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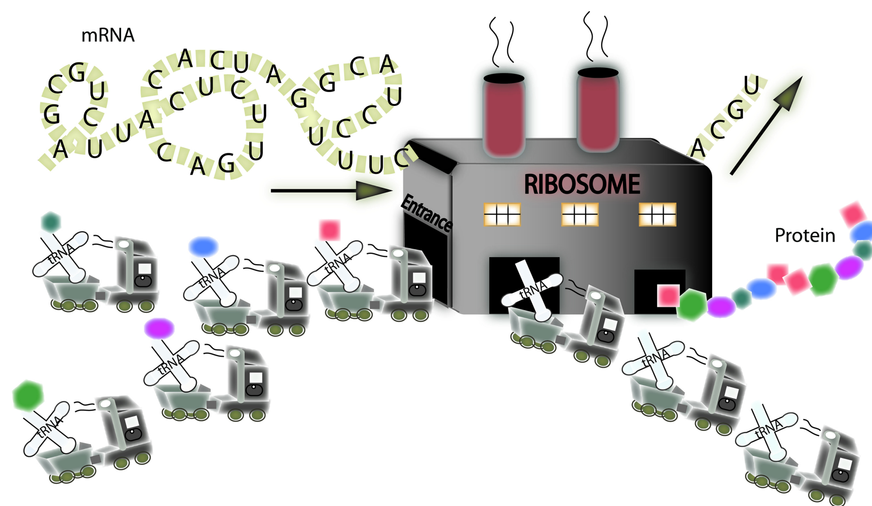
Lund Protein Production Platform
LP3: (www.lu.se/lp3)

Outline today:

A. Protein Production

B. LP3



Protein being manufactured according to mRNA instructions
Credit: Sean Studer, UCSD
(picture from: http://ucsdnews.ucsd.edu/archive/newsrel/science/santibiotarget.asp)

Department of Biology
FACULTY OF SCIENCE | LUND UNIVERSITY

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Biological Museum
Current student
Internal Biology – for staff

Start | About us | Education | Research | Collaboration | Services | Contact | Search SEARCH

Start › Services › LP3 - Lund Protein Production Platform

- DNA Sequencing Facility
- Field stations
- Greenhouses and cultivation spaces
- Instrumental Chemistry
- LP3 - Lund Protein Production Platform
  - LP3 news
  - Crystallization
  - People working at LP3
  - Related facilities
- Lund Wind Tunnel
- Microscopy
- National Bioinformatics Infrastructure
- Workshop

LP3 - Lund Protein Production Platform

Lund Protein Production Platform, LP3, is a cross-faculty facility for protein production, purification and crystallization, primarily directed at academic research groups based at Lund University.

The services available include

- Cloning - design of a vector construct, ordering of a synthetic gene, cloning and plasmid preparation.
- Protein production in bacterial (*E. coli*) or eukaryotic (insect) cells.
- Microbiological growth monitoring (Bioscreen C).
- Protein labeling (seleno-methionine incorporation, labeling with stable isotopes (^{2}H, ^{13}C, ^{15}N), biotinylation, phosphorylation).
- Protein purification using state of the art chromatography equipment.
- Development of expression and purification protocols.
- High-throughput protein crystallization.

LP3 works as an intellectual hub for exchange of scientific ideas and for dissemination and assimilation of new methodologies for protein production, purification and analysis. The service facility is integrated with research training and development of skills in experimental protein science for PhD students and postdocs.

Beyond LP3

For access to additional protein characterization methods (CD, DLS, MS, NMR, etc) we can help you come in contact with the appropriate experts.

What are the costs?

In view of the customized nature of the services provided it is necessary to obtain a quotation from LP3 for the desired work.

A price list for protein crystallization and characterization can be found here.

Postgraduate course
A one-week intensive course about recombinant protein production.

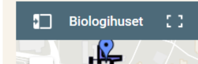
CONTACT INFORMATION

The LP3 labs are located on floor 1 in Biology building A, Sölvegatan 35, Lund.

Lund Protein Production Platform
Biology Building A
Sölvegatan 35

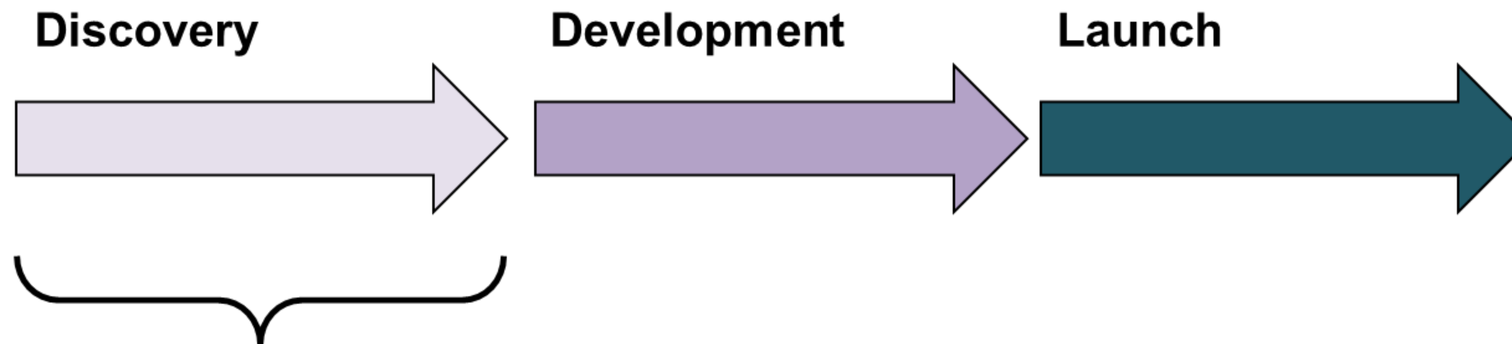
Phone:
+46 46 2227785

E-mail:
lp3@biol.lu.se



Why produce proteins ?

Drug development process



Target (Protein) Production

Need of mgs amounts for drug discovery
- **High throughput screening**
- **Structural studies**

Natural expression levels are often low (µg/L) / human proteins needed

Recombinant protein production needed

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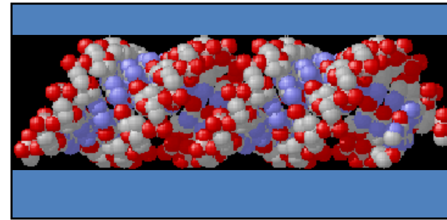
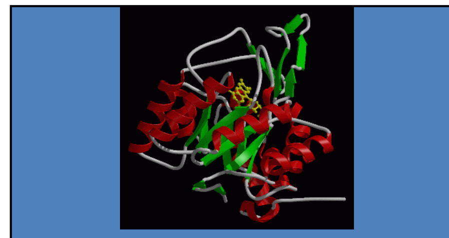
What is protein production?

- cDNA
- Expressionsystem
- Purification
- Characterisation



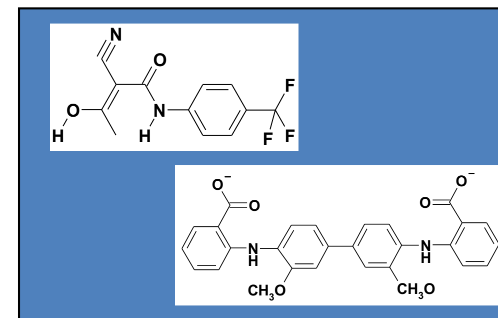
Structure

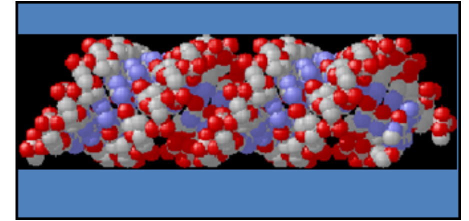


Product (vaccine, antibody, enzyme, hormone)

Compound screening

Assays





cDNA

If sequence is known

- Amplify from cDNA library
- Buy EST (expressed sequence tag)
- Synthesize (native or optimised)

If sequence is not known

- Clone yourself
- Purify native protein

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Expressionsystem

- Procaryotic (*E. coli*)
- Yeast (*S. cerevisae*; *P. pastoris*)
- Insect cells (Baculovirus expression vector system(BEVS))
- Mammalian cells (CHO; HEK)
- Cell-free expression

	PTM			Speed			Resources		
	None	Some	Complete	Fast	Medium	Long	Few	Some	Many
Bacteria	●			●			●		
Yeast		●			●			●	
Insect		●			●			●	
Mammalian			●			●			●

Cell free expression - Challenge: Large scale expression > 3 mg & PTM

Choice of expressionsystem and -vector:

- Posttranslational modifications/ processing?
- Secreted protein?
- Membrane protein?
- Co-factor requirements?
- Literature?

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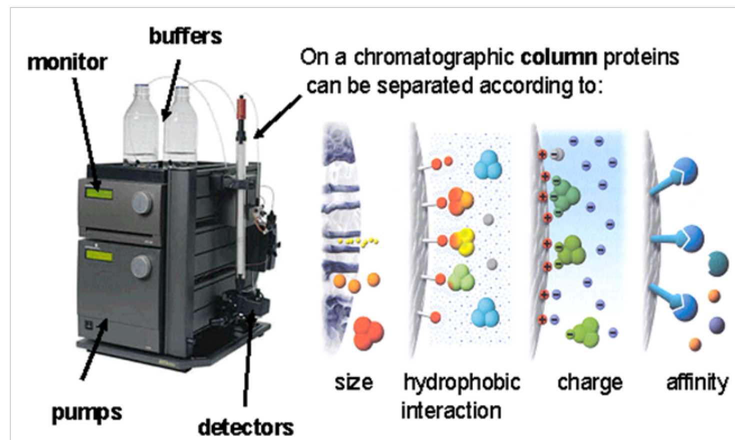
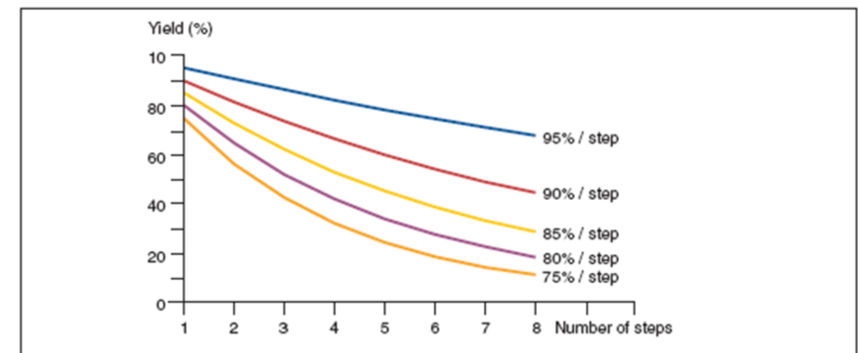
Purification

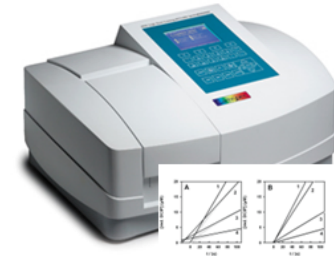
- Affinity tags (IMAC, GST, MBP, Streptag…)
- Classical (Gelfiltration, Hydrophobicity, Ion exchange)





- Refolding (on column; by dilution (dialysis))

Characterisation



- Activity (assay development)
- Purity (no contaminants, no degradation)
- Homogenous (no aggregation)
- Stability (of activity and conformation overtime)
- Solubility (in different buffers, at different concentrations)
- N-terminal sequencing; Mass
- ……………..

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Characterisation

Raynal *et al. Microbial Cell Factories* (2014) 13:180
DOI 10.1186/s12934-014-0180-6

REVIEW Open Access

Quality assessment and optimization of purified protein samples: why and how?

Bertrand Raynal[1,2*], Pascal Lenormand[1,2], Bruno Baron[1,2], Sylviane Hoos[1,2] and Patrick England[1,2*]

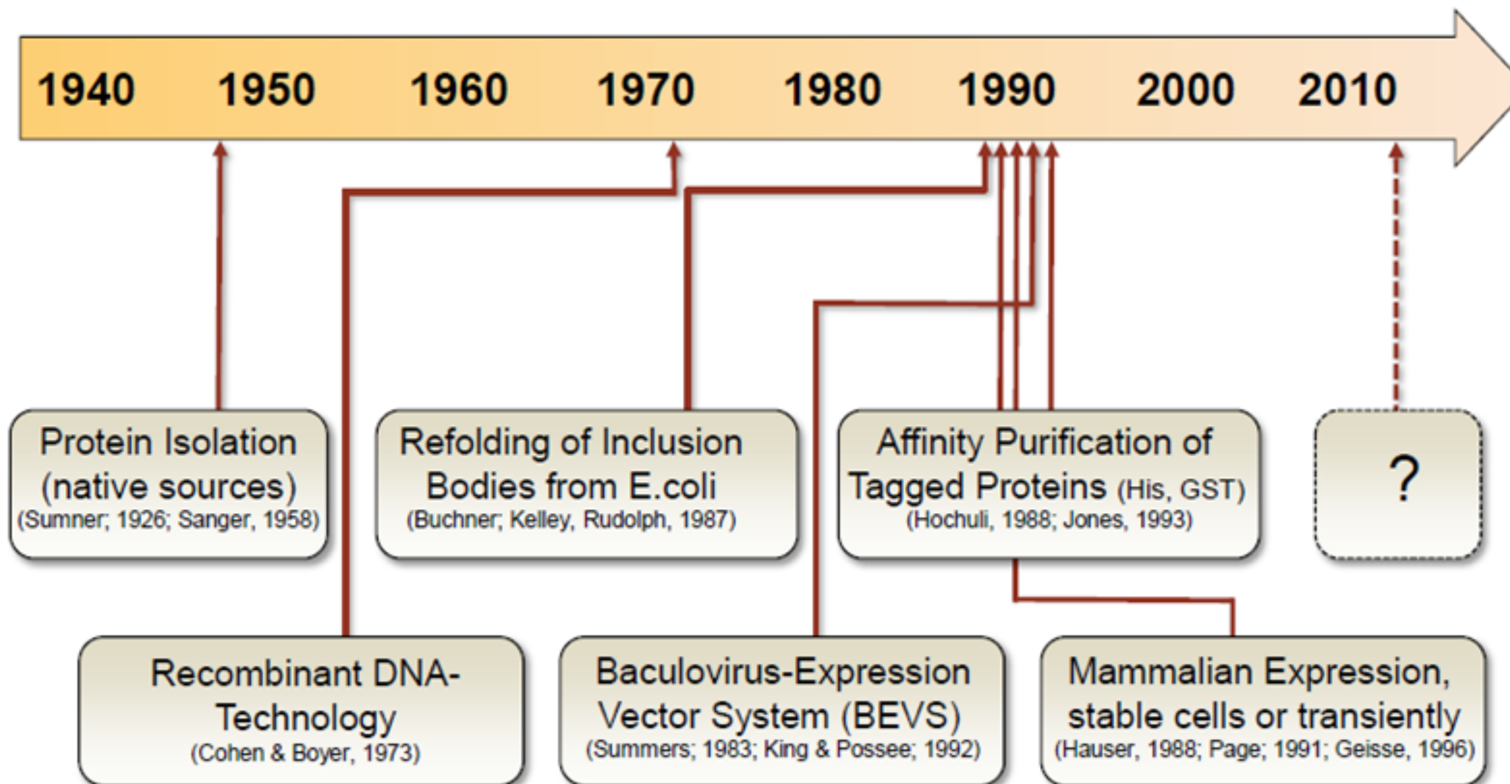
Lebendiker *et al. BMC Research Notes* 2014, 7:585
http://www.biomedcentral.com/1756-0500/7/585

CORRESPONDENCE Open Access

The Trip Adviser guide to the protein science world: a proposal to improve the awareness concerning the quality of recombinant proteins

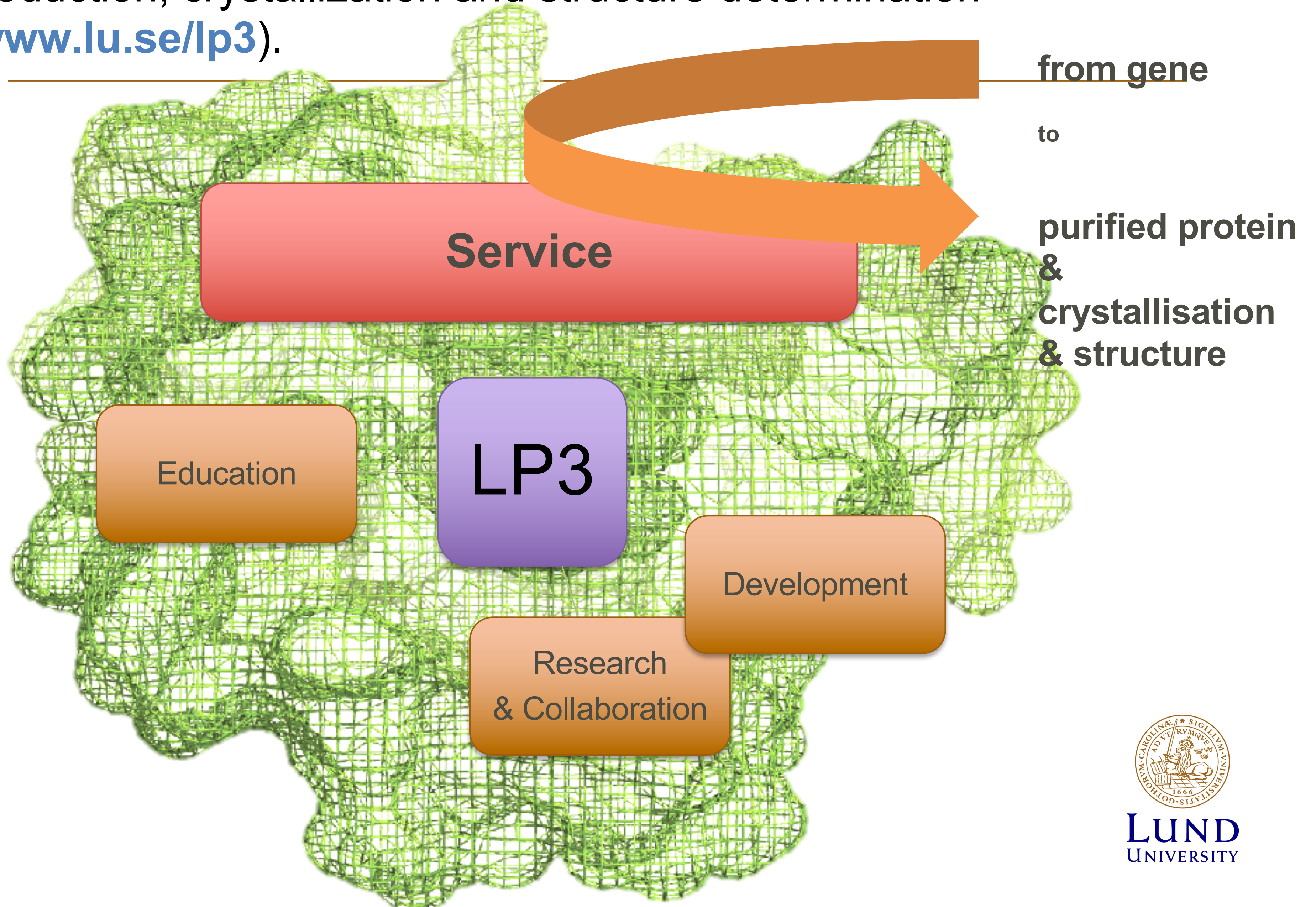
Mario Lebendiker[1†], Tsafi Danieli[1†] and Ario de Marco[2*]

A short history of protein production



From: Protein Expression | Lorenz Mayr | November 3, 2011 | ComplexINC Kick-Off Munich

LP3 is a cross-faculty facility of Lund University for protein production, crystallization and structure determination (**www.lu.se/lp3**).



from gene

to

purified protein & crystallisation & structure

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What is protein production?

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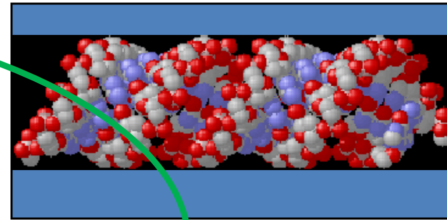
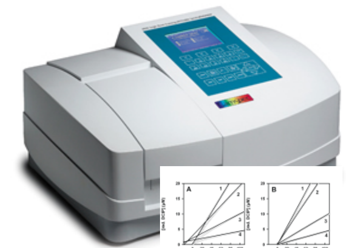
- Characterisation

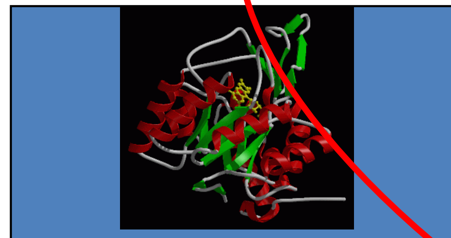




Formulation

Crystallisation

Structure



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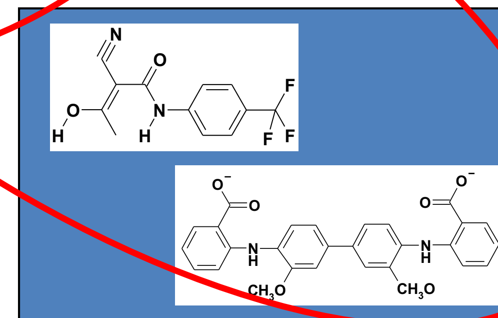
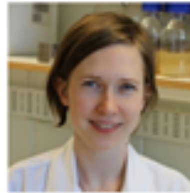


LP3: Location (www.lu.se/lp3)

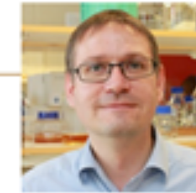
People at LP3

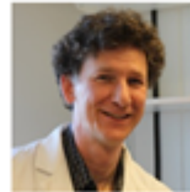

Annika Rogstam
PhD, Scientist

Ewa Krupinska
MSc, Scientist

Therese Lindvall
MSc, Scientist

Maria Gourdon
PhD, Scientist


Wolfgang Knecht
PhD, Senior Lecturer, LP3 manager


Claes von Wachenfeldt
PhD, Senior Lecturer

Derek Logan
PhD, Senior Lecturer

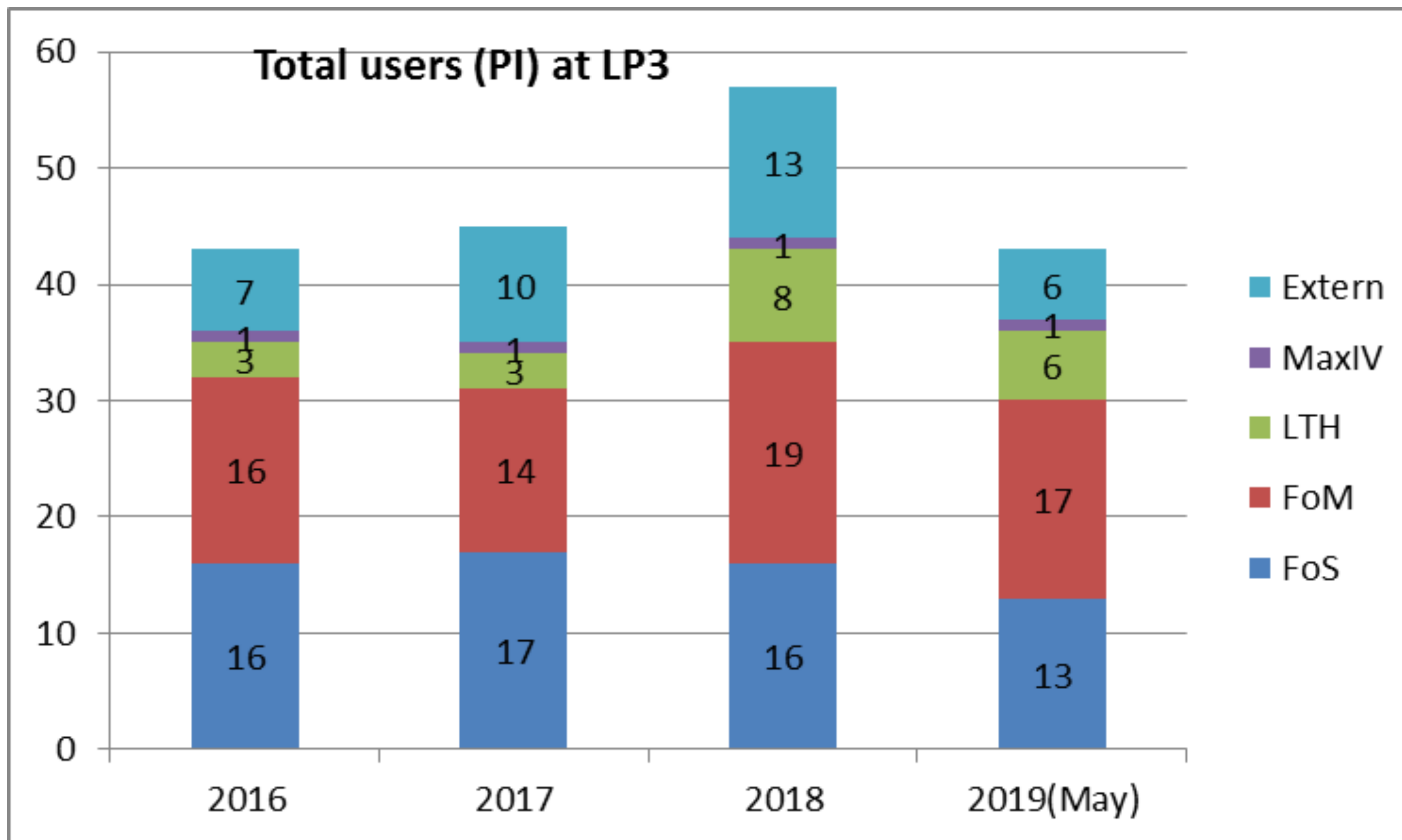
Zoe Fisher
PhD (ESS & LU)

Vladimir Talibov
Postdoc
FragMAX project

Celeste Sele
PhD, Scientist

Hasan Cicek

LP3 –users



LP3 - Lund Protein Production Platform

LP3 can help with:

Protein production

- Plasmids for protein production
- Recombinant protein production:
    a. in bacteria (*E. coli*)
    b. in eukaryotic (insect) cells
- Protein labeling with stable isotopes (2H, 13C, 15N)
- Protein purification

High-throughput crystallization

- SEC, DSF and DLS
- Mosquito nanoliter pipetting robot with LCP
- UV imaging system with plate hotel

Structure determination

- BAG beamtime at BioMAX
- Collect and process x-ray data

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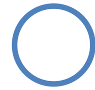
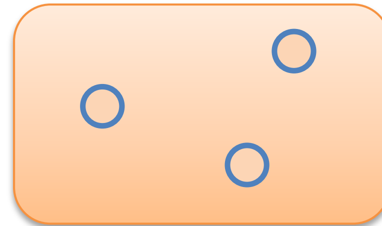
Protein Production at LP3

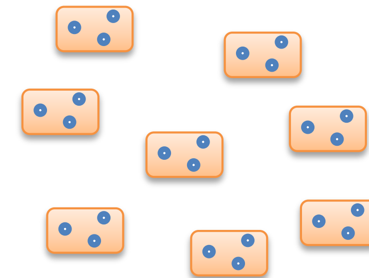
Order the gene, clone in an expression/donor vector

Create the expression strain or expression vector

Grow under optimal growth conditions







E. coli

Baculovirus expression vector system

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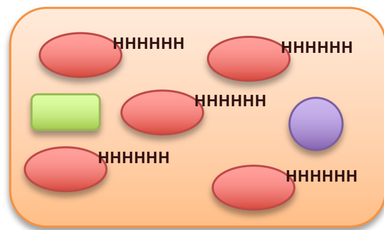
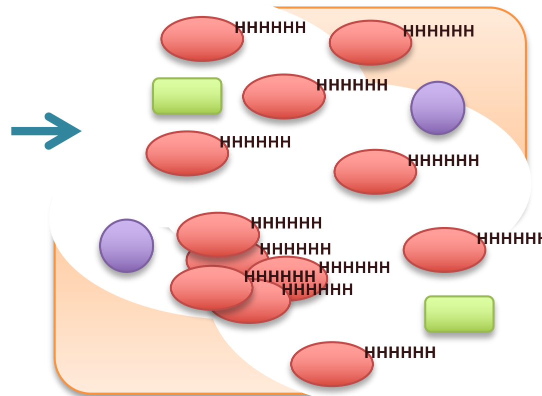
Protein Production at LP3

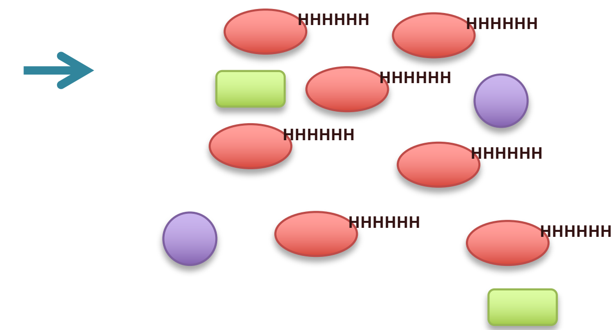
Harvest (Centrifugation, 8 000 x g)

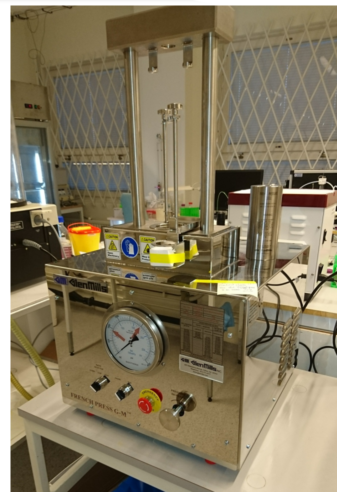
Cell disruption (French Press, 18000 psi)

Remove cell debris and insoluble proteins (Ultracentrifugation, 100 000 x g)



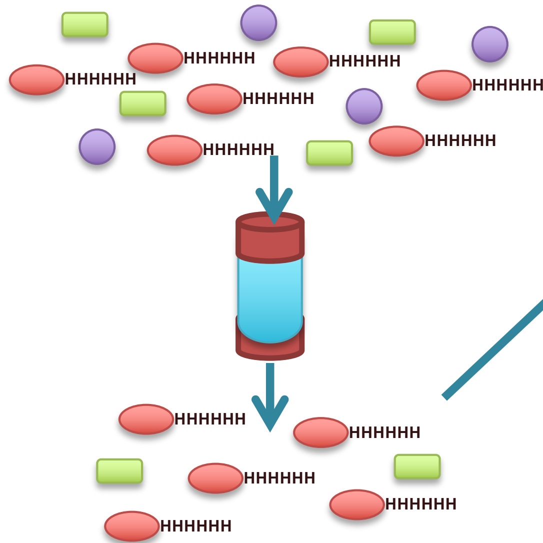




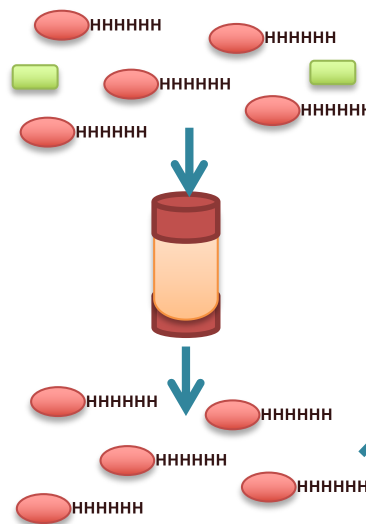


Protein Production at LP3

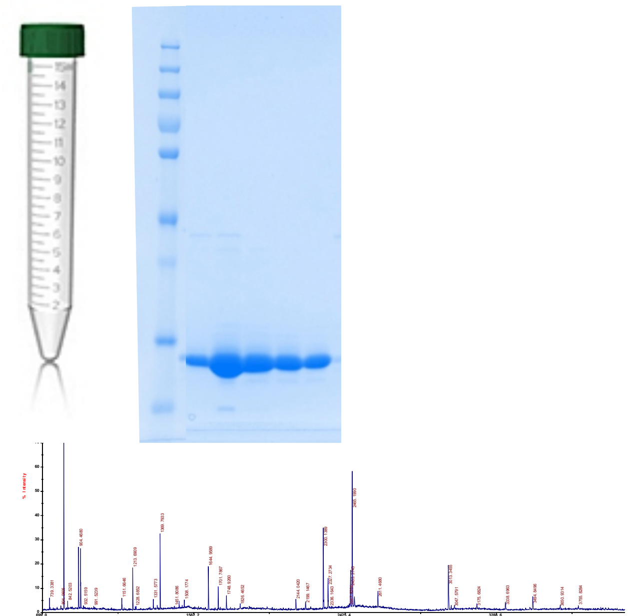
1st purification step (affinity chromatography)



2nd purification step (gel filtration)



Quality control (mass spectrometry, SDS-PAGE)



or any other combinations and chromatography types

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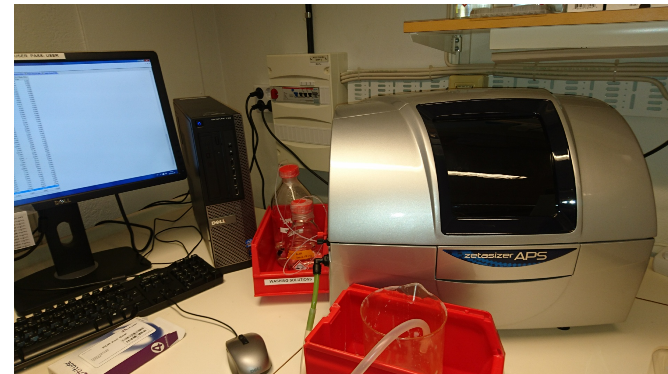
Protein crystallization at LP3

- Differential scanning fluorimetry (DSF) enables identification of maximally stabilising conditions for crystallisation
- Dynamic light scattering (DLS) enables analysis of effects of buffers and additives on aggregation



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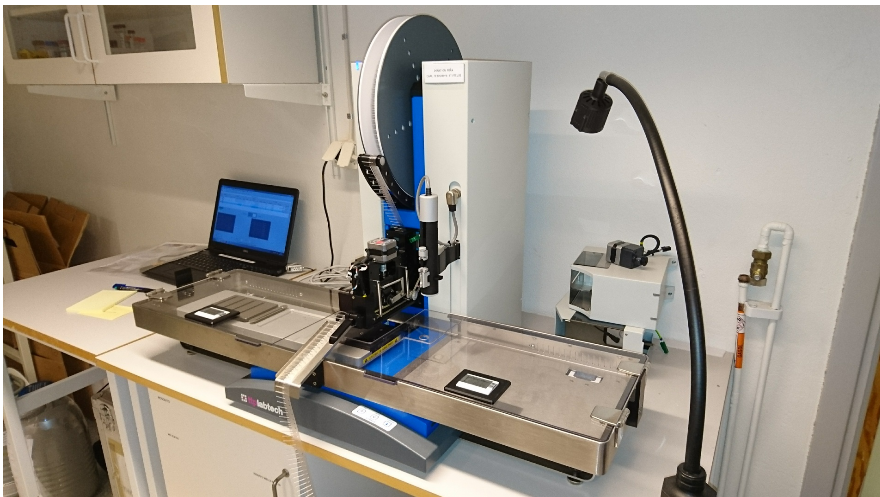
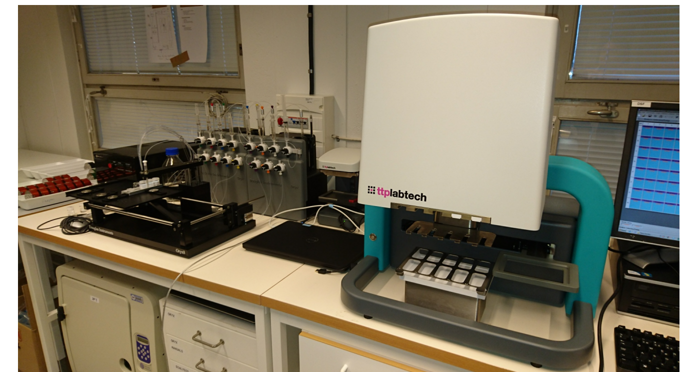
Protein crystallization at LP3

- Nanolitre dispensing robots enable low-volume, high density crystallisation trials in 96-well plates, with 1-3 protein drops per well, with minimal sample consumption





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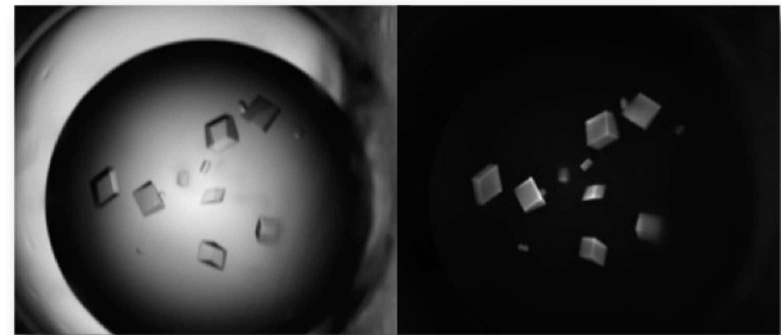
Protein crystallization at LP3

- UV imaging enables identification of protein crystals
- Images stored in web-accessible database

Figure 2.



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Crystall screening at BioMAX beamline (MAX IV laboratory)

How to access LP3?
What does it cost?
What are the timelines?

Hit identification

Fragment-based Lead Generation

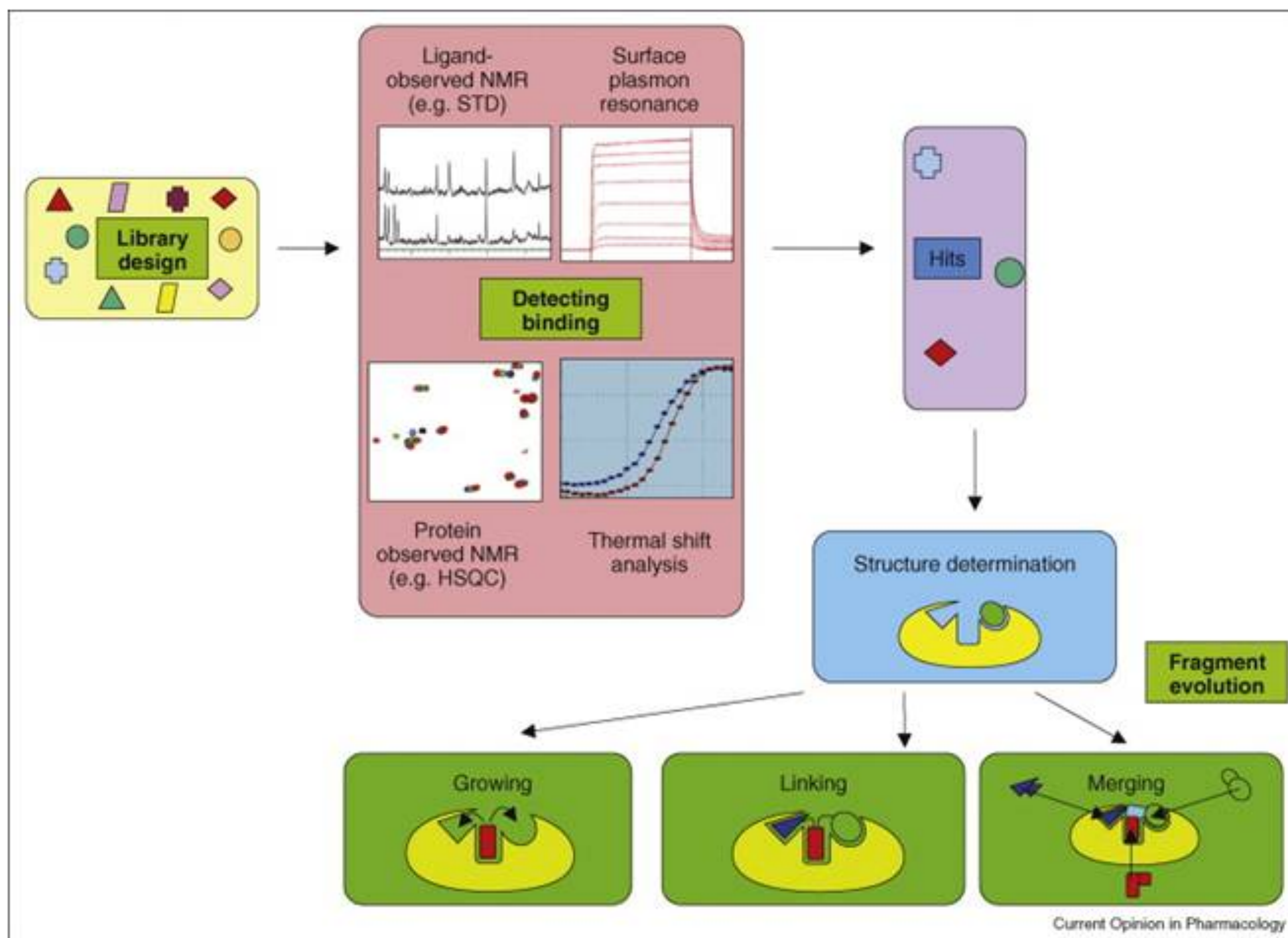


Figure 1. Taken from Schulz and Hubbard, *Current Opinion in Pharmacology* 2009, **9**:615–621

FragMAX

MAX IV Laboratory

Fragment Screening using X-ray Crystallography combines great hit rate and extensive information

Fragment-based Lead Generation

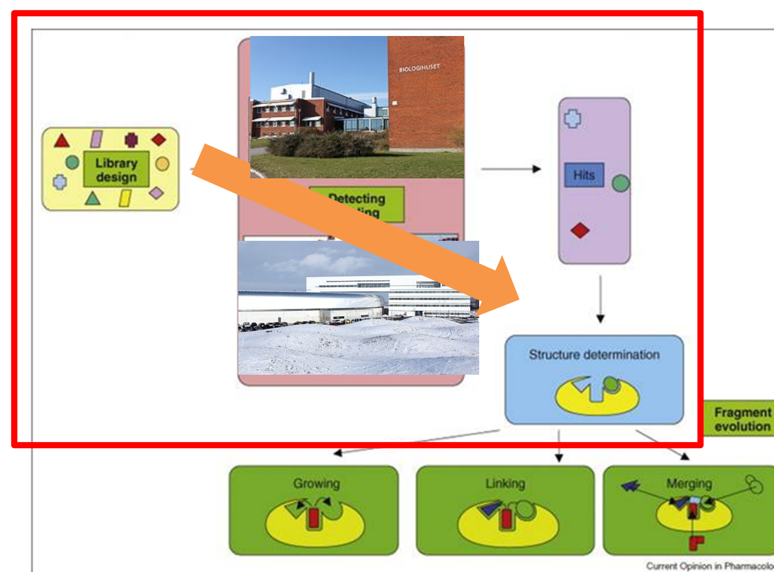
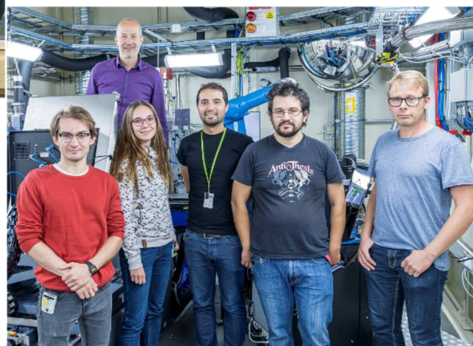


Figure 1. Taken from Schulz and Hubbard, *Current Opinion in Pharmacology* 2009, **9**:615–621

FragMAX team



Education

Postgraduate course

A one-week intensive course about recombinant protein production.

Courses

From the 3 – 7 December, 2018, LP3 host the practical postgraduate course *Protein Factories*

In this course the participants will get hands-on experience of protein production and purification using bacterial and insect cell-based expression systems (baculovirus expression vector system (BEVS)). The course presents methods to express genes, which have products that are known, unknown or not well characterized, and to analyze the gene products. Theories behind the methods are discussed and experiments are performed. The methods to be presented include: optimization of recombinant gene expression in *Escherichia coli* and in the BEVS; affinity purification of tagged proteins; using proteases to for the removal of protein fusion tags; generation and analysis of gene fusions; molecular cloning; metabolic engineering; seleno-methionine incorporation for MAD phasing; ^{2}H-, ^{13}C-, ^{15}N-labeling for NMR studies; production of perdueterated proteins for neutron-scattering experiments. Strategies for improving production of "difficult" proteins will be presented. The lectures will cover the current status of cell-based protein production systems, and theoretical aspects of the methodology.

Page Manager: Claes.von_Wachenfeldt@biol.lu.se | 10 July 2019

Protein Production Network Sweden

a network of protein production facilities hosted by Swedish universities

https://ppns.ki.se/

Mammalian cells
Mammalian Protein Expression core facility
University of Gothenburg

Swedish NMR Centre,
University of Gothenburg
Cell free

E. coli
Protein Expertise Platform
Umeå University

Lund Protein Production platform
Lund University
E. coli,
BEVS
(Per)deuteration

Protein Science Facility
Karolinska Institutet, SciLifeLab
E. coli

Protein Expression and Characterization, DDD
SciLifeLab
E. coli, BEVS, Mammalian cells
(only for DDD projects)

PPNS

The Protein Production Network Sweden (PPNS) is an informal network formed in 2013 between protein production facilities at Swedish universities. The overall aim is to make competence and protein production methodologies available to the Swedish academic life science community and to make best use of our available resources.

PPNS managers

Göran Karlsson
Professor
NMR for life
Gothenburg University

Helena Berglund
Senior Lecturer
Protein Science Facility
Karolinska Institutet

Wolfgang Knecht
Senior Lecturer
Lund Protein Production Platform
Lund University

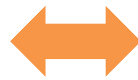
Anders Ohlson
Head of Facility
Protein Expression and Characterizatio n
SciLifeLab

Malin Bäckström
Researcher
Mammalian Protein Expression
Gothenburg University

Mikael Lindberg
Senior research engineer
Protein Expertise Platform
Umeå University

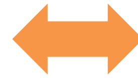
The core facility dilemma….

Providing on demand and cost effective service.



Offer the latest technologies and protocols to scientific questions.

Ideally occupies all time of facility personal.



Requires % time and new expertise.

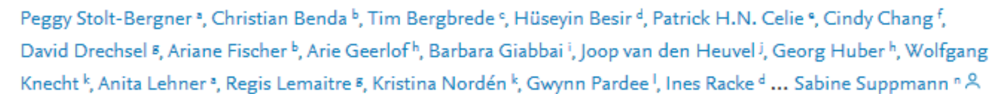
Strategy & Developments

Better use of BEVS

E.g. what virus backbone to use.

Journal of Structural Biology
Volume 203, Issue 2, August 2018, Pages 71-80

Baculovirus-driven protein expression in insect cells: A benchmarking study


Peggy Stolt-Bergner [a], Christian Benda [b], Tim Bergbrede [c], Hüseyin Besir [d], Patrick H.N. Celie [e], Cindy Chang [f], David Drechsel [g], Ariane Fischer [b], Arie Geerlof [h], Barbara Giabbai [i], Joop van den Heuvel [j], Georg Huber [h], Wolfgang Knecht [k], Anita Lehner [a], Regis Lemaitre [g], Kristina Nordén [k], Gwynn Pardee [l], Ines Racke [d] ... Sabine Suppmann [n]

Stable isotope labeling of proteins and lipids

- adapting expression systems
- implementing effective methods
- understand cellular responses to isotope labeling

Structural biology with neutrons

FragMAX

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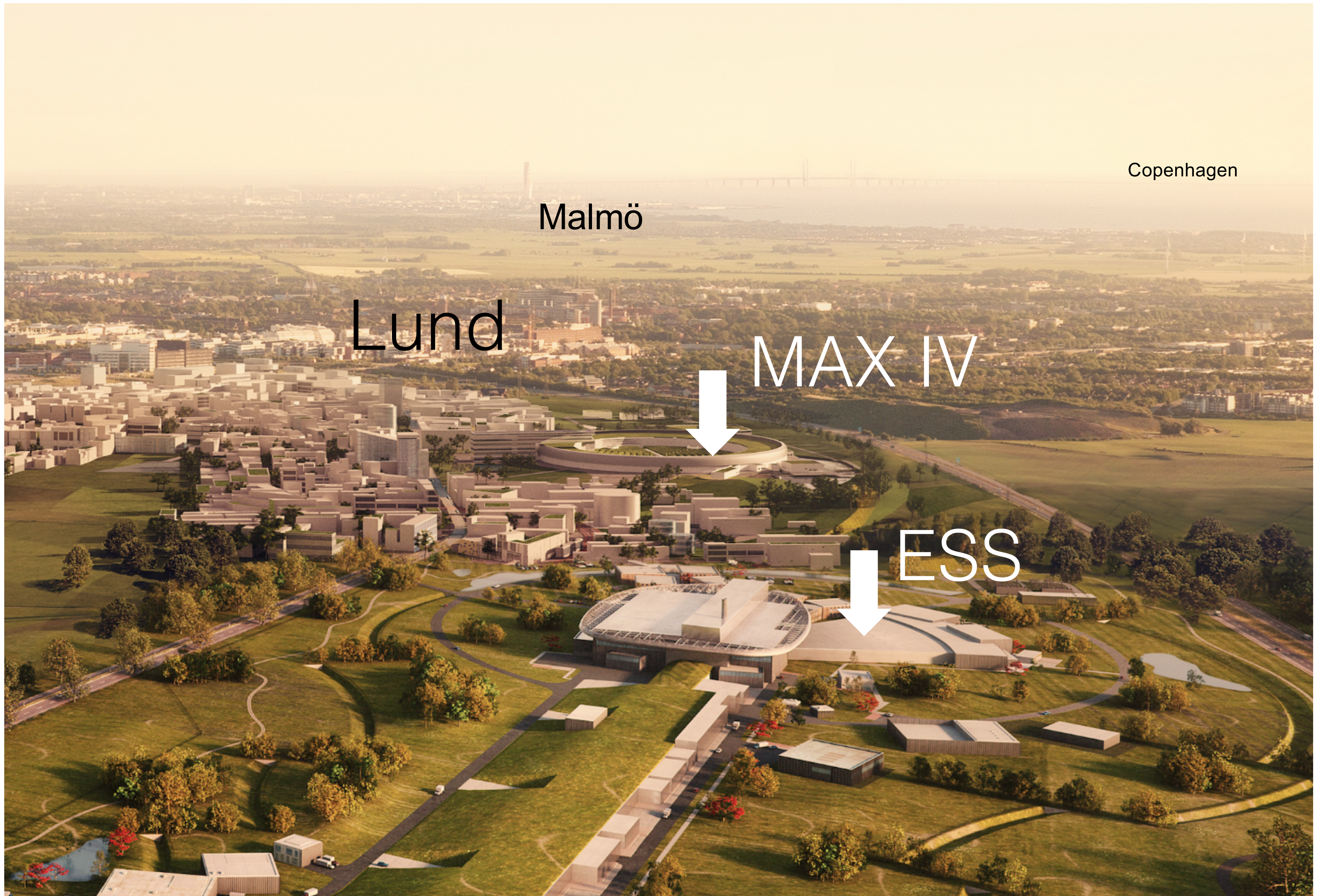

Picture from: https://adfs.esss.lu.se/adfs/ls/

Lund University: Strategic plan 2017–2026

Vision

A world-class university that works to understand, explain and improve our world and the human condition

Priority areas

- Education and research are to be intertwined
- Stimulating active collaboration to solve societal challenges
- Continued development as an international university
- Well-developed leadership and collegiality
- Students, employees and visitors are to be offered attractive environments
- **The potential of MAX IV and ESS is to be fully exploited**

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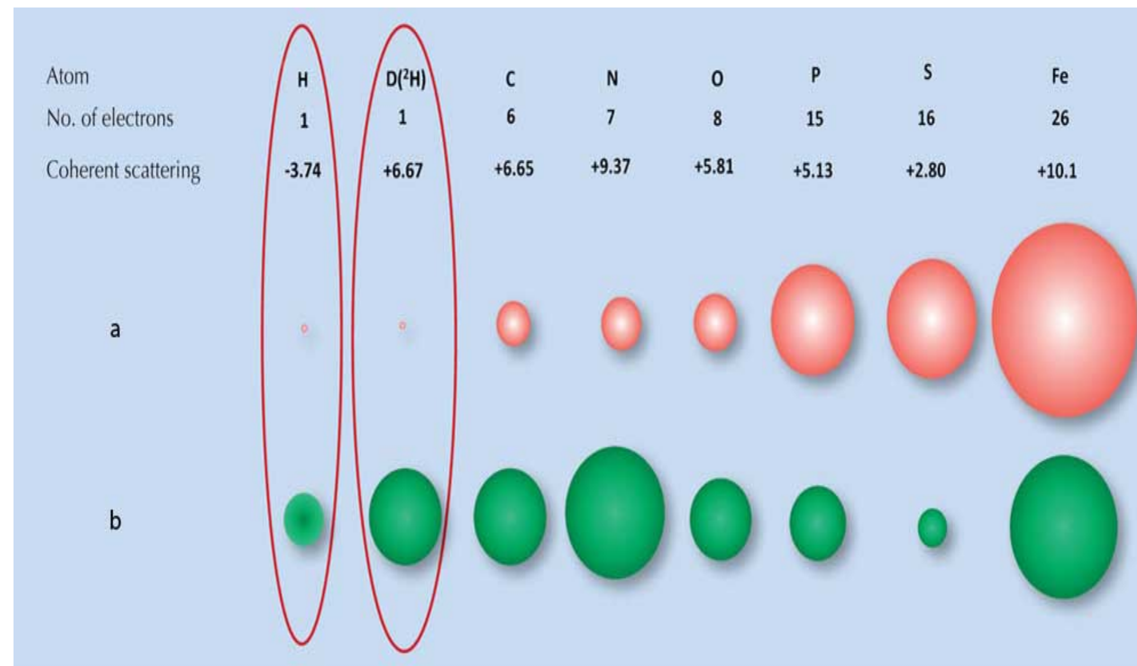
Neutron & X-ray scattering cross sections

Neutrons have wavelengths appropriate to study inter-atomic distances. With energies comparable to molecular motions, they interact weakly with materials, and can penetrate into the bulk in a non-destructive manner.

Neutron scattering lengths for different atom types found in biological materials:



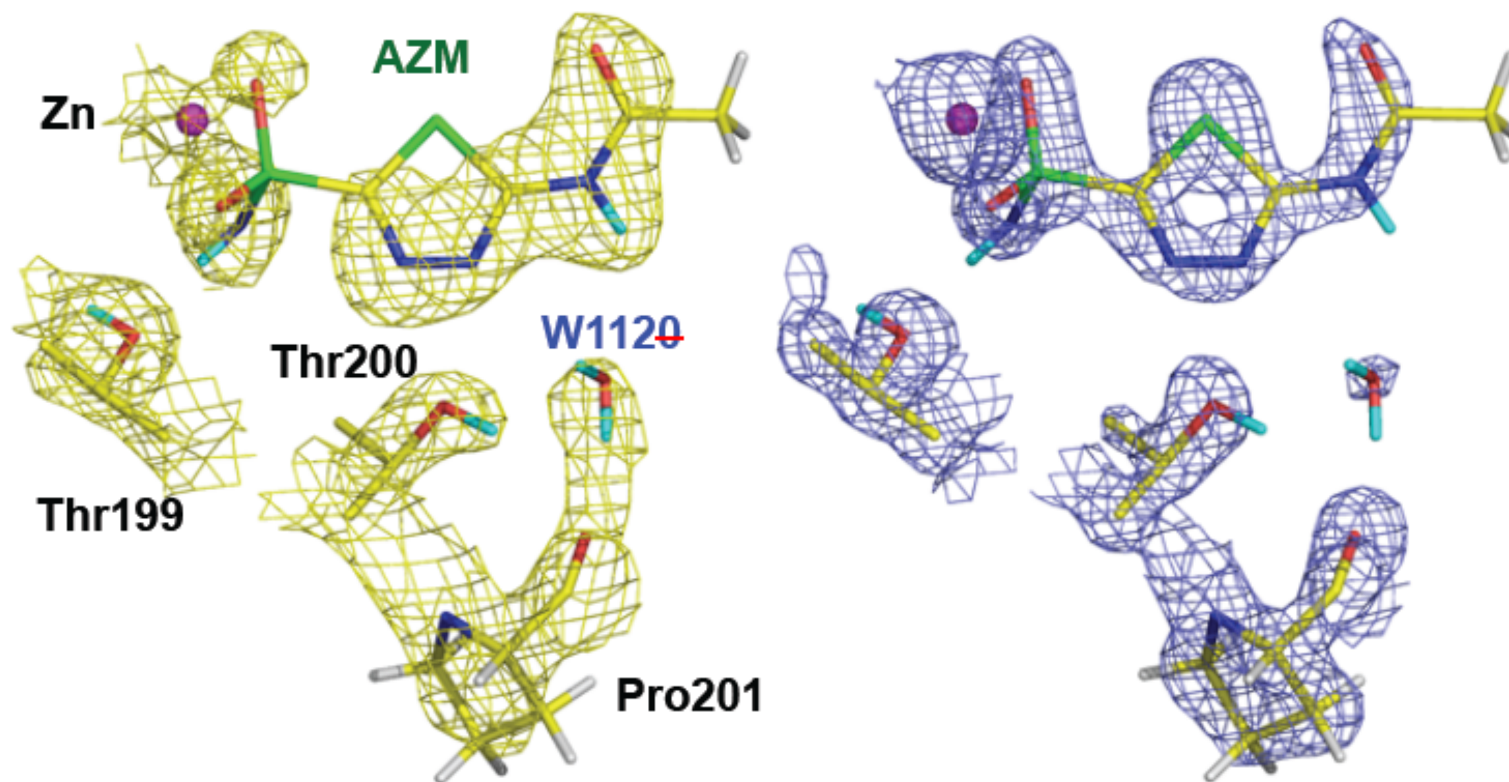
H atoms make up ~50% of atoms of biological macromolecules (lipids, proteins, nucleic acids, carbohydrates).

Protein-ligand interactions with neutrons

Elucidate specific binding interactions between ligand and protein

Can observe protonation/charged state of compounds

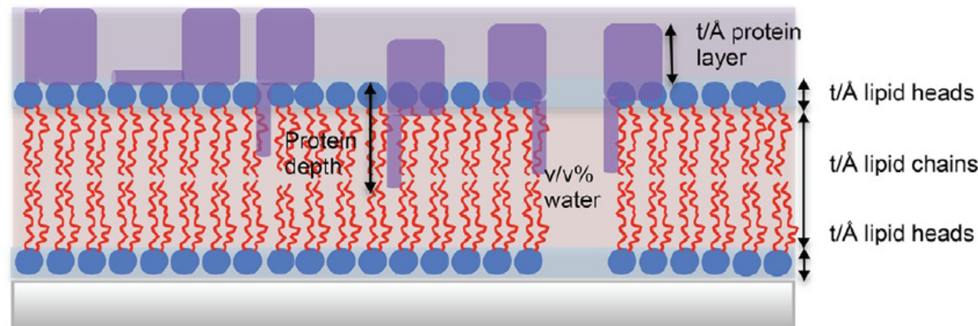
Directly observe how ligand binds – H-bonding, role of solvent, which groups of the protein are involved

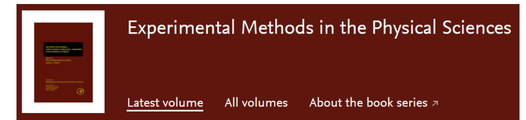


Complementary X-ray and neutron structures show the "full" details of ligand binding: Neutron data (yellow) show unambiguous data for H/D atoms, while X-ray (blue) data shows unambiguous data for the "heavier" atoms, such as S.

REF: Fisher et al. (2012) JACS 134, p.14726.

Neutron reflectometry – the importance of deuteration





Neutron Scattering - Applications in Biology, Chemistry, and Materials Science

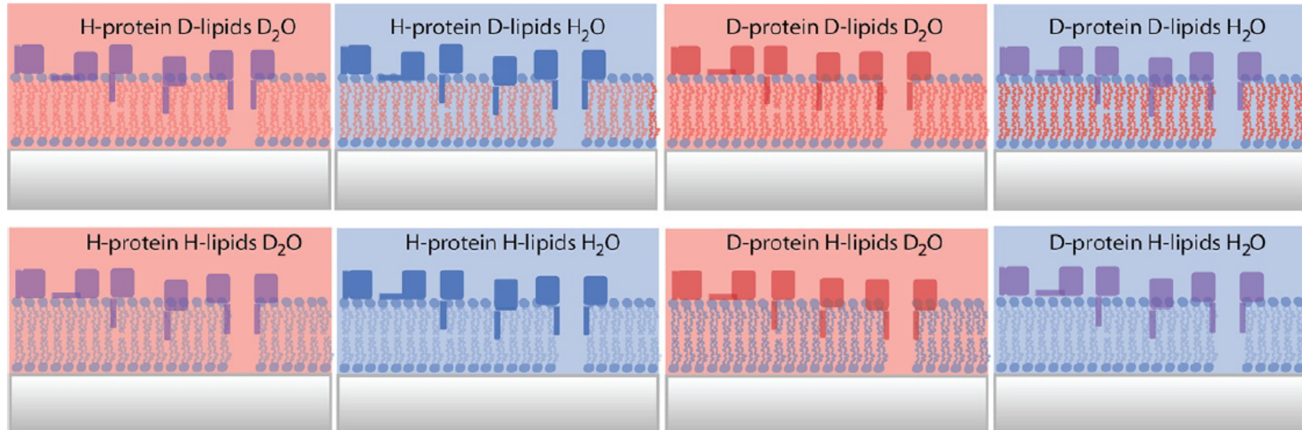
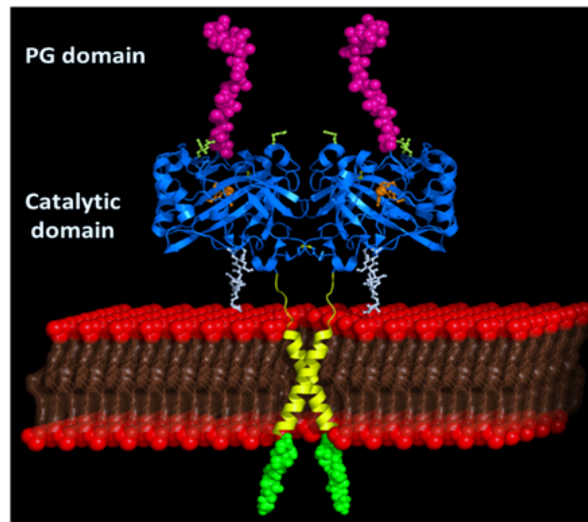


FIGURE 6 Modeling neutron reflectivity data from a protein-membrane system using contrast variation by deuterating lipids and proteins in turn. *Adapted with permissions from [207].*

Deuterated reagents are essential for neutron scattering, but not readily available.

Method development: Protein Deuteration

Carbonic anhydrase IX



Carbonic anhydrases – some facts

- Metalloenzymes that use a Zn-hydroxide mechanism to catalyze reversible conversion of CO_2 and $HCO_3^- + H^+$

- Mammalian CAs are involved with everything: respiration, acid/base homeostasis, gluconeogenesis, ureagenesis, bone resorption, and other processes

- In humans there are 16 expressed isoforms – found in all tissue types with varying distribution

- 30-80% sequence identity.

- Exist as monomers or dimers with different subcellular distributions – some cytosolic, membrane (extracellular CA domain), mitochondrial, some are secreted.

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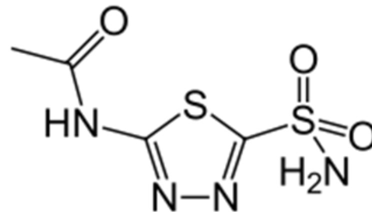
• Dysfunction/regulation of them is involved with a range of disease (eg. CA IX and its role in cancer).

• CAIs developed in the 1950s as a treatment for glaucoma. Today they are prescribed to treat a variety of things, including ***glaucoma***, epilepsy, altitude sickness, hypertension.

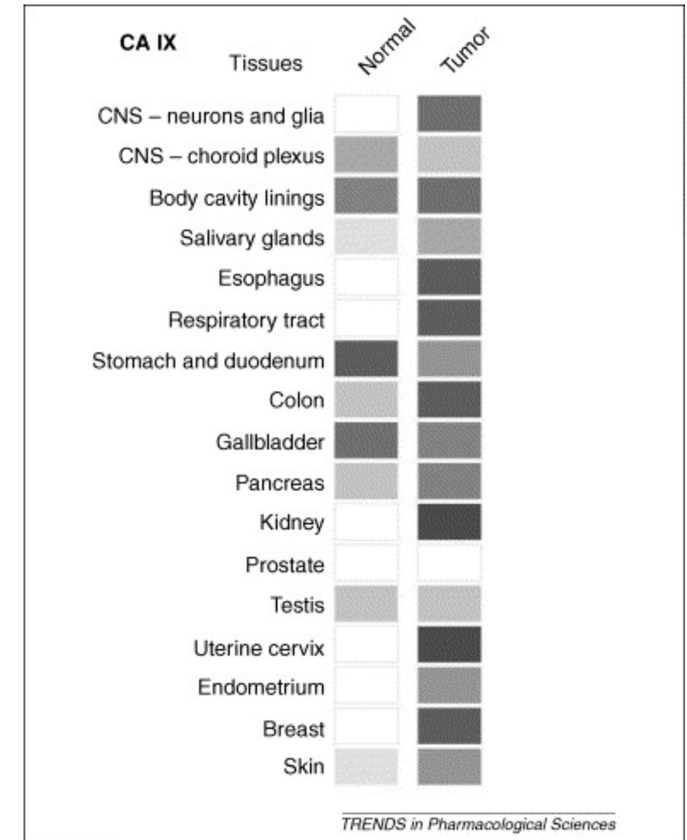
• CAIs have sulfonamide groups that coordinate to the Zn in the active site, displacing the catalytic water. They bind reversibly but have low K_i (low nM range).



Acetazolamide
(Diamox)

Human CA IX and its role in cancer

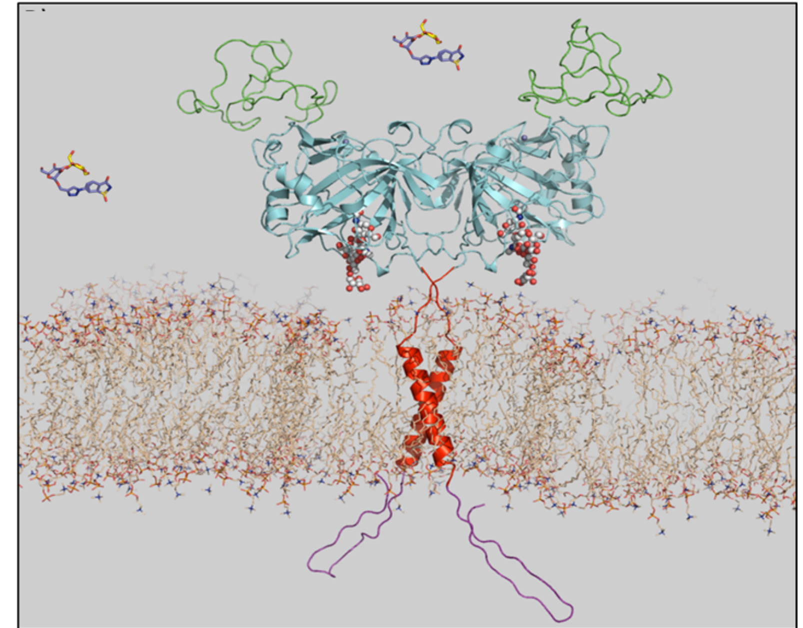
- In the 90s human CA IX was discovered as a surface protein in HeLa cells
- Subsequent analysis showed it to be a 459 aa protein, multiple domains (incl membrane spanning domain), dimeric with extracellular CA.
- Expression is hypoxia-controlled and in “normal” tissues is only found in selected areas of the GI
- Everywhere else is it only expressed in solid tumors.



Thiry A. et. al. *Trends in Pharmacological Sciences* (2006)

HCA IX role in solid tumors

- CA IX found at high levels in solid tumors, expression induced under hypoxia (HIF-1 mediated).
- The EC domain serves to acidify ECM, IC domain interacts with cell-cell adhesion proteins.
- Multiple effects lead to metastasis.



Model of membrane protein CA IX

→ Attractive drug and diagnostic target

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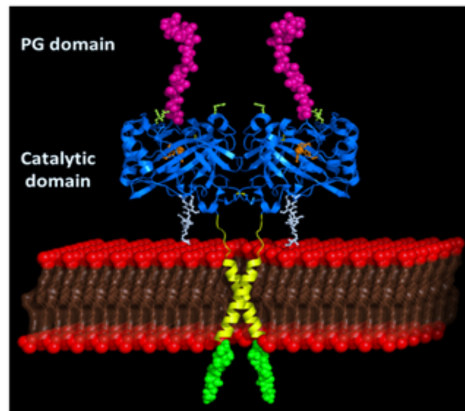
Isoform specific drugs and diagnostic tools needed

- High sequence and structural conservation in HCAs complicates drug design, causes cross reactivity & side effects (depression, exhaustion, metallic taste, pins-and-needles in lips and fingers, kidney stones).

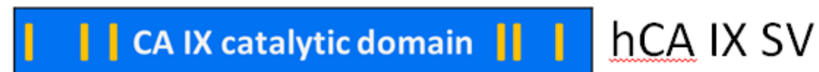
Unmet medical need and an innovation to be made

Can a combination of X-ray and neutron structure information of CA IX with new ligands be a model for future structure based inhibitor design?

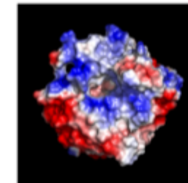


Carbonic anhydrase IX

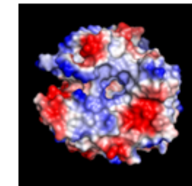
hCA IX fl



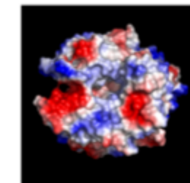
hCA IX SV



WT hCA II



hCA IX_{mimic}



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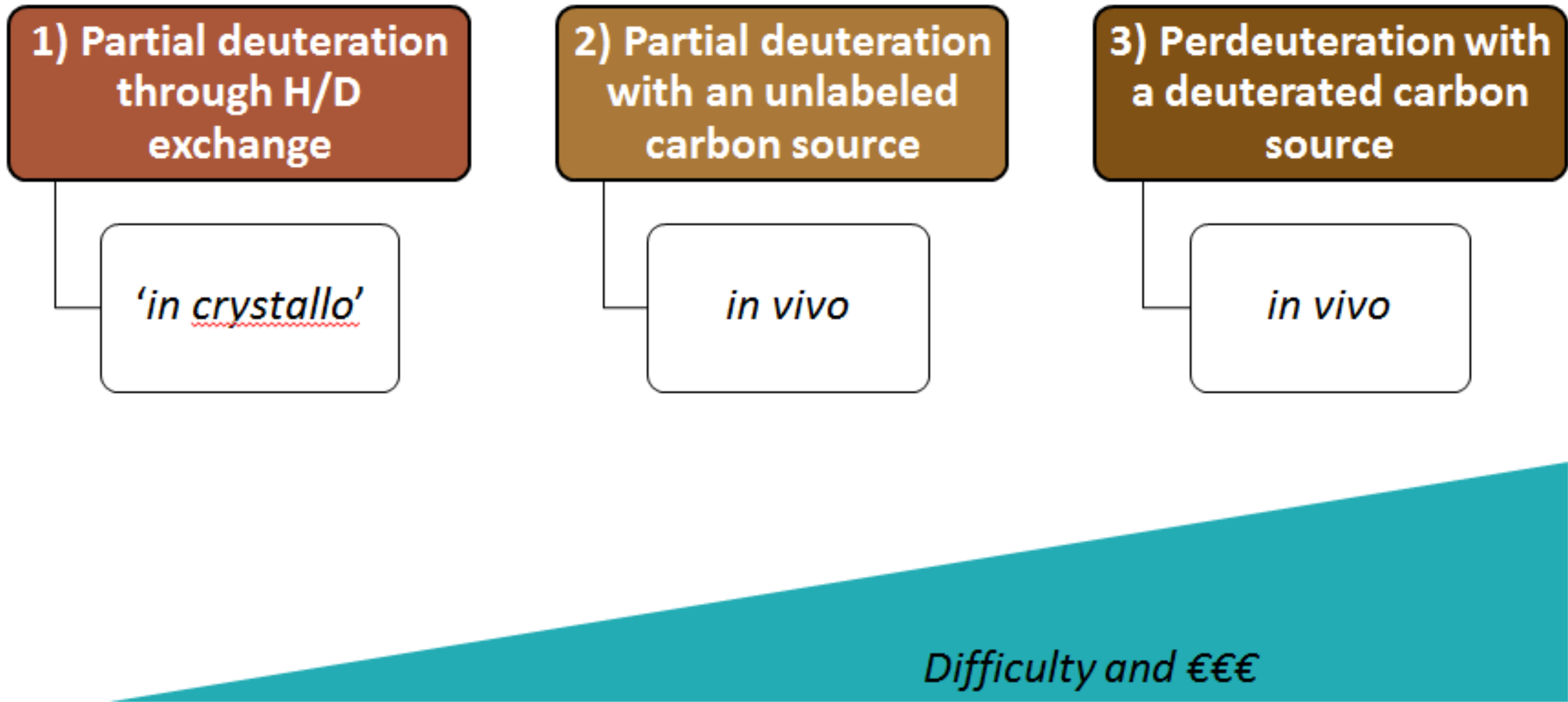
Deuteration for neutron protein crystallography:
Main methods and limitations (toxicity, stress, cost)



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Deuteration of hCAs in *E. coli*

Protein production

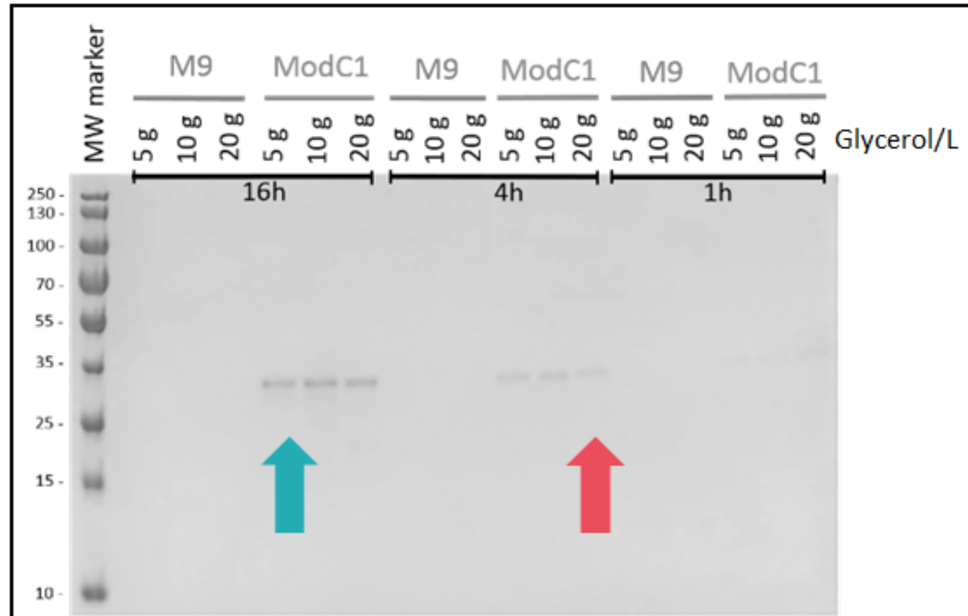
Compare, optimize media & condition

Mass spectrometry (MALDI-TOF and ESI MS)

Protein thermal Stability (DSF)

Crystallization

WT hCA II expression in ModC1 vs. M9 minimal media using different amount of glycerol as carbon source.



Conclusions:

- Poor expression in M9 minimal media
- Using **more glycerol** (20 g/L) results in good protein yields for shorter induction time while using **less glycerol** (5 g/L) led to sufficient yields when protein was expressed overnight.

Comparison of deuterated M9 medium and deuterated ModC1

M9

	Component	g/L
bulk solution	NH_4Cl	2,00
	KH_2PO_4	2,99
	Na_2HPO_4	6,06
	Unlabeled glycerol	20
	NaCl	5,84
		mg/L
Additives	Thiamine	2
	$MgSO_4{\cdot}7H_2O$	250
	$FeCl_3$	2,92
	$CaCl_2$	11,1
	D_2O	up to 1 L

ModC1

	Component	g/L
bulk solution	NH_4Cl	2,58
	KH_2PO_4	2,54
	Na_2HPO_4	4,16
	Unlabeled glycerol	20
	K_2SO_4	1,94
		mg/L
Additives	Thiamine	48
	$MgSO_4{\cdot}7H_2O$	670
	$FeSO_4{\cdot}7H_2O$	20
	Trisodium citrate	88
	$MnSO_4{\cdot}H_2O$	5
	$ZnSO_4{\cdot}7H_2O$	8,6
	$CuSO_4{\cdot}5H_2O$	0,76
	D_2O	up to 1 L

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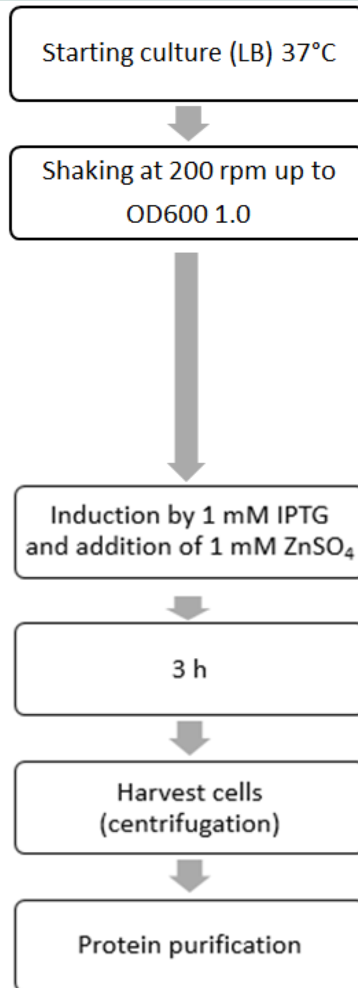
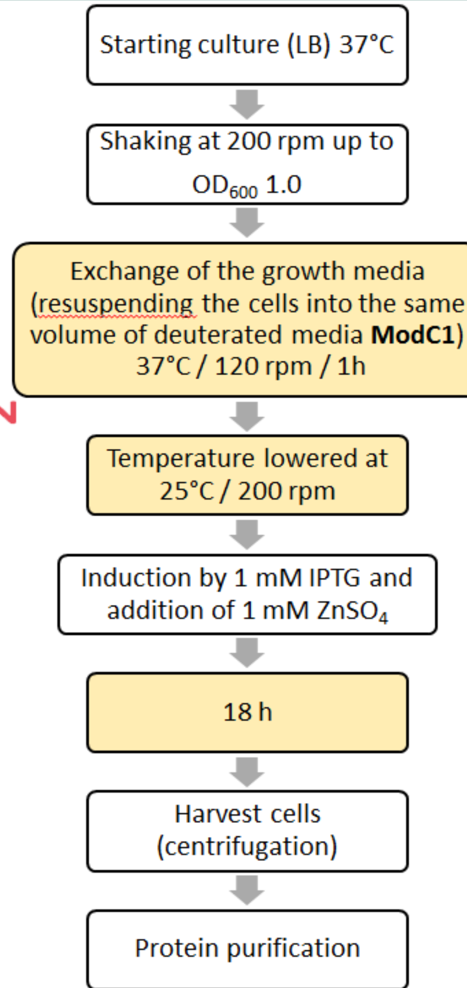
Protein expression in *E. coli* BL21 (DE3) cells.



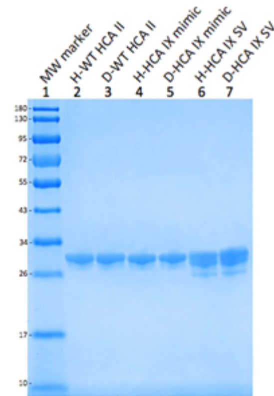


- D_2O recycled with rotary evaporation from media that was previously used to grow bacteria.
- Unlabeled glycerol

Results: Comparison of growth media and purification

Comparison of yields for hydrogenous LB media and deuterated ModC1 medium

Hydrogenous (LB media)	**Yield (mg/L)**
WT hCA II	49
hCA IX mimic	54
hCA IX SV (pk.2 only)	13
Deuterated ModC1	
WT hCA II	24
hCA IX mimic	16
hCA IX SV (pk.2 only)	4.4



Conclusions:

- Expression in ModC1 minimal media gives lower yields in comparison with H-LB media
- From the SDS-Page we do not observe any size difference between hydrogenous or deuterated protein

Results: Mass spectrometry (MALDI-TOF and ESI MS)

	MALDI TOF	ESI MS
WT hCA II	**51%**	**65%**
HCA IX mimic	**62%**	**77%**

Conclusions:

- MALDI TOF gave lower values for both proteins - due to the use of hydrogenous matrix and rapid back-exchange.
- ESI-MS better reflects the actual degree of deuteration

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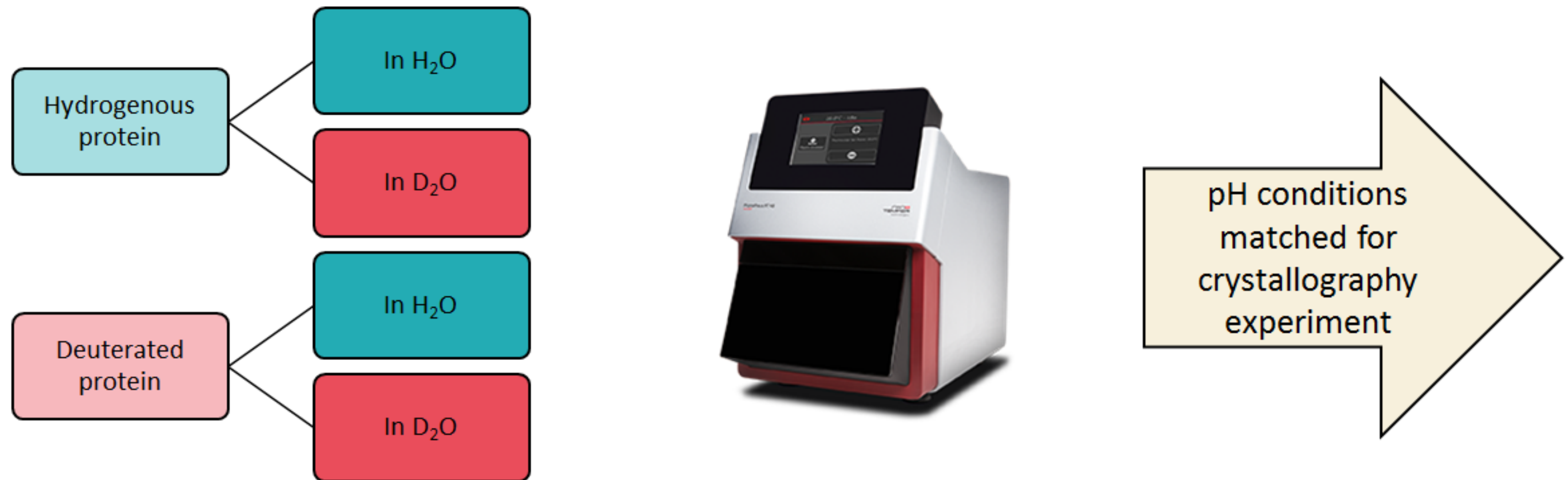
Results: Protein stability measurements with differential scanning fluorometry:



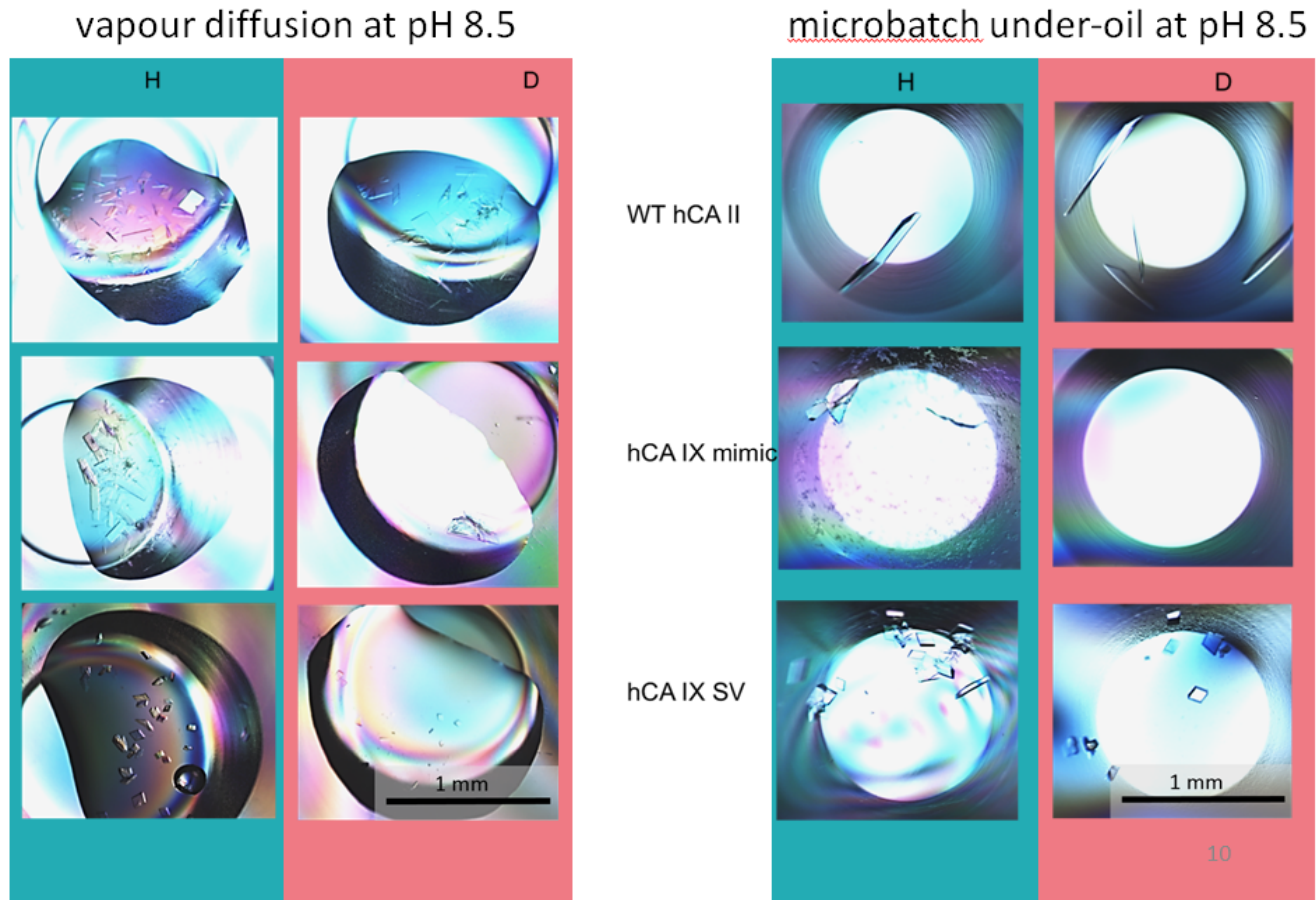
Conclusions:

- Deuterated proteins are 1 - 4 °C less stable than hydrogenous, depending on pH
- D-protein in D_2O is more stable than D-protein in H_2O

Results: Crystallization trials of H vs. D protein

Conclusions:

- No crystals appeared for either H or D version at low pH (5.5 and 6.5) and the best crystals always grew **at pH 8.5**
- Differences in the size and number of the crystals or no crystallization
- Optimization of conditions needed



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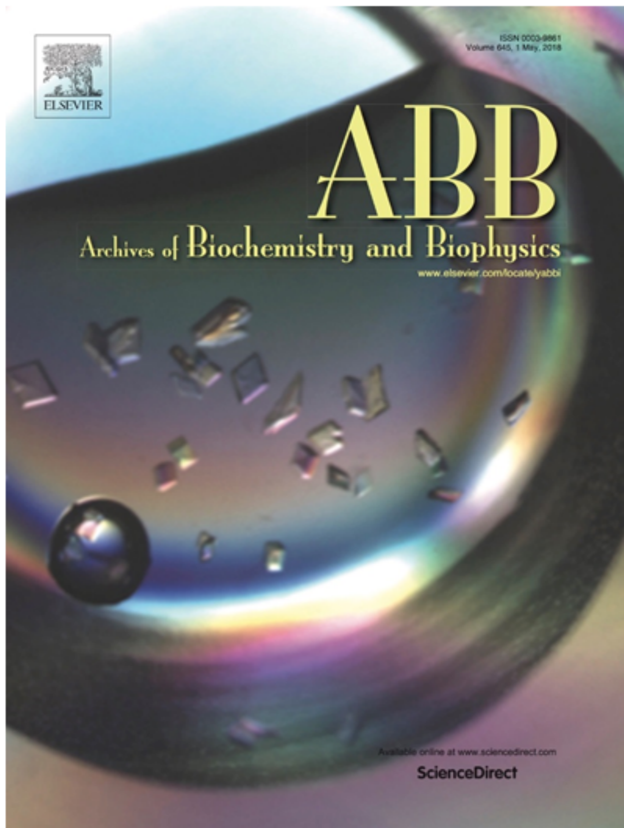

- 65-77% D incorporation when using unlabeled C-source and recycled D_2O
- Good yields of protein, cost effective simple method for production of deuterated proteins for different techniques (crystallization)
- If both fresh D_2O and labeled glycerol is used, the cost increases 4-fold
- Protein solubility is unaffected (in the ranges used here), thermal stability and crystallization behaviour are affected

REFERENCE: Koruza K, Lafumat B, Végvári Á, Knecht W, Fisher SZ (2018) “Deuteration of human carbonic anhydrase for neutron crystallography: Cell culture media, protein thermostability, and crystallization behavior”, *Arch Biochem Biophys*. **645**, p.26-33.

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ESS Deuteration and Macromolecular Crystallization (DEMAX) Platform

Chemical Deuteration
- Organic (chemical) synthesis
- Enzymatic Synthesis
- Separate, analyze a range of molecules
- Yeast-derived total lipid preparation
- Future: extraction of lipids, oils and other small molecules from biomass

Biological Deuteration
- Deuterated biomass production (algae, bacteria)
- Protein expression & purification
- Biophysical characterization (DLS, thermofluor, nanotemper, purity)
- D incorporation with ESI-MS
- Future: yeast biomass

Macromolecular Crystallization
- High- and low-throughput screening
- Large volume crystallization
- Optimization (seeding, fine-screening, temperature)
- X-ray testing 100 K @ MAX lab (with LP3)
- Support for xtal mounting & H/D exchange for RT measurements

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