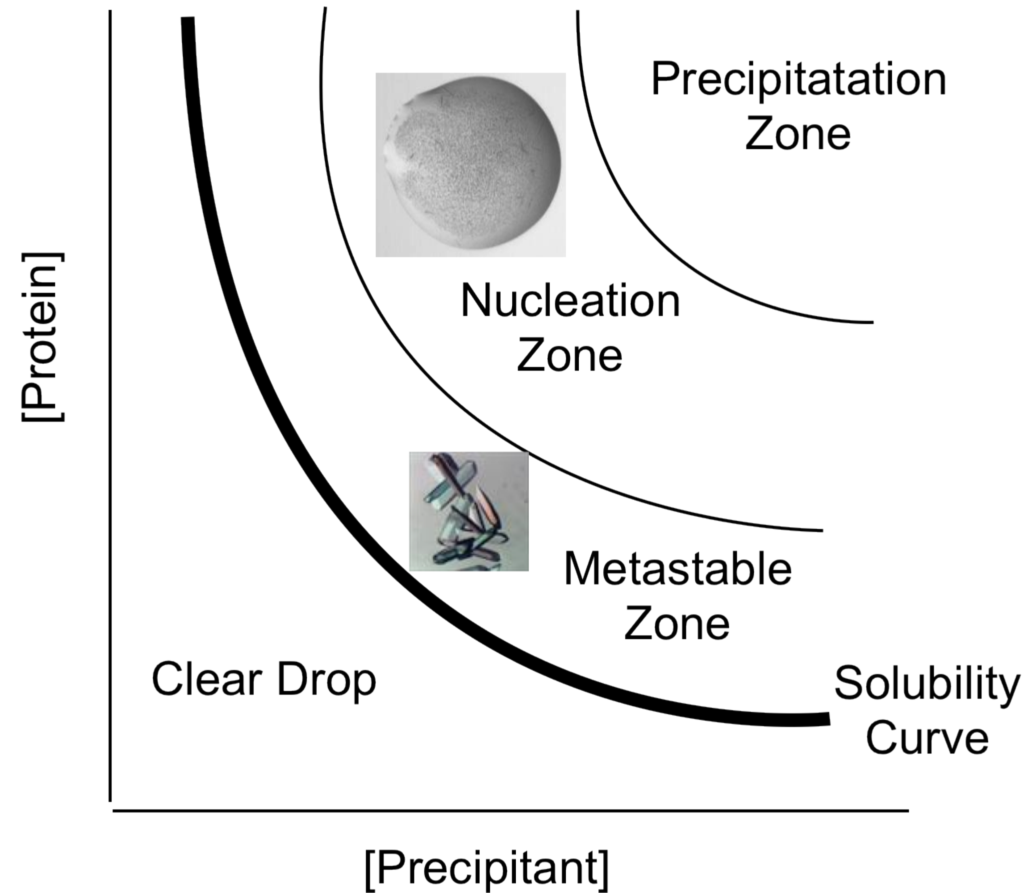
Synchrotron-based methods for diffraction, scattering and imaging

• Protein crystallization is achieved by slowly changing the solution conditions to precipitate the protein in an ordered fashion.



When is your protein ready to go into crystallization trials?

- Should be as pure as possible
  - Simple his-tag purification may not be sufficient
  - Overload your gel to check for minor contaminants
  - Consider polishing by gel filtration or ion exchange
- Check for activity where possible
- Spectroscopic checks
  - CD – folded?
  - UV/Vis, Fluorescence etc. – cofactor/ligand present?
- Dynamic light scattering or analytical ultracentrifugation
  - Checks for aggregation + homogeneity
- SAXS
  - Very sensitive to aggregation and inhomogeneity

What concentration to use?

- Pre-crystallization tests available – maybe useful sometimes

- Various rules of thumb
  - The highest concentration you can achieve without your protein crashing out or aggregating
  - The concentration at which it elutes off an ion exchange or affnity column
  - 10 mg/ml

- Arwen's rule of thumb
  - You know your protein best
  - The highest concentration your protein is comfortable at

- Note – if you set down a tray and all the drops precipitate (cloud over) immediately then check straight away on a microscope for microcrystals. If there are none, quickly open each drop and add additional crystallization solution to each droplet or to each well.

Where to start?

- Multiple commercial screens exist
  - All based on a factorial approach to cover a wide range of phase space
  - All will vary:
    - Salt
    - pH
    - Precipitant
  - Some will include additives

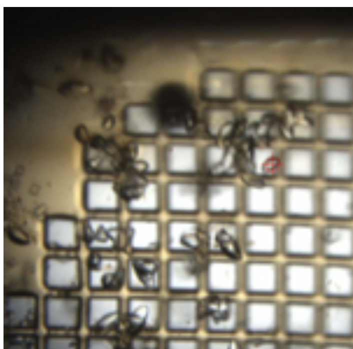
- How many trials you set is really dependent on how much material you have
  - Robotic crystallization now allows routine small drops (nl-ul)
  - Don’t feel you have to screen against everything available
    - Often a simple magic 50, or equivalent, will give you an idea of where your protein might crystallize
    - Structural genomics initiatives are now providing optimised screens backed up with huge amounts of data

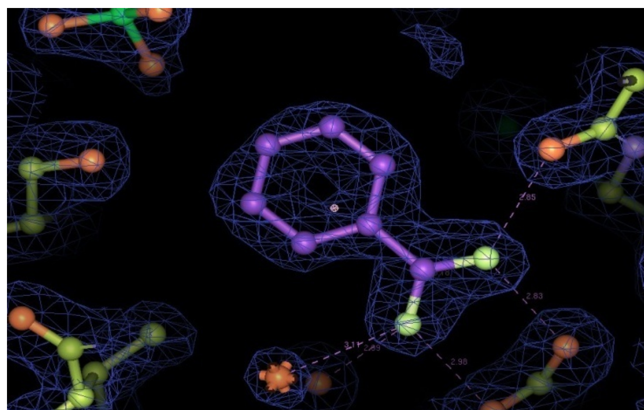
- Don’t forget to include temperature, DVR, [protein] as variables!

I have a hit! What next?

- Optimisation
  - Usually by grid screen
  - Can include additives
  - Exact strategy will depend on your hit
    - Lots of tiny crystals?
      - Consider dropping precipitant or protein concentration a little to reduce nucleation
    - Single small or ugly crystal
      - Consider seeding

- Always worth checking diffraction – with modern microbeams it's possible to get amazing data from teeny-tiny crystals!



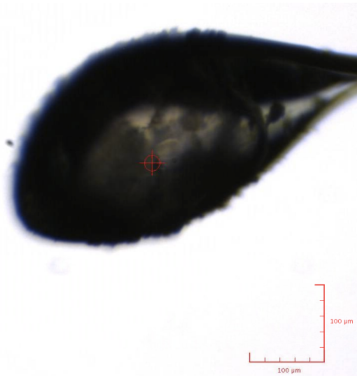


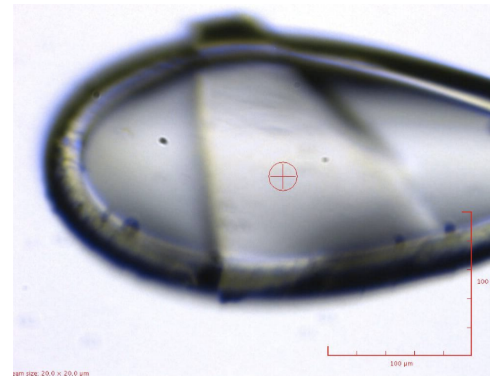
Resolution	47.4-1.65 (1.74-1.65)
Completeness	96.6 (96.5)
R_{merge}	0.084 (0.564)
$\langle I \rangle / \sigma \langle I \rangle$	12.9 (2.3)
Multiplicity	4.3 (4.2)
Rwork	0.20 (0.24)
Rfree	0.25 (0.34)

Cryo-cooling

- Why cryo-cool?
  - To minimise radiation damage
  - Hydroxyl radicals are not mobile at 100K
    - Note electrons still mobile
  - Considerable gain in "dose" lifetime (10-100x)
  - Makes sample shipping easier!
  - Reduces background scatter

- Must minimise ice-formation
  - Cool fast
  - Find solution additives that form a vitreous glass





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Cryo Protection: First thing to try?

do you have another one?
- no → get one
- yes → mother liquor > 30% cryo?
  - yes → loop up and cool
  - no → mother liquor component soluble in oil?
    - no → cool in oil
    - yes → mother liquor contains potential cryo?
      - yes → add more
      - no → add glycerol

Cryo testing

does it “freeze clear”?

vs.

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What can be used as a cryo-protectant?

Anything organic ~30%
- Glycerol
- Ethylene glycol
- Sorbitol
- sugars
- trehalose (>50%)
- low-MW PEGs (100 – 400)
- MPD
- Alcohols

Salts
- Malonate
- Formate
- Li_2SO_4
- $(NH_4)_2SO_4$ (> 3.5 M)

Oils
- Paratone-N
- Fomblin
- Silicone
- Parafin
- ….

high-MW PEGs

Can also use physical processes
- Speed
  - hyperquenching
- Pressure

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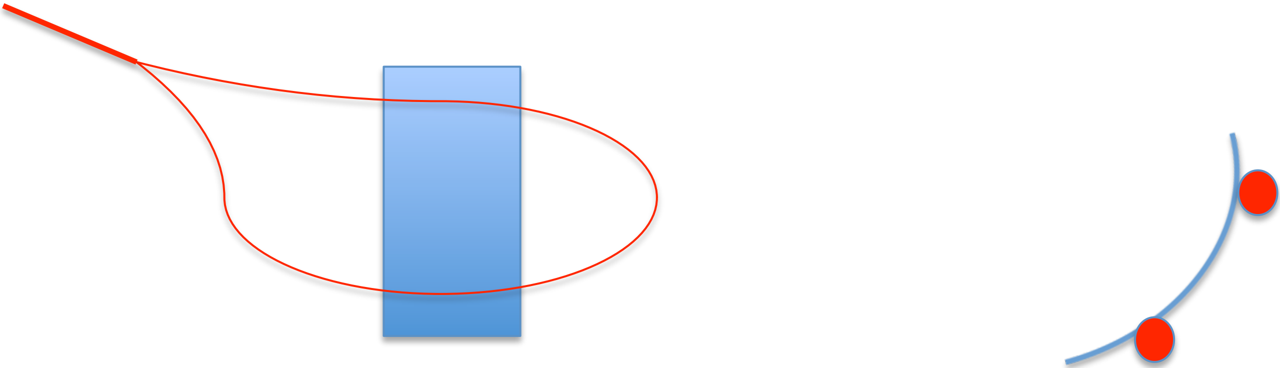
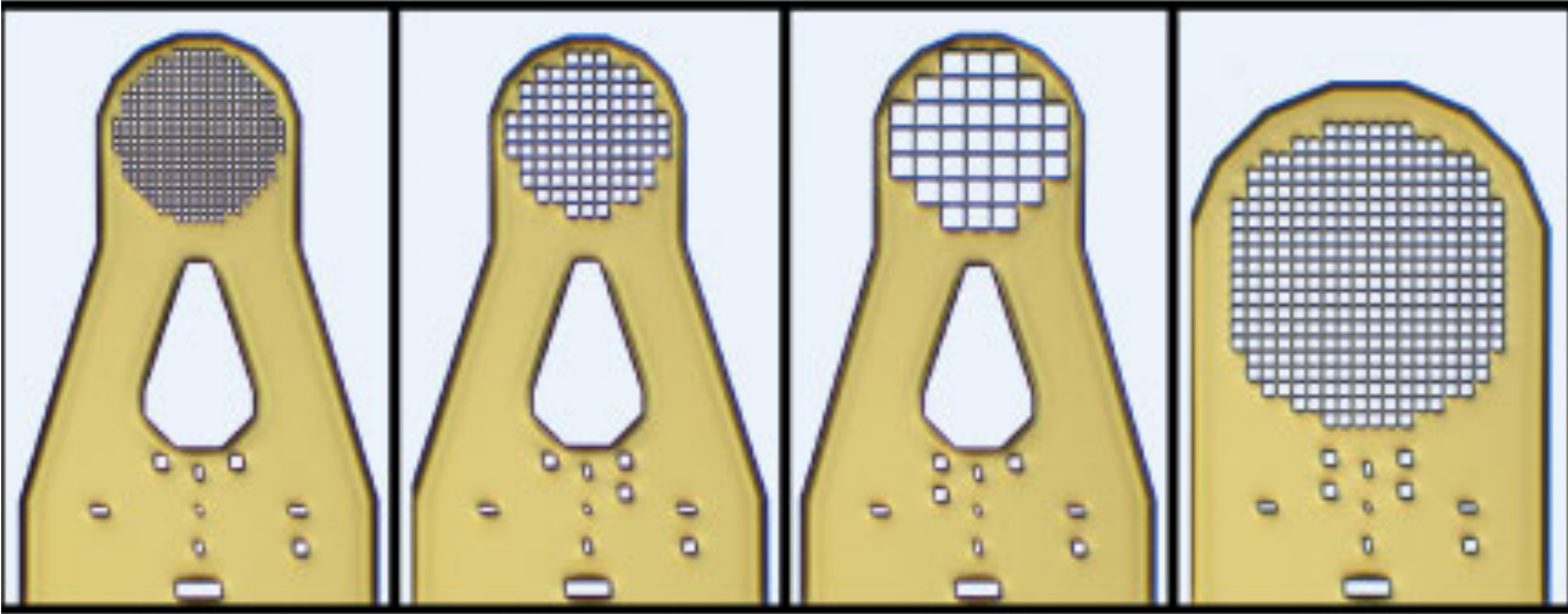
Consideration for membrane proteins that are needles or plates





Mitegen mesh mounts

How to collect your diffraction data

- Depends very much on the question you are interested in
- Direct Fourier (i.e a Ligand complex of a known structure)
  - Don't need masses of redundancy
  - Can get away with not brilliant completeness
  - Resolution needed depends on the question
    - Just approximate position?
    - Detailed information on binding mode and bonding?
  - Note: some ligands may be radiation labile!

How to collect your diffraction data

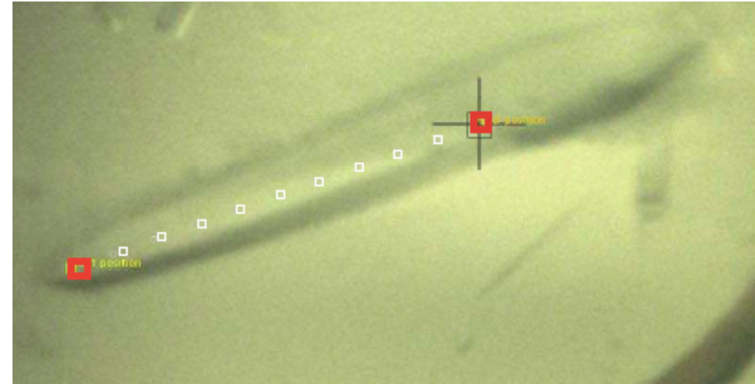
- Depends very much on the question you are interested in
- Molecular replacement
  - Low resolution data should be good (give information about solvent boundary)
  - Completeness should be good
  - Ideally good resolution, but ease of solution will depend on the quality of the model

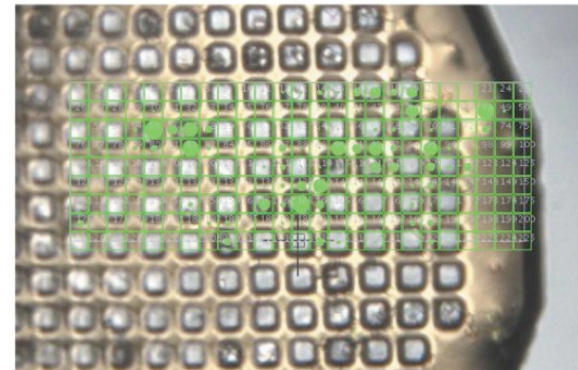
How to collect your diffraction data

- Depends very much on the question you are interested in
- Experimental Phasing
  - Low resolution data should be good (give information about solvent boundary)
  - Completeness should be good
  - Ideally good resolution, but signal often fades at high resolution
  - Avoid radiation damage!
    - You're looking for tiny changes in F!

How to collect your diffraction data

- Make use of the beamline!
  - Microbeams
  - Helical scans
  - Grid scans
  - Humidifier/Cryo
  - In plate diffraction
  - Fast detectors – OK to screen!





I24, Diamond

- Scaling and merging

- Examining the merging statistics by batch (image) can identify poorly merging images

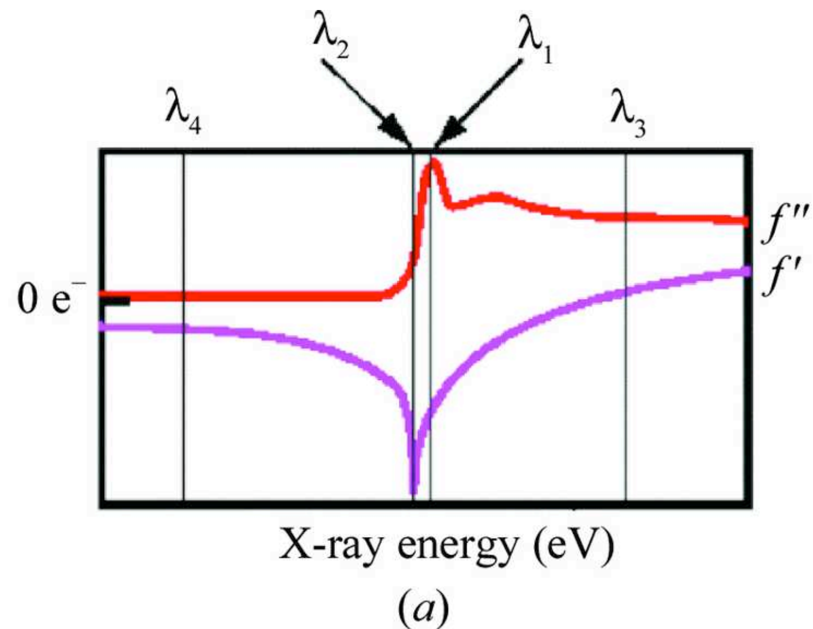
    - Ice rings
    - Radiation damage
    - Poorly diffracting region of the crystal
    - Crystal slippage

- Removing these can make a HUGE difference to your dataset quality

- Different programmes provide this information in different ways – it is always worth checking!

- Practical experimental phasing
- High redundancy is good
- Radiation damage is usually bad
- Precise measurements are vital
- Can be useful to do an X-ray fluorescence spectrum to be sure your heavy atom scatterers are in the crystal!

- Practical experimental phasing
- Collect peak data first with at least multiplicity = 4
- If Radiation damage apparent
  - Stop and try SAD phasing
  - If crystal is big enough move to a new bit
- If no damage – measure high energy remote
- Then measure inflection point (so f' is maximised)



- Practical experimental phasing
- Collect peak data first with at least multiplicity = 4
- If Radiation damage apparent
  - Stop and try SAD phasing
  - If crystal is big enough move to a new bit
- If no damage – measure high energy remote
- Then measure inflection point (so f' is maximised)
- General data quality should be good
  - Check your scaling statistics!
- Can use correlation coefficients or midslope to determine if you have anomalous signal present

Small Angle X-ray Scattering (SAXS)

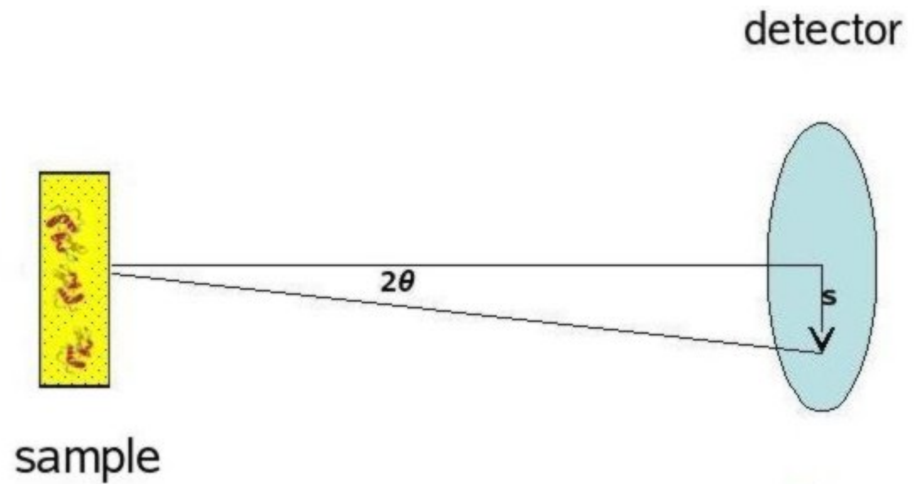
Four main uses:

1. Characterization
2. Hypothesis generating or testing
3. Constraining other data
4. Generating a molecular envelope

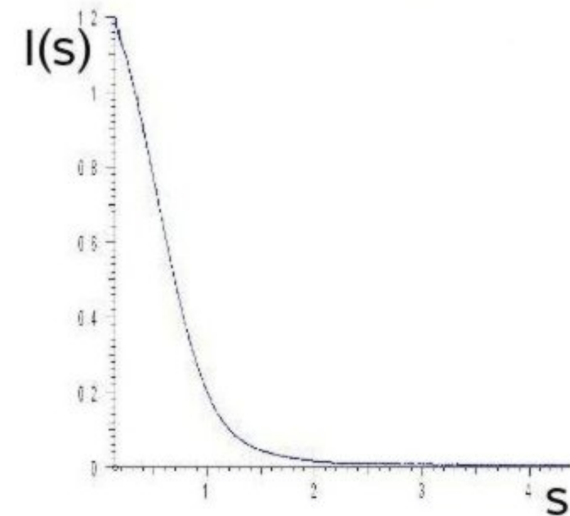
All of which need good data.

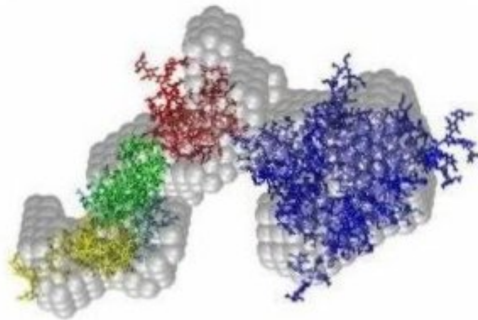
However, unlike crystallography, bad data can still give a result.

Monochromatic beam



Radiation sources:
- X-ray generator (λ=0.1 – 0.2 nm)
- Storage ring (λ=0.03 – 0.4 nm)
- Neutron reactor (λ=0.1 – 1 nm)





Basic Assumptions in analyzing SAXS data

- A single species exists in solution
- The sample minus the buffer equals the buffer
- That there is no inter-particle interaction
- That there is no radiation damage

SAXS is *deceptively* simple; the self deception comes with failure to achieve these criteria

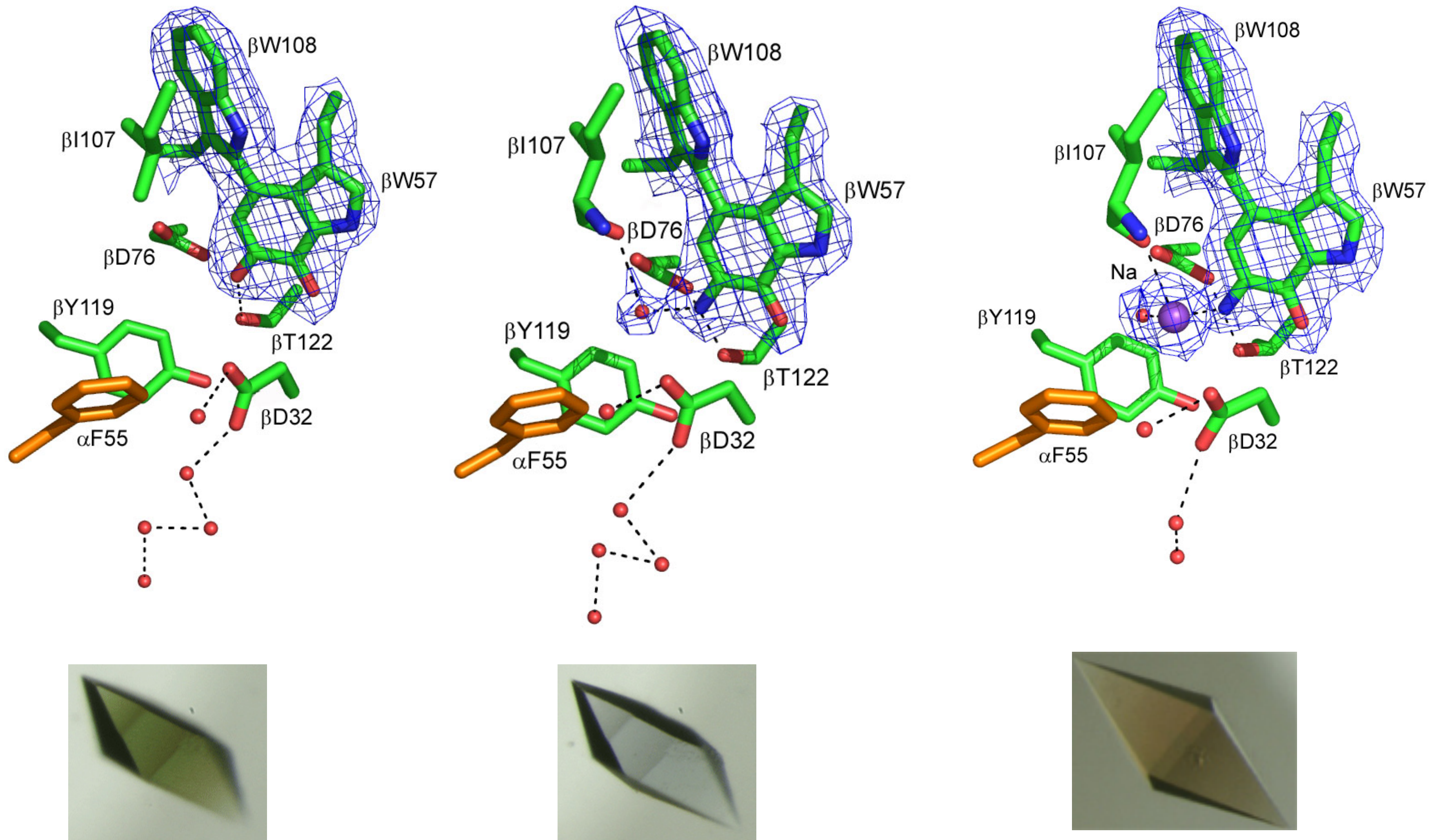
Cryo-X-ray microscopy

- Works just like electron microscopy
- Greater penetration depth means you can image whole cells (~10 μm thick samples)
- Uses soft X-rays (~510 eV) in the water window between carbon and oxygen
- Cells need to be vitrified (as for EM)
- Resolution ~ 40 nm

Trapping methodologies can be used to determine the structures of intermediates

- Mechanistic trapping
    - Alter reaction conditions to prevent full turnover
    - Use mutants to prevent full turnover
    - Use altered substrates to prevent full turnover
    - Drive the system into steady state

Isosteric but chemically distinct structures can often not be distinguished



- Single crystal optical spectroscopy

UV-visible & fluorescence

- quick ✓
- must have a reporter chromopore ✗
- relatively easy to assign spectral features ✓

- (resonance) Raman
  - don't need a chromophore ✓
  - complex spectrum ✗
  - can be quick if you know what feature you need to follow ✓

- IR, fluorescence, fluorescence life-time

Cryostream

Sample alignment microscope

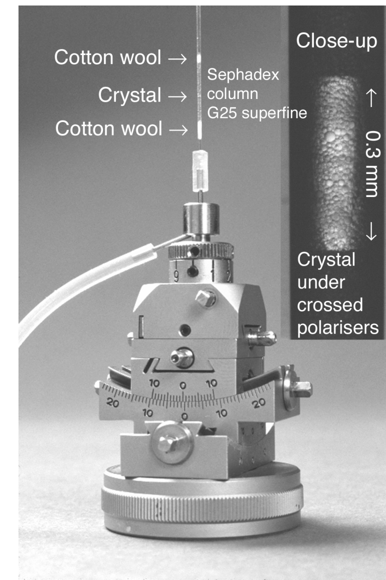
Sample position

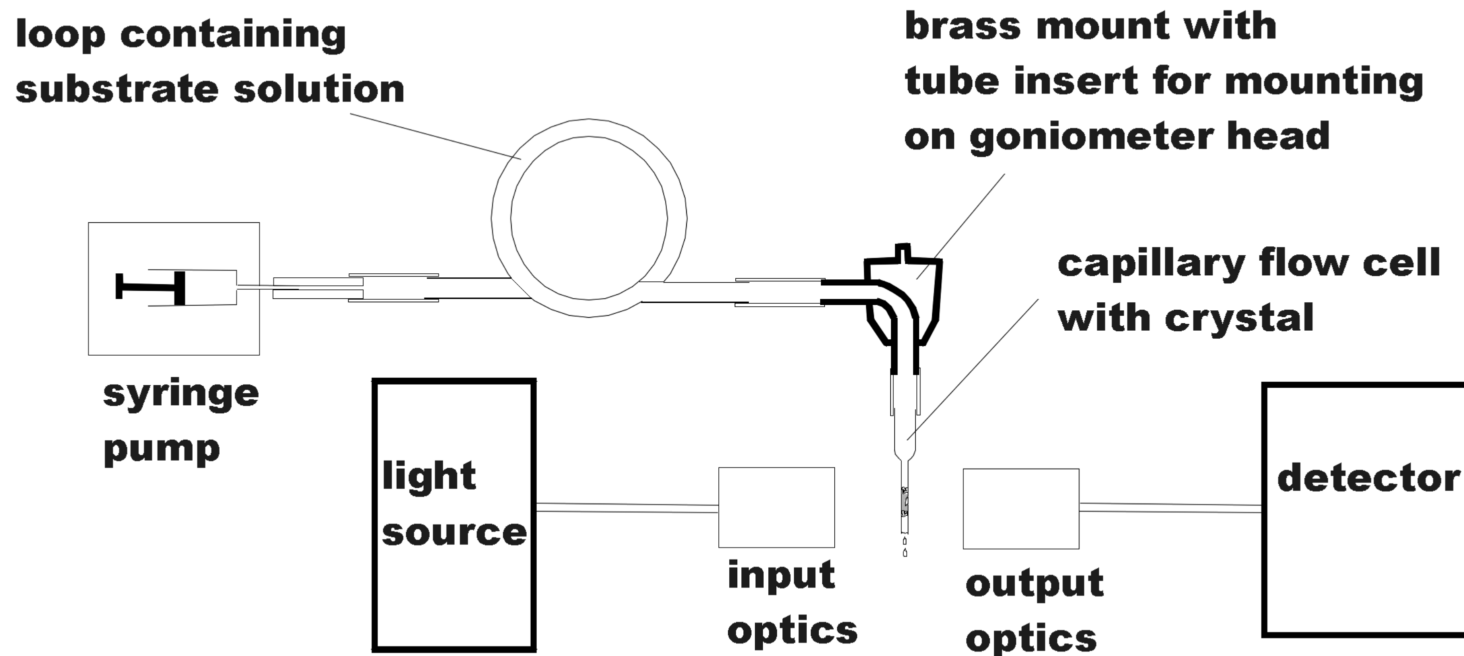
Focusing optics

To spectrograph

White light in

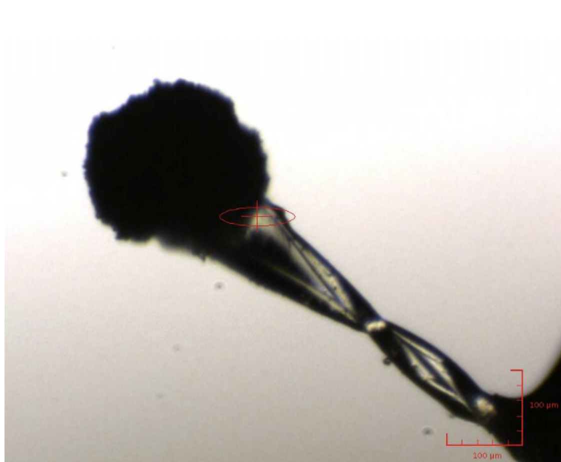
- Kinetics in the crystal are not always equivalent to solution
- Often major differences in rate
- Sometimes differences in equilibrium
- Off-pathway intermediates may form
- Define conditions in which desired intermediate accumulates

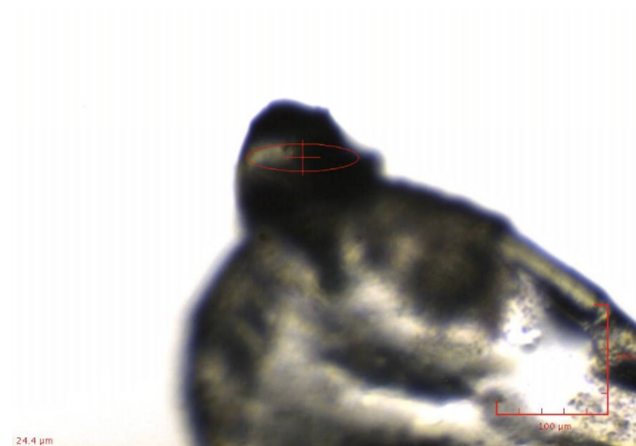




Tips for good quality spectra

- Minimise solvent around the crystal
- Optimised cryoprotection is critical!
  - Avoid glycerol if looking at features around 500-700nm
  - Ice reduces transparency and scatters light
  - Can try and wash ice off, or use a different bit of crystal





Tips for good quality spectra

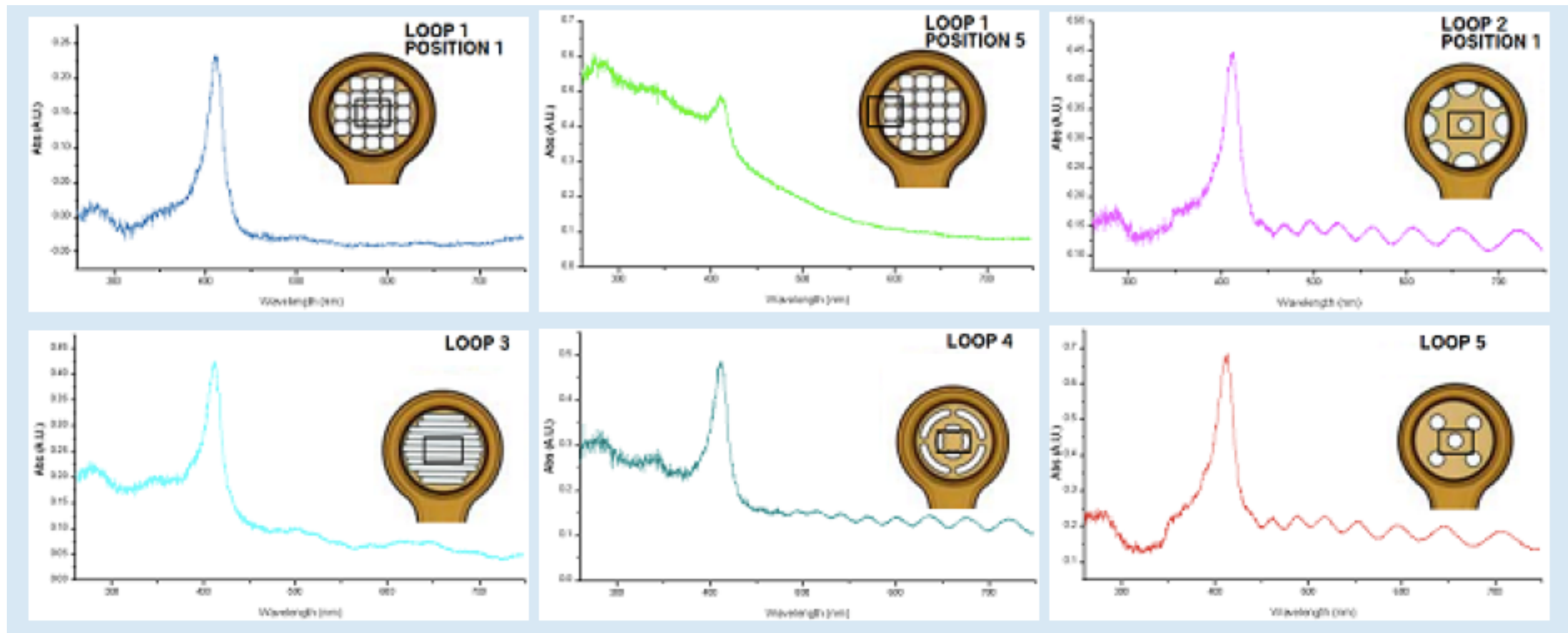
- Try different parts of the crystal for the “best” spectrum
- Use a loop that is big enough to fit the focal spot of the light source
    - Ideal to have a loop big enough to be able to take reference off the mother liquor
    - Useful for high optical density samples

Tips for good quality spectra

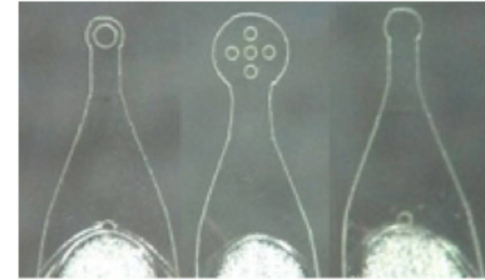
- Dealing with high optical density samples
  - Smaller crystals are better
  - If you can’t see through it neither can the spectrometer!
  - Try and find the thinnest part (battle here with best bit for XRD)
  - Bandpass filters can allow you to increase exposure time without saturating the detector

Tips for good quality spectra

- Mesh Mounts
  - best are the simple meshes, not more featured options



Tips for good quality spectra



- Mesh Mounts
  - UV-vis transparent meshes also available

Mitegen's UV-Clear™ Loops

280 nm

Standard Polyimide Loops

Optical Transmission (%)

100
80
60
40
20
0

150 300 450 600 750 900

Wavelength (nm)

- If you have a redox centre (or other electrophilic site) in radiation induced changes should be a consideration

- Radiation induced redox change is FAST

- Several instruments/spectroscopies now available in-house and at synchrotrons to investigate

- If you have a radiation problem may need to compromise your resolution by attenuating, shorter exposures etc.

- "Old School" composite datasets may be the best way forward

- Changing wavelength does not make a useful difference in reduction rate

- Avoid high Z atoms in crystal stabilising solution may slow reduction (under further investigation)

Useful reading

“Protein Crystallography for non-crystallographers, or how to get the best (but not more) from published macromolecular structures”Wlodaweret al., FEBS Journal 275 1-121 (2008)

"Combining X-ray crystallography and single crystal spectroscopy to probe enzyme mechanisms" Pearson, & Owen, (2009) *Biochem Soc Trans* **37** 378-381

“Defining redox-state of X-ray crystal structures by single crystal ultraviolet-visible microspectrophotometry” Wilmot et al., (2002) Methods in Enzymology 355 301-318

“Cryocooling and radiation damage in macromolecular crystallography” Garman & Owen (2006) Acta Cryst. D 62 32-47

“Radiation damage in macromolecular crystallography: what is it and why should we care?” Garman (2010) Acta D. 66 339-351

“Biological applications of cryo-soft X-ray tomography” Duke et al., Journal of Microscopy (2014) 255 65-70

“Small angle X-ray scattering as a complementary tool for high throughput structural studies” Grant et al., 2011 Biopolymers 95 517-530

CCP4 study weekend proceedings! www.ccp4.ac.uk/ccp4course.php