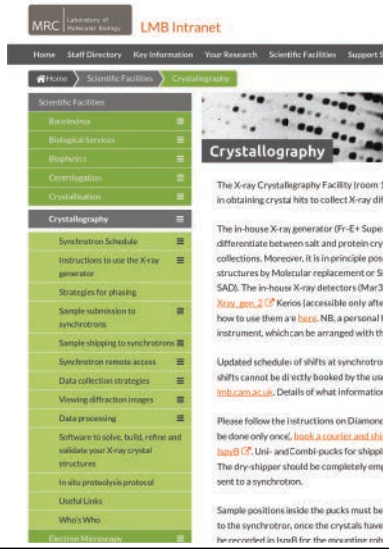
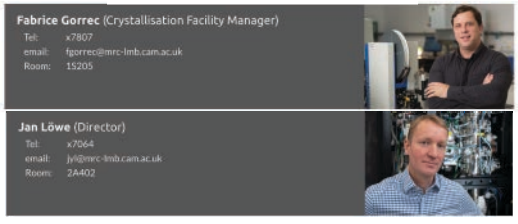
X-ray crystallography facility at the LMB

Room 1S205

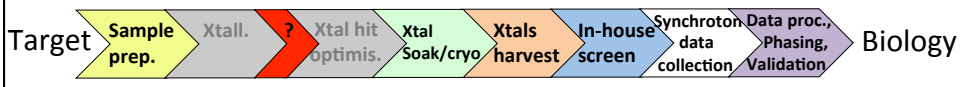


1S205 lab is shared between Crystallization and Crystallography facilities

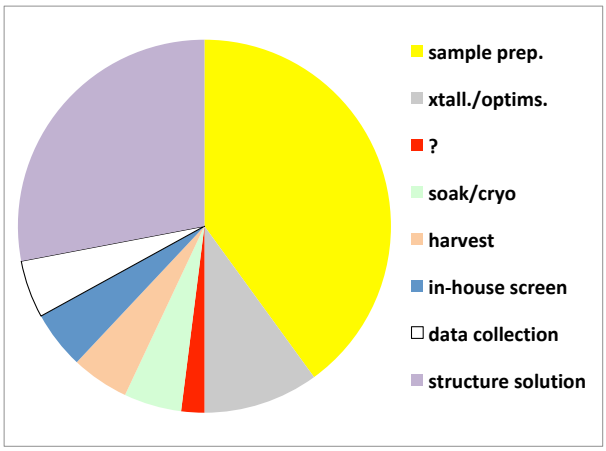


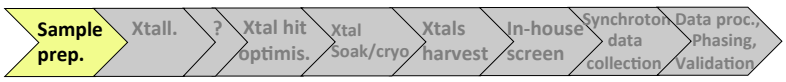
"talk to us" = Dom and Fabrice

Crystallographic project workflow



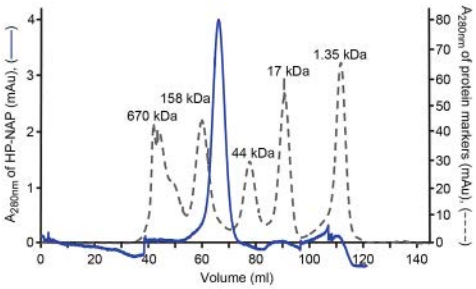
Typical time consumption



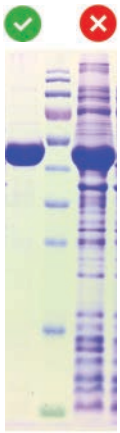


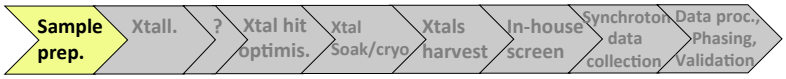
Sample quality: what to aim for?

Gel filtration (SEC-MALLS)



SDS-PAGE gel





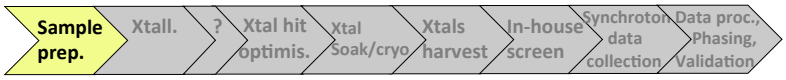
Two crystallization key factors of protein samples: solubility and stability

Proteins, unlike DNA, have a large variation in both structural and physicochemical properties, which in turn will affect **solubility** and **stability** (key factors in crystallization).

These two key factors are defined in the "crystallography urban dictionary" as follows:

- **Soluble sample** = a single peak in gel filtration chromatograms that can be concentrated to at least around 5-10 mg/ml
- **Stable sample** = a 5-10 mg/ml soluble sample that produces around 50% clear drops in a crystallization screening experiment

If the ratio of clear/precipitation drops differs significantly from 50:50 either way, you can discuss with us what to do next



How to obtain soluble and stable samples

- Skills
- Experience
- Creativity

Skills = lab skills/tricks in working with proteins (e.g., construct design, different overexpression cell strains, different inductions levels or growth temperatures of cultures, different tags for purification, …), which can make the difference between obtaining soluble protein rather than inclusion bodies.

Experience = literature knowledge of specific protein families and insights into stabilising conditions, such as right detergent/chemicals, salt concentrations, phosphorylation state, cofactors (e.g., small molecule, peptide, protein-protein interactions, …), …

Once you decide on a target to crystallize, you can come to talk to us for advice on best strategies and/or we can suggest who in the LMB may have the expertise to help you

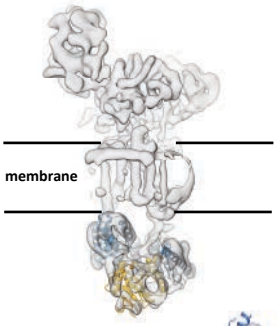
Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → Data proc., Phasing, Validation

Creativity = your talent

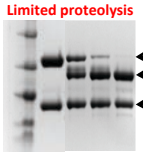
Example 1: Lowe's group

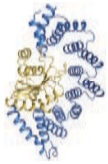
Crystallized monomer (2.1 Å)(unpublished)

Example 2: Hegde's group



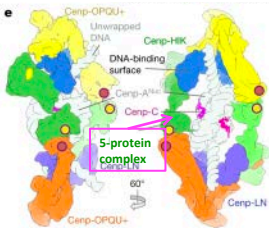
Limited proteolysis



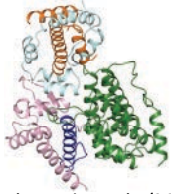


Crystallized P1*-P2 complex (2.2 Å) (unpublished)

Example 3: Barford's group



multiple sequence alignment and secondary structure predictions



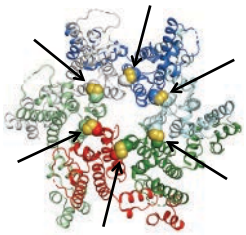
Crystallized 5-protein complex (2.9 Å) (unpublished)

Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → Data proc., Phasing, Validation

Creativity = your talent

Example 4: James' group

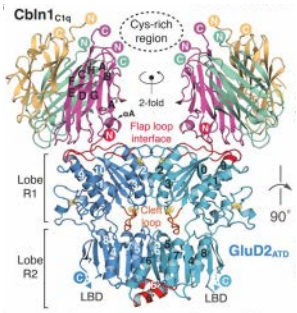
Stabilization of HIV hexameric capsid by engineered disulfide bridges for crystallization



Price *et al.*, 2009 (NSMB)

Example 5: Aricescu's group

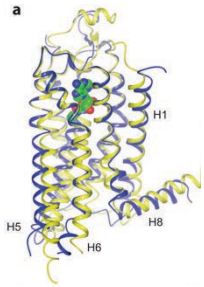
Tethered construct to crystallize a protein complex connecting pre- and post-synaptic axons



Elegheert *et al.*, 2016 (Science)

Example 6: Tate's labs

Thermostabilization of membrane proteins by systematic mutagenesis (6-12 months!)



Magnani *et al.*, 2016 (Nat. protocols)

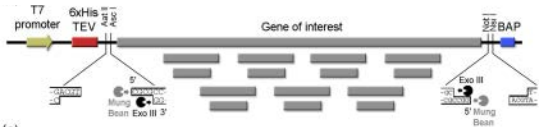
Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | Xtals harvest | In-house screen | Synchroton data collection | Data proc., Phasing, Validation

Creativity = your talent

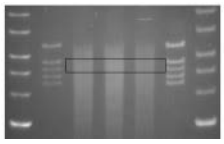
Example 7: ESRF's labs

ESPRIT (Expression of Soluble Proteins by Random Incremental Truncation)

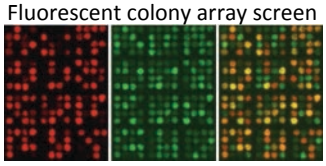
Gene bidirectional digestion



Library size selection



Fluorescent colony array screen

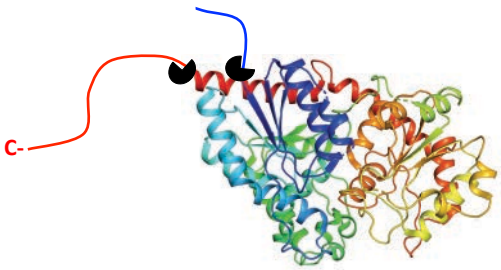


Up to 30,000 constructs can be screened in a few weeks

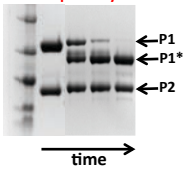
ESPRIT helps to identify soluble expression constructs in difficult cases with no useful sequence homology to other proteins (e.g., the high valuable target influenza virus polymerase subunit PB2, Guilligay *et al*., 2008, NSMB)

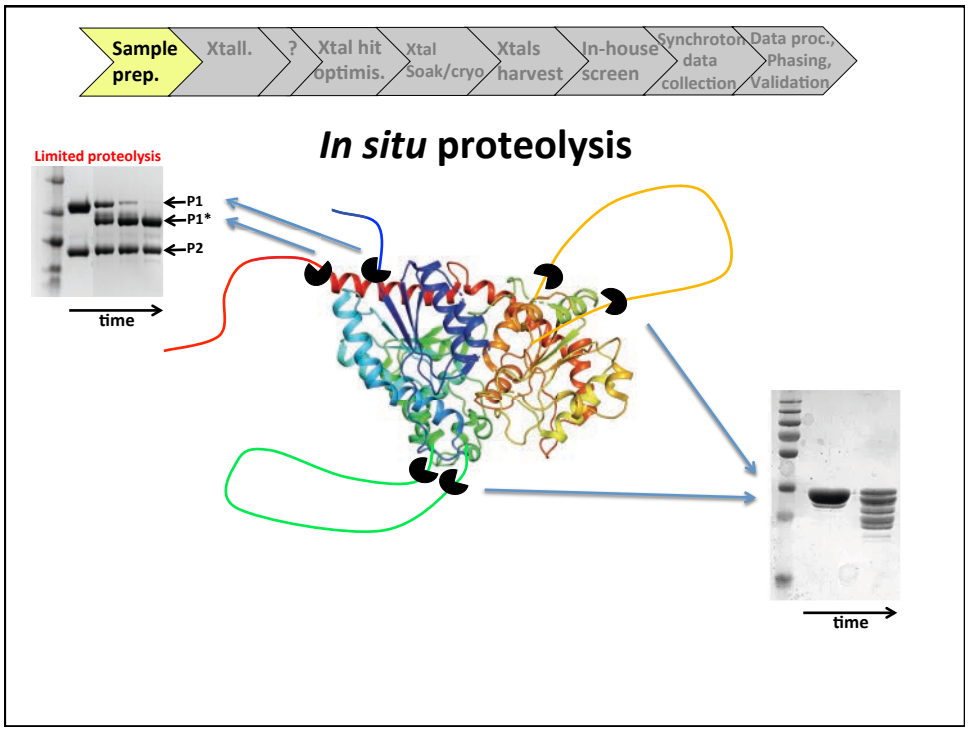
Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | Xtals harvest | In-house screen | Synchroton data collection | Data proc., Phasing, Validation

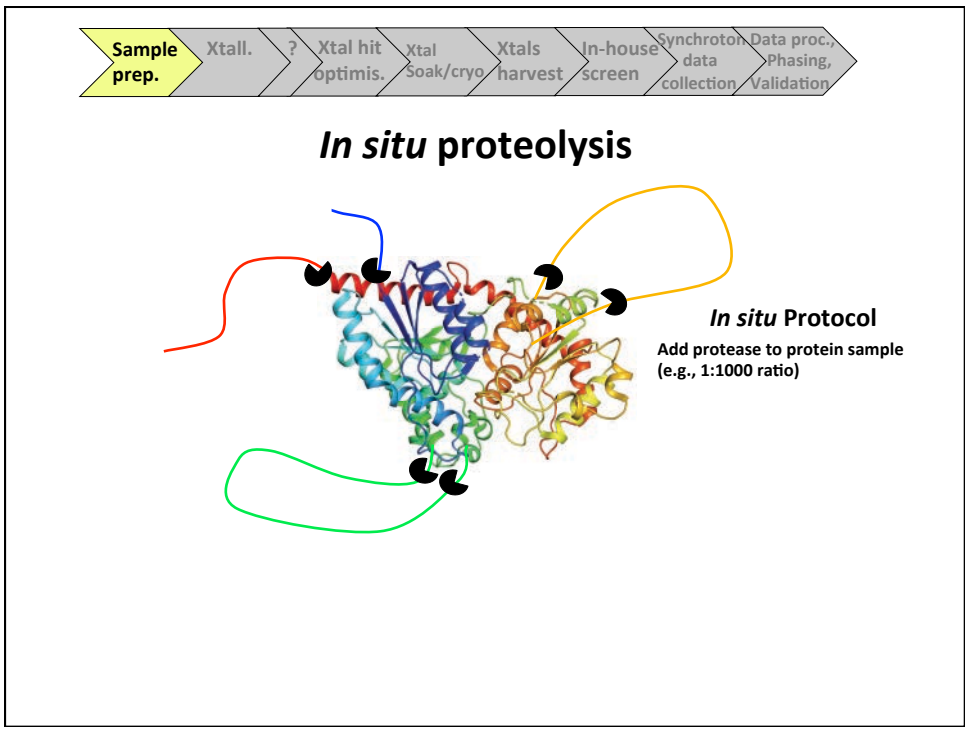
In situ proteolysis

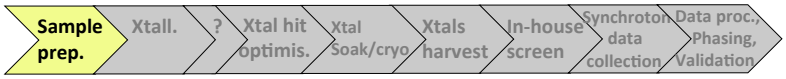


Limited proteolysis

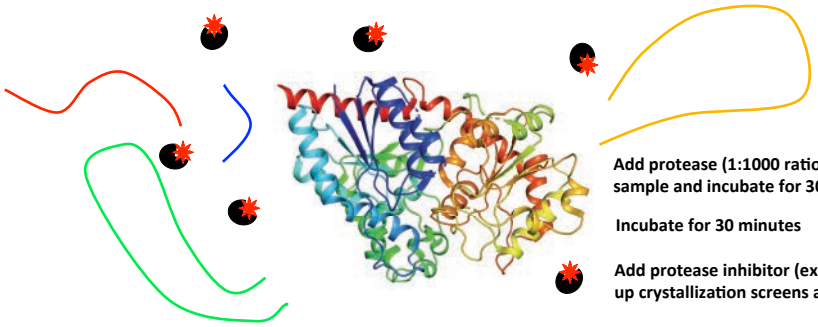



Sample prep.
Xtall.
?
Xtal hit optimis.
Xtal Soak/cryo
Xtals harvest
In-house screen
Synchroton data collection
Data proc., Phasing, Validation
In situ proteolysis
Limited proteolysis
P1
P1*
P2
time
time


Sample prep.
Xtall.
?
Xtal hit optimis.
Xtal Soak/cryo
Xtals harvest
In-house screen
Synchroton data collection
Data proc., Phasing, Validation
In situ proteolysis
In situ Protocol
Add protease to protein sample
(e.g., 1:1000 ratio)



In situ proteolysis

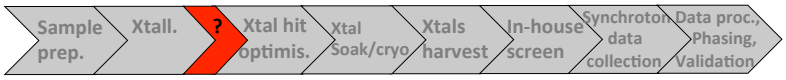


Add protease (1:1000 ratio) to protein sample and incubate for 30 minutes

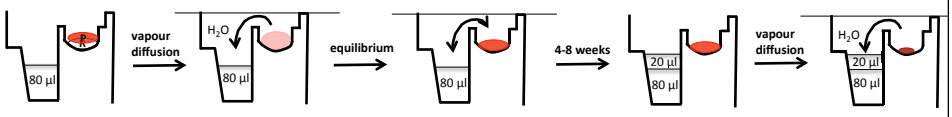
Incubate for 30 minutes

Add protease inhibitor (excess) and set up crystallization screens as usual

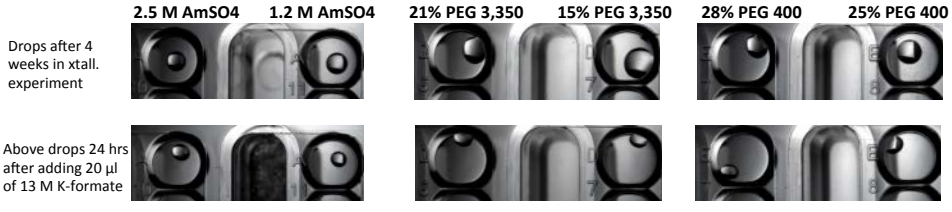
- *In situ* proteolysis can be performed at the X-ray facility with 3 available proteases to test: trypsin, chymotrypsin and subtilisin
- The protocol (quick and easy) is available on the facility internal webpage
- If you would like help with this you can talk to us



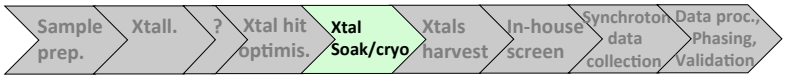
Dehydration of clear drops to promote crystallization in old plates



10-20 µl of 13 M K-formate can shrink drops from all different conditions in xtall. plates:



- Drop dehydration can be performed at the X-ray facility with concentrated K-formate solutions readily available
- The protocol (quick and easy) is available on the facility internal webpage
- If you would like help with this you can talk to us



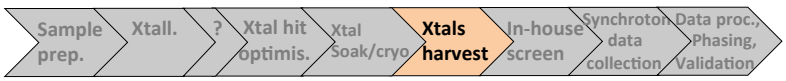
Crystal soaking and cryoprotection are underestimated obstacles that lie between crystallization and crystallography.
Difficulties in soaking and cryoprotection can vary greatly depending on a number of factors:

Crystal robustness
- anything will work with some really robust crystals
- others (majority) will require systematic screening of many cryoprotectants and/or ligand concentrations at different time courses

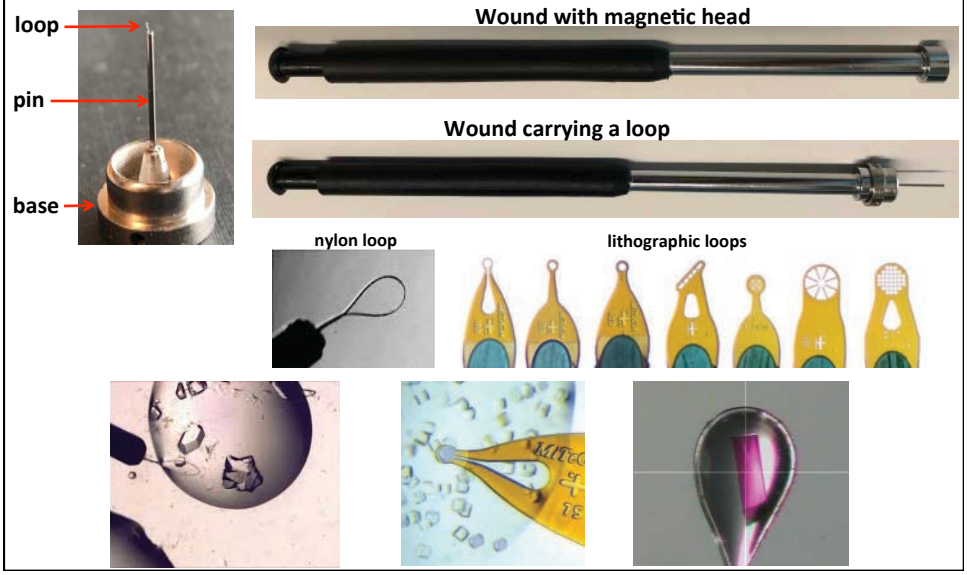
Ligand solubility
- the ideal ligand is highly soluble in aqueous solutions (however, the ligand should always be solved in a crystal compatible solution)
- poor solubility requires experimenting with chemicals/conditions suitable for both ligand and crystal (it can range from very easy to very hard to impossible)

Heavy atom soaks (for *ab initio* phasing)
- many different strategies and too long topic for this talk
- some suggestions/protocols are available on the X-ray facility webpage

Different levels of expertise can be required to optimise the above procedures depending on the case.
Once you have optimised your crystal hits, you can talk to us for advices on how to prepare the crystals for data collection.

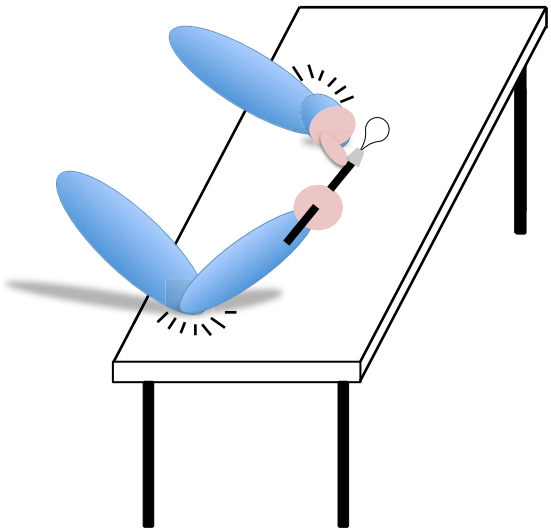


Crystal fishing



Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | **Xtals harvest** | In-house screen | Synchroton data collection | Data proc., Phasing, Validation

Avoid sudden acceleration while fishing crystals

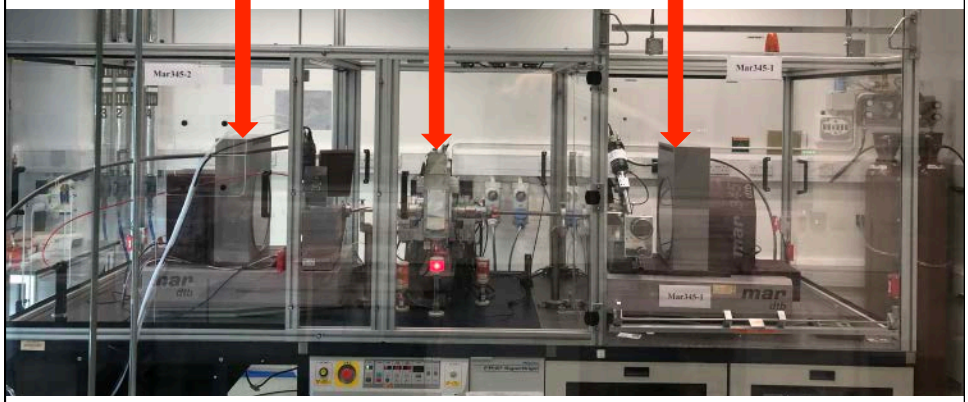


Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | Xtals harvest | **In-house screen** | Synchroton data collection | Data proc., Phasing, Validation

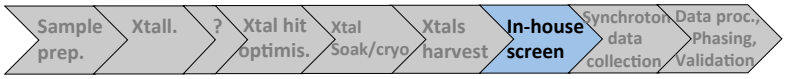
Rigaku FrE+ X-ray generator using a Cu rotating anode

Mar345dtb image plate

Mar345dtb image plate



- Quick training session on technical and safety issues required before using the instrument (email me to arrange training)
- Access to online booking system (Kerio account) is granted after training
- Instructions on how to use it available both on site and on the X-ray facility webpage



How to take advantage of an in-house X-ray generator

1 − Quickly discriminate between protein crystal hits and false positives (e.g., salt crystals)

- **LV cryo-oil** (i.e., universal cryoprotectant) available at the X-ray facility

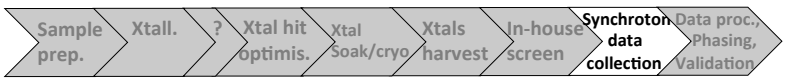
2 − Optimise cryoprotection conditions (checking for ice rings and/or loss of diffraction)

3 − Minimise the time you have to spend to collect at synchrotrons (limited time available and unsocial working hours often at week-ends)

4 − Structures of large well diffracting crystals can be solved in-house (5-20 minutes per frame = 15-60 hrs for 180 images)

5 − Select the best crystals for experiments at low throughput beamlines (e.g., longwavelength beamline, I23, at Diamond)

6 − If there is no imminent synchrotron beamtime available (e.g., maintenance shutdown or overbooking), ligand or heavy atom soaks can be optimised in-house by checking different time courses and ligand concentrations



Diamond (Oxford, UK)

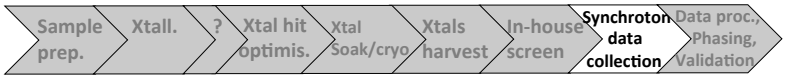
ESRF (Grenoble, FR)

MX beamlines:
I03 − tunable
I04 & **I24** − tunable microfocus
I04-1 − fixed wavelength at Se K-edge
I23 − long wavelength (S-SAD and light atoms, K, Ca, Cl and P)
VMXi − fixed wavelength *in-situ* data collection
VMXm − tunable nanofocus
B21 − Small Angle X-ray Scattering (SAXS)

MX beamlines:
ID23-1, ID29 & **ID30B** − tunable
ID23-2 & **ID30A** − fixed wavelength microfocus
MASSIF-1 − fully automatic data collection
BM29 − Small Angle X-ray Scattering (SAXS)

The schedule for synchrotron trips/sessions can be found on the LMB crystallography webpage.

To request time for experiments at synchrotrons please email to: xraymgr@mrc-lmb.cam.ac.uk



Two data collection options at synchrotons

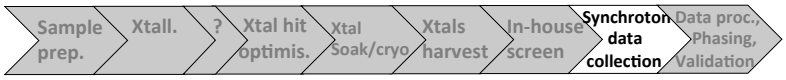
1 – travelling to the sites

Beamline ID30B, ESRF

2 – remote data collection

Remote workstations at the X-ray facility

- The X-ray facility can help with data collection both remotely and at synchrotrons (please contact me if you need help with your data collection)
- Remote collection requires shipping samples by courier to synchrotrons and instructions to do it can be found on the crystallography webpage (if you are not sure about the instructions for preparing shipping airway bills please contact me)



Data collection strategies

A few basic concepts of crystallography

- Theory of crystallography is outside the scope of this talk; moreover, it is not essential to carry out experiments at the LMB facilities

Schools:

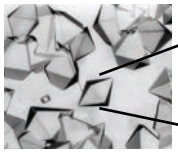
- Cold Spring Harbor (New York, USA) – Crystallography course (2 weeks during autumn)

Books:

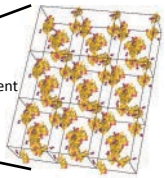
- Outline of crystallography for biologists (David Blow)
- Protein Crystallography (Blundell & Johnson)
- Biomolecular Crystallography (Bernhard Rupp)
- Structure determination by X-ray crystallography (Ladd and Palmer) (Chemistry level)
- Elements of Modern X-ray physics (Als-Nielsen & McMorrow) (Physics level)
- Fabrice and I are always happy to discuss any issue/question you may have about crystallography

Crystals

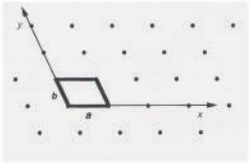
Protein crystals



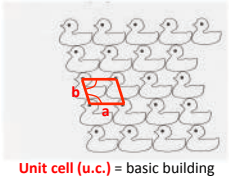
crystalline arrangement



A crystalline arrangement is defined by a **lattice**, which is an infinite array of points with identical environment

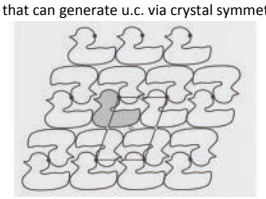


Associating a molecule with every lattice point in the same way = **Crystal**

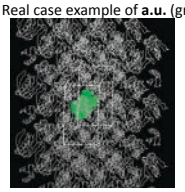


Unit cell (u.c.) = basic building (repeat) block of a crystal

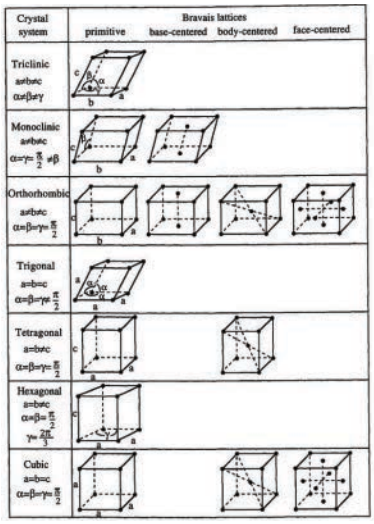
asymmetric unit (a.u.) = smallest unit that can generate u.c. via crystal symmetry



Real case example of **a.u.** (green)



14 Bravais lattices
(after Auguste Bravais, 1811-1863)



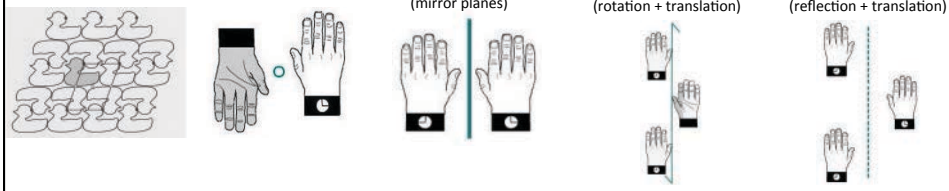
symmetry

Spacegroups

The **spacegroup** is a concise description of all the symmetries in a given 3-dimensional crystal

Crystal symmetries:

Rotations | Inversions | Reflections (mirror planes) | Screw axes (rotation + translation) | Glide planes (reflection + translation)



Example: spacegroup $P4_122$ = 4-fold rotation along c, 2-fold rotation along a and b, and a screw axis along c (the letter in front represents the type of Bravais lattice, in this case P = Primitive)

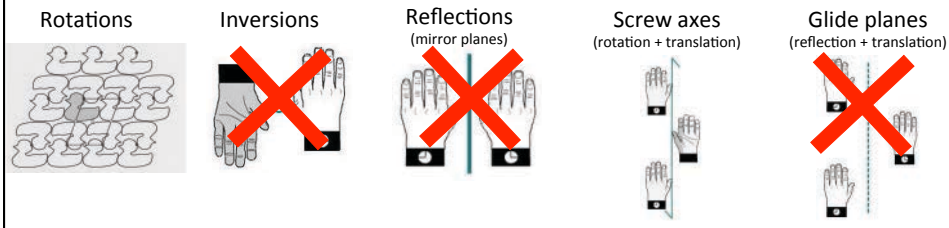
The total number of possible spacegroups = 230

Spacegroups

The **spacegroup** is a concise description of all the symmetries in a given 3-dimensional crystal

Crystal symmetries:

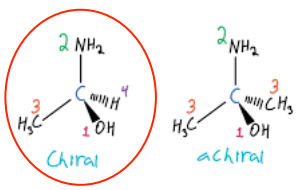
Rotations | Inversions | Reflections (mirror planes) | Screw axes (rotation + translation) | Glide planes (reflection + translation)



Example: spacegroup $P4_122$ = 4-fold rotation along c, 2-fold rotation along a and b, and a screw axis along c (the letter in front represents the type of Bravais lattice, in this case P = Primitive)

The total number of possible spacegroups = 230

Only **65** spacegroups are possible in protein crystals



The 65 chiral spacegroups

TRICLINIC
P 1

MONOCLINIC
P 2 P 2_1
C 2

ORTHORHOMBIC
P 2 2 2 P 2 2 2_1 P 2_1 2_1 2 P 2_1 2_1 2_1
C 2 2 2 C 2 2 2_1
I 2 2 2 I 2_1 2_1 2_1
F 2 2 2

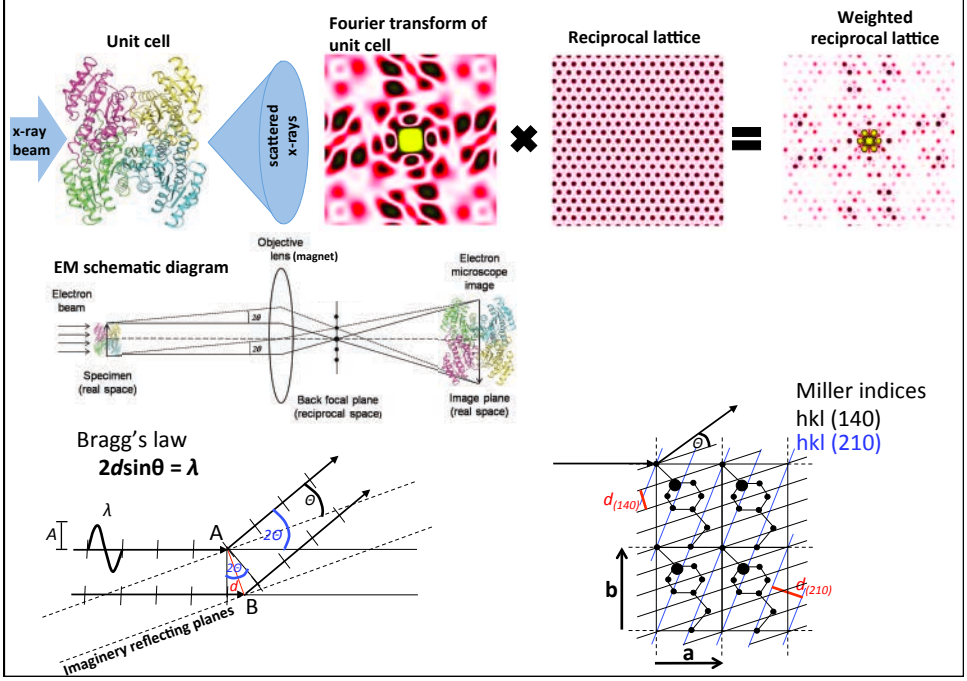
TETRAGONAL
P 4 P 4_1 P 4_2 P 4_3
I 4 I 4_1
P 4 2 2 P 4 2_1 2 P 4_1 2 2 P 4_1 2_1 2
P 4_2 2 2 P 4_2 2_1 2 P 4_3 2 2 P 4_3 2_1 2
I 4 2 2 I 4_1 2 2

TRIGONAL
P 3 P 3_1 P 3_2
R 3
P 3 1 2 P 3 2 1 P 3_1 1 2 P 3_1 2 1
P 3_2 1 2 P 3_2 2 1
R 3 2

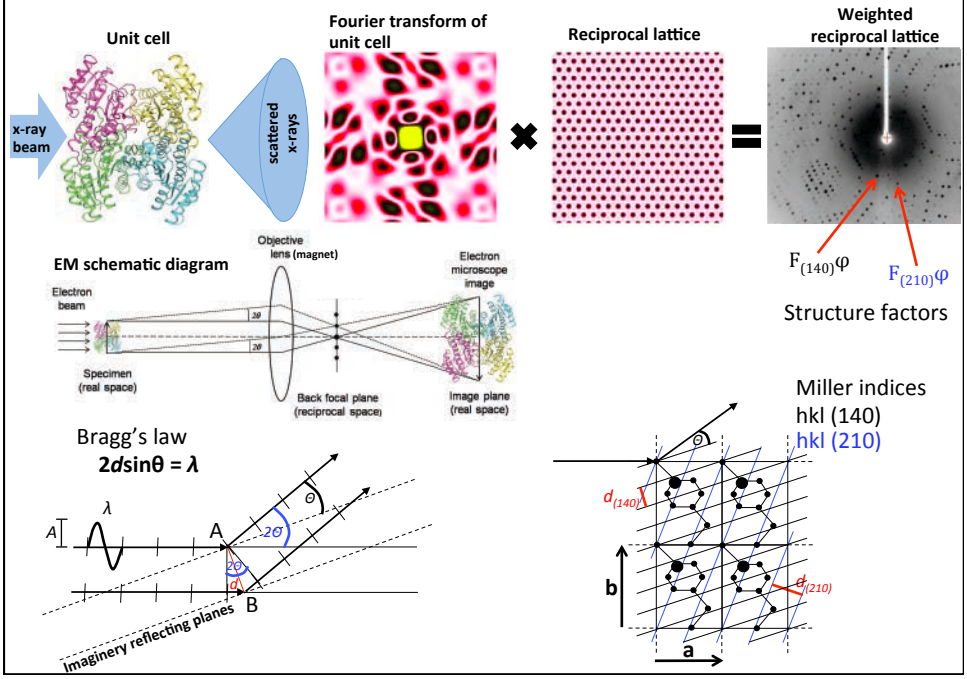
HEXAGONAL
P 6 P 6_1 P 6_5 P 6_2
P 6_4 P 6_3
P 6 2 2 P 6_1 2 2 P 6_5 2 2 P 6_2 2 2
P 6_4 2 2 P 6_3 2 2

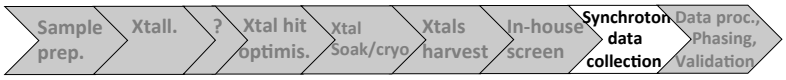
CUBIC
P 2 3 I 2 3 F 2 3
P 2_1 3 I 2_1 3
P 4 3 2 I 4 3 2 F 4 3 2
P 4_2 3 2 P 4_3 3 2 P 4_1 3 2
F 4_1 3 2 I 4_1 3 2

X-ray crystal diffraction



X-ray crystal diffraction





Data collection strategies

in-house
(5-20 mins per image)

1. Bravais lattice estimation from only 1-2 images with **iMOSFLM** (Andrew Leslie, LMB)
2. Mosaicity (Fabrice's talk) estimation (also depending on beam divergence and resolution)
3. Radiation damage is not an issue

Symmetry	Complete data (°)
1	180 (any axis)
2	180 (b), 90 (ac)
222	90 (ab, ac, bc)
4	90 (c, ab)
422	45 (c), 90 (ab)
3	60 (c), 90 (ab)
32	30 (c), 90 (ab)
6	60 (c), 90 (ab)
622	30 (c), 90 (ab)
23	~60
432	~35

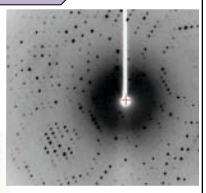
at synchrotron
(0.08-0.1 secs per image)

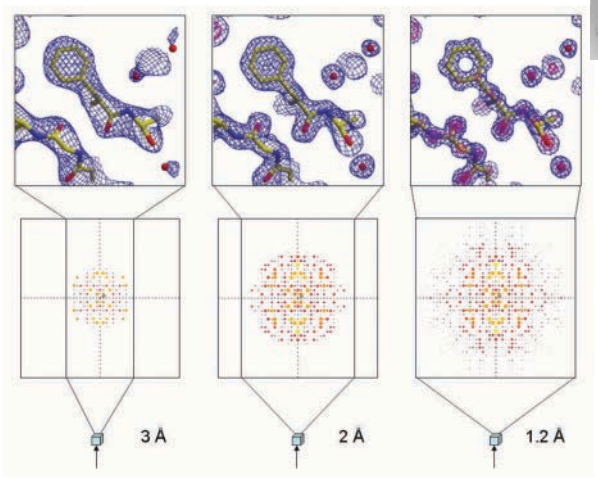
1. Lattice knowledge is no longer critical due to speed (unless crystals are very sensitive to radiation damage)
2. Mosaicity knowledge not critical due to fine φ slicing (0.1-0.2°)
3. Radiation damage always to be checked (on-the-fly tools and/or sacrificial crystal)

Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | Xtals harvest | In-house screen | Synchroton data collection | **Data proc., Phasing, Validation**

Fourier transform of electron density of crystal unit cell

$$\rho(x,y,z) = 1/V_c \sum_{h=-\infty}^{\infty} \sum_{k=-\infty}^{\infty} \sum_{l=-\infty}^{\infty} |F_{(hkl)}| \cos[2\pi(hx + ky + lz) - \varphi_{(hkl)}]$$

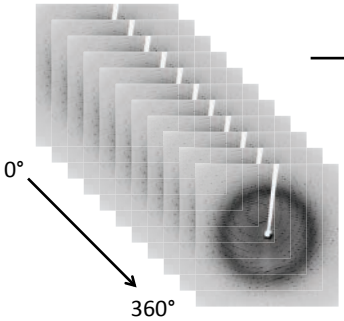




Rupp (2003)

Sample prep. | Xtall. | ? | Xtal hit optimis. | Xtal Soak/cryo | Xtals harvest | In-house screen | Synchroton data collection | **Data proc., Phasing, Validation**

Data processing of diffraction images



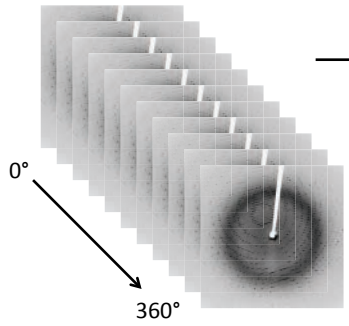
→ Spot finding

↓

Image header info
- Detector distance
- Beam centre
- Wavelength (λ)

Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → **Data proc., Phasing, Validation**

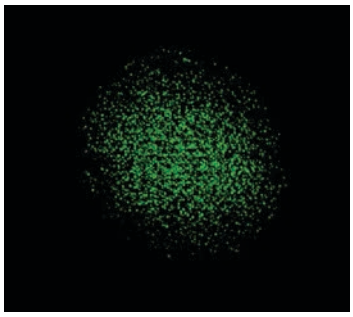
Data processing of diffraction images



→ Spot finding

↓

Image header info
- Detector distance
- Beam centre
- Wavelength (λ)



rs_mapper, Takanori Nakane (LMB)

← Index

- Unit cell
- Bravais lattice (cubic, trigonal, monoclinic, …)
- Crystal orientation

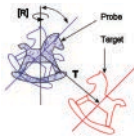
→ hkl → Spot prediction and integration → Scaling and merging → $\sqrt{I_{hkl}}$ = F_{hkl}

Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → **Data proc., Phasing, Validation**

Finding the phases

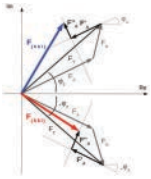
$$\rho(x,y,z) = 1/V_c \sum_{h=-\infty}^{\infty} \sum_{k=-\infty}^{\infty} \sum_{l=-\infty}^{\infty} |F_{(hkl)}| \cos[\, 2\pi(hx + ky + lz) - \varphi_{(hkl)}]$$

Molecular Replacement



- Experimentally the simplest method with a single native dataset required
- Search homolog not always available
- Ideally 40% or more sequence identity between search model and target is required

Single-wavelength anomalous diffraction (**SAD**)



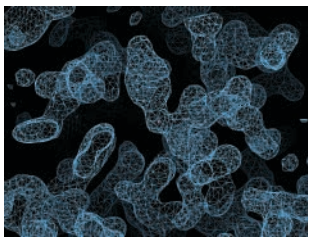
- Anomalous scatterers are part of the protein (e.g., **sulfur**, **zinc**, **iron**, …) or can be incorporated (e.g., **SeMet**)

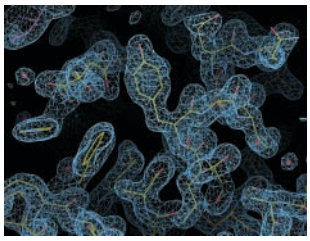
- Multi-wavelength anomalous dispersion (**MAD**)
- Single isomorphous replacement with anomalous signal (**SIRAS**)
- Multiple isomorphous replacement (**MIR**) with anomalous signal (**MIRAS**)

- Produce better starting phases (i.e., better initial maps for building)
- Experiments are more difficult [either for data collection (MAD) or soaking the heavy atoms (SIRAS/MIR)]

Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → **Data proc., Phasing, Validation**

$$\rho(x,y,z) = 1/V_c \sum_{h=-\infty}^{\infty} \sum_{k=-\infty}^{\infty} \sum_{l=-\infty}^{\infty} |F_{(hkl)}| \cos[2\pi(hx + ky + lz) - \varphi_{(hkl)}]$$





Model building in **Coot** (Paul Emsley, LMB)

Model **refinement** to:

1) fit chemically sensible models into observed data (electron density maps)
2) calculate the best electron density to criticise the model

Software for refinement in crystallography:

- **Refmac5** in CCP4 (Garib Murshudov, LMB)
- **Phenix**
- **BUSTER**

Model validation in **MolProbity**

Clashscore, all atoms:	9.22	
Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1		
Poor rotamers	3	0.12%
Favored rotamers	2285	92.40%
Ramachandran outliers	0	0.00%
Ramachandran favored	2678	99.81%
MolProbity score	1.49	
Cβ deviations >0.25Å	0	0.00%
Bad bonds:	0 / 22231	0.00%
Bad angles:	7 / 29764	0.02%
Cis Prolines:	0 / 56	0.00%
CaBLAM outliers	0	0.0%
CA Geometry outliers	0	0.00%
Chiral volume outliers	0/3318	
Waters with clashes	0/0	0.00%

Sample prep. → Xtall. → ? → Xtal hit optimis. → Xtal Soak/cryo → Xtals harvest → In-house screen → Synchroton data collection → **Data proc., Phasing, Validation**

Crystallography software

- Data collected using the in-house X-ray generator are usually processed with MOSFLM through the **iMOSFLM** graphical user interface
- Synchrotron data at Diamond are automatically processed by **Xia2** using three different software, **DIALS**, **XDS** and **autoPROC/Staraniso**
- Instructions on how to launch **Xia2** and scripts/tutorials for **iMOSFLM**, **DIALS** and **XDS** can be found on the X-ray crystallography webpage
- For Molecular Replacement the following software is available via **CCP4**: **Phaser** and **MolRep** as stand alone programs and **Balbes**, **MrBump** and **MoRDa** as automatic pipelines
- For anomalous/heavy atom search is available **ShelxD** via **CCP4** and **HySS** via **Phenix** (or automatic phasing pipeline such as **autoSHARP** and **Crunk2** in **CCP4** or **Autosol** in **Phenix**)
- Density modification programs include **Parrots** and **ShelxE** via **CCP4** and **Resolve** in **Phenix**
- For model building is available **Coot** (Paul Emsley, LMB) and **ChimeraX**
- Refinement programs include **Refmac5** (Garib Murshudov) via **CCP4** and **phenix.refine** in **Phenix**
- Structure validation is generally assessed with **MolProbity** (Duke University)

All software necessary from data processing to phasing to structure refinement is available on the **LMB linux servers (hex, hal and max)**

For any problem or help with the software please do not hesitate to contact me

Tutorials

Crystallography explanatory videos

Recorded movies of seminars on data processing given by people who wrote codes for data integration (Harry Powell and Andrew Lesley), experimental phasing (Andrea Thorn) and refinement (Garib Murshudov):
https://www2.mrc-lmb.cam.ac.uk/research/scientific-training/crystallography-course-2013/

Two-day data collection and processing training

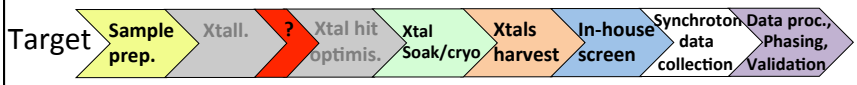
Three times a year (usually Feb, Jul and Dec) a two-day users' training is run at Diamond (one day to visit all the MX beamlines and the other day is a workshop on data processing

Seven-day data collection and structure solution workshop

Diamond-CCP4 jointly run a seven-day workshop (Dec) ranging from data collection to data processing, analysis and structure solution

If you are interested in the training courses above and would like more information please do not hesitate to contact me

Summary and advices



- Produce the most "crystallizable" construct/sample (skills, experience and creativity) — Talk to us ➔ LMB
- Prepare two 10 mg/ml protein samples for crystallization screening:
  1. **low** salt (NaCl) concentration (50 mM)
  2. **high** salt (NaCl) concentration (500 mM)
- Set up **1 or 2 sparse-matrix screens** with the above samples and check that about **50% of drops are clear** before proceeding to use all **23 different available screens**
  If that is not the case, especially with the low salt concentration samples, try adding **10% glycerol** or other stabilizers until 50% of drops will be clear in this pre-screening
- If no crystals appear after 4-6 weeks in any of the 23 screens, **dehydration of drops** and/or **in situ proteolysis** are an option before moving on to different constructs
- Once crystal hits are obtained, talk to Fabrice for crystal optimisation
- Ligand/heavy atoms soaking and cryoprotection ➔ talk to us
- Using in-house X-ray generator or synchrotrons ➔ talk to me
- Data processing, phasing and other structure related issues ➔ talk to me ➔ LMB

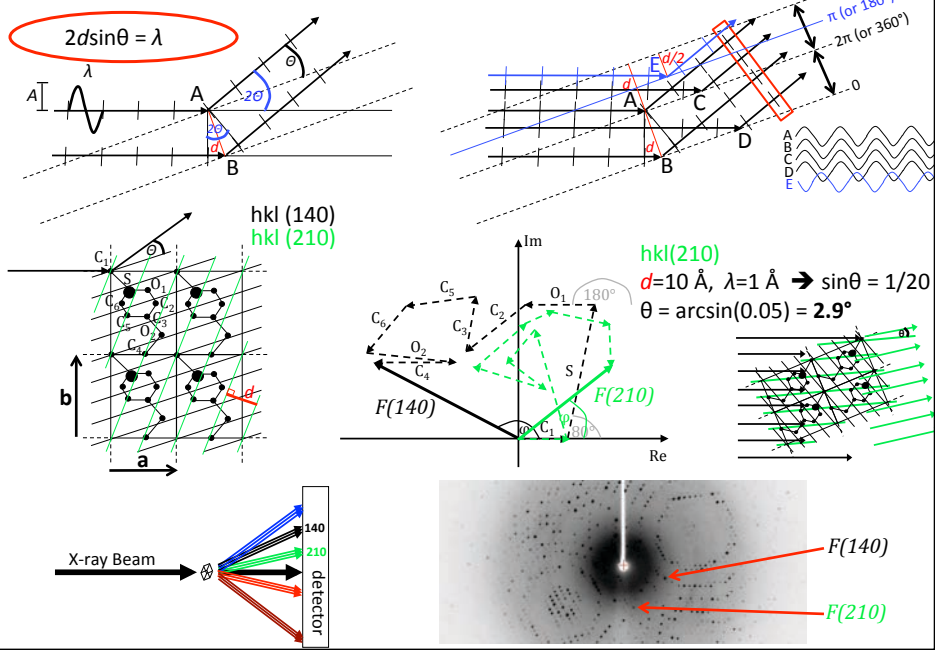
X-ray crystallography or single particle cryo-EM?

X-ray crystallography	Cryo-EM
no size limit (though MWs higher than 300 kDa are rarely crystallized, unless they are really stable, e.g. viruses)	Lower limit currently of about 50 kDa
Quick output (1 hour to set up and few days to get an answer)	Lower throughput
Majority of useful crystals produce data at resolutions of about 1-3 Å	Majority of useful EM grids maps are >3.5 Å

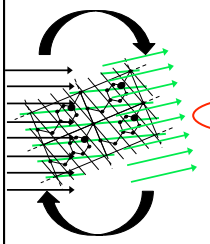
- Regions of interest in cryo-EM maps of large complexes that cannot be resolved properly due to flexibility can be investigated separately by X-ray crystallography for high resol. data
- X-ray crystallography as method of choice for drug discovery for high-throughput capabilities to screen a large amount of compounds in very short time

<u>Final remark, if sample amount is not an issue, why not to spend 1 hour to set up crystallization screens before spending months on optimizing EM grids?</u>

Real space crystal diffraction

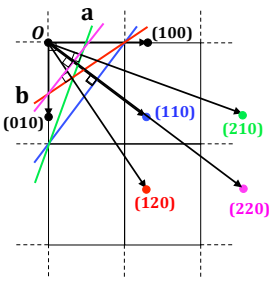


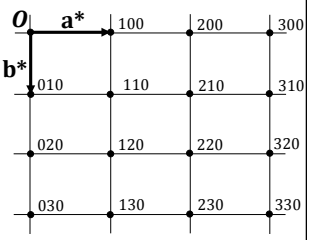
Reciprocal space crystal diffraction



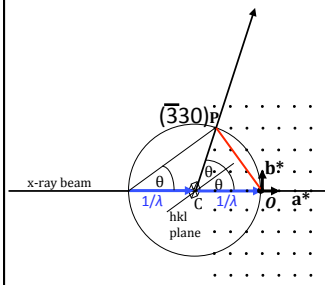
$2d\sin\theta = \lambda$

$2\sin\theta/\lambda = 1/d$



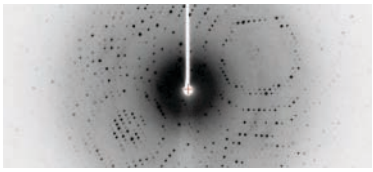


Reciprocal lattice
a*=1/**a**, **b***=1/**b** and **c***=1/**c**



$2(1/\lambda)\sin\theta = \overline{PO} = 1/d$

$2\sin\theta/\lambda = 1/d$



2D projection of the reciprocal lattice points lying on the **Ewald sphere** at a given orientation