SINE2020 General Assembly

Parma, 4 June 2018

WP6: Macromolecular Crystallogenesis

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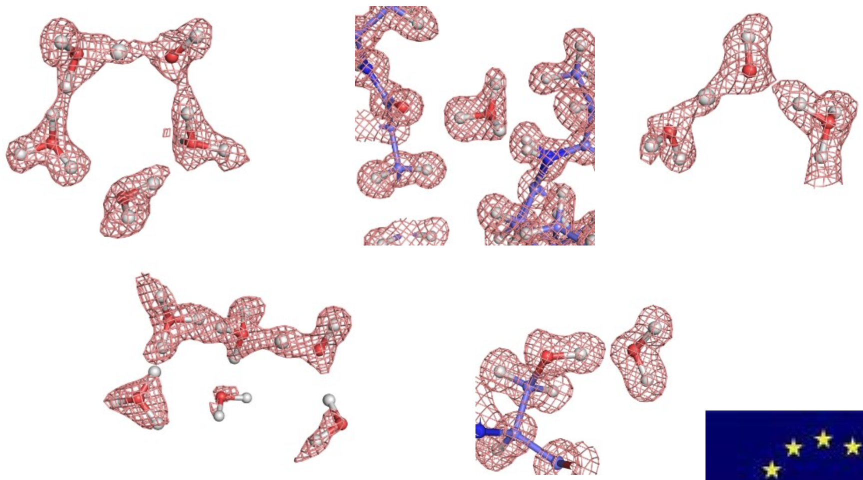
This project is funded by the European Union (GA no. 654000)

0. Background and Context of WP6

Neutron protein crystallography can provide *crucial* information on biological systems that is inaccessible by other methods. Key issues are protonation states, hydrogen bonding, hydration, redox proteins – all central issues for structural biology and drug design *eg*:

Yee et al *J. Appl. Cryst.* (2017)
Gerlits et al, *J. Med Chem.* (2017)
Kwon *et al*, *Nature Communications* (2016)
Howard et al, *IUCrJ* (2016)
Gerlits *et al*, *Angewandte C.* (2016)
Blakeley et al, *IUCrJ* (2015)
Casadei *et al*, *Science* (2014)
Langan et al *Structure* (2014)
Cuypers *et al*, *Angewandte C.* (2014)
Oksanen et al, *PloS one* (2014)



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1. Objectives

- Development of methods whereby large crystal growth can become a routine service-orientable capability for neutron protein crystallography
- Apply these methods to both model systems and challenging target systems
- Plans for implementation of viable methods at neutron beam sources (eg ILL, FRM-II and eventually ESS).

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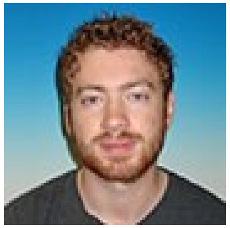
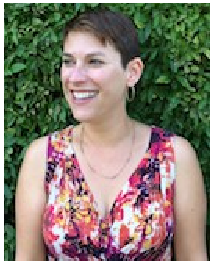
Work package tasks

Task 6.1.1: Development of a robotic system for large crystal growth Task leader: ILL



Task 6.1.2: Development of a flow crystallisation system
Task leader: ILL



Task 6.2.1: Phase diagram characterisation for proteins
Task leader: ESS

Task 6.3.1: Phase diagram characterisation for proteins
Task leader: FZJ

Task 6.3.2: Application of vapour diffusion approaches
Task leader: FZJ

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- Influence of the degree of Deuteration on the crystallizaiton of proteins

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Deuteration for neutron protein xtallography:

- Survey of the PDB, we looked at deposited neutron crystal structures and associated publications
- *(1) used the most, followed by (3) and (2) appears in the literature a few times*

1) Partial deuteration through H/D exchange
- *'in crystallo'*

2) Partial deuteration with an unlabeled carbon source
- *in vivo*

3) Perdeuteration with a deuterated carbon source
- *in vivo*

Difficulty and $$$

Improve (2), save costs & still get good yield and sufficient D incorporation?

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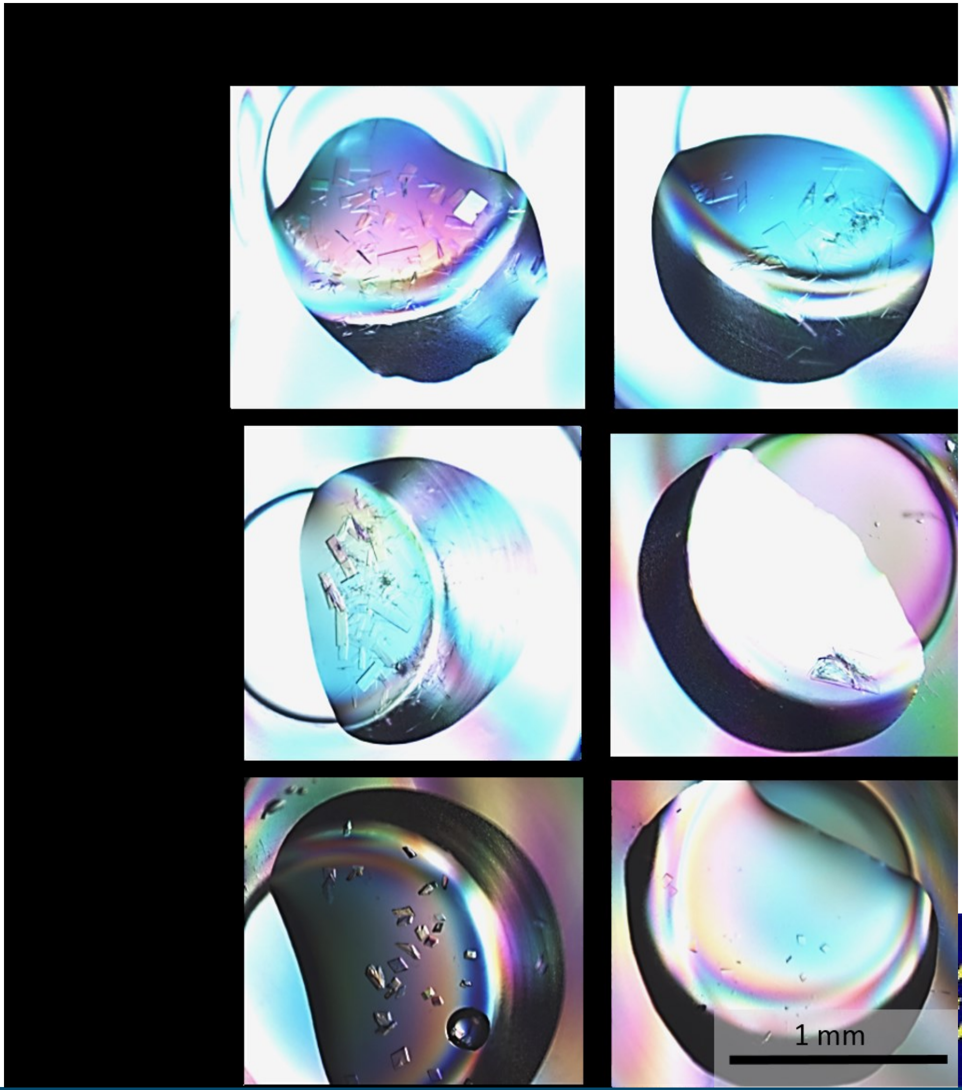
Crystallization trials of H vs. D protein - vapour diffusion

Conclusions:

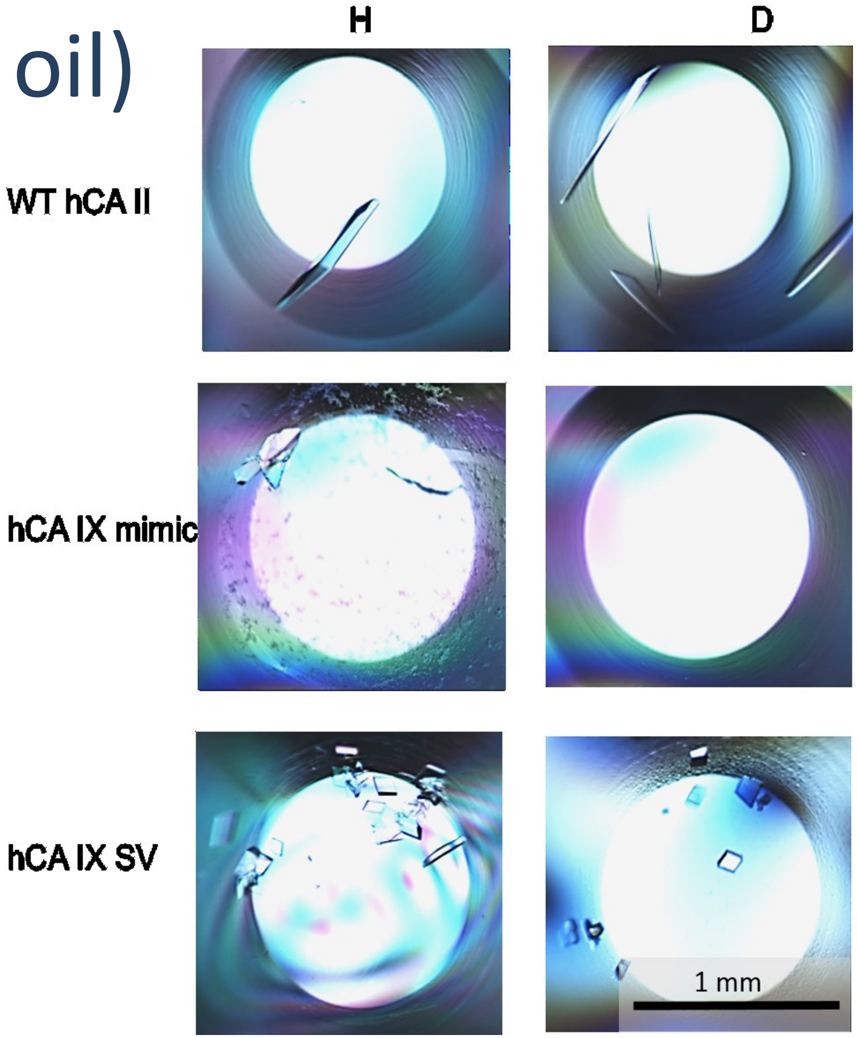
- **No crystals appeared for either H or D version at low pH (5.5 and 6.5) and the best crystals always grew at pH 8.5**

- **Differences in the size and number of the crystals or no crystallization**

- ***Optimization of conditions needed***



Crystallization trials of H vs. D protein – microbatch (under oil)



Conclusions:

- **Much nicer crystal in batch for both H and D**
- **Again, we see differences in the *size* and *number* of the crystals or *no crystallization***
- ***Optimization of conditions needed***

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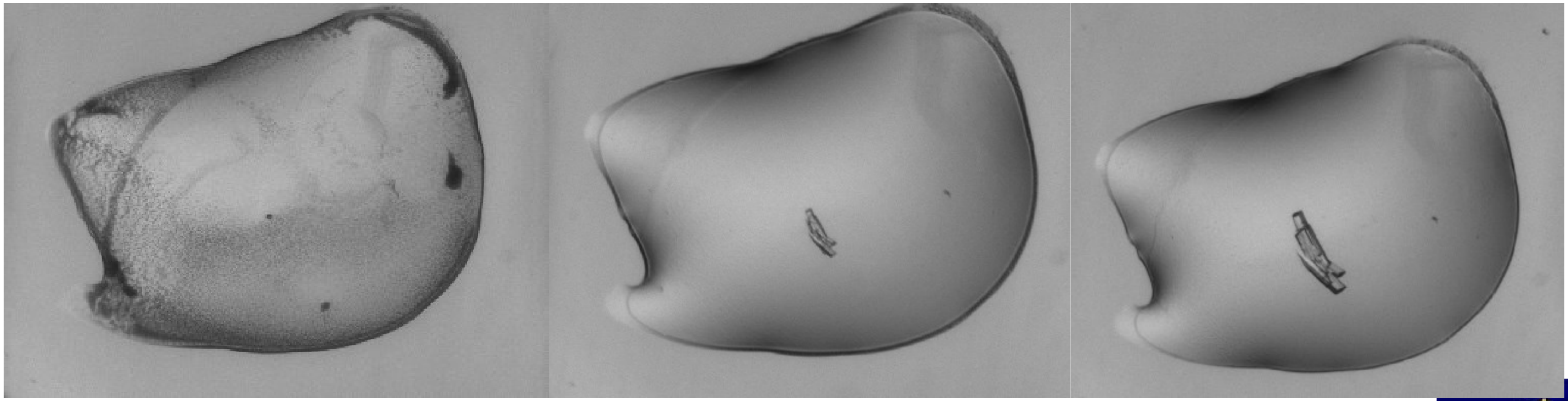
Crystallization trials and tribulations of H and D versions of hCA IX SV

- 12 mg/mL prep
- Sparse matrix screen (commercial: JCSG+ and Morpheus), set-up 300 nL drops on Mosquito
- Condition A8: 20% PEG 3350, 0.2 M ammonium formate (no buffer)



1 day 30 days 60 days

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- Building and testing crystallization devices

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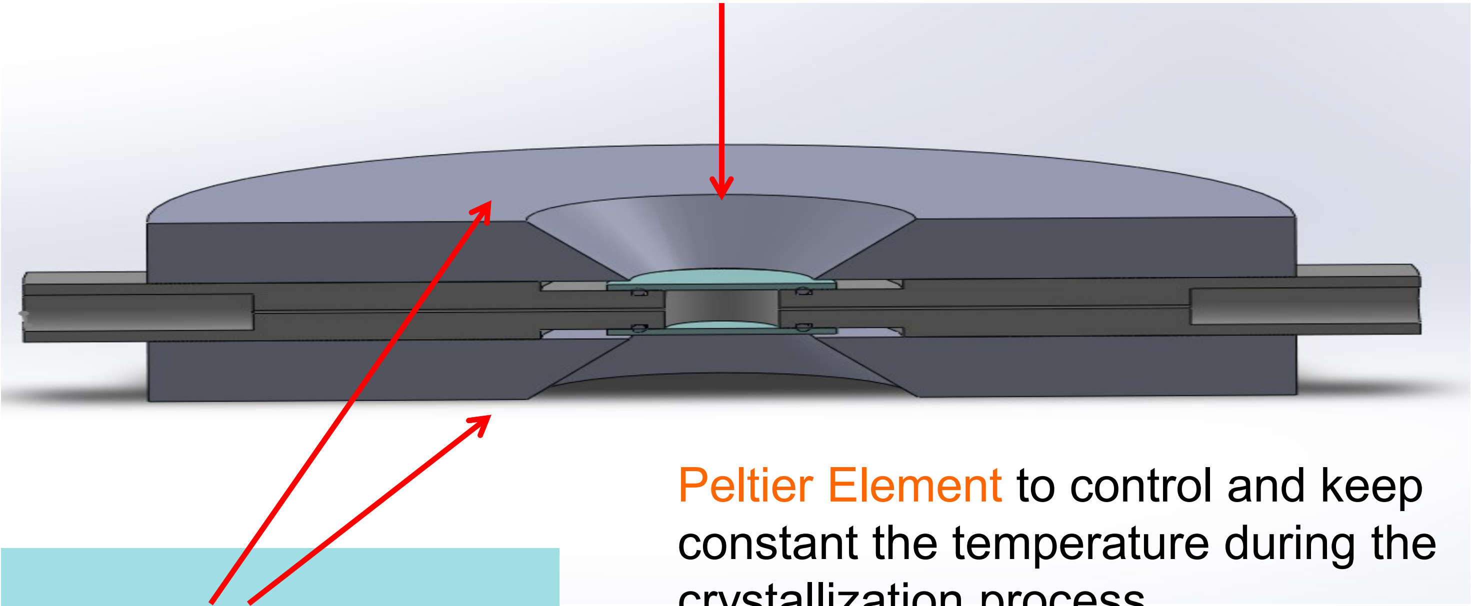
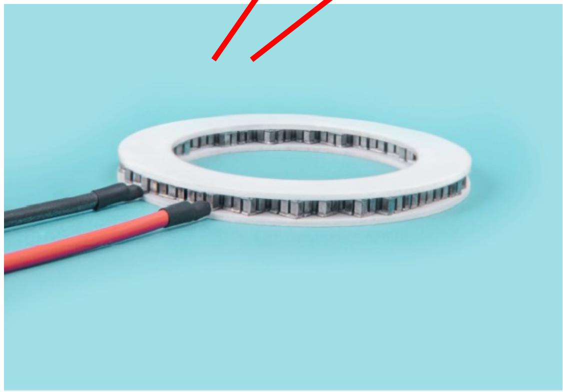
Inverted microscope to visualize the crystal during the growth





Peltier Element to control and keep constant the temperature during the crystallization process

Symmetric Round shape: isotropic diffusion of heat

Central hole: inverted microscope use

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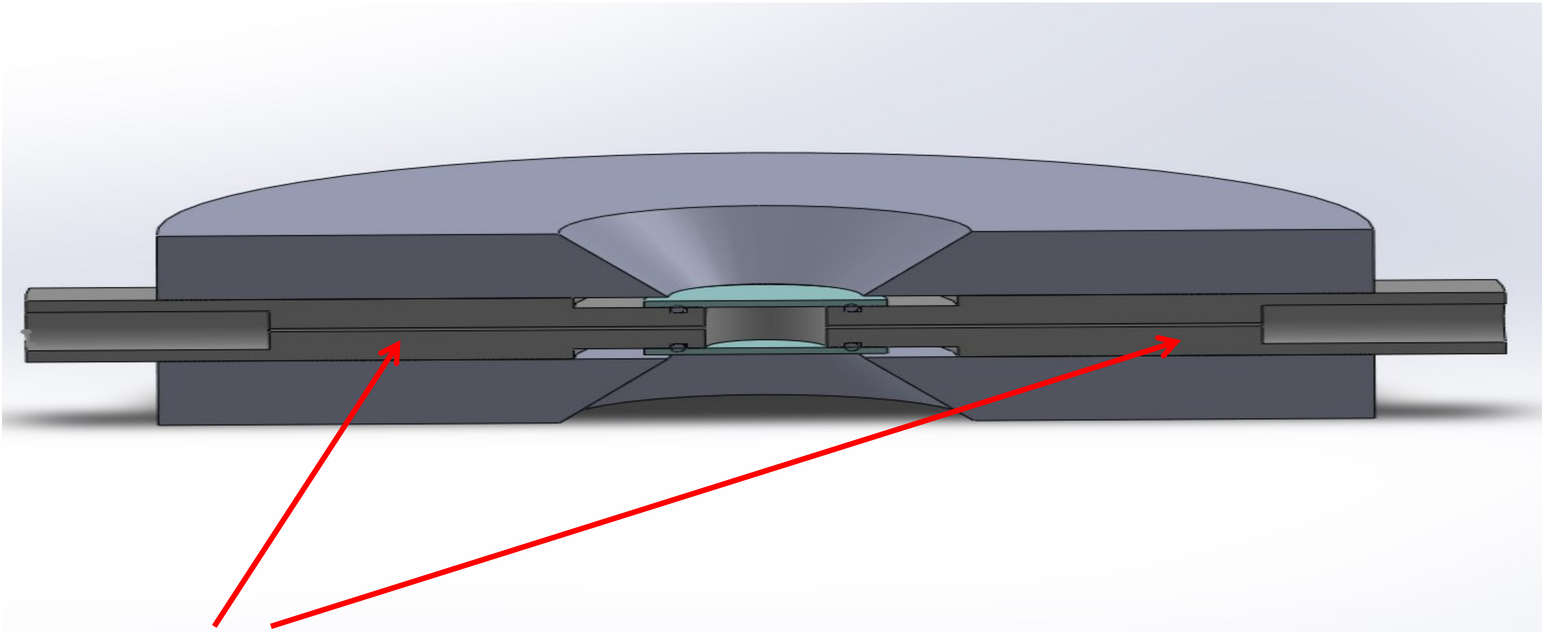
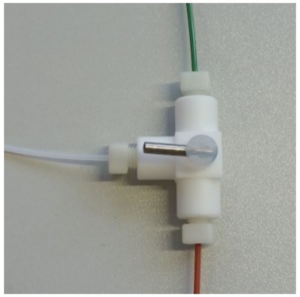
In-situ macroseeding apparatus:



The exchange of the mother liquor is allowed by means of two capillarity built in the spacer

Prevent osmotic shock:

Continuous variation from solution 1 to solution 2 with a slow gradient



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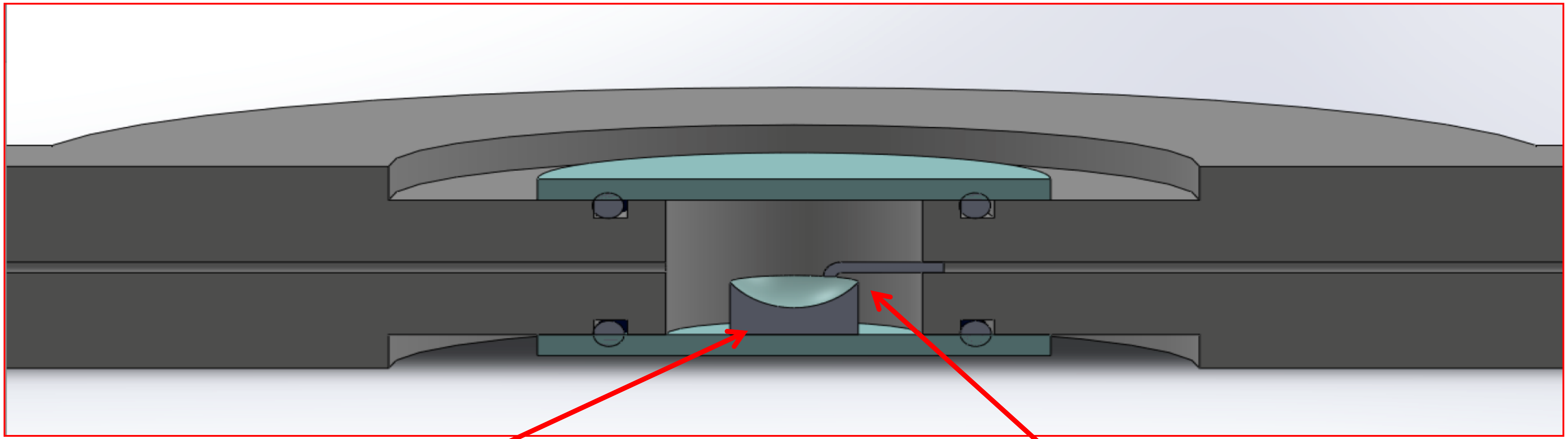
How can we avoid microconvective flux?



Sitting drop bridge in the crystallization chamber

Micro-pipe to change the drop Crystallization condition e.g. more protein
(3D built)

Powerful flexibility of the set-up due to the 3D printing option

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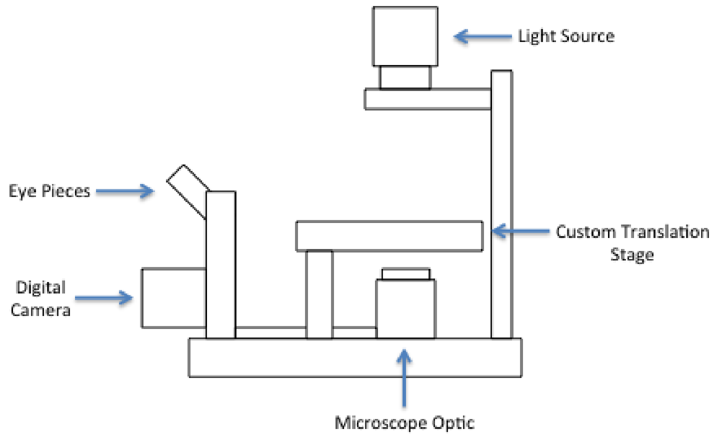
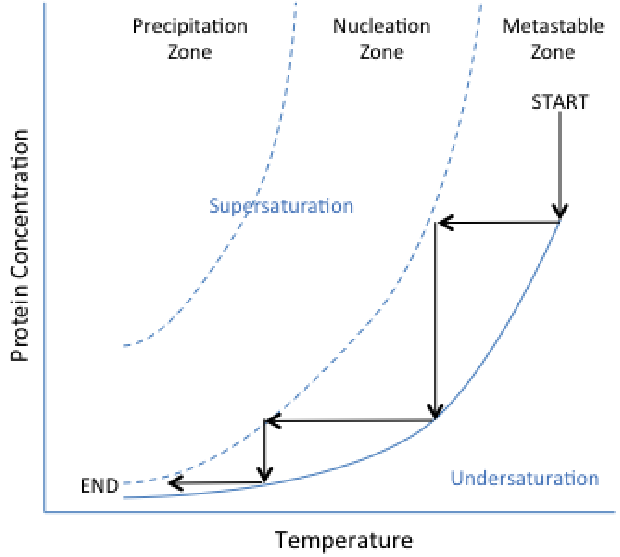
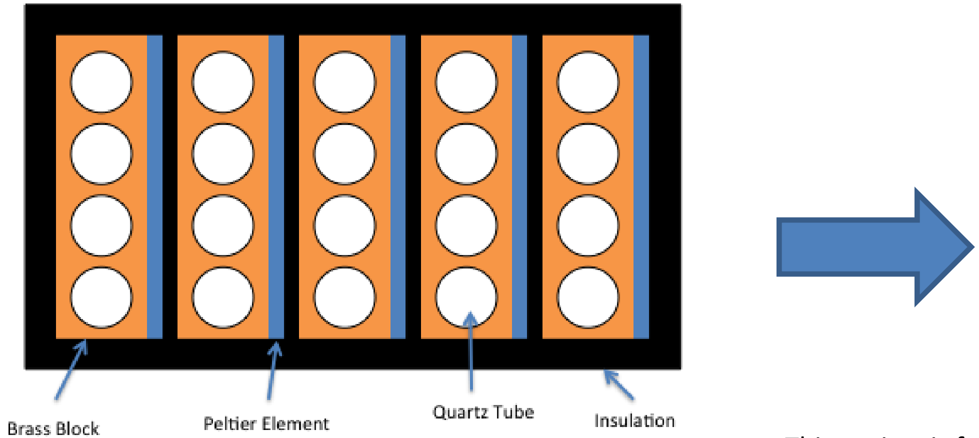
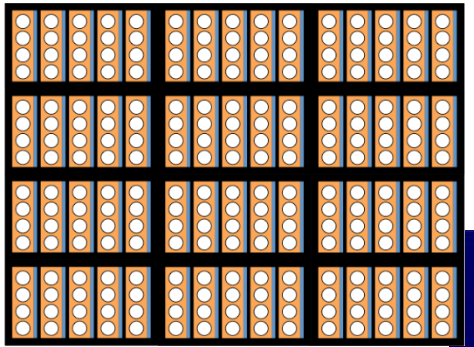
Task 6.1.1: Development of a robotic system for large crystal growth

Concept









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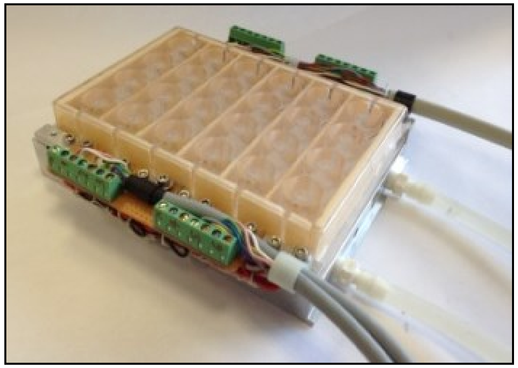
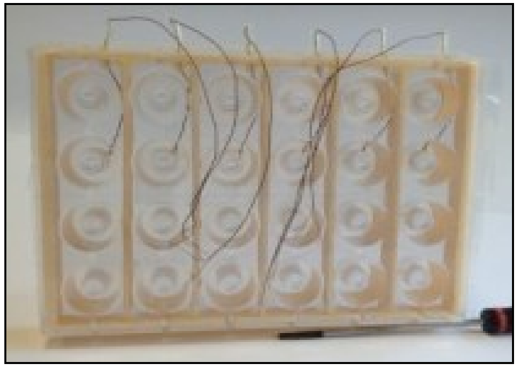
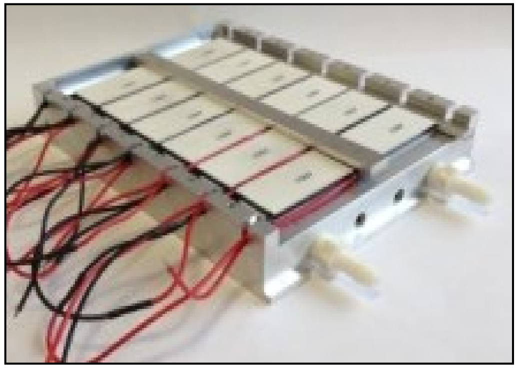
Task 6.1.1: Development of a robotic system for large crystal growth







Crystallisation plate:

- Based on readily available 24-well sitting drop vapor diffusion crystallization plate (Hampton Research).
- Strips of wells are insulated with foam for 6 strips of 4 wells, each at different temperatures, controlled by 2 Peltier elements.
- The plate is filled with thermally conducting resin, Stycast, for heat transfer from Peltier element to the droplet.
- A thermistor is embedded in the resin per strip of wells to readout the temperature experienced by a row of wells.

Temperature Control Base:

- Peltier elements (TE Technology, TE-63-1.4-1.15) are aligned and grouped in 6 strips of 2 (each group of 2 with independent temperature control).
- Aluminium cooling base with channels for water as coolant fluid.
- Single thermistor attached to external surface of cooling base to register its temperature with failsafe function.

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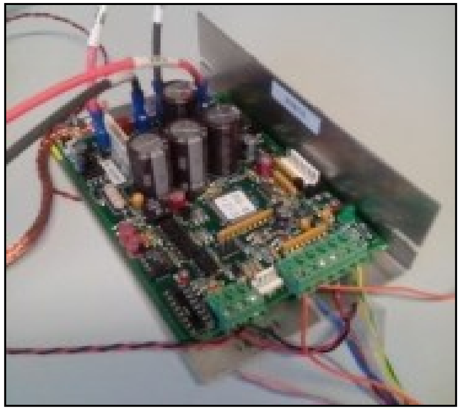
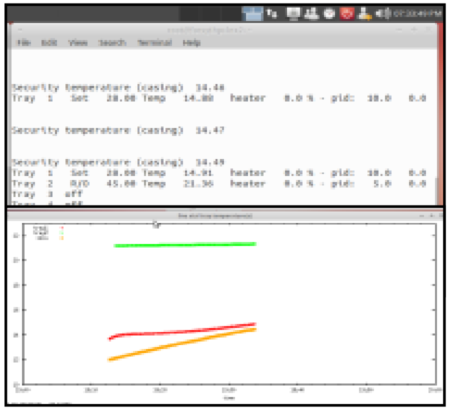
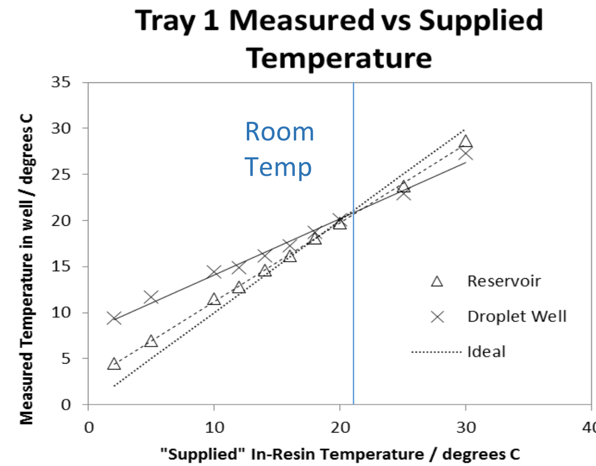
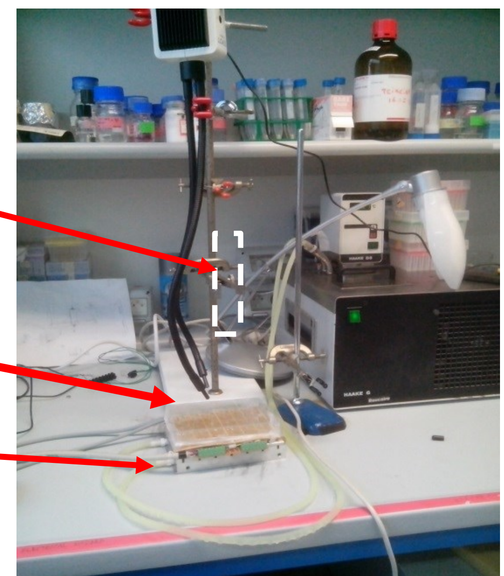
Task 6.1.1: Development of a robotic system for large crystal growth







- Camera from on top
- Two point LED lights
- Water coolant inlet/outlet



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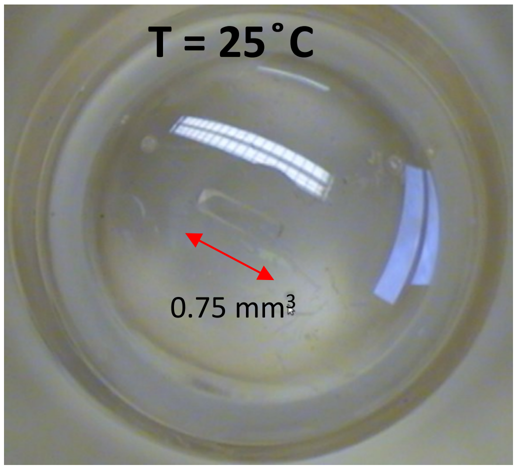
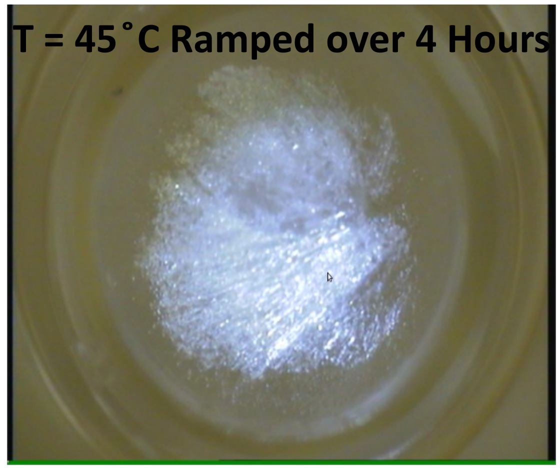
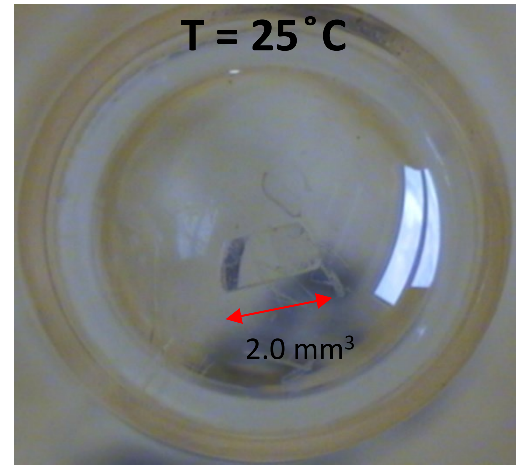
Task 6.1.1: Development of a robotic system for large crystal growth

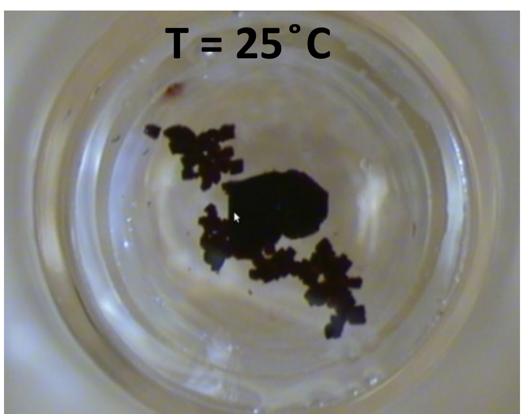
Trypsin

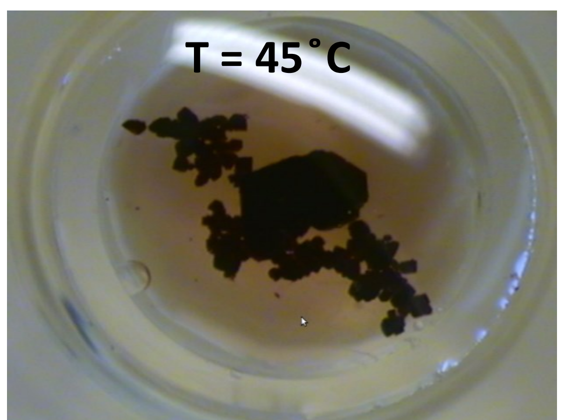


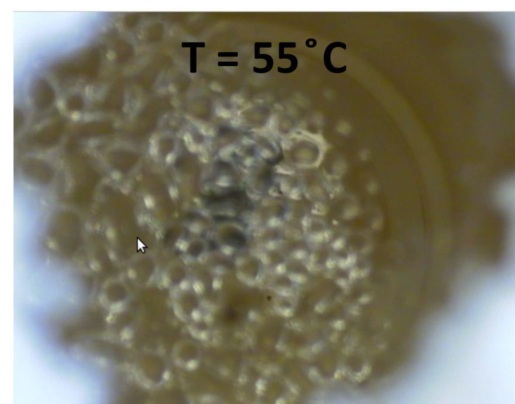




Rubredoxin







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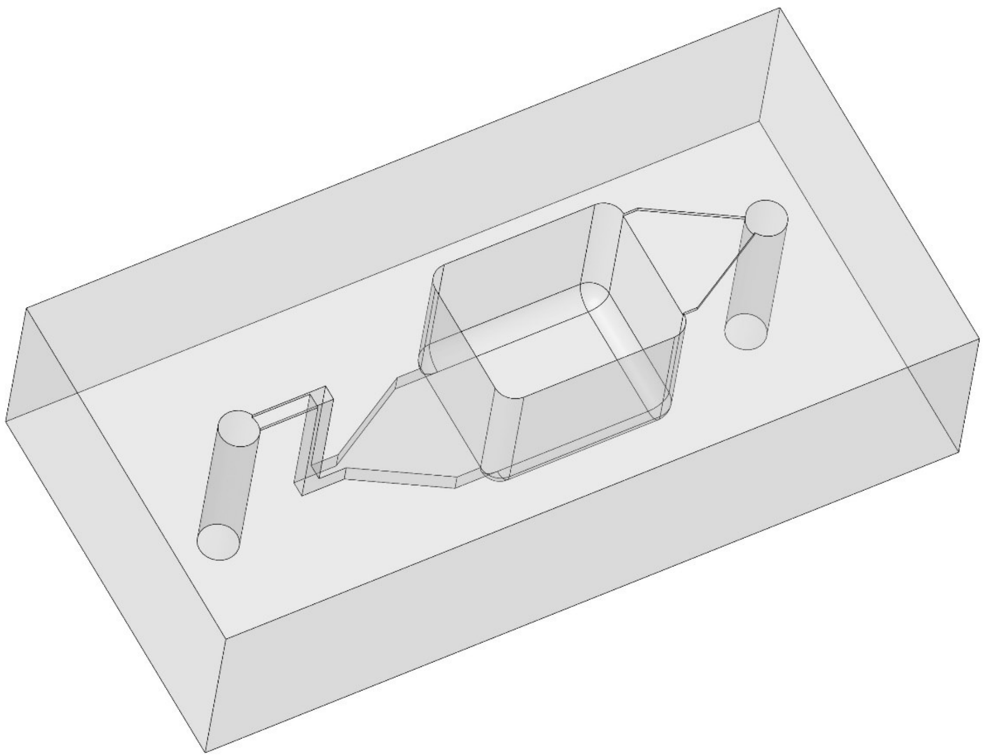
Prototype Chip Design

Inlet



Outlet

Internal well dimension

L x W x H

5mm x 4mm x 4mm

$80mm^3$ Total Vol

3D Printed Chip

UV cured resin

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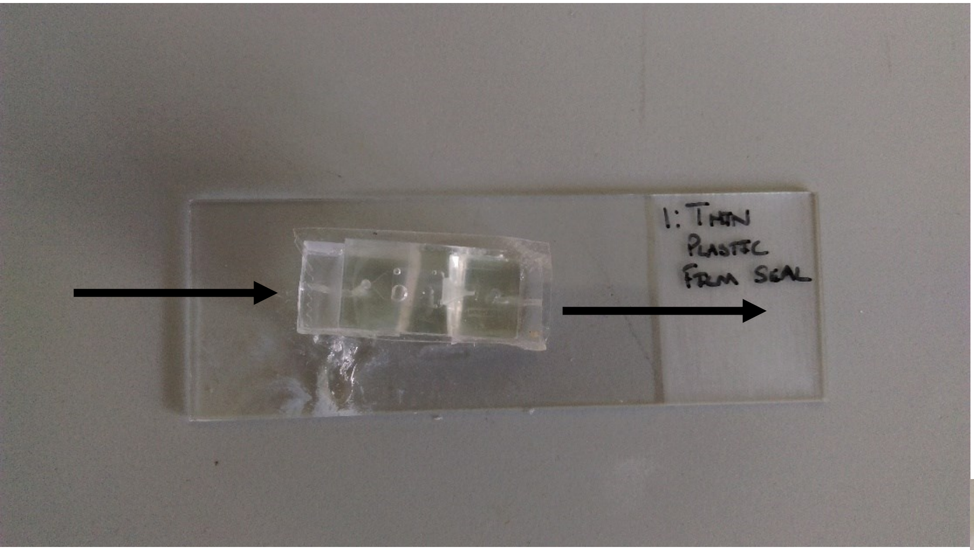
Prototype Chip Testing



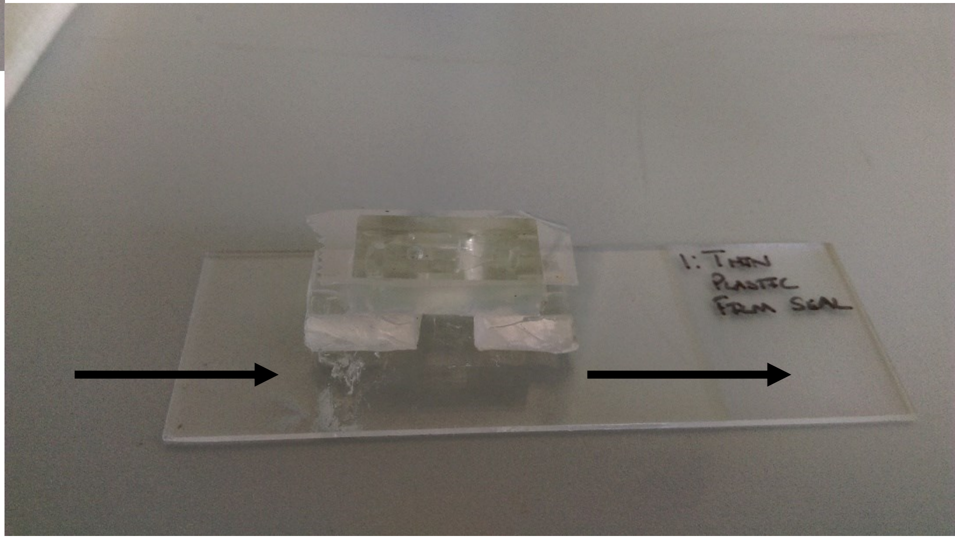
Arrows indicate where tubing enters/exits and direction of flow

PDMS Polymer blocks used to connect tubing with inlets/outlets of chip

Two channels punched at 90 degrees from one another.

Horizontal channel for tubing inlet/outlet connection

Vertical channel for chip inlet/outlet connection



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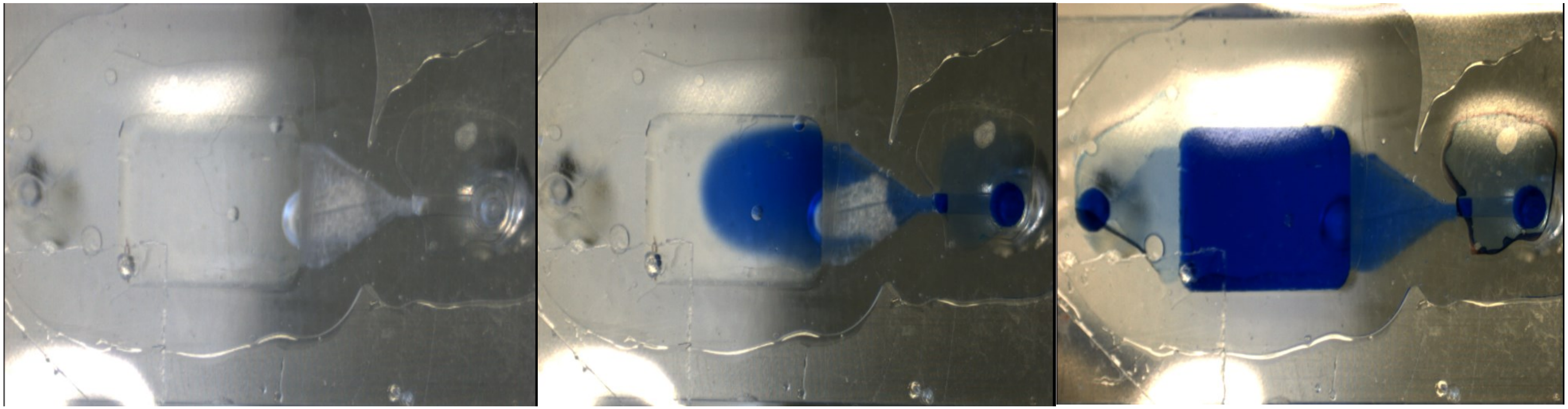
Prototype Chip Testing



- Tape layer prone to leaks
- Pressure build up in cell – seal failure
- Errors in printing process could cause malformed channels/blockages
- More robust cell device needed

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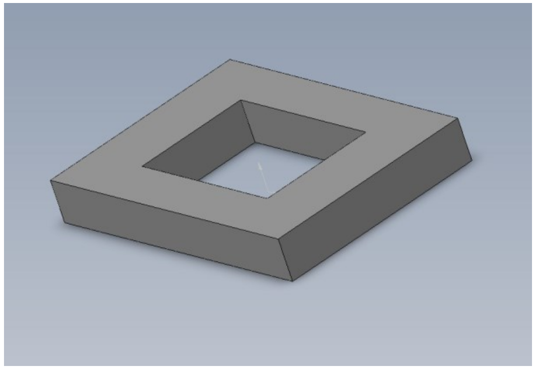
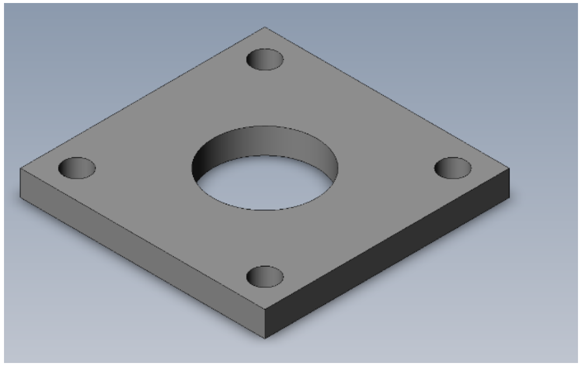
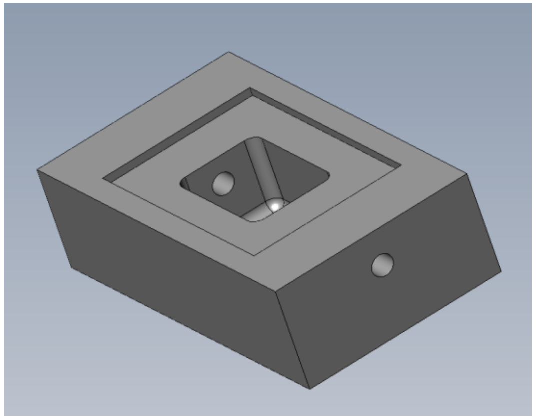
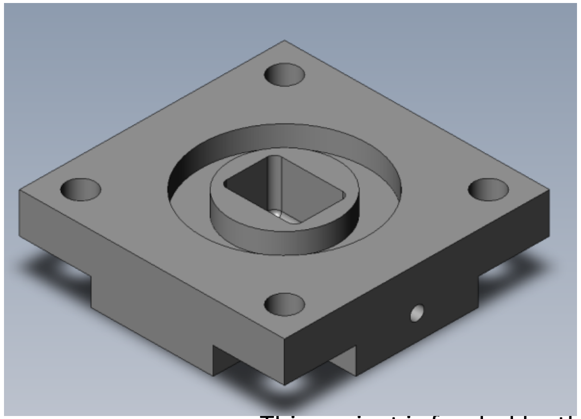
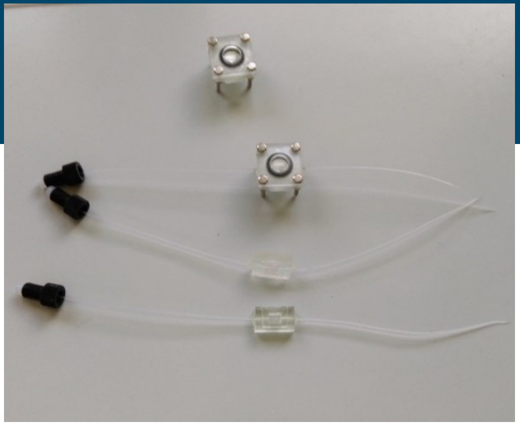
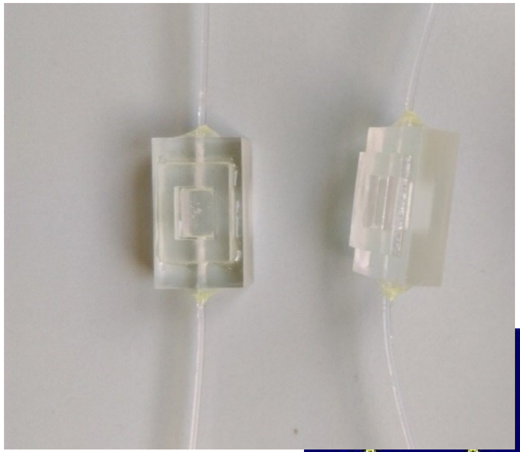
Design evolution

2 chip designs

- Resealable O-ring design
- Sealed sandwich design



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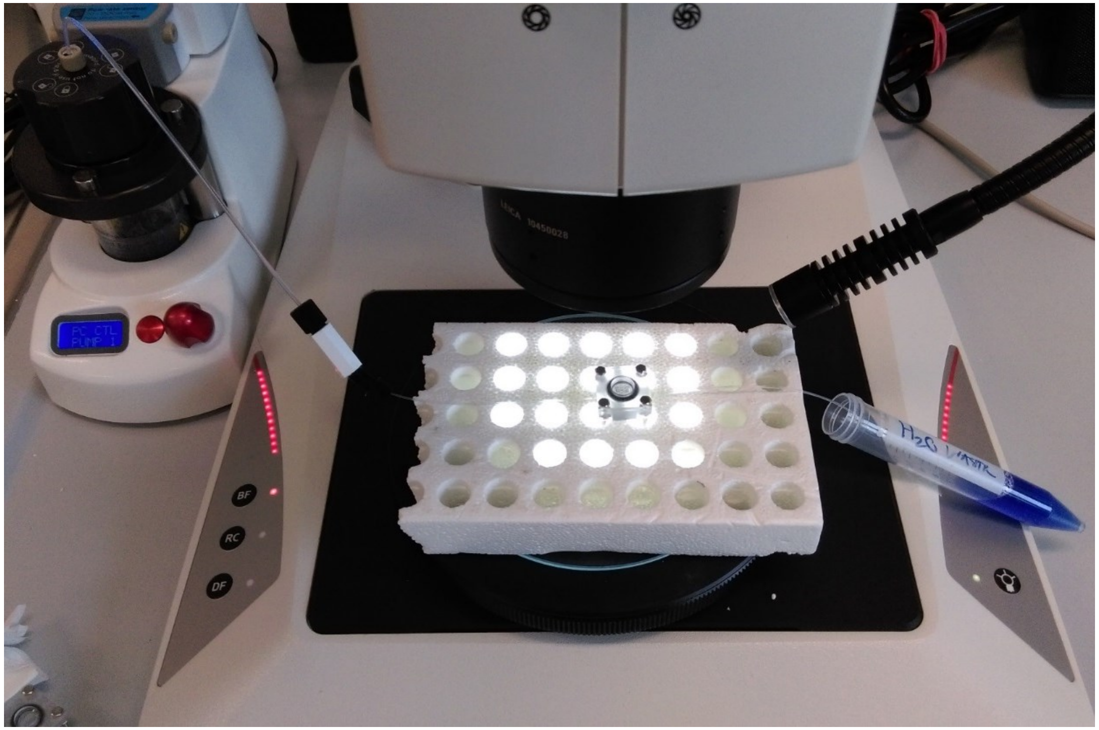
Prototype Chip Testing

Resealable O-ring design



- Double sided crystallisation tape sandwiched between O-ring and top assembly to create seal.
- Fluid flows through cell BUT does not fill cell completely (air gap)
- Difficult to see interior of well over time due to condensation build up on surface of well
- Adjusting tape position, tightening screws (sloping) , lubricating O-ring did not fix this

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- Magnetic ordering of small crystals in a gel matrix

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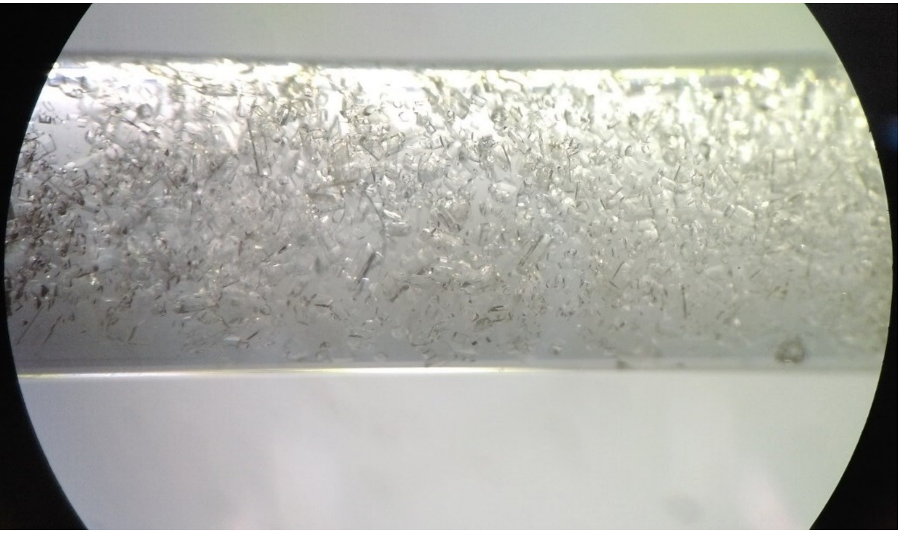
Optimisation

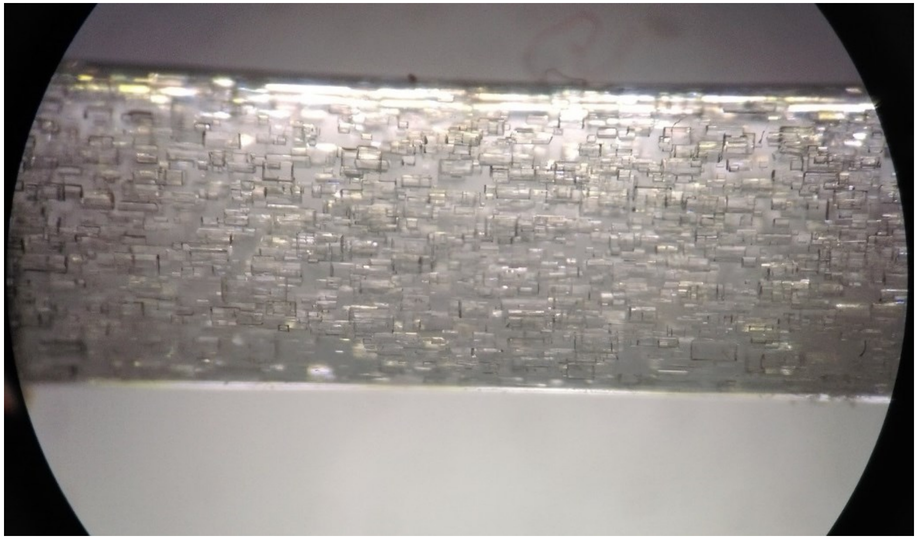
Time in field = 24 Hrs

Control (x3)



- Control sample disordered and random
- Very dense sample

Magnet (x9)



- Sample imaged immediately after taken out of field (above)
- Highly ordered crystals in line with Magnetic field
- No sign of secondary nucleates

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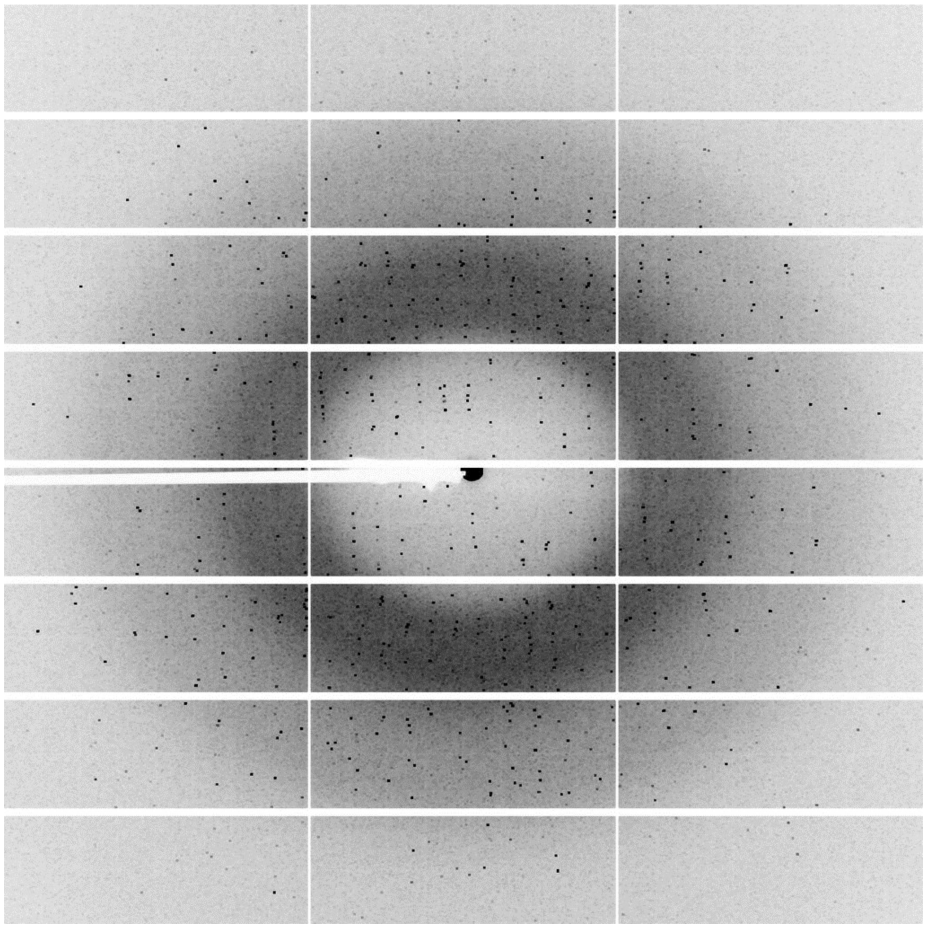
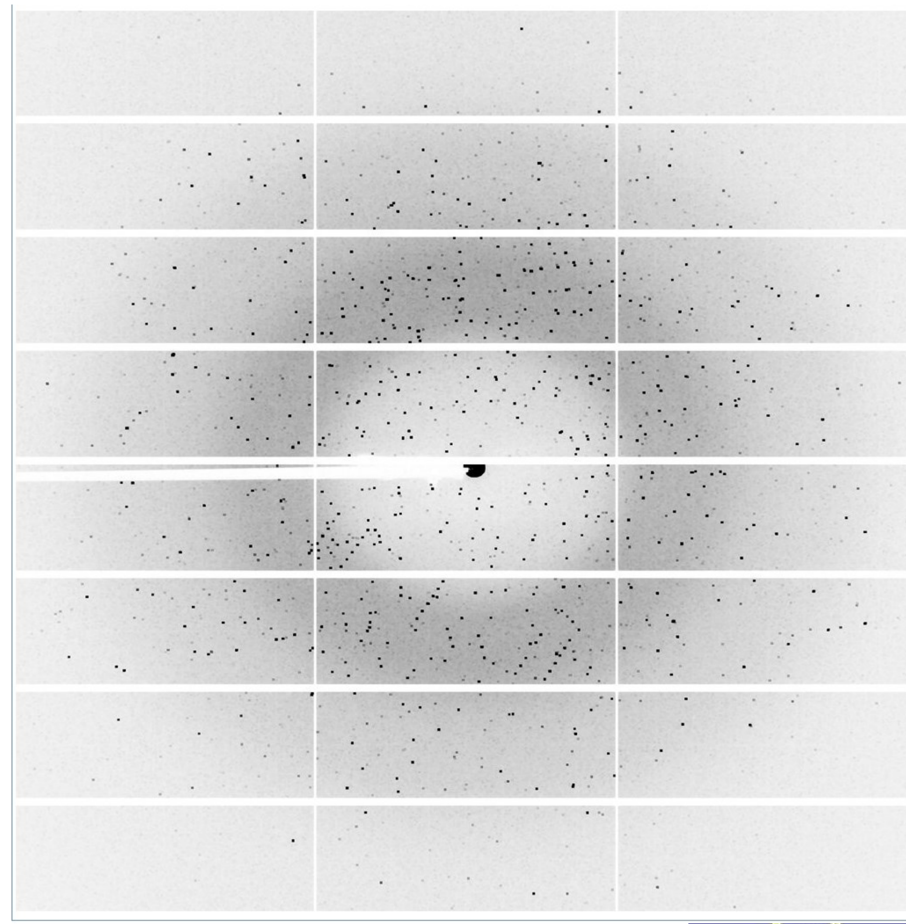
X-Ray Diffraction tests MASSIF-1

Sample



Control



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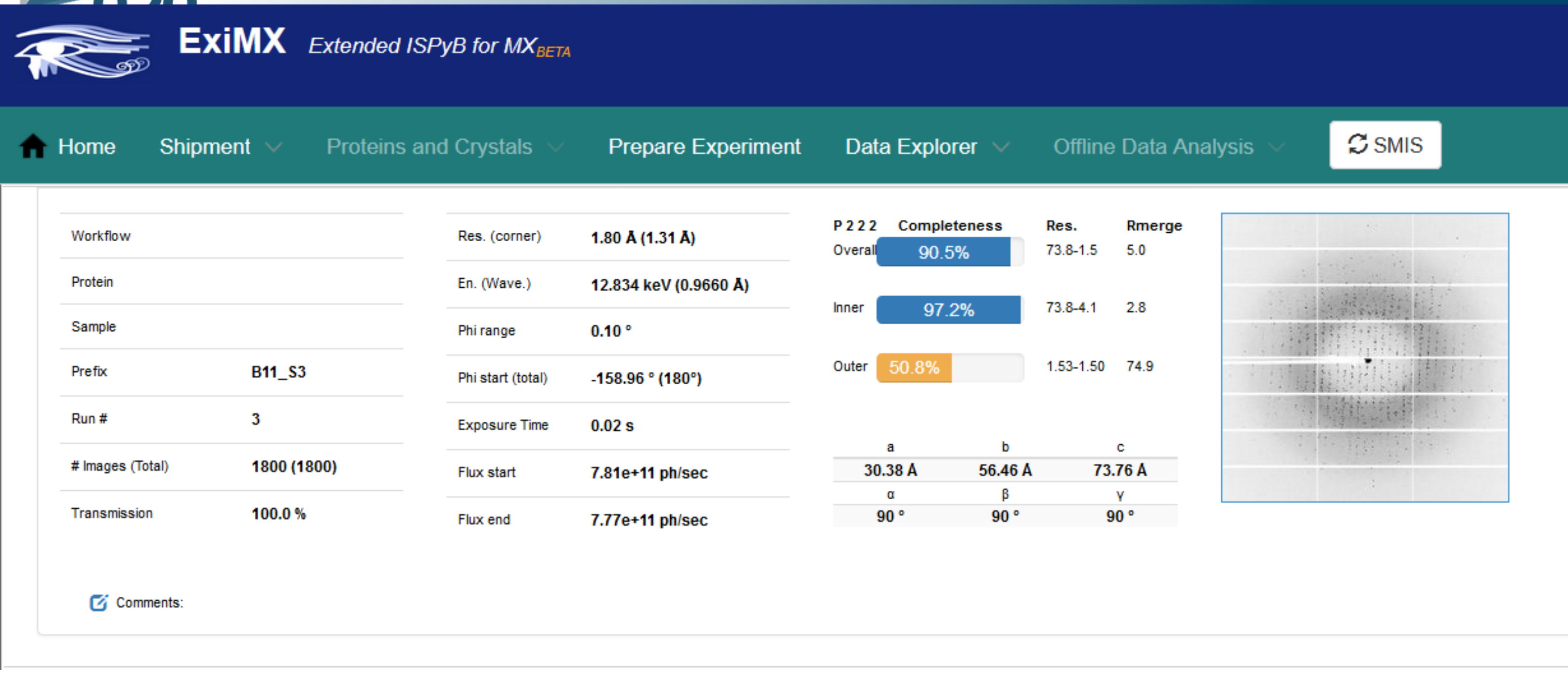
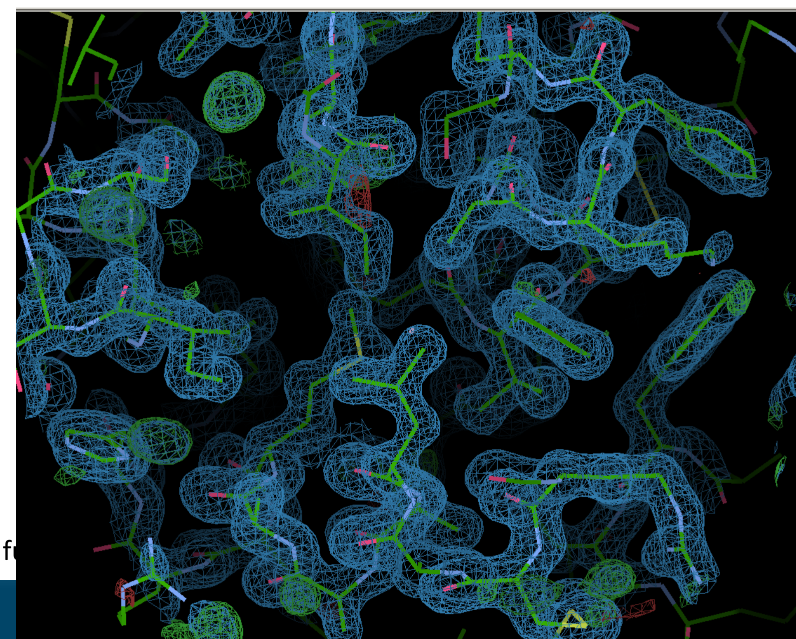
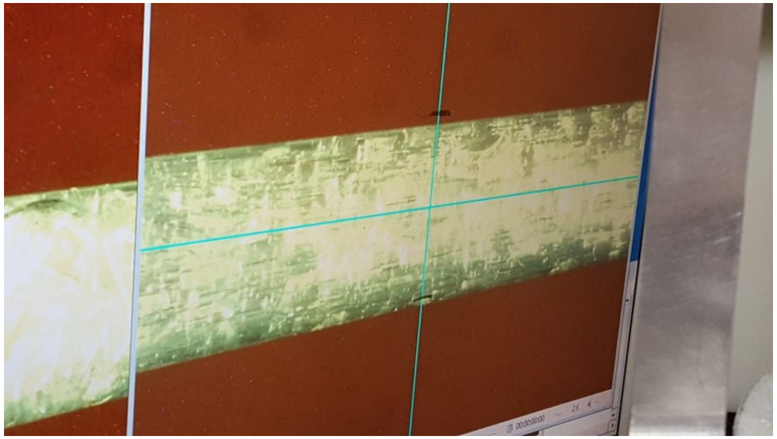
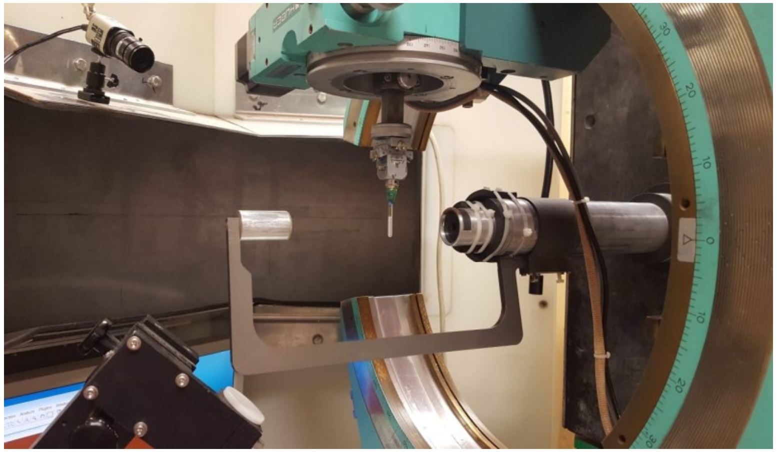
X-Ray Diffraction tests MASSIF-1



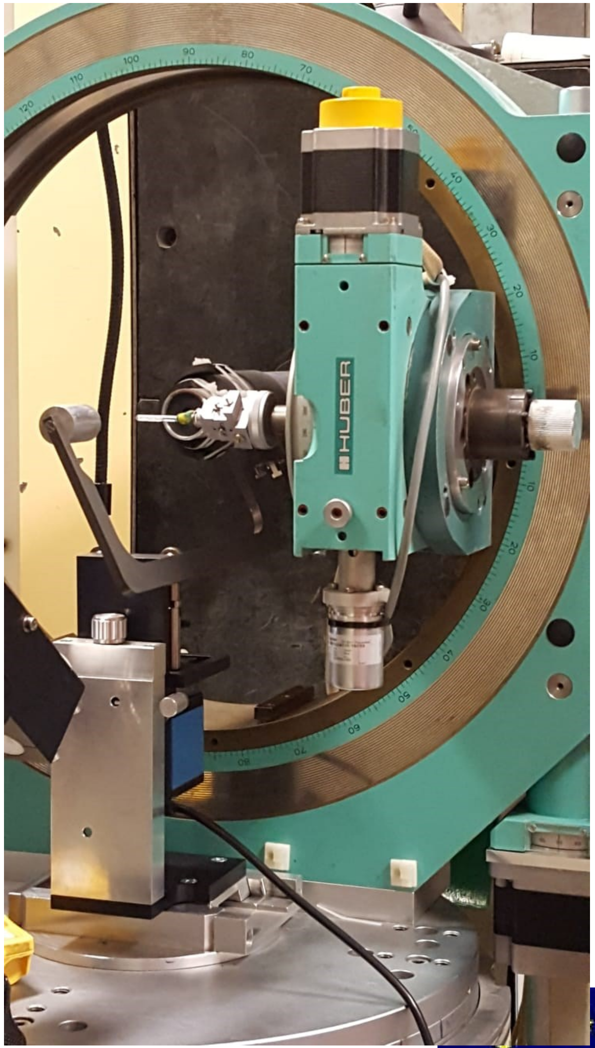


D19 testing





TOA set 2.42Å



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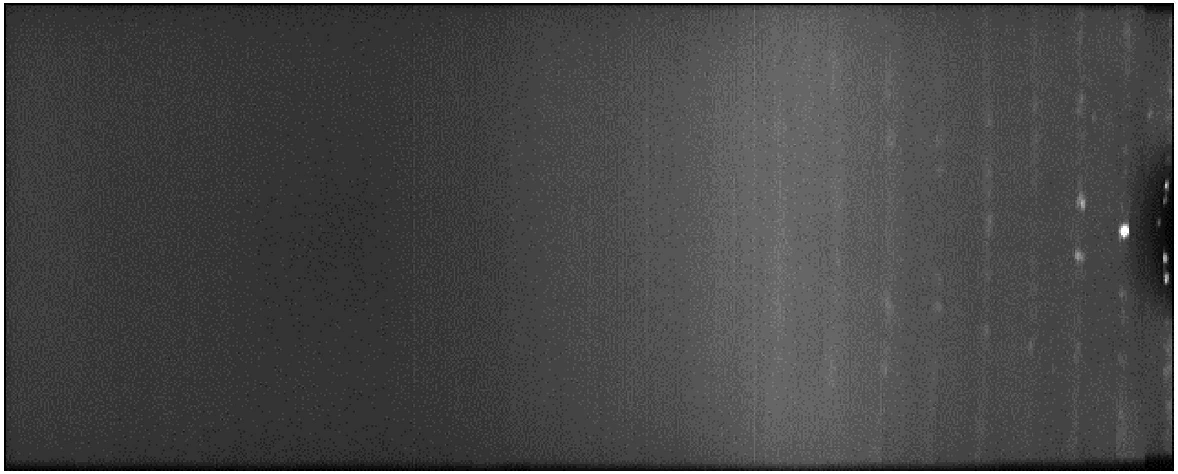
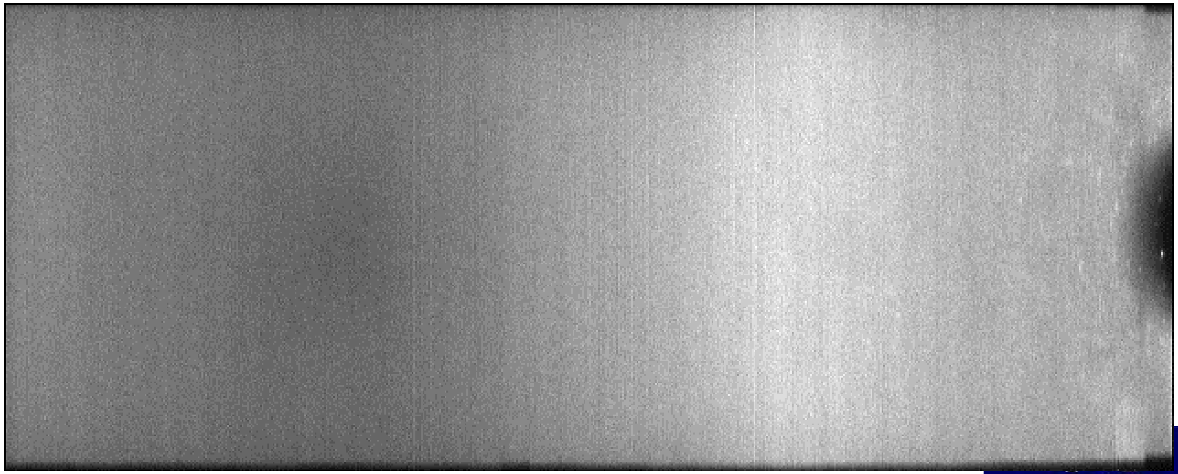
D19 testing

b2s4.
15 min exposure @ chi 90
#155713.



b2c2. Non aligned
15 min exposure @ chi 90
#155716.



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Just a choice of recent publications made with the help of the SINE2020 project…

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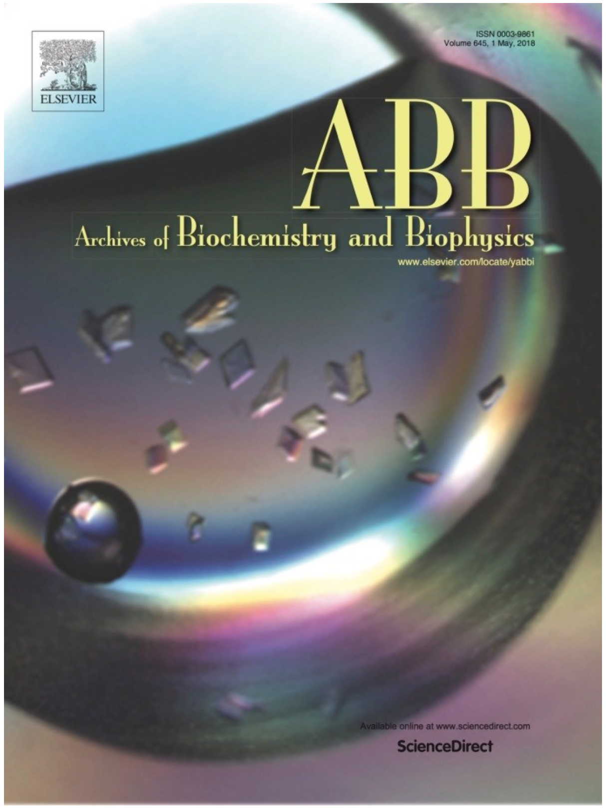

- 65-77% D incorporation when using unlabeled C-source and recycled D_2O
- Good yields of protein, cost effective simple method for production of deuterated proteins for different techniques (crystallization)
- If both fresh D_2O and labeled glycerol is used, the cost increases 4-fold
- Protein solubility 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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CRYSTAL GROWTH & DESIGN

Article

Cite This: Cryst. Growth Des. 2018, 18, 1483−1494

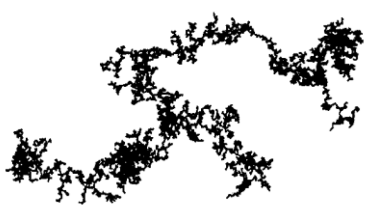
pubs.acs.org/crystal

Crossover from a Linear to a Branched Growth Regime in the Crystallization of Lysozyme

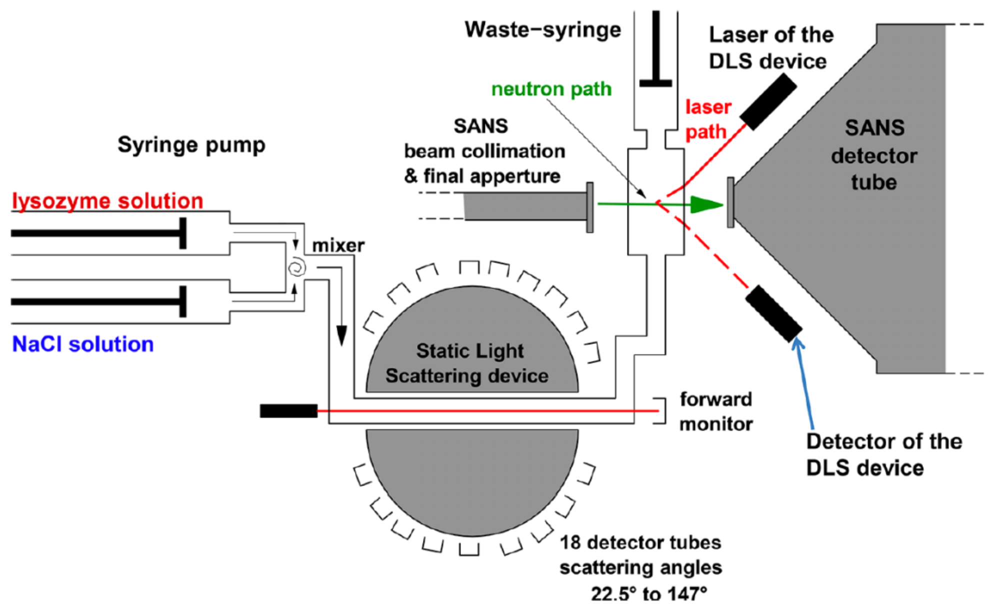
R. J. Heigl,[†] M. Longo,[†] J. Stellbrink,[‡] A. Radulescu,[†] R. Schweins,[§] and T. E. Schrader*,[†]

DLCA (Diffusion Limited Cluster-Cluster Aggregation)

$$E_B \ll K_B T$$



DIFFUSION-LIMITED Cl-Cl-3d
M=10,732



Every collision results in aggregation

This project is funded by the European Union (GA no. 654000)

Summary:

- Many model systems tested and used for testing the respective methods
- challenging proteins to crystallize identified and partly successfully crystallized to yield large crystals
- Using partially deuterated proteins increases protein yield at the expression step, so more protein is available to crystallize
- Using (per-)deuterated proteins has effect on crystallization conditions
- Microseeding helps to explore phase diagrams
- Microseeding helps to decouple nucleation from crystal growth
- Crystallization apparatuses will allow feeding of protein sollution to a growing crystal without the need of transferring the crystal.

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Next steps

- Prepare more deuterated proteins and investigate their crystal growth for future neutron beam times this year for testing
- submit and write manuscripts…
- Further development of the crystallization apparatuses
- Further studies on magnet ordering of protein crystals

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