Image processing in 3D

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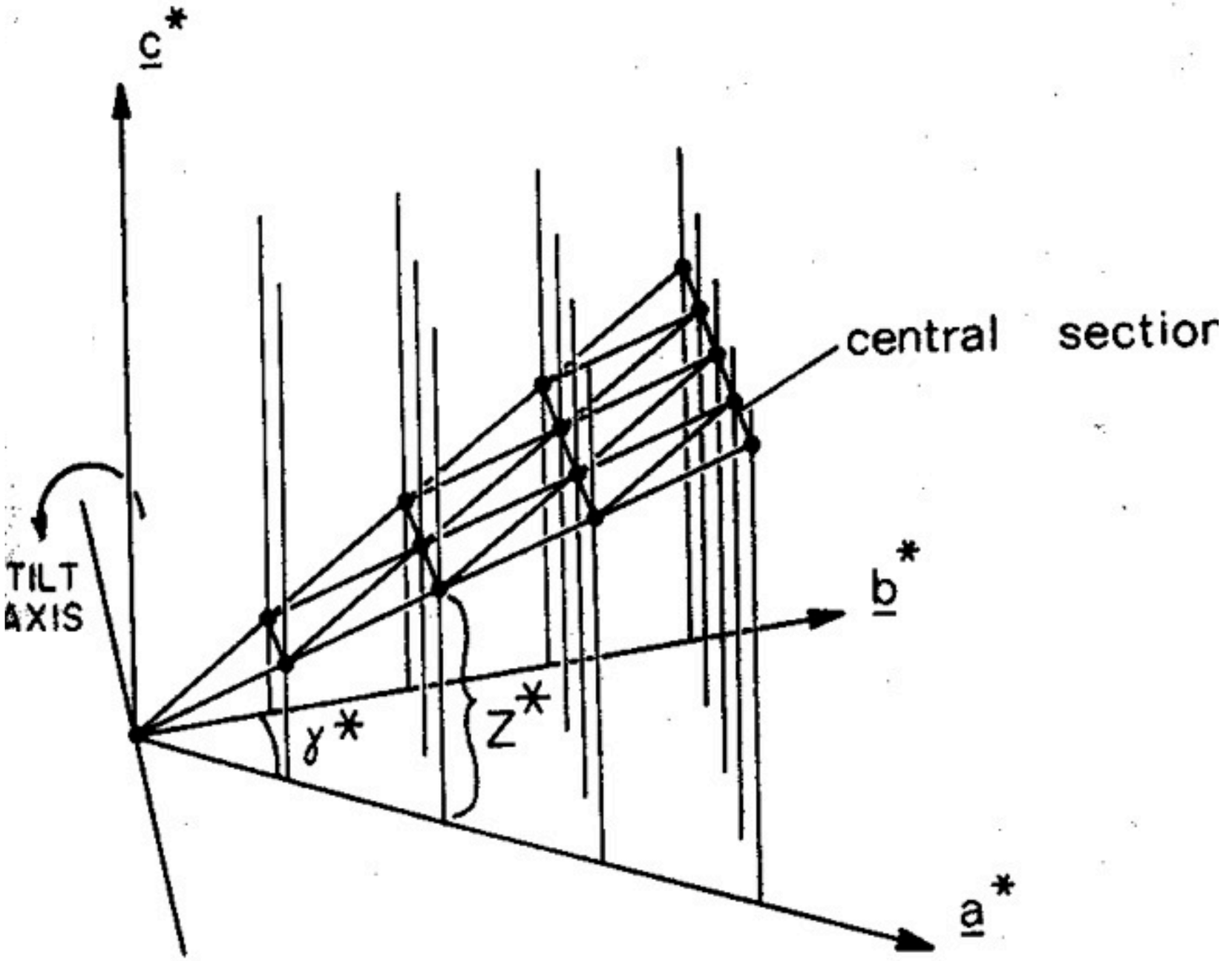
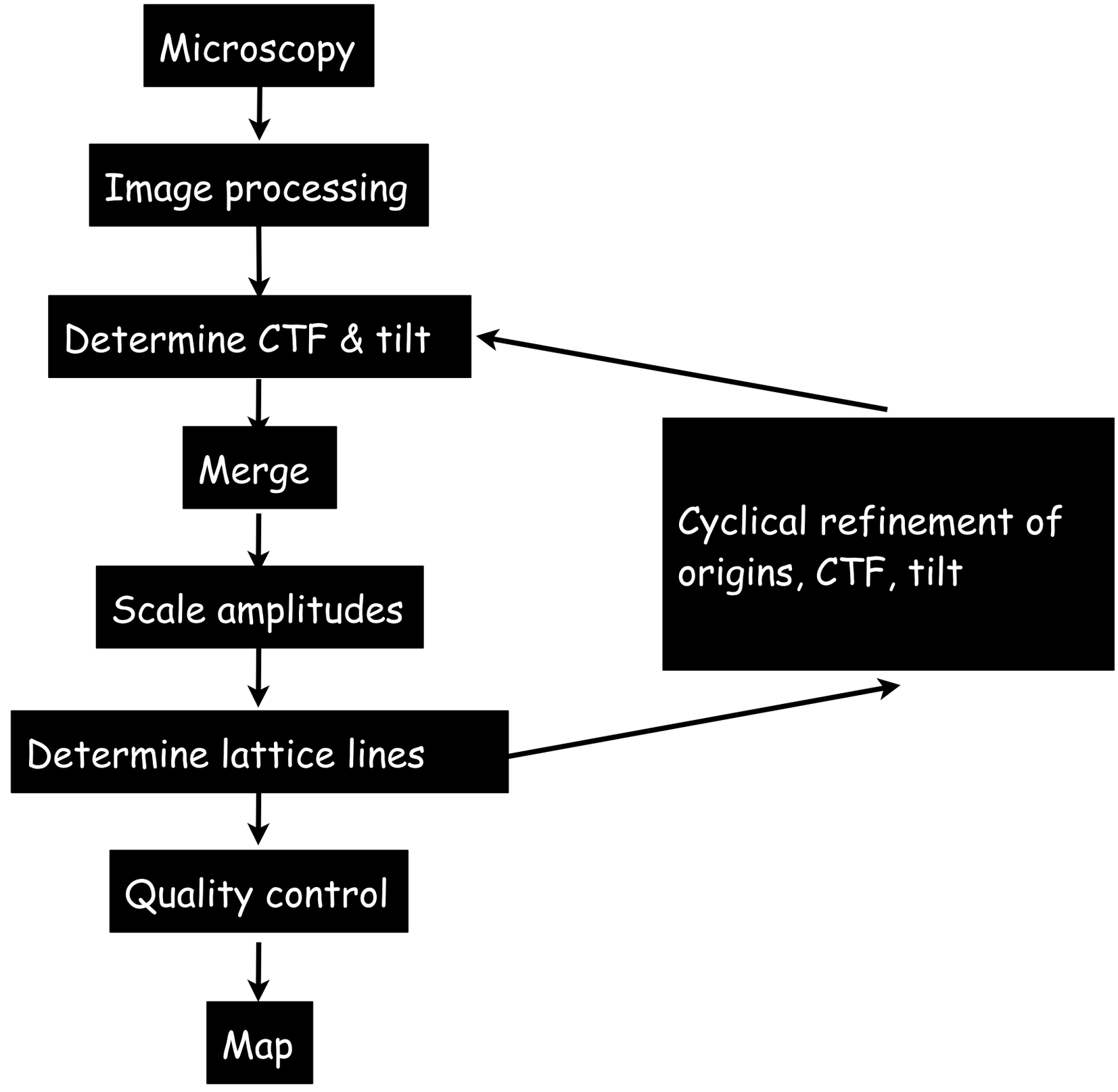
Useful References

- Amos, L.A., Henderson, R. and Unwin, P.N.T. (1982) Prog. Biophys. Molec. Biol. **39**:183-231.
- Agard, D. (1983) J. Mol. Biol. **167**:849-52
- Henderson, R., Baldwin, J.M., Downing, K.H., Lepault, J. and Zemlin, F. (1986) Ultramicroscopy **19**:147-178
- Henderson, R.,Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. **213**:899-929.
- Havelka, W.A., Henderson, R. and Oesterhelt, D. (1995) J. Mol. Biol. **247**:726-738.

Central Section Through a 3D Transform

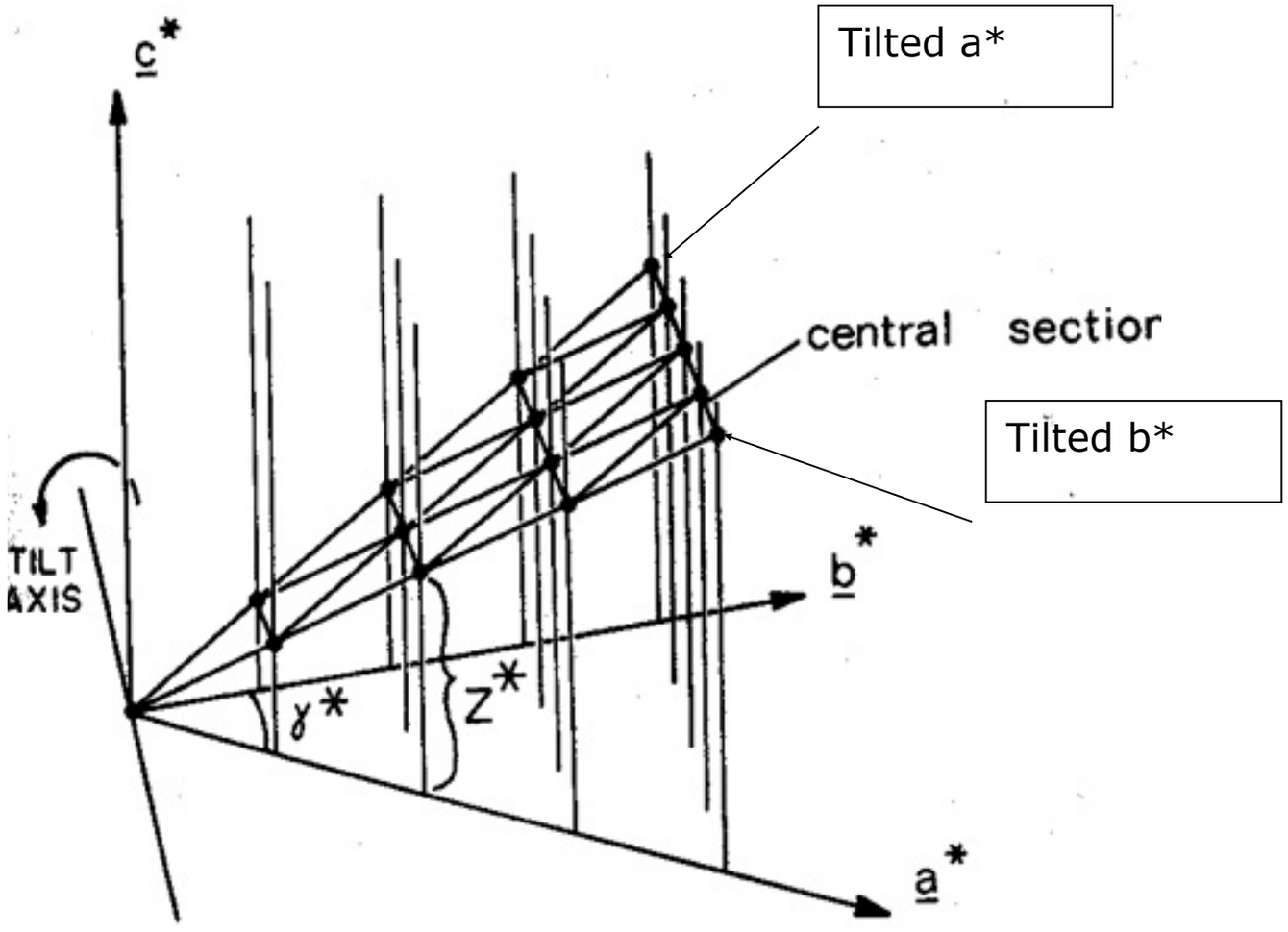


The Sequence of Steps- Small Images/Low Resolution

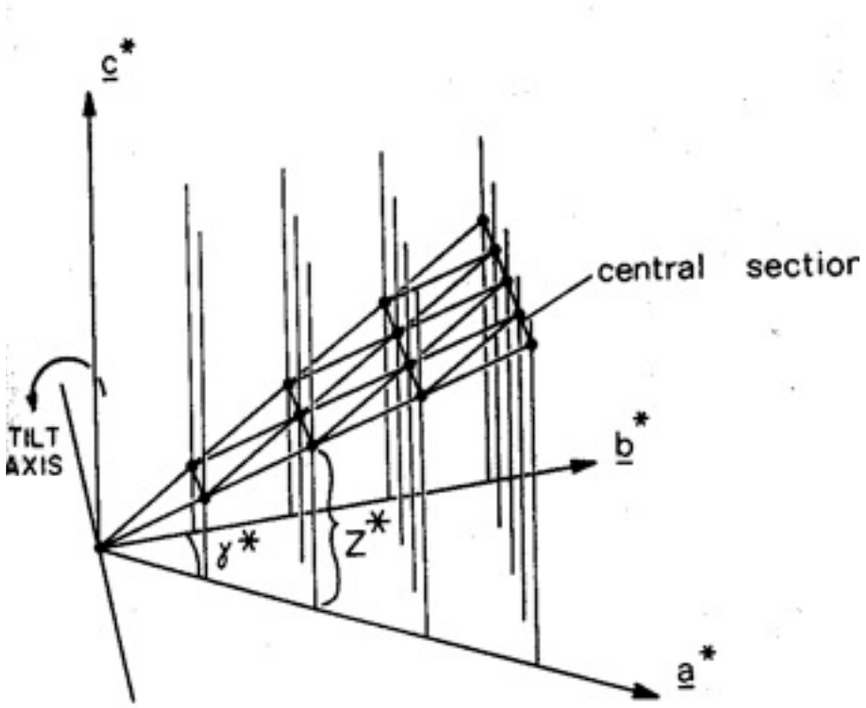


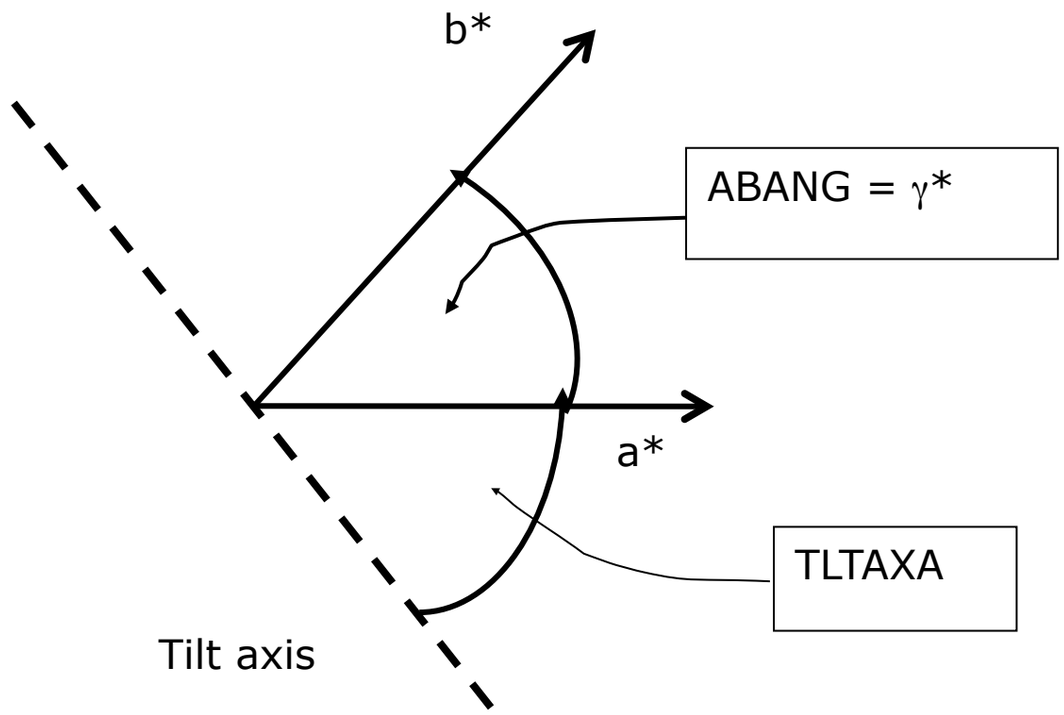
Estimate of Tilt Axis and Angle

- Determine defocus in 4 corners of film- good choice for small tilt angles- '**CTFTILT**'
- Compare distortion of lattice with that of untilted specimen- works best for higher tilt angles- '**EMTILT**'



Tilt Axis Conventions in MRC





Check the absolute hand!

More underfocus = lower in microscope

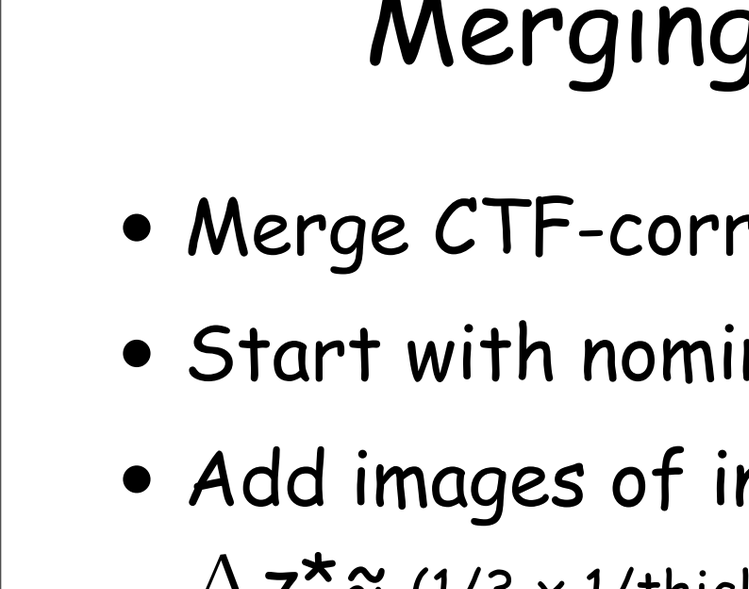
Less underfocus = higher in microscope

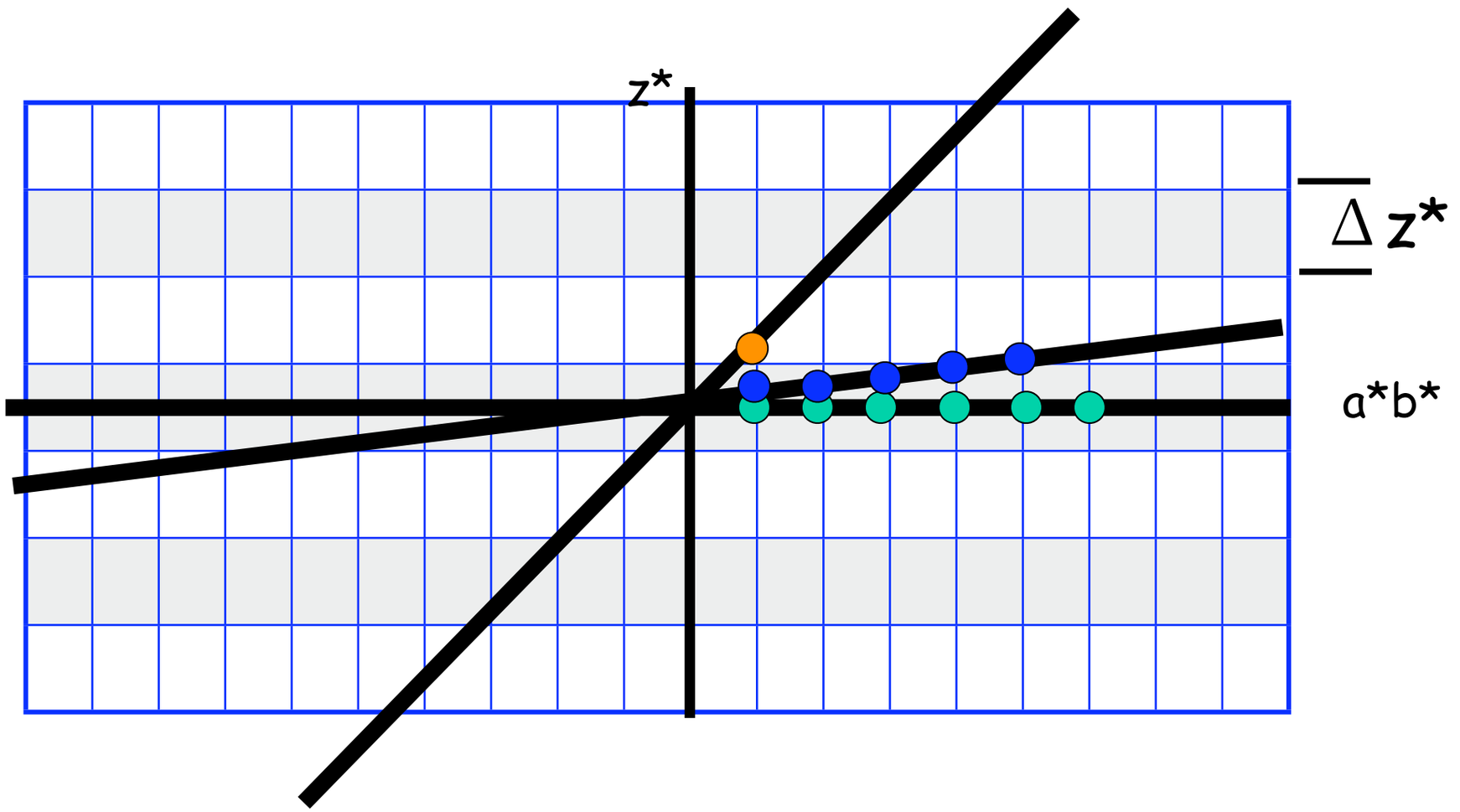
Z* +ve

Z* -ve

Merging and Phase Origin Refinement

- Merge CTF-corrected data in ORIGTILT
- Start with nominally untilted image set to correct phase origin
- Add images of increasing tilt and compare phases within
  $\Delta z^* \approx$ (1/3 x 1/thickness)





Merging and Phase Origin Refinement

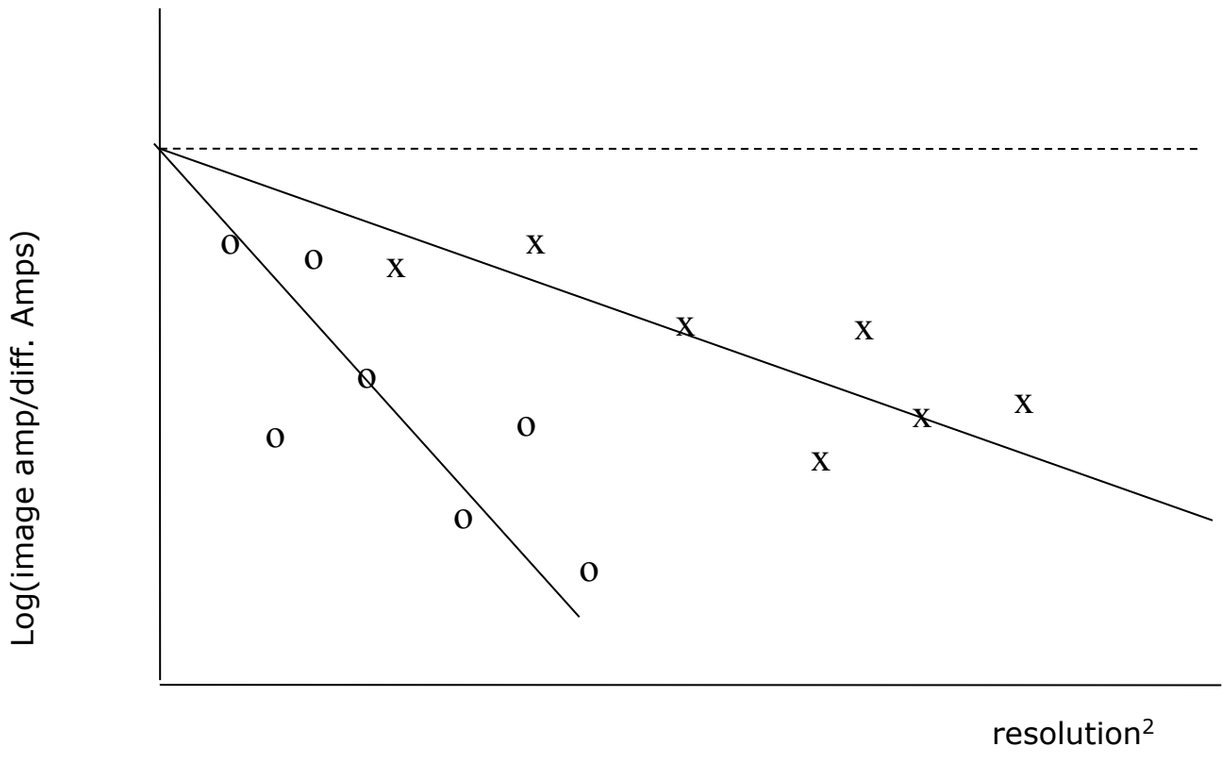
- By looking for best agreement this generally puts z origin at centre of mass by minimizing phase gradients
- Check for a clear minimum in refinement
- Do phase errors make sense? Are they significantly less than 90°?
- Follow by cycles of origin, tilt and CTF refinement

	7	7	7					
7	8	8	8	7				
7	8	9	8	7				
7	8	8	8	7				
	7	7	7					

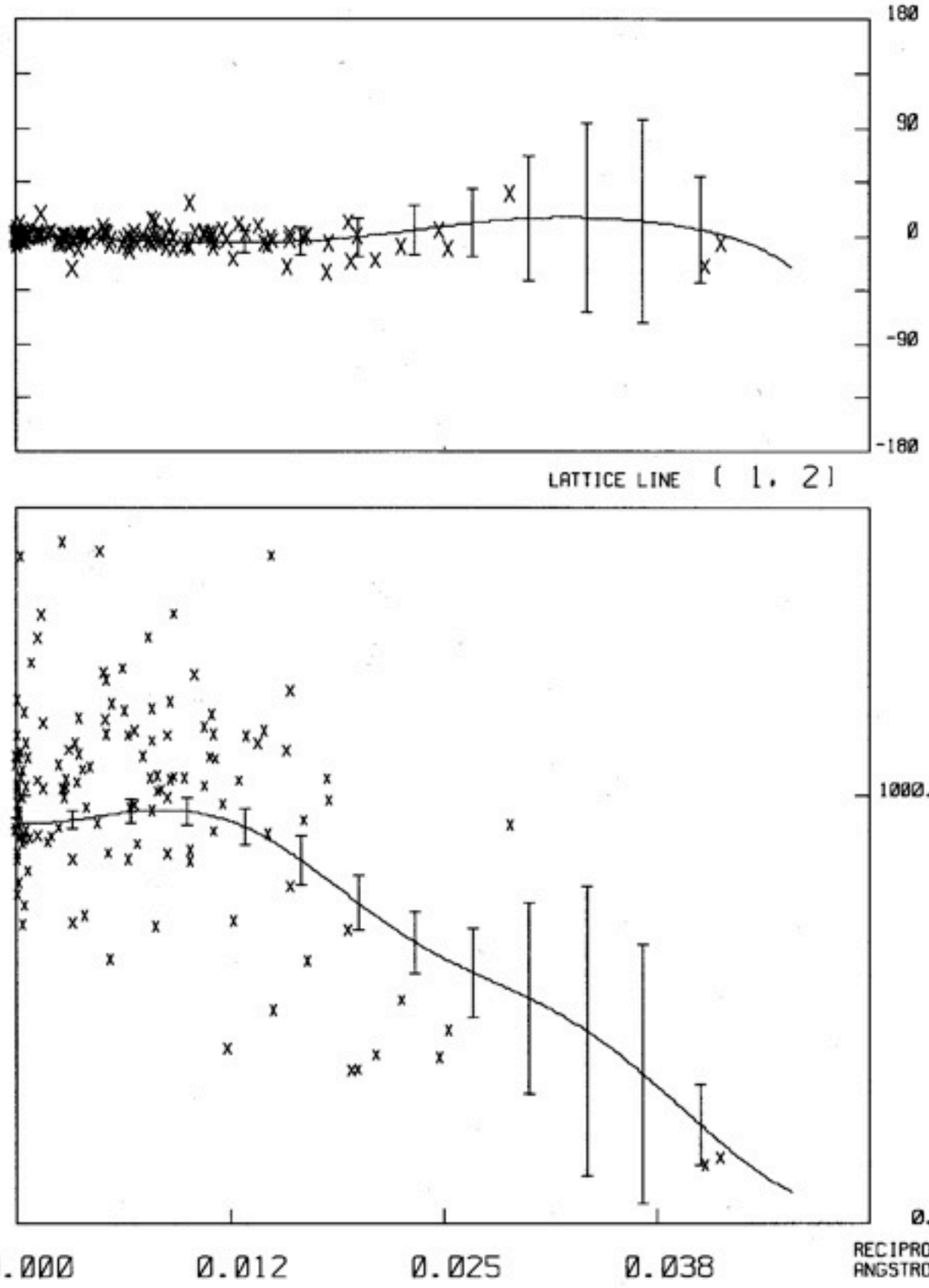
	2	3	4	4	4	4		
	2	4	5	5	5	4		
	3	4	5	5	5	3	3	3
	3	4	5	5	4	2	4	4
	3	4	4	4	3	4	5	5
2	4	5	5	5	4	4	5	5
3	4	5	5	4	4	4	5	5
3	4	4	4	3	2	3	4	4
4	4	3	3	2				

Amplitude Scaling

- Correct for CTF- important if no electron diffraction data.
- Determine temperature factors to minimize $\Sigma(F_{ref}-kF_{obs}e^{0.25B_{xy}(X^2+Y^2)+0.25B_zZ^2})$.
- Best to limit B-factors to $0<B<1000$ Å^2.
- MRC program- SCALIMAMP3D

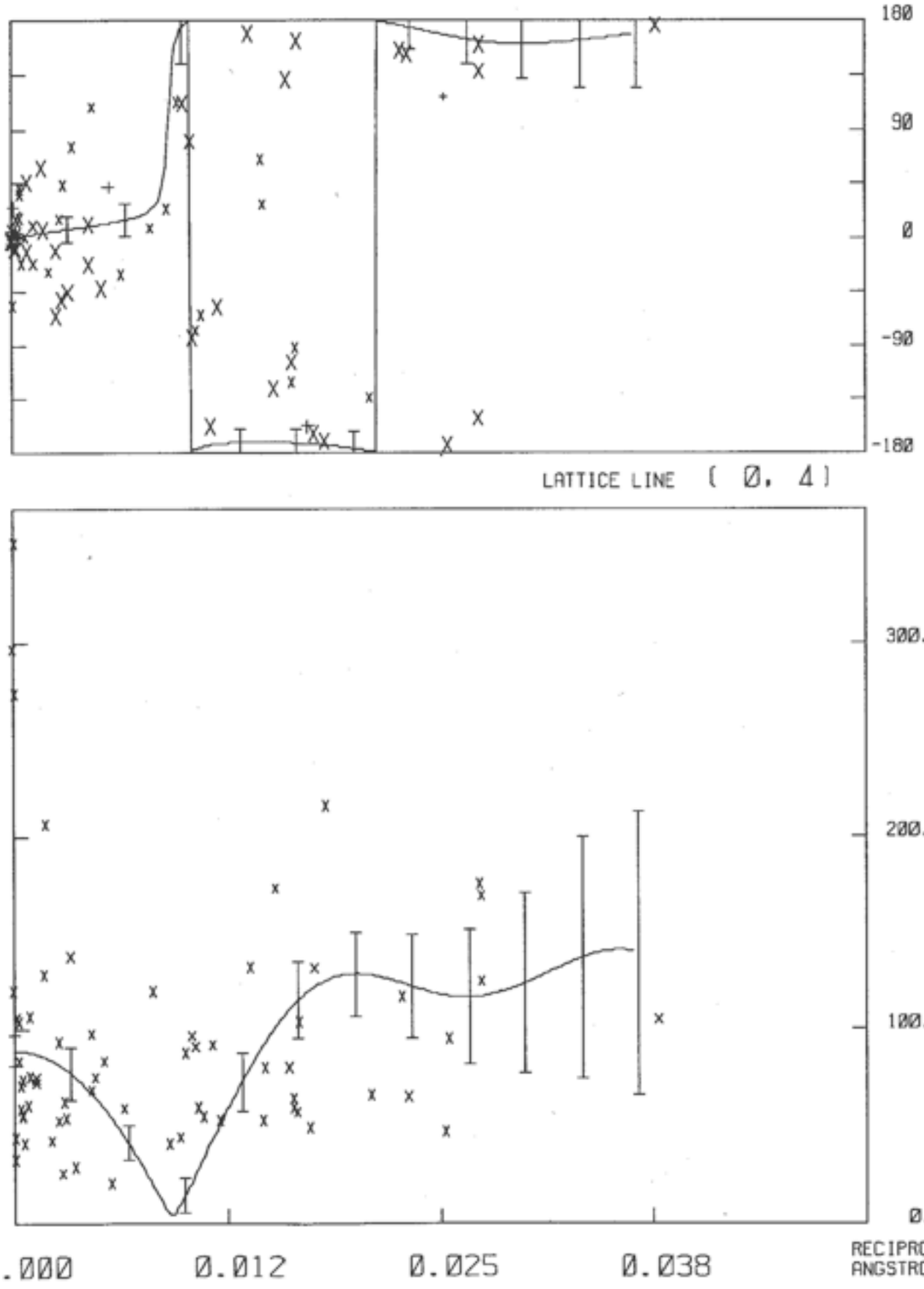


Determination of Lattice Lines

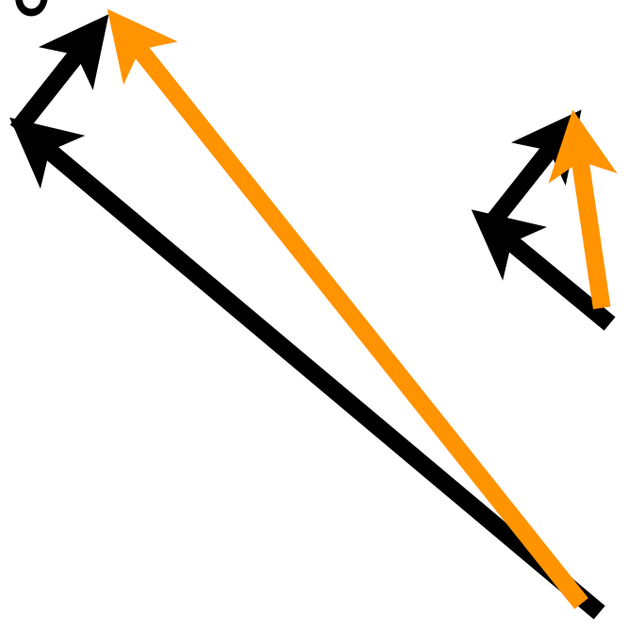


- Can interpolate by hand
- Often best with limited and noisy data
- Automated procedures fit sinc functions at intervals of e.g. 1/thickness- be very careful!
- Are there any extreme outliers (particularly in phase)
- Image amplitudes generally have a lot of scatter.

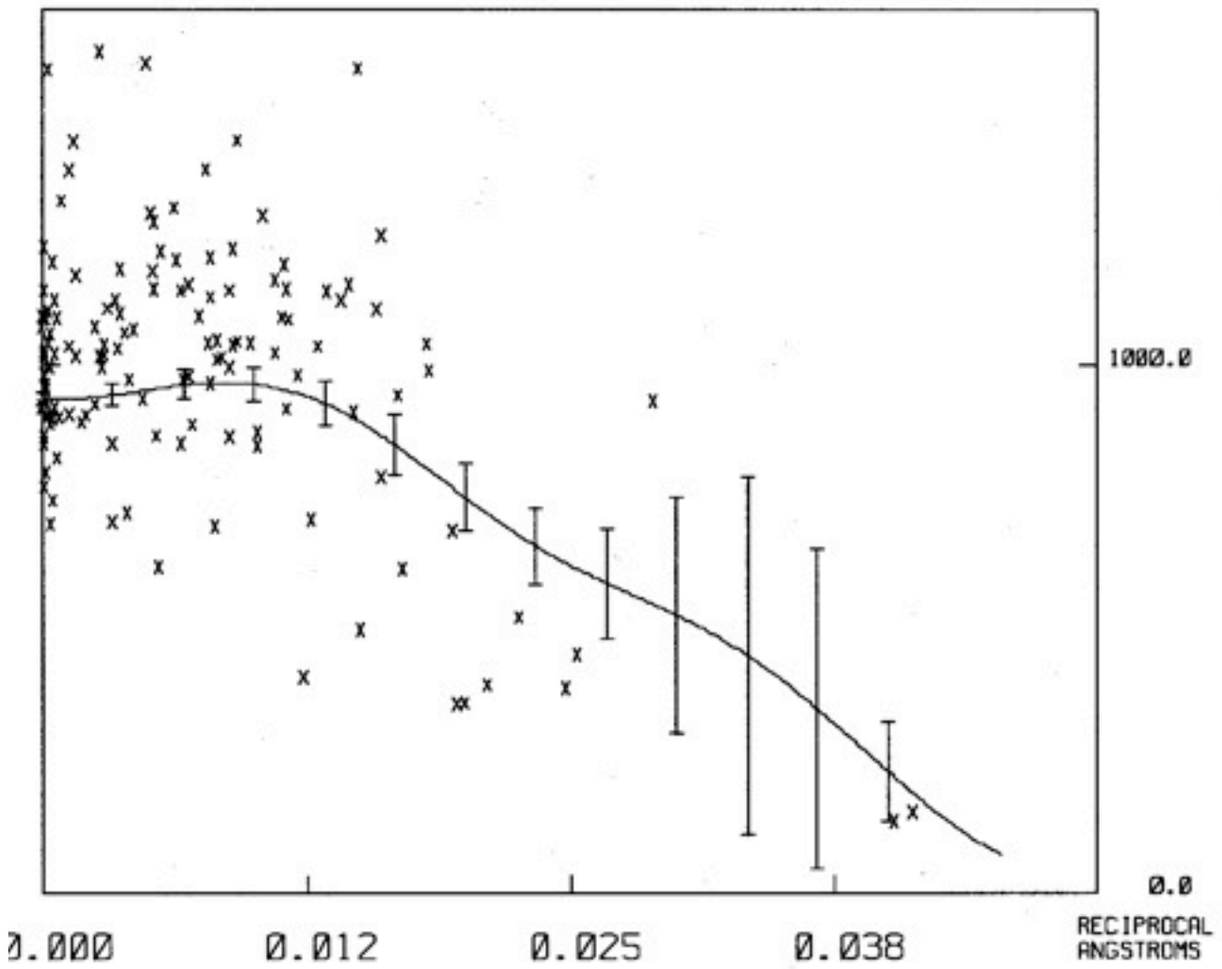
Hand Drawing Lattice Lines



- Try to take into consideration the relationship between amplitude and phase in the complex plane
- e.g. Phases can change more rapidly along z* when amplitudes are small
- e.g. If phase changes by 180° amplitude must pass through zero

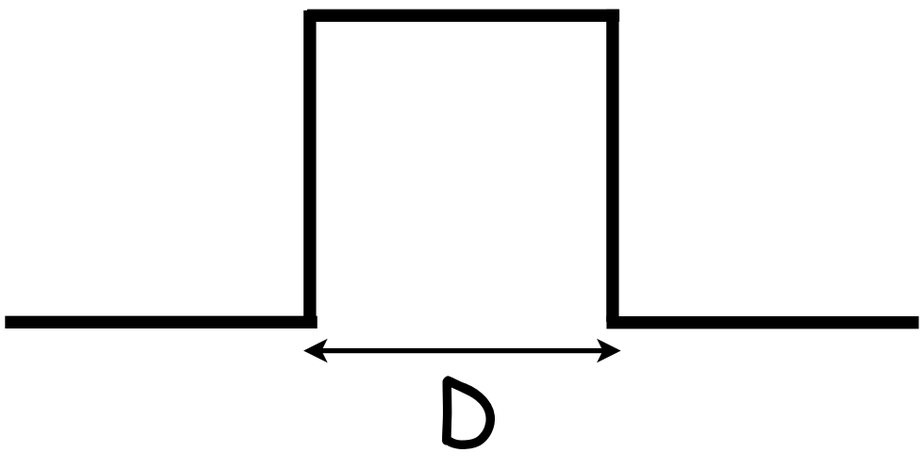


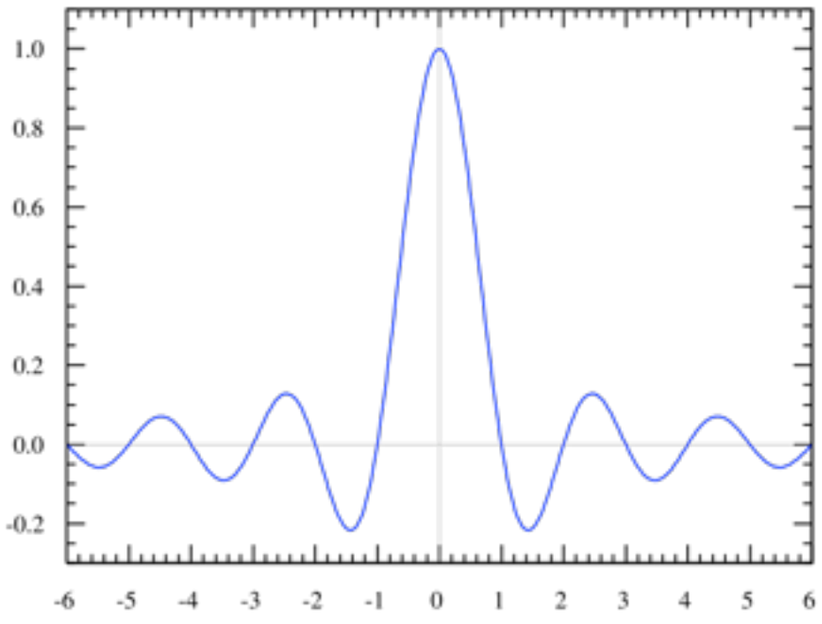
Automated determination of lattice lines



- 2D crystal = 3D crystal X square pulse.
- Fourier transform of 2D crystal = convolution of Fourier transform of 3D crystal with sinc function.
- MRC program LATLINE

$$\mathrm{sinc}(x) = \sin(\pi x)/\pi x$$

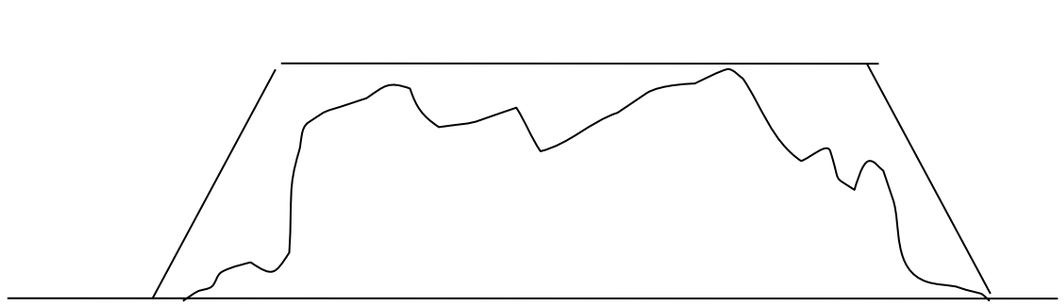




1/D

Automatic Lattice Line Fitting

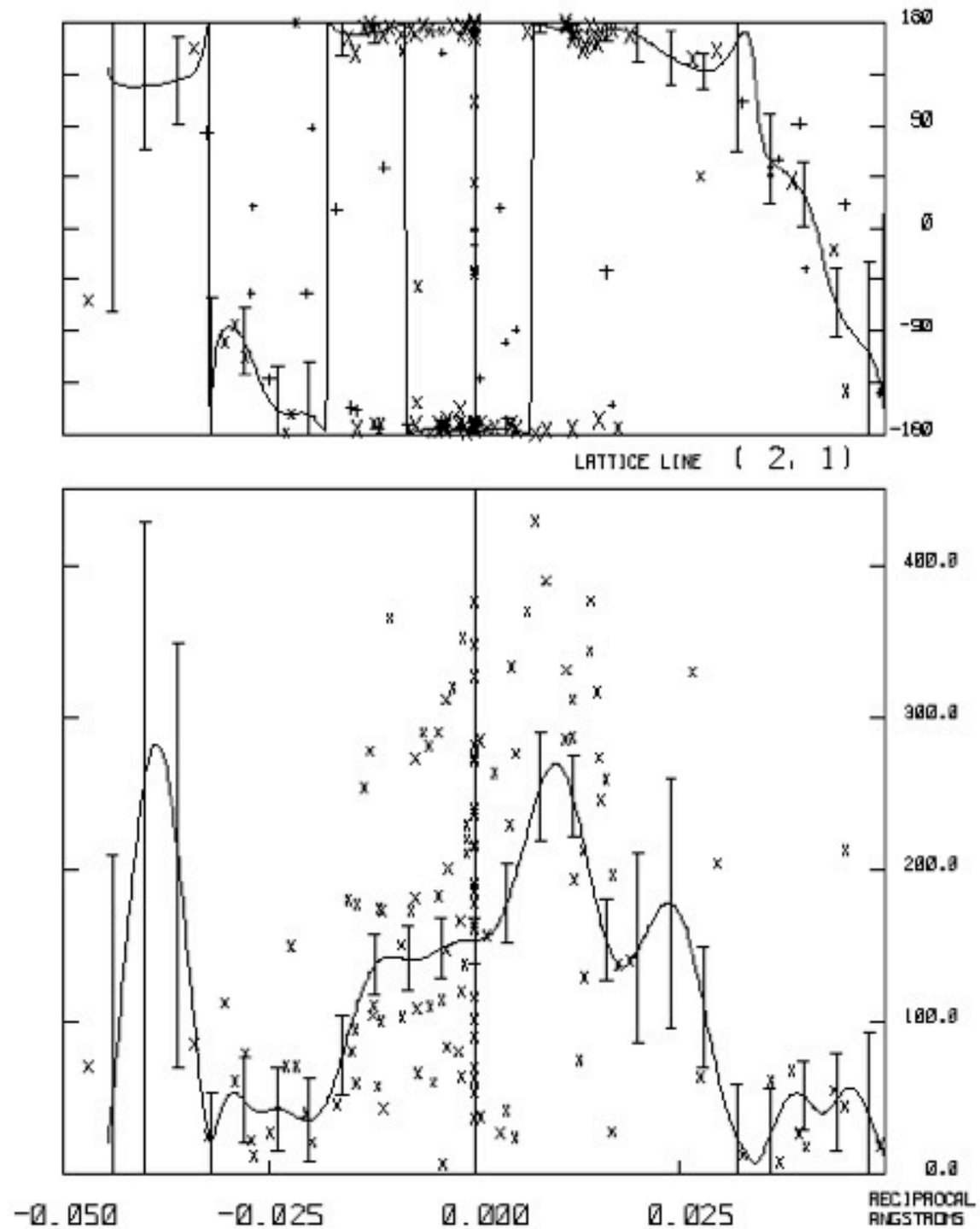
- Least squares fitting of curves to data.
- Constraint given by profile function e.g.



- $1/\sigma^2$ weighting of amplitude and phase data σ = rms local background or estimated phase error

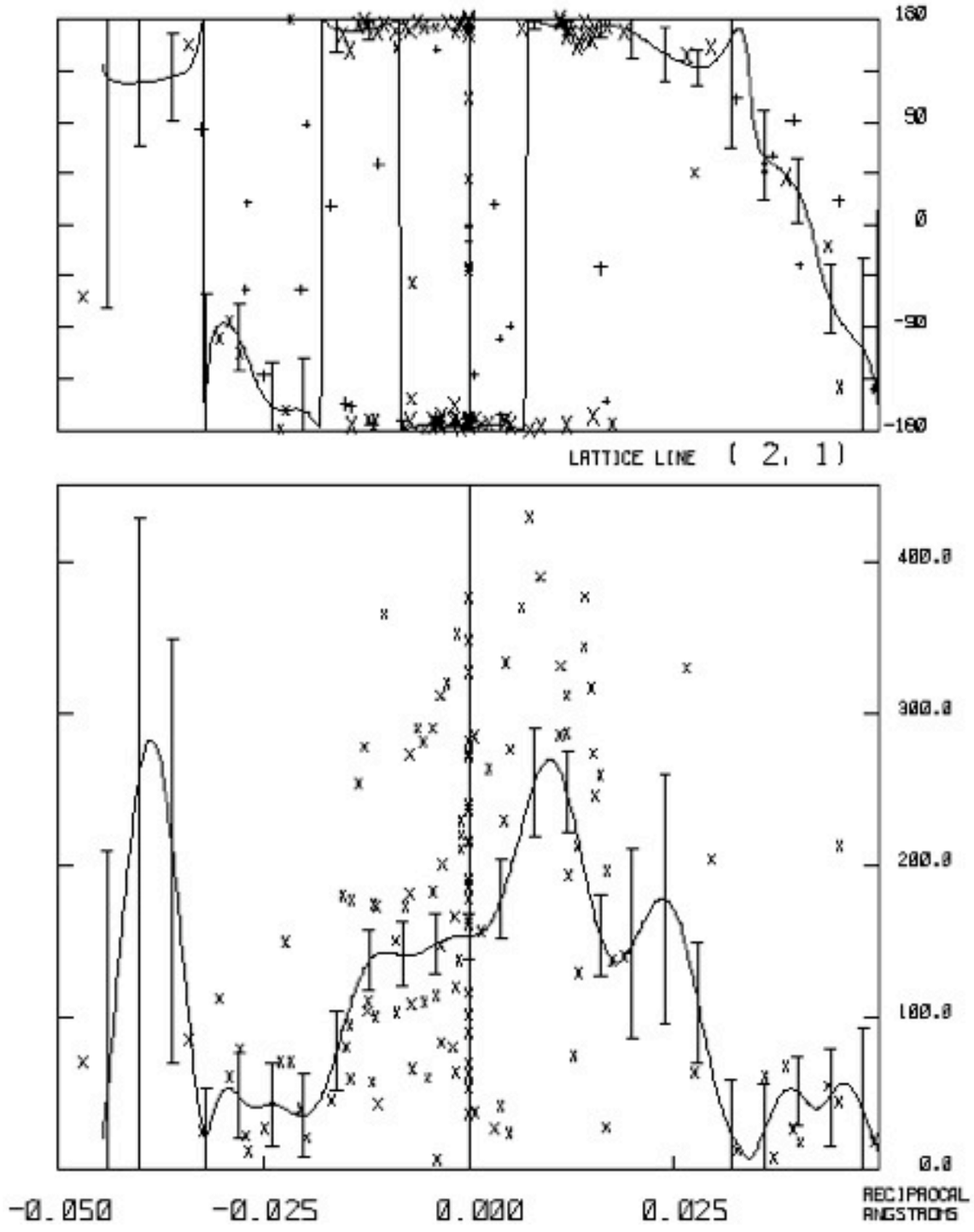
Automatic Lattice Line Fitting

- You must check the results very carefully on graphical plots
- Poorly determined parts at higher z* may best be deleted- these commonly have large error bars and/or unrealistically high amplitudes compared to those at lower z*



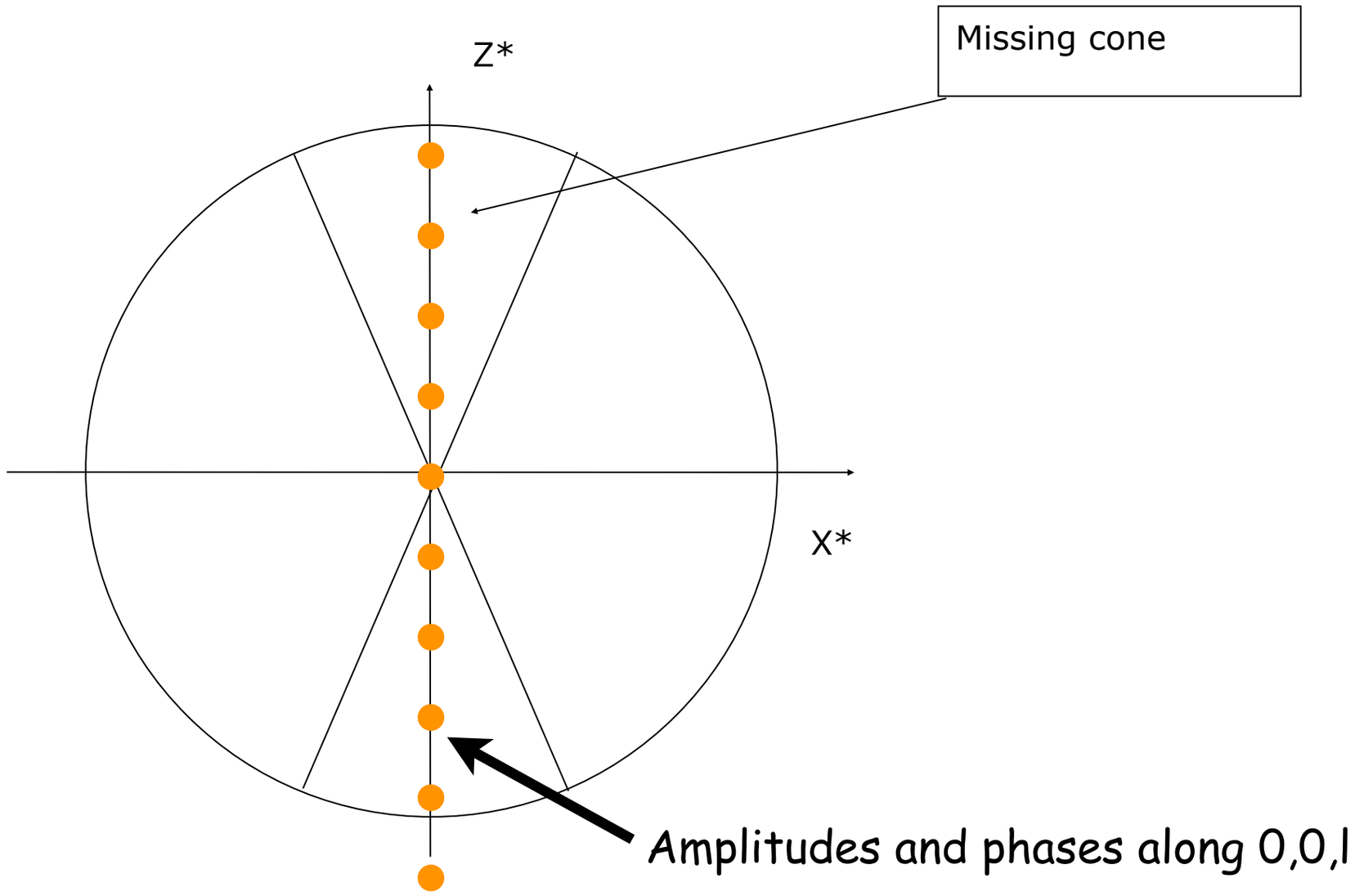
Calculating the Structure

- Sample lattice lines at a fine enough (≡ 1/c) interval to accurately follow variations in transform- must be finer than 1/thickness.
- Sampling will give list of h,k,l,F, ϕ , σ_F, σ_ϕ.
- Calculated density map using standard crystallographic Fourier program with correct 3D symmetry and unit cell a,b,c,90°,90°, γ .
- Least error map uses Fourier coefficients $mFe^{i\phi}$ where m = figure-of-merit = cos(phase error).



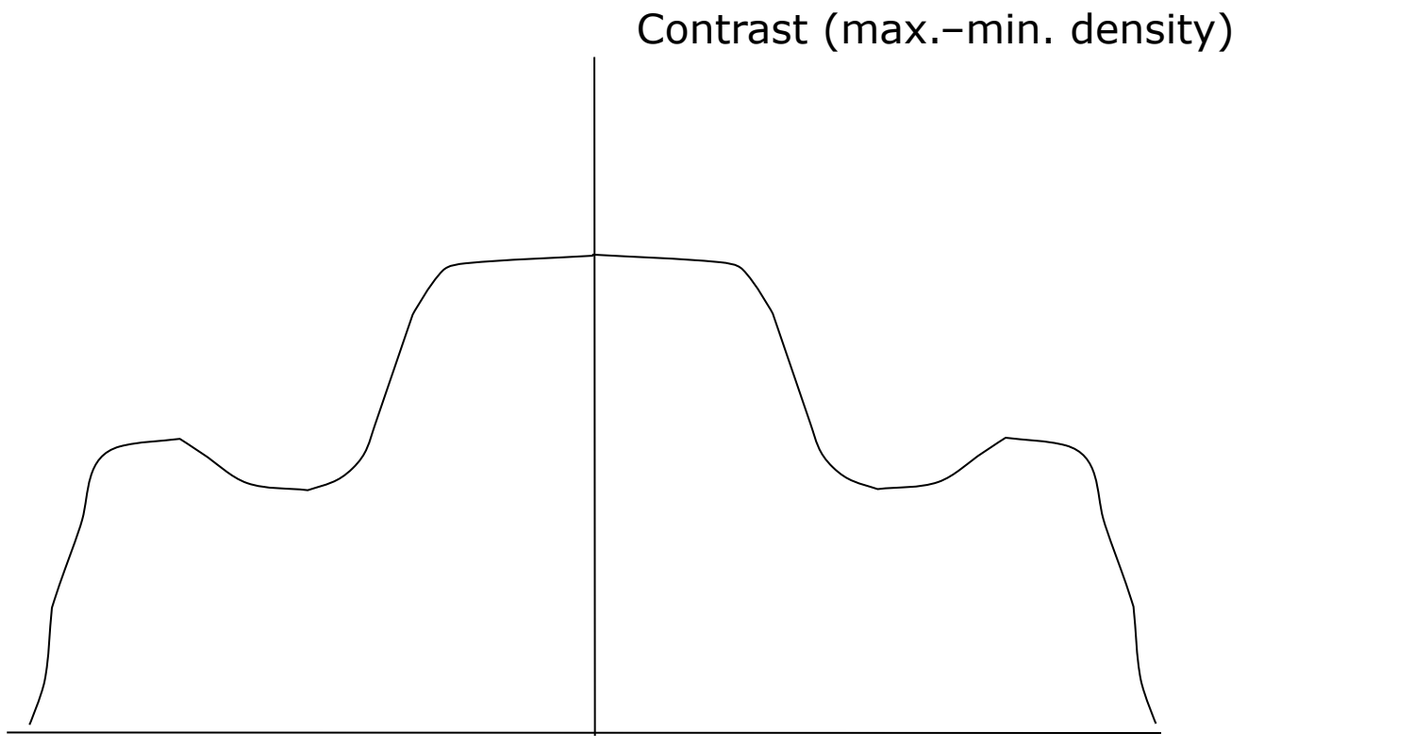
The Missing Cone Problem

- Usually not serious if high tilts available- 60° tilts cover 87% of reciprocal space.
- Missing data along (0,0,l) means that each section has mean density=0 ⇒ incorrect density profile.

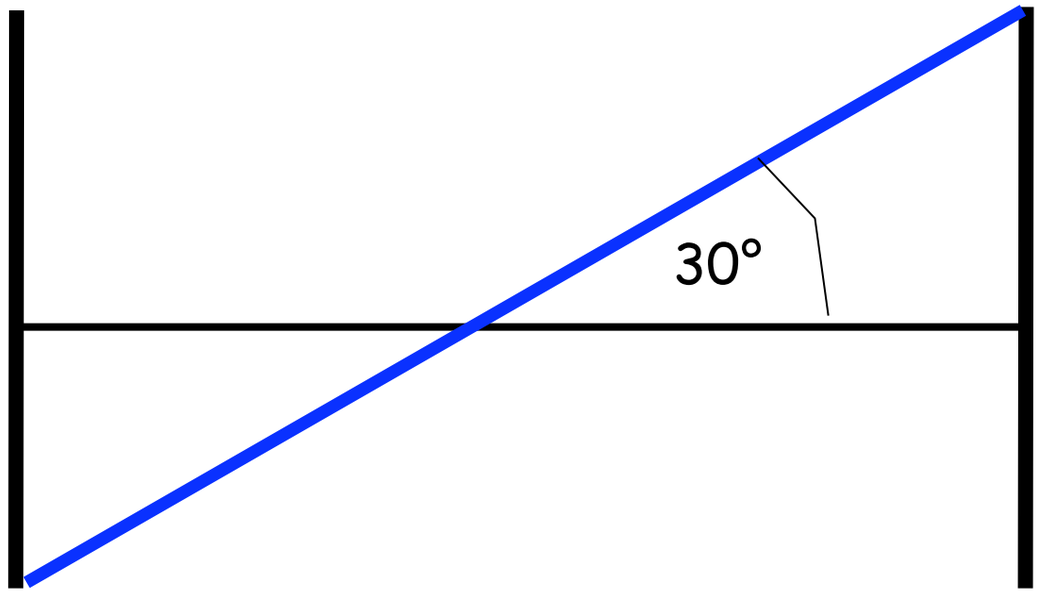


Estimating (0,0,l)

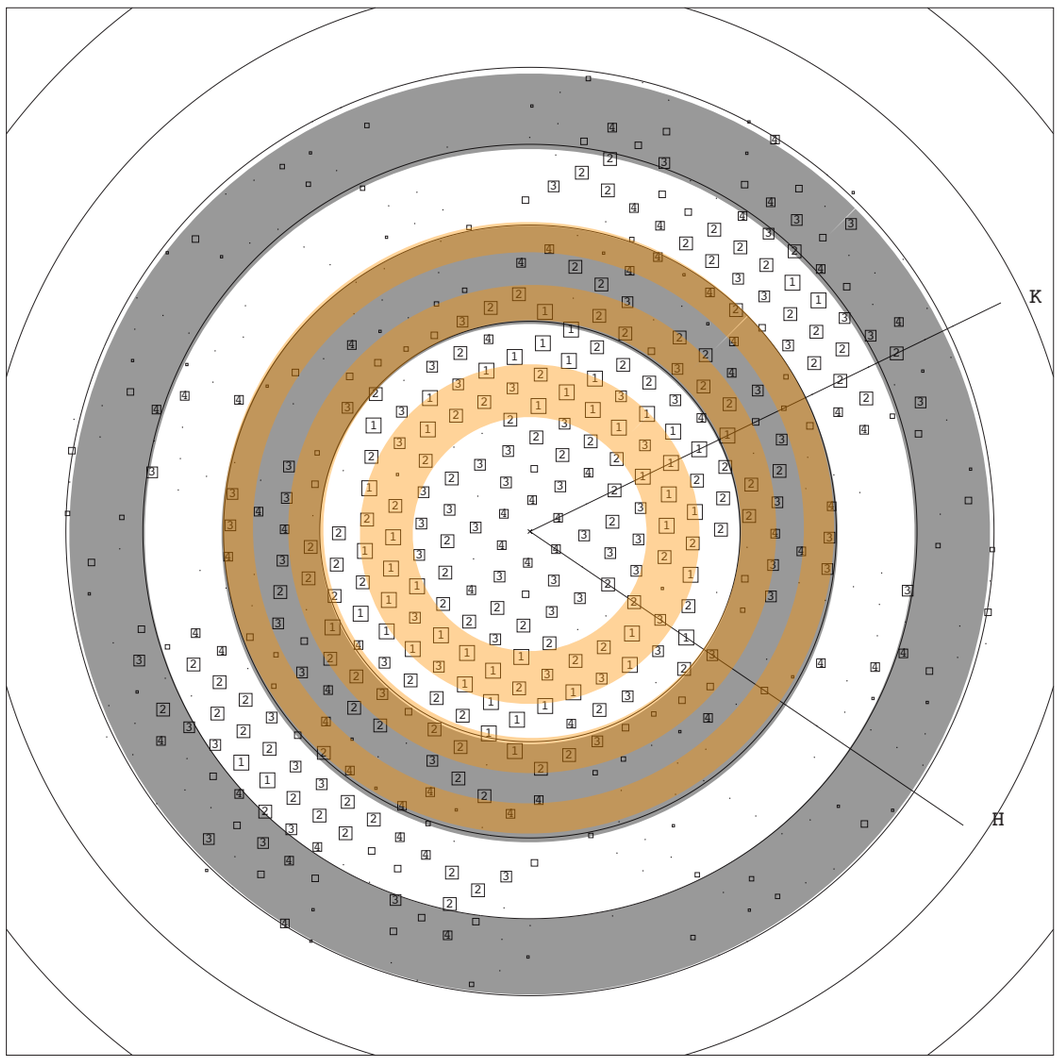
- X-ray powder diffraction
- Thin sections cut perpendicular to crystal
- Side views from folds in crystal
- Contrast profile

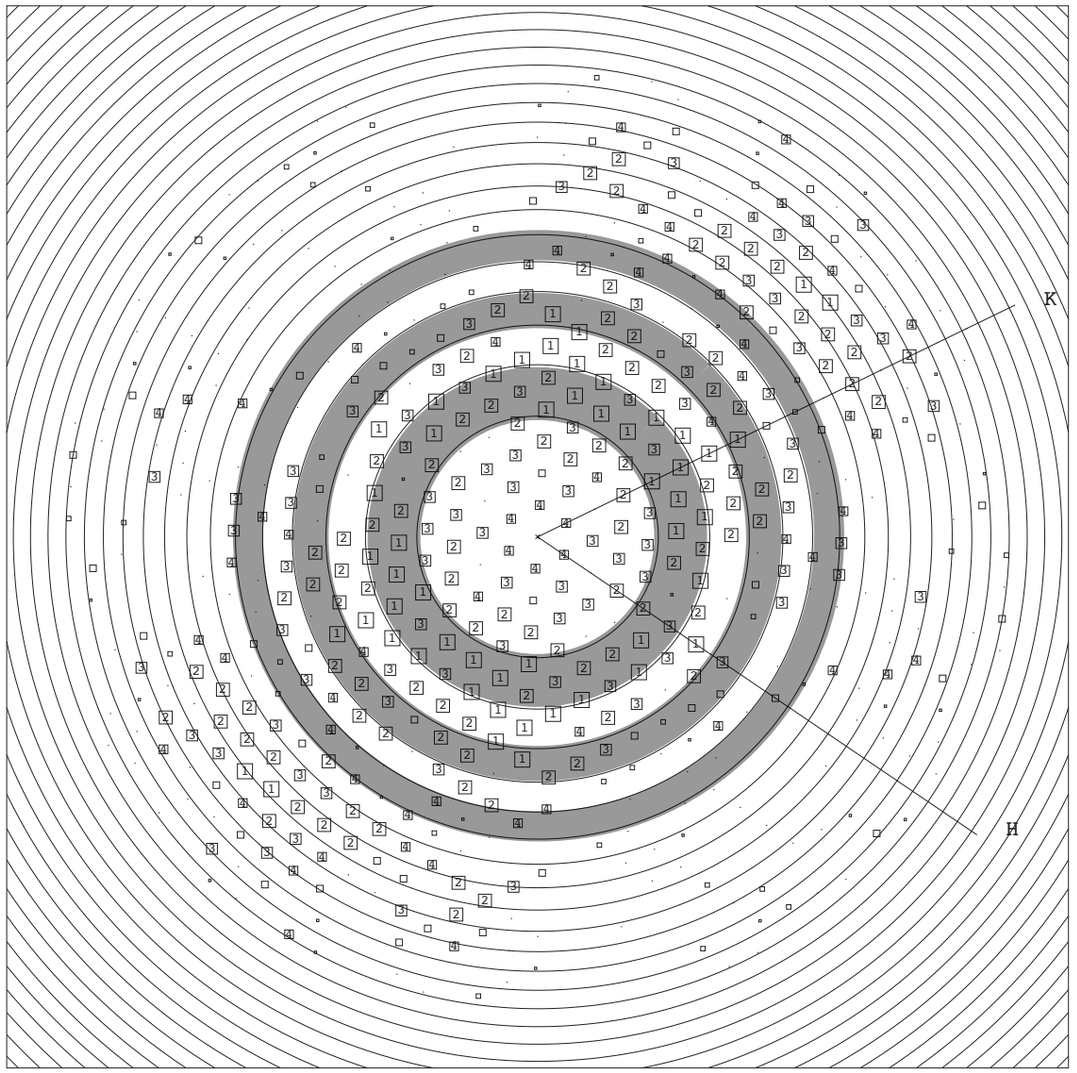


High Resolution and Large Crystals

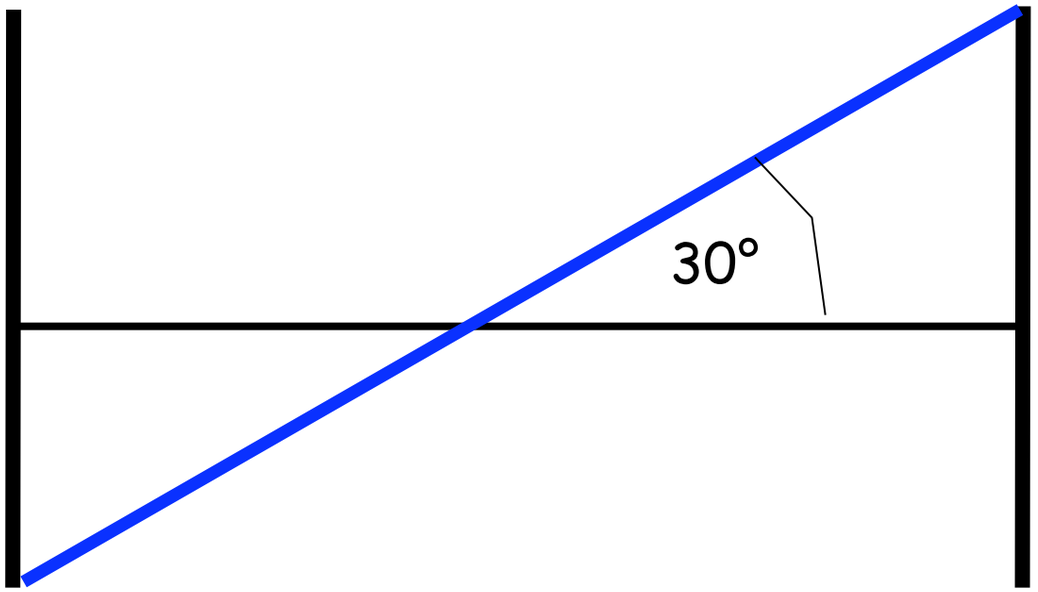


Δf = 5000 Å for 1 μm crystal

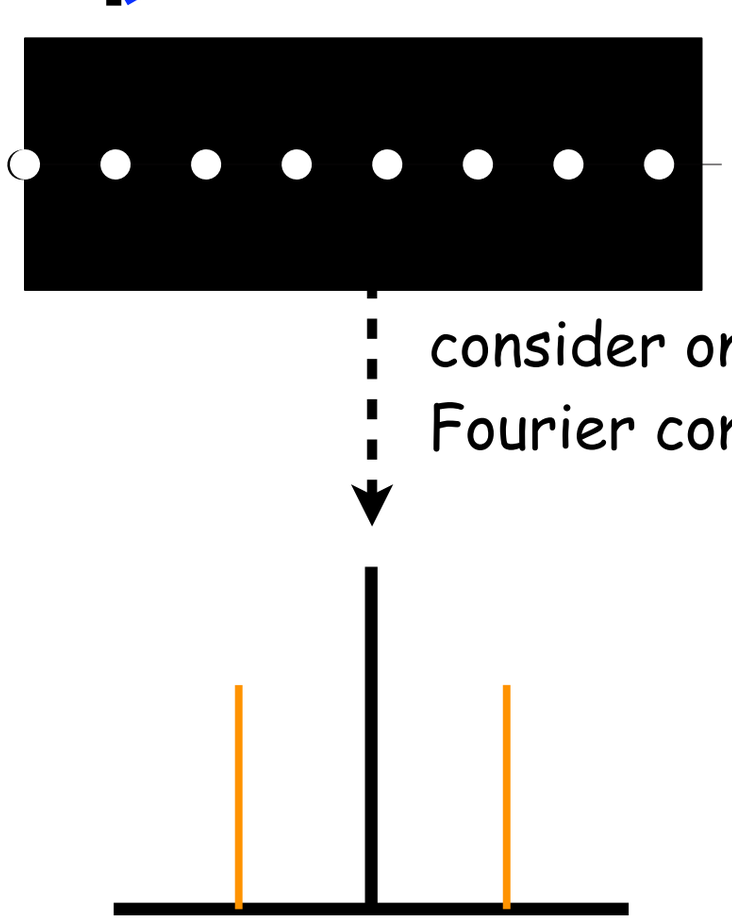




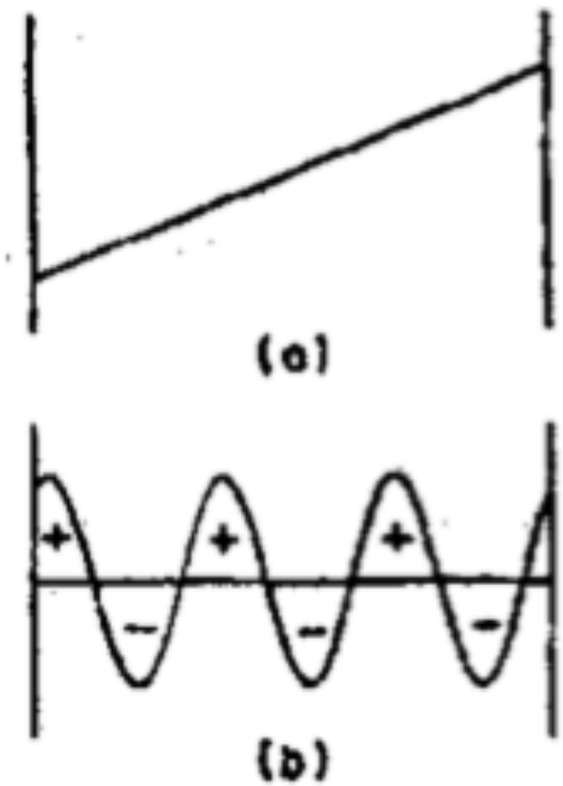
High Resolution and Large Crystals



Δf = 5000 Å for 1 μm crystal

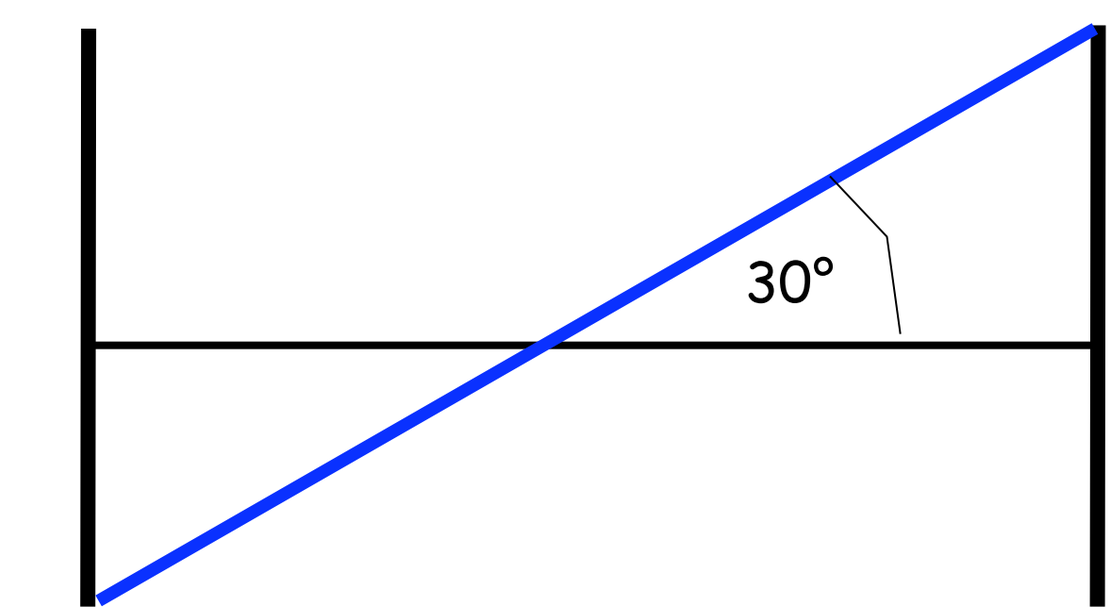


consider one
Fourier component

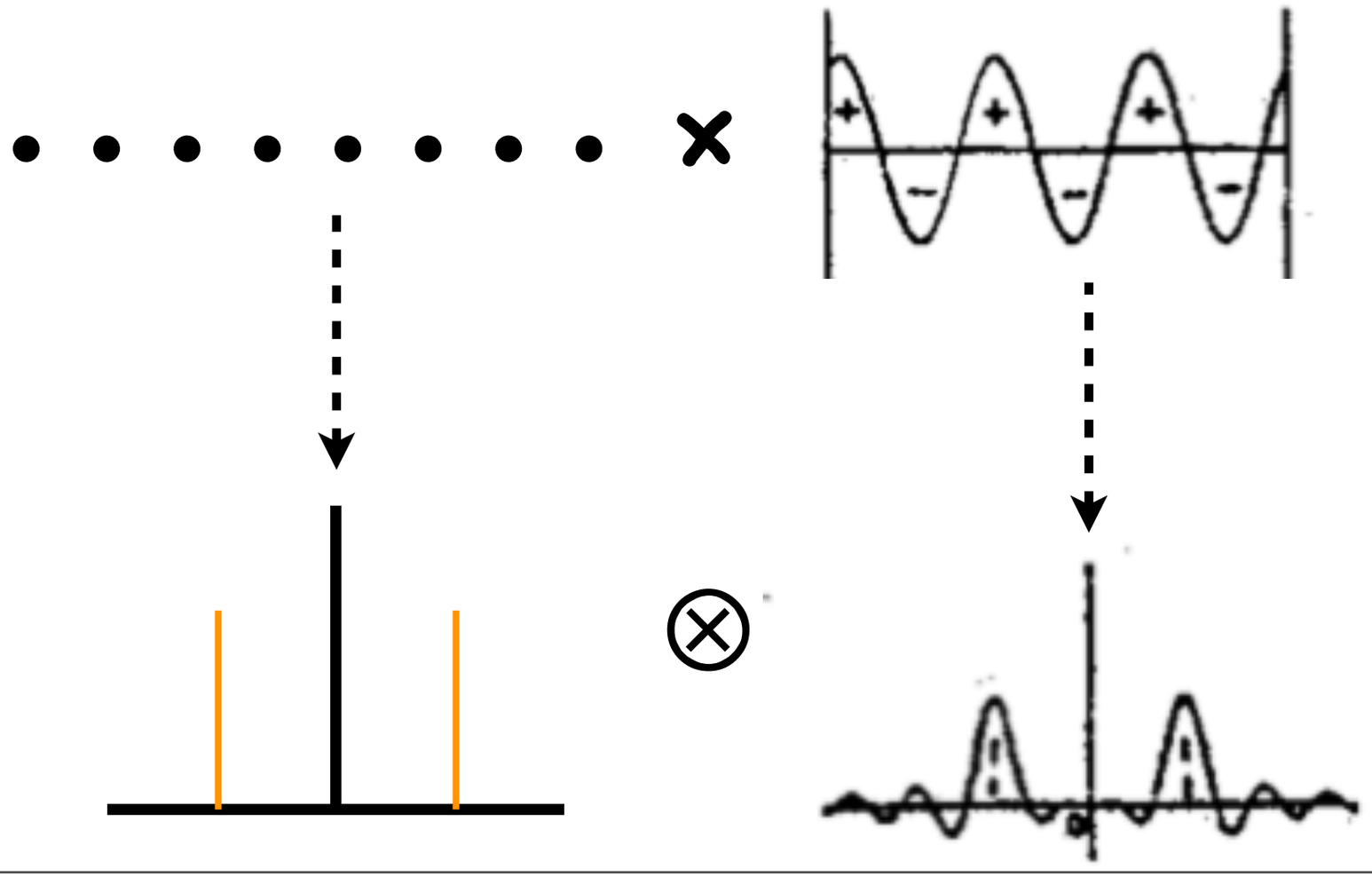


Contrast for each Fourier component varies with height of crystal- described by TILT TRANSFER FUNCTION or TTF

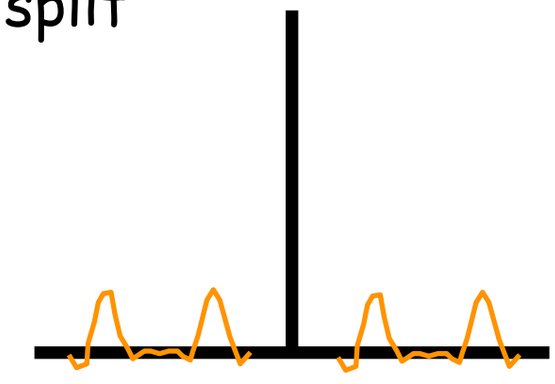
High Resolution and Large Crystals



Δf = 5000 Å for 1 μm crystal

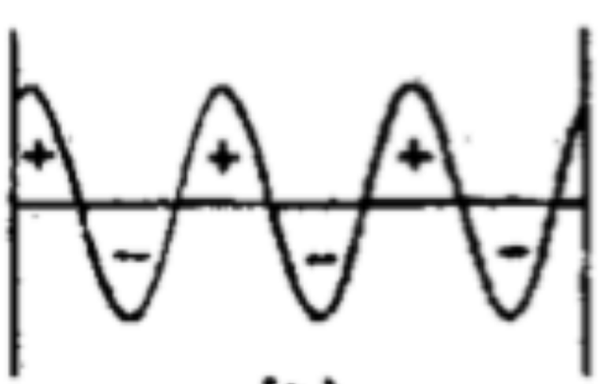


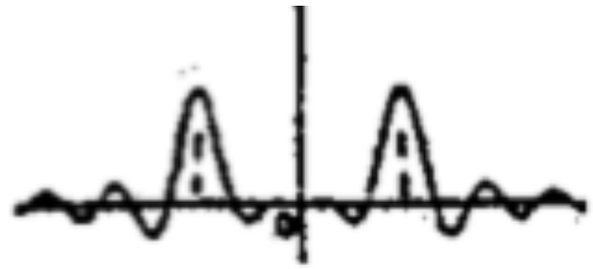
consider one Fourier component- each spot becomes split



High Resolution and Large Crystals

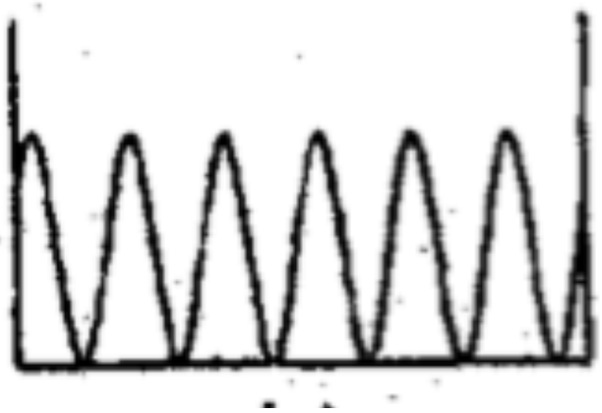
TTF

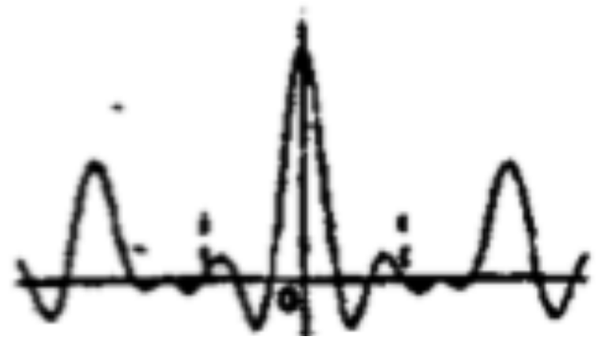




split spot

correction applied

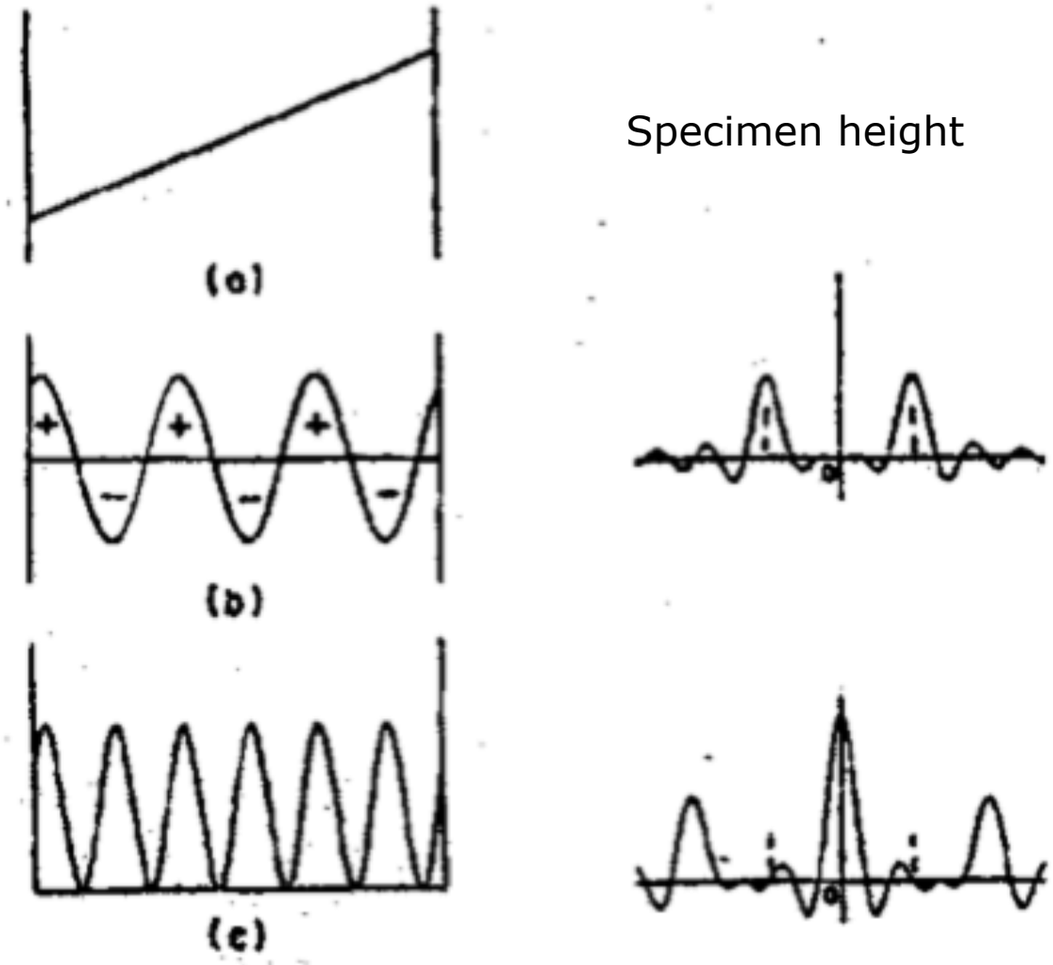




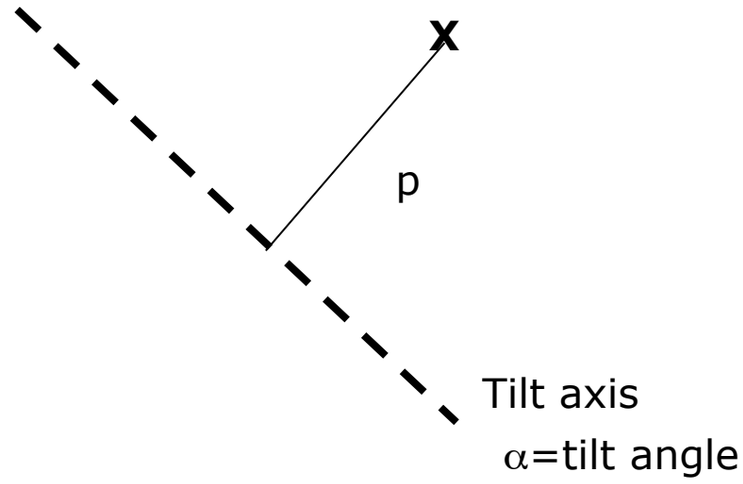
single spot restored to correct spatial frequency with small satellite spots

High Resolution and Large Crystals

Contrast for each Fourier component varies with height of crystal- described by TILT TRANSFER FUNCTION



Specimen height



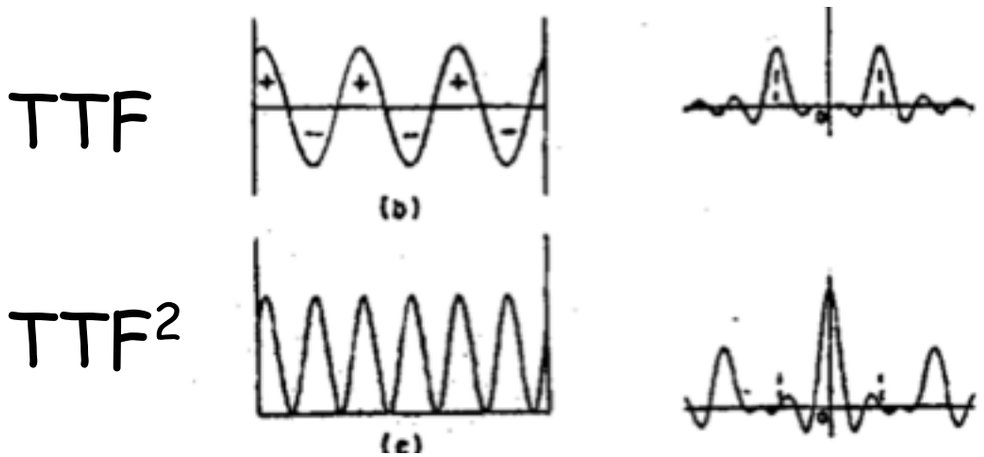
$$TTF(\theta, p) = -2 \sin (cp + \gamma_o),$$

where c and γ_o are constants for given θ, defocus and tilt:

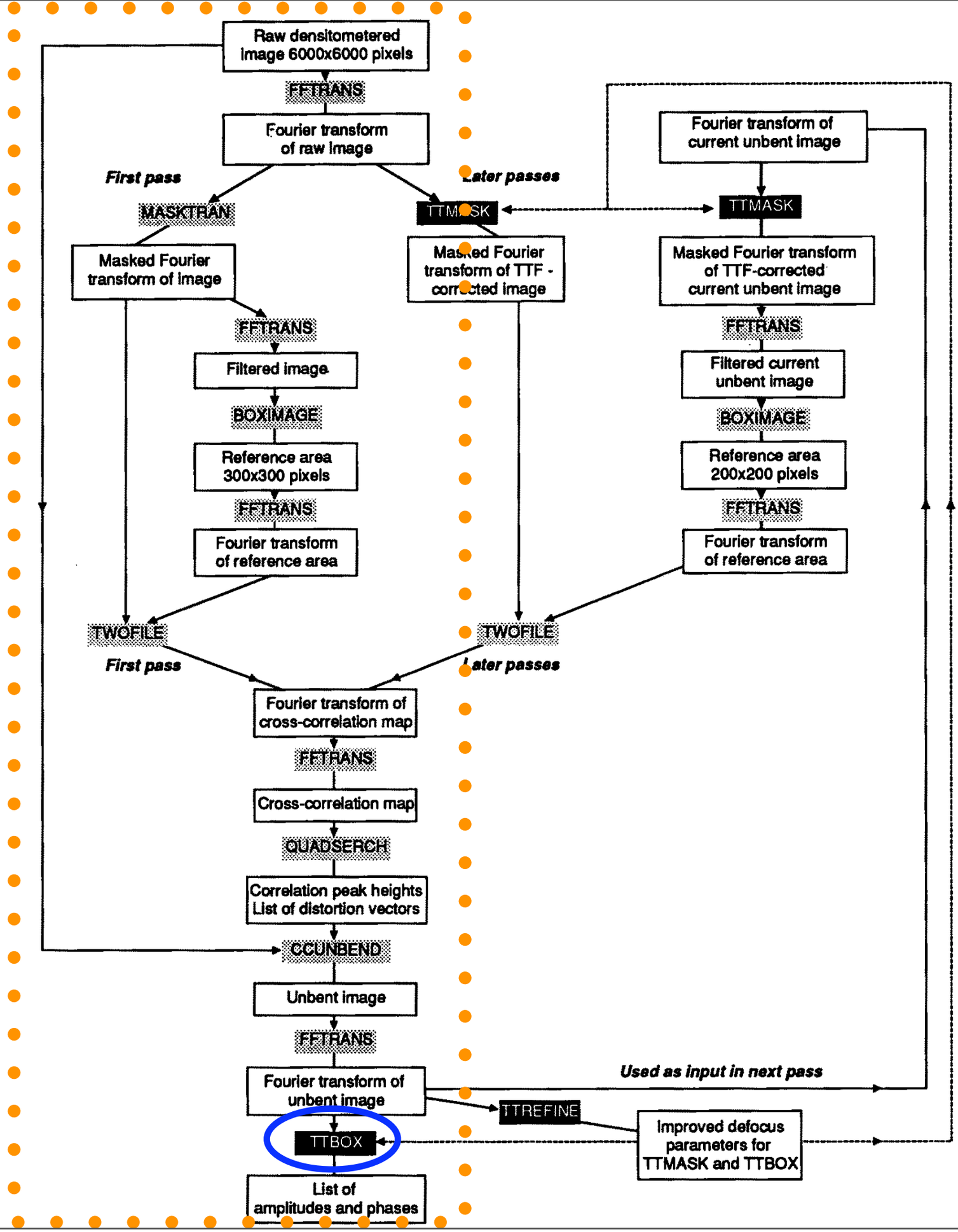
$$c = \frac{2\pi}{\lambda}\left(\frac{\theta^2}{2}\tan\alpha\right); \quad \gamma_o = \frac{2\pi}{\lambda}\left(\Delta F_o \frac{\theta^2}{2} - c_s \frac{\theta^4}{4}\right).$$

Correction of CTF for Tilt

- Multiply image by TTF

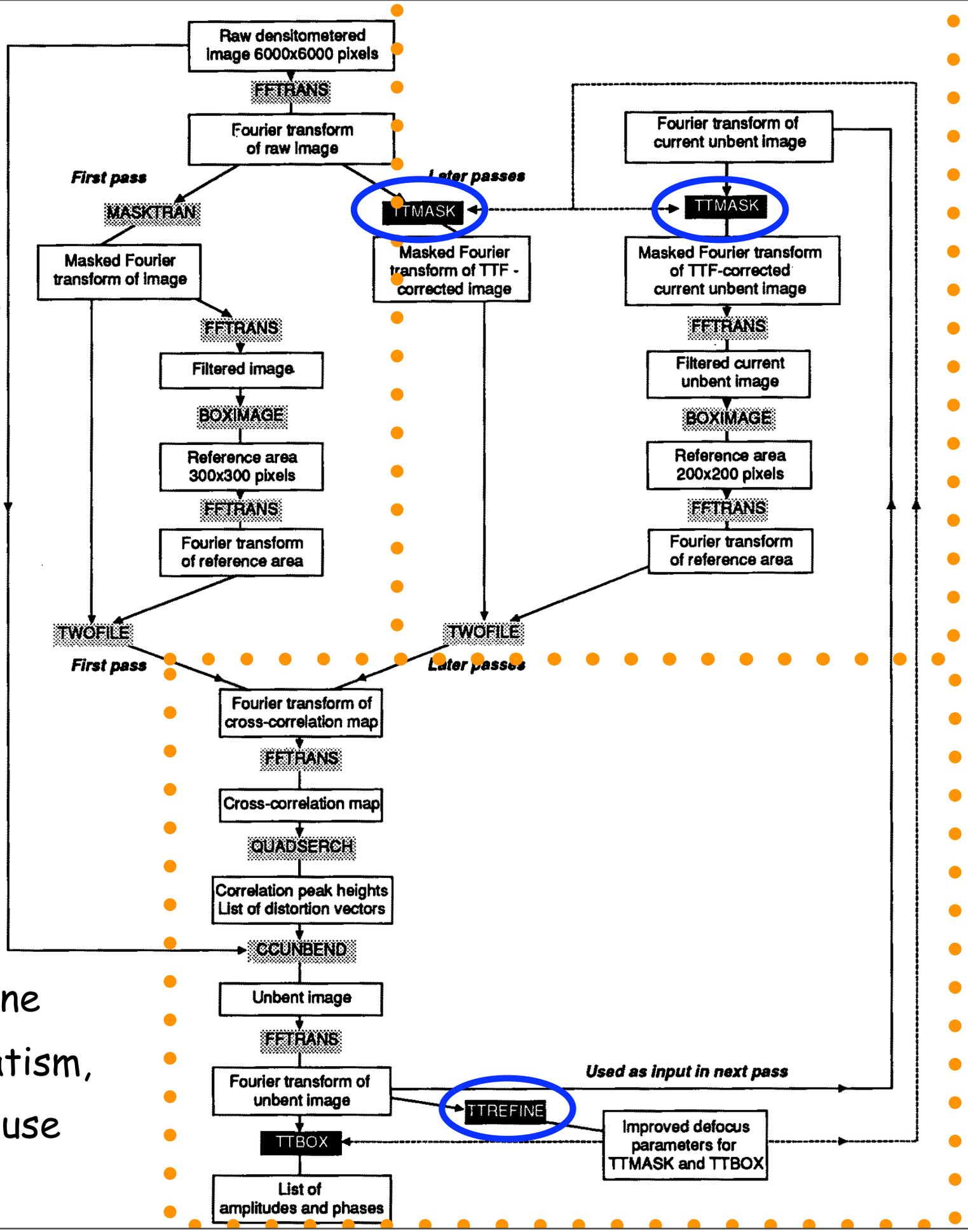


- In practice perform convolution in Fourier space
- Unbending is still essential and interdependent with TTF correction- cycles of TTF refinement and unbending require care!
- TTBOX- reads amp and phase like MMBOX but applies TTF-correction
- **The output of TTBOX consists of a list of amplitudes and phases for the Fourier components of the crystal, fully corrected for tilt, defocus and astigmatism, and ready to be merged with data from other images.**

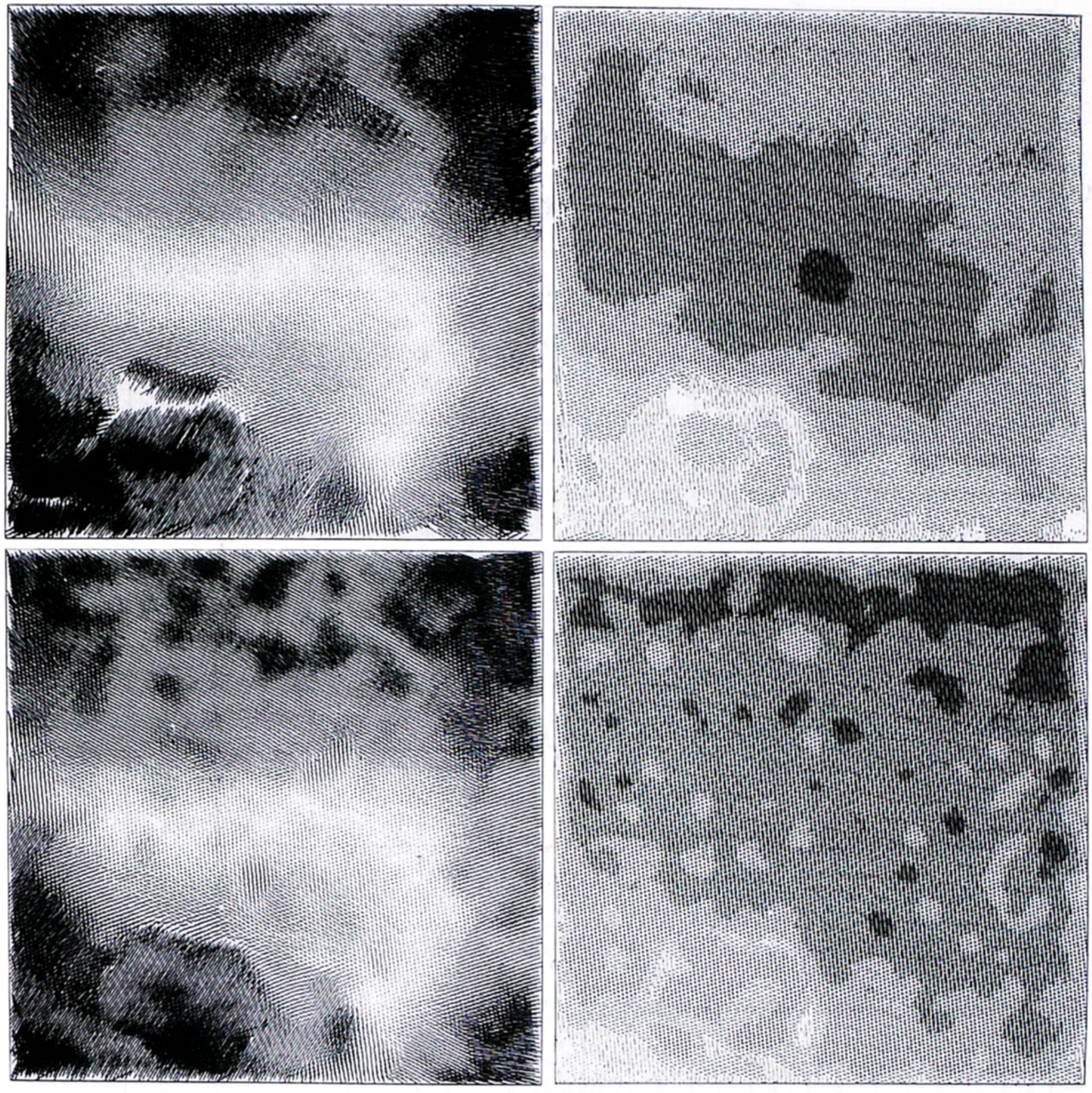


TTBOX essentially takes the place of MMBOX

TTMASK- analagous to MASKTRAN but corrects for defocus and tilt



TTREFINE- refine defocus, astigmatism, (tilt-geometry)- use with care!


(a)
(b)
(c)
(d)

High Resolution and Large Crystals

Parallel																Perpendicular															
6	5	5	7	6	10	8	11	8	7	7	6	6	7	8		6	6	5	10	5	7	7	6	8	6	7	9	7	11	8	
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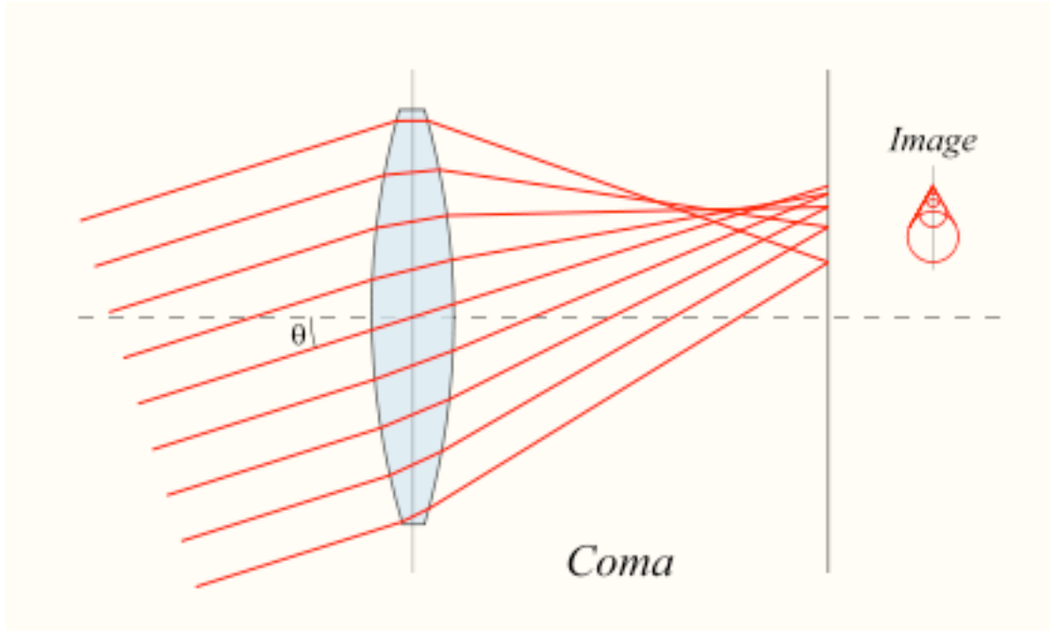
17	41	37	11	8	6	5	8	6	8	5	5	6	9	6		8	11	6	6	7	8	11	8	6	8	7	6	6	8	8	
8	6	19	9	7	8	7	10	8	10	7	8	7	6	6		8	6	8	7	8	6	8	7	5	7	5	8	5	8	7	
5	7	7	8	9	10	8	41	18	8	10	8	5	8	5		7	6	6	8	8	7	8	7	6	9	8	7	5	7	6	
7	9	9	10	10	12	37	227	50	10	9	7	10	6	8		8	7	7	7	6	7	15	35	5	6	5	5	8	6	7	5·5 Å
7	8	6	9	7	11	12	46	15	9	9	7	10	8	6		7	6	9	5	8	6	6	6	6	8	8	9	8	7	10	
8	7	5	10	10	5	7	11	9	7	7	9	17	11	8		8	5	7	9	5	7	6	9	9	6	8	6	9	8	6	
7	5	6	6	5	6	6	13	10	9	6	6	19	31	16		7	6	7	6	6	8	9	7	6	7	6	7	10	9	6	
5	8	9	6	7	10	8	9	7	10	5	11	9	23	17		8	8	8	7	8	9	7	9	7	9	8	7	7	8	7	
6	6	9	7	7	6	7	9	6	6	6	8	7	9	9		5	10	7	7	7	7	8	7	7	7	7	10	9	7	9	
9	7	8	7	7	11	7	10	6	6	8	5	8	7	5		10	6	7	7	8	8	6	7	5	9	7	6	7	5	9	
7	7	8	5	9	6	8	10	9	9	8	6	7	8	5		7	8	9	11	11	8	7	8	7	5	8	8	6	9	6	
7	8	6	7	6	9	7	7	6	6	7	7	5	8	6		7	6	6	6	7	8	7	7	8	8	6	7	7	7	8	
6	8	7	7	8	9	6	8	7	7	7	7	6	7	6		9	8	8	7	6	7	7	7	7	8	7	6	6	7	7	
8	7	7	8	6	6	8	7	6	7	7	6	8	8	7		7	6	6	7	7	7	6	6	7	7	6	7	8	7	8	
8	7	8	7	8	8	7	6	8	8	6	6	5	8	7		7	8	9	6	7	7	7	7	6	7	8	7	7	7	6	
7	8	8	8	7	6	8	8	7	7	7	6	6	7	7		8	8	8	8	7	7	7	7	6	7	7	7	8	7	7	
7	7	6	8	7	7	6	8	7	7	7	6	7	7	7		6	7	8	7	7	6	7	6	6	7	7	6	6	7	7	
8	8	7	6	6	7	11	14	8	7	7	7	7	8	7		7	8	7	6	7	7	8	11	9	6	7	6	6	7	6	
7	7	9	7	8	7	10	47	17	8	7	7	7	8	6		7	9	7	7	7	7	8	16	10	8	8	7	7	9	7	3·5 Å
6	6	7	7	7	7	8	7	9	7	7	8	6	7	6		8	7	8	7	6	5	7	8	10	7	7	9	7	8	7	
8	6	6	7	8	7	7	8	8	6	6	9	7	7	7		7	7	7	6	6	7	7	7	8	8	8	8	7	6	7	
7	7	7	7	7	6	7	8	7	7	7	6	7	9	7		8	8	7	7	6	7	8	6	6	7	7	7	7	7	6	
7	6	8	7	6	9	6	7	7	6	7	7	7	7	7		7	7	7	7	8	7	6	7	7	8	8	7	8	7	7	
8	8	7	7	6	7	7	8	8	7	6	7	7	6	6		8	7	7	6	7	7	7	7	7	6	8	6	7	8	7	
6	6	6	6	8	8	8	8	7	7	8	8	6	7	7		7	6	7	7	6	7	7	8	6	7	7	6	6	7	7	
5	7	6	6	8	8	7	6	7	8	8	6	7	6	8		7	8	6	9	8	6	7	10	8	8	7	8	5	6	6	

5·5 Å

3·5 Å

Beam tilt misalignment

- Analagous to axial coma in optics
- Causes a small change in defocus and astigmatism and a resolution-dependent shift of the position of the image components.
- Defocus and astigmatism taken care of in the CTF correction.
- The image shift can be separated into a component which is proportional to the beam tilt and (resolution)^3 together with a simple shift of the whole image.
- Correction for beam tilts of magnitude from 1 to 3 milliradians often necessary.
- Spot at 3.5 Å resolution typically has between 200 ° and 400 ° phase shift in the beam tilt direction.



$$\Delta\alpha = -\frac{2\pi}{\lambda} C_s \theta^2 (\boldsymbol{\theta} \cdot \boldsymbol{\theta}_0)$$

resolution^3

beam tilt

Correcting for beam tilt misalignment

- Determination of the beam tilt is done entirely from the phases.
- Two methods:
  - phases of spots related within one image by the crystallographic symmetry compared with one another, or
  - the phases from a new image compared with phases from images already corrected for beam tilt.
- ORIGTILT- refinement of phase origin (OX, OY) and beam tilt (TX, TY) done jointly by minimisation of the phase difference.
- Effect of beam tilt is proportional to resolution^3, therefore determine (OX, OY) from the low resolution spots and (TX, TY) from high resolution spots.
- **CHECK- beam tilt magnitude and direction should be similar within one imaging session when conditions have not changed**
- **DO THE VALUES MAKE SENSE?**

Correcting for beam tilt misalignment

- Determination of beam tilt for tilted specimens requires an iterative procedure e.g. start with merged list of 0° (corrected) with 20° tilts (uncorrected).
- This merged list used as the reference for preliminary refinement of beam tilt by comparing data from each image against data from all the others.
- Subsequent merges should result in beam tilt and phase origin converging to, unambiguous values with convincing phase residuals.
- Then add e.g. 45° tilts with preliminary beam tilts derived by comparison with the 20° merged data, followed again by iterative refinement.

Film number	Beam tilt/milliradians
509	1.9
510	2.3
511	2.0
522	2.4
526	2
527	3.2
549	3.9

Final thoughts and observations

Map Interpretation

- The missing cone and generally poorer measurements at high z* cause blurring along the c-axis.
- For 60° tilts resolution is ~1.5 times worse in C-direction.
- At 20 Å a 25-50 kDa protein will appear as a single blob.
- Number of blobs $\propto$ 1/res.
- 10-7 Å resolution will reveal α-helices.

Map Interpretation

- Beware of data in the 4-5 Å range- these are notoriously difficult to interpret. You will probably get it wrong!
- Even well determined X-ray maps at 2.5 Å resolution have been traced backwards!
- Try to use all the biochemical and genetic knowledge available to you.

Things to Be Aware Of

- Make as many amplitude and phase measurements as you can- this will substantially reduce noise in your map
- Do not overestimate your resolution- a single spot at 4 Å does not imply 4 Å resolution

Things to Be Aware Of

- Programs may have bugs.
- Check your log files for error messages
- Make sure all crystals have same thickness
- Crystals more than 1 unit cell thick are very difficult to process

Things to Be Aware Of

- Do not use programs blindly- check that you are getting sensible and consistent results when comparing independent images
- Be patient and thorough