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:
  \[
  (\vec{S}_o - \vec{S}_i) \cdot \vec{a} = h \\
  \text{and } (\vec{S}_o - \vec{S}_i) \cdot \vec{b} = k \\
  \text{and } (\vec{S}_o - \vec{S}_i) \cdot \vec{c} = l
  \]

- Monochromatic 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 $I_{\text{exp}}(hkl)$

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<th>Step</th>
<th>Data Integration</th>
<th>Data Scaling</th>
<th>Refinement</th>
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<tbody>
<tr>
<td>Concept</td>
<td>Frames $\rightarrow I_{\text{exp}}(hkl)$</td>
<td>$I_{\text{exp}}(hkl) \rightarrow I_{\text{ideal}}(hkl)$</td>
<td>Match atom coordinates to $I_{\text{ideal}}(hkl)$</td>
</tr>
<tr>
<td>Requirement</td>
<td>Signal vs. background</td>
<td>Error Model</td>
<td>$(x, y, z) \leftrightarrow \rho(x, y, z) \leftrightarrow F(hkl)$</td>
</tr>
</tbody>
</table>
Data Processing and Scaling

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

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

For X-rays*:

$$I_{\text{exp}}(hkl) = \frac{e^4}{m e^2 c^4} \frac{\lambda^3 V_{\text{crystal}}}{V^2_{\text{unit cell}}} I_0 L P T E I_{\text{ideal}}(hkl)$$

Differences between Types of Radiation

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

Two theories for structure factor calculation from atom coordinates:

<table>
<thead>
<tr>
<th>kinematic scattering</th>
<th>dynamic scattering</th>
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</thead>
<tbody>
<tr>
<td>only one scattering event</td>
<td>multiple scattering events</td>
</tr>
<tr>
<td>valid for X–rays, neutrons</td>
<td>valid for electrons</td>
</tr>
<tr>
<td>$</td>
<td>F_{\text{ideal}}(hkl)</td>
</tr>
<tr>
<td>calculation via form factors</td>
<td></td>
</tr>
<tr>
<td>$F(hkl) = \sum_{\text{atoms}} f_j(\theta)e^{-2\pi ihx+ky+lz}$</td>
<td></td>
</tr>
</tbody>
</table>
Types of Radiation — X–rays

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

2. typical wavelength: $\lambda = 0.8–1.9\text{Å}$

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)
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

Types of Radiation — electrons

1. strong interaction compared with X–rays: good for very small crystals (≪ 1µm thickness)

2. typical wavelength: $200keV = 0.0251\text{Å}$

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

4. new phasing possibilities

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

Diffraction of nanocrystals (van Genderen *et al.*, Acta Cryst A72 (2016))
3 - Electron Diffraction
The (seemingly) Empty Drop

Drops viewed through TEM
Nanocrystals

Novartis I:
\[ \varnothing = 1,700\text{nm} = 1.7\mu\text{m} \]

Novartis II:
\[ \varnothing = 500\text{nm} = 0.5\mu\text{m} \]

Thermolysin:
\[ \approx 2 \times 1 \times \text{very thin } \mu\text{m}^3 \]
Solvent reduces contrast
How small is “nano”?

Typical protein crystal size for X-rays: 0.2 mm

Typical protein crystal size for electrons: 100x140x1,700 nm³

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

Detector noise and radiation sensitivity require low contrast images

Electron Microscope: Diffraction Mode

Plane Wave  Object  Lense
Electron Microscope: Diffraction Mode

Image at Backfocal Plane = \| Fourier transform of object \|

If object = crystal:

diffraction spots according to Laue condition:

Backfocal Plane

Rays of *equal direction* focus on detector
Types of Distortions

- Pincushion
- Barrel
- Spiral
- Elliptical

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

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

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

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.

<table>
<thead>
<tr>
<th></th>
<th>Uncorrected</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X–Shifts Δx</strong></td>
<td><img src="image1" alt="Uncorrected X-shifts" /></td>
<td><img src="image2" alt="Corrected X-shifts" /></td>
</tr>
<tr>
<td><strong>Y–Shifts Δy</strong></td>
<td><img src="image3" alt="Uncorrected Y-shifts" /></td>
<td><img src="image4" alt="Corrected Y-shifts" /></td>
</tr>
<tr>
<td>P1 cell</td>
<td>12.0811 11.9496 11.8249 89.986 90.481 89.780</td>
<td>12.0665 12.1757 12.0574 90.048 90.026 90.065</td>
</tr>
</tbody>
</table>
5 - Experimental Considerations

- Ewald sphere or “plane”

- dynamic scattering

- Instrumental limitations
X-rays: The Ewald Sphere

\[ \lambda = 1 \text{Å}, \text{“normal” resolution: } 2\theta_{\text{max}} = 40^\circ \]

- 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 $\lambda_X = 1\text{Å}$
- Typical $e^-$ wavelength $\lambda_e = 0.025\text{Å}$
- 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 ≈ 1°
- Ewald sphere virtually flat
- Without curvature: impossible to refine both detector distance and cell

\[
\frac{1}{\lambda_e} = \frac{1}{0.025\text{Å}}
\]

“normal” resolution: 
\[2\theta_{\text{max}} = 1°\]
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: $\lambda = 2d \sin \theta$; $d, \lambda$ are known
Elliptical Distortion introduces Errors

- Bragg’s law: \( \lambda = 2d \sin \theta \); \( d, \lambda \) are known

Image courtesy M. Clabbers
Dynamic Scattering

- Kinematic Theory of Diffraction: Every photon / electron / neutron scatters once in the crystal

\[ |F_{\text{ideal}}(hkl)| \propto \sqrt{I_{\exp}(hkl)} \]

- Dynamic Scattering: Multiple Scattering events occur

- Electron Diffraction: Multiple Scattering occurs even with nanocrystals
Dynamic Scattering
### Multiple (Dual) Scattering

- Outgoing ray $\vec{S}_1^1$ acts as incoming ray for reflection $\vec{S}_0^2$.
- 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 $I(h_2, k_2, l_2)$

- $I(h_1k_1l_1)$ must be strong
- $I(h',k',l')$ must be strong
- $I(h_2k_2l_2)$ must be weak
- $I(h_1k_1l_1)$ and $I(h_2k_2l_2)$ 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 designed for $180^\circ$ rotation
6 - Example Structures
Pharmaceutical I: Visualisation of Hydrogen Atoms

H–atom positions can be refined against electron diffraction data

- Field of view: 3µm
- Crystal: 1.6µm × 400nm
- \( d_{\text{min}} < 0.8\text{Å} \)
- \( I/\sigma_I(0.91 - 0.81\text{Å}) : 1.8 \)
- \( P2_12_12_1 \): 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: 3µm
- Crystal: 400nm diameter
- $d_{\text{min}} = 0.80\text{Å}$
- $I/\sigma_I(0.90 - 0.80\text{Å}) : 2.5$
- $P2_12_12_1$: 92% completeness with 6 crystals ($d_{\text{min}} > 0.84\text{Å}$: 96%)
- Direct methods: only 1 wrong atom type
- Visualisation of hydrogen atoms
- 1806 refl., 258 param., 267 restraints
- $R_1 = 18.5\%, R_{\text{complete}} = 21.9\%$
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

### Data integration

<table>
<thead>
<tr>
<th></th>
<th>Single crystal</th>
<th>Merged data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>$P2_12_12$</td>
<td></td>
</tr>
<tr>
<td><strong>Unit cell dimensions</strong></td>
<td>a, b, c (Å)</td>
<td>104.56, 68.05, 32.05</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>32.05-2.50</td>
<td>57.04-2.50</td>
</tr>
<tr>
<td><strong>$R_{	ext{merge}}$ (%)</strong></td>
<td>31.7 (107.3)</td>
<td>35.7 (113.2)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>41.0 (40.5)</td>
<td>69.0 (51.3)</td>
</tr>
<tr>
<td><strong>Unique reflections</strong></td>
<td>9518 (817)</td>
<td>25148 (1373)</td>
</tr>
<tr>
<td><strong>Redundancy</strong></td>
<td>2.76 (3.46)</td>
<td>4.33 (4.59)</td>
</tr>
</tbody>
</table>

### Refinement

- **$R_1$ (%)**: 25.90, 23.54
- **$R_{	ext{merge}}$ (%)**: 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 Crystallography 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

<table>
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<th>Instrumentation</th>
<th>Proessing</th>
<th>Analysis</th>
</tr>
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<tr>
<td>+ from Powder</td>
<td>++ Detector*</td>
<td>+ Integration</td>
<td>++ Direct Methods</td>
</tr>
<tr>
<td>- from Solution</td>
<td>- Rot^n Axis*</td>
<td>- Param. Stability</td>
<td>+ Molec. Repl.</td>
</tr>
<tr>
<td>- Data sets / day</td>
<td>- Lenses</td>
<td>+/- Scaling</td>
<td>+ Refinement</td>
</tr>
<tr>
<td></td>
<td>- Crystal Orient^n*</td>
<td></td>
<td>- Potential Repr.</td>
</tr>
</tbody>
</table>

* Current project at LBR / PSI
9 - Acknowledgements

- Prof. J. P. Abrahams, Dr. E. van Genderen, M. Clabbers, Dr. T. Blum, C. Borsa, J. Heidler (group members at PSI / C-CINA, Basel)

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- Dr. W. Kabsch (XDS)

- Dr. D. Waterman (DIALS)

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