Biomolecular Structure and Mechanics

14th of May 2025

Structural Biology
X-ray Crystallography

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LBM

LAB AI 2351

LBM

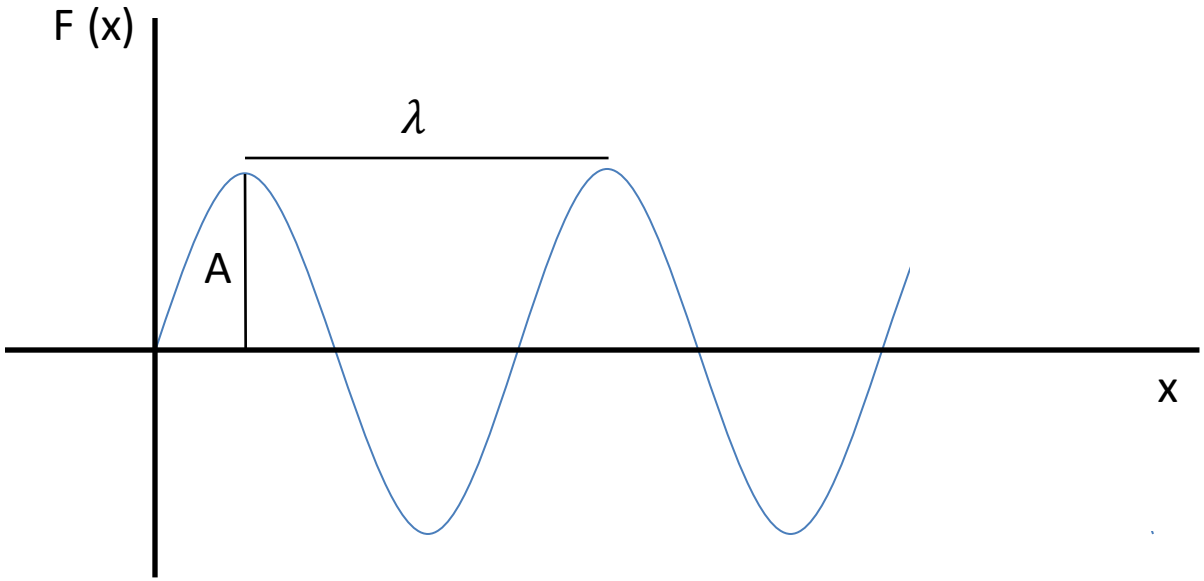
Content of lectures

- **Why x-rays and why crystals?**
- **Macromolecular crystallization**
- **Crystal packing**
- **The diffraction experiment**
- The phase problem
- Molecular replacement
- Refinement and validation

Content of lectures

- **Why x-rays and why crystals?**
- **Macromolecular crystallization**
  - **Need large amount of chemically and conformationally pure macromolecule**
  - **Test ∞ parameters to produce intermolecular interactions that lead to crystals using vapor diffusion method, for example.**
  - **Protect the crystals against ice formation using cryoprotectant and expose them to x-rays in the synchrotron.**
- **Crystal packing**
  - **Unit cell**
  - **Asymmetric unit (AU) and space group symmetry**
  - **Differentiate between biological oligomeric form and crystal contacts and AU content**
- **The diffraction experiment**
  - **Collect all possible scattered x-rays in all possible directions before the x-rays damage the crystal**
  - **Structure factor (F(S)) equation describes the diffracted x-ray, where the amplitude is dependent on the type of atom (# of electrons) doing the scattering and the phase depends on the direction of scattering.**
  - **Bragg's law determines the angle of diffraction of the x-rays diffracted from a crystal**
- The phase problem
- Molecular replacement
- Refinement and validation

X-rays as Waves



$F(x)=A \sin(2\pi\nu x + \alpha)$

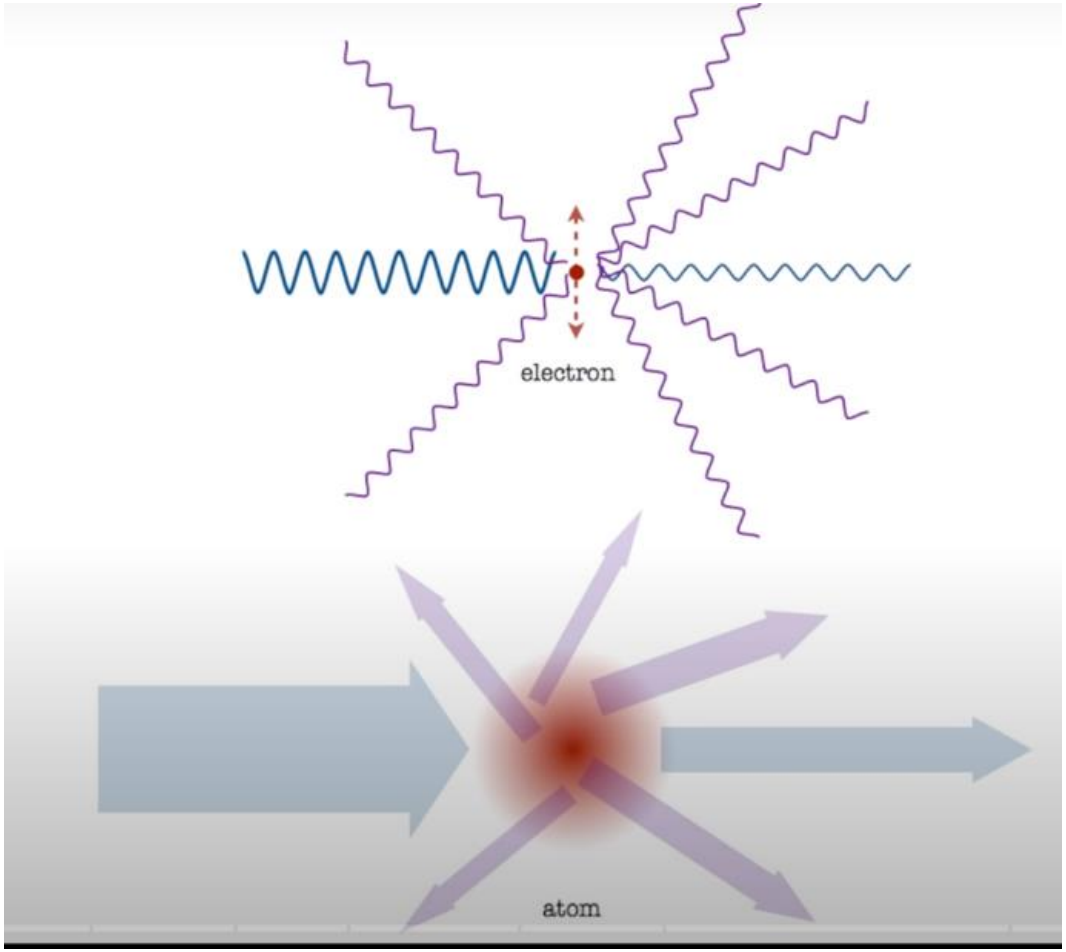
Where:
A: amplitude
$\nu = 1/\lambda$: frequency
λ = wavelength
α = phase

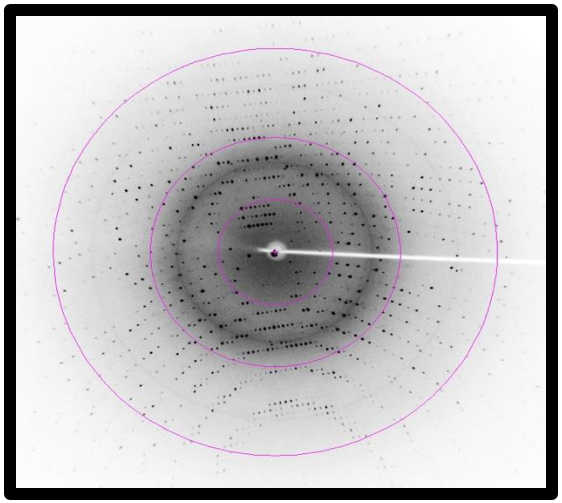
The energy of the radiation is related to its λ like this:

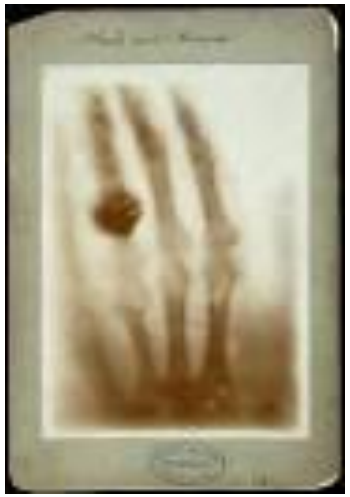
$E=hc/\lambda$

Where h is Plank's constant and c is the speed of light

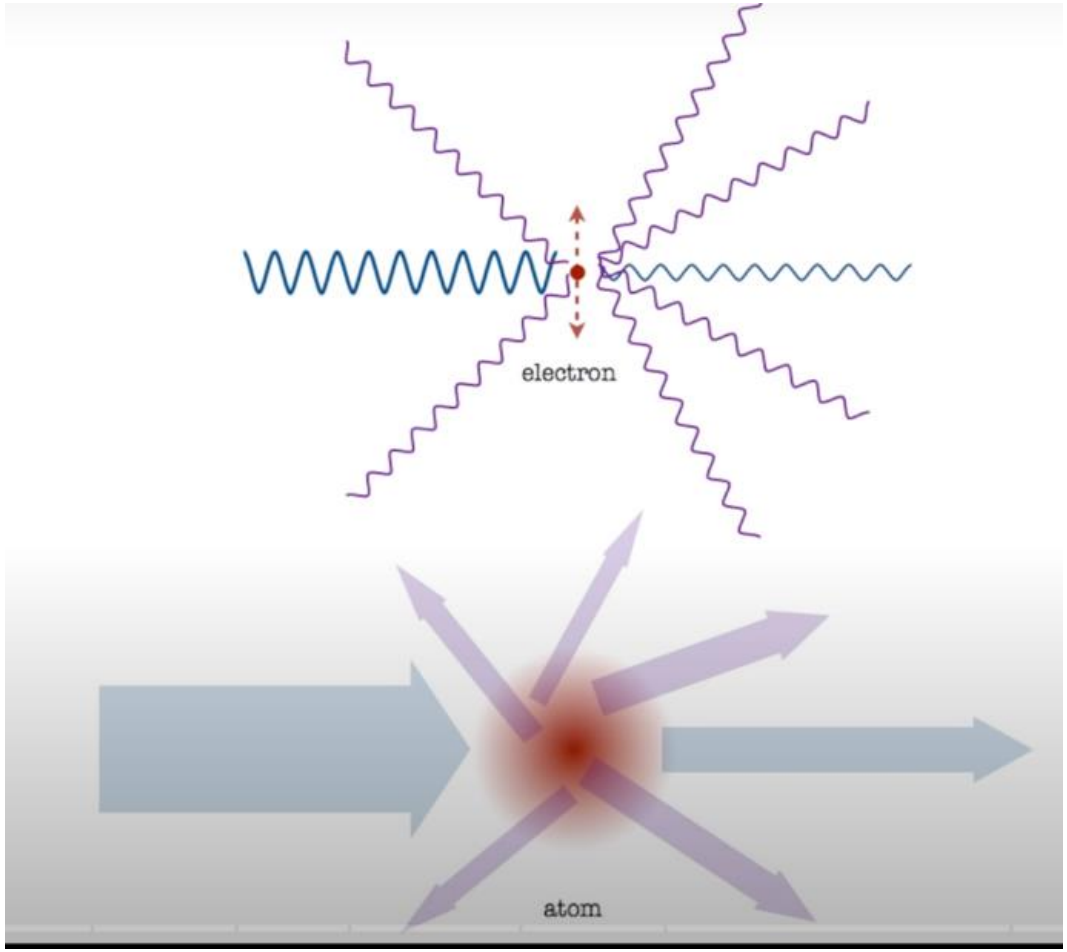
X-ray scattering







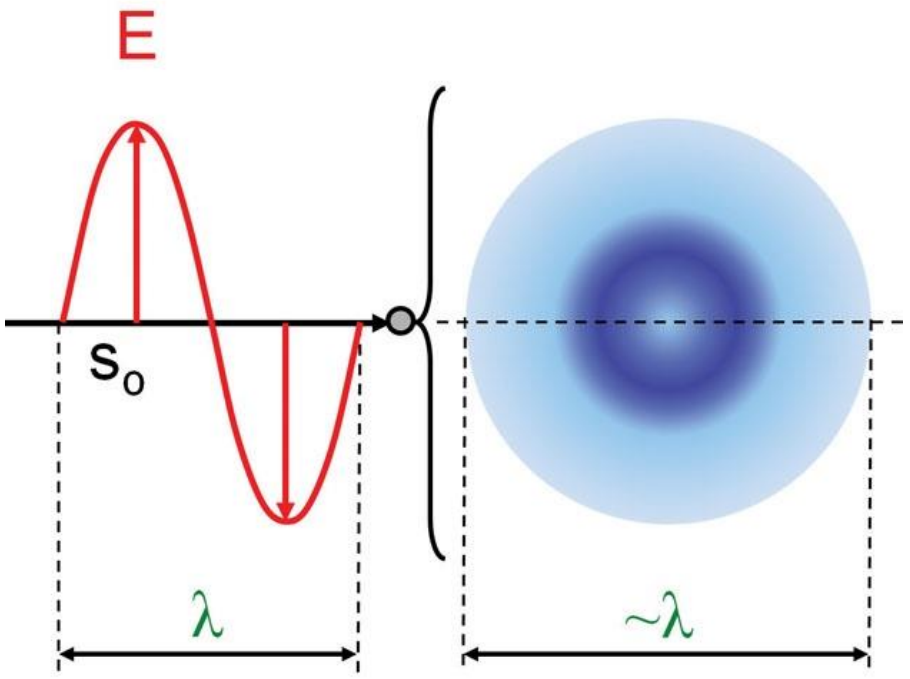
X-ray scattering



DIFFRACTION experiment

- What we measure is the intensity of the scattered X-rays
  $\lambda out = \lambda in$
- What we calculate is the electron position - electron density - of the object doing the scattering

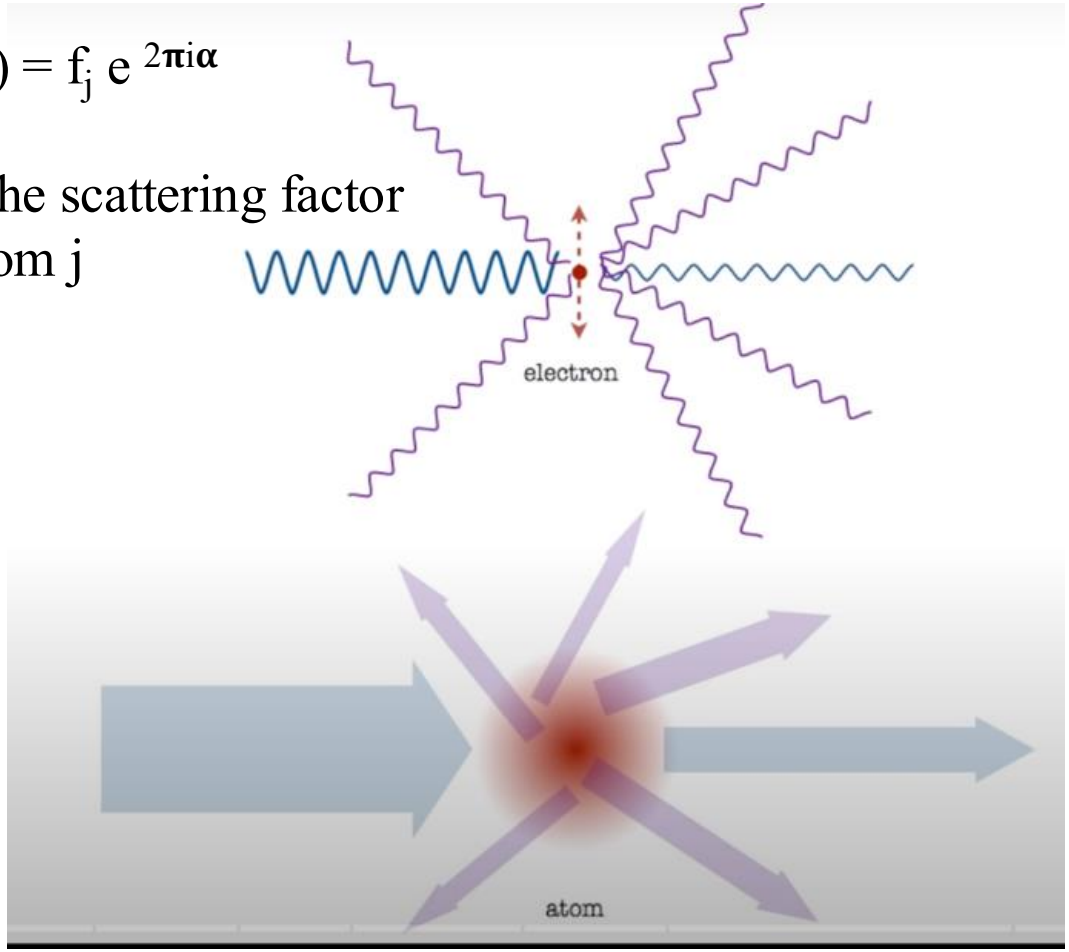
Why x-rays?

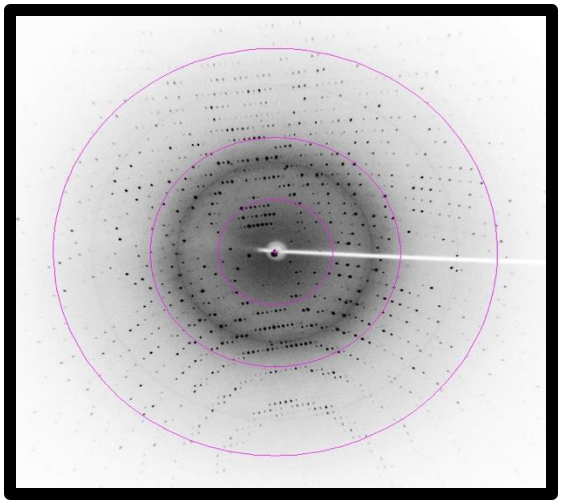


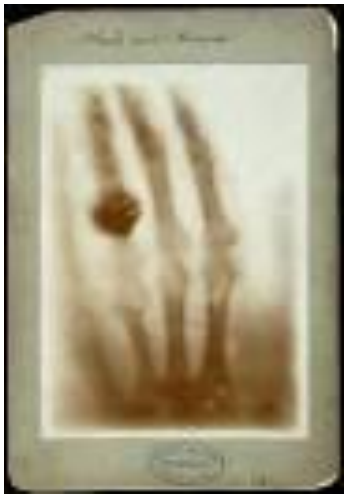
X-ray scattering intensity depends on the atom type

$F_j(S) = f_j e^{2\pi i \alpha}$

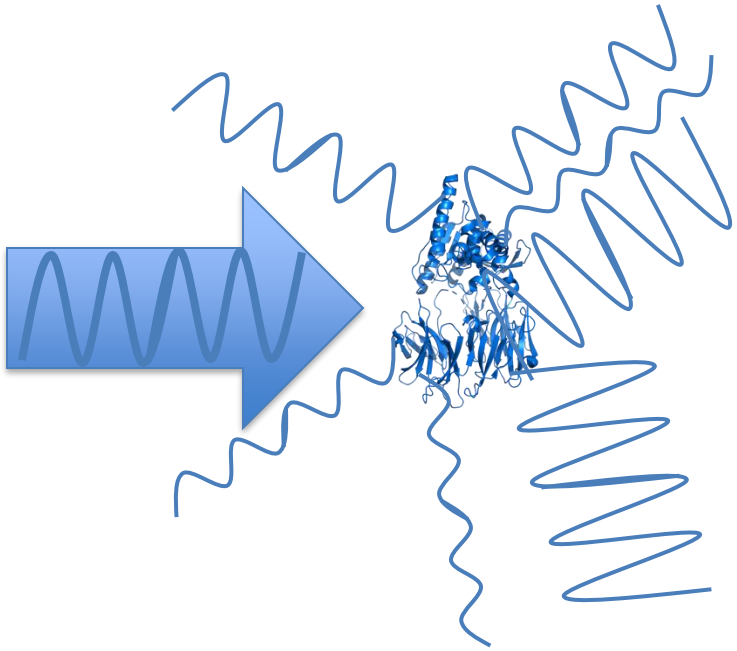
f_j is the scattering factor of atom j



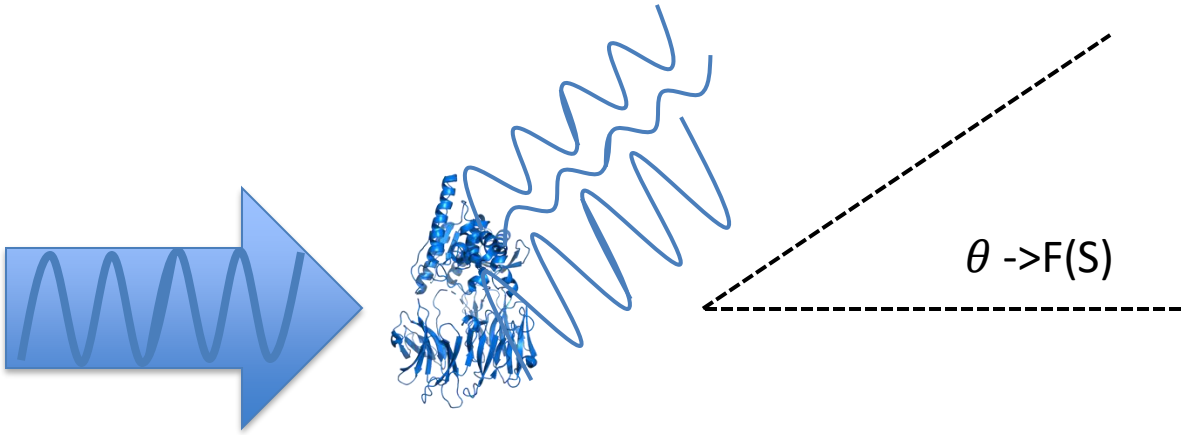




Diffraction from a molecule



Diffraction from a molecule



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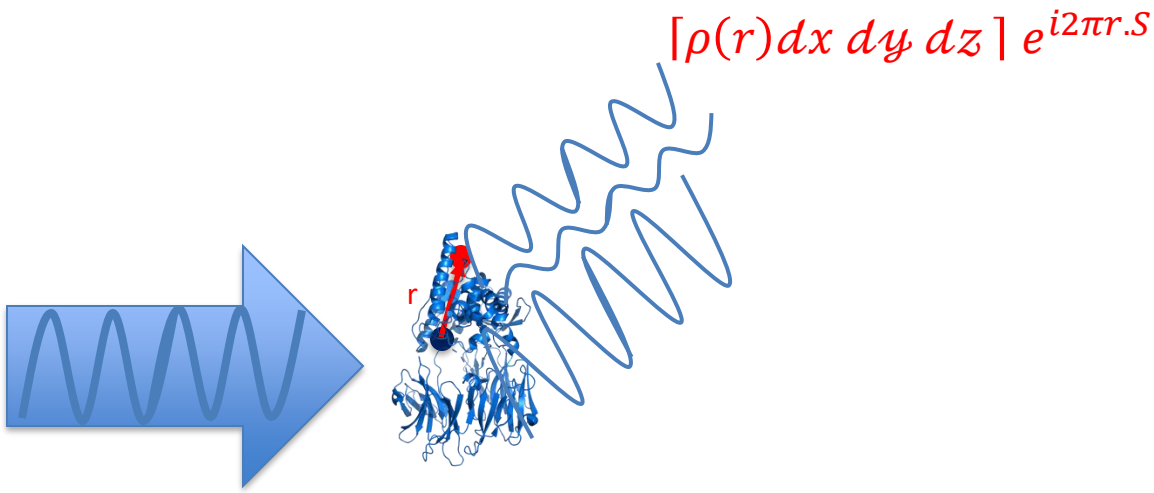
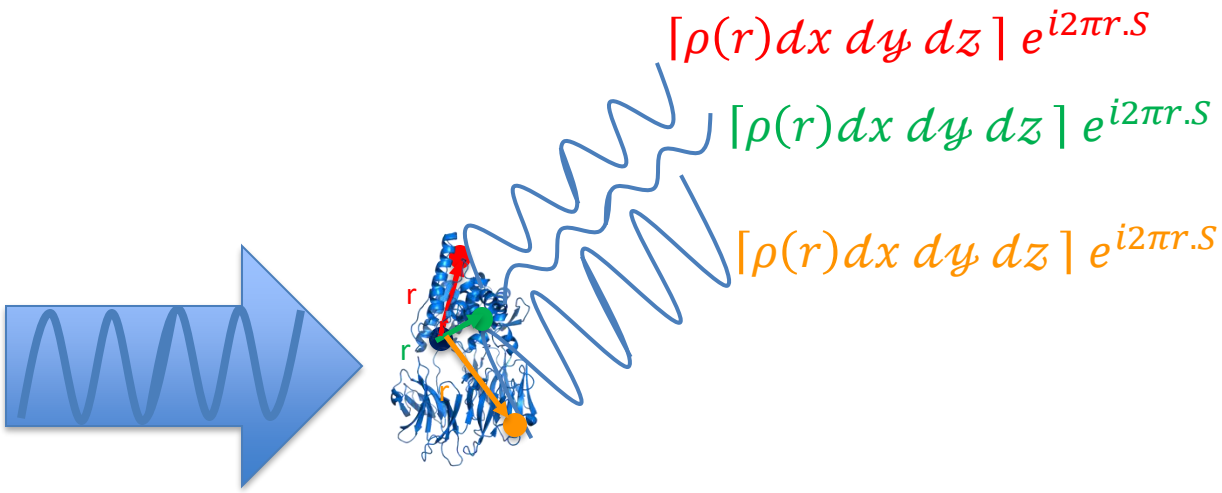
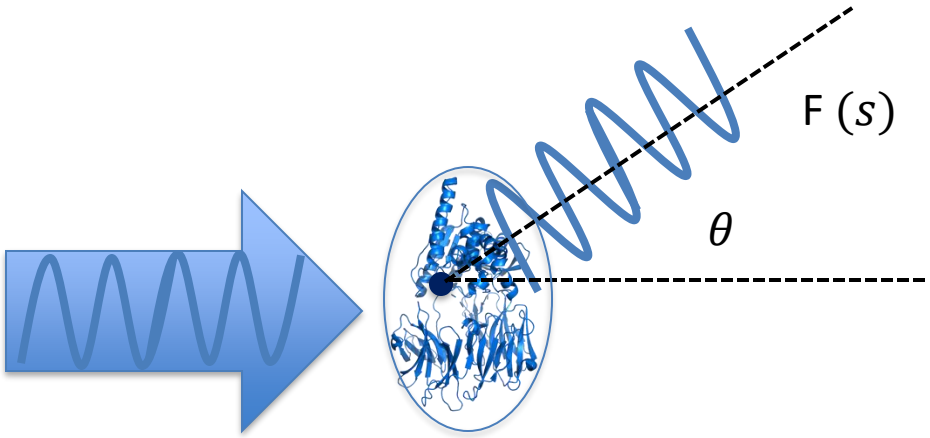


Diffraction from a molecule

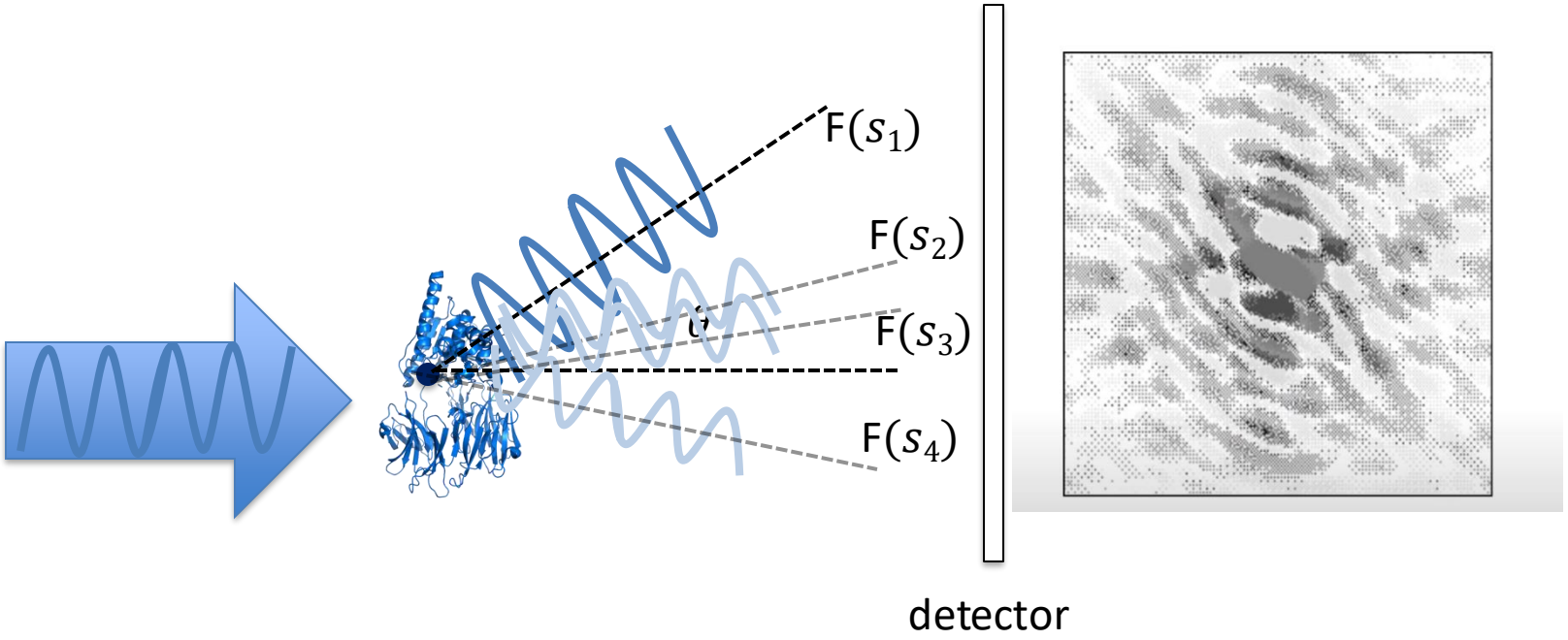


Diffraction from a molecule



$$\mathrm{F}\,(s) = \iiint [\rho(r) dx\, dy\, dz\,]\, e^{i2\pi r.S}$$

Diffraction from a molecule



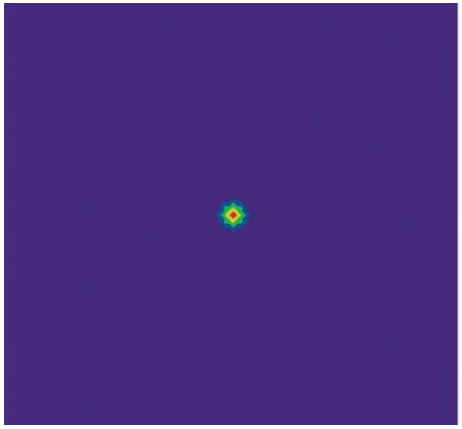
$$F(s) = \iiint \rho(r)\, e^{i2\pi r.S}\, dxdydz$$

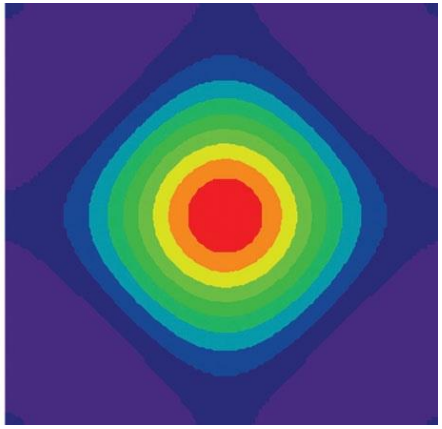
All atoms in the molecule contribute to each diffracted X-ray and to the calculation of each structure factor

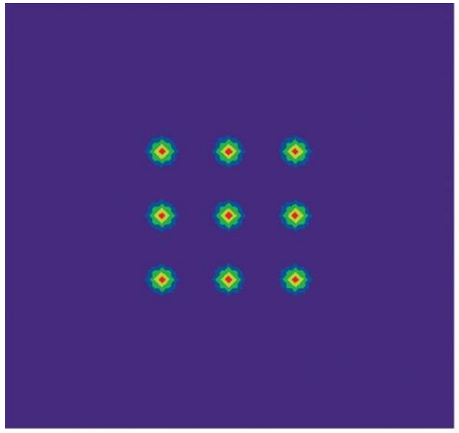
Why do we need protein crystals?

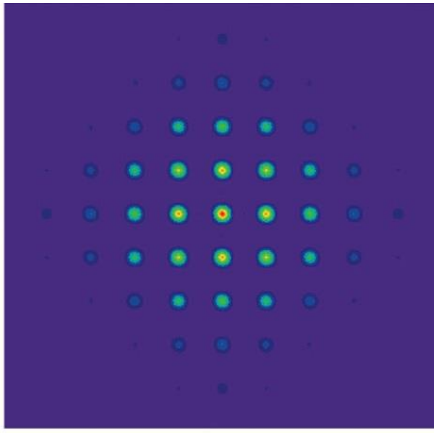
- X-ray diffraction of a single molecule is very weak and yields limited structural information
- By having protein molecules in repeating units in a 3-D array, scattered X-rays cancel each other out in most directions except for discrete diffraction spots ("amplified signal")

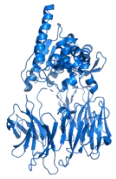
2-D diffraction pattern

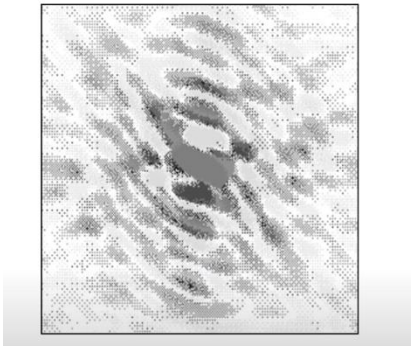


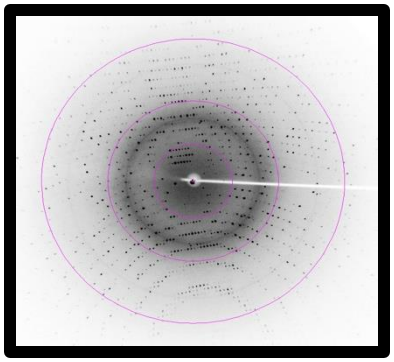












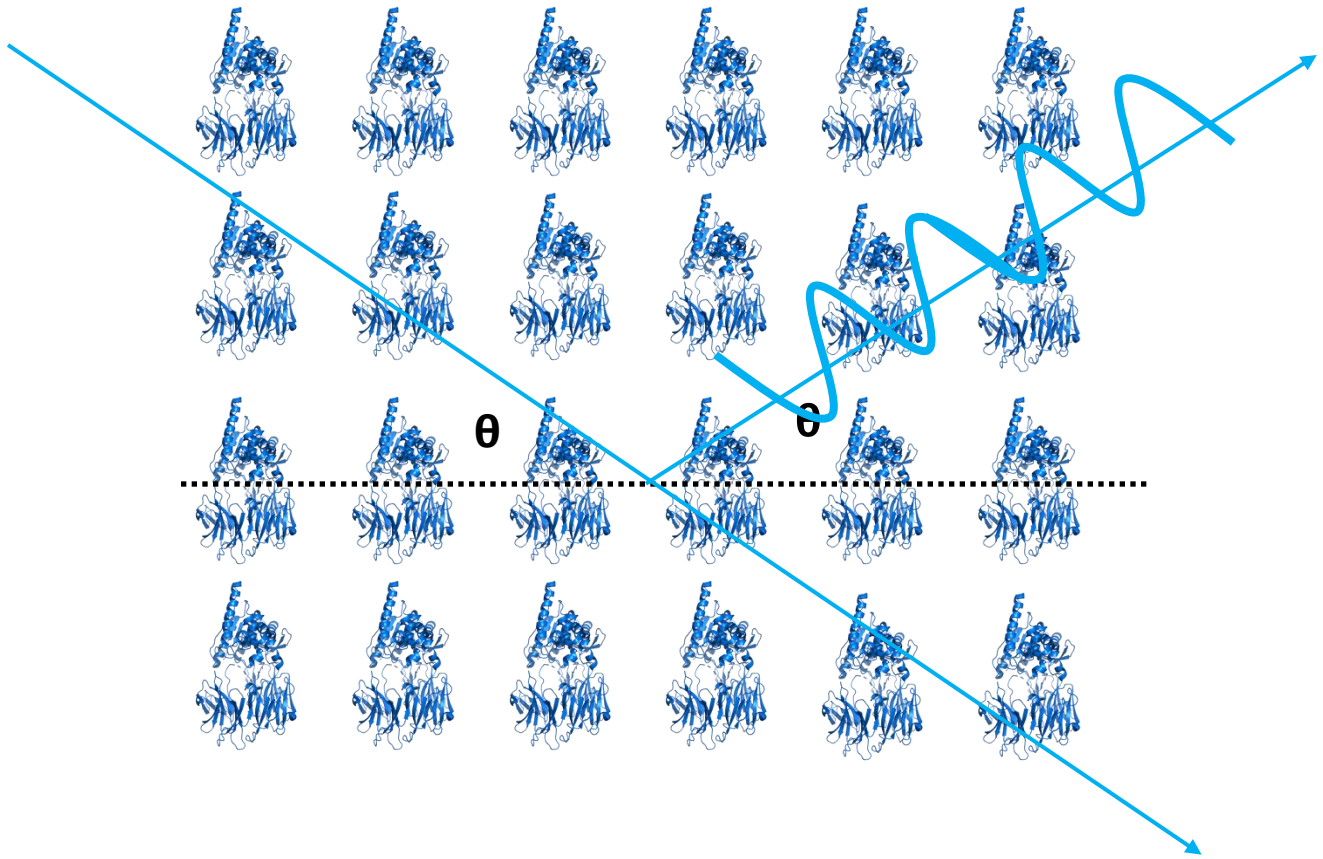
object

Diffraction pattern

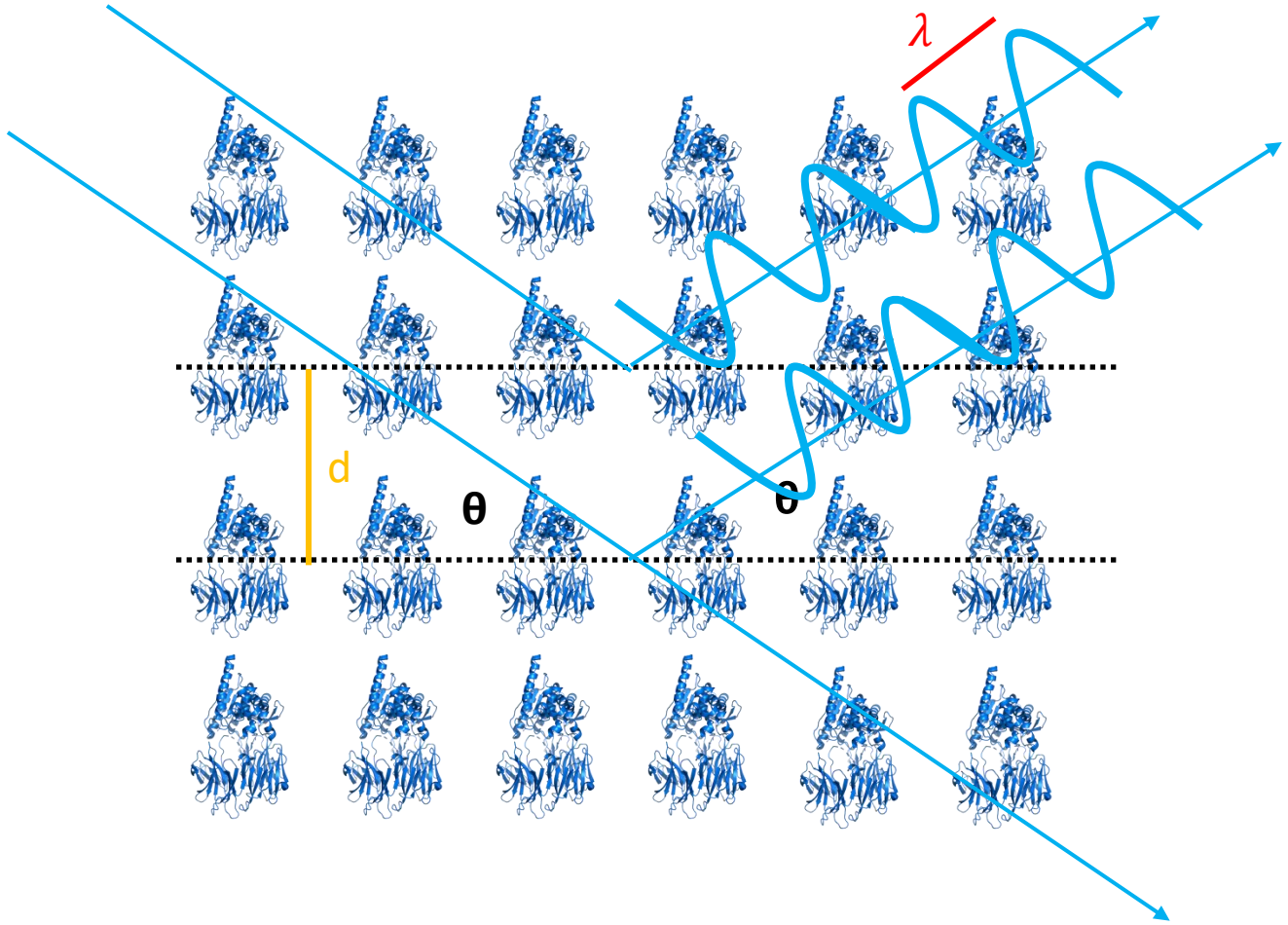
object

Diffraction pattern

Diffraction from a protein crystal

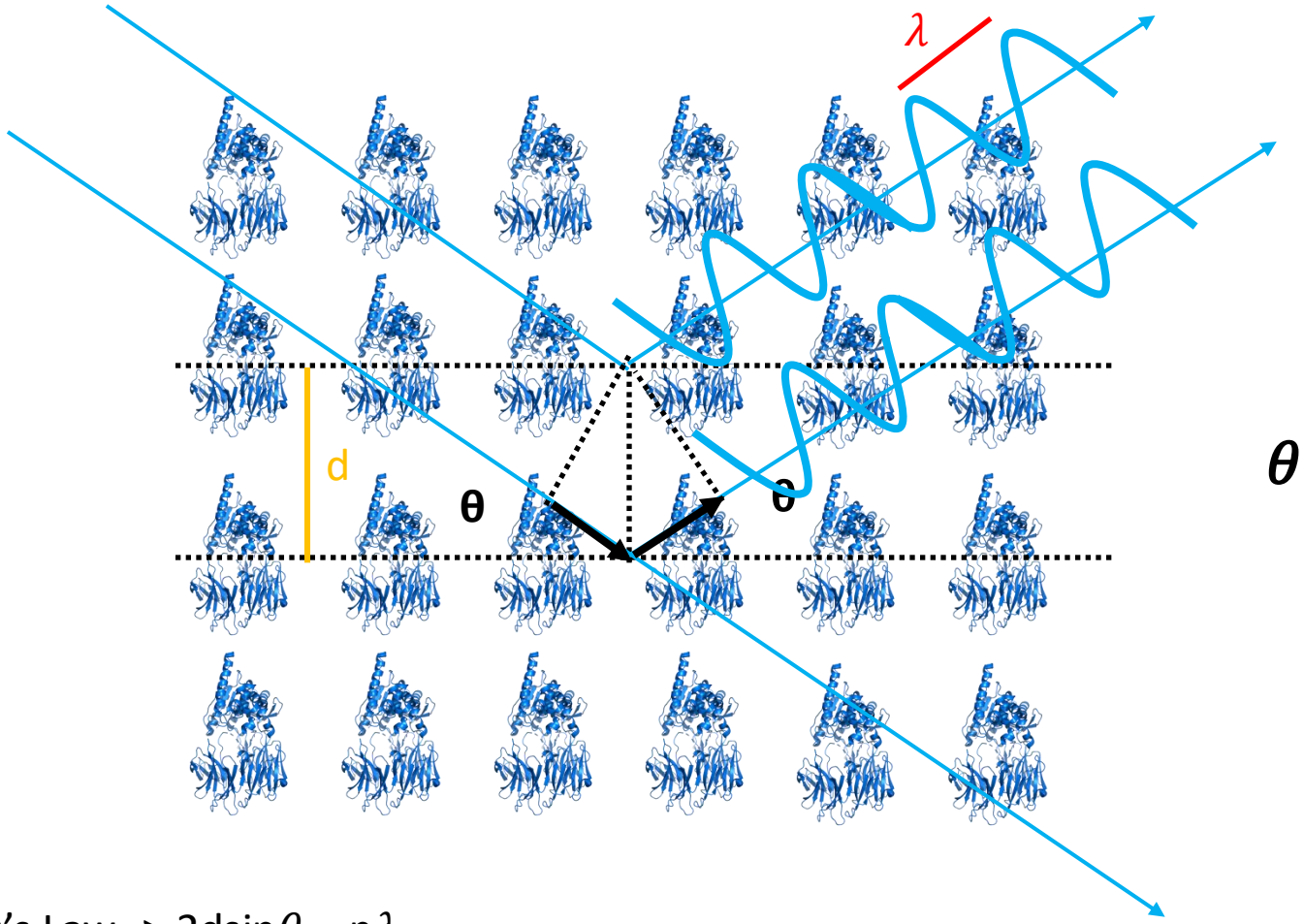


Diffraction from a protein crystal



Bragg's Law

Diffraction from a protein crystal



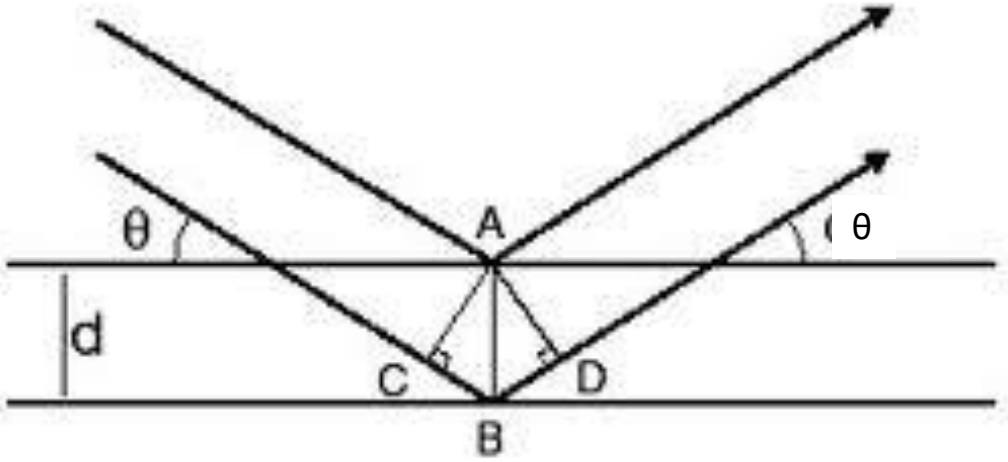
f (S)

$\theta \propto n\lambda/d$

Bragg's Law -> $2d\sin\theta = n\lambda$

X-ray diffraction – Bragg's law

Bragg's Law determines the position of spots



$$\sin\theta = \frac{BD}{AB}$$

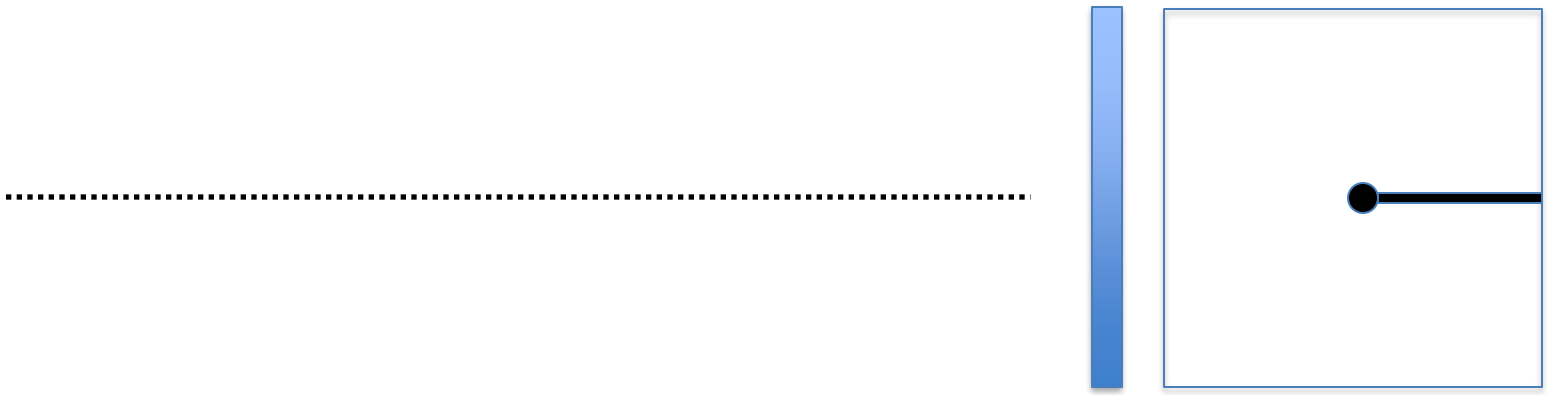
$$BD = AB\sin\theta = d\sin\theta$$

Condition for constructive interference
Path length difference = nλ

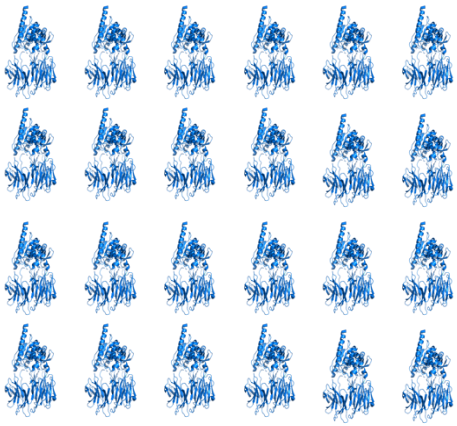
CB+BD=2BD=nλ

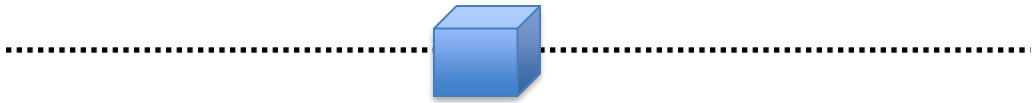
2 d sinθ = nλ

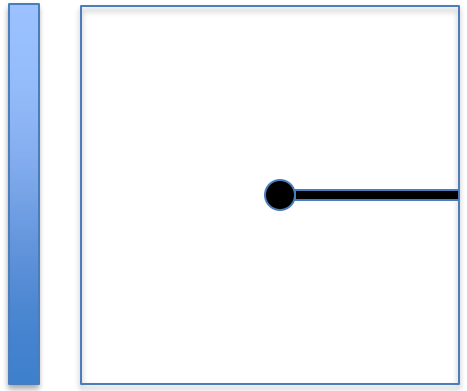
Diffraction from a protein crystal



Diffraction from a protein crystal

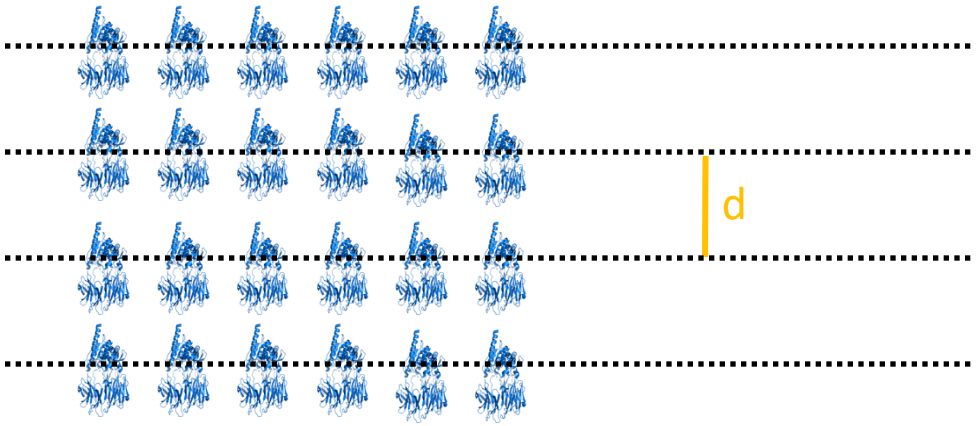


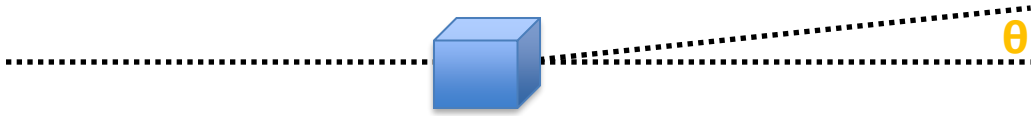


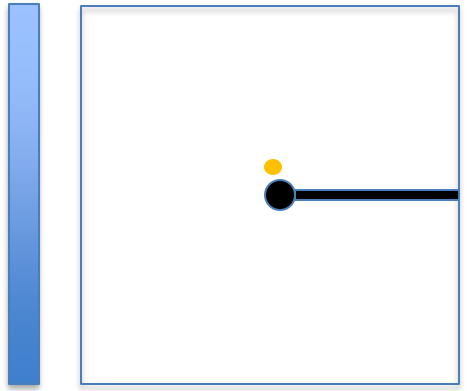


Diffraction from a protein crystal

$\theta \propto n\lambda/d$







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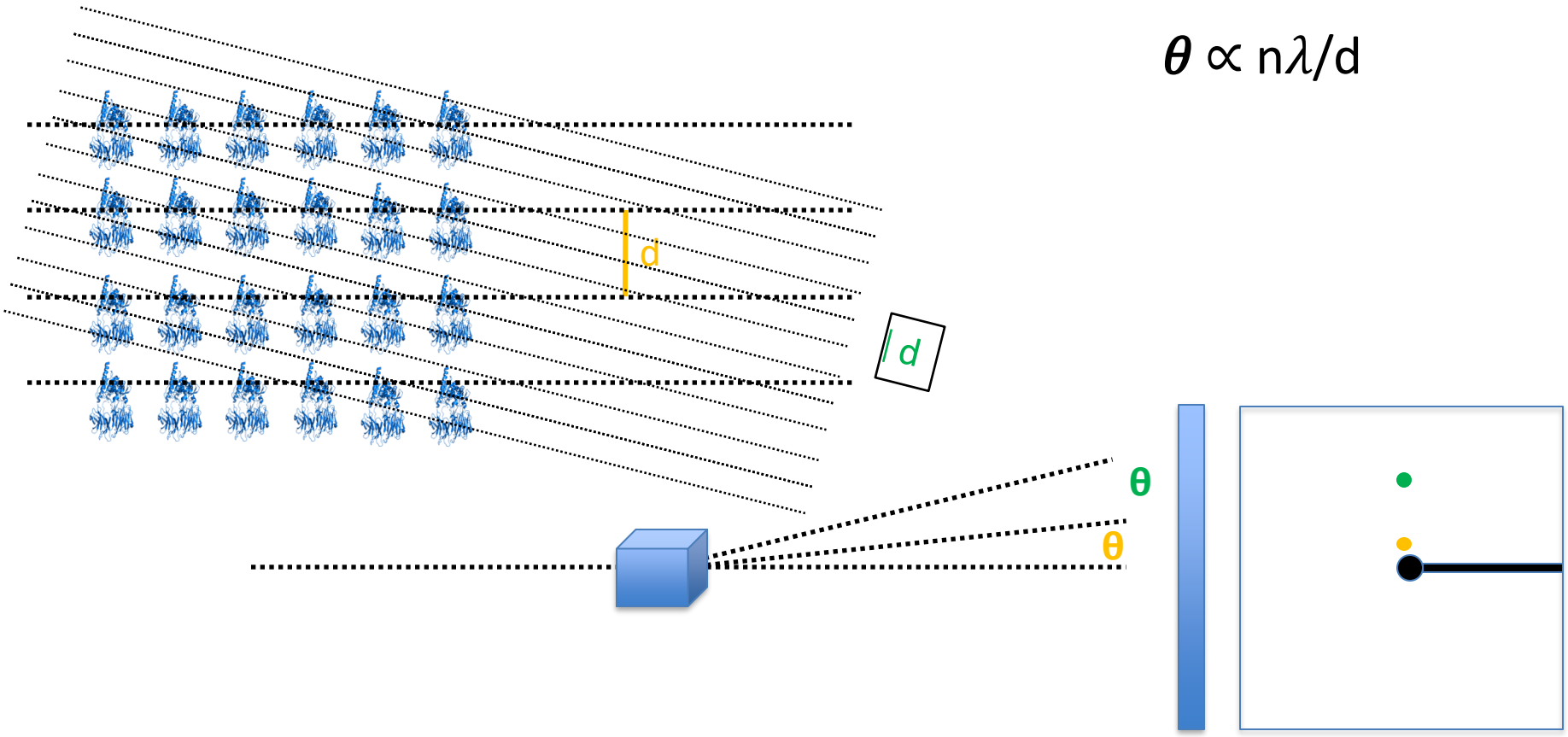
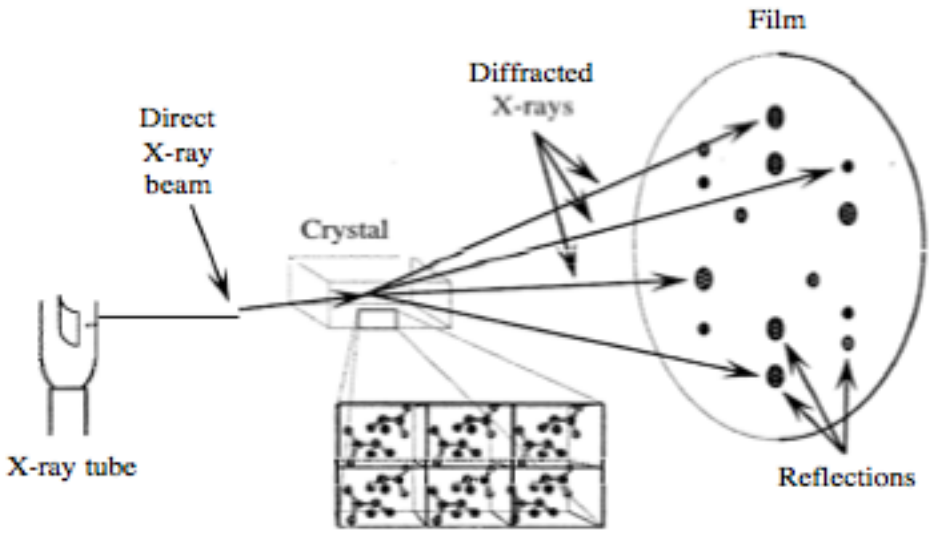


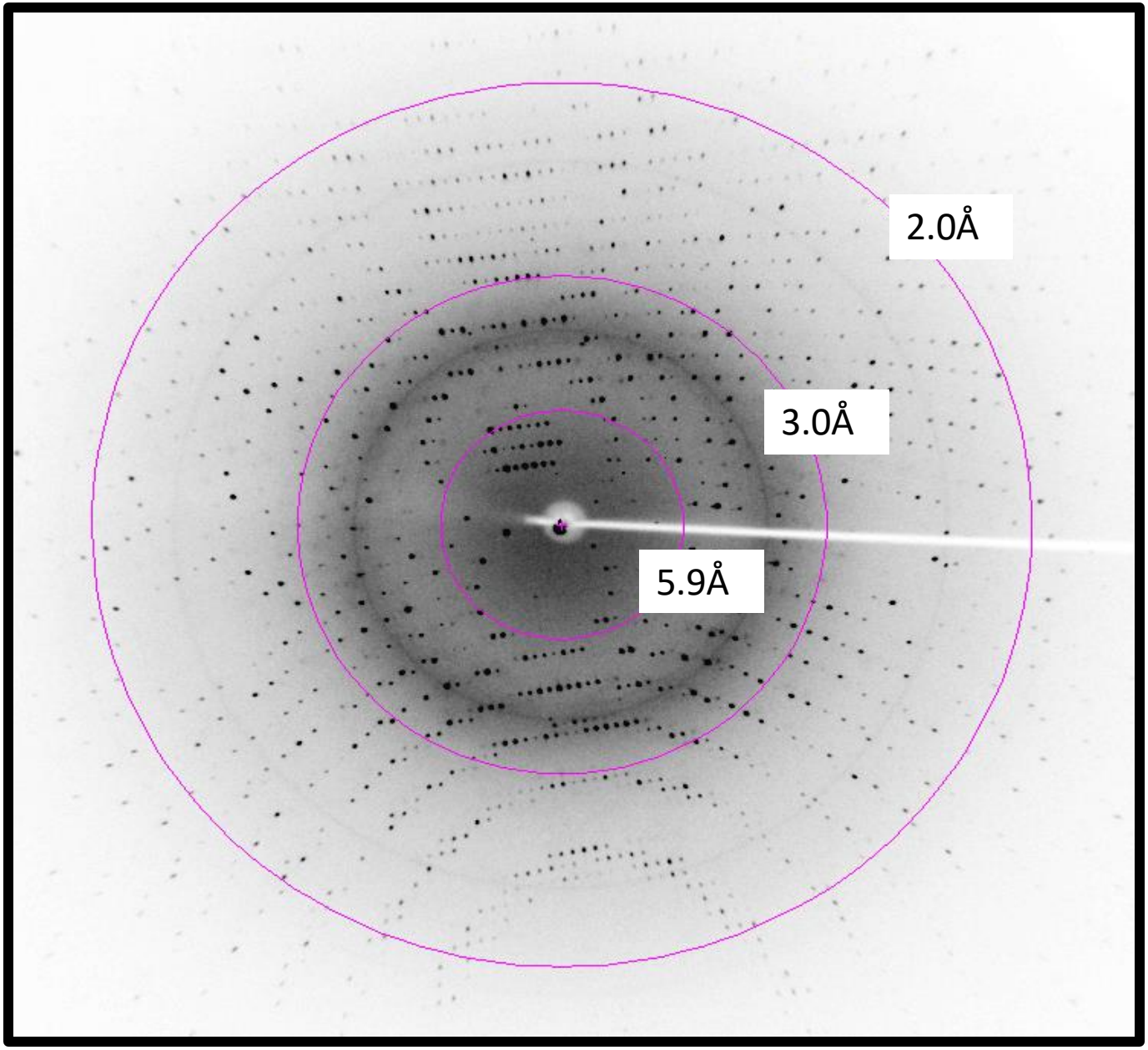
Diffraction from a protein crystal



Rhodes, G. (2006)

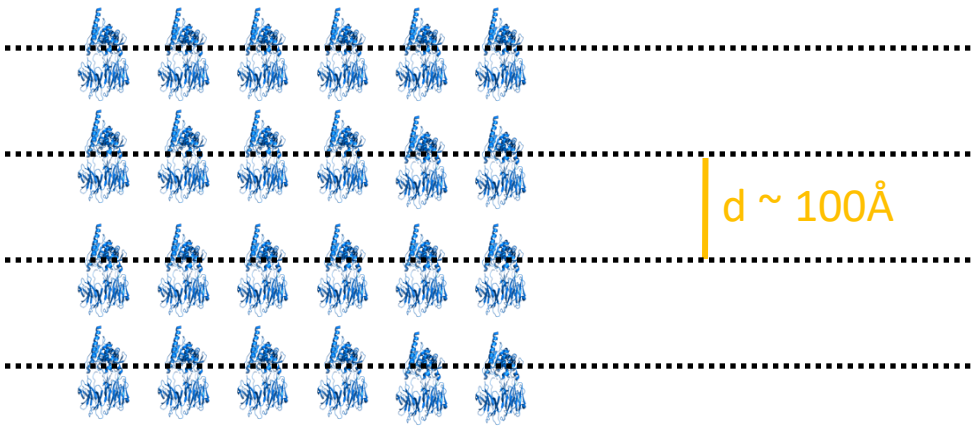
The diffraction pattern

- Diffracted X-rays – discrete points originate from the constructive interference from scattered X-rays by atoms repeated in the crystal lattice
- Bragg's law determines the position of the spots and the content of the crystal determines their intensity.

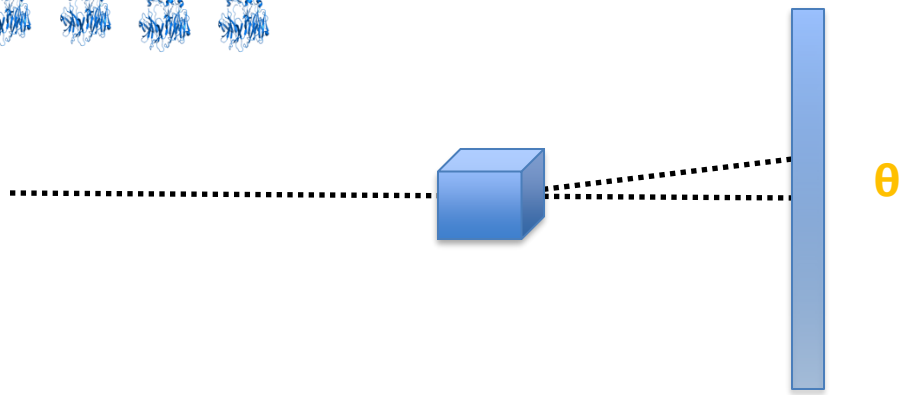

2.0Å
3.0Å
5.9Å

Diffraction from a protein crystal

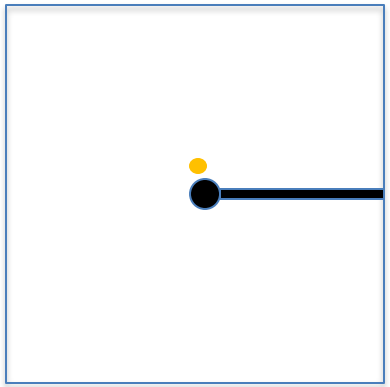
Protein crystal



d ~ 100Å



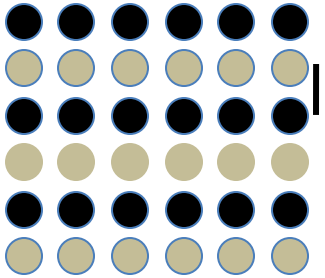
θ



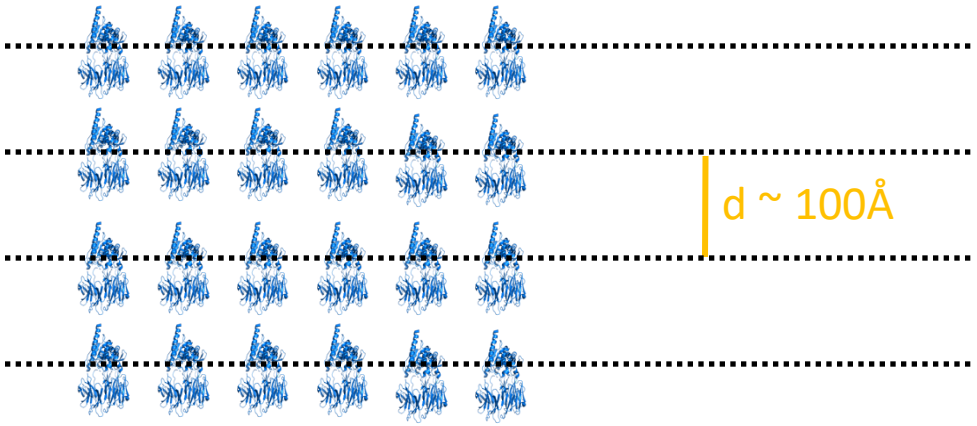
$\theta \propto n\lambda/d$

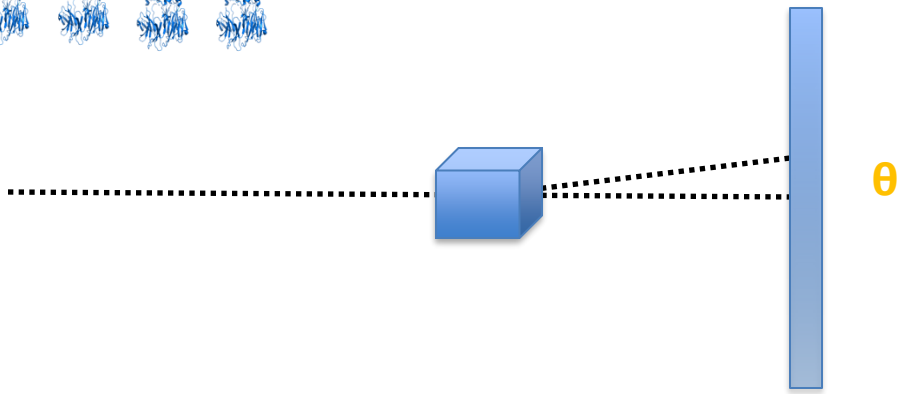
Diffraction from a protein crystal

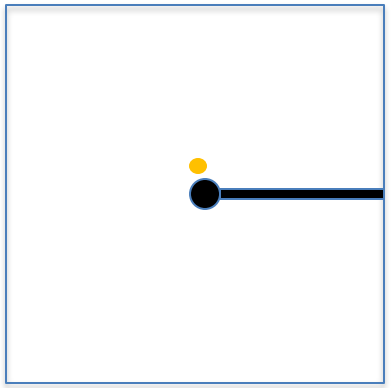
Salt crystal



Protein crystal







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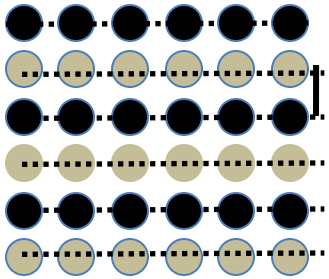
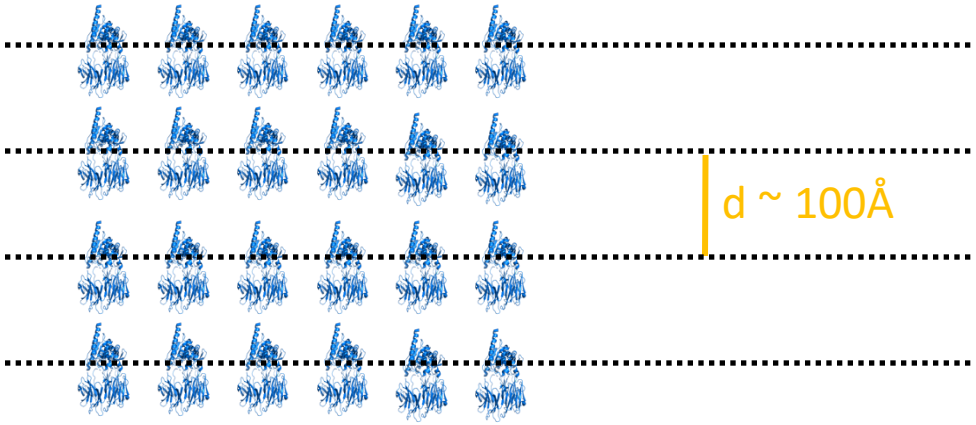
Diffraction from a protein crystal

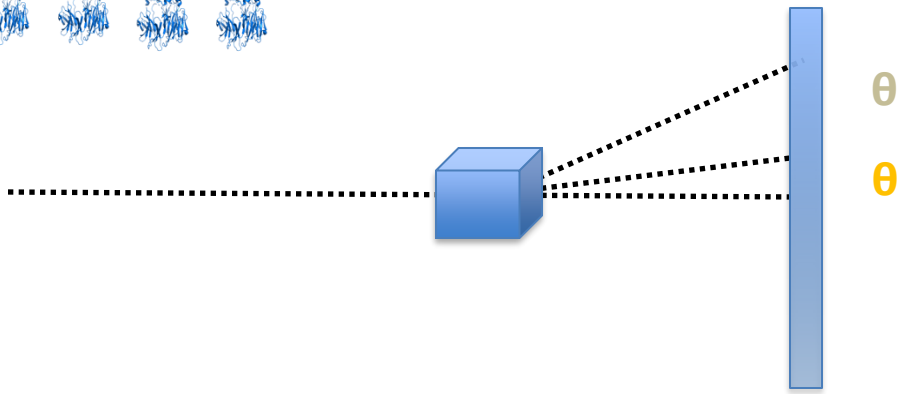
Salt crystal

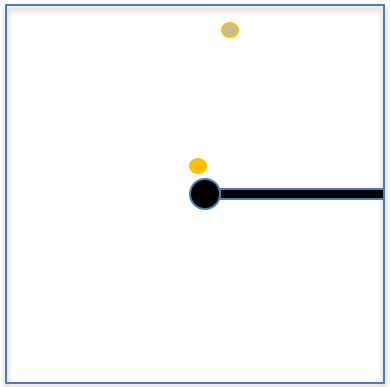


d ~ 1Å
(MAX INTERPLANAR
spacing)

Protein crystal



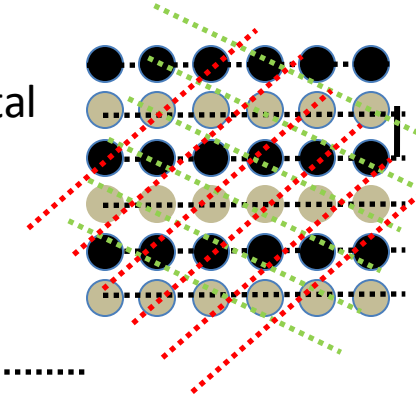




$\theta \propto n\lambda/d$

Diffraction from a protein crystal

Salt crystal



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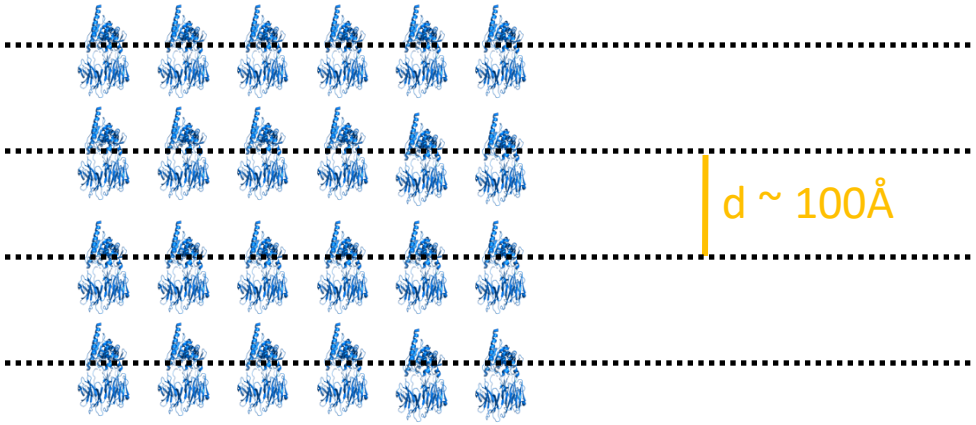
Protein crystal



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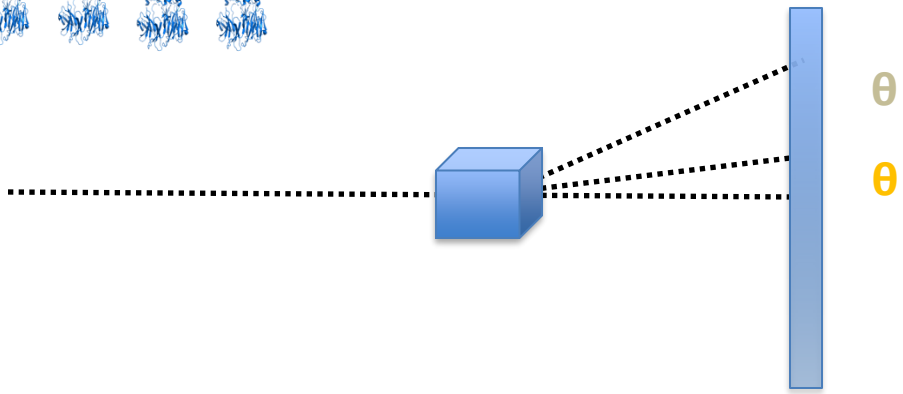
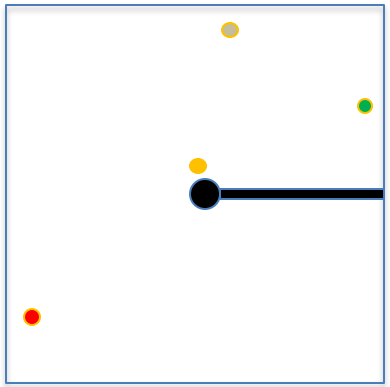


θ

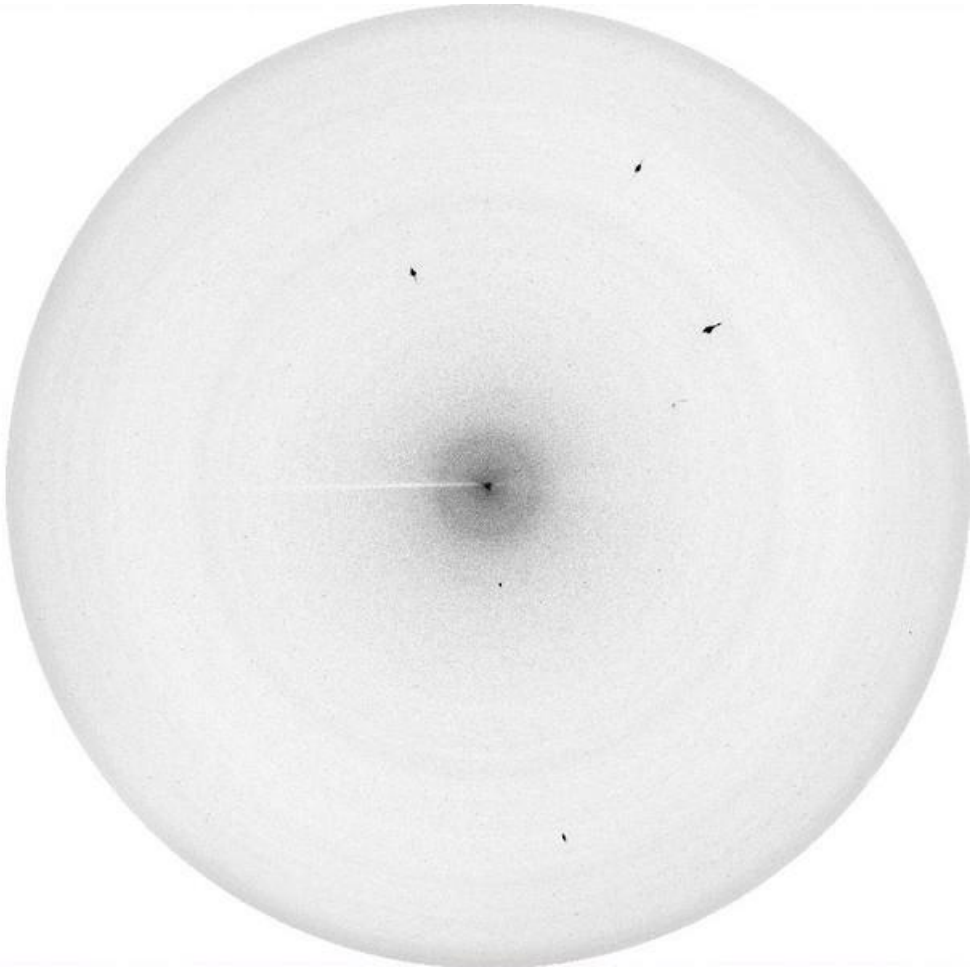
θ



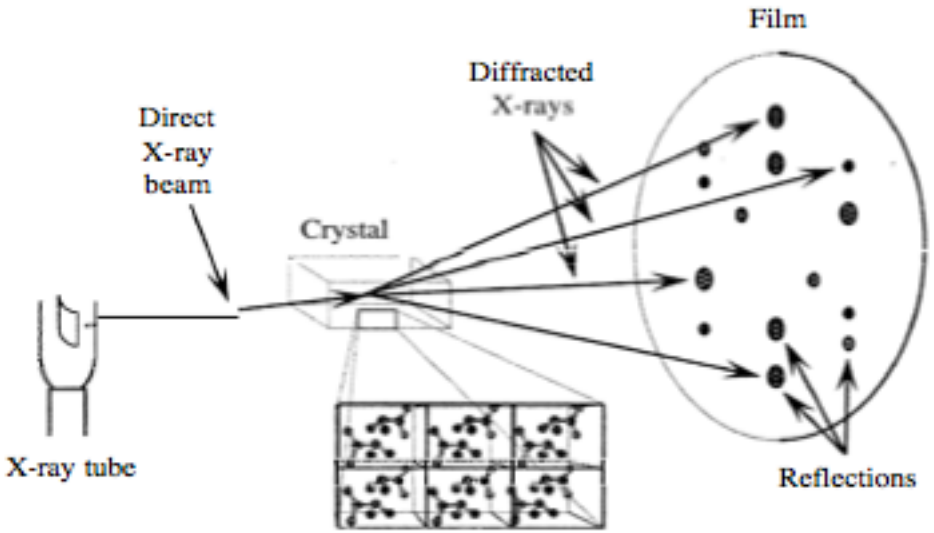
$\theta \propto n\lambda/d$

Salt diffraction

- Only small d, big diffracting angles only:



The diffraction experiment– data collection

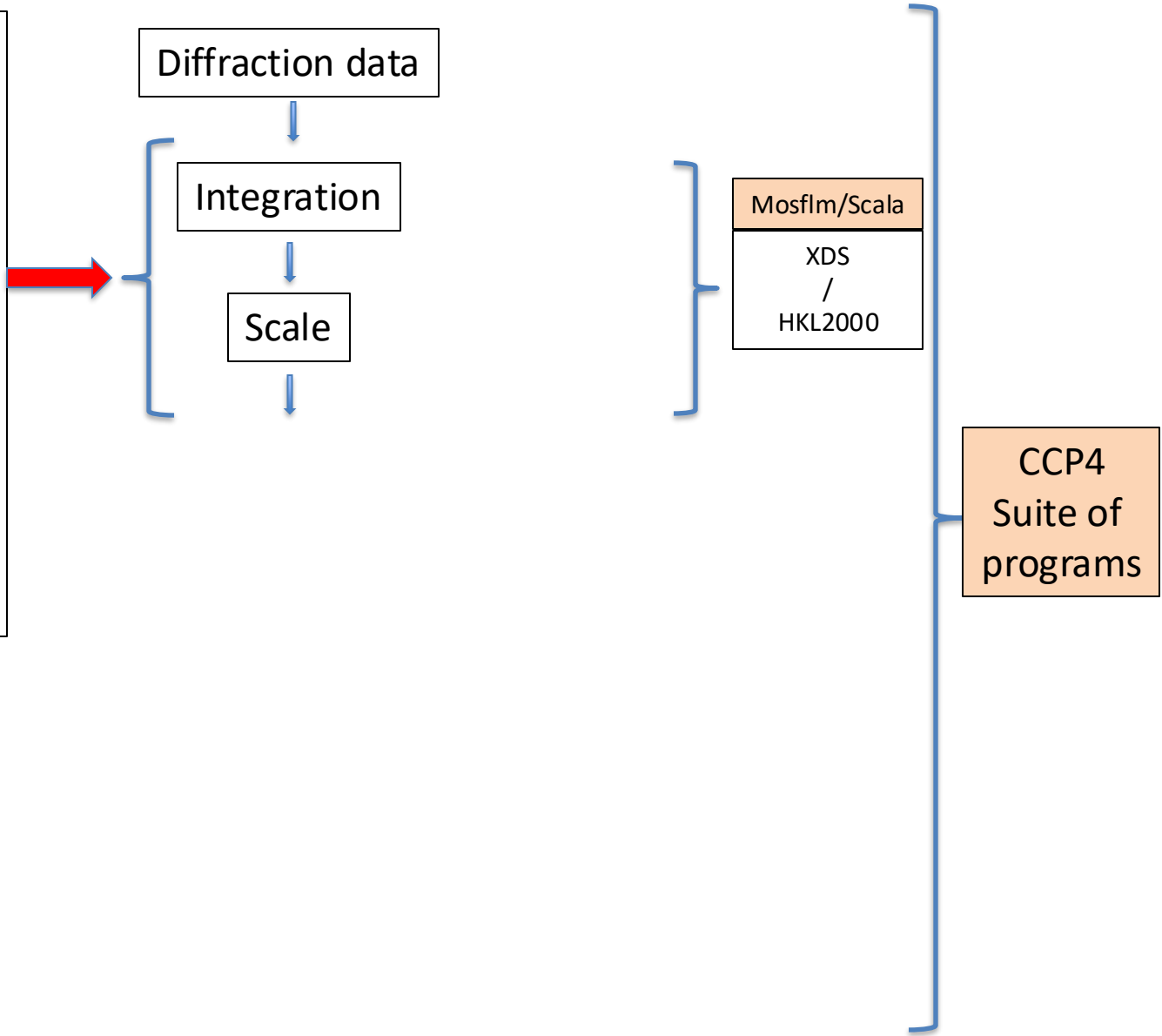


Rhodes, G. (2006)

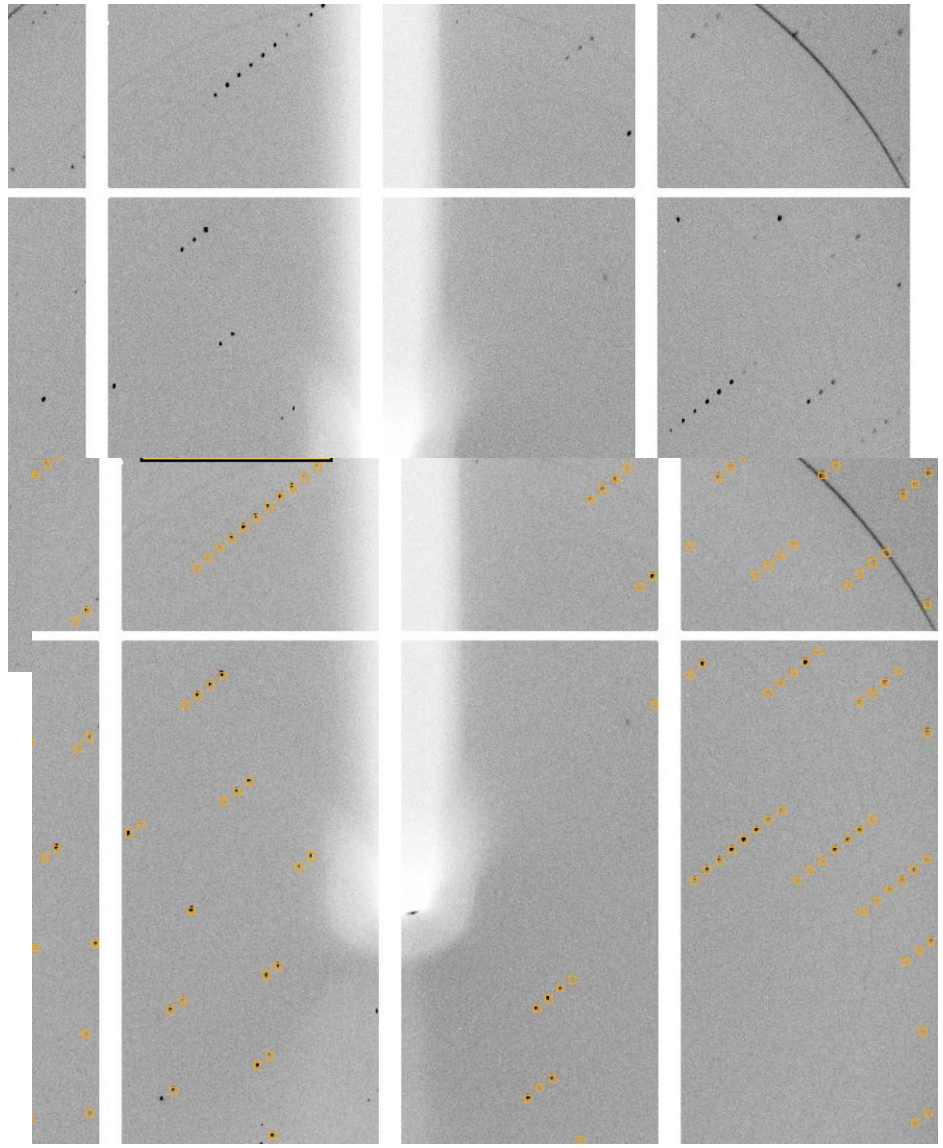
Crystal rotates around itself in order to collect all possible reflections – rotate the Ewald Sphere- collect a full data set
The rotation range depends on the symmetry of the crystal – higher symmetry less rotation range necessary

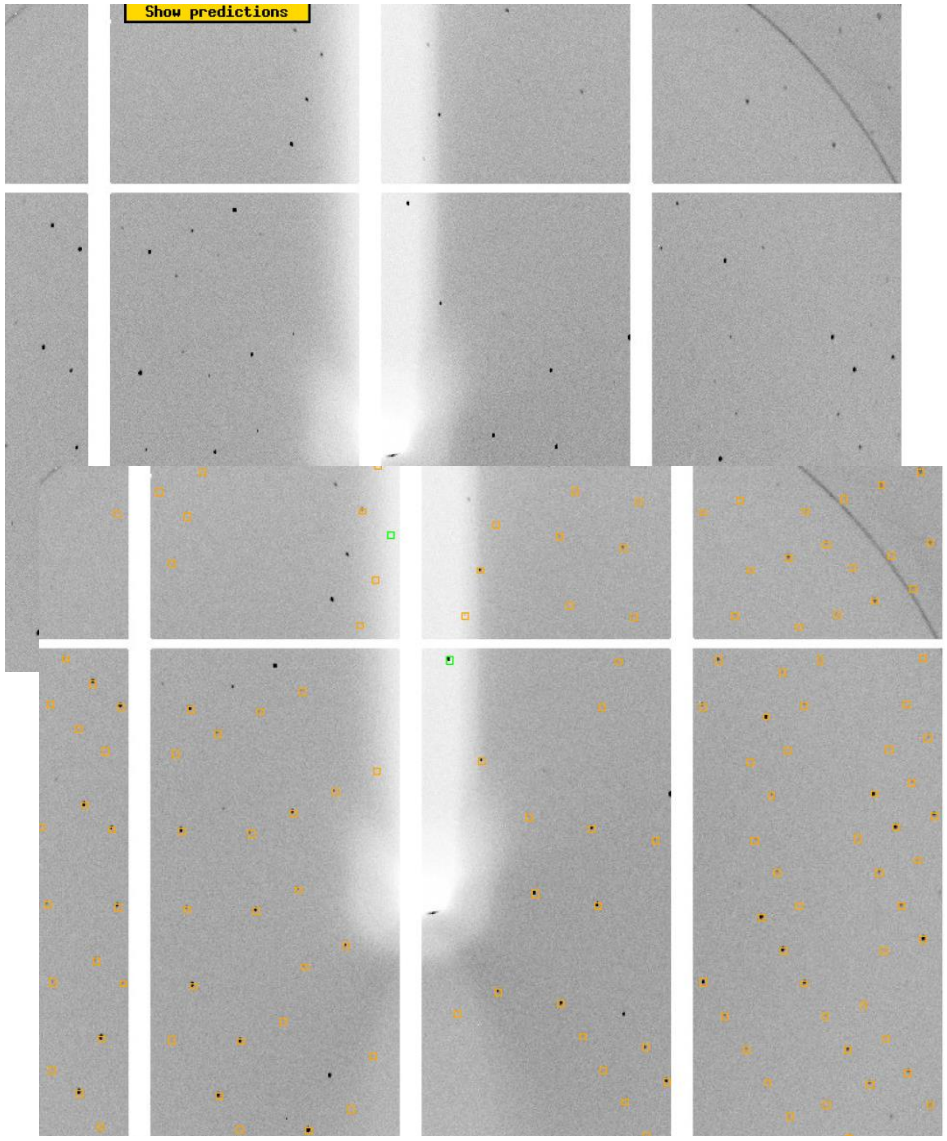
Data processing and statistics

-Calculate cell parameters
-Refine cell parameters
-Calculate space group;
-Scale intensities and errors;
-Transform intensities to structure factor amplitudes;
-OUTPUT file: list of measured reflections with their intensities and their errors. 'Mtz file'



Data processing and statistics





Data processing and statistics

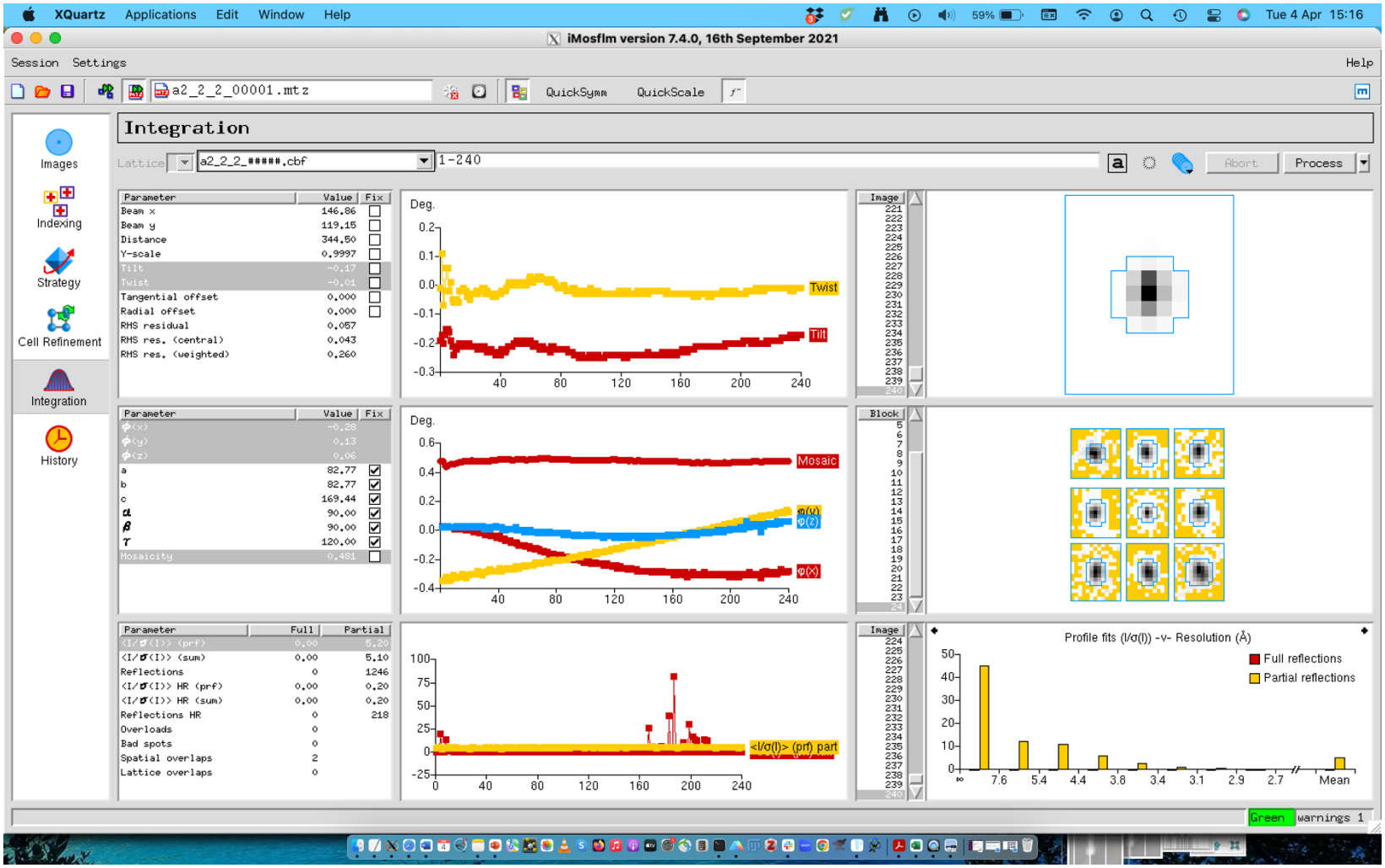
X-ray source & Detector
Wavelength (Å)
Space group
Cell (Å °)
Resolution range (Å)
Number of observations
Unique reflections
Completeness
Rmerge
Mean I/σ(I)

Statistics for data set and high resolution shell

Completeness should be around 95% and most of the time 100%
Rmerge should be ≤10%
Mean I/σ(I) (signal to noise ratio) should be ≥2

$$R_{merge} = \frac{\sum_{hkl}\sum_{i=1}^{N}\left| I_{(hkl)i} - \bar{I}_{(hkl)} \right|}{\sum_{hkl}\sum_{i=1}^{N} I_{(hkl)I}}$$

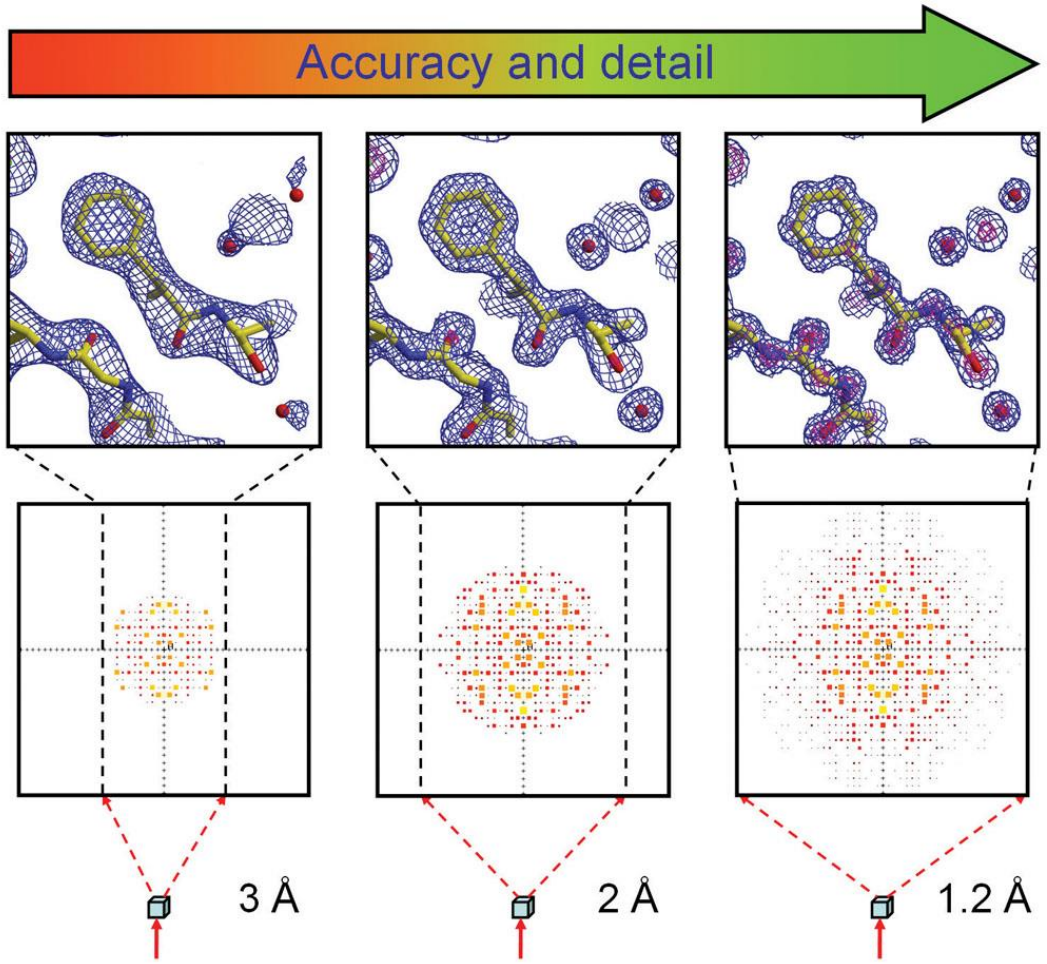
Data processing and statistics



Data processing and statistics

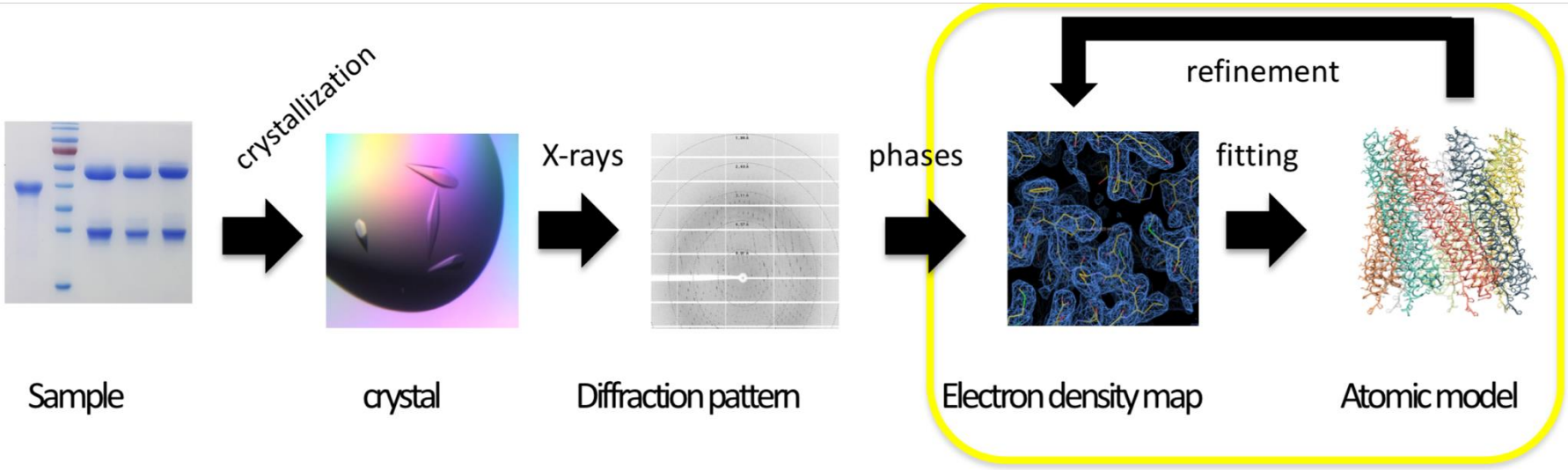
	Aurora A	**SPF**
X-ray source & Detector	SLS PXIII Pilatus	SLS PXIII Pilatus
Wavelength (Å)	1	1
Space group	$P6_122$	$P2_1$
Cell (Å °)	82.3 82.3 169.4 90 90 120	71.9 73.8 94.4 90 96.3 90
Resolution range (Å)	66-2.9 (3-2.9)	30 -1.6 (1.7-1.6)
Number of observations	92281	401202
Unique reflections	8204	128933
Completeness	100%	99%
Rmerge	12.5% (90%)	5% (30%)
Mean I/σ(I)	14.2 (2.5)	11.5 (2.8)

X-ray diffraction - resolution

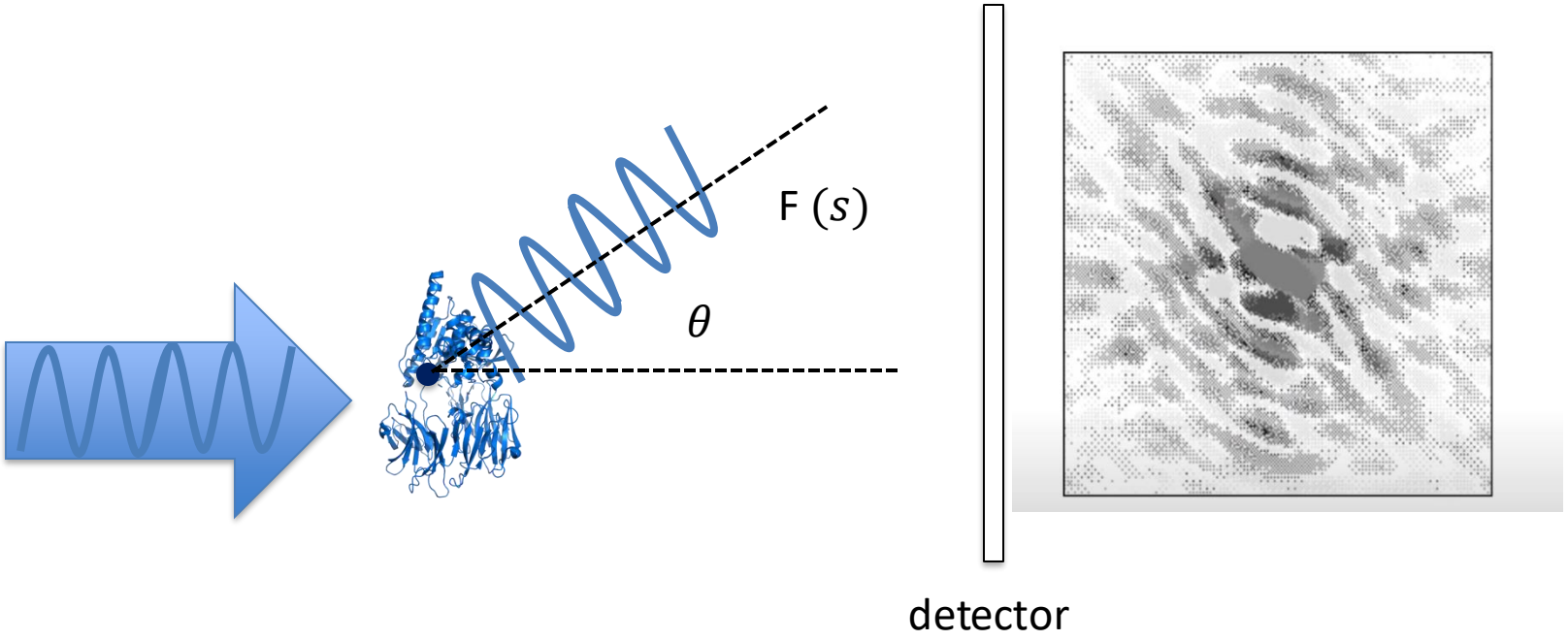


Content of lectures

- **Why x-rays and why crystals?**
- **Macromolecular crystallization**
- **Crystal packing**
- **The diffraction experiment**
- The phase problem
- Molecular replacement
- Refinement and validation


crystallization
X-rays
phases
refinement
fitting
Sample
crystal
Diffraction pattern
Electron density map
Atomic model

Diffraction from a molecule



$$F(s) = \iiint \rho(r)\, e^{i2\pi r.S}\, dxdydz$$

All atoms in the molecule contribute to each diffracted X-ray and to the calculation of each structure factor

Fourier Transform

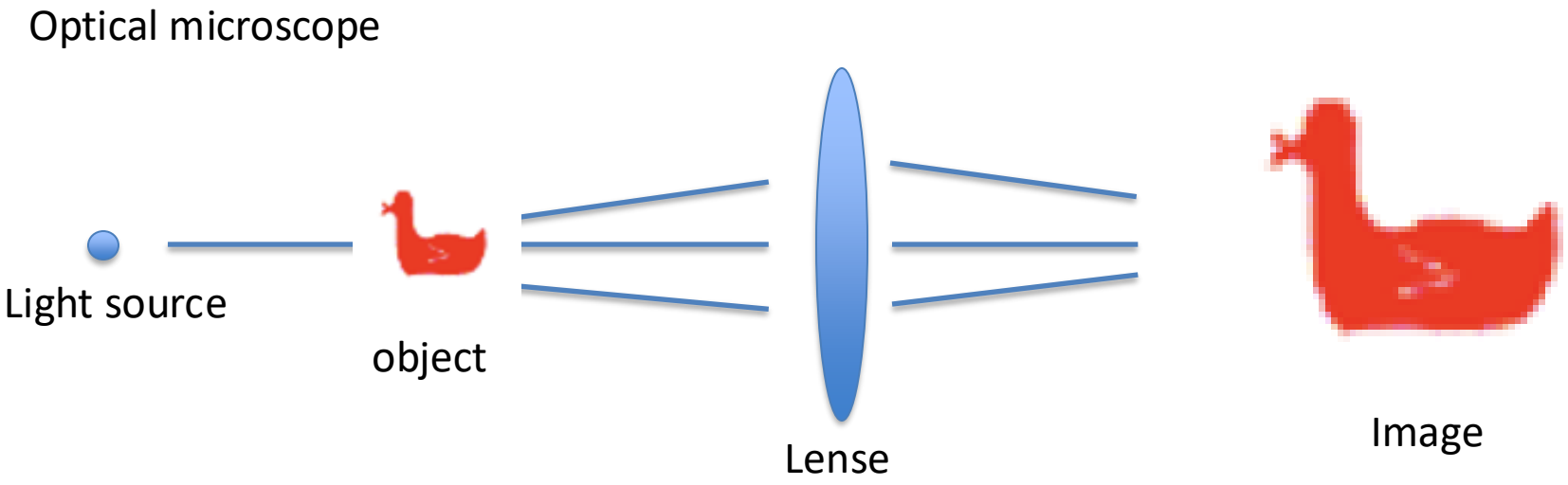
- The structure factor is the Fourier transform of the electron density. The electron density is the inverse Fourier transform of the structure factor.

$$F(s) = \iiint \rho(r)\, e^{i2\pi r.S}\, dxdydz \qquad F(S) = FT\ \rho\ (x,y,z)$$

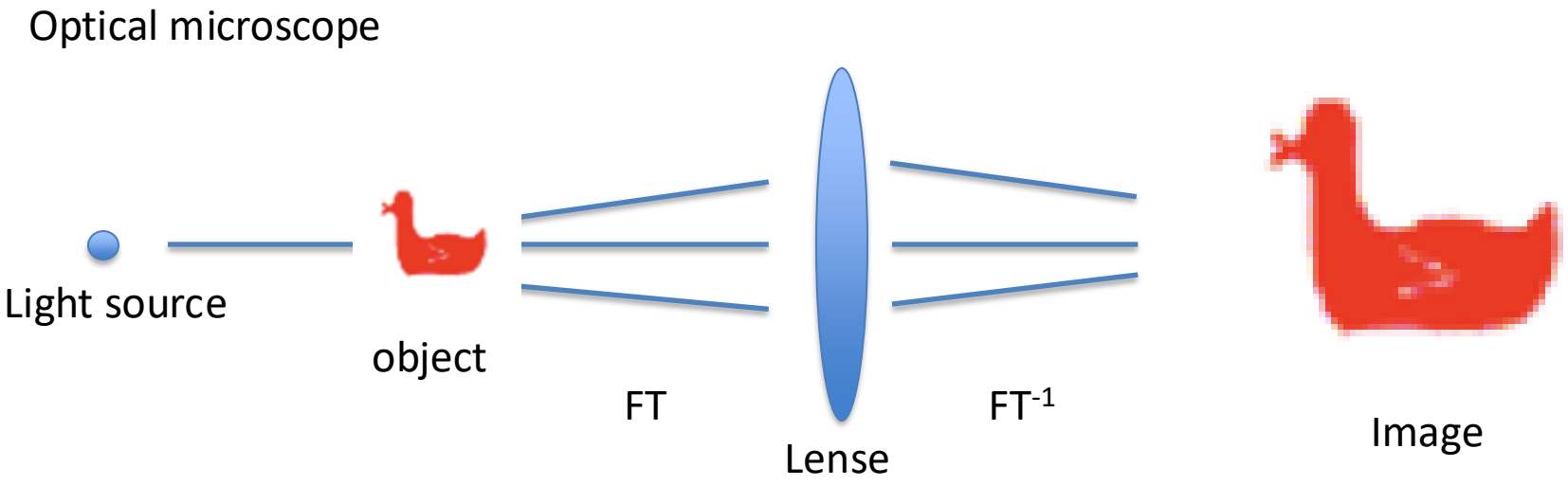
$$\rho(r) = \iiint F(s)\, e^{-i2\pi r.S}\, dS \qquad \rho\ (x,y,z) = FT^{-1}\ F(S)$$

ρ(r) or ρ(x,y,z) is in real space (Å), F (S) is in reciprocal space ($Å^{-1}$).

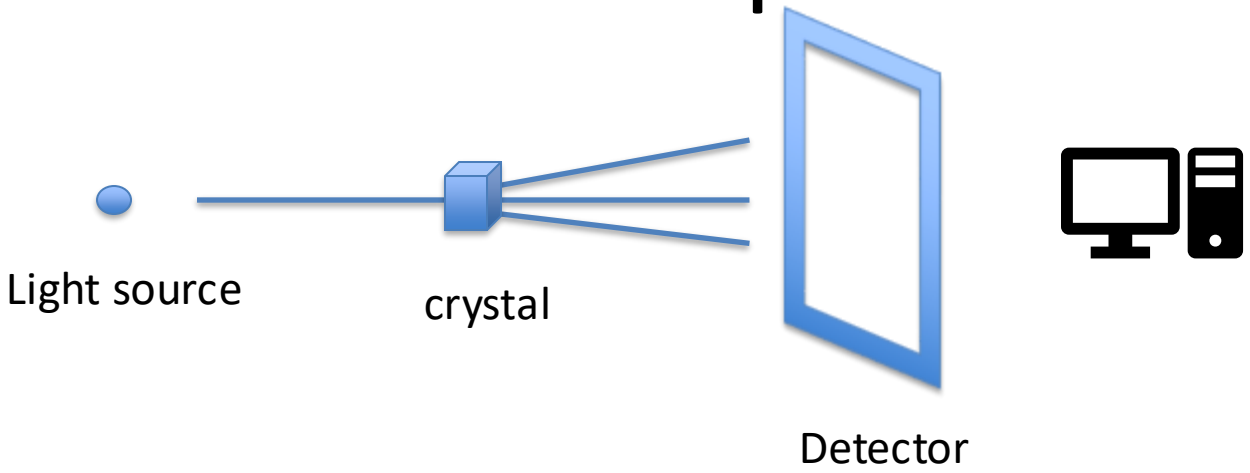
The diffraction experiement



The diffraction experiement

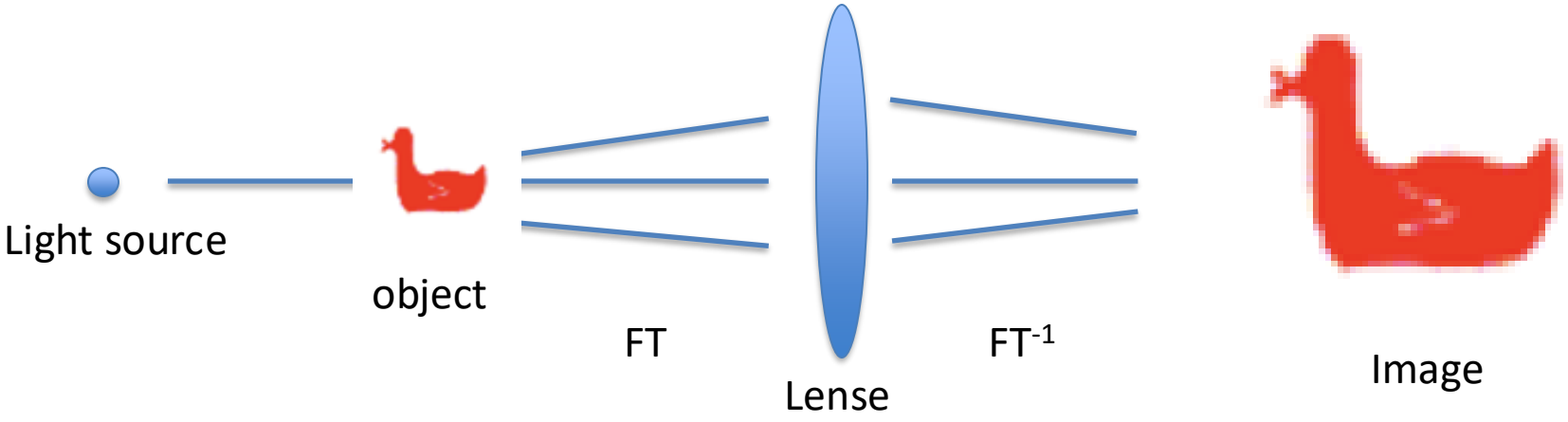


The diffraction experiement



Optical microscope

NO lense that can focus x-rays



The electron density calculation

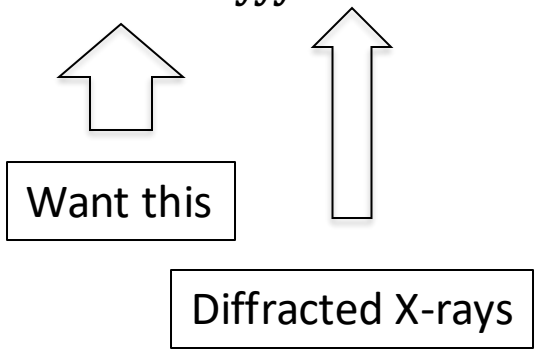
- The structure factor is the Fourier transform of the electron density. The electron density is the inverse Fourier transform of the structure factor.

$$F(s) = \iiint \rho(r)\, e^{i2\pi r.S}\, dxdydz$$

F(S) = FT ρ (x,y,z)

$$\rho(r) = \iiint F(s)\, e^{-i2\pi r.S}\, dS$$

ρ (x,y,z) =FT $^{-1}$ F(S)



So what is the problem??

The phase problem

$$\rho(xyz) = FT^{-1}\, F(S)$$

The phase problem

$$\rho(xyz)=FT^{-1}\, f(S)$$

- F(S) is the structure factor that represents the diffracted x-rays and it is a wave with amplitude and phase (a complex number)

$$F(S)=A\, e^{2\pi i\alpha}$$

The phase problem

$$\rho(xyz)=FT^{-1}\,F(S)$$

- $\mathbf{F}_{hkl}$ is the structure factor that represents the diffracted X-rays and it is a wave with amplitude and phase (a complex number)

$$F(S)=A\,e^{2\pi i\alpha}$$

- In the diffraction experiment , we collect the intensity of the diffracted X-ray which is proportional to the module squared of the structure factor

$$I\approx |F(S)|^2 \approx A\,e^{2\pi i\alpha}\cdot A\,e^{-2\pi i\alpha}\approx A^2$$

$A=A_o$

The phase problem

$$\rho(xyz)=FT^{-1}\,F(S)$$

- $\mathbf{F}_{hkl}$ is the structure factor that represents the diffracted X-rays and it is a wave with amplitude and phase (a complex number)

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$$I\approx |F(S)|^2 \approx A\,e^{2\pi i\alpha}\cdot A\,e^{-2\pi i\alpha}\approx A^2$$

- We only record the amplitude of the structure factor, the phase information is lost!!

$$A=A_o$$

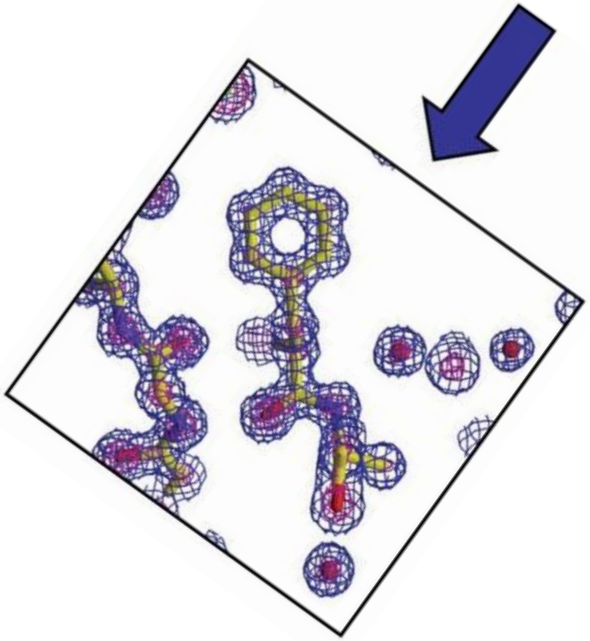
The phase problem

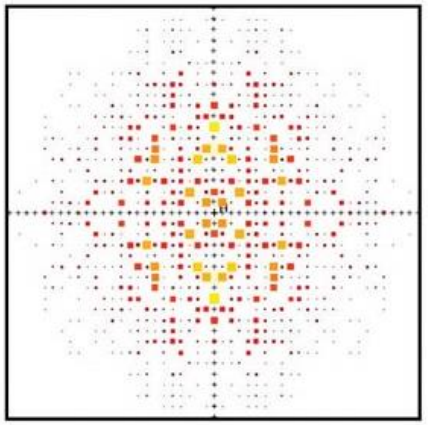
$$\rho(xyz) = FT^{-1}\, F(S)$$

$$\rho(xyz) = FT^{-1}\, A_o\, e^{2\pi i \alpha}$$

α

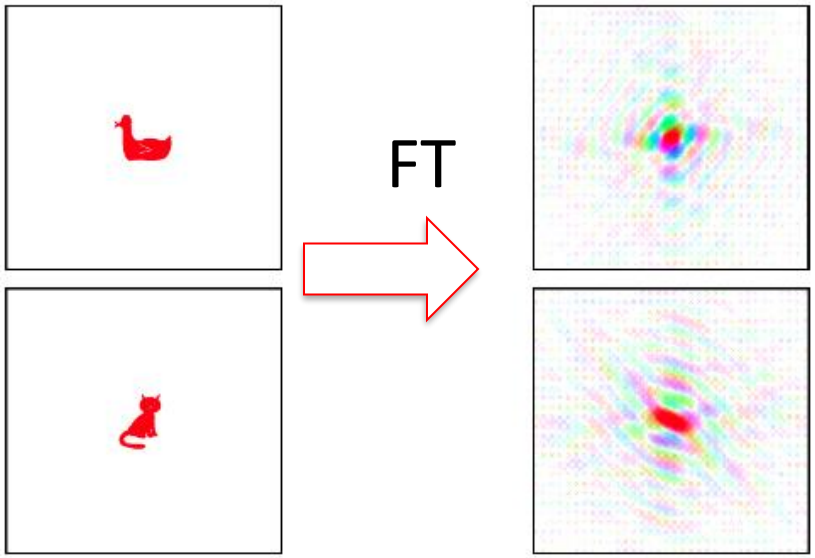
???





The phase problem

$F(S) = A_o\, e^{2\Pi i \alpha}$

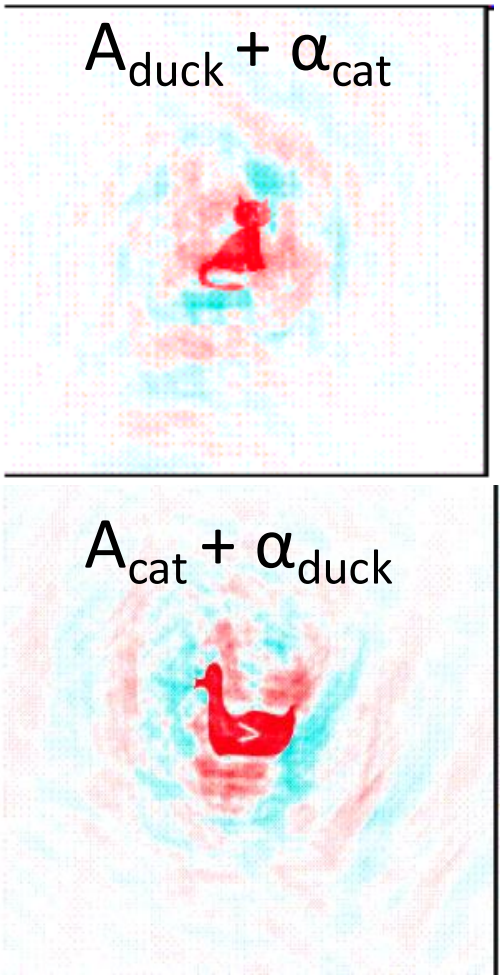


FT

Real space

Imaginary space

FT^{-1}



Without phases you cannot reconstruct image of original object

Third challenge in macromolecular crystallography!!

Methods to obtain α_c

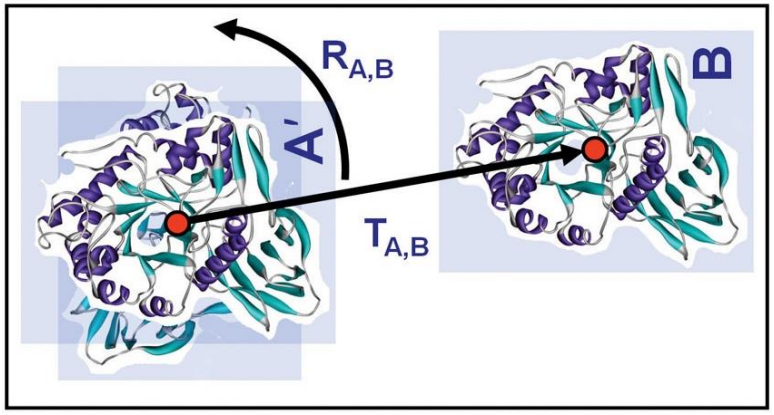
- Direct methods (used for small molecules)
- MR - molecular replacement
- MIR- multiple isomorphous replacement
- MAD/SAD – multi- or single wavelength anomalous dispersion
  - For MIR and MAD/SAD, the phases are calculated from experimental data – less (model) bias but more time in the wet lab!

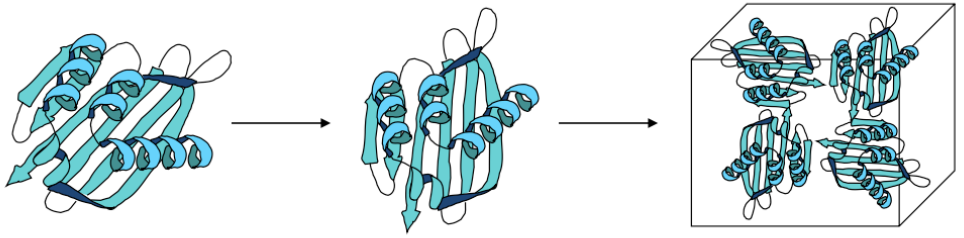
Molecular replacement

- Solve the phase problem by placing an already solved homologous structure (based on sequence identity of ~30% or higher) in our lattice.
- Alpha-fold models have helped to solve the phases of previously failed jobs.

Molecular replacement

How? Rotate and translate the model (3D searches) until the calculated diffraction data (A_c) from the relocated model fits the observed diffraction data (A_o)





MR examples

- Aurora A kinase – is ligand in ATP pocket?

Structure solution and refinement

1. Have observed amplitudes A_{obs} or A_o

Structure solution and refinement

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2. Use the MR solution to calculate $\mathbf{F}_c = A_c\, e^{2\Pi i \alpha c}$ (first estimate of the phase, α_c)

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    1. R-factors (compare A_o and A_c)

R-factors

- R-factor and R_{free} as criteria for convergence and quality of model – this is resolution dependent!!
- A good value for the R factor is between 15-25%

$$R = \frac{\sum_{hkl} \left| A_{obs}(hkl) - kA_{calc}(hkl) \right|}{\sum_{hkl} A_{obs}(hkl)}$$

R-factors

- R_{free} is calculated using a set of intensities (around 5%) that are excluded from the refinement process, such that they are not affected by model bias.
- It is usually 1-10% higher than the R factor

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$\rho(x,y,z) = FT^{-1}\, (2A_o - A_c)\, e^{2\Pi i \alpha c}$

$\rho(x,y,z) = FT^{-1}\, (A_o - A_c)\, e^{2\Pi i \alpha c}$ ---> difference map

The electron density map

- The electron density ρ(xyz) is represented as a map, where we model the atomic coordinates

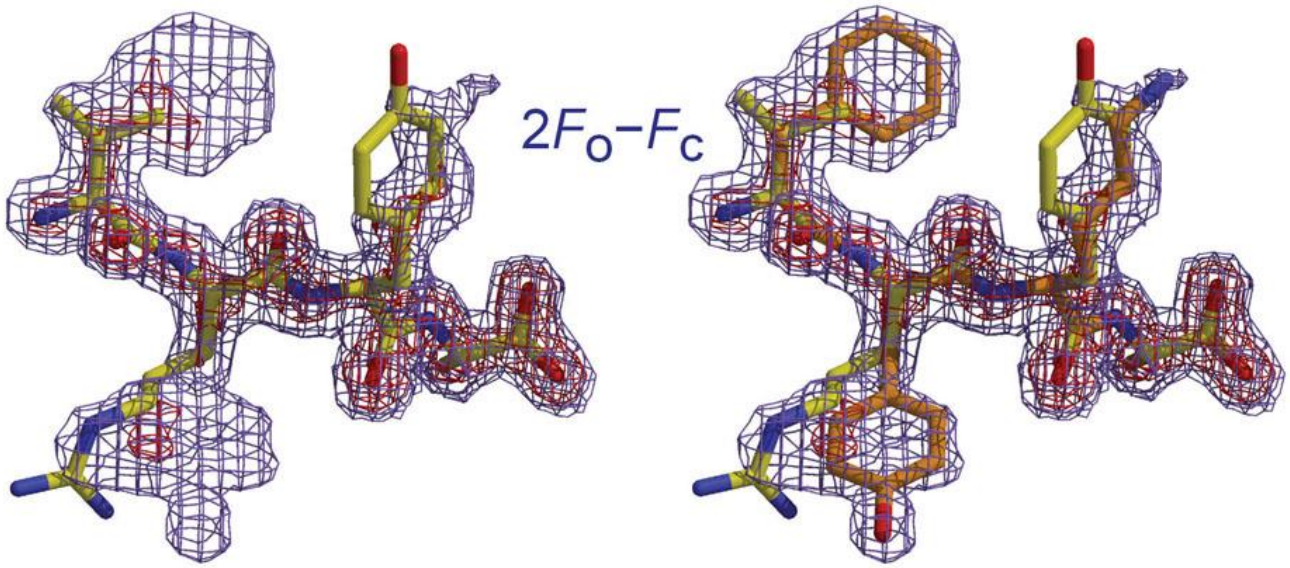
The electron density map

- The *Ao – Ac* (Fo-Fc) difference map shows negative difference density (where there should not be any density) in red and positive difference density (where there should be density) in green.
- Both regions correctly reveal the difference between the starting model and the correct model.

$$\rho(x,y,z) = FT^{-1} (A_o - A_c)\, e^{2\Pi i \alpha c} \text{ ---> difference map}$$

The electron density map

The 2Ao – *Ac* (2Fo-Fc) map can be interpreted as a combination of the previously mentioned difference map (Fo – *Fc)* and a Fo map. The 2Fo – *Fc* map is well suited to early model building stages



$\rho(x,y,z) = FT^{-1}\,(2A_o - A_c)\,e^{2\Pi i \alpha c}$

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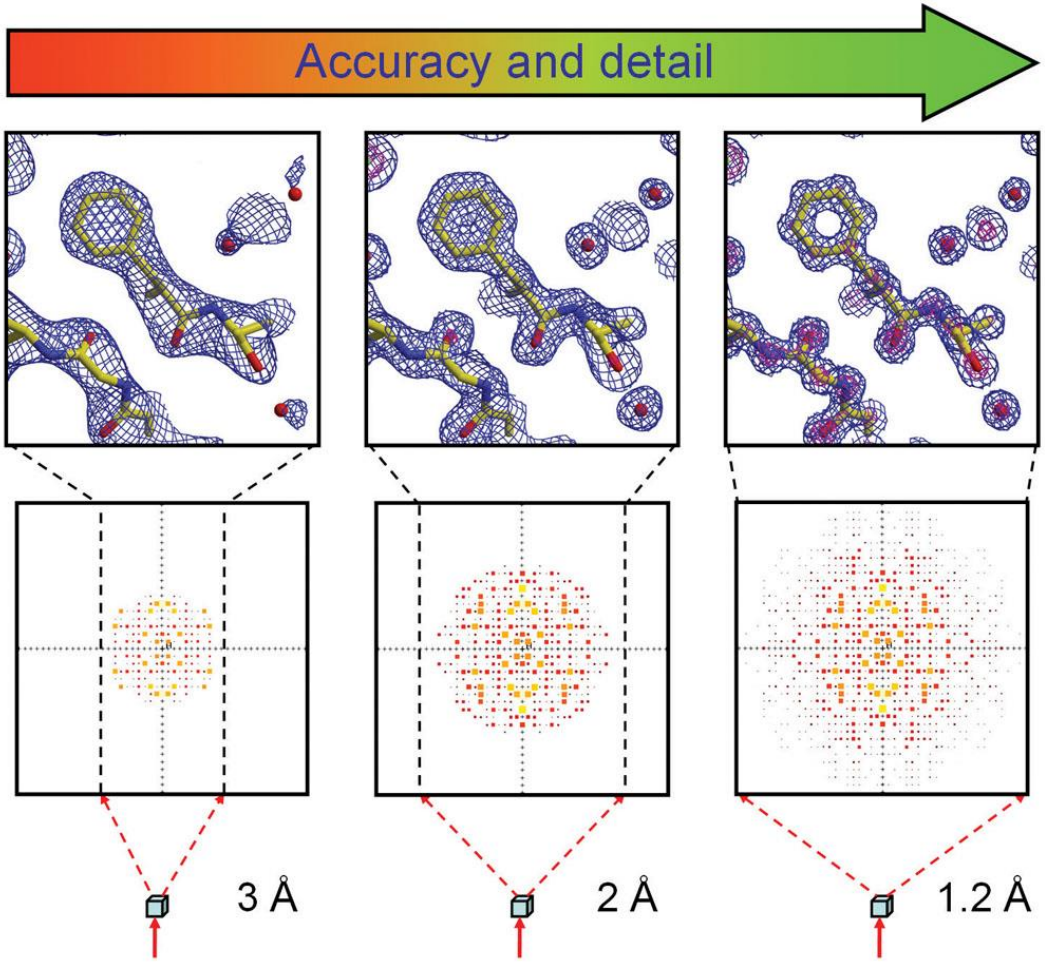
4. Visualize $\rho(x,y,z)$ using a map and optimize molecular model

Model building

- Con


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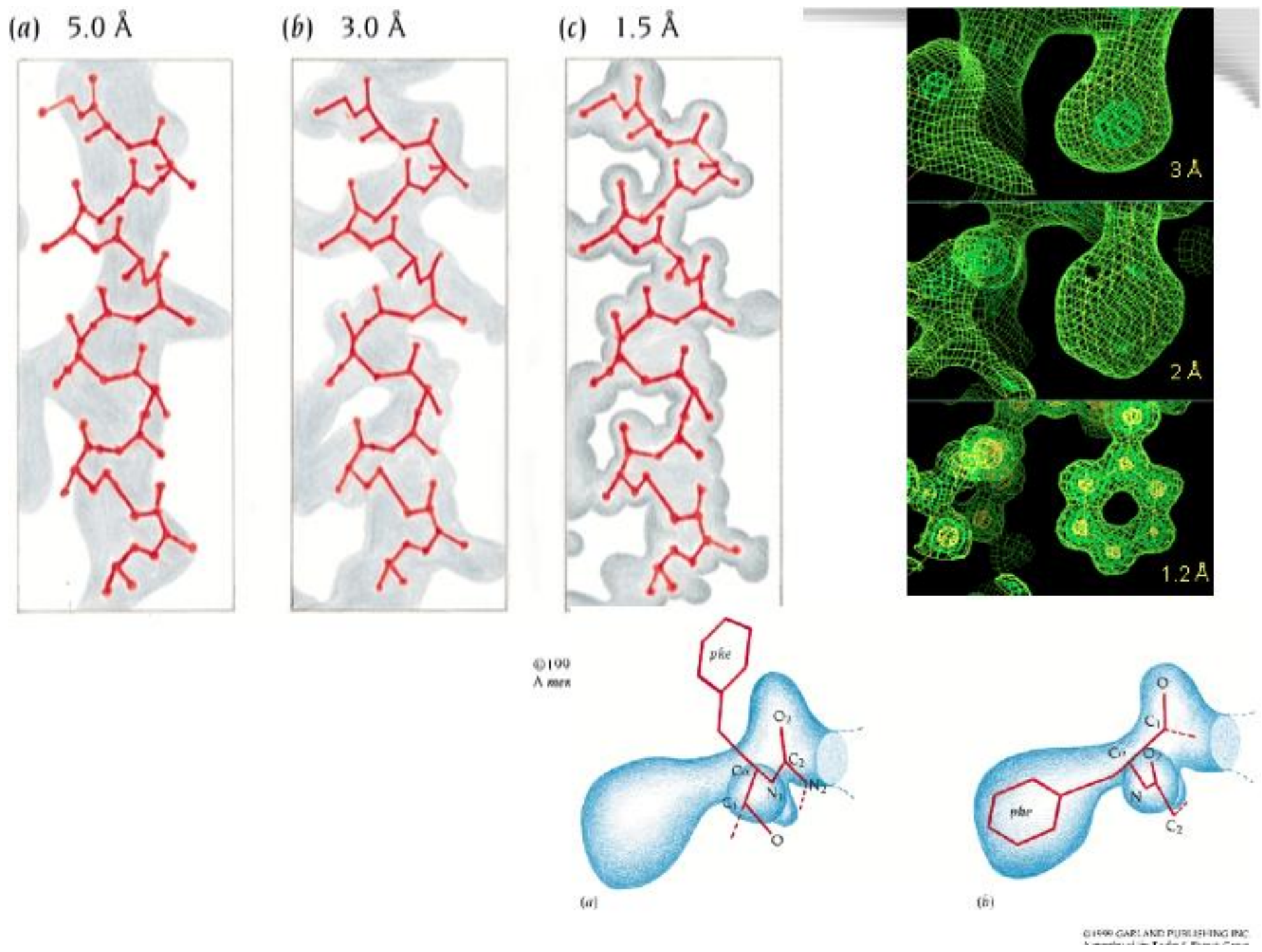
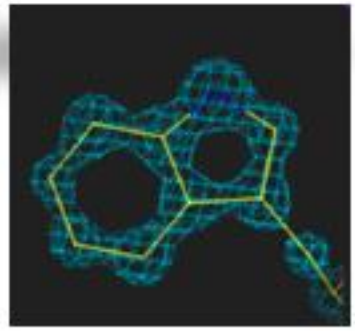
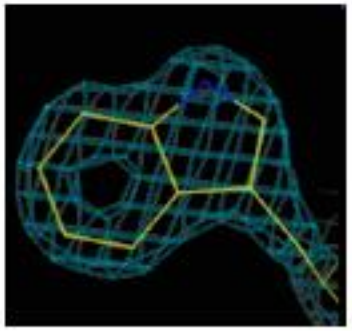
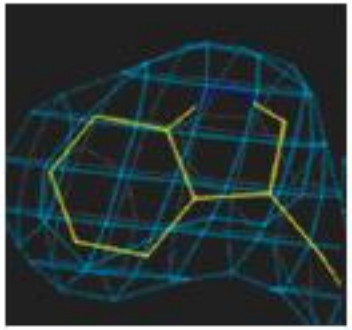

(a) 5.0 Å
(b) 3.0 Å
(c) 1.5 Å
3 Å
2 Å
1.2 Å
phe
phe
(a)
(b)



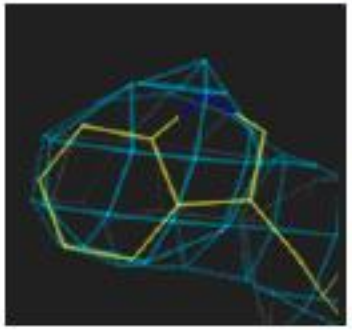
1.0 Å



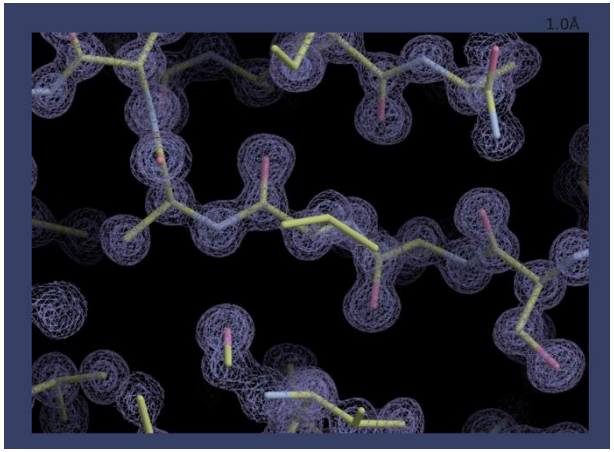
1.5 Å

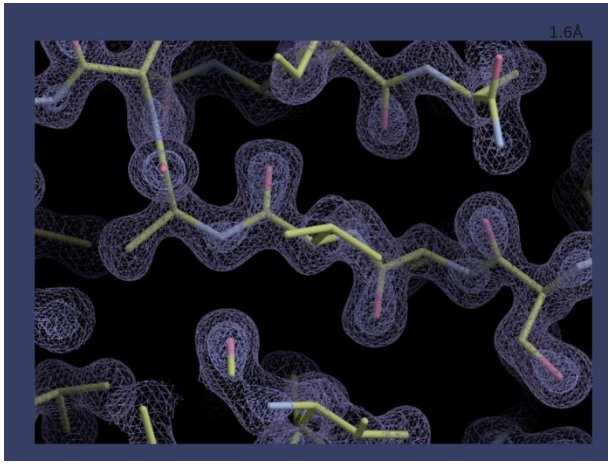


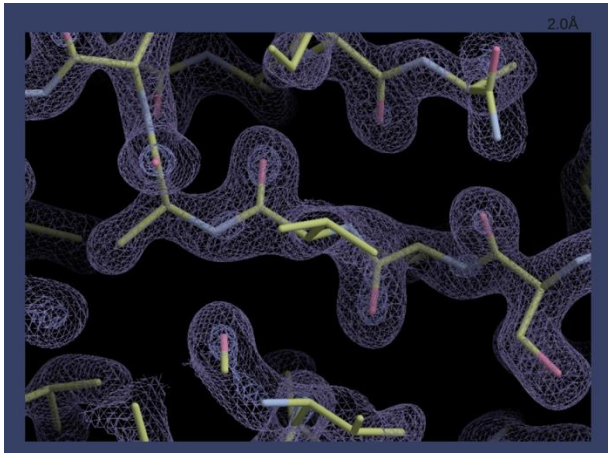
3.0 Å

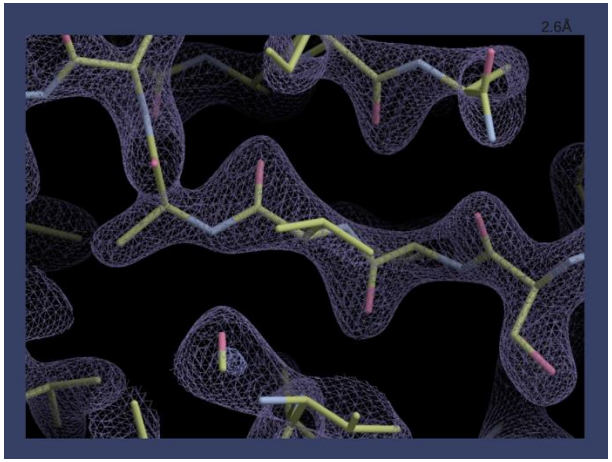


4.0 Å


1.0Å


1.6Å


2.0Å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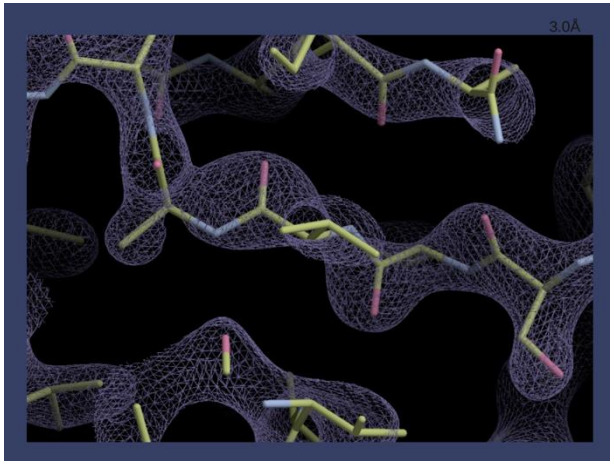
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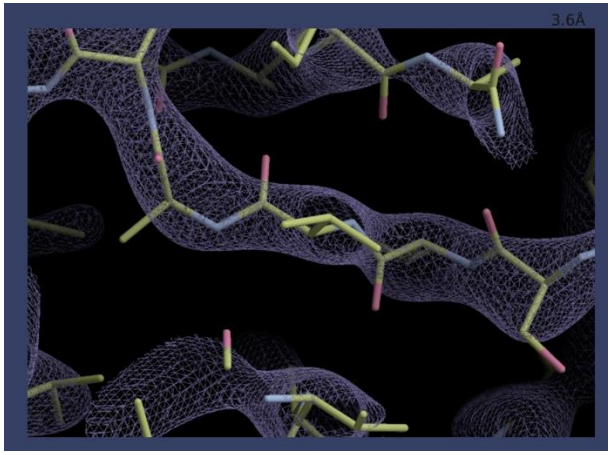
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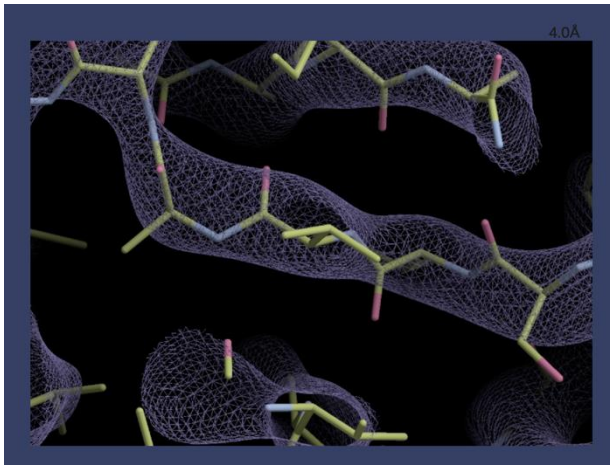
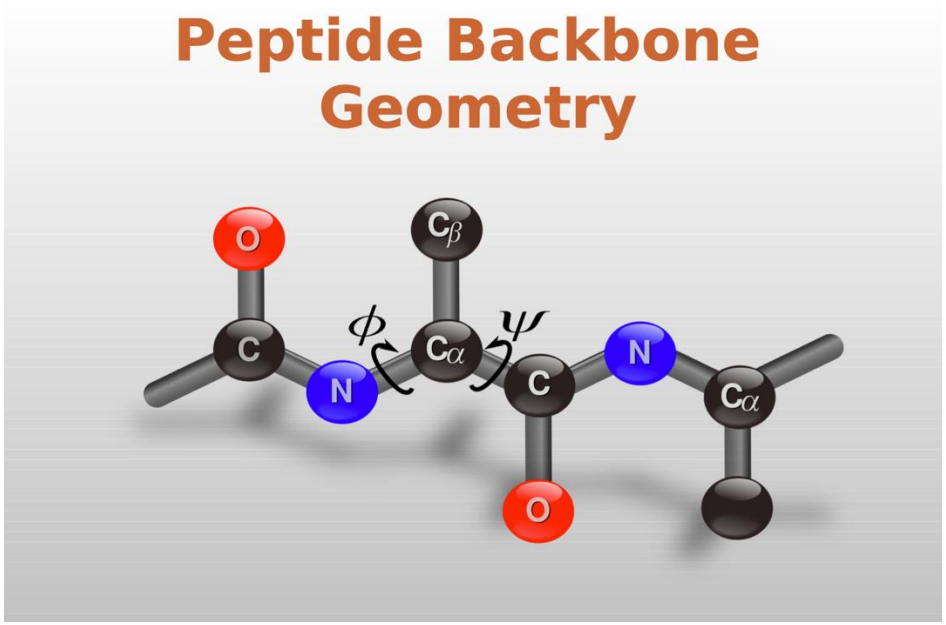
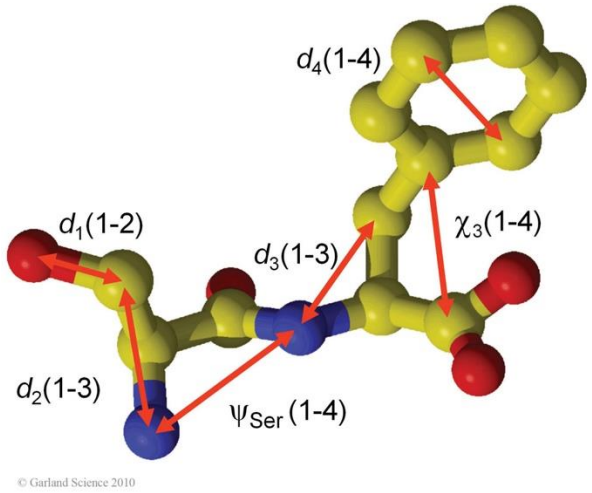

4.0Å

Model building and refinement

- Chemical laws have to be kept – restrains in refinement





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    $\rho(x,y,z) = FT^{-1}\, (A_o - A_c)\, e^{2\Pi i \alpha c}$ ---> difference map
4. Visualize $\rho(x,y,z)$ using a map and optimize molecular model
5. Use the new model to calculate new $\mathbf{F}_c = A_c\, e^{2\Pi i \alpha c}$ (new α_c, hopefully improved!)

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   $\rho(x,y,z) = FT^{-1}\, (A_o - A_c)\, e^{2\Pi i \alpha c}$ ---> difference map
4. Visualize $\rho(x,y,z)$ using a map and optimize molecular model
5. Use the new model to calculate new $\mathbf{F}_c = A_c\, e^{2\Pi i \alpha c}$ (new α_c, hopefully improved!)
6. Calculate new R-factors and new $\rho(x,y,z)$ from A_o and latest α_c

Structure solution and refinement

1. Have observed amplitudes A_{obs} or A_o
2. Use the MR solution to calculate $\mathbf{F}_c = A_c\, e^{2\Pi i \alpha c}$ (first estimate of the phase, α_c)
3. Calculate two things:
   1. R-factors (compare A_o and A_c)
   2. $\rho(x,y,z)$ from $\rho(x,y,z) = FT^{-1}\, A_o\, e^{2\Pi i \alpha c}$

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NOT IN cryoEM

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8. Repeat steps 4 to 7 until A_c approaches A_o (= Refinement)

Model building & Refinement

- "Repeat the process until A_c approaches A_o "
- "The key to successful protein structure modelling is the cycling between local real space model building and model correction and global reciprocal space refinement. The molecular model is built in real space into electron density using computer graphics. The parameters of the model atoms (coordinates x, y, z for example) are corrected in restrained reciprocal space refinement by optimizing the fit between observed and calculated structure factor amplitudes (A_o and A_c)" (from Rupp, B. (2010) Biomolecular Crystallography).
- Successive rounds of rebuilding, error correction, and refinement are needed to obtain a good final protein model.

Model building & Refinment

```
CRYST1  107.134   70.634  106.955  90.00 119.85  90.00 P 1 21 1
SCALE1      0.009334  0.000000  0.005357        0.00000
SCALE2      0.000000  0.014157  0.000000        0.00000
SCALE3      0.000000  0.000000  0.010780        0.00000
ATOM      1  N   ASN A   4     -28.919  13.478  40.870  1.00141.12           N
ATOM      2  CA  ASN A   4     -28.597  14.680  40.049  1.00138.30           C
ATOM      3  CB  ASN A   4     -28.136  14.256  38.645  1.00138.55           C
ATOM      4  CG  ASN A   4     -27.099  15.204  38.053  1.00139.73           C
ATOM      5  OD1 ASN A   4     -25.970  15.295  38.549  1.00135.90           O
ATOM      6  ND2 ASN A   4     -27.482  15.914  36.987  1.00137.13           N
ATOM      7  C   ASN A   4     -29.649  15.816  39.943  1.00132.40           C
ATOM      8  O   ASN A   4     -29.347  16.824  39.296  1.00125.34           O
ATOM      9  N   GLU A   5     -30.877  15.702  40.467  1.00131.12           N
ATOM     10  CA  GLU A   5     -31.728  14.496  40.484  1.00132.68           C
ATOM     11  CB  GLU A   5     -31.895  13.757  41.834  1.00132.56           C
ATOM     12  C   GLU A   5     -32.818  14.340  39.402  1.00134.58           C
ATOM     13  O   GLU A   5     -33.441  13.281  39.259  1.00135.69           O
ATOM     14  N   ASN A   6     -33.054  15.450  38.688  1.00122.42           N
```

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ATOM    898  CA  GLY A 112     -43.737   5.641  33.440  1.00 45.10           C
ATOM    899  C   GLY A 112     -44.317   4.270  33.133  1.00 43.23           C
ATOM    900  O   GLY A 112     -44.720   4.106  32.005  1.00 42.05           O
ATOM    901  N   LEU A 113     -44.354   3.337  34.129  1.00 41.68           N
ATOM    902  CA  LEU A 113     -45.044   2.059  34.013  1.00 47.56           C
ATOM    903  CB  LEU A 113     -44.985   1.212  35.307  1.00 47.03           C
ATOM    904  CG  LEU A 113     -45.143  -0.305  35.124  1.00 49.28           C
ATOM    905  CD1 LEU A 113     -44.042  -0.887  34.271  1.00 48.80           C
ATOM    906  CD2 LEU A 113     -45.151  -0.971  36.491  1.00 54.29           C
ATOM    907  C   LEU A 113     -46.480   2.276  33.578  1.00 38.77           C
ATOM    908  O   LEU A 113     -46.940   1.617  32.663  1.00 35.72           O
ATOM    909  N   TYR A 114     -47.170   3.241  34.195  1.00 42.29           N
ATOM    910  CA  TYR A 114     -48.602   3.456  33.893  1.00 41.59           C
ATOM    911  CB  TYR A 114     -49.333   4.362  34.904  1.00 40.95           C
ATOM    912  CG  TYR A 114     -50.841   4.137  34.909  1.00 38.66           C
```

Dynamic information of the crystal

$$B = \frac{8\pi^2}{3}\left\langle u_r^2 \right\rangle$$

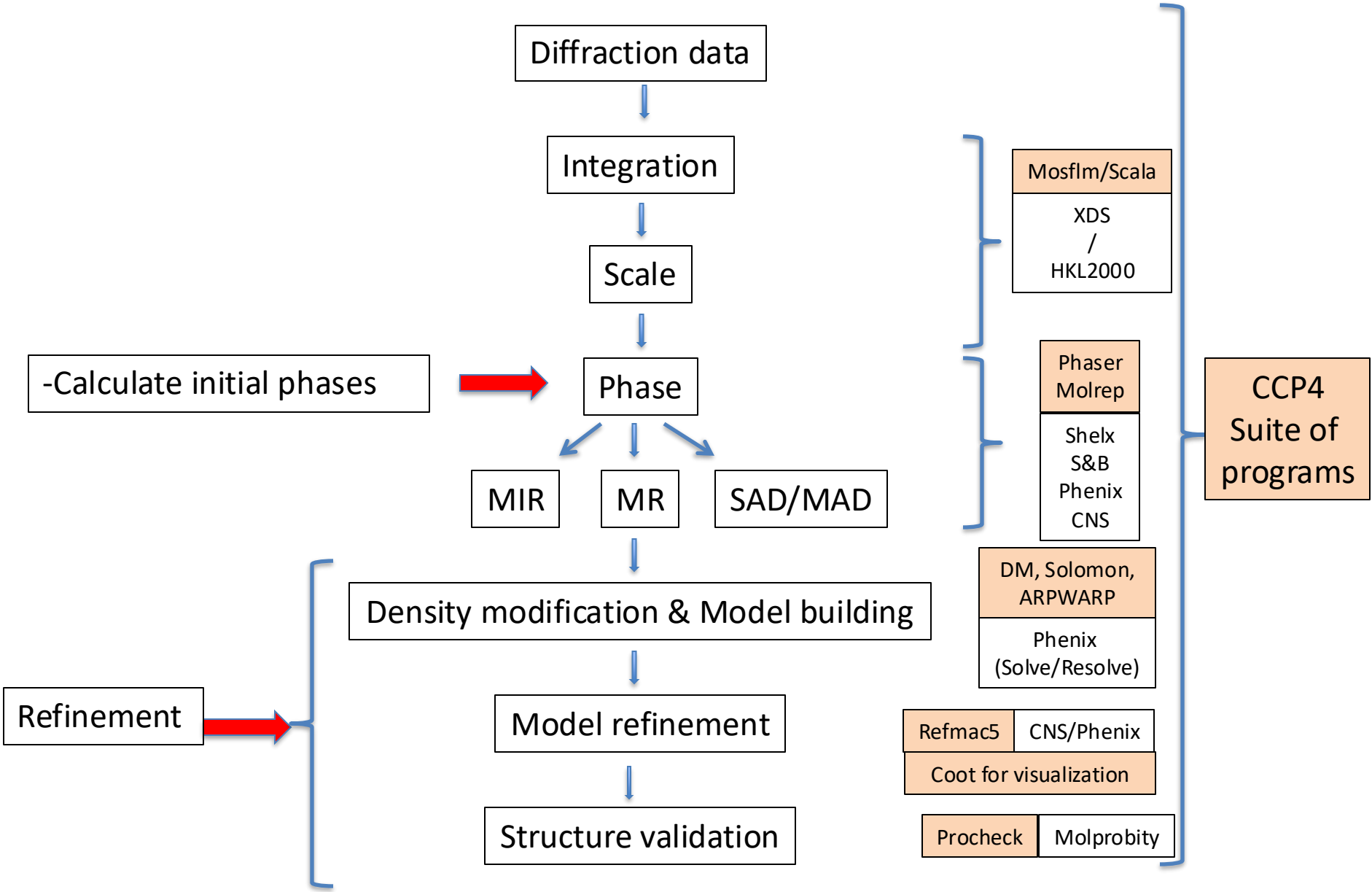
Example:
B = 20 -- 0.5 Å displacement
B = 80 -- 1 Å displacement

B: B-factor, temperature factor, or Debye-Waller factor: dynamic disorder ~5-30(Å^2)

$$\langle u_r^2 \rangle = \frac{1}{T}\sum_{t_j=1}^{T}(x_i(t_j) - \tilde{x}_i)^2$$

mean square atomic fluctuation ~0.25-0.60(Å)

General overview of how to solve a macromolecular structure



Crystallographic table

Crystallization condition	
X-ray source & Detector	
Wavelength (Å)	
Space group	
Cell (Å °)	
Resolution range (Å)	
Number of observations	
Unique reflections	
Completeness%	Around 100%
R_{merge}%	Bellow 10%
Mean I/σ(I)	>2

Refinement statistics (Phenix Refine)	
Resolution	
R (working set)%	15-25%
R_{free}%	5-10%>R-factor
Ramachandran outliers	Should be 0
RMSD Bond lengths (Å) Bond angles (°)	Small deviations from ideal values
Model	
Ligands	
Number of water molecules	
B factor (Å^2)	