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Structure Bioinformatics Course – Basel 2004

Introduction to X-ray crystallography

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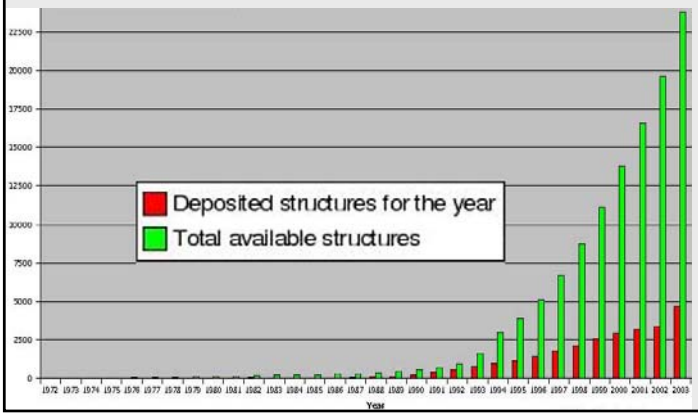
Intro – why protein crystallography

Methods to study protein structure:

1. X-ray

85% of atomic structures in PDB were determined by X-ray crystallography

2. NMR
3. 3D modelling



PDB statistics
~27'000 structures
Sept 2004

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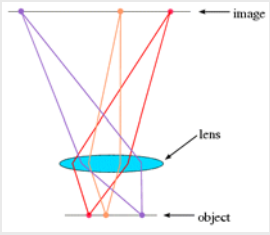
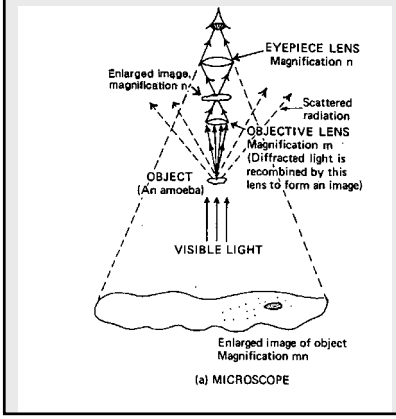
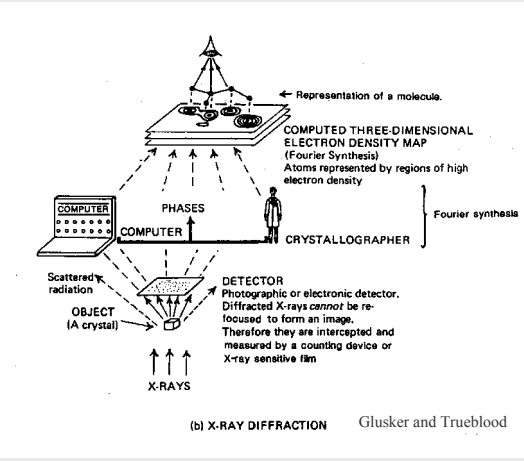
Microscope *vs* X-ray diffraction







Glusker and Trueblood

same principle, no lenses

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1. Why X-rays?

Dimensions:

- **Chemical bond ~1 Å (C-C bond 1.5 Å)**
- **Protein domain ~50 Å**
- **Ribosome ~250 Å**
- **Icosahedral virus ~700 Å**

Wavelengths:

- **Visible light λ = 200 - 800 nm**
- **X-rays λ = 0.6 - 3 Å**
- **Thermal neurons λ = 2 - 3 Å**
- **Electron beam λ = 0.04 Å (50 keV electron microscope)**

2. Why crystals?

to be answered later…

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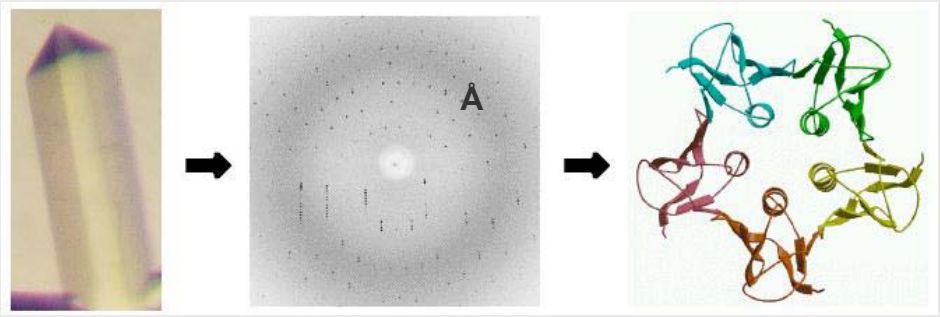
Four steps to a crystal structure

Protein purification
(usually after cloning/recombinant expression)



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What you get – a PDB file

```
...
ATOM    216  N   ARG D 351       4.388  68.438  23.137  1.00 43.02
ATOM    217  CA  ARG D 351       4.543  69.520  22.185  1.00 44.67
ATOM    218  CB  ARG D 351       4.967  69.042  20.821  1.00 44.90
ATOM    219  CG  ARG D 351       6.398  68.654  20.761  1.00 51.64
ATOM    220  CD  ARG D 351       6.868  68.340  19.302  1.00 63.98
ATOM    221  NE  ARG D 351       7.166  66.901  19.052  1.00 73.04
ATOM    222  CZ  ARG D 351       6.372  66.035  18.349  1.00 76.38
ATOM    223  NH1 ARG D 351       5.205  66.430  17.818  1.00 75.53
ATOM    224  NH2 ARG D 351       6.754  64.767  18.165  1.00 75.80
ATOM    225  C   ARG D 351       3.271  70.311  22.056  1.00 44.67
ATOM    226  O   ARG D 351       3.326  71.535  21.975  1.00 44.20
ATOM    227  N   MET D 352       2.145  69.620  22.040  1.00 43.72
ATOM    228  CA  MET D 352       0.880  70.278  21.909  1.00 45.59
ATOM    229  CB AMET D 352      -0.260  69.244  21.726  0.50 44.00
ATOM    230  CB BMET D 352      -0.337  69.338  21.761  0.50 44.14
ATOM    231  CG AMET D 352      -0.395  68.734  20.260  0.50 45.54
ATOM    232  CG BMET D 352      -1.699  70.119  21.628  0.50 47.21
ATOM    233  SD AMET D 352      -1.370  67.186  19.986  0.50 51.17
ATOM    234  SD BMET D 352      -1.768  71.563  20.386  0.50 50.67
ATOM    235  CE AMET D 352      -2.900  67.856  19.848  0.50 46.38
ATOM    236  CE BMET D 352      -3.556  71.823  20.152  0.50 50.17
ATOM    237  C   MET D 352       0.646  71.204  23.118  1.00 46.70
ATOM    238  O   MET D 352       0.276  72.366  22.923  1.00 49.10
...
ATOM    532  O   HOH W   4       2.840  93.717  24.656  1.00 34.14
ATOM    533  O   HOH W   5      -6.598  98.596  19.494  1.00 37.63
ATOM    534  O   HOH W   7       3.016  64.018  27.662  1.00 49.04
ATOM    535  O   HOH W   8       4.775  77.762  16.985  1.00 56.39
...
```

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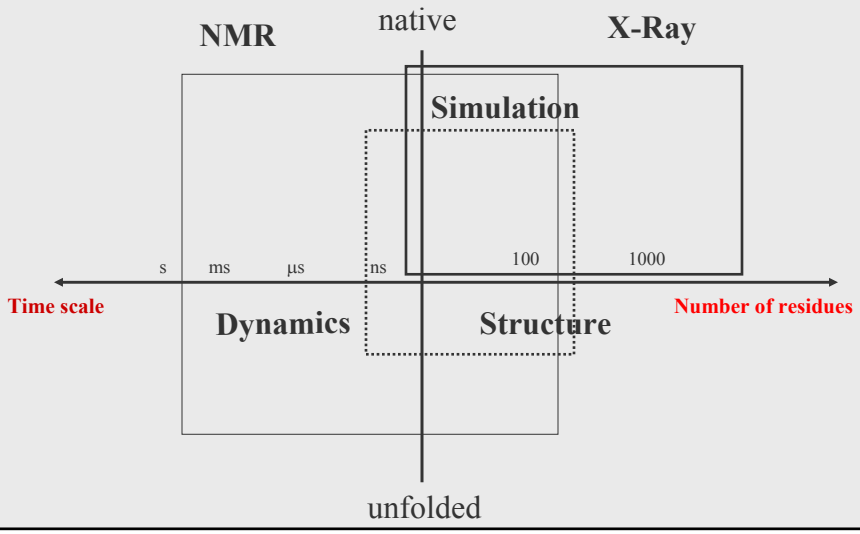
X-ray *vs* NMR *vs* Simulation

15% of protein structures are determined by NMR,
75% of these proteins were never crystallised



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Protein crystallography

Advantages:

- Is *the* technique to obtain an atomic resolution structure
- Yields the correct atomic structure *in solution*
  Caveat: is the structure in crystal the same as in solution? Yes!
- Atomic structure is a *huge amount of data* compared to what any other biochemical/biophysical technique could provide

-> This is why X-ray structures get to Cell and Nature…

Disadvantages:

- Needs crystals
- Is laborous in any case:
  cloning/purification 3-6 months *per structure*
  crystallisation 1-12 months
  data collection 1 month
  phasing/structure solution 3 months

-> This is why it is so expensive…

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Content of this lecture

I. Protein crystals and how to grow them

II. A bit of theory – diffraction

III. Practice -- X-ray diffraction experiment, phase problem and structure calculation

Suggested reading:

http://www-structmed.cimr.cam.ac.uk/course.html

http://www-structure.llnl.gov/Xray/101index.html

(two excellent online courses)

Books

• Cantor, C.R., and Schirmer, P.R. Biophysical Chemistry, Part II. Freeman, NY (1980)

• Rhodes, G. *Crystallography made crystal clear: A guide for users of macromolecular models.* Academic Press, N.Y. (2000)

• Drenth, J. *Principles of protein X-ray crystallography*. Springer (1995)

• Blundell, T.L. and Johnson, L.N. *Protein Crystallography*. Academic Press: N.Y., London, San Francisco (1976)

• Ducroix & Giege. *Protein crystallisation*

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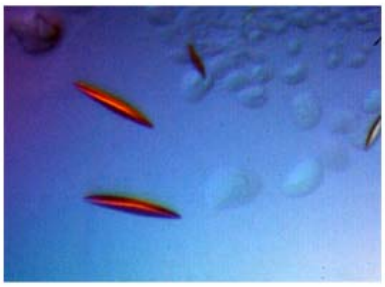
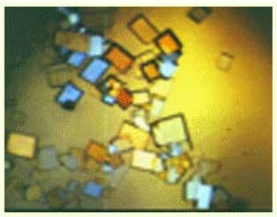
I. Protein crystals



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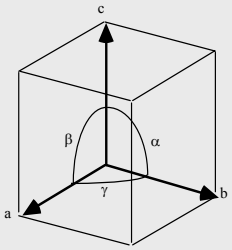
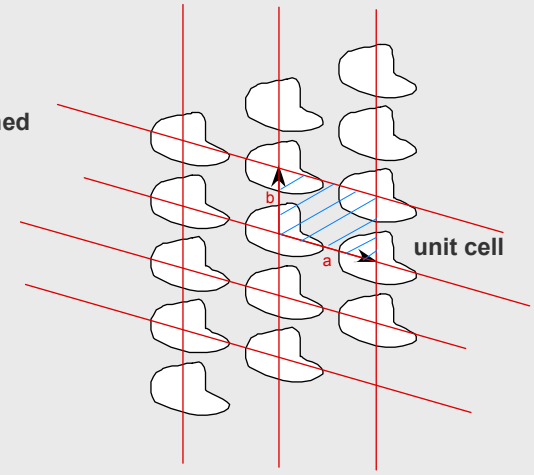
Crystal lattice

Periodic **arrangement in 3 dimensions**

A crystal *unit cell* is defined by its *cell constants* a, b, c, α, β, γ





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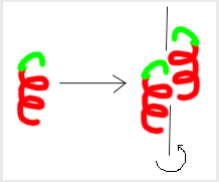
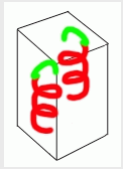
Crystal symmetry

Besides lattice translations, most crystals contain *symmetry elements* such as rotation axes

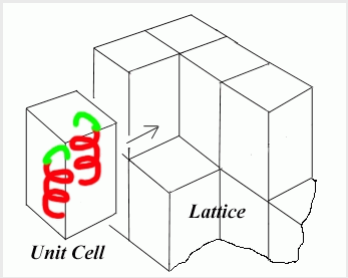
2-fold symmetry axis

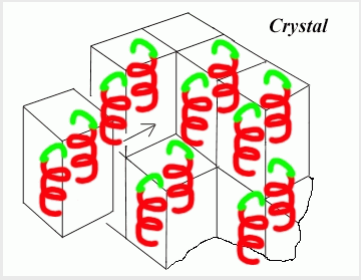




asymmetric unit

unit cell





Crystal symmetry obeys to one of the *space groups*

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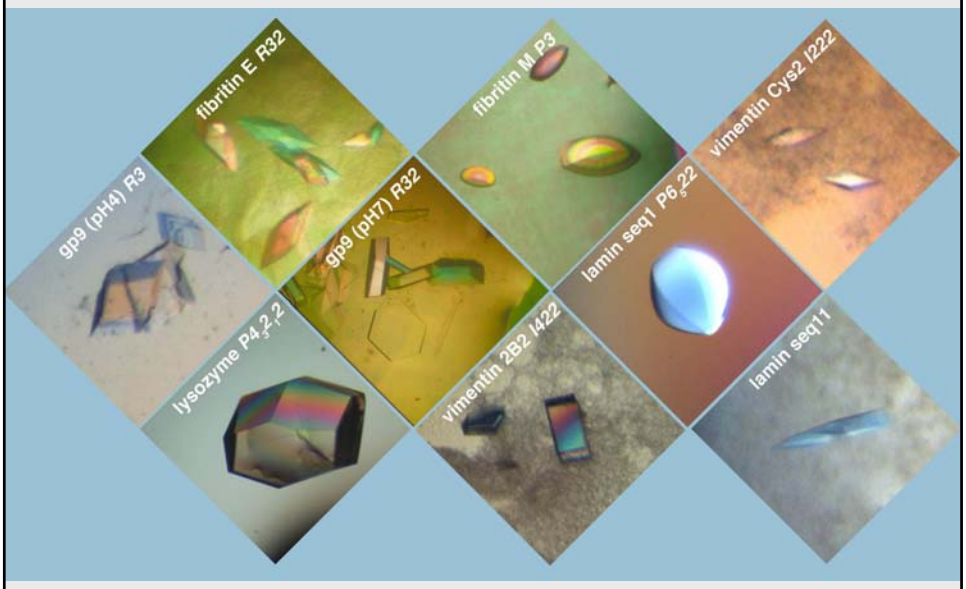
Protein crystals



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Protein crystallisation

“Kristallographen brauchen Kristalle”

Principle

- **Start with protein as a solution**
- **Force protein to fall out of solution as solid phase**
  -> amorphous precipitate or crystal

How to decrease protein solubility

- **Add precipitating agent (salt, PEG, …)**
- **Change pH**
- **…**

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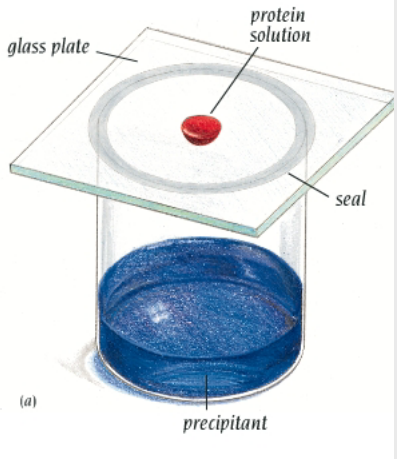
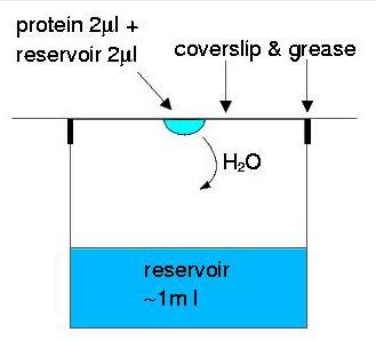
Protein crystallisation

'Hanging drop':





Example:

Protein: 10mg/ml
in 10 mM Tris buffer, pH7.5

Reservoir solution:
2M ammonium sulphate
in 100mM citrate buffer, pH5.5

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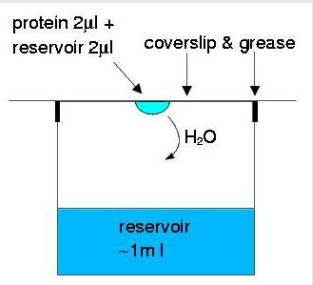
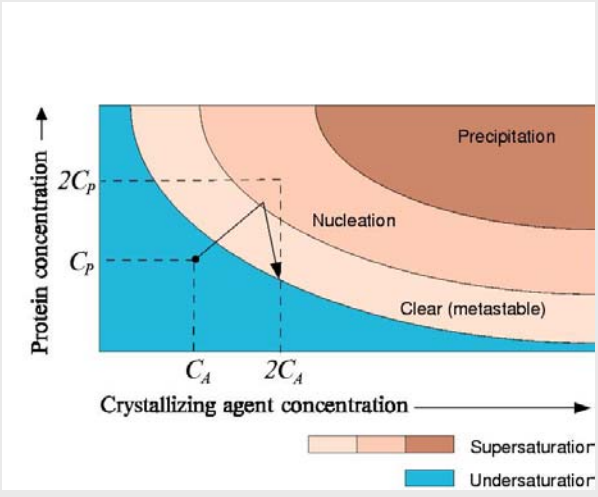
Phase diagram of protein crystallisation





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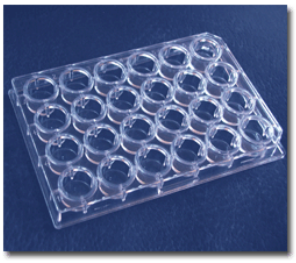
How to find crystallisation conditions

Step 1: Screening

- **Trial and error: different precipitants, pH, etc**
- **Miniaturise: 1 μl protein / experiment per hand, 50 nl by robot**
- **Automatise**

100-1000 different conditions



Step 2: Grow large crystals

- **Optimise quantitive parameters (concentrations, volumes)**

Step 3: Check whether your crystal diffracts X-rays

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Requirements for crystallisation

Protein has to be:

- **Pure (chemically and 'conformationally')**
- **Soluble to ~10 mg/ml**
- **Available in mg quantities**
- **Stable for at least days at crystallisation temperature**

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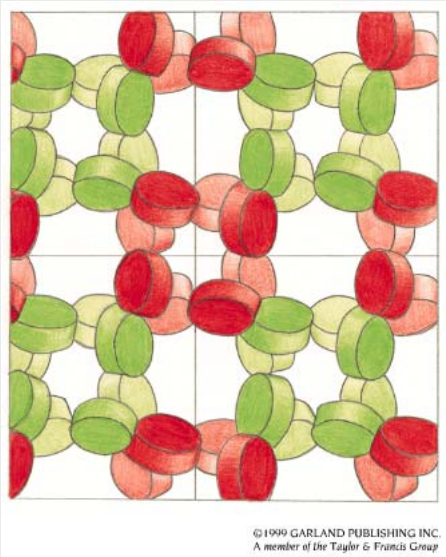
Protein crystals contain lots of solvent



typically 30 to 70% solvent by volume

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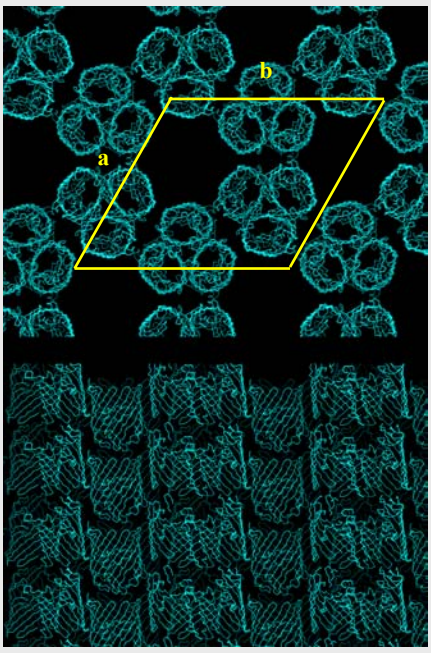
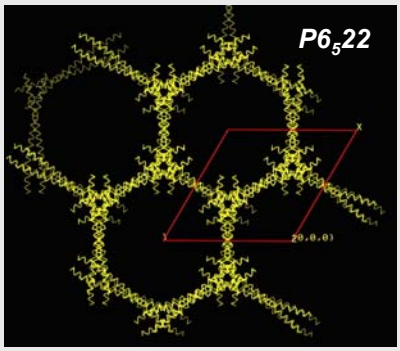
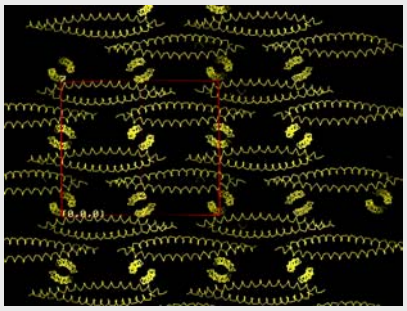
Packing of protein molecules into crystal lattice







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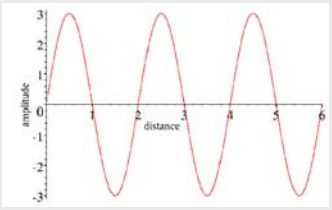
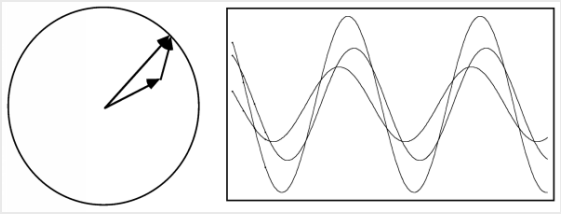
A bit of theory – diffraction of waves

A wave:
wavelength, speed,
amplitide (F), phase (φ)



The result of a two waves' summation depends on their amplitudes and (relative) phase



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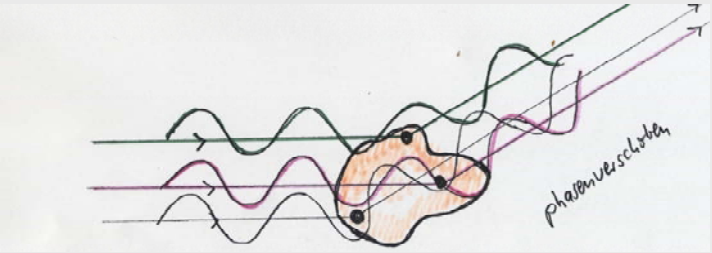
Diffraction from any object

X-rays will scatter on each atom of the object:

- scatter predominantly on electron shells, not nuclei
- elastic (=same energy)
- in all directions



The intensity of diffracted radiation in a particular direction will depend on the interference (=sum) of scattered waves from every atom of the object

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Diffraction as Fourier transform

Real space (x,y,z):	'Reciprocal space' (h,k,l):
electron density $\rho(x,y,z)$	diffracted waves $F(h,k,l)$, $\varphi(h,k,l)$

Physics tells us that the diffracted waves are Fourier transforms of the electron density:

$$F(h,k,l)e^{i\varphi(h,k,l)} = \int_{xyz} \rho(x,y,z)e^{2\pi i(hx+ky+lz)}dxdydz$$

Moreover, a backward transform (synthesis) should bring us from waves back to the electron density:

$$\rho(x,y,z) = const \cdot \int_{hkl} F(h,k,l)e^{-2\pi i(hx+ky+lz)+i\varphi(h,k,l)}dhdkdl$$

I.e. once we know the amplitudes and phases of diffracted waves we can calculate the electron density!

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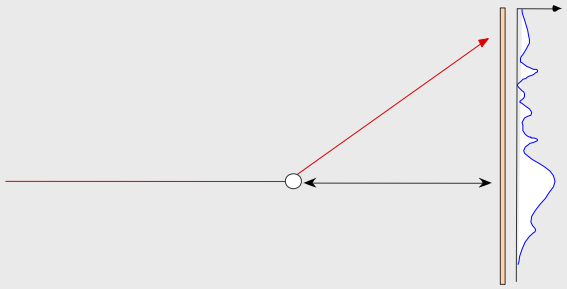
Diffraction on a single (protein) molecule



Will we see anything? Theoretically, YES: spread diffraction, no reflections

But practically:

- **Very low intensity of diffracted radiation**
- **Radiation would kill the molecule before satisfactory diffraction data are collected**
- **Orientation of a single molecule would have to be fixated somehow**

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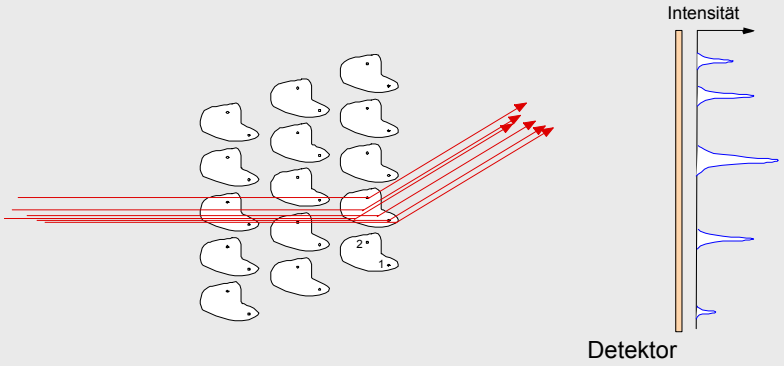
Diffraction on a crystal



Here we start seeing sharp peaks:

the Fourier transform becomes nonzero only for integer values of h,k,l

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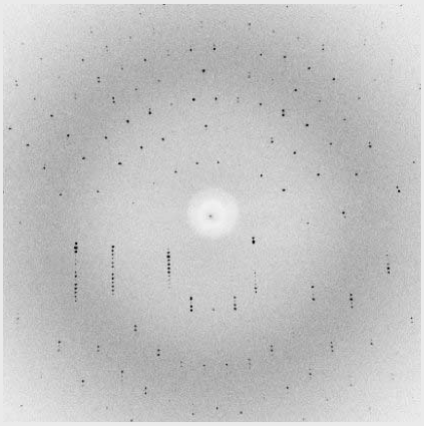
What do we see in a crystal diffraction pattern?



<u>Locations of reflections</u>

depend on the crystal lattice parameters and crystal orientation

<u>Intensities of reflections</u>

correspond to the squared amplitudes of diffracted waves

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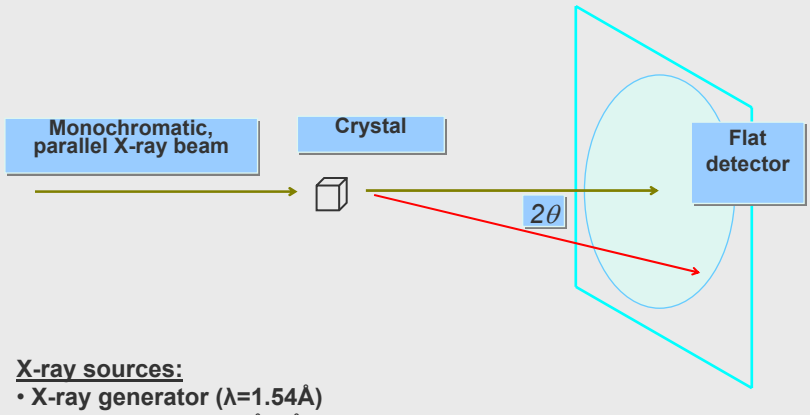
III. Practice. A. Diffraction data collection



X-ray sources:
- X-ray generator (λ=1.54Å)
- Synchrotron (λ=0.6Å-2Å)

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Diffraction geometry

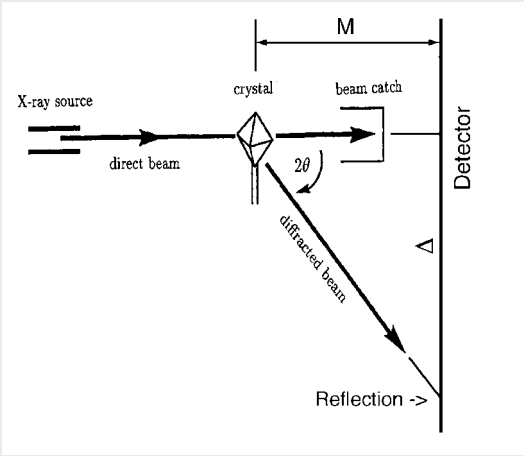
Diffraction angle:

$2\Theta = \arctan \Delta / M$

Bragg's formula:

$d = \lambda / (2 \sin \Theta)$

d is *resolution* in Å
~ the smallest spacing that will be resolved



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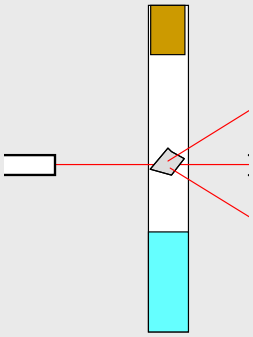
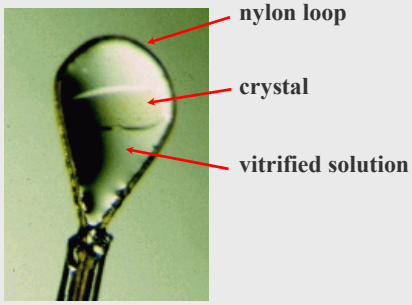
Crystal mount

Old:
sealed capillary
-> crystal stays at 100% humidity



Modern:
"flash cooling" to T=100°K in nitrogen stream



Problem: Radiation damage

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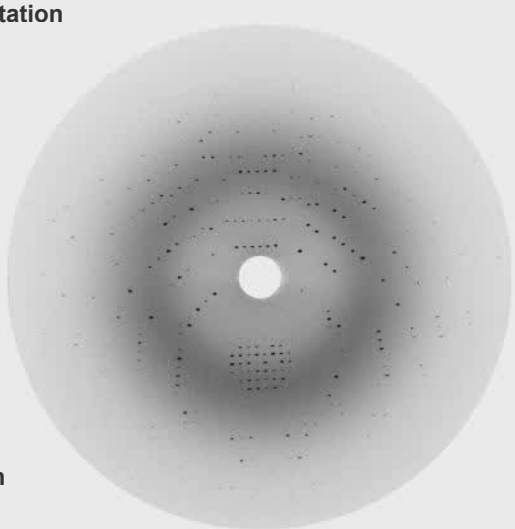
Data collection

Slowly rotate the crystal by the (horisontal) axis, record one image per each ~1° rotation



~ 100 images with
~100-1000 reflections each
= ~ 10^4 – 10^5 reflections

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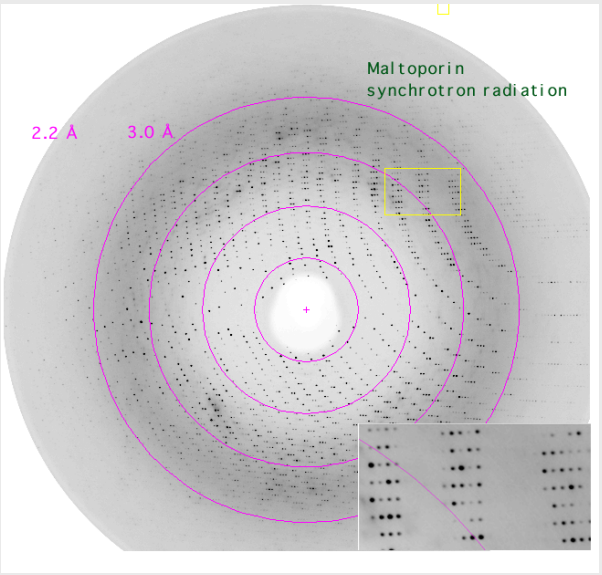
Diffraction quality



1. What is the maximal resolution?
2. Is it a nice single lattice?

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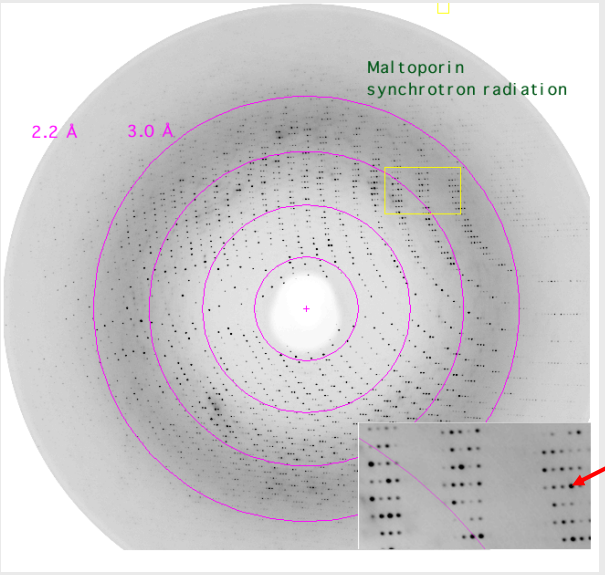
Indexing and integration



1. Assign indices h,k,l to each reflection
2. Record intensity of each reflection

h = 8
k = 12
l = 13

I = 12345 -> F = 111.2

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B. Phase problem

Fourier synthesis:

$$\rho(x,y,z) = const \cdot \sum_{hkl} F_{hkl} e^{-2\pi i(hx+ky+lz)+i\varphi_{hkl}}$$

However, there is a problem:
experiment yields amplitudes of reflections but *not phases*:
Amplitude F = sqrt(I)
Phase φ - ?

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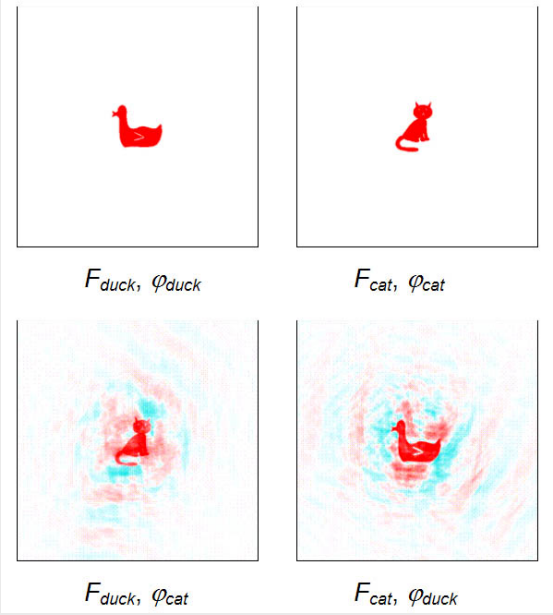
Phases are more important than amplitudes



http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

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Methods to solve the phase problem

1. Isomorphous replacement by heavy atoms (MIR)
2. Molecular replacement by a similar structure (MR)
3. Anomalous X-ray scattering on a heavy atom (MAD)
4. Direct methods -> 'guess the phase'

We will only discuss the first two…

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Multiple isomorphous replacement

1. Soak a heavy atom (U, Hg, Pt, Au, Ag…) into your crystal
2. Hope that (a) the heavy atom is specifically binding to a few positions on the protein and (b) the binding does not change the protein conformation or crystal cell parameters ('isomorphism')
3. Collect a new diffraction data set from the derivatised crystal -> F_{PH1}
4. Repeat for at least one another derivative -> F_{PH2}
5. Then there is a computation procedure that yields an estimate of protein ('native') phases:

F_P (native protein crystal)
F_{PH1} (derivative 1)
F_{PH2} (derivative 2)

} -> φ_P (estimate)

6. Do a Fourier synthesis with F_P and φ_P

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Molecular replacement

1. You have to know the 3D structure of a related protein
2. If the two structures are close, there is a computational procedure that finds the correct position/orientation of the known structure in the new cell
3. Use the measured amplitudes F_P and the phases calculated from the model φ_{model} for Fourier synthesis

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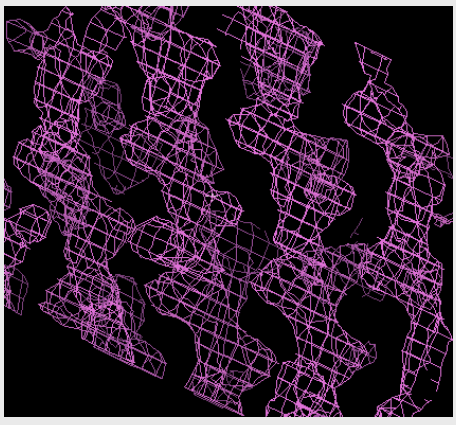
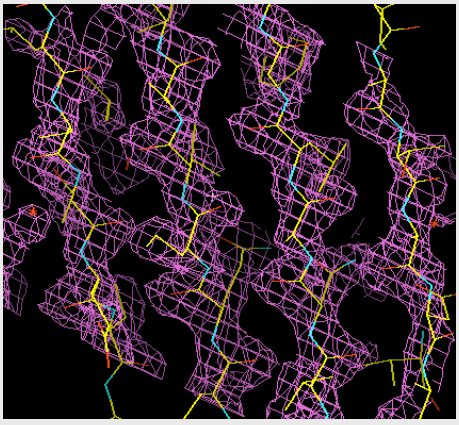
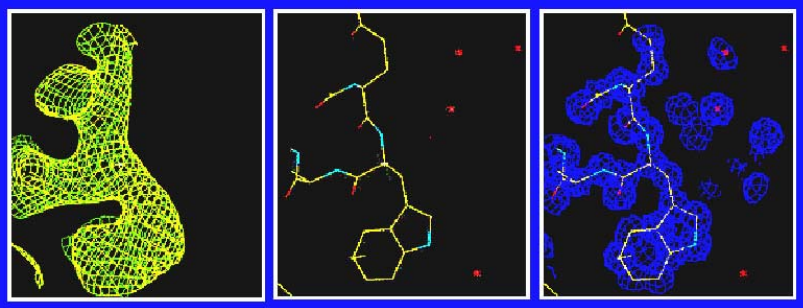
C. From electron density to atomic model





Once initial phases are obtained, a protein model (your idea of what is right) is fitted into the initial electron density map (the actual and sometimes marginal experimental reality)



what you see → what you think → what you get

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Building and refining atomic model

Observed amplitudes, initial phases

⬇ FS

Initial electron density map

⬇ model build

Initial model

⬇ FT

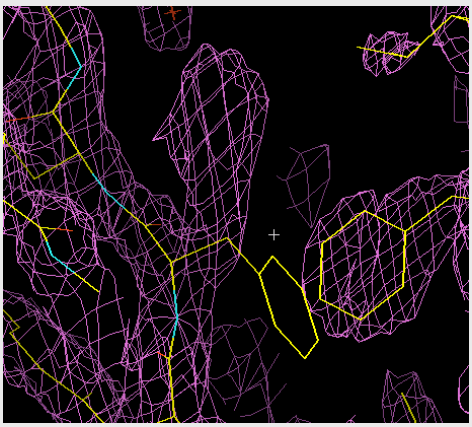
Observed amplitudes, phases calculated from the model

⬇ FS

Better map

⬇ model build

Final model



Automated refinement:

Program attempts to minimise the discrepancy between the observed amplitudes and those calculated from the model by adjusting the positions of atoms as well as their occupancies and temperature factors

Restraints: stereochemistry

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Model quality

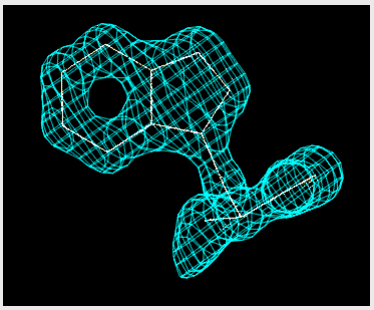
1. Model should match experimental data

F_{obs} – observed amplitudes

F_{calc} – calculated from the model

R-factor

$$R = \sum |F_{obs} - F_{calc}| / \sum F_{obs}$$



2. Model should have good stereochemistry

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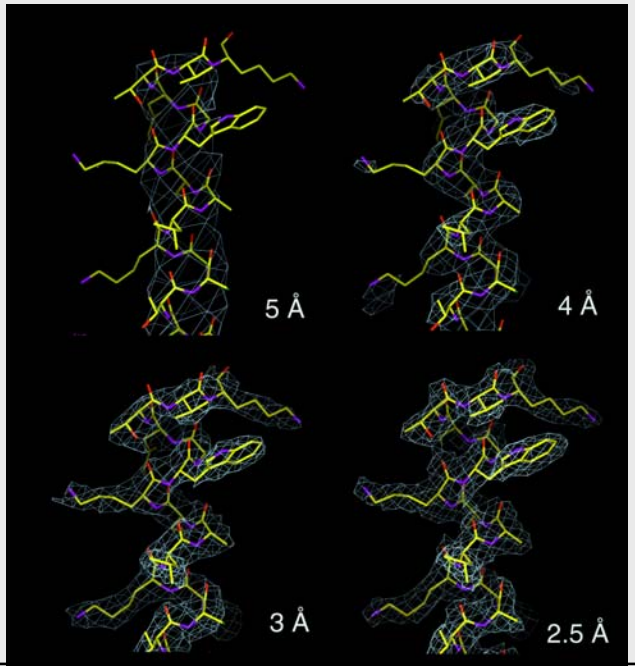
Resolution



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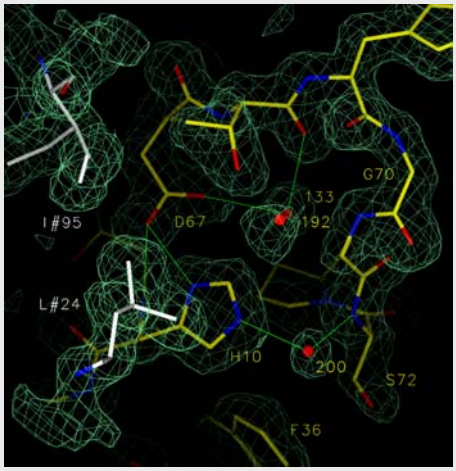
Resolution and accuracy

• Once resolution is better than ~3Å, building (and refinement) of a full atomic model (except hydrogens) becomes possible

• But the accuracy in atoms positions is much better (~ few tens of Å), especially since the model is stereochemically restrained



<u>Ultrahigh resolution</u>

Current record is about 0.6Å:

• hydrogens seen

• valent electrons seen

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Atomic temperature factor

May either reflect the true thermal motion of the molecule

or

a conformation variability from unit cell to unit cell