Macromolecular X-ray Crystallography

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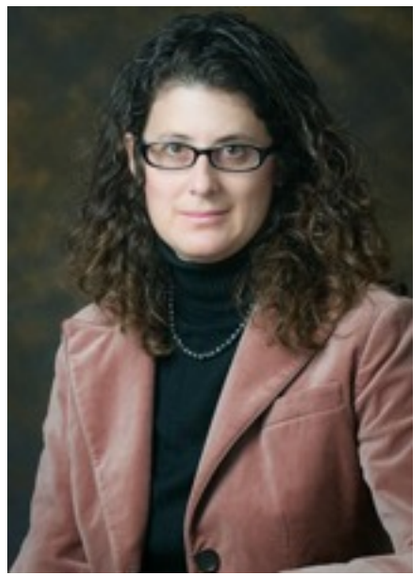
Rosenzweig Lab

References

- Rhodes, G. Crystallography Made Crystal Clear. 3rd ed. 2006. Academic Press, Burlington.
- Drenth, J. Principles of Protein X-ray Crystallography. 1994. Springer-Verlag, New York.
- Hampton Research Catalog, volume 18
- McPherson, A. Crystallization of Biological Macromolecules. 1998. CSHL Press, Cold Spring Harbor.
- http://skuld.bmsc.washington.edu/scatter

The real experts...



Amy Rosenzweig

Tom Lawton

Why X-ray Crystallography?

- Widely-used method for molecular-level 3-D structure determination of biomolecules (proteins, nucleic acids)

Why X-ray Crystallography?

- For bioinorganic chemists:
  - Visualize how biomolecules interact with metals
    - Structures of complex metallocofactors
    - Identification of metal centers
      - Stoichiometry

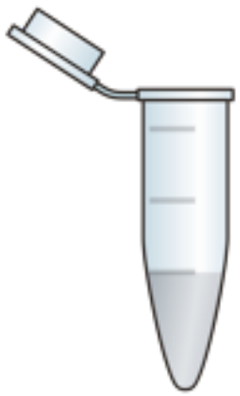
Sommerhalter *et al.* (2005) *Inorg. Chem. 44*, 770-778.

Why X-ray Crystallography?

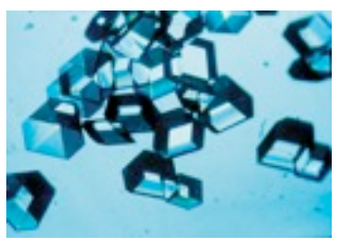
- Limitations
  - Oxidation states often unknown
    - Can be affected by X-ray exposure
  - Occupancy of metal binding sites can be altered
  - An ensemble technique

Sommerhalter *et al.* (2005) *Inorg. Chem. 44*, 770-778.

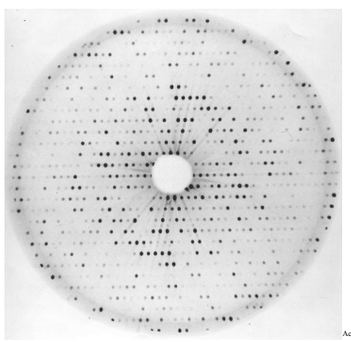
A crystallographic experiment



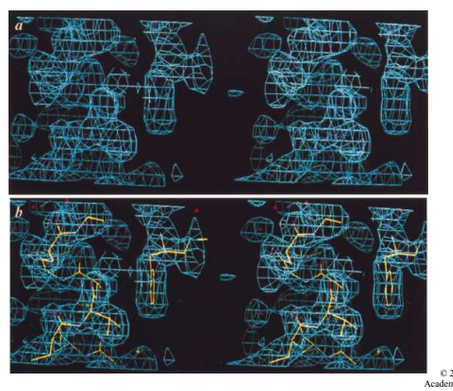
Protein



Crystals



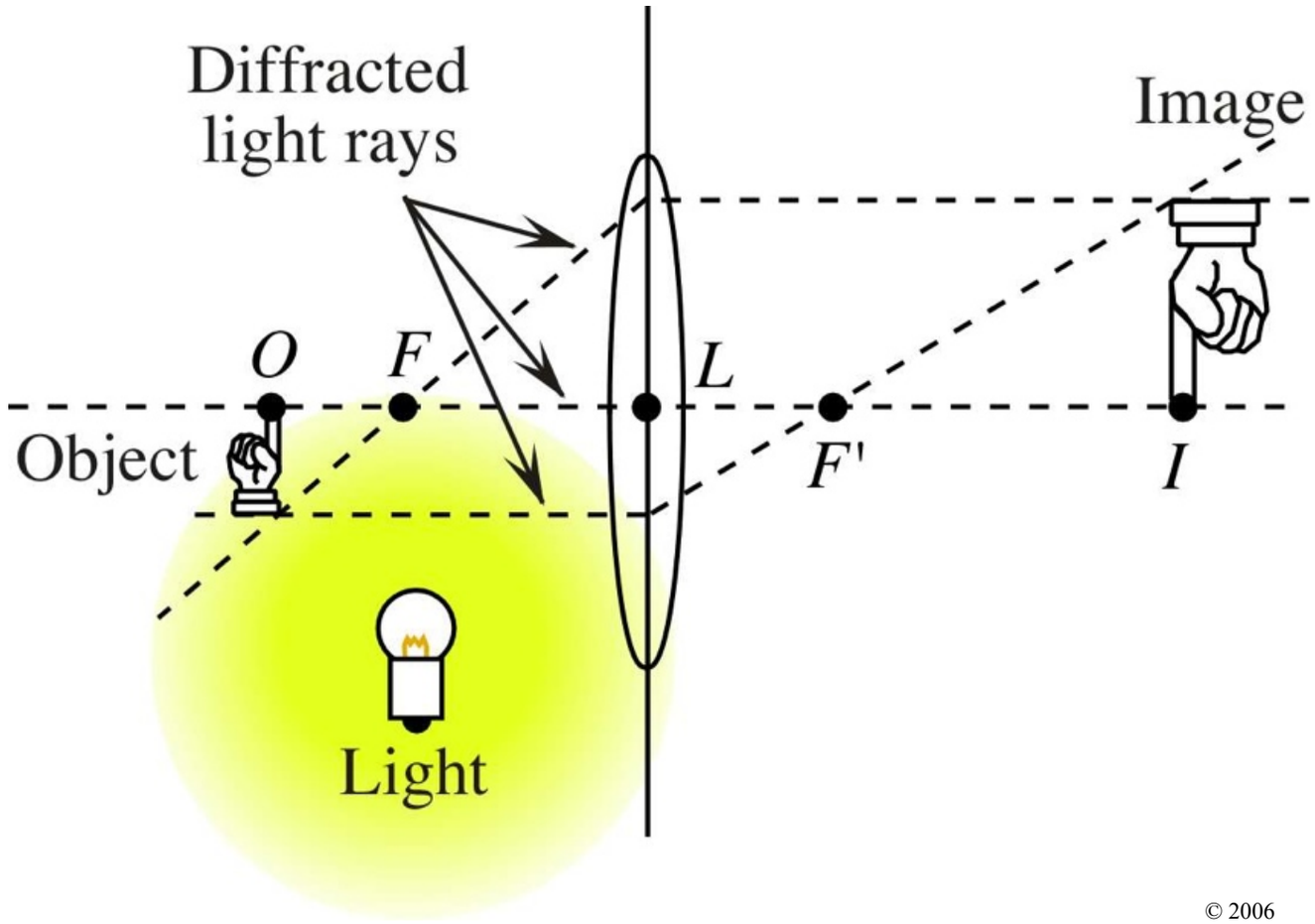
X-ray diffraction



Electron density map
and
Molecular model

Why use this approach?

Microscopy analogy



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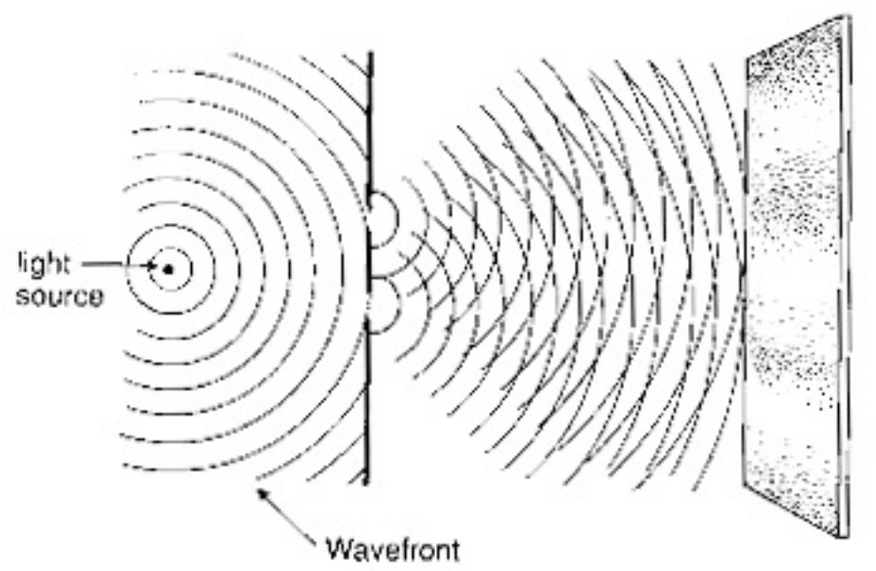
Diffraction

- Bending or scattering of waves around obstacles
- Object and wavelength (λ) must be of similar size
- Scattered waves can interfere constructively or destructively

double slit experiment

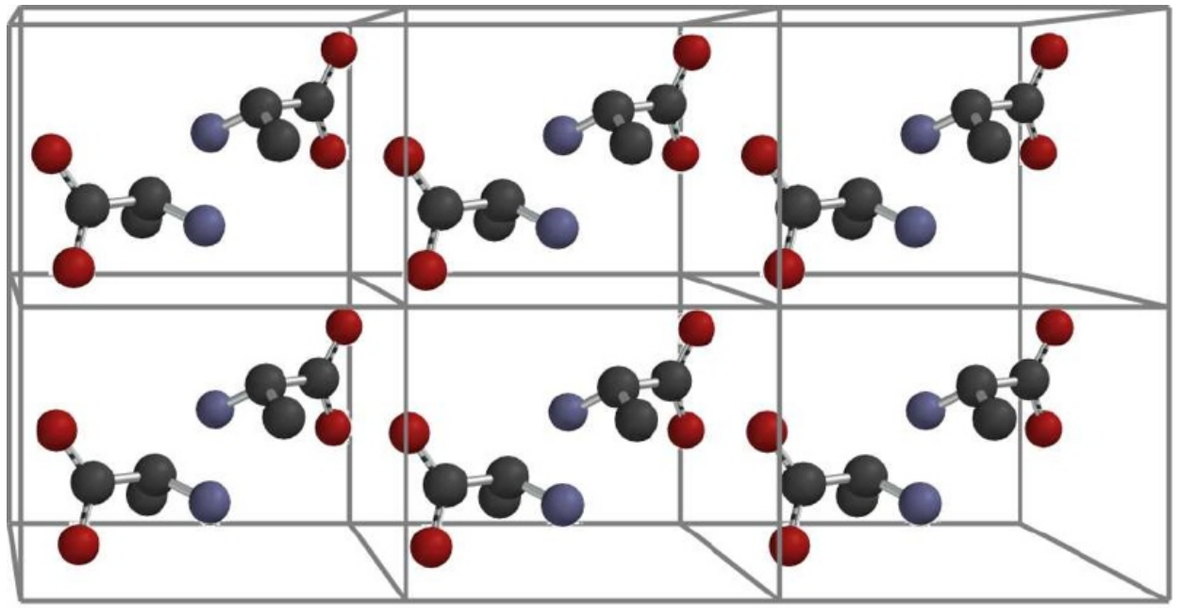


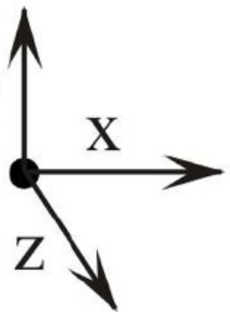
Diffraction and structure

- In microscopy, light is diffracted by objects and is focused by lenses to create a magnified image
  - λ of incident light ~ object
    - Visible light is 400-700 nm
    - Bonded atoms are 0.1 nm (~1 Å)
    - Appropriate λ is in X-ray range (0.1-100 Å)

Two problems:

1. Single molecules diffract X-rays weakly





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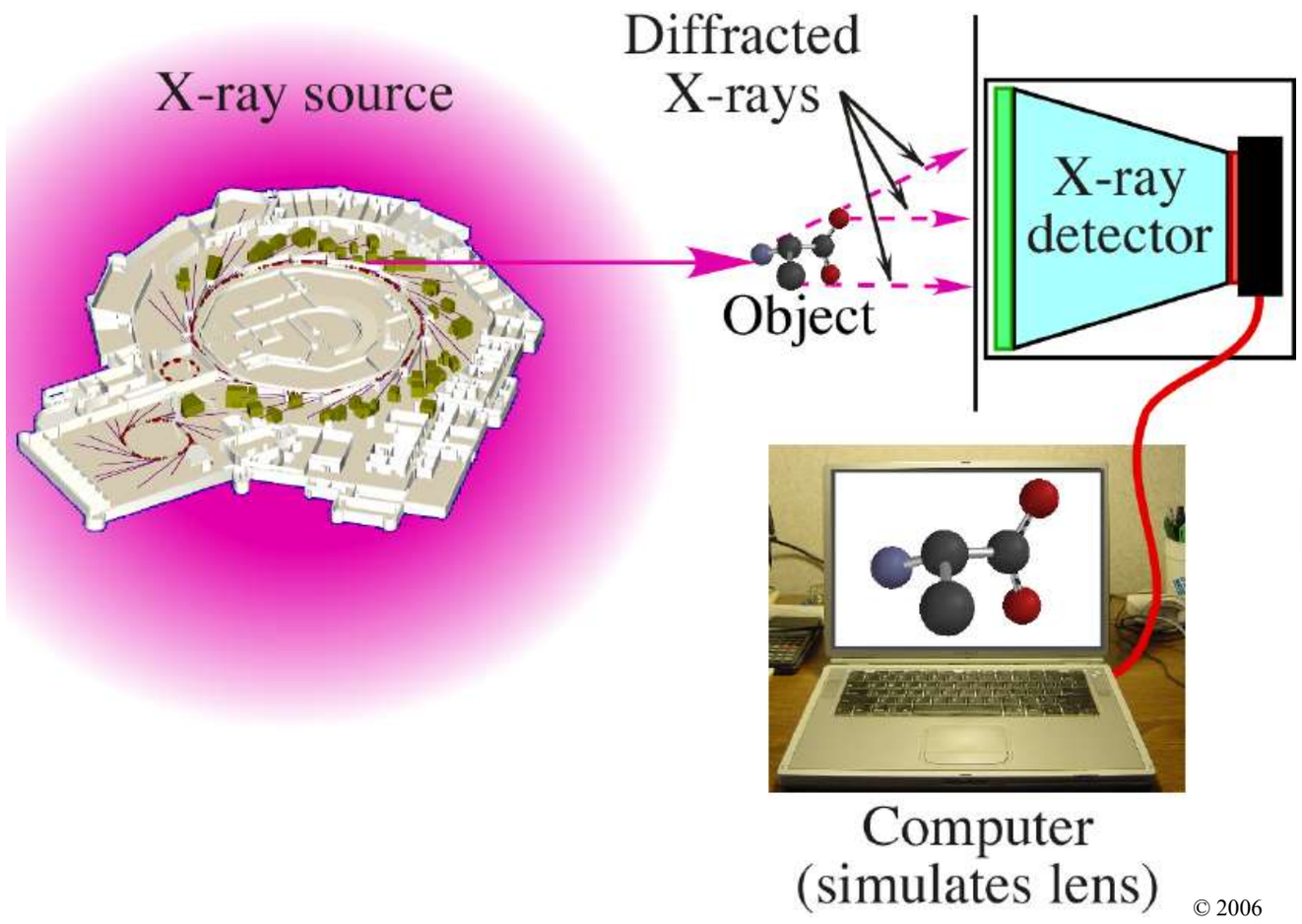

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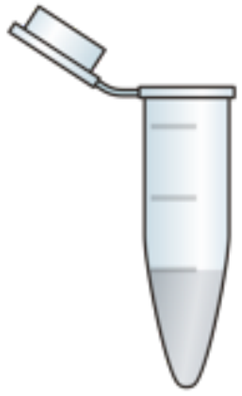
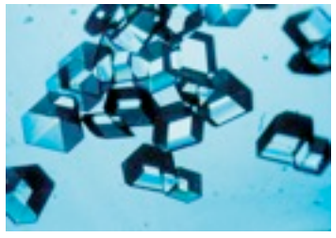
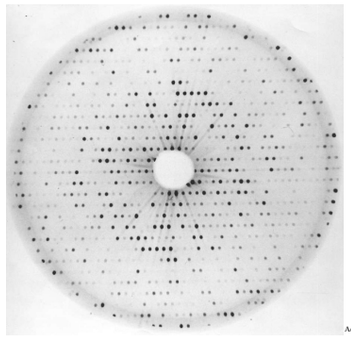
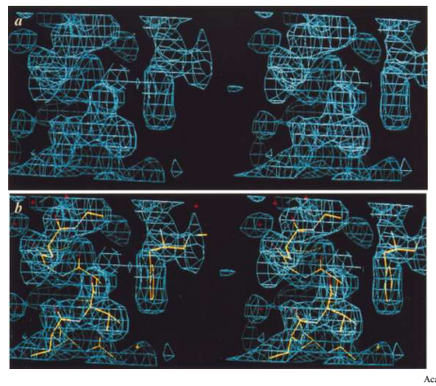
11. X-rays can't be focused by lenses

A crystallographic experiment



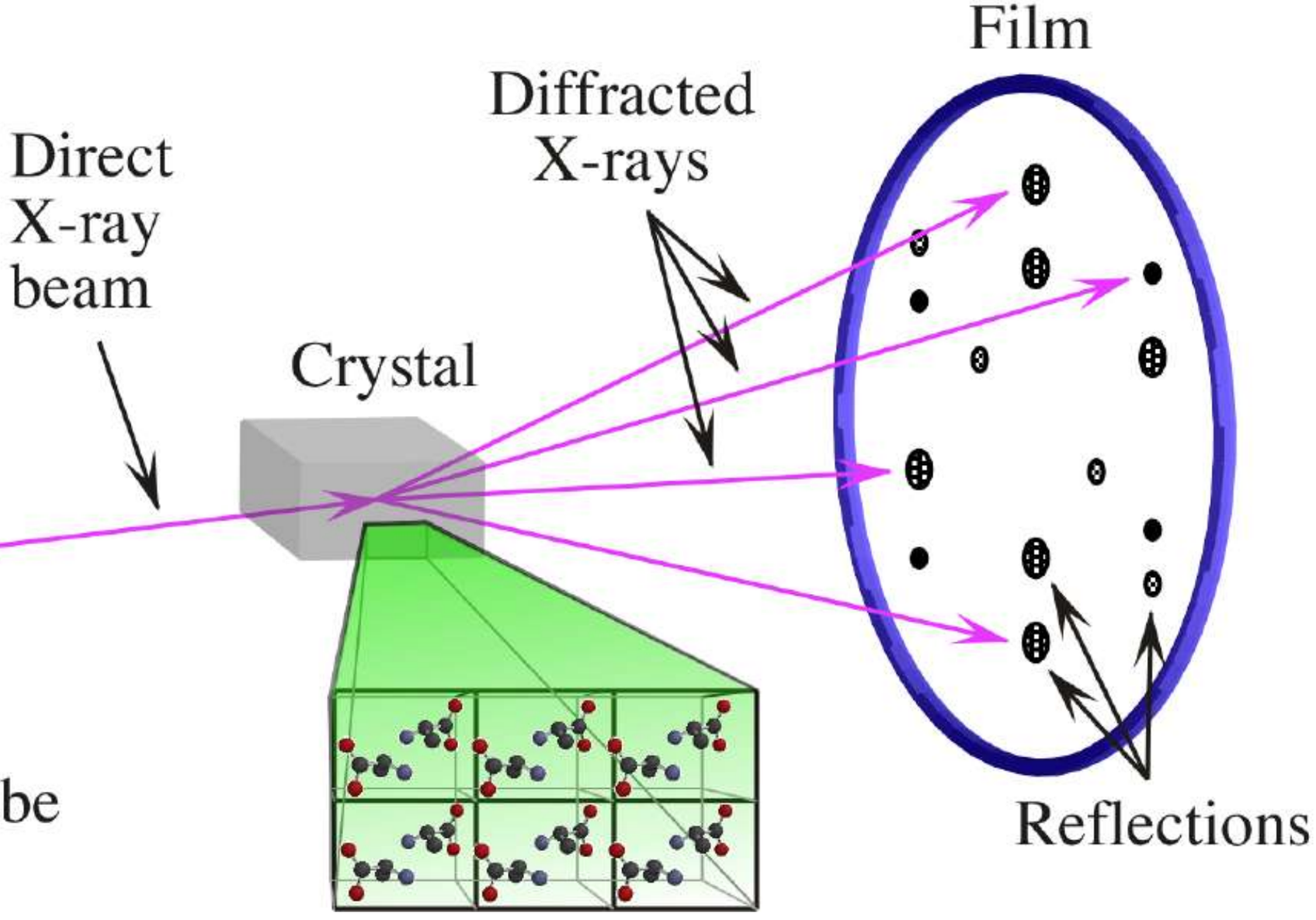






- Crystallization
- Diffraction data collection
- Structure solution
- Model building and refinement

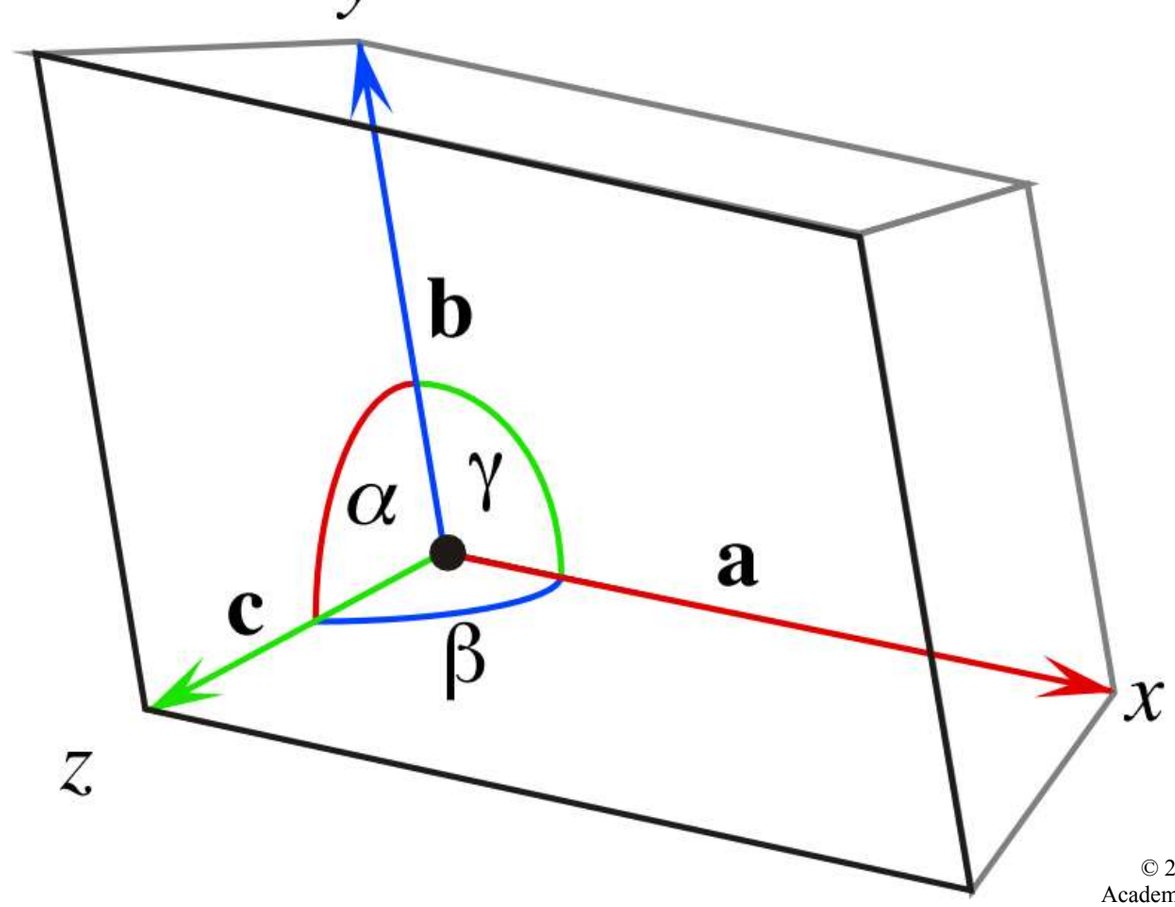
An X-ray diffraction experiment



Rigaku in-house generator

I. 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



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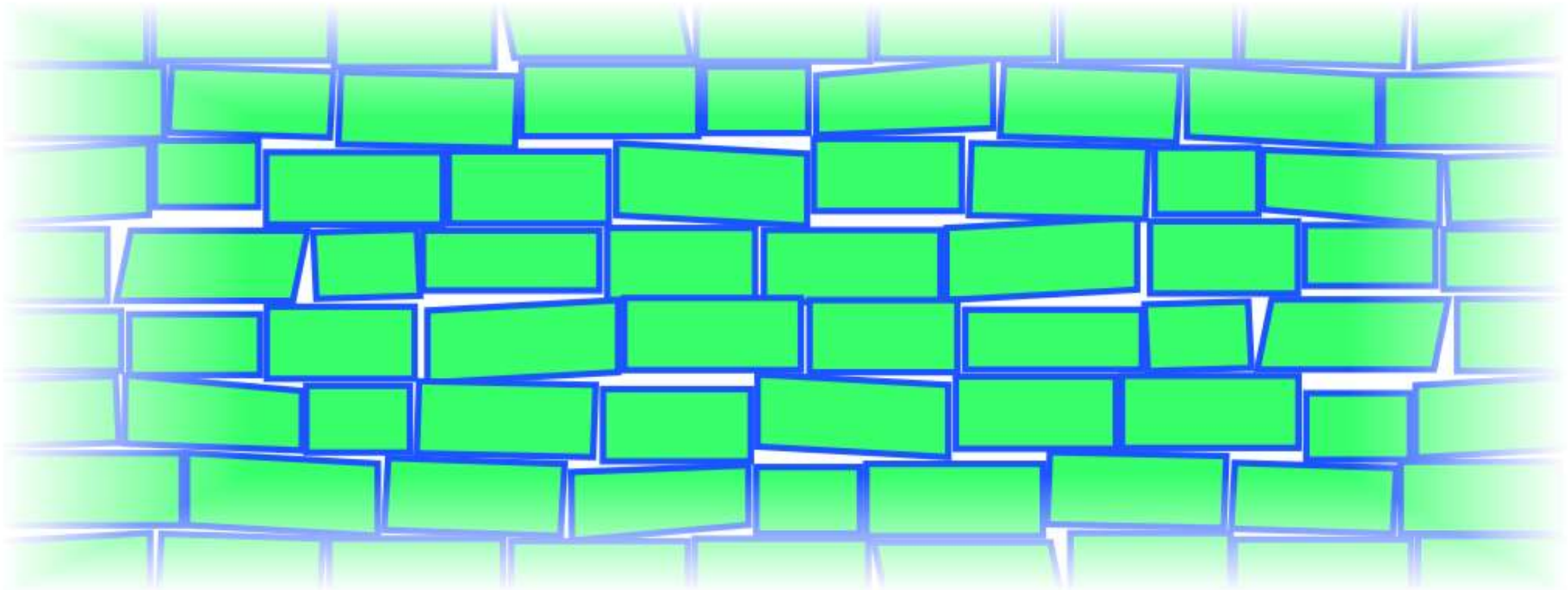
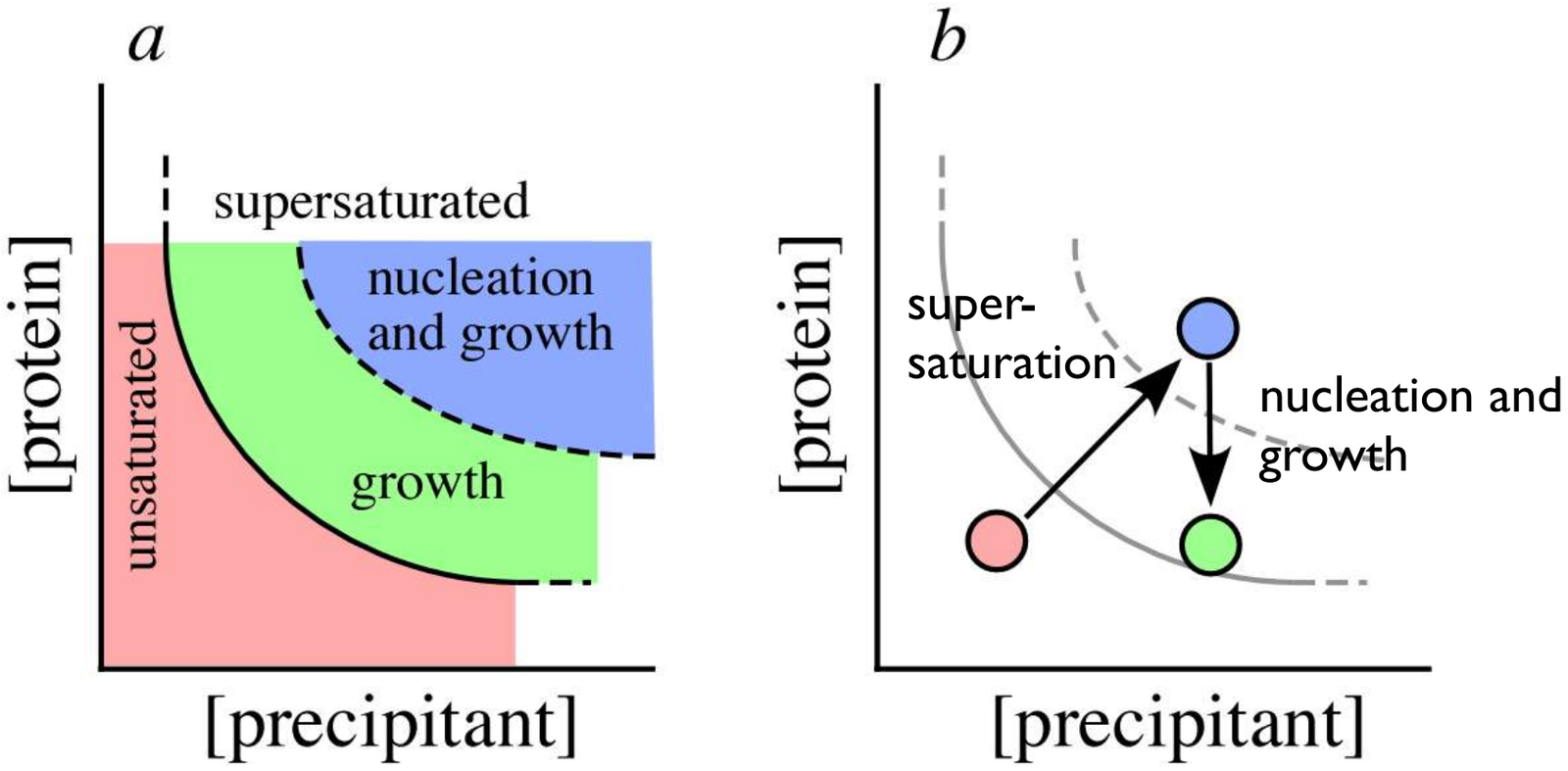
I. Crystallization



- Lattice is array of unit cells
- Crystallographic experiment provides an image of average electron density in a unit

1. Growing crystals



- Theory: supersaturation, nucleation, growth
- Practice: slow controlled precipitation from solution without unfolding the protein

1. Growing crystals

- Supersaturation can be achieved by addition of precipitating agents
  - Salts (ammonium sulfate, NaCl)
  - Polymers (polyethylene glycol)
  - Organic solvents (alcohols, 2-methyl-2,4-pentanediol)

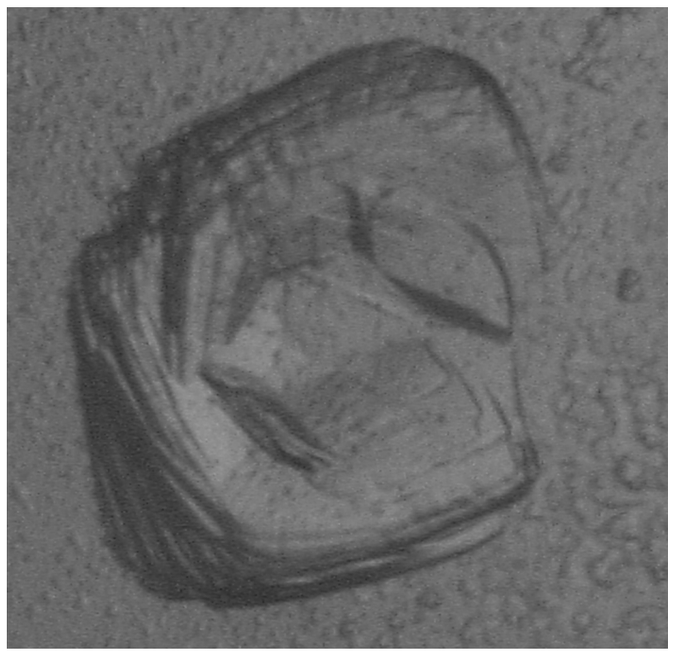
I. Growing crystals

- Nucleation and growth
  - An aggregate of sufficient size and order initiates crystal growth
  - Usually a random event but techniques exist to drive nucleation (i.e., seeding)
  - Additives can influence growth and nucleation

I. Growing crystals

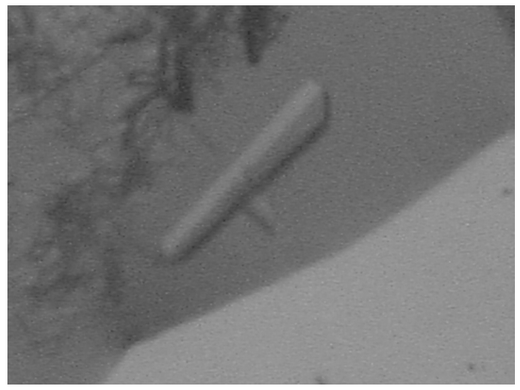
- Other variables
  - Protein purification, preparation, stability
  - Structural homogeneity
  - Temperature
  - pH

I. Improved purification



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

20-30% PEG 4000
0.2-0.4 M lithium sulfate
0.1 M HEPES pH 6.5-7.0



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

same crystallization conditions

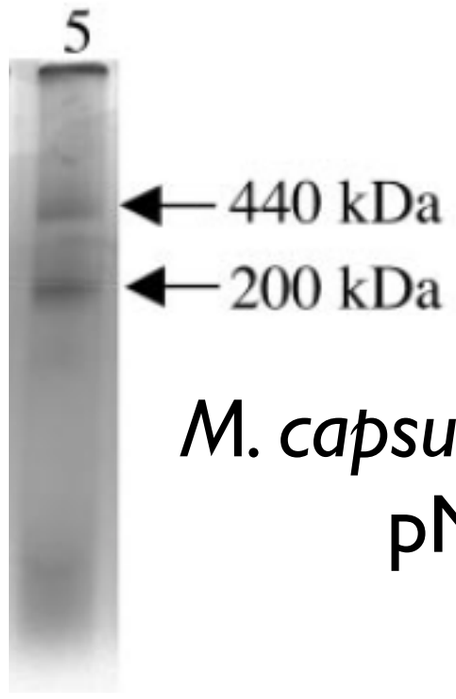
1. Growing crystals

- Other variables
  - Protein purification, preparation, stability
  - Structural homogeneity
  - Temperature
  - pH

of metal cofactors

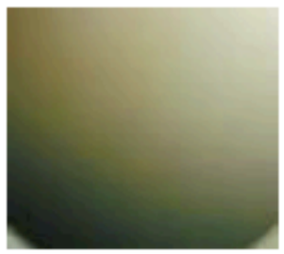
1. Growing crystals

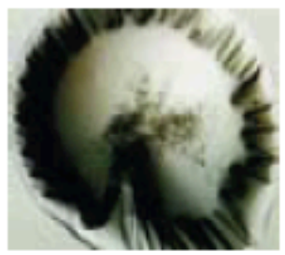
- Other variables
  - Protein purification, preparation, stability
  - Structural homogeneity
  - Temperature
  - pH

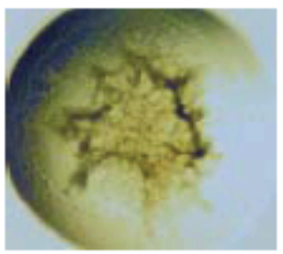


M. capsulatus (Bath) pMMO

I. Growing crystals


Clear Drop


Skin / Precipitate


Precipitate

Precipitate / Phase

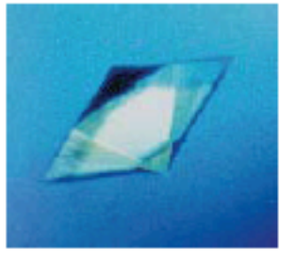
Quasi Crystals

Microcrystal

Needle Cluster

Plates

Rod Cluster


Single Crystal

- can’t predict crystallization conditions
- extensive screening by trial and error required

1. Growing crystals

Vapor diffusion is the most commonly used method

low [ppt]

figure 1

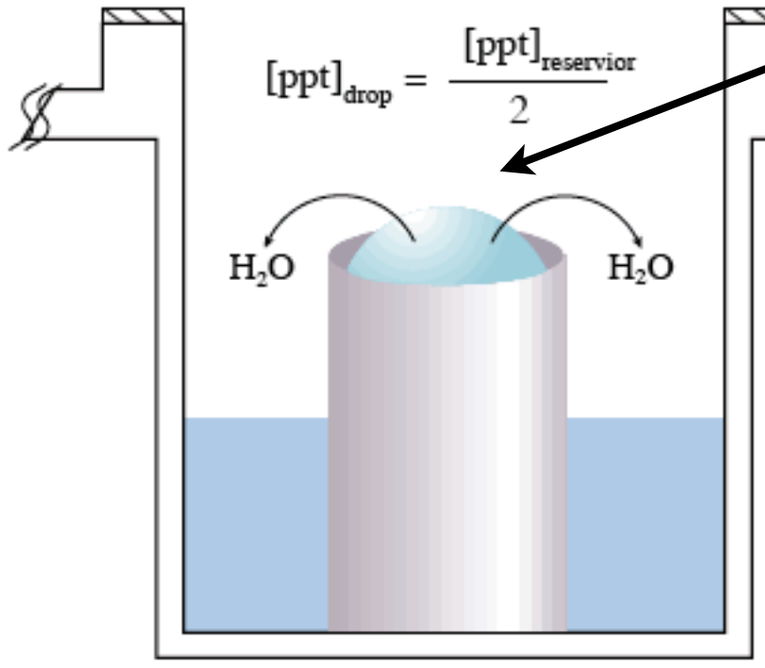
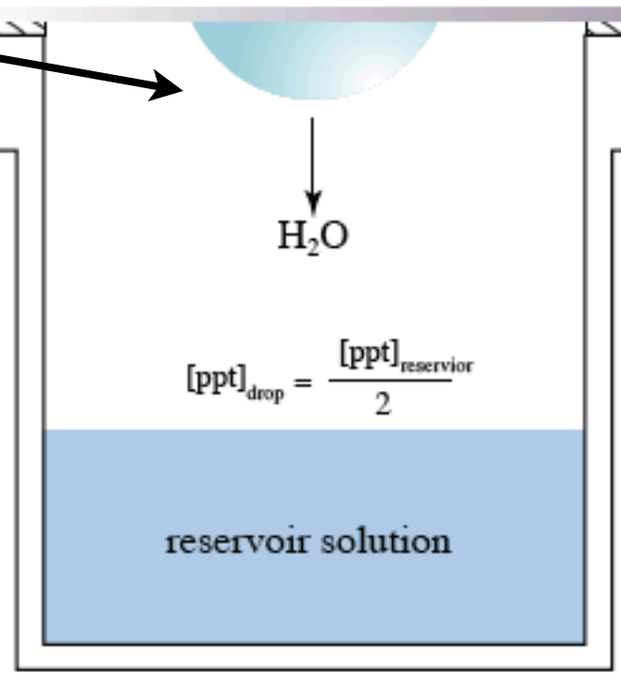


figure 2

high [ppt]



Sitting drop

Hanging drop

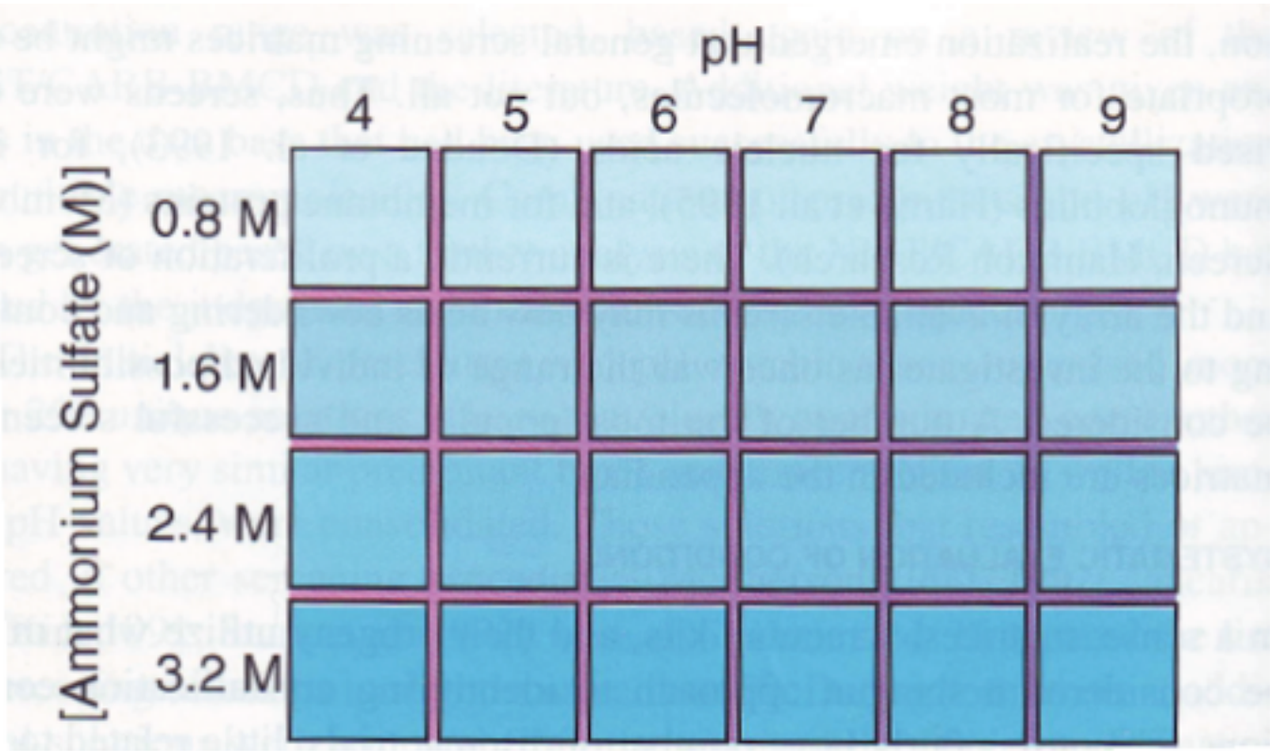
1. Growing crystals

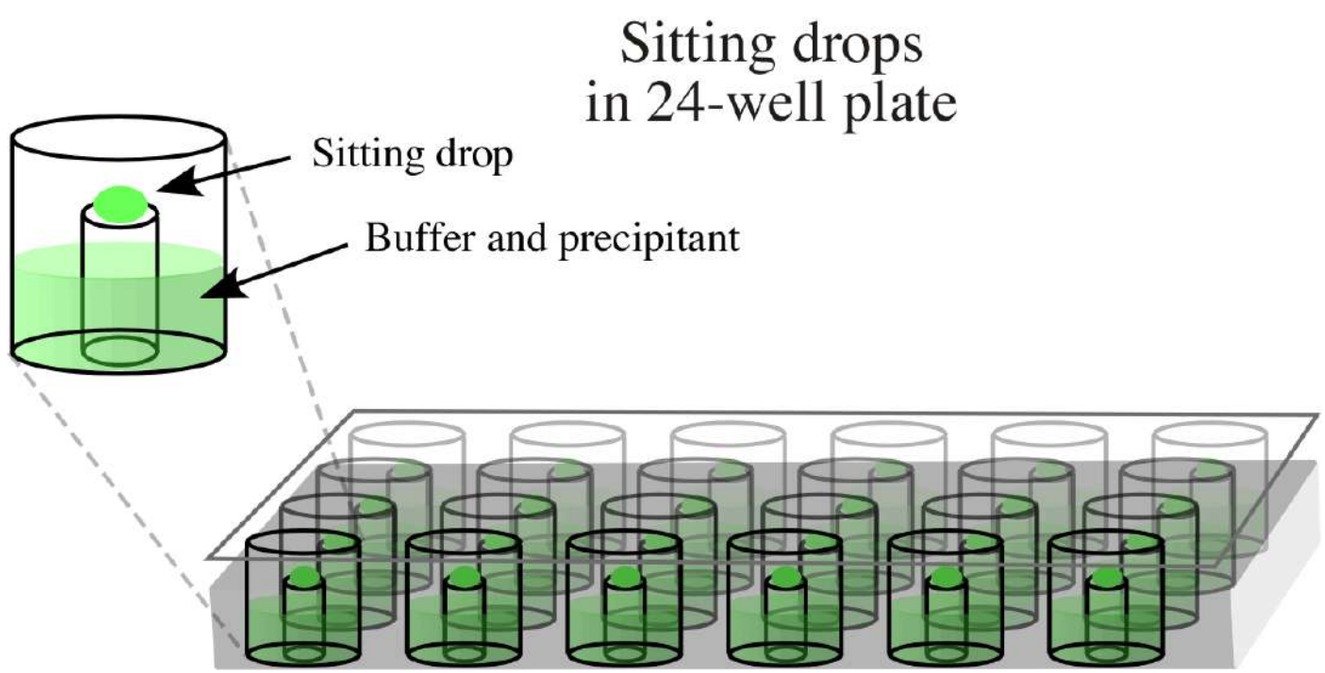
- Sparse matrix screens are typical first step
  - Diverse sets of conditions based on database mining of published crystallization conditions
  - Intentionally biased screen towards conditions that have worked previously
  - Commercially available, 96-well format

I. Once you have crystals...

- Are they protein crystals?
- Are they crystals of the protein of interest?
- Is it a single crystal of sufficient size?
- Can the crystals be harvested and frozen?
- Do the crystals diffract X-rays?
- Can enough data be collected to solve the structure?

I. Optimization





Secondary screens:

24-well format

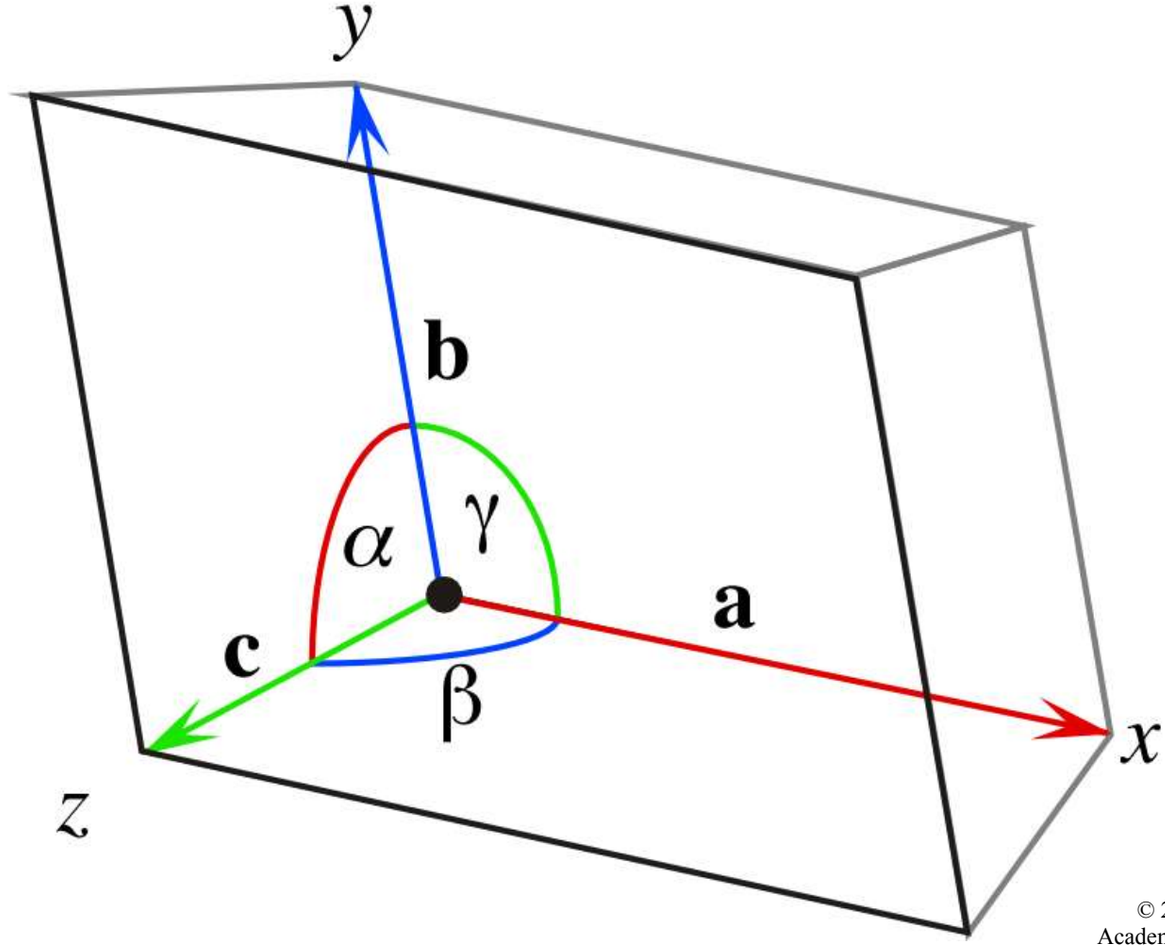
Homemade screens

pH vs. [ppt] is a typical starting point

Other variables come into play

I. Crystal lattices

- A crystal lattice is made up of unit cells
- Unit cells have a defined shape
- x, y, z coordinate system



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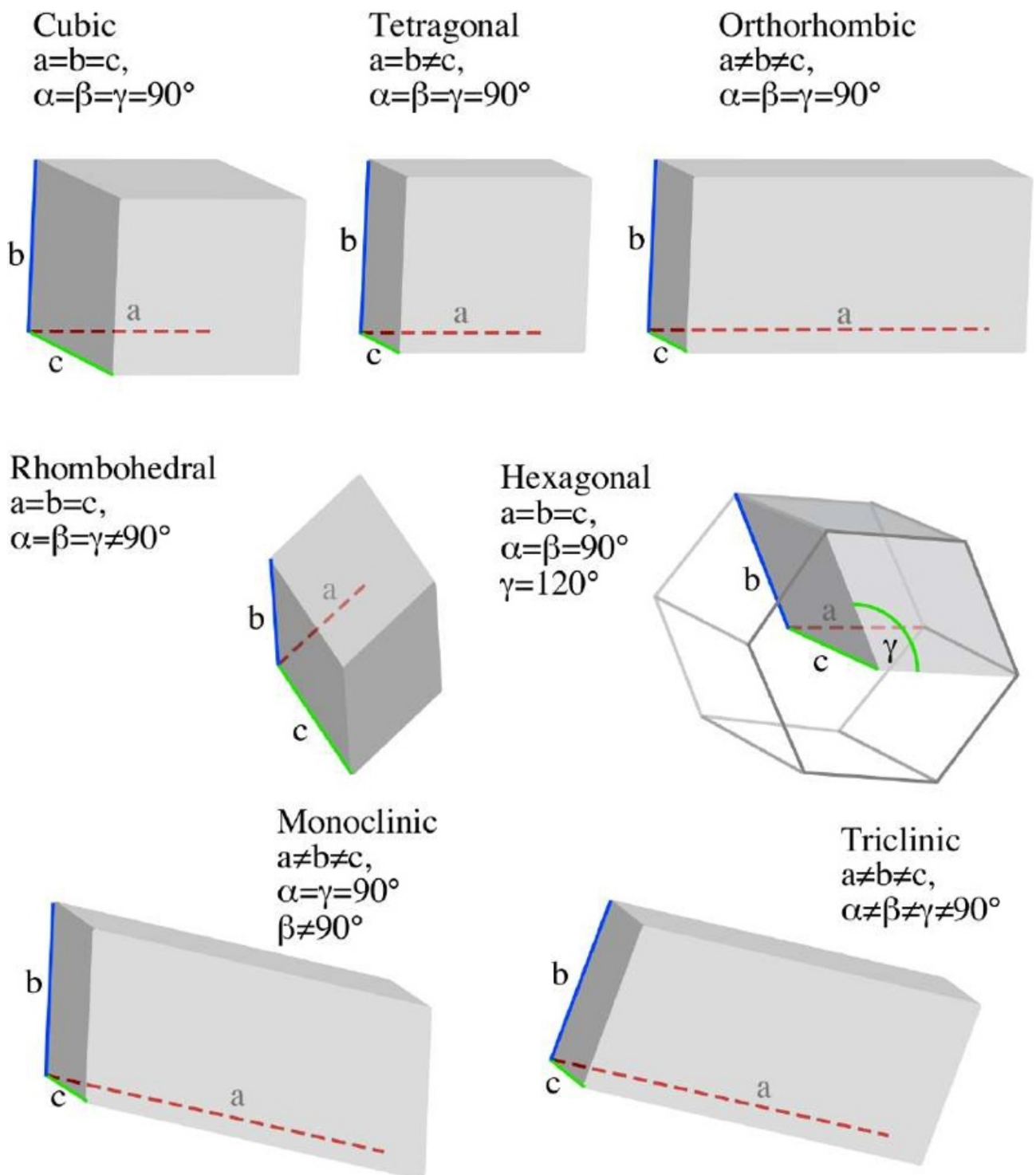
I. Lattice types



14 different lattice systems

Defined by unit cell shape

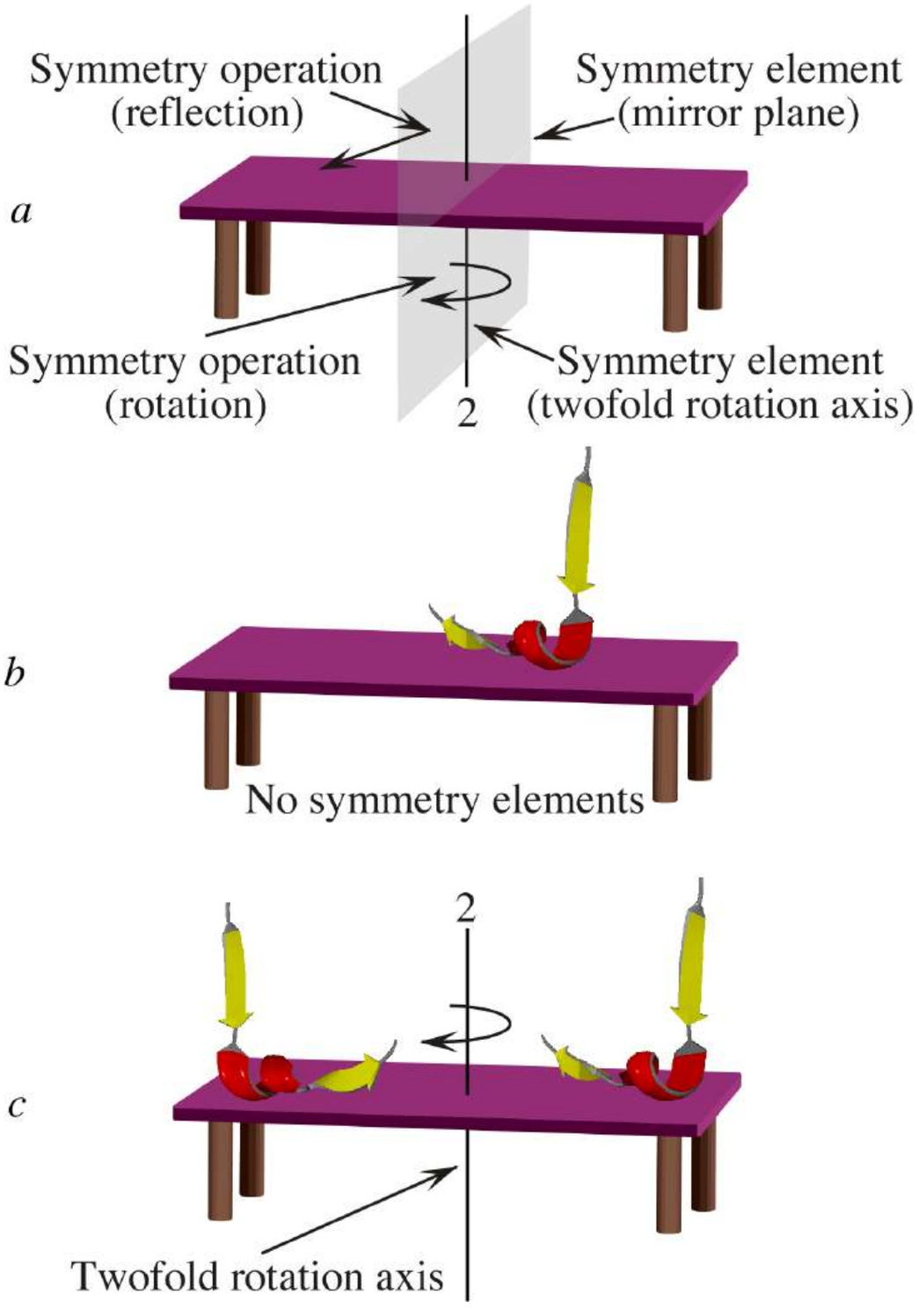
a = b = c relationships are identities

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

Can have internal symmetry
- multiple copies of protein in unit cell
- higher order oligomerization state related by symmetry

Final model only describes structure of asymmetric unit

I. Symmetry operations



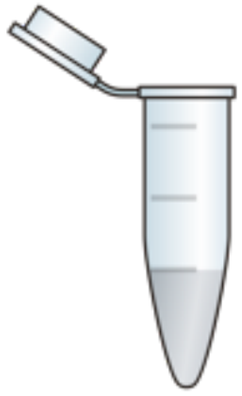
Symmetry operations classify lattices into space groups

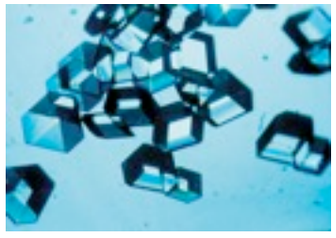
230 different space groups

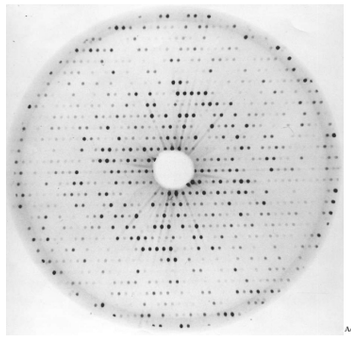
Chiral molecules restricted to 65 possible space groups

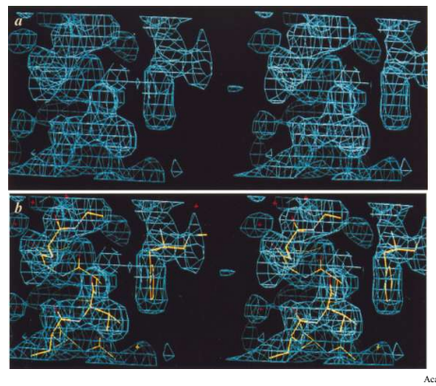
Assigning the correct space group is really important!

A crystallographic experiment







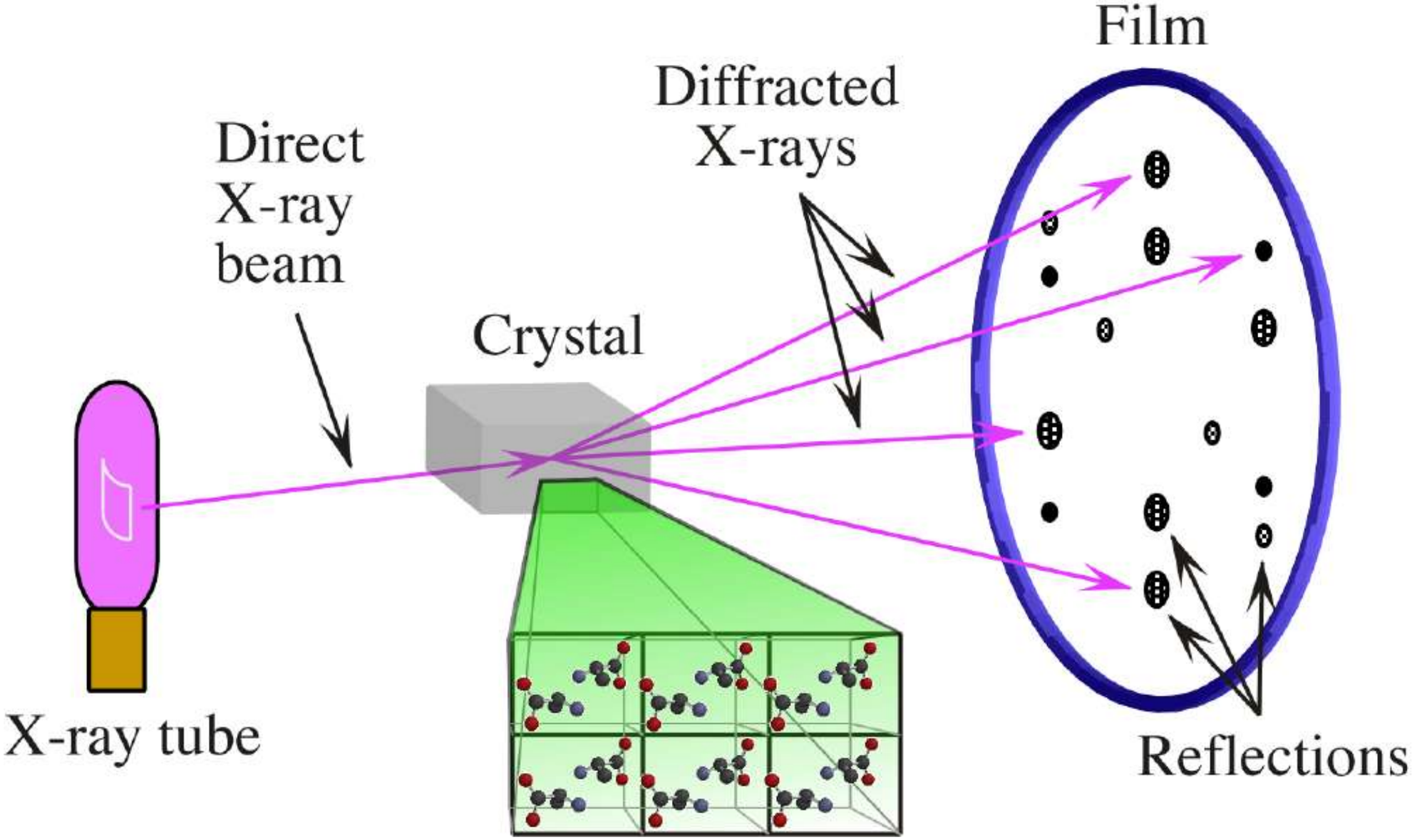


- Crystallization
- Diffraction data collection
- Structure solution
- Model building and refinement

II. A diffraction experiment

- Harvest and freeze crystals
- Take to X-ray source
- Screen for diffraction
- Collect datasets

II. A diffraction experiment

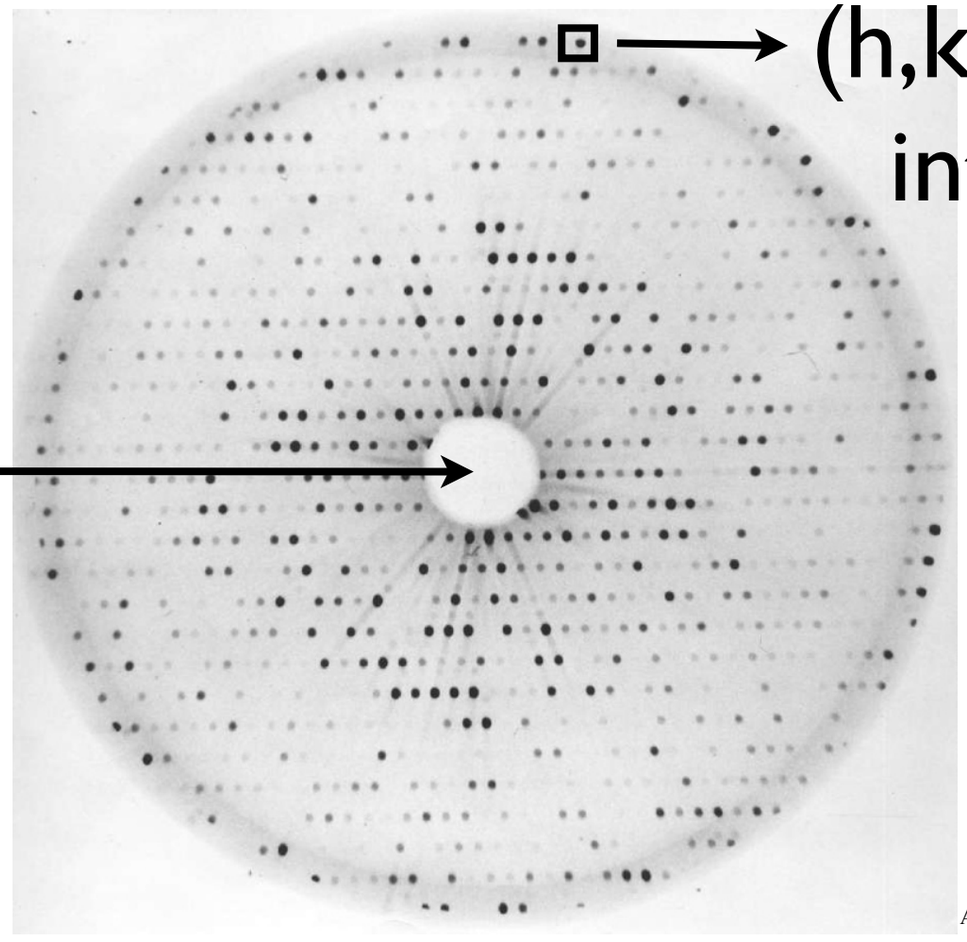


II. Diffraction data

- Each spot is a reflection with an index
- Indices tell us lattice type and unit cell dimensions

indices increase

(0,0,0)



(h,k,l) index, intensity

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II. X-ray diffraction

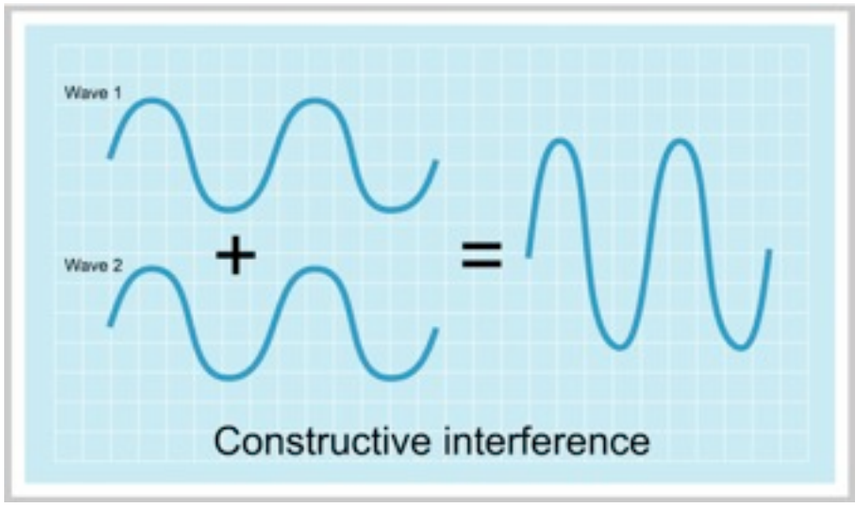
- In single crystal diffraction, only diffracted X-rays with strong constructive interference are observed

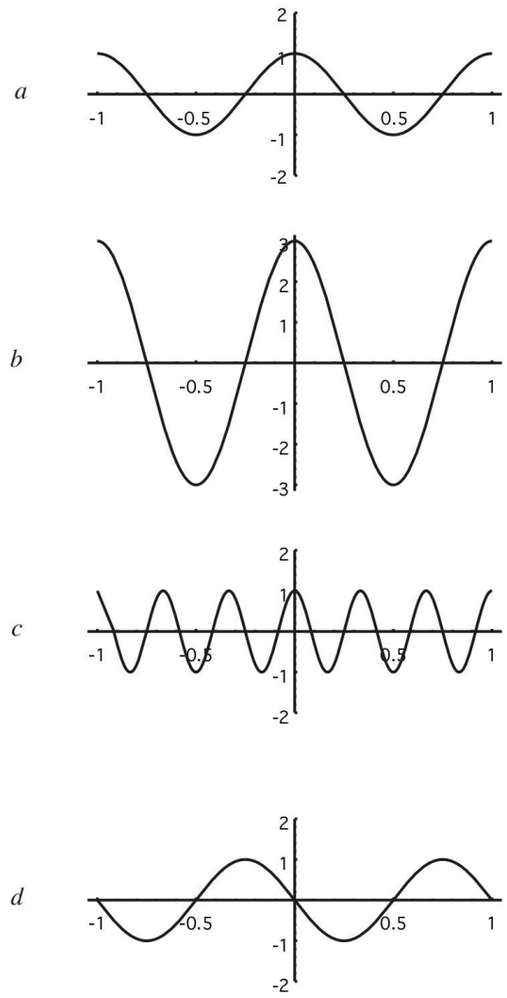
II. X-rays are waves

- Have an amplitude, phase, frequency

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

- Constructive interference increases amplitude





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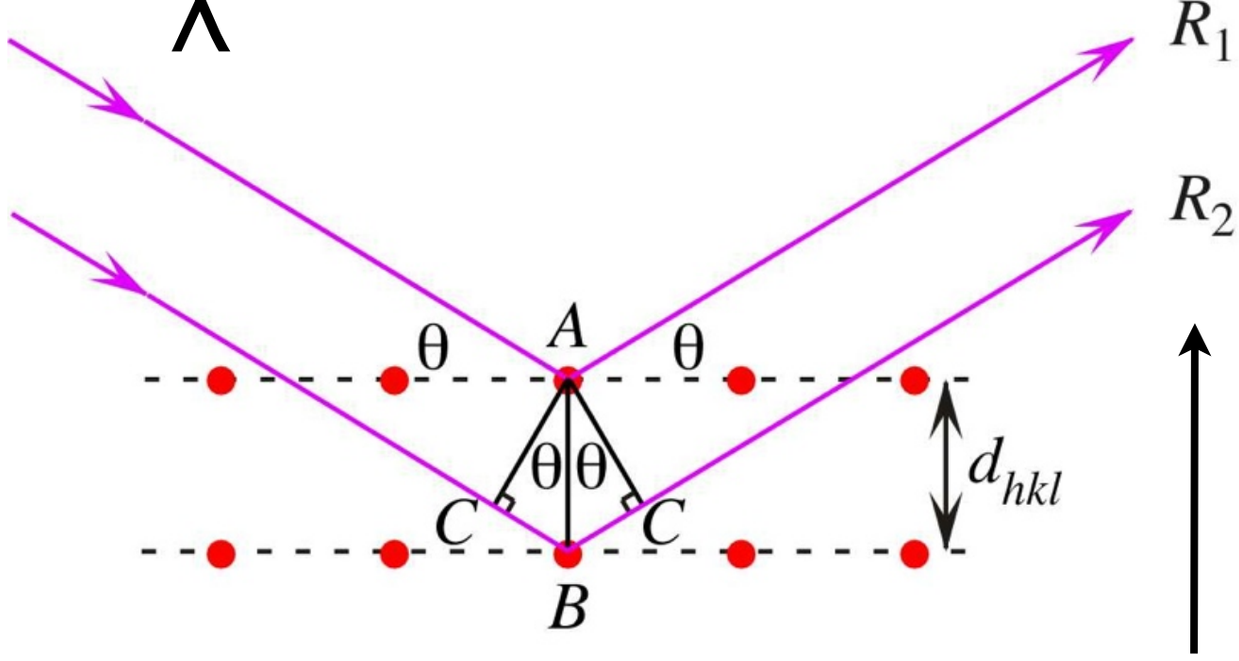
II. Bragg's law

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

- Conditions that satisfy Bragg's law lead to diffraction

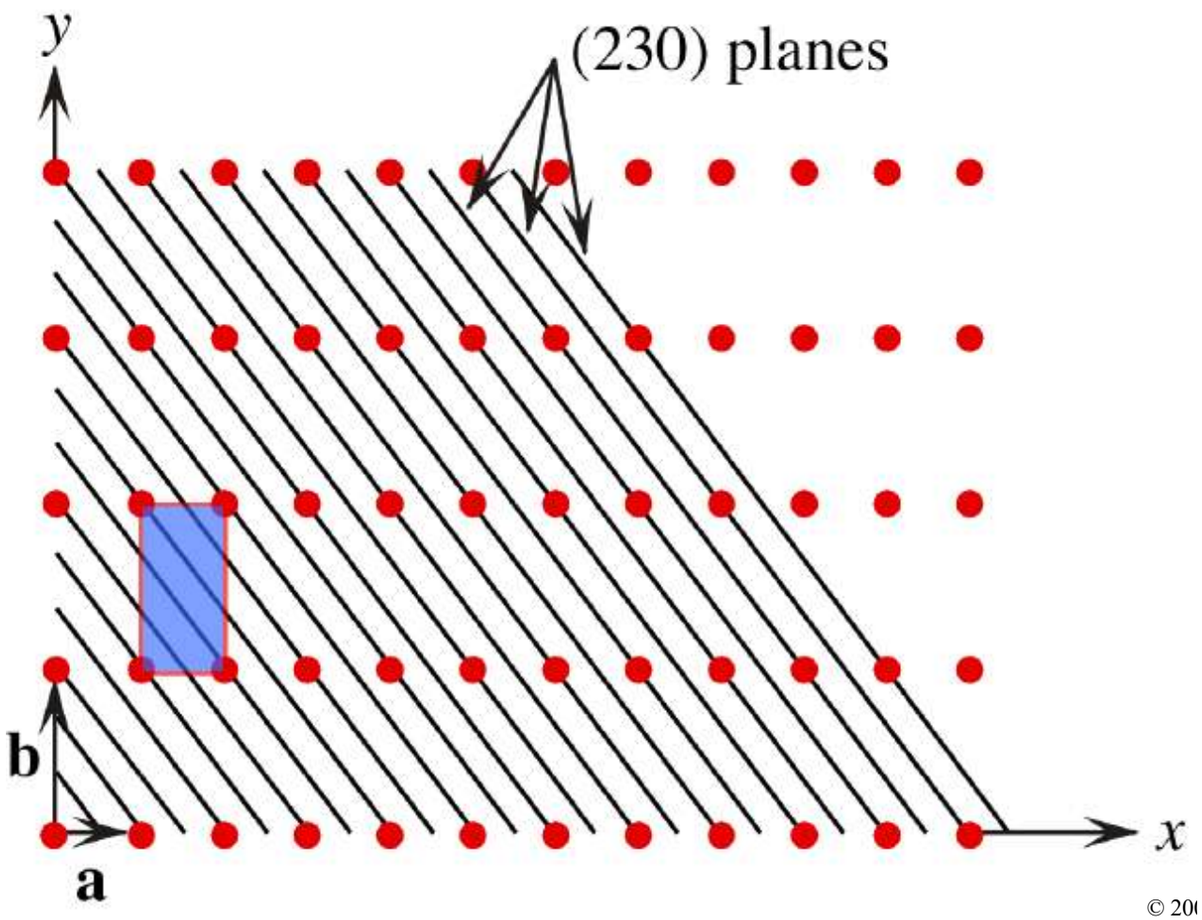


$2d_{hkl} \sin \theta = n\lambda$ in phase

II. Planes??

- A crystal lattice can be divided into 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 is the same as the index of corresponding reflection in the diffraction pattern

II. Examples of lattice planes



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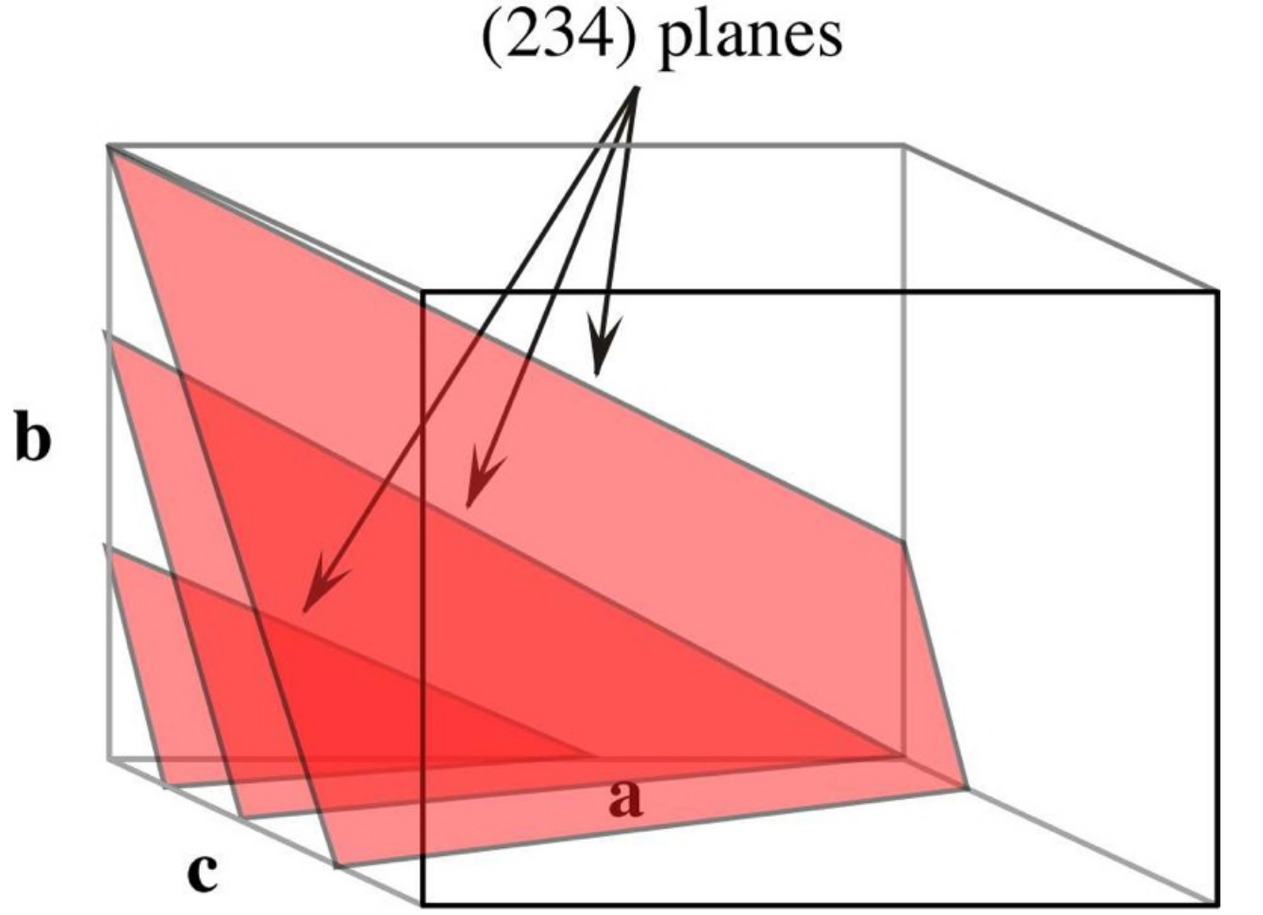

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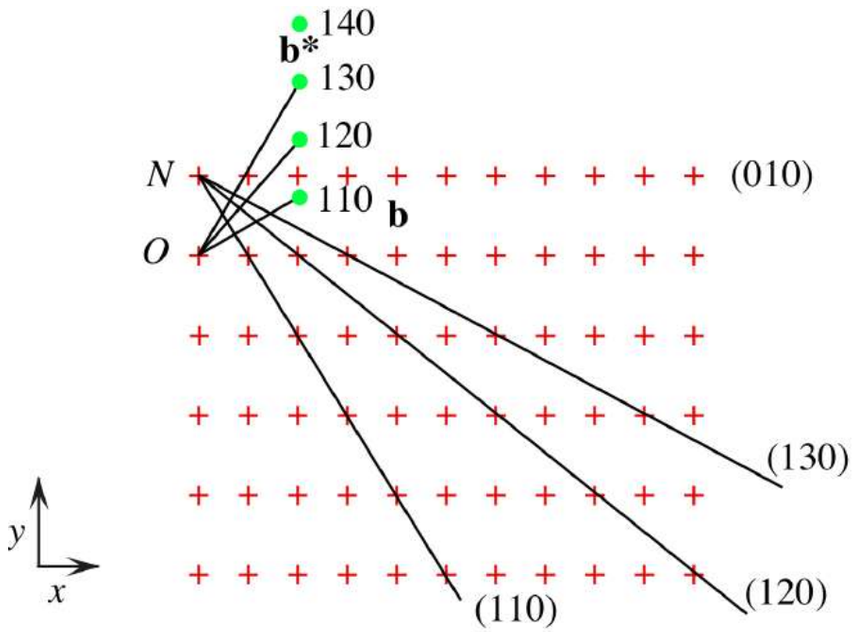
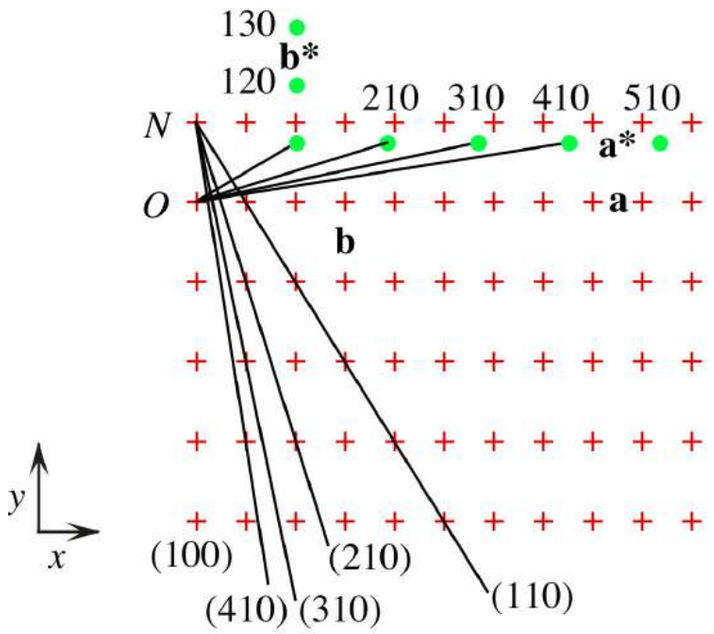
II. Planes II

- Each set of planes gives rise to a single reflection
- Reflections form a reciprocal lattice
- The reciprocal lattice is what we see in diffraction data - but related to crystal lattice via indices of lattice planes.

II. Real vs. reciprocal space

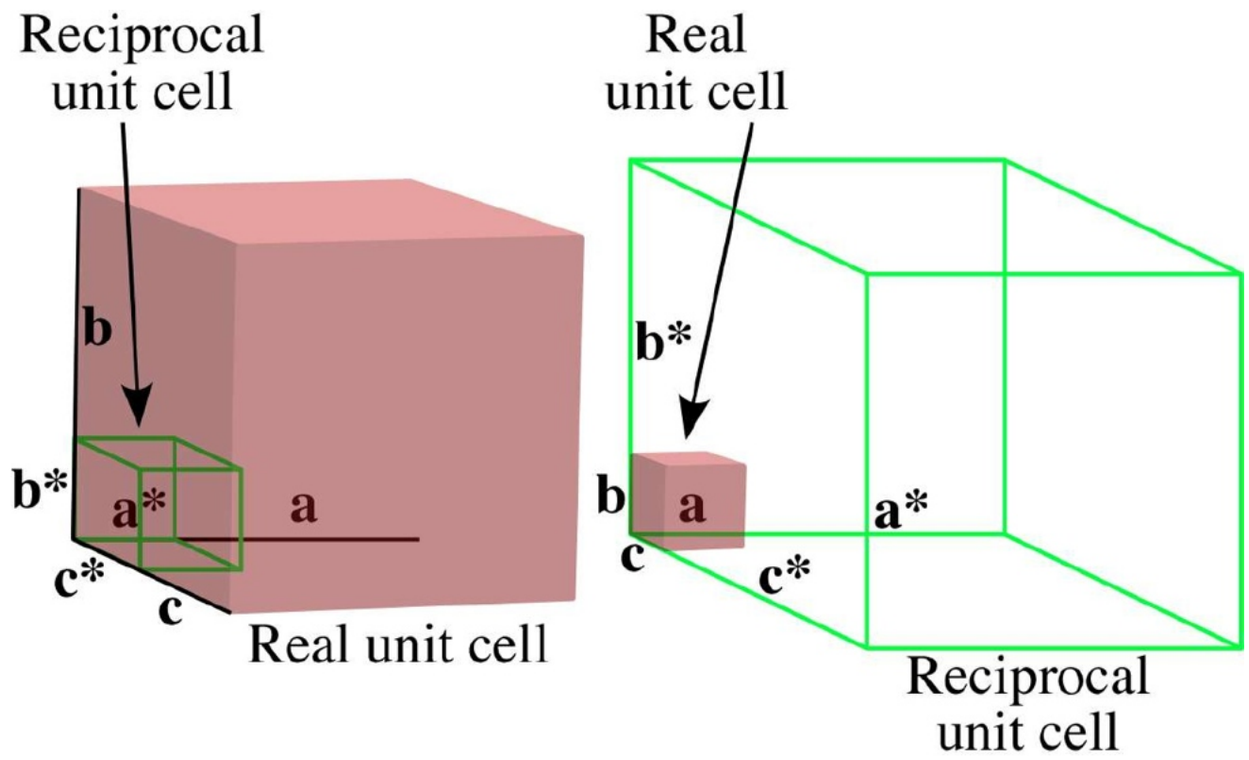


a



b

In crystals...



$a = 1/a^*$

$b = 1/b^*$

$c = 1/c^*$

II. Diffraction patterns

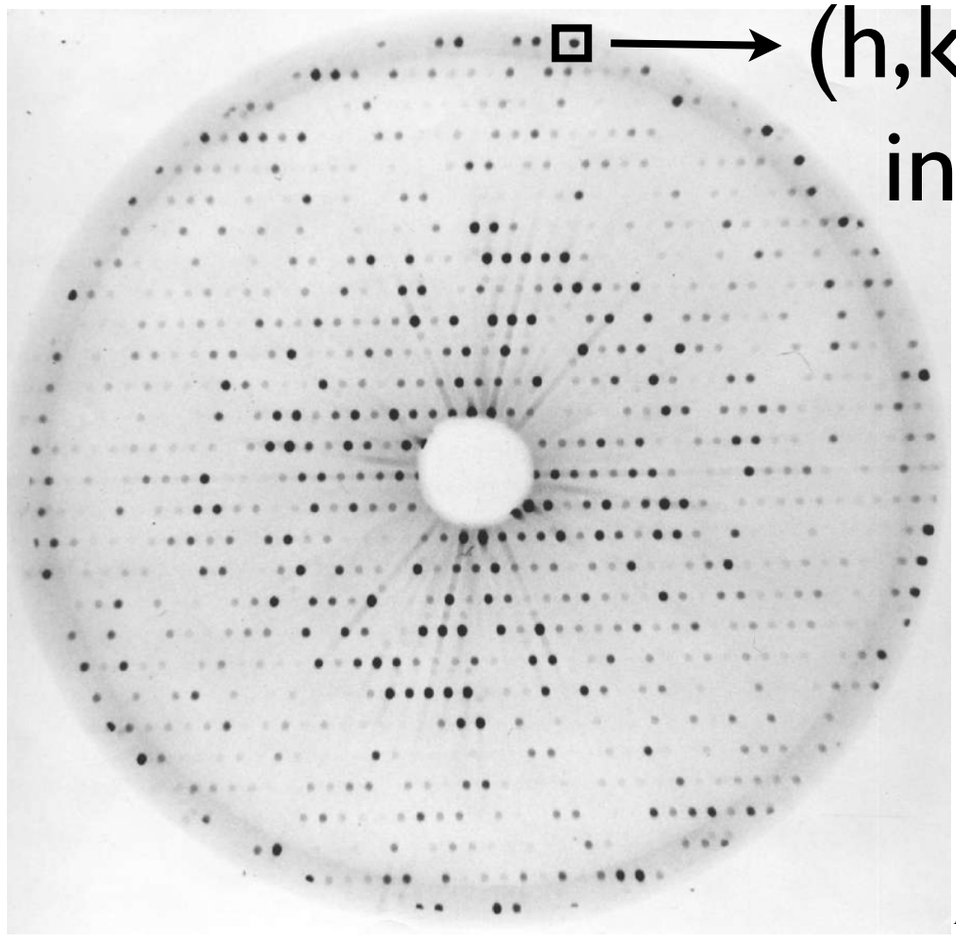
- Reciprocal lattice spacing is related real lattice spacing
- Can calculate unit cell dimensions from h,k,l indices alone
- Position of spots in diffraction pattern contains information about unit cell and its symmetry

II. What about intensities?

- Intensity reports on amount of electron density associated with given set of planes
- Electron density associated with every atom in the unit cell contributes to the intensity of a single reflection

II. Diffraction data

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



□ ⟶ (h,k,l) index, intensity

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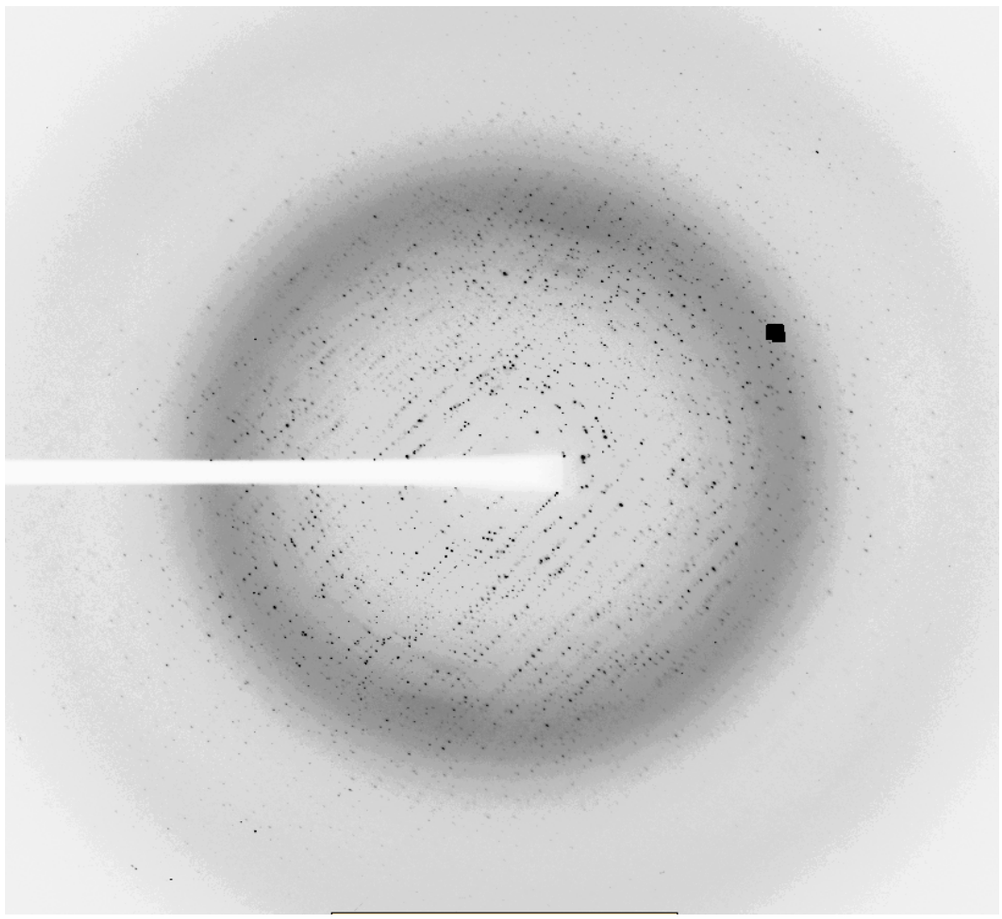
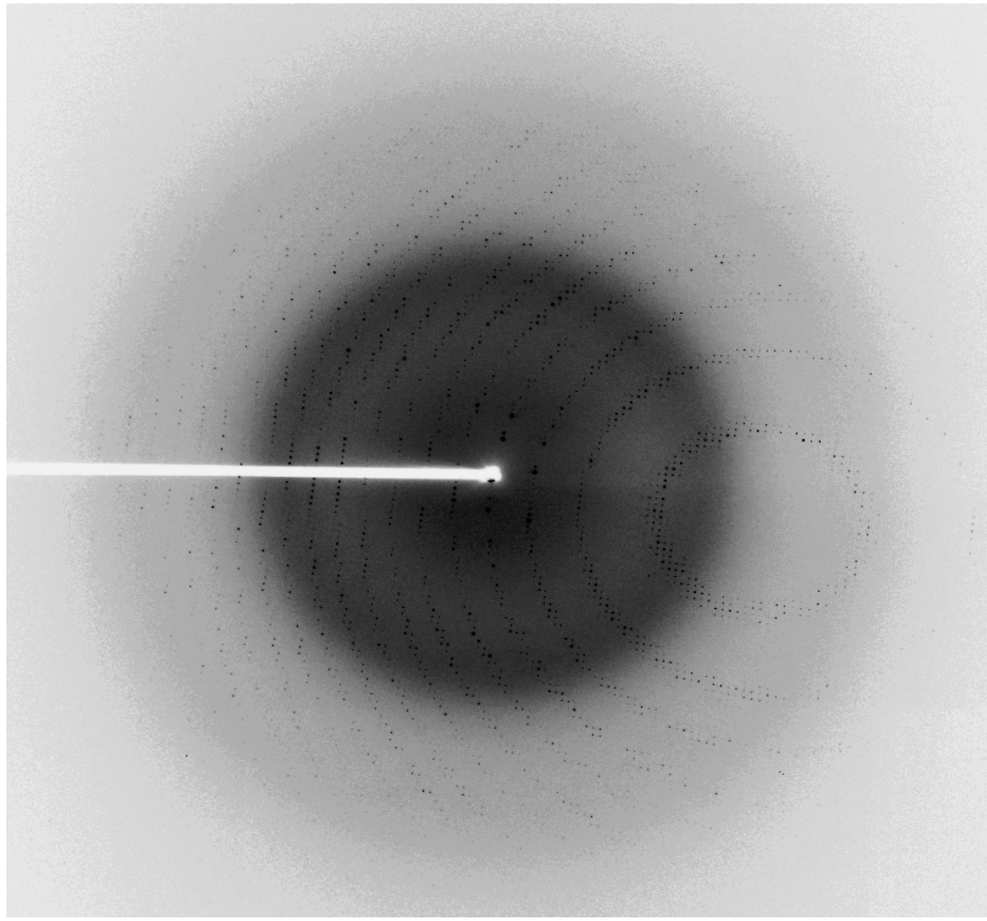
Academic Press

Multiple vs. single lattice

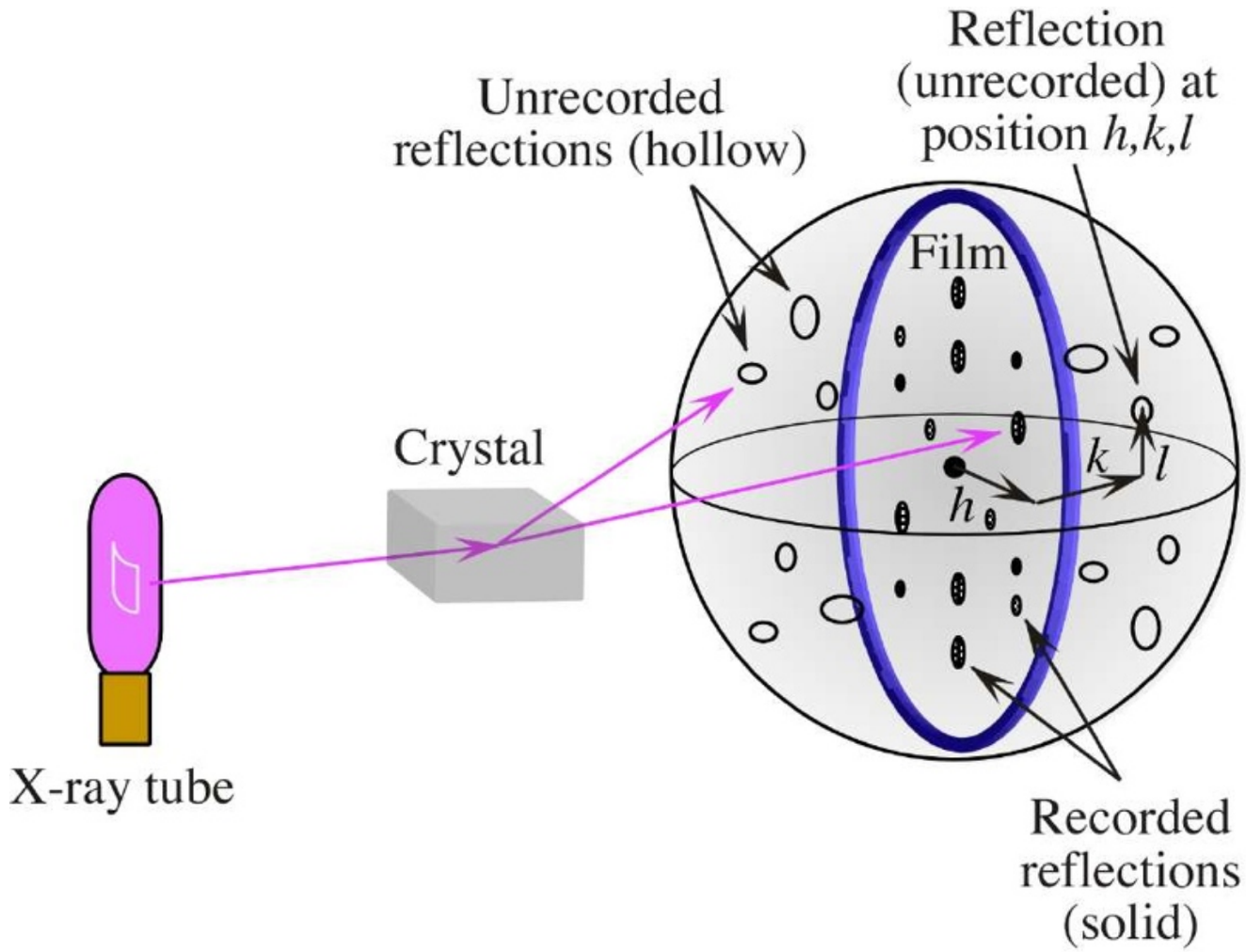




edge 1.7 Å

II. Data collection

- What is a 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



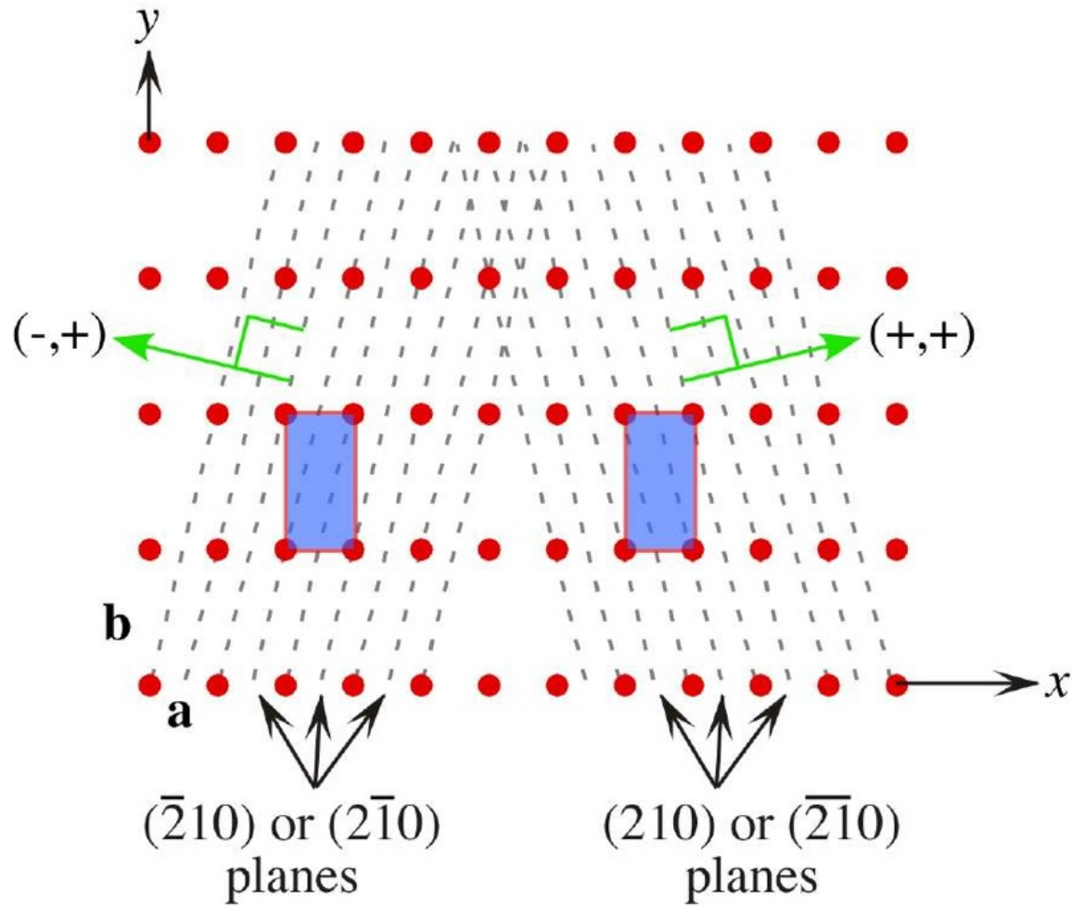
Crystal rotation during data collection captures unrecorded reflections

II. Data collection variables

- X-ray exposure time
    - Avoid overexposure/saturation
- Crystal-to-detector distance
    - Use resolution limit as guide
- Frame width (ω)
    - Partial vs. full reflections and overlaps
- How much data to collect?

II. How much data to collect?

- # of unique reflections depends on internal symmetry of lattice
- Higher symmetry = less data
- 180° is a complete set
- Friedel's law: $I_{hkl} = I_{\overline{hkl}}$
  - exceptions...



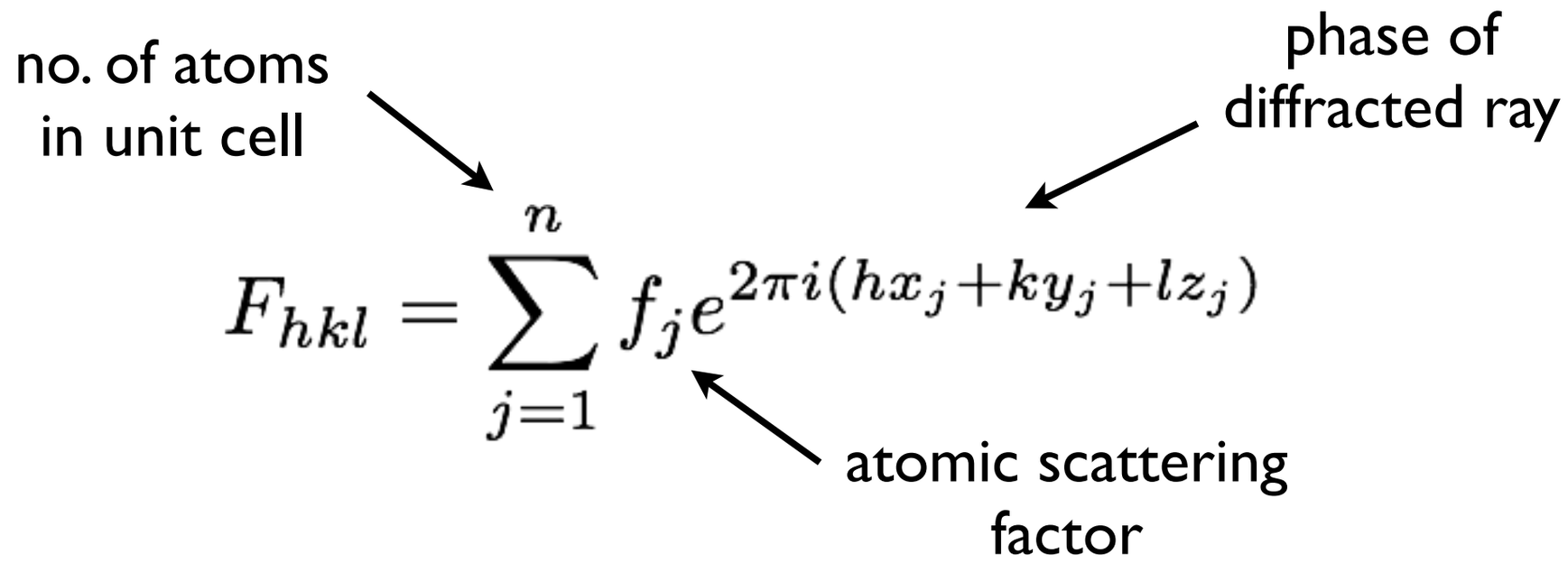
II. Data processing

- Peak search
- Indexing
- Integration
- Scaling
- Postrefinement

Final output is a list of reflections (h, k, l), and intensities (I)

III. Structure solution

- Structure factors
  - 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



III. Structure solution

- Structure factors can be expressed as sum of contributions from volume elements in crystal
- electron density of volume element centered on $(x, y, z) = \rho(x, y, z)$

electron density function

$$F_{hkl} = \int_V \rho(x, y, z) e^{2\pi i(hx_j + ky_j + lz_j)} dV$$

volume of unit cell

III. Structure solution

- Electron density map is 3D plot of $\rho(x, y, z)$
- 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

III. Structure solution

- How do we compute $\rho(x, y, z)$ 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 = ??

III. Solving the phase problem

- Three common methods
  - Molecular replacement
  - Isomorphous replacement
  - Anomalous scattering

III. Solving the phase problem

- Molecular replacement
  - Use similar protein of known structure to compute phases
  - Structure can be solved from single, native dataset

III. Solving the phase problem

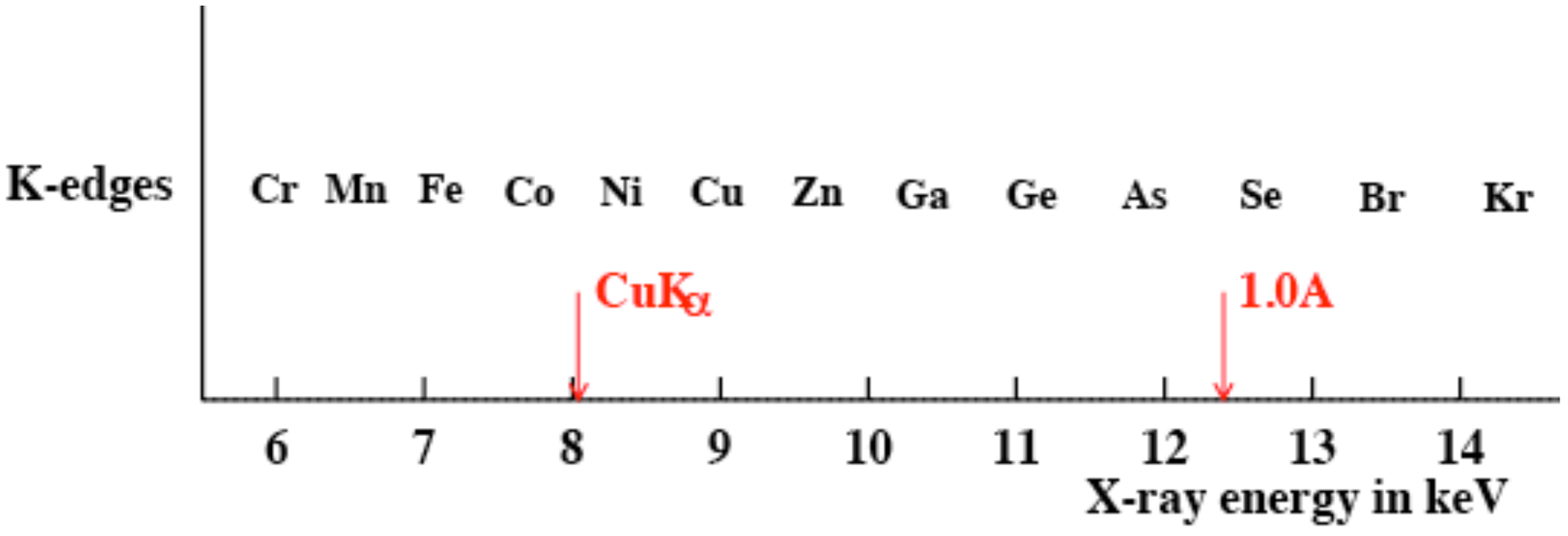
- Isomorphous replacement and anomalous scattering
  - Make use of heavy atom sites (i.e., metals) in crystals (derivative or native)
  - Typically requires collection of multiple datasets

III. Anomalous Scattering

- X-ray absorption by heavy atoms alters diffraction
  - Friedel's law does not hold $I_{hkl} \neq I_{\overline{hkl}}$
  - Must be able to tune λ to absorption edge
  - Intensity difference in Friedel pairs locates heavy atom sites in unit cell - used to compute phases
  - Additional datasets are collected near heavy atom absorption edge
  - Requires tunable X-ray source and data must be highly redundant with minimal X-ray damage

III. Anomalous scattering

- Can also be used to identify metals in crystal structures



IV. Model generation

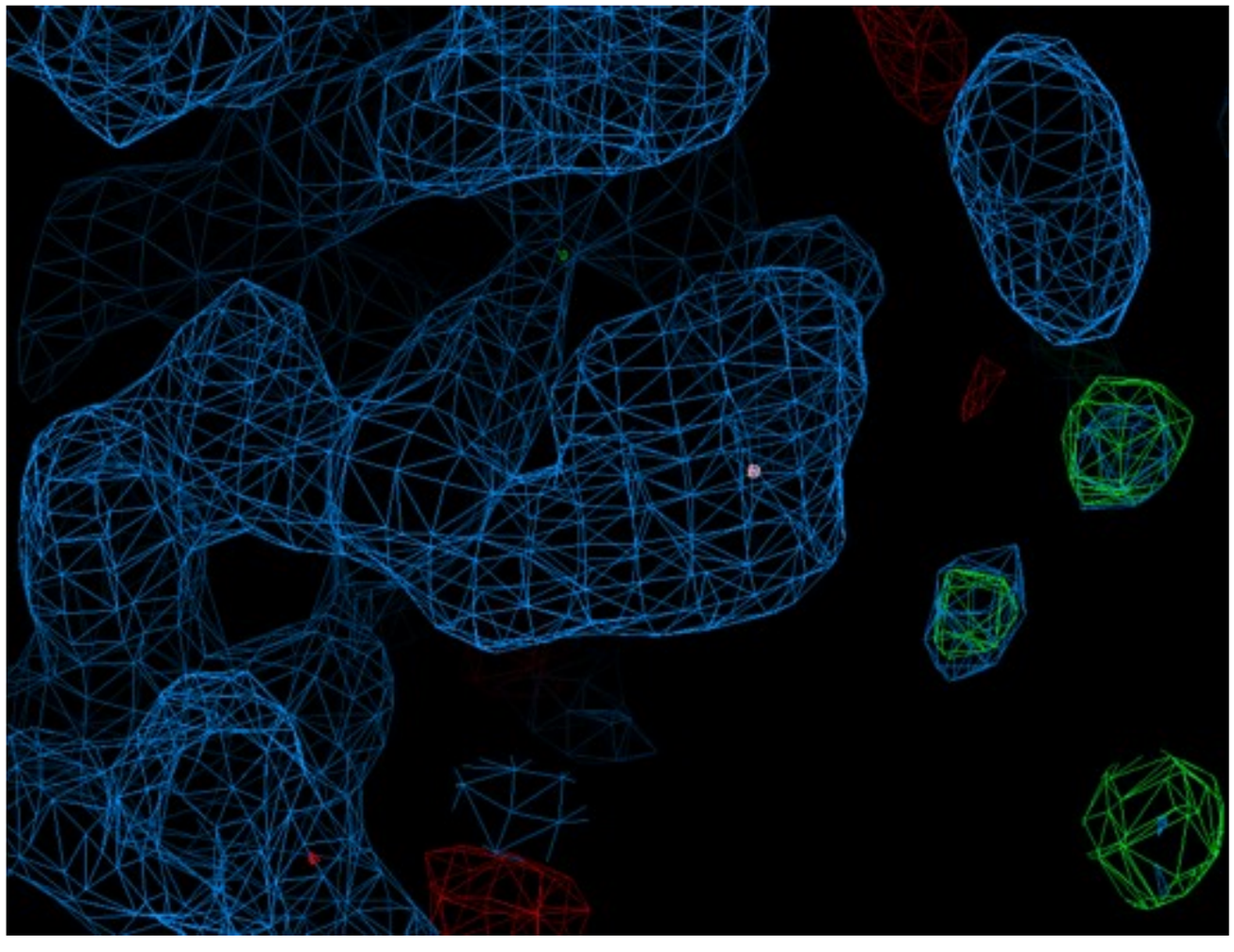
- 3D plot of $\rho(x, y, z)$ function produces electron density map
- Phase improvement
- Map interpretation
- Refinement of coordinates

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

phases

amplitudes (from intensities)

Map Interpretation

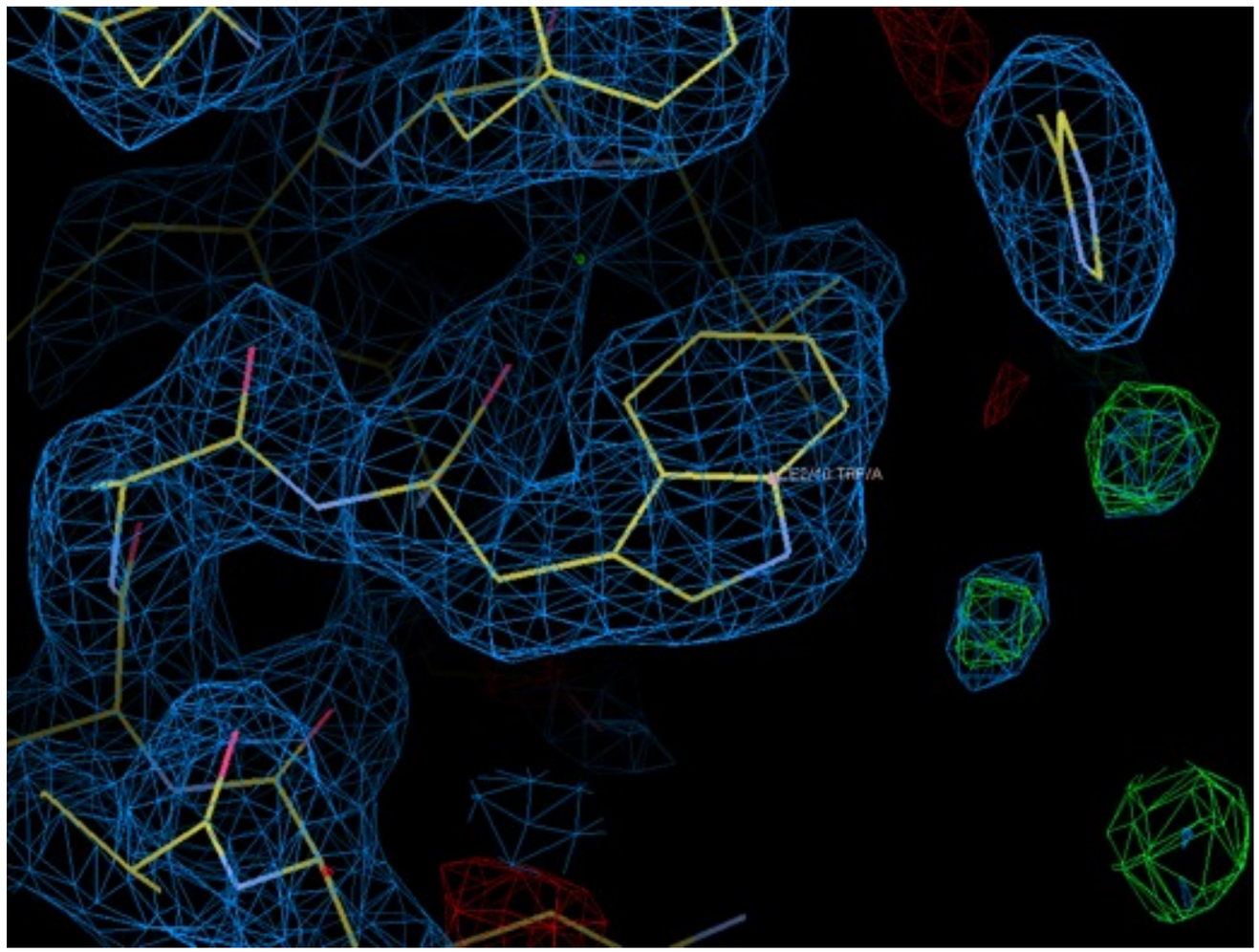


Blue = $2F_o$-F_c map

Red/Green = F_o-F_c map

1.8 Å resolution map

Map Interpretation



Blue = $2F_o$-F_c map

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IV. Map interpretation

- 3D plot of $\rho(x, y, z)$ function produces electron density map
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$$\rho(x,y,z) = \frac{1}{V}\sum_h\sum_k\sum_l |F_{hkl}|e^{-2\pi i(hx_j+ky_j+lz_j-\alpha'_{hkl})}$$

phases

amplitudes (from intensities)

IV. Model evaluation

- Data collection statistics
  - Resolution
  - $R_{sym}/R_{merge} < 0.1$
  - $I/\sigma I > 2$
  - Completeness/redundancy ~ 100%

IV. Data collection statistics

	Pt-Atox1
Data collection	
Space group	$P3_221$
Cell dimensions	
a, *b*, *c* (Å)	54.07, 54.07, 55.58
α, β, γ (°)	90.00, 90.00, 120.00
Resolution (Å)	50.00-1.60 (1.63-1.60)
R_{sym} or R_{merge}	0.118 (0.503)
$I / \sigma I$	24.9 (3.3)
Completeness (%)	99.7 (99.7)
Redundancy	19.5 (9.5)

IV. Model evaluation

- Model refinement statistics
  - R-factor
  - R_{free} statistic
  - RMS deviation (bond lengths, angles)
  - Ramachandran statistics

IV. Data collection statistics

Refinement	
Resolution (Å)	46.83-1.60
No. reflections	12060
R_{work} / R_{free}	0.179/0.210
No. atoms	609
Protein	496
Ligand/ion	21
Water	92
B-factors	
Protein	17.0
Ligand/ion	33.9
Water	39.6
R.m.s. deviations	
Bond lengths (Å)	1.252
Bond angles (°)	0.010

IV. Model evaluation

- Considerations for structures with transition metals
  - Metal ion occupancy
  - Oxidation state and influence by X-rays
  - Ligand order and occupancy
  - Anomalous scattering data

Evaluate by inspecting electron density maps

Three case studies...

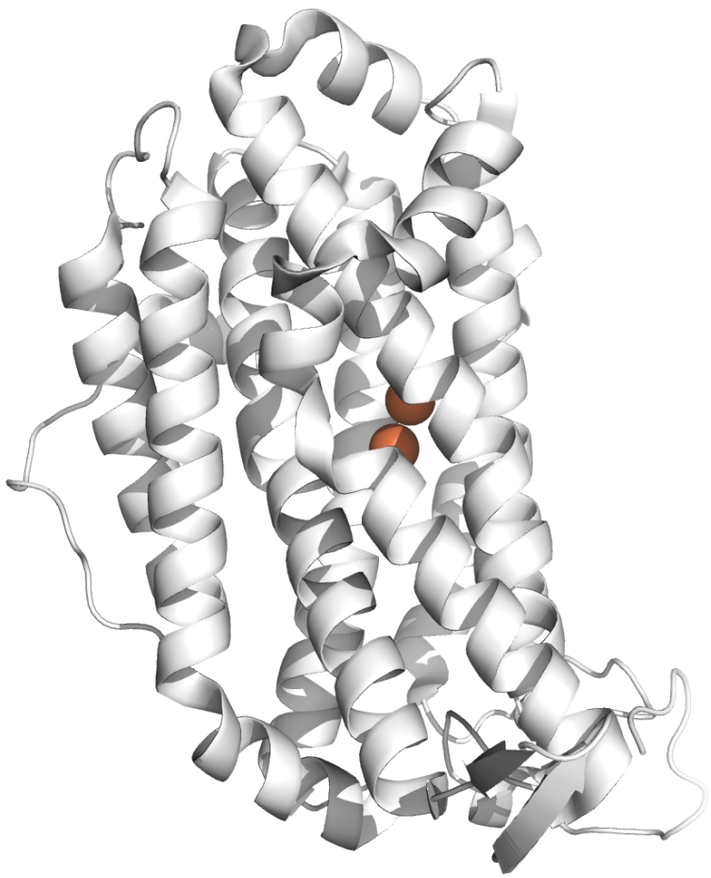
- Crystallographic site assignment in the Mn/Fe cofactor of class Ic RNR

- Högbom, M., Stenmark, P., Voevodskaya, N., McClarty, G., Gräslund, A., and Nordlund, P. (2004) *Science 305,* 245-248.

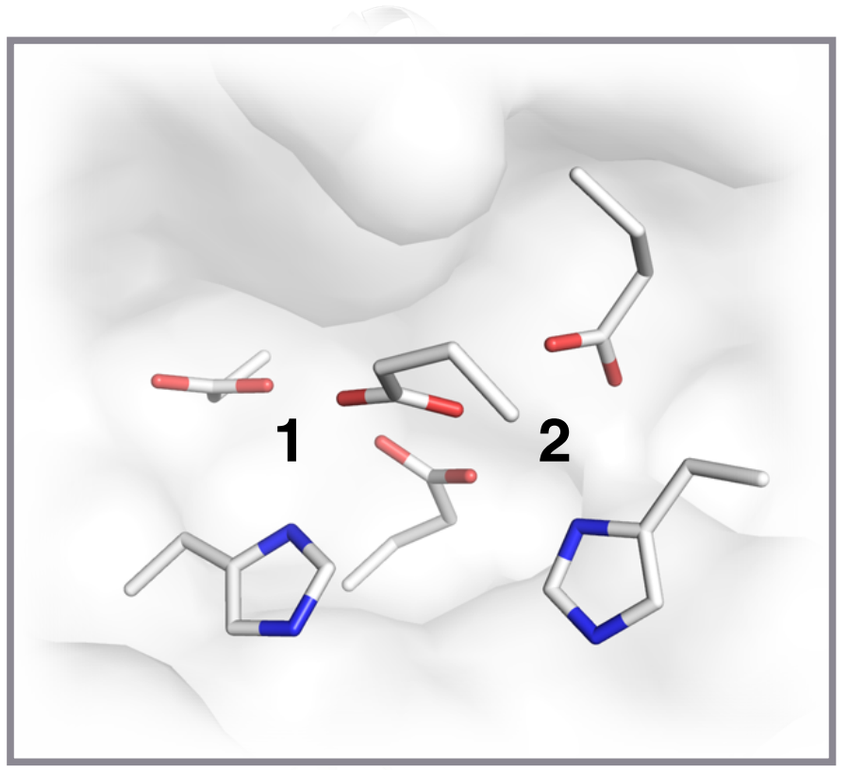
- Andersson, C.S., Öhrström, M., Popović-Bijelić, A., Gräslund, A., Stenmark, P., and Högbom, M. (2012) *J. Am. Chem. Soc. 134*, 123-125.

- Dassama, L.M.K., Boal, A.K., Krebs, C., Rosenzweig, A.C., and Bollinger, J.M., Jr. (2012) *J. Am. Chem. Soc. 134*, 2520-2523.

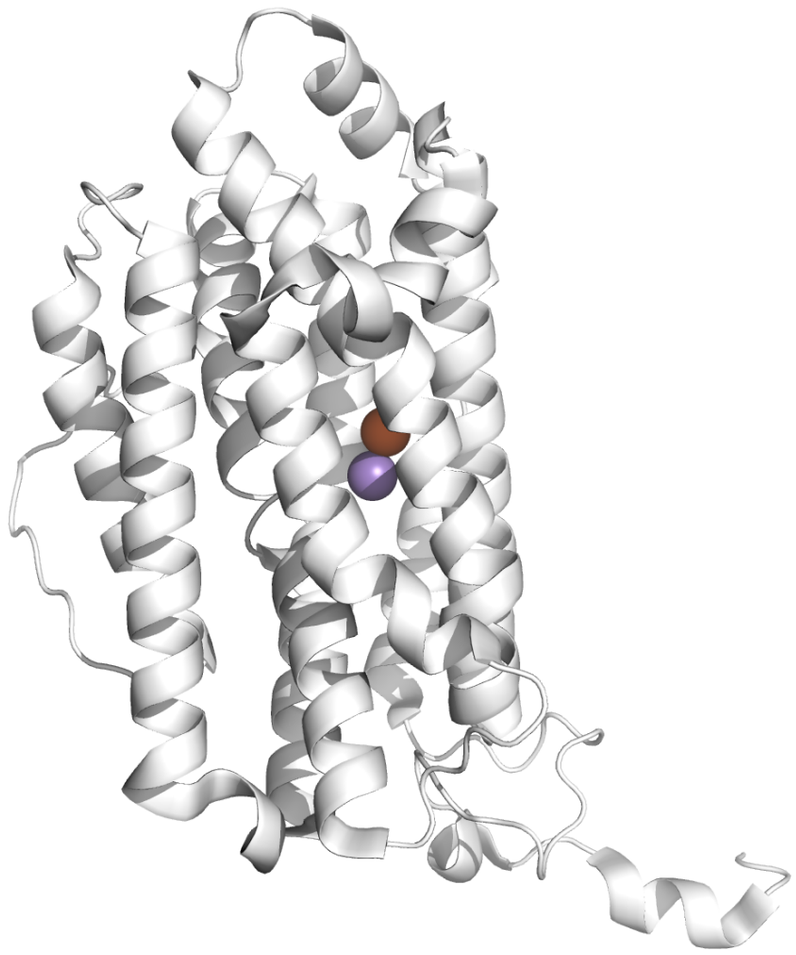
Class I β2 structures



Class Ia



Class Ib



Class Ic

Three case studies...

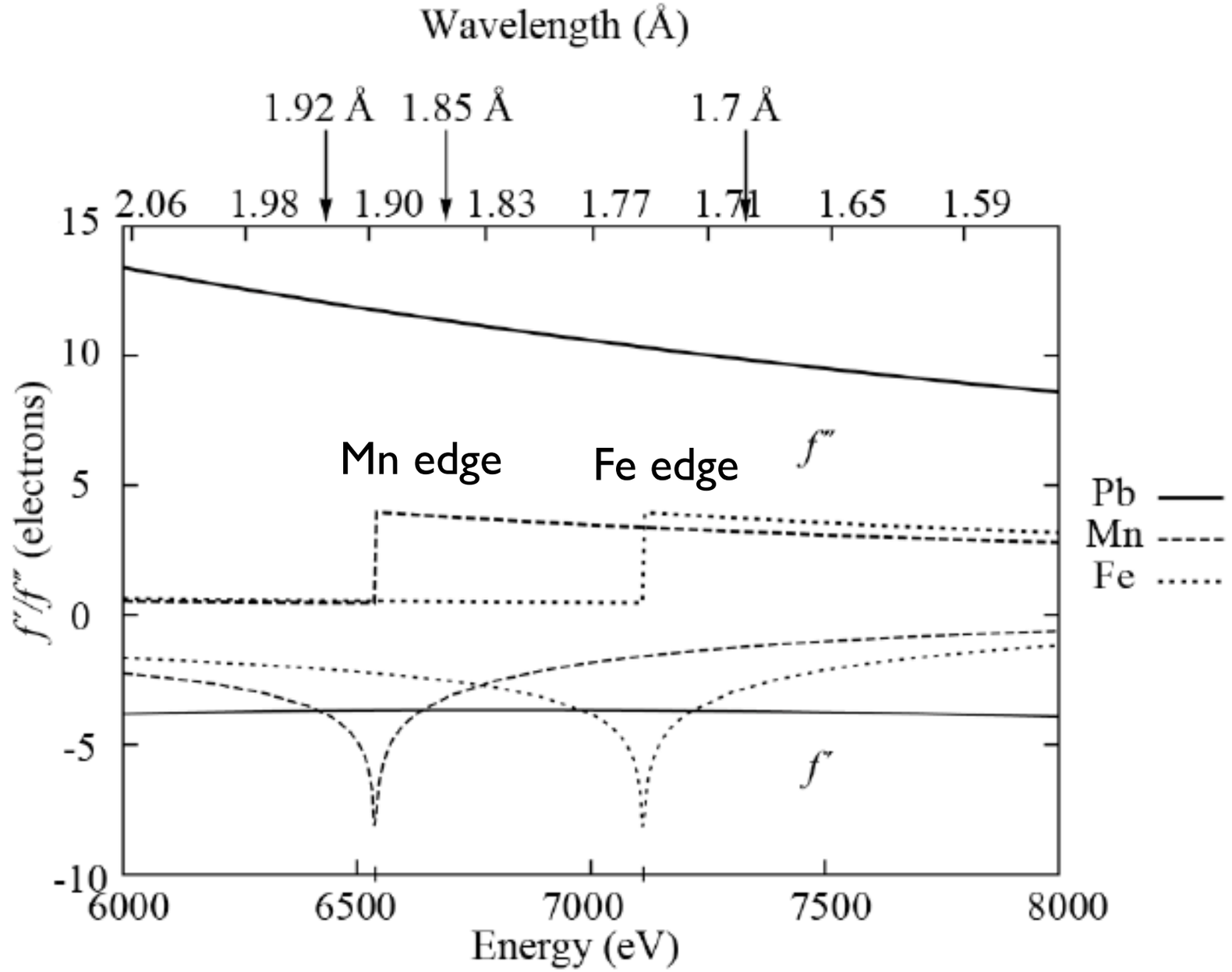
- Crystallographic site assignment in the Mn/Fe cofactor of class Ic RNR

- Högbom, M., Stenmark, P., Voevodskaya, N., McClarty, G., Gräslund, A., and Nordlund, P. (2004) *Science 305,* 245-248.

- Andersson, C.S., Öhrström, M., Popović-Bijelić, A., Gräslund, A., Stenmark, P., and Högbom, M. (2012) *J. Am. Chem. Soc. 134*, 123-125.

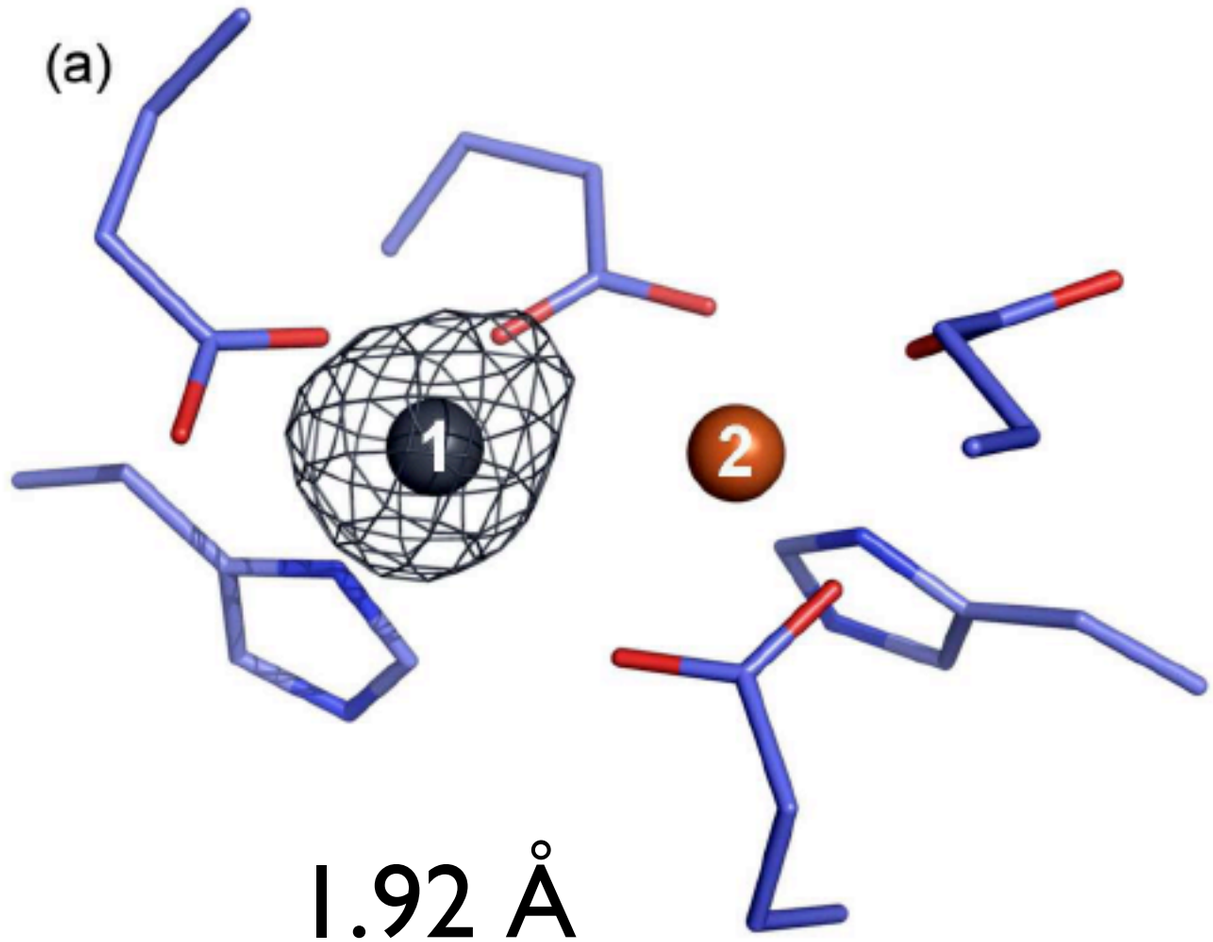
- Dassama, L.M.K., Boal, A.K., Krebs, C., Rosenzweig, A.C., and Bollinger, J.M., Jr. (2012) *J. Am. Chem. Soc. 134*, 2520-2523.

Mn/Fe anomalous scattering



Andersson, C.S., Öhrström, M., Popovic-Bijelic, A., Gräslund, A., Stenmark, P., and Högbom, M. (2012) *J. Am. Chem. Soc. 134*, 123-125.

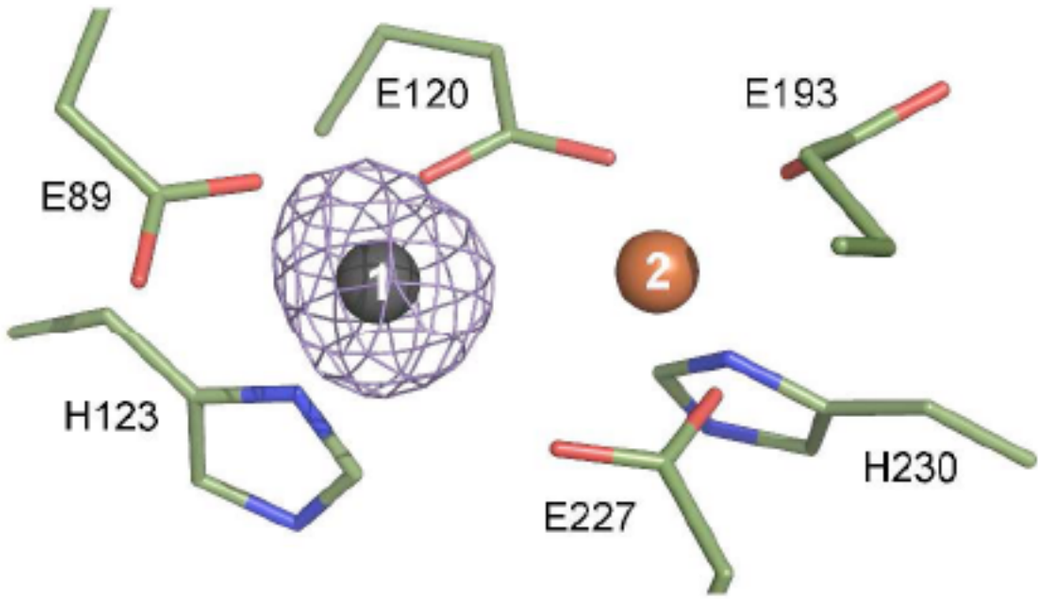
Initial crystal conditions

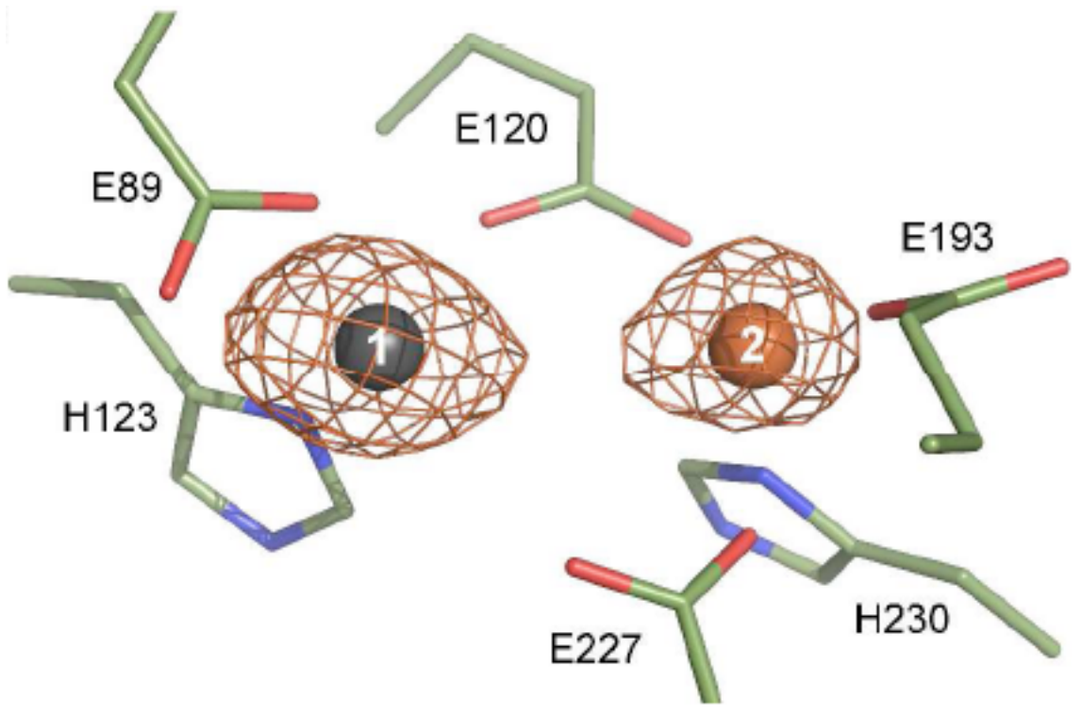


1.92 Å

Andersson *et al.* (2012) *J. Am. Chem. Soc. 134*, 123-125.

New crystal conditions





Mn edge 1.85 Å

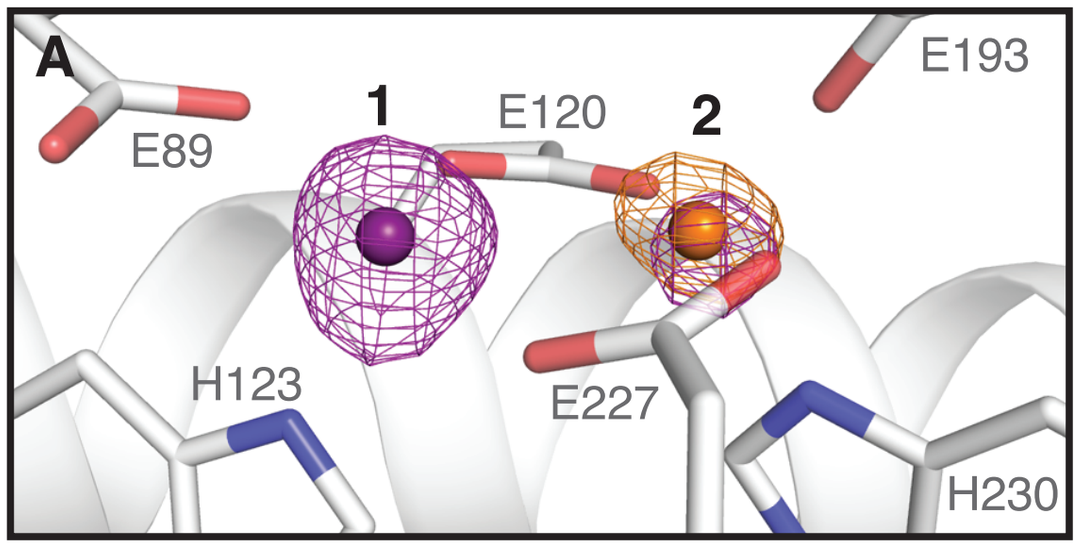
site 1 binds Mn, Fe (or Pb)

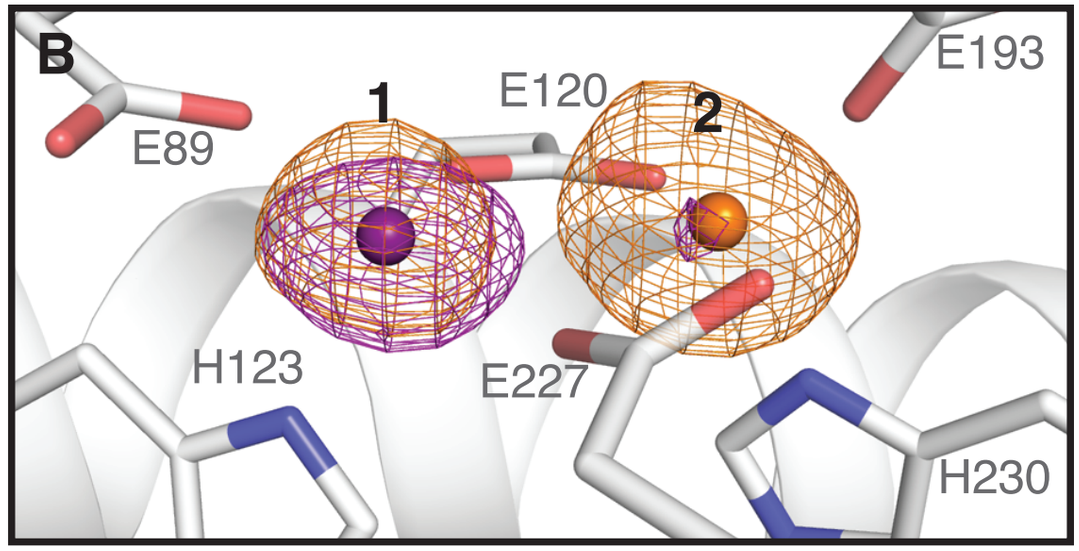
Fe edge 1.7 Å

site 2 binds Fe

Andersson *et al.* (2012) *J. Am. Chem. Soc. 134*, 123-125.

Site occupancy and loading





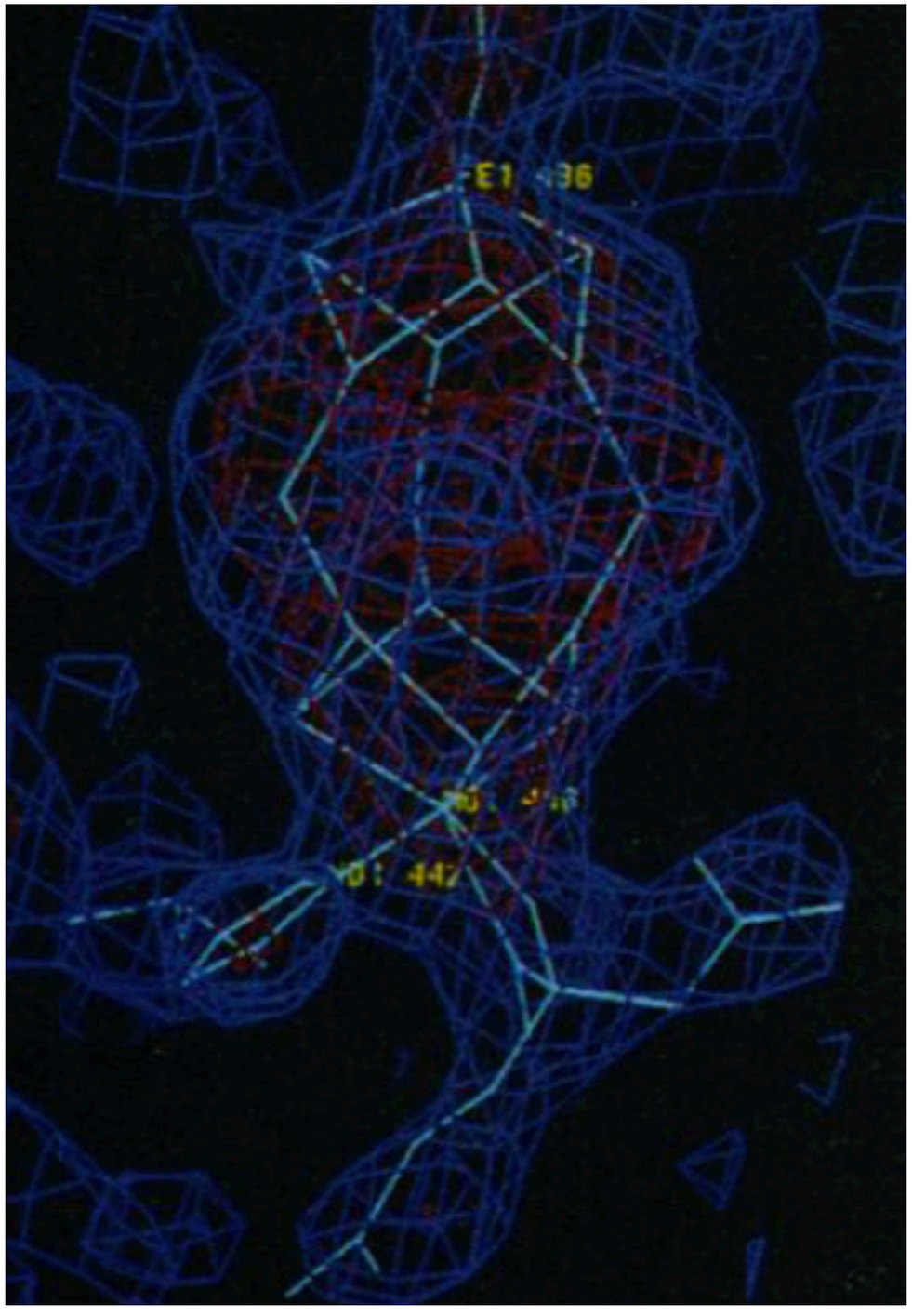
- Apo protein loaded with 1.5 eq Mn^{II} and < 0.5 eq Fe^{II} + O_2

- <1 eq Mn^{II} and 1.5 eq Fe^{II} + O_2

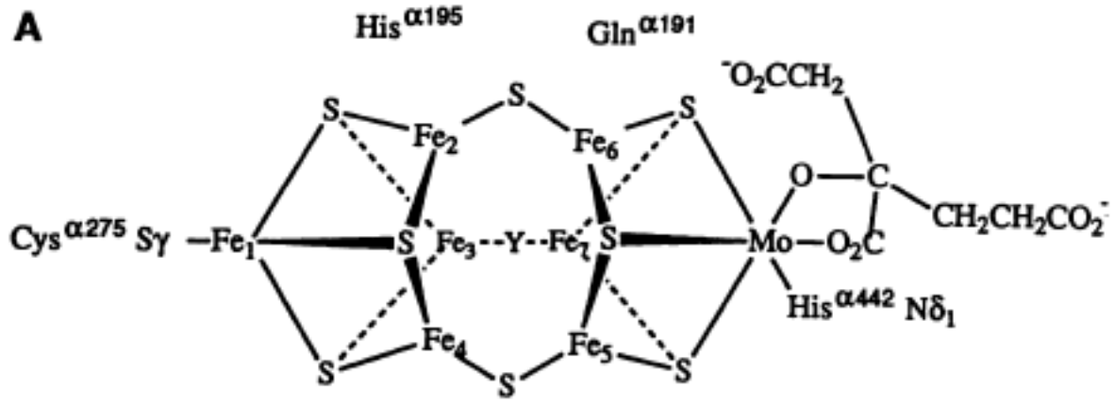
Dassama, *et al.* (2012) *J. Am. Chem. Soc. 134*, 2520-2523.

Three case studies...

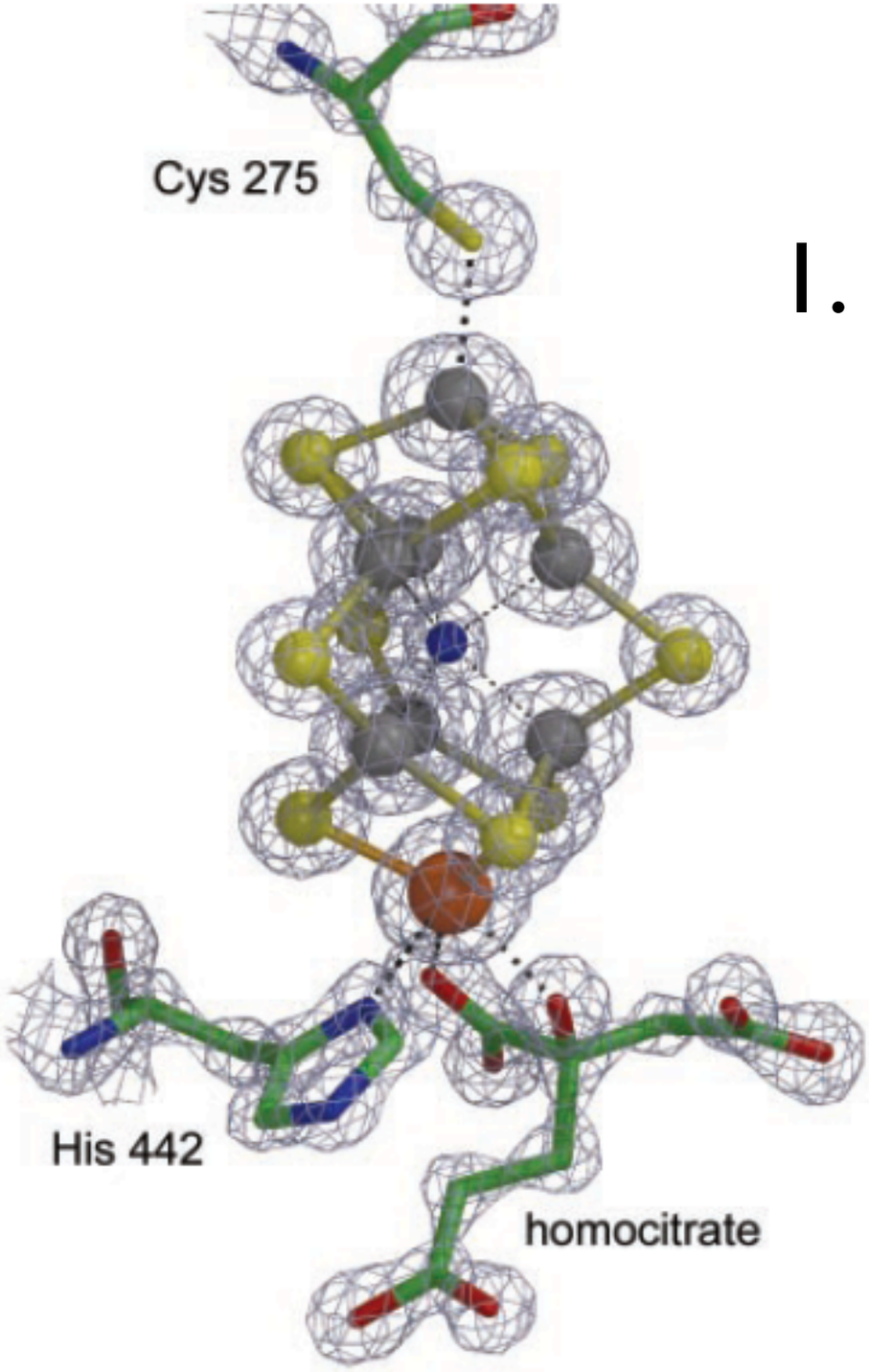
- Structure of FeMoCo in nitrogenase
  - Kim, J., and Rees, D.C. (1992) *Science 257*, 1677-1682.
  - Einsle, O., Tezcan, F.A., Andrade, S.L., Schmid, B., Yoshida, M., Howard, J.B., and Rees, D.C. (2002) *Science 297*, 1696-1700.
  - Spatzal, T., Aksoyoglu, M., Zhang, L., Andrade, S.L., Schleicher, E., Weber, S., Rees, D.C., and Einsle, O. (2011) *Science 334*, 940.



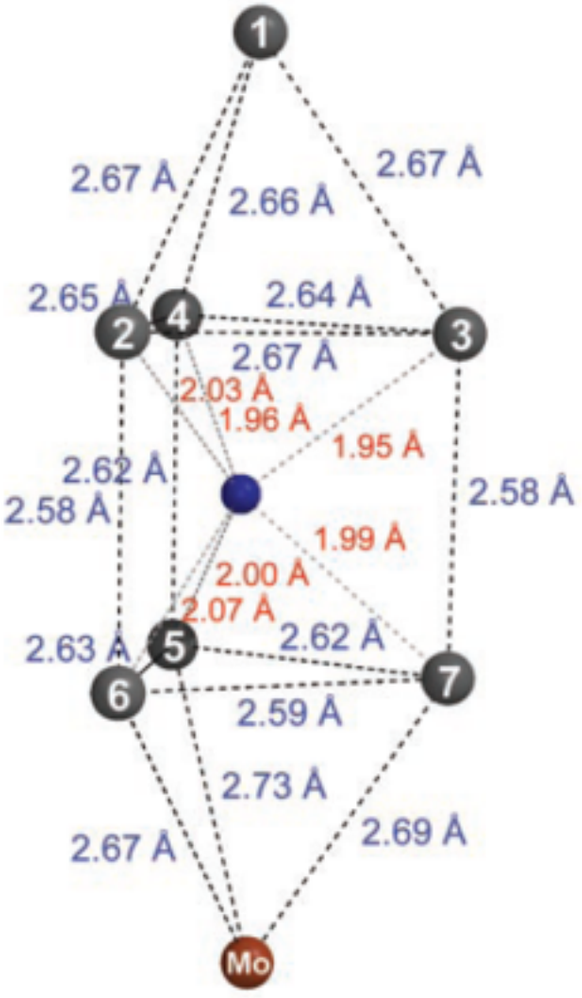
2.75 Å resolution



Kim, J., and Rees, D.C. (1992) *Science 257*, 1677-1682.



1.16 Å resolution



Einsle, O., Tezcan, F.A., Andrade, S.L., Schmid, B., Yoshida, M., Howard, J.B., and Rees, D.C. (2002) *Science 297*, 1696-1700.

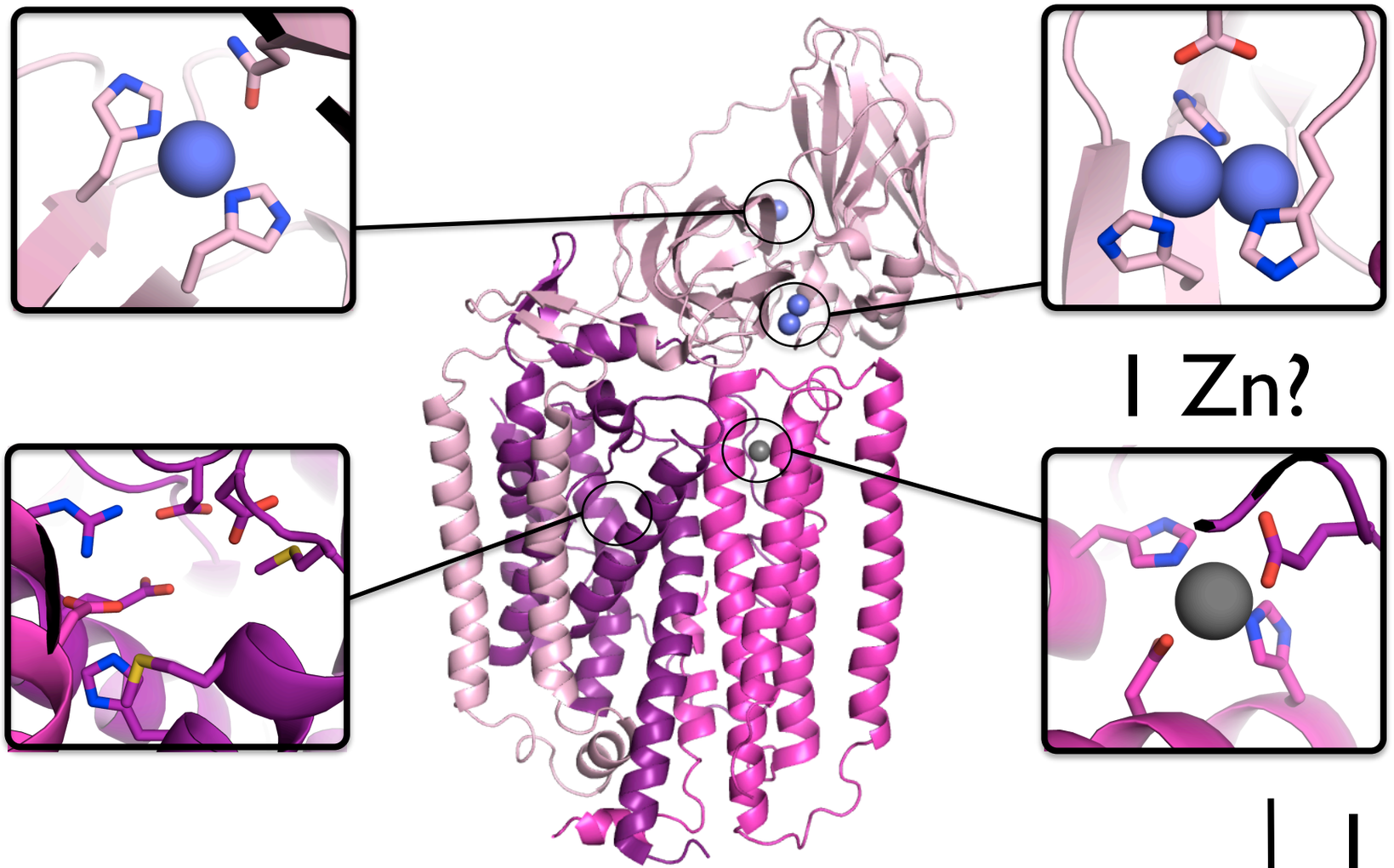
Three case studies...

- Identification of the metal sites in pMMO
  - Lieberman, R.L., and Rosenzweig, A.C. (2005) *Nature 434*, 177-182.
  - Hakemian, A.S., Kondapalli, K.C., Telser, J., Hoffman, B.M., Stemmler, T.L., and Rosenzweig, A.C. (2008) *Biochemistry 47*, 6793-6801.
  - Smith, S.M., Rawat, S., Telser, J., Hoffman, B.M., Stemmler, T.L., and Rosenzweig, A.C. (2011) *Biochemistry 50*, 10231-10240.

pMMO X-ray structures

1 Cu

2 Cu → XAS



1 Zn?

→ *M. caps*

→ sp. M

0.2 M ZnAc

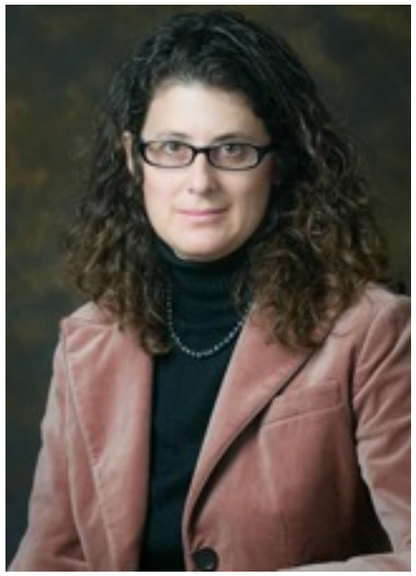
↓ 1 Cu

OB3b

$$CH_4 \xrightarrow[O_2]{\text{2-15 Cu?}} CH_3OH$$

Tom Lawton

Acknowlegements



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