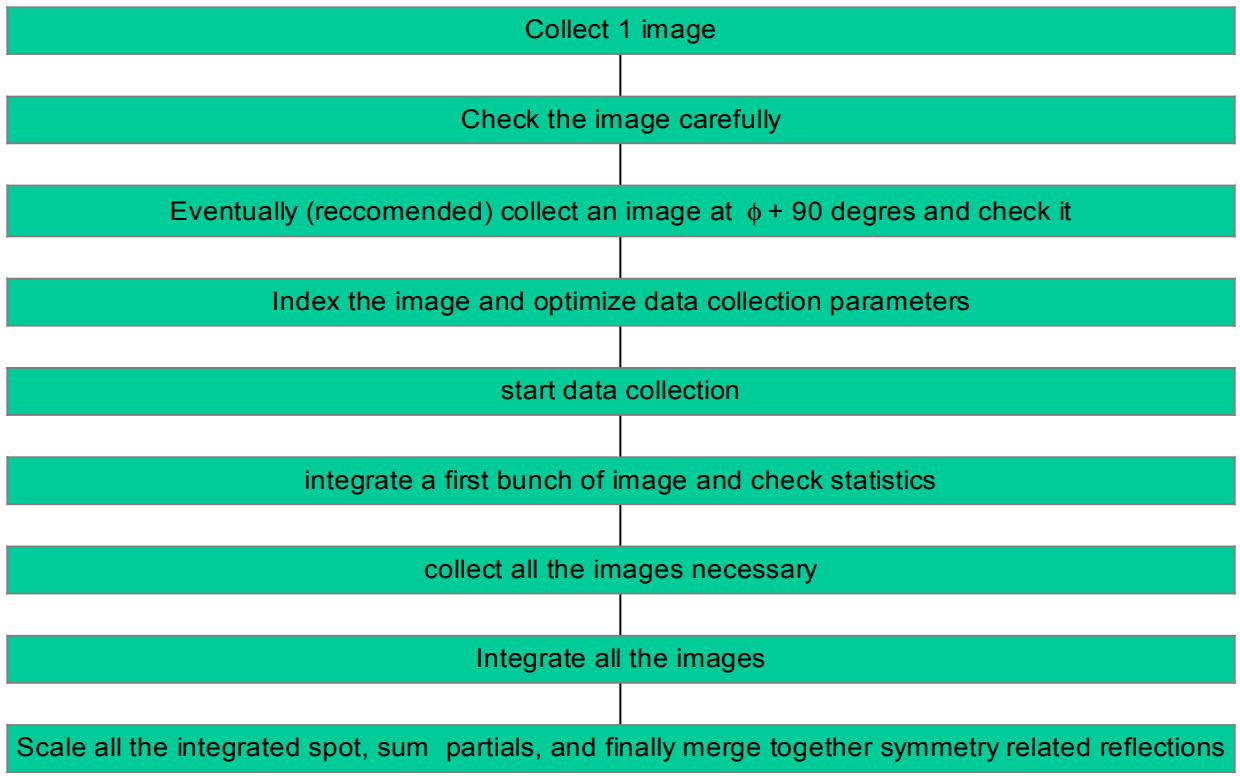
Rotation Method –Exposure Time

- In order to achieve an higher resolution data set, the exposure time could be increased, taking care that a complete dataset is still possible. In any case consider that:
- Long exposure times can result in many spots being saturated
- To double $\langle I/\sigma \rangle$ of the spots the exposure time must be increased by a factor of four
- Long exposure times can be critical for radiation sensitive crystals
- Sometimes it is better to have a shorter exposure time but collect more images. In this case the disadvantages are: 1) many images, 2) more readout noise, 3) worse duty cycle (important with Imaging Plate).

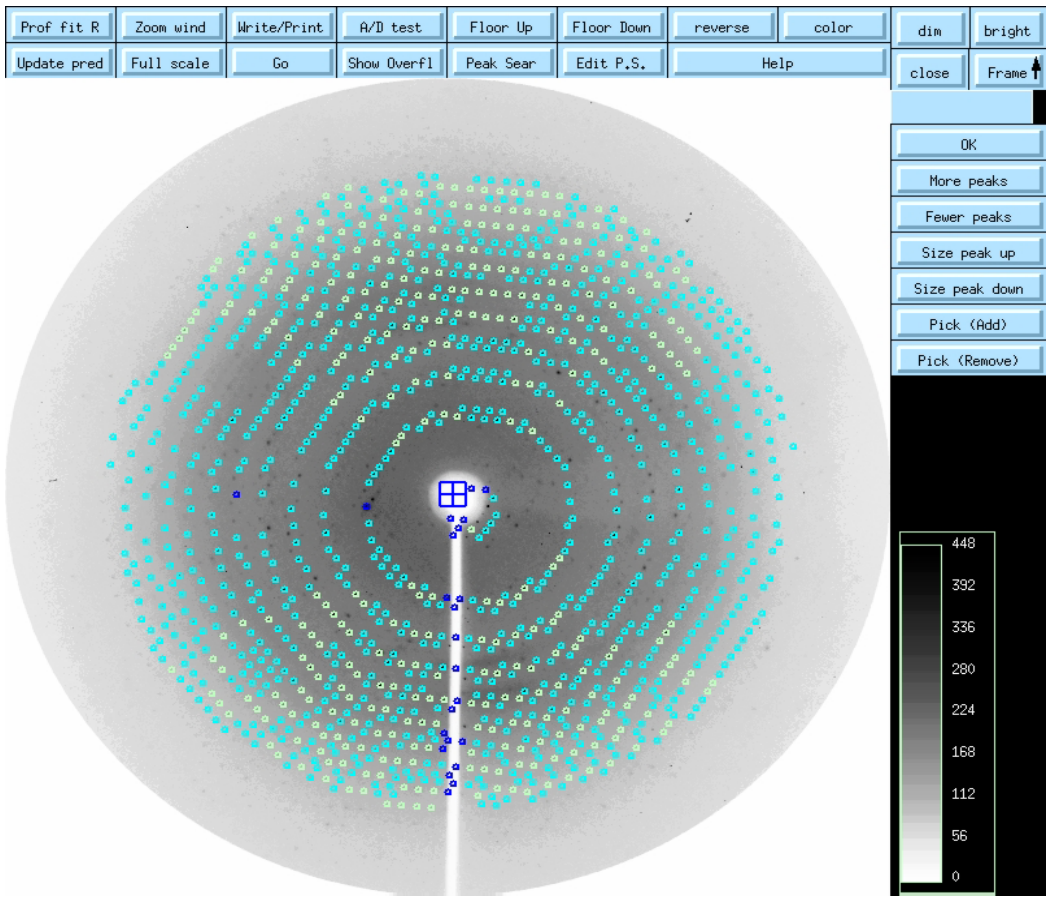
Rotation Method – General Checks

- Spots not well resolved: check $\Delta\phi$ and crystal to film distance
- Spots are split or streaked: check cryo conditions, crystallization conditions or protein homogeneity
- Ice rings: Check cryo conditions
- No spots in the outer region of the detector: Increase (not a large amount) the exposure time if you are confident in your resolution limits, otherwise adjust the crystal to film distance to fill the detector (better $\langle I/\sigma \rangle$ at longer distance)
- High mosaic spread: Try annealing or check cryo conditions or crystallization conditions
- Always check images at 0 and 90 degrees for anisotropic behavior
- Strong background: Try smaller collimation (be careful of alignment of the crystal and/or experimental setup). If possible use an Helium purging path.

Data Collection – General layout

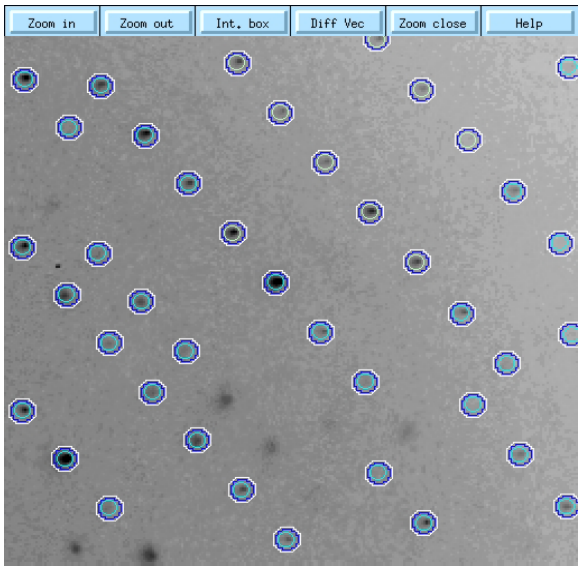


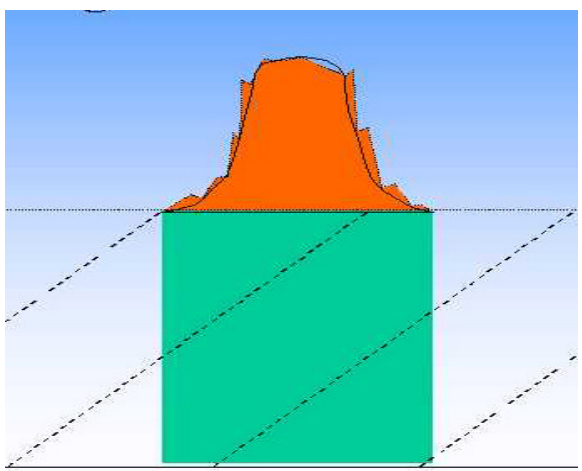
Data Processing - Indexing



- At the indexing stage we determine the lattice symmetry of the crystal and the orientation of the lattice with respect to the laboratory orthogonal coordinate system
- In general this is done finding the highest order lattice symmetry that well predicts as much spots as possible on the diffraction pattern

Data Processing - Integration





- Once the first image has been indexed, on the basis of refined indexing parameters such as unit cell, detector and beam geometry, mosaicity and integration box, the intensities for all the predicted spots is determined; this process is then automatically repeated for all the subsequent images.
- The spot intensity is in general estimated by using a profile function obtained by fitting the intensity profile of strong reflections in the same detector zone.
- Correction factors are applied to intensities (Lorentz and Polarization)

Data Collection – Scaling and Merging

- All the integrated spots from different images are put on a common scale.
- The partial reflections are summed together.
- Better estimate of the unit cell parameters are determined (Postrefinement)
- Symmetry related reflections are merged together giving a final dataset useful for the subsequent structure solution and refinement.

- Several figures of merit are issued at the end of the scaling and merging stage, giving an idea of the goodness of the dataset.

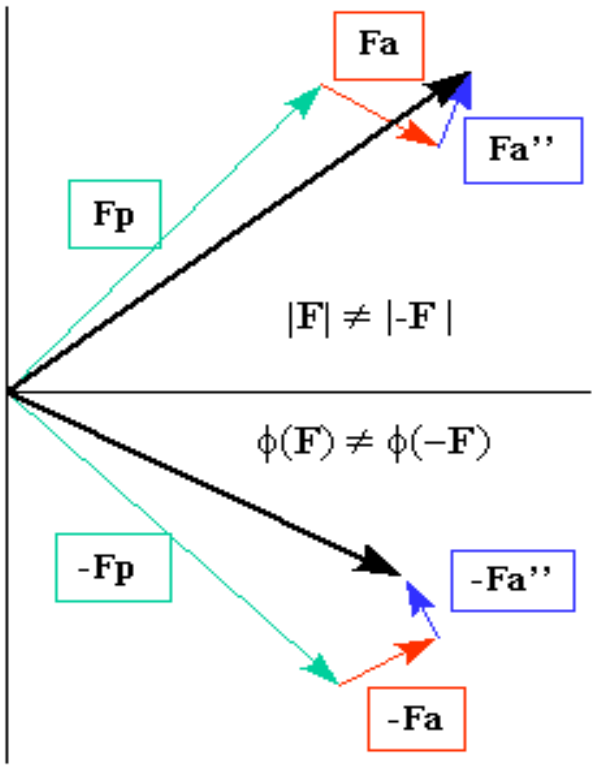
Rotation Method – Quality Indicator

- Completeness: indicates the percentage of reflections experimentally determined with respect to the theoretical ones for that resolution (should be as near as possible to 100 %, check the low resolution completeness.
- $\langle I/\sigma \rangle$: It gives an indication of the signal to noise ratio, and then how strong the diffraction from the crystal is compared to the backround. It is very important in deciding the high resolution limit. A suggested limit for the high resolution shell is $\langle I/\sigma \rangle = 2$.
- Redundancy: Indicates the number of times that the reflections are measured as such or as symmetry related. In general the higher the redundancy is, the better the reflections intensity estimation.
- R_{merge}: One definition is

  $R_{merge} = \Sigma_{hkl} \Sigma_i | I_i(hkl) - \langle I(hkl) \rangle | / \Sigma_{hkl} | \langle I(hkl) \rangle |$

  In general the lower the better, but strongly depends on the redundancy (other indicators exist that take redundancy into account)

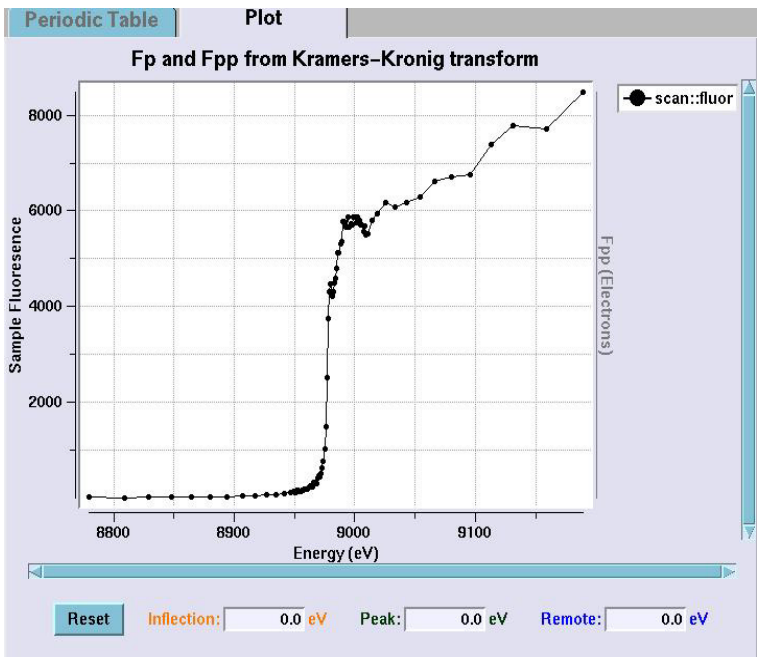
Multiwavelenght Anomalous Dispersion

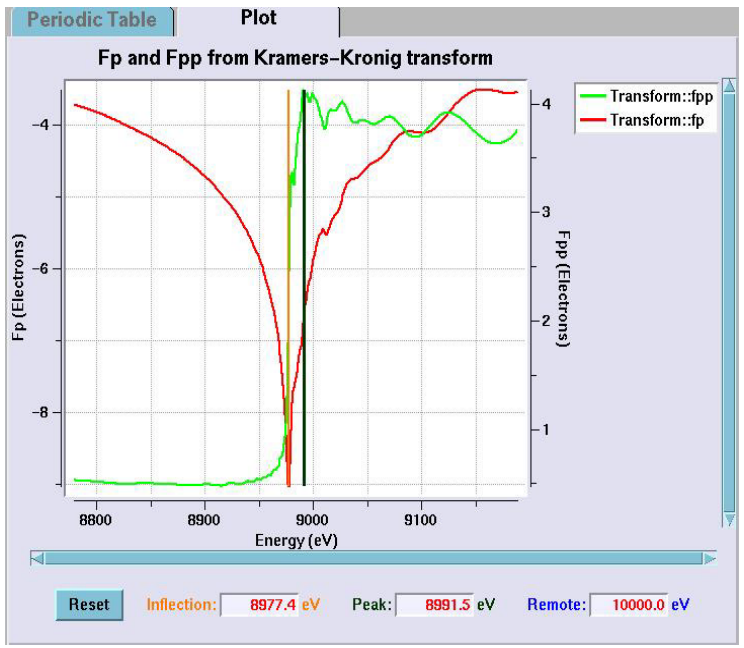


- The MAD (Multiwavelength Anomalous Dispersion) phasing method exploits the abrupt changes in scattering power of heavy atoms (such as transition metals, lanthanides, Se or Br) in the vicinity of absorption edges. The changes in scattering power result in differences between the diffracted intensities measured at different wavelengths as well as differences between reflections of the type (hkl) and (-h-k-l) (anomalous pairs) measured at the same wavelength. These differences can be usefully exploited in order to gain phase informations.

The MAD experiment

- The first step in a MAD experiment, is the acquisition of a fluorescence spectrum from the protein sample, in order to exactly determine the wavelengths of the absorption peak (where *f''* is maximized) and the inflection point (*f'* is minimized). Once the the peak and inflection point are determined, at least three data collection must be carried out (the third one is the so called 'remote', at a wavelength far away from the absorption edge (usually at lower wavelength).





Structure Solution

In order to calculate the electron density inside the crystal, we need the complex structure factors *F(hkl)* but from a diffraction experiment, we know only their magnitude |*Fhkl*|

$$\rho(x\ y\ z) = 1/V\ \Sigma_{hkl}\ F(h\ k\ l)\ \exp[-2\pi i(hx+ky+lz) + i\alpha(h\ k\ l)]$$

Phases can be obtained by means of different alternative techniques:

Experimental Techniques:

Multiple Isomorphous Replacement (MIR, Requires the introduction of heavy atoms in the crystal)

Multiwavelength Anomalous Dispersion (MAD, Requires a tunable source of X-ray and an anomalous scatterer

Computational Techniques

Molecular Replacement (MR, Requires a suitable starting model)

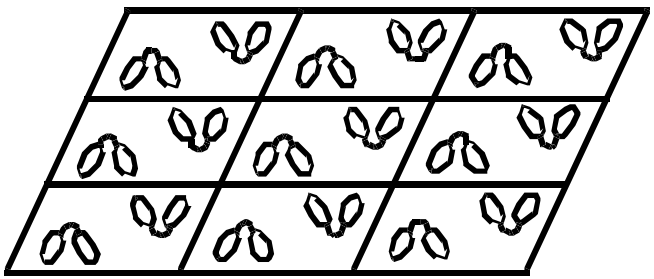
Ab-initio (Requires very high resolution data, few cases solved up to now)

Molecular replacement

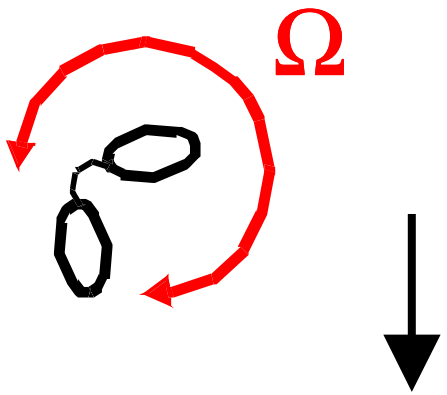
- **Molecular replacement can be used when you have a good model for a reasonably large fraction of the structure in the crystal.**
- To carry out molecular replacement, you need to place the model structure in the correct orientation and position in the unknown unit cell.
- To orient a molecule you need to specify three rotation angles and to place it in the unit cell you need to specify three translational parameters. So if there is one molecule in the asymmetric unit of the crystal, the molecular replacement problem is a 6-dimensional problem.
- It turns out that it is usually possible to separate this into two 3-dimensional problems. A **rotation function** can be computed to find the three rotation angles, and then the oriented model can be placed in the cell with a 3D **translation function**.

Molecular Replacement Stages

Crystal

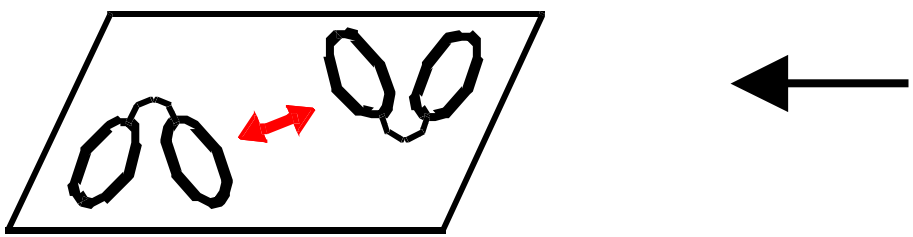


1. Rotation Search



2. PC Refinement

3. Translation Search



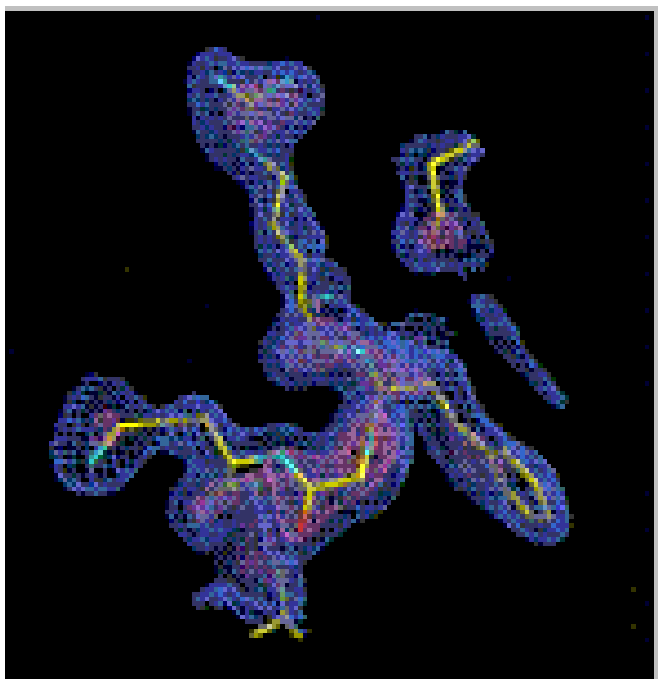
Refinement

- Once the phase problem is solved, it is possible to calculate the electron density inside the crystal and to fit an initial molecular model into it.

- **Refinement** is the process of adjusting the model to find a closer agreement between the calculated and the observed structure factors.

- Refinement technique are based on the principle of least-squares, or maximum likelihood. Molecular model coordinates x, y, z and thermal parameters are changed in order to improve the agreement between the calculated $|F_c|$ and the observed $|F_o|$
- Given the unfavorable ratio between the number of observations and the refined parameters, it is necessary to introduce some geometrical restrains (expected bond length, bond angle and torsional angle).

Refinement –Quality indicator



- The quality of the final model can be established on the base of several quality indicator:
  - Connectivity of the resulting electron density.
  - R-factor/Free R-factor (least square residual for the working and test dataset).
  - Final model geometry.

$$\text{R-factor} = \Sigma_{hkl} \left| |Fo| - k|Fc| \right| / \Sigma_{hkl} |Fo|$$

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