FONDAZIONE EL.B.A. - NICOLINI
Electronics Biotechnology Advanced

The role of LB thin protein film in protein crystal growth by LB nanotemlate and robot

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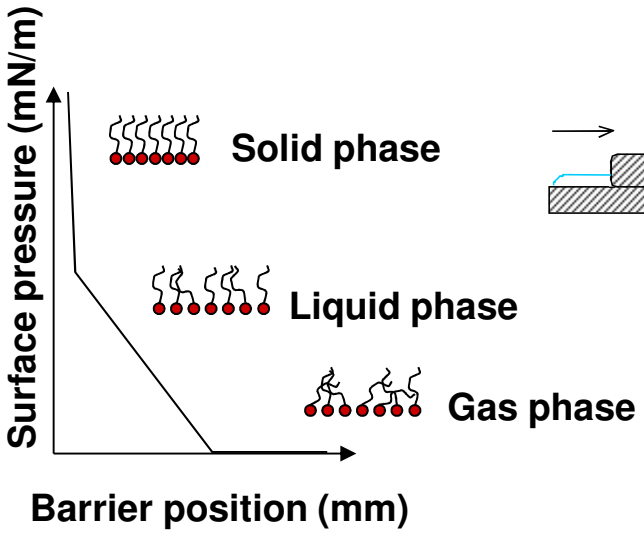
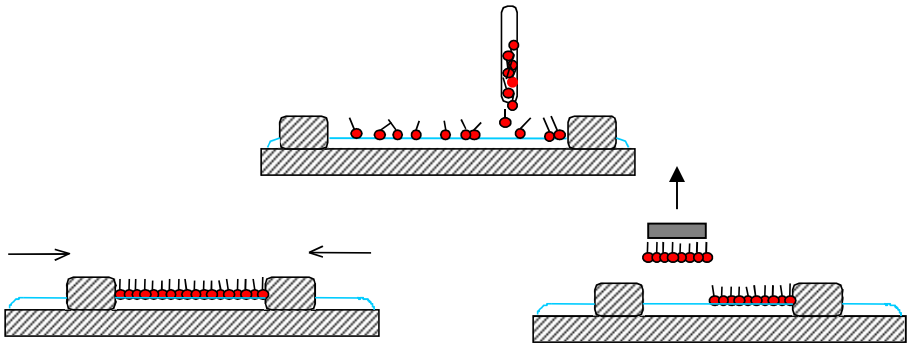
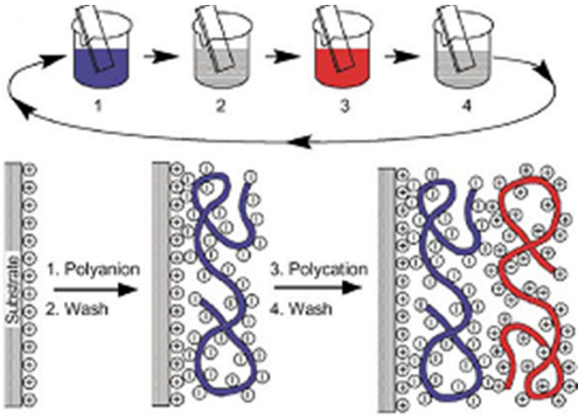
Thin Film Technologies

Langmuir-Blodgett

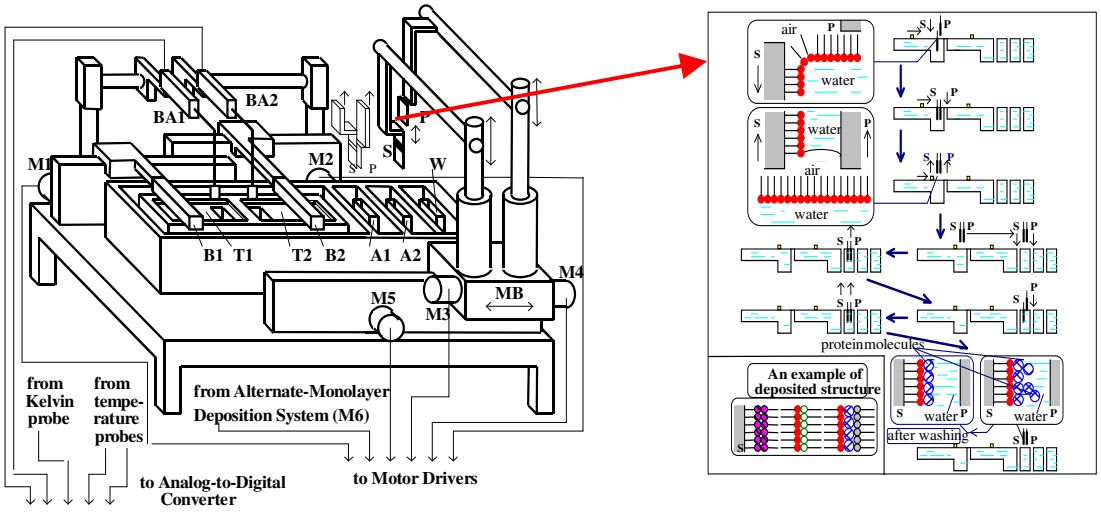




Layer-by-Layer self assembly

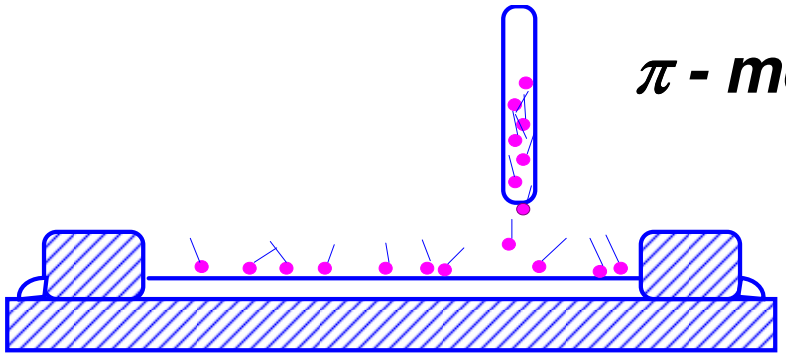


Protective plate



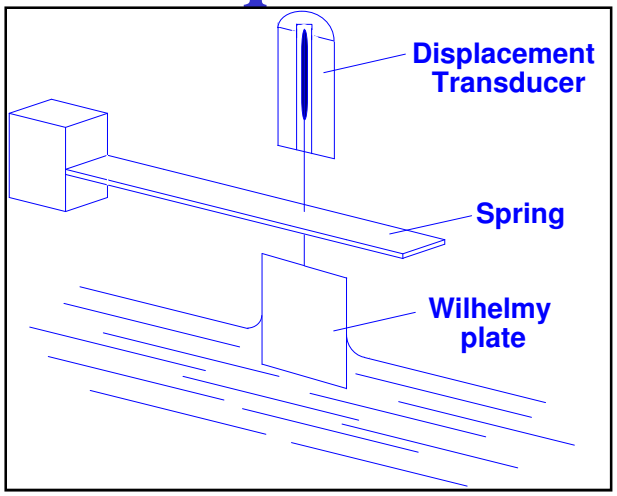
M1-M6 - stepper motors; BA1, BA2 - Wilhelmy balances; B1,B2 - barriers; T1,T2 – Langmuir troughs; A1, A2 - compartments for adsorption; W - compartment for washing; S - substrate; MB-mobile block

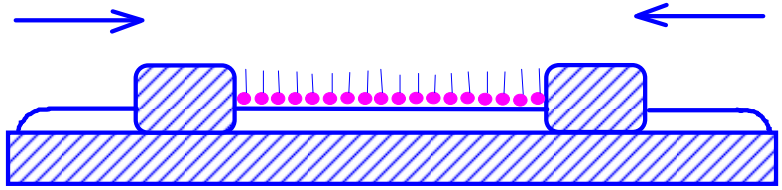
The Langmuir-Blodgett Technique



Spreading

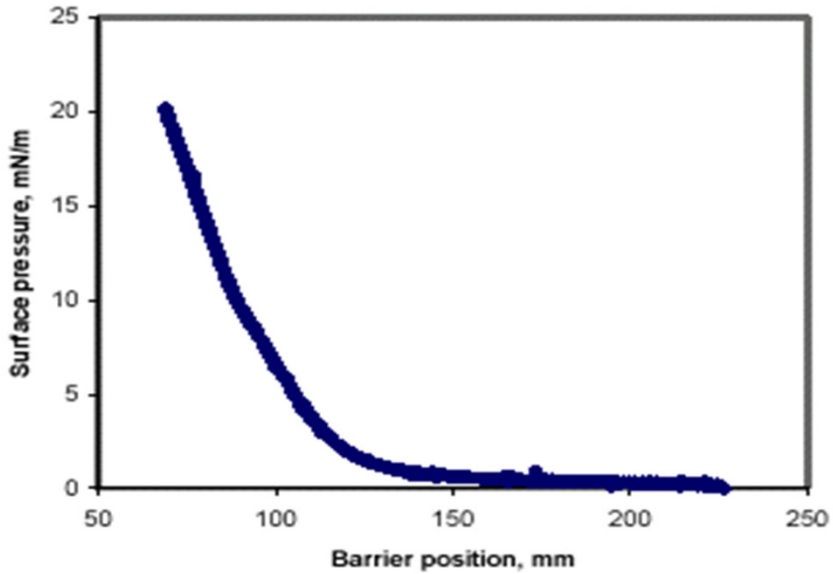
π - measurements



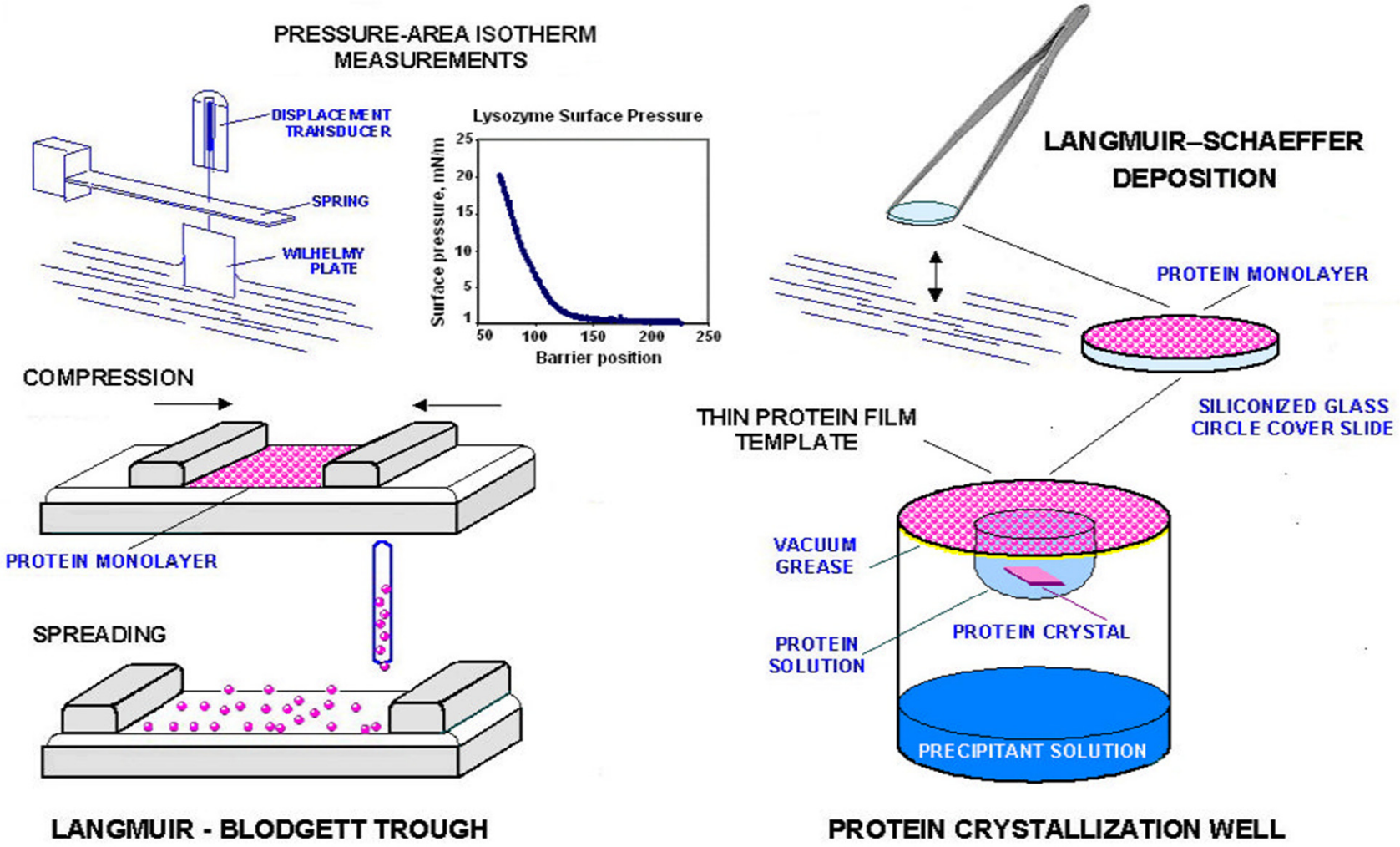


Compression

π - isotherm

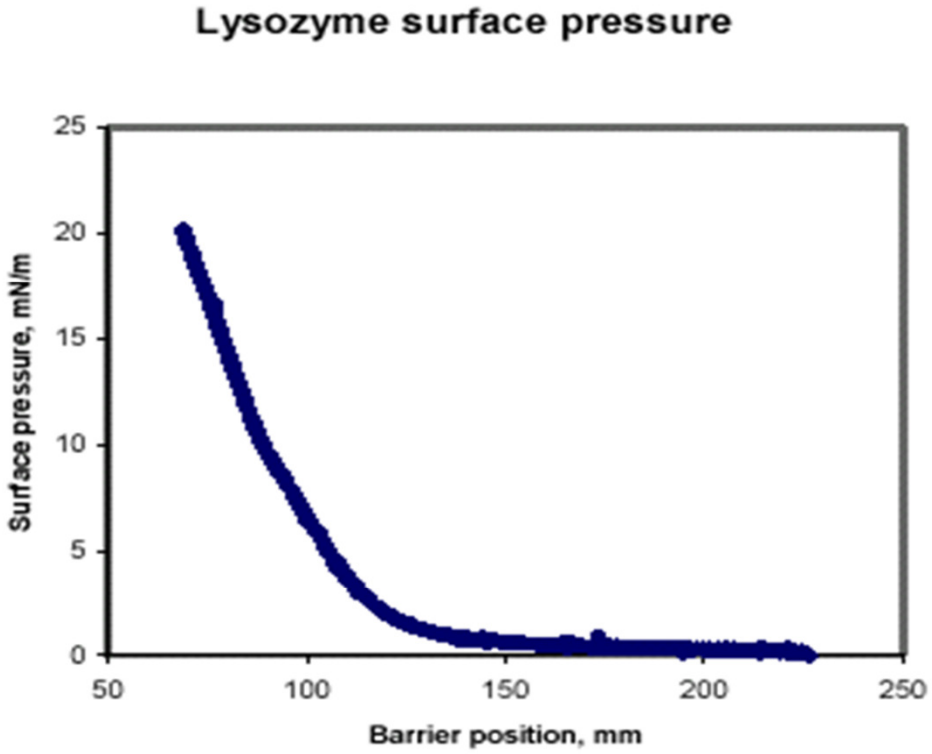


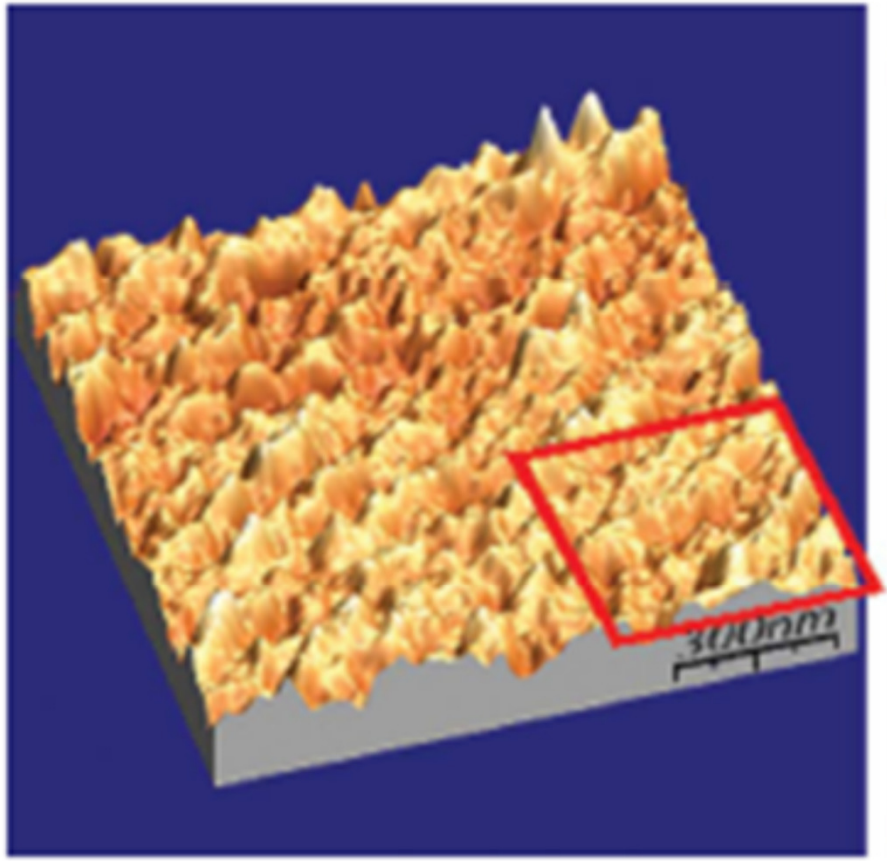
LB nanotemplate protein crystallization method



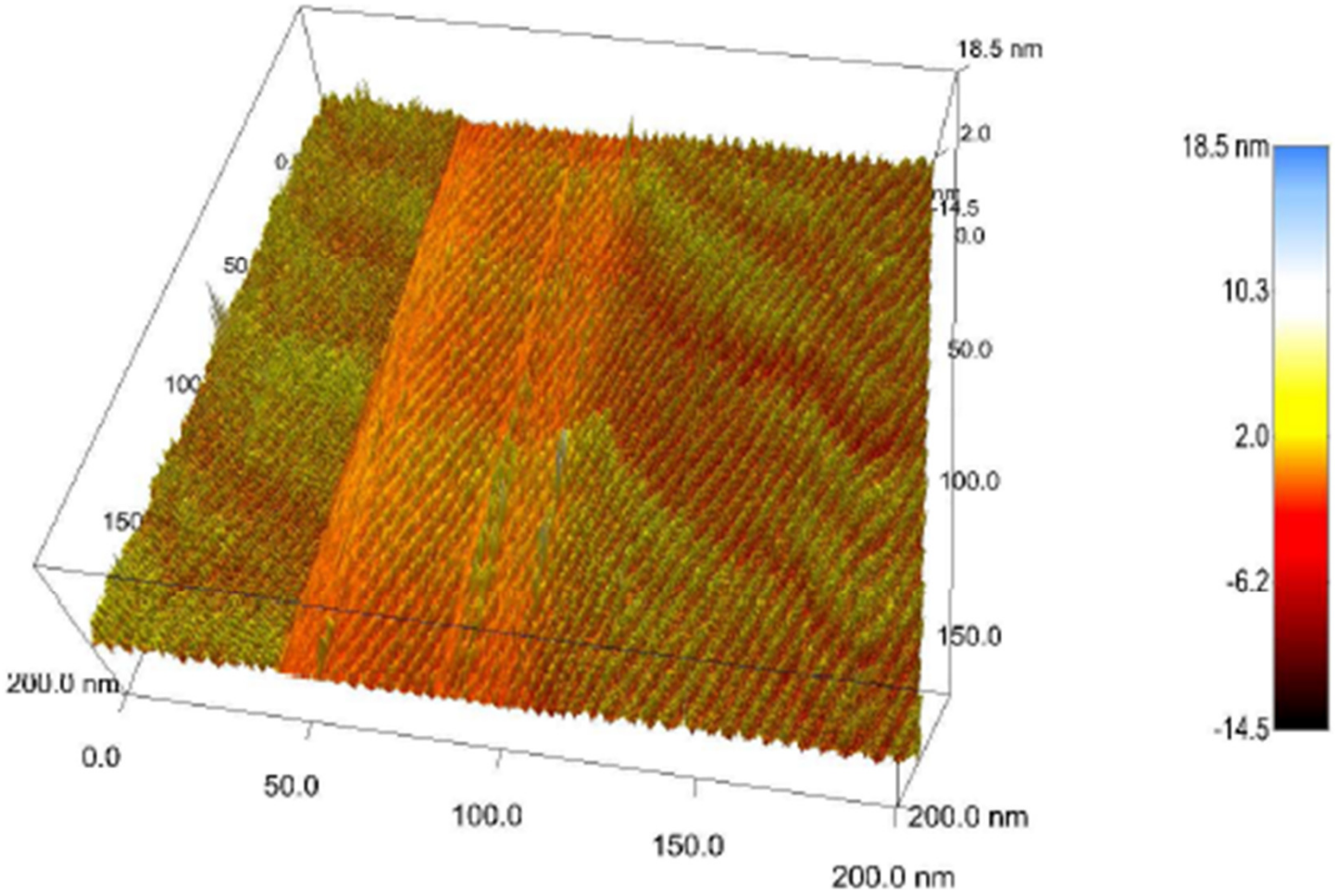
Pechkova E, Nicolini C., *Protein nanocrystallography: a new approach to structural proteomics*, Trends in Biotechnology 22, 117-122, 2004.

Isotherm and Atomic Force Microscopy of Lysozyme Langmuir-Blodgett film.



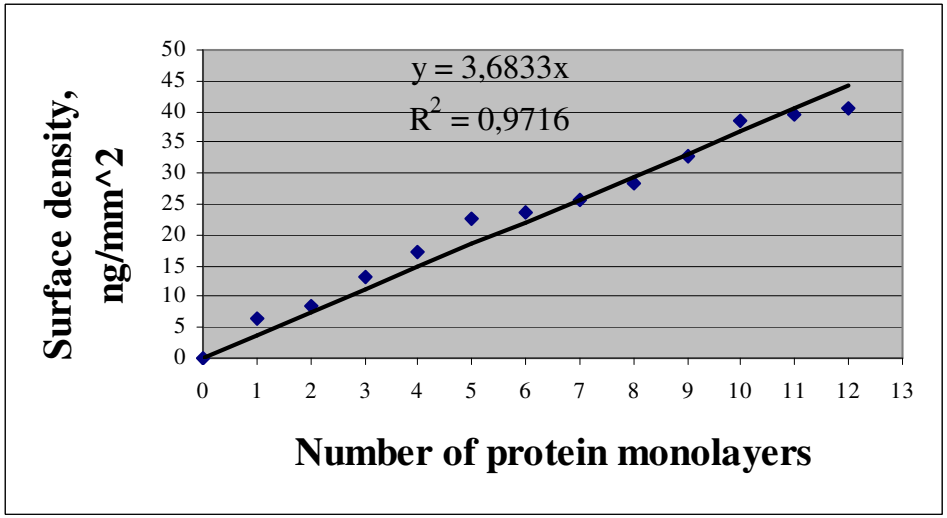


AFM images (in air):
LB film (2 monolayers) of thaumatin (24 kDa) at 200 nm scale

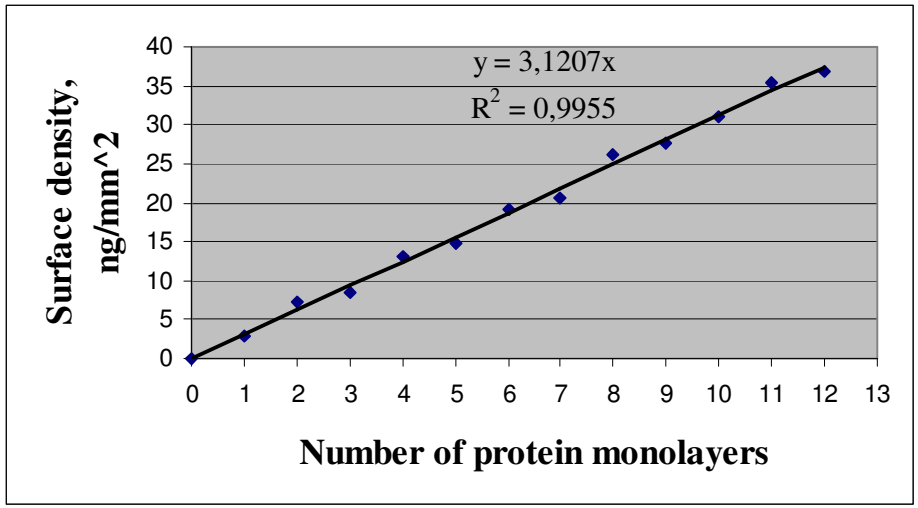


Nanogravimetric measuarments

Protein surface density



Human protein kinase CK2α
(MW 45 kDa)



Cytochrome P450 scc
(MW 56 kDa)

Film packing (area per molecule)

20,36 nm²	**Experimental**	29,52 nm²
17,8 nm²	**Close parking system**	30 nm²

Test proteins for classical vs LB nanotemplate crystallization

Lysozyme
Chicken Egg White *MW≅14KDa*

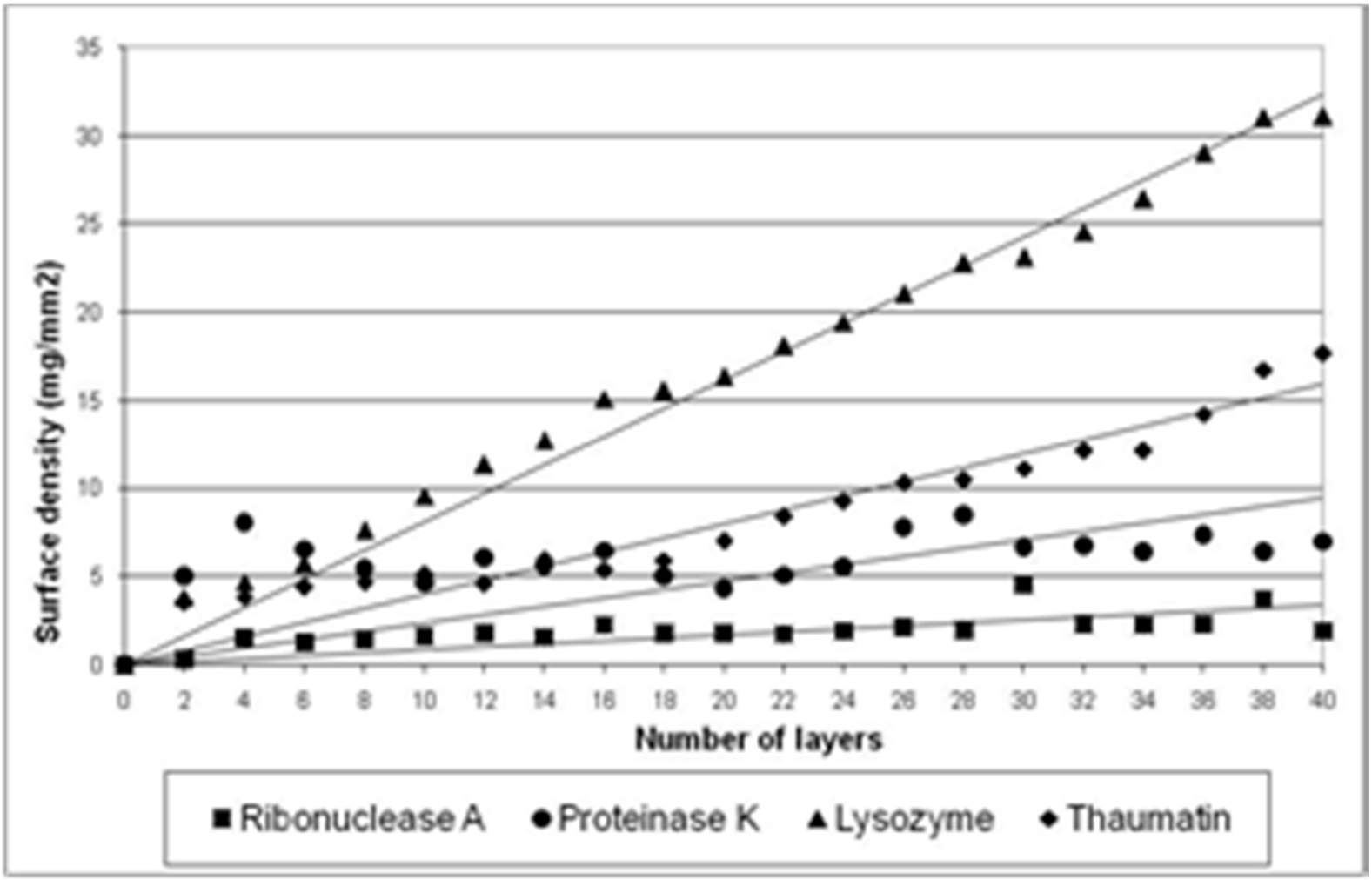
Thermolysin
Bacillus thermoproteolyticus *MW≅35KDa*

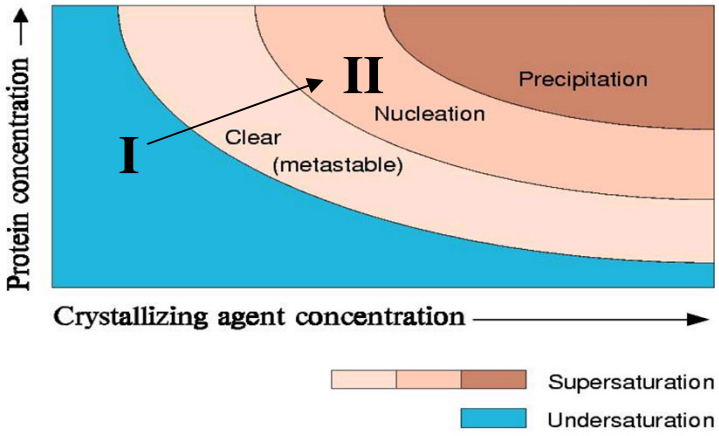
Thaumatin
Thaumatococcus Daniellii *MW≅22KDa*

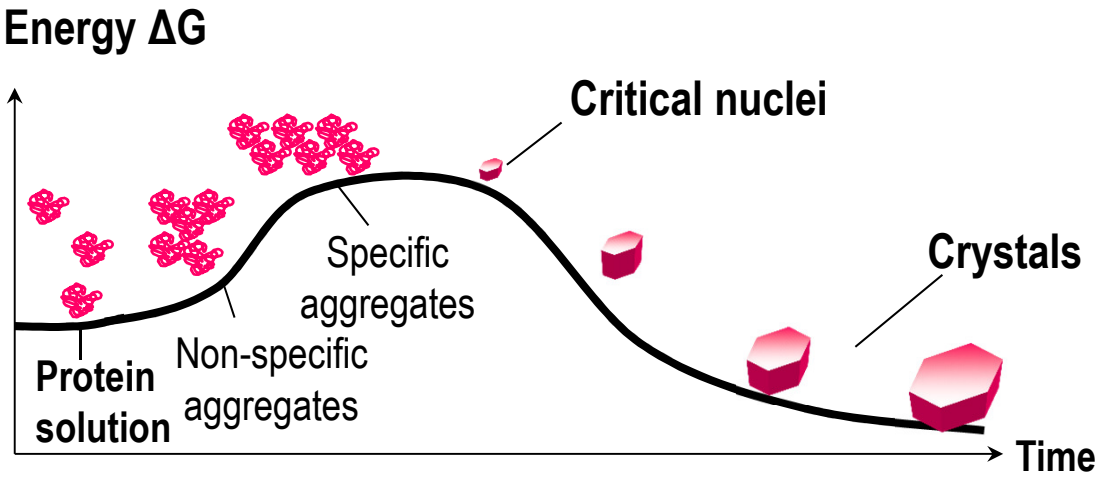
Ribonuclease A
Bovine Pancreas *MW≅14KDa*

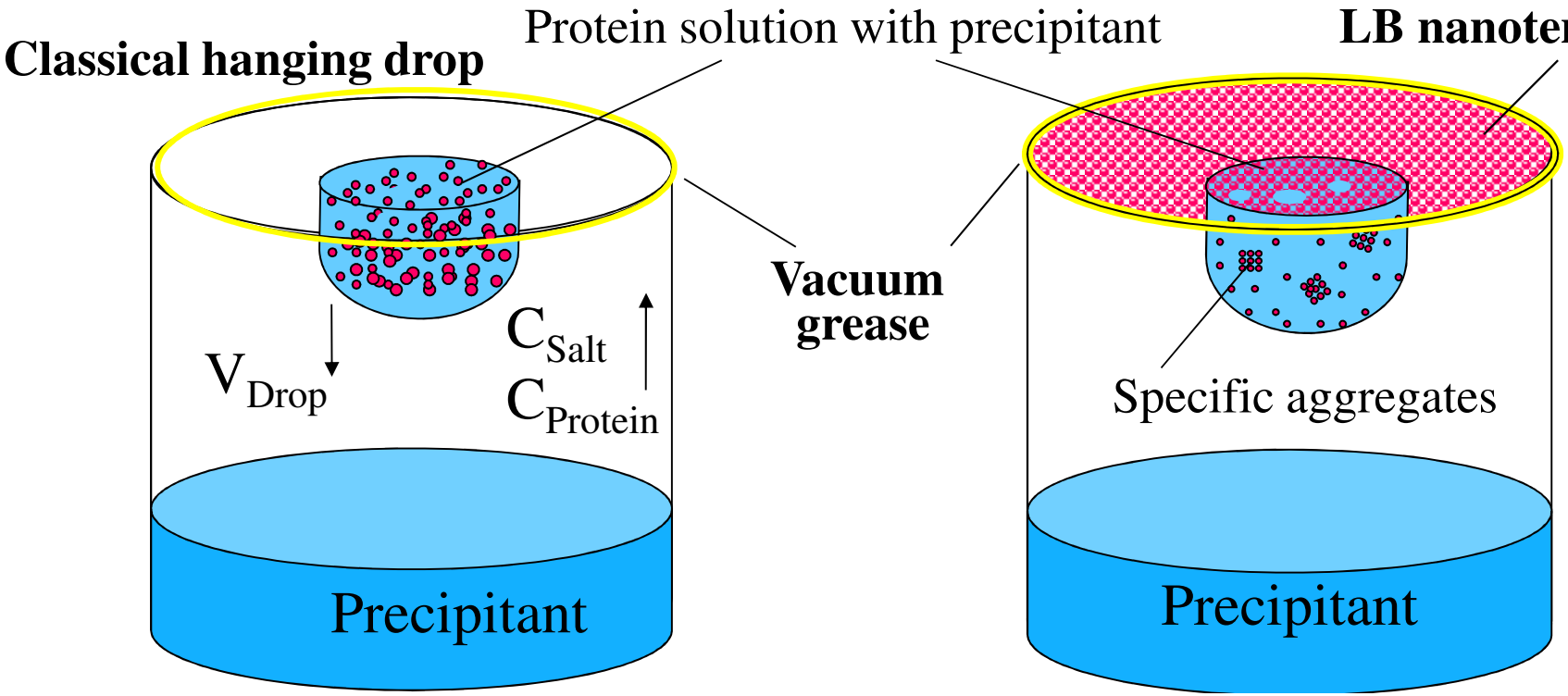
Proteinase K
Tritirachium album *MW≅29KDa*

Dependence of the surface density of deposited ribonuclease A, thaumatin, proteinase K and lysozyme nanostructured films upon the number of transferred protein mono-layers, at 20 mN/m of pressure.







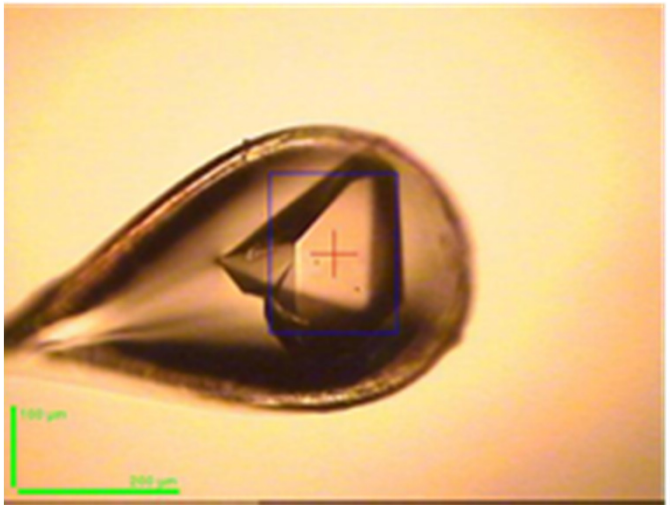


Pechkova E, Nicolini C, From art to science in protein crystallization by means of thin film technology, **Nanotechnology 13**, 460-464, 2002.

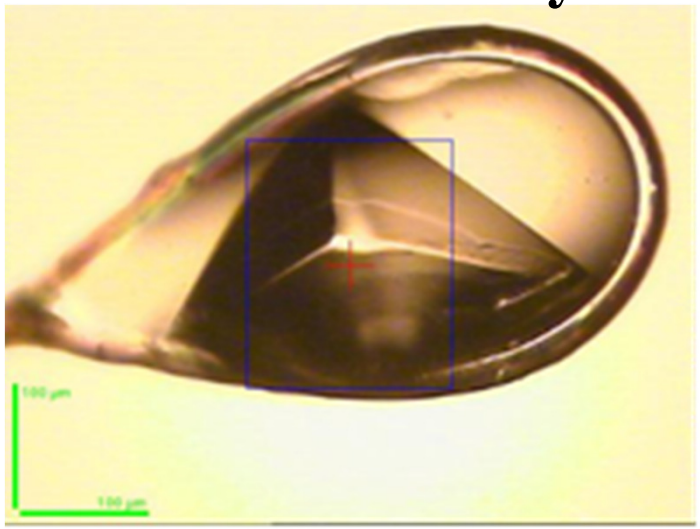
Protein crystallization conditions used for both LB and classical hanging drop vapour diffusion method. The cryo-protectant used during the X ray diffraction data collection is also indicated

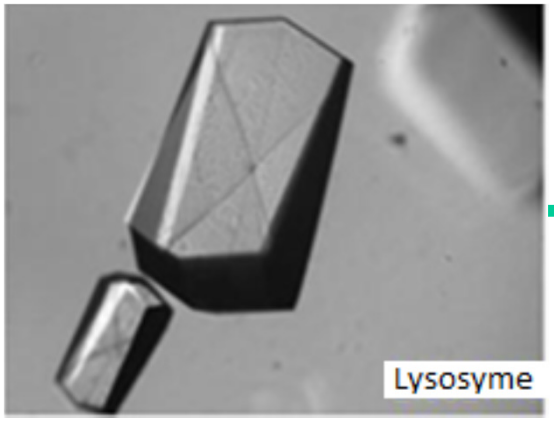
Protein	*Protein solution*	*Reservoir solution*	*Drop*	*Cryoprotectant*
Proteinase K	20 mg/ml in 25 mM HEPES buffer pH 7	400 mM Na/K-tartrate in 25 mM HEPES buffer pH 7	1:1	4M TMAO
Ribonuclease A	10 mg/ml in 50 mM Na-acetate pH 5.5	1.75 M $(NH_4)_2SO_4$ 2M NaCl in 100mM Na-acetate pH 5.5	1:1	Mother liquid
Thaumatin	15 mg/ml in 100 mM ADA buffer pH 6.5	1 M Na/K in 100 mM ADA buffer tartrate pH 6.5	1:1	30% Glycerol, 0.7M Na/K in 100 mM ADA buffer tartrate pH 6.5
Thermolysin	100 mg/ml in 50 Mm MES pH 6.0	35% saturated $(NH_4)_2SO_4$	1:1 with 1M NaCl in 50mM MES pH 6.0	Dry Paraton-N (0.5h in vacuum centrifuge)
Insulin (Zn-free)	18 mg/ml in 50 mM Na_2HPO_4 pH 10.4 1 mM EDTA	400 mM Na_2HPO_4 pH 10.4 10 mM EDTA	1:1	30% Glycerol in400 mM Na_2HPO_4 pH 10.4 10 mM EDTA
Lysozyme	40 mg/ml in 50 mM NaAc buffer pH 4.5	0.9 M NaCl	1:1	20% Glycerol in 0.9 M NaCl

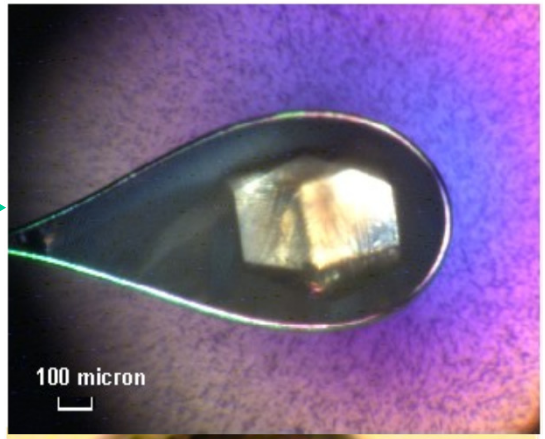
Classical Proteinase K crystal

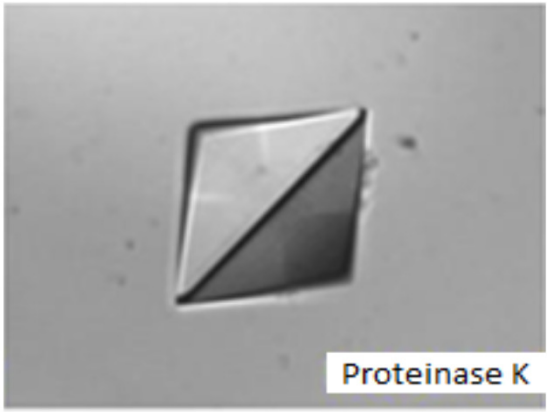


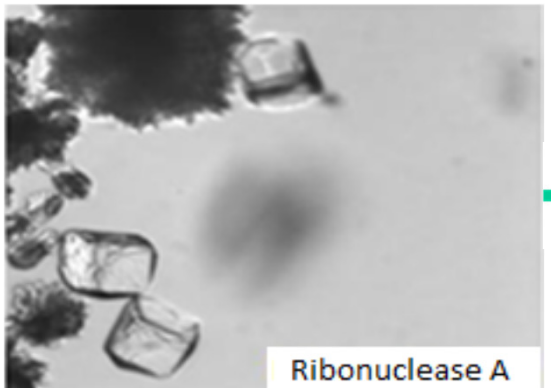
LB Proteinase K crystal

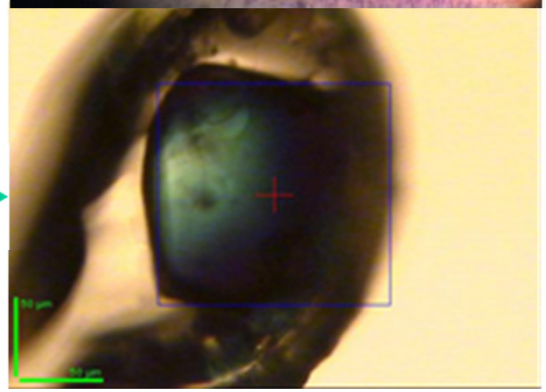


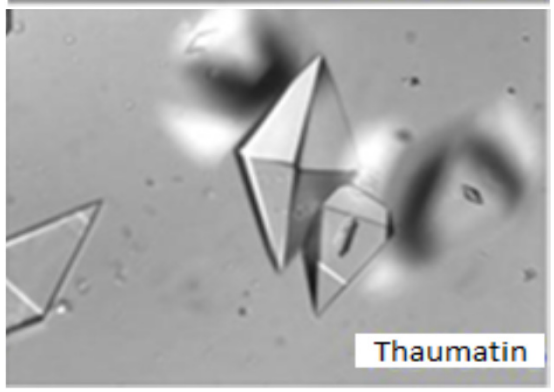




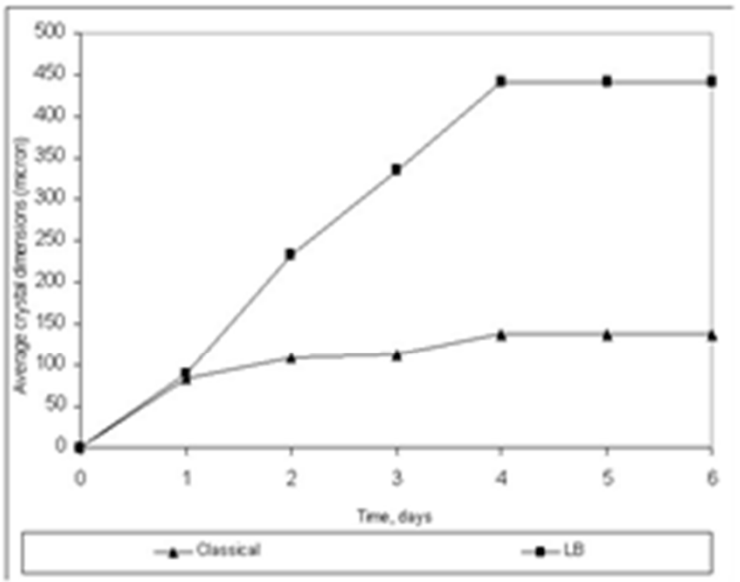




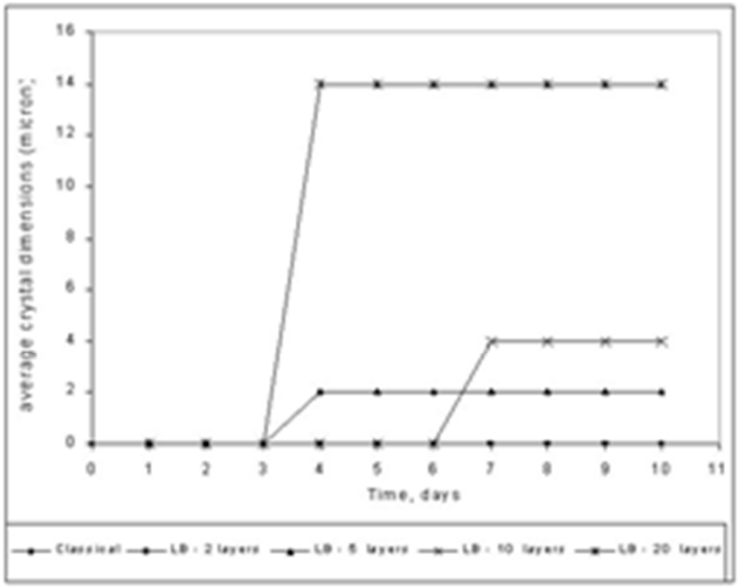




Screening test for thaumatin “critical” concentration. A: screening test for ribonuclease A “critical” concentration. B: screening test for proteinase K “critical” concentration

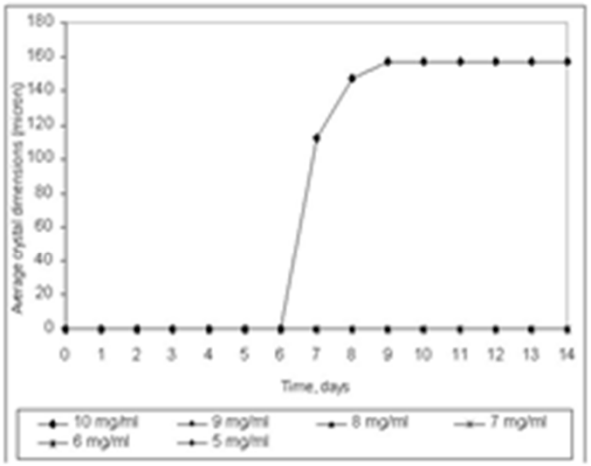


A

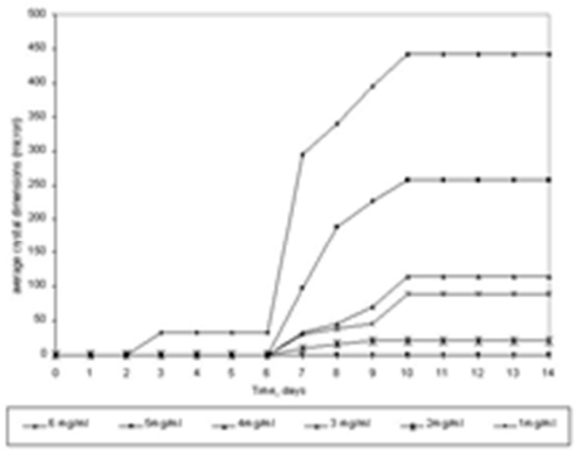


B

A: critical concentration for proteinase K with classical vapor diffusion method. B: critical concentration for thaumatin with classical vapor diffusion method. C: critical concentration for ribonuclease A with classical vapor diffusion method.



A



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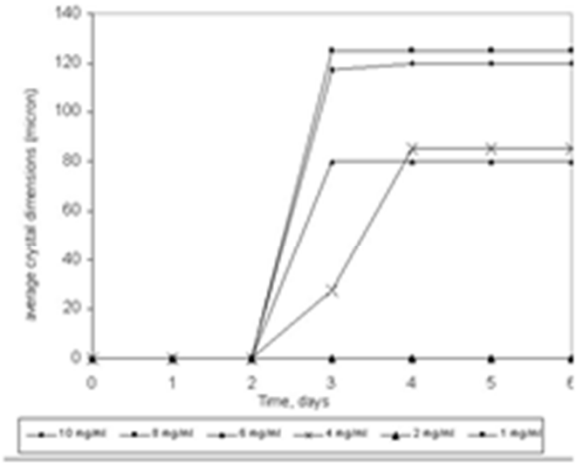
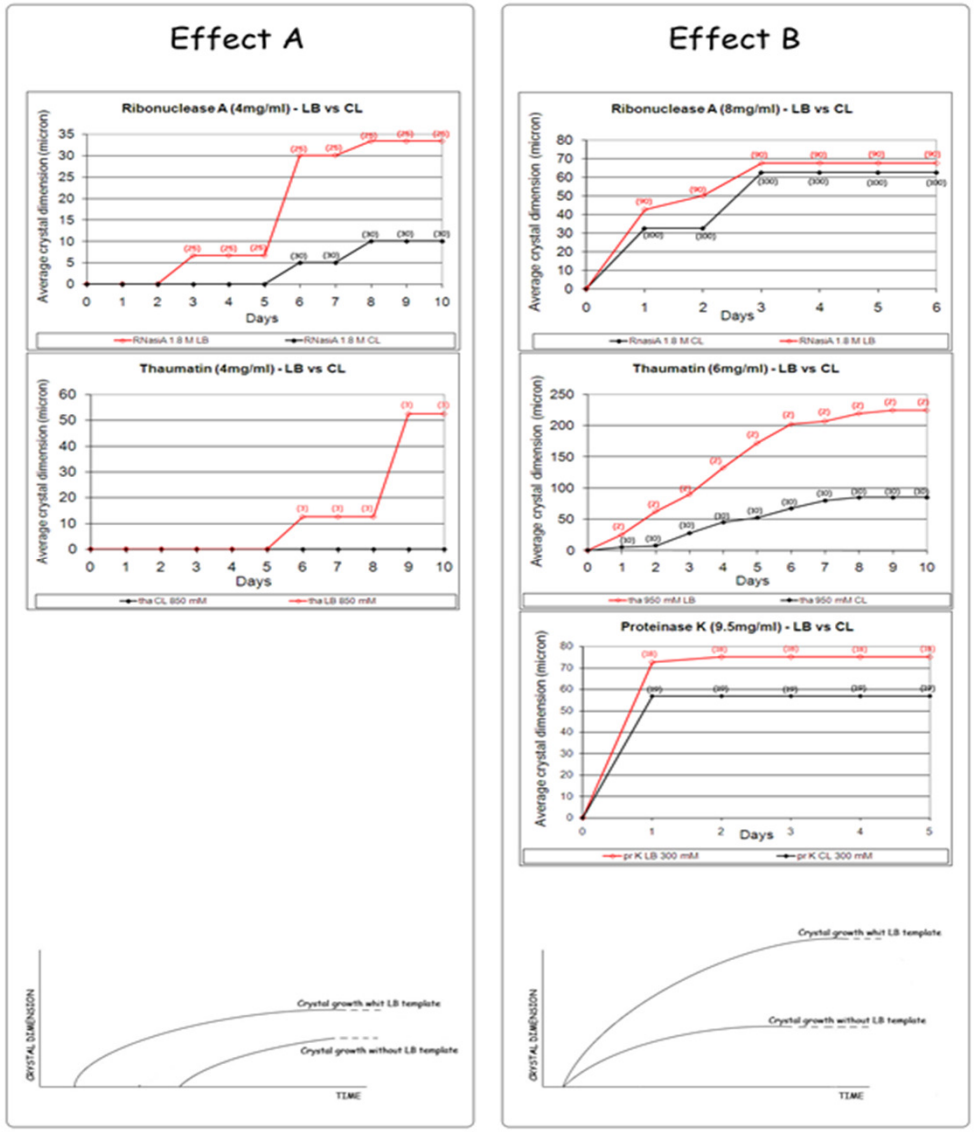


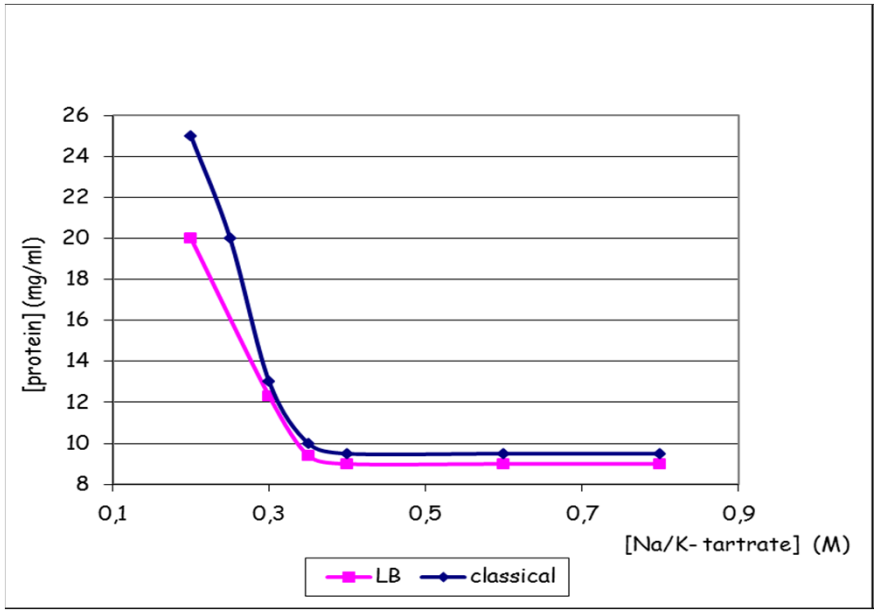
C

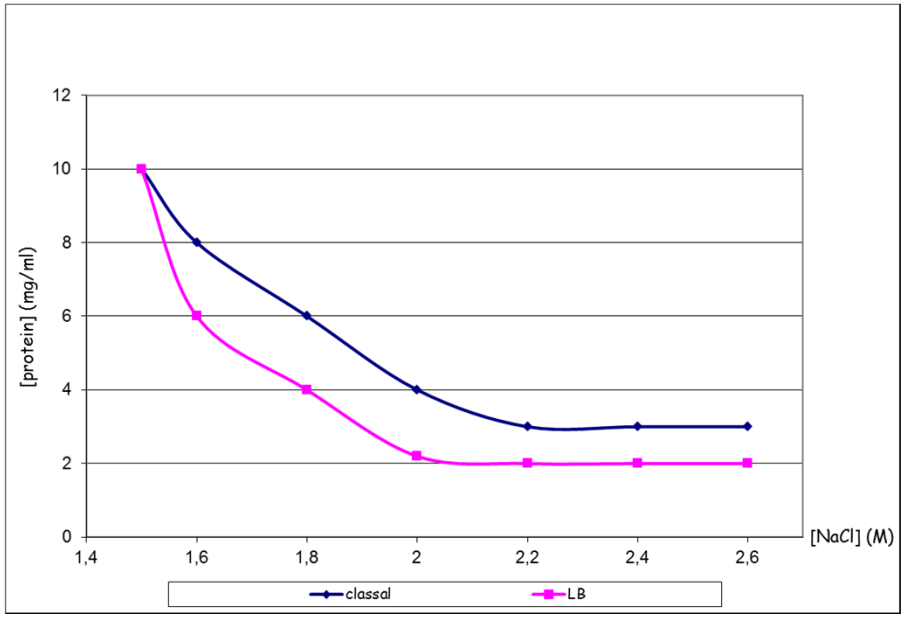
RESULTS	**Standard protocol concentration**	**Critical concentration**
Lysozyme	40 mg/ml	5 mg/ml
Proteinasi K	20 mg/ml	9.5 mg/ml
Thaumatin	15 mg/ml	2 mg/ml
Ribonucleasi A	10 mg/ml	4 mg/ml

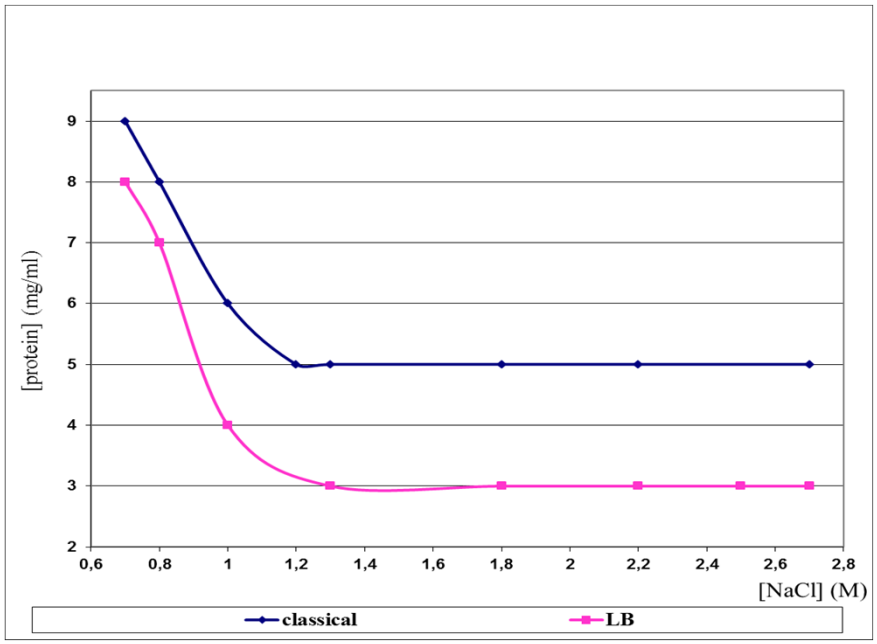
Effects of LB template in crystal growth (in brackets number of crystals), Effect A: template facilitates nucleation step. LB crystals grow earlier than classical, Effect B: template facilitates crystal growth. LB crystals grow larger than classical.

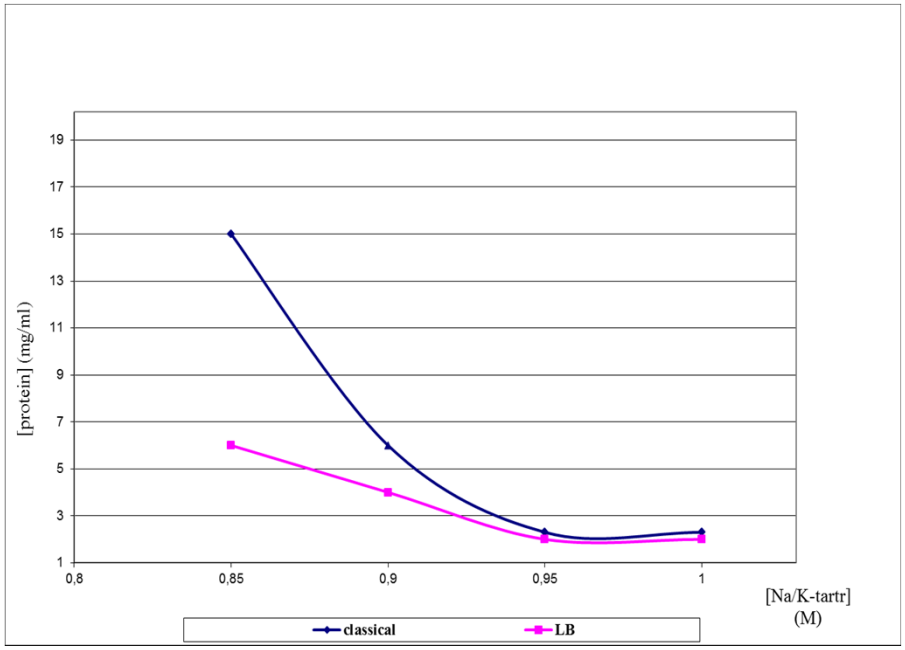


Phase Diagrammes of Proteinase K, Ribonuclease K, Thaumatin and Lysozyme (clockvise)

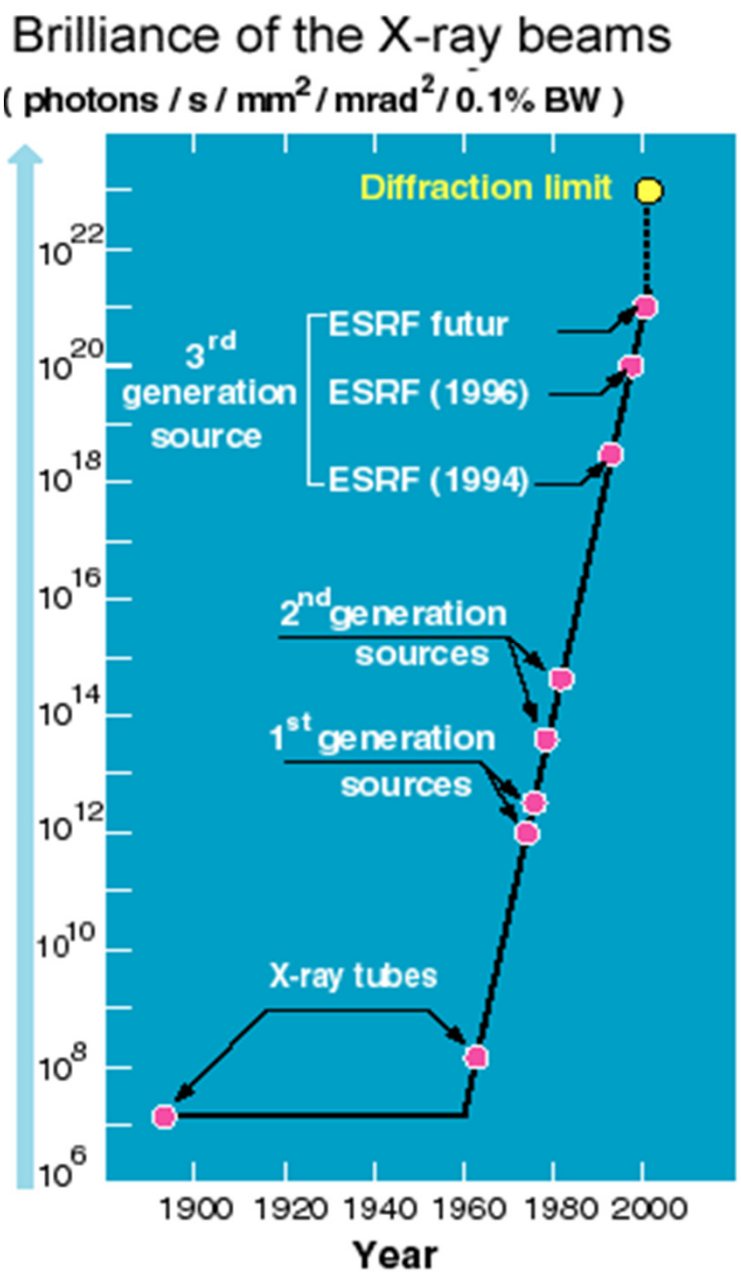








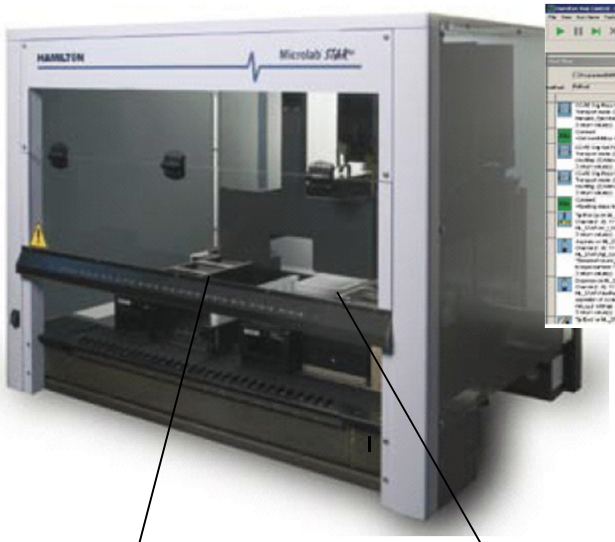
European Synchrotron Radiation Facility
(ESRF), Grenoble, France

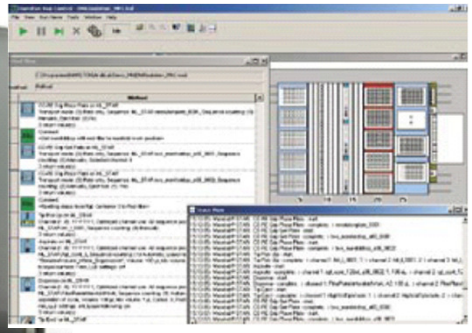


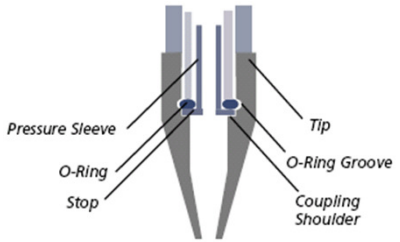
LB and classical protein crystals analyzed at the different ESRF beamlines.

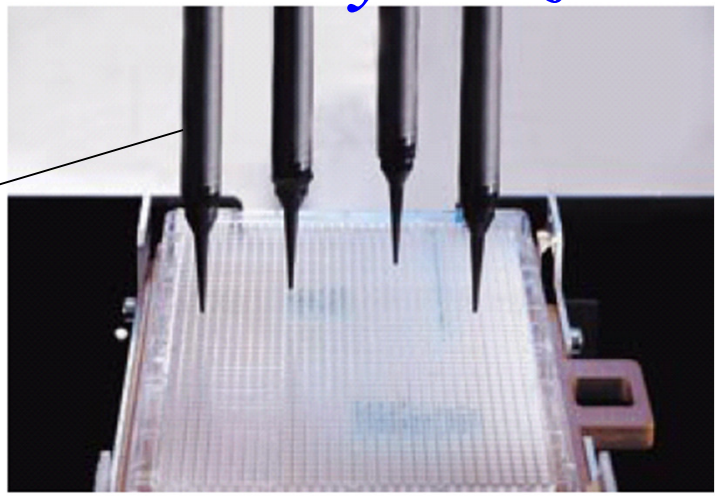
Beamline at ESRF	*Protein crystal and method Of preparation*	*Number of crystal being studied*	*Crystal size (μm)*	*Beam size (μm)*	*Total radiation dose (MGy)*
ID13	Lysozyme classical	2	400×200×100	20×20	10 (500s exposure)
ID13	Lysozyme LB	2	750×375×190	20×20	100 (4800s exposure)
	Lysozyme LB	2	Less than 1 μm	0.5×0.5	
ID29	Thaumatin classical	1	100×200×150	100×100	9.5
ID29	Thaumatin LB	3	100×300×150	100×100	9.5
ID23–1	Proteinase K classical	1	200×100×150	80×60	44,1
ID23–1	Proteinase K LB	1	200×100×150	80×60	44,1
ID14–2	Proteinase K classical	1	200×300×100	160×200	2.4
ID14–2	Proteinase K LB	3	160×200×50 300×200×200 350×300×300	160×200 100×100 100×100	2.43 2.22 2.16
ID14–2	Ribonuclease classical	1	140×140×50	140×140	4.06
ID14–2	Ribonuclease LB	1	140×140×50	140×140	4.96

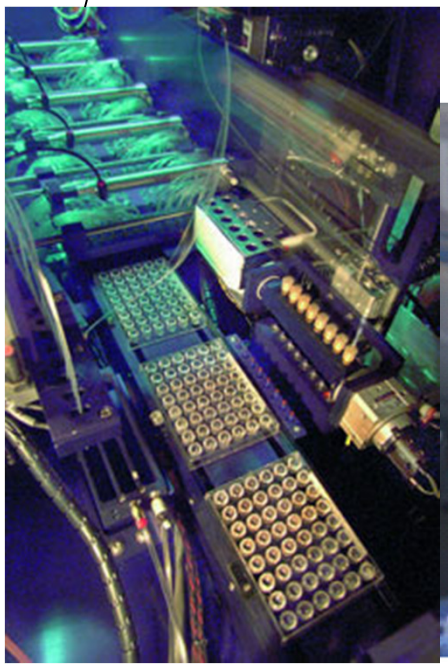
High throughput protein crystallization

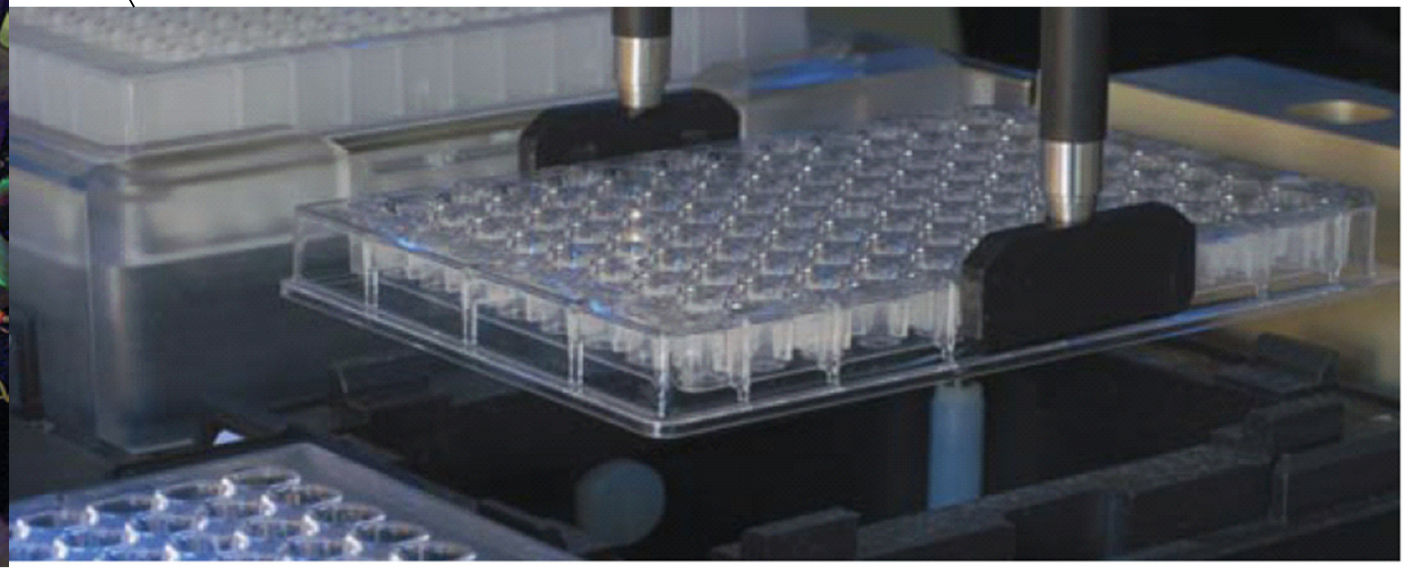




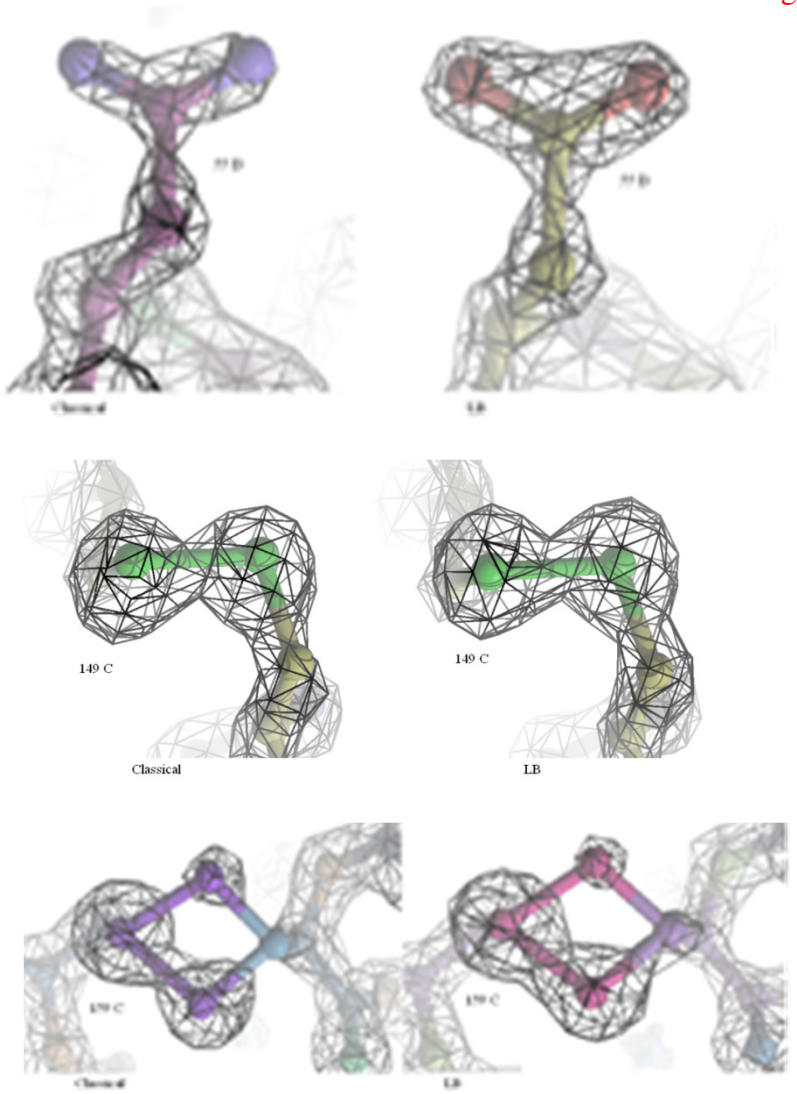


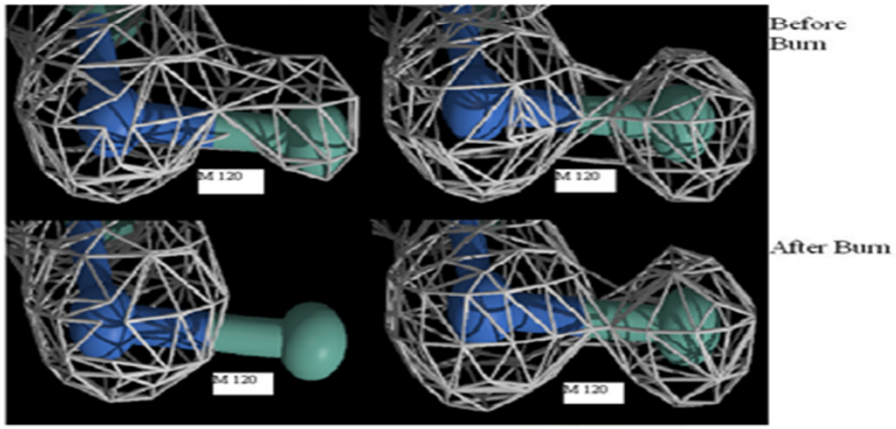




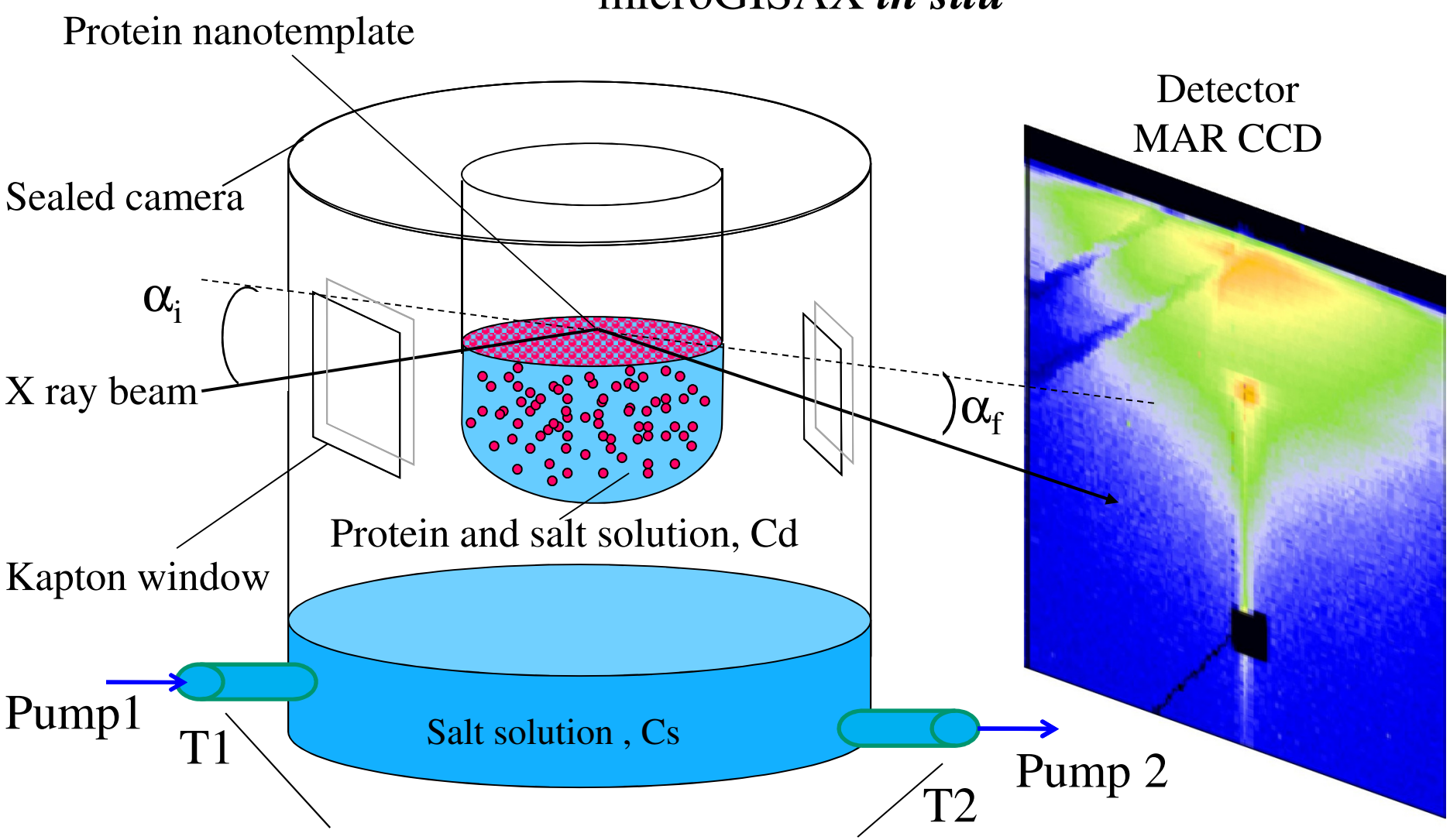


Electron density of crystals grown using robotic technique for LB-based nanotemplate method and classical method.. A: comparison of electron density map for carboxilic acid group of aspartic acid at 55 position contoured at 2.01sigma. B: comparison of electron density map of disulphide bond of cystein residue at 71 position contoured at 2.5 sigma. C: comparison of electron density map of disulphide bond of cystein residue at 149 position contoured at 2.5 sigma. D: comparison of electron density map of proline residue at 135 position of classical and LB crystal contoured at 2.5 sigma. E: comparison of electron density map of cysteine residue at 159 position of classical and LB crystal contoured at 2.5 sigma. From left to right and from top down.

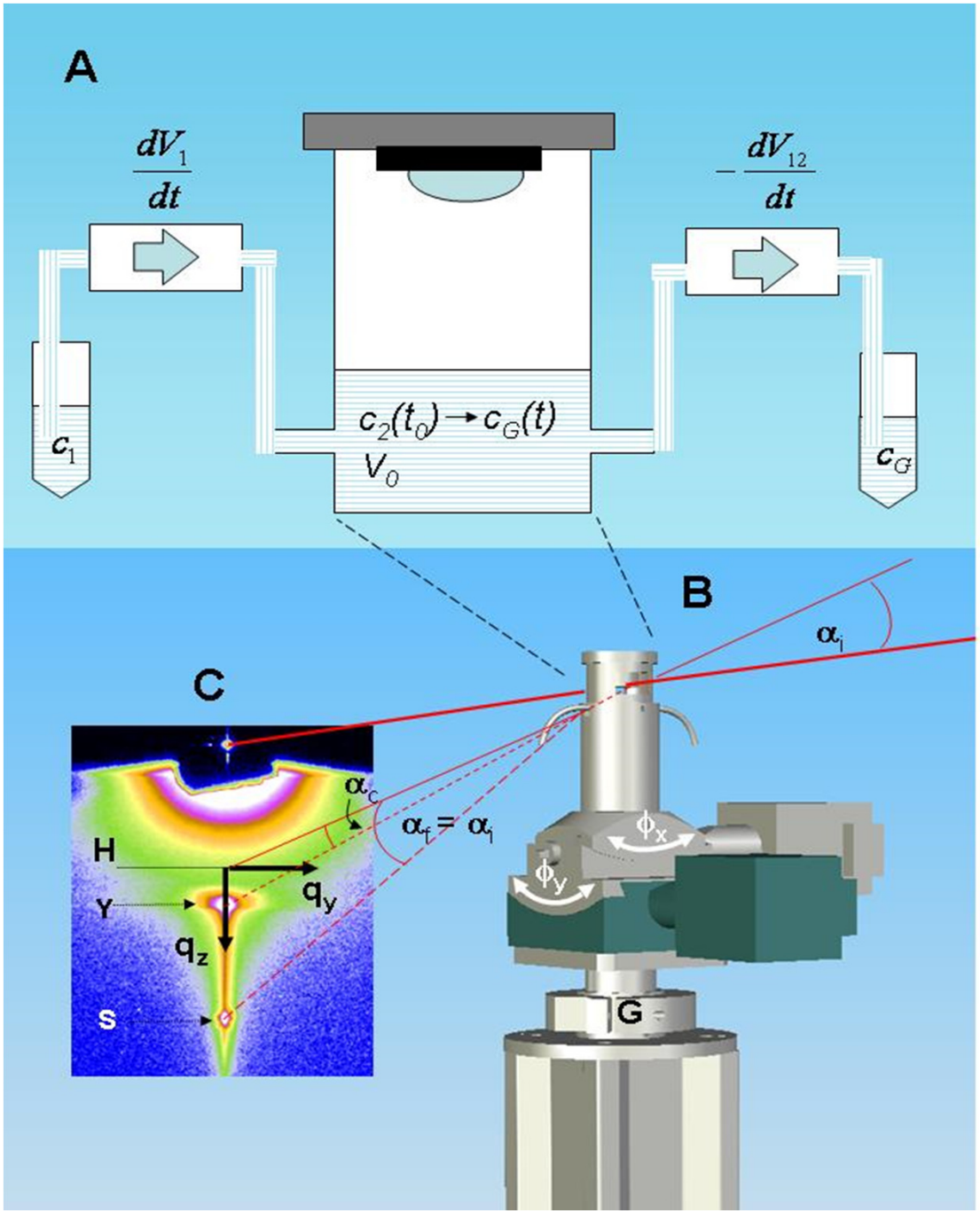




microGISAX *in situ*

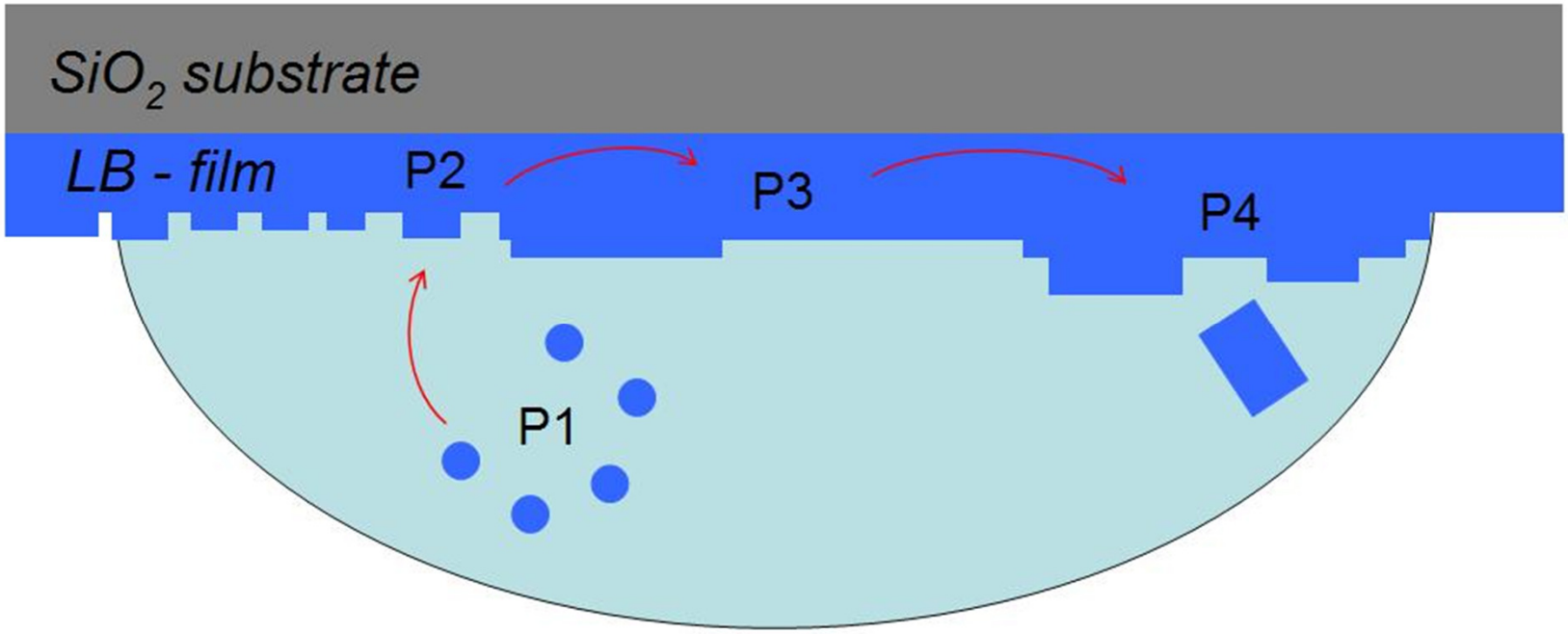


The tubes connected with pamps fo the salt solution exchange:
Cs=Cd "stop solution" for aliement procedure
Cs»Cd for accelerated nucleation
Cs=2Cd for controlled grouth



Setup of μGISAXS setup at ID13 beamline/ESRF. The flow through crystallisation cell is tilted by ϕy to adjust a fixed angle of incidence (αi). As typical features the Yoneda Peak (Y) at α_c and the specular peak at $\alpha_f=\alpha i$ are shown in the 2d GISAXS pattern.

Figure 3. Actual experimental configuration



MODEL for reaction pathways on LB-film

Pechkova, Gebhardt, Riekel, Nicolini, Biophysical Journal, Part I, 2010

Gebhardt, Pechkova, Rielel, Nicolini, Biophysical Journal, Part II, 2010