NGP-net
Non-globular proteins in molecular physiopathology

NGP-net Winter School on Experimental Methods for Protein Disorder & Aggregation
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Protein disorder studied by a synergy of experiment and simulation: X-ray crystallography and biophysics

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Brno, 9.1. 2019

Outline of the Presentation

- Interplay of dynamics and statics in conformational ensemble of intrinsically disordered proteins (IDPs)
- The concept of synergic study of IDPs (and their aggregation)
- “Frozen” IDP structural features: crystallography of IDPs
- Principles, execution and evaluation of crystallographic experiment
- Crystallography of IDP dynamic features: surrogate IDP binding partners
- Crystallography of IDP aggregation

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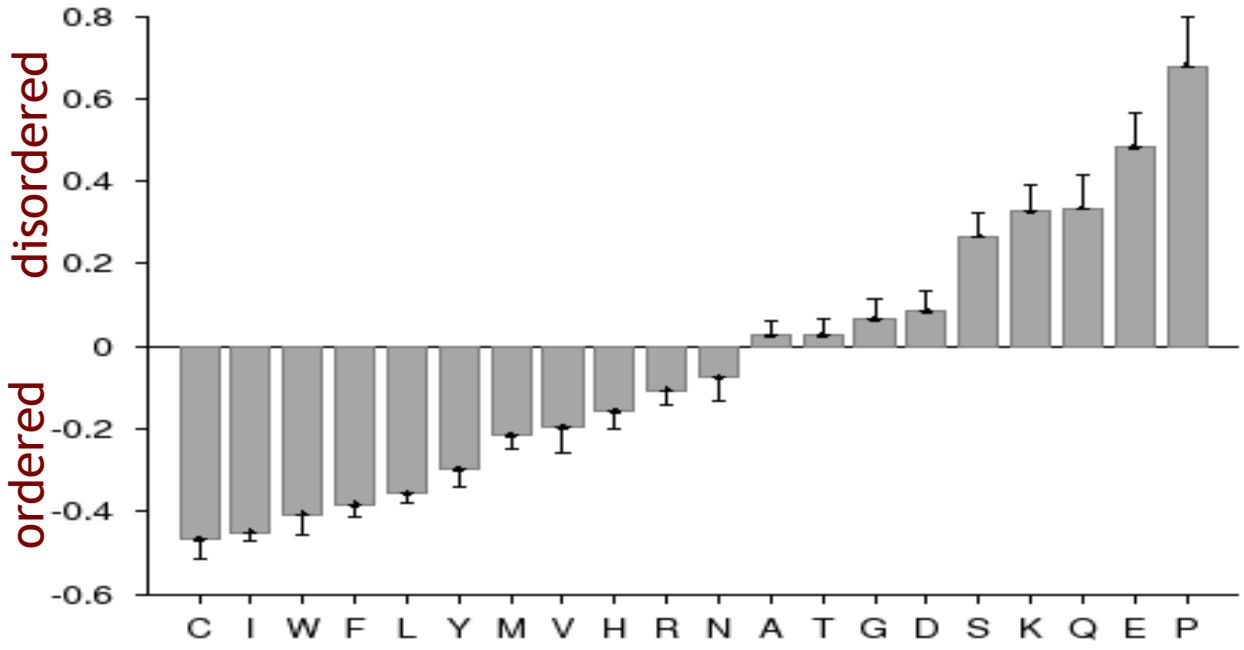
Nature of IDP conformational ensemble

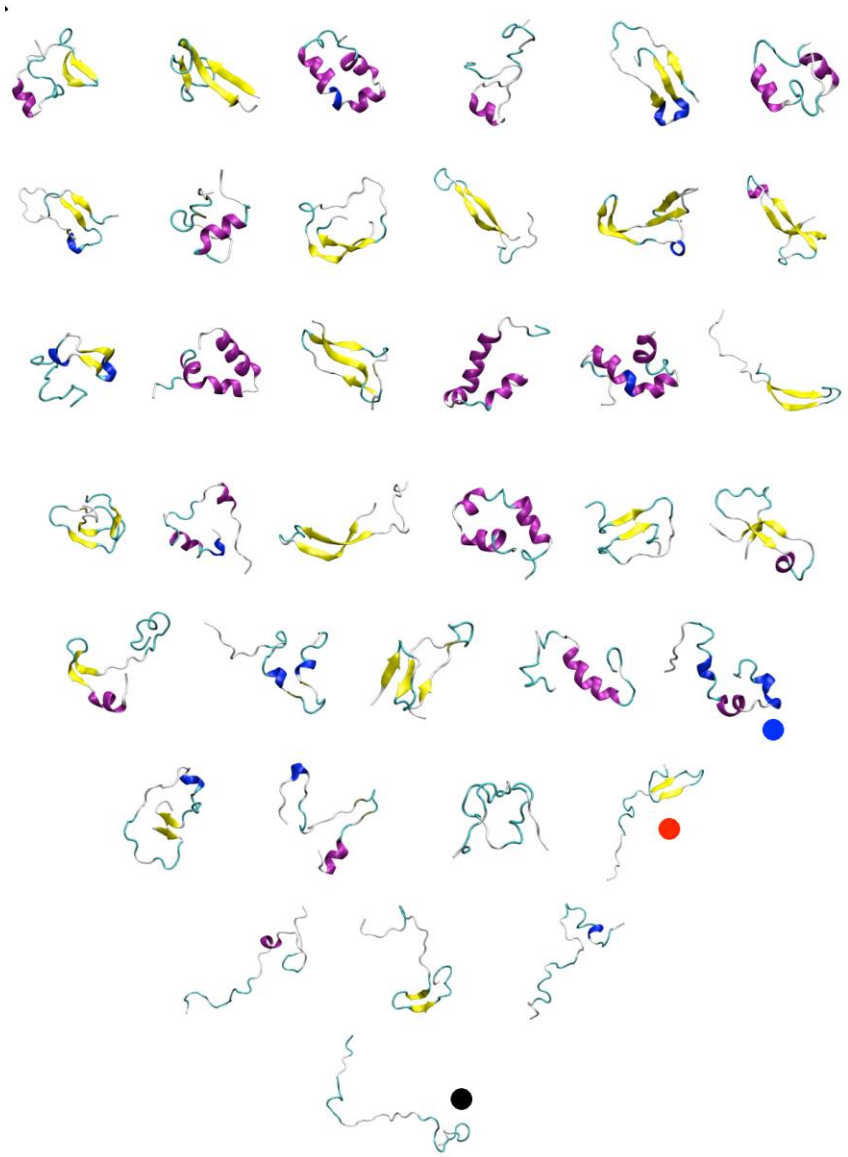
IDPs are **compositionally** and **sequentially** biased: they are enriched in polar/charged amino acids whereas depleted in hydrophobic aa.

Consequently, IDP can be fairly well predicted from sequence (around 80 % accuracy)



Uversky 2011

Conformational ensemble of a disordered protein

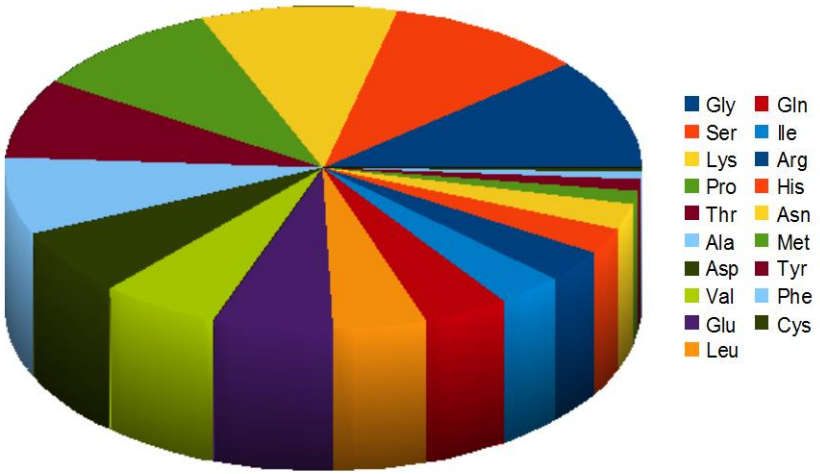


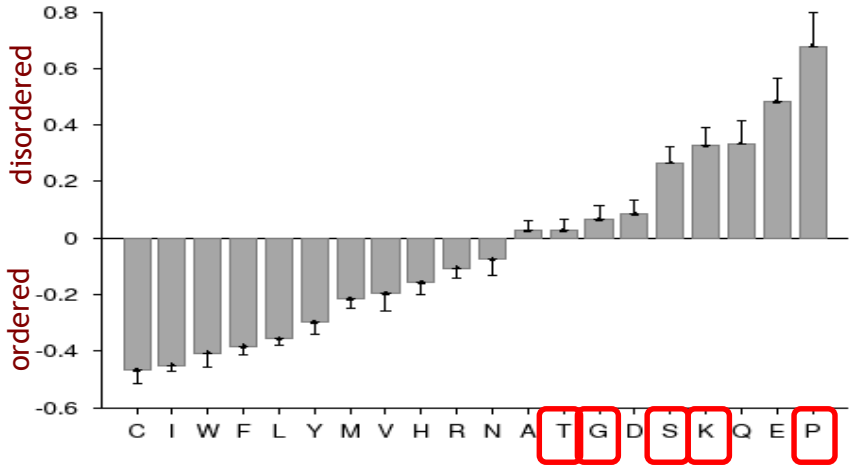
Vendruscolo 2017

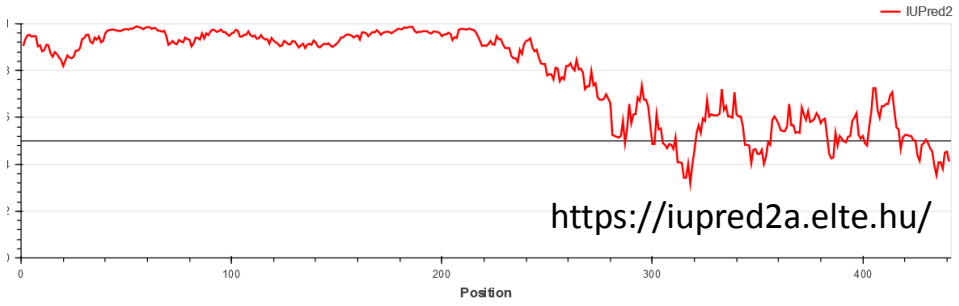
Nature of IDP conformational ensemble

Example: neuronal protein tau

Longest human tau40 isoform		
Gly	49	11.1%
Ser	45	10.2%
Lys	44	10.0%
Pro	43	9.8%
Thr	35	7.9%
Ala	34	7.7%
Asp	29	6.6%
Val	27	6.1%
Glu	27	6.1%
Leu	21	4.8%
Gln	19	4.3%
Ile	15	3.4%
Arg	14	3.2%
His	12	2.7%
Asn	11	2.5%
Met	6	1.4%
Tyr	5	1.1%
Phe	3	0.7%
Cys	2	0.5%
Trp	0	0.0%



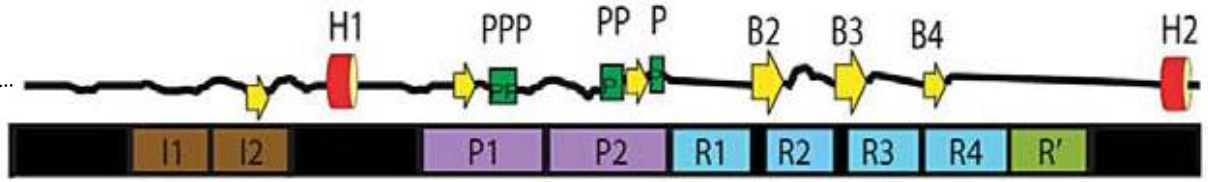


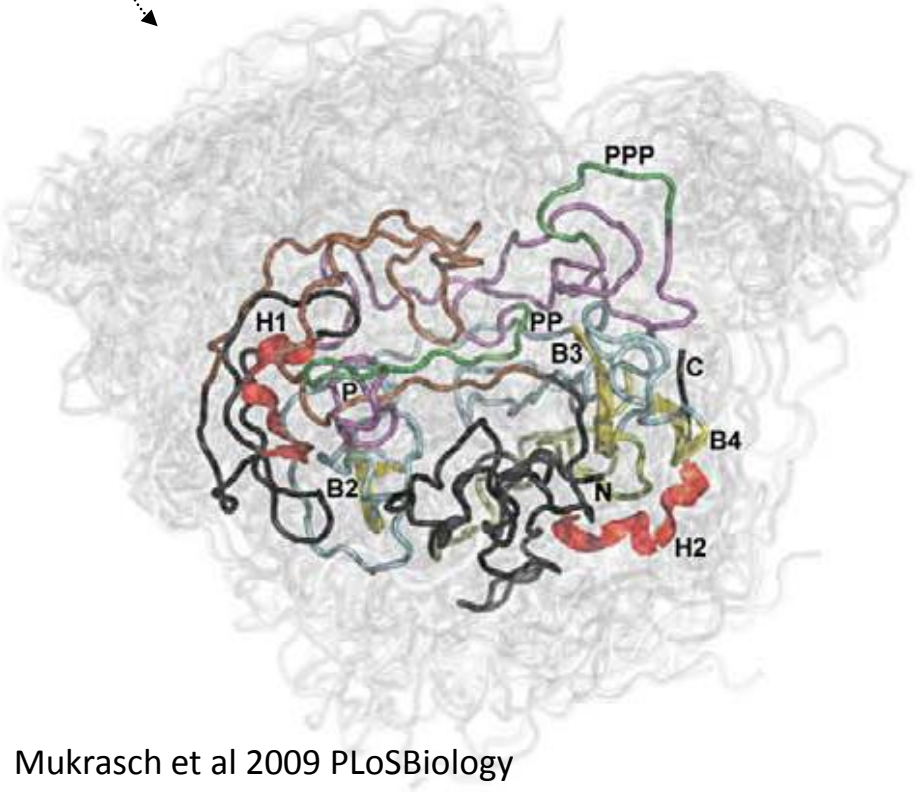


https://iupred2a.elte.hu/

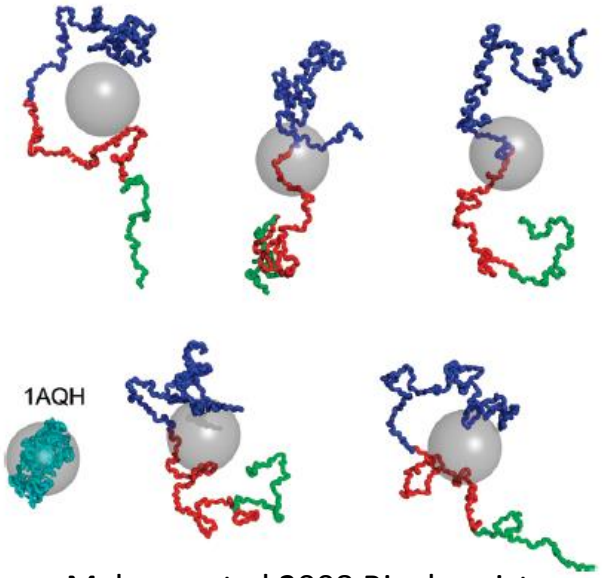
Nature of IDP conformational ensemble

Comformational ensemble of tau as seen by NMR and SAXS:





Mukrasch et al 2009 PLoSBiology

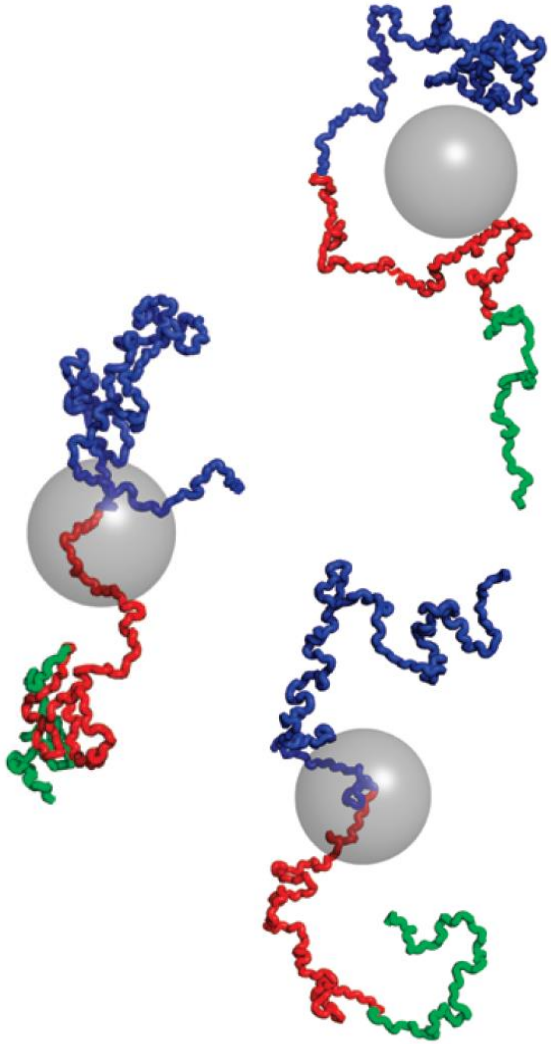


Mylonas et al 2008 Biochemistry

Conformation sorts and their destiny

Ensemble:

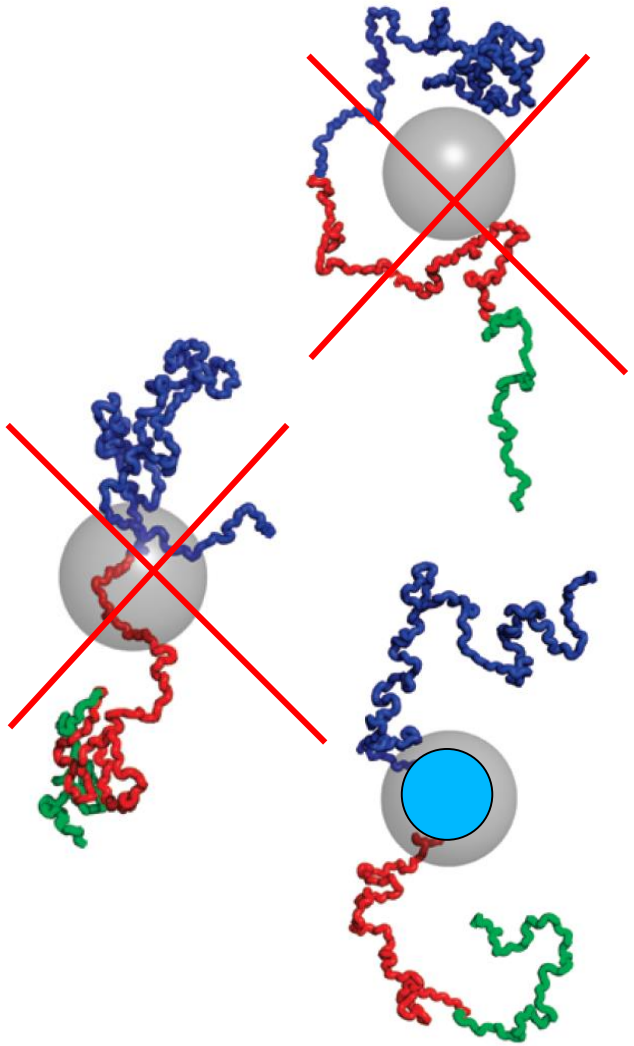
- Long and short-range conformations (tertiary and secondary structures)
- Paper-clip model of tau
- **small 3D motifs (S/T turns, Asx turns …)**
- propensity to larger secondary structures (polyproline stabilized by n-> pi interaction, extended - stabilized in beta structure, helical stabilized by H-bonds)
- Slowly or rapidly forming
- Long and short living



Destiny of CE after complex formation

Interaction of CE with a binding partner (hetero/homo dimerization):

- Freezing of interaction surface conformation
- Distant parts remain in CE but modified
- Kinetics of complex formation may contain informations about contact site of CE
- Fold(s) of binding interface may contain info about free state propensities



Conformational ensemble and allostery further reading

Tompa, P. (2014). "Multisteric Regulation by Structural Disorder in Modular Signaling Proteins: An Extension of the Concept of Allostery." Chemical Reviews **114**(13): 6715-6732.

Ohhashi, Y. et al (2018). "Molecular basis for diversification of yeast prion strain conformation." Proc Natl Acad Sci U S A 115(10): 2389-2394.

Berlow, R. B.et al (2018). "Expanding the Paradigm: Intrinsically Disordered Proteins and Allosteric Regulation." J Mol Biol 430(16): 2309-2320.

Lucato, C. M. et al (2017). "Amyloidogenicity at a Distance: How Distal Protein Regions Modulate Aggregation in Disease." J Mol Biol 429(9): 1289-1304.

Motlagh, H. N. et al (2014). "The ensemble nature of allostery." Nature 508(7496): 331-339.

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- Dynamic IDP structural features: light scattering experiments and binding studies

Output of crystallographic experiments

- (static) coordinates of atoms (deduced from the maxima of electron density, assayed in diffraction experiments) – **model** of structure
- (dynamic) features of structure model:
    a) alternative conformations of side chains, main chain, loops (depending on resolution, deduced from electron density)
    b) B-factors of atoms (atomic position displacements) – refined as parameters during structure solution
    c) Errors of coordinates – depends on resolution and the mode of computational refinement (not all programs give these parameters)
    d) "invisible" parts of structure = too weak, non-interpretable electron density due to **disorder,** missing chain (wrong construct, cleavage during crystallization …)

Crystallographic studies of IDP

Bacterial toxin-antitoxin modules

Complexes of therapeutic antibodies against IDPs involved in neurodegeneration

Amyloid aggregation of IDPs

http://www.xtal.iqfr.csic.es/Cristalografia/index-en.html

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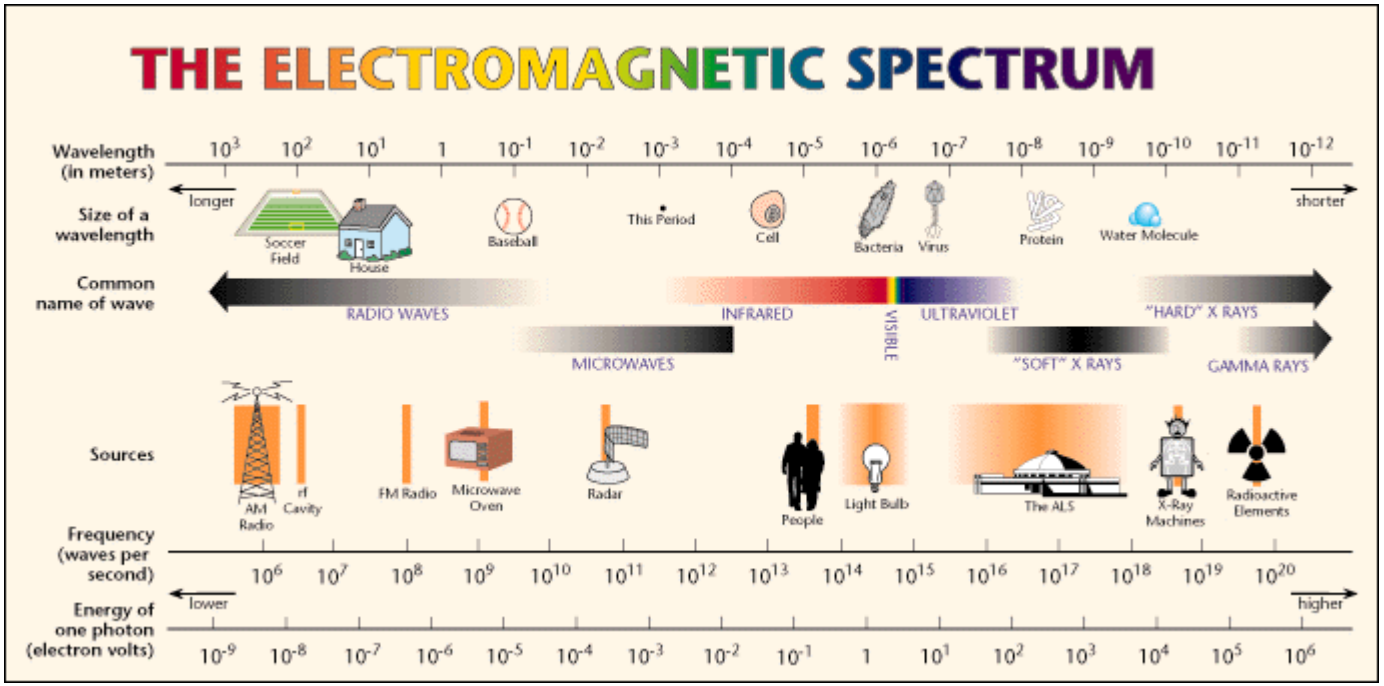
Interaction of a wave with matter

Electromagnetic waves and matter waves (wave-particle duality) are widely used probes for determining properties of matter

Interaction of waves with matter (examples):

- Absorption, reflection, refraction, diffraction …
- Diffraction is due to wave **scattering** (**elastic**, non-elastic)



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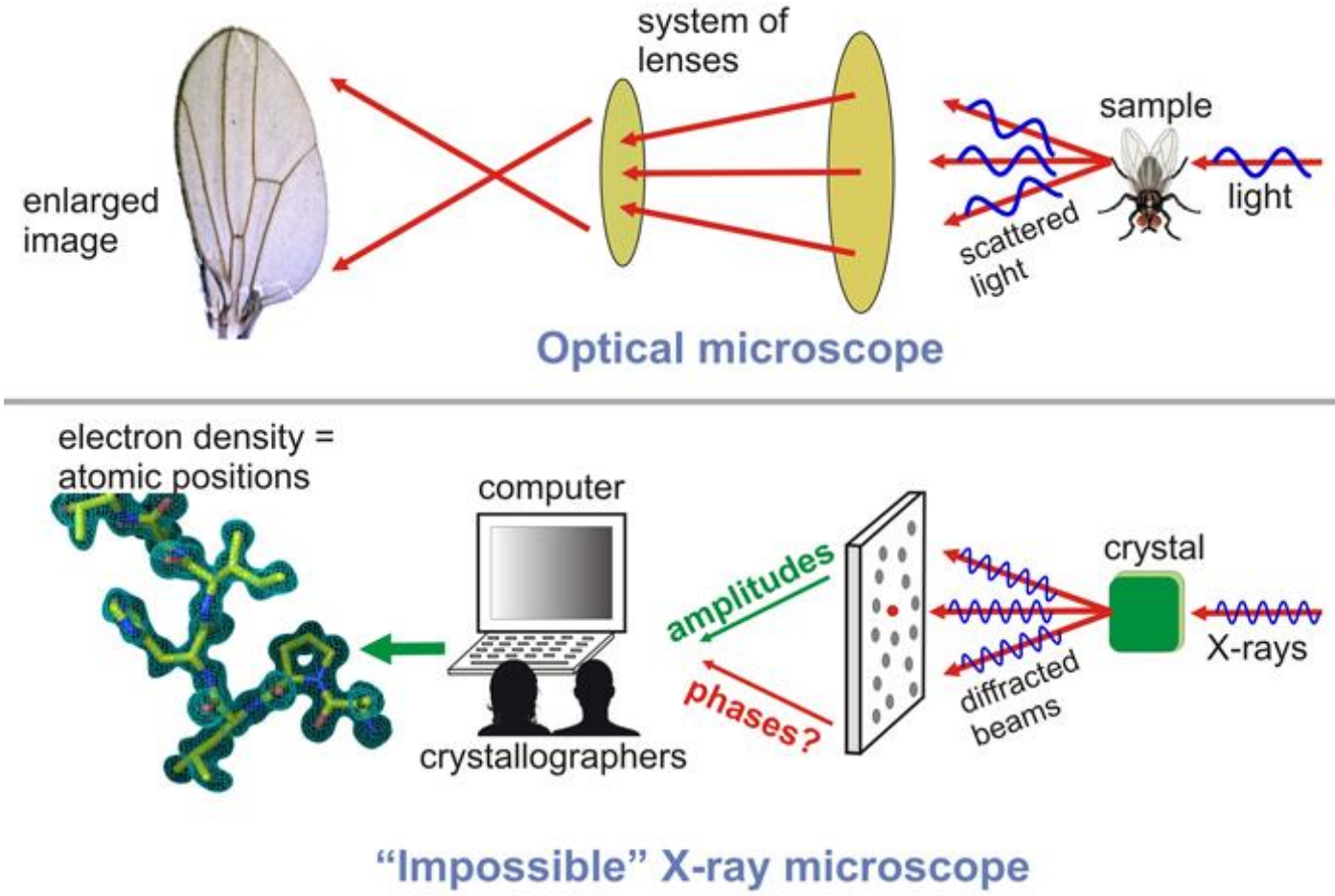
Determination of atomic details



http://www.xtal.iqfr.csic.es/Cristalografia/archivos_07/problema-fases-en.jpg

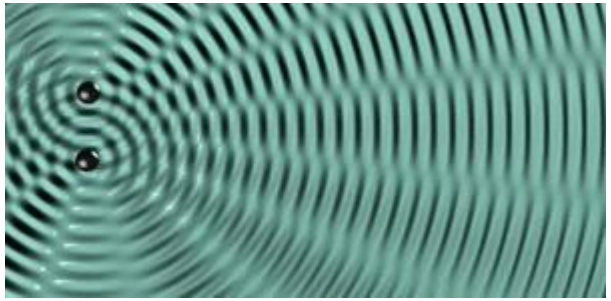
Scattering and Diffraction

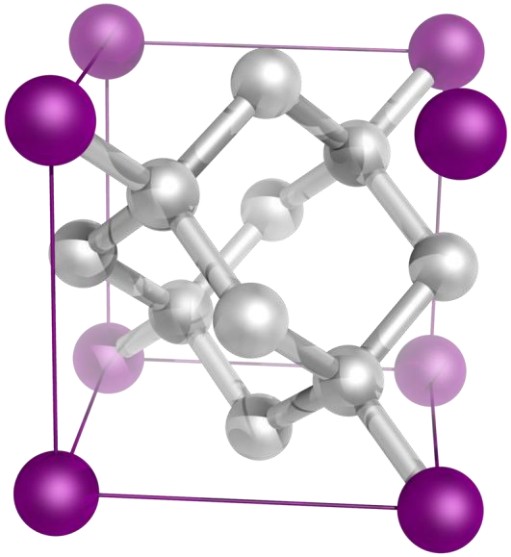
Matter, particles may scatter the waves by various mechanisms:

- X-ray –Thomson scattering: elastic scattering of electromagnetic radiation (X-ray photons) by a free charged particle (“free” electron cloud of atoms)
- Neutrons – scattering of thermal neutrons via nuclear forces, interaction with nuclei
- Electrons – scattering of electron beam via electrostatic interactions with atoms

Crystals and Diffraction

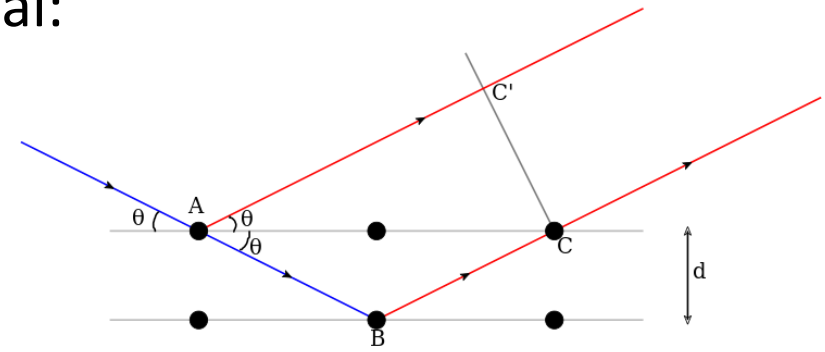
Scattering from a regularly arranged particles in a crystal lead to mutual interferences leading to diffraction of waves



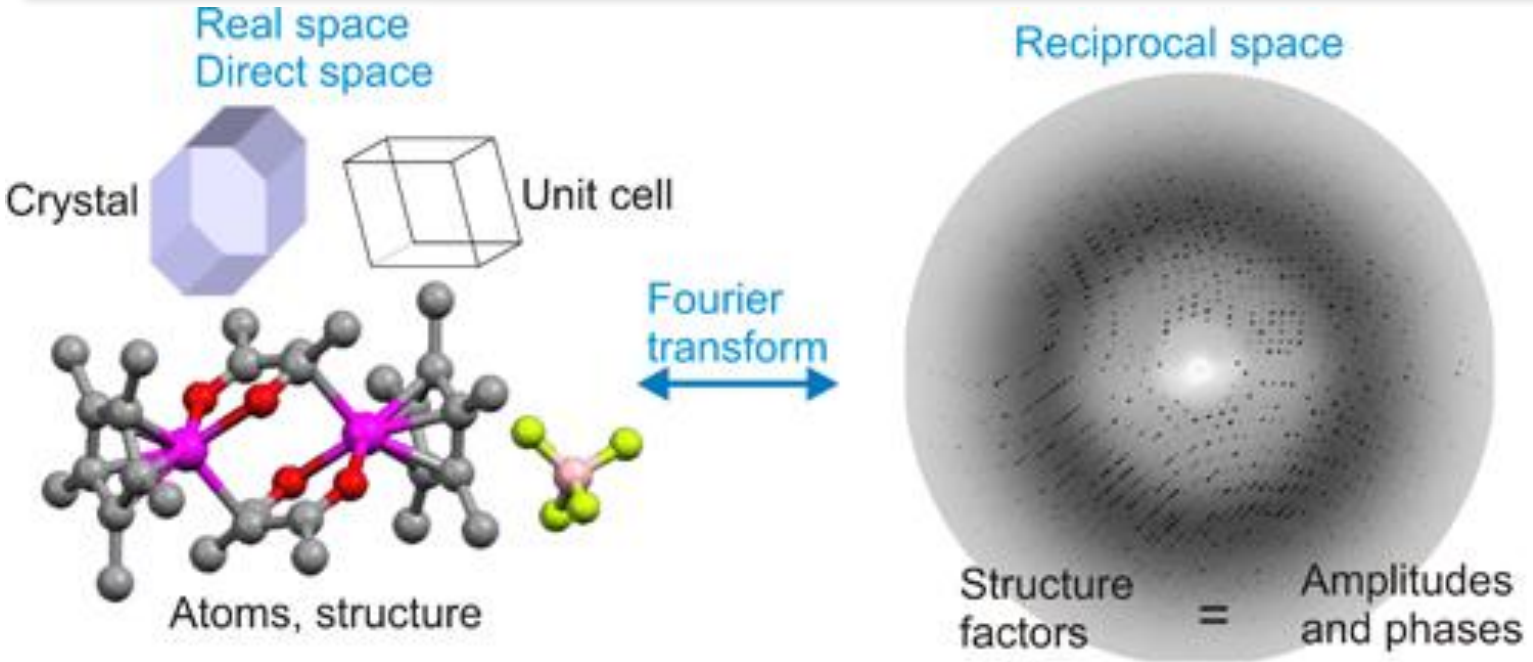


Bragg law of diffraction on crystal:

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



Crystals and Diffraction



$$\rho(xyz) = \frac{1}{V} \sum_{hkl=-\infty}^{+\infty} \underbrace{|F(hkl)|}_{\text{Amplitudes}} \cdot e^{-2\pi i[hx+ky+lz-\underbrace{\phi(hkl)}_{\text{Phases}}]}$$

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Diffraction from crystal planes gives a regular pattern of X-ray spots on detector. From their position, intensity and phase the electron density can be reconstructed. Phases are lost in the experiments and have to be acquired separately

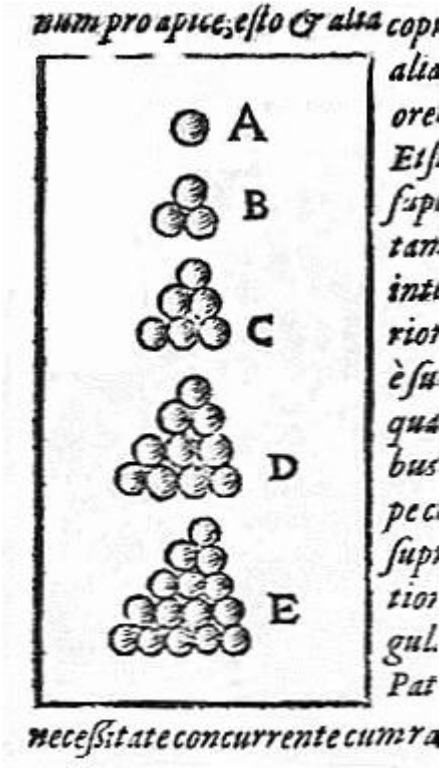
Historical excursus: dawn of modern crystallography

Prague, 1611 – Johannes Kepler:

Strena Seu de Nive Sexangula (*A New Year's Gift of Hexagonal Snow*)

- The first proposal of the internal arrangement of a crystal

Johannes Kepler



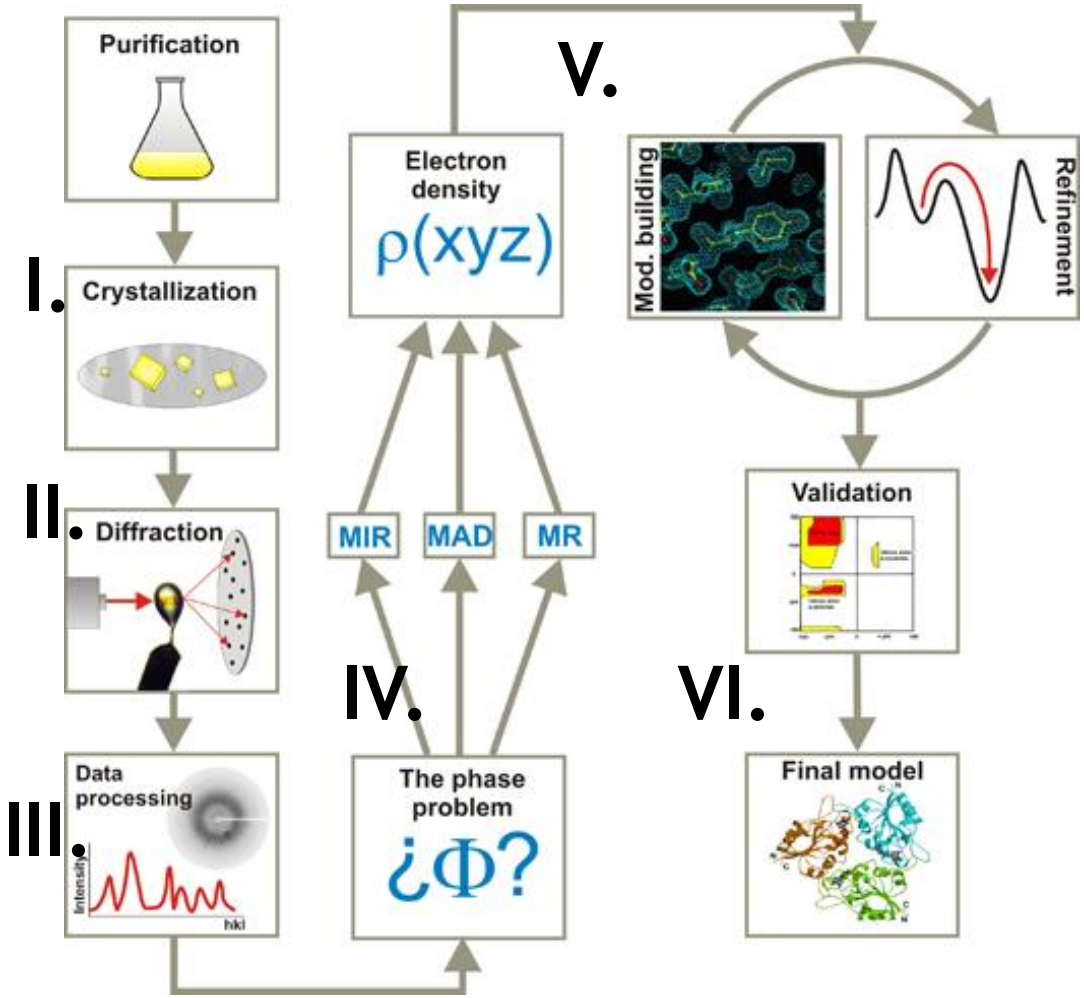
Crystallography by Diffraction

Crystallography: probing crystals by waves:

- X-ray diffraction: intensity depends on number of electrons (hydrogens largely invisible)
- Neutron diffraction – by nuclei (also hydrogens), but need for large crystals
- Electron diffraction: need for thin small crystals

Question of radiation damage by various methods

Pipeline of X-ray crystallography experiment



During evolution of the method, different steps represented the main bottleneck of the method

The protein preparation and mainly crystallization remained the main time-limiting steps also today.

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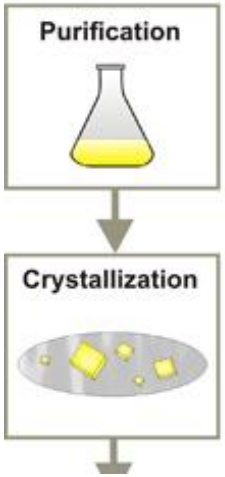
Pipeline of X-ray crystallography: I. Crystallization

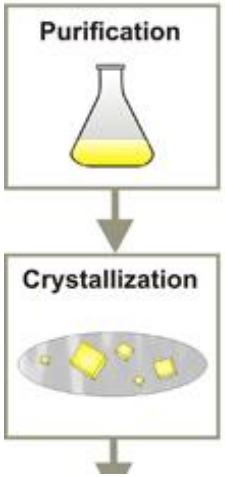


Crystals of hemoglobin were prepared nearly a century before Max Perutz solved the first X-ray structure, but structure solution take him 22 years.

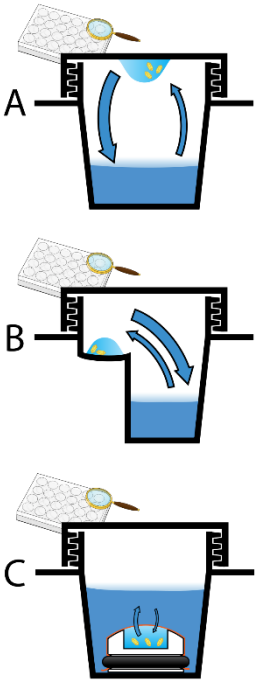
Structure solution today take in average a week, but obtaining diffracting crystals in a new project can not be guaranteed beforehand (ribosome ~20 years – Ada Yonath).

Finding crystallization conditions requires a large-scale screening experiment of various combination of concentrations, precipitants, additives and pH

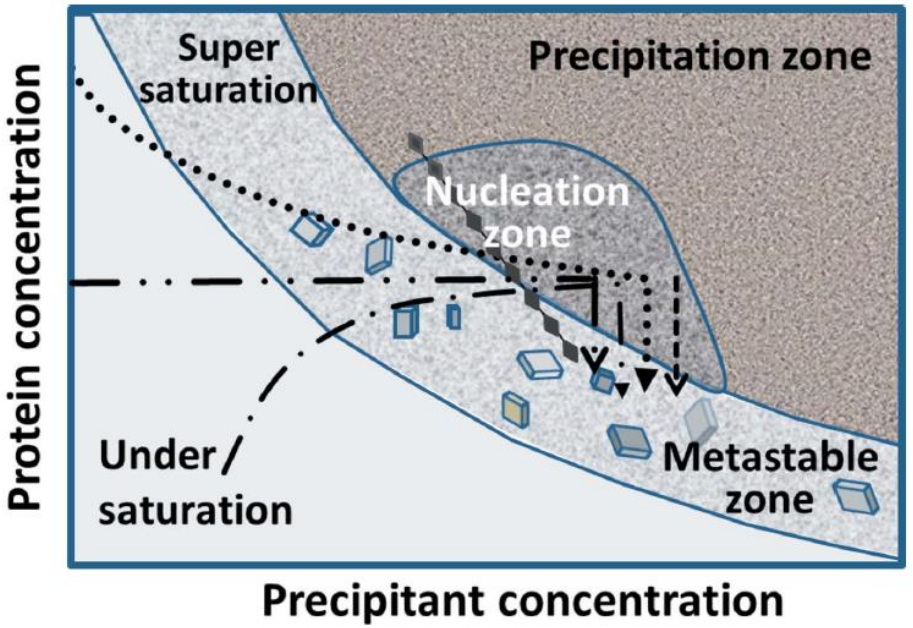
The birth of the Crystals



Various crystallization experiment designs imply different trajectories in phase diagram towards well-diffracted crystal forms



https://upload.wikimedia.org/wikipedia/commons/thumb/e/e5/CrystalDrops.svg/800px-CrystalDrops.svg.png



Schlichting, I. (2015). IUCrJ 2, 246-255.

High-throughput protein crystallization

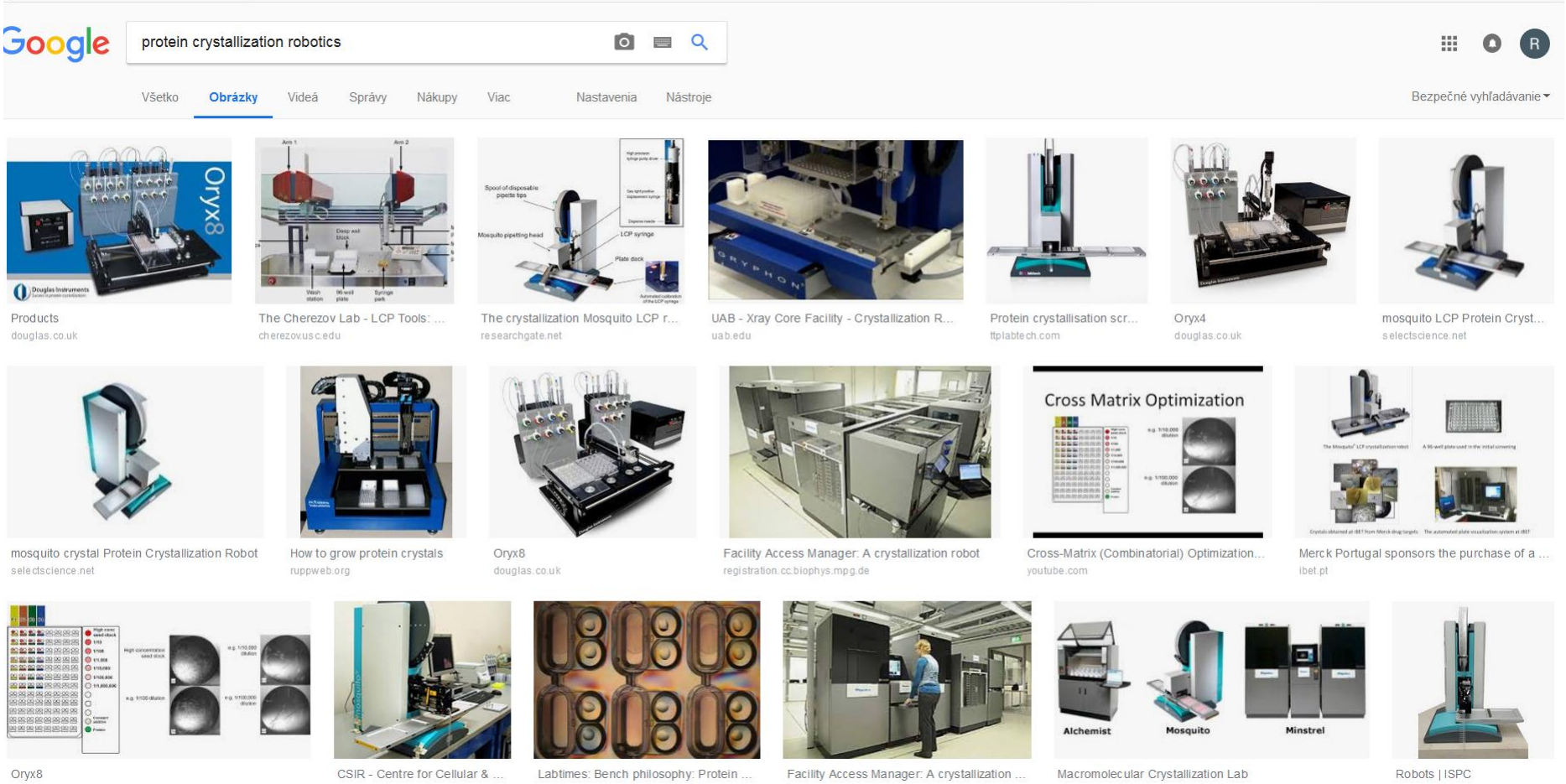
Pros:

- Easy examination of a large area of phase diagram
- Lowering volume drop = lower protein and precipitant consumption
- Reducing time and work space requirements
- Efficient crystal nucleation in smaller drop volumes
- *In situ* on-the-plate crystal testing

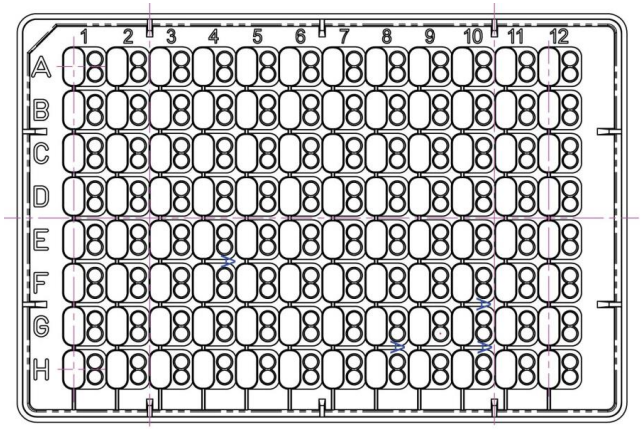
Cons:

- Required adoption of costly robotics pipetting systems
- Intrinsic large dead volume

COSTLY high-throughput robotics …



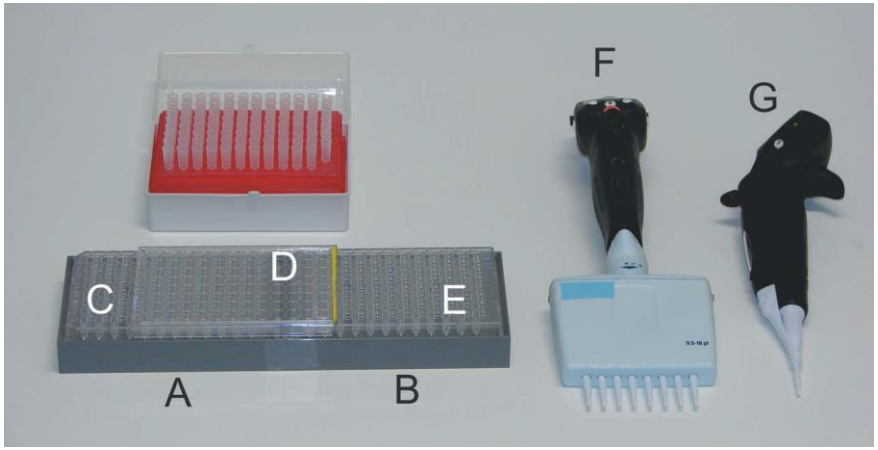
… alternative: Manual high-throughput system



Crucial is the use of motorized handheld pipettes, able to (repetitively) dispense sub-microliter volumes with disposable tips

Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

Non-robotic manual assembly of nanoliter drops



Key components:

- MRC 96/192 well plates for sitting drops assembly
- Eight-channel motorized handheld pipette
- Single-channel motorized handheld pipette

Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

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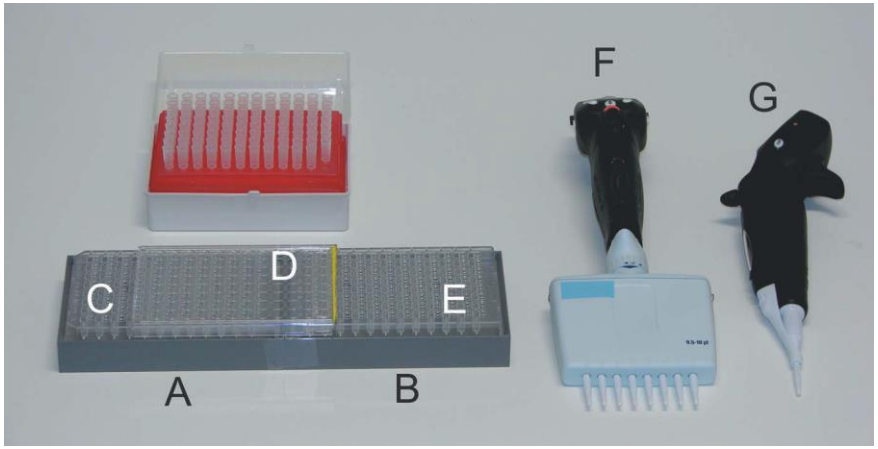
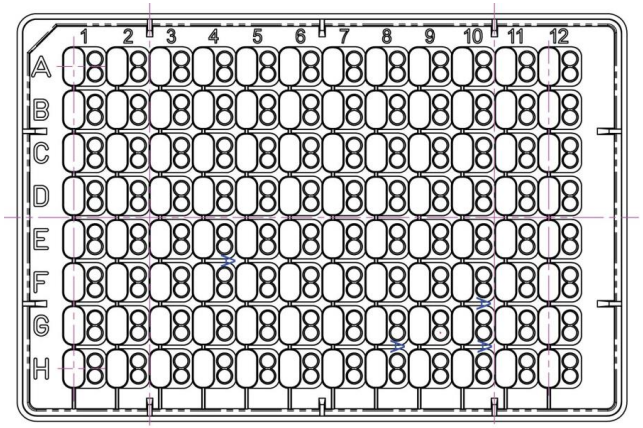
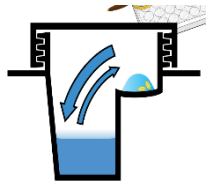






Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

Procedure:

1. Pipette precipitant screen solutions into MRC plate reservoirs
2. Dispense precipitant (**200-500 nl**) into sitting drop depression, using repetitive mode of multi-channel motorized pipette – this volume determines the final volume of the drop
3. Add 500 nl of protein solution to the precipitant droplets using repetitive mode of single-channel motorized pipette; sixteen drops with one aspiration-dispensing cycle
4. Check the plate under microscope and seal

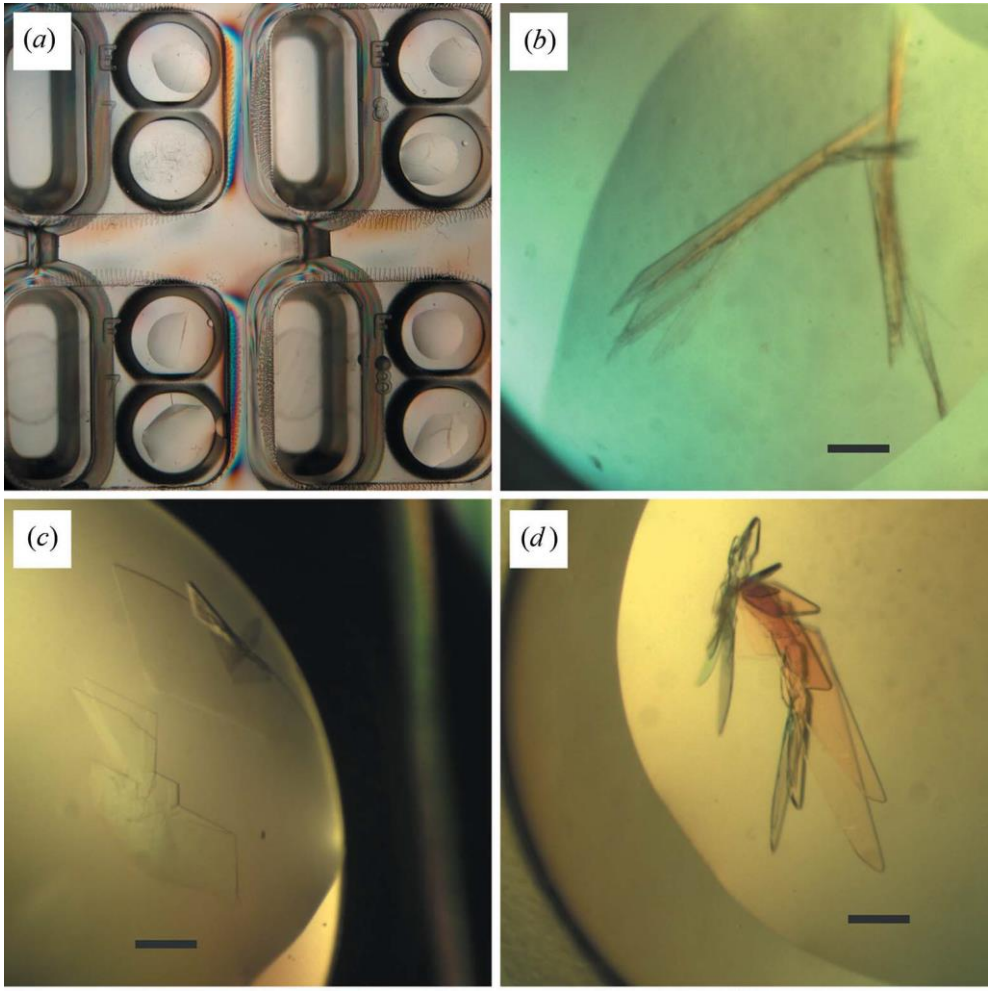
Loading of 192 sitting drops (steps 2-4) takes ~ 12 min

Non-robotic manual assembly of nanoliter drops

Advantages:

1. Easy to perform, does not require an extraordinarily skilled operator
2. Exceptionally low dead volume for protein solution dispensing (less than 2 µl for 96 drops)
3. The method could be extended for crystallization of small organic molecules using vapour diffusion, solvent evaporation or antisolvent liquid diffusion techniques
4. **No costly robotics needed**

Results: antibody Fab complexes



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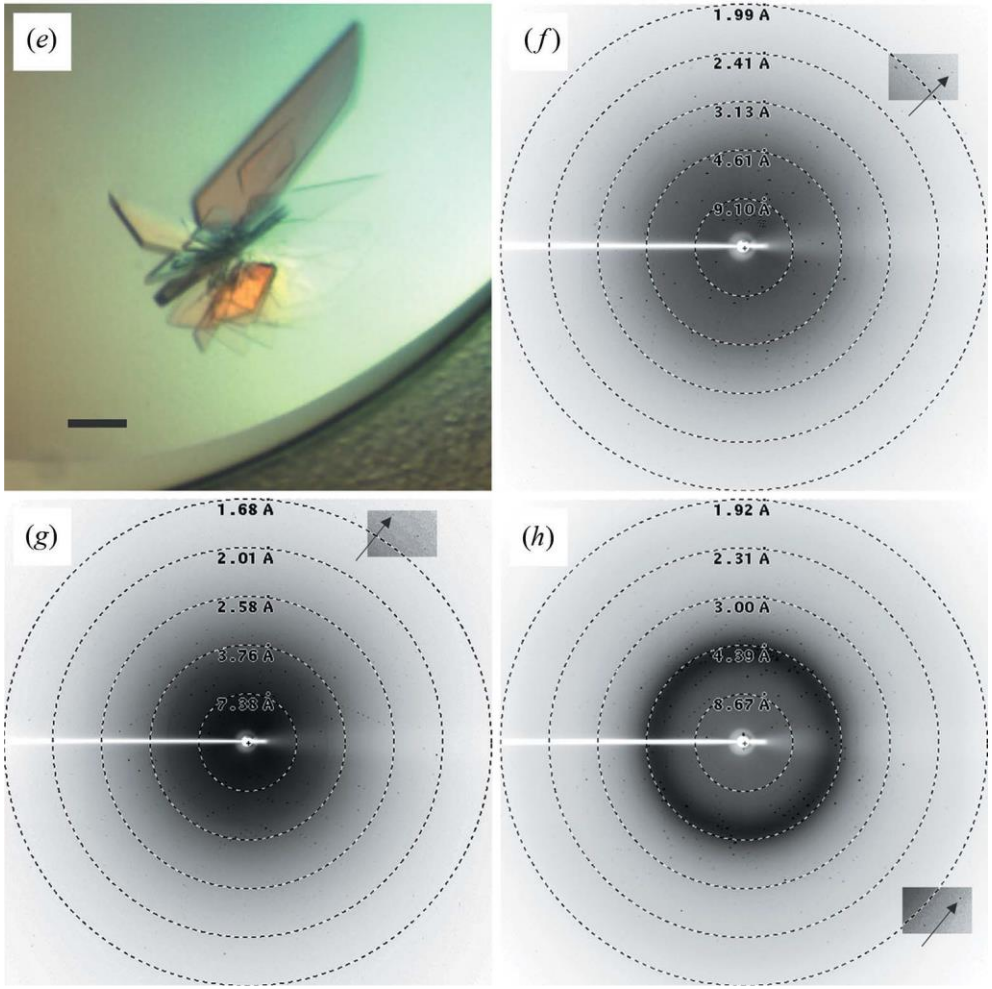


Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

Validation of manual dispensing

Accuracy and precision of the multichannel motorized pipette Pipetman Concept 8x10 in dispensing of nanolitre volumes of PBS and 25%(*w*/*w*) PEG 8000.

The values of means and standard deviations (SDs) are calculated from 80 replicates.

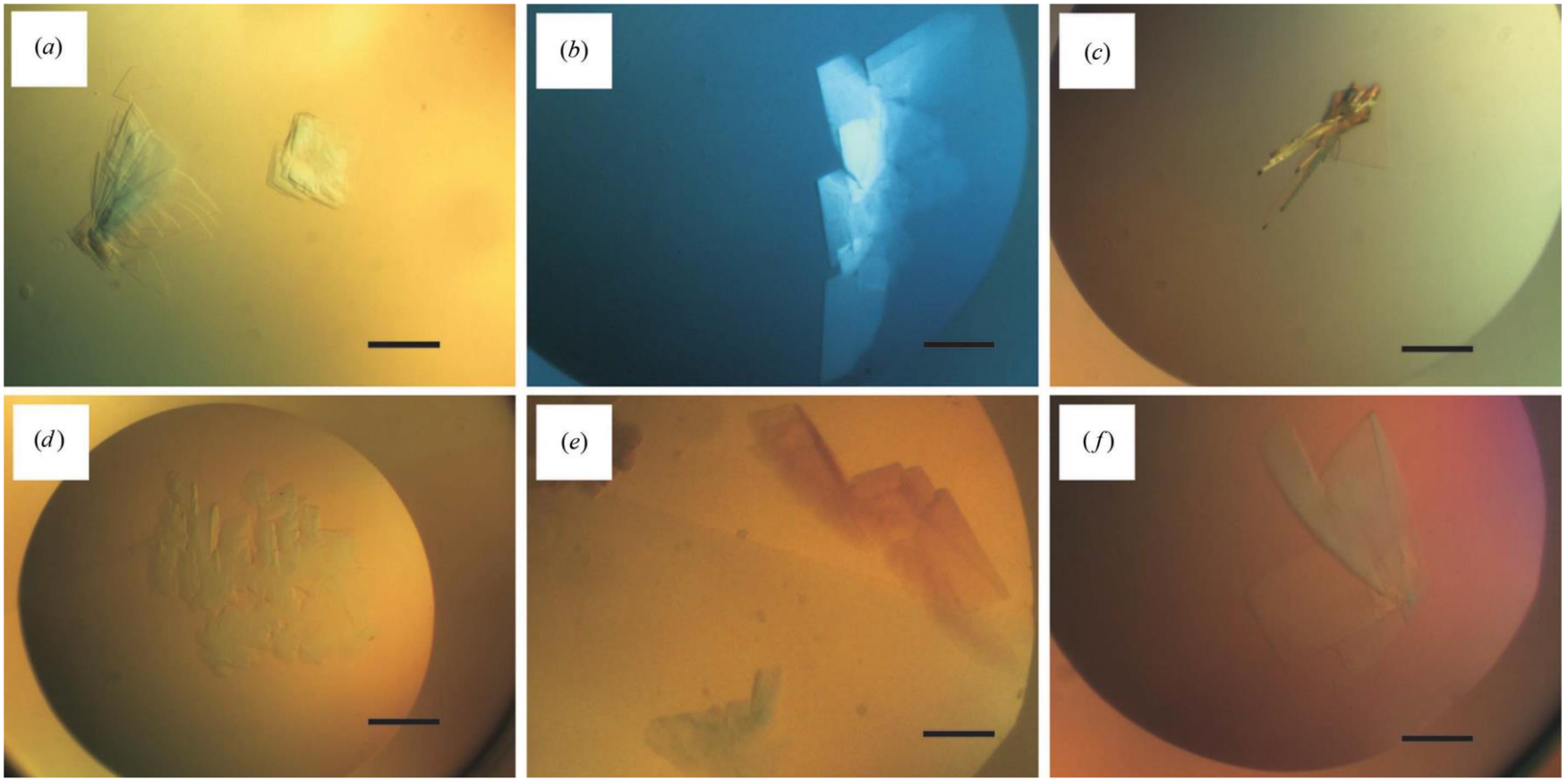
	PBS (nl)		PEG 8000 (nl)	
Pre-set volume (nl)	Mean	SD	Mean	SD
200	179	20	248	33
300	275	23	320	30
400	405	30	370	31
500	531	32	453	37

For a **500** nl pre-set volume, single-channel pipette C10 used for protein dispensing has a maximal systematic error equal to **± 40 nl**, with the random error equal or inferior to 13 nl (**2.6%** coefficient of variation).

Manual dispensing of the solutions of variable viscosity by a motorized multichannel pipette has similar variability and error as dispensing by robotics or by a handheld nanoject pipettor.

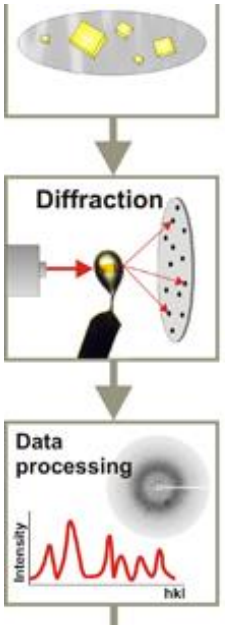
Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

Repeatability of the method



Skrabana, R. et al. (2012). J. Appl. Cryst. 45, 1061-1065.

II. X-ray data collection

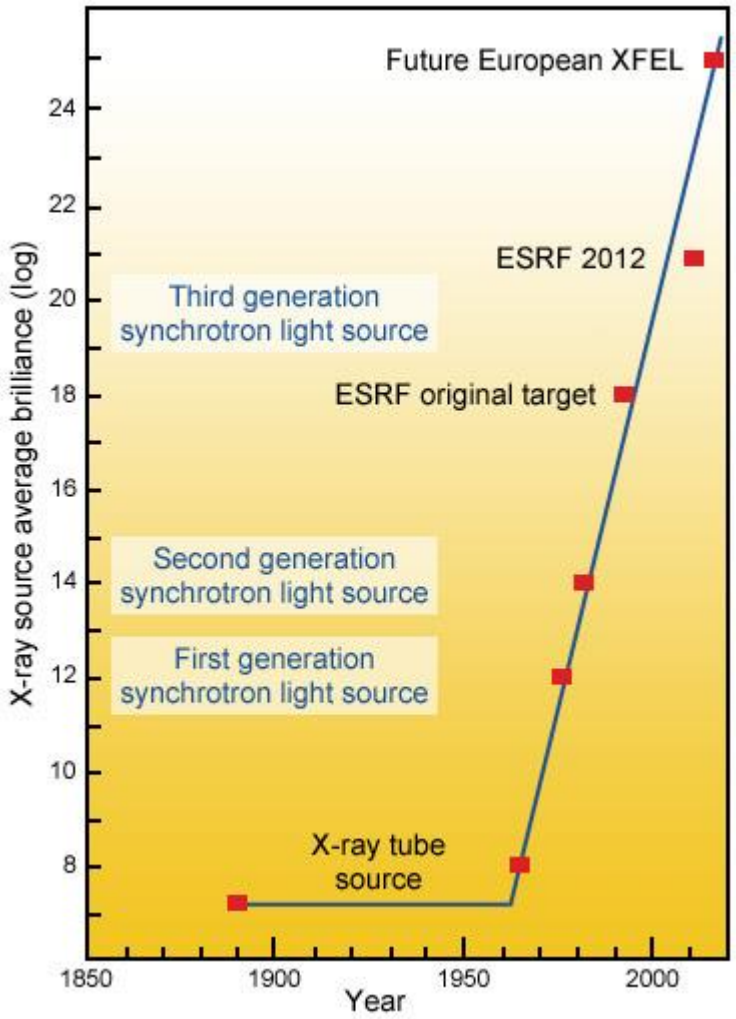


Data collection step is the last lab experiment in the structure determination pipeline

It has to be carefully optimized in terms of:

- crystal preparation (cooling)
- data collection strategy (crystal size, orientation, symmetry)
- source selection and tuning (beam dimension, wavelength, intensity, detector type and distance)
- Time of exposition versus ongoing radiation damage
- planned phasing method

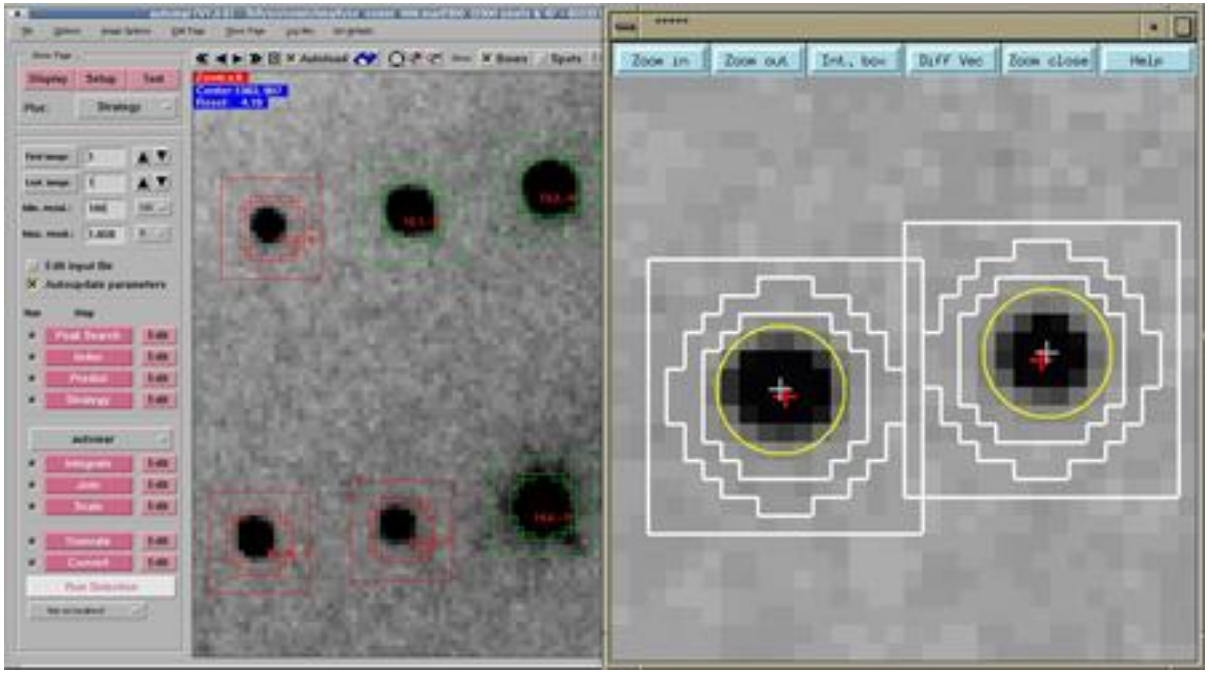
The panel of available X-ray sources



http://www.xtal.iqfr.csic.es/Cristalografia/archivos_02/brilliance-lg.jpg

Synchrotron beam time is nowadays “easily” available, but modern home sources may confer some advantages

III. Diffraction data processing

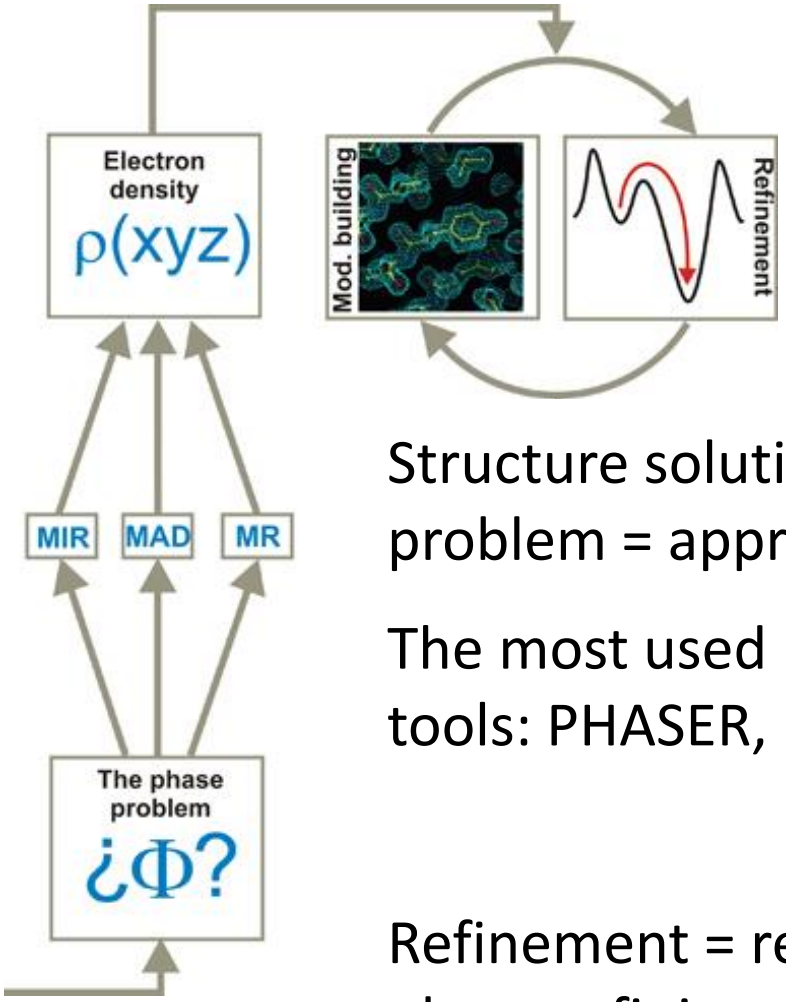


The first step in computational data analysis:

- its success or errors influence final model quality and reliability

Tools: XDS, MOSFLM, HKL3000, …

IV. + V. Structure solution and refinement

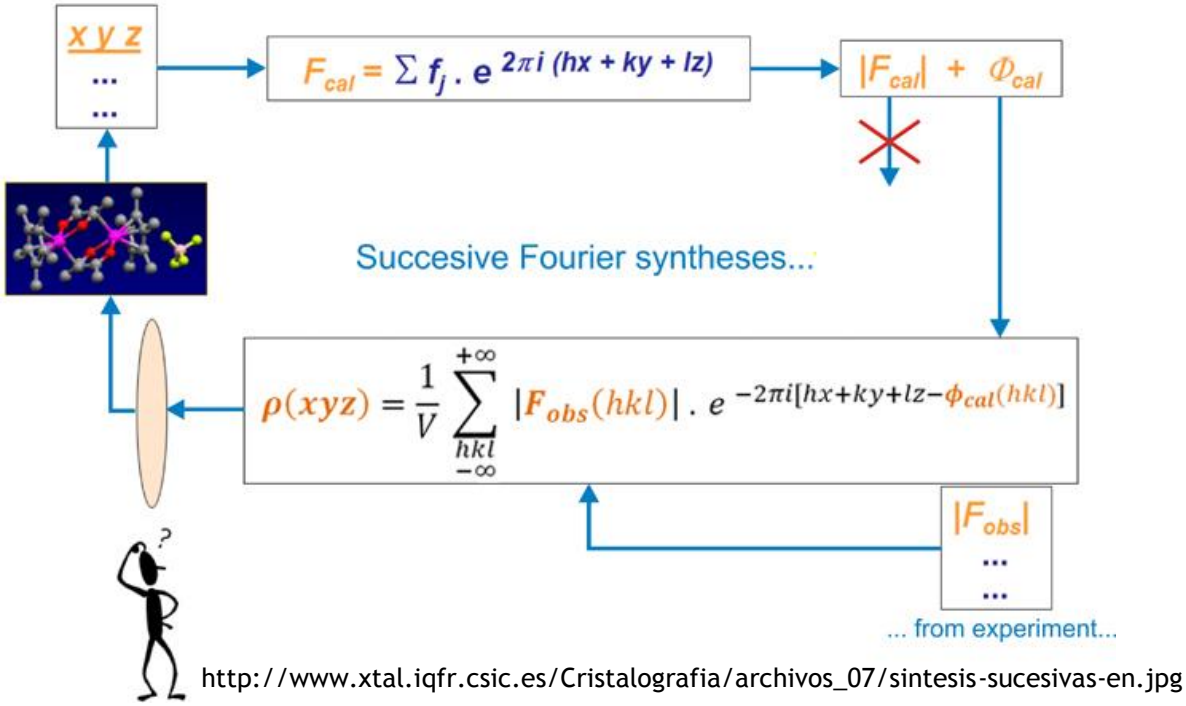


Structure solution = initial solution of the phase problem = approximate intial phases

The most used method is molecular replacement – tools: PHASER, MOLREP, …

Refinement = repetitive cycles of model building and phase refining

Iterative refinement of phases

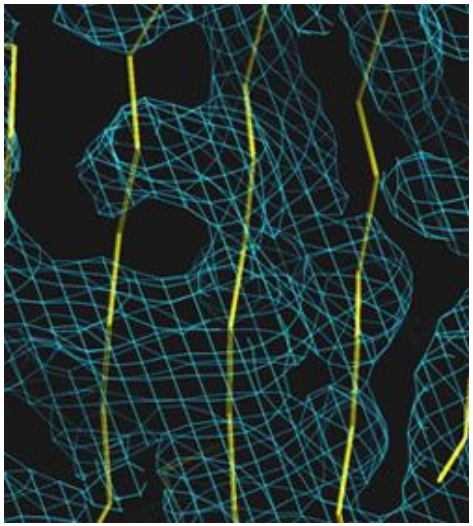


http://www.xtal.iqfr.csic.es/Cristalografia/archivos_07/sintesis-sucesivas-en.jpg

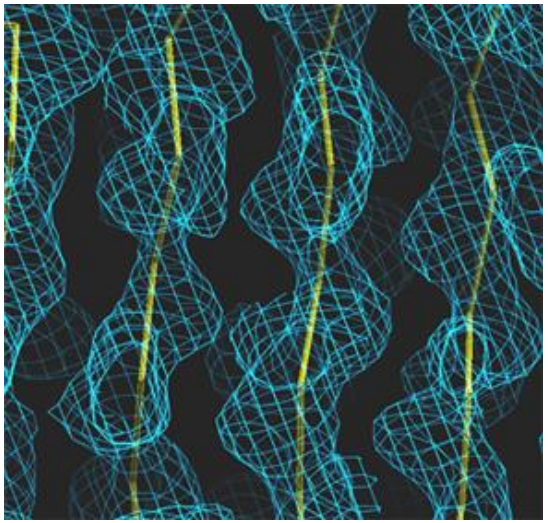
Manual model building require knowledge of stereochemistry of atoms and molecules under refinement, experience is of advantage …

Errors in placement of atoms in the electron density may escape purely numeric validation of refinement progress

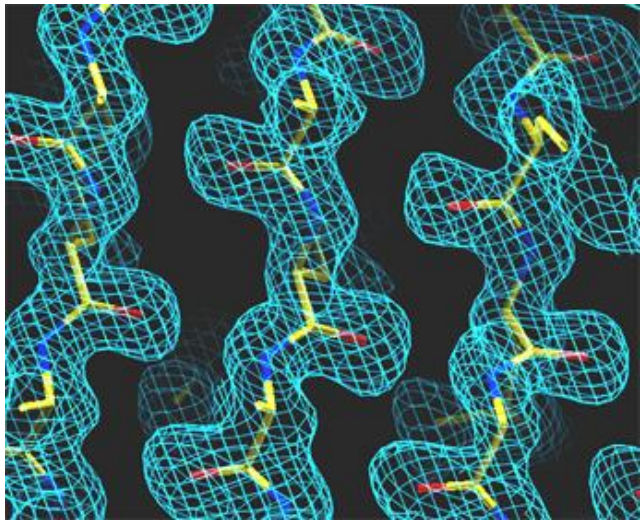
Reliability of details vs diffraction data resolution



5 Angstr.



3 Angstr.



1.7 Angstr.

Manual model building into available electron density:

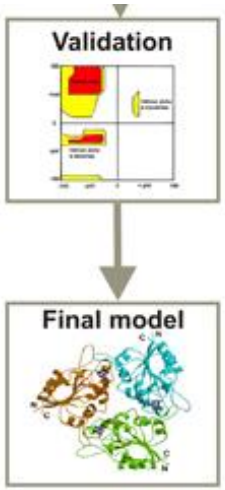
- A conservative approach needed

Tools for manual model building: COOT

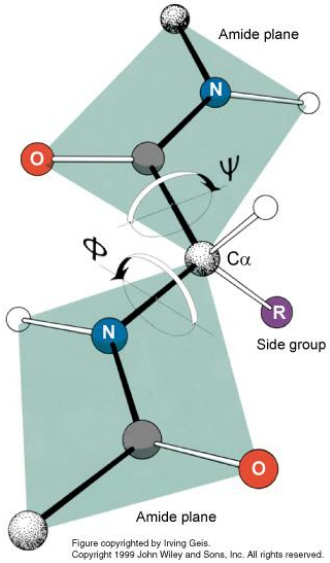
Tools for refinement in reciprocal space: REFMAC (CCP4), PHENIX, SHELX, …

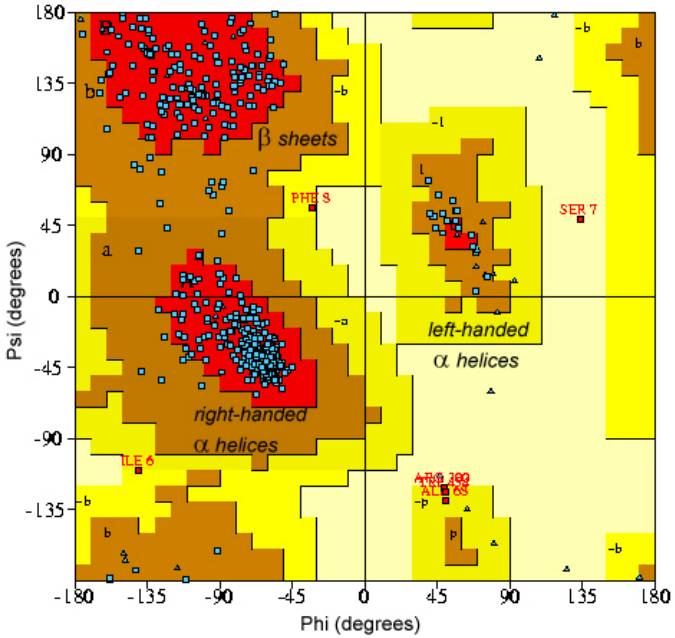
http://www.xtal.iqfr.csic.es/Cristalografia/archivos_08

VI. Model validation and deposition



Validation of the model is indispensable step after (**and during**) iterative structure refinement, before the deposition in PDB

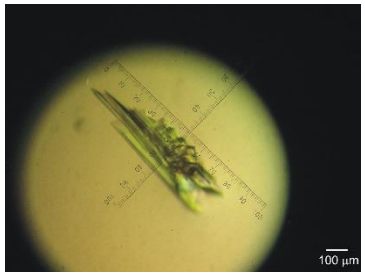
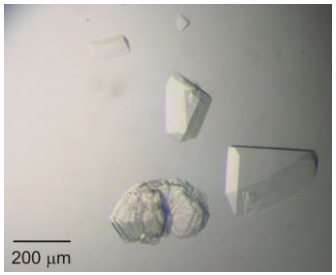




Meaningful bond lengths and angles, side chain rotamers, dihedral angles, stereochemistry, correspondence with electron density.

Tools: COOT, MOLPROBITY, …

Example of Refinement statistics

	2V17	3L10
		
Space group	P21	P21
Unit cell		
a (Å)	71.5	41.3
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c (Å)	85.5	72.7
β (°)	113.9	92.9
Protein molecules in a.u.	1	1
Resolution (Å)	1.65	2.0
R (%)[a]	16.0	16.2
Rfree (%)[b]	21.8	24.9
Model – atom sites	3323	3330
Solvent molecules	702	434
Number of zinc/sodium atoms	0	1/2

J Struct Biol 2010

Output of crystallographic experiments

- (static) coordinates of atoms = **model** of structure – containing various amount of **dynamic** features:

  a) alternative conformations of side chains, main chain segments, loops

  b) B-factors of atoms (atomic position displacements) – a proxy for relative evaluation of flexibility of individual part of molecule

  c) Errors of coordinates – direct measure of atomic detail reliability (not all refinement pipelines give these parameters)

  d) “invisible” parts of structure = too weak, non-interpretable electron density due to **disorder, or** missing chain (wrong construct, cleavage during crystallization …)

Useful links to web resources

Tutorials, theory, lectures:

- http://www.ruppweb.org/default.htm
- Wikipedia
- http://reference.iucr.org/dictionary/Main_Page - online dictionary of crystallography
- https://chem.libretexts.org/Bookshelves/Inorganic_Chemistry/Supplemental_Modules_(Inorganic_Chemistry)/Crystallography
- http://www.xtal.iqfr.csic.es/Cristalografia/index-en.html - A comprehensive online textbook
- https://www.phenix-online.org/documentation/dictionary.html

Useful links to courses, conferences, journals

Page of IUCr

https://www.iucr.org/

https://www.iucr.org/calendar/events

Further reading

Karplus, P. A. and K. Diederichs (2015). "Assessing and maximizing data quality in macromolecular crystallography." Curr Opin Struct Biol 34: 60-68.

Wlodawer, A., et al (2018). "Detect, correct, retract: How to manage incorrect structural models." FEBS J 285(3): 444-466.

Dauter, Z., et al (2014). "Avoidable errors in deposited macromolecular structures: an impediment to efficient data mining." IUCrJ 1(Pt 3): 179-193.

Wlodawer, A., et al (2013). "Protein crystallography for aspiring crystallographers or how to avoid pitfalls and traps in macromolecular structure determination." FEBS J 280(22): 5705-5736.

Minor, W. et al (2016). “The young person’s guide to the PDB.” Postepy Biochem. 62(3):242-249.

Outline of the Presentation

- Interplay of dynamics and statics in conformational ensemble of intrinsically disordered proteins (IDPs)
- The concept of synergic study of IDPs (and their aggregation)
- “Frozen” IDP structural features: crystallography of IDPs
- Principles, execution and evaluation of crystallographic experiment
- Crystallography of IDP dynamic features: surrogate IDP binding partners
- Crystallography of IDP aggregation

Freezing of IDPs in complex

- Crystallography of IDP dynamic features: surrogate IDP binding partners

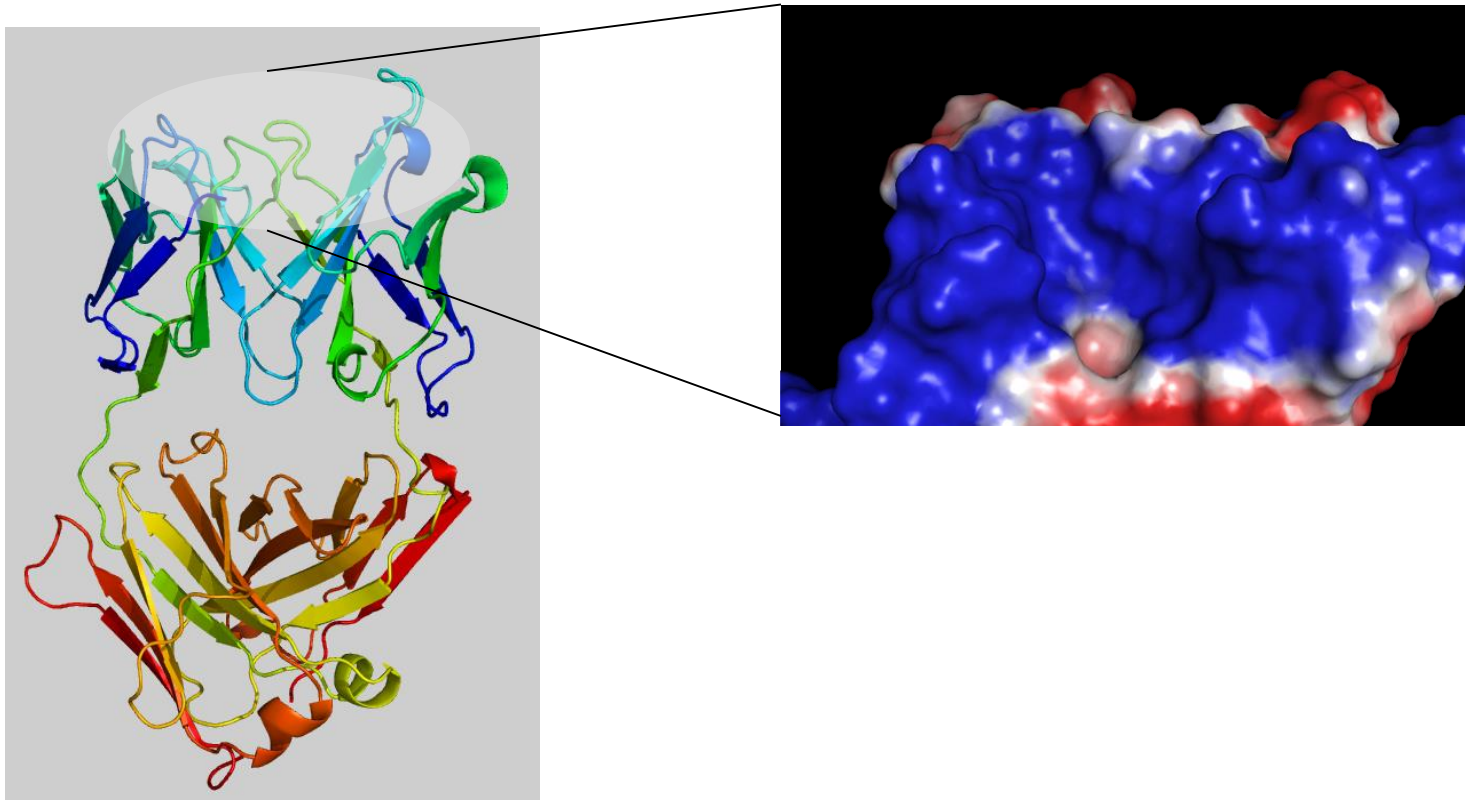
• Disordered tau alone is not forming crystals, but using binding partners it is possible to stabilize distinct functional fold of tau and likely crystallize the complex

• specific monoclonal antibodies can serve as surrogate tau protein binding partners to aid tau crystallization

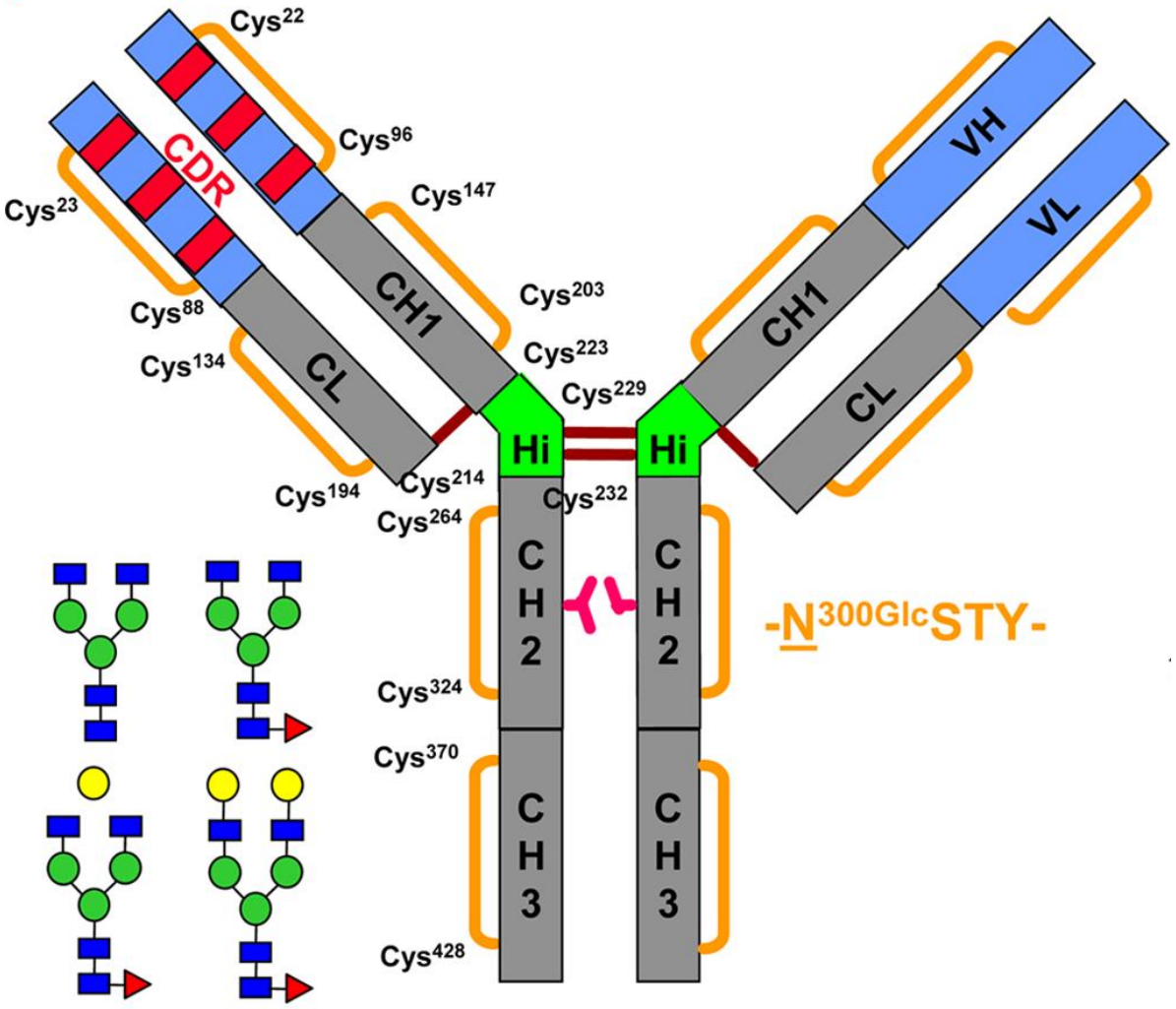
• conformation-dependent mAb can serve as a molecular mold

Antibodies for tau structural studies

Monoclonal antibody represents the surrogate binding partner of tau which permits study of the epitope

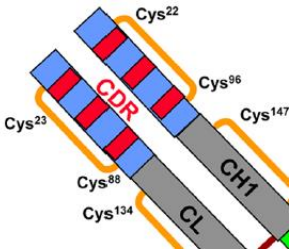


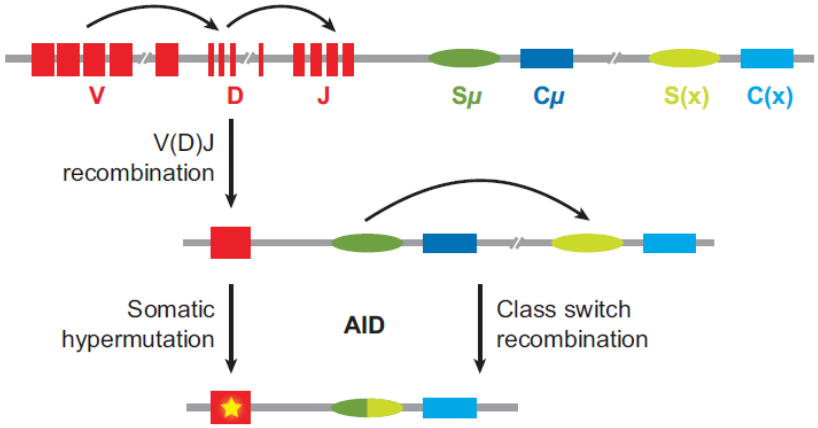
Main features of monoclonal antibody



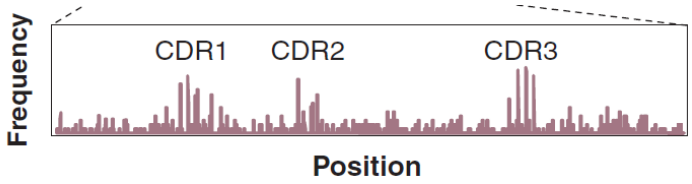
Beck et al (2013) Analytical Chemistry

Antibody is secreted by B-lymphocytes





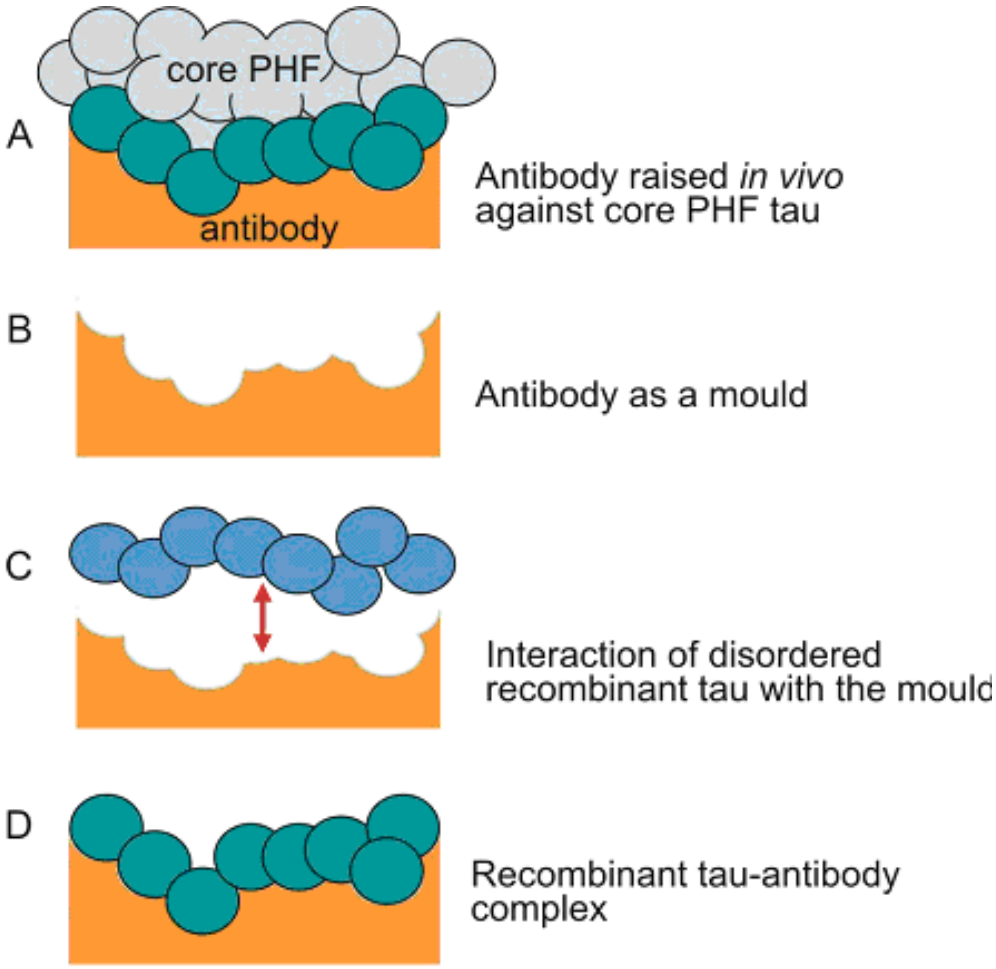
Teng & Papavasiliou (2007) AnnRev Genetics



The large variability (= multireactivity) of naïve B-lymphocyte Immunoglobulins is due to V-, D- and J- gene recombination producing variable domains of light and heavy chain

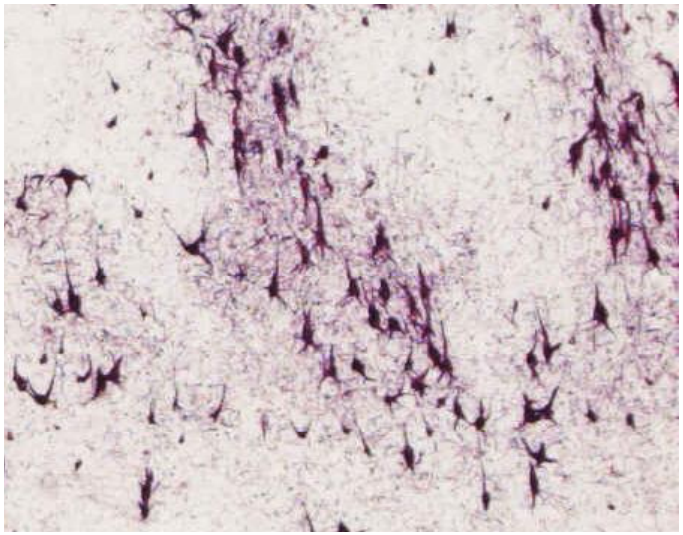
Mature antibodies acquire specificity by somatic hypermutations

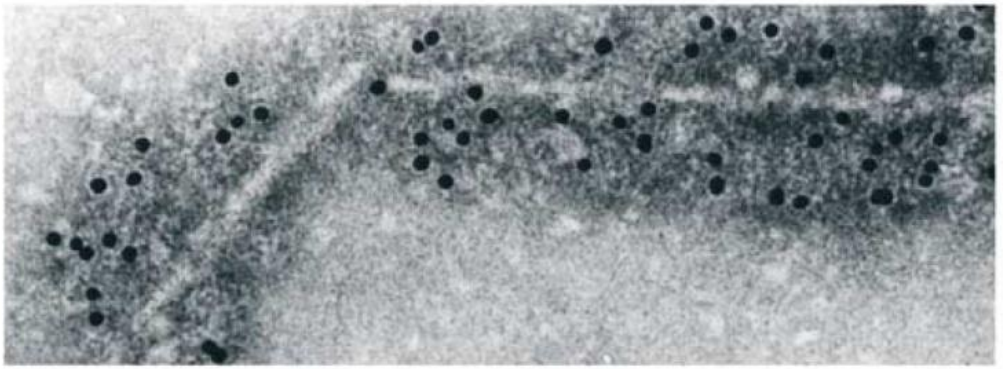
Recombinant tau acquire stabile conformation after binding to the antibody combining site

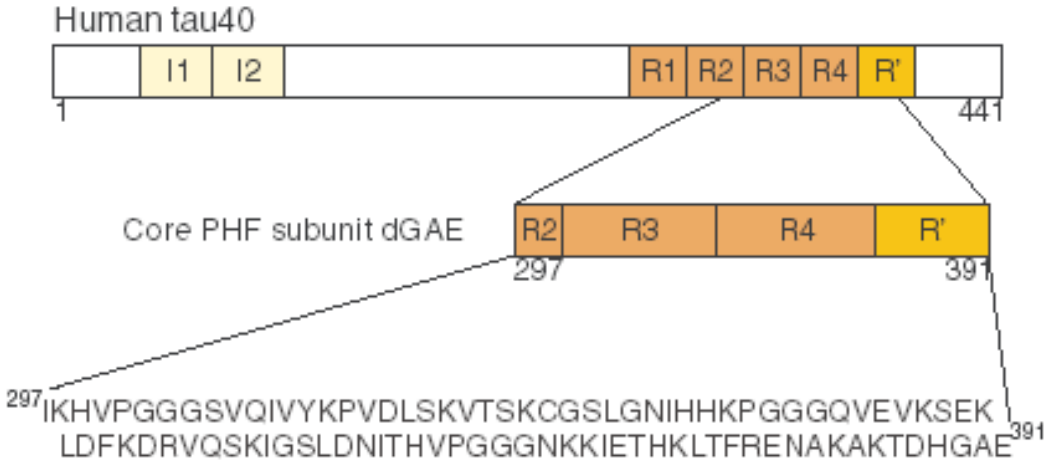


Sevcik et al *Prot&Pept Lett. 2009*

Antibody MN423 as an imprint of PHF core

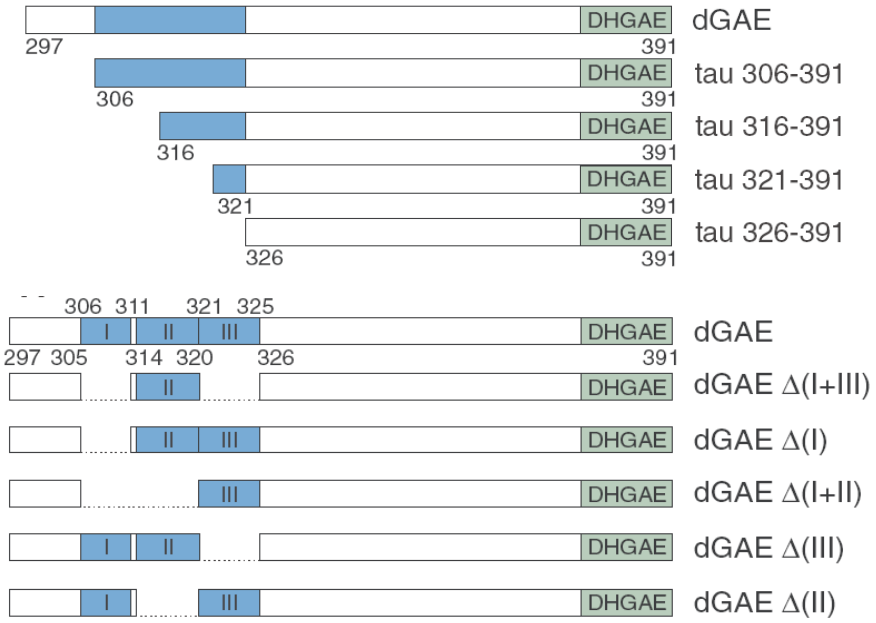


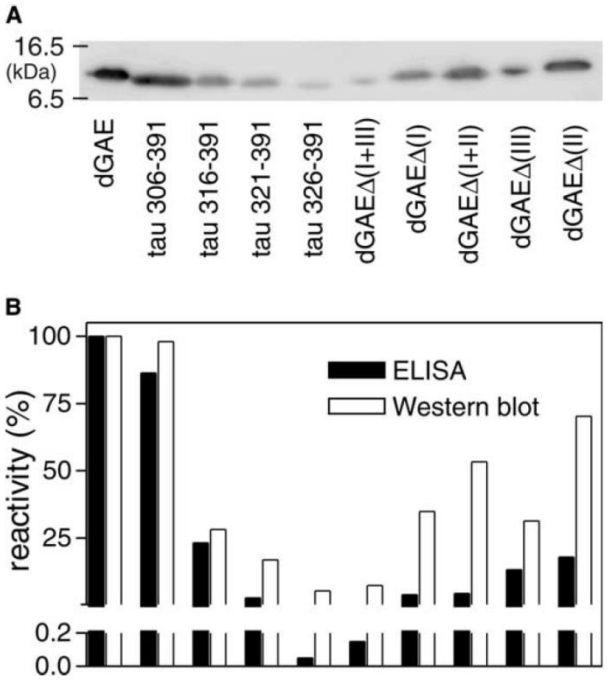




Skrabana et al *Febs Lett. 2004*

Antibody MN423 as an imprint of PHF core

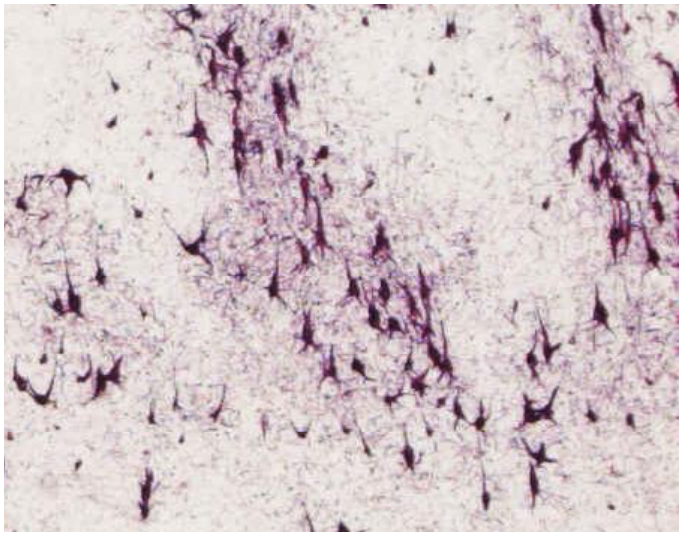


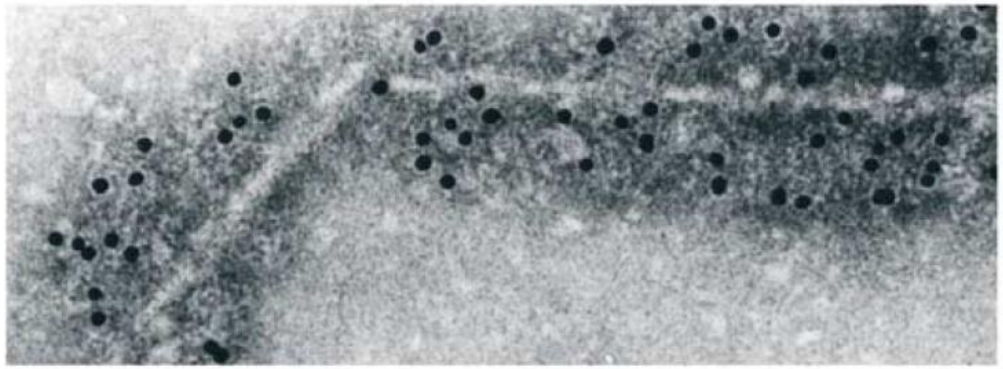


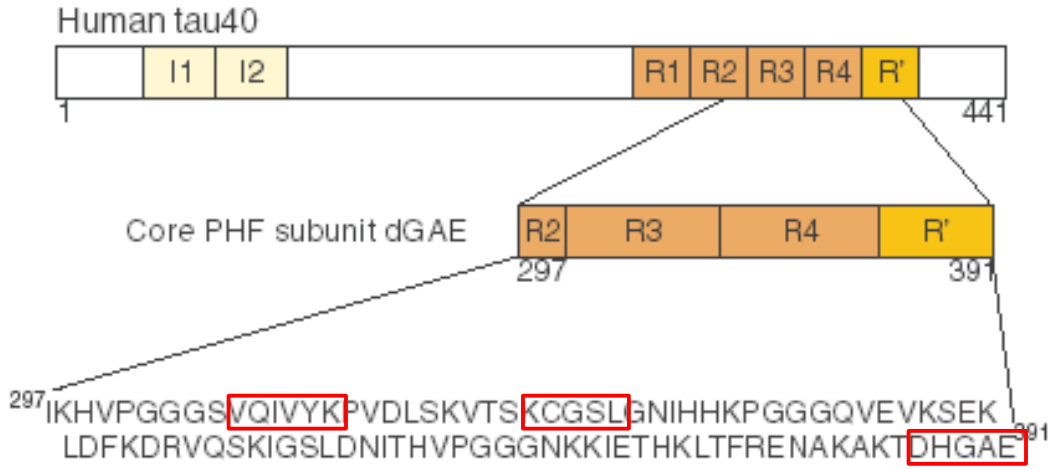
Antibody MN423 recognizes discontinuous epitope on tau from PHF core

Skrabana et al *Febs Lett. 2004*

Antibody MN423 as an imprint of PHF core





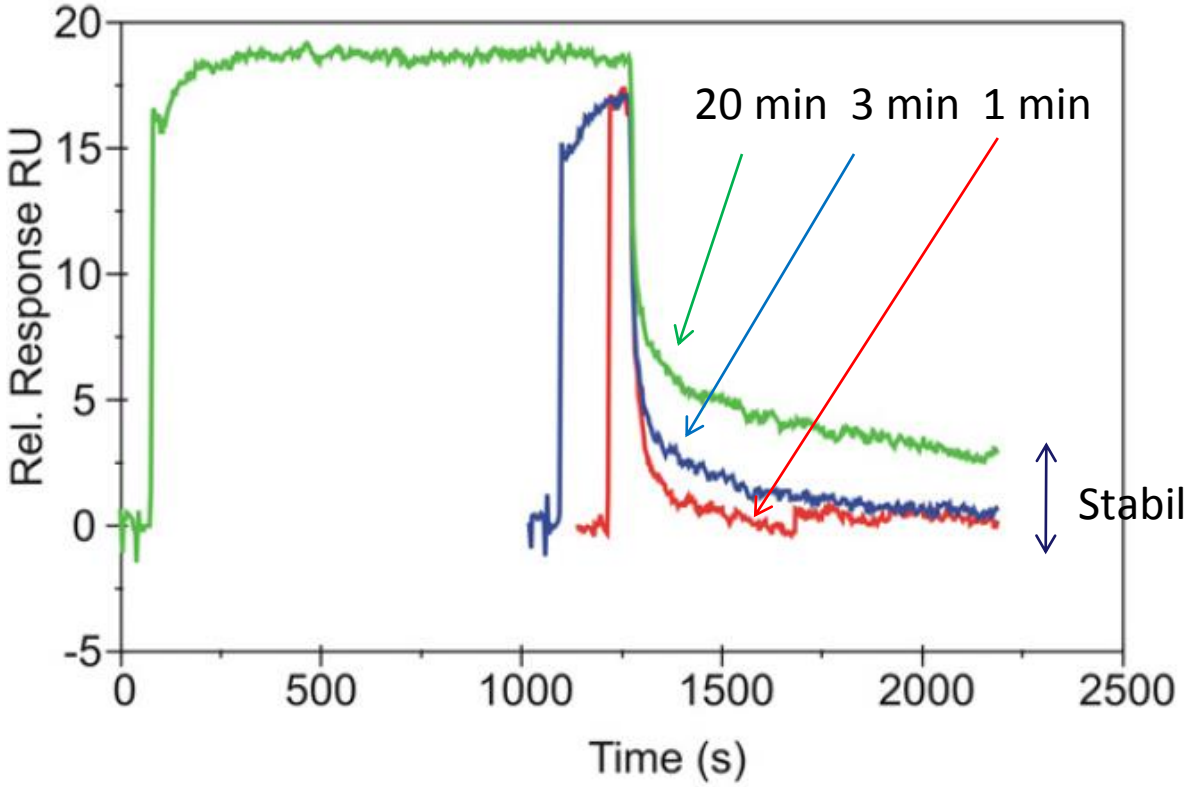


Epitope of MN423 is conformational

Skrabana et al *Febs Lett. 2004*

Binding to MN423 induces conformational change on tau

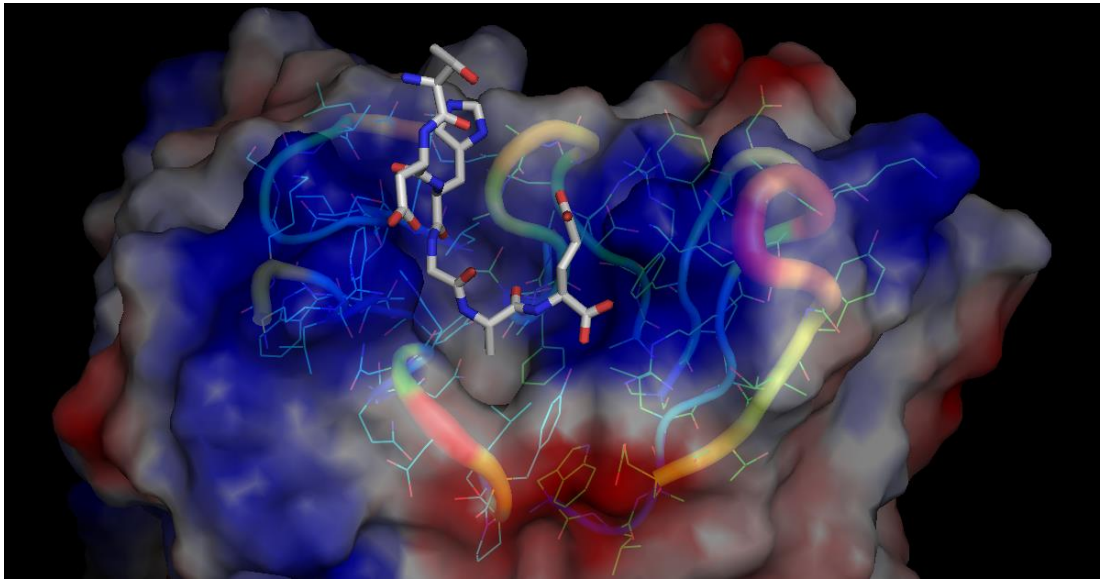
Interaction of MN423 with tau297-391 measured on optical biochip

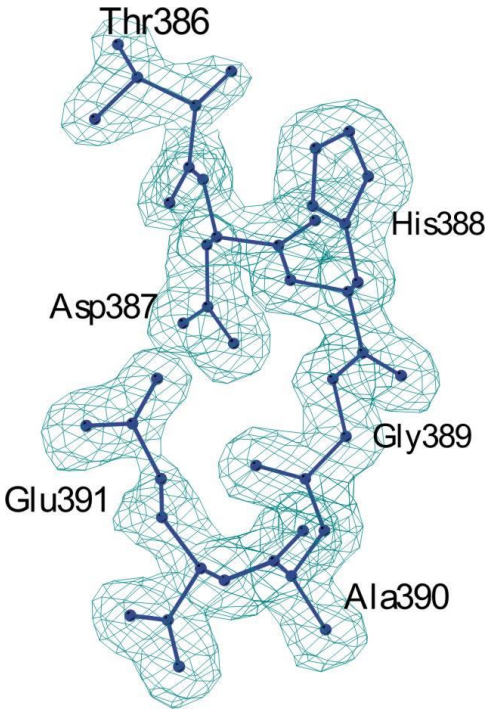


Recombinant analogues of PHF core tau likely change the conformation after binding to MN423

FEBS Letters 2007

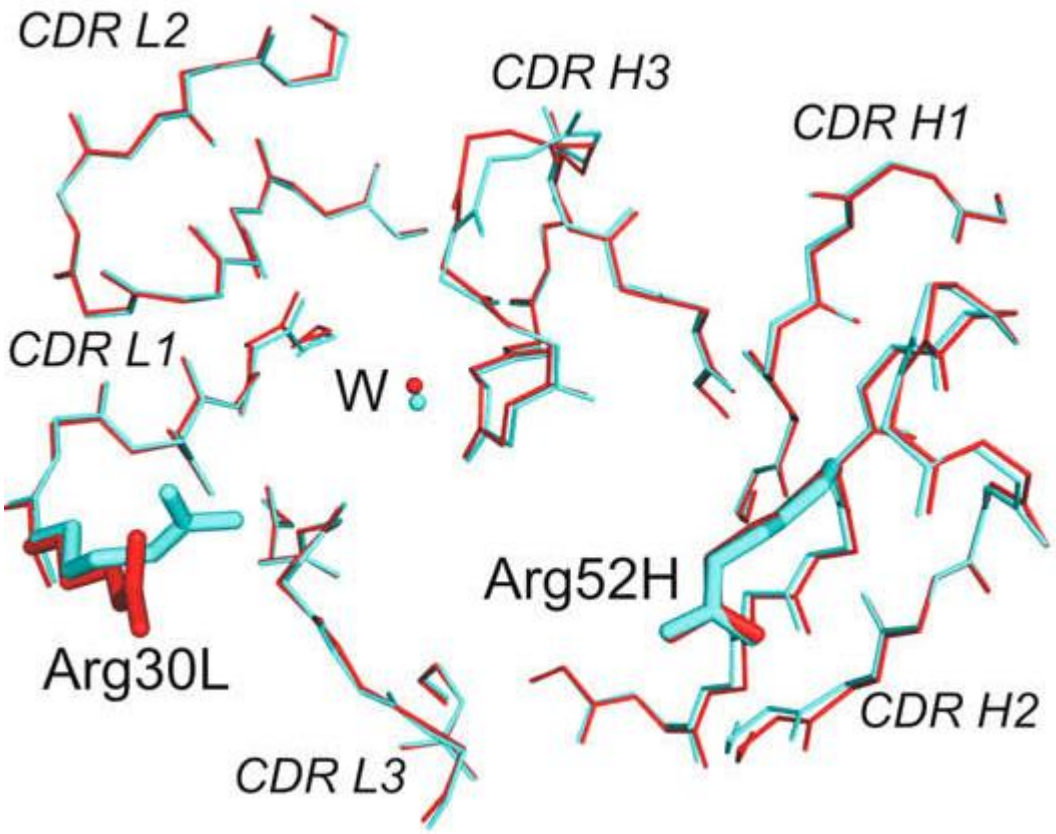
Atomic structure of PHF core C-terminus





Sevcik et al *Febs Lett. 2007*

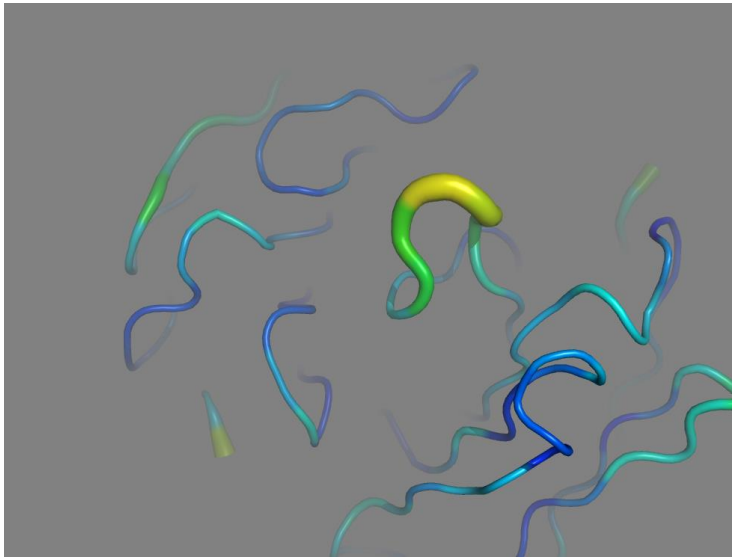
Validation: Combining site of MN423 is really a rigid mould

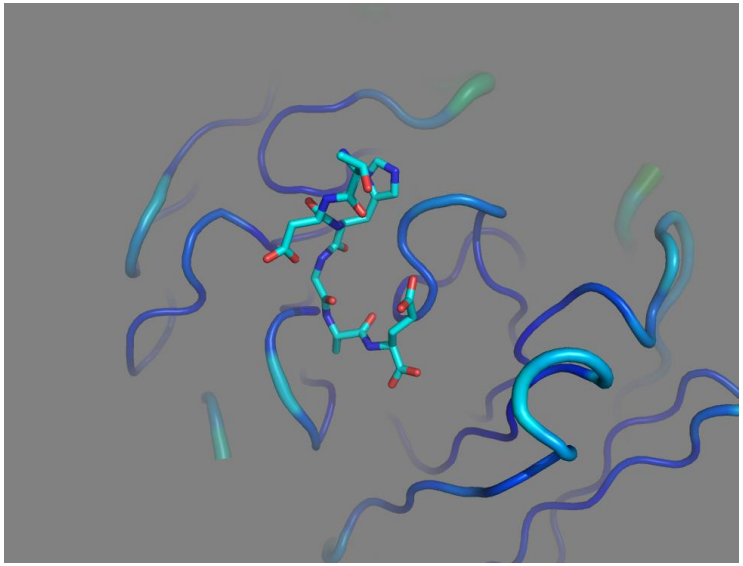


Red trace – free MN423; cyan trace – complexed MN423

J Struct Biol 2010

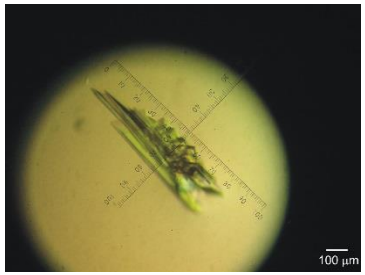
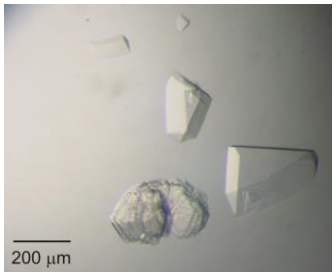
Comparison of free and bound MN423 antibody





No large change in antibody CDRs mobility (B-factor values) after complex formation – is it due to the **rigid tau antigen?**

Refinement statistics: complexed (2V17) and free (3L10) MN423

	2V17	3L10
		
Space group	P21	P21
Unit cell		
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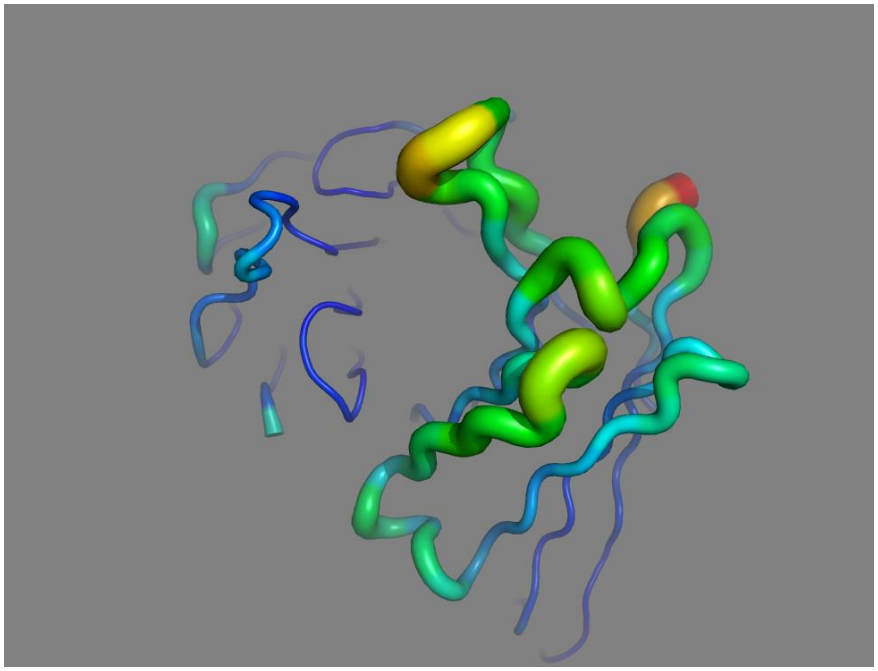
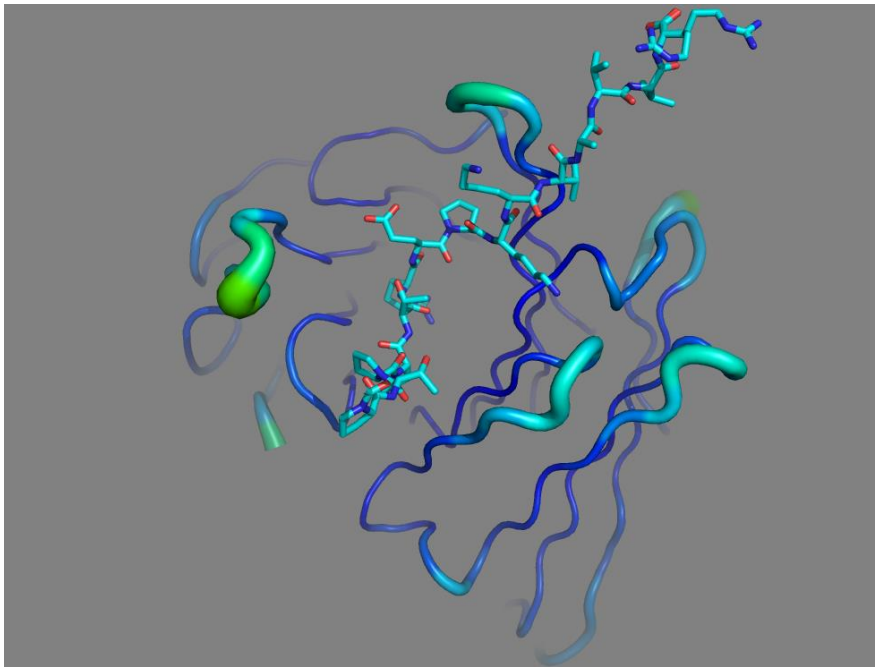
Model – atom sites	3323	3330
Solvent molecules	702	434
Number of zinc/sodium atoms	0	1/2

J Struct Biol 2010

Binding of tau and tau5 flexibility

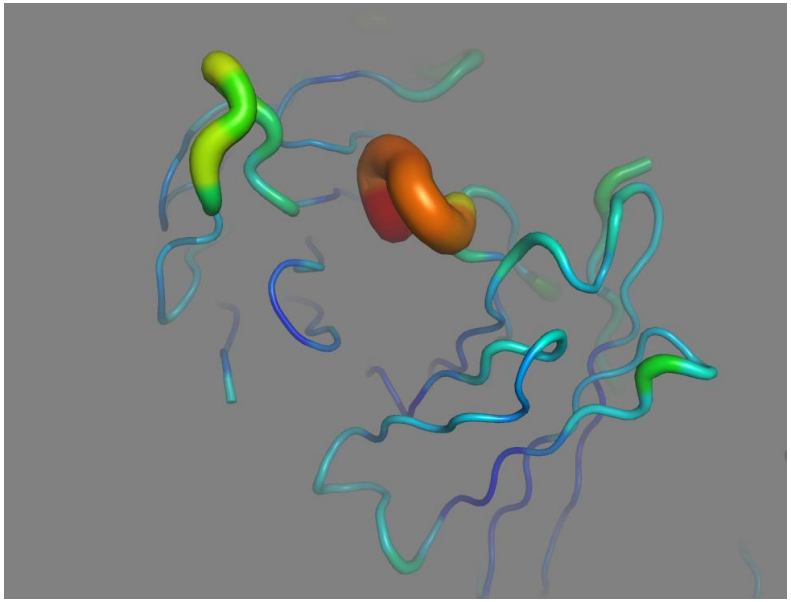


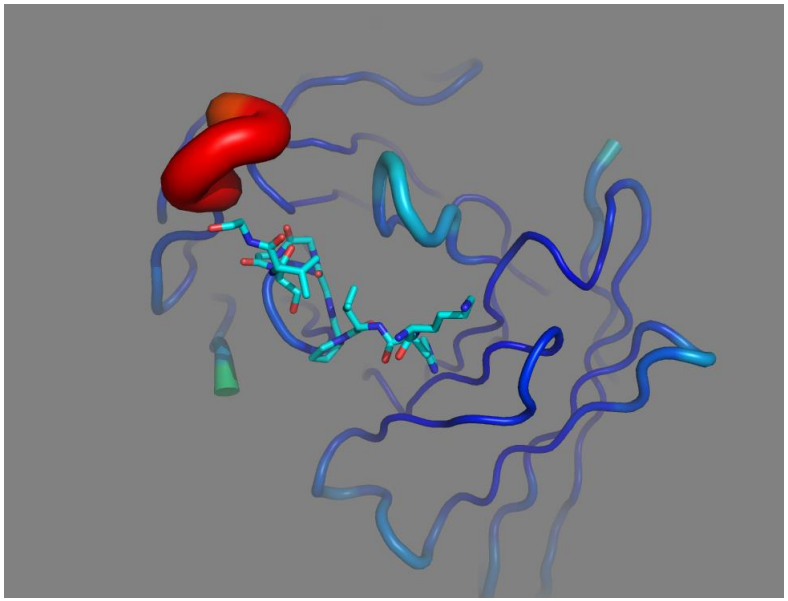


Again, CDRs (in the heavy chain) are largerly stabilized after complex formation.

Is the CDR flexibility a general feature for regonition of intrinsically disordered antigens?

DC8E8 has exceptionally flexible CDRL1 and H3 loops





CDR H3 is largely stabilized after complex formation, CDR L1 remains flexible and may exert inhibition of tau-tau interaction

Is the CDR flexibility important for recognition of four homologous tau sequences?

Conclusions

Antibody MN423 raised against a rigid tau PHF core exhibits no large change in antibody CDRs mobility after complex formation

Antibodies AX1 and Tau5, recognizing a flexible, disordered tau chain, exhibit stabilization of CDR loops after complex formation.

Is the CDR flexibility important for recognition of four homologous tau sequences?

Is the CDR flexibility a general feature for recognition of intrinsically disordered antigens?

Outline of the Presentation

- Interplay of dynamics and statics in conformational ensemble of intrinsically disordered proteins (IDPs)
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- “Frozen” IDP structural features: crystallography of IDPs
- Principles, execution and evaluation of crystallographic experiment
- Crystallography of IDP dynamic features: surrogate IDP binding partners
- Crystallography of IDP aggregation

Anatomy of amyloid fibrils

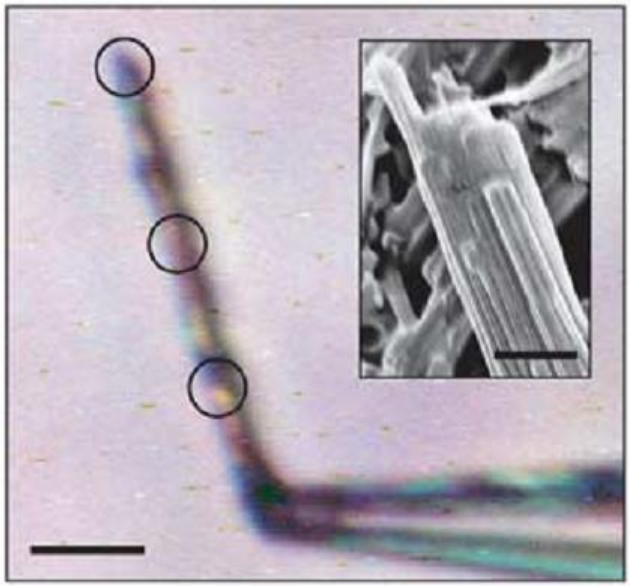
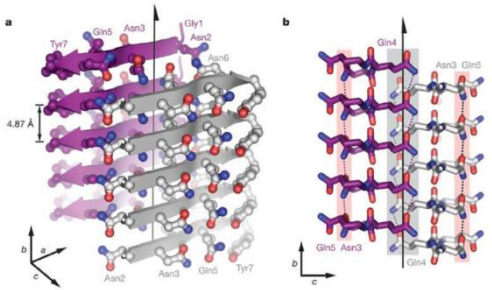
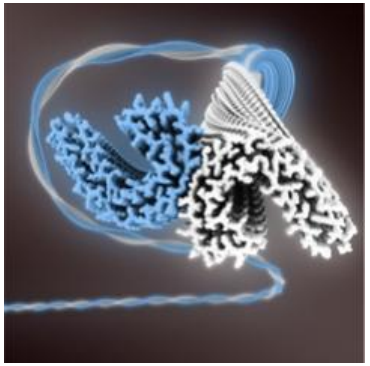
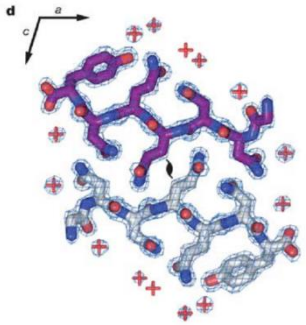
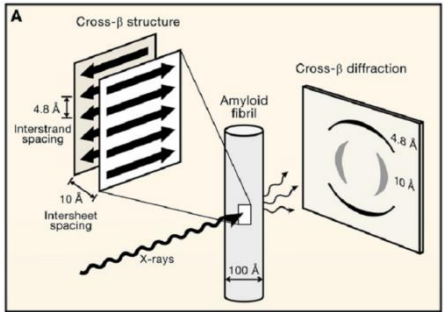


Figure 1 | The NNQQNY microcrystal used for X-ray diffraction data collection, held to the tip of a glass capillary by cryoprotectant (50% ethylene glycol/water). Scale bar, 10 mm. X-rays were focused on the encircled areas. Separate data sets were collected for each and were merged to provide the final data set. The inset shows a scanning electron micrograph of NNQQNY crystals, suggesting that the 'large' microcrystals used for data collection are composed of several aligned, nanometre-sized blocks. Scale bar of inset, 1 mm.





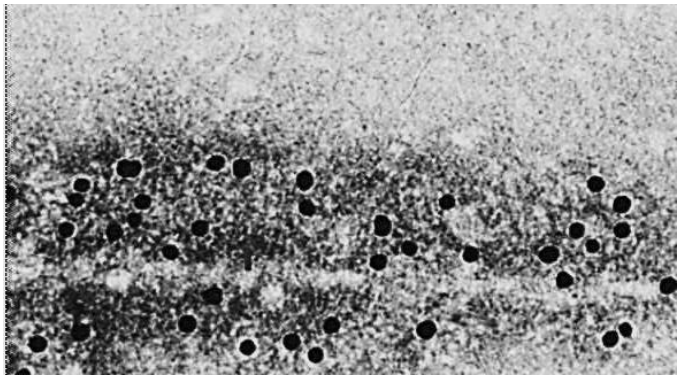


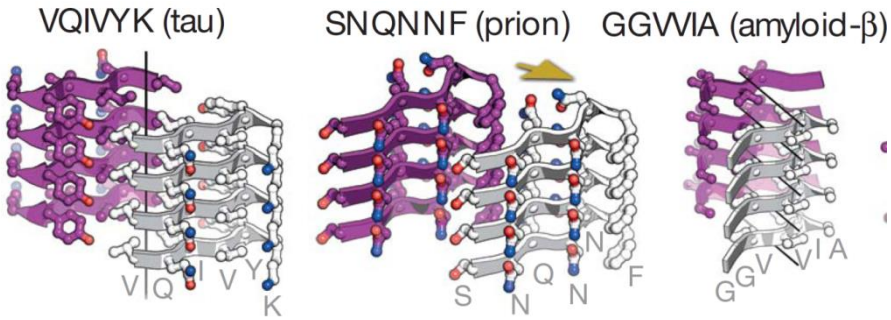


Eisenberg D and Jucker M (2012) Cell

65

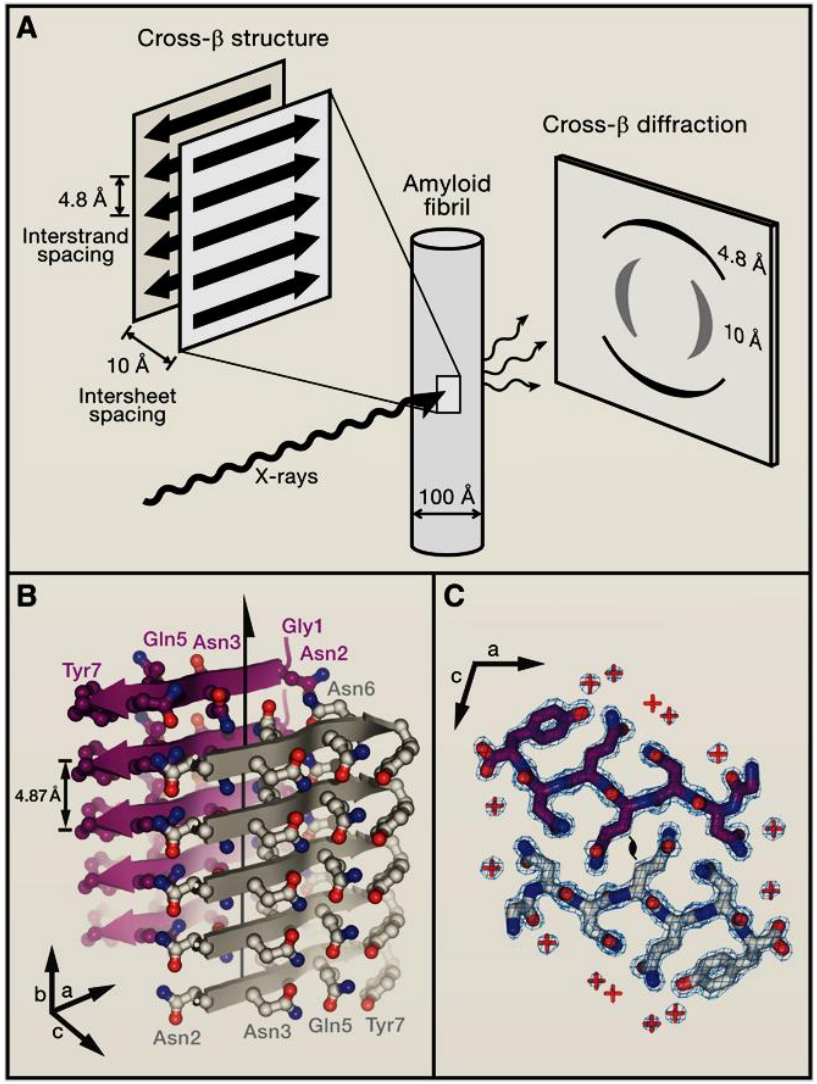
Common fold observed in amyloidoses revealed by X-ray crystallography





Sawaya et al 2007

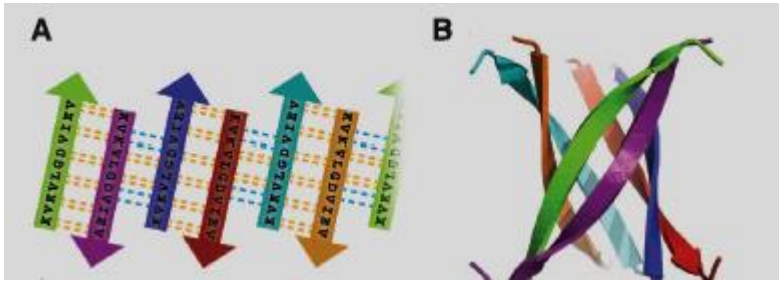
Spine of amyloid fibrils is formed by steric zipper from amyloidogenic sequences



Eisenberg and Jucker 2012

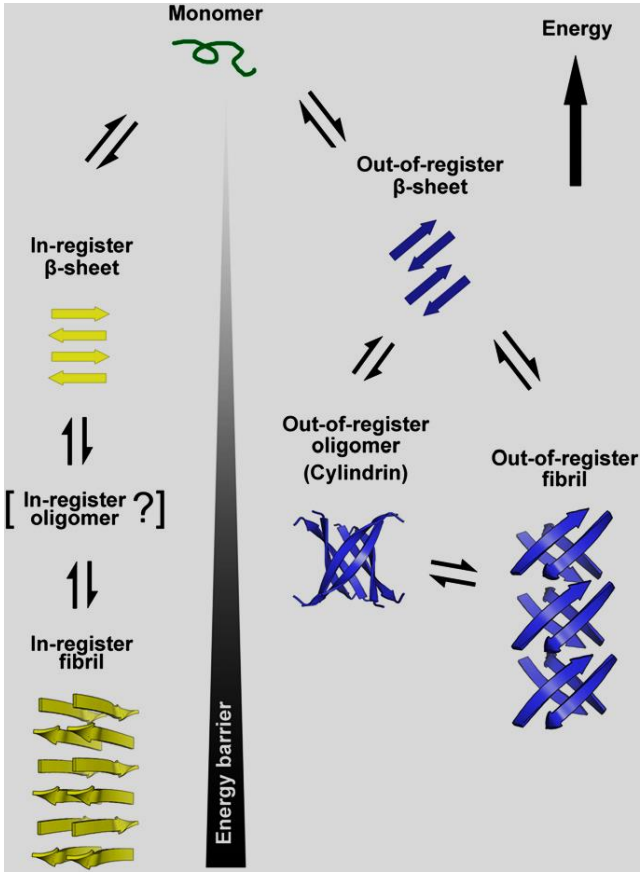
Toxic gain of function of assembled proteins?

Amyloid-prone sequences can form small oligomeric cylindrins



Laganowsky et al (2012) Science

Out-of-register oligomers and fibrillar aggregates are toxic to cells



Liu et al (2012) PNAS

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