X-ray Crystallography Lectures

1. Biological imaging by X-ray diffraction. An overview.
2. Crystals. Growth, physical properties and diffraction.
3. Working in reciprocal space. X-ray intensity data collection and analysis.
4. Crystallographic phasing. Molecular replacement, isomorphous replacement, multiwavelength methods.
5. Crystals won't grow? Don't despair, try small angle x-ray scattering.

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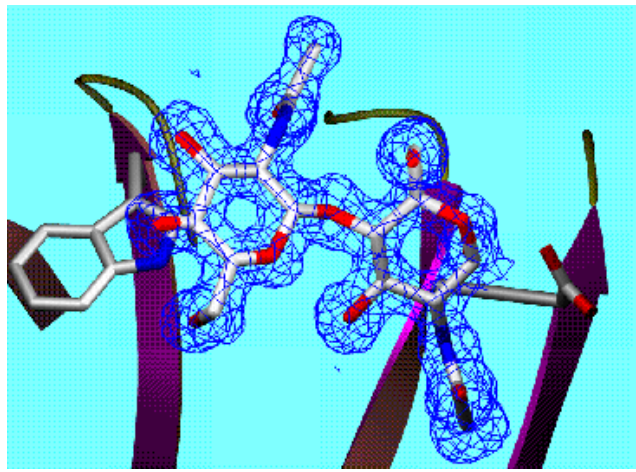
Bio5325, Fall 2006

Biological Imaging by X-ray Diffraction

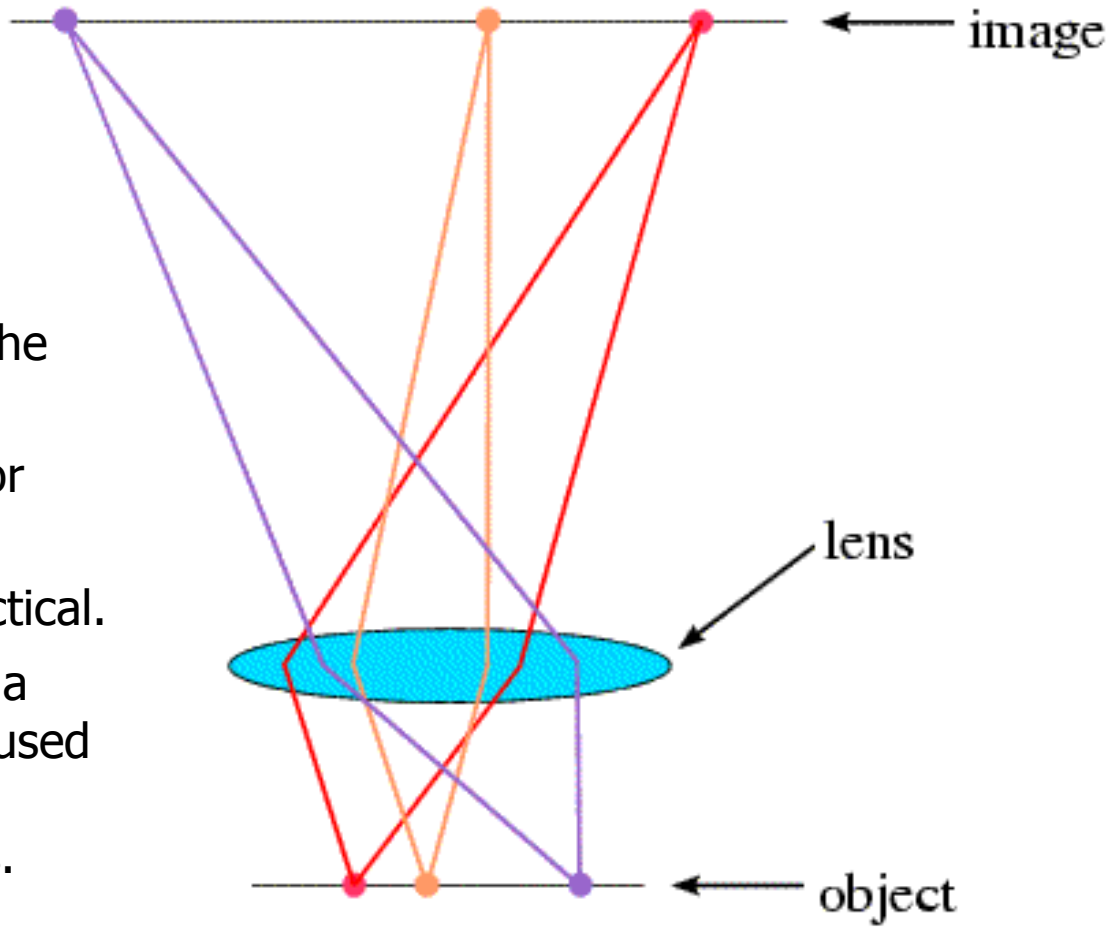
- Use of x-rays and crystals for high resolution imaging
- X-ray data collection, processing, and interpretation
- Basic principles of x-ray scattering
- The Fourier transform
- Growing crystals
- Resources for further study



materials from - http://www-structmed.cimr.cam.ac.uk/course.html and other sources

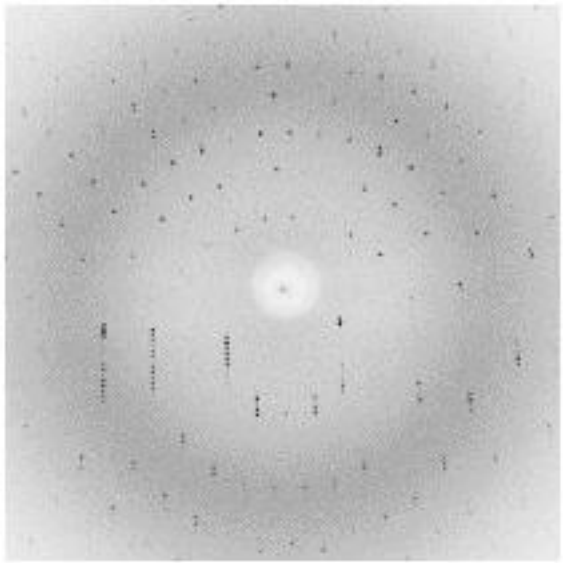
Optical Microscopy

- Resolution is limited by the diffraction limit.
- X-rays are appropriate for atomic-scale resolution.
- X-ray lenses are not practical.
- The Fourier transform is a mathematical operation used in lieu of a lens for x-ray crystallographic analyses.

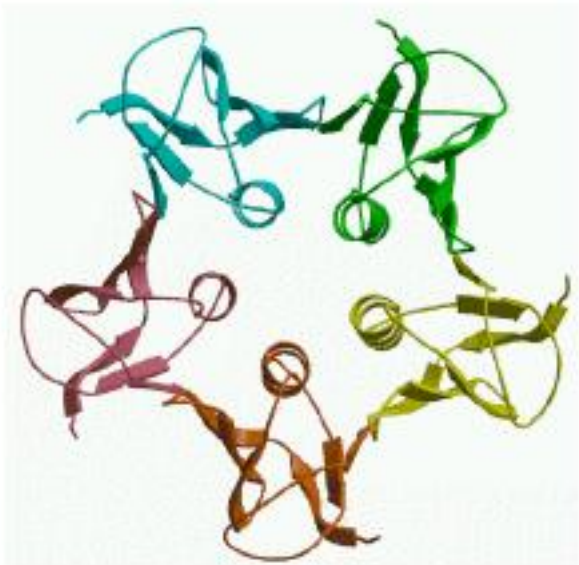


Steps of X-ray Crystallographic Structure Determination

sample



data collection



model building

X-ray Sources

Cu Kα radiation

- Cathode ray tube (at the dentist).
- Rotating anode generator (in the laboratory).
- Monochromatic radiation; λ = 1.5418 Å produced by accelerating electrons at a copper target.

Synchrotron radiation (brightness, coherence, low crossfire)

- Particle storage ring (electrons, positrons).
- Bending magnet or insertion device to harvest EM radiation (x-rays).
- Si crystal monochrometer to select x-ray energy.

X-ray Crystallography

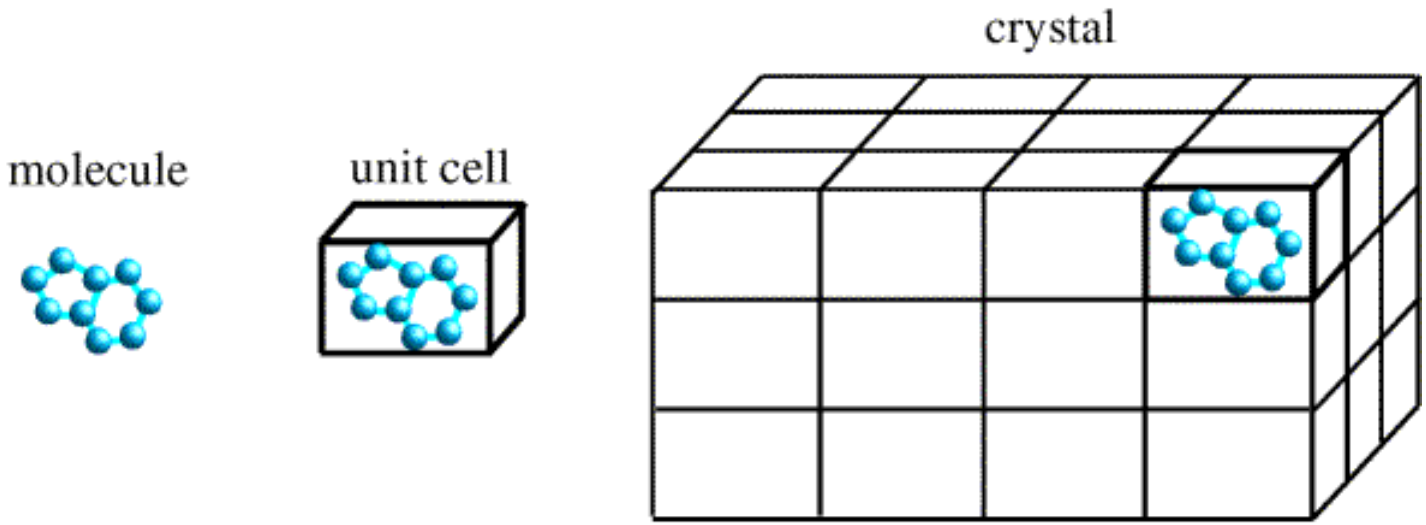
Electron Density

- X-rays are scattered by the electrons of the sample.
- X-ray imaging reveals the time-averaged distribution of electrons in a molecule, or the "electron density."
- The sharpness of features in the electron density, and our certainty about the positions of atoms, depends on the resolution of the x-ray experiment.

Crystals Are Required

- The intensity of x-ray scattering by a single molecule is unimaginably weak.
- A typical protein crystal (0.2 mm cube) aligns ~10^{15} molecules so they scatter x-rays in phase (constructive interference).
- Crystals of biological samples have imperfections (disorder) that limit the resolution of the diffraction measurements.

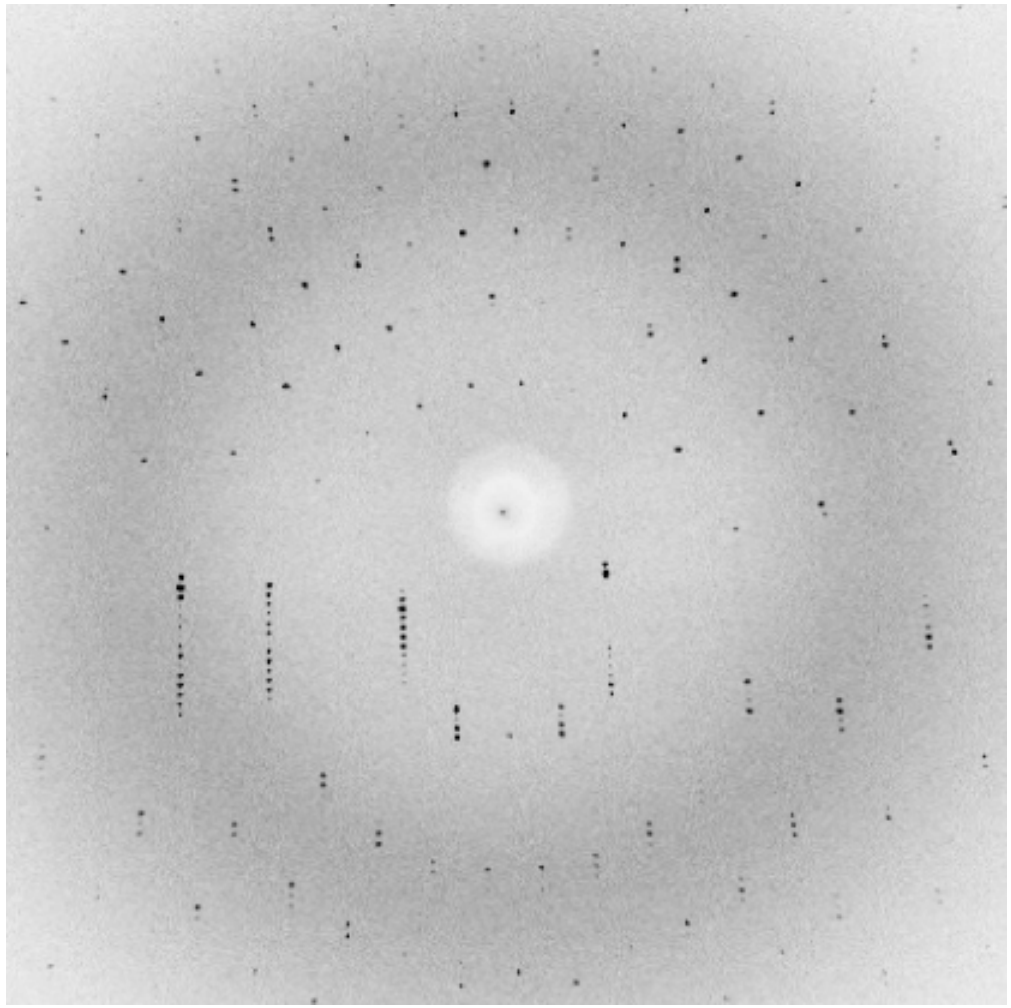
A Crystal is an Amplifier of X-ray Scattering



X-ray Diffraction from Crystals

- Diffracted x-rays are emitted from collisions with electrons.
- The x-ray waves can add up in phase (constructively) or out of phase (destructively), depending on the orientation of molecules with respect to the incoming and outgoing waves.
- The molecules within a crystal are aligned so that their (continuous) diffraction patterns are in phase only at discrete positions that depend on the internal dimensions and symmetry of the crystals.
- The resulting x-ray diffraction pattern is recorded on a 2D detector as discrete data points known as "reflections." We can consider the diffraction pattern to arise from x-rays "reflecting" off of discrete planes (Bragg planes) within the crystal. These planes consisting of equivalent atoms of the structure aligned with the incident x-ray beam.
- The positions of x-ray reflections depend upon crystal parameters and the wavelength of the incident x-rays. The relative intensities of the reflections contain information about the structure of the molecule.

The Oscillation Method



Fourier Transforms and the Phase Problem

- The x-ray diffraction pattern is related to the scattering object by a mathematical operation known as a Fourier transform.

$$\mathbf{F}(\mathbf{s}) = \int_{space} \rho(\mathbf{r}) \exp(2\pi i \mathbf{s} \cdot \mathbf{r}) d\mathbf{r}$$

- The objective lens of a light microscope performs the same function as the Fourier transform used in x-ray crystallography.
- Fourier transforms can be inverted – the Fourier transform of the diffraction pattern will reveal the structure of the scattering object.
- The problem with phases:

  We need to know the amplitudes of the diffracted x-rays as well as their relative phases to compute the Fourier transform. The x-ray experiment measures only the amplitudes (reflection intensities are the square of the Fourier amplitudes).

Solving the Phase Problem

Perturbing the X-ray Scattering in a Predictable Way

- Isomorphous replacement with heavy atoms.
- Anomalous scattering of x-rays by endogenous or added scatterers.
  - inelastic scattering of x-rays causes shift in phases of scattered rays.
  - extremely useful in conjunction with tunable (synchrotron) radiation

Guessing the Phases

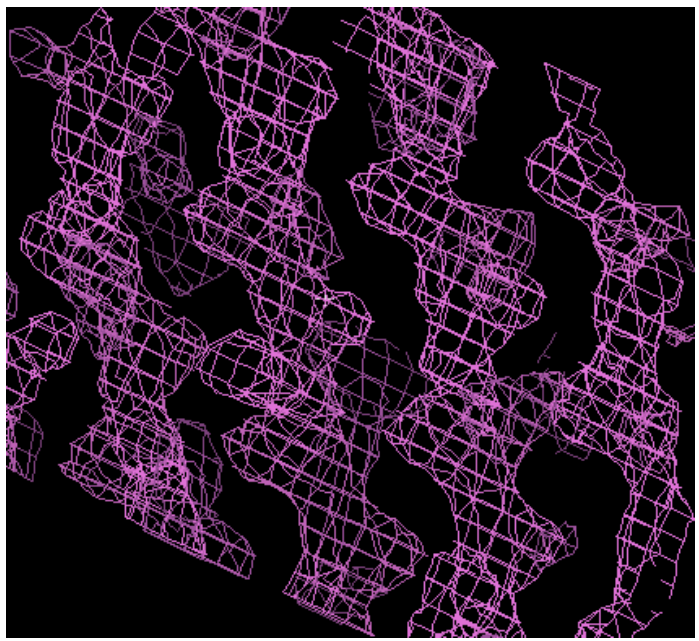
- Molecular replacement using a model of a related object.
- Direct methods – phase relationships for triplets of reflections.

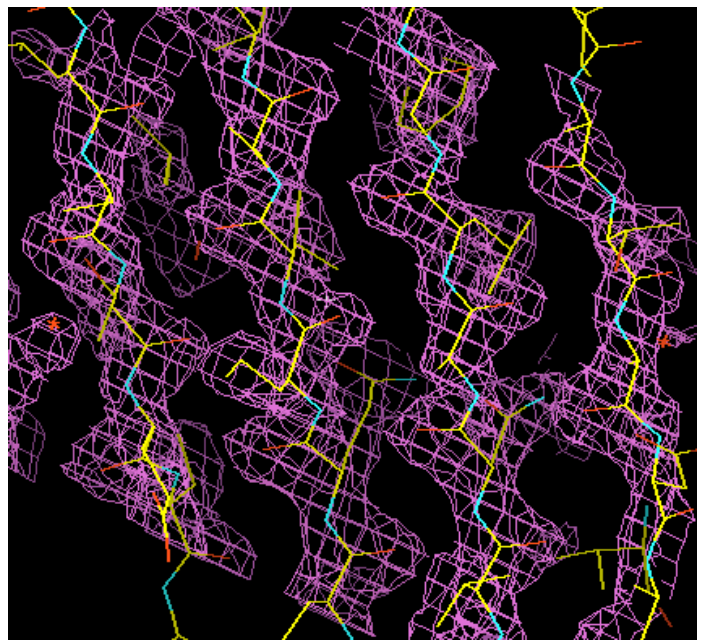
The Interpretation of X-ray Diffraction Data

- Our (un)certainty about an x-ray structure is directly related to the quality of the x-ray diffraction data.
- The electron density revealed by the Fourier transform of the diffraction data (actually, the square root of the intensities) has resolution-dependent features.
- Errors in measurement of the reflection intensities and the phases (estimated from isomorphous replacement methods) degrade the quality of the "x-ray image" of the molecule.

The Interpretation of X-ray Diffraction Data

- A crystallographic model of a protein is built on the basis of the shape of the electron density, the known amino acid sequence, standard chemical constraints/restraints for polypeptides (bond angles and lengths, allowed torsions, etc.), and the agreement between the measured x-ray data and the diffraction pattern calculated from the model (the R-factor).





Judging the Quality of X-ray Structures

X-ray Data Quality

- R_{sym}– the error in measured intensities of equivalent reflections (typically ranging from 3% at low resolution to 35% at the high resolution limit).
- Resolution, signal-to-noise ratio (I/sigma > 3-4 for useful data)

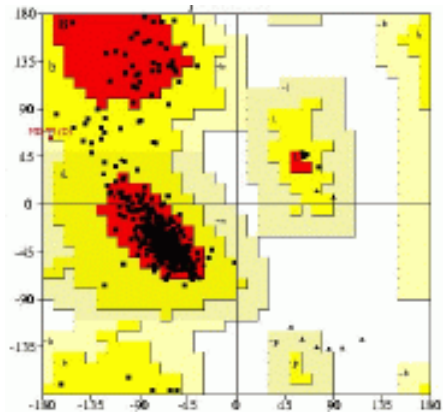
Crystallographic Model Quality

- R_{cryst} – the error in agreement between the model and experimental structure factor amplitudes (typically ranging from 16% (high resolution structure) to 28% (lower resolution).
- Free R-factor (R_{free}) – a crystallographic R-factor calculated from a small set (5-10%) of reflections that are reserved and not used during model refinement (R_{free} is typically larger (+ 2-4%) than R_{cryst}). Over-refinement causes an artificial decrease in R_{cryst} with little or no change in R_{free}.

Judging the Quality of X-ray Structures

Crystallographic Model Quality (cont)

- Agreement between the model and known structures.
  - Ramachandran plot.
  - Deviation from standard geometry (bond angles, lengths, etc.).
  - Fold recognition – does the model look like any other proteins in the protein data bank?



Does the model satisfy other experimental constraints/data?

- Locations of functionally important residues.
- Shape consistent with known function(s).

"Table 1" : A Standard for X-ray Publications

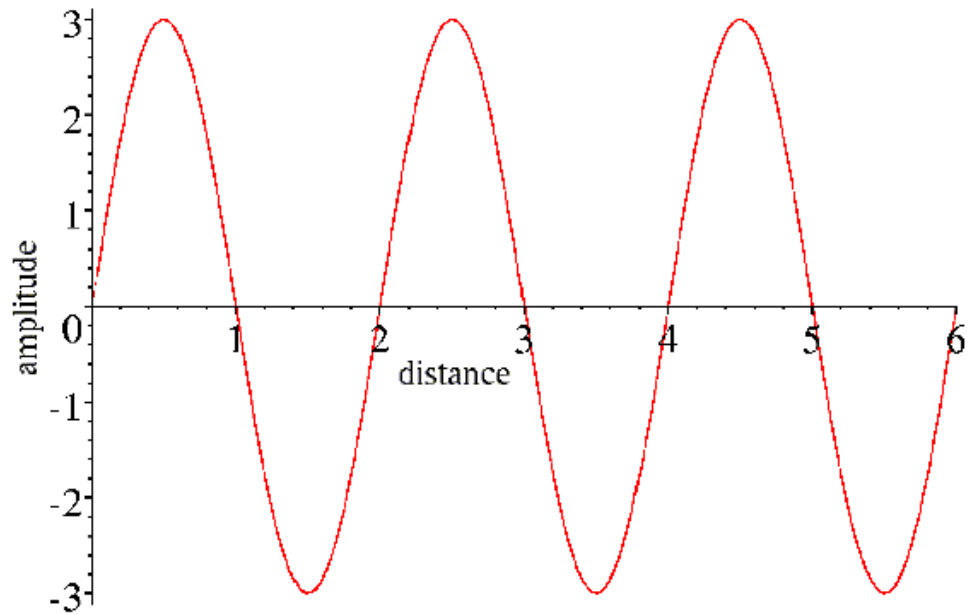
Table 1. Summary of crystallographic data analysis

Data and phasing statistics							
space group		$P2_1$					
unit cell parameters		a=59.0, b=106.2, c=73.2, β=92.1°					
Dataset	native	Hg1	Hg2	iodo1	iodo2	iodo3	iodo4
source	X25[a]	F1[b]	X12C[c]	F1	A1[d]	F1	X12C
λ (Å)	1.100	0.950	1.015	0.950	0.945	0.950	1.200
resolution(Å)	2.95(3.06-2.95)	3.20	3.20	3.50	3.50	3.50	3.20
completeness(%)	99.8(99.9)	97.5	96.6	97.8	99.6	99.4	99.7
redundancy	3.8	3.2	4.0	3.1	3.4	3.7	6.6
R_{merge} (%)	7.0(33.8)	8.3	10.3	9.5	11.3	9.2	9.7
I/σ	18.8(3.6)	13.1	8.0	10.8	12.0	14.2	12.0
number of sites		2	2	4	2	6	2
R_{cullis} (iso,acent/cent)		0.86/0.83	0.82/0.77	0.97/0.97	0.90/0.92	0.91/0.91	0.89/0.90
R_{cullis} (anomalous)		0.99					0.99
figure of merit (MLPHARE)		0.42/0.63/0.43 (acentric/centric/all)					
figure of merit (RESOLVE)		0.60/0.71/0.61					
Refinement statistics							
resolution range(Å)		50-2.95(3.01-2.95)					
total reflections/test set		19010/929					
R_{work} /R_{free} (%)		23.1(40.1)/25.9(42.8)					
rms deviations from ideality							
bond(Å)/angle(deg)		0.0067/1.29					

[a,c] Beamlines X25 and X12C, Brookhaven National Synchrotron Light Source.
[b,d] Stations F1 and A1, Cornell High Energy Synchrotron Source.

X-ray Scattering Basics

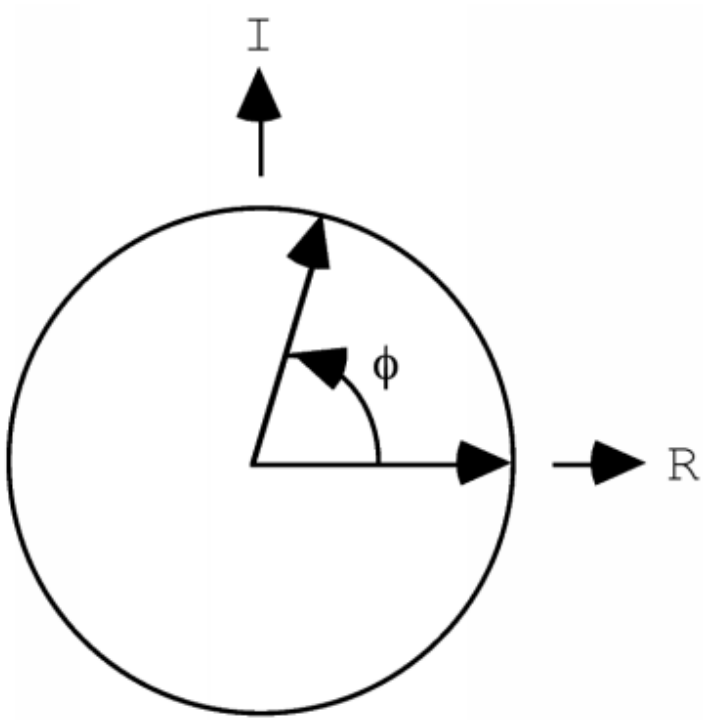
- X-ray diffraction results from the interaction of waves (x-rays) with matter (electrons bound atoms of our protein).
- Electromagnetic waves have electrical and magnetic components oriented perpendicular to one another and to the direction of travel.
- A wave can be described by a cosine function with an amplitude and period (wavelength):



$A \bullet \cos(2\pi\nu\tau)$
or
$A \bullet \cos(2\pi x/\lambda)$

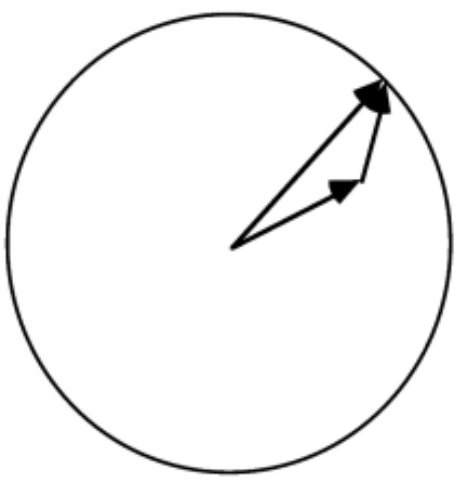
X-ray Scattering Basics

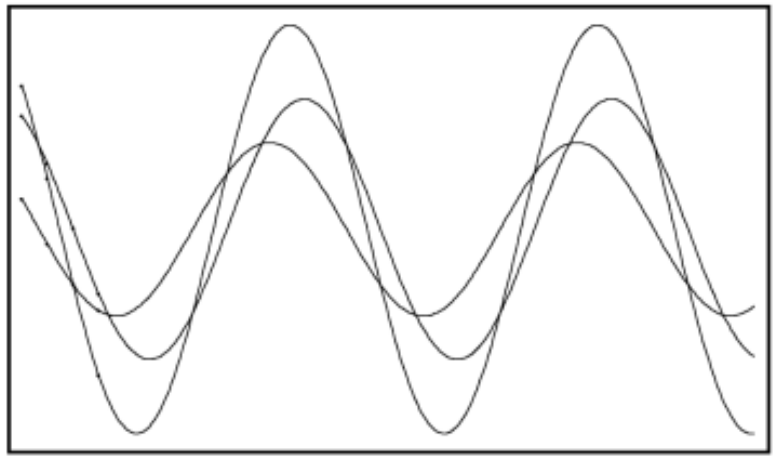
- Waves can be described as vectors.
- The length of vector R corresponds to the amplitude of the wave.
- The phase at a given time/position in space (ϕ) is the position of the vector around the circle swept out by rotation of the vector.



X-ray Scattering Basics

- Waves can be added (as cosine waves $\Rightarrow$ with difficulty, or as vectors $\Rightarrow$ easily).

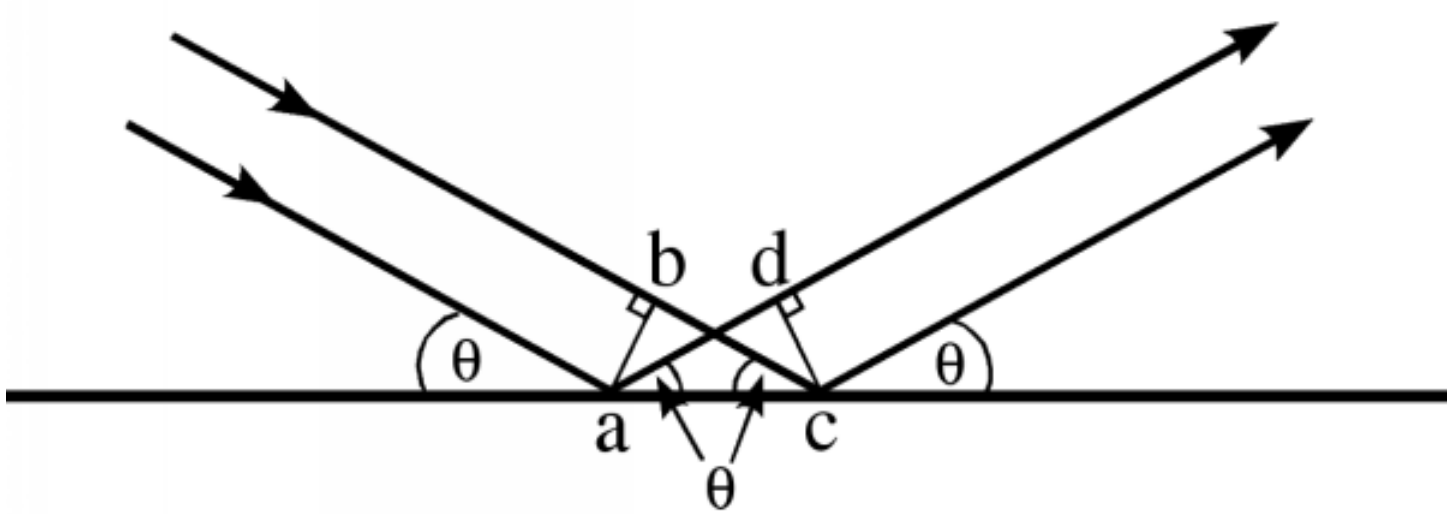




- Waves scatter in phase when they travel the same distance or over distances differing by exactly an integral number of wavelengths.
- Light is reflected by a mirror at the same angle as the angle of incidence. The same is true of the Bragg reflecting planes in a crystal.

X-ray Scattering Basics

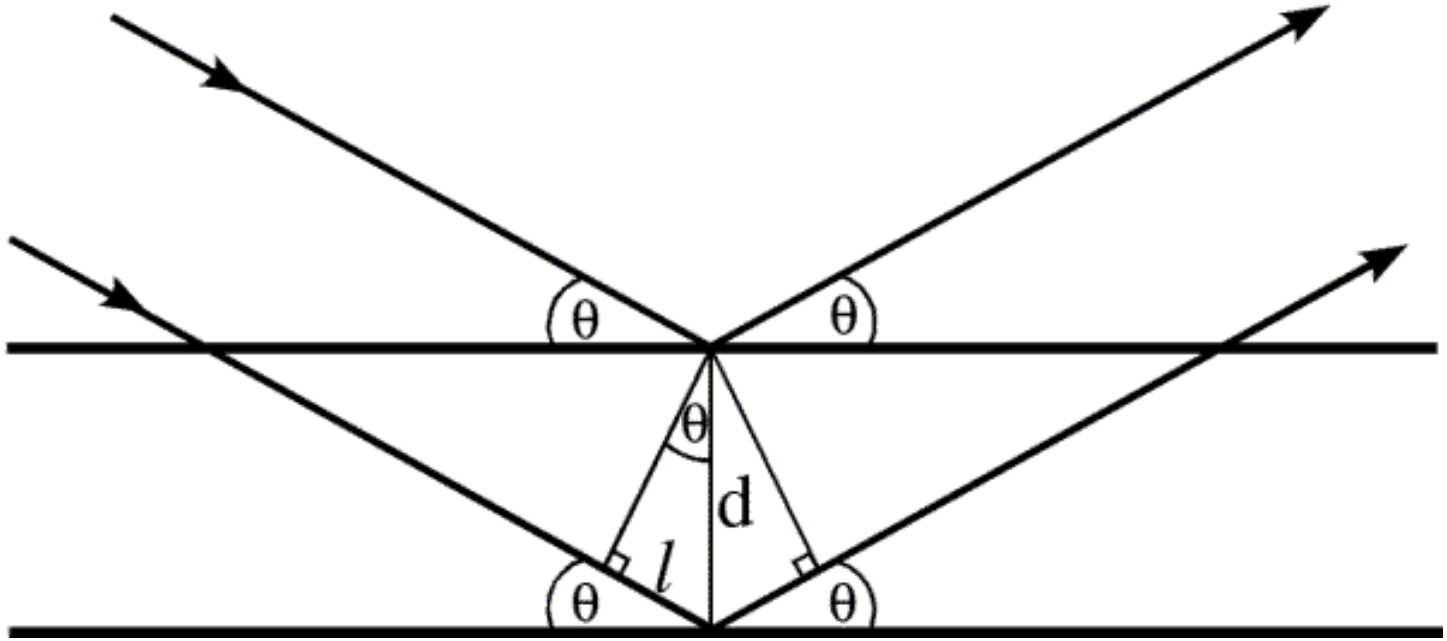
Constructive scattering from a Bragg plane:



- The angle (θ) of the incident radiation is the same as the angle of the reflected ray.
- Because triangles abc and cda share side ac, it is easy to show that bc is equal to da. Thus, the 2 rays that are reflected from this plane travel along identical path lengths and scatter in phase.

X-ray Scattering Basics

Scattering from two different Bragg planes:



- In this case, in-phase scattering of the 2 rays will depend upon the distance d and the wavelength (λ) of the incident radiation.
- The distance l is equal to d sinθ. For the 2 rays to scatter in phase, λ = 2d sinθ. This is Bragg's law in its simplest form.

Bragg Scattering Simulator

http://www.eserc.stonybrook.edu/ProjectJava/Bragg/index.html

Nota bene:

- When the wavelength is lengthened, the diffracted intensity becomes less sensitive to d-spacing. Short wavelengths are needed to resolve features corresponding to small (atomic) distances.
- With the wavelength fixed, as d-spacing is shortened (resolving higher resolution features) you have to move to a higher value of theta in order to see the first peak of diffraction intensity. Small distances in the crystal show up at high angles of diffraction. This is one consequence of the diffraction pattern having a reference frame of "reciprocal space" whereas the crystal is in the frame of "laboratory (real) space."

X-ray Scattering Basics

Bragg's Law: A Condition for Scattering In Phase:

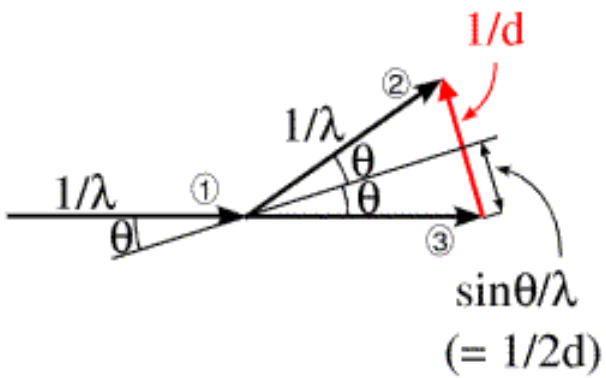
$$\lambda = 2d \sin\theta$$

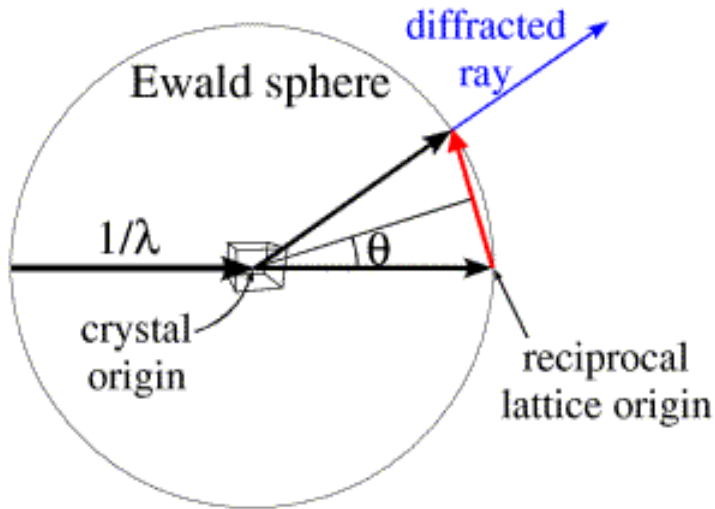
- For a given λ, in-phase scattering at higher angles of diffraction (θ) is accommodated by reflecting planes having smaller spacings (d) between them.
- This is one manifestation of the reciprocal relationship between real distances in the crystal and the dimensions of the diffraction pattern. High resolution information (short d-spacings between equivalent atoms lying on reflecting planes) is recorded at higher diffraction angles.
- Poorly-ordered crystals yield diffraction information only at low angle, indicative of the limited resolution attainable.
- Crystallographers refer to measurements in "real space" (the crystal frame of reference) and "reciprocal space" (the diffraction geometry frame of reference).

X-ray Scattering Basics

Ewald's Sphere

- An artificial geometrical construction that illustrates the geometry of a diffraction experiment.
- Two separate origins, one for real space (centered on the crystal) and one for reciprocal space (at the intersection of the direct (unreflected) beam with Ewald's sphere).
- Points on the reciprocal lattice are in reflecting condition when they intersect Ewald's sphere.

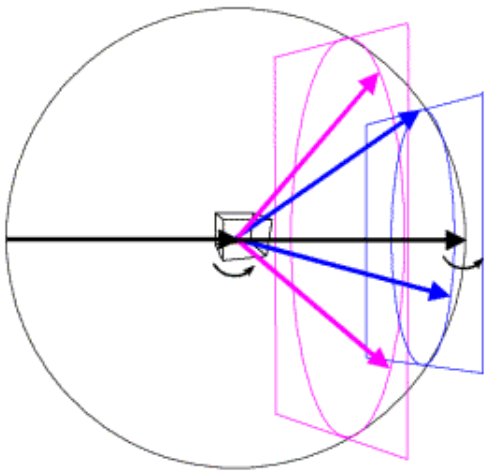


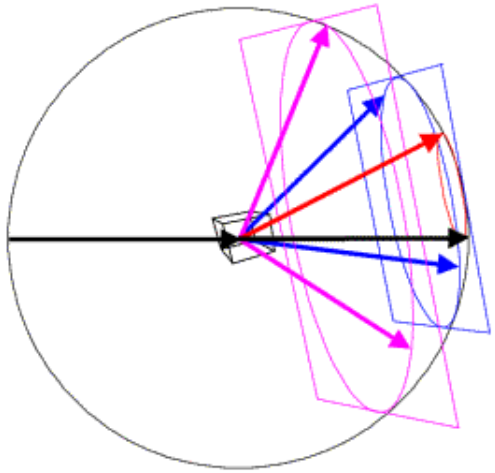


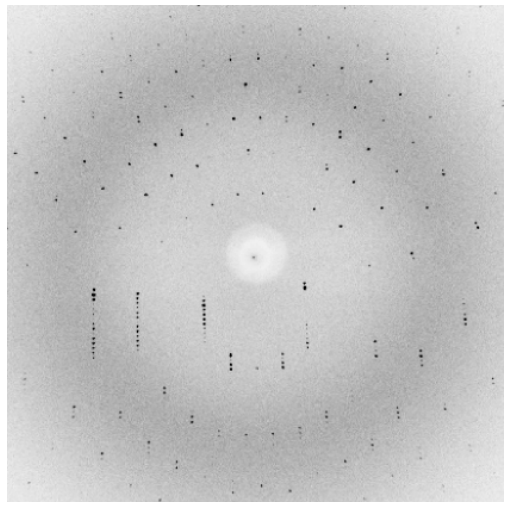
X-ray Scattering Basics

Ewald's Sphere

- The intersection of planes of the reciprocal lattice with Ewald's sphere corresponds to circles of reflections. Each circle is populated by the reflections from one plane of the reciprocal lattice. These circles are is referred to as reflection "lunes."
- Different reflections are observed as the crystal is rotated by the corresponding (coupled) rotation of the reciprocal lattice.







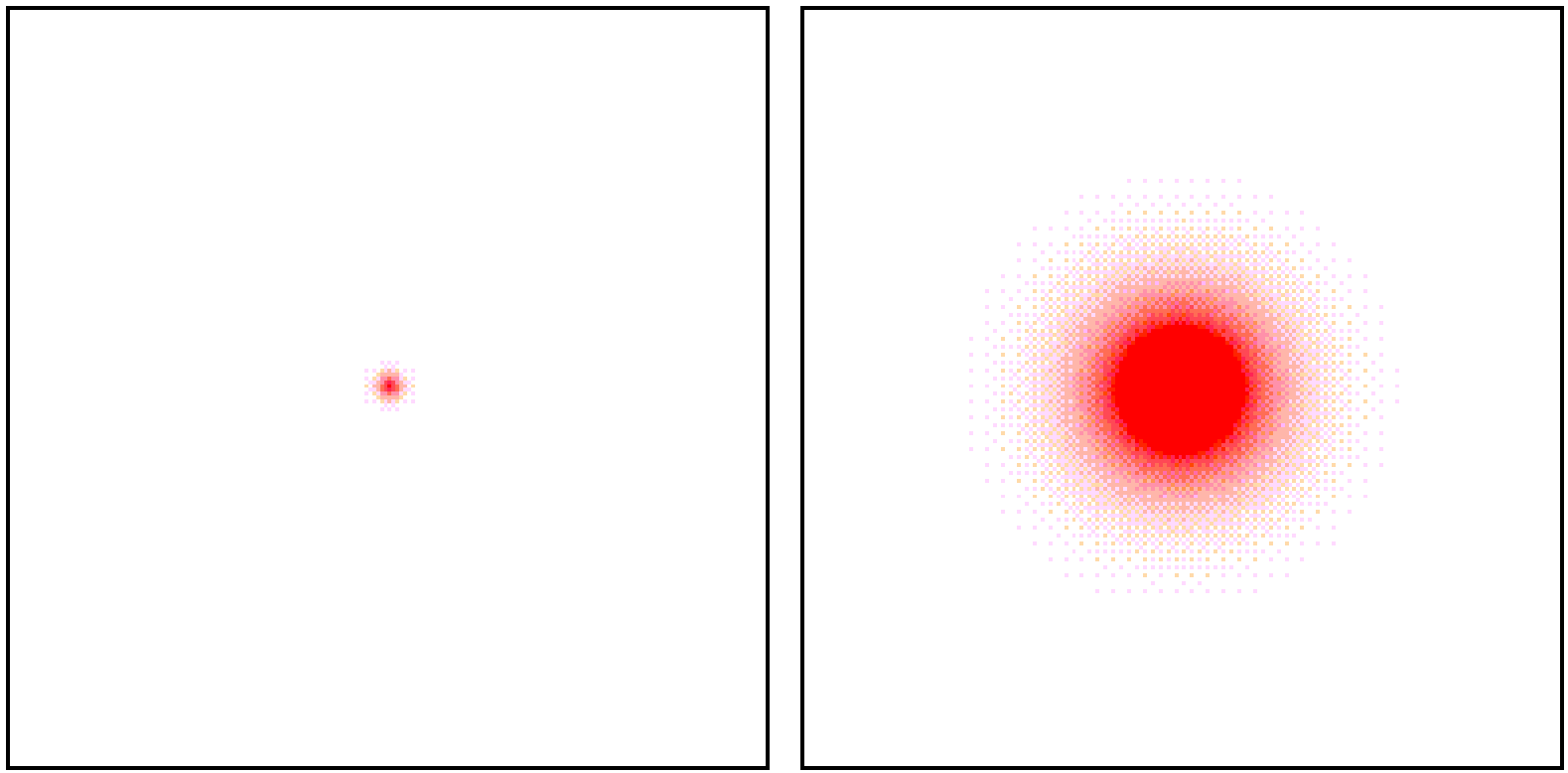
Ewald's Sphere Simulator

X-rayView software download (Java script):

http://phillips-lab.biochem.wisc.edu/software.html

Kevin Cowtan's Book of Fourier

an atom & its Fourier transform

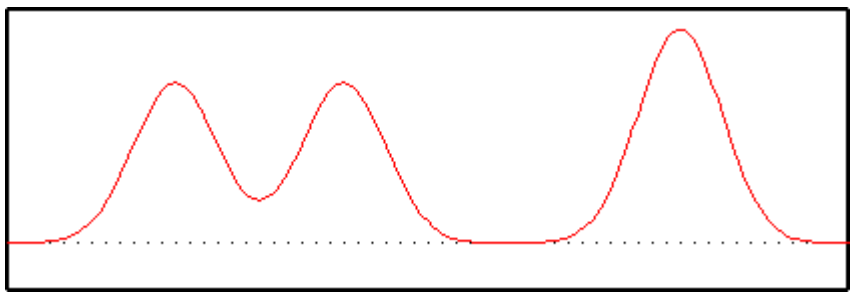


http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

Fourier Transforms in Crystallography

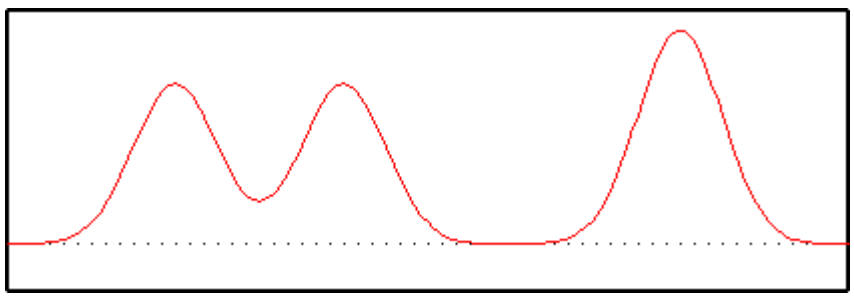
Consider an imaginary 1-dimensional crystal consisting of 3 atoms, 1 oxygen and 2 carbon atoms.

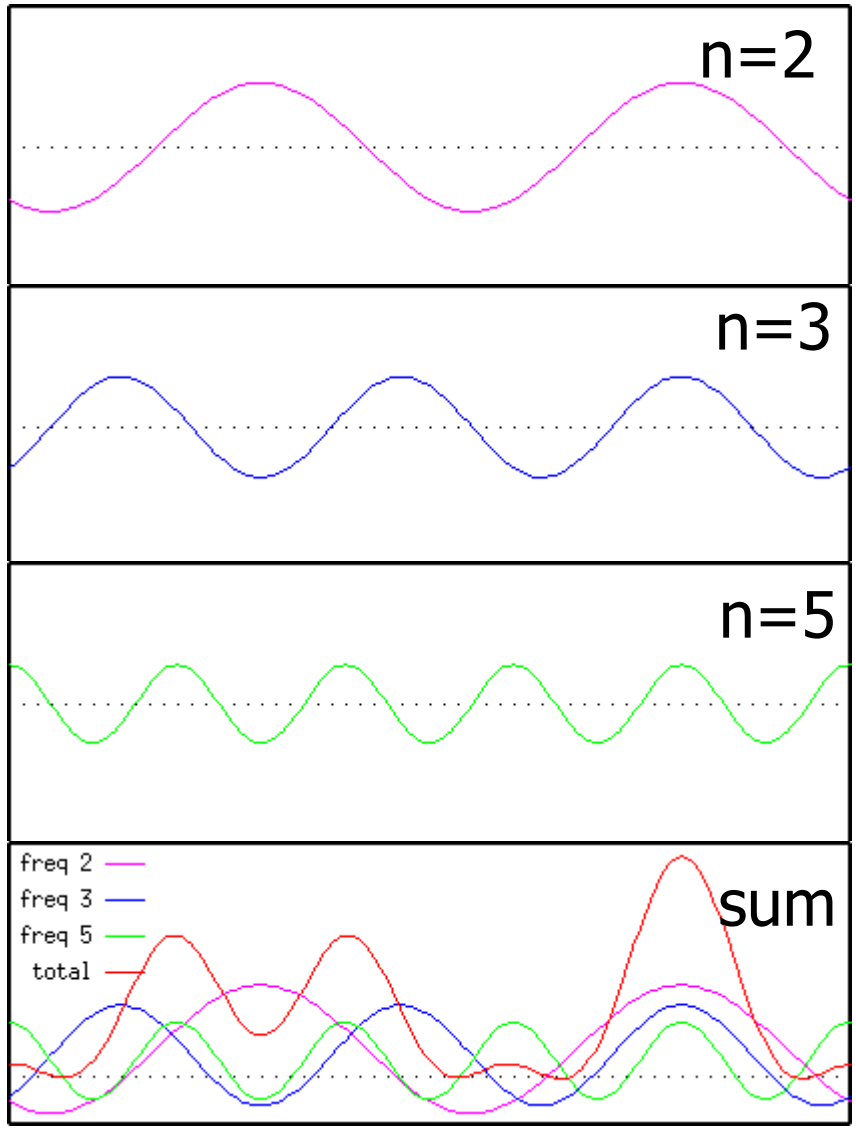
- The resulting electron density in the unit cell is:



Fourier Transforms in Crystallography

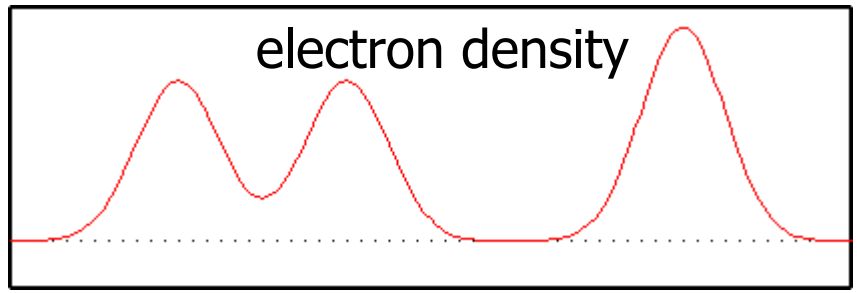
- We can represent the electron density of the crystal as a series of sin waves with different frequencies, whose sum approximates the original distribution of density.

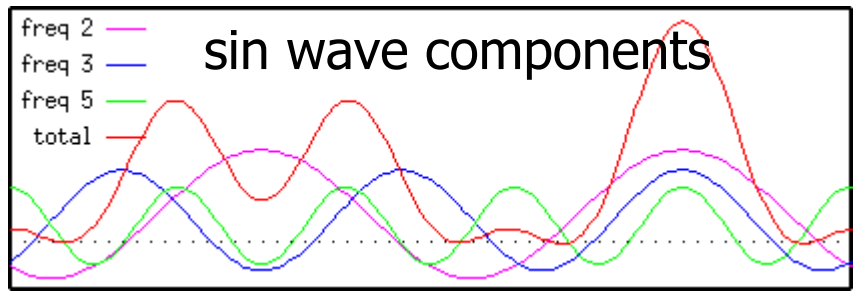


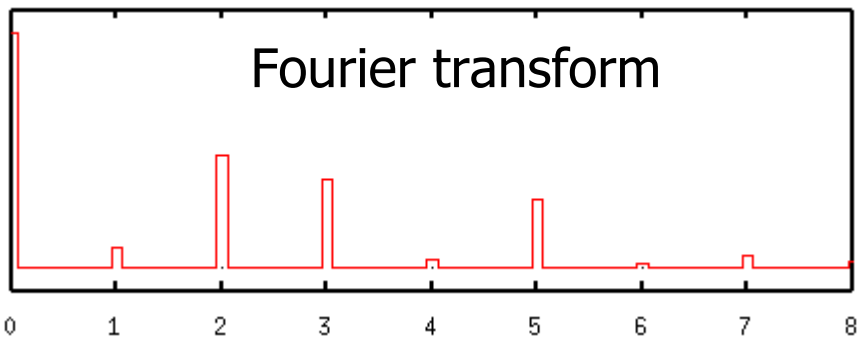


Fourier Transforms in Crystallography

- The Fourier transform of the crystal (below) reflects the strong contributions of the n = 2, 3, 5 waves.





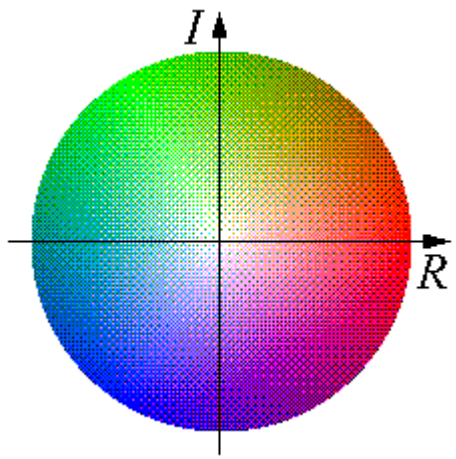


http://www.ysbl.york.ac.uk/~cowtan/fourier/ftheory.html

Complex Numbers

We will view Fourier transforms consisting of complex numbers with amplitudes (represented here by color saturation) and a phase (represented by hue).

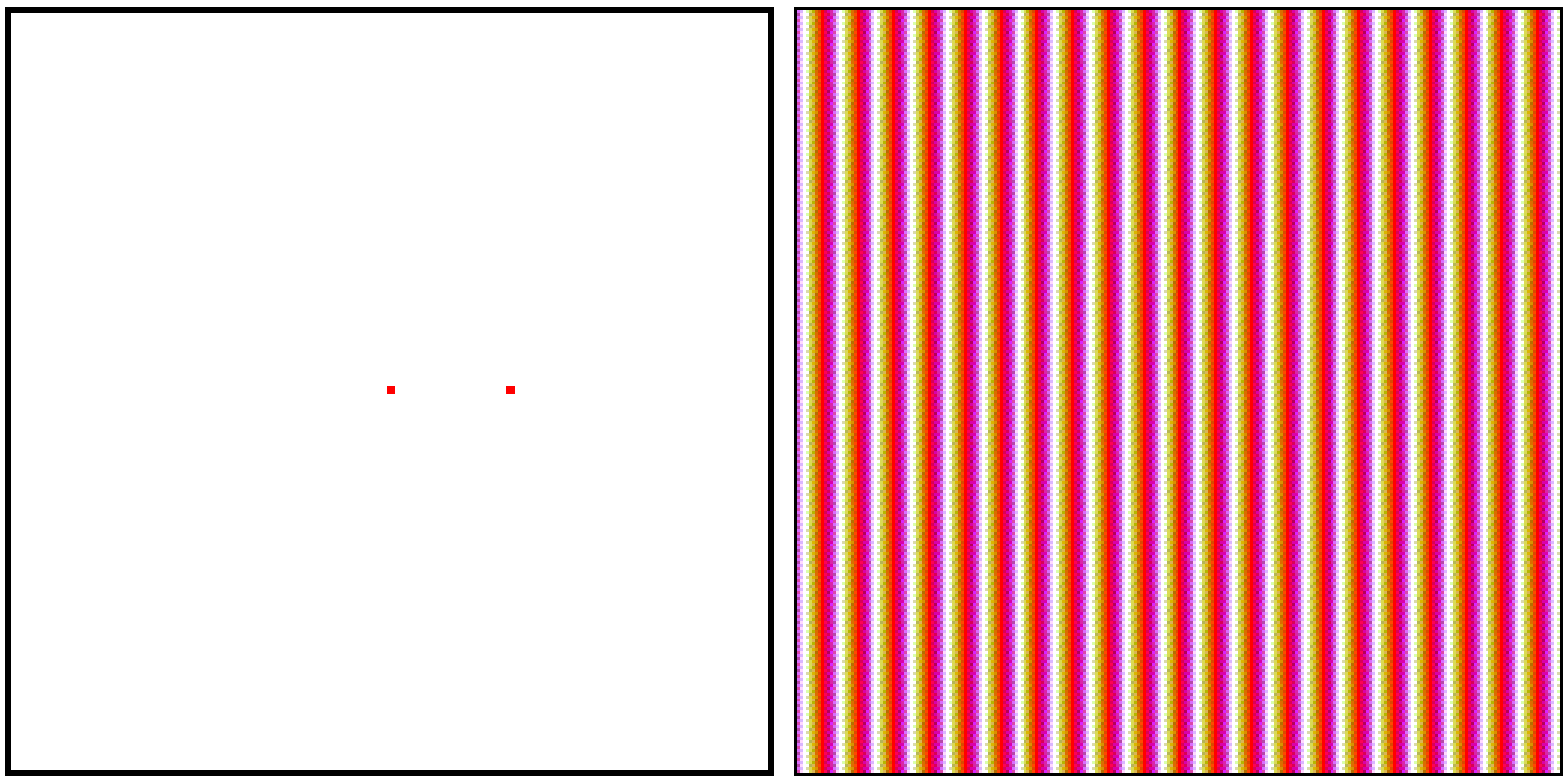
- A phase of π/2 (90 deg.) is symbolized by yellow-green, a phase of π (180 deg.) is green-blue, etc.



http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

A 1D Array & Its Fourier Transform

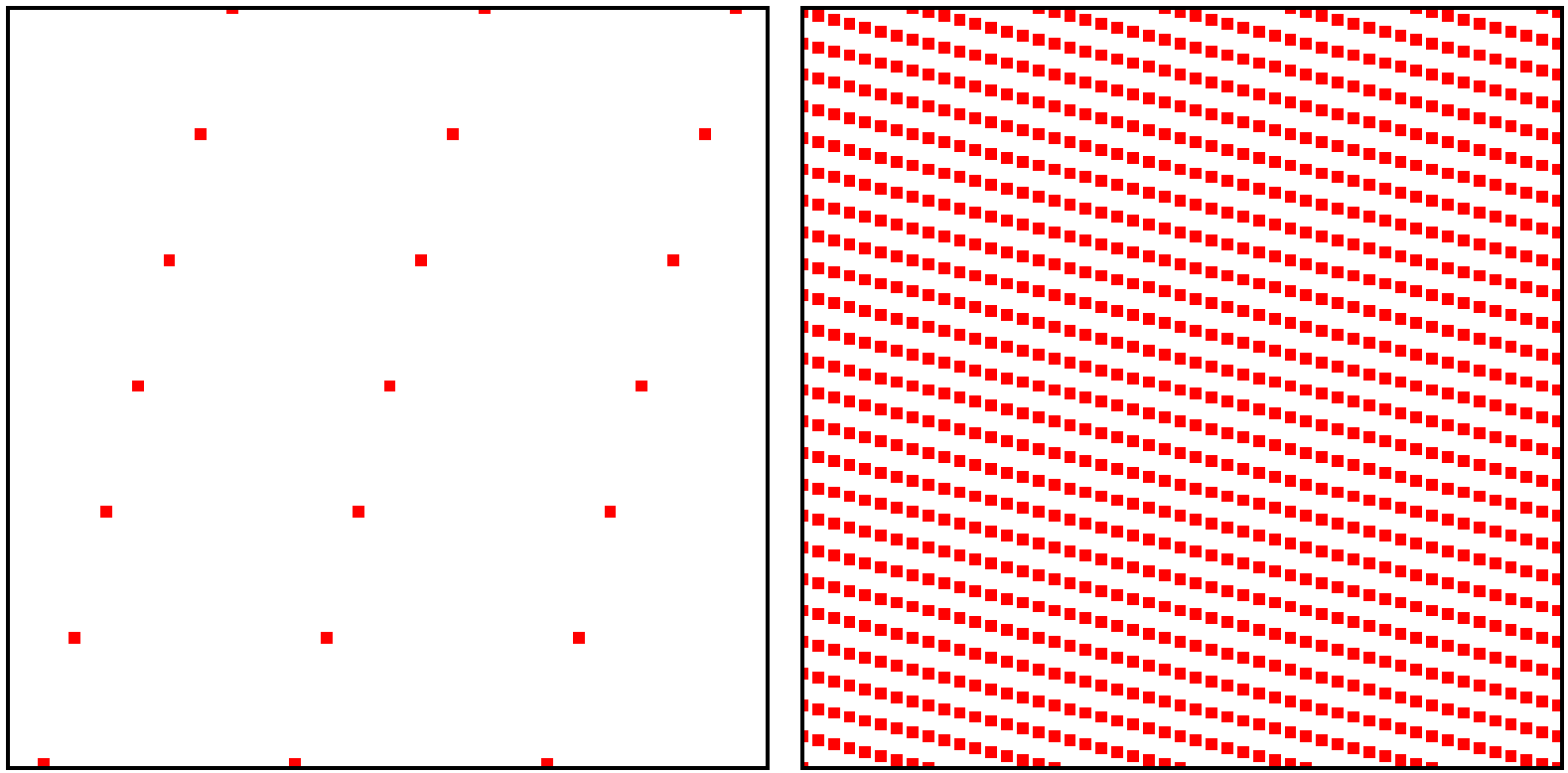
The electron density distribution inside a crystal is constrained by the crystal lattice and its symmetry.



http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

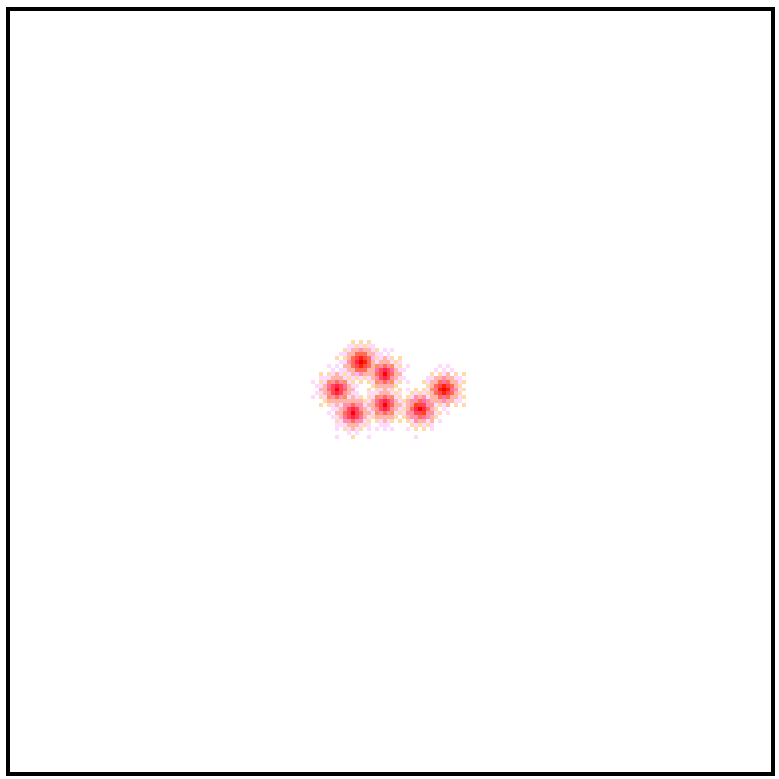
A 2D Lattice & Its Fourier Transform

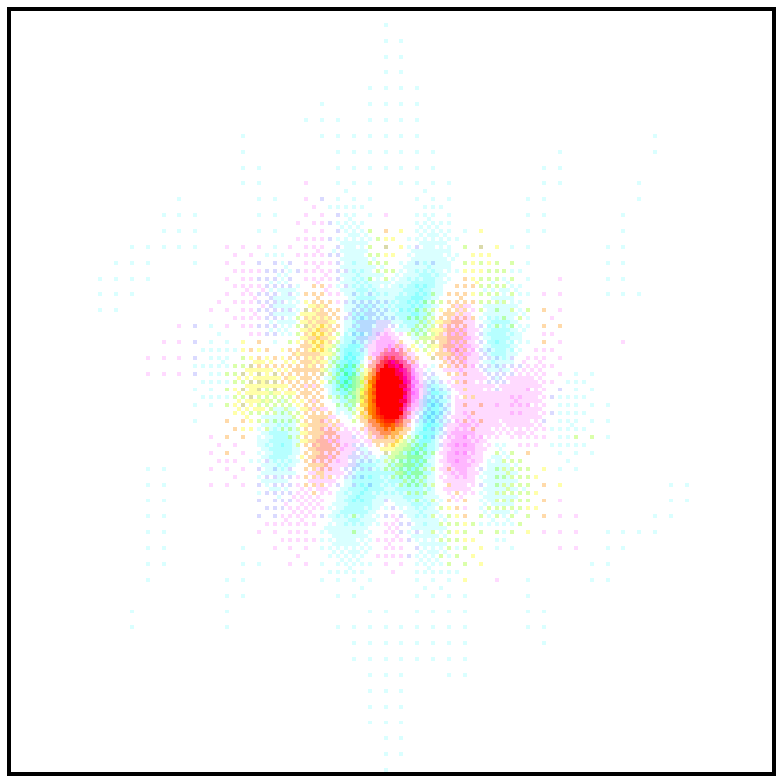
Notice the reciprocal directions and spacings of the transformed lattice (right) compared to the starting lattice (left).



http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

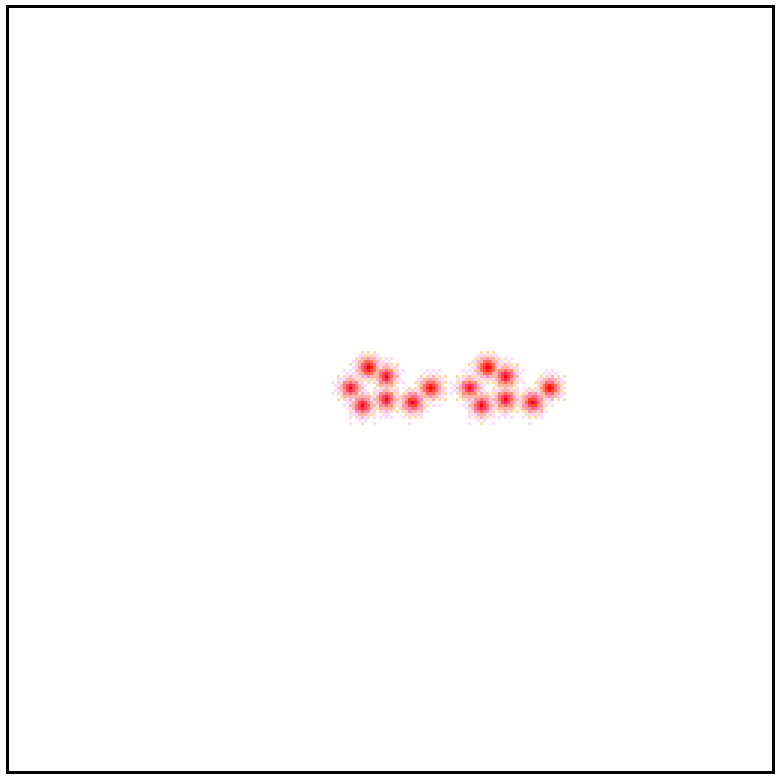
A Molecule & Its Fourier Transform

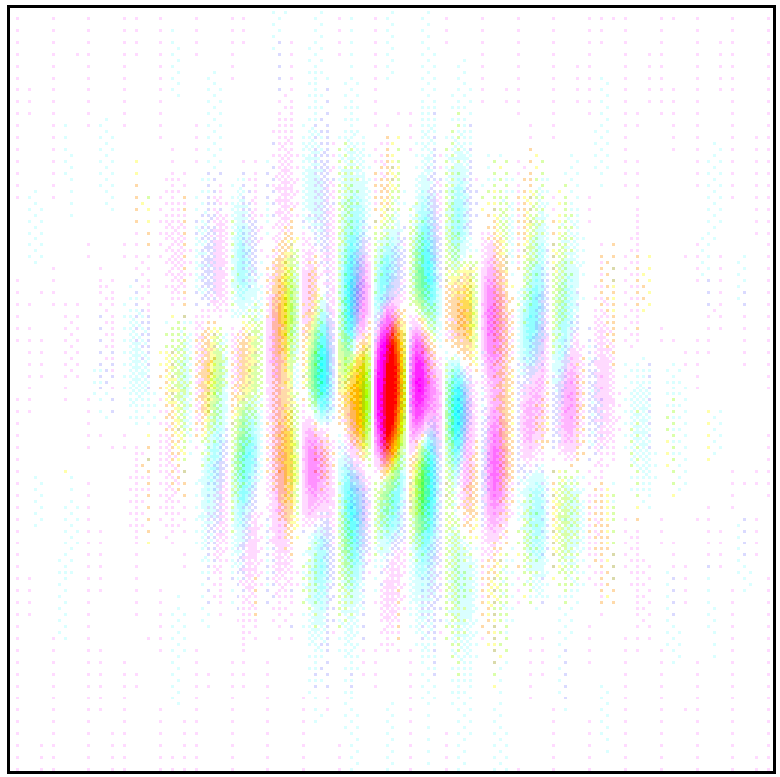




http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

Two Molecules & Their Fourier Transform





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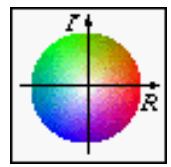
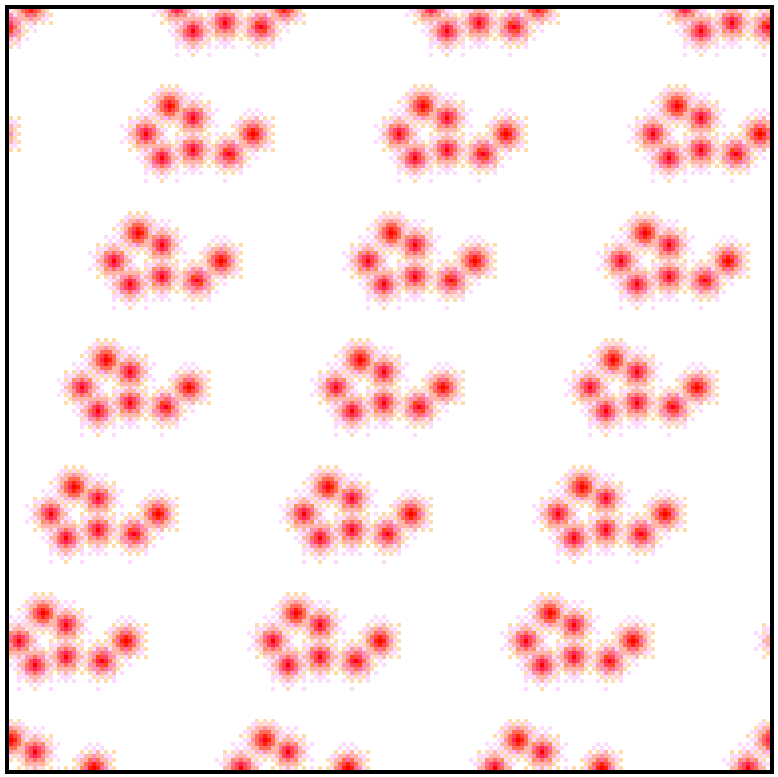
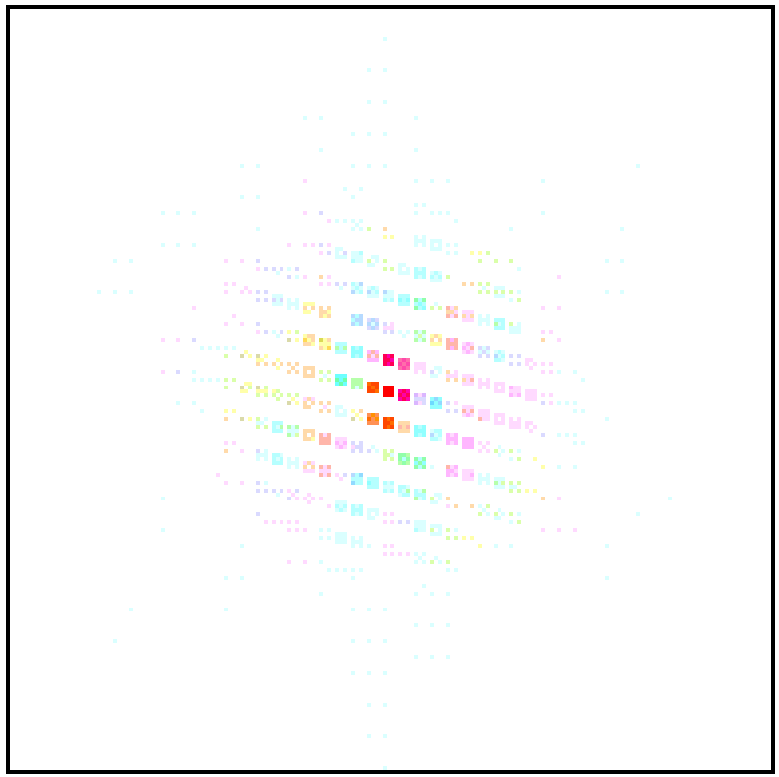


A Crystal & its Fourier Transform





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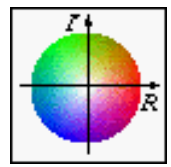
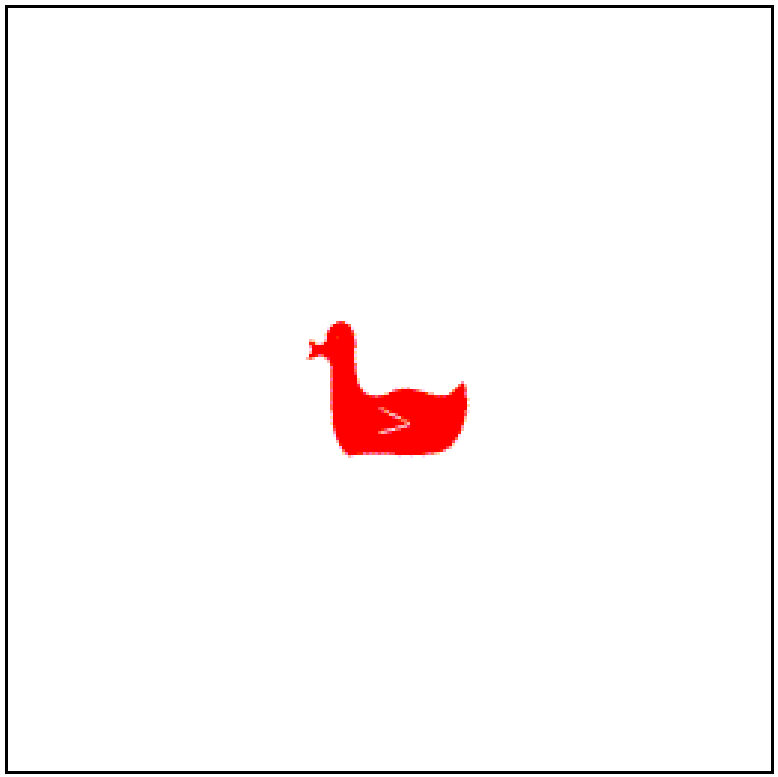
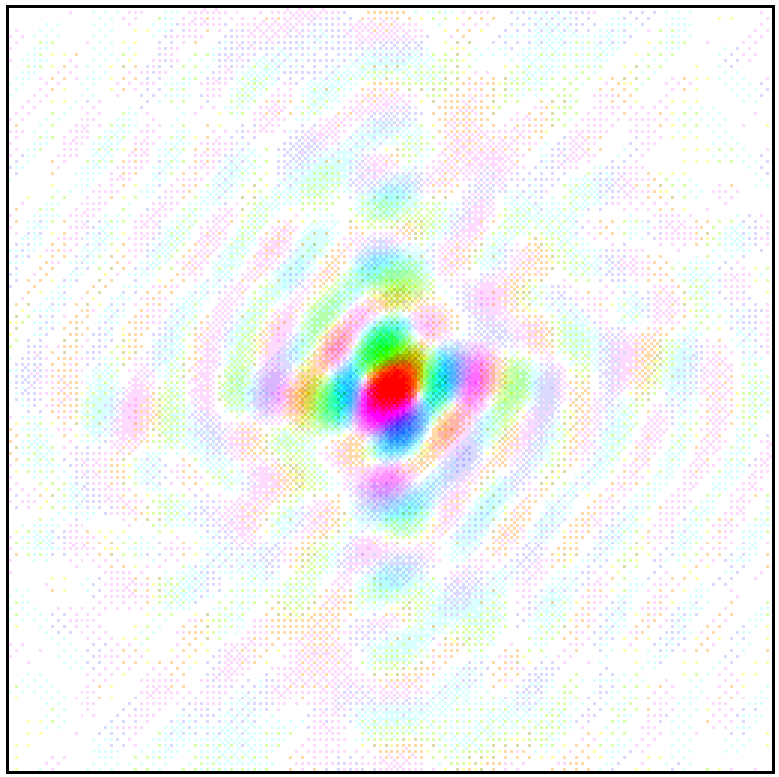


The Fourier Duck





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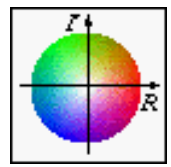
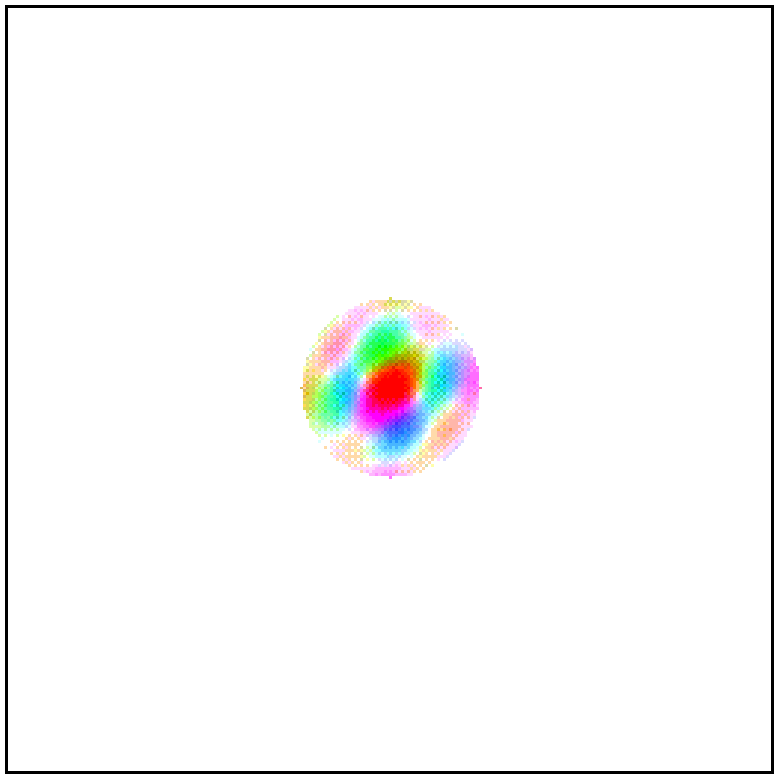
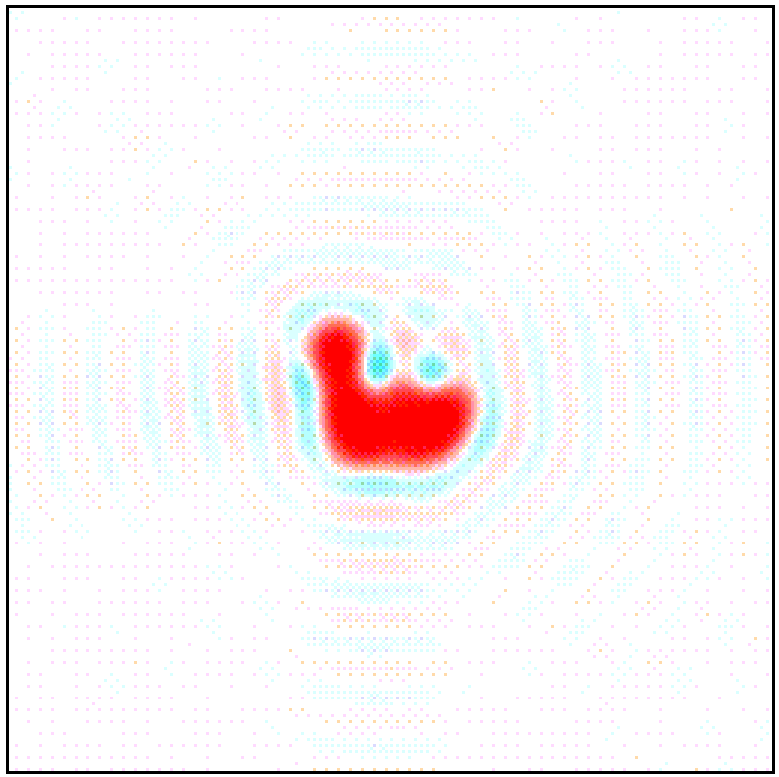


Low Resolution Duck





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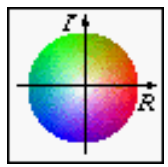
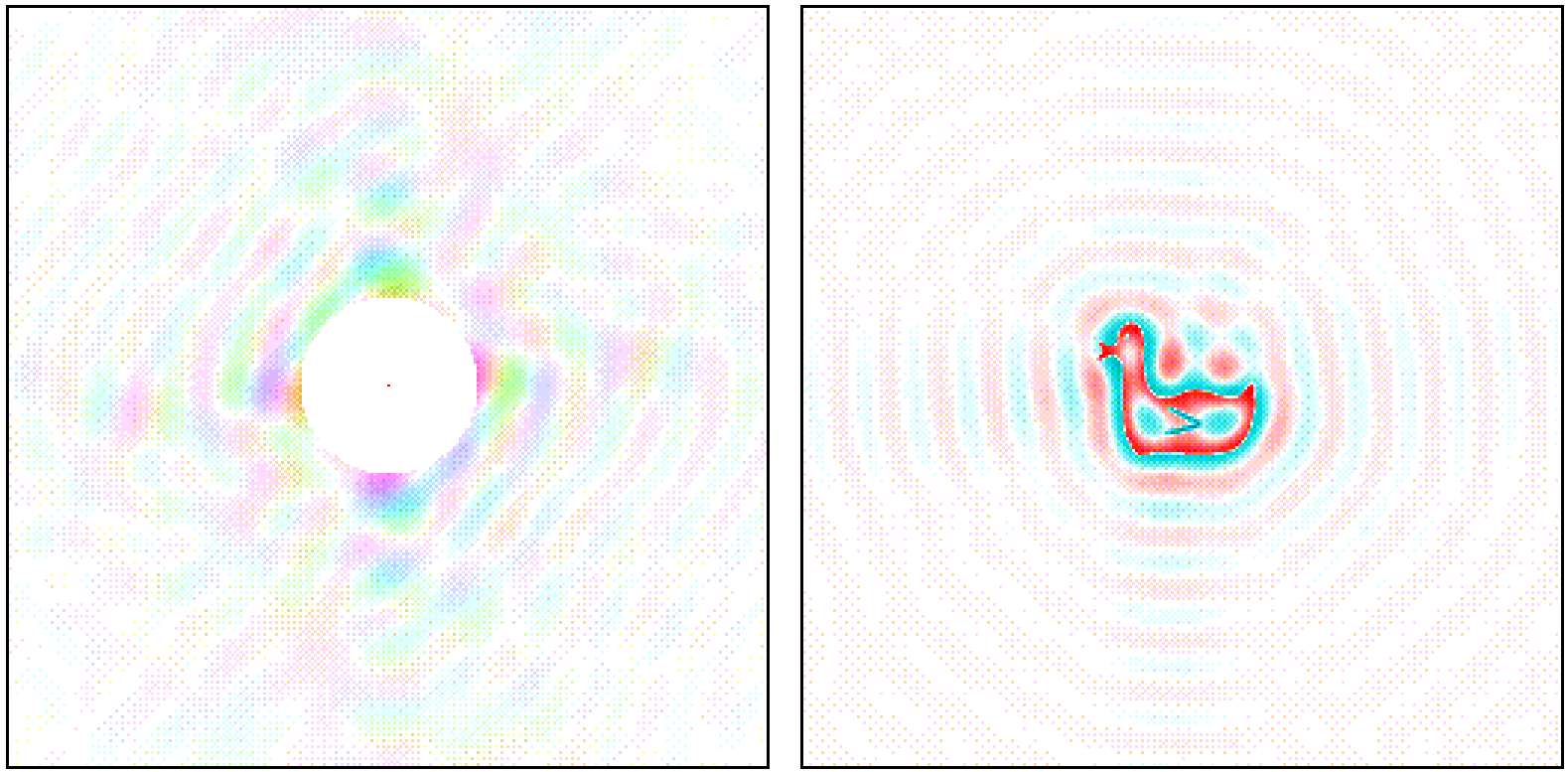
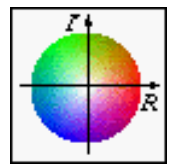


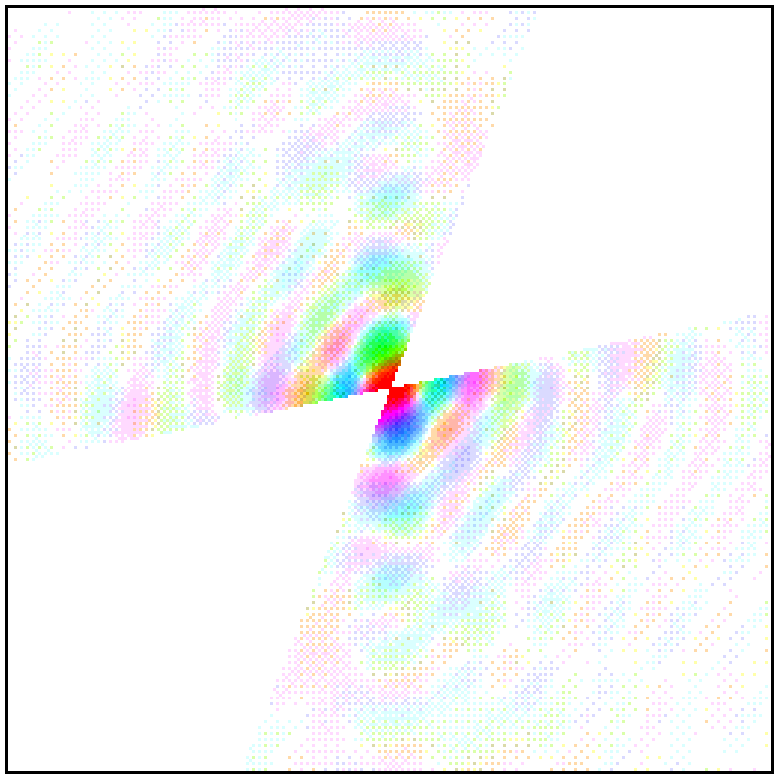
Only High Resolution Data

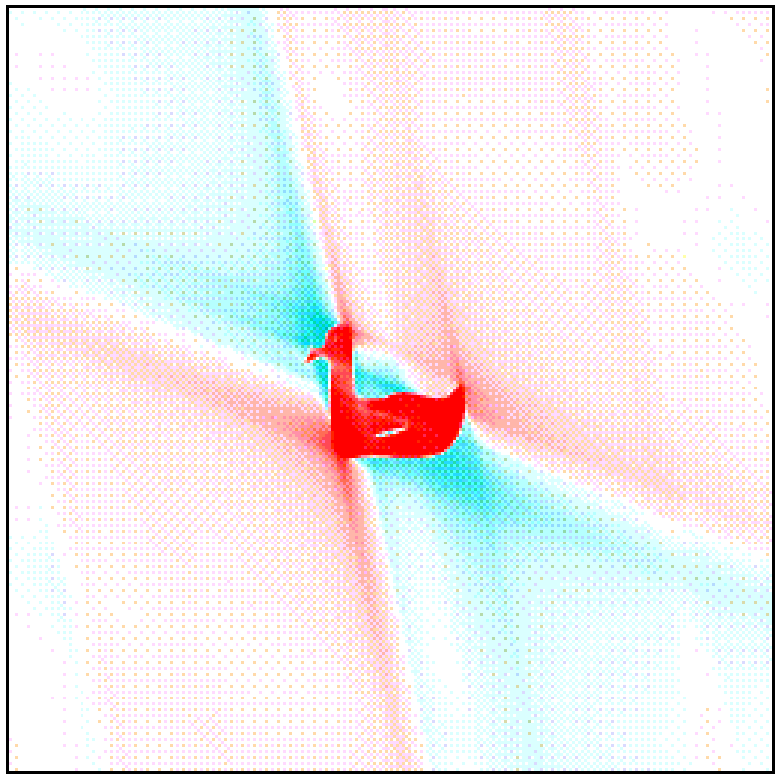


http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

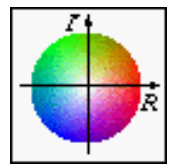


Missing Wedge of Data

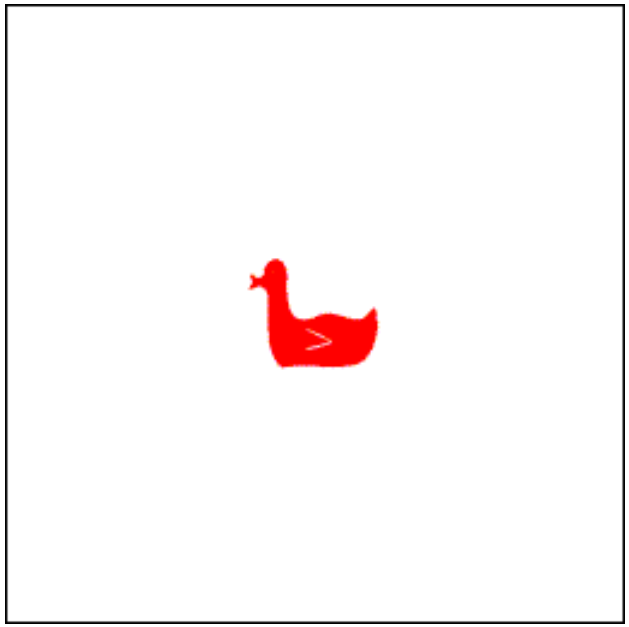


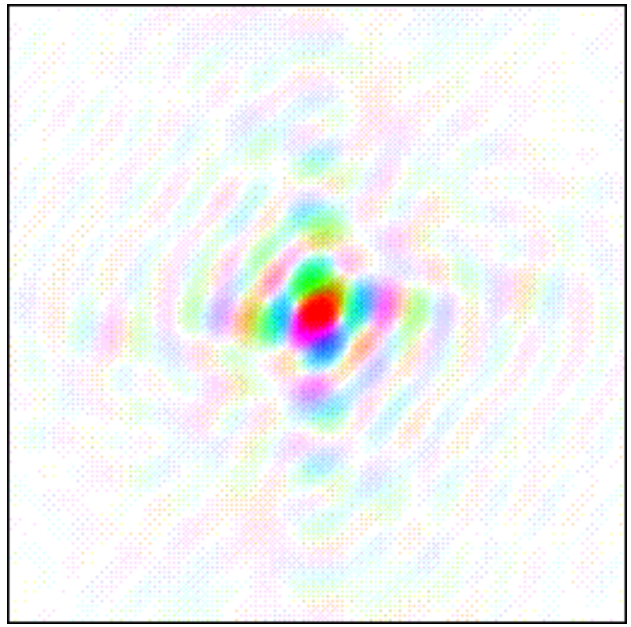


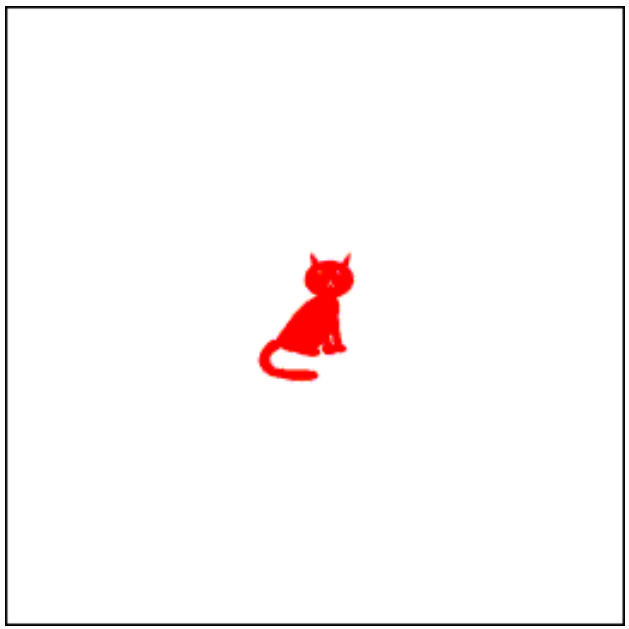
http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

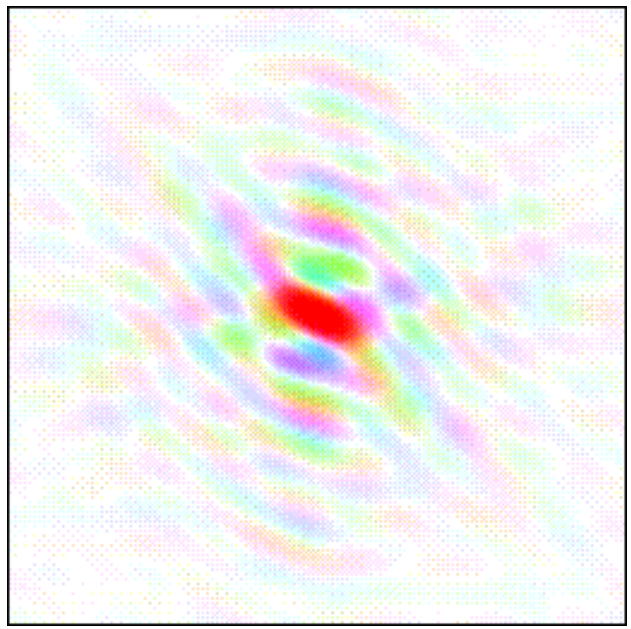


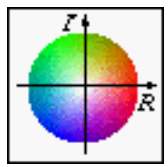
Animal Transformations



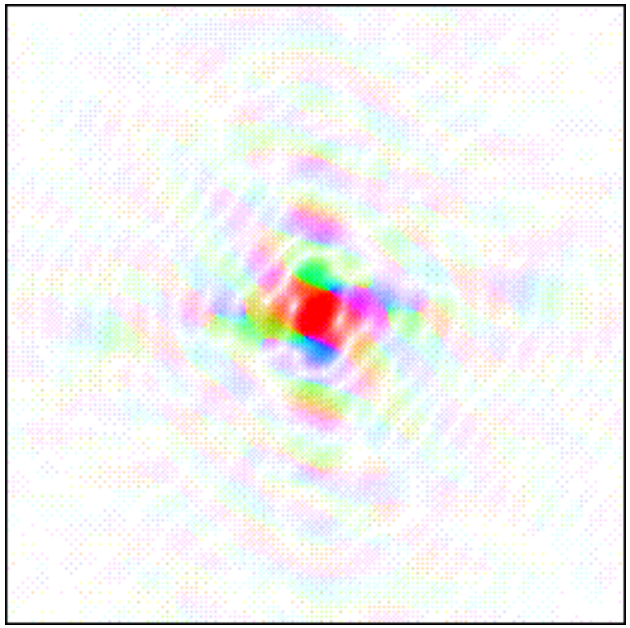


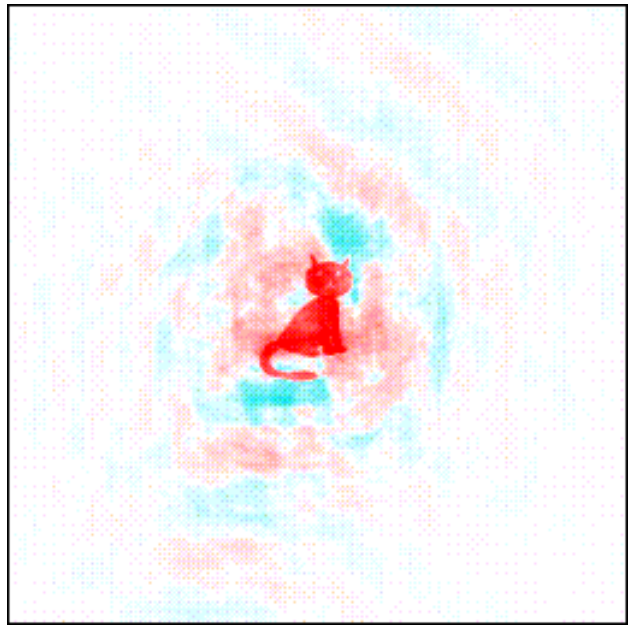




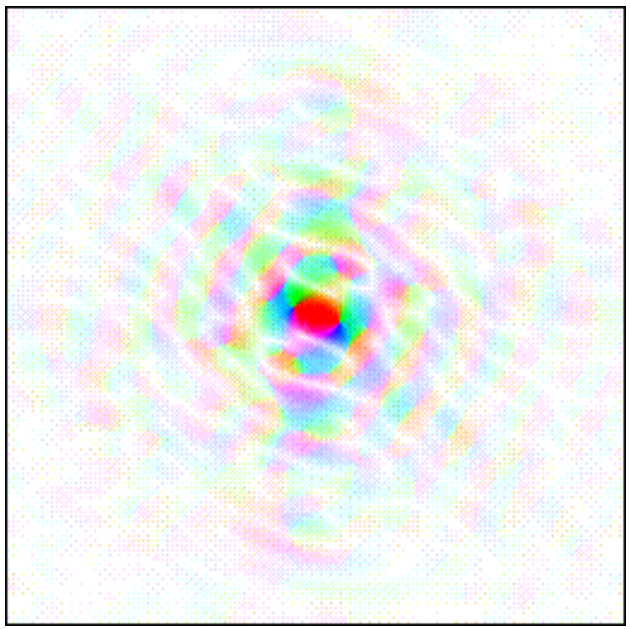


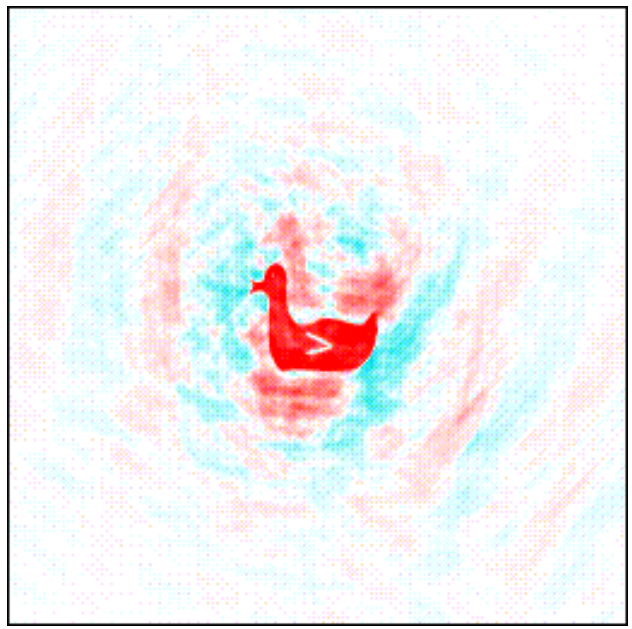
Mixing duck amplitudes with cat phases

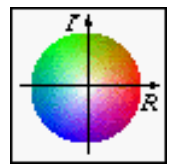




Mixing cat amplitudes with duck phases

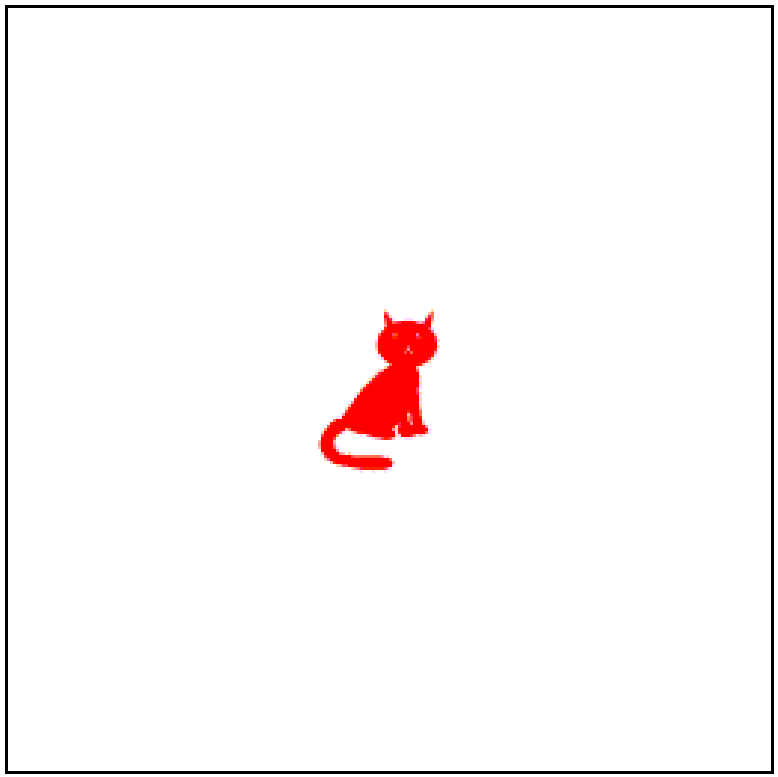


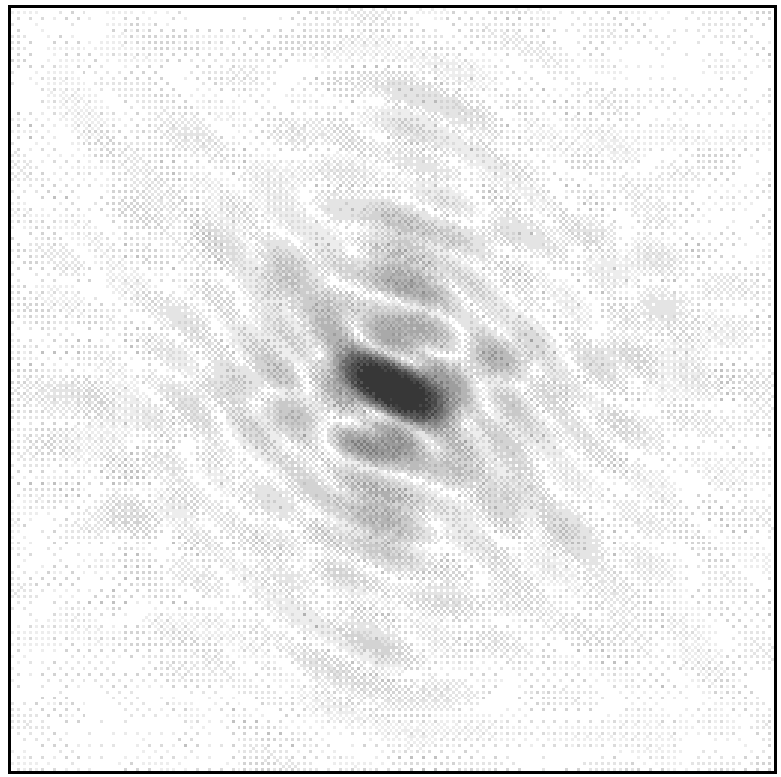




http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

A Tail of Two Cats

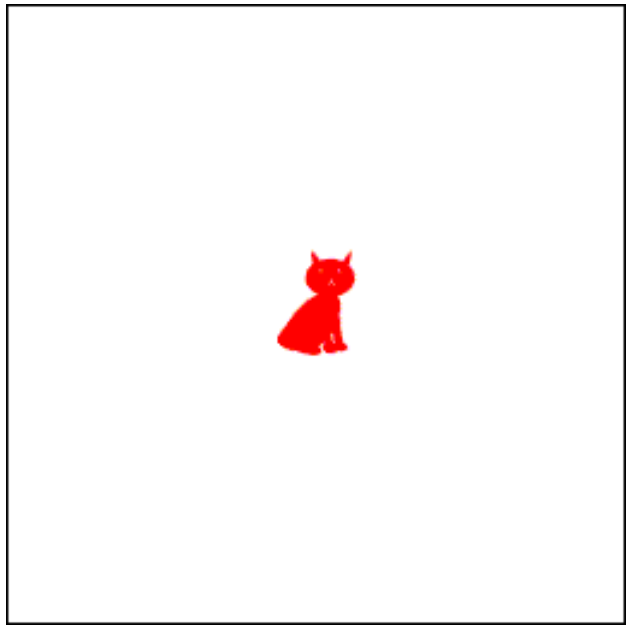


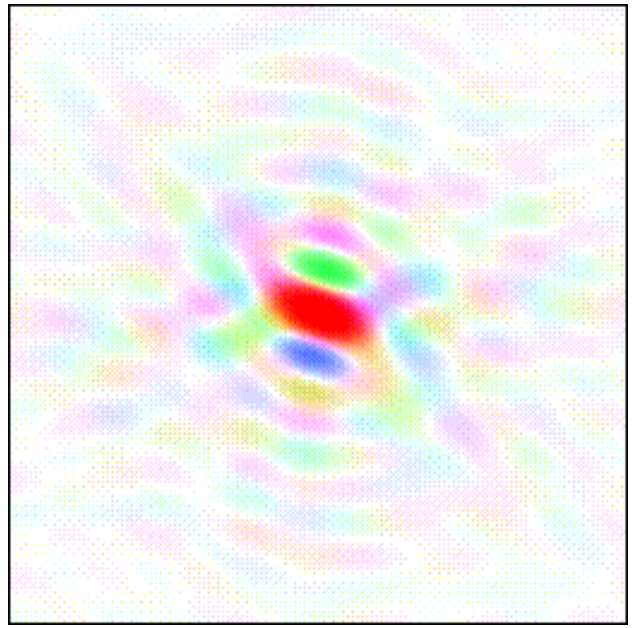


Fourier amplitudes recorded without phases

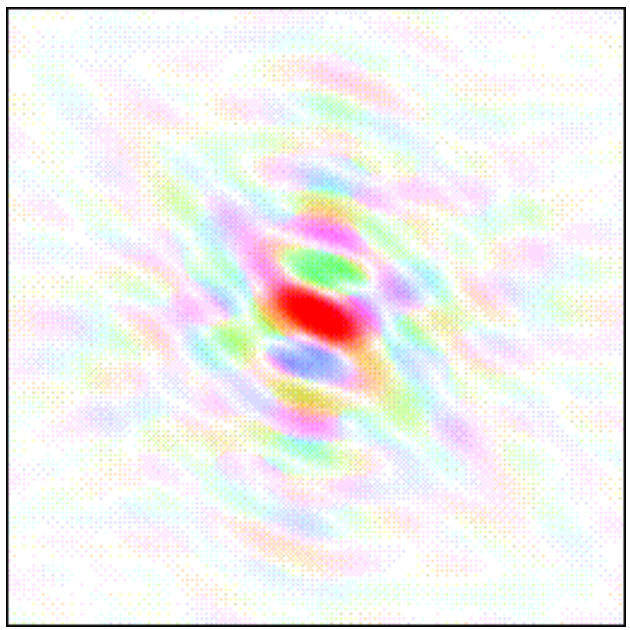
http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html

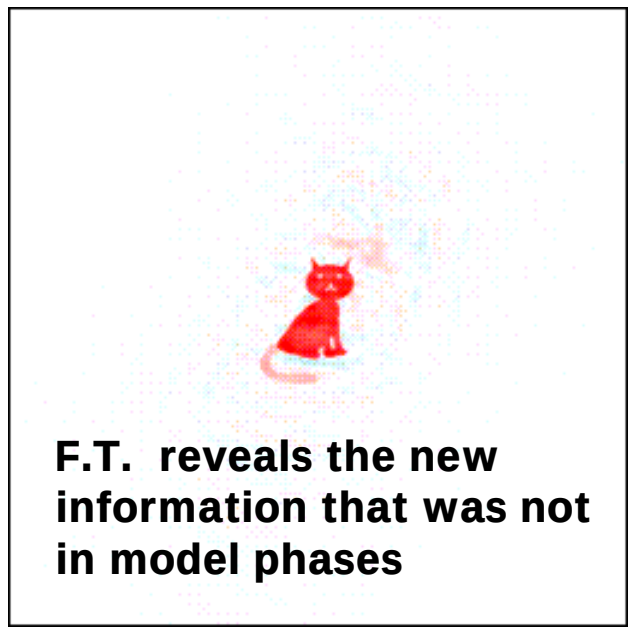
A Manx Cat (incomplete model for molecular replacement)



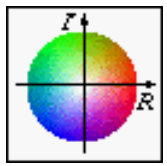


Apply Manx Phases to Cat Amplitudes





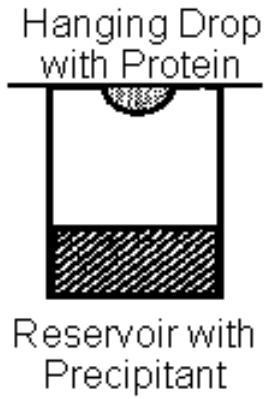
F.T. reveals the new information that was not in model phases

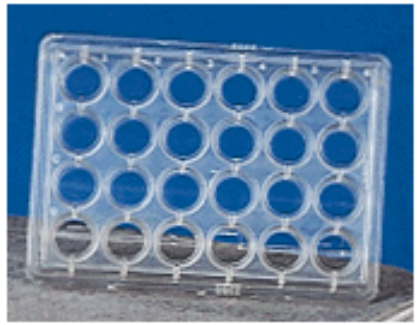


Growing Protein Crystals

Methods

- Vapor diffusion
- Dialysis
- Protein droplet under oil

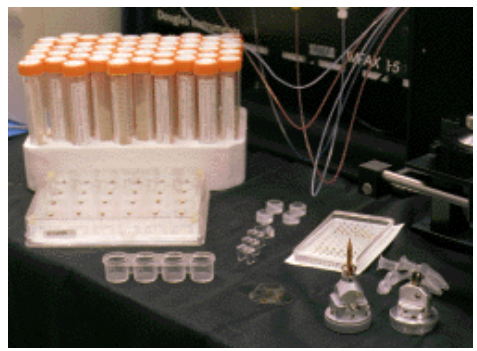




Sample Requirements

- Purity (more is usually better)
- Amount of protein (>= 1 mg. of pure protein)

Resources/Strategies



- Buy a crystallization kit:
  http://www.hamptonresearch.com/
  http://www.nextalbiotech.com/

- Let someone else do the experiment:
  http://www.decode.com/Services/Structural-Biology.php
  http://www.hwi.buffalo.edu/Research/Facilities/CrystalGrowt.html

Additional Reading

1. "Crystal Structure Analysis. A Primer." by J.P. Glusker and K.N. Trueblood. 2nd ed. (1985. Oxford University Press). (for beginners)
2. "Crystallography Made Crystal Clear." by Gale Rhodes (2nd ed. 2000. Academic Press). (for beginners)
3. "Principles of Protein X-ray Crystallography." by Jan Drenth. (1994. Springer). (advanced/beginner)
4. "Protein Crystallography." by T.L. Blundell and L.N. Johnson. (1976. Academic Press). (dated, but still useful for protein heavy atom methods)
5. Randy Read's Online Course:

   http://www-structmed.cimr.cam.ac.uk/course.html
6. Kevin Cowtan's "Book of Fourier":
   http://www.ysbl.york.ac.uk/~cowtan/fourier/fourier.html
7. Bernhard Rupp's "Crystallography 101" Course:
   http://ruppweb.dyndns.org/Xray/101index.html
8. Many Crystallographic References
   http://www.iucr.org/cww-top/edu.index.html