



Center for
Cellular Imaging
and Nano Analytics

C-CINA.org

Electron Crystallography of 2D crystals of membrane proteins:

Towards side-chain resolution from badly-ordered 2D crystals of potassium channels

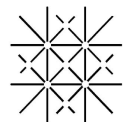
Henning Stahlberg

Center for Cellular Imaging and NanoAnalytics (C-CINA)

Biozentrum, University of Basel, Switzerland

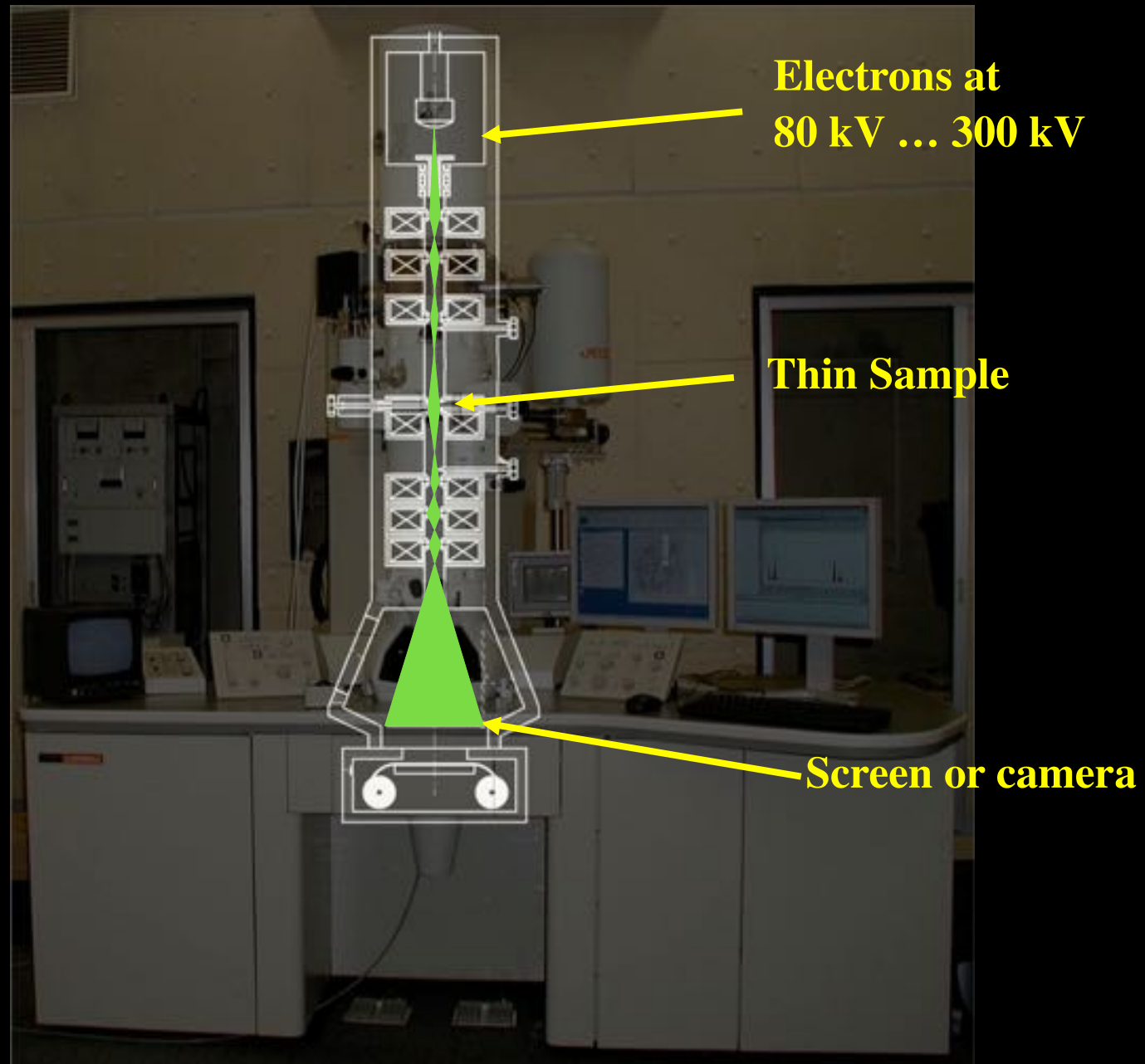
BIOZENTRUM

Universität Basel
The Center for Molecular Life Sciences



Uni Basel

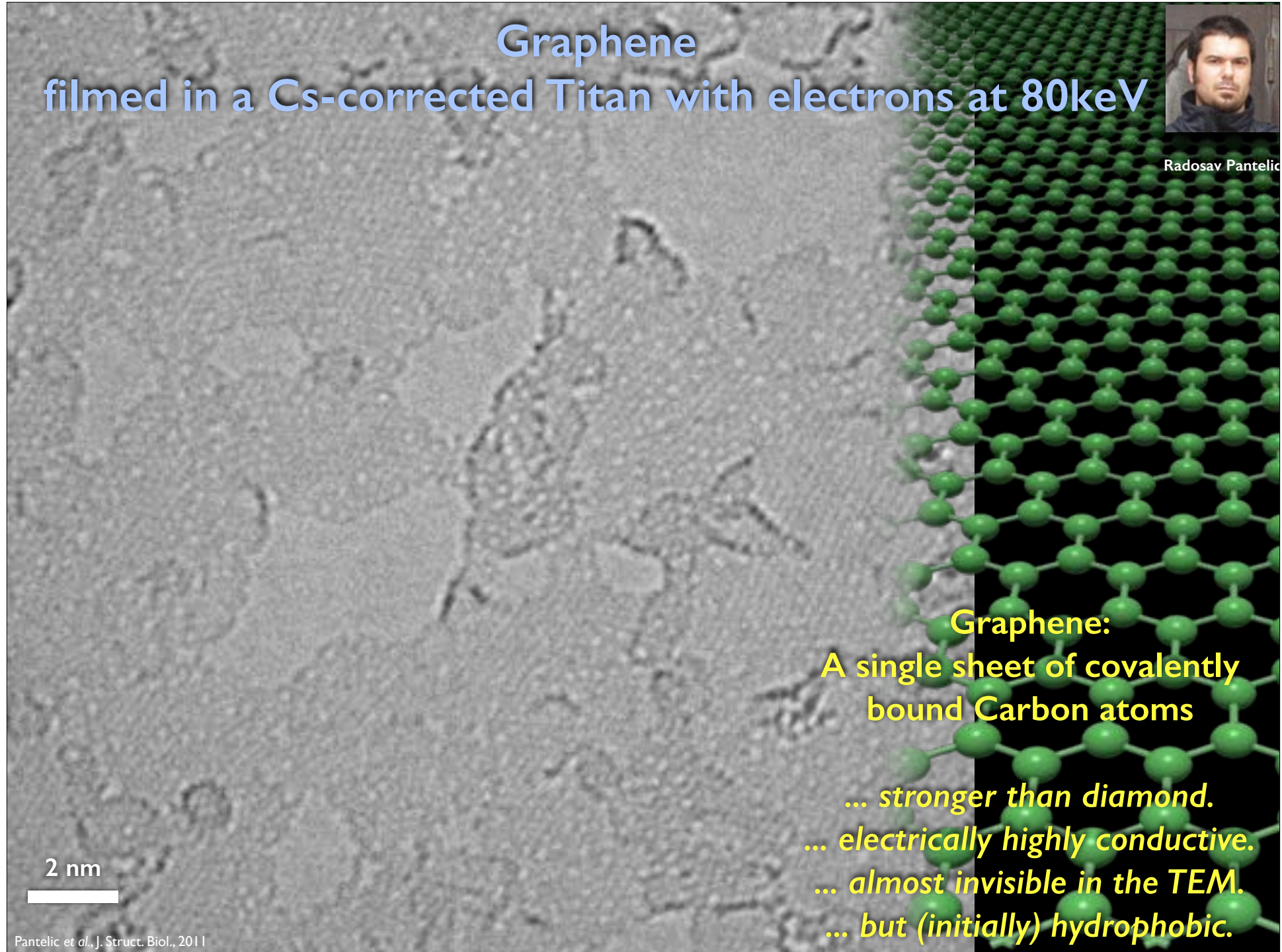
The Transmission Electron Microscope



Graphene filmed in a Cs-corrected Titan with electrons at 80keV



Radosav Pantelic



Graphene:
A single sheet of covalently bound Carbon atoms
... stronger than diamond.
... electrically highly conductive.
... almost invisible in the TEM.
... but (initially) hydrophobic.

viruses frozen in vitrified ice

Imaging
biological
specimens
is more
difficult...

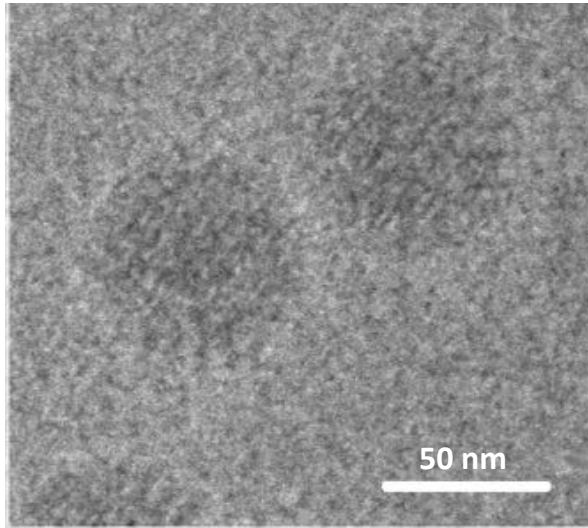
50 nm



Rotavirus, mockup images adapted from Grigorieff, *eLife*, 2013;2:e00573.

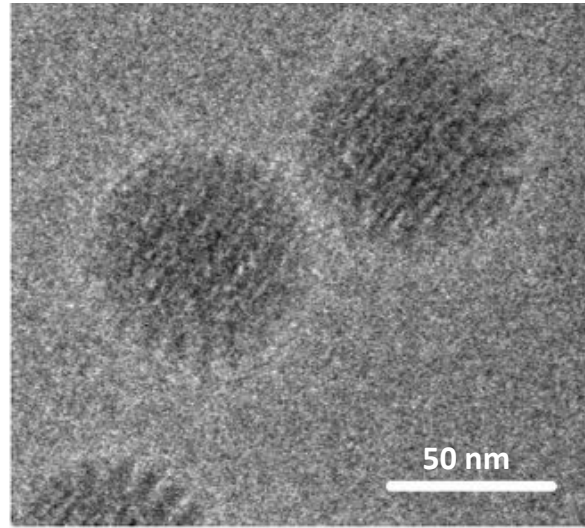
New Camera Technology in TEM

< 2012
Imaged on a
CCD camera or on film



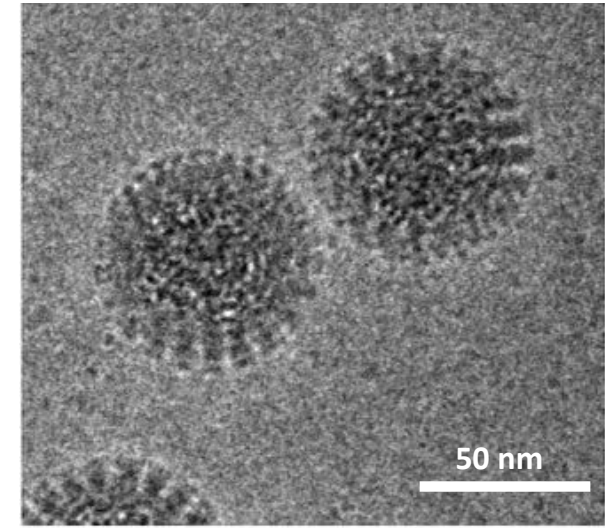
Typical Parameters:
Exposure Time: 1 second
Dose: 10 electrons / \AA^2

> 2013
Imaged on a
direct electron detector



Typical Parameters:
Exposure Time: 1 second
Dose: 20 electrons / \AA^2

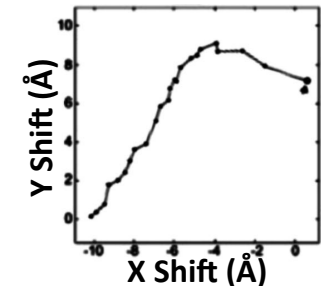
Imaged on a
direct electron detector
with dose-fractionation
and drift correction



Typical Parameters:
Sub-Frames: 40
Frame Exposure Time: 0.5 seconds
Total Exposure Time: 20 seconds
Total Dose: 40 electrons / \AA^2



Sub-Frames
are aligned
and averaged
by software.



Rotavirus, mockup images adapted from Grigorieff, *eLife*, 2013;2:e00573.

Old Camera

Calculated Fourier transform of an image
(contains Amplitudes and Phases)



Movie mode image recording:

- No more drifted images
- Dose fractionation

↑
~ 3.5 Å

↑
7 Å

↑
14 Å

↑
14 Å

↑
7 Å

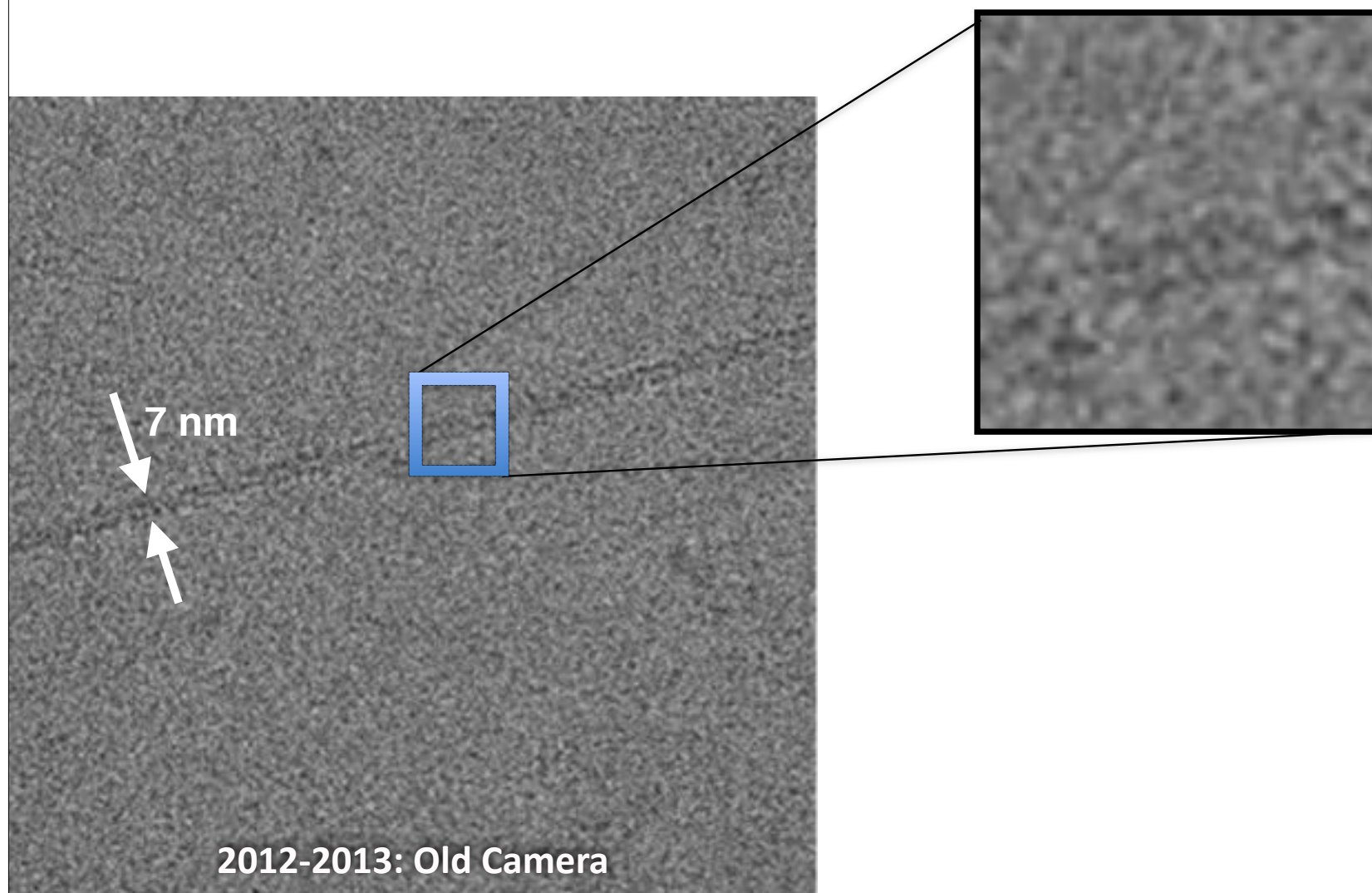
↑
~ 3.5 Å

Direct Electron Detector

Old Camera

Pyrin Domain (PYD) fibrils

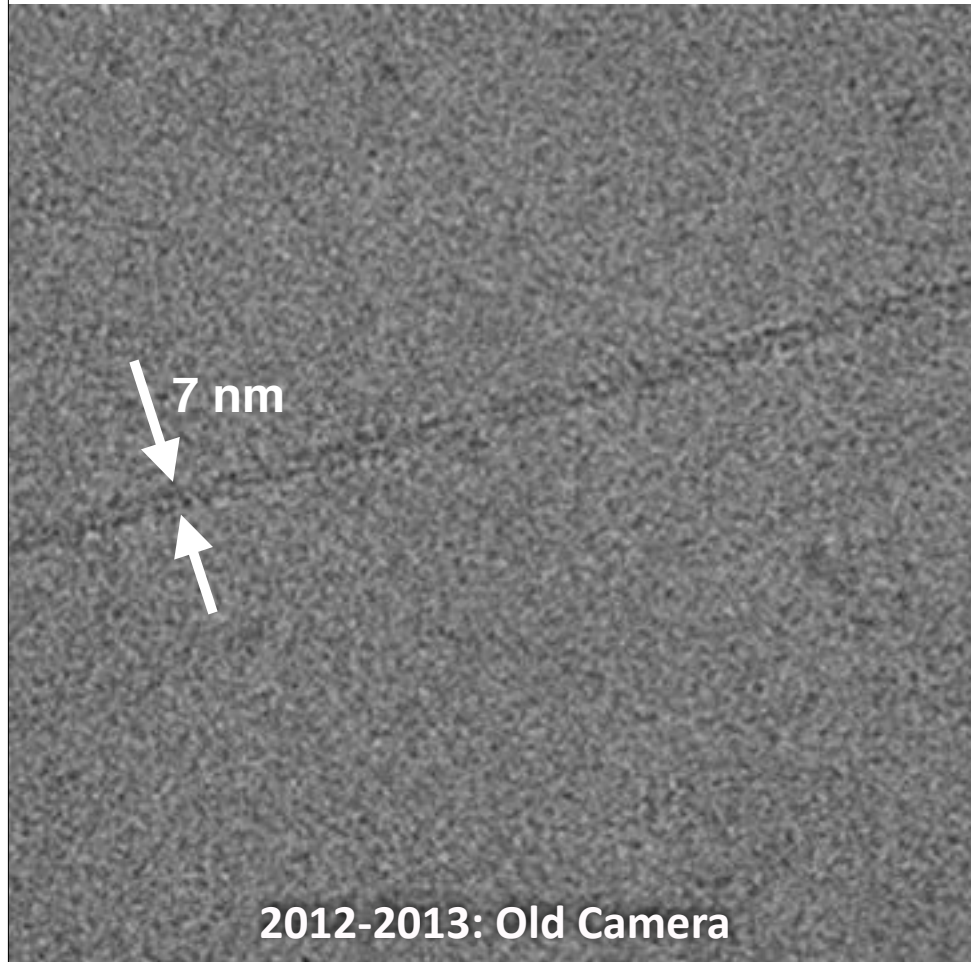
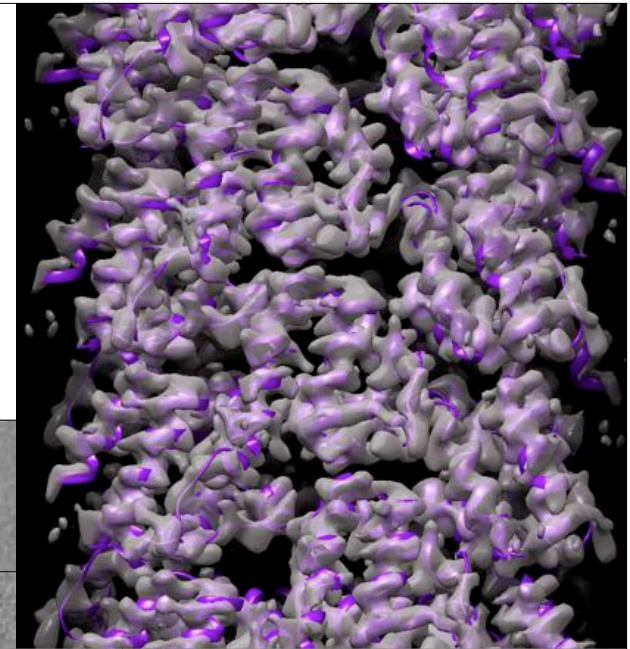
(an interacting protein of the adaptor sensor protein (ASC)
and caspase-1, involved in apoptosis)



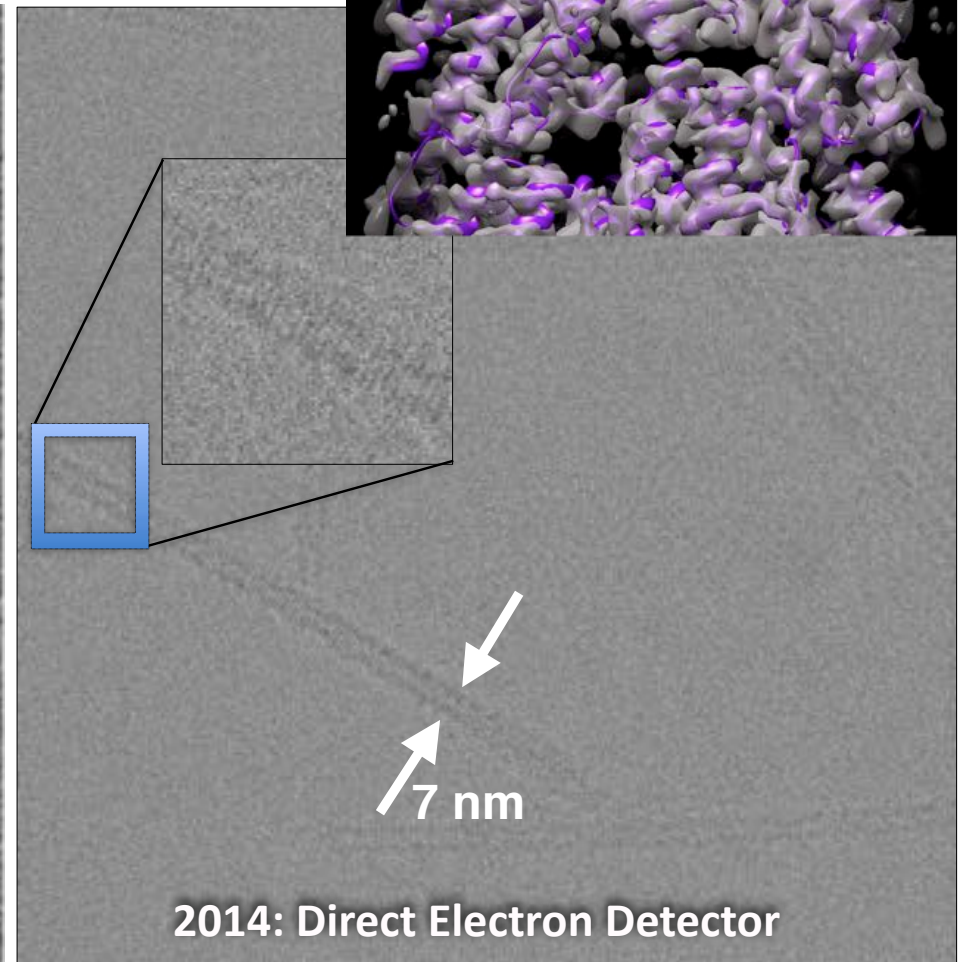
With Profs. Ed Egelman (Virginia), Sebastian Hiller, Petr Broz (Biozentrum)

Pyrin Domain (PYD) fibrils

(an interacting protein of the adaptor sensor protein (ASC) and caspase-1, involved in apoptosis)



2012-2013: Old Camera



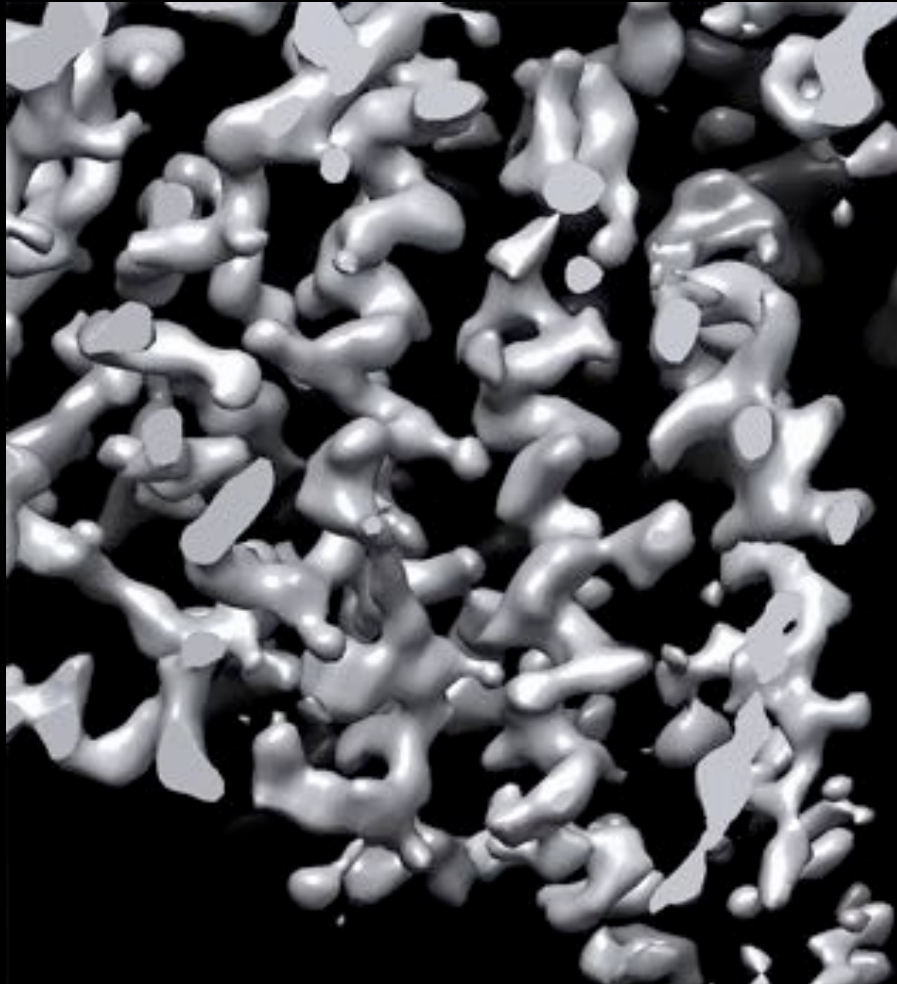
2014: Direct Electron Detector

With Profs. Ed Egelman (Virginia), Sebastian Hiller, Petr Broz (Biozentrum)

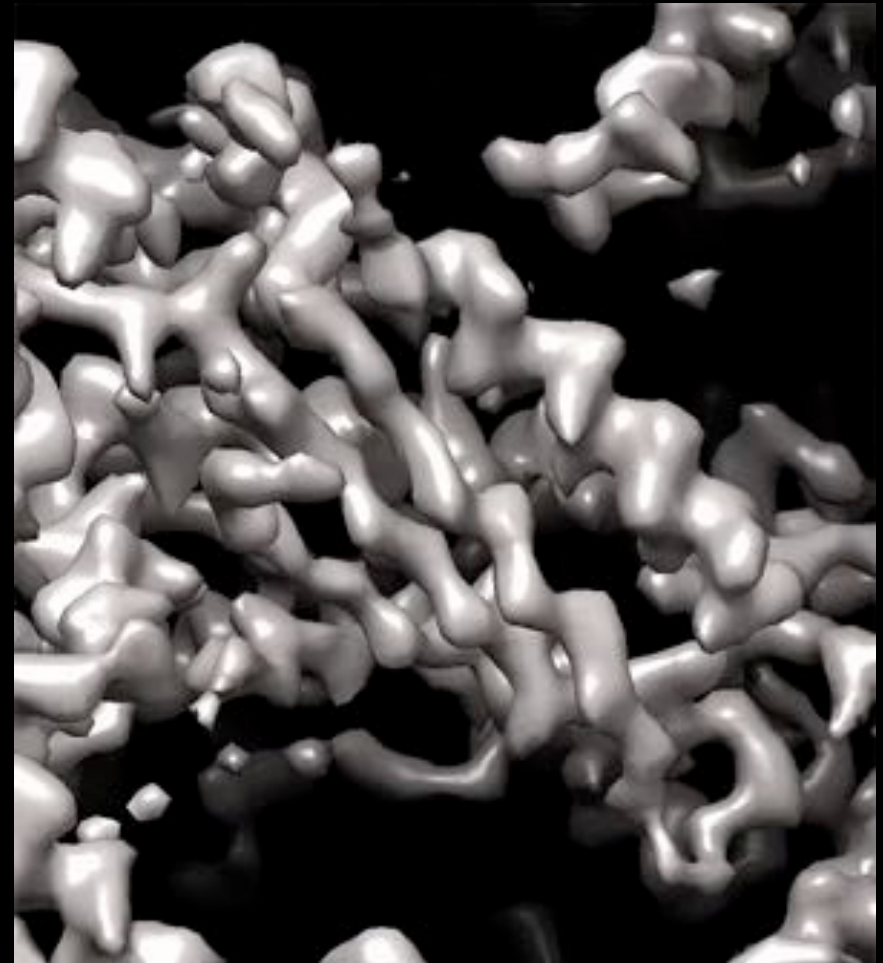
Structures from Transmission Electron Microscopy Images in C-CINA

Here at 3.5 Å resolution

Alpha-Helices

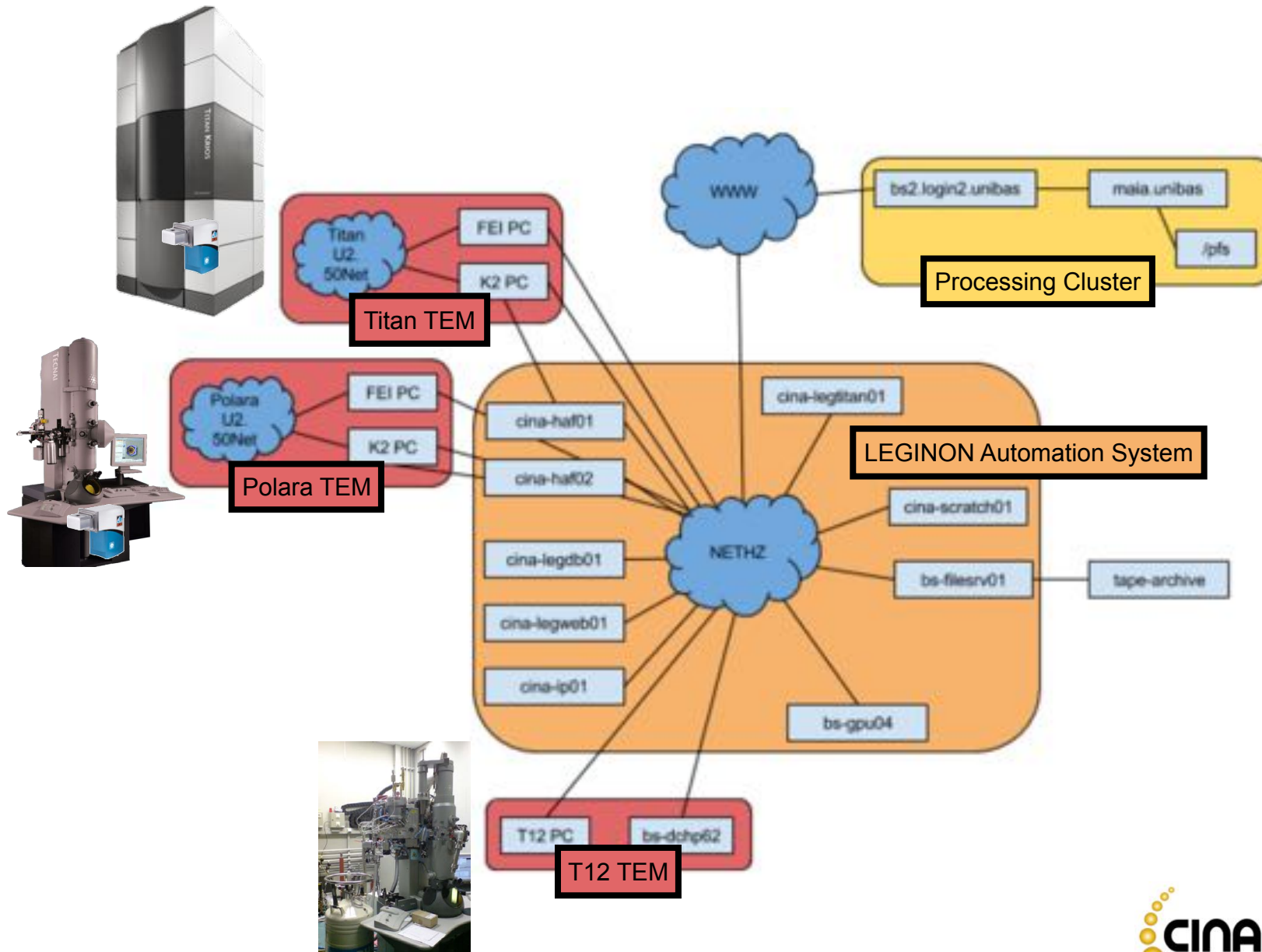


Beta-Sheet



(Image Processing: Ed Egelman)

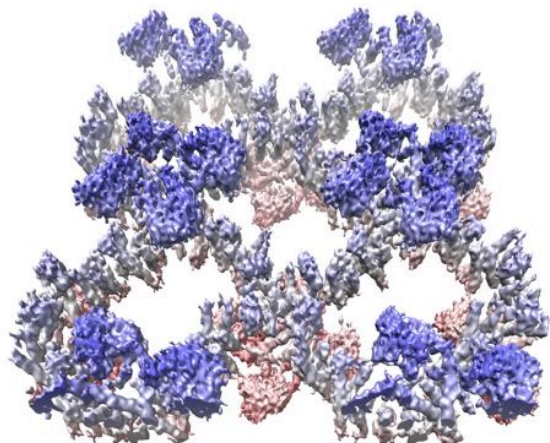
Automation of Cryo-EM in C-CINA



Recent (2014) High-Resolution Structures from C-CINA

MloK1

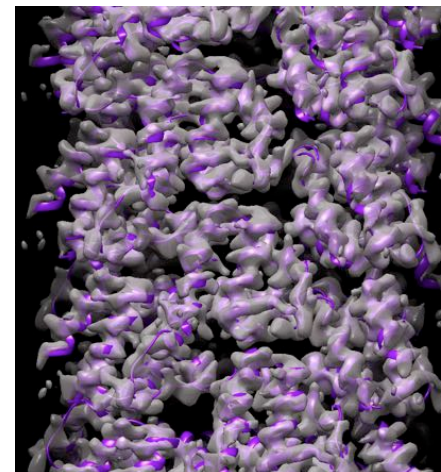
cAMP modulated K⁺ channel with voltage sensors



4.5 Å resolution, Membrane Protein 2D Crystal in Lipid Membrane

PYD fibrils

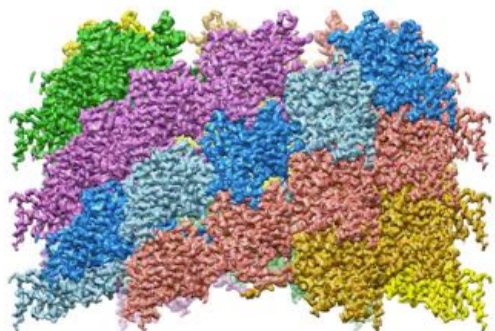
A fibrillar protein involved in triggering apoptosis



3.4 Å resolution, Helical cryo-EM, with Hiller

T6SS Sheath

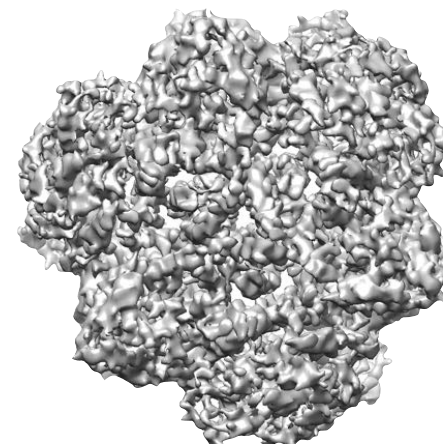
The bacterial type-VI secretion system stalk



3.2 Å resolution, Helical cryo-EM, with Basler

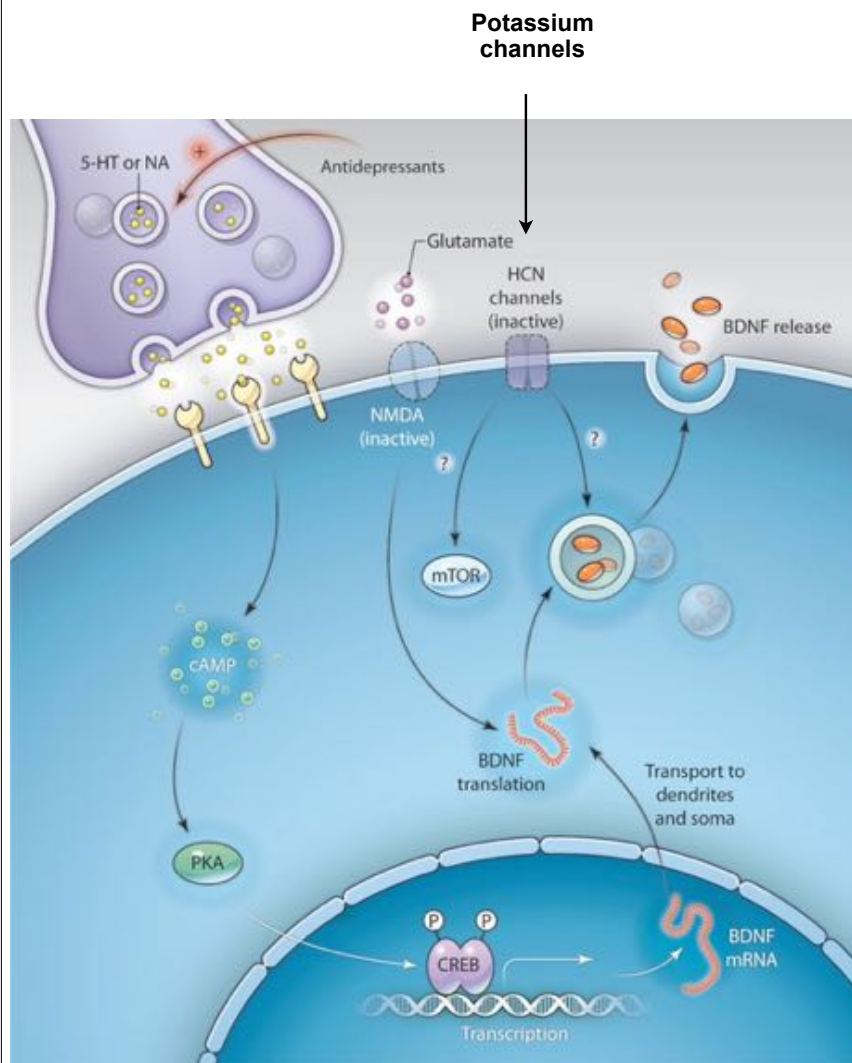
CBP

Cerura Biliprotein

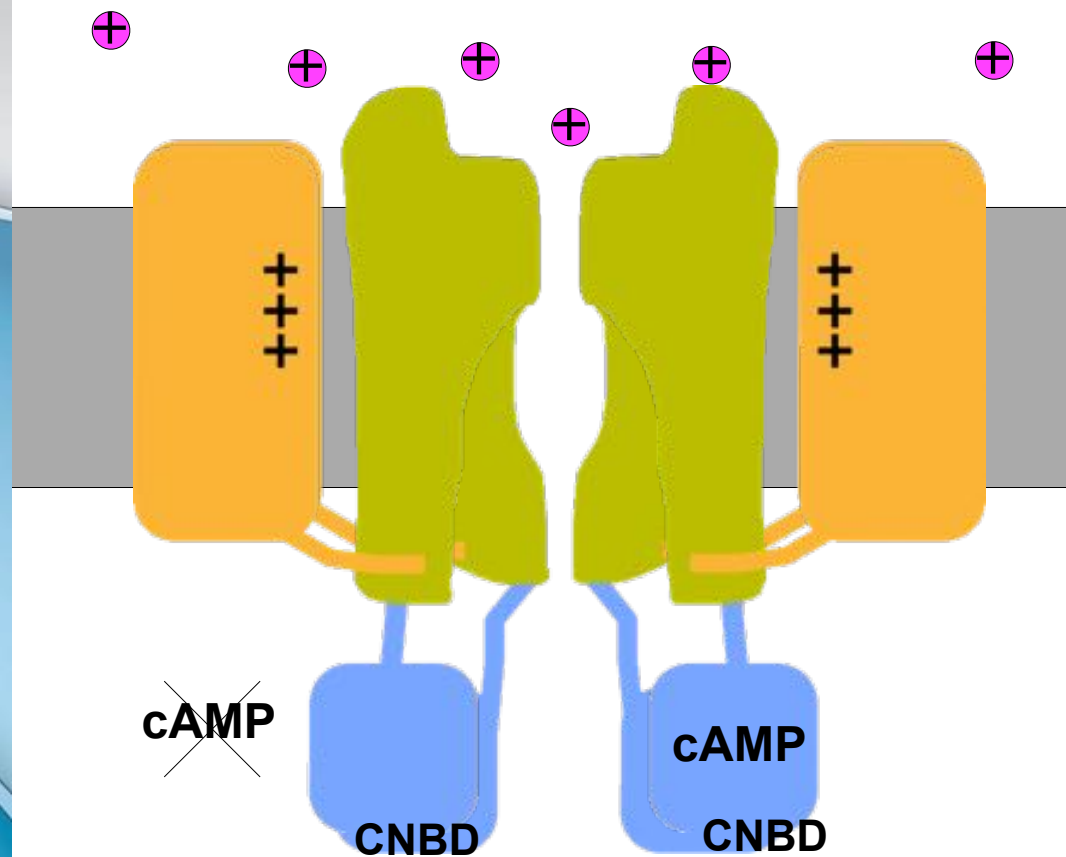


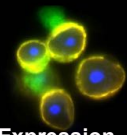
up to 3.0 Å resolution, Single Particle cryo-EM

MloK I: prokaryotic cAMP-modulated potassium channel



Shah, Science Signaling 5(244), 1-3 (2012)

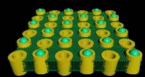




Expression



Purification



2D Crystallization



Sample Prep



Imaging

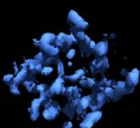
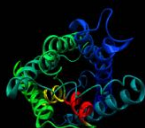
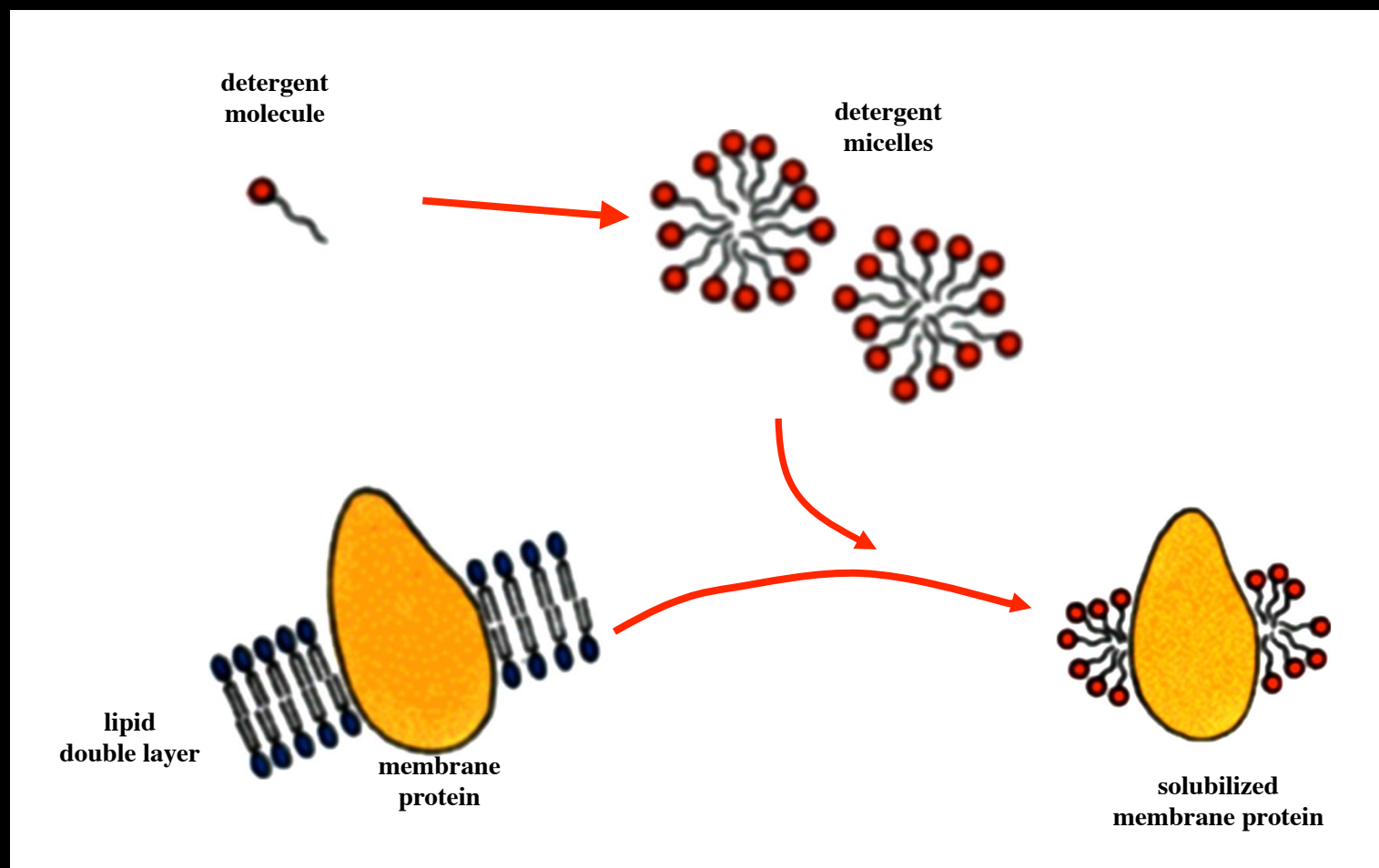
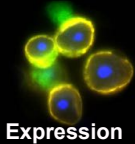


Image Processing



Model Building

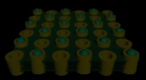




Expression



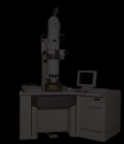
Purification



2D Crystallization



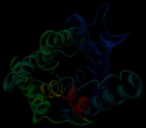
Sample Prep



Imaging

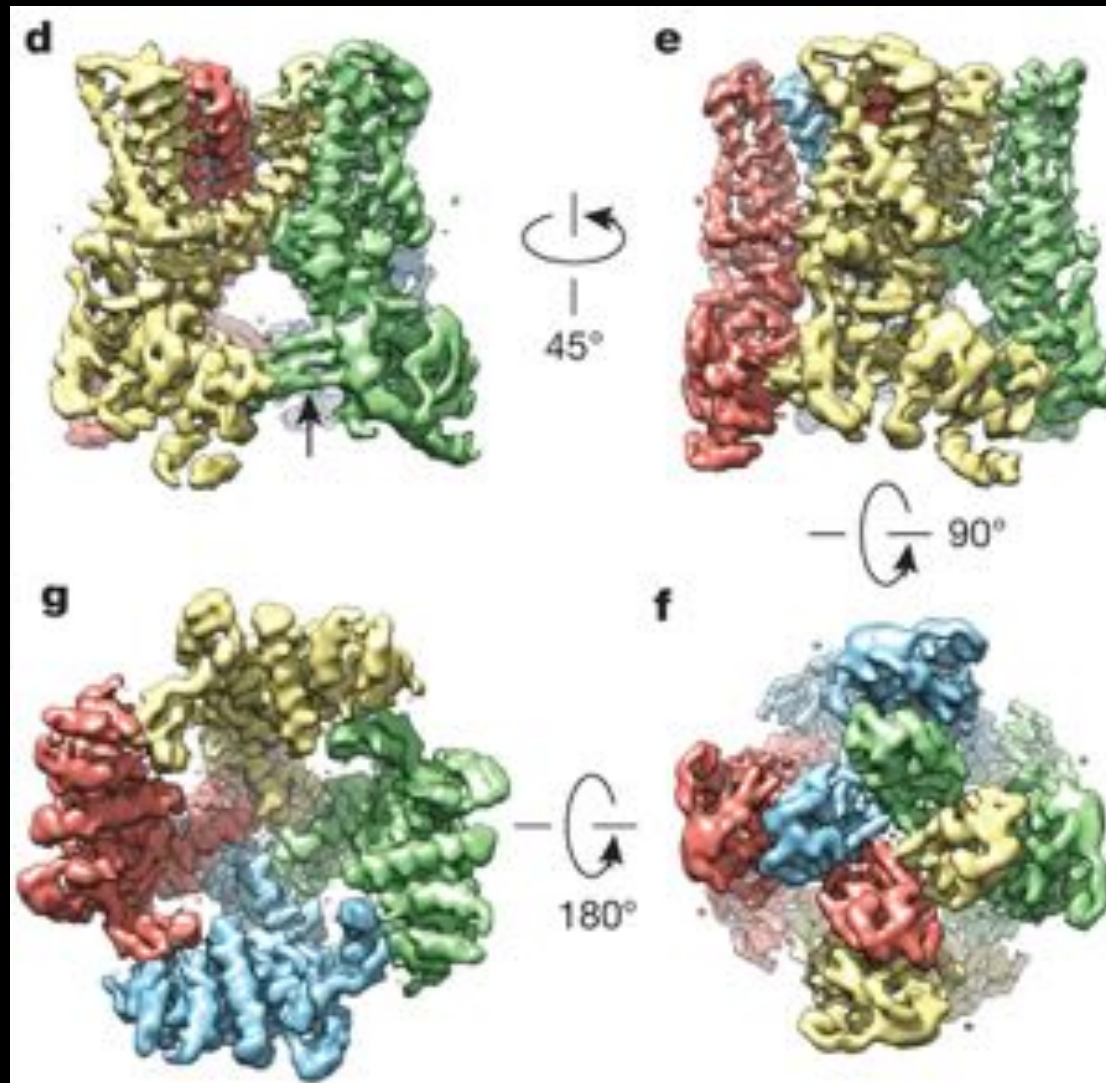


Image Processing



Model Building

Yifan Cheng, UCSF: 3D reconstruction of TRPV1 determined by single-particle cryo-EM.



Parameters:

TEM:
Polaris, 300kV

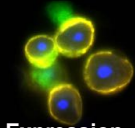
Sample:
Purified Membrane
Protein in Amphipols
(instead of detergent)

Particles in final map:
10'357 (out of 170'000)

Image processing:
RELION (Bayesian
Maximum Likelihood)

Resolution:
3.4 Å (isotropic 3D)

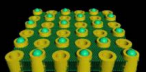
Maofu Liao *et al.*, & Yifan Cheng, *Nature* **504**, 107-112 (2013)



Expression



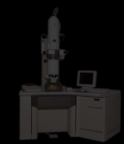
Purification



2D Crystallization



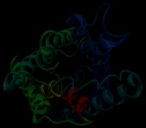
Sample Prep



Imaging

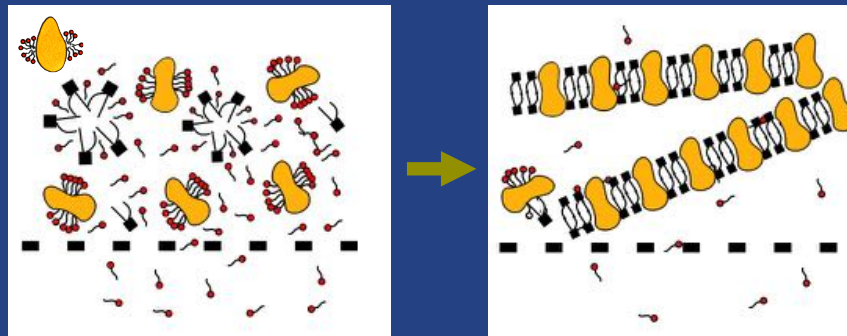


Image Processing

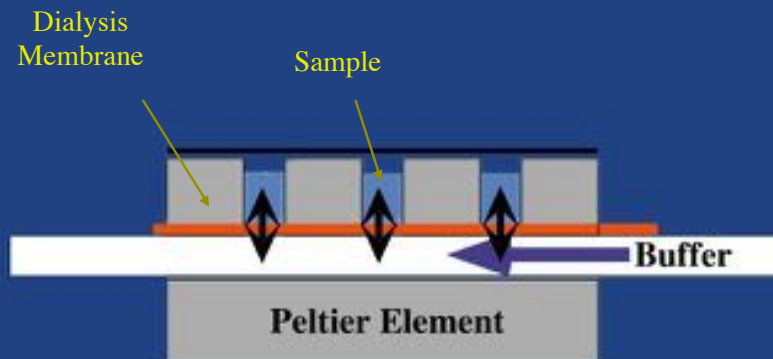
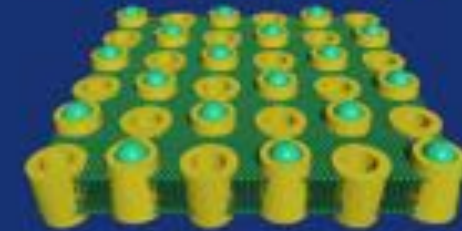


Model Building

2D crystallization: removal of detergent by dialysis

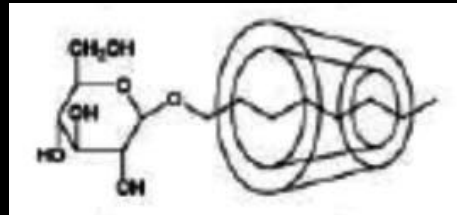
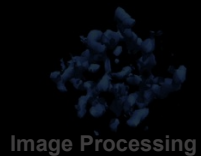
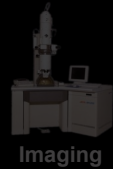
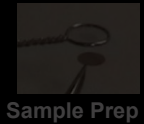
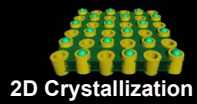
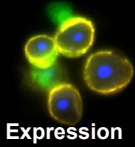


2D crystal

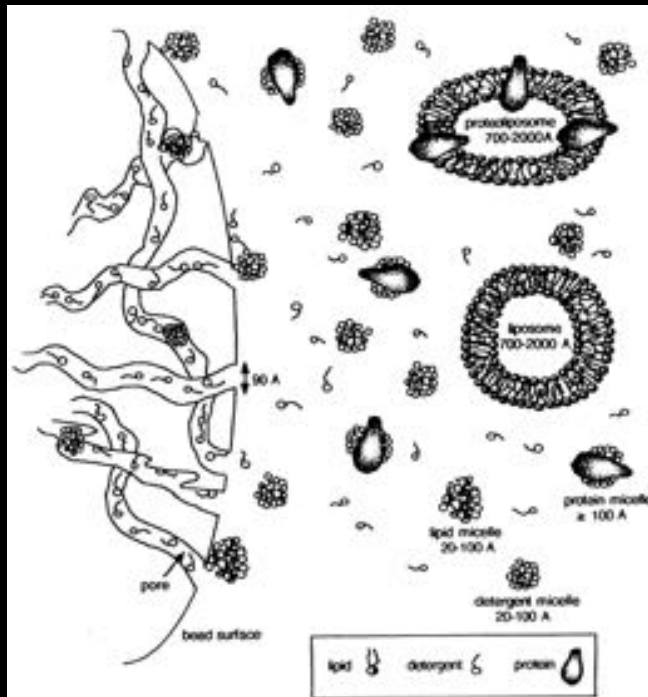


Jap *et al.*, *Ultramicroscopy* (1992)

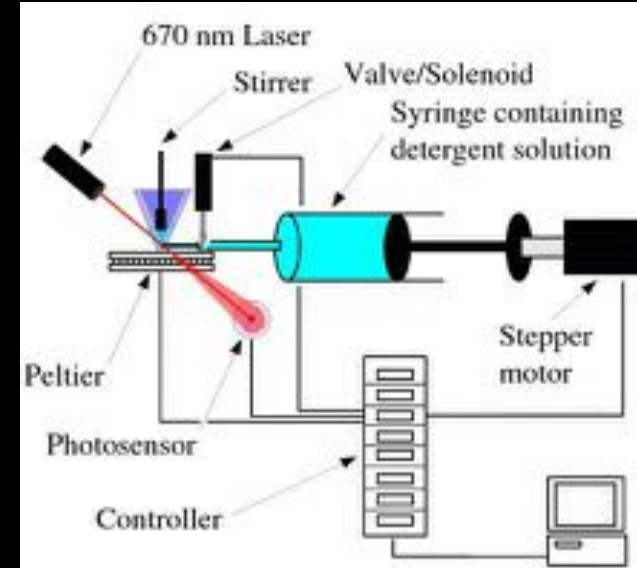
Alternative methods to remove detergent:



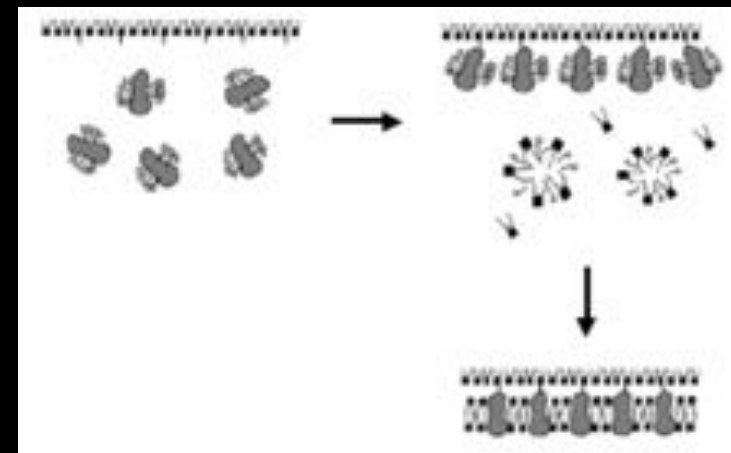
Adding Cyclodextrin



Adding Biobeads



Dilution



Adsorbing to a lipid monolayer

Tricks from Tom Walz to 2D crystallize membrane proteins by dialysis (2010)

Primary Screens – Reconstitution of Protein

- protein concentration: 1 mg/ml
- standard buffer (10 mM MES, pH 6, 150 mM NaCl)
- Lipid to Protein Ratio (LPR): 0.5, 1, 2 w/w
- vary lipids: DMPC, DOPC, POPC, *E. coli* lipids
PE and PS lipids, other lipid mixtures

Identify the lipid
Identify the approximate LPR

Secondary Screens – Produce 2D Crystals

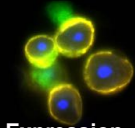
- protein concentration: 1 mg/ml
- vary buffer (divalent cations, pH, salt)
- vary LPRs

Identify the buffer conditions
Identify the LPR

Ternary Screens – Produce the “Golden Batch”

- increase protein concentration
- vary LPR around the identified LPR
- vary divalent cation concentration

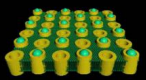
Get the perfect 2D crystals



Expression



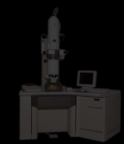
Purification



2D Crystallization



Sample Prep



Imaging



Image Processing



Model Building

Electron Microscopy Analysis of 2D Crystals of Membrane Proteins

Priyanka D. Abeyrathne¹, Marcel Arheit¹, Fabian Kebbel¹, Daniel Castano-Diez¹, Kenneth N. Goldie¹, Mohamed Chami¹, Ludovic Renault², Werner Kühlbrandt^{3,*}, and Henning Stahlberg^{1,*}

Comprehensive Biophysics 1.19 (2012)

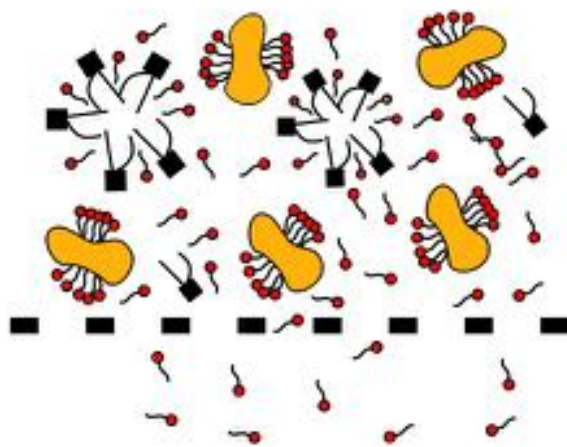
Table 1. Structures of membrane proteins analyzed by electron crystallography, and the protein production, purification, and crystallization conditions. Future, updated versions of this table will be maintained at <http://2dx.org>. Crystallization Method: DI = Dialysis; BB = Biobeads; LM = Lipid Monolayer; SP = Salt Precipitation; FU = Fusion.

Family	Protein	Resolution		PDB / EMDB	Origin	Expressed in	Conc [mg/ml]	Lipid added	LPR [w/w]	Detergent	T		Salt [mM]	Non-ionic agents [%]	Crystallization		References		
		2D [Å]	3D [Å]								[°C]	pH			Time	Method		Crystal type	
Ion Channels	ApoB ₅₀ - Adenovirus gas ion channel	12			E. coli	E. coli	0.4	DOPC	1	DM	20	8	250 NaCl, 0.6 Na ₂ S ₂ O ₈	-	10d	DI	sheets	(Conroy et al., 2004)	
	Anomalous A5 - Ca ²⁺ -specific ion channel	6.5			Rat	E. coli	0.1	DOPC / DOPS	+	none	20	7.8	150 NaCl, 2 CaCl ₂ , 3 Na ₂ S ₂ O ₈	+	3-4 d	LM	sheets	(Jiang et al., 2000)	
	Max1 - mechanosensitive ion channel	15			E. coli	E. coli	1	E. coli lipid	0.45	Tris, N-300	4	8	100 KCl	-	5 d	BB	vesicle	(Saito et al., 1999)	
	VDAC - voltage-dependent anion channel	18			Porcine											-	tubes	(Sveinbjornsson et al., 2002)	
Potassium Channels	KcsA potassium channel	8			E. coli	E. coli	1-2	DMPG / Sodium cholate	+	DDM	37	7.5	100 KCl, 1 EDTA	-	3 d	DI	-	(Li et al., 1998)	
	KirBac3.1 potassium channel	9			M. magnetotactans	E. coli	1	DOPC	0.8 - 1	DM	20 / 37	8	100 KCl, 3 Na ₂ S ₂ O ₈ , 75 MgCl ₂	-	7 d	BB	sheets	(Kao et al., 2005)	
	MinK3 - Cyclic Nucleotide-Modulated K ⁺ Channel	10			M. Jost	E. coli	0.5	E. coli lipid		DM	20 / 37	6.7	20 KCl, 1 BaCl ₂	-	5 d	DI	sheets	(Chiu et al., 2007)	
Ion Anticouplers	CIC-ec1 - chloride-pyrenyl anticoupler from E. coli	6.5			E. coli	E. coli		POPC	0.4	DM	4	7	20 NaCl, 20 MgCl ₂ , 0.8 Na ₂ S ₂ O ₈	-	several days	DI	sheets	(Mondillo et al., 2001)	
	NhaA - Na ⁺ - H ⁺ antiporter from E. coli	4			E. coli	E. coli	0.8	E. coli lipid	0.2 - 0.5	DDM	37	4	25 KAc, 150 KCl, 0.1 GdCl ₃ , 3 Na ₂ S ₂ O ₈	5-10 glycerol	4 - 6 d	DI	tubes	(Williams et al., 1999)	
		7			E. coli	E. coli	0.5	E. coli lipid	0.2 - 0.5	DDM	37	4	25 KAc, 150 KCl, 0.1 GdCl ₃ , 3 Na ₂ S ₂ O ₈	5-10 glycerol	4 - 6 d	DI	tubes	(Williams, 2000)	
	NhaP1 - Na ⁺ - H ⁺ antiporter from M. jannaschii	7			HTI	E. coli	E. coli	0.5	E. coli lipid	0.2 - 0.5	DDM	37	4	25 KAc, 150 KCl, 0.1 GdCl ₃ , 3 Na ₂ S ₂ O ₈	5-10 glycerol	4 - 6 d	DI	tubes	(Nappell et al., 2008)
		8			M. jannaschii	E. coli	E. coli	1	E. coli lipid	0.4 - 0.55	DDM	37	4	200 NaCl, 25 Acetate	10 glycerol	3 - 7 d	DI	tubes	(Vimolthanasri et al., 2002)
				M. jannaschii														(Kleinermann et al., 2011)	
	TmA - secondary amine channel	17			E. coli		1	DMPG/POPC	0.1 - 1.5	DDM (lipids in situ)		7.8	30 Tris, 150 NaCl, 40 MgCl ₂			DI		(Yin et al., 2000)	

2D crystallization of membrane proteins by dialysis

Factors influencing 2D crystallization

- Protein quality & concentration
- Type of lipids (acyl chain length, saturation, charges)
- Type of detergent
- Lipid-protein ratio (LPR)
- Way/speed of detergent removal
- Temperature (stability, diffusion, membrane fluidity)
- pH (charges, conformation, stability)
- Ionic strength (protein charges)
- Bivalent cations (interaction with proteins / lipids)
- Inhibitors / binding partner (conformational stability)



Detergent removal

2D crystal formation



2D crystal

Grid conditioning and handover

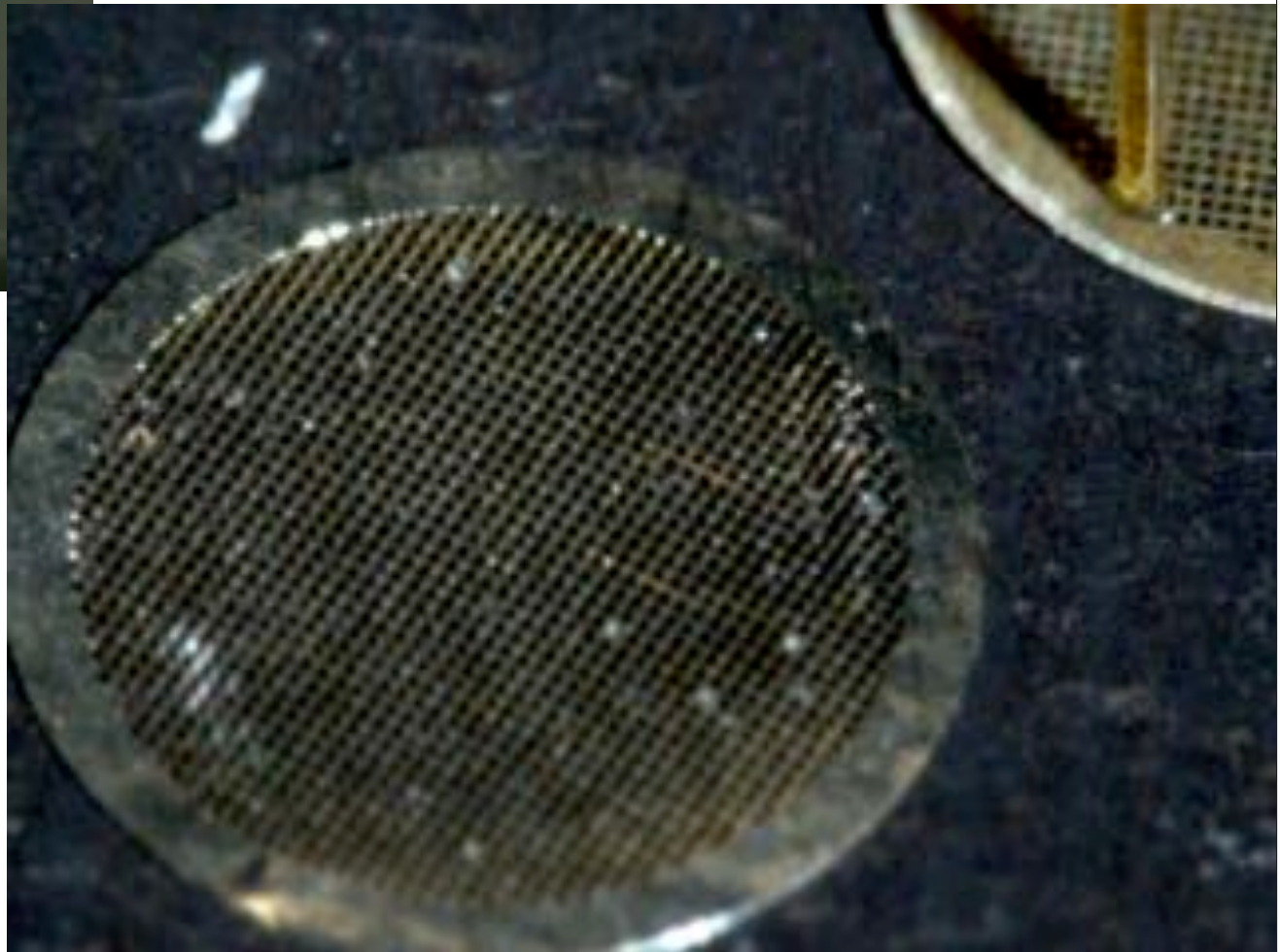
Kemmerling *et al.*, J Struct Biol. 177(1):128–134 (2012)



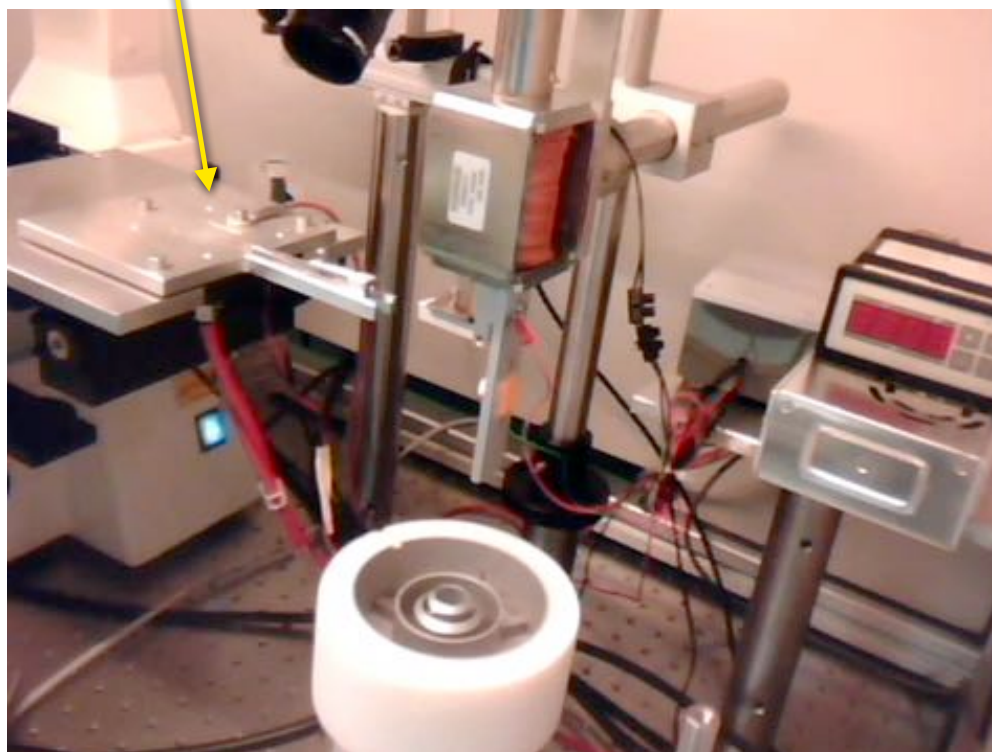
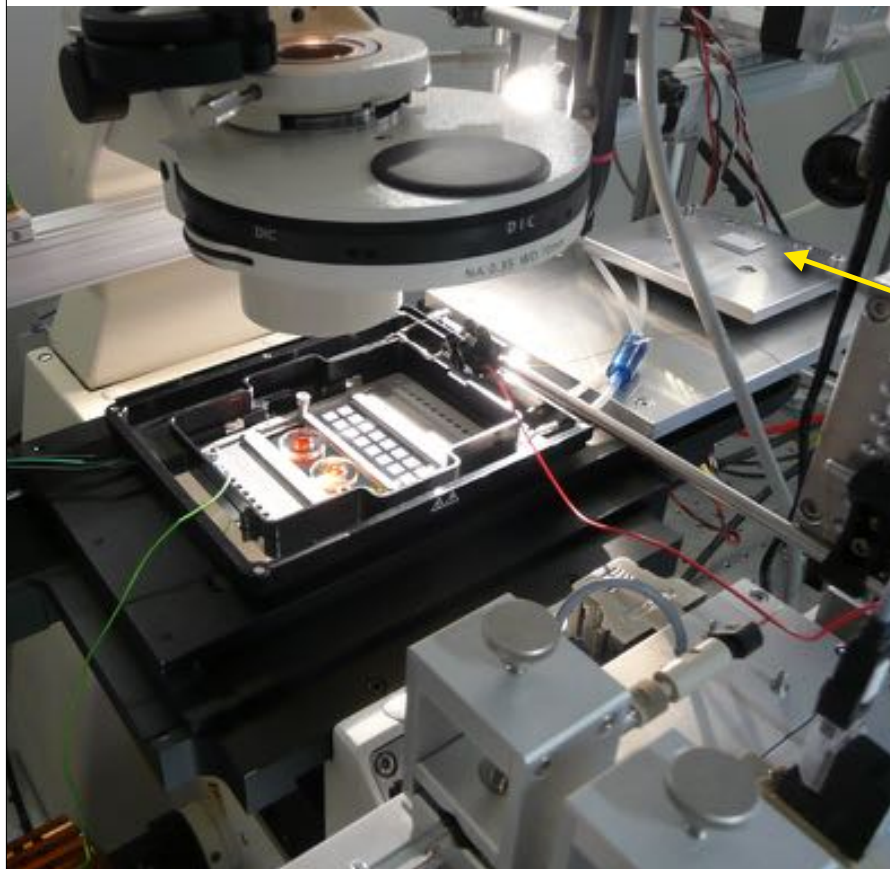
**Grid activation by a
Helium plasma beam
(without vacuum)**

**Writing on an EM grid
with a microfluidic capillary**

Team headed by
Thomas Braun,
C-CINA, Basel

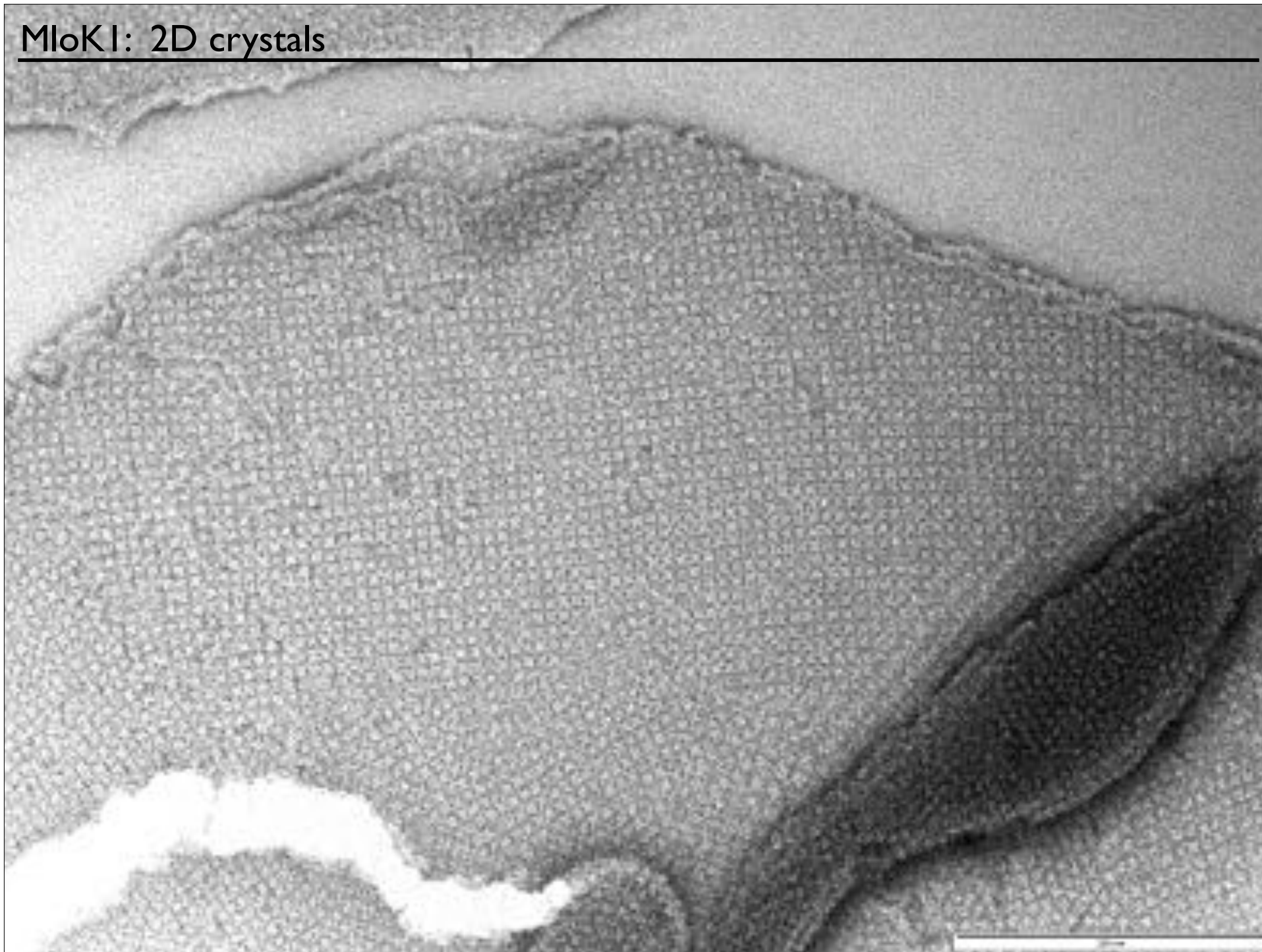


Microfluidic Cryo-EM Grid Preparation

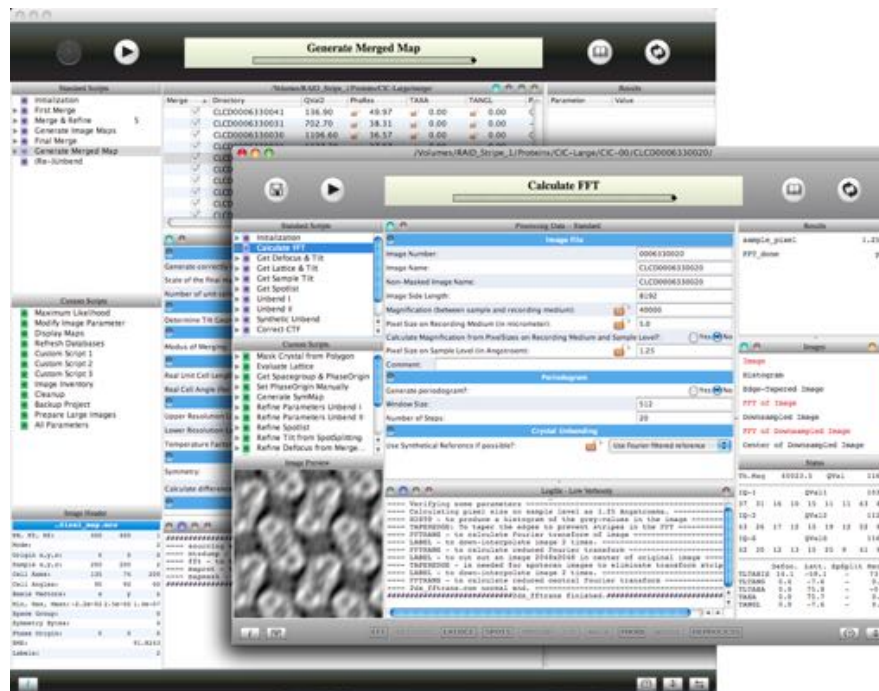
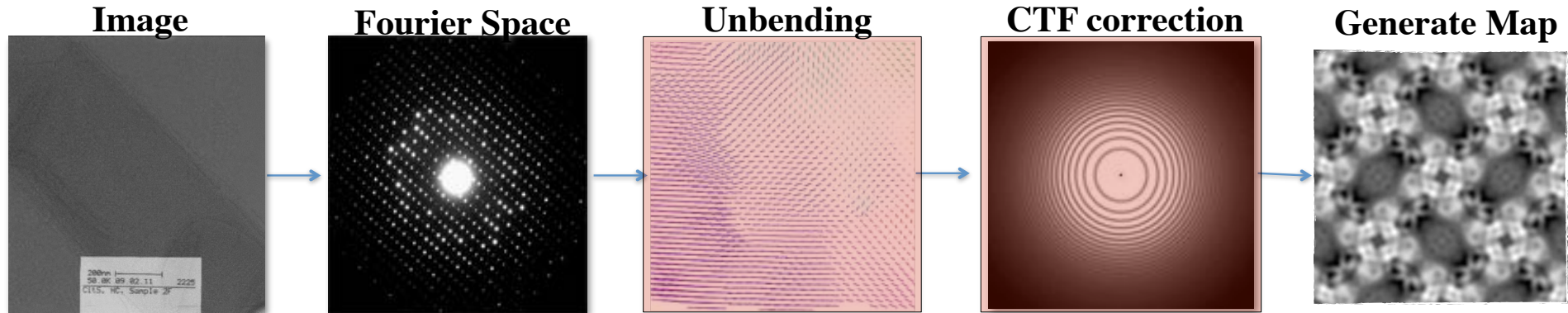


Team headed by
Thomas Braun,
C-CINA, Basel

MloKI: 2D crystals

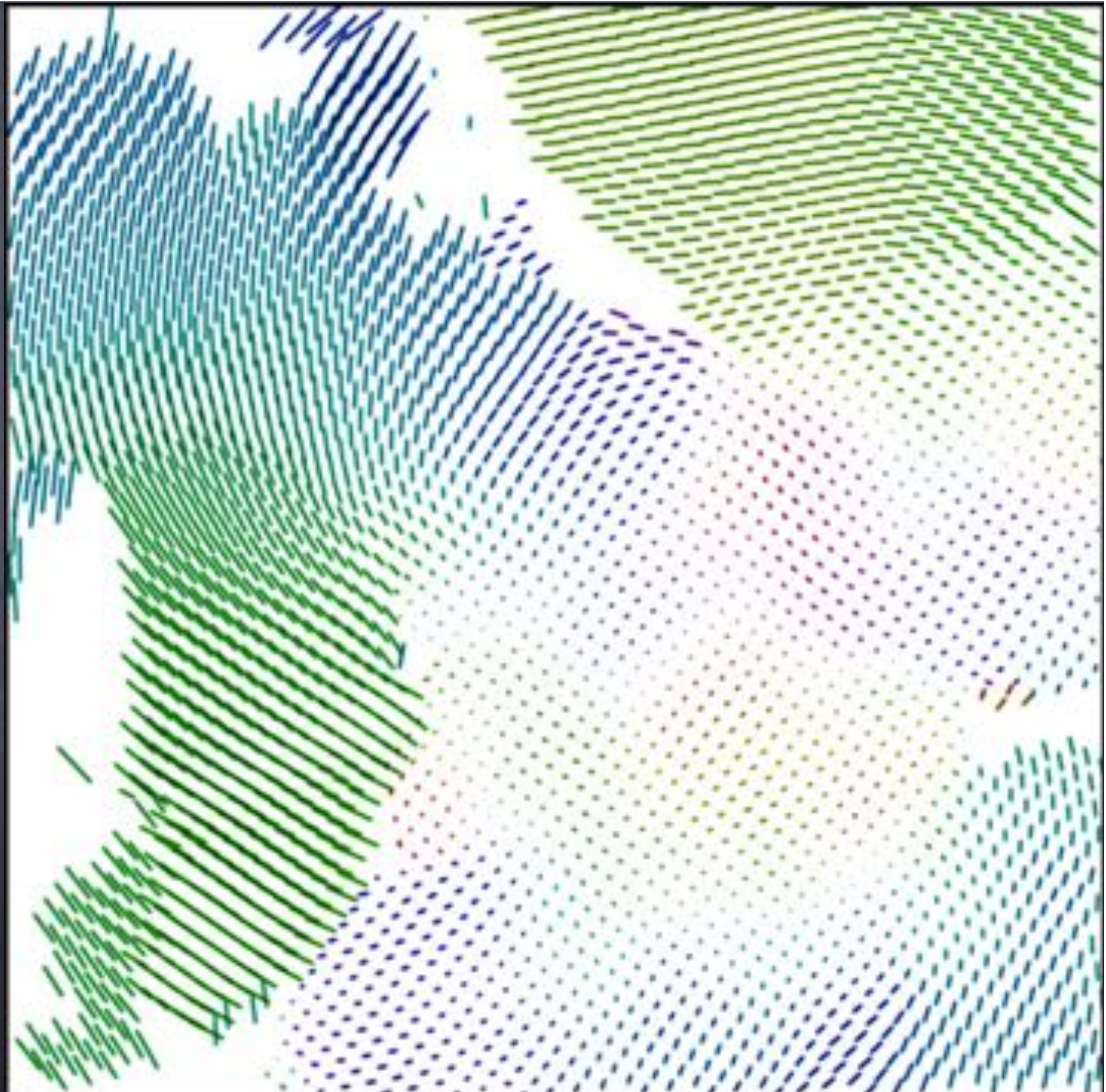


The 2dx software: user-friendly data processing in electron crystallography



Unbending Profile to correct crystal distortions

These lines show
10x exaggerated
vectors that
indicate how specific
areas of the image
have to be "warped"
to produce a
perfect crystal
image.



Fourier Transformation
of the
original 2D crystal image

(ZOOM)

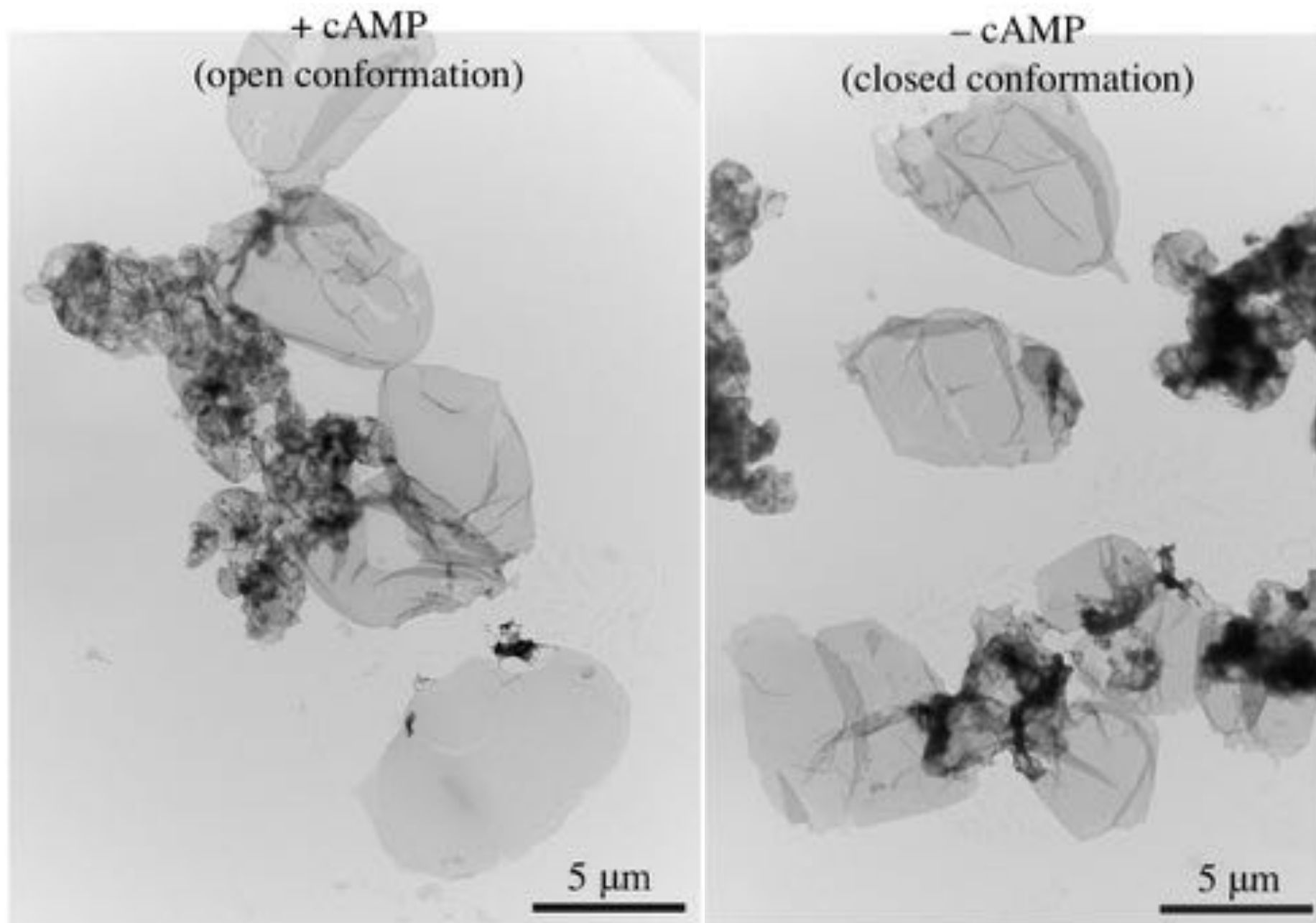


Fourier Transformation
of the
unbent 2D crystal image

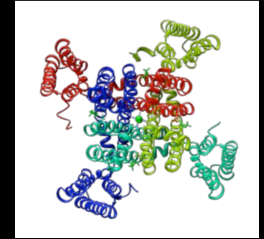
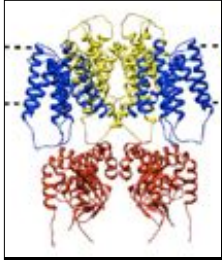
(ZOOM)



MloKI: 2D crystals

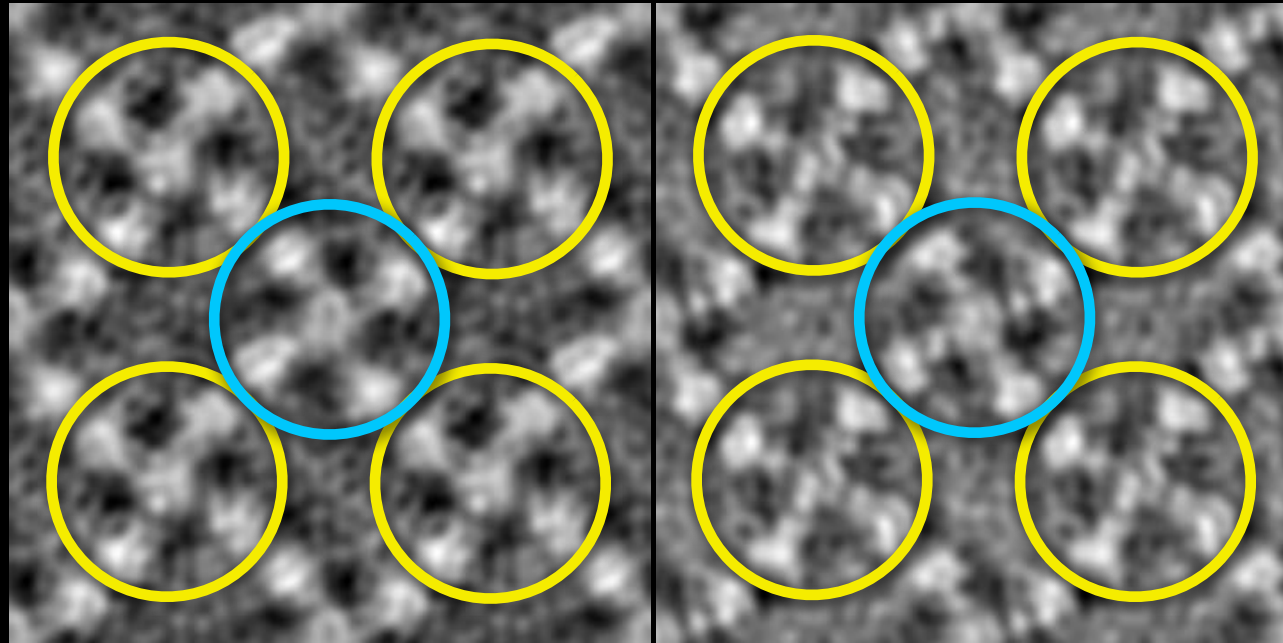


MloK I – Cryo-EM data collection of 2D crystals

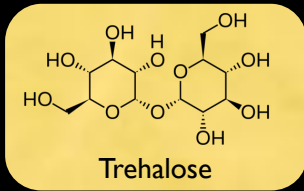


-cAMP

+cAMP

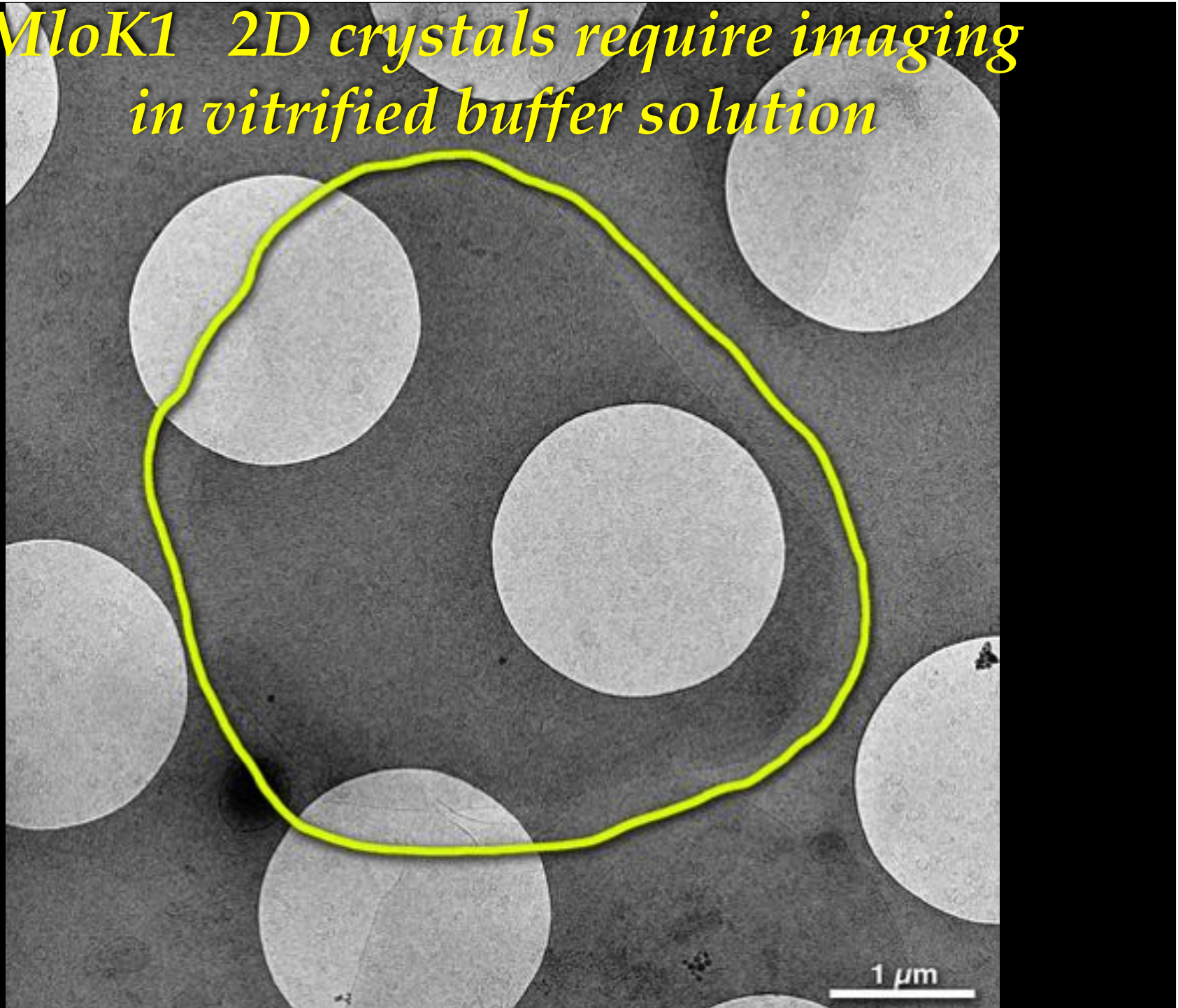


Sugar
Embedding

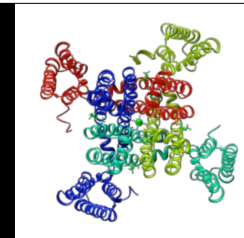
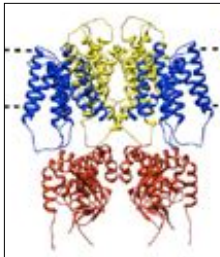


Julia Kowal
Paul Baumgartner
Mohamed Chami
Marcel Arheit
Sebastian Scherer

*MloK1 2D crystals require imaging
in vitrified buffer solution*



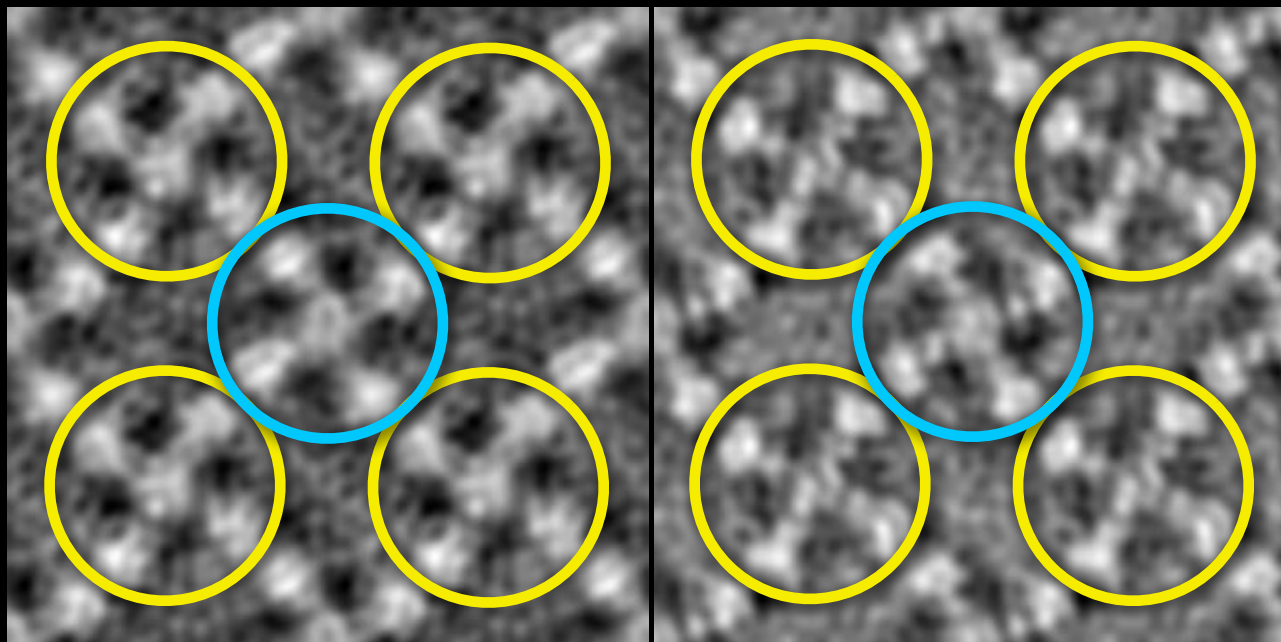
MloK I – Cryo-EM data collection of 2D crystals



-cAMP

+cAMP

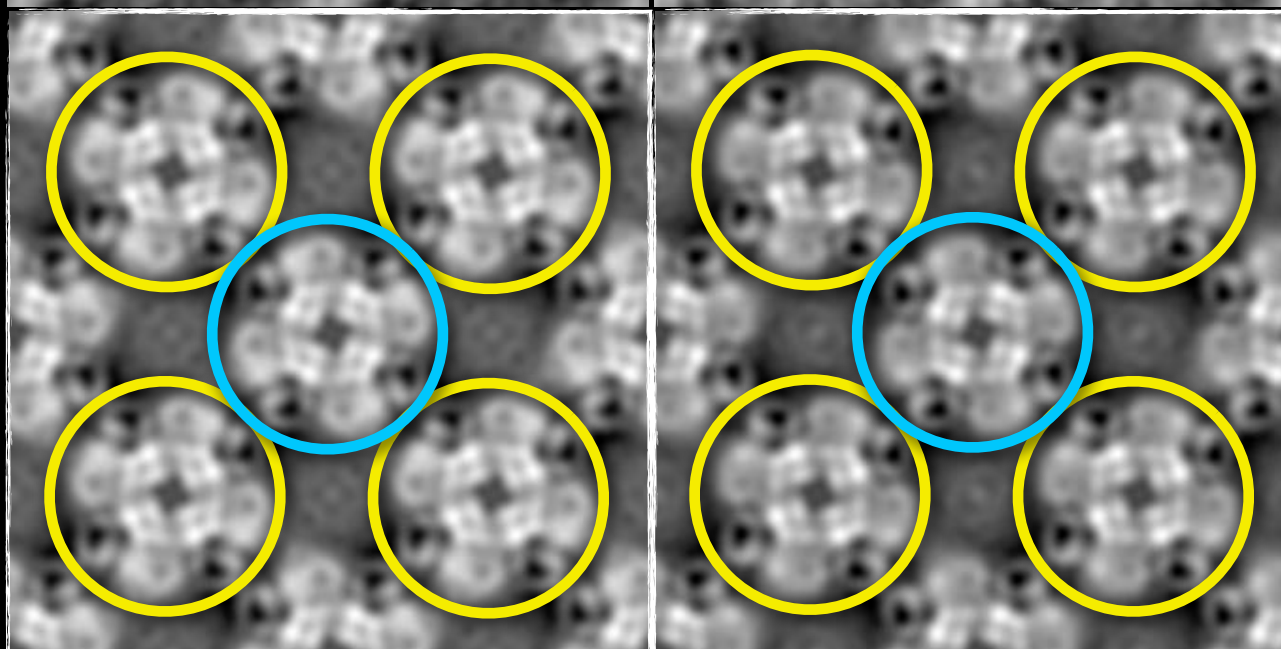
Sugar
Embedding



Plain
Cryo-EM

H₂O

Water



Julia Kowal
Paul Baumgartner
Mohamed Chami
Marcel Arheit
Sebastian Scherer

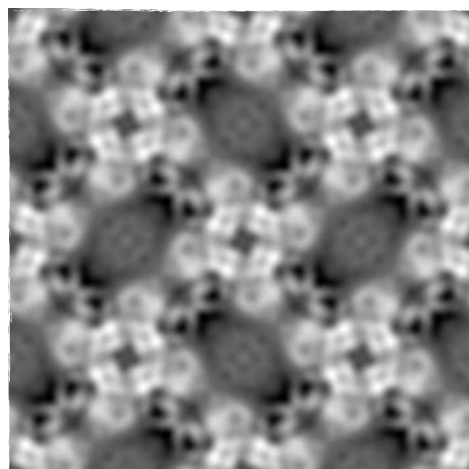
MloK I: electron crystallography



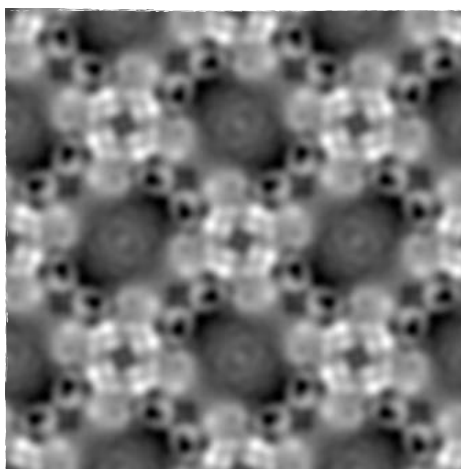
Julia Kowal

Paul Baumgartner
Mohamed Chami
Marcel Arheit
Sebastian Scherer

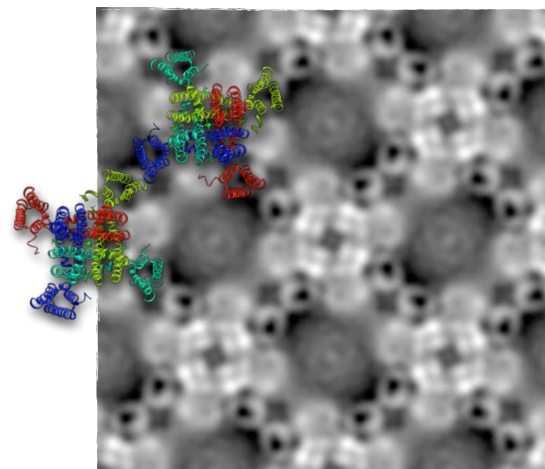
2D projection maps



MloK1 with ligand

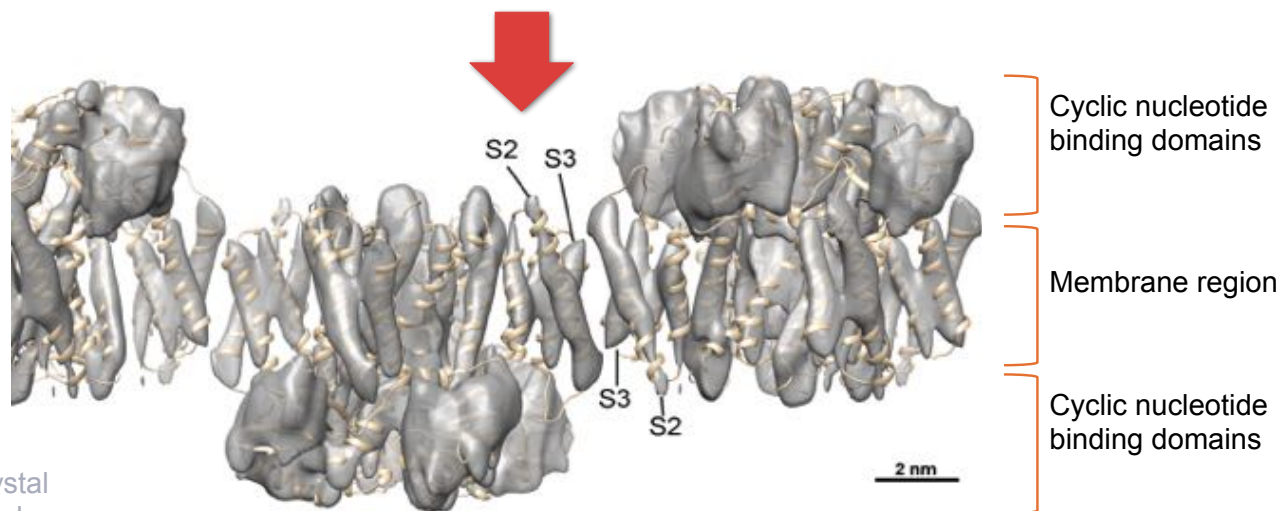


MloK1 without ligand



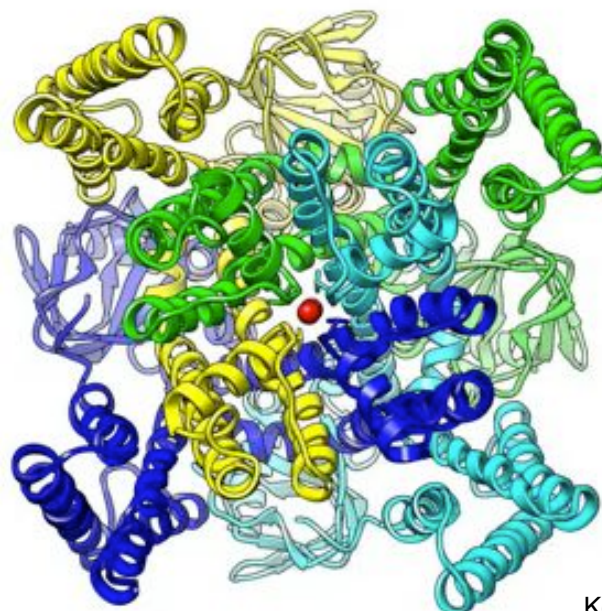
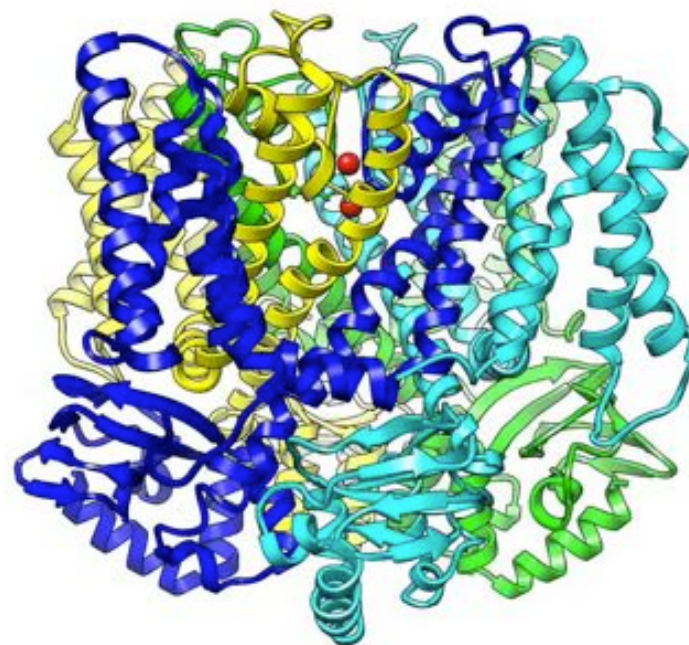
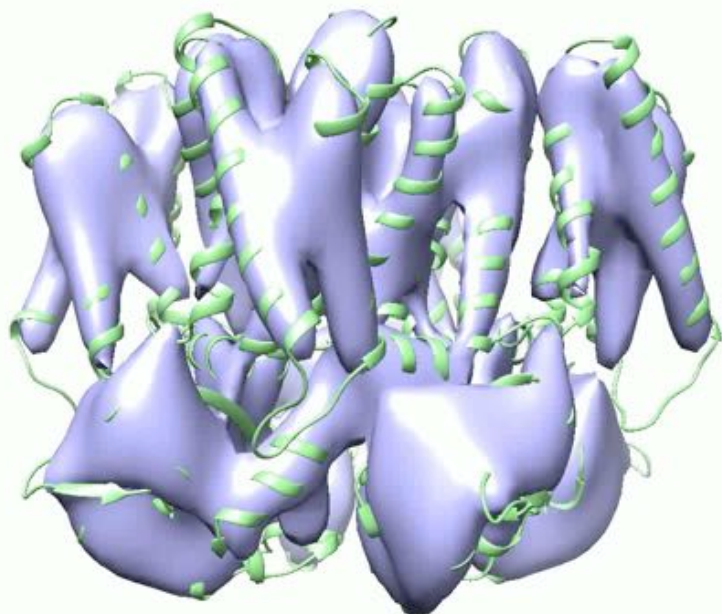
Both Maps

3D volumes



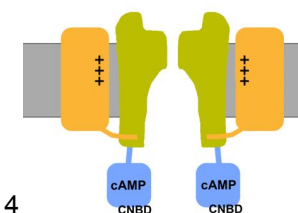
Side view of 2D crystal
MloK1 without ligand
Resolution: 7Å (12Å vertical)

MloKI with and without cAMP



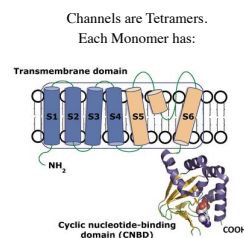
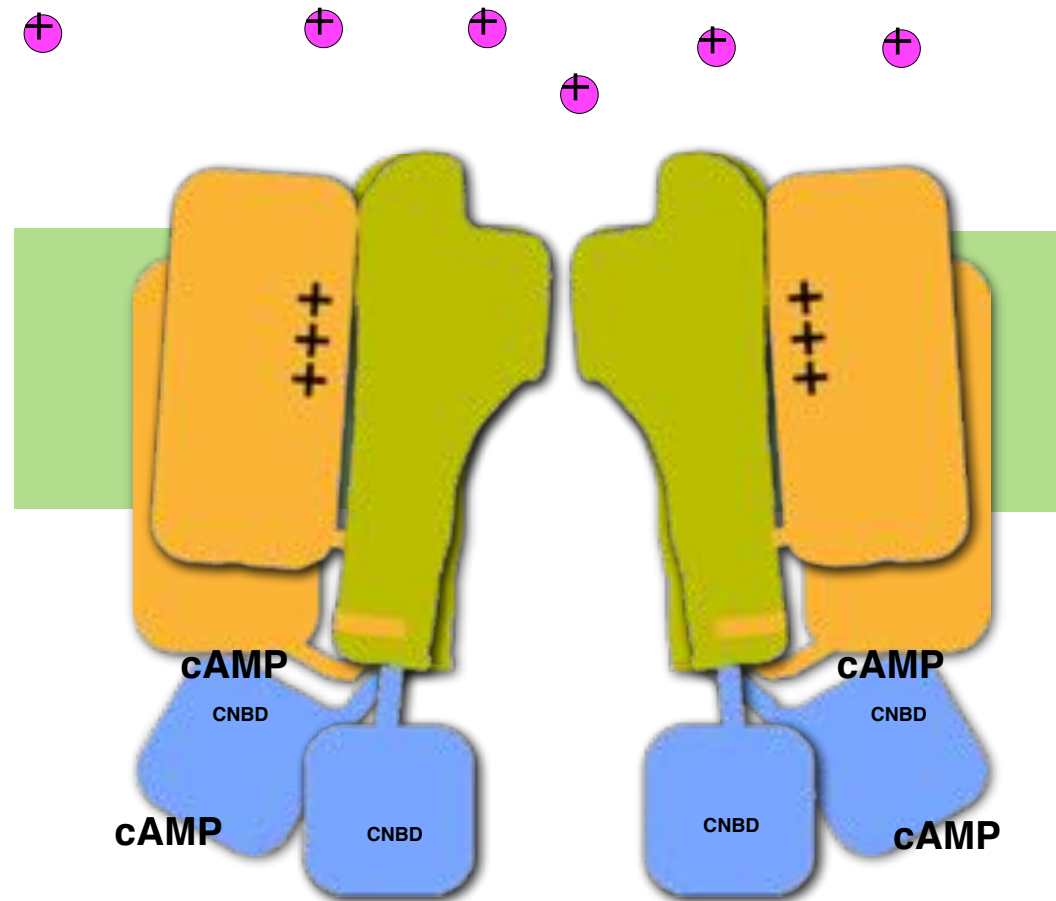
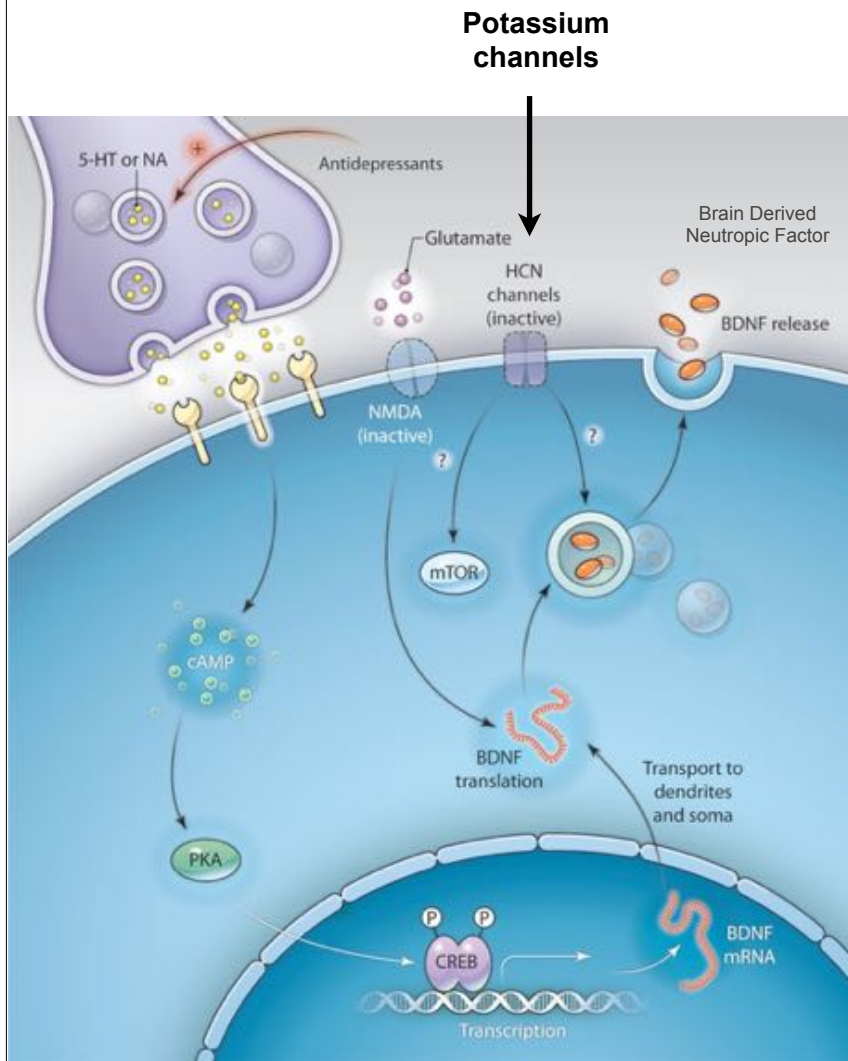
Gunnar Schröder, Jülich, Germany.
DireX
Deformable Elastic Network Fitting

G.F. Schröder, A.T. Brunger, and M. Levitt.
Structure (2007) 15:1630-1641



Kowal et al., Nature Comm. 2014

Mechanism of HCN channels?

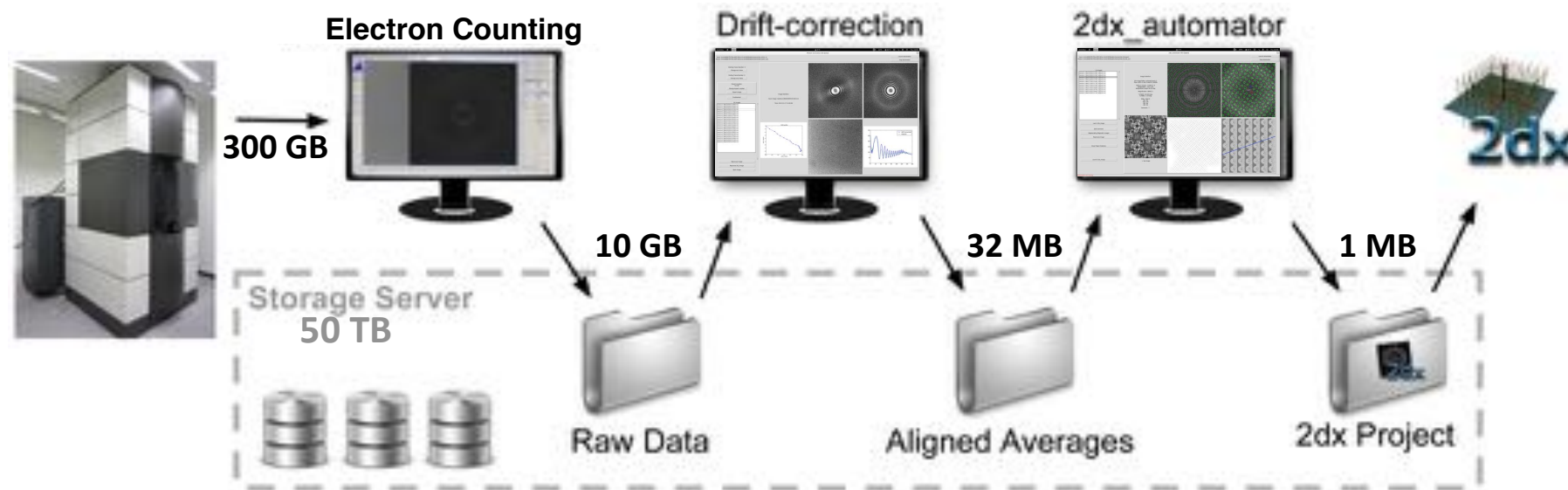


Shah, Science Signaling 5(244), 1-3 (2012)

J. Kowal *et al.*, Nature Communications (2014)

A K2 Summit camera arrived in Basel Nov. 2013

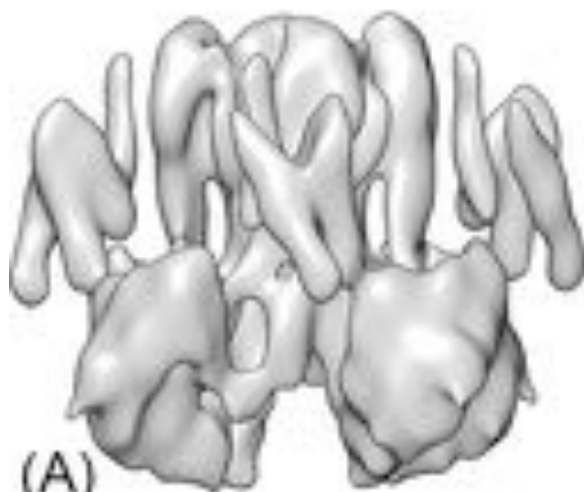
Automated data processing directly at the Titan



Automated movie alignment is using on Xueming Li's (Yifan Cheng lab, UCSF) alignment tool.

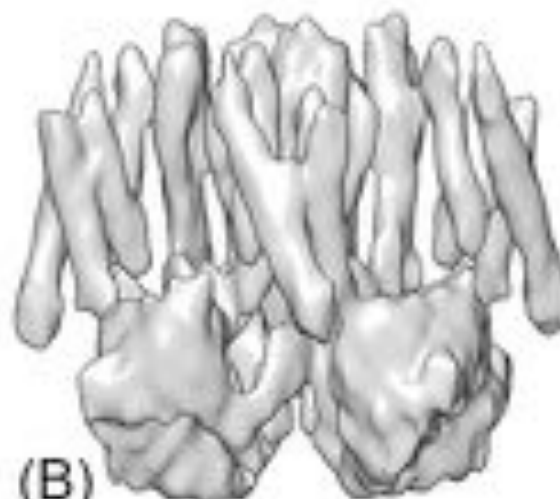
MloK I cryo-EM maps

Film



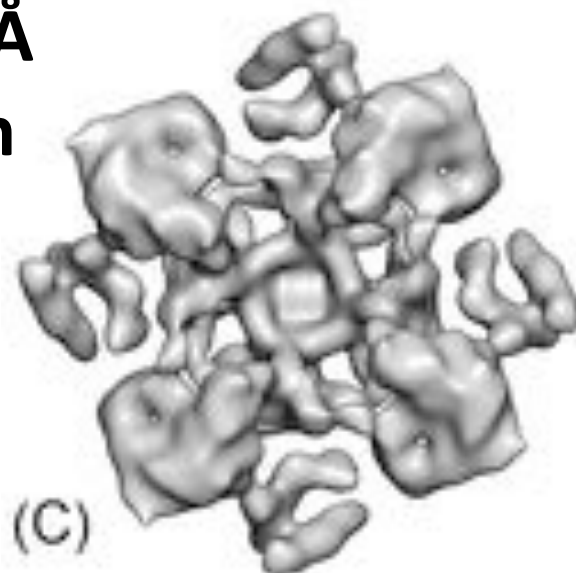
(A)

K2 Summit



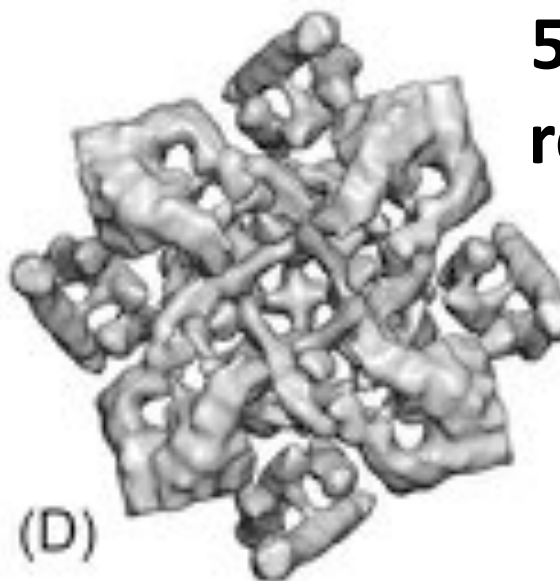
(B)

3 years:
7 x 7 x 12 Å
resolution



(C)

2 days:
5 x 5 x 9 Å
resolution



(D)

Scherer *et al.*, JSB 2014

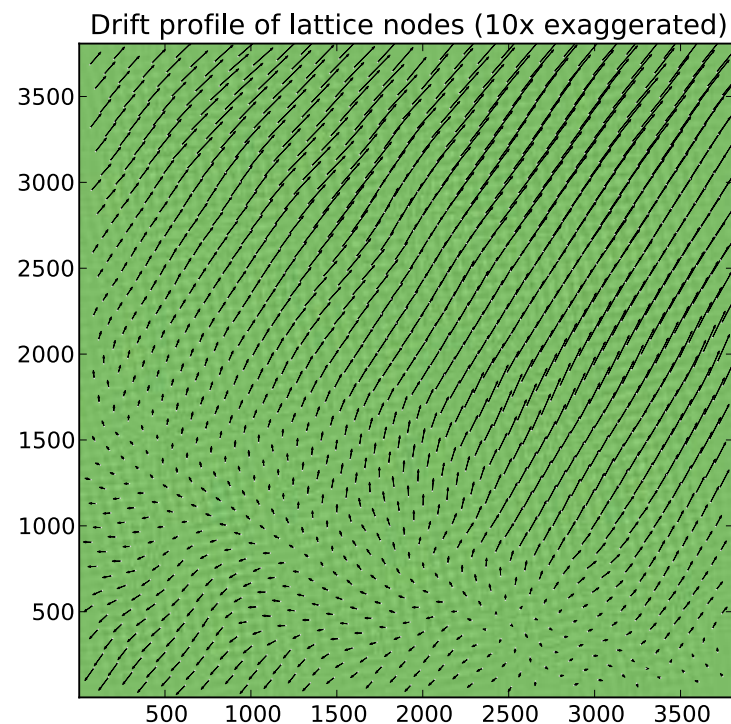
Movie-Mode image processing for 2D crystals

2D crystals locally move and distort under the electron beam!

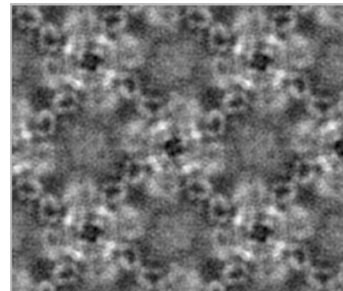
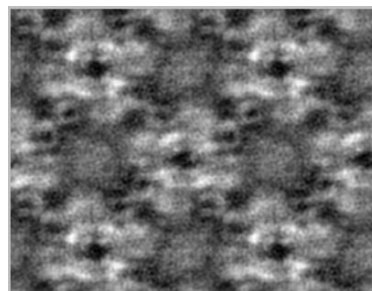
Crystal distortion vectors for 38 frames
from the same 2D crystal



The drift profile
for the movie sequence



Reconstruction
without
movie-mode
processing

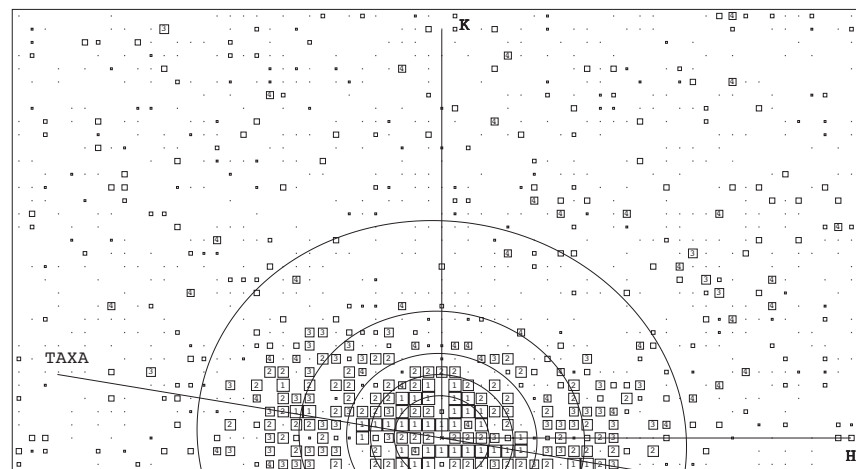
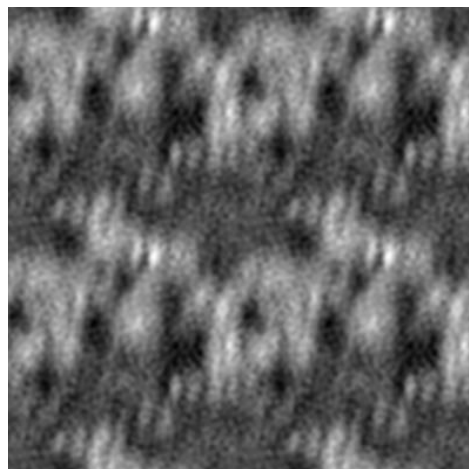


Reconstruction
with
movie-mode
processing

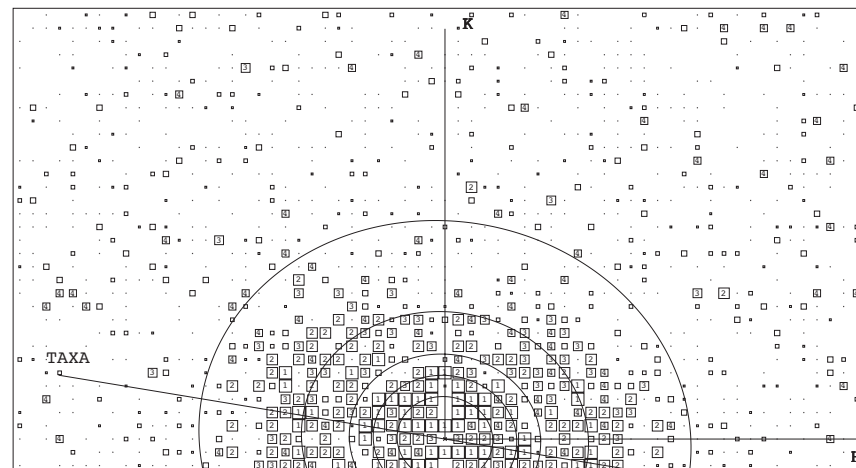
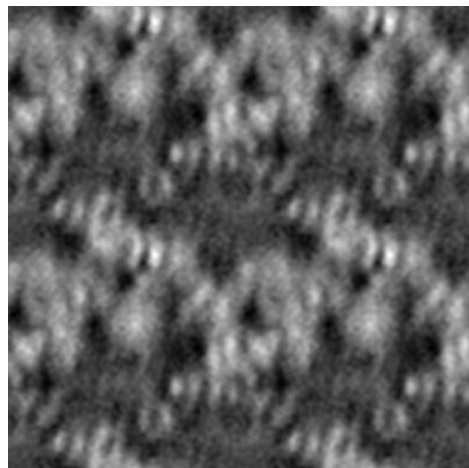
Movie-Mode image processing for 2D crystals

Higher resolution, especially for tilted 2D crystals
perpendicularly to the tilt axis.

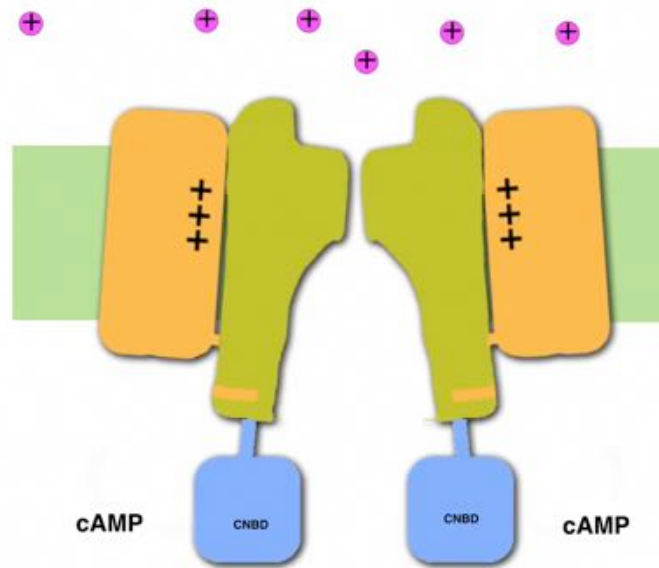
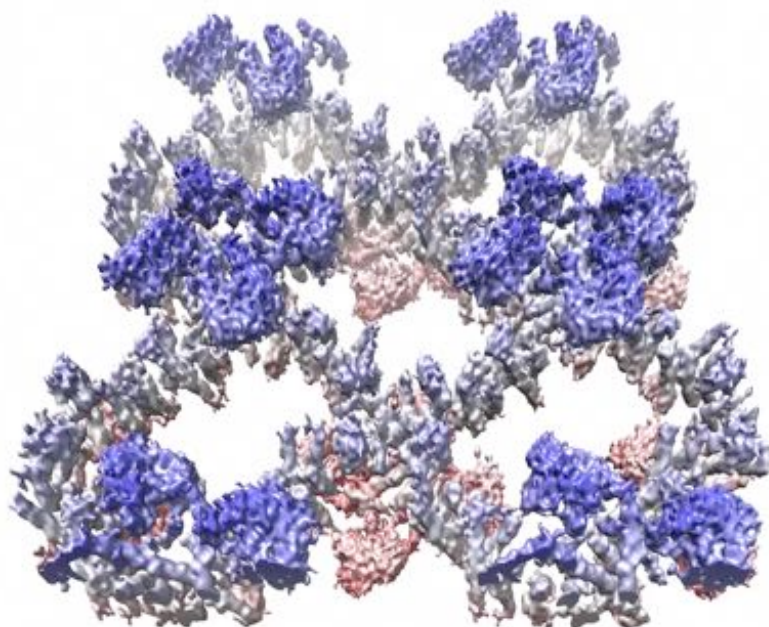
Classical image
processing



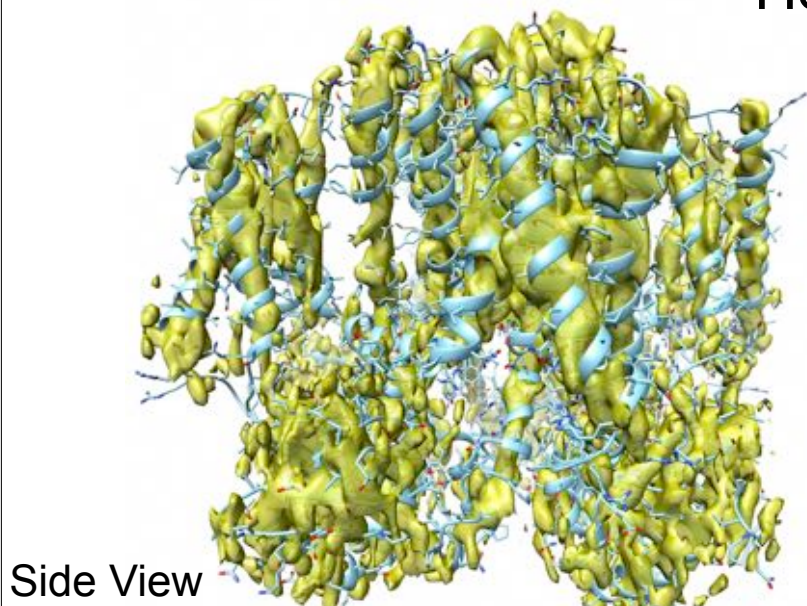
Movie-mode frame
processing



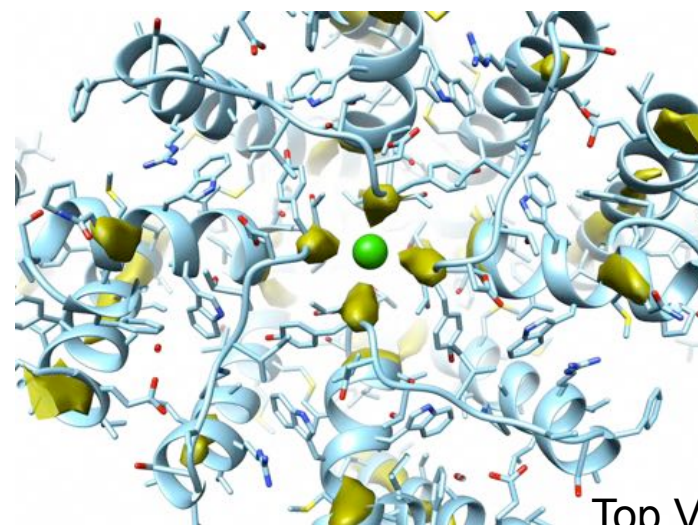
MloK I: cAMP-modulated K⁺ channel with putative voltage sensors



Here: with cAMP



Side View



Top View

Conclusions

- **2D crystals of membrane proteins that diffract electrons to 10Å can be grown within 6 to 12 months in most cases.**
- **Electron Diffraction only works on 2D crystals that are well-ordered and >1 μm diameter.**
- **Massive advantage from direct electron detectors:**
 - 3x better SNR, 3x smaller PSF
 - Drift correction (movie mode)
 - Dose fractionation in movies (dose-dependent resolution filter)
 - 3x improved final resolution (e.g.: 9Å => 3Å)
- **3.0Å resolution is (almost) possible by cryo-EM imaging with direct electron detection of 2D crystals >100nm diameter that diffract to at least 1nm resolution.**
- **2.0 Å resolution from cryo-EM of membrane proteins should be possible after:**
 - correction for the curvature of the Ewald Sphere
 - correction for the limited flatness of the 2D crystals
 - correction for the effect of beam tilt.
 - more precise determination of the defocus.
- **What SNR(q) can an instrument give us before target destruction?**
 - TEM @ 300kV: 30 e/Å² total dose to measure up to 3Å.
 - TEM @ 300kV: 120 e/Å², when using dose-dependent resolution filter (<30 e/Å²: 3Å, <50 e/Å²: 7Å, <120 e/Å²:15Å).

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MloK1:

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