LEADING INNOVATION FOR A HEALTHIER WORLD
uOttawa
Faculté de médecine
Faculty of Medicine
CHEF DE FILE EN INNOVATION POUR UN MONDE EN SANTÉ

Lecture 7, BCH 8102, 2021 Winter

Membrane Protein Structural Biology: Methods and Applications

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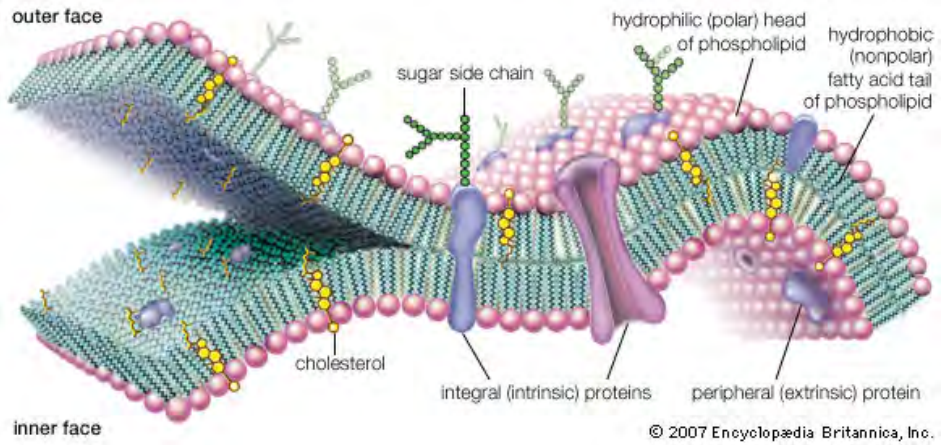
Lecture Outline

Part I: Technology

a) Importance of membrane proteins
b) A brief history
c) Where to start?
d) Old techniques and new discovery
e) New methodology and old problems

Part II: Structural Determination

a) X-ray crystallography: bolts and nuts
b) Cryo-electron microscopy: bolts and nuts

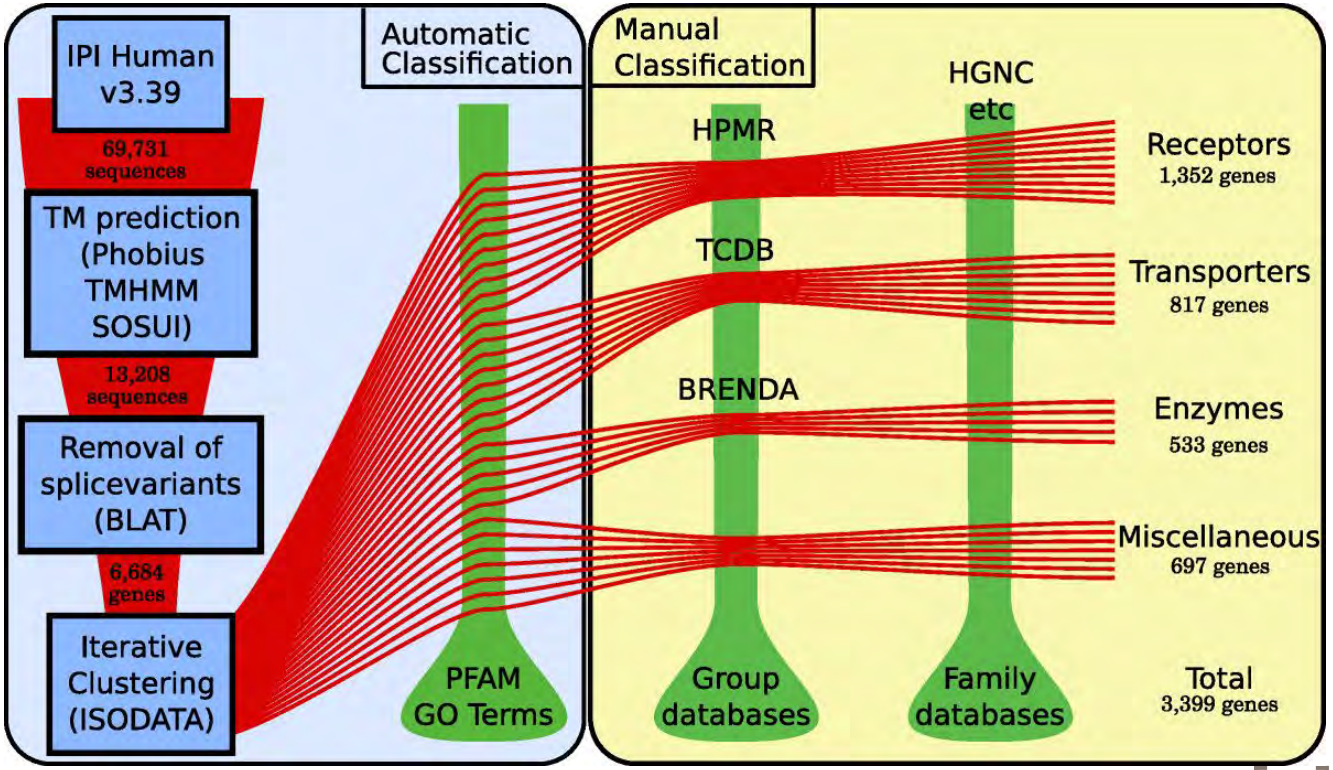


Part I:
TECHNOLOGY

Importance of membrane proteins

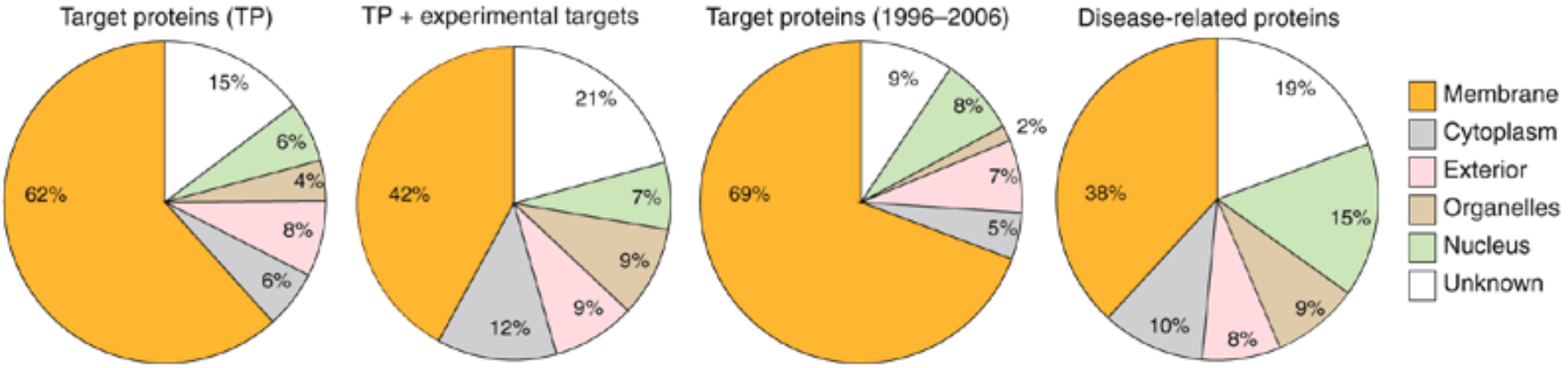
- Encoded by some 20-30% genes in typical genome.
- Major components of the mosaic lipid bilayers in cellular membranes
- Mediate cell-to-cell communication and signaling events.
- Disruptions or mutations in humans have been implicated in diseases, such as cardiovascular and metabolic diseases, cancer, rare genetic diseases, ...

Human Membrane Proteome



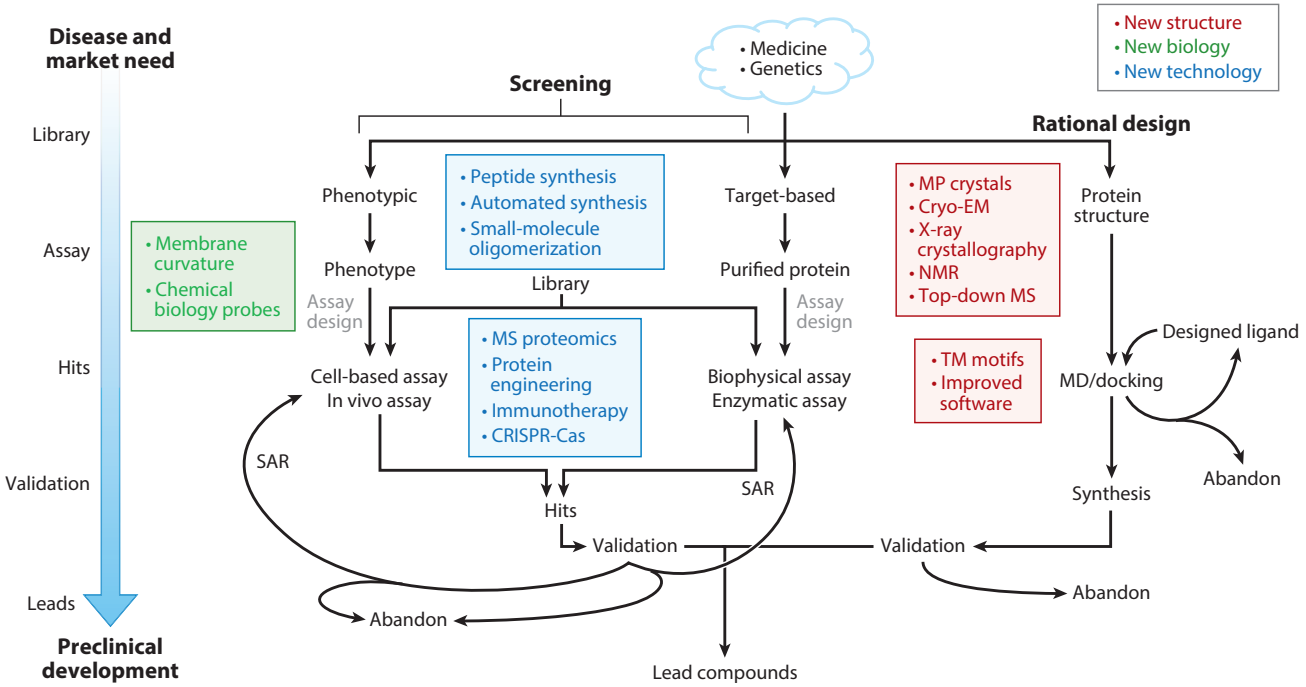
(Almén et al, BMC Biol, 2009)

Half Drug Targets are Membrane Proteins.



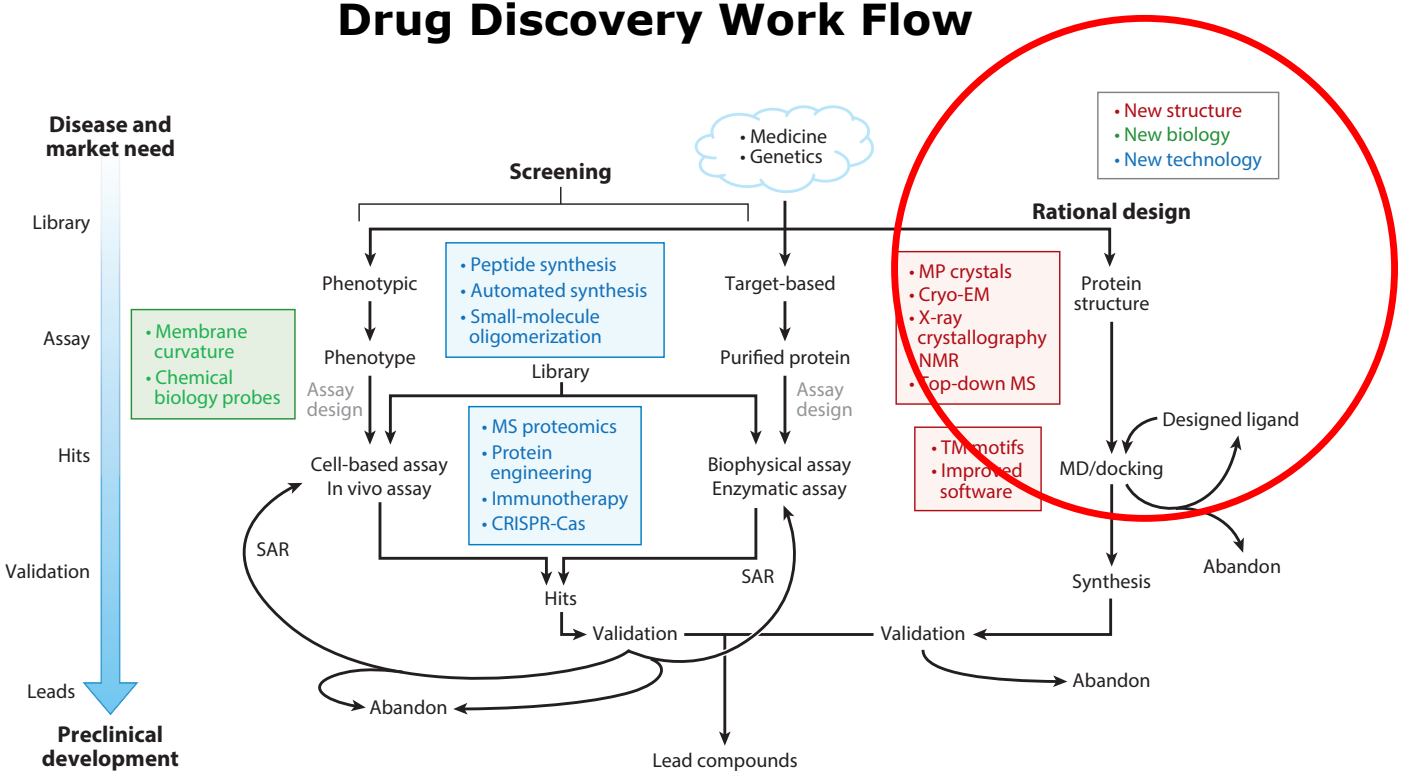
(Yildirim et al, Nat Biotech, 2007)

Drug Discovery Work Flow



(Yin & Flynn, Annu Rev Biomed Eng, 2016)

Drug Discovery Work Flow



(Yin & Flynn, Annu Rev Biomed Eng, 2016)

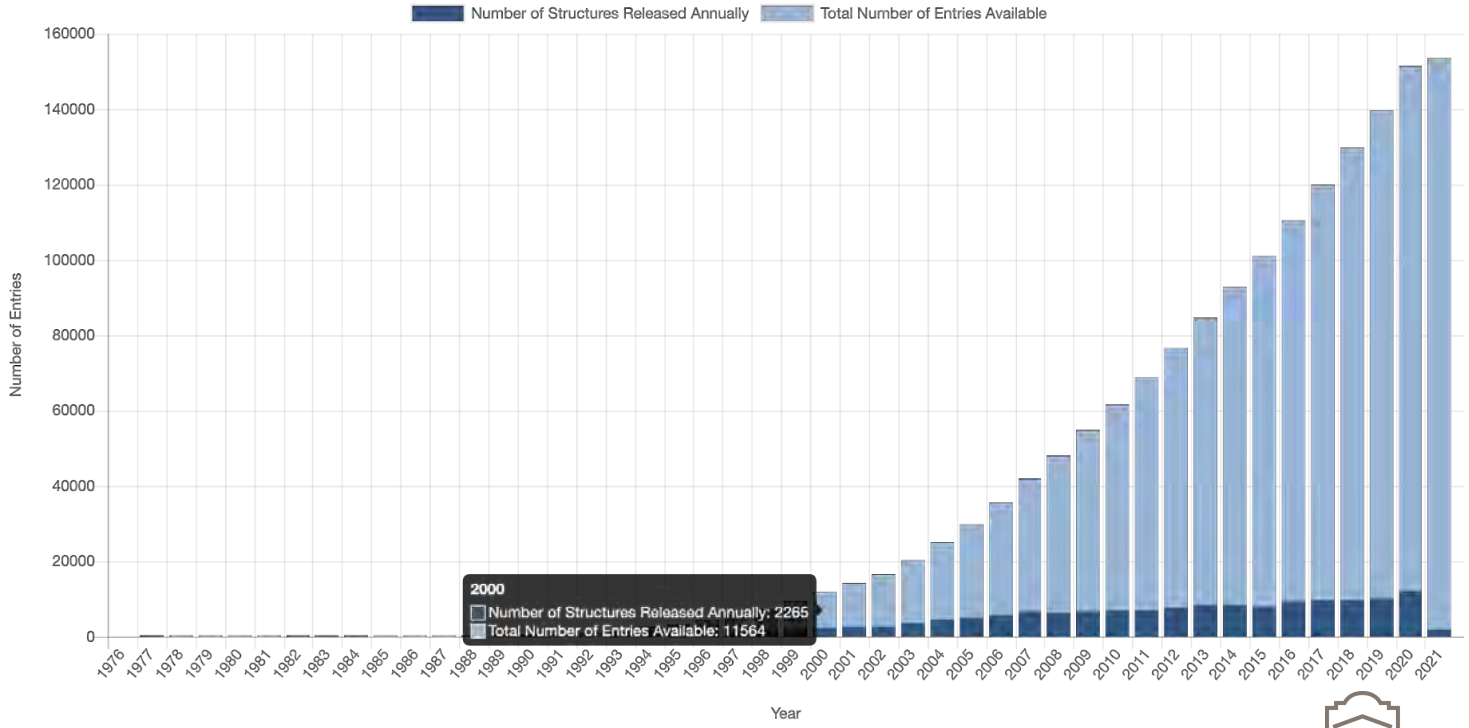
Structural Biology

- Understanding biology by examining three dimensional (3-D) molecular architectures and their changes.
- Learning life in action with the eyes of atoms: chemical and physical properties of biological matters.
- Structures of biological molecules determine their functions.

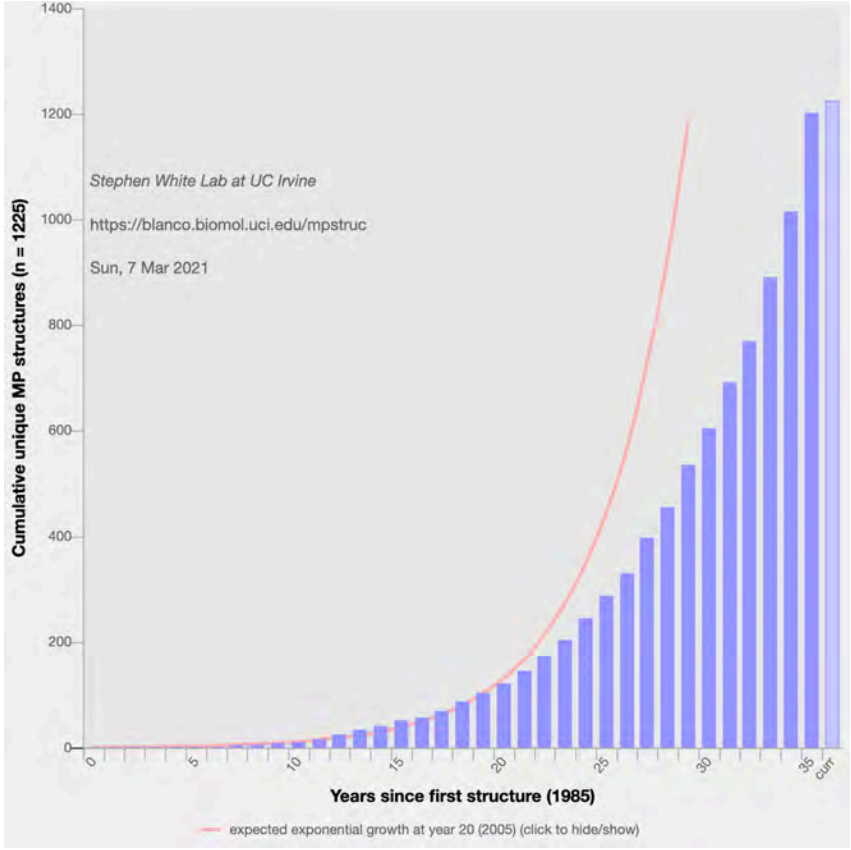
Central dogma:

Sequence ➜ Structure ➜ Function

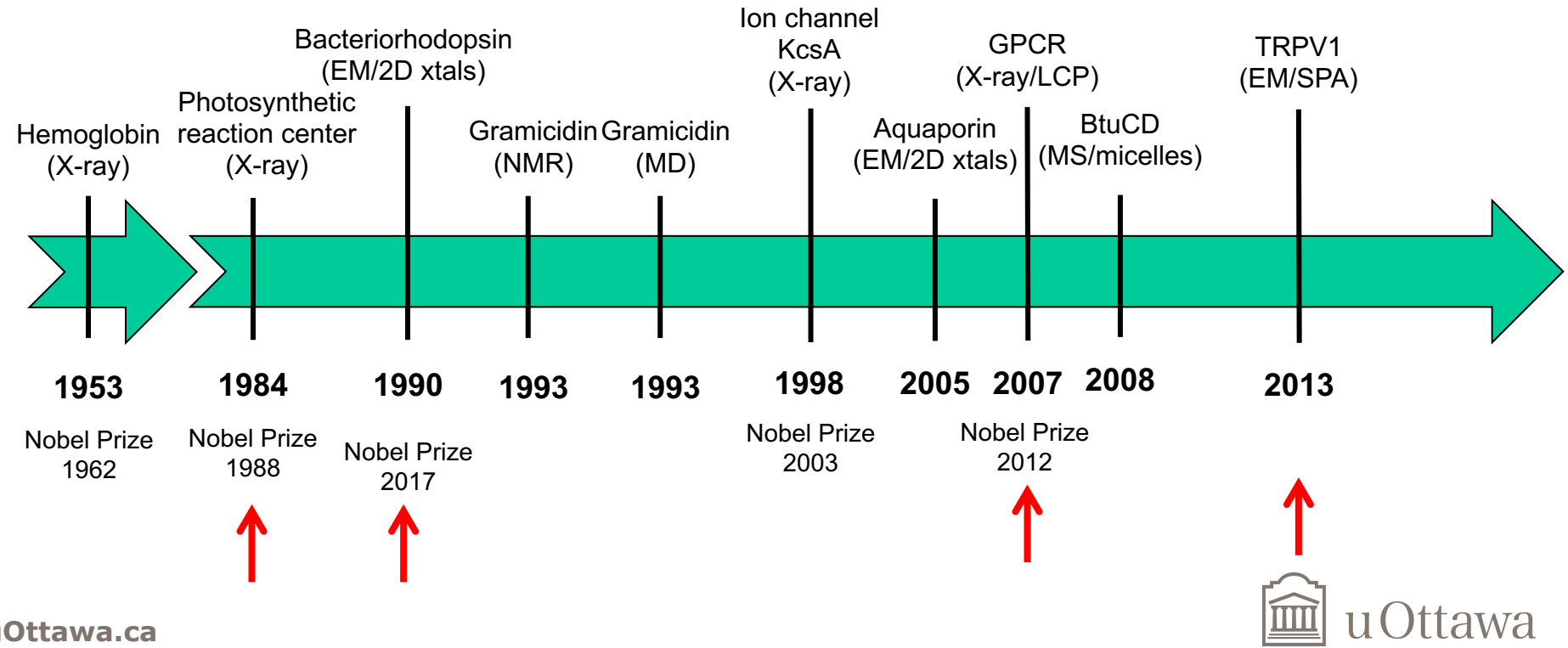
Atomic/Near-Atomic Models of Proteins (2021-3-7)



Atomic/Near-Atomic Models of Membrane Proteins (2021-3-7)

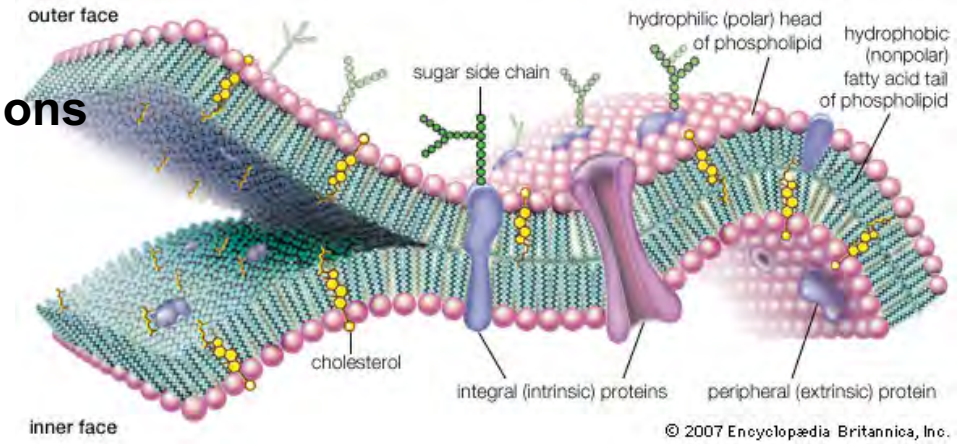


A brief history: some "atomic" milestones



Where to start?

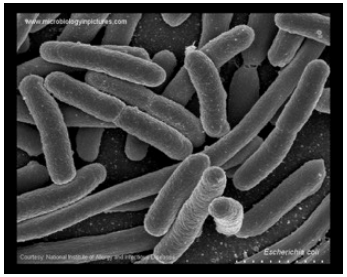
- Challenges
- Things to consider
- Right ways to address right questions
- Optical basics



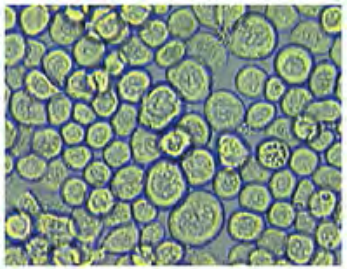
Challenges in Membrane Protein Structural Biology

- Naturally occurred proteins exist in low abundance, with only a few exceptions (*e.g.*, bacteriorhodopsin or aquaporin), and form complexes.
- *E. coli* is often not suitable for producing recombinant membrane proteins of eukaryotic origins.
- No so-called standard protocol of protein extraction, largely due to the complexity of protein-lipid interaction.
- Protocols of purification, crystallization, and *in vitro* reconstitution remain empirical for individual cases.

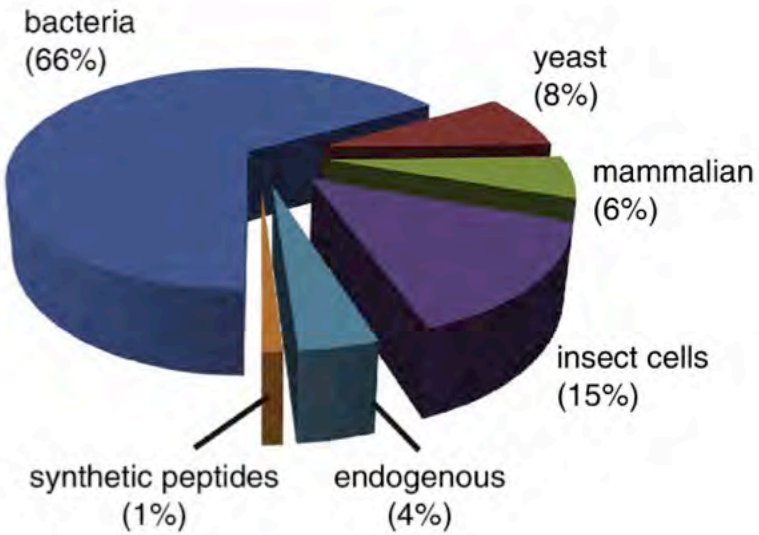
Choosing the appropriate expression hosts for recombinant proteins.



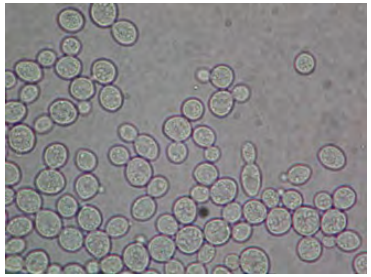
E. coli



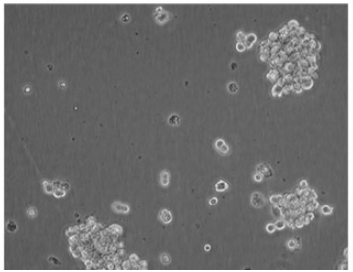
Sf9 insect cells



(Zorman et al, Curr Opin Struct Biol, 2015)



Budding yeast



HEK 293sus

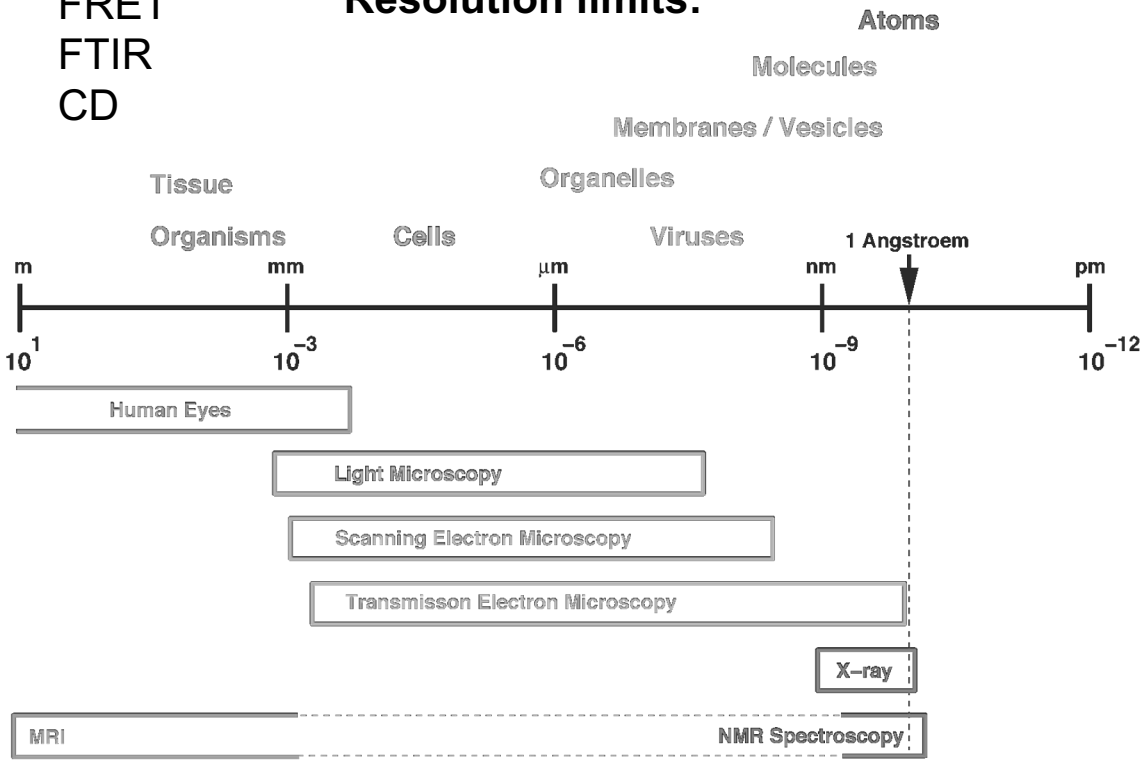
Things to consider for membrane protein extraction and purification.

- Cell disruption
- Solubilization agent
  - Detergents
  - Polymers
- Protein engineering
- Column chromatography
- *In vitro* reconstitution

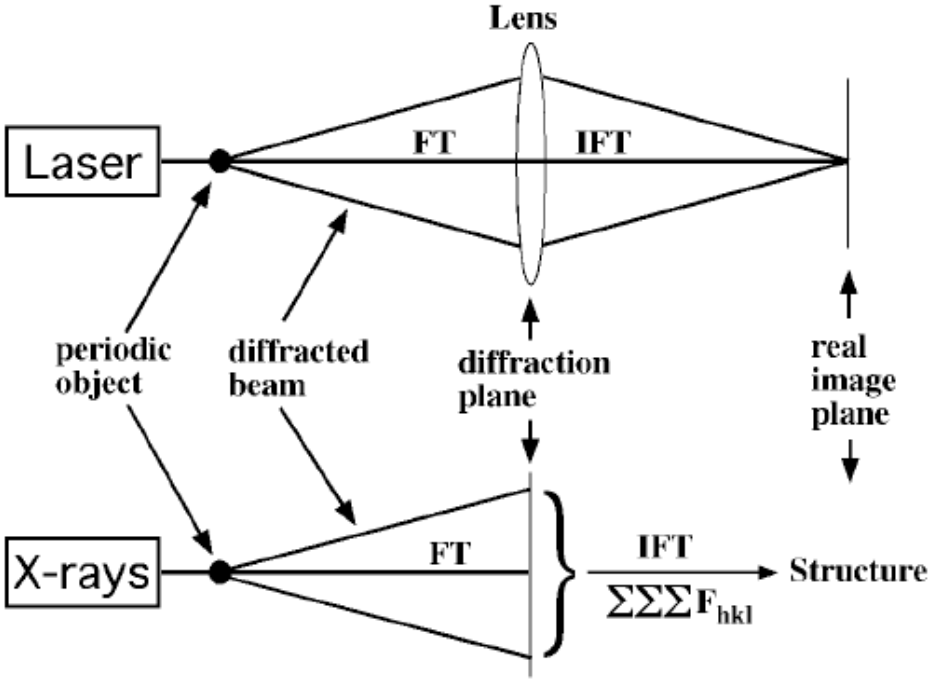
Ways to study membrane protein structures: optics & spectroscopy.

- FRET
- FTIR
- CD

Resolution limits:



Optical diffraction & X-ray diffraction.



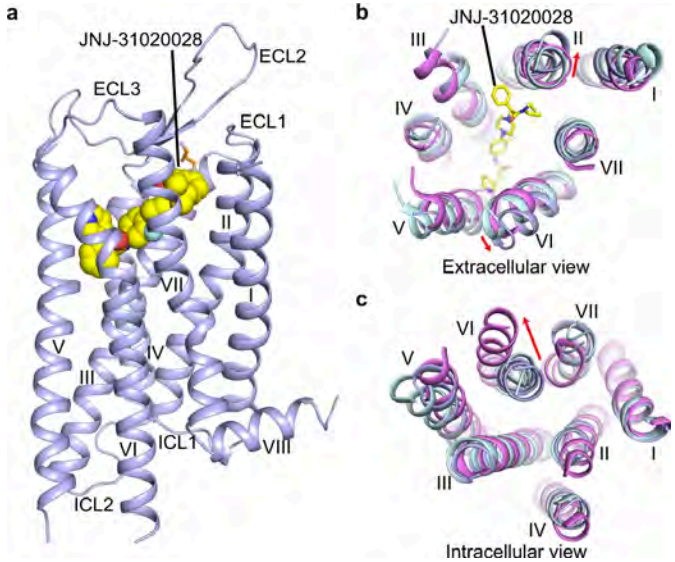
If F(X)=FT[f(x)], then f(x)=IFT[F(X)]

where FT=Fourier transform & IFT=Inverse Fourier transform.

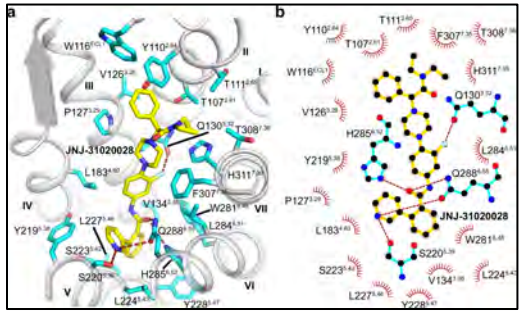
Note: important in X-ray crystallography and 3D reconstruction algorisms.

Old techniques and new discovery

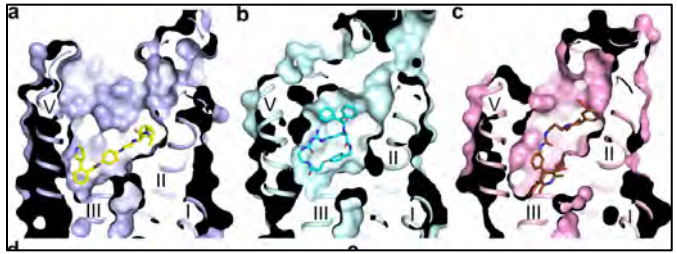
X-ray Crystallography: ≤ 3.0



Neuropeptide Y Y_2 Receptor
(Tang et al, Nat Comm, 2021)



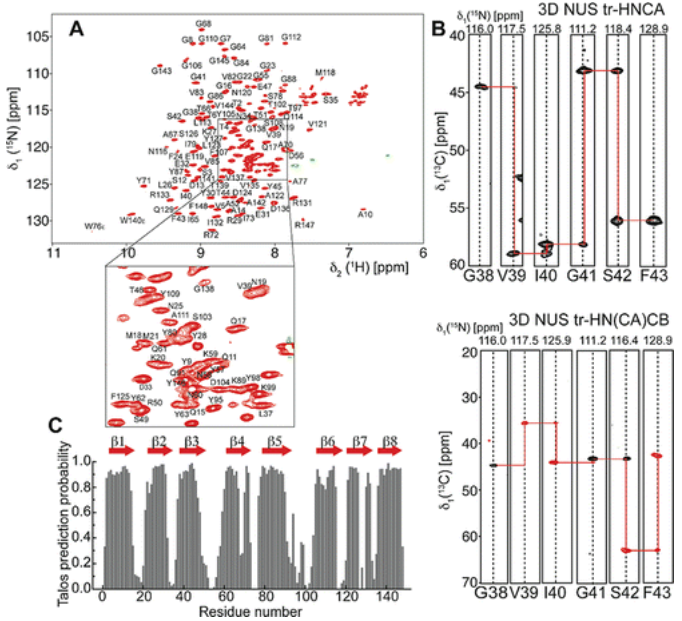
Protein-ligand interaction



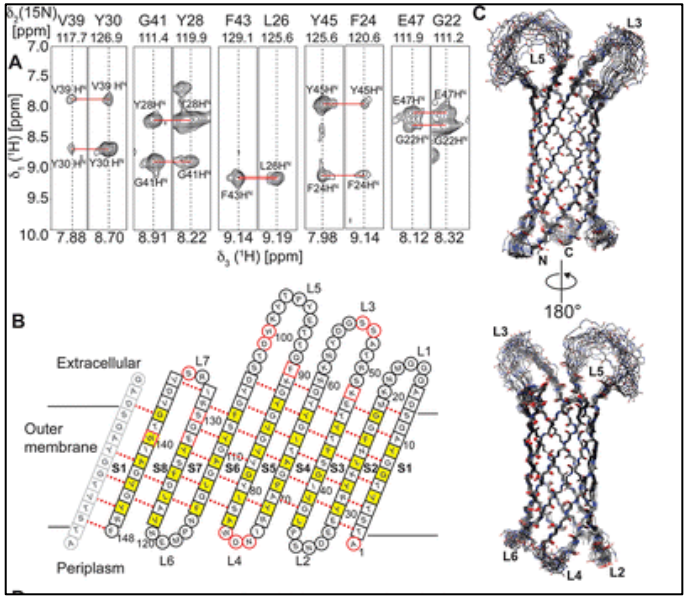
Drug discovery/design

Old techniques and new discovery

Nuclear magnetic resonance (NMR): small/median MP



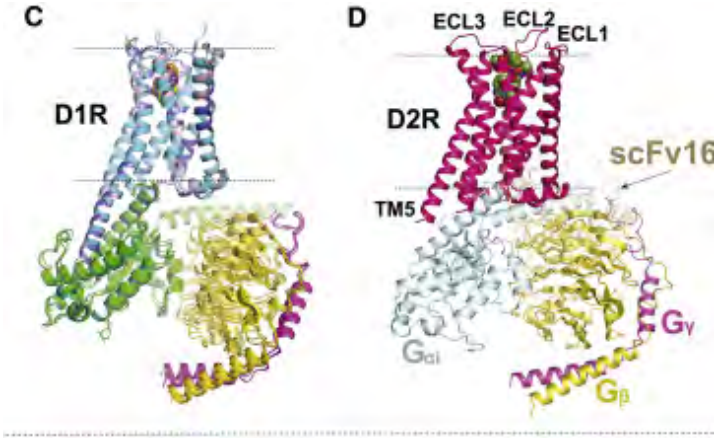
Bacterial OMPX
(Hagn et al, JACS, 2013)



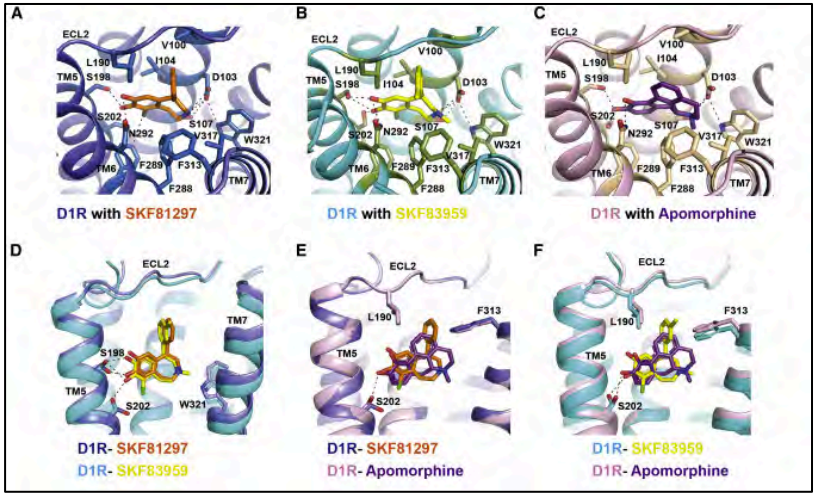
Protein dynamics

Old techniques and new discovery

Cryo-electron microscopy (cryo-EM): ≥ 100 kD



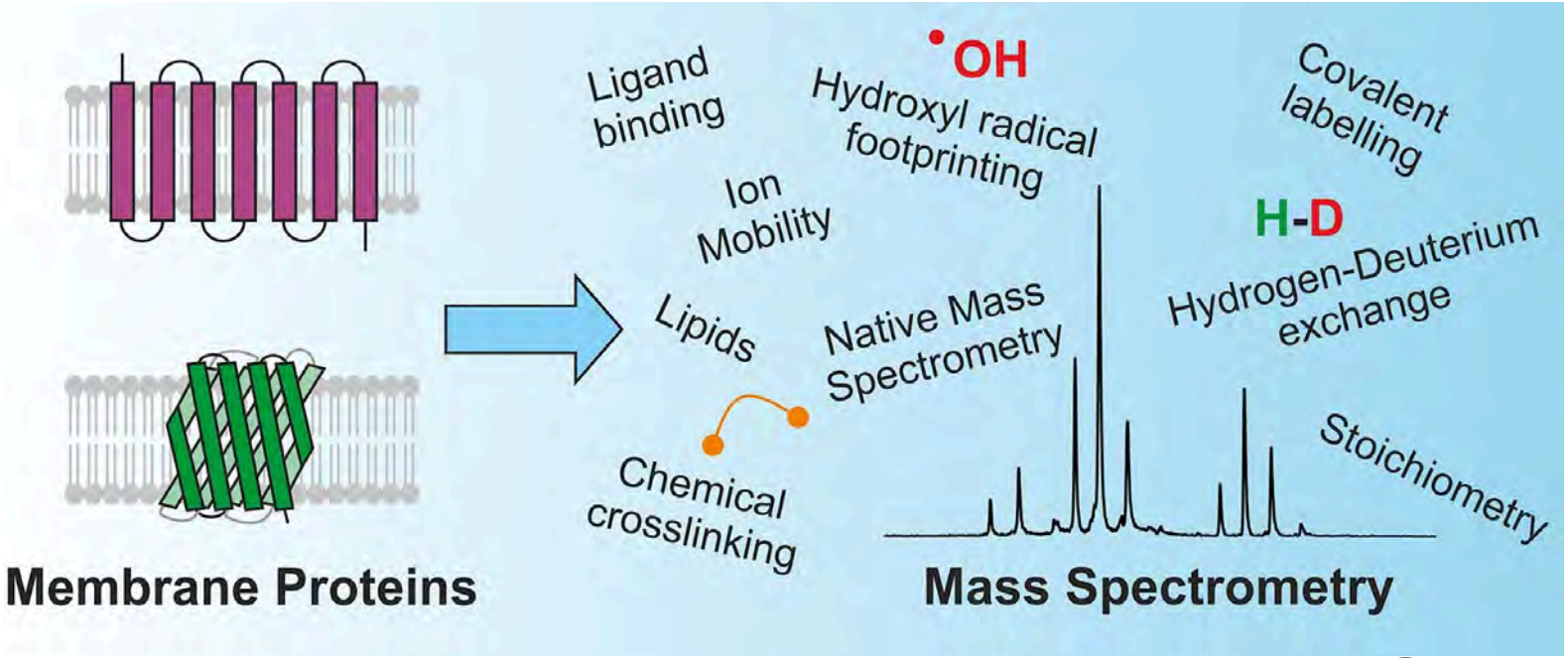
Human D1 and D2 Dopamine Receptors (Zhuang et al, Cell, 2021)



- Ligand/drug-binding sites/kinetics
- Receptor signaling mechanism

Old techniques and new discovery

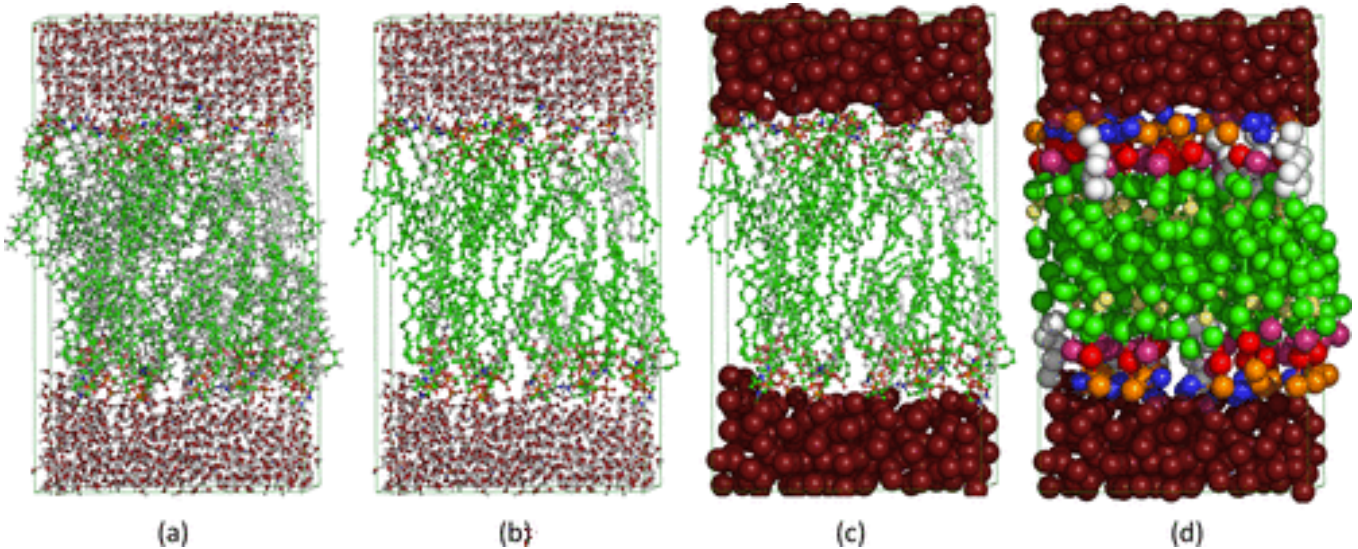
Mass spectrometry: native conditions



Native Mass Spestrometry
(Calabrese & Radford, Methods, 2018)

Old techniques and new discovery

Molecular dynamics (MD) simulation: membrane-embedded MP

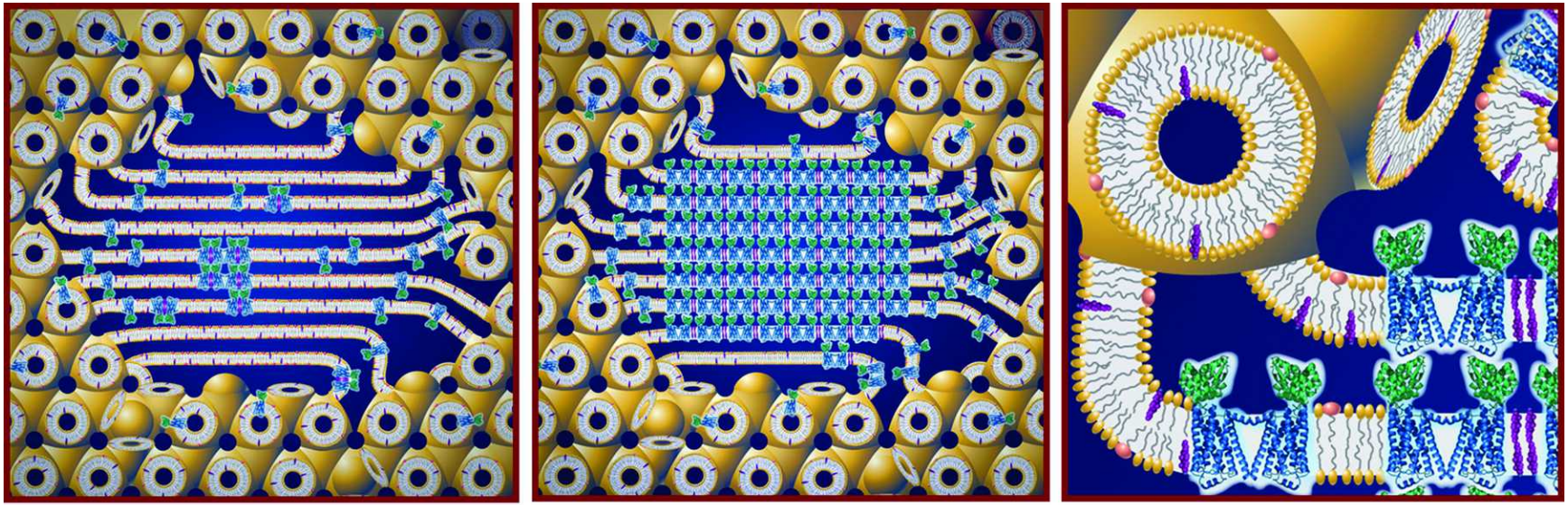


Full-atomistic | United-atom (Cα-H) | Hybrid | Coarse-grained

Four scales of MD simulations
(Goossens & Winter, J Chem Inf Model, 2018)

New methodology and old problems

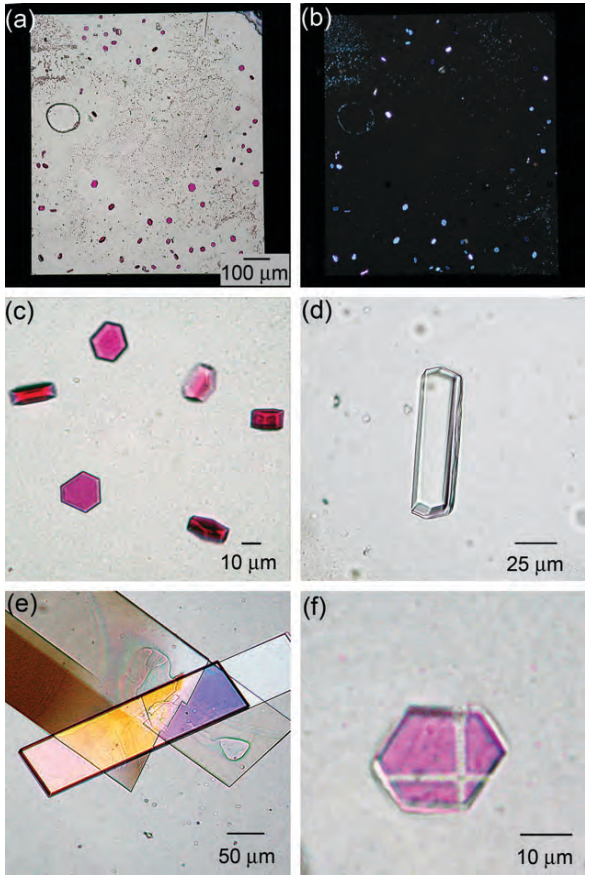
Lipid cubic phase (LCP) / microdiffraction



(a) (b) (c)

(Caffrey, Acta Cryst F, 2015)

Bacteriorhodopsin



Bacteriorhodopsin (Birefringence)

Bacteriorhodopsin

Lysozyme

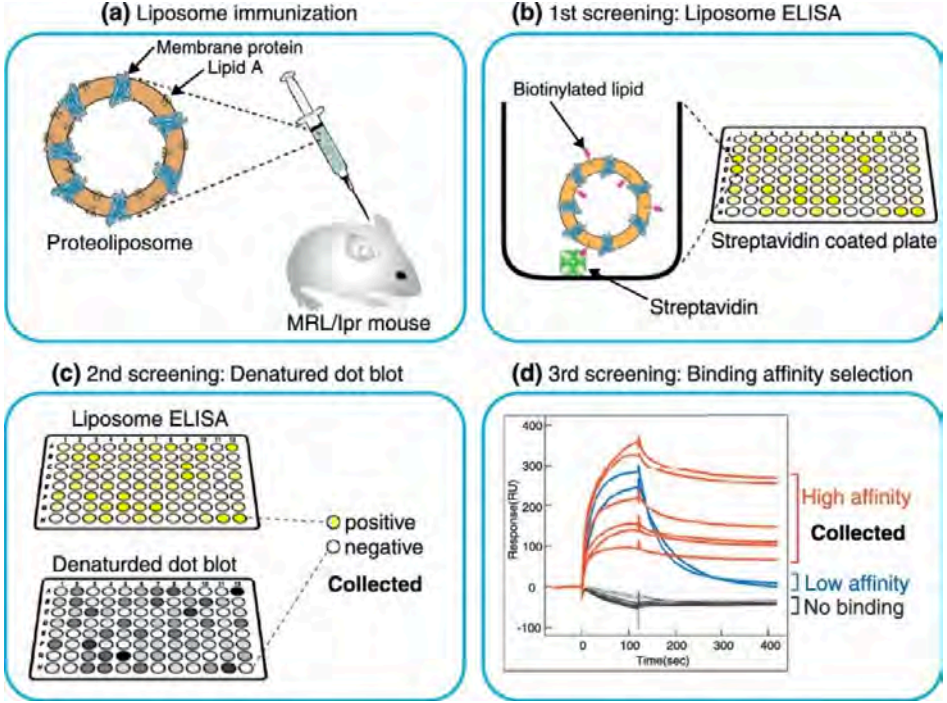
Cholesterol

Bacteriorhodopsin (X-ray damaged)

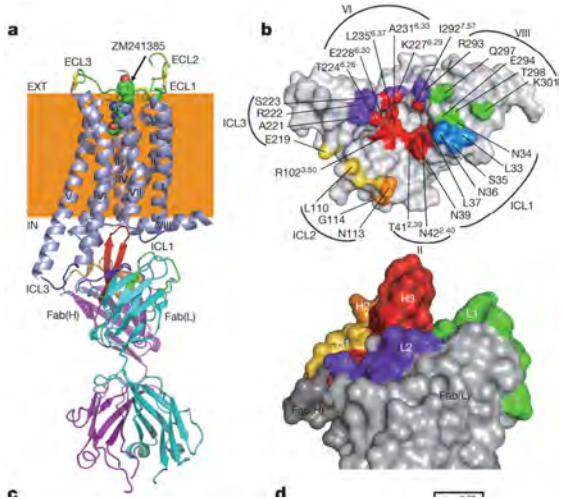
(Cherezov & Caffrey, Faraday Discuss, 2007)

New methodology and old problems

Antibodies: antibody fragment (F_{ab})



(Hino et al, Curr Opin Struct Biol, 2013)

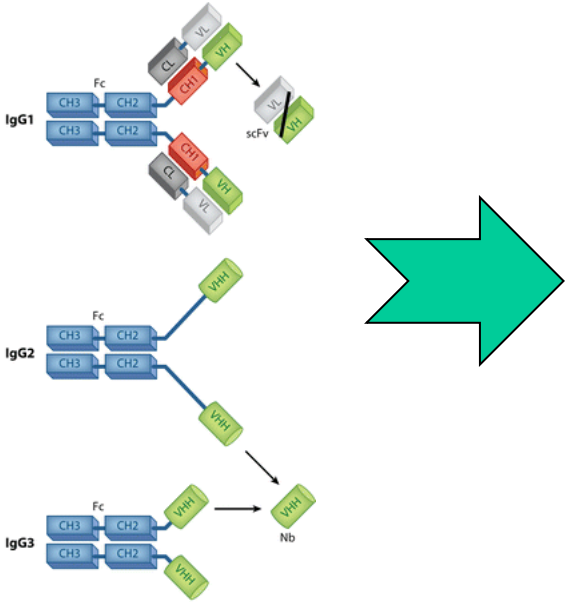


A_{2A} adenosine receptor (A_{2A}AR) G-protein coupled receptor)

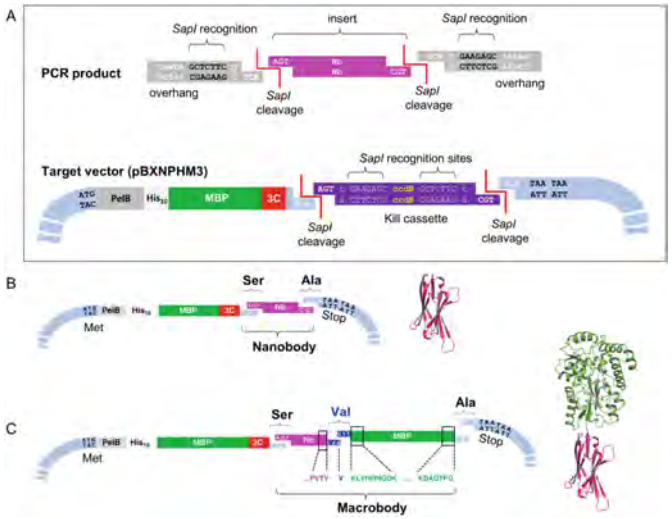
(Hino et al, Nature, 2012)

New methodology and old problems

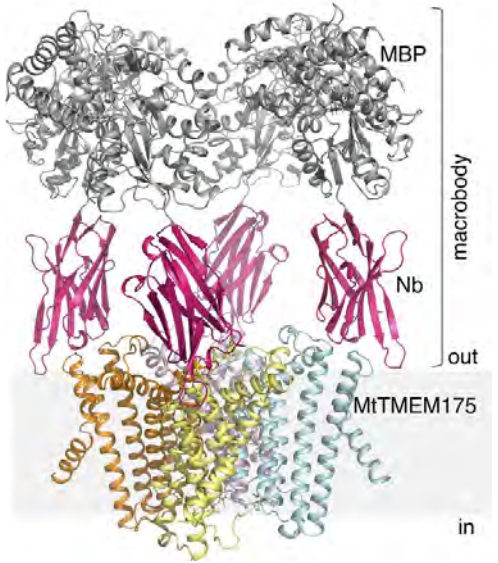
Antibodies: single-chain nanobody



Camelidae antibodies
(Muyldermans, Annu Rev Biochem, 2013)



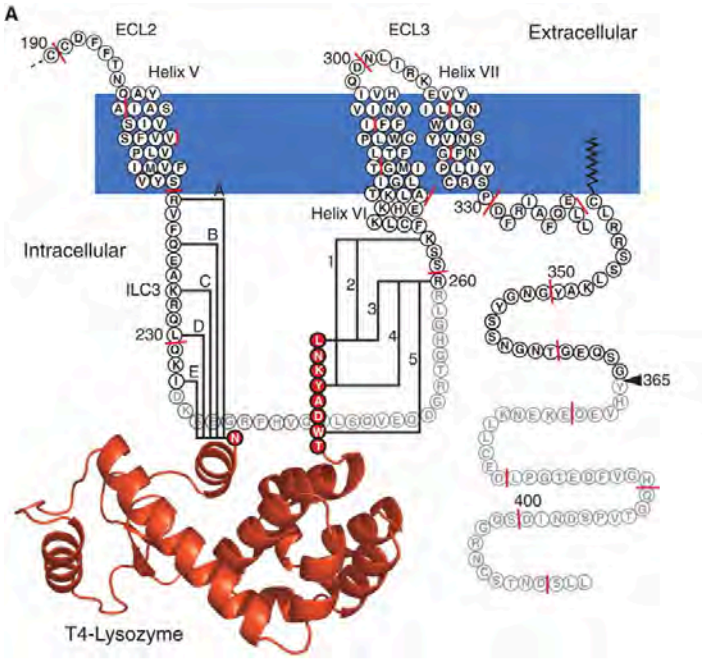
(Brunner & Schenck, Methods Mol Biol, 2020)

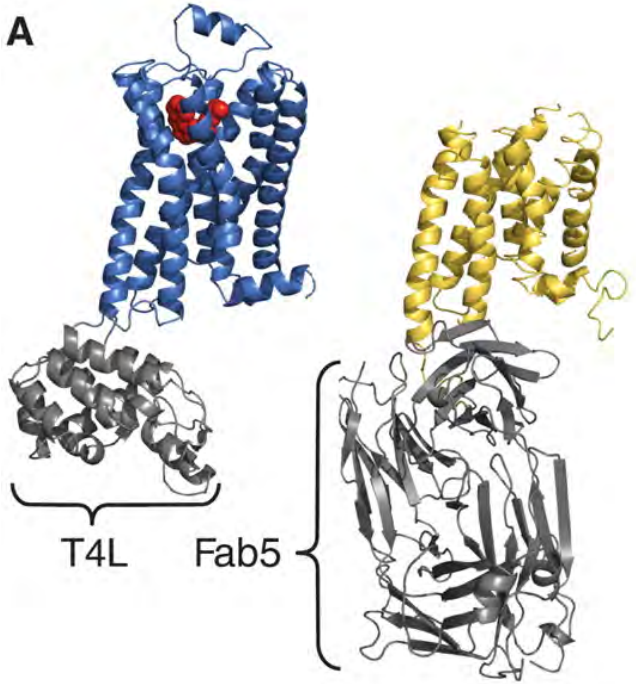


(Brunner et al, eLife, 2020)

New methodology and old problems

Protein engineering: fusion proteins

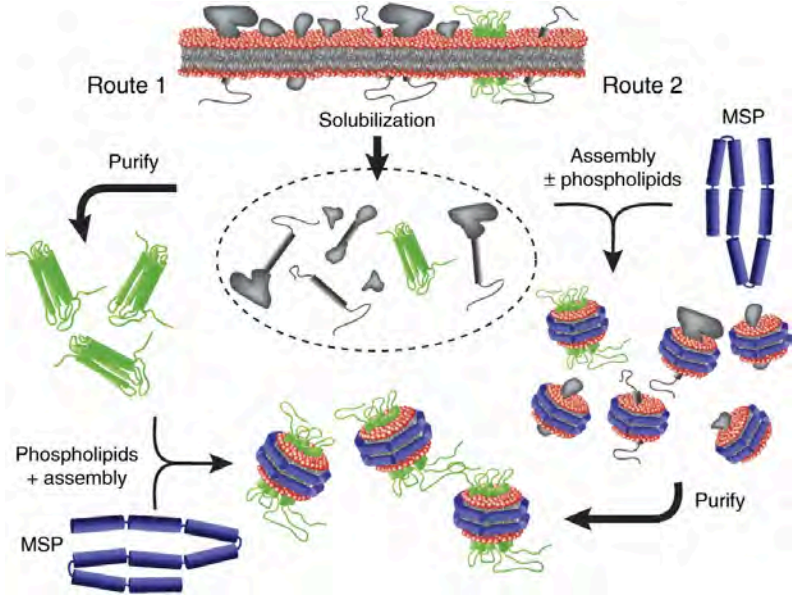




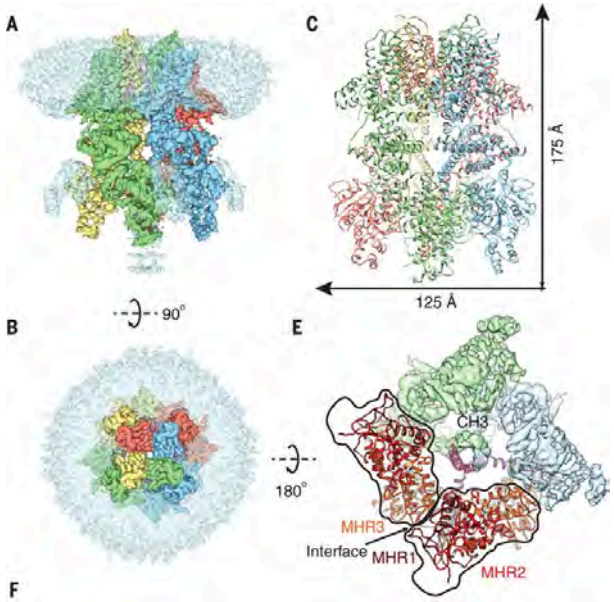
β2-adrenergic receptor (β2-AR)
(Rosenbaum et al, Science, 2007)

New methodology and old problems

Reconstitution: nanodiscs



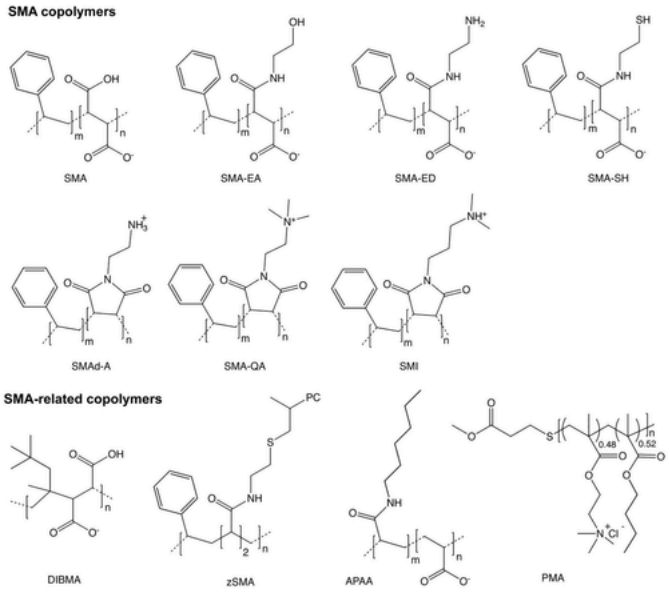
(Denisov & Sligar, Nat Struct Mol Biol, 2016)



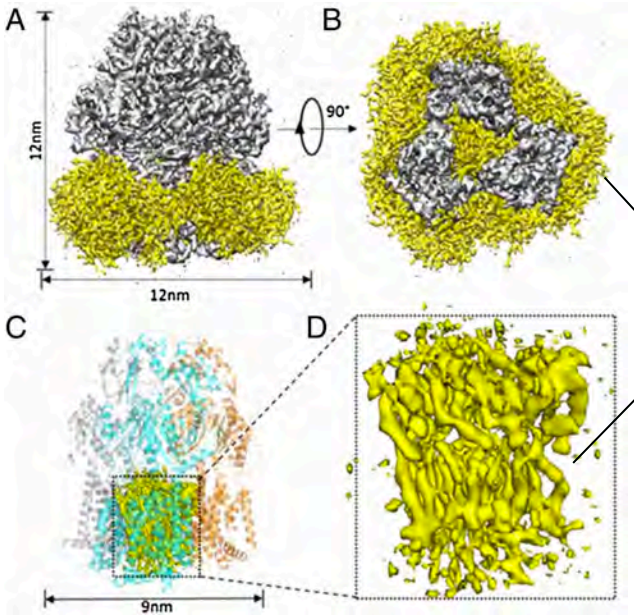
TRPM4
(Autzen et al, Science, 2018)

New methodology and old problems

Reconstitution: styrene maleic acid lipid particles (SMALPs)



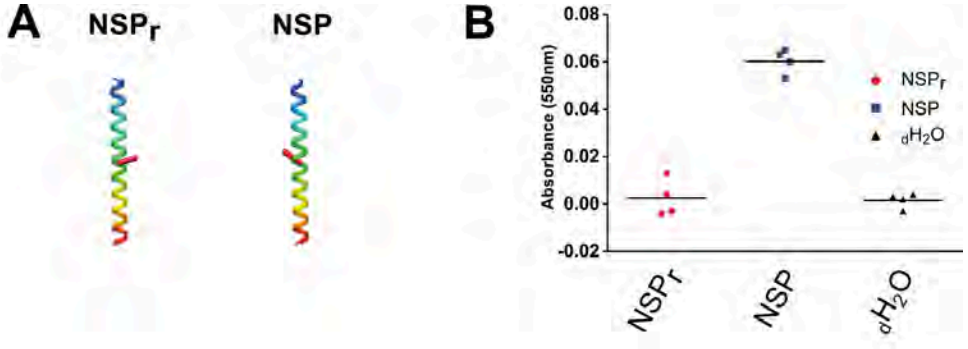
(Overduin & Esmaili, SLAS Discovery, 2009)



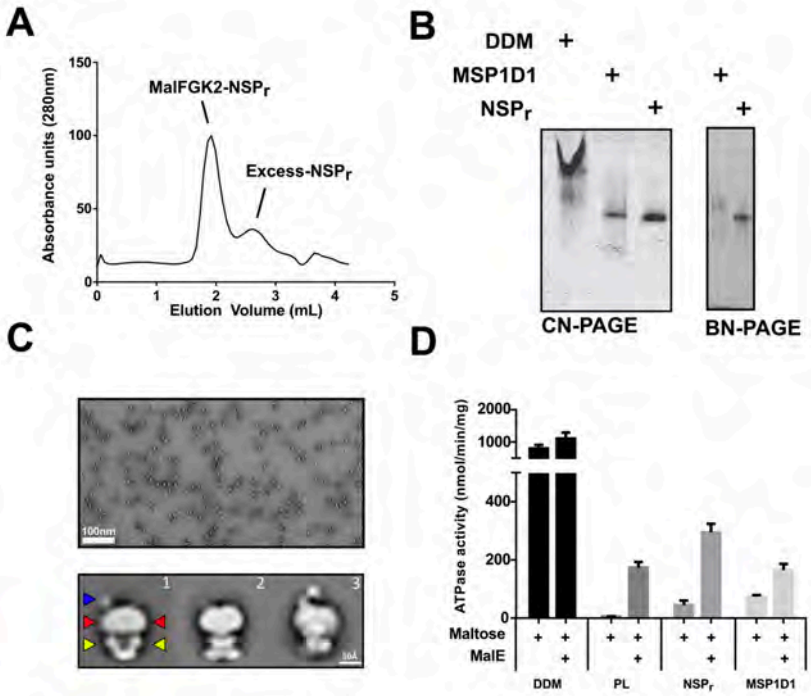
(Qiu et al, PNAS, 2018)

New methodology and old problems

Reconstitution: peptidisc



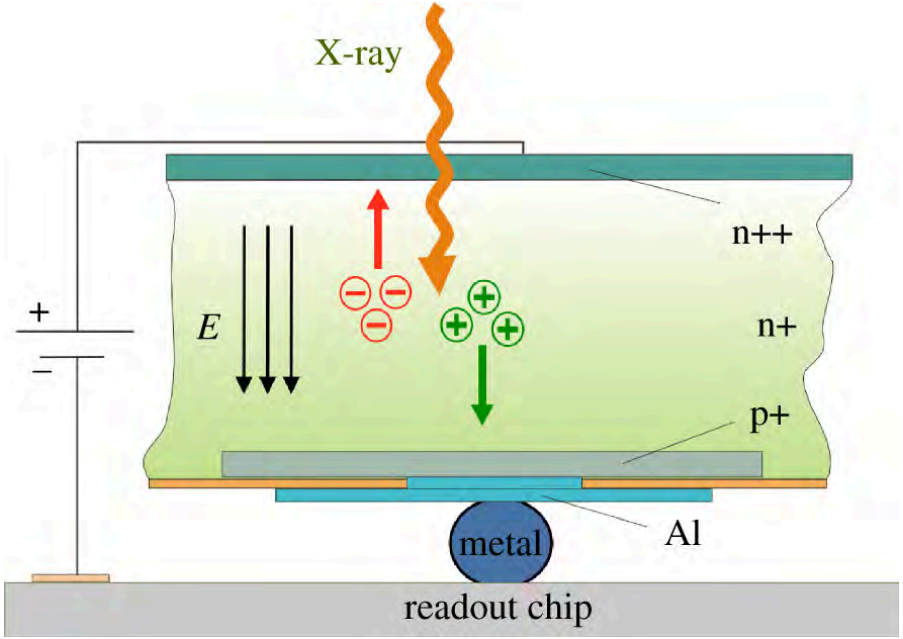
NSP: nanodisc scaffold peptide



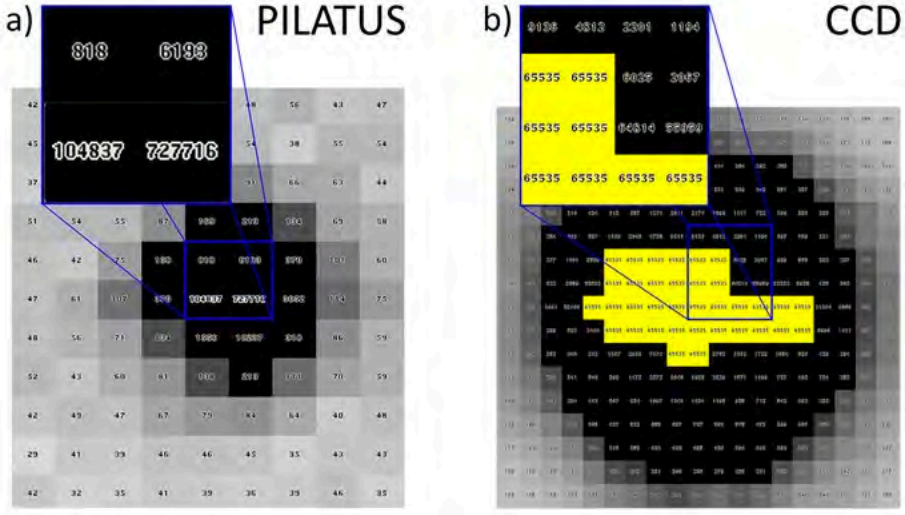
(Carlson et al, eLife, 2018)

New methodology and old problems

Direct detector: X-ray



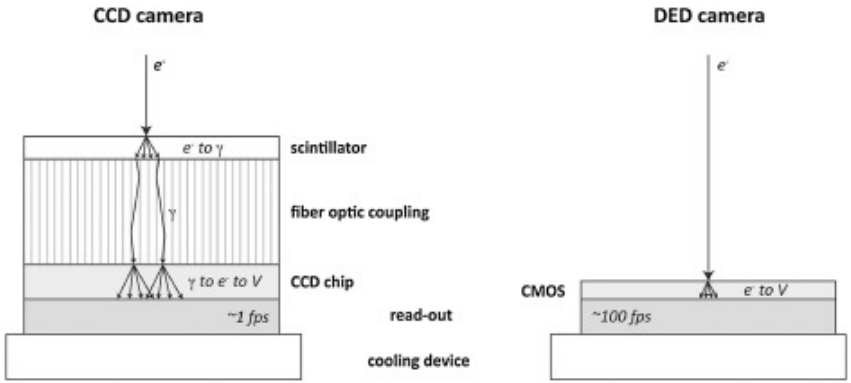
(Föster et al, Philos Trans A Math Phys Eng Sci, 2019)



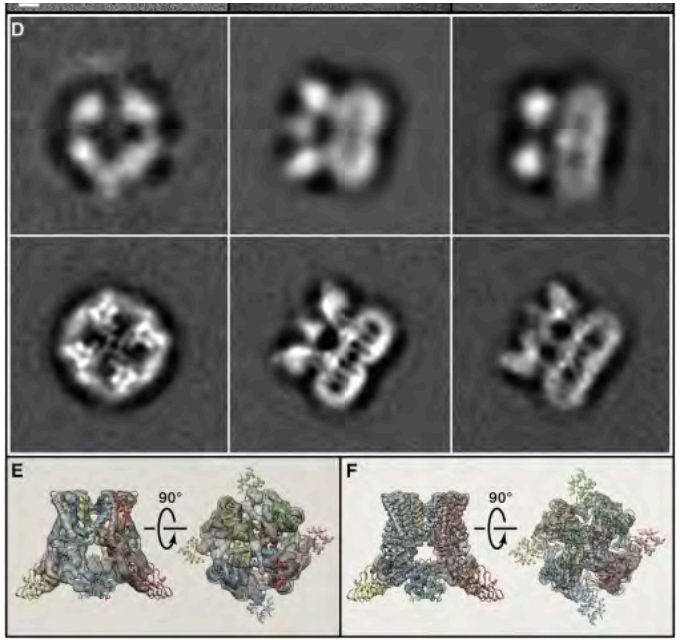
(https://www.dectris.com/features/features-pilatus3-r/high-dynamic-range/)

New methodology and old problems

Direct detector: cryo-EM



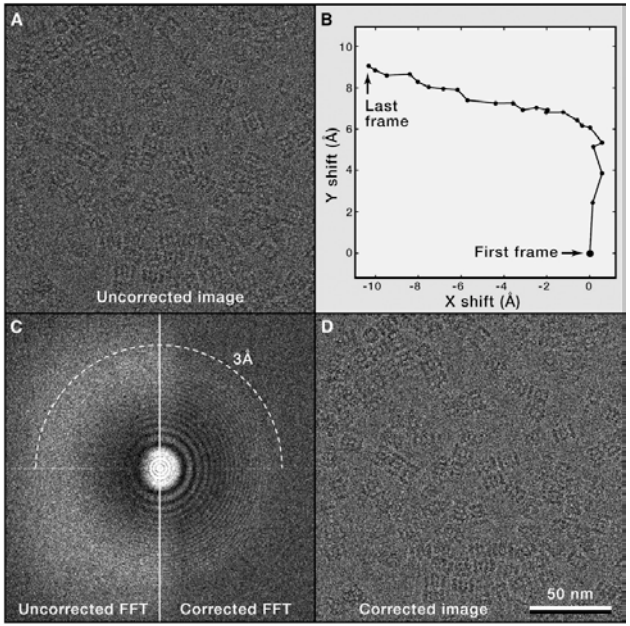
(Koning et al, Annals Anatomy, 2018)



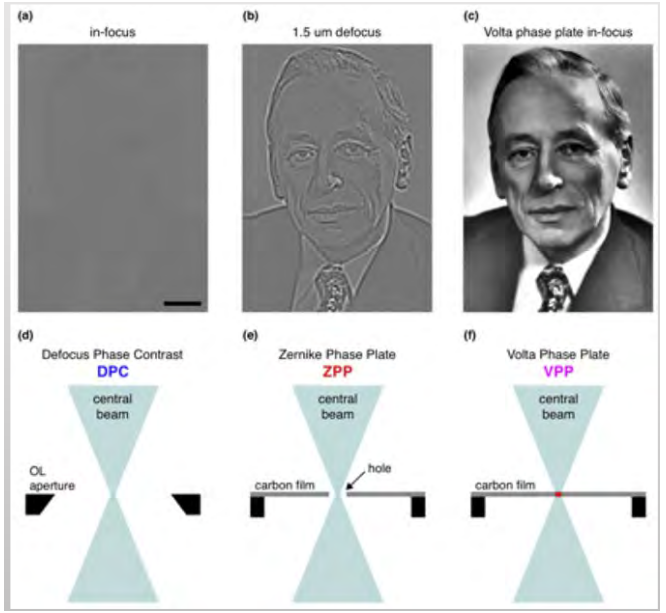
(Yifan Cheng, Cell, 2015)

New methodology and old problems

Motion correction / phase plates: cryo-EM



(Yifan Cheng, Cell, 2015)



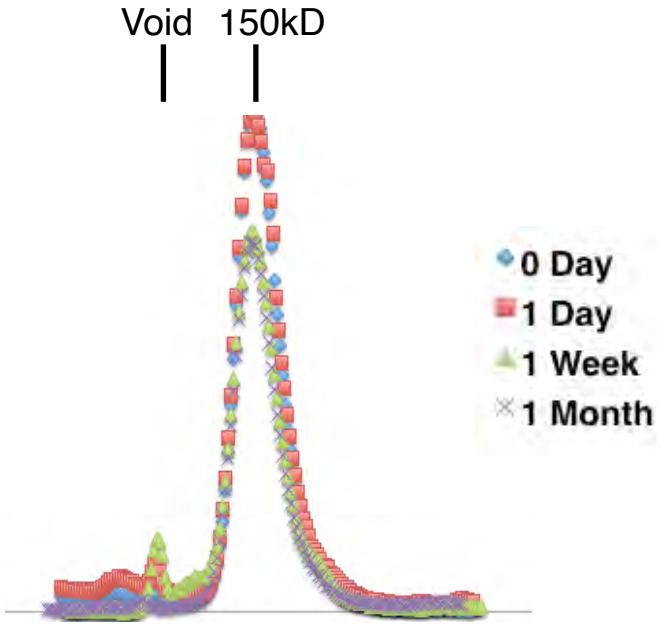
(Danevet & Baumeister, Curr Opin Struct Biol, 2017)

Part II:
STRUCTURAL DETERMINATION

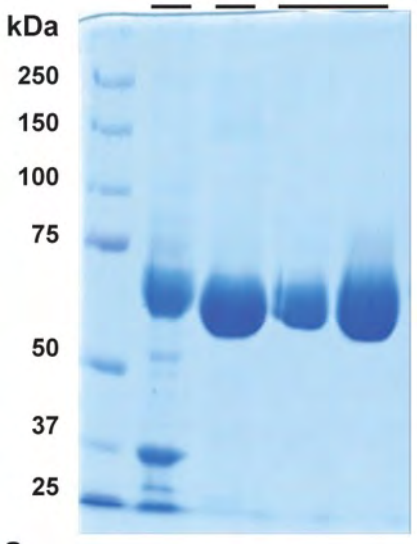
X-ray crystallography: bolts and nuts

- Protein preparation
- Crystallization
- Data collection
- Data processing
- Model building

Protein preparation



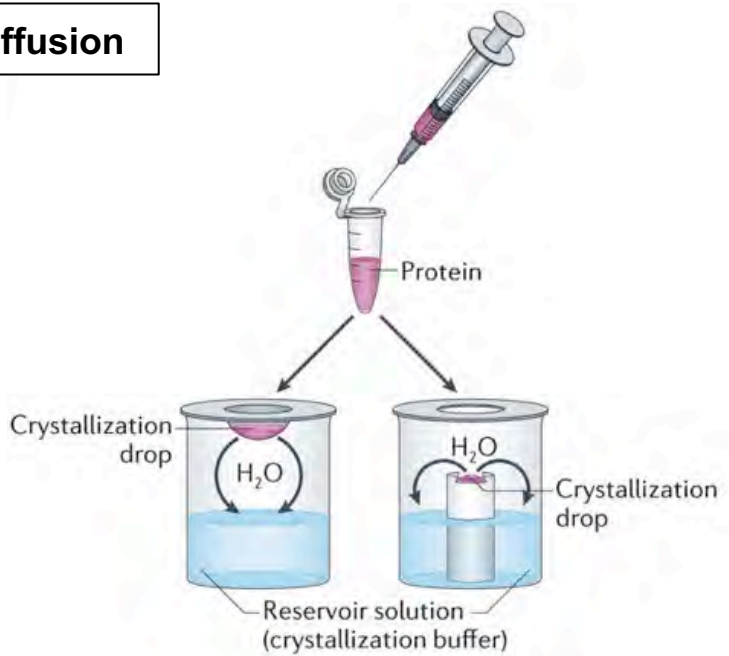
Mono-disperse



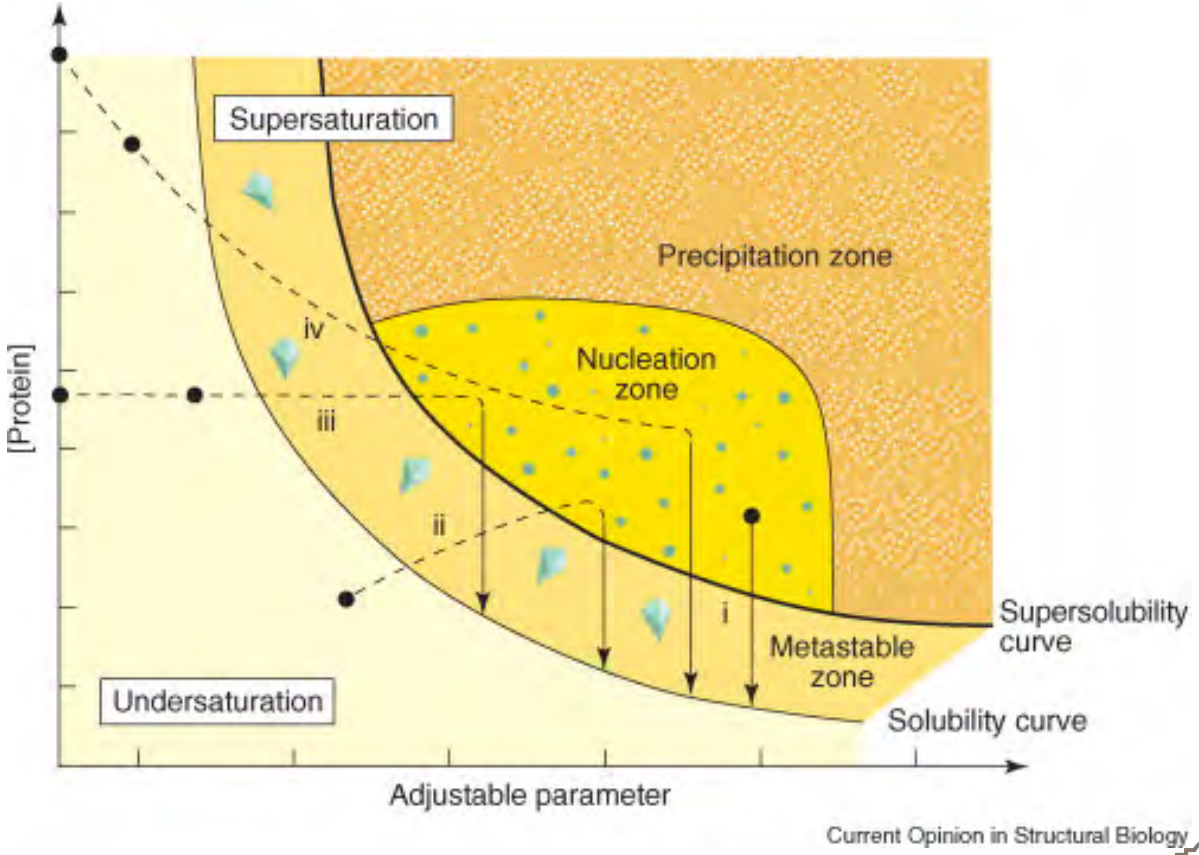
High purity

Crystallization

Coarse vapor diffusion



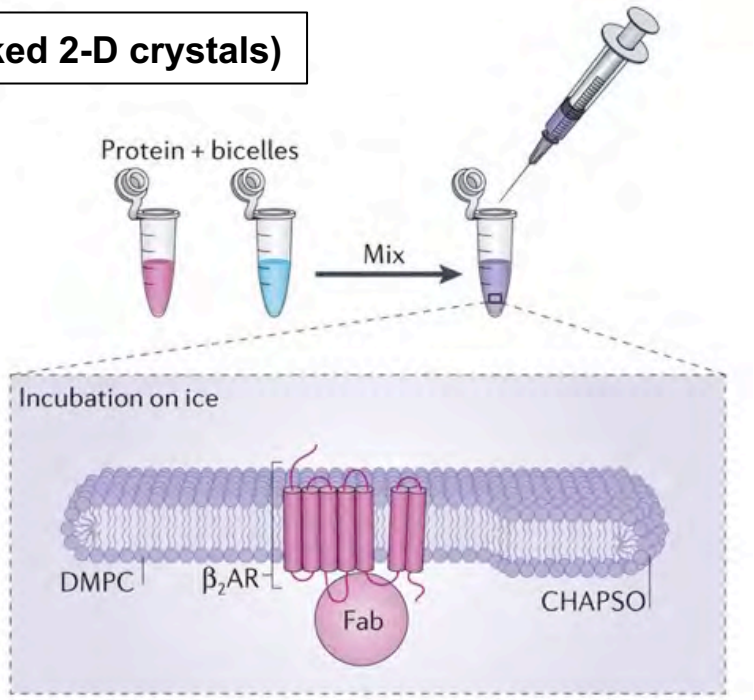
(Ghosh et al, Nature Rev Mol Cell Biol, 2015)



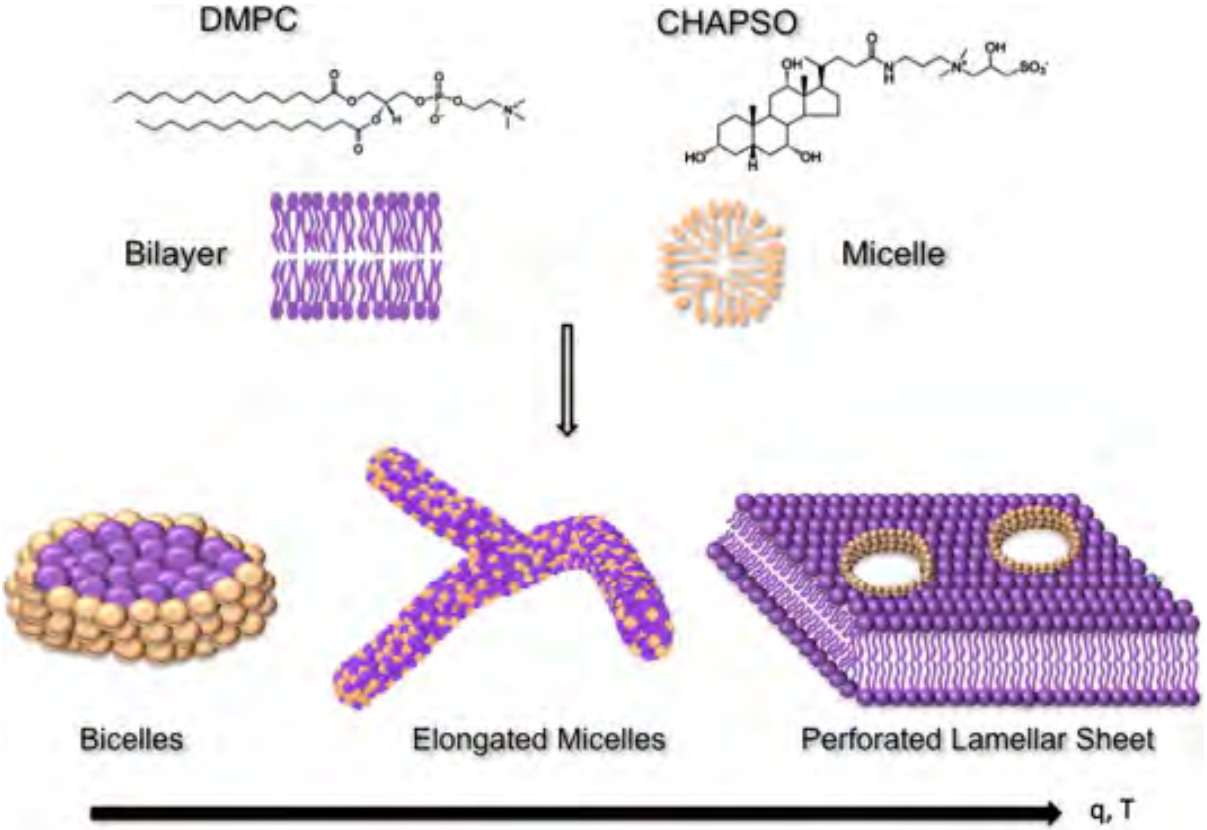
(Naomi Chayen, Curr Opin Struct Biol, 2004)

Crystallization

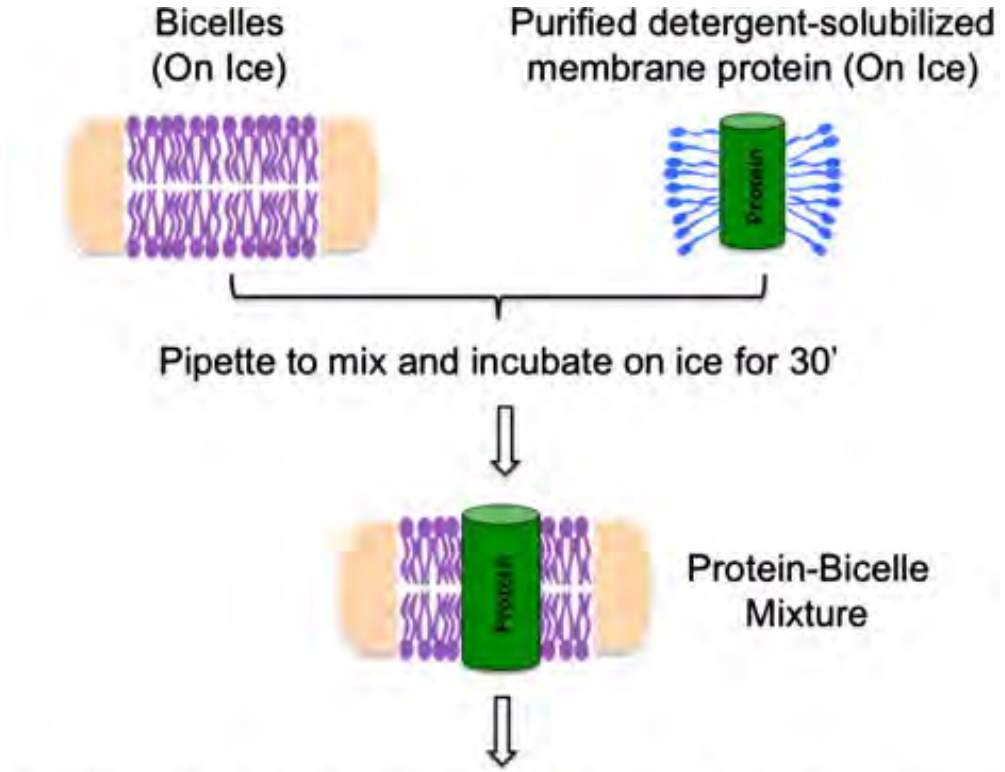
Bicelle (stacked 2-D crystals)



(Ghosh et al, Nature Rev Mol Cell Biol, 2015)

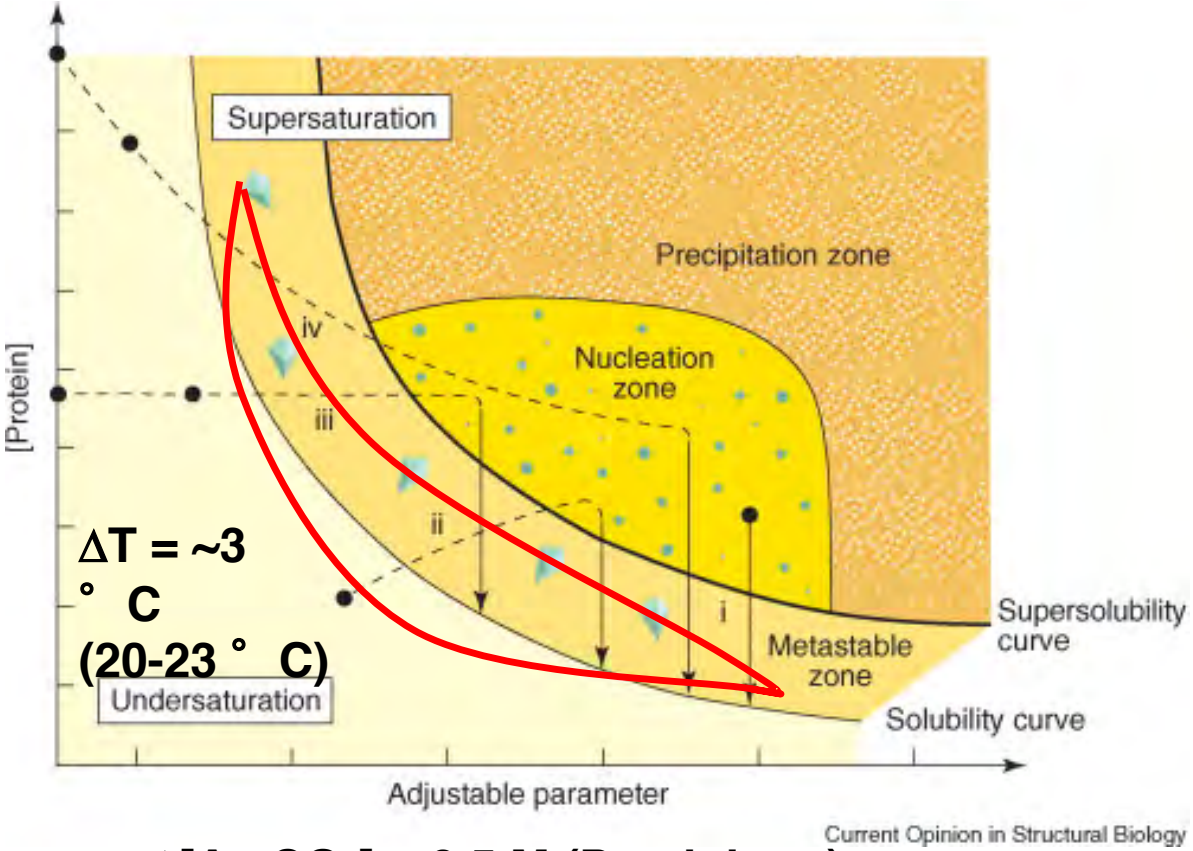


(Ujwal & Bowie, Methods, 2011)



(Ujwal & Bowie, Methods, 2011)

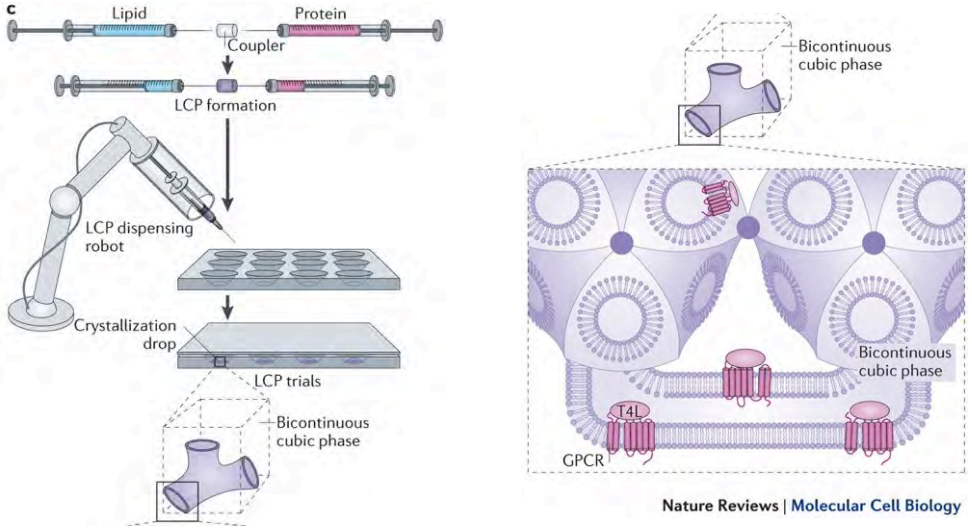
[Protein] = 4-10 mg/mL



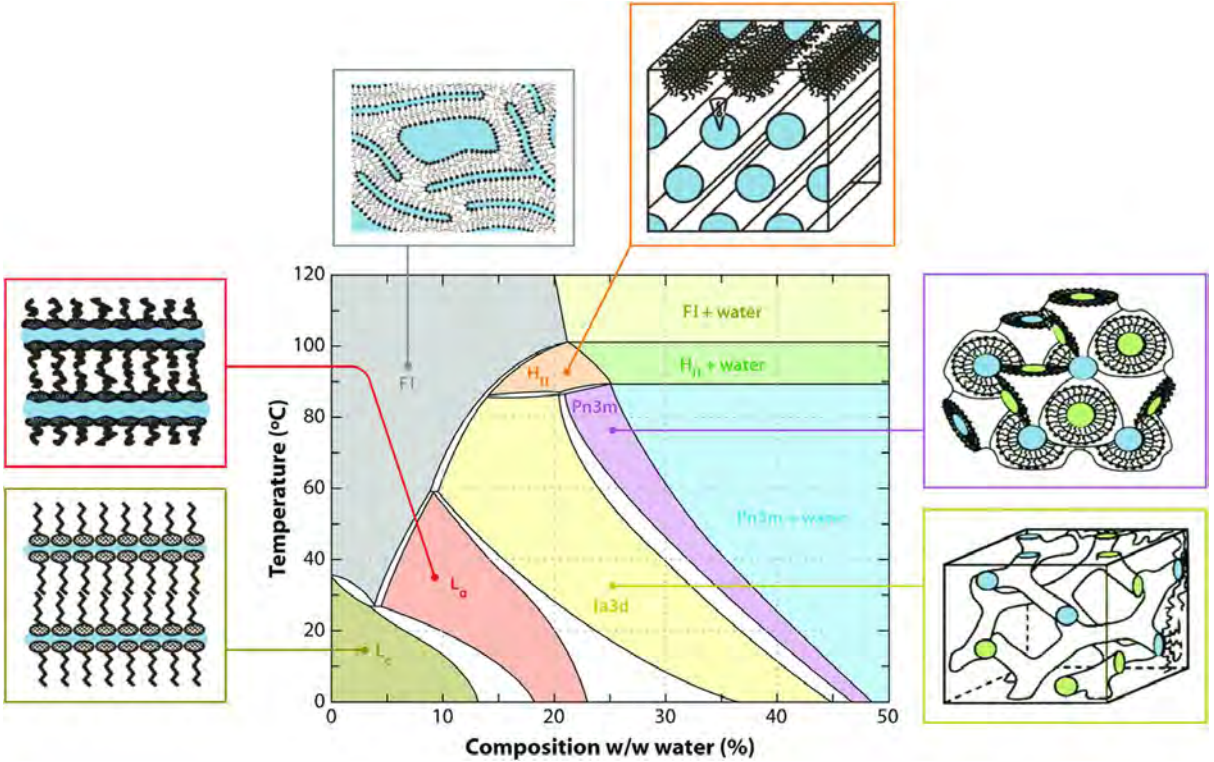
$\Delta[AmSO_4] < 0.5$ M (Precipitant)

Crystallization

In meso lipid cubic phase

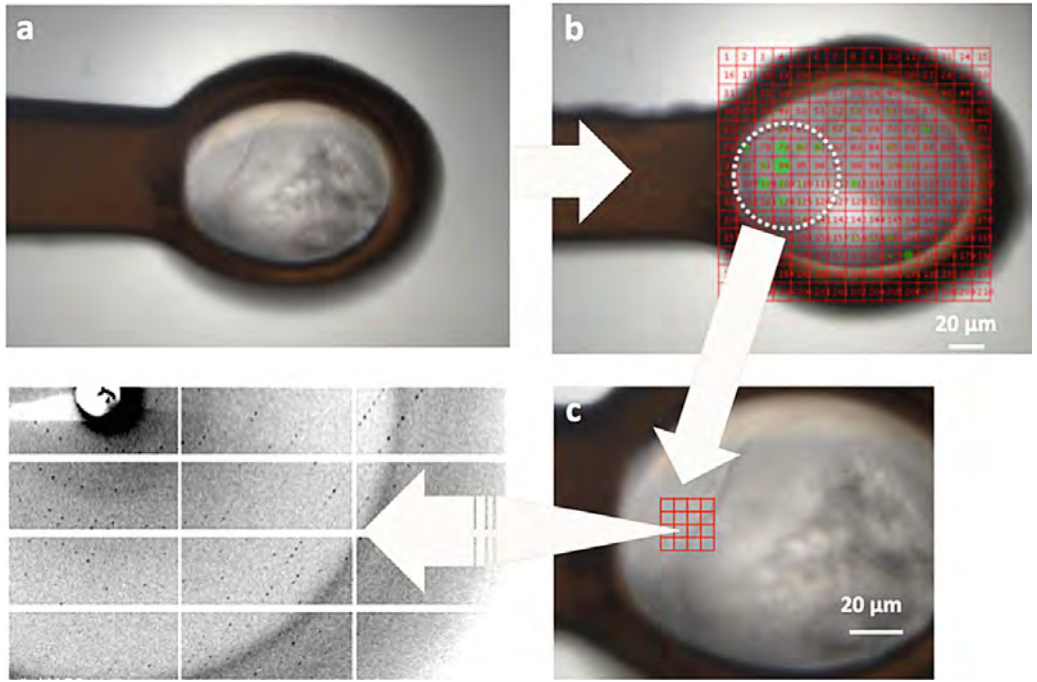


(Ghosh et al, Nature Rev Mol Cell Biol, 2015)



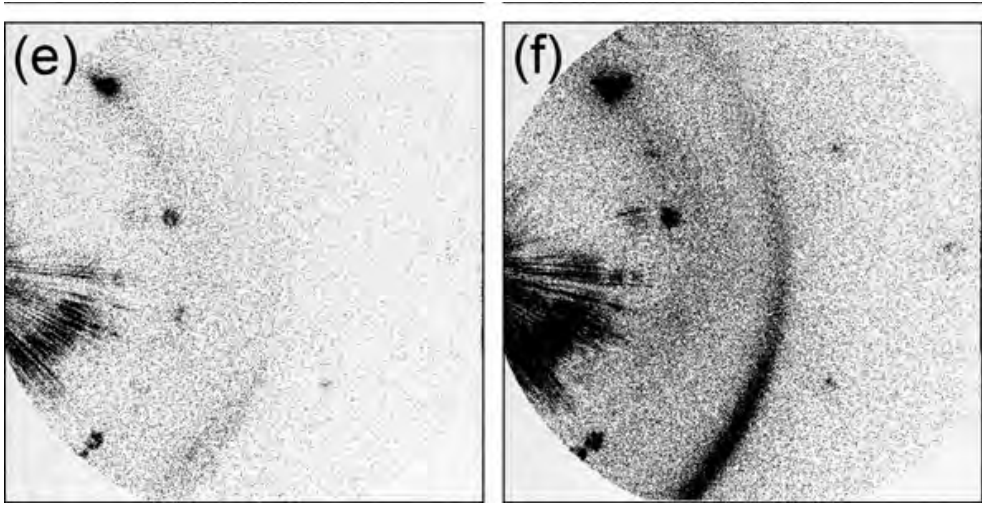
(Caffrey, Acta Cryst F, 2015)

Data Collection



(Warren et al, in "The Next Generation in Membrane Protein Structure Determination", 2016)

Data Collection



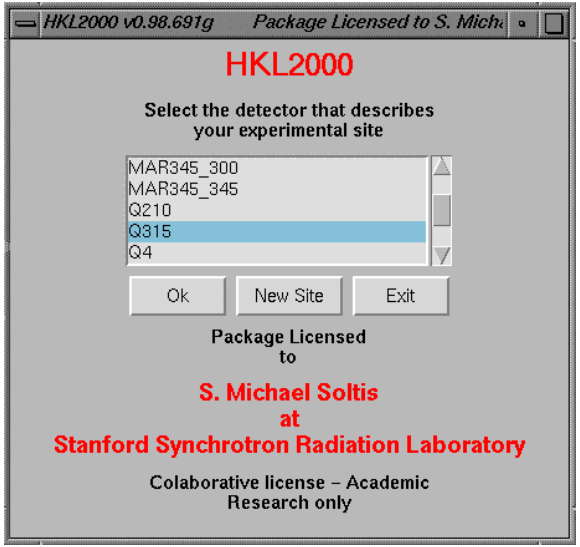
1s exposure

10s exposure

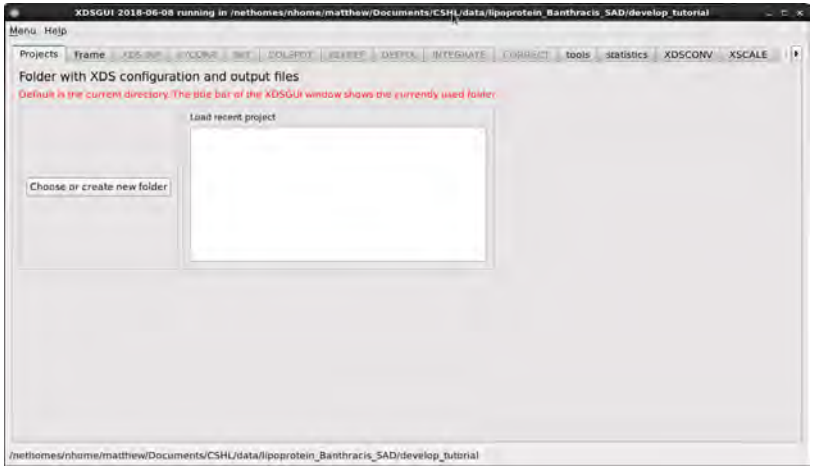
(Cherezov & Caffrey, Faraday Discuss, 2007)

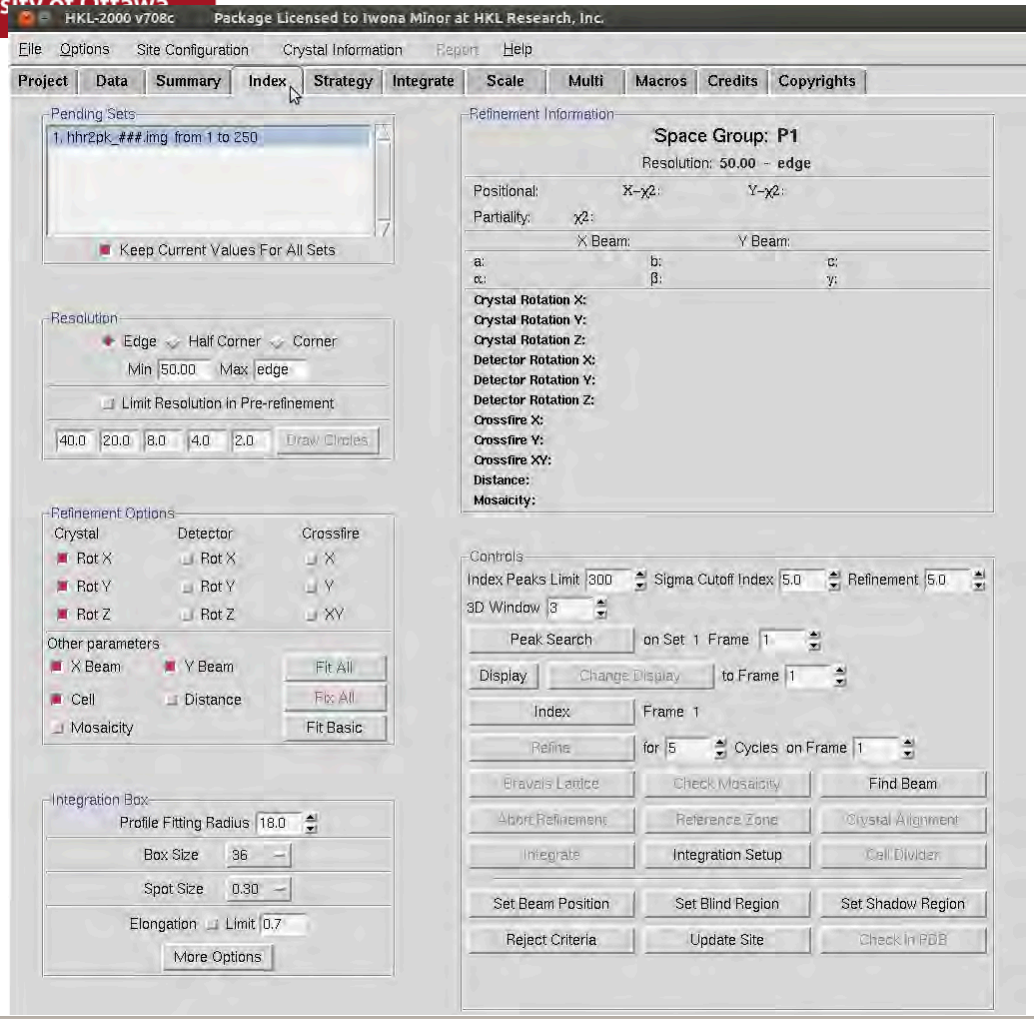
Data Processing

HKL 2000/3000



XDS




HKL-2000 v708c
Package Licensed to Iwona Minor at HKL Research, Inc.
File Options Site Configuration Crystal Information Report Help
Project Data Summary Index Strategy Integrate Scale Multi Macros Credits Copyrights
Pending Sets
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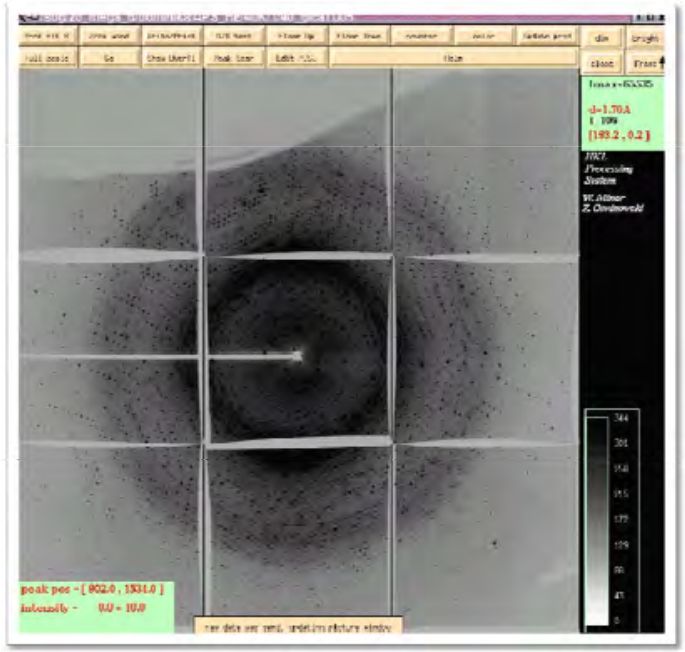
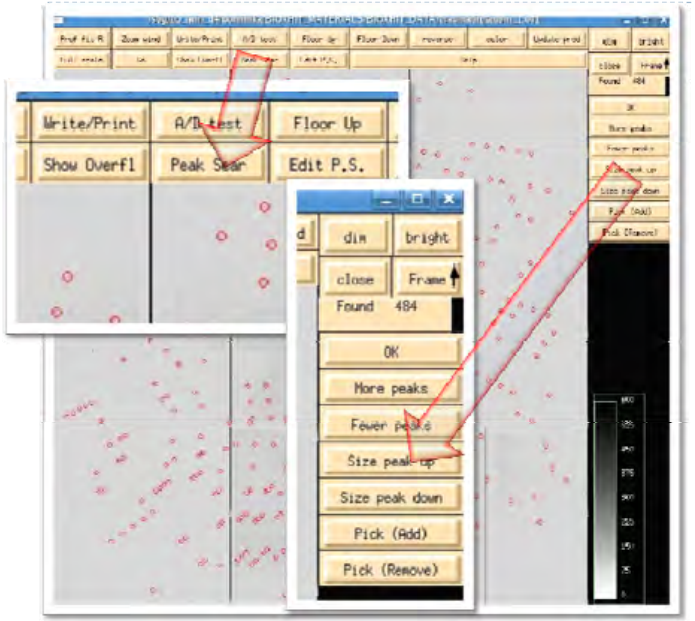
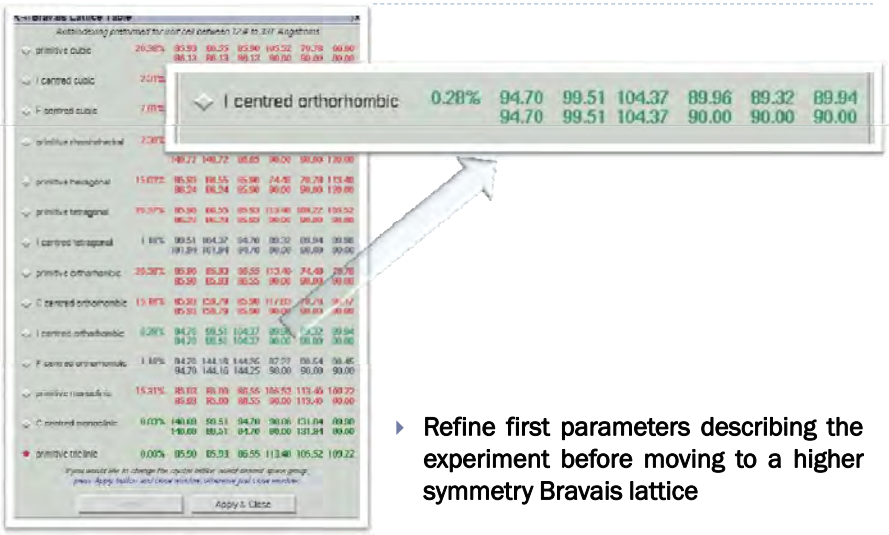
Keep Current Values For All Sets
Resolution
Edge Half Corner Corner
Min 50.00 Max edge
Limit Resolution in Pre-refinement
40.0 20.0 8.0 4.0 2.0 Draw Circles
Refinement Options
Crystal Detector Crossfire
Rot X Rot X X
Rot Y Rot Y Y
Rot Z Rot Z XY
Other parameters
X Beam Y Beam Fit All
Cell Distance Fix All
Mosaicity Fit Basic
Integration Box
Profile Fitting Radius 18.0
Box Size 36
Spot Size 0.30
Elongation Limit 0.7
More Options
Refinement Information
Space Group: P1
Resolution: 50.00 - edge
Positional: X-χ2: Y-χ2:
Partiality: χ2:
X Beam: Y Beam:
a: b: c:
α: β: γ:
Crystal Rotation X:
Crystal Rotation Y:
Crystal Rotation Z:
Detector Rotation X:
Detector Rotation Y:
Detector Rotation Z:
Crossfire X:
Crossfire Y:
Crossfire XY:
Distance:
Mosaicity:
Controls
Index Peaks Limit 300 Sigma Cutoff Index 5.0 Refinement 5.0
3D Window 3
Peak Search on Set 1 Frame 1
Display Change Display to Frame 1
Index Frame 1
Refine for 5 Cycles on Frame 1
Bravais Lattice Check Mosaicity Find Beam
Abort Refinement Reference Zone Crystal Alignment
Integrate Integration Setup Cell Divider
Set Beam Position Set Blind Region Set Shadow Region
Reject Criteria Update Site Check in PDB

Inspection of diffraction data / Preparation for data merging

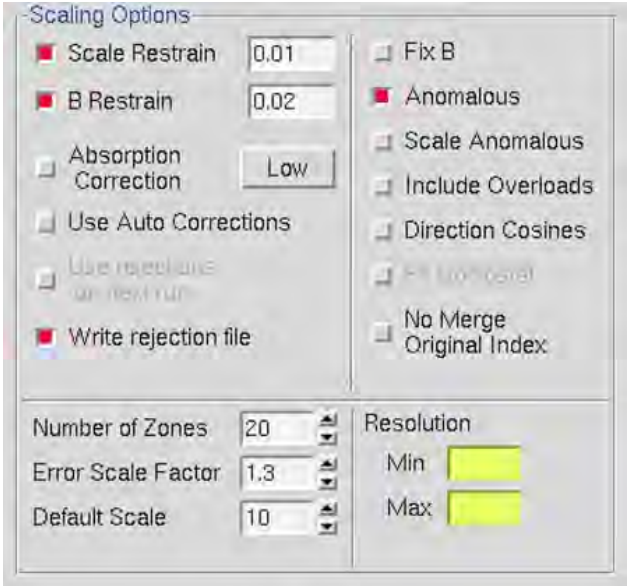


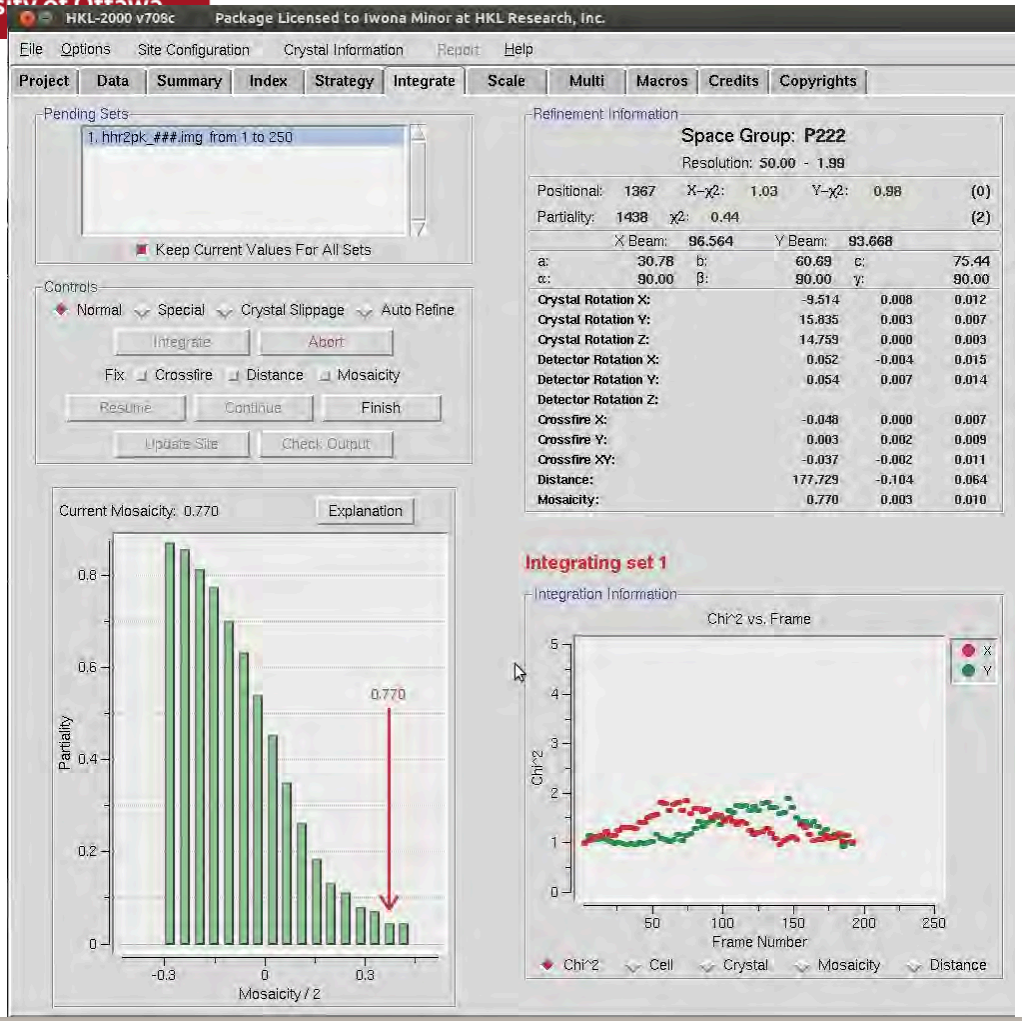


Space group / Scaling / Integration



- Refine first parameters describing the experiment before moving to a higher symmetry Bravais lattice




HKL-2000 v708c
Package Licensed to Iwona Minor at HKL Research, Inc.
File Options Site Configuration Crystal Information Report Help
Project Data Summary Index Strategy Integrate Scale Multi Macros Credits Copyrights
Pending Sets
1. hhr2pk_###.img from 1 to 250
Keep Current Values For All Sets
Controls
Normal Special Crystal Slippage Auto Refine
Integrate Abort
Fix Crossfire Distance Mosaicity
Resume Continue Finish
Update Site Check Output
Refinement Information
Space Group: P222
Resolution: 50.00 - 1.99
Positional: 1367 X-χ2: 1.03 Y-χ2: 0.98 (0)
Partiality: 1438 χ2: 0.44 (2)
X Beam: 96.564 Y Beam: 93.668
a: 30.78 b: 60.69 c: 75.44
α: 90.00 β: 90.00 γ: 90.00
Crystal Rotation X: -9.514 0.008 0.012
Crystal Rotation Y: 15.835 0.003 0.007
Crystal Rotation Z: 14.759 0.000 0.003
Detector Rotation X: 0.052 -0.004 0.015
Detector Rotation Y: 0.054 0.007 0.014
Detector Rotation Z:
Crossfire X: -0.048 0.000 0.007
Crossfire Y: 0.003 0.002 0.009
Crossfire XY: -0.037 -0.002 0.011
Distance: 177.729 -0.104 0.064
Mosaicity: 0.770 0.003 0.010
Current Mosaicity: 0.770
Explanation
Partiality
0.770
Mosaicity / 2
Integrating set 1
Integration Information
Chi^2 vs. Frame
Chi^2
Frame Number
X
Y
Chi^2 Cell Crystal Mosaicity Distance

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reading from a file: C3_2_0001.x
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reading from a file: B13_1_0039.x
reading from a file: B13_1_0040.x

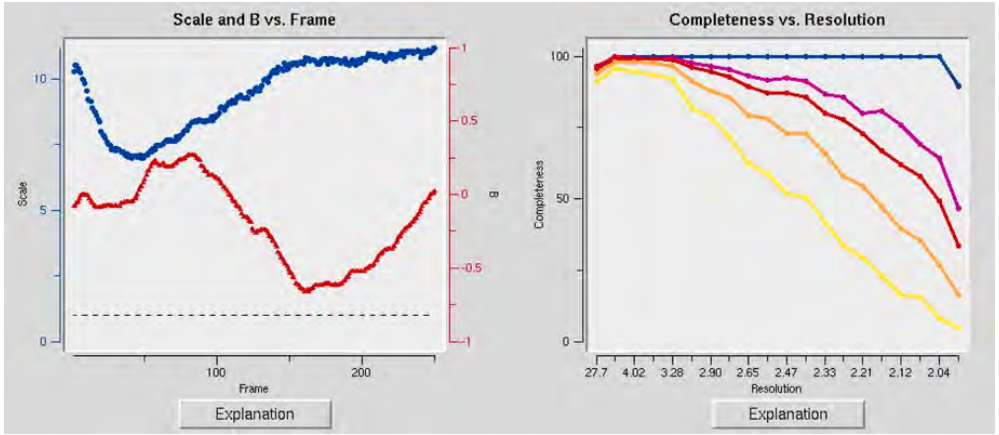
Output / Statistics

Crystal - Global Refinement

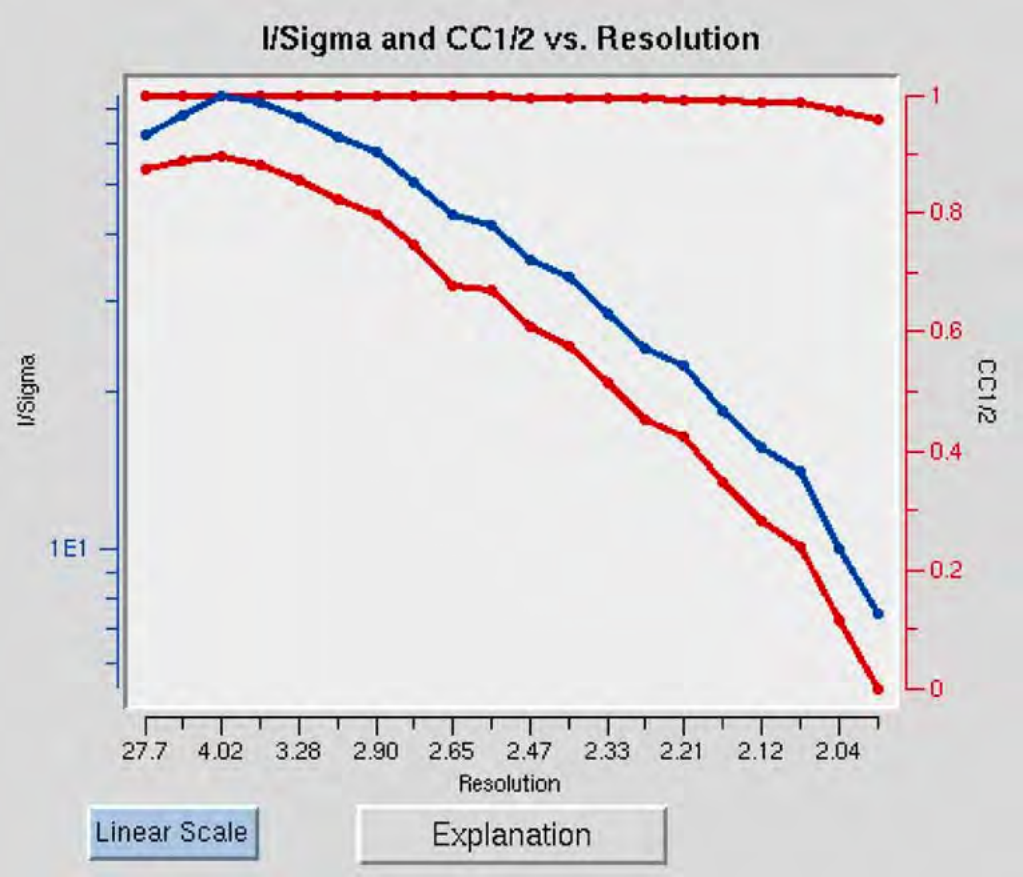
Space Group	P21212				
Resolution	50.00 - 1.99				
a	30.78	± 0.01	α	90	±
b	60.72	± 0.01	β	90	±
c	75.44	± 0.01	γ	90	±
Cell Volume	140989.5				
Mosaicity Range	0.64 - 0.75				

Global Statistics

Error Scale Factor	1.619	Total Number of Reflections	68932
Error Systematic	0.034	Number of Reflections Marked for Rejection	33
Radiation Damage	0.016	Percentage of Reflections Marked for Rejection	0.05
Anomalous Signal	0.038	Percentage of Reflections Rejected	0
Internal Non-isomorphicity	0.005	Total Rmerge / Rmeas / Rpim	0.033 / 0.036 / 0.014

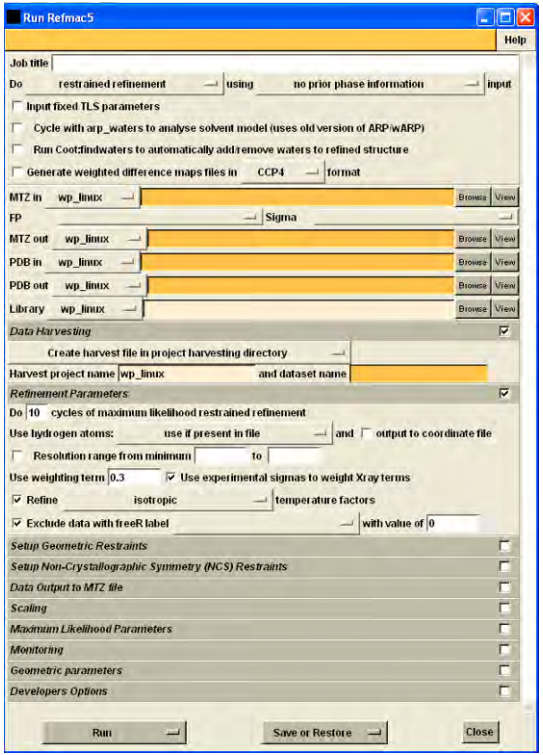


Output / Statistics

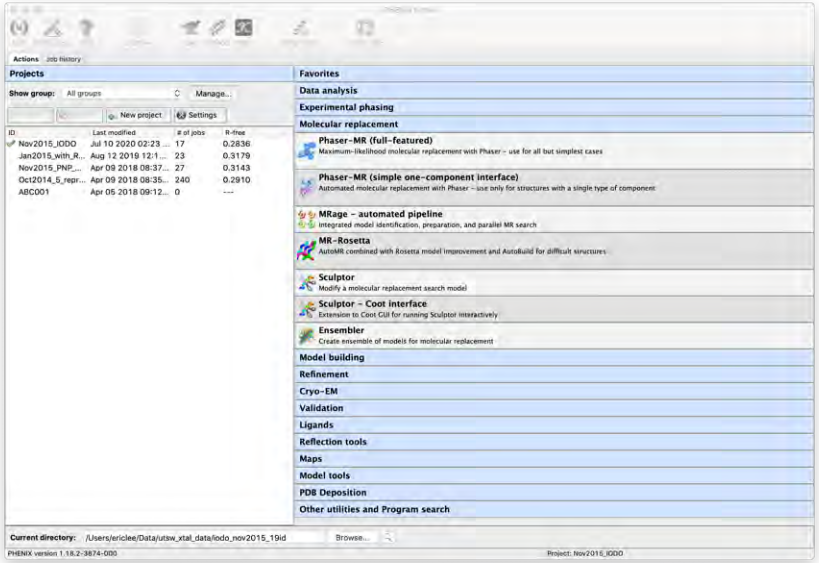


Model Building

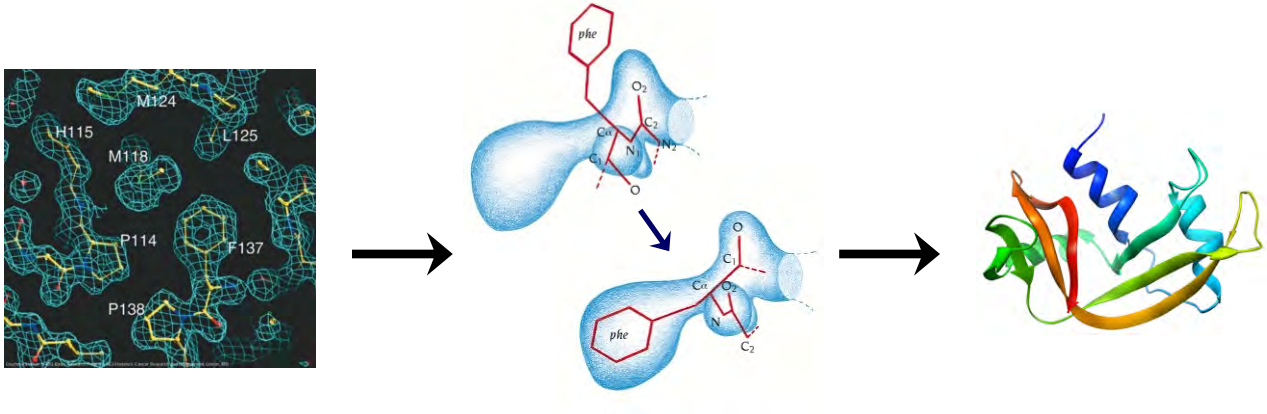
REFMAC



PHENIX



1. Fitting the electron density to build a model



Electron Density Map

Fitting the aa sequence into the map

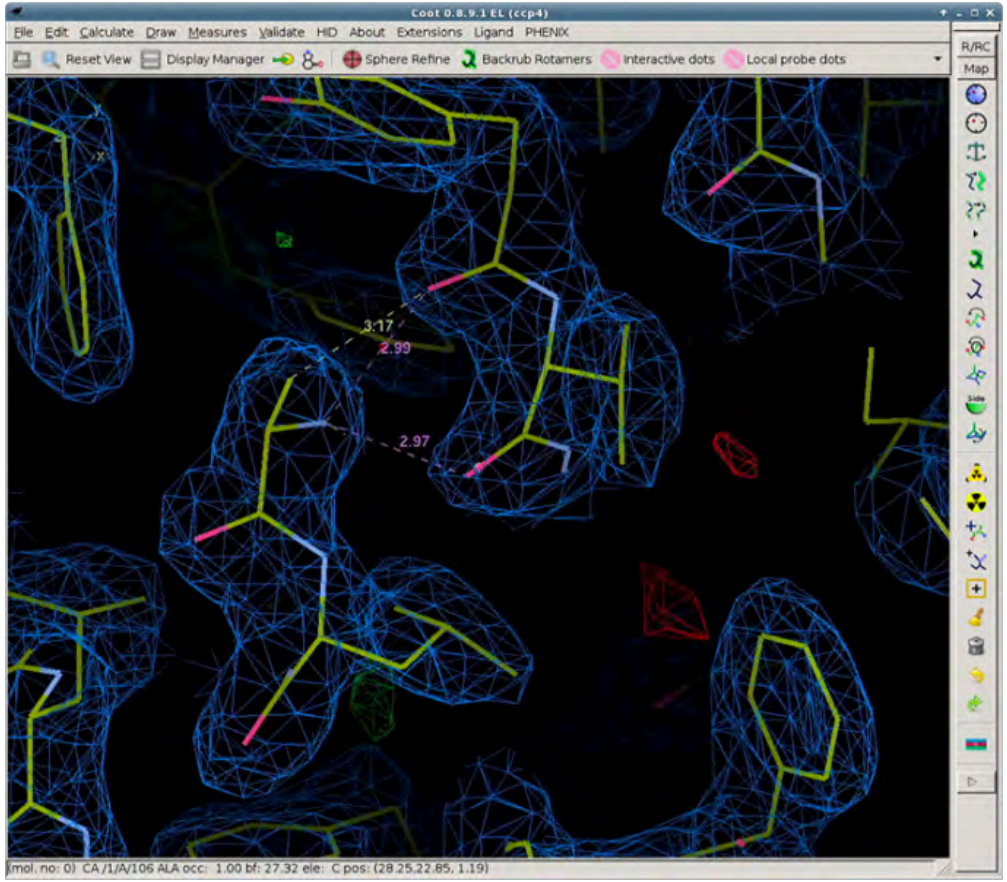
Starting Protein Model

The next step is to fit the amino acid sequence of the protein into the electron density map. You do this "by hand" *in silico*, i.e. using a computer.

Once you have a reasonable fit – i.e. a starting model - you then use computer programs to rotate bond angles, adjust bond lengths, etc. to improve fit between the amino acid sequence and the electron density to arrive at the final protein model.

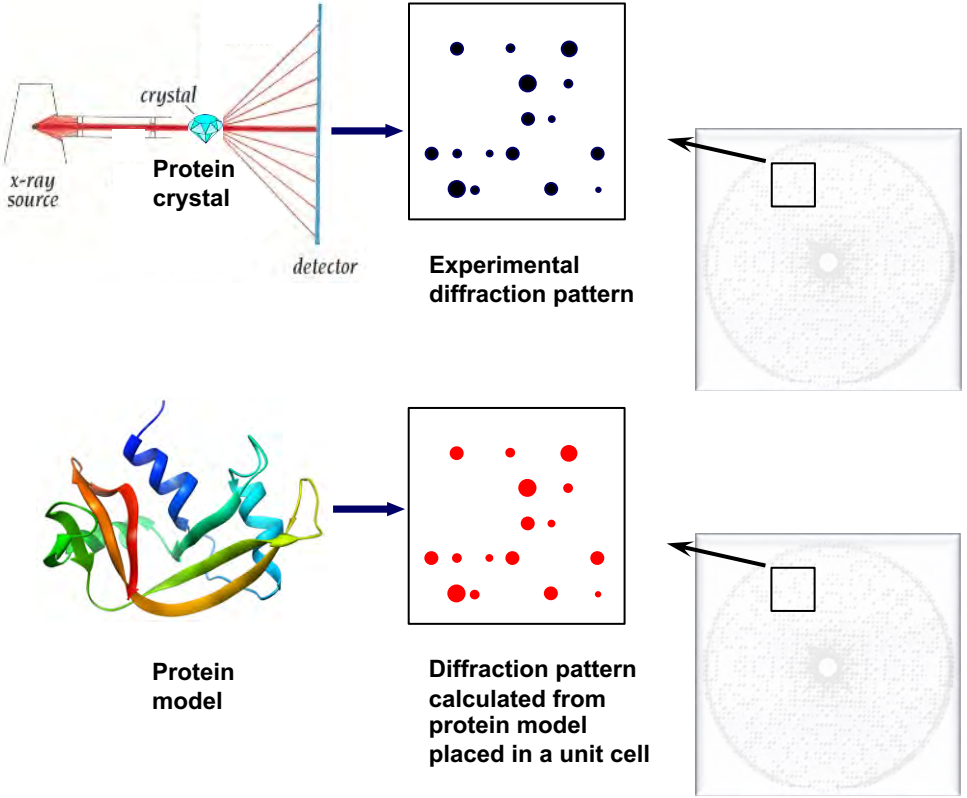
Model Building

COOT



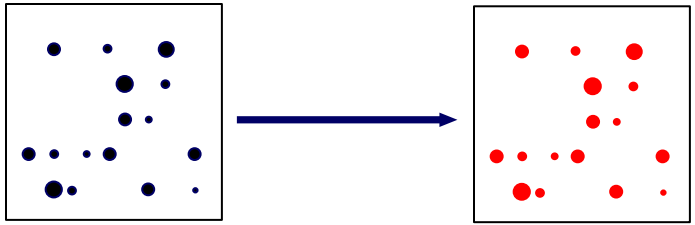
2. Parameters used to assess accuracy of a protein model

ii) R-factor (accuracy of "fit")



2. Parameters used to assess accuracy of a protein model

ii) R-factor (accuracy of "fit")



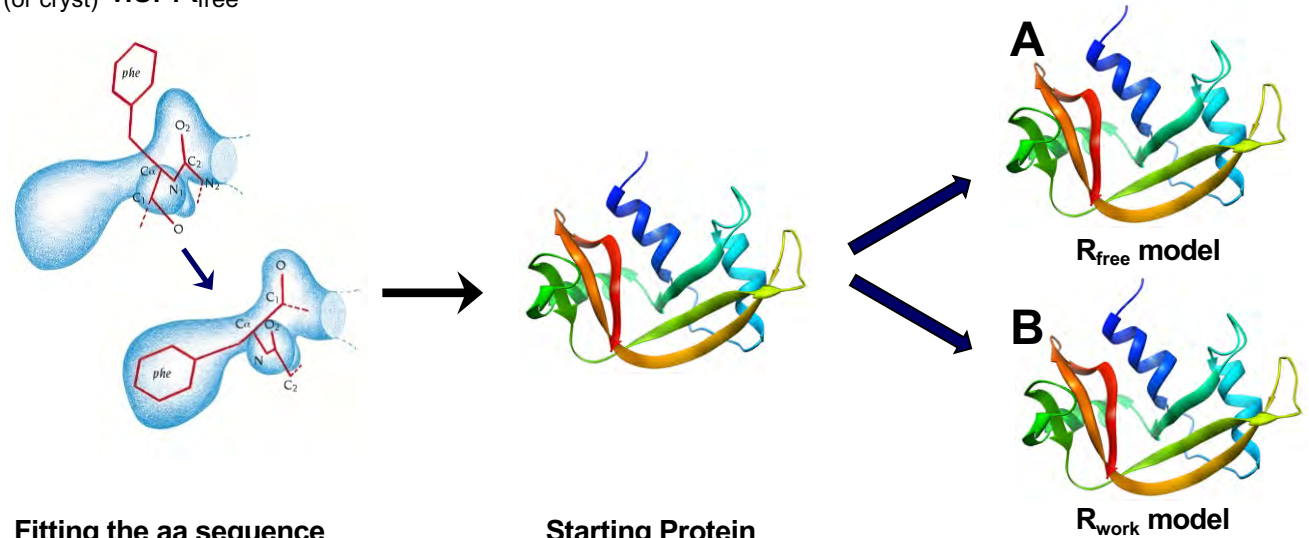
Experimental (observed) Diffraction Pattern

Calculated Diffraction Pattern

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

2. Parameters used to assess accuracy of a protein model

ii) $R_{work\ (or\ cryst)}$ v.s. R_{free}



Fitting the aa sequence into the map

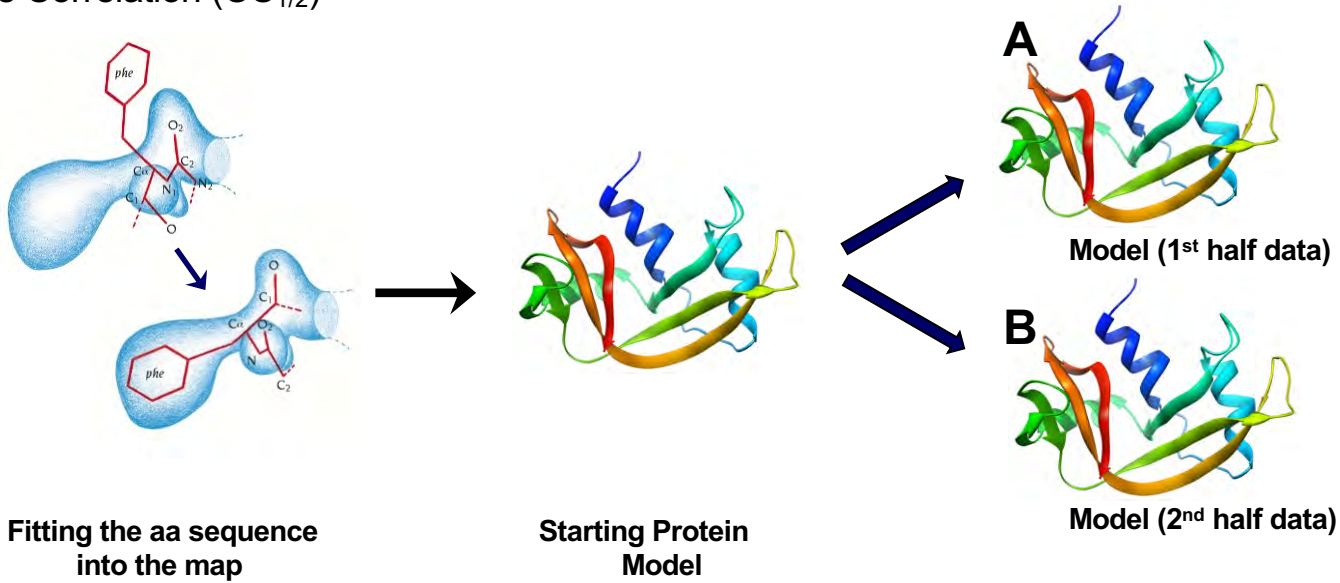
Starting Protein Model

To avoid over-modeling, we assess by setting aside a small set of data and measuring free R-factor (**R_{free}**), how well the model predicts experimental observations that are not themselves used to fit the model. Then compare with the R-factor calculated for the working set (**R_{work}**).

In a good model, R_{free} is close in value to R_{work} (i.e. within ~10% difference).

2. Parameters used to assess accuracy of a protein model

iii) Cross Correlation ($CC_{1/2}$)

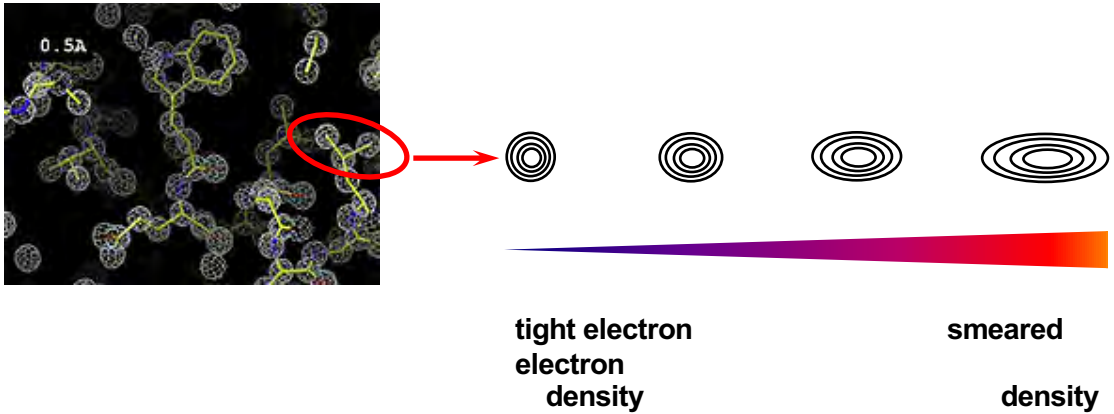


$$CC_{1/2} = \frac{\sigma_\tau^2}{\sigma_\tau^2 + \sigma_\varepsilon^2} = \frac{\langle I^2 \rangle - \langle I \rangle^2}{\langle I^2 \rangle - \langle I \rangle^2 + \sigma_\varepsilon^2}$$

$CC_{1/2}$ between intensity estimates from half data sets. Primary indicator for use for selecting high resolution cutoff for data processing. Is related to the effective signal to noise of the data

2. Parameters used to assess accuracy of a protein model

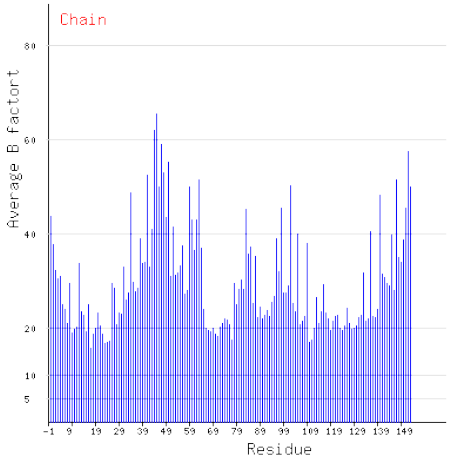
iv) B-factor



Even in a high resolution electron density map, some atoms have tight density around each atom (often in the protein core), while others have "smeared" electron density (often on the protein surface). The smearing of density can arise due to different factors, including movement of the side chain in the crystal. The B-factor or "temperature" fact defines the amount of smearing and thus how accurately one can define the position of a side chain.

2. Parameters used to assess accuracy of a protein model

iv) B-factor

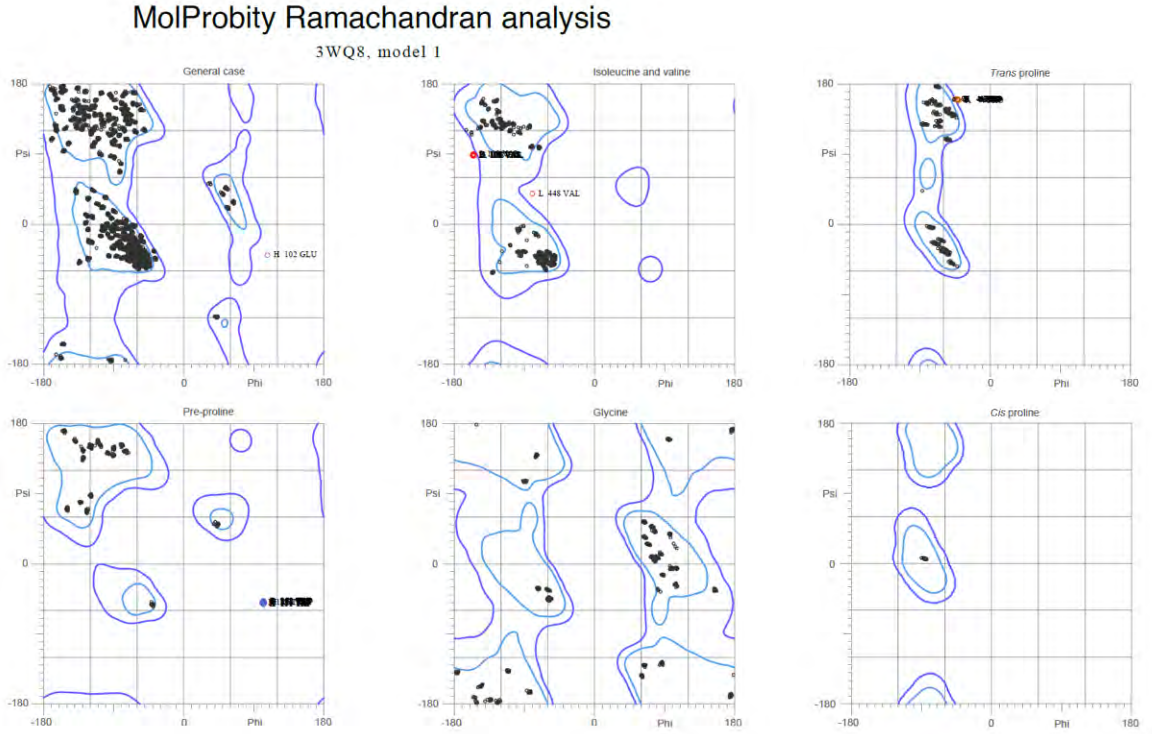


Low B-factor **High B-factor**

B-factor values of less than 30 Å^2 signify confidence in an side chains position due to tight electron density, while a temperature value of greater than 60 Å^2 signifies disorder due to smearing of the density. The Protein Data Bank has plots of B-factor versus residue number. Residues on the surface of proteins tend to have higher B-factors!

2. Parameters used to assess accuracy of a protein model

v) Ramachandran Plots

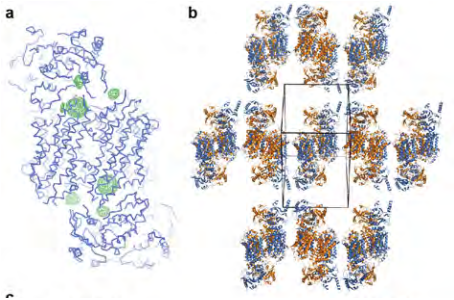


When solving a structure, one always checks the Ramachandran Plots to ensure that residues are found within the "allowed regions".

"Table 1" of structural biology works

Extended Data Table 1 | Data processing and refinement statistics

	Native*	$[PW_{12}O_{40}^{3-}]^{\dagger}$	$[(CH_3)_3Pb^-]$	$(Ta_6Br_{12}^{2+})$
Data collection				
Beamline	19-ID-D/23-ID-D‡	19-ID-D	19-ID-D	19-ID-D
Space group	I 222	I 222	I 222	I 222
Cell dimensions				
a, *b*, *c* (Å)	173.6, 224.8, 253.3	175.5, 227.5, 254.5	174.6, 225.9, 253.4§ 173.6, 225.9, 252.7‖	176.0, 228.0, 253.7
Resolution (Å)	50-3.9 (3.93-3.9)	50-5.0 (5.04-5.0)	50-4.5 (4.54-4.5)	50-5.0 (5.04-5.0)
R_{sym} or R_{merge}	16.1 (NA)	13.5 (33.5)	8.7 (NA)§ 7.1 (NA)‖	8.8 (NA)
$\langle I \rangle / \langle \sigma I \rangle$	8.8 (0.15)	5.1 (1.4)	8.0 (0.45)§ 6.1 (0.18)‖	8.6 (0.50)
Completeness (%)	99.4 (84.2)	94.4 (47.3)	97.4 (55.9)§ 94.7 (54.7)‖	81.1 (18.3)
Redundancy	18.9 (2.5)	3.1 (1.7)	6.0 (2.3)§ 4.3 (2.5)‖	3.7 (1.3)
Refinement				
Resolution (Å)	25-3.94			
No. reflections	34889			
R_{work}/ R_{free}	24.5 / 32.9			
No. atoms				
Protein	18151			
R.m.s deviations				
Bond lengths (Å)	0.010			
Bond angles (°)	1.64			



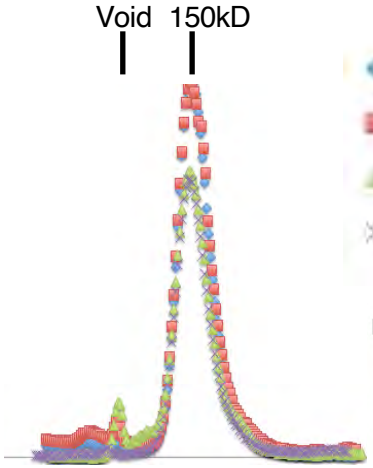
(Human ABCG5/G8, 2016)

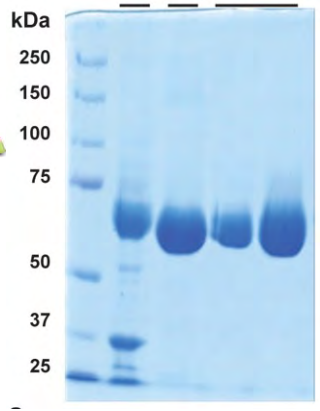
Cryo-electron microscopy: bolts and nuts

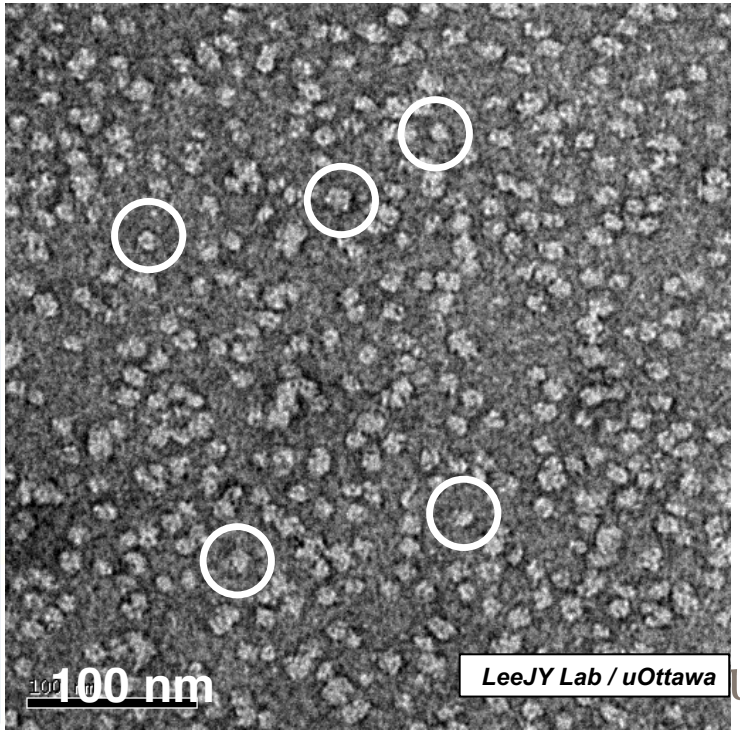
- Protein preparation.
- Negative-stain screenig
- Data collection
- Data processing
- Model building

Protein preparation

(Same standard as X-ray crystallography)

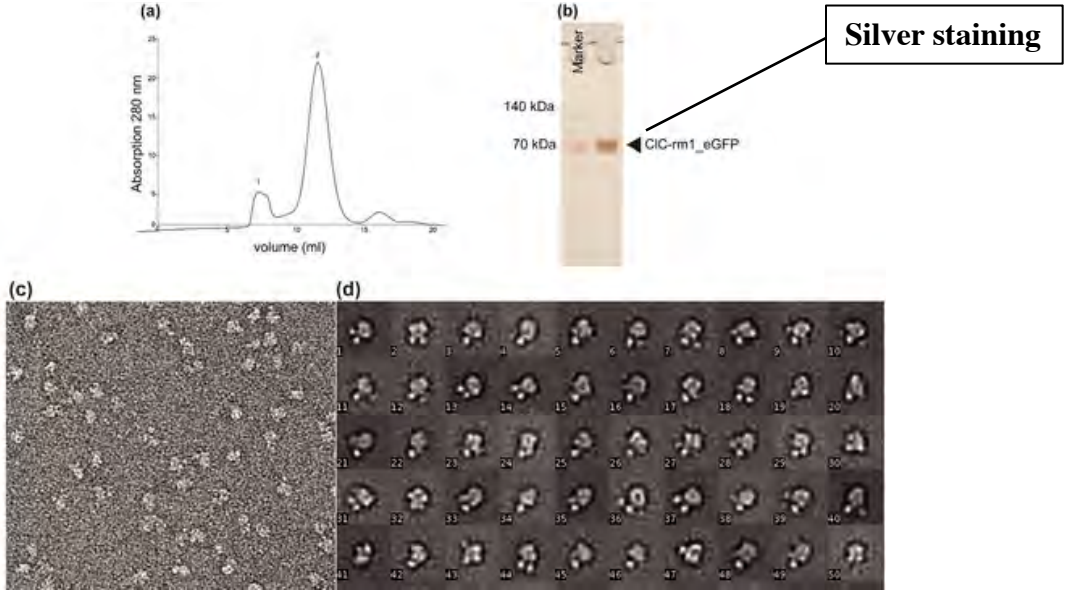






Protein preparation

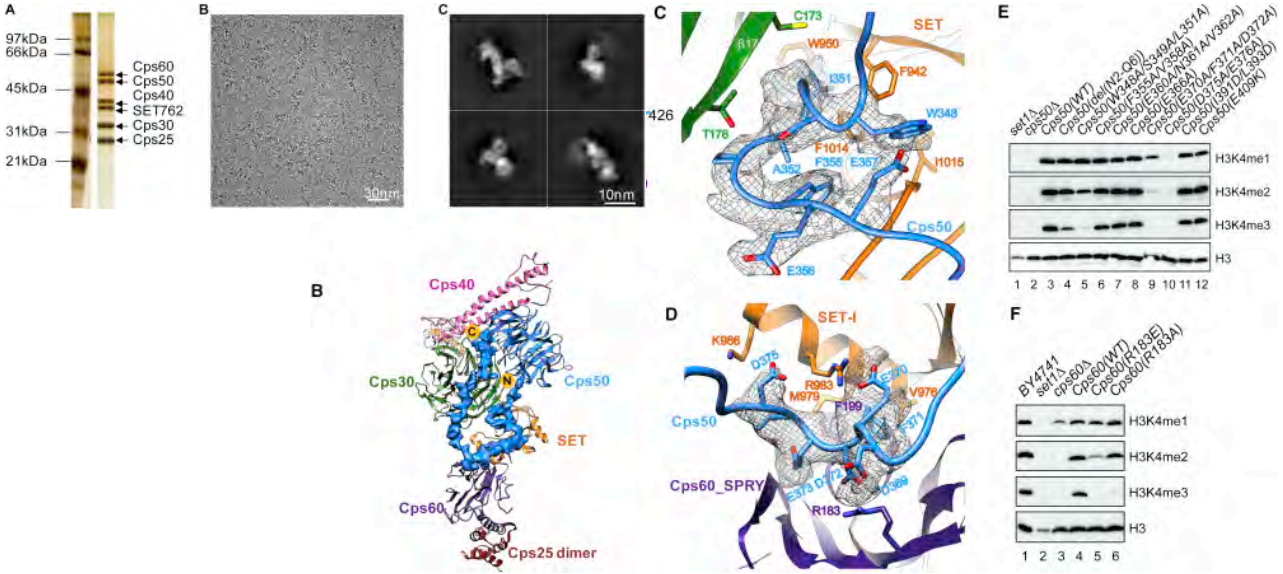
(But no need to purify several mg proteins!)



(Abeyrathne & Grigorieff, PLOS ONE, 2017)

Protein preparation

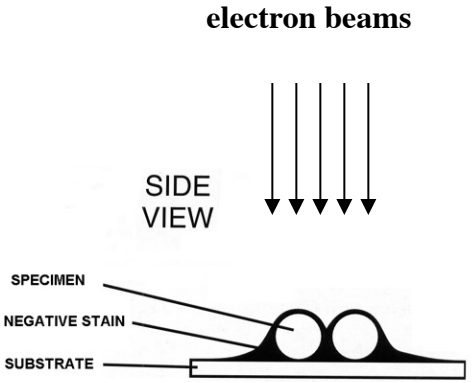
(Functionally characterized)



(Qu/Takahashi/Yang et al, Cell, 2018)

EM Samples: Negative Staining

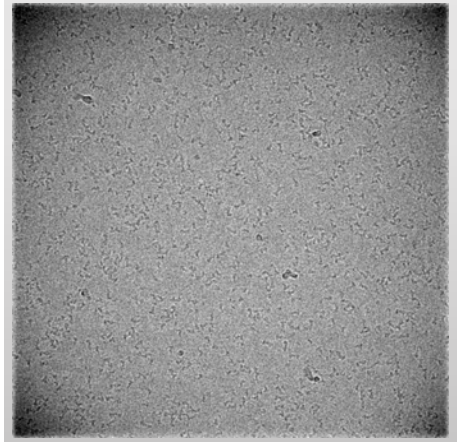
- Principle:
  - Embedding objects in a layer of heavy-metal salts that surround the proteins like a shell.
  - Shape of objects are visible in contrast to the optically opaque stains.
- Benefits:
  - Small amount of proteins (0.01 mg/mL)
  - Easy and quick (preparation and imaging)
  - No need of high-end microscope; diagnostic
- Downsides:
  - Low resolution (*e.g.*, high noise from stains)
  - Artifacts (lack of hydration)



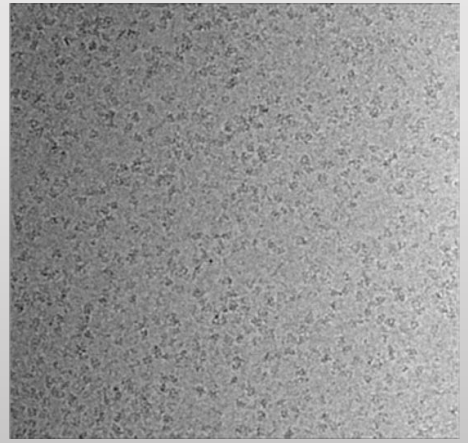
(Brenner & Horne, BBA, 1959)

EM Samples: Protein Concentrations

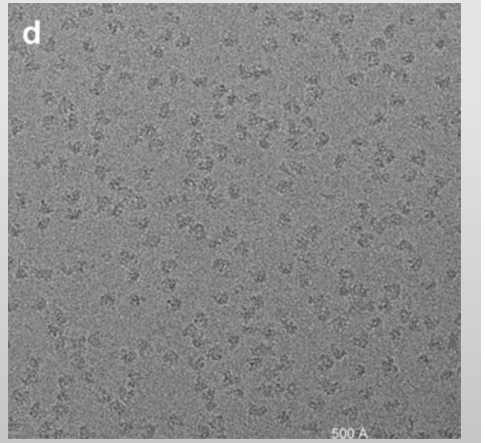
- Negative staining: 0.01-0.05 mg/mL
- Cryo: 0.1-5 mg/mL



CTF3 complex, 130 kDa
0.2 mg/ml



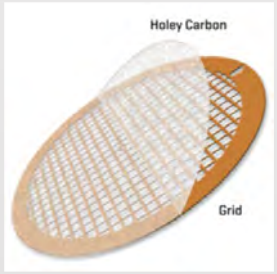
Cas12a-AcrVA4/5 complex, 200 kDa
3 mg/ml



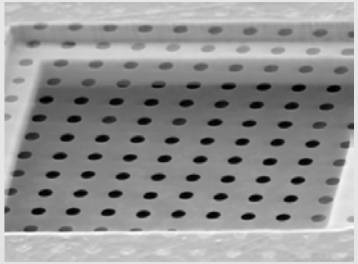
APC/C complex, 1.2 MDa
0.1 mg/ml on continuous carbon film

Preparation of cryo-EM Grids

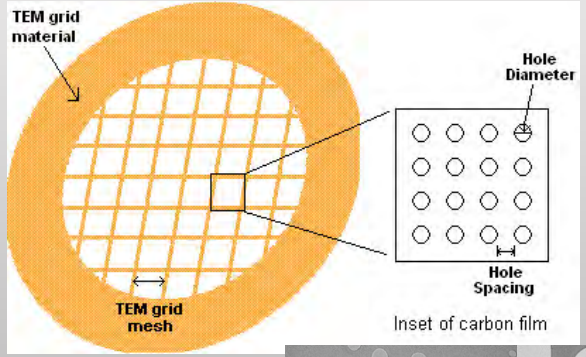
- Supporting grids for cryo-EM
  - Holey carbon grids
    - Quantifoil
    - C-Flat
    - Lacey
  - Gold grids (Quantifoil UltrAuFoil® Holey Gold Films)

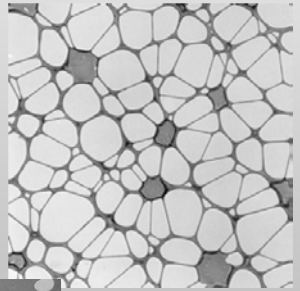


Holey carbon

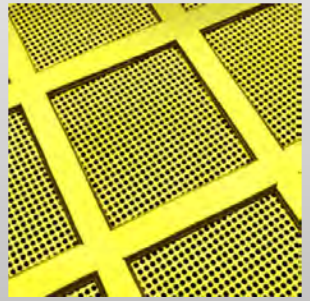


Quantifoil

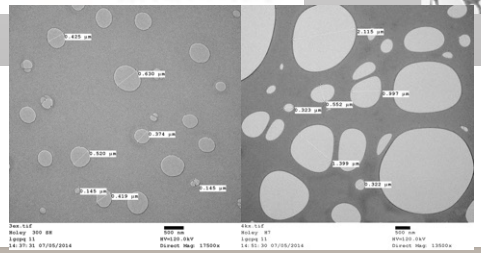




Lacey

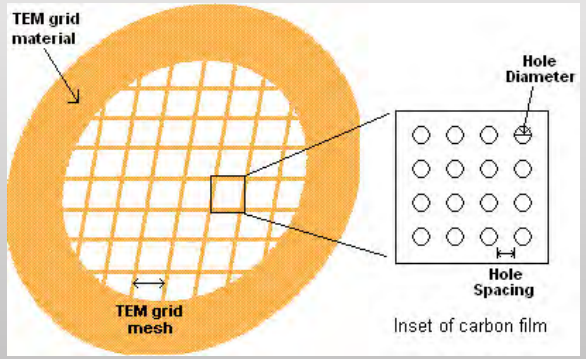


Gold grids (Quantifoil UltrAuFoil)



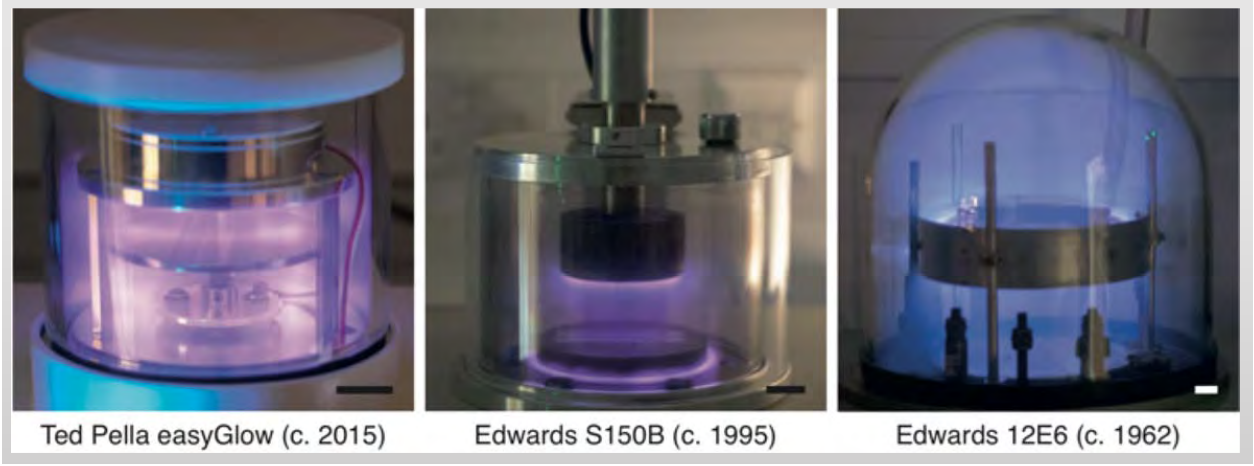
Preparation of cryo-EM Grids

- Supporting grids for cryo-EM
  - Holey carbon grids
    - Quantifoil
    - C-Flat
    - Lacey
  - Gold grids (Quantifoil UltrAuFoil® Holey Gold Films)



Preparation of cryo-EM Grids

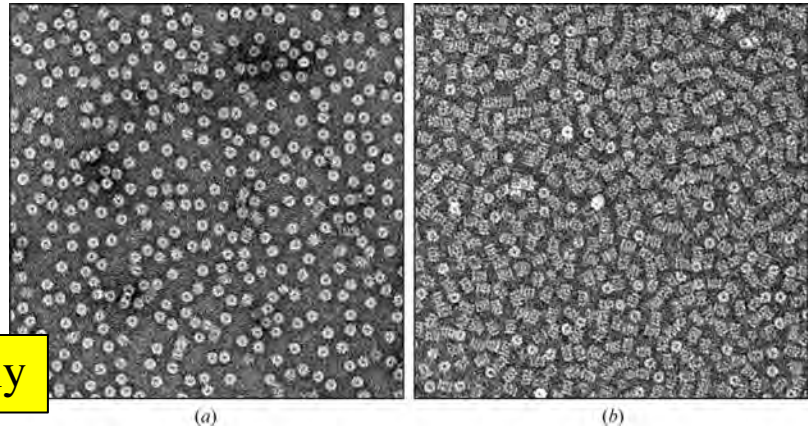
Glow-Discharging



- **Ionization-based plasma.**
- **Remove the organic contamination.**
- **Make surface hydrophilic.**

Preparation of cryo-EM Grids

Glow-Discharging



air only

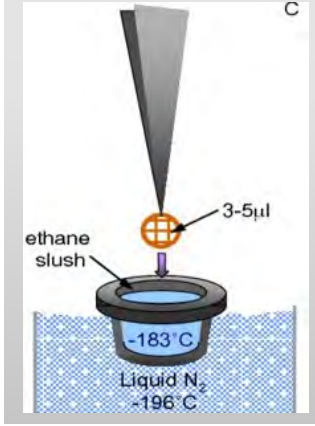
air + pentylamine

(Proteasome, Morris & Fonseca, Acta Cryst D, 2017)

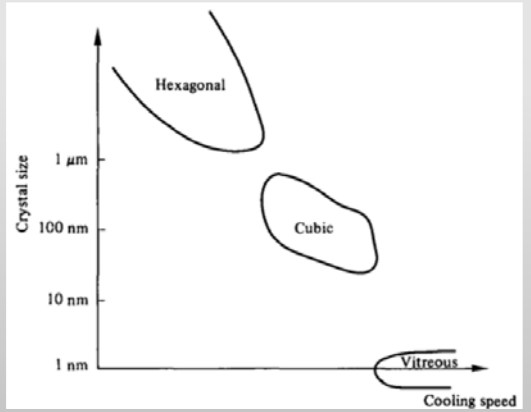
- **Different machines.**
- **Different discharging duration.**
- **Different air conditions.**

Preparation of cryo-EM Grids

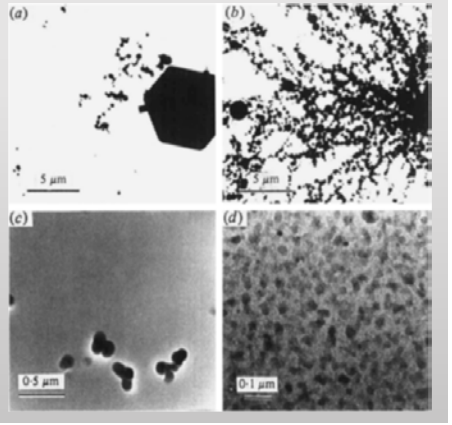
Sample Freezing with a Plunger



Liquid ethane
(Liquid N_2 boils
on contact)



Cooling speed
v.s.
Ice forms



Ice
contamination

(Dubochet et al, Q Rev Biophys, 1988)

Preparation of cryo-EM Grids

Home-made Plungers

FEI Vitrobot

✓

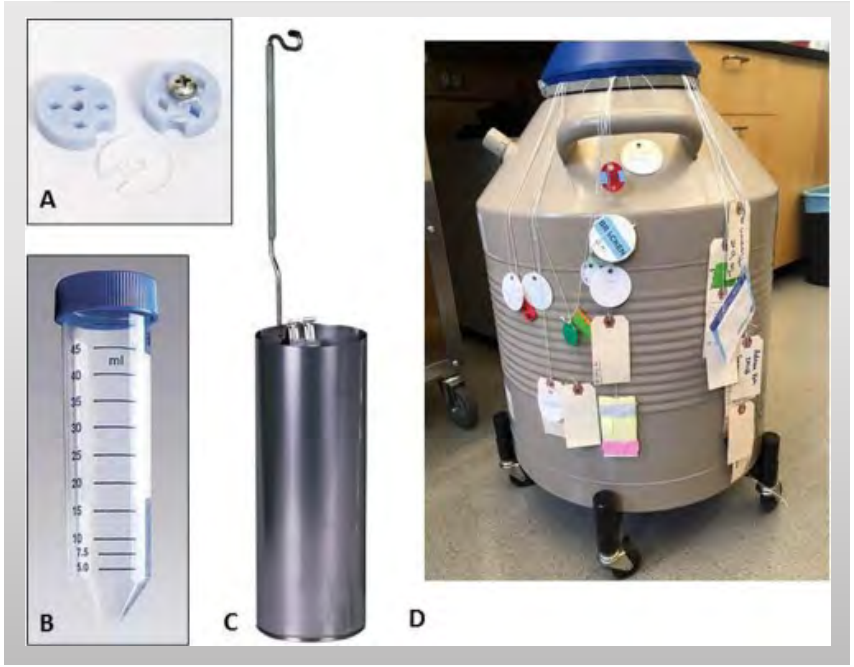
Gatan CP3

(Commercially available)

Leica EM GP2

✓

Storage and Transfer



Gatan 626 holder

Toolset with the autoloader

Data Collection

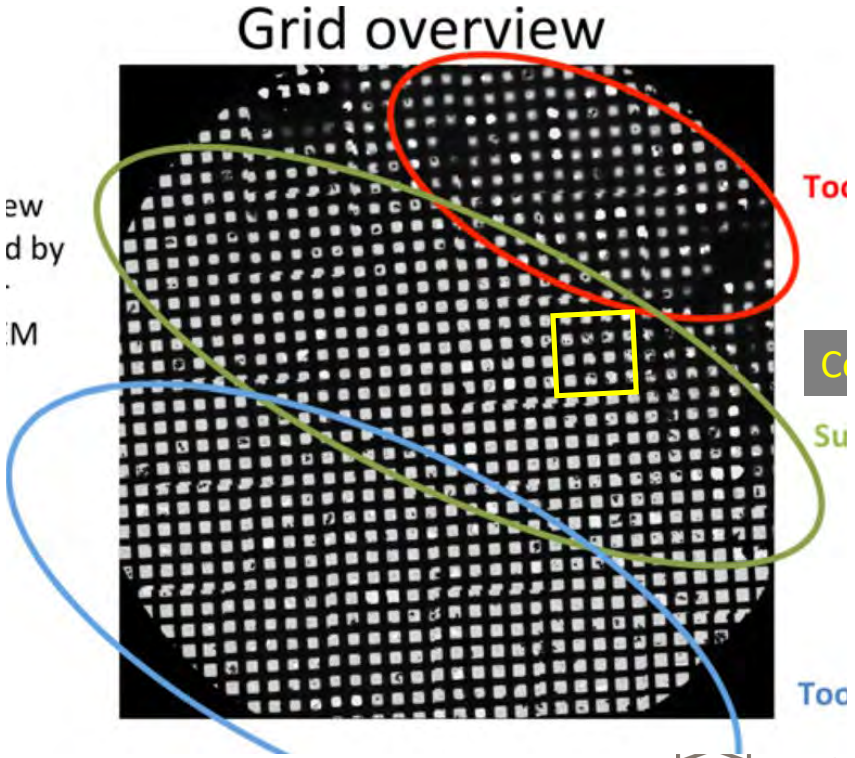
- Very important for data collection
  - Learn to compare different areas (squares and holes) on the grids
  - Good grids, good and fast data collection

- Literature search for similar cases

- Types of EM grids
- Protein concentrations ± additives
- Blotting conditions: time, force, humidity & ethane temperature

Data Collection

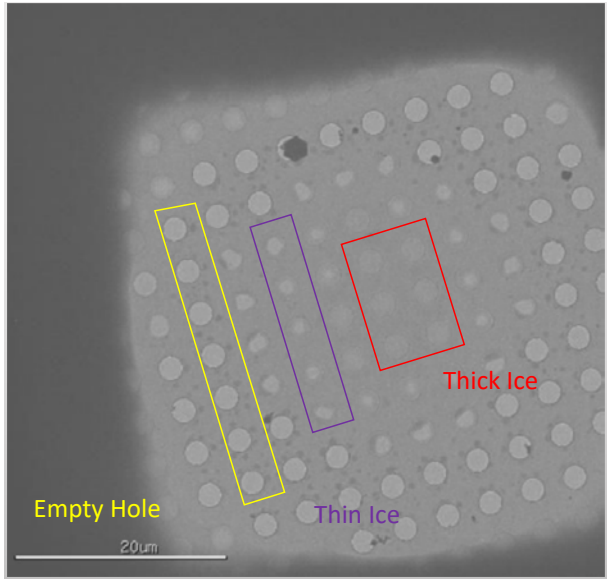
- Most area of a whole grid is likely not ideal.
  - Red: too thick
  - Blue: too thin
  - Green: suitable



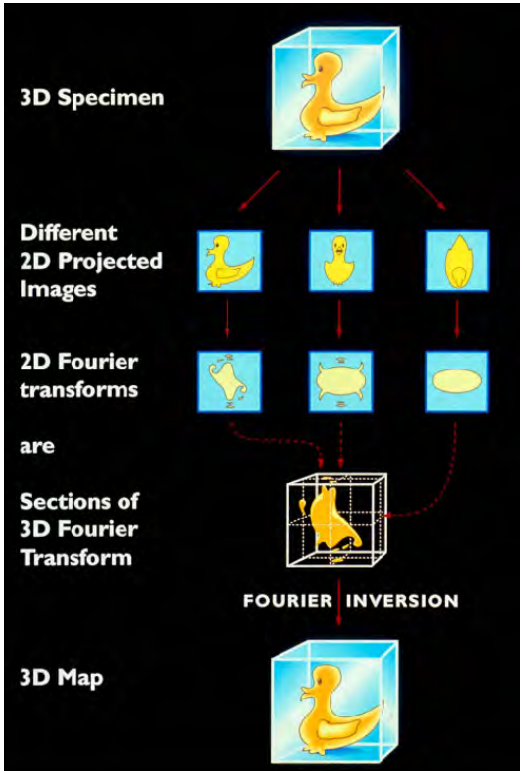
Data Collection

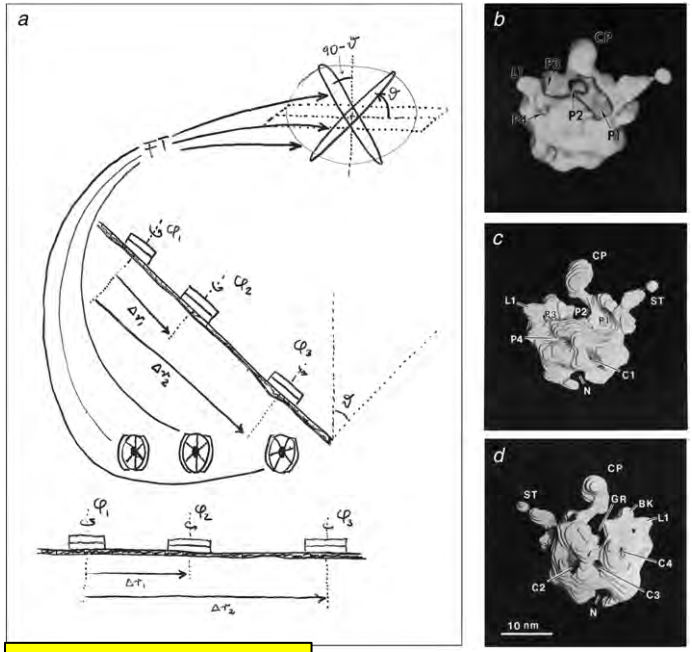
- Too thick
  - Low contrast
  - Low S/N ratios

- Too thin
  - Not thick enough to accommodate particles
  - Protein denaturation
  - More preferred orientation
  - Poor support and large motion during imaging
  - Vulnerable to radiation damage



Data Processing



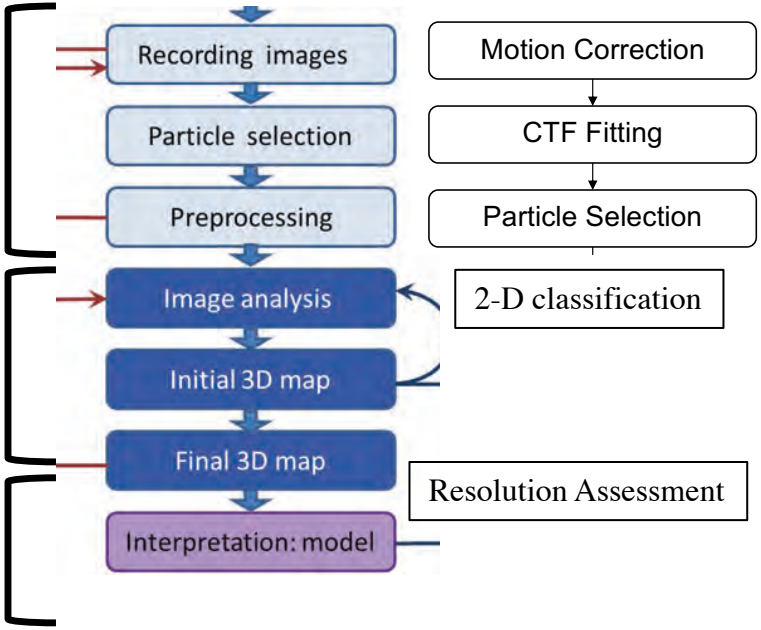


1979 hand-drawing

(Frank, Q Rev Biophysc, 2009)

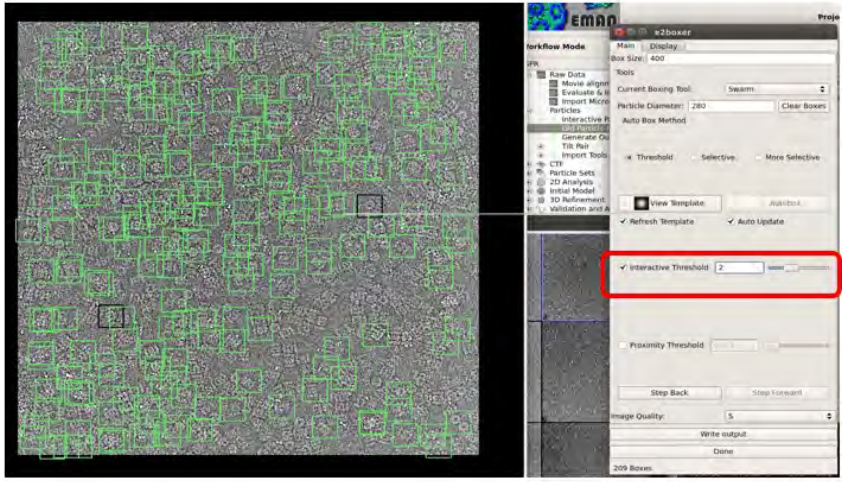
Data Processing

- cryoSPARC (GPU)
- Relion (GPU)
- EMAN/EMAN2
- Frealign/cisTEM
- Xmipps/Scipion
- Spider
- IMAGIC
- MRC/2dx (2-D crystals/MicroED)
- …



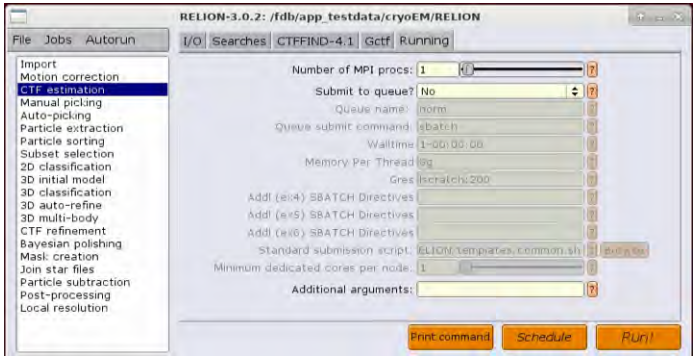
Data Processing

EMAN

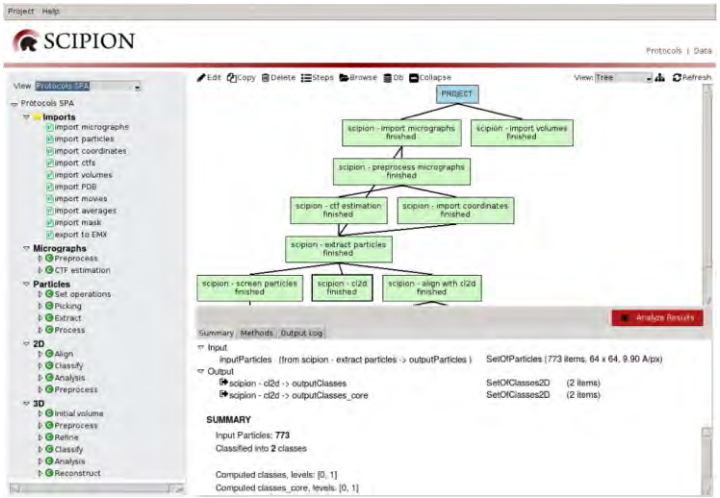


Data Processing

RELION

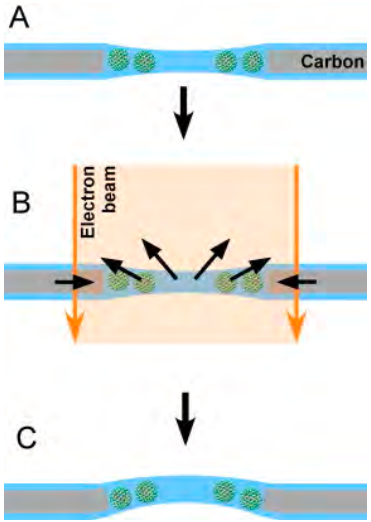


SCIPION



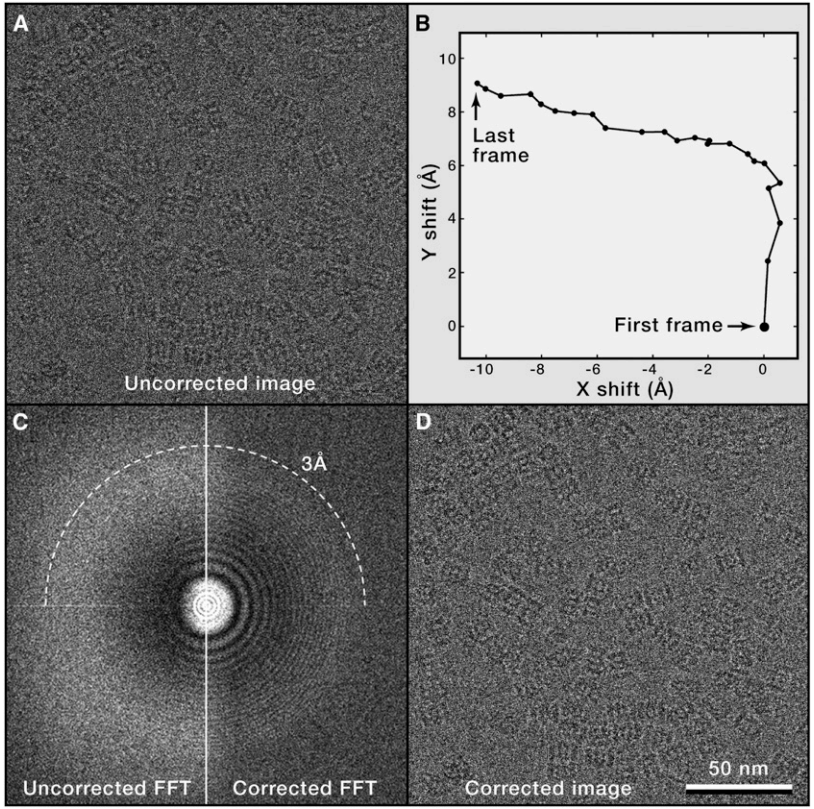
Motion Correction

- Stage drift
- Beam-induced sample motion



(Brilot et al, J Struct Biol, 2012)

(Zivanov et al, IUCrJ, 2019)



(Cheng et al, Cell, 2015)

CTF Assessment (Power Spectra)

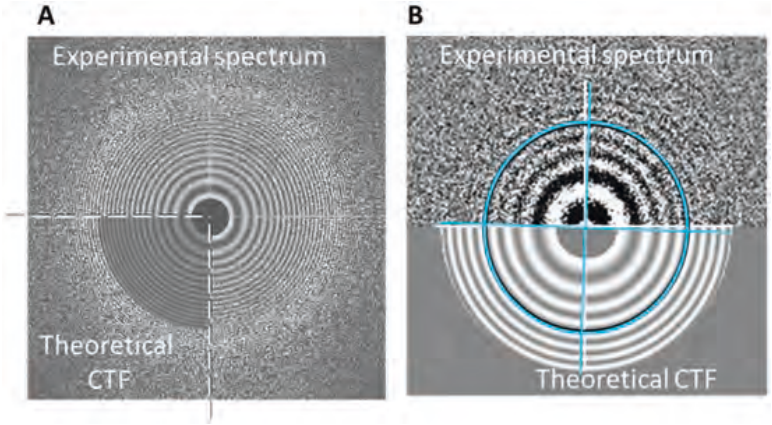
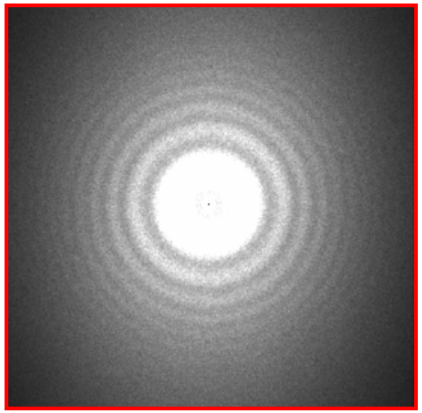


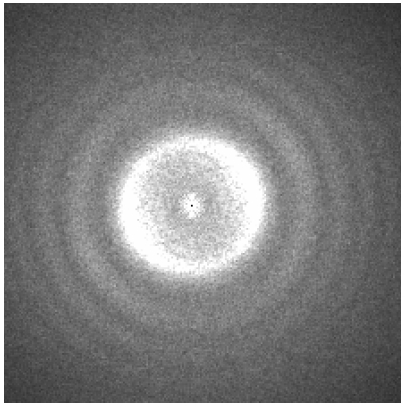
Fig. 8 Assessment of CTF parameters. (**a**) Comparison of theoretically calculated CTF (*left bottom quadrant*) with CTF seen in experimental spectrum. For an accurate CTF determination the Thon rings from both image parts should match accurately. (**b**) Identification of axes of astigmatism which are superimposed over Thon rings of an actual observed power spectrum and compared with the theoretical spectrum. The spectrum of a micrograph shown here indicates that there is a small astigmatism, ~2%, and the axes of ellipse are slightly tilted, shown in *light blue*

(Costa et al, Meth Mol Biol, 2017)

CTF Assessment (Power Spectra)

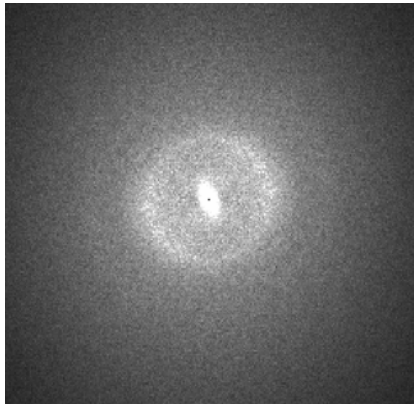
Good:
- Isotropic
- Thon rings at high resolution

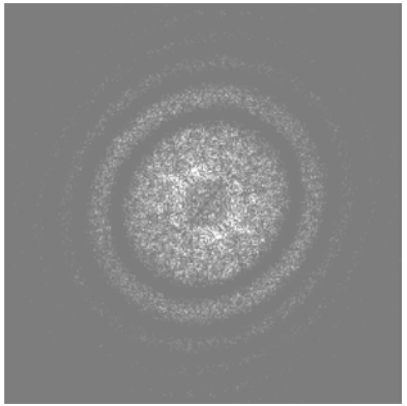




Bad:
Missing Thon rings at certain direction due to drift (*can be corrected if movies are recorded*)

Bad:
Thon rings only at low resolution

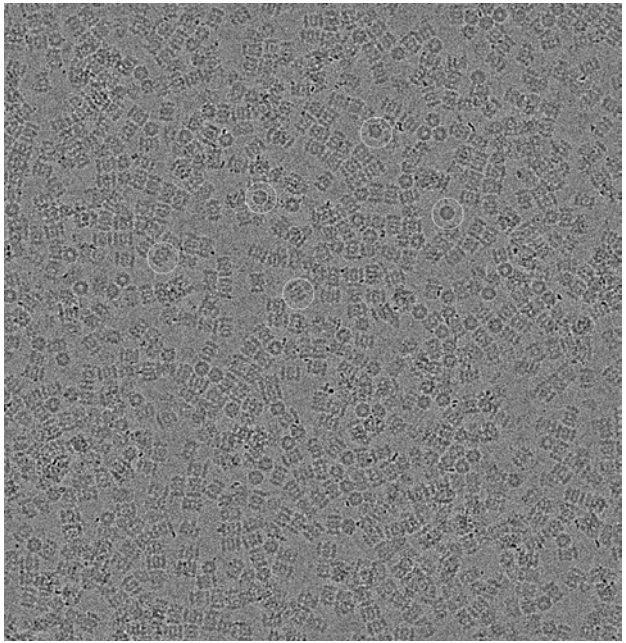




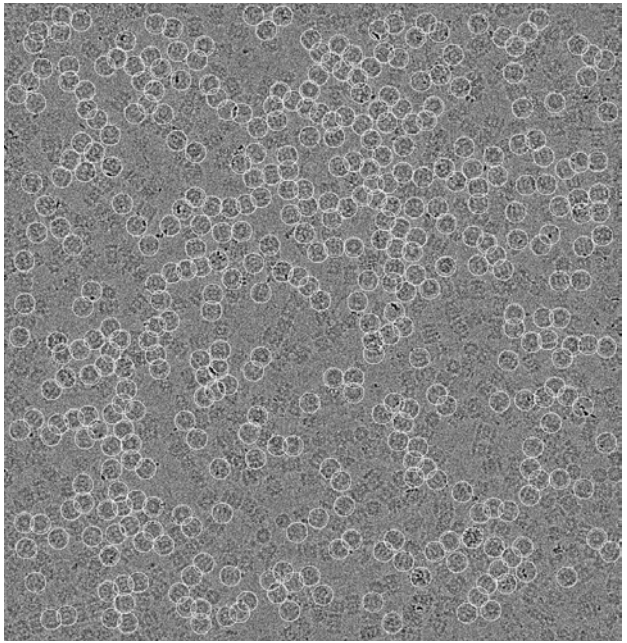
Bad:
Elliptic Thon rings due to astigmatism (*can be useful if properly processed*)

Particle Selection & 2-D Classes

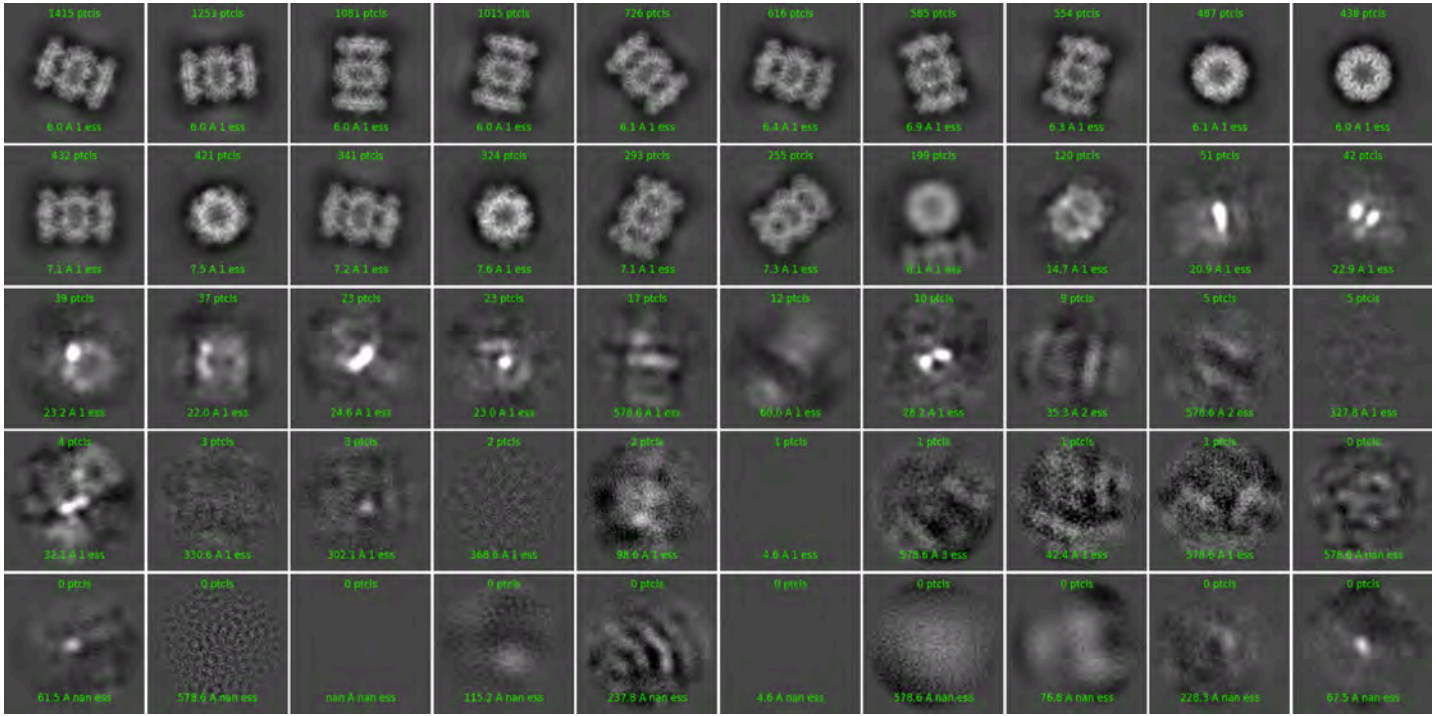
Manual



Automated
(template/deep-learning)

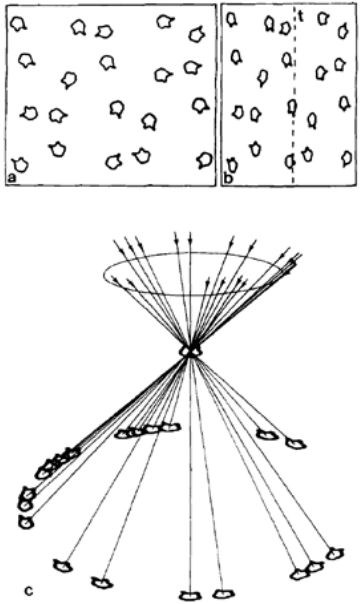


Particle Selection & 2-D Classes



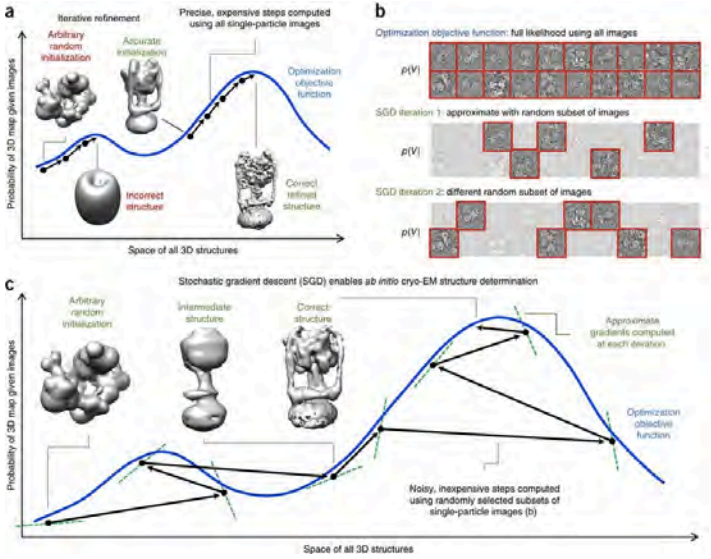
Initial Model & 3-D Classes

Random Conical Tilt

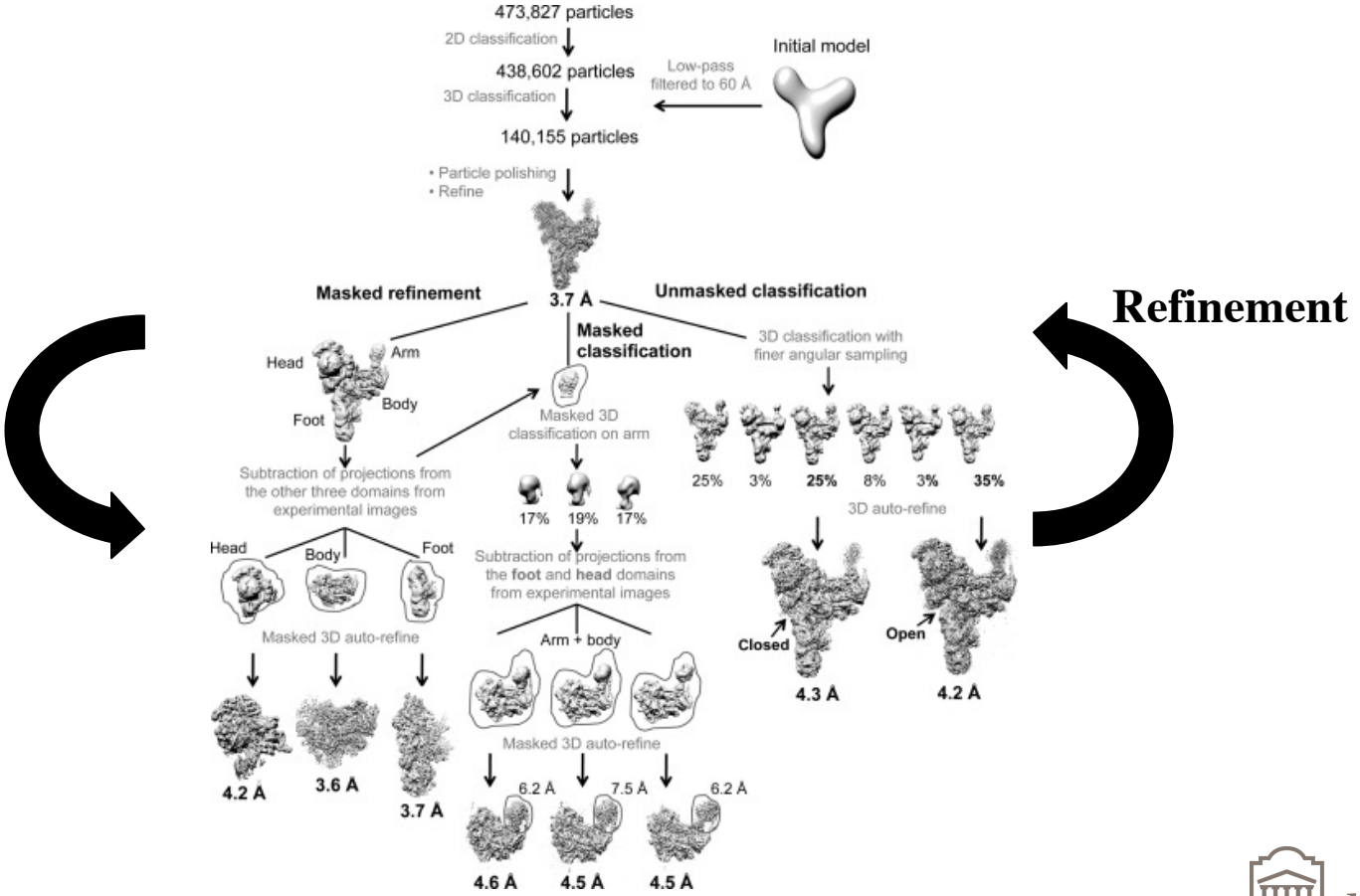


(Radermacher et al, J Microsc, 1986)

Stochastic Gradient Descent



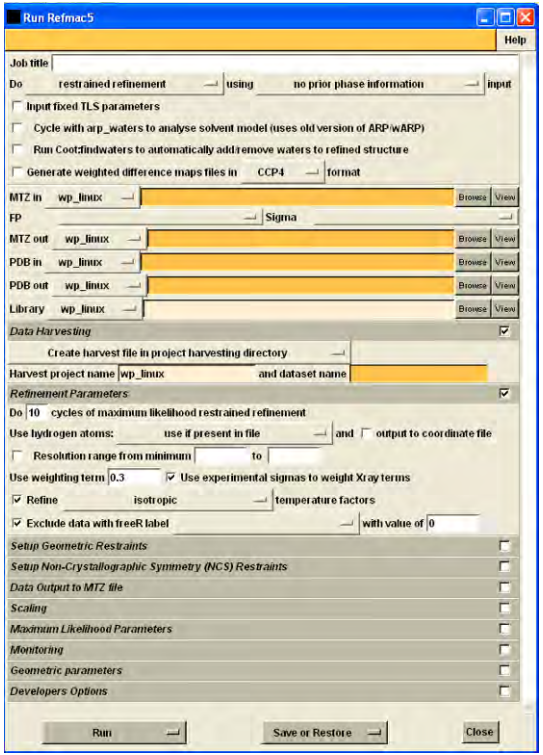
(Punjani et al, Nat Methods, 2017)



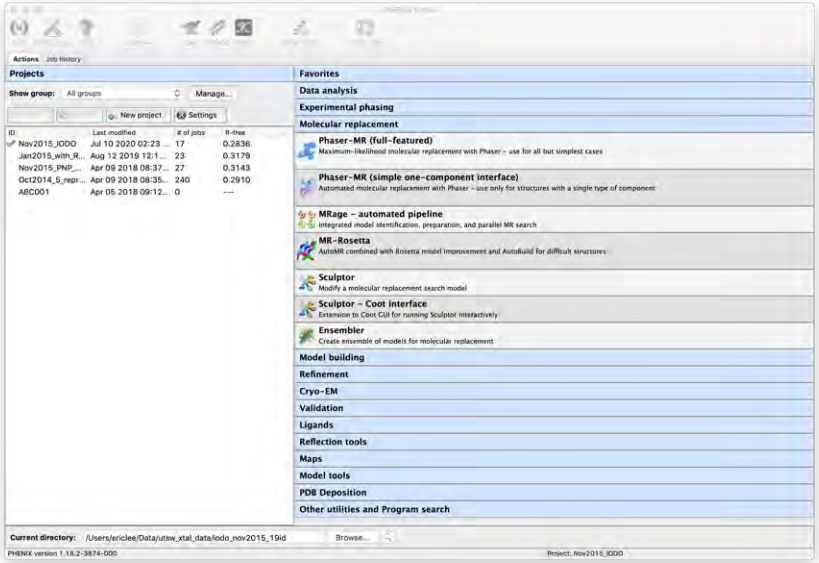
(Scheres, Meth Enzymol, 2016)

Model Building

REFMAC



PHENIX



Model Building

COOT