

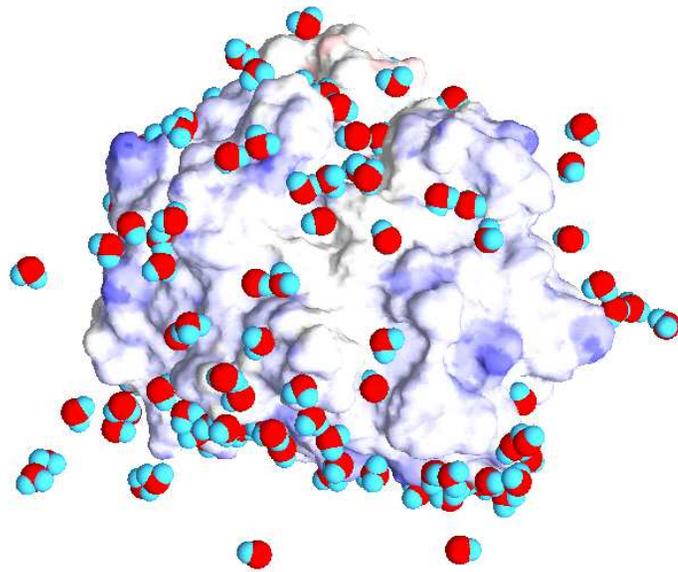
Preparation of Micro Crystallization Experiments Using the Cubic Phase Method

Peter Nollert

 **Emerald BioSystems**

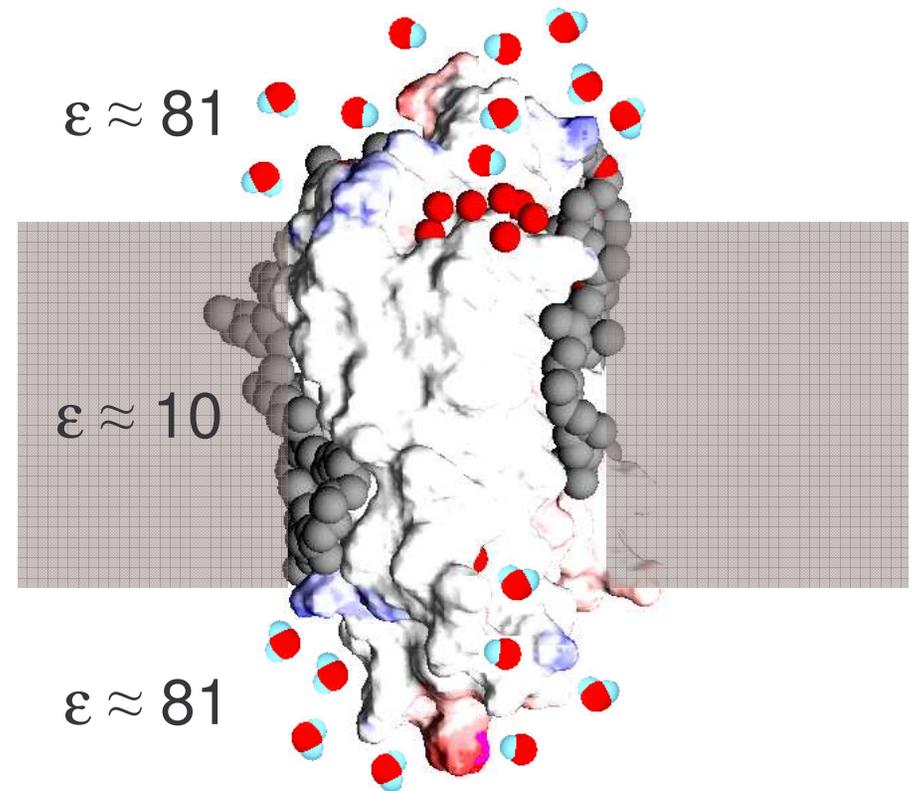
- Membrane proteins & crystallization
- LCP concept
- LCP track record
- Technology

Why is it so difficult to work with membrane proteins?



$\epsilon \approx 81$

soluble protein
interaction with water & ions



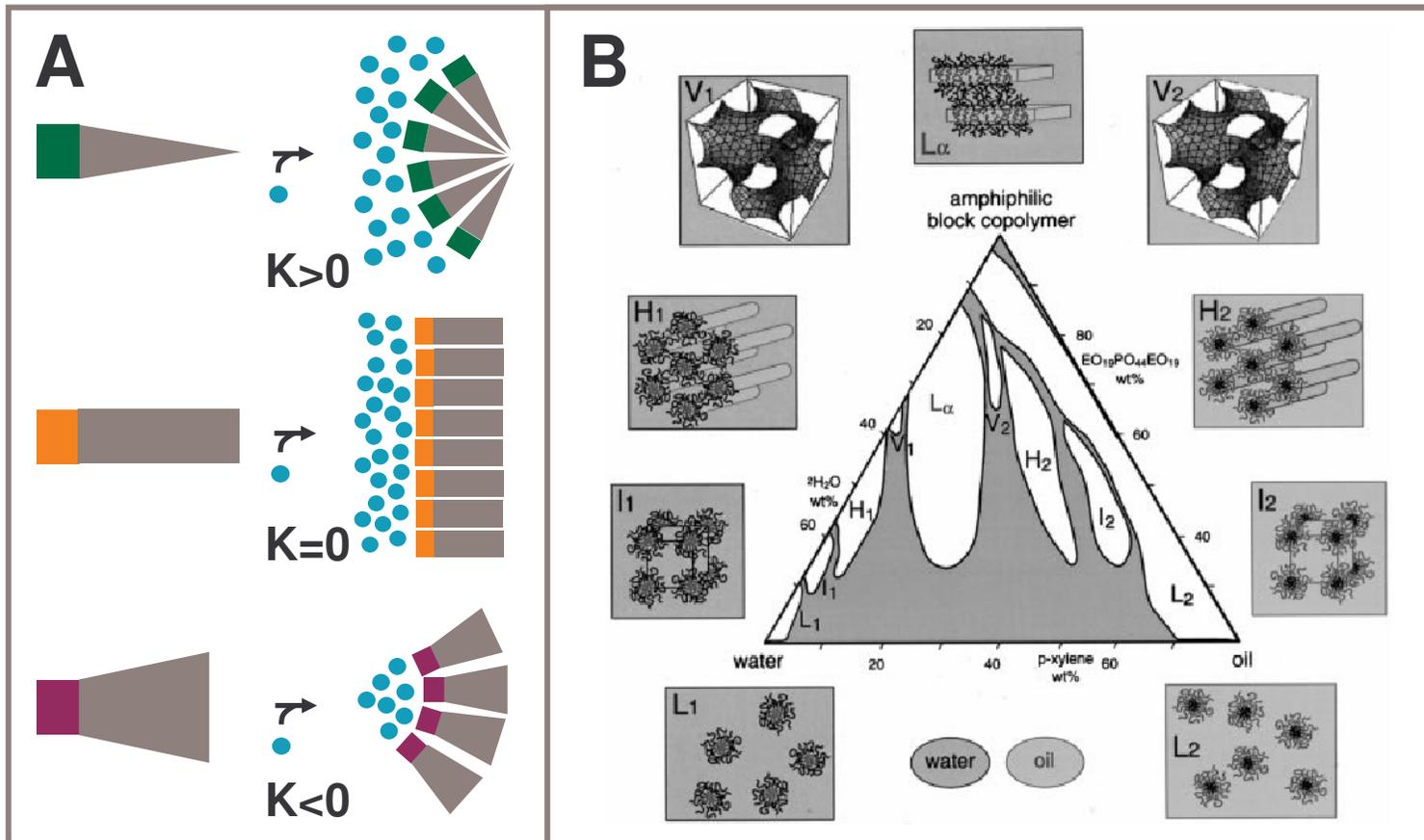
$\epsilon \approx 81$

$\epsilon \approx 10$

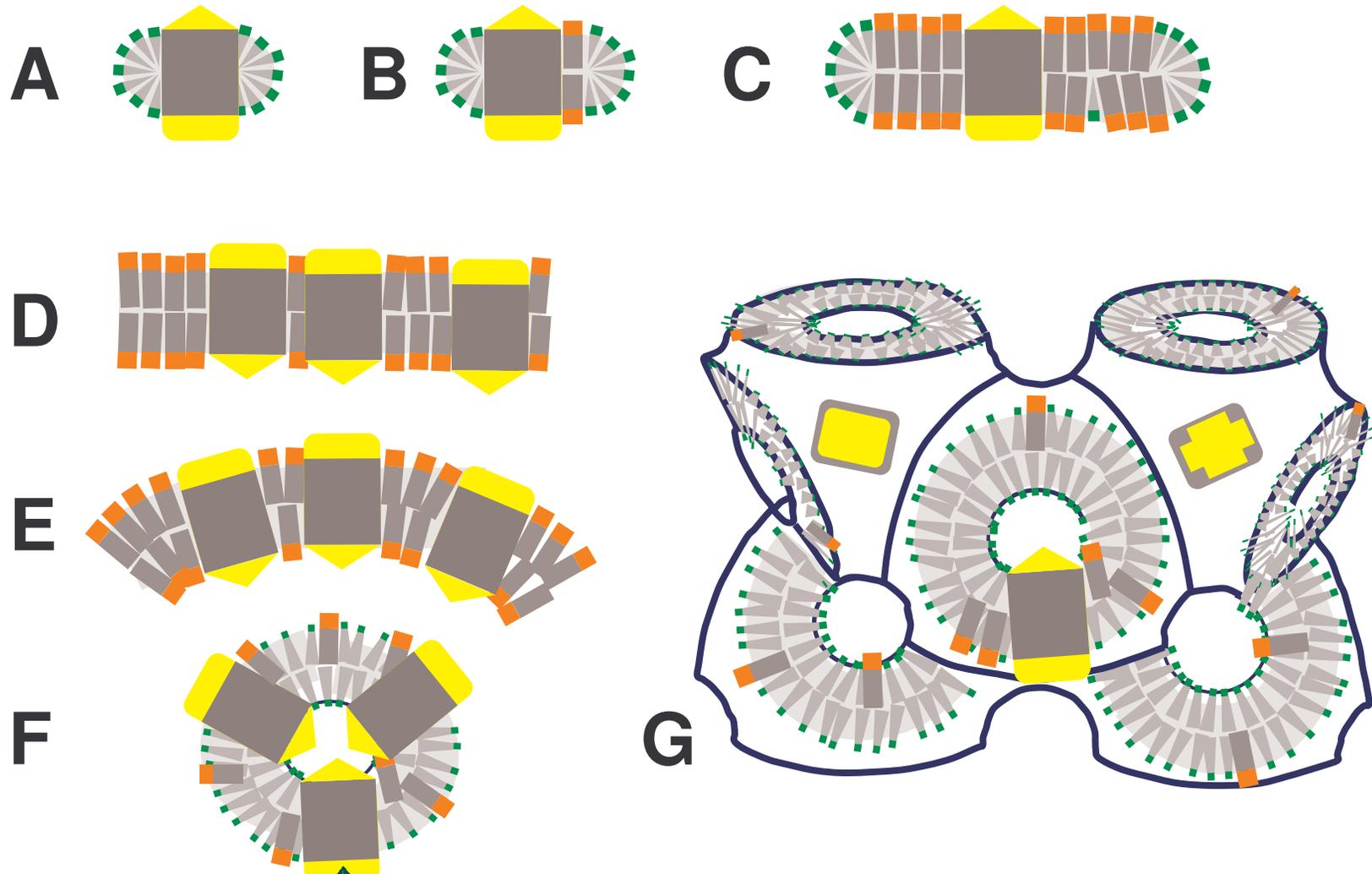
$\epsilon \approx 81$

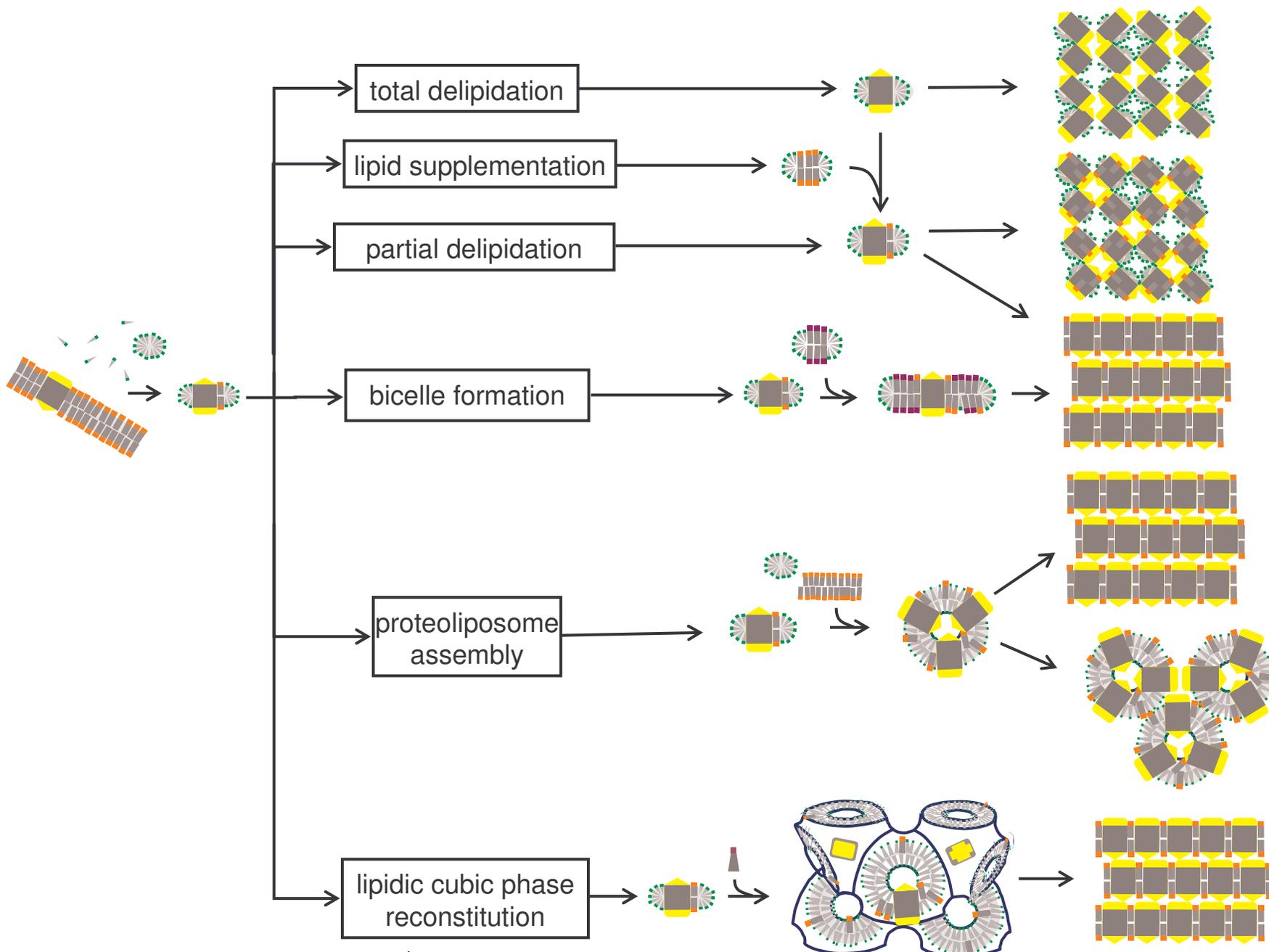
membrane protein
interaction with lipid / detergent
& water & ions

Amphipile polymorphism

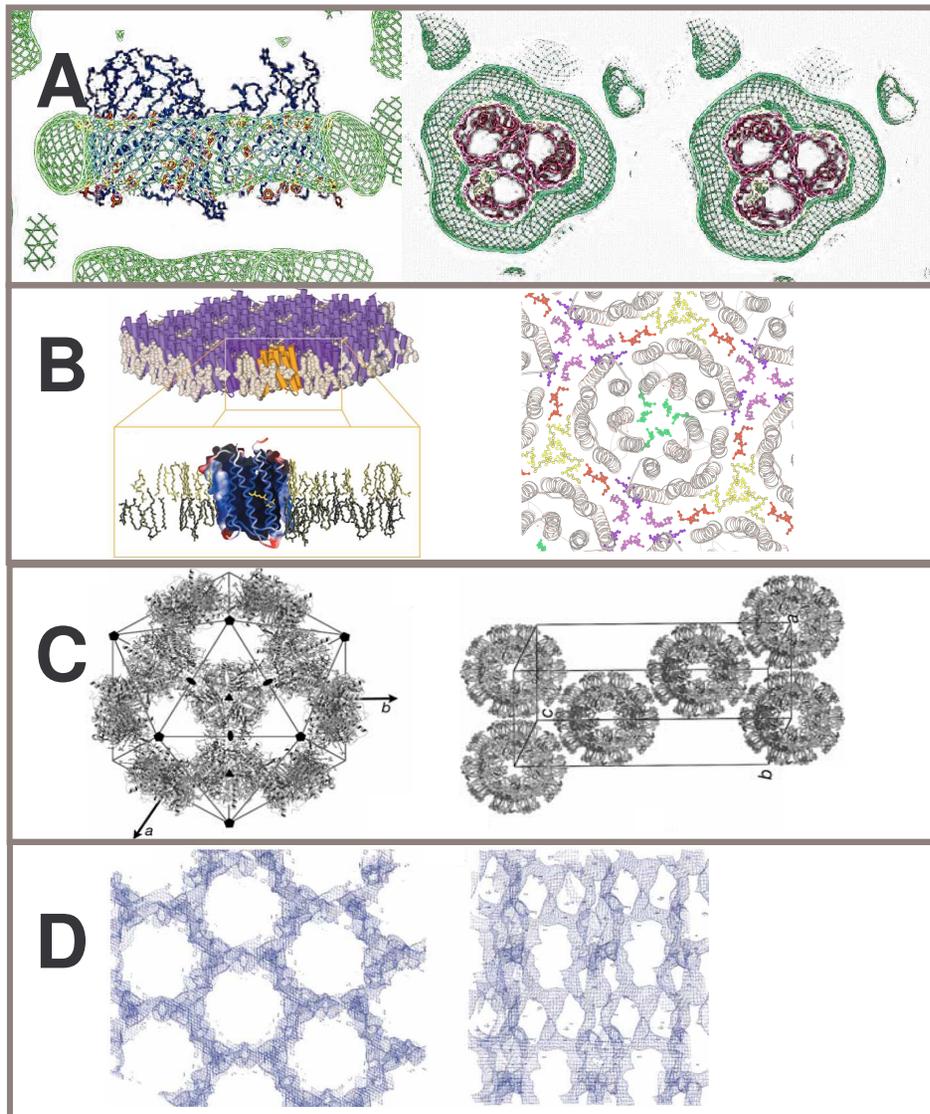


Membrane protein / amphipile microstructures





Experimentally determined amphiphile packing within membrane protein crystals



Type II
micellar

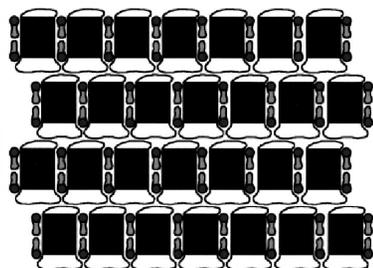
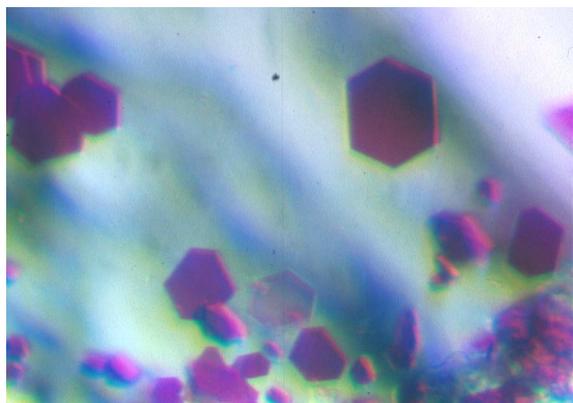
Type I
2D continuous
Lamellar stacks

2D discontinuous

3D continuous

8 membrane proteins that have been crystallized in LCP:

1. bias towards 7TM
2. layered architecture
3. good diffractors

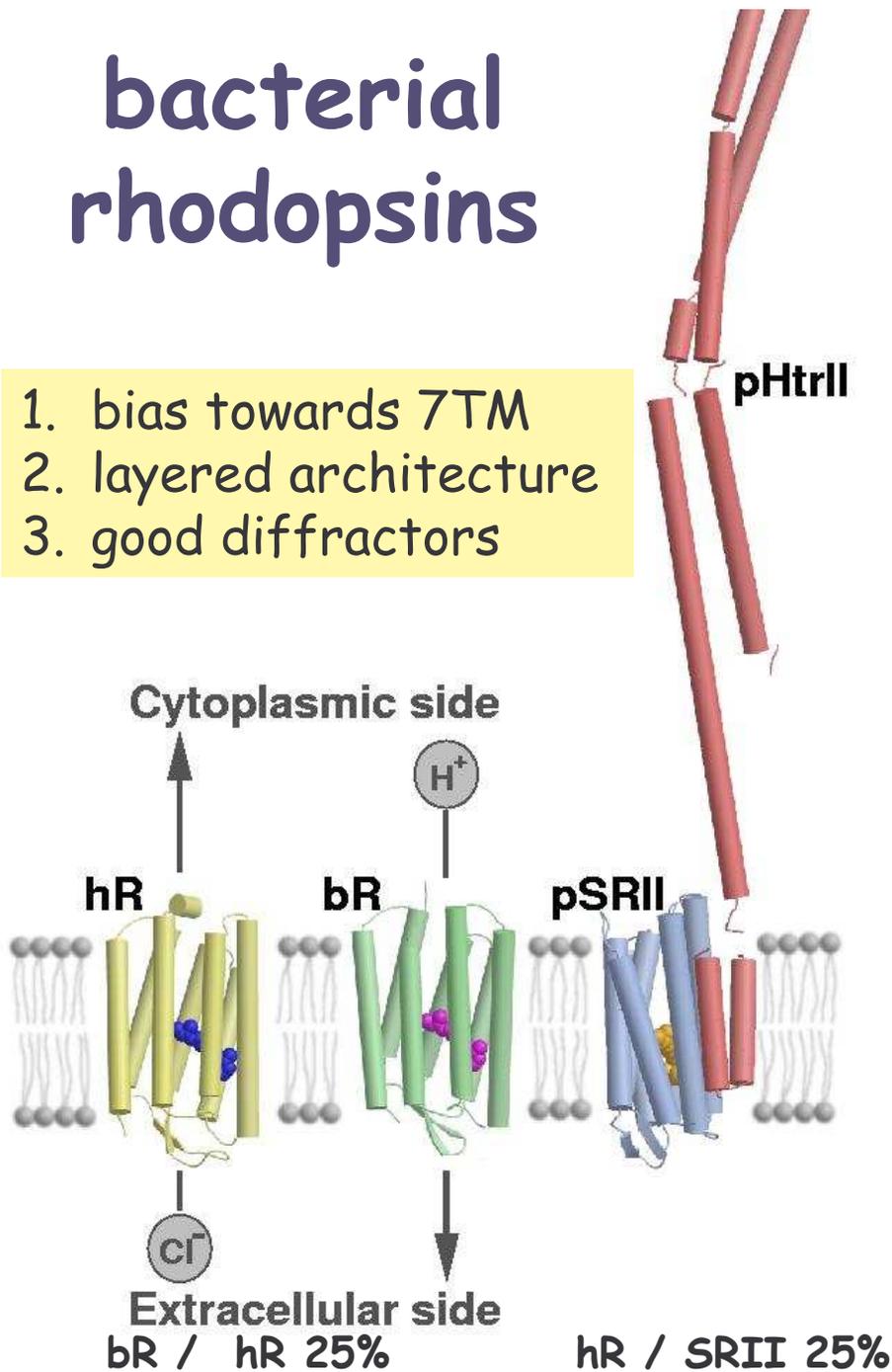


protein	resolution [Å]	access code
bacteriorhodopsin	2.35	1AP9
bacteriorhodopsin	2.3	1BRX
bacteriorhodopsin	1.55	1C3W
bacteriorhodopsin D96M	2.0	1C8S
bacteriorhodopsin, K-state	2.5	1DZE
bacteriorhodopsin, K-state	2.6	1IXF
bacteriorhodopsin, L-state	2.1	1EOP
bacteriorhodopsin E204Q	1.8	1F4Z
bacteriorhodopsin E204Q	1.7	1F50
bacteriorhodopsin	2.3	1IW6
bacteriorhodopsin D85S/F219L	2.0	1JV6
bacteriorhodopsin early M state	2.0	1KG8
bacteriorhodopsin D85S O state	2.25	1JV7
bacteriorhodopsin mock early M	1.81	1KG9
bacteriorhodopsin	1.65	1KGB
bacteriorhodopsin, K-state	1.43	1MOK
bacteriorhodopsin	1.47	1MOL
bacteriorhodopsin M-1	1.43	1MOM
bacteriorhodopsin	1.9	1QHJ
bacteriorhodopsin early state	2.1	1QKO
bacteriorhodopsin early state	2.1	1QKP
sensory rhodopsin II	2.4	1JGJ
sensory rhodopsin II K state	2.27	1GU8
sensory rhodopsin II K state	2.27	1GUE
sensory rhodopsin II	2.1	1H68
sensory rhodopsin II	2.4	1JGJ
SRII transducer complex	1.93	1H2S
halorhodopsin	1.8	1E12
photosynthetic reaction centre RCvir	3.7	
photosynthetic reaction centre RC sph	6	
light harvesting complex		

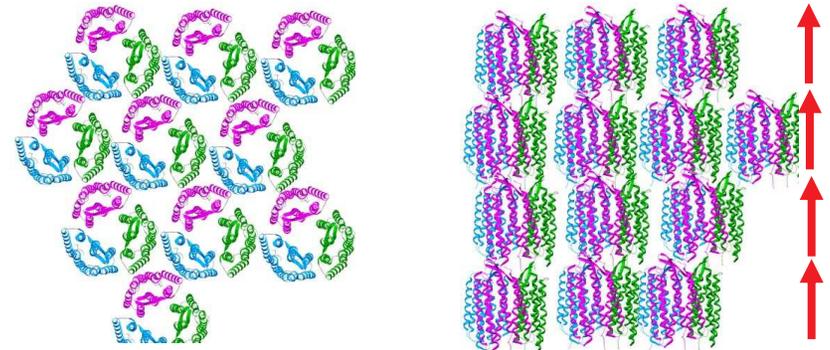
Anaebana Sensory rhodopsin 2 A 1 XIO

bacterial rhodopsins

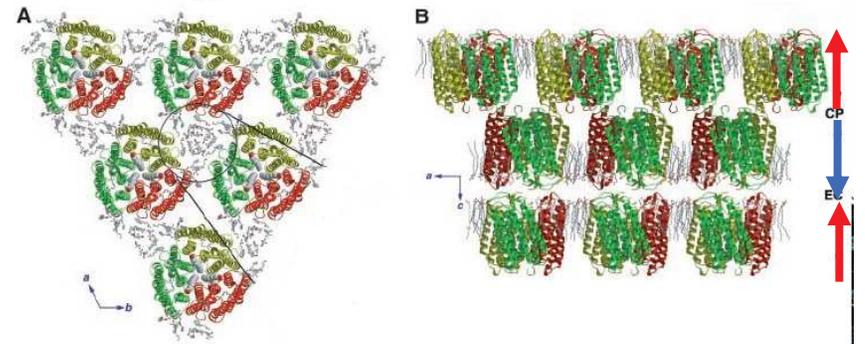
1. bias towards 7TM
2. layered architecture
3. good diffractors



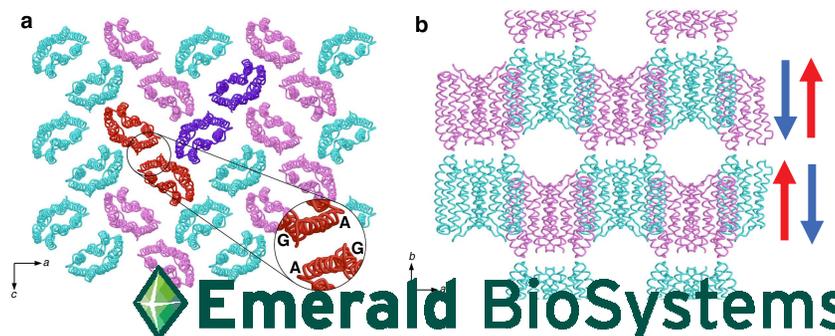
Bacteriorhodopsin 1.55 Å
P63, solvent 33%



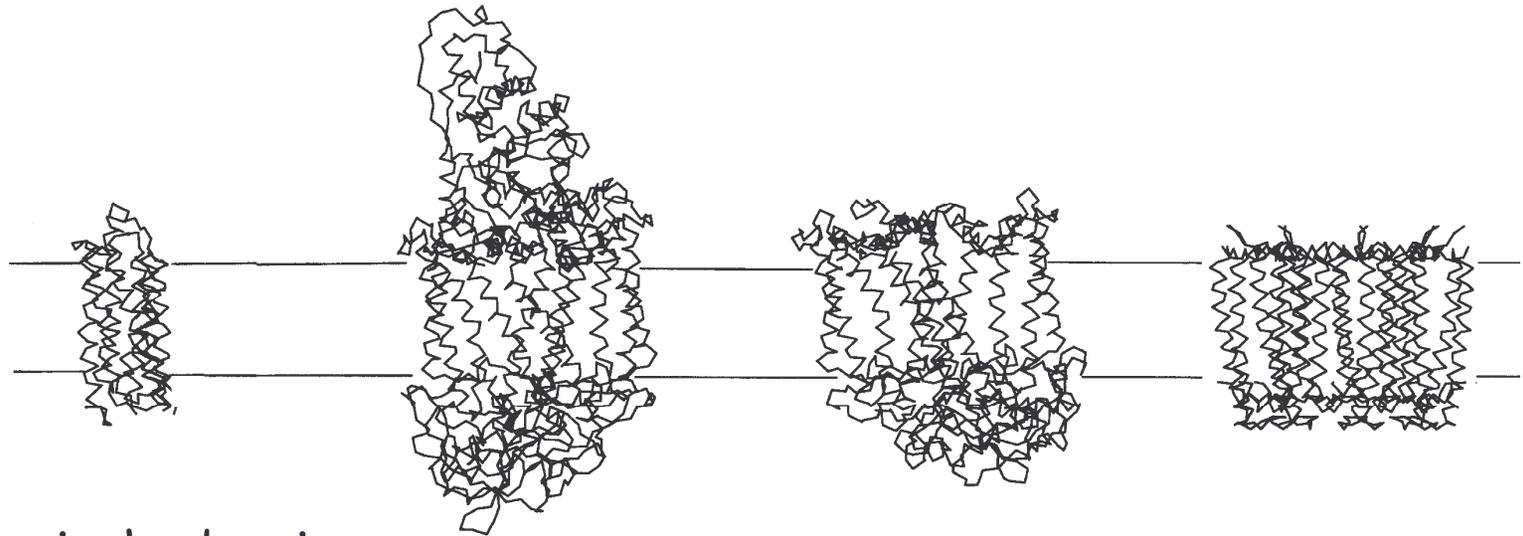
Halorhodopsin 1.8 Å
P63₂₂, solvent 33%



Sensory rhodopsin II 2.1 Å,
C222₁, solvent 43%



size does not matter...



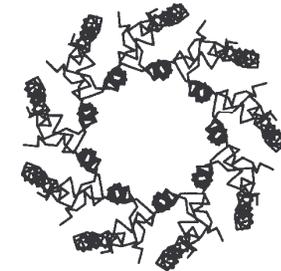
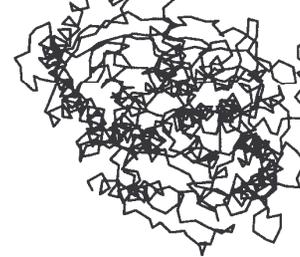
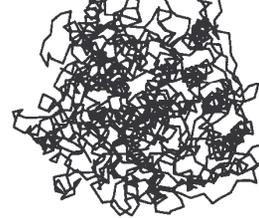
bacteriorhodopsin
halorhodopsin
sensory rhodopsin II

photosynthetic reaction
centres

R. viridis

R. sphaeroides

light harvesting
complex 2



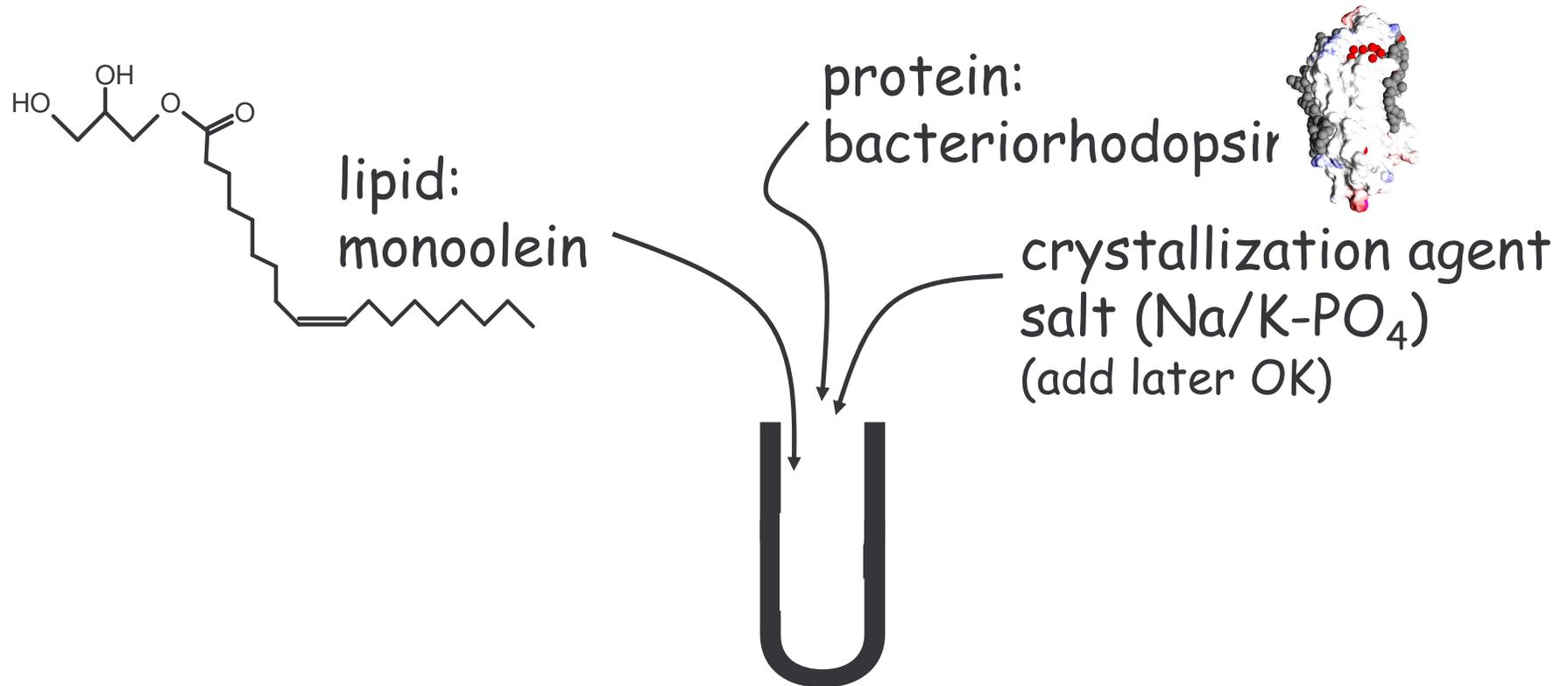
mass [kDa] 26.8
subunits 1 \ 3

132
4

88.6
3

90.4
9

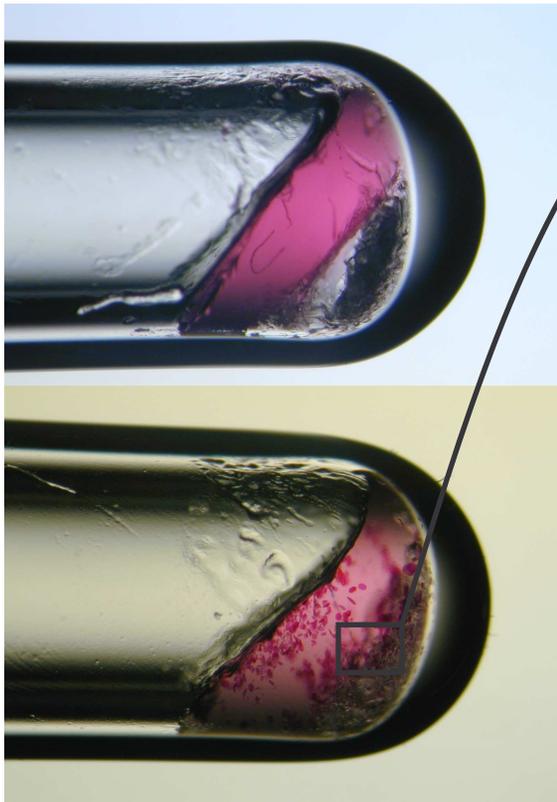
Crystallization in lipidic cubic phases (LCP)



Nollert, P., Navarro, J., Landau, E.M.
Crystallization of Membrane Proteins *in cubo*
Methods in Enzymology, 343 pp, 2001

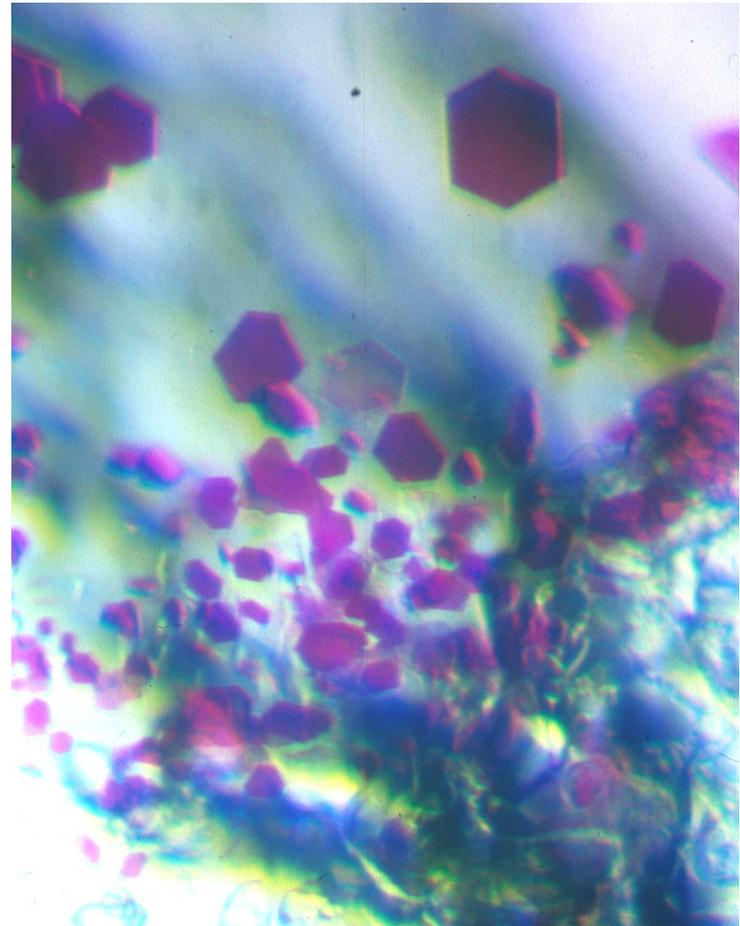
Landau, E.M., Rosenbusch, J.P.
Lipidic cubic phases: a novel concept for the crystallization of membrane
proteins. PNAS, 10, 93(25):1432-5. 1996

in cubo: the crystallization process



day 1

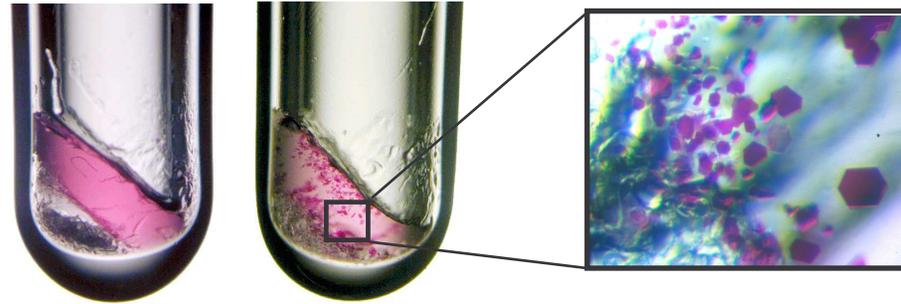
day 30



Evolution of LCP technology

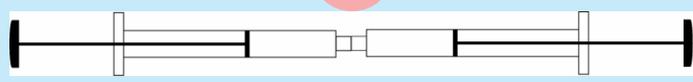
complication:
LCP is highly viscous,
impossible to pipet

1st generation
test tube
weigh & centrifuge
~10 ul



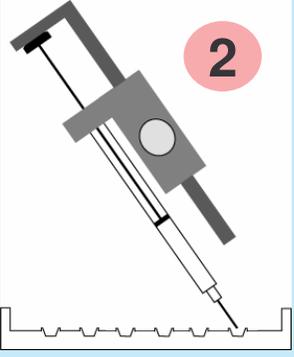
2nd generation
micro method
well, manual dispense
"drop": 200 nl + 2 ul

1



two steps process
1. prepare LCP
2. dispense LCP

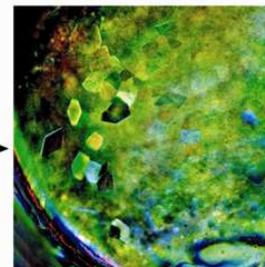
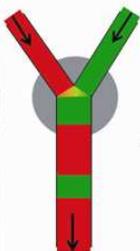
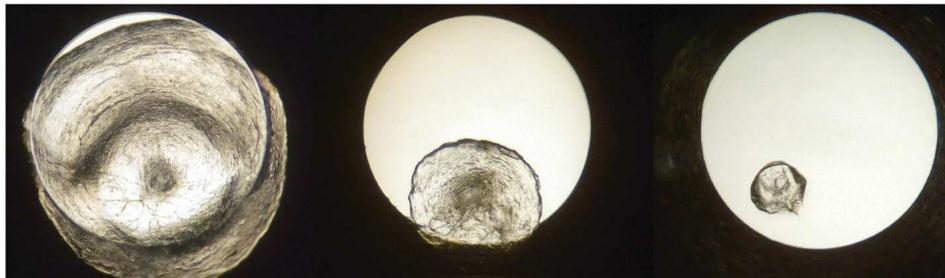
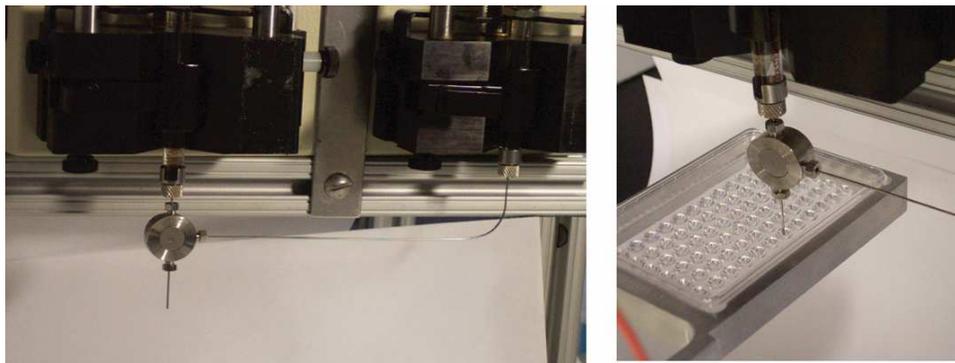
2



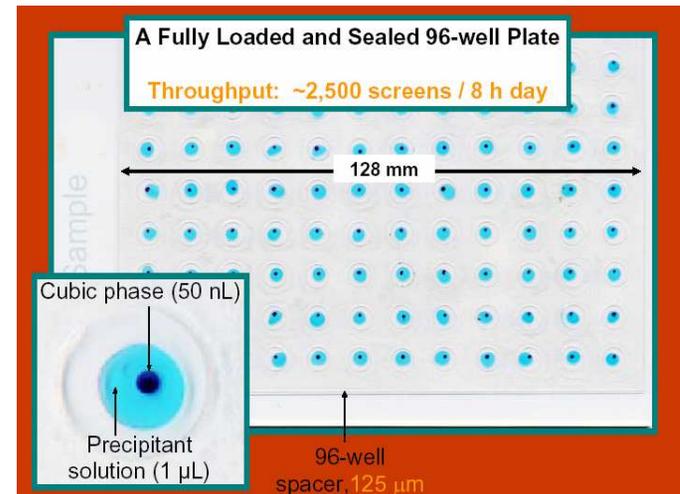
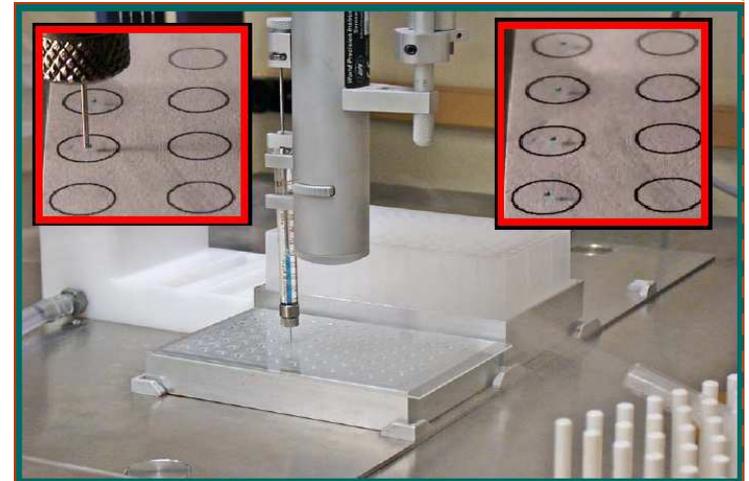
J. Appl. Cryst.
(2002) **35**, 637-640

3rd generation
automated dispense
10-25 nl

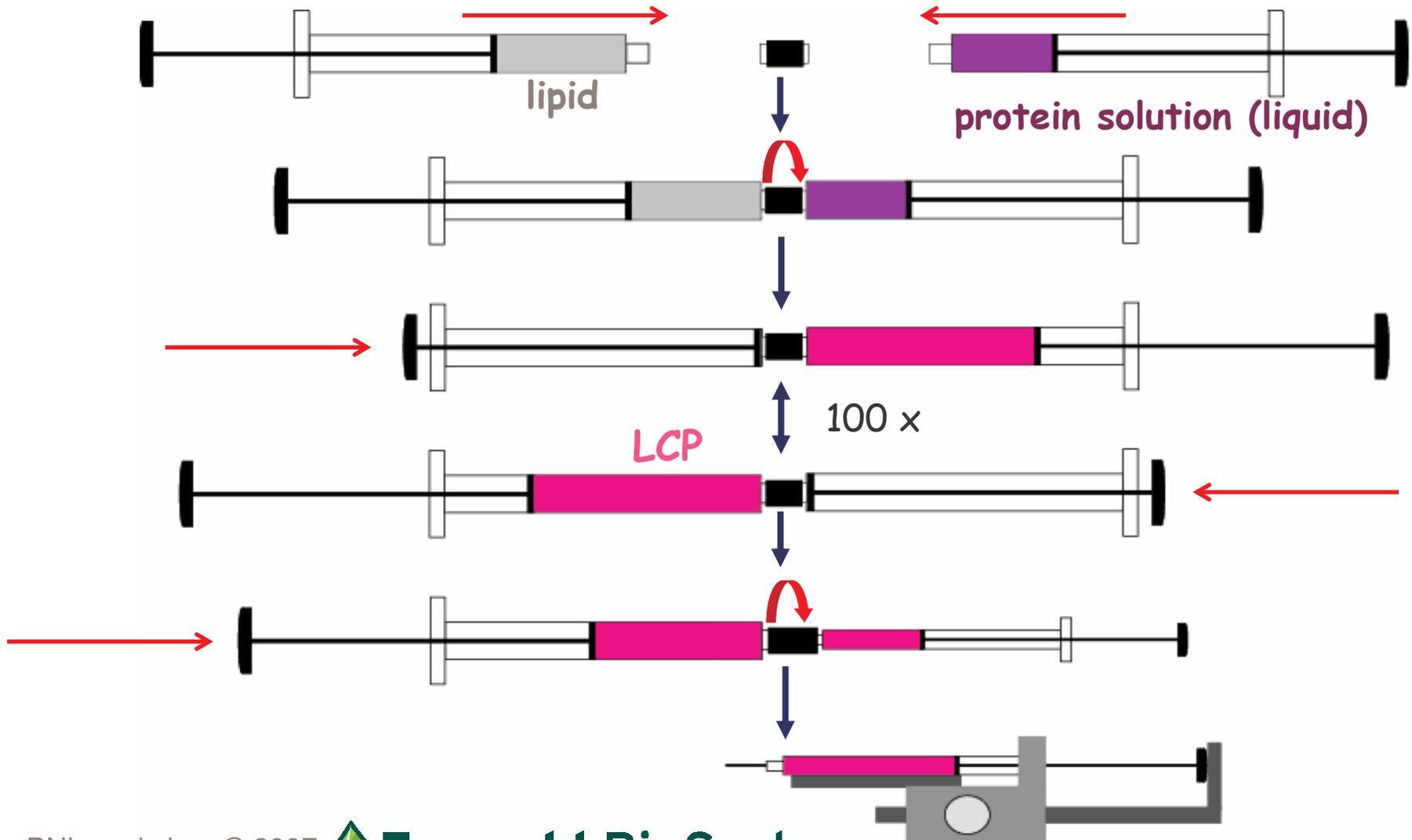
Automated LCP dispensation



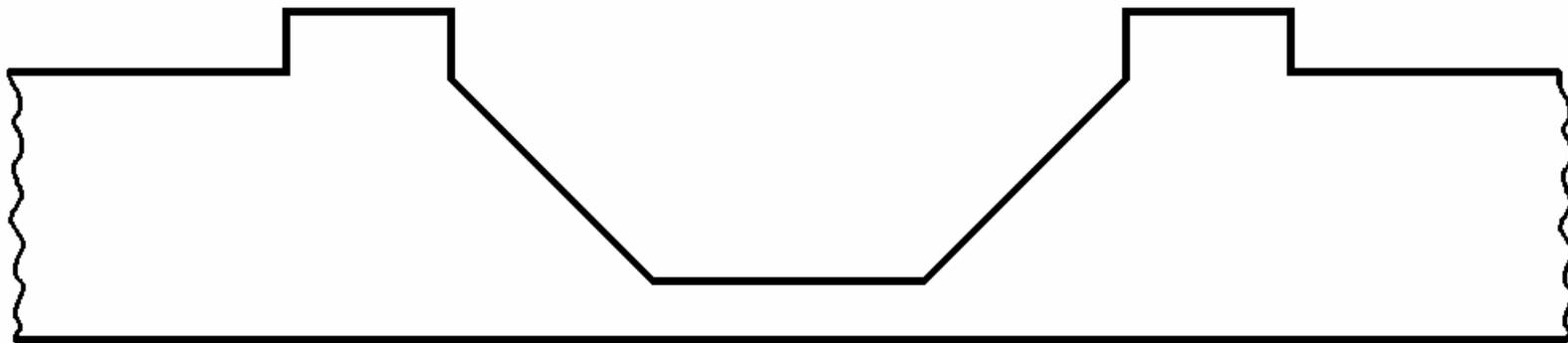
Caffrey *et al.* 'in meso robot'



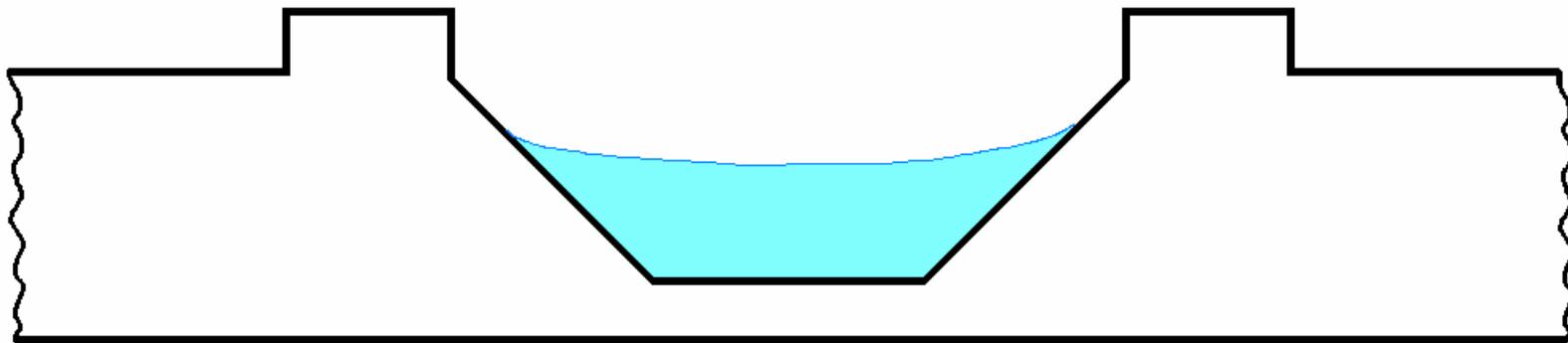
Preparation of the LCP using syringes



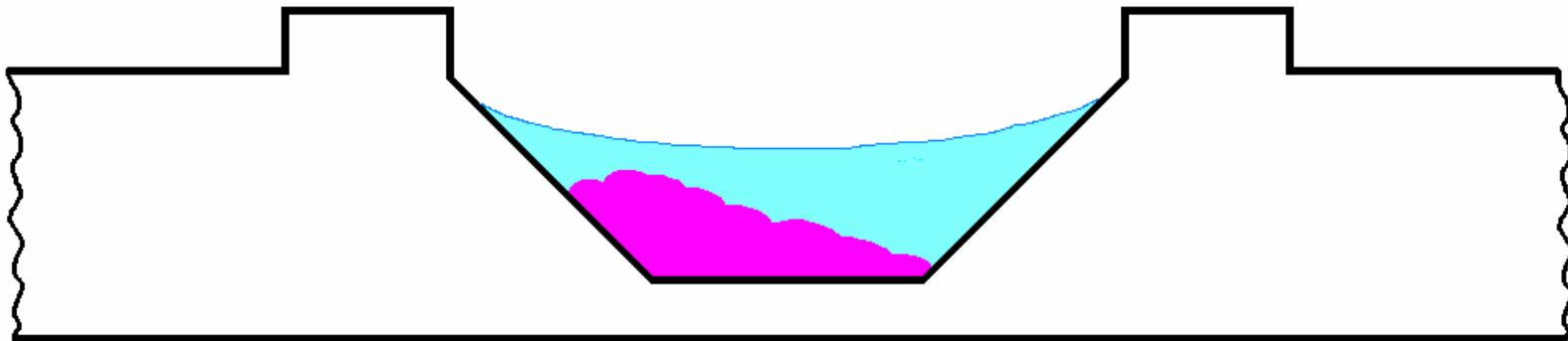
empty well



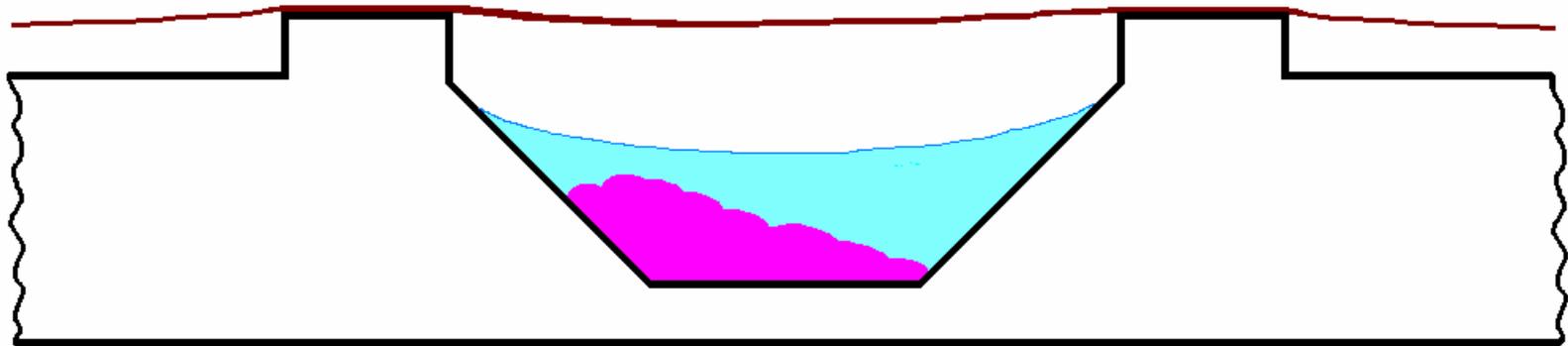
fill well with 2 μL of liquid crystallant



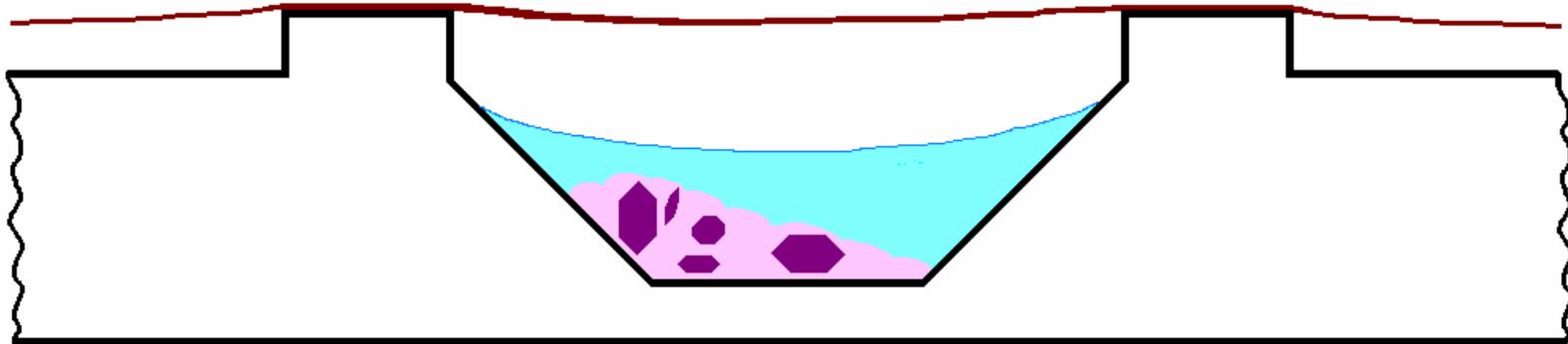
dispense 100 - 200 nL LCP (contains protein)



cover well with tape
wait

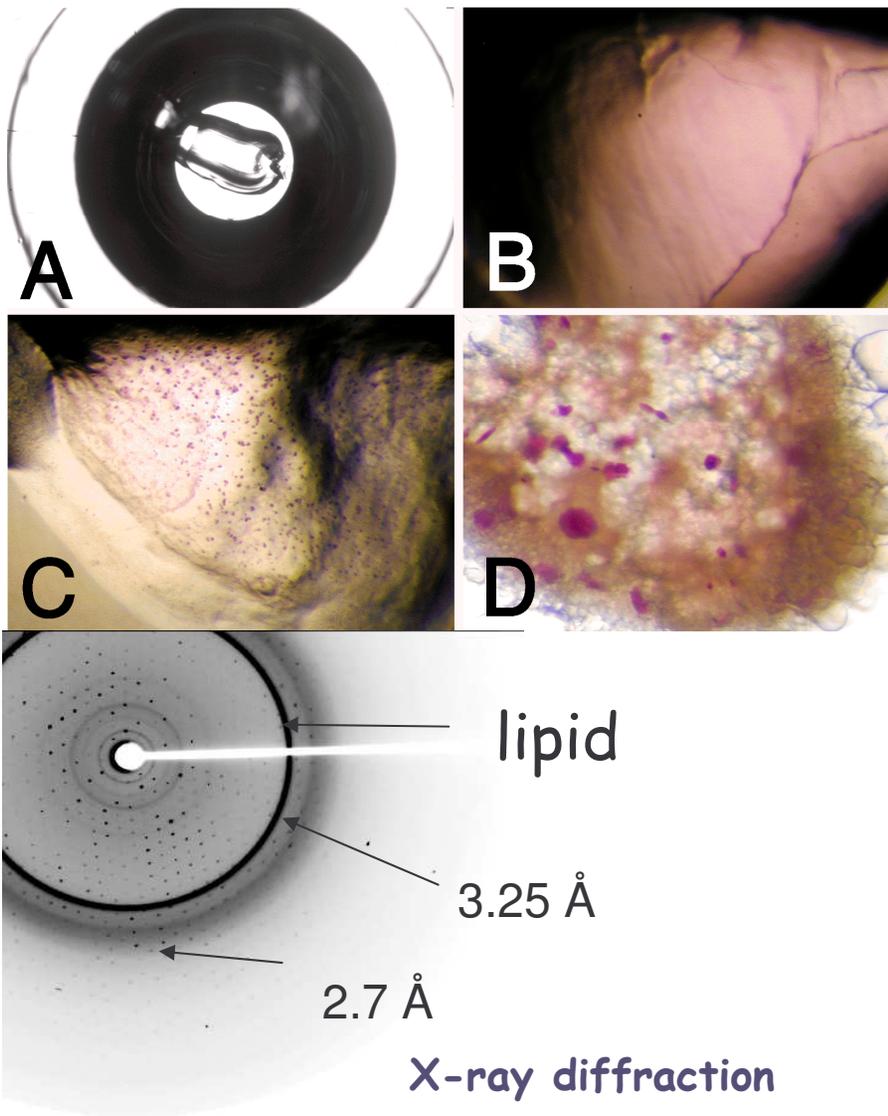


observe setup



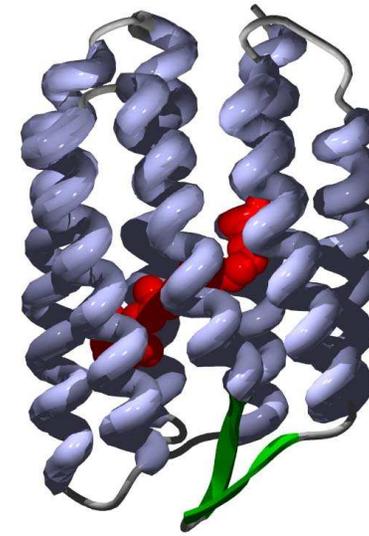
- take out crystal
- cool
- diffract
- determine structure

Proof of principle experiment: crystallization of **Bacteriorhodopsin** & **Sensory Rhodopsin II**



- crystal fished directly
- no cryoprotection

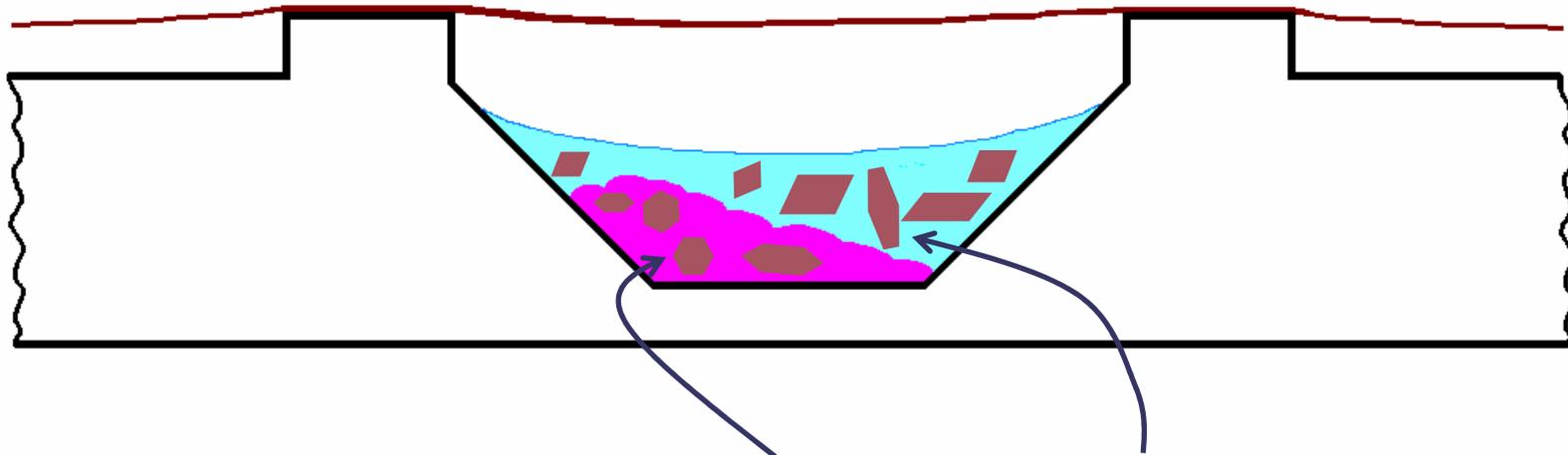
BNL workshop © 2007



- 300 setups \approx 300 μ g protein
- time frame (for ca. 2 persons):
expression,
purification,
crystallization,
structure determination 2.1 Å: < 1year

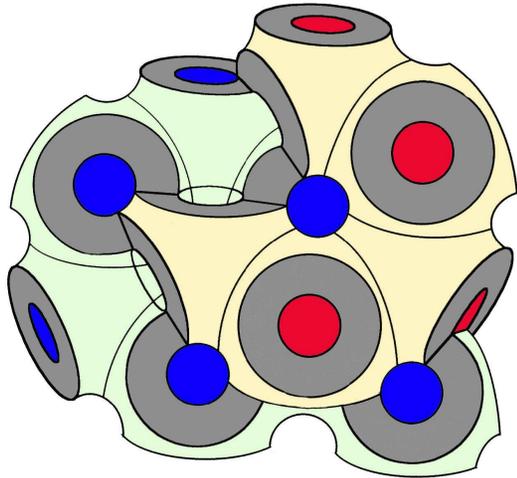
 **Emerald BioSystems**

for soluble proteins:



- crystals may appear inside LCP or/and in overlaying solution

Putative locations of proteins in LCP

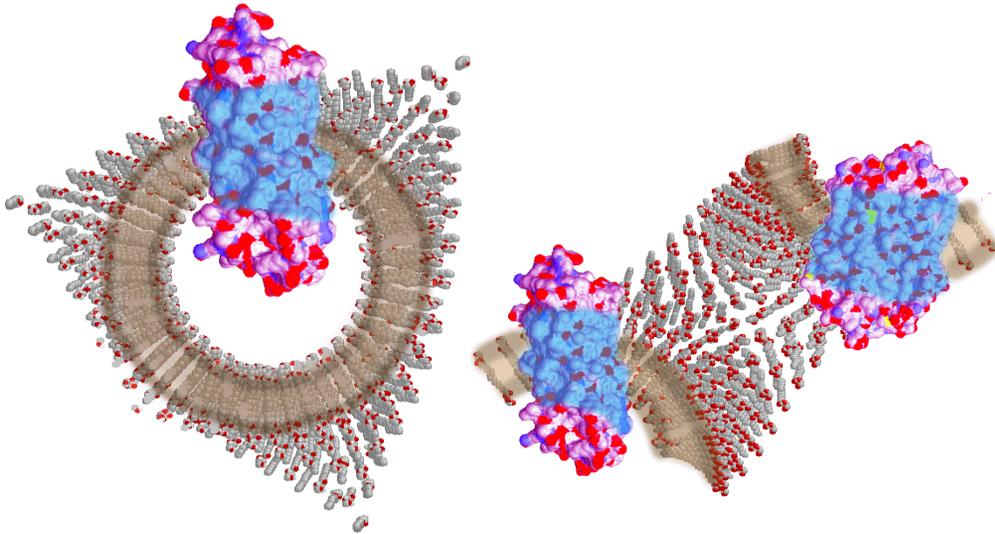


Pn3m unit cell 80-120 Å

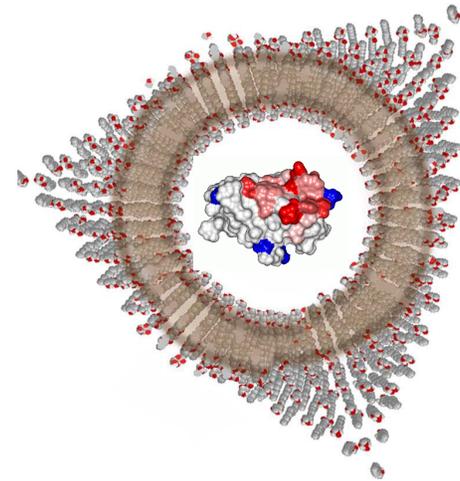
monoolein bilayer thickness...17.3 Å

bacteriorhodopsin $\approx 25 \times 35 \times 45$ Å

membrane protein: embedded in bilayer



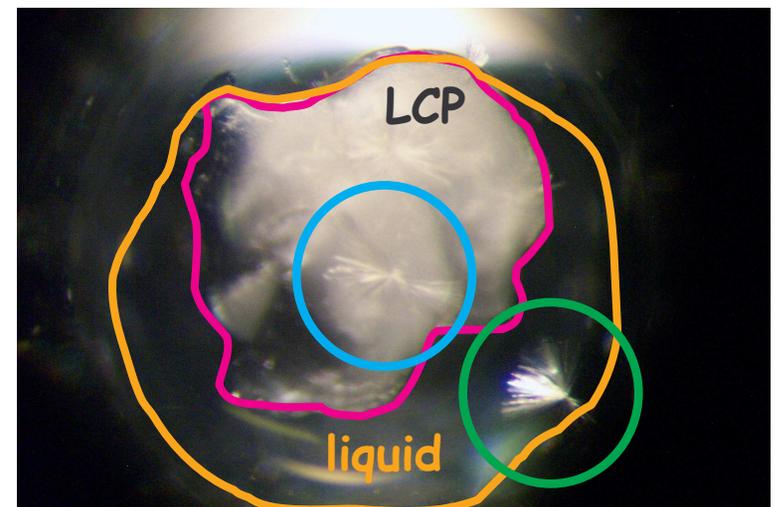
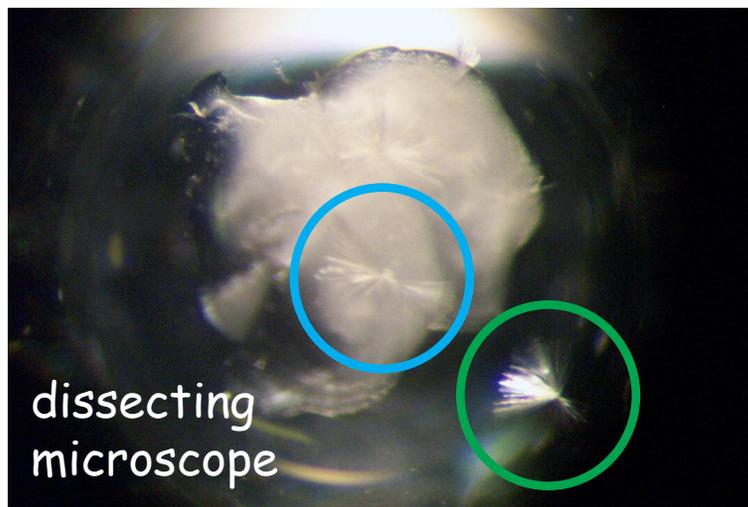
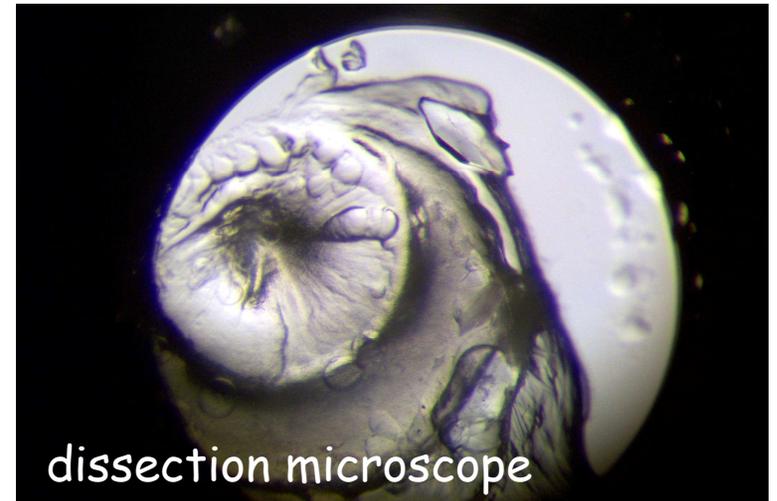
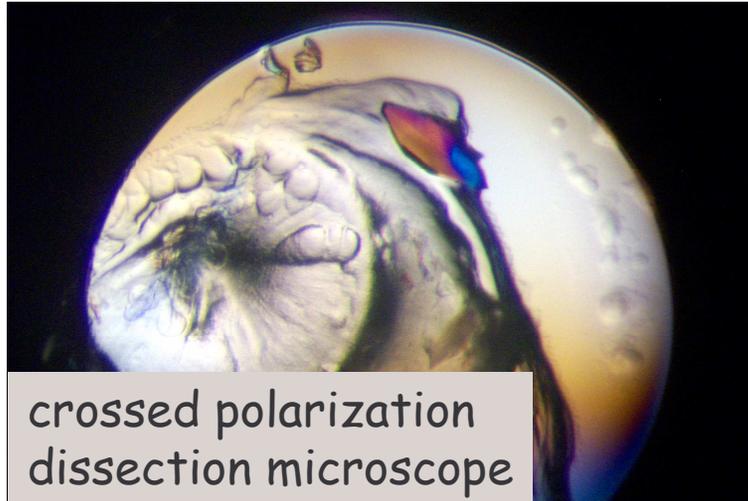
soluble protein: in channels



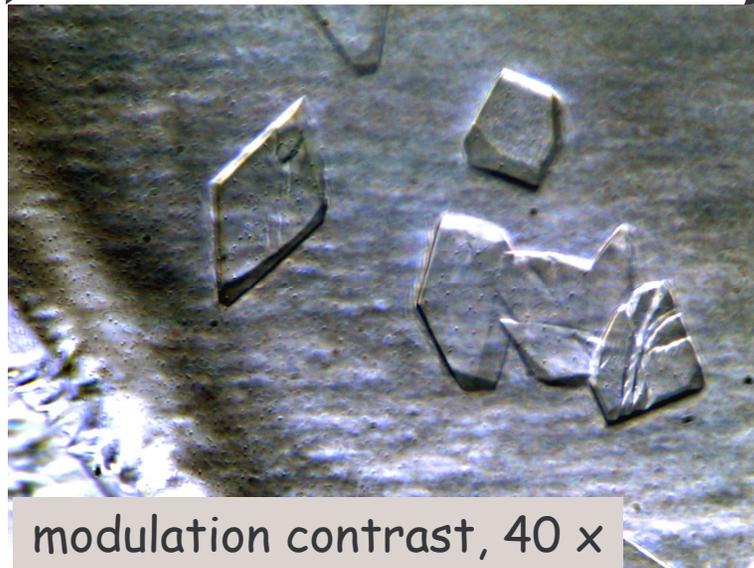
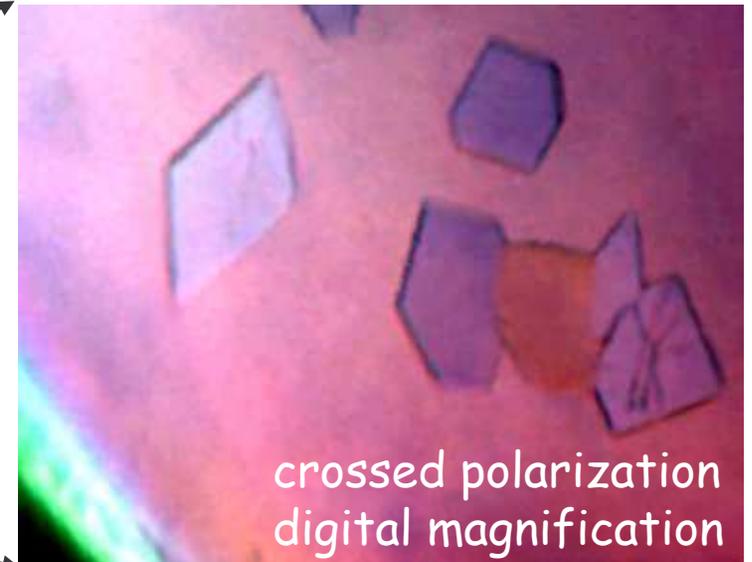
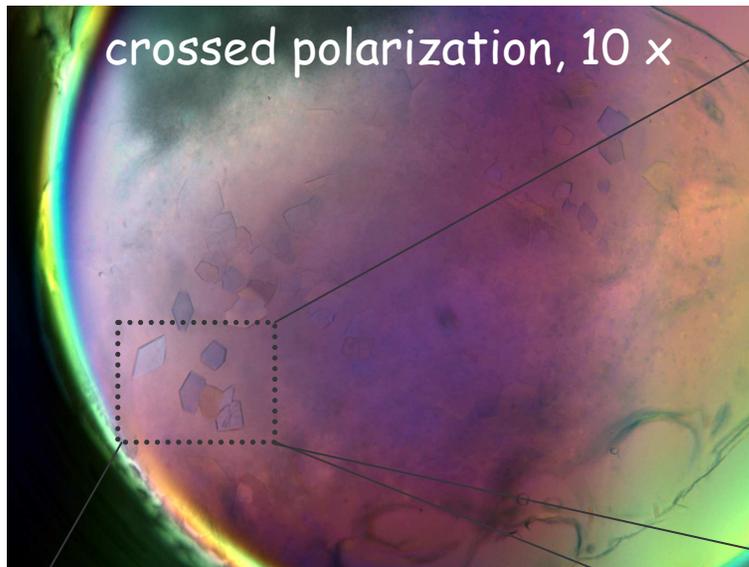
Crystal growth of soluble proteins: Lysozyme

Lysozyme

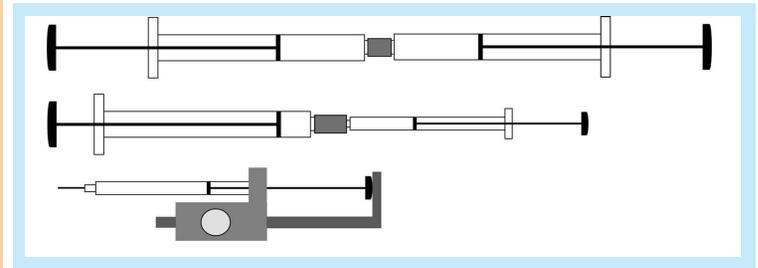
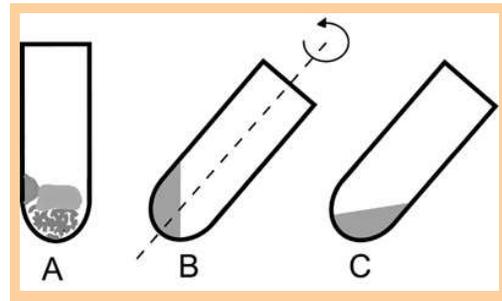
conditions from:
J. Struct. Biol.
121, 82-91 (1998)



Crystal growth of soluble proteins: Thaumatin



Comparison of glass vial based procedure with the micro method



1st generation

→ 2nd generation

	glass vial method	micro method
setup volume	5 - 20 uL	LCP 0.1 - 0.2 uL + 2 uL solution
protein amount ^a	17 - 70 ug	0.38 - 0.76 ug
setups / 1 mg protein ^a	14 - 28	2072 ^b - 1036 ^b
setups / person / day	ca. 48	> 1000
applicable observation modes	dissecting microscopy polarization microscopy	dissecting microscopy bright and darkfield light microscopy fluorescence microscopy polarization microscopy

^a 60 % monoolein, final protein concentration 3.5 mg/mL

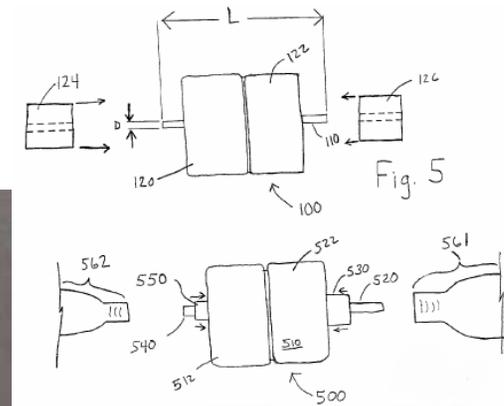
^b including 20 % loss of material BNL workshop © 2007

micro LCP on a shoestring budget

"Investment": Cubic Kit Available through Emerald BioSystems

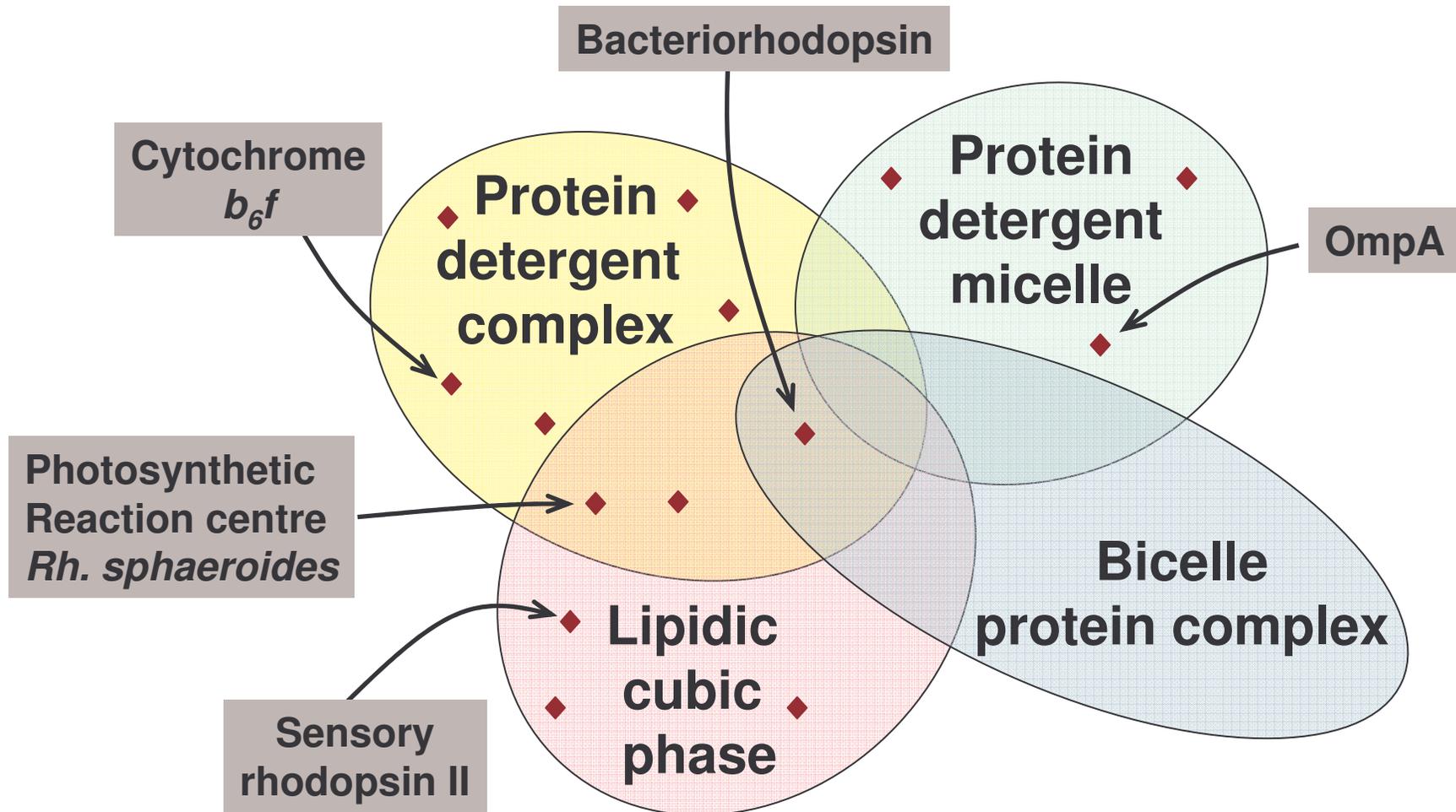
- Syringes
- Dispenser
- Coupler
- Lipid
- Trays
- Tape

Consumables



cat. Nr.	manufacturer	item	number	1000 setups	1 setup
1007-00-0	Robbins	Terasaki microtray (96 wells)	11	\$7.26	0.73 ct
EBS-WIZ-F	Emerald BioSystems	Wizard solutions (96 cond., 2 ul / well)	11	\$50.00	5.0 ct
	Manco	crystal clear tape (72 yds)	1	\$1.99	0.2 ct
M-239	Nu-check	monoolein lipid 250 mg		\$15.00	1.5 ct
		total		\$74.25	~7.5 ct

Schematic coverage of 'crystallization space' with different crystallization methodologies



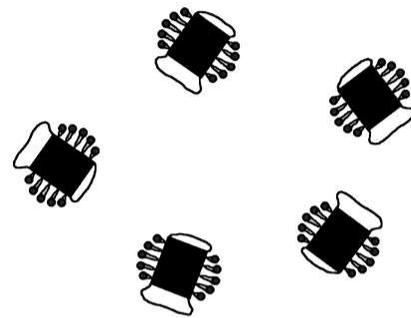
Take home message:

micro-LCP is

1. different (crystallization inside a liquid crystal)
2. small (>1000 setups / mg protein)
3. cheap (8 ct / setup)

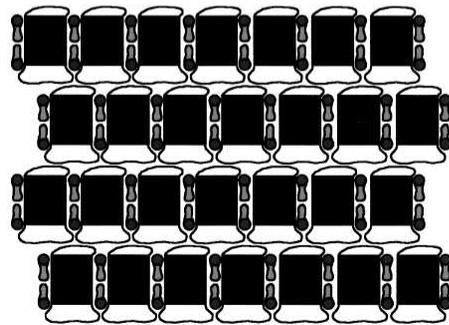
→ **give it a try**

How does this work?



protein detergent micelle

crystal nucleation & growth



layered crystal architecture

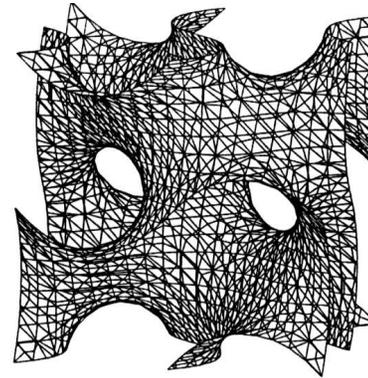
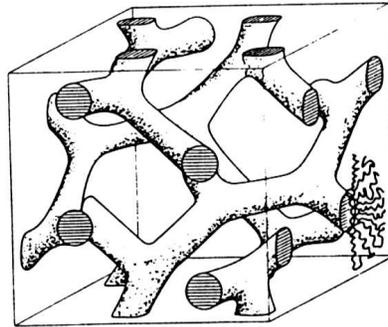
→ contribution of the cubic phase?

Models of Bicontinuous Cubic Phases

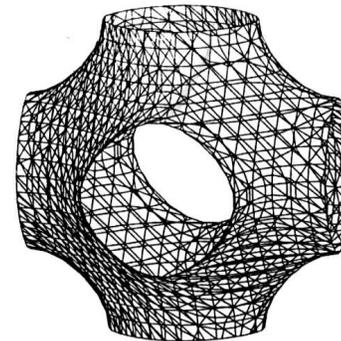
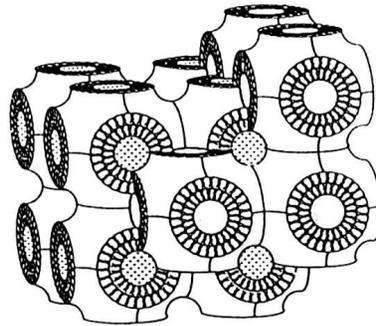
membrane structure

IPMS

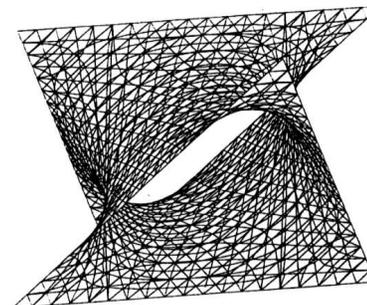
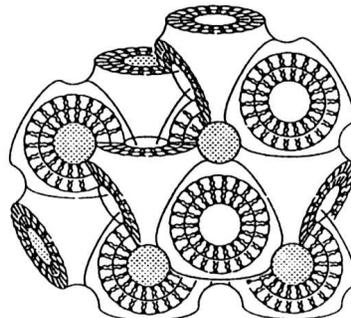
G (gyroid)
 $Ia3d$
Q230



P (primitive)
 $Im3m$
Q229

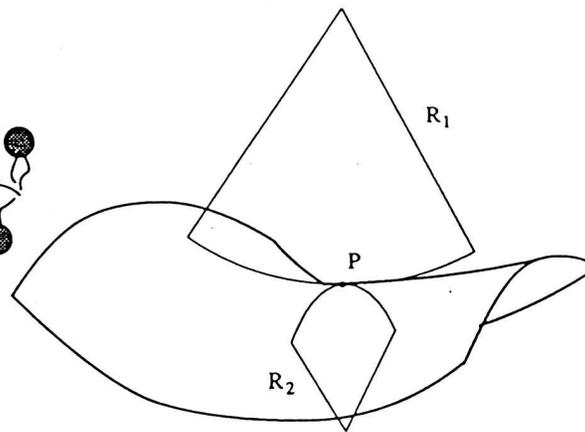
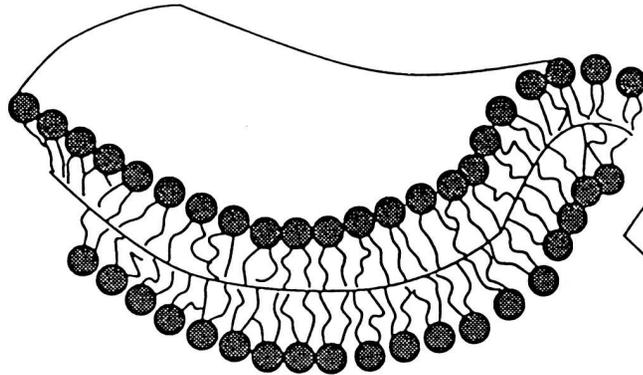


D (diamond)
 $Pn3m$
Q224

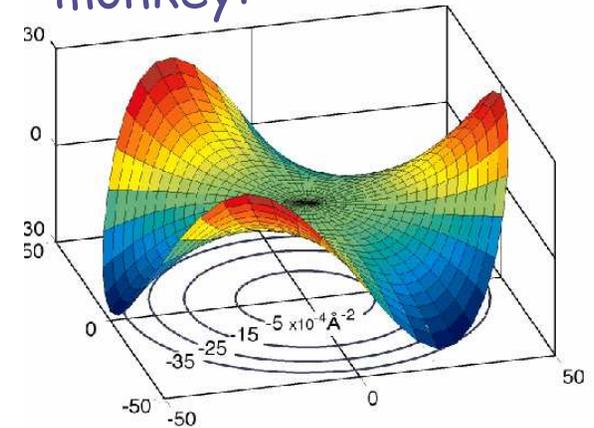


Model of a saddle surface

horse:



monkey:



local curvature

- principal curvatures C_1 & C_2 at points on surface are equal, but opposite in sign: $C_1 = -C_2$
 $C = 1/R$
- mean curvature $H \equiv 0$
 $H = 1/2(1/R_1 + 1/R_2)$
- Gaussian curvature $K \leq 0$
 $K = 1/(R_1 \cdot R_2)$

average curvature

$$K_{\text{ave}} = 2\pi\chi/A_0 a^2$$

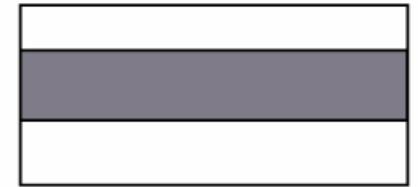
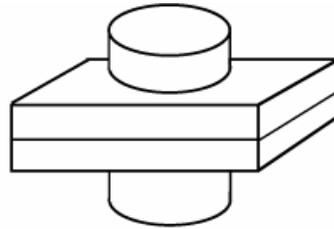
χ ... Euler-Poincaré characteristic

a ...lattice parameter

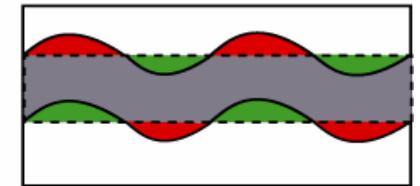
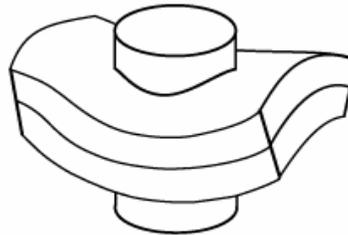
$$A_0 \dots A/V^{2/3}$$

Interactions of a cylindrical particle with membranes

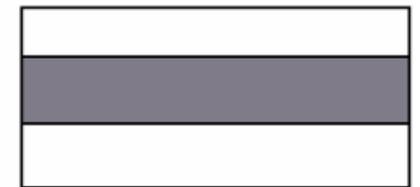
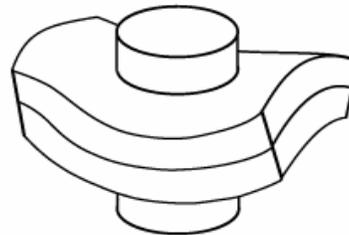
planar membrane



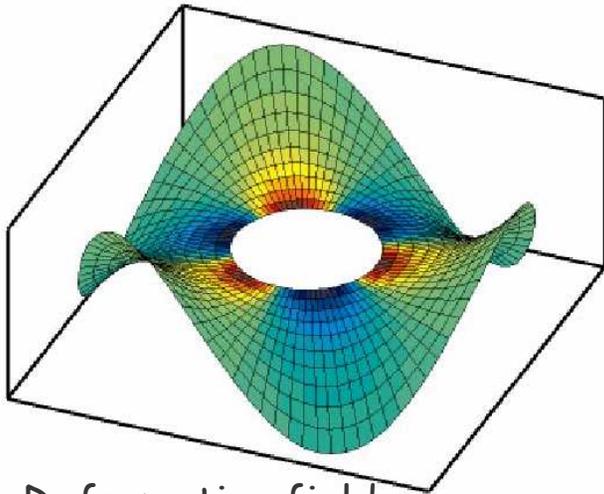
curved membrane
(horse saddle)



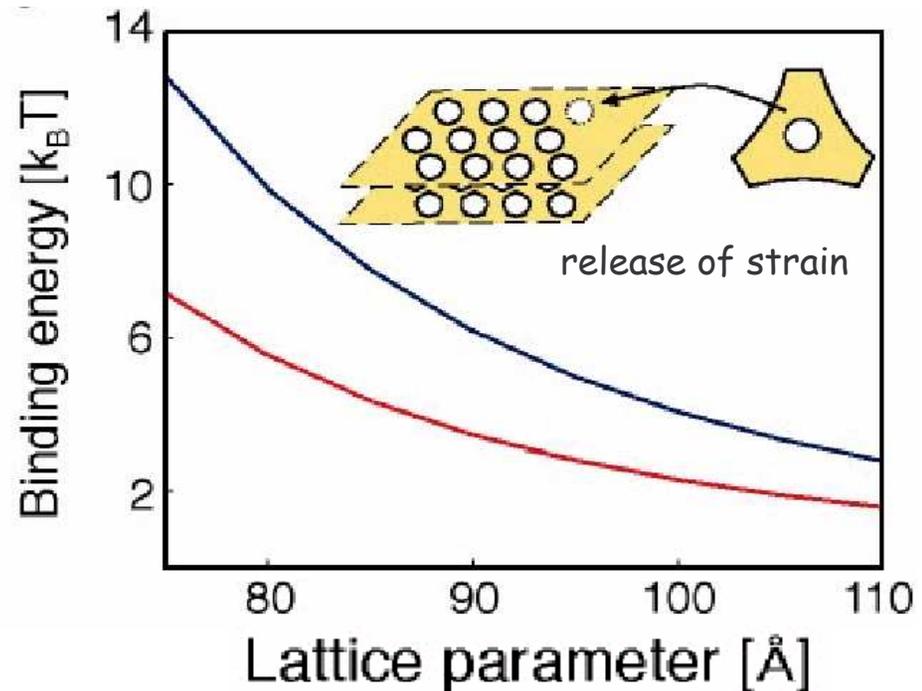
compensation:
• reduced exposure
• strain



Energetics of a protein in a curved membrane (Elastostatics)



Deformation field caused by embedded protein



— $\kappa_m = 2.8 k_B T$ (Vacklin et al., 2000)

— $\kappa_m = 5.0 k_B T$ (Chung and Caffrey, 1994)

⇒ preferential occupation of monkey saddle locations

⇒ large proteins favor formation of lamellar stacks much more than smaller ones

e.g. 4TM ($\frac{1}{2}$ diameter of bR), @ $a=93\text{\AA}$ $\epsilon_{\text{elas}} \sim 0.3-0.6 k_B T$

Total mean curvature energy

horse saddle:

$$\epsilon_{\text{elas.}} = 72\pi(p_3^\pi \pi^2)^2 \left(\frac{R}{a}\right)^4 \kappa$$

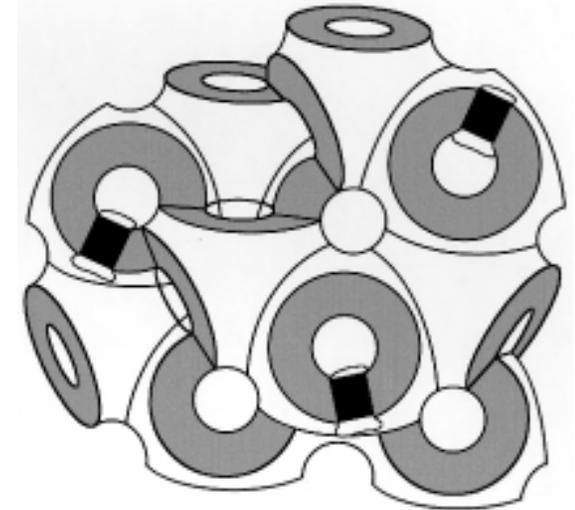
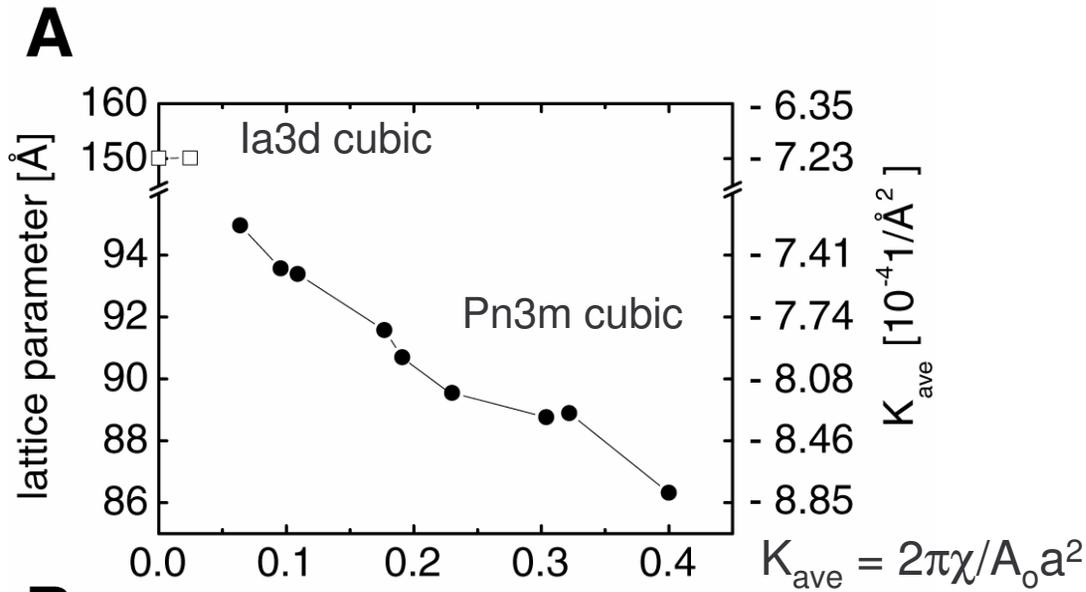
monkey saddle:

$$\epsilon_{\text{elas.}} = 16\pi(p_2^\pi \pi^2)^2 \left(\frac{R}{a}\right)^2 \kappa$$

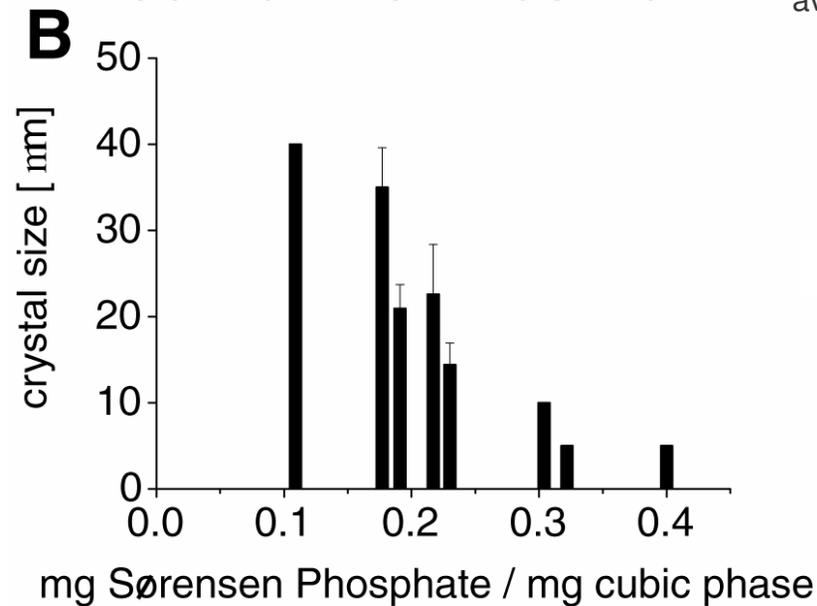
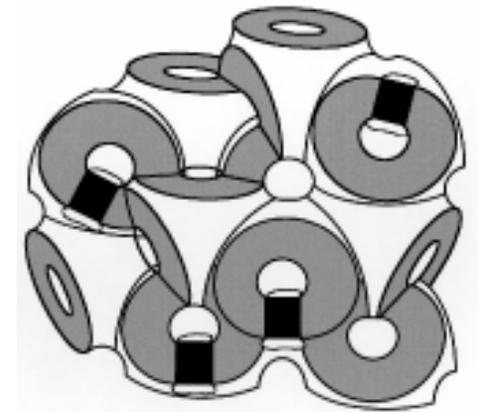
p... thickness, R...protein radius,
a...lattice parameter, κ ...bilayer bending
modulus

Michael Grabe, John Neu, George Oster

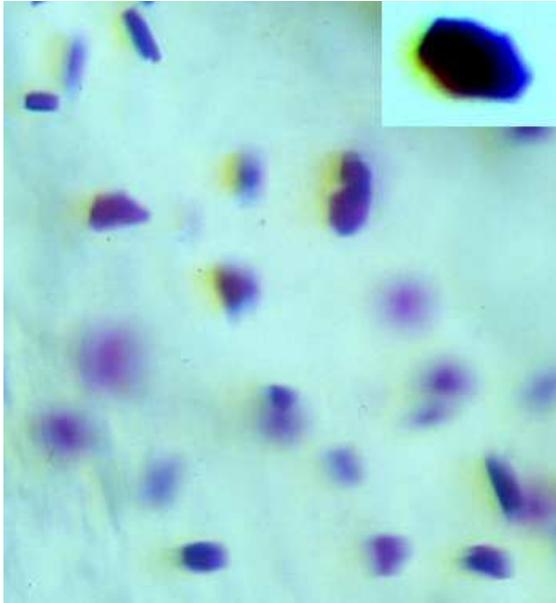
SAXS on bR-LCP crystallization setups



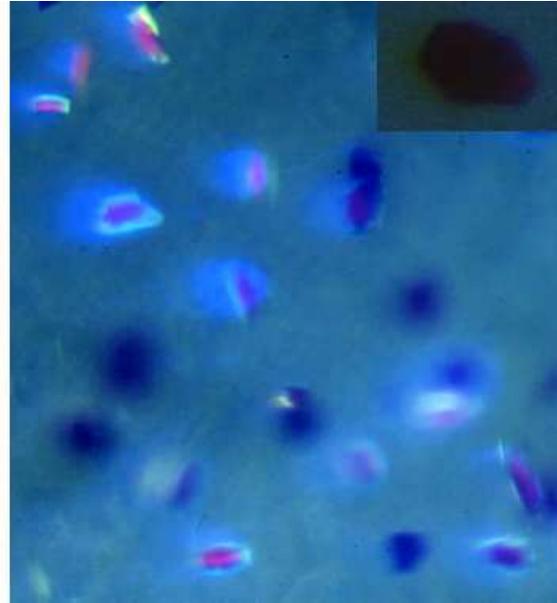
Soerensen salt
↓



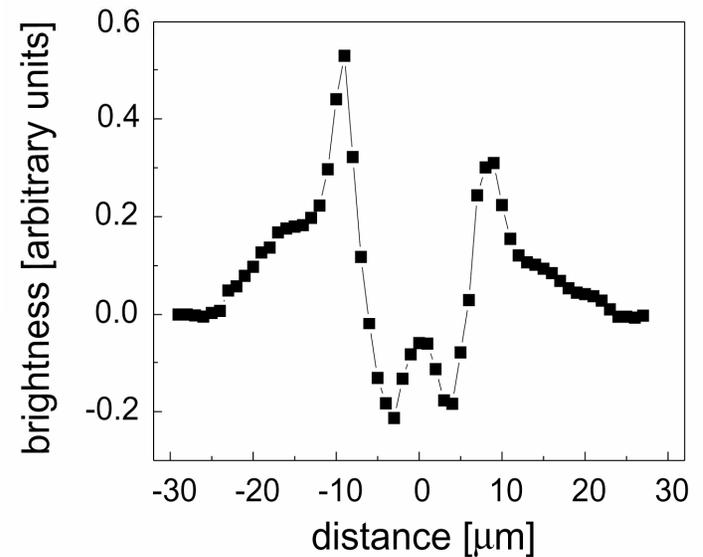
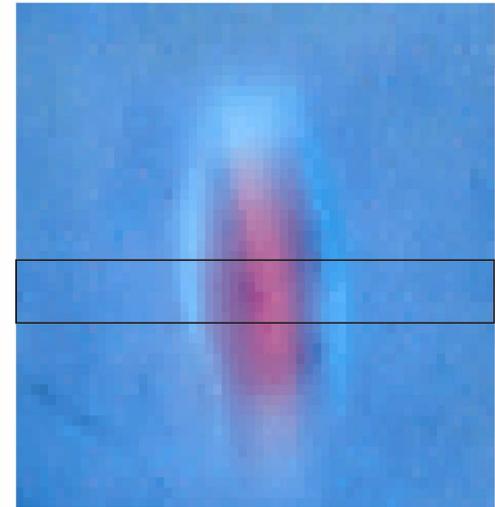
Lipid phase around crystals is not a cubic phase



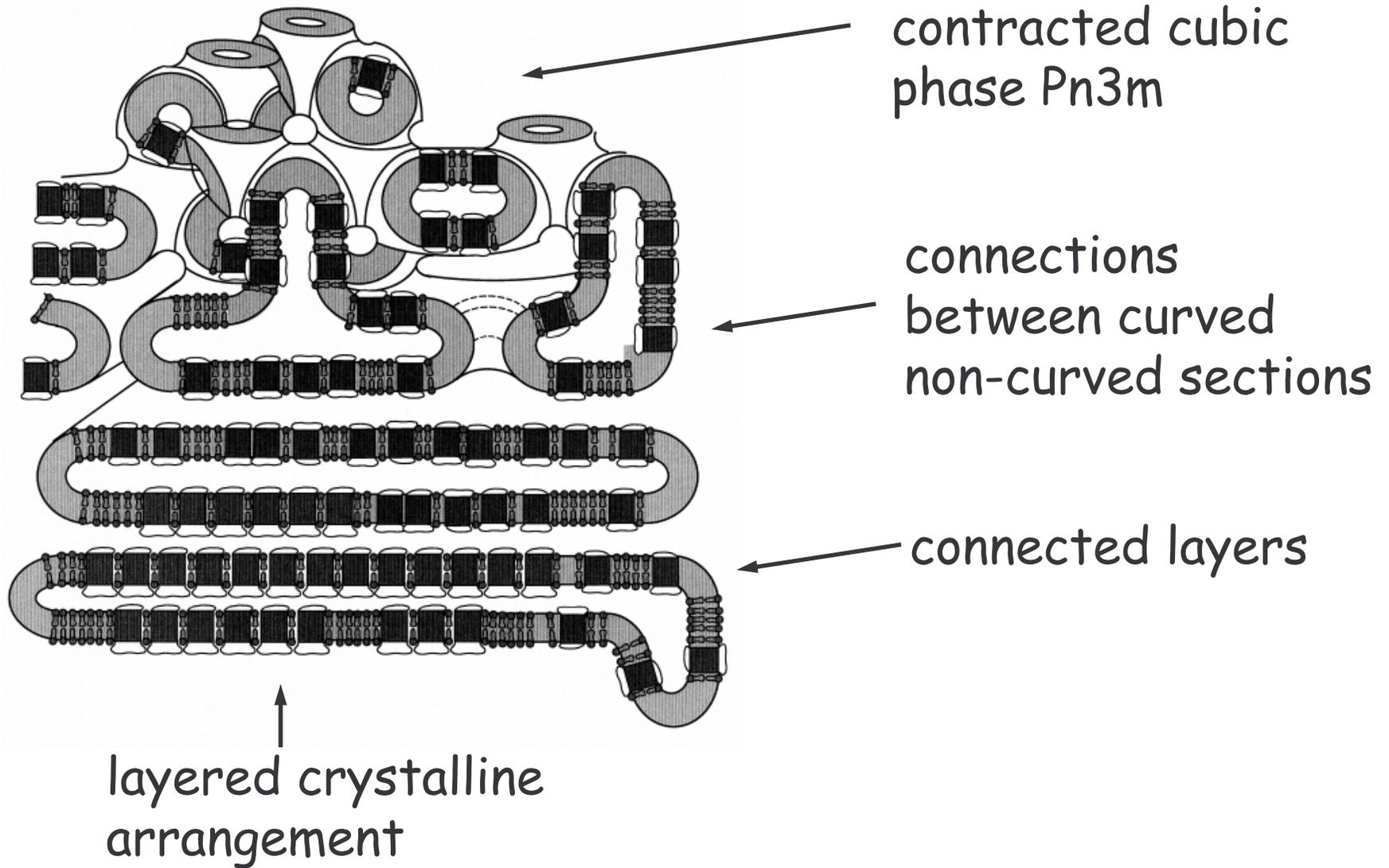
without
polarizer



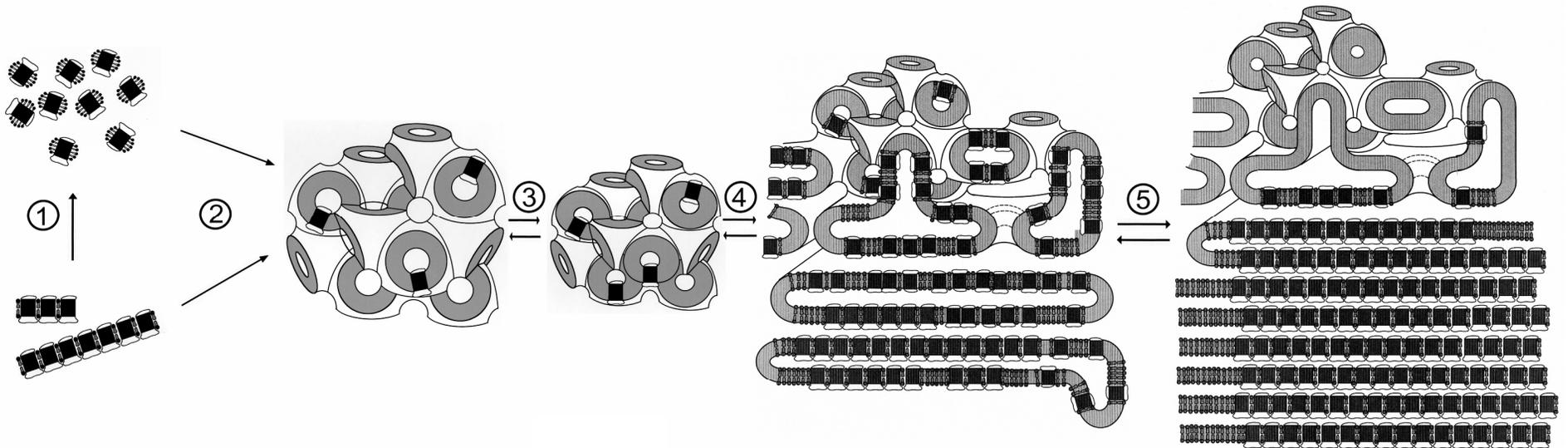
with crossed
polarizer



hypothetical interface of cubic phase with growing protein crystal



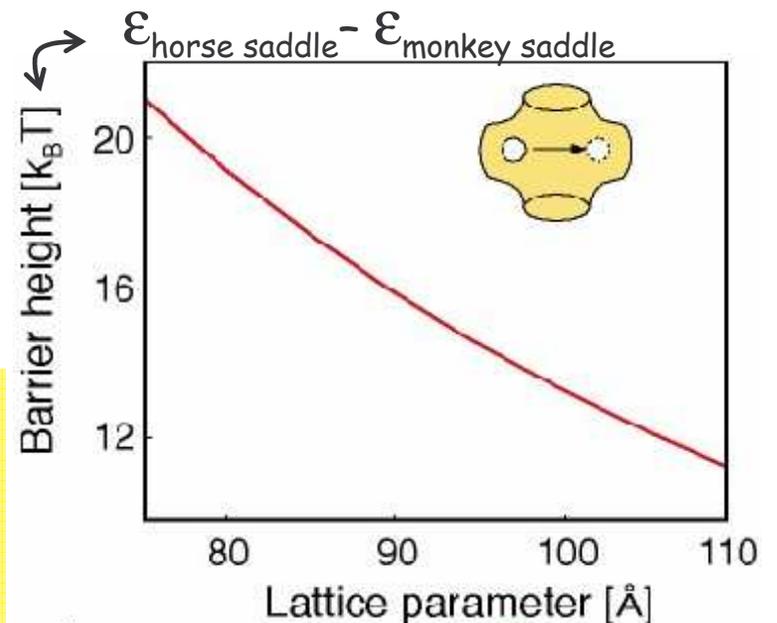
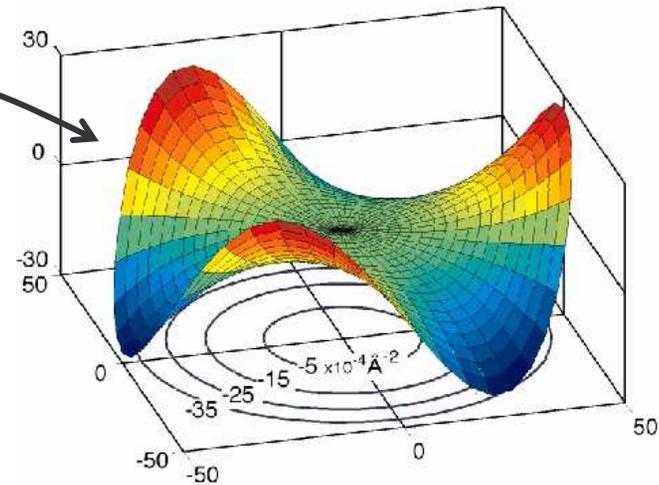
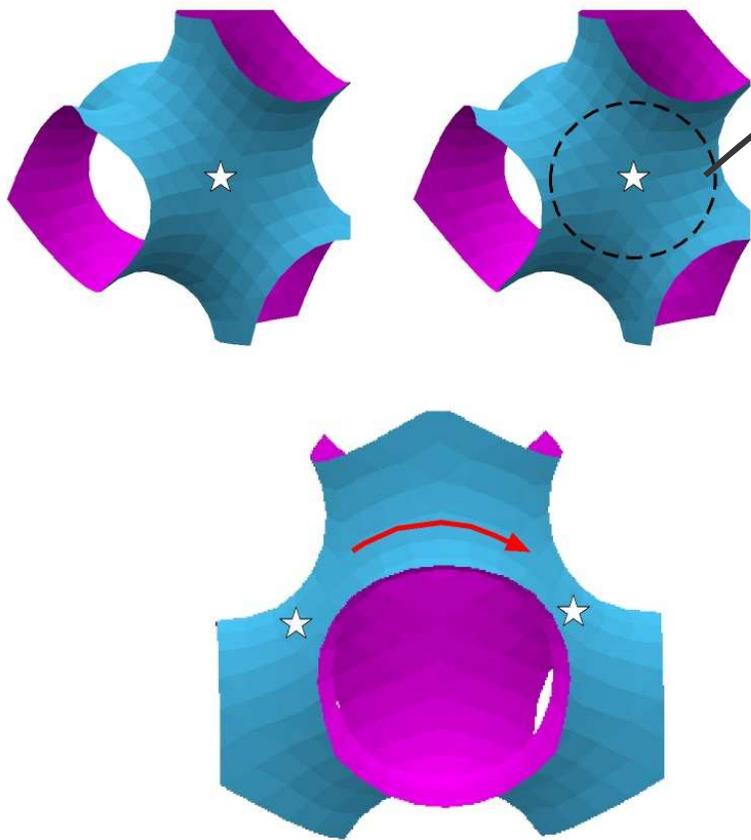
Hypothesis: *in cubo* crystallization mechanism



Hypothesis for the Crystallization Mechanism

- Incorporation of bacteriorhodopsin into lipid matrix
- Addition of salt contracts cubic phase = increases curvature
- Nucleation by local phase separation of components
- Growth of crystals by lateral diffusion of protein in membrane

Diffusion of membrane protein in lipidic cubic phase



⇒ horse saddles are local energy barrier to diffusion

⇒ modified net diffusion rate: $D(a) = D_0 e^{(-u(a))/k_B T}$

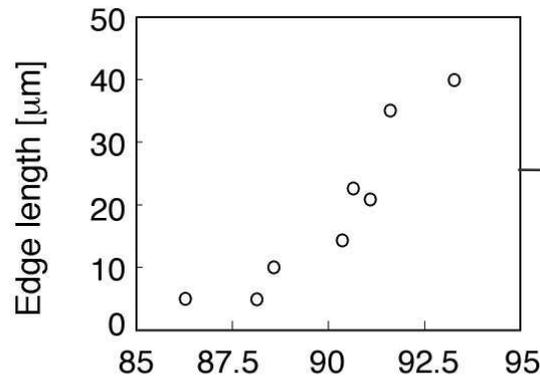
for bR: $D_0 \sim 3.3 \mu\text{m}^2/\text{s}$

D_0 ... flat bilayer diffusion constant

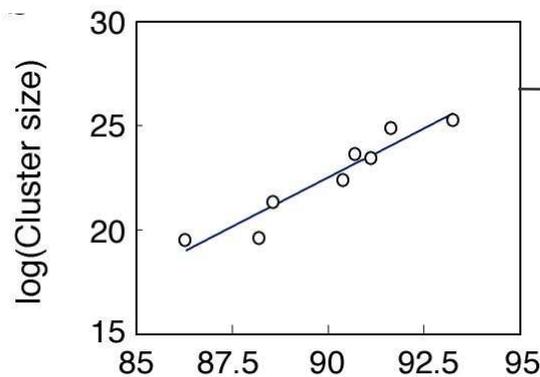
u ... barrier crossing energy

Free energy barrier to crystallization & critical cluster size

from
experimental
data:



geometrical
considerations:



crystal nucleation theory*

$$\epsilon_{\text{electrostatic}} = 4.1 k_B T$$

Free energy barrier to crystallization

$$G_0(93.3\text{\AA}) = 43.6 k_B T$$

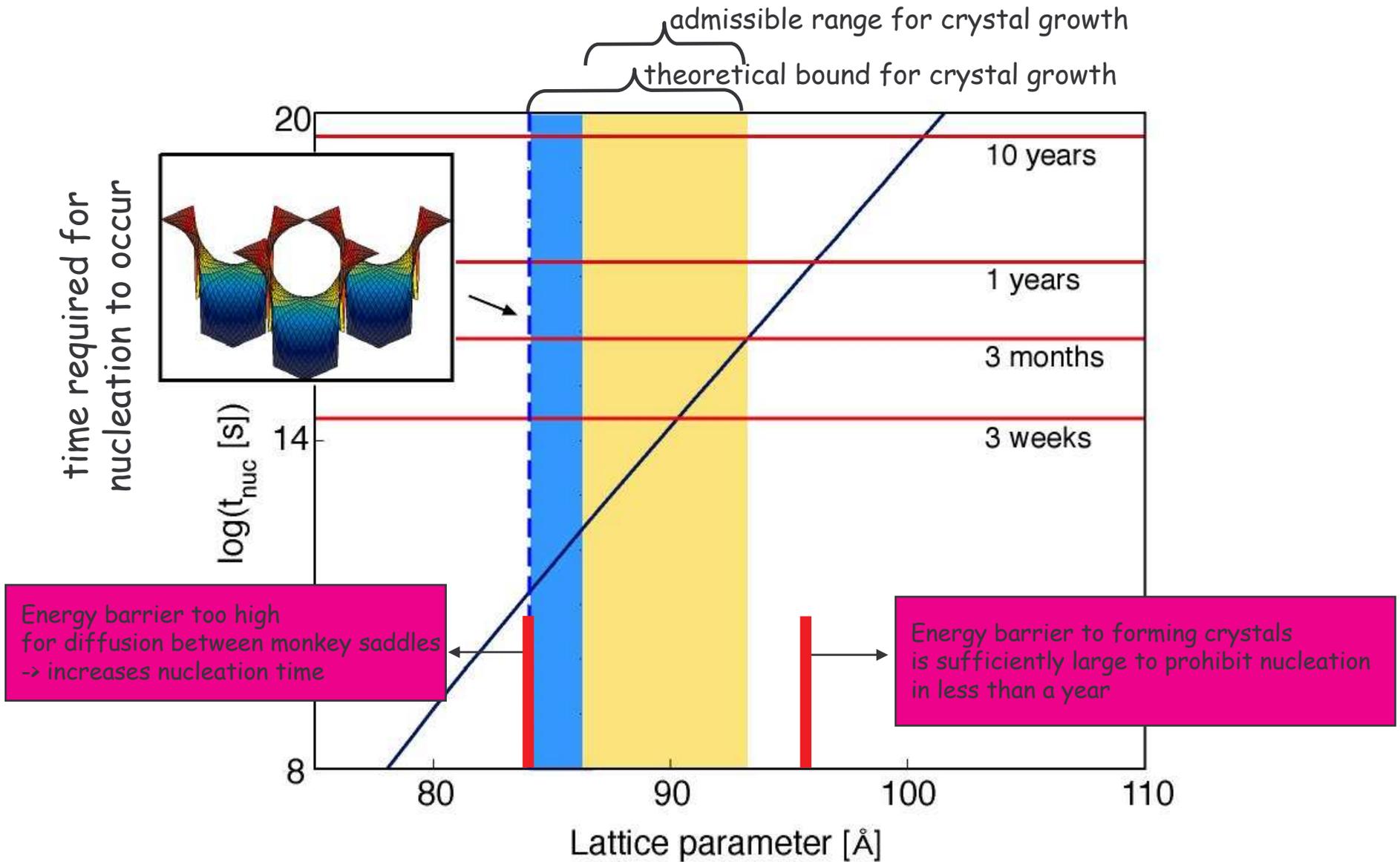
(compare to $\sim 50 k_B T$ for soluble proteins,
ten Wolde et al., 1997)

critical cluster size

$$S_{\text{crit}} = 29$$

* see appendices of Grabe, Neu, Oster, Nollert, Biophys.J. 84, 854-868, 2003

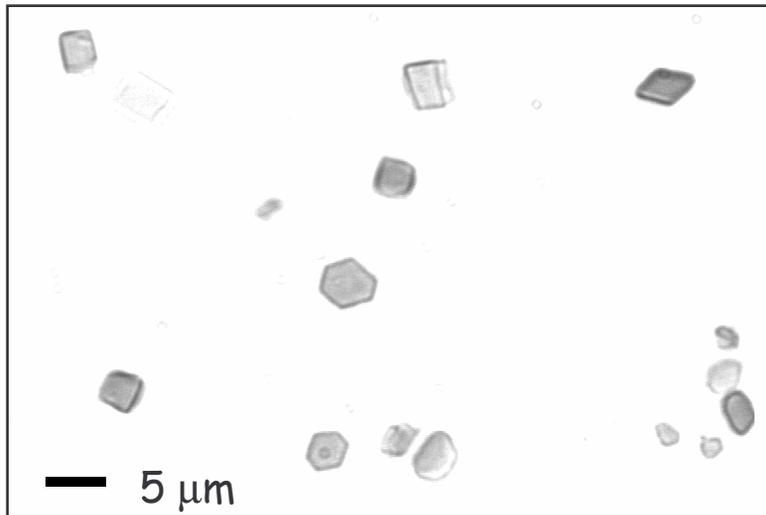
Upper and lower bounds for crystallization



Test & Predictions of Hypothesis

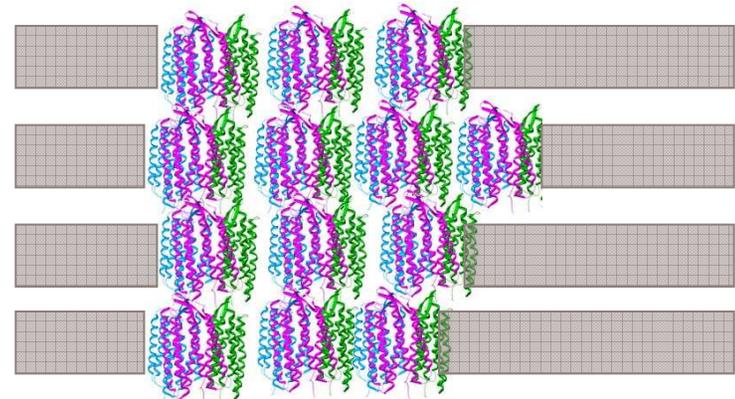
crystallization by other means *layered crystal architecture*
that contract cubic phase

- PEG
- Deyhydration



observed in:

- Photosynthetic Reaction Center
- Bacteriorhodopsin
- Halorhodopsin
- Sensory Rhodopsin II



- non-crystallizability of <5 TM
- crystallization occurs only in window a $\sim 85 - 95\text{\AA}$