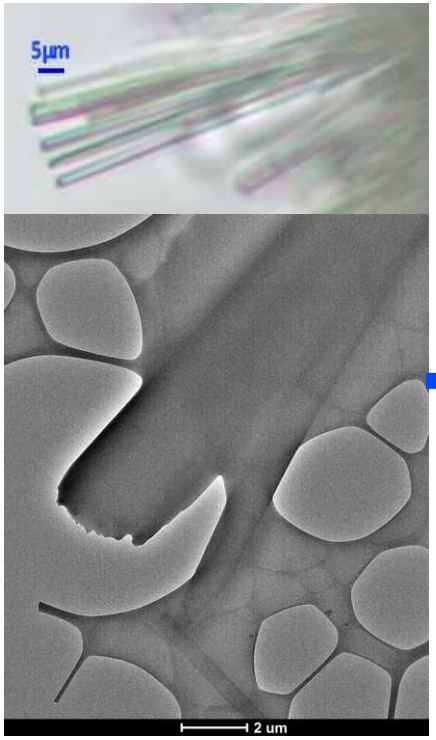
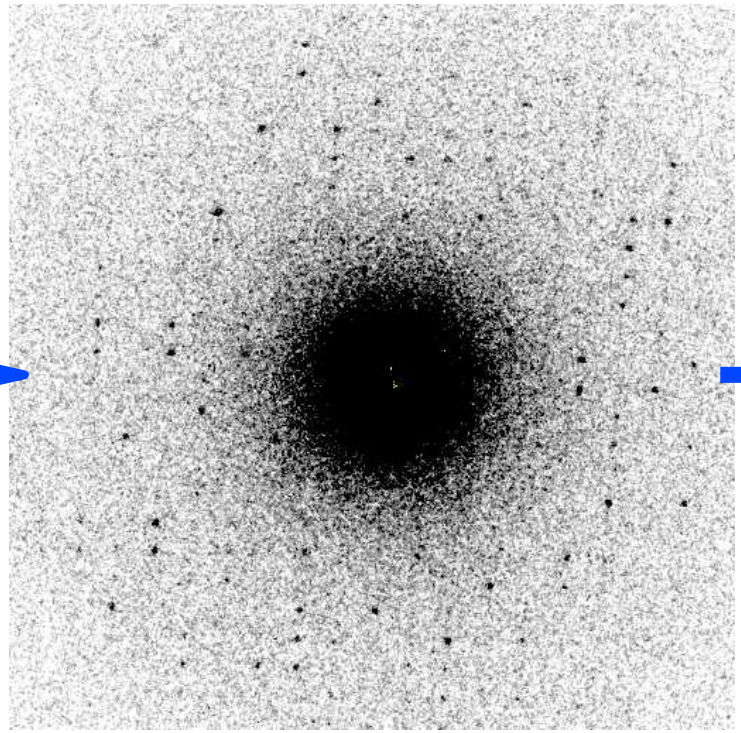
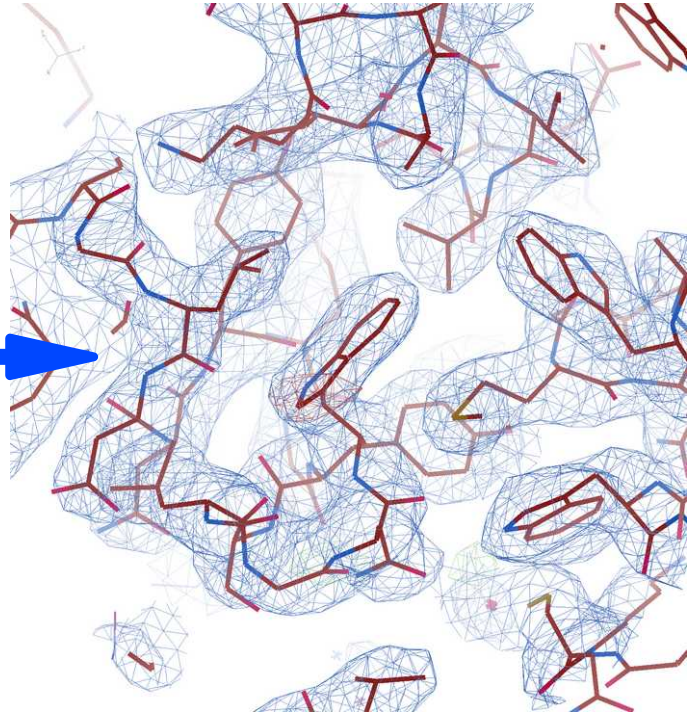
Electron diffraction of protein nano-crystals: a new emerging technique in structural biology



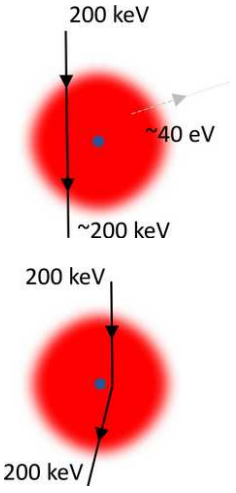


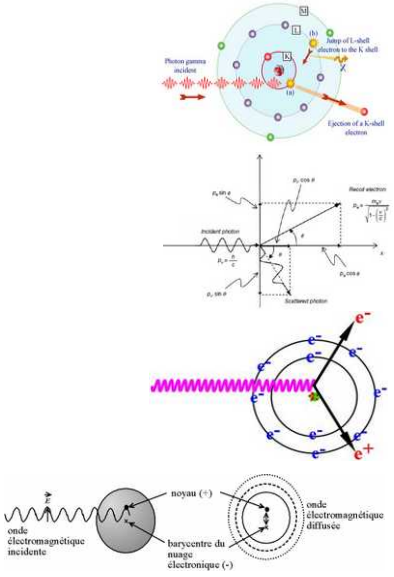


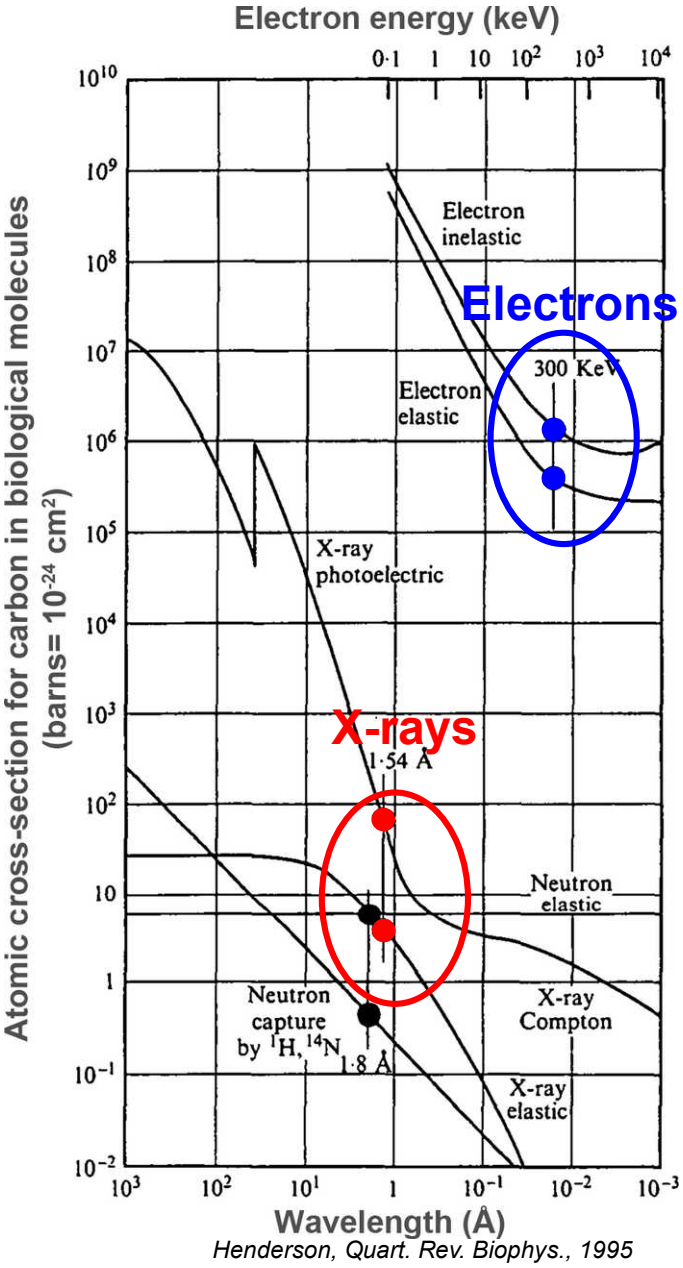
Why using electrons for diffraction experiments ?

- Strongly interact with matter
  - About 10^4 times more than X-rays
  - Elastic scattering represents 25% of scattered electrons (5 % for X-rays)

- Electrons deposit less energy per diffracting particle
  - Electron : 60-120 eV
  - X-ray : 80-240 keV







Henderson, Quart. Rev. Biophys., 1995

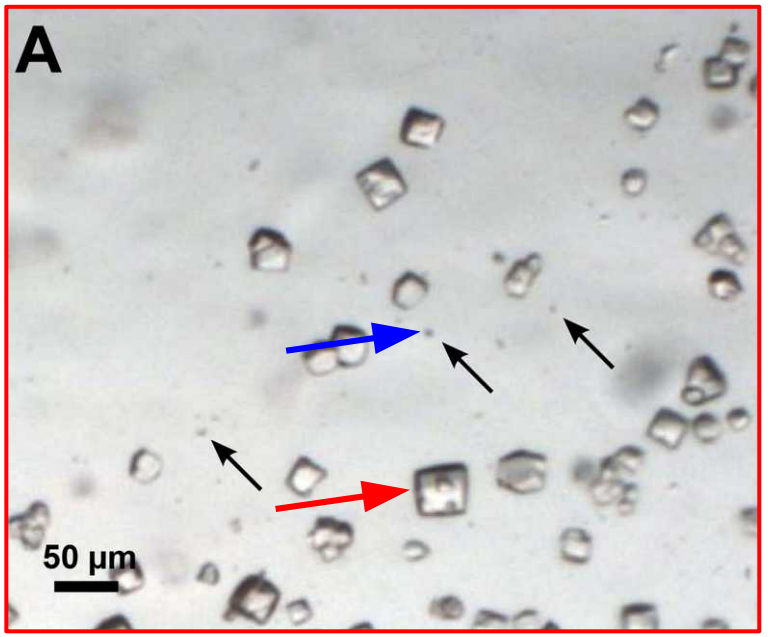
- => Damage per diffracting particle are significantly lower with electrons

X-rays versus electrons

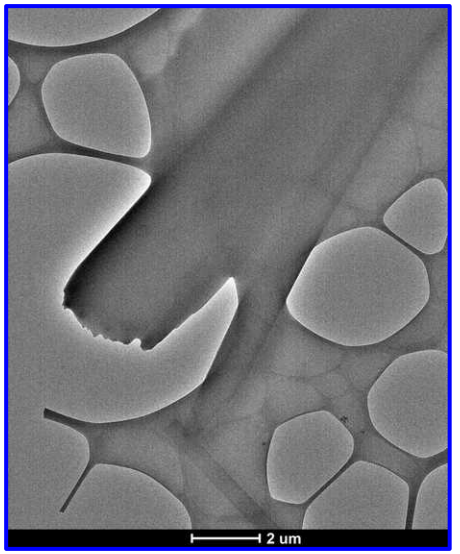
- How many molecules in a crystal?
  - Typical lysozyme (129 aa, 14 kDa) crystal
    - Unit cell a=b=77 Å c=39 Å, 8 molecules per unit cell
  - in X-ray crystallography:
    - Crystal size 50 µm x 50 µm 50 µm
    - 4.3 10^{12} molecules
  - in electron crystallography
    - Crystal size 200 nm x 200 nm 200 nm
    - 2.8 10^{5} molecules
  - Difference : 7 orders of magnitude
- If large crystals are available
  - Lower energy per molecules deposited by X-rays
  - X-ray crystallography will be more efficient

$$I(\vec{s}) \propto N_{cell}^2$$

- If only sub-micrometer crystals are available
  - Electron crystallography should be more efficient



Shi et al., eLife, 2013



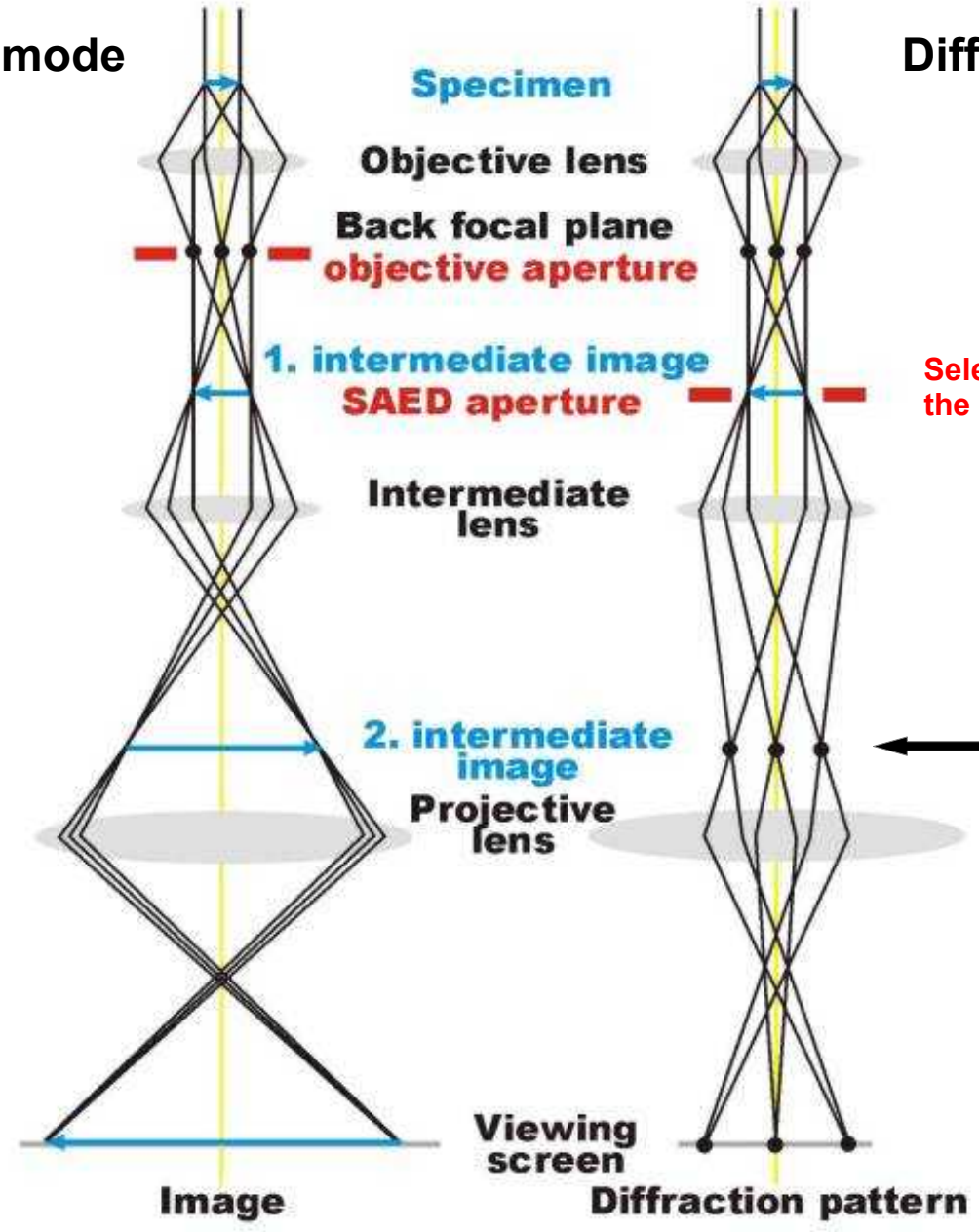
W. L. Ling, M. Bacia, D. Housset, IBS, 2017

Drawback of a stronger interaction?

- Problem of multiple scattering with electrons
  - When an electron interact with more than one atom before leaving the sample
    - The second, third, … interaction can be
      - Inelastic
      - Elastic
  - Increase with sample thickness
    - However not critical for biological samples, if crystals are thin enough
  - Difficult to take into account in data processing
    - Introduce errors in diffracted intensities if not taken into account

- Optimal thickness for maximal Bragg intensities and minimal multiple scattering

Diffraction with an electron microscope



Selection of the area contributing to the diffraction pattern

Back focal plane of intermediate lens

Camera length (equivalent of crystal detector distance in X-ray crystallography) may be changed by tuning intermediate or projective lenses)

Illustrations taken from Irina Gutsche's presentation

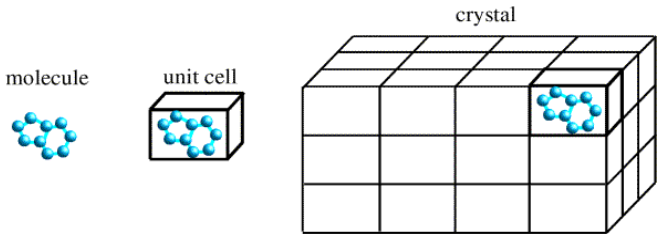
Comparison with X-ray beam lines

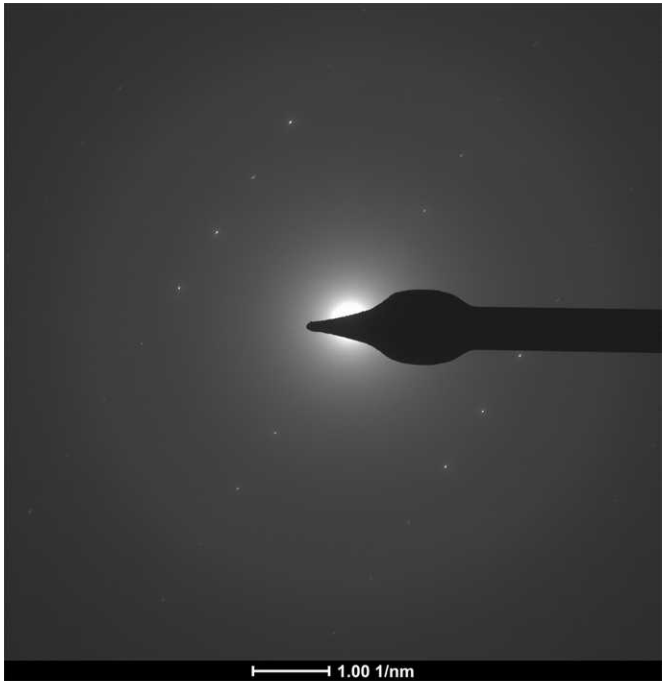
- The detector does not move
- Camera length (equivalent of crystal-detector distance) can be adjusted by playing with intermediate or projective lenses
- The wavelength is much smaller than with X-rays
  - λ = 0.02508 Å for 200 keV, 0.01969 Å for 300 keV
- Small angle diffraction
  - 2θ = 0.72° for 2 Å resolution for 200 keV electrons (29° for 2 Å resolution with 1 Å X-rays)
- Lenses may introduce distortion
- Sample holder limits rotation of the crystal to ~ 80°

Diffraction and crystals

- Diffraction experiments can be made
  - On a single particle
  - On a crystal
- In both cases, structural information can be obtained
- If the sample is a crystal, diffracted beams are concentrated in discrete directions defined by Laue equations:

$$\vec{a} \cdot \vec{s} = h \, , \vec{b} \cdot \vec{s} = k \, , \vec{c} \cdot \vec{s} = l$$





- Here, we will focus on diffraction of electrons by crystalline samples

Why to use diffraction instead of imaging?

- Having a crystal drastically amplifies the signal
  - $I \propto N^2$, with N the number of molecules in your crystal
  - $I \propto N$ for imaging
- Diffraction signal is not "blurred" by translation movement of the sample
  - Translating the sample only changes the phases of diffracted beams, not the intensities

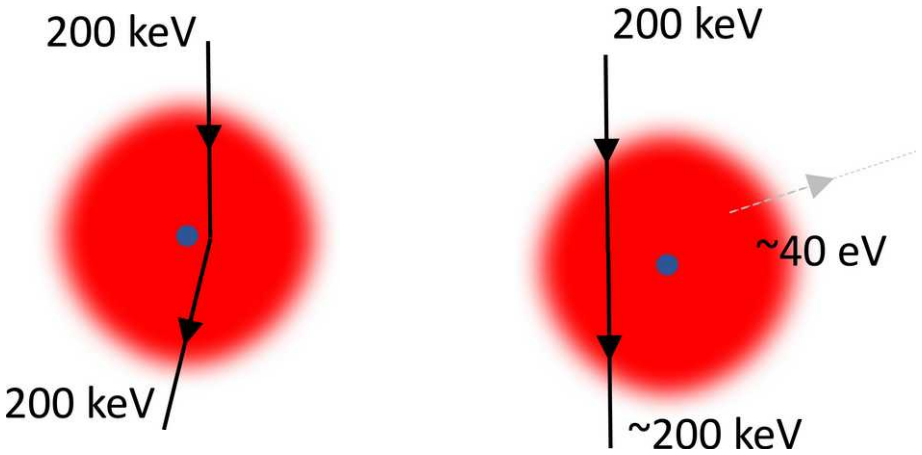
$$I(\vec{s}) \propto \left| \sum_J f_J . \exp[2i\pi \vec{s} . (\vec{r}_J + \vec{t})] \right|^2 = \left| \sum_J f_J . \exp[2i\pi \vec{s} . \vec{r}_J] \right|^2 . \underbrace{\left| \exp[2i\pi \vec{s} . \vec{t}] \right|^2}_{=1}$$

  - Small movements of the sample do not affect resolution of the data

- About 2 Å resolution data can be recorded on a protein crystal with an F20 200 keV electron microscope
  - In imaging mode, resolution is limited to about 10-15 Å
- => If you have 100 000 molecules, better form a crystal with them!

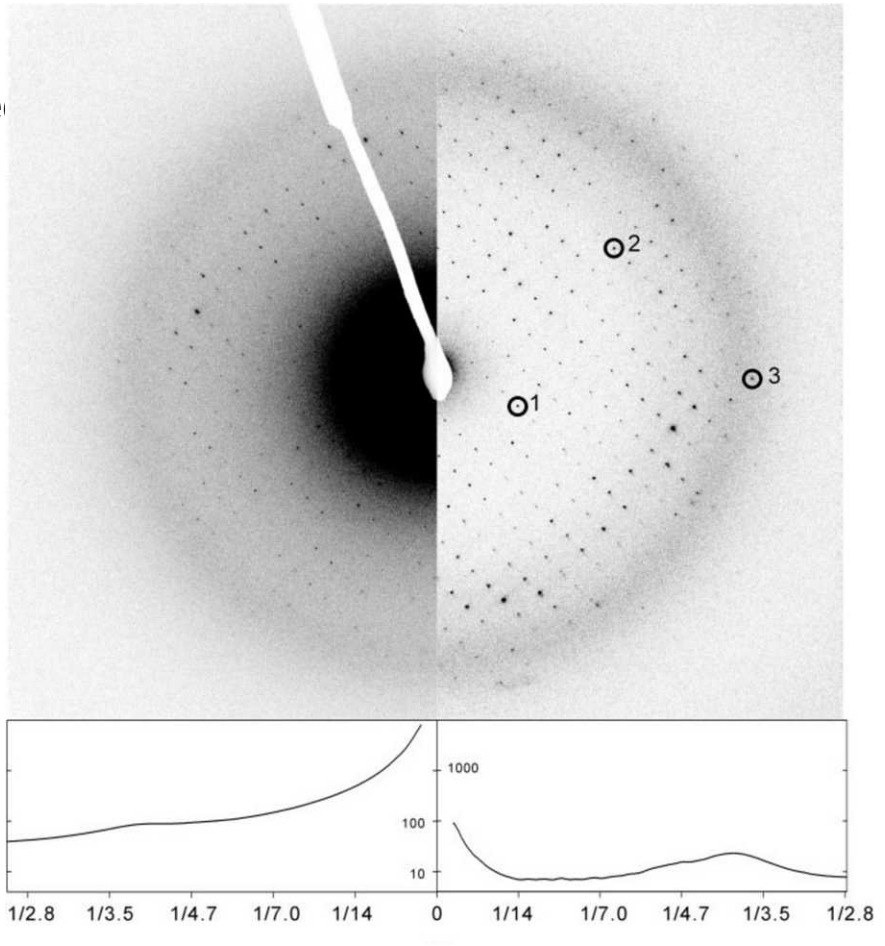
Energy filtering improve signal to noise ratio

- Energy filtering remove inelastically scattered electrons
  - Electrons with energy loss > 10 eV are remove
  - No or small deviation of inelastic electrons
    - Essentially present in the central region of the diffraction pattern



25 %

75 %



Diffraction pattern of F41 flagellin crystal (41 kDa) collected without (left) and with (right) energy filter. Unit cell dimensions : a=52 Å, b=37Å, c=119 Å, β=90.8°

Yonekura et al., Biophys. J., 2002

Pioneering studies in the 70'

- First electron diffraction patterns of protein crystals (catalase) in the 70'

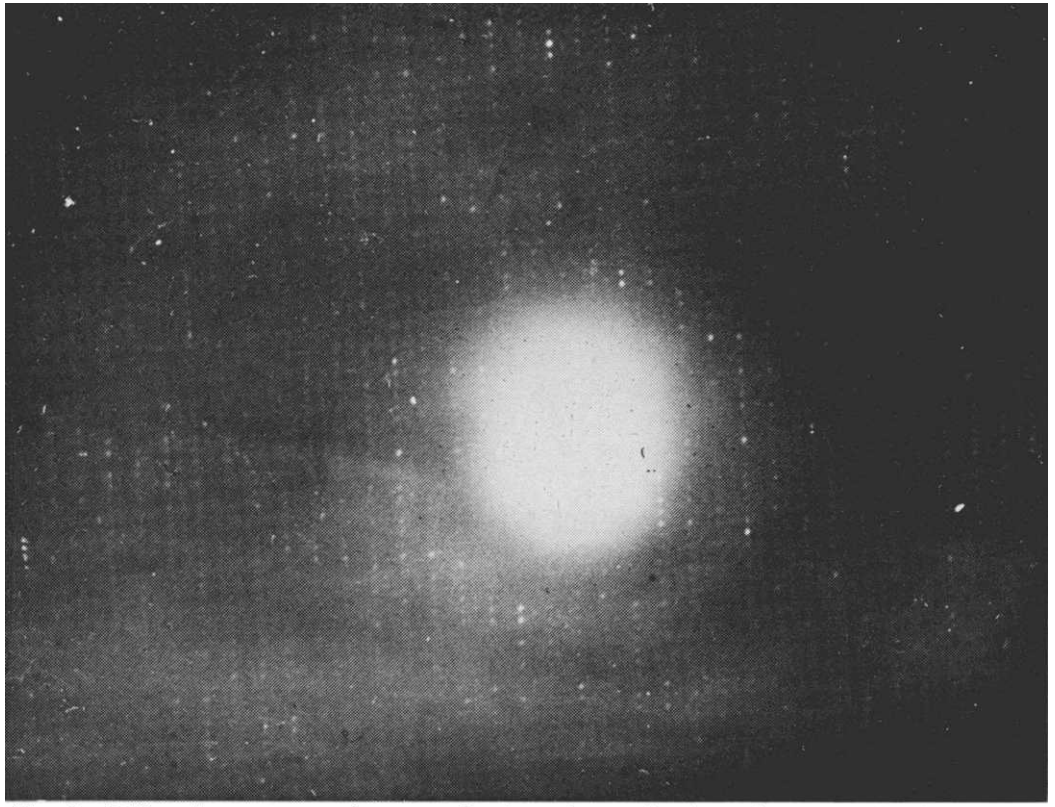


Fig. 2. Diffraction pattern of wet, unfixed, and unstained catalase crystal taken in the hydration stage (26 torr, 200 kev) showing the *a* and *c* axes. The photograph has been dodged to show the higher orders.

The crystal is put in a hydratation chamber at room temperature. About 3500 reflections up to 2 Å. Unit cell : a=73 Å, c=184Å. Maximum e dose : ~ 0.6 e/Å^2 (10^{-3} C/cm^2)

Matricardi, Moretz & Parsons, Science, 1972

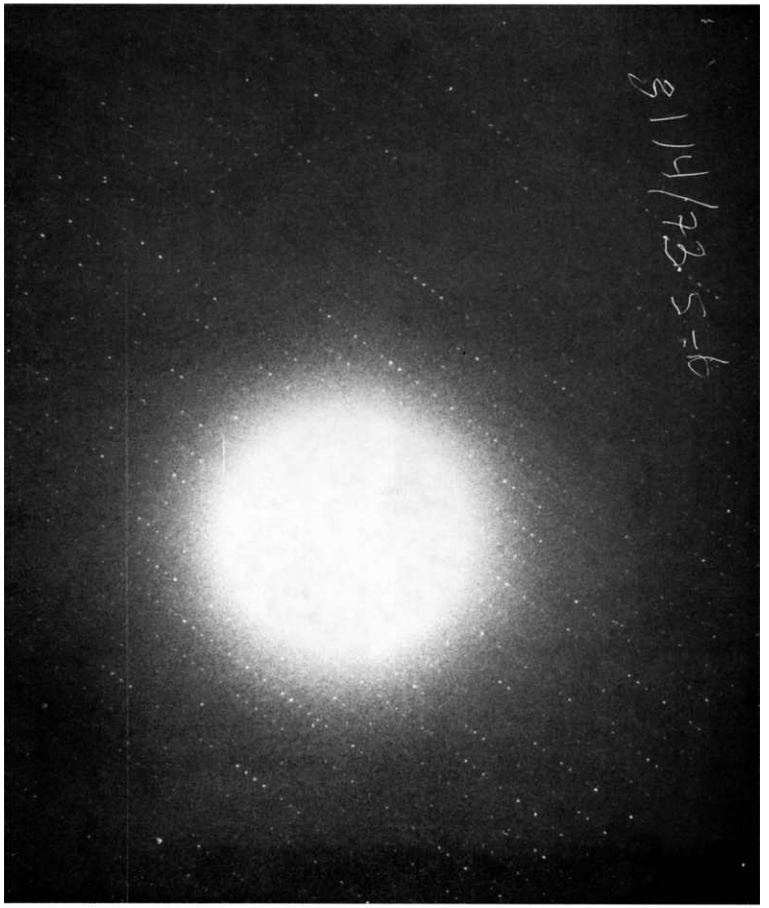


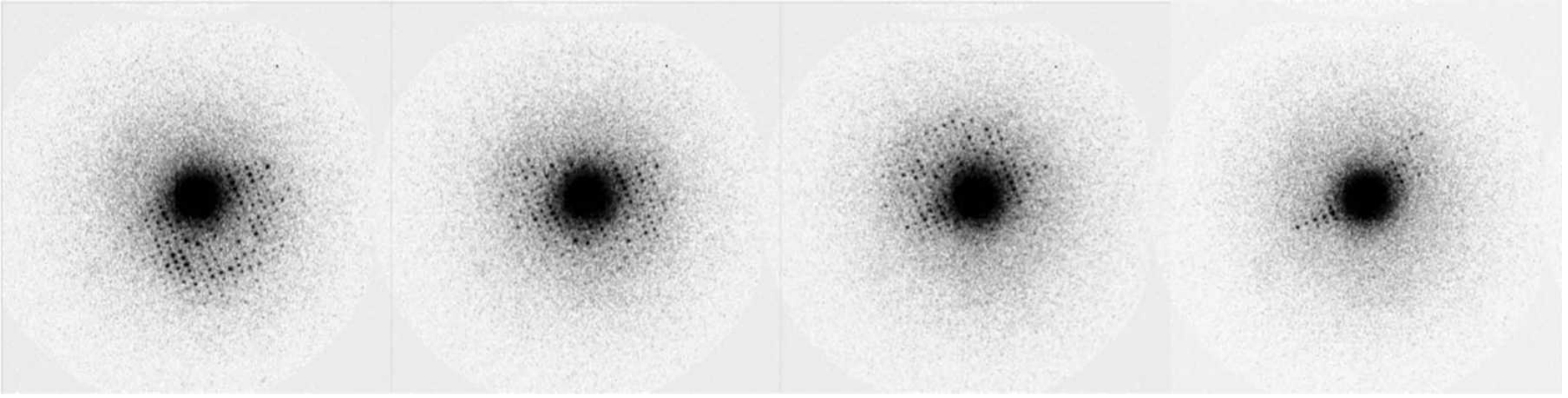
Fig. 2. Electron diffraction pattern (*h*0*l* net) from an untilted catalase crystal.

Same conditions. 3.2 Å data. Crystal size of 10 x 20 x 0.05 µm^3. Unit cell : a=70 Å, c=177 Å

Dorset & Parsons, Acta cryst A, 1975

First high resolution data set from 3D protein crystals by ED in 2013

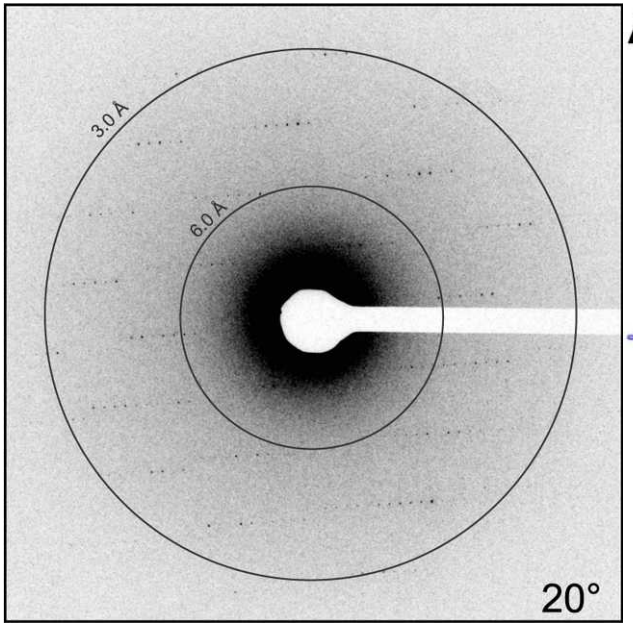
- Lysozyme at 1.8 Å
  - 200 kV electron microscope
  - Medipix hybrid detector
  - Oscillation of 0.05° / image and exposure time of 1s / image
  - Damage observed at ~ 3 e / $Å^2$
  - No structure solve as data were not complete

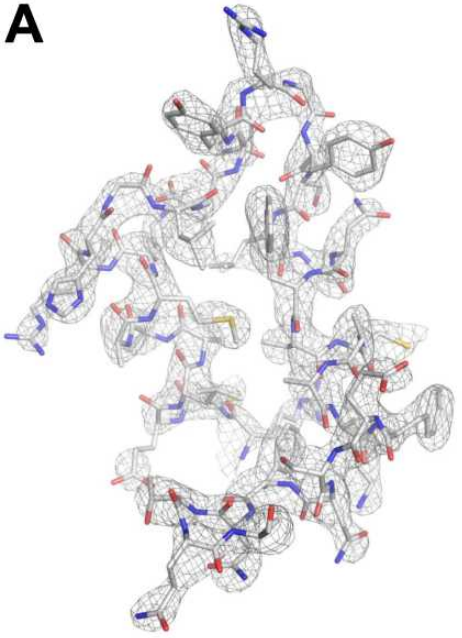


Nederlof *et al.*, Acta Crystallogr D, 2013

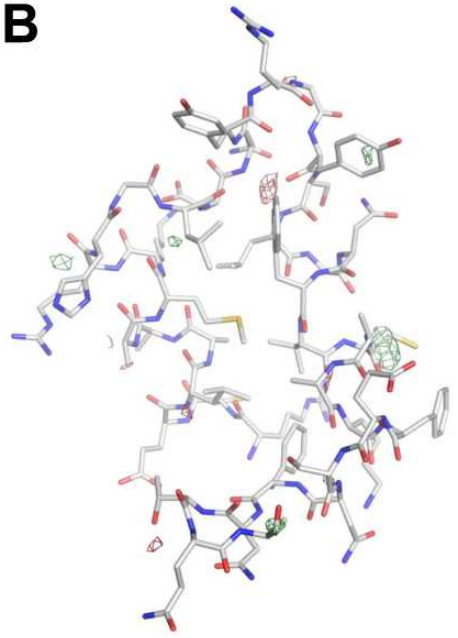
First protein structure by ED in 2013

- Lysozyme at 2.9 Å
  - 200 kV electron microscope
  - CMOS TVIPS detector
  - Based on still images but high reduncy (34)
  - Exposure time of 10 s, at 0.01 e / $Å^2$
  - Up to 10 e / $Å^2$ per crystal

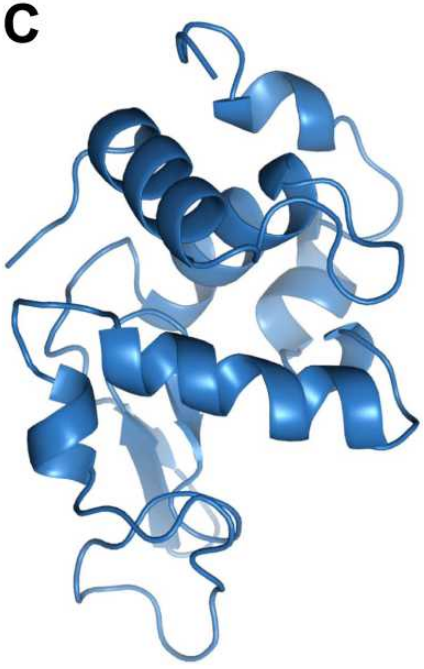




$2F_{obs} - F_{calc}$



$F_{obs} - F_{calc}$



Final Structure

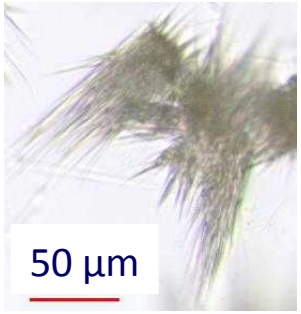
Shi et al., eLife, 2013

ED protein structures deposited so far

- PDB of protein structures in September 2020
  - 50 protein structures from esssentially 4 labs: T. Gonen, K. Yonekura, J. P. Abrahams & T. Grüne
  - Mainly « test » proteins
  - Only 1 new structure (the model shares ~ 35 % sequence identity)

Protein	Nb of aa	Resolution	PDB entry	year	Reference
Lysozyme	129	2.9	3J4G	2013	Shi et al.
Lysozyme	129	2.5	3J6K	2014	Nannenga et al.
Catalase	4x527	3.2	3J7B	2014	Nannenga et al.
Ca ATPase	994	3.4	3J7T	2015	Yonekura et al.
Lysozyme	129	2.5	5A3E	2014	Nannenga et al.
Catalase	4x527	3.2	5GKN	2017	Yonekura et al.
Proteinase K	279	1.75	5I9S	2016	Hatnee et al.
Lysozyme	129	1.8	5K7O	2017	De la Cruz et al.
Xylanase	190	2.3	5K7P	2017	De la Cruz et al.
Taumatin	207	2.5	5K7Q	2017	De la Cruz et al.
Trypsin	223	1.7	5K7R	2017	De la Cruz et al.
Proteinase K	279	1.6	5K7S	2017	De la Cruz et al.
Thermolysin	316	2.5	5K7T	2017	De la Cruz et al.
Lysozyme	129	2.11	5O4W	2017	Clabbers et al.
Lysozyme	129	2.11	5O4X	2017	Clabbers et al.
Lysozyme	129	2.2	5OCV	2017	Xu et al.
TGFβ-TGFβ-RII	200	2.9	5TY4	2017	De la Cruz et al.
Proteinase K	279	1.71	6CL7	2018	Hattne et al.
Proteinase K	279	2	6CL8	2018	Hattne et al.
Proteinase K	279	2.2	6CL9	2018	Hattne et al.
Proteinase K	279	2.8	6CLA	2018	Hattne et al.
Proteinase K	279	3.2	6CLB	2018	Hattne et al.
NA/K ion channel	2x91	2.5	6CPV	2018	Liu & Gonen
Lyzozyme	129	1.9	6H3B	2018	Duyvesteyn et al.
Lysozyme	129	2.8	6HU5	2019	Lanza et al.
Catalase	4X527	3	6JNT	2019	Yonekura et al.
Catalase	4X527	3	6JNU	2019	Yonekura et al.
HIV Gag fragment	110	3	6N3J	2018	Purdy et al.
HIV Gag fragment	110	2.9	6N3U	2018	Purdy et al.
Proteinase K	279	2.75	6N4U	2018	Martynowycz et al.
Proteinase K	279	2.17	6PKJ	2019	Martynowycz et al.
Proteinase K	279	2.18	6PKK	2019	Martynowycz et al.
Proteinase K	279	2.59	6PKL	2019	Martynowycz et al.
Proteinase K	279	2.17	6PKM	2019	Martynowycz et al.
Proteinase K	279	2.08	6PKN	2019	Martynowycz et al.
Proteinase K	279	2.07	6PKO	2019	Martynowycz et al.
Proteinase K	279	1.91	6PKP	2019	Martynowycz et al.
Proteinase K	279	1.85	6PKQ	2019	Martynowycz et al.
Proteinase K	279	1.79	6PKR	2019	Martynowycz et al.
Proteinase K	279	2.16	6PKS	2019	Martynowycz et al.
Proteinase K	279	1.85	6PKT	2019	Martynowycz et al.
Proteinase K	279	2.1	6PU4	2019	Hattne et al.
Proteinase K	279	2.7	6PU5	2019	Hattne et al.
R2-like ligand binding oxidase	328	3	6QRZ	2019	Xu et al.
Lysozyme	129	1.8	6S2N	2019	Büker et al.
Granulin	248	1.55	6S2O	2019	Büker et al.
CypA	165	2.5	6U5G	2019	Wolff et al.
Carbonic anhydrase II / azm	260	2.5	6YMA	2020	Clabbers et al.
Carbonic anhydrase II	260	2.5	6YMB	2020	Clabbers et al.
Granulin	248	2.83	6YNG	2020	Bunker

In practice : putting crystals on a Grid



Mechanical fracture

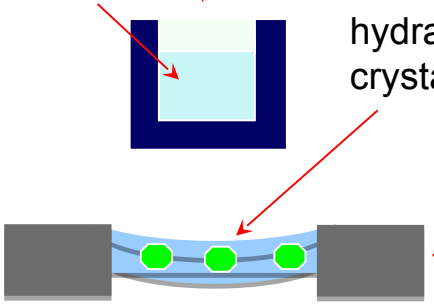
(vortexed with beads)

2- 3 µl of crystal solution deposited on a grid & bloting

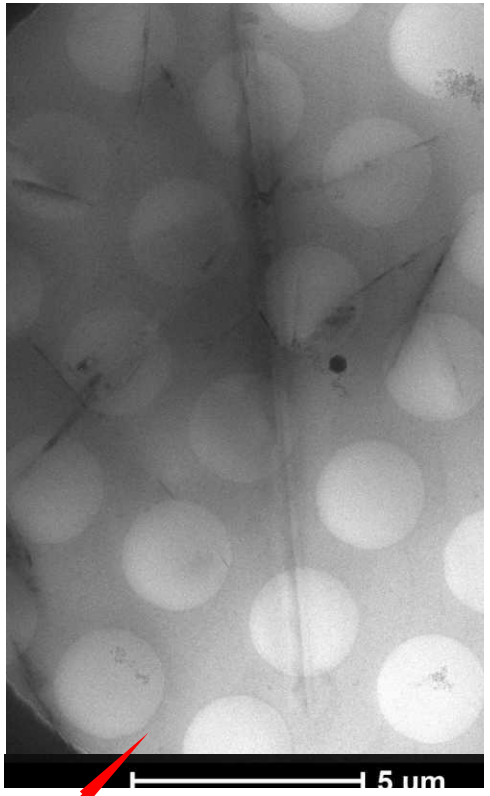
Vitrification

Liquid ethane

Frozen hydrated crystals



- Ideal crystal size is 100-200 nm



Adapted from Wai Li Ling

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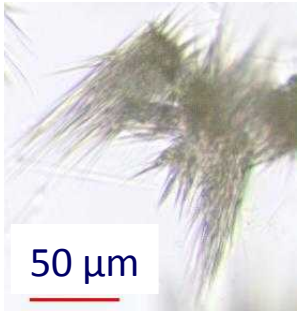

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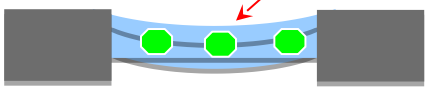

Adapted from Wai Li Ling

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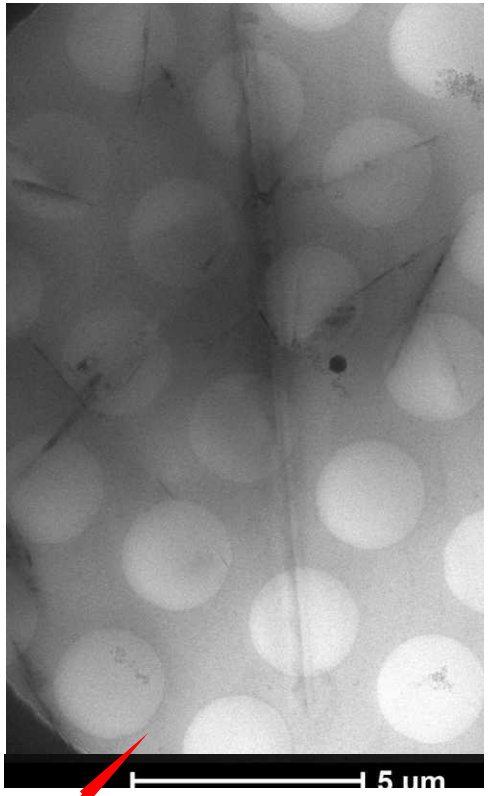
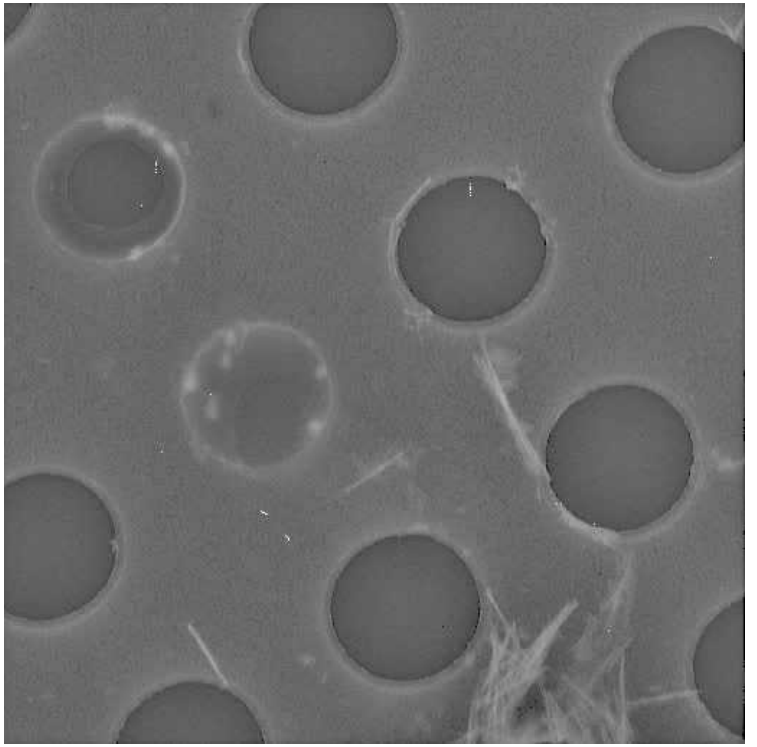


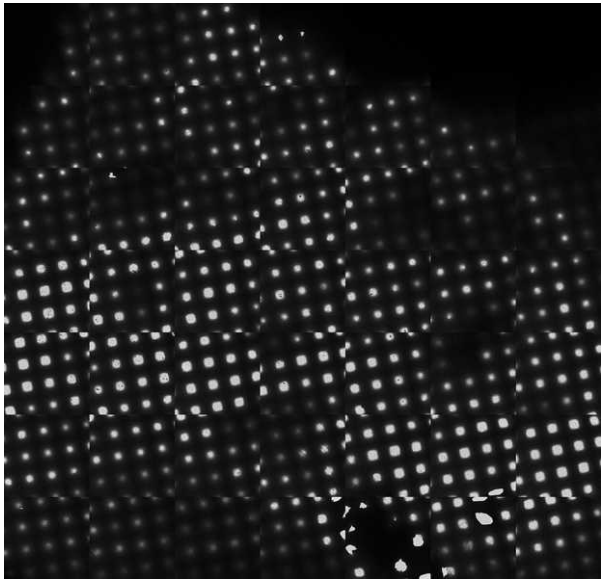
- Center the crystal (tuning of the eucentric height)



Sample preparation

- Ideal crystal size is 100-200 nm
  - Electrons do not cross the sample if too thick
  - Signal is too weak is crystal is too small
    - Not enough unit cells
- Obtaining nano-sized crystals is not easy
  - Optimization of crystallization
    - Favouring nucleation (lot of small crytals instead of a few big ones)
    - Stoping crystal growth (taking out the crystals at a given time)
    - Seeding to control nucleation
  - Breaking larger crystals
    - Sonication
    - With a needle
    - Vortexing with a bead (0.5 to 1 mm bead, teflon, steeel)
- Obtaining a thin solvent layer on the grid
    - Blot optimization

Sample preparation (followed)



- Often end up with a certain distribution of sizes
  - Presence of “large” crystals may be a problem
    - Solvent too thick around large crystals
    - May mask smaller crystals
    - Difficult to separate large and small crystal
- Difficult to assess the size of obtained crystals
  - Crystals smaller than 200 nm can barely be seen with visible light microscope
  - Final check can only be done with the electron microscope
- Viscous crystallization conditions may cause problems (e.g. PEG, glycerol)
  - Difficult to blot => solvent too thick on the grid
  - Transfer to a less viscous solution may be necessary

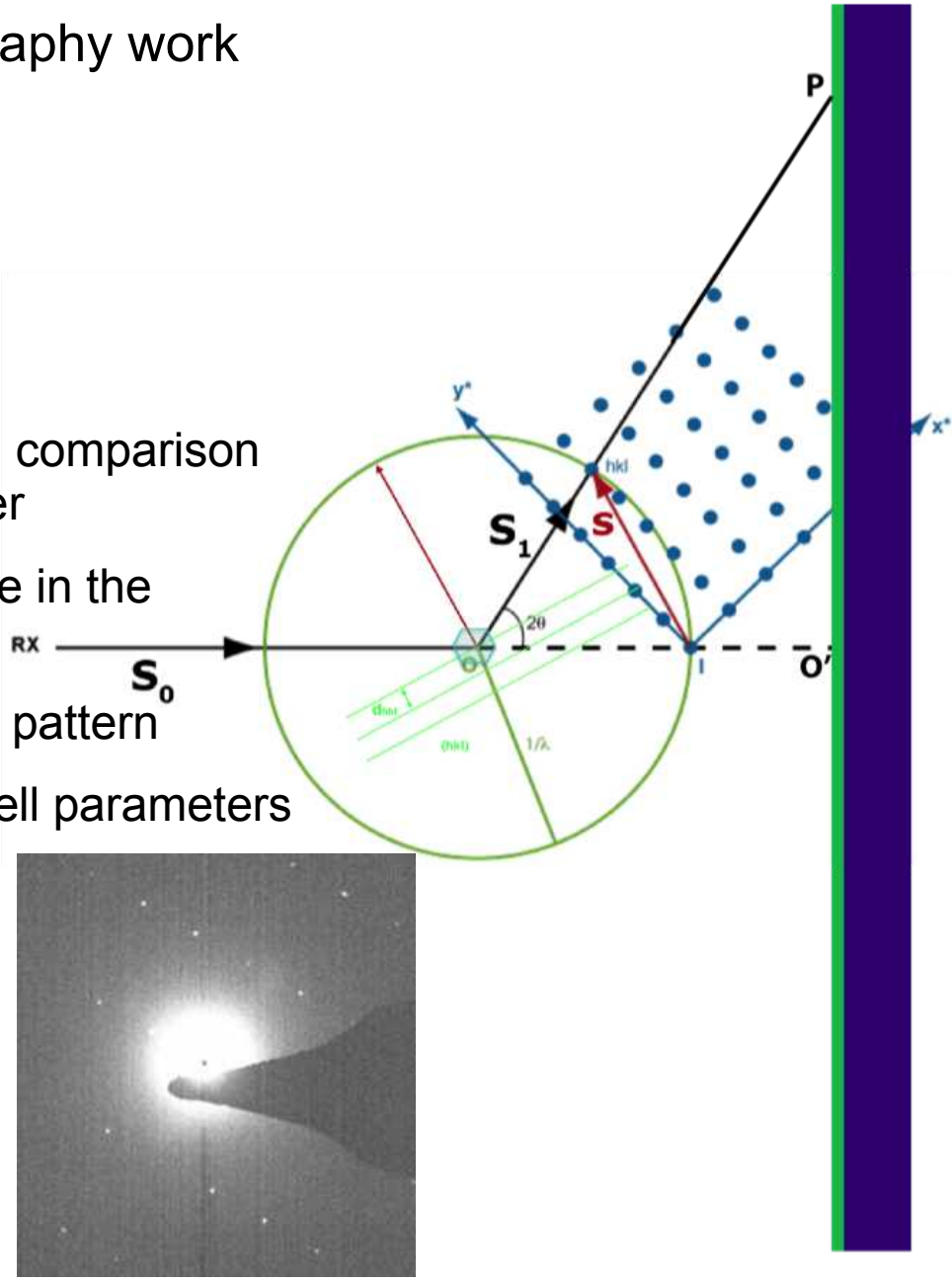
Collecting data

- Very similar to X-ray crystallographic data collection
- The crystal is centred (tuning of the eucentric height)
- A small area around the crystal is selected (SAED)
- The grid (and the crystal) is rotated continuously from -50° up to + 50°
- Images a recorded in a shutterless mode
  - Requires a fast readout detector
  - Ideally a Hybrid pixel detector (equivalent to Pilatus for X-rays)
- Each image correspond to a 0.05 to 0.5° wedge
- Exposure time is about 0.5 s per image
  - A full data set take about 2-3 mn
- In practice, about 30 to 40° can be collected before the crystal becomes too highly damaged

Data processing

- Softwares developped for X-ray crystallography work fine!
  - XDS or DIALS can be used

- A few differences:
  - Due to much shorter wave length (0.02 Å in comparison with 1 Å for X-rays), the Ewald is much flater
  - Diffraction patterns represent almost a plane in the reciprocal space
  - You often see Fridel's pair on the diffraction pattern
  - You cannot refine camera-length and unit cell parameters simultaneously
    - Too highly correlated parameters
  - Statistics are somehow different from X-ray crystallography standards



Solving structure

- As for X-rays, phasing is an essential issue
- Methods used successfully:
  - Molecular replacement
    - With previously known structure
    - With lower resolution single particle EM map
  - Direct methods
    - For peptides, when subatomic resolution is obtained (d < 1 Å)
- Still to be tested:
  - Multiple isomorphous replacement
    - Could work with some heavy atoms
    - Weaker signal expected (scattering factor ~ $Z^{4/3}$ for electrons, ~Z^2 for X-rays)
    - May be impaired by higher errors on structure factor (in comparison with X-ray diffraction)

Structure refinement

- With your favorite refinement program for X-ray crystallography
  - Refmac in CCP4
  - Phenix

- The only difference is to use electron scattering factors instead of X-ray scattering factors
  - Easy to do with ccp4i and ccp4i2

- Overall very similar to refinement against X-ray crystallographic data

Electron atomic scattering factors

- Calculated from the X-ray atomic scattering factor (Mott-Bethe formula)

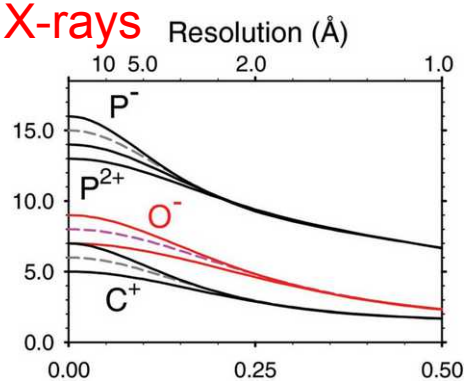
$$f_{el}(s,Z)=\frac{m_0 e^2}{8\pi h^2 \varepsilon_0}\left(\frac{Z_0-f_x(s,Z_0)}{s^2}+\frac{\Delta Z}{s^2}\right)$$

with Z_0 electrons and $Z=Z_0+\Delta Z$ nuclear charges

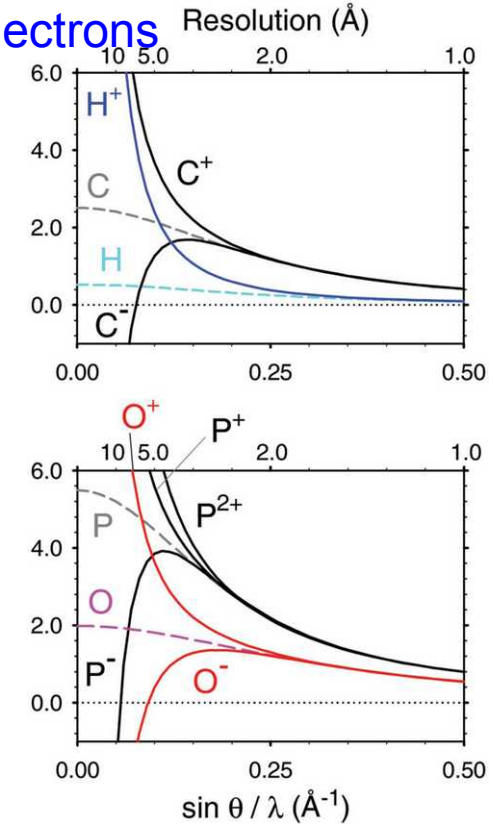
  - In Refmac use: source EC MB
  - Calculated from atomsf.lib file

- Tabulated in the International Tables of Crystallography vol. C (pp 226-244)
  - Includes neutral and charged species
  - In Refmac use: source EC
  - Atomsf-electron.lib used
  - May be better if ions are present

X-rays

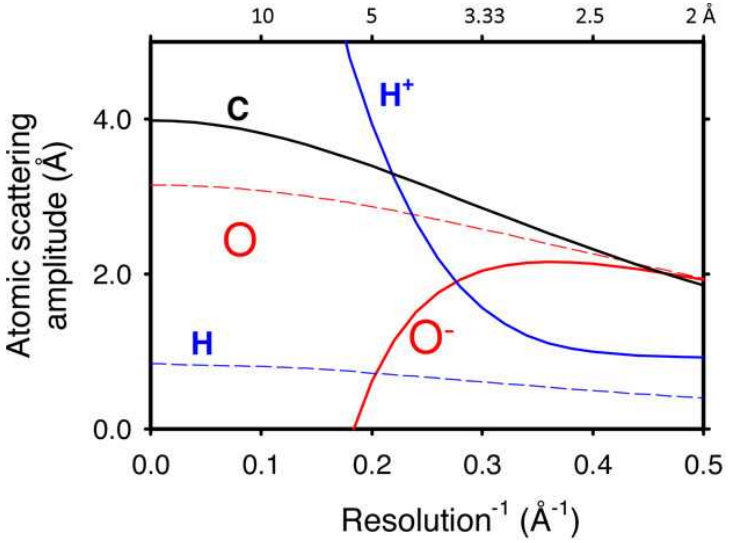


Electrons



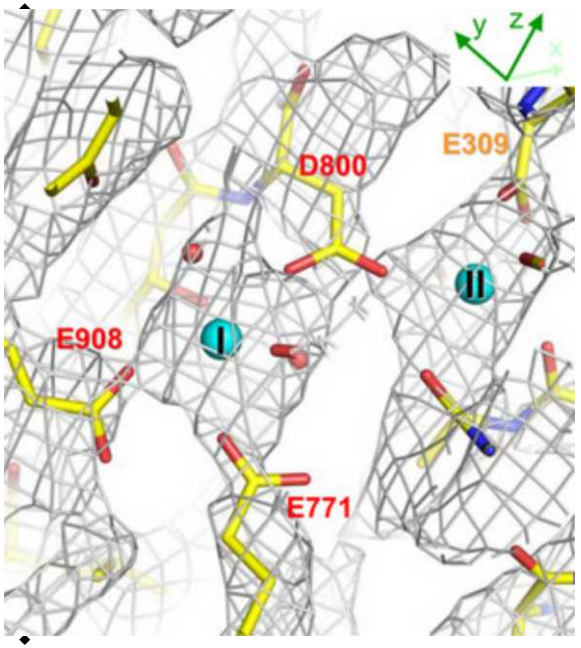
Yonekura *et al.*, *IUCrJ*, **5**, 348-353, 2018

Charged chemical group can be identified

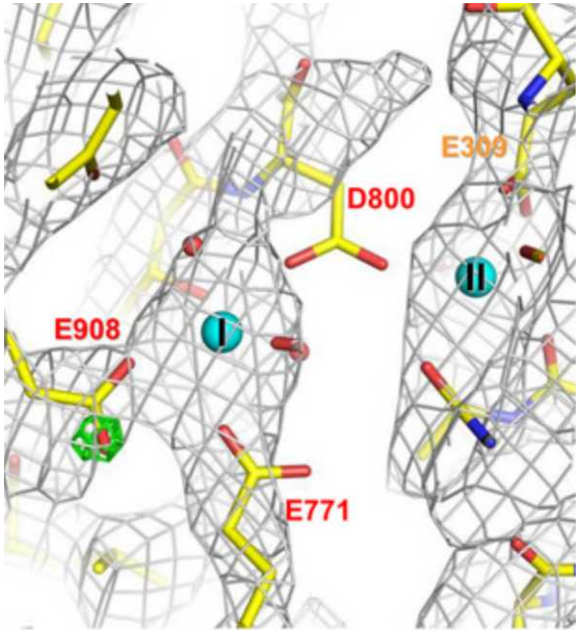


- Exemple of Ca^{2+} ATPase
  - Comparison of maps computed either:
    - In the 5 – 3.4 Å resolution range (small impact of O^-)
    - In the 8 – 3.4 Å resolution range (large impact of O^-)

5 – 3.4 Å



8 – 3.4 Å

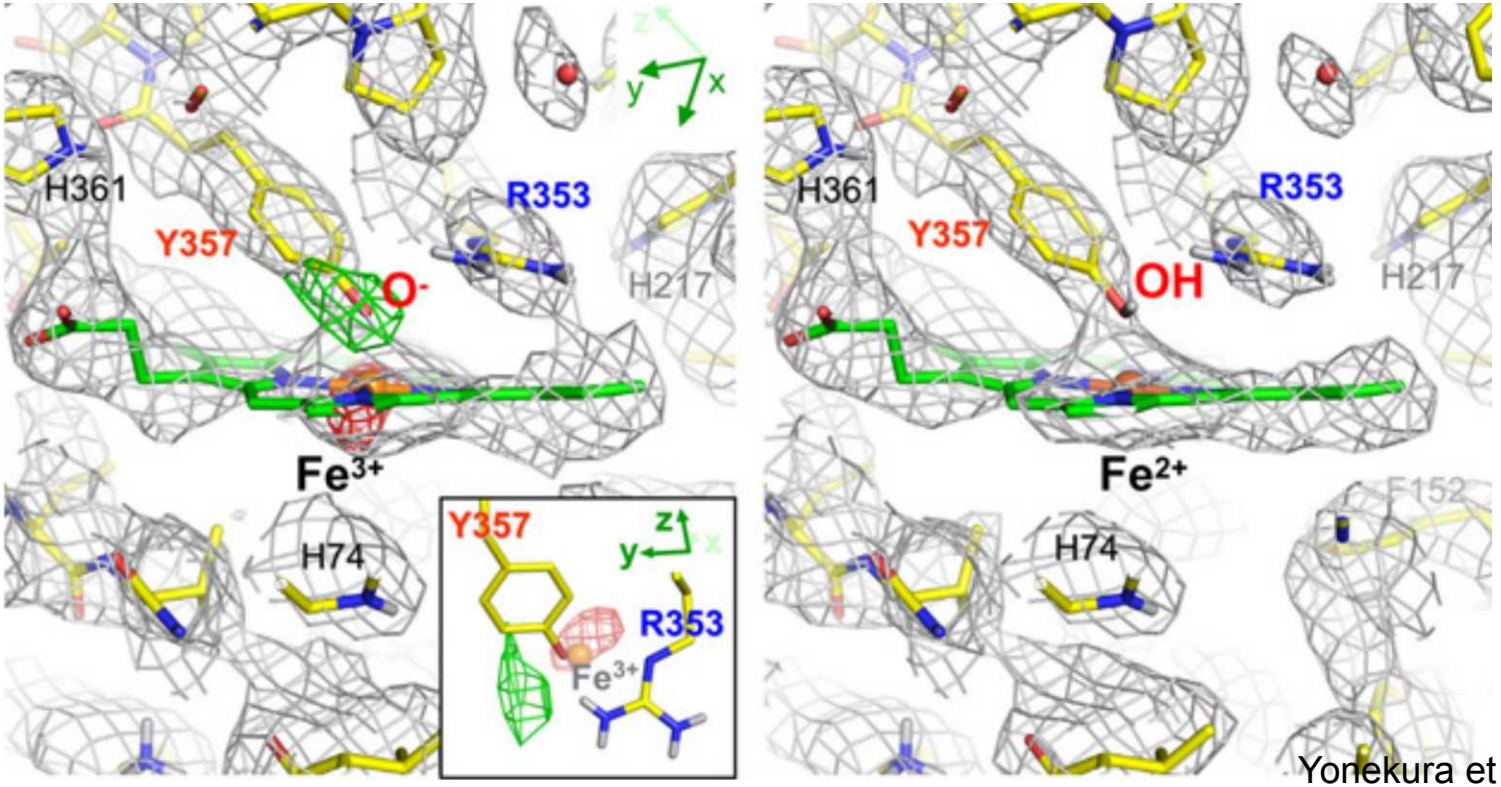


- D800 is clearly negatively charged => calculated pKa < 3
- E908 is uncharged and protonated

Yonekura et al., PNAS, 2015

Oxidation state of metals can be identified

- Heme group of catalase is supposed to contain an Fe^{3+} and a deprotonated tyrosine in its vicinity
- Electron diffraction data demonstrate the presence of an Fe^{2+} and a protonated tyrosine
- This unexpected result may be induced by electrons => radiation damage



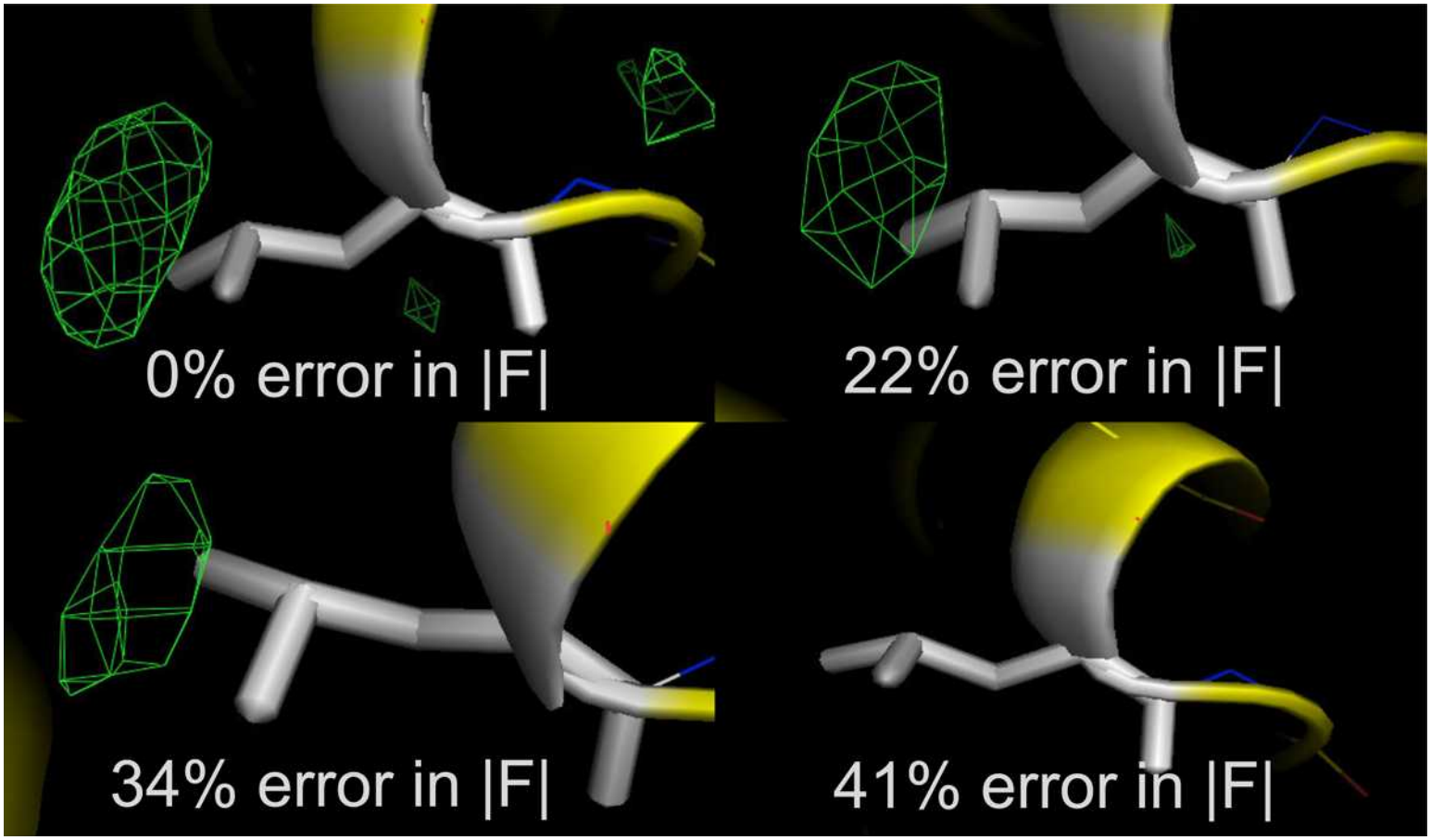
Yonekura et al., PNAS, 2015

Quality of electron diffraction data?

Protein	PDB entry	Res. (Å)	Compl. (%)	redundancy	R_{merge}	$CC_{1/2}$	I/σ(I)	R_{work}	R_{free}
NA/K ion channel	6CPV	2.5	81.7 (69.5)	4.9 (3.6)	np	98.4 (24.3)	4.7 (1.6)	0.218	0.263
QYNNQNNFV	6AXZ	0.75	97.0 (96.2)	5.8 (4.4)	0.232 (0.638)	98.2 (20.9)	4.57 (1.77)	0.242	0.246
Lysozyme	5OCV	2.20	79.7 (64.3)	42.4 (25.9)	0.614 (1.45)	95.5 (53.3)	5.07 (2.13)	0.237	0.270
Lysozyme	5O4X	2.11	61.7 (49.8)		0.388 (0.640)		2.7 (1.0)	0.264	0.279
TGFβ-TGFβ-RII	5TY4	2.9	68.8	3.8 (3.9)	np	95.1 (25.5)	3.3 (0.8)	0.292	0.328
VQIVYK	5K7N	1.11	83.0 (79.4)	1.9 (1.8)	np	98.7 (63.9)	2.4 (1.1)	0.210	0.223
Lysozyme	5K7O	1.8	97.6 (93.2)	8.6 (4.1)	np	90.1 (0)	3.7 (0.8)	0.240	0.284
Xylanase	5K7P	2.3	85.4 (66.1)	4.2 (2.9)	np	91.8 (0.1)	3.5 (1.0)	0.230	0.267
Taumatin	5K7Q	2.5	94.2 (90.0)	4.4 (3.6)	np	84.8 (0.1)	3.5 (1.8)	0.251	0.295
Trypsin	5K7R	1.7	90.1 (61.2)	6.9 (2.8)	np	72.2 (0.1)	2.6 (0.3)	0.248	0.281
Proteinase K	5K7S	1.6	96.8 (86.8)	8.2 (5.5)	np	91.2 (0.1)	3.4 (0.9)	0.224	0.255
Thermolysin	5K7T	2.5	97.1 (90.3)	12.3 (12.0)	np	84.7 (0.2)	5.6 (3.8)	0.290	0.310

Impact of error in structure factors ?

Above 40 % error in |F|, impossible to distinguish Hen and Turkey lysozyme



- Not dramatic for refinement
  - If phase information is accurate enough (good pdb model)
- May prevent experimental phasing
  - Rely on difference between structure factors

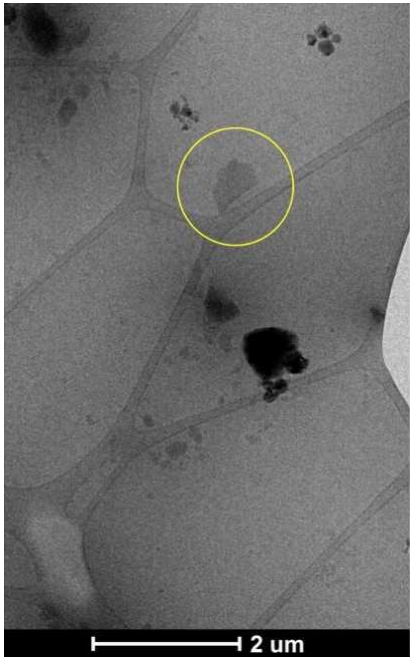
Adapted from John Spence

Data collection on bovine insulin crystals

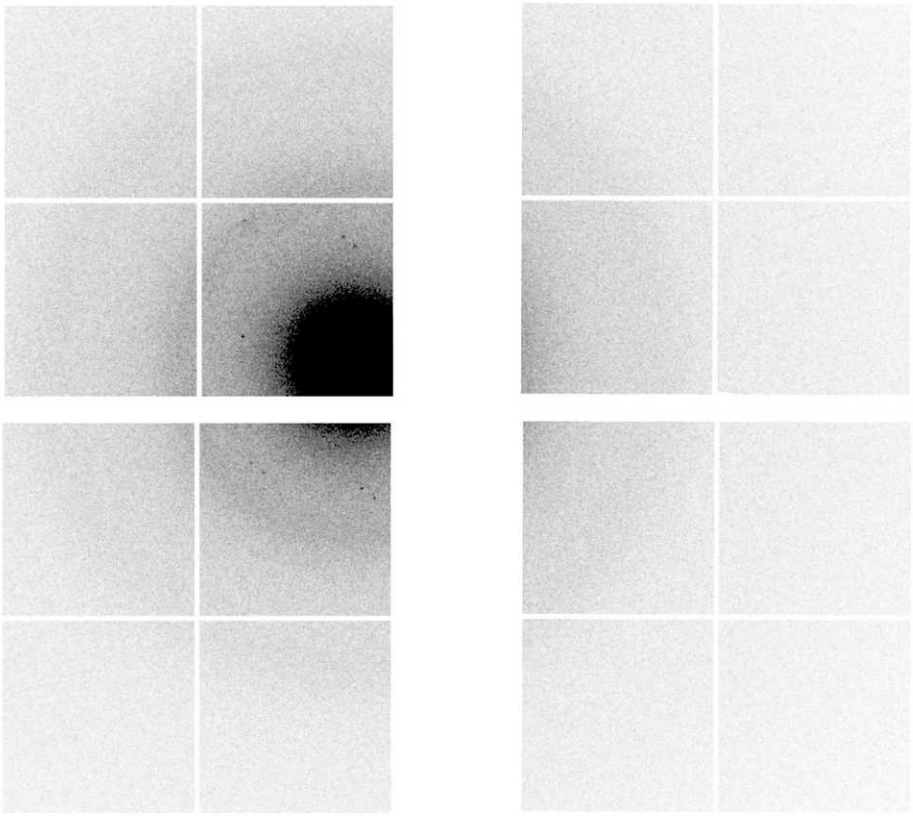
Electron diffraction data (in Basel):

- Thalos 200 kV electron microscope

- Timepix – Hybrid Pixel Detector



Insulin crystal 07 :
102 frames, 1.2 s/frame, 0.37°/frame
60 useful images (22°)
3.02 Å resolution



Data collection : Collaboration Thorsten Blum, Max Clabbers, Jan Pieter Abrahams (Biozentrum Basel)

5 insulin data sets merged

Space group : ***R***3
Unit cell : a=b=82.40 Å c=33.46 Å
Data processing with XDS

Crystal #	**1**	**2**	**3**	**4**	**5**
Frame exposure (s)	1.2	1.2	0.6	0.6	0.6
Number of frames	65	60	80	70	90
Φ_{total} (°)	17	22	30	27	34
Resolution max.	3.12	3.02	3.30	3.17	3.67
R_{meas}	0.231	0.238	0.205	0.196	0.133
I/σ(I)	3.55	3.88	3.11	11.2	4.47

Merged insulin data

- Electron and X-ray diffraction data on the same batch of crystals

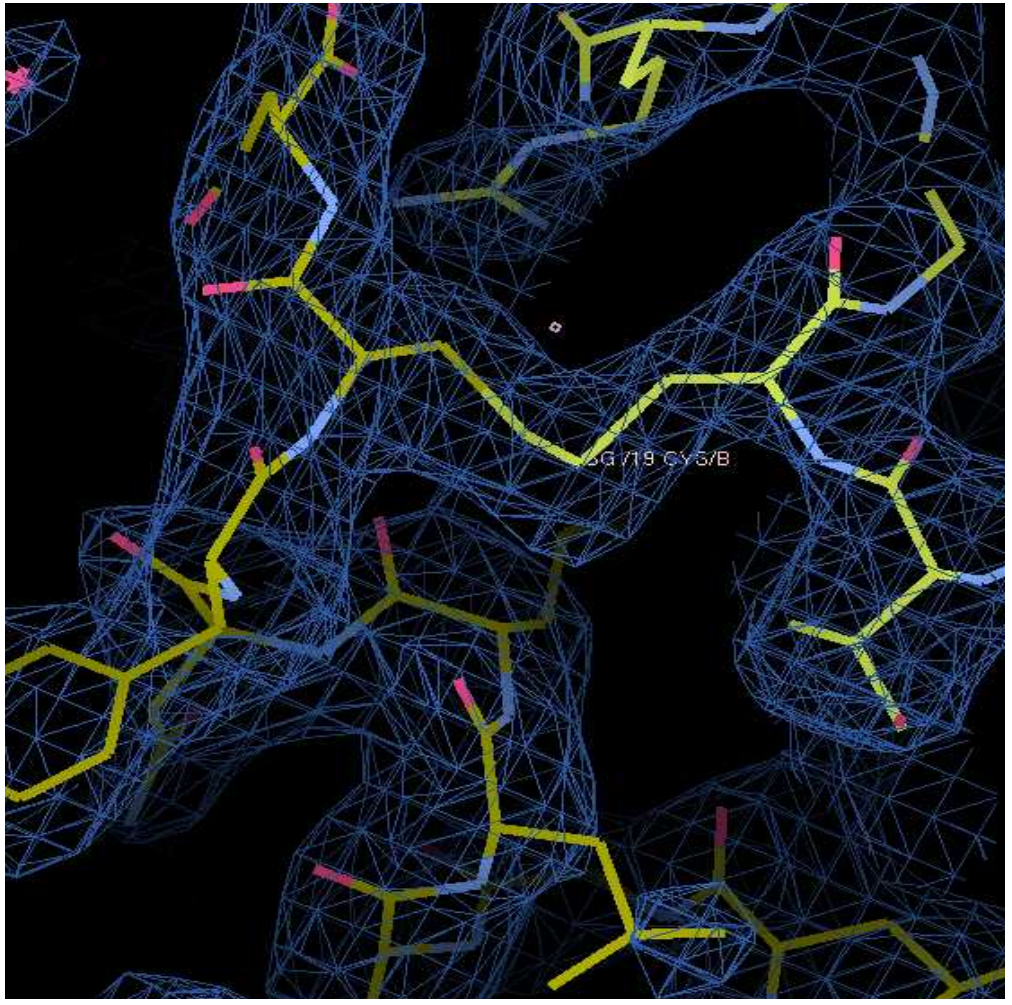
	Electron diffraction	X-ray diffraction
Resolution (Å)	30 – 3.25	30 – 2.30
Nb observations	3194	16882
Nb unique reflections	1201	3613
Completeness (%)	90.2	99,3
R_{meas}	0.328	0.107
$CC_{1/2}$	0.923	0.993
I/σ(I)	2.86	11.8
Crystal volume (μm^3)	0.16	4.2 10^5

Refined structure

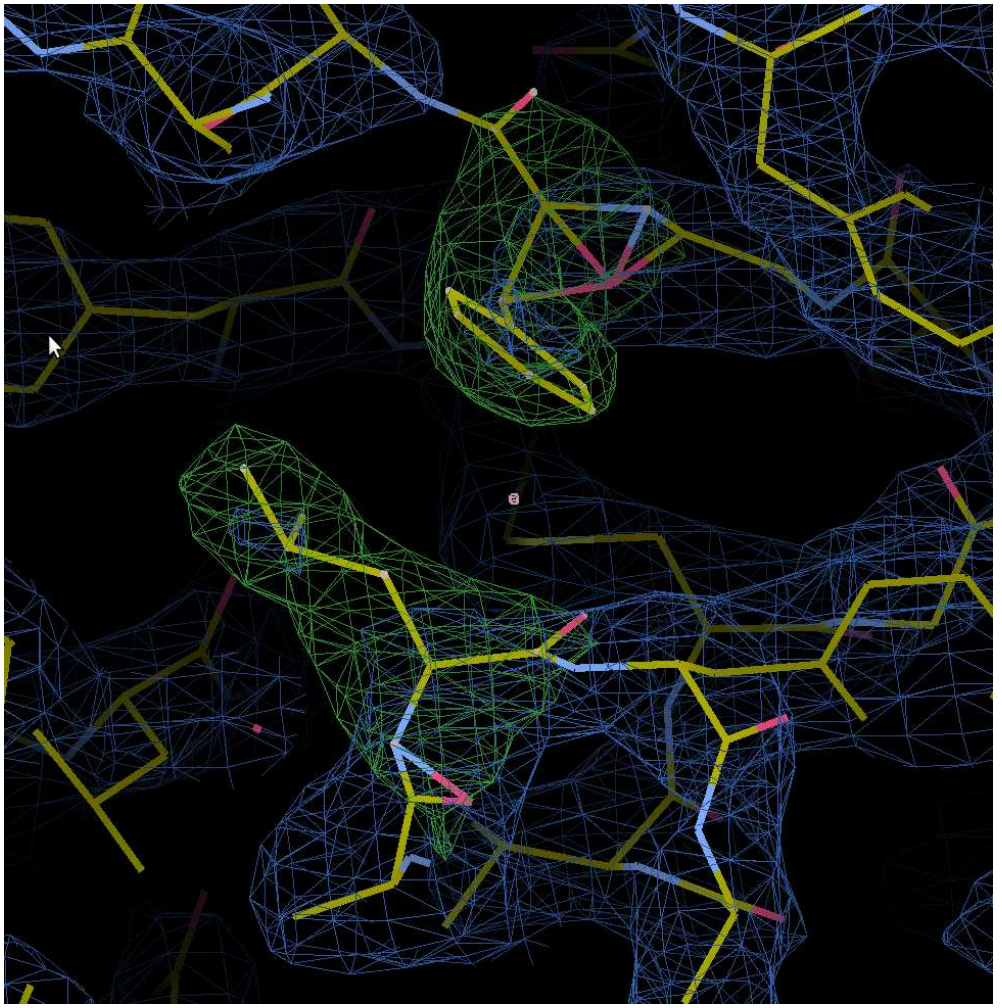
- Structure solved by molecular replacement (100 % identity model)
- Refinement performed with Refmac (ccp4) and proper atomic scattering factors

Refinement	X-ray	ED
Resolution range	24.20 – 2.30 (2.36 – 2.30)	30.30 – 3.25 (3.33 – 3.25)
Nb of refl. (work set)	3213 (217)	1030 (63)
Nb of refl. (free set)	180 (9)	97 (3)
Number of atoms	805	770
Nb refl. / Nb param	0.998	0.334
R-factor	0.166	0.194
R_{work}	0.162 (0.130)	0.181 (0.349)
R_{free}	0.238 (0.333)	0.319 (0.501)
σ bond (Å)	0.008	0.006
σ angle (°)	1.174	1.034

Coulomb potential map



Insulin Coulomb potential map contoured at 1 σ



Insulin Coulomb potential omit map. residual map (contoured at ± 3 σ) shows where to place the missing residues

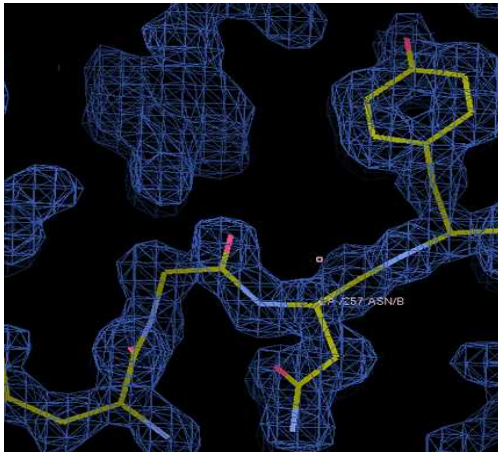
Data on a few other proteins or peptide…

	Insulin	Thermolysin	Thaumatin	LGNY
Resolution (Å)	30 – 3.25	14.5 – 3.26	12.3 – 2.76	11.0 – 1.0
Nb observations	3194	18806	21293	4386
Nb unique reflections	1201	4536	4597	971
Completeness (%)	90.2	84.3	65.6	58.0
R_{meas}	0.328	0.618	0.593	0.254
$CC_{1/2}$	0.923	0.867	0.878	0.976
$I/\sigma(I)$	2.86	2.14	2.09	5.14

A few other proteins or peptide…

- Refinement statistics on insulin, thermolysin, thaumatin & peptide

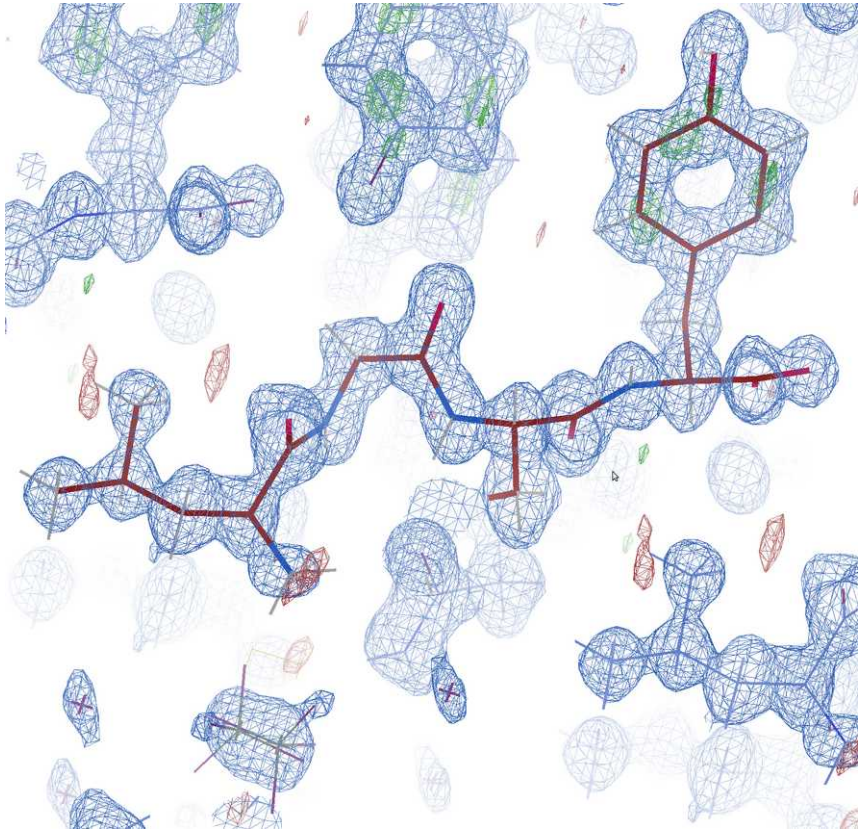
Refinement	Insulin	Thermolysin	Thaumatin	LGNY
Resolution range	30.30 – 3.25 (3.33 – 3.25)	37.52 – 3.16 (3.34 – 3.16)	12.34-2.76 (2.83-2.76)	11.0 – 1.00 (1.03-1.00)
Nb of refl. (work set)	1030 (63)	4293 (262)	4364 (108)	861 (39)
Nb of refl. (free set)	97 (3)	237 (12)	231 (3)	99 (5)
Number of atoms	770	2438	1551	39
Nb refl. / Nb param	0.33	0.44	0.70	2.45
R-factor	0.194	0.214	0.283	0.265
R_{work}	0.181 (0.349)	0.210 (0.347)	0.281 (0.160)	0.262 (0.44)
R_{free}	0.319 (0.501)	0.292 (0.541)	0.320 (0.176)	0.296 (0.438)
σ bond (Å)	0.006	0.007	0.005	0.004
σ angle (°)	1.034	1.156	1.005	1.057

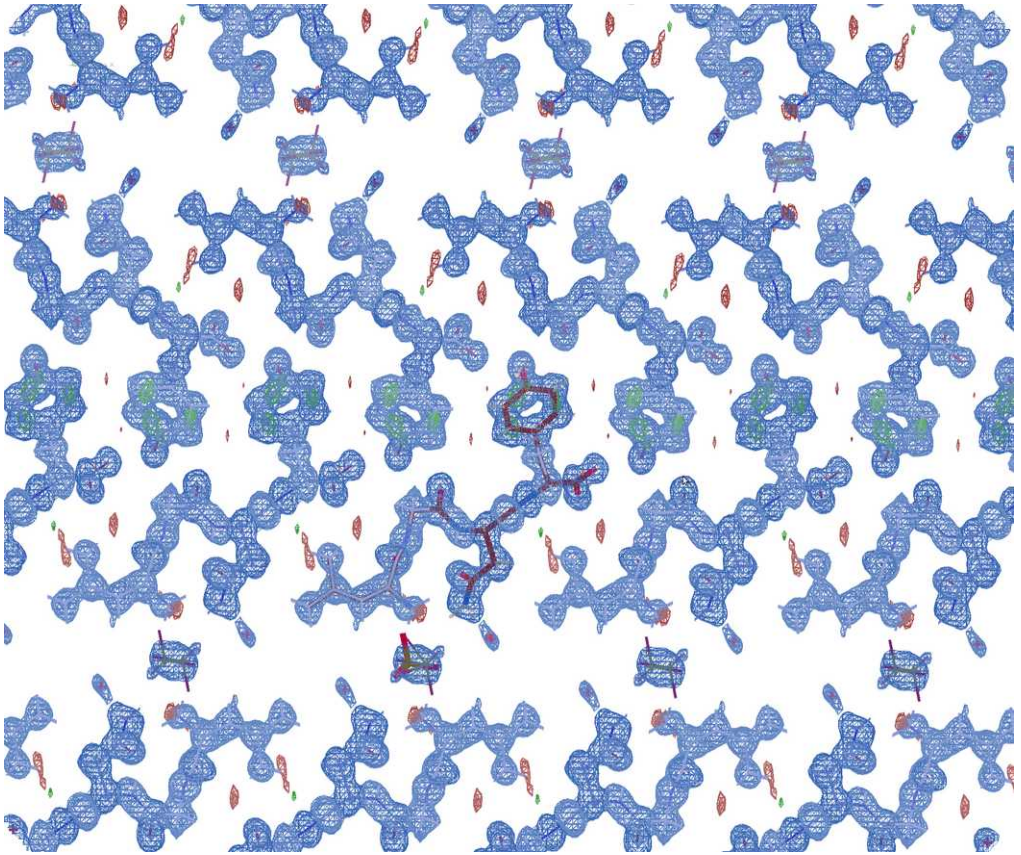


LGNY

LGNY Coulomb potential map

- Good fit with the peptide model
- Several peaks above 3σ in the residual map
- No information to complete the model



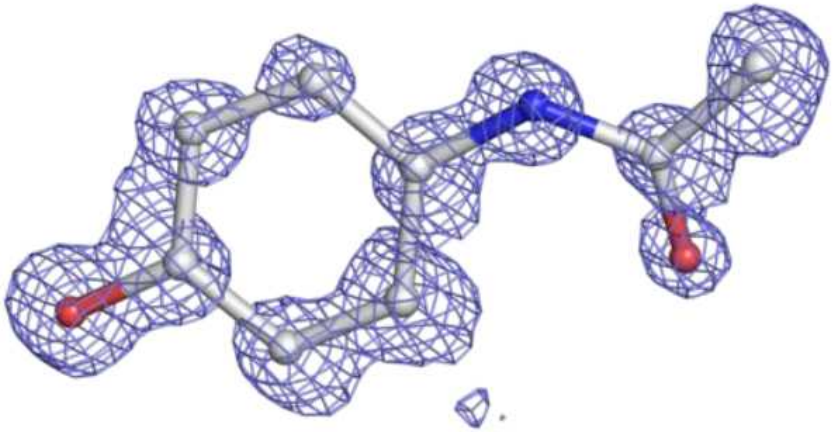


Dynamical or multiple scattering

- X-rays interact weekly with matter
  - The probability for a photon to be diffracted twice is negligible
    - Kinematical approximation
  - $$I(\vec{s}) \propto |F(\vec{s})|^2$$
- Electrons strongly interact with matter
  - \+ get information out of small samples
  - \- an electron can be scattered several times while travelling through the sample
  - $$I(\vec{s}) \neq |F(\vec{s})|^2$$
  - Dynamical theory of diffraction
    - Can be taken into account during refinement
    - Only feasible for small molecules

Taking multiple diffraction into account

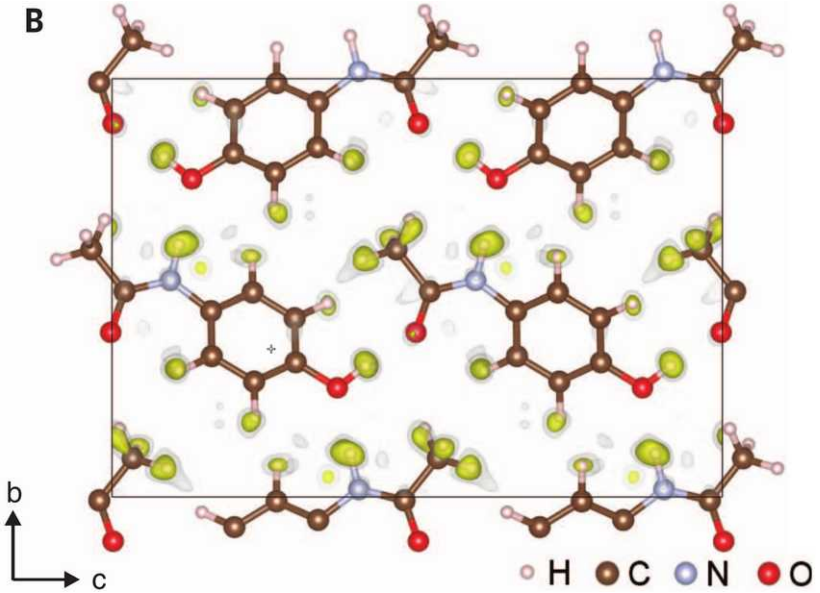
- Feasible on small molecule (paracetamol at 0.8 Å resolution)
- Tiny details such as hydrogen atoms can be seen
- Untractable on protein crystals



Kinematical refinement : R_{fact} = 22 %

Jones et al., *ACS Cent. Sci*, **4**:1587-1592(2018)

Coulomb potential difference map
contoured at 2σ (grey) and 3 σ (green)



Dynamical refinement : R_{fact} = 8.8 %

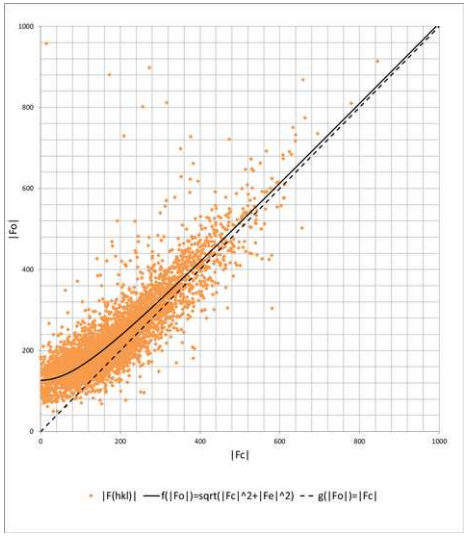
Palatinus et al., *Science*, **355**:166-169 (2017)

A simpler way of correcting data for dynamical scattering ?

- Multiple scattering induces overestimation of weak reflections

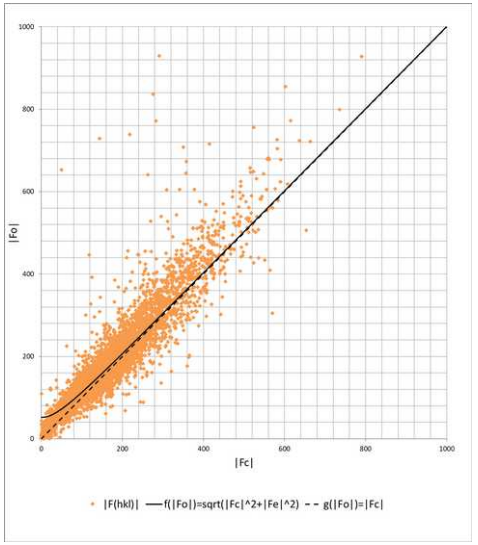
$$|F_{obs}| = \left(|F_{calc}|^2 + |F_e|^2\right)^{1/2}$$

$<|F_e(d)|>$ Is estimated for about 10 resolution shells



- The corrected intensities is then calculated by :

$$|F_{obs,corr.}(\vec{s})|^2 = \frac{|F_{obs}(\vec{s})|^2}{1 + \frac{<|F_e(d)|>^2}{|F_{calc}(\vec{s})|^2}}$$



Clabbers et al. *Acta Cryst A*, 75:82-93 (2019)

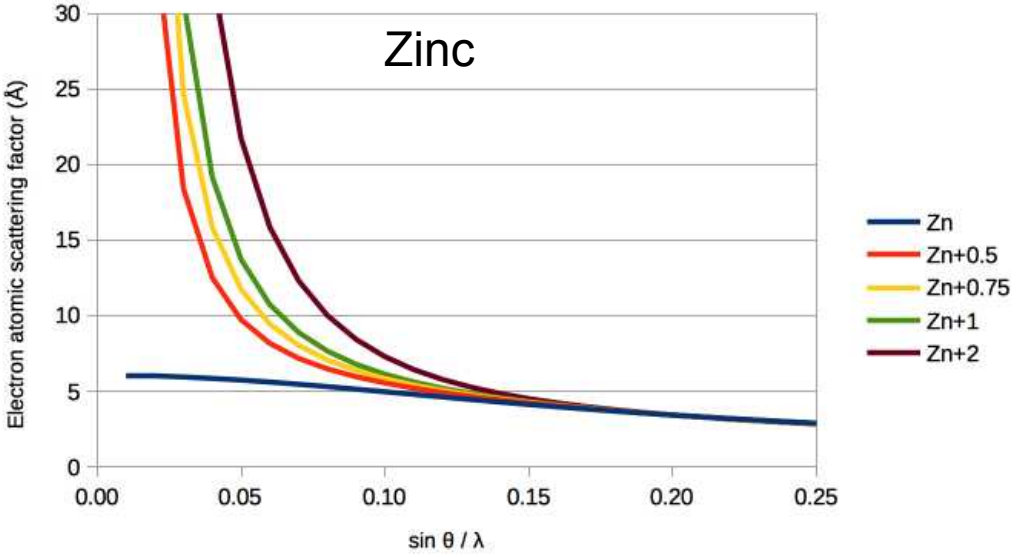
Corrected data lead to improved refinement statistics ?

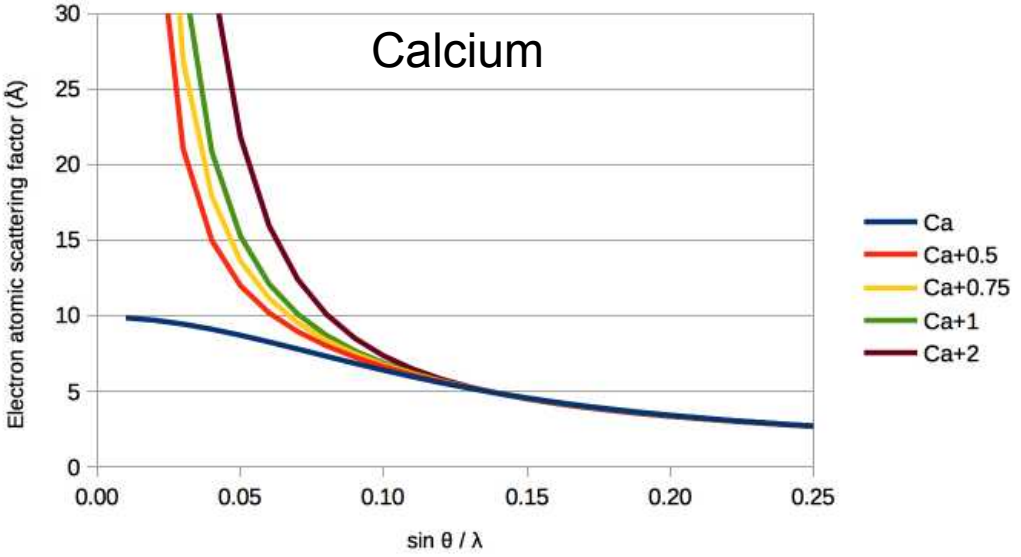
- Refinement statistics on insulin, thermolysin and thaumatin

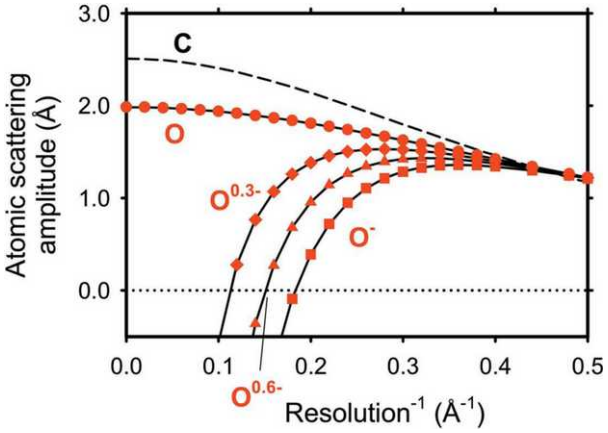
	Insulin		Thermolysin		Thaumatin	
Refinement	Uncorrected data	Corrected for dynamical scattering	Uncorrected data	Corrected for dynamical scattering	Uncorrected data	Corrected for dynamical scattering
Resolution range	30.30 – 3.25 (3.33 – 3.25)	30.30 – 3.25 (3.33 – 3.25)	37.52 – 3.16 (3.34 – 3.16)	37.49 – 3.16 (3.34 – 3.16)	12.34-2.76 (2.83-2.76)	12.34-2.76 (2.83-2.76)
Nb of refl. (work set)	1030 (63)	1030 (63)	4293 (262)	4292 (261)	4364 (108)	4363 (107)
Nb of refl. (free set)	97 (3)	97 (3)	237 (12)	237 (12)	231 (3)	231 (3)
Number of atoms	770	770	2438	2438	1551	1551
Nb refl. / Nb param	0.334	0.334	0.440	0.440	0.703	0.703
R-factor	0.194	0.186	0.214	0.152	0.283	0.252
R_{work}	0.181 (0.349)	0.177 (0.312)	0.210 (0.347)	0.149 (0.172)	0.281 (0.160)	0.249 (0.145)
R_{free}	0.319 (0.501)	0.298 (0.349)	0.292 (0.541)	0.215 (0.197)	0.320 (0.176)	0.293 (0.337)
σ bond (Å)	0.006	0.006	0.007	0.006	0.005	0.006
σ angle (°)	1.034	1.007	1.156	1.019	1.005	1.128

Estimating charges of ions

- Electrons « sense » the charge of atoms
  - Electron atomic scattering factor vary with atomic charge
- Electron diffraction data (or single particle EM) can be used to estimate atomic charges
  - Atomic scattering factors of charge species parametrized with 5 gaussians and used in Refmac

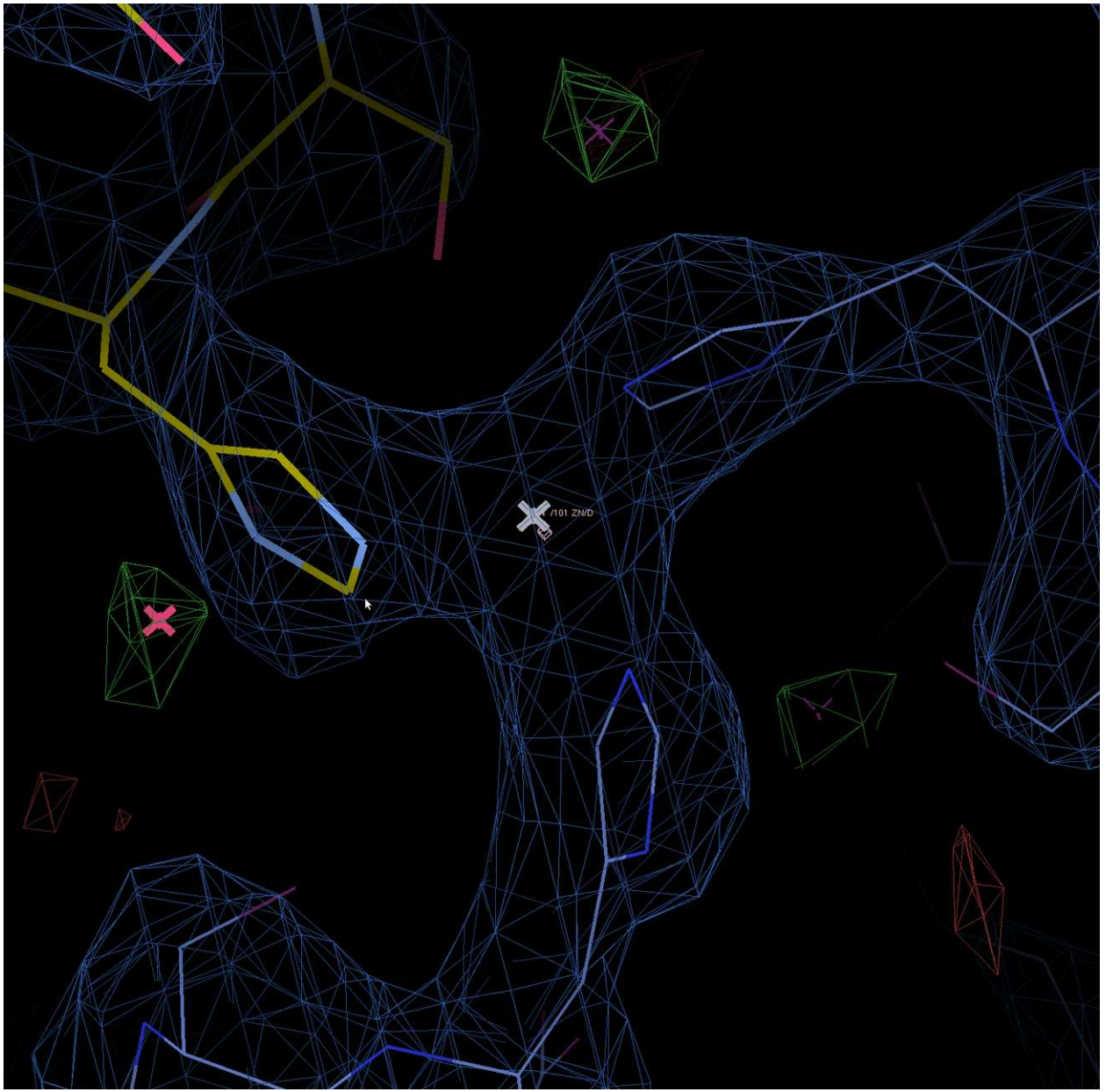






International Tables for crystallography (2006) Volume C, Chapt 4.3, pp 259-429
Yonekura et al. (2016) J. Appl. Cryst., 49:1517-1523

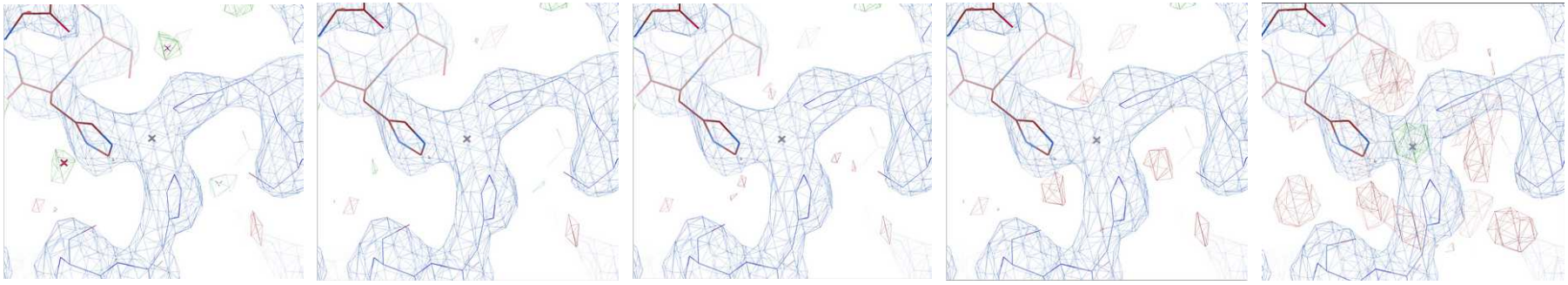
Zinc in insulin crystal



Test of different charge states for zinc in insulin

- Electron atomic scattering for Zn and Zn^{2+} taken from the International table for crystallography
  - Parametrization of Zn, $Zn^{+0.5}$, Zn^{+1}, Zn^{+2} atomic scattering factor (5 gaussians model)
  - Refinement with different charges

	Zn		$Zn^{+0.5}$		$Zn^{+0.75}$		Zn^{+1}		Zn^{+2}	
	Uncorr. data	Corrected data	Uncorr. data	Corrected data	Uncorr. data	Corrected data	Uncorr. data	Corrected data	Uncorr. data	Corrected data
Zn B-factor (Å^2)	19.39	19.49	23.31	23.96	23.65	27.83	39.36	34.33	82.41	418.6
<B-factor> H10D-NE2	31.07	33.48	32.32	34.41	32.70	34.58	32.95	34.85	29.95	38.79
R_{work}	0.181	0.177	0.185	0.184	0.184	0.184	0.188	0.186	0.196	0.193
R_{free}	0.319	0.298	0.313	0.275	0.327	0.278	0.308	0.279	0.318	0.283



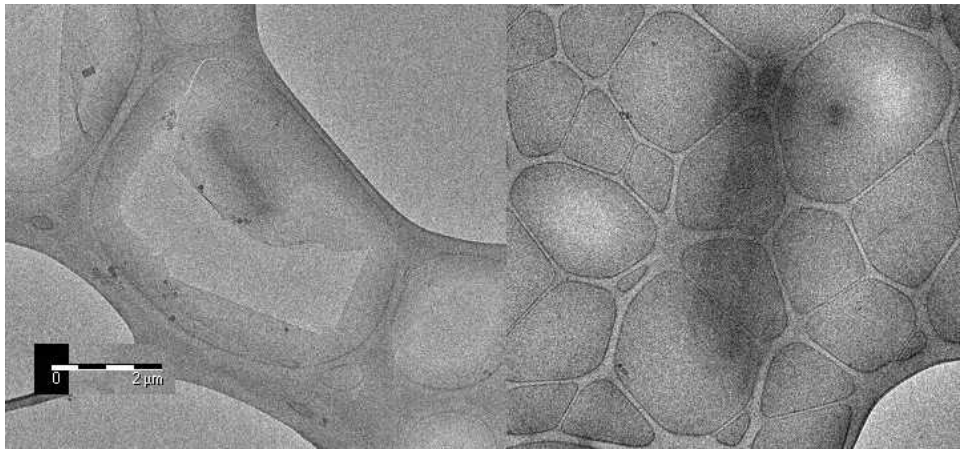
- Charge of zinc ion close to +1 => in the range of values by *ab initio* calculation or electronegativity equalization methods (0.55 – 1.1)

A new detector on the F20 at IBS

- Hybrid pixel Medipix 3RX direct detector (Amsterdam Scientific Instruments)
- 512x512 pixels, pixel size 55 µm
- No readout noise, High dynamic range, resistant to the direct beam (no beam stop required)

First test on lysozyme crystals

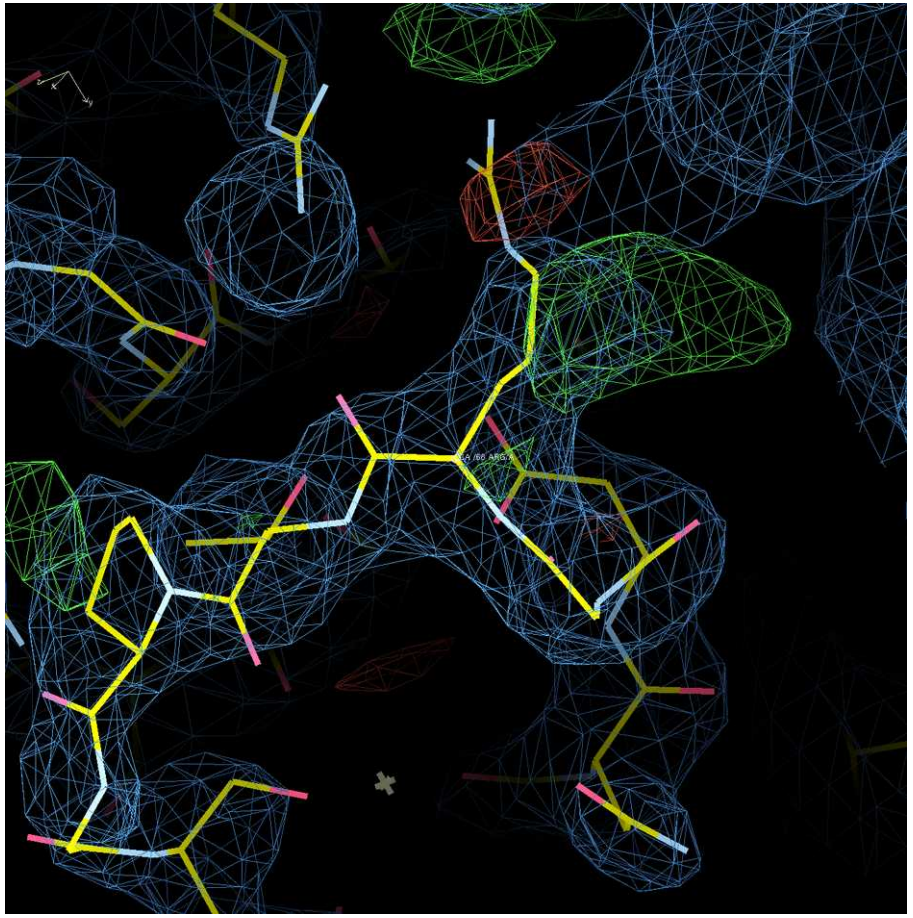
- Small and thin crystals
- Good data

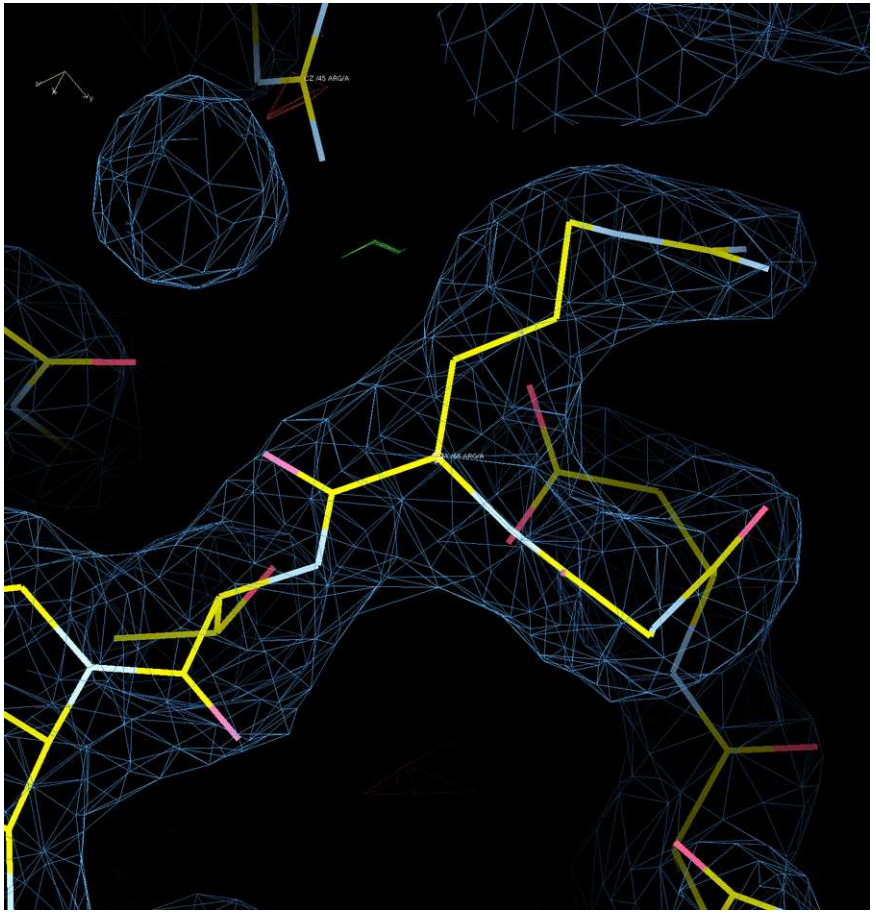


	Electron diffraction
Resolution (Å)	30 – 2.9
Nb observations	33900
Nb unique reflections	3046
Completeness (%)	56.4
R_{meas}	0.329
$CC_{1/2}$	0.976
I/σ(I)	6.68
Crystal volume (µm^3)	~ 0.13

Informative difference fourier maps

- **{2F_{obs} – $\underline{F}_{calc}$; φ_{calc}} and {F_{obs} – $\underline{F}_{calc}$; φ_{calc}} Coulomb potential maps allow to correct the model**





Conclusions

- Electron diffraction should become a useful tool in structural biology
  - For submicrometer crystals
  - For proteins up to 100kDa
  - When information on charge, hydrogens are useful
  - For preparing X-fel experiments
- Provides high resolution information (up to 1 Å)
- Only requires a few crystals (5 to 20, in general)
- Requires a standard cryo-electron microscope (200 kV is fine) but an appropriate detector to minimize radiation damages
  - Designing a dedicated electron diffractometer can be helpful

- Sample preparation is a difficult step
- Dynamical scattering is observed but does not prevent structure solution and refinement
- **Electron microscopes dedicated to electron diffraction are available (IBS, Grenoble) or will be available soon (CBS, Montpellier ; IGBMC, Illkirch)**

Acknowledgements

- ***IBS-MEM - Grenoble***
  - Wai Li Ling
  - Maria Bacia
  - Guy Schoehn
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  - Julie Lopes
  - Jacques-Philippe Colletier
  - Martin Weik
- ***Biozentrum - Basel***
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  - Max Clabbers
  - Jan Pieter Abrahams
- ***IBS-ISBG EM platform***
  - Emmanuelle Neuman
  - Daphna Fenel
  - Christine Moriscot
  - Benoit Gallet
  - Guy Schoehn
- ***ESRF - Grenoble***
  - Ulrich Zander
  - Andrew McCarthy
- ***Amsterdam Scientific***
  - Igor Nederlof