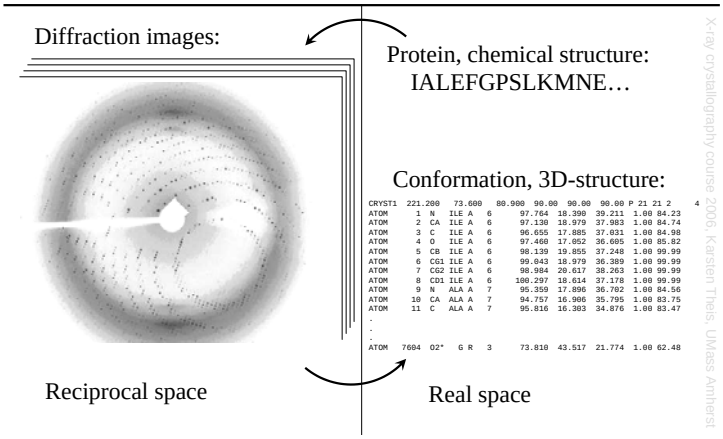
Protein structure from X-ray diffraction

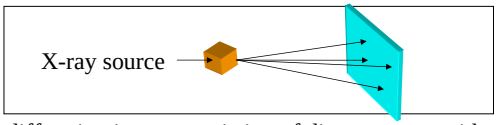


X-ray crystallography intensive course March 2006

- Friday hands-on sessions
  - Crystallization
  - X-ray diffraction
- Monday hands-on sessions
  - X-ray data processing
  - Crystal follow-up
  - Modelling into e-density
- Friday lecture 9 – 11:30 am
  - Crystals
  - basics of diffraction
- Monday lecture 9-11:30am
  - Crystal symmetry
  - Phase problem
  - Model quality

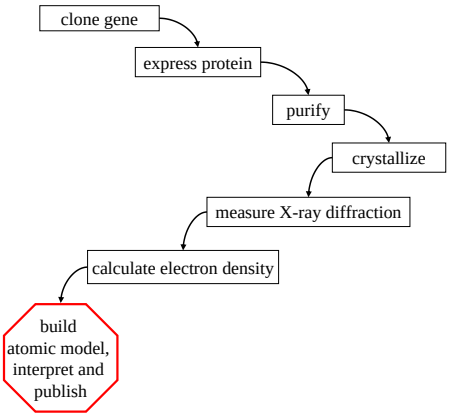
Diffraction experiment

- Ingredients: X-ray beam, crystal, detector



- a diffraction image consisting of discrete spots with distinct intensities I_{hkl} is observed on the detector
- diffraction: constructive interference of scattered light by objects that are related through translational symmetry

Steps in solving a structure

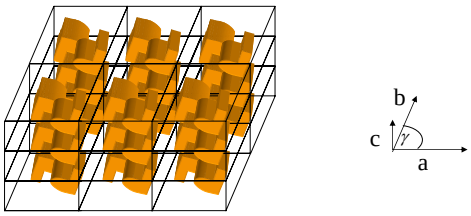


Obtaining well-diffracting crystals

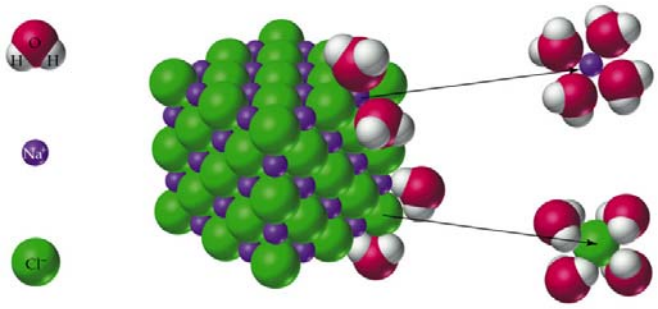
- Take-home message: This is the hard part
- definition: three-dimensional single crystal
- a good protein sample
- principles of crystal growth
- crystallization techniques
- strategies to obtain well-diffracting crystals (quickly?)
- Practical considerations

Three dimensional crystals

- periodic array of atoms, molecules, viruses...
- translational symmetry along three vectors a, b, c
- unit cell with edges a, b, c and angles α, β, γ is the building block for the whole crystal



NaCl crystal



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Problem set 1: protein crystals

Describe the differences between ionic crystals and protein crystals in terms of

- size of objects
- shape of objects
- space between objects
- number and nature of contacts
- expected mechanical stability
- requirement for mother liquor

- kinetics of nucleation
- kinetics of growth

Handling protein crystals

- Protein crystals contain large solvent channels, typically making up 40% - 70% of its volume
- The crystalline order is destroyed by exposing a crystal to air (solvent evaporates) or to mechanical stress (behaves somewhat like watermelon flesh)
- Crystals may be transferred surrounded by mother liquor in a capillary (used to soak crystals in solutions containing ligands, heavy atoms or cryoprotectants).
- Best long-term storage is in liquid nitrogen on a loop affixed to a support (you will learn this technique this afternoon)

Know your protein

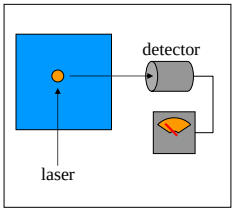
- Sequence, molecular weight
- Extinction coefficient
- Disulfides, glycoprotein?, phosphorylated?
- Maximum stability/activity, degradation?
- Cofactors
- Tags used in purification
- Substances encoutered during purification
- Secondary structure prediction
- Homologs with known structure

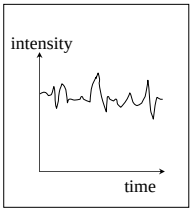
A good protein sample

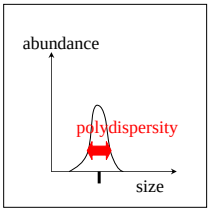
- pure (SDS gel, Mono Q, IEF, mass spec)
- defined buffer (DTT, volatiles, pH)
- defined concentration (by UV at 280 nm)
- stocks flash-frozen at -80 °C
- as little aggregation as possible
  - check with dynamic light scattering (sample requirements: 0.5 mg/ml, 20 µl)
  - output: molecular mass and polydispersity
  - to improve: salt, pH, temperature, detergent, batch, cofactors, binding partners, mutagenesis

Dynamic Light Scattering

- Measures the fluctuation of light scattered by the protein in solution
- Fluctuation is due to Brownian motion
- Big particles are slow, small particles are fast

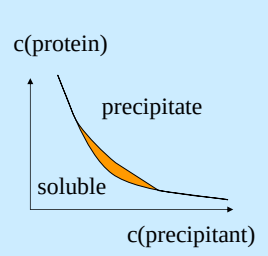






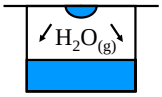
Crystallization: solubility

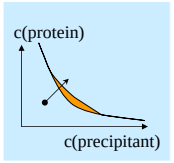
- protein solubility varies with the concentration of salts, polyethylene glycols and other substances in the protein solution
- a protein will quickly form an amorphous precipitate if the solubility is lowered drastically
- a protein might crystallize if the concentration is slightly above the solubility limit



Crystallization: vapor diffusion

- slowly increases protein and precipitant concentrations (12 h to 4 days to equilibrate)
- mix protein solution with precipitant solution 1:1 and equilibrate against excess of the latter
- takes 1 µl of 10 mg/ml protein sol. per experiment





Finding initial conditions

- Crystal screens I (original) and II (copy cat)
- Check crystal setups every day in first week
- Possible results: clear, precipitate, crystal and many others (turbid, bubbles, clothing fibers)
- If almost all or almost no drops are clear, raise or lower protein concentration, respectively
- Focus on setups that show some precipitate, but not a heavy yellow or brown precipitate indicative of protein denaturation
- For each experiment, find precipitant concentration that precipitates protein after 2-3 days (by series of follow-up experiments)

Practical considerations

- To efficiently screen different pH values, the buffer strength of the protein sample should be low (10 mM) compared to the screen's (100mM)
- Vapor diffusion is affected by high concentrations of salt or glycerol in the protein sample. Check that the drops actually decrease in size with time.
- Volatiles will not stay in the drop
- If the setup is not closed off completely, the well solution will eventually dry out
- Dirt (fibers, dust, fingerprints) might influence nucleation and make experiments irreproducible.

Improving size and diffraction

- Systematic variation of all concentrations and pH
- Additive screens and detergent screens
- Temperature
- Seeding with crushed crystals (micro seeding)
- Dialysis, batch, sitting drop
- … check old setups for different crystal form

It does not crystallize…

- Check purity and stability
- Remove cysteins and other trouble makers
- Remove flexible parts
- Try thermophilic orthologs
- Try single domains
- Try physiologically relevant complexes
- Try complex with antibody FAB domain

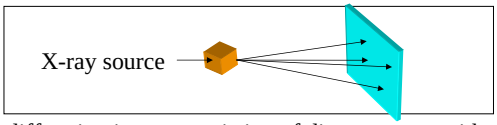
Problem set 2: Crystallization

- You equilibrate 1µl protein (c=10mg/ml) and 1µl precipitant (30% PEG) against an excess of 30% PEG
  - what are the initial concentrations?
  - what are the final concentrations?
- You get a crystal containing 50% solvent, 50% protein
  - what is the protein concentration in the crystal?
  - what is the maximal volume of the crystal?
- You crystal grows three times as fast along the C-axis as compared to the A-axis and B-axis. What shape will your protein crystal have when it has finished growing?

Stretch!

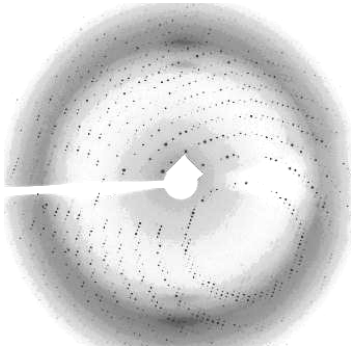
Diffraction experiment

- Ingredients: X-ray beam, crystal, detector



- a diffraction image consisting of discrete spots with distinct intensities I_{hkl} is observed on the detector
- diffraction: constructive interference of scattered light by objects that are related through translational symmetry

Features of diffraction images



- Discrete spots
- Intensities are different from spot to spot
- In general, intensities decrease from the center to the edge

Structure ↔Diffraction data

- First step: understand how given structure leads to diffraction pattern
- Second step: measure diffraction data
- Third step: Solve structure based on diffraction data

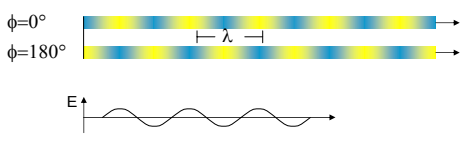
- If we know how to calculate the diffraction pattern from a given structure, we can use the measured diffraction data to check if a putative structural model is correct

X-rays

- Electromagnetic radiation, $\lambda \approx 1$ Å
- Produced by copper anode or synchrotron
- X-ray beam described by
  - wavelength λ
  - direction

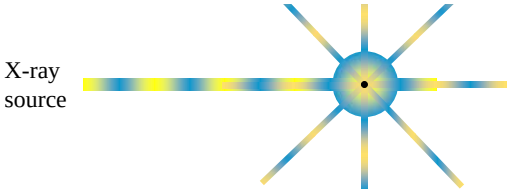
  } wave vector **k**, $|\mathbf{k}| = 1/\lambda$
  - amplitude |F| (how strong)
  - phase ϕ (peaks and valleys)

  } $A=|A| \exp(i\phi)$

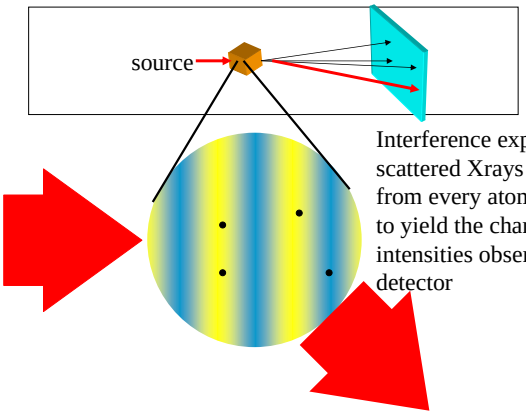


Elastic scattering

- weak interaction of X-rays with electrons (most of the beam passes straight through)
- emission of scattered X-rays in all directions
- wavelength does not change
- phase does not change

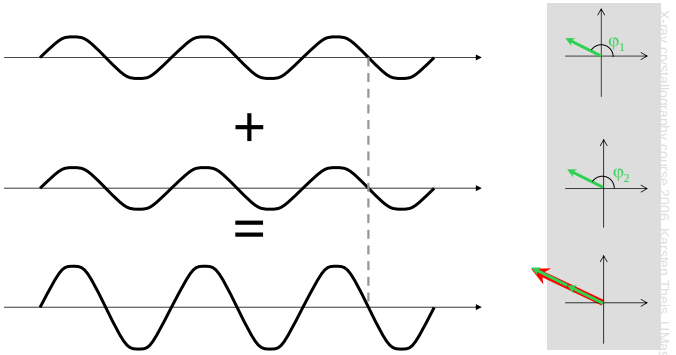


Every atom contributes to each spot



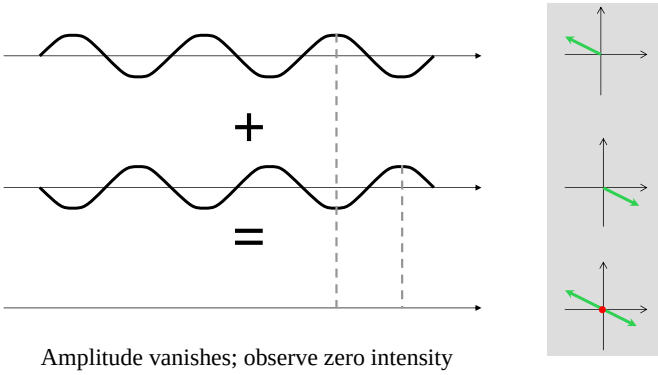
Interference explains how scattered Xrays originating from every atom combine to yield the characteristic intensities observed on the detector

Constructive Interference: $\Delta\varphi=0$



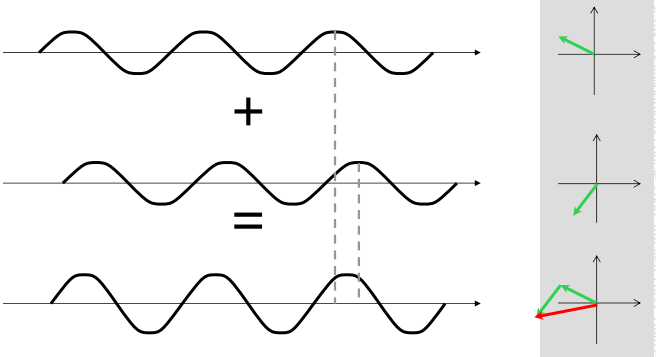
Amplitude doubles; Intensity quadruples

Destructive Interference: $\Delta\varphi=\pi$



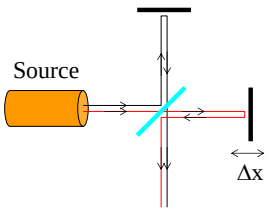
Amplitude vanishes; observe zero intensity

Interference with arbitrary $\Delta\varphi$



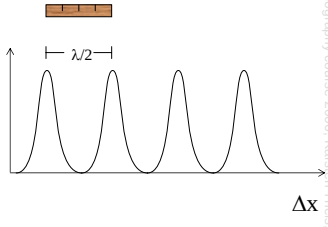
Measuring distances using light

Michelson Interferometer



Detector

Light is used as a yardstick that has a length of λ/2



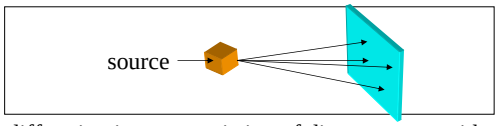
To measure atomic distances through interference and thus determine the structure of molecules, our yardstick must have atomic dimensions.
This is why X-rays are required for crystallographic structure determinations

Interference

- Interference describes superposition ("addition") of X-rays with identical wavelength and direction
- Depending on the positions of the scatterers, the scattered X-rays have certain phase differences resulting in amplification or cancelation
- Interference leads to diffraction patterns on the detector that contain structural information

Diffraction experiment

- Ingredients: X-ray beam, crystal, detector

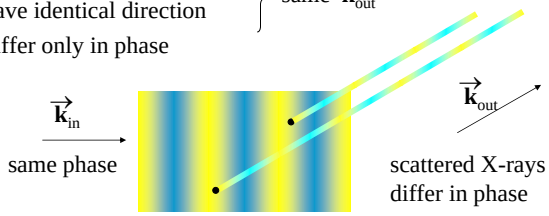


- a diffraction image consisting of discrete spots with distinct intensities I_{hkl} is observed on the detector
- diffraction: constructive interference of scattered light by objects that are related through translational symmetry

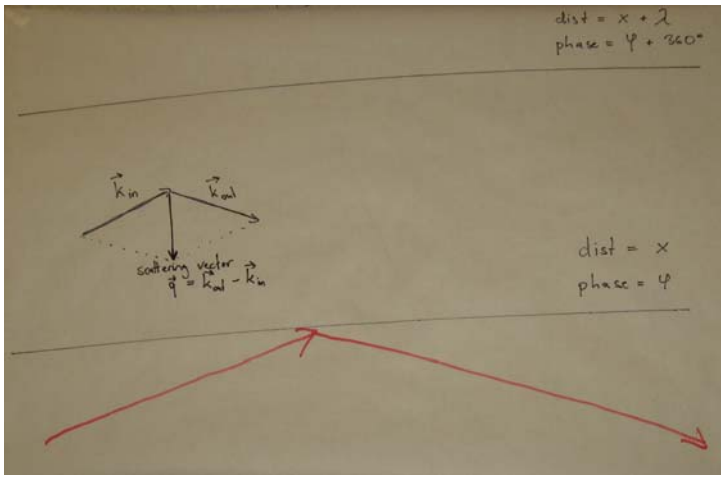
Atomic position determines phase

- Scattered X-rays hitting a certain location on the detector
  - originate from different atoms
  - have identical wavelength
  - have identical direction
  - differ only in phase

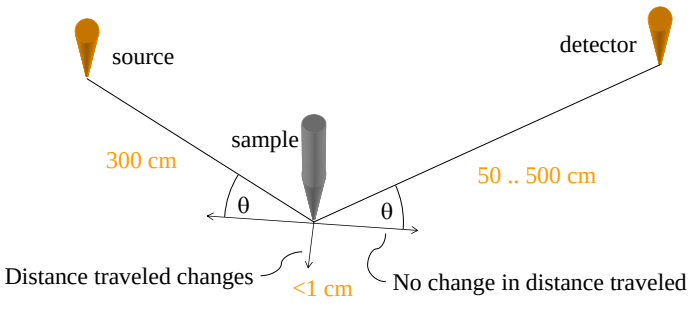
same $\vec{k}_{out}$



Difference in phase results from difference in distance traveled between source and detector



A geometry exercise

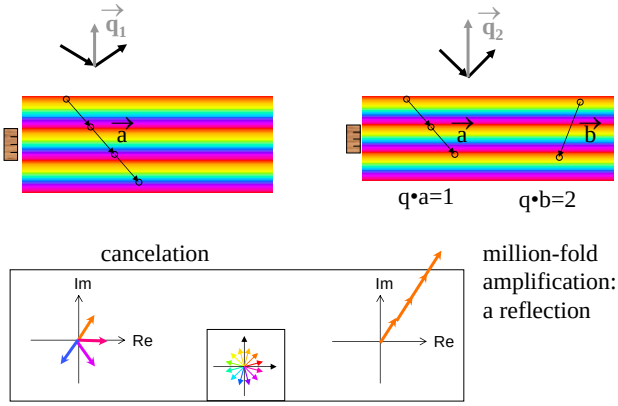


Problem set 3: diffraction basics

In the movie, you saw that X-rays scattered from points on certain **planes** have identical phase at the detector. By experimenting with different "wavelengths" and diffraction angles (i.e. detector positions), answer the following questions

- What determines the orientation of these **planes**?
- How is the distance between these planes (the d-spacing or resolution) affected by wavelength and diffraction angle?

Laue diffraction conditions (I)



Laue diffraction conditions (II)

q•a no whole number

↓

$\Delta\phi = 2\pi\ q \bullet a \neq 0$

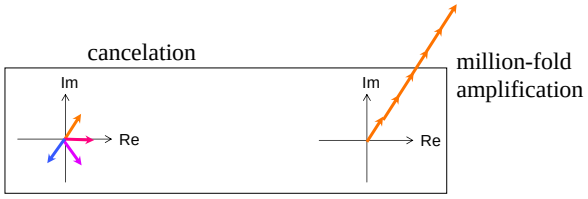
↓

no constructive interference: no spot (reflection) observed

q•a = h (whole number)
q•b = k (whole number)
q•c = l (whole number)

↓

constructive interference: spot (reflection) observed



h,k,l: Miller indices

- Three integers, h, k, l, to define reflection
- Assigning Miller indices requires knowledge of the cell parameters and crystal orientation
- We want to know how to measure reflection with certain Miller indices h,k,l in order to measure complete data set (all reflections up to a given resolution)

Take-home message

A single crystal hit by X-rays diffracts X-rays in certain directions with certain intensities. The directions depend on the crystal lattice; the intensities tell us about the protein structure.

Problem set 4: reciprocal space

Given a crystal with the unit cell vectors **a**, **b**, and **c**, answer the following questions:

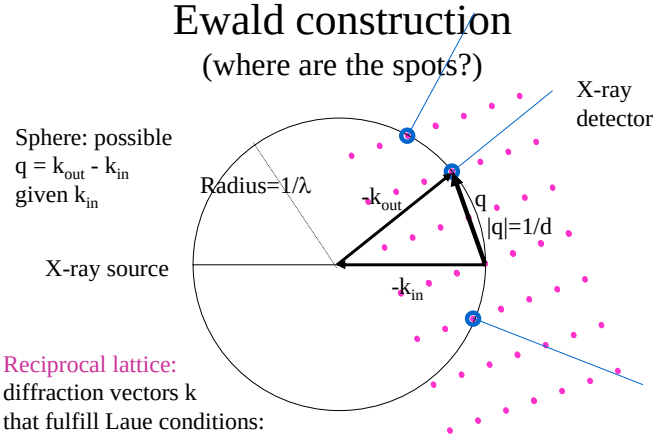
- What are the Laue conditions for the (100) reflection? (we'll call that scattering vector **a***)
- What are the Laue conditions for the (010) reflection? (we'll call that scattering vector **b***)
- For a scattering vector q=2**a***, do we expect constructive interference?
- For a scattering vector q=2**a*** + **b***, do we expect constructive interference? What are the Miller indices?

We have a new crystal form with **a'**=0.5**a**.

- What are the new **a*'**, **b*'**, **c*'**?
- Will you observe more reflections for the large or the small unit cell?

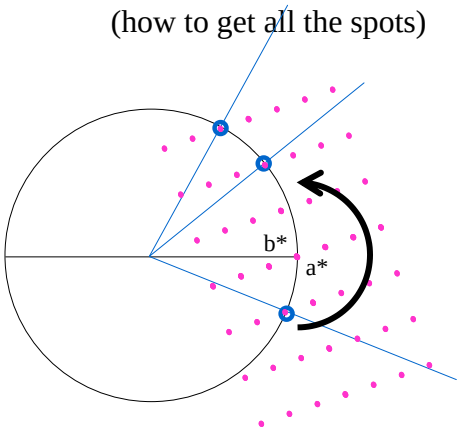
Ewald construction

(where are the spots?)

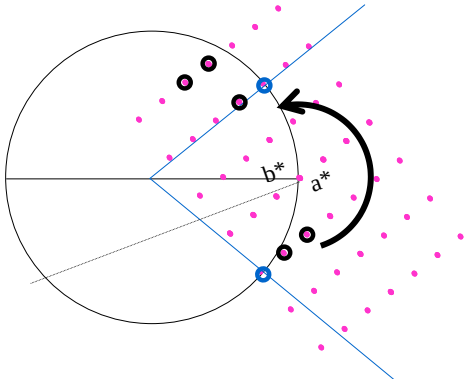


Reciprocal lattice:
diffraction vectors k that fulfill Laue conditions:
q·a, q·b, q·c are integers

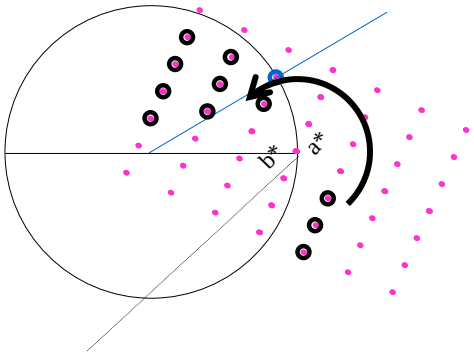
Rotation method
(how to get all the spots)



Rotation method
(how to get all the spots)

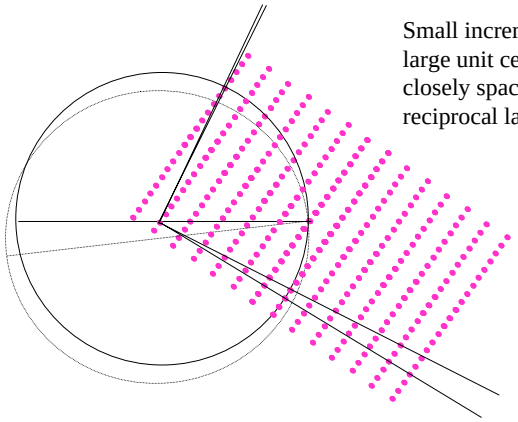


Rotation method
(how to get all the spots)

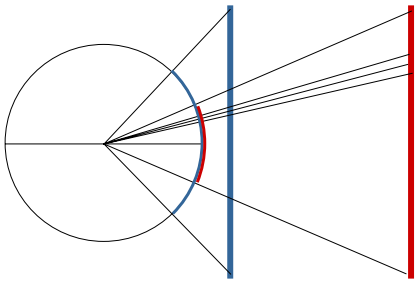


Rotation increment

Small increments for large unit cells (i.e. closely spaced reciprocal lattice)



Detector distance



small distance: more reflections; spots might overlap because they are closely spaced

large distance: less reflections; neighboring spots are better separated