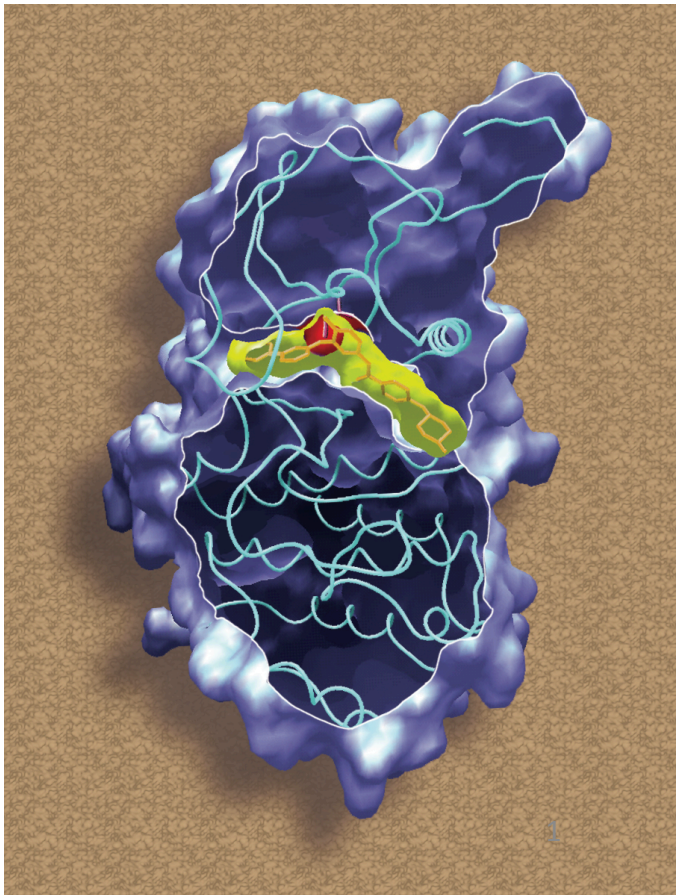
Bi 204 Methods: X-Ray diffraction

. Calibrating Non-covalent Molecular Interactions

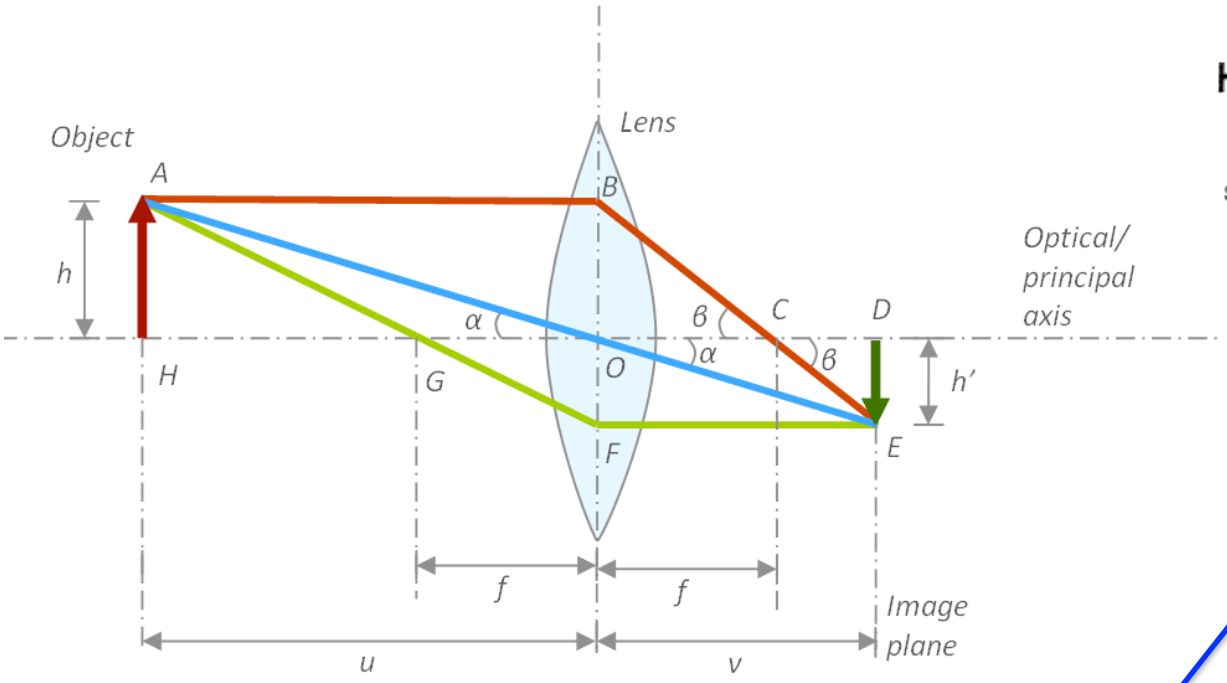
Stroud
2017

stroud@msg.ucsf.edu



A ‘Ligand’ the cancer drug imatinib (Gleevec) bound to the tyrosine kinase Abl.

Optical image formation, - without lenses



Functional Mimicry of a Protein Hormone by a Peptide Agonist: The EPO Receptor Complex at 2.8 Å

Oded Livnah, Enrico A. Stura, Dana L. Johnson, Steven A. Middleton, Linda S. Mulcahy, Nicholas C. Wrighton, William J. Dower, Linda K. Jolliffe, Ian A. Wilson*

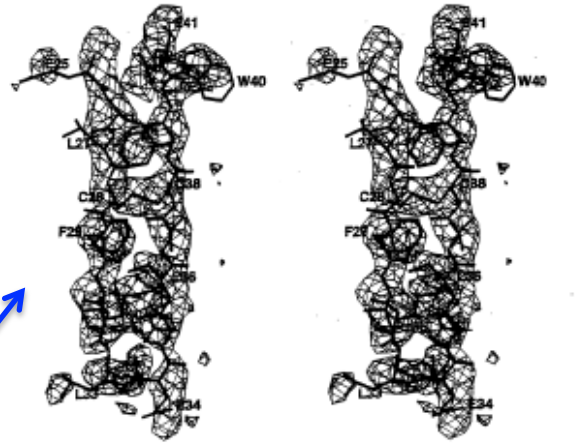
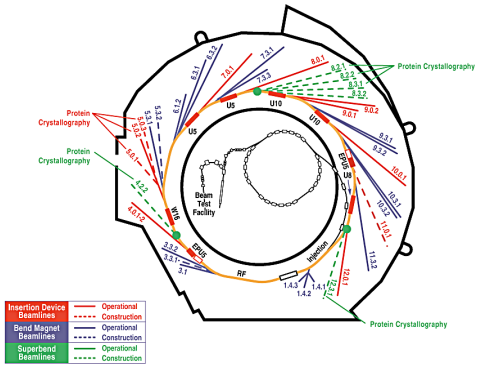
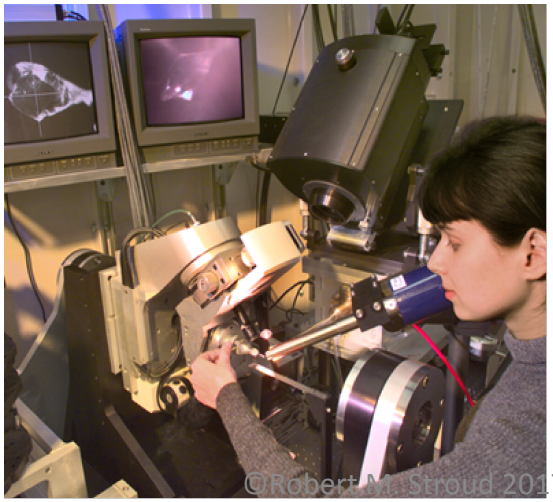


Fig. 1. Stereoview of the initial experimental solvent-flattened MIR electron density map at 25.0 to 3.1 Å resolution, contoured at 1.3 σ for residues 25 to 41 with superimposed coordinates from the final refined structure. This segment covers β strands A and B in D1 and shows one of the two characteristic disulfide bridges [Cys^{26}–Cys^{38}] in the first domain of the cytokine receptor superfamily.

Type of light	wavelength	what we see?	character	speed
Light	5000Å	dielectric	em. waves-good lenses++	speed of light
X-rays	1 to 3 Å	electron density; f ~ n_e	em. waves-NO lenses	speed of light
neutrons	1 to 5 Å	nuclei	particles NO lenses	slow speed thermal neutrons
electrons	0.01 - 0.1 Å	electric fields	particles Poor lenses.	eV~0.5mv^2.





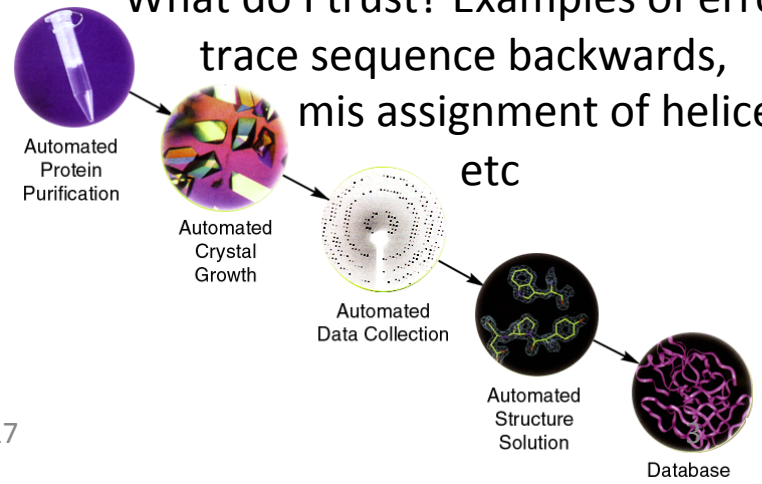
If automated- why are there errors?

What do I trust? Examples of errors

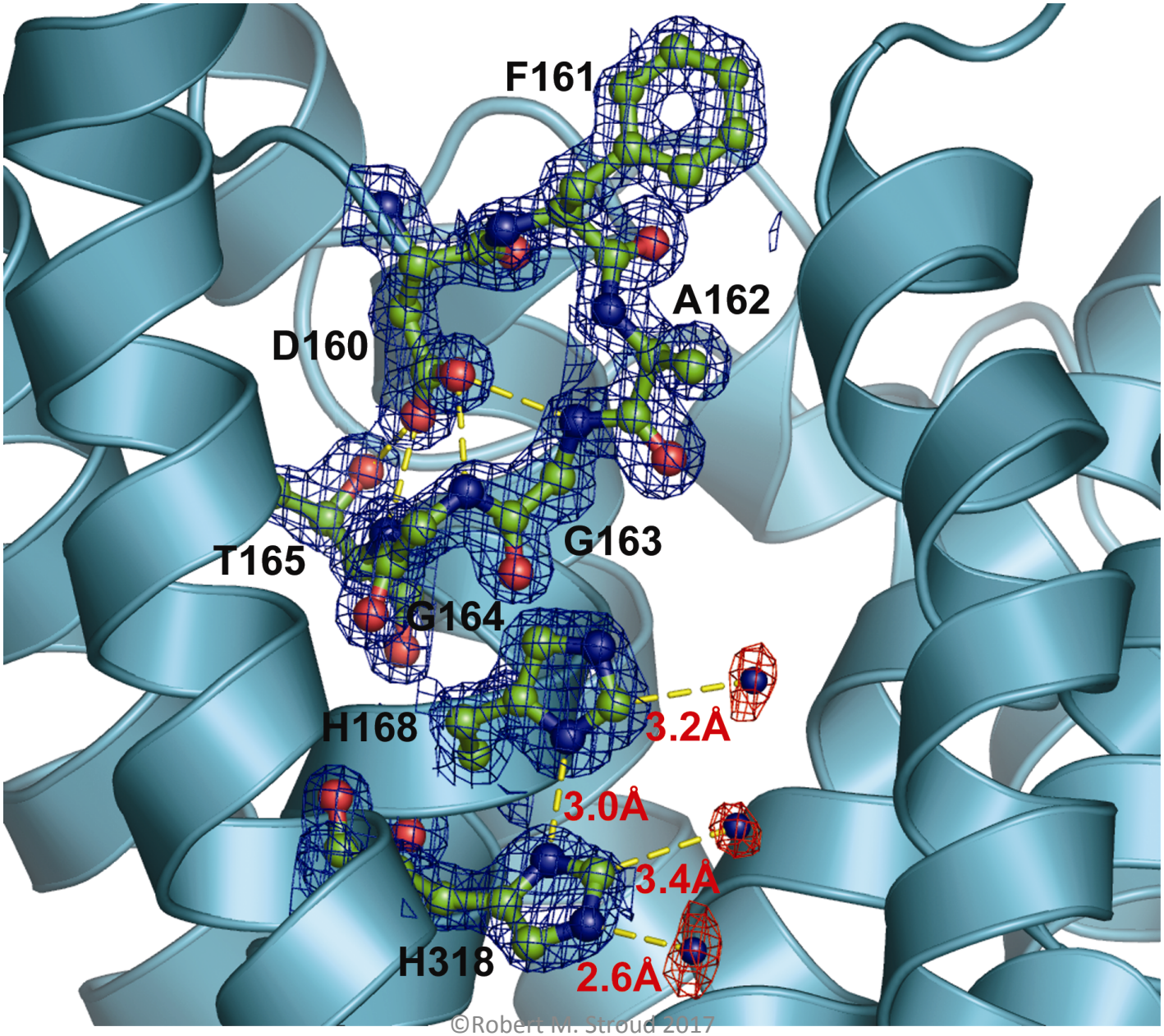
trace sequence backwards,

mis assignment of helices

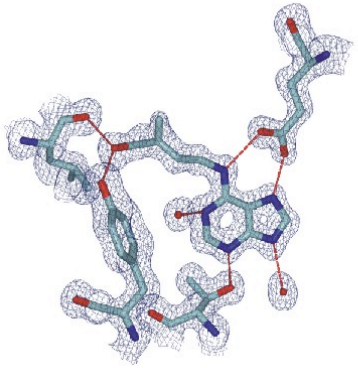
etc

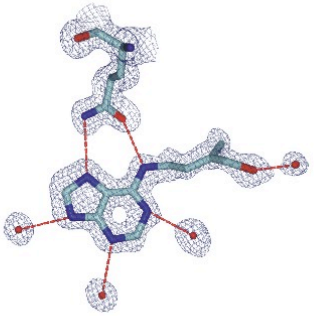


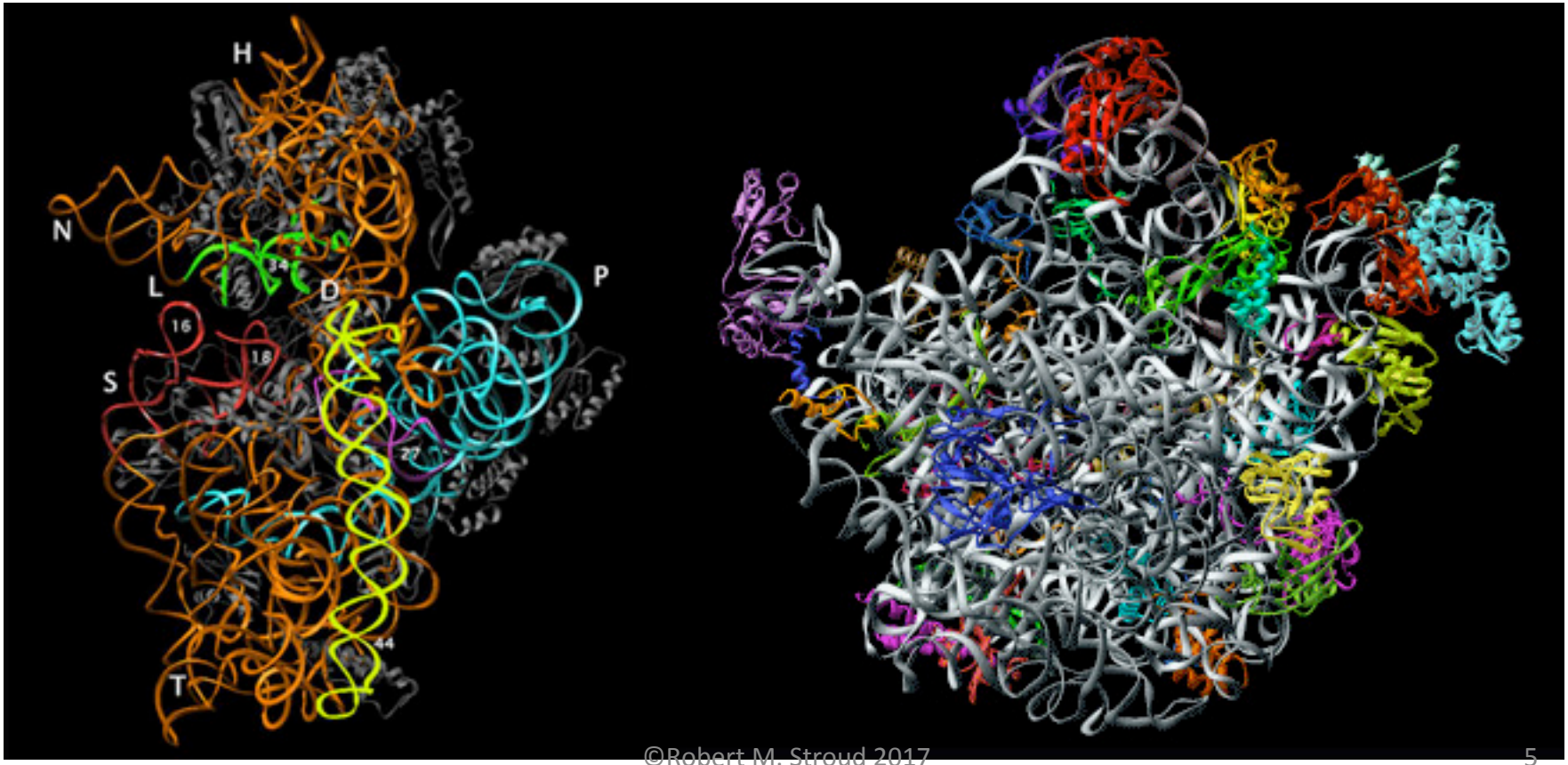
NH3 sites and the role of D160 at 1.35Å Resolution



Data/Parameter ratio is the same for all molecular sizes at the same resolution d_{min} ie. quality is the same!







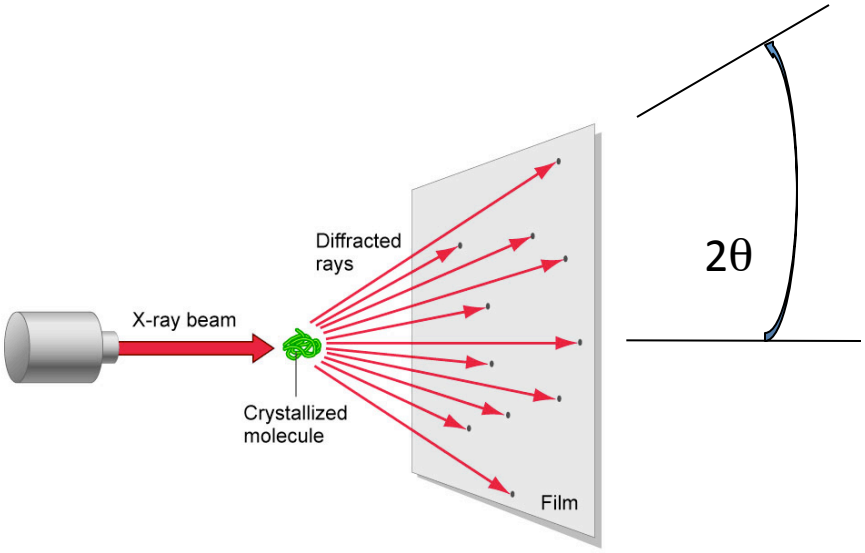
30S

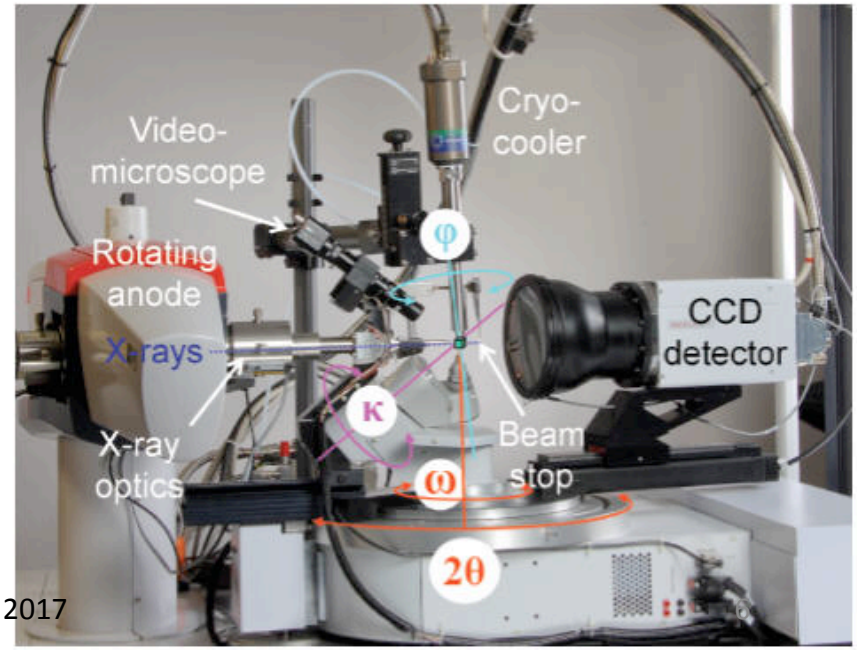
50S

Determining Atomic Structure

- X-ray crystallography = optics λ ~ 1.5Å (no lenses)
- Bond lengths ~1.4Å
- Electrons scatter X-rays; X-rays 'see electrons'
- Resolution –Best is λ/2 Typical is 1 to 3 Å
- Accuracy of atom center positions ±1/10 Resolution

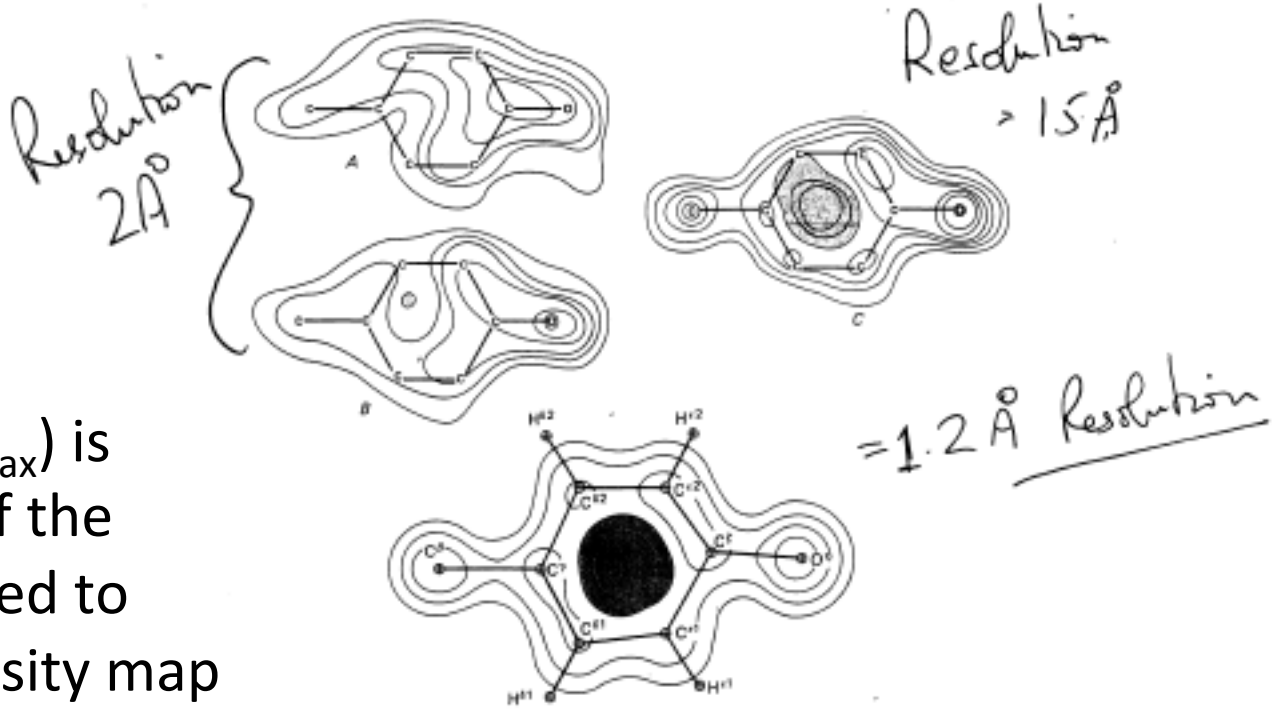
$$d_{\min} = \frac{\lambda}{2 \sin \theta_{\max}}.$$





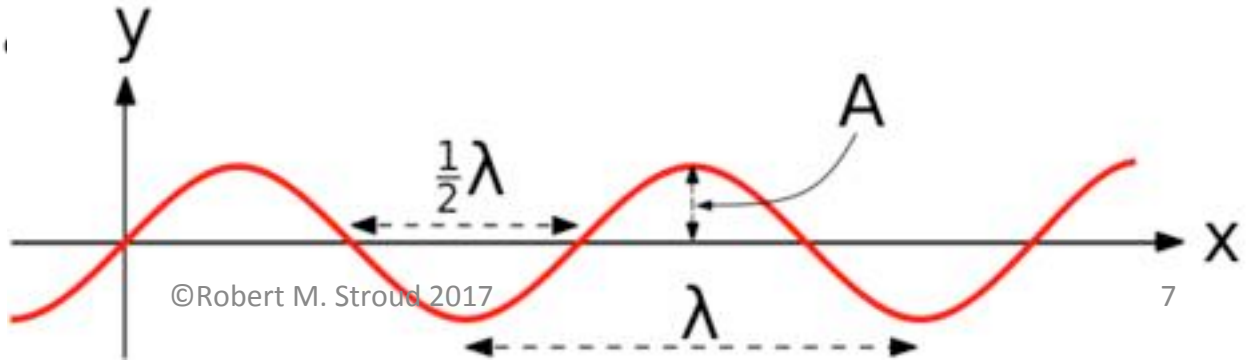
Resolution $d_{min} = \lambda / 2 \sin(\theta_{max})$

differs from Rayleigh criterion



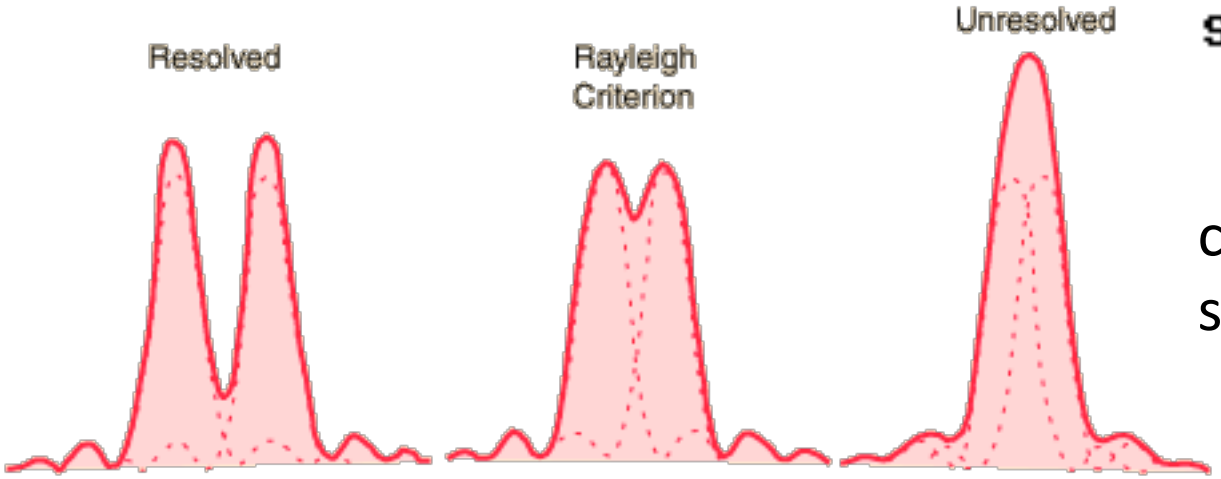
$d_{min} = \lambda / 2 \sin(\theta_{max})$ is the wavelength of the shortest wave used to construct the density map

FIGURE



The Rayleigh Criterion

- The Rayleigh criterion is the generally accepted criterion for the minimum resolvable detail - the imaging process is said to be diffraction-limited when the first diffraction minimum of the image of one source point coincides with the maximum of another.



$$\sin\theta_R = 1.22\,\frac{\lambda}{d}$$

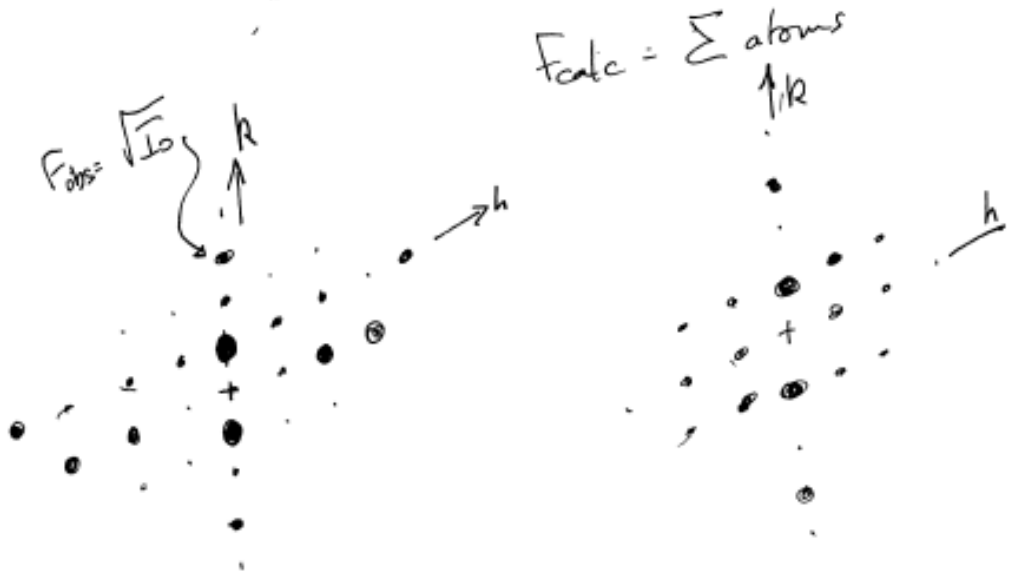
compared with

$\sin(\theta_{max}) = \lambda / 2\, d_{min}$

Bottom Lines: $I_{nke} = |F_{nke}|^2$

- "R factor" = Agreement between Amplitudes calculated, $F_{calc}^{hkl} = \sum_j f_j e^{2\pi i(hx+ky+lz)}$ and Amplitudes observed $\sqrt{I_{obs}}_{hkl} = Fobs_{hkl}$

$$R = \frac{\sum_{hkl} ||F_o| - |F_c||}{\sum_{hkl} |F_o|}$$



How do we judge the Quality of structure?

2. Overall quality criteria: agreement of observations with diffraction calculated from the interpreted structure.

3. Since we refine the structure To match the I_{hkl} overfitting ?

Define R_{free} for a 'hold-out ' set of observations.

4. OK? R < 20%, R free< 25%

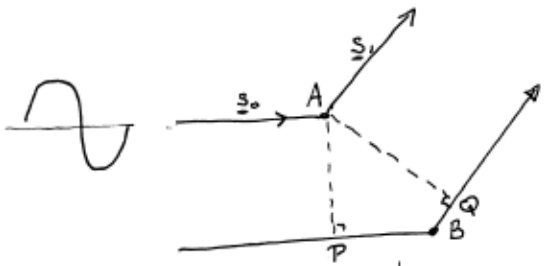
5. But the experimental errors in measuring Fo are ~ 3%. inadequate models of solvent, atom motion, anharmonicisity

6 Accuracy ~ 0.5*res*R

Scattering by matter – (interference of a single wavelength X-ray)

Scattering from a point is equal in all directions.

add a second point, scattering in some direction $\underline{s}_1$



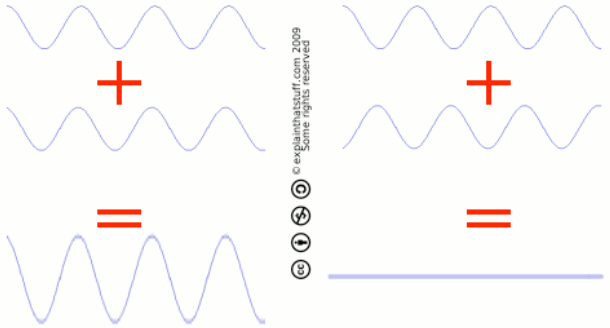
The second wave, scattered by B travels further by the distance PB + BQ.
Its scattered wave lags in phase by

$$= \frac{2\pi}{\lambda}\left([P-B] + [B-Q]\right)$$

$$= \frac{2\pi}{\lambda}\left(\underline{\Phi}\right)$$

where $\underline{\Phi}$ = path length extra for B versus the reference A.

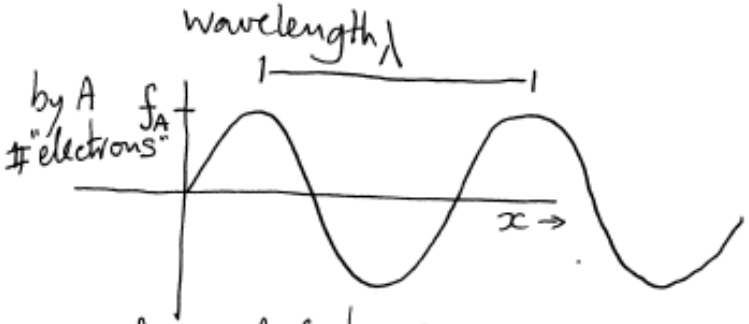
Adding up the scattering of Atoms: 'interference' of waves



Constructive Interference

Destructive Interference

Waves add out of phase by 2π[extra path/λ]



f_A = # of electrons

$$F = A \sin 2\pi\left(\frac{x}{\lambda} - \omega t\right)$$

For $f_A = f_B = 1$ say, total 2 electrons scatter

$$F = A \sin 2\pi\left(\frac{x}{\lambda} - \omega t\right) + A \sin 2\pi\left(\frac{x+\phi}{\lambda} - \omega t\right)$$

$$F = 2A \cos 2\pi\left(\frac{\phi}{2\lambda}\right) \sin 2\pi\left(\frac{x+\phi/2}{\lambda} - \omega t\right)$$

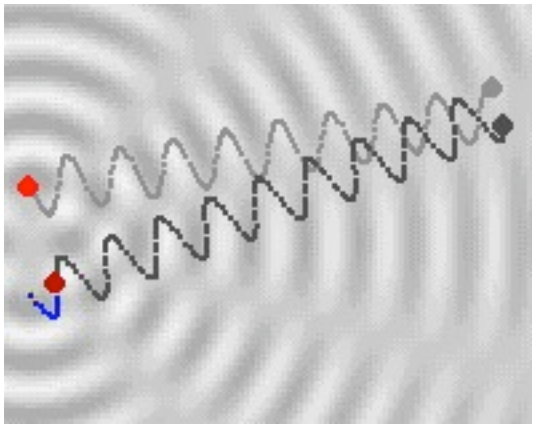
Amplitude of F, Always less than 2 (less than the sum of all electrons in object)

the wave is 'phase shifted by $\left(\frac{2\pi\phi}{2\lambda}\right)$

In general they add up to something amplitude In between -2f and +2f.

For n atoms

$F^2 = f \times \sqrt{n}$



This sum corresponds to a wave of amplitude less than 2, (ie < the number of (electrons / points))

Amp. = $|F| = 2\cos 2\pi\left(\frac{\phi}{2\lambda}\right)$

which has the same periodicity in time

$\sin 2\pi\left(\frac{x+\phi/2}{\lambda} - \omega t\right)$

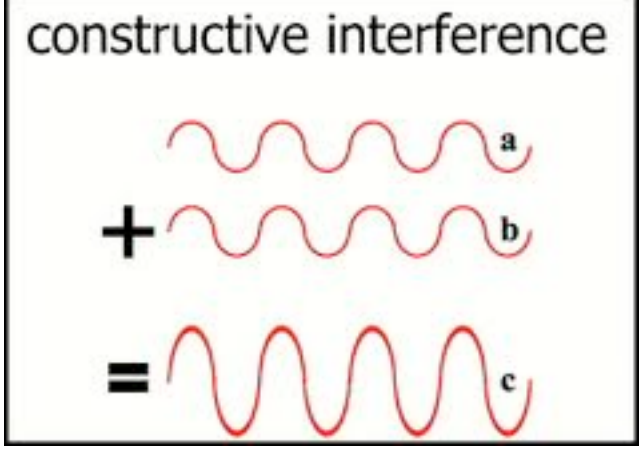
and is 'phase shifted' by $\frac{\phi/2}{\lambda}$

We only observe intensity of scattering = (Amplitude)^2, - or the number of photons scattered at any one position. Thus the 'time' dependent component is irrelevant. But, the relative 'phase shift' is crucial.

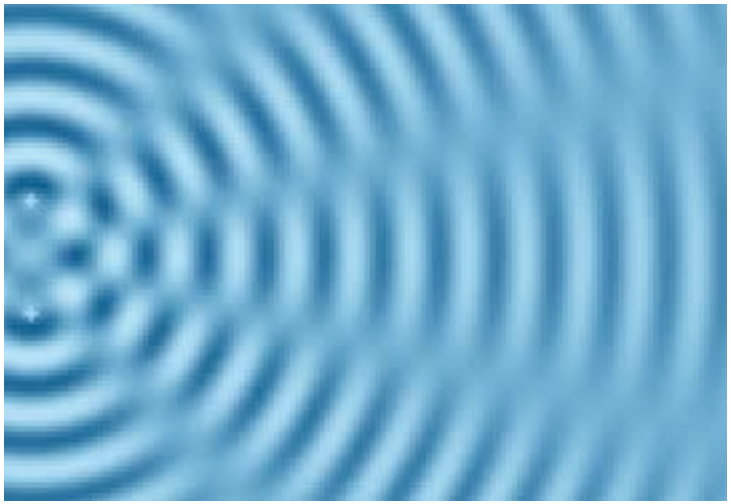
If the incoming wave is represented by a line = amplitude along the x-axis, -

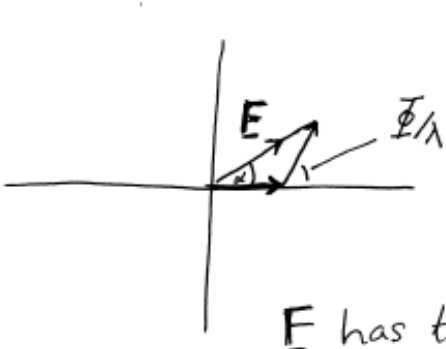
The scattered wave can be represented by the sum of another arrow that represents the scattered wave from A, plus another from B.

If x-rays, the amplitude of each component is the number of electrons in that atom, f.



Just 2 atoms…

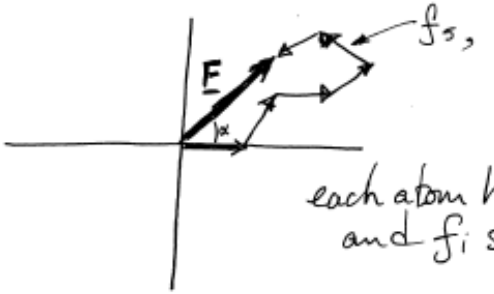




α = relative phase to the 1st atom.

F has the required amp. & phase.

If we use this method we can add $i = 1$ to n different atoms; each amplitude f_i



each atom has $f_i \cos\alpha_i$ along x and $f_i \sin\alpha_i$ along y

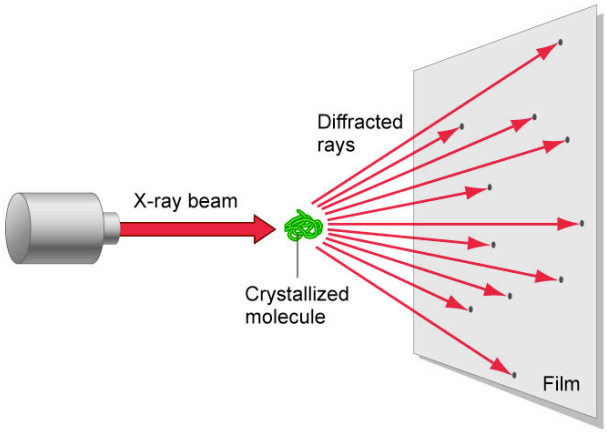
If we put 'units' on the axes, we can add up the 'x' and 'y' components to write the sum over "x", the sum over "y", — hence calculate $\underline{F}$ as a wave of amplitude

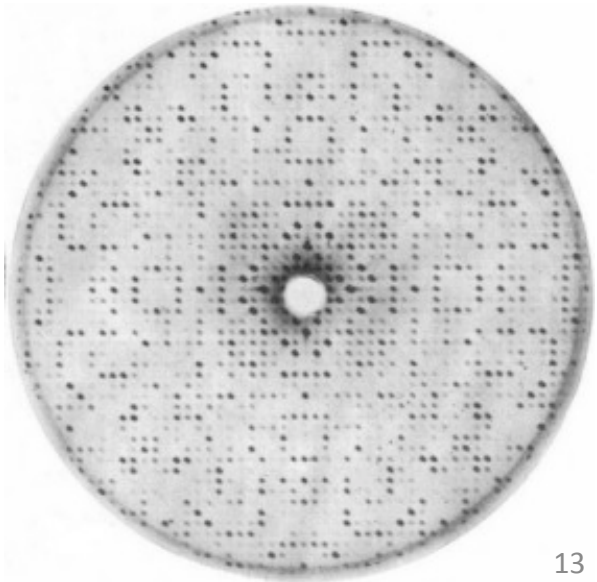
$$|\underline{F}| = \sqrt{\left(\sum_x f_i \cos\alpha_i\right)^2 + \left(\sum_y f_i \sin\alpha_i\right)^2}$$

and $$\alpha = \tan^{-1}\left(\frac{\sum_y f_i \sin\alpha_i}{\sum_x f_i \cos\alpha_i}\right)$$

Many atoms add by the same rules.

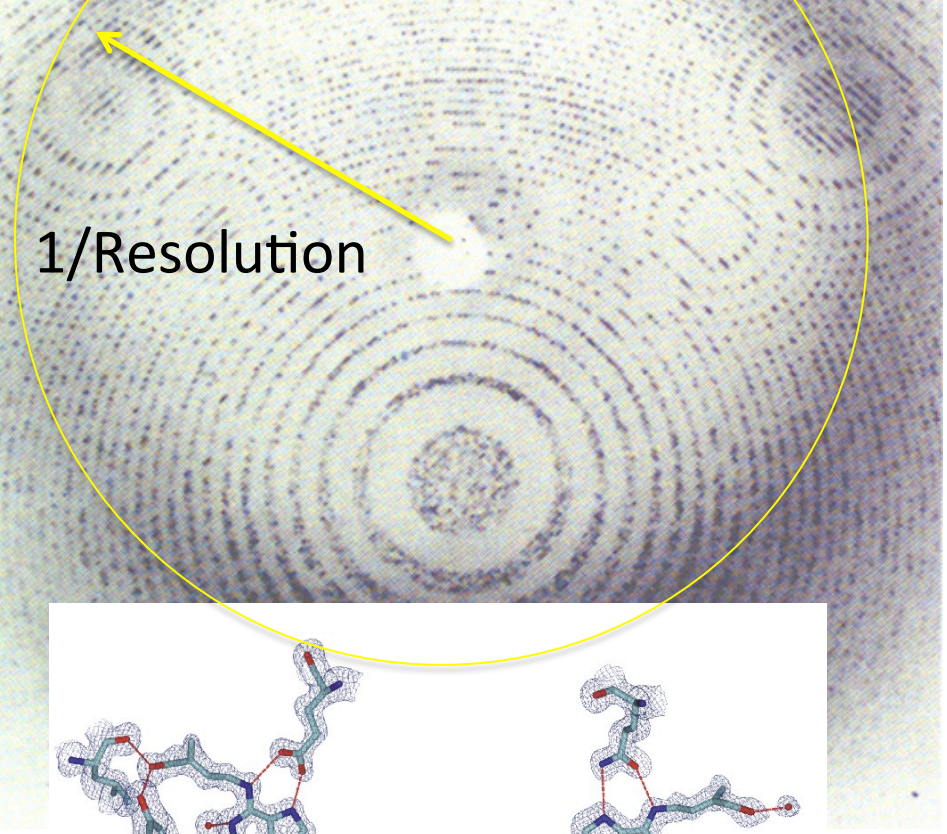
Different in every direction.

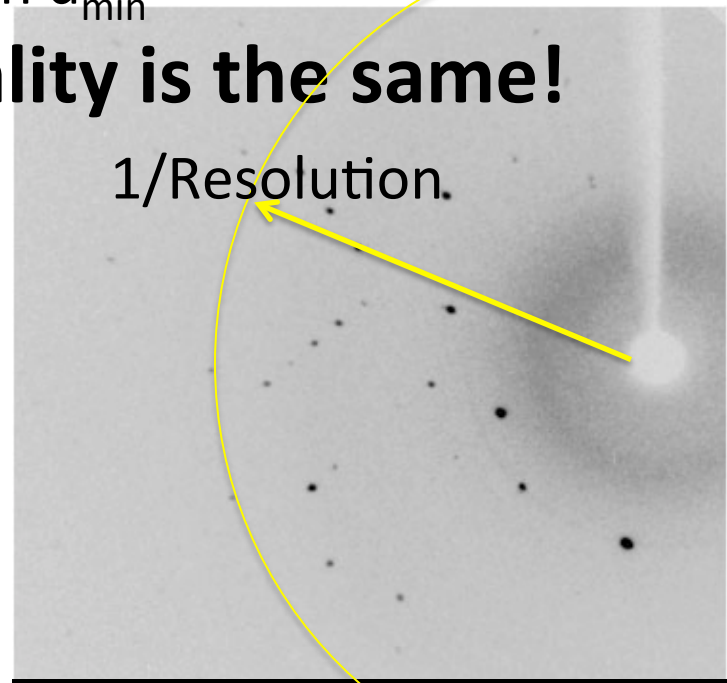


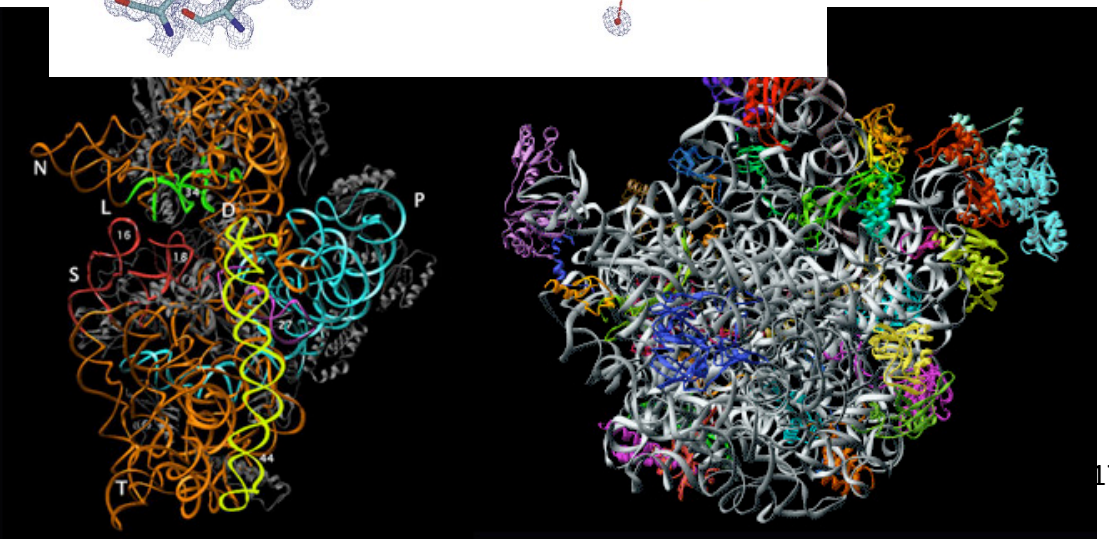


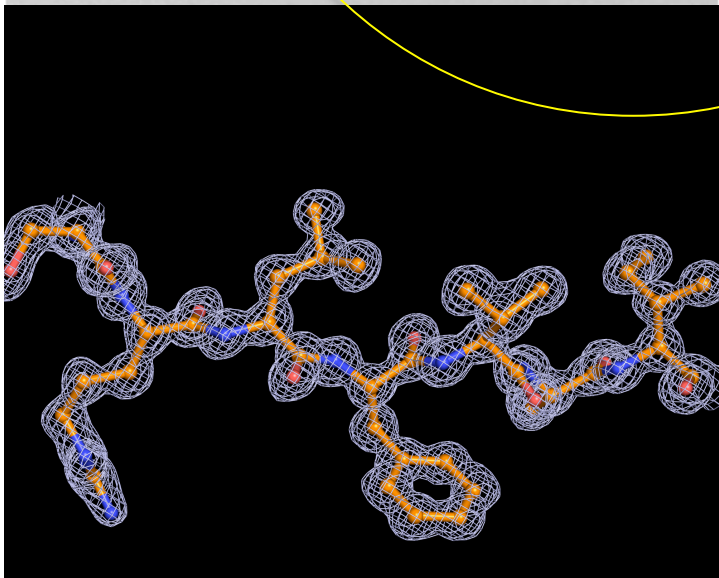
Data/Parameter is the same for all molecular sizes at the same resolution d_{min}

ie. quality is the same!



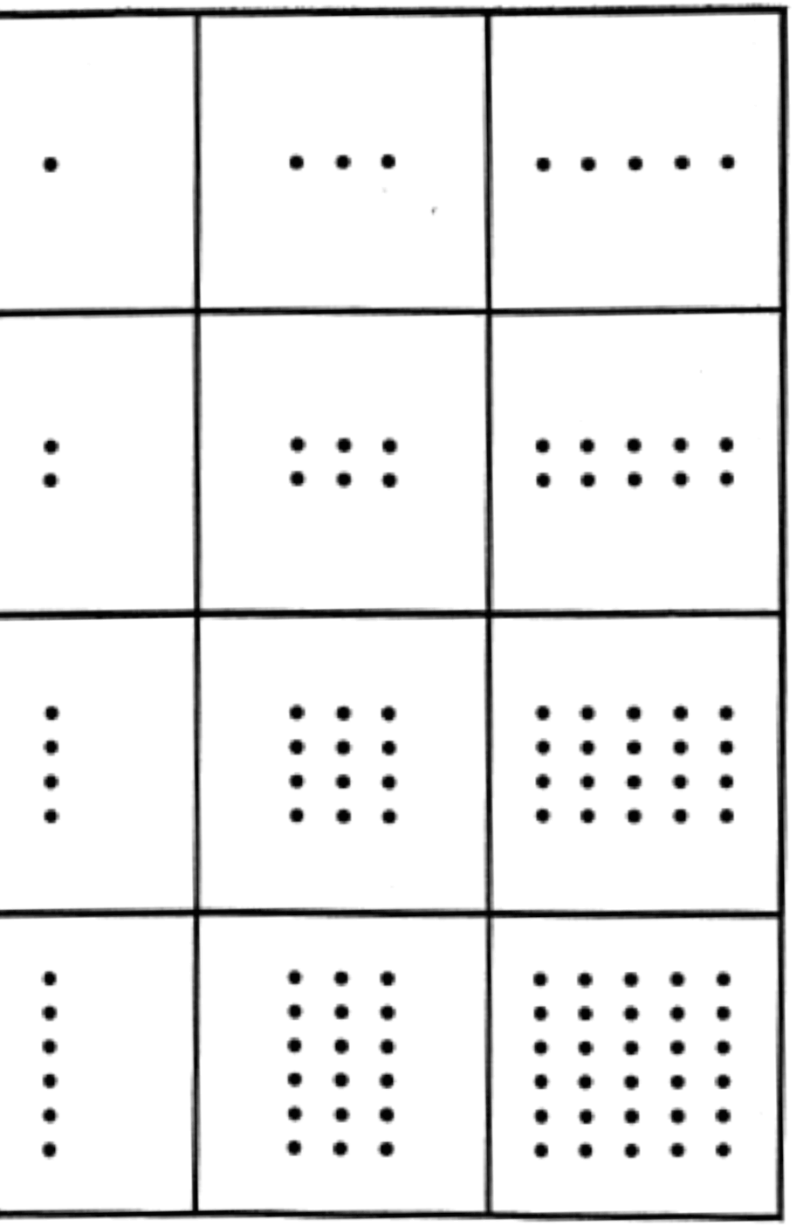


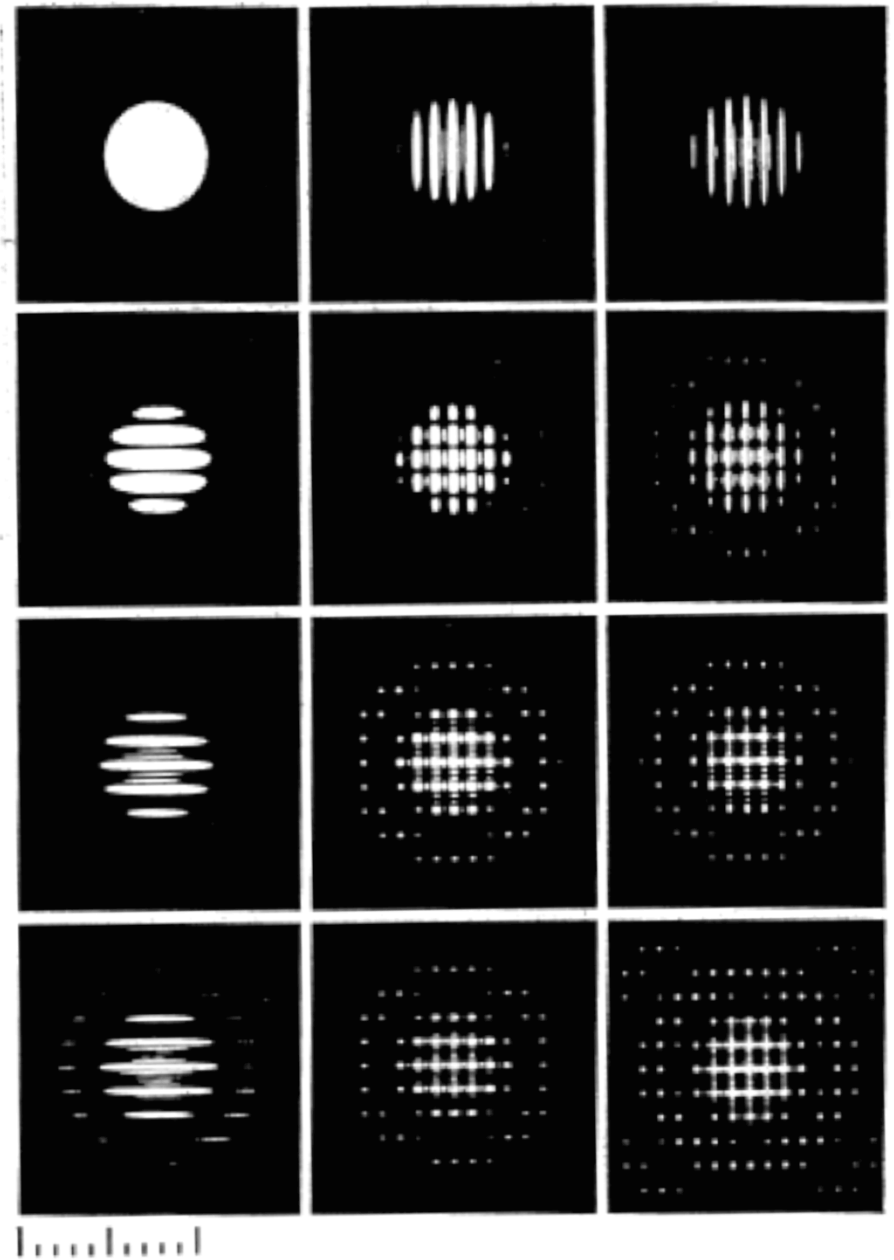




duce the lattice of 12. Subsidiary diffraction maxima due to the small number of apertures can be seen clearly.

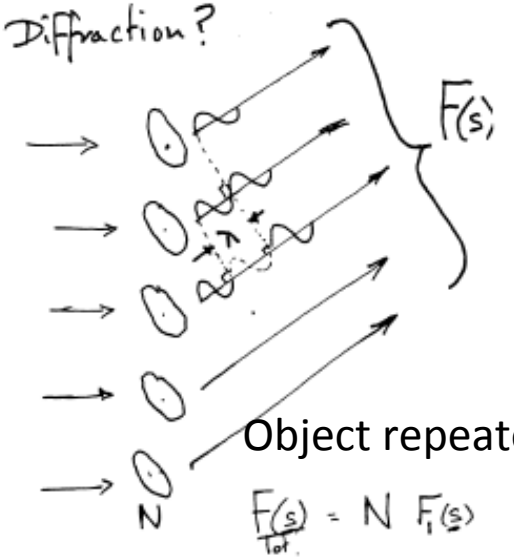
Plate 10





(8)

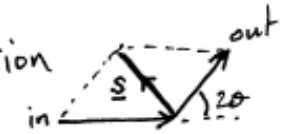
Why Diffraction?



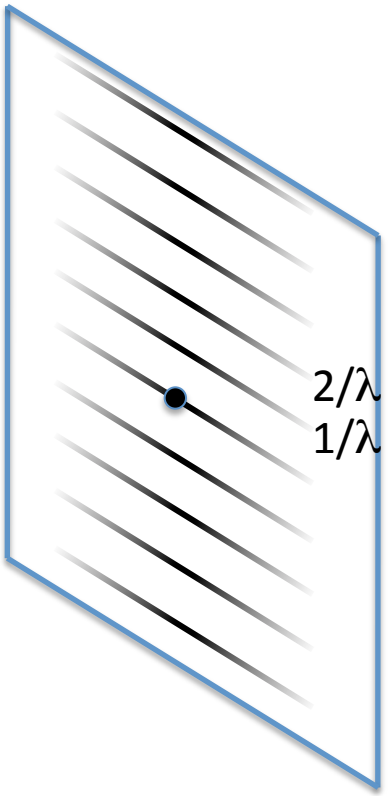
Object repeated

$F_{Tot}(\underline{s}) = N\, F_1(\underline{s})$

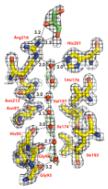
$\underline{s}$ refers to a scattering direction



so $|\underline{s}| = \frac{2\sin\theta}{\lambda}$



This is all there is? YES!!



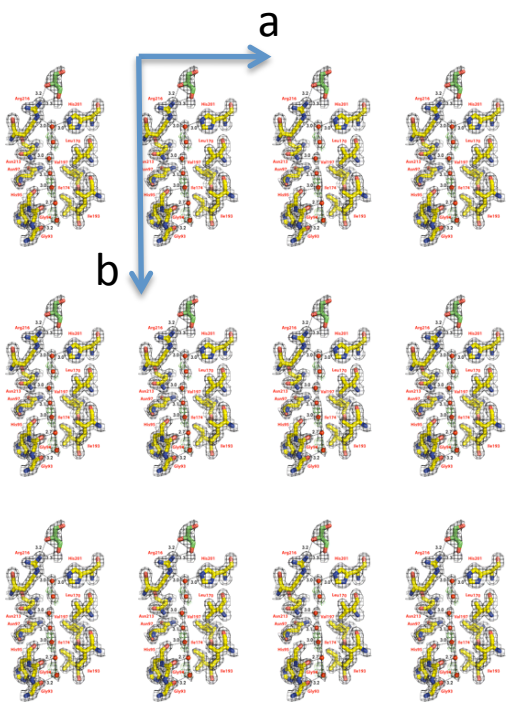
FT

FT^{-1}

Scattering pattern is the Fourier transform (FT) of the structure: Amplitude and phase of waves is a sum of waves from each atom

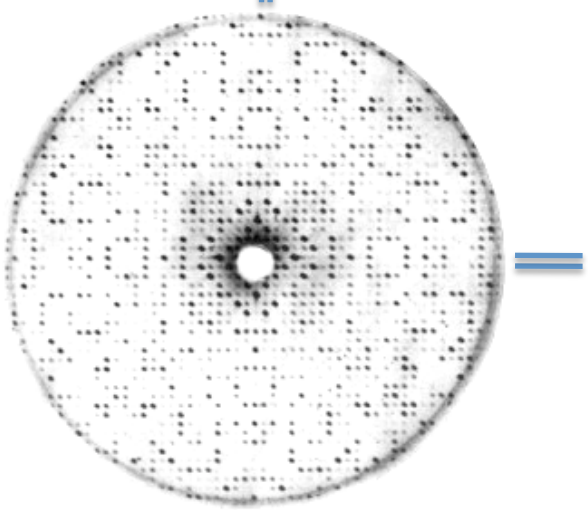
$$F(\underline{S}) = \Sigma_j f_j e^{(2\pi i \mathbf{r_j}.\mathbf{S})}$$

Structure is the 'inverse' Fourier transform of the Scattering pattern



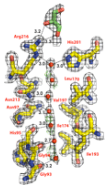
FT

FT^{-1}



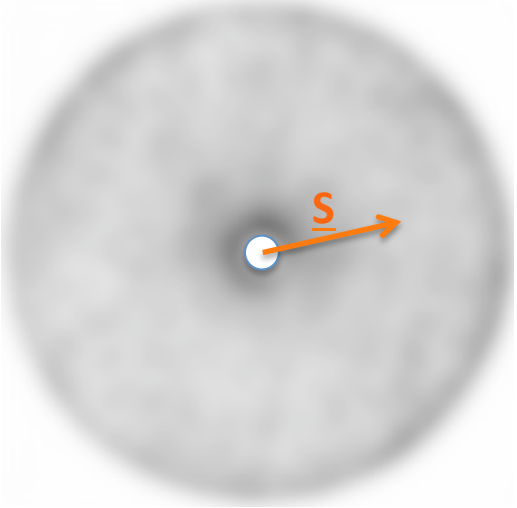
$$\rho(\underline{r}) = \Sigma F(\underline{S}) e^{(-2\pi i \mathbf{r}.\mathbf{S})}$$

This is all there is?



FT

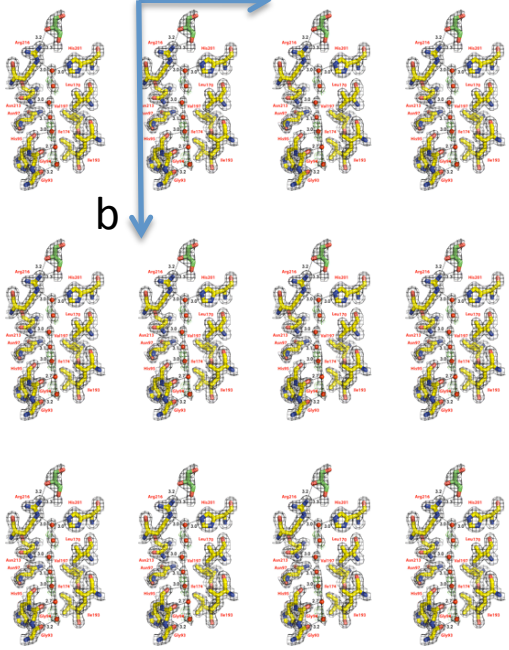
FT^{-1}



Scattering pattern is the Fourier transform of the structure

$$\underline{F}(\underline{S}) = \Sigma_j f_j e^{(2\pi i \mathbf{r_j.S})}$$

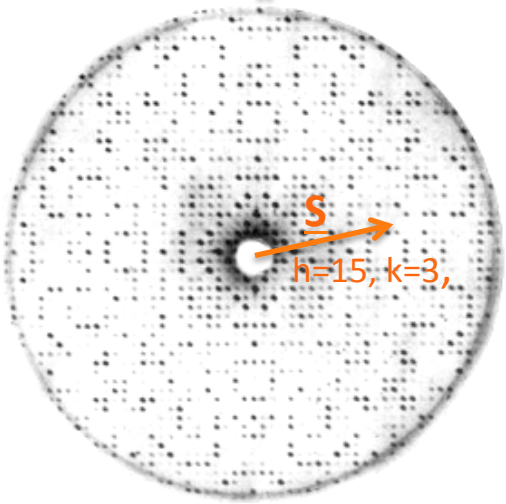
Structure is the 'inverse' Fourier transform of the Scattering pattern



FT

FT^{-1}

1/a



1/b

$$\rho(\underline{r}) = \Sigma \underline{F}(\underline{S}) e^{(-2\pi i \mathbf{r.S})}$$

$$\underline{F}(h,k,l) = \Sigma_j f_j e^{(2\pi i\ (hx+ky+lz))}$$

$$\rho(x,y,z) = \Sigma \underline{F}(h,k,l) e^{(-2\pi i \mathbf{r.S})}$$

The sequence shows how the diffraction pattern of a simple object is built up by superposition of sets of fringes.

Plate 2

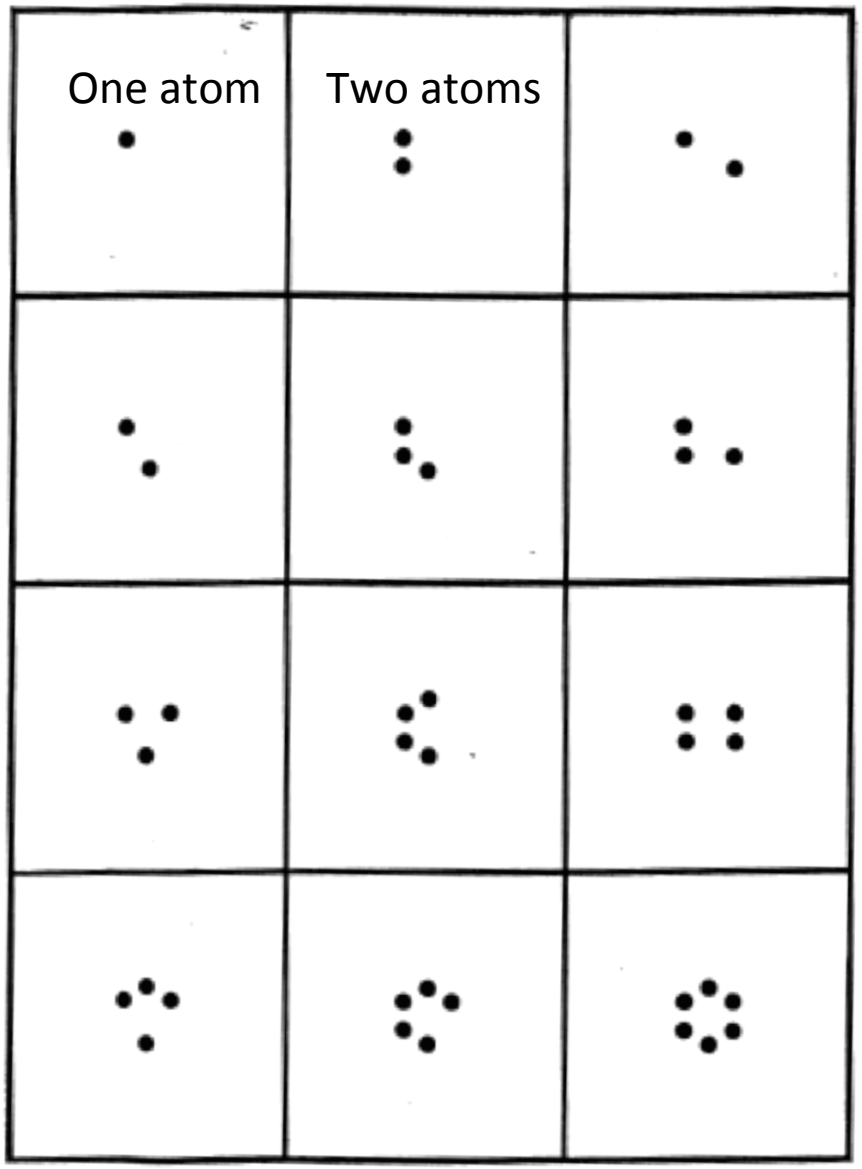
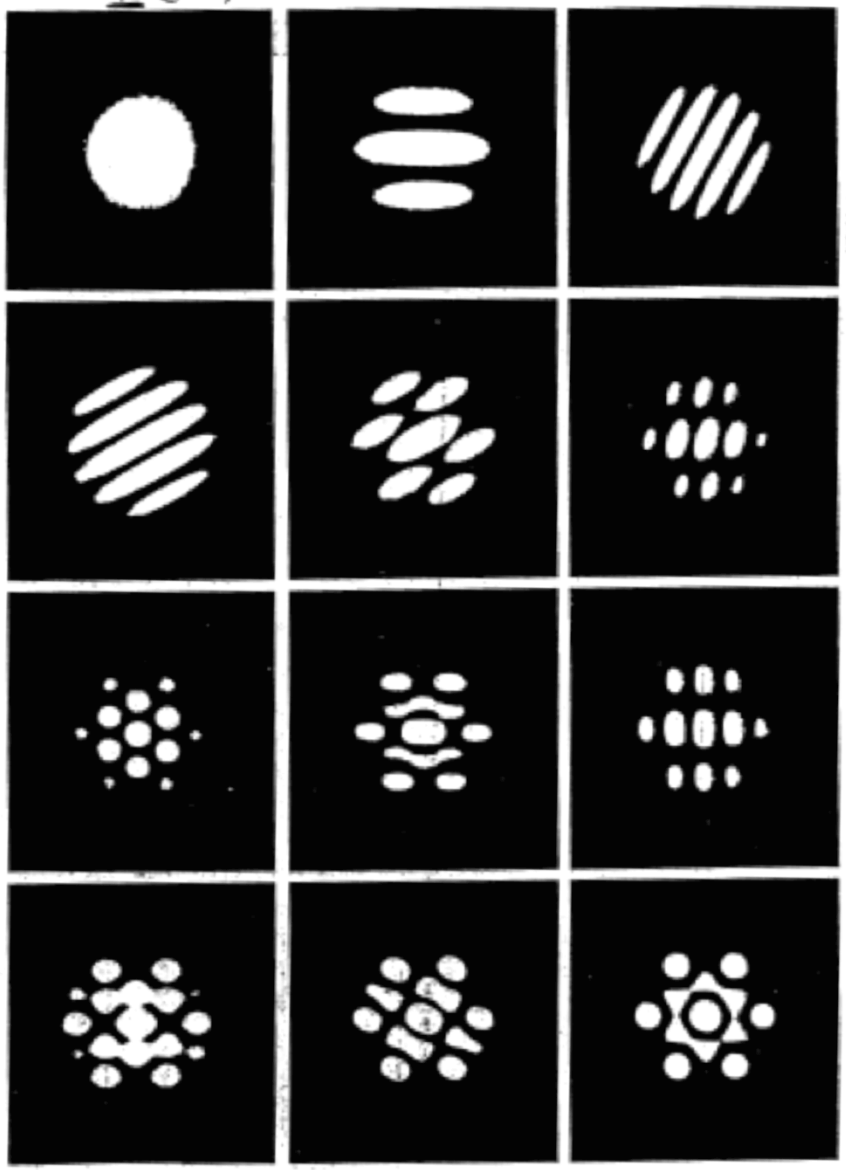


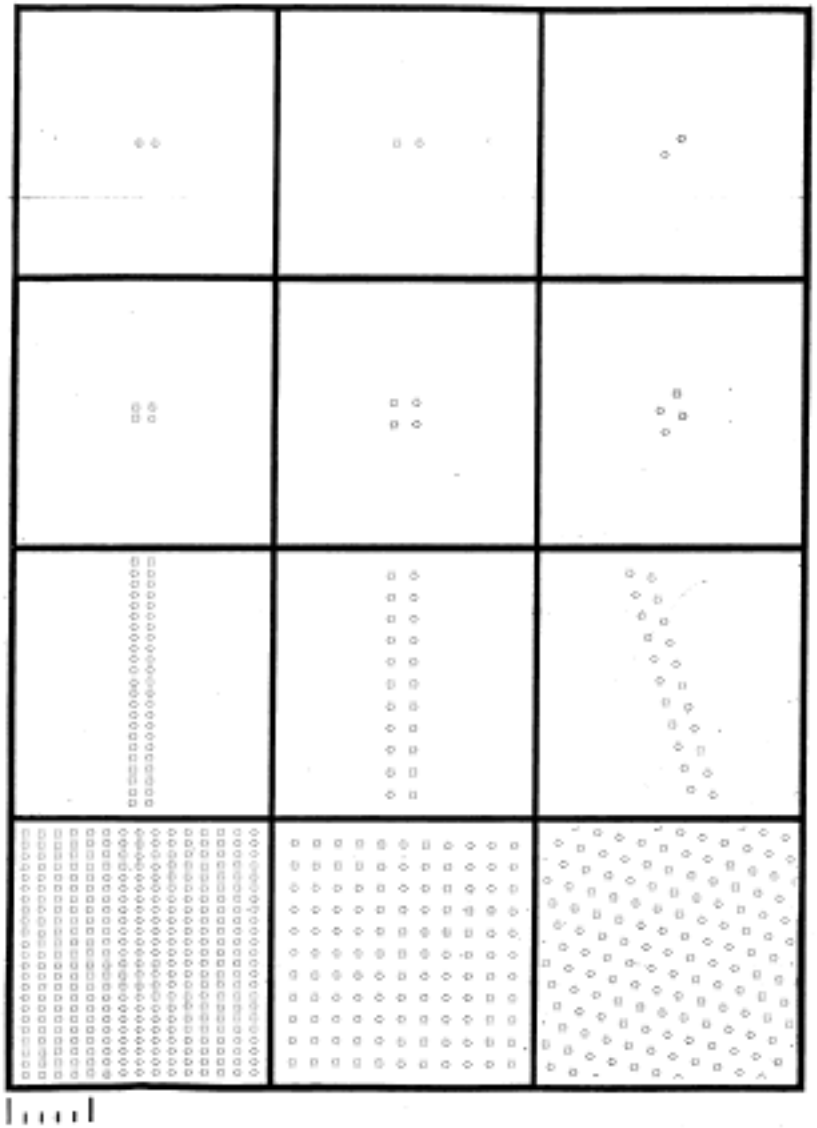
Plate 2



Build a crystal

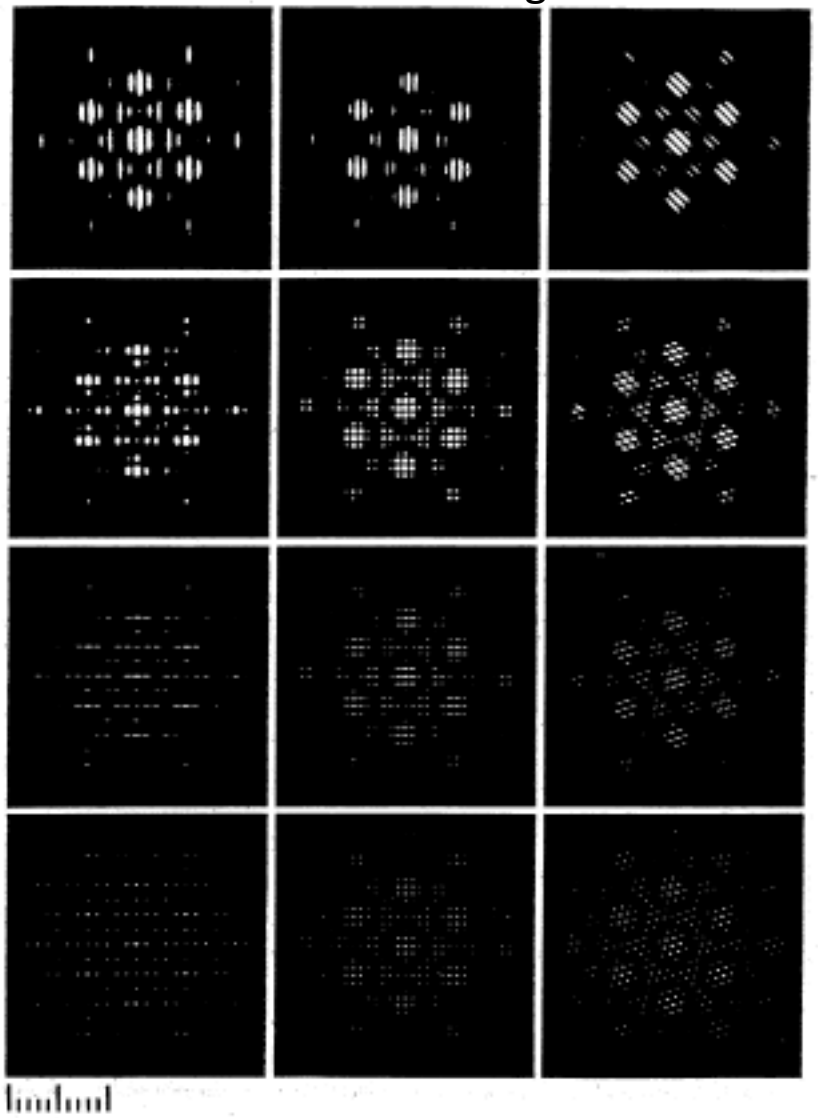
Plate 11

Object



Scattering

Plate 11



$$\underline{F}_{(h,k,l)} = \Sigma_j f_j e^{(2\pi i (hx_j+ky_j+lz_j))}$$

Every X-ray reflection (h,k,l) has a contributing wave from all j atoms .

$$\rho(x,y,z) = \Sigma_{hkl} \quad \underline{F}_{(h,k,l)} e^{(-2\pi i(hx+ky+lz))}$$

or $$\rho(x,y,z) = \Sigma_{hkl} \left| \underline{F}_{(h,k,l)} \right| e^{(-2\pi i(hx+ky+lz) + \phi_{hkl})}$$

Every point in the density map has contributions from every reflection

PROOF OF INVERSE FOURIER TRANSFORM

(7)

Once we measure

Intensity = $|F(\underline{s})|^2$ we calculate $|F(\underline{s})|$

We need to determine $\alpha(\underline{s})$ (it's phase)

We can invert the "observations", to calculate the 'scattering density' map.

For X-rays ≡ electron density map.

by

$$\rho(\underline{r}) = \sum \underline{F}(\underline{s})\, e^{-2\pi i\, \underline{r}_i \cdot \underline{s}} = \int_S \underline{F}(\underline{s})\, e^{-2\pi i\, \underline{r}\cdot\underline{s}}$$

Just as all atoms contribute to each amplitude $\underline{F}(\underline{s})$, so each $F(\underline{s})$ contributes to $\rho(\underline{r})$. called "fourier inversion". Note vectors are underlined.

Proof? postulate true, then apply –

$$\text{for some } \underline{s}' \quad \underline{F}(\underline{s}') = \int_{r=}^{V_r} \left(\int_S \underline{F}(\underline{s})\, e^{-2\pi i\, \underline{r}\cdot\underline{s}}\, d\underline{s} \right) e^{2\pi i\, r s'}\, dV_r$$

$$= \int_r \int_s \underline{F}(\underline{s})\, e^{2\pi i r(\underline{s}' - \underline{s})}\, d\underline{s}\, dV_r$$

This $\int = 0$ unless $\underline{s}' = \underline{s}$, then $= 1$

$$\underline{F}(\underline{s}') = \underline{F}(\underline{s}')$$

QED

Scattering pattern is the Fourier transform of the structure

$$\mathbf{F(\underline{S}) = \Sigma_j\, f_j\, e^{(2\pi i \mathbf{rj}.\mathbf{S})}}$$

Structure is the 'inverse' Fourier transform of the Scattering pattern

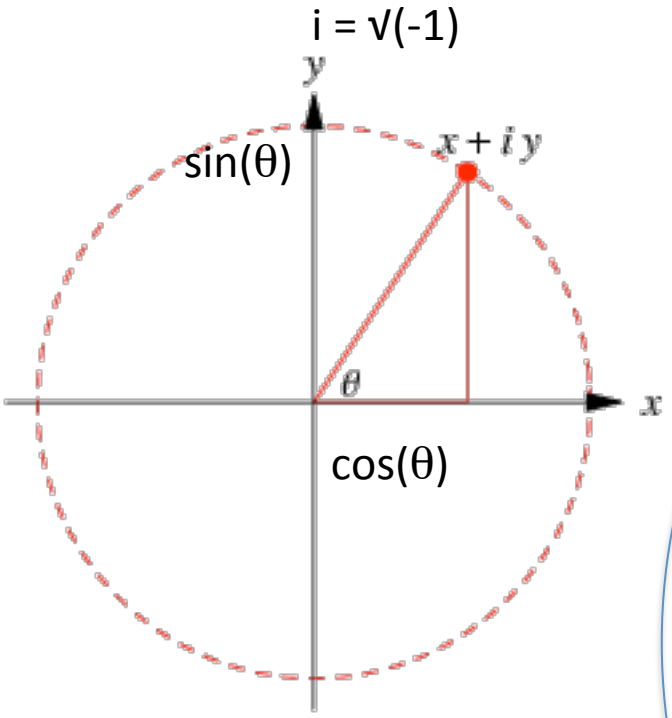
$$\mathbf{\rho(\underline{r}) = \Sigma\, F(\underline{S})\, e^{(-2\pi i \mathbf{r}.\mathbf{S})}}$$

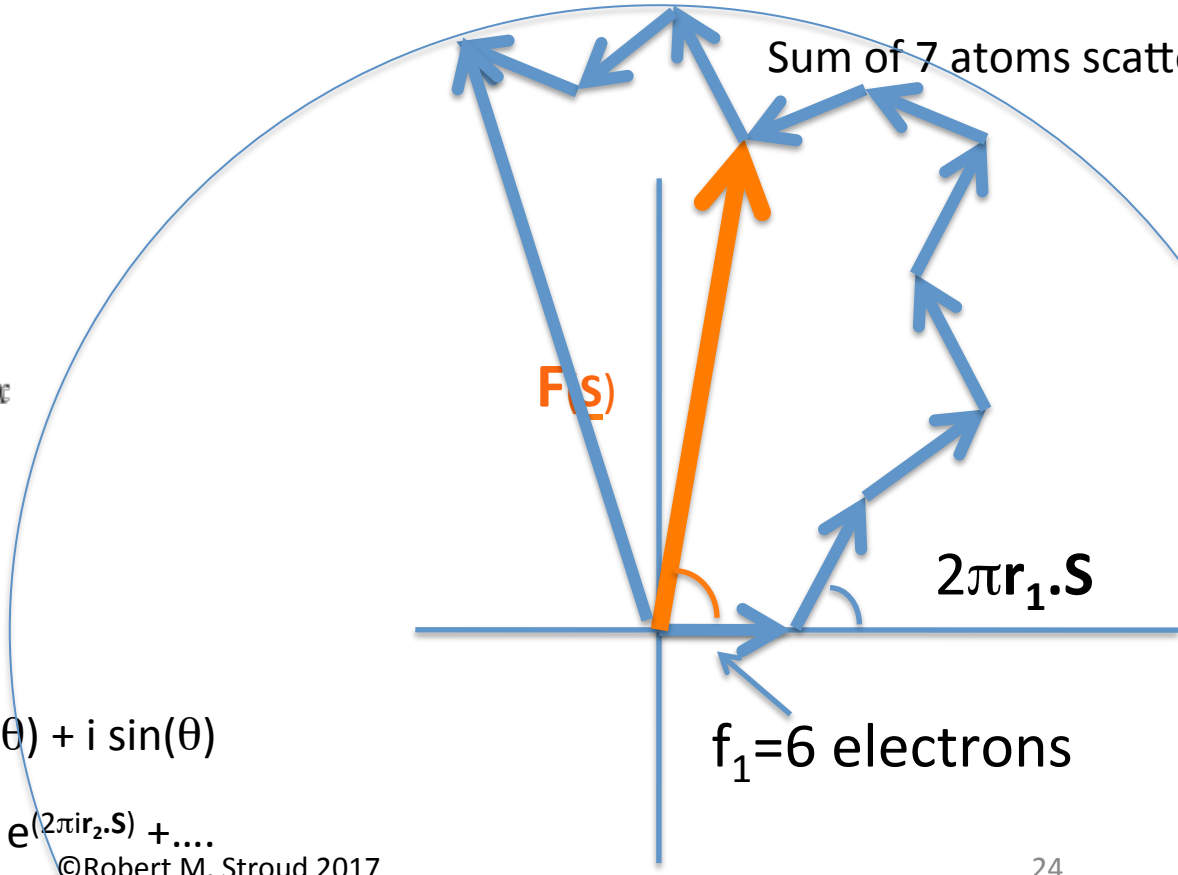
‘Difference maps’
-Define bound ligands
-to find any missing atoms during refinement,
-to find ligands
-define movements of protein or water
-determine ion positions
-determine changes in dynamic motion

Suppose we interpret 7 atoms; but 3 remain to be found in density

Result is a wave of
amplitude $|\mathbf{F}(\underline{\mathbf{S}})|$
phase $\Phi(\underline{\mathbf{S}})$

In reality, maybe 3 atoms are missing.
How to see what is missing?





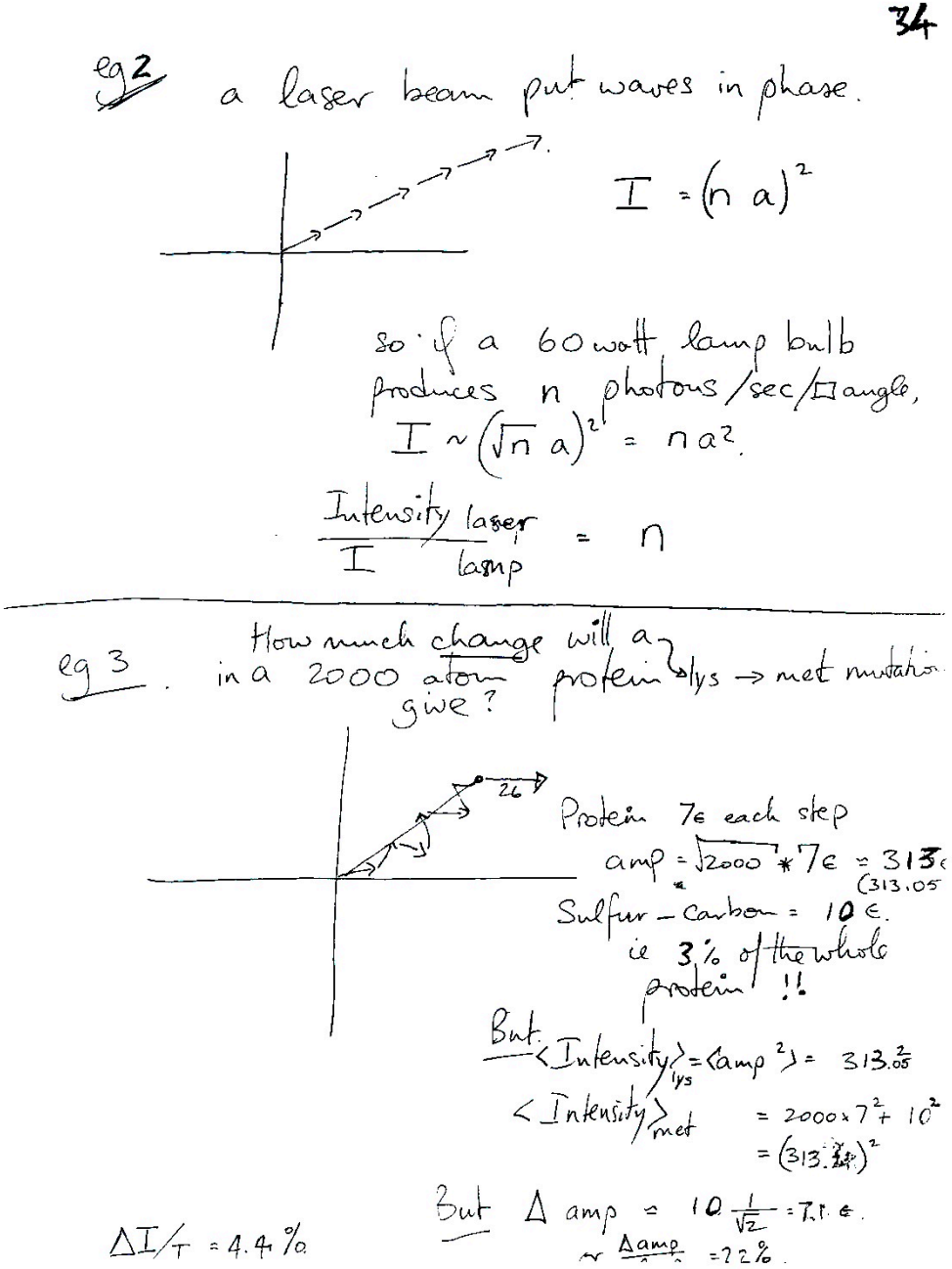
$$e^{(i\theta)} = \cos(\theta) + i \sin(\theta)$$

$$\mathbf{F}(\underline{\mathbf{S}}) = f_1 e^{(2\pi i \mathbf{r}_1.\mathbf{S})} + f_2 e^{(2\pi i \mathbf{r}_2.\mathbf{S})} + \ldots$$

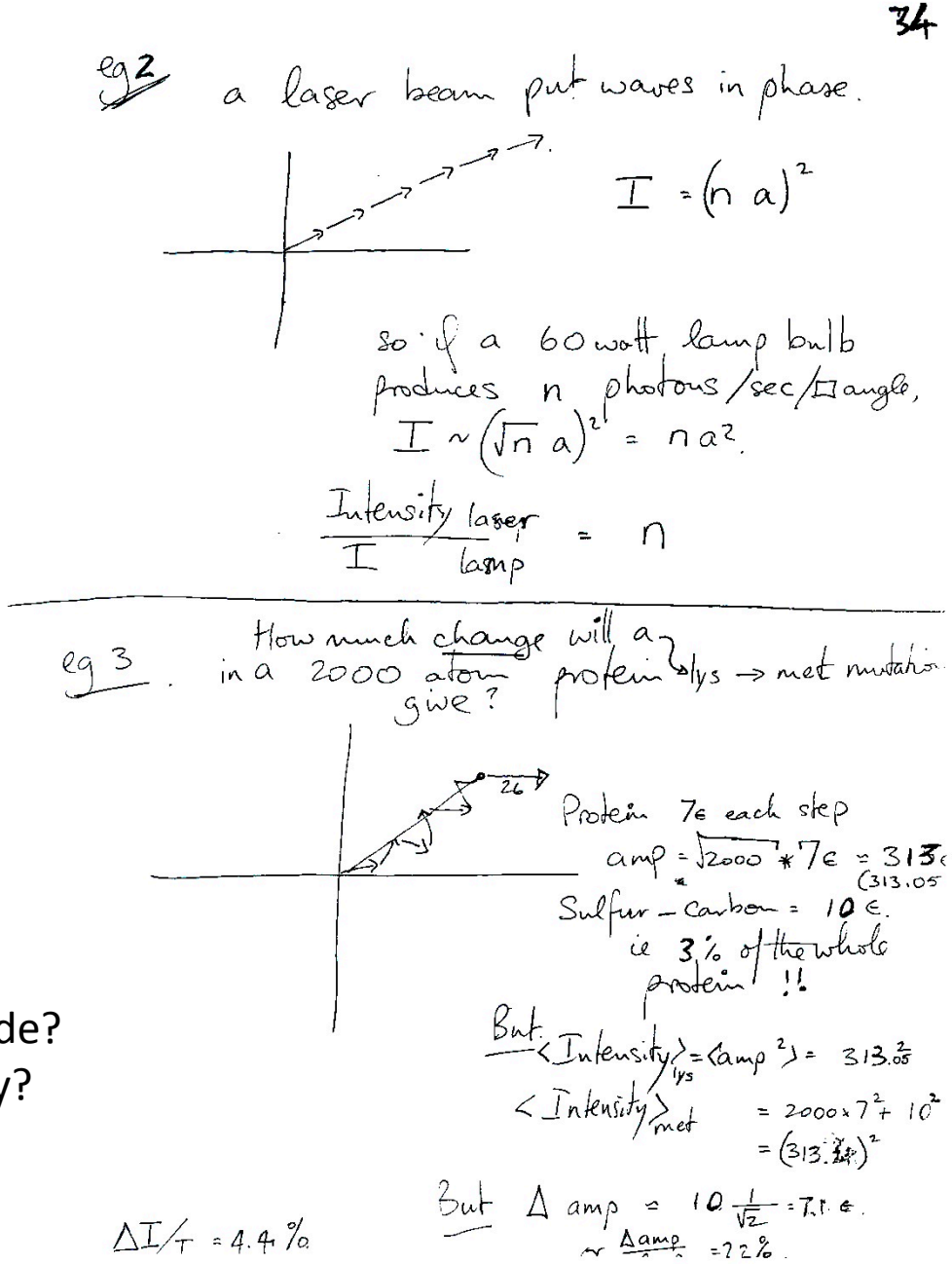
and why do we care?

How much difference will it make to the average intensity?
average amplitude?

if we add a single Hg atom?



and why do we care?



How much difference will 10 electrons make to the average intensity? 98,000 e^2
average amplitude? 313

average difference in amplitude?
average difference in intensity?

if we add a single Hg atom?

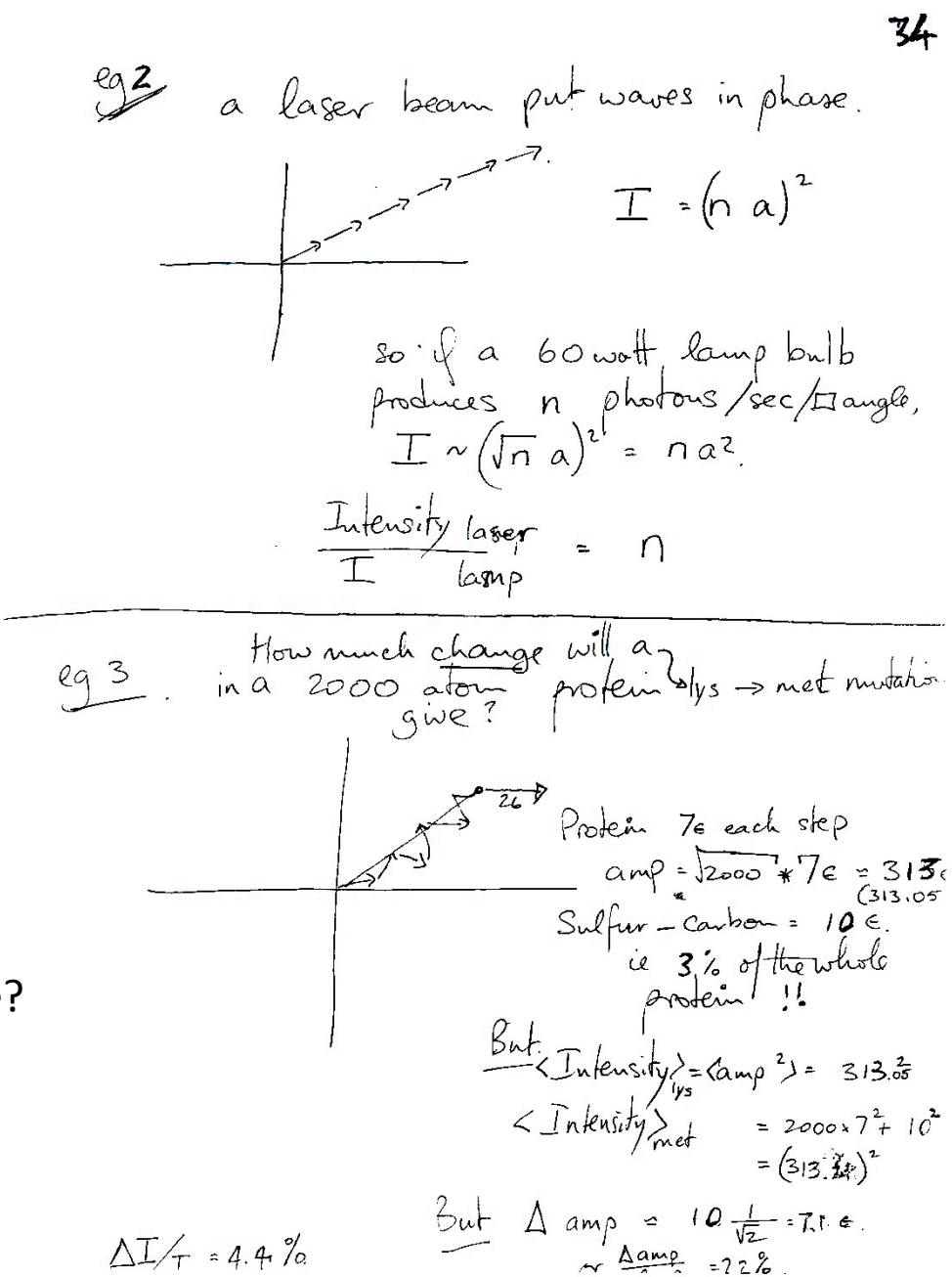
and why do we care?

or Hg atom n=80e

How much difference will 80 electrons make to the average intensity? 98,000 e^2
average amplitude? 313 e

average difference in amplitude?
18 % of each amplitude!
36% of each Intensity

difference in 'intensity?'
104,400-98000=6400 (6.5%)



USES: 2. Add a substrate, Grow a new crystal

Measure New $|\mathbf{F}(\underline{\mathbf{S}})|_{obs+substrate}$ Compare with the apo-protein.

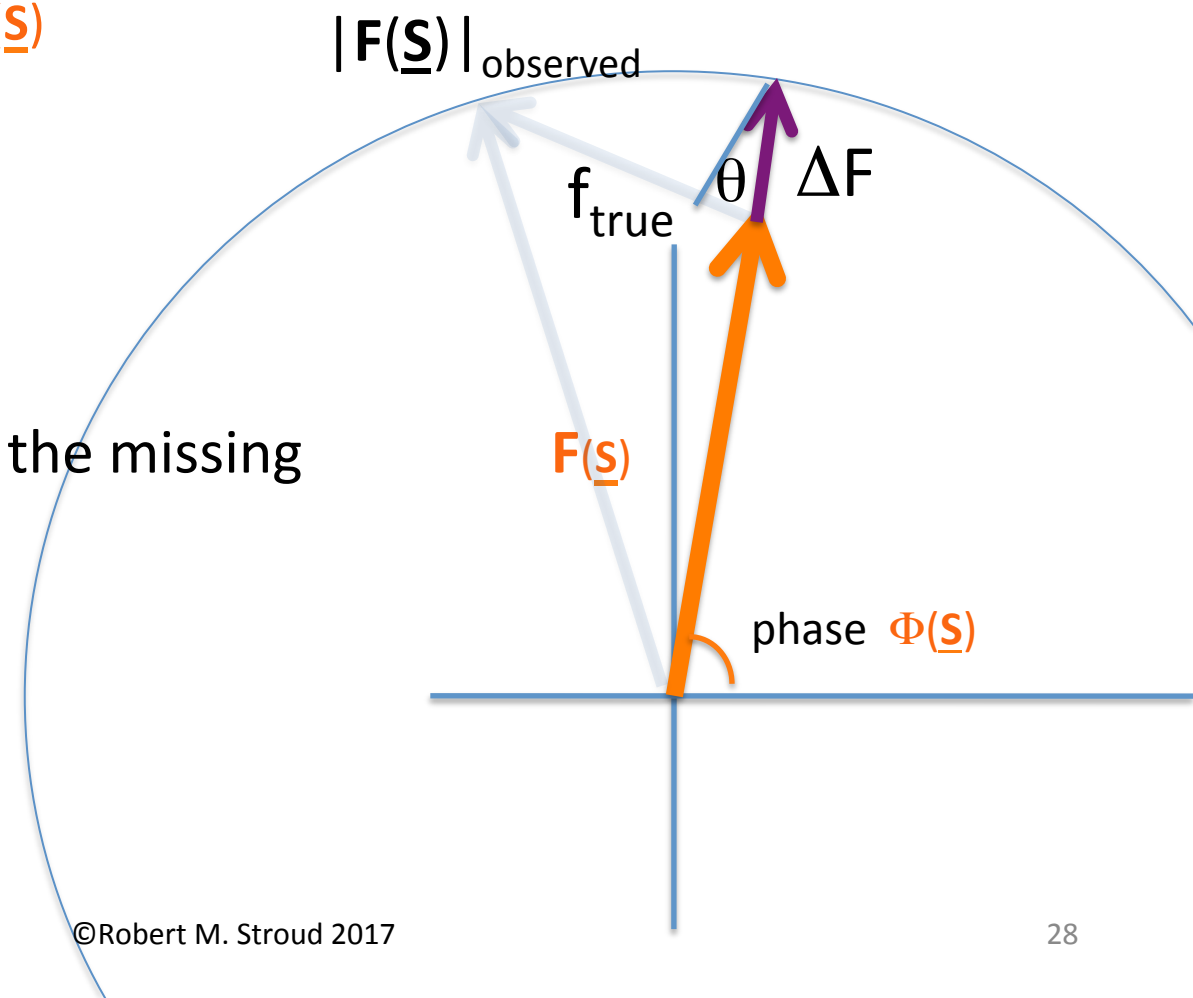
Transform $\Delta F = \left| |\mathbf{F}(\underline{\mathbf{S}})|_{obs+substrate} - |\mathbf{F}(\underline{\mathbf{S}})|_{obs} \right| \Phi(\underline{S})$

or

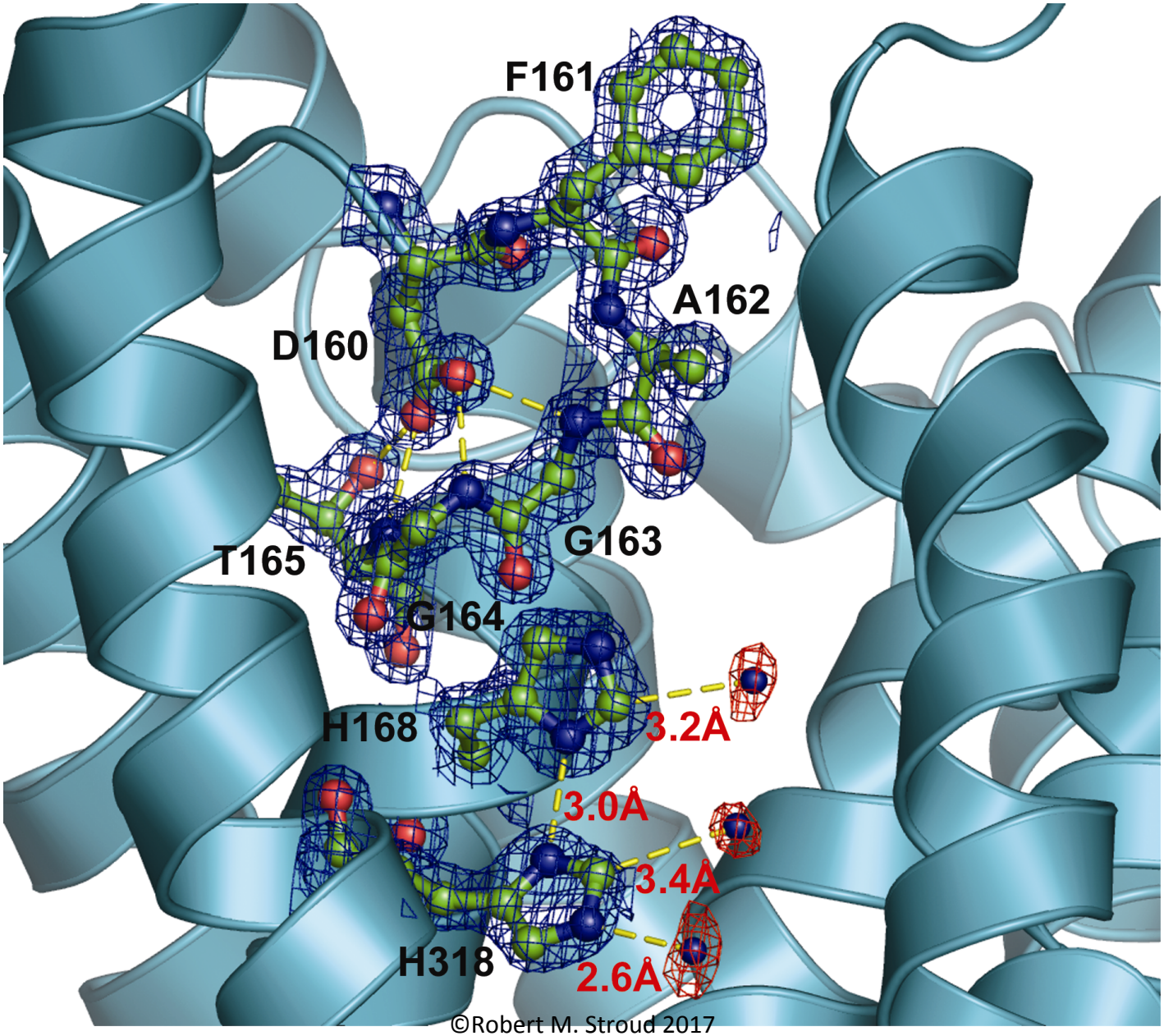
$[2|\mathbf{F}(\underline{\mathbf{S}})|_{obs+substrate} - |\mathbf{F}(\underline{\mathbf{S}})|_{obs}] \Phi(\underline{S})$

= a '$2F_0$-F_o map'

It is **unbiased** as to where the missing substrate is.



A Dfference map shows 1/3 occupied NH3 sites and the role of D160 at 1.35Å Resolution. Here are 0.3 NH_3 peaks!



Khademi..Stoud 2003

Fo-Fc maps identify everything ordered that is 'missing'

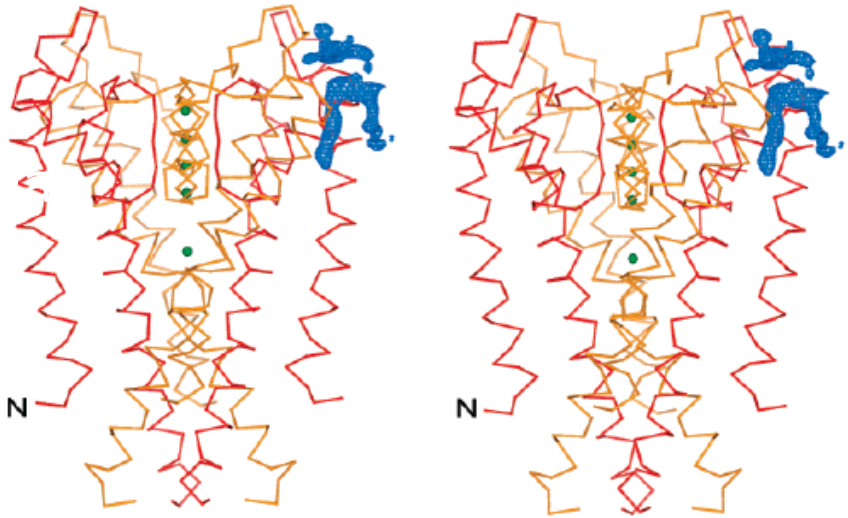


FIGURE 1: Lipid molecules in KcsA crystals. A stereoview of the KcsA structure with electron density corresponding to the lipid molecule. The backbone of KcsA is shown as a red and yellow trace. Green spheres represent potassium ion binding sites. The $F_o - F_c$ map (contoured at 3σ) was calculated using a model that does not contain lipid molecules. For clarity, density corresponding to only one of the lipid molecules is shown. The KcsA monomer consists of an N-terminal outer helix, a central pore helix, and a C-terminal inner helix. This figure was prepared with MOLSCRIPT (*31*) and Raster3D (*32*).

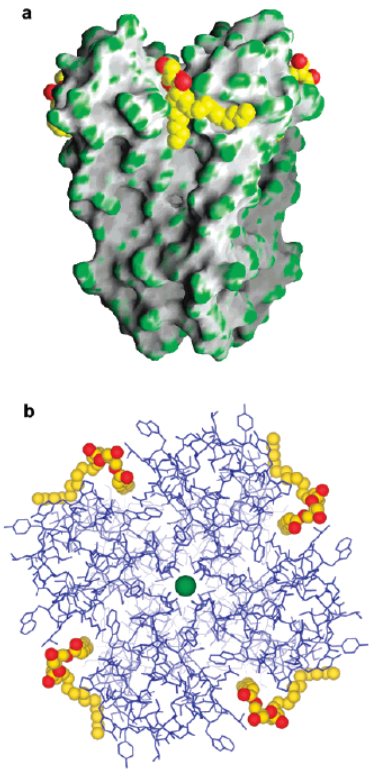


FIGURE 3: Structural analysis of lipid binding to KcsA. (a) Binding surface of the lipid molecule. The surface of KcsA is colored according to curvature (green, convex; gray, concave). The lipid molecule, built as 1,2-diacylglycerol, is shown in CPK representation with oxygen atoms colored red and carbon atoms colored yellow. (b) Lipid-binding site viewed from the extracellular side along the 4-fold axis of KcsA. The channel is colored blue. The green sphere represents the potassium ion. The lipid molecule is in CPK representation colored as in panel a. Panel a was prepared with GRASP (*33*). Panel b was prepared with MOLSCRIPT (*31*) and Raster3D (*32*).

-Eliminate Bias
-Half electron content
-See electrons

The closer you get –the lower the noise.
Can see single electrons.

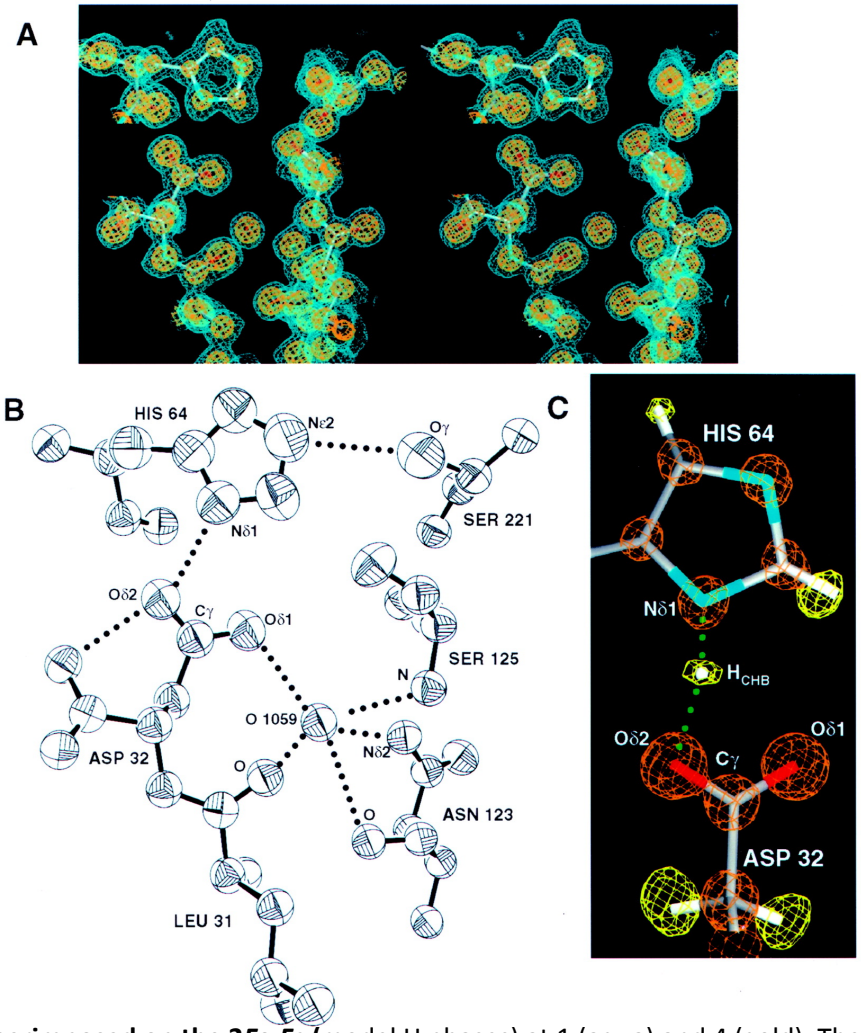
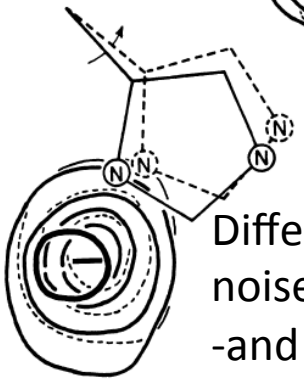


Figure 3 The catalytic triad. **(A) Stereoview displaying Model H superimposed on the 2*Fo Fc* (**model H phases) at 1 (aqua) and 4 (gold). The densities for C and N in His 64 are weaker than in Asp 32. The Asp 32 CO2 bond at 4 is continuous, while the density for the C and O1 are resolved. (B) Schematic of the catalytic residues and hydrogen bonded neighbors with thermal ellipsoid representation countered at 50% probability (*29*). Catalytic triad residues Ser 221 and His 64 show larger thermal motion than the Asp 32. Solvent O1059 appears to be a relatively rigid and integral part of the enzyme structure. (C) Catalytic hydrogen bond (CHB)**. A *Fo Fc* (model H phases) difference map** contoured at +2.5 (yellow) and 2.5 (red) and a 2*Fo Fc* (model H phases) electron density map contoured at 4 (gold). The position of the short hydrogen atom (labeled HCHB) in the CHB is positioned in the positive electron density present between His 64 N1 and Asp 32 O2.

Published in: Peter Kuhn; Mark Knapp; S. Michael Soltis; Grant Ganshaw; Michael Thoene; Richard Bott; *Biochemistry* **1998,** 37, 13446-13452.
DOI: 10.1021/bi9813983

Seeing small shifts
-down to ±0.1Å



Difference Map
noise ~20% of noise in the parent protein
-and only two peaks!

FIG. 7. The peaks associated with His57 on the difference map. The lower peak is negative density (−) while the other one is positive (+). The latter peak is a composite with a solvent molecule density (see text).

FIG. 7. The peaks associated with His57 on the difference map. The lower peak is negative density (−) while the other one is positive (+). The latter peak is a composite with a solvent molecule density (see text).

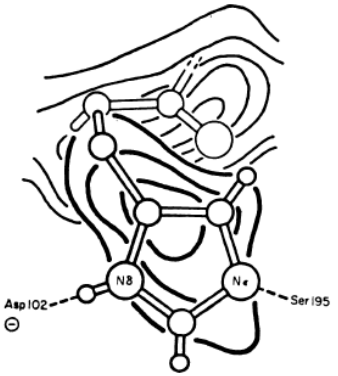


FIG. 8. Electron density for His57 in the DIP-trypsin Fourier map, computed for the plane parallel to the imidazole ring.

TABLE 1

Analysis of Fourier maps

Map	$\langle\lvert F_{obs}\rvert\rangle$ (e)	σ (e)	Calculated† $\langle\Delta\rho^2\rangle^{\frac{1}{2}}$ (e Å^{-3})	Observed‡ r.m.s. error (e Å^{-3})	Observed highest noise (e Å^{-3})	S.D.§	Observed highest peak (e Å^{-3})	S.D.
BA-trypsin—DIP-trypsin	84·7	2·3	0·069	0·059	0·17	2·5	0·75	11
DIP-trypsin	573·0	21·0	0·38	—	—	—	—	—

† ΔF: $\langle\Delta\rho^2\rangle = \frac{1}{2V^2}\sum_{hkl}\Delta F^2\,(2-m^2)$,

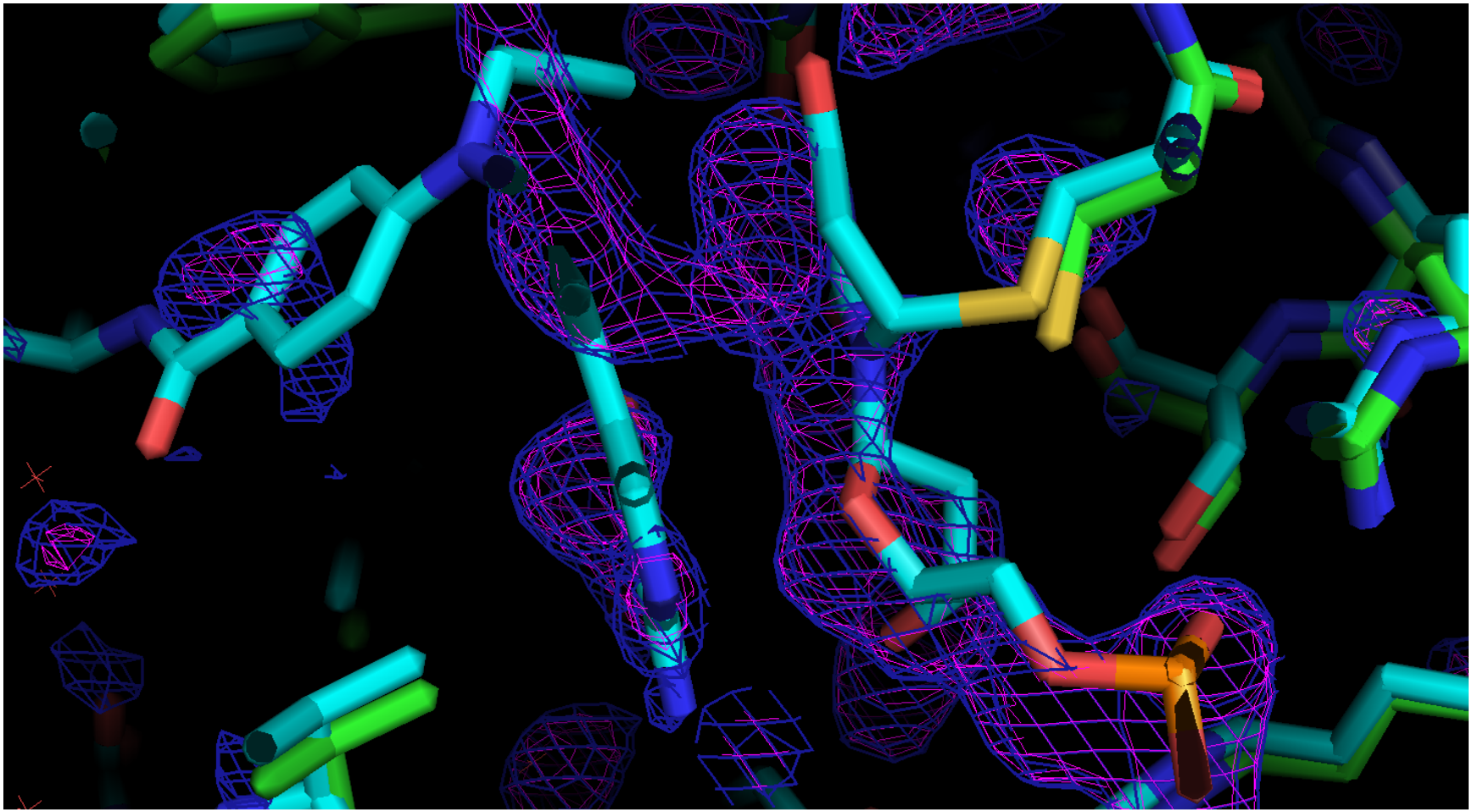
F_{DIPT}: $\langle\Delta\rho^2\rangle = \frac{1}{V^2}\sum_{hkl}F^2_{DIPT}\,(1-m^2)$,

(after Henderson & Moffat, 1971).

‡ The observed root mean-square density error is based on a relatively featureless region of the map.

§ S.D., the electron density given as a multiple of the calculated r.m.s. error.

Fo-Fc density at 2.5 and 2.0 σ
Green = IntB analog complex,
Cyan =dUMP-CB3717 complex



Relative Information in Intensities versus phases

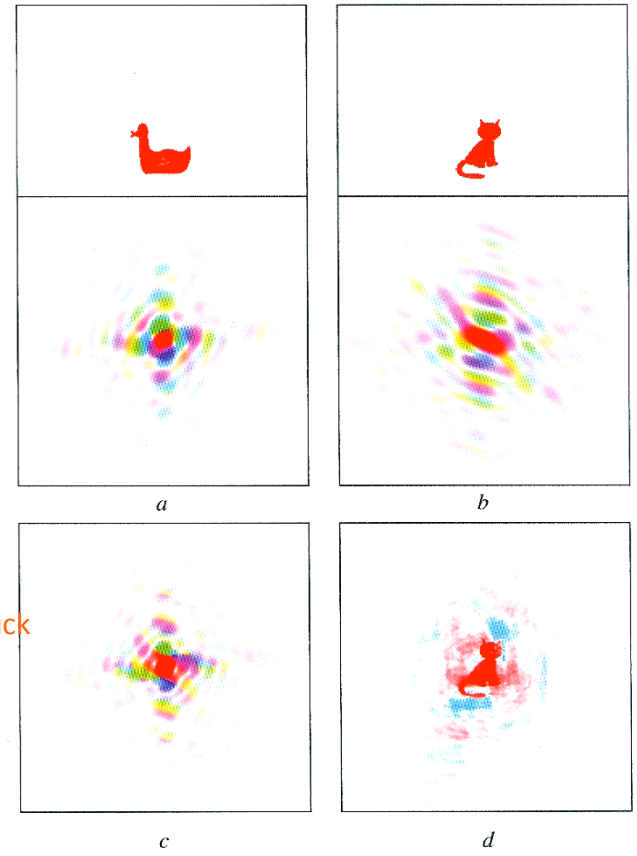
$\rho(\underline{r})$ duck

$\rho(\underline{r})$ cat

$\underline{F}_{(\underline{S})}$ duck → $|F_{(\underline{S})}|$, $\phi_{(\underline{s})}$

$\underline{F}_{(\underline{S})}$ cat

$$\underline{F}_{(\underline{S})} = \Sigma_j f_j e^{(2\pi i \mathbf{rj.S})}$$



$|F_{(\underline{S})}|$ duck, $\phi_{(\underline{s})}$ cat

$$\rho(\underline{r}) = \Sigma \underline{F}_{(\underline{S})} e^{(-2\pi i \mathbf{r.S})}$$

Looks like a .....

Figure 6.1 ► Relative amounts of information contained in reflection intensities and phases. (*a*) and (*b*) Duck and cat, along with their Fourier transforms. (*c*) Intensity (shading) of the duck transform, combined with the phases (colors) of the cat transform. (*d*) Back-transform of (*c*) produces recognizable image of cat, but not duck. Phases contain more information than intensities. Figure generously provided by Dr. Kevin Cowtan.

Relative Information in Intensities versus phases

$\rho(\underline{r})$ duck

$\rho(\underline{r})$ cat

$\underline{F}_{(\underline{S})}$ duck → $|F_{(\underline{S})}|$, $\phi_{(\underline{s})}$

$\underline{F}_{(\underline{S})}$ cat

$\underline{F}_{(\underline{S})} = \Sigma_j f_j e^{(2\pi i \mathbf{rj.S})}$

$|F_{(\underline{S})}|$ duck, $\phi_{(\underline{s})}$ cat

$\rho(\underline{r}) = \Sigma \underline{F}_{(\underline{S})} e^{(-2\pi i \mathbf{r.S})}$

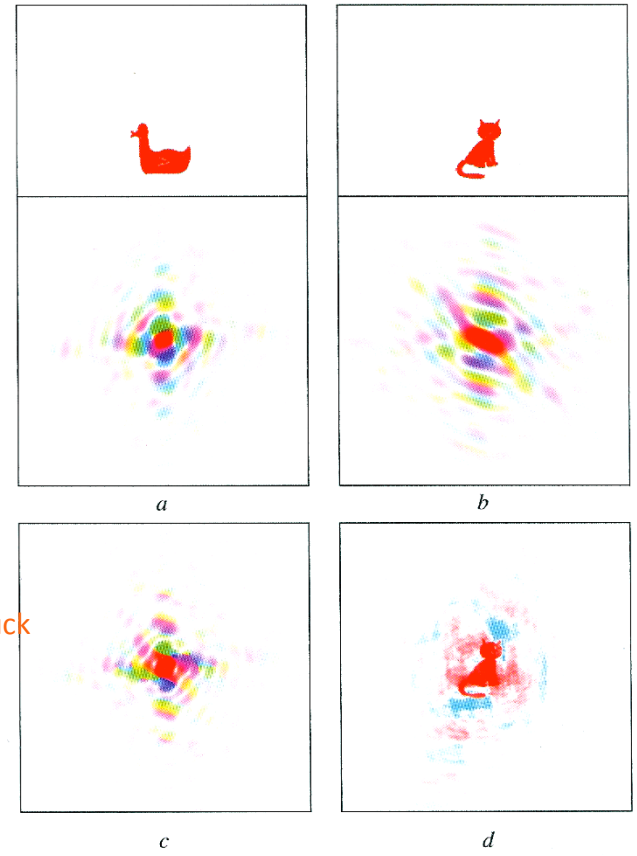


Figure 6.1 ▶ Relative amounts of information contained in reflection intensities and phases. (*a*) and (*b*) Duck and cat, along with their Fourier transforms. (*c*) Intensity (shading) of the duck transform, combined with the phases (colors) of the cat transform. (*d*) Back-transform of (*c*) produces recognizable image of cat, but not duck. Phases contain more information than intensities. Figure generously provided by Dr. Kevin Cowtan.

Looks like a CAT

PHASES DOMINATE:

-Incorrect phases = incorrect structure

-incorrect model = incorrect structure

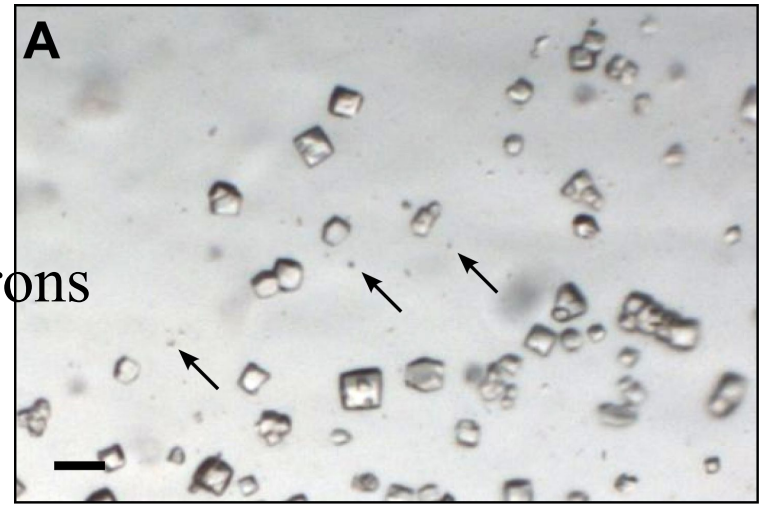
-incorrect assumption = incorrect structure

Electron Diffraction

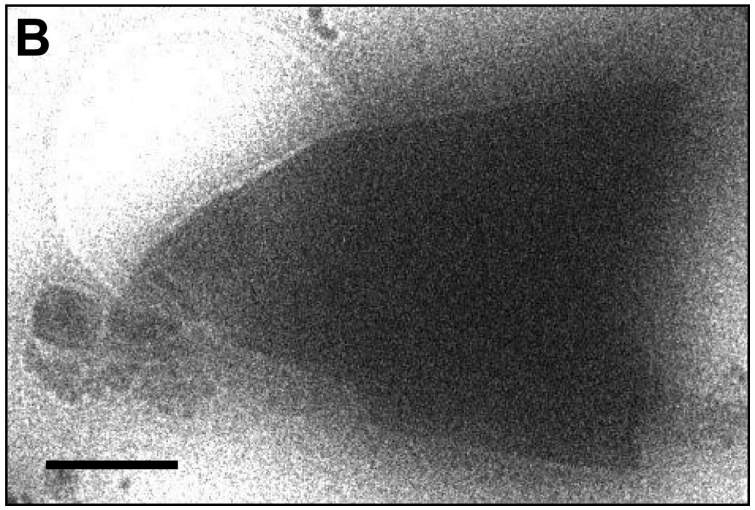
- -Electrons are scattered more than X-rays:
  - Every diffracted beam is a new incident ray!!
- -from Electric fields in atoms
  - Scatter goes up as square root of atom number
- Absorption is high, - so very thin crystals only!
  - 100Å -5000Å
- Phasing? Molecular replacement? Guess?
  - because Heavy metals are less different.
- Tilting specimen to 'scan' diffraction

Images of lysozyme microrystals.(A) Light micrograph showing lysozyme microcrystals (three examples indicated by arrows) in comparison with larger crystals of the size normally used for X-ray crystallography.

Bar =50 microns

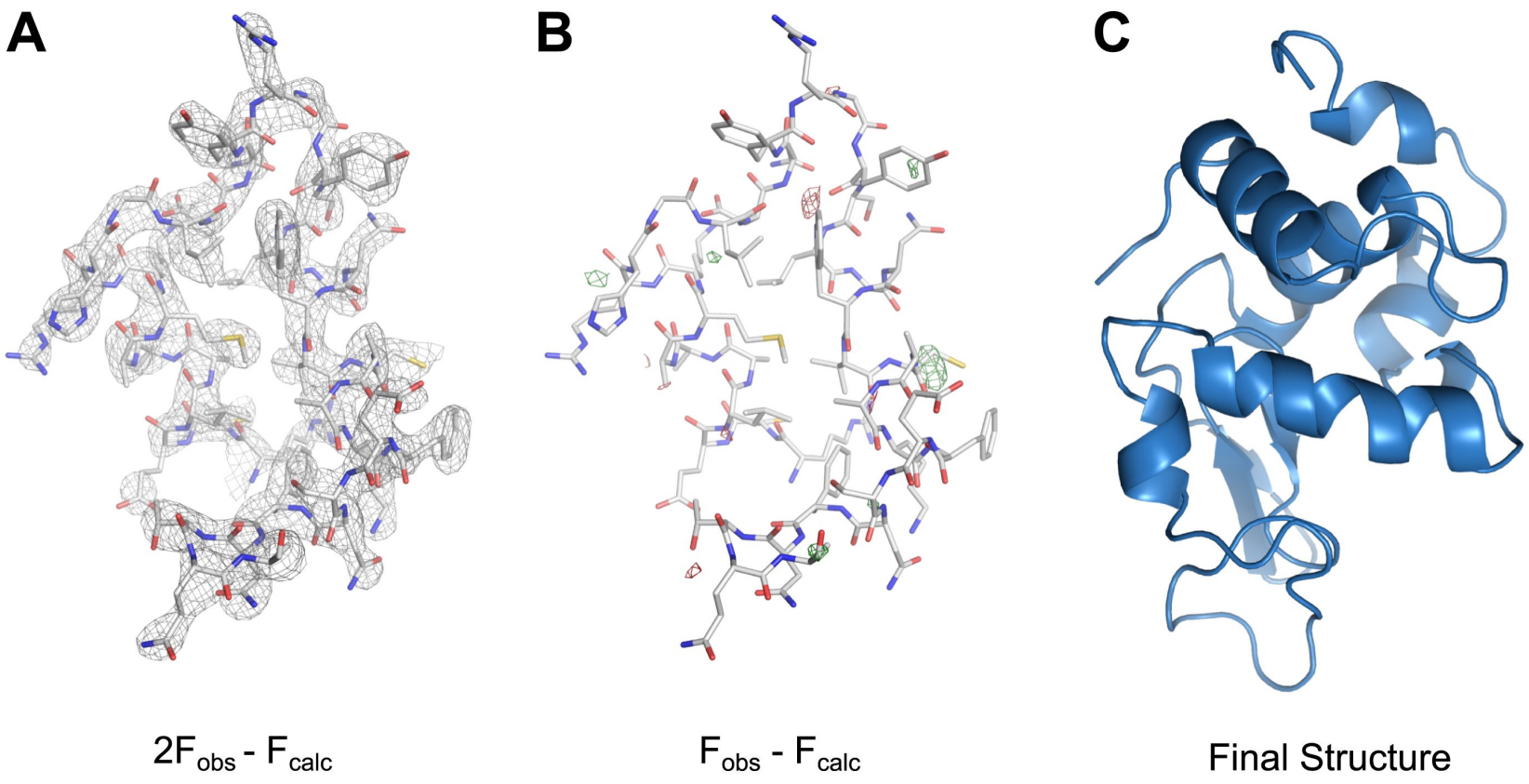


Bar = 1 micron
crystal thickness
0.5-1 micron
ie. 5000Å



Dan Shi et al. eLife Sciences 2013;2:e01345

MicroED structure of lysozyme at 2.9 Å resolution.(A) The 2Fobs−Fcalc (contoured at 1.5σ) map covers protein residues 5–45 of lysozyme.



Dan Shi et al. eLife Sciences 2013;2:e01345

Supplementary Proof of the ‘Random Walk’ calculation

The 'Random Walk' problem? (p33.1-33.3)

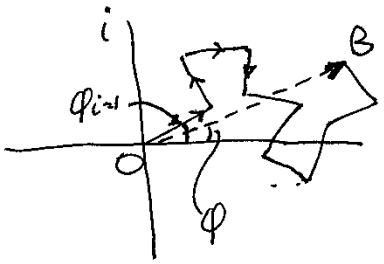
What is the average sum of
n steps in random directions?

(What is the average amplitude
<|F(s)|> from an n atom structure?)

-AND why do we care?!.....

How much difference from adding a
mercury atom (f=80).

"Random Walk", in 2d - -
(What is scattering amplitude from
n atoms, each with f electrons)



n steps, of length f
relatively randomly
different angles.
ie. Probability
$P(\varphi)$

Easiest to consider as the expected value
maximum for $|OB|^2$ (rather than OB)

$$|OB|^2 = \underline{OB} \cdot \underline{OB}^*$$

[Review: $\underline{OB} = |OB|(\cos(\varphi) + i\sin\varphi)$
$\underline{OB}^* = |OB|(\cos(\varphi) - i\sin\varphi)$

so $\underline{OB} \cdot \underline{OB}^* = |OB|^2(\cos^2\varphi + \sin^2\varphi) = |OB|^2$]

So $\underline{OB} = \sum_{i=1}^{n} f\, e^{2\pi i \varphi_i}$

$\underline{OB}^* = \sum_{i=1}^{n} f\, e^{-2\pi i \varphi_i}$

The average intensity for an n atom structure, each of f electrons is $\langle I \rangle = nf^2$

The average amplitude is Square root of n, times f

$$|OB|^2 = \left(\sum_{i=1}^{n} e^{2\pi i \varphi_i}\right)\left(\sum_{j=1}^{n} e^{-2\pi i \varphi_j}\right) \cdot f^2$$

$$= \sum_i \sum_j e^{2\pi i(\varphi_i - \varphi_j)} \cdot f^2$$

since $P(\varphi_i) = P(\varphi_j)$ = constant (all equally probable)

the sums..

- for $i \neq j$ $P(\varphi_i)$ all equally probable

$$\text{so } \sum_i \sum_j \Rightarrow \int_0^{2\pi} \cos 2\pi(\varphi_i - \varphi_j) + i \sin 2\pi(\varphi_i - \varphi_j)$$

$$\cos \to 0 \qquad i \sin \to 0$$

- for $i = j$ $e^{2\pi i(\varphi_i - \varphi_j)} = e^{2\pi i 0} = 1$

$$\langle |OB|^2 \rangle = nf^2$$

$$OB = \sqrt{n} f \quad \text{---------- (1)}$$