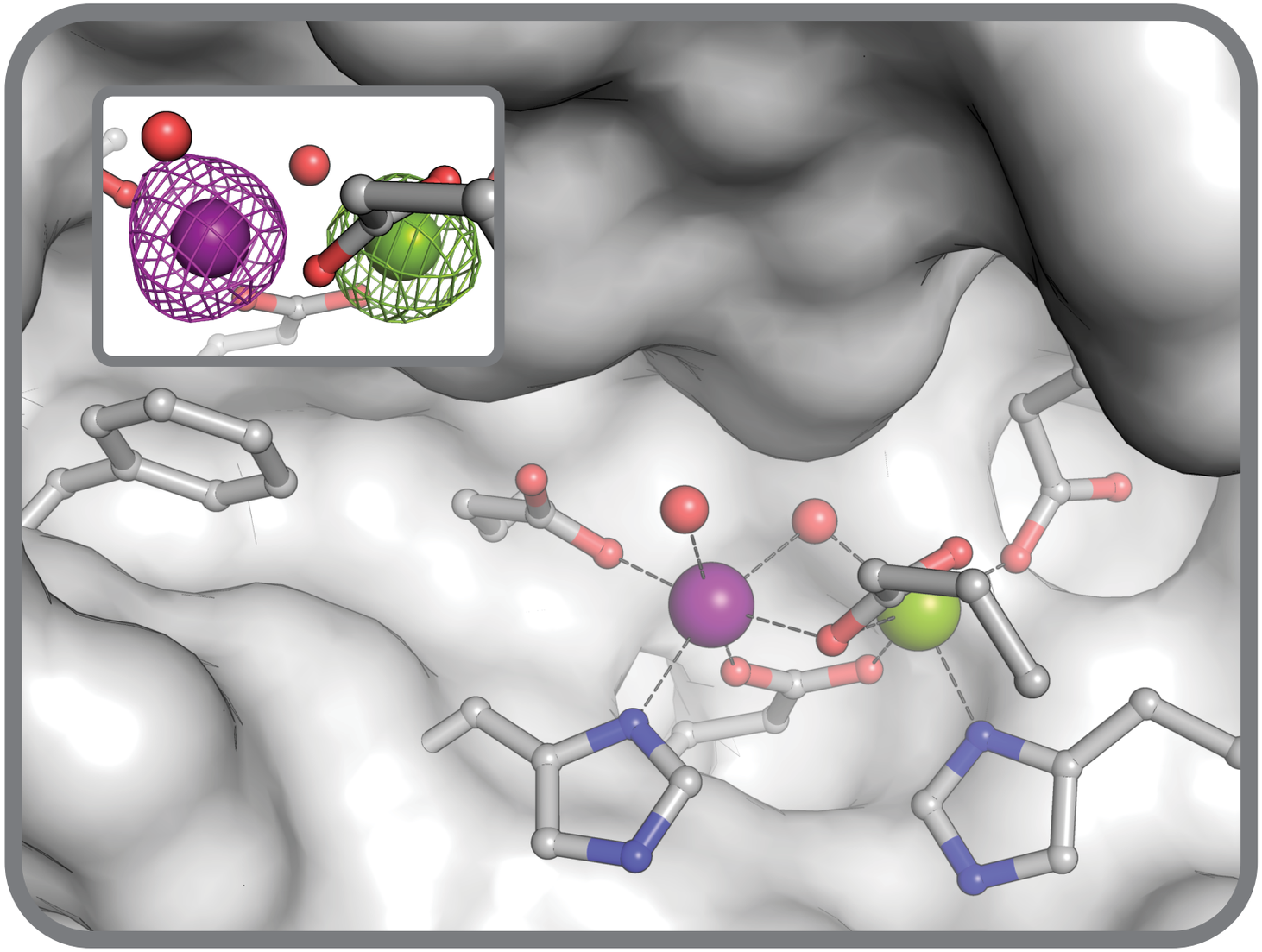
X-ray crystallography



Amie Boal
Penn State University
Oliver Einsle
University of Freiburg

May 31, 2014
2014 Penn State Bioinorganic Workshop

How do we visualize macromolecules?

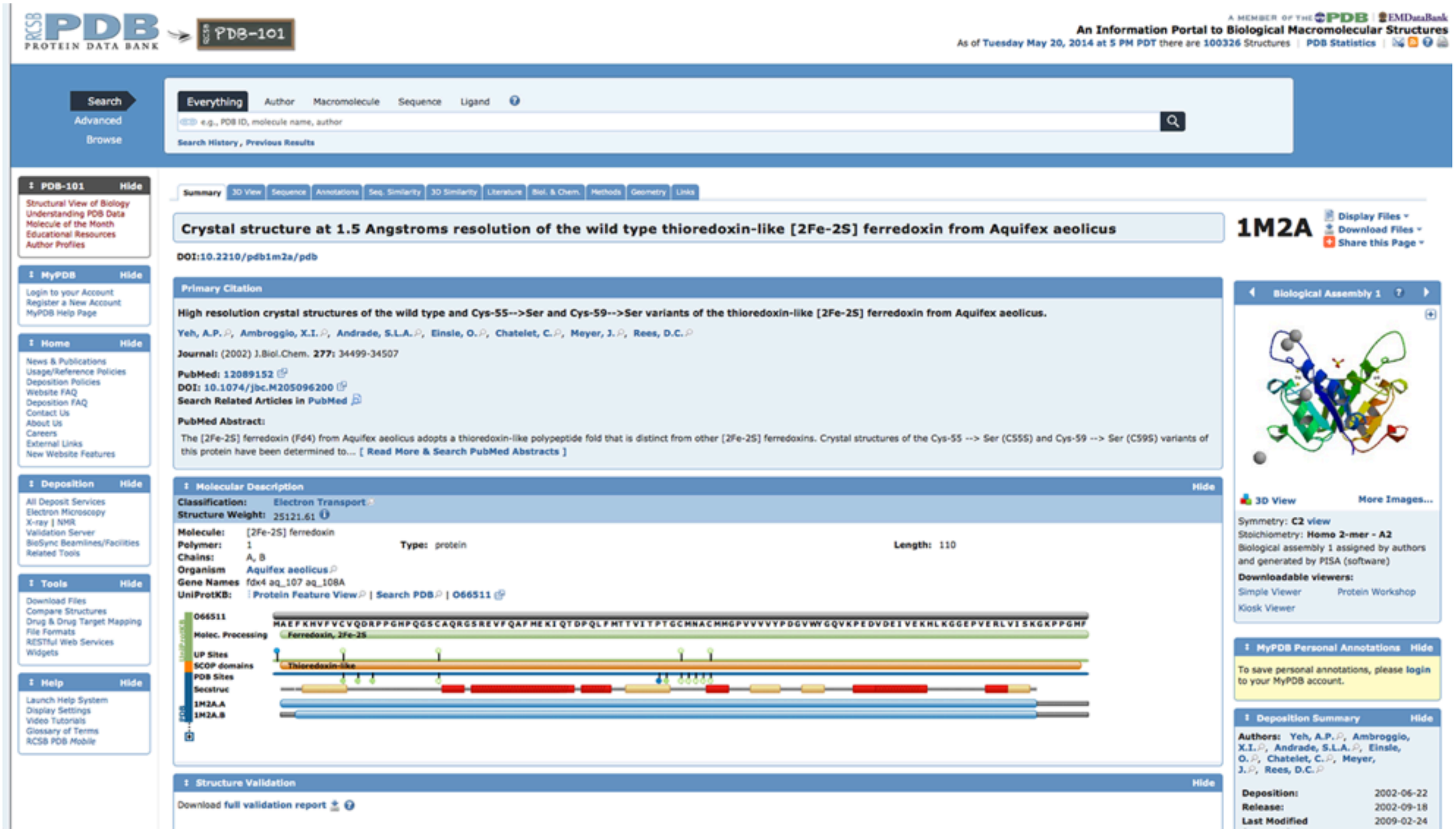
- X-ray crystallography
- NMR spectroscopy
- Electron microscopy
- Small-angle X-ray scattering
- Circular dichroism, analytical ultracentrifugation

Resolution, size range, ease of use

X-ray crystallography

- Useful with a large range of molecular sizes
- High-resolution information possible
- Ongoing effort to improve user accessibility
- Many available tools to view and assess results
  - Molecular models
  - Electron density maps

Protein Data Bank



Protein Data Bank

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ATOM      3  C   ALA A   1       8.846  -3.642  47.425  1.00 84.01           C
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ATOM     13  OE1 GLU A   2       5.478  -2.199  44.371  1.00 91.34           O
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ATOM     35  N   HIS A   5       5.218   4.321  40.862  1.00 12.52           N
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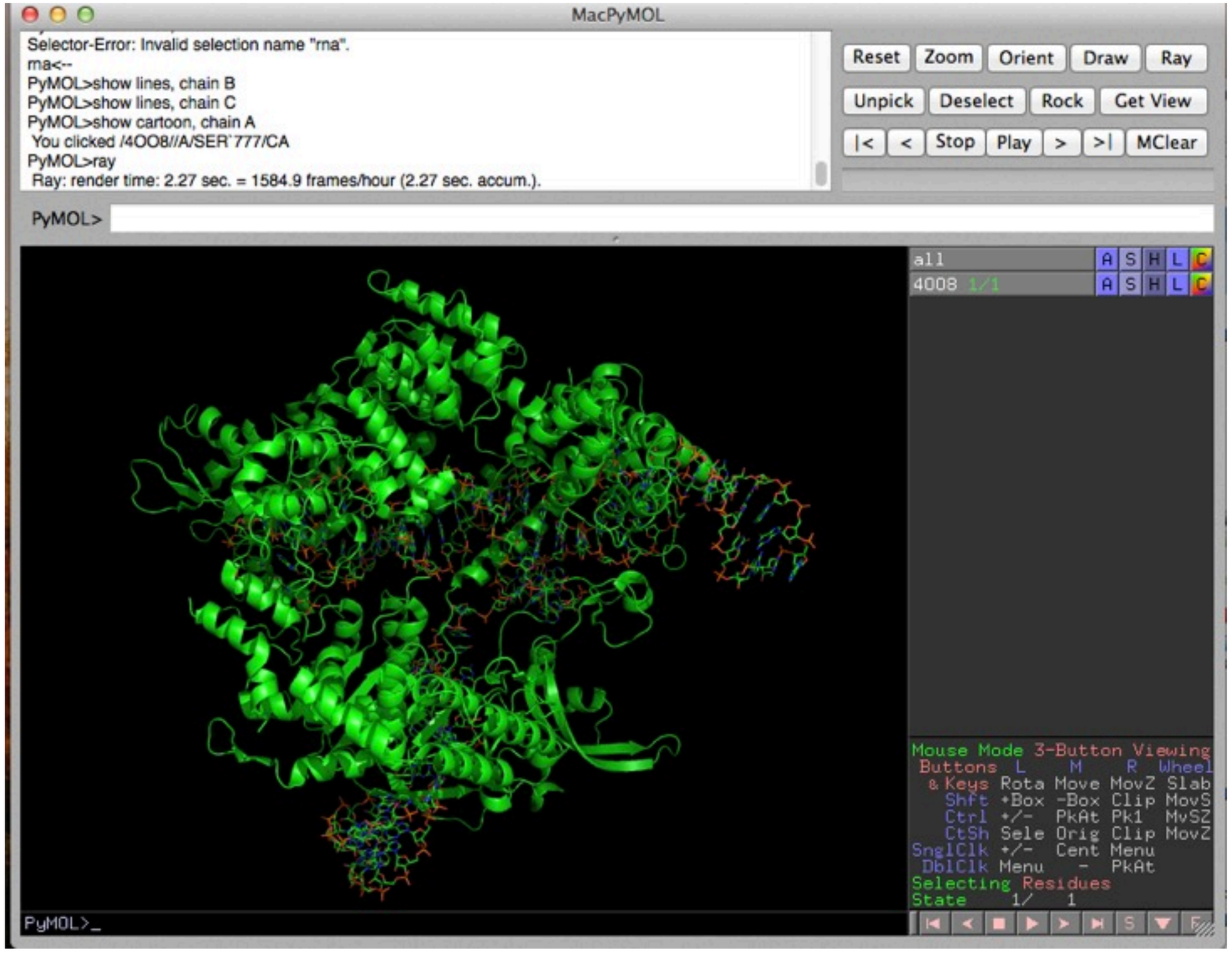
ATOM     37  C   HIS A   5       4.027   6.216  40.137  1.00  8.86           C
```

PDB accession code 1M2A

Model viewing

- PyMOL Molecular Graphics System
  - www.pymol.org
- Chimera
  - www.cgl.ucsf.edu/chimera/
- Coot
  - www2.mrc-lmb.cam.ac.uk/Personal/pemsley/coot/
- Other resources
  - www.rcsb.org/pdb/static.do?p=software/software_links/molecular_graphics.html

Model viewing



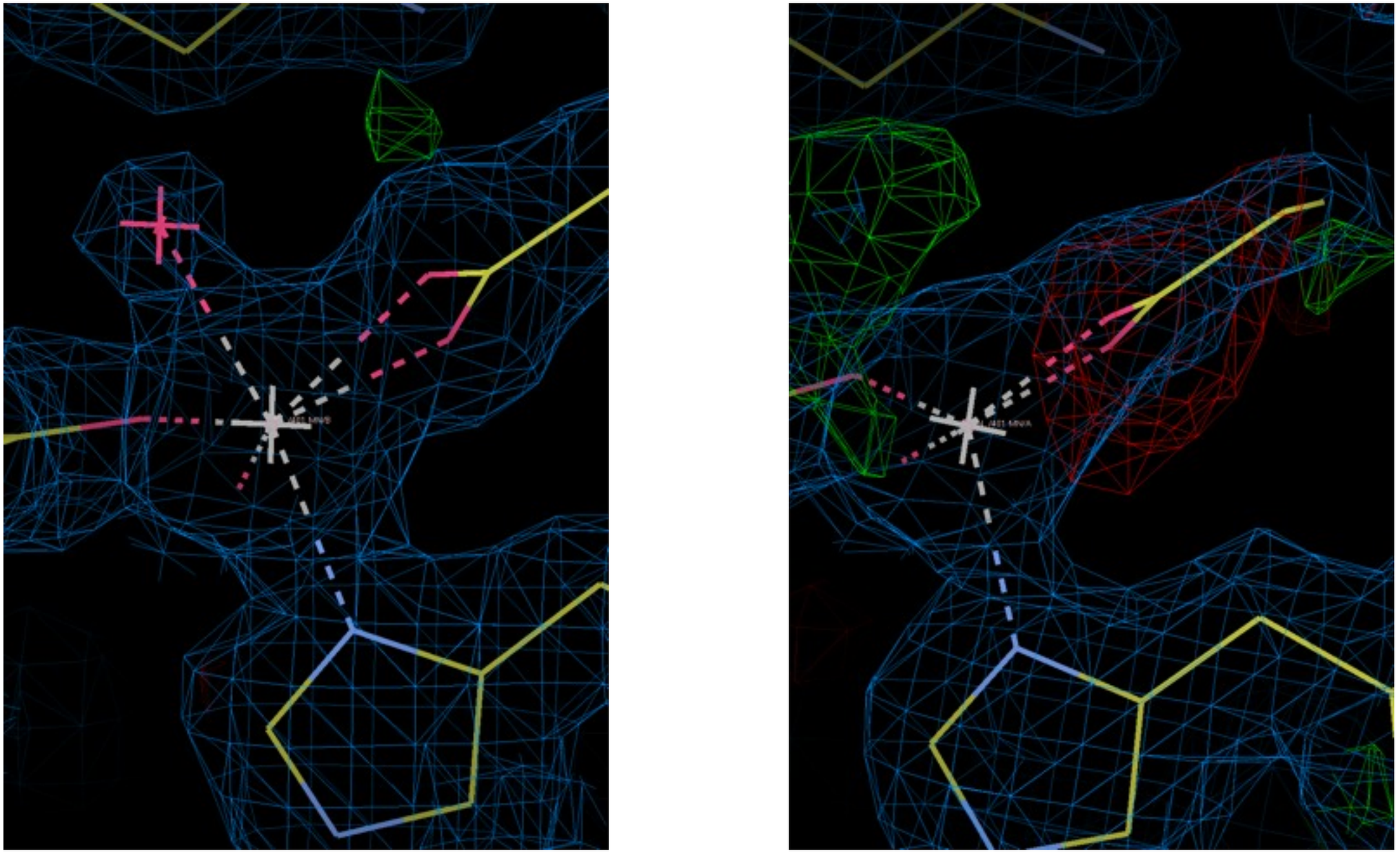
Limitations

- Static snapshot of dynamic molecules
- Model is an average of all molecules in crystal lattice
- Obtained under specific conditions - possibly far from biological relevance
- Structure can be influenced by crystallization and data collection process
- Resulting model is an interpretation of data

Electron density map viewing

- Electron density server
  - eds.bmc.uu.se/eds/
- Swiss PDB viewer
  - http://spdbv.vital-it.ch/
- Coot
  - www2.mrc-lmb.cam.ac.uk/Personal/pemsley/coot/

Map viewing



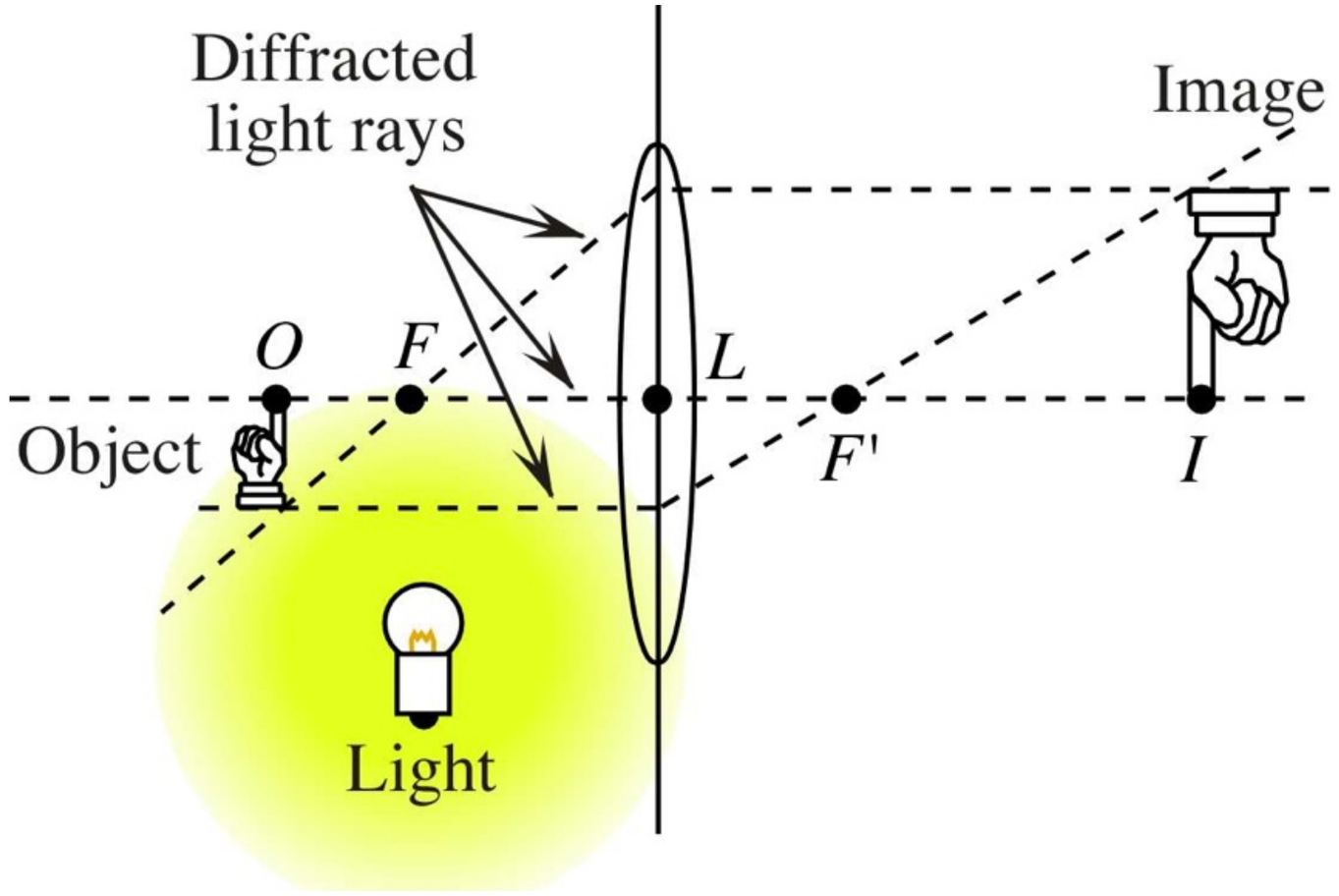
PDB accession code 4DR0

Metalloprotein crystallography

- Starting point for mechanistic/computational study
- Inspiration for synthetic models
- Models for other biophysical techniques
- Location, identity, stoichiometry of metallocofactors
- Geometry and identity of ligands
- Structures of complex metallocofactors

Sommerhalter, M., Lieberman, R.L., and Rosenzweig, A.C. (2005) *Inorg. Chem. 44*, 770-778.

Microscopy analogy



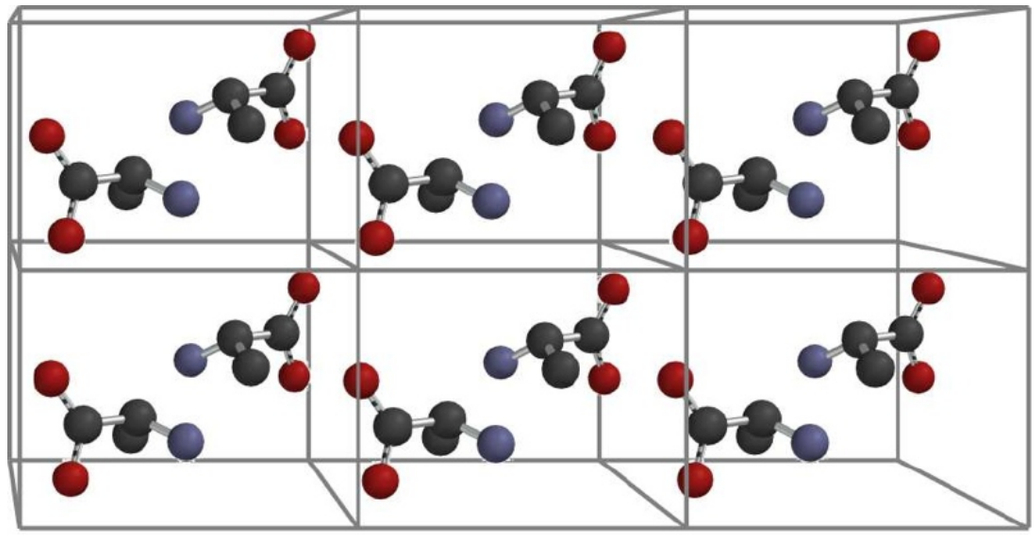
Wavelength of light rays must be appropriate for object size

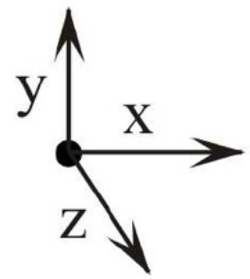
X-rays are used to visualize atomic-level structure in macromolecules

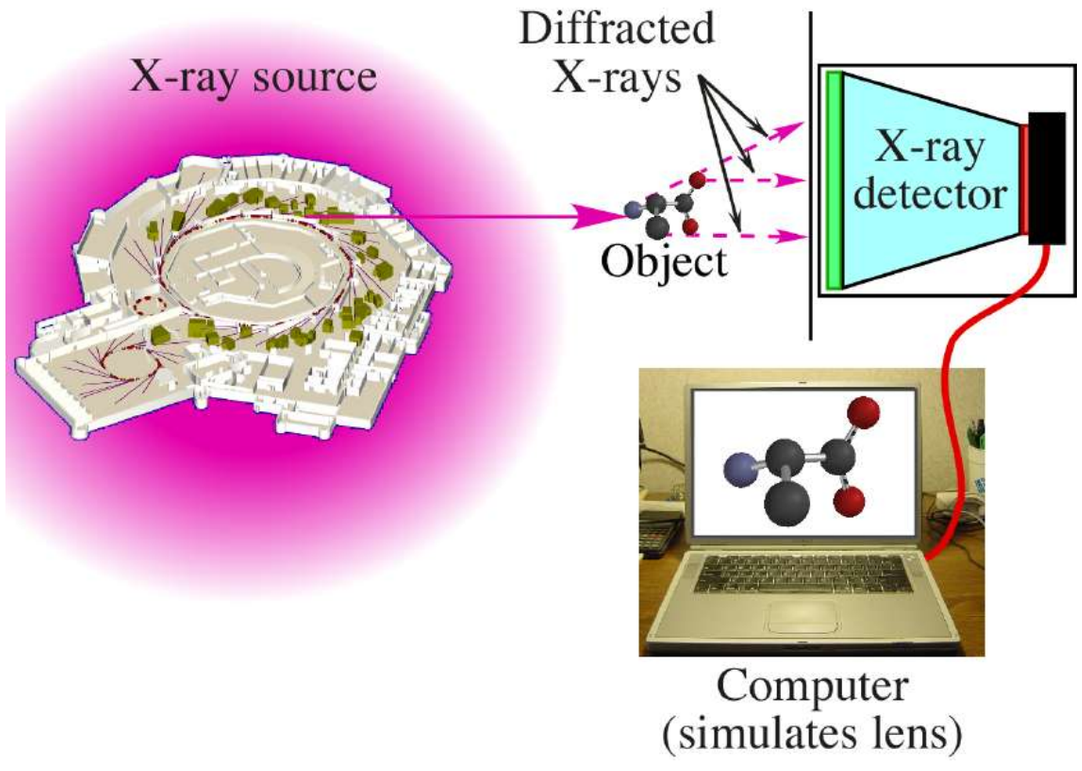
Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

1. Single molecules diffract X-rays weakly

Crystal lattice amplifies signal







2. X-rays can't be focused by lenses

Structure solution facilitated by mathematical methods

Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Crystallography workflow

- Biological sample production
- Crystallization
- X-ray diffraction data collection
- Structure solution/phase determination
- Model building and validation

Other resources

- Workshops and courses
  - APS CCP4 crystal school - www.ccp4.ac.uk/schools/APS-school/
  - NSLS RapidData - www.bnl.gov/rapidata/content/announcement.asp
  - CSH course - meetings.cshl.edu/courses/2014/c-crys14.shtml

Other resources

- Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed.
- Rupp, B. (2010) Biomolecular Crystallography
- Metalloprotein crystallography
    - Sommerhalter, M., Lieberman, R.L., and Rosenzweig, A.C. (2005) *Inorg. Chem. 44*, 770-778.

Crystallography workflow

- Biological sample production
- Crystallization
- X-ray diffraction data collection
- Structure solution/phase determination
- Model building and validation

Biological sample production

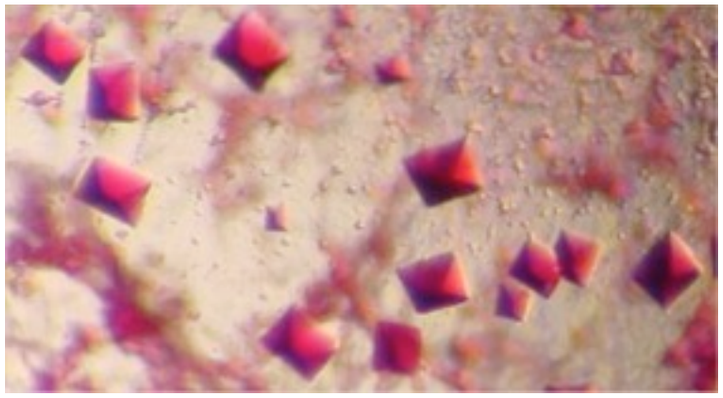
- Construct/sequence selection
- Source organism
- Metallocofactor incorporation
- Affinity tags and purification scheme

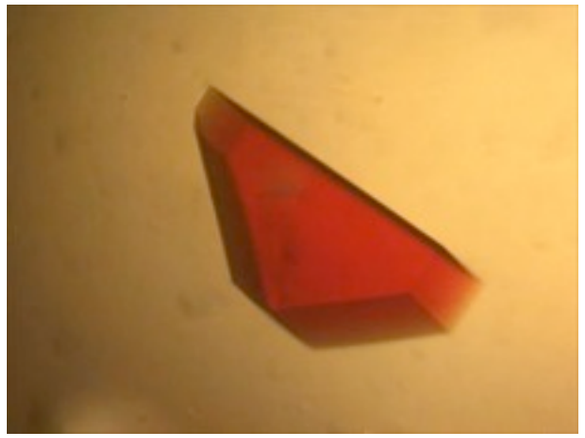
Must assess purity, polydispersity, activity

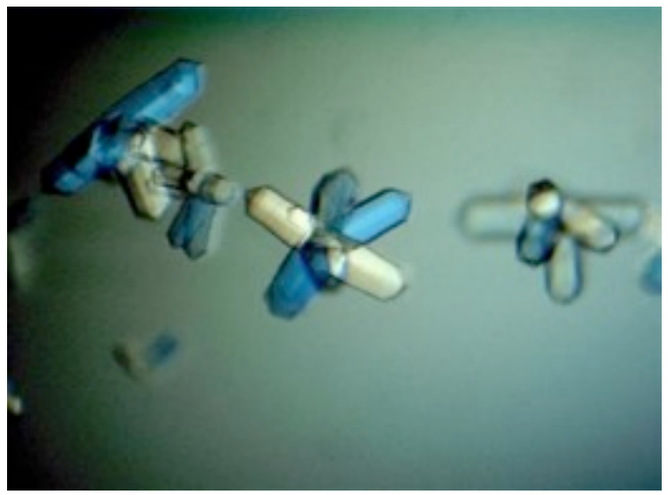
Crystallography workflow

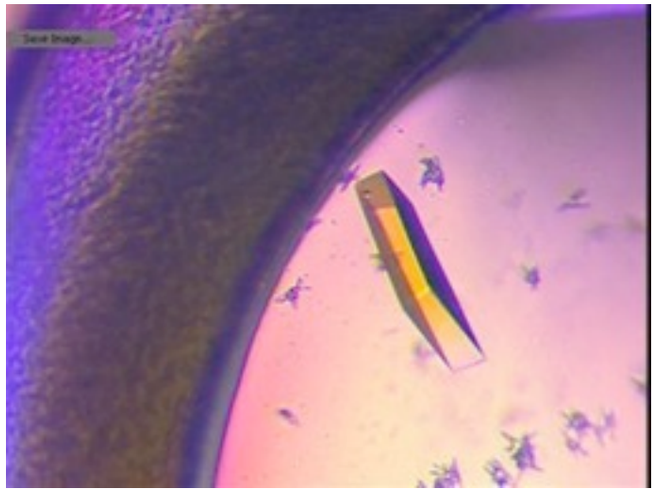
- Biological sample production
- Crystallization
- X-ray diffraction data collection
- Structure solution/phase determination
- Model building and validation

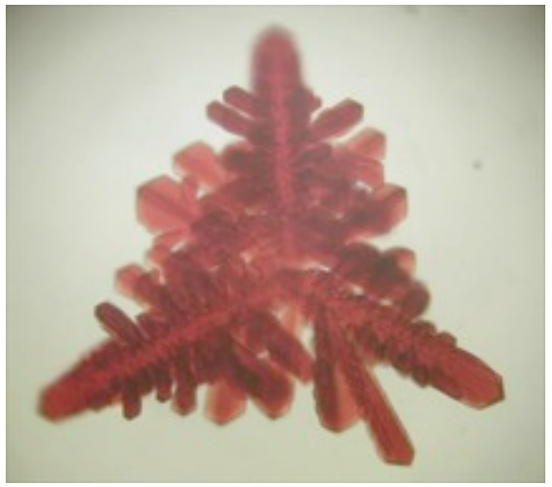
Macromolecular crystals







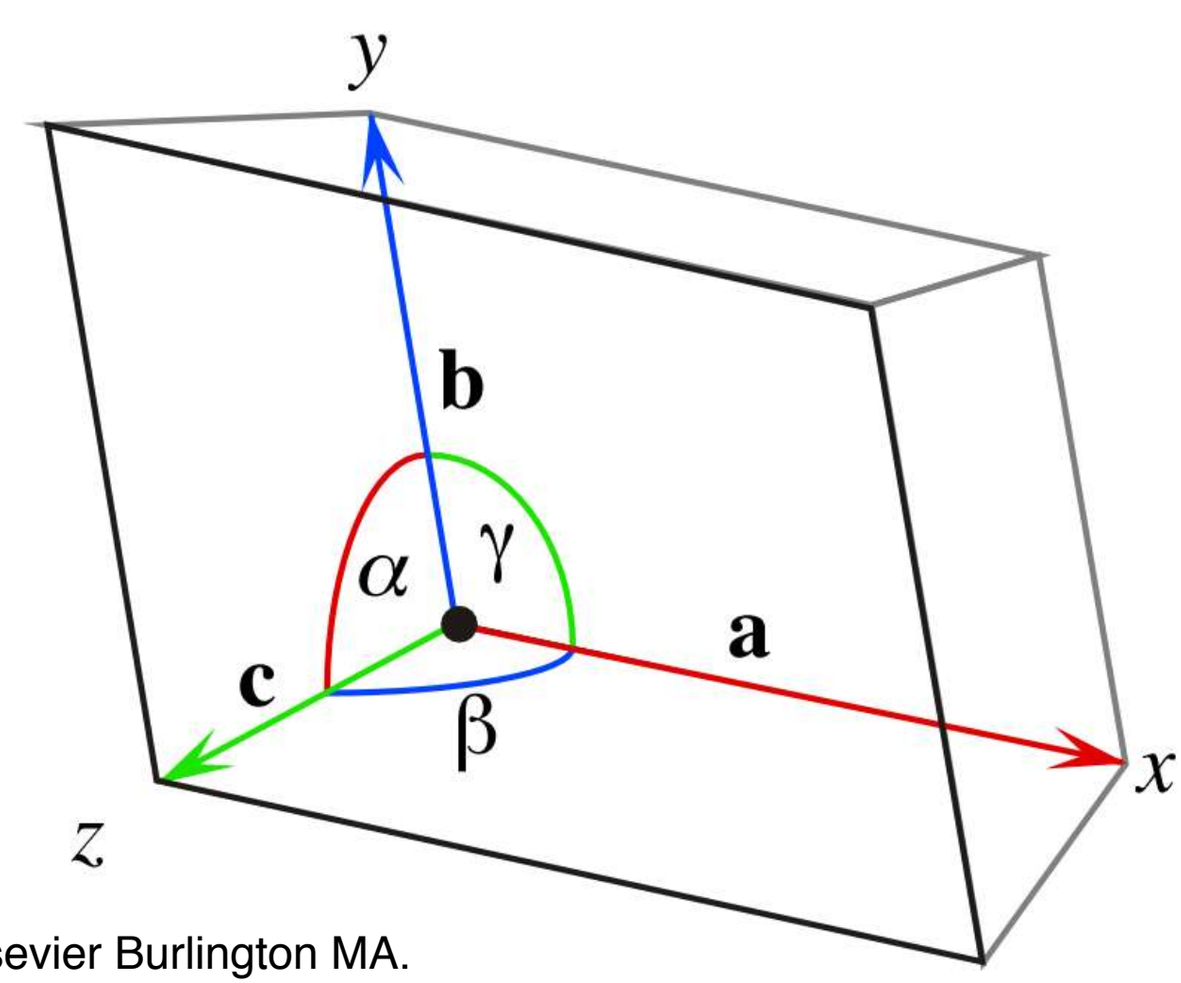




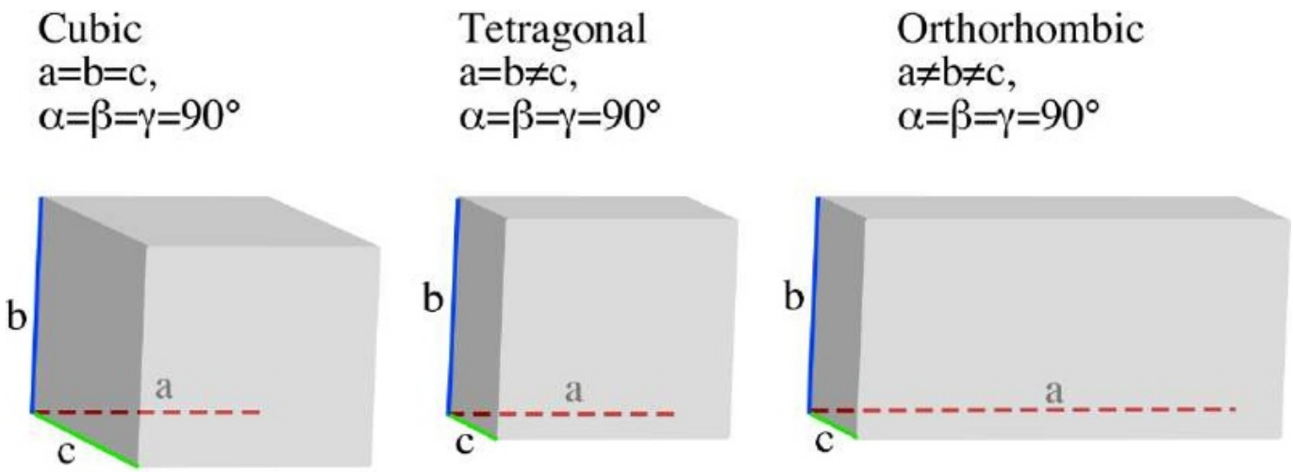
Hampton Research

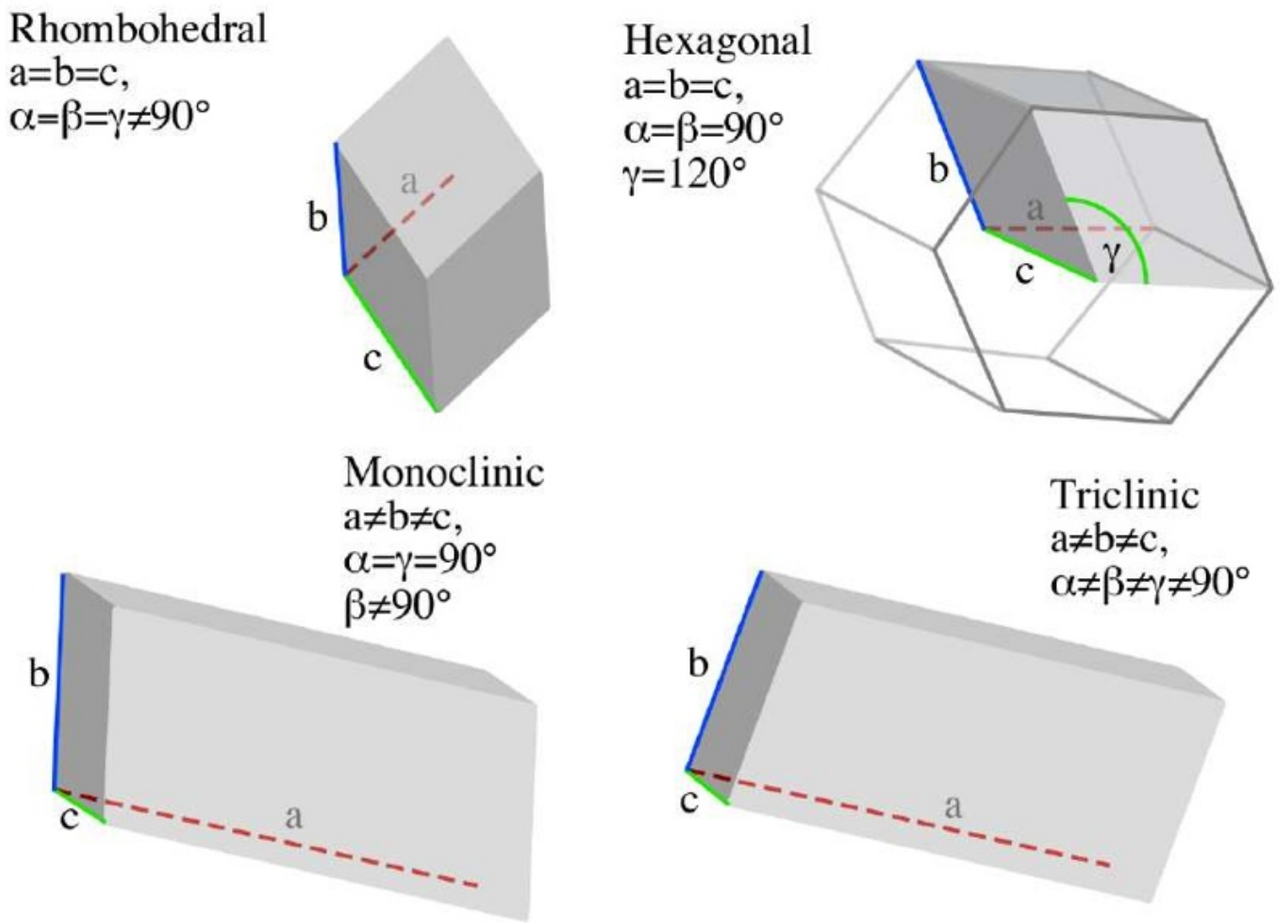
Crystallization

- A macromolecular crystal is an ordered 3D array of molecules held together by weak interactions
- Made up of unit cells
- Cells are the repeating unit
- Unit cells have a defined shape
- x, y, z coordinate system



Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.





14 different lattice systems
- Bravais lattice

Defined by unit cell shape

a = b = c relationships are identities

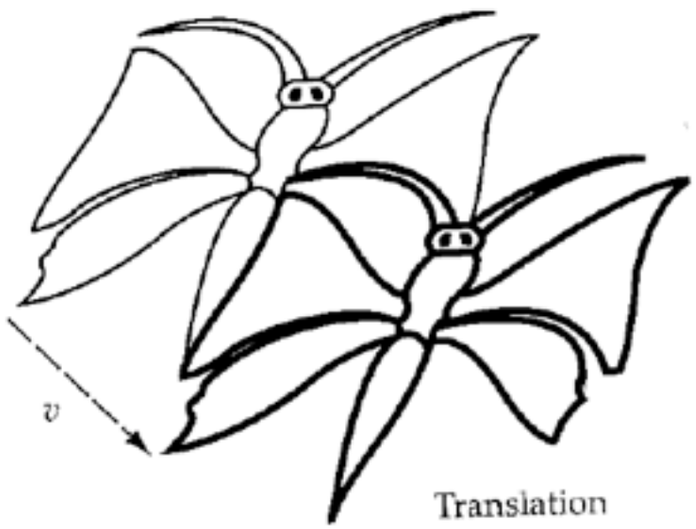
If a = b, unit cell contents along axes are identical

Can have internal symmetry
- multiple copies of protein in unit cell
- related by symmetry operators

Final model only describes structure of asymmetric unit

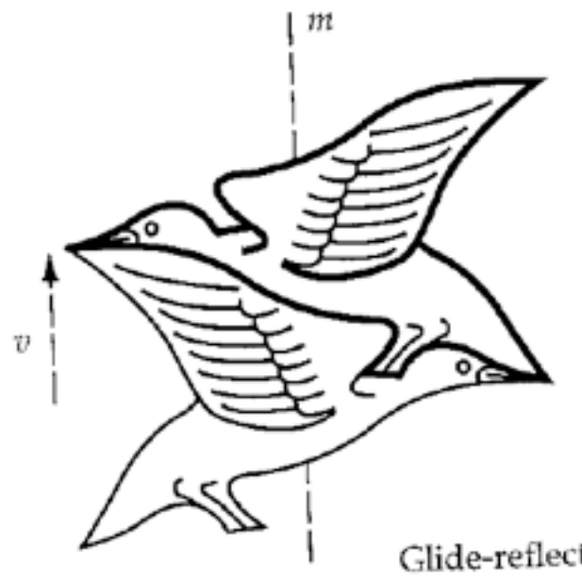
Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Symmetry operators



Translation

Reflection



Glide-reflection

Rotation

Schattschneider, D. (2004) M.C. Escher: Visions of Symmetry, 2nd Ed. University of Michigan Press.

Space groups

- 230 possible space groups
- 65 for chiral molecules
- Designated by symbols that describe lattice type and symmetry operators
  - $P2_12_12_1$
  - $C2$
- Must assign space group correctly to solve structure

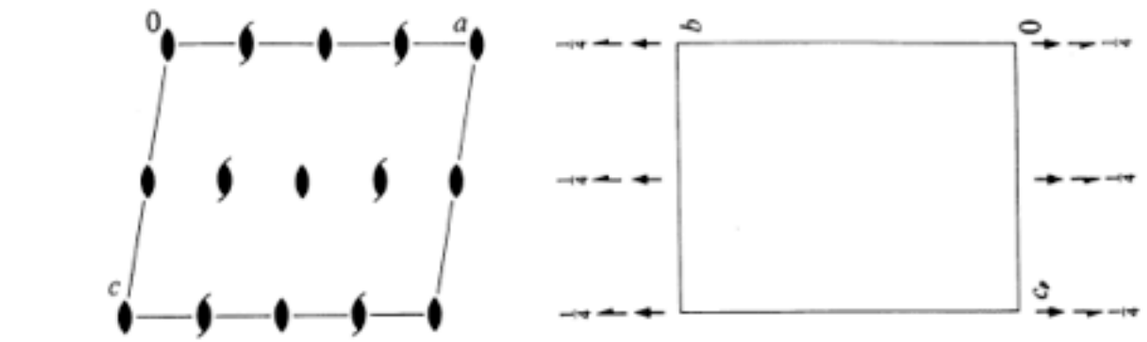
Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

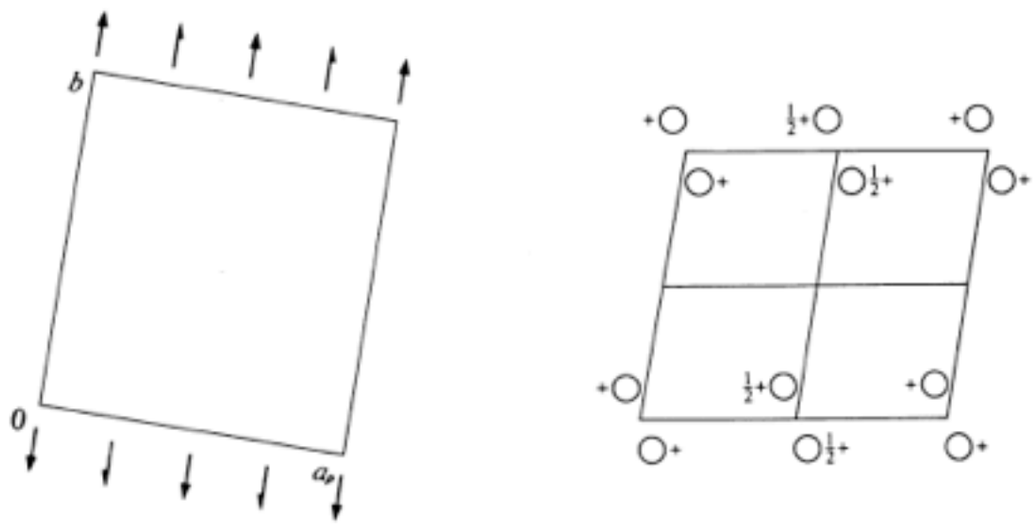
Wukovitz, S., and Yeates, T. (1995) *Nat. Struct. Biol. 2*, 1062-1067.

C2 space group

$C2$	C_2^3	2	Monoclinic
No. 5	$C121$		Patterson symmetry $C12/m1$

UNIQUE AXIS b, CELL CHOICE 1





Origin on 2

Asymmetric unit $0 \le x \le \frac{1}{2}$; $0 \le y \le \frac{1}{2}$; $0 \le z \le 1$

Symmetry operations

For $(0,0,0)+$ set

(1) 1 (2) 2 0,y,0

For $(\frac{1}{2},\frac{1}{2},0)+$ set

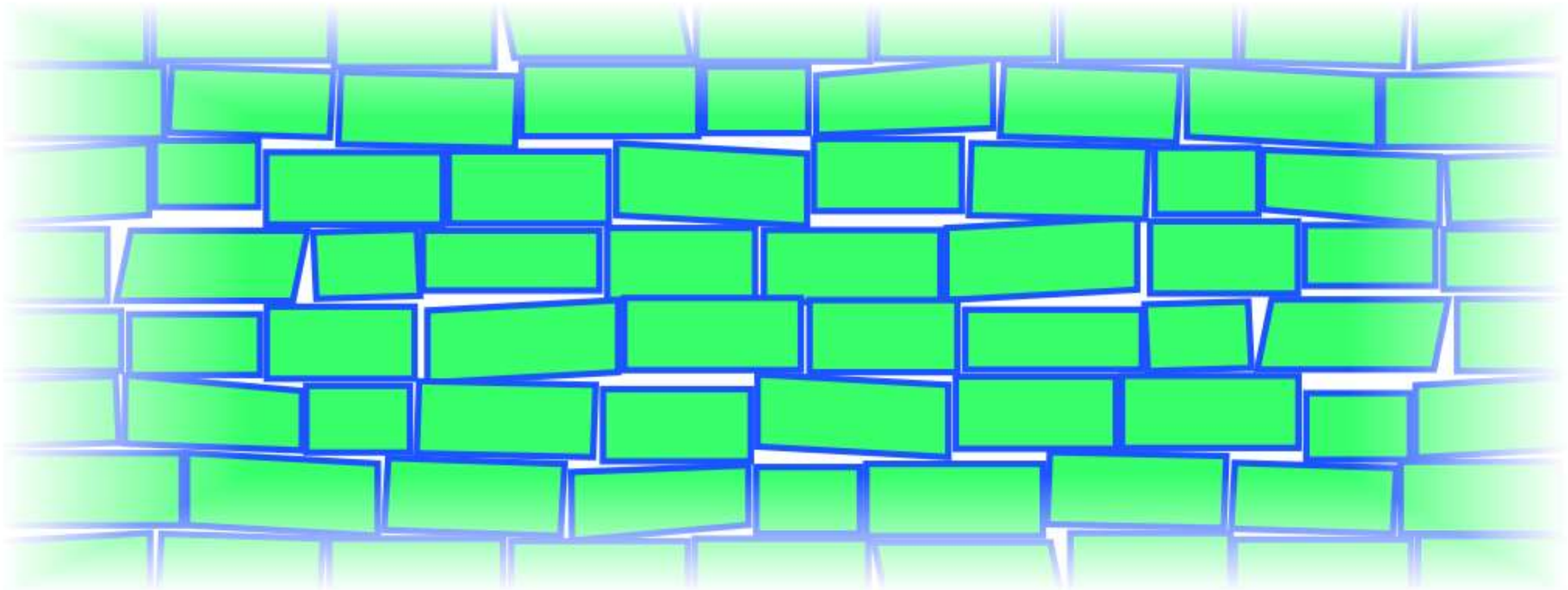
(1) $t(\frac{1}{2},\frac{1}{2},0)$ (2) $2(0,\frac{1}{2},0)$ $\frac{1}{4}$,y,0

Positions

Multiplicity, Wyckoff letter, Site symmetry		Coordinates	
		$(0,0,0)+$	$(\frac{1}{2},\frac{1}{2},0)+$
4 c 1	(1) x,y,z	(2) $\bar{x},y,\bar{z}$	

International Union of Crystallography (2006) Vol. A, pp. 124-131.

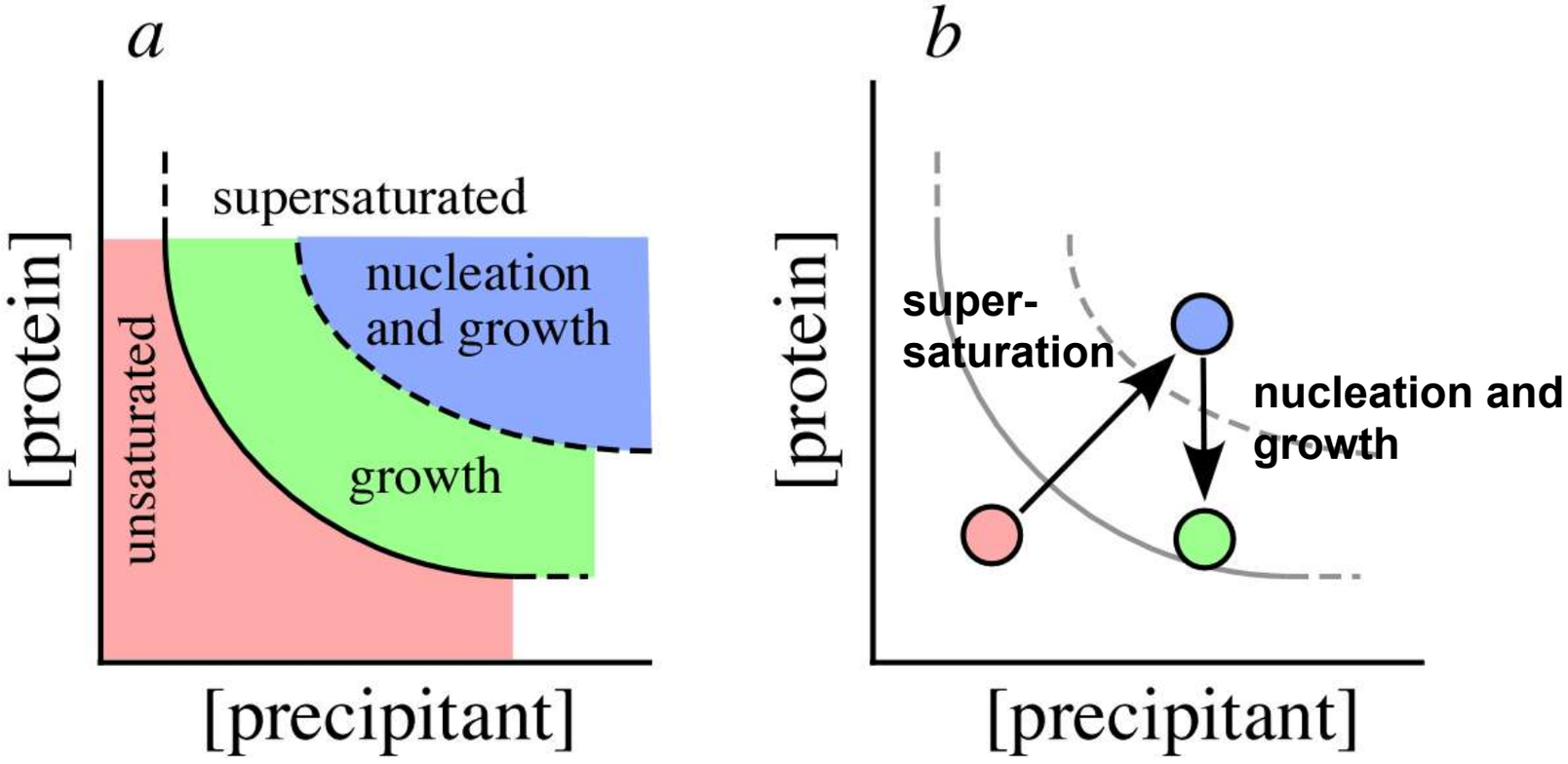
Crystallization



- Lattice is array of unit cells - an imperfect one!
- Crystallographic experiment provides an image of average electron density in the asymmetric unit

Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Crystallization



- Supersaturation, nucleation, growth
- Slow controlled precipitation from solution without unfolding the molecule

Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Precipitants

- Salts (ammonium sulfate)
- Polyethylene glycol
- Small organic compounds
- Additives, pH, temperature important

Disrupt biomolecule hydration layer

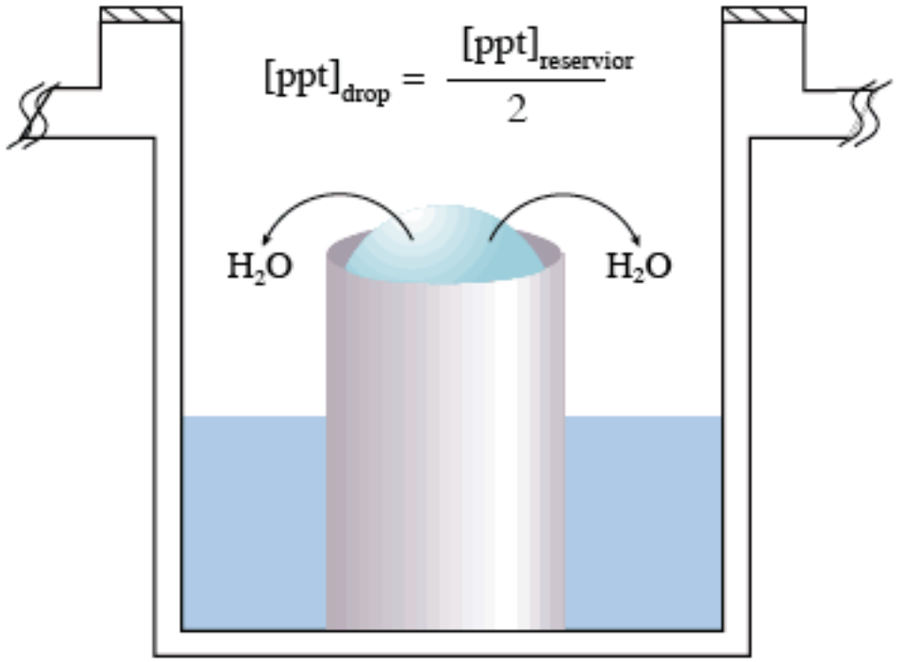
Force self-association without denaturation

Can affect structure

Crystallization

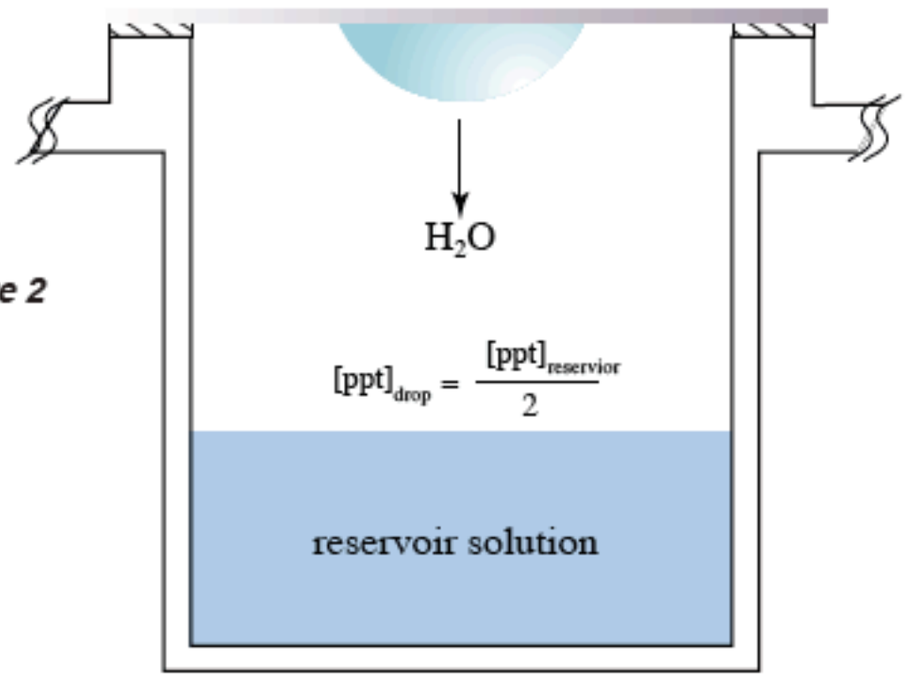
Vapor diffusion is the most commonly used method

figure 1



Sitting drop

figure 2



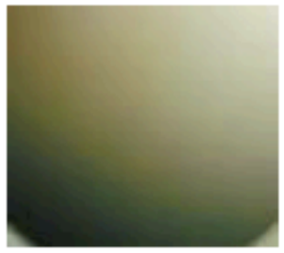
Hanging drop

A crystallization experiment takes days/weeks/months

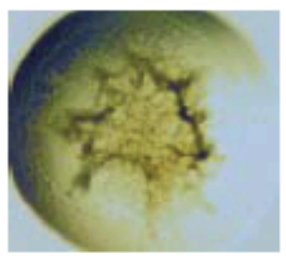
Hampton Research

Crystallization

Spectrum of possible outcomes in a crystallization screen


Clear Drop


Skin / Precipitate


Precipitate


Precipitate / Phase

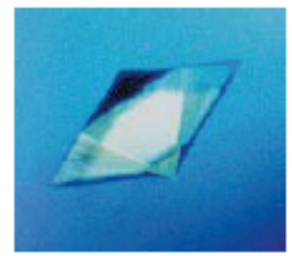

Quasi Crystals


Microcrystal


Needle Cluster


Plates

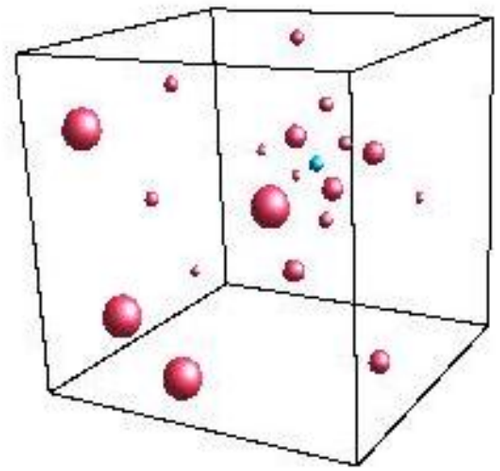

Rod Cluster

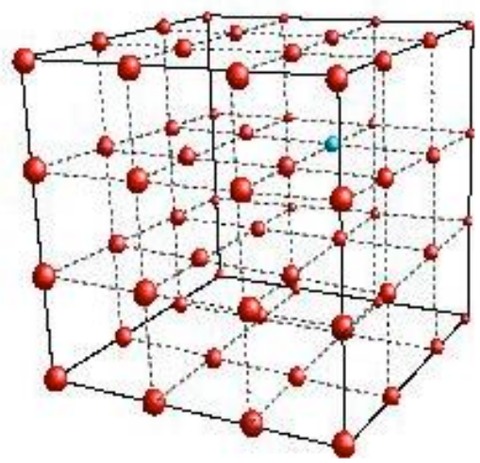

Single Crystal

Hampton Research

Finding the precipitant

- Trial-and-error approach
- Sparse-matrix screens sample “condition space”
- May also require sampling of “sequence space”

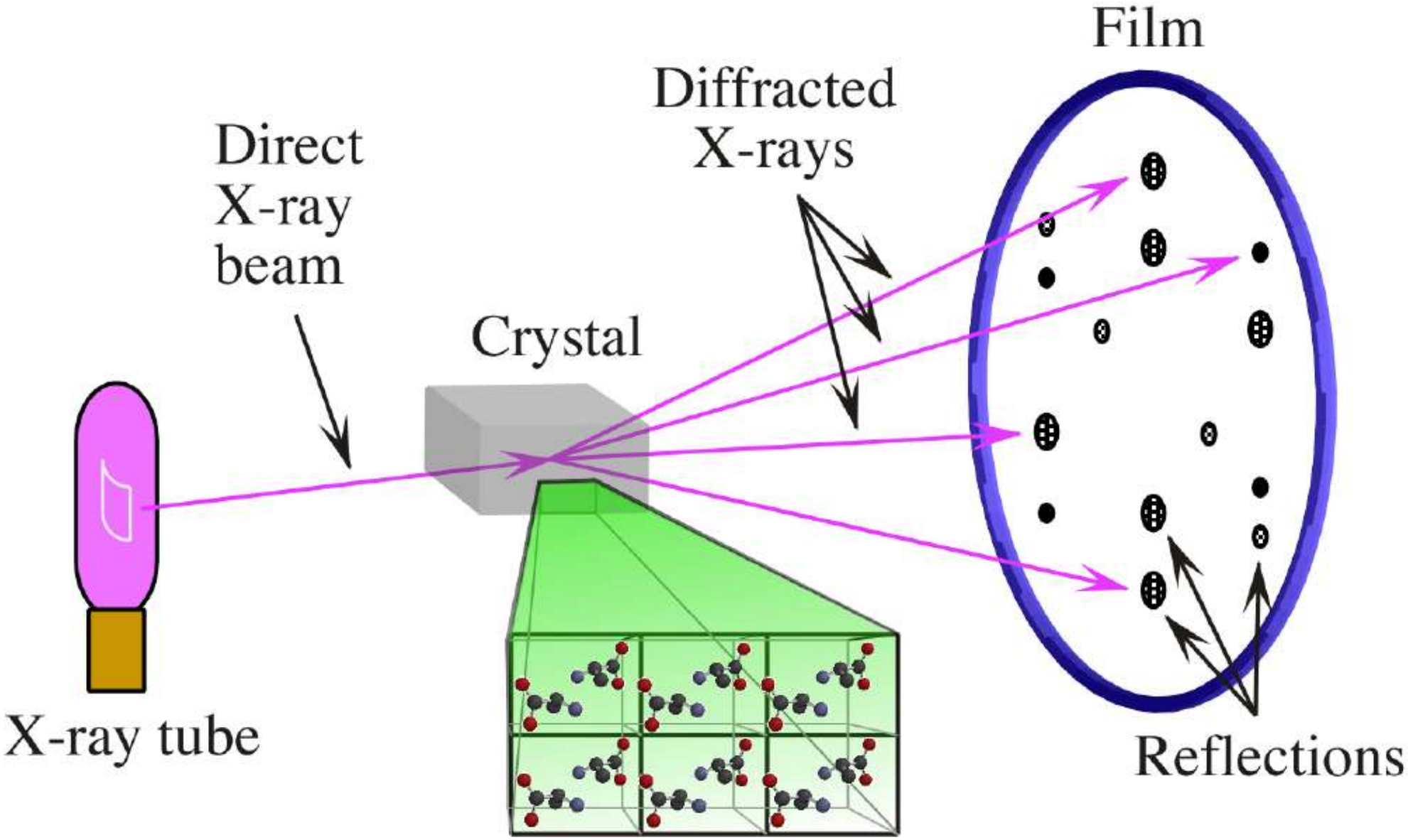




Crystallography workflow

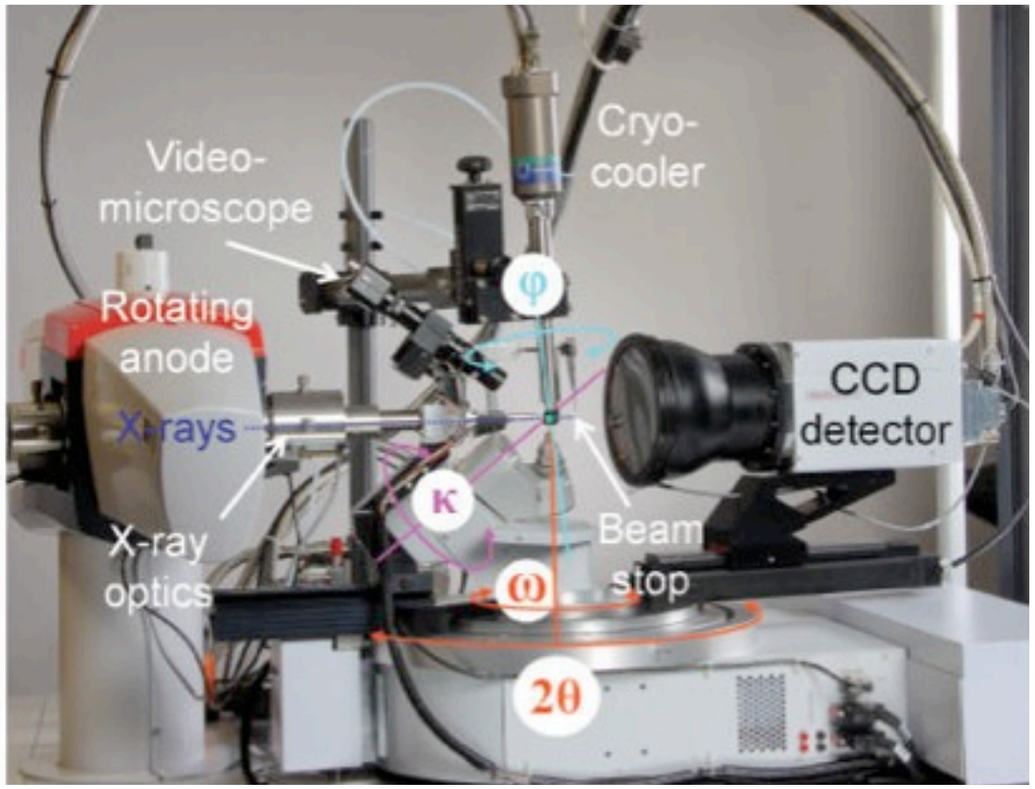
- Biological sample production
- Crystallization
- X-ray diffraction data collection
- Structure solution/phase determination
- Model building and validation

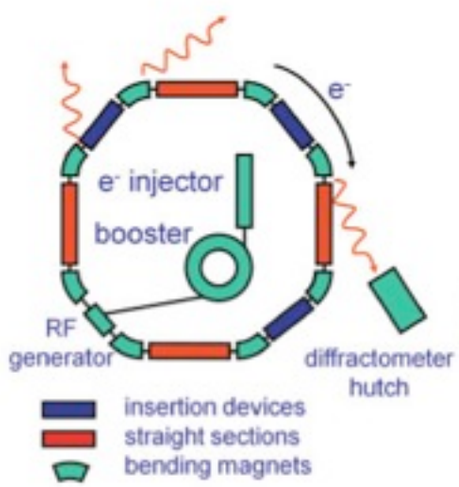
X-ray diffraction data

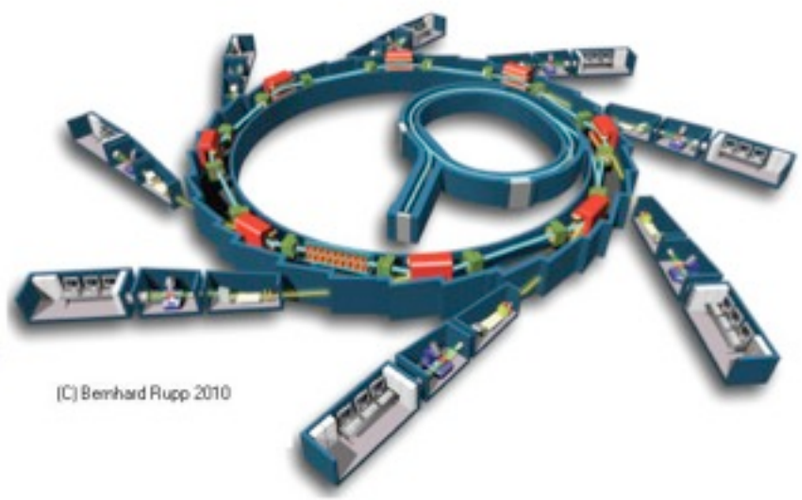


Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Where are data collected?

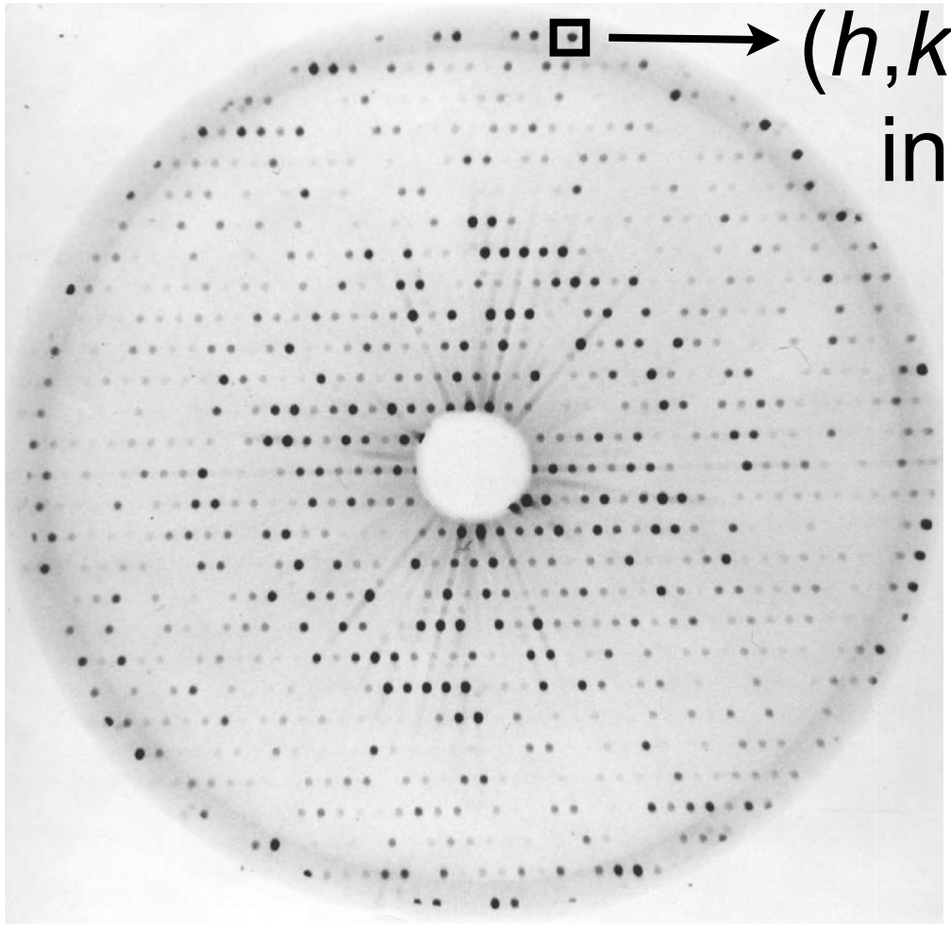






X-ray diffraction data

- What do we look for in a diffraction pattern?
  - spot shape
  - single lattice
  - resolution limit



⊡ ⟶ (*h*,*k*,*l*) index, intensity

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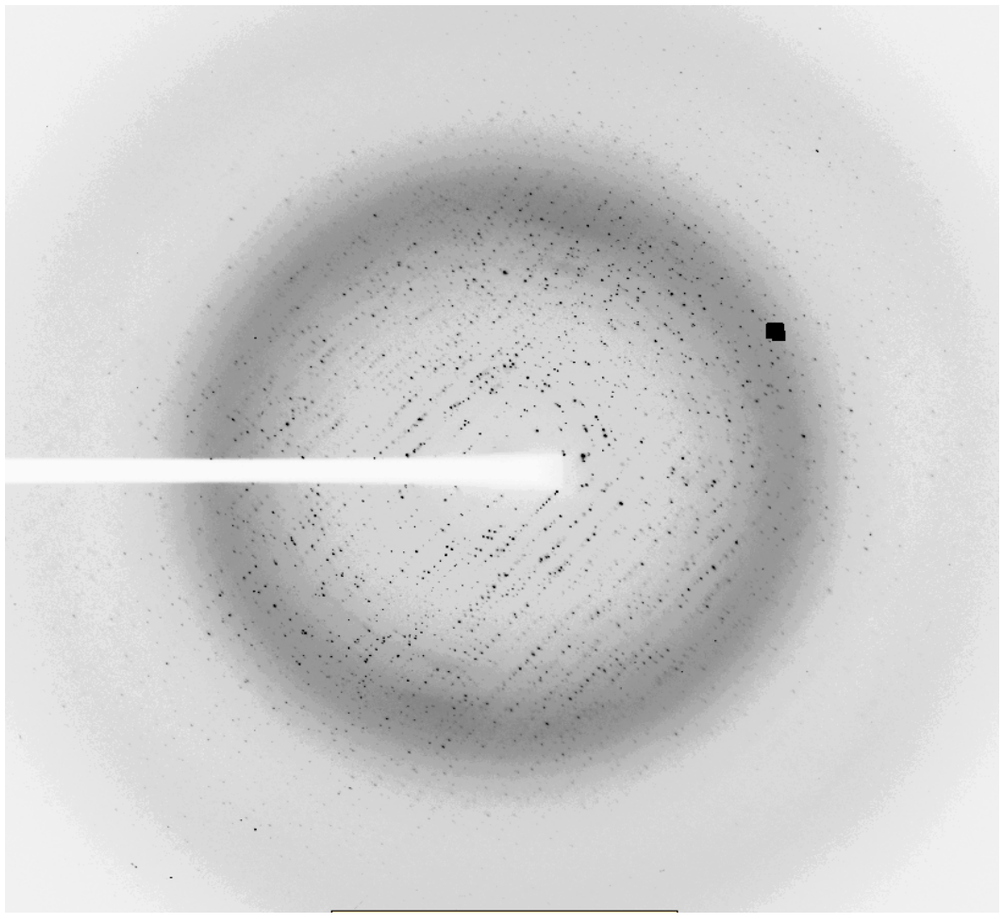
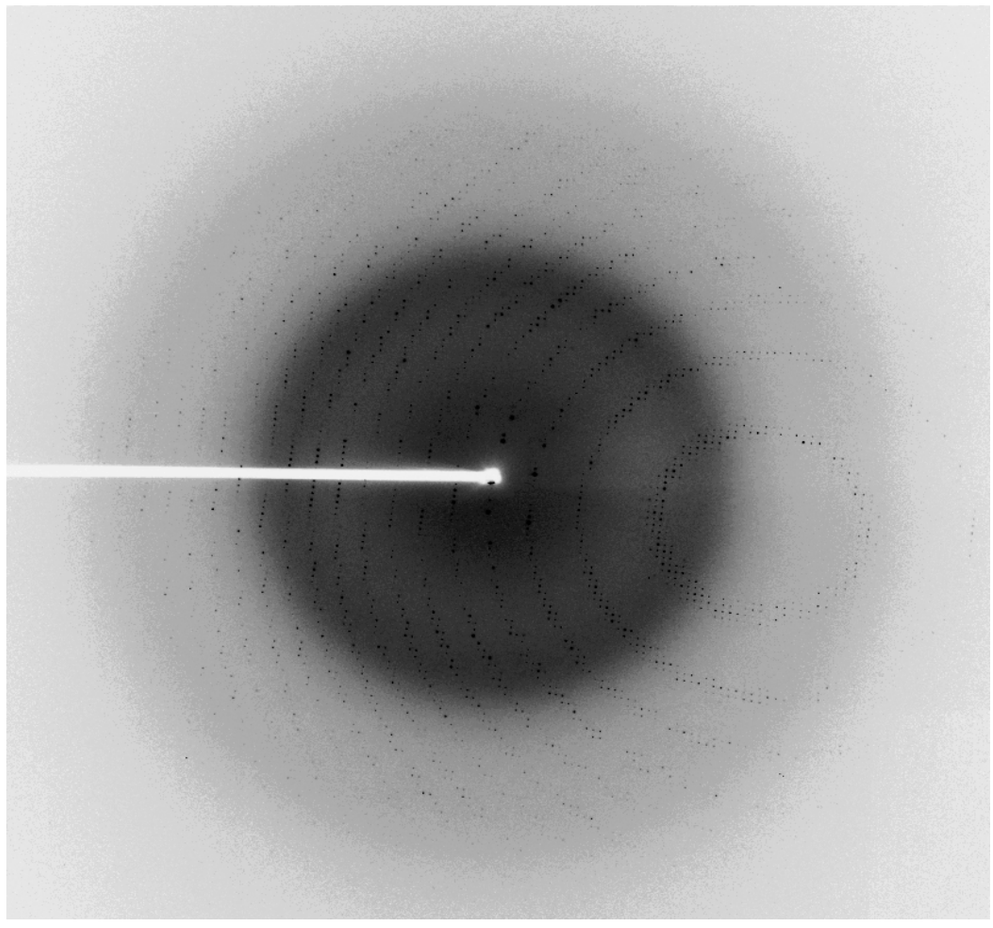
Good and bad diffraction



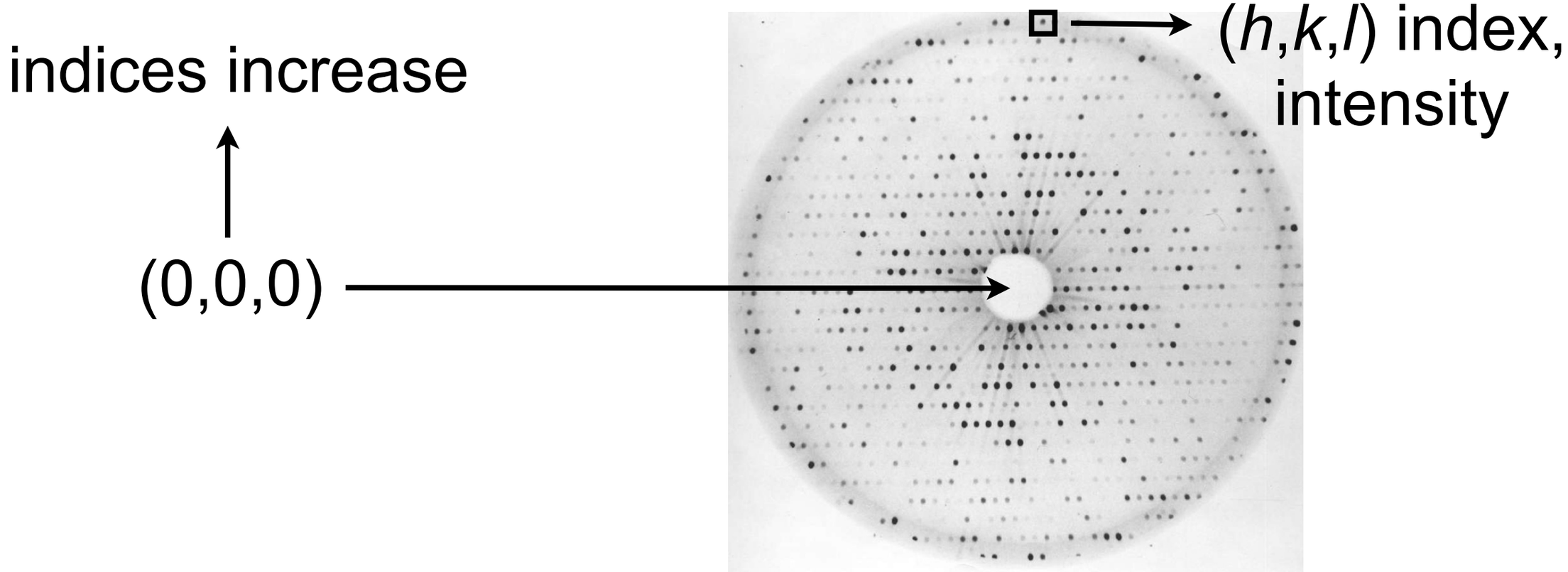
N-terminal His-tagged *B. subtilis* NrdF old prep



N-terminal His-tagged *B. subtilis* NrdF new prep
+ MonoQ purification step

X-ray diffraction data

- Each spot is a reflected X-ray that is given an index
- Indices provide information about the crystal lattice
- Intensities provide information about positions of atoms



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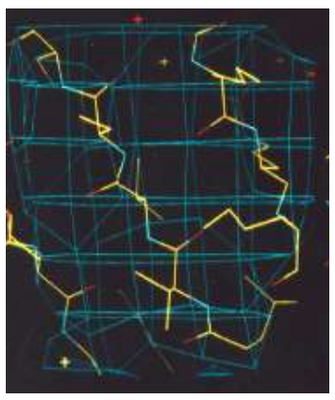
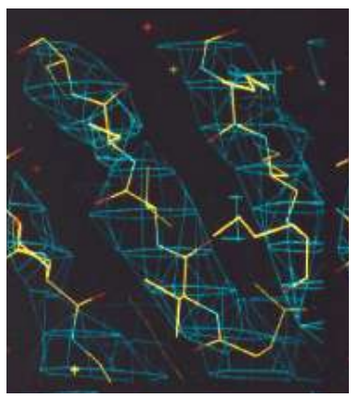
Importance of resolution

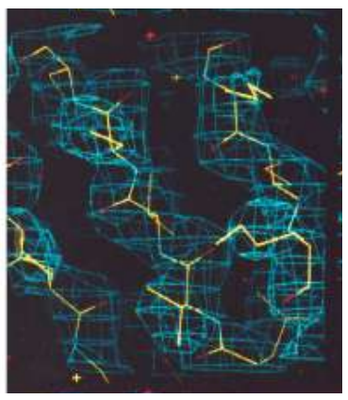
- Reflections far away from the origin provide high-resolution structural information
- Resolution affects level of detail in electron density maps



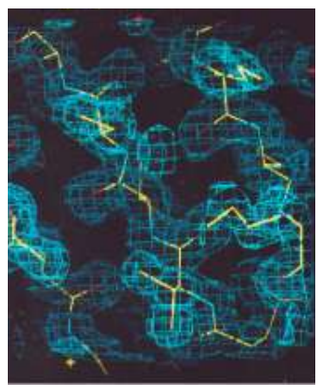
6.0 Å



4.5 Å



3.0 Å



1.6 Å

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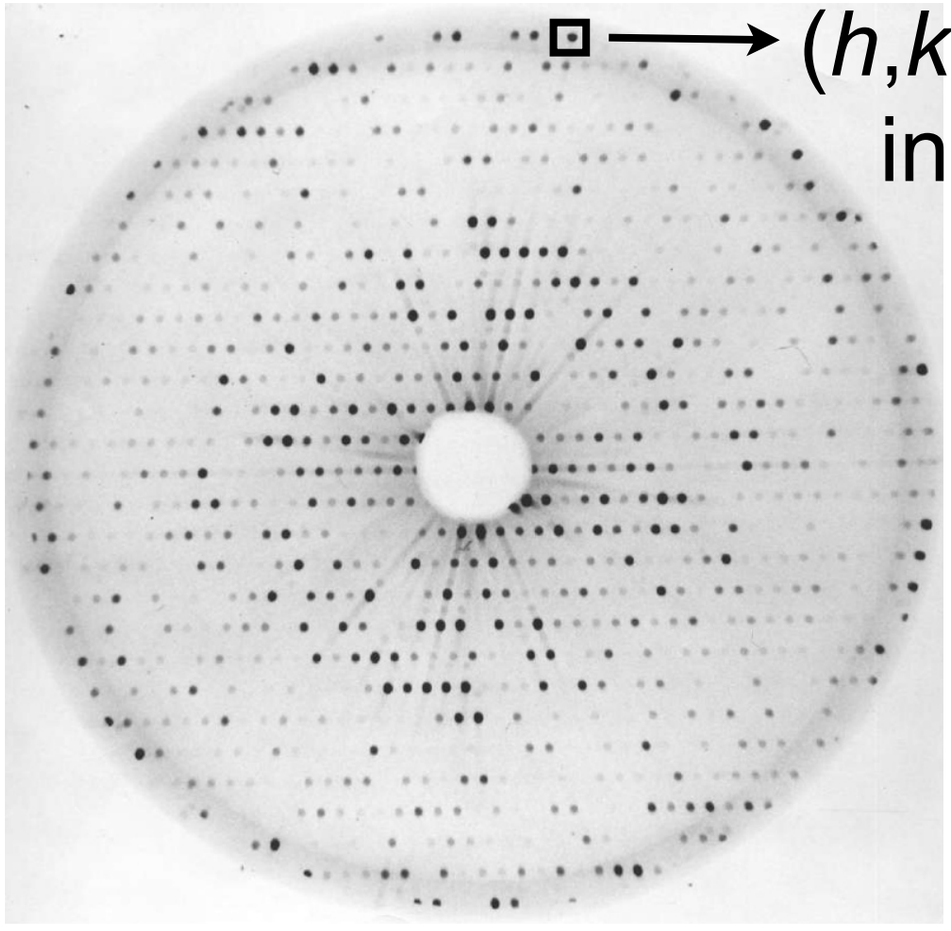
X-ray diffraction data

- What do we look for in a diffraction pattern?
  - spot shape
  - single lattice
  - resolution limit



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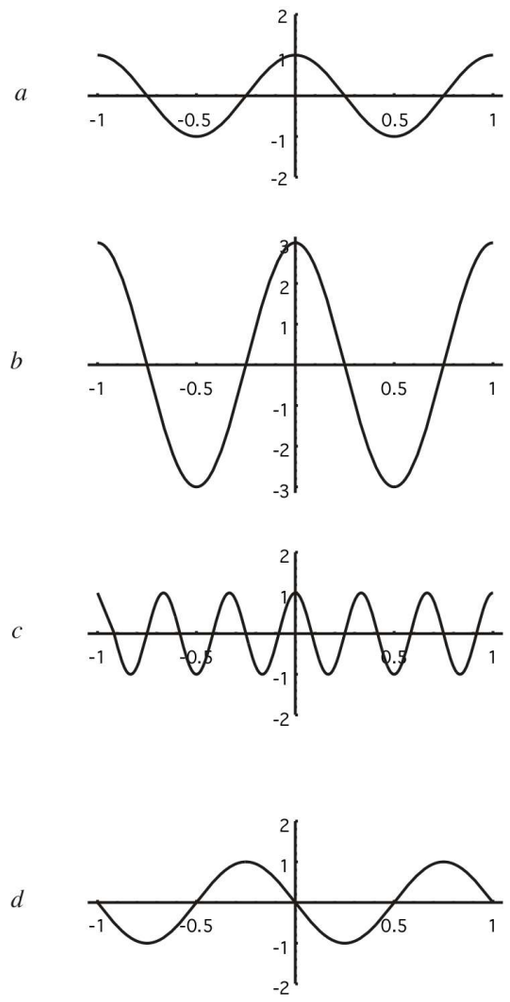
X-ray diffraction

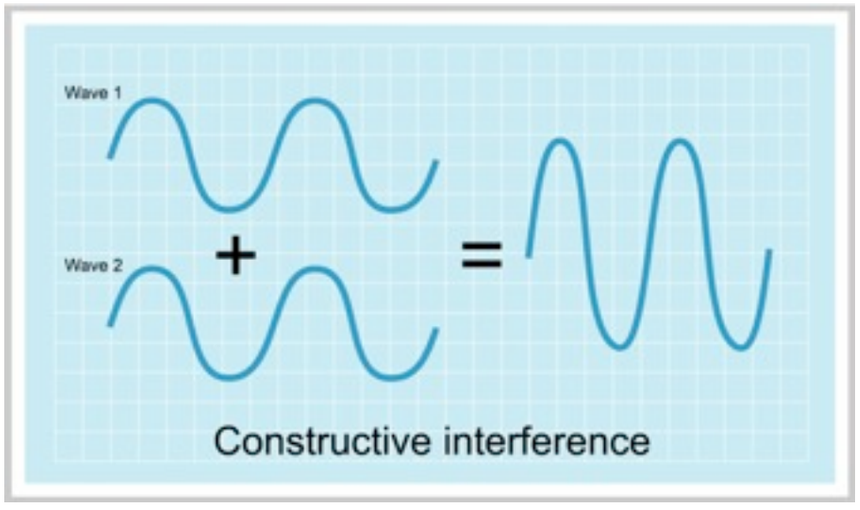
- Diffracted rays with strong constructive interference are observed
- Bragg’s law describes constructive interference in X-ray diffraction by crystals

X-ray diffraction

- X-rays have an amplitude, phase, frequency
- Constructive interference increases amplitude

$$f(x) = F \cos 2\pi(hx+\alpha)$$





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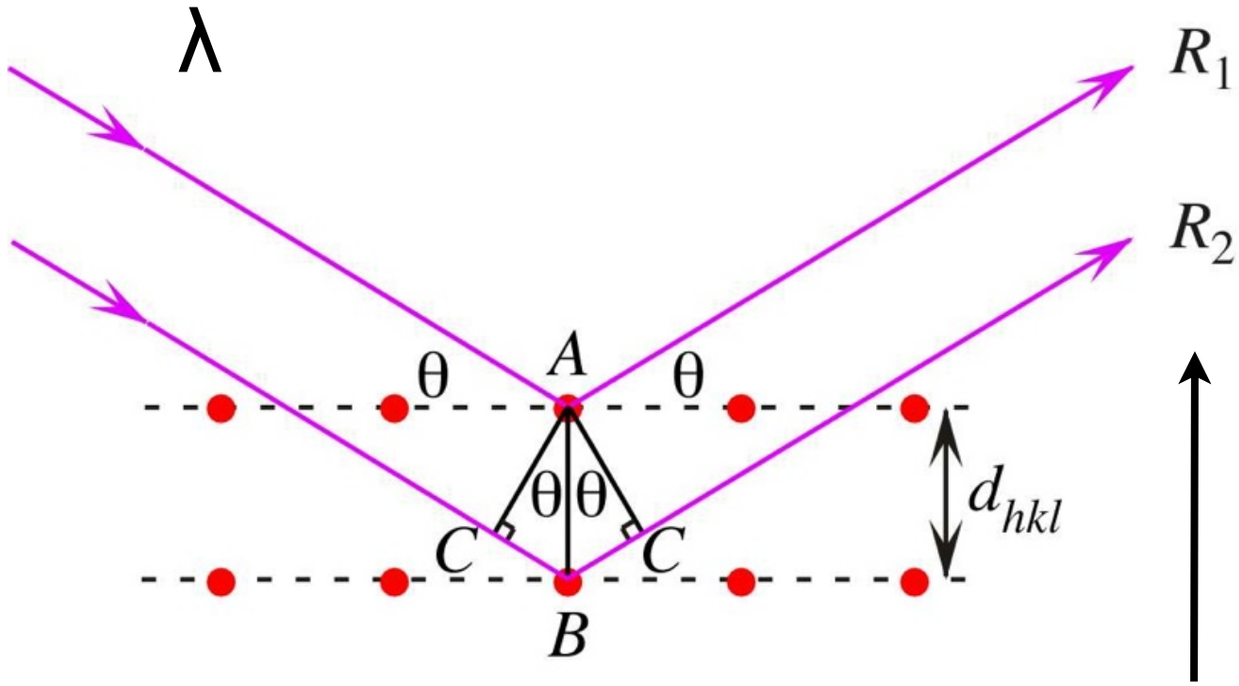
Bragg's law

- Parallel planes of atoms in crystals produce diffracted X-rays with strong constructive interference

- Conditions that satisfy Bragg's law lead to diffraction



$2d_{hkl} \sin \theta = n\lambda$ in phase

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Bragg's law

- A crystal lattice can be divided into many different regularly spaced sets of parallel planes
- Indexed based on number of times planes intersect the unit cell on each a,b,c edge
- Index notation is *h*, *k*, *l* and corresponds the index of a spot in the diffraction pattern

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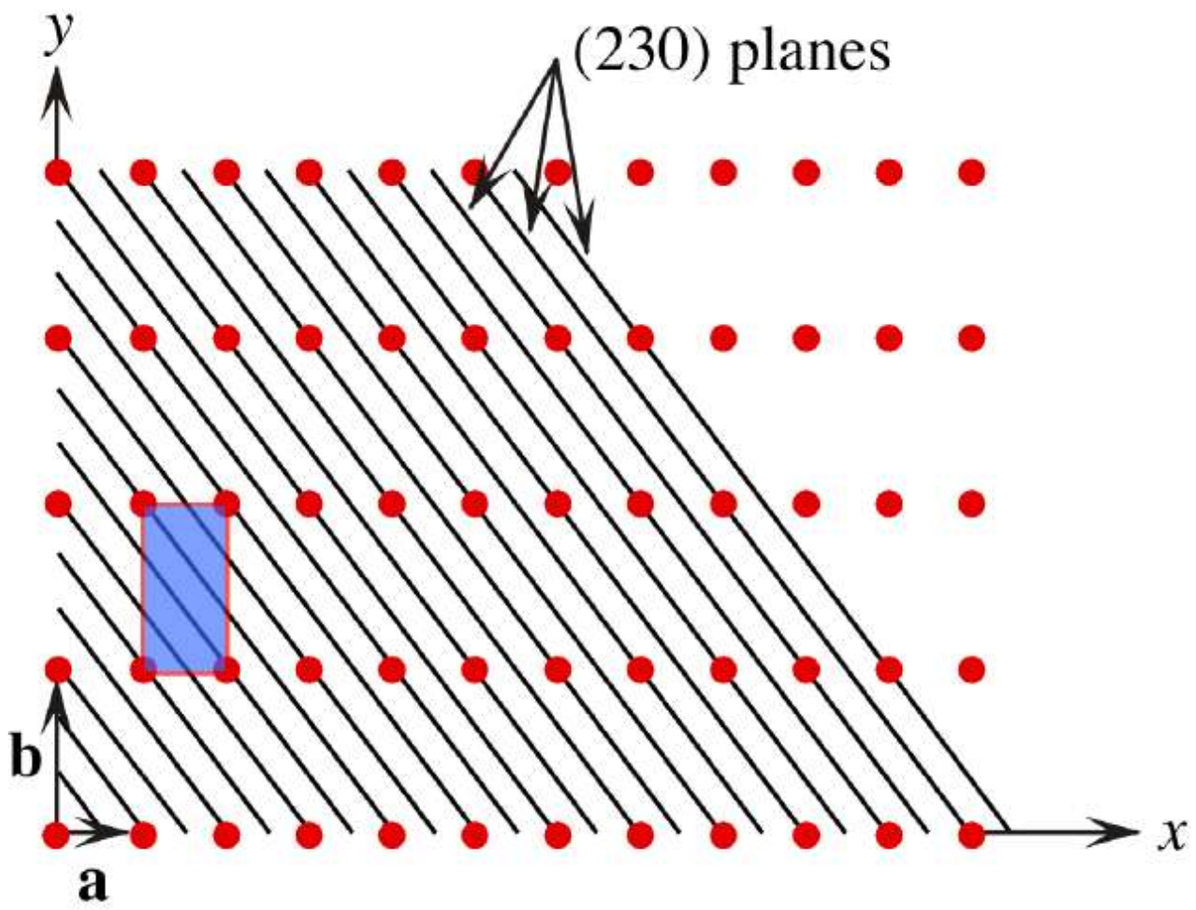
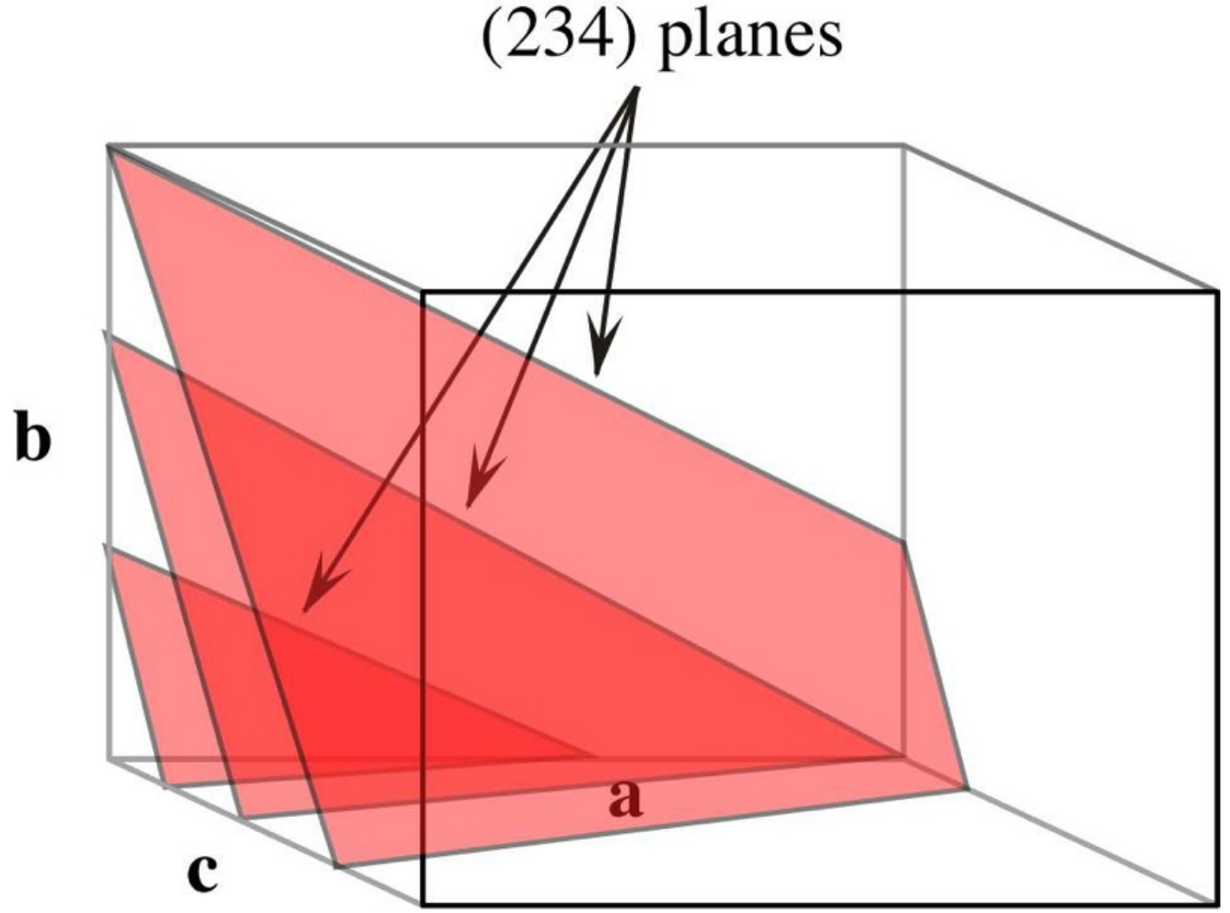
Examples of lattice planes





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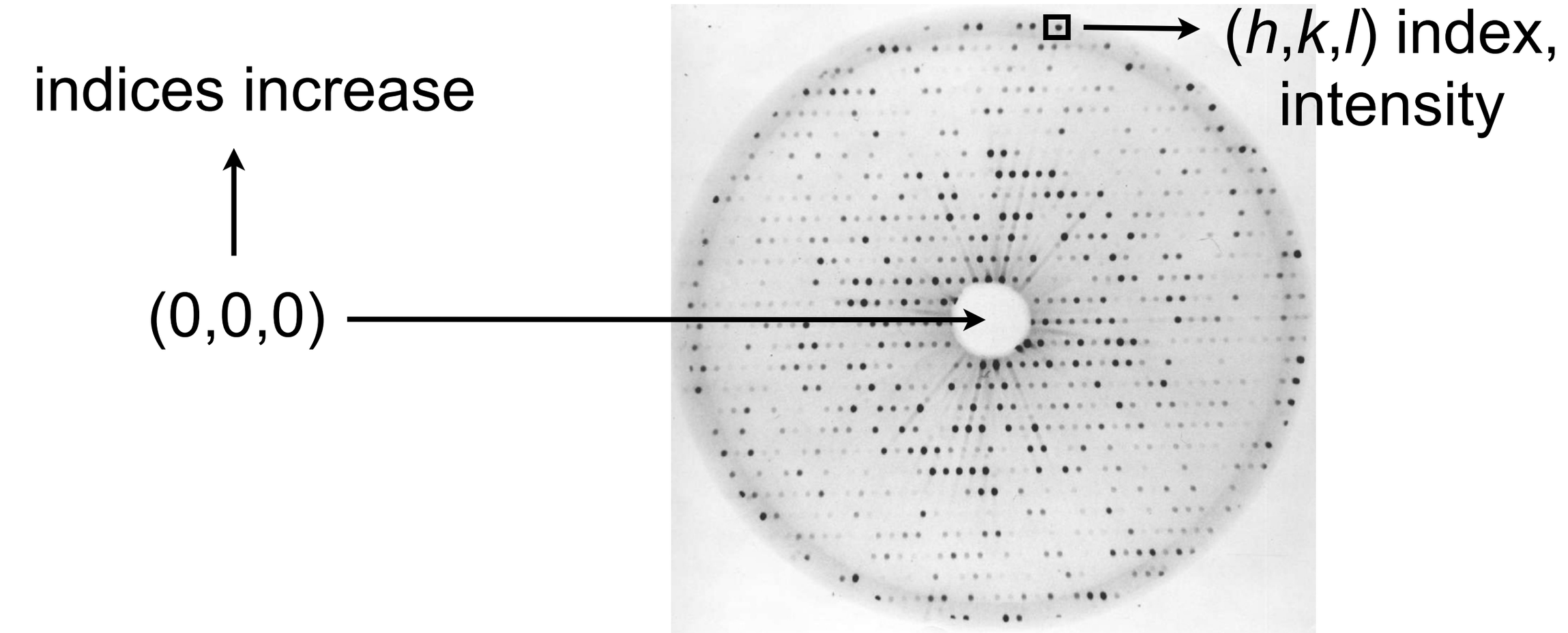
Diffraction data

- Each spot is a reflection with an index
- Indices provide information about the crystal lattice



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More about planes

- Each set of planes gives rise to a single reflection or spot
- Reflections form a reciprocal lattice
- The reciprocal lattice is related to the crystal lattice via indices of lattice planes
- Closely spaced planes provide more detailed information about electron density of atoms that lie on the planes

Diffraction patterns

- Can calculate unit cell dimensions from *h*,*k*,*l* indices
- Positions of spots in diffraction pattern contain information about lattice type and symmetry
- Intensity is a reporter of the electron density associated with given set of planes

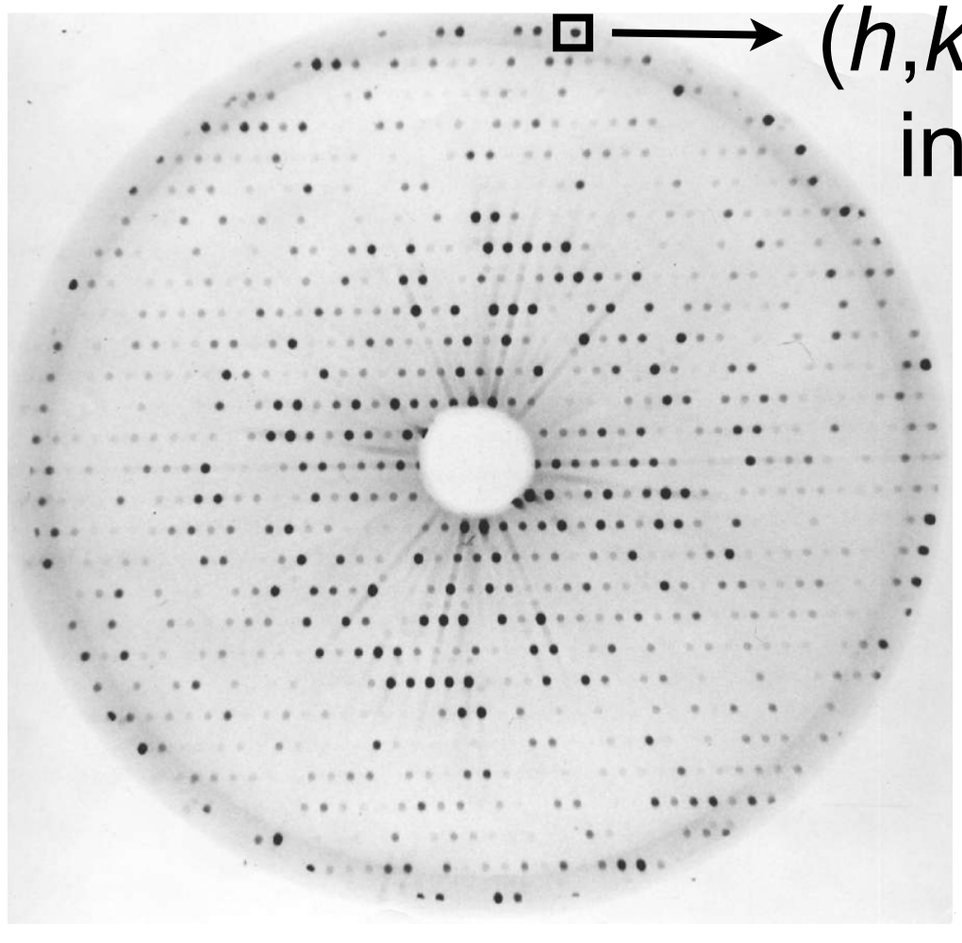
Diffraction data

- Each spot is a reflection with an index
- Indices provide information about the crystal lattice

Unit cell dimensions

Lattice type

Resolution



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Matthews coefficient

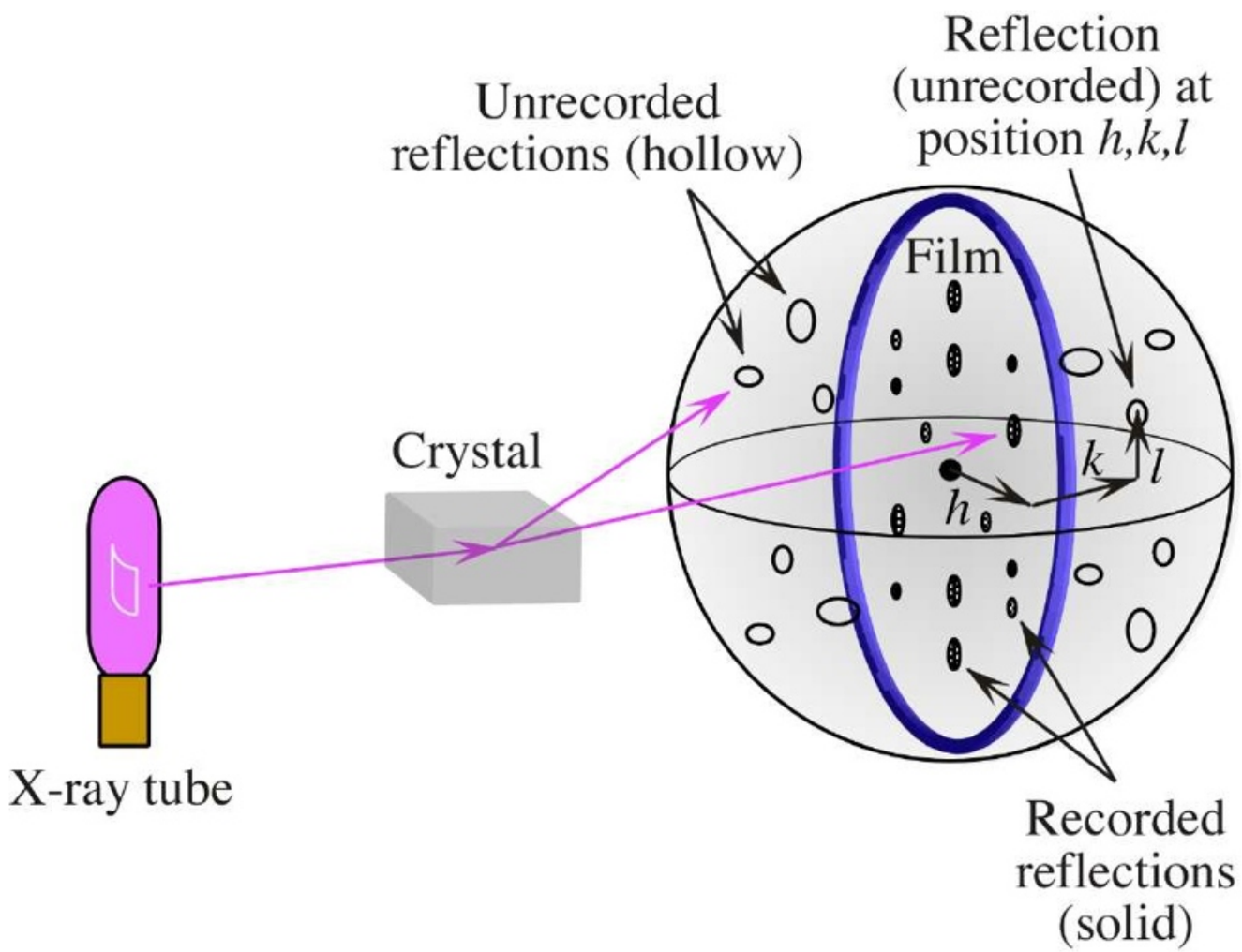
- Use unit cell dimensions to estimate number of residues in asymmetric unit
- Assume ~50% solvent content in protein crystals
- Provides useful information for structure solution and phasing

Kantardjieff, K.A., and Rupp, B. (2003) *Protein Science 12*, 1865-1871.

X-ray dataset

- Set of diffraction pattern images collected while rotating the crystal
- Goal is to collect a complete and redundant set of reflections and their intensities
- Diffraction patterns are typically not reported
- Use data collection statistics to judge quality
- Datasets are large (1-10 GB)

Data collection



Crystal rotation during data collection captures unrecorded reflections

Important parameters:

- oscillation angle
- number of images
- exposure time
- detector position

X-ray dataset

- Set of diffraction pattern images collected while rotating the crystal
- Goal is to collect a complete and redundant set of reflections and their intensities
- Diffraction patterns are typically not reported
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- Datasets are large (1-10 GB)

X-ray data processing

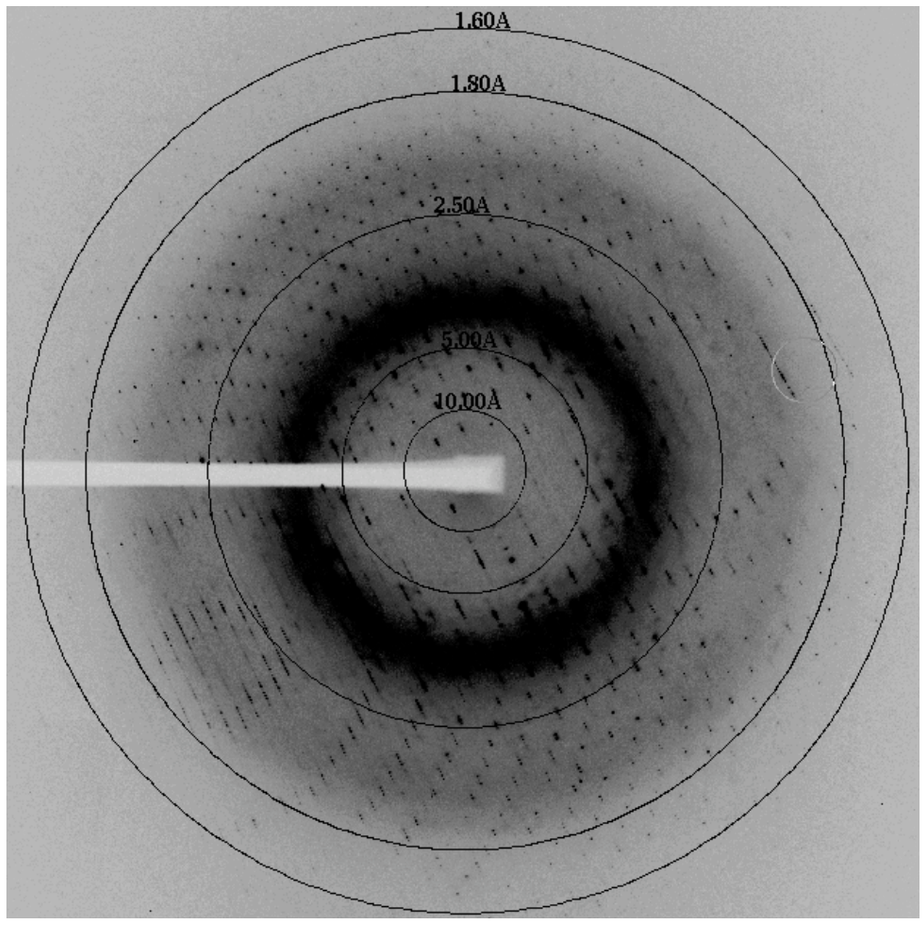
- Indexing
- Bravais lattice and unit cell assignment
- Integration
- Scaling and analysis of statistics
- Space group and resolution limit determination

Data collection statistics

- **Resolution**
  - ~3.5-4.0 Å to resolve secondary structure
  - ~2.5-3.5 Å to identify side chains
  - ~1.5-2.5 Å to model side chains/backbone confidently
- **Intensity to background ratio (*I*/sigma*I*)**
  - Sets resolution limit
- **R_{merge} or R_{sym}**
  - Measure of intensity differences between reflections with same index (<0.1 overall)

Resolution bins

- Spots in each diffraction image are binned by resolution
- Statistics are compiled for overall dataset and individual resolution bins



Data collection statistics

- **Resolution**
  - ~3.5-4.0 Å to resolve secondary structure
  - ~2.5-3.5 Å to identify side chains
  - ~1.5-2.5 Å to model side chains/backbone confidently
- **Intensity to background ratio (*I*/s*I*), completeness, redundancy**
  - Sets resolution limit
- **R_{merge} or R_{sym}**
  - Measure of intensity differences between reflections with same index (<0.1 overall)

Data collection statistics

	WT
Data collection statistics	
Maximum resolution (Å)	1.50
Wavelength (Å)	0.9918
Total reflections	98,554
Unique reflections	27,758
Completeness (%)[a]	98.9 (97.1)
$I/\sigma(I)$	7.8 (1.5)
R_{sym} (%)[b]	5.1 (40.4)
Refinement statistics	
Resolution limits (Å)	31.5–1.50
R-factor[c]	0.184
R-free	0.216
Estimated coordinate error (Å)[d]	0.09
RMS deviations from ideal values	
Bond lengths (Å)	0.024
Bond angles (°)	2.192
Dihedral angles (°)	25.11
Improper torsion angles (°)	1.60
Average temperature factor (Å^2)	
Protein	22.1, 20.6
Iron-sulfur	13.0, 12.8
Water	36.8
Zinc	37.1
Sulfate	44.3
Ramachandran plot,[e] residues in	
Most favored regions (%)	90.2
Additional allowed regions (%)	9.1
Generously allowed regions (%)	0.6
Disallowed regions (%)	0.0

Overall

< 0.1 overall

High res. bin

> 2.0 high res. bin

Yeh, A.P., Ambroggio, X.I., Andrade, S.L.A., Einsle, O., Chatelet, C., Meyer, J., and Rees, D.C. (2002) *J. Biol. Chem. 277*, 34499-34507.

Structure solution

- Structure factors relate diffraction intensities to electron density and atom position
  - Each reflection can be described by a structure factor (F_{hkl}) function
  - F_{hkl} is the sum of diffractive contributions of all atoms in the unit cell

no. of atoms in unit cell

$$F_{hkl} = \sum_{j=1}^{n} f_j e^{2\pi i(hx_j + ky_j + lz_j)}$$

atomic scattering factor

Structure solution

- Electron density map is 3D plot of ρ(*x, y, z*)
- ρ(*x, y, z*) is a Fourier sum

electron density function

structure factor

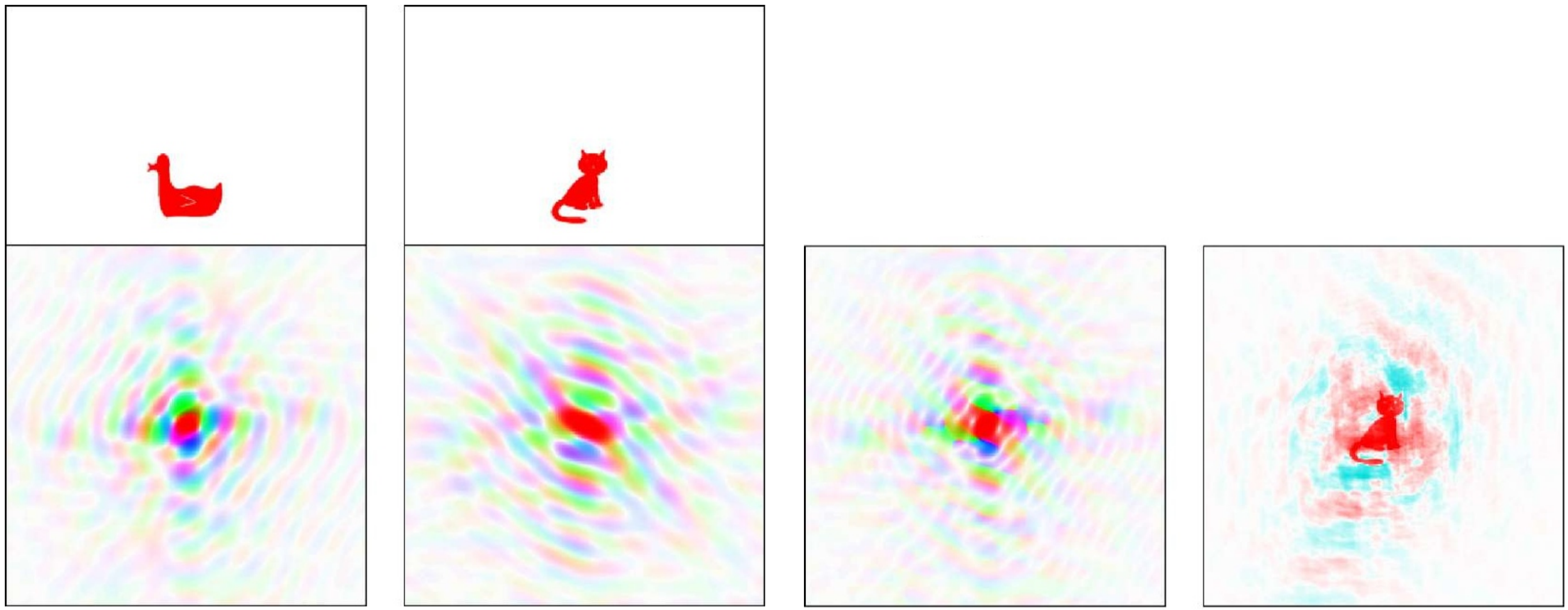
$$\rho(x,y,z) = \frac{1}{V}\sum_{h}\sum_{k}\sum_{l} F_{hkl} e^{-2\pi i(hx_j+ky_j+lz_j)}$$

sum over all reflections in diffraction pattern

Structure solution

- How do we plot ρ(*x, y, z*) from diffraction data?
- F_{hkl} is a periodic function and has an amplitude, frequency, and phase
  - amplitude ~ $\sqrt{I_{hkl}}$
  - frequency = $\frac{1}{d_{hkl}}$
  - phase = ??

The phase problem



- Phases contain most of the information necessary for structure determination

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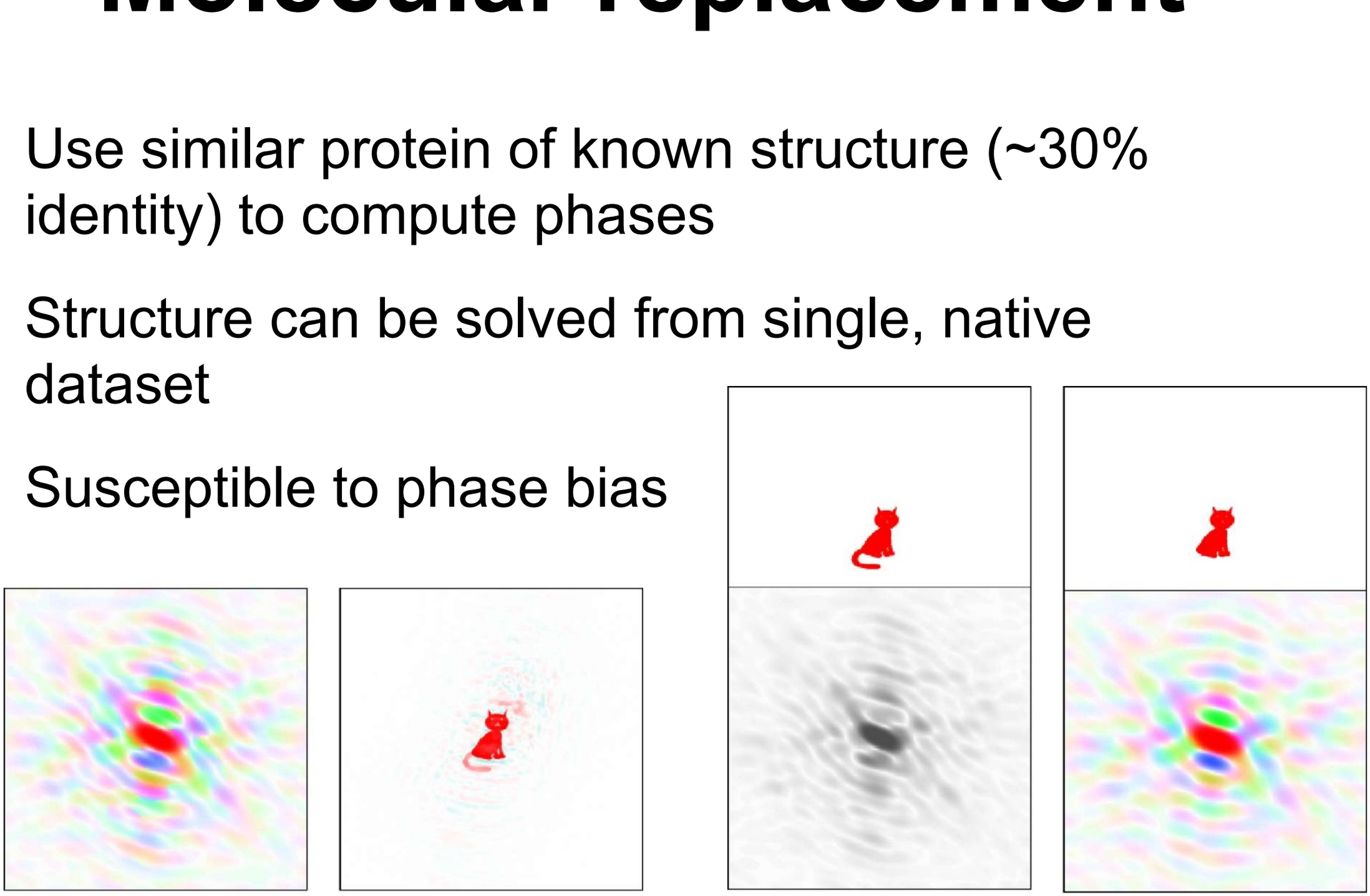
Solving the phase problem

- Molecular replacement
- Anomalous diffraction methods
- Multiple isomorphous replacement

Molecular replacement

- Use similar protein of known structure (~30% identity) to compute phases
- Structure can be solved from single, native dataset
- Susceptible to phase bias



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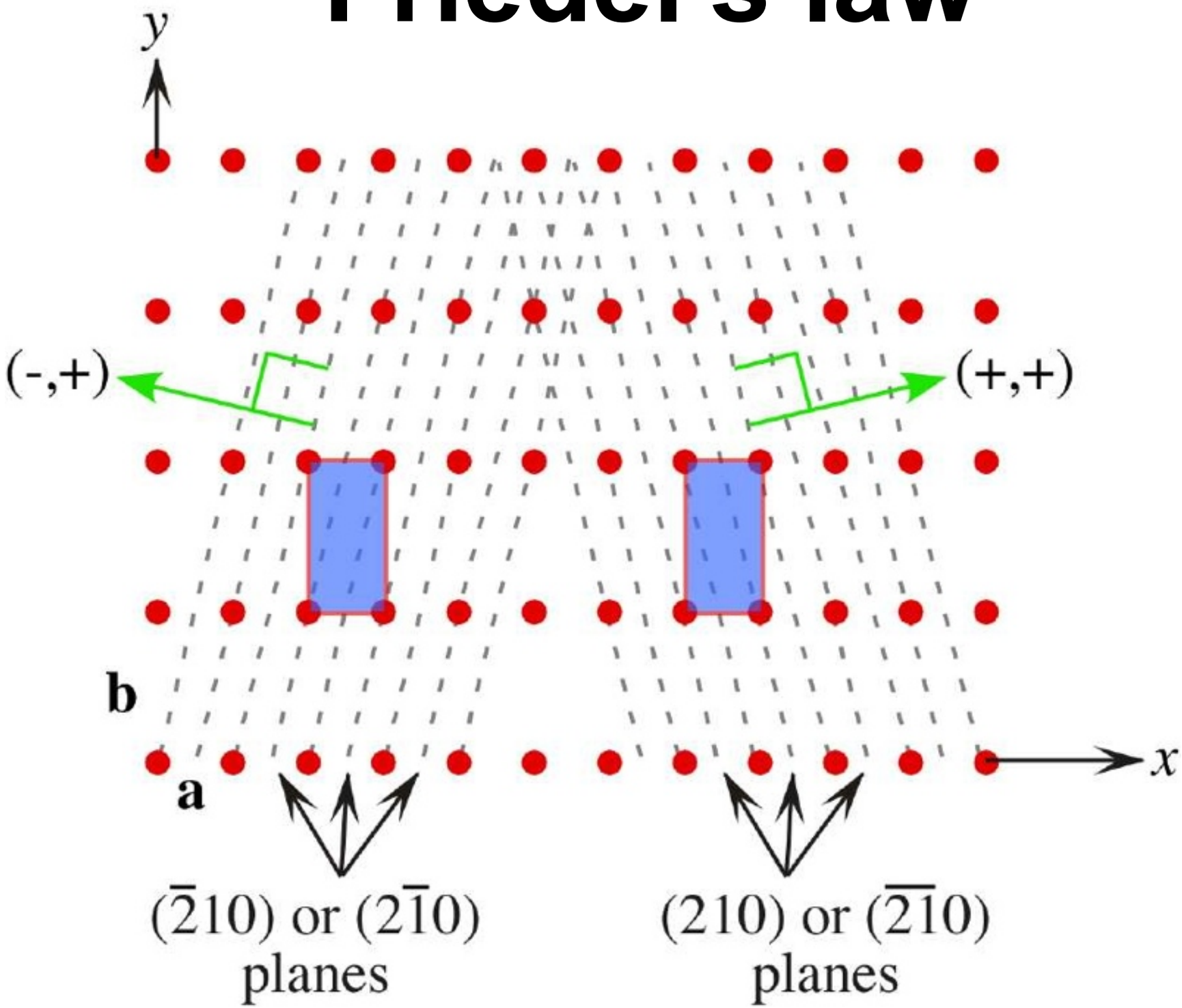
Experimental phasing

- Use heavy atom sites (transition metals or halogens) in crystals (derivative or native)
- Determine position of heavy atoms in unit cell
- Use phase information from heavy atoms to compute protein phases
- Typically requires collection of multiple datasets and/or crystals
- Generates electron density map without a model

Anomalous diffraction

- X-ray absorption by heavy atoms alters diffraction
  - Friedel's law does not hold $I_{hkl} \neq I_{\overline{hkl}}$
- Intensity differences in Friedel pairs allows for heavy atom location in unit cell
- Datasets are collected near heavy atom absorption edge
- SeMet substitution or native metal cofactors used as anomalous scatterers
- Requires a tunable X-ray source

Friedel's law



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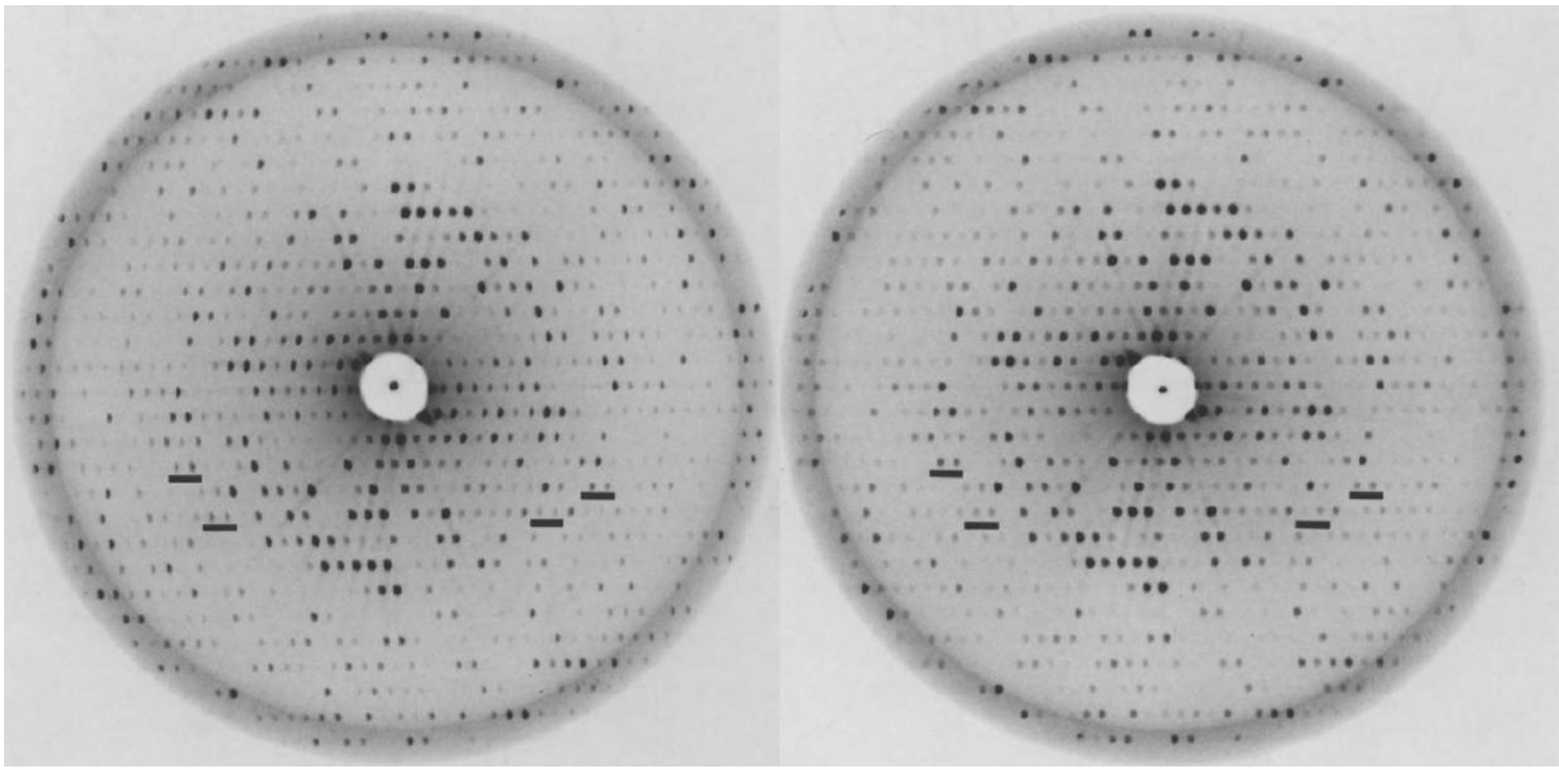
Isomorphous replacement



- Requires many crystals
- Heavy atoms must bind to same site
- Crystals must be isomorphous

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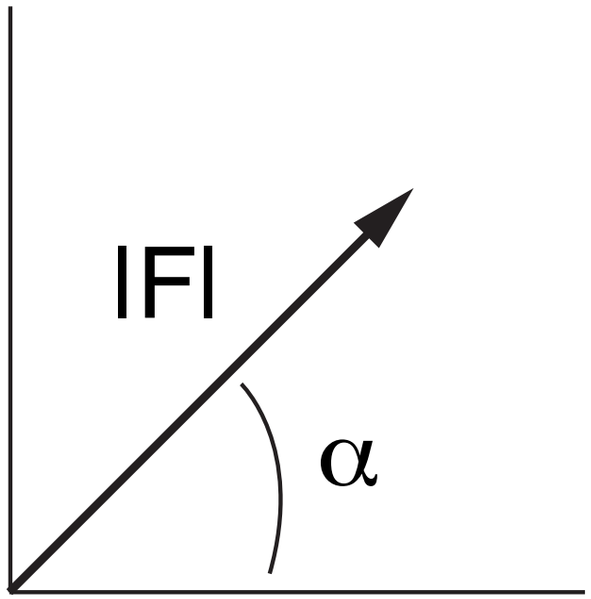
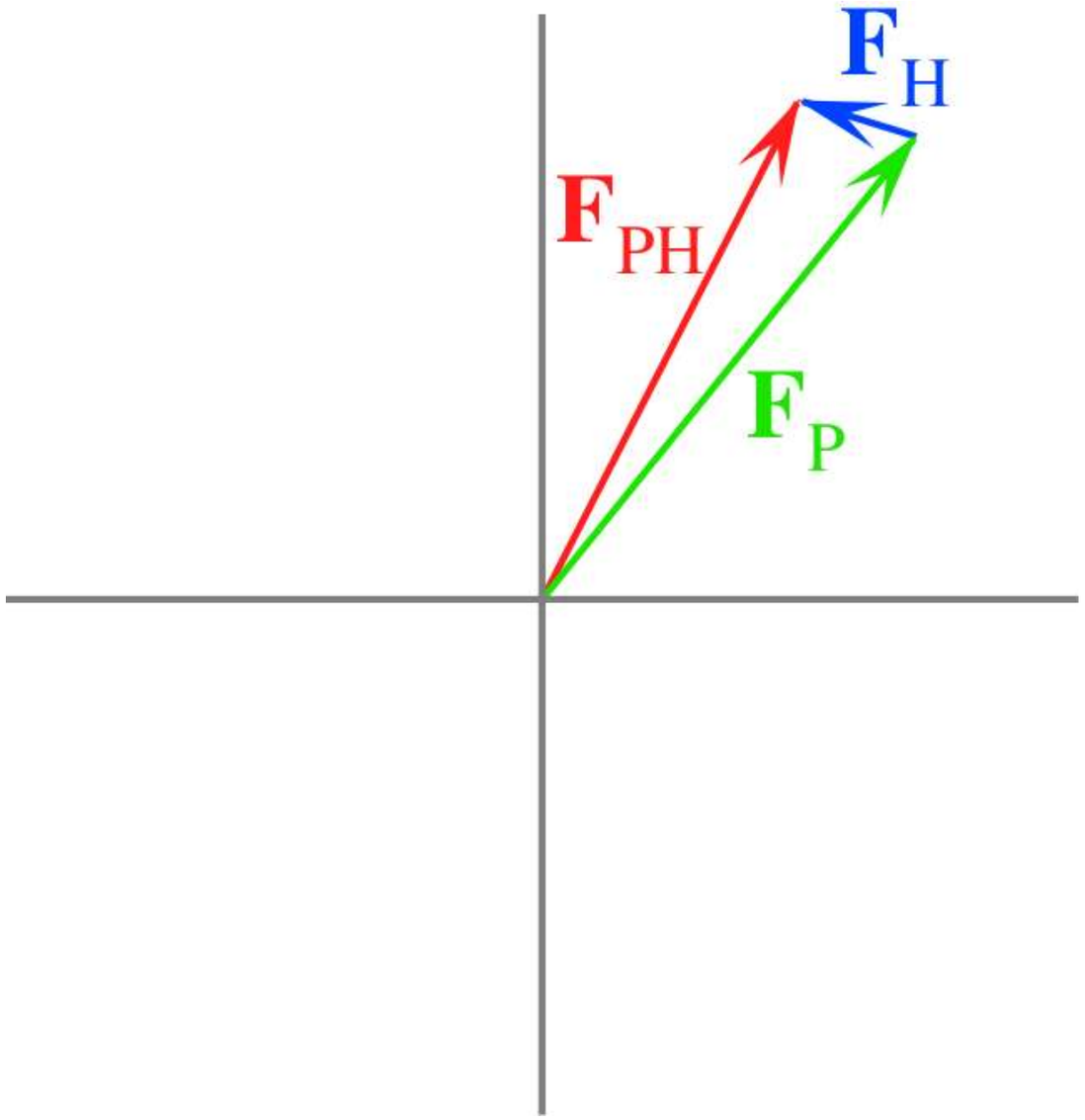
Experimental phasing





Structure factors can be represented as vectors

$$\mathbf{F}_P = \mathbf{F}_{PH} - \mathbf{F}_H$$

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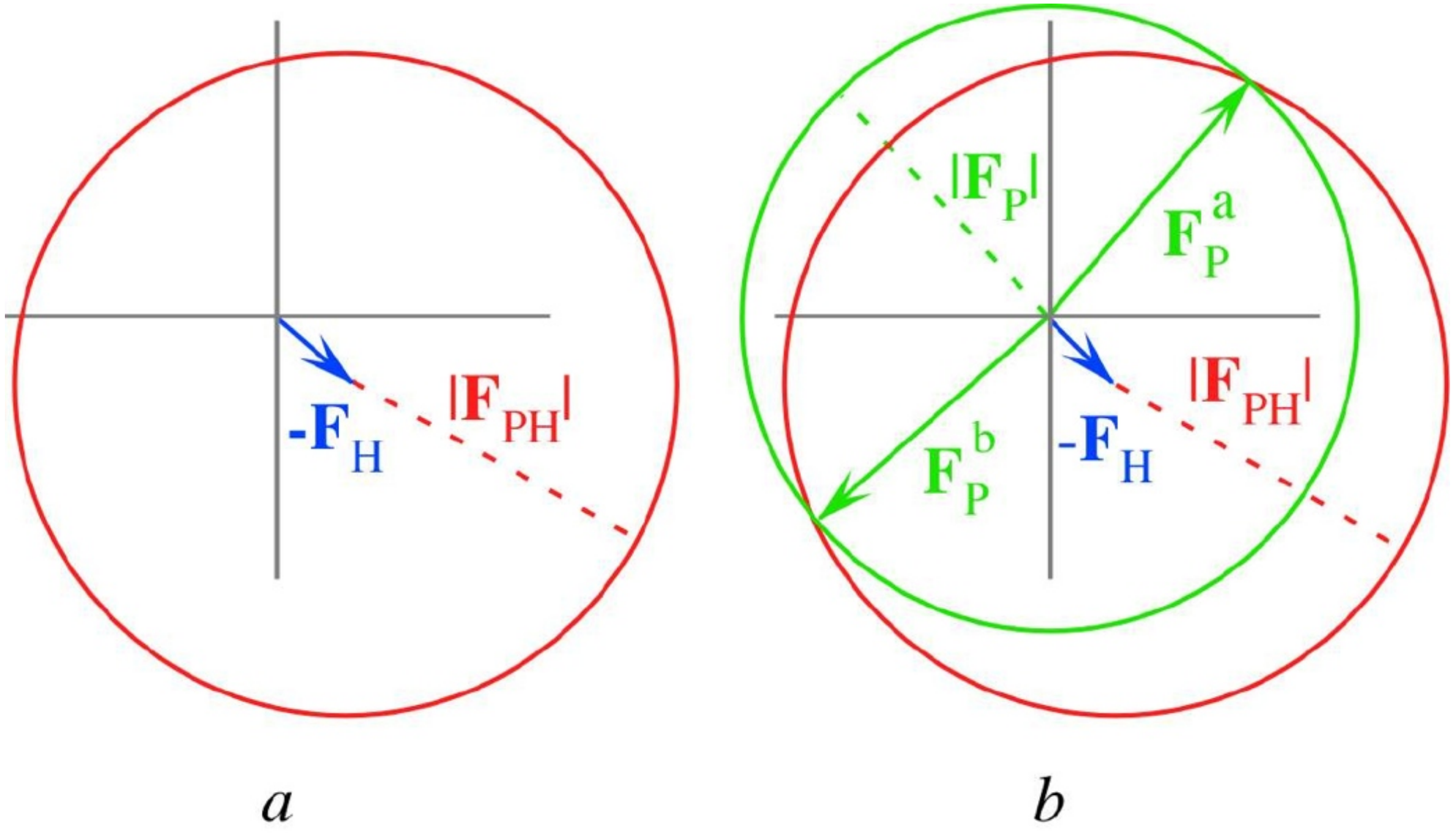
Experimental phasing



Harker diagrams provide vector solutions for $\mathbf{F_P}$

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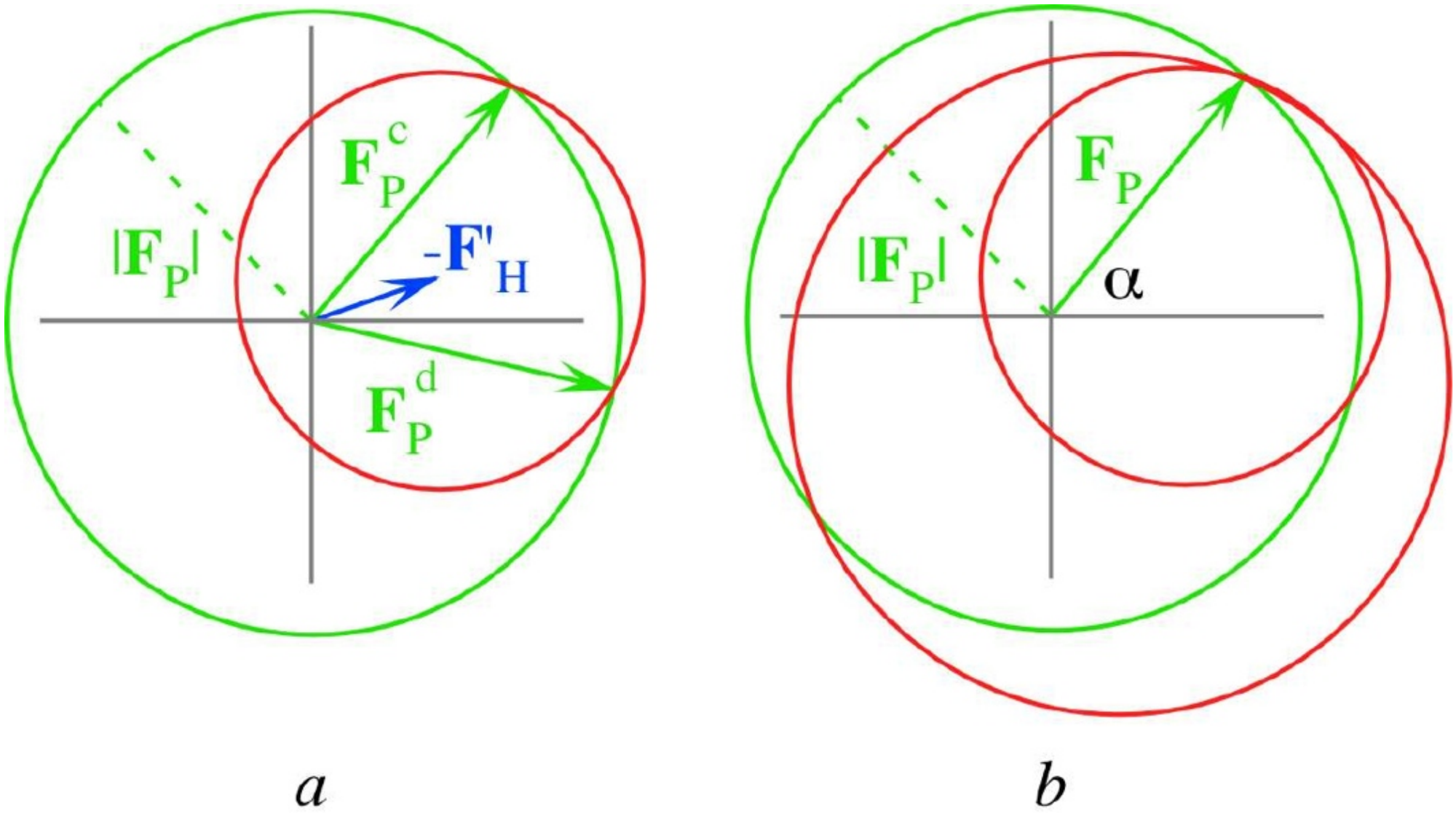
Experimental phasing



Phase ambiguity resolved by additional derivatives

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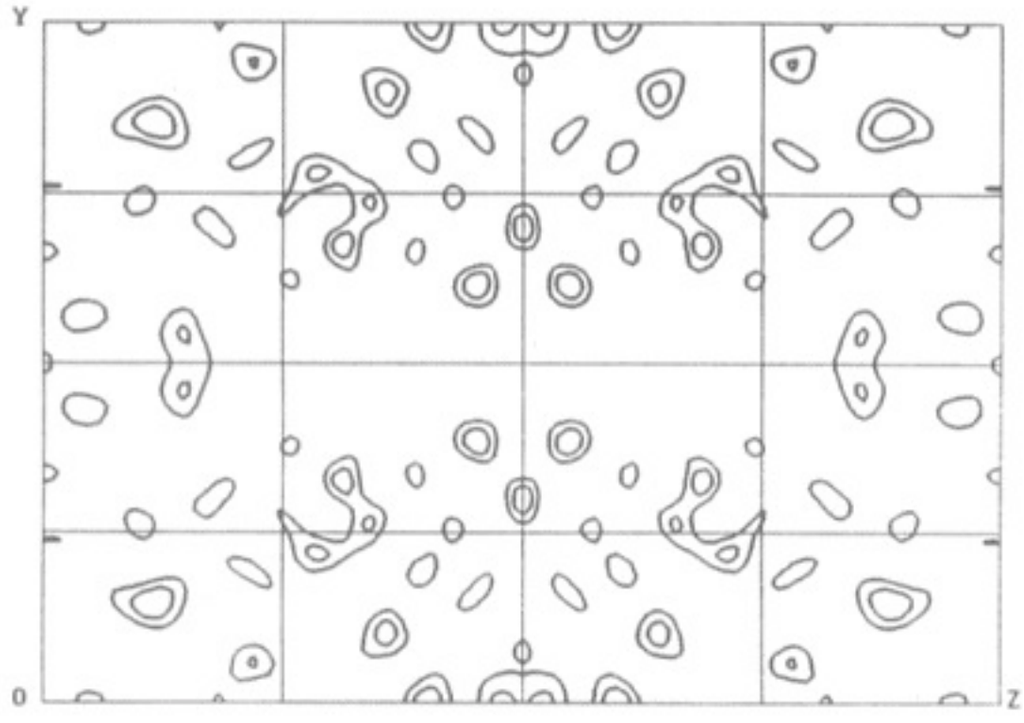
Heavy atom location

$$P(u,v,w) = \frac{1}{V}\sum_h\sum_k\sum_l |\mathbf{F}_{hkl}|^2 e^{-2\pi i(hu+kv+lw)}$$

Contour map of Patterson function *P*(*u*, *v*, *w*) shows peaks corresponding to vectors between atoms



Cell symmetry simplifies 3D search for Patterson atoms

Harker sections contain Patterson vectors for symmetry-related atoms

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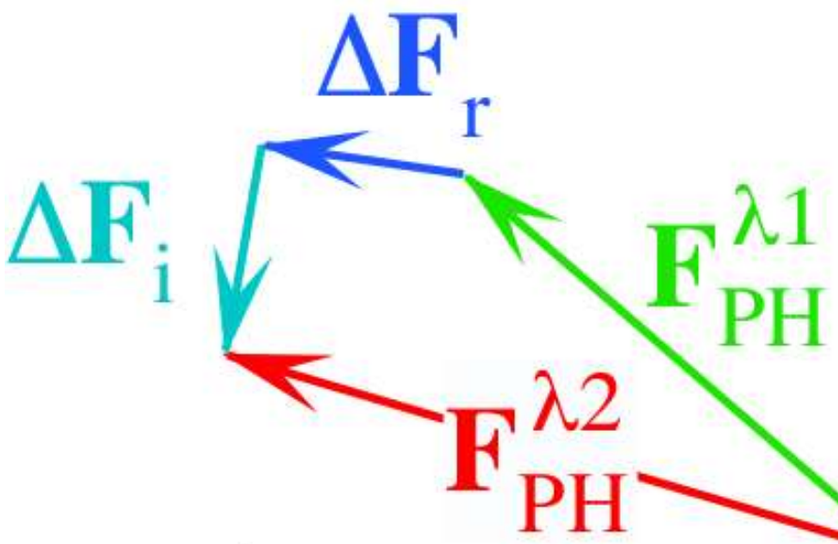
Anomalous diffraction



$$\mathbf{F}_{PH}^{\lambda 2} = \mathbf{F}_{PH}^{\lambda 1} + \Delta\mathbf{F}_r + \Delta\mathbf{F}_i$$

$F_{PH}^{\lambda 2}$ w/ anomalous scattering

$F_{PH}^{\lambda 1}$ w/o anomalous scattering

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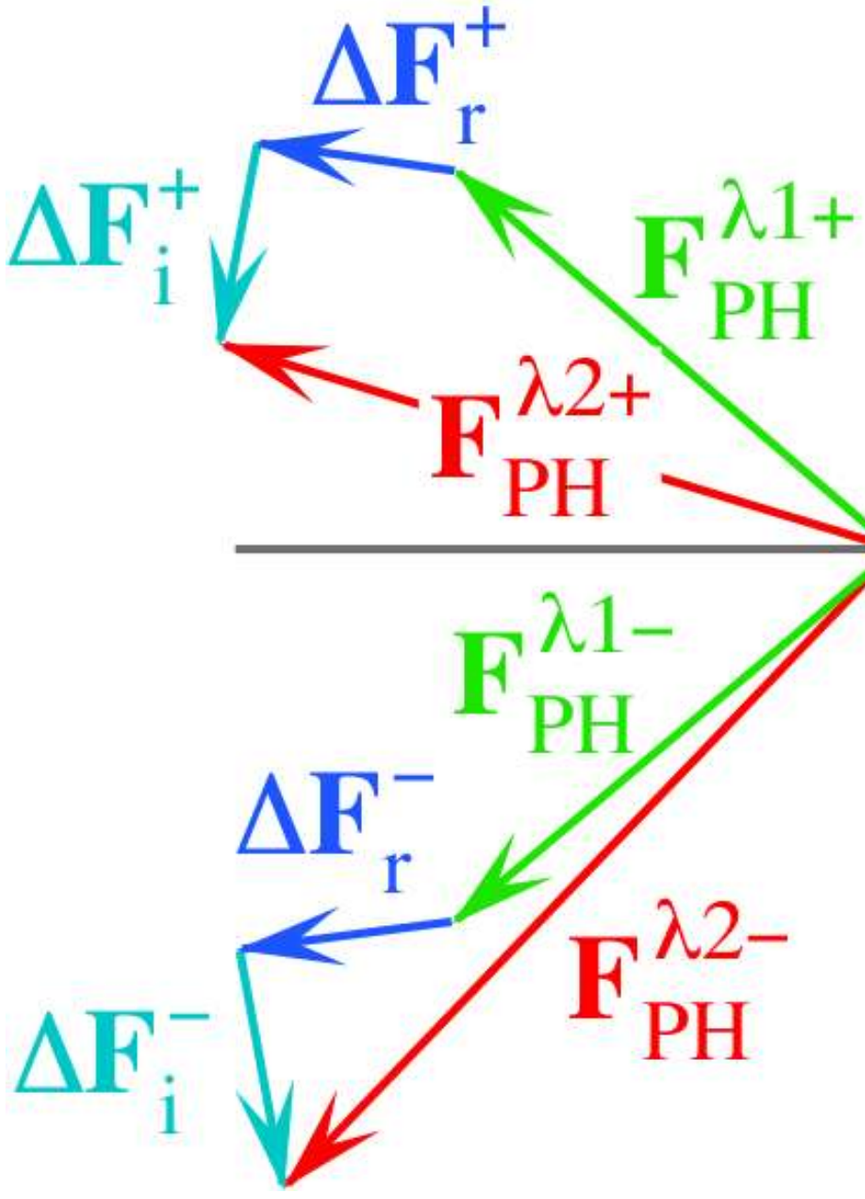
Anomalous diffraction



$F_{PH}^{\lambda 1}$ Friedel pairs are equal in intensity and with phase opposite in sign

$F_{PH}^{\lambda 2}$ Friedel pairs have different intensity due to change in imaginary scattering factor

Magnitude of anomalous scattering contributions are known quantities

Phases depend only on atom positions

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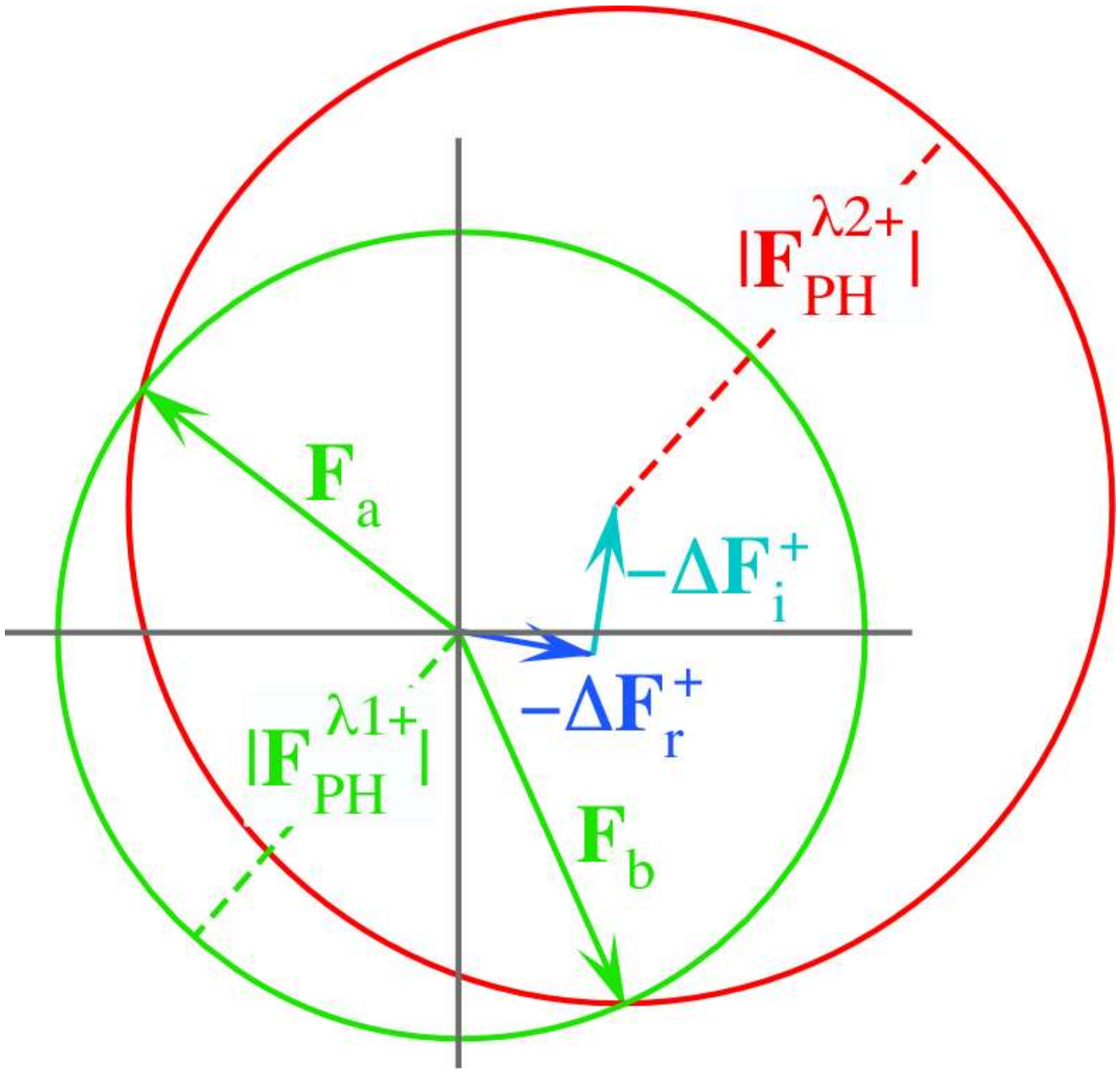
Anomalous diffraction



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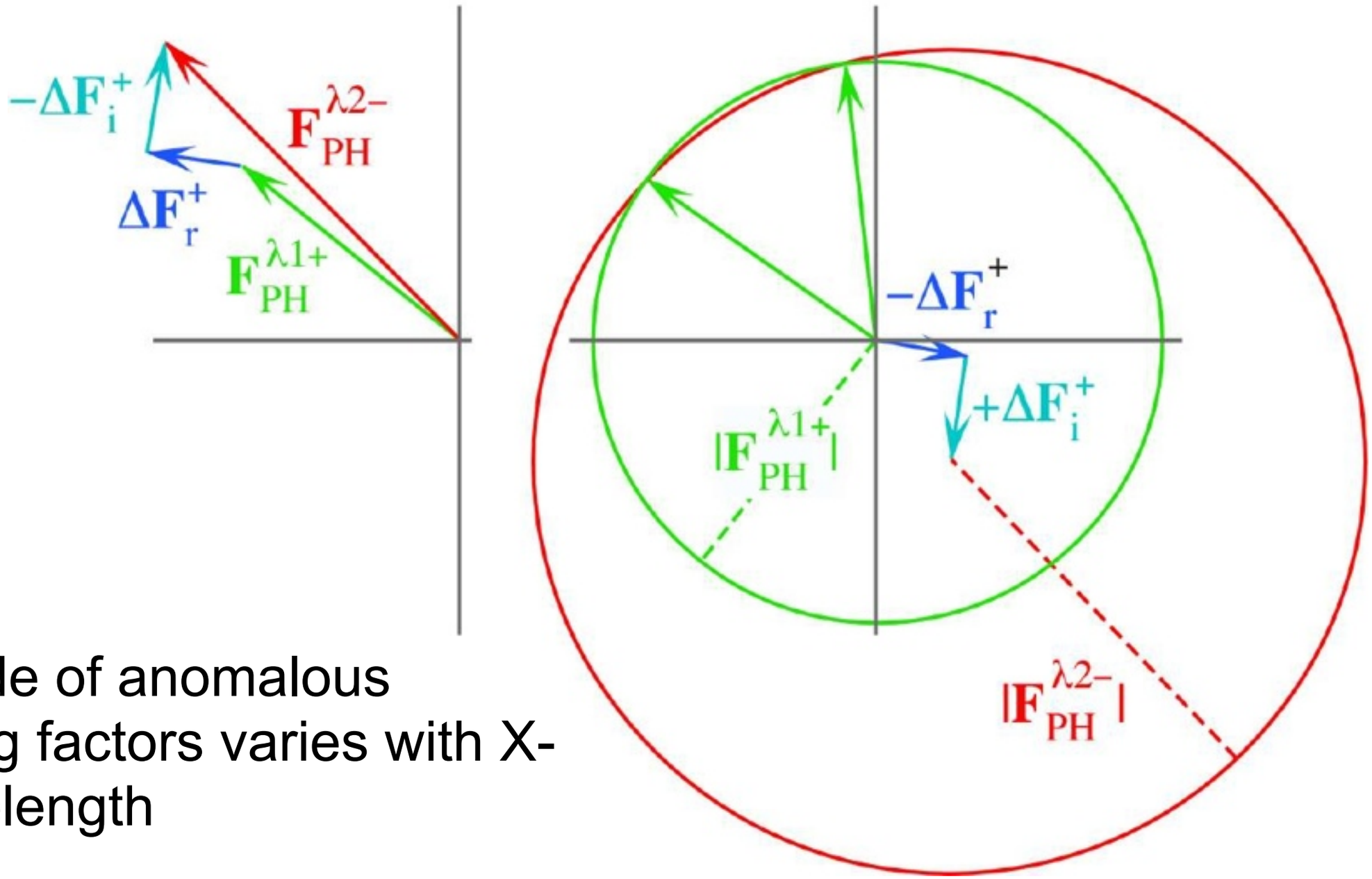
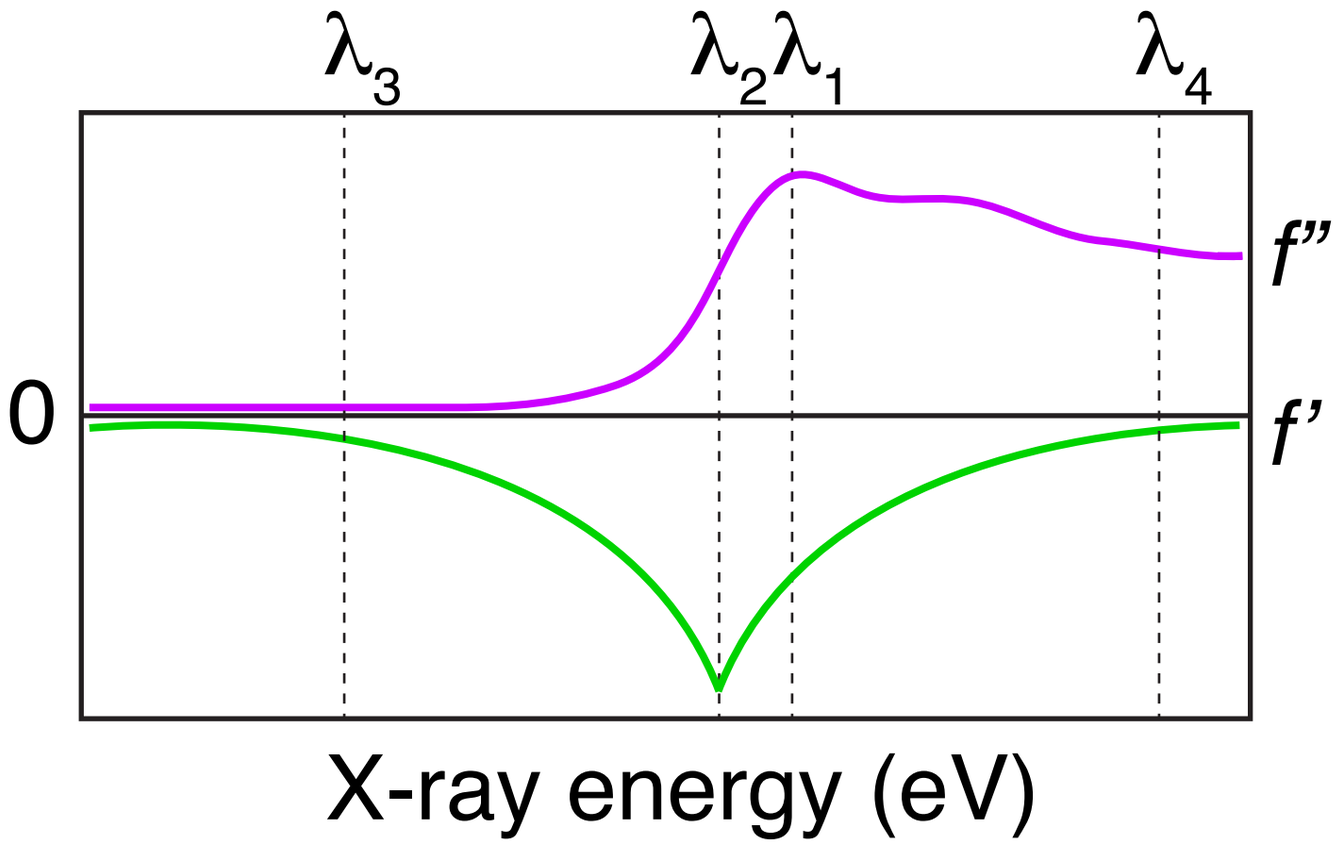
Anomalous diffraction



Magnitude of anomalous scattering factors varies with X-ray wavelength

Rhodes, G. (2006) Crystallography Made Crystal Clear, 3rd Ed. Elsevier Burlington MA.

Wavelength selection



λ_1 at maximum *f”* has largest anomalous signal (peak)

λ_2 chosen at maximum *f’* (inflection)

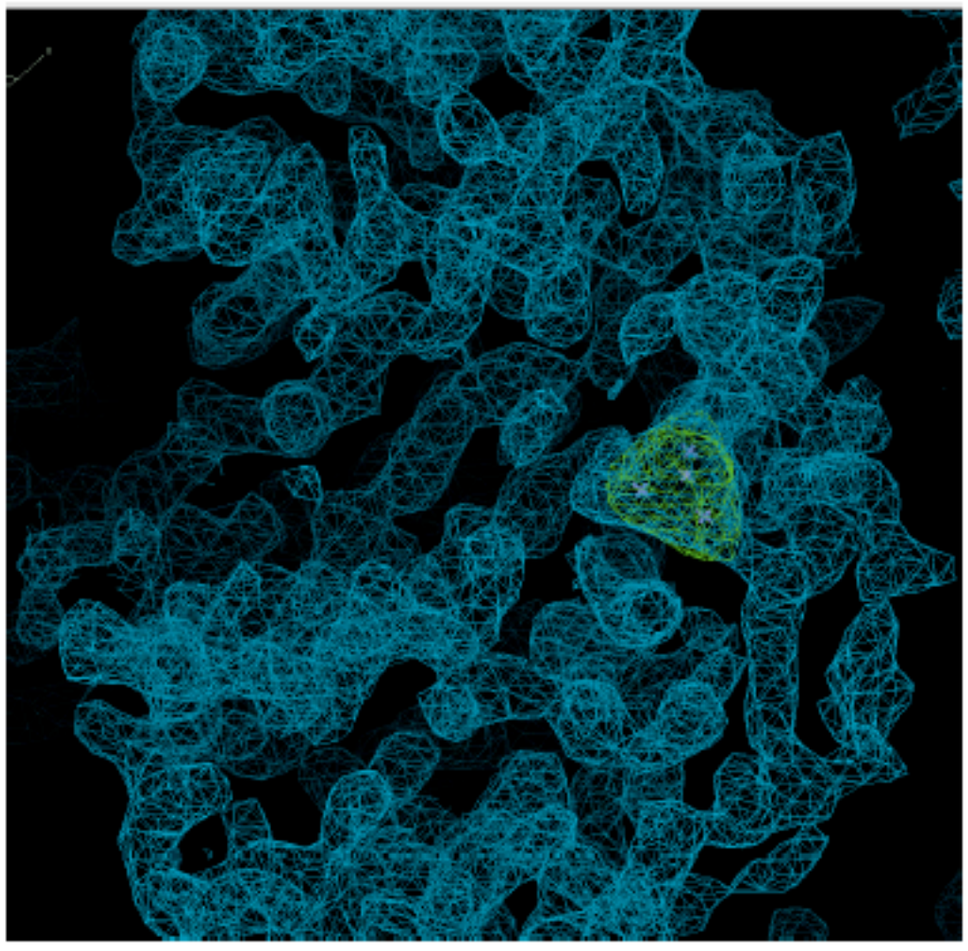
λ_3, λ_4 range between 100-1000 eV of edge (remote)

http://skuld.bmsc.washington.edu/scatter/

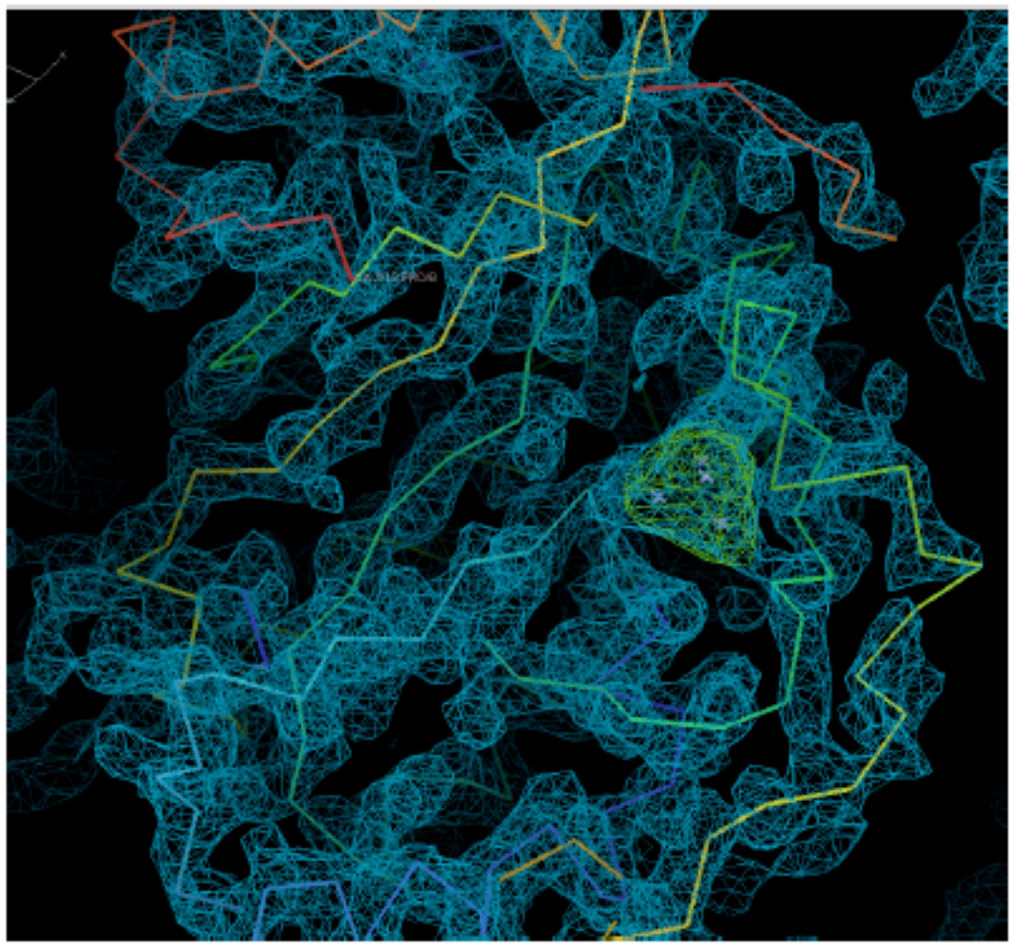
The phase problem

- Often a trial-and-error process
- Phasing methods can be combined
- Phase information is iteratively improved during model building and refinement
- How do we assess the quality of phase determination?

The phase problem



$2F_o$-F_c map (blue) = 1.4σ
Fe anomalous difference map (green) = 5.0σ



$2F_o$-F_c map (blue) = 1.4σ
Fe anomalous difference map (green) = 5.0σ

- Positive outcome is an interpretable electron density map

Model building and validation

- 3D plot of ρ(*x, y, z*) function produces electron density map
- Map interpretation
- Refinement of coordinates
- Validation of final model

phases

$$\rho(x,y,z) = \frac{1}{V}\sum_h\sum_k\sum_l |F_{hkl}| e^{-2\pi i(hx_j+ky_j+lz_j-\alpha'_{hkl})}$$

amplitudes (from intensities)

Electron density maps

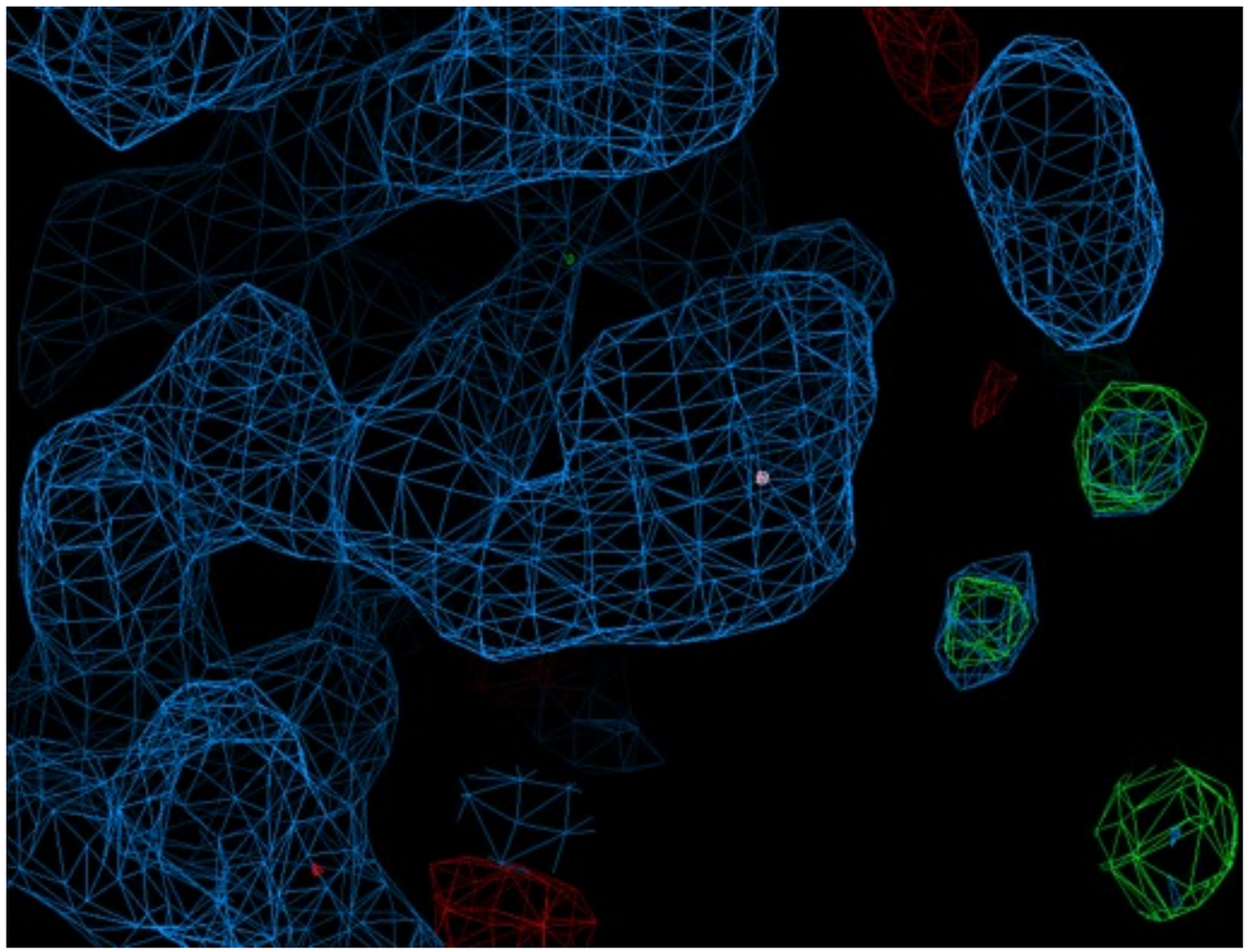
$$\rho(x,y,z) = \frac{1}{V}\sum_h\sum_k\sum_l |F_{hkl}|e^{-2\pi i(hx_j+ky_j+lz_j-\alpha'_{hkl})}$$

F_{obs} from diffraction data

F_{calc} from molecular model

- $2F_o$-F_c electron density map
  - Single color molecular surface map
- F_o-F_c electron density map
  - Difference map highlighting errors in the model

Map Interpretation

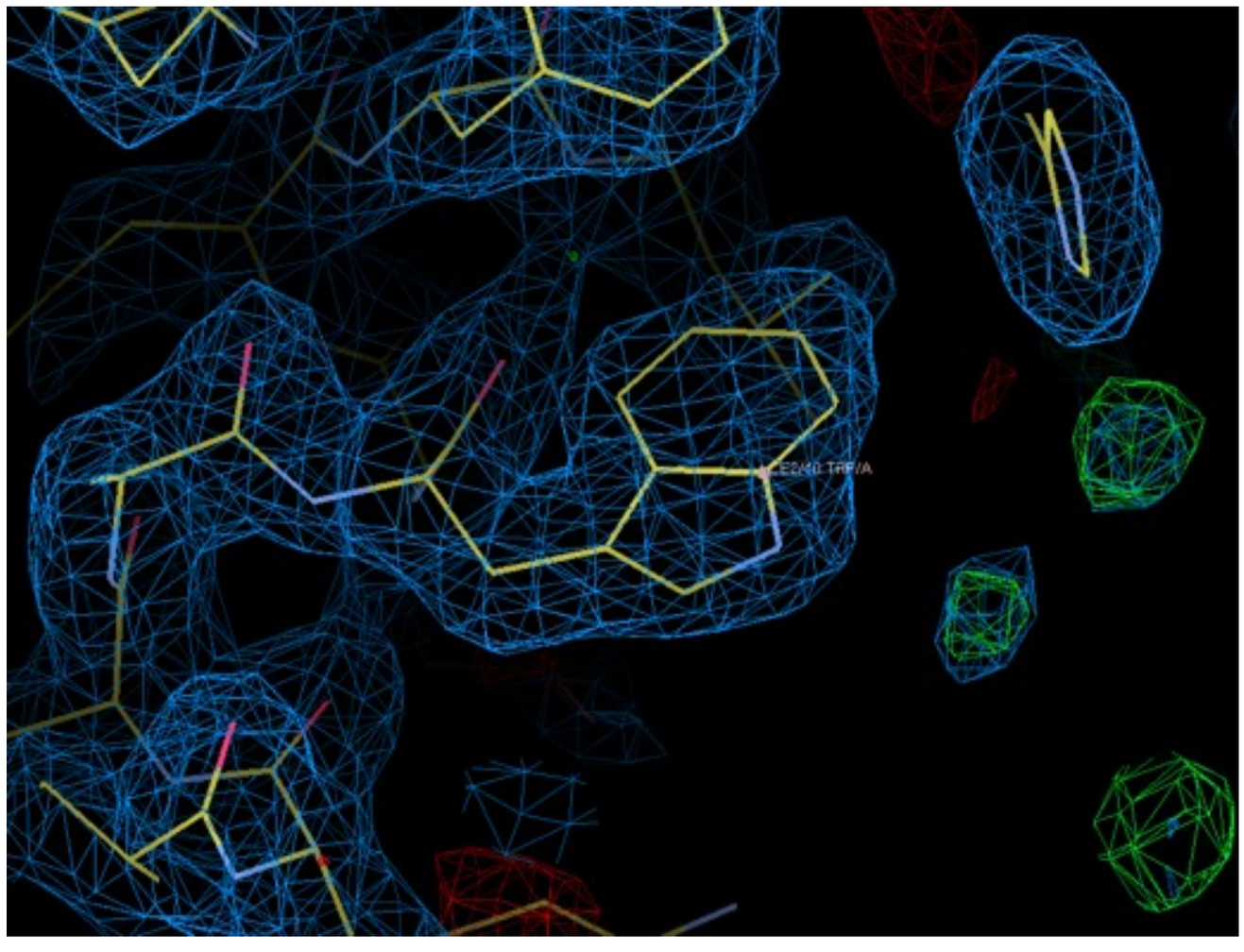


Blue = $2F_o$-F_c map

Red/green = F_o-F_c map

1.8 Å resolution map

Map Interpretation



Blue = $2F_o$-F_c map

Red/Green = F_o-F_c map

1.8 Å resolution map

Crystal structure refinement

- Computer-assisted improvement of the agreement between model and diffraction intensity data
- Least-squares/maximum likelihood methods
- Refine model parameters to improve fit of F_{calc} to F_{obs}
  - Atom positions (x_j, y_j, z_j)
  - Temperature factors (B)
  - Occupancy

Model evaluation

- **How well does the model predict the X-ray data?**
  - *R*-factor
  - R_{free} statistic

$$R = \frac{\sum ||F_{obs}| - |F_{calc}||}{\sum |F_{obs}|}$$

- **How well does the model conform to known geometric parameters?**
  - RMS deviation (bond lengths, angles)
  - Ramachandran statistics
  - Molprobity server

Model quality is a local property

Refinement statistics

	WT
Data collection statistics	
Maximum resolution (Å)	1.50
Wavelength (Å)	0.9918
Total reflections	98,554
Unique reflections	27,758
Completeness (%)[a]	98.9 (97.1)
$I/\sigma(I)$	7.8 (1.5)
R_{sym} (%)[b]	5.1 (40.4)
Refinement statistics	
Resolution limits (Å)	31.5–1.50
R-factor[c]	0.184
R-free	0.216
Estimated coordinate error (Å)[d]	0.09
RMS deviations from ideal values	
Bond lengths (Å)	0.024
Bond angles (°)	2.192
Dihedral angles (°)	25.11
Improper torsion angles (°)	1.60
Average temperature factor (Å^2)	
Protein	22.1, 20.6
Iron-sulfur	13.0, 12.8
Water	36.8
Zinc	37.1
Sulfate	44.3
Ramachandran plot,[e] residues in	
Most favored regions (%)	90.2
Additional allowed regions (%)	9.1
Generously allowed regions (%)	0.6
Disallowed regions (%)	0.0

→ *R* should be between 0.1-0.3 and within 0.05 of R_{free}

Yeh, A.P., Ambroggio, X.I., Andrade, S.L.A., Einsle, O., Chatelet, C., Meyer, J., and Rees, D.C. (2002) *J. Biol. Chem. 277*, 34499-34507.

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  - Ramachandran statistics
  - Molprobity metrics and other validation tools

Model quality is a local property

Model validation resources

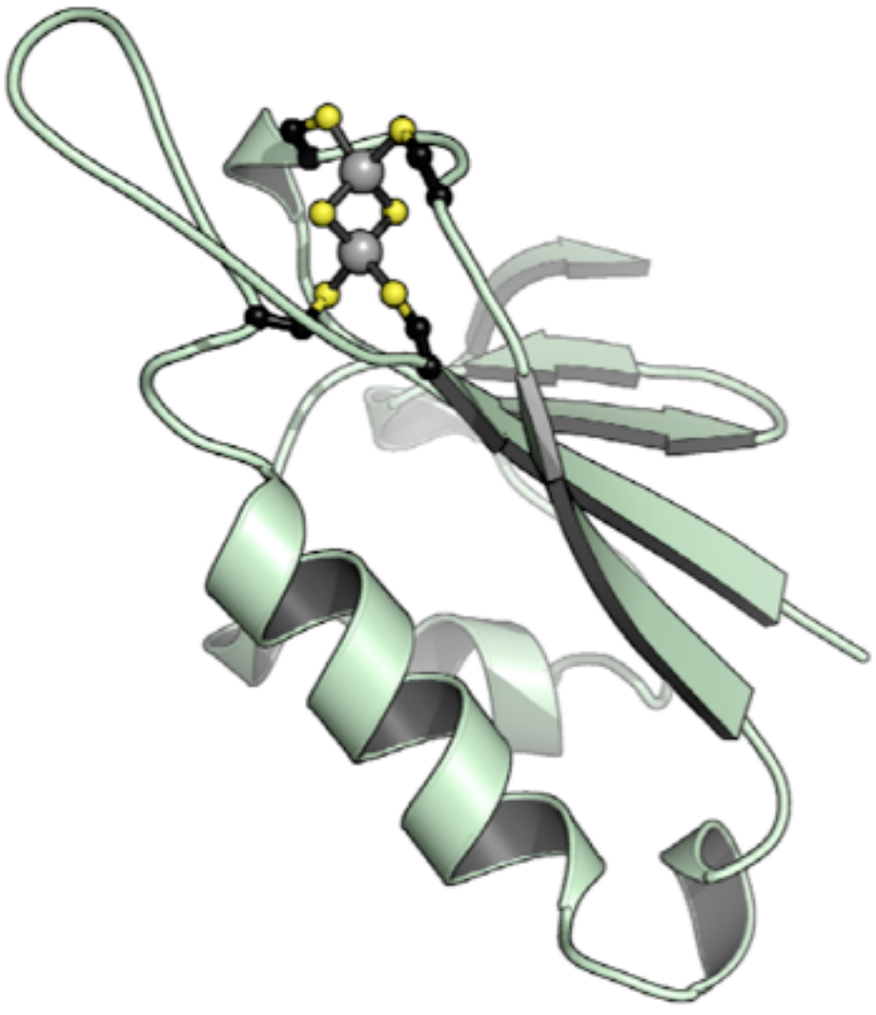
- Molprobity server
  - molprobity.biochem.duke.edu/
- PDB Validation Server
  - validate.rcsb.org/
- Other resources
  - www.rcsb.org/pdb/static.do?p=software/software_links/analysis_and_verification.html

Crystallography workflow

- Biological sample production
- Crystallization
- X-ray diffraction data collection
- Structure solution/phase determination
- Model building and validation

A preview of the tutorial

Complete de novo structure determination of a thermophilic [2Fe-2S] ferredoxin by MAD phasing



Diffraction data processing

Derive heavy atom positions

Use automated phasing pipeline

Model building and refinement

Oliver Einsle, Amie Boal, Andrew Mitchell