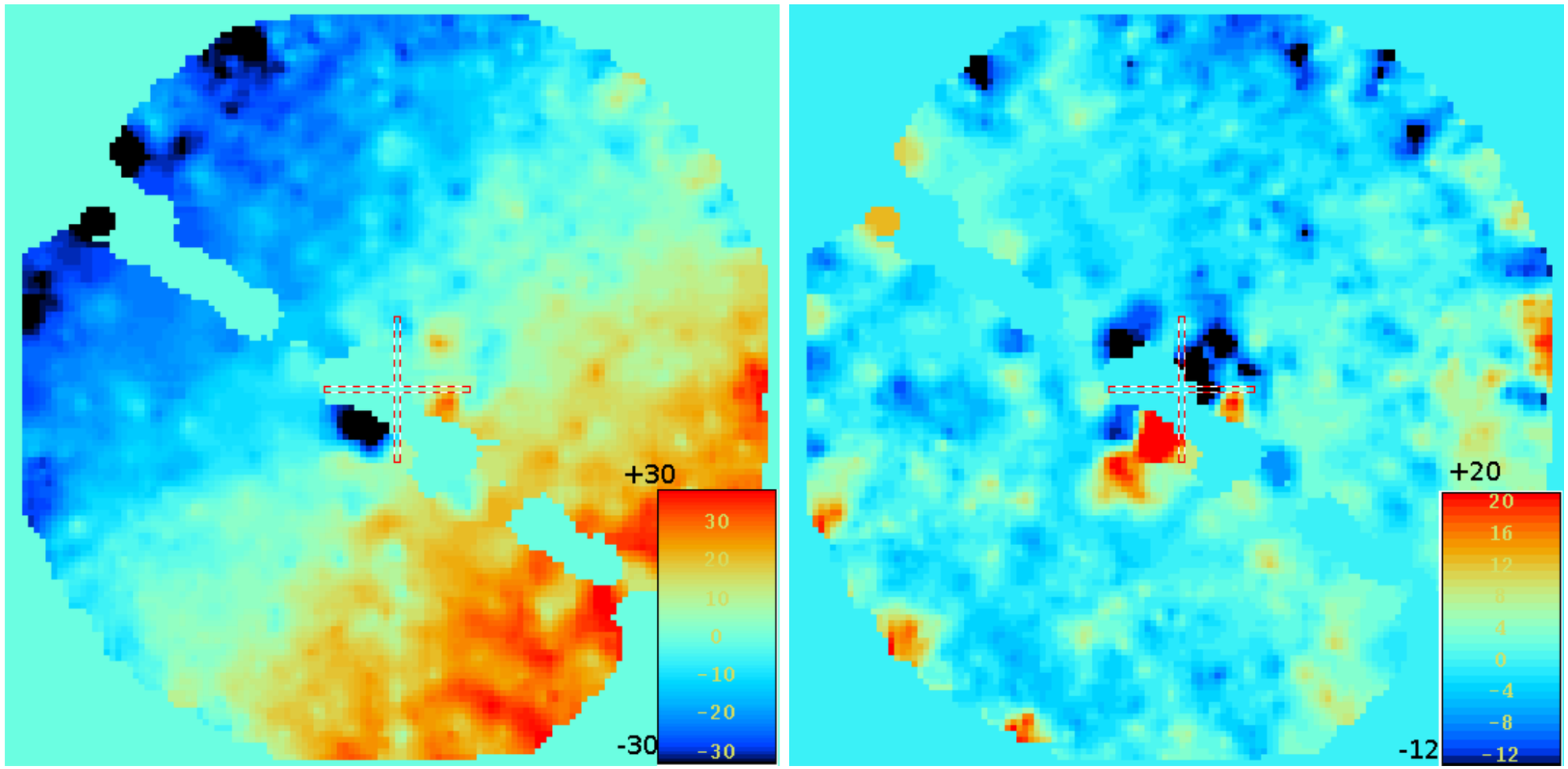
- Spot positions **calculated** from Laue Conditions

$$\vec{S}.\vec{a} = h$$
$$\vec{S}.\vec{b} = k$$
$$\vec{S}.\vec{c} = l$$

- Data Processing: Deviations between **calculated** and **observed** positions
- *e.g.* XDS: per–pixel look-up tables for X– and Y–coordinates
- **Independent** of Source of Error

Spatial Correction for the Detector Surface

XDS Correction Table X–coordinate and Y–coordinate



Directly Visible Improvements

Garnet Data set processed before spatial correction:

BEAM_DIVERGENCE: 0.16°
REFLECTING_RANGE: 0.47°

Garnet Data set processed after spatial correction:

BEAM_DIVERGENCE: 0.15°
REFLECTING_RANGE: 0.28°

Improved Cell Accuracy with Look–up Tables

1. Collect data from garnet
2. Change as little as possible
3. Collect data from target sample
4. Process using garnet correction tables

Sample Courtesy Roche $C_{31}H_{29}Cl_2F_2N_3O_4$**, SG** $P2_1$

Data Collection and Processing: **Max Clabbers**

	a	b	c	α	β	γ
XRPD	6.405	18.206	25.829	90.000	92.180	90.000
XDS uncorrected	6.556	18.728	26.276	90.500	92.243	90.540
XDS corrected	6.564	18.721	26.254	90.064	92.171	90.137

4 - Conclusions

- Electron Diffraction = Structures from very small crystals $< 1\mu m$
- Applications: Single Crystal Structures, where X–rays only see powder
- High quality data + structures for organic compounds
- Proteins: Radiaton damage currently limits competing data resolution: new ways of data collection required

5 - Acknowledgements

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