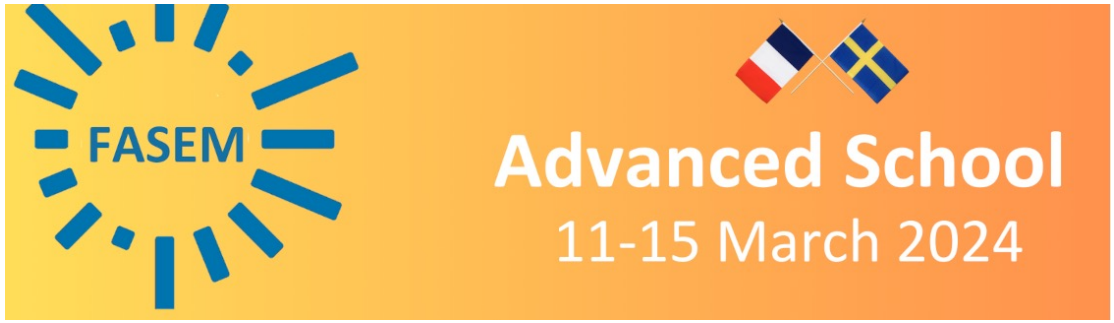
Sample preparation for neutron scattering: biodeuteration & protein crystallization.

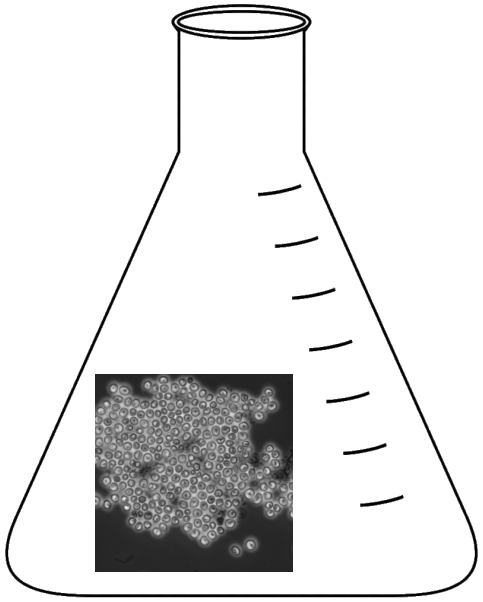


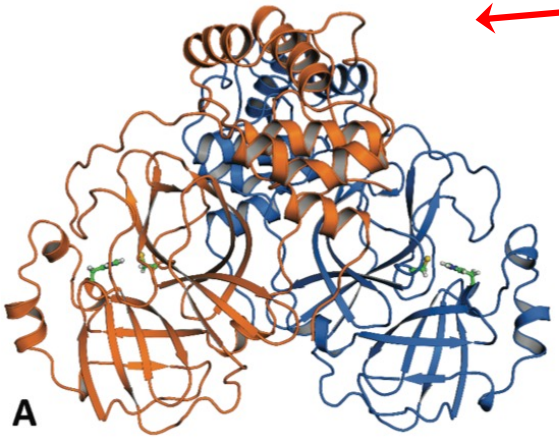
Zoë Fisher

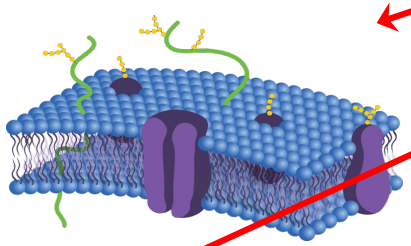
DEMAX platform @ ESS & Biology Dept @ LU

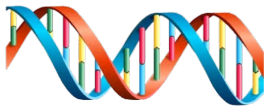
Deuterated biomaterials are in demand!

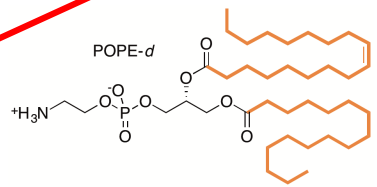
- Used in neutron scattering, NMR, mass spectrometry
- We can extract from whole cells (biomass): Proteins, peptides, DNA, lipids, carbohydrates (e.g. cellulose)

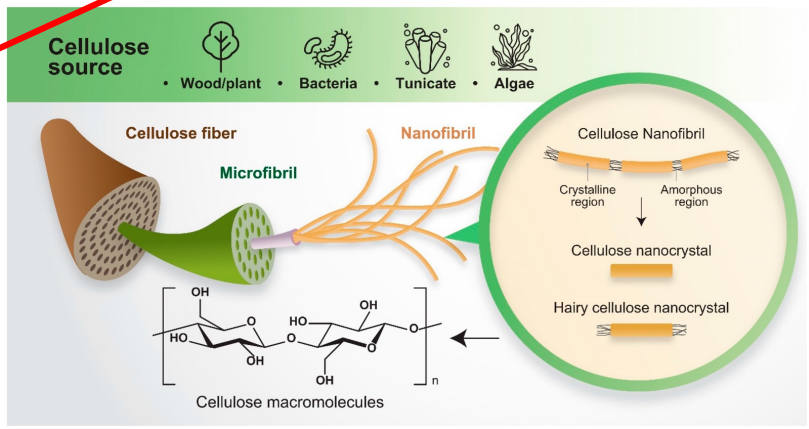










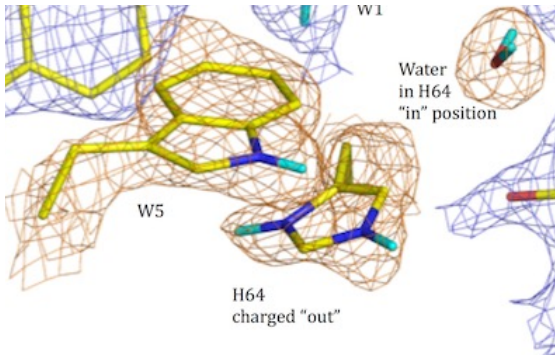


https://doi.org/10.3390/ma16103856

Biomolecules in neutron scattering are used for:

Single crystal diffraction

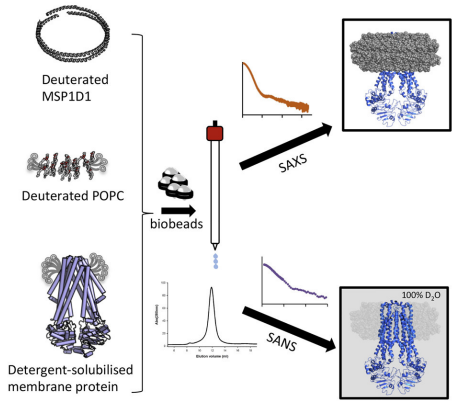
Macromolecular structures:
Localization of hydrogen atoms



Enzyme mechanism, effect of mutations, drug binding

Small angle neutron scattering

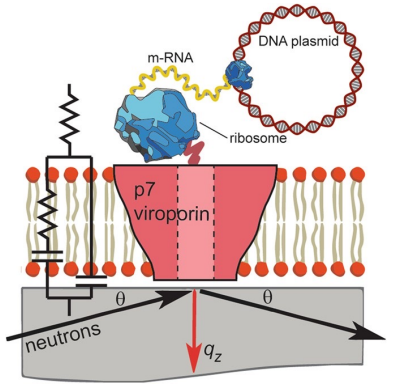
Nanoscale structures: localization of components using H/D contrast



Proteins, DNA/RNA, liposomes/ nanodiscs, membrane proteins, drug delivery systems

Neutron reflectometry

Nanoscale structure membranes, and surfaces using H/D contrast



Membrane proteins/peptides, drug delivery systems, insertion, fusion

Atomic structures

3D structure of protein molecules

Solution Structures

Size and shape of complexes in solution

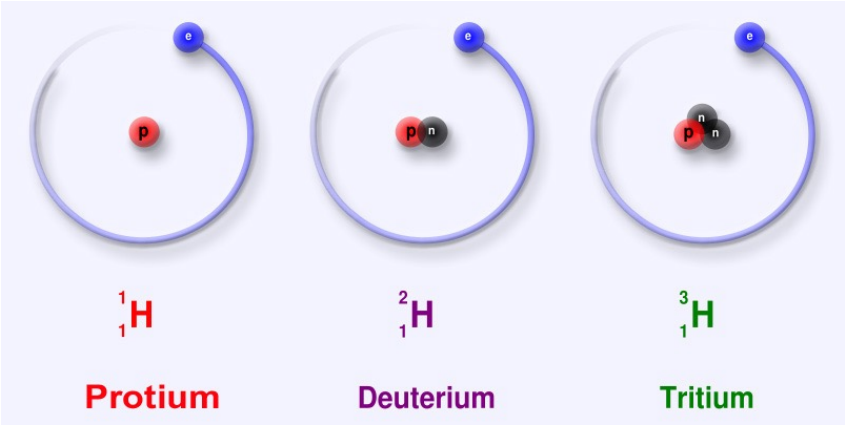
Surface structures

Structure & composition of surfaces

Borrowed & adapted from Hanna Wacklin-Knecht

What do we mean by deuterated biomaterials?

- Molecules from living organisms are abundant in hydrogen, spec. ^{1}H isotope
- Deuteration: replacing endogenous ^{1}H with ^{2}H to greater or lesser extent through a variety of methods
- Nomenclature: *deuterated, perdeuterated, H/D exchange*



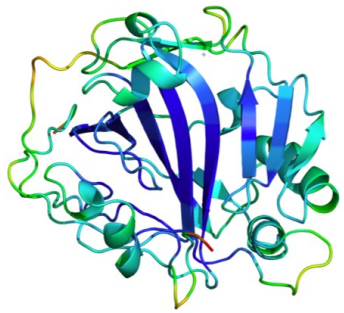
1 in 6420 H atoms are ^{2}H

Carbon	C	1647
Hydrogen	H	2565
Nitrogen	N	465
Oxygen	O	517
Sulfur	S	21

Formula: $C_{1647}H_{2565}N_{465}O_{517}S_{21}$

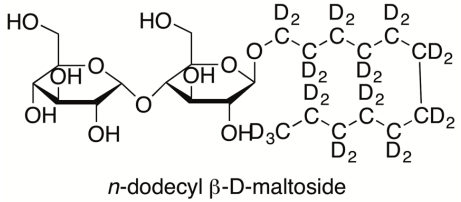
Total number of atoms: 5215

~ 350 amino acids

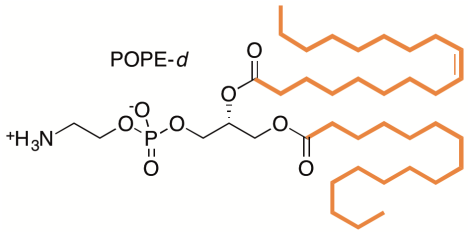


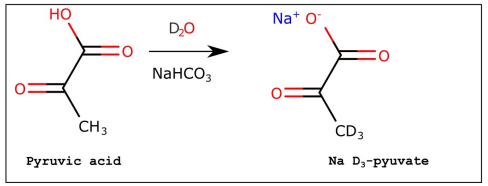
Different kinds of deuteration: chem vs. bio

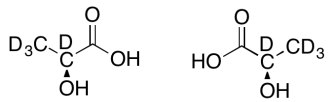
- **Chemical deuteration**: organic synthesis of small molecules using either commercial deuterated precursors and deuterated solvents, or make the precursors/monomers in the lab using Parr reactor (pressure, temp, catalyzed H/D exchange).



n-dodecyl β-D-maltoside

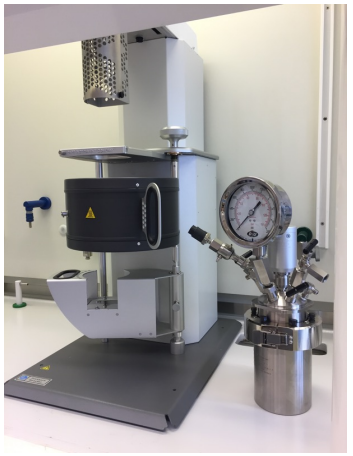


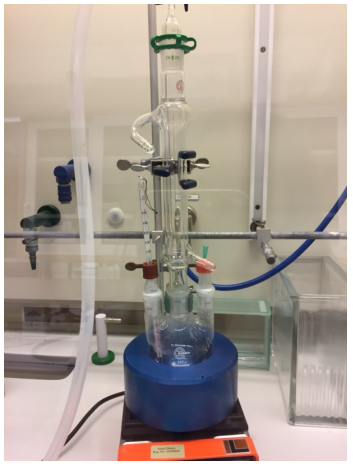




L-lactic acid-d_4 D-lactic acid-d_4

- **Biological deuteration**: production of molecules under deuterated conditions in living cells (the rest of this talk)





H atoms have special neutron scattering properties

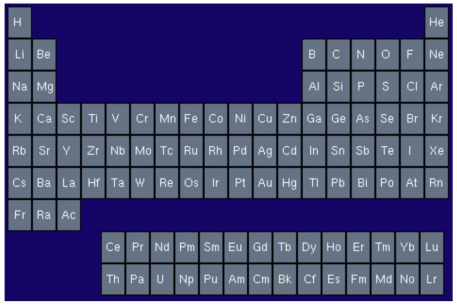
^{1}H has relatively small coherent scattering and very large background, while ^{2}H (deuterium) has the opposite!
^{1}H also has negative scattering – leads to signal cancellation of neighboring atom;
^{2}H (D) has positive scattering & low background – gives strong peaks in density maps

NIST Center for Neutron Research

NIST National Institute of Standards and Technology

Home | Live Data | Instruments | CHRNS | Proposals

Neutron scattering lengths and cross sections



NOTE: The above are only thermal neutron cross sections. I do not have any energy dependent cross sections. For energy dependent cross sections plea
National Lab.

Select the element, and you will get a list of scattering lengths and cross sections. All of this data was taken from the Special Feature section of neutron sc
their isotopes in *Neutron News*, Vol. 3, No. 3, 1992, pp. 29-37.

The scattering lengths and cross sections only go through element number 96 Cm (Curium)

A long table with the complete list of elements and isotopes is also available.

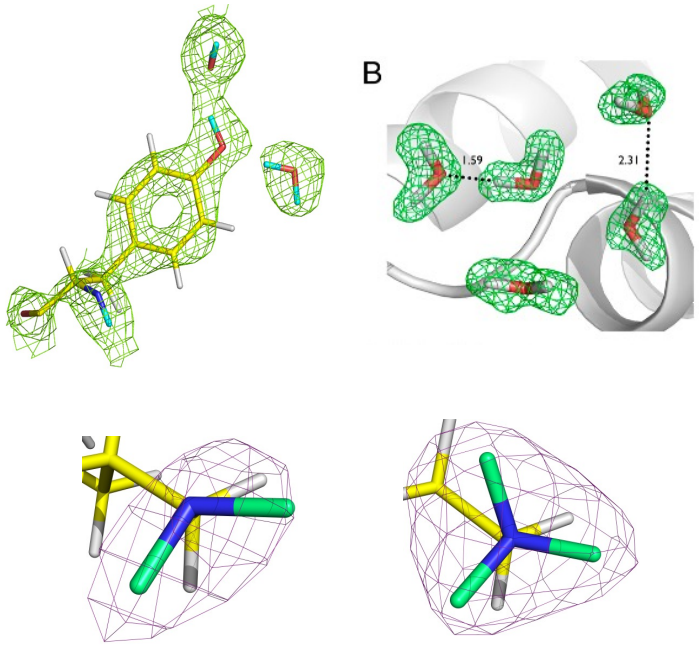
Neutron scattering lengths and cross sections

Isotope	conc	Coh b	Inc b	Coh xs	Inc xs	Scatt xs	Abs xs
H	---	-3.7390	---	1.7568	80.26	82.02	0.3326
1H	99.985	-3.7406	25.274	1.7583	80.27	82.03	0.3326
2H	0.015	6.671	4.04	5.592	2.05	7.64	0.000519
3H	(12.32 a)	4.792	-1.04	2.89	0.14	3.03	0

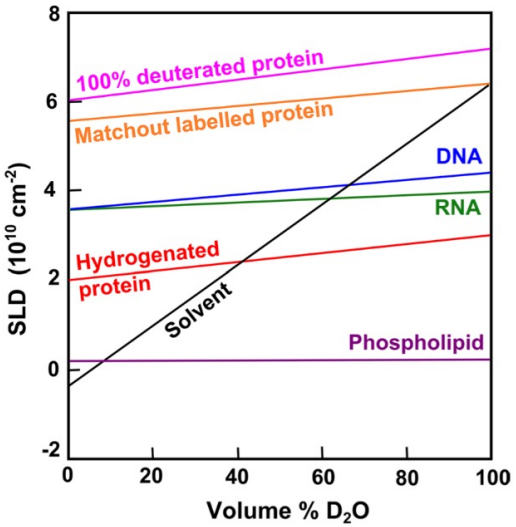
https://www.ncnr.nist.gov/resources/n-lengths/

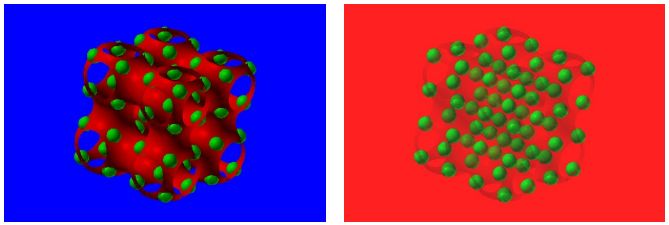
Purpose and extent of deuteration depends on technique

Determine position of hydrogen atoms in macromolecular structures



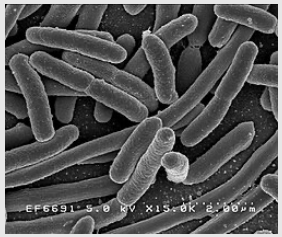
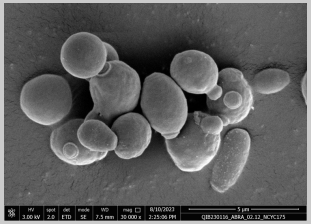
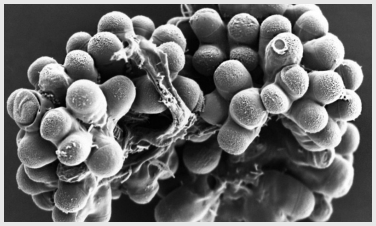
Neutrons enable contrast variation through selective deuteration of materials (SANS, NR, Imaging):



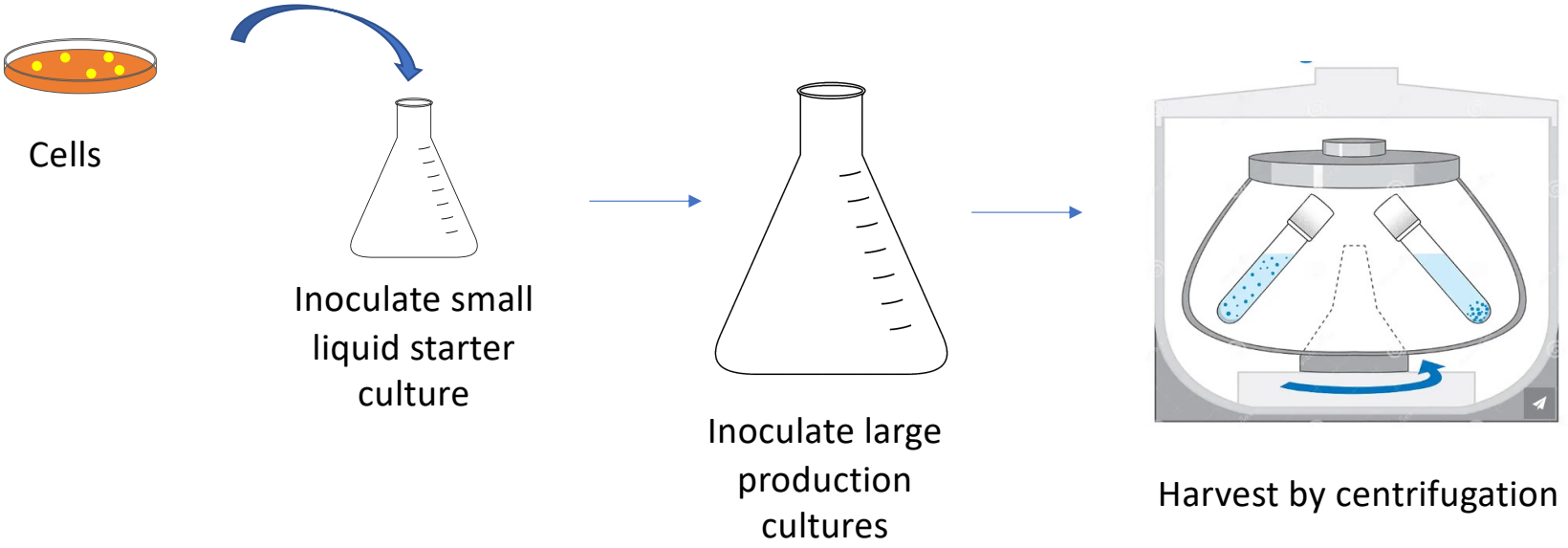


Different organisms are used for different molecules

*All of these can tolerate up to 99% D

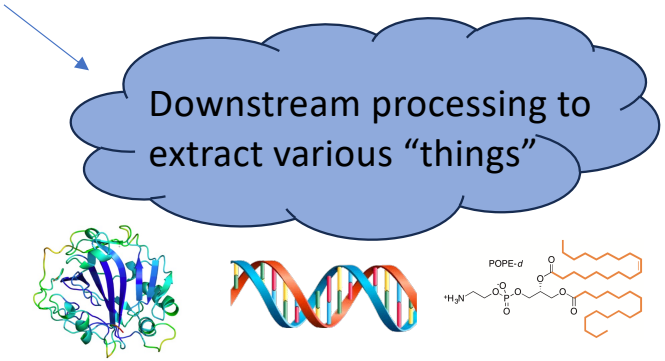
Bacteria ***Escherichia coli* (*E. coli*)** ***Acetobacter xylinus* (*A. xylinus*)**	**prokaryote**		**Recombinant proteins** **Plasmid DNA** **Cardiolipin** **Cellulose**
Yeast ***Pichia pastoris* (*P. pastoris*)**	**eukaryote**		**Lipids** **Cholesterol, ergosterol** **Membranes** **Recombinant proteins**
Algae ***Botryococcus braunii* (*B. braunii*)**	**eukaryote**		**Total cell extract** **Endogenous proteins** **Lipids? Oil?**

Biomass production in different amounts of D



1) Partial deuteration (65-85% D)
2) Full(per)deuteration (~99% D)

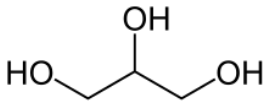
More D, more $$

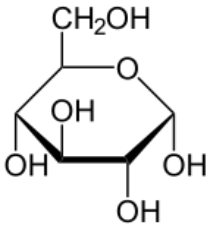


Liquid growth media components – *E. coli*

	Component	g/L
Bulk solution	NH_4Cl	2.58
	KH_2PO_4	2.54
	Na_2HPO_4	4.16
	K_2SO_4	1.94
Carbon source – choose 1	Glycerol	5
	Glucose	5
	d-algal extract	10 mL
		mg/L
Additives	$FeSO_4 \cdot 7H_2O$	20.0 (72 uM final)
	Trisodium citrate	88.0 (0.3 mM final)
	$MnSO_4 \cdot H_2O$	5.0 (30 uM final)
	$ZnSO_4 \cdot 7H_2O$	8.60 (30 uM final)
	$CuSO_4 \cdot 5H_2O$	0.76 (3 uM final)
	Thiamine chloride	48.0
	$MgSO_4 \cdot 7H_2O$	670 (2.7 mM final)
H_2O/D_2O		up to 1 L

- Replace H_2O with D_2O
- Replace “normal” carbon source with deuterated carbon source (e.g. glucose or glycerol or cell extracts)
- A combination of these things to get partial labeling





Limitations in biodeuteration

- Limited number of species tolerate D_2O – highly toxic in higher organisms (insects, mammals, plants) >30%
- Cells are not happy in D_2O: slow growth, low yields
- Requires a lot of very expensive D_2O and carbon source (e.g. glycerol-d8)

<u>2019</u>

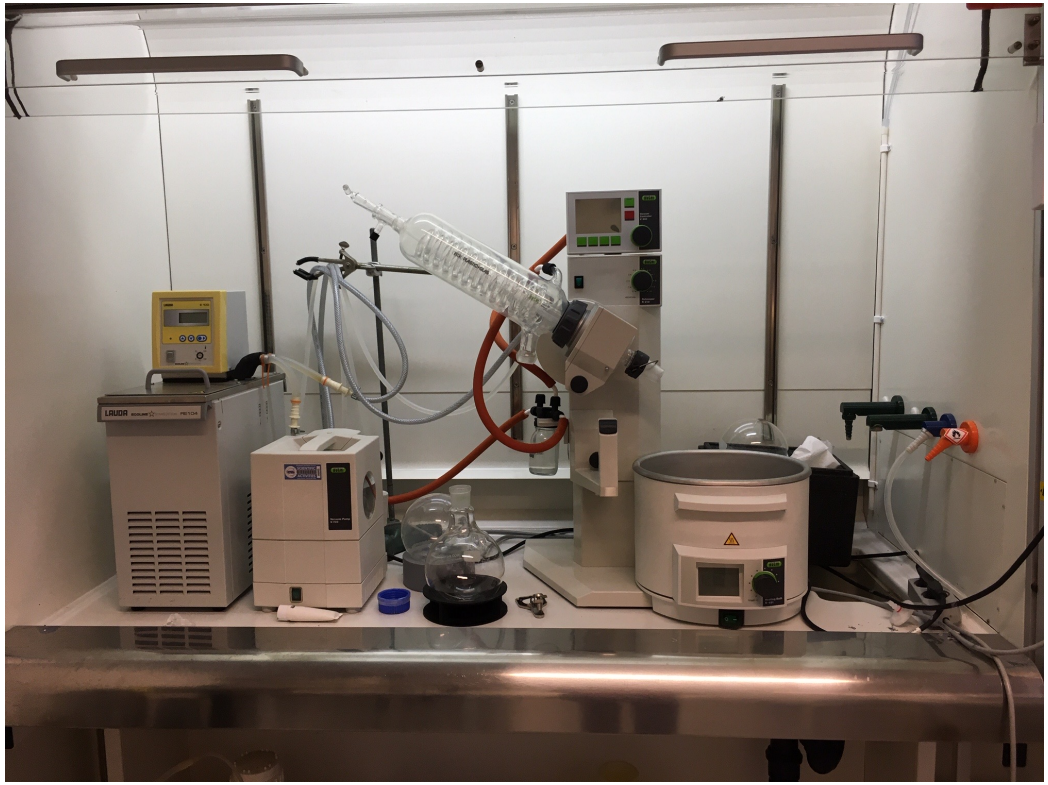
1g of glycerol-d8 for 300 SEK 1 kg of D_2O for 3200 SEK

<u>2023</u>

1g of glycerol-d8 for 2160 SEK 1 kg of D_2O for > 16,600 SEK

(Lead times up to 26 weeks in some cases)

- D_2O – we re-use it multiple times by rotary evaporation of spent cell media to recover the D_2O
- It is not 100% “clean” and some D is lost over time

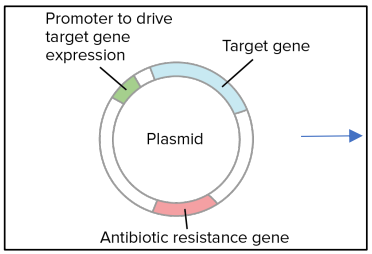


- Carbon source: let’s grow our own!

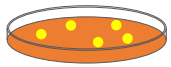
Botrycoccus braunii growing in D_2O

- Biodeuteration is needed, in demand but expensive
- *Not technically difficult if you can grow the right microbe in deuterated media*

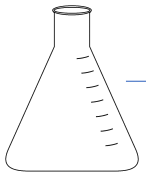
Workflow: from gene to structure



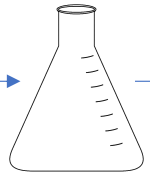
Transformation of plasmid into bacteria



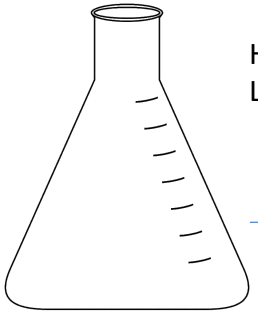
Single colony pick



Liquid culture starter



Test expression

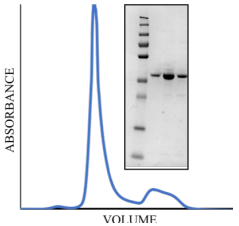


Large scale growth 6-9 L

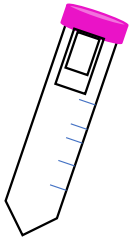
Harvest & Lysis

Liquid chromatography to extract protein

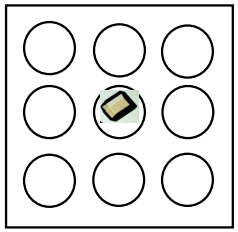
Purified protein



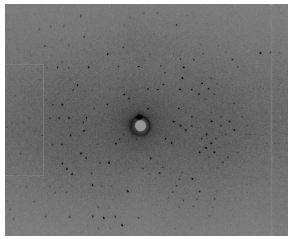
Analyze purity, quality by SDS-PAGE and MS; Biophysical characterization



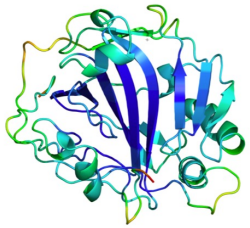
Concentrate & buffer exchange ~ 10 mg/mL



Crystallization set-up



Diffraction data collection

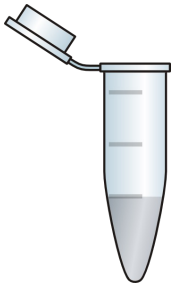


Solve crystal structure & analyze, interpret

Borrowed & adapted from Swati Aggarwal
doi: 10.1016/j.pep.2021.105954

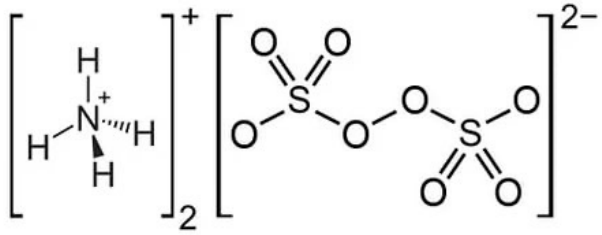
So we need crystals to get measurable diffraction data….but how do you *make* a protein crystal?

- Need mg amounts of ~95% pure, 1-50 mg/mL concentrated protein.
- Beneficial to characterize protein for solubility, purity, stability, and treat it gently (use fresh, don't lyophilize, don't repeat freeze/thaw etc).

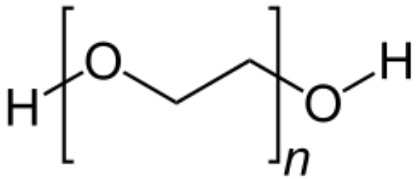


Precipitant solution

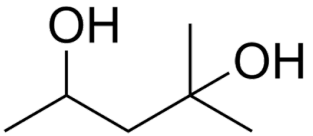
- This is the liquid that you mix your protein with & that promotes crystal nucleation
- They contain chemicals that promote taking water away from the protein, effectively increases [protein] e.g. PEG, NaCl
- Usually includes a buffer (e.g. Tris) and sometimes additives (e.g. divalent cations, reducing agents, detergents)
- Often includes ‘additives’ that help improve crystals e.g. divalent cations, organics such as glycerol, metal salts like $ZnCl_2$, $MgCl_2$ and often reducing agents (DTT)



Ammonium sulfate

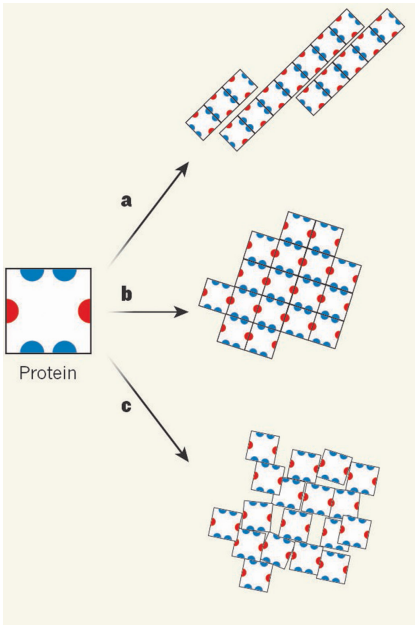


Polyethylene glycol (PEG)
n=200-20 000

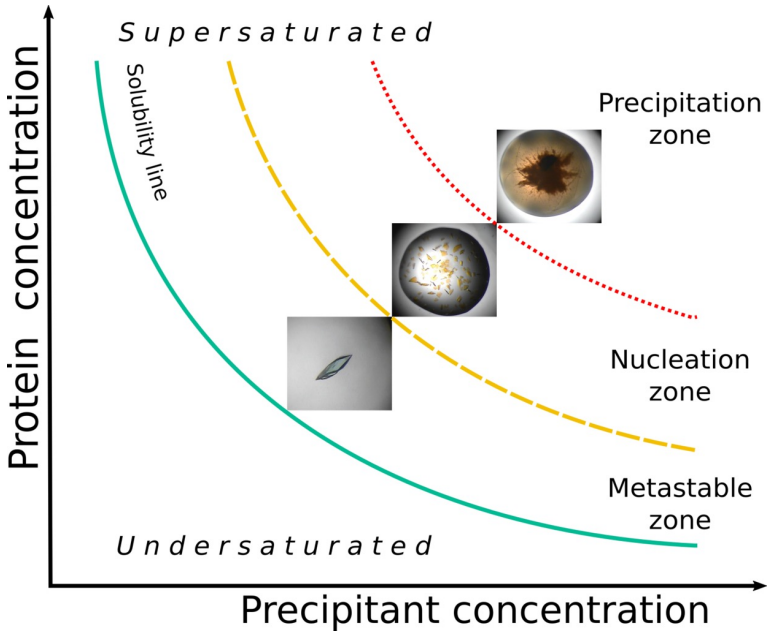


Methyl pentanediol (MPD)

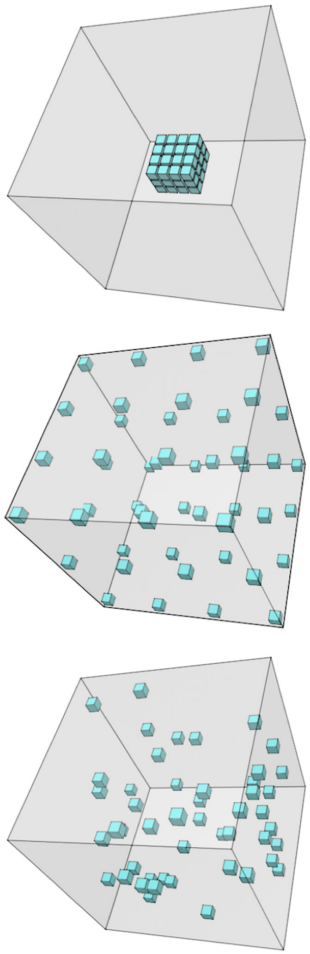
Crystal formation: the Phase Diagram



https://doi.org/10.1042/bio_2020_108



https://doi.org/10.1016/bs.mie.2019.11.015

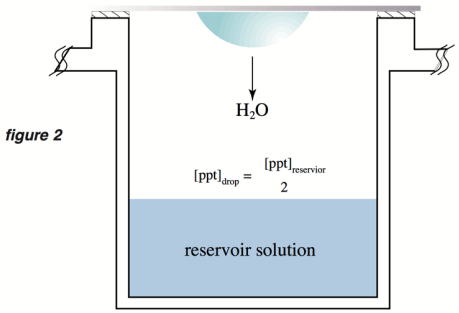


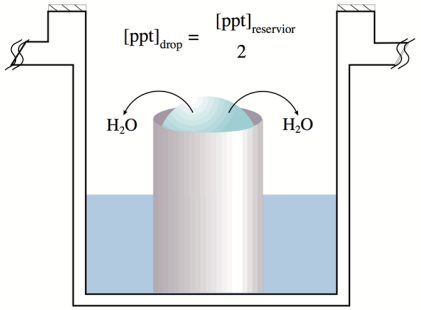
Commercial screens can explore a vast crystallization “space” in the phase diagram (or be rather narrow).

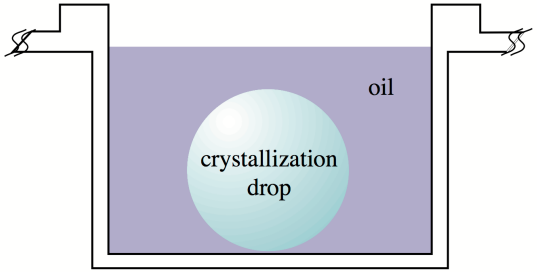
Crystallization methods

- Vapour diffusion

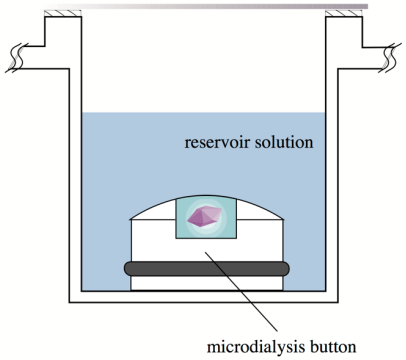
Hanging vs sitting drop







- Batch (under oil)



- Dialysis

https://hamptonresearch.com/growth_101_lit.aspx

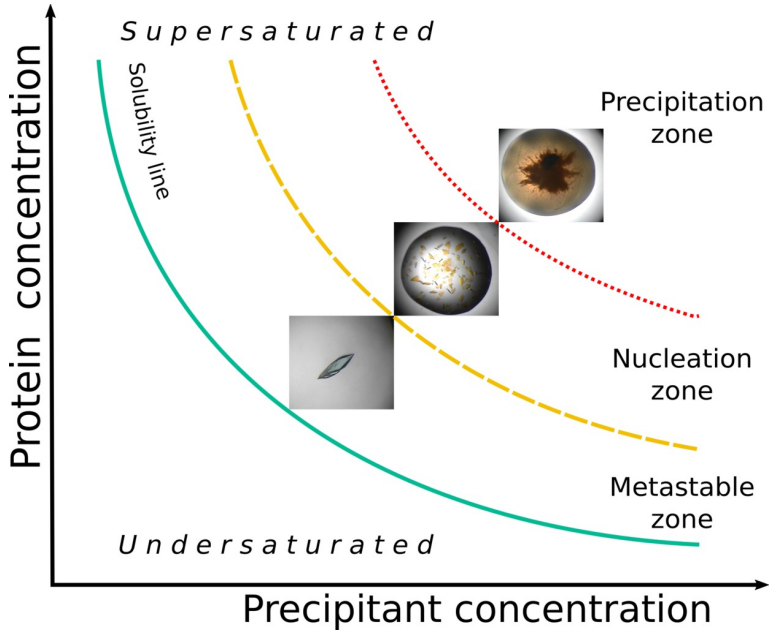
Modifications to basic methods

Can modify or adjust these methods by doing things that promote nucleation (formation of new crystals):

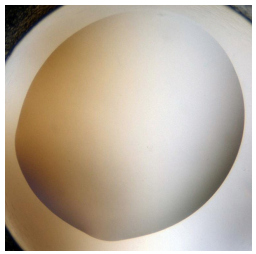
- Crystal seeding (micro or macro)

or simply growth:

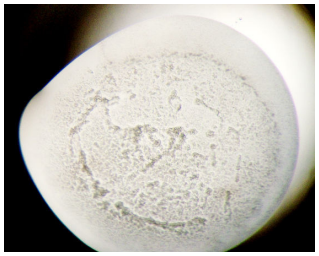
- Crystal feeding



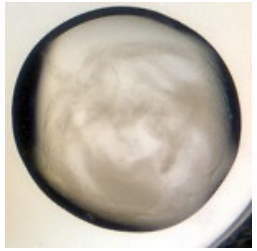
Crystal evaluation & imaging



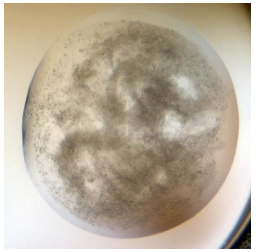
Clear drop



Phase separation



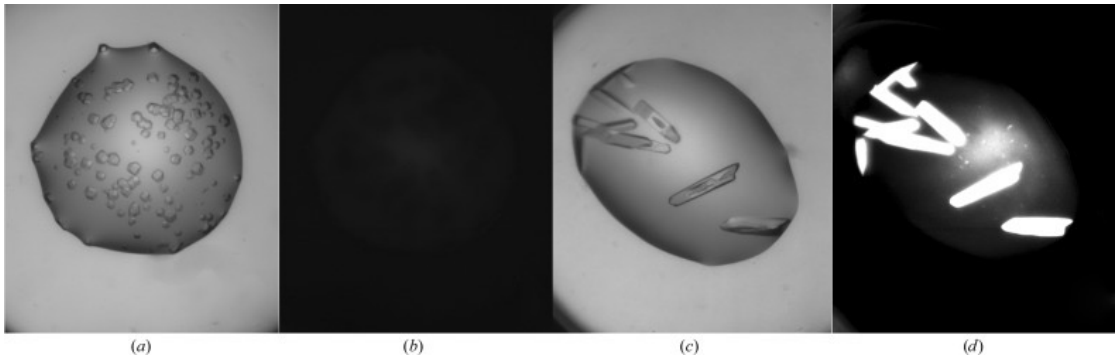
Heavy precipitation



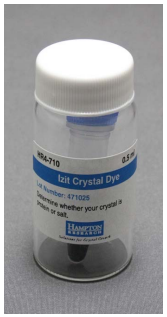
Microcrystals

Single 3D crystal

- Manual inspection with microscope
- Or plate hotel with automatic imaging
- Visible light & sometimes UV option

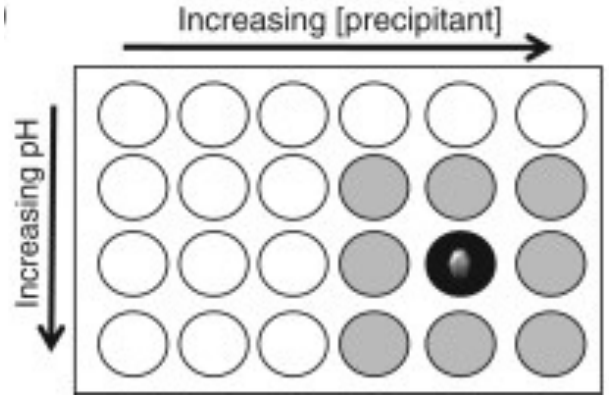


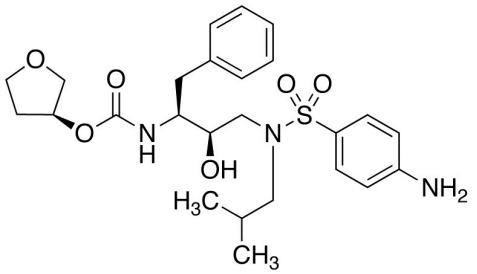
- UV helps us to distinguish protein crystals from salt crystals using protein intrinsic fluorescence



Optimization of crystallization conditions

- Fine grid searches around initial conditions
- E.g. vary pH or precipitant concentration
- Try additives (e.g. ions, organics)
- Try substrates, inhibitors, ligands
- Metal chelators or reducing agents
- Detergents
- Vary temperature
- Different crystallization method
- Seeding methods (see earlier slides!)

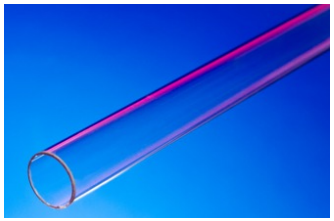


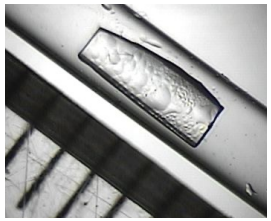


amprenavir

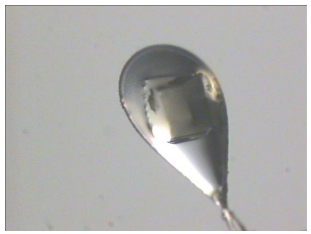
Preparing crystals for data collection

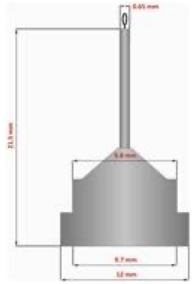
- Can do diffraction measurements at room temperature or at cryo conditions (100 K, N2 gas)
- Various different crystal supports for harvesting and data collection





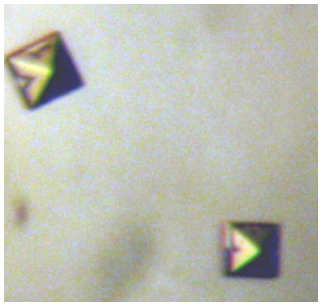
Pros for RT	Cons for RT
No damage from freezing	Technically challenging (need to practice!)
No cryoprotectants, SEE or LN2	Sensitive proteins degrade, radiation damage!
Observe structure closer to physiological conditions	Can't make complexes or trap reaction intermediates



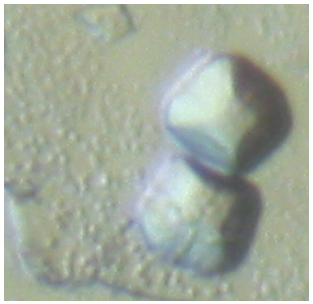


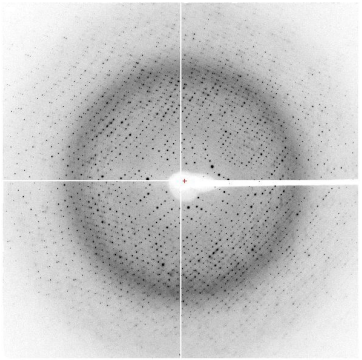
Pros for cryo	Cons for cryo
Easy to do, standardized mounts	Need cryo conditions, freezing itself can damage xtal
Easy to store, preserve sensitive samples	Cryo-induced artefacts (glycerol, freeze-in conformations)
Protect from radiation damage	Need for special SEE, LN2 consumables

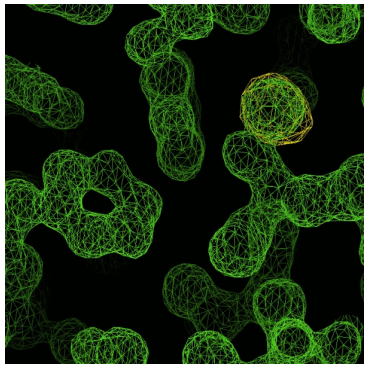
Always test them with X-rays
since looks can be deceiving

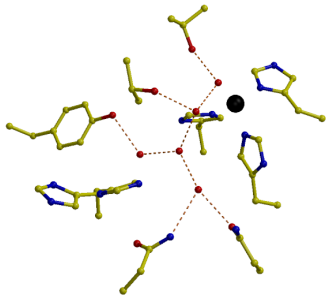

Nice crystals!


No diffraction




Good diffraction!





…and it all starts with high quality protein production and crystallization!

DEMAX Platform

Chemical Deuteration

- Small organic molecules, monomers
- Lipids (e.g. POPC, SOPC, POPE)
- Surfactants (e.g. sugar-based)
- Novel organic molecules for various applications
- Separation & analysis of yeast-derived lipids

Biological Deuteration

- Cell culture to produce deuterated biomass from *E. coli*, *B. braunii*, *P. pastoris*
- Extraction of recombinant soluble proteins, lipids, plasmid DNA, "other"
- Biophysical characterization of products

Protein Crystallization

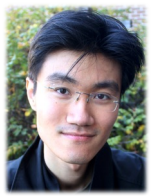
- High- and low-throughput screening
- Fine screening & optimization in large volumes
- Support for room temperature crystal mounting & data collection
- X-ray testing (LU BAG at MAX lab)

Core team with technical support from LU & ILL
+ postdocs


Zoë


Anna


Hanna


Jia-Fei

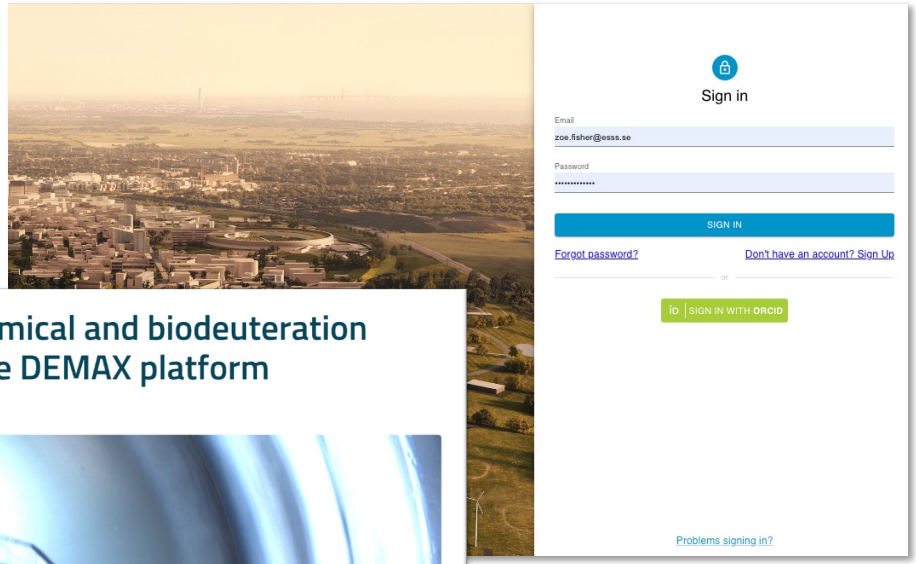
+ 0.7 RE @ LU

+ 0.2 analysis @ ILL

User proposals

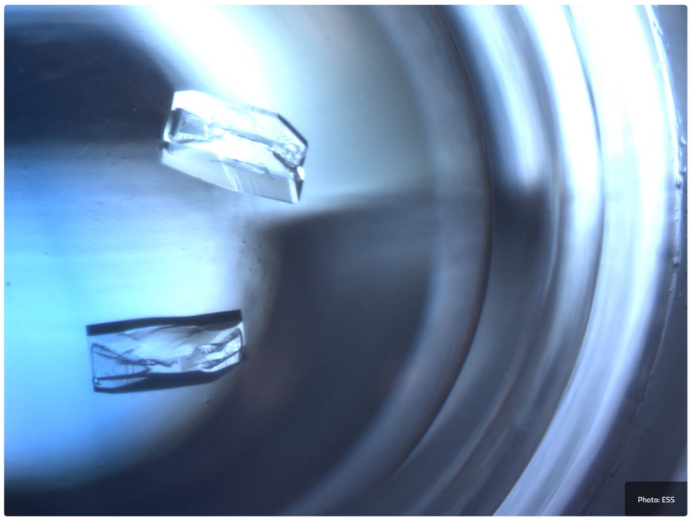
useroffice.ess.eu

- Issued 3 pilot calls for user proposals (2019, 2020, 2022)
- Rolling access is currently open until end of 2024
- User should register and submit proposals online (URL above)
- Please reach out to us before submitting a proposal!



Pilot call for chemical and biodeuteration support from the DEMAX platform

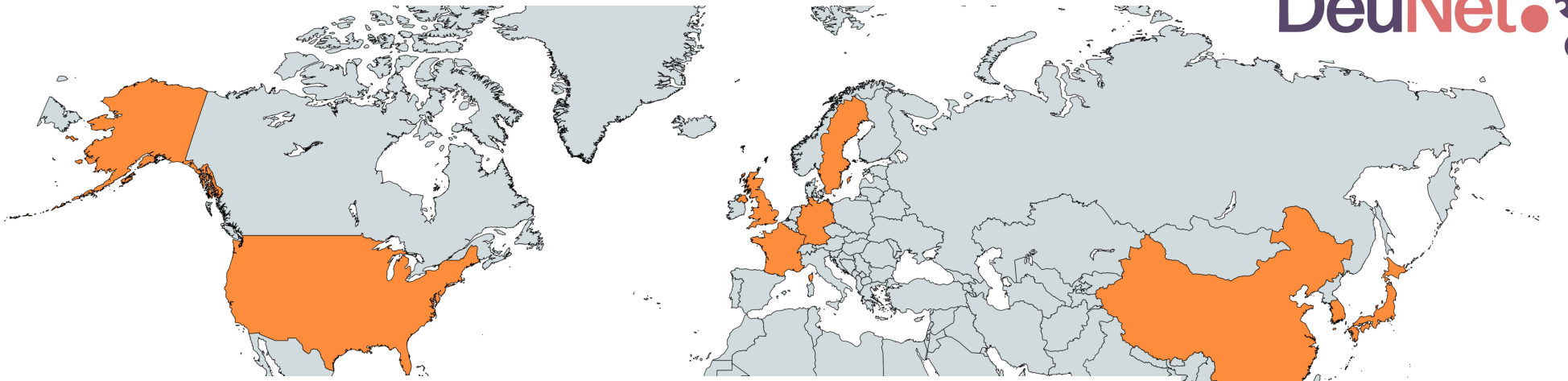
JANUARY 10, 2022



The Deuteration and Macromolecular Crystallisation (DEMAX) platform at ESS supports neutron users from the soft matter, biology, life sciences and chemistry research areas. The neutron techniques that these communities typically use include small angle scattering, reflectometry, single crystal diffraction, and spectroscopy. For steady state ESS operations, DEMAX is currently developing three areas of support: Biological deuteration (e.g. cell paste, soluble proteins, lipids, membranes), Chemical deuteration (e.g. small organic molecules, surfactants, phospholipids), and Crystallisation (large protein crystal growth).

https://deuteration.org

DeuNet



OAK RIDGE National Laboratory

EUROPEAN SPALLATION SOURCE

LUND UNIVERSITY

Australian Government ANSTO Science. Ingenuity. Sustainability.

UNIVERSITY OF MARYLAND | NIST INSTITUTE FOR BIOSCIENCE & BIOTECHNOLOGY RESEARCH

NIST National Institute of Standards and Technology U.S. Department of Commerce

J-PARC MLF

Kumatori Deuteration Station

Department of Organic Chemistry
Graduate School of Pharmaceutical Sciences,
Kyoto University

JÜLICH FORSCHUNGSZENTRUM

SNS 中國散裂中子源 China Spallation Neutron Source

NEUTRONS FOR SCIENCE

GIFU PHARMACEUTICAL UNIVERSITY

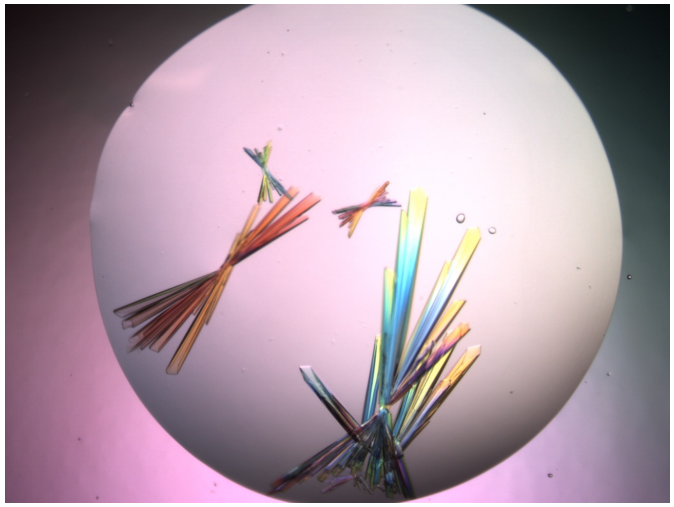
Science & Technology Facilities Council ISIS

Support for deuterated materials

- DeuNet is an international network of ~15 labs/facilities
- DeuNet aims to facilitate access to deuteration services and customised d-labelling of molecules for use in a wide range of research areas.
- DeuNet promotes collaborations between labs, supports development of new methods for deuteration, and increases the visibility of labs to funders, users.
- DeuNet facilitates communication between each members and collaborators through regular meetings and user workshops.
- For information on members, access modes, please visit

  https://deuteration.org

Thank you for your attention!



- Questions? Comments?

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