# Advanced biochemistry and its methods
# Lecture 5

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**Žídek: Strukturní biochemie (skripta k prednášce C9530), kapitoly 11–14**

# Methods of protein structure determination

**COMPUTATIONAL:**

De novo structure calculation not reliable

- **Homology modeling**
  using a similar known structure as a starting model
- **Prediction based on Machine Learning**
  using sequence alignment and database including known structures (AlphaFold)
  using a similar known structure as a starting model
- **Structure determination based on experiment**
  usually MD simulation with experimental restraints

# Methods of protein structure determination

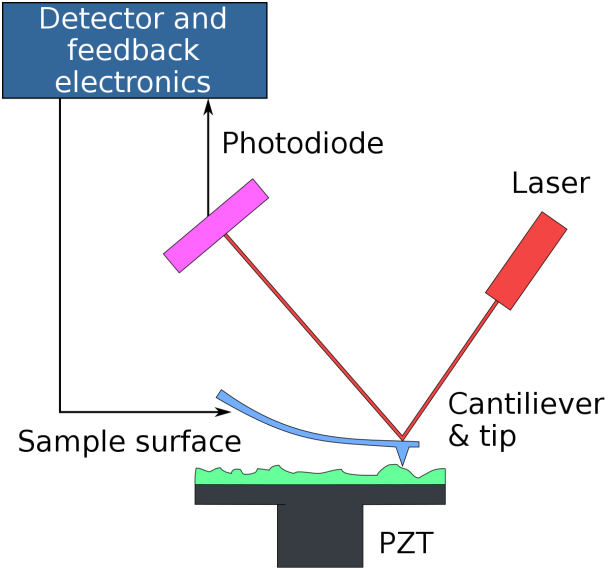
**EXPERIMENTAL:**

- **Mass Spectrometry**
  mostly analysis of cross-linked fragments
- **Scanning surface**
  e.g. Atomic Force Microscopy (various modes)

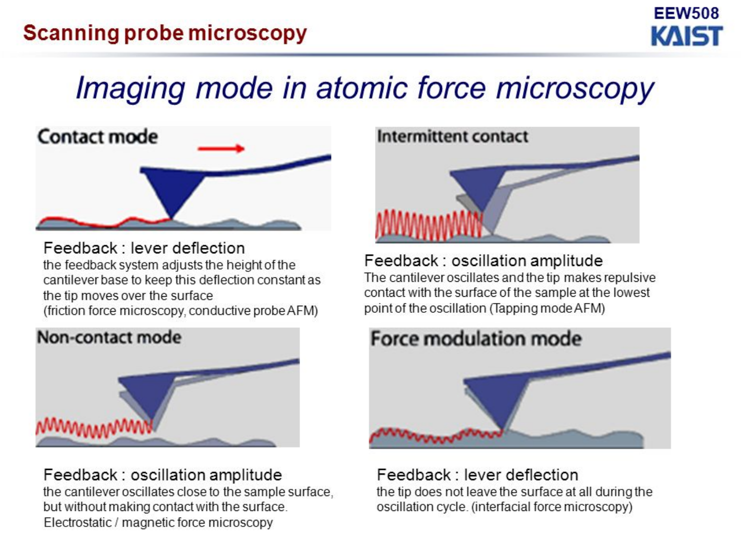
Interactions of electromagnetic (other) waves with molecules:

- **Spectroscopy**
  how molecules change characteristics of the wave (intensity, phase, polarization, frequency)
- **diffraction methods (Microscopy)**
  how molecules change direction of wave in space

# Atomic force microscopy



# Atomic force microscopy



**Scanning probe microscopy**

EEW508
KAIST

## *Imaging mode in atomic force microscopy*

**Contact mode**

Feedback : lever deflection
the feedback system adjusts the height of the cantilever base to keep this deflection constant as the tip moves over the surface
(friction force microscopy, conductive probe AFM)

**Intermittent contact**

Feedback : oscillation amplitude
The cantilever oscillates and the tip makes repulsive contact with the surface of the sample at the lowest point of the oscillation (Tapping mode AFM)

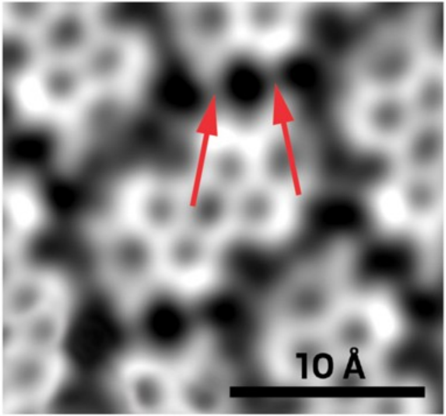
**Non-contact mode**

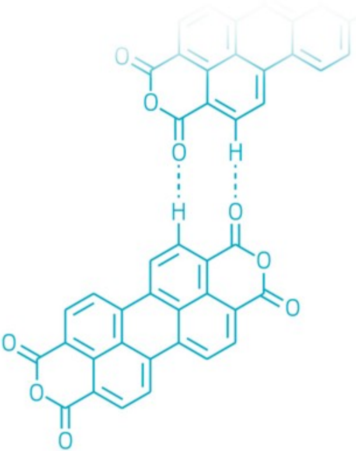
Feedback : oscillation amplitude
the cantilever oscillates close to the sample surface, but without making contact with the surface.
Electrostatic / magnetic force microscopy

**Force modulation mode**

Feedback : lever deflection
the tip does not leave the surface at all during the oscillation cycle. (interfacial force microscopy)

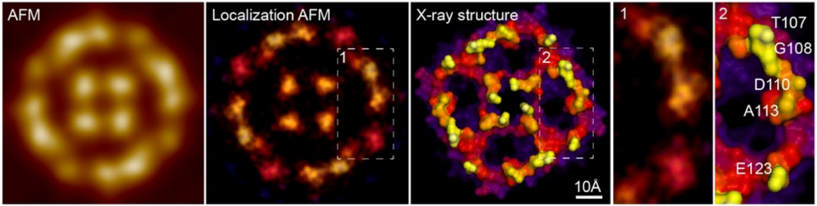
# Atomic force microscopy





# Atomic force microscopy

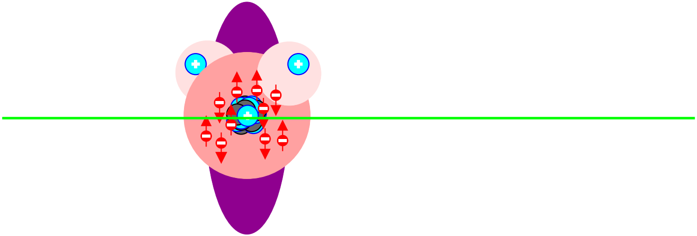
image reconstruction ”localization AFM”
cf. super-resolution microscopy



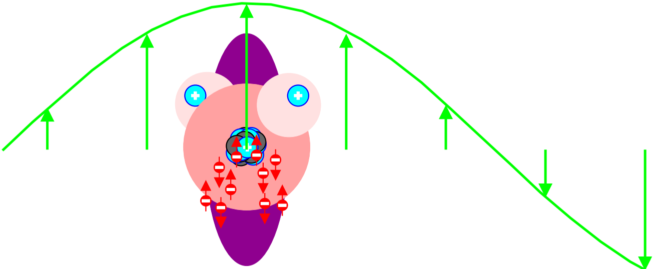
# Spectroscopy

- **UV/VIS spectrophotometry**
  absorption, transition of electrons to higher orbitals
  concentration, content of aromatic amino acids, heme, prosthetic groups
- **IR spectroscopy**
  absorption, transition of nuclei to higher vibration states

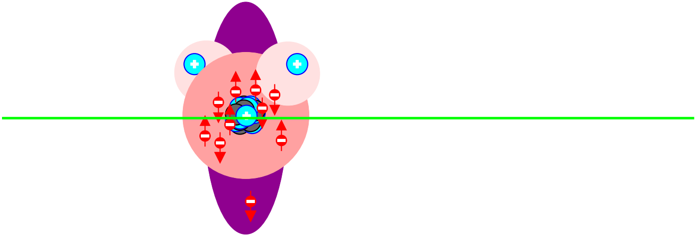
# Absorption by molecules



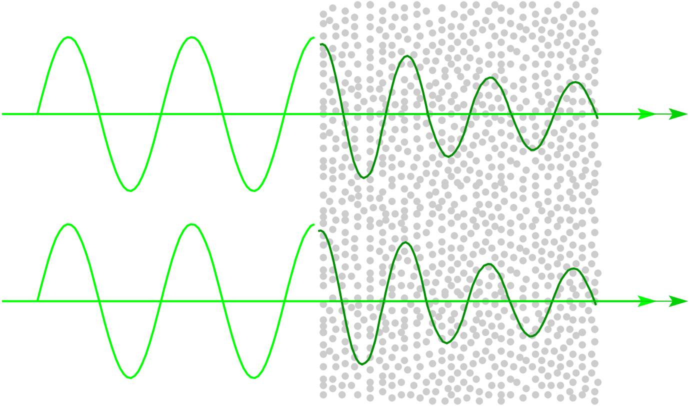
# Absorption by molecules



# Absorption by molecules

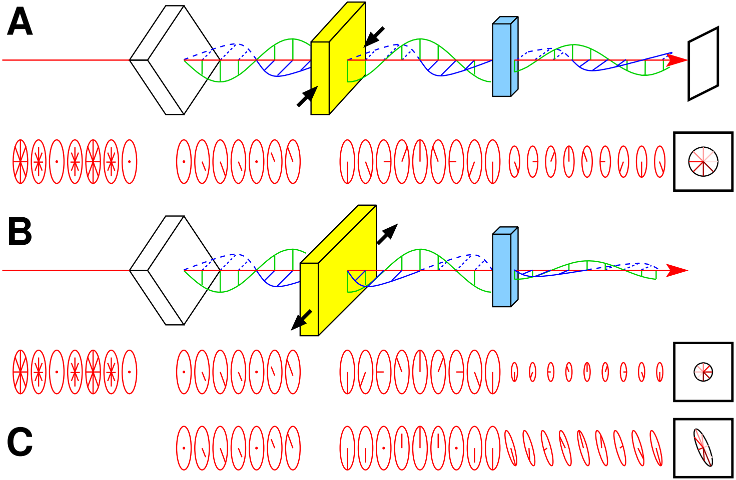


# Absorption by molecules



# CD Spectroscopy

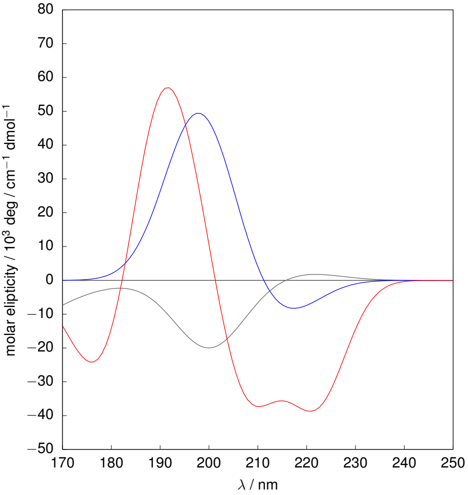
- **CD spectroscopy**
  absorption differences of polarized light by chiral molecules



# CD spectroscopy and secondary structure

overall content of secondary structures

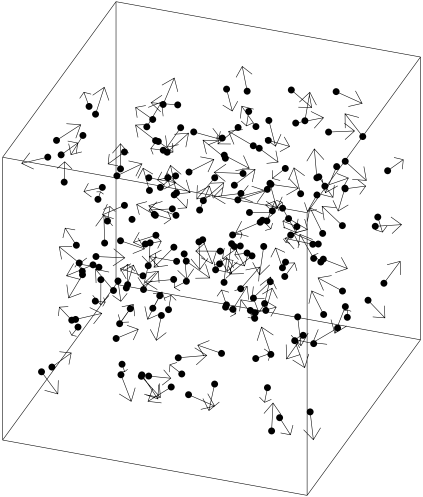
Random coil $\alpha$-helix $\beta$-sheet



# Spectroscopy

- **NMR spectroscopy**
  precession of magnetic moments of nuclei in magnetic field
  magnetic moments are slightly aligned in a static magnet
  axis of alignment is tilted by electromagnetic (radio) waves
  aligned magnetic moments precess about the static field
  resulting oscillating magnetic field is measured
  we do not observe the applied electromagnetic waves
  interactions of magnetic moments (mutual, with electrons)
  ⇒ resolution, structural information
  atomic resolution structure determination,
  dynamics, interactions

# Nuclear magnetic resonance



● molecule

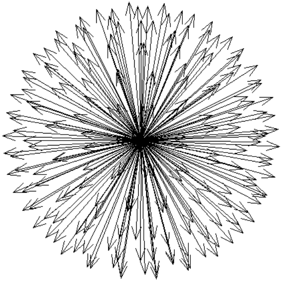
↗ magnetic moment

| | $S$ | $\frac{10^{-9}\gamma}{\mathrm{rad\,s^{-1}T^{-1}}}$ | % |
|---|---|---|---|
| $e^-$ | 1/2 | −182.000 | 100 |
| $^{1}$H | 1/2 | 0.277 | 99.98 |
| $^{13}$C | 1/2 | 0.067 | 1.1 |
| $^{14}$N | 1 | 0.019 | 99.6 |
| $^{15}$N | 1/2 | −0.027 | 0.4 |
| $^{17}$O | 5/2 | −0.036 | 0.04 |
| $^{19}$F | 1/2 | 0.252 | 100 |
| $^{31}$P | 1/2 | 0.108 | 100 |
| $^{129}$Xe | 1/2 | −0.075 | 24.4 |

quadrupolar (relax fast)

rare isotopes (enrichment)

# NMR sample outside magnet

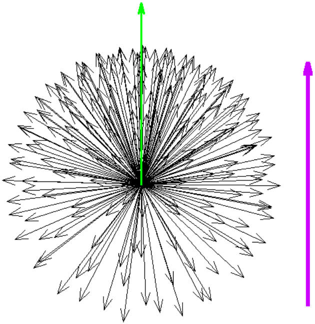


in equilibrium (spherical symmetry)

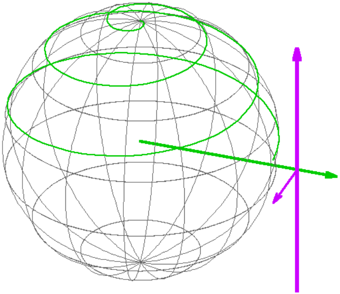
# Polarization

Boltzmann distribution: $P(\theta) \propto \mathrm{e}^{-\frac{E}{k_\mathrm{B}T}} = \mathrm{e}^{\frac{\vec{\mu}\cdot\vec{B}}{k_\mathrm{B}T}} \Rightarrow M_z = \frac{N}{V}\frac{\mu^2 B}{3k_\mathrm{B}T}$

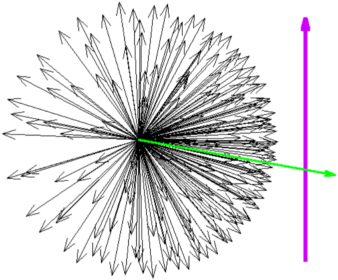
Precession (angular momentum in a field): $\vec{\omega} = -\gamma B$



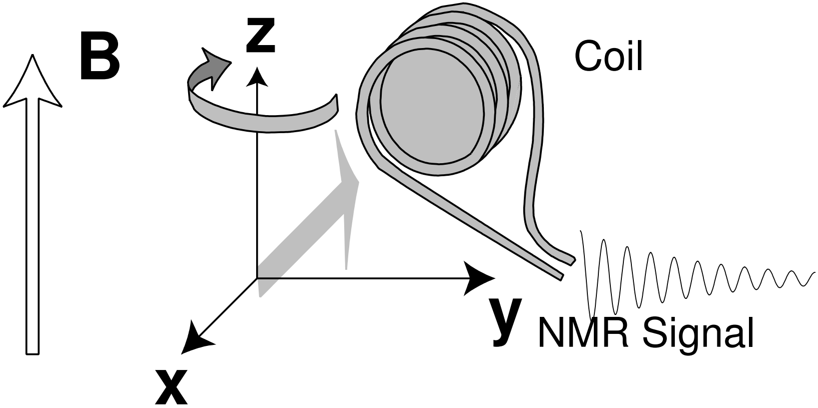
# Excitation



# Coherent evolution

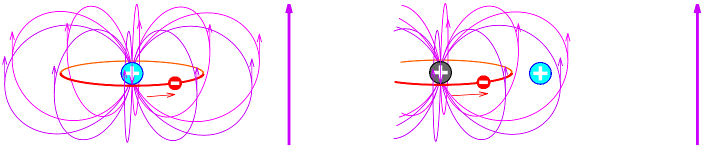


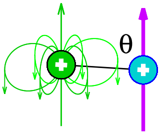
# NMR signal detection



reproduced from M. H. Levitt: Spin Dynamics

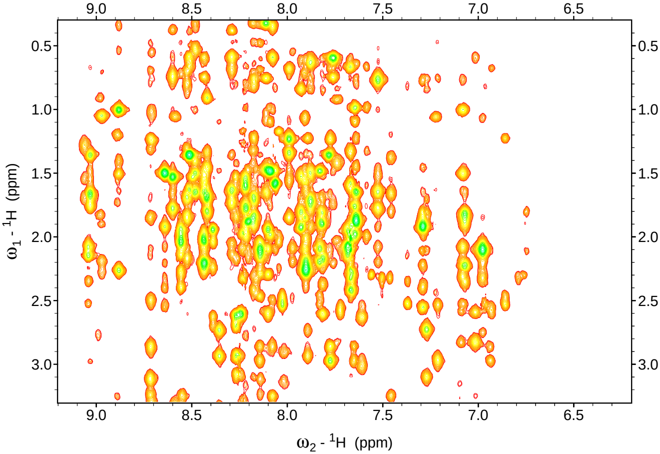
# Interactions with other nuclei and electrons





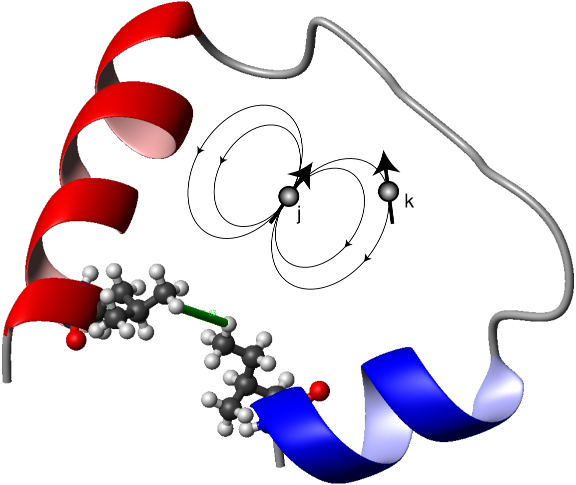
# Nuclear Overhauser effect from NOESY spectra

Nuclear Overhauser effect: result of dipole-dipole interactions
Peak intensities are proportional to nuclear Overhauser effect



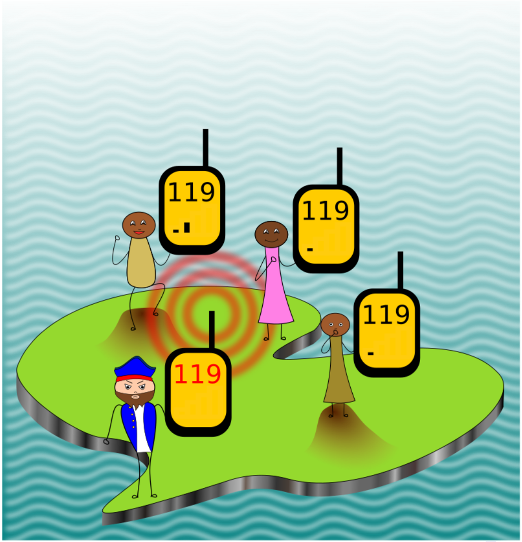
# Distances from nuclear Overhauser effect

Nuclear Overhauser effect proportional to $1/d^6$ ($d$ = distance)



# Distance based structure calculation

Model of the protein built from known distances



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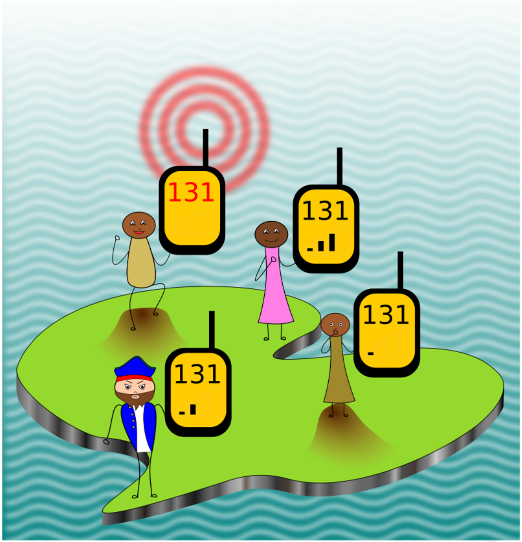

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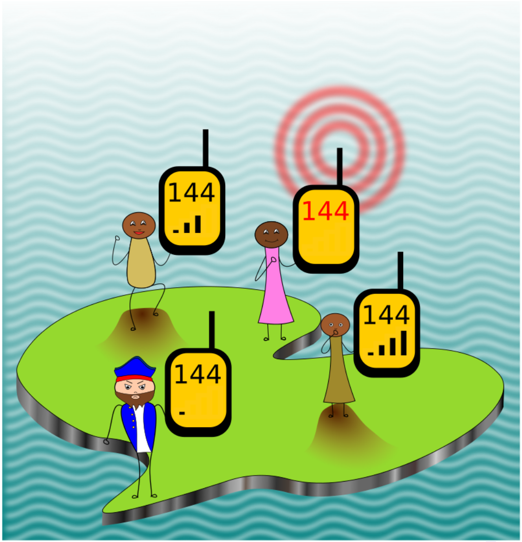

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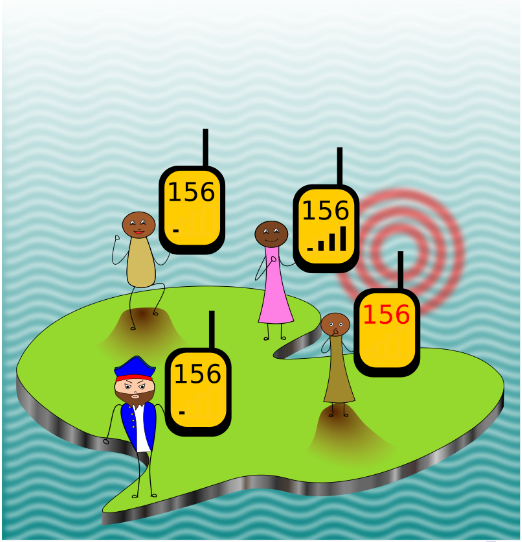
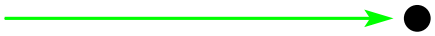


# Scattering by small molecules



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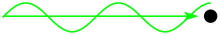

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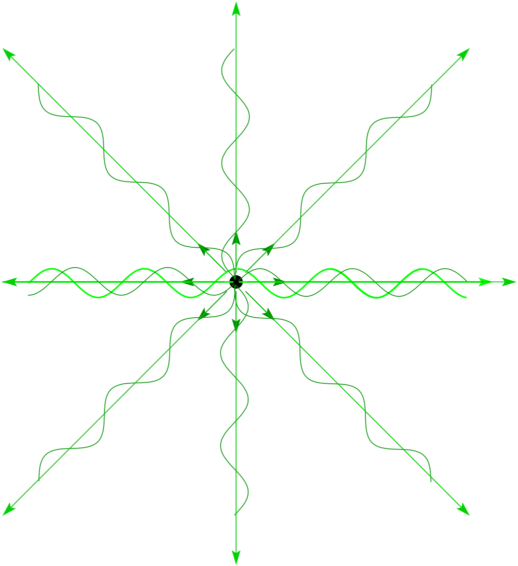

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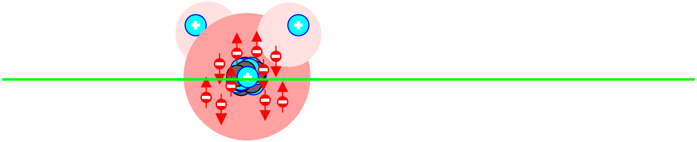

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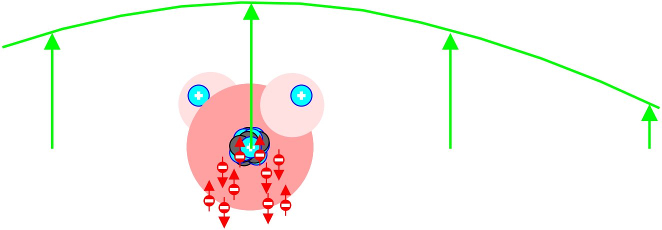

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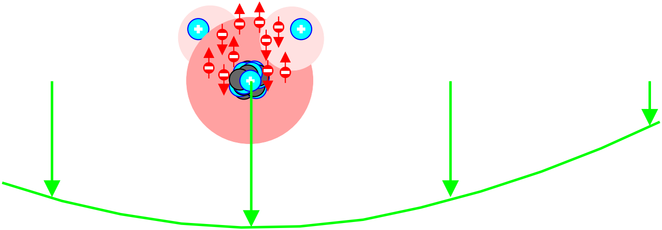
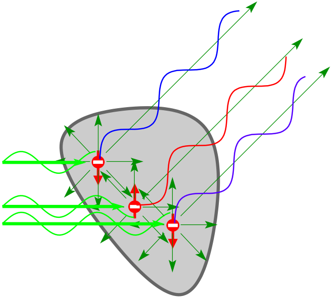
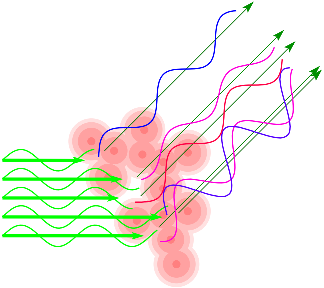


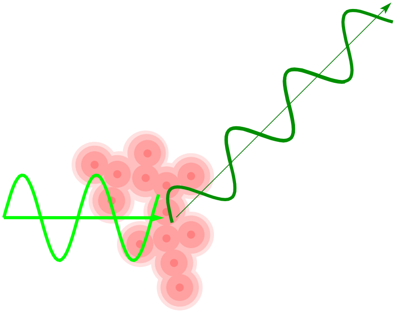
# Scattering by large molecules



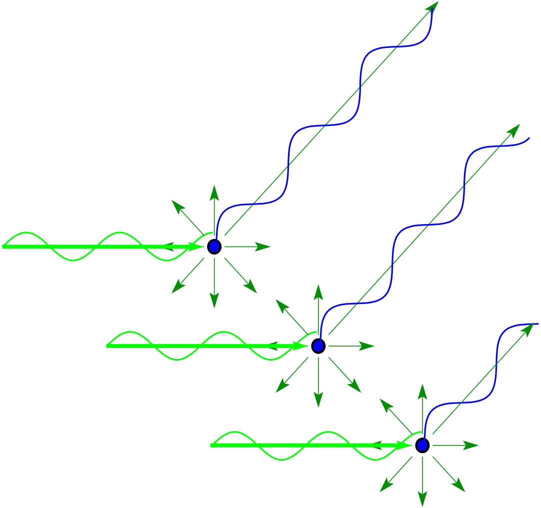
# Scattering by large molecules



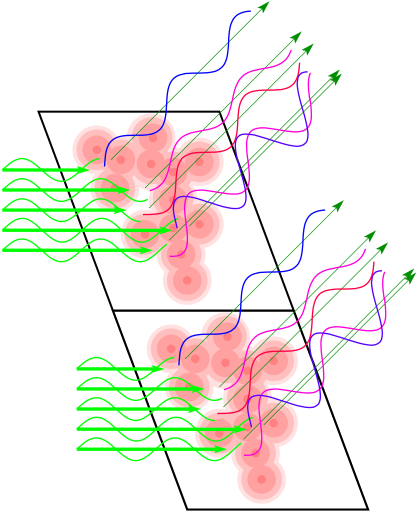
# Scattering by large molecules



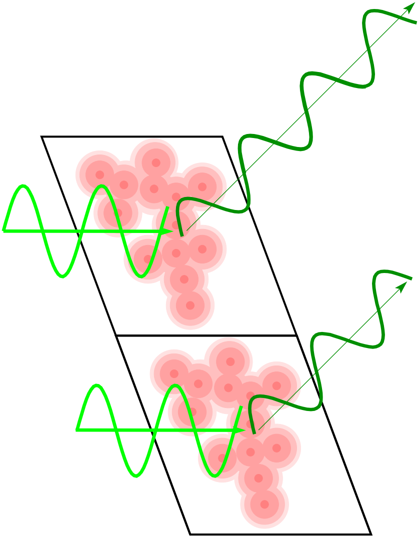
# Constructive interference in crystals



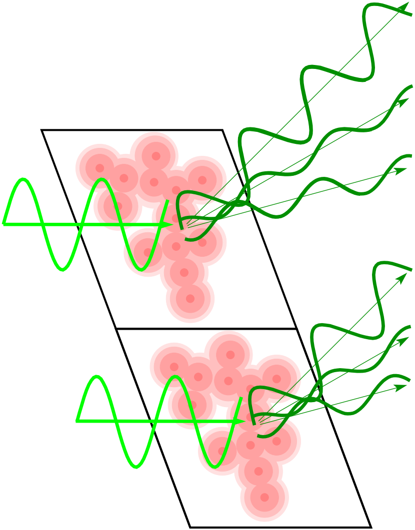
# Electron density



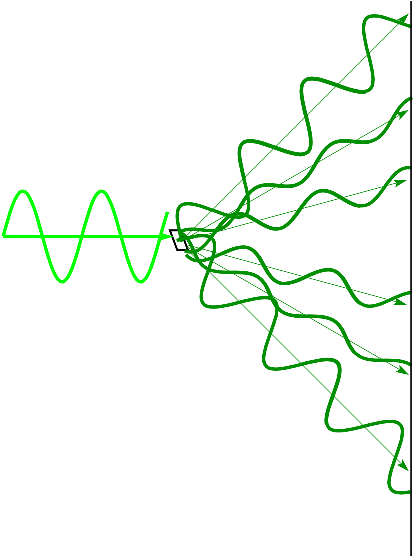
# Structure factor



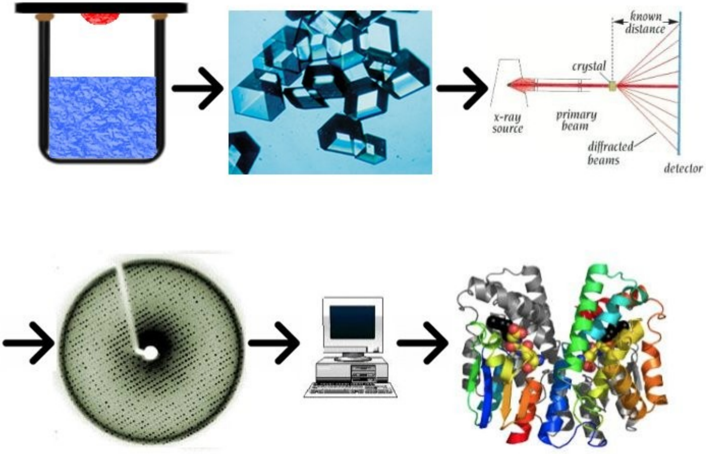
# Structure factor



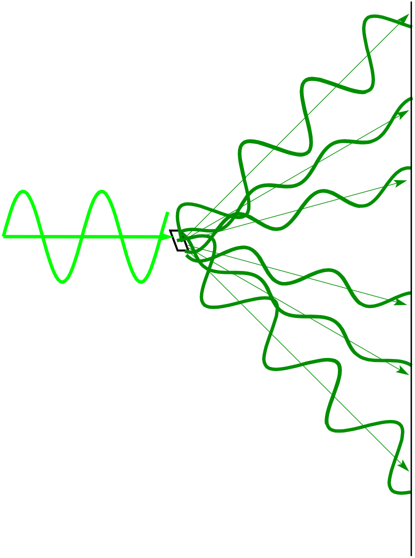
# Structure factor



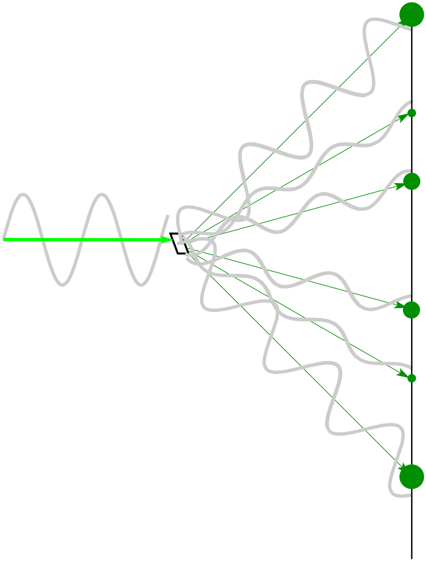
# X-ray crystallography



# Structure factor



# Structure calculation



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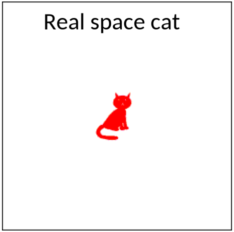
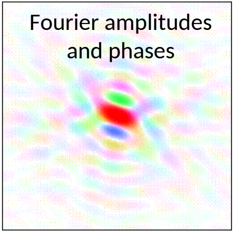


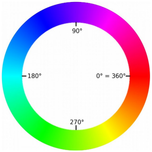
Real space cat

Fourier transform

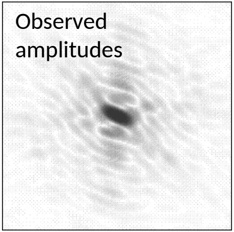


Fourier amplitudes and phases

Circular rainbow scale of phases



Linear intensity scale of amplitude size



Observed amplitudes

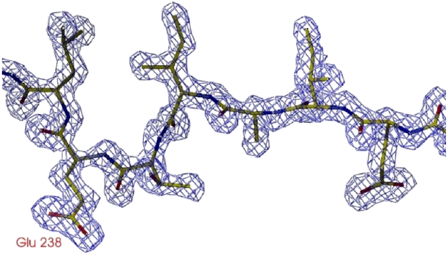
$$F(h\,k\,l) = V \int_{x=0}^{1}\int_{y=0}^{1}\int_{z=0}^{1} \rho(x\,y\,z)\exp[2\pi i(hx+ky+lz)]\,dx\,dy\,dz$$

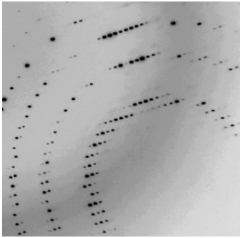
# Phase problem

## Electron density equation
## & PHASE PROBLEM

$$\rho(x\,y\,z) = \frac{1}{V}\sum_h\sum_k\sum_l \left|F(h\,k\,l)\right| \exp\left[-2\pi i(hx+ky+lz) + i\alpha(h\,k\,l)\right]$$

$$\boxed{F(h\,k\,l) = |F(h\,k\,l)|e^{i\alpha(h\,k\,l)}}$$

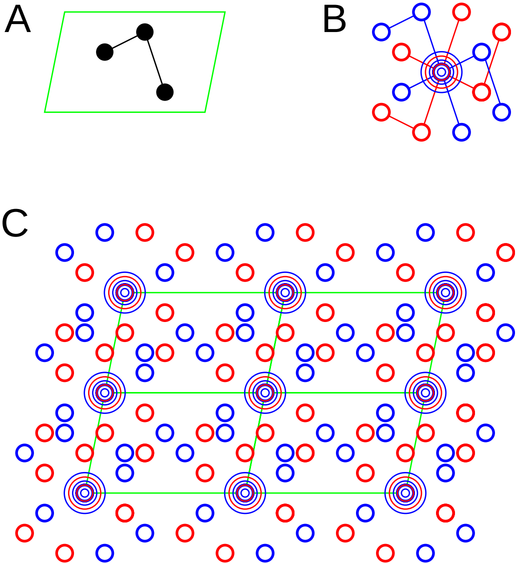




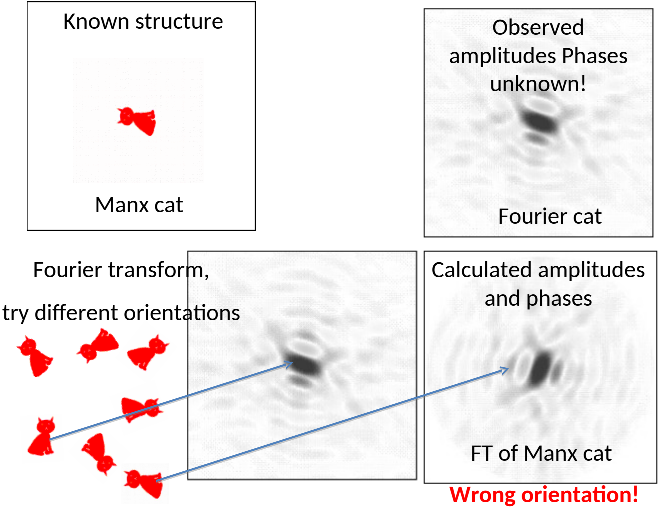
# Phase problem

- **Direct interpretation of amplitudes**
  mutual positions of atoms calculated from amplitudes
  for simple molecules (Patterson function/map)
- **Using heavy atoms**
- **Molecular replacement**
  Diffraction back-calculated from a known structure
  similar to the studied proteins
  Orientation and position of the molecule in the crystal
  obtained by searching for the match of diffraction patterns
  (measured vs. back-calculated)
  Calculated phases used for the unknown molecule

# Patterson maps



# Molecular replacement



Known structure

Manx cat

Observed amplitudes Phases unknown!

Fourier cat

Fourier transform,

try different orientations

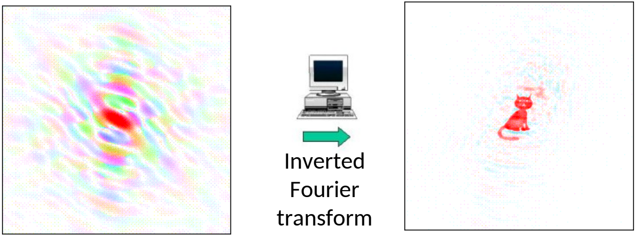
Calculated amplitudes and phases

FT of Manx cat

**Wrong orientation!**

# Molecular replacement

Observed **amplitudes** (tailed cat),
calculated **phases** (Manx cat)

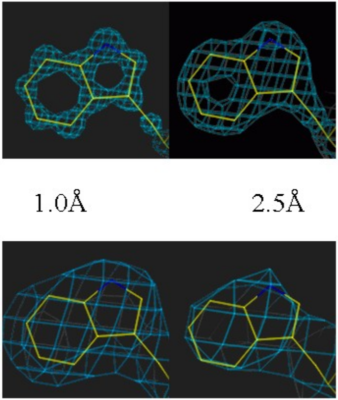


Inverted
Fourier
transform

Even the tail becomes visible!

# Model building

## Model building & resolution

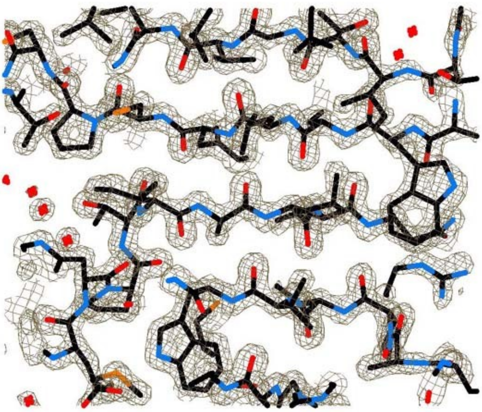


1.0Å 2.5Å

3.0Å 4.0Å

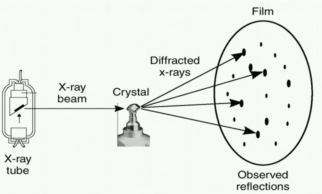
Model building

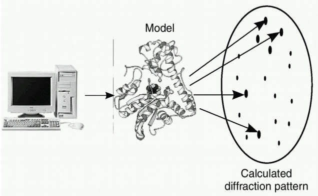
# Model building & refinement



# Validation

## R-factor, $R_{free}$ factor





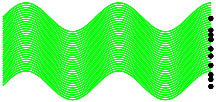
### R-factor

$$R = \frac{\sum_{hkl} ||F_{\mathrm{obs}}| - k|F_{\mathrm{calc}}||}{\sum_{hkl} |F_{\mathrm{obs}}|}$$

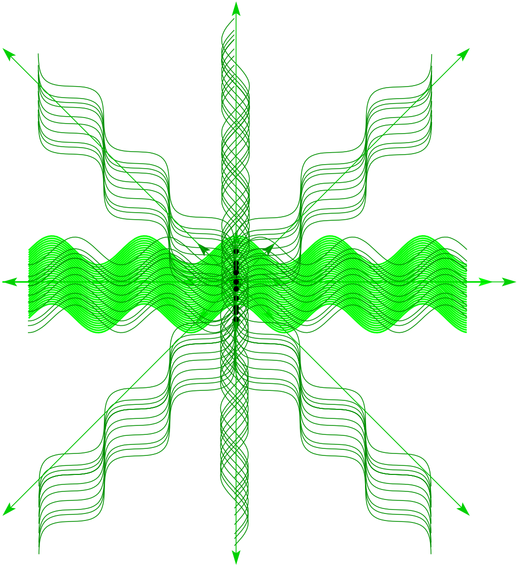
### $R_{free}$ factor

$$R_{\mathrm{free}} = \frac{\sum_{hkl \subset T} ||F_{\mathrm{obs}}| - k|F_{\mathrm{calc}}||}{\sum_{hkl \subset T} |F_{\mathrm{obs}}|}$$

# Scattering by molecules



# Scattering by molecules



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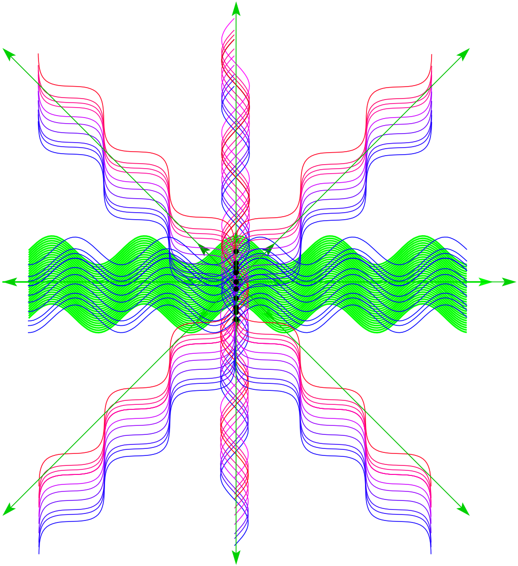
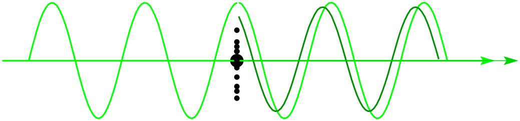
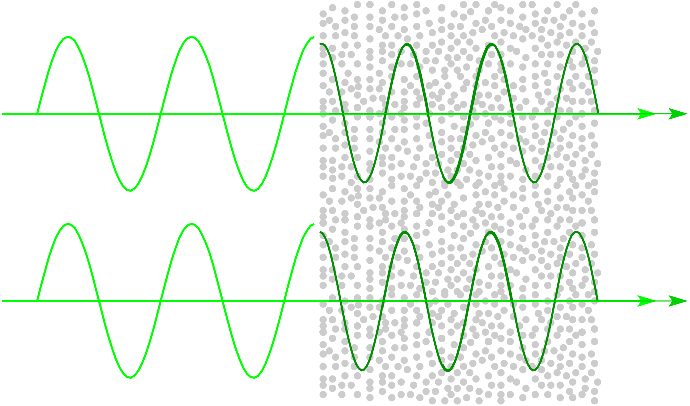


# Scattering by molecules



# Refractive index



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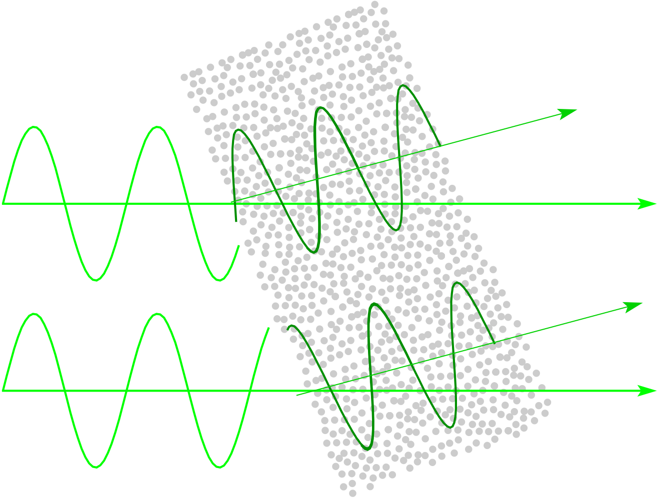
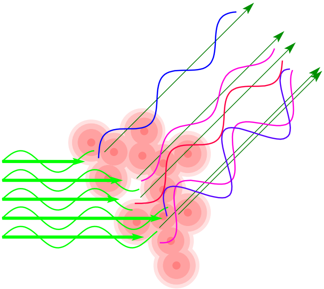


# Small angle X-ray scattering (SAXS)



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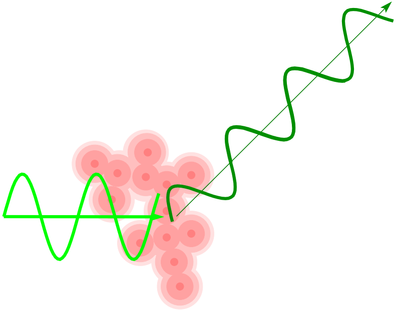

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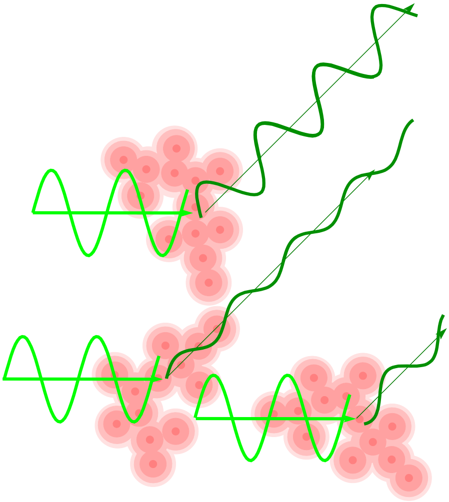

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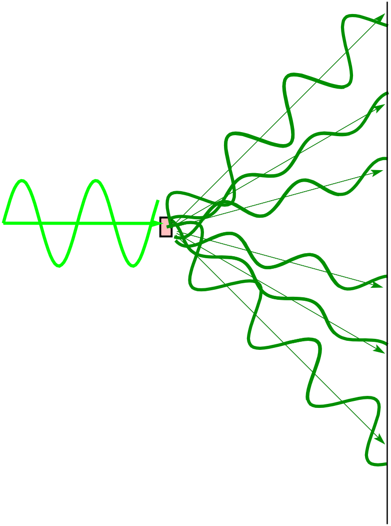

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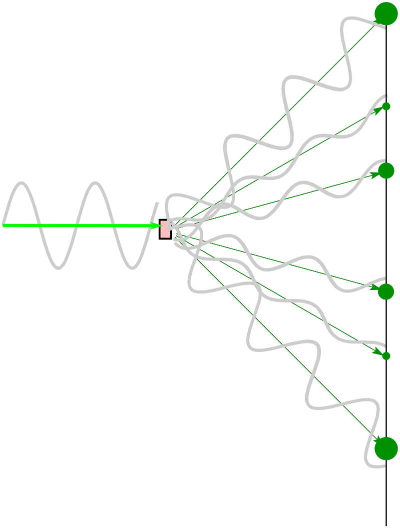


# Small angle X-ray scattering (SAXS)

crystal diffraction:

$I(\vec{q}) = \int_V \int_V \Delta\rho(\vec{r})\Delta\rho(\vec{r}+\Delta\vec{r})\mathrm{e}^{\mathrm{i}\vec{q}\cdot\Delta\vec{r}}\mathrm{d}\vec{r}\mathrm{d}(\vec{r}+\Delta\vec{r})$

SAXS: $I(q) = \left\langle \int_V \int_V \Delta\rho(\vec{r})\Delta\rho(\vec{r}+\Delta\vec{r})\mathrm{e}^{\mathrm{i}\vec{q}\cdot\Delta\vec{r}}\mathrm{d}\vec{r}\mathrm{d}(\vec{r}+\Delta\vec{r}) \right\rangle_{\theta_q,\phi_q}$

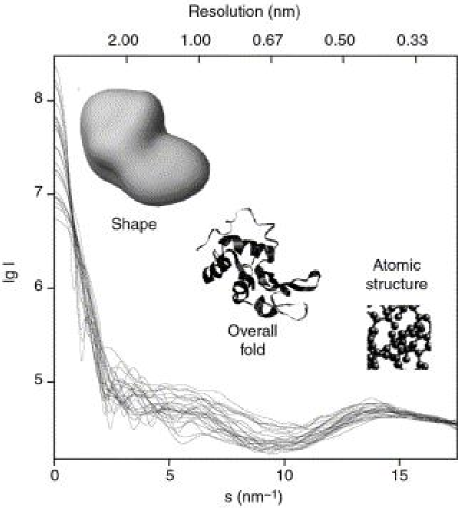
$$= 4\pi \int_0^{D_{\max}} \underbrace{r^2 \left\langle \int \Delta\rho(\vec{r})\Delta\rho(\vec{r}+\Delta\vec{r})\mathrm{d}\Delta\vec{r} \right\rangle_{\theta_r,\phi_r}}_{P(r)=r^2 V\rho^2\gamma_0(r)} \frac{\sin(qr)}{qr}\mathrm{d}r$$

Guinier law: $I(q) \approx I(0)\mathrm{e}^{-R_\mathrm{g}^2 q^2/3}$ for $q \to 0$

$\theta$: scattering angle
$\vec{q}$: scattering vector
$q = |\vec{q}| = 4\pi\sin(\theta/2)/\lambda$: momentum transfer
$I(q)$: scattering intensity in direction given by $q$
$\gamma_0$: probability of finding a point at $r$ from a given point
$R_\mathrm{g}$: radius of gyration

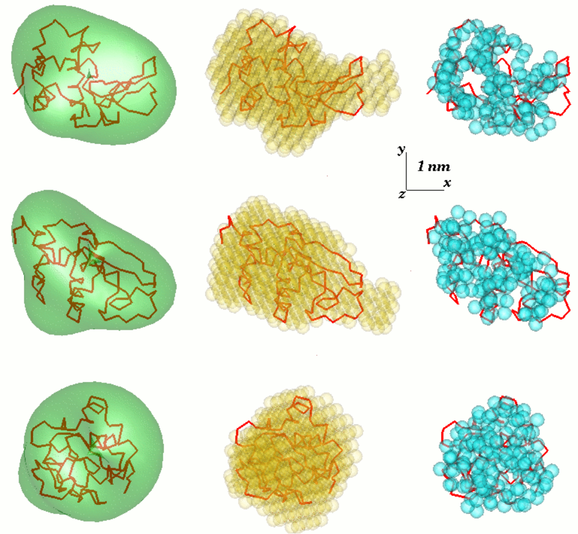
Svergun and Koch, Rep. Prog. Phys. 66 (2003) 1735, stacks.iop.org/RoPP/66/1735

# Small angle X-ray scattering (SAXS)



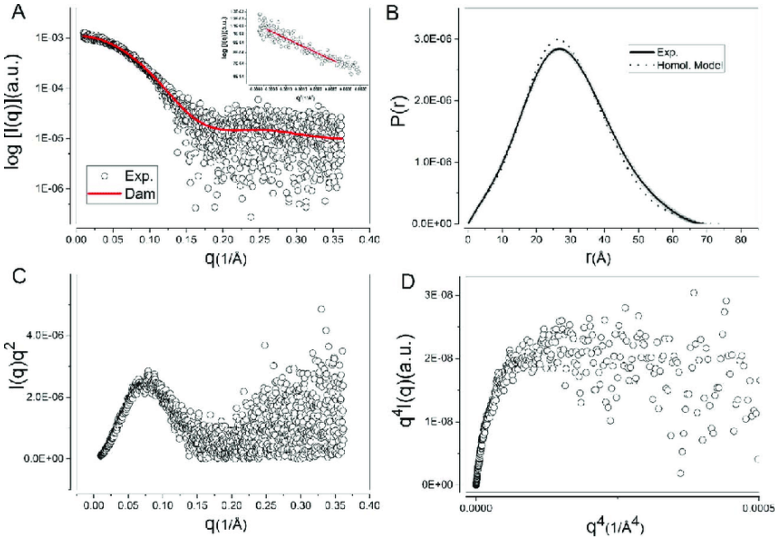
from Wikipedia

# Small angle X-ray scattering (SAXS)



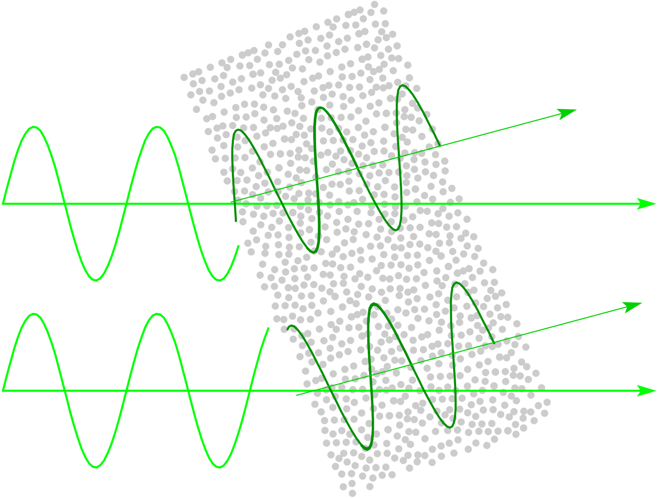
from Wikipedia

# Small angle X-ray scattering (SAXS)

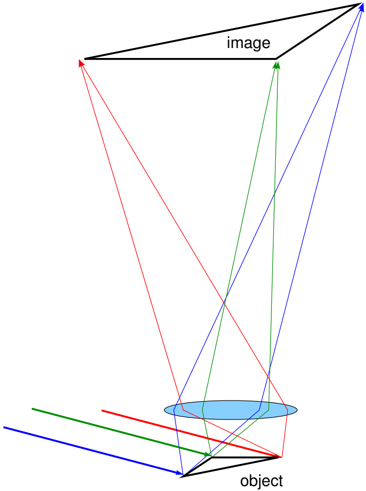


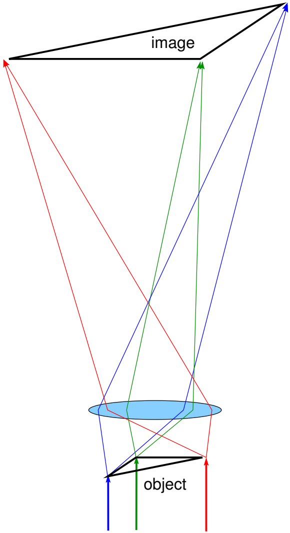
Evangelista, D., New Biotechnology 2017, DOI: 10.1016/j.nbt.2017.10.001

# Refractive index

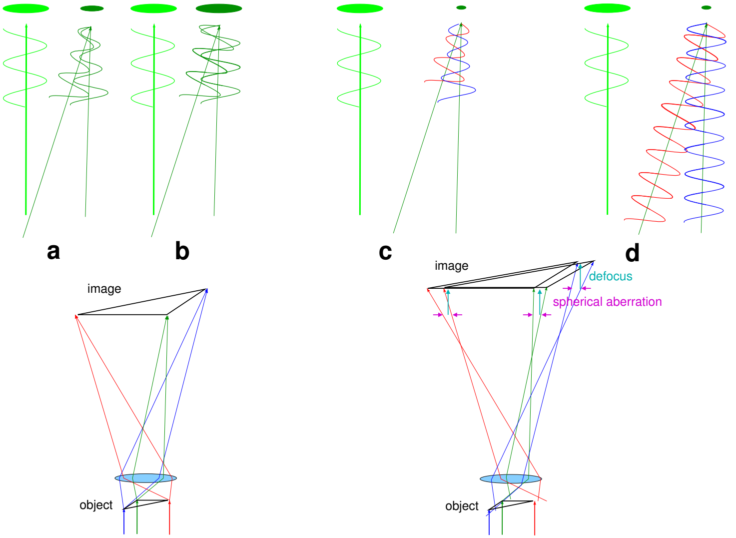


# Microscopy





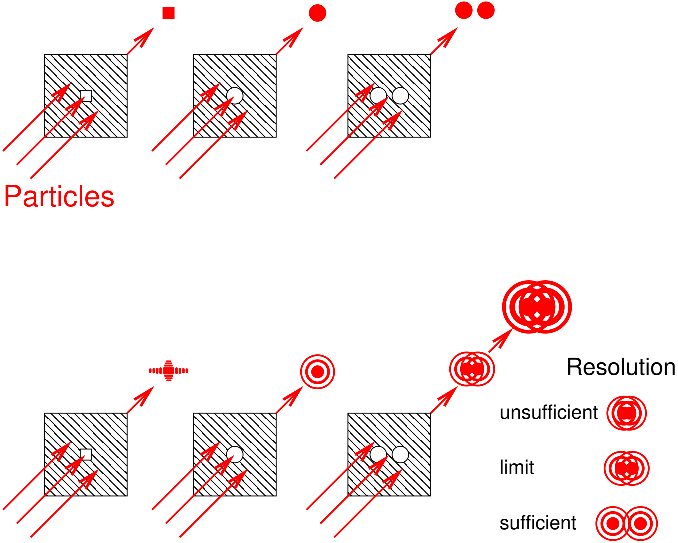
# Phase vs amplitude objects



# Phase vs amplitude objects

- **a** Perfect lens in focus provide contrast in intensity of scattered vs. original wave if the scattered wave is absorbed by the sample
- **b** Perfect lens in focus provide no contrast in intensity of scattered vs. original wave if the scattered wave is not absorbed by the sample, but only phase-shifted by 90$^\circ$
- **c** Imperfect and defocused lens provide contrast in intensity of scattered vs. original wave if the scattered wave is absorbed by the sample
- **d** Defocus and spherical aberration of lens provide contrast in intensity of scattered vs. original wave if the scattered wave is not absorbed by the sample, but only phase-shifted by 90$^\circ$. Defocus and spherical aberration introduce another phase shift ⇒ scattered have oposite phase and cancel each other.

# Diffraction by aperture



Particles

Waves

Resolution

unsufficient

limit

sufficient

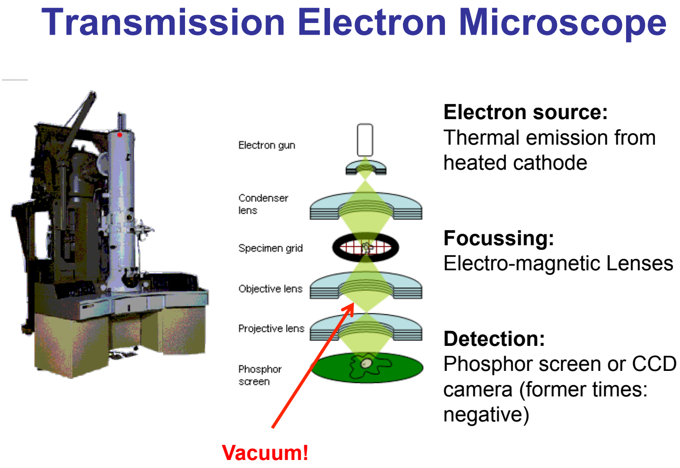
# Limitation of optical microscopy

Photons scatter as waves ⇒ limited resolution
resolution $< 0.61\lambda/n$ ($\lambda =$ wavelegth, $n =$ refractive index
⇒ $\lambda \approx 0.1\,\mathrm{nm}$ (distances of atoms in molecules), X-rays
Lens are not available for X-rays ($\lambda \approx 0.1\,\mathrm{nm}$)
no material has sufficient refractive index

SOLUTIONS:

- **Analysis of diffraction patterns**
  intensity enhanced if molecules are aligned in crystals
  ⇒ **X-ray crystallography**
  applicable also to electron and neutron waves
- **Microscopy with electrically charged waves**
  electrons scatter as waves
  electron beams are bent in electromagnetic field
  ⇒ **Electron microscopy**

# Transmission electron microscope



## Transmission Electron Microscope

**Electron source:**
Thermal emission from heated cathode

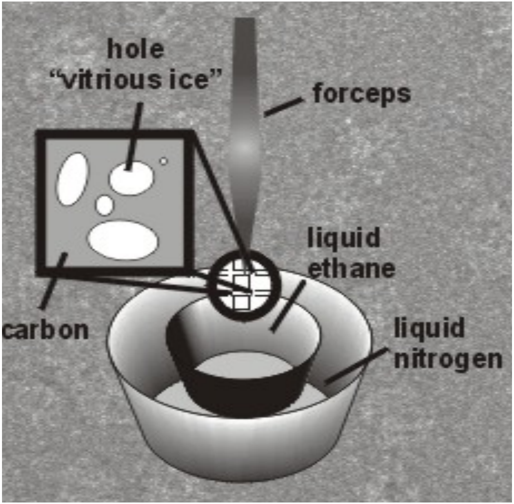
**Focussing:**
Electro-magnetic Lenses

**Detection:**
Phosphor screen or CCD camera (former times: negative)

**Vacuum!**

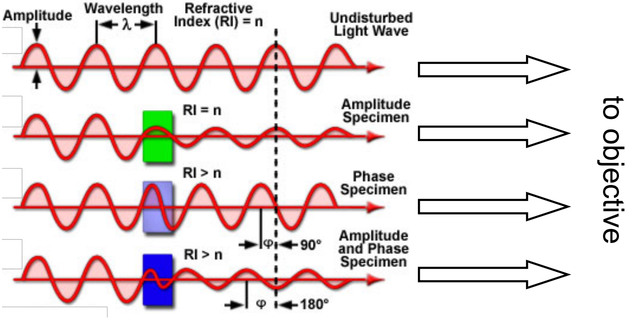
# Cryo-electron microscopy

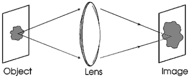
Proteins in vitrous ice, can reach atomic resolution



# Amplitude vs. phase objects

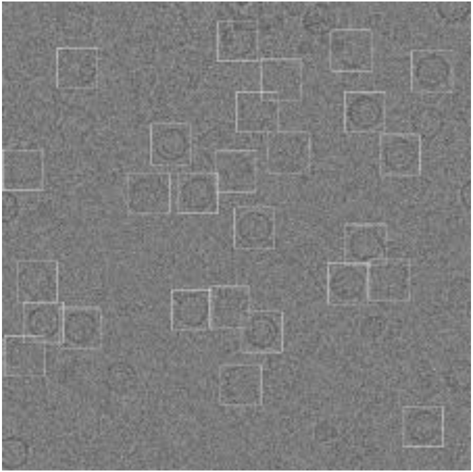
Macromolecules in water / vitreous ice
are phase objects





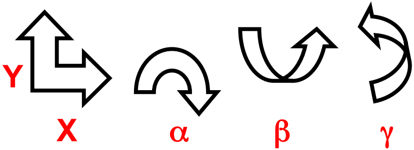
# Samples damaged by electrons

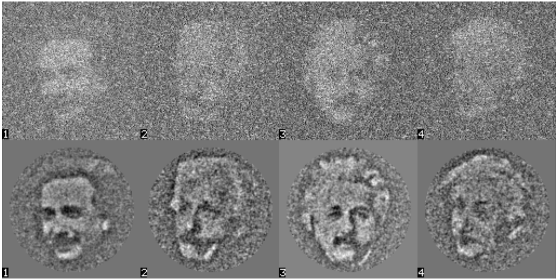
## Signal to noise ratio



# Alignment and classification

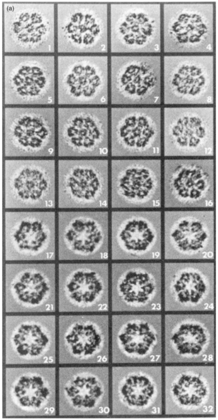
Images contain diffrent views of possibly different molecules

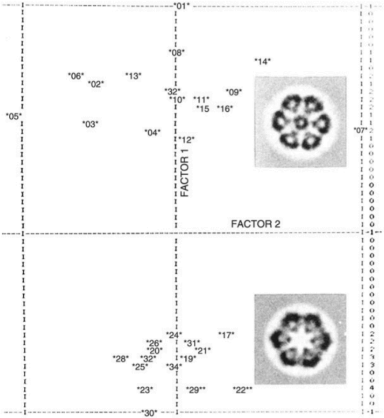




# Alignment and classification

## Classification and averaging (principal component analysis)





# 3D reconstruction

Iterative process:
2D projections are calculated from a 3D model
alignment and classification are improved iteratively