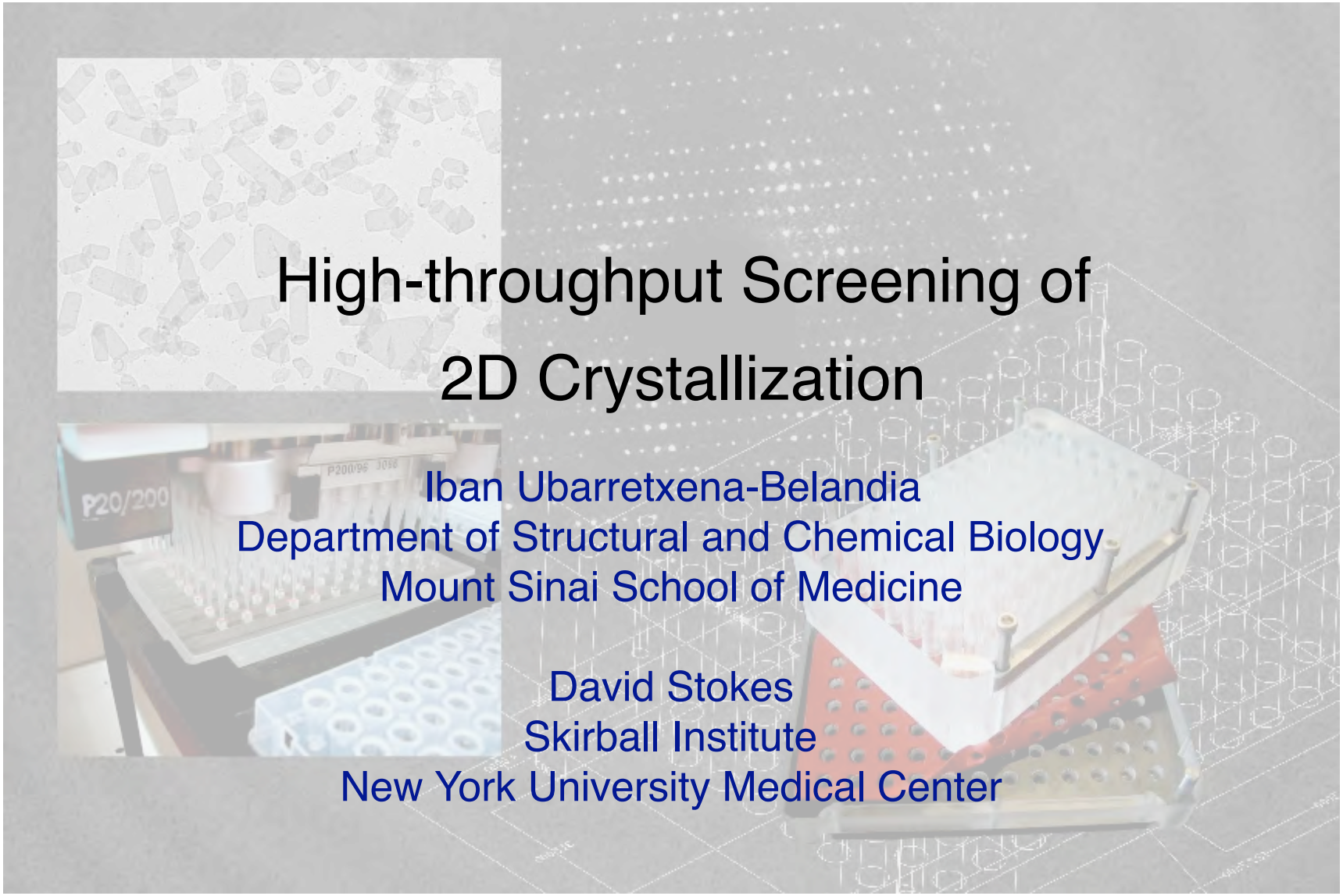

High-throughput Screening of 2D Crystallization

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Mount Sinai School of Medicine

David Stokes
Skirball Institute
New York University Medical Center

2DX has been applied to a number of membrane proteins

Table 1. 3D structures of membrane proteins determined by electron crystallography. Atomic-resolution structures are highlighted in bold.

Integral Membrane Protein	Resol. (Å)	Year	Reference
Bacteriorhodopsin	7.0	1975	(Henderson and Unwin)
Bacteriorhodopsin	6.5	1983	(Leifer and Henderson)
Bacteriorhodopsin	3.5	1990	(Henderson, Baldwin et al.)
Plant LHC-II	3.4	1991	(Kühlbrandt and Wang)
Porin PhoE	6.0	1991	(Jap, Walian et al.)
Acetylcholine receptor	9.0	1993	(Unwin)
Frog Rhodopsin frog	6.5	1997	(Unger, Hargrave et al.)
Bacteriorhodopsin	3.0	1997	(Kimura, Vassylyev et al.)
Plant photosystem II RC	8.0	1998	(Rhee, Morris et al.)
Neurospora H^+-ATPase	8.0	1998	(Auer, Scarborough et al.)
Gap junction channel	7.5	1999	(Unger, Kumar et al.)
NhaA Na/ H^+ antiporter	7.0	2000	(Williams)
Glycerol channel GlpF	6.9	2000	(Stahlberg, Braun et al.
Halorhodopsin	5.0	2000	(Kunji, von Gronau et al.)
Aquaporin-1	3.8	2000	(Murata, Mitsuoka et al.)
Glutathione transferase	6.0	2002	(Holm, Morgenstern et al.)
Oxalate transporter OxlT	6.5	2002	(Hirai, Heymann et al.)
Ca^{2+}-ATPase	6.5	2002	(Xu, Rice et al.)
Bovine Rhodopsin	5.5	2003	(Krebs, Edwards et al.)
EmrE multidrug transporter	7.0	2003	(Ubarretxena-Belandia et al.)
Eye lens Aquaporin 0	1.9	2005	(Gonen, Cheng et al.)
Acetylcholine receptor	4.0	2005	(Unwin)
Human aquaporin 2	4.5	2005	(Schenk, Werten et al.)
Plant Aquaporin SoPIP2	5.0	2005	(Kukulski, Schenk et al.)
Sec YEG complex	8.0	2005	(Bostina, Mohsin et al.)
Glutathione transferase	3.2	2006	(Holm, Bhakat et al.)
Gap junction channel	7.0	2007	(Oshima, Tani et al.)
Aquaporin-4	2.8	2009	(Tani, Mitsuma et al.)
hCTR1 Cu transporter	7.0	2009	(De Feo, Aller et al.)

Pros and cons of electron crystallography of 2DX

PROS

Membrane proteins studied in their natural lipid bilayer environment.

Molecular packing is less constrained and can accommodate conformational changes.

Membrane proteins in a 2DX are fully accessible to the aqueous medium.

CONS

Screening of 2D crystallization trials is slow and optimal 2D crystals for structure determination at high-resolution are relatively rare.

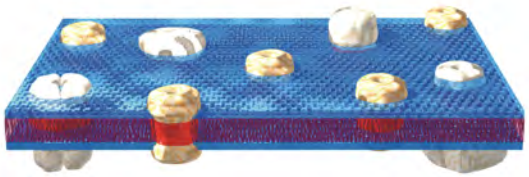
Data collection is tedious as many crystals and different tilts have to be merged in dataset.

2D crystals are flimsy.

Obtained 3D map suffers from anisotropic resolution.

General strategy to 2D crystallize membrane proteins

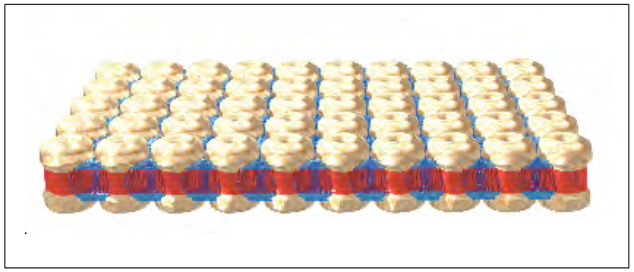
Expression



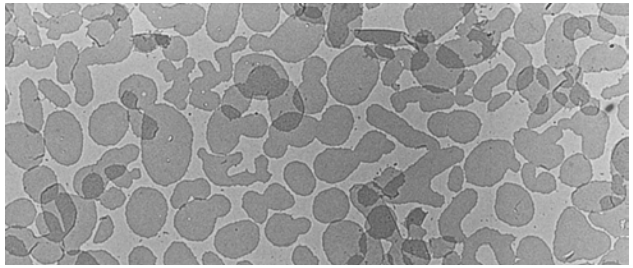
Solubilization

Purification

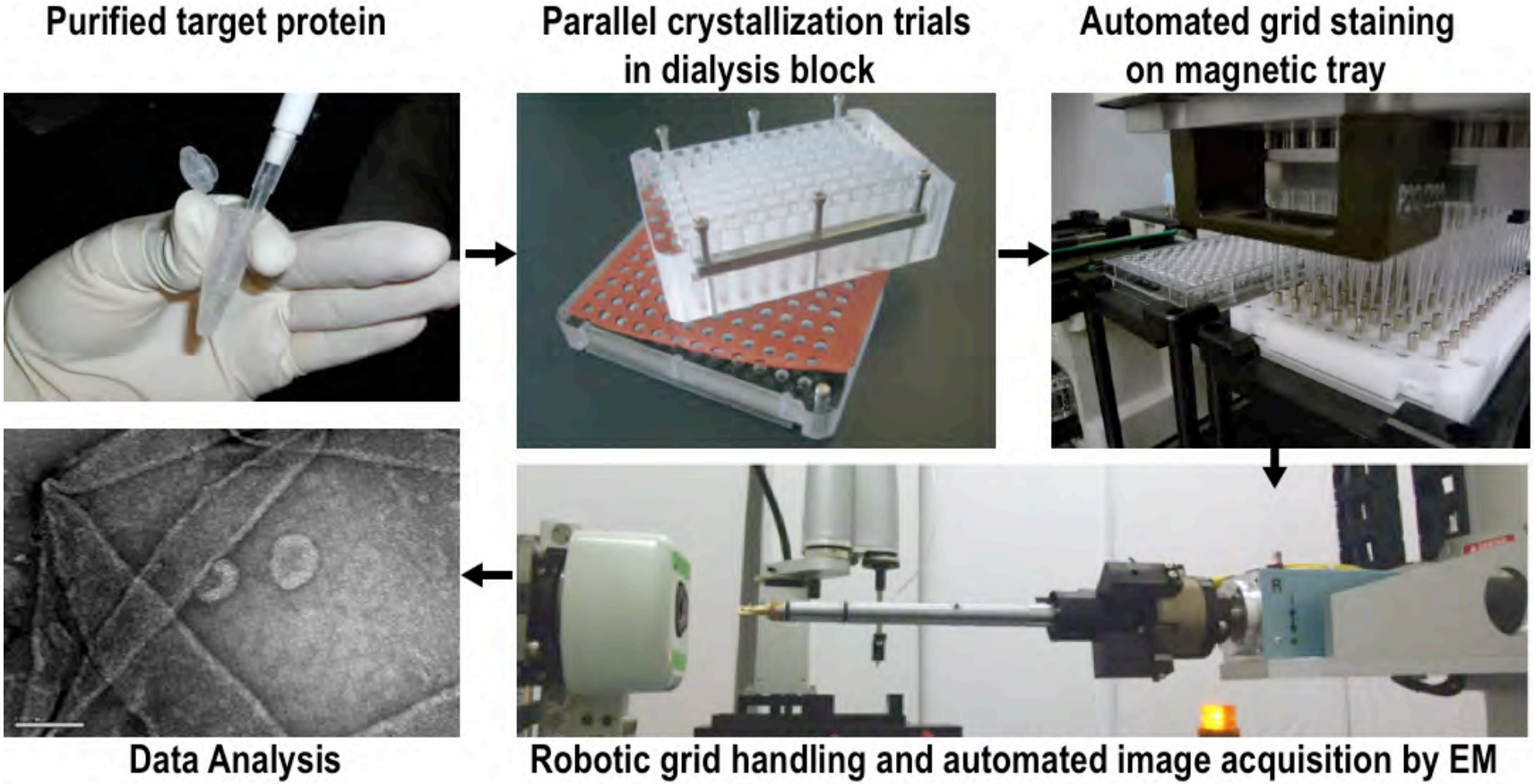
2D crystallization



Imaging



Our current 2DX pipeline

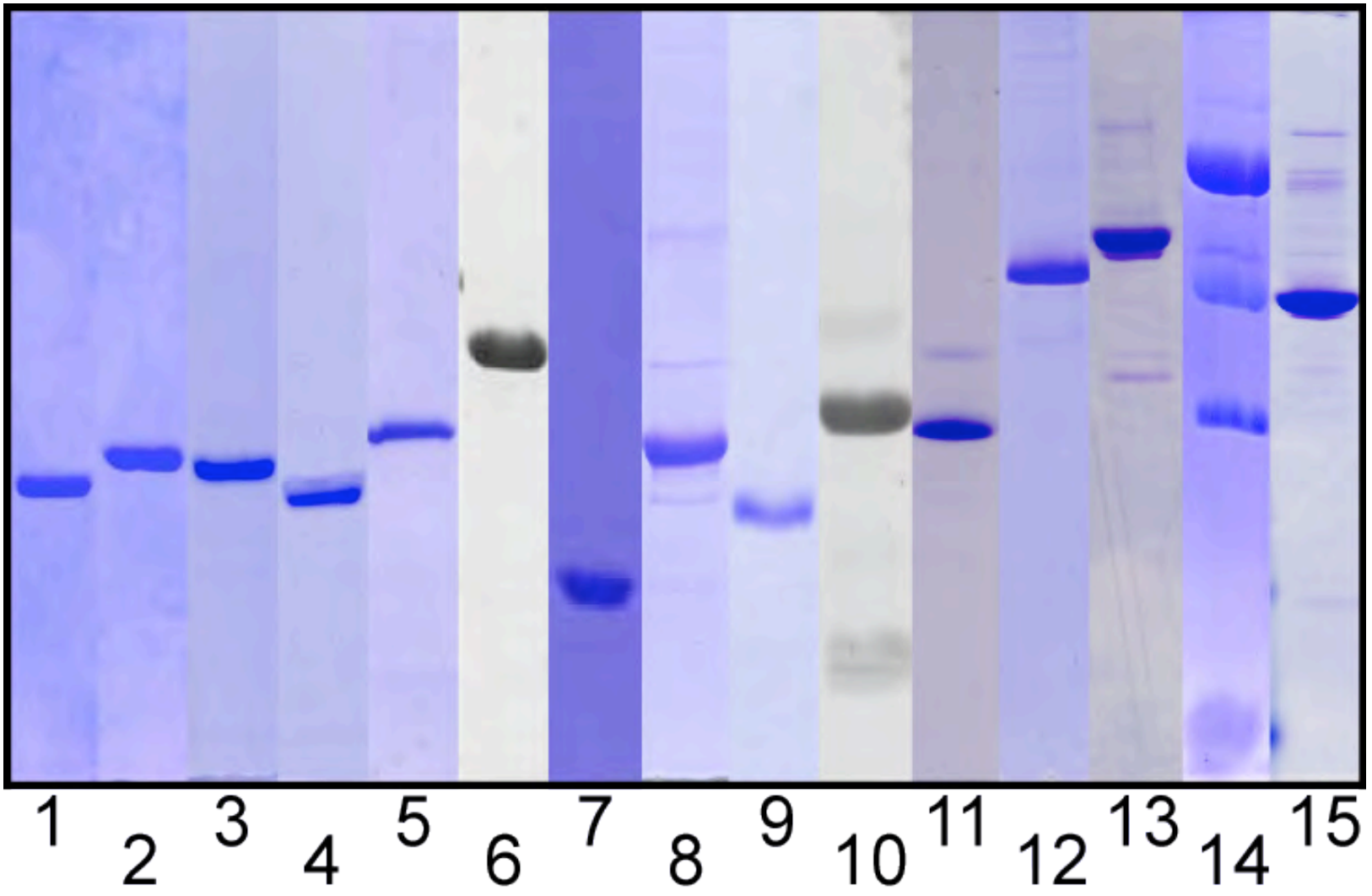


The membrane protein targets

Protein	Organism	(Putative) Function	MW (kDa)	pI	TMD	N-term	% TM	Screens
E2P1	*M.voltae*	Protease	25.9	8.7	4	Inside	45	4
E2P2	*M.maripaludis*	Protease	25.9	5.0	4	Inside	39	3
E2P3	*M.marisnigri*	Protease	31.8	5.3	7	Inside	59	2
E2P4	*M.hungatei*	Protease	32.9	7.8	9	Inside	61	2
P2A3	*S.oneidensis*	Cation efflux family protein	32.5	5.4	5	Inside	36	5
Rhomboid PA3086	*P.aeruginosa*	Intramembrane protease	31.8	9.9	6	Inside	45	4
YkgB-D332	*E.coli*	Unknown	21.9	6.0	3	Inside	38	1
YkgB-D36	*E.coli*	Unknown	21.8	5.7	3	Inside	37	4
β1-adrenergic receptor	*M.gallopavo*	G-protein coupled receptor	54.1	9.3	7	Outside	33	3
Rhomboid GlpG	*E.coli*	Intramembrane protease	31.3	9.2	6	Inside	46	1
Cytochrome b561	*P.aeruginosa*	Electron carrier activity	20.6	9.6	4	Inside	47	1
E1 protein	Semliki Forest Virus	Inducer of membrane fusion	47.4	7.6	0 (1)	Outside	5	4
P40B7	*B.subtilis*	Sporulation kinase C	48.8	6.3	2	Inside	10	1
Kdp-ATPase (4 subunits)	*E.coli*	High-affinity K-pump	159.2	5.2-9.4	17	3 in, 1out	31	1
P39H10	*K. pneumoniae*	Diguanylate cyclase	45.7	6.5	2	Inside	11	1

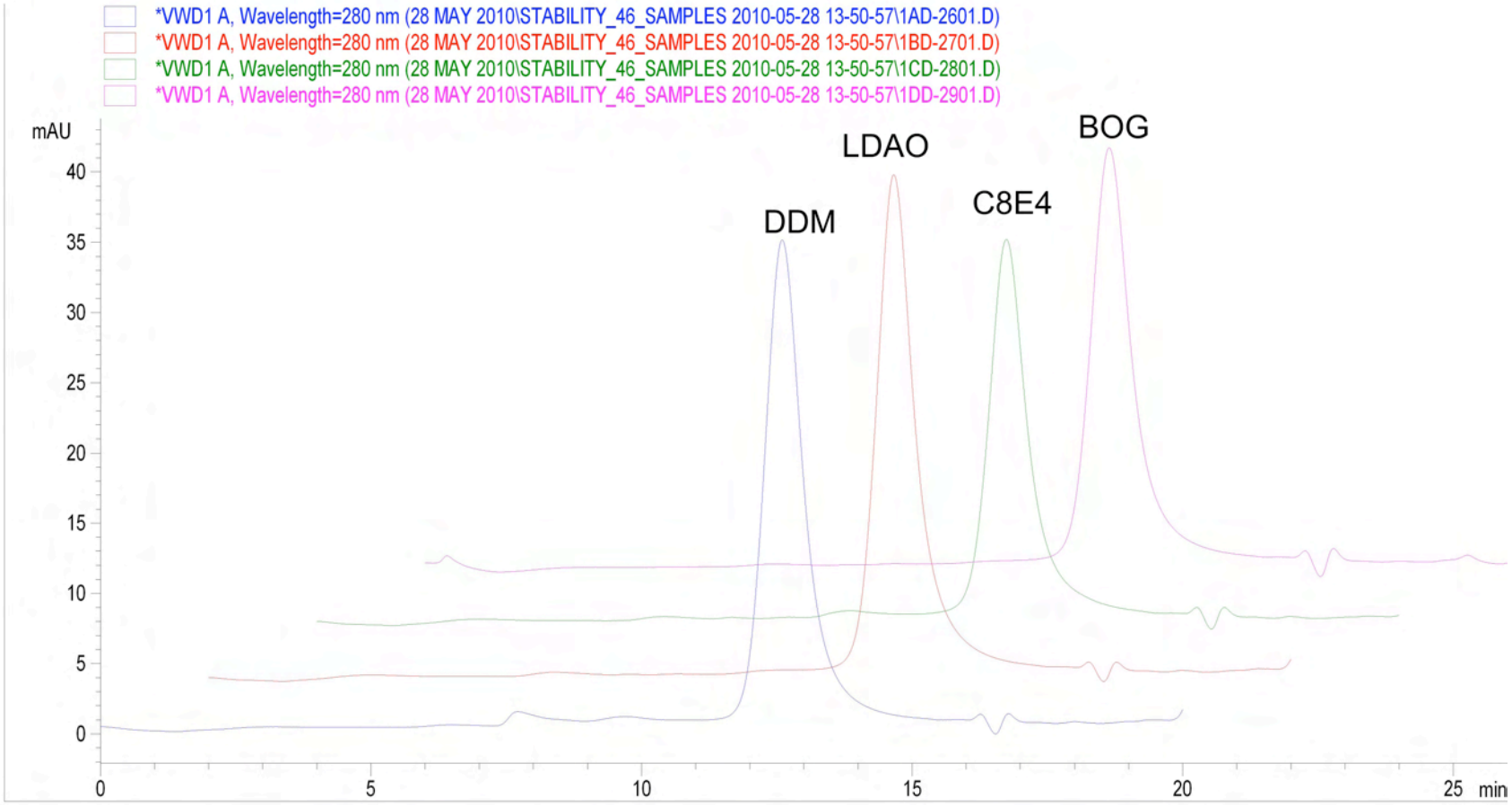
PI: isoelectric point; TMD: transmembrane domain; N-Term: location of N-teminus; %TM: % of transmembrane sequence ; Screens: Numl

Purity of the membrane protein targets



Behavior of membrane protein targets in detergent

A given protein and its homologs are selected based on their behavior on SEC



Making sure we know the detergent concentration of the membrane protein

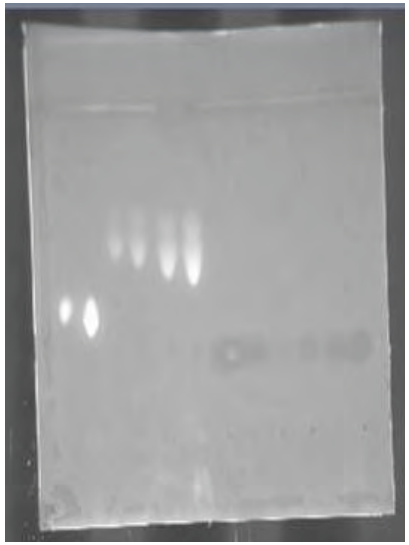
Colorimetric detection

Condensation reaction of furfural derivatives (generated by dehydration of sugars in concentrated H_2SO_4) with aromatic molecules such as phenol > spectrophotometer

- Nasty reactants
- Only possible to use on glucosidic/maltosidic or bile-salt derived detergents.

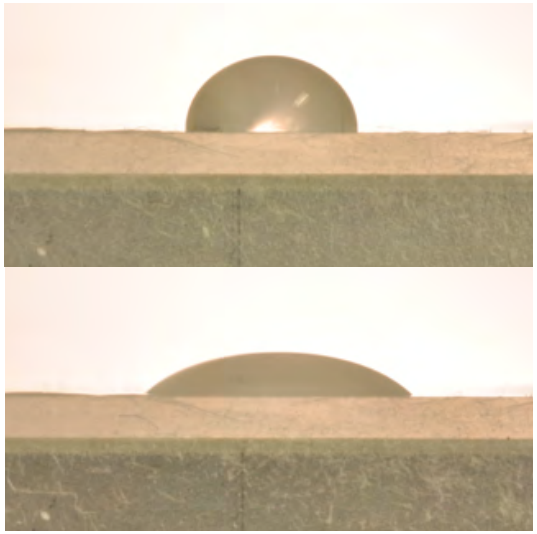
TLC – Thin layer chromatography

Based on mobile phase and detection method, detergents and lipids can be both identified and quantified.

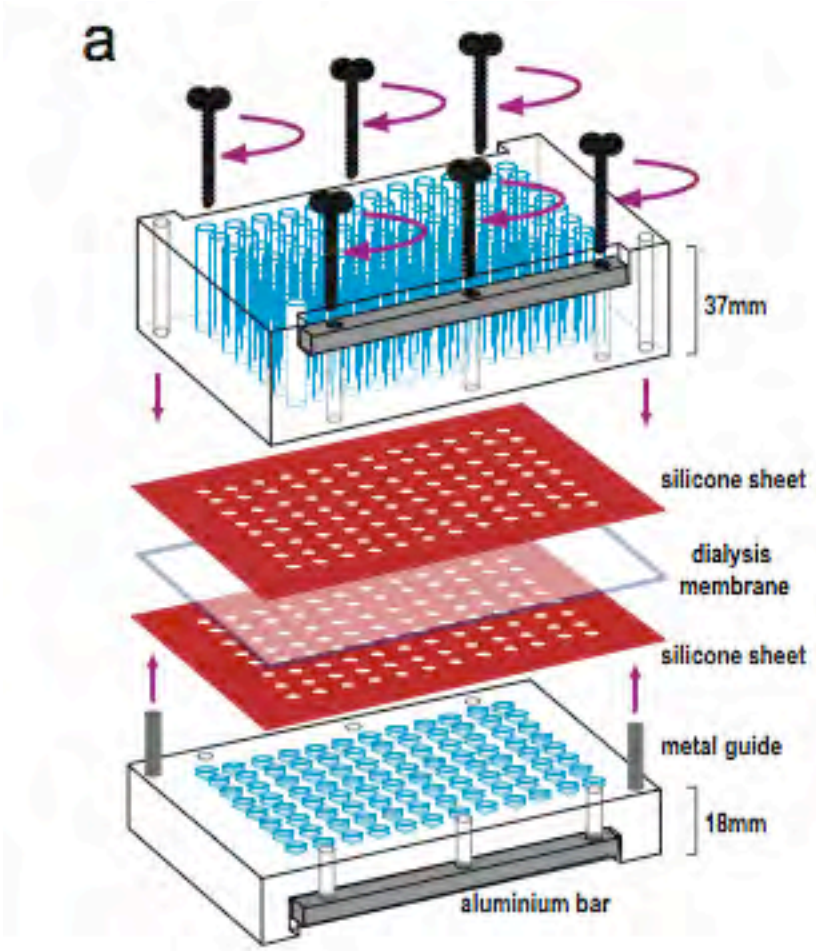


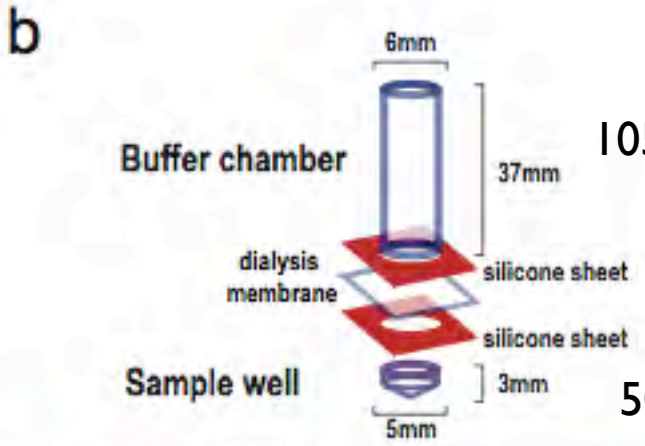
Drop shape

Detergents modify surface tension of aqueous solutions. Both shapes and contact angles of drops are affected.



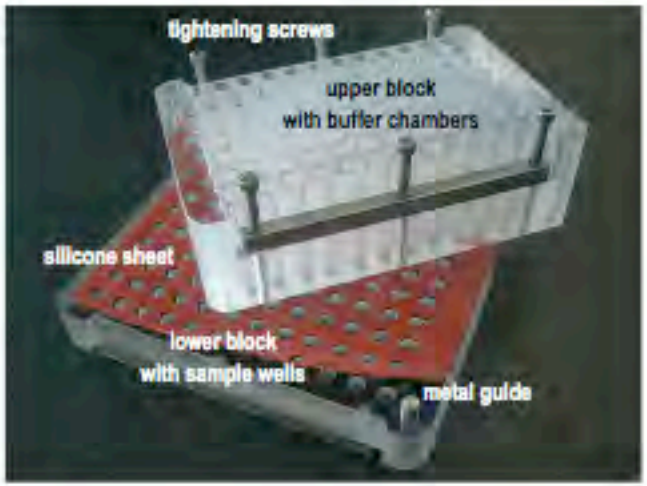
2D crystallization in parallel





1050 µl buffer

50 µl sample



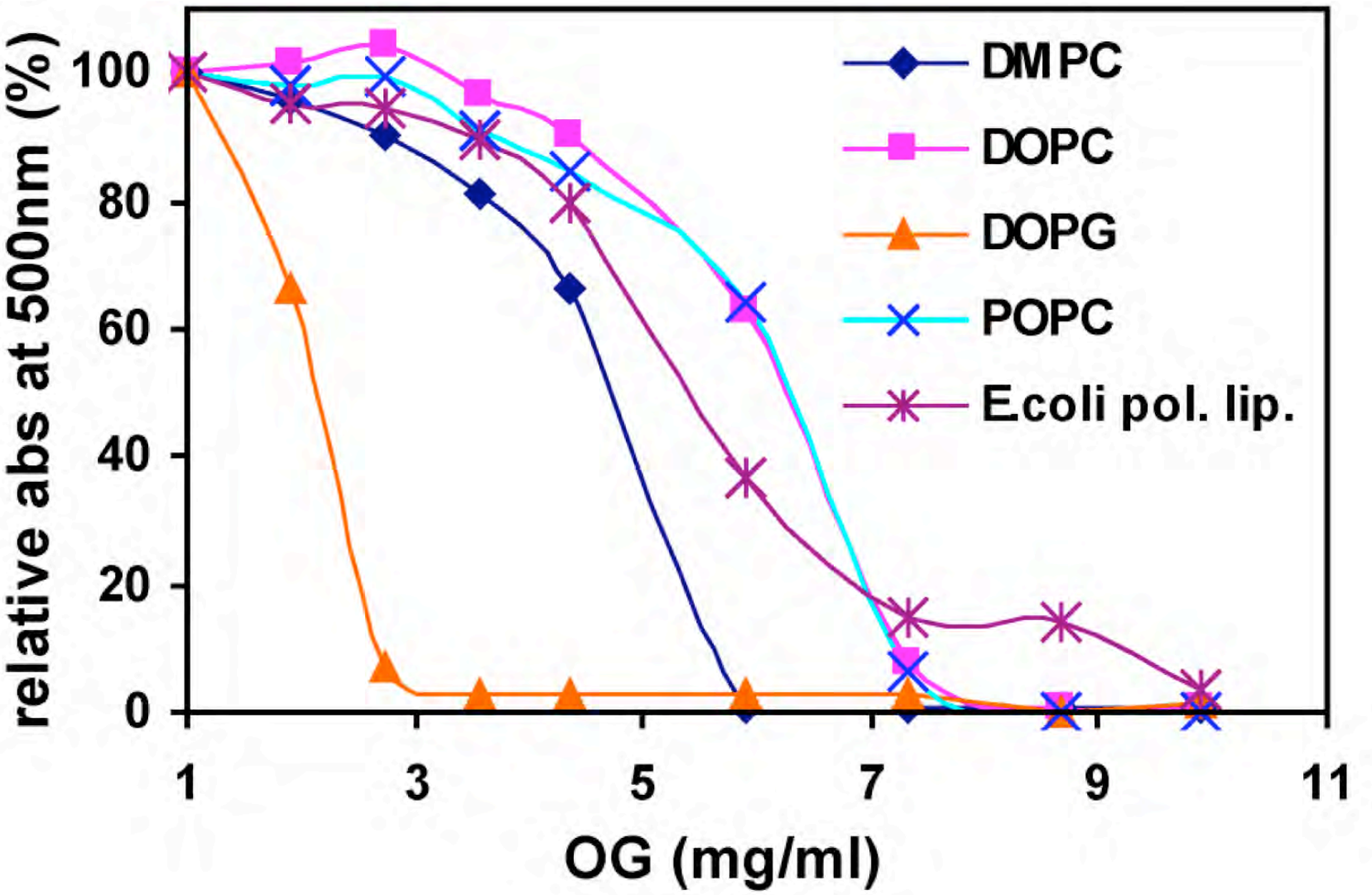
Standard conditions for initial screen

Lipids: DMPC, DOPC, POPC, DOPG, E.coli polar lipid extract
pH: 6.0 (20mM MES), 7.0 (20mM TES), 8.0 (20mM TES)
LPRs: 0.25, 0.75, 1.5
Cations (mM): Na^{+} (100), Mg^{2+} (10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	DOPC 0.25	POPC 0.75	DOPG 1.5	X	DOPC 0.25	POPC 0.75	DOPG 1.5	X	DOPC 0.25	POPC 0.75	DOPG 1.5
B	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25
C	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75
D	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5
E	X	DOPC 0.25	POPC 0.75	DOPG 1.5	X	DOPC 0.25	POPC 0.75	DOPG 1.5	X	DOPC 0.25	POPC 0.75	DOPG 1.5
F	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25	DMPC 0.25	DOPC 0.75	POPC 1.5	E.coli 0.25
G	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75	DMPC 0.75	DOPC 1.5	DOPG 0.25	E.coli 0.75
H	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5	DMPC 1.5	POPC 0.25	DOPG 0.75	E.coli 1.5

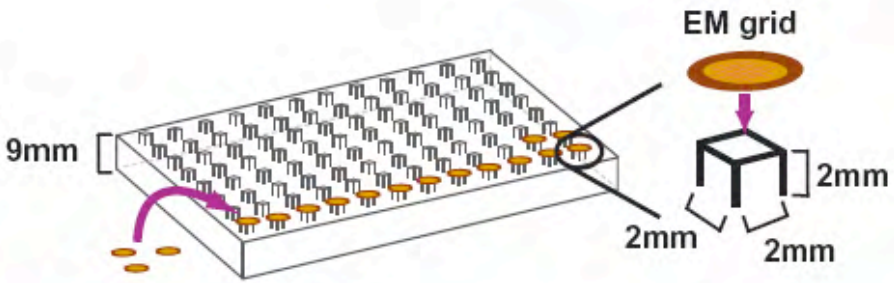
pH6.0, pH7.0, pH8.0
Rows A-D contain 100mM Na^{+}; Rows E-H contain 10mM Mg^{2+}

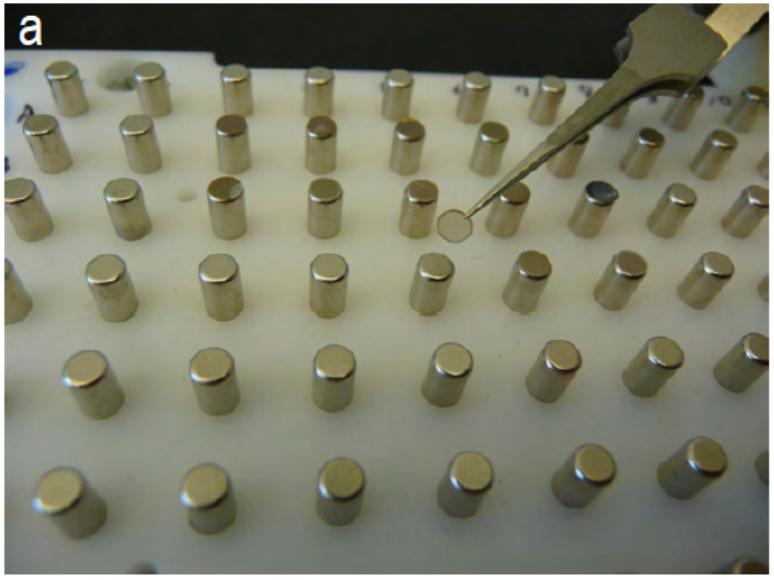
Making mixed micelles in the optimal detergent

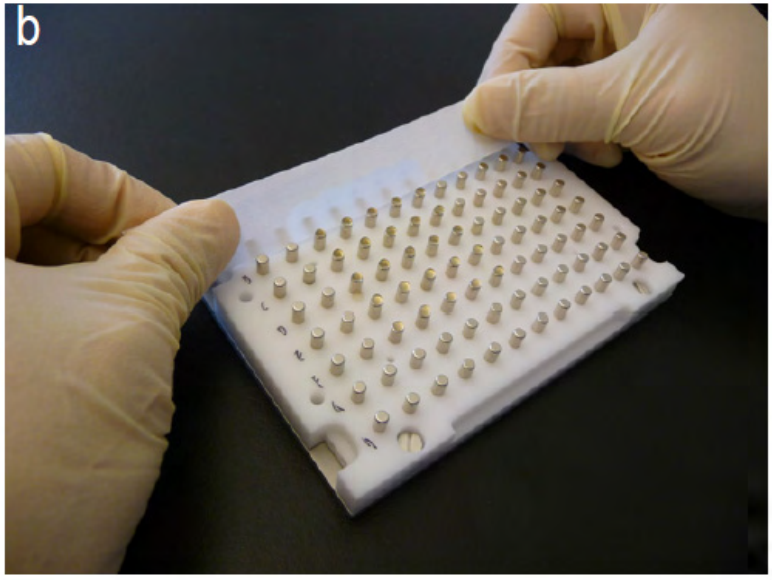


Negative staining on a 96-well format

Negatively staining of 96
specimens for evaluation by EM

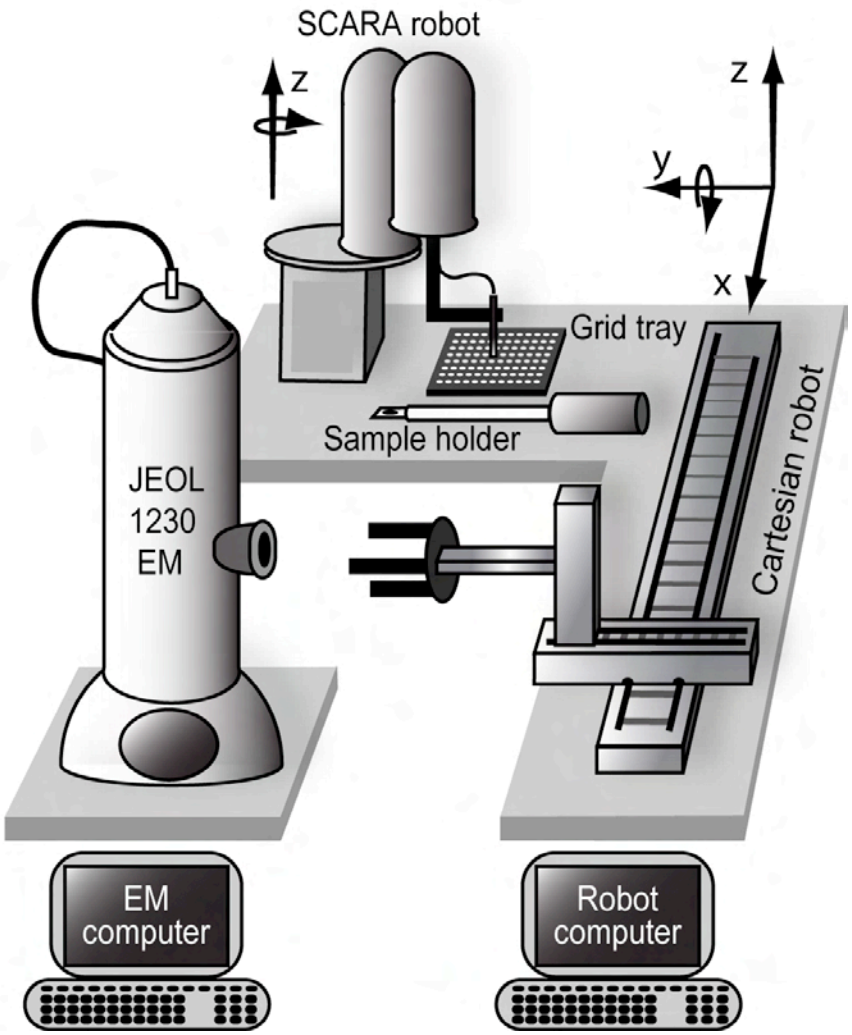




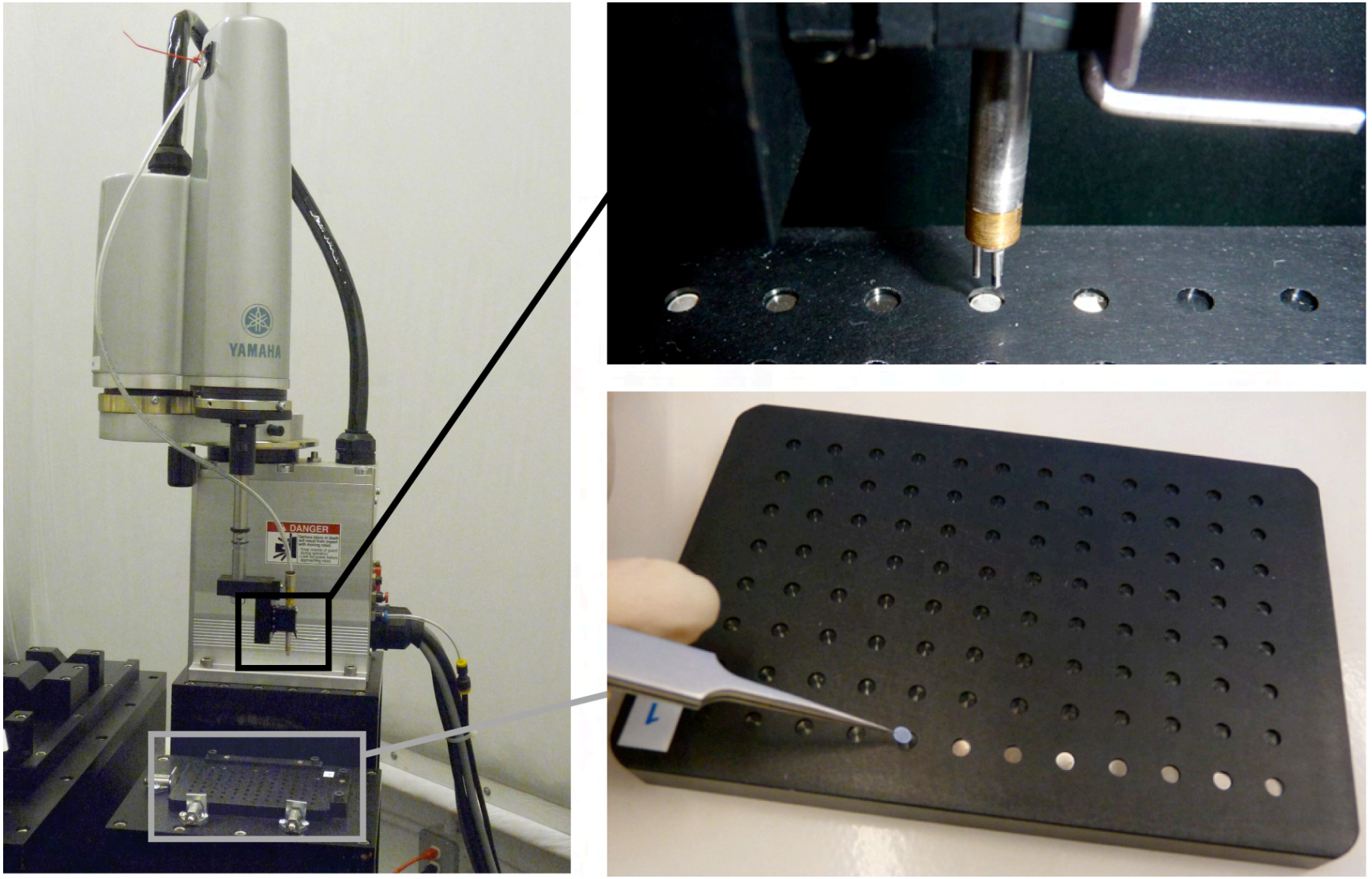


Automated loading and imaging of specimens

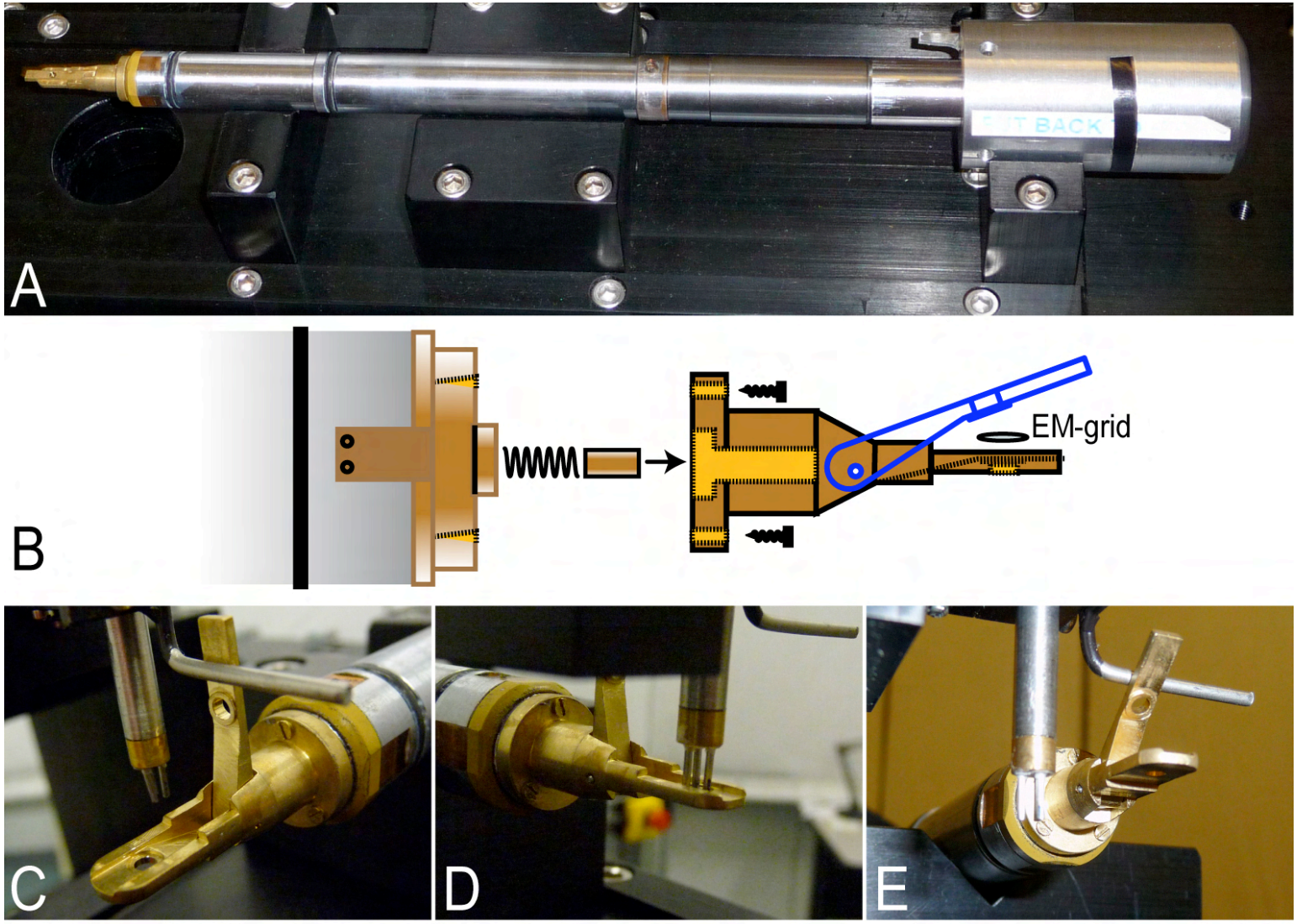
Need to load and image 96 grids
per crystallization block



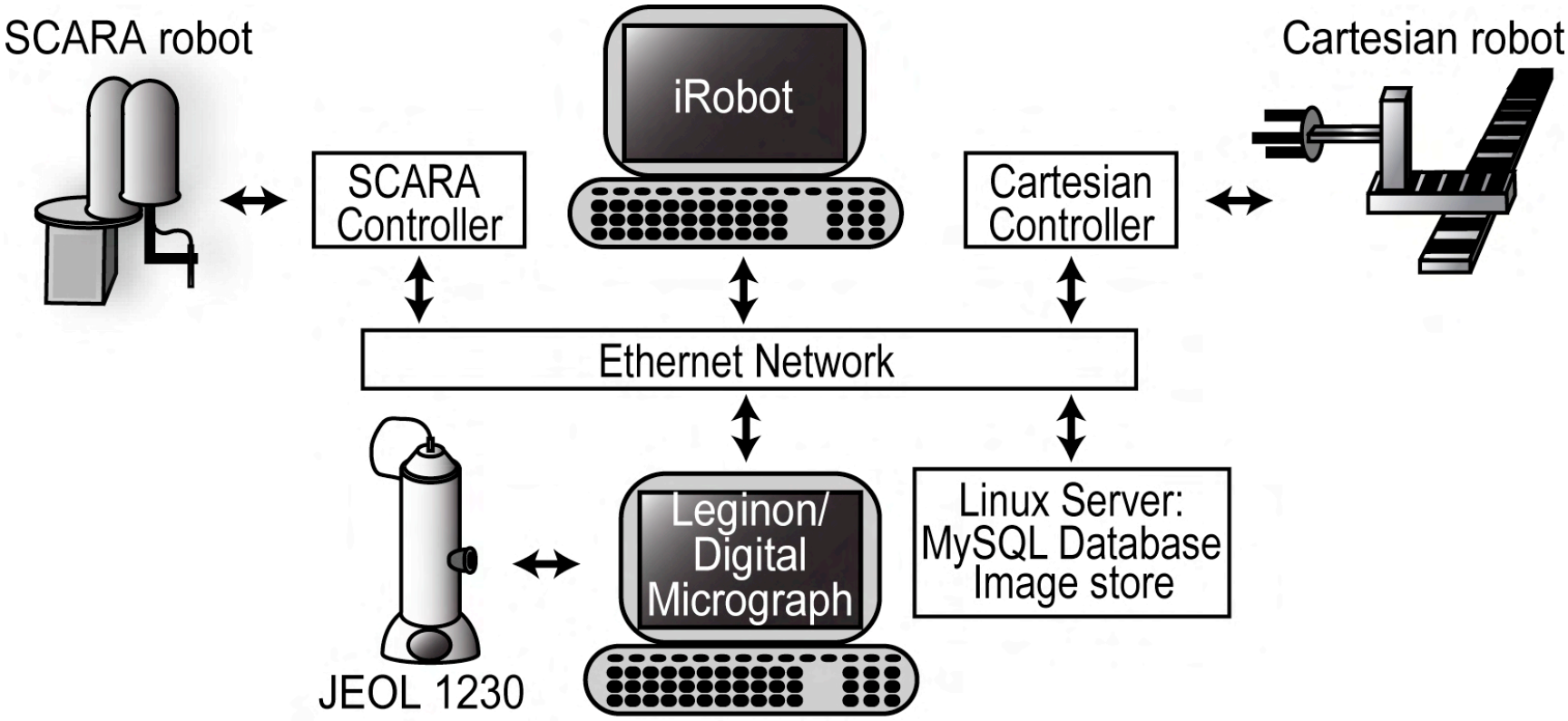
Automated loading and imaging of specimens



Automated loading and imaging of specimens

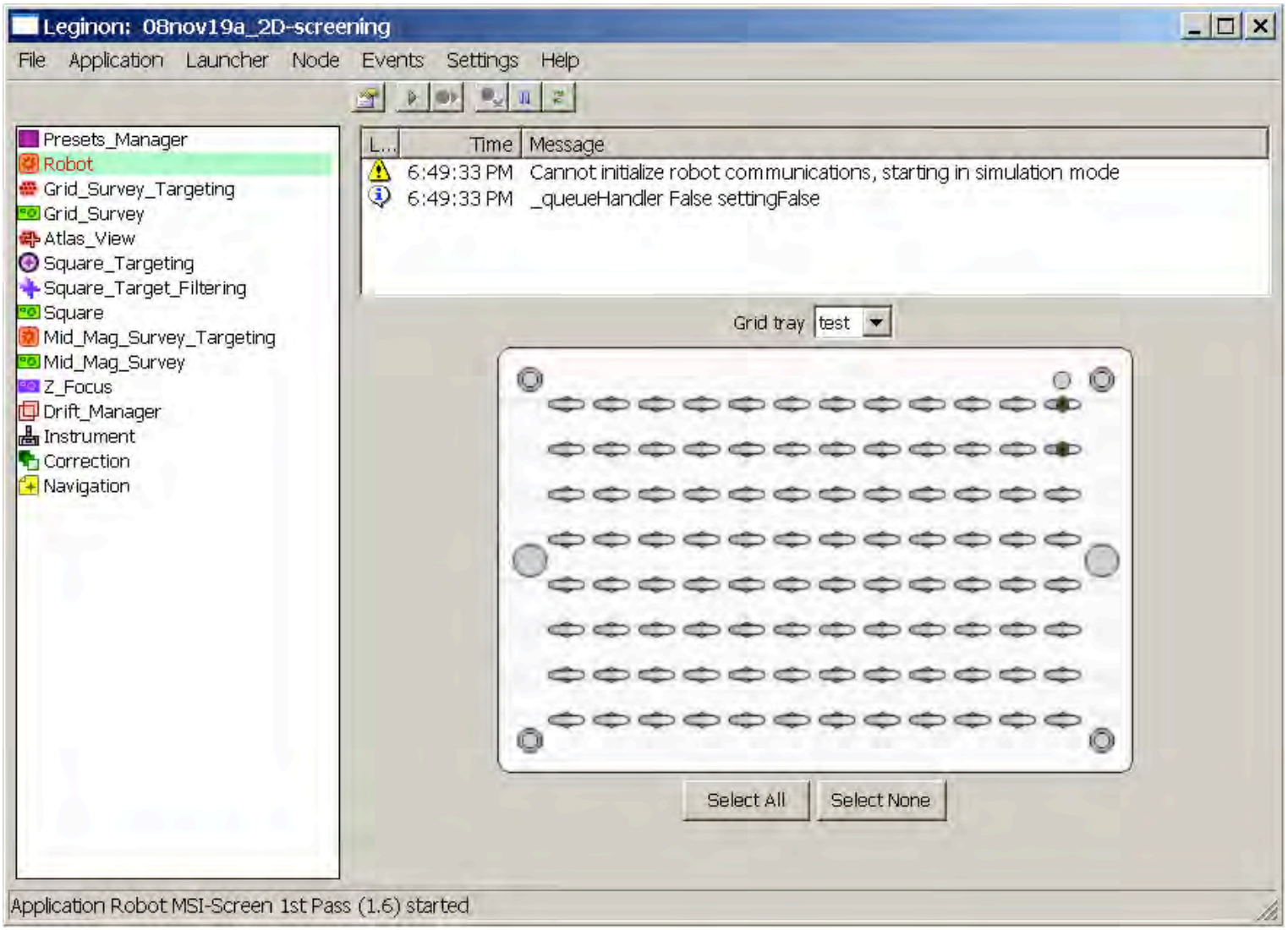


Automated loading and imaging of specimens



Automated loading and imaging of specimens

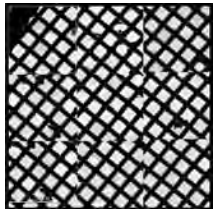
Leginon controls robot and 2DX imaging



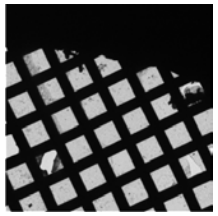
LEGINON is a system designed for automated collection of images by TEM (NRAMM at Scripps)

NRAMM
National Resource for Automated Molecular Microscopy
Supported by National Center for Research Resources

Define montage image area (e.g. 3x3)

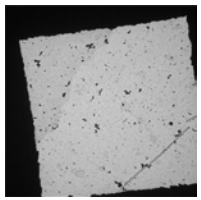


Take a images at low mag

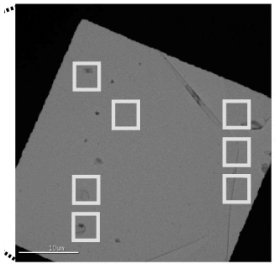


Pick target squares using square finder (histogram criteria)

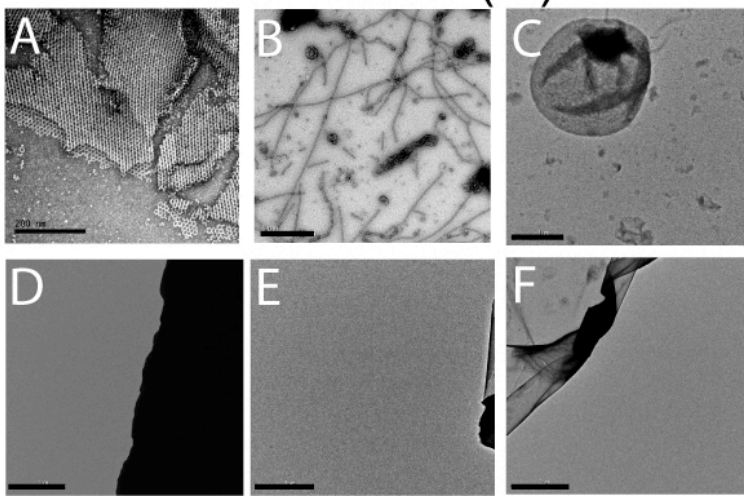
Take images of selected squares



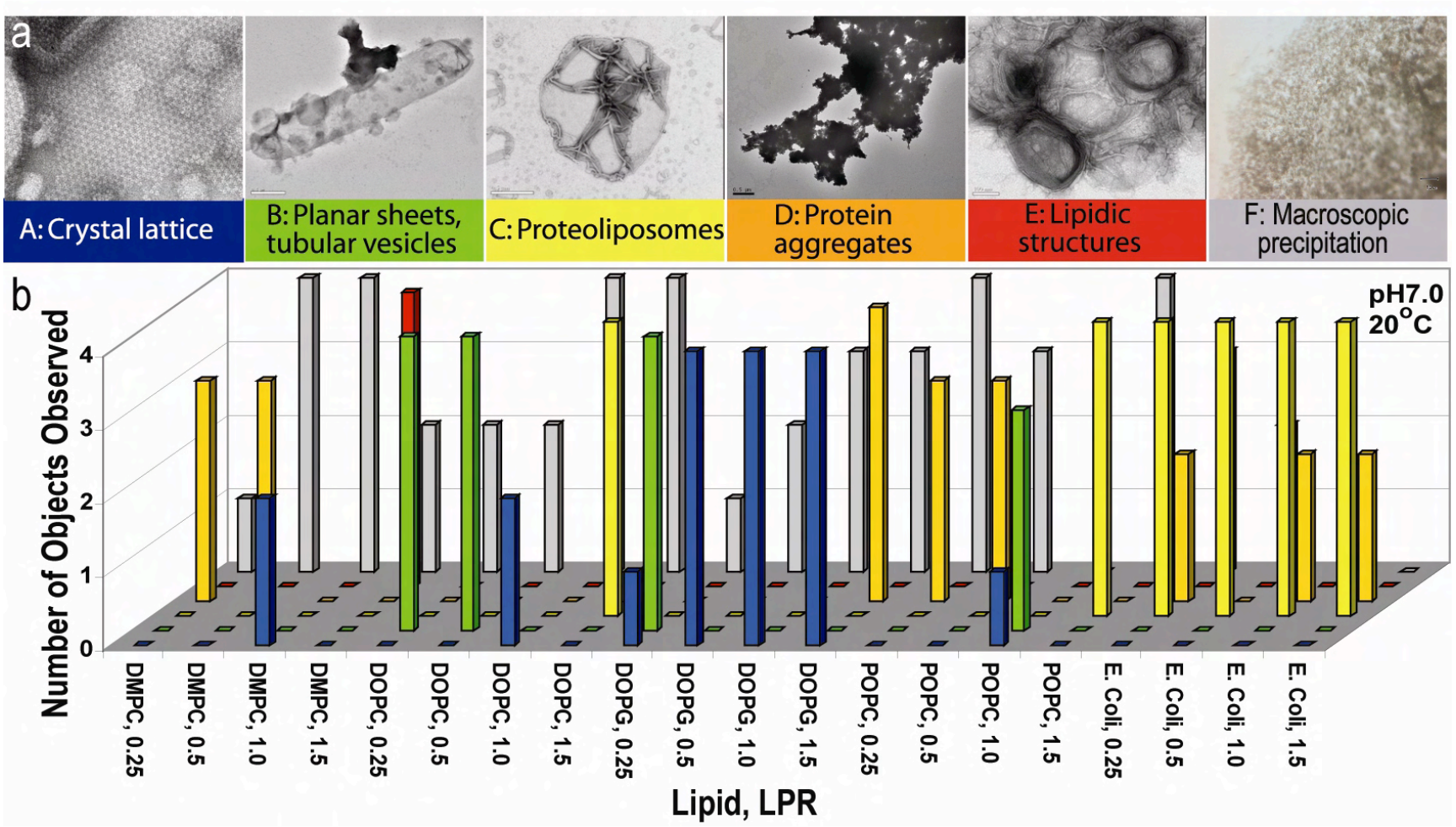
Pick targets from square images (histogram criteria)



Take images at intermediate mag for evaluation

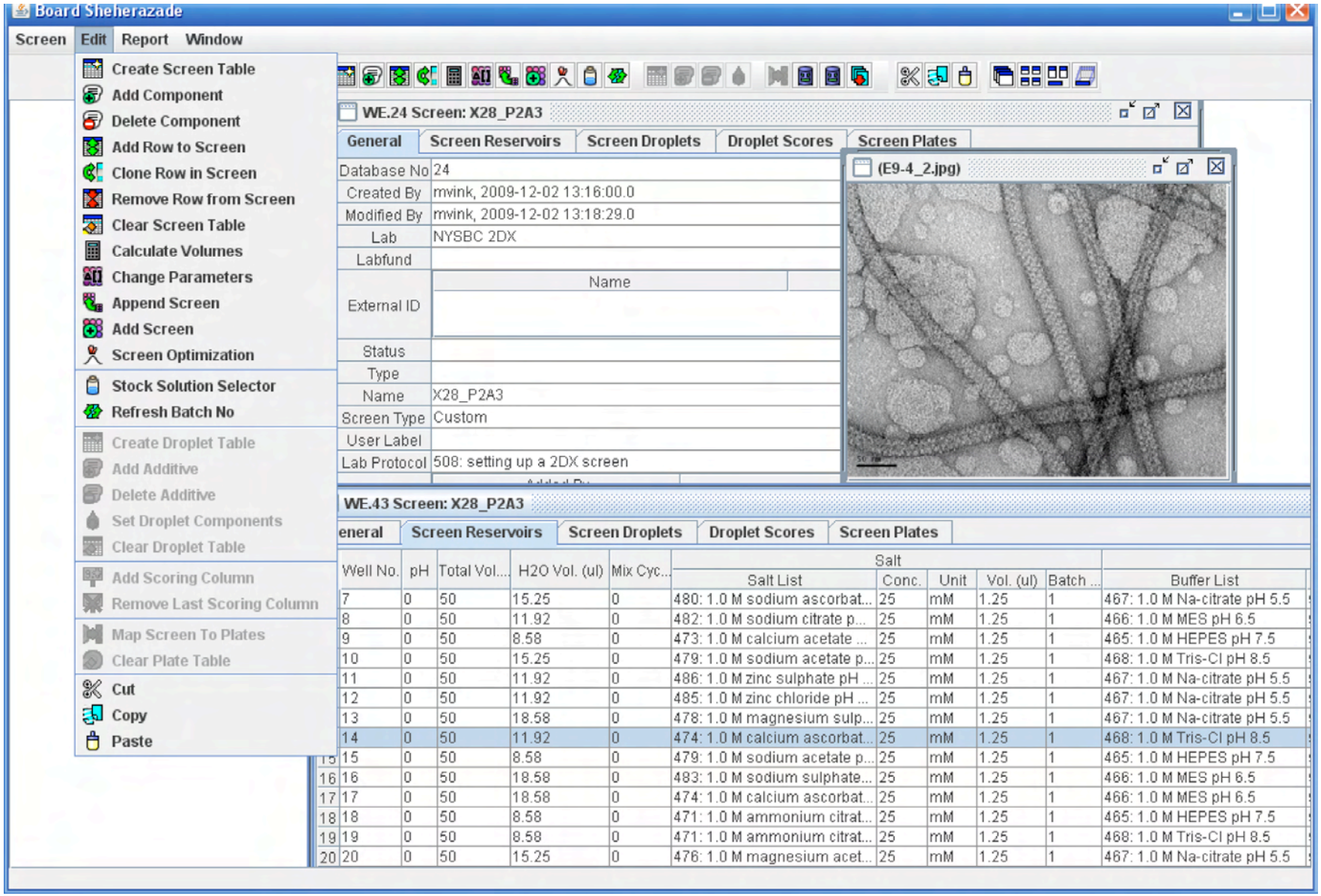


2DX screening evaluation and outcome



Currently evaluation is carried out by an expert. We need to develop algorithms for automated evaluation of the screens.

Laboratory Information Management System (LIMS)



SESAME by Zsolt Zolnai at the University of Wisconsin-Madison

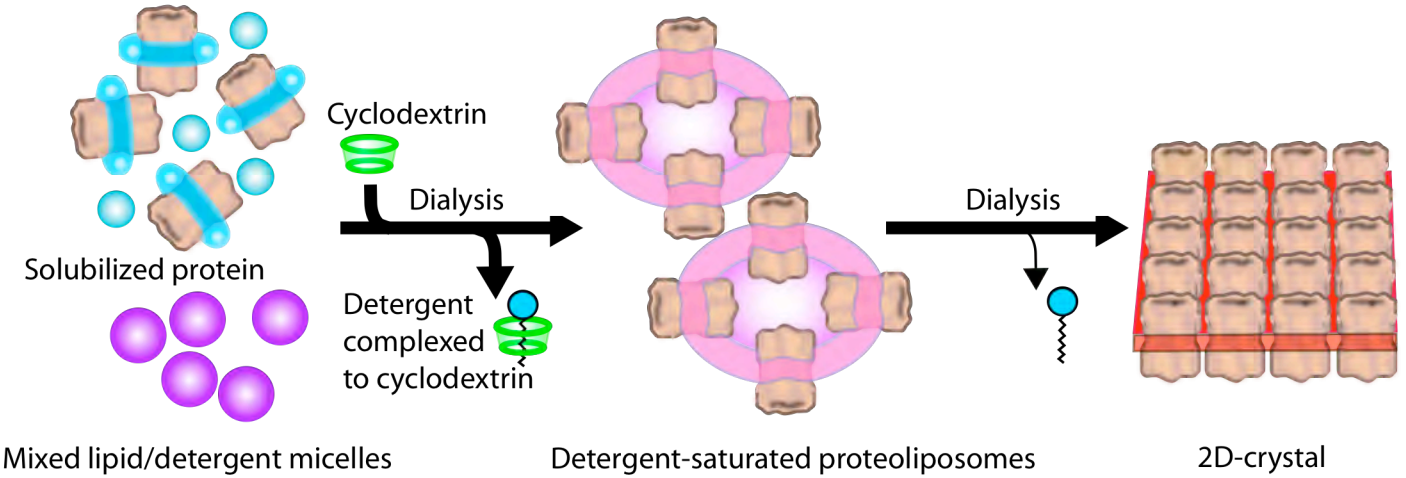
After a long story, how did we perform?

Protein	Organism	(Putative) Function	MW (kDa)	pI	TMD	N-term	% TM	Screens	Best Outcome
E2P1	*M.voltae*	Protease	25.9	8.7	4	Inside	45	4	Sheets, Tubes
E2P2	*M.maripaludis*	Protease	25.9	5.0	4	Inside	39	3	Sheets, Tubes,
E2P3	*M.marisnigri*	Protease	31.8	5.3	7	Inside	59	2	Sheets, Tubes
E2P4	*M.hungatei*	Protease	32.9	7.8	9	Inside	61	2	Sheets, Tubes
P2A3	*S.oneidensis*	Cation efflux family protein	32.5	5.4	5	Inside	36	5	Helical crystals
Rhomboid PA3086	*P.aeruginosa*	Intramembrane protease	31.8	9.9	6	Inside	45	4	Lipidic structures
YkgB-D332	*E.coli*	Unknown	21.9	6.0	3	Inside	38	1	Tubes
YkgB-D36	*E.coli*	Unknown	21.8	5.7	3	Inside	37	4	Helical crystals
β1-adrenergic receptor	*M.gallopavo*	G-protein coupled receptor	54.1	9.3	7	Outside	33	3	Sheets, Tubes
Rhomboid GlpG	*E.coli*	Intramembrane protease	31.3	9.2	6	Inside	46	1	Aggregates
Cytochrome b561	*P.aeruginosa*	Electron carrier activity	20.6	9.6	4	Inside	47	1	Lipidic structures
E1 protein	Semliki Forest Virus	Inducer of membrane fusion	47.4	7.6	0 (1)	Outside	5	4	Sheet crystals
P40B7	*B.subtilis*	Sporulation kinase C	48.8	6.3	2	Inside	10	1	Vesicles, Sheets
Kdp-ATPase (4 subunits)	*E.coli*	High-affinity K-pump	159.2	5.2-9.4	17	3 in, 1out	31	1	Sheets, Tubes
P39H10	*K. pneumoniae*	Diguanylate cyclase	45.7	6.5	2	Inside	11	1	Sheets, Tubes

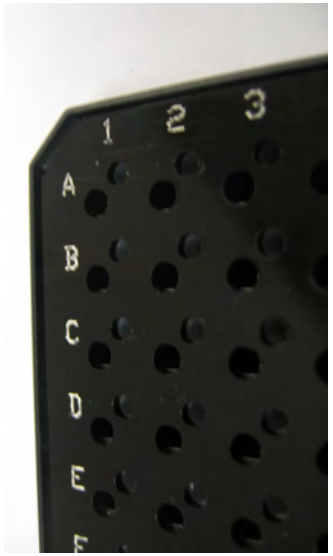
PI: isoelectric point; TMD: transmembrane domain; N-Term: location of N-teminus; %TM: % of transmembrane sequence ; Screens: Number of screens

Current developments

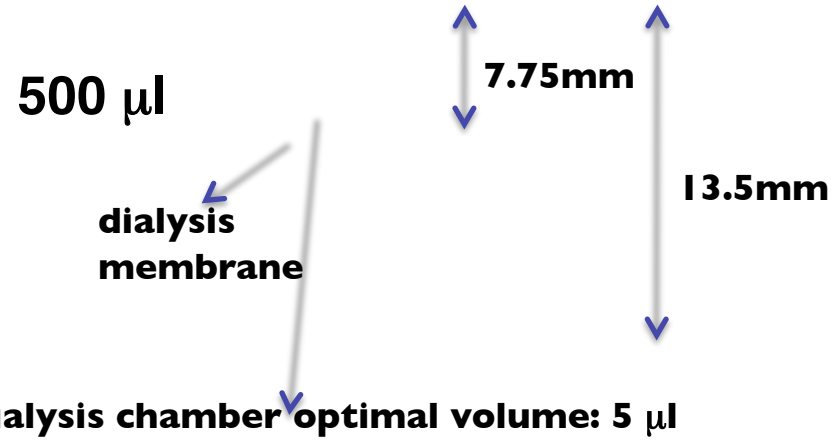
1) Following on Remigy's steps



2) Do dialysis with much less protein sample: 0.3 mgs for 96 conditions



Cross-section of the wells



TRANSCONTINENTAL EM INITIATIVE FOR MEMBRANE PROTEIN STRUCTURE (TEMIMPS)

NIH funded:

PSI-Biology: Centers for Membrane Protein Structure Determination (U54)

Team of 6 investigators with extensive experience in 2DX of membrane proteins:

Stokes, David L. - NYU SoM/NYSBC - Program Director

Engel, Andreas - Case Western Reserve - Co-PI

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Goals:

- Solve structures of eukaryotic membrane proteins that are relevant for human health and disease.
- Study their molecular mechanisms in their natural lipid environment.
- Accelerate membrane protein structure determination from 2DX.

Multiple positions available (http://temimps.nysbc.org/jobs/):

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