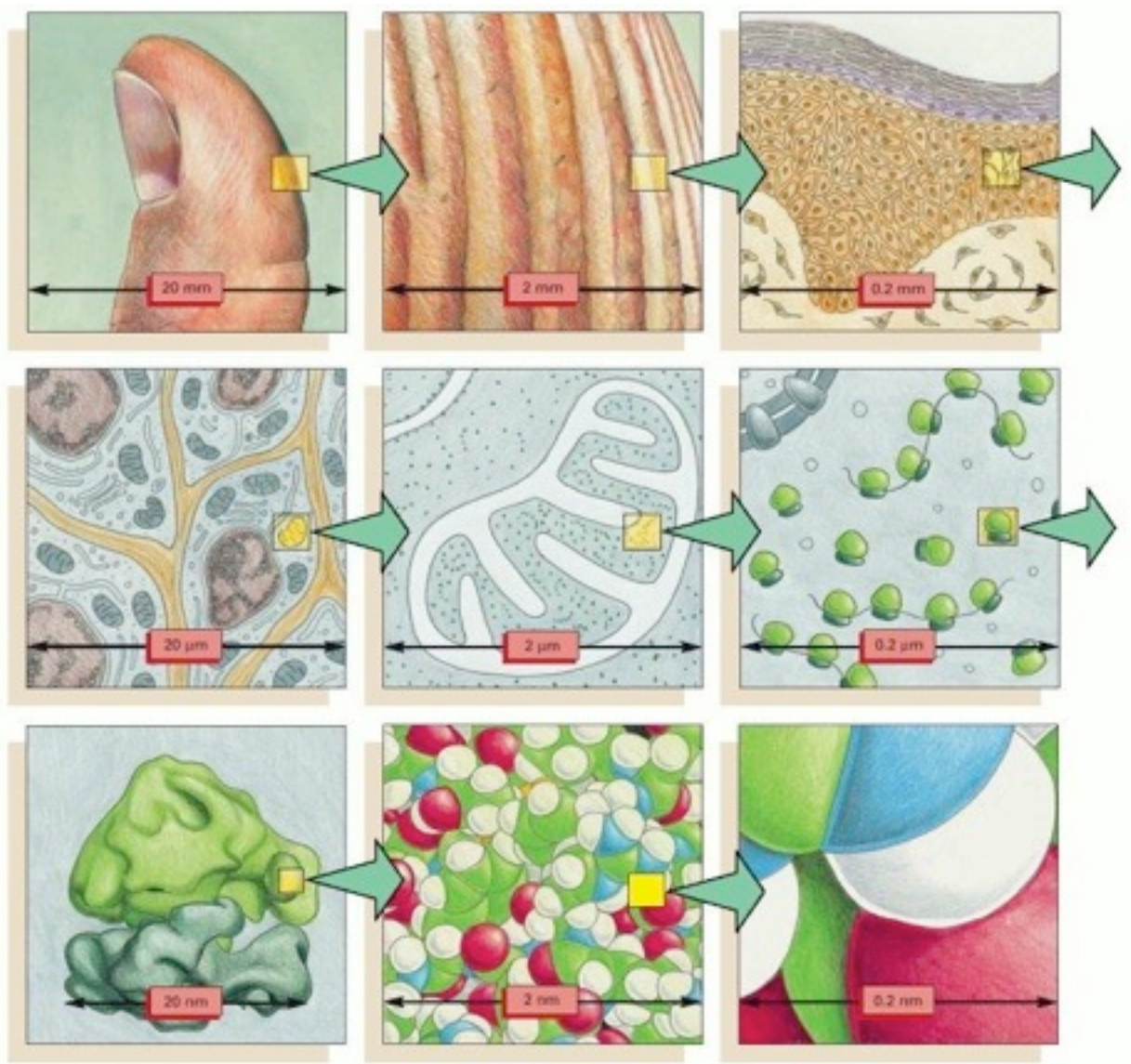
X-ray Protein Crystallography on Membrane Proteins

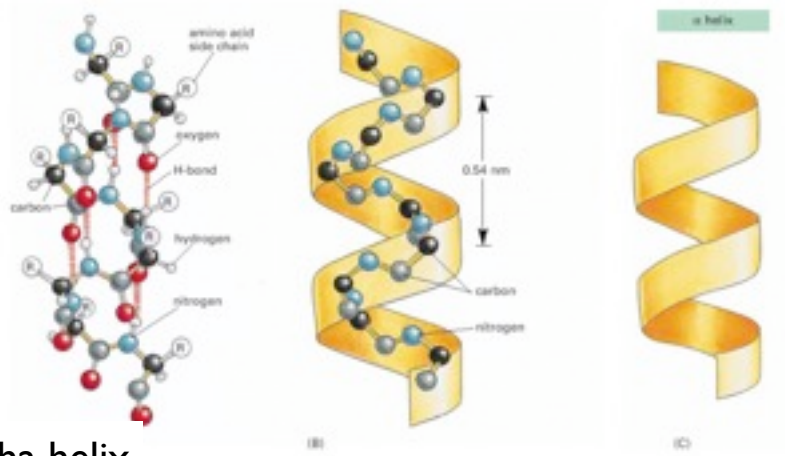
Riken Harima Institute/Kyoto University

So Iwata

All Proteins in Our Body Have Regular 3D Structures at the Atomic Level

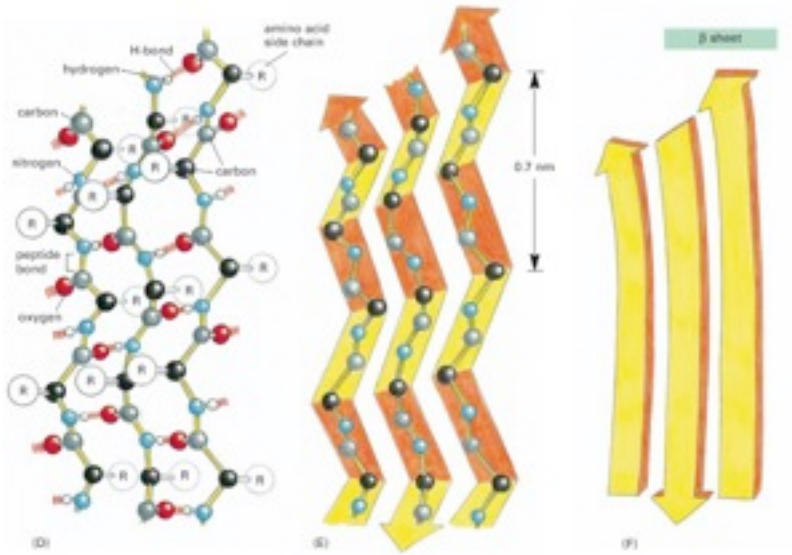


Structural elements of proteins

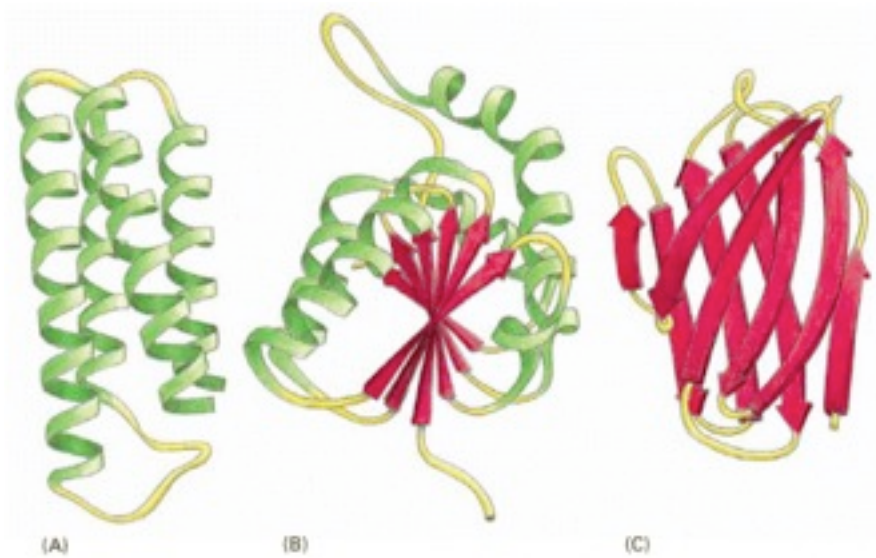


Alpha helix

Proteins are made of polypeptide chains. Depending on the amino acid sequence of the chain, proteins can take various local structures like alpha helix or beta sheet.



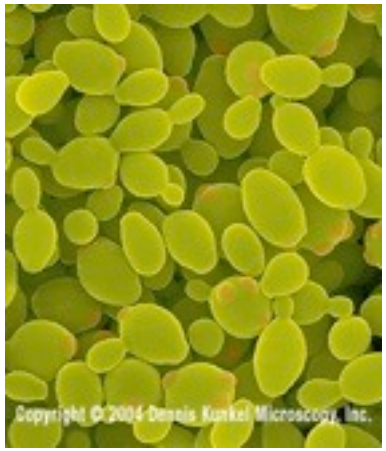
beta sheet



Example of proteins

Molecular Anatomy: X-ray Crystallography Helps to Solve the Mystery of Protein Structure and Function

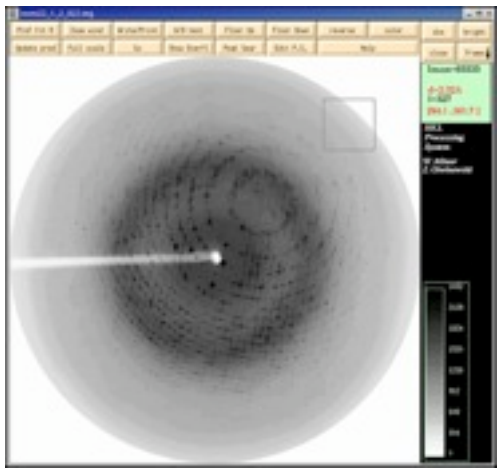
DNA Cloning



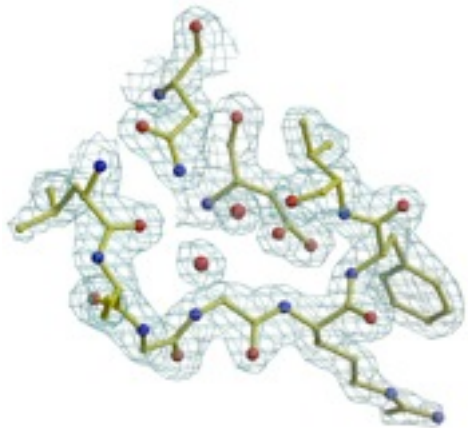
Protein Expression

Purification

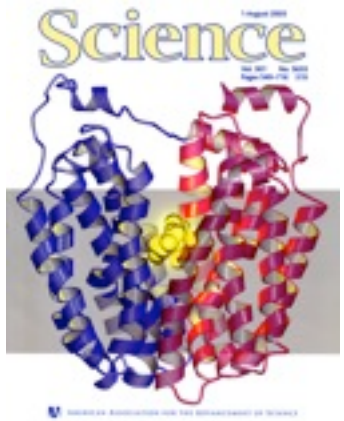
Crystallization



X-ray Data Collection

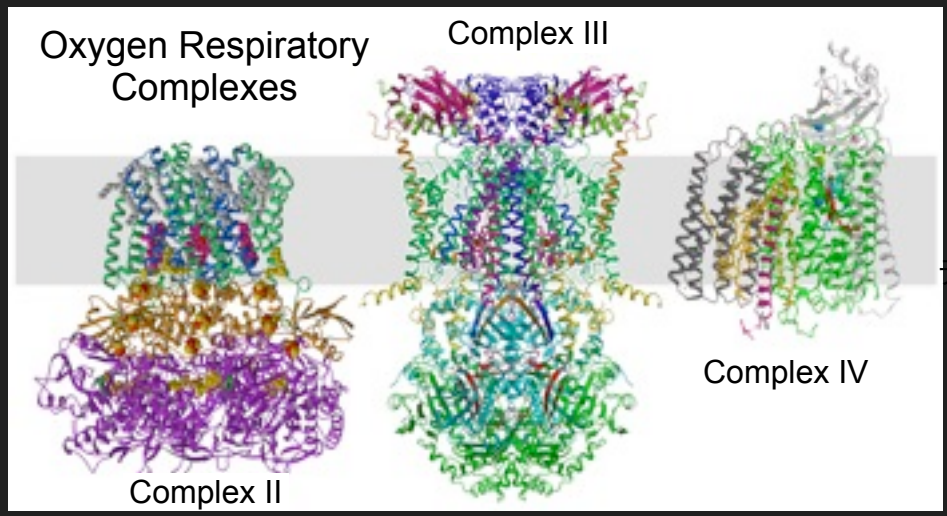


Phasing & Electron Density Map



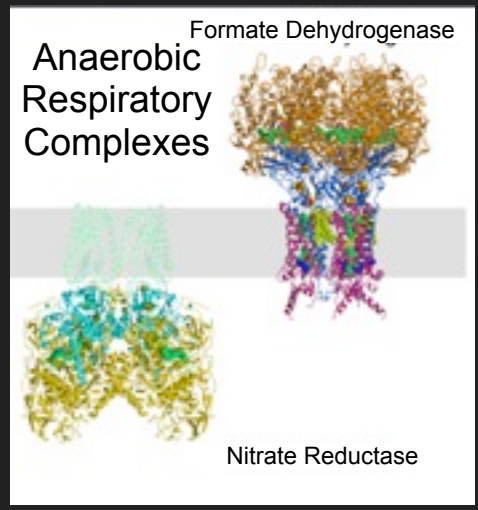
Structure Model

Membrane Protein Structures Solved in our Group

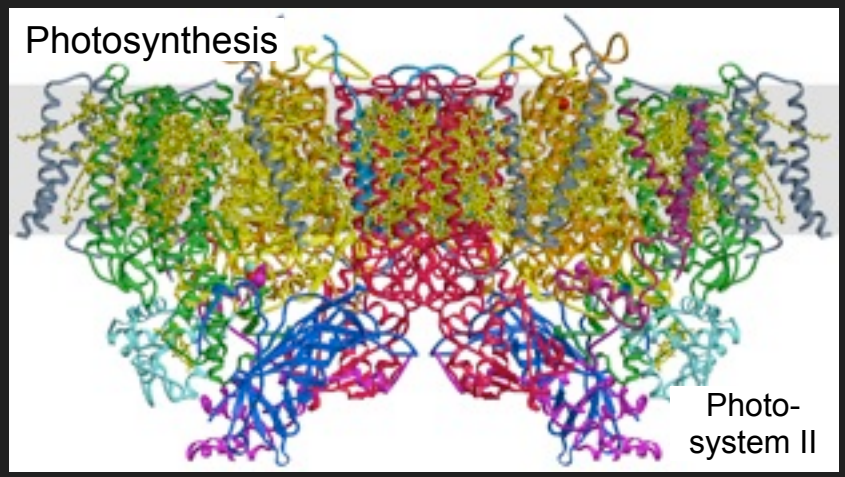


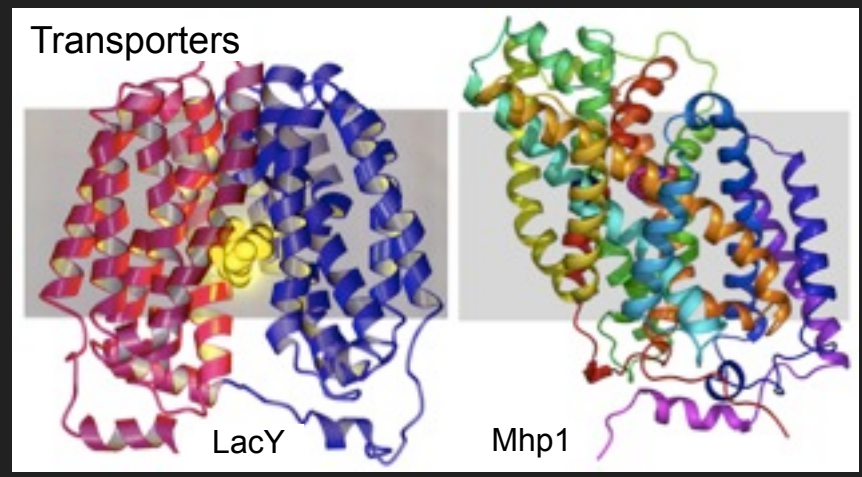
テキスト

Complex II: Science 299 2003
Complex III: Science 281, 1998, N. Engl. J. Med. 341,1999
Complex IV: Nature 376, 1995, Nature Str. Biol. 7, 2000



Formate Dehydrogenase: Science 295 2002
Nitrate Reductase: Structure 12, 2004
Polysulfide reductase: Nat. Str. Mol. Biol. 15, 2008



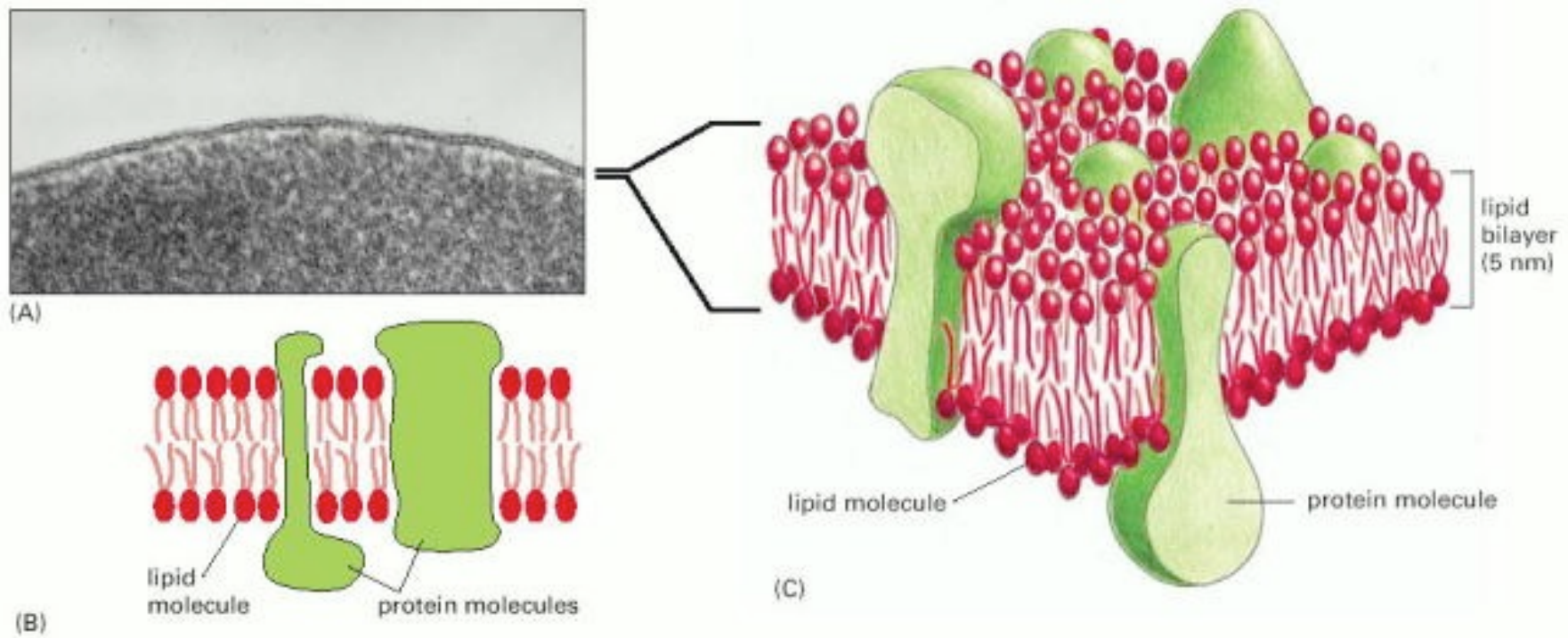


Photosystem II: Science 299 2003
LacY: Science 301, 2003, EMBO J. 25, 2006, PNAS 104, 2007
Mhp1: Science 322, 2008

Membrane Proteins

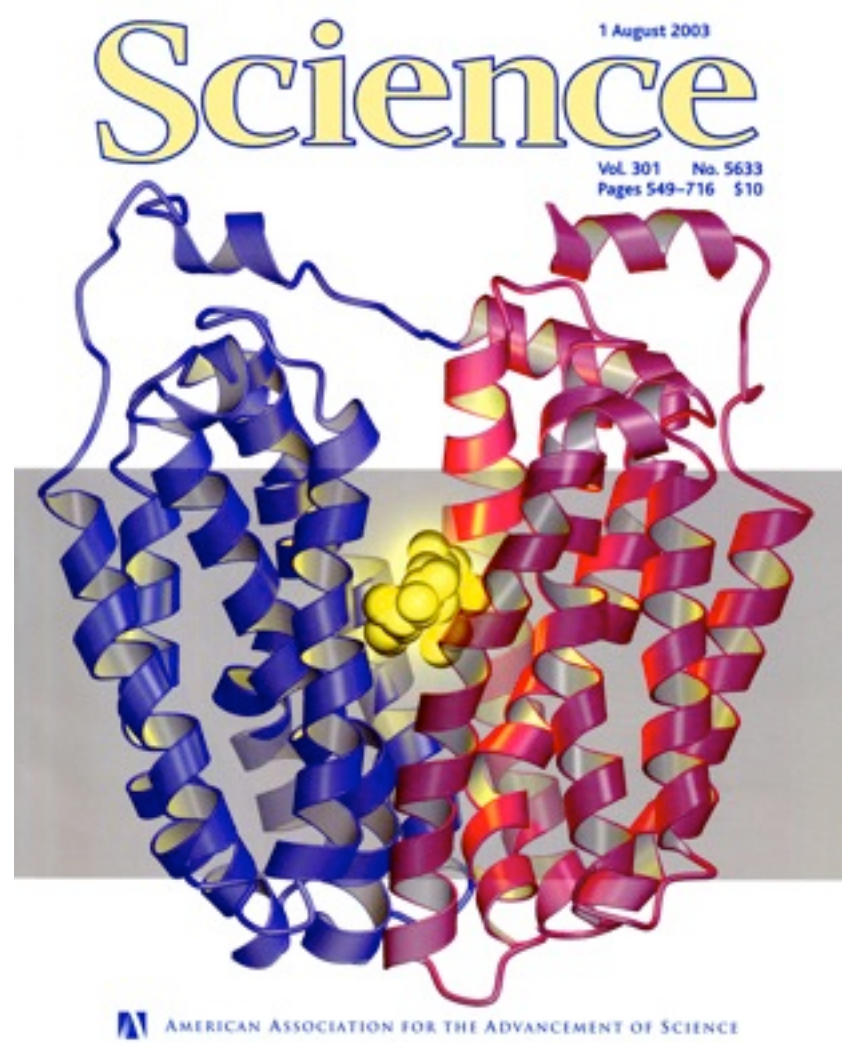
Various functions: oxygen respiration, photo synthesis, signal transduction, molecular transport and so on.

Over 30% of proteins in the human genome are membrane proteins (up to 10,000).



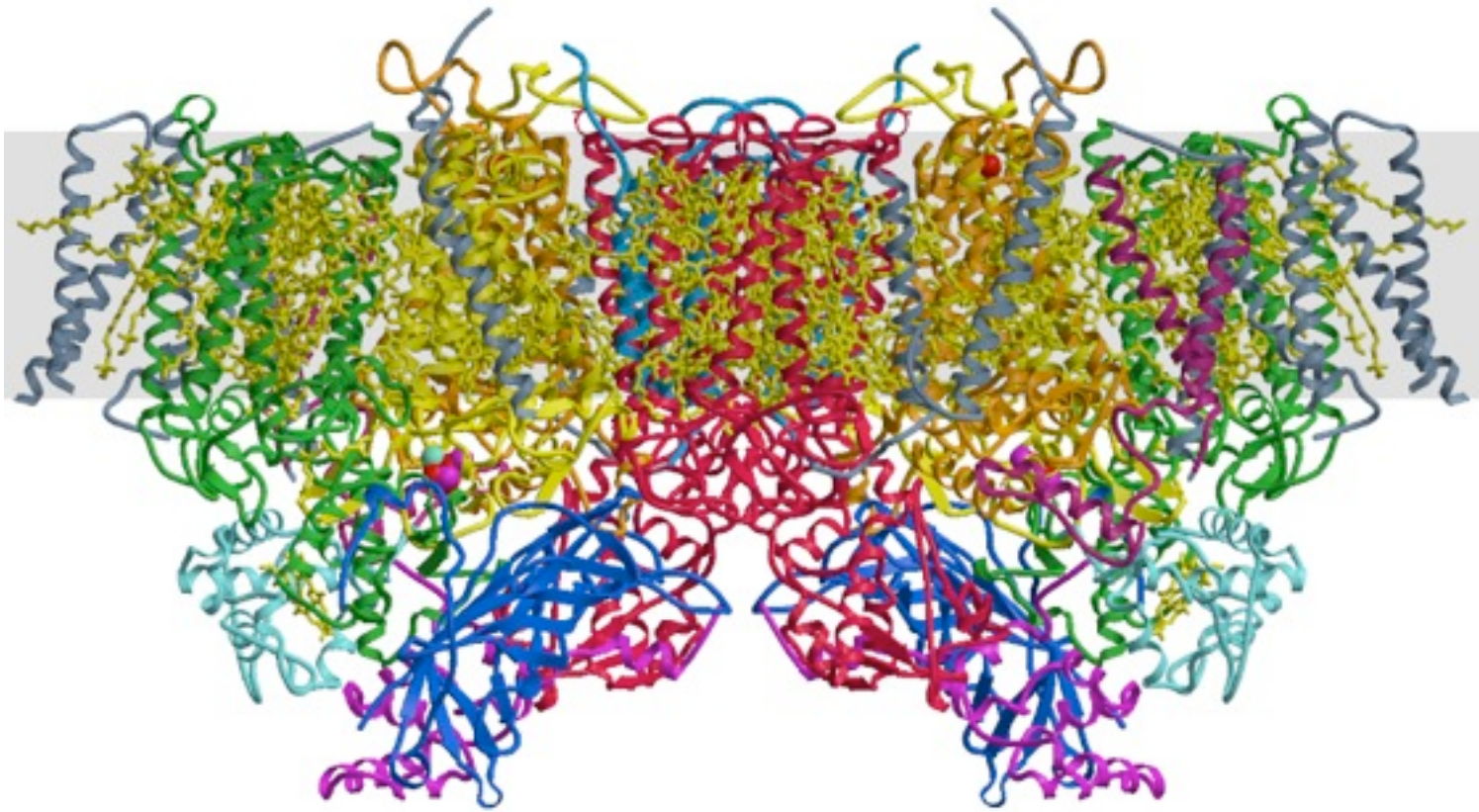
Example Membrane Protein Structures from Our Group: Lactose Permease (Sugar Transporter)

Responsible for sugar uptake into the cells. Homologue of the human glucose transporter, which dysfunction causes diabetes (Abramson *et al,* Science 301,610, 2003).



Photosystem II (PS II)

All oxygen in the atmosphere is generated from water using light energy by PS II. (Ferreira *et al*, Science 308, 2004).

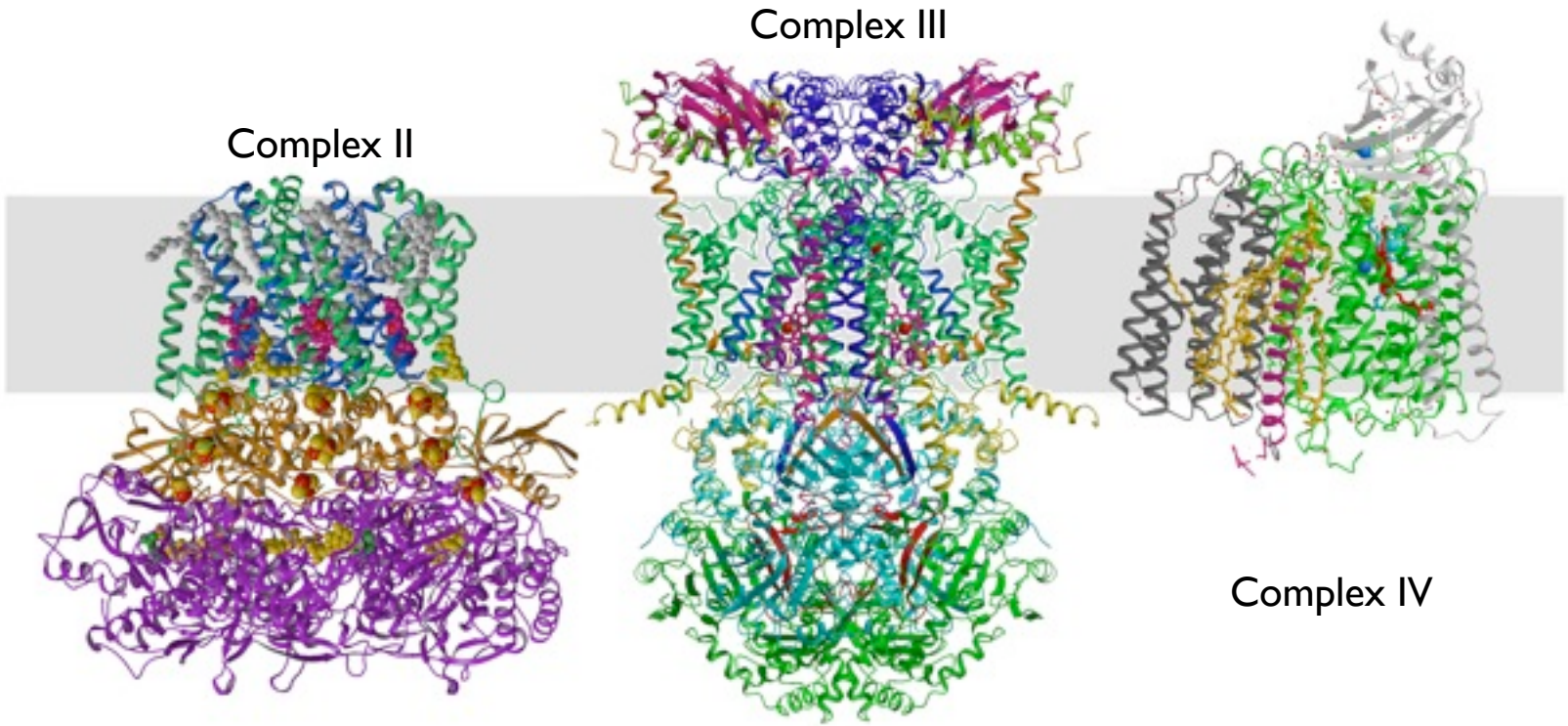


Oxygen Respiratory Enzymes

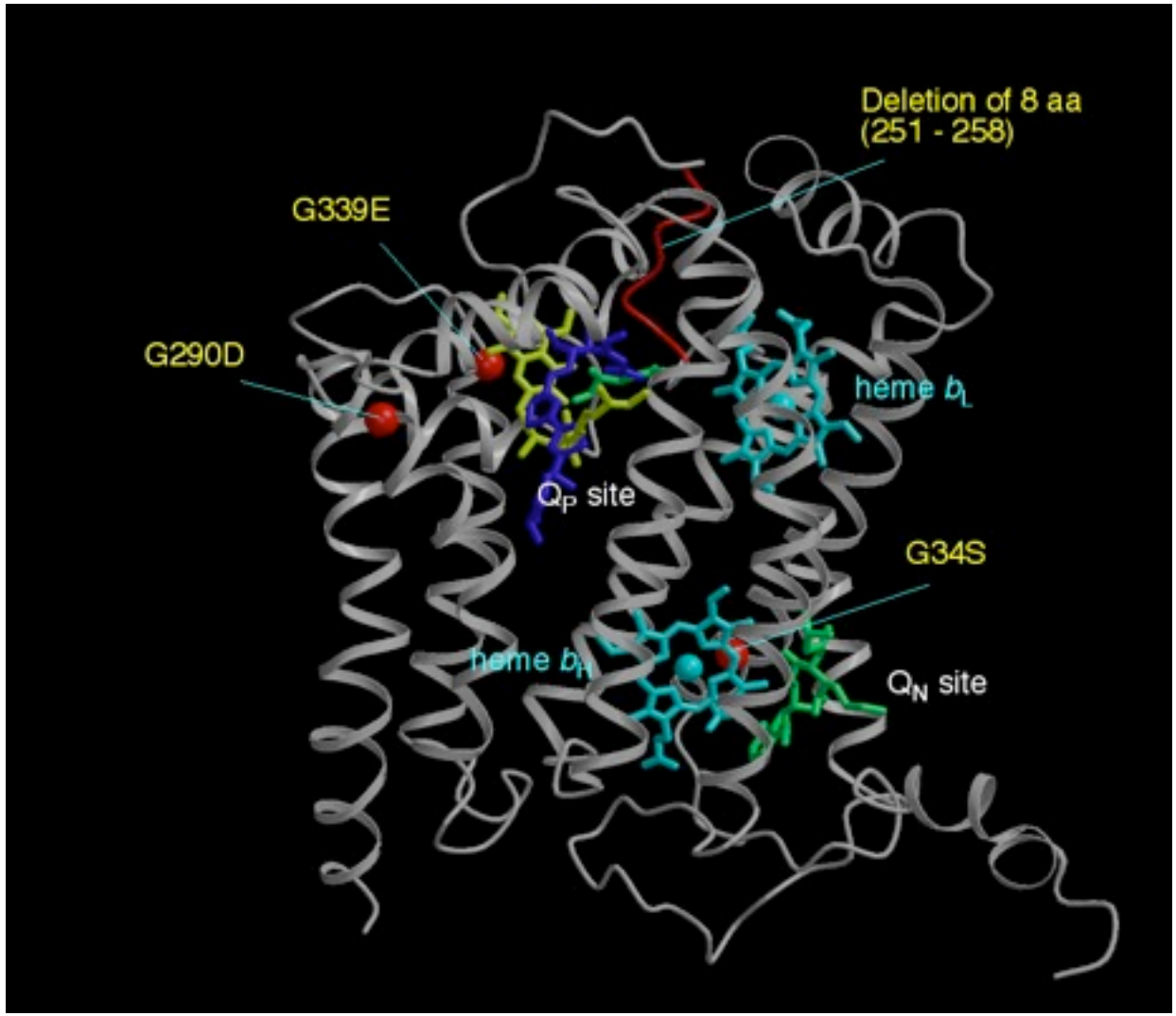
Complex II: Mutations Cause Tumors and Premature Aging (Yankovskaya et al., Science 299 2003).

Complex III: Mutations cause some human genetic disorders (Iwata et al., Science 281, 1998, Andreu et al, N. Engl. J. Med. 341,1999)

Complex IV: "Burns" nutrients using O_2 for energy production (Iwata et al., Nature 376, 1995, Abramson et al., Nature Str. Biol. 7, 2000)



Analysis of Exercise Intolerance Mutations of Complex III (Andreu *et al,* N. Engl. J. Med. 341,1999)



Protein Crystallography to improve QOL

<u>Reduce Cholesterol Level</u>

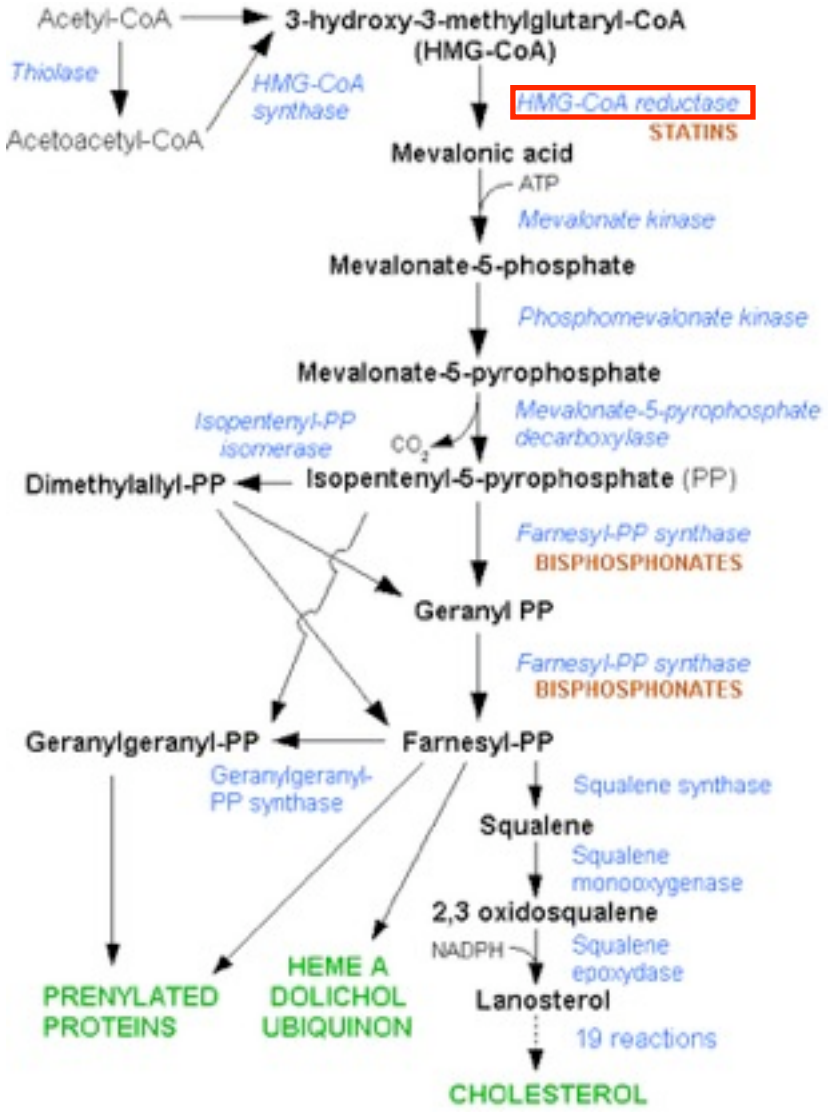
Simvastatin (statin): HMG CoA Reductase Blocker

<u>Lower Blood Pressure</u>

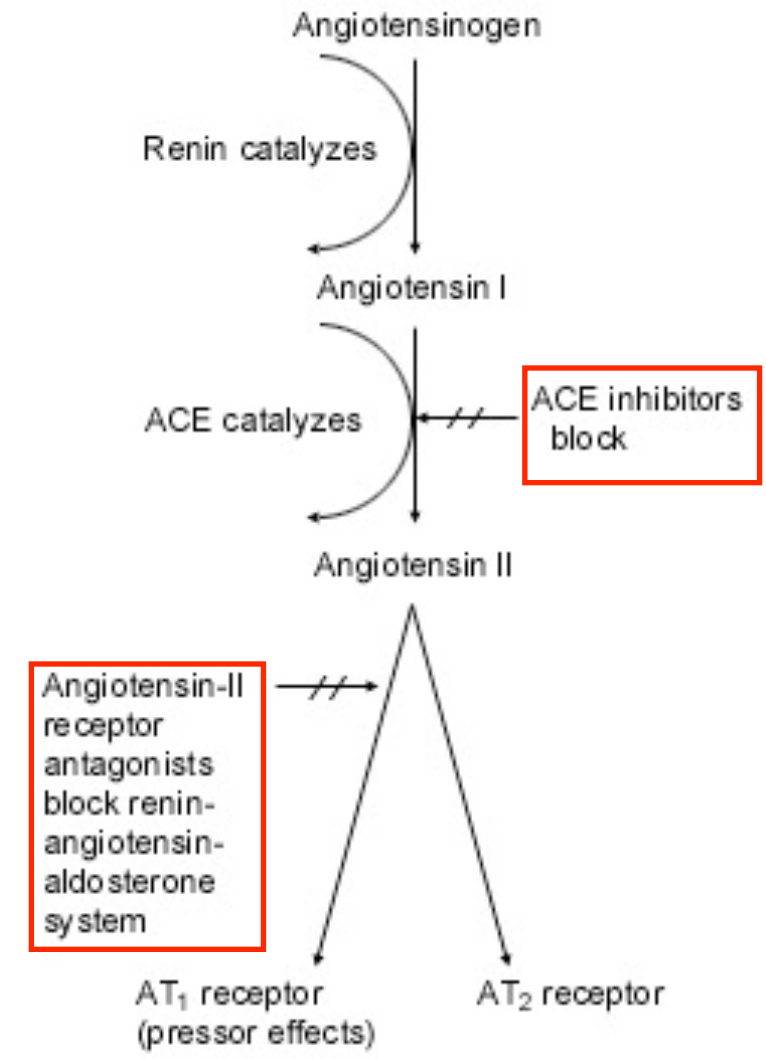
Cozaar: Angiotensin II Receptor Antagonist

Lisinopril: Angiotensin Converting Enzyme (ACE) Blocker

Cholesterol Synthesis (Mevalonate Pathway)

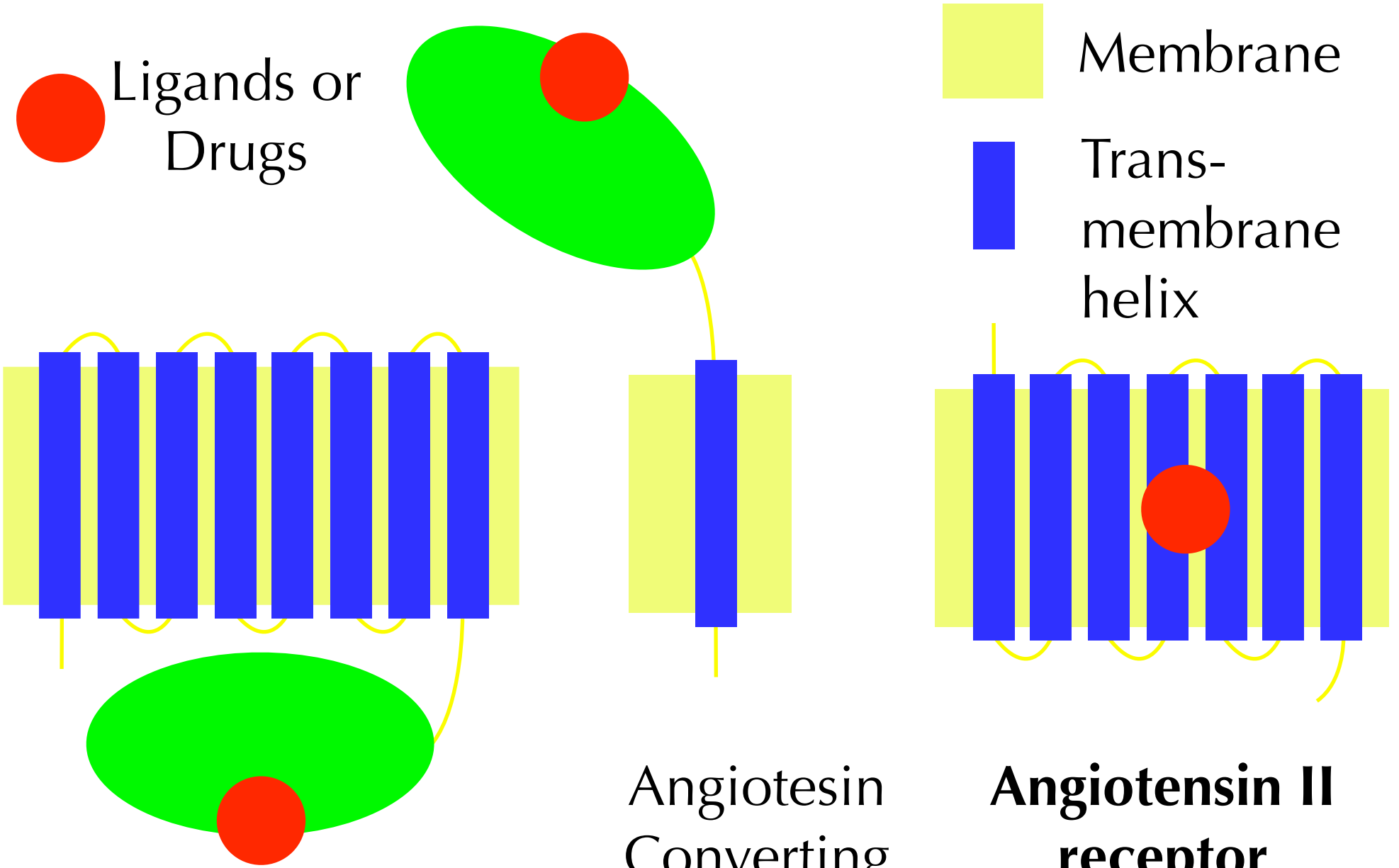


Blood Pressure Control by Angiotensin



Blood Vessel Constriction

All Three Targets Are Membrane Proteins



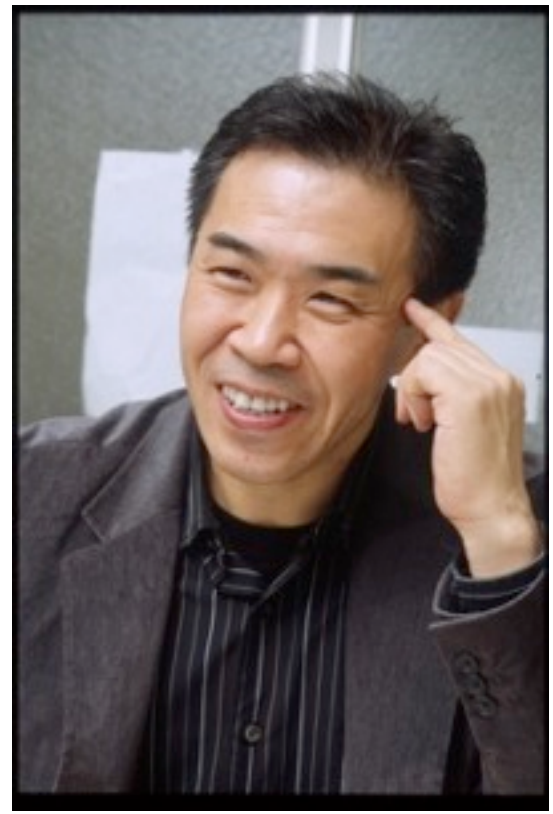
HMG CoA Reductase

Angiotesin Converting Enzyme (ACE)

Angiotensin II receptor (GPCR)

Here Comes Another Problem...

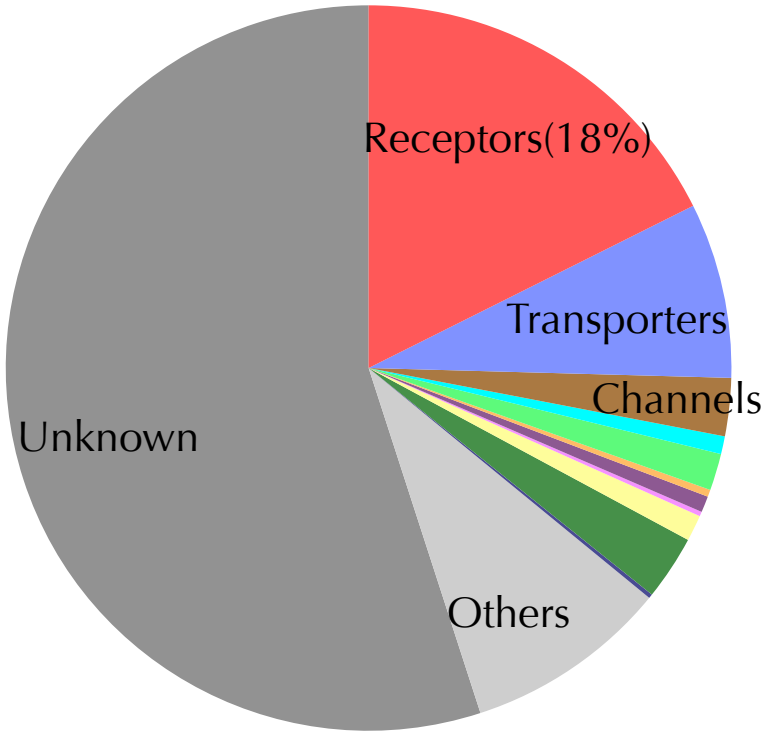
Before (2003)

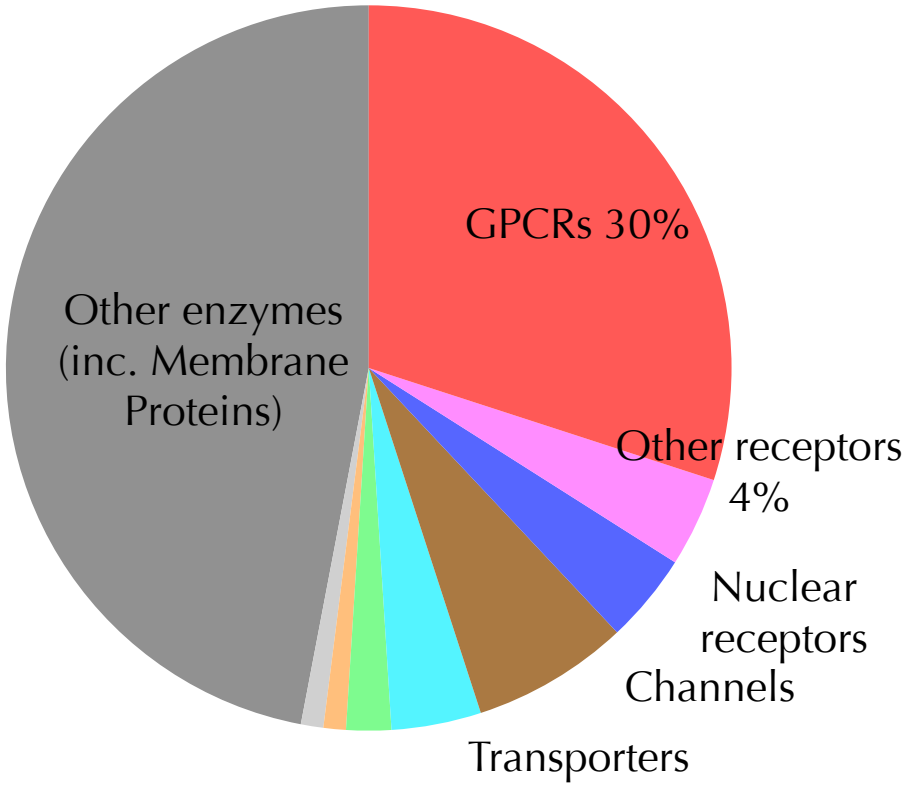


After (2006)

Androgen receptor in follicle cells

Membrane Proteins are Major Drug Targets



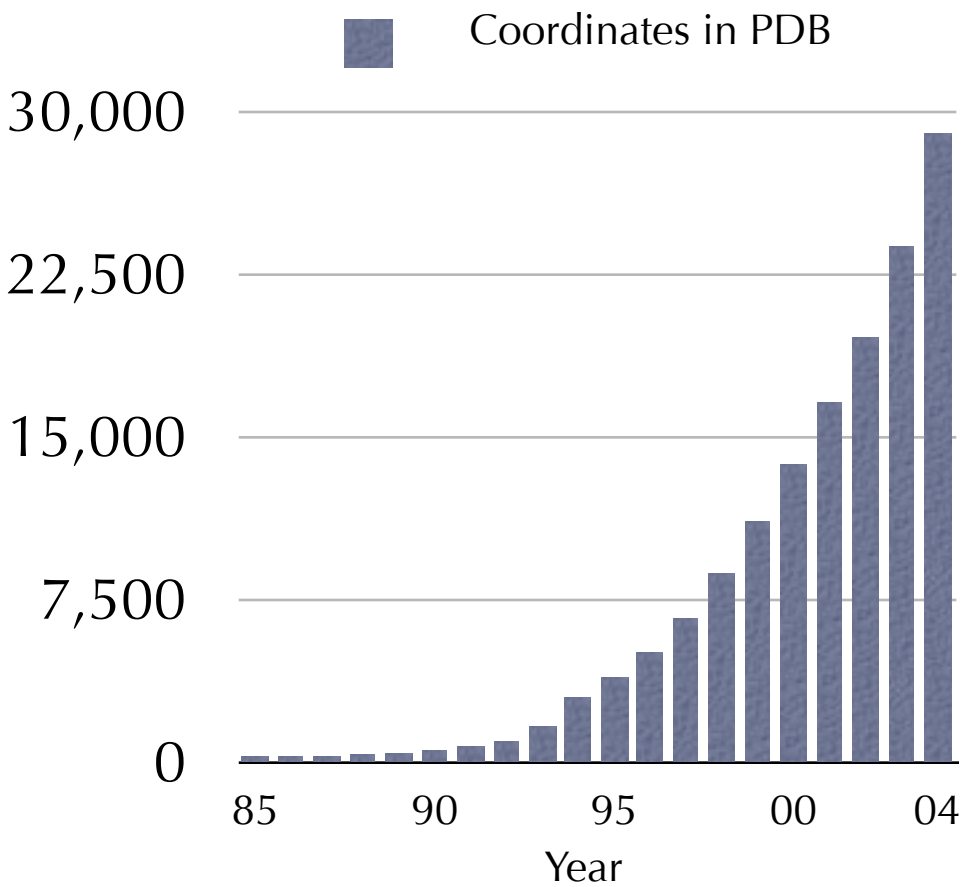


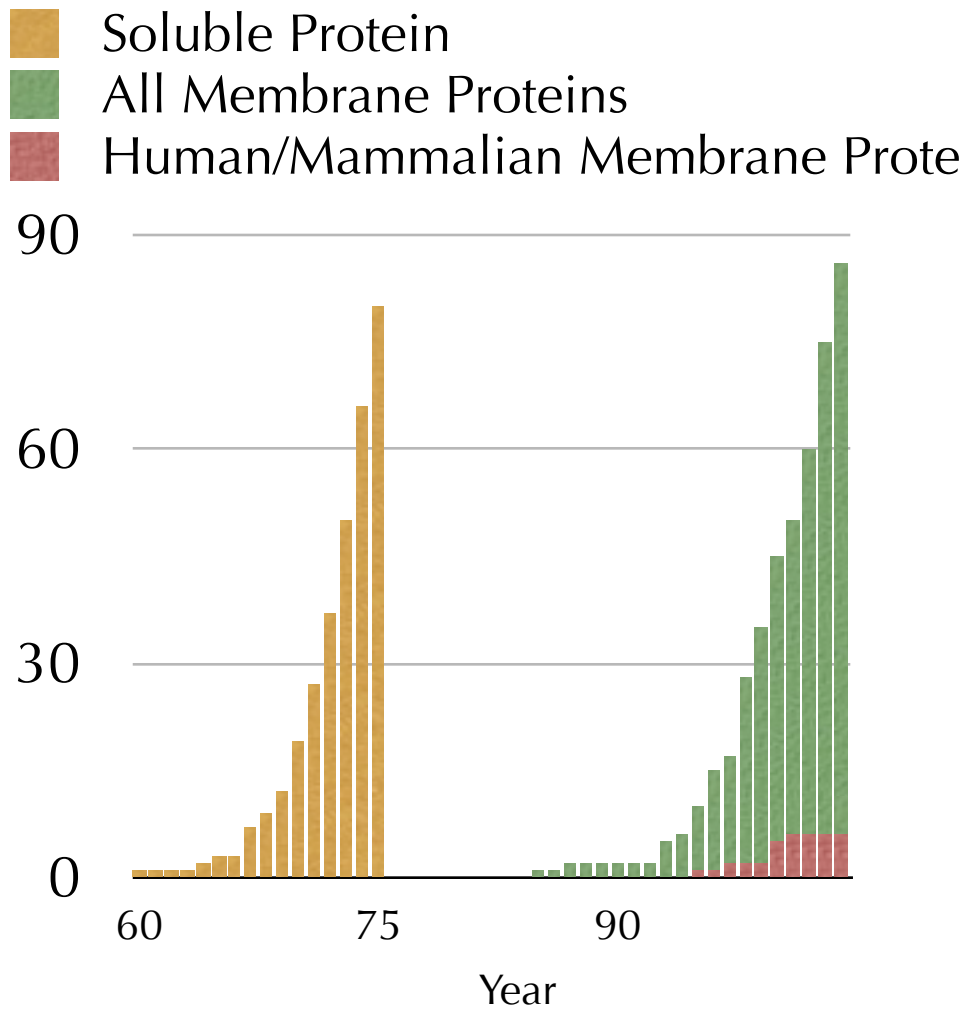
Human Membrane Proteins

Drug targets

Human Membrane Proteins are Major Targets of Structural Biology

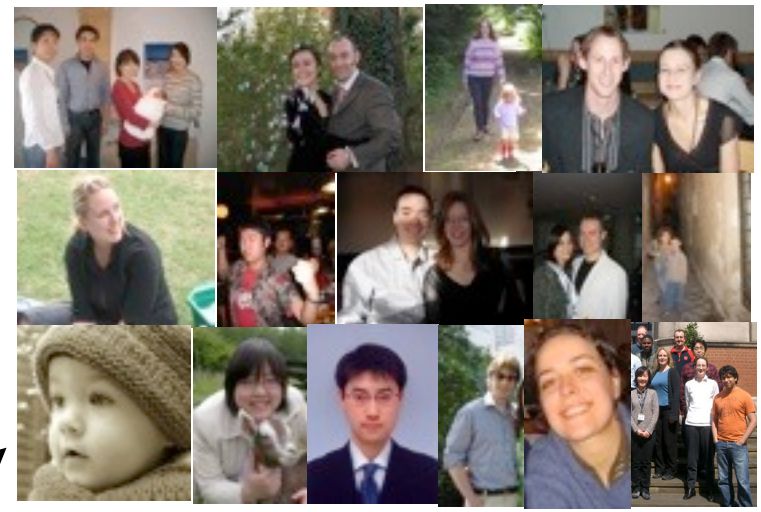
- No human GPCR structure





Establishing a Membrane Protein Crystallography Pipeline

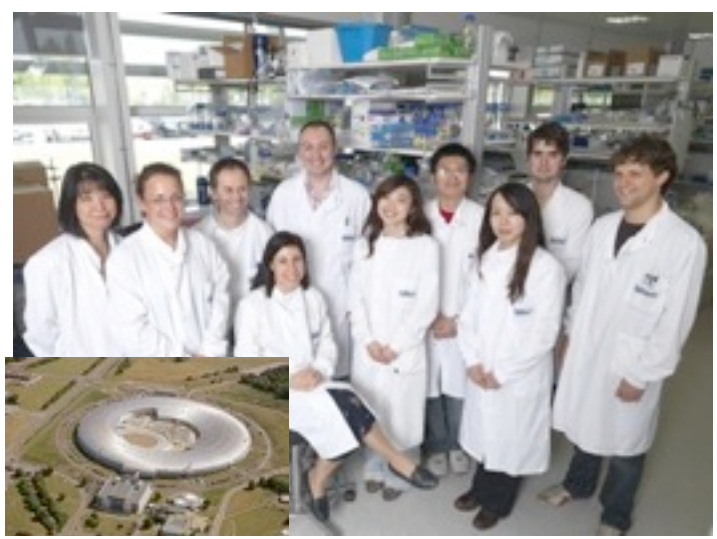
Imperial College London (Membrane Protein Crystallography group, MPC)



Bernadette Byrne
David Drew

-Membrane protein biotechnology
-Membrane protein production
- Transporter biochemistry

Diamond-MPL



Kostas Beis
Alex Cameron
Isabel Moraes
So Iwata

- Automated crystallization
- Data collection
- Structure determination

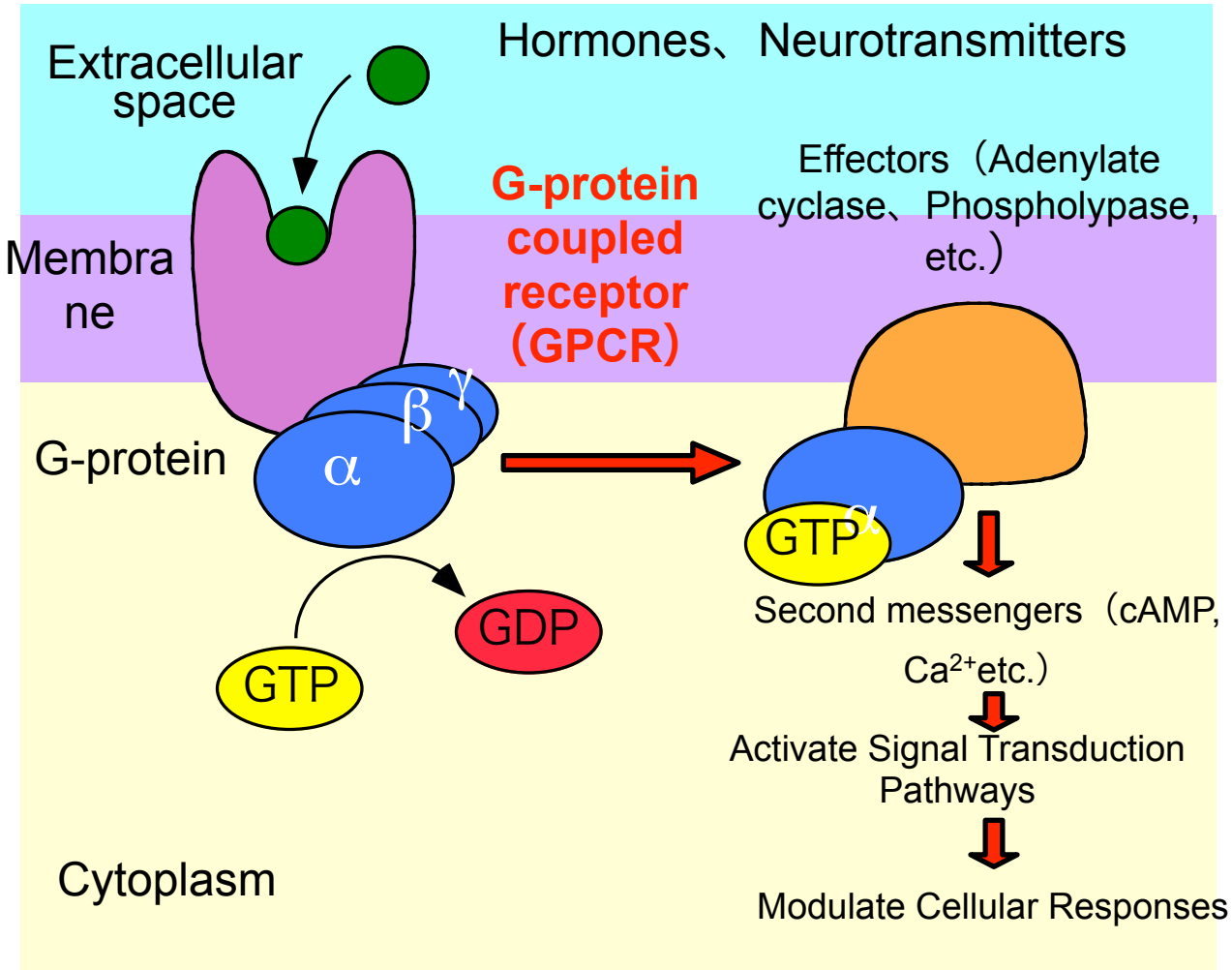
ERATO-Kyoto Lab

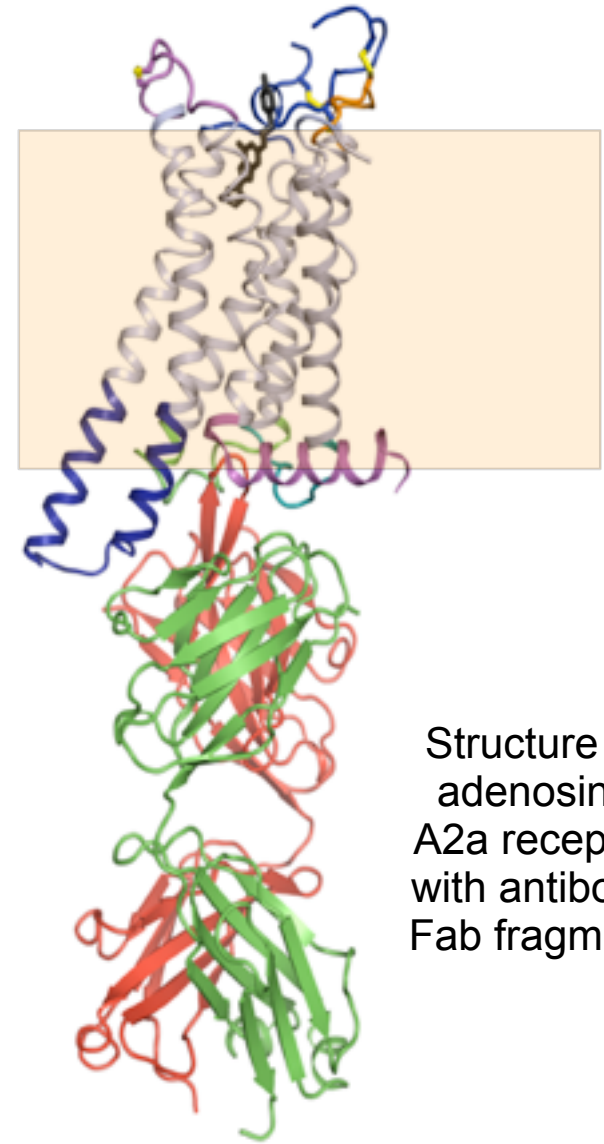
Takuya Kobayashi
Takeshi Murata
Tatsuro Shimamura
Norimichi Nomura

- Production facilities (Insect and Mammalian cells)
- Antibody technology
- GPCR biochemistry

G-protein Coupled Receptors (GPCRs)

- Transmembrane receptors that sense hormones, neurotransmitters and other molecules.
- >30% drugs are targeting GPCRs

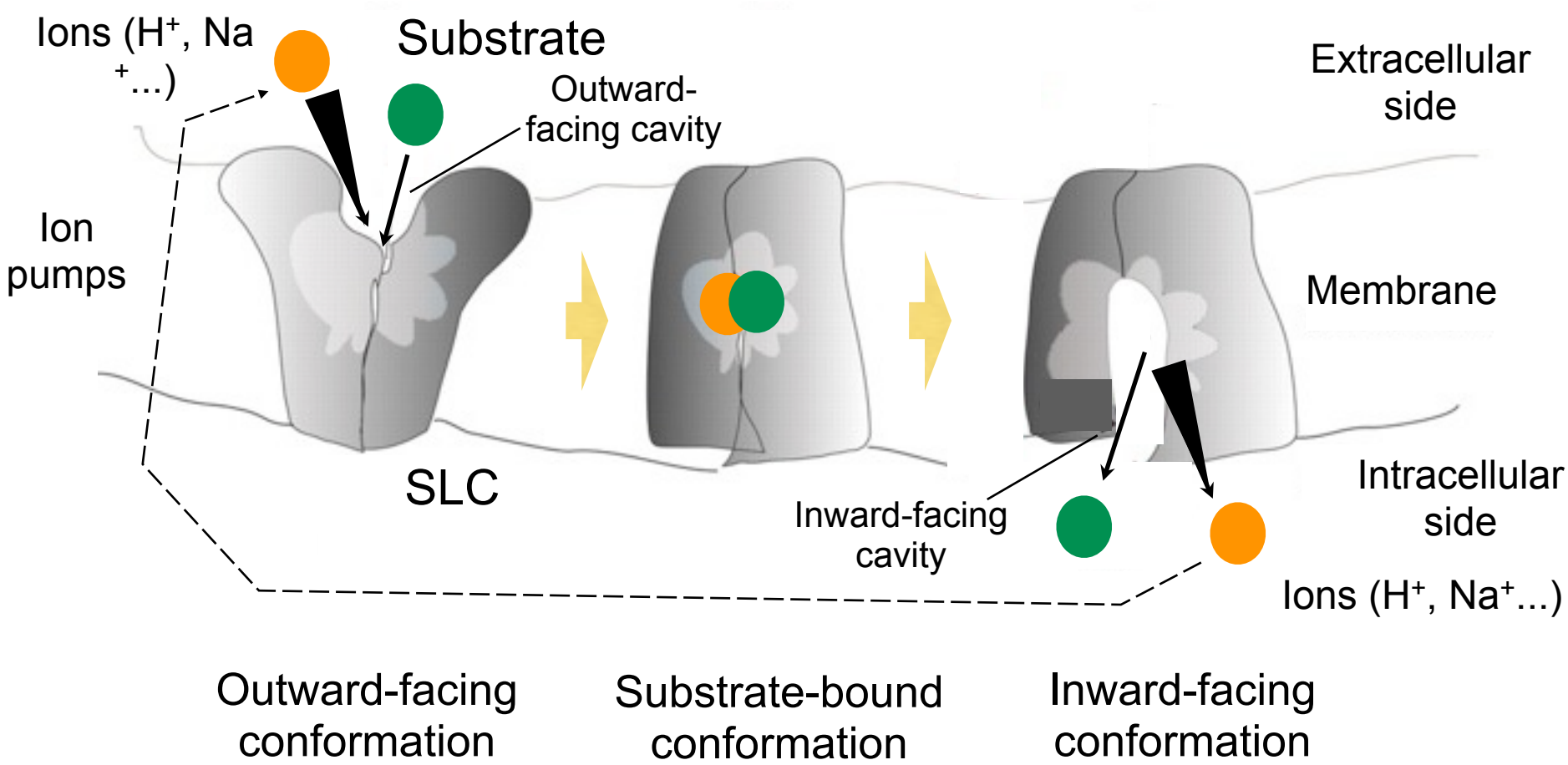




Structure of adenosine A2a receptor with antibody Fab fragment

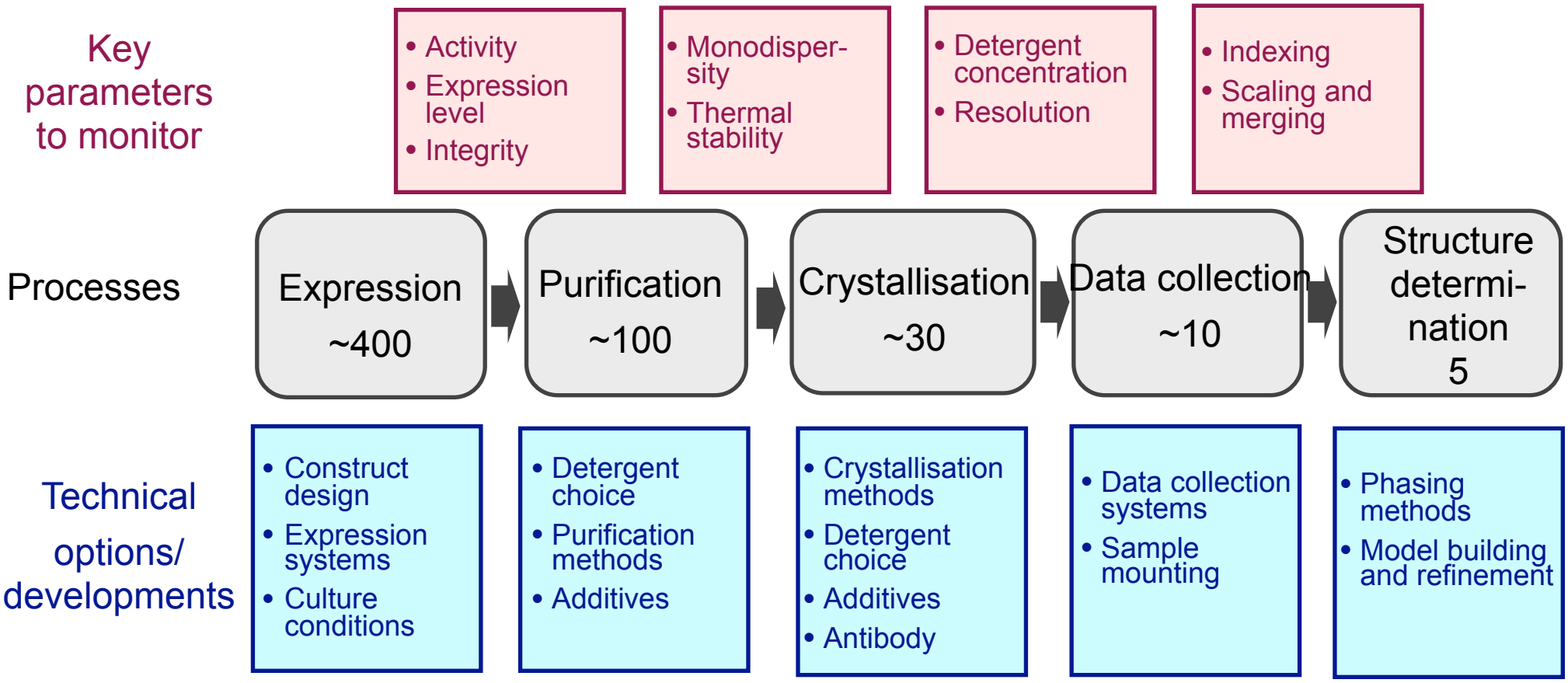
Solute Carrier Family (SLC) Transporters

- Membrane transporters including over 300 members organized into 47 families (by Human Genome Organization)
- Composed of facilitative transporters (passive transporters) and secondary active transporters (use an ion gradient for energy).

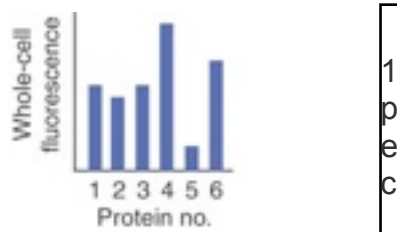


Membrane Protein Crystallography Pipeline

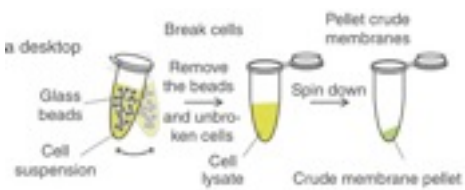
- Method development to address major bottlenecks for membrane protein crystallography.



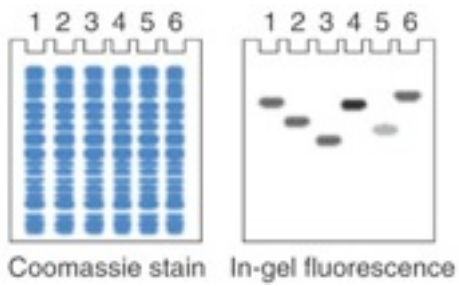
GFP-based Membrane Protein Expression and Purification System in *S. cerevisiae* (1)



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

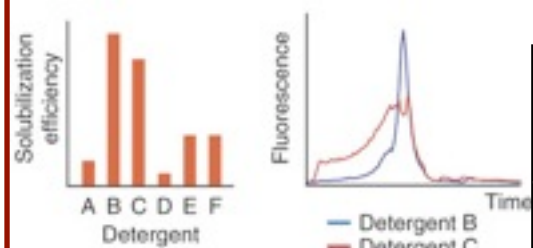


2. Isolate crude membranes

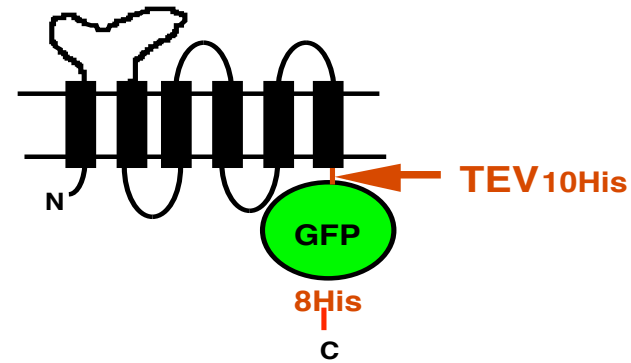


3.Check integrity using in-gel fluorescence

4. Check cellular localization by confocal micro-scopy

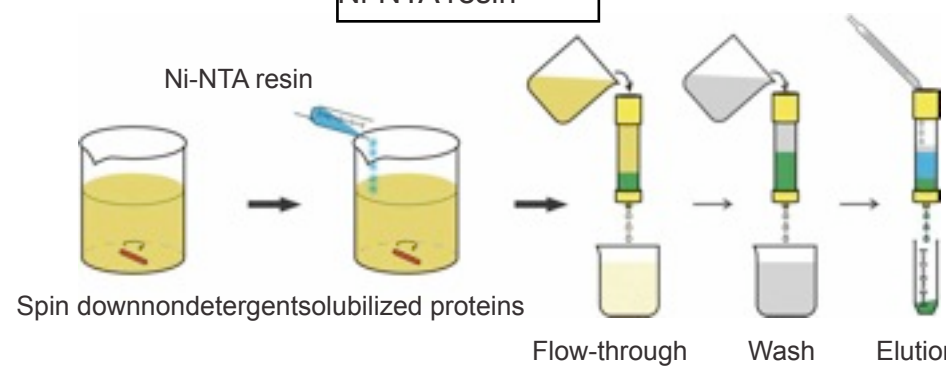


5.Check mono-disperisty in different deter-gent by fluorescence size-exclusion chromatography

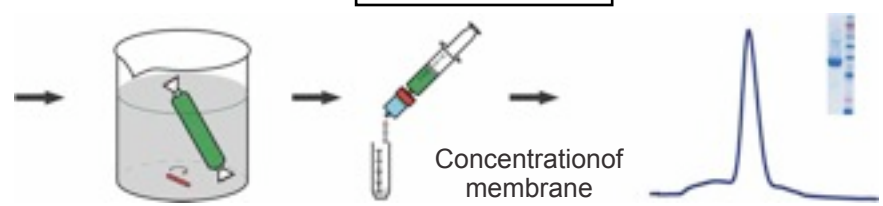


Drew *et. al.* Nat. Methods. 2, 2006
Newstead *et. al.* PNAS 104, 2007
Drew *et al.*, Nat. Protoc. 3, 2008

1.Detergent-solubilization → 2.Incubation of supernatant with Ni-NTA resin → 3 . I M A C purification

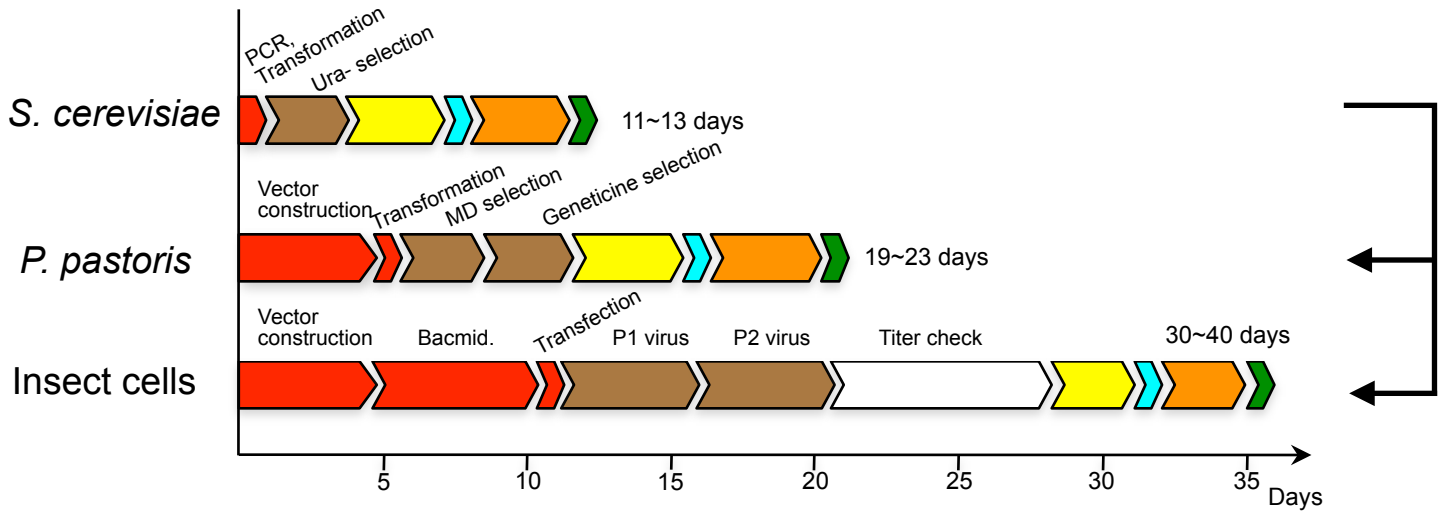


4.Dialysis and cleavage of GFP-8His → 5.Reverse IMAC to remove GFP-8His and his-tagged TEV → 6.Gel filtration of membrane protein (option)



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

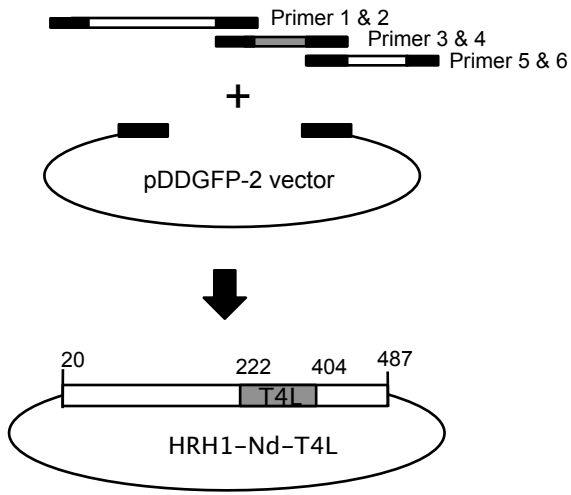
- Suitable for fast screening of the most stable/expressed constructs

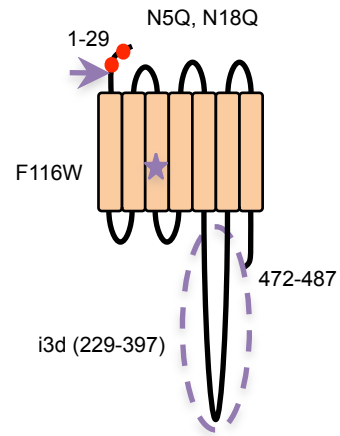


Easy to transfer to the systems to produce better proteins

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

- Easy to construct variants by combining multiple fragments



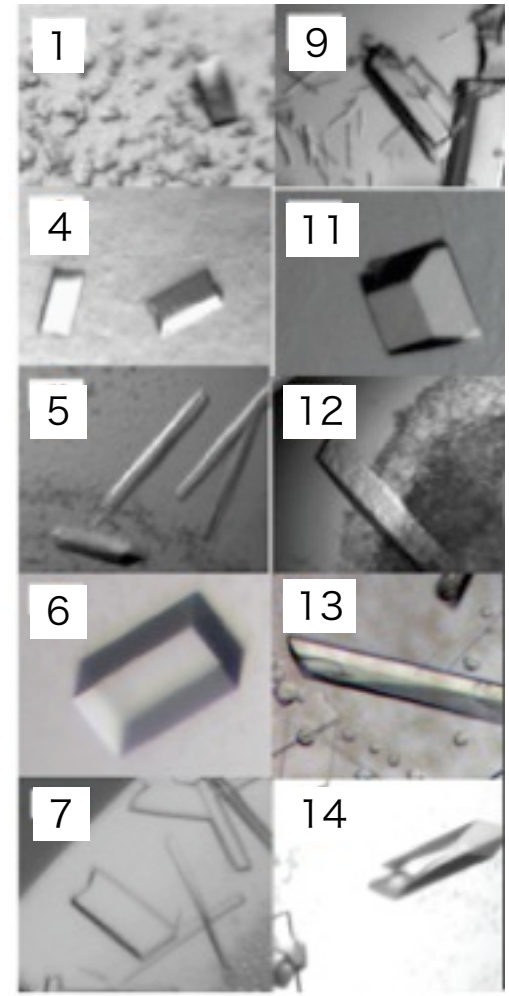


Name	Deletions and mutations
HRH1-WL	HRH1 whole length
HRH1-Nd-i3d	HRH1(20-487), i3d(229-397)
HRH1-Nd-T4L	HRH1(20-487), T4L
HRH1-Nd-F116W-i3d	HRH1(20-487), F116W, i3d(229-397)
HRH1-Nd-L123W-T4L	HRH1(20-487), F116W, T4L

Examples: Crystallisation of SLCs

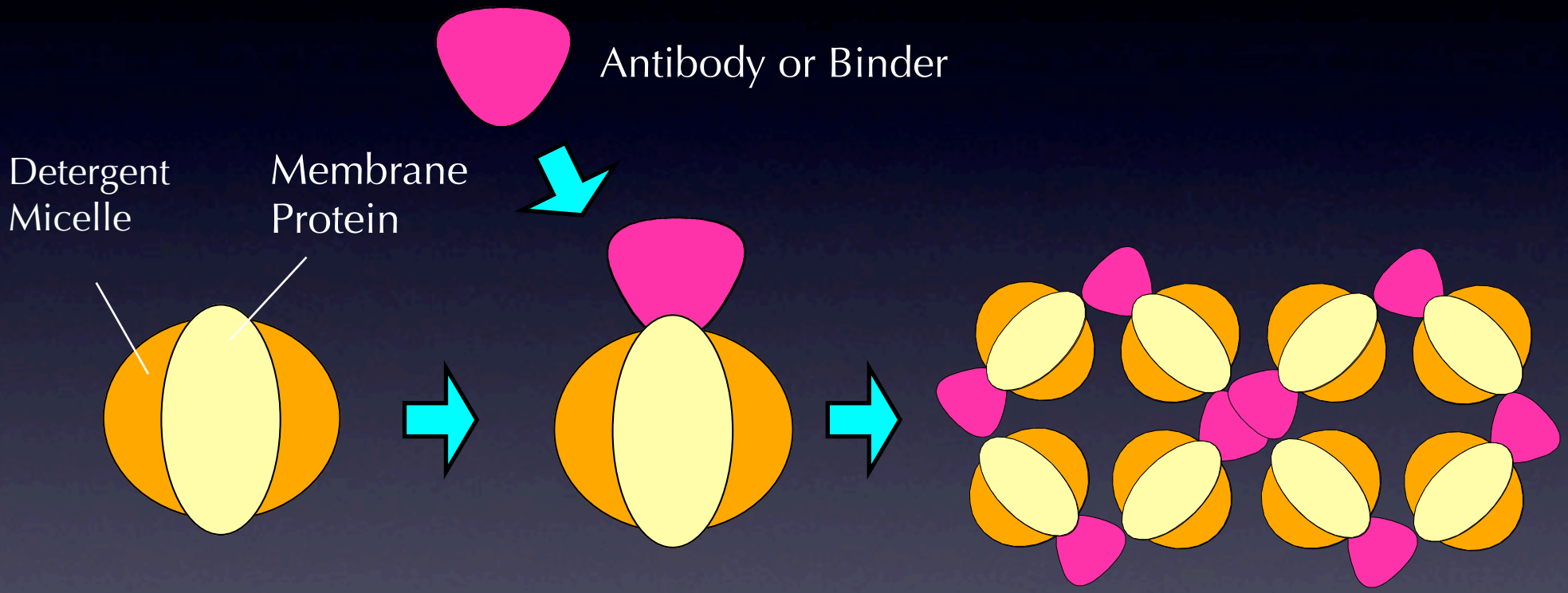
- Crystals from 16 SLC proteins and their orthologues were obtained
- Only six crystals diffracted better than 4.0 Å, one better than 3.0 Å.
- Problem: extremely difficult to obtain high-quality crystals.

			Substrate(s)	Res. (Å)
	Glucose transporters (SLC2)			
1	GLUT5	Rat	Fructose, Glucose	5.0
2	GLUT7	Human	Fructose, Glucose	10.0
3	LacY	*Bacterial*	Lactose	3.5
4	FucP	*Bacterial*	Fucose	3.8
	Bicarbonate transporters (SLC4)			
5	Band3	Human	$Bicarbonate/Cl^-$	5.0
6	BOR1	Rice	Borate/?	3.6
7	BOR1	Arabidopsis	Borate/?	10.0
	Sodium/proton exchangers (SLC9)			
8	NhaA	*Bacterial*	Na^+/H^+	4.5
9	NapA	*Bacterial*	Na^+/H^+	3.3
	Sodium/bile salt cotransporters (SLC10)			
10	NtcP	Rat	Bile salt/Na^+	10.0
11	NtcP	*Bacterial*	Bile salt/Na+	2.8
	Proton/oligopeptide cotransporters (SLC15)			
12	PepT	*Bacterial*	oligopeptide	3.8
13	PepT	*Bacterial*	oligopeptide	3.0
14	PepT	*Bacterial*	oligopeptide	6.5



Mammalian Plant Bacterial

Crystallization of Membrane Proteins using Antibody Fragment or Artificial Binder

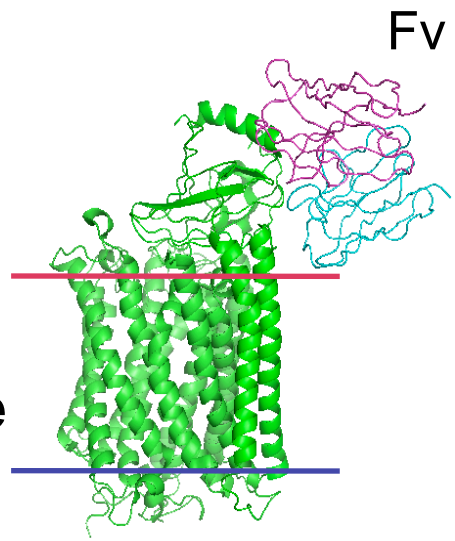


Detergent solubilised membrane proteins (difficult to crystallize because of the detergent micelle).

Membrane protein Antibody/ binder complex

Crystal of the membrane protein - antibody/binder complex

Antibody fragments for membrane protein crystallisation

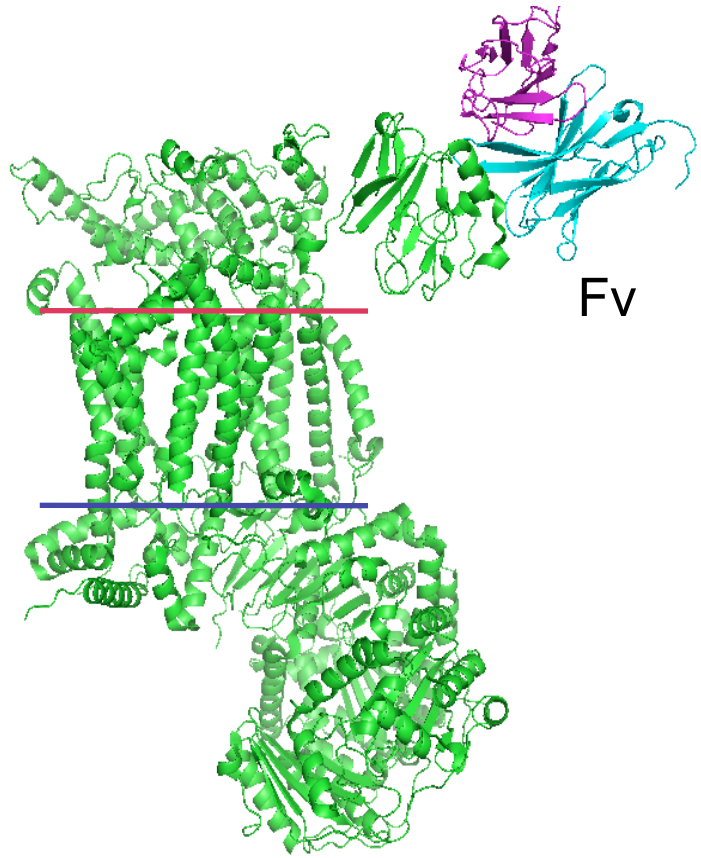


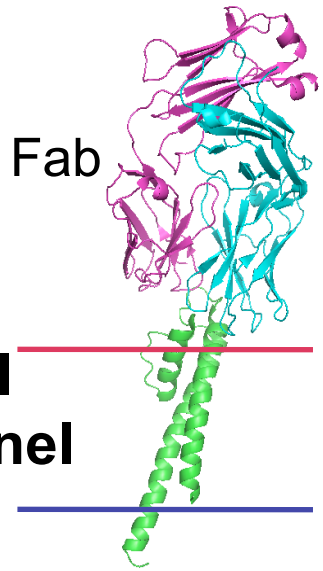
bacterial cytochrome oxidase

Iwata, Michel et al. (1995)

yeast cytochrome bc_1 complex

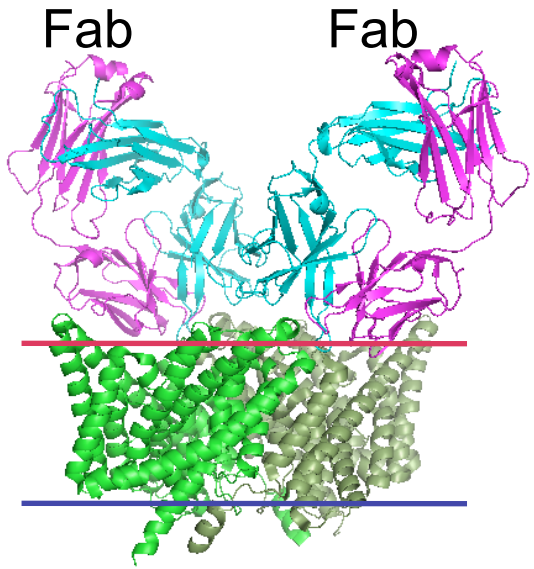
Hunte, Michel et al. (2000)



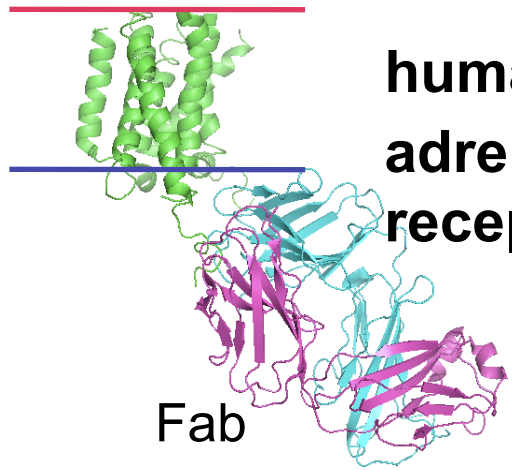


bacterial K^+ channel (KcSA)

MacKinnon et al. (2001)



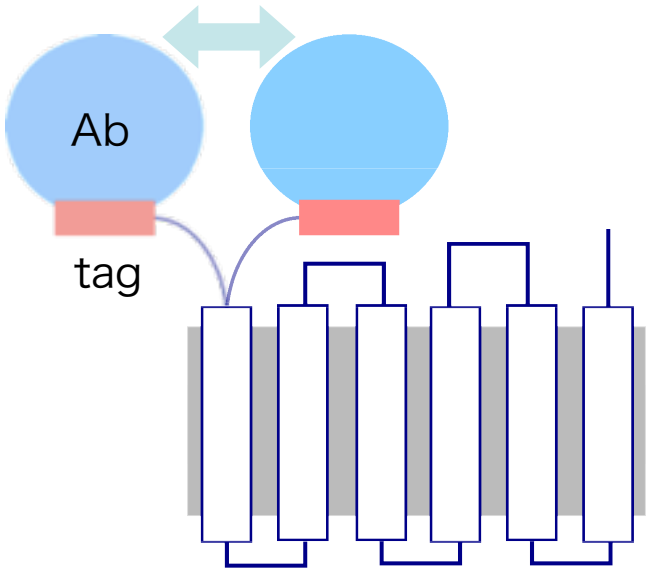
bacterial chloride channel (CIC) MacKinnon et al. (2003)



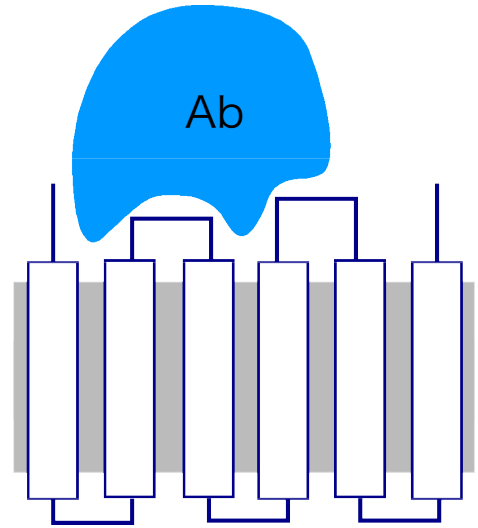
human β_2 adrenergic receptor

Kobilka et al. (2007)

Antibodies for Membrane Protein Crystallization

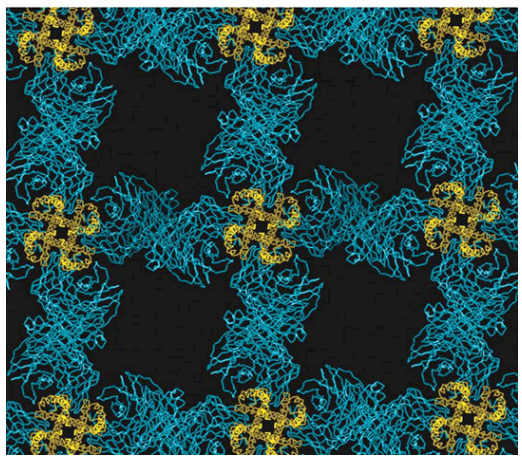


Antibody NOT suitable for crystallisation recognizing a sequential epitope

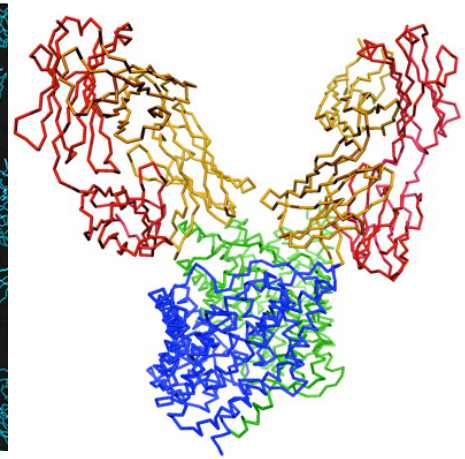


Antibody suitable for crystallisation recognizing a conformational epitope

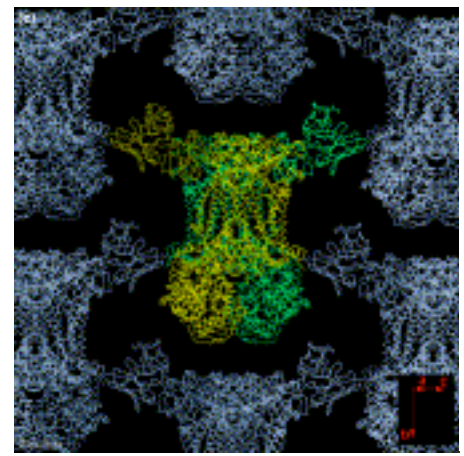
Problem: extremely difficult to raise antibodies against native mammalian membrane proteins



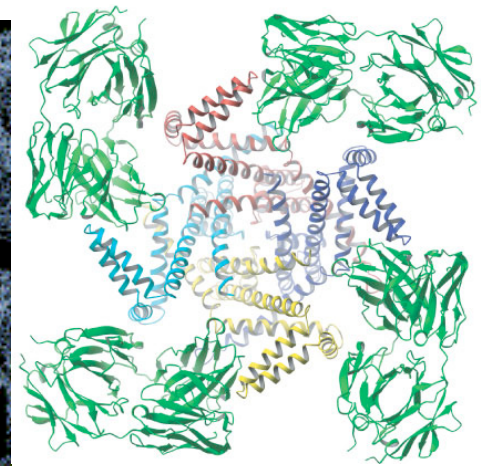
KcsA K-channel



CLC CL cannel



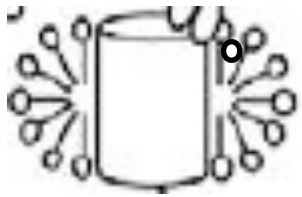
Cytochrome bc 1 complex



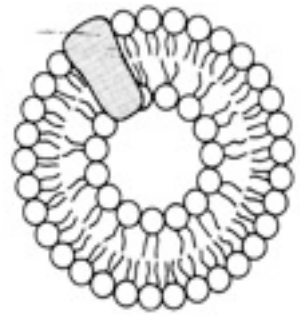
KvAP K-channel

Improved Immunization Strategies

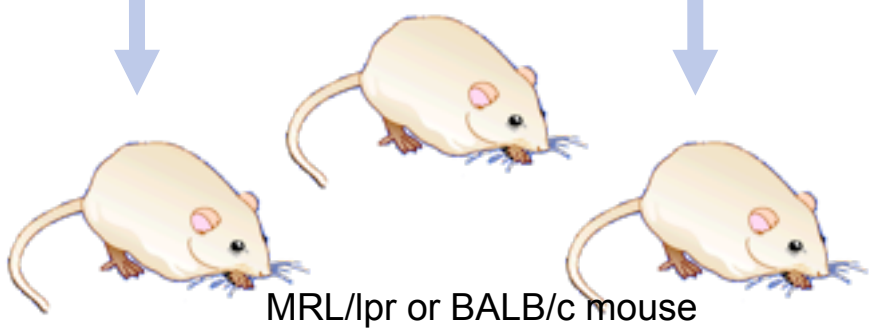
purified protein	protein-liposome	protein-baculovirus



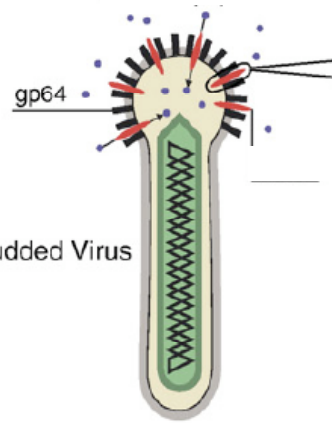
+ Adjuvant
(pertussis toxin)

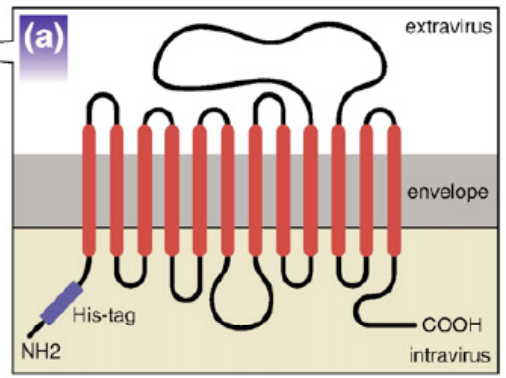


+ Adjuvant
(Lipid A)

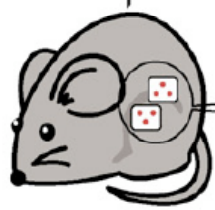


MRL/lpr or BALB/c mouse

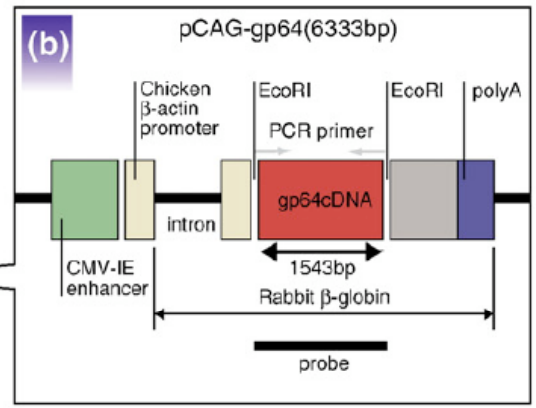




immunize

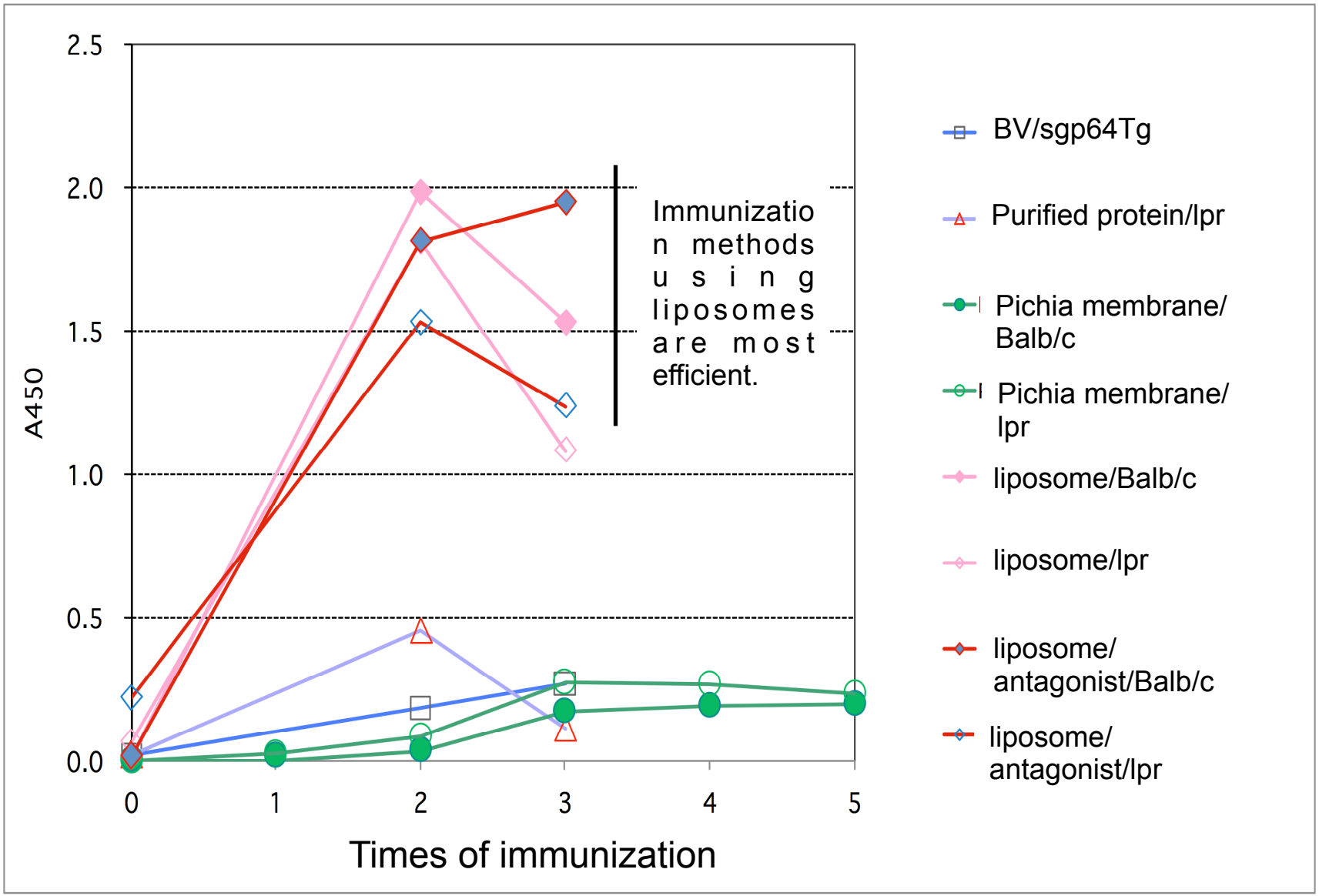


gp64 transgenic mouse



Hybridoma / phage display

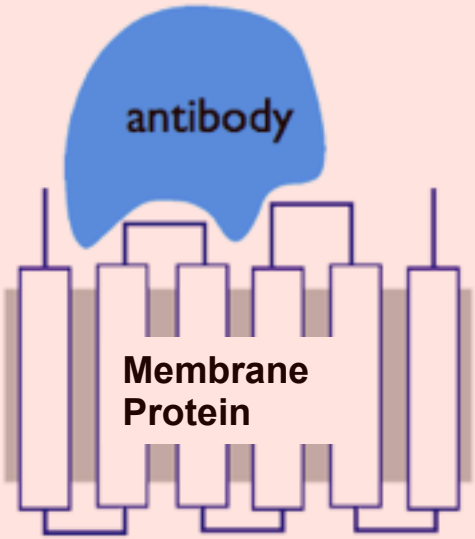
Comparison of Immunization Strategies



Antibody Screening for Membrane Protein Crystallization

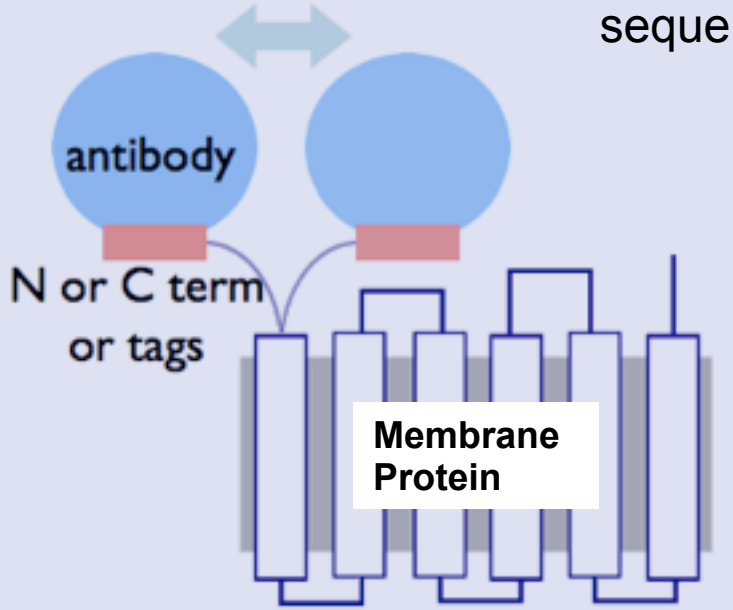
- Combining ELISA with native membrane proteins and the Western/DOT blots with denatured membrane proteins.

Antibodies suitable for crystallisation: recognizing conformational epitopes

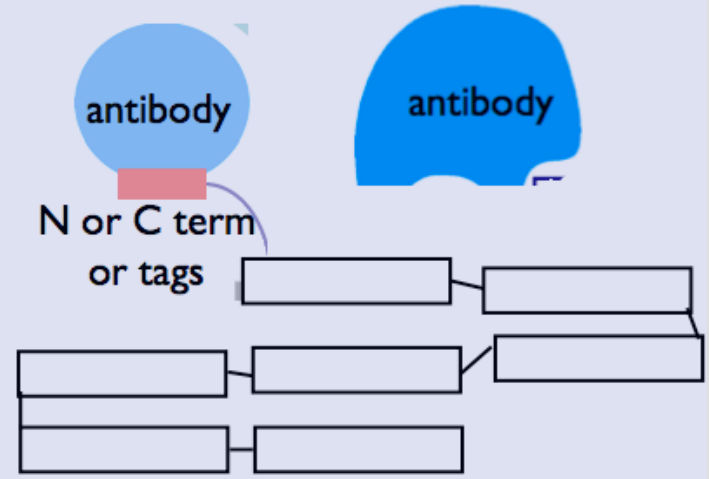


ELISA +, WB/DOT -

Antibodies NOT suitable for crystallisation: recognizing sequential epitopes



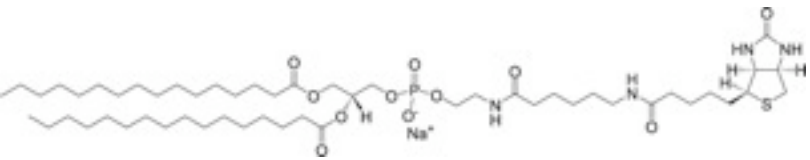
ELISA +, WB/DOT+



ELISA -, WB/DOT +

Liposome ELISA (L-ELISA)

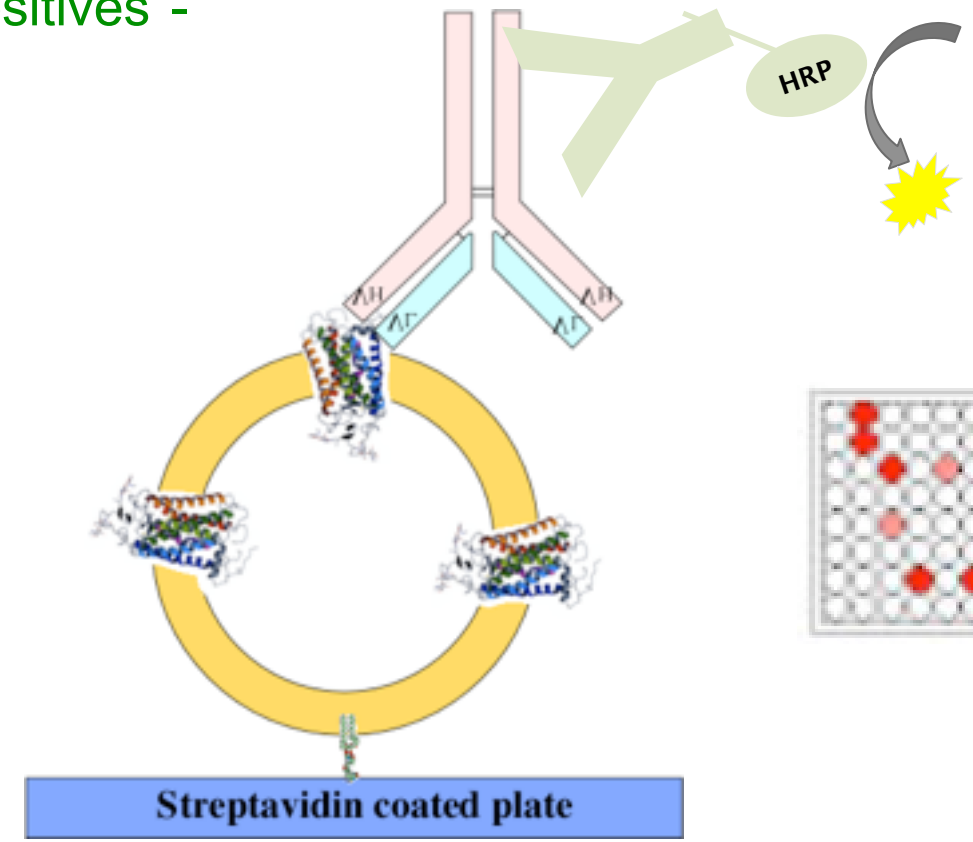
- Prevent membrane protein denaturation - causes false positives - on the ELISA plate.

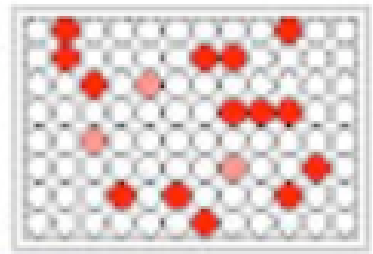


Lipids with 16:0 Biotinyl CAP PE

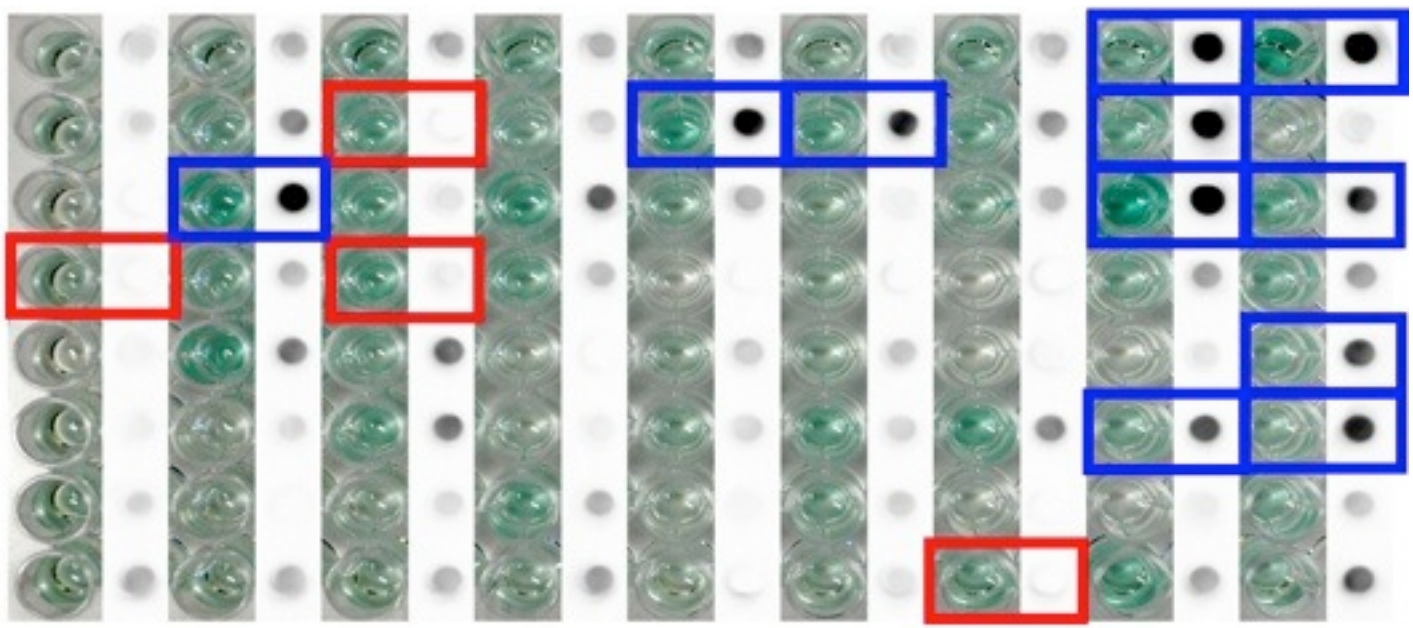
Antigen reconstituted into proteoliposome

Testing culture sup.





Antibody Screening using L-ELISA and DOT blot

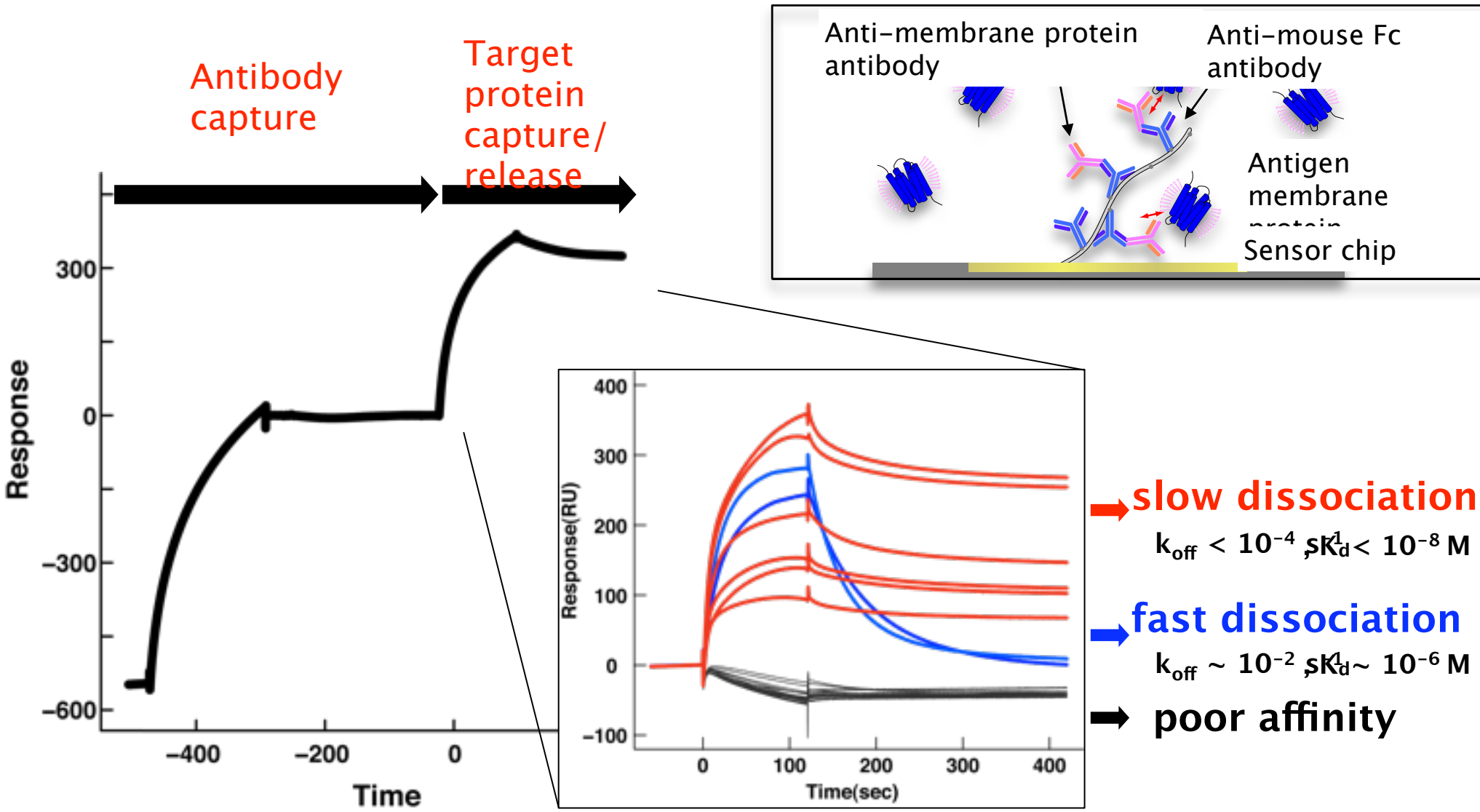


ELISA + & Dot - = **Conformational epitope**

ELISA + & Dot + = **Sequential epitope**

Affinity Test using Biacore

- Select antibodies with a low Kd (high affinity) and a slow k_{off} (slow dissociation)



➡ **slow dissociation**

$k_{off} < 10^{-4}$ s^{-1}, $K_d < 10^{-8}$ M

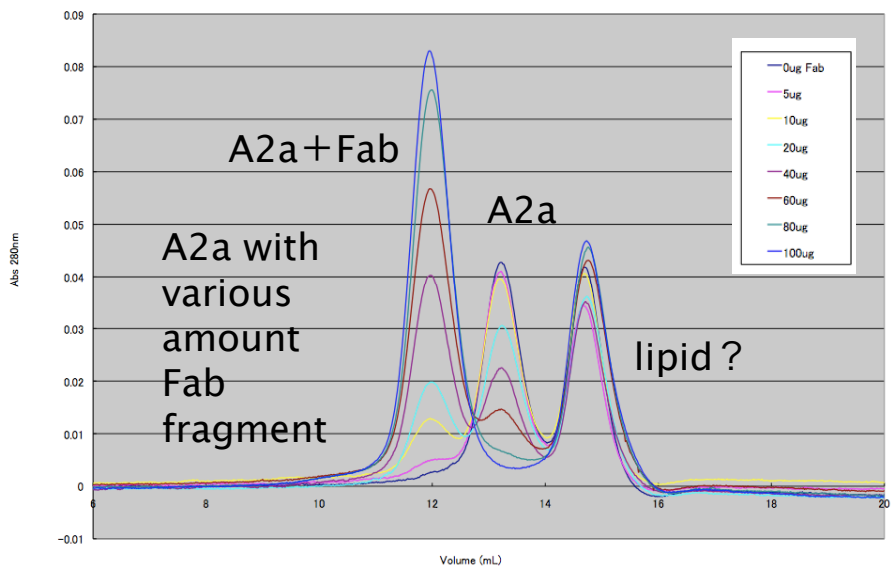
➡ **fast dissociation**

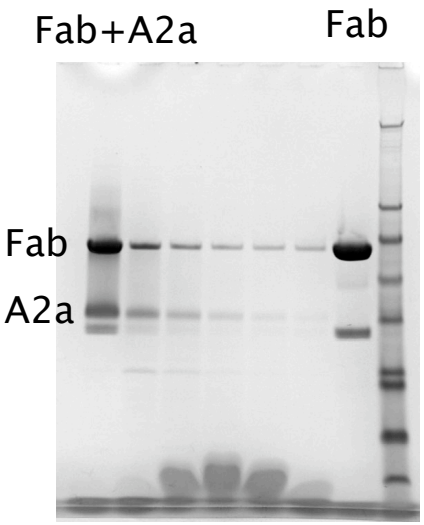
$k_{off} \sim 10^{-2}$ s^{-1}, $K_d \sim 10^{-6}$ M

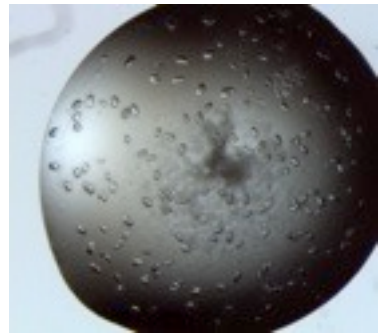
➡ **poor affinity**

Membrane Protein - Antibody Fab Fragment Complex Preparation

Adenosine A2a

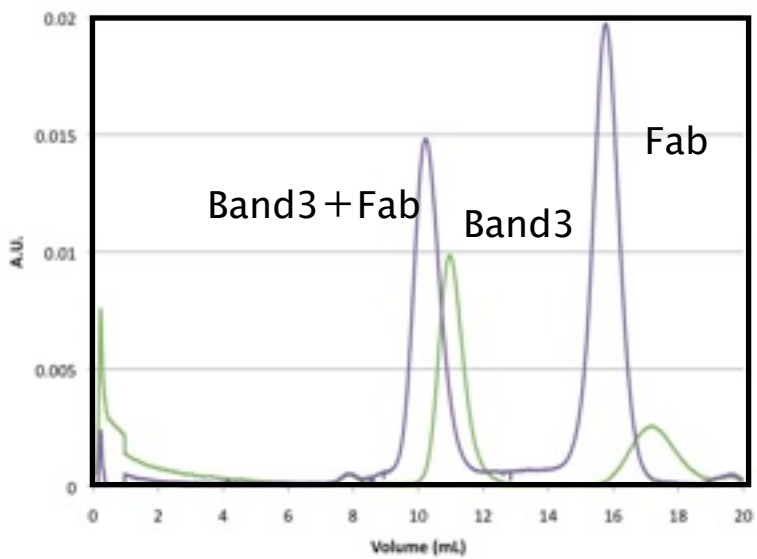


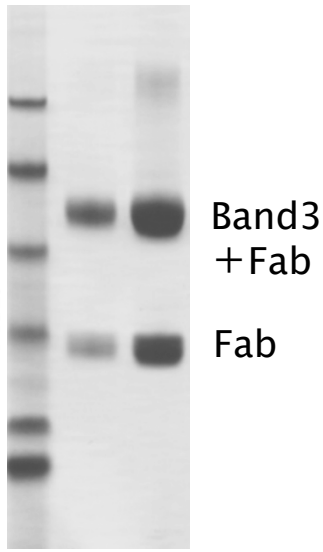




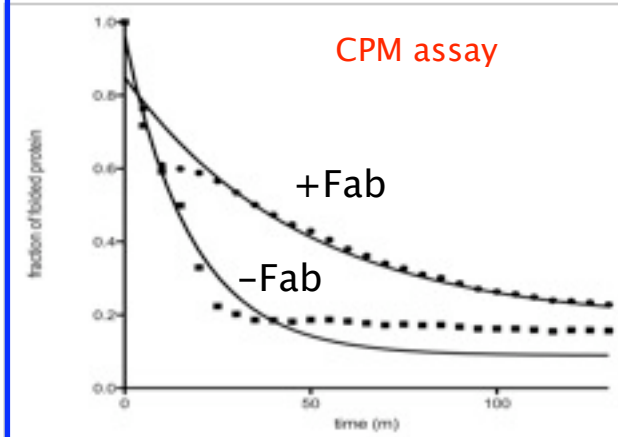
Complex crystals

Human Anion Exchanger





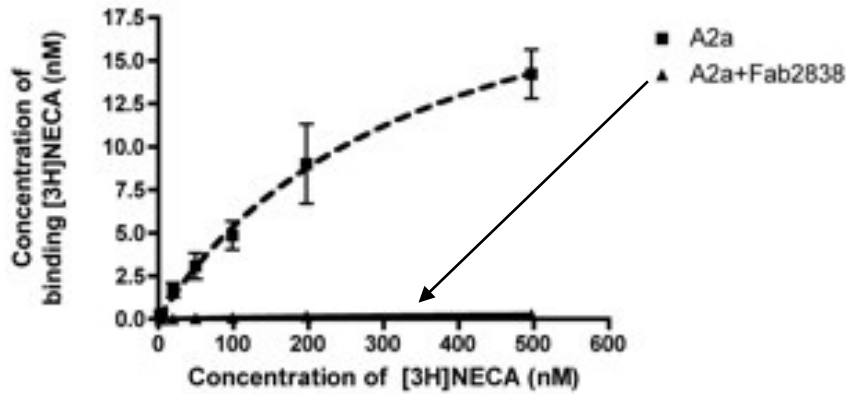
Antibody significantly improves the thermostability of A2a



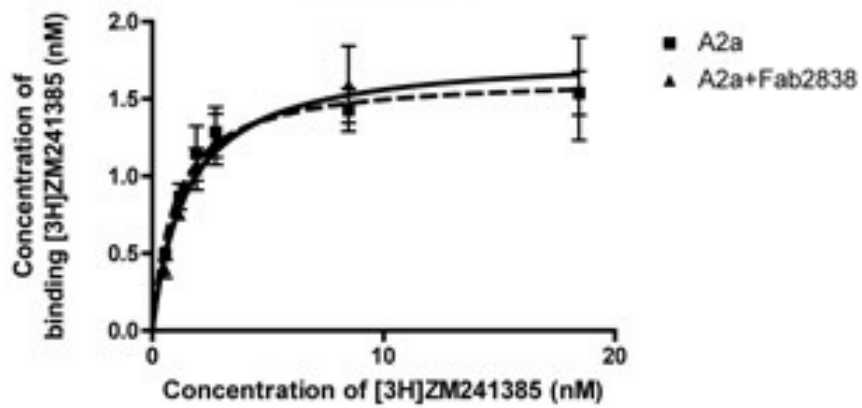
Applications for Functional/Therapeutic Antibodies and for Imaging

-Fab2838 locks adenosine A2a receptor into the inactive conformation

NECA(Agonist, only binds to the active form)

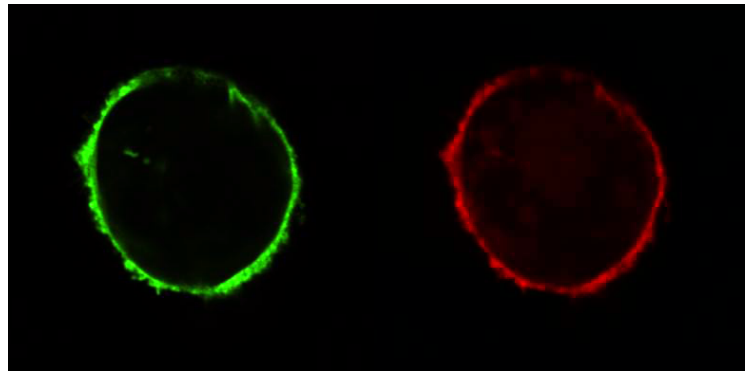


ZM241385(Antagonist, can bind to the inactive form)



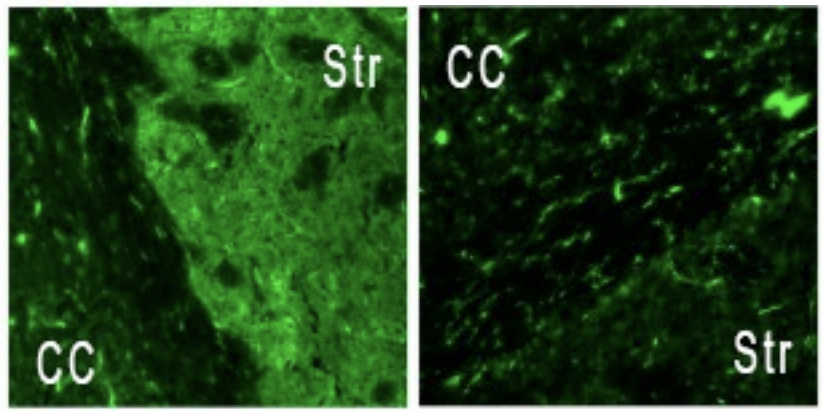
-Cell imaging using antibodies against adenosine A2a receptor

Cultured cells



GFP(control) C40010+2nd Ab(+Alexa546)

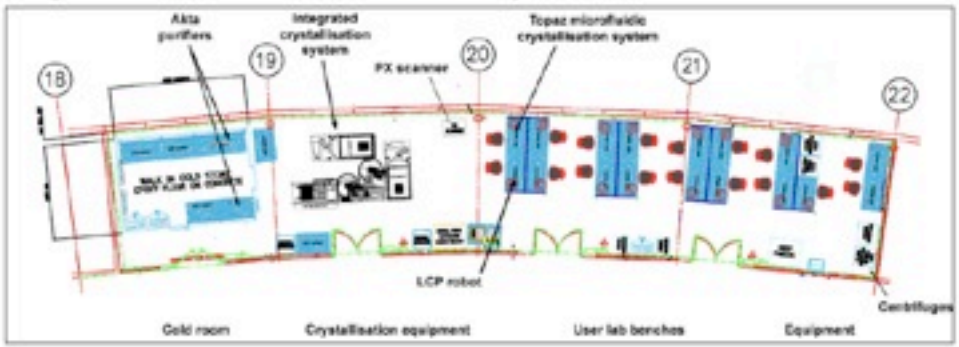
Mouse brain



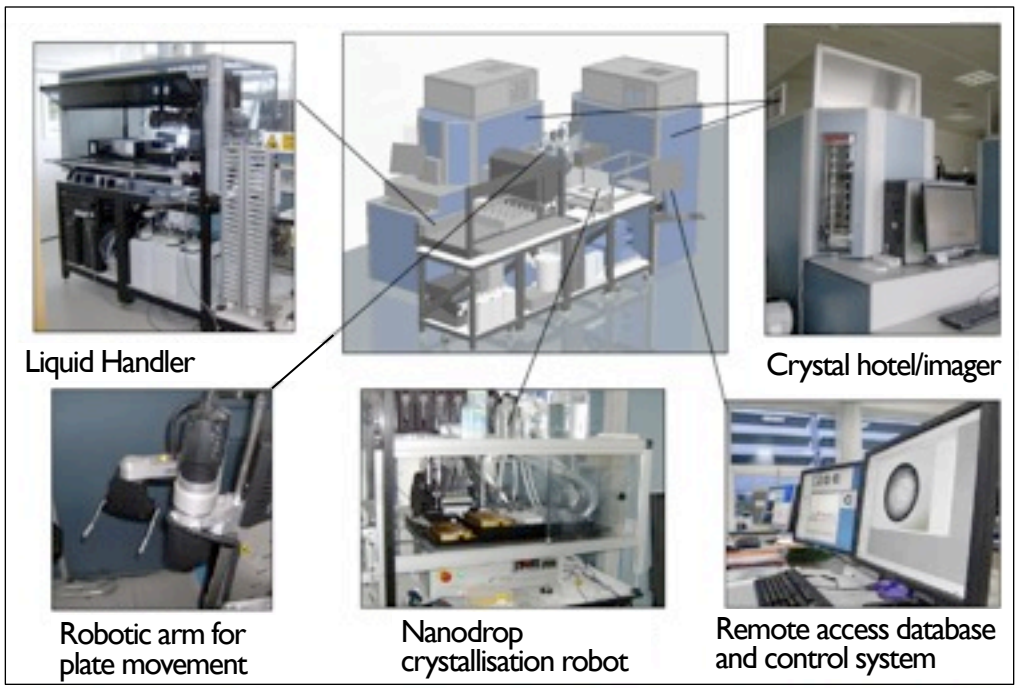
with 1ry antibody Control

Automated Crystal Optimisation at Diamond Membrane Protein Laboratory (MPL)

Plan of the MPL Facility



Fully integrated sitting-drop Rhombix crystallisation robot



Fluidigm Toparz microfluidics system

Cubic phase crystallization robot

PX scanner in-plate X-ray diffraction system

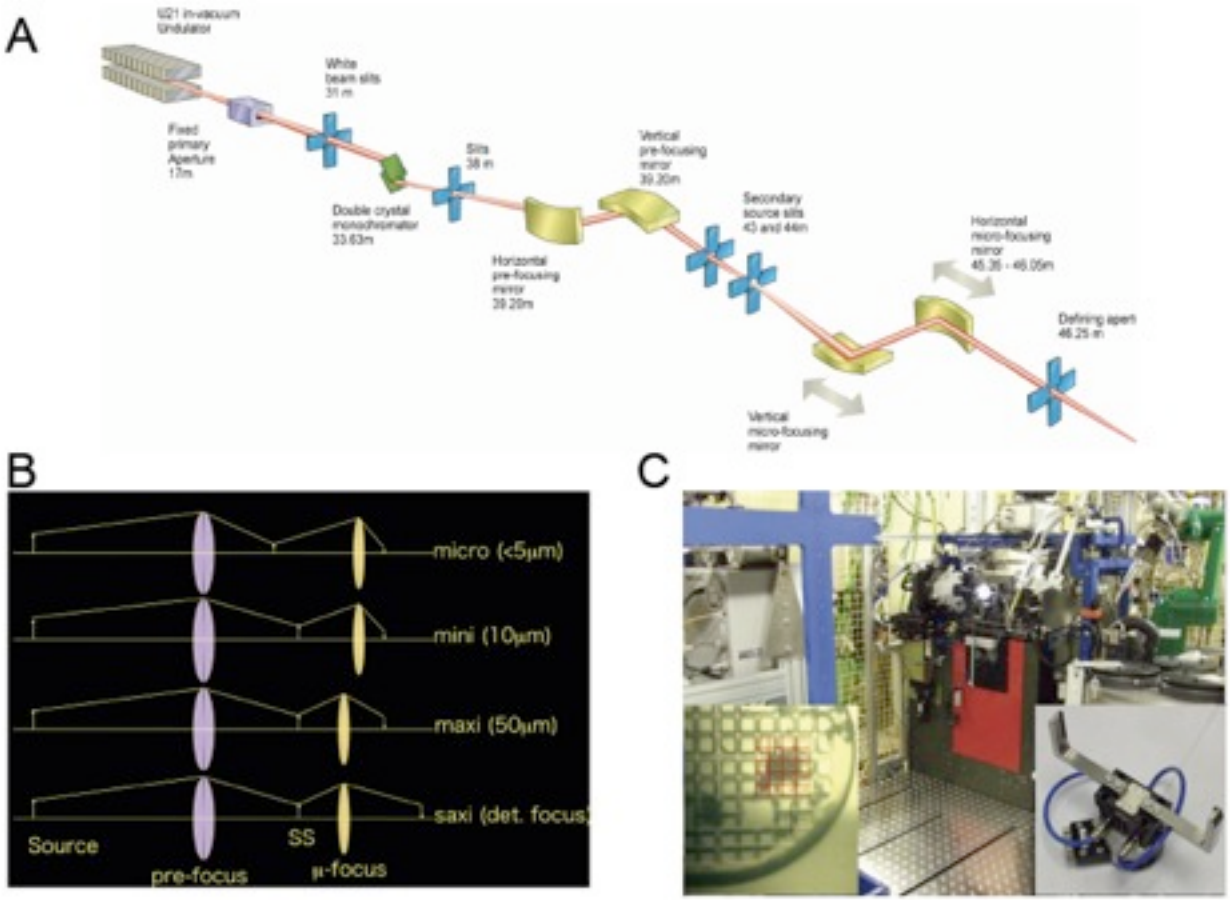
Diamond-MPL

- Funded by Wellcome Trust/ Japan Science and Technology Agency/BBSRC
- Imperial/Diamond joint project
- Methods development for automated membrane protein crystallisation and high quality X-ray data collection
- User training and research facility for membrane protein crystallisation and data collection

diamond

Imperial College London

X-ray Data Collection at the Diamond Microfocus Beamline I24



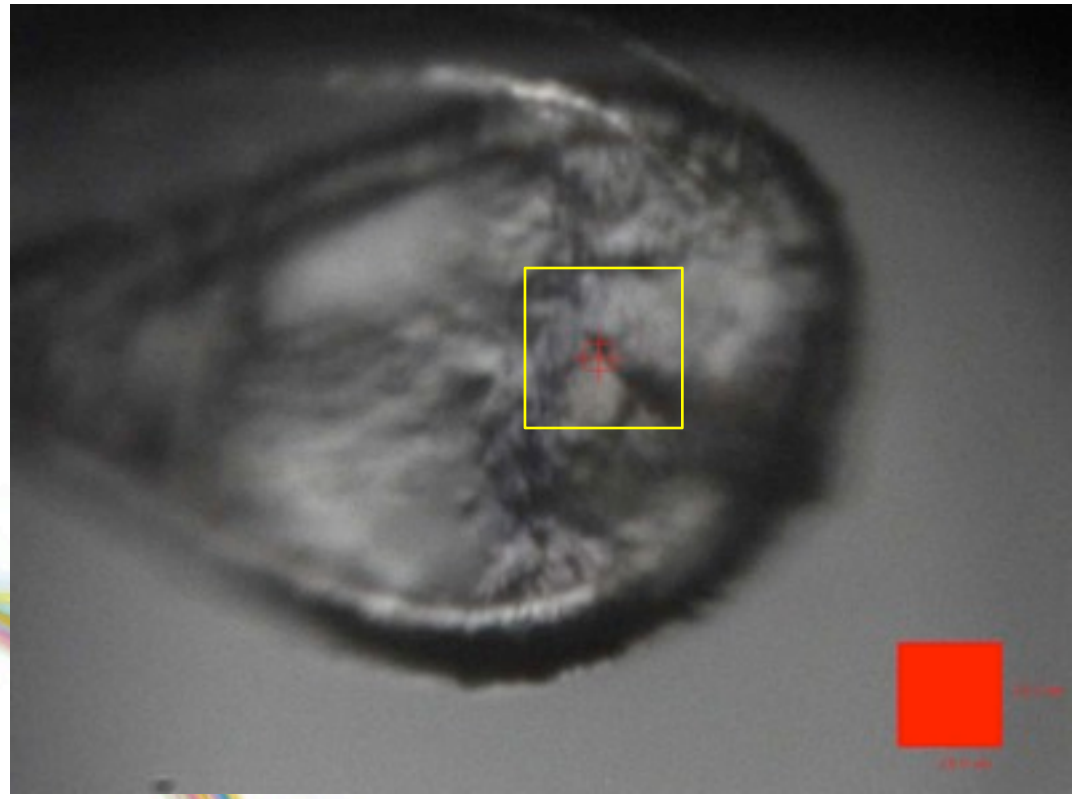
Beamline I24 at Diamond. A. Beamline optics. B. Beam focus options. C. Sample environment with Irelec CATS robotic sample changer, grid scanning system (left) and the plate gripper (right). D. Aperture array.

- Membrane protein crystals are often weakly diffracting, small and radiation sensitive.
- Diamond beamline I24 is designed to collect high quality data from micro crystals (5-50 μm).
- Developing a new crystal mounting system and software to complete a dataset from multiple crystals to avoid radiation damage.
- Collaboration with Dr. Evans, I24 principal beamline scientist

diamond

Adenosine A2a Receptor-Fab Complex Crystal

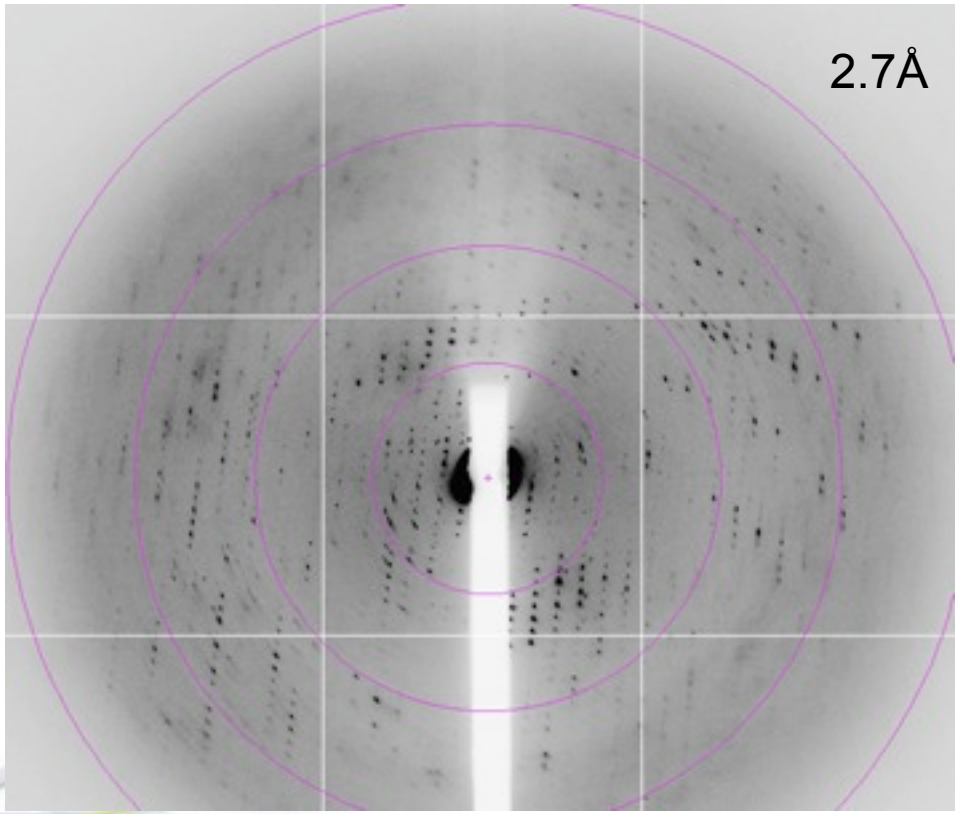
- Maximum resolution < 2.5Å
- Difficult to freeze. A large mosaicity.

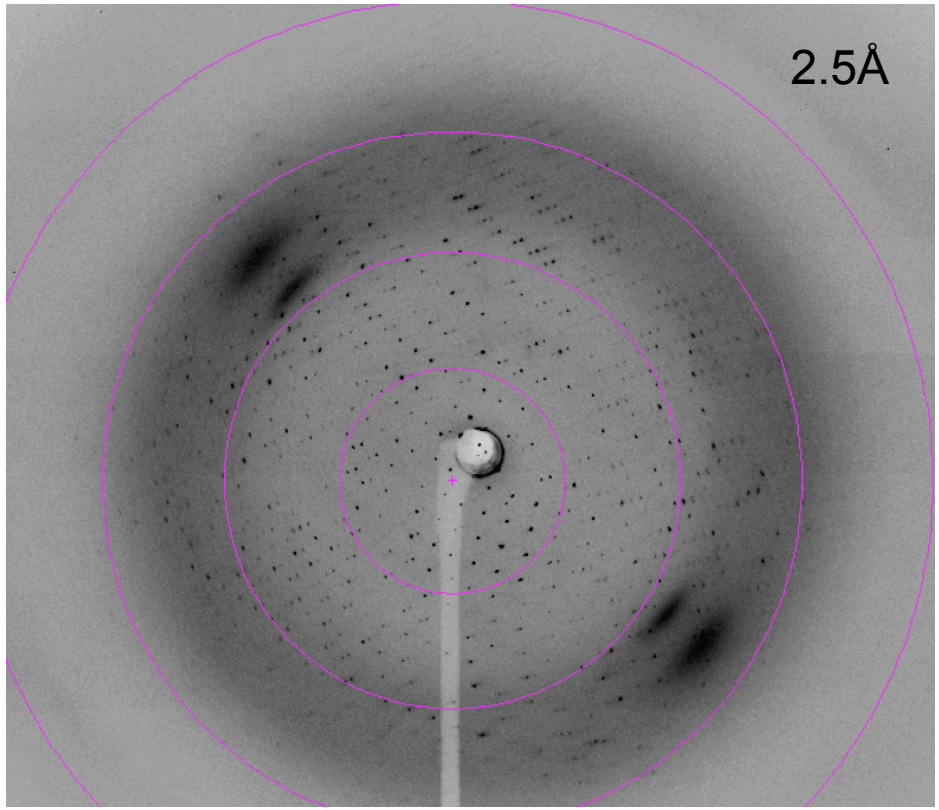


Standard size Beam (50µ x 50µ)

Micro Beam (10µ x 10µ)

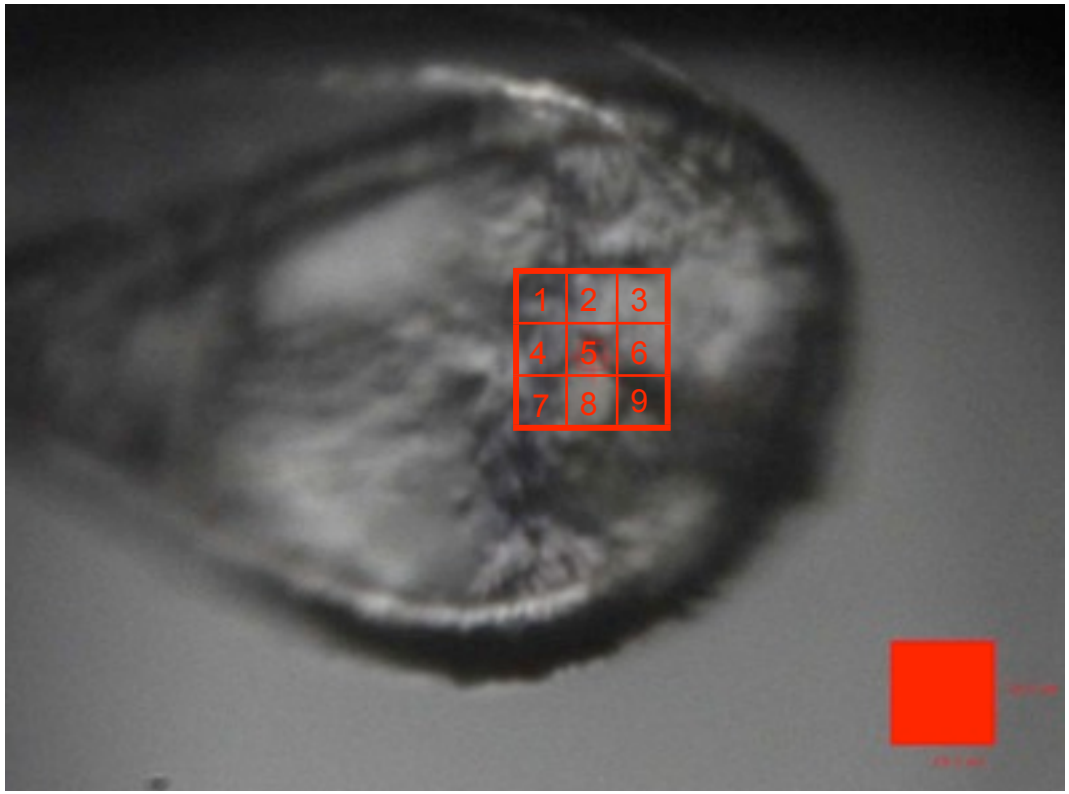
Standard Size Beam vs Micro Beam





Standard size beam (IO4: 50µ x 50µ, 50% transmission, 5 sec)

Micro Beam (I24: 10µ x 10µ, 30% transmission, 1sec)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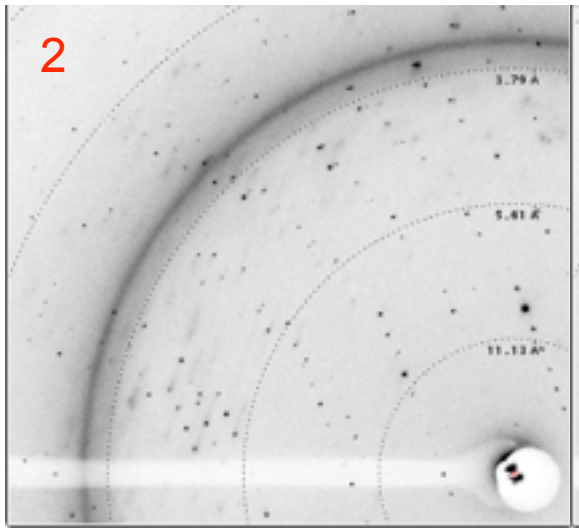
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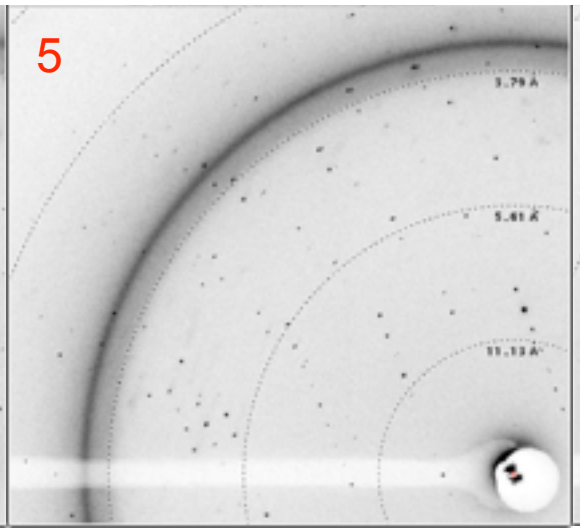
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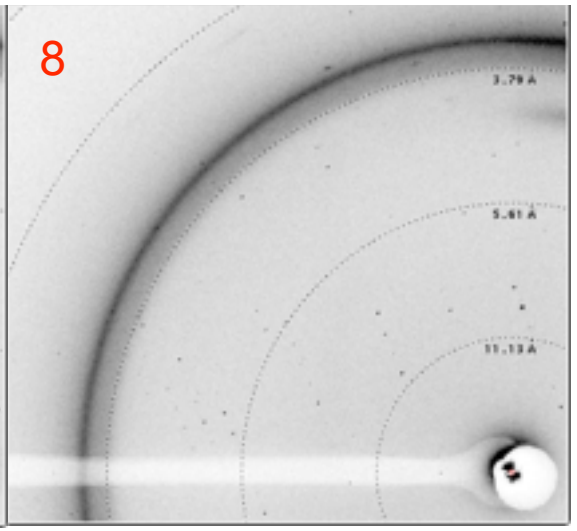
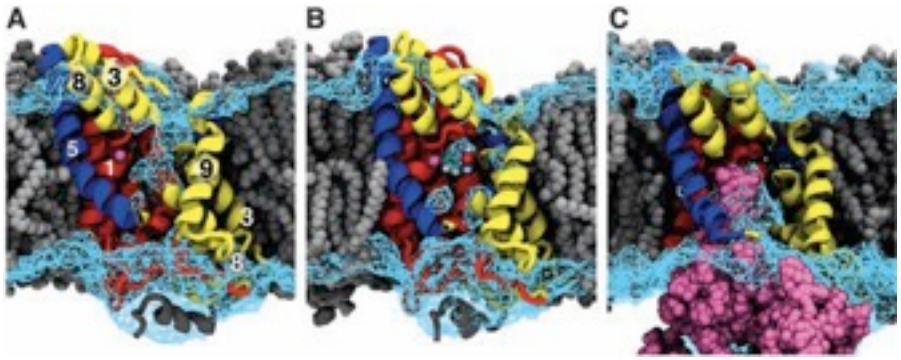
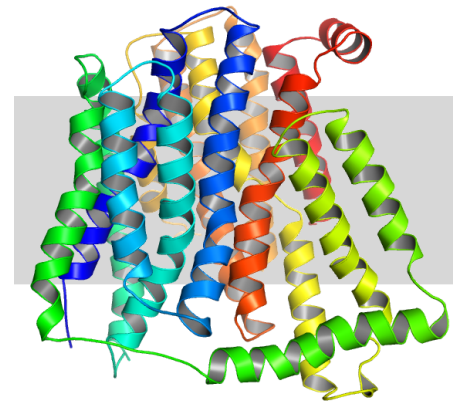

8

Membrane transporters & respiratory enzymes

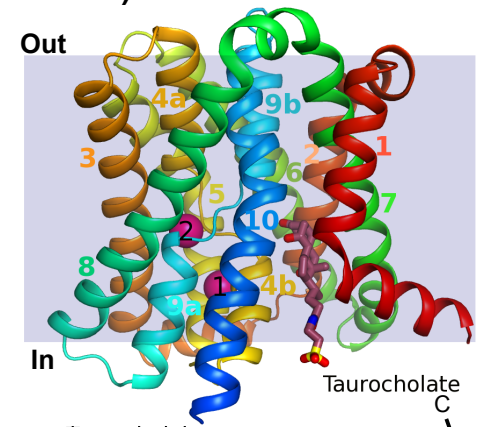
Mhp1 hydantoin transporter (Science 2008, Science 2010)



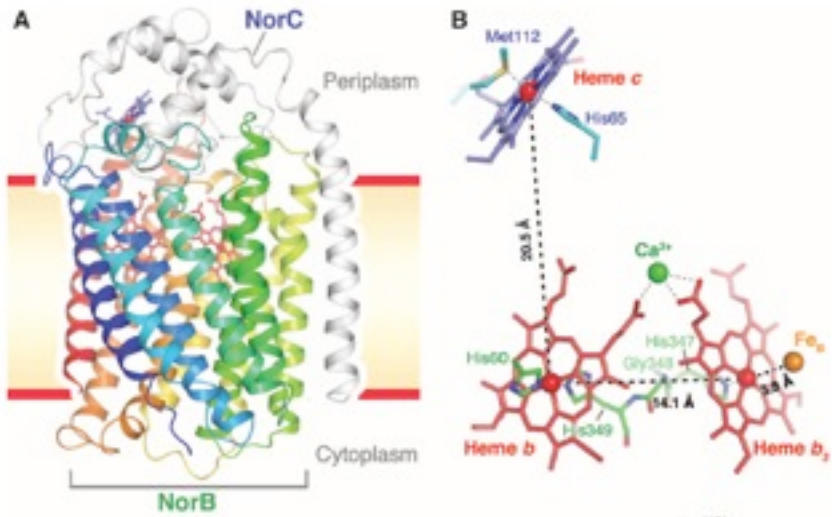
Oligopeptide transporter (EMBO J. 2010)



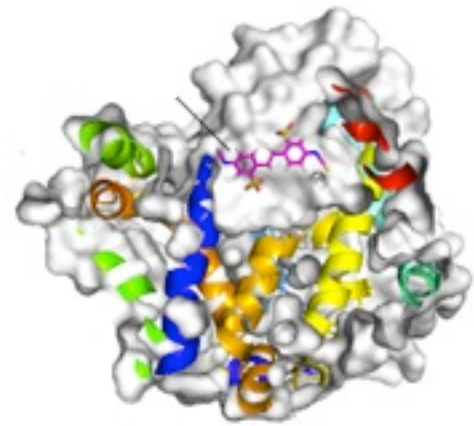
ASBT bile acid transporter (Nature 2011)

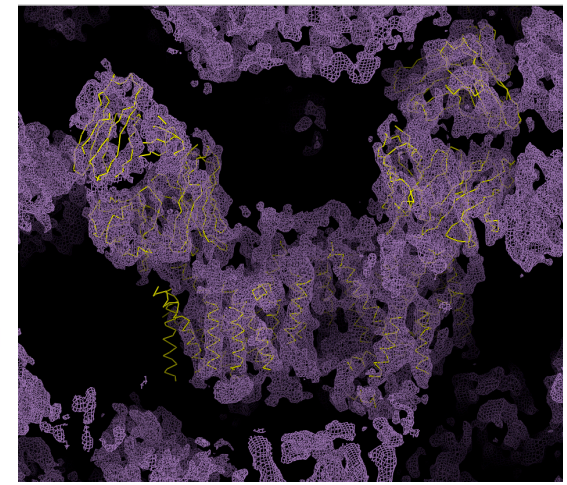


NO reductase- Fab complex (Science 2010)



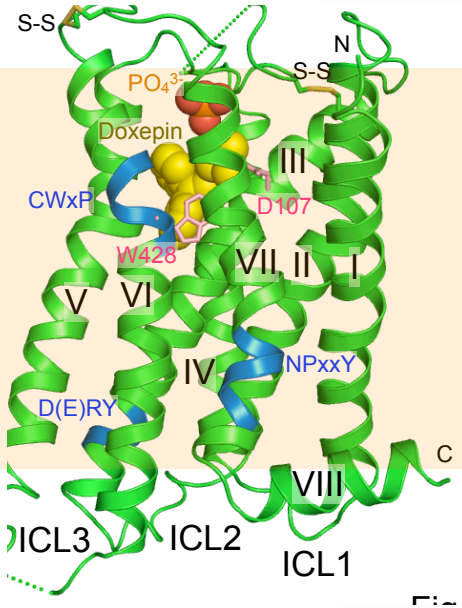
Human anion exchanger - Fab complex

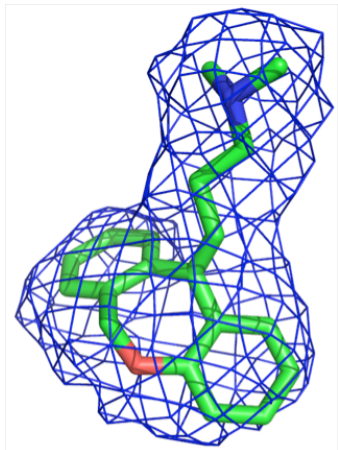




G-protein coupled receptors

Human Histamine H_1R (Nature 2011)





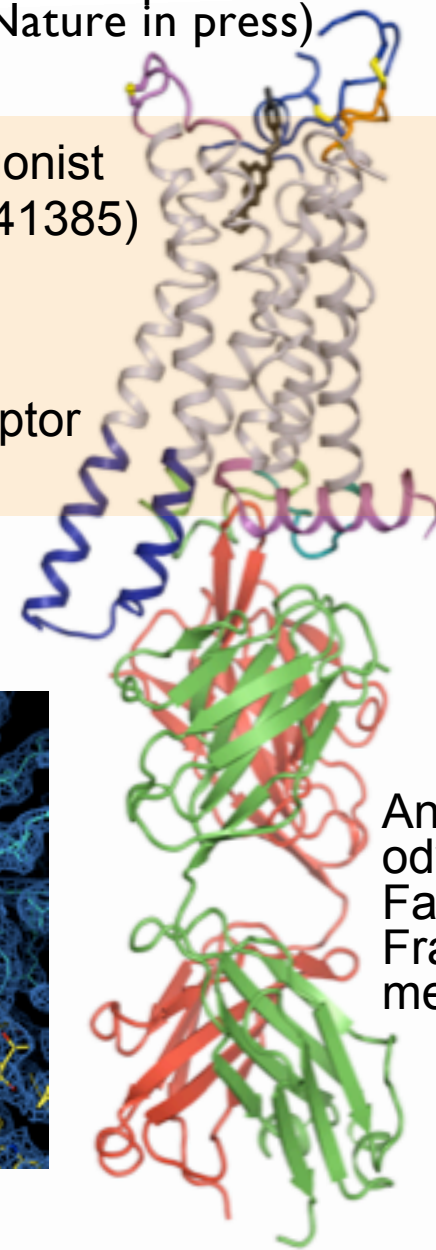
Antihistamine (Doxepin)

Human Adenosine A2aR-Fab complex (Nature in press)

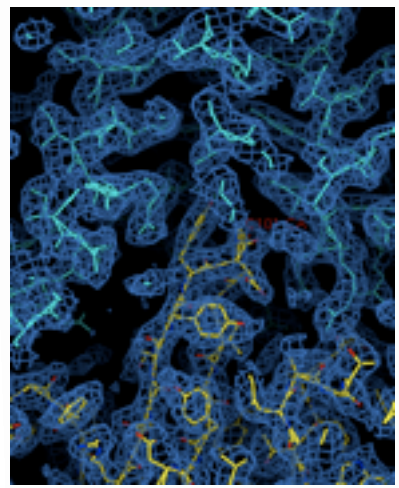
Antagonist (ZM241385)

A2a receptor

i3-loop

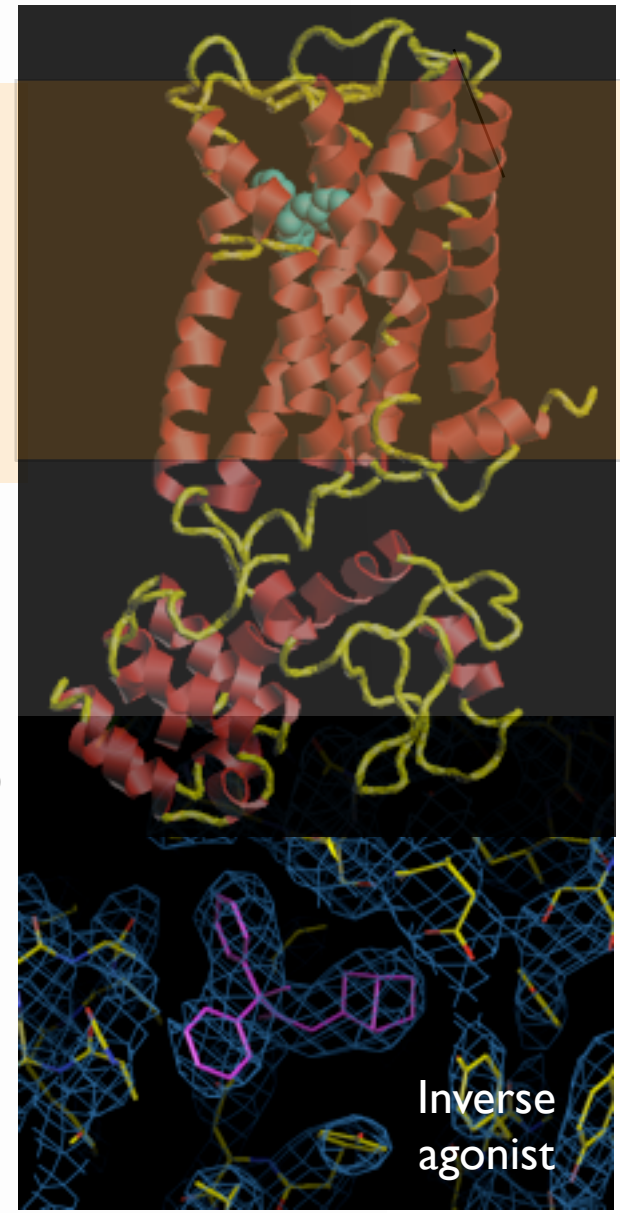


Antibody Fab Fragment



CDR-H3

Human Muscarinic acetylcholine M_2R (Nature in press)



Histamine H_1 receptor

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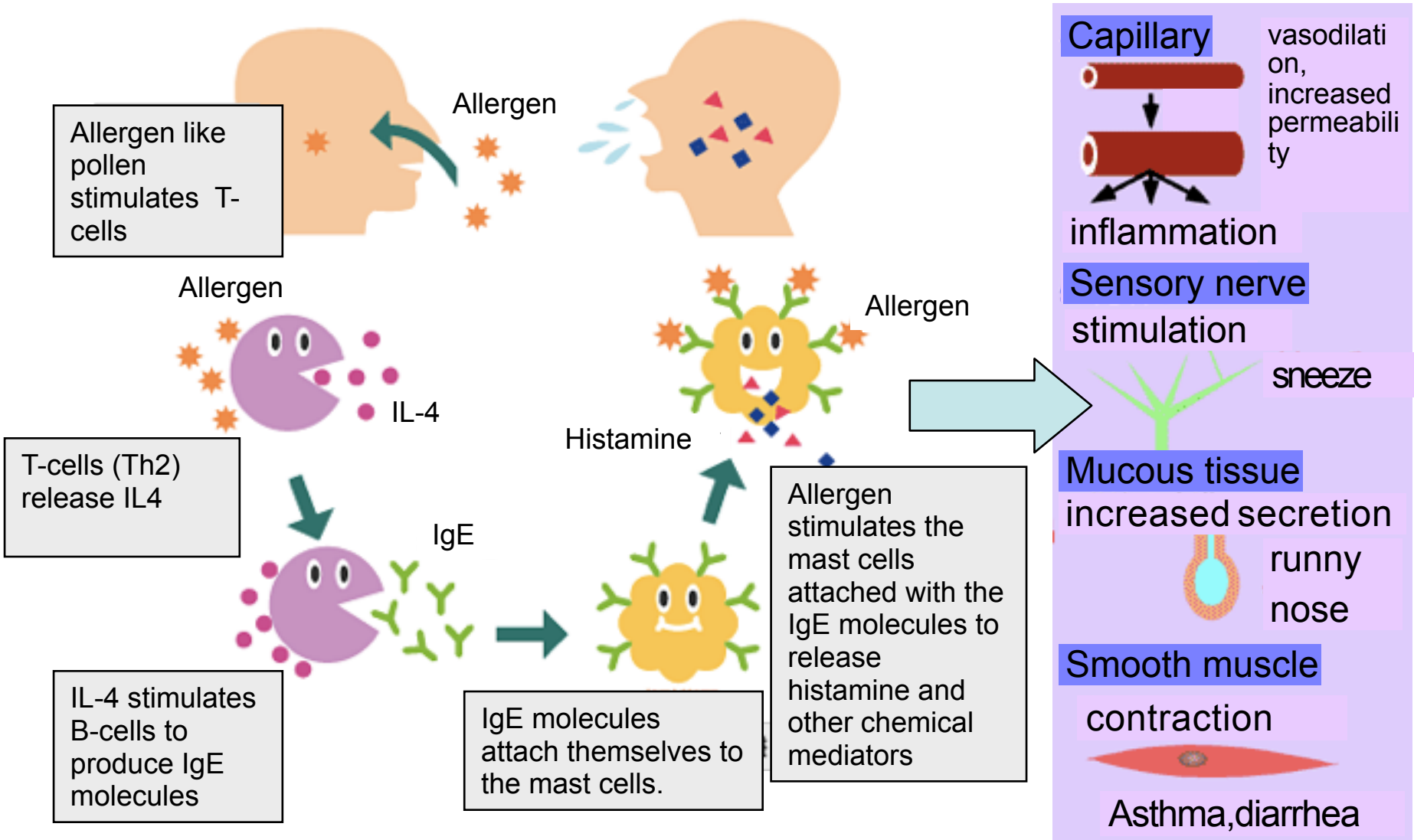
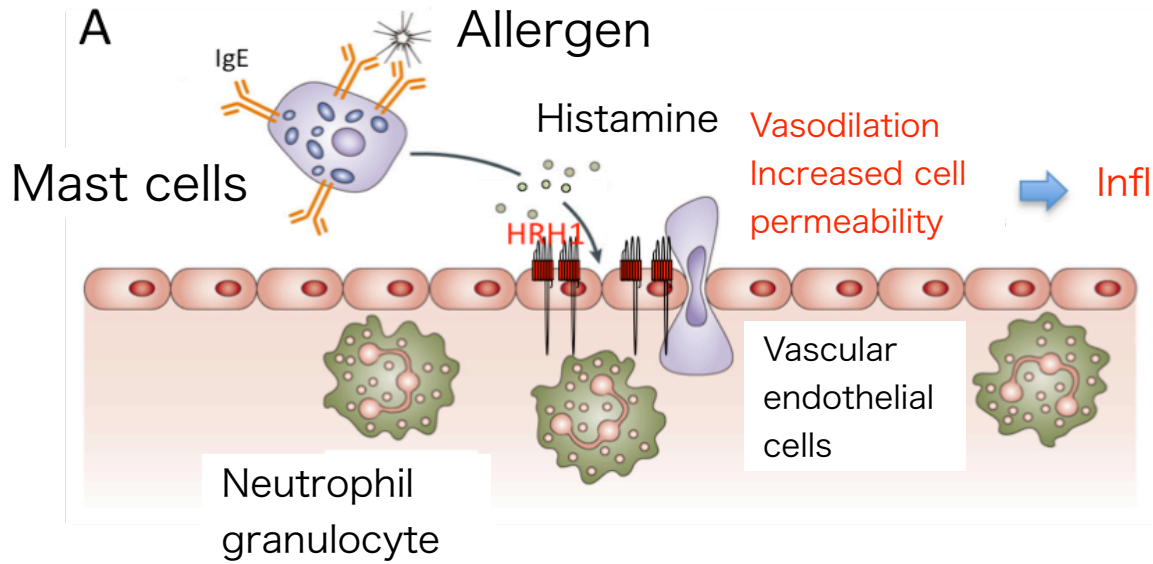
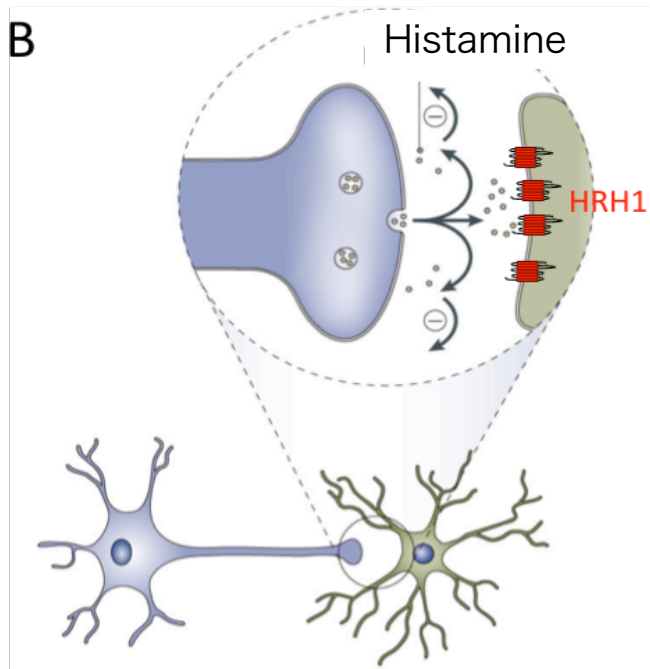
Allergic reactions and histamine



Histamine H_1 Receptor (H_1R)

- Expressed in smooth muscles, on vascular endothelial cells, in the heart.
- Cause vasodilation, along with increased cell permeability when activated.
- In CNS, involved in histaminergic neuron activation.



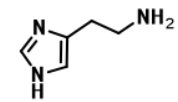


CNS
(histaminergic neurons)

Histamine H_1 Receptor Antagonists (Inverse agonists, Antihistamines)

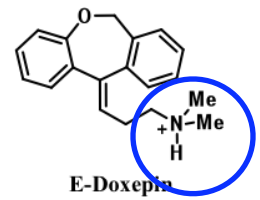
- Inhibit H_1R to alleviate allergic reactions.
- 1st gen. drugs show side effects (sedation, dry mouth, arrhythmia) because of BBB permeability and low H_1R selectivity.
- 2nd gen. drugs with a carboxylic moiety show reduced BBB permeability and higher H_1R selectivity.

Histamine



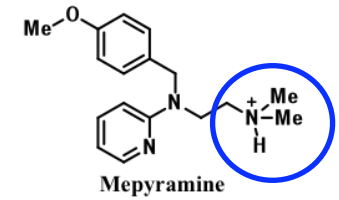
Histamine

1st generation antihistamines



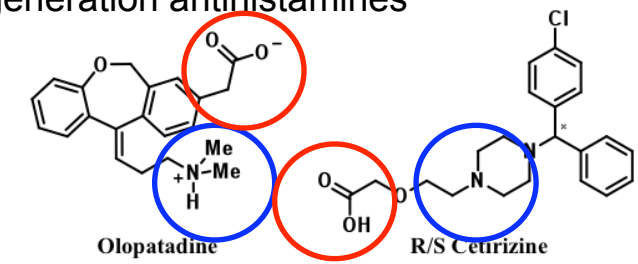
E-Doxepin

Z-Doxepin

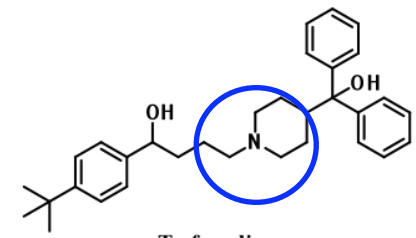


Mepyramine

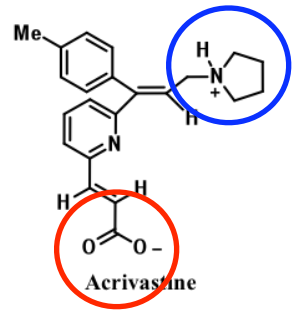
2nd generation antihistamines



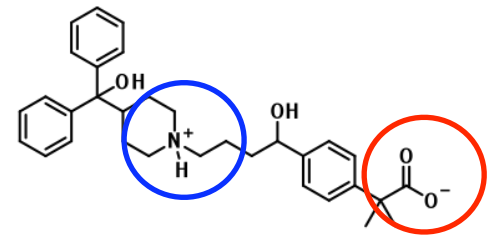
Olopatadine R/S Cetirizine



Terfenadine

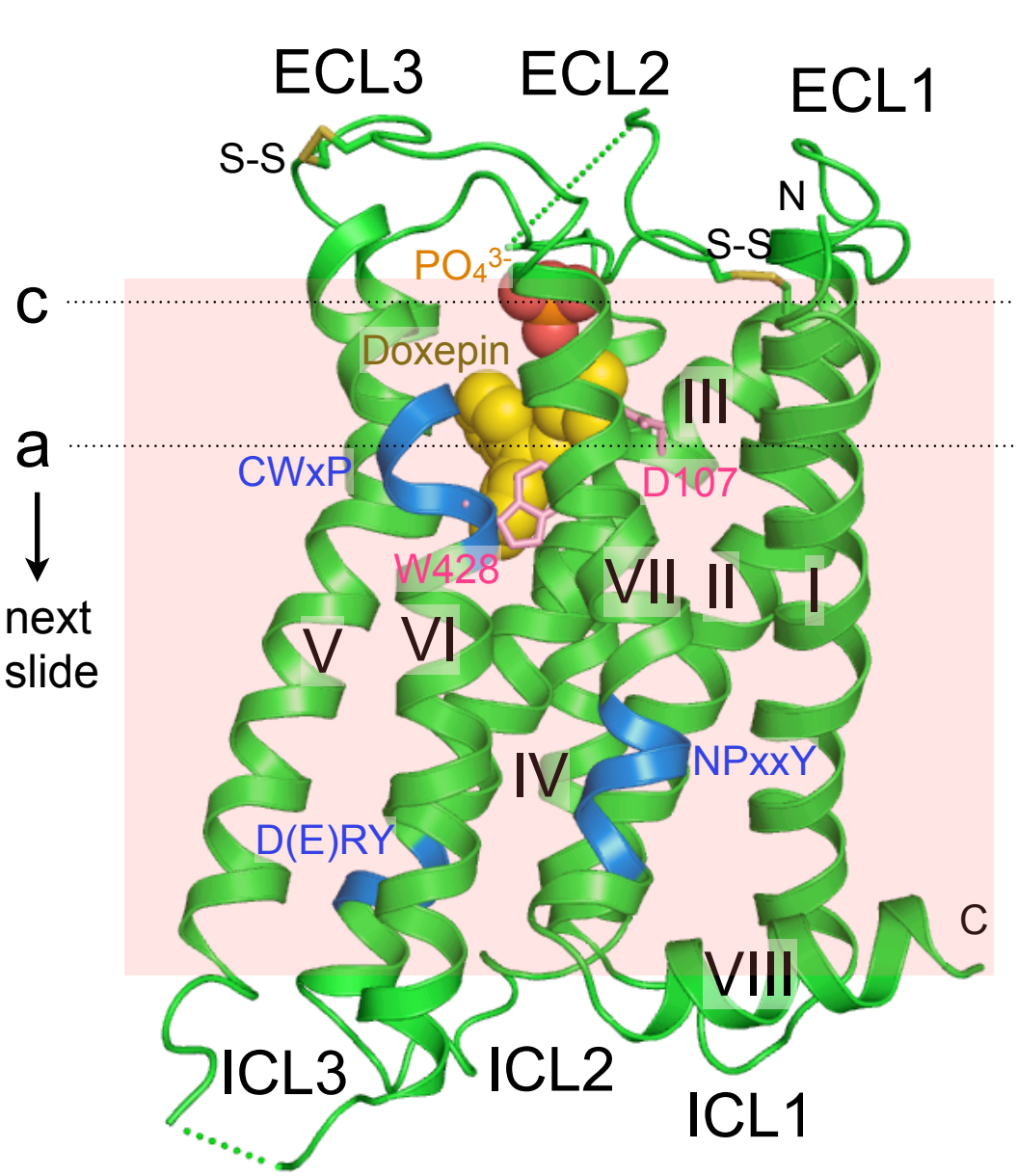


Acrivastine

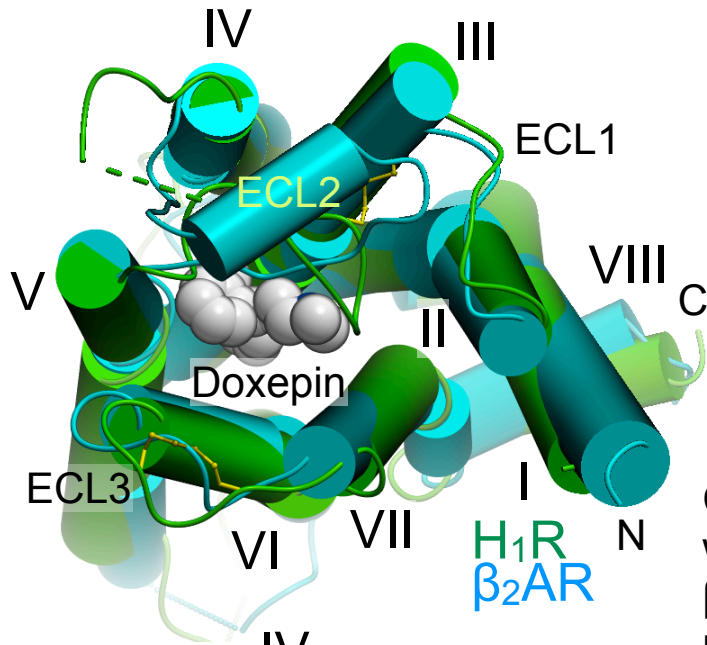


Fexofenadine

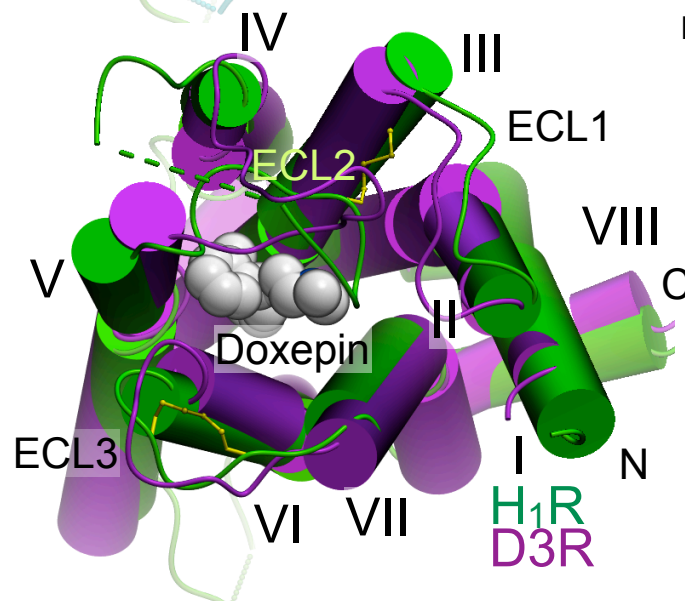
Structure of H_1R



Histamine H_1R



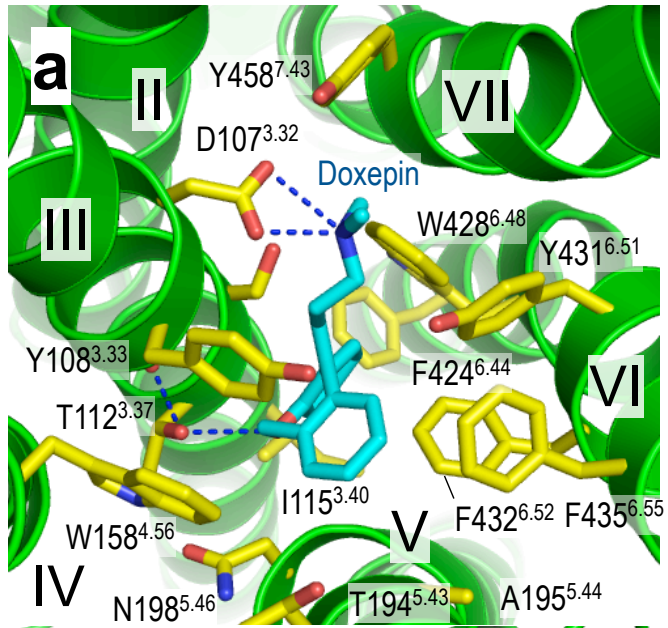
Comparison with β_2-adrenergic receptor (β_2 AR)

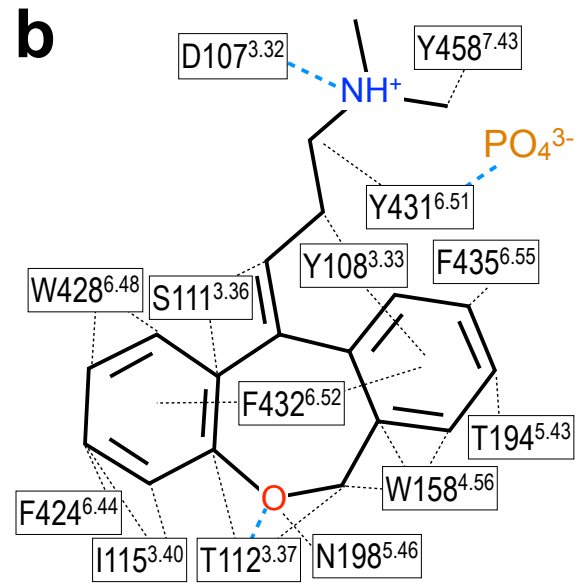


Comparison with dopamine D_3 receptor (D3R)

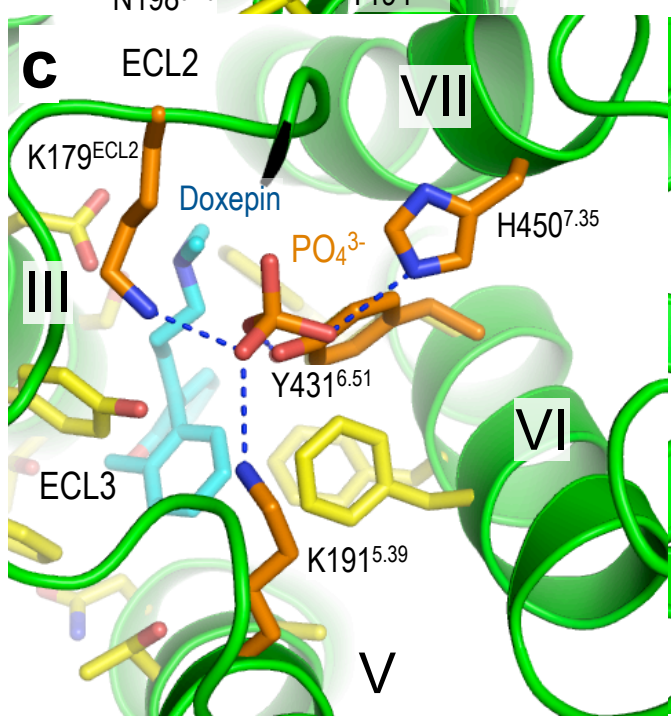
Binding interactions of doxepin

Doxepin binding site (1st gen. antihistamine)

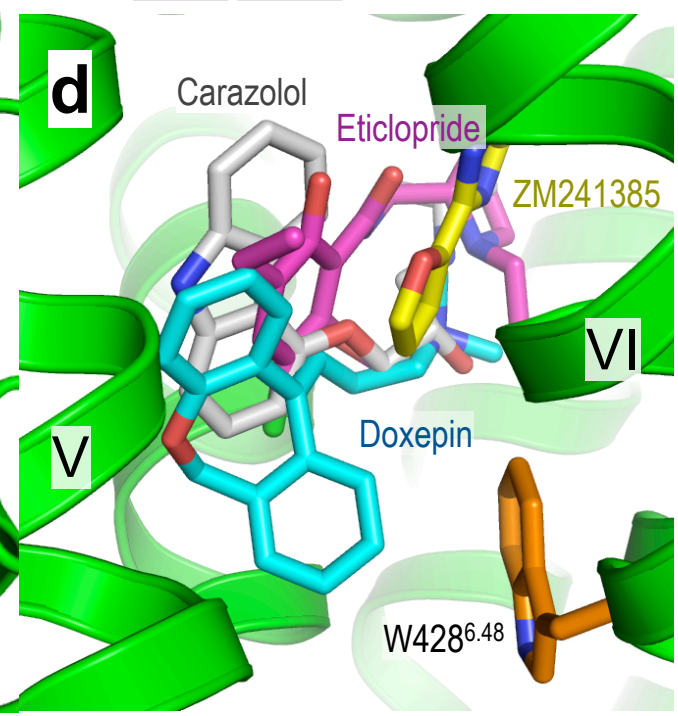




Doxepin binding interactions



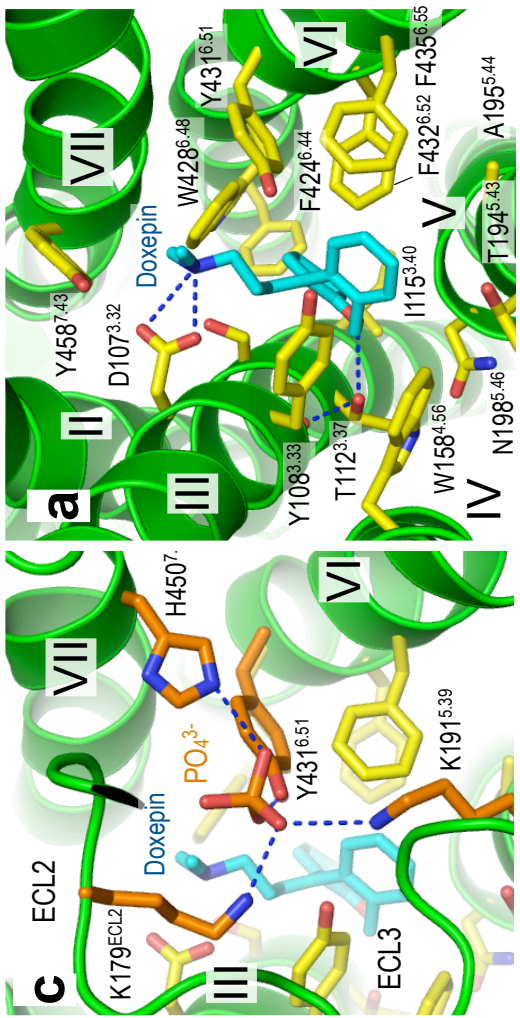
Anion-binding region with PO_4^{3-}



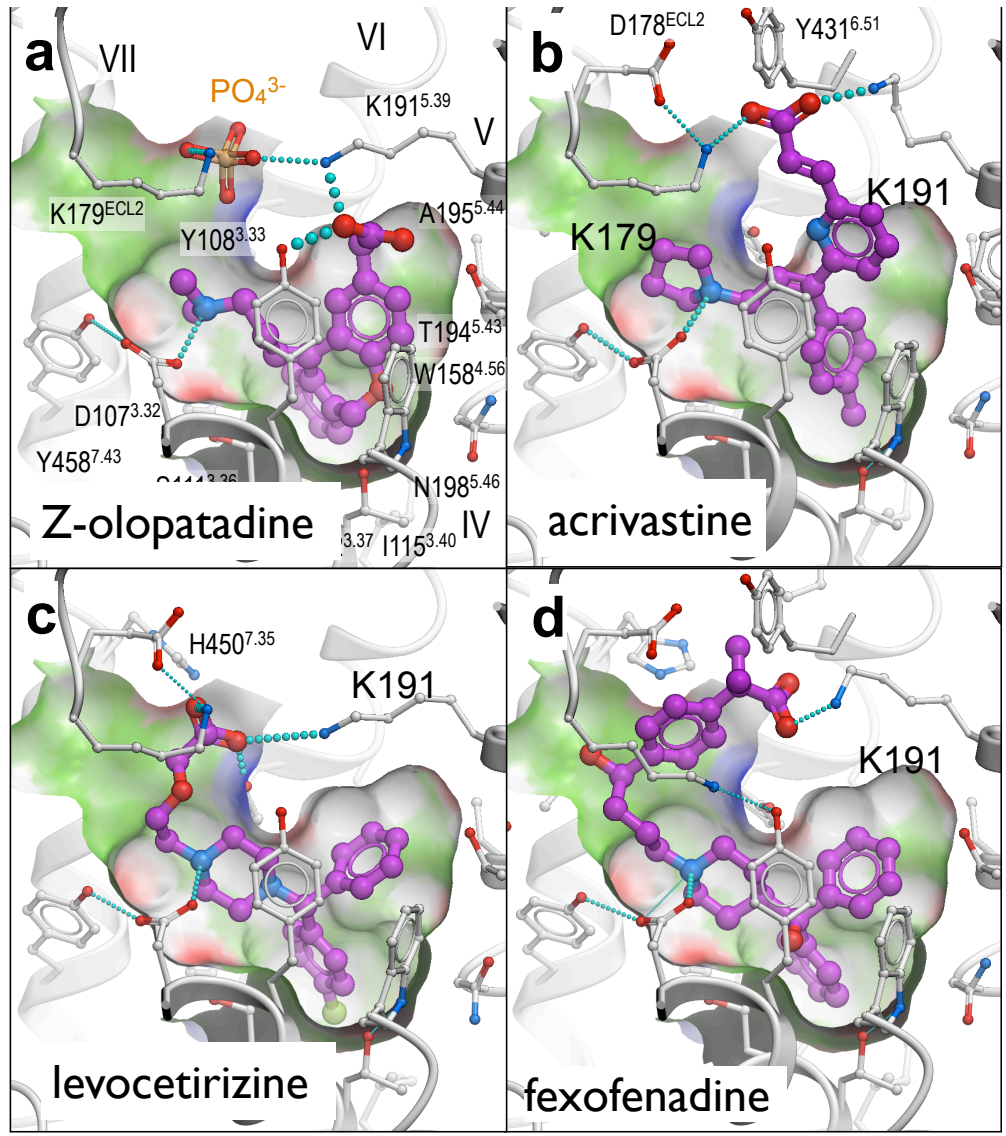
Ligand-binding positions in GPCRs

Interactions of 2nd generation antihistamines with H_1R

1st gen. antihistamine (doxepin)



2nd gen. antihistamines (models)



Residues in PO_4^{3-} binding site, Lys 179, Lys 191, Tyr 431 are unique to H_1R.

They are involved in the 2nd gen. antihistamine binding.

histamine H_1	K191
histamine H_2	G
serotonin	T/S
acetylcholine	T/S
α adrenaline	V
β adrenaline	A
D3 dopamine	V

Action mechanisms of 1st and 2nd generation antihistamines