Electron Diffraction of Biological Macromolecules

Bioinformatics and X–Ray Structural Analysis

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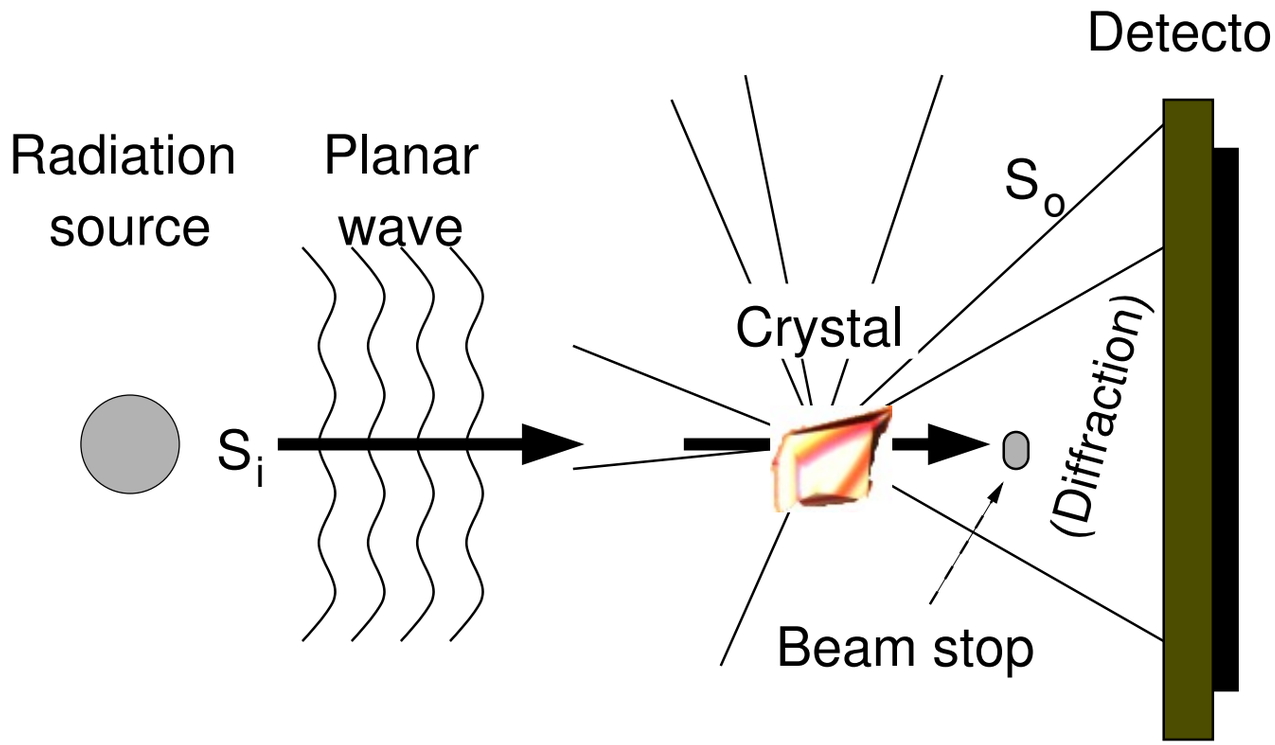
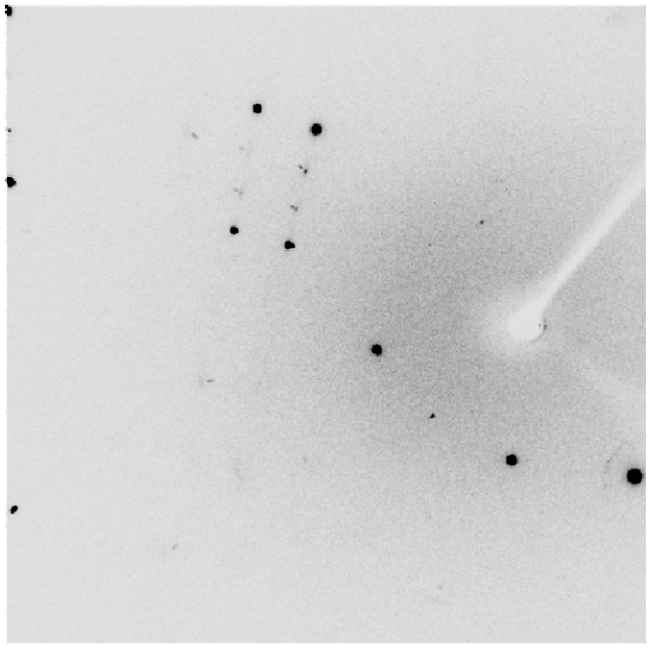
1 Crystals and Diffraction

1.1 Structure Determination by Single Crystal Diffraction





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

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

1.2 Spot Position and Spot Intensity

- 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

- Spot intensity $\Leftrightarrow$ Unit cell content

1.3 Data Collection . . . → . . . 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 $I_{\text{exp}}(hkl)$
- Data processing: from detector signal to amplitudes

1.4 Data Processing and Scaling

For X–rays*:

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

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

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

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

2 Types of Radiation

For atomic structure solution by crystallography:

1. X–rays

2. neutrons

3. electrons

3 Differences between Types of Radiation

1. Calculation of $|F_{\text{calc}}(hkl)|$ from atom coordinates

2. Conversion from $I_{\text{exp}}(hkl)$ to $|F_{\text{ideal}}(hkl)|$

3. X–rays and neutrons: $|F_{\text{ideal}}(hkl)| \propto \sqrt{I_{\text{exp}}(hkl)}$

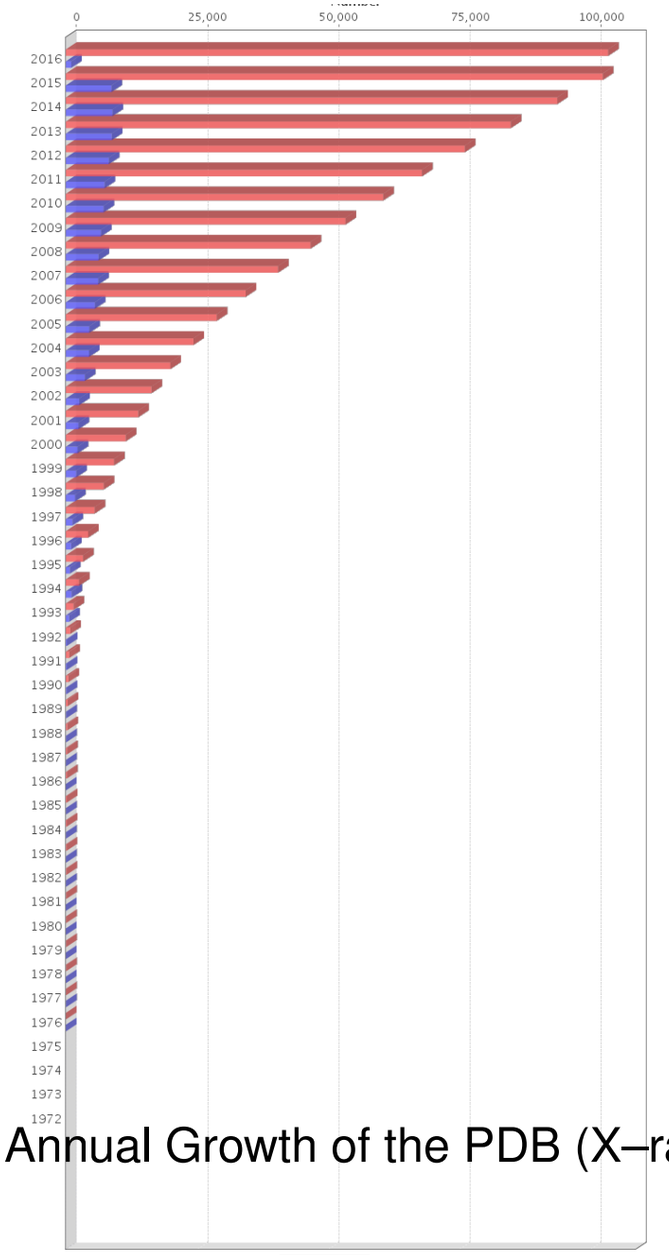
3.1 Types of Radiation — X–rays

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

2. typical wavelength: λ =0.8–1.9Å

3. standard structure determination

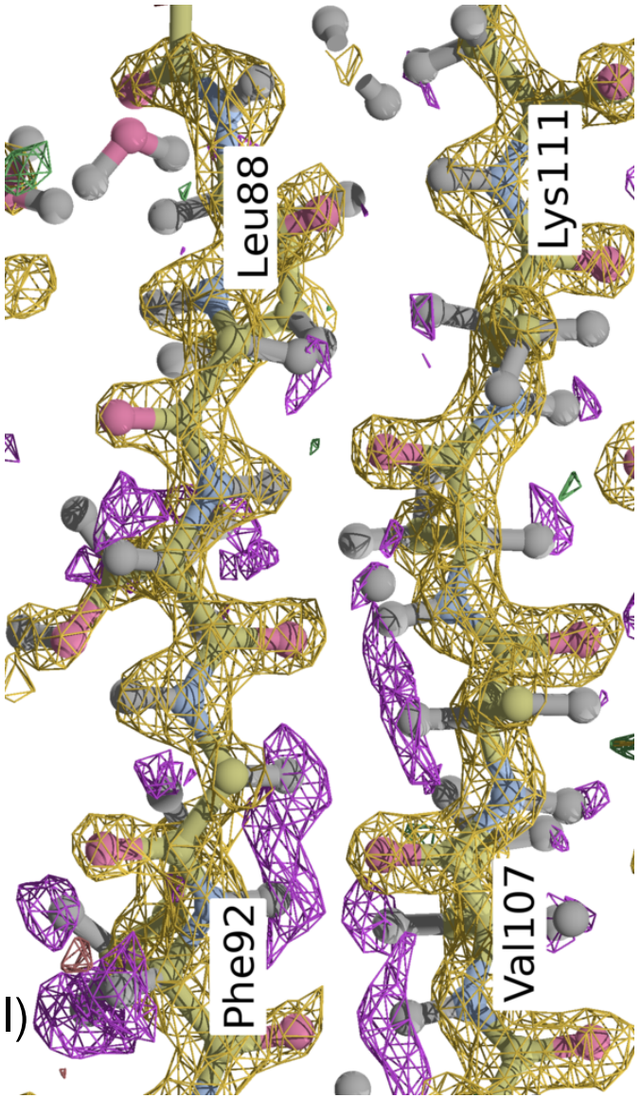
4. PDB (Protein Data Base):
   - 80,000 X–ray structures
   - 80 neutron structures
   - 60 electron structures



Annual Growth of the PDB (X–ray)

3.2 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

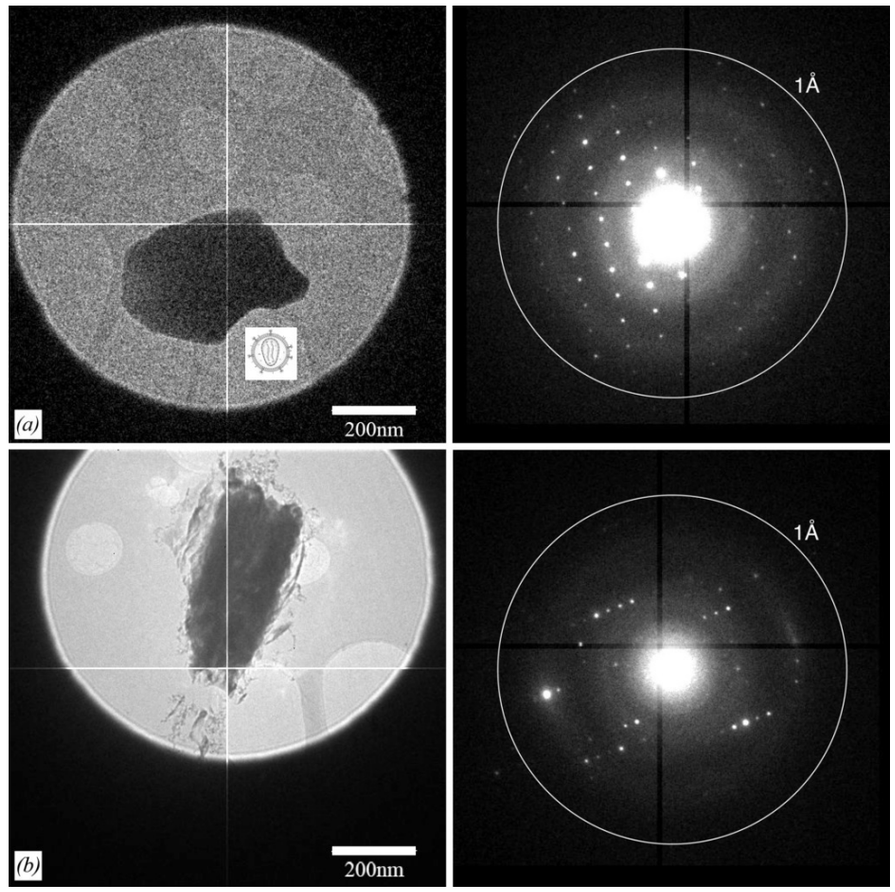
3.3 Types of Radiation — electrons

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

2. typical wavelength: $200keV = 0.0251$Å: flat Ewald sphere

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

3.4 Goal of Diffraction Experiment

- Fit molecule into density $\rho(x,y,z)$ to determine atomic structure

- $\rho(x,y,z) = \sum_{h,k,l} |F_{\text{ideal}}(hkl)| e^{i\phi(hkl)} e^{-2\pi i(hx+ky+lz)}$

3.5 Crystallographic Maps

- After phasing, diffraction data provide *density maps* $\rho(x,y,z)$

- The type of map depends on the interaction

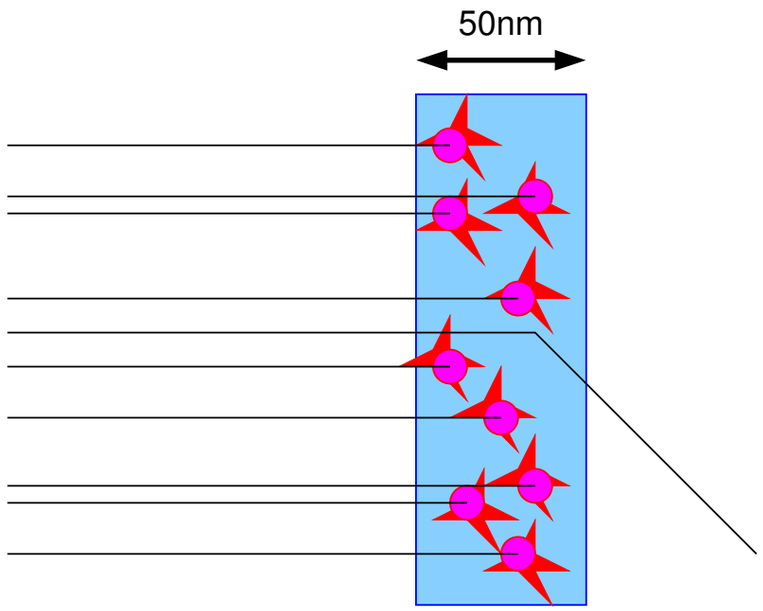
Radiation	Interaction	Map type
X–ray	e^-	electron density map
n	nucleus	nucleic “density” map
e^-	$p+e^-$	Coulomb potential $\approx$ electron density map

- Macromolecules can be built into the maps “as usual”

4 Applications for Electron Diffraction

1. Diffraction & Radiation Damage
2. Nanocrystals have less Defects
3. Powder contains Single Nanocrystals
4. Seemingly failed Crystallisation Attempts contain Nanocrystals

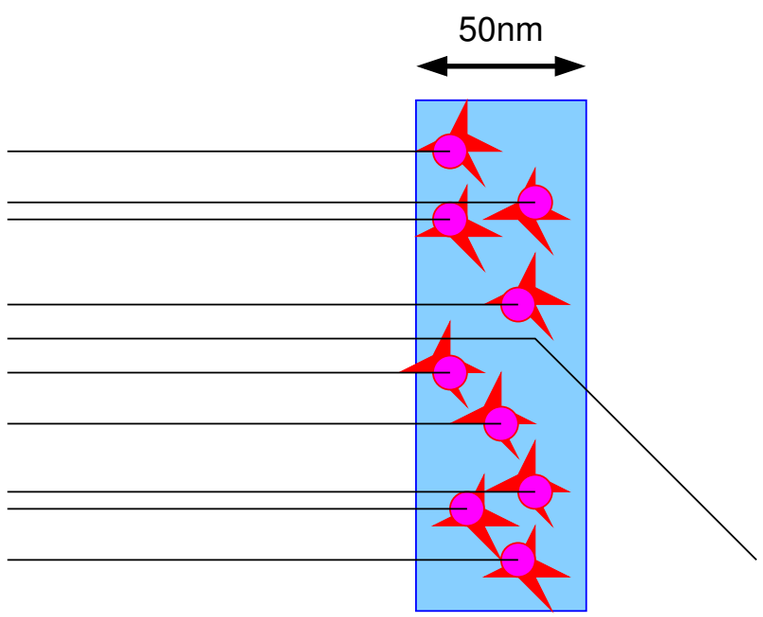
4.1 X–rays Scattering and Electron Scattering



X–rays (10keV)

- Probability of inelastic scattering: 10^{-4}
- Deposited energy: 10keV
- Probability of elastic scattering: $10^{-5} = 10^{-4}/10$
- Damage per diffracted photon: 100keV

4.2 X–rays Scattering and Electron Scattering



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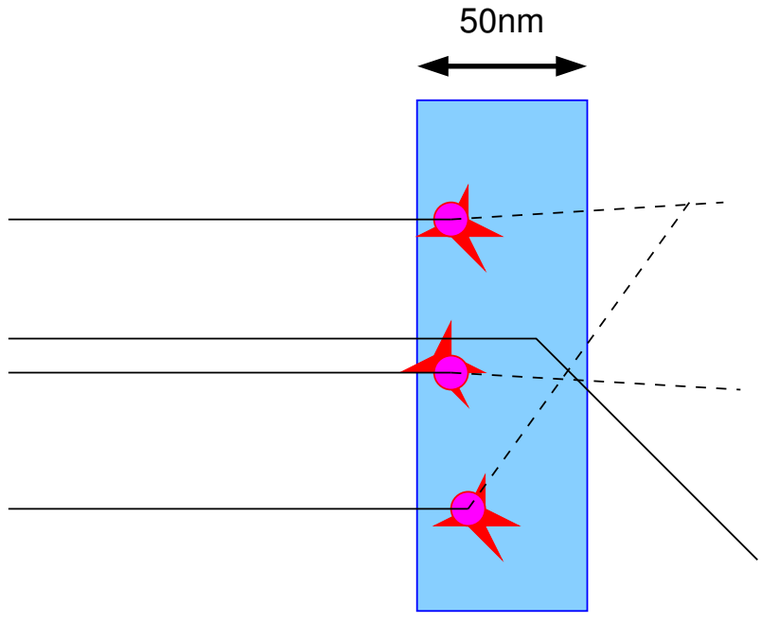
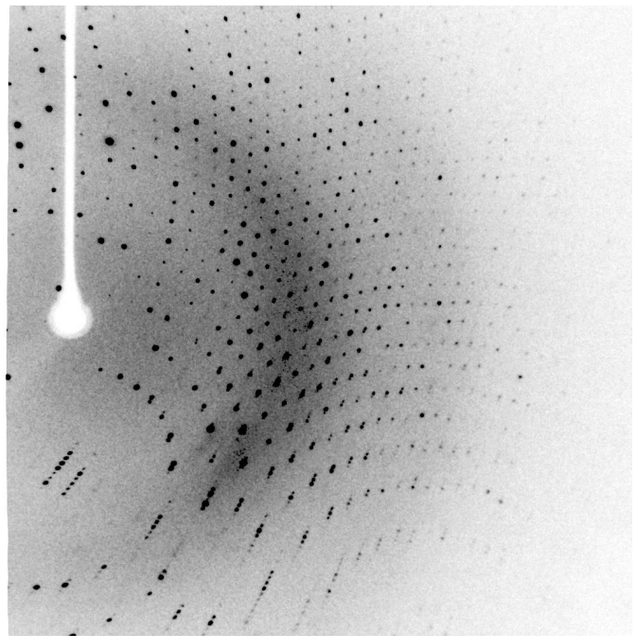


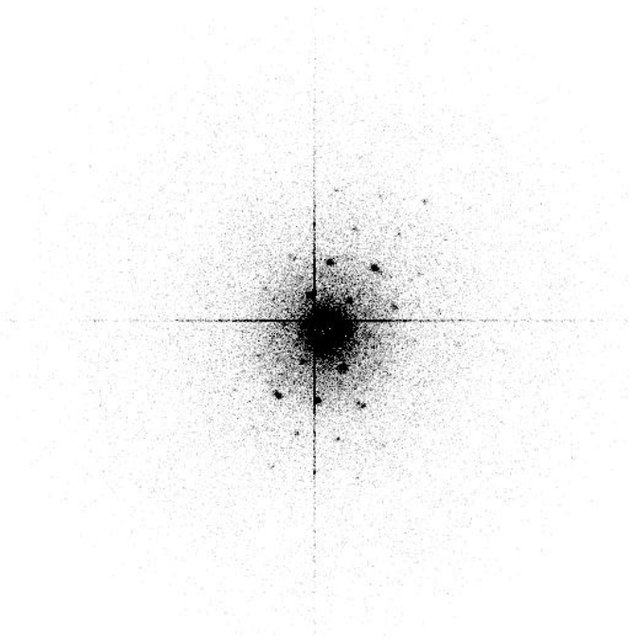
e^- (200keV)

- Probability of inelastic scattering: 30%
- Deposited energy: 20eV
- Probability of elastic scattering: 10%
- Damage per diffracted electron: 60eV = 0.06keV

2,000 times more damage with X–rays

4.3 X–rays Scattering and Electron Scattering





- Small Crystals very radiation sensitive
- X–rays mostly pass through (99.99%): beamstop
- X–rays mostly damage (10:1).

4.4 Small Crystals

X–rays

You **can** measure nanocrystals. You need

- Free Electron Laser (XFEL, SwissFEL, . . .)
- 10,000 – 100,000 crystals, $V \approx 5\ ml$
- Special Software, Computational Demands

Electrons

You **must** measure nanocrystals. You need

- Electron Microscope
- 1–2 nanocrystals
- standard software (XDS, SHELX, Refmac5,. . .)
- Direct Pixel Detector helps (Timepix, Dectris Eiger, . . .)

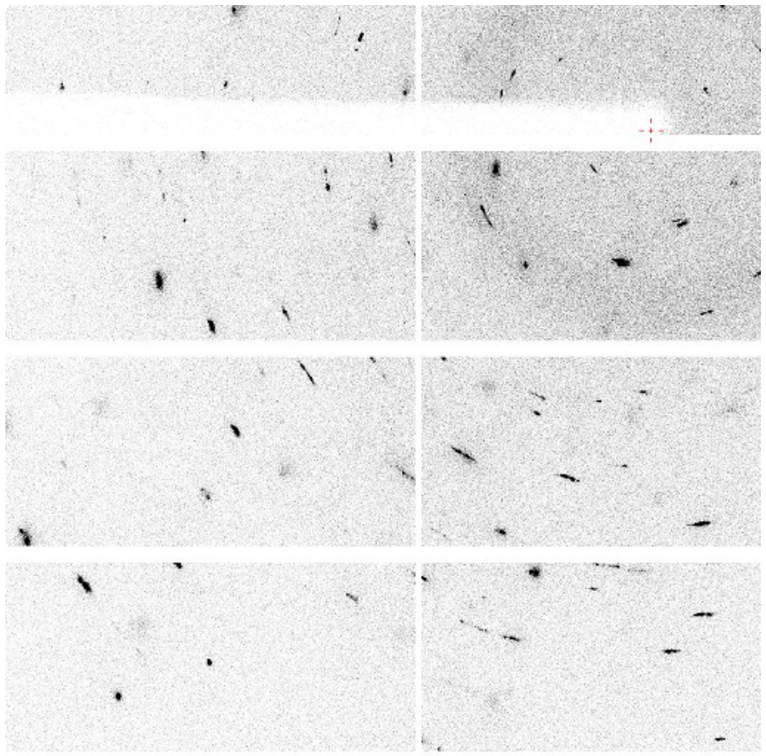
4.5 Joint Venture: Free Electron Lasers and Electron Diffraction

- Beamtime for FELs will be very competitive
- Only few end stations available
- Electron Microscopes are “more abundant”
- Sample quality can be pre-assessed with Electron Diffraction
- Structures can be solved from Electron Diffraction

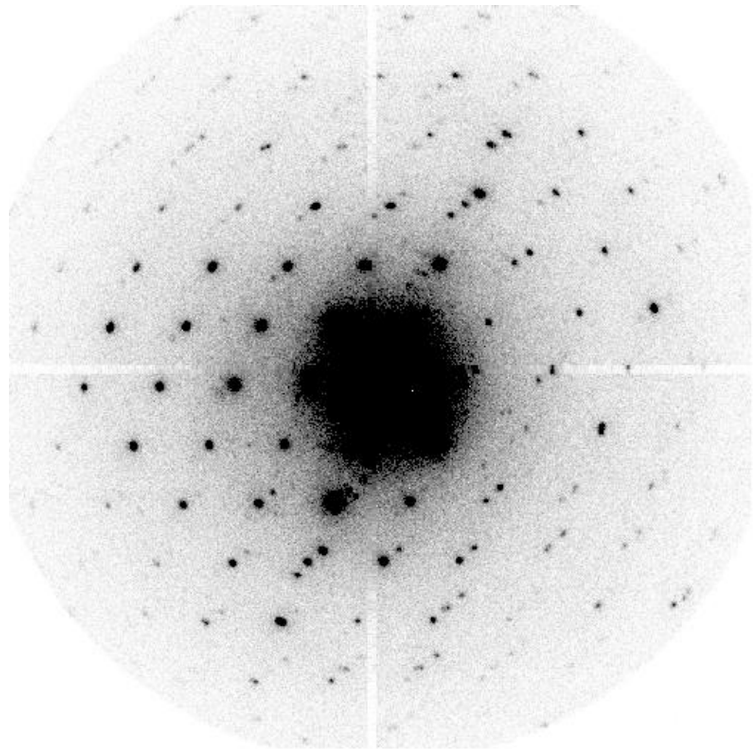
$\Rightarrow$ FEL have more time to time–resolved studies

$\Rightarrow$ Electron diffraction enhances the through-put of FELs

4.6 Applications: Better Ordered Crystals



- “long” range disorder
- worse with larger crystals
- worse with freezing
- nanocrystals: often better defined spots



4.7 Applications: Single Crystals

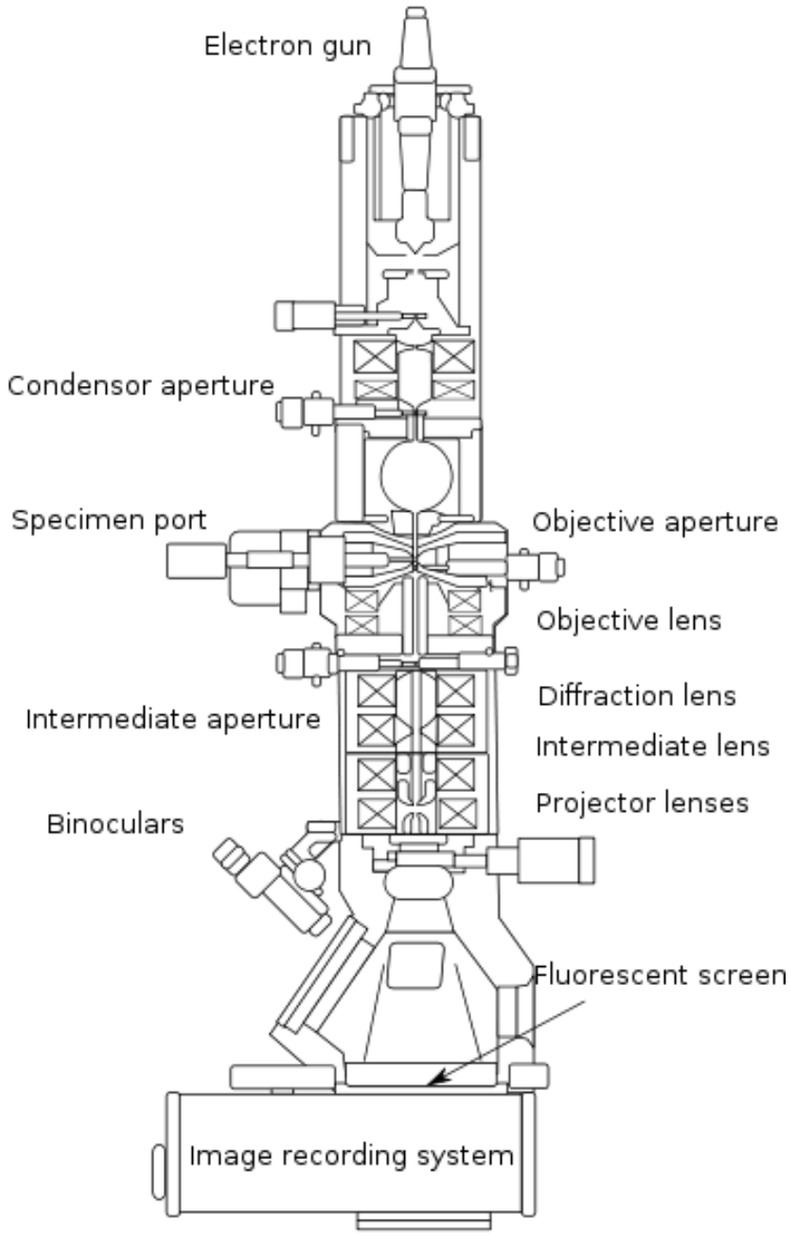
- Powder samples often contain single nanocrystals suitable for electron diffraction
- Usually too small for conventional crystallography
- Highly interesting for the pharmaceutical industry

4.8 Applications: Crystals at all!

- macromolecules are difficult to crystallise
- in particular: membrane proteins
- Large fraction of clear drops actually contains nanocrystals —Stevenson *et al.*, PNAS (2014) 111, 8470–8475

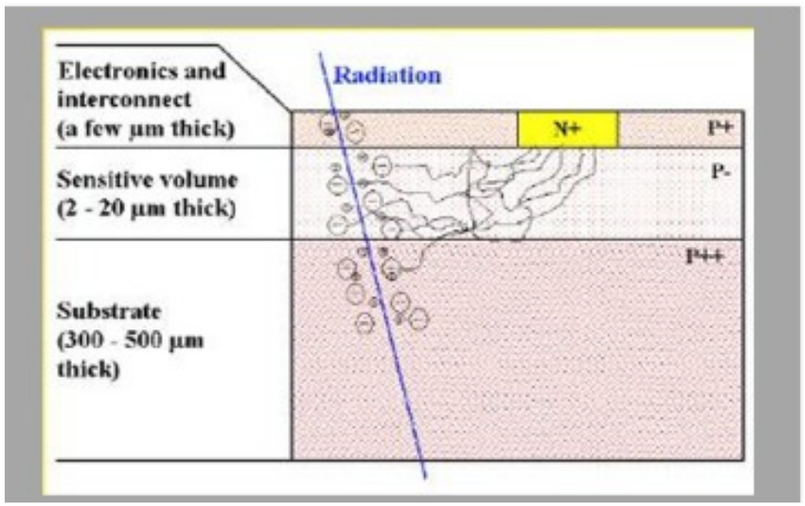
5 Instruments for Electron Diffraction

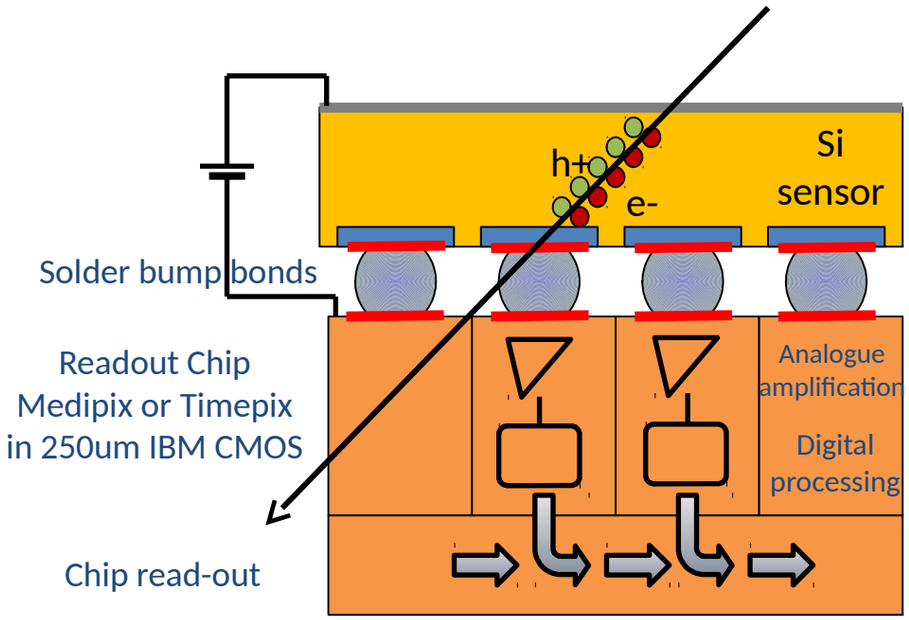
5.1 Electron Microscopes



(Wikipedia)

5.2 Direct Pixel Detectors





Monolithic direct electron detector:
- damage prone
- Small point spread
- Low dynamic range

Ideal for imaging

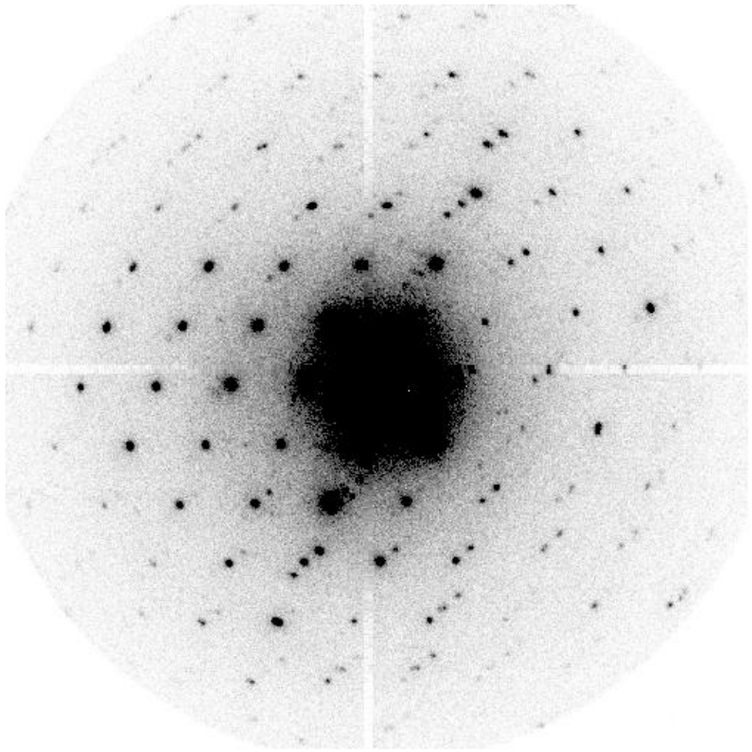
Hybrid pixel detector:
- radiation hard
- Larger point spread
- High dynamic range

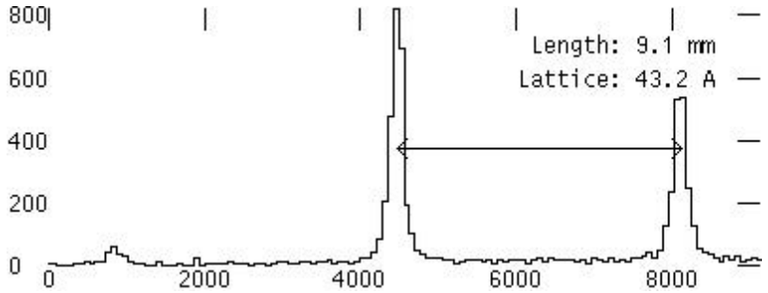
Ideal for diffraction

(Courtesy Prof. Abrahams)

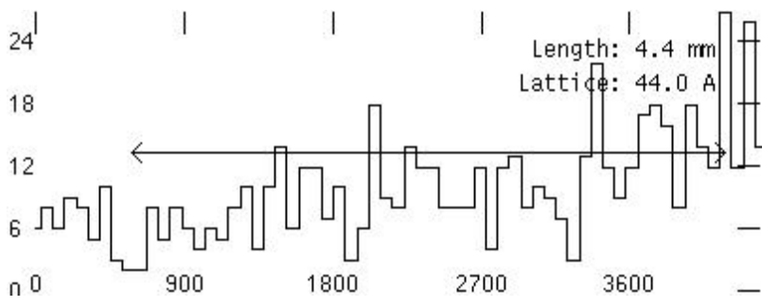
5.3 Direct Pixel Detectors

Direct Pixel Detectors have no electronic noise, only background scattering



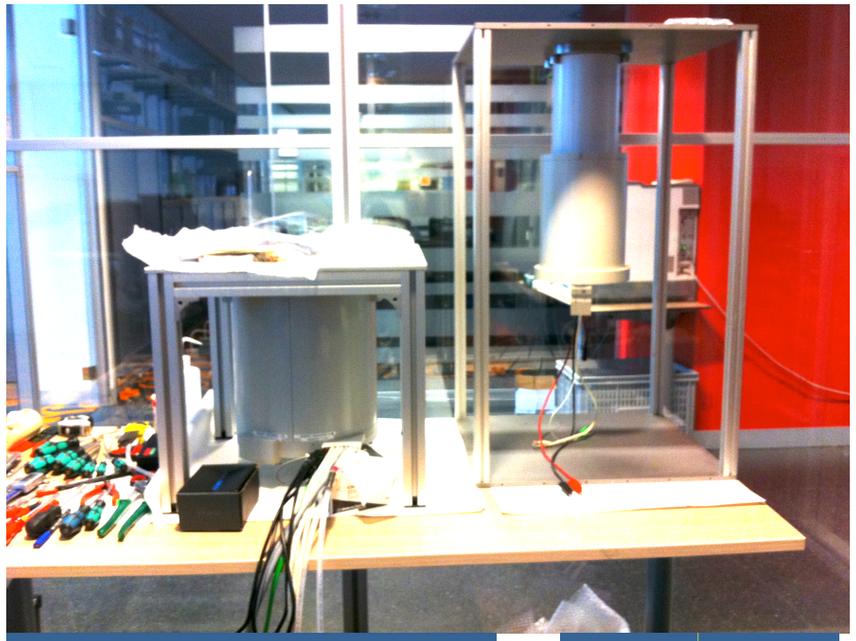


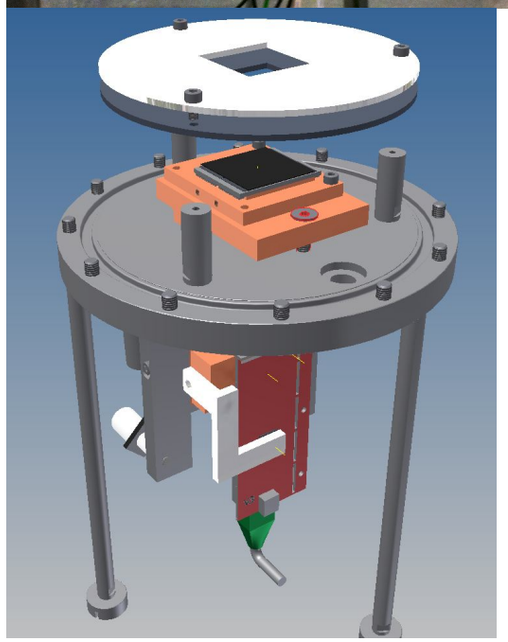
Cross–section with spots

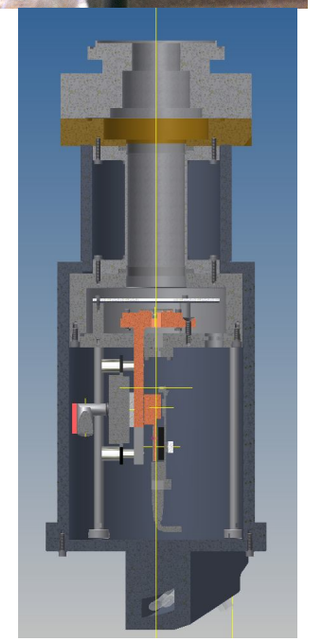


Cross–section without spots

5.4 The Timepix Detector

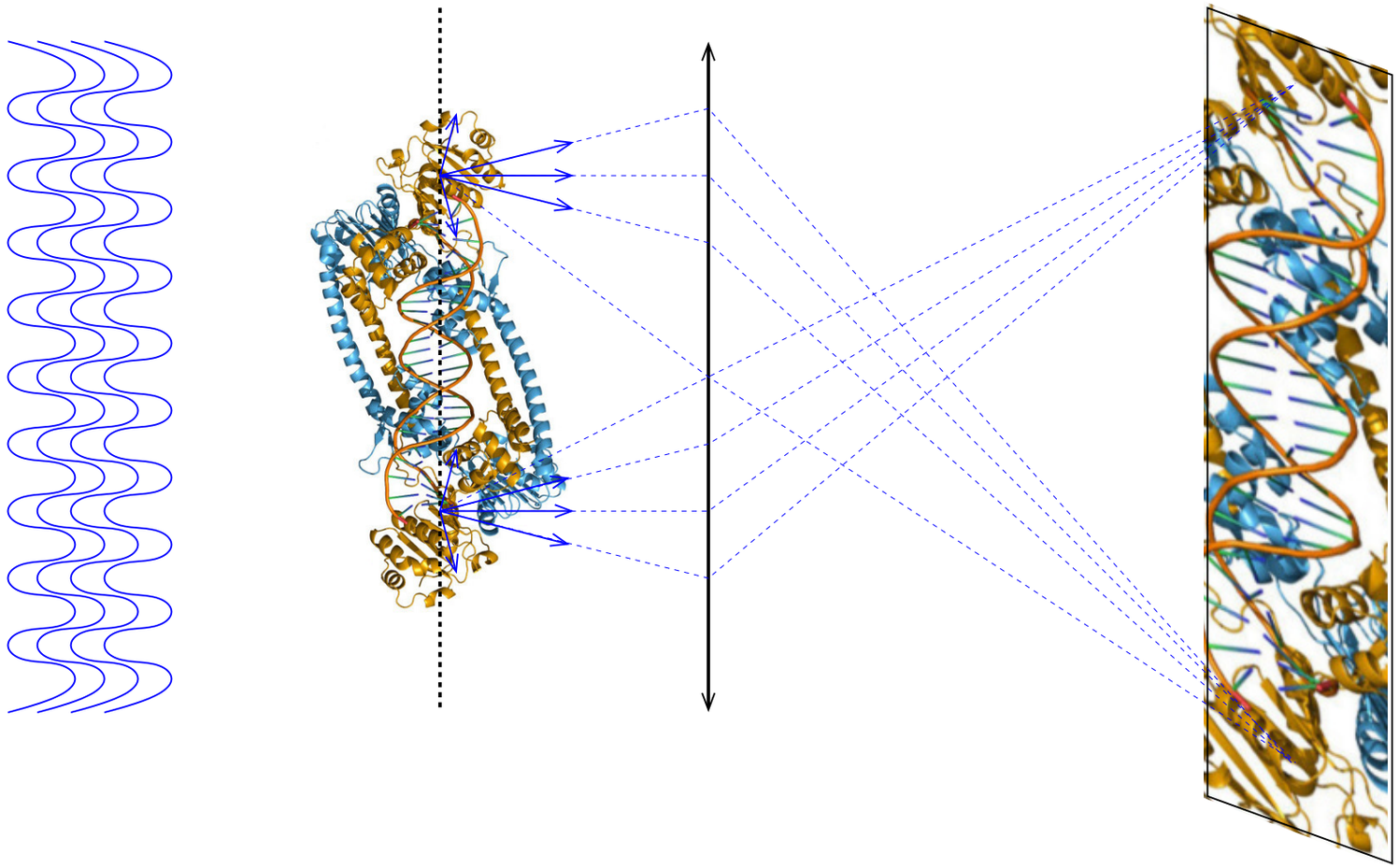






- Timepix assembly:
- ASI read–out
- Electronics outside vacuum
- Peltier cooling of detector $\pm 0.1K$
- 512×512 and 1024×1024 pixel versions
- linear: 1–10,000 e^- / frame
- read–out: up to 120 frames /s
- radiation hard

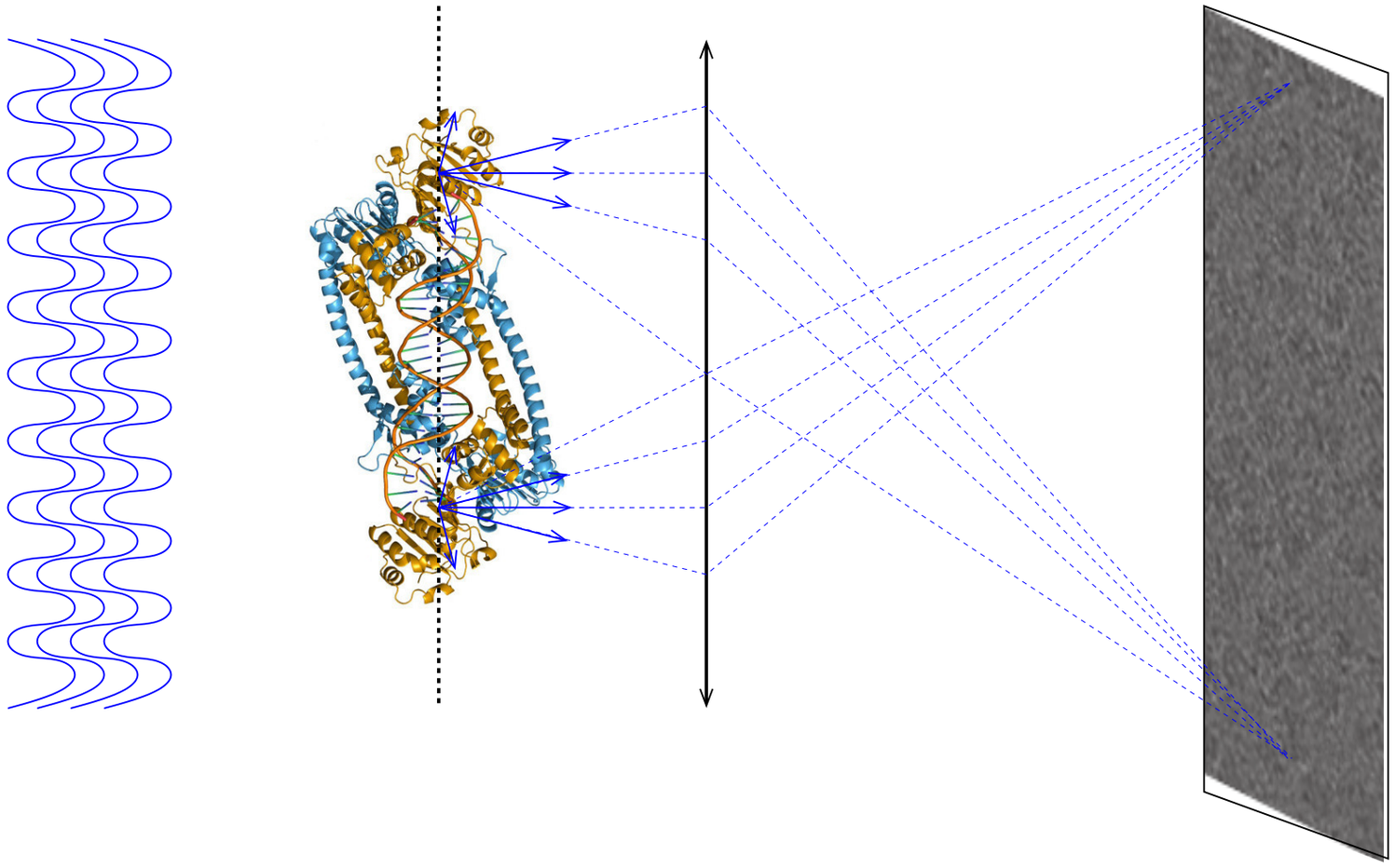
5.5 Electron Microscope: Imaging Mode



Plane Wave Object Lense Image Plane (Detector)

Rays of **equal origin** focus on detector

5.6 Electron Microscope: Imaging Mode

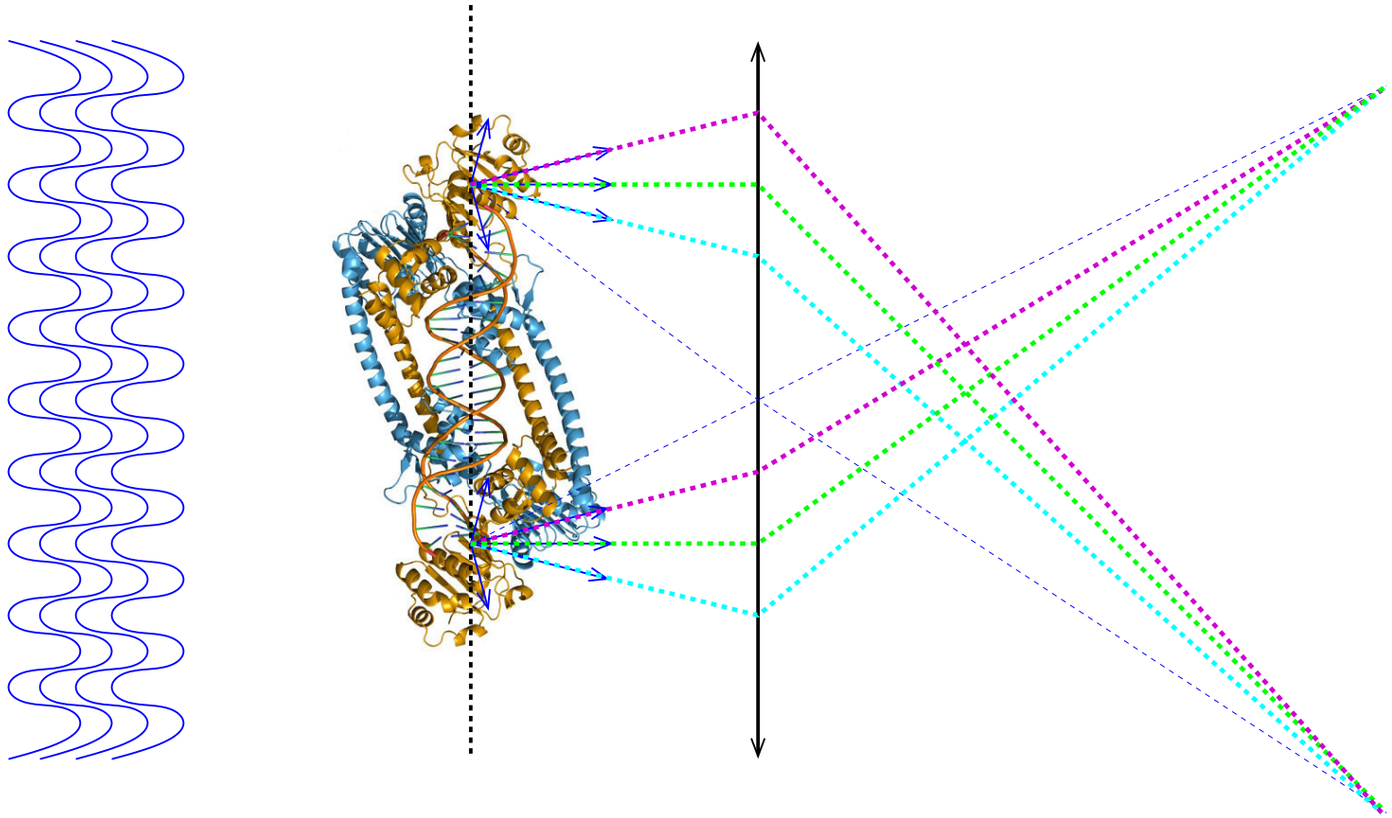


Plane Wave Object Lense Image Plane (Detector)

Detector noise and radiation senstivity require low contrast images

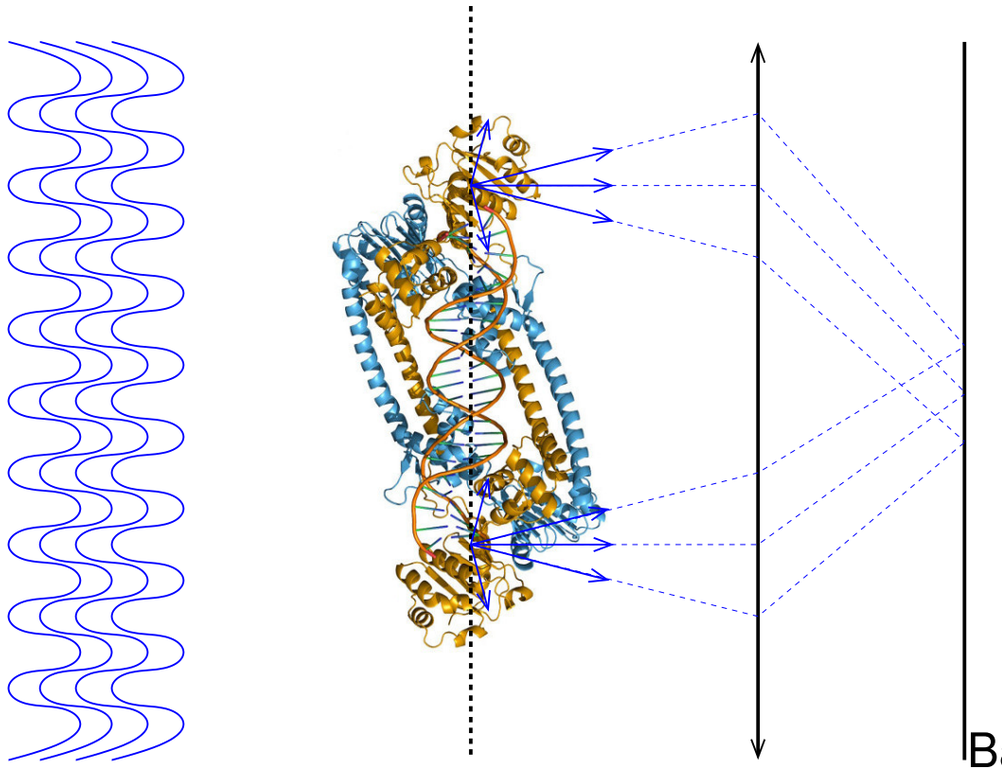
Martinez-Rucobo *et al.* Molecular Cell (2015) 58, 1079–1089

5.7 Electron Microscope: Diffraction Mode



Plane Wave Object Lense

5.8 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

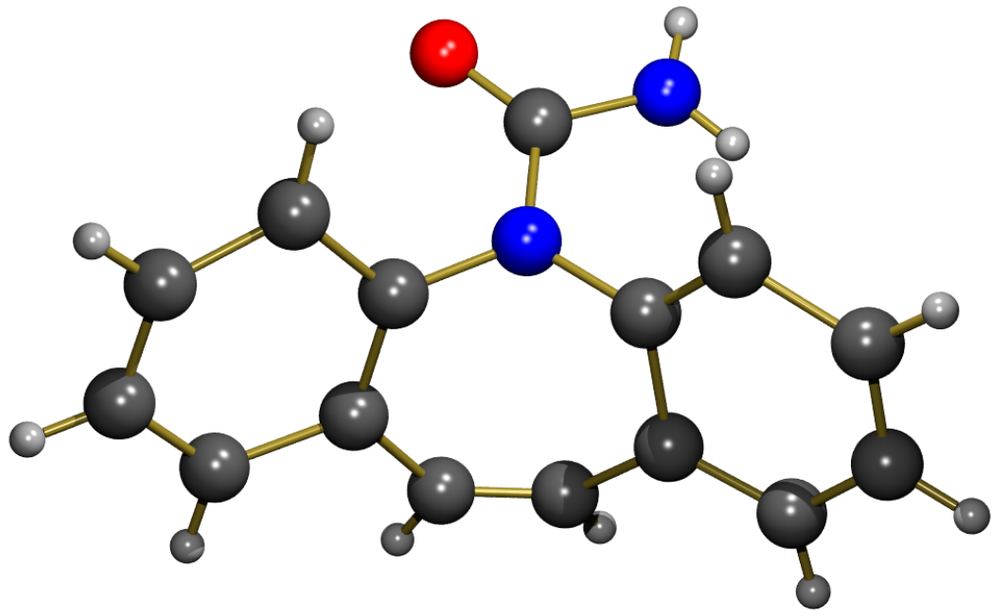
6 Example Data and Example Structures

1. Carbamazepine (van Genderen *et al.*, Acta Cryst (2016) A72 (2))

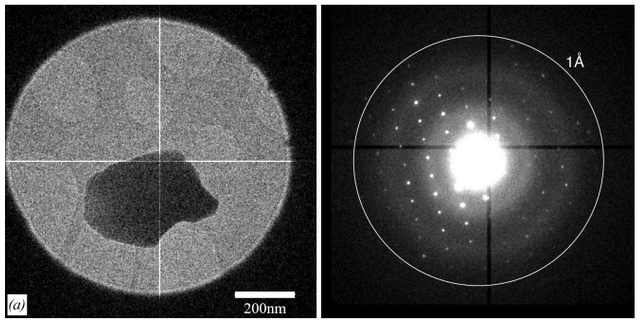
2. Lysozyme (Manuscript in preparation)

6.1 Carbamazepine

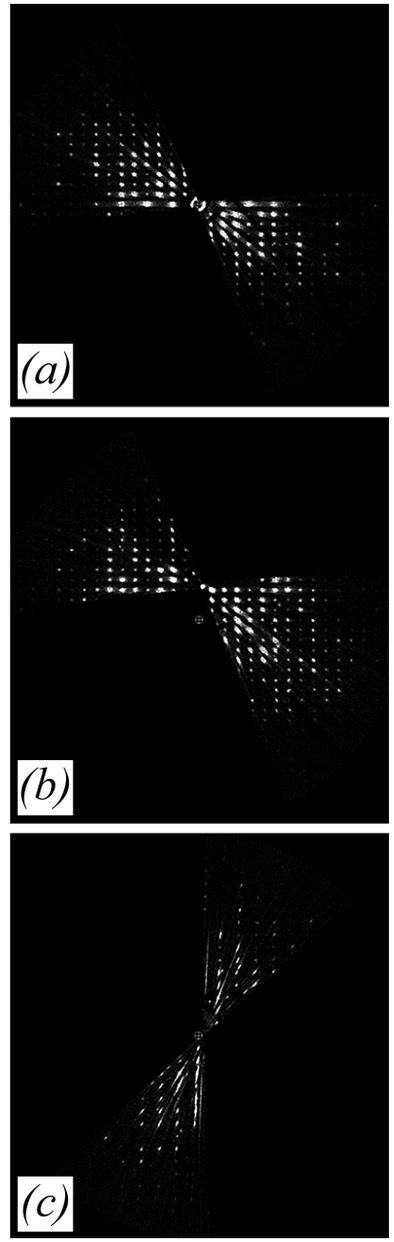
- Drug for epilepsy
- Small organic compound $C_{15}H_{12}N_2O$
- Well know strucuture used as test case (El Hassan *et al.*, Crystal Growth and Design (2013), 13, 2887–2896)



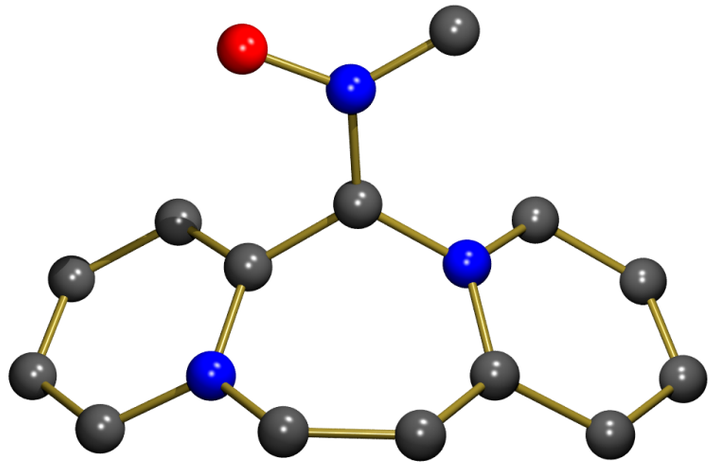
6.2 Carbamazepine Data

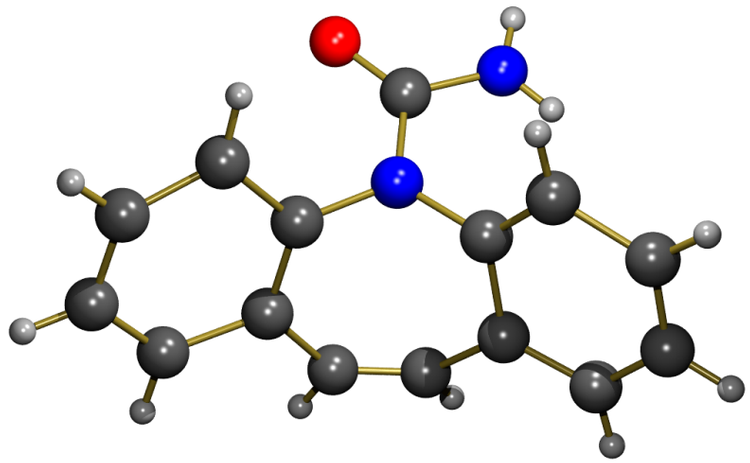


Crystal Size	$1.2 \times 0.8 \times 0.2$
Rotation range	51°
Dose	$4.0 e^- / \text{Å}^2$
Space group	$P2_1/n$
Resolution	8.7–0.8 (0.85–0.80)Å
Completeness	45% (46%)
R_{merge}	8.4% (35.8%)
I/σ_I	5.6 (1.8)
R_{complete}	28.0 %



6.3 Carbamazepine Structure Solution

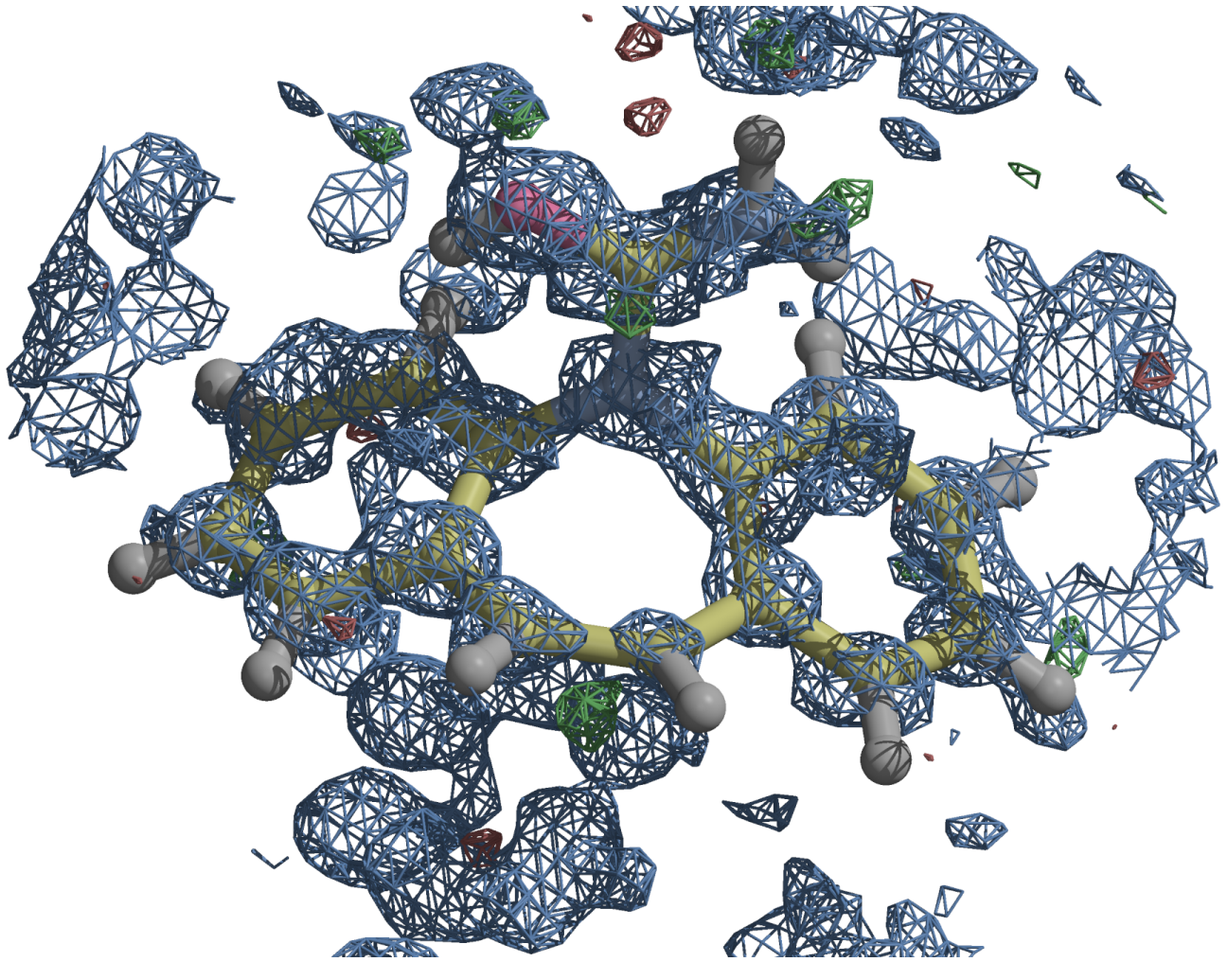




shelxt solution **final model**

- Solved with direct methods, *i.e.* no chemical information
- No atoms missed, no atoms too many
- Only 4 wrongly assigned atom types

6.4 Carbamazepine Structure Solution



Low data completeness affects map quality despite atomic resolution

6.5 Solving Macromolecular Data: Lysozyme

- Currently PDB holds three entries from 3D electron diffraction

1. 4ZNN: peptide involved in Alzheimers disease, $P2_1$, 1.4Å [Rodriguez *et al.* Nature (2015) 525, 486–490]
2. 5A3E: Lysozyme, $P2_1$, 2.5Å [Nannenga *et al.* Nat. Meth. (2014) 11, 927]
3. 3J7U: Catalase, $P2_12_12_1$, 3.2Å [Yonekura *et al.* PNAS (2015) 112, 3368–3373]

Structures 1+2 are collected from μ crystals; Structure 3 was solved by merging data from 99 crystals.

6.6 Data from a single Lysozyme nanocrystal

- Collected 40° before radation damage destroyed crystal
- Crystal thickness $\approx 100nm$
- Data processed with RED and with XDS in $P1$

	RED	XDS
Cell	32.3Å 69.7Å 105.6Å	32.1Å 70.9Å 104.0Å
	93.6° 92.0° 90.1°	93.4° 91.9° 91.1°
Resolution	32.3–2.4 (2.5–2.4)	32.1 –2.2 (2.3–2.2)
I/σ_I	21.2 (8.8)	6.7 (1.4)
R_{merge}	7.9% (13.7%)	28.9% (49.8%)
Completeness	4.1% (0.1%)	20.7% (20.8%)
# refl.	1897	14148 (2571)
# unique refl.	1568	9542 (1539)

6.7 Spacegroup of Lysozyme nanocrystal

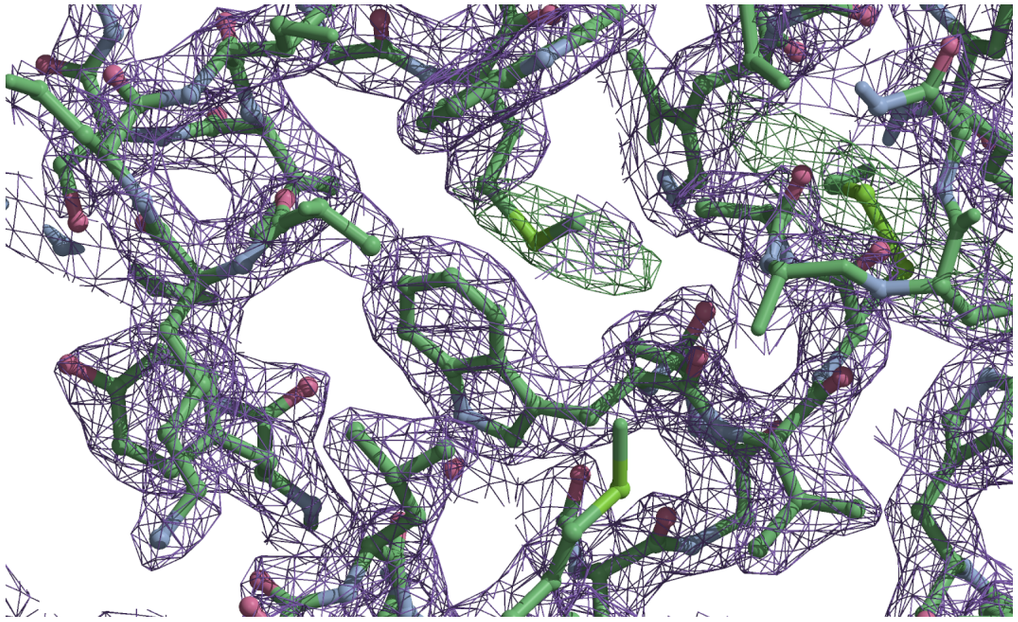
- Cell: $32.1 \times 70.9 \times 104.0$, 93.4° 91.9° 91.1°
- XDS suggests: P 2 1 1
- PDB ID 4R0F: $P2_12_12$ with $104.63 \times 66.49 \times 31.65$

Possible explanations:

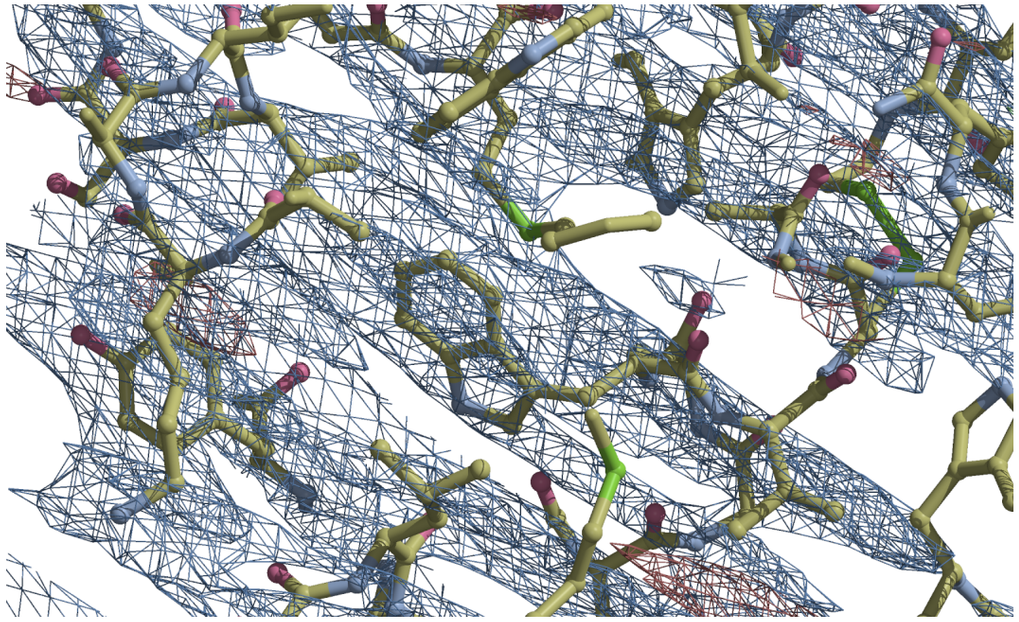
1. α angle distorted because of erroneous parameters (distance, frame width, rotation range, image distortions)
2. Macrocrystal induces more rigid packing $\Rightarrow$ enforces higher symmetry

$\Rightarrow$ Currently an open question

6.8 Lysozyme: Model Bias

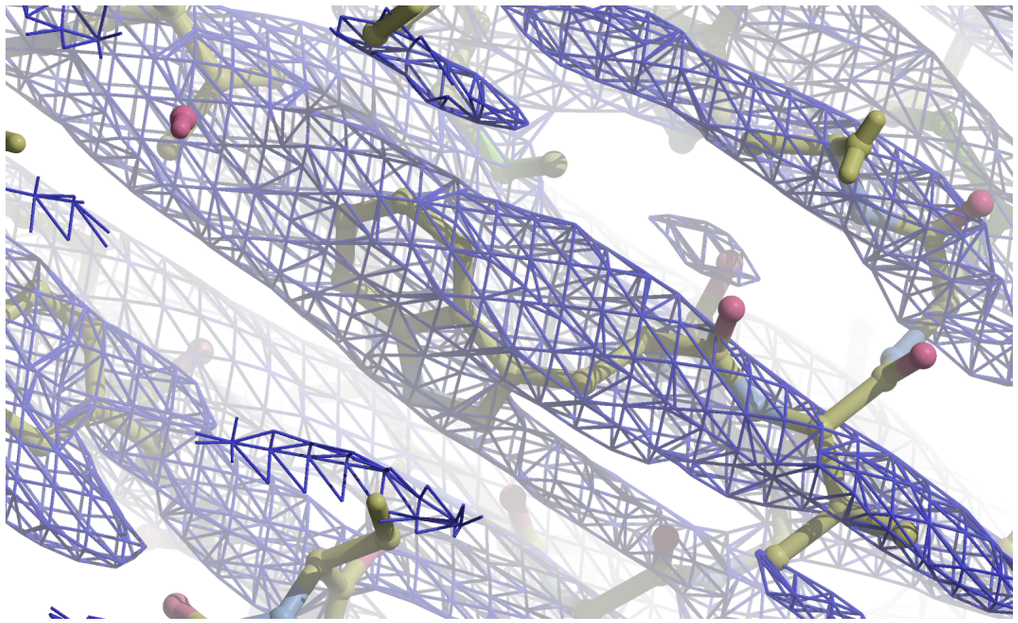


Refined map from Refmac5

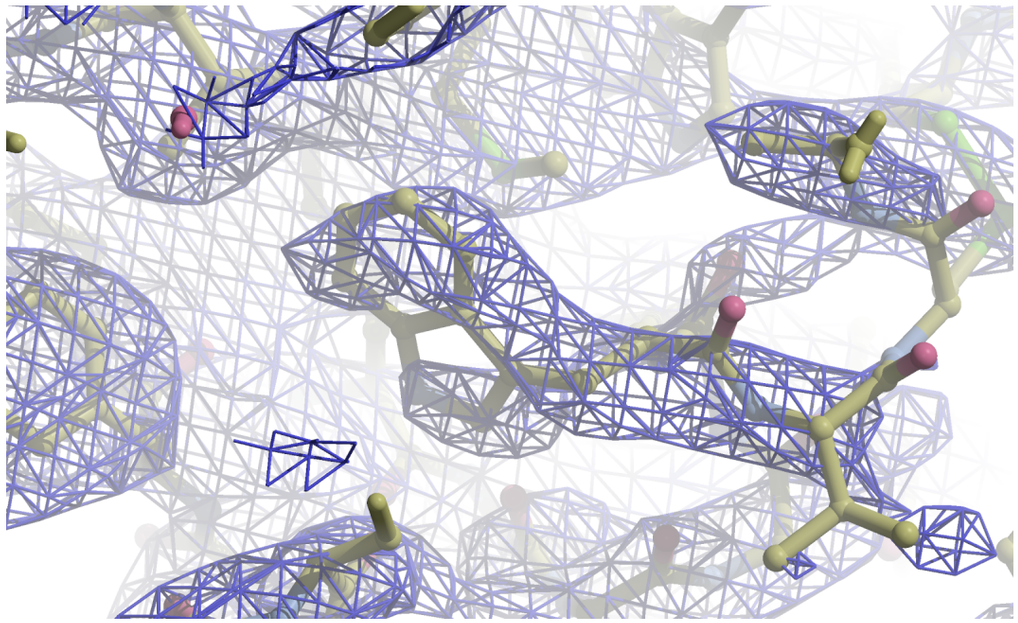


Refined map from ShelxI

6.9 Lysozyme: is it Real? (I)

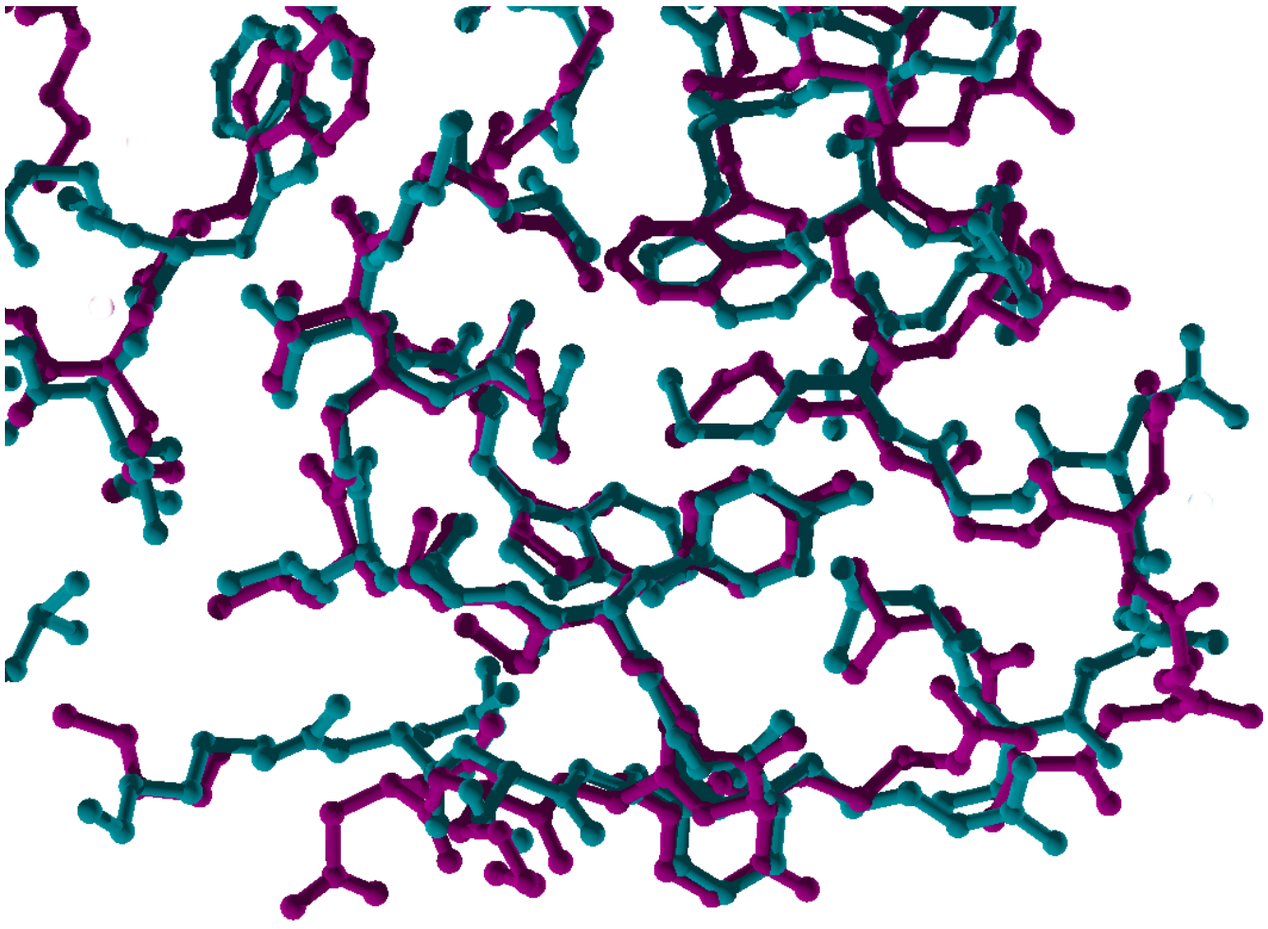


Refined map from ShelxI (zoomed)



Same map 4x NCS averaged

6.10 Lysozyme: is it Real? (II)



- Purple: Molecular replacement including side chains
- Green: Molecular replacement with poly-Ala model; side chains autobuilt with Buccaneer
- Autobuilding uses sequence information and data.
- Many side chains consistent

7 Phasing with Images

7.1 The Crystallographic Phase Problem

$$\rho(x,y,z) = \sum_{h,k,l} |F_{\text{ideal}}(hkl)| e^{i\phi(hkl)} e^{-2\pi i(hx+ky+lz)}$$

- Diffraction experiment measured amplitudes $|F_{\text{ideal}}(hkl)|$

- Phases $\phi(hkl)$ “get lost”

- Phasing methods:

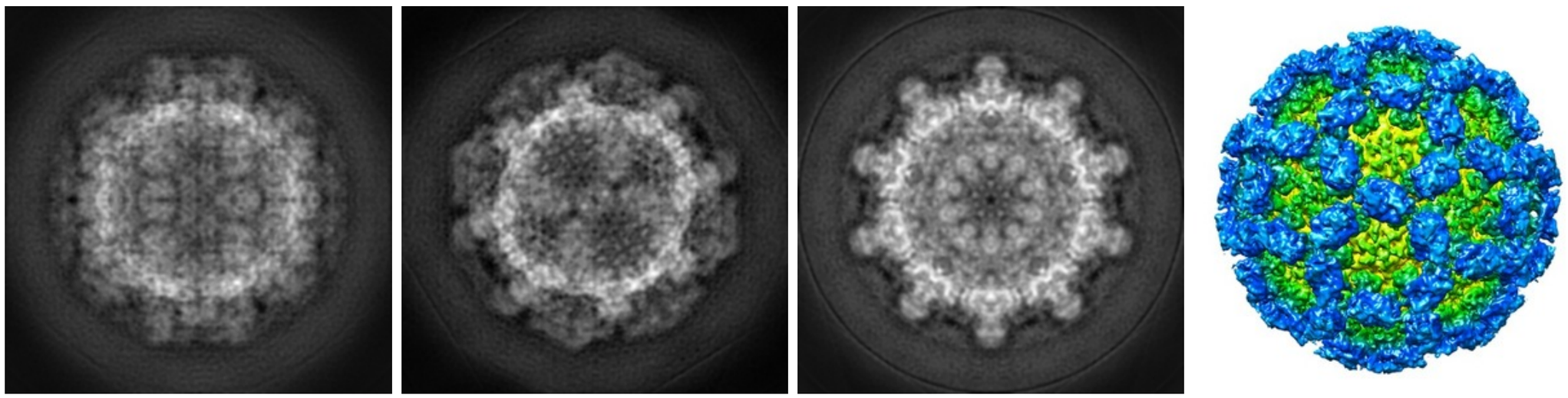
  1. Molecular Replacement

  2. SAD/MAD — Single–/Multi–wavelength anomalous dispersion

  3. SIRAS — Isomorphous replacement with anomalous dispersion

7.2 Electron Microscope Imaging

1. Record *many* images
2. Classify, group, and reduce noise
3. Find orientations
4. Reconstruct 3D electron density



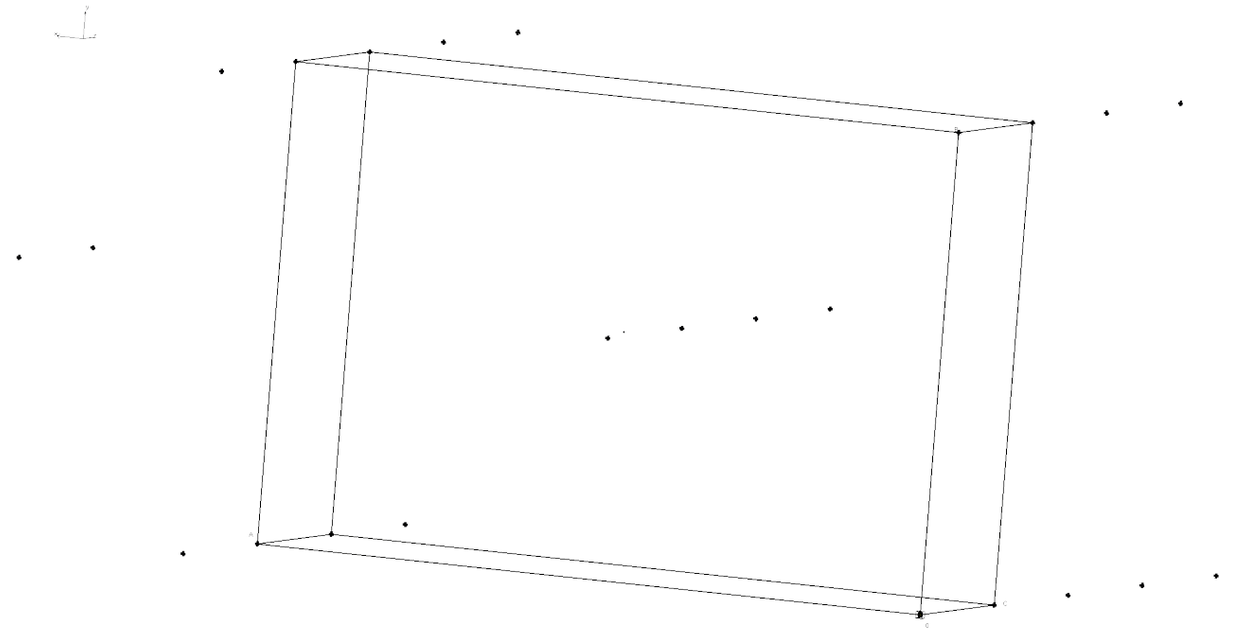
(EMDB 3281, *A chimeric sapovirus capsid*)

7.3 Indexing Diffraction Data

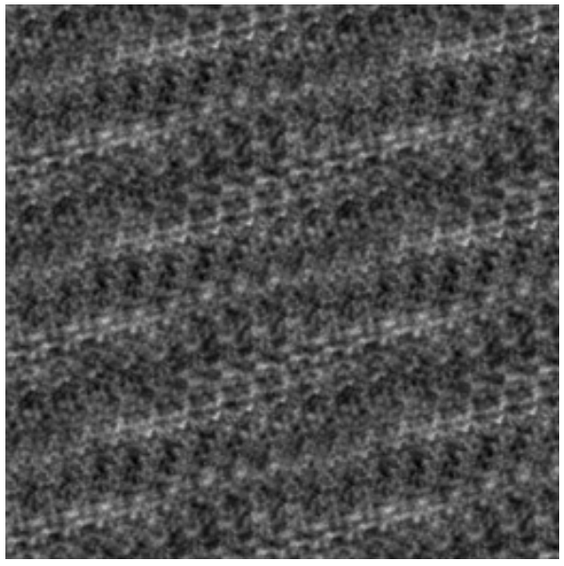
- Diffraction Data can be indexed

$\Rightarrow$ Unit Cell Dimensions and (often) Space group are known without solving the structue

$\Rightarrow$ Place single atom at unit cell corners and create projections from “single atom map” from all orientations



7.4 EM Imaging from Crystals



Projected Lysozyme Density
210Å × 210Å

Projected Single Atom Density
210Å × 210Å
Same Orientation
Match: 3.6%

Projected Single Atom Density
210Å × 210Å
Different Orientation
Match: 0.8%

In Image Mode, Contrast between 3.6% and 0.8% too low
(Simulated Data)

7.5 EM Imaging from Crystals in Fourier Space

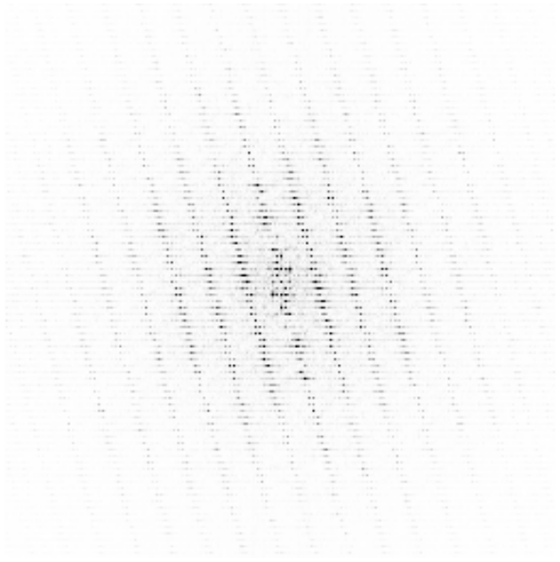
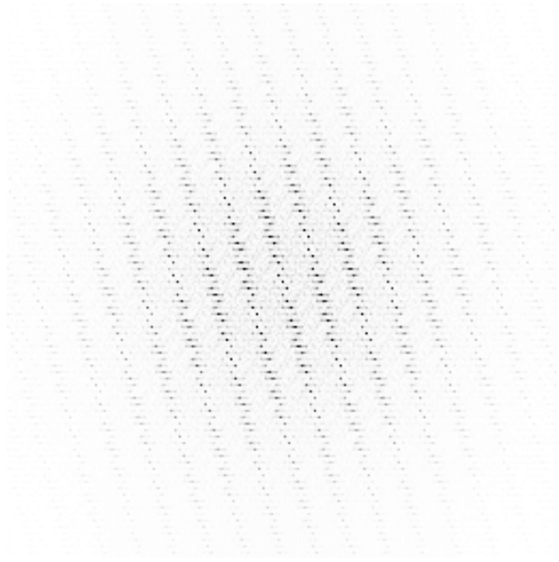
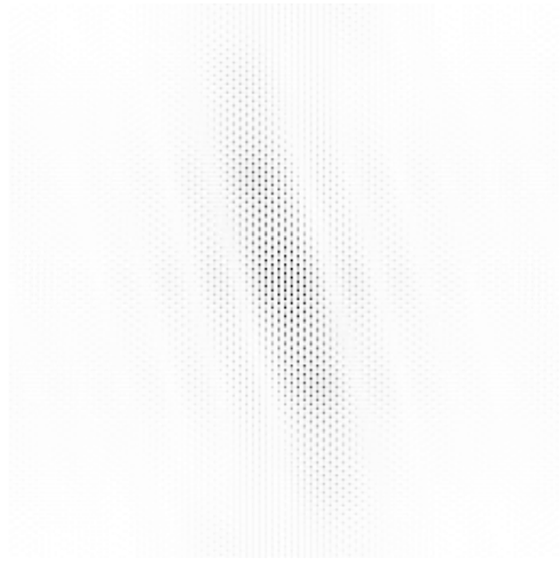


Image of Lysozyme Crystal
after Fourier Transform
210Å × 210Å



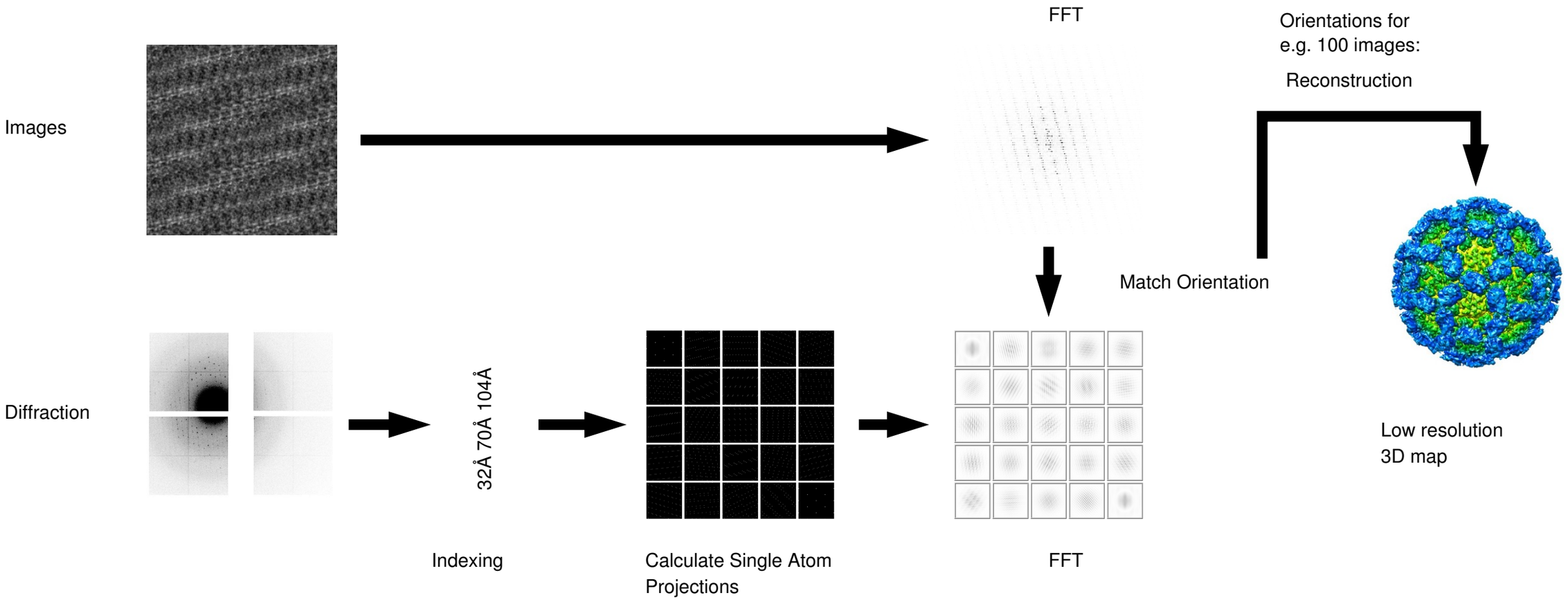
Projected Single Atom Density
after Fourier Transform
210Å × 210Å
Same Orientation
Match: 65%



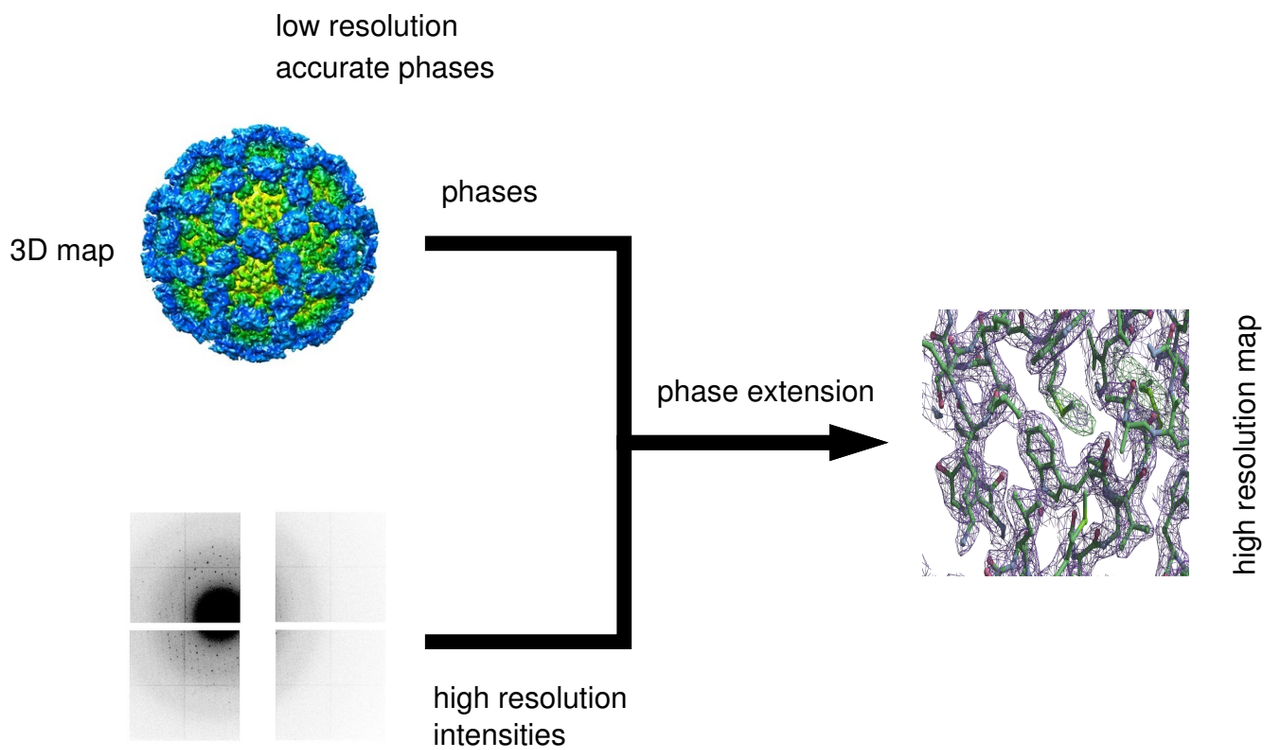
Projected Single Atom Density
after Fourier Transform
210Å × 210Å
Different Orientation
Match: 14%

Contrast after Fourier Transformation enables selection of correct orientation
(Simulated Data)

8 Phasing with EM Images I



9 Phasing with EM Images II



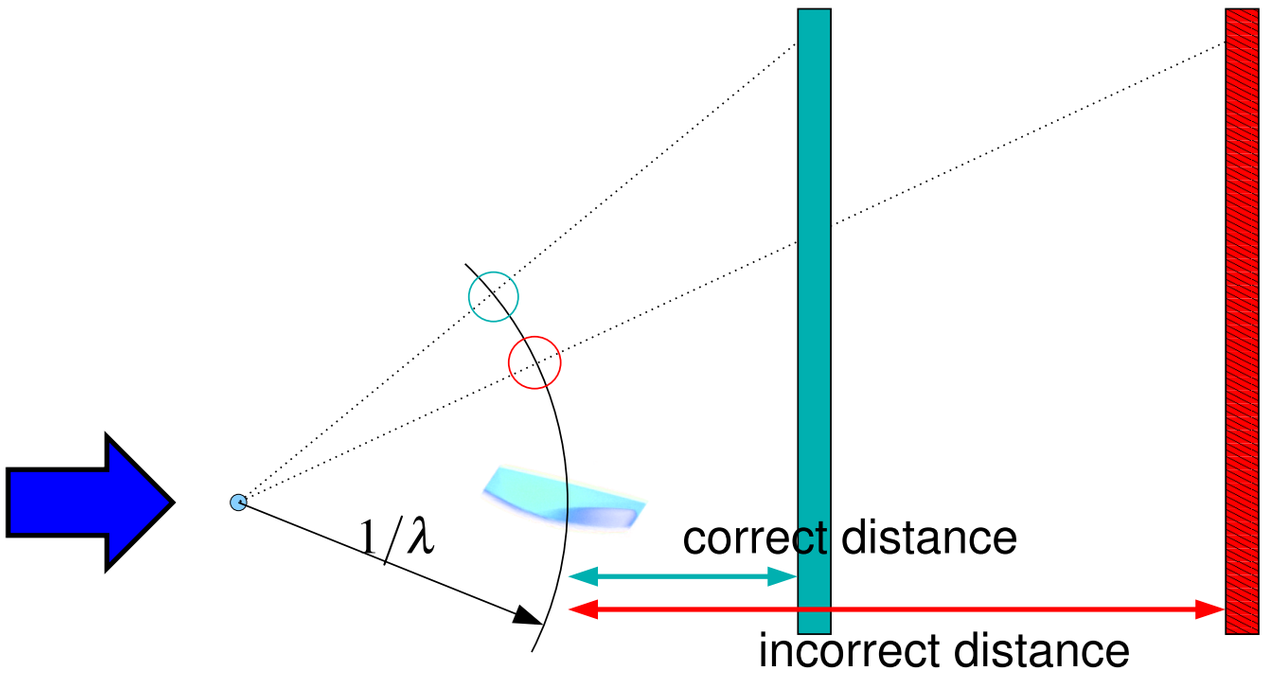
- Phases from Electron imaging **low resolution** but **accurate**
- Phases can be extended to high resolution refle

10 Experimental Considerations

- Ewald “plane”
- dynamic scattering
- Instrumental limitations

10.1 X–rays: The Ewald Sphere

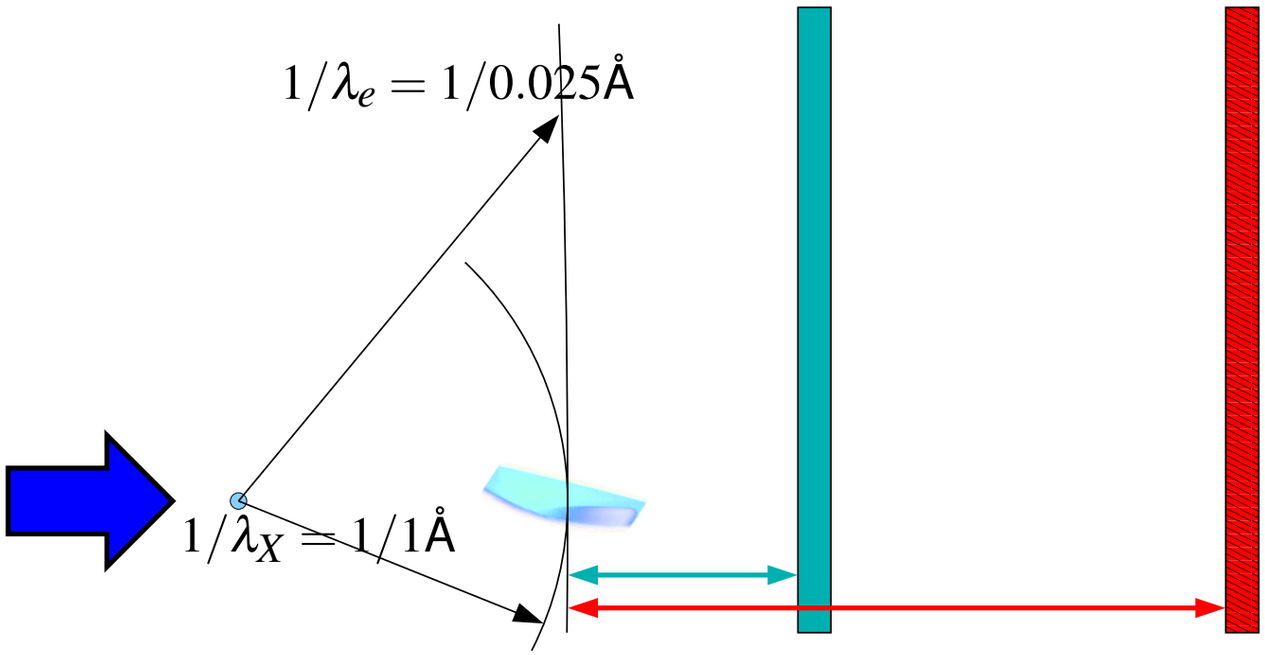
$\lambda = 1$Å, “normal” resolution: $2\theta_{\max} = 40°$



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

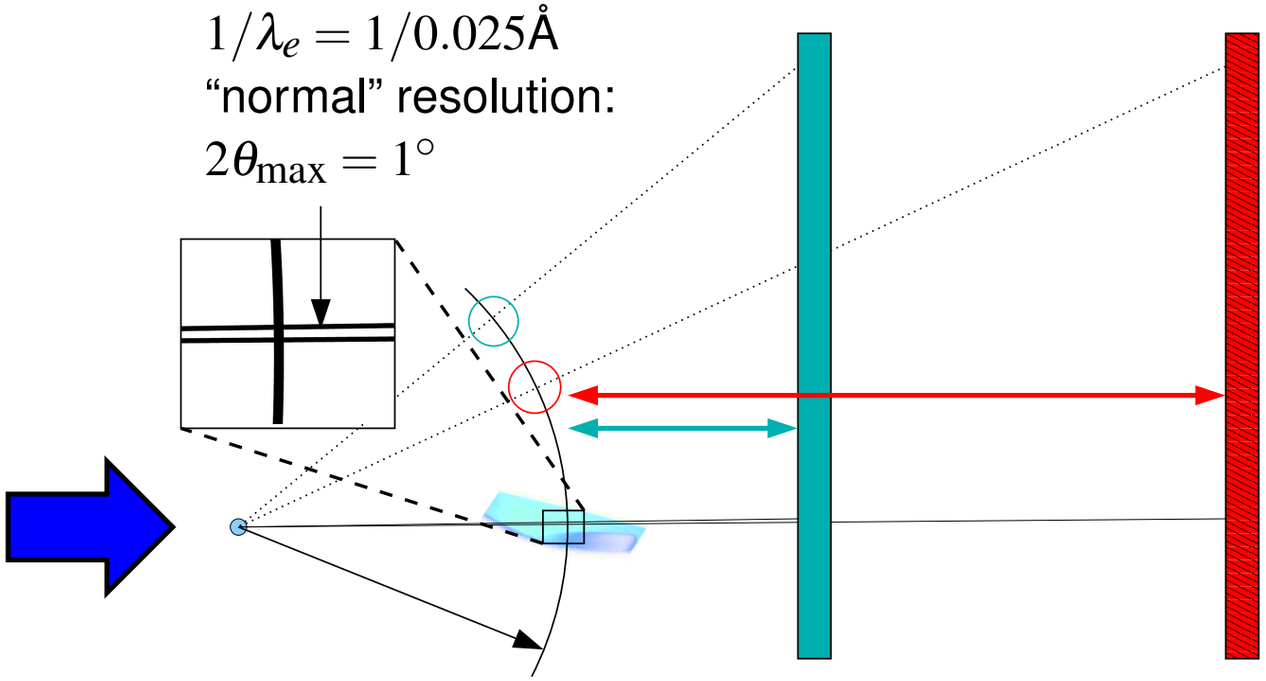
Curvature of the Ewald sphere **gauges** the diffraction geometry

10.2 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

10.3 Electrons: The Ewald “Plane”



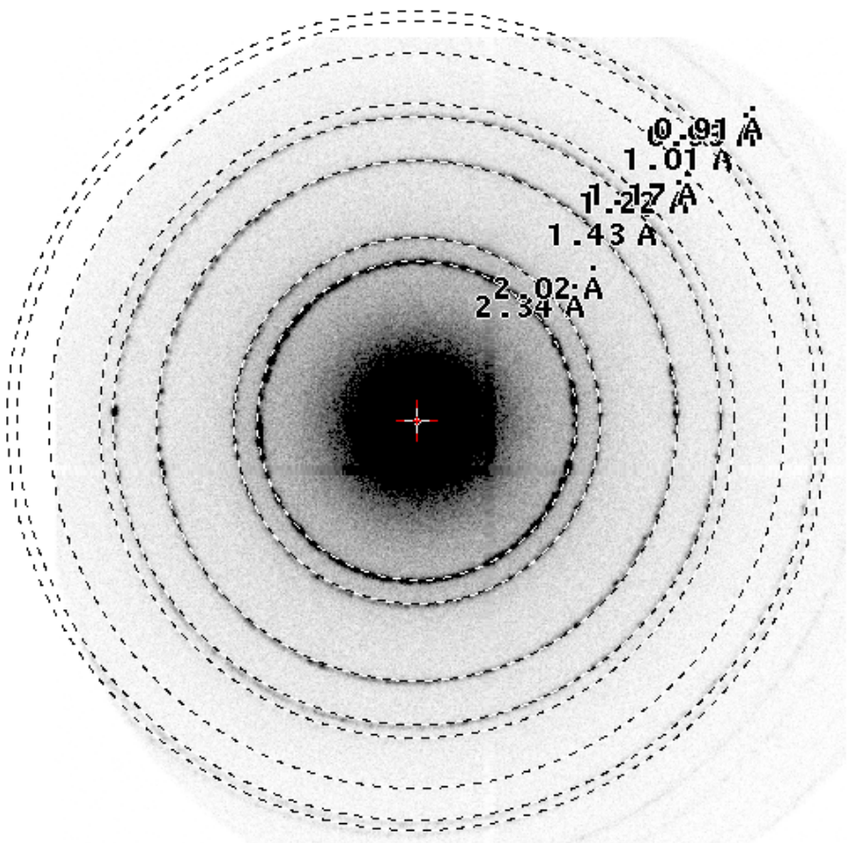
- opening angle of highest resolution reflections $\approx 1°$
- Ewald sphere virtually flat
- Without curvature: impossible to refine both detector distance and cell

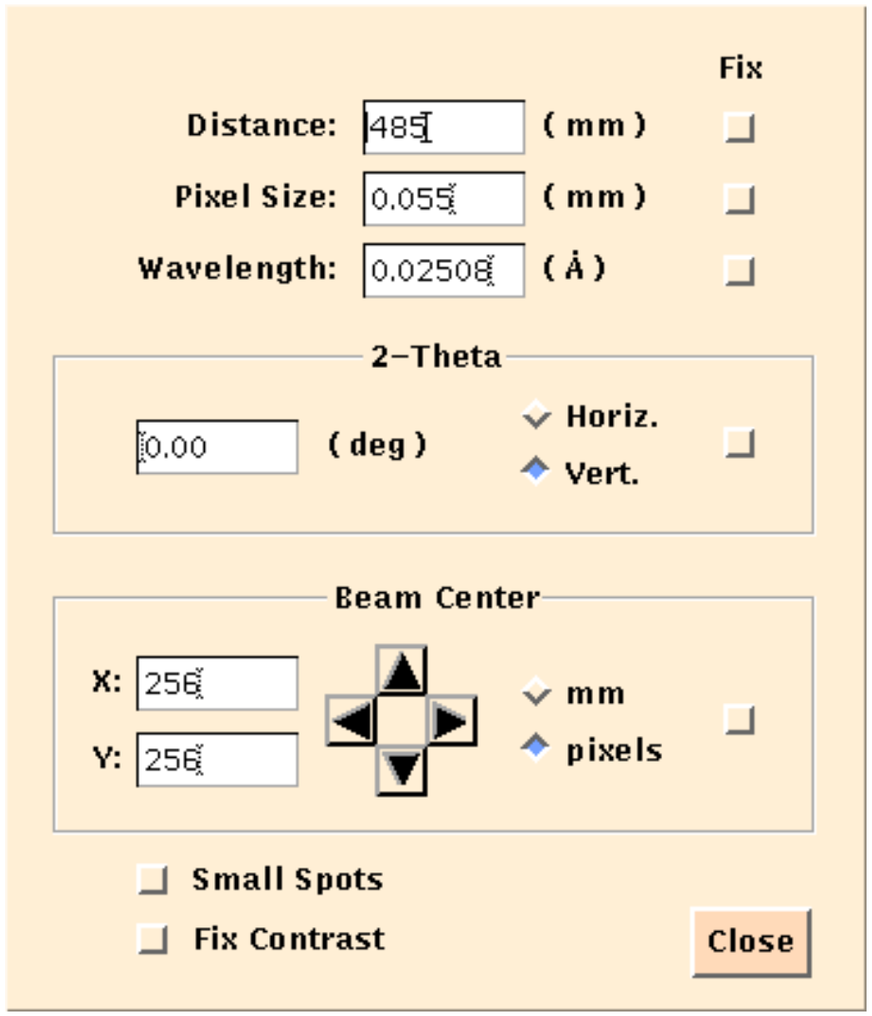
10.4 Electrons: The Ewald “Plane”

- 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
- Distance calibration from powder sample

10.5 Distance Calibration

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

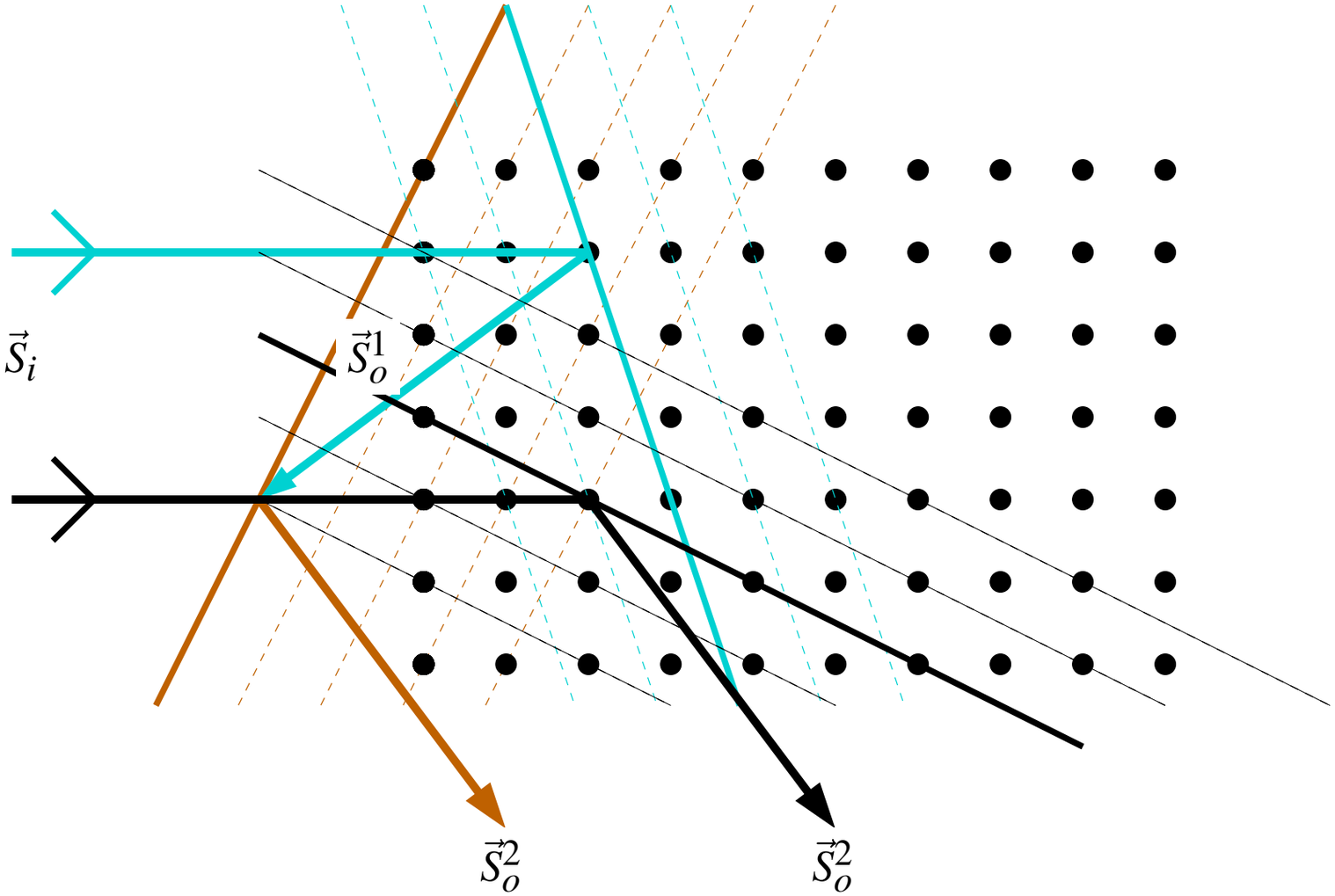




10.6 Dynamic Scattering

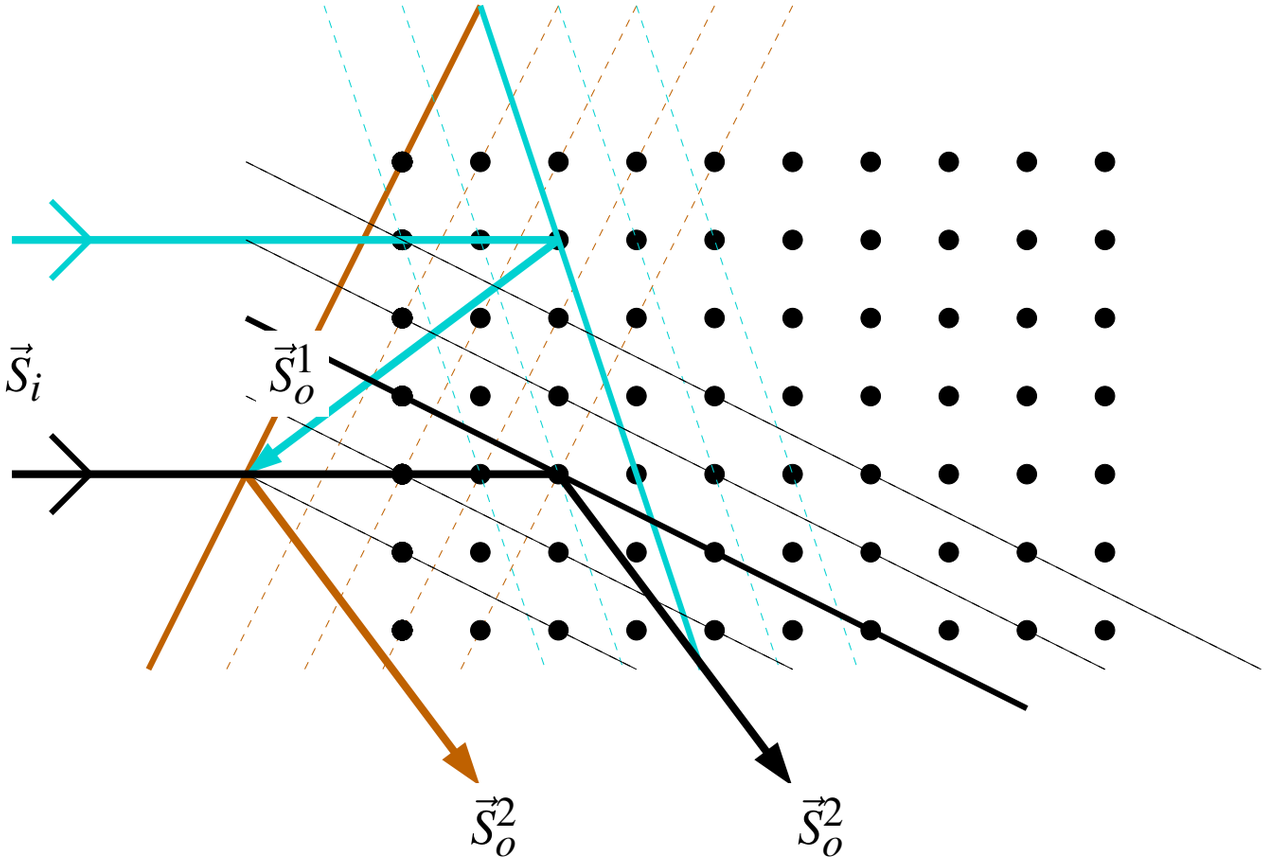
- Kinematic Theory of Diffraction: Every photon / electron / neutron scatters once in the crystal
- $|F_{\text{ideal}}(hkl)| \propto \sqrt{I_{\text{exp}}(hkl)}$
- Dynamic Scattering: Multiple Scattering events occur
- Electron Diffraction: Multiple Scattering occurs even with nanocrystals

10.7 Dynamic 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

10.8 Dynamic Scattering



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

$$\begin{aligned}(\vec{S}_o^1 - \vec{S}_i)\cdot\vec{a} &= h_1\\ (\vec{S}_o^2 - \vec{S}_o^1)\cdot\vec{a} &= h_2\\ \hline (\vec{S}_o^2 - \vec{S}_i)\cdot\vec{a} &= h_1 + h_2\end{aligned}$$

Experimental Intensities by superposition of two reflections:

$$I_{\text{exp}}(h_2k_2l_2) = |F_{\text{ideal}}(h_2k_2l_2) + \alpha F_{\text{ideal}}(h_1k_1l_1)|$$

- $\alpha < 1$: 0.1 for 50 nm path length
- $(h_1k_1l_1)$ strong and $(h_2k_2l_2)$ weak $\Rightarrow$ wrong estimate for $|F_{\text{ideal}}(h_2k_2l_2)|$
- affects high resolution data

10.9 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

10.10 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

10.11 SwissFEDI

Swiss Free Electron Diffraction Instrument

- Horizontal beam
- 15–18m instrument length
  1. Reduced Cross–talk between magnetic lenses
  2. No optical enlargement of detector distance: $1 - -2^\circ$ opening angle covers $20 \times 20\text{cm}^2$ detector area
- Sample holder designed for sample rotation

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