Protein Crystallisation & Neutron Crystallography

SwedNESS PhD school:

Neutrons in Life Science and Biomaterials

Lund 2021-06-14

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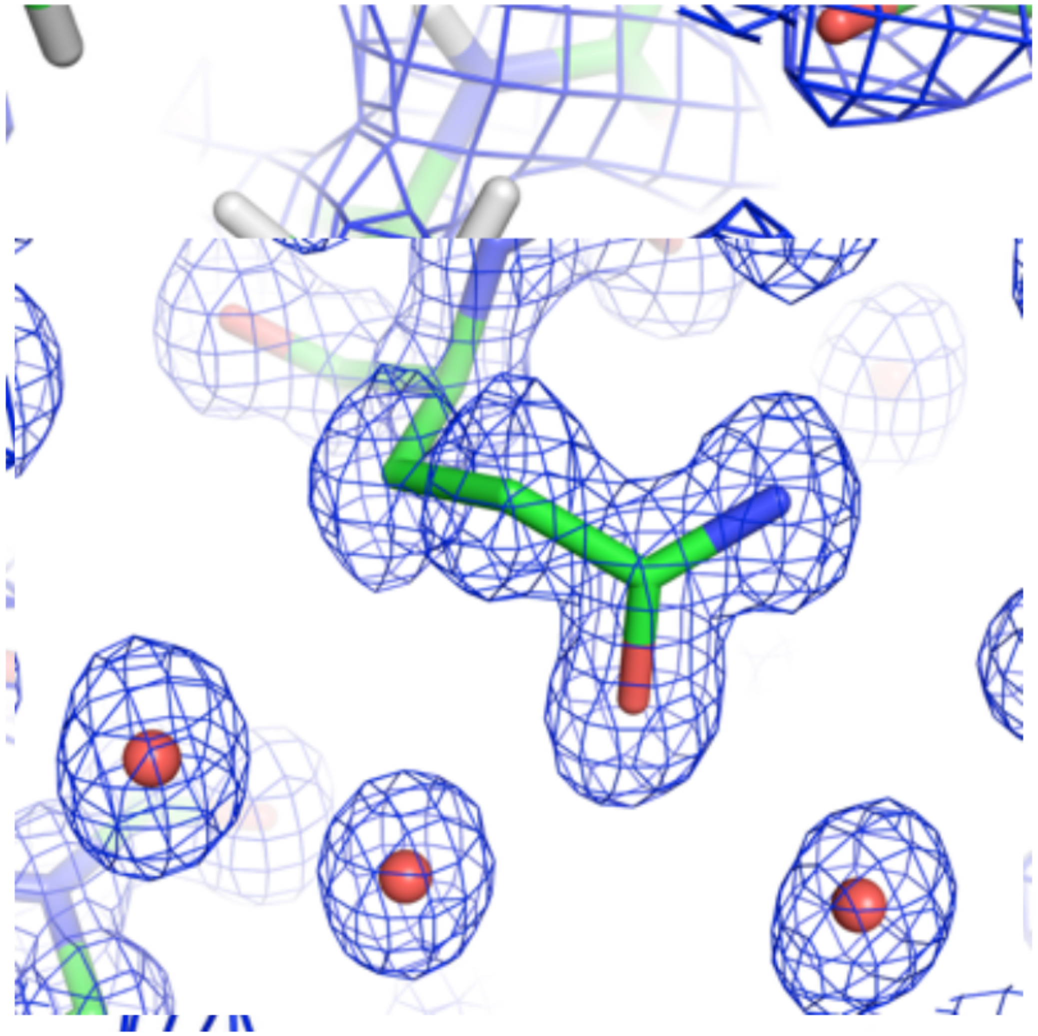
Macromolecular Crystallography

Outline

- **Why use neutrons for crystallography?**
- How is it done in practice
    - Protein crystallisation
    - Large crystal growth
    - Protein diffractometers
    - Data processing & refinement

Neutron scattering from hydrogen

- Hydrogen has comparable scattering lengths to C, N or O
- ^{1}H has high incoherent scattering
- This means we can see H as well as heavier atoms

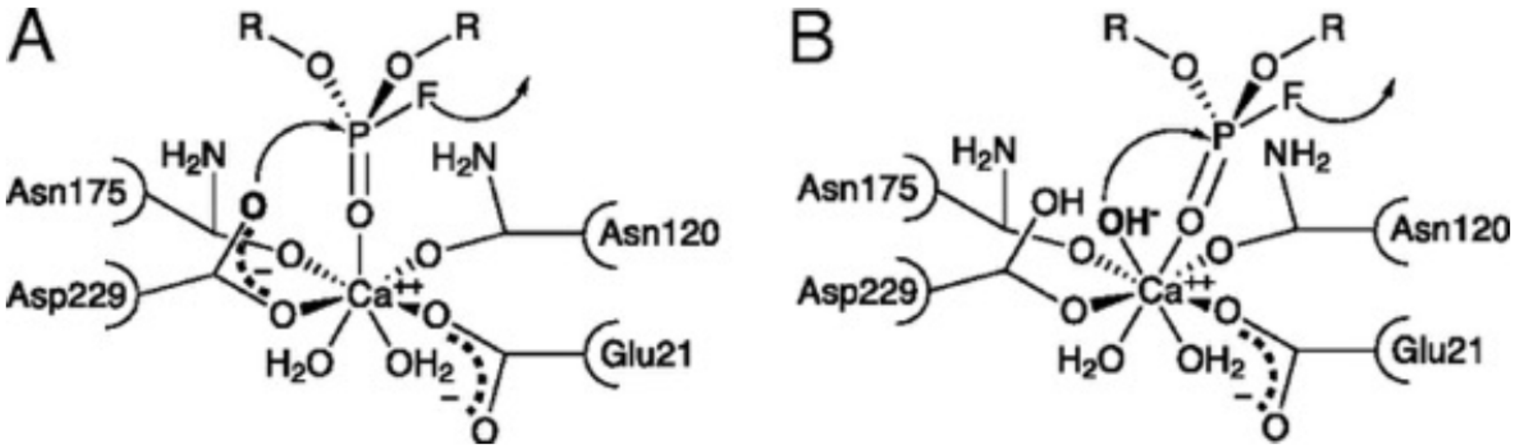


Neutron 1.9 Å

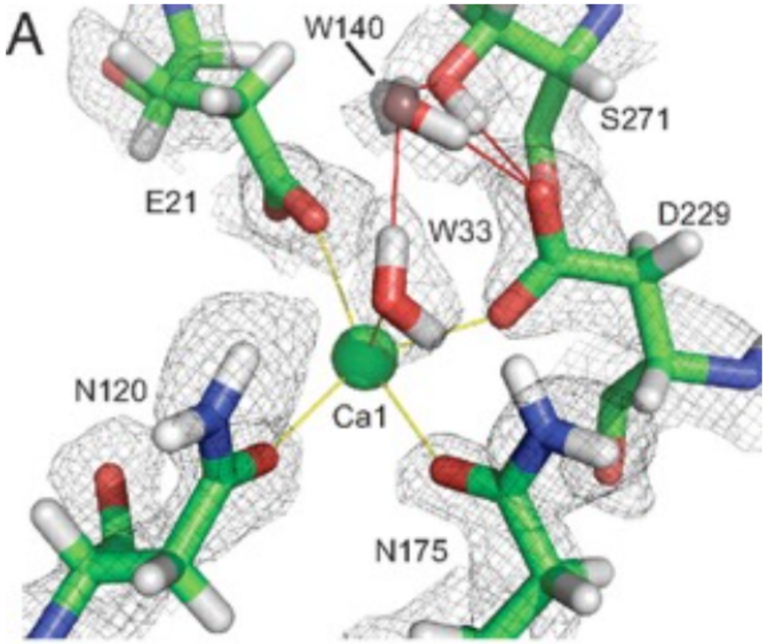
X-ray (1.1 Å)

Why is hydrogen interesting?

1. Enzyme mechanism



H_2O vs. OH^-



Di-isopropyl fluorophosphatase detoxifies nerve agents like sarin

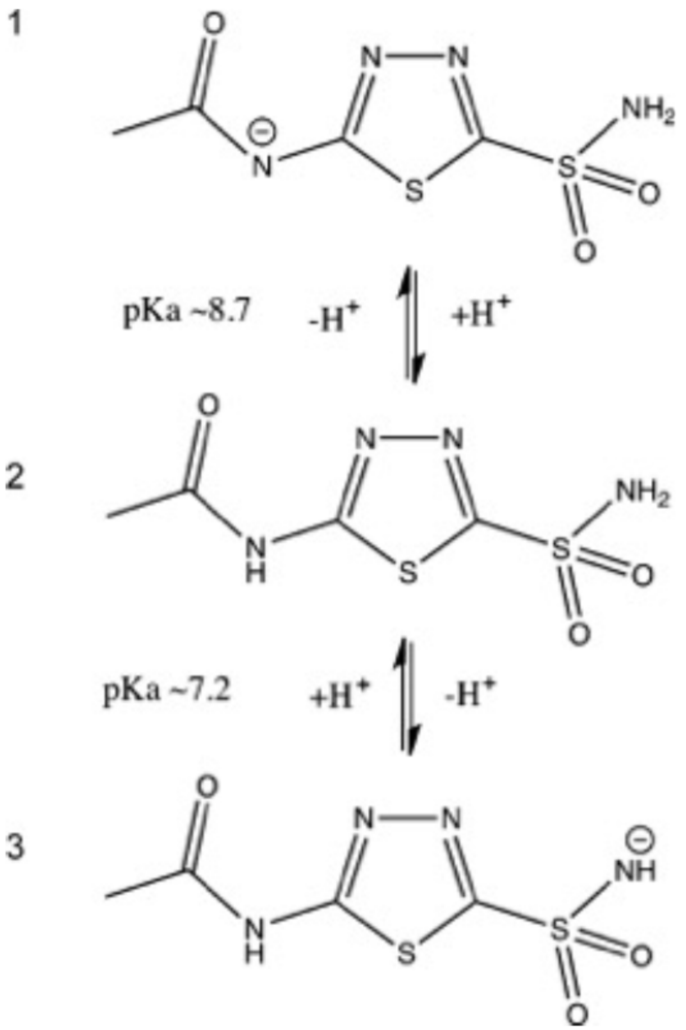
Blum M et al. PNAS 2009:106:713-718

Why is hydrogen interesting?

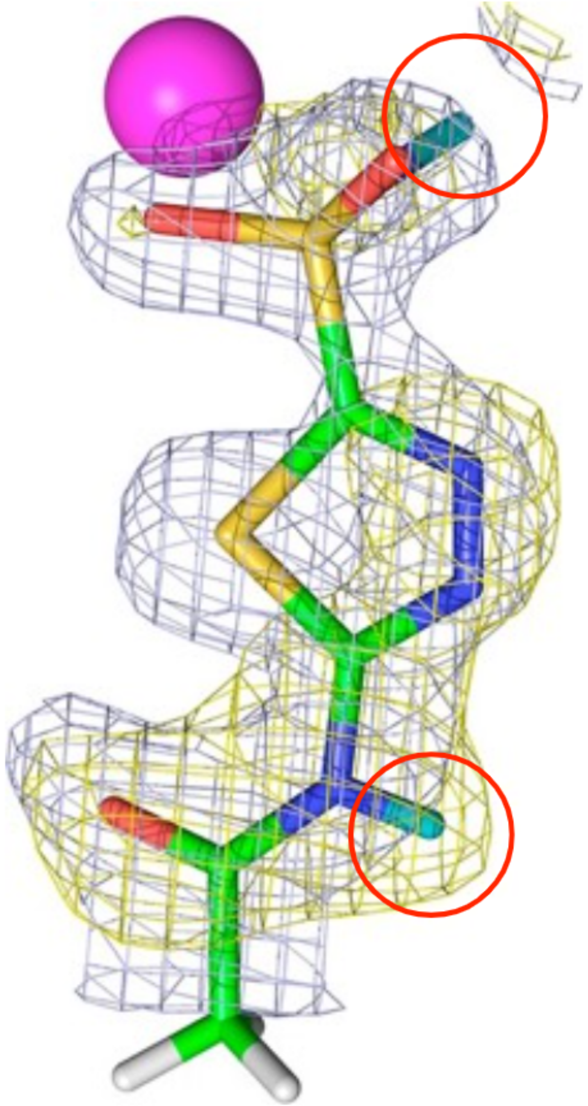
2. Ligand binding and protonation states

Acetazolamide in Human Carbonic Anhydrase II

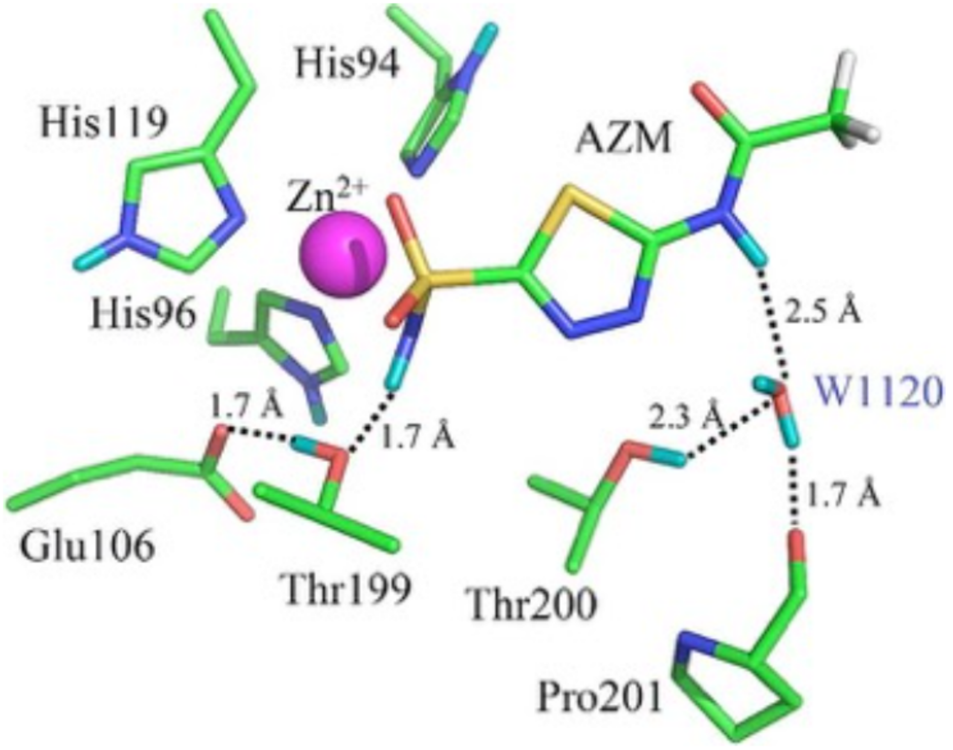
Three possible protonation states at physiological pH



Protonation state clearly determined by neutrons

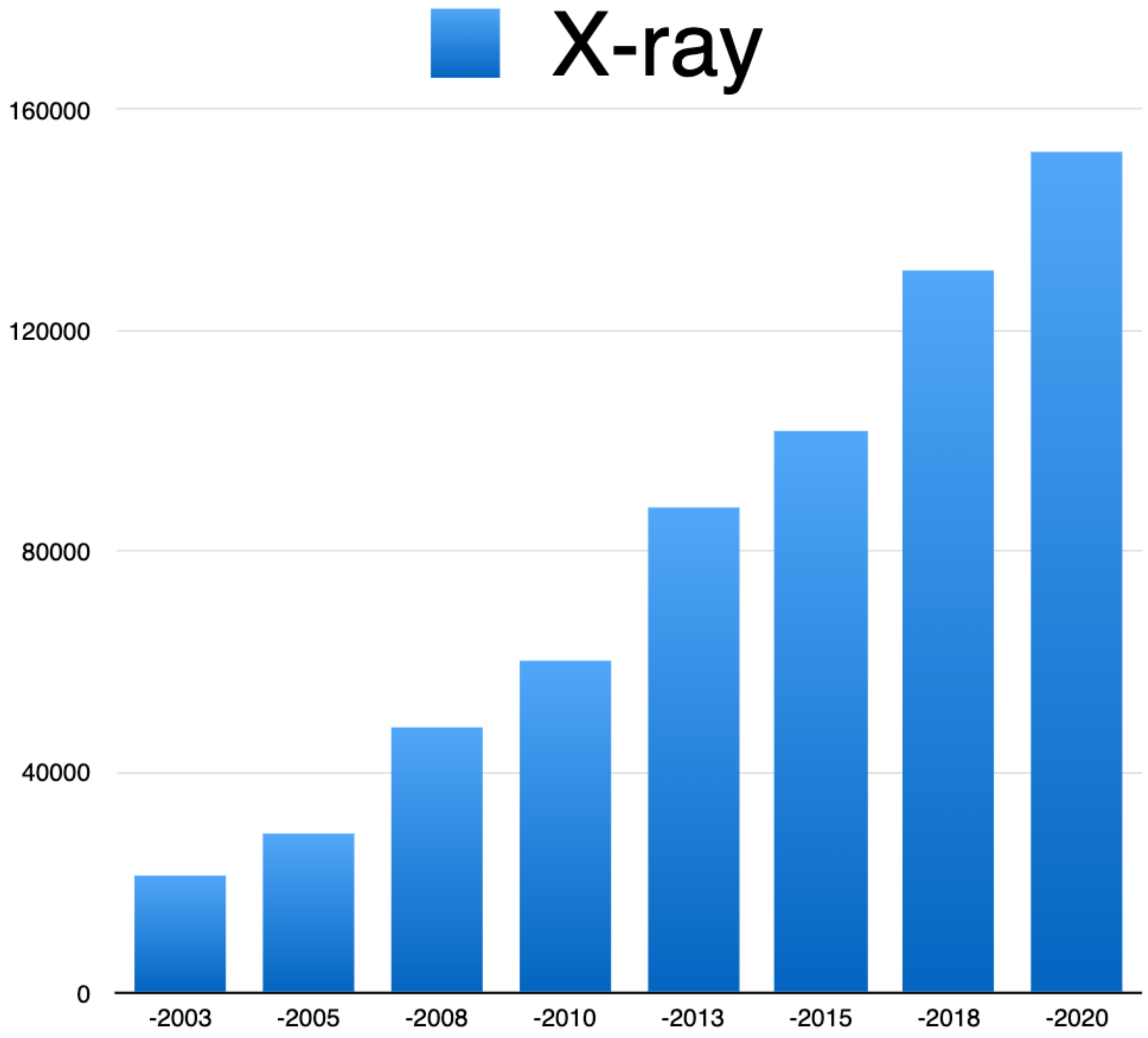


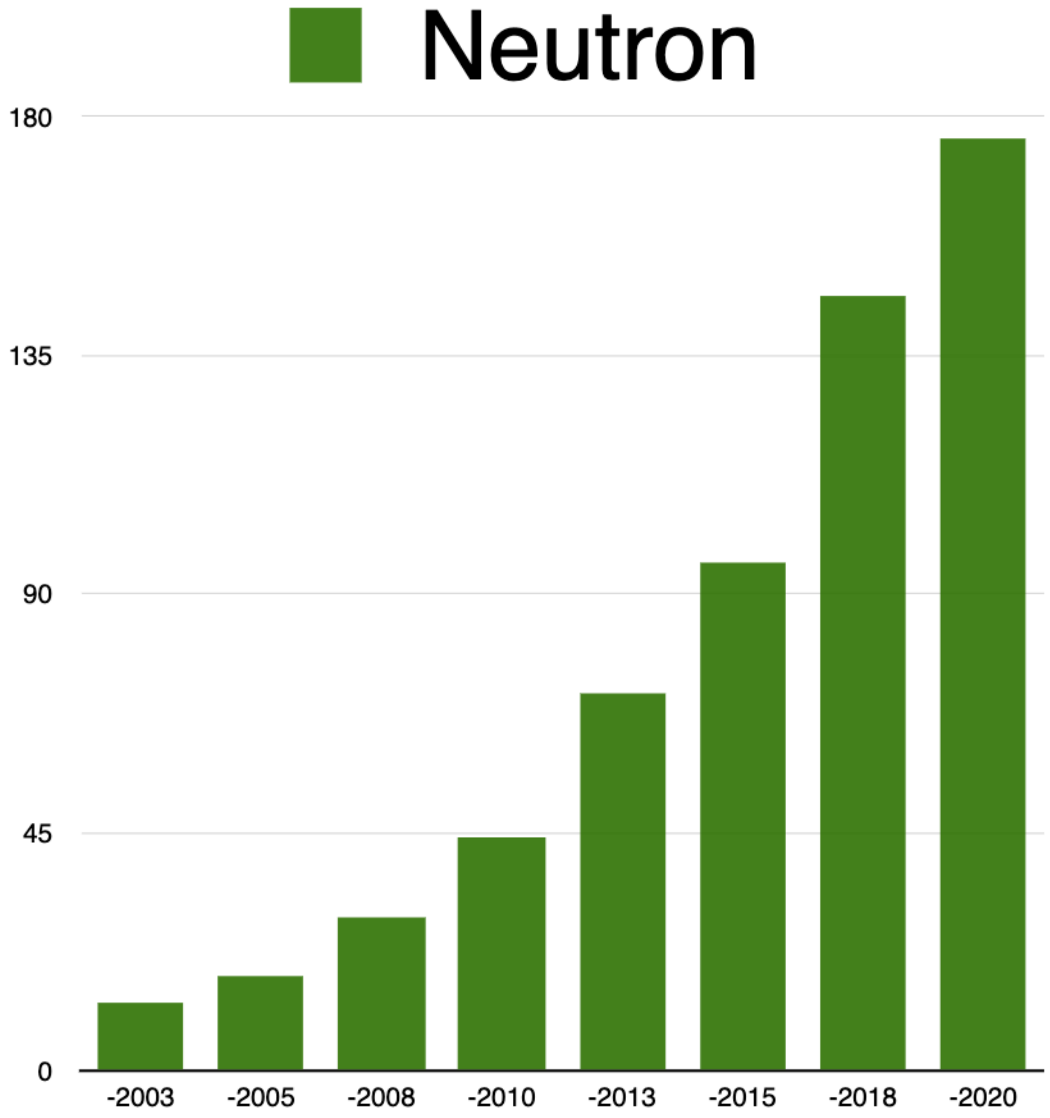
Provides full picture of ligand binding



Fisher SZ et al. JACS 2012;134:14726-14729

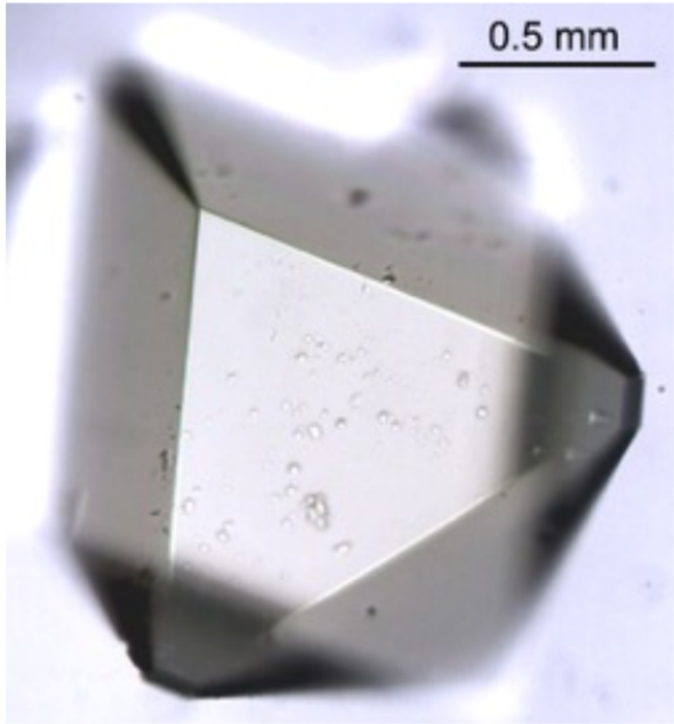
Neutron crystallography is still difficult





Challenges for neutron crystallography

- **Weak neutron sources**
  - Bigger crystals → more diffracting volume
  - Use Laue geometry → make all neutrons count
- **Incoherent scattering**
  - Exchange ^{1}H to ^{2}H (deuterium)
  - Produce perdeuterated protein



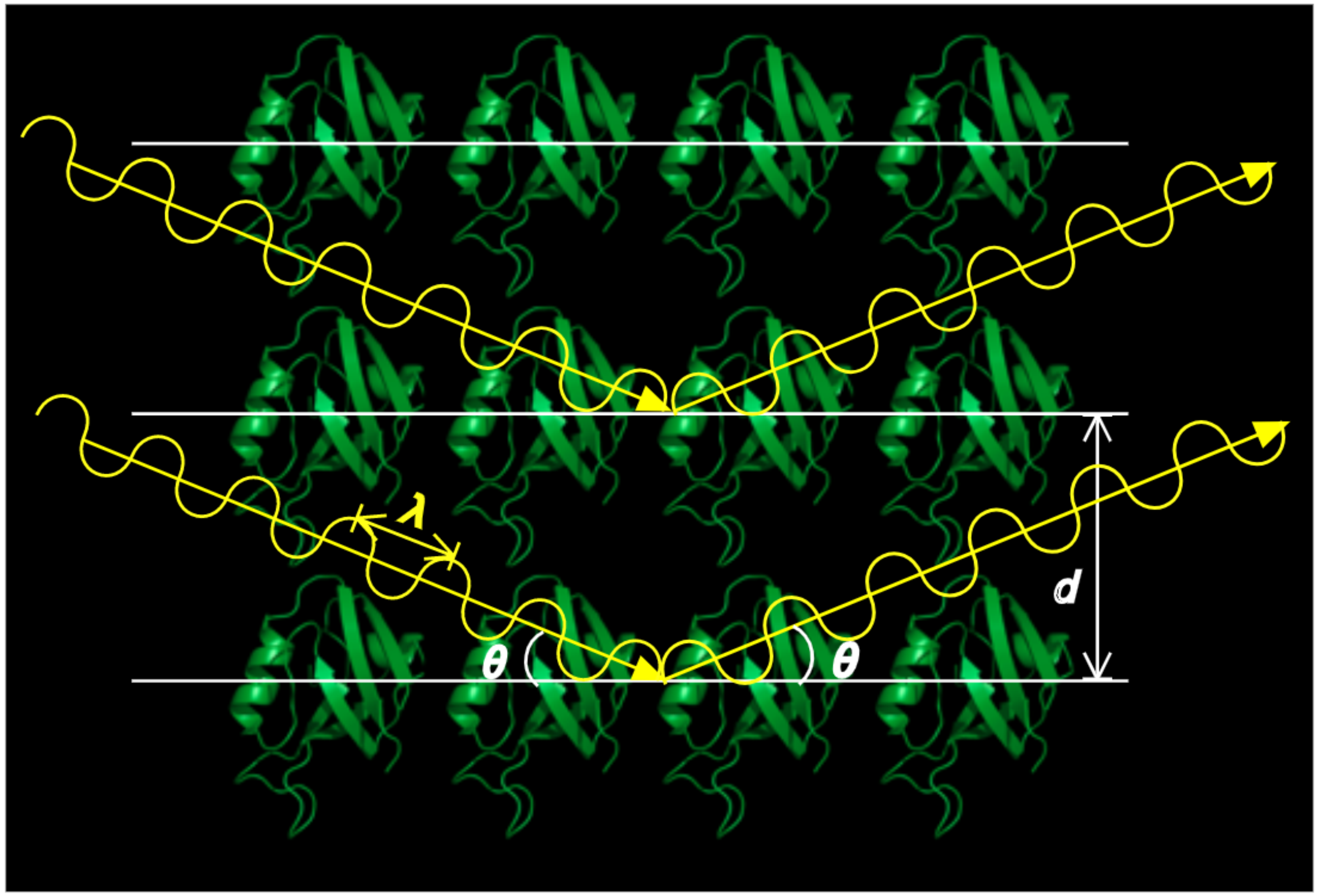
Oksanen, E *et al. J. R. Soc. Interface* **2009**, *6 Suppl 5*, S599-610.

Incoherent scattering

- Incoherent scattering contributes *only* to background!

Some scattering cross sections

Nucleus	σ_{coh} (10^{-28} m^2)	σ_{incoh}(10^{-28} m^2)
^{1}H	1,76	82,03
^{2}H	5,59	7,64
^{12}C	5,56	0
^{14}N	11,03	0,5
^{16}O	4,23	0



Phase change random in scattering

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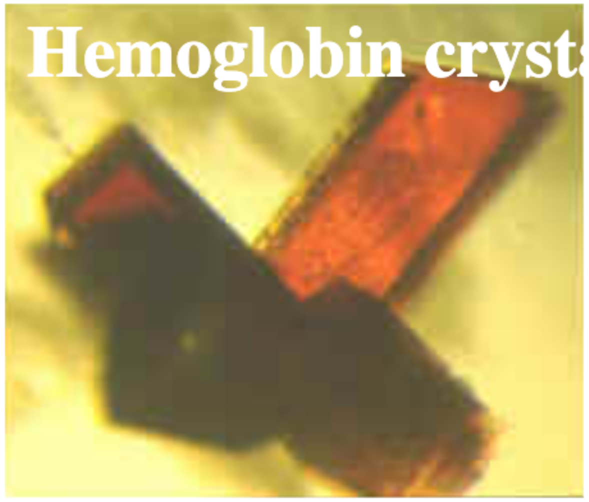
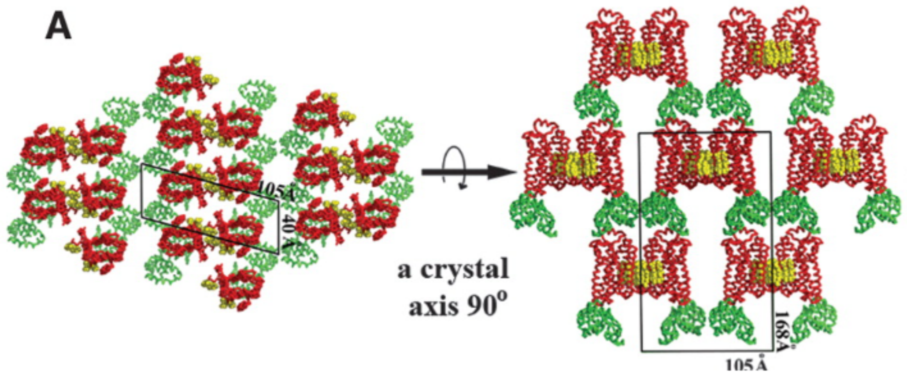
  - Large crystal growth
  - Protein diffractometers
  - Data processing

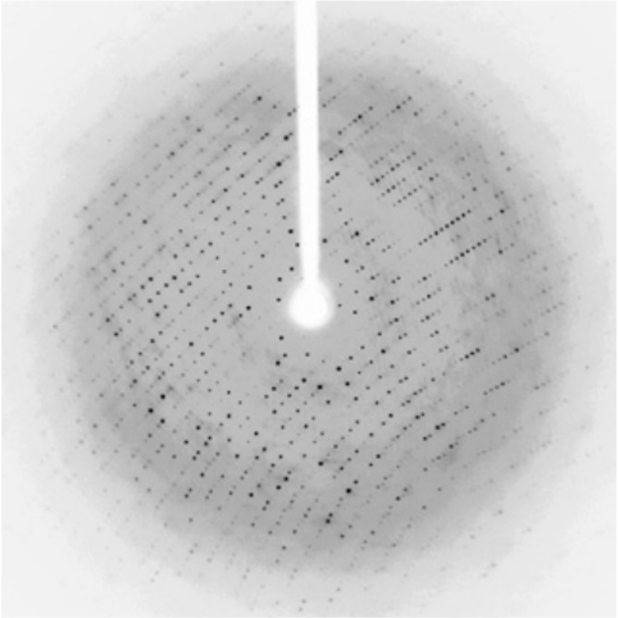
Protein crystals

- First reported in 1840 – hemoglobin
- Originally a purification method
- First protein crystal diffraction – 1934
- First protein crystal structure – 1957





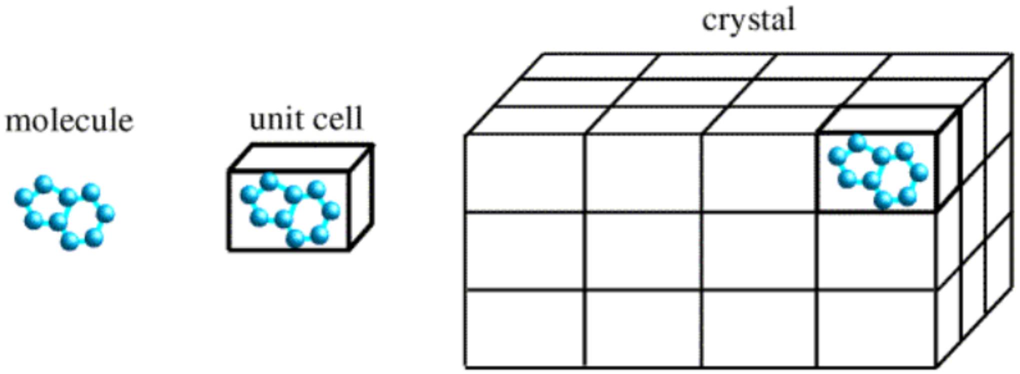
Human adrenergic receptor

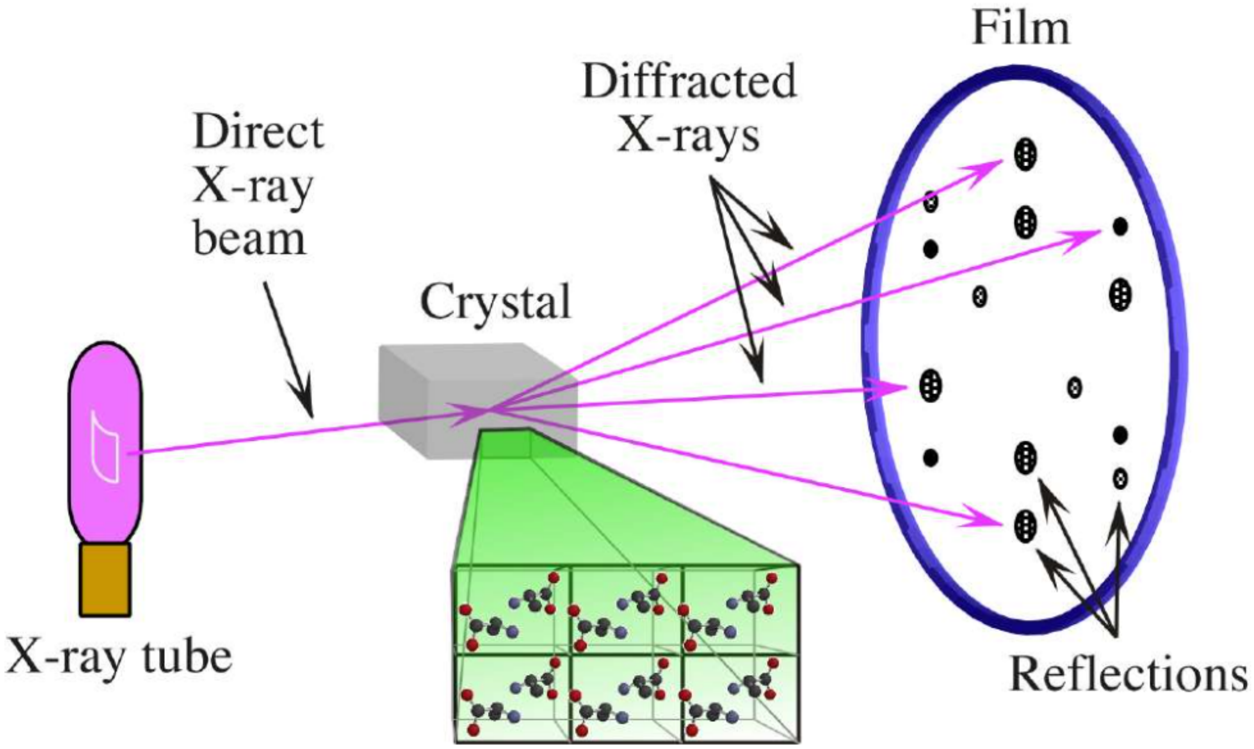


Myoglobin

Why do we need crystals?

- Scattering from individual molecules would be far too weak
- Crystals order the molecules in 3D and they serve as amplifiers – crystal lattice
- Crystal are composed of many unit cells, they contain the same thing and produce the same signal.

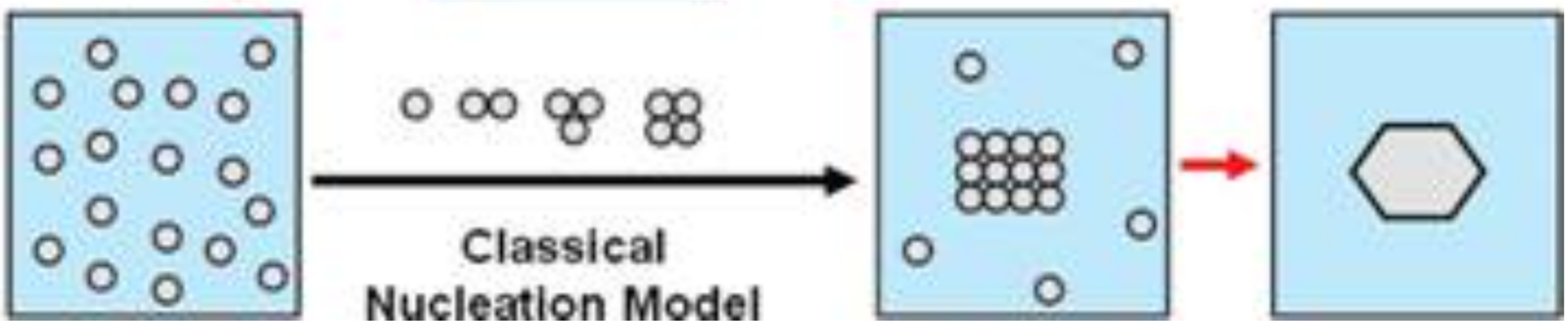




Crystal formation

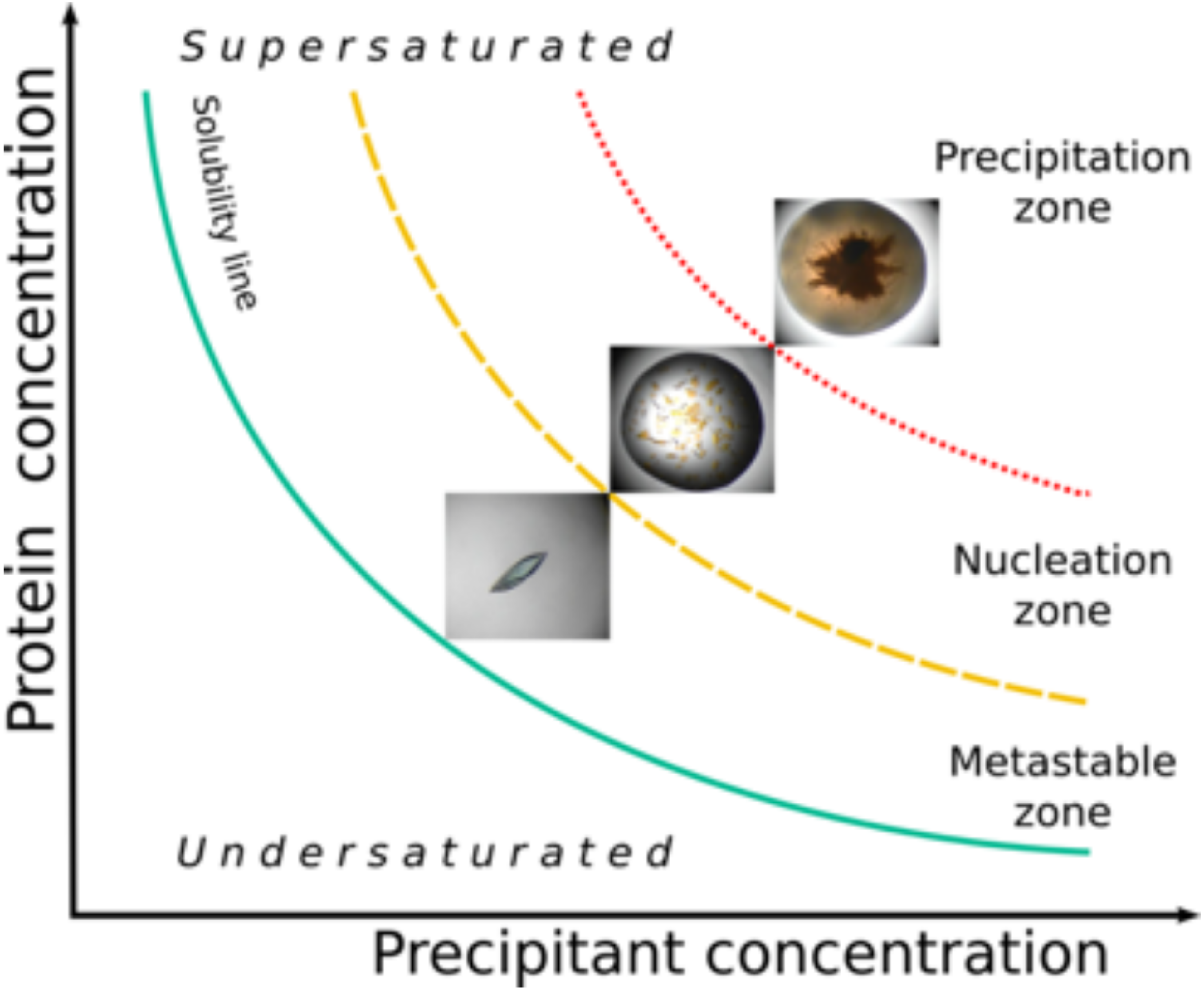
Crystals form in two phases:

1. Nucleation
2. Growth



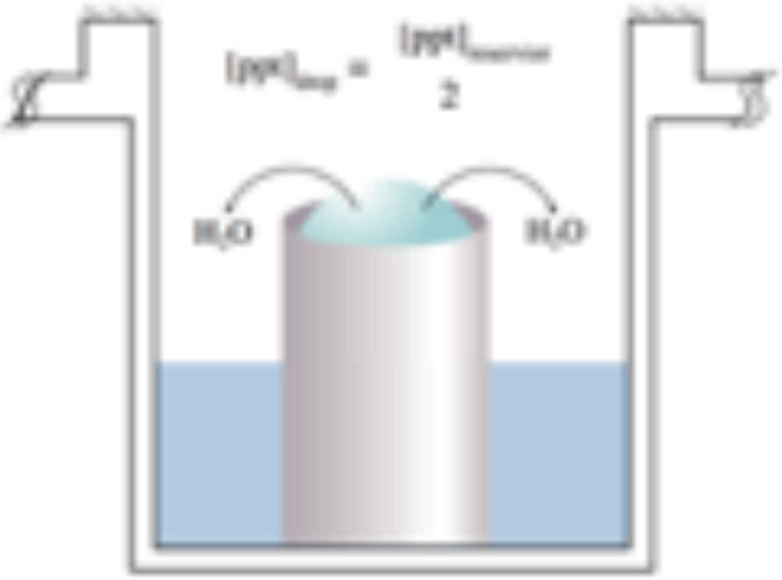
Stable nucleus: a molecular aggregate that attracts new molecules from the solution faster than others are lost to the solution

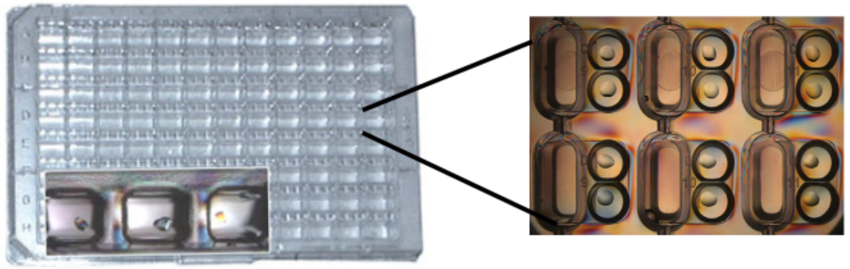
The Phase Diagram

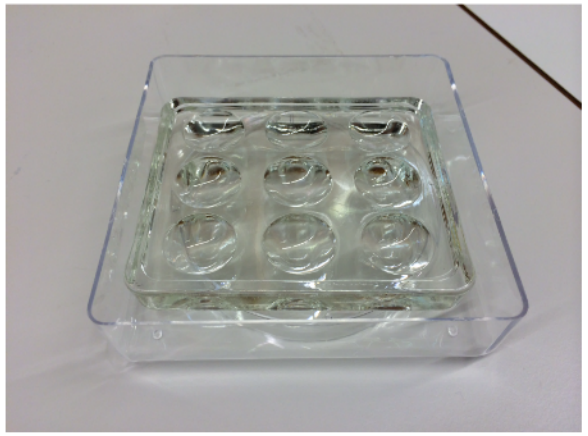


Vapour Diffusion

- Sitting and hanging drop formats
- Mix protein and precipitant (ppt) solution – initial [ppt] lower in drop than in reservoir . Over time water is pulled to the reservoir through vapour phase until equilibrium is reached. This increases the [protein].
- Scaleable! Small: 96 well plates for screening, typically use robot, sitting drop; Medium: Linbro 24 well plate, can do hanging drop and sitting drop; Large: 9-well glass plate, sitting drop.







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How big crystals do we need?

$$I_{pk} = t\phi(\lambda)\varepsilon(\lambda)\kappa N_s \frac{\lambda^4}{2V_{cell}\sin^2\theta} T_{DW}\langle |F_{hkl}|^2 \rangle$$

$$N_s = \frac{V_{cryst}}{V_{cell}} \qquad I_{pk} = t\phi(\lambda)\varepsilon(\lambda)\kappa \frac{V_{cryst}\lambda^4}{2V_{cell}^2\sin^2\theta} T_{DW}\langle |F_{hkl}|^2 \rangle$$

I_{pk} = integrated intensity for an average Bragg peak (n) = duration of measurement (s),

t = duration of measurement (s)

$\phi(\lambda)$= incident spectral flux at sample ($n.cm^{-2}.s^{-1}.Å^{-1}$)

$\varepsilon(\lambda)$ = detector efficiency

κ = conversion factor 1×10^{24} ($cm^2.bn^{-1}$)

N_S = no. of unit cells in sample,

2θ = Bragg angle for reflection,

V_{cell} = unit cell volume ($Å^3$)

V_{cryst} = crystal volume ($Å^3$)

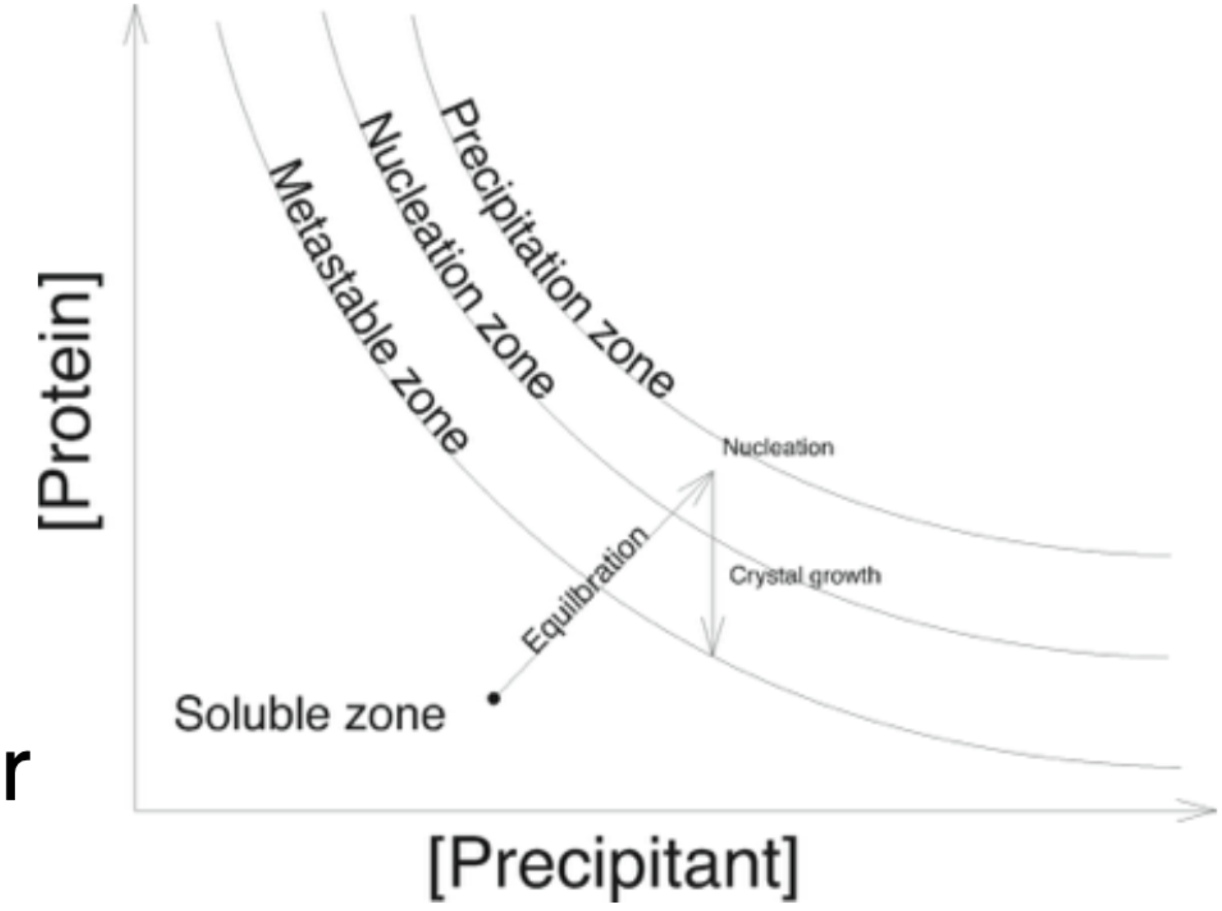
T_{DW} = temperature factor

$|F_{hkl}|^2$ = structure factor modulus of reflection hkl squared (bn)

Growing large crystals

1. Feeding drops

- Crystal growth is limited by the amount of crystallisable protein in the drop
- Adding protein solution allows growth to continue
- Will also dilute the mother liquor and restart vapour diffusion
- Often causes extra nucleation
- Works well when metastable zone is broad

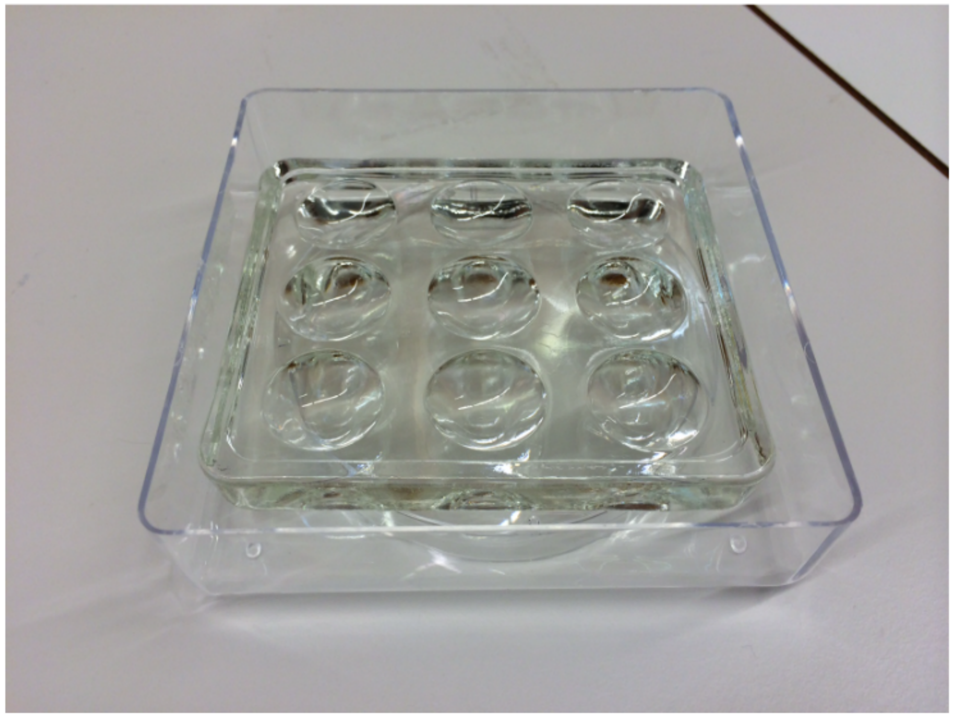


Often we are in the metastable zone even before vapour diffusion happens!

Growing large crystals

2. Increasing volume

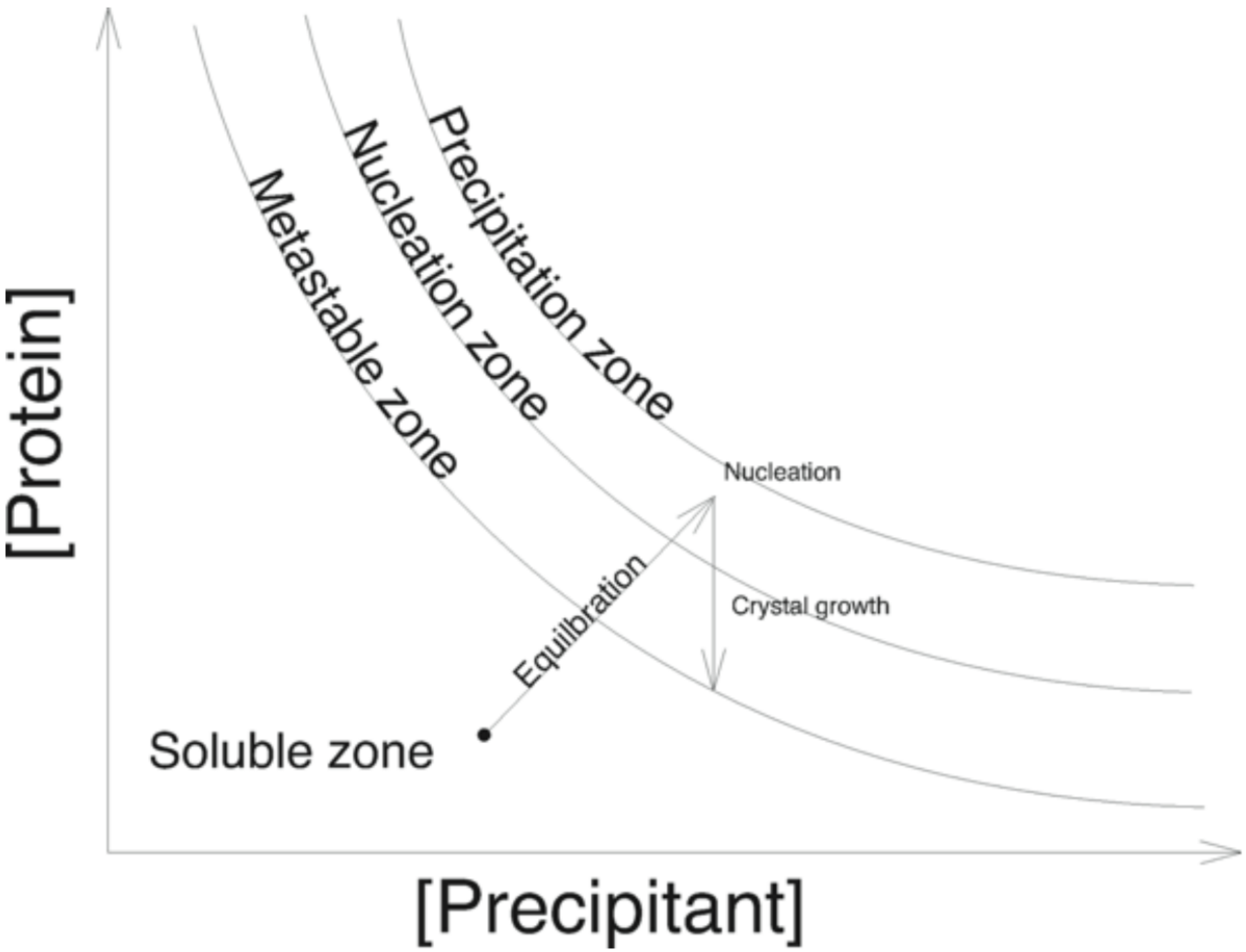
- Drop volume is limited by plates (*e.g.* ~ 50 µl for the CrysChem plate)
- A nine-well glass plate is a sandwich box allows easily > 200 µl drops
- Equilibration is very slow – a good thing for large crystals



In vapour diffusion the equilibration rate is strongly dependent on drop volume (surface -to-volume ratio)

Often we are in the metastable zone even before vapour diffusion happens!

Growing large crystals – limitations of vapour diffusion



Crystallisable protein = $([\mathrm{Protein}]_{max}-[\mathrm{Protein}]_{solubility})V_{drop}$

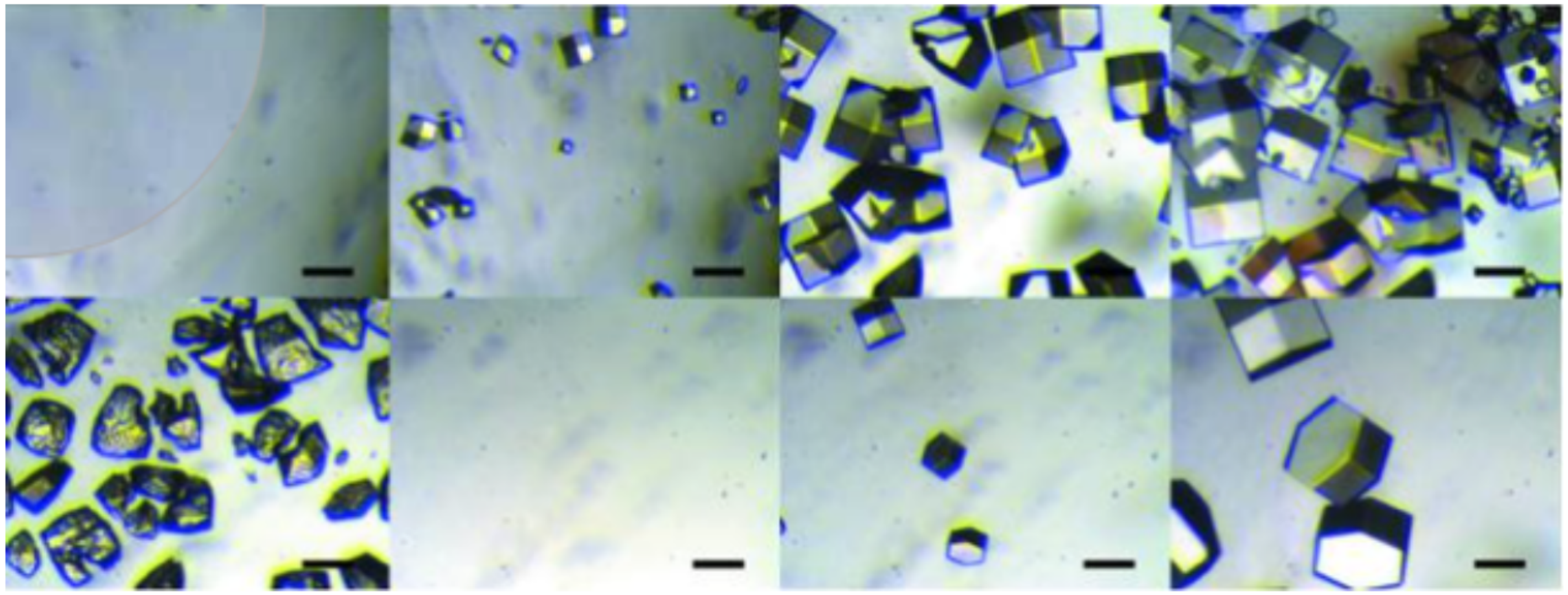
- Difficult to control path along phase diagram → often difficult to avoid extra nucleation
- Not reversible
- Repeated macro seeding adds mosaicity
- Feeding drops works when the metastable zone is wide

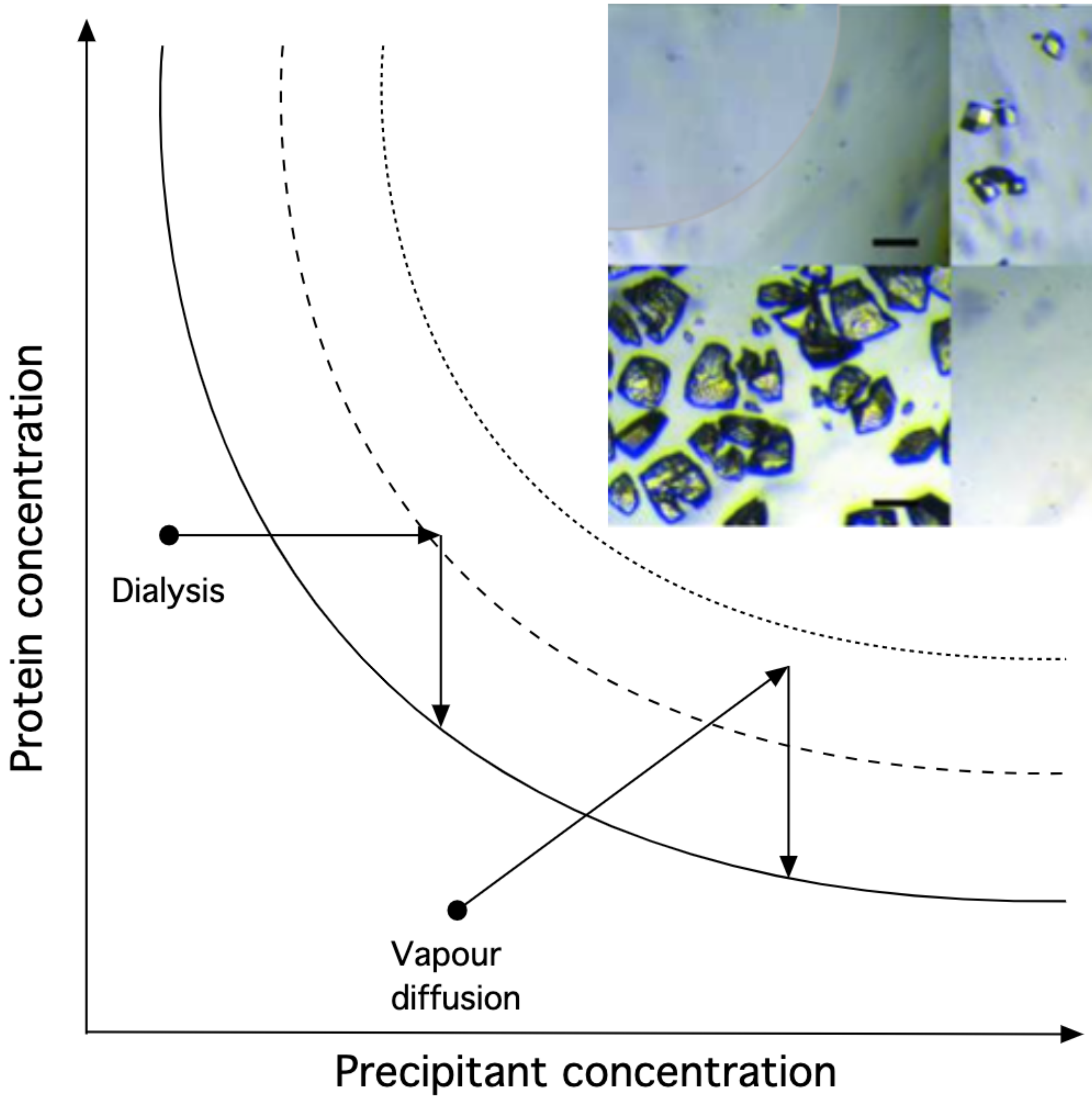
Growing large crystals

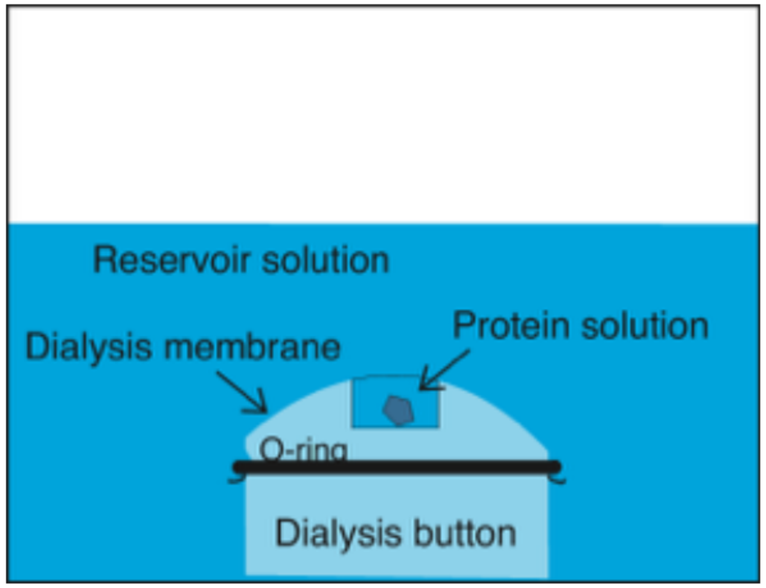
3. Dialysis

Junius N., Oksanen E., Terrien M., Berzin C., Ferrer J.-L., Budayova-Spano M., 2016 *J. Appl. Cryst.* 49 806-813

Reversibility by changing reservoir

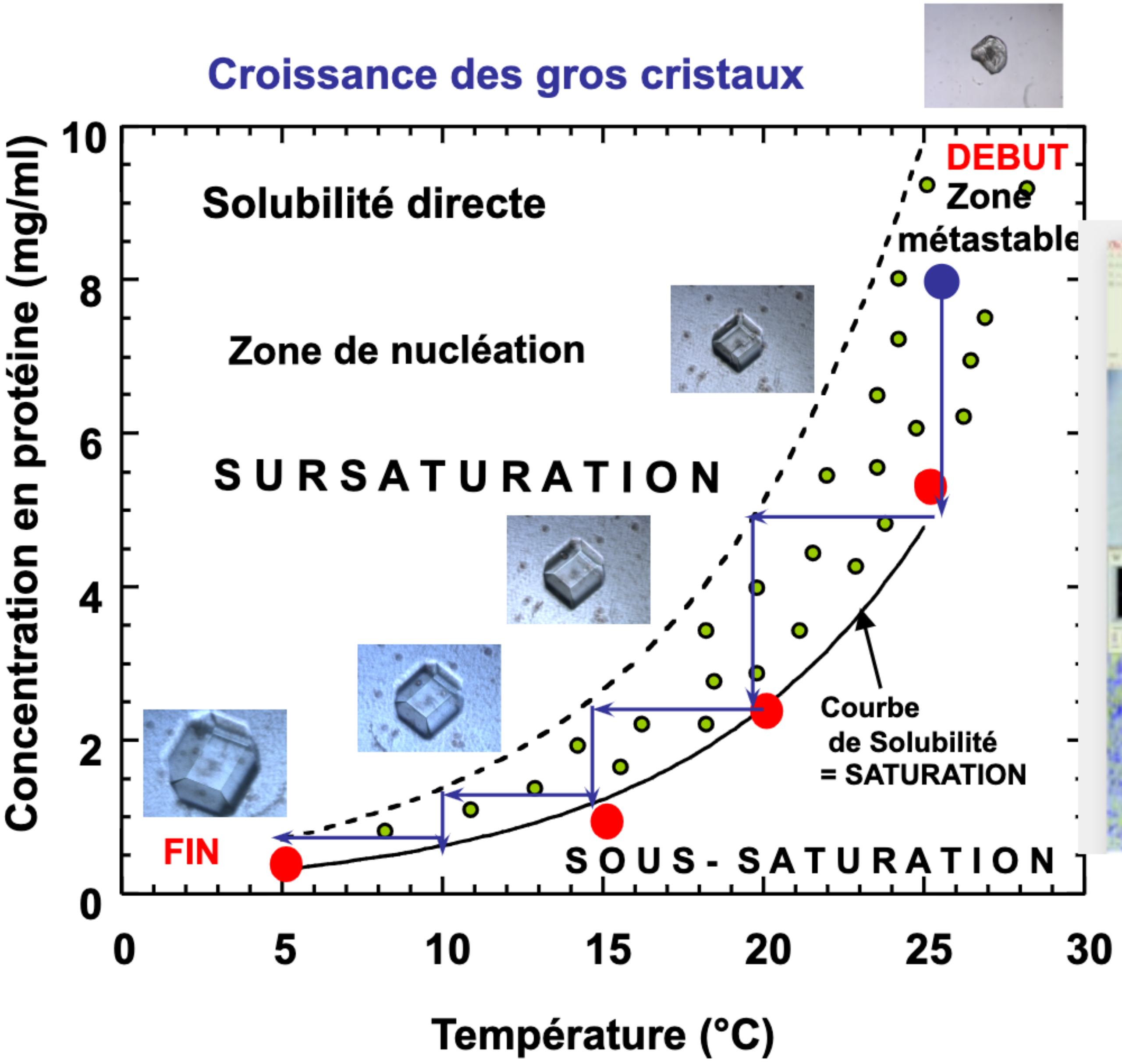




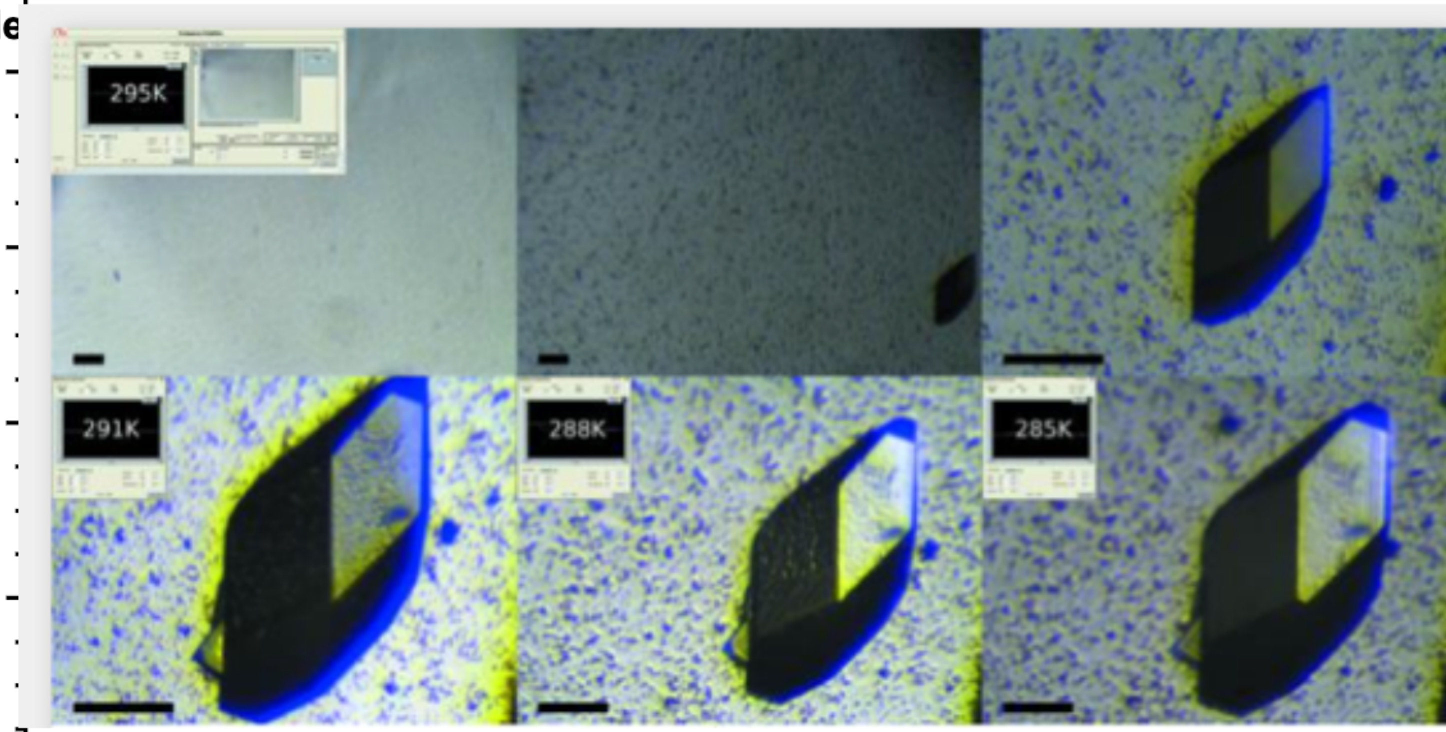


Growing large crystals

4. Temperature



Budayova-Spano, M.; Dauvergne, F.; Audiffren, M.; Bactivelane, T.; Cusack, S. 2007 *Acta Cryst.*, *D***63**, 339-347

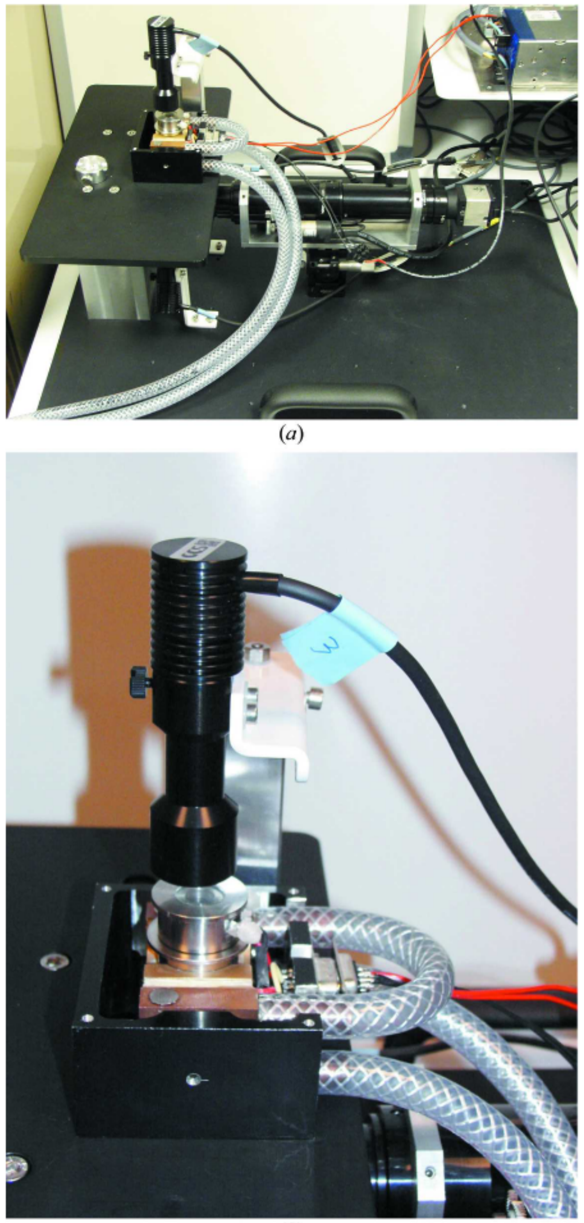


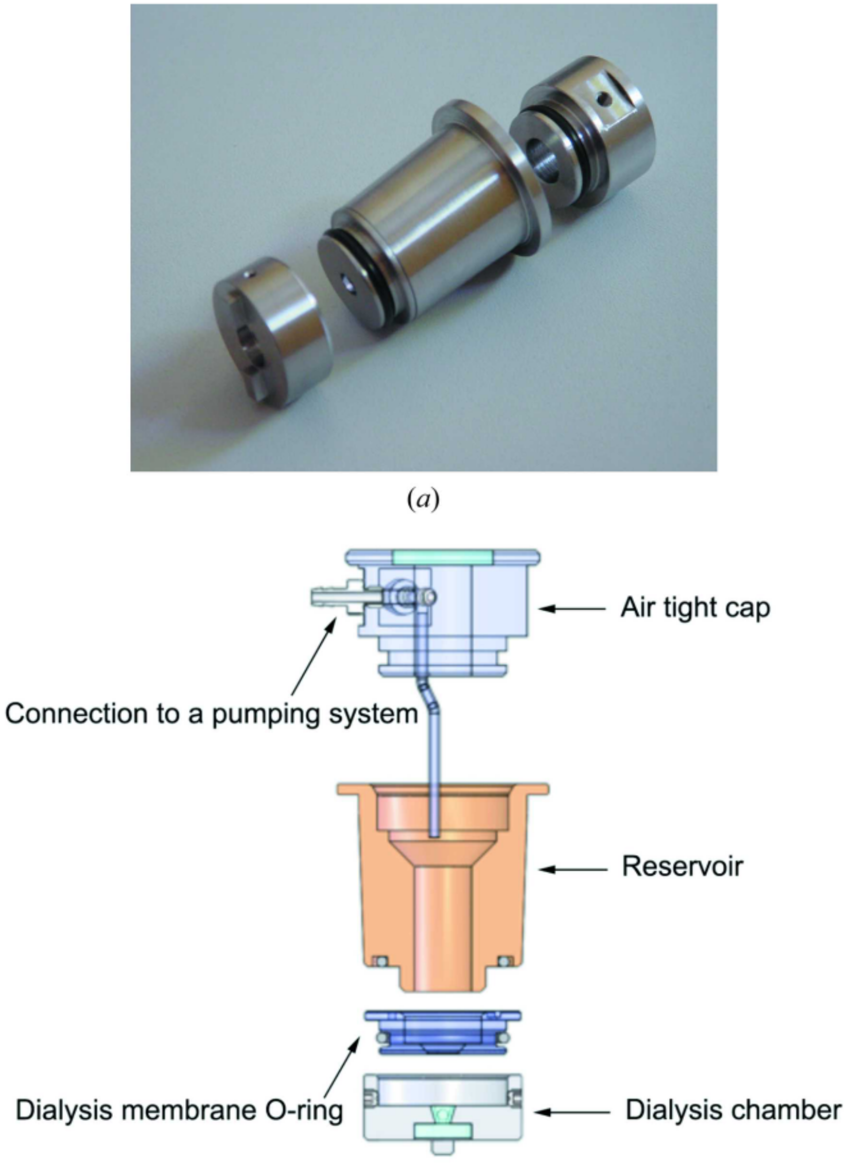
Junius N., Oksanen E., Terrien M., Berzin C., Ferrer J.-L., Budayova-Spano M., 2016 *J. Appl. Cryst.* **49** 806-813

Combining dialysis with temperature control

Junius N., Oksanen E., Terrien M., Berzin C., Ferrer J.-L., Budayova-Spano M.,
2016 *J. Appl. Cryst.* **49** 806-813

- The OptiCrys device allows flowing-reservoir dialysis with temperature control
- Real time visualisation allows understanding of the phase diagram
- Serial instead of parallel


(*a*)
(*b*)



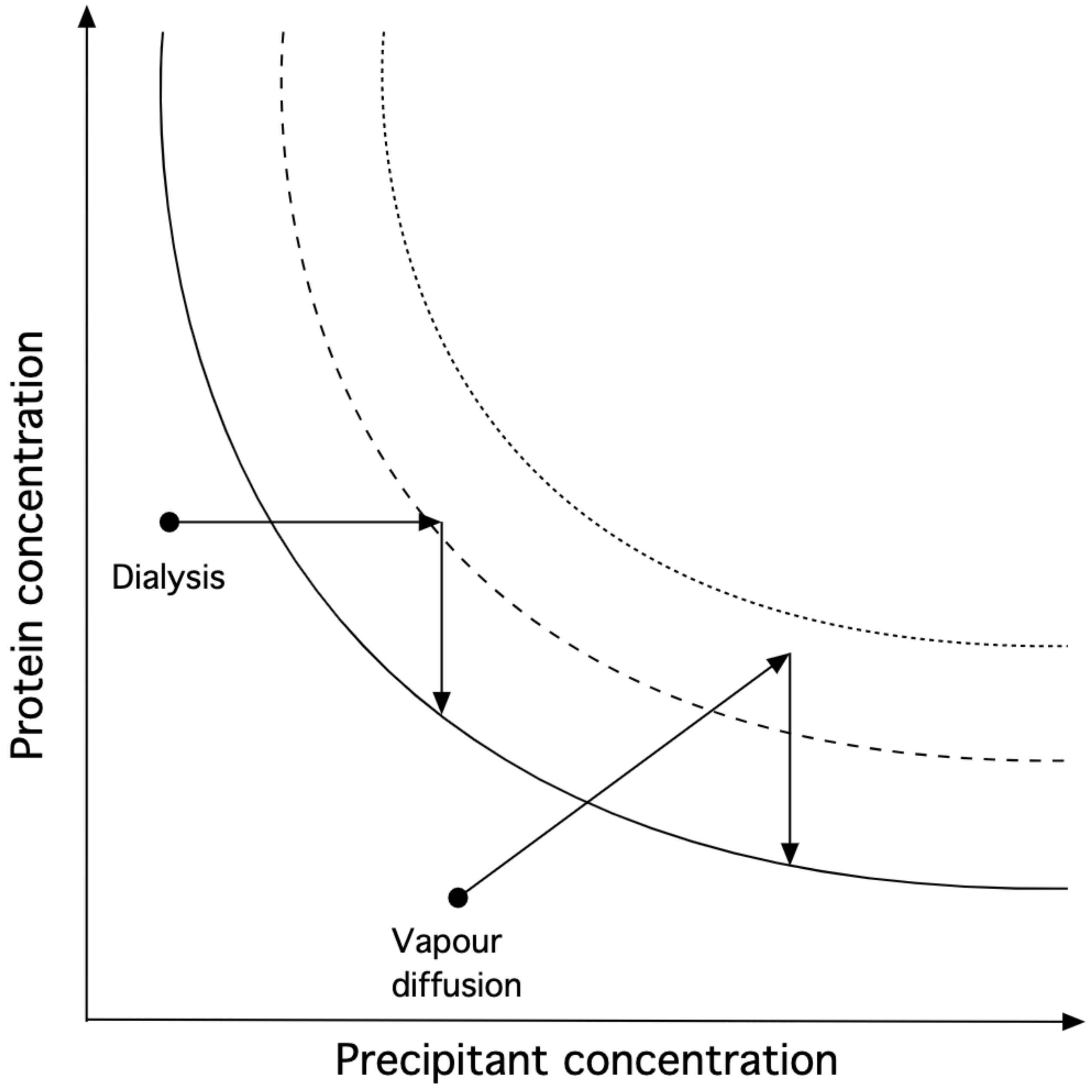
(*a*)
(*b*)

What can we do about incoherent scattering?

EUROPEAN SPALLATION SOURCE

- Replace all hydrogen by deuterium
- Exchange of mother liquor by D_2O & deuterated reagents ← Indispensable!
- Perdeuteration of protein = expression in D_2O & deuterated carbon source ← Very helpful

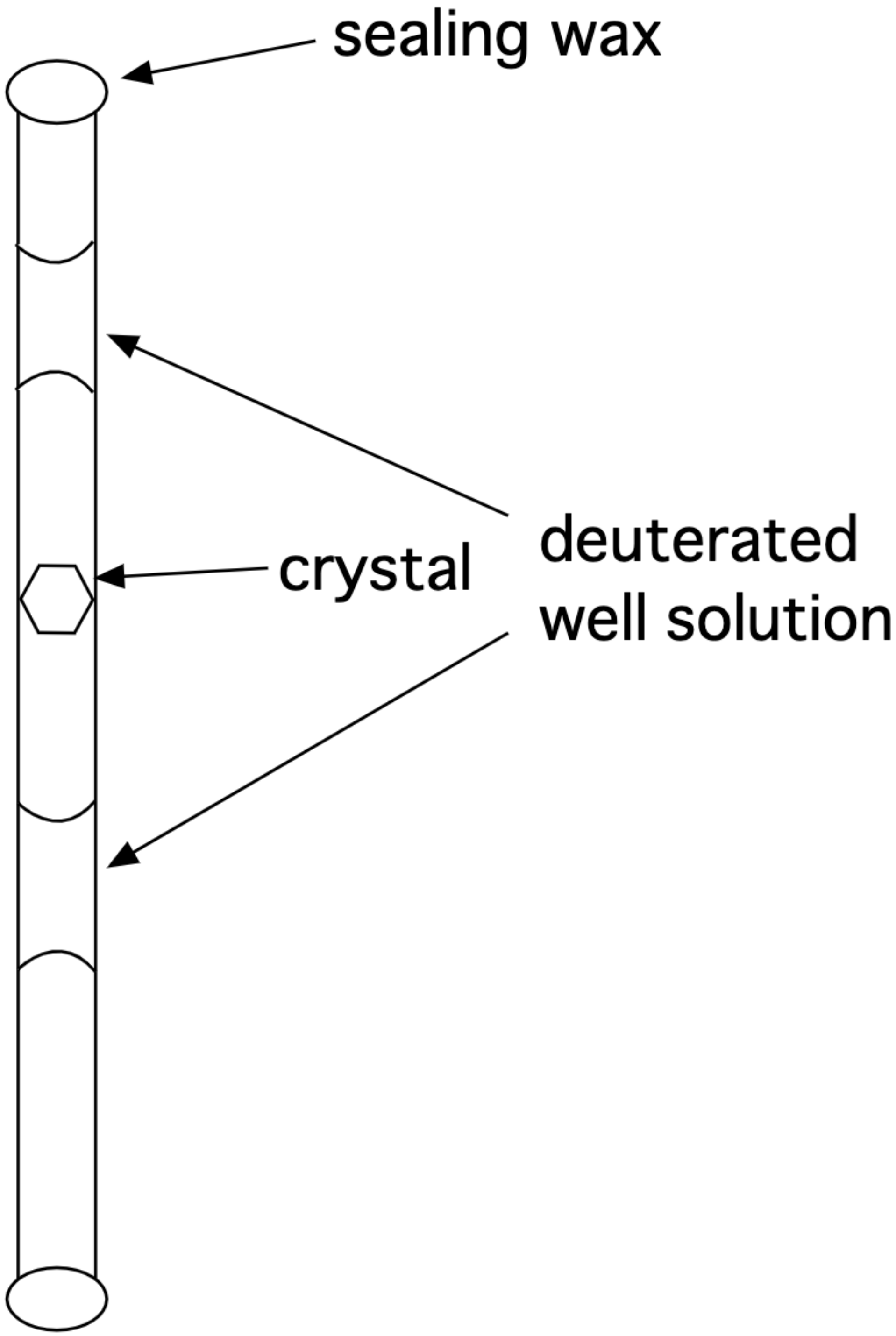
Crystallisation under deuterated conditions



- Protein solubility is lower in D_2O →
- Not as much protein available for crystal growth
- More nucleation on the side
- pH is not pD
- Deuterated reagents can be difficult to get and/or expensive

Exchange in capillary

- The crystal is mounted in a capillary with deuterated liquid plugs
- Gentle exchange through vapour phase
- Works well for crystals grown with vapour diffusion



sealing wax

crystal

deuterated well solution

Outline

- Why use neutrons for crystallography?
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  - **Protein diffractometers**
  - Data processing & refinement

Neutron data collection methods

Reactor source		Spallation source
Monochromatic	Continuous Laue	TOF-Laue
Oscillation method	Still images at regular ϕ	
☹Less neutron flux -> larger crystals and more time needed ☺Can use monochromatic X-ray software (modified for detector geometry, *e.g.* HKL at FRM-II) ☺Low background	☺More neutron flux -> smaller crystals and less time needed ☹Data processing more complicated; use modified X-ray Laue software *e.g.* Daresbury Laue suite ☹Higher background	☹Less neutron flux -> Spallation sources are weak ☹Data processing not mature; each facility has their own software *e.g.* StarGazer (J-PARC), Mantid (SNS) ☺Low background ☺Combines advantages of Laue and monochromatic

What wavelength to use?

$$I_{pk} = t\phi(\lambda)\varepsilon(\lambda)\kappa \frac{V_{cryst}\lambda^4}{2V_{cell}^2\sin^2\theta} T_{DW}\langle|F_{hkl}|^2\rangle$$

I_{pk} = integrated intensity for an average Bragg peak (n) = duration of measurement (s),

t = duration of measurement (s)

$\phi(\lambda)$= incident spectral flux at sample ($n.cm^{-2}.s^{-1}.Å^{-1}$)

$\varepsilon(\lambda)$ = detector efficiency

κ = conversion factor 1×10^{24} ($cm^2.bn^{-1}$)

N^S = no. of unit cells in sample,

2θ = Bragg angle for reflection,

vcell = unit cell volume ($Å^3$)

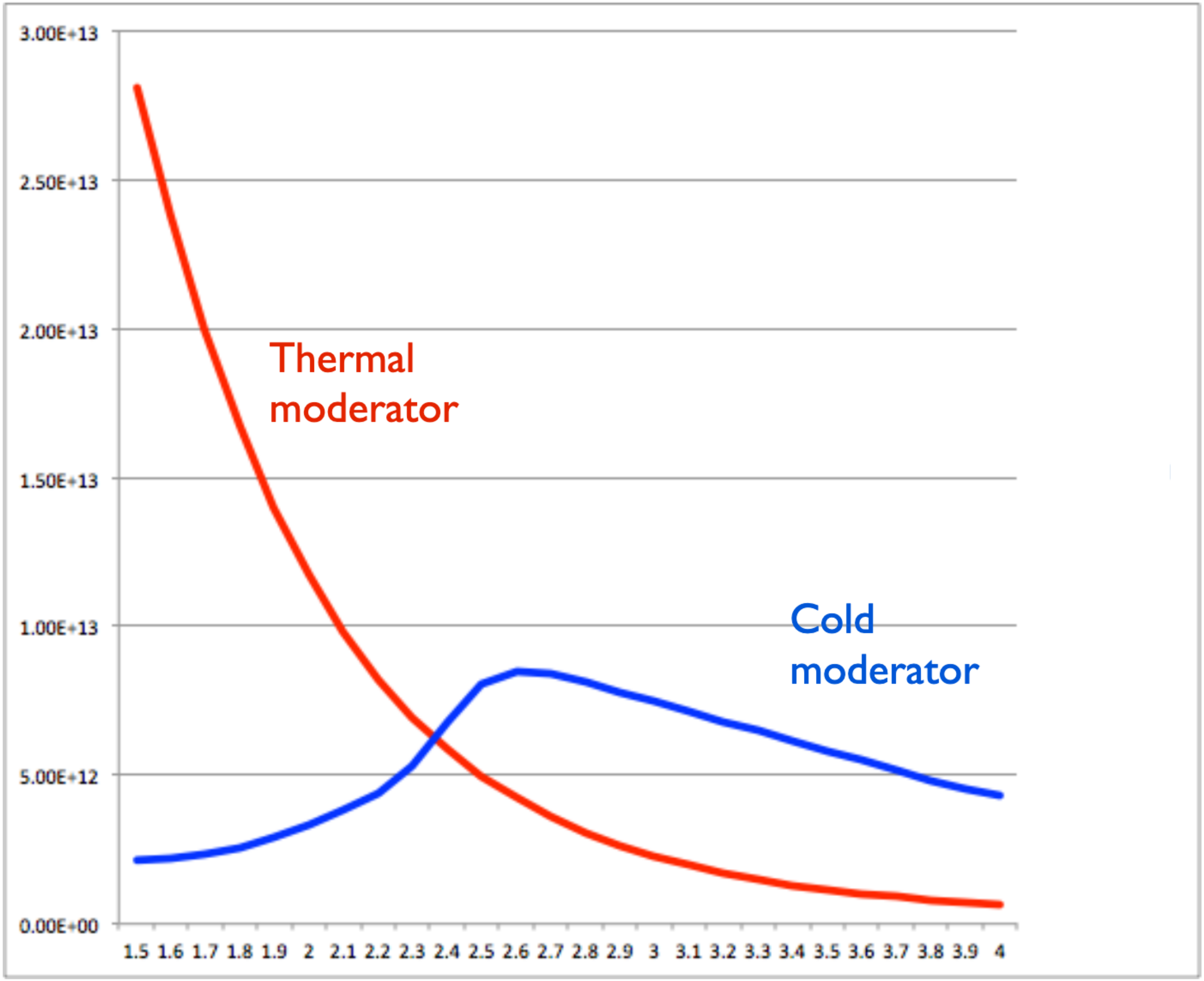
vcryst = crystal volume ($Å^3$)

T_{DW} = temperature factor

$|F_{hkl}|^2$ = structure factor modulus of reflection hkl squared (bn

Air absorption is not a problem → Go for long wavelengths!

Moderator spectra



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

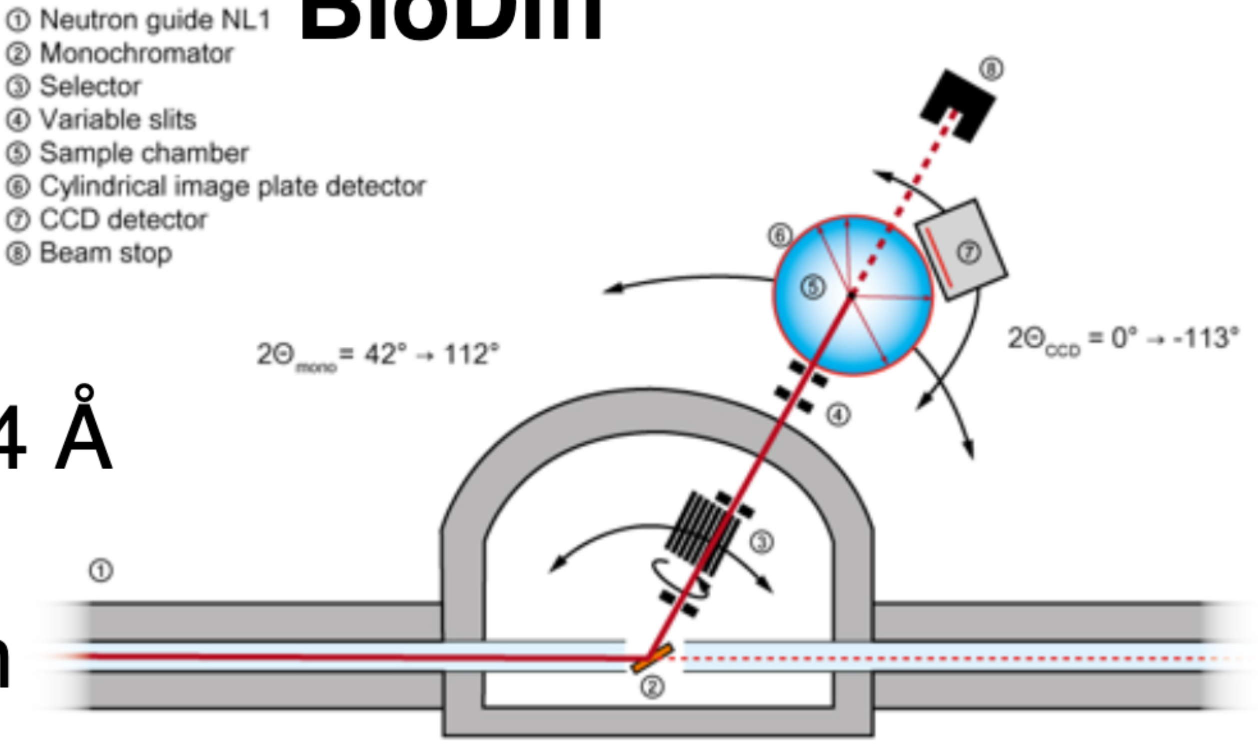
Reactor instruments – Monochromatic

- **BIX-3 & 4** @ JRR3, Tokai-mura, Japan
  - Cold, λ=2.9 & 2.6 Å
  - Cylindrical geometry
  - Image plate detector

Currently closed

- **BioDiff** @ FRM-II, Munich, Germany
  - Cold, variable 2.4 < λ < 5.6 Å
  - Cylindrical geometry
  - Image plate detector
- **D19** @ ILL, Grenoble, France
  - Thermal, variable 0.8 < λ < 2.4 Å
  - ‘Banana’ ^{3}He detector
  - For small cells, high resolution

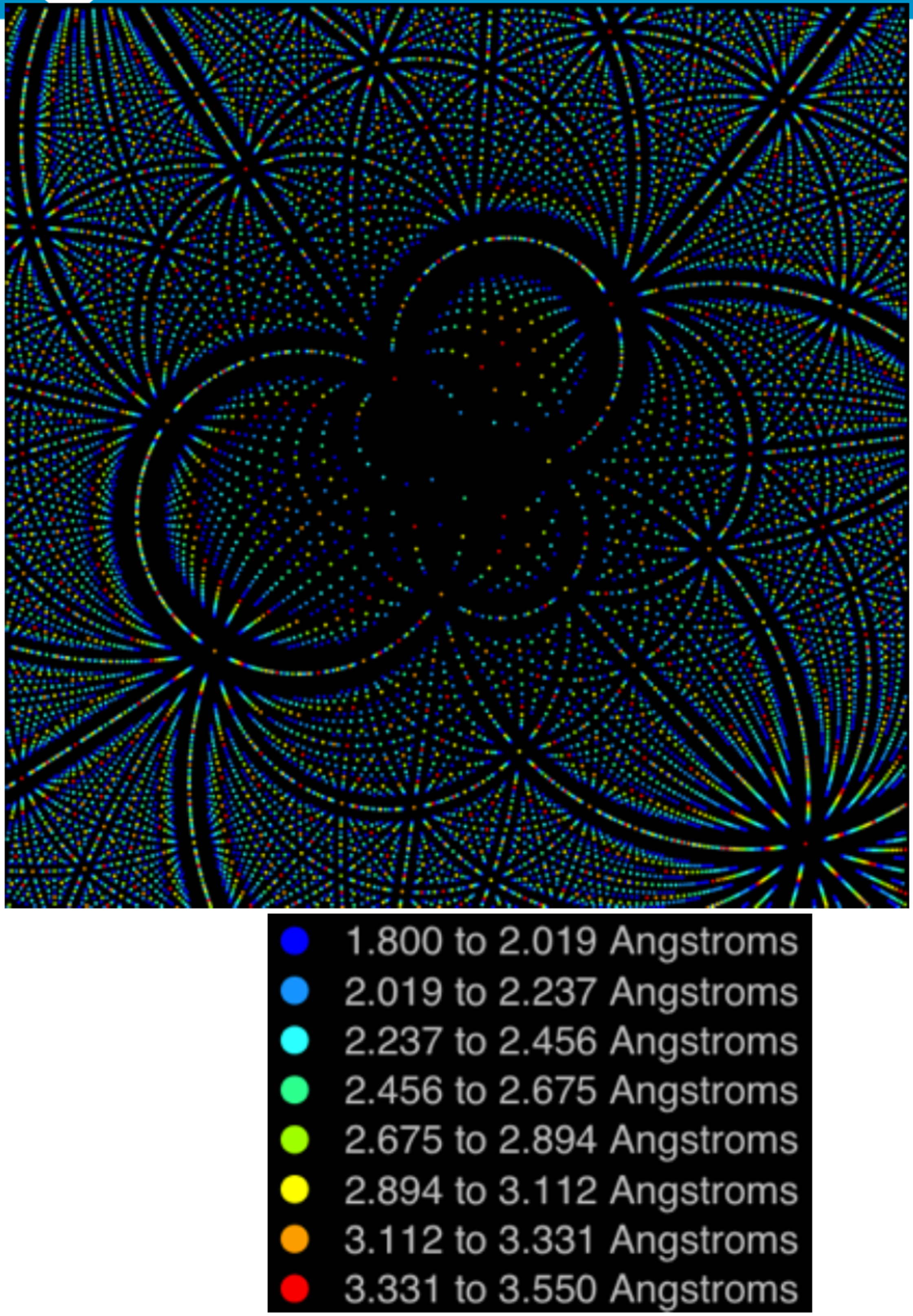
BioDiff



① Neutron guide NL1
② Monochromator
③ Selector
④ Variable slits
⑤ Sample chamber
⑥ Cylindrical image plate detector
⑦ CCD detector
⑧ Beam stop

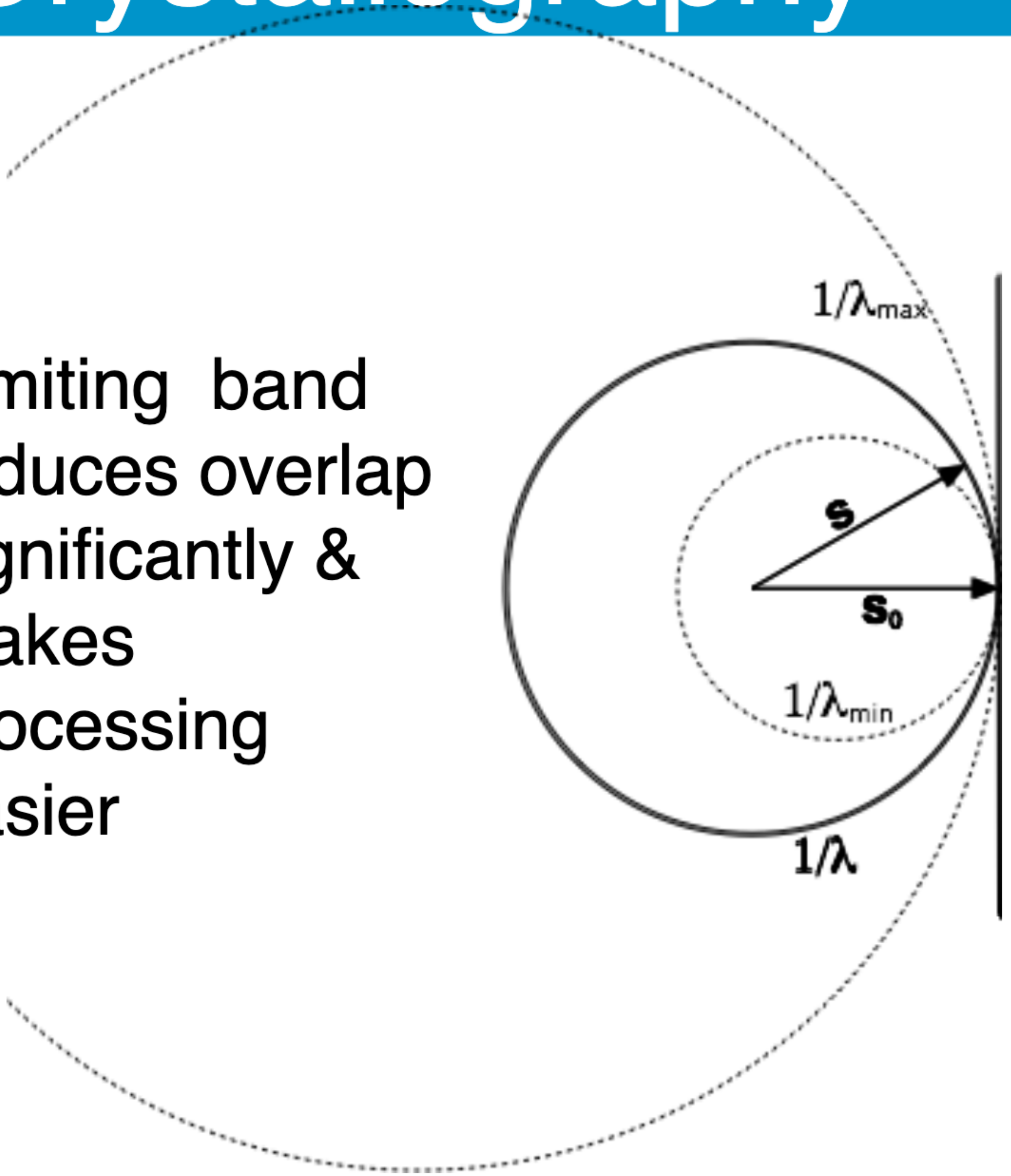
Laue Crystallography - using more wavelengths

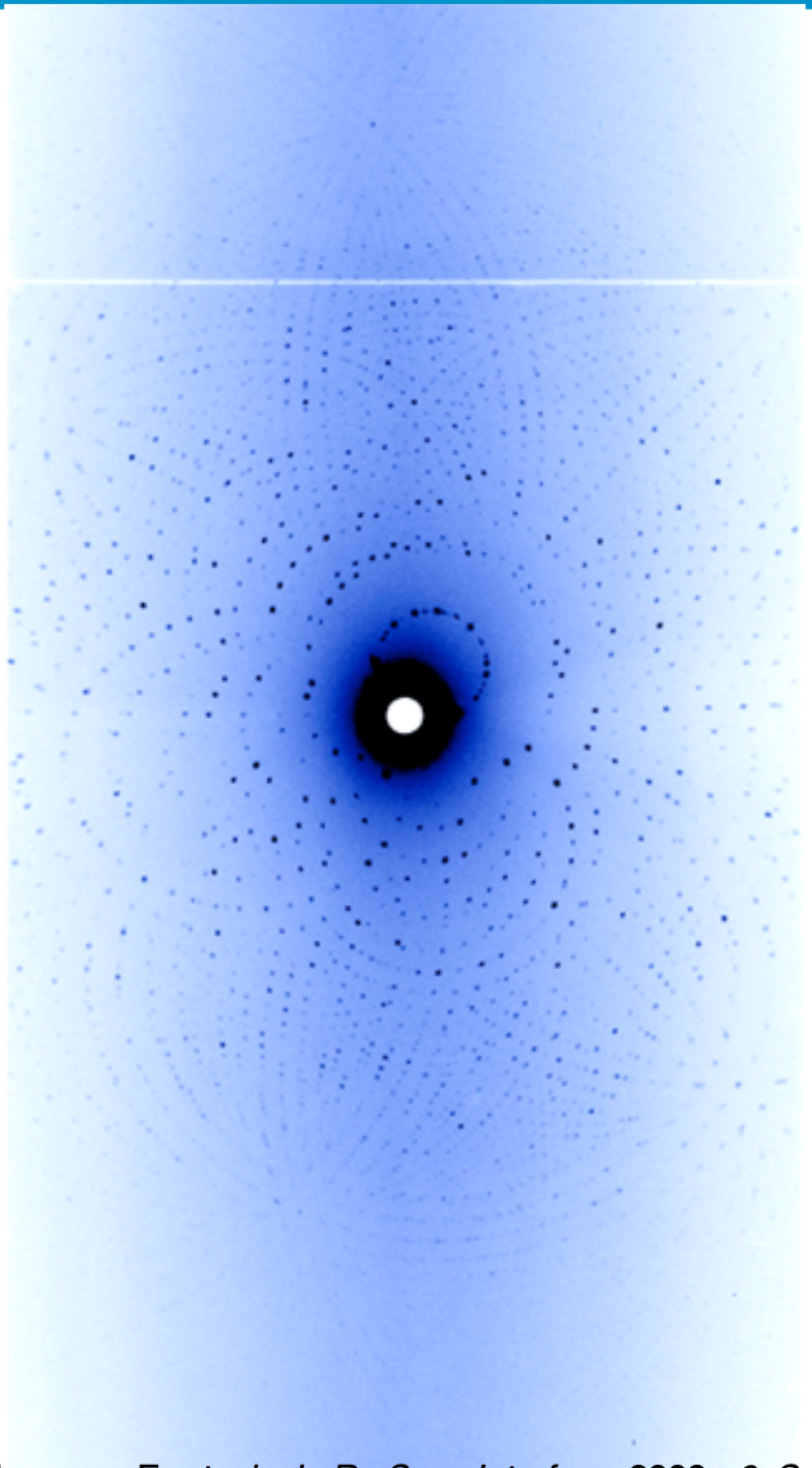
- Uses more of the available flux than monochromatic methods
- Signal at one λ- background at all
- Data processing is more complicated → harmonic & spatial overlap
- Very sensitive to crystal mosaicity



Quasi-Laue Crystallography

Limiting band reduces overlap significantly & makes processing easier

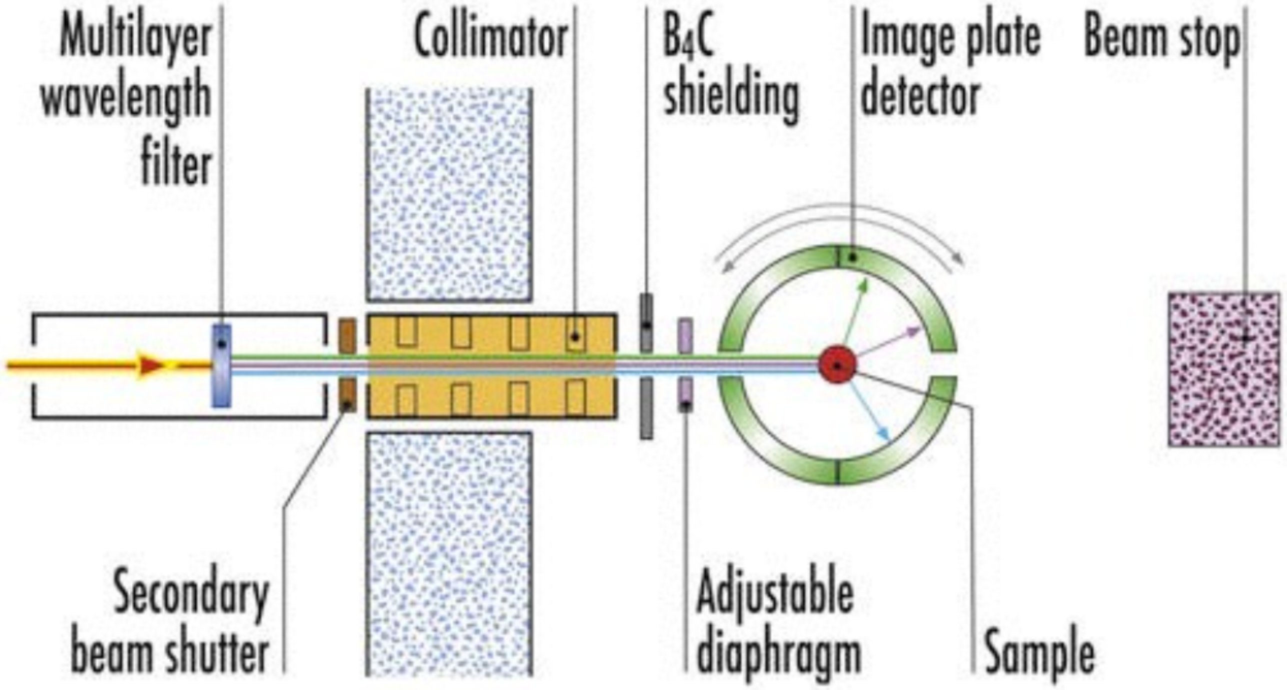




Oksanen, E *et al.* *J. R. Soc. Interface* **2009**, *6 Suppl 5*, S599-610.

Reactor instruments – Laue

- **LADI-III** @ ILL, Grenoble, France
  - Cold, Quasi-Laue, filters with δλ/λ=30%
  - λ-ranges 2.9-3.9 Å, 3.6-4.7 Å, 4.1-5.3 Å
  - Cylindrical geometry
  - Image plate detector
- **IMAGINE** @ HFIR, TN, USA
  - Cold, Quasi-Laue, mirrors
  - λ-ranges 2.0-3.0 Å, 2.8-4.0 3.3-4.5 Å
  - Cylindrical geometry
  - Image plate



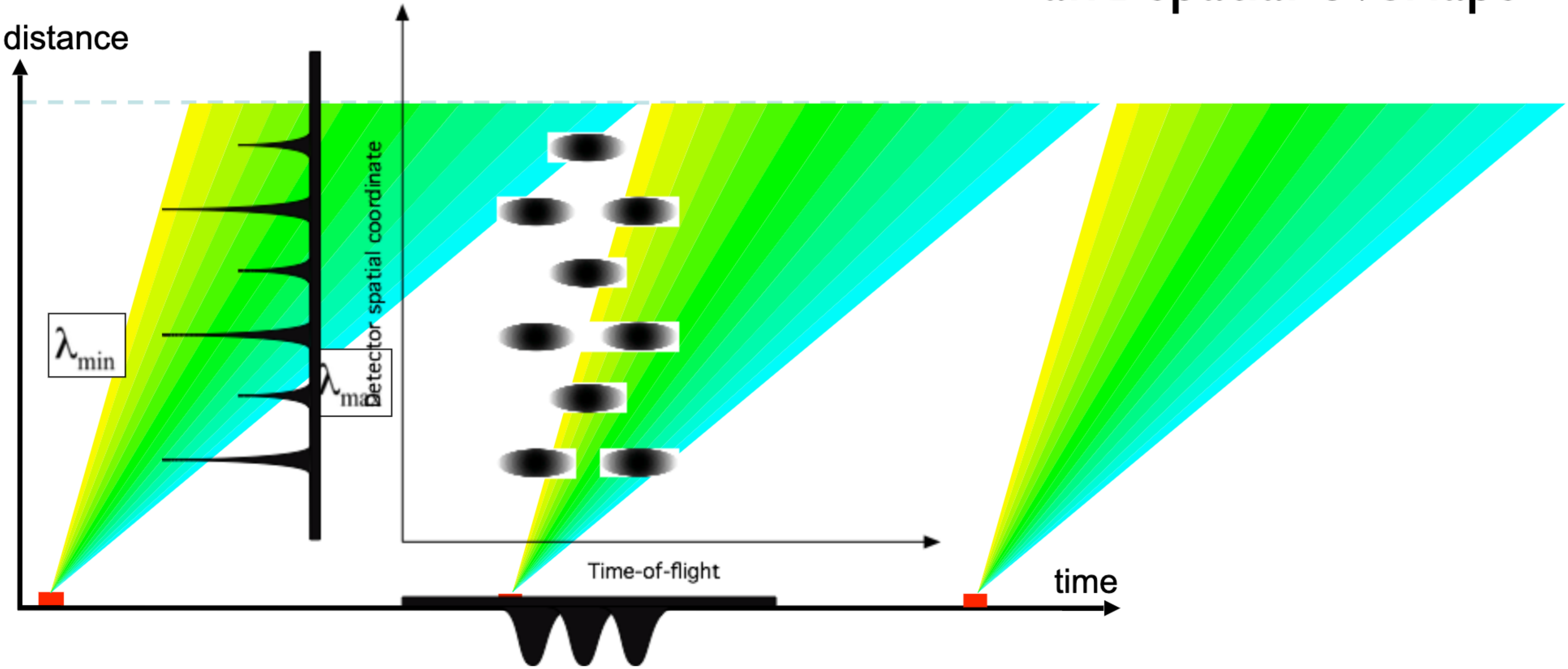
LADI-III layout

TOF Laue crystallography

Spreads background in many time bins

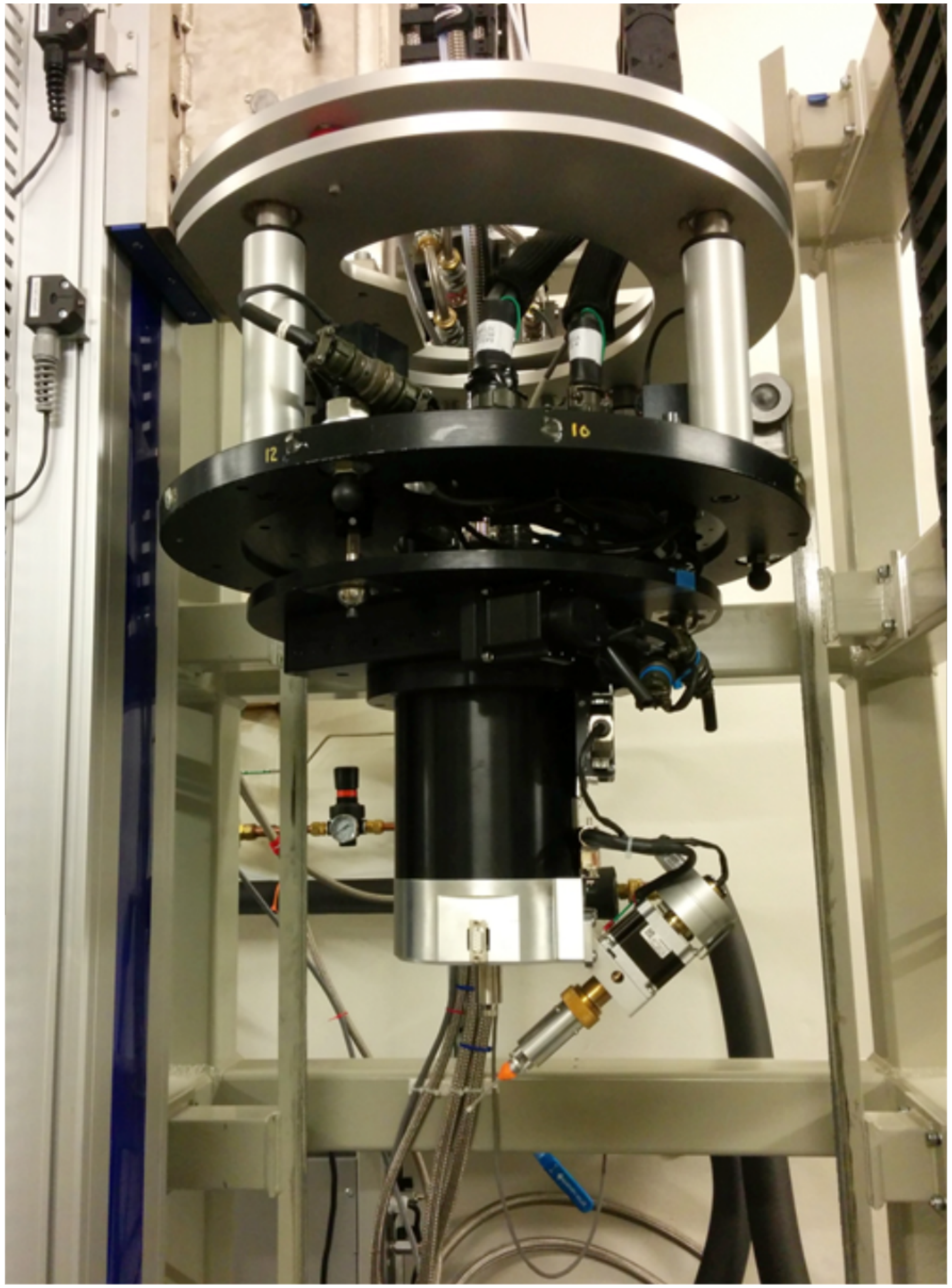
Can resolve harmonic and spatial overlaps

At pulsed spallation sources we can resolve time-of-flight → energy

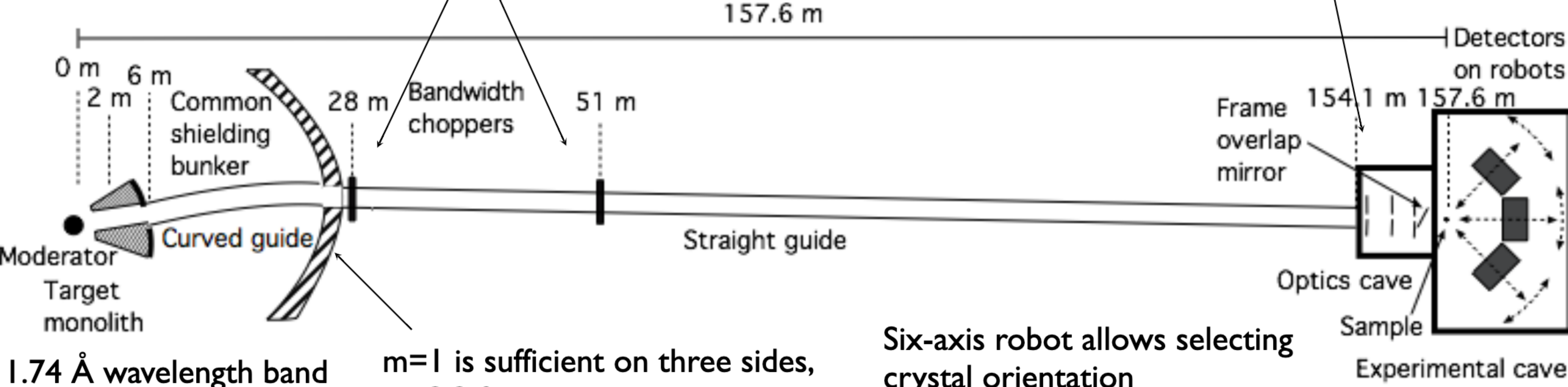


Spallation Source Instruments – TOF Laue

- **MaNDi** @ SNS, TN, USA
  - Cold, TOF-Laue, Δλ=2.15/4.3 Å
  - 30 m length, λ-range 1-10 Å
  - Polyhedral geometry
  - Anger camera detector
- **iBIX** @ J-PARC, Tokai-mura, Japan
  - Cold, TOF-Laue, Δλ=3 Å
  - 30 m length, λ-range 0.8-3.85 Å
  - Polyhedral geometry
  - Wavelength-shifting fibre detector



NMX – conceptual view



Adjustable opening chopper allows flexible bandwidth selection.

Collimation section giving flexibility in beam size and divergence

Cold moderator

1.74 Å wavelength band (1.8-3.55 Å)

m=1 is sufficient on three sides, m=2.2 for curving

Six-axis robot allows selecting crystal orientation

Three 60 x 60 cm detectors with 0.2 mm spatial resolution Variable sample-detector distance (0.2-1.0 m)
Variable 2θ angle (0-110°)

- **NMX** @ ESS, Lund, Sweden
  - Cold, TOF-Laue, Δλ<1.75 Å
  - 158 m length, λ-range 1.8-10 Å
  - Flexible geometry (robots)
  - Gd-GEM detector

NMX – Macromolecular diffractometer at ESS

EUROPEAN
SPALLATION
SOURCE

Key advantages of ESS Macromolecular Diffractometer

Smaller crystals needed (200 μm vs. 1 mm)

0

Partne

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Data processing workflow – challenges with neutrons

Find spots

Weak diffraction, crowded Laue pattern

Spot x,y (TOF)
(detector coordinates)

Index spots

In Laue unit cell and UB-matrix are coupled – we have to know the cell

h,k,l

Integrate spot intensities

High background (deuteration helps)
In Laue harmonic and spatial overlap

I(h,k,l)

Scale intensities

Multiple wavelength scaling

Not needed with neutrons – we know the X-ray structure

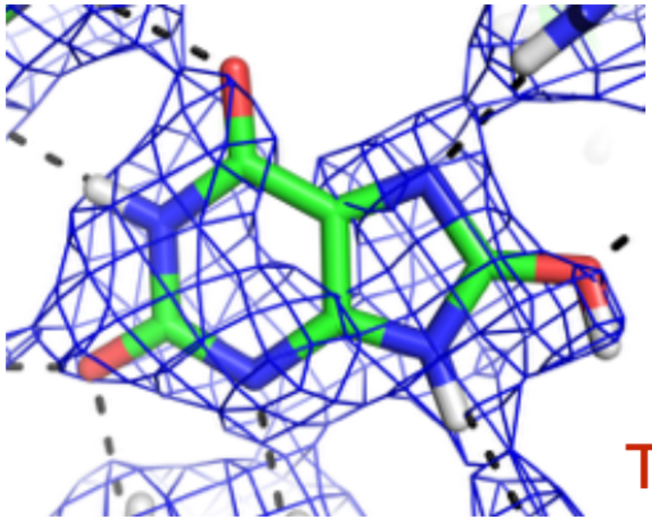
~~Determine phases~~

I(h,k,l),ϕ(h,k,l)

Refine structure

Atom x,y,z

Hydrogens increase number of parameters



Typically low completeness reduces map quality

Joint X-ray neutron refinement

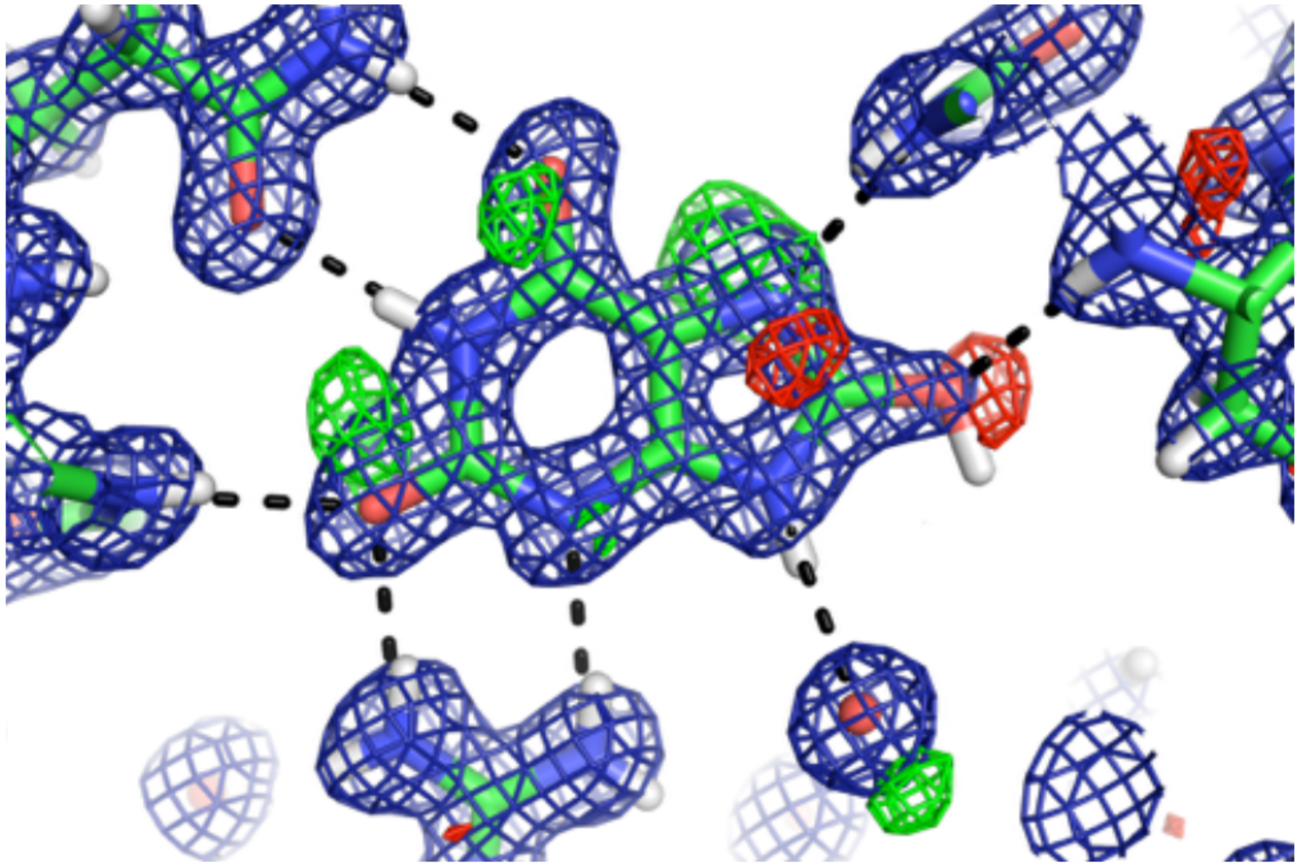
- Refining hydrogens doubles number of parameters
- A joint neutron-X-ray target function remedies the data/parameter ratio
- Needs X-ray data collected from the ‘same’ crystal and same temperature
- Implemented in *e.g.* phenix.refine

No neutron structure without an X-ray structure!

Geometry target values for hydrogens are not as precise as heavy atoms

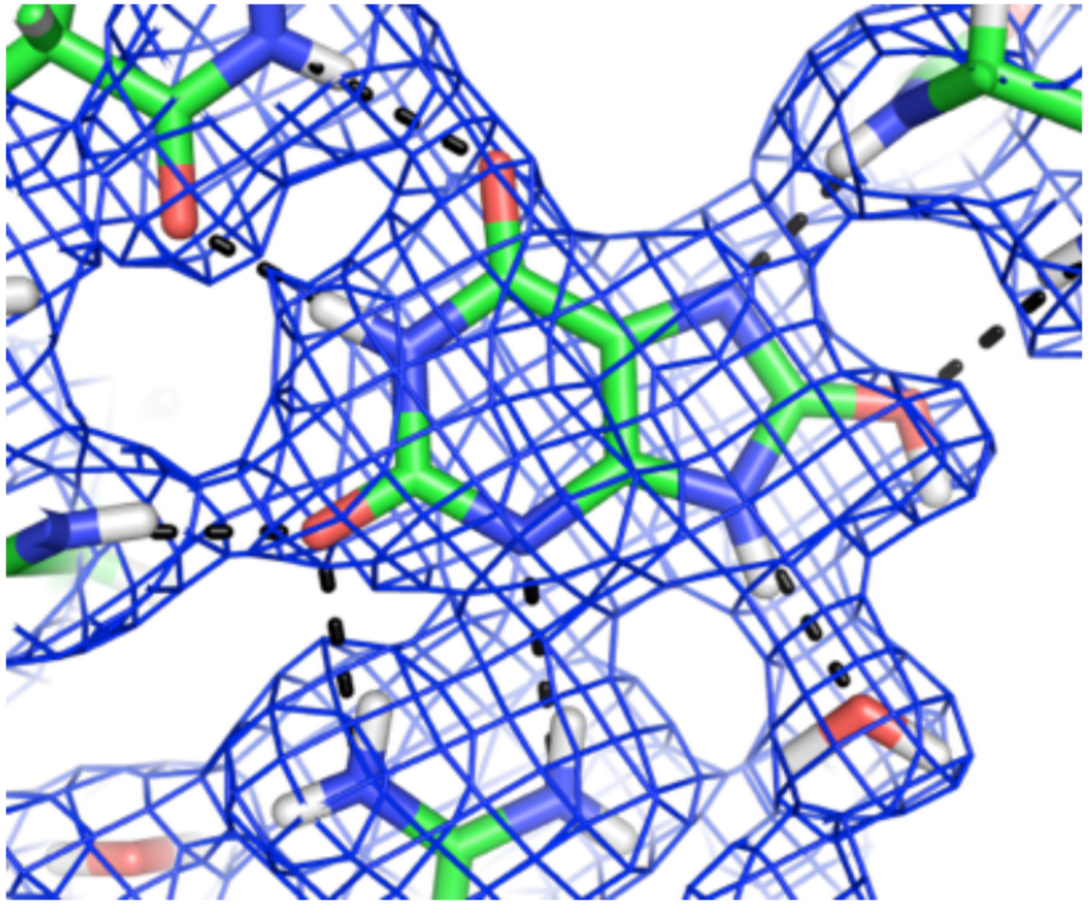
Map interpretation

X-ray 1.05 Å



Locate ‘heavy’ atoms

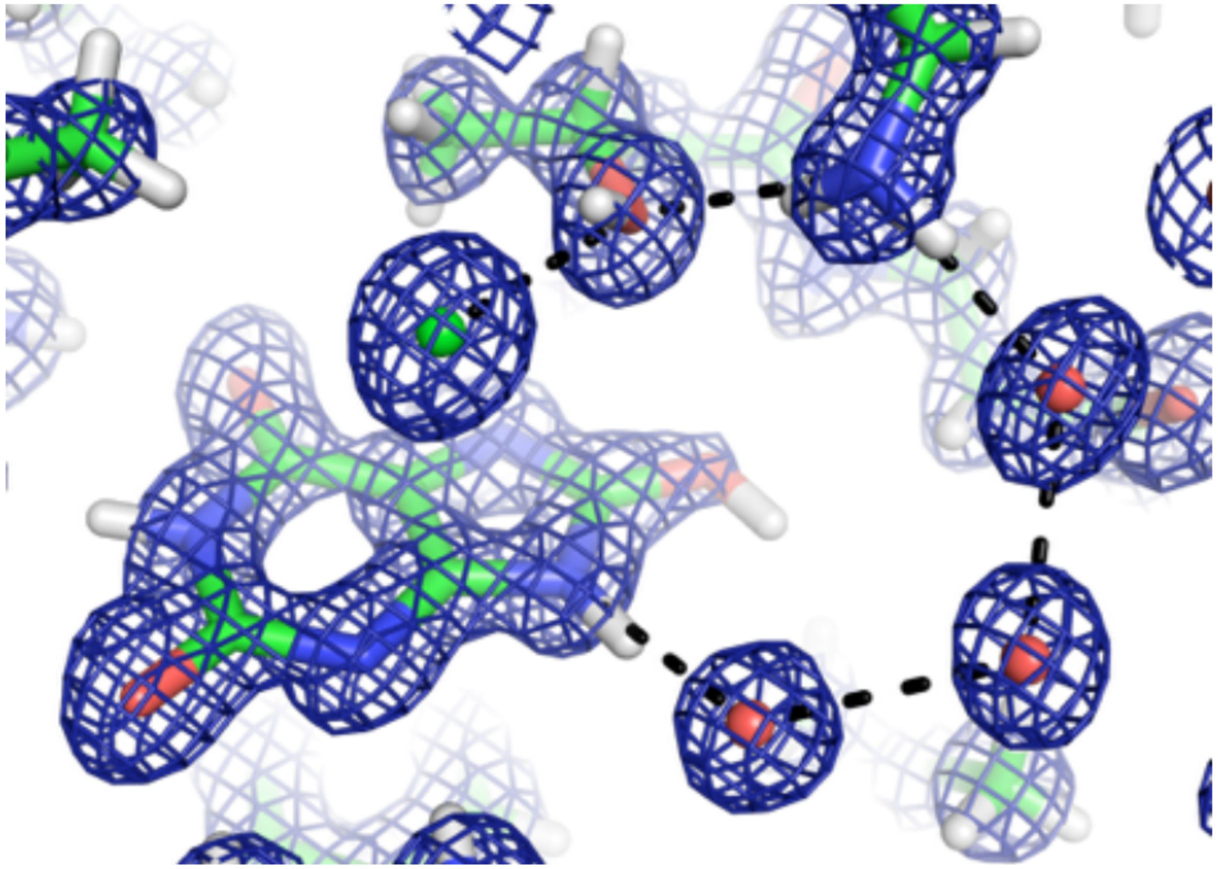
Neutrons 2.3 Å



Locate hydrogens

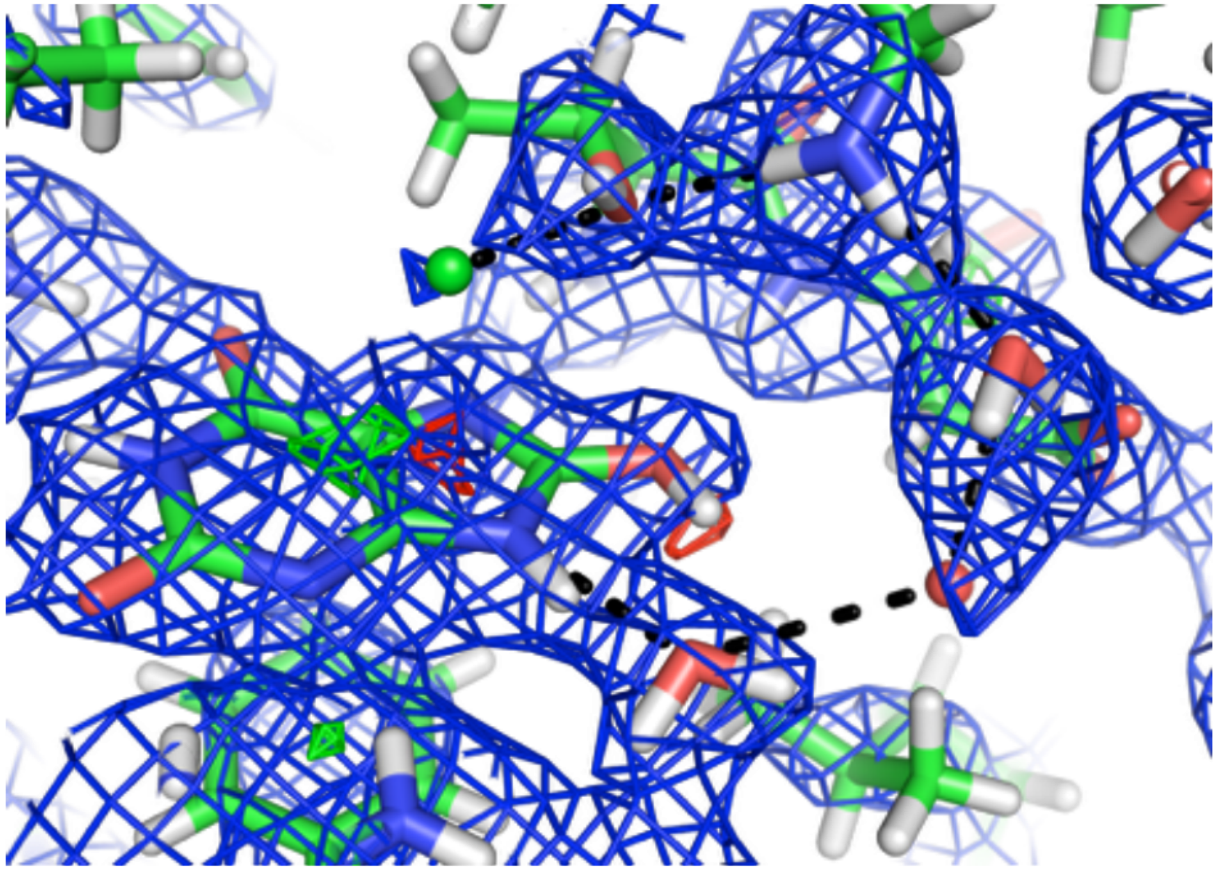
Map interpretation - water molecules

X-ray 1.05 Å



Locate ‘heavy’ atoms

Neutrons 2.3 Å



Locate hydrogens

Questions?

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