Towards the femtosecond crystallography using SACLA on membrane protein crystals

So Iwata

Kyoto University/Riken SPring-8 Center (SACLA)

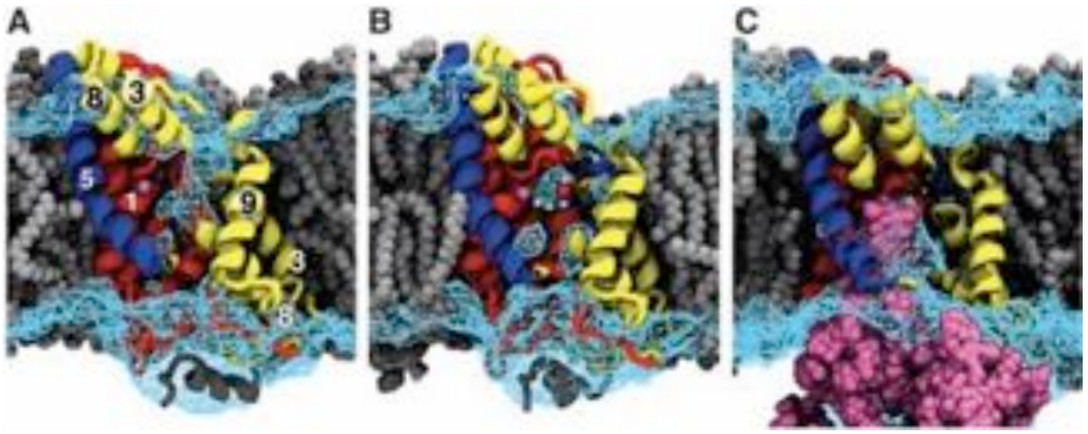
Membrane Protein Crystallography Pipeline based on Recombinant Expression Systems Focusing on Mammalian Targets

- Method development to address major bottlenecks for membrane protein crystallography.

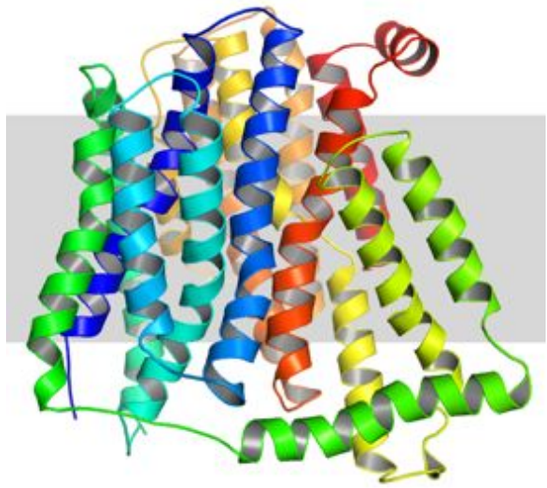
Key parameters to monitor	• Activity • Expression level • Integrity	• Monodisper-sity • Thermal stability	• Detergent concentration • Resolution	• Indexing • Scaling and merging	
Processes	Expression ~500	Purification ~200	Crystallisation ~60	Data collection ~30	Structure determi-nation 10
Technical options/ developments	• Construct design • Expression systems • Culture conditions	• Detergent choice • Purification methods • Additives	• Crystallisation methods • Detergent choice • Additives • Antibody fragments	• Data collection systems • Sample mounting • XFEL	• Phasing methods • Model building and refinement

Membrane transporters & respiratory enzymes

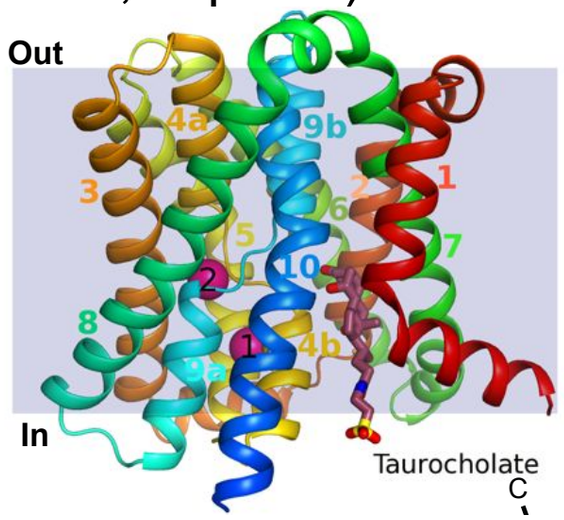
Mhp1 hydantoin transporter (Science 2008, Science 2010)



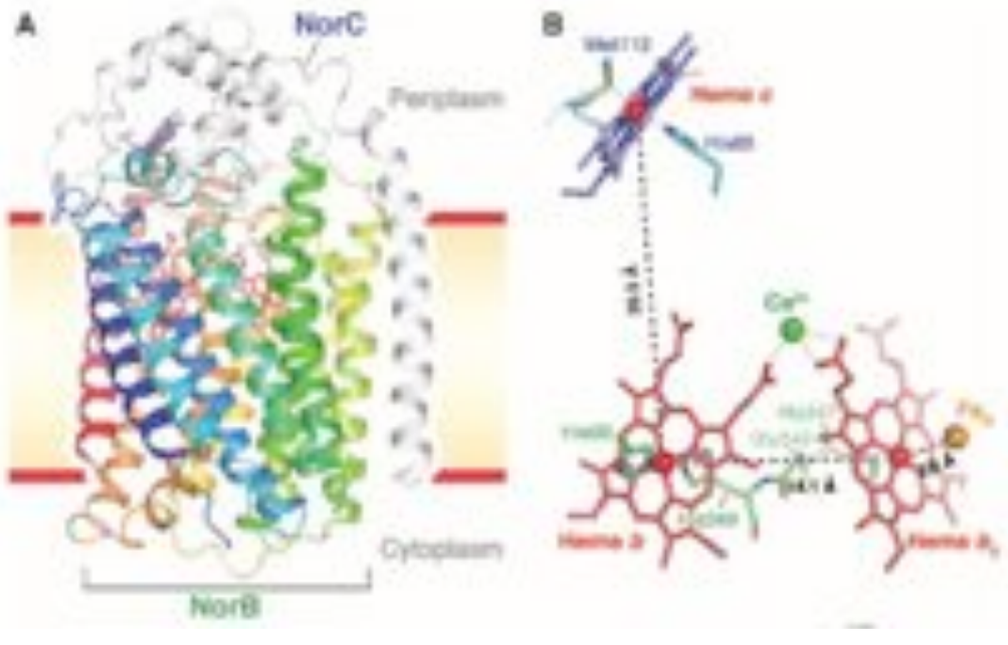
Oligopeptide transporter (EMBO J. 2010)



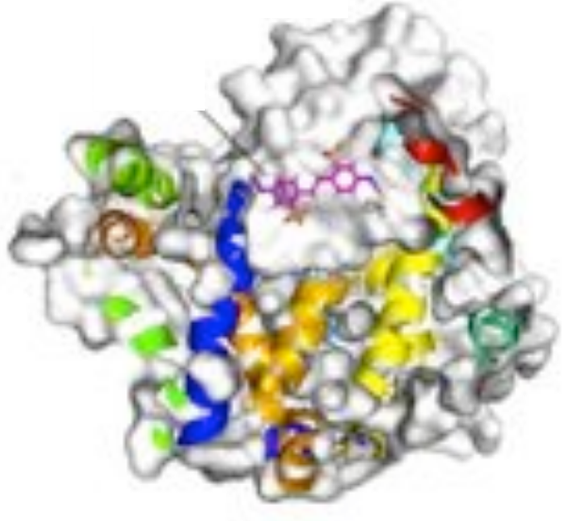
ASBT bile acid transporter (Nature 2011, in press)

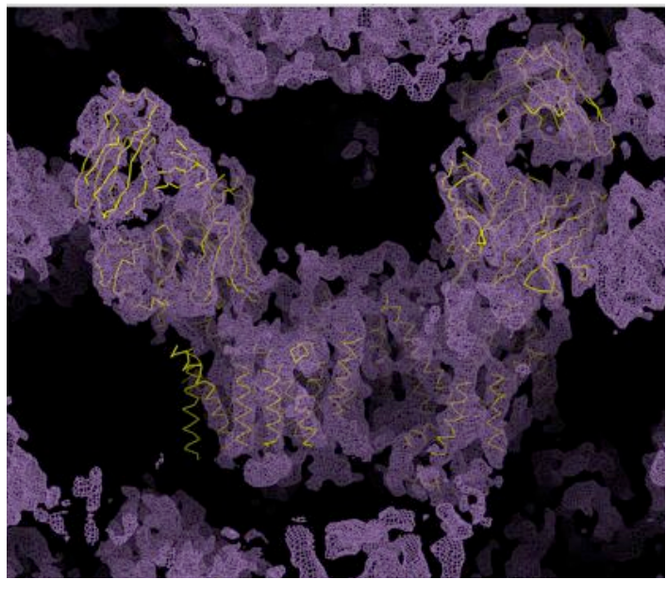


NO reductase- Fab complex (Science 2010)



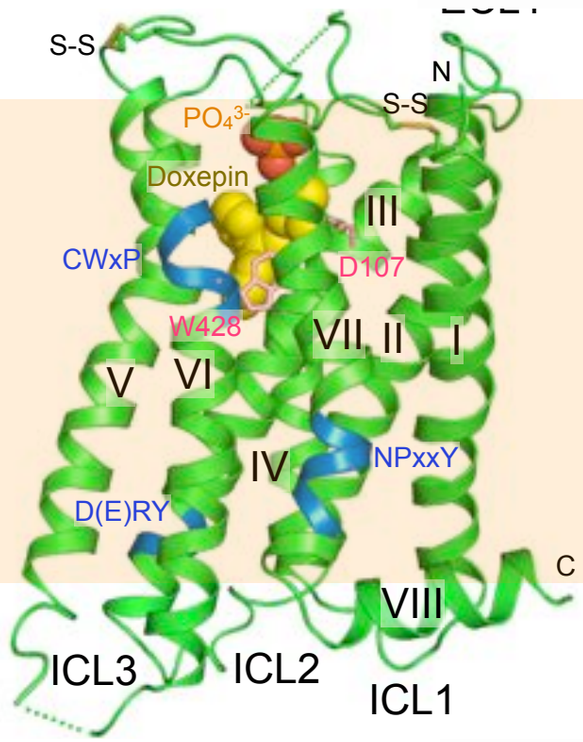
Human anion exchanger - Fab complex

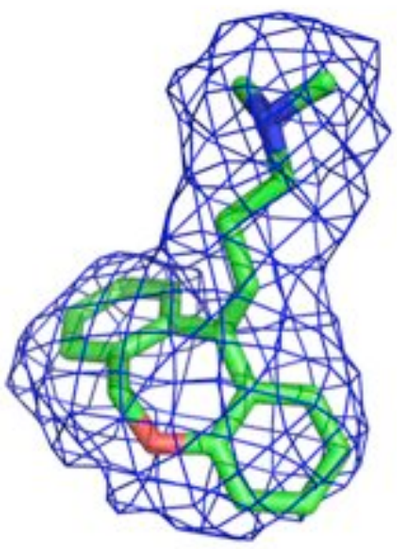




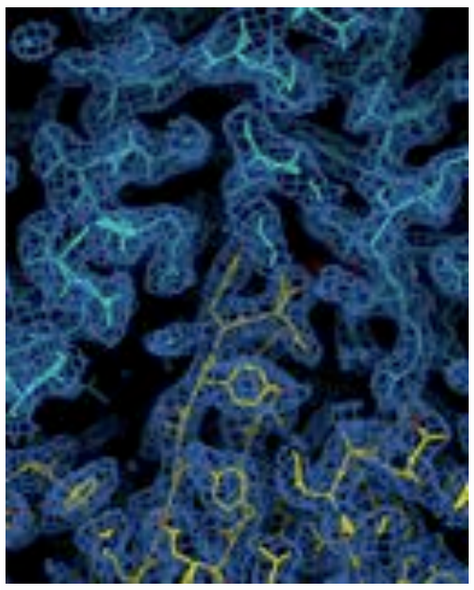
G-protein coupled receptors

Human Histamine H_1R (Nature 2011)





Antihistamine (Doxepin)



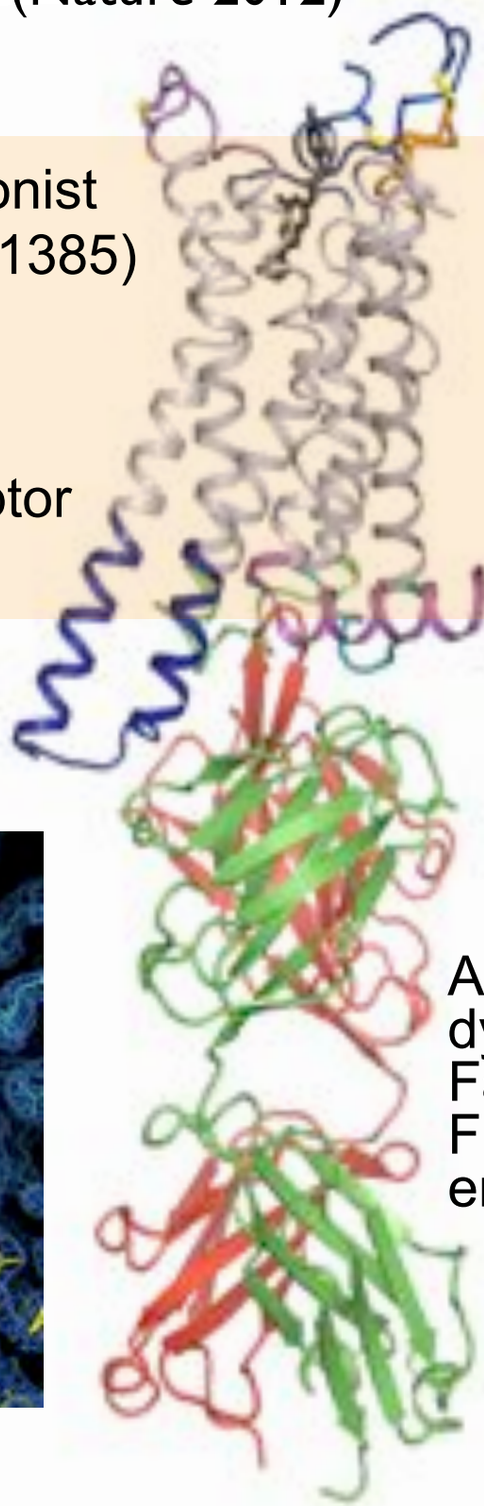
CDR-H3

Human Adenosine A2aR-Fab complex (Nature 2012)

Antagonist (ZM241385)

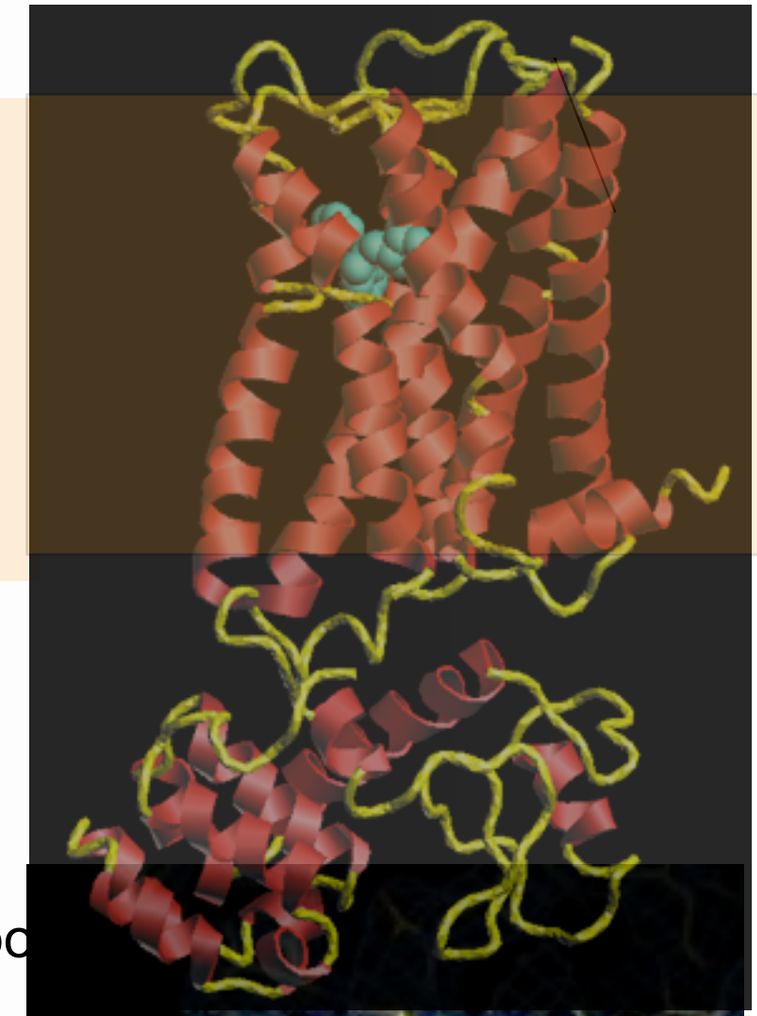
A2a receptor

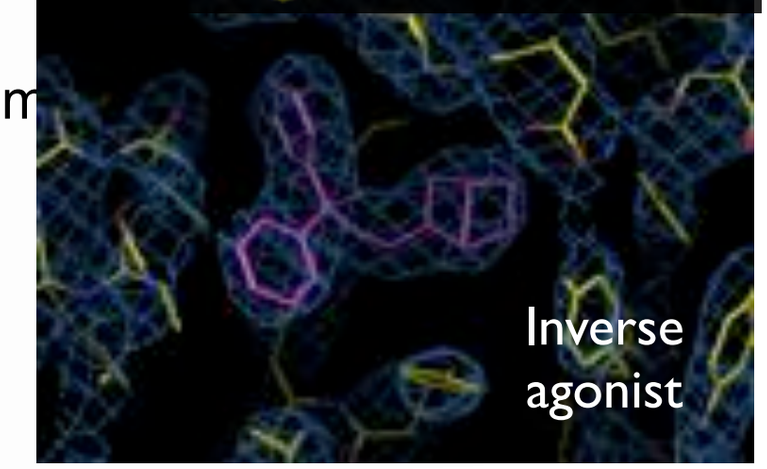
i3-loop



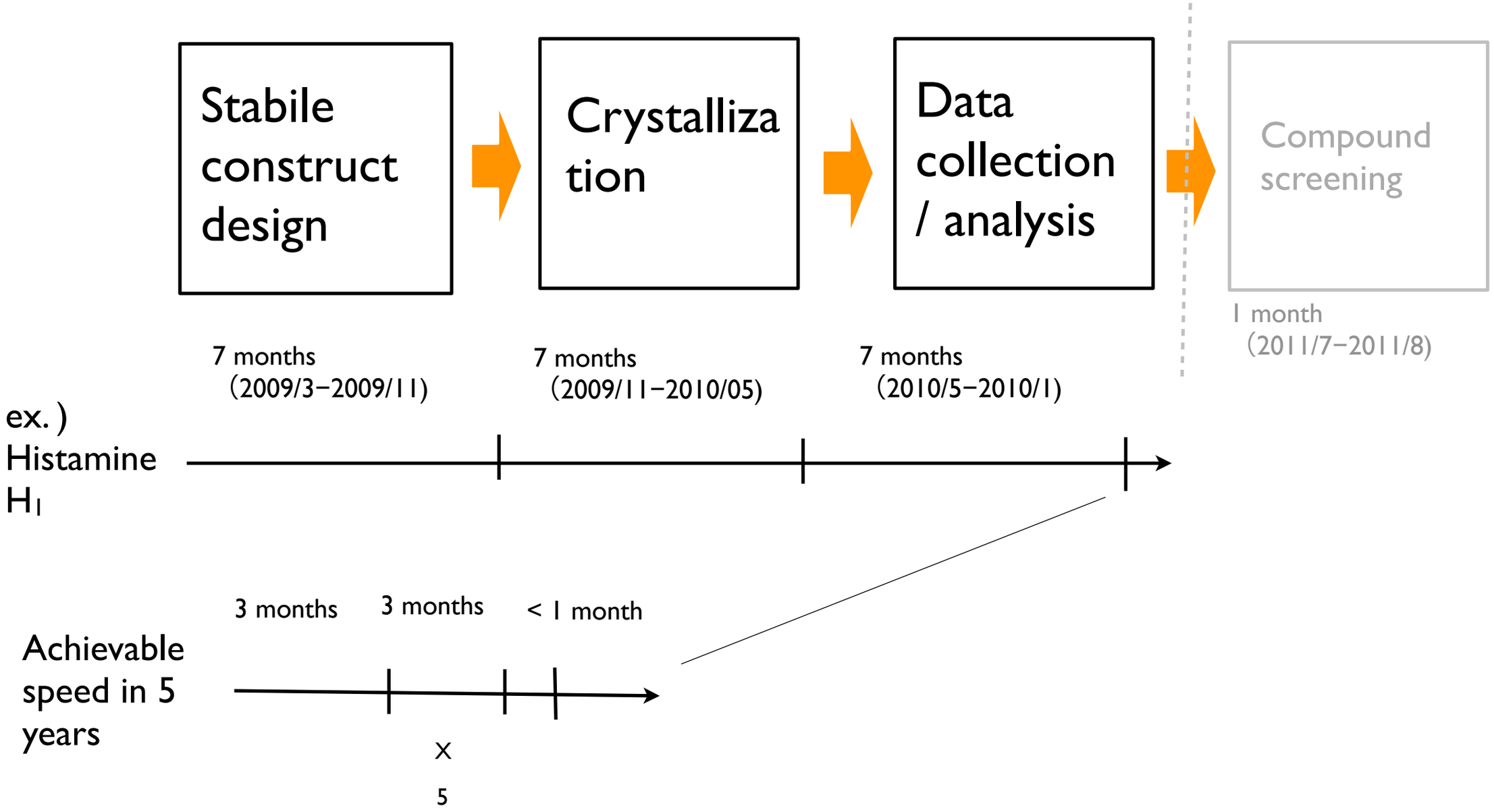
Antibody Fab Fragment

Human Muscarinic acetylcholine M_2R (2012)



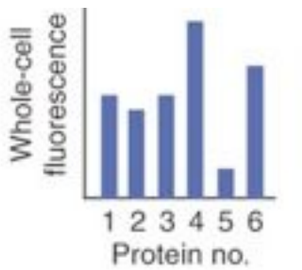


How fast can we solve a membrane protein structure?

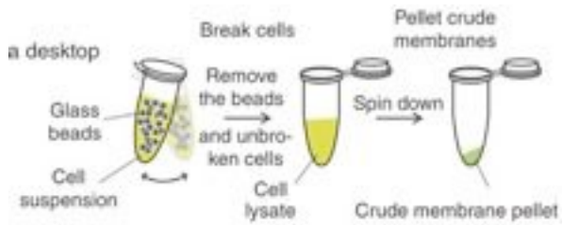


GFP-based Membrane Protein Expression and Purification System in *S. cerevisiae* (1)

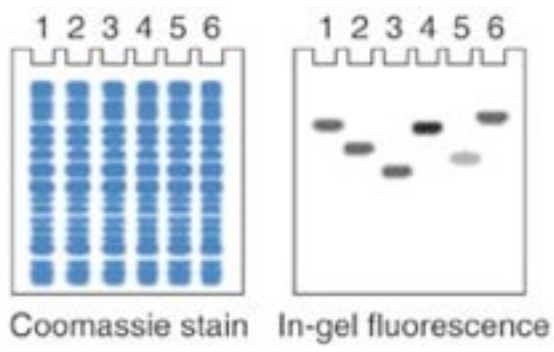
Imperial College London



1.Screen membrane protein-GFP over-expression by whole-cell fluo-rescence

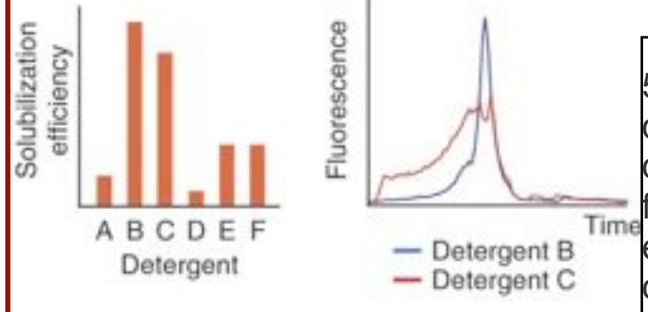


2. Isolate crude membranes

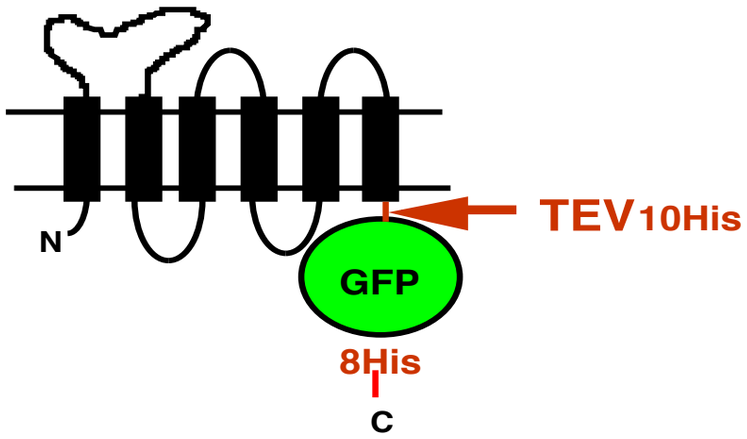


3.Check integrity using in-gel fluorescence

4. Check cellular localization by confocal micro-scopy

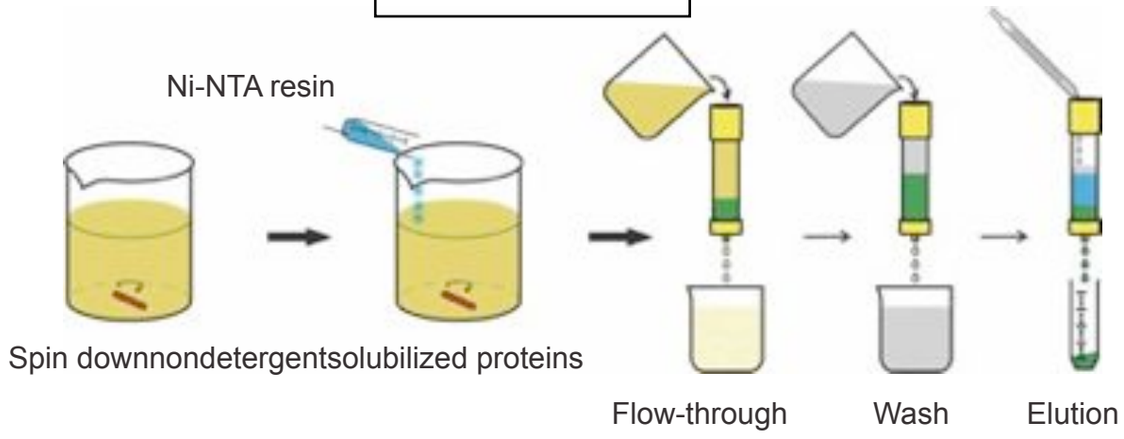


5.Check mono-disperisty in different deter-gent by fluorescence size-exclusion chromatography

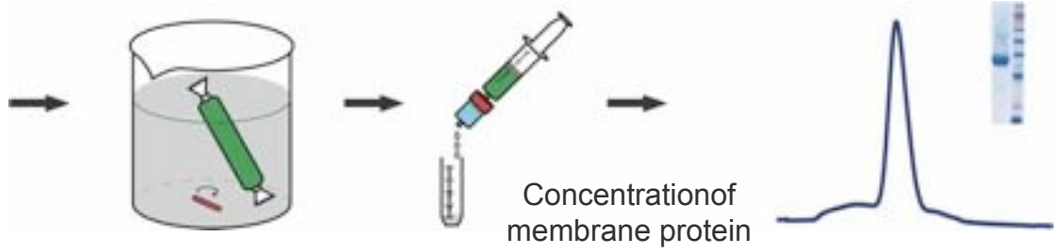


Drew *et. al.* Nat. Methods. 2, 2006
Newstead *et. al.* PNAS 104, 2007
Drew *et al.*, Nat. Protoc. 3, 2008

1.Detergent-solubilization → 2.Incubation of supernatant with Ni-NTA resin → 3.IMAC purification

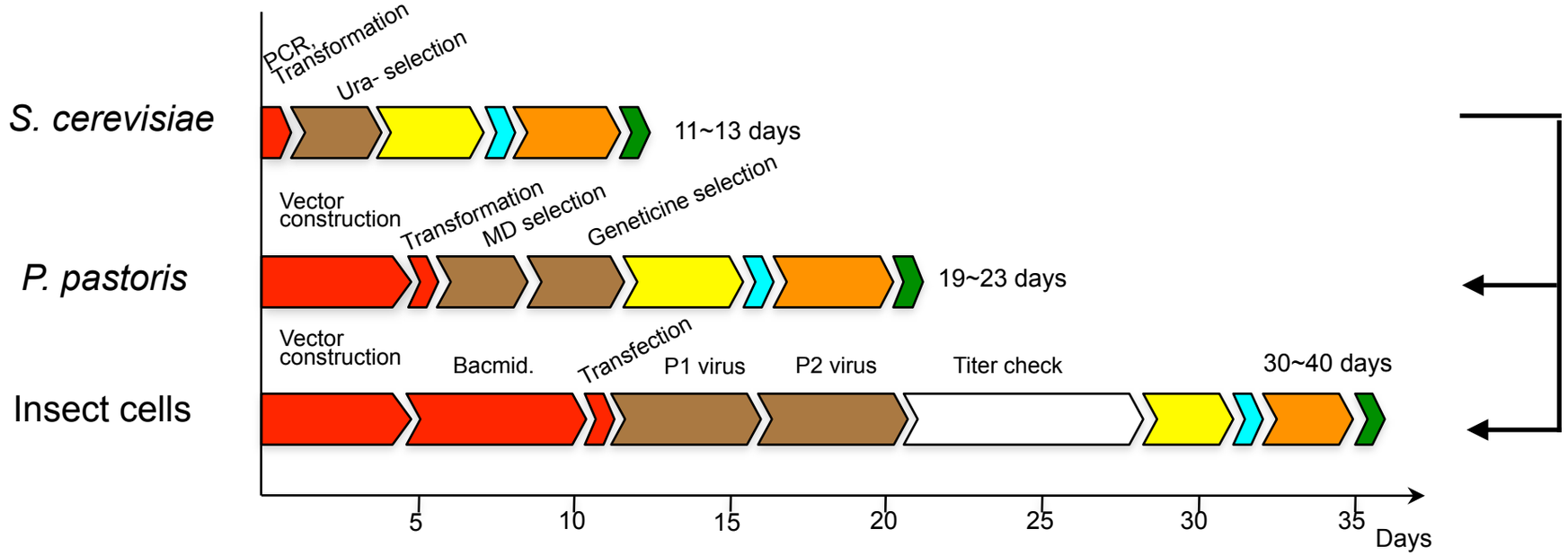


4.Dialysis and cleavage of GFP-8His → 5.Reverse IMAC to remove GFP-8His and his-tagged TEV → 6.Gel filtration of membrane protein (option)



GFP-based Membrane Protein Expression and Purification System in *S. cerevisiae* (2)

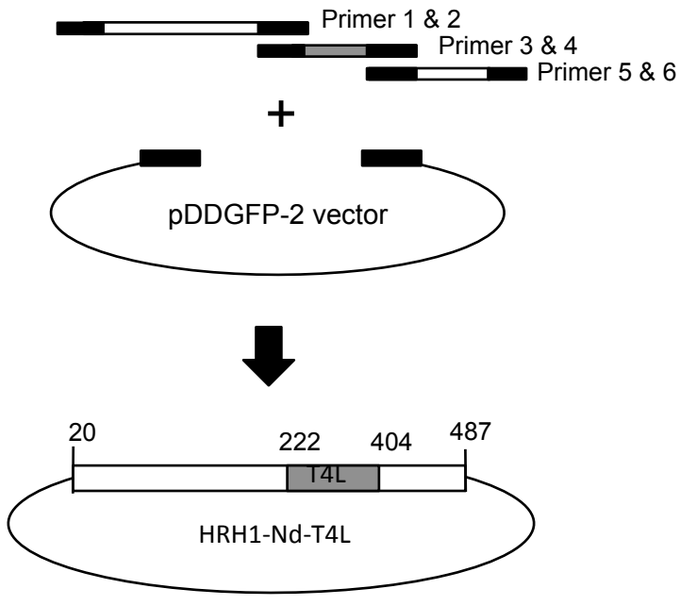
- Suitable for fast screening of the most stable/expressed constructs

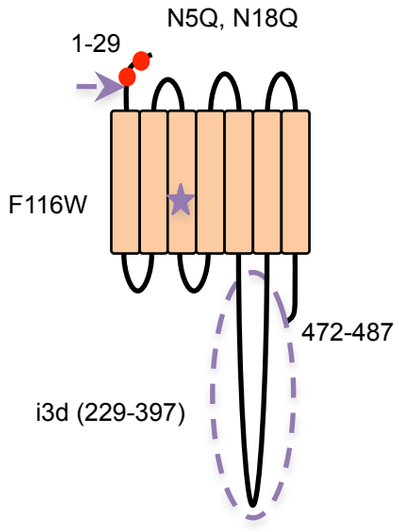


Easy to transfer to the systems to produce better proteins

Sugawara *et. al.* BBRC. 704, 2009

- Easy to construct variants by combining multiple fragments

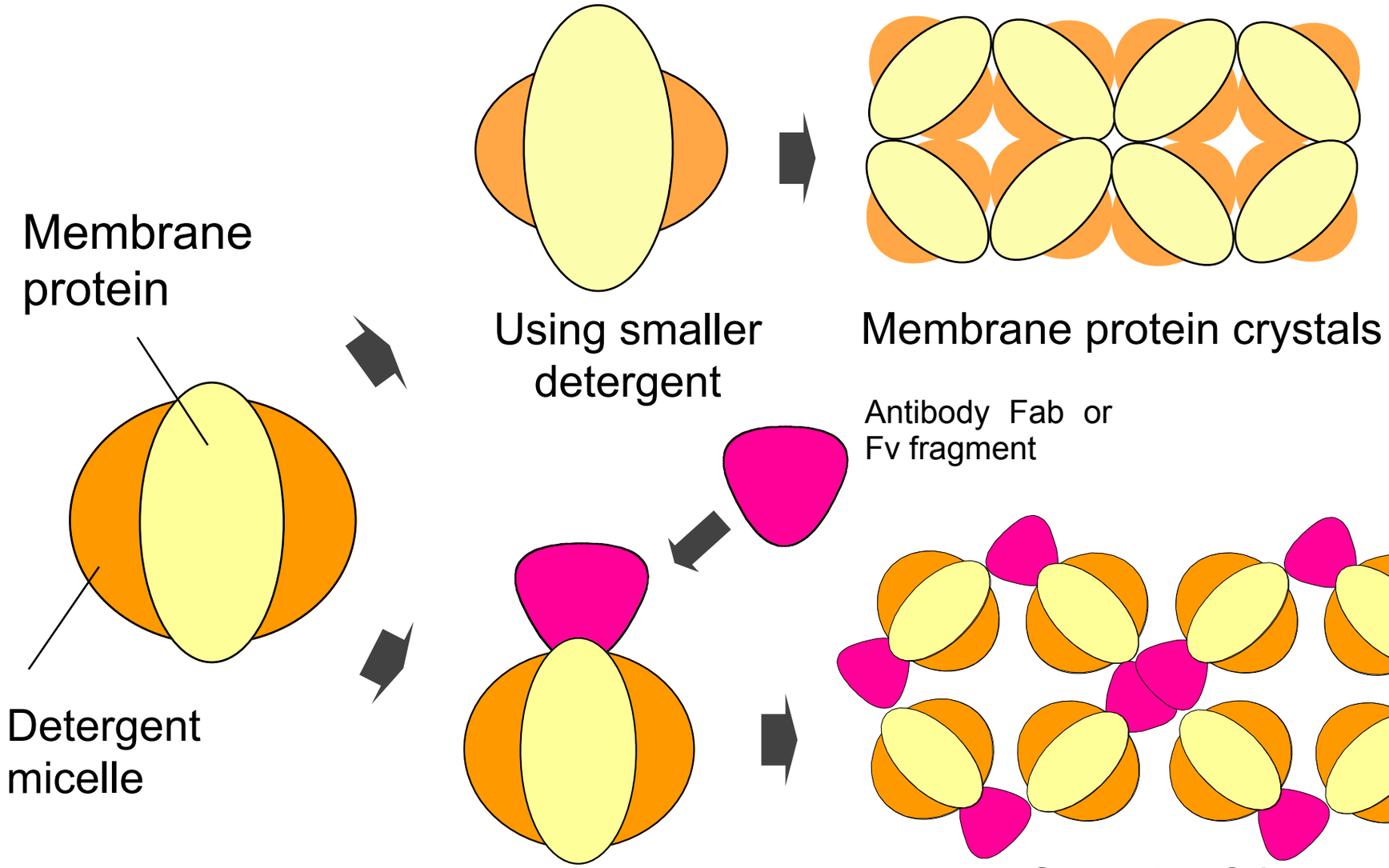


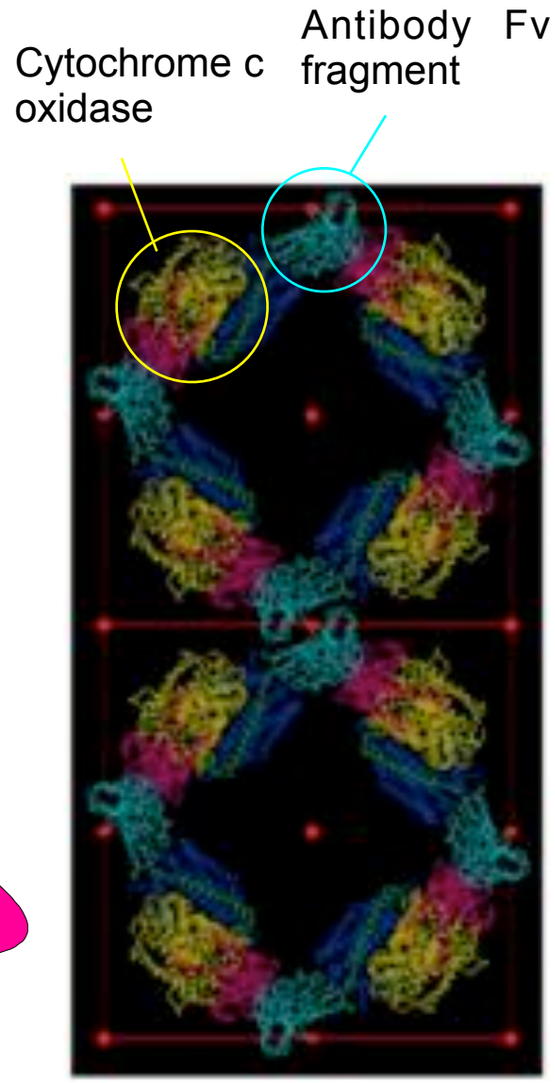


Name	Deletions and mutations
HRH1-WL	HRH1 whole length
HRH1-Nd-i3d	HRH1(20-487), i3d(229-397)
HRH1-Nd-T4L	HRH1(20-487), T4L
HRH1-Nd-F116W-i3d	HRH1(20-487), F116W, i3d(229-397)
HRH1-Nd-L123W-T4L	HRH1(20-487), F116W, T4L

Crystallisation of Membrane Proteins using Detergents

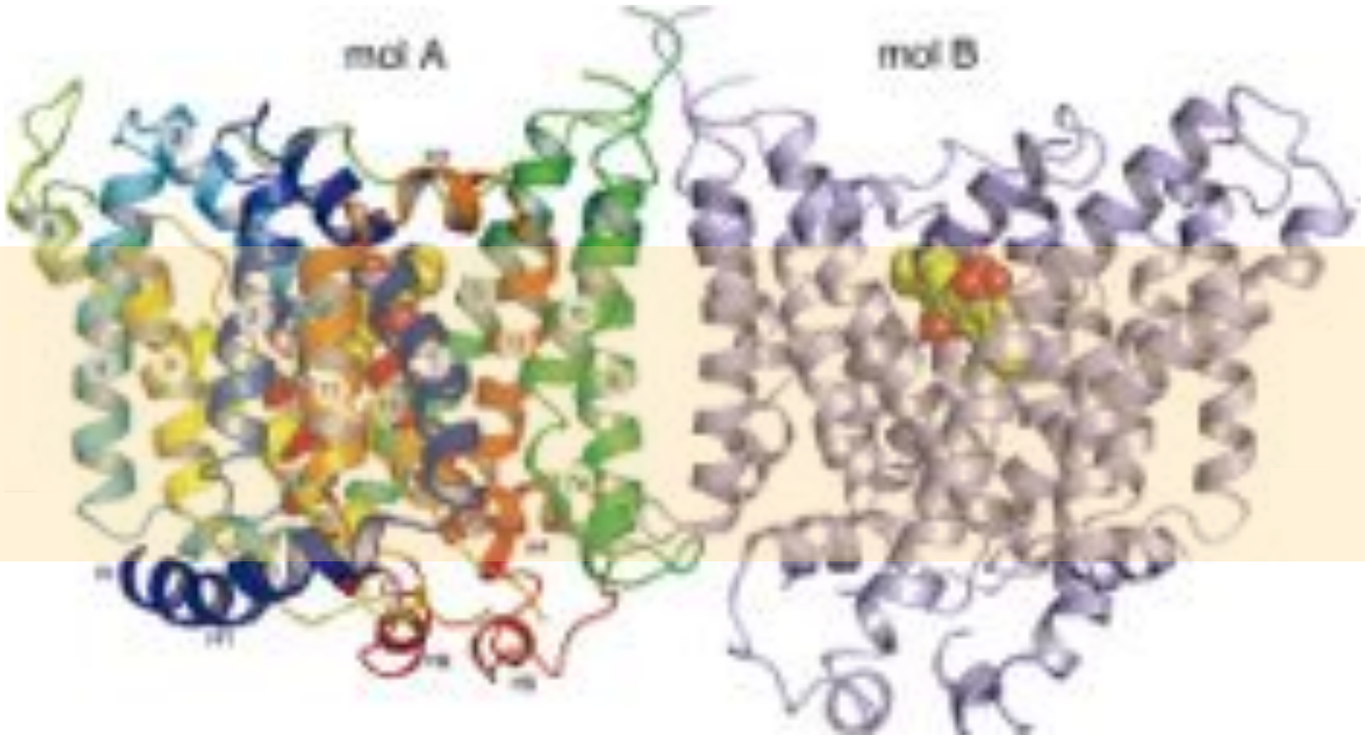
- Large micelle detergents stabilise membrane proteins
- Small micelle detergents are more suitable for crystallisation
- Antibody fragments facilitate membrane protein crystallisation even in large micelle detergents

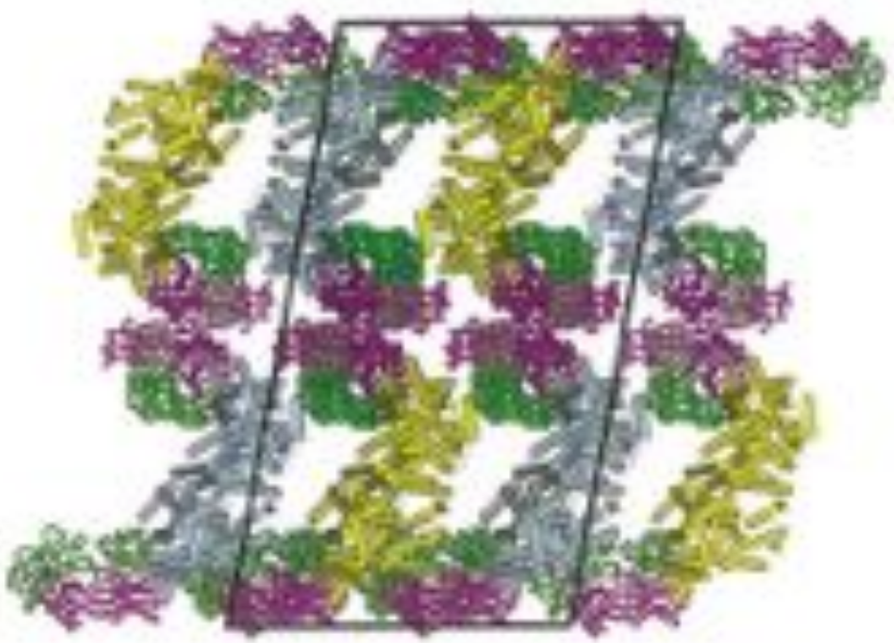




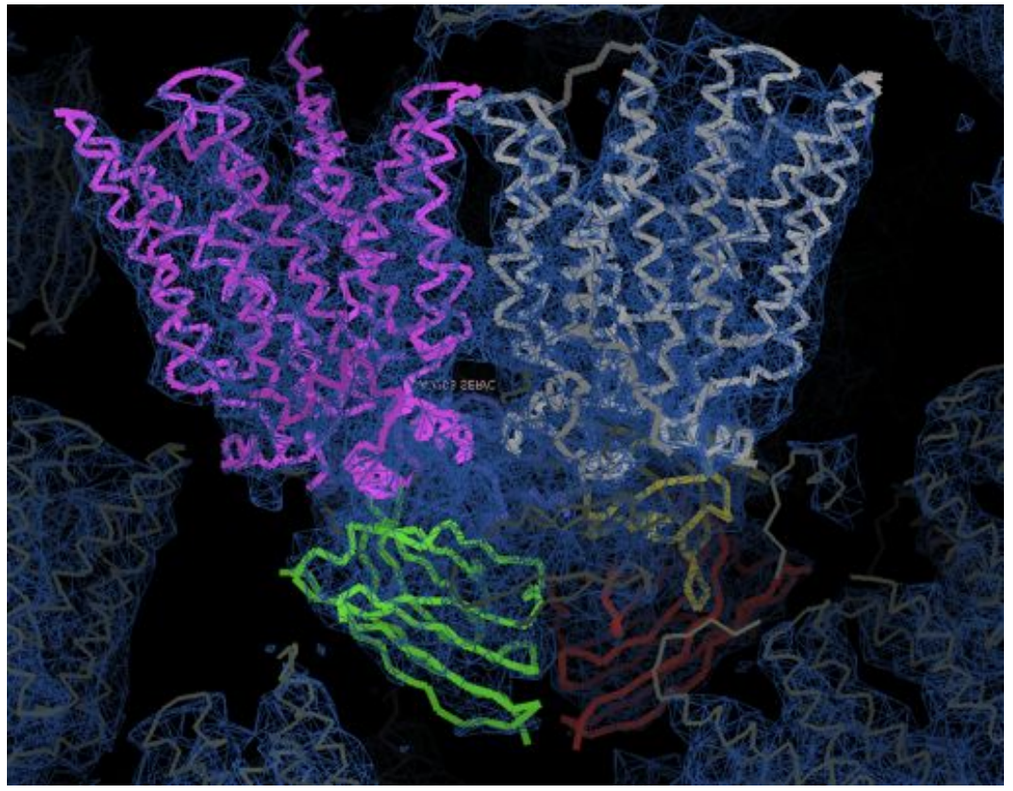
Cytochome c oxidase - Antibody Fv fragment crystal (Iwata *et al.* Nature 376, 1995).

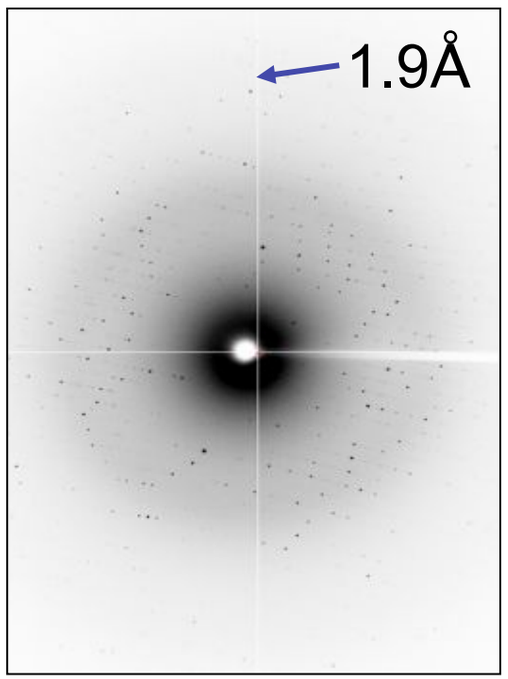
New structures (1)





Human erythrocyte anion exchanger 1 (Band 3) - Fab complex

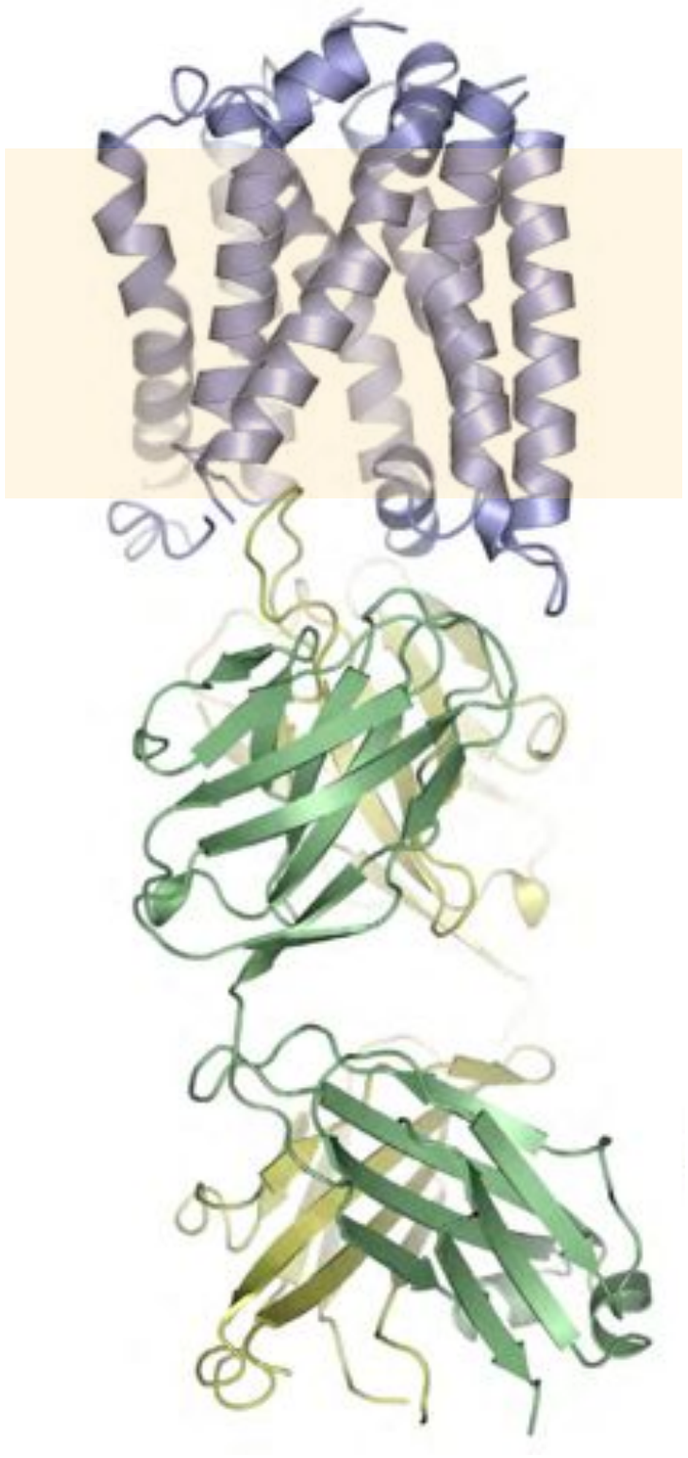




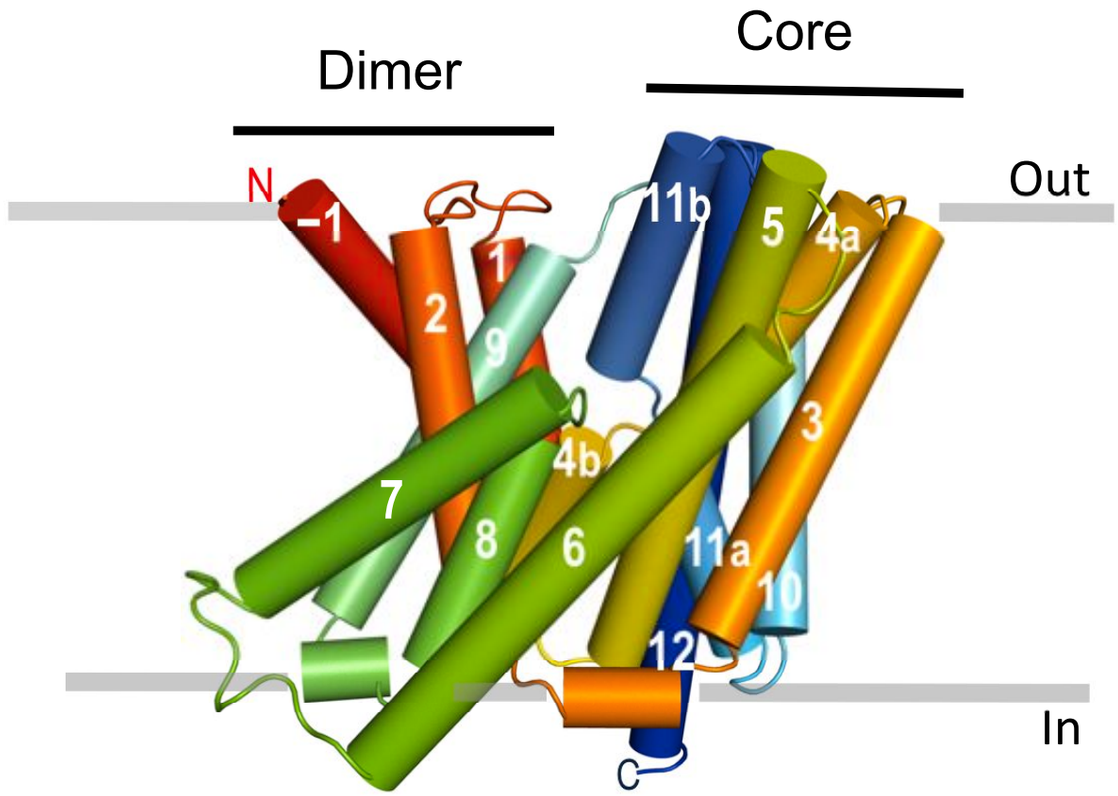
Mouse sugar transporter - Fv complex

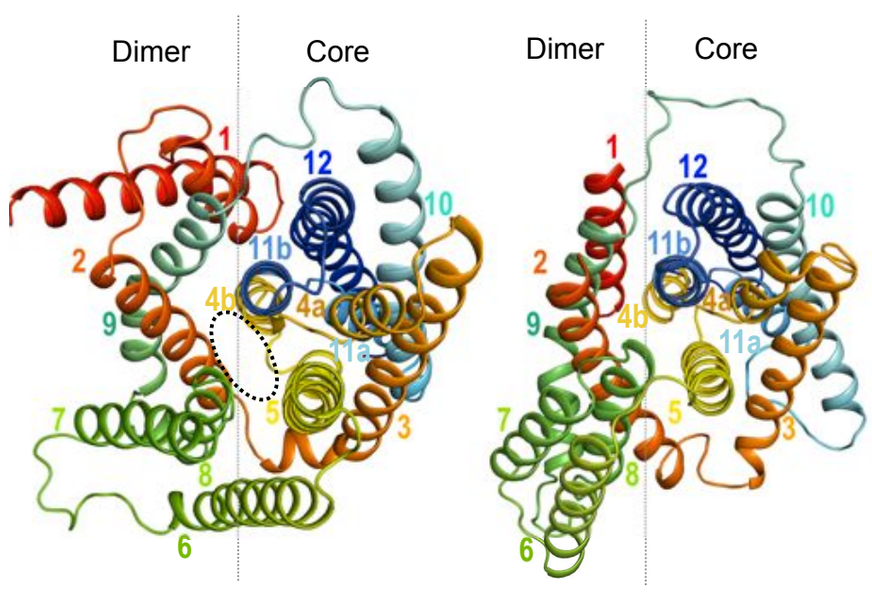
Diffraction image at SACLA

New structures (2)



Membrane protease - Fab complex
(Nature in press)





NapA (outward-facing) NhaA (inward-facing)

NapA sodium/H^+ antiporter
(Nature in press)

SACLA @SPring-8

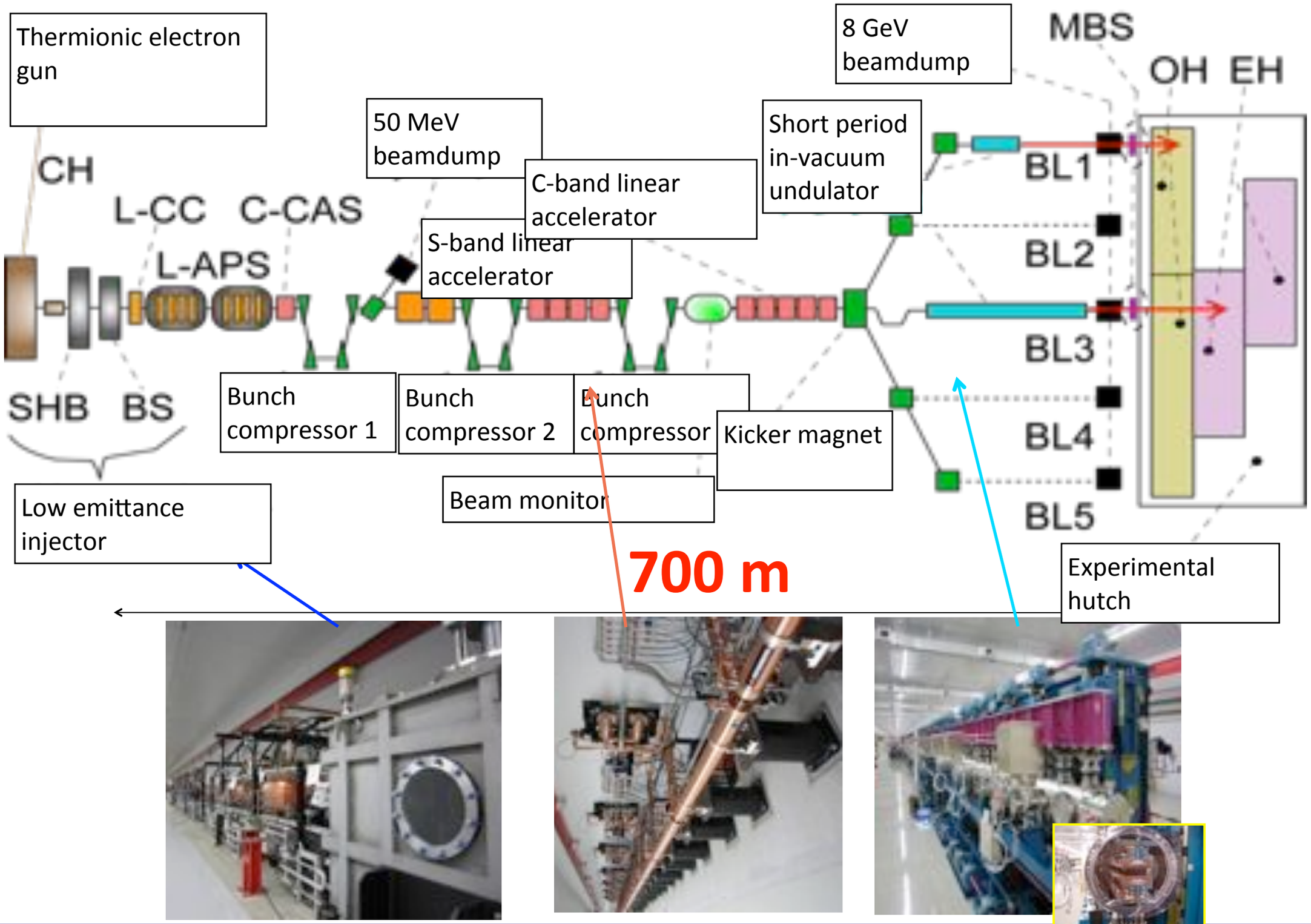
Pulse Energy*	0.3 mJ @10 keV
Peak Power*	>30 GW
Pulse duration	~10 fs or less
Photon energy range	4.5 to 15 keV
Stability Intensity $\sigma_{\delta I/I}$ *	≤ 10%
Repetition rate	20 Hz (Max. 60 Hz)

Summary of Performance

Pulse Energy*	0.3 mJ @10 keV
Peak Power*	>30 GW
	(1 billion times stronger than synchrotron)
Pulse duration	~10 fs or less
	(Shorter than chemical bond breakage time)
Photon energy range	4.5 to 15 keV
	(Hard X-ray suitable for X-ray crystallography)
Stability Intensity $\sigma_{\delta I/I}$ *	≤ 10%
	(Very stable)
Repetition rate	10 Hz (Max. 60 Hz)
	(Fast)

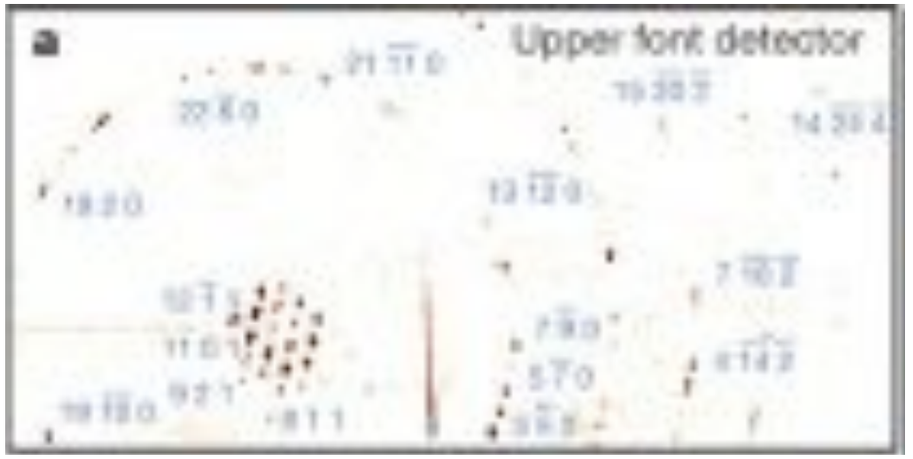
* depending on the lasing wavelength

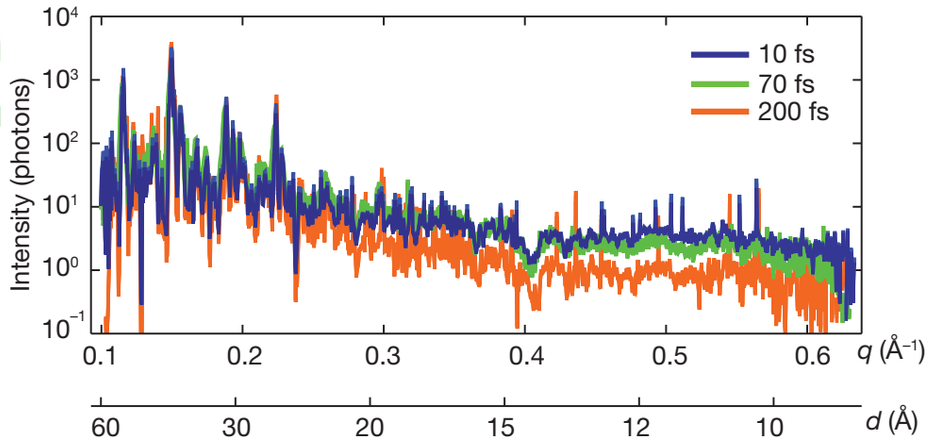
SACLA design

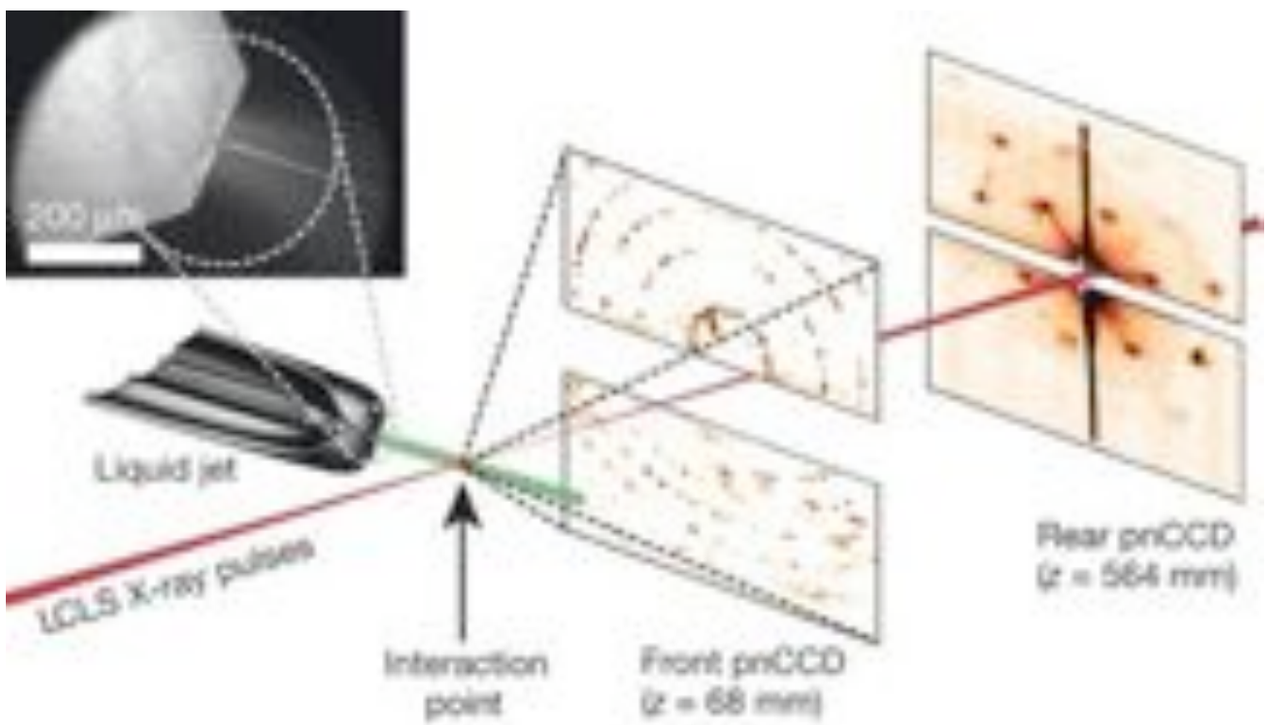


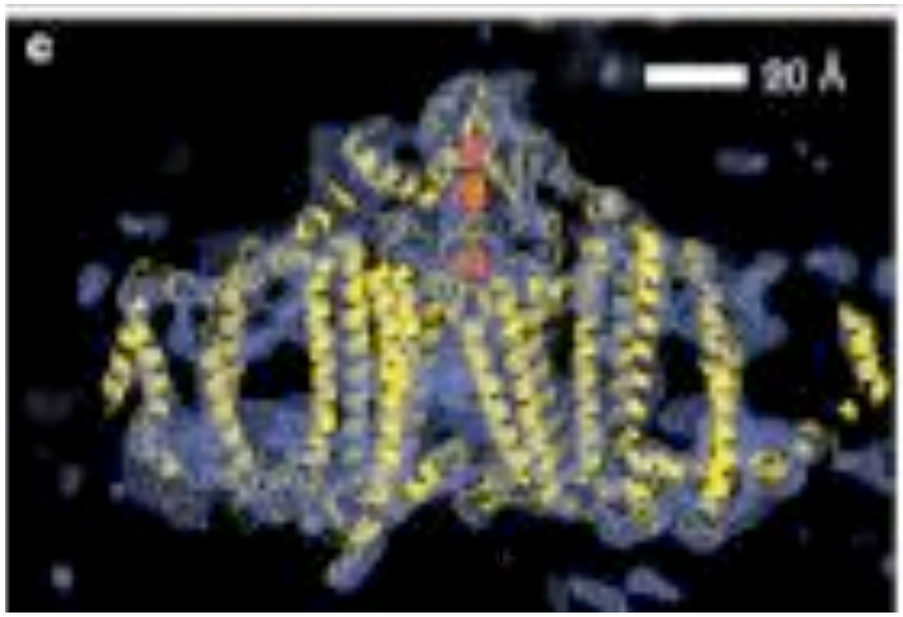
Protein nanocrystallography using X-FEL

- Photosystem I structure has been solved at LCLS(Stanford, US) using nano crystals (200 nm to 2 μm in size) at 8.5 Å resolution (Chapman *et al.,* Nature 2010).
- Using liquid jet system, 112,725 images were collected (15,445 were used) .
- 70 fs pulses cause little radiation damage. Sample dose is *ca* 700 MGy (cf. 30MGy limit for conventional synchrotron experiments) and this can be significantly improved with shorter puls.

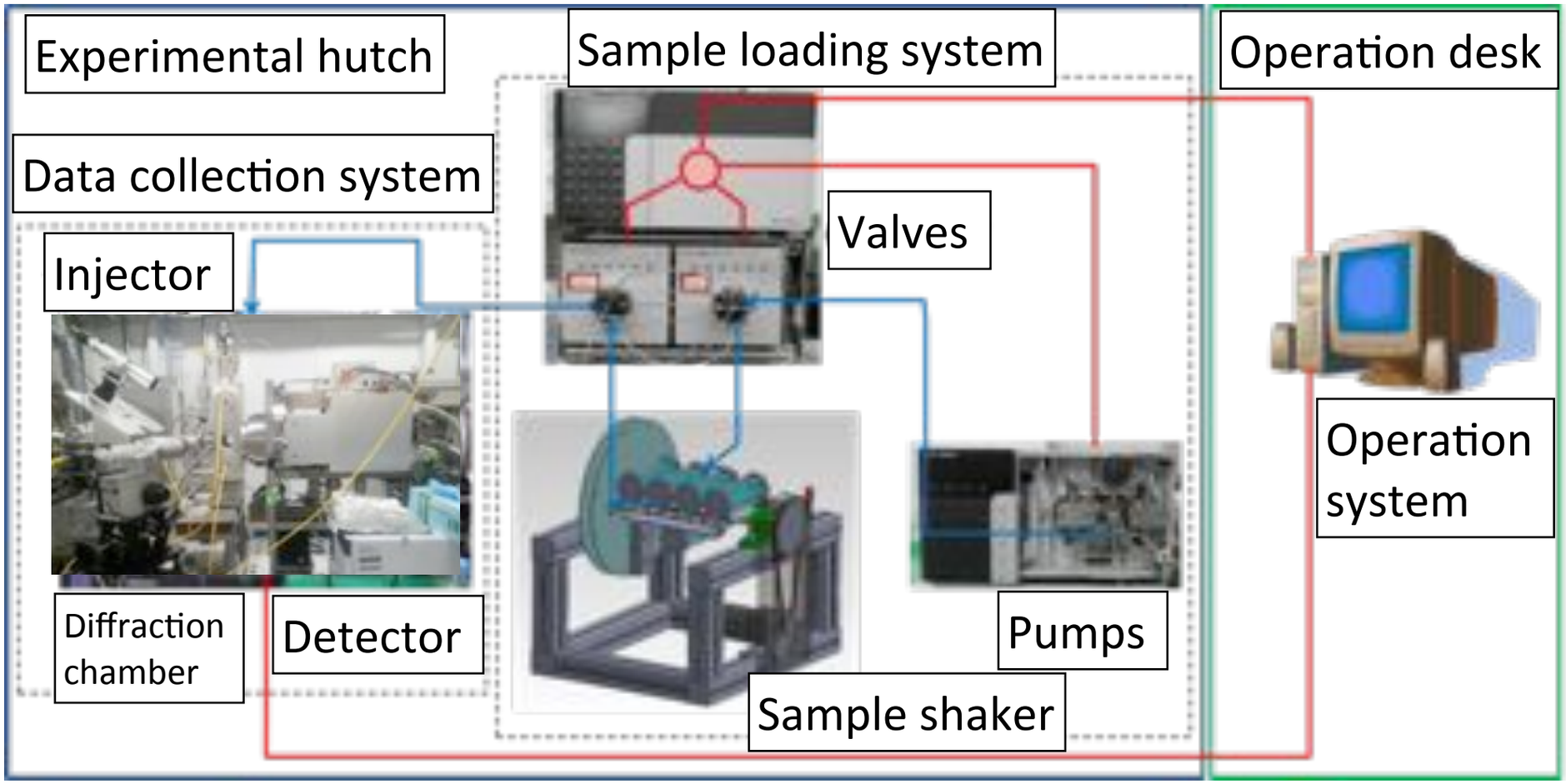








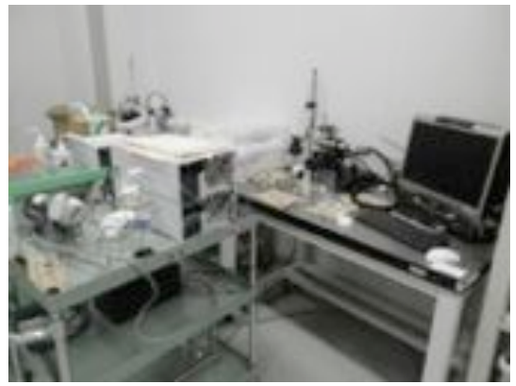
Data collection system



Offline test bench

Sample preparation room

offline injector



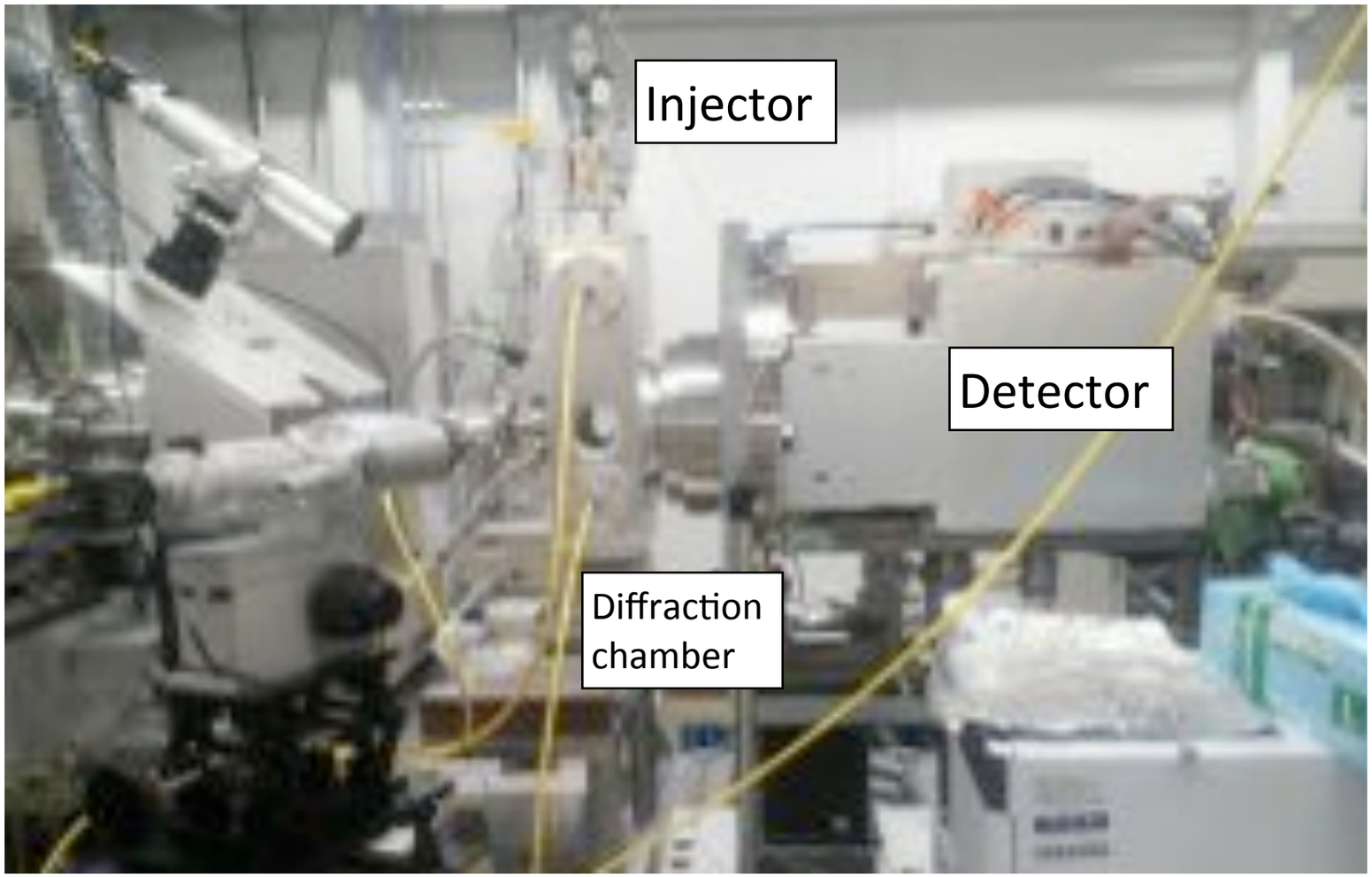
Offline test bench

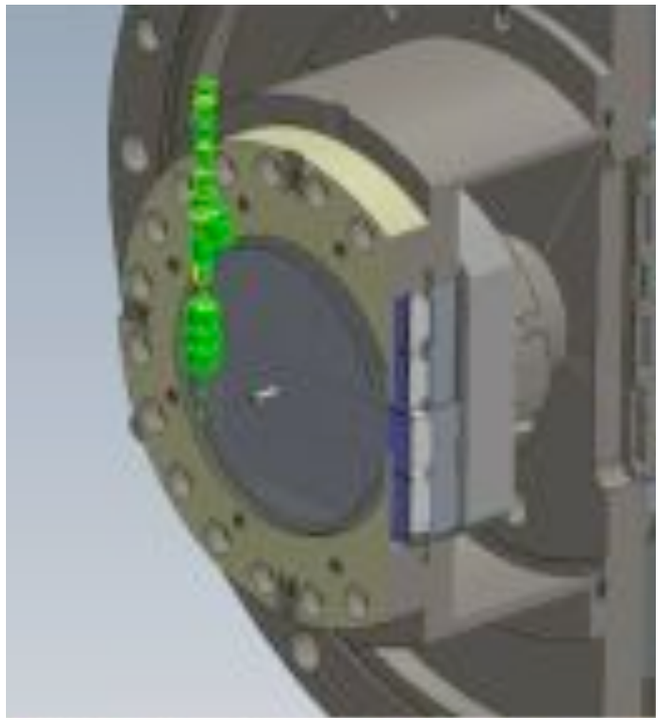
- To facilitate injector (or any sample loader) development, an offline injector test-bench has been setup next to the sample preparation room.

Data collection system

- A sample loading system, an injector and an mpCCD detector have been integrated with a diffraction chamber to construct the data collection system for the SFX experiments.
- We have tested the system using Lysozyme as a test sample (March 2013) to evaluate the system.

Serial Femtosecond Crystallography (SFX) data collection system at SACLA

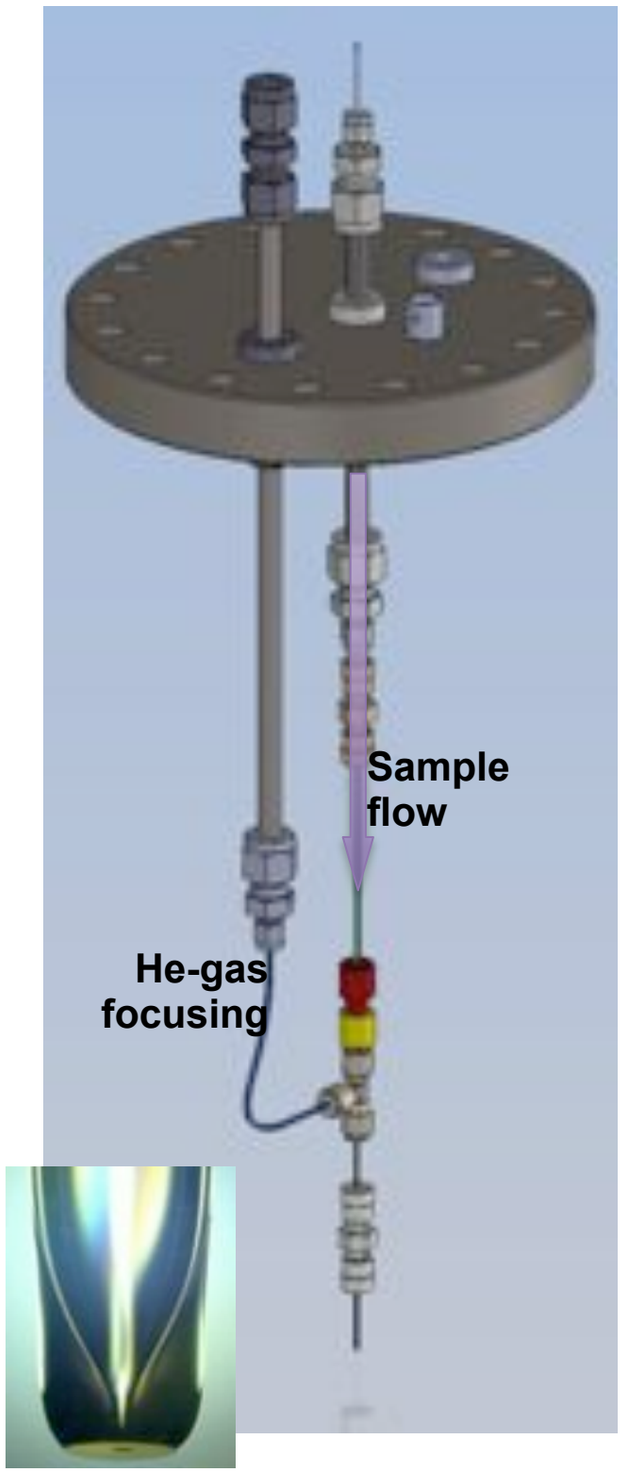




Nam & Song, *et al., Imaging fully hydrated whole cells by ..* (*Phys. Rev. Lett*. **110**:098103, 2013);
Park & Song, *et al*., *Assessment of radiation damage in single ..* (*Phys. Rev.* E. **86**:042901, 2012).

SWD-mpCCD
Image format:
2048 x 2048 (110mm x 110mm)
Sample-detector distance: min. 50mm
Frame-rate: max. 60 Hz
Quantum efficiency:
80%@6 keV, 20%@12 keV

Liquid jet injector for SFX

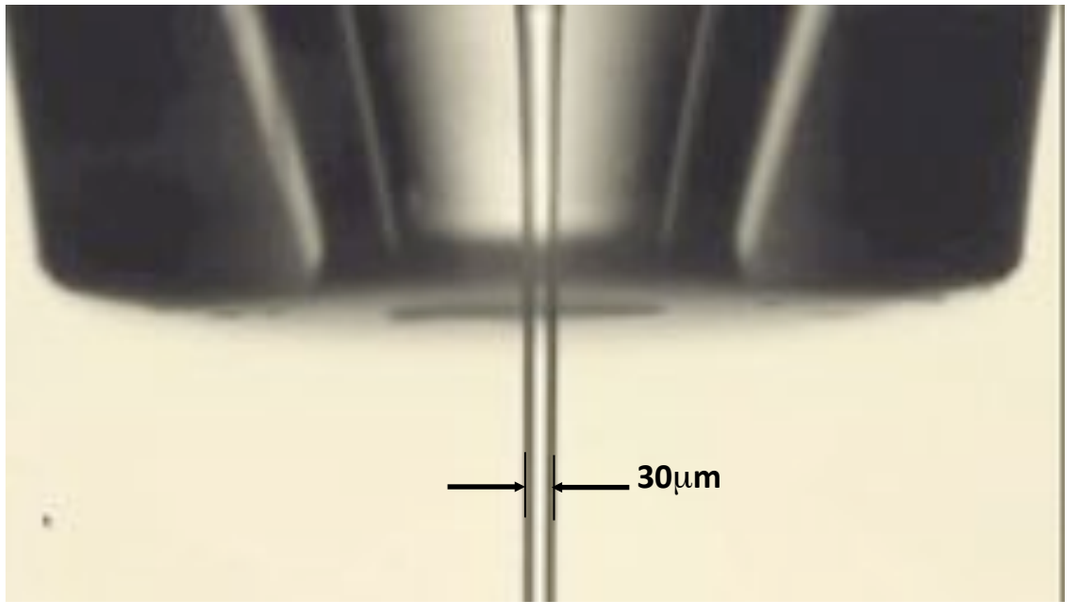


Features

- X-ray diffraction from proteins in buffer solution (native)
- Quick adjustment of liquid beam diameter (3~30μm)
  : adaptable to crystals in various sizes
- Lower sample clogging at the nozzle

Typical liquid flow rate (sample consumption):

- 20μL/min for the 3μm liquid beam: ~ mL/hour
- 0.2mL/min for the 30μm liquid beam: ~ 10mL/hour



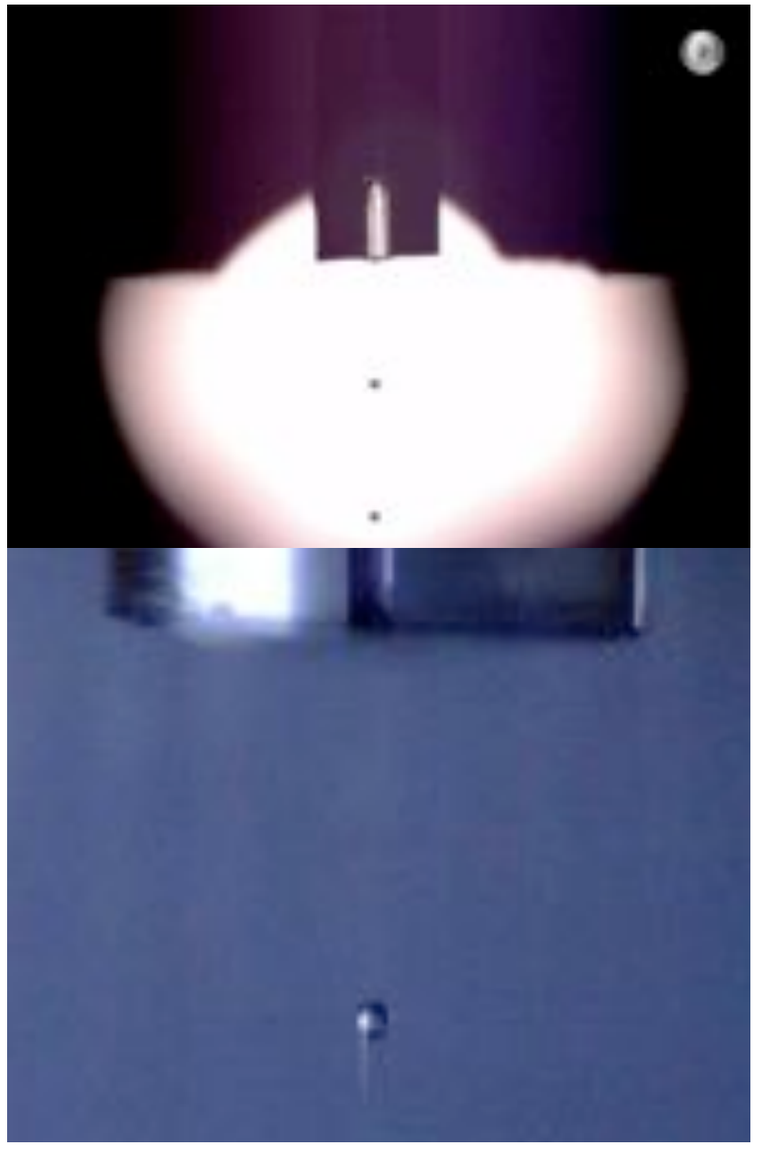
Plan for FY 2014: advanced SFX

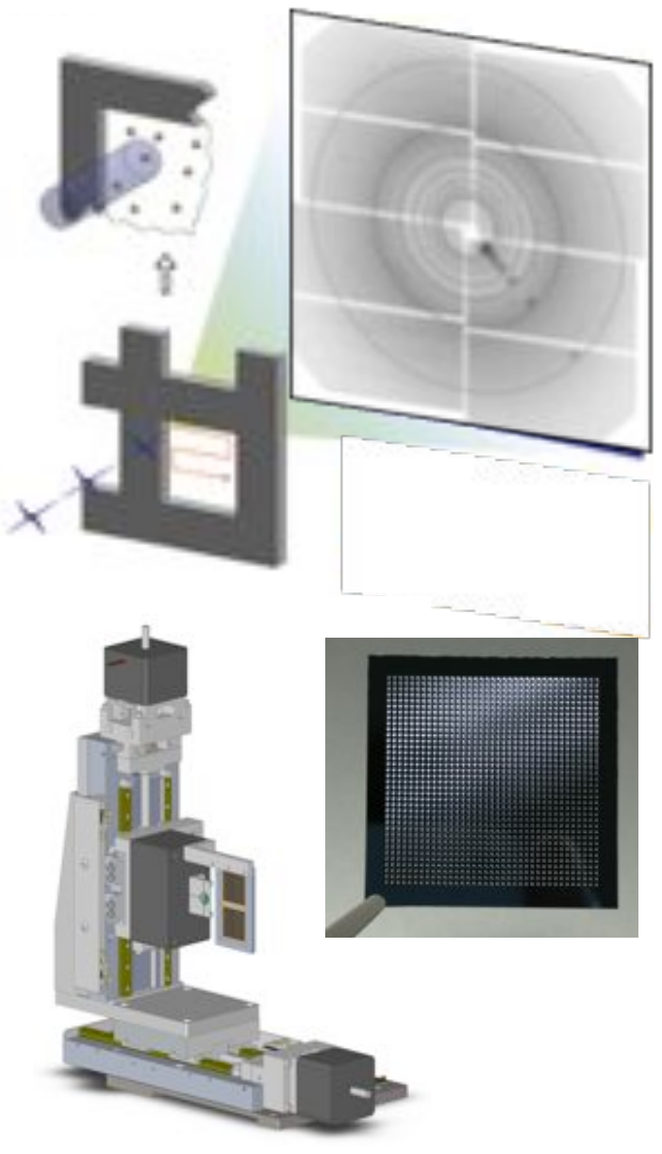
Advanced injectors under development

Recirculate samples:
Save sample consumption (requires down to a few ml of samples)
- Recirculate the sample reservoir

Synchronised droplet injector
Save sample consumption significantly (1/10,000 of the current injector or a few µg of samples)
- Easy to control the sample environment

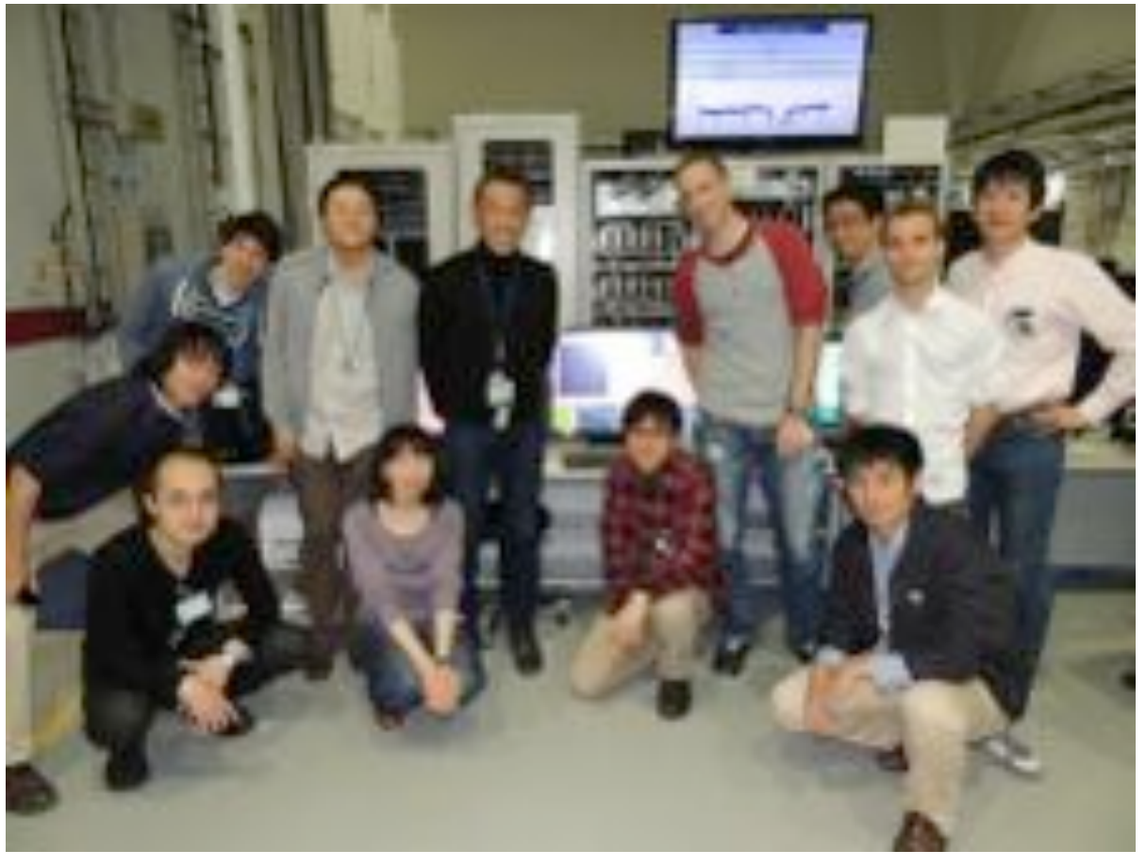
Hybrid fixed target:
- For lipidic cubic phase
- 30~60 Hz raster scan





Protein nanocrystallography at SACLA（1）

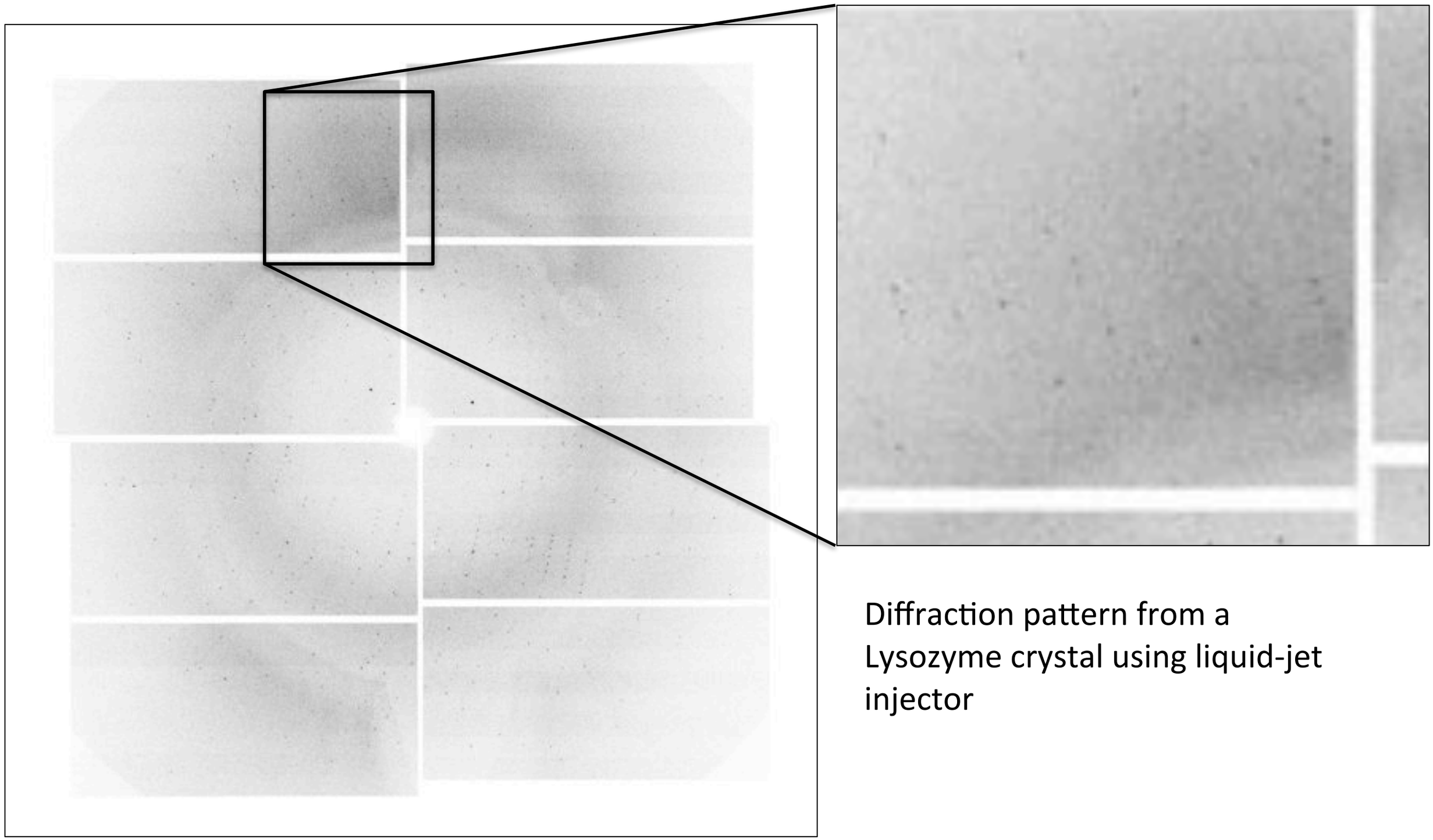
- Data collection system with a liquid jet and a fast CCD (mpCCD) has been constructed (March 2013)
- 10 fs pulses cause almost no radiation damage because it is faster than chemical bond breakage.
- More than 200,000 images can be collected in one hour at 60Hz (currently 36,000 images at 10Hz). It means we can finish data collection within 30min or less.
- Data from 5-10 μm lysozyme crystals has been collected at 1.5 Å resolution.



Protein nanocrystallography at SACLA（2）

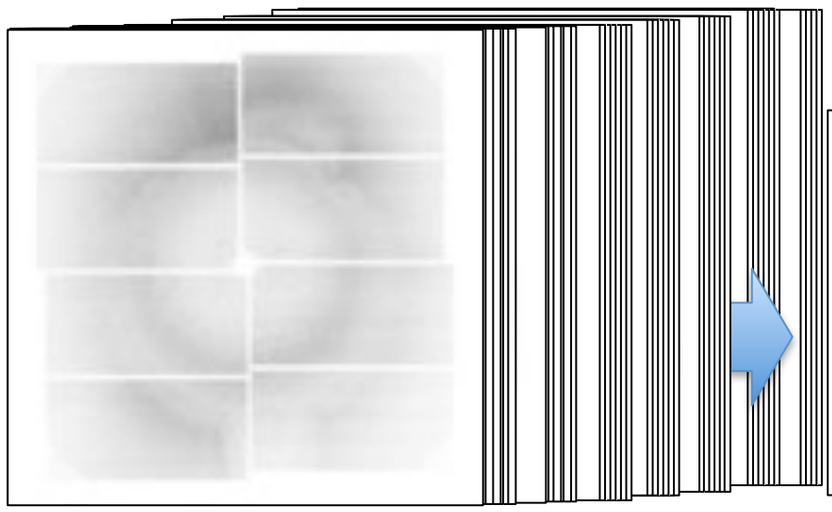
Only one in every 10 images are shown

First SFX data at SACLA（Mar 25th, 2013）



Diffraction pattern from a Lysozyme crystal using liquid-jet injector

Data processing



7 runs (14,000 images)
12min at 10 Hz

Fast selection

Pick-up the images which have pixels more than 10,000 counts within the water ring by a Python script (6148images)

Indexing and integration

Program : Indexamajig

3520 images were indexed using CrystFEL ver. 0.5.0 (Thomas A. White et al.)

Merging

Program: process_hkl

Calculate mean intensities of the diffractions with the same index.

Statistics

Program: check_hkl

Evaluate completeness, S/N, redundancy and so on.

Data processing of Lysozyme data

Run94362- 94368 7datasets （14,000 images）

↓

6148 were selected （over 10,000 count pixels）

↓

3520 were indexed then processed

1/d centre	# refs	Possible	Compl	Meas	Red	S/N	d(A)
1.584	2068	2068	100.00	97604	47.2	7.88	6.31
3.438	1943	1943	100.00	76769	39.5	7.94	2.9
4.111	1906	1906	100.00	69701	36.6	6.91	2.43
4.609	1885	1886	99.95	60245	32.0	5.54	2.17
5.016	1871	1871	100.00	49939	26.7	4.48	1.99
5.366	1864	1866	99.89	33923	18.2	3.73	1.86
5.675	1845	1857	99.35	17821	9.7	2.97	1.76
5.953	1792	1867	95.98	10594	5.9	3.11	1.68
6.207	1361	1847	73.69	5052	3.7	4.26	1.61
6.442	411	1852	22.19	1055	2.6	6.64	1.55

Electron density map（2Fo-Fc, 1σ）

20 cycle refinement using refmac with the TLS. Without solvent molecules.

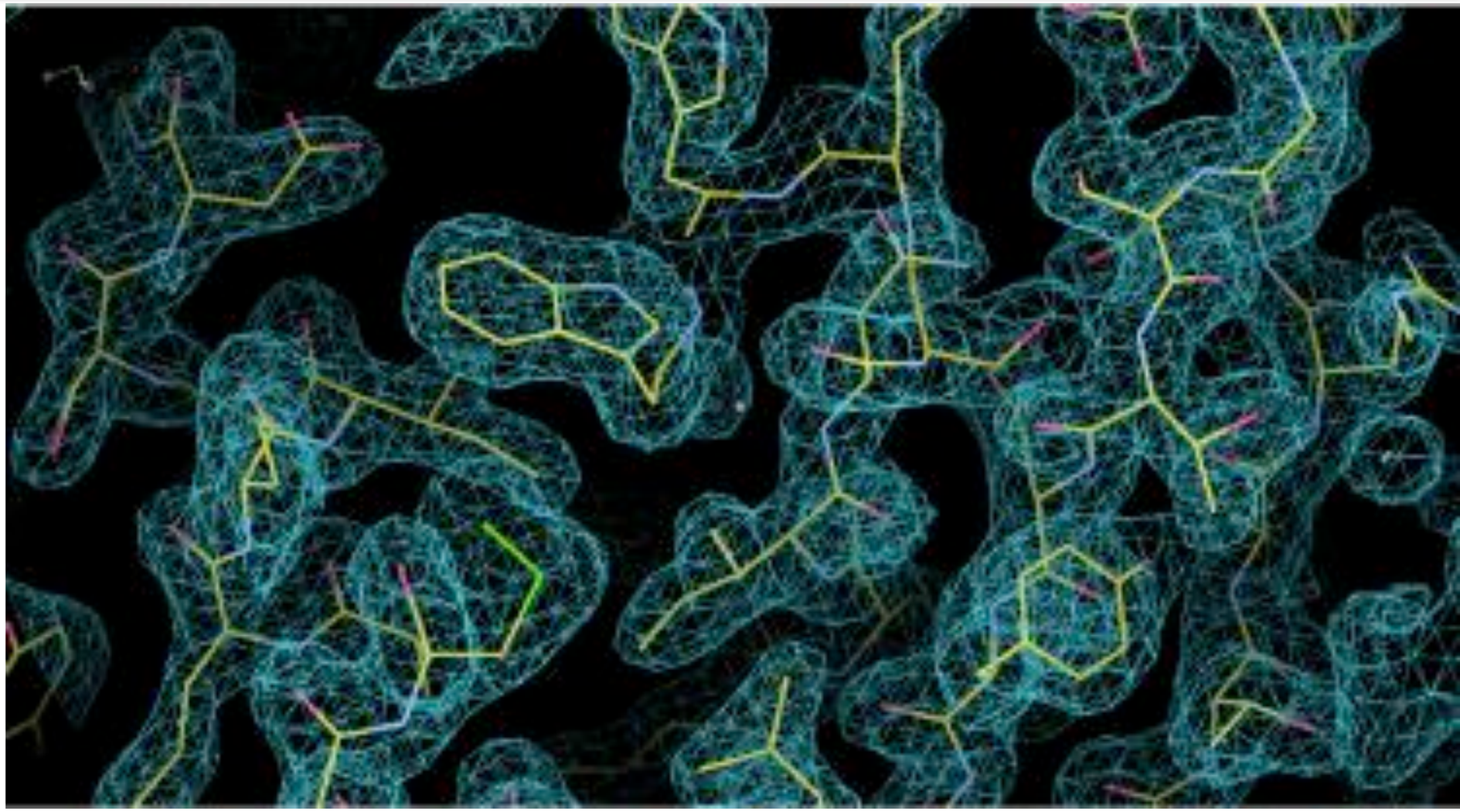
Res = 30-1.7 Å (1.744-1.700 Å)

***C*ompl = 99.8% (98.8%)**

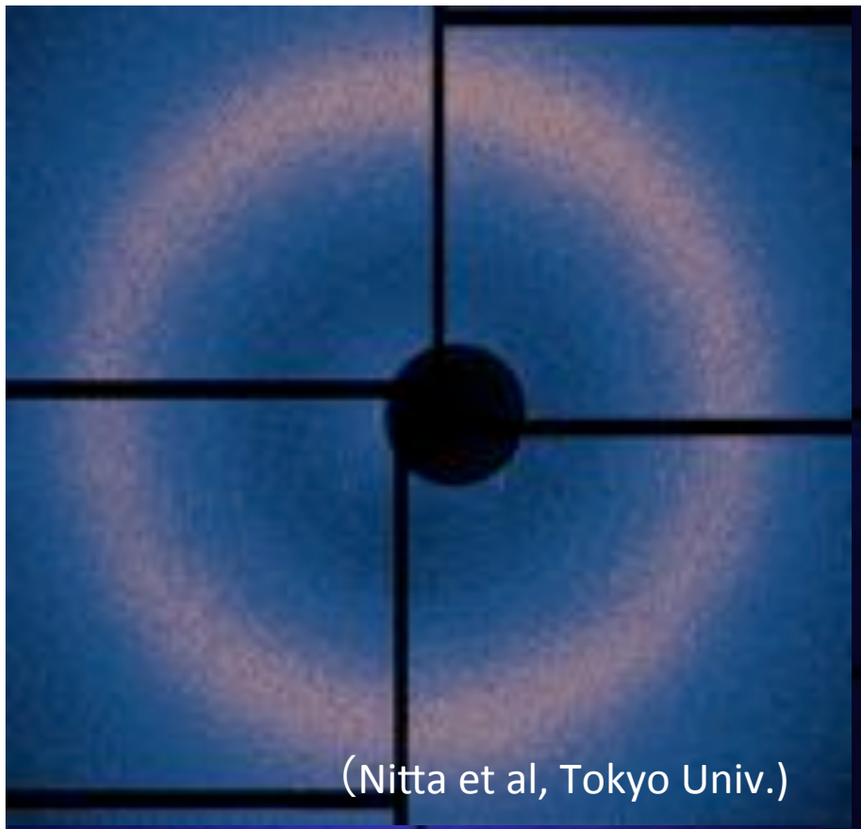
***R*work = 21.3% (44.1%)**

***R*free = 25.3% (53.9%)**

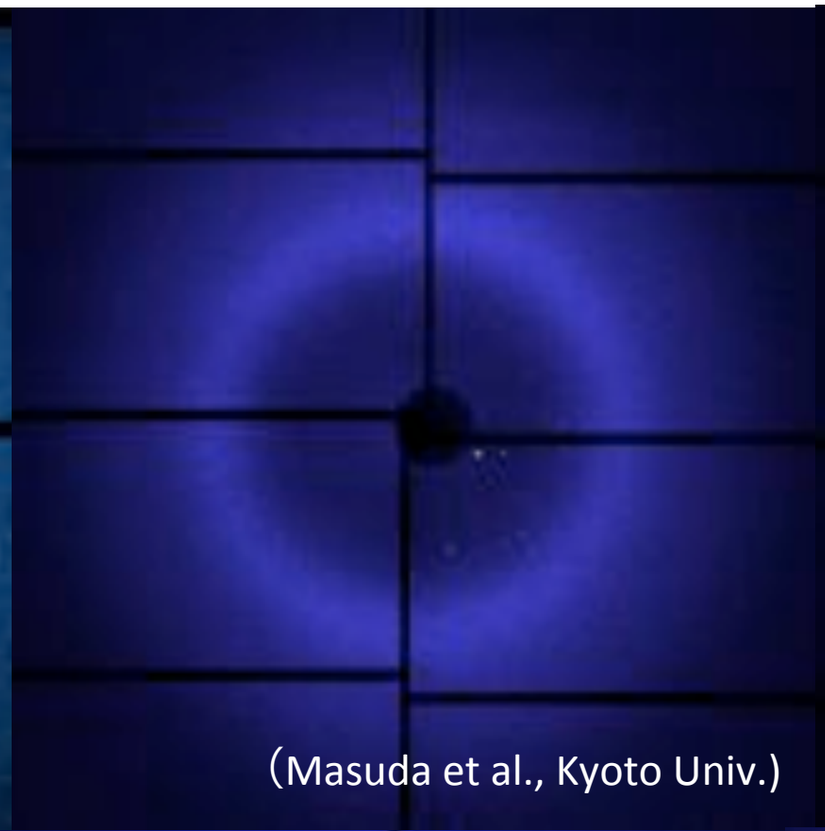
Mean *B* = 29.9 Å^2



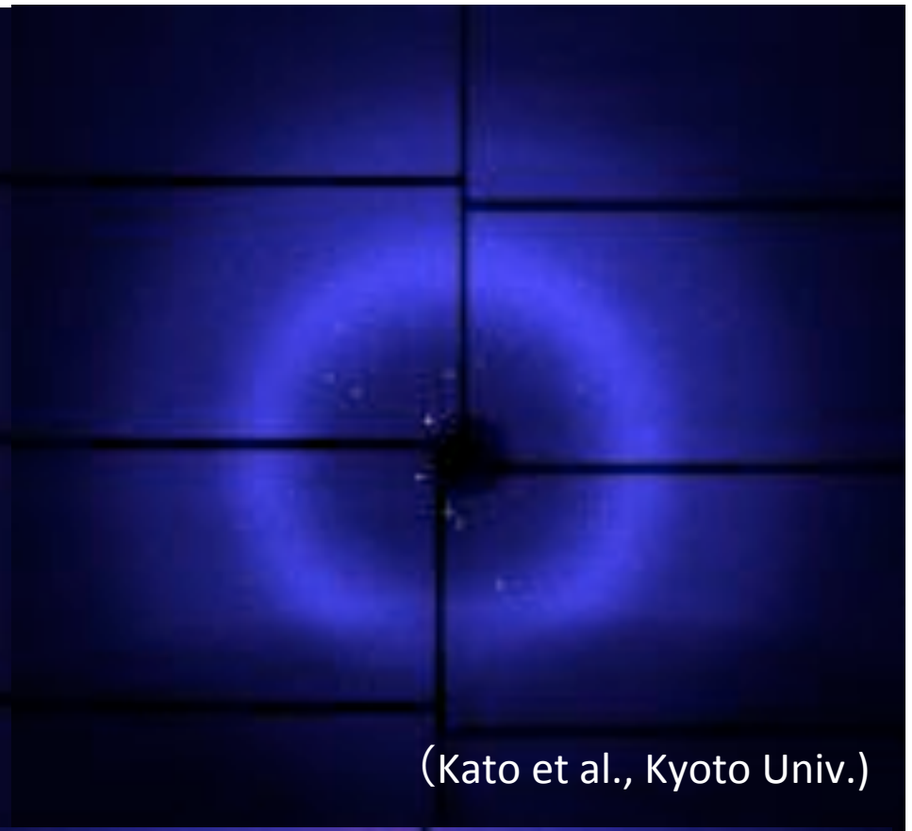
Diffraction patter from various protein samples (including those structures are unknown)



(Nitta et al, Tokyo Univ.)



(Masuda et al., Kyoto Univ.)



(Kato et al., Kyoto Univ.)

(Chavas et al、 KEK)

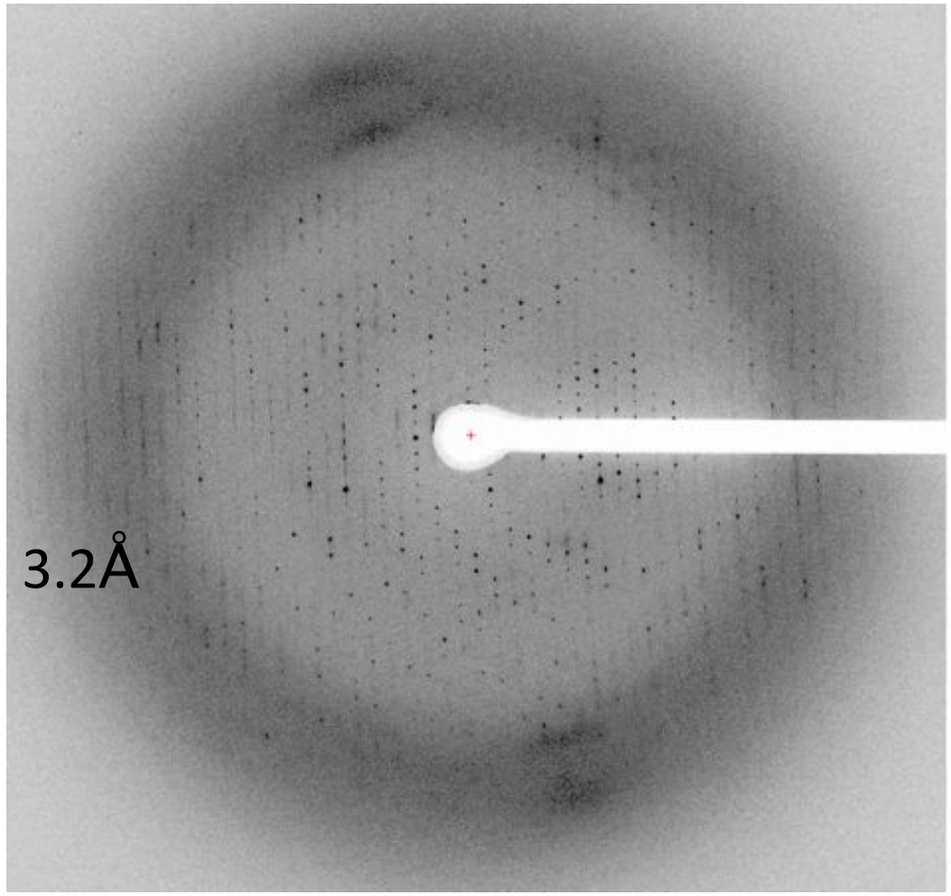
(Chavas et al、 KEK)

(Mizohata et al、 Osaka Univ.)

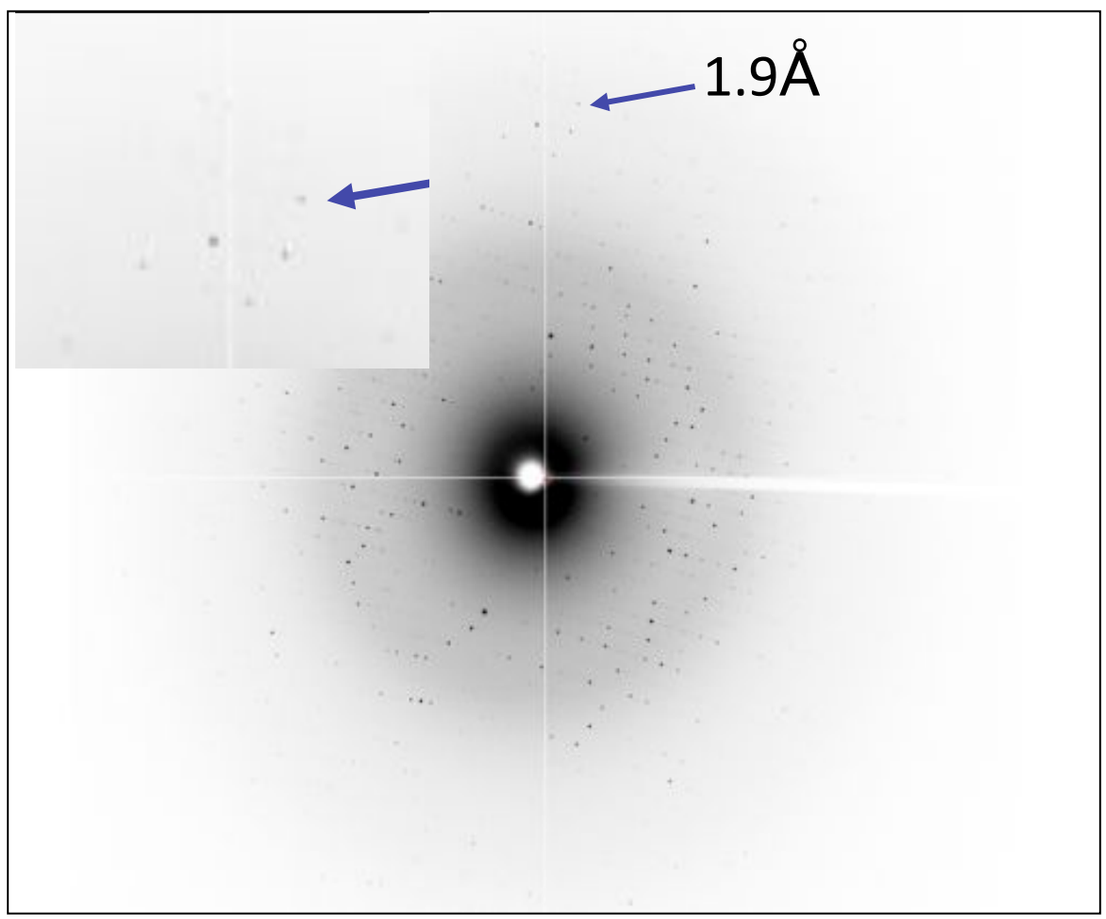
Improved X-ray Diffraction of Mammalian Sugar Transporter-Fv Complex Crystal at SACLA

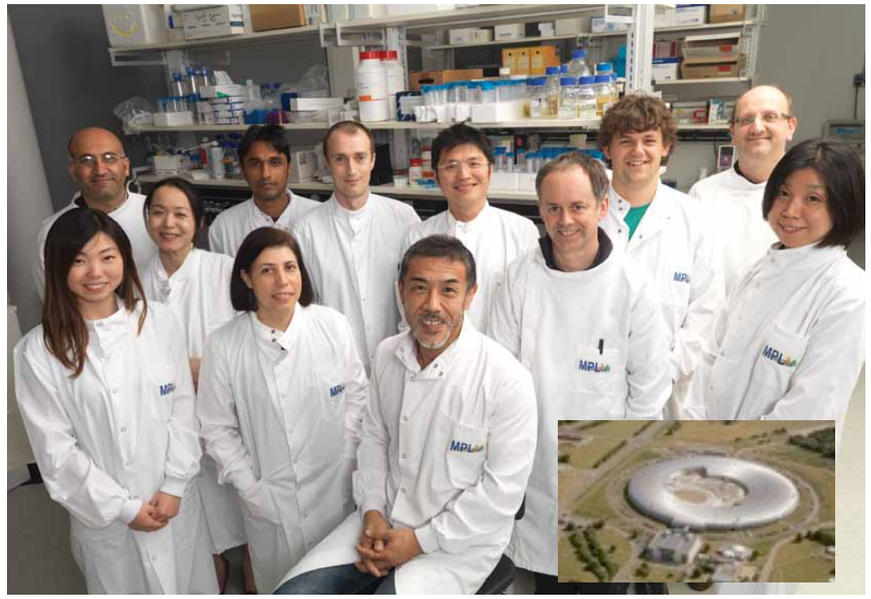
- Sugar-transporter - Fab complex crystals diffracted up to 3.2Å at BL32XU of SPring-8.
-Diffraction limit was significantly improved when a large crystals was used at SACLA.

SPring-8



SACLA





Diamond-MPL
(Supported by
Wellcome trust and
BBSRC)
David Drew
Alex Cameron
Simon Newstead

Kyoto University (supported by JST
Research acceleration program)
Satoshi Ogasawara, Norimich Nomura,
Takuya Kobayashi, Takeshi Murata, Tatsuro
Shimamura, Tomoya Hino, Takatoshi
Arakawa

Riken SPring-8 Centre (supported by X-ray
Free Electron Laser Priority Strategy
Program (MEXT))
Eriko Nango, Rie Tanaka, Jaehyun Park,
Changyong Song, Takaki Hatsui, Eriko
Nango, Rie Tanaka (RIKEN)
Kensuke Tono, Yasumasa Joti, Takashi
Kameshima (JASRI),
Mamoru Suzuki (Osaka Univ.), Fumitaka
Makune (Tokyo U.)