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Macromolecular Electron Crystallography

Bioinformatics and X–Ray Structural Analysis — Universität Konstanz
13th February 2017

1 - Outline

1. Structure Determination with Crystallography

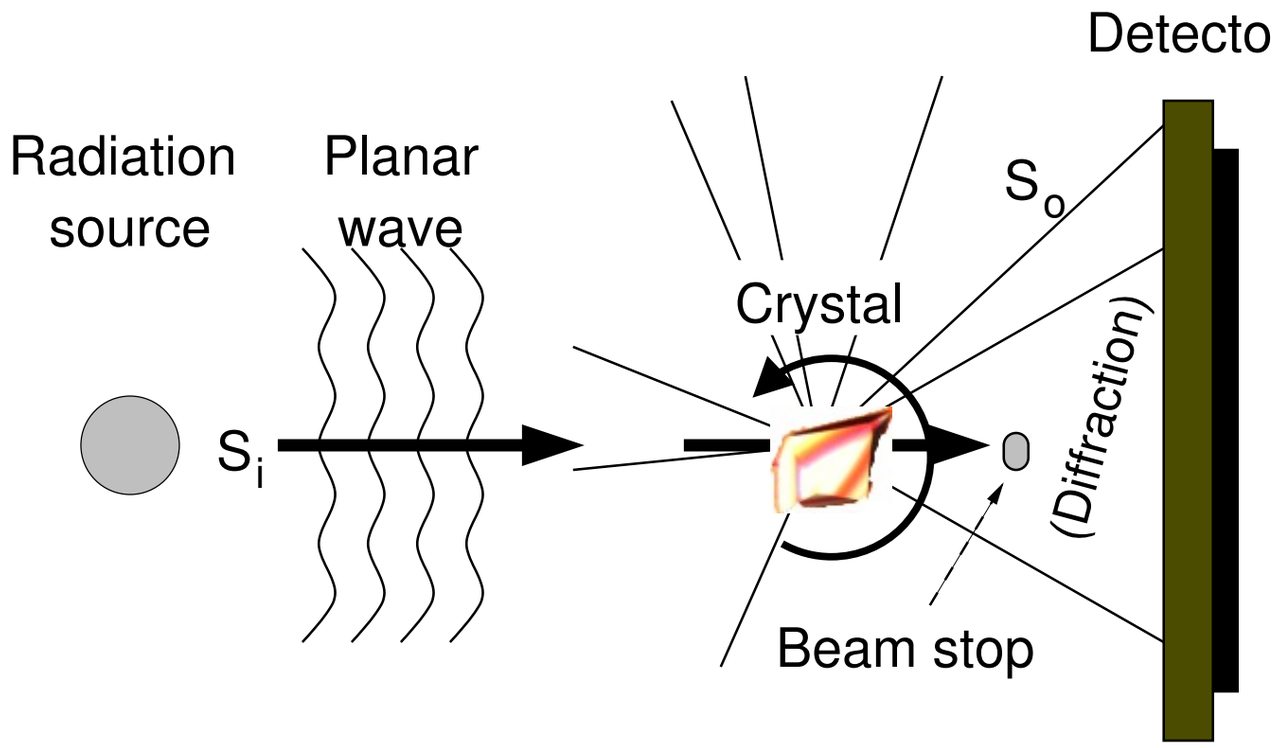
2. Electron Diffraction

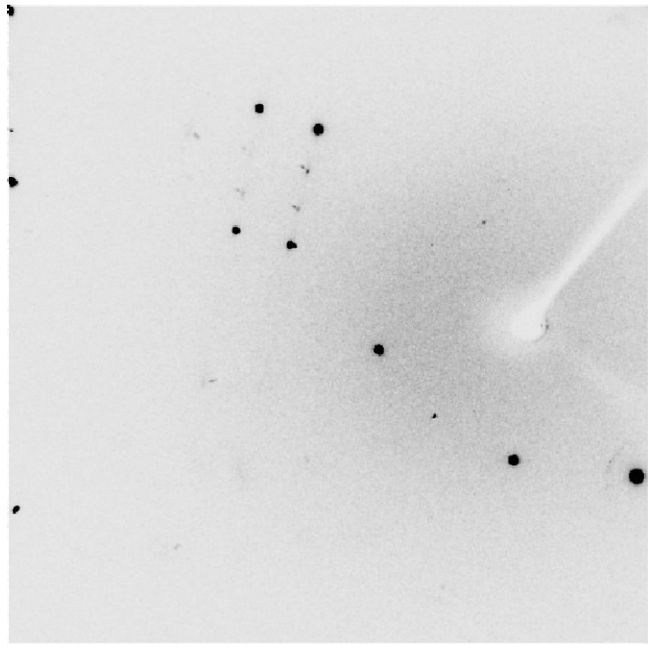
3. Radiation Damage

4. Dynamic Scattering

5. Examples: Lysozyme & Thermolysin

2 - Structure Determination by Single Crystal Diffraction





- Diffraction spots: interaction between **wave** and **crystal**

- Experimental result: **Position** and **Intensity** for each spot

Spot Position

- Spots positions according to Laue Conditions and orientation of Unit Cell:

$$(\vec{S}_o - \vec{S}_i).\vec{a} = h$$
$$\textbf{and } (\vec{S}_o - \vec{S}_i).\vec{b} = k$$
$$\textbf{and } (\vec{S}_o - \vec{S}_i).\vec{c} = l$$

- Monochhromatic wave: $\vec{S} = (S_o - S_i)$ can be calculated from experimental geometry

- Spot position $\Leftrightarrow$ Crystal lattice, independent from radiation type

Spot Intensity

- Spots intensity depends on physics of interaction

  X–rays interact with electrons, crystallographic map corresponds to electron density (number of electron per Volum, e^-/A^3).

  Electrons interact with electrostatic potential from electrons + nuclues $(\varphi(\vec{r}))$

  Neutrons interact with nucleus *via* weak interaction, and magnetic moment. Map units **= ?**

- Spot intensity $\Leftrightarrow$ Unit cell content: where are the atoms, what type of atoms are they

From Data Collection to Structure Refinement

- Structure determination: atom coordinates refined against idealized amplitudes $|F_{\text{ideal}}(hkl)|$

- Relationship amplitudes and intensities: $|F_{\text{ideal}}(hkl)|^2 \propto I_{\text{ideal}}(hkl)$

- Detector signal = experimental intensity $I_{\text{exp}}(hkl)$

Step	Data **Integration**	Data **Scaling**	**Refinement**
Concept	Frames $\rightarrow I_{\text{exp}}(hkl)$	$I_{\text{exp}}(hkl) \rightarrow I_{\text{ideal}}(hkl)$	Match atom coordinates to $I_{\text{ideal}}(hkl)$
Requirement	Signal vs. background	Error Model	$(x,y,z) \leftrightarrow \rho(x,y,z) \leftrightarrow F(hkl)$

Data Processing and Scaling

Integration Extraction of I_{exp} from detector: intensity counts after background subtraction — largely **independent** from radiation source

Scaling Conversion from I_{exp} to I_{ideal}: reduction of experimental errors, crystal shape, detector properties, ... — **depends** on type of radiation

For X–rays*:

$$I_{\text{exp}}(hkl) = \frac{e^4}{{m_e}^2 c^4} \underbrace{\frac{\lambda^3 V_{\text{crystal}}}{V^2_{\text{unit cell}}} I_0 LPTE}_{\text{exp. Parameter}} I_{\text{ideal}}(hkl)$$

*C. Giacovazzo, *Fundamentals of Crystallography*, Oxford University Press

Differences between Types of Radiation

Detector $\rightarrow I_{\text{ideal}}(hkl)$ $\Leftarrow$ Refinement $\Rightarrow$ $|F_{\text{calc}}(hkl)| \leftarrow$ Model

Two theories for structure factor calculation from atom coordinates:

kinematic scattering	**dynamic scattering**
only one scattering event valid for X–rays, neutrons $\lvert F_{\text{ideal}}(hkl)\rvert \propto \sqrt{I_{\text{ideal}}(hkl)}$ calculation via form factors $F(hkl) = \sum_{\text{atoms j}} f_j(\theta) e^{-2\pi i hx+ky+lz}$	multiple scattering events valid for electrons

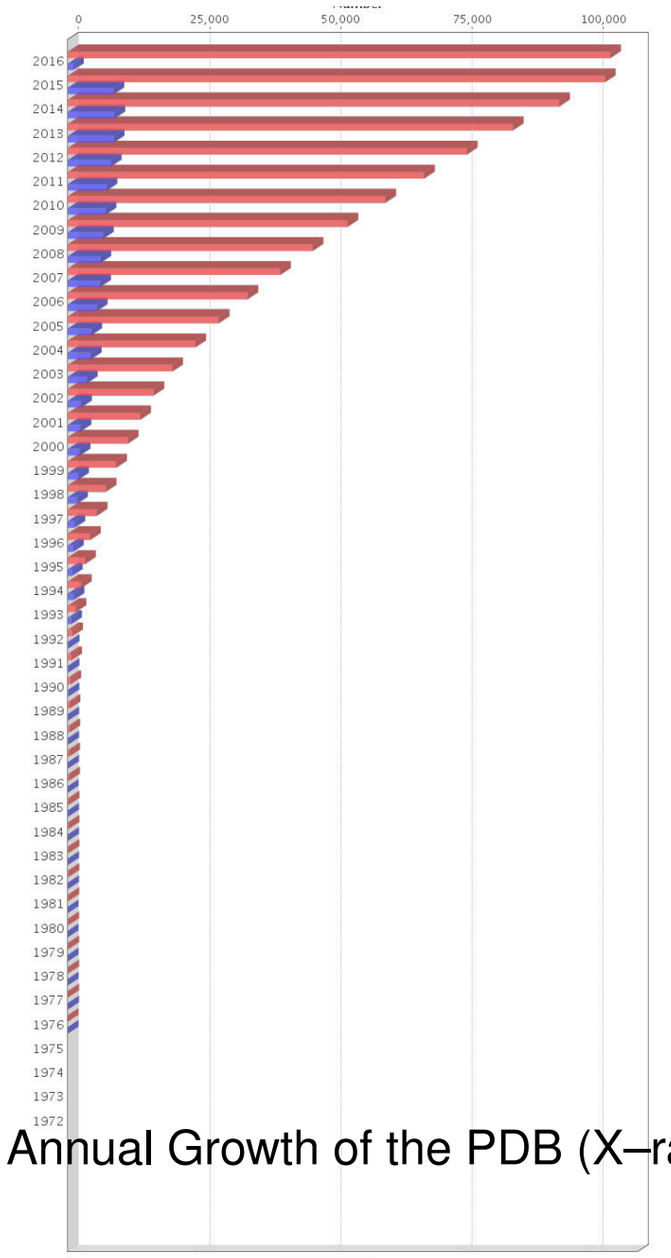
Types of Radiation — X–rays

1. most advanced (pipelines from data collection to structure refinement)

2. typical wavelength: λ =0.8–1.9Å

3. standard structure determination

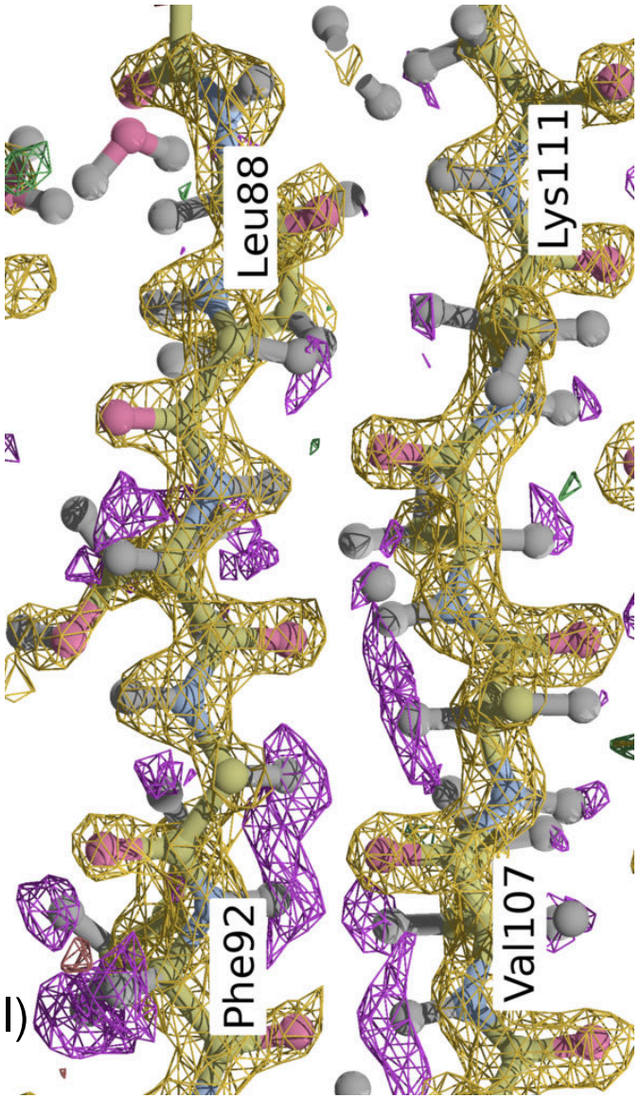
4. PDB (Protein Data Base):
   - 113,000 X–ray structures
   - 112 neutron structures
   - 57 electron structures (mostly 2D crystals and false positives)



Annual Growth of the PDB (X–ray)

Types of Radiation — neutrons

1. (virtually) no radiation damage
2. requires large crystals ($\geq 1\text{mm}^3$)
3. visualisation of hydrogen atoms
4. adjacent elements (e.g. K^+ vs. Cl^-, Zn^{2+} vs. Cu^+)
5. structure determination from radiation sensitive samples (Photosystem II)



PDB ID 2ZOI: D/H exchange in β–strand

(Gruene *et al*, J. Appl. Cryst. 47 (2014), 462–466)

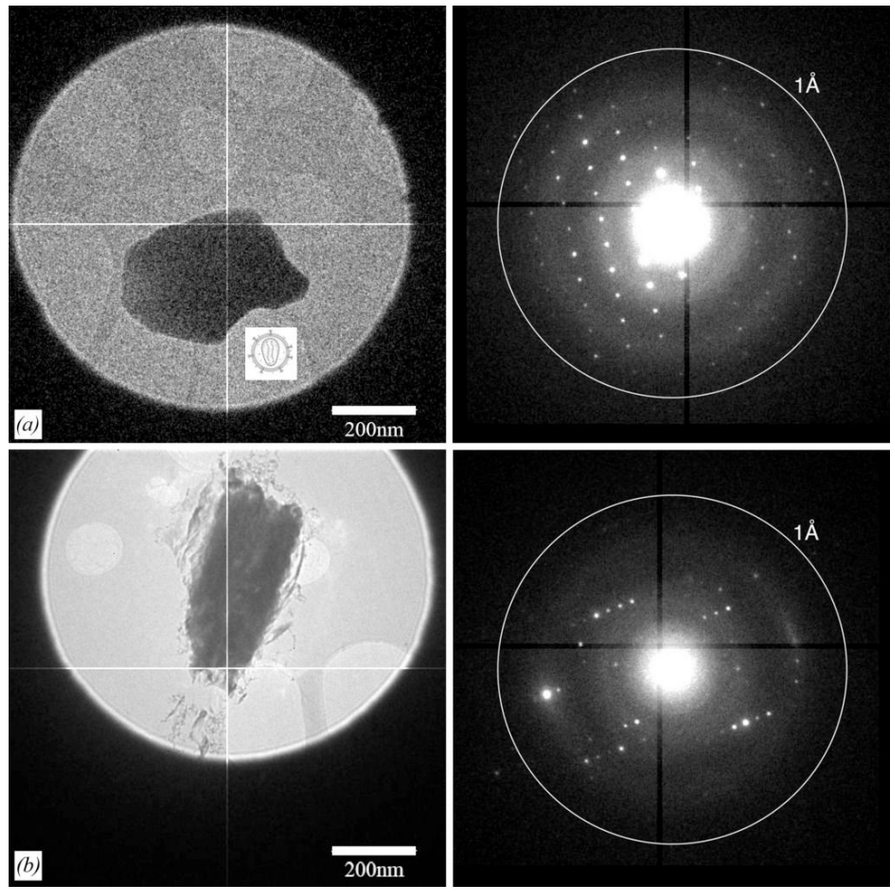
Types of Radiation — electrons

1. strong interaction compared with X–rays: good for very small crystals ($\ll 1\mu m$ thickness)

2. typical wavelength: $200keV = 0.0251$Å

3. charge enables electron optics: imaging **and** diffraction

4. new phasing possibilities



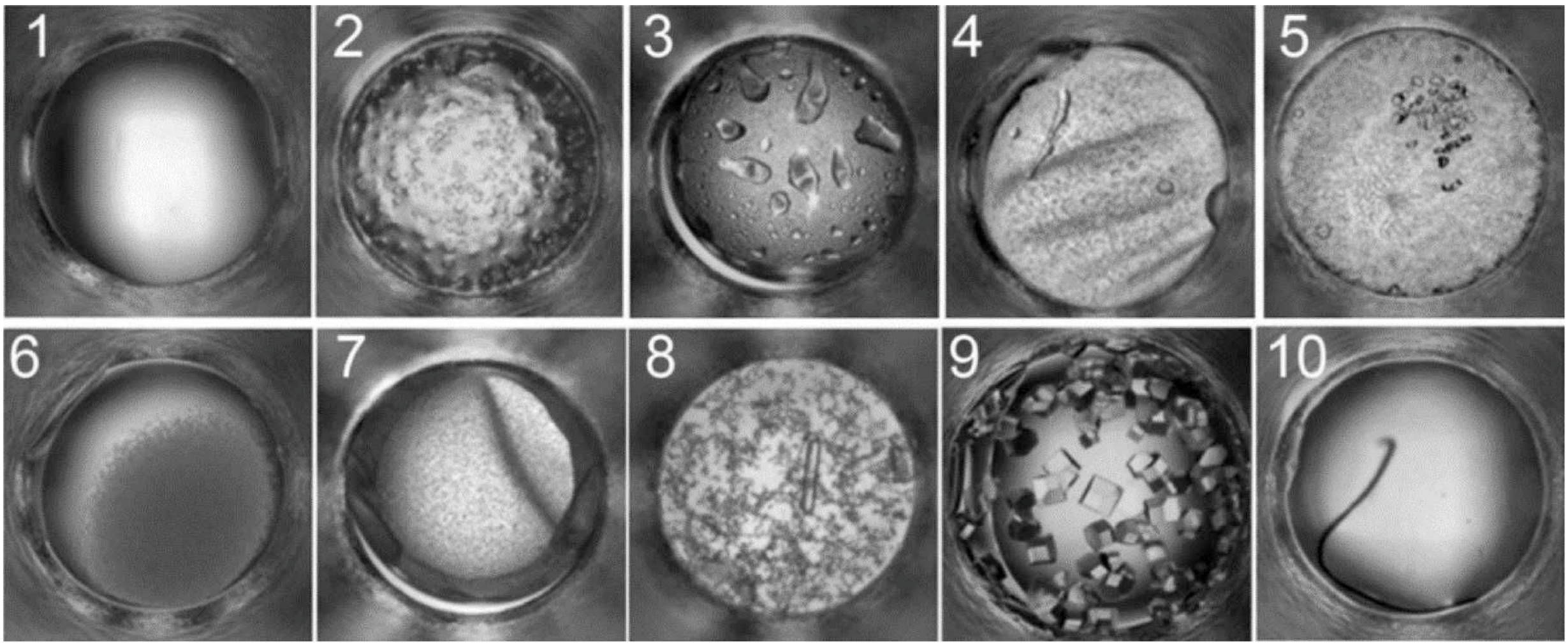
Diffraction of nanocrystals

(van Genderen *et al.*, Acta Cryst A72 (2016))

Inset: HIV to scale, courtesy Thomas Splettstoesser, en.wikipedia.org

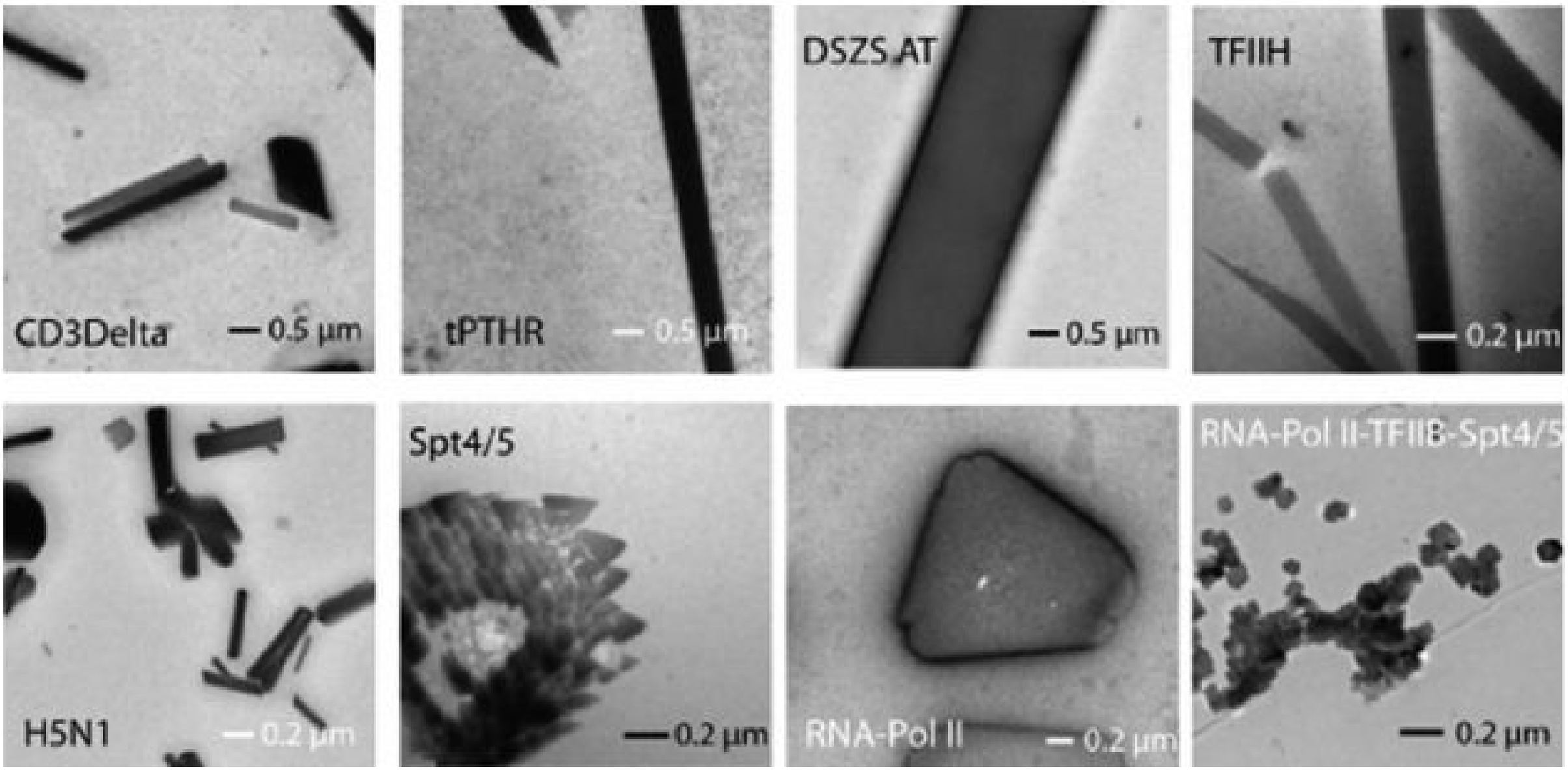
3 - Electron Diffraction

The (seemingly) Empty Drop



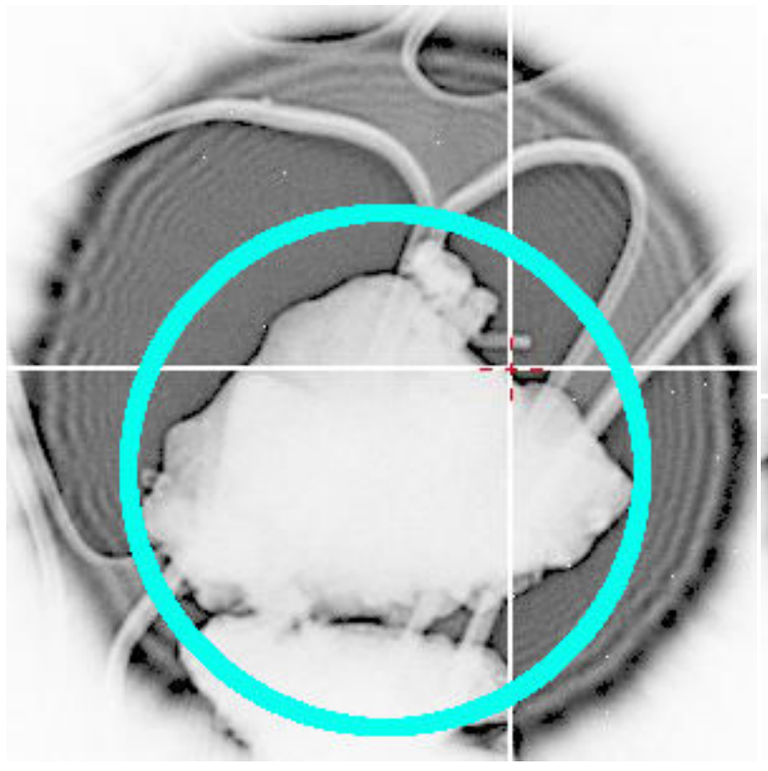
Luft, Wolfley, Snell, Crystal Growth& Design (2011), 11, 651–663

Drops viewed through TEM

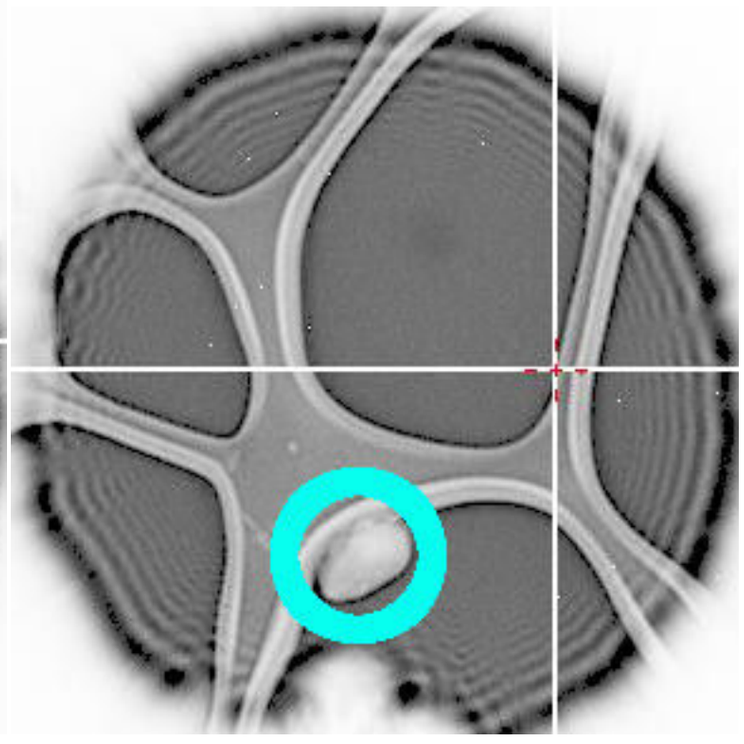


Stevenson,. . . , Calero, PNAS (2014) 111, 8470–8475 / Calero, . . . , Snell, Acta Cryst (2014) F70, 993–1008

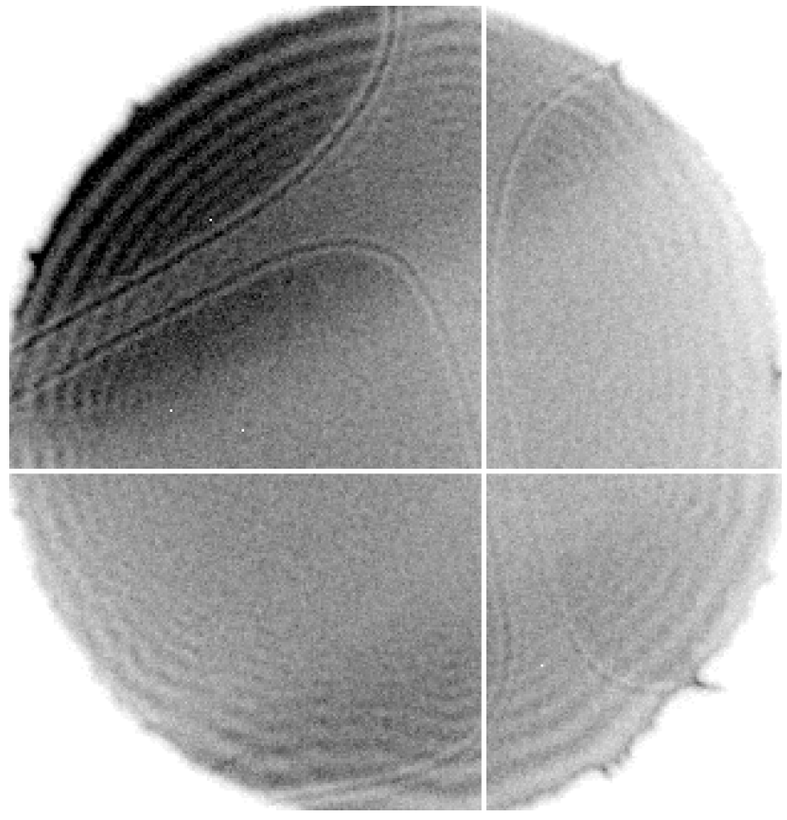
Nanocrystals



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

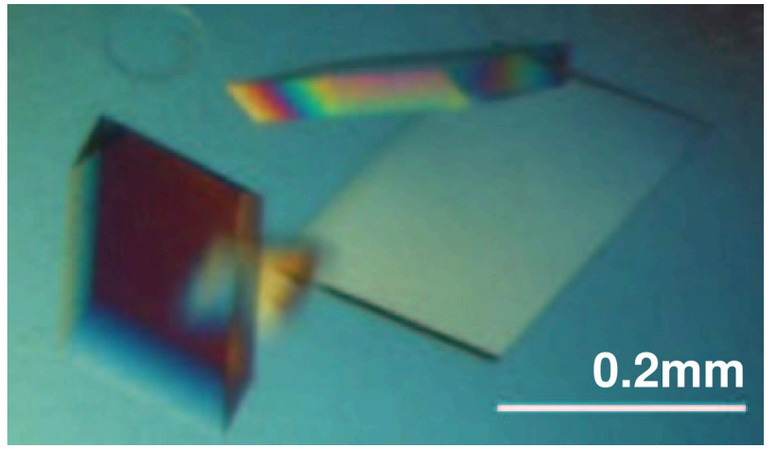


Novartis II:
$\varnothing = 500nm = 0.5\mu m$

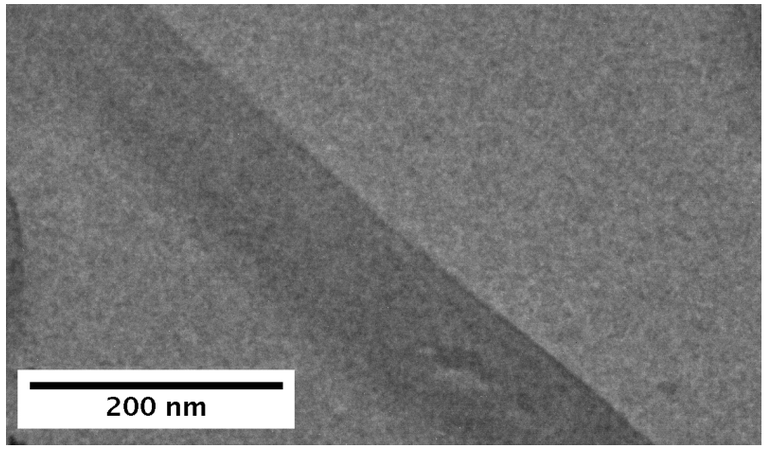


Thermolysin:
$\approx 2 \times 1 \times$ very thin μm^3
Solvent reduces contrast

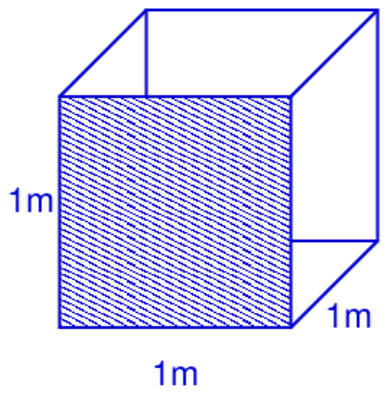
How small is “nano”?

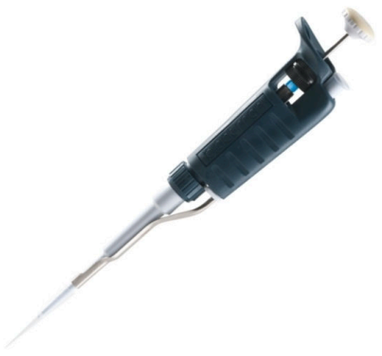


typical protein crystal size for X-rays



typical protein crystal size for electrons, 100x140x1,700 nm^3





volumes compare like $1m^3$ or 6 bath tubs of water *vs.* $10\mu l$

Applications for 3D Electron Crystallography

- You cannot get bigger crystals
  - Membrane Proteins
  - Protein needle crystals
  - Organic / Pharmaceutics: often only powder available
- Inorganic Applications
  - Catalyst chemistry: structure determination at “original size”
- Crystal Disorder

Effects of Crystal Volume on Diffraction Data

Reducing crystal volume reduces the resolution by (at least) two effects:

1. $I(hkl) \propto V_{\text{crystal}}$: 1/10 volume = 1/10 intensity
2. Henderson / Garman limit: maximum dose per volume before resolution is halved: 1/10 volume = 1/10 dose before radiation damage destroys crystal

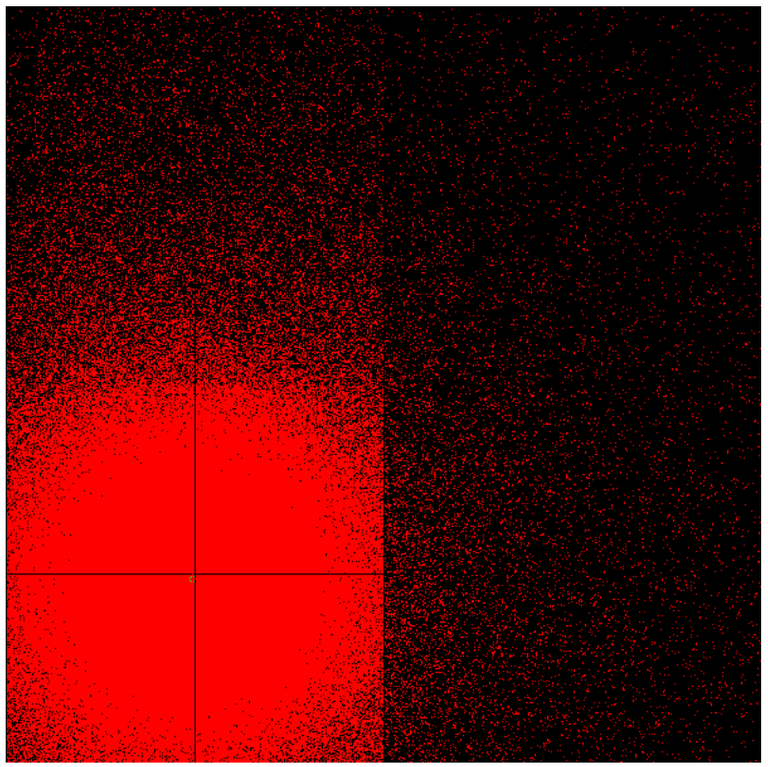
From (1): In order to record the same quality diffraction pattern from a 10 times smaller crystal requires 10 times more intense beam.

From (1)+(2): This makes the crystal die 100 times faster

4 - Instruments for Electron Diffraction

Medipix / Timepix Detector Family

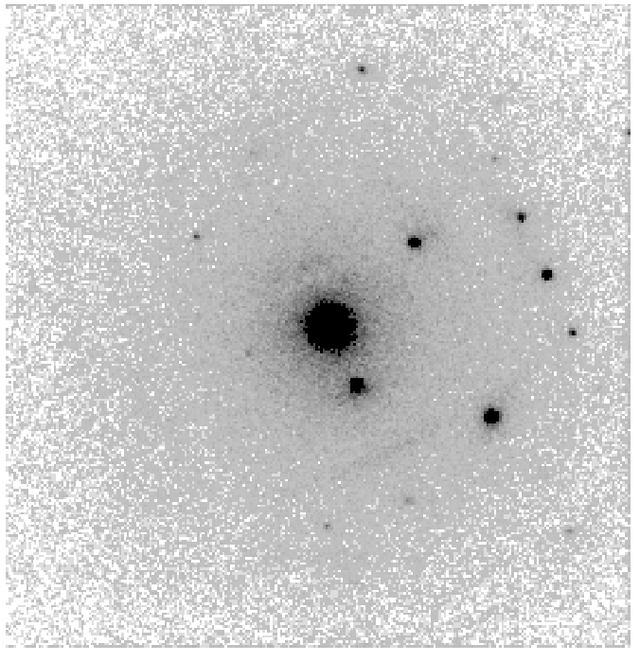
- hybrid pixel detector for electrons (*cf.* Pilatus / Eiger)
- no read–out noise
- high dynamic range
- fast read–out: non–stop sample rotation (“shutterless data collection”)
- 512x512 and 1024x1024 pixel cameras installed in Basel (and Pisa (Prof. Mauro Gemmi) and Stockholm (Prof. Sven Hovmöller))



Diffraction image from a MFI type zeolite:
black = 0 counts
red $\geq$ 1 (carbon scatter + crystal signal) count

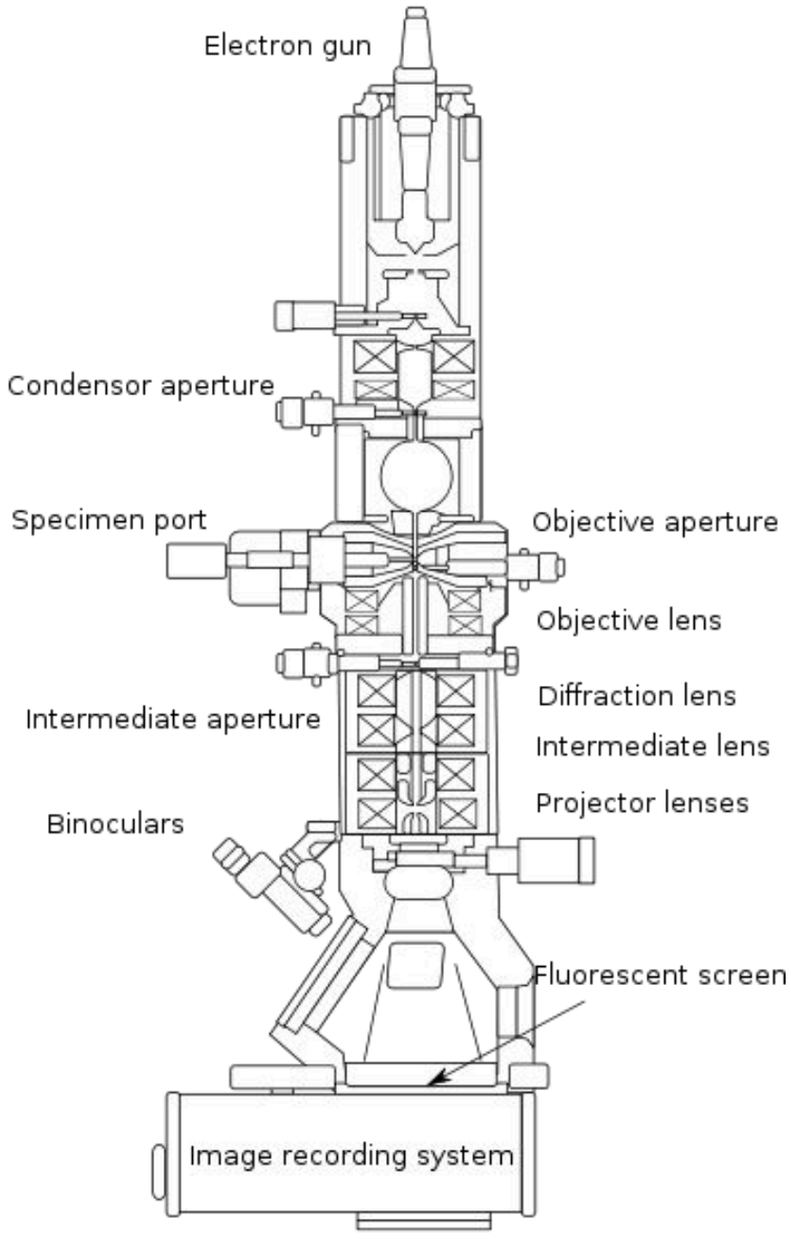
Eiger Chip

- Developed at PSI
- 256x256 pixel test chip with 200keV instrument
- pilot for improving phosphor to higher energies $\geq$ 300 keV
- fast read–out (up to 8kHz), very low dead time
- Next: Jungfrau and Mönch with Si, $GaAs$, or $CdTe$



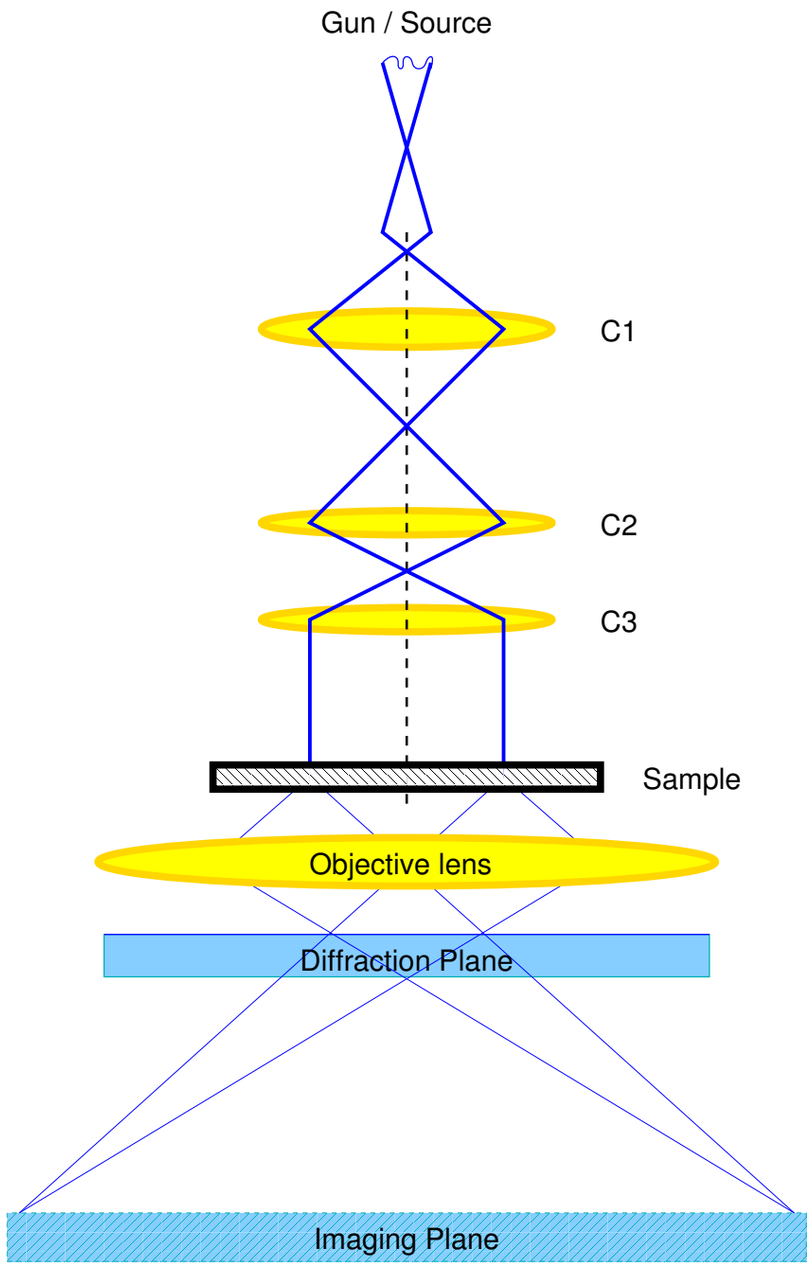
Electron diffraction (from an inorganic compound) on a 256x256 Eiger chip

Electron Microscopes



(Wikipedia)

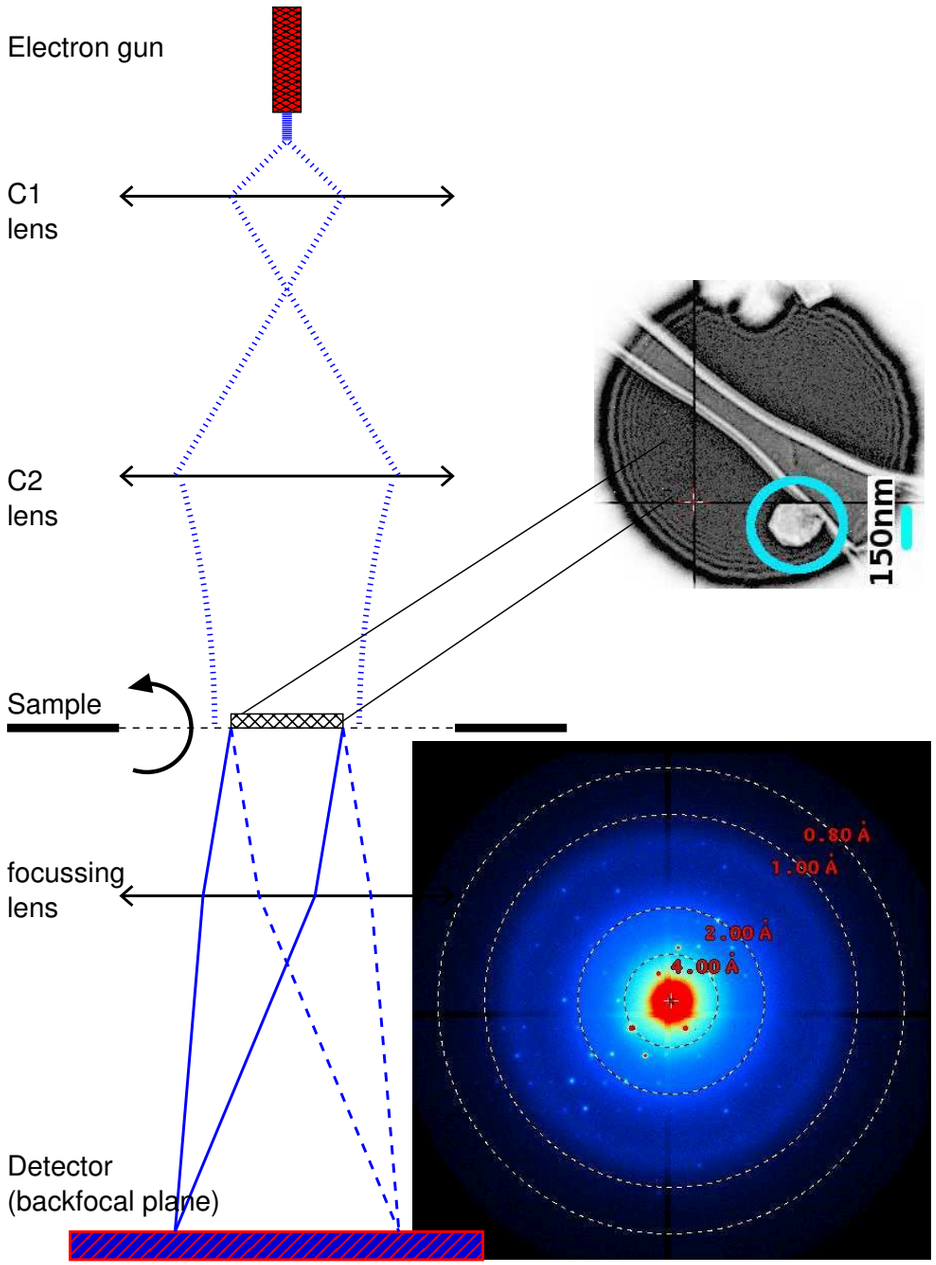
The Lens System



- 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

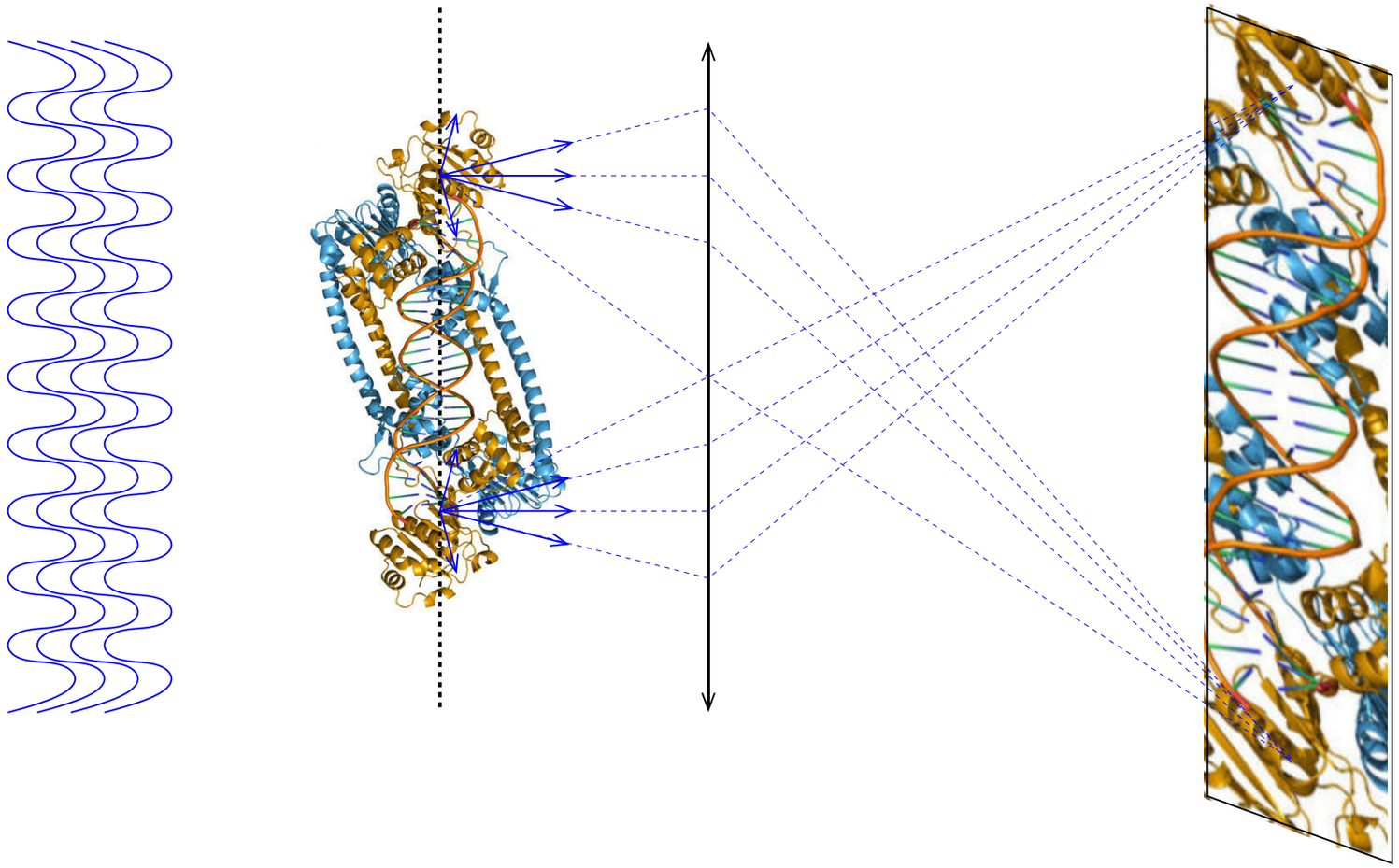
Lenses cause distortions.

Modern Instruments lack C3 Lens



- Without C3–lens
- Beam describes an arc
- Sample height must be well positioned

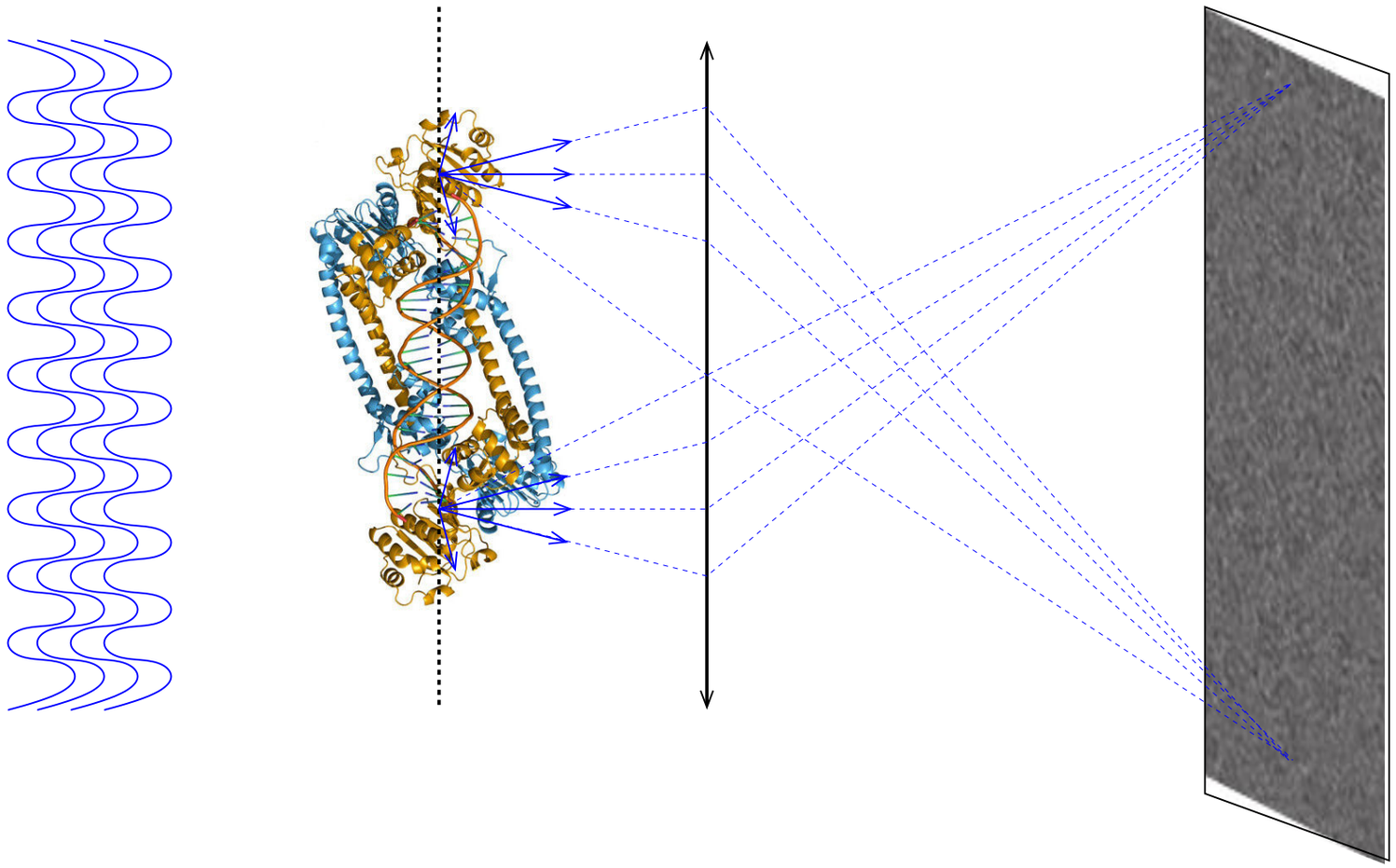
Electron Microscope: Imaging Mode



Plane Wave Object Lense Image Plane (Detector)

Rays of **equal origin** focus on detector

Electron Microscope: Imaging Mode

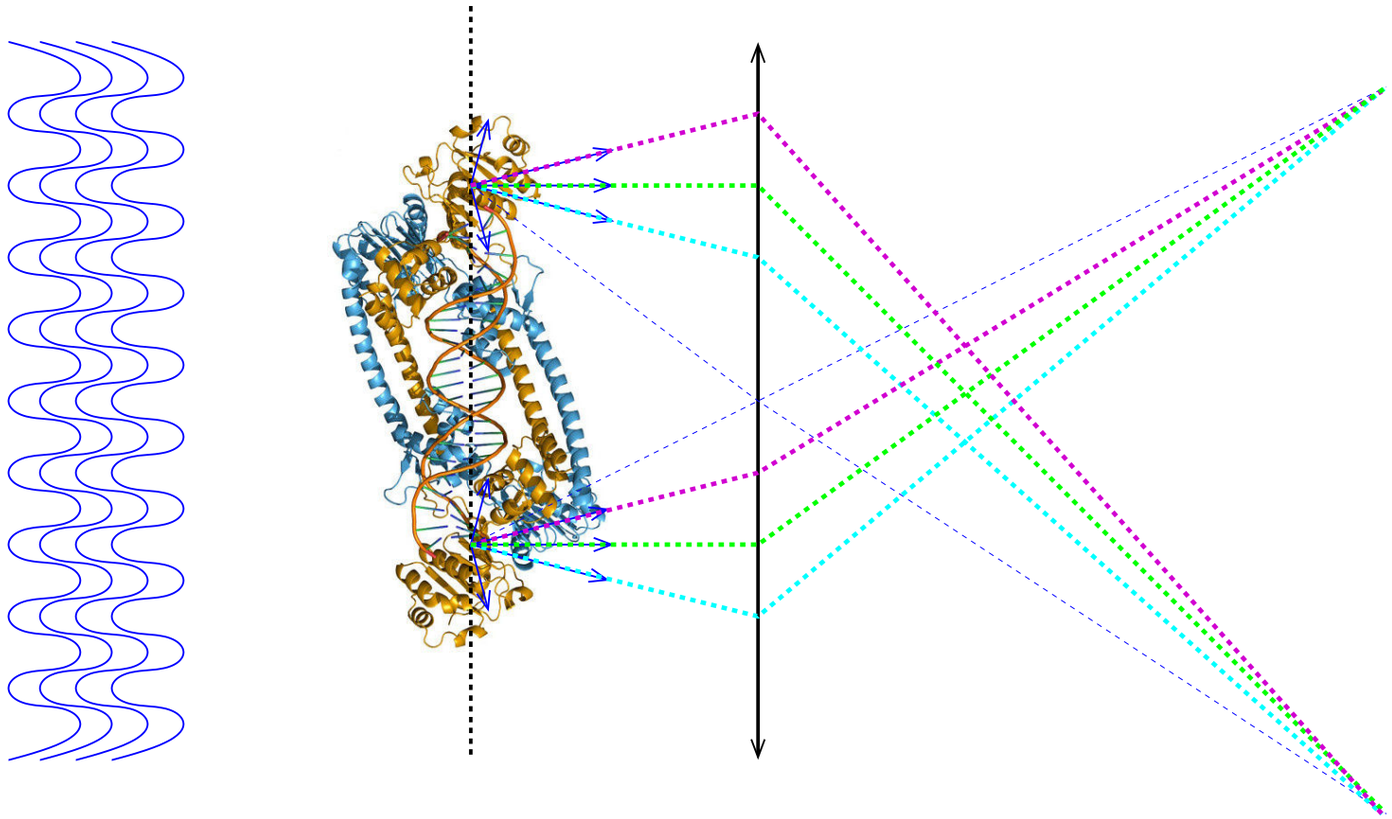


Plane Wave Object Lense Image Plane (Detector)

Detector noise and radiation senstivity require low contrast images

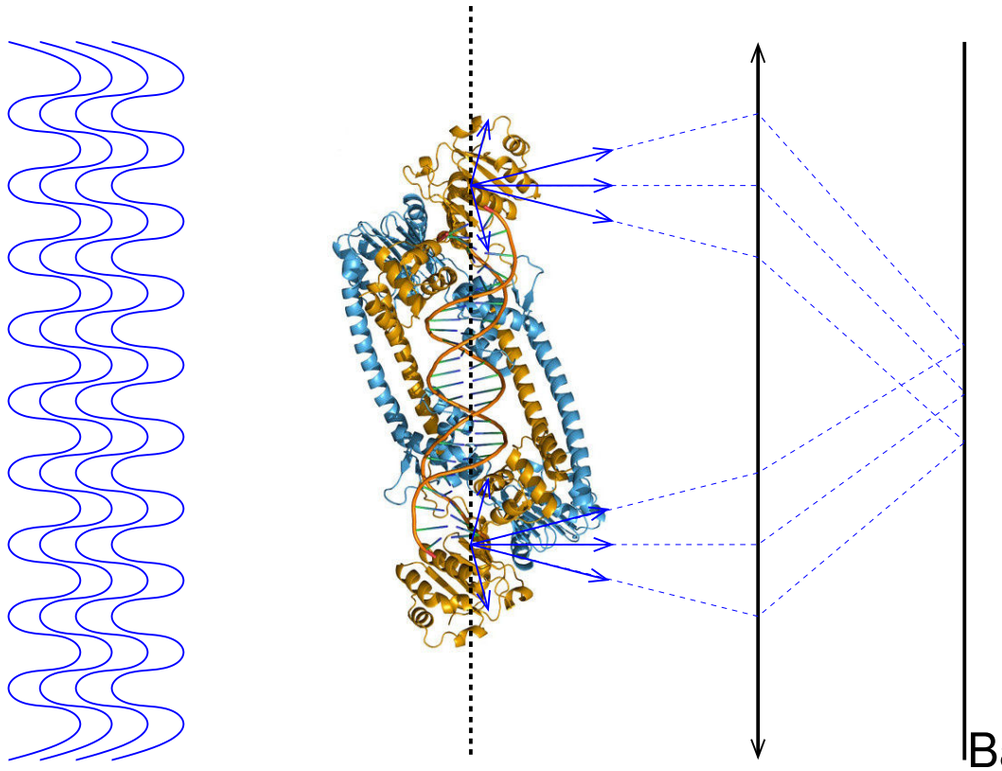
Martinez-Rucobo *et al.* Molecular Cell (2015) 58, 1079–1089

Electron Microscope: Diffraction Mode



Plane Wave Object Lense

Electron Microscope: Diffraction Mode



Plane Wave Object Lense Backfocal Plane

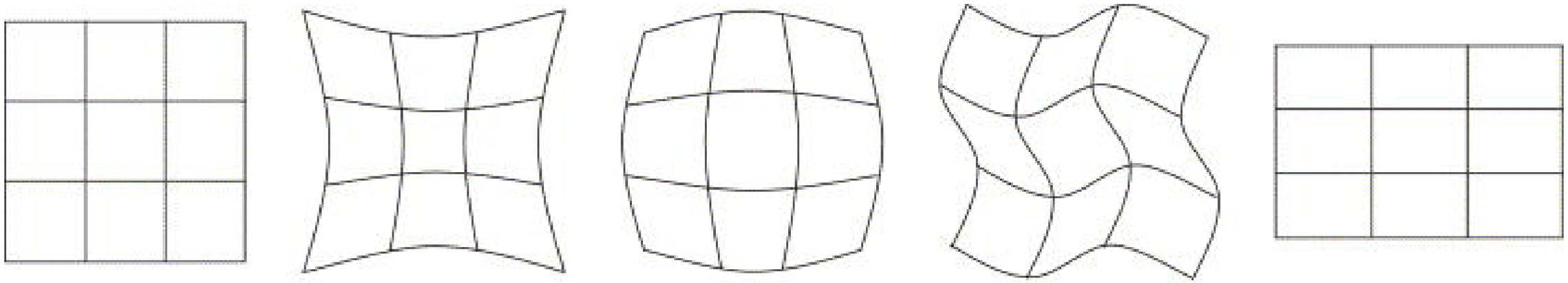
Image at Backfocal Plane =
||Fouriertransform of object||

If object = crystal:

diffraction spots according to Laue condition

Rays of **equal direction** focus on detector

Types of Distortions



Capitani, Oleynikov, Hovmöller, Mellini, *A practical method to detect and correct for lens distortion in the TEM* Ultramicroscopy (2006), 106, 66–74

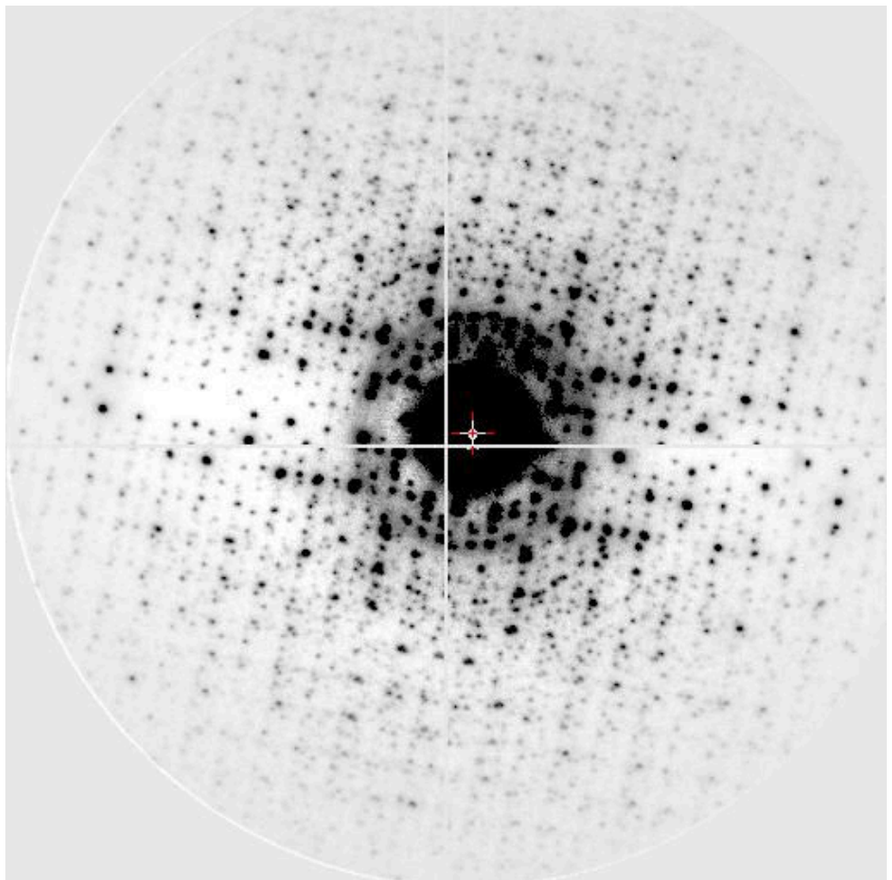
Pincushion Barrel Spiral Elliptical

- one–to–one distortions: every pixel maps back onto undistorted grid
- causes of distortion:
  1. lenses
  2. imperfect detector surface

Crystal Glasses

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

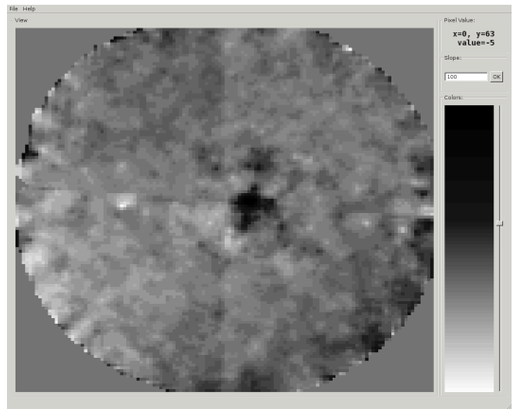
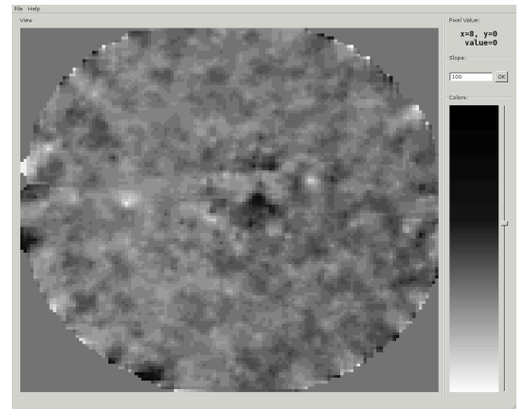
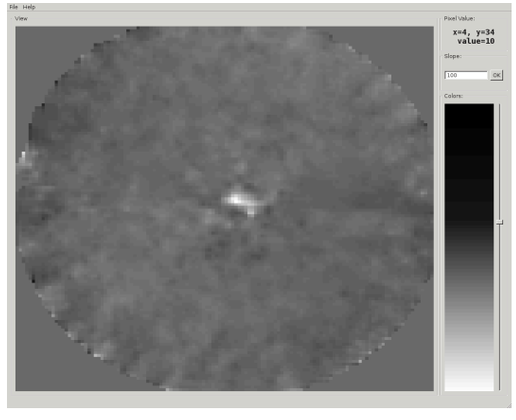
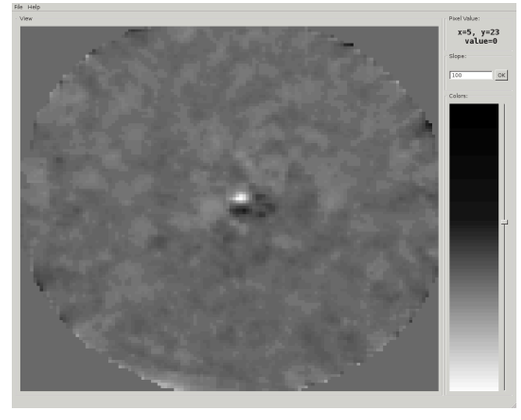
(Wikipedia)



1. Collect and process data set from garnet
2. Predict spot positions
3. Calculate per–spot deviation: correction tables
4. Use X/Y correction tables for target sample
   - Readily available with XDS
   - Unit cell dimensions must be comparable

Preliminary Results

Use correction tables from crystal 1 during integration of crystal 2.

	Uncorrected	**Corrected**
X–Shifts Δx		
Y–Shifts Δy		

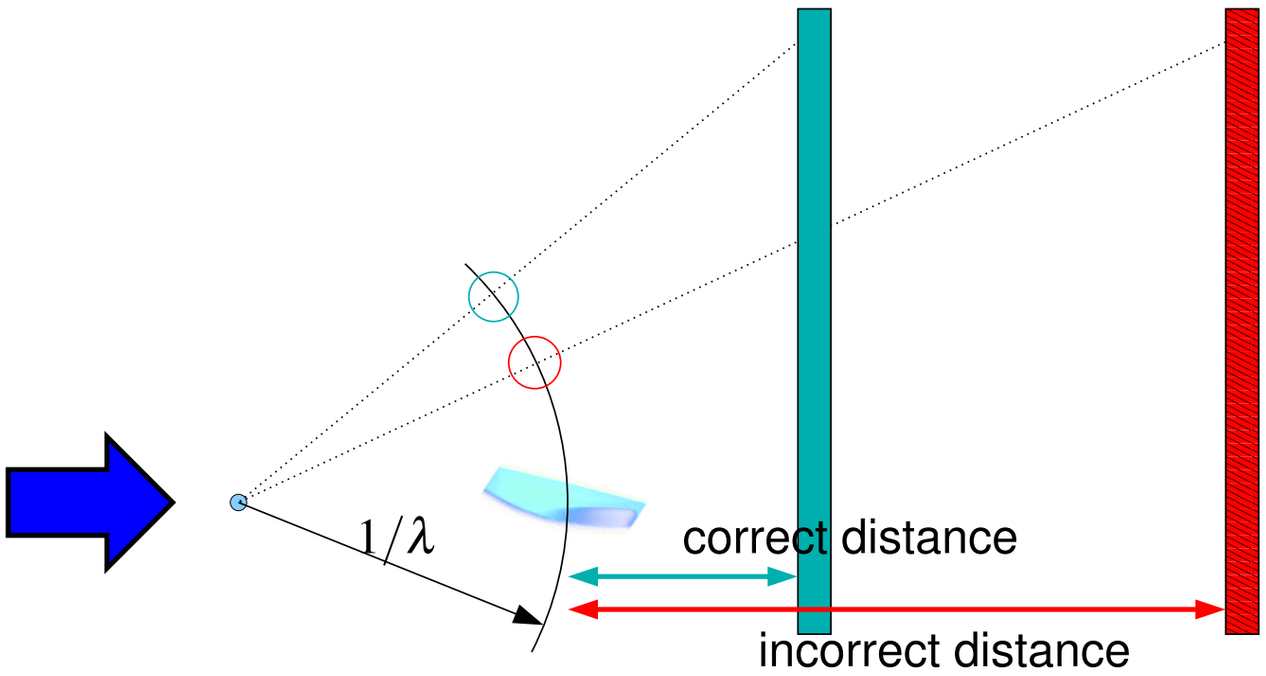
P1 cell	12.0811	11.9496	11.8249	89.986	90.481	89.780	12.0665	12.1757	12.0574	90.048	90.026	90.065

5 - Experimental Considerations

- Ewald sphere or “plane”
- dynamic scattering
- Instrumental limitations

X–rays: The Ewald Sphere

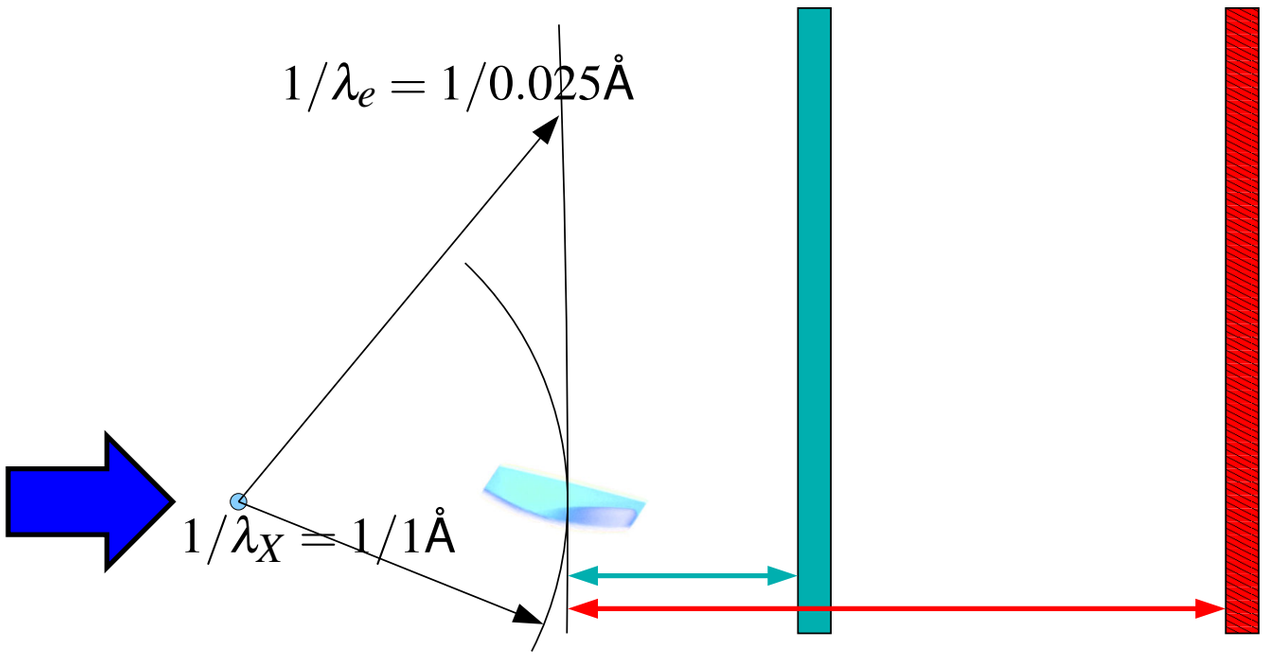
$\lambda = 1$Å, “normal” resolution: $2\theta_{\max} = 40°$



- Assume: wrong detector distance
- Diffraction spot calculated wrongly (red circle)
- Reciprocal lattice becomes distorted

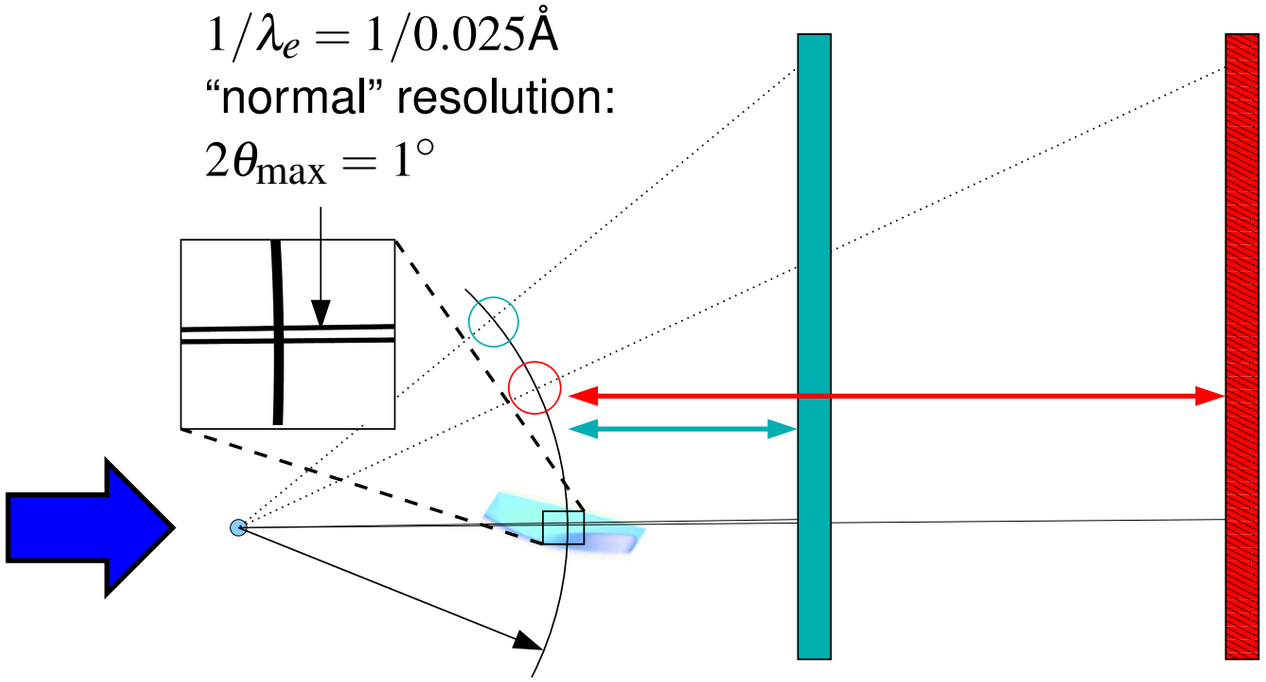
Curvature of the Ewald sphere **gauges** the diffraction geometry

Electrons: The Ewald “Plane”



- Typical X–ray wavelength $\lambda_X = 1$Å
- Typical e^- wavelength $\lambda_e = 0.025$Å
- Radius of Ewald sphere 40x greater
- Ewald sphere nearly flat

Electrons: The Ewald “Plane”



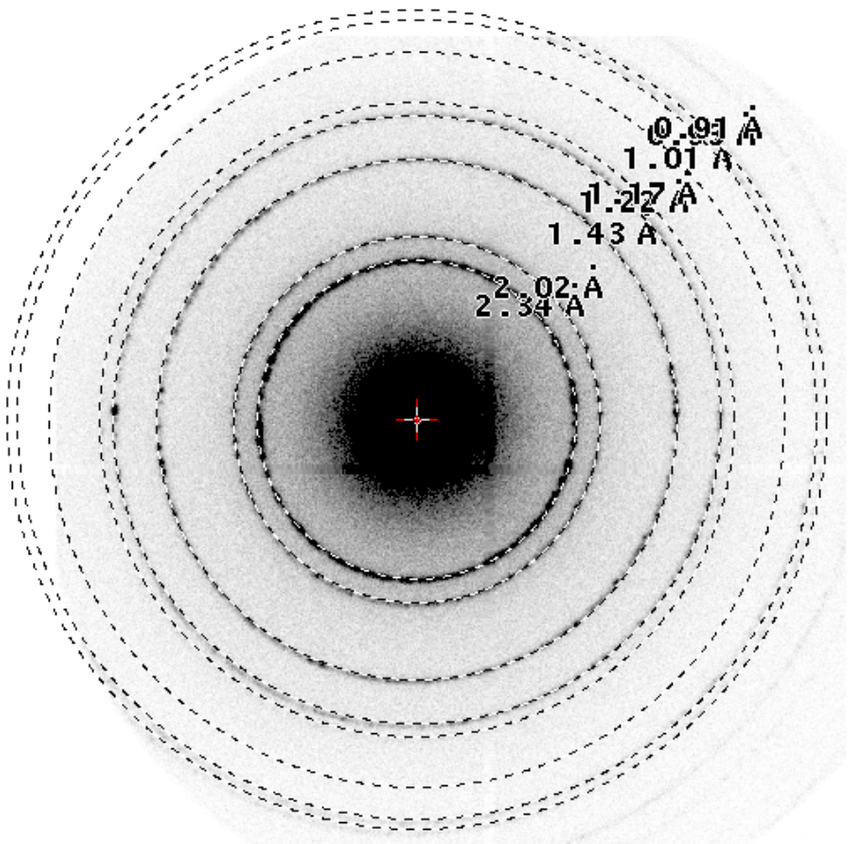
- typical wavelength with X–rays: 1Å
- typical wavelength with electrons: 0.025Å
- opening angle of highest resolution reflections $\approx 1°$
- Ewald sphere virtually flat
- Without curvature: impossible to refine both detector distance and cell

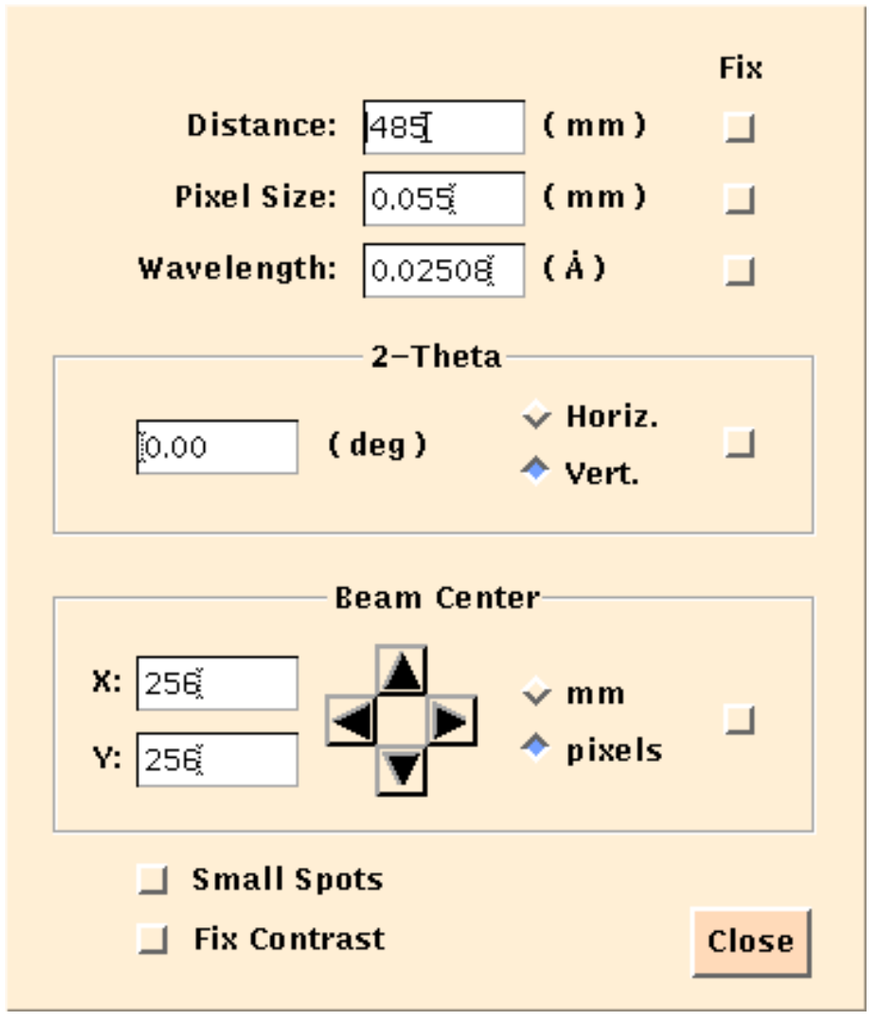
Electrons: The Ewald “Plane”

- Detector distance and unit cell parameters are strongly related
- Wrongly set distance can lead to incorrect bond lengths
- Distance refinement with X–ray data routine
- Distance refinement with electron data = unstable
- good: Distance calibration from powder sample
- better: Distance calibration from chemical bond lengths

Distance Calibration

- Bragg's law: $\lambda = 2d \sin\theta$; d, λ are known





Elliptical Distortion introduces Errors

- Bragg's law: $\lambda = 2d \sin\theta$; d, λ are known

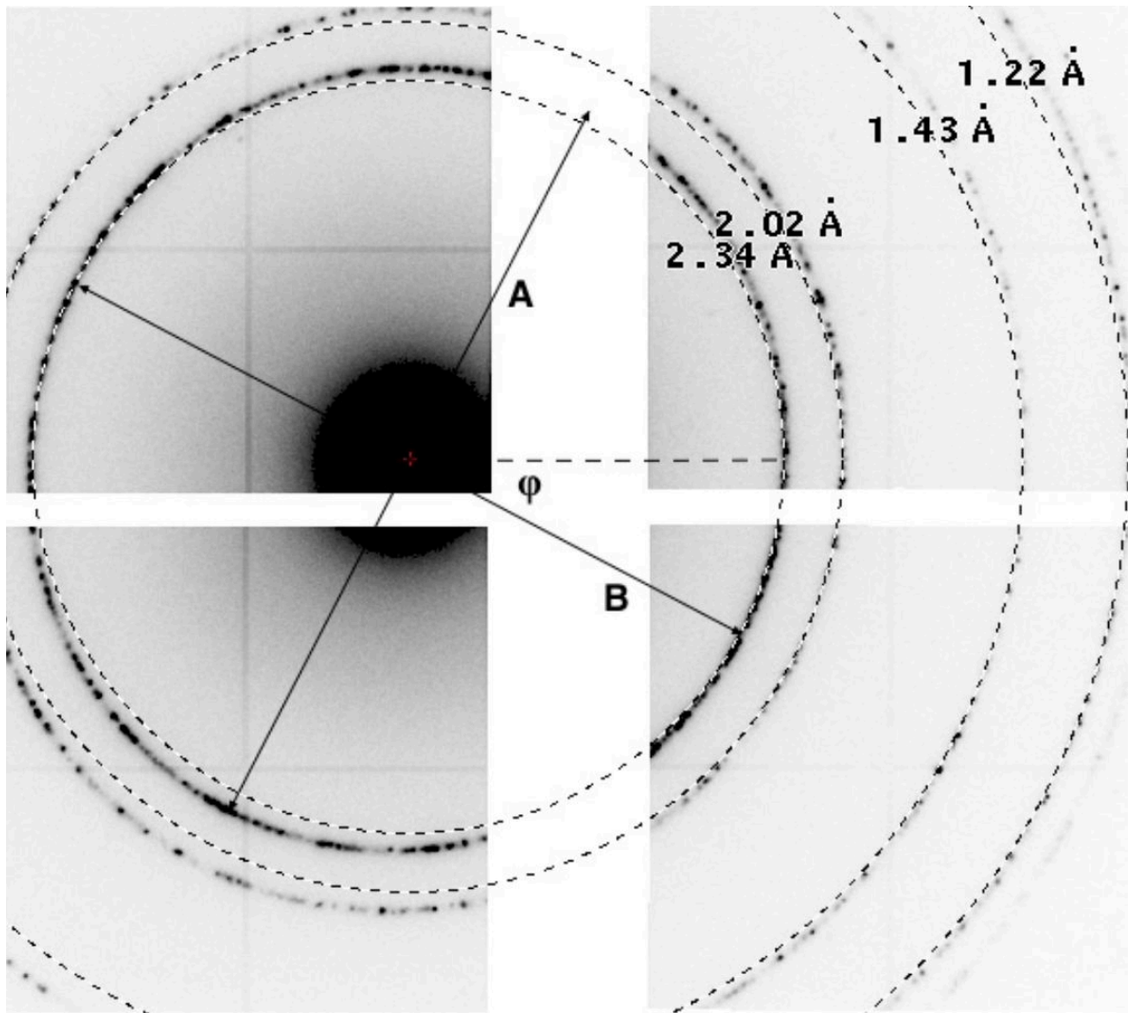
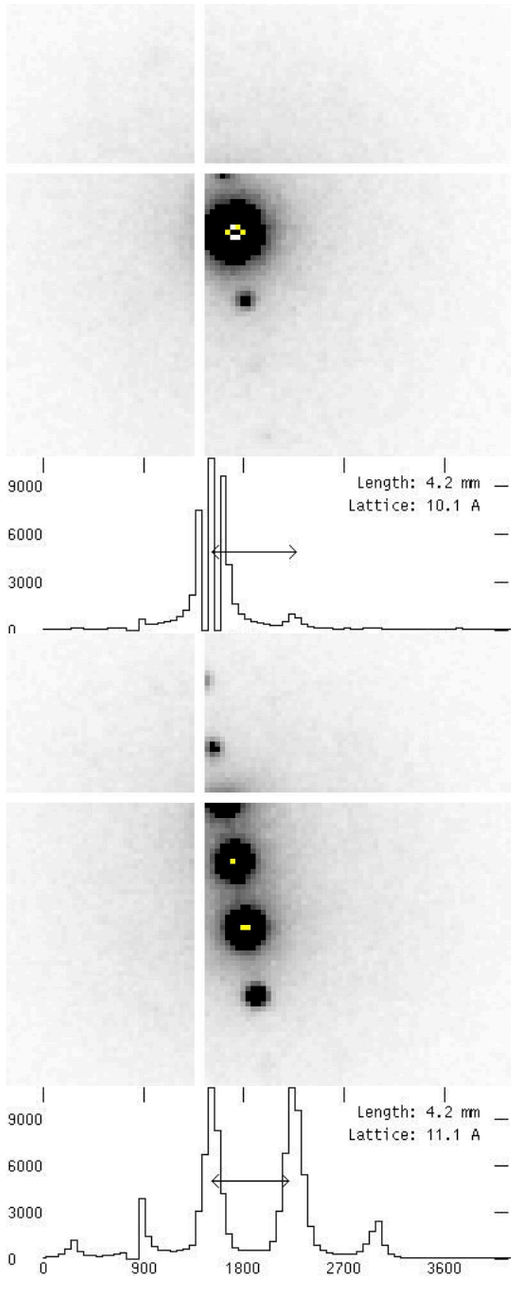


Image courtesy M. Clabbers

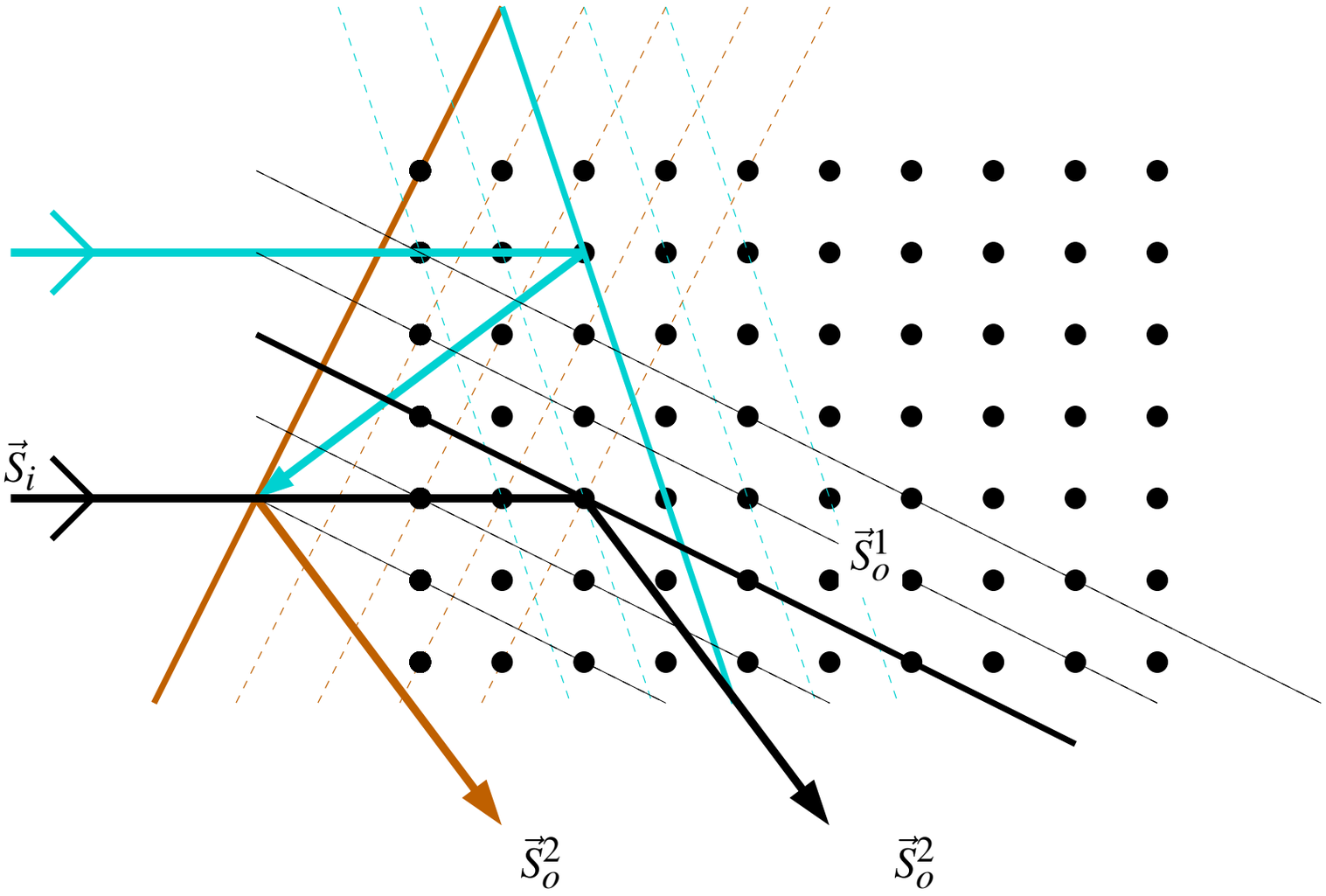
Dynamic Scattering

- 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

Dynamic Scattering

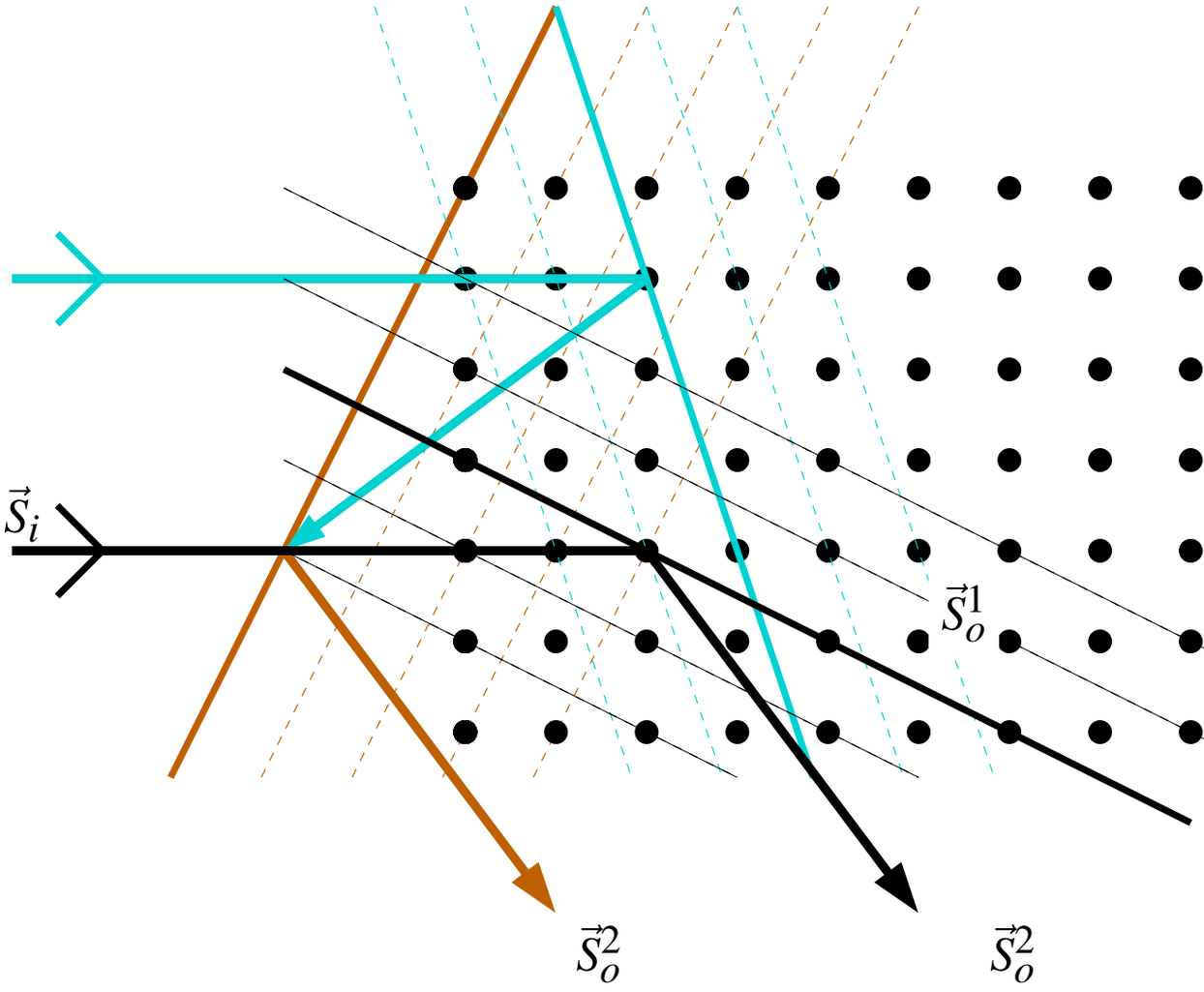


Multiple (Dual) Scattering



- Outgoing ray $\vec{S}_o^1$ acts as incoming ray for reflection $\vec{S}_o^2$.
- Re–reflection with 10% probability at 50 nm path length

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
- Therefore: Measured intensities “shifted” from strong to weak
- Low resolution reflection under–, high resolution reflections overestimated
- Covered during refinement by reduced B–factor: electron diffraction includes map–sharpening

Dynamic Scattering for Organic Crystals

- Presence in Macromolecular Diffraction data currently discussed in literature
- Some claim it is negligible
- Experimental evidence equivocal
- Treatment (scaling / refinement) should be improved

Other Instrumental limitations

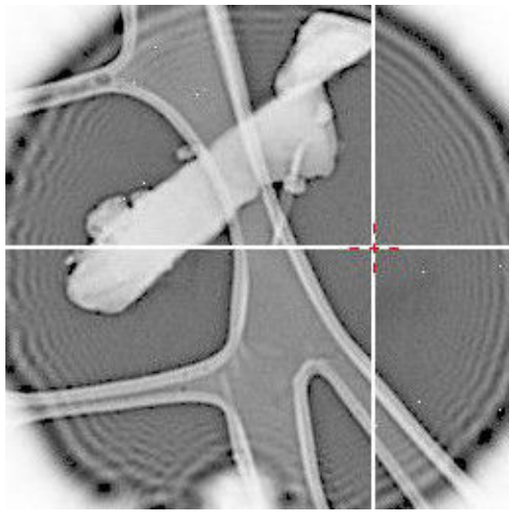
- Electron Microscopes not designed for accurate sample rotation
- Rotation axis not linked to Camera read–out
- Lense system rotates (diffraction) image: rotation axis unknown
- Sample holder not desiged for 180° rotation

6 - Example Structures

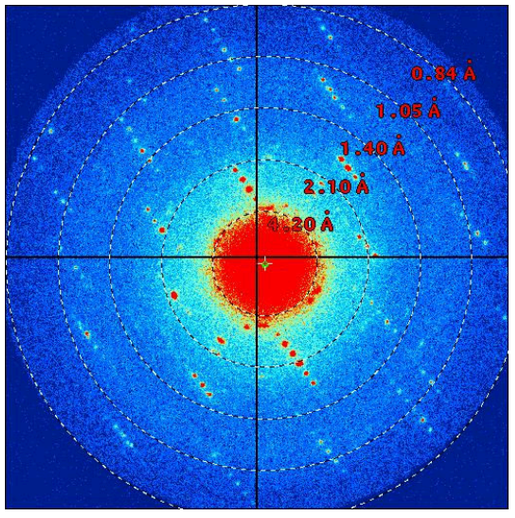
Pharmaceutical I: Visualisation of Hydrogen Atoms

H–atom positions can be refined against electron diffraction data

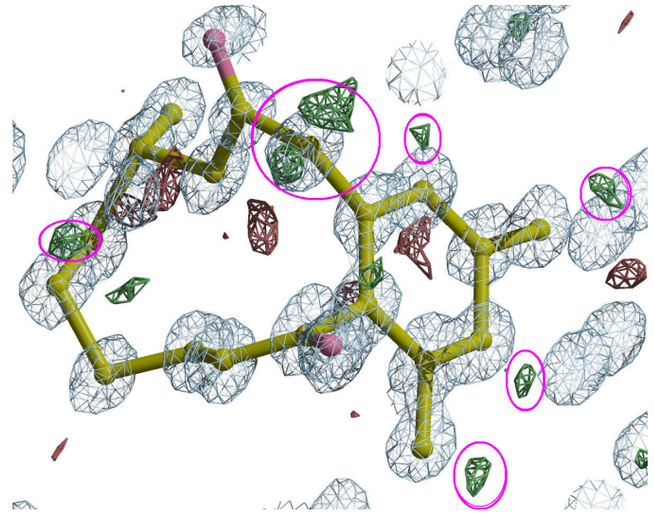
CCDC: IRELOH, Dai et al., Eur. J. Org. Chem (2010), 6928-6937



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



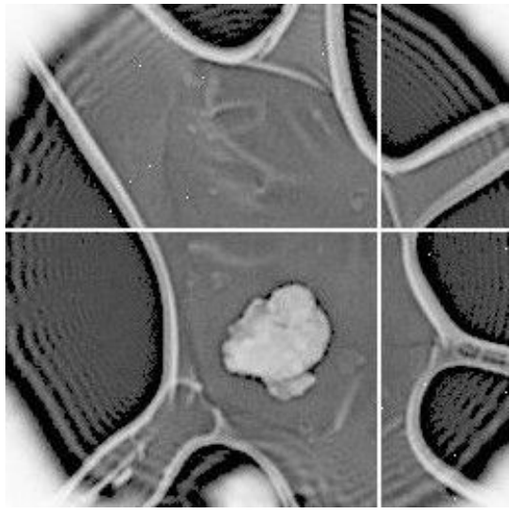
- $d_{\text{min}} < 0.8$Å
- $I/\sigma_I(0.91 - 0.81\text{Å}) : 1.8$
- $P2_12_12_1$: 85% completeness with 3 crystals
- a=8.06Å b=10.00Å c=17.73Å



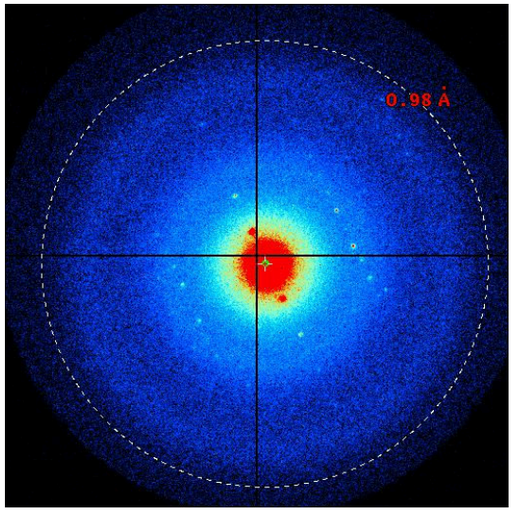
- **Refinement** of hydrogen atom positions with mild restraints (SADI))
- 1334 reflections, 195 parameters, 156 restraints (RIGU)
- $R1 = 15.5\%, R_{\text{complete}} = 18.5\%$

Pharmaceutical II: Differentiation of Atom Types

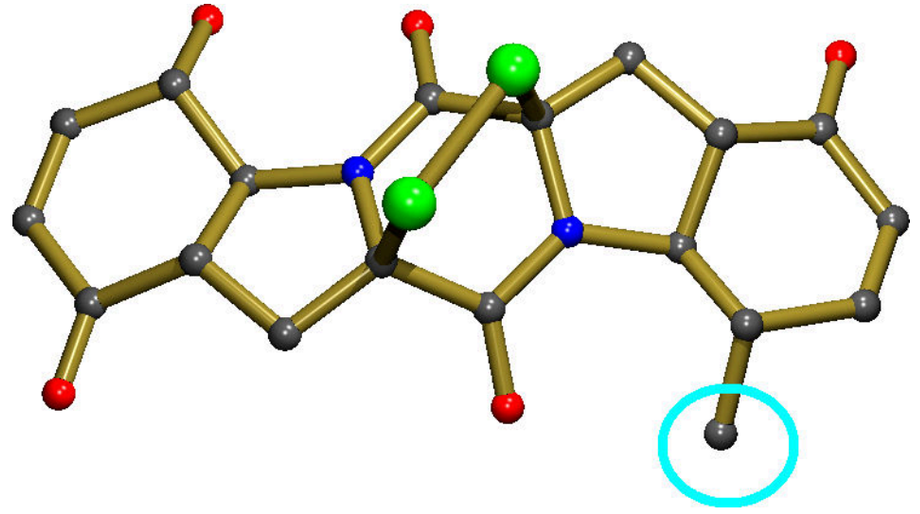
Data quality: recognition of atom types, C *vs.* O *vs.* N *etc.* (CCDC: EPICZA)



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

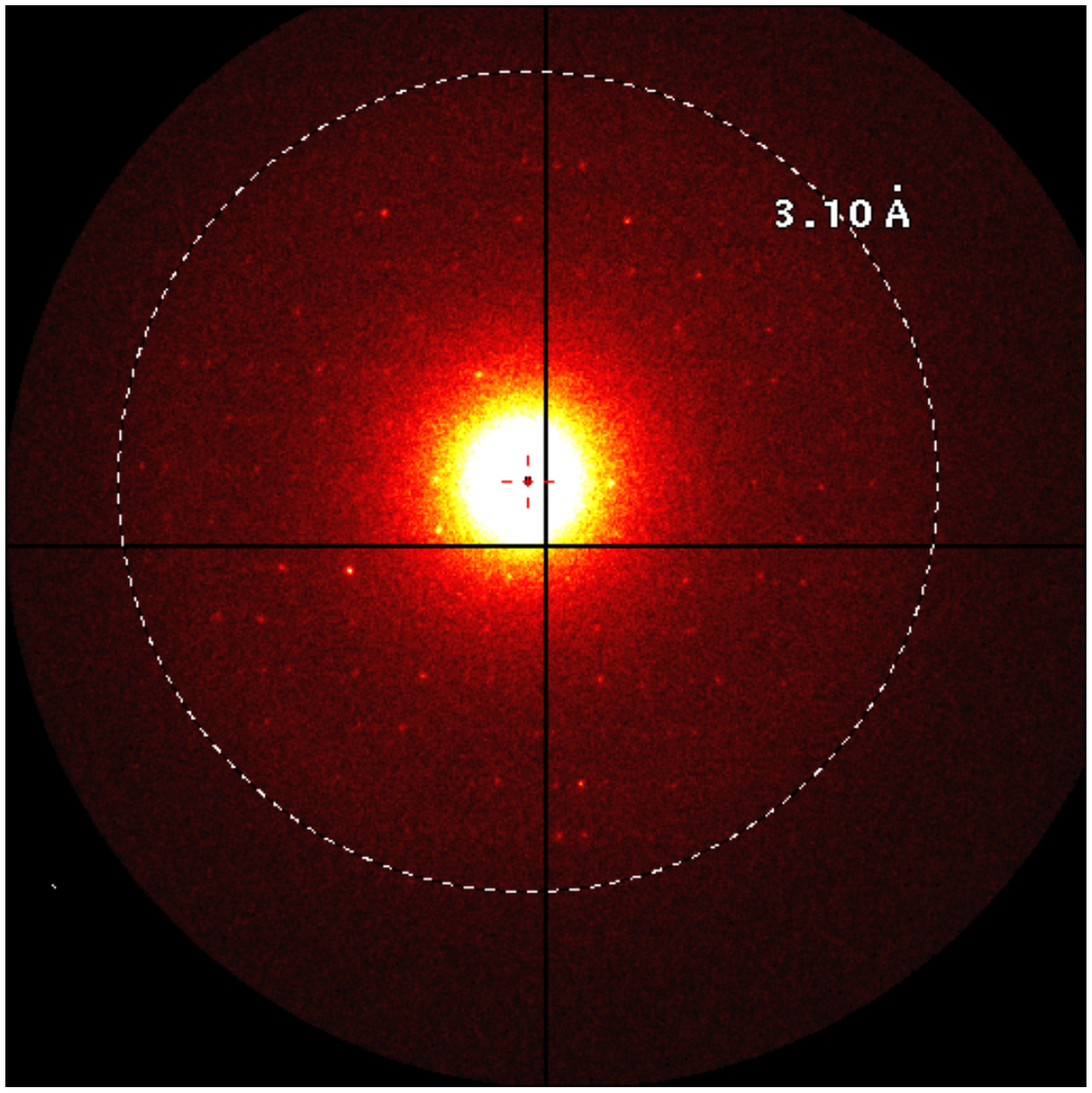


- $d_{\text{min}} = 0.80$Å
- $I/\sigma_I(0.90 - 0.80\text{Å}) : 2.5$
- $P2_12_12_1$: 92% completeness with 6 crystals ($d_{\text{min}} > 0.84$Å : 96%)

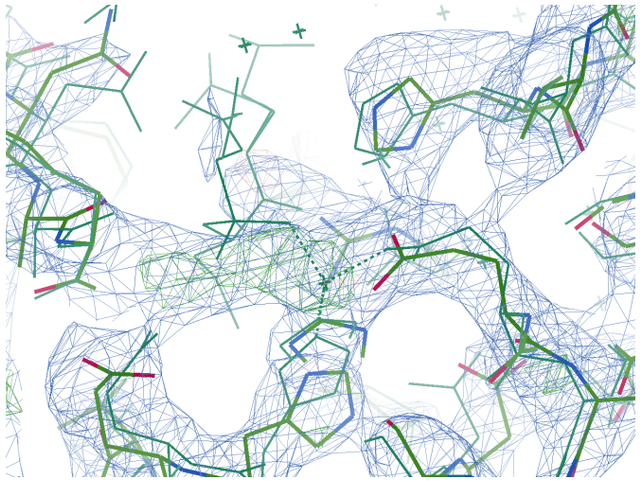


- Direct methods: only 1 wrong atom type
- Visualisation of hydrogen atoms
- 1806 refl., 258 param., 267 restraints
- $R1 = 18.5\%, R_{\text{complete}} = 21.9\%$

Thermolysin (sample courtesy Ilme Schlichting)



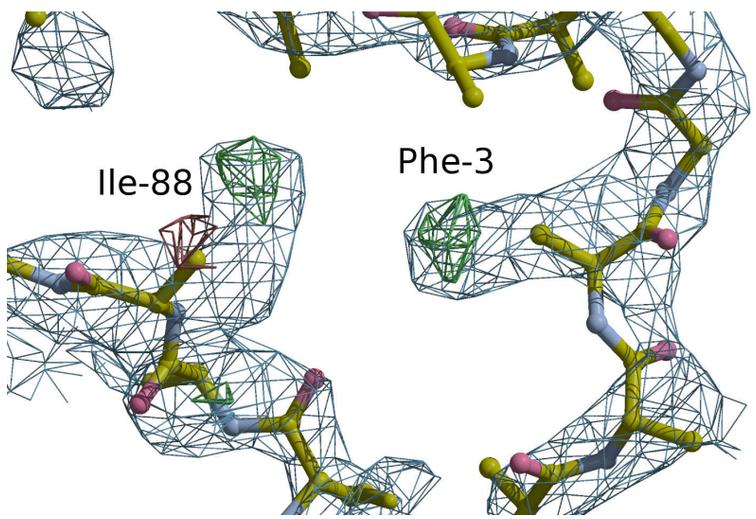
- Spacegroup $P6_122$
- Unit Cell 94.3 94.3 130.4 90° 90° 120°
- $d_{\text{min}} = 3.5$Å
- 72.4% completeness
- MR with 3DNZ poly Alanine: TFZ=26.4, LLG=433
- Buccaneer: side chain extension 315/316
- Refmac5: R1/“Rfree” = 28.0% / 29.9% (4N5P w/o water)



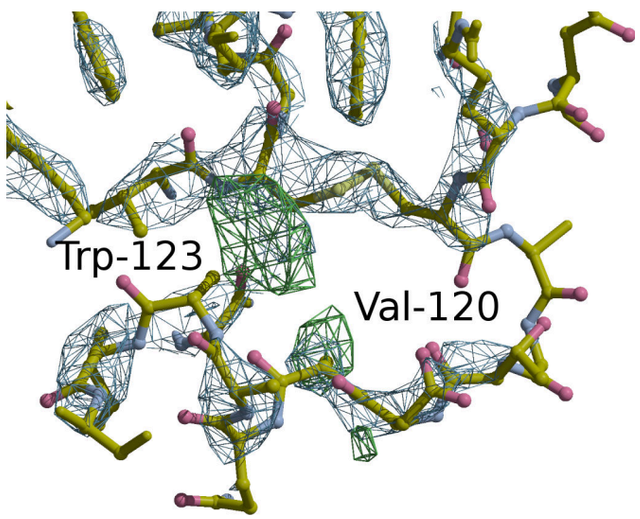
Lysozyme

	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

7 - Electron Crystallogaphy in CCP4

1. Data processing: DIALS (with D. Waterman)
2. Scaling: Aimless
3. MR: Phaser / Molrep
4. Autobuilding: Buccaneer
5. Refinement: Refmac5
   - `SOURCE ELECTRON MB`
   - `MAPC FREE EXLCUDE`
6. Model Building: Coot

8 - Summary: Electron Crystallography for non–Material Scientists

Sample Prep	Instrumentation	Proessing	Analysis
+ from Powder - from Solution - Data sets / day	++ Detector* - Rot^{n} Axis* - Lenses - Crystal Orient^{n}*	+ Integration - Param. Stability +/- Scaling	++ Direct Methods + Molec. Repl. + Refinement - Potential Repr.
* Current project at LBR / PSI			

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- Dr. B. Schmitt, PSI Detector group
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- Dr. W. Kabsch (XDS)
- Dr. D. Waterman (DIALS)
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