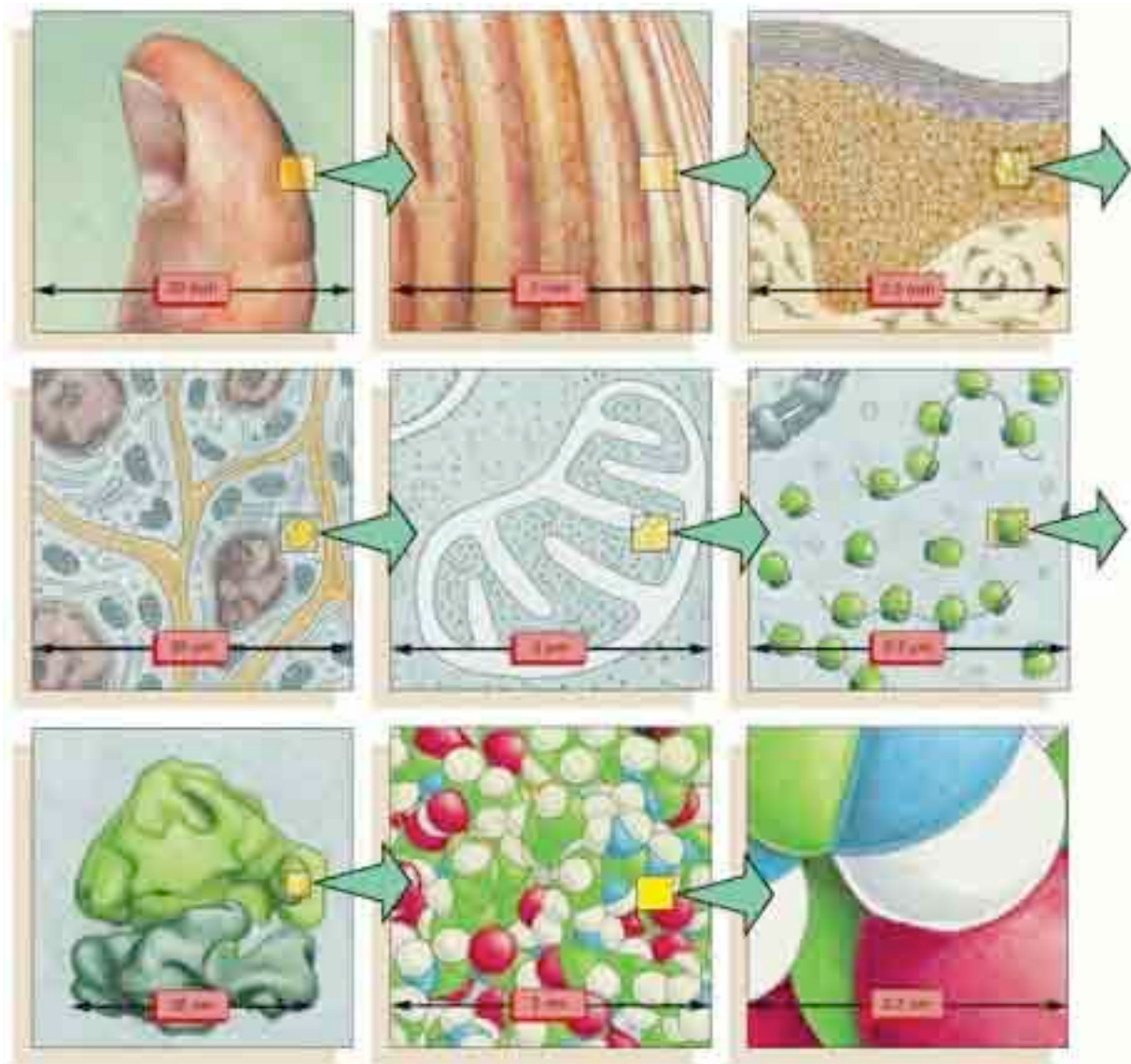


X-ray Protein Crystallography on Membrane Proteins

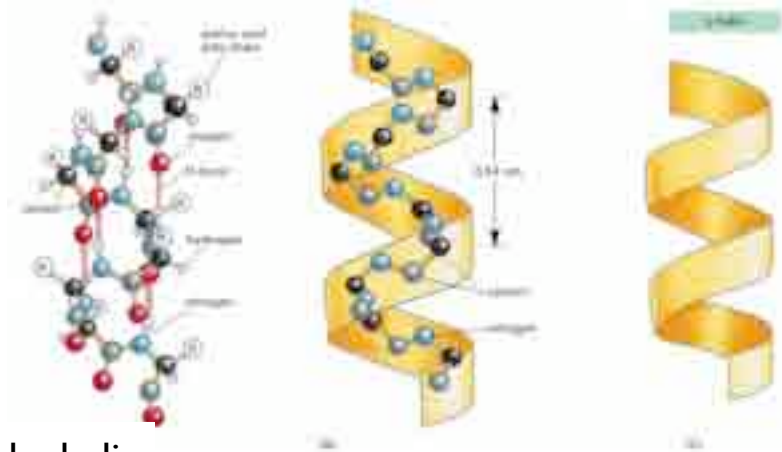
Riken Harima Institute/Kyoto
University

So Iwata

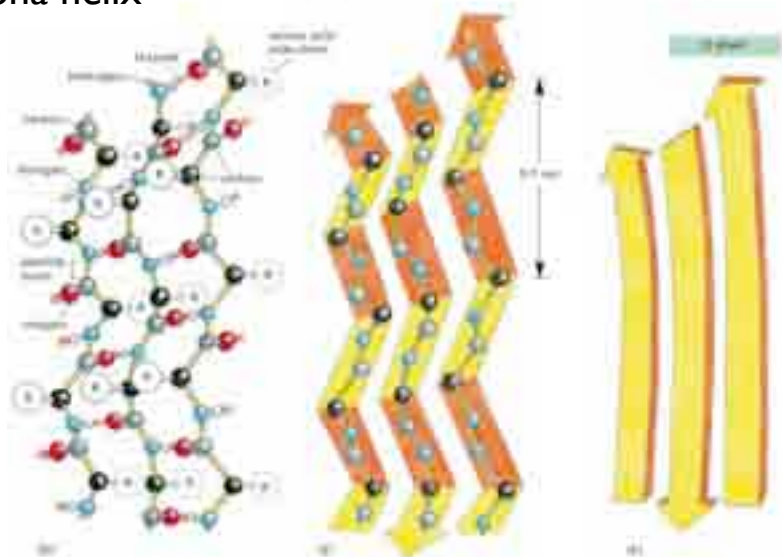
All Proteins in Our Body Have Regular 3D Structures at the Atomic Level



Structural elements of proteins

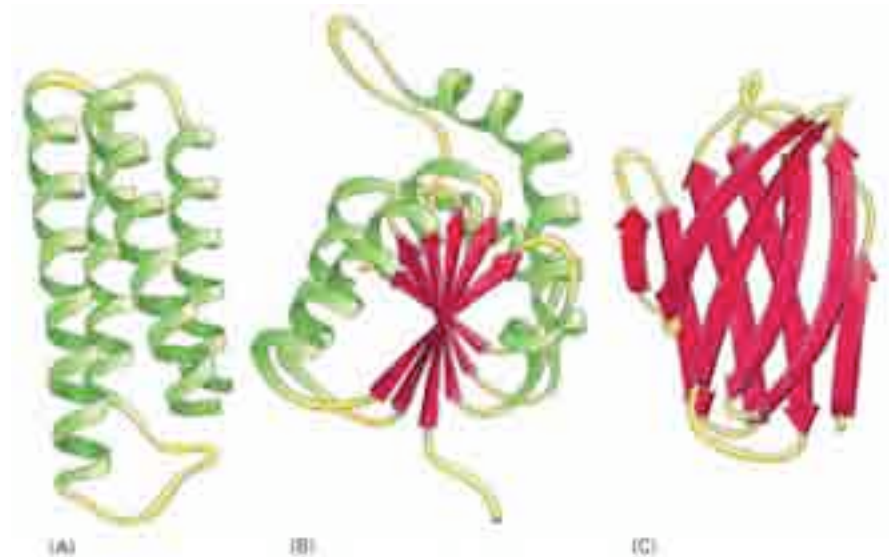


Alpha helix



beta sheet

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.



Example of proteins

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

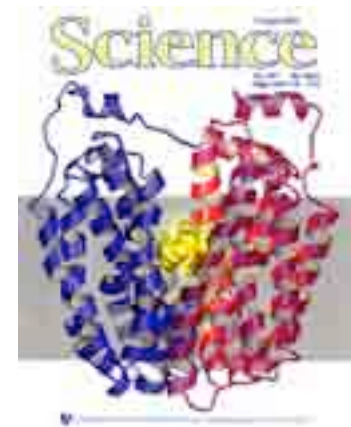
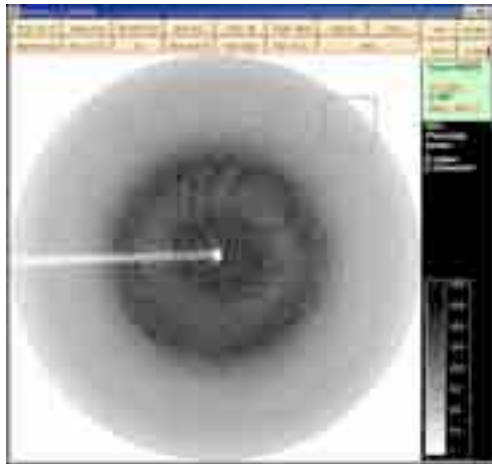


DNA Cloning

Protein Expression

Purification

Crystallization

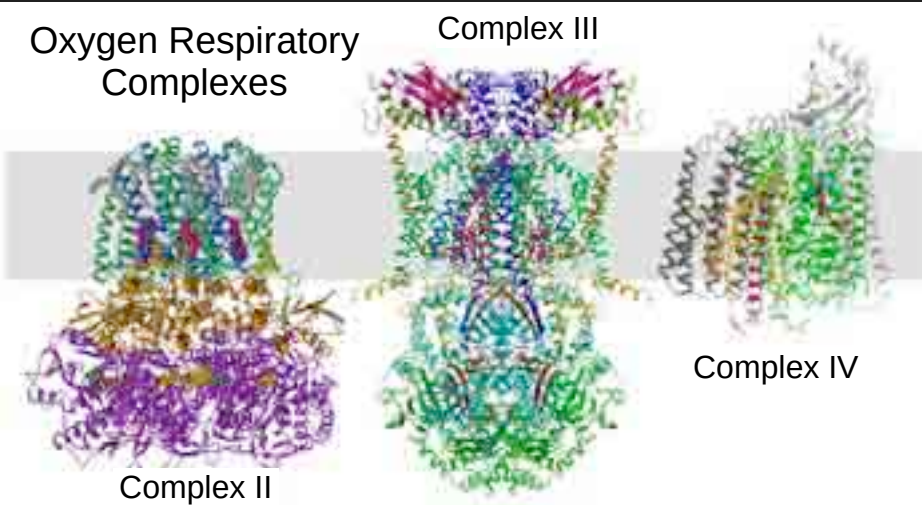


X-ray Data Collection

Phasing & Electron Density Map Structure Model

Membrane Protein Structures Solved in our Group

Oxygen Respiratory Complexes

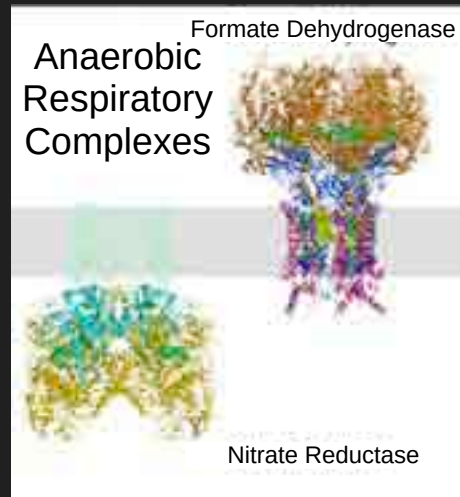


Complex II: Science 299 2003

Complex III: Science 281, 1998, N. Engl. J. Med. 341, 1999

Complex IV: Nature 376, 1995, Nature Str. Biol. 7, 2000

Anaerobic Respiratory Complexes

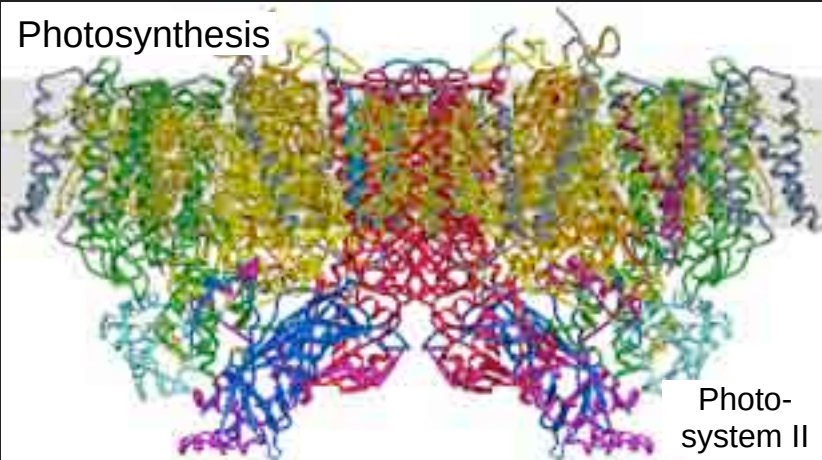


Formate Dehydrogenase: Science 295 2002

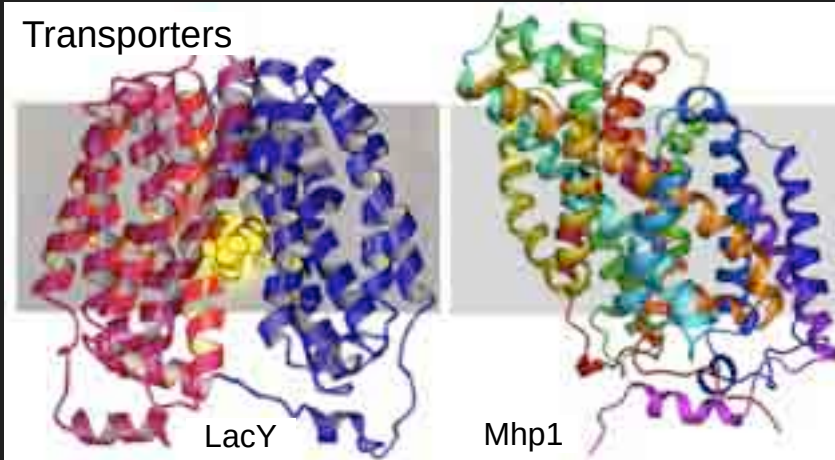
Nitrate Reductase: Structure 12, 2004

Polysulfide reductase: Nat. Str. Mol. Biol. 15, 2008

Photosynthesis



Transporters



Photosystem II: Science 299 2003

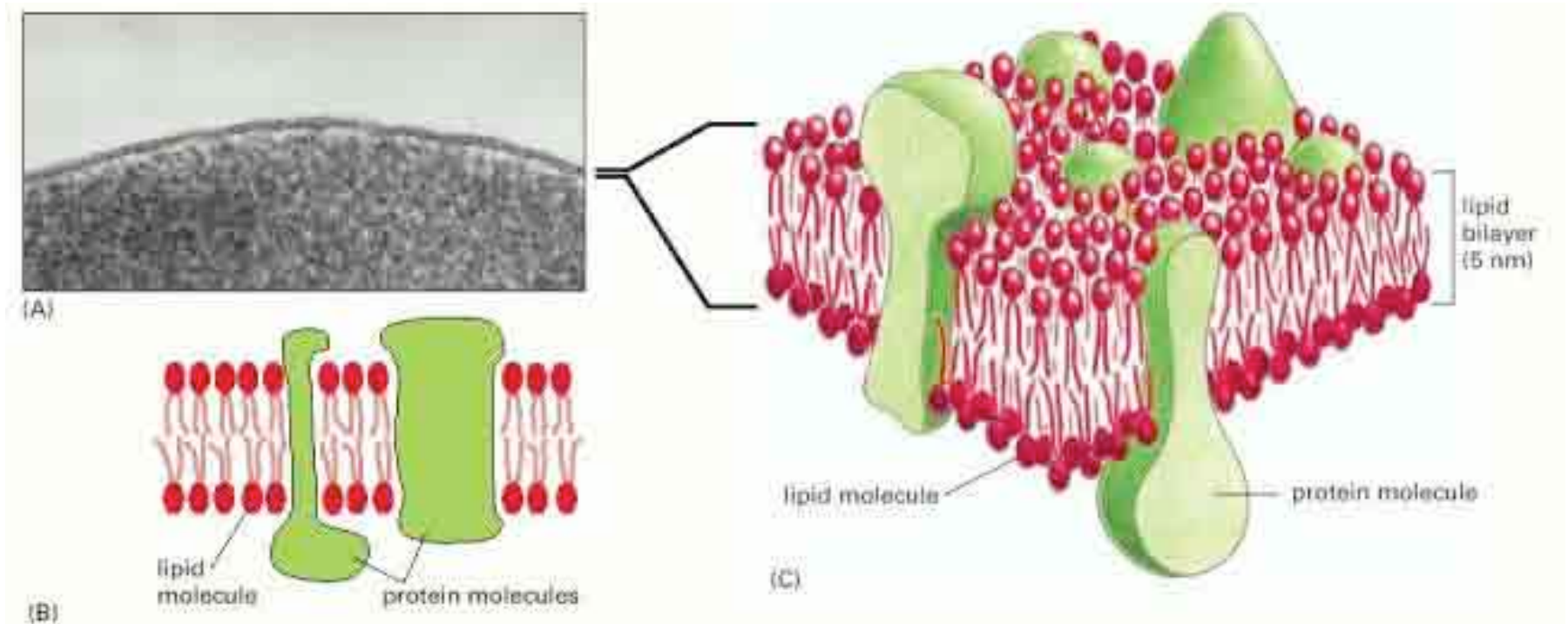
LacY: Science 301, 2003, EMBO J. 25, 2006, PNAS 104, 2007

Mhp1: Science 322, 2008

Membrane Proteins

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

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



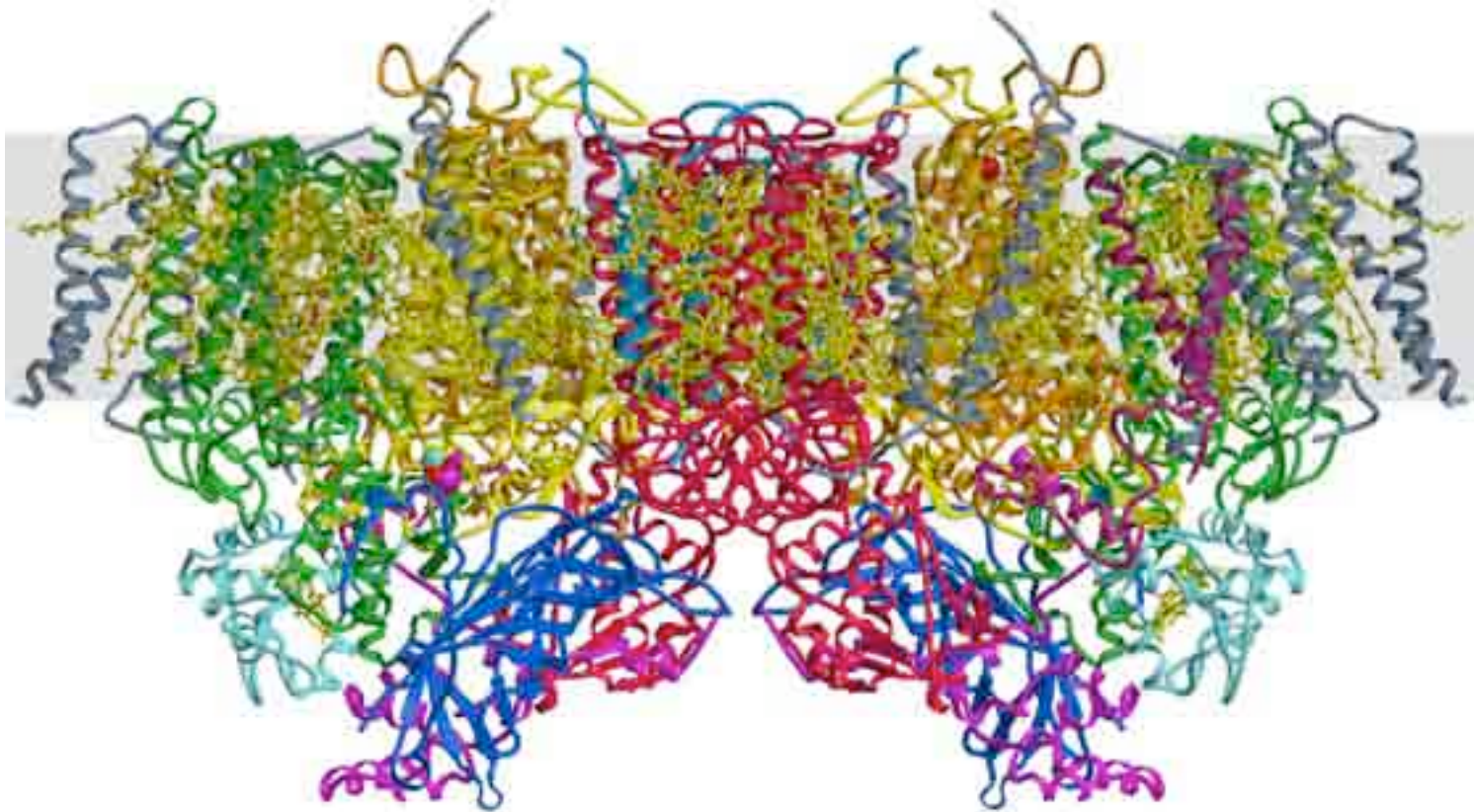
Example Membrane Protein Structures from Our Group: Lactose Permease (Sugar Transporter)

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).

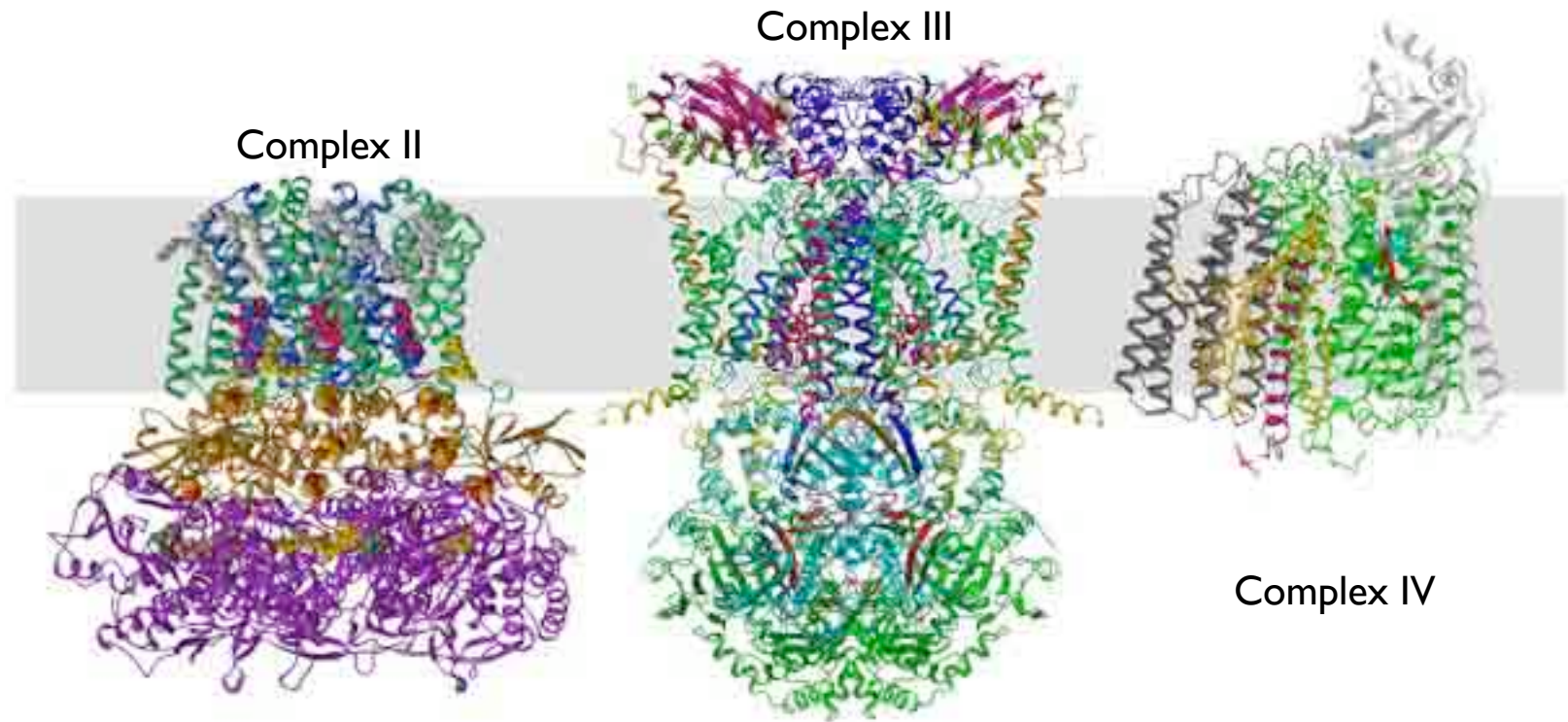


Oxygen Respiratory Enzymes

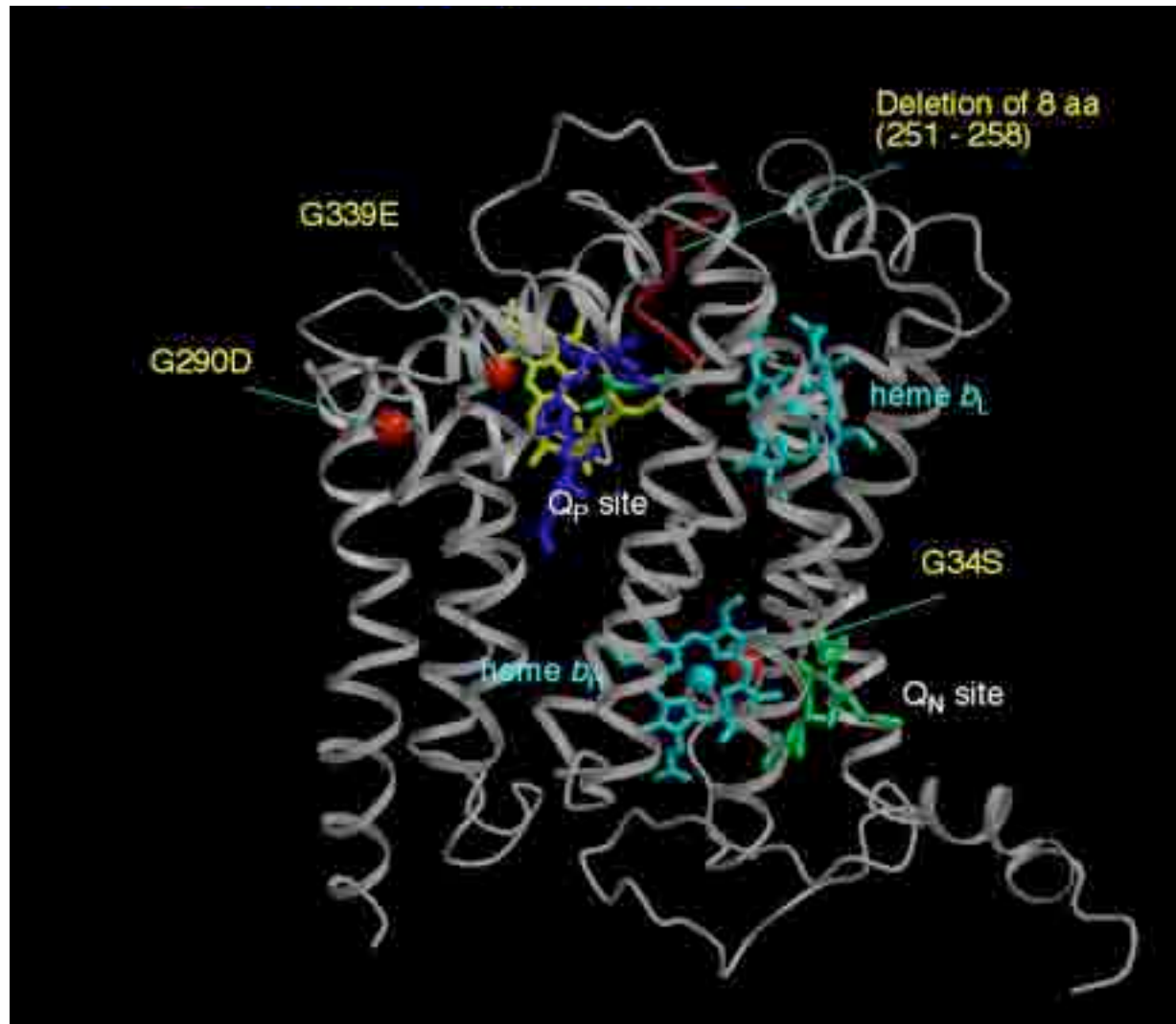
Complex II: Mutations Cause Tumors and Premature Aging (Yankovskaya et al., Science 299 2003).

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 O₂ 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



Reduce Cholesterol Level

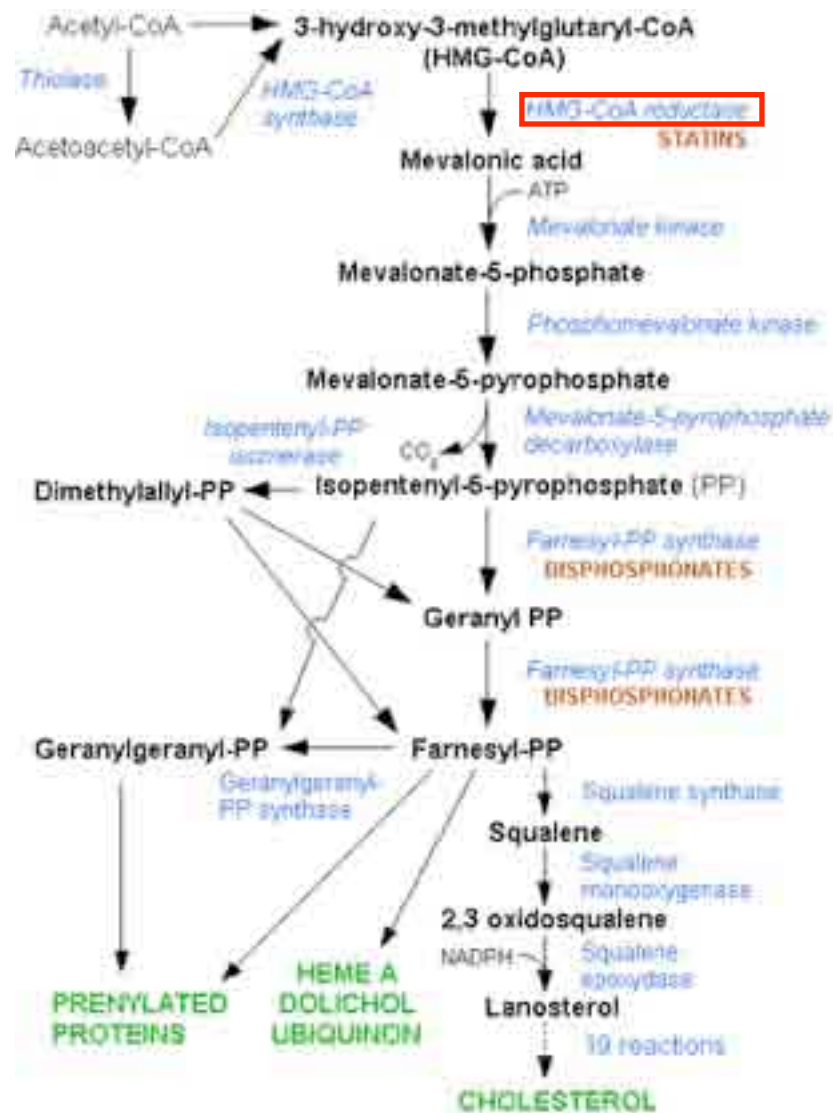
Simvastatin (statin): HMG CoA Reductase Blocker

Lower Blood Pressure

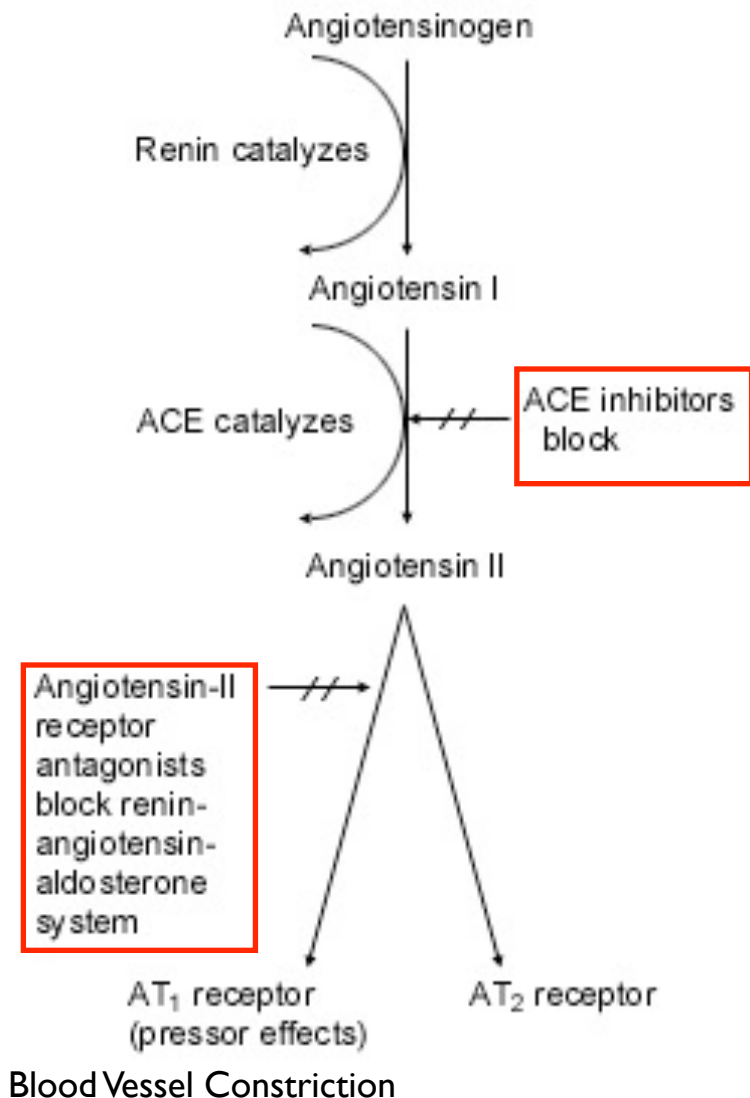
Cozaar: Angiotensin II Receptor Antagonist

Lisinopril:
Angiotensin
Converting Enzyme
(ACE) Blocker

Cholesterol Synthesis (Mevalonate Pathway)

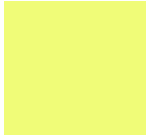


Blood Pressure Control by Angiotensin

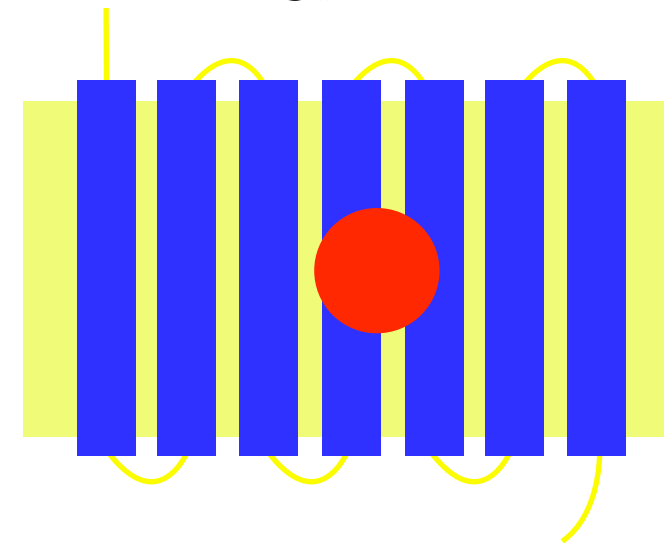
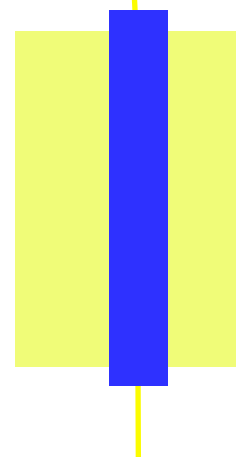
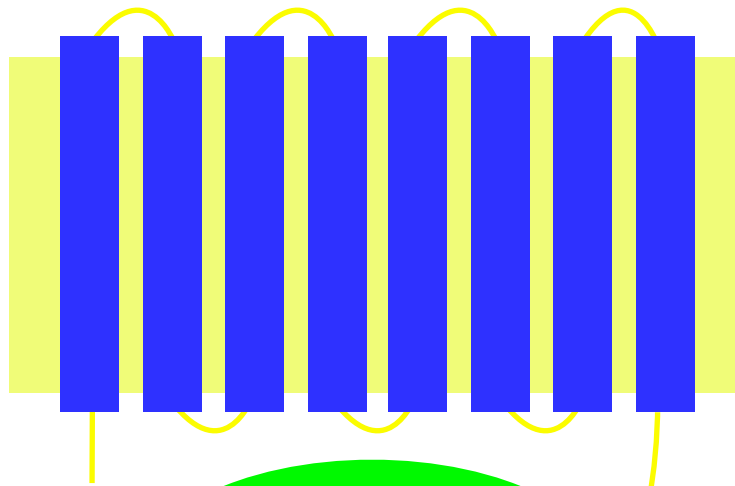


All Three Targets Are Membrane Proteins

 Ligands or Drugs

 Membrane

 Trans-membrane helix



HMG CoA Reductase

Angiotensin
Converting
Enzyme (ACE)

**Angiotensin II
receptor
(GPCR)**

Here Comes Another Problem...



Before (2003)

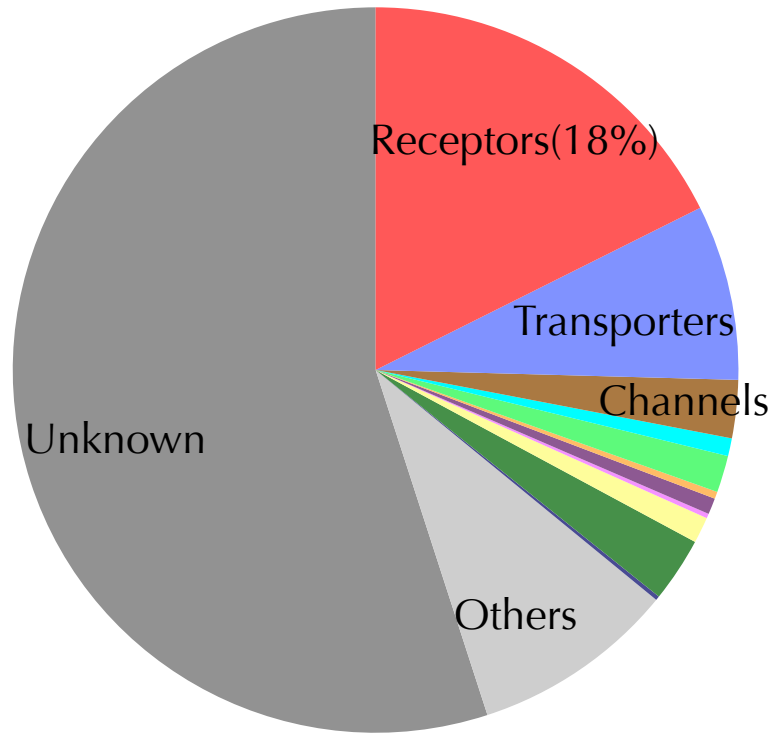


After (2006)

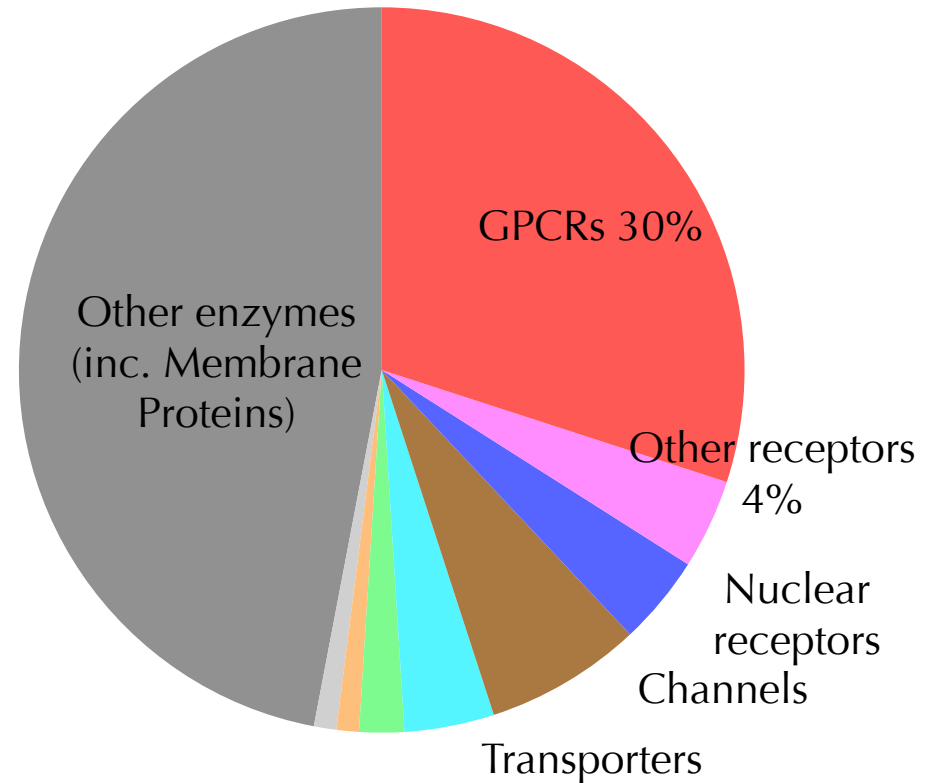
Androgen
receptor
in follicle
cells



Membrane Proteins are Major Drug Targets



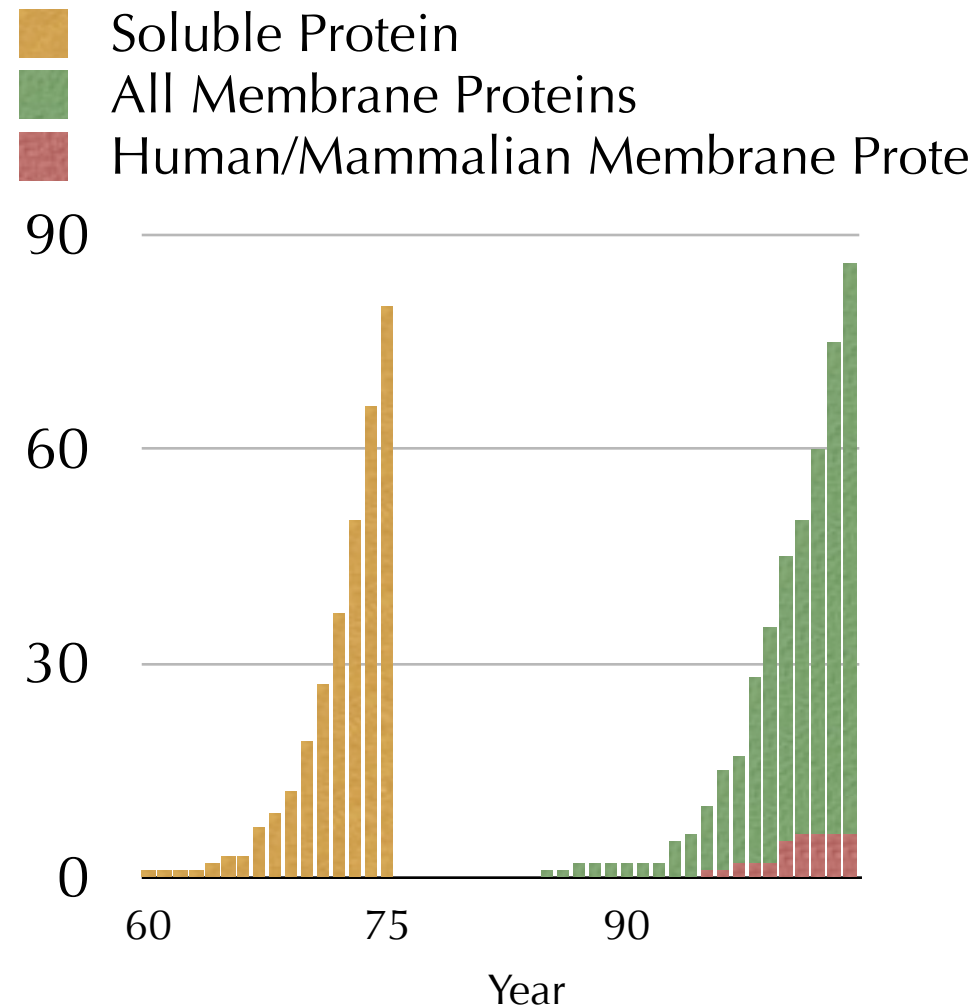
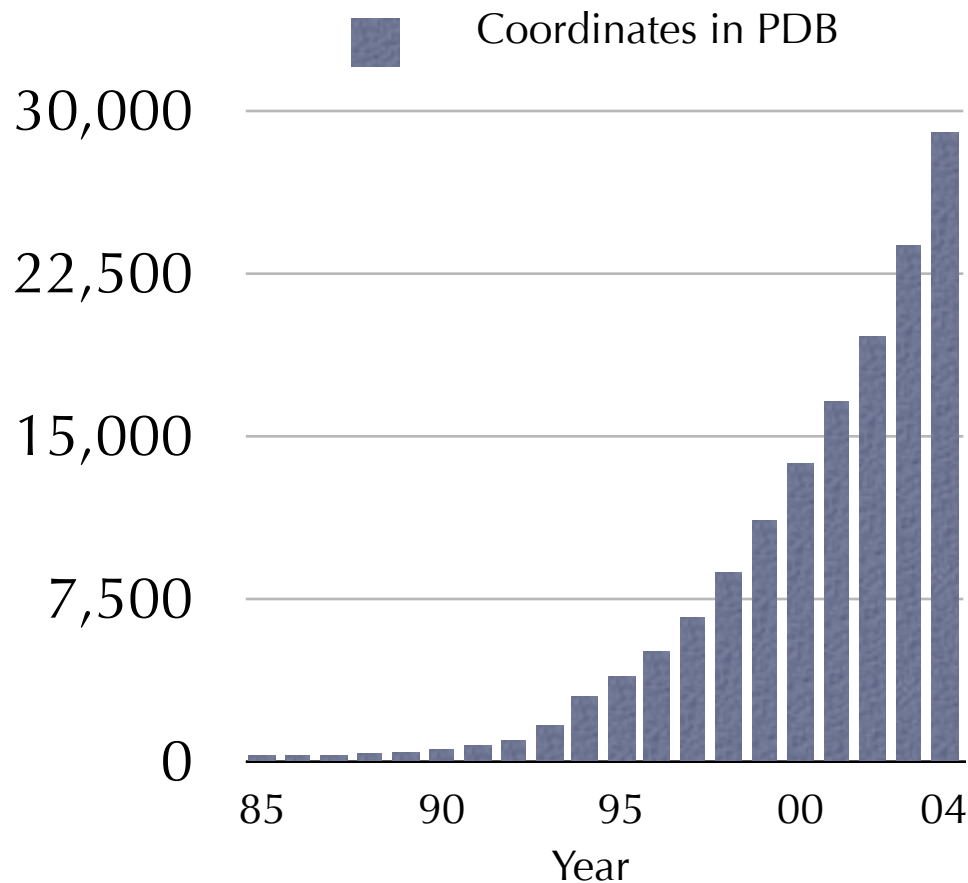
Human Membrane Proteins



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

ERATO-Kyoto Lab



Kostas Beis
Alex Cameron
Isabel Moraes
So Iwata

- Automated crystallization
- Data collection
- Structure determination

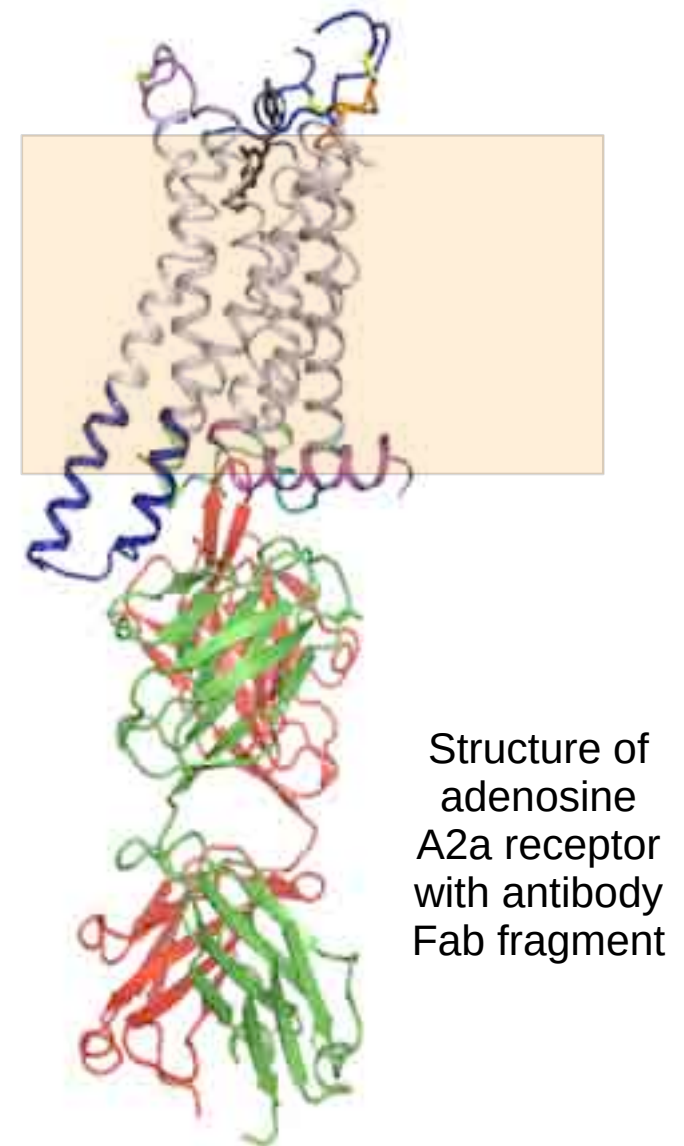
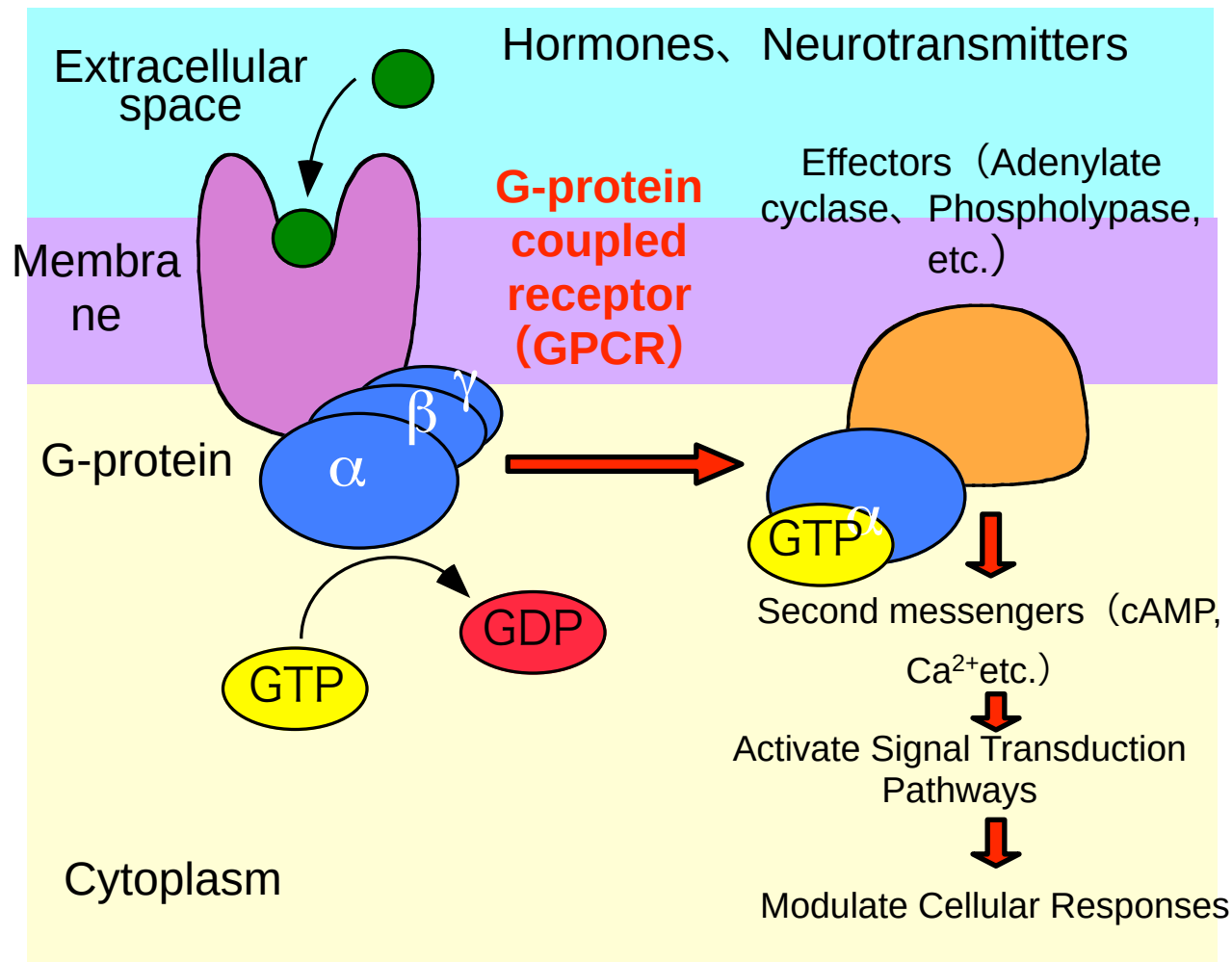


Takuya Kobayashi
Takeshi Murata
Tatsuro Shimamura
Norimichi Nomura

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

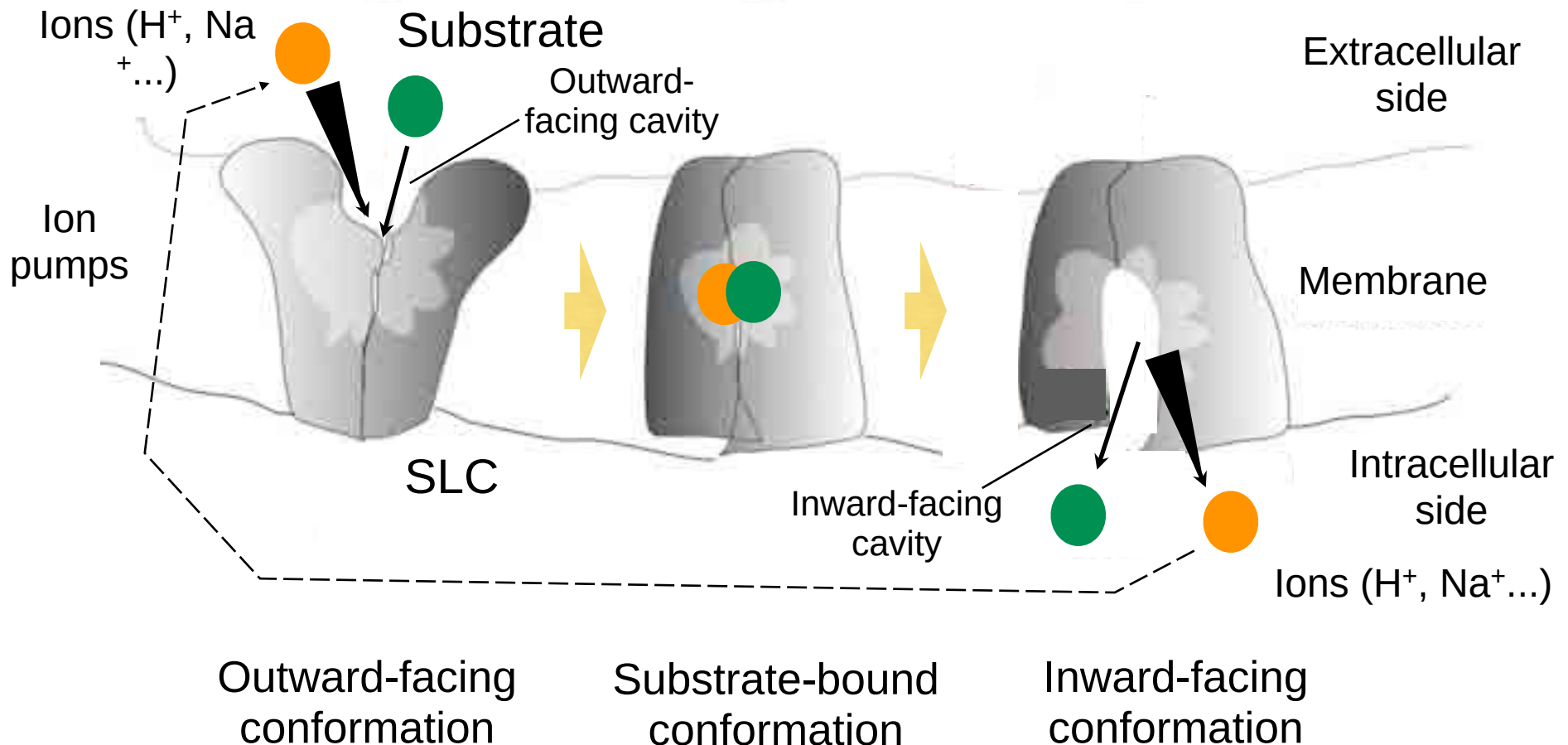
G-protein Coupled Receptors (GPCRs)

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



Solute Carrier Family (SLC) Transporters

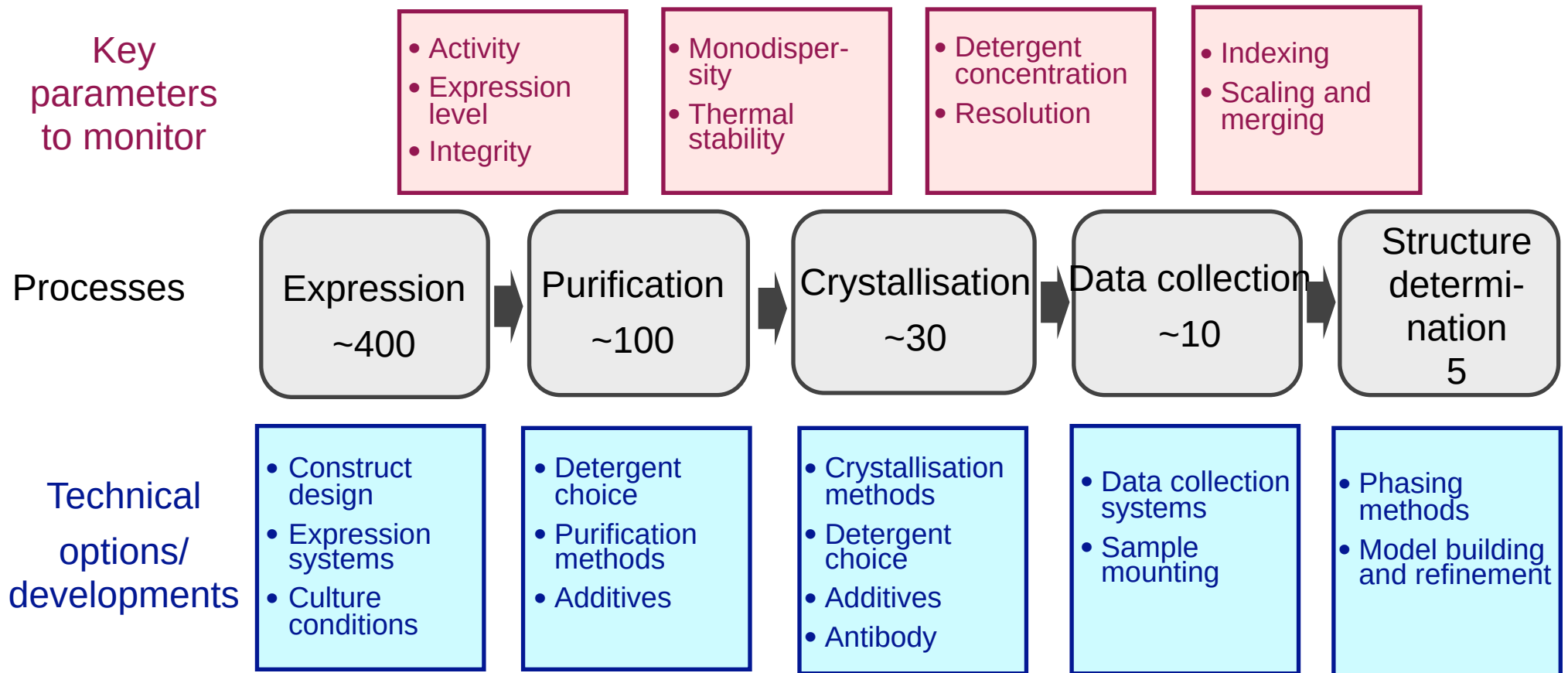
- Membrane transporters including over 300 members organized into 47 families (by Human Genome Organization)
- Composed of facilitative transporters (passive transporters) and secondary active transporters (use an ion gradient for energy).



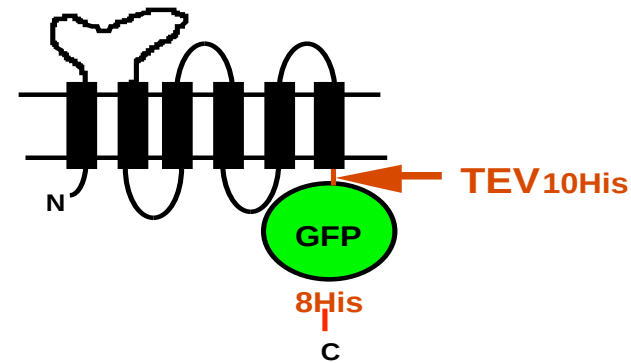
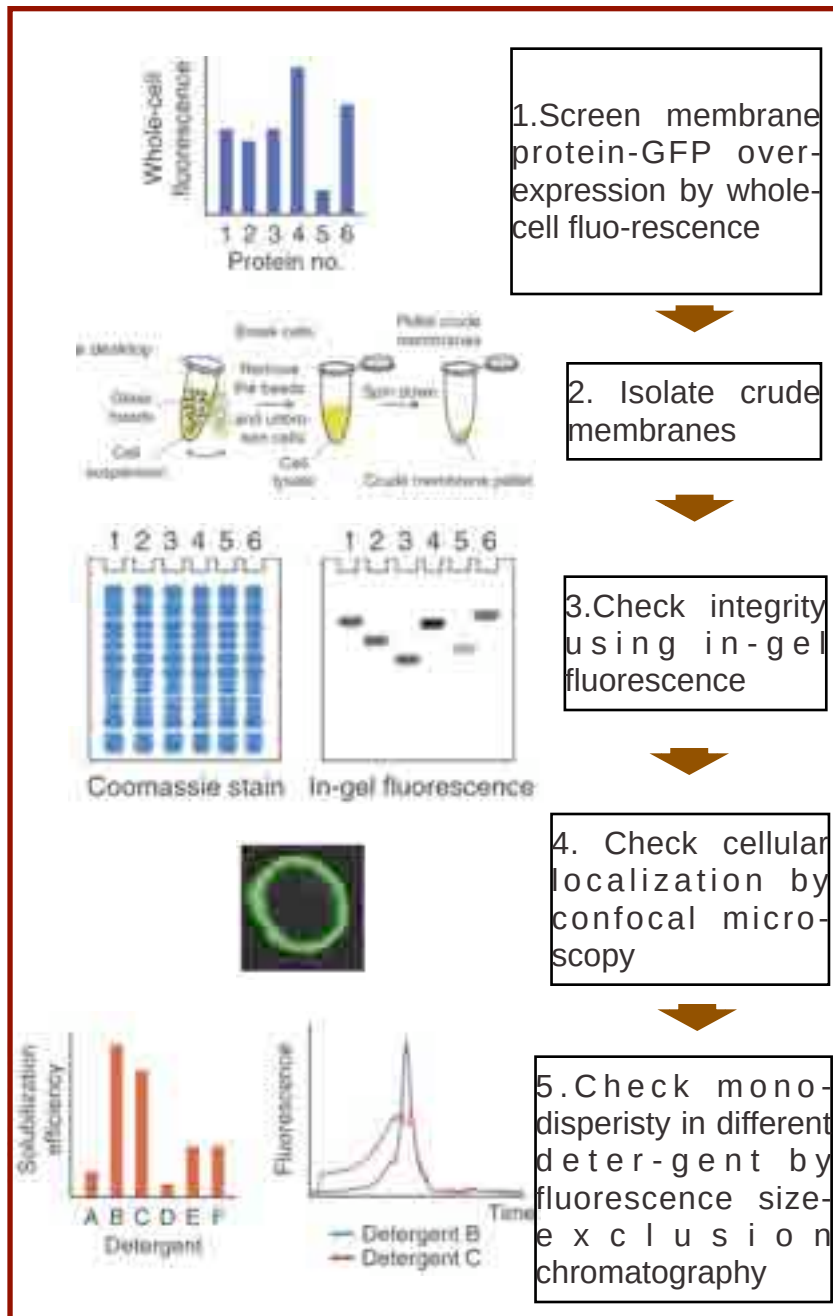


Membrane Protein Crystallography Pipeline

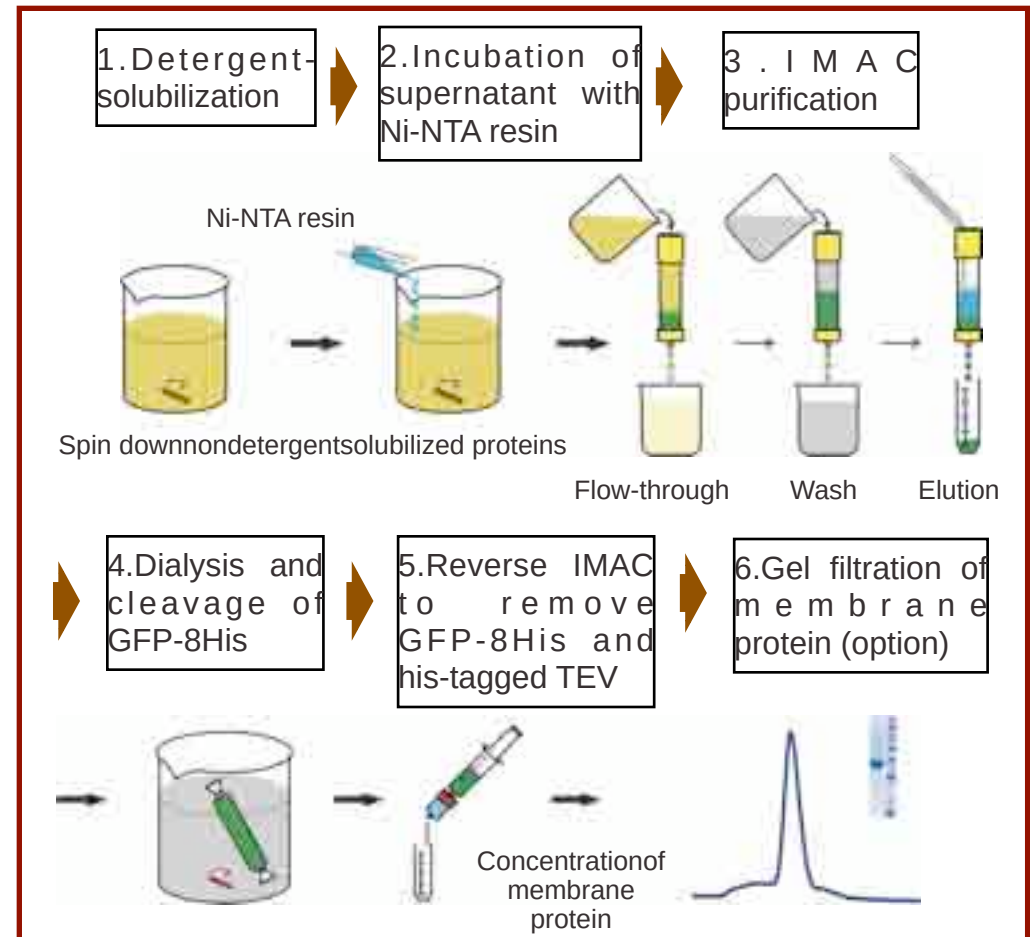
- Method development to address major bottlenecks for membrane protein crystallography.



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

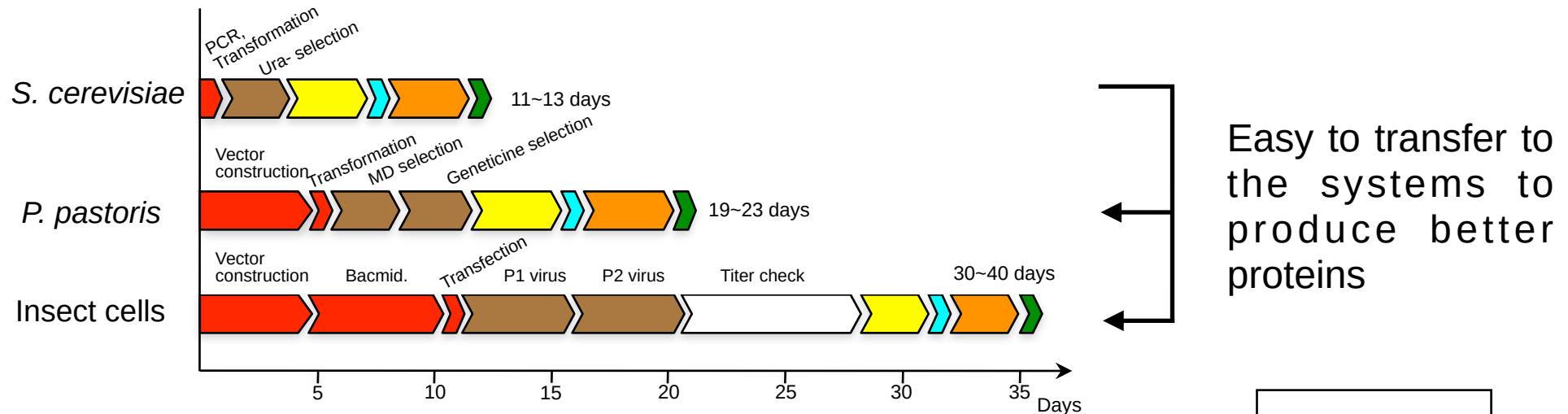


Drew *et al.* Nat. Methods. 2, 2006
Newstead *et al.* PNAS 104, 2007
Drew *et al.*, Nat. Protoc. 3, 2008



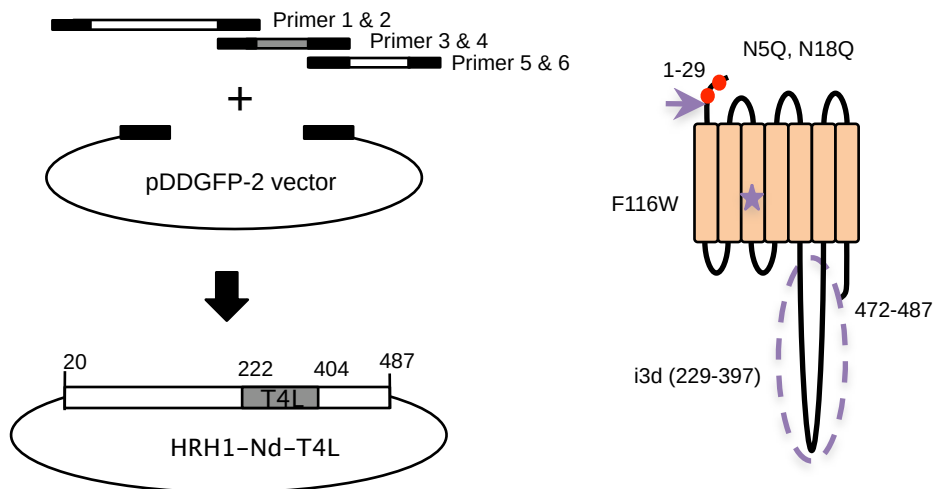
GFP-based Membrane Protein Expression and Purification System in *S. cerevisiae* (2)

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



- Easy to construct variants by combining multiple fragments

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

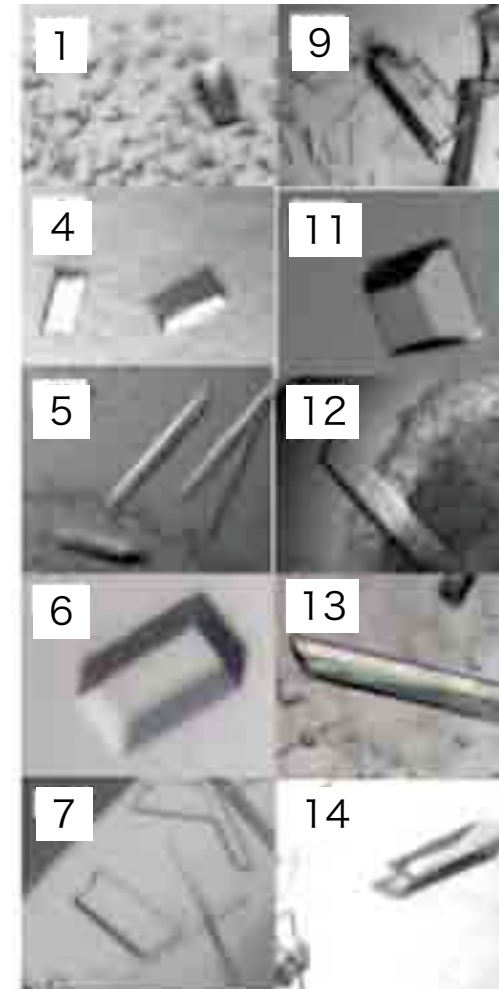


Name	Deletions and mutations
HRH1-WL	HRH1 whole length
HRH1-Nd-i3d	HRH1(20-487), i3d(229-397)
HRH1-Nd-T4L	HRH1(20-487), T4L
HRH1-Nd-F116W-i3d	HRH1(20-487), F116W, i3d(229-397)
HRH1-Nd-L123W-T4L	HRH1(20-487), F116W, T4L

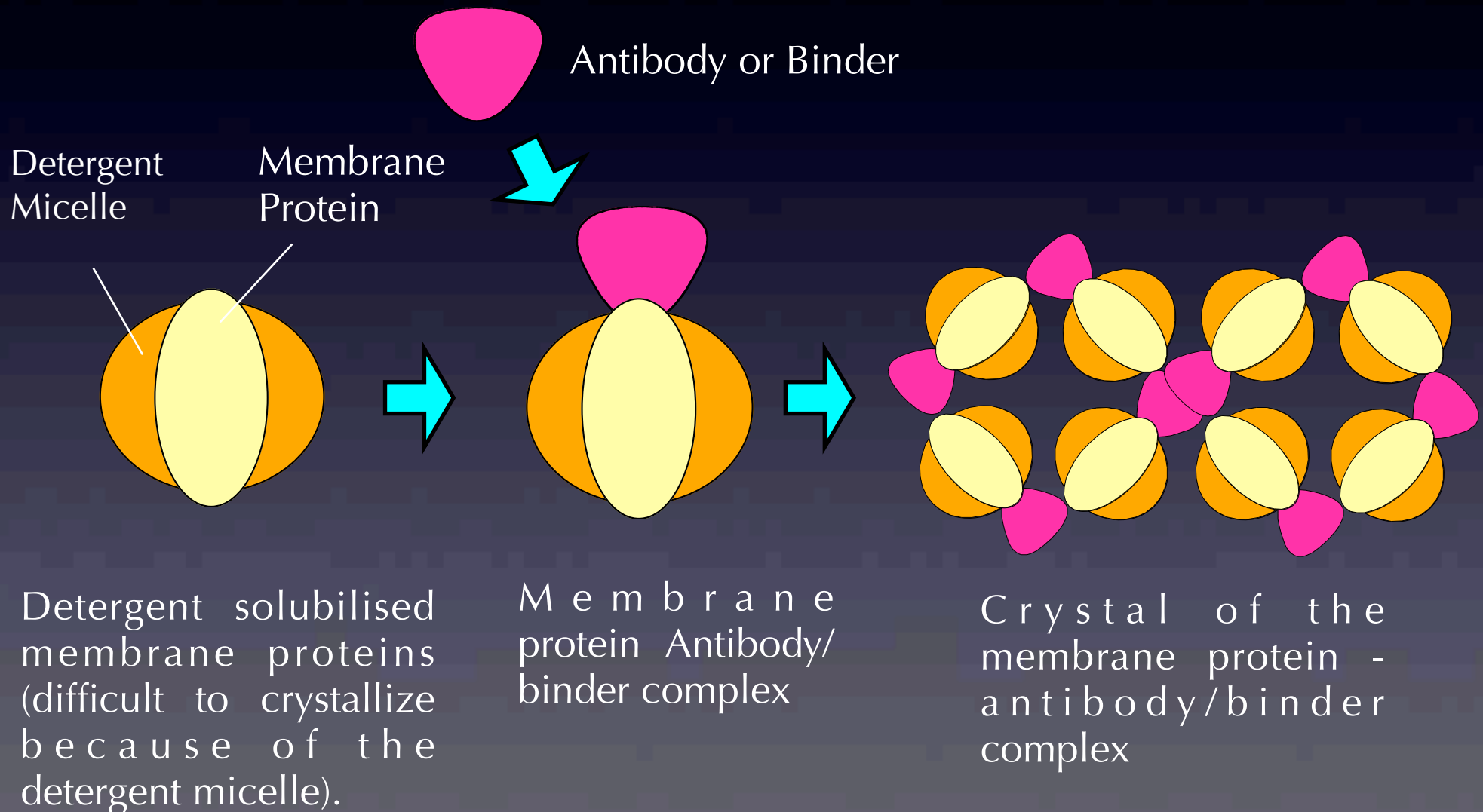
Examples: Crystallisation of SLCs

- 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



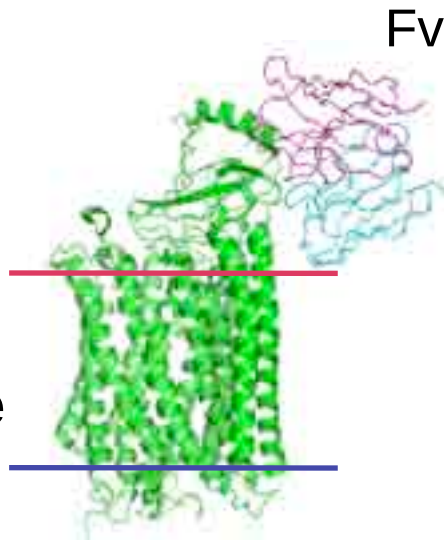
Crystallization of Membrane Proteins using Antibody Fragment or Artificial Binder



Antibody fragments for membrane protein crystallisation

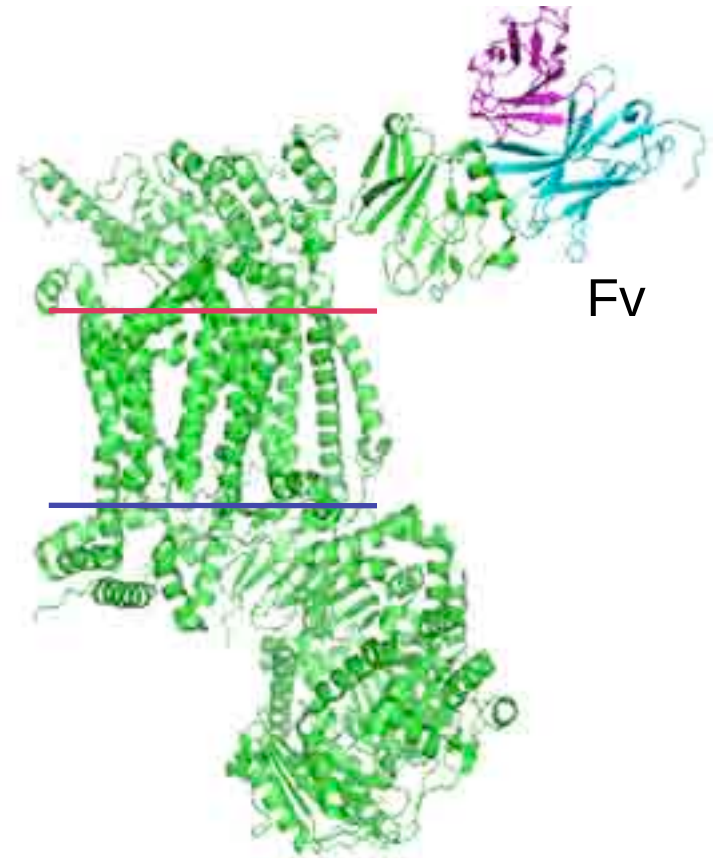
**bacterial
cytochrome
oxidase**

Iwata, Michel et al. (1995)



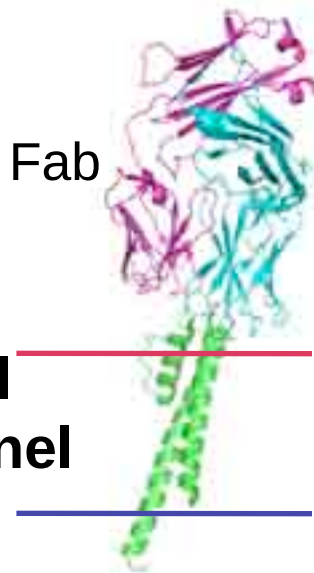
**yeast
cytochrome bc_1
complex**

Hunte, Michel et al. (2000)



**bacterial
 K^+ channel
(KcSA)**

MacKinnon et al. (2001)

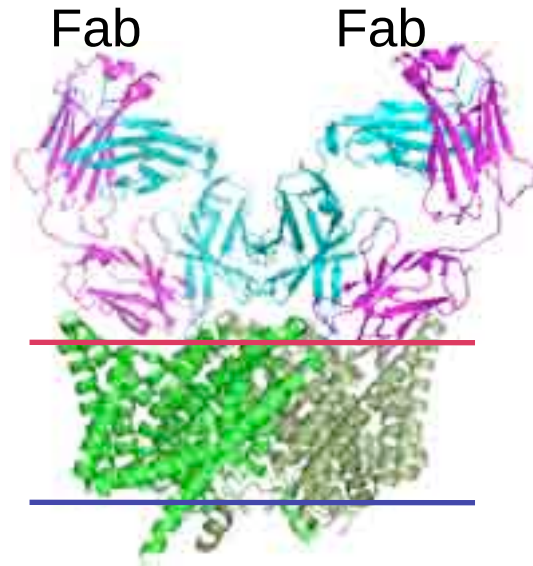


Fab

Fab

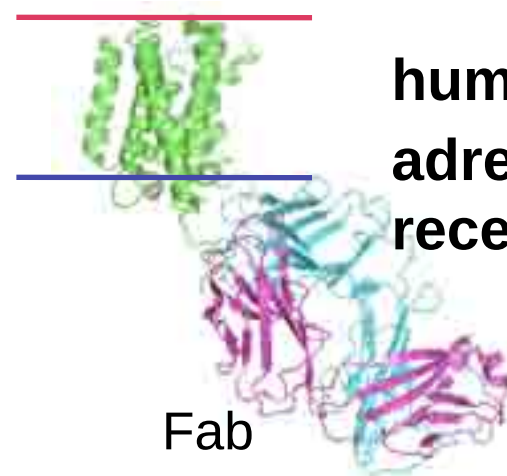
**bacterial
chloride channel
(ClC)**

MacKinnon et al. (2003)

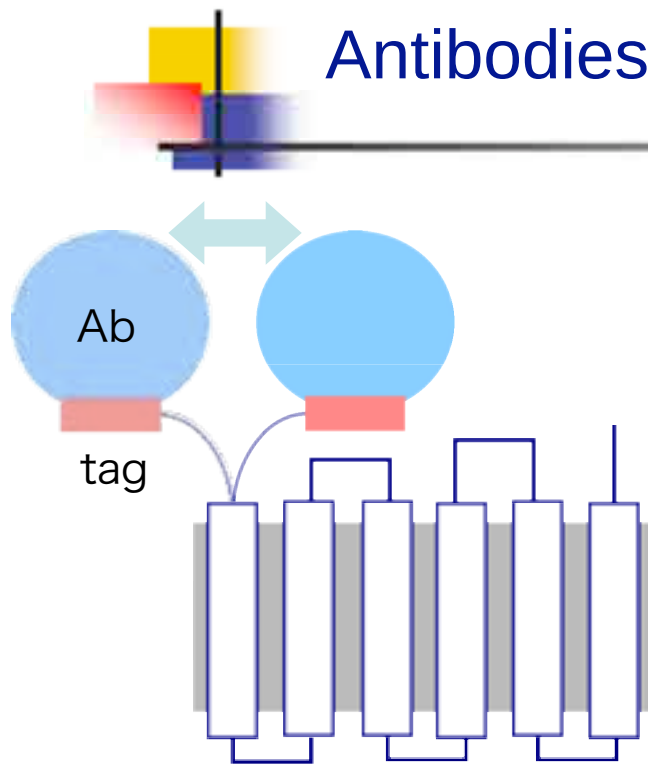


**human β_2
adrenergic
receptor**

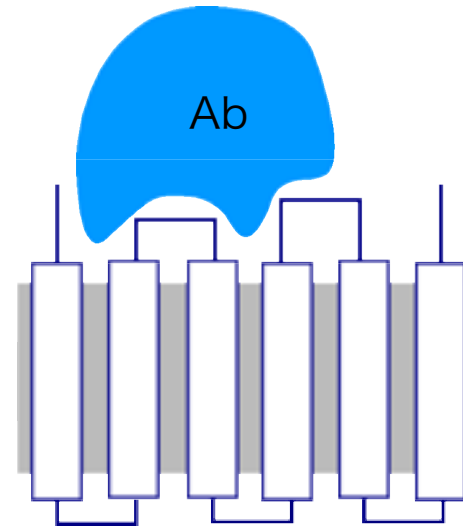
Kobilka et al. (2007)



Antibodies for Membrane Protein Crystallization



Antibody
NOT suitable
for
crystallisation
recognizing a
sequential
epitope



Antibody
suitable for
crystallisation
recognizing a
conformational
epitope

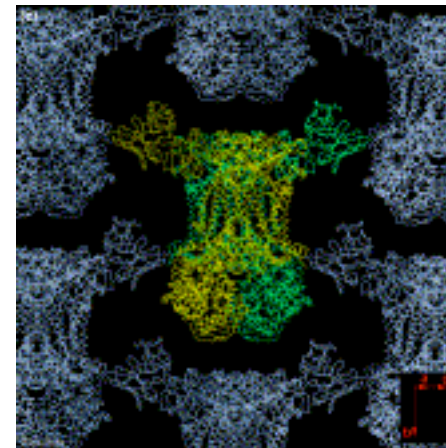
Problem: extremely difficult to raise antibodies against native mammalian membrane proteins



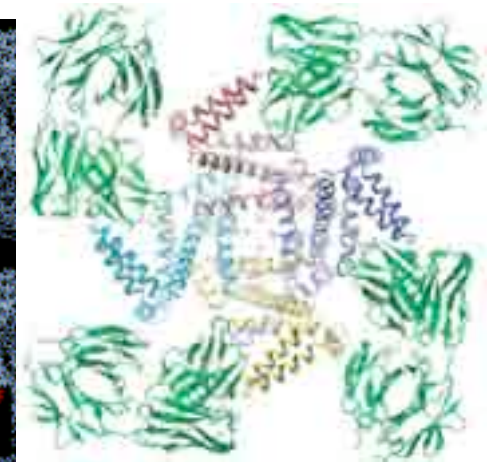
KcsA K-channel



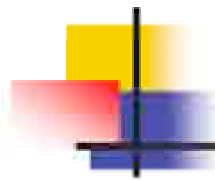
CLC CL channel



Cytochrome bc 1 complex



KvAP K-channel

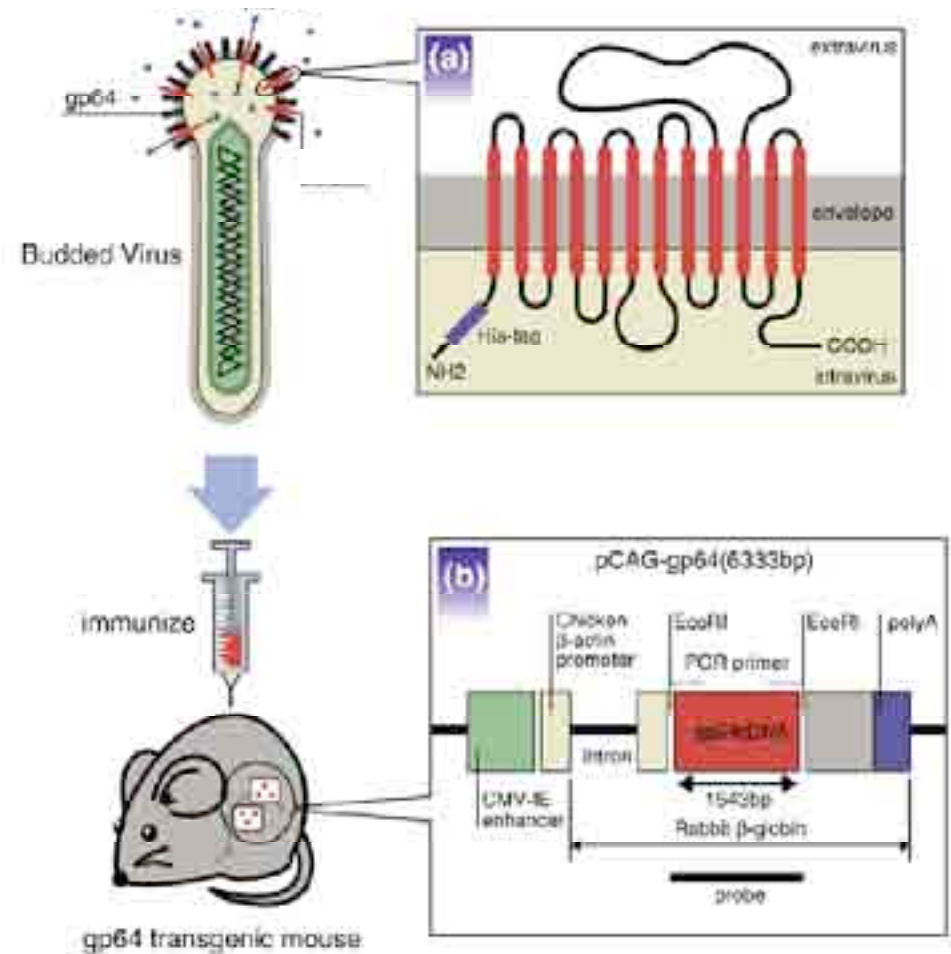
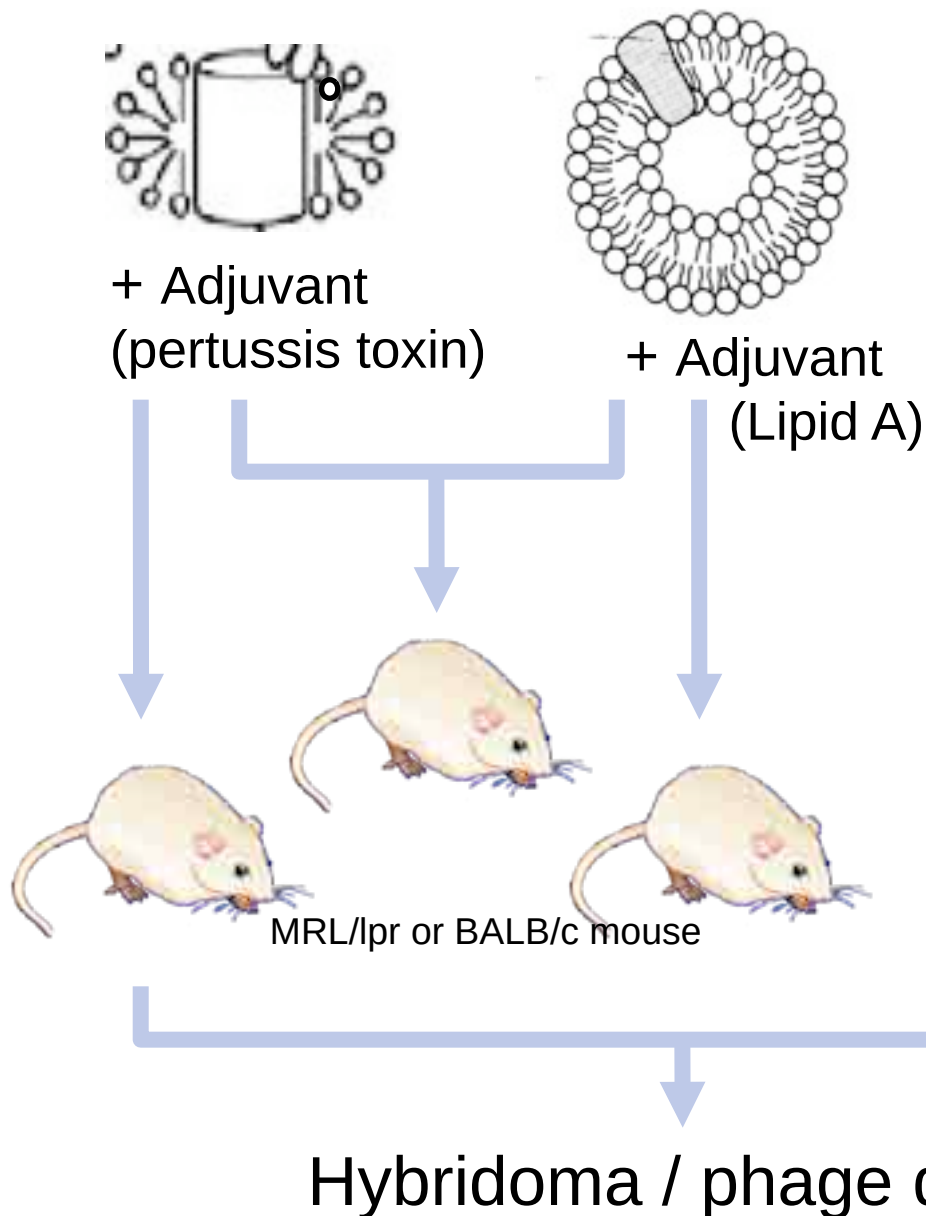


Improved Immunization Strategies

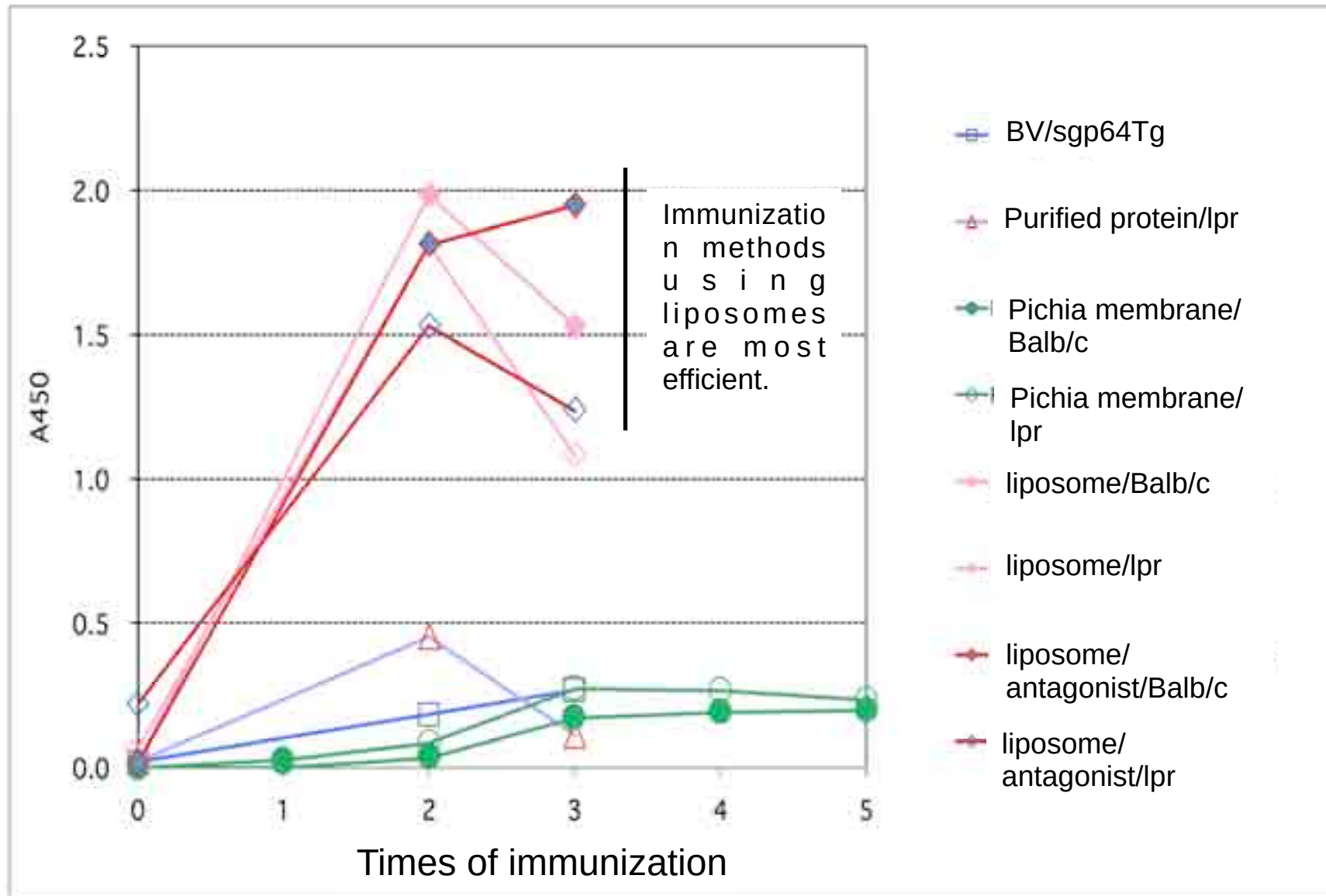
purified protein

protein-liposome

protein-baculovirus



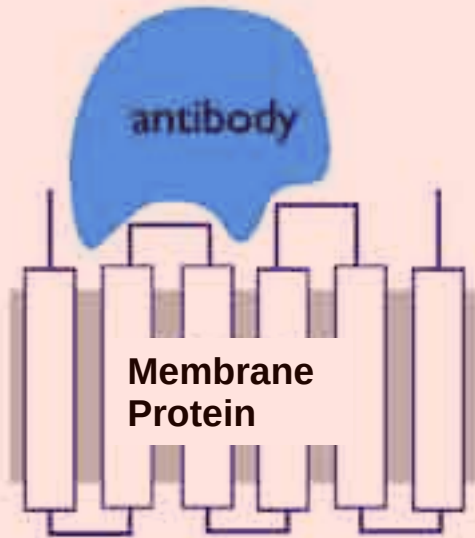
Comparison of Immunization Strategies



Antibody Screening for Membrane Protein Crystallization

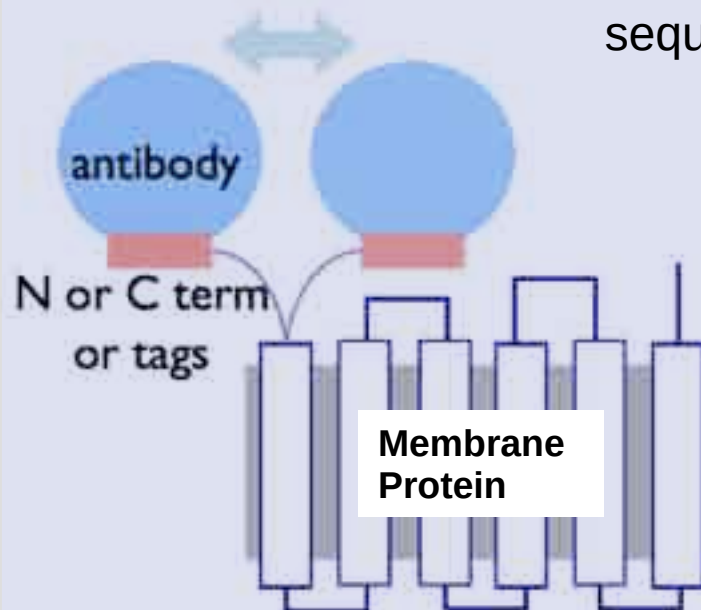
- Combining ELISA with native membrane proteins and the Western/DOT blots with denatured membrane proteins.

Antibodies suitable for crystallisation: recognizing conformational epitopes

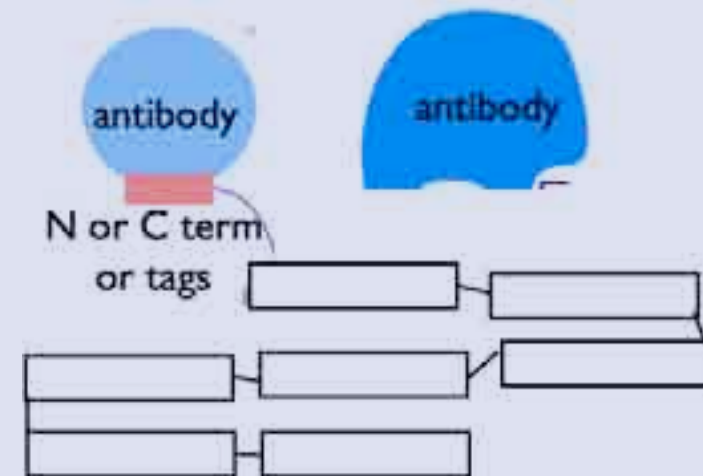


ELISA +, WB/DOT -

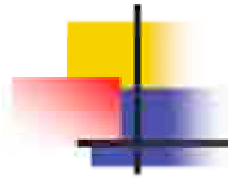
Antibodies NOT suitable for crystallisation: recognizing sequential epitopes



ELISA +, WB/DOT +

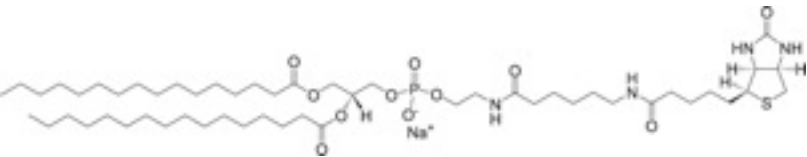


ELISA -, WB/DOT +



Liposome ELISA (L-ELISA)

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

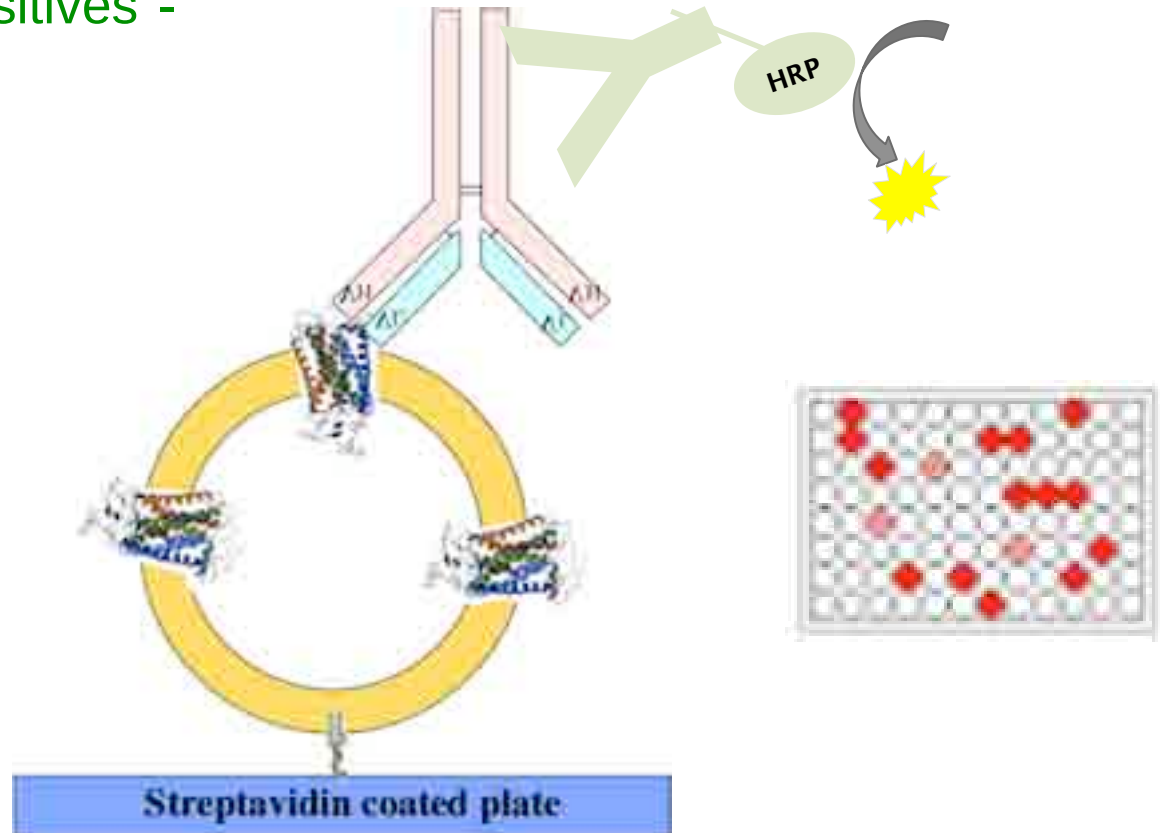


Lipids with 16:0 Biotinyl CAP PE

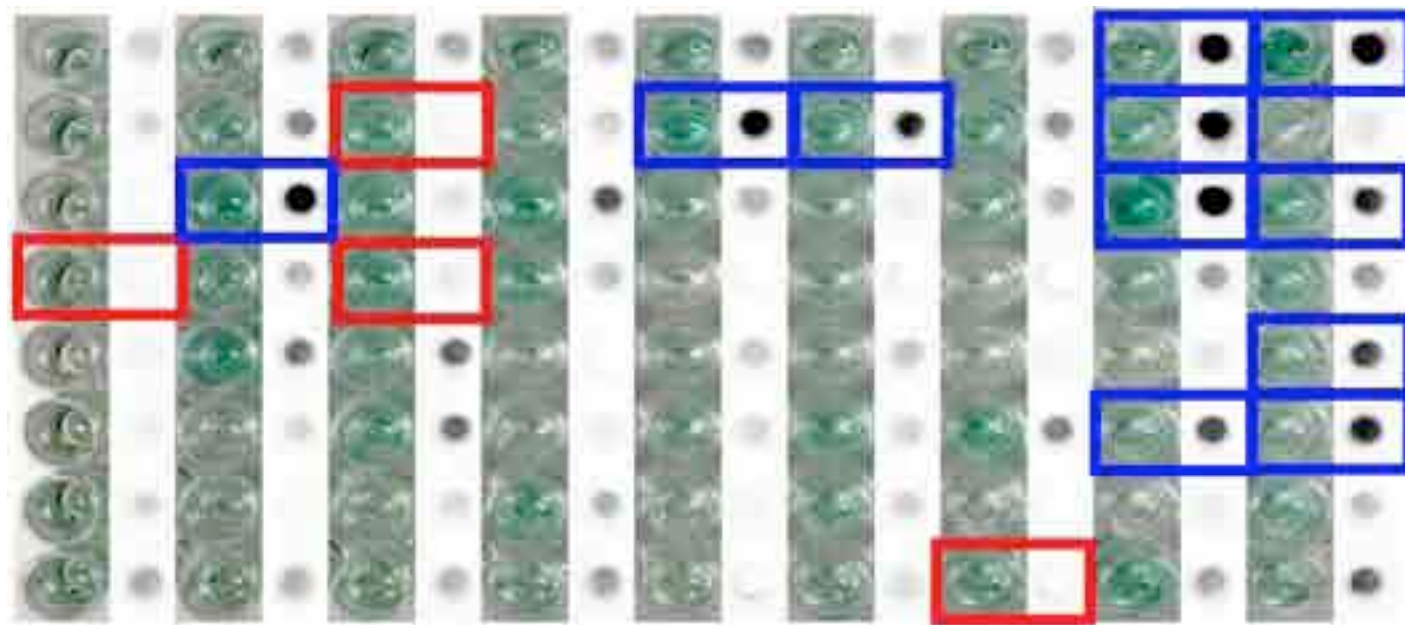


Antigen reconstituted into proteoliposome

Testing culture sup.



Antibody Screening using L-ELISA and DOT blot



ELISA + & Dot -

=

Conformational epitope



ELISA + & Dot +

=

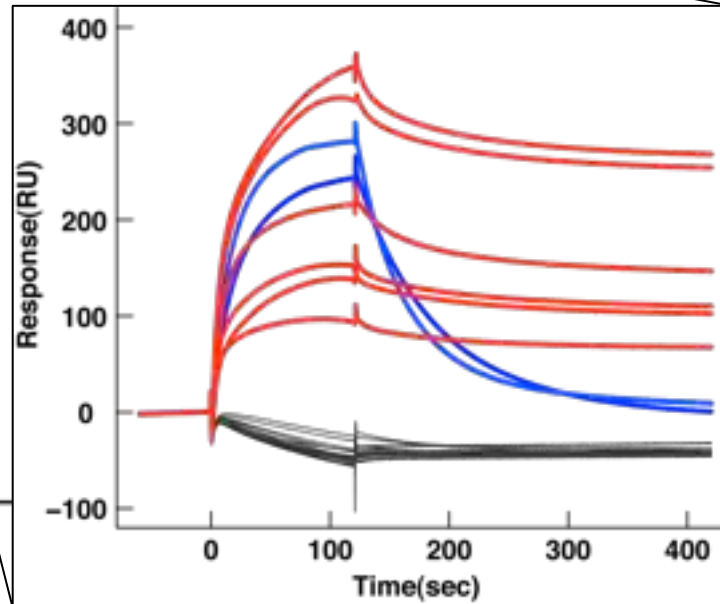
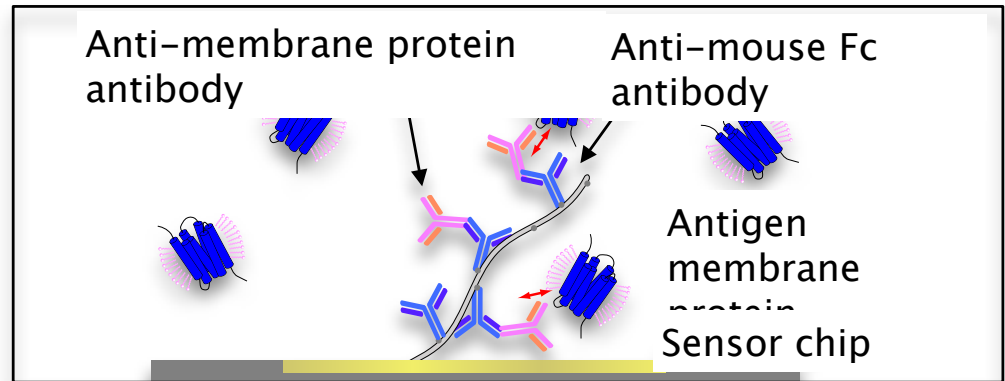
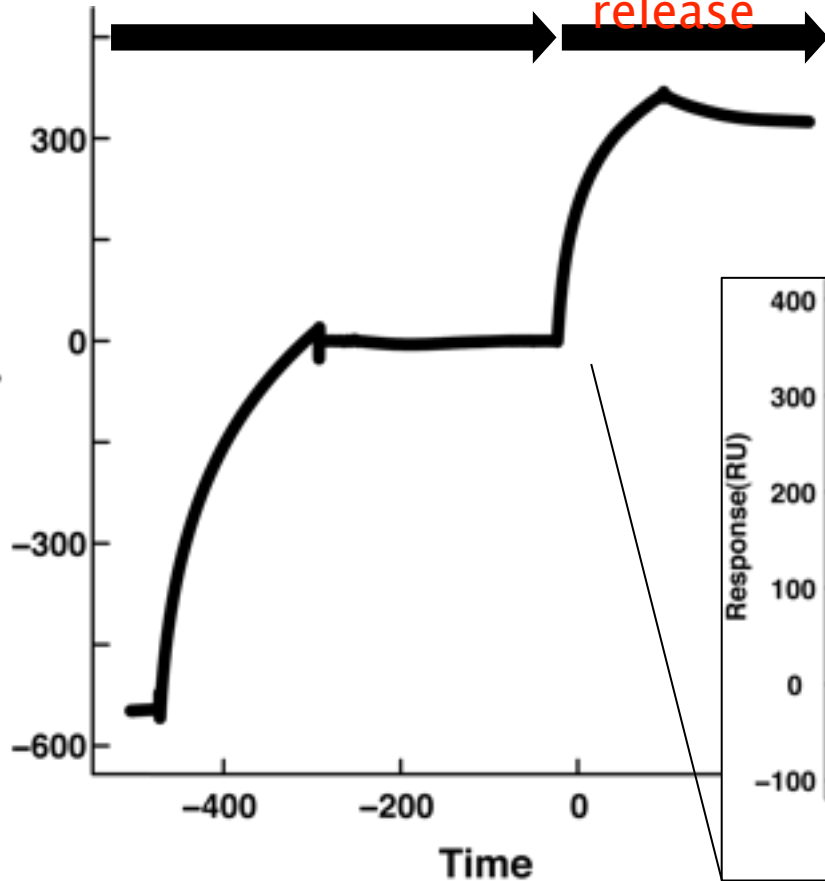
Sequential epitope

Affinity Test using Biacore

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

Antibody capture

Target protein capture/release



→ **slow dissociation**

$$k_{off} < 10^{-4}, K_d < 10^{-8} \text{ M}$$

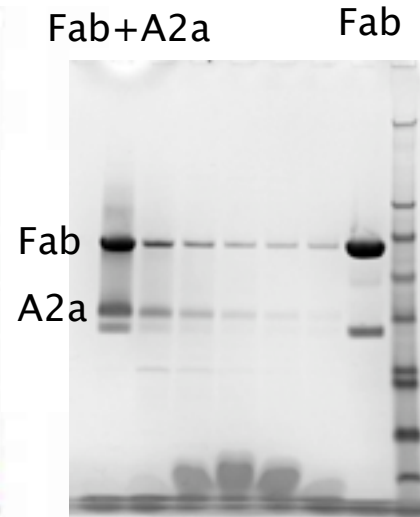
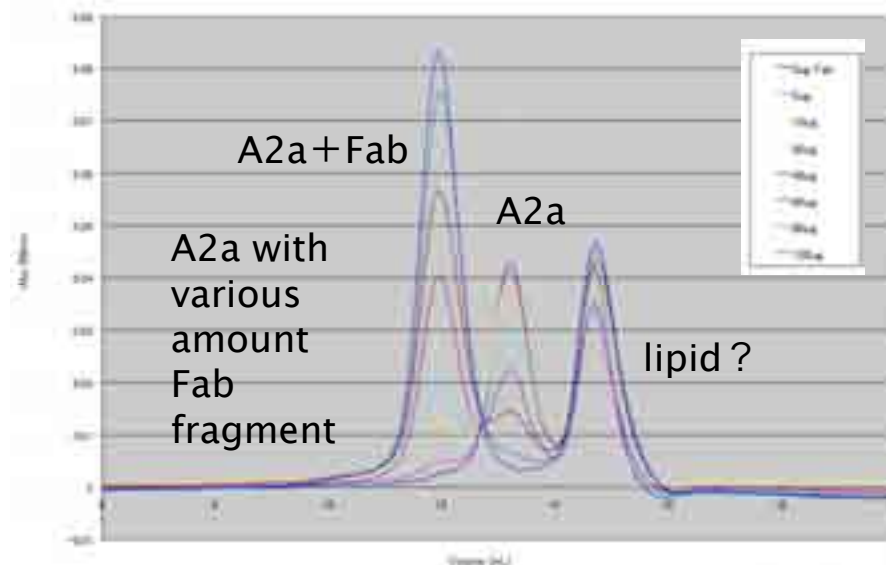
→ **fast dissociation**

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

→ **poor affinity**

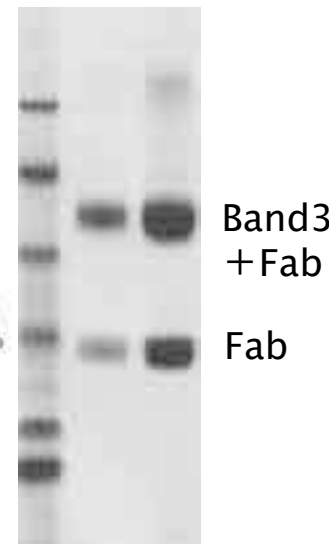
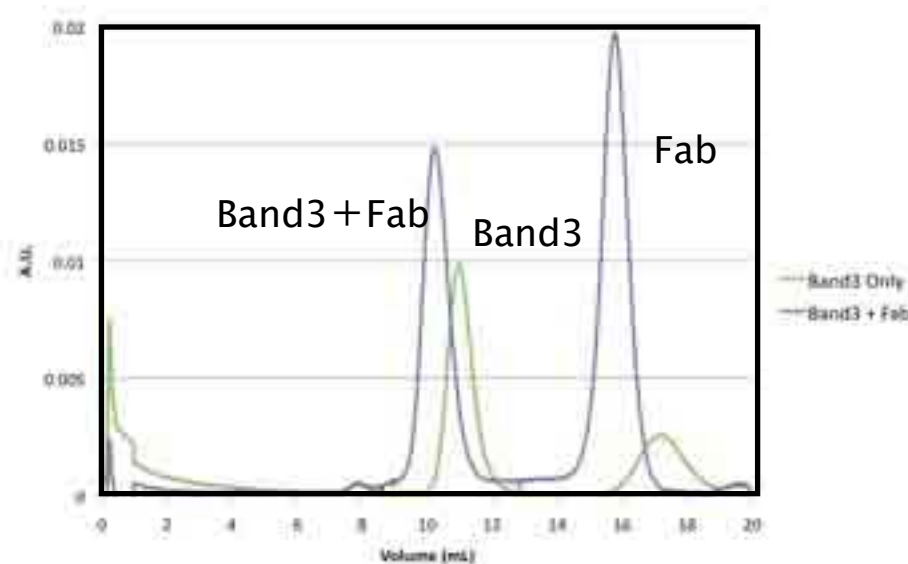
Membrane Protein - Antibody Fab Fragment Complex Preparation

Adenosine A2a

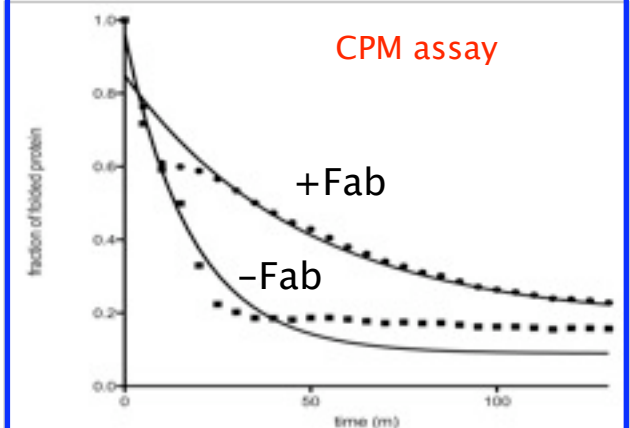


Complex crystals

Human Anion Exchanger



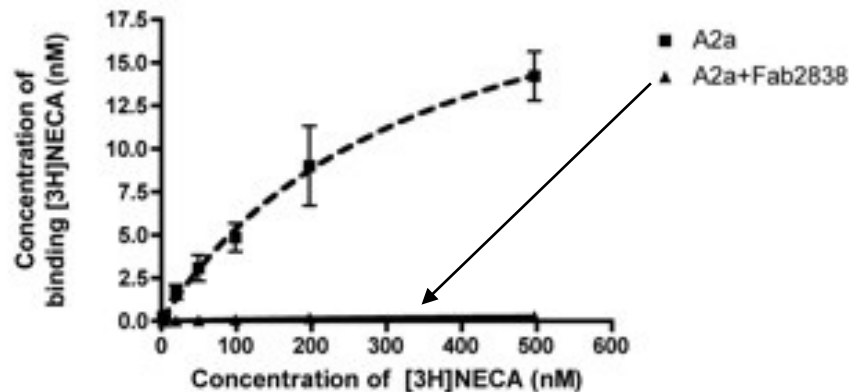
Antibody significantly improves the thermostability of A2a



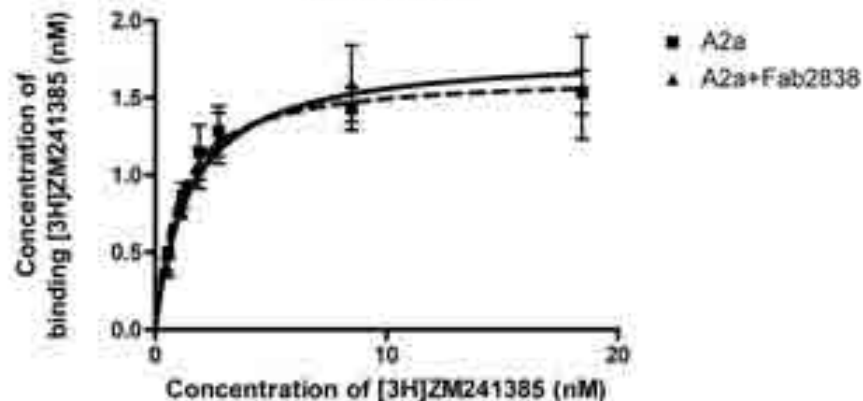
Applications for Functional/Therapeutic Antibodies and for Imaging

-Fab2838 locks adenosine A2a receptor into the inactive conformation

NECA(Agonist, only binds to the active form)

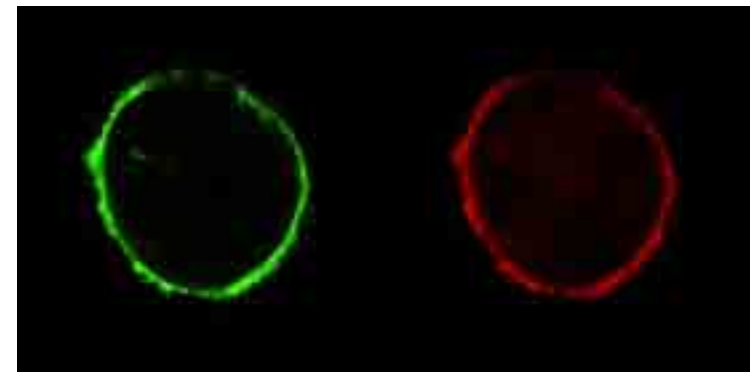


ZM241385(Antagonist, can bind to the inactive form)



-Cell imaging using antibodies against adenosine A2a receptor

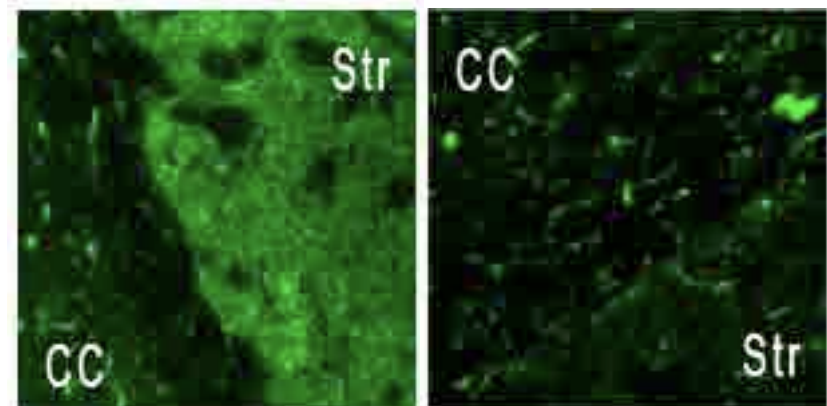
Cultured cells



GFP(control)

C40010+2nd Ab(+Alexa546)

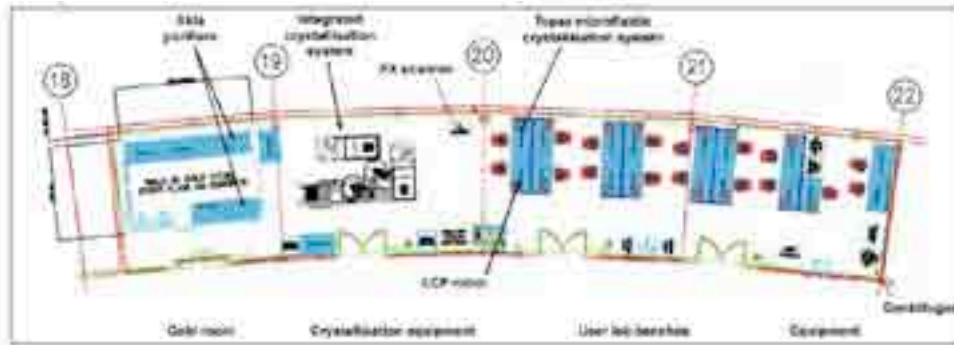
Mouse brain



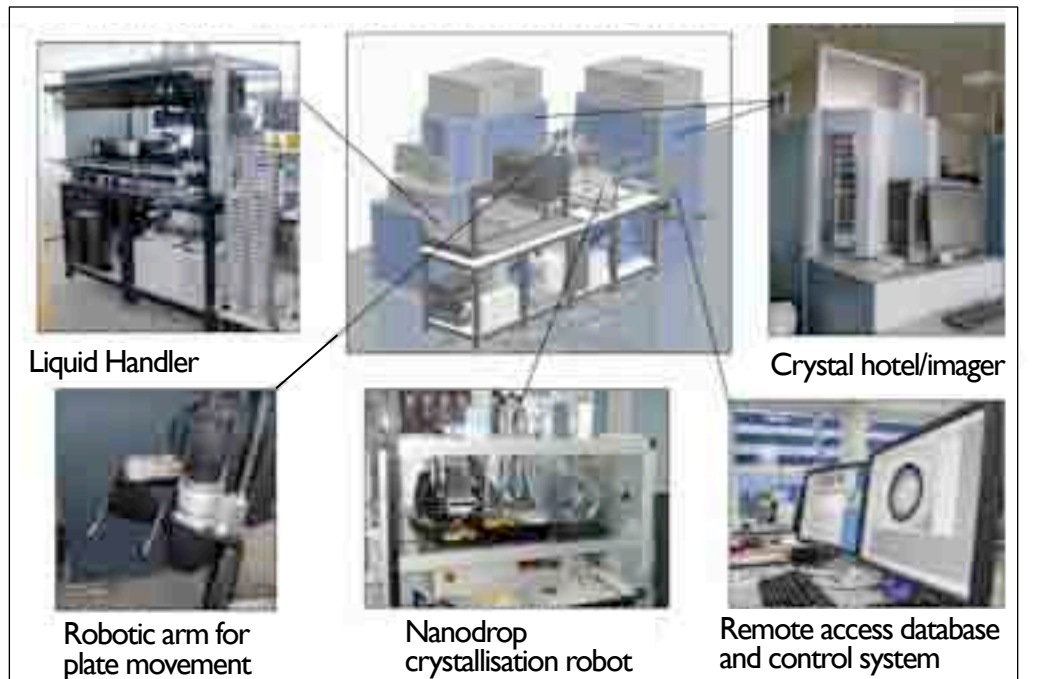
with 1ry antibody

Control

Plan of the MPL Facility



Fully integrated sitting-drop Rhombix crystallisation robot



Fluidigm Topariz microfluidics system

Cubic phase crystallization robot

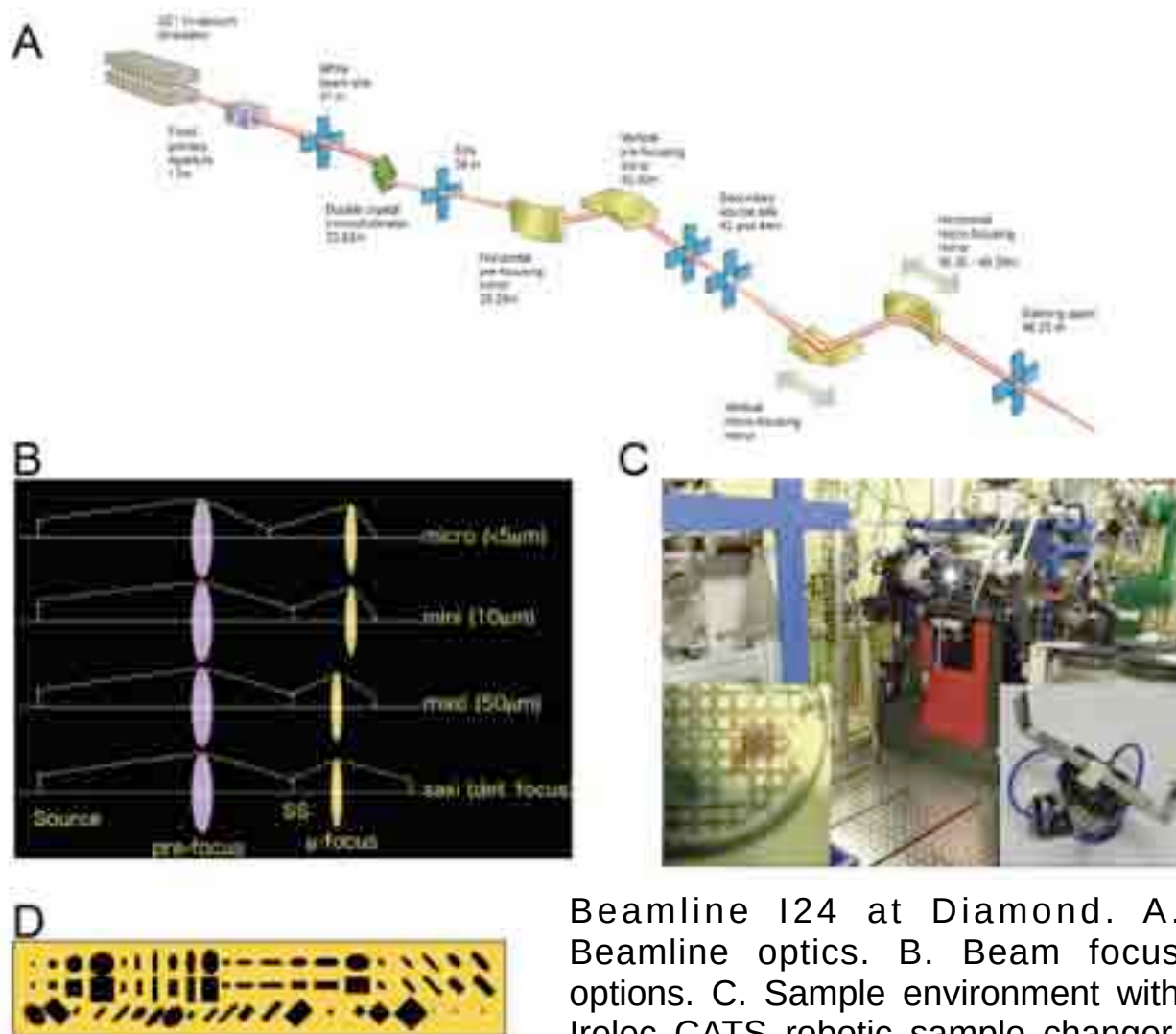
PX scanner in-plate X-ray diffraction system

Automated Crystal Optimisation at Diamond Membrane Protein Laboratory (MPL)

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

X-ray Data Collection at the Diamond Microfocus Beamline I24

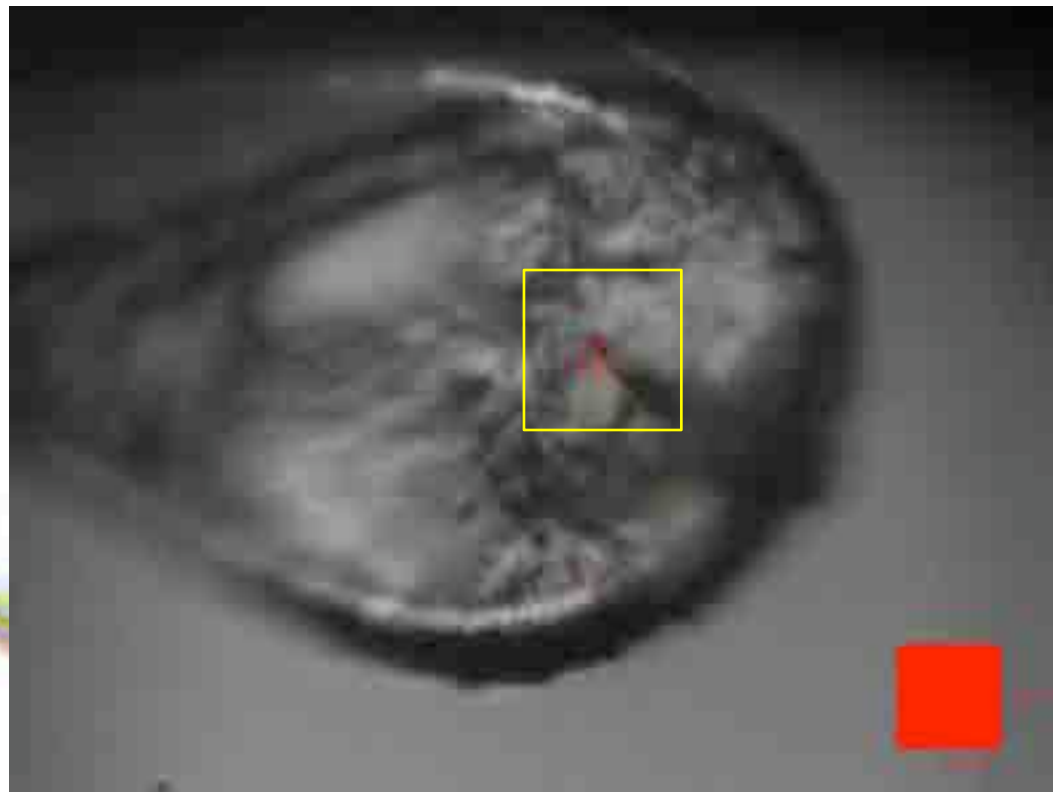


Beamline I24 at Diamond. A. Beamline optics. B. Beam focus options. C. Sample environment with Irelec CATS robotic sample changer, grid scanning system (left) and the plate gripper (right). D. Aperture array.

- 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

Adenosine A2a Receptor-Fab Complex Crystal

- Maximum resolution $< 2.5\text{\AA}$
- Difficult to freeze. A large mosaicity.

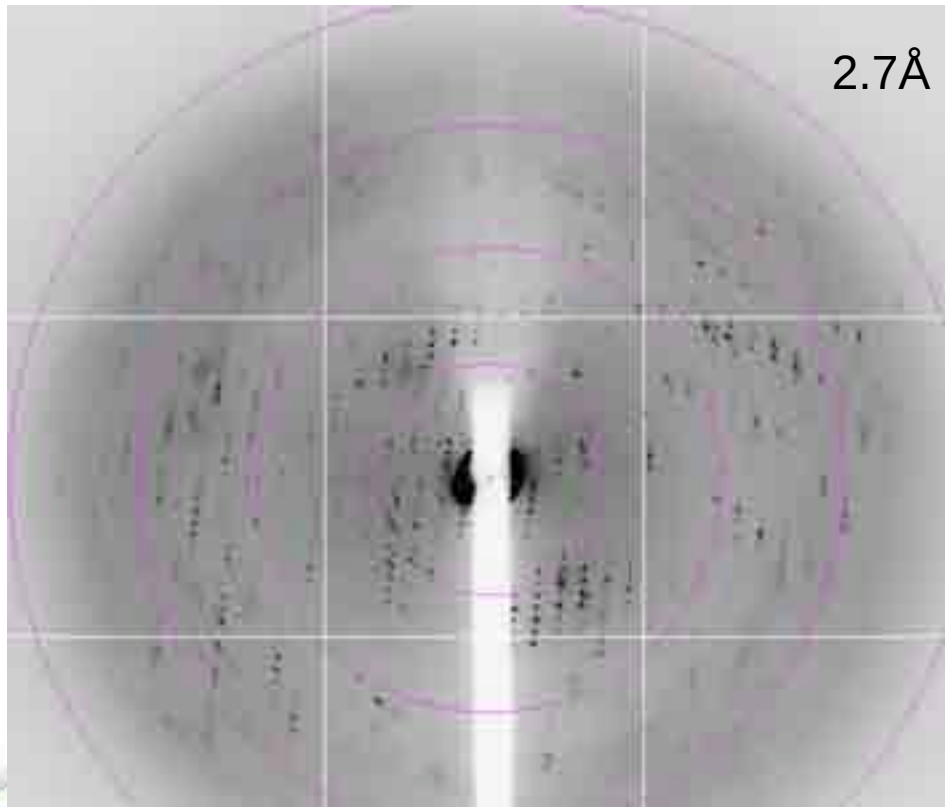


Standard size
Beam ($50\mu \times 50\mu$)

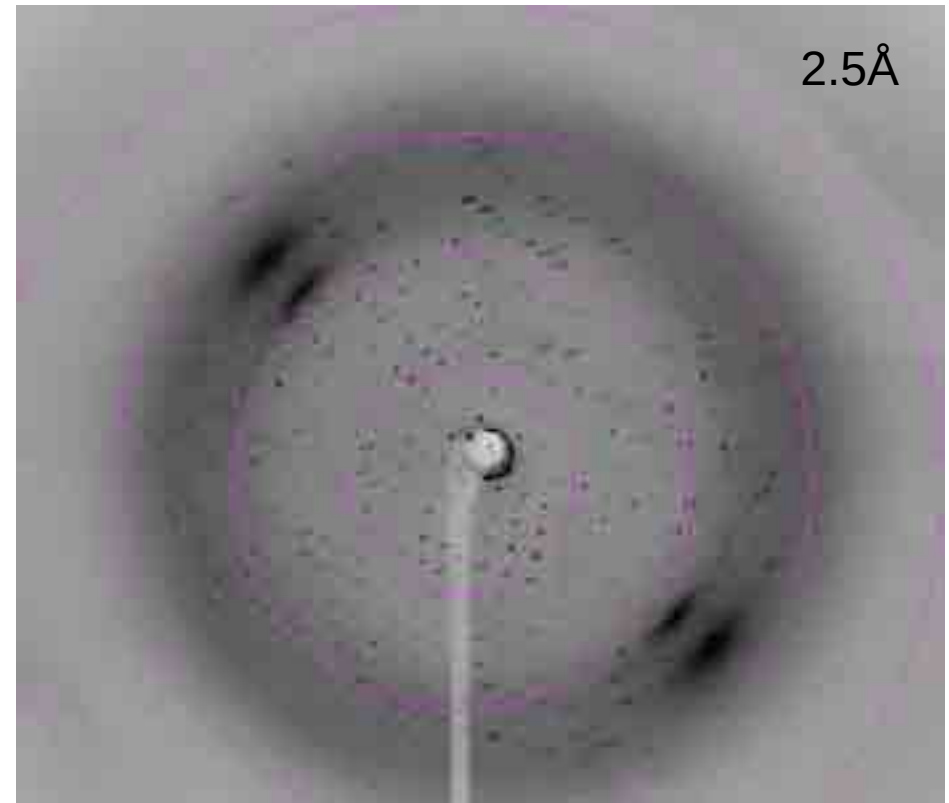


Micro Beam
($10\mu \times 10\mu$)

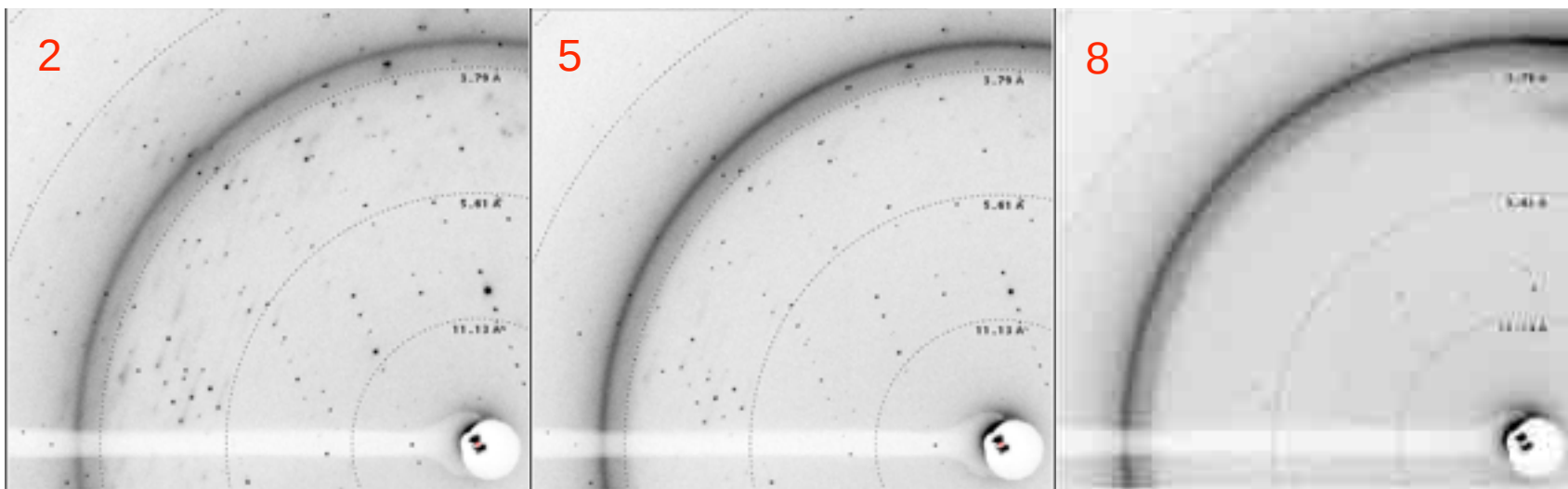
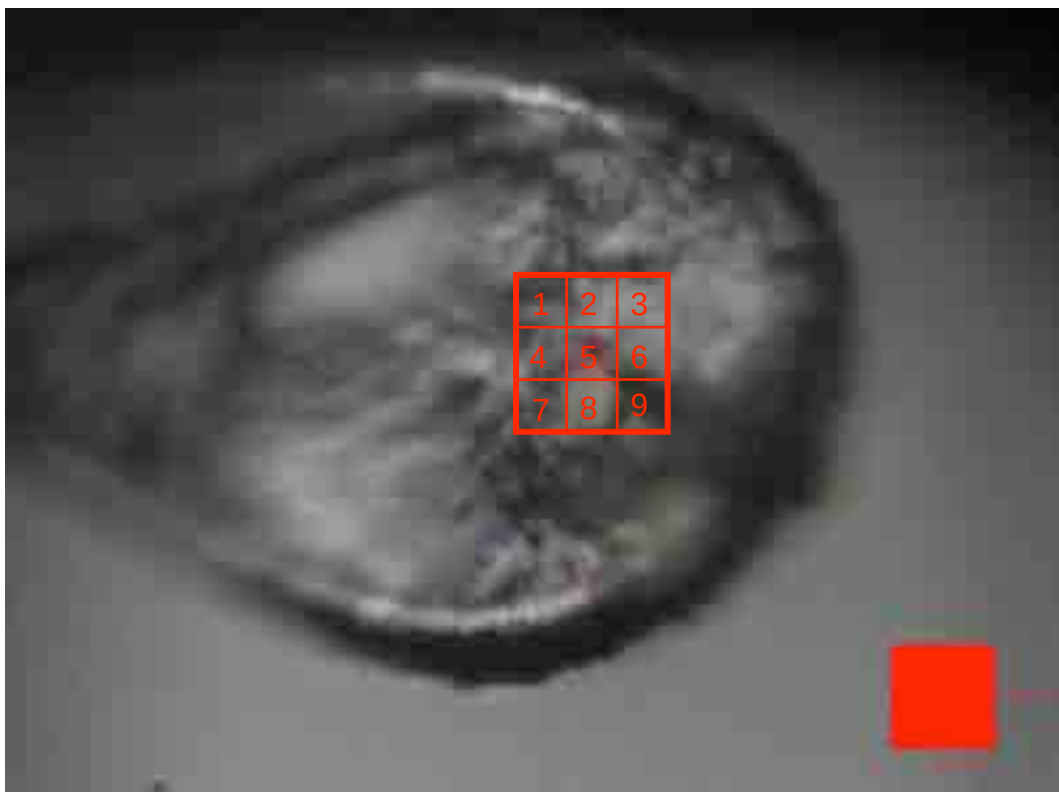
Standard Size Beam vs Micro Beam



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



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



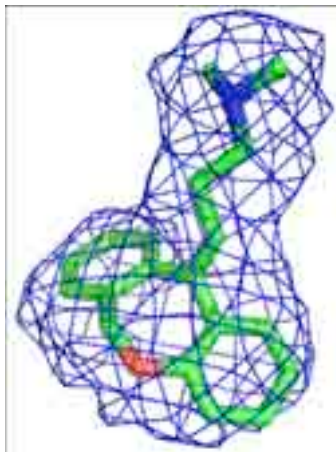
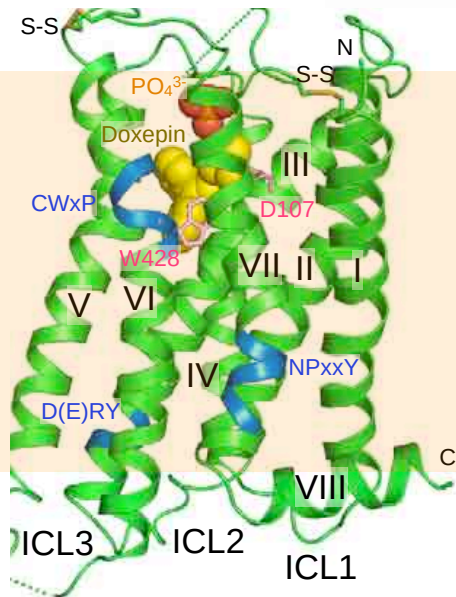


A 3D ribbon diagram of the ABC transporter structure, showing the Out (outside) and In (inside) orientations, and the Taurocholate molecule.

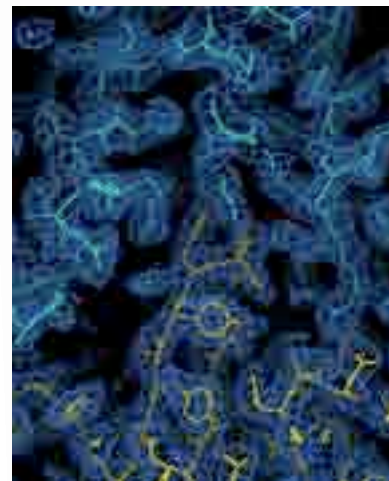


G-protein coupled receptors

Human Histamine H₁R
(Nature 2011)



Antihistamine (Doxepin)



CDR-H3

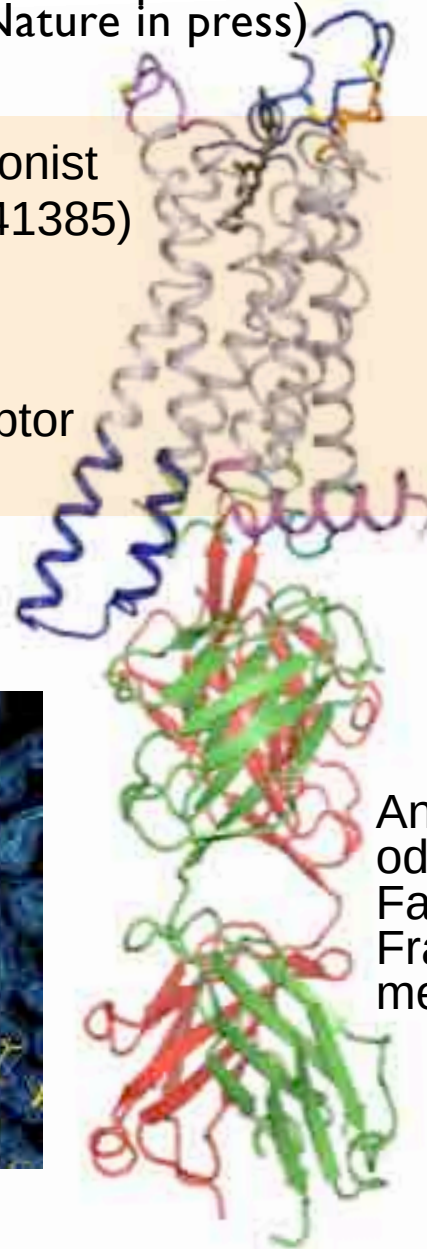
Human Adenosine A2aR-Fab
complex
(Nature in press)

Antagonist
(ZM241385)

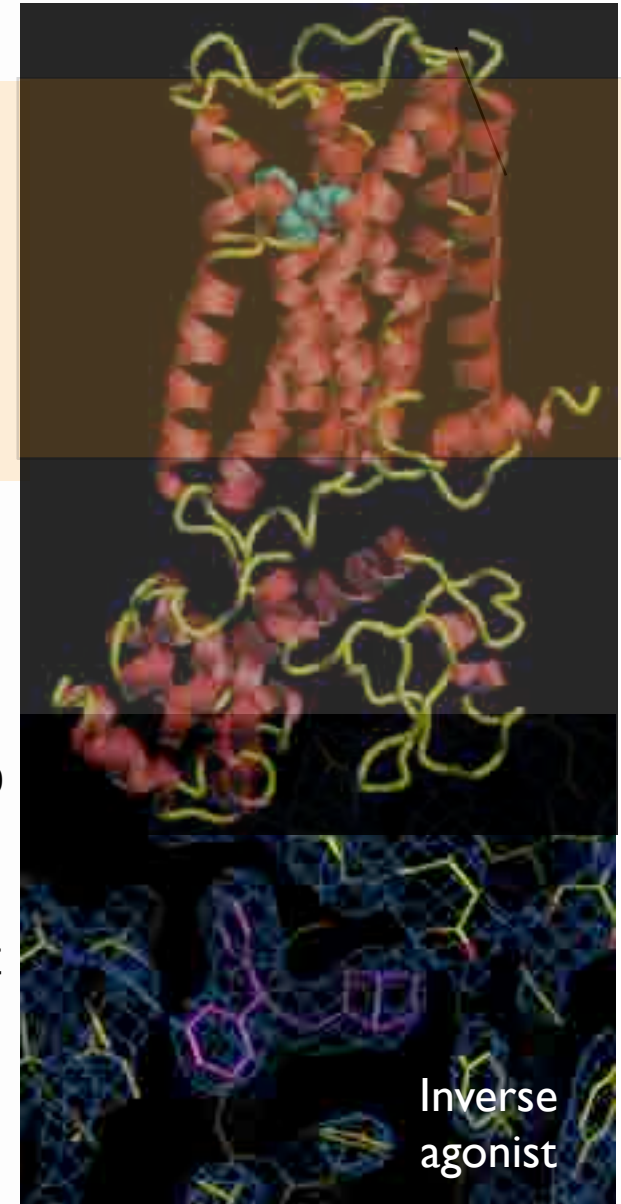
A2a
receptor

i3-loop

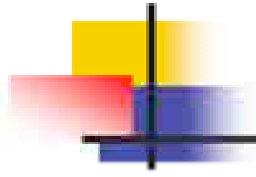
Antibody
Fab
Fragment



Human Muscarinic acetylcholine
M₂R (Nature in press)



Inverse
agonist



Histamine H₁ receptor

Collaborators

Imperial College London
/ Diamond Light Source (MPL)
/ Kyoto University (ERATO)

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(ERATO)

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(ERATO)

Hirokazu Tsujimoto (ERATO)

Simone Weyand (MPL)

Takuya Kobayashi (ERATO)

So Iwata

Diamond Light Source
Graeme Winter

The Scripps Research Institute

Vadim Cherezov

Wei Liu

GyeWon Han

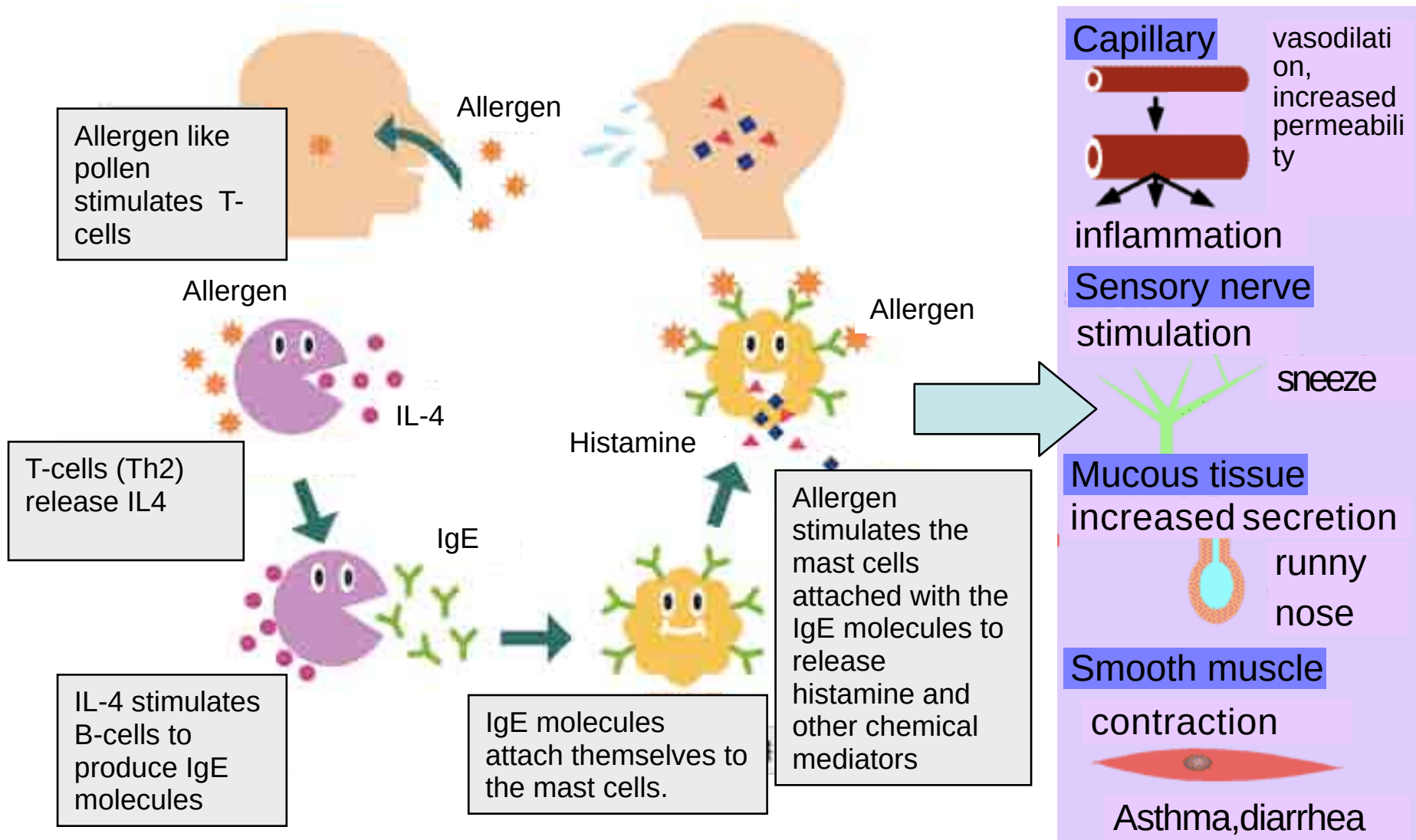
Raymond C. Stevens

University of California, San
Diego

Vsevolod Katritch

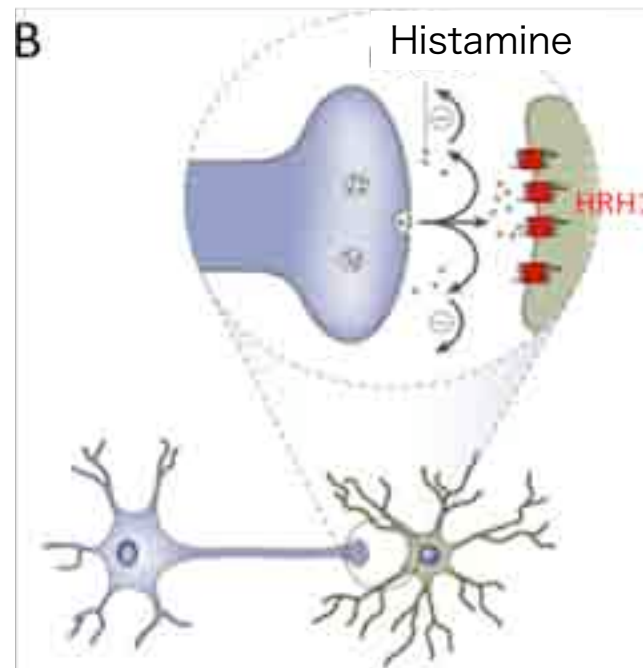
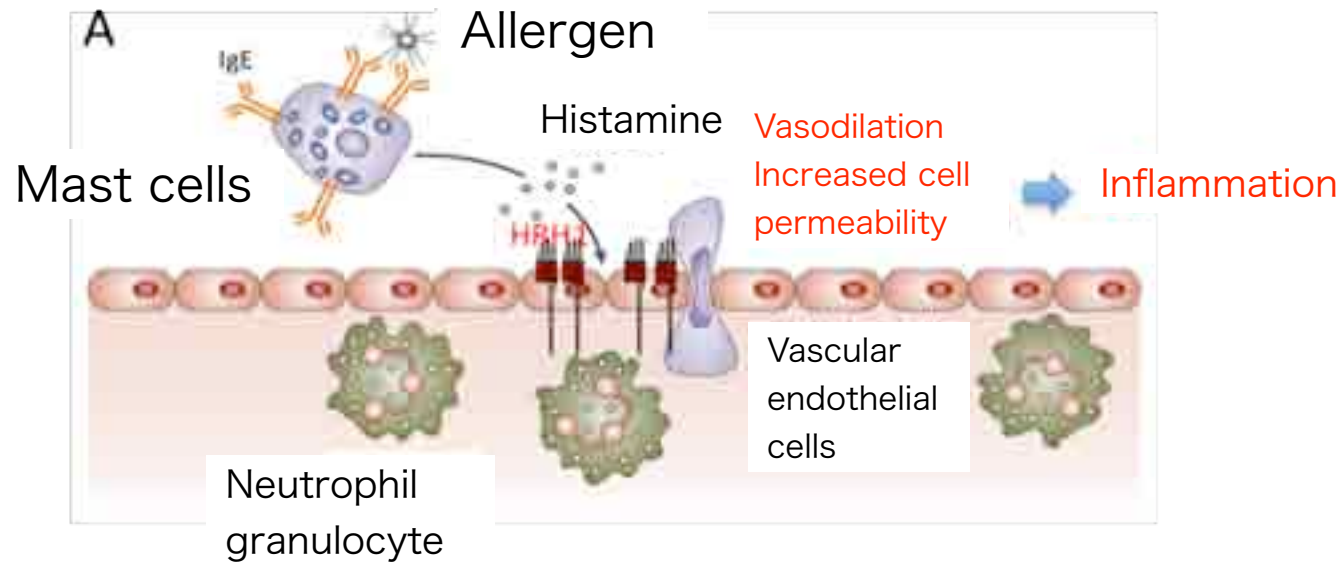
Ruben Abagyan

Allergic reactions and histamine



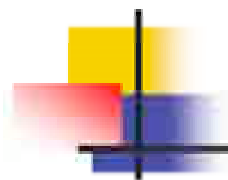
Histamine H₁ Receptor (H₁R)

- 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.



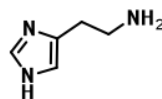
CNS
(histaminergic
neurons)

Histamine H₁ Receptor Antagonists (Inverse agonists, Antihistamines)



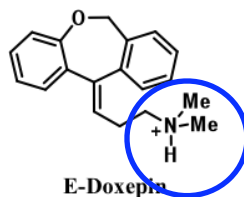
- Inhibit H₁R to alleviate allergic reactions.
- 1st gen. drugs show side effects (sedation, dry mouth, arrhythmia) because of BBB permeability and low H₁R selectivity.
- 2nd gen. drugs with a carboxylic moiety show reduced BBB permeability and higher H₁R selectivity.

Histamine

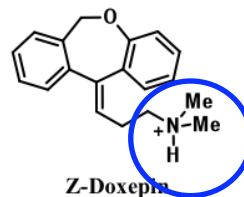


Histamine

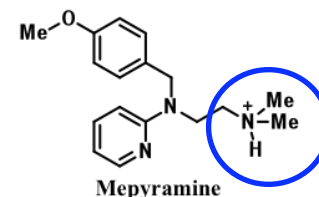
1st generation antihistamines



E-Doxepin

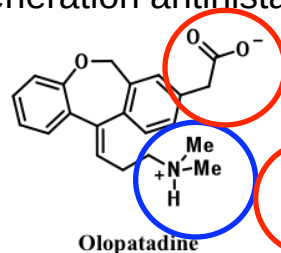


Z-Doxepin

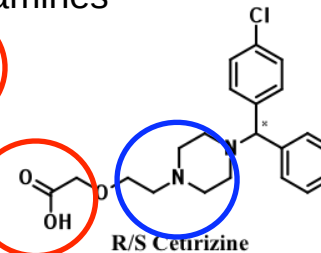


Mepyramine

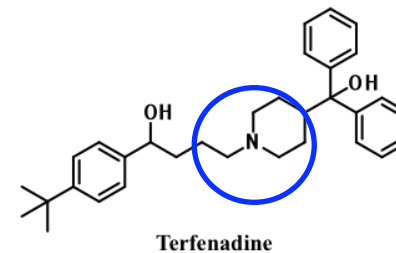
2nd generation antihistamines



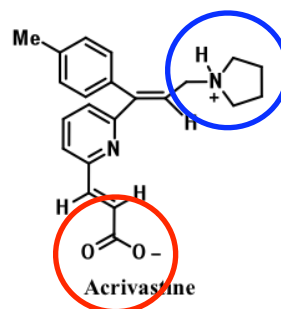
Olopatadine



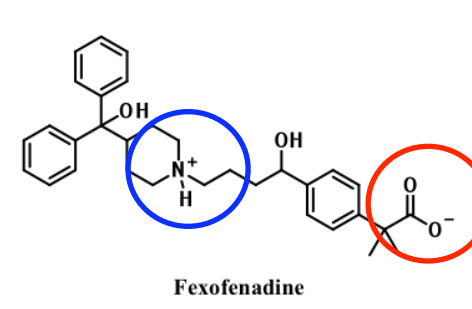
R/S Cetirizine



Terfenadine

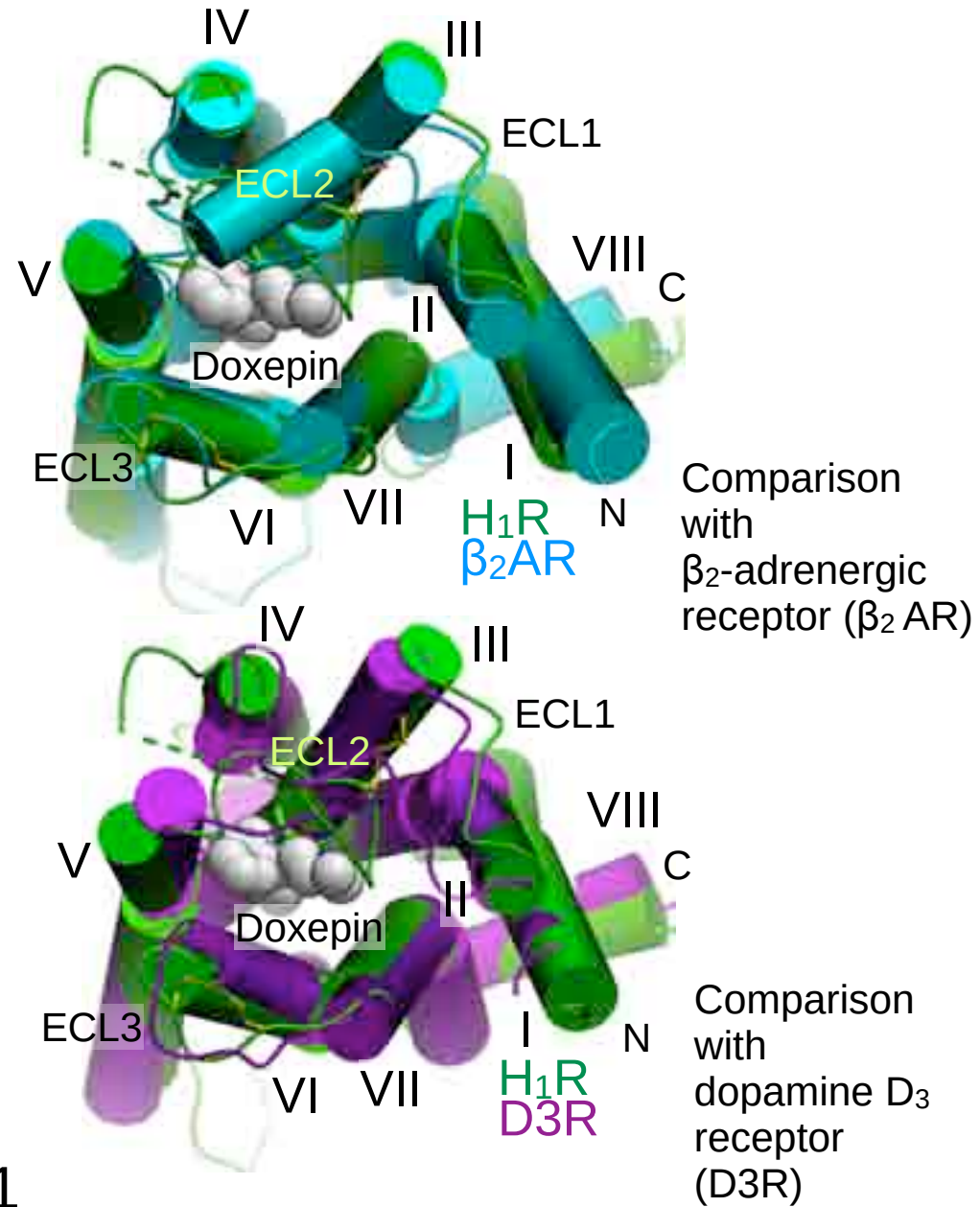
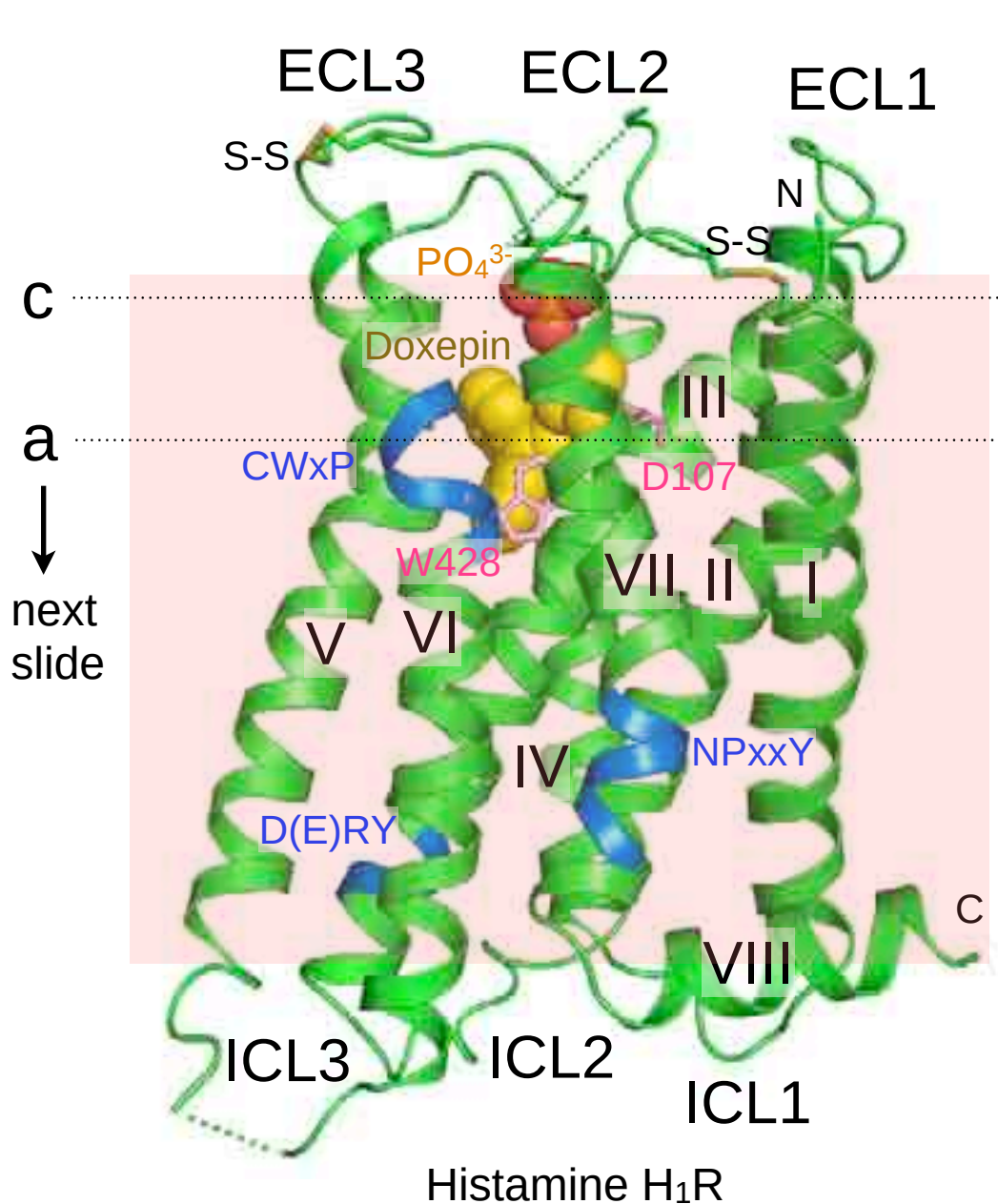


Acrivastine



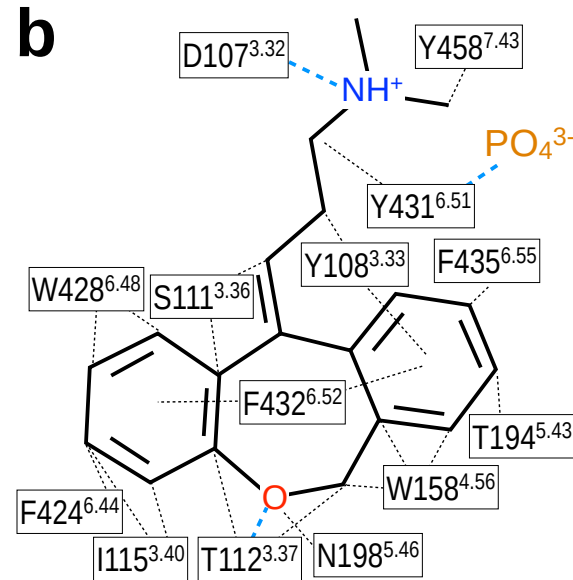
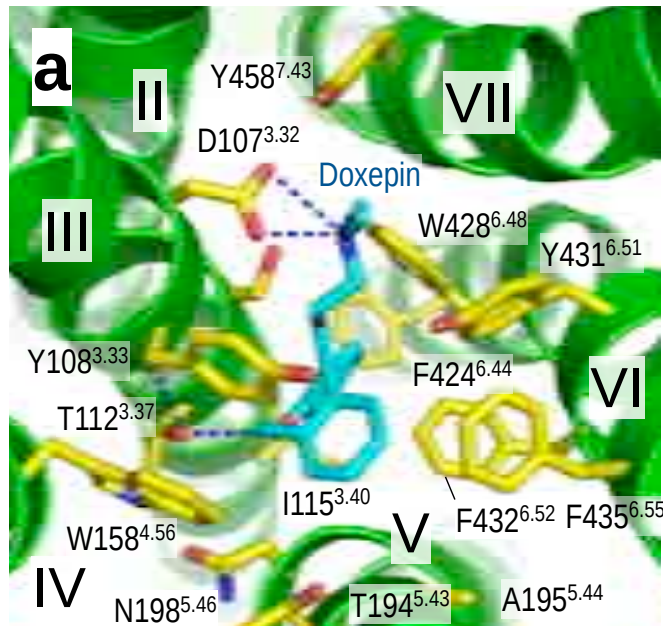
Fexofenadine

Structure of H₁R

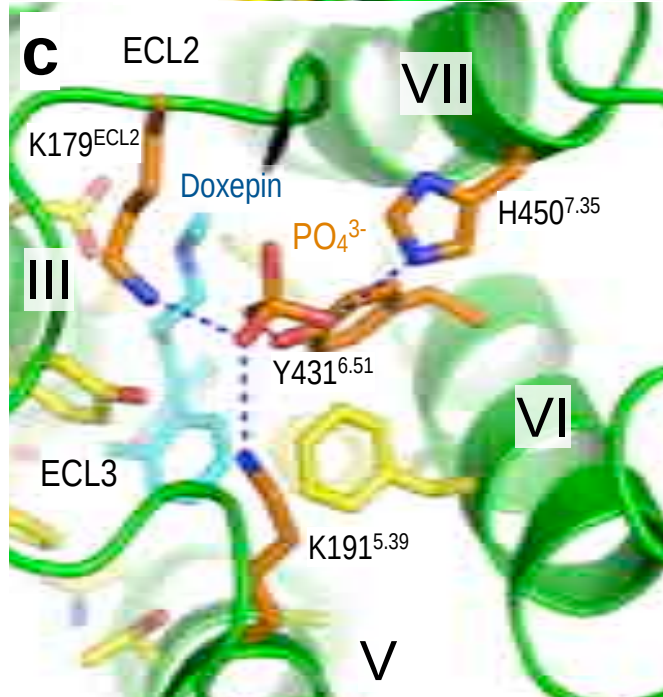


Binding interactions of doxepin

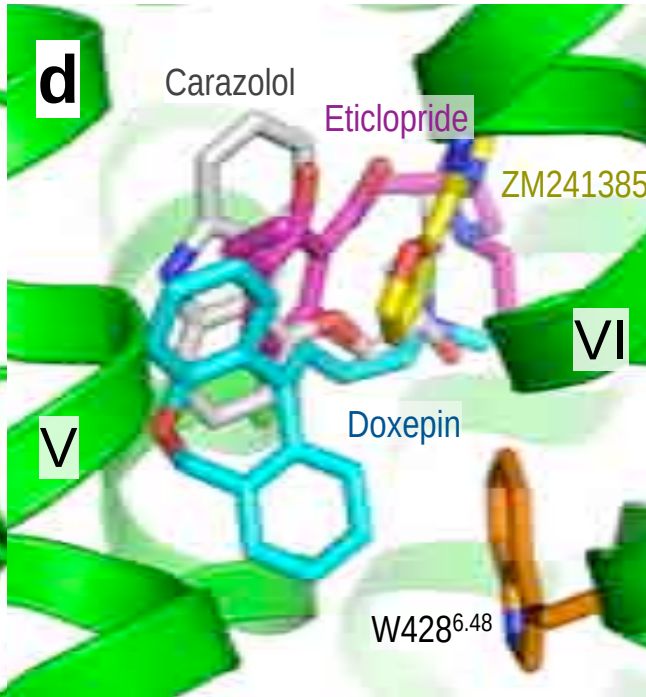
Doxepin binding site (1st gen. antihistamine)



Doxepin binding interactions



Anion-binding region with PO₄³⁻

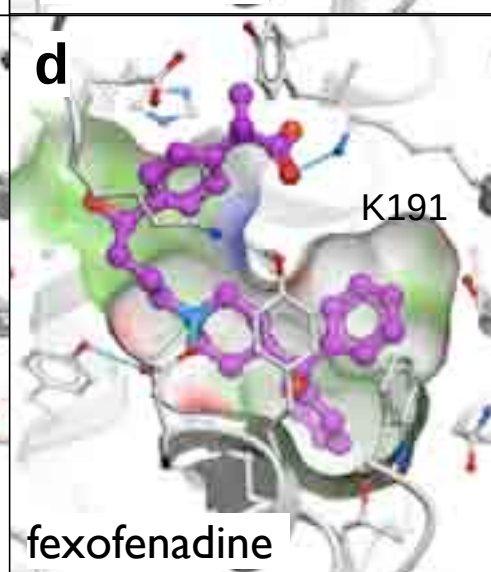
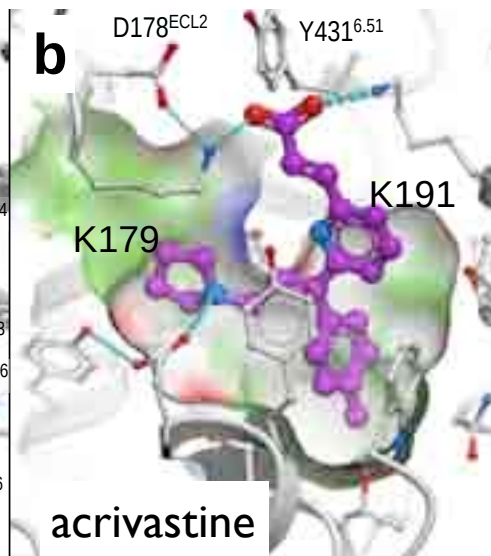
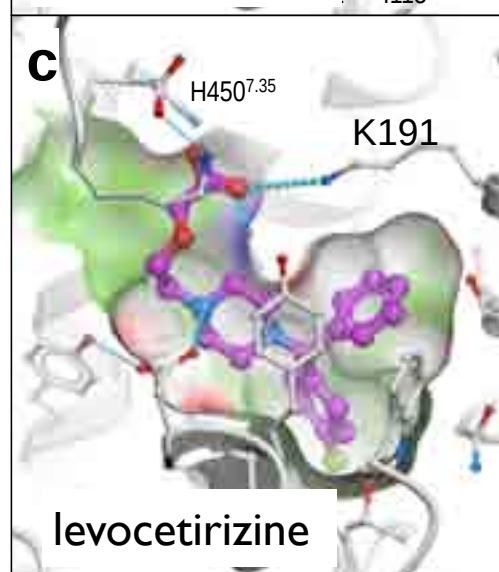
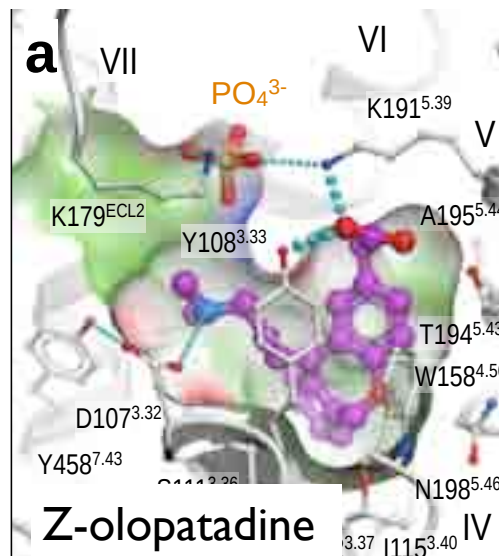
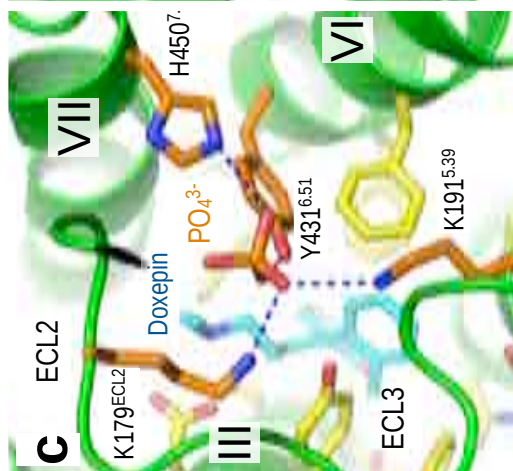
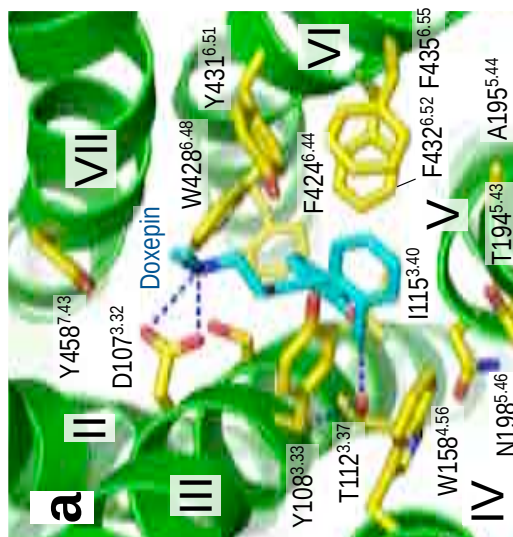


Ligand-binding positions in GPCRs

Interactions of 2nd generation antihistamines with H₁R

1st gen. antihistamine
(doxepin)

2nd gen. antihistamines (models)



Residues in PO₄³⁻ binding site, Lys 179, Lys 191, Tyr 431 are unique to H₁R.

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

