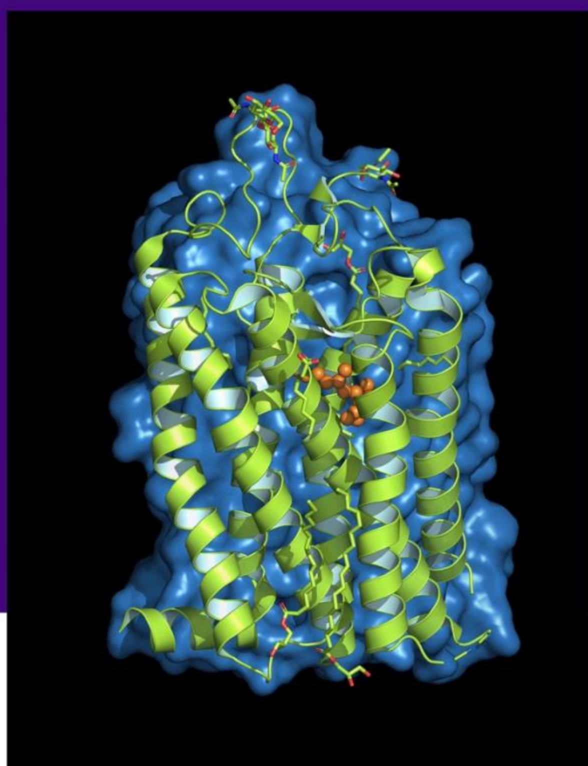
ICRP2024
Abstract book



20th International Conference on Retinal Proteins

17-21 Nov. 2024

Interlaken, Switzerland

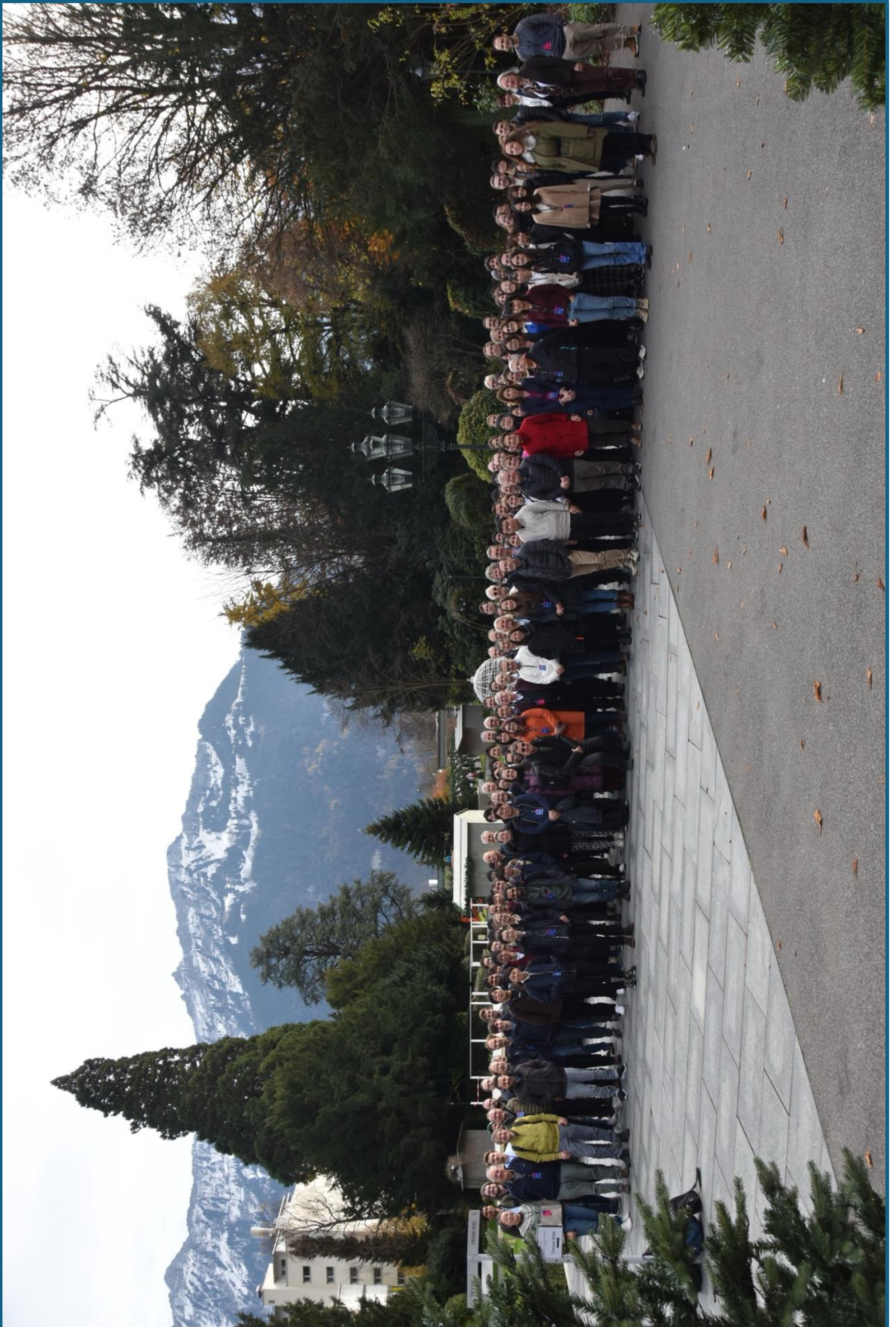
https://indico.psi.ch/event/15612/

Welcome to the 20th International Conference on Retinal Proteins.

We thank all the speakers, chairs and poster presenters for your engagement for the upcoming outstanding exchange and discussion. Your participation will definitely bring the field one step forward.

We thank all the conference participants for your support and exchange of ideas.

Enjoy the conference.



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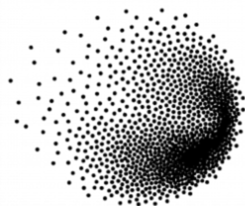

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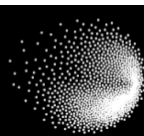

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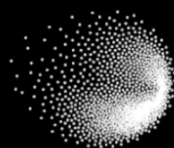

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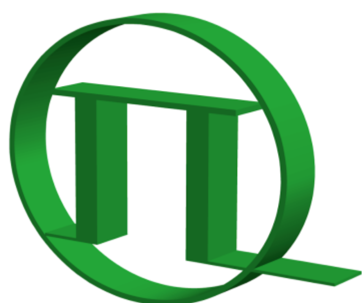

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Biophysical Journal Special Issue – Call for papers

We are thrilled to announce a special issue of *Biophysical Journal* dedicated to the 20th Retinal Protein Conference. This issue will showcase cutting-edge research and advancements in the study of retinal proteins and their vital role in light-sensing mechanisms in living organisms. We invite all conference participants and researchers to contribute their original research articles and invited reviews, allowing us to collectively celebrate four decades of innovation in this important field. Join us in publishing impactful contributions that enhance our understanding of retinal proteins and their functionality in light perception!

Detailed information: https://www.biophysics.org/Portals/0/BPSAssets/Publications/Documents/BJ_CallForPapers_RetinalProteins.pdf

Deadline for submission: June 30, 2025

Editor: Ana-Nicoleta Bondar, Gebhard Schertler, Ching-Ju Tsai

Biophysical Journal
Call for Papers

Special Issue: Retinal Proteins: Experiment and Computation

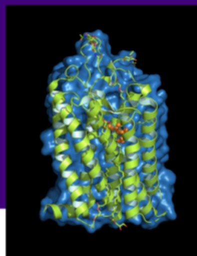
Editors: ***Ana-Nicoleta Bondar, University of Bucharest and Forschungszentrum Jülich***
Gebhard Schertler, Paul Scherrer Institut
Ching-Ju Tsai, Paul Scherrer Institut

Biophysical Journal will publish a special issue titled "Retinal Proteins: Experiment and Computation," dedicated to high-quality research on retinal proteins. We welcome submissions on all topics addressed at the 20th International Conference on Retinal Proteins, ICRP 2024, Interlaken, Switzerland. This includes cell signaling with retinal proteins; retinal proteins for optogenetics applications; discovery of new retinal proteins; physiology of retinal proteins; structure, function, and dynamics of retinal proteins; and advanced experimental and computational methods to study retinal proteins.

The Journal aims to publish the highest-quality work; articles should have sufficient importance to be of general interest to biophysicists regardless of their research specialty. Please contact the editors with queries about scope and suitability.

Deadline for submission: June 30, 2025

- Instructions for authors can be found at: https://www.cell.com/biophysj/authors.
- Please include a cover letter stating that you would like to contribute to the Retinal Proteins special issue and please describe why the work fits into the special issue.
- Normal publishing charges will apply.
- Questions can be addressed to the BJ Editorial Office at BJ@biophysics.org or to (240) 290-5600.



20th International Conference on Retinal Proteins

17 – 21 November 2024
Congress Kursaal
Interlaken, Switzerland

To submit, visit https://www.editorialmanager.com/biophysical-journal/

Announcement

Conference website

ICRP2024 = https://indico.psi.ch/event/15612/

ICRP archive = https://www.retinalproteins.org/

During the conference

About the abstract website

This website will only be available during the conferences +/- 7 days. The abstracts and the information on this website will be compiled into a single PDF file and distributed to all the participants by email. This PDF file will not be published online for search.

Photo shooting

We will take photos during the conference. When the conference is finished, photos will be deposited to the registration website https://indico.psi.ch/event/15612/.

If you wish not to appear in any photos, please inform us immediately.

Data Confidentiality

Please respect the scientific output and presentation. Photo shooting of posters or presentation content is not allowed. All content of the conference is confidential and not public disclosure.

Liability Statement

Please take care of your own personal items. We take no responsibility for the loss, theft, or damage of personal belongings brought onto the conference venue. Attendees are advised to take appropriate measures to secure their personal items.

Local Information

Medical emergency number	144
Police emergency number	117
Closest pharmacy	Apotheke Dr. Portmann AG, Höheweg 4, 3800 Interlaken (600 meter away)
Tourist Information	https://www.interlaken.ch/en/
Cold tap water	Drinkable

News 17-Nov:

Please mount your poster immediately. The assigned poster board number is written on your Welcome Envelope.

News 18-Nov:

1) Group photo shooting at 12:30.

2) Advisory board dinner: @Board_members, please meet at the entrance at 19:15.

News 19-Nov:

1) Dr. Emanuel Katzmann from JEOL will give a talk "Cryo-EM – Solutions by JEOL" at 12:30 in the Lecture Hall.

2) Tomorrow's excursion: visit Castle Spiez

News 20-Nov:

1) Excursion to the Castle Spiez. **Please come to the main entrance at 13:10.**

2) Personal items can be left at the cloakroom area downstairs during excursion.

3) Conference dinner will be held in a different room at 18:30.

Abstract Book and Photo Album download links

Abstract book:

https://indico.psi.ch/event/15612/attachments/27861/56918/ICRP2024_abstract_book.pdf

Photo Day 1 (17 Nov):

https://indico.psi.ch/event/15612/attachments/27861/56890/Photo_2024-11-17.zip

Photo Day 2 (18 Nov):

https://indico.psi.ch/event/15612/attachments/27861/56904/Photo_2024-11-18.zip

Photo Day 3 (19 Nov):

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Photo Day 4 (20 Nov):

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Photo Day 5 (21 Nov):

https://indico.psi.ch/event/15612/attachments/27861/56915/Photo_2024-11-21.zip

Timetable

Time\Date	Sunday-17-Nov-2024	Monday-18-Nov-2024	Tuesday-19-Nov-2024	Wednesday-20-Nov-2024	Thursday-21-Nov-2024
08:30-09:00		Preparation	Preparation	Preparation	Preparation
9:00	*(ERC SOL; ~20 ppl)*	10-min Session B Intro	10-min Session F Intro	10-min Session J Intro	10-min Session L Intro
9:10		20-min Talk B1	20-min Talk F1	20-min Talk J1	20-min Talk L1
9:20					
9:30		20-min Talk B2	20-min Talk F2	20-min Talk J2	20-min Talk L2
9:40					
9:50		20-min Talk B3	20-min Talk F3	20-min Talk J3	20-min Talk L3
10:00					
10:10		20-min Talk B4	20-min Talk F4	20-min Talk J4	20-min Talk L4
10:20					
10:30		Coffee break	Coffee break	Coffee break	20-min Talk L5
10:40					
10:50					Coffee break
11:00		10-min Session C Intro	10-min Session G Intro	10-min Session K Intro	
11:10		20-min Talk C1	20-min Talk G1	20-min Talk K1	
11:20					10-min Session M Intro
11:30		20-min Talk C2	20-min Talk G2	20-min Talk K2	20-min Talk M1
11:40					
11:50		20-min Talk C3	20-min Talk G3	20-min Talk K3	20-min Talk M2
12:00	Conference Start 12:00				
12:10	Register	20-min Talk C4	20-min Talk G4	20-min Talk K4	20-min Talk M3
12:20	mount poster				
12:30		Group Photo	Lunch + Poster	Light lunch + Poster	20-min Talk M5
12:40		Lunch + Poster			
12:50					Lunch + Poster
13:00					
13:10					
13:20				Bus ride	
13:30		10-min Session D Intro	10-min Session H Intro	Bus ride	
13:40		20-min Talk D1	20-min Talk H1	Bus ride	
13:50				Bus ride	10-min Session N Intro
14:00		20-min Talk D2	20-min Talk H2	Excursion	20-min Talk N1
14:10				Excursion	
14:20		20-min Talk D3	20-min Talk H3	Excursion	20-min Talk N2
14:30	Welcome speech			Excursion	
14:40		20-min Talk D4	20-min Talk H4	Excursion	20-min Talk N3
14:50	10-min Session A Intro			Excursion	
15:00	20-min Talk A1	Coffee break	Coffee break	Excursion	20-min Talk N4
15:10				Excursion	
15:20	20-min Talk A2			Excursion	20-min Talk N5
15:30		10-min Session E Intro	10-min Session I Intro	Excursion	
15:40	20-min Talk A3	20-min Talk E1	20-min Talk I1	Excursion	Coffee break
15:50				Excursion	unmount poster
16:00	20-min Talk A4	20-min Talk E2	20-min Talk I2	Excursion	
16:10				Excursion	
16:20	Coffee break	20-min Talk E3	20-min Talk I3	Bus ride	Keynote IV
16:30	mount poster			Bus ride	
16:40		20-min Talk E4	20-min Talk I4	Bus ride	
16:50				Bus ride	
17:00	Keynote I	Coffee break	Coffee break	X empty X	
17:10				X empty X	
17:20				X empty X	Plenary discussion
17:30		Keynote II	Keynote III	Poster and social hour	
17:40					
17:50					
18:00	Talk "Past --- Future"				
18:10					
18:20	20-min Flash poster Talks				
18:30		20-min Flash poster Talks	20-min Flash poster Talks	Conference dinner	Conference End 18:30
18:40	X empty X				
18:50	Poster and social hour	X empty X	X empty X		
19:00	light dinner provided	Poster and social hour	Poster and social hour		
19:10					
19:20					
19:30		(Advisory board dinner)			
...					
...					
...					
...					
22:00	bar open til 22:00	bar open til 22:00	bar open til 22:00	bar open til 22:00	
	Sunday-17-Nov-2024	Monday-18-Nov-2024	Tuesday-19-Nov-2024	Wednesday-20-Nov-2024	Thursday-21-Nov-2024

Program

Time\Date		Chair/Speaker	Title
Time\Date		Sunday-17-Nov-2024	
12:00-14:30			Registration
14:30-14:50		Gebhard Schertler, Massimo Olivucci	Welcome speech
14:50-15:00	**Session A**	**Chair = Steven Smith**	**Session A = Trends of retinal protein signaling research**
15:00-15:20	Talk A1	Oliver Ernst	Structural insights into light-gating of potassium-selective channelrhodopsin
15:20-15:40	Talk A2	Martha E. Sommer	What rhodopsin shows us about arrestin coupling at 7TMRs
15:40-16:00	Talk A3	Josef Wachtveitl	Near-UV und IR spectroscopic markers for retinal configuration during the photocycle of microbial rhodopsins
16:00-16:20	Talk A4	Ching-Ju Tsai	Active state structures of a bistable visual opsin bound to G proteins
16:20-17:00			Coffee break & mount poster
17:00-18:00	Keynote 1	Brian Kobilka (chaired by Gebhard Schertler)	The role of protein dynamics in G protein coupled receptor signaling
18:00-18:20		Gebhard Schertler	Linking the past to the future
18:20-18:40	Flash Talk	Poster presenters	Flash poster Talks. 6 poster presenters (Aditya Lakshminarasimhan; Satoshi Tsunoda; Songhwan Hwang; Andreea Pantiru; Anika Spreen; Jonas Mühle)
18:40-18:50			<<buffer time>>
18:50-22:00			Poster session at the bar, light dinner provided
Time\Date		Monday-18-Nov-2024	
08:30-09:00			<<preparation>>
09:00-09:10	**Session B**	**Chair = Kwang-Hwan Jung**	**Session B = Structural mechanism of microbial rhodopsins**
09:10-09:30	Talk B1	Clemens Glaubitz	Molecular mechanisms and evolutionary robustness of a color switch in proteorhodopsins – a solid-state NMR and computational approach
09:30-09:50	Talk B2	Hideaki Kato	Structural diversity of channelrhodopsins
09:50-10:10	Talk B3	Matthias Broser	Structural elucidation of the far-red absorbing and highly fluorescent retinal chromophore in fungal neorhodopsins
10:10-10:30	Talk B4	Ritsu Mizutori	Structural basis for proton transporting mechanism in viral heliorhodopsin, V2HeR3
10:30-11:00			Coffee break
11:00-11:10	**Session C**	**Chair = Xavier Deupi**	**Session C = Discovery of new animal rhodopsins**
11:10-11:30	Talk C1	Zuzana Musilova	See you in the dark: rhodopsin-based visual system in the deep-sea fishes
11:30-11:50	Talk C2	Marjorie Lienard	Functional evolution and spectral tuning mechanisms of insect visual Gq opsins
11:50-12:10	Talk C3	Polina Isaikina	Characterization of Butterfly Long-Wavelength Opsin for Advanced Optogenetics
12:10-12:30	Talk C4	Alina Pushkarev	Crustaceans as a source of new bistable rhodopsins for optogenetic applications
12:30-13:30			Group photo Lunch + Poster
13:30-13:40	**Session D**	**Chair = Josef Wachtveitl**	**Session D = Dynamics of retinal proteins**
13:40-14:00	Talk D1	Stefan Haacke	New Insights on the Ultrafast Photophysics of Archaerhodopsin-3 and its Fluorescent Mutants
14:00-14:20	Talk D2	Gerrit Lamm	The photochemistry of a microbial rhodopsin from Cryobacterium levicorallinum investigated by time-resolved optical spectroscopy
14:20-14:40	Talk D3	Giorgia Ortolani	Structural Basis for the Prolonged Photocycle of Sensory Rhodopsin II Revealed by Serial Synchrotron Crystallography
14:40-15:00	Talk D4	Yosuke Mizuno	Light-induced FTIR spectroscopy of microcrystals of visual rhodopsin grown in LCP
15:00-15:30			Coffee break
15:30-15:40	**Session E**	**Chair = Keiichi Inuoe**	**Session E = Advanced methods for retinal proteins** **Pioneering the Next Revolution in Protein Mechanistic Insights with Cutting-Edge Methodologies**
15:40-16:00	Talk E1	Miroslav Kloz	Femtosecond Stimulated Raman Spectroscopy: A Tool Tailored for the Study of Rhodopsin Dynamics
16:00-16:20	Talk E2	John Kennis	Reaction Dynamics and Mechanisms of Newly Discovered Bistable Microbial Rhodopsins
16:20-16:40	Talk E3	Thomas Perkins	Quantifying a light-induced energetic change in a single molecule of bacteriorhodopsin by atomic force microscopy
16:40-17:00	Talk E4	Feng-jie Wu	Elucidating GPCR conformational dynamics by a novel NMR method

17:00-17:30			Coffee break
17:30-18:30	Keynote 2	Rich Mathies (chaird by Massimo Olivucci)	Evolution of a Coherent Picture of Visual Photochemistry
18:30-18:50	Flash Talk	Poster presenters	Flash poster Talks. 6 poster presenters (Hartmut Oschkinat; Takashi Nagata; Tillmann Utesch; Keiichi Kojima; Ramprasad Misra; Masae Konno)
18:50-19:00			<< buffer time >>
19:00-22:00			Poster session at the bar (Advisory board dinner at 19:30 in a restaurant)
Time\Date		Tuesday-19-Nov-2024	
08:30-09:00			<<preparation>>
09:00-09:10	**Session F**	**Chair = Martha Sommer (replacing Thomas Sakmar)**	**Session F = Function of animal rhodopsins and related proteins**
09:10-09:30	Talk F1	Takahiro Yamashita	Characterization of red-sensitive non-visual opsins
09:30-09:50	Talk F2	Yuji Furutani	Structural key elements crucial for function of Krokinobacter rhodopsin 2 and dynamics of heliorhodopsin
09:50-10:10	Talk F3	Kota Katayama	How far can structure-spectroscopy studies of cone pigments approach the essence of the spectral tuning mechanism?
10:10-10:30	Talk F4	Lee Harkless	The role of RGS proteins in determining melanopsin signaling outcomes
10:30-11:00			Coffee break
11:00-11:10	**Session G**	**Chair = Judith Klein-Seetharaman**	**Session G = Physiology of animal retinal proteins**
11:10-11:30	Talk G1	Stephan Neuhauss	From Light to Sight: Retinal Proteins in the Regulation of Photoreceptor Signaling in the Zebrafish Retina
11:30-11:50	Talk G2	Deborah Walter	Engineering an OptoGPCR based on a bistable rhodopsin for optogenetic applications
11:50-12:10	Talk G3	W Ajith Karunarathne	Melanopsin Governs Wavelength-Dependent Cell Signaling and Animal Behavior
12:10-12:30	Talk G4	Thomas Mager	ChReef – An improved ChR for Future Optogenetic Therapies
12:30-13:30		Emanuel Katzmann	Cryo-EM – Solutions by JEOL & Lunch + Poster
13:30-13:40	**Session H**	**Chair = Richard Neutze**	**Session H = Photopharmacology and dynamics of retinal proteins**
13:40-14:00	Talk H1	Amadeu Llebaria	Photopharmacology: light and molecules for dynamic structural crystallography
14:00-14:20	Talk H2	Jörg Standfuss	Photopharmacology the Movie: How Rhodopsins Pave the Way for a Dynamic Future in Structural Biology
14:20-14:40	Talk H3	Eriko Nango	Structural Dynamics of Microbial Rhodopsins Captured by X-ray Free Electron Lasers
14:40-15:00	Talk H4	Valerie Panneels	Ultrafast Dynamics of Our Light-Receptor for Vision Rhodopsin, Using an X-ray Free Electron Laser
15:00-15:30			Coffee break
15:30-15:40	**Session I**	**Chair = Igor Schapiro**	**Session I = Theoretical approaches in retinal proteins**
15:40-16:00	Talk I1	Massimo Olivucci	Comparative Computational Studies of Animal Rhodopsins
16:00-16:20	Talk I2	Flurin Hidbar	LAMBDA: Light Absorption Modeling via Binding Domain Analysis
16:20-16:40	Talk I3	Ana-Nicoleta Bondar	Graph-based methodologies for direct comparisons of protein-water hydrogen-bond networks in visual and microbial rhodopsins
16:40-17:00	Talk I4	Xavier Deupi	Rhodopsin Activation at Different Time Scales
17:00-17:30			Coffee break
17:30-18:30	Keynote 3	Richard Neutze (chaied by Jörg Standfuss)	Structural mechanism of proton pumping by bacteriorhodopsin: an historical overview
18:30-18:50	Flash Talk	Poster presenters	Flash poster Talks. 5 poster presenters (Mako Aoyama; Xuchun Yang; Michal Koblizek; Camille Brouillon; Alexey Alekseev)
18:50-19:00			<< buffer time >>
19:00-22:00			Poster session at the bar
Time\Date		Wednesday-20-Nov-2024	
08:30-09:00			<<preparation>>
09:00-09:10	**Session J**	**Chair = Yuji Furutani**	**Session J = Carotenoids in retinal protein function** **Microbial rhodopsins inevitably meet carotenoids for fully utilizing sunlight**
09:10-09:30	Talk J1	Andrey Rozenberg	Carotenoid antennas in proton-pumping rhodopsins from bacteria and archaea
09:30-09:50	Talk J2	Keiichi Inoue	Spectroscopic study on carotenoid binding ion-transporting microbial rhodopsins

09:50-10:10	Talk J3	María del Carmen Marín Pérez	Light-harvesting by antenna-containing xanthorhodopsin from an Antarctic cyanobacterium
10:10-10:30	Talk J4	Shin-Gyu Cho	Heliorhodopsin-mediated light-modulation of ABC transporter
10:30-11:00			Coffee break
11:00-11:10	Session K	Chair = Joerg Standfuss	Session K = Ion channel rhodopsins
11:10-11:30	Talk K1	Quentin Clement Bertrand	Structural effects of high laser power densities on an early bacteriorhodopsin photocycle intermediate
11:30-11:50	Talk K2	Matthias Mulder	Structural insights into the opening mechanism of Channelrhodopsin C1C2
11:50-12:10	Talk K3	Han Sun	Channel opening and ion conduction mechanism in channelrhodopsin C1C2, ChR2, and iChloC
12:10-12:30	Talk K4	Joachim Heberle	Mechanism of the chloride pump NmHR in protein crystals, detergent micelles, and living cells
12:30-13:20			Lunch
13:20-17:00			Excursion
17:00-17:30			<< buffer time >>
17:30-22:00			Poster session at the bar Conference dinner at 18:30
Time\Date		Thursday-21-Nov-2024	
08:30-09:00			<<preparation>>
09:00-09:10	**Session L**	**Chair = Andreea Pantiru**	**Session L = Optogenetics with bistable rhodopsins**
09:10-09:30	Talk L1	Akihisa Terakita	Diverse coral opsins and their molecular properties
09:30-09:50	Talk L2	Mitsumasa Koyanagi	Evolution of jumping spider rhodopsin for optimizing depth perception from image defocus
09:50-10:10	Talk L3	Sonja Kleinlogel	A visual opsin from jellyfish enables precise temporal control of G protein signaling
10:10-10:30	Talk L4	Johannes Vierock	pHRoG: pH Regulating optoGenes for all-optical control of subcellular pH
10:30-10:50	Talk L5	Richard McDowell	Spectral tuning of mammalian melanopsins
10:50-11:20			Coffee break
11:20-11:30	**Session M**	**Chair = Ana-Nicoleta Bondar**	**Session M = Ion-transporting mechanism in microbial rhodopsins**
11:30-11:50	Talk M1	Kirill Kovalev	4D structural studies of the light-driven sodium pump ErNaR
11:50-12:10	Talk M2	Moran Shalev-Benami	'Light Up the Dance Floor' – Cryo-EM Studies of Bestrhodopsins Provide New Snapshots of Light-Based Activation Mechanisms
12:10-12:30	Talk M3	Przemysław Nogły	Key residues in the transport mechanism of chloride pumping rhodopsin
12:30-12:50	Talk M4	Kwang-Hwan Jung	Dual roles of proton pumping rhodopsin in Gloeobacter: Energy production and gene regulation
12:50-13:50			Lunch + Poster
13:50-14:00	**Session N**	**Chair = Peter Hegemann**	**Session N = From structure to physiology of retinal proteins**
14:00-14:20	Talk N1	Shunki Takaramoto	ApuRhs, a new family of anion channelrhodopsin from apusomonads
14:20-14:40	Talk N2	Shoko Hososhima	Proton transport mechanism of viral heliorhodopsin, V2HeR3
14:40-15:00	Talk N3	Wayne Busse	Localization of the Fluorescent Rhodopsin NeoR in Fungal Zoospores with Insights into Its Enzymatic Functionality
15:00-15:20	Talk N4	Judith Klein-Seetharaman	A Comprehensive Rhodopsin Dataset and Quantitative Molecular Docking Analysis of Rhodopsin-Retinal Interactions
15:20-15:40	Talk N5	Phyllis Robinson	Melanopsin, from Molecule to Behavior
15:40-16:20			Coffee break unmount poster
16:20-17:20	Keynote 4	Robert J Lucas (chaired by Akihisa Terakita)	Animal opsins, from understanding unconventional vision to optogenetic application(s)
17:20-18:20			Plenary discussion
18:20-18:30			<< buffer time >>
18:30			Conference End

Keynote

◉ Keynote 1 >> Brian Kobilka (Stanford University, USA)

The role of protein dynamics in G protein coupled receptor signaling

Chaired by Gebhard Schertler

G protein coupled receptors (GPCRs) conduct the majority of transmembrane responses to hormones and neurotransmitters and mediate the senses of sight, smell and taste. Thanks to advances in protein engineering, crystallography and cryo-electron microscopy (cryoEM) over the past 14 years there are now more than 500 deposited high-resolution structures of GPCRs including inactive states, active states and GPCR-G protein complexes. These structures have provided important insights into common mechanisms of G protein activation for Family A and Family B GPCRs. However, they don't fully explain the complex behavior of many GPCRs that signal through more than one G protein isoform, and through G protein independent pathways. Moreover, we still do not fully understand the mechanism of G protein coupling specificity. This complex functional behavior provides evidence for the existence of multiple functionally distinct conformational states that may be too transient or unstable to be captured by crystallography or cryoEM. We have used fluorescence, EPR and NMR spectroscopy to study the dynamic properties of several GPCRs. I will discuss a few examples of what these studies have taught us about the role of protein dynamics in GPCR signaling.

◉ Keynote 2 >> Richard Mathies (University of California, Berkeley, USA)

Evolution of a Coherent Picture of Visual Photochemistry

Chaired by Massimo Olivucci

The evolution of our understanding of the mechanism of the cis-to-trans photochemical isomerization in rhodopsin that initiates the key sense of vision is presented. Resonance Raman intensity analysis provided the earliest spectroscopic indications of the femtosecond nuclear isomerization dynamics in rhodopsin. Subsequent femtosecond (10^{-15} s, fs) transient absorption experiments showed that the transition from the 11-cis reactant to a high-energy all-trans photoproduct was complete in only 200 fs. Higher time resolution transient absorption experiments revealed coherent vibrational oscillations in the photoproduct absorption demonstrating that after impulsive excitation the reaction was so fast that "vibrational quantum beats" survived the internal conversion to ground state product. The mechanistic importance of the observed vibrational coherence was revealed by novel isotopic labeling demonstrating the role of the relative phase of the retinal hydrogen-out-of-plane or HOOP modes for the efficiency of surface crossing from cis-reactant to trans-photoproduct. Together this work presents a “vibrationally coherent model” for the reactive surface crossing in rhodopsin and a novel Vibrational Phase Isotope Effect in chemical reactivity.

My presentation will also briefly recognize the important contributions of Professor Johan Lugtenburg (Leiden, 1942-2023) and Prof. Dr. Lubert Stryer (Stanford, 1938-2024) to the Retinal Proteins field. Johan and his group played a critical role using physical organic chemical synthesis of novel isotopically-labeled retinals to reveal photochemical mechanisms in retinal proteins. In addition to his many contributions to understanding Biochemistry, Lubert and his colleagues revealed the role of transducin in the G-protein cyclic nucleotide activation mechanism of visual pigments. It was a privilege to work with both of these talented and creative scientists and individuals.

◉ Keynote 3 >> Richard Neutze (University of Gothenburg, Sweden)

Structural mechanism of proton pumping by bacteriorhodopsin: an historical overview

Chaired by Jörg Standfuss

In the half-century since its discovery (1), bacteriorhodopsin has been studied extensively so as to understanding how light energy may be utilized to pump protons up-hill across an energy transducing biological membrane. Numerous biophysical methods have been developed to explore the function of bacteriorhodopsin and these have had major impact on many fields of life-science research. In this lecture I will review historical developments towards understanding the structural biology of bacteriorhodopsin, beginning from the first structural insights from this integral membrane protein (2). I will emphasize the development of synchrotrons and X-ray free electron lasers as user facilities for macromolecular crystallography, and overview the development of time-resolved X-ray diffraction methods at these sources (3). I will summarize structural findings that emerged from low-temperature trapping studies of the reaction intermediates of bacteriorhodopsin (4), and relate these to later time-resolved serial crystallography studies of real-time light-initiated structural changes in bacteriorhodopsin (5-7). A remarkably consistent picture for the structural mechanism of proton pumping by bacteriorhodopsin emerges from this very large body of work.

(1) D. Oesterhelt, W. Stoeckenius, Rhodopsin-like protein from the purple membrane of Halobacterium halobium. Nat New Biol 233, 149-152 (1971).

(2) R. Henderson, P. N. Unwin, Three-dimensional model of purple membrane obtained by electron microscopy. Nature 257, 28-32 (1975).

(3) G. Branden, R. Neutze, Advances and challenges in time-resolved macromolecular crystallography. Science 373, (2021).

(4) C. Wickstrand et al., Bacteriorhodopsin: Structural Insights Revealed Using X-Ray Lasers and Synchrotron Radiation. Annu Rev Biochem 88, 59-83 (2019).

(5) E. Nango et al., A three-dimensional movie of structural changes in bacteriorhodopsin. Science 354, 1552-1557 (2016).

(6) P. Nogly et al., Retinal isomerization in bacteriorhodopsin captured by a femtosecond x-ray laser. Science 361, (2018).

(7) T. Weinert et al., Proton uptake mechanism in bacteriorhodopsin captured by serial synchrotron crystallography. Science 365, 61-65 (2019).

◉ Keynote 4 >> Robert Lucas (University of Manchester, UK)

Animal opsins, from understanding unconventional vision to optogenetic application(s)

Chaired by Akihisa Terakita

Animals have solved the problem of how to make cells photosensitive by evolving light-activated G protein coupled receptors (animal rhodopsins). Thus, studying the animal rhodopsin family holds the promise of understanding the diverse array of light-dependent behaviours and physiologies that animals display, and achieving optogenetic control over G-protein signalling events for experimental and therapeutic purposes. I will provide an overview of my laboratory's efforts to exploit this opportunity first to gain a richer understanding of mammalian photobiology and second to explore the challenges and opportunities of animal opsin optogenetics.

SESSION A – Trends of retinal protein signaling research

► Session A Indroduction >> Steven Smiths (Stony Brook University, USA)

Retinal – A Versatile Light-Driven Engine

This session covers cutting-edge research on retinal-containing proteins, spanning a wide range from microbial rhodopsins to vertebrate and invertebrate rhodopsins. The talks by Oliver Ernst and Joseph Wachtveit focus on the structure and function of microbial rhodopsins. When the first Retinal Proteins Conference was held in 1984, bacteriorhodopsin and halorhodopsin were the only microbial rhodopsins that had been discovered. There are now hundreds of unique microbial membrane proteins that are known to use light energy absorbed by a covalently attached retinal prosthetic group to drive protein function. The current talks will discuss retinal proteins that function as both ion pumps and ion channels. Oliver Ernst makes use of a range of methods to elucidate the mechanism of light-gating by a potassium-selective channel-rhodopsin. Using near-UV and IR spectroscopy, Joseph Wachtveit describes mechanistic features in the photoreaction of several microbial rhodopsin ion pumps. Together, these studies will highlight the way that microbial rhodopsins laid the foundation for and have become essential tools in the field of optogenetics.

Ching-Ju Tsai and Martha Sommers will showcase their studies on visual rhodopsins and their associated effector proteins. Almost 40 years ago, visual rhodopsins emerged as one of the founding members of the large G protein-coupled receptor (GPCR) superfamily. Prior to this time there was a lively debate as to whether the visual rhodopsins functioned as calcium channels or GPCRs. Brian Kobilka and Robert Lefkowitz settled the debate through comparisons of the rhodopsin and β2 adrenergic receptor sequences. As with the microbial rhodopsins, there are now hundreds of known, unique vertebrate and invertebrate rhodopsins that function as either visual or non-visual receptors. A glimpse of the complexity of these signaling systems will be provide by Ching-Ju Tsai and Martha Sommers. Ching-Ju Tsai will describe a bistable opsin from the jumping spider in complex with its downstream G protein signaling partners, while Martha Sommers will review the intriguing arrestin structure-function relationships in the visual receptor rhodopsin.

Talk A1 >> Oliver Ernst (University of Toronto, Canada)

Structural insights into light-gating of potassium-selective channelrhodopsin

Takefumi Morizumi, Kyumhyuk Kim, Hai Li, Probal Nag, Tal Dogon, Oleg A. Sineshchekov, Y. Wang, Leonid S. Brown, Songhwan Hwang, Han Sun, Ana-Nicoleta Bondar, Igor Schapiro, Elena G. Govorunova, John L. Spudich, & **Oliver P. Ernst**

Structural information on channelrhodopsins' mechanism of light-gated ion conductance is scarce, limiting its engineering as optogenetic tools. We used single-particle cryo-electron microscopy of peptidisc-incorporated protein samples to determine the structures of the slow-cycling mutant C110A of kalium channelrhodopsin 1 from Hyphochytrium catenoides (HcKCR1) in the dark and upon laser flash excitation. Upon photoisomerization of the retinal chromophore, the retinylidene Schiff base NH-bond reorients from the extracellular to the cytoplasmic side. This switch triggers a series of side-chain reorientations and merges intramolecular cavities into a transmembrane K+ conduction pathway. Molecular dynamics simulations confirm K+ flux through the illuminated state but not through the resting state. The overall displacement between the closed and the open structure is small, involving mainly side-chain rearrangements. Asp105 and Asp116 play a key role in K+ conductance. Structure-guided mutagenesis and patch-clamp analysis reveal the roles of the pathway-forming residues in channel gating and selectivity.

Co-authors

Ana-Nicoleta Bondar (University of Bucharest, Faculty of Physics); Leonid Brown (University of Guelph); Tal Dogon (The Hebrew University of Jerusalem); Elena Govorunova (University of Texas Medical School at Houston); Songhwan Hwang (Leibniz-Forschungsinstitut für Molekulare Pharmakologie); Kyumhyuk Kim (University of Toronto); Hai Li (University of Texas Medical School at Houston); Takefumi Morizumi (University of Toronto); Probal Nag (Hebrew University of Jerusalem); Igor Schapiro (The Hebrew University of Jerusalem); Oleg Sineshchekov (University of Texas Medical School at Houston); John Spudich (University of Texas Medical School at Houston); Han Sun (Leibniz-Forschungsinstitut für Molekulare Pharmakologie); Yumei Wang (University of Texas Medical School at Houston)

Talk A2 >> Martha Sommer (ISAR Bioscience, Germany)

What rhodopsin shows us about arrestin coupling at 7TMRs

The seven transmembrane helical receptor (7TMR) architecture appeared early in evolution, for example the retinal-binding microbial rhodopsins that harness light to pump ions across the cell membrane. The 7TMR design emerged again independently in multicellular life, and many of these early receptors also bound retinal to enable photoreception. However, instead of pumping ions, these new 7TMRs harnessed existing cytosolic proteins as signal transducers. The increase in system complexity was obviously advantageous, since today the G protein-coupled receptors (GPCRs) represent one of the largest gene families in mammals.

GPCRs number in the hundreds, and the diversity of ligands bound by these receptor subtypes is huge. Despite this, the intracellular GPCRs signal transduction machinery is

composed of relatively few components, e.g. ~20 G proteins, 7 GPCR kinases, and 4 arrestins. While it is unknown which of these components "arrived first" at the primordial receptor, arrestin-mediated regulation of GPCR signalling is nearly universal. Arrestins stem from an ancient lineage of membrane-associated scaffold proteins, and there are (increasingly) notable examples of 7TMR to do not couple to G proteins but do bind arrestins. Therefore, arrestin-coupling at ancient 7TMRs was a key event in the evolution of GPCRs.

Within this context, I will present a current assessment of the arrestin structure-function relationship based on our structural and biophysical studies of arrestin-1 and its dynamic interactions with vertebrate rhodopsin. Arrestin activation is not a simple switch but a complex interplay of different functional inputs (e.g. phosphorylated receptor C-terminus, 7TM core, membrane, etc.) that functionally synergize to modulate the conformational equilibrium of arrestin. In this way, the limited number of arrestin family members are able to regulate the substantially larger and diverse family of GPCRs.

Talk A3 >> Josef Wachtveitl (Goethe University Frankfurt, Germany)

Near-UV und IR spectroscopic markers for retinal configuration during the photocycle of microbial rhodopsins

Microbial rhodopsins form a large class of photoreceptive membrane proteins that contain a co- valently bound all-trans retinal chromophore in the dark state. Despite their global structural similarity, microbial rhodopsins exhibit a remarkable functional variability and can, for example, act as sensors, ion channels or (inward or outward) ion pumps. Not least because of their central role in optogenetics, it is important to identify overarching principles of the photoreaction through appropriate spectroscopic markers that combine seamless temporal observation with spectral sen- sitivity to the configurational changes of the chromophore during the photocycle.

The second bright state (SBS) of 13-cis retinal is a spectral signature in the near-UV region, ranging from femtoseconds up to seconds, originally described for the light-driven sodium pump KR2.[1]

Its spectral distinctiveness during the KR2 photocycle helped to cnfirm that the retinal adopts an all-trans configuration in the O(2)-state and also allowed blue light quenching (BLQ)-like optical control experiments over an expanded time scale.

We have now extended and generalized this concept in the framework of a systematic, time resolved spectroscopic study on H^+- (HsBR, (G)PR), Na^+- (KR2, ErNaR) and Cl^-- (NmHR) pumps utilizing both the UV/vis and the IR spectral range. In all cases the SBS serves as an excellent marker for retinal configuration, retinal torsion and - being sensitive

to the electrostatic environment - even transient ion binding, which allows us to pinpoint and follow mechanistic nuances of the respective proteins. The combination with studies in the mid-IR fingerprint region provides a spectroscopic analysis tool that allows a detailed, precise and temporally fully resolved description of retinal configurations during all stages of the photocycle.

[1] Asido, M., Kar, R.K., Kriebel, C. N., Braun, M., Glaubitz, C., Schapiro, I. and Wachtveitl, J. (2021) J. Phys. Chem. Lett., 12, 6284.

Talk A4 >> Ching-Ju Tsai (Paul Scherrer Institute, Switzerland)

Active state structures of a bistable visual opsin bound to G proteins

Opsins are G protein-coupled receptors that have evolved to detect light stimuli and initiate intracellular signaling cascades. Their role as signal transducers is critical to light perception across the animal kingdom. Opsins covalently bind to the chromophore 11-cis retinal, which isomerizes to the all-trans isomer upon photon absorption, causing conformational changes that result in receptor activation. Monostable opsins, responsible for vision in vertebrates, release the chromophore after activation and must bind another retinal molecule to remain functional. In contrast, bistable opsins, responsible for non-visual light perception in vertebrates and for vision in invertebrates, absorb a second photon in the active state to return the chromophore and protein to the inactive state. Structures of bistable opsins in the activated state have proven elusive, limiting our understanding of how they function as bidirectional photoswitches. Here we present active state structures of a bistable opsin, jumping spider rhodopsin isoform-1 (JSR1), in complex with its downstream signaling partners, the human Gi and the chimeric human Gi/jumping spider Gq heterotrimers. The active-state JSR1 is either reconstituted with 9-cis retinal followed by illumination, or with the analogue all-trans retinal 6.11, where C6 and C20 are cyclized to avoid isomerization to 11-cis configuration. These structures elucidate key differences in the activation mechanisms between monostable and bistable opsins, offering essential insights for the rational engineering of bistable opsins into diverse optogenetic tools to control G protein signaling pathways.

SESSION B – Structural mechanism of microbial rhodopsins

►Session B Introduction >> Kwang-Hwan Jung (Sogang University, Seoul Korea)

Structural mechanism of microbial rhodopsins

Talk B1 >> Clemens Glaubitz (Goethe University Frankfurt, Germany)

Molecular mechanisms and evolutionary robustness of a color switch in proteorhodopsins – a solid-state NMR and computational approach

Proteorhodopsins are widely distributed photoreceptors from marine bacteria. Their discovery revealed a high degree of evolutionary adaptation to ambient light, resulting in blue-and green-absorbing variants that correlate with a conserved glutamine/leucine at position 105. On the basis of an integrated approach combining DNP-enhanced solid-state NMR spectroscopy and linear-scaling quantum mechanics/molecular mechanics (QM/MM) methods, this single residue is shown to be responsible for a variety of synergistically coupled structural and electrostatic changes along the retinal polyene chain, ionone ring, and within the binding pocket. They collectively explain the observed color shift. Furthermore, analysis of the differences in chemical shift between nuclei within the same residues in green and blue proteorhodopsins also reveals a correlation with the respective degree of conservation. Our data show that the highly conserved color change mainly affects other highly conserved residues, illustrating a high degree of robustness of the color phenotype to sequence variation.

While PR is an outward proton pump, the second part of the presentation will address the inward proton pump XeR and the role of histidines in proton transfer.

Mao, J.; Jin, X.; Shi, M.; Heidenreich, D.; Brown, L. J.; Brown, R. C. D.; Lelli, M.; He, X.; Glaubitz, C., Molecular mechanisms and evolutionary robustness of a color switch in proteorhodopsins. Sci Adv 2024, 10 (4), eadj0384

Authors

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Talk B2 >> Hideaki Kato (The University of Tokyo, Japan)

Structural diversity of channelrhodopsins

Channelrhodopsins (ChRs) are light-activated ion channels that serve as pivotal tools in optogenetics for neuroscience research. Recent advancements in structural biology, spectroscopy, electrophysiology, and computational biology have significantly deepened our understanding of this fascinating protein family. However, the molecular mechanisms behind specific properties of ChRs—such as conductance, kinetics, absorption/action spectrum, and light sensitivity—remain elusive.

In this talk, we present cryo-electron microscopy structures of ~10 distinct ChRs, including both well-established optogenetic tools and those not yet utilized in optogenetics but exhibiting unique properties. This structural information allows us to develop a standardized numbering system, facilitating easier comparison and interpretation of ChR structures. Furthermore, our findings identify some key mechanisms that regulate the diverse properties exhibited by ChRs. These insights not only advance our fundamental understanding of ChR function but also inform the design of enhanced optogenetic applications.

Talk B3 >> Matthias Broser (Humboldt University of Berlin, Germany)

Structural elucidation of the far-red absorbing and highly fluorescent retinal chromophore in fungal neorhodopsins

Fungal neorhodopsins (NeoRs) exhibit unique spectral properties among rhodopsins, including far-red light absorption with maxima close to or even above 700 nm, high fluorescence, and an unusual photoisomerization from all-trans to 7-cis. These extreme photochemical characteristics are attributed to a specific protein environment surrounding the protonated retinal Schiff base (pRSB), which facilitates extensive delocalization of the positive charge along the chromophore in the electronic ground state. However, due to the lack of structural data, the precise interactions between the pRSB and surrounding amino acid side chains have remained elusive, and previous theoretical studies have relied on homology models.

Here, we present the crystal structure of the rhodopsin domain of NeoR from Rhizoclosmatium globosum in its far-red absorbing all-trans state, at 2.2 Å resolution. Our findings reveal an unexpectedly twisted retinal chromophore and provide definitive identification of all side chain rotamers as well as the precise locations of water molecules. The three carboxylate residues of the formal counterion triad—E136, D140, and E262—are tightly interconnected, forming an efficient barrier that prevents water from penetrating

the active site. These residues are embedded within an extensive hydrogen-bond network, ensuring strong interactions between adjacent helices via amino acid side chains. This rigid configuration excludes water-mediated ground state dynamics, explaining the narrow absorption band of NeoR. Notably, the closest carboxylate to the pRSB nitrogen is E136, located at a distance of 3.2 Å. Glutamate E141, proposed in a recent computational study as a key residue influencing the extreme photophysics of NeoR, is in close proximity (~3.2 Å) to the polyene chain. A single point mutation, D140T, in NeoR leads to a significant blue shift and broadening of the absorption spectrum, along with reduced fluorescence, and facilitates photoisomerization toward 9-cis and 11-cis. These altered properties are linked to increased water accessibility to the pRSB active site, owing to the less effective shielding.

Our results position NeoR as a valuable model for exploring the fundamental mechanisms of retinal photochemistry at the molecular level and as a promising template for the rational design of advanced optogenetic tools. Additionally, they provide a remarkable example of how natural evolution has fine-tuned the versatile rhodopsin scaffold to achieve extreme spectral properties of retinal.

Co-authors

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Talk B4 >> Ritsu Mizutori (Nagoya Institute of Technology, Japan)

Structural basis for proton transporting mechanism in viral heliorhodopsin, V2HeR3

V2HeR3 is the first heliorhodopsin with its function clarified as a proton transporter across the membrane. Patch-clamp measurements identified E191, E205, and E215 as important amino acids for proton transport. However, the detailed molecular mechanism remains unknown. In this study, we investigated the proton transport mechanism of V2HeR3 by X-ray crystallography, spectroscopic measurements, patch clamp measurements, and theoretical calculations.

We obtained the structure of the "dark state" under dim red light and the structure of the "light state" under laboratory light, both at a resolution of 2.1 Å. Significant differences between the two structures are observed. In the light state, the α-helix around the retinal Schiff base of TM7 is unwound, and two additional water molecules are inserted. To determine which photointermediate corresponds to the light state structure, we performed structural analysis of K and M intermediates using infrared spectroscopy. Focusing on the structure changes of protein backbone (amide I), an up-shifted band was observed in K intermediate compared to the ground state, indicating a weakening of the hydrogen bond strength of protein backbone. On the other hand, in the M intermediate, we observed a

down-shifted band, indicating a strengthening of the hydrogen bond strength in the protein backbone. Generally, the hydrogen bond strength of the protein backbone is considered weaker when the α-helix is unwound, suggesting that the light state structure reflects the K intermediate.

Furthermore, in both the K intermediate and M intermediate, we captured the C=O stretching vibration band corresponding to changes in the hydrogen bonding of protonated carboxylic acids derived from E191. From the results of transient absorption spectroscopy measurements, we found that mutating E191 to glutamine prolonged the lifetime of the M intermediate. Additionally, considering that water molecules situated near E191 in the light-state structure, it is suggested that E191 plays an important role in proton transport.

In the presentation, we propose a proton transport model for V2HeR3 based on the structures of the dark and light states, along with spectroscopic measurements, quantum chemical calculations, and the patch-clamp measurements on various mutants.

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SESSION C – Discovery of new animal rhodopsins

► Session C Introduction >> Xavier Deupi (Paul Scherrer Institute, Switzerland)

Discovery of new animal rhodopsins

This session focuses on the discovery and functional diversity of rhodopsins in various animal groups—fish, insects, and crustaceans—with an emphasis on their evolutionary adaptations, spectral properties, and potential applications in optogenetics. It brings together studies in genomics, molecular biology, and protein engineering, reflecting the conference's goal of exploring the many roles rhodopsins play in biological systems and technology.

The first two talks cover evolutionary aspects of animal rhodopsins, emphasizing ecological and behavioral adaptations. **Zuzana Musilova** opens by examining rhodopsins in deep-sea fishes, which have adapted through gene duplications to function in the low light and blue-green wavelengths of the deep ocean. Her studies show how unique molecular modifications in these rhodopsins represent evolutionary strategies for vision in challenging, light-limited environments. Following this, **Marjorie Lienard** discusses the evolution of insect visual systems, showing how molecular changes in opsins allow insects to fine-tune light sensitivity and color perception. Her findings highlight the relationship between environmental adaptations and opsin diversity, linking specific visual adaptations to behaviors like mate recognition and pollination, which are critical for survival and reproduction in these species.

The final two talks focus on transforming these naturally evolved opsins into tools for optogenetics. **Polina Isaikina** examines the butterfly long-wavelength opsin PxRh3, which she has characterized and engineered for optogenetic applications. This work explores how PxRh3's signaling properties can be modulated to control cellular functions, offering new possibilities for research and therapeutic innovations. **Alina Pushkarev** concludes with an exploration of crustacean rhodopsins, particularly those that respond to far-red light. These opsins are promising for deep-tissue optogenetics due to their ability to penetrate biological tissues effectively, making them valuable tools for neural and physiological research.

Together, these studies highlight the remarkable diversity and adaptability of animal rhodopsins, from natural visual adaptations to novel applications in biotechnology. This session illustrates how understanding rhodopsins across different contexts can lead to innovative tools and insights, aligning with the conference's broader focus on advancing research and applications in retinal proteins.

Talk C1 >> Zuzana Musilova (Charles University in Prague, Czechia)

See you in the dark: rhodopsin-based visual system in the deep-sea fishes

In extreme environments like the deep ocean, fish face significant challenges and have adapted to the harsh conditions. The absence of light and a narrower colour spectrum have shaped evolution of numerous adaptations of the visual system in the deep-water fishes. Here we focus on molecular adaptations of the deep-sea fish using genomics and transcriptomics to explore genes essential for vision. We specifically focus on rhodopsins, the pigments of rods sensitive in the dim light conditions. While most fish species have one (or two) copies of the rod rhodopsin genes in their genome, an extreme diversity of rhodopsins has raised in several deep-sea fishes by multiple gene duplications. Spinyfins (Diretmidae), as an extreme example, have multiple (up to 38) rod opsins sensitive to blue-green light spectrum of 444 to 519 nm, and at the same time they have one of the shortest-sensitive rhodopsin known in vertebrates. The differentially sensitive rhodopsins have evolved by accumulating mutations in the key amino-acid sites, and some of them show quite drastic modifications, such as loss of the disulfid bridge. Yet, these rhodopsins seem to be functional and are highly expressed in the retina. This extraordinary set up - although not as extreme as in the spinyfin - has convergently evolved also in two other unrelated deep-sea groups (lanternfishes and tube-eyes) and suggests unique mode of vision (with a potential for colour vision using rod cells), albeit its exact mechanism and function remain elusive.

Talk C2 >>Marjorie Lienard (University of Liège, Belgium)

Functional evolution and spectral tuning mechanisms of insect visual Gq opsins

The ability to capture incoming wavelengths of light and perceive the colours of the world stems from peripheral light-sensitive G-protein coupled opsin receptors in the eye, ultimately informing visually guided behaviours. Here we explore the molecular and functional basis of visual adaptations in lepidopteran and odonate insect groups with multifaceted visual ecologies. By leveraging a cell-based opsin expression platform, we start mapping Gq opsin genotype-phenotype relationships, pinpointing spectral residues and revealing molecular mechanisms underlying shifts in insect ultraviolet (UV), short-wavelength (SW), and long-wavelength (LW) light sensitivity. We also investigate the interplay between regulatory phototransduction networks, gene expression plasticity, retinal mosaic and adaptive opsin evolutionary trajectories. For instance, we find that in diurnal lycaenid and riodinid butterflies equipped with duplicate SW or LW opsins, coordinated functional opsin shifts and specialized opsin mosaic patterns contribute to fine discrimination of intraspecific long-wavelength coloration. Opsin tuning in trichromatic hawkmoth pollinators follows lineage-specific diel adaptations for enhanced short or long-

wavelength light capture, whereas LW opsin expression plasticity contributes to colour-tuning potentially aiding mate-search recognition behaviours in damselflies. By merging advances in molecular genetics research on peripheral visual receptors, our results contribute to disentangling the functional and regulatory basis of evolutionary adaptations underlying sensory visual phenotypes in invertebrates.

Talk C3 >> Polina Isaikina (Paul Scherrer Institute, Switzerland)

Characterization of Butterfly Long-Wavelength Opsin for Advanced Optogenetics

Butterfly long-wavelength opsin (PxRh3) from Papilio xuthus displays unique spectral properties, notably a red-shifted absorption maximum, making it a promising candidate for optogenetic applications (1,2,3).

Here, I will discuss the functional characterization of PxRh3 expressed in HEK293 cells, including its activation and inactivation wavelengths, G protein selectivity, and bistability. Our experimental data reveal that PxRh3 can be activated at 620 nm, demonstrating exceptional sensitivity even at far-red wavelengths.

Additionally, through targeted engineering, we successfully redirected PxRh3 native Gi/q signaling pathway to the Gs pathway, enabling precise light-controlled modulation of cAMP levels. The engineering expands the utility of red-shifted optogenetic tools for inhibiting and stimulating various G proteins within cells.

These results pave the way for developing versatile optogenetic tools capable of precise GPCR signaling control in diverse tissues, holding significant potential for research and therapeutic applications. Further optimization will enhance PxRh3's biochemical and structural properties, expanding the opsin toolkit for optogenetic research and enabling more precise control over cellular processes.

References:

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2. Saito et al., Zoological Letters, 2019
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Talk C4 >> Alina Pushkarev (Humboldt University of Berlin, Germany)

Crustaceans as a source of new bistable rhodopsins for optogenetic applications

Although crustaceans have intrigued the scientific world for a long time. In order to prove that animals such as the mantis shrimp indeed see beyond the visible spectrum, absorbance measure- ments were made directly on the eyes complemented with behavioral studies. Crustacean rhodopsins are especially interesting since they can detect wavelengths beyond the visual spectrum. Humans call the visual spectrum this way since it is our limitation as a species. In reality, different insects, marine animals, and fungi can detect light waves beyond this range. Rhodopsins that absorb at the far end of the visible spectrum are important for use in the field of optogenetics for controlling neuronal activity by expressing light-activated proteins in otherwise blind neurons. Infrared-absorbing rhodopsins are useful since the red wavelengths penetrate the tissue and scatter more in the neuronal tissue. Far-red-absorbing rhodopsins are scarce in nature, and one of their bearers are stomatopod crustaceans (a famous member is the peacock mantis shrimp), which can detect wavelengths ranging from UV (310 nm wavelength) to infrared (over 700 nm) by using different rhodopsins. In this work, I will present the opsins we were able to express and characterize from crustaceans, many of which are bistable.

Authors

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SESSION D – Dynamics of retinal proteins

► Session D Introduction >>Josef Wachtveitl (Goethe University Frankfurt, Germany)

Dynamics of retinal proteins

Time resolved probing of the interaction between the retinal and its binding pocket enables the investigation of fundamental principles of light conversion. Retinal isomerization disrupts the interaction network of the ground state ("protein quake") and propagates in the protein to establish certain functions (light-induced ion transfer / channel gating, ...). For a complete molecular understanding, effects such as quantum coherence, ground state heterogeneity or branching of the reaction pathway in the excited state, which contribute to the photoinduced dynamics, are important. The importance of oligomeric states and the limits of color tuning are also the subject of discussion. In this session, central issues such as determinants for the ratio between productive isomerization and non-productive back reaction to all-trans will be discussed (S. Haacke). The evolutionary adaptation to different environments or the introduction of different functionalities and the underlying optimization principles will also be addressed from a spectroscopic (G. Lamm) and a structural perspective (G. Ortolani), here in both talks with a focus on the determinants for extremely long-lived photocycles. The last contribution of the session (Y. Mizuno) also brings together structural and spectroscopic investigations, since for an integrated experimental approach sample dynamics must be measured under the same conditions.

Talk D1 >> Stefan Haacke (University of Strasbourg - CNRS, France)

New Insights on the Ultrafast Photophysics of Archaerhodopsin-3 and its Fluorescent Mutants

Archaerhodopsin-3 (AR-3) is a light-driven transmembrane proton pump found in Halorubrum sodomense, that has emerged as an interesting platform for optogenetics, since the wild-type (wt) form and a large variety of mutants display good fluorescence levels, depending on the transmembrane voltage. Multiple mutants were reported over the years, with fluorescence quantum yields (FQY) reaching up to 1.2%, in the best cases, which is still low as compared to the most fluorescent natural neorhodopsin (NeoR). Experimental and theoretical studies aiming at a rational understanding of the mutation-induced ≈100-fold increase of FQY, as compared to wt AR-3, were disclosed recently for the Quasar and Archon families. In the present contribution, we focus on the double mutant DETC and the quintuple mutant Arch-5. A combination of different spectroscopic techniques (fluorescence, transient absorption, and Raman scattering), either steady-state or with time resolution down to the 50 fs, allows us to establish that the long-lived fluorescence in the mutant comes from a one-photon excitation process, unlike wt AR-3. Both all-trans/15-anti and 13-cis/15-syn isomers of the protonated Schiff base retinal

(PSBR) cation contribute to the fluorescence in the mutants with slightly different excited state lifetimes (ESL). The temperature-dependence of the ESL's allows us to determine the exc. state barrier height, which prevents photo-isomerisation. Indeed, as compared to wt AR-3, the isomerization QY is found to be > 15 times smaller for DETC, and close to zero for Arch-5.

In a recent quantum chemistry treatment, some us showed that the extended ESLs of AR-3 mutants and of NeoR are due to unusual mixture of charge-transfer and covalent biradical character of the first excited state, which give rise to the formation of an energy barrier for increasing C13=C14 dihedral angle. In this contribution, we will show that the same electronic properties are found for wt AR-3 as well, but **without** the existence of an excited state barrier. Indeed, our experiments show that the photo-isomerisation occurs on a 300-fs time scale with vibrational coherences surviving in the photo-products. As the simulations show, the latter are due to excited state wavepackets along the C13-methyl and C14-H wag motions, the phase of which controls the outcome of the reaction, productive 13-cis isomerization vs. non-productive all-trans backreaction.

In summary, we will present new fundamental results, which allow to rationalize the exceptional fluorescent properties of AR-3 mutants, and which explain why AR-3 is an outstanding platform for genetical engineering of fluorescent rhodopsins.

Co-authors

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Talk D2 >> Gerrit Lamm (Goethe University Frankurt am Main, Germany)

The photochemistry of a microbial rhodopsin from Cryobacterium levicorallinum investigated by time-resolved optical spectroscopy

Microbial rhodopsins form a versatile group of photoreceptors that are found in almost all areas of live. While the main structural features are largely conserved, they differ in key aspects that ultimately determine protein properties and functionality. Recently, we discovered novel microbial rhodopsins, originating from organisms mostly living in cold environments.1 They exhibit several unique properties, such as an elongated C-terminus, novel 3- and 7-letter motifs and a within this sub-family conserved arginine residue not previously observed in microbial rhodopsins. Spectroscopic characterization of CryoR family members revealed extremely slow photocycle kinetics under physiological

conditions (pH 8.0), while normal photocycle durations in the range of seconds were observed under acidic conditions (pH 3.5). In this study, CryoR1 from Cryobacterium levicorallinum was investigated in more detail. pH titration experiments revealed an 80 nm spectral shift between two subpopulations, one of which has an absorption maximum at 620 nm under acidic conditions, whereas absorption maxima >600 nm are rarely observed among microbial rhodopsins. This subpopulation is furthermore accompanied by strong coherent oscillations on the ultrafast timescale and a decrease in photoproduct yield. By combining structural and spectroscopic data a model was finally developed that explains the extremely slow photocycle dynamics of CryoRs under physiological conditions. The highly conserved arginine (R57 in CryoR1) plays a key role in this model, which is reinforced by the spectroscopic data on the CryoR1 R57T variant.

References

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Talk D3 >> Giorgia Ortolani (University of Gothenburg, Sweden)

STRUCTURAL BASIS FOR THE PROLONGED PHOTOCYCLE OF SENSORY RHODOPSIN II REVEALED BY SERIAL SYNCHROTRON CRYSTALLOGRAPHY

Microbial rhodopsins are a family of light-responsive proteins featuring seven transmembrane helices and a retinal chromophore linked to a lysine residue on helix seven. These proteins operate as proton or ion pumps, light-gated ion channels, and photosensors.

Research on Bacteriorhodopsin (bR) from *Halobacterium salinarum* and Sensory Rhodopsin II (NpSRII) from *Natronomonas pharaonis* has provided valuable insights into energy transduction and phototaxis, but while both proteins exhibit similar structural characteristics and coordinated photocycles, bR photocycle duration is significantly faster than in NpSRII.

In our study, we performed serial synchrotron X-ray crystallography (SSX) studies to better understand the mechanisms of light sensing in NpSRII and, then, compared our results with those in bR to elucidate the structural factors contributing to the differences in the photocycle durations.

While we observed several common structural changes, NpSRII differs from bR in that its helix G does not unwind near the lysine residue, and transient water molecule binding sites do not quickly appear on the cytoplasmic side of the retinal. These structural distinctions prolong NpSRII's photocycle due to the need of this sensory protein to extend the light-initiated signal.

This research offers a structural rationale for how a common protein framework can be modified for diverse functions and highlights the insights that time-resolved crystallography can provide. Comparative time-resolved diffraction studies reveal how minor structural variations influence protein function, paving the way for new understandings of protein-catalyzed reactions.

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Talk D4 >> Yosuke Mizuno (Nagoya Institute of Technology, Japan)

Light-induced FTIR spectroscopy of microcrystals of visual rhodopsin grown in LCP

Visual rhodopsin is a light-sensitive G-protein-coupled receptor (GPCR) responsible for twilight vision. Vision is initiated by the photoisomerization of the retinal chromophore from 11-cis to all-trans, which is bound to rhodopsin via a protonated Schiff base linkage. Rhodopsin is one of the most extensively studied GPCRs at the molecular level. The three-dimensional structures of the dark state, the initial intermediate Batho, and subsequent intermediates Lumi and the active Meta-II have been determined by X-ray crystallography at low temperatures. In 2023, time-resolved X-ray crystallography at room temperature captured structural changes up to the Batho intermediate in real-time for the first time. However, it remains unclear whether the structural changes observed in crystal are identical to those occurring in the natural lipid environment.

In this study, we performed light-induced difference FTIR spectroscopy on LCP microcrystals of rhodopsin used for time-resolved structural analysis. Since there have been no prior examples of light-induced FTIR spectroscopy using microcrystal samples of photoreceptive proteins, we carefully optimized conditions such as film sample preparation, measurement temperature, and data analysis to obtain highly accurate spectra. As a result, we successfully measured the spectra of intermediates formed during the photoreaction process of rhodopsin within the microcrystals. We then compared these spectra with those obtained in the lipid environment to discuss the differences in structural changes during the photoreaction process.

Co-authors

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SESSION E – Advanced methods for retinal proteins

► Session E Introduction >> Keiichi Inoue (The University of Tokyo, Japan)

Pioneering the Next Revolution in Protein Mechanistic Insights with Cutting-Edge Methodologies

New methodologies not only serve as a foundation but also act as catalysts for expanding our understanding of protein mechanisms. For over 50 years, bovine rhodopsin (bvRh) and *Halobacterium salinarum* bacteriorhodopsin (*Hs*BR) have served as model systems for the application of newly developed techniques across a broad range of fields, including spectroscopy, structural analysis, and advanced imaging, largely due to their availability in large quantities and remarkable thermal stability. While these pioneering technologies have unveiled novel mechanistic insights into bvRh and *Hs*BR, the proteins themselves have significantly contributed to shaping and refining the development of these methodologies. In recent years, the discovery of new proteins with diverse and complex functionalities has highlighted gaps in our understanding of the underlying mechanism creating this functional diversity. Addressing these gaps requires the development of innovative methodologies capable of providing deeper and more comprehensive insights. In this session, groundbreaking research utilizing cutting-edge spectroscopic techniques, atomic force microscopy, and an innovative NMR method will be presented, shedding light on new facets of rhodopsin and GPCR. It will offer an opportunity to consider how new methodologies will lead the future revolution in protein research, unlocking new frontiers in our understanding of protein dynamics and function.

Talk E1 >> Miroslav Kloz (ELI ERIC, Czech)

Femtosecond Stimulated Raman Spectroscopy: A Tool Tailored for the Study of Rhodopsin Dynamics

The research on Rhodopsin proteins has become so extensive that there is a demand for advanced techniques that enable detailed characterization and study of Rhodopsins with high throughput and minimal sample requirements. Femtosecond stimulated Raman spectroscopy (FSRS) is a technique that allows the examination of conformational changes of the retinal cofactor at the molecular level, with time resolution on the order of approximately 100 femtoseconds, matching the timescales of molecular dynamics.

Utilizing Raman resonance, the vibrational states of the retinal cofactor can be observed with minimal interference from the protein environment. When employing a synchronized laser pair, the experimental setup enables the recording of dynamics spanning from femtoseconds to seconds—covering 12 orders of magnitude in time—within a single

experiment. This capability allows for comprehensive characterization of the activation processes of various rhodopsins.

At the ELI Beamlines facility in Prague, we operate such a setup, which has successfully characterized the dynamics of rhodopsins such as “Bestrhodopsin,” “Neorhodopsin,” and several other systems. We continuously strive to improve experimental sensitivity to minimize the required sample volume, which is particularly relevant for rhodopsins that cannot undergo repeated photocycles in vitro, such as the rhodopsin responsible for human vision.

Currently, we are developing methods to collect data from single-shot acquisitions and using spatial light displacement to extend the time window to seconds and minutes. We will present the experimental technique and results demonstrating the potential of our setup in advancing rhodopsin science.

Talk E2 >> John Kennis (Vrije Universiteit Amsterdam, Netherlands)

Reaction Dynamics and Mechanisms of Newly Discovered Bistable Microbial Rhodopsins

Here we present the reaction dynamics and mechanisms of two newly discovered bistable microbial rhodopsins: Bestrhodopsin, which is red-green metastable, and Neorhodopsin, which is red-UV metastable. Bestrhodopsin and Neorhodopsin are unusual microbial rhodopsins in that they form 11-cis and 7-cis metastable RSB species, respectively, rather than canonical 13-cis RSB.

Application of femtosecond-to-millisecond transient absorption and femtosecond stimulated Raman spectroscopy showed that in the green-absorbing state of Bestrhodopsin, photoinduced 11-cis to all-trans RSB isomerization occurs in 240 fs and proceeds in part directly, and in part through a 13-cis ground state photointermediate that thermally converts to all-trans in 120 ps. Hence the Bestrhodopsin all-trans to 11-cis forward reaction (Kaziannis et al. PNAS 2024) as well as the 11-cis to all-trans reverse reaction both proceed via 13-cis RSB species, suggesting that the Bestrhodopsin binding pocket transiently favors 13-cis RSB upon photoisomerization and that protein relaxation is required to form the metastable all-trans and 11-cis RSB species.

In Neorhodopsin, the forward reaction to 7-cis RSB involves formation of a primary photoproduct P715 in 1.1 ns at an exceedingly low quantum yield of 0.001, which evolves into a secondary product P760 in 18 ns at a 0.5 quantum yield. P760 evolves into the deprotonated UV absorbing metastable state on timescales longer than 1 ms. The Neorhodopsin reverse photoreaction from the 7-cis UV absorbing state is characterized by ultrafast internal conversion from an optically allowed S2 excited state to an optically

forbidden S1 excited state in 40 fs. The S1 state evolves to a primary ground state photoproduct P460, which is presumable all-trans RSB, on the 10 - 100 ps timescale at a quantum yield of 8%. The blue absorption of P460 indicates that it involves a protonated RSB, indicating that 7-cis to all-trans isomerization is followed immediately by Schiff base protonation. P460 evolves to a secondary product P420 in microseconds, which finally evolves to the metastable red-absorbing state in milliseconds. Intriguingly, we resolved an excitation energy transfer (EET) process from excited UV absorbing Neorhodopsin to red-absorbing Neorhodopsin, which is assigned to EET among a heterogeneous population of red and UV absorbing Neorhodopins within homodimers. To our knowledge this is the first observation of EET between RSBs in a oligomeric rhodopsin assembly.

Talk E3 >> Thomas Perkins (JILA, NIST & University of Colorado, USA)

Quantifying a light-induced energetic change in a single molecule of bacteriorhodopsin by atomic force microscopy

Ligand-induced conformational changes are critical to the function of many membrane proteins and arise from numerous intramolecular interactions. In the photocycle of the model membrane protein bacteriorhodopsin (bR), absorption of a photon by retinal triggers a conformational cascade that results in pumping a proton across the cell membrane. While decades of spectroscopy and structural studies have probed this photocycle in intricate detail, changes in intramolecular energetics that underlie protein motions have remained elusive to experimental quantification. Here, we measured these energetics on the millisecond time scale using atomic-force-microscopy–based single-molecule force spectroscopy. Precisely timed light pulses triggered the bR photocycle while we measured the equilibrium unfolding and refolding of the terminal 8-amino-acid region of bR's G-helix. These dynamics changed when the EF-helix pair moved ~9 Å away from this end of the G helix during the "open" portion of bR's photocycle. In ~60% of the data, we observed abrupt light-induced destabilization of 3.4 ± 0.3 kcal/mol, lasting 38 ± 3 ms. The kinetics and pH-dependence of this destabilization were consistent with prior measurements of bR's open phase. The frequency of light-induced destabilization increased with the duration of illumination and was dramatically reduced in the triple mutant (D96G/F171C/F219L) thought to trap bR in its open phase. In the other ~40% of the data, photoexcitation unexpectedly stabilized a longer-lived state distinct from the canonical photocycle. Through this work, we establish a general single-molecule force spectroscopy approach for measuring millisecond-scale ligand-induced energetics and lifetimes in membrane proteins, in general, and light-driven dynamics, in particular.

Co-authors

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Talk E4 >> Feng-Jie Wu (University of Basel, Switzerland)

Elucidating GPCR conformational dynamics by a novel NMR method

NMR chemical shift changes can report on the functional dynamics of biomacromolecules in solution with sizes >1 MDa. However, their interpretation requires chemical shift assignments to individual nuclei, which for large molecules often can only be obtained by tedious point mutations that may interfere with function.

Recently we in silico predicted and in practice created a global positioning system (GPS) that relies on pseudocontact shifts (PCSs) induced by paramagnetic thulium tag attached at various sites to an antibody in order to precisely position magnetic nuclei at distances >60 Å within a protein of interest (1). This information is subsequently used to obtain the NMR assignment for the nuclei. Using the GPS-PCS method, we have successfully assigned close to a hundred of 1H-15N NMR resonances of a G protein-coupled β1-adrenergic receptor (β1AR) in various functional forms. This amount of probes thoroughly report the details of how a GPCR undergo conformational transitions between different states.

Such method can be applied to any protein system with an existed binder protein. The understanding of a protein dynamics can be applied to fine-tune its signaling output.

1. Wu, F.-J. et al. Nanobody GPS by PCS: An Efficient New NMR Analysis Method for G Protein Coupled Receptors and Other Large Proteins. J. Am. Chem. Soc. (2022) doi:10.1021/jacs.2c09692.

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SESSION F – Function of animal rhodopsins and related proteins

► Session F Introduction >> Martha Sommer (ISAR Bioscience, Germany)

Function of animal rhodopsins and related proteins

This session explores the diverse roles and mechanisms of rhodopsins across various species, spanning visual and non-visual systems in both microbes and animals. The presentations cover topics such as spectral tuning, structural determinants of function, and regulatory pathways in rhodopsin signalling. These discussions contribute to a deeper understanding of retinal proteins, their evolutionary adaptations, and their applications in fields like optogenetics, thereby supporting the conference's goal of advancing knowledge in retinal protein research.

Takahiro Yamashita (Kyoto University, Japan) presents work on the arthropsins, which are Gq-coupled non-visual opsins like the vertebrate melanopsins, that span UV to red spectral regions. Here the spectral tuning mechanisms of arthropsin are compared to vertebrate cone pigments and insect visual opsins. **Yuji Furutani** (Nagoya Institute of Technology, Japan) presents work on two microbial rhodopsins, the sodium pumping rhodopsin from Krokinobacter eikastus (KR2) as well as the recently discovered and intriguingly strange heliorhodopsin. **Kota Katayama** (Nagoya Institute of Technology, Japan) presents work exploring the spectral tuning of cone pigments. **Lee Harkless** (University of Maryland Baltimore County, USA) wraps up the session highlighting how signalling outcomes in melanopsin, a non-visual opsin, are shaped by regulatory proteins (RGS) and downstream signalling pathways. This adds a layer of complexity to rhodopsin function, demonstrating how interactions with cellular components diversify physiological roles.

Talk F1 >> Takahiro Yamashita (Kyoto University, Japan)

Characterization of red-sensitive non-visual opsins

Vertebrate visual opsins are categorized into two types, rhodopsin and cone visual pigment. Cone pigments show their characteristic spectral sensitivities ranging from the UV to the red region to contribute to color vision. Also in invertebrate species, insects have visual opsins with various spectral sensitivities for color discrimination. In the phylogenetic tree of opsins, vertebrate cone pigments and insect visual opsins are classified into different opsin groups. Thus, these opsins have convergently acquired their spectral diversity. In the classification of opsins, arthropod (including insects) and mollusk visual opsins belong to Gq-coupled opsin group together with vertebrate non-visual opsin, Opn4. In many vertebrate species, Opn4 is generally sensitive to blue light, in contrast to the spectral diversity of insect visual opsins. In addition, recent analysis revealed that Gq-coupled opsin group contains a different type of protostome opsin, arthropsin. Arthropsin was first

identified from the water flea (arthropod) genome and subsequently identified from many protostome species. In this study, we show that arthropsins, which function in extraocular tissues, have the spectral diversity from the UV to the red region. To our knowledge, long wavelength-sensitive arthropsins exhibit most red-shifted spectral sensitivity among the non-visual opsins characterized so far. I would like to discuss the spectral tuning mechanism of arthropsin to compare with vertebrate cone pigments and insect visual opsins.

Talk F2 >> Yuji Furutani (Nagoya Institute of Technology, Japan)

Structural key elements crucial for function of Krokinobacter rhodopsin 2 and dynamics of heliorhodopsin

Microbial rhodopsins have been discovered in various organisms and been elucidated to exhibit various functions, such as proton pump, light sensor, ion pump and channel, and light-regulated enzyme. The molecular mechanisms underlying these functions have been extensively studied by various structural and spectroscopic analyses. Especially, vibrational spectroscopic techniques played important roles for elucidating key elements involving the molecular mechanisms of the various functions. In this talk, I'd like to discuss the molecular mechanisms of two rhodopsins: a sodium pumping rhodopsin from *Krokinobacter eikastus* (KR2) and a new type of rhodopsin, heliorhodopsin.

KR2 pumps sodium ions during the photocycle, but it does not bind a substrate sodium ion near the protonated Schiff base (PRSB) in the dark state. Thus, interaction change between the PRSB and its counter ion would be crucial for the pumping function. Spectroscopic analysis on Lys255 mutants with alkyl amine PRSB elucidated that the covalent bond between the protein backbone and the PRSB is prerequisite for the stability and the sodium pumping function of KR2 [1]. In addition, combination of infrared and Raman spectroscopy revealed that hydration near the PRSB is important for keeping deprotonation state of the counter ion Asp116 and suppression of the retinal distortion [2].

Heliorhodopsin has almost no sequence homology with type-I and type-II rhodopsins and exhibits inverted membrane topology. O intermediate state is considered as a functional state. By using time-resolved spectroscopic methods including a new type of infrared spectroscopy with quantum cascade laser, it was elucidated that rise and decay kinetics of the O intermediate is regulated by hydrogen-bonding interactions between Ser on the 3rd helix and Asn on the 4th helix (SNap bond), which unexpectedly corresponds to the DC gate of channelrhodopsins [3].

[1] S. Ochiai et al. *Biocheistry* 62, 1849-1857, 2023.

[2] S. Tomida et al. *Photochem. Photobiol. Sci.* 22, 2499-2517, 2023.

[3] T. Nakamura et al. *J. Mol. Biol.* 436, 168666, 2024.

Talk F3 >> Kota Katayama (Nagoya Institute of Technology, Japan)

How far can structure-spectroscopy studies of cone pigments approach the essence of the spectral tuning mechanism?

To understand the photoreaction processes involved in the functional expression of cone pigments (which control the spectral tuning of light absorption and signal transduction), it is necessary to: 1) determine the energy level of the excited state of chromophore governed by quantum chemistry, and 2) elucidate the dynamic structural change that can be described by Newtonian mechanics. In this study, we present the latest findings that reveal some of the structural factors causing a 30 nm difference in λmax between red and green pigments by combining light-induced FTIR difference spectroscopy, systematic amino acid mutation, and quantum chemical calculations. Additionally, we will discuss the progress in structural analysis aimed at determining the three-dimensional structure of cone pigments and strategies for developing therapeutic drugs for color blindness.

Talk F4 >> Lee Harkless (University of Maryland Baltimore County, USA)

The role of RGS proteins in determining melanopsin signaling outcomes

Melanopsin is a photosensitive G-Protein Coupled Receptor (GPCR) and is expressed in 6 distinct Intrinsically Photosensitive Retinal Ganglion Cell (ipRGC) subtypes in the mouse retina. IpRGCs are responsible for a myriad of non-image and image forming behaviors. Many of these subtypes differ in their light response amplitude and kinetics, as well as utilize heterogeneous phototransduction components downstream of Gαq. The mechanisms governing how a single GPCR, melanopsin, signals through different second messengers or channels is unclear. To understand how ipRGC subtypes signal differently from one another, we explored whether differential expression of relevant phototransduction cascade components exists between subtypes. Analysis of an available RNAseq dataset reveals that individual ipRGC subtypes differ in their G-protein expression and additionally are enriched for specific RGS proteins. Using RNAscope, we show that the M1 ipRGC is enriched for a Gαq selective RGS protein and that the M4 ipRGC expresses multiple Gαi/o selective RGS proteins. Cell-based BRET assays reveal a promiscuous G-protein signaling profile of mouse melanopsin, and that RGS proteins then refine the downstream signaling of melanopsin in various ways. We further show that melanopsin can

increase cAMP via Gαq alone, Gαq-mediated cAMP production is enhanced by a class II adenylate cyclase, and Gαq-cAMP signaling is eliminated when Gbeta-gamma is inhibited. To directly test whether ipRGCs respond to increases in intracellular cAMP, we expressed the Gs-DREADD in M1 and M4 ipRGCs. M1 and M4 ipRGCs fire action potentials driven by Gαs-DREADDs. Our data informs how melanopsin signaling is regulated by RGS proteins, which may offer clues to how melanopsin signals differentially in vivo and provides new evidence for the role of cAMP in ipRGCs.

Co-authors

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SESSION G – Physiology of animal retinal proteins

► Session G Introduction >> Judith Klein-Seetharaman (Arizona State University, USA)

Physiology of animal retinal proteins

This session is focused on the translation of retinal protein structure to physiology and function of the organism in which it is found. I would like to dedicate this session to the late Prof. Ulrike Alexiev who deeply cared about the significance of studying the structure and dynamics of retinal proteins to functions with rigorous biophysical approaches.

In the opening presentation by Stephan Neuhauss we will hear about cone function and circadian regulation in zebrafish, which is a model organism important for the study of photoreceptors due to the zebrafish' unique capability of regenerating their retinas. This is particularly important in the light of photoreceptor degeneration diseases in humans that progressively lead to blindness and affect more than 2 million people worldwide. This presentation will set the stage to several presentations in the important field of optogenetics. This field has great promise in developing therapeutic interventions for retinopathies and other diseases.

The presentation by Deborah Walter focuses on the optimization of the bistable jumping spider rhodopsin (JSR1) with respect to manipulating G protein signaling. The bistable properties of bacteriorhodopsins have been a major advantage for both, the study of their fundamental biophysical properties and use in optogenetics, and the relatively recent discovery of bistable properties in animal rhodopsins has created major opportunities for studying non-microbial rhodopsins.

We will then hear from Ajith Karunarathne about a novel role for the non-visual opsin melanopsin in intrinsically photosensitive retinal ganglion cells (ipRGCs) governing circadian regulation in Seasonal Affective Disorder (SAD), affecting the mental health of millions of people. The high complexity of ipRGCs and melanopsin signaling is currently unraveling. Deciphering the details of G protein coupling is therefore critical in this clinically important area.

We will then return to the practical application of retinal proteins for optogenetic therapies. Thomas Mager will describe their efforts on optimizing photoexcitation properties while minimizing cellular stress and phototoxicity through genetic engineering. The long-term goal of this work is to use the improved construct in therapeutic approaches, closing the loop to the first presentation in this session, as vision and hearing are closely related, as is evidenced by the rare disease, Usher syndrome, which is characterized by both, vision and hearing loss caused by mutations in a single gene.

This session highlights the need to understand the commonality of disease and signaling mechanisms in different physiological functions carried out by retinal proteins as well as their therapeutic potential.

Talk G1 >> Stephan Neuhauss (University of Zurich, Switzerland)

From Light to Sight: Retinal Proteins in the Regulation of Photoreceptor Signaling in the Zebrafish Retina

In this presentation, I will explore the mechanisms regulating the cone visual transduction cascade in the zebrafish retina, with a particular focus on the processes involved in its negative regulation. These regulatory mechanisms regulate the shut-off kinetics and are crucial for maintaining temporal resolution of vision. Notably, several of these mechanisms and many of the proteins involved are specific to cone photoreceptors, highlighting the unique aspects of cone-mediated vision. I will also discuss the role of circadian rhythms in modulating cone photoreceptor activity that contribute to the adaptive responses of the retina. Zebrafish, with their well-characterized visual system and close parallels to human retinal function, offer an ideal model for investigating these processes. By using zebrafish, we can gain insights into the fundamental biology of the retina that may have implications for understanding vertebrate vision and human retinal diseases.

Talk G2 >> Deborah Walter (Paul Scherrer Institute, Switzerland)

Engineering an OptoGPCR based on a bistable rhodopsin for optogenetic applications

Jumping spider rhodopsin (JSR1) is a light-sensitive G protein-coupled receptor (GPCR). Due to its bistable properties, it is an interesting target for optogenetic applications, which allows rapidly switching the GPCR signal on and off. Here, we use JSR1 as a model to derive strategies for engineering bistable rhodopsin with different primary G protein selectivity. JSR1 couples to the jumping spider Gq protein and can couple to human Gi and Gq proteins. Since Gs signaling is most distinct from Gq signaling, we exchanged parts of the intracellular and G protein-interacting sequences with the sequence of known Gs-coupled receptors. These engineered chimeras are validated with expression tests and time-resolved cellular signaling assays. The first results show strong cAMP activation that can be switched on and off with different wavelengths. In the next step, chimeric proteins will be purified to obtain biophysical characteristics and assess the binding to purified G proteins. With structural and biophysical analysis of those bistable chimeric receptors, we hope to improve the overall understanding of GPCR signaling selectivity and the design of engineered light-controlled bistable GPCRs that mimic specific GPCR signaling profiles.

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Talk G3 >> Ajith Karunarathne (Saint Louis University, USA)

Melanopsin Governs Wavelength-Dependent Cell Signaling and Animal Behavior

Over 10 million people in the United States are affected by Seasonal Affective Disorder (SAD), a form of depression more common among young adults and women. SAD is also linked with other mental health diseases. Although the interrupted sleep-wake cycle and the perturbation of the endogenous circadian rhythm have been named as major contributors, there is a critical knowledge gap underlying the pathophysiology of SAD. Despite the vague molecular rationale, the potential of bright light therapy, listed as a SAD treatment strategy by many institutes, including the National Institute of Mental Health (NIHM), offers hope and optimism for the future. The non-visual opsin melanopsin is proposed as the primary circadian photopigment in intrinsically photosensitive retinal ganglion cells (ipRGCs) that extend axonal projections to the master circadian pacemaker in the brain, suprachiasmatic nuclei (SCN). Our research, along with others, has shown that melanopsin activates multiple major G protein heterotrimers, including Gq, Gi/o, and Gs. Our novel data suggests that while the signaling properties of melanopsin are wavelength-dependent, the melanopsin-knockout mice lack the wavelength-dependent circadian shift exhibited by control mice. Our work may provide a molecular foundation for how environmental light exposure influences human behavior and shed light on light therapy.

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Talk G4 >> Thomas Mager (Institute for Auditory Neuroscience, University Medical Center Goettingen, Germany)

ChReef – An improved ChR for Future Optogenetic Therapies

Optogenetic approaches carry huge potential for future therapies, because they enable remote control of excitable cell activity with unique spatiotemporal resolution. Due to the low single-channel conductance of ChRs (1), which can be employed for excitable cell photostimulation, the optogenetic control of cellular activity relies on a combination of strong ChR expression and high light intensity stimulation. This is particularly relevant for clinical applications as it bears a risk for proteostatic stress and phototoxic effects. In this regard, ChRmine, a recently identified so-called bacteriorhodopsin-like-cation channelrhodopsin (BCCR; (2)) is of great interest. ChRmine is optimally activated with green light and shows large photocurrents. Using a high performance automated patch-clamp system (Syncropatch 384, Nanion), which we operated in synchrony with LED-based illumination, we recently showed by noise analyses that the single channel conductance of ChRmine is considerably bigger than the single channel conductance of the state-of-the-art ChR CatCh. However, ChRmine utility is impaired by a strong, light dependent desensitization, which can be mainly attributed to a light-dependent inactivation process, which resembles a substrate inhibition of the partial type. The noise analyses moreover revealed that photocurrent inhibition by light is likely associated with the presence of a parallel photocycle open state of lower conductance and/or low open probability. We recently engineered the ChRmine mutant T218L/S220A, which we named ChReef ("ChR that excites efficiently"). In ChReef the light-dependent inactivation process that resembles a substrate inhibition of the partial type, was abrogated by the mutations, which led to a pronounced reduction of photocurrent desensitization. Moreover, comparative experiments revealed that the stationary photocurrent of the ChRmine mutant ChReef was considerably larger than the photocurrents of other widely-used ChR variants. We therefore anticipated an increase in the efficiency of sustained excitable cell photostimulation and accordingly assessed the suitability of ChReef for future optogenetic therapies, thereby focusing on cardiac defibrillation, vision restoration and hearing restoration.

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SESSION H – Photopharmacology and dynamics of retinal proteins

► Session H Introduction >>Richard Neutze (University of Gothenburg, Sweden)

Photopharmacology and dynamics of retinal proteins

Time-resolved X-ray crystallography has undergone a rapid growth since the development of serial crystallography at an X-ray free electron laser. By combining X-ray diffraction data collection at room-temperature with a mechanism of reaction triggering, it is possible to observe changes in protein structure along the reaction pathway of interest. Several fascinating structural results have been recorded from retinal proteins describing conformational changes spanning the time-domain from femtoseconds to seconds, since these reactions can be initiated within microcrystals using an external laser pulse. Despite these advances, the field is hamstrung by the limitation of studying proteins that are naturally light-driven. One pathway for overcoming this limitation is to use photopharmacology, and to engineer chromophores that interact with crystallized protein. In this manner, a structural response in the protein of interest can be initiated by a photoisomerization event within a bound molecule, and be followed using time-resolved serial X-ray crystallography.

Amadeu Llebaria will introduce the field of photopharmacology and discuss how azobenzene photo-switches may be designed and synthesized so as to serve as tools for rapidly initiating a reaction within protein crystals.

Jörg Standfuss will illustrate the potential of this strategy by presenting results from time-resolved serial femtosecond X-ray crystallography experiments that used photoactive azobenzene to mimic retinal in rhodopsin. In this manner, structural details of concerning the trajectory as a bound ligand dissociates from a protein could be explored.

Eriko Nango will highlight the power of time-resolved serial femtosecond X-ray crystallography by presenting a number of structural results from diverse retinal proteins. These successful studies include the proton pump bacteriorhodopsin, the sodium pump KR2, the light-gated ion channel channelrhodopsin, and the chloride pump NM-R3.

Valerie Panneels will close the session by describing the ultrafast response of bovine visual rhodopsin to the isomerization of 11-cis retinal to an all-trans conformation. These structural observations provide a remarkable glimpse into the first events of mammalian vision.

Talk H1 >> Amadeu Llebaria (Institut de Química Avançada de Catalunya, IQAC-CSIC, Spain)

Photopharmacology: light and molecules for dynamic structural crystallography

Azobenzene photoisomerization can be chemically implemented in protein ligands to actuate on biological receptors and to manipulate their activity with light. Azobenzene small molecule photoswitches can be designed and synthesized to serve for real-time regulation of receptors with high spatiotemporal accuracy using specific illumination patterns. The basis for this is a different interaction mode of the ligand isomers with the biological receptor. Therefore, light can be used for a precise control of physiology, on/off drug activation and targeting localized organs in free behaving animals. Strikingly, the photomolecular isomerization can also be employed in structural studies. Photoswitchable ligands co-crystallized with biomolecules can be used for triggering molecular actions in the crystal upon illumination. The bound ligand can be very fast photoisomerized, sharply generating a new state, which induces a receptor rearrangement that can be experimentally measured. This light switch in the crystallized receptor, which is reminiscent of some photon activated endogenous receptor systems, opens unprecedented possibilities to measure structural changes at atomic resolution and at very short-time scale. This approach will involve cooperative work of chemists, biologists, physicists and engineers, and may open a new perspective in dynamic studies of biological processes that can change our understanding of life and applied to invent radically new therapeutic approaches.

Talk H2 >> Jörg Standfuss (Paul Scherrer Institute, Switzerland)

Photopharmacology the Movie: How Rhodopsins Pave the Way for a Dynamic Future in Structural Biology

Structural biology has been critical for our understanding of how proteins work on the molecular level. However, resolving the temporal evolution of biological macromolecules in response to activating stimuli—such as the binding of small molecular ligands or drug molecules—remains a challenge. In recent years, our research group has leveraged X-ray Free Electron Laser facilities to experimentally investigate how rhodopsins, acting as pumps, channels, or light sensors, are activated at the atomic level across a wide range of time scales.

In our latest experiments at the Swiss X-ray Free Electron Laser, we explored how photoactive azobenzene compounds, mimicking retinal, can be used to "rhodopsinize" other proteins for structural studies. My presentation will focus on the dissociation dynamics of the photopharmacological drug candidate azo-combretastatin A4 from tubulin, capturing events from the initial photochemical reaction in the femtosecond range, through

the disruption of high-affinity protein-ligand interactions in nanoseconds, adaptation of the binding pocket in microseconds, and release of the compound in milliseconds. I will discuss the relevance of these findings for our understanding of how anti-cancer drugs destabilize the microtubule network. Furthermore, I will propose the use of azobenzene-based photoswitches to trigger G protein-coupled receptors and other pharmacologically relevant targets in time-resolved structural biology experiments.

Talk H3 >> Eriko Nango (Tohoku University, Japan)

Structural Dynamics of Microbial Rhodopsins Captured by X-ray Free Electron Lasers

Microbial rhodopsins are a light-sensitive protein with an arrangement of seven-transmembrane helices and a retinal chromophore. The retinal photoisomerizes from the all-trans to the 13-cis configuration, inducing a conformational change and ion transport. While the ternary structures of microbial rhodopsin families are similar, they have distinct functions, such as pumps and channels which transport different ions. Thus, the structure-activity relationship of microbial rhodopsin has garnered significant interest.

Serial femtosecond crystallography (SFX) is a protein determination method using X-ray free electron lasers (XFEL). In SFX, a myriad of microcrystals are ejected from a sample injector and delivered to the intersection point with XFEL, resulting in a diffraction pattern as a still image. Combining a reaction trigger such as light, time-resolved serial femtosecond crystallography (TR-SFX) allows to observe structural dynamics of protein at work at high spatial and temporal resolution. The technique has revealed dynamic structures of various proteins including microbial rhodopsins. We reported the first TR- SFX structures of bacteriorhodopsin (bR) in 2016 [1]. Subsequently, ultrafast dynamics studies of bR and dynamic structures of sodium pump rhodopsin KR2 were published [2-4]. We also determined TR-SFX structures of channelrhodopsin (ChR) and chloride pump rhodopsin NM-R3 [5,6]. Comparing the dynamic structures that have been revealed so far, there are common movements in rhodopsins that transport different ions while some differences are found [7].

Serial millisecond crystallography (SMX) at synchrotrons is also an important tool for visualizing structural dynamics. In SMX, slower structural changes can be observed at a longer delay time, and the process of ion transport can be traced by anomalous dispersion signals. Recently, we have introduced systems for SMX at SPring-8. I will present the results of dynamic structure of microbial rhodopsins obtained at SACLA/SPring-8.

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Talk H4 >> Valérie Panneels (Paul Scherrer Institute, Switzerland)

ULTRAFAST DYNAMICS OF OUR LIGHT-RECEPTOR FOR VISION RHODOPSIN, USING AN X-RAY FREE ELECTRON LASER

Mammalian rhodopsin is our light receptor for vision. It belongs to the druggable G protein-coupled receptor family. Located in the retina, it is the site of one of the fastest events in our body. The protein core of rhodopsin hosts the retinal chromophore which, like a switch, isomerises in less than 200 femtoseconds upon photoabsorption. This triggers, over decades of time, intramolecular conformational changes in the rhodopsin, towards the intracellular side, initiating the signalling cascade, generating in milliseconds the vision event to the brain via the optic nerve. The intramolecular initial events transforming the rhodopsin resting state[1-2] (dark state) into the transducin-binding activated state[3-5] (Meta II state) are not completely understood.

We experimentally determined with spatial and temporal resolution the ultrafast changes of native rhodopsin at room temperature using time-resolved serial femtosecond crystallography (TR-SFX)[6] at the japanese and swiss X-ray free electron lasers (XFELs), SACLA and SwissFEL. Thousands of rhodopsin microcrystals grown in the dark were successively injected in the light of a pump laser and probed after various time-delays from femtoseconds to milliseconds using an XFEL. After 1 picosecond, we observe a distorted all-trans retinal that has induced a few changes in its binding pocket while the excess energy of the absorbed 480 nm-photon dissipates inside rhodopsin through an anisotropic protein breathing motion towards the extracellular domain. Interestingly, some amino acids known to be key elements of the transduction of the signal are involved in the protein breathing motion. Complementary analyses like QM/MM and molecular dynamics help to complement the characterization of our new models at 1, 10 and 100 picoseconds of photoactivation.

The same type of experiment was applied on later time-delays from picoseconds to early microseconds showing a relaxation of the whole structure followed by the first major

retinal conformational change modifying its binding pocket. Complementary analyses with time-resolved spectroscopy are supporting our findings.

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SESSION I – Theoretical approaches in retinal proteins

► Session I Introduction >>Igor Schapiro (Hebrew University of Jerusalem, Israel)

Theoretical approaches in retinal proteins

Theoretical and computational studies of retinal proteins have a long history. One example is the prediction of 11-cis to all-trans photoisomerization by Arieh Warshel in 1976. This pioneering study showed that this process could occur on an ultrafast timescale, before personal computers and structural data were available. Another important contribution to retinal proteins is the point charge model of Barry Honig, which is still used today to estimate the effect of the protein environment on the light absorption of the protonated Schiff base of retinal.

Massimo Olivucci will present recent progress in automating multiscale simulations of retinal proteins, including absorption maxima and excited state dynamics.

Flurin Hidber will talk about the use of neural networks to predict absorption maxima.

Ana-Nicoleta Bondar will present graph-based methods for the analysis of hydrogen bonding networks. This will be demonstrated for animal and microbial rhodopsins.

Xavier Deupi will conclude the session with simulations of different stages of the photocycle of animal rhodopsins. The simulations will include excited state dynamics as well as later intermediates.

Talk I1 >> Massimo Olivucci (University of Siena, Italy)

Comparative Computational Studies of Animal Rhodopsins

The development of a computational protocol for the automated construction of congruous models of different retinal proteins, has allowed for a systematic study of the changes of specific properties of rhodopsins belonging to different organisms. Such properties include color, excited state lifetime, photoproduct appearance time and isomerization quantum yield. Here I will discuss the application of such techniques to a series of visual pigments including variants living in very different illumination conditions. The comparative analysis of the results unveils previously unnoticed relationship between the above fundamental spectral and photochemical properties.

Talk I2 >> Flurin Hidber (Paul Scherrer Institute, Switzerland)

LAMBDA: Light Absorption Modeling via Binding Domain Analysis

Opsins are light-sensitive proteins essential for light-perception. Predicting their peak wavelength of absorbance (λmax) from sequence data is challenging due to the complex interactions within the retinal binding site. We introduce LAMBDA (Light Absorption Modeling via Binding Domain Analysis), a computational framework that uses Message Passing Neural Networks (MPNNs) to accurately predict λmax values for animal and microbial opsin for dark and activated states. LAMBDA integrates residue embeddings from large language models with generic residue numbering (GRN) systems to build detailed graphs of the retinal binding site. By mapping GRNs, our model infers structural positions from sequence data, enabling comparisons across species and protein folds. We have used LAMBDA to release a dataset comprising over 40,000 opsin sequences with predicted λmax values. This resource provides new tools to study the evolution and functional diversity of opsins.

Authors

Xavier Deupi i Corral (Paul Scherrer Institute); Flurin Hidber (Paul Scherrer Institute)

Talk I3 >> Ana-Nicoleta Bondar (University of Bucharest, Faculty of Physics, Romania)

Graph-based methodologies for direct comparisons of protein-water hydrogen-bond networks in visual and microbial rhodopsins

High-resolution structures and atomic-level simulations of visual and microbial rhodopsins have revealed protein-water hydrogen-bond networks at key functional sites. Understanding the general principles of how rhodopsins use hydrogen-bond networks during reaction cycles will guide the choice of residues for site-directed mutagenesis to probe functional mechanisms and, in the case of microbial pump rhodopsins, to attempt to change the ion specificity of the pumps. The talk will focus on the development and applications of our recent graph-based methodologies for visual rhodopsins and microbial pump rhodopsins.

To enable direct comparisons between the hydrogen-bond networks of microbial pump rhodopsins with distinct amino acid residues sequences we implemented the unique numbering scheme NS-mrho, and graph-based computations of pair-wise similarities between the hydrogen-bonding groups and hydrogen bonds of microbial pump rhodopsins. The same scheme enables comparisons of the hydrophobic interaction networks of microbial pump rhodopsins. We applied NS-mrho to a dataset of 30 unique experimental

structures microbial pump rhodopsins and identified hydrogen-bond fingerprints of proton and chloride pumps. From pair-wise similarity computations we found that, compared to hydrogen bonds, the similarity between hydrogen-bonding residues varies less with the phylogenetic distance, which suggests distinct hydrogen-bond partners. Computations of a proton pump mutated to mimic interactions of a sodium pump indicate drastically altered hydrogen bonding at the retinal pocket, which could help explain experimental observations on lack of activity of the mutant.

The graph-based analyses of static visual rhodopsin structures and other class A GPCRs reveal that about half of a protein's internal hydrogen-bond network is mediated by water molecules. The inactive receptor hosts extensive hydrogen-bond networks such that relatively few new hydrogen bond would need to be recruited or rearranged to establish long-distance paths to relay changes in receptor structure and dynamics.

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Talk I4 >> Xavier Deupi (Paul Scherrer Institute, Switzerland)

Rhodopsin Activation at Different Time Scales

Activation of rhodopsins occurs across a broad range of time scales, from ultrafast light-induced events in retinal within picoseconds to slower protein conformational changes and subsequent protein-protein interactions over milliseconds. Recent studies have provided

detailed atomic-level insights into these processes, contributing to a comprehensive understanding of how light energy is captured and converted into biological signals.

In one study, we investigated the photoisomerization mechanism in an isolated retinal model using advanced quantum mechanical methods to describe excited-state processes occurring on the femtosecond to picosecond scale **(1)**. By analyzing charge redistribution and the topography near the conical intersection, we gained deeper insights into the factors influencing isomerization selectivity and photoproduct formation at the earliest stages of activation.

Following retinal isomerization, a cascade of conformational changes leads to protein activation. We used quantum mechanics calculations and molecular dynamics simulations to interpret time-resolved structural data on bovine rhodopsin activation obtained at the SwissFEL free-electron laser at the Paul Scherrer Institute **(2)**. Our calculations helped determine that rhodopsin reaches the *batho* state within one picosecond of light absorption and supported a mechanism in which absorbed energy is released through anisotropic breathing motions toward extracellular regions critical for activating other class A G protein-coupled receptors.

Later in the activation process, on the millisecond scale, rhodopsins form signaling complexes with G proteins. Analysis of the cryo-electron microscopy structure of jumping spider rhodopsin isoform-1 (JSR1) in complex with its G protein partners **(3)** suggests that selectivity for G proteins may be modulated by changes in the plasticity of the G protein binding site.

In summary, this talk will provide an overview of rhodopsin activation across time scales, emphasizing their functional diversity and highlighting both the similarities and differences compared to ligand-binding G protein-coupled receptors.

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SESSION J – Carotenoids in retinal protein function

► Session J Introduction >> Yuji Furutani (Nagoya Institute of Technology, Japan)

Microbial rhodopsins inevitably meet carotenoids for fully utilizing sunlight

Microbial rhodopsins generally bind an all-trans retinal as the chromophore. The absorption maximum is tuned by surrounding amino acid residues according to the environmental light condition for maximizing the efficiency of the functions. However, as suggested by quantum chemistry, it is not possible to gather sunlight fully in the wide spectral range from UV to infrared region with one chromophore. Therefore, it is inevitable for microbial rhodopsins to bind the second chromophore for widening the range of the absorption wavelength. The first example was found in Sarinibacter ruber, where xanthorhodopsin (XR) binds a kind of carotenoid, salinixanthin, and the light energy captured by the carotenoid is transferred to the retinal chromophore for evoking the proton pumping function. Recently, it was found that microbial rhodopsins capable of carotenoids binding exist widely in aquatic environments. In this session, microbial rhodopsins binding carotenoids will be extensively discussed based on metagenomic analysis, spectroscopic and structural studies from genetical, structural, functional and ecological viewpoints. The session also invites a talk regarding another new rhodopsin heliorhodopsin and its light-modulation of ABC transporter.

Talk J1 >> Andrey Rozenberg (Technion - Israel Institute of Technology, Israel)

Carotenoid antennas in proton-pumping rhodopsins from bacteria and archaea

The phenomenon of light-harvesting antennas in microbial rhodopsins, although known for two decades now, has remained an idiosyncrasy of two related proteins binding ketolated carotenoids from the xanthorhodopsin family: Salinibacter ruber xanthorhodopsin and Gloeobacter rhodopsin. By exposing diverse rhodopsin proton pumps to environmental samples of carotenoids, we discovered that a range of rhodopsins from freshwater and marine environments bind common hydroxylated carotenoids and that such antenna-rhodopsin complexes are much more widespread in aquatic environments. Fluorescence measurements show that the energy is transferred between the antenna carotenoids bound non-covalently to the surface of the protein and the retinal moiety, itself a carotenoid derivative, inside the protein. A total of three different rhodopsin families: xanthorhodopsins, proteorhodopsins and a distinct proteorhodopsin-like family from Asgard archaea (HeimdallRs) are found to be able to recruit hydroxylated carotenoid antennas. Different proteins demonstrate varying specificities with respect to the type of carotenoids with HeimdallRs appearing to bind the broadest range of antennas from lutein to the epoxycarotenoid fucoxanthin. We demonstrate that antennas increase pumping

activity in these rhodopsins and given a ubiquitous distribution of rhodosin pumps with the potential to bind such antennas, we hypothesize that this phenomenon has a global impact on the amount of sunlight utilized by photoheterotrophs in aquatic microbial communities.

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Talk J2 >> Keiichi Inoue (The University of Tokyo, Japan)

Spectroscopic study on carotenoid binding ion-transporting microbial rhodopsins

Ion-transporting microbial rhodopsins are the most abundant members of the microbial rhodopsin family. These proteins use an all-*trans*-retinal chromophore to capture light energy to initiate subsequent conformational change in the protein moiety, which facilitates active (ion pumps) or passive (ion channels) ion transport. Recently, it was revealed that many ion-pumping rhodopsins also bind a carotenoid molecule in the protein in addition to retinal (1,2). In xanthorhodopsin (XR), the best characterized carotenoid-binding rhodopsin from *Salinibacter ruber*, the 4-keto type carotenoid salinixanthin functions as an antenna pigment, transferring light energy to the retinal to promote outward H^+ transport (1). In 2023, it was discovered that hydroxylated carotenoids, such as lutein and zeaxanthin, also bind to outward H^+-pumping rhodopsins (2). We investigated the antennae function of hydroxylated carotenoids in Kin4B8, a newly identified member of the xanthorhodopsin family, using transient absorption spectroscopy. Kin4B8 bound to lutein exhibited a stronger transient absorption signal compared to the protein without lutein, indicating energy transfer from the lutein to the retinal. Interestingly, the signal enhancement increased with shorter excitation wavelengths, differing from the energy transfer between salinixanthin and retinal in XR, where the energy-transfer quantum yield remains nearly constant across the entire absorption spectrum of salinixanthin. Additionally, we observed the transient absorption change in lutein in the middle of the photocycle, indicating that the structure of lutein is altered by the helical movement of the Kin4B8 protein. In this presentation, we will discuss these findings along with results from other new carotenoid-binding rhodopsins.

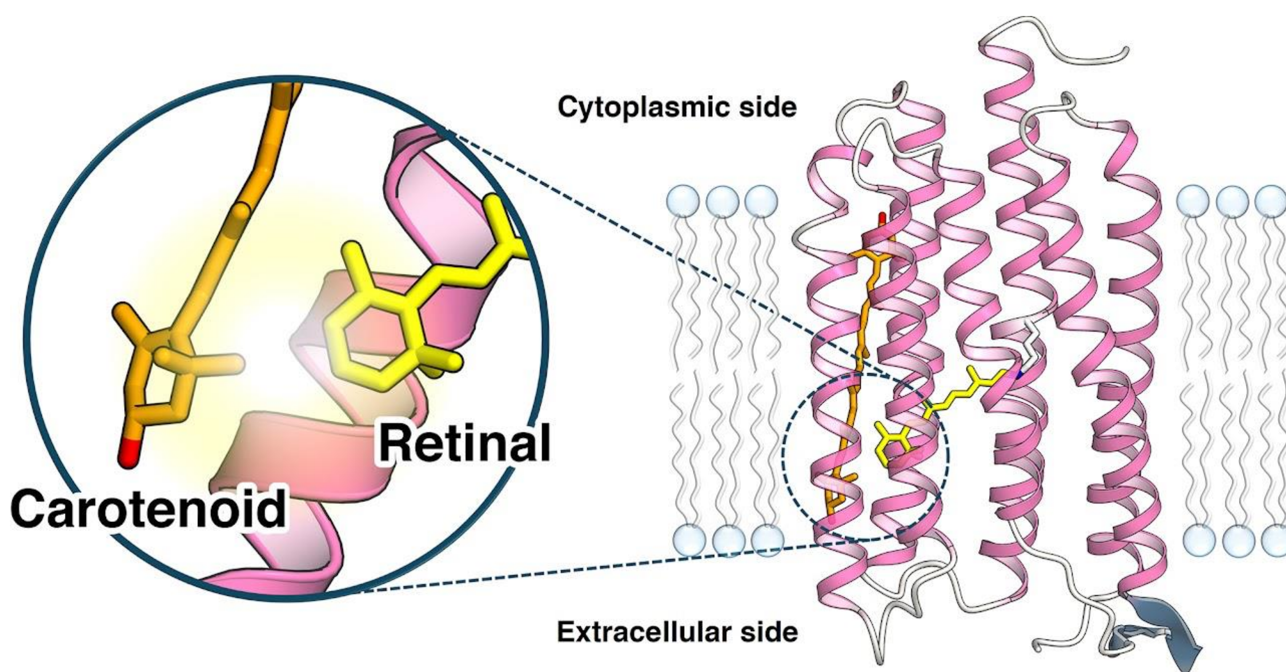

Figure 1: The structure of hydroxylated carotenoid-binding rhodopsin

[1]: https://inoue.issp.u-tokyo.ac.jp/img/content/inoue_icrp.jpg

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Talk J3 >> María del Carmen Marín Pérez (Technion-Israel Institute of Technology, Israel)

Light-harvesting by antenna-containing xanthorhodopsin from an Antarctic cyanobacterium

Microbial rhodopsins are a versatile family of light-sensitive proteins crucial to various phototrophic and sensory processes in microorganisms. Xanthorhodopsins, notable for their dual chromophore system involving retinal and carotenoids, have been predominantly studied in halophilic bacteria where they facilitate light-driven outward proton pumping and enhanced light-harvesting. However, there is a significant gap in understanding their presence and function in cyanobacteria. Here, we report the discovery and characterization of a novel xanthorhodopsin in Antarctic cyanobacteria that uniquely binds a hydroxylated carotenoid. Utilizing bioinformatic analysis, spectroscopic techniques, and functional assays, we elucidate the properties and ecological role of this xanthorhodopsin. Our findings reveal that this xanthophyll-binding xanthorhodopsin represents an evolutionary adaptation, enhancing the ability of cyanobacteria to thrive in diverse and fluctuating light environments, potentially contributing to their ecological success. Differently to the previously characterized xanthorhodopsin from the cyanobacterium *Gloeobacter violaceus*, which binds a ketolated carotenoid (echinenone), the newly identified xanthorhodopsin binds lutein, a hydroxylated carotenoid. Our discovery highlights the potential ecological significance of xanthorhodopsins in supporting the success of these organisms in their natural habitats.

Co-authors

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Talk J4 >> Shin-Gyu Cho (Sogang University, Seoul Korea)

Heliorhodopsin-mediated light-modulation of ABC transporter

Rhodopsins are light-driven seven-transmembrane proteins with a retinal chromophore that binds to a specific Lys residue, and are found in various organisms. Six years ago, heliorhodopsins (HeRs) had been discovered and formed a distinct clade, separate from the rhodopsins. Especially, HeRs have been hypothesized to have widespread functions. Recently, the functions for few HeRs have been revealed; however, the hypothetical functions remain largely unknown. Here, we investigate light-modulation of heterodimeric multidrug resistance ATP-binding cassette transporters (OmrDE) mediated by *Omithinimicrobium cerasi* HeR (OcHeR). In this study, we classified genes flanking the HeR-encoding genes in the same operon into 10 groups and identified highly conservative residues for protein–protein interactions. Next, we investigated relationship between OcHeR and OmrDE in one of the 10 groups. Our results revealed a binding between OcHeR and OmrDE, as determined by isothermal titration calorimetry analysis to derive thermodynamic parameters. Specifically, positively charged residues within an intracellular loop of OcHeR played a crucial role in the binding. Based on the thermodynamic parameters, we propose a positive cooperatively sequential binding mechanism. Moreover, a conformational change induced by light in OcHeR enhanced OmrDE drug transportation, as measured by changes in enzyme kinetic parameter, drug transport capacity in membrane vesicle, cell viability assay, and half-life of conformational changes. These results suggest that the conformational change derives dynamic energy and further leads protein structural change in OmrDE. Hence, the binding may be crucial to drug resistance in *O. cerasi* as it survives in a drug-containing habitat. Overall, we revealed a function of HeR as regulatory rhodopsin for multidrug resistance. Our findings suggest potential applications in anticancer therapy and optogenetic technology.

Authors

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SESSION K – Ion channel rhodopsins

► Session K Introduction >> Jörg Standfuss (Paul Scherrer Institute, Switzerland)

Ion channel rhodopsins

Ion channel rhodopsins are light-sensitive proteins that combine ion channel and photoreceptor functions, allowing cells to respond directly to light by regulating ion flow across cell membranes. Found in microorganisms like algae, these proteins open in response to specific wavelengths of light, enabling ions such as sodium, potassium, or chloride to pass through. This light-triggered ion movement initiates signaling processes that help organisms navigate and adapt to their environment. Their precise light sensitivity makes ion channel rhodopsins essential tools for understanding and manipulating cellular processes using optogenetic techniques.

The first presentation by Quentin Bertrand investigates how intense laser power affects time-resolved X-ray laser studies of bacteriorhodopsin, focusing on structural dynamics in crystals versus solution and the implications for biological interpretations. The second presentation by Matthias Mulder explores channelrhodopsin through time-resolved crystallography, revealing the structure of an early deprotonated intermediate with a partially open channel. The third presentation by Han Sun extends this research with a computational study of the channelrhodopsin opening mechanism, offering deeper insights into ion conduction and comparisons with two other channelrhodopsins. The session concludes with Joachim Heberle's presentation on chloride transport by NmHR, where his team examines structural dynamics across various environments—protein crystals, detergent micelles, and living cells.

Talk K1 >> Quentin Clément Bertrand (Paul Scherrer Institute, Switzerland)

Structural effects of high laser power densities on an early bacteriorhodopsin photocycle intermediate

Time-resolved serial crystallography at X-ray Free Electron Lasers offers the unique opportunity to observe ultrafast photochemical reactions at the atomic level. The new technique has yielded exciting molecular insights into various biological processes including light sensing and photochemical energy conversion. However, to achieve sufficient levels of activation within an optically dense crystal, high laser power densities are often used, which has led to an ongoing debate to which extent photodamage may compromise interpretation of the results. Here we compare time-resolved serial crystallographic data of the bacteriorhodopsin K-intermediate collected at laser power densities ranging from 0.04 to 2493 GW/cm2 and follow energy dissipation of the absorbed photons logarithmically

from picoseconds to milliseconds. Although the effects of high laser power densities on the overall structure are small, in the upper excitation range we observe significant changes in retinal conformation and increased heating of the functionally critical counterion cluster. We compare light-activation within crystals to that in solution and discuss the impact of the observed changes on bacteriorhodopsin biology.

Talk K2 >> Matthias Mulder (Paul Scherrer Institute, Switzerland)

Structural insights into the opening mechanism of Channelrhodopsin C1C2

Channelrhodopsins are light-gated cation channels that enable mobile algae cells to locate suitable conditions for photosynthesis. Beyond their natural role, scientists have shown interest in channelrhodopsins as tools in optogenetics. The first crystal structure of a channelrhodopsin was that of C1C2, a chimera of channelrhodopsin 1 and channelrhodopsin 2 from Chlamydomonas reinhardtii. The channel opening of C1C2 is controlled by two gates, the intracellular and central gates, which connect to an extracellular vestibule to form a continuous channel upon activation. Although C1C2 remains one of the most extensively studied channelrhodopsins, the molecular details of its channel opening are still largely unknown.

In this study, we have used serial crystallography to resolve the structural changes upon light-induced retinal isomerization in C1C2. We observe large conformational movements in the retinal binding pocket and the adjacent central gate. Rearrangements in the counterion network suggest that the structure represents an early deprotonated intermediate, where the channel has partially opened, but significant bottlenecks remain at the extracellular gate. Our study provides direct structural insights into the initial rearrangements upon photoactivation, bringing us closer to an atomistic understanding of cation conduction in channelrhodopsins.

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Talk K3 >> Han Sun (Leibniz-Forschungsinstitut für Molekulare Pharmakologie, Germany)

Channel opening and ion conduction mechanism in channelrhodopsin C1C2, ChR2, and iChloC

Channelrhodopsins are photoreceptors that act as ion channels upon illumination. Over the last two decades, channelrhodopsins have become an essential tool in optogenetics, enabling precise manipulation of neurons, neural circuits, and animal behavior through the use of light. Although structural biology studies have provided important structural insights into channelrhodopsins, a detailed study of their ion permeation mechanism has been challenging due to the lack of the experimentally resolved open-state structures.

In this study, we employed a multi-scale simulation approach comprising molecular dynamics (MD) based computational electrophysiology, quantum-mechanics/molecular mechanics (QM/MM), and constant pH simulations to obtain the fully open state of three channelrhodopsins. This approach was facilitated by leveraging the experimentally resolved protonation state of the open-state in the simulations. Starting from the open-state simulations, a significant number of spontaneous ion permeation events were observed in C1C2, ChR2 and iChloC, where the ion conduction mechanism differs from that of most prokaryotic and eukaryotic cation channels, which function as multi-ion, single-file pores. Analyses of the ion binding sites in these open-state channelrhodopsins provided key insights into their ion selectivity. Our study presents a robust computational approach to establish the fully open-state structures of channelrhodopsins, addressing a formidable challenge in structural biology.

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Talk K4 >> Joachim Heberle (Freie Universität Berlin, Germany)

Mechanism of the chloride pump NmHR in protein crystals, detergent micelles, and living cells

In our previous work (Mous et al. Science. 2022, 375:845-851), we traced chloride pumping in a Halorhodopsin from the marine bacterium Nonlabens marinus (NmHR) by

applying time-resolved serial crystallography, time-resolved molecular spectroscopy, and multiscale simulations. In the present work, we used variants in which residues that are involved in (transient) chloride binding in NmHR, have been replaced by other residues and applied time-resolved UV/Vis and IR spectroscopies to scrutinize the role of these residues. We recently developed in-cell IR spectroscopy to probe the transient structural changes of NmHR in living cells and compared these with the kinetics of detergent-solubilized NmHR (Oldemeyer et al. J Am Chem Soc. 2024, 146:19118-19127). The development of in-cell molecular spectroscopy is of general relevance, given the successful development of optogenetic tools from photoreceptors to interfere with enzymatic and neuronal pathways in living organisms.

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SESSION L – Optogenetics with bistable rhodopsins

►Session L Introduction >> Andreea Pantiru (University of Manchester, UK)

Optogenetics with bistable opsins

The first attempts at optogenetics employed animal opsins as photoactivators. Since then microbial opsins have vastly outperformed them in terms of breadth and depth of experimental application but animal rhodopsins have distinct advantages for optogenetics. Moreover, exploring the optogenetic potential of animal rhodopsins has provided a focus for studying the fascinating diversity of this family of proteins that the >1.5 million known species of animal rely on to detect light.

Talk L1 >> Akihisa Terakita (Osaka Metropolitan University, Graduate School of Sciecne, Japan)

Diverse coral opsins and their molecular properties

Many animals capture light through photoreceptor proteins, opsins and the light signals are utilized for various biological functions, both vision and non-visual function after conversion into biological signals such as neural ones. Therefore, it is generally understood that the opsins are situated at the "entrance" for light reception and one of the important determinants for photoreceptive properties of photoreceptor cells and/or organs. So far, thousands of opsins have been identified from a wide variety of animals, mainly from bilaterians, and they are classified into several groups.

We have been characterizing opsins from cnidarians including corals and jellyfish because in addition to the fact that cnidarians are phylogenetically distant from bilaterians, cnidarians with only simple diffuse nervous systems may have diverse opsins exhibiting different characteristics from those of opsins from bilaterians with more developed nervous systems, such as centralized nervous systems that can integrate and extract light information. Actually, we identified a novel Gs-coupled opsin which has unique molecular properties in the box jellyfish [1, 2]. Here we show that some molecular characteristics of opsins isolated from an anthozoan animal, reef-building coral (under the phylum Cnidaria). Reef-building corals exhibit physiological responses and behaviors to light and possess various opsins that divided into three groups, i.e. they have anthozoa-specific opsins (ASO) that are divided into two groups, ASO1 and ASO2 groups, which are phylogenetically distinct from other opsins groups, in addition to the members that belong to the group composed of cnidarian Gs-coupled opsins including the box jellyfish opsin. We previously reported that an opsin named acropsin4, a member from ASO2 group, light-dependently elevated calcium ion in mammalian cultured cells [3]. In this study, we investigated spectroscopic and biochemical characteristics of several opsins from a reef-building coral

and found some molecular properties unique to the coral opsins. We would present some findings on the coral opsins and also discuss optogenetic potentials of these opsins.

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Talk L2 >> Mitsumasa Koyanagi (Osaka Metropolitan University, Japan)

Evolution of jumping spider rhodopsin for optimizing depth perception from image defocus

Animal opsins, a member of the G protein-coupled receptor (GPCR) family form photoreceptor proteins by binding the chromophore retinal to underlie photoreception of animals, such as vision. Animal opsins have been identified from varied animals, and the diversity in molecular property, such as absorption spectrum, photoreaction manner and G protein selectivity has been well studied [1]. Accumulating evidence indicates close relationships between the molecular properties of opsins and characteristics of the underlying physiology and ecology, like color vision and visual adaptation.

Jumping spiders are highly vision-dependent animals and jump accurately to capture their prey. We previously reported that the measurement of distances to the targets, so-called depth perception, is achieved based on defocused images captured by the second deepest layer (L2) of their unique four-layered retina, where a green-sensitive rhodopsin (Rh1) is localized but green light is not focused [2, 3]. Accordingly, in addition to the unique retinal structure, the absorption spectrum of Rh1 that enables to capture defocused images in L2 is suggested to be a basic requirement for the mechanism. Here we investigated a significance of the spectral sensitivity of jumping spider Rh1 in the depth perception mechanism by analyzing absorption spectra of Rh1s of various jumping spider and “non-jumping” spider species. Mutational analysis of recombinant spider Rh1s with the aid of ancestral sequence inference uncovered the spectral tuning sites for the evolution of the absorption spectrum of jumping spider Rh1. Based on these results, we will discuss the spectral optimization of jumping spider Rh1.

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Talk L3 >> Sonja Kleinlogel (Hoffmann La Roche Pharma Ltd, Switzerland)

A visual opsin from jellyfish enables precise temporal control of G protein signaling

Phototransduction is mediated by distinct types of G protein cascades in different animal taxa: bilateral invertebrates typically utilise the Gαq pathway whereas vertebrates typically utilise the Gαt(i/o) pathway. By contrast, photoreceptors in jellyfish (Cnidaria) utilise the Gαs intracellular pathway, similar to olfactory transduction in mammals1. How this habitually slow pathway has adapted to support dynamic vision in jellyfish remains unknown. Here we study a light-sensing protein (rhodopsin) from the box jellyfish Carybdea rastonii and uncover a mechanism that dramatically speeds up phototransduction: an uninterrupted G protein-coupled receptor – G protein complex. Unlike known G protein-coupled receptors (GPCRs), this rhodopsin constitutively binds a single downstream Gαs partner to enable G-protein activation and inactivation within tens of milliseconds. We use this GPCR in a viral gene therapy to restore light responses in blind mice.

Authors

Michiel van Wyk (University of Bern); Sonja Kleinlogel (Hoffmann La Roche Pharma Ltd)

Talk L4 >> Johannes Vierock (Charité Universitätsmedizin Berlin, Germany)

pHRoG: pH Regulating optoGenes for all-optical control of subcellular pH

Life is organized in cells and even smaller compartments that create functional environments for signal processing and enzymatic reactions. In most subcellular organelles, such as mitochondria and lysosomes, proton concentrations are tightly regulated, and prolonged changes in lysosomal pH are associated with neurodegenerative diseases such as Alzheimer's or Parkinson's, cellular aging, and the adaptation of different types of cancer to their increased metabolic activity. Despite the importance of subcellular pH for cell homeostasis and its role in various diseases, molecular tools for organelle-specific, time-resolved, and quantitative manipulation of subcellular pH remain limited. Chemical drugs like bafilomycin A1 or hydroxychloroquine, used to manipulate endolysosomal pH, affect all organelles along the pathway simultaneously and are slowly taken up and cleared by the cell.

By comparing the targeting and performance of different ion-transporting opsins in subcellular organelles, we developed a suite of pH-Regulating optoGenes, called pHRoG, that, in combination with spectrally complementary sensors for voltage and pH, allow

spatially and temporally precise manipulation of organelle-specific physiology. In lysosomes, we show how these tools can be used to manipulate subcellular enzyme activity and study the pH and buffering capacity of individual lysosomes under physiological conditions. We demonstrate bidirectional control of local pH levels in different cell lines and neurons, and quantify both the potential and limitations of subcellular optogenetic pH manipulation using state-of-the-art opsins.

Co-authors

Jakob Walther (Charite Universitätsmedizin Berlin)

Talk L5 >> Richard McDowell (University of Manchester, UK)

Spectral tuning of mammalian melanopsins

Melanopsin (OPN4) is a key photopigment that is involved in the regulation of essential non-visual functions such as circadian phase setting in mammals. To date, the peak spectral sensitivity (λmax) of melanopsin has been determined for only a limited number of species. These results indicate very little divergence in λmax (~480nm), despite the differing temporal niches they inhabit. The molecular mechanisms underpinning the spectral tuning rules for melanopsin are poorly understood, with further study limited by technical difficulties in isolating and characterising melanopsin pigment.

We have developed an in vitro heterologous cell assay system for the high-throughput acquisition of spectral sensitivities of photopigments. Here, we used this assay system to provide the spectral sensitivities of melanopsin for 22 mammalian species. Spectral sensitivities were collected for many of the domesticated species of mammal, as well as evolutionarily distinct species from a range of diverse environmental niches. Corroborating prior data, melanopsin λmax is remarkably consistent (~480nm), with few exceptions. We next used the insights gained from the screen to identify potential spectral tuning sites. Mutagenesis was used to shift λmax of melanopsin to better adapt it for use as an optogenetic tool. Finally, mutagenesis was conducted to assess putative counterion sites for melanopsin. This data provides the first comprehensive screen of peak spectral sensitivities for melanopsins from mammalian species and offers novel experimental evidence for the molecular mechanisms underpinning the spectral sensitivity of melanopsin.

Co-authors

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SESSION M – Ion-transporting mechanism in microbial rhodopsins

► Session M Introduction >> Ana-Nicoleta Bondar (University of Bucharest, Faculty of Physics, Romania)

Ion-transporting mechanisms in microbial rhodopsins

Ion-transporting microbial rhodopsins use sidechains placed at specific locations and discrete water molecules to orchestrate conformational changes coupled to stepwise translocation of ions across cellular membranes. The session 'Ion-transporting mechanisms in microbial rhodopsins' has four talks:

Kirill Kovalev (EMBL Hamburg) will discuss the outward sodium pump ErNaR, the Na+ Rhodpsin from Erythrobacter sp. HL-111. Compared to KR2, the sodium pump rhodopsin from Krokinobacter eikastus, ErNaR has a Glu sidechain (E64) instead of KR2-L74. This residue replacement is important, because in KR2 reorientation of L74 associates with reorientation of N112, a residue that helps coordinate the sodium ion. Having E64 as a second counterion near the protonated retinal Schiff base contributes to ErNaR being more efficient (1).

Moran Shalev-Benami (Weizmann Institute of Science, Rehovot) will discuss bestrhodopsins, which consist of two rhodopsin domains, 8-TM helices each, covalently bound to each other via a linker and fused to a bestrophin-like ion channel. The photoycle starts with retinal isomerization from all-trans to 11-cis and, depending on the organism, results in the transfer of anions in a calcium-dependent or calcium-independent manner. In Tara-RBB bestrhodopsin, both rhodopsin domains have three carboxylic sidechains at the retinal binding pocket, and ion coordination is thought to involve three key residues (N1152, D1157, and H1160) that are relatively close to each other in the polypeptide chain (2).

Przemyslaw Nogly (Jagiellonian University, Krakow) will discuss sodium and chloride ion pumping microbial rhodopsins. Whereas KR2 pumps sodium from the intracellular to the extracellular side, the chloride ion pumps from Nonlabens marinus, NmHR, pumps chloride ions in the opposite direction. X-ray diffraction and time-resolved crystallography data identified internal sites visited by chloride ions as they pass through the pump, the timescales for the discrete chloride transfer steps, and internal protein-water hydrogen-bond networks that assist ion passage (3).

Kwang-Hwan Jung (Sogang University, Seoul) will discuss the proton pumping rhodopsin from the unicellular cyanobacterium Gloeobacter violaceus. Gloeobacter lacks a thylakoid membrane and oxygen evolution takes place in the periplasmic space. Its efficient proton pumping rhodopsin, GHR, is thought important to provide energy; non-covalent binding of canthaxanthin, a carotenoid, to GHR, enhances the speed of proton pumping, and heat resistance (4).

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Talk M1 >> Kirill Kovalev (EMBL Hamburg, Germany)

4D structural studies of the light-driven sodium pump ErNaR

Light-driven sodium-pumping rhodopsins are unique natural active ion transporters also being a great model for the understanding of the molecular mechanism of transmembrane sodium translocation against strong electrochemical gradients. The main type of sodium-pumping rhodopsins are proteins with the NDQ motif, such as KR2 from bacteria Krokinobacter eikastus[1]. However, even in the case of KR2, the mechanism of sodium translocation remain under debate[2], [3]. Recently, we reported a new subgroup of NDQ rhodopsins comprising an additional glutamic acid residue in close proximity of the retinal Schiff base (RSB), and presented the in-depth characterization of one of its members, ErNaR from Erythrobacter sp. HL-111[4]. The additional glutamic acid (E64 in ErNaR) allowed to make the spectroscopic and functional properties of ErNaR almost pH-insensitive in the wide range of proton concentration. The structural basis for this affect is suggested to be the low-barrier hydrogen bond (LBHB) between E64 and the main counterion of the RSB, D105 in ErNaR, which likely results in a very low pKa value of D105. However, the mechanism of light-driven sodium pumping in the new subgroup of NDQ rhodopsins involving transient protonation the RSB counterion remains unknown. We present the current progress of the structural investigations of ErNaR using cryo-electron microscopy and X-ray crystallography, including time-resolved serial femtosecond and millisecond crystallography methods. The ensemble of the high-resolution ErNaR structures under various conditions and at fs-ms time delays to the optical excitation of the protein allowed us to propose the molecular mechanism of the ErNaR functioning and to show the conformational changes associated with the increase of the pKa value of D105 allowing for proton translocation from the RSB to the counterion. With the example of ErNaR, we will also demonstrate current progress on use of 4D crystallography on microbial rhodopsins at the PETRAIII synchrotron beamlines.

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Talk M2 >> Moran Shalev-Benami (Weizmann Institute of Science, Israel)

‘Light Up the Dance Floor’ – Cryo-EM Studies of Bestrhodopsins Provide New Snapshots of Light-Based Activation Mechanisms

Rhodopsins are integral membrane proteins that bind retinal chromophores to become light sensitive. These proteins are ubiquitous in nature and play key roles in microbial physiology as well as in animal visual perception. All microbial rhodopsin species described to date share a similar architecture with a large fraction combining their light sensitive unit with an ion transporting activity integrated into a single protein domain. Recently, our group, in collaboration with eight groups around the globe, discovered and characterized a novel family of rhodopsins in marine unicellular algae – the bestrhodopsins – in which one or two rhodopsin domain(s) are C-terminally fused to a separate bestrophin channel domain. Bestrhodopsins form pentameric megacomplexes (~700 kDa) with ten rhodopsins surrounding and modulating a central anion conducting channel with visible and/or near infrared light. In the present talk, we will discuss our current understanding of the molecular mechanisms governing rhodopsin ion-channel fusions, including light-control and ion-selectivity, and provide snapshots of the bestrhodopsins captured in action.

Co-authors

Jakob Walther (Charite Universitätsmedizin Berlin)

Talk M3 >> Przemysław Nogły (Jagiellonian University, Poland)

Key residues in the transport mechanism of chloride pumping rhodopsin

Chloride transport is an essential process maintaining ion balance across cell membranes, cell growth, and neuronal action potentials. However, the molecular mechanism of the transport remains elusive. Among chloride transporters, light-driven rhodopsins have gained attention as optogenetic tools to manipulate neuronal signaling.

We combined time-resolved serial crystallography (SwissFEL and SLS synchrotron) to provide a comprehensive view of chloride-pumping rhodopsin's structural dynamics and molecular mechanism throughout the transport cycle from 10 ps to 50 ms [1]. We traced transient anion binding sites, obtained evidence for the mechanism of light energy utilization in transport and identified steric and electrostatic molecular gates ensuring unidirectional transport. These structural insights provided the basis for mutagenesis and functional study of the mechanistic features enabling finely controlled chloride transport across the cell membrane.

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Talk M4 >> Kwang-Hwan Jung (Sogang University, Seoul Korea)

Dual roles of proton pumping rhodopsin in Gloeobacter: Energy production and gene regulation

Light is a significant factor for living organisms with photosystems, like microbial rhodopsin - a retinal protein that functions as ion pumps, channels, and sensory transducers. *Gloeobacter violaceus* PCC7421, has a proton-pumping rhodopsin called the *Gloeobacter* rhodopsin (GR). It was involved in energy production by green light with photosynthetic machinery. The helix-turn-helix family of transcriptional regulators has various motifs, and they regulate gene expression in the presence of various metal ions. We show that active proton outward pumping rhodopsin interacted with the helix-turn-helix transcription regulator and regulated gene expression. This interaction is confirmed using ITC analysis (KD of 8 μM) and determined the charged residues required. Using *in vitro* expression experiments with fluorescent and luciferase reporter systems, we confirmed that ATP-binding cassette (ABC) transporters and the self-regulation of *G. violaceus* transcriptional regulator (GvTcR) are regulated by light, and gene regulation is observed in *G. violaceus* using the real-time PCR. These results could expand our understanding of microbial rhodopsin function's natural potential and limitations.

Co-authors

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SESSION N – From structure to physiology of retinal proteins

► Session N Introduction >> Peter Hegemann (Humboldt University of Berlin, Germany)

From structure to physiology of retinal proteins

Talk N1 >> Shunki Takaramoto (The Institute for Solid State Physics, The University of Tokyo, Japan)

ApuRhs, a new family of anion channelrhodopsin from apusomonads

Current understanding of light-sensing systems and rhodopsin diversity in heterotrophic flagellates (HFs) is limited, with notable examples in marine stramenopile species, non-photosynthetic algae, and choanoflagellates. One under-researched group is apusomonads, bacterivorous biflagellate protists found in freshwater and marine sediments worldwide. Apusomonads are closely related to opisthokonta, which includes animals, fungi, and their unicellular relatives, retaining ancestral traits valuable for studying early opisthokont evolution.

Recently, two extensive culturing efforts isolated a significant diversity of apusomonads, introducing four new genera and seven new species. These studies generated transcriptomic data to resolve phylogenetic relationships and examine key traits through comparative genomics. This study has aimed to uncover hidden microbial rhodopsin diversity in HFs by screening all available apusomonad omics data and environmental data. We discovered 14 microbial rhodopsins forming a new family, ApuRhs, unique to apusomonads. Phylogenetic analysis revealed that ApuRhs constitute a new, independent family within type-1 microbial rhodopsins. They possess conserved DAQ and (A/I/M/C)TQ motifs in the transmembrane helix 3.

Here, we tested their photoactivity by electrophysiological experiments, which demonstrated that ApuRhs have absorption peaks between blue and UV ranges (385–489 nm). Patch clamp experiments showed that ApuRhs function as ion channels transporting anions showing particularly high ion permeability for monovalent anions such as Cl^- and NO_3^-. An interesting point is that ApuRhs with the DAQ motif exhibit absorption maxima in the UV region, suggesting that the retinal Schiff base is deprotonated. Despite this, the excitation in the UV region induces ion channel activity, indicating that the photoreaction starts from the deprotonated retinal Schiff base and structural changes during this process lead to channel gating. Furthermore, transient absorption measurements on ApuRh with the DAQ motif revealed that the photocycle is initiated upon excitation in the UV region. These findings suggest that ApuRhs represent a new, deep-branching family of microbial rhodopsin channels, independently evolved from the well-known channelrhodopsins family.

This research highlights that significant microbial rhodopsin diversity remains undiscovered within heterotrophic flagellates.

Co-authors

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Talk N2 >> Shoko Hososhima (Department of Life Science and Applied Chemistry, Nagoya Institute of Technology, Japan)

Proton transport mechanism of viral heliorhodopsin, V2HeR3

Rhodopsins convert light into signals and energy in animals and microbes. Heliorhodopsins (HeRs), a recently discovered new rhodopsin family, are widely present in archaea, bacteria, unicellular eukaryotes and giant viruses, but their function remained unknown [1, 2]. Using electrophysiological measurements, we revealed that a viral HeR from *Emiliania huxleyi* virus 202 (V2HeR3) is a light-activated proton transporter [3]. Recently, novel functions of non-ion-transporting HeRs have been reported [4]. These studies suggest that HeR functions would be diverse similar to the type-1 microbial rhodopsins.

Here we report the proton transport mechanism of V2HeR3. Photocurrents of V2HeR3 were measured by using whole-cell patch clamp under different ion conditions. Lowering the extracellular pH from 7.4 to 6.0 shifted the reversal potential. In contrast, replacing the solutions with sodium or potassium showed no significant reversal potential shift, suggesting that V2HeR3 is a light-gated proton channel. Site-directed mutagenesis study showed that E191 in TM6 constitutes the gate together with the retinal Schiff base. E205 and E215 form a proton accepting group of the Schiff base, and mutations at these positions converted the protein into an outward proton pump. On the other hand, our previous study suggested that V2HeR3 contains a binding site for a monovalent anion at the extracellular side, and that anion binding affects proton transport. Here, we used site-directed mutagenesis study to show that several amino acids of a loop region at the extracellular side are important for a monovalent anion effect. Based on the present electrophysiological results, we propose the monovalent anion-sensitive and proton-transporting mechanism of viral heliorhodopsin, V2HeR3.

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Talk N3 >> Wayne Busse (Humboldt University of Berlin, Germany)

Localization of the Fluorescent Rhodopsin NeoR in Fungal Zoospores with Insights into Its Enzymatic Functionality

Rhodopsin guanylyl cyclases (RGCs) control phototactic behavior in motile zoospores of unicellular aquatic fungi. Based on their dimerization, RGCs can be divided into two groups: homo- and heterodimeric. In heterodimeric RGCs, a conventional green-absorbing rhodopsin (RGC1) pairs with the far-red absorbing rhodopsin NeoR to form a functional enzyme. NeoR stands out with its remarkable absorption around the 700 nm mark. In addition, the red-absorbing state is intensely fluorescent, which is reversibly switched upon illumination into a deprotonated UV-absorbing state. Given that the enzyme is activated by green light, the role of NeoR within the heterodimeric assembly remains unclear.

To elucidate the enzymatic mechanism, we performed functional studies with recombinantly expressed proteins combined with introduced point mutations in the pseudo-symmetric enzymatic active centers. By manipulating each binding site individually, we demonstrate that RGC1 is primarily responsible for functionality, while NeoR affects the overall catalytic turnover without contributing to cGMP synthesis. Notably, replacing the metal ion-binding aspartate in the degenerate catalytic center of NeoR led to a six-fold increase in enzyme activity. Additionally, we spectroscopically characterized the photocycle dynamics of RGC1 and two other rhodopsins, the most blue- and red-absorbing NeoR partners known to date. Neither enters a deprotonated M-state, which is expected to be the main signaling state due to its long lifetime. However, the design of various chimeras shows that the heterodimeric RGCs can be converted into functional homodimers by exchanging their catalytic domains without altering the photochemistry, thus giving the rhodopsins a secondary role in the enzymatic functionality. Furthermore, we aimed to study the protein under native conditions. Therefore, we used the original fungus Rhizoclosmatium globosum and observed an intrinsic fluorescence inside the cell that we could experimentally assign to NeoR. We localized NeoR adjacent to the lipid-rich droplet, in a sickle-shaped organelle of the chytrid fungus known as the rumposome, which together form the fungal eye. The photophysical properties further allowed us to study local structure of the rumposome using single-molecule localization microscopy. The ongoing study aims to reveal the number of fluorophores per zoospore and provide further subcellular structural information.

Taken together, we provide an overview of the functionality of each particular binding site in the enzyme, with a detailed spectroscopic characterization of various green-absorbing

RGCs, and present the first sensory rhodopsin that can be observed inside its native organism based on its intrinsic fluorescence properties.

Co-authors

Matthias Broser (Humboldt Universität zu Berlin); Yousef Kamrani; Enrico Klotzsch; Peter Hegemann (Humboldt-Universität zu Berlin)

Talk N4 >> Judith Klein-Seetharaman (Arizona State University, USA)

A Comprehensive Rhodopsin Dataset and Quantitative Molecular Docking Analysis of Rhodopsin-Retinal Interactions

Rhodopsin is a retinal protein and G-Protein Coupled Receptor critical for vertebrate vision. Extensive crystallization efforts of rhodopsin since the publication of its first structure in 2000 has generated 66 structures of different conformational states of rhodopsin with and without its binding partners arrestin, rhodopsin kinase and the G-protein, transducin. This provides us with an opportunity to quantify the interactions of retinal with rhodopsin in different structures on a large scale. We created a database of all bovine rhodopsin structures and classified them into 16 dark-state rhodopsin, 26 opsin, 19 Metarhodopsin (Meta)-II, and 5 other photo-intermediate structures. Across these groups, we also identified 21 entries contain the N2C/D282C substitution mutation that stabilizes the structure. In addition, there are 8 Meta II structures with G-Protein, 4 with arrestin (3 Meta-II and 1 opsin with arrestin), 4 Meta-II with kinase, 9 opsin with transducin. Then, we quantified binding of retinal to these structures through prediction of binding affinities. Using DiffDock and GNINA Minimized Affinity, which are protein-ligand pose prediction and binding affinity calculation tools, we docked each of the 66 rhodopsin proteins against 11-cis-retinal and all-trans-retinal. As expected, Meta-II structures bind better to all-trans-retinal while dark structures bind better to 11-cis retinal conformations. However, we also find that the flexibility of the binding pocket during photo-intermediate states – lumi-rhodopsin and batho-rhodopsin – enable these conformations to dock with higher affinity to both retinal chromophores. Furthermore, opsin and active forms with proteins bound, the binding affinity profile of retinal weakens on average despite adopting conformations similar to canonical inactive and active states. These results provide a novel, quantitative perspective into the rhodopsin activation process.

Authors

Aditya Lakshminarasimhan (Arizona State University); Chase Harms (Arizona State University); Judith Klein-Seetharaman (Arizona State University)

Talk N5 >> Phyllis Robinson (University of Maryland, Baltimore County, USA)

Melanopsin, from Molecule to Behavior

Melanopsin is a fly-like visual pigment (R-type) expressed in a small subset of light-sensitive retinal ganglion cells (ipRGCs) in the mammalian retina. It is involved in regulating non-image forming visual behaviors, such as circadian photoentrainment and the pupillary light reflex (PLR), as well as aspects of image-forming vision. ipRGCs act as autonomous photoreceptors via the intrinsic melanopsin-based phototransduction pathway and as a relay for rod/cone input via synaptically driven responses. The regulation of melanopsin by phosphorylation profoundly effects it's signaling kinetics and amplitude. C-terminal phosphorylation of melanopsin by a GRK determines the recovery kinetics of the intrinsic melanopsin-based photoresponse in ipRGCs, the duration of the PLR, and the speed of entrainment. The PKA dependent phosphorylation of melanopsin's intracellular loops attenuates it's response. Understanding the biochemistry of the visual pigment melanopsin leads to insights into the physiology and behavior associated with ipRGCs.

POSTERS

Poster 1 >> Anne Mayer (Goethe Universität Frankfurt am Main, Germany)

Investigation of the Role of Histidines in the inward directed proton-pump Xenorhodopsin by Solid-state NMR

The regulation of ion gradients across cell membranes is essential for life. One way of regulation in unicellular organisms are microbial rhodopsins (MRs), photosensitive retinylidene membrane proteins. Xenorhodopsin from Nanosalina sp. (NsXeR) is a MR and light-driven inward proton pump [1, 2]. Its unusual pumping behaviour could probably have a preventing character from alkaline environments or it could play a role as regulator of intracellular signalling [3].

Histidine is an amino acid with special properties, due to the imidazole side chain group [4, 5]. In some MRs, it serves as hydrogen bond or salt bridge partner in direct vicinity of the retinal Schiff base (RSB) or is part of a proton donor or acceptor complex [6, 7, 8]. Furthermore, it can act as pH sensor or ion selector [9, 10].

In NsXeR, H48 is part of the hydrogen bond network of the RSB and could probably function as proton acceptor of it. H94 seems to belong to the proton release group at the cytoplasmic side [2]. H225 is also located towards the cytoplasmic side [1] but its function is still unclear. We investigated the histidine residues in NsXeR using solid-state NMR at ambient temperatures as well as under frozen conditions to enable DNP enhanced data acquisition and cryo-trapping. The data of the dark state protein shows, that all three histidines react differently to pH changes. First photocycle intermediate trapping experiments indicate that H225 already undergoes some changes during the K-state.

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Poster 2 >> Robin Torben Stipp (Paul Scherrer Institute, Switzerland)

Investigation of binding and function of a photoswitchable β2AR antagonist using time-resolved crystallography

G protein-coupled receptors (GPCRs) are a superfamily of membrane-bound proteins involved in key signaling pathways such as immune response, vision, smell, and taste. The β2 adrenergic recep- tor (β2AR) is an adrenaline-activated member of the rhodopsin-like subgroup of GPCRs, playing a crucial role in cardiovascular and pulmonary regulation. This makes β2AR a key drug target, with a dedicated group of antagonists, so-called beta-blockers, developed to treat diseases such as heart dysfunction, asthma, angina, migraine, glaucoma, and many more. Despite being one of the most extensively studied GPCRs, with over 35 structures depicting various ligand-bound states, the dynamic mechanisms behind ligand binding and affinity remain poorly understood.

This project employs time-resolved X-ray crystallography, custom-designed photochemical lig- ands, and a novel sample delivery method to investigate β2AR dynamics during photon-induced ligand affinity shifts. Photoswitches based on the beta-blocker Propranolol were developed and evaluated for suitability for time-resolved crystallographic studies. After identifying a satisfac- tory compound, the ligand-protein complex was crystallized and ligand binding in the orthosteric pocket was confirmed. Time-resolved data was collected at X-ray Free Electron Lasers (SwissFEL and LCLS) using novel solid support and conventional jet-based delivery methods. The resulting structures reveal ligand isomerization and β2AR rearrangements, elucidating the molecular mech- anisms determining ligand affinity at near-atomic resolution.

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Poster 3/Talk B3 >> Matthias Broser (Humboldt University of Berlin, Germany)

Structural elucidation of the far-red absorbing and highly fluorescent retinal chromophore in fungal neorhodopsins

Fungal neorhodopsins (NeoRs) exhibit unique spectral properties among rhodopsins, including far-red light absorption with maxima close to or even above 700 nm, high fluorescence, and an unusual photoisomerization from all-trans to 7-cis. These extreme photochemical characteristics are attributed to a specific protein environment surrounding the protonated retinal Schiff base (pRSB), which facilitates extensive delocalization of the positive charge along the chromophore in the electronic ground state. However, due to the lack of structural data, the precise interactions between the pRSB and surrounding amino acid side chains have remained elusive, and previous theoretical studies have relied on homology models.

Here, we present the crystal structure of the rhodopsin domain of NeoR from Rhizoclosmatium globosum in its far-red absorbing all-trans state, at 2.2 Å resolution. Our findings reveal an unexpectedly twisted retinal chromophore and provide definitive identification of all side chain rotamers as well as the precise locations of water molecules. The three carboxylate residues of the formal counterion triad—E136, D140, and E262—are tightly interconnected, forming an efficient barrier that prevents water from penetrating the active site. These residues are embedded within an extensive hydrogen-bond network, ensuring strong interactions between adjacent helices via amino acid side chains. This rigid configuration excludes water-mediated ground state dynamics, explaining the narrow absorption band of NeoR. Notably, the closest carboxylate to the pRSB nitrogen is E136, located at a distance of 3.2 Å. Glutamate E141, proposed in a recent computational study as a key residue influencing the extreme photophysics of NeoR, is in close proximity (~3.2 Å) to the polyene chain. A single point mutation, D140T, in NeoR leads to a significant blue shift and broadening of the absorption spectrum, along with reduced fluorescence, and facilitates photoisomerization toward 9-cis and 11-cis. These altered properties are linked to increased water accessibility to the pRSB active site, owing to the less effective shielding.

Our results position NeoR as a valuable model for exploring the fundamental mechanisms of retinal photochemistry at the molecular level and as a promising template for the rational design of advanced optogenetic tools. Additionally, they provide a remarkable example of how natural evolution has fine-tuned the versatile rhodopsin scaffold to achieve extreme spectral properties of retinal.

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Poster 4 >> Shin Nakao (Okayama University, Japan)

Optogenetic induction of cancer cell death using an outward proton pump rhodopsin

Medicines used for cancer treatment often lead to side effects by causing damage to normal cells through diffusion. To address this issue, we aimed to develop an optogenetic method for inducing cell death with light, which has the advantage of high spatiotemporal resolution. So far, we have successfully developed an optogenetic cell death method through intracellular pH alkalization using an outward proton pump rhodopsin, Archaerhodopsin-3 (AR3), and demonstrated the applicability of the method to human cultured cells and the nematode *Caenorhabditis elegans* [Ref. 1]. In this study, we applied this optogenetic cell death method to cancer cells and tumors to demonstrate its antitumor effects.

The gene for AR3 was introduced into mouse cancer-derived cell lines (MC38, B16F10) using a lentiviral/retroviral vector to establish stable cell lines. Upon green light irradiation of AR3-expressing cells in *in vitro* experiments, propidium iodide (PI) signals clearly indicated cell death. Subsequently, AR3-expressing cells were transplanted subcutaneously into C57BL/6 mice, and tumor volume changes were monitored following green light irradiation (7 mW/mm2 for 1 hour). The volume of AR3-expressing MC38 and B16F10 tumors increased in a time-dependent manner, similar to the growth pattern of non-expressing tumors without green light irradiation. Notably, significant reductions in growth rate were observed in AR3-expressing MC38 and B16F10 tumors. These findings strongly suggest antitumor effects through optogenetic cell death. Consequently, the optogenetic cell death method demonstrates high applicability to cancer cells, both *in vitro* and *in vivo*, indicating its potential for the development of a new cancer therapy with fewer side effects.

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Poster 5 >> Taito Urui (Osaka University, Japan)

Structural Evolution of Retinal Chromophore in Primary Intermediates of Inward and Outward Proton-Pumping Rhodopsins

Proton-pumping rhodopsins, which consist of seven transmembrane helices and have a retinal chromophore bound to a lysine side chain through a Schiff base linkage, offer valuable insights for developing unidirectional ion transporters. Despite identical overall structures and membrane topologies of outward and inward proton-pumping rhodopsins, these proteins transport protons in opposing directions, suggesting a rational mechanism that enables protons to move in different directions within similar protein structures. In the present study, we clarified the chromophore structures in primary intermediates of inward proton-pumping rhodopsin, schizorhodopsin 4 (SzR4), and outward proton-pumping rhodopsin, Gloeobacter rhodopsin (GR) using time-resolved resonance Raman spectroscopy. A large distortion of the retinal chromophore was observed at the K intermediate in GR, whereas the distortion was small at the K intermediate in SzR4. The large distortion at the K intermediate in GR suggested the steric interaction between the retinal chromophore and amino acid residues and would result in the pKa change of a proton acceptor. The hydrogen bond strength at the K and L intermediates was weak and strong, respectively, in both pumps. Most importantly, it was common to both pumps that the hydrogen bond of the Schiff base became stronger in the L intermediate than in the unphotolyzed state. Although the stronger hydrogen bond likely facilitates the deprotonation of the Schiff base, the movement of the dissociated proton differed in two pumps. The similarity in the chromophore structures of the L intermediates of the two pumps indicated that the direction of proton release from the Schiff base during the L-to-M transition is determined not by the structure of the retinal chromophore but by the number of negative charges on the extracellular side of the Schiff base. This is contrast to that the chromophore configuration is a determinant for the direction of proton uptake. The present study, together with our previous studies, clarified the determining factors of the transport direction in inward and outward proton-pumping rhodopsins.

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Poster 6/Talk B4 >> Ritsu Mizutori (Nagoya Institute of Technology, Japan)

Structural basis for proton transporting mechanism in viral heliorhodopsin, V2HeR3

V2HeR3 is the first heliorhodopsin with its function clarified as a proton transporter across the membrane. Patch-clamp measurements identified E191, E205, and E215 as important amino acids for proton transport. However, the detailed molecular mechanism remains unknown. In this study, we investigated the proton transport mechanism of V2HeR3 by X-ray crystallography, spectroscopic measurements, patch clamp measurements, and theoretical calculations.

We obtained the structure of the "dark state" under dim red light and the structure of the "light state" under laboratory light, both at a resolution of 2.1 Å. Significant differences between the two structures are observed. In the light state, the α-helix around the retinal Schiff base of TM7 is unwound, and two additional water molecules are inserted. To determine which photointermediate corresponds to the light state structure, we performed structural analysis of K and M intermediates using infrared spectroscopy. Focusing on the structure changes of protein backbone (amide I), an up-shifted band was observed in K intermediate compared to the ground state, indicating a weakening of the hydrogen bond strength of protein backbone. On the other hand, in the M intermediate, we observed a down-shifted band, indicating a strengthening of the hydrogen bond strength in the protein backbone. Generally, the hydrogen bond strength of the protein backbone is considered weaker when the α-helix is unwound, suggesting that the light state structure reflects the K intermediate.

Furthermore, in both the K intermediate and M intermediate, we captured the C=O stretching vibration band corresponding to changes in the hydrogen bonding of protonated carboxylic acids derived from E191. From the results of transient absorption spectroscopy measurements, we found that mutating E191 to glutamine prolonged the lifetime of the M intermediate. Additionally, considering that water molecules situated near E191 in the light-state structure, it is suggested that E191 plays an important role in proton transport.

In the presentation, we propose a proton transport model for V2HeR3 based on the structures of the dark and light states, along with spectroscopic measurements, quantum chemical calculations, and the patch-clamp measurements on various mutants.

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Poster 7 >> Zikun Lyu (University of Tokyo, Japan)

Electrophysiological study of the mechanism of acetate and chloride binding effects on an artificial inward proton pumping rhodopsin

Inward proton-pumping rhodopsins are members of the microbial rhodopsin family. They use light energy captured by the retinal chromophore to transport proton (H+) from the outside to the inside of the cells. Recently, an outward proton pumping rhodopsin from Pseudomonas putida, PspR, was artificially converted into an inward proton pump (PspR FWSCG) by mutating three amino acid residues [1]. On the other hand, the pump activity of PspR is enhanced by extracellular weak organic acid (WOAs) at pH 5.0, similar to fungal rhodopsin. However, there is no report on the effect of WOAs on inward proton-pumping rhodopsin, and the mechanism of WOA effect is still unclear. Here, we studied the mechanism of the WOA effect (acetic acid, Ac) on the H+ transport activity of PspR FWSCG and other mutants through patch clamp measurements. While Ac did not enhance the inward proton-pumping activity of PspR FWSCG at neutral pH, interestingly, PspR FWSCG exhibited a channel-like photocurrent at pH 5.0, and this channel-like photocurrent is enhanced by Ac, with a solvent including NaCl. While the channel-like photocurrent was also observed for PspR D73A/S/T mutants, however, it disappeared by replacing NaCl/NaAc with Na2SO4, indicating the Ac/Cl− bindings play a critical role. A Cl− titration experiment with purified PspR FWSCG revealed that its absorption wavelength is 27-nm blue-shifted by Cl− binding with Kd = 2.3 mM. While the Cl− binding induces the channel-like current, pump activity measurement using Escherichia coli cells indicated that the bound Cl− reduces the inward H+ activity of PspR FWSCG. Interestingly, the absorption wavelength was blue-shifted by 23 nm by the binding of Ac (Kd = 324 mM). Because the Ac binding was clarified to compete with the Cl− binding by a Cl− titration experiment in the presence of NaAc with Kd = 4.5 mM, both of them are considered to bind at the same site in the protein. In the presentation, we will discuss the mechanism of the ion transport of PspR and its mutants regulated by the Cl− and Ac binding.

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Poster 8 >> Keiichi Kojima (Okayama University, Japan)

A repertoire of visible light-sensitive opsins in a deep-sea hydrothermal vent shrimp

In contrast to the terrestrial environment where humans reside, sunlight cannot reach the deep-sea. Instead, only very dim near-infrared and visible light from the black-body radiation is present around the hydrothermal vent in the deep-sea. The deep-sea hydrothermal vent shrimp is believed to detect this dim light using its enlarged dorsal eye; however, the molecular basis of the photoreception remains unexplored. Here, we characterized the molecular properties of opsins from the vent shrimp *Rimicaris hybisae*. Transcriptomic analysis identified six opsins (three Gq-coupled opsins, one Opn3, one Opn5 and one peropsin), and functional analysis revealed that five of these opsins exhibited light-dependent G protein activity, while peropsin exhibited the ability to produce 11-cis retinal from all-trans retinal like photoisomerases. Noteworthy, all *R. hybisae* opsins, including Opn5, convergently show visible light sensitivity (around 457 – 522 nm), whereas most opsins categorized as Opn5 have been demonstrated to be UV-sensitive. Our mutational analysis revealed that the unique visible light sensitivity of *R. hybisae* Opn5 is achieved through the stabilization of the protonated Schiff base by the counterion residue at position 83 (Asp83), which is different from the position identified in other opsins. Our results suggest that the vent shrimp *R. hybisae* has adapted to preferentially maintain a repertoire of visible light-sensitive opsins, including the uniquely visible light-sensitive Opn5, for the efficient reception of dim visible light in the deep-sea environment.

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Poster 9/Talk C2 >> Marjorie Lienard (University of Liège, Belgium)

Functional evolution and spectral tuning mechanisms of insect visual Gq opsins

The ability to capture incoming wavelengths of light and perceive the colours of the world stems from peripheral light-sensitive G-protein coupled opsin receptors in the eye, ultimately informing visually guided behaviours. Here we explore the molecular and functional basis of visual adaptations in lepidopteran and odonate insect groups with multifaceted visual ecologies. By leveraging a cell-based opsin expression platform, we start mapping Gq opsin genotype-phenotype relationships, pinpointing spectral residues and revealing molecular mechanisms underlying shifts in insect ultraviolet (UV), short-wavelength (SW), and long-wavelength (LW) light sensitivity. We also investigate the interplay between regulatory phototransduction networks, gene expression plasticity, retinal mosaic and adaptive opsin evolutionary trajectories. For instance, we find that in diurnal lycaenid and riodinid butterflies equipped with duplicate SW or LW opsins, coordinated functional opsin shifts and specialized opsin mosaic patterns contribute to fine discrimination of intraspecific long-wavelength coloration. Opsin tuning in trichromatic hawkmoth pollinators follows lineage-specific diel adaptations for enhanced short or long-wavelength light capture, whereas LW opsin expression plasticity contributes to colour-tuning potentially aiding mate-search recognition behaviours in damselflies. By merging advances in molecular genetics research on peripheral visual receptors, our results contribute to disentangling the functional and regulatory basis of evolutionary adaptations underlying sensory visual phenotypes in invertebrates.

Poster 10 >> Kazumi Sakai (Kyoto University, Japan)

Transient optical control of intracellular cAMP level using photocyclic visual rhodopsin

Opsins are photoreceptive proteins in animals and function as GPCR. Thus, opsins can evoke the intracellular second messenger systems by light to lead to cellular responses. Vertebrate visual opsin, rhodopsin, couples to transducin (Gt) and triggers the hyperpolarization response in rod cells of the retina. In addition, vertebrate rhodopsin is known to couple to Gi type of G protein, which leads to the prolonged decrease of the intracellular cAMP level in mammalian cells. Thus, vertebrate rhodopsin has a potential as an optogenetic tool for regulating the intracellular cAMP level. However, vertebrate

rhodopsin forms a metastable active state after photoreception and cannot thermally self-regenerate to the dark state. This molecular property makes it difficult to induce repetitive cellular responses by vertebrate rhodopsin. Recently, in the wake of the first identification of photocyclic non-visual opsin (1,2), we successfully created photocyclic vertebrate rhodopsin mutant by a single mutation (3). This vertebrate rhodopsin mutant photo-converted to the active state and spontaneously reverted back to the dark state. This photocyclic mutant could induce the repetitive transient decrease in cAMP level by light. In this study, we designed a different type of photocyclic vertebrate rhodopsin mutant by the change of the G protein coupling selectivity. The photocyclic vertebrate rhodopsin whose cytoplasmic domains are replaced by those of Gs-coupled GPCR induced the repetitive transient increase in cAMP level by light. We would like to discuss the potential of photocyclic vertebrate rhodopsin mutants for an optogenetic tool.

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Poster 11 >> Noa Nairner (Weizmann Institute of Science, Israel)

Structural Studies of the Betrhodopsins Capture Wavelength Specific Mechanism of Action

Many organisms sense light using rhodopsins, which are photoreceptive membrane proteins containing a retinal chromophore. This family of proteins shares a similar structure comprised of 7 transmembrane domains to which the retinal is covalently bound, but beyond these shared features they exhibit a vast diversity of functions, e.g. light-driven ion transporting in the case of many microbial rhodopsins. Recently, our group characterized a novel family of rhodopsins in marine unicellular algae – the bestrhodopsins, where two rhodopsin domains in tandem are fused to a bestrophin channel domain. Cryo-EM analysis of one bestrophin specie, Tara-RRB (Rhodopsin-Rhodopsin-Bestrophin), revealed that it forms a pentameric megacomplex, in which the channel center is comprised of five bestrophin monomers surrounded by five rhodopsin pseudo-dimers. Electrophysiological measurements further revealed that in accordance with their structure, bestrhodopsins are light activated ion channels. Here, we show the cryo-EM analysis of two additional RRB variants from different algae clades, capturing the complexes in action in response to differential light conditions. These studies reveal their mechanism of activation and demonstrates the wavelength specific channel opening and closing mechanism.

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Poster 12/Talk C4 >> Alina Pushkarev (Humboldt University of Berlin, Germany)

Crustaceans as a source of new bistable rhodopsins for optogenetic applications

Although crustaceans have intrigued the scientific world for a long time. In order to prove that animals such as the mantis shrimp indeed see beyond the visible spectrum, absorbance measure- ments were made directly on the eyes complemented with behavioral studies. Crustacean rhodopsins are especially interesting since they can detect wavelengths beyond the visual spectrum. Humans call the visual spectrum this way since it is our limitation as a species. In reality, different insects, marine animals, and fungi can detect light waves beyond this range. Rhodopsins that absorb at the far end of the visible spectrum are important for use in the field of optogenetics for controlling neuronal activity by expressing light-activated proteins in otherwise blind neurons. Infrared-absorbing rhodopsins are useful since the red wavelengths penetrate the tissue and scatter more in the neuronal tissue. Far-red-absorbing rhodopsins are scarce in nature, and one of their bearers are stomatopod crustaceans (a famous member is the peacock mantis shrimp), which can detect wavelengths ranging from UV (310 nm wavelength) to infrared (over 700 nm) by using different rhodopsins. In this work, I will present the opsins we were able to express and characterize from crustaceans, many of which are bistable.

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Poster 13 >> Shota Itakura (Nagoya Institute of Technology, Japan)

FTIR study of bistable rhodopsin

Animal rhodopsins are light-responsive G protein-coupled receptors (GPCRs) that function in both vision and non-visual processes such as circadian rhythms. They bind retinal as a chromophore within the protein moiety, and the photoisomerization reaction of retinal induced by light absorption triggers structural changes in the receptor, activating signal transduction pathways mediated by G proteins. Animal rhodopsins are largely classified

into two groups based on the stability of their light-activated states: monostable and bistable types. The former exhibits a bleaching reaction where the retinal dissociates from the protein moiety upon activation by the cis-to-trans isomerization of retinal. In contrast, the latter returns to its initial state through a re-isomerization reaction from the trans to cis forms upon absorbing light again in the activated state.

Structural and spectroscopic studies on the photoreaction of monostable animal rhodopsins have advanced, deepening our understanding of the molecular mechanisms of retinal isomerization, structural changes in the receptor, and activation at the atomic level. However, research on bistable animal rhodopsins has lagged behind. In this study, we aimed to elucidate the differences in photoreaction dynamics between monostable and bistable types of animal rhodopsins. By modifying the sample chamber of the Attenuated Total Reflection FTIR spectroscopy (ATR-FTIR), we successfully captured the structural changes accompanying the binding of the receptor with G protein-interacting peptides or domains under light illumination. In the presentation, we will also discuss the differences in the activation mechanisms between monostable and bistable types, based on spectroscopy measurements conducted by varying the length and concentration of the peptides.

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Poster 14 >> Songhwan Hwang (Humboldt University of Berlin, Germany)

Computational investigation of pH-dependent conformational dynamics of bistable fish louse rhodopsin

The vision of the fish louse Argulus foliaceus is crucial for searching and infecting its host, with increased swimming activity in darkness enhancing its infection success.[1] Additionally, the fish louse is sensitive to pH, as elevated acidity levels enhance its mortality, while its host fish can survive in the same conditions.[2] Therefore, pH is a critical environmental factor affecting the survival and mortality of the fish louse. We have found that fish louse rhodopsin, a G-protein coupled receptor, exhibits bistability: the 11-cis retinal bound state shows pH-sensitive absorption of wavelengths in a range of pH 4 to 9, whereas all-trans retinal bound state does not exhibit spectral changes within this pH range. Currently, the atomistic mechanism behind the profound bistability of fish louse

rhodopsin, which could be of interest for optogenetic applications, remains uncharted. The presented study employed a combination of different computational techniques to elucidate conformational dynamics of fish louse rhodopsin, focusing on the pH-dependent spectral shifts.

To predict and explore the conformational space of fish louse rhodopsin, we conducted AlphaFold calculations[3] based on variations of multiple sequence alignments through alanine scanning, which revealed four distinct conformational clusters[4]. Subsequent constant pH molecular dynamics simulations[5,6] on these clusters were performed to predict pH changes of titratable residues upon conformational variation. Finally, a hybrid quantum mechanics/molecular mechanics (QM/MM) approach, along with vertical excitation energy calculations, was employed to predict the UV/Vis absorption spectra of retinal in different conformational states.

Our simulation studies identified Asp288 in transmembrane (TM) helix 7, one of the seven TM helices, as pH-dependent. This prediction, along with the associated structural alterations related to the retinal binding site, provides intriguing insights into the molecular mechanisms governing the spectral properties of rhodopsin, which is currently being verified experimentally.

In conclusion, this integrated computational study predicts the conformational landscape of fish louse rhodopsin coupled with the pH sensitivity, potentially enhancing our knowledge of allosteric regulation. Our study further paves the way for future research on pH-dependent conformational dynamics in other rhodopsins.

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Poster 15/Talk D1 >> Stefan Haacke (University of Strasbourg - CNRS, France)

New Insights on the Ultrafast Photophysics of Archaerhodopsin-3 and its Fluorescent Mutants

Archaerhodopsin-3 (AR-3) is a light-driven transmembrane proton pump found in Halorubrum sodomense, that has emerged as an interesting platform for optogenetics, since the wild-type (wt) form and a large variety of mutants display good fluorescence levels, depending on the transmembrane voltage. Multiple mutants were reported over the years, with fluorescence quantum yields (FQY) reaching up to 1.2%, in the best cases, which is still low as compared to the most fluorescent natural neorhodopsin (NeoR). Experimental and theoretical studies aiming at a rational understanding of the mutation-induced ≈100-fold increase of FQY, as compared to wt AR-3, were disclosed recently for the Quasar and Archon families. In the present contribution, we focus on the double mutant DETC and the quintuple mutant Arch-5. A combination of different spectroscopic techniques (fluorescence, transient absorption, and Raman scattering), either steady-state or with time resolution down to the 50 fs, allows us to establish that the long-lived fluorescence in the mutant comes from a one-photon excitation process, unlike wt AR-3. Both all-trans/15-anti and 13-cis/15-syn isomers of the protonated Schiff base retinal (PSBR) cation contribute to the fluorescence in the mutants with slightly different excited state lifetimes (ESL). The temperature-dependence of the ESL's allows us to determine the exc. state barrier height, which prevents photo-isomerisation. Indeed, as compared to wt AR-3, the isomerization QY is found to be > 15 times smaller for DETC, and close to zero for Arch-5.

In a recent quantum chemistry treatment, some us showed that the extended ESLs of AR-3 mutants and of NeoR are due to unusual mixture of charge-transfer and covalent biradical character of the first excited state, which give rise to the formation of an energy barrier for increasing C13=C14 dihedral angle. In this contribution, we will show that the same electronic properties are found for wt AR-3 as well, but **without** the existence of an excited state barrier. Indeed, our experiments show that the photo-isomerisation occurs on a 300-fs time scale with vibrational coherences surviving in the photo-products. As the simulations show, the latter are due to excited state wavepackets along the C13-methyl and C14-H wag motions, the phase of which controls the outcome of the reaction, productive 13-cis isomerization vs. non-productive all-trans backreaction.

In summary, we will present new fundamental results, which allow to rationalize the exceptional fluorescent properties of AR-3 mutants, and which explain why AR-3 is an outstanding platform for genetical engineering of fluorescent rhodopsins.

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Poster 16 >> Arno Munhoven (Humboldt University of Berlin, Germany)

Are Rhodopsins Involved in Avian Magnetosensitivity?

For centuries, sailors have navigated unknown waters using star charts and compasses. Similarly, Earth's magnetic field guides various migratory bird species over thousands of kilometers to their feeding or wintering grounds. Despite 60 years of research, the light-dependent molecular mechanisms underlying avian magnetosensitivity remain elusive (Nimpf & Keays, 2022). Recent studies have shown that the cryptochrome 4 (Cry4) photoreceptor in migratory songbirds forms radical pairs that can be influenced by magnetic fields 500 times stronger than Earth's (Xu et al., 2021). However, proving the causality between Cry4 and avian navigation is challenging due to the lack of suitable genetically modifiable model organisms. Additionally, Cry4's photochemical properties, such as partial irreversibility and lack of red light absorption (under which birds orient), cast doubt on its role as a magnetodetector (Kutta et al., 2017).

In this context, rhodopsins have been overlooked as potential magnetodetectors due to their inability to form radical pairs, a requirement based on a theory from the 1970s (Schulten et al., 1978). Nevertheless, recent findings suggest rhodopsins might interact with Cry4 (Nießner et al., 2011; Wu et al., 2020). This project aims at investigating whether specific rhodopsins interact with cryptochrome in vitro or facilitate energy transfer. Additionally, we will explore the photochemistry of several rhodopsins for radical pair characteristics. To address these questions, we will heterologously express and purify these GPCRs in order to subject them to comprehensive spectroscopic studies. The opsin genes originate from genetically modifiable birds, thereby opening the door to behavioural experiments with knockout lines.

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Poster 17 >> Sayaka Ohashi (Nagoya Institute of Technology, Japan)

Structural analysis approaching the origin of spectral tuning in cone pigments

Three cone pigments; red, green, and blue consist of a different protein bound to a common chromophore, 11-cis-retinal. Structure determination of cone pigments is needed for a precise understanding of spectral tuning. The principal obstacle to solving the structures is their inherent instability in detergent micelles and photo-bleach reaction. To overcome these multiple bottlenecks, we have engineered primate red/green pigments (MR/MG) with BRIL-fusion and thermostabilizing mutations to obtain stable structures. In addition to engineering MR/MG, we used an antibody fragment against BRIL to increase the protein size for cryo-EM single-particle analysis. We aim to discuss further improvements to achieve the goal of structure determination.

FTIR spectroscopy is also crucial as a structural analysis method that elucidates the chemical interactions between cone pigments and retinal. We have previously conducted high-accurate FTIR spectroscopy to investigate the structural factors behind the 30 nm difference in λmax between MR and MG, revealing that the polar environment, including water molecules near the β-ionone ring, is involved. In this study, we focused on the differences in three amino acids around retinal between MR and MG, performed FTIR spectroscopy on mutants in which each amino acid was swapped between them to analyze the differences in hydrogen bonding network in more detail, In the presentation, we will discuss the latest data on the spectral tuning mechanism, along with the results of quantum chemical calculations.

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Poster 18/Talk D2 >> Gerrit Lamm (Goethe University Frankurt am Main, Germany)

The photochemistry of a microbial rhodopsin from Cryobacterium levicorallinum investigated by time-resolved optical spectroscopy

Microbial rhodopsins form a versatile group of photoreceptors that are found in almost all areas of live. While the main structural features are largely conserved, they differ in key aspects that ul- timately determine protein properties and functionality. Recently, we discovered novel microbial rhodopsins, originating from organisms mostly living in cold environments.1 They exhibit sev- eral unique properties, such as an elongated C-terminus, novel 3- and 7-letter motifs and a within this sub-family conserved arginine residue not previously observed in microbial rhodopsins. Spec- troscopic characterization of CryoR family members revealed extremely slow photocycle kinetics under physiological conditions (pH 8.0), while normal photocycle durations in the range of sec- onds were observed under acidic conditions (pH 3.5). In this study, CryoR1 from Cryobacterium levicorallinum was investigated in more detail. pH titration experiments revealed an 80 nm spec- tral shift between two subpopulations, one of which has an absorption maximum at 620 nm un- der acidic conditions, whereas absorption maxima >600 nm are rarely observed among microbial rhodopsins. This subpopulation is furthermore accompanied by strong coherent oscillations on the ultrafast timescale and a decrease in photoproduct yield. By combining structural and spectro- scopic data a model was finally developed that explains the extremely slow photocycle dynamics of CryoRs under physiological conditions. The highly conserved arginine (R57 in CryoR1) plays a key role in this model, which is reinforced by the spectroscopic data on the CryoR1 R57T variant.

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Poster 19 >> Tatsuya Sakamoto (Nagoya institute of technology, Japan)

Attenuation of light-induced structural changes of SRII-HtrII complex from Natronomonas pharaonis analyzed by surface-enhanced infrared spectroscopy

Sensory Rhodopsin II is a microbial rhodopsin derived from *Natronomonas pharaonis*, functioning as a light sensor for negative phototaxis by forming a complex with the transducer protein *p*HtrII. *p*SRII absorbs blue light, which isomerizes the retinal chromophore from all-*trans* to 13-*cis*. The photoreaction leads to formation of intermediates in the order of K, L, M, and O, which finally returns to the initial state in the photocycle. During the formation of the M intermediate, helix F opens outwardly in the cytoplasmic side, and second transmembrane helix (TM2) of *p*HtrII rotates clockwise following the movement of the helix F. Various spectroscopic studies have been conducted to elucidate key structural changes by comparing *p*SRII alone and the *p*SRII-*p*HtrII complex. The previous experiments were basically performed under conditions where the proteins were not aligned.

Surface-enhanced infrared absorption spectroscopy (SEIRA) is a measurement method utilizing the phenomenon that infrared absorption intensity is enhanced by about 10 to 100 times on a gold thin film. SEIRA has three characteristics. First, the effective range of enhancement by the gold film is limited to about 10 nm from the gold surface. This allows selective measurement of proteins adsorbed onto the gold thin film. Furthermore, the

closer the vibration is to the gold thin film, the stronger the infrared absorption is enhanced. Second, it selectively enhances the infrared absorption of vibrational modes moving perpendicular to the gold film. Third, SEIRA enables measurement of protein under similar orientation in a cell membrane by forming a monolayer of proteins attached on the gold thin film.

Using SEIRA, comparative experiments were carried out on the M intermediates of *p*SRII and *p*SRII-*p*HtrII complex. *p*SRII and *p*HtrII were connected by a linker sequence, which enabled that *p*SRII and *p*HtrII domains were closely attached on a gold thin film, and the light-induced structural changes were measured by SEIRA. The amplitude of the light-induced infrared absorption change was increased in accordance with increase of surface concentration of *p*SRII, while the *p*SRII-*p*HtrII complex exhibited decrease of the light-induced infrared absorption change as the surface concentration increased. The condensed *p*SRII and *p*HtrII interactions probably suppress the light-induced structural changes of *p*SRII and *p*HtrII, which can be monitored by SEIRA.

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Poster 20 >> Tillmann Utesch (Leibniz-Institut für Molekulare Pharmakologie (FMP), Germany)

Understanding the voltage-sensitivity of Arch3-based microbial rhodopsins

In neuroscience, the visualization of membrane potentials in living cells is fundamental to understand the function of neurons. Although extensive research in recent years led to significant progress, the low signal intensity remains a very challenging problem for monitoring electric currents. In this context, voltage-dependent fluorescent archaerhodopsin-3 (Arch3) was identified as solid basis for developing new optogenetic sensors. Arch3 has, however, two major limitations for the application in living cells: a) its low quantum yield and b) its low voltage sensitivity.

In our study, we aimed to understand and predict the voltage response of known Arch3 derivatives, including Arch2, Arch3, and Archon1, as well as selected mutants using the molecular dynamics (MD) based computational electrophysiology approach. This technique allows us to capture dynamic processes on the nano- to microsecond time scale upon voltage. These observed structural changes include the influx of water, the rearrangement

of the hydrogen bonding network including the retinal Schiff base, and minor structural alterations in the protein. We systematically predicted the roles of key amino acids by mutation and suggested variants with improved voltage sensing properties based on this knowledge. Subsequently, the computational predictions were validated through experiments.

We showed that besides to the well-known arginine R92 adjacent to the counter ions, also intracellular aspartic acid D125 in helix D plays a key role in the voltage sensing process. The role of D125 was further investigated by mutation. In D125N, the increased flexibility of helix D disrupted the hydrogen bonding network around N125, resulting in a higher voltage sensitive fluorescence in the validation experiments.

This knowledge on the molecular level will be further deepened in the future and aid in the rational design of new optogentic sensors with enhanced properties.

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Poster 21/Talk D3 >> Giorgia Ortolani (University of Gothenburg, Sweden)

STRUCTURAL BASIS FOR THE PROLONGED PHOTOCYCLE OF SENSORY RHODOPSIN II REVEALED BY SERIAL SYNCHROTRON CRYSTALLOGRAPHY

Microbial rhodopsins are a family of light-responsive proteins featuring seven transmembrane helices and a retinal chromophore linked to a lysine residue on helix seven. These proteins operate as proton or ion pumps, light-gated ion channels, and photosensors.

Research on Bacteriorhodopsin (bR) from *Halobacterium salinarum* and Sensory Rhodopsin II (NpSRII) from *Natronomonas pharaonis* has provided valuable insights into energy transduction and phototaxis, but while both proteins exhibit similar structural characteristics and coordinated photocycles, bR photocycle duration is significantly faster than in NpSRII.

In our study, we performed serial synchrotron X-ray crystallography (SSX) studies to better understand the mechanisms of light sensing in NpSRII and, then, compared our results with those in bR to elucidate the structural factors contributing to the differences in the photocycle durations.

While we observed several common structural changes, NpSRII differs from bR in that its helix G does not unwind near the lysine residue, and transient water molecule binding sites do not quickly appear on the cytoplasmic side of the retinal. These structural distinctions prolong NpSRII's photocycle due to the need of this sensory protein to extend the light-initiated signal.

This research offers a structural rationale for how a common protein framework can be modified for diverse functions and highlights the insights that time-resolved crystallography can provide. Comparative time-resolved diffraction studies reveal how minor structural variations influence protein function, paving the way for new understandings of protein-catalyzed reactions.

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Poster 22 >> Ori Berman (Weizmann Institute of Science, Israel)

Spectral and Functional Analysis of the Bestrhodopsins - a Family of Light Activated Ion Channels

Rhodopsins are integral membrane proteins that bind retinal chromophores to form light-absorbing pigments. These proteins are ubiquitous in nature and play critical roles in microbial physiology as well as animal visual perception. Our lab recently discovered and characterized a novel family of rhodopsins in alga - the bestrhodopsins, in which one- (or often two-) rhodopsin domain(s), are C-terminally fused to a bestrophin channel. Bestrophins are ion channels known for their involvement in developing the retinal pigment epithelium in humans and other animals. Cryo-EM analysis of bestrhodopsins revealed that it forms a pentameric megacomplex (~700 kDa) with ten rhodopsin units surrounding the channel in the center. We further showed that bestrhodopsins are light dependent anion channels. Here we will present a thorough analysis of different family members from the bestrhodopsin family. These members greatly differ in their domain composition, light response and channel activation properties.

Authors

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Poster 23 >> Yusuke Sakai (The University of Manchester, UK)

Mechanism of spectral sensitivity and G protein signalling in mouse and human Opn5s

Opsins are members of a large family of G protein-coupled receptors (GPCRs) and serve as photoreceptive proteins by binding chromophore retinal. Recent progress in biochemical and physiological studies of "non-visual" opsins expressed outside photoreceptor cells has shown a great diversity in their spectral sensitivities, signalling properties, and physiological functions. Opn5, a type of non-visual opsins, is found expressed in a wide range of mammalian tissues such as retina, brain, spinal cord, outer ear, and testis (1,2). All mammalian Opn5s identified thus far have their spectral sensitivity peaks in UV region. The characterisation of G protein signalling of mammalian Opn5s provide evidence that they couple with Gi/o- and Gq-type (especially G14 subtype) G proteins (1,3,4). While knowledge regarding their spectral sensitivity and G protein signalling has been accumulating greatly, the mechanism underlying the unique molecular properties is still unexplored. In this study, we focused on human and mouse Opn5s to clarify the amino acid residues responsible for their UV sensitivity and G protein selectivity. Substituting a single amino acid near the retinal Schiff base in transmembrane helix II which is conserved among mammalian Opn5s successfully shifted their peak spectral sensitivities from UV to visible light region, showing the residue is at least a necessary condition for achieving UV sensitivity in human and mouse Opn5s. We compared overall G protein activation profiles of human and mouse Opn5 using BRET-based G protein activation biosensors and found that human Opn5 showed more specific activation of Gq than mouse Opn5 which exhibited the promiscuous G protein activation profile. Based on this result, we identified amino acid residues responsible for Gq specificity in human Opn5. These results could serve as a stepping stone to uncover the physiological functions of Opn5 in mammals, which are not yet fully understood, and may also provide important insights for the development of new optogenetic tools.

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Poster 24/Talk D4 >> Yosuke Mizuno (Nagoya Institute of Technology, Japan)

Light-induced FTIR spectroscopy of microcrystals of visual rhodopsin grown in LCP

Visual rhodopsin is a light-sensitive G-protein-coupled receptor (GPCR) responsible for twilight vision. Vision is initiated by the photoisomerization of the retinal chromophore from 11-cis to all-trans, which is bound to rhodopsin via a protonated Schiff base linkage. Rhodopsin is one of the most extensively studied GPCRs at the molecular level. The three-dimensional structures of the dark state, the initial intermediate Batho, and subsequent intermediates Lumi and the active Meta-II have been determined by X-ray crystallography at low temperatures. In 2023, time-resolved X-ray crystallography at room temperature captured structural changes up to the Batho intermediate in real-time for the first time. However, it remains unclear whether the structural changes observed in crystal are identical to those occurring in the natural lipid environment.

In this study, we performed light-induced difference FTIR spectroscopy on LCP microcrystals of rhodopsin used for time-resolved structural analysis. Since there have been no prior examples of light-induced FTIR spectroscopy using microcrystal samples of photoreceptive proteins, we carefully optimized conditions such as film sample preparation, measurement temperature, and data analysis to obtain highly accurate spectra. As a result, we successfully measured the spectra of intermediates formed during the photoreaction process of rhodopsin within the microcrystals. We then compared these spectra with those obtained in the lipid environment to discuss the differences in structural changes during the photoreaction process.

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Poster 25 >> Da Som Kim (Jeounbuk national university, South Korea)

The study of proton pumping actinorhodopsin with 3D structure

Actinorhodopsin, a bacterial rhodopsin derived from Candidatus Rhodoluna planktonica MWH-DarI (GenBank: ACN42849.1), is a light-driven outward H+ pump in freshwater environments. To study this protein, we chemically synthesized the actinorhodopsin gene based on the Candidatus Rhodoluna planktonica MWH-DarI sequence and cloned it for expression. We successfully expressed actinorhodopsin in an E. coli expression system, incorporating all-trans retinal, which imparted a red coloration to the protein. The expressed actinorhodopsin closely resembles Gloeobacter rhodopsin and contains residues that bind carotenoids.

Previous analyses of bacterial rhodopsins, such as xanthorhodopsin, archaerhodopsin, and Gloeobacter rhodopsin, have explored their functional properties and interactions with carotenoids. In this study, we crystallized the expressed actinorhodopsin to determine its 3D protein structure. This structural information provides insights into the carotenoid-binding capabilities of actinorhodopsin compared to other carotenoid-binding rhodopsins.

Our study further characterized actinorhodopsin in terms of pH-dependent absorbance, proton pumping activity, and photocycle dynamics under light adaptation. The results demonstrate that actinorhodopsin effectively absorbs green light and exhibits a rapid photocycle rate conducive to outward proton pumping. Structural analysis reveals that actinorhodopsin forms a homopentameric complex with a central pore. This unique pentameric structure is stabilized by five strong salt bridges between glutamic acid and arginine residues, which hold each protomer together from the cytoplasmic side. The occurrence of such salt-bridged pentameric structures is relatively rare among rhodopsins, highlighting the distinctive nature of actinorhodopsin's structural configuration.

[This research was supported by 2022R1A6A3A01087301 and 2022R1A6A1A03055869]

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Poster 26 >> Masaki Tsujimura (The University of Tokyo, Japan)

Strong hydrogen bonds and proton transfer in microbial rhodopsins

Hydrogen bonds (H-bonds) play roles in stabilizing the protein higher-order structure and in acting as sites for chemical reactions, including proton transfer. [OH...O] H-bond can be characterized by the O–H bond distance (γO–H), the O...O distance (γO...O), the O–H stretching vibrational frequency (νO–H) and the ^{1}H NMR chemical shift (δH). An H-bond can also be characterized by the free energy difference between the [OH...O$^-$] and [O$^-$...HO] species (ΔG). The difference in the pK_a values between the H-bond donor (D) and acceptor (A) in water, $\Delta pK_a = pK_a(DH/D^-) - pK_a(AH/A^-)$, is linearly related to ΔG empirically (Huyskens and Zeegers-Huyskens. J. Chim. Phys. 61, 1964). ΔpK_a is a simple but an important parameter which is related not only to proton transfer via ΔG, but also to the strength of the H-bond ("pKa slide rule"; Gilli et al. Acc. Chem. Res. 42, 2009).

In this study, we analyze the relationships between ΔpK_a and γO–H, γO...O, νO–D, and δH, using X-ray crystal structures of microbial rhodopsins and a quantum mechanical/molecular mechanical (QM/MM) approach (Tsujimura et al. Biophys. J. 122, 2023). The correlations can be explained by introducing a model potential for the H-bond and using previously reported empirical correlations (e.g., valence bond order model; Steiner and Saenger. Acta Cryst. B50, 1994). Specifically, the correlation between ΔpK_a and γO...O is validated using crystal structures of small molecules obtained from the Cambridge Structural Database and atomic-resolution structures of proteins obtained from the Protein Data Bank. Finally, we discuss the strong H-bonds associated with proton transfer in microbial rhodopsins, including (i) the strong H-bonds of water molecules in proton pumping rhodopsins with νO–D < 2400 cm^{-1} (Shibata and Kandori. Biochemistry 44, 2005), and (ii) the strong H-bond between carboxylic acid residues (e.g., Glu64...Asp105 in sodium pumping rhodopsin *Er*NaR; Podoliak et al. Nat. Commun. 15, 2024).

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Poster 27/Talk E1 >> Miroslav Kloz (ELI ERIC, Czech)

Femtosecond Stimulated Raman Spectroscopy: A Tool Tailored for the Study of Rhodopsin Dynamics

The research on Rhodopsin proteins has become so extensive that there is a demand for advanced techniques that enable detailed characterization and study of Rhodopsins with high throughput and minimal sample requirements. Femtosecond stimulated Raman spectroscopy (FSRS) is a technique that allows the examination of conformational changes

of the retinal cofactor at the molecular level, with time resolution on the order of approximately 100 femtoseconds, matching the timescales of molecular dynamics.

Utilizing Raman resonance, the vibrational states of the retinal cofactor can be observed with minimal interference from the protein environment. When employing a synchronized laser pair, the experimental setup enables the recording of dynamics spanning from femtoseconds to seconds—covering 12 orders of magnitude in time—within a single experiment. This capability allows for comprehensive characterization of the activation processes of various rhodopsins.

At the ELI Beamlines facility in Prague, we operate such a setup, which has successfully characterized the dynamics of rhodopsins such as "Bestrhodopsin," "Neorhodopsin," and several other systems. We continuously strive to improve experimental sensitivity to minimize the required sample volume, which is particularly relevant for rhodopsins that cannot undergo repeated photocycles in vitro, such as the rhodopsin responsible for human vision.

Currently, we are developing methods to collect data from single-shot acquisitions and using spatial light displacement to extend the time window to seconds and minutes. We will present the experimental technique and results demonstrating the potential of our setup in advancing rhodopsin science.

Poster 28 >> Shunya Murakoshi (The University of Tokyo, Japan)

Structural Insights into Xanthophyll Binding in Proteorhodopsin

Proteorhodopsins, members of the out-ward proton pump rhodopsin family, are widely utilized by marine microorganisms to capture light energy, which is then used to power cellular processes. Traditionally, these proteins utilize retinal molecules as their sole chromophore, absorbing light in specific wavelengths. However, the discovery of xanthorhodopsins, which bind additional hydrocarotenoid molecules as antenna chromophores, expanding their light absorption spectrum, has opened new avenues of research into the functional diversity of rhodopsins.

In this study, we extend our previous research on Kin4B8, a newly discovered xanthorhodopsin that uniquely binds hydroxycarotenoid molecules. Here, we focus on a proteorhodopsin that, like xanthorhodopsin, utilizes xanthophyll as an additional chromophore. Using cryo-electron microscopy, we performed high-resolution structural analysis of the proteorhodopsin-xanthophyll complex, achieving a resolution of 2.7Å. This structural elucidation provides insights into the molecular mechanisms by which xanthophylls contribute to the photobiological functions of proteorhodopsins.

Our findings have significant implications for understanding the energy conversion processes in marine microorganisms and expand the potential applications of rhodopsins in optogenetics and synthetic biology. The detailed structural information gained from this study enhances our knowledge of light-driven energy utilization in diverse environmental conditions, offering new possibilities for bioengineering and biotechnological innovations.

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Poster 29 >> Ramprasad Misra (Humboldt University of Berlin, Germany)

On elucidating cryo-EM structure and function of rhodopsin-guanylyl cyclase from Catenaria anguillulae

Enzymerhodopsins are a novel class of natural rhodopsin-based photoreceptor proteins known for their light-regulated enzyme activity. To date, three types of these fusion proteins with an N-terminal type-1 rhodopsin and a C-terminal enzyme domain, namely, histidine kinase rhodopsins (HKR), rhodopsin phosphodiesterase (RhoPDE) and rhodopsin guanylyl cyclase (RhGC) have been identified, although their physiological relevance is largely unknown. In spite of their immense potential in applications in optogenetics, the structure of a full-length enzymerhodopsin also still remains elusive.

In this presentation, we wish to discuss the function and state-of-the-art of cryo-EM structure of rhodopsin-guanylyl cyclase from *Catenaria anguillulae* (CatRhGC). This protein converts guanosine triphosphate (GTP) to cyclic GMP when irradiated with green light with high light to dark activity ratio. The full-length CatRhGC is expressed either in yeast (Pichia pastoris) or in insect (sf21) cells, followed by studies of their light-regulated enzyme activity and structure elucidatation. This protein is known to be functional in dimeric forms, although the 2D classification of cryo-EM micrograph revealed that the detergent solubilized protein mostly remained as pentamer with a mixture of other oligomeric forms, viz., dimer, tetramer and monomer. Efforts are made to obtain the cryo-EM structure of the protein in presence of either GTP or GTP-inhibitor supplemented by a suitable divalent metal ion that are expected to facilitate dimer formation of the protein. Although a high-resolution structure of the full-length CatRhGC is yet to be resolved, we shall discuss the steps taken to control the protein quality to achieve the active, functional form of the protein for functional and structural analyses.

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Poster 30/Talk E3 >> Thomas Perkins (JILA, NIST & University of Colorado, USA)

Quantifying a light-induced energetic change in a single molecule of bacteriorhodopsin by atomic force microscopy

Ligand-induced conformational changes are critical to the function of many membrane proteins and arise from numerous intramolecular interactions. In the photocycle of the model membrane protein bacteriorhodopsin (bR), absorption of a photon by retinal triggers a conformational cascade that results in pumping a proton across the cell membrane. While decades of spectroscopy and structural studies have probed this photocycle in intricate detail, changes in intramolecular energetics that underlie protein motions have remained elusive to experimental quantification. Here, we measured these energetics on the millisecond time scale using atomic-force-microscopy–based single-molecule force spectroscopy. Precisely timed light pulses triggered the bR photocycle while we measured the equilibrium unfolding and refolding of the terminal 8-amino-acid region of bR's G-helix. These dynamics changed when the EF-helix pair moved ~9 Å away from this end of the G helix during the "open" portion of bR's photocycle. In ~60% of the data, we observed abrupt light-induced destabilization of 3.4 ± 0.3 kcal/mol, lasting 38 ± 3 ms. The kinetics and pH-dependence of this destabilization were consistent with prior measurements of bR's open phase. The frequency of light-induced destabilization increased with the duration of illumination and was dramatically reduced in the triple mutant (D96G/F171C/F219L) thought to trap bR in its open phase. In the other ~40% of the data, photoexcitation unexpectedly stabilized a longer-lived state distinct from the canonical photocycle. Through this work, we establish a general single-molecule force spectroscopy approach for measuring millisecond-scale ligand-induced energetics and lifetimes in membrane proteins, in general, and light-driven dynamics, in particular.

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Poster 31 >> Teppei Sugimoto (Nagoya institute of technology, Japan)

Spectroscopic analysis of the photoreaction scheme of TAT rhodopsin in the presence of calcium ion

TAT rhodopsin is a new type of microbial rhodopsin found in SAR11 HIMB114, a marine bacterium. A unique feature of TAT rhodopsin is the presence of two absorption states, visible- and UV-absorbing, at physiological pH. The visible-absorbing state contains a protonated Schiff base, like many other rhodopsins. However, this state is non-functional due to thermal back-isomerization following the primary K intermediate [1]. In contrast, the UV-absorbing state exhibits a long-lived photocycle, suggesting that TAT rhodopsin may act as a UV-dependent environmental pH sensor. Moreover, recent findings reveal that calcium ions shift the equilibrium toward the UV-absorbing state, making it the dominant form of TAT rhodopsin under physiological ocean conditions.

In this study, we analyzed the photoreaction of TAT rhodopsin in the presence of calcium ions using various spectroscopic techniques. First, we performed flash photolysis measurements to clarify the photocycle in the presence of calcium ions. Without calcium (at pH 10), the longest-lived intermediate was M intermediate, characterized by a deprotonated Schiff base. However, in the presence of calcium ions, the red-shifted O intermediate became the longest-lived. We found that the photoreaction involved four proton transfer reactions via Schiff bases. Next, we examined whether proton transfer during the photoreaction occurred through the solvent, using a pH-sensitive dye. We also employed low-temperature FTIR spectroscopy to investigate the structural changes occurring during the formation of each intermediate. In particular, we studied how the hydrogen bonding environment around the Schiff base changes during the formation of each intermediate, using isotope-labeled samples. Based on the findings from these spectroscopic techniques, we will discuss the role of calcium ions and the specific proton transfer mechanism during the photoreaction of TAT rhodopsin.

Co-authors

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Poster 32 >> Ryu Sato (Osaka Metropolitan University, Japan)

Spectral optimization of jumping spider Rh1 for depth perception from image defocus

Jumping spiders measure the distance and jump to capture their prey. We previously reported that jumping spiders perceive the depth based on defocused images captured by the second deepest layer (L2) of their unique four-layered retina. The image defocus is generated by a mismatch between color of lights focused on the L2 and received by the expressing rhodopsin [1, 2]. Accordingly, in addition to the unique retinal structure, the absorption spectrum of Rh1 that enables to receive defocused images in L2 is suggested to be a basic requirement for the mechanism. Here we investigated a significance and stringency of the spectral de-tuning of jumping spider rhodopsin (Rh1) in the depth perception mechanism. Identification and spectroscopic analysis of several kinds of jumping spider and "non-jumping" spider Rh1s revealed that the wavelength of maximum absorbance (λmax) of all analyzed jumping spider Rh1s are identical (535 nm), and any of λmax of "non-jumping" spider Rh1s are shorter than 535 nm, showing the stringency of the spectral de-tuning for the mismatch. We also inferred the ancestral sequence of jumping spider Rh1s (Anc2) as well as that of Anc2 and the outgroup (Anc1) and conducted a series of mutational analysis of Anc1 to identify sites involved in the spectral tuning. We determined that the substitutions at 16 sites including 4 predominant sites, are additively responsible for the spectral shift to Anc2. Theoretical calculation estimated that longer wavelength sensitive Rh1 could provide higher depth resolution, supporting that the longest 535 nm-λmax of jumping spider Rh1 is optimized for the depth perception. Furthermore, we sought the reason why λmax does not exceed 535 nm within protein function. We found some artificial Rh1 mutants at the site 292 have λmax longer than 535 nm (~545 nm) with a machine learning-based λmax estimation of possible Rh1 mutants at the four sites and experimental confirmation, but their G protein activation abilities are significantly decreased, indicating a disadvantage of Rh1 having the amino acid residue that can shift the λmax to longer than 535 nm. Based on the results, we will discuss the spectral tuning mechanism of jumping spider Rh1 that underlies the depth perception from image defocus and a trade-off between adaptations for the depth perception and structural constraints at protein level, providing a new insight into optimization of the absorption spectrum of visual pigment.

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Poster 33/Talk E4 >> Feng-Jie Wu (University of Basel, Switzerland)

Elucidating GPCR conformational dynamics by a novel NMR method

NMR chemical shift changes can report on the functional dynamics of biomacromolecules in solution with sizes >1 MDa. However, their interpretation requires chemical shift assignments to individual nuclei, which for large molecules often can only be obtained by tedious point mutations that may interfere with function.

Recently we in silico predicted and in practice created a global positioning system (GPS) that relies on pseudocontact shifts (PCSs) induced by paramagnetic thulium tag attached at various sites to an antibody in order to precisely position magnetic nuclei at distances >60 Å within a protein of interest (1). This information is subsequently used to obtain the NMR assignment for the nuclei. Using the GPS-PCS method, we have successfully assigned close to a hundred of 1H-15N NMR resonances of a G protein-coupled β1-adrenergic receptor (β1AR) in various functional forms. This amount of probes thoroughly report the details of how a GPCR undergo conformational transitions between different states.

Such method can be applied to any protein system with an existed binder protein. The understanding of a protein dynamics can be applied to fine-tune its signaling output.

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Poster 34 >> Takashi Nagata (The University of Tokyo, Japan)

Spectroscopic study on the absorption spectra and photoreaction of retinal G-protein-coupled receptors, RGRs

Animal rhodopsins typically function as light-sensing G-protein-coupled receptors. These animal rhodopsins, including vertebrate visual rhodopsins, bind 11-cis-retinal (11cR) as a chromophore. It isomerizes into all-trans-retinal (atR) upon light absorption, leading to the formation of the active form that drives intracellular signaling cascades. Vertebrate visual rhodopsins lose their photosensitivity after light absorption until they release atR and bind new 11cR. Therefore, supplying visual rhodopsins with 11cR is critical for maintaining photosensitivity. Retinal G-protein-coupled receptors (RGRs) play a crucial role in the supply system of 11cR in the eyes of vertebrates [1]. RGRs covalently bind the atR and re-isomerize it to 11cR upon light absorption, and then, the 11cR is released from RGRs and transported to visual rhodopsins. Hence, RGRs function as retinal-photoisomerases, not light sensors, for maintaining vision. Although most vertebrates possess RGRs, their absorption spectra were unknown except for bovine RGR, which maximally absorbs blue light [2]. To understand the spectroscopic properties of different RGRs, we expressed RGRs from other vertebrate species with mammalian cultured cells and investigated their absorption spectra by UV–visible spectroscopy. We found that purified chicken RGR as well as bovine RGR exhibited a reversible photoreaction in which the chromophore 11cR produced by the photo-isomerization from atR absorbs light again and re-isomerizes to atR. Most RGRs, including chicken and human RGRs, showed absorption maxima in the blue region [3], suggesting that blue light is essential in maintaining vision in vertebrates. Interestingly, teleost fishes possess two RGRs, RGRa and RGRb, which were duplicated in a teleost lineage. RGRa was a blue-absorbing pigment like other RGRs but RGRb formed a green-absorbing pigment. Based on the site-directed amino-acid mutagenesis, we investigated amino acid residues that are responsible for the spectral shift between the two teleost RGRs. In the presentation, we will discuss the spectral tuning mechanism for the two teleost RGRs.

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Poster35 >> Koichiro Kishi (The University of Tokyo, Japan)

Cryo-EM Analysis of a Diverse Channelrhodopsin Collection

Channelrhodopsins (ChRs) have revolutionized neuroscience as powerful optogenetic tools. Recent discoveries of new variants and engineered mutants have expanded ChR diversity, enhancing optogenetic capabilities. However, the structural basis for ChRs' diverse properties remains mysterious. To address this knowledge gap, this study aimed to clarify the relationship between ChR structure and function. Here, we performed cryo-electron microscopy (cryo-EM) structural analysis for a diverse set of 10 ChRs, comprising 7 cation-conducting ChRs (including C1C2, KnChR, TsChR) and 3 anion-conducting ChRs (including GtACR1). Binding antibody Fab fragments approach allowed us to obtain high-resolution structures (ranging from 2.23 Å to 3.10 Å) for all 10 ChRs and the obtained maps were clear enough to model water molecules. Compared to the previously reported crystal structures of C1C2 (PDB ID: 3UG9) and GtACR1 (PDB ID: 6CSM), our cryo-EM analysis revealed more water molecules in both cases, indicating that even at similar resolutions, cryo-EM can provide higher quality structural information compared to crystal structural analysis.

Focusing on absorption wavelengths, KnChR and TsChR exhibit blue-shifted absorption spectrum at 456 nm and 436 nm, respectively. These blue-shifted spectra are likely caused by the isomerization of retinal from 6-s-trans to 6-s-cis configuration, triggered by steric clash with the retinal pocket. To investigate whether this blue-shift mechanism is general, we focused on the key residue G202 (in C1C2) and identified 5 ChRs from metagenomic data where this position is not occupied by Glycine (non-G). Upon measuring their action spectra, we found that all 5 non-G ChRs exhibited blue-shifted spectra below 470 nm, indicating that the non-G strategy may be a general mechanism for creating blue-shifted ChRs.

Furthermore, to facilitate comparison among the expanded set of ChR structures in this study, we propose a numbering scheme based on the conservation of amino acid residues in ChRs. Details about the ChR numbering system and deep structural comparisons using this numbering system will be discussed in the presentation.

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Poster 36/Talk F4 >> Lee Harkless (University of Maryland Baltimore County, USA)

The role of RGS proteins in determining melanopsin signaling outcomes

Melanopsin is a photosensitive G-Protein Coupled Receptor (GPCR) and is expressed in 6 distinct Intrinsically Photosensitive Retinal Ganglion Cell (ipRGC) subtypes in the mouse retina. IpRGCs are responsible for a myriad of non-image and image forming behaviors. Many of these subtypes differ in their light response amplitude and kinetics, as well as utilize heterogeneous phototransduction components downstream of Gαq. The mechanisms governing how a single GPCR, melanopsin, signals through different second messengers or channels is unclear. To understand how ipRGC subtypes signal differently from one another, we explored whether differential expression of relevant phototransduction cascade components exists between subtypes. Analysis of an available RNAseq dataset reveals that individual ipRGC subtypes differ in their G-protein expression and additionally are enriched for specific RGS proteins. Using RNAscope, we show that the M1 ipRGC is enriched for a Gαq selective RGS protein and that the M4 ipRGC expresses multiple Gαi/o selective RGS proteins. Cell-based BRET assays reveal a promiscuous G-protein signaling profile of mouse melanopsin, and that RGS proteins then refine the downstream signaling of melanopsin in various ways. We further show that melanopsin can increase cAMP via Gαq alone, Gαq-mediated cAMP production is enhanced by a class II adenylate cyclase, and Gαq-cAMP signaling is eliminated when Gbeta-gamma is inhibited. To directly test whether ipRGCs respond to increases in intracellular cAMP, we expressed the Gs-DREADD in M1 and M4 ipRGCs. M1 and M4 ipRGCs fire action potentials driven by Gαs-DREADDs. Our data informs how melanopsin signaling is regulated by RGS proteins, which may offer clues to how melanopsin signals differentially in vivo and provides new evidence for the role of cAMP in ipRGCs.

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Poster 37 >> Yuma Matsuzaki (The University of Tokyo, Japan)

Crystal structure of the rhodopsin in Asgard archea

Recent studies have reported the energy transfer from carotenoids to retinal in several rhodopsin proton pumps. This kind of energy transfer is also observed in the newly identified Heimdallarchaeial rhodopsin 1 (HeimdallR1) and carotenoid fucoxanthin. However, the binding mode of fucoxanthin to HeimdallR1 and the underlying energy transfer mechanism remain uncertain. To address this, we determined the crystal structure

of HeimdallR1 at 2.1 Å resolution. Although the electron density of fucoxanthin was not observed, likely due to its dissociation during crystallization, we identified several structural features of HeimdallR1. These include a membrane extended α-helix of intracellular loop 3 (ICL3) , a unique feature not observed in other rhodopsins, and a fenestration similar to those seen in other carotenoid-binding rhodopsins. These findings offer valuable insights into the possible fucoxanthin binding mode and the energy transfer mechanism in HeimdallR1.

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Poster 38 >> Taiki Nakamura (Osaka University, Japan)

Unusual Interaction of Schiff Base in the Retinal Chromophore of Sodium Ion-Pumping Rhodopsins

A Schiff base in the retinal chromophore of microbial rhodopsin is crucial for its ion-transport mechanism. In our presentation, we will report the discovery of an unprecedented isotope effect caused by deuteration on the C=N stretching frequency of the protonated Schiff base (PSB) in sodium ion (Na+)-pumping rhodopsins, *Krokinobacter* rhodopsin 2 (KR2) and *Bellilinea* sodium ion-pumping rhodopsin, indicating an unusual interaction in the protonated Schiff base. For these two Na+-pumping rhodopsins, we discovered that the frequency of the C=NH stretching vibrational band differed between H2O and the mixed buffer (H2O:D2O=1:1). The frequency difference was caused by deuteration. However, the shift cannot be explained by considering only the vibrational modes of the chromophore because the only exchangeable proton for the retinal chromophore is that of the PSB. This suggests that the shift observed for the Na+-pumping rhodopsins was caused by the interaction of the PSB with amino acids containing exchangeable protons and/or water molecules. Observations using KR2 mutants showed that no amino acid residues were responsible for the isotope effect, suggesting that the shift was caused by the interaction of water molecules with PSB. The proximity of the H–O–H bending vibrational frequency and the C=N stretching vibrational frequency of the H2O molecule suggests that this isotope effect originates from the coupling between the H–O–H bending and C=N stretching vibrations. In the mixed buffer, the fraction of H2O, which couples to the C=NH stretching vibration, decreased to 25%, resulting in a difference in the C=NH stretching frequency. Crystallographic data of wild-type KR2

showed that a water molecule was located perpendicular to the C=N–C plane of the PSB. Water molecules are accessible near the Schiff base, because the chromophore is largely twisted; otherwise, they cannot. Interestingly, we observed no isotope shift for a KR2 mutant in which the chromophore was less twisted, supporting our hypothesis that the isotope shift observed for the Na+-pumping rhodopsins is due to the H2O molecule located at a position perpendicular to the C=N–C plane of the PSB. The present study provides new insights into the interaction network of the PSB in the Na+-pumping rhodopsins.

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Poster 39/Talk G3 >> Ajith Karunarathne (Saint Louis University, USA)

Melanopsin Governs Wavelength-Dependent Cell Signaling and Animal Behavior

Over 10 million people in the United States are affected by Seasonal Affective Disorder (SAD), a form of depression more common among young adults and women. SAD is also linked with other mental health diseases. Although the interrupted sleep-wake cycle and the perturbation of the endogenous circadian rhythm have been named as major contributors, there is a critical knowledge gap underlying the pathophysiology of SAD. Despite the vague molecular rationale, the potential of bright light therapy, listed as a SAD treatment strategy by many institutes, including the National Institute of Mental Health (NIHM), offers hope and optimism for the future. The non-visual opsin melanopsin is proposed as the primary circadian photopigment in intrinsically photosensitive retinal ganglion cells (ipRGCs) that extend axonal projections to the master circadian pacemaker in the brain, suprachiasmatic nuclei (SCN). Our research, along with others, has shown that melanopsin activates multiple major G protein heterotrimers, including Gq, Gi/o, and Gs. Our novel data suggests that while the signaling properties of melanopsin are wavelength-dependent, the melanopsin-knockout mice lack the wavelength-dependent circadian shift exhibited by control mice. Our work may provide a molecular foundation for how environmental light exposure influences human behavior and shed light on light therapy.

Authors

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Poster 40 >> Tatsuki Tanaka (The University of Tokyo, Japan)

The high-light-sensitivity mechanism of the bacteriorhodopsin-like channelrhodopsin GtCCR4.

Channelrhodopsins are microbial light-gated ion channels that can control the firing of neurons in response to light. The cation channelrhodopsins from the cryptophyte *Guillardia theta* (GtCCRs) possess a DTD motif, similar to the proton pump bacteriorhodopsin (BR), and are thus referred to as BR-like channelrhodopsins. Among the GtCCRs, GtCCR4 exhibits more than 10 times higher light sensitivity compared to GtCCR1-3 and the typical channelrhodopsin CrChR2. Additionally, GtCCR4 possesses advantageous properties as an optogenetic tool, such as selectivity for monovalent cations other than protons and small desensitization under prolonged exposure. To understand how GtCCR4 achieves its high photosensitivity, we compared GtCCR2 and GtCCR4 by structural, electrophysiological, and spectroscopic analyses. Our structural analysis revealed that GtCCR4 has a significantly bent TM6 in its initial state, whereas many microbial rhodopsins have been suggested to bend their TM6 upon activation. Additionally, our FTIR difference spectra revealed that the structural changes in the peptide backbone of GtCCR4 during activation are smaller compared to those of GtCCR2. Lastly, our flash laser-based electrophysiological and time-resolved spectroscopic analyses demonstrated that, in contrast to GtCCR2 and other channelrhodopsins, GtCCR4 rapidly returns to its initial state almost simultaneously with channel closure, making it immediately ready to receive light again. These results suggest that the bent TM6 of GtCCR4 enables high light sensitivity by omitting the conformational change upon activation and eliminating the channel recovery time.

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Poster 41 >> Satoshi Tsunoda (Nagoya Institute of Technology, Japan)

Driving forces of proton-pumping rhodopsins

Proton-pumping rhodopsins are light-driven proton transporters that have been discovered from various microbiota. They are categorized into two groups, outward-directed and inward-directed proton pumps. Although the directions of transport are opposite, they are active proton transporters that create a H+ gradient across a membrane. Here, we aimed to study the driving force of the proton-pumping rhodopsins, and the effect of membrane voltage and pH gradient on their pumping functions. We systematically characterized the H+ transport properties of nine different rhodopsins, six outward-directed H+ pumps, and three inward-directed pumps by patch clamp measurements after expressing them in mammalian cells. The driving force of each pump was estimated from the slope of the current-voltage relations (I-V plot). Notably, among the tested rhodopsins, we found a large variation in driving forces, ranging from 83 to 399 mV. The driving force and decay rate of each pump current exhibited a good correlation. We determined driving forces under various pHs. pH dependency was less than predicted by the Nersnt potential in most of the rhodopsins. Our study demonstrates that the H+-pumping rhodopsins from different organisms exhibit various pumping properties in terms of driving force, kinetics, and pH dependency, which could be evolutionarily derived from adaptations to their environments.

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Poster 42/Talk G4 >> Thomas Mager (Institute for Auditory Neuroscience, University Medical Center Goettingen, Germany)

ChReef – An improved ChR for Future Optogenetic Therapies

Optogenetic approaches carry huge potential for future therapies, because they enable remote control of excitable cell activity with unique spatiotemporal resolution. Due to the low single-channel conductance of ChRs (1), which can be employed for excitable cell photostimulation, the optogenetic control of cellular activity relies on a combination of strong ChR expression and high light intensity stimulation. This is particularly relevant for clinical applications as it bears a risk for proteostatic stress and phototoxic effects. In this regard, ChRmine, a recently identified so-called bacteriorhodopsin-like-cation

channelrhodopsin (BCCR; (2)) is of great interest. ChRmine is optimally activated with green light and shows large photocurrents. Using a high performance automated patch-clamp system (Syncropatch 384, Nanion), which we operated in synchrony with LED-based illumination, we recently showed by noise analyses that the single channel conductance of ChRmine is considerably bigger than the single channel conductance of the state-of-the-art ChR CatCh. However, ChRmine utility is impaired by a strong, light dependent desensitization, which can be mainly attributed to a light-dependent inactivation process, which resembles a substrate inhibition of the partial type. The noise analyses moreover revealed that photocurrent inhibition by light is likely associated with the presence of a parallel photocycle open state of lower conductance and/or low open probability. We recently engineered the ChRmine mutant T218L/S220A, which we named ChReef ("ChR that excites efficiently"). In ChReef the light-dependent inactivation process that resembles a substrate inhibition of the partial type, was abrogated by the mutations, which led to a pronounced reduction of photocurrent desensitization. Moreover, comparative experiments revealed that the stationary photocurrent of the ChRmine mutant ChReef was considerably larger than the photocurrents of other widely-used ChR variants. We therefore anticipated an increase in the efficiency of sustained excitable cell photostimulation and accordingly assessed the suitability of ChReef for future optogenetic therapies, thereby focusing on cardiac defibrillation, vision restoration and hearing restoration.

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Poster 43 >> Toshiki Nakamura (Nagoya Institute of Technology, Japan)

Time-resolved infrared dual-comb spectroscopy using quantum cascade lasers reveals differences in conformational changes of two heliorhodopsins found from a bacterium and an archaeon

Heliorhodopsin is a novel rhodopsin that binds an all-trans retinal as a chromophore and is composed of seven transmembrane helices, which are same features as previously discovered microbial rhodopsins have. However, it has low sequence homology and inverted orientation of the transmembrane helices. The functions and molecular mechanisms of microbial rhodopsins, such as ion transport and optical signaling, have been discussed in detail, while the functions and molecular mechanisms of heliorhodopsins are less understood.

48C12 and TaHeR are two representative heliorhodopsins, which have been considered as models of heliorhodopsin. The amino acid sequence homology between the two heliorhodopsins is high and their tertiary structures are also very similar. These heliorhodopsins were thought to transmit signals to other proteins by conformational changes associated with the formation of the O intermediate. Interestingly, the two heliorhodopsins were reported to have very different time constants for the formation and decay of the O intermediate, although the difference has not been examined well.

In this study, we identified the factors that cause differences in the time constants of the O intermediates of the heliorhodopsins based on the differences in the conformational changes elucidated by using infrared dual-comb spectroscopy with quantum cascade lasers, which is the latest type of time-resolved infrared spectroscopy method. From differences in the infrared spectra we obtained, we clarified that the Ser(112) – Asn(138) hydrogen bond connecting TM3 and TM4, which is unique to 48C12, is the determinant of the time constants for the formation and decay of the O intermediate. Furthermore, by swapping the two amino acids in both heliorhodopsins, inter-conversion of the time constants between 48C12 and TaHeR was also achieved.

To examine the conservation of the SNap bond in heliorhodopsins, we compared the sequences of 3612 heliorhodopsins in public databases and literature, and found that the SNap bond is conserved in 72% of the heliorhodopsins. Moreover, by extending the sequence comparison to microbial rhodopsins, we found that the SNap bond is located at the same site with the DC gate of channelrhodopsins and enzymerhodopsins. The similarity between the SNap bond and the DC gate in function of controlling dynamics of the key intermediates implies that a new commonality exist between microbial rhodopsins and heliorhodopsins that are distant from each other in the molecular phylogenetic tree.

Reference: T. Nakamura et al. J. Mol. Biol. 436 (16), 168666, 2024 (DOI: 10.1016/j.jmb.2024.168666)

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Poster 44 >> Makoto Iwasaki (Osaka Metropolitan University, Japan)

Characterization of Gq activation by coral opsins.

Animal opsin-based pigments are light-sensitive G-protein-coupled receptors (GPCRs), which drive G protein-mediated signal transduction cascades. Thousands of opsins have been identified from a wide variety of animals thus far, and they are phylogenetically classified into eight groups, which is well matched to a grouping based on molecular properties of the opsins; opsins belonging to different groups generally couple to different types of G proteins. Recently, anthozoan cnidarians, such as corals and sea anemones, contain multiple opsins that are phylogenetically classified into two different groups, namely ASO (Anthozoan specific opsin) I and ASO II groups. We recently found that a member of ASO II group from a reef-building coral, *Acropora millepora*, named acropsin 4, light-dependently increases Ca2+ levels in mammalian cultured cells [1], suggesting that opsins in ASO II group drive Gq-signaling.

In this study, we heterologously expressed several coral opsins in the ASO II group other than acropsin 4 in mammalian cultured cells to investigate whether the ASO II group opsins basically activate Gq. Additionally, we comparatively analyzed specific activation of Gq against Gi/Go among the ASO II group opsins and jumping spider Rh1 belonging to the large Gq-coupled opsin group, the invertebrate visual opsin/melanopsin group [2], because the spider Rh1 activates Gi as well as Gq [3, 4]. The results suggested that ASO II group is a novel Gq-coupled opsin group containing opsins each of which has different selectivity for Gq and Gi/Go activation, distinguished from the known Gq-coupled opsin group. We also discuss optogenetic potentials of the members of ASO II group.

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Poster 45/Talk H4 >> Valérie Panneels (Paul Scherrer Institute, Switzerland)

ULTRAFAST DYNAMICS OF OUR LIGHT-RECEPTOR FOR VISION RHODOPSIN, USING AN X-RAY FREE ELECTRON LASER

Mammalian rhodopsin is our light receptor for vision. It belongs to the druggable G protein-coupled receptor family. Located in the retina, it is the site of one of the fastest events in our body. The protein core of rhodopsin hosts the retinal chromophore which, like a switch, isomerises in less than 200 femtoseconds upon photoabsorption. This triggers, over decades of time, intramolecular conformational changes in the rhodopsin, towards the intracellular side, initiating the signalling cascade, generating in milliseconds the vision event to the brain via the optic nerve. The intramolecular initial events transforming the rhodopsin resting state[1-2] (dark state) into the transducin-binding activated state[3-5] (Meta II state) are not completely understood.

We experimentally determined with spatial and temporal resolution the ultrafast changes of native rhodopsin at room temperature using time-resolved serial femtosecond crystallography (TR-SFX)[6] at the japanese and swiss X-ray free electron lasers (XFELs), SACLA and SwissFEL. Thousands of rhodopsin microcrystals grown in the dark were successively injected in the light of a pump laser and probed after various time-delays from femtoseconds to milliseconds using an XFEL. After 1 picosecond, we observe a distorted all-trans retinal that has induced a few changes in its binding pocket while the excess energy of the absorbed 480 nm-photon dissipates inside rhodopsin through an anisotropic protein breathing motion towards the extracellular domain. Interestingly, some amino acids

known to be key elements of the transduction of the signal are involved in the protein breathing motion. Complementary analyses like QM/MM and molecular dynamics help to complement the characterization of our new models at 1, 10 and 100 picoseconds of photoactivation.

The same type of experiment was applied on later time-delays from picoseconds to early microseconds showing a relaxation of the whole structure followed by the first major retinal conformational change modifying its binding pocket. Complementary analyses with time-resolved spectroscopy are supporting our findings.

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Poster 46 >> Hana Maruyama (Nagoya Institute of Technology, Japan)

The cytoplasmic domain is involved in the channel gating of anion channelrhodopsin, GtACR1

GtACRs are light-gated anion channels from the cryptophyte Guillardia theta1. GtACR1 possesses 438 amino acids and consists of a membrane-embedded 7-TM rhodopsin domain (295 amino acids) and a cytoplasmic domain (143 amino acids). Recent study revealed that the cytoplasmic domain modulates the anion channel activity of GtACR1, in which the channel-open state is prolonged depending on the anion species i.e. NO3- and Cl- 2. However, the molecular mechanism of the cytoplasmic domain remains unknown.

We here measured the photocurrent of the GtACR1 variants by using a whole-cell patch-clamp to characterize the channel gating mechanism. The channel opening rate of GtACR1 with the cytoplasmic domain is about 2.5 times slower than that of GtACR1 without the cytoplasmic domain. In contrast, the channel closing rate of GtACR1 with cytoplasmic domain is about 2 times faster than that of GtACR1 without cytoplasmic domain. Thus, the cytoplasmic domain regulates the channel gating of GtACR1. We then focused on two regions in the cytoplasmic domain, K256-D260 and R309-D318 where several charged residues are involved. Site-directed mutagenesis study showed that the specific charged residues in the cytoplasmic domain affect the channel gate-on and -off. Based on these results, we propose a functional model of the channel regulation by the cytoplasmic domain in GtACR1.

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Poster 47 >> OFER FILIBA (The Hebrew University of Jerusalem, Israel)

Photoisomerization of deprotonated Retinal Schiff Base

Rhodopsins are transmembrane proteins which function as photoreceptors and have a wide variety of functionalities ranging from sensors to ion pumps. Universally, they share a common chromophore, the retinal as well as a similar secondary structure of seven transmembrane helices.

Recently, a new rhodopsin was discovered, which comprise of eight transmembrane helices: Histidine kinase rhodopsin (HKR). The discovery of HKR established a novel sub class of the microbial rhodopsins that is coined enzymerhodopsins. Spectroscopic studies of HKR revealed two switchable and thermally stable isoforms: ultraviolet light-absorbing (Rh-UV) and blue light-absorbing (Rh-Bl).

It was determined spectroscopically that the Rh-UV form of HKR carries a 13-cis-retinal Schiff base which is deprotonated and undergoes a photoisomerization. Due to the deprotonation of the Schiff base, a lone pair is localized around its nitrogen atom which give rise to n-π^* excited state. This excited state could facilitate an intersystem crossing (ISC) between singlet and triplet states. A small energy gap between singlet and triplet state is essential for successful ISC. In this contribution I will present the results of a hybrid quantum mechanics/molecular mechanics (QM/MM) simulation in the excited state. The energy gap between the low-lying singlet and triplet state is calculated along the isomerization pathway.

Co-authors

Igor Schapiro (The Hebrew University of Jerusalem)

Poster 48/Talk I2 >> Flurin Hidber (Paul Scherrer Institute, Switzerland)

LAMBDA: Light Absorption Modeling via Binding Domain Analysis

Opsins are light-sensitive proteins essential for light-perception. Predicting their peak wavelength of absorbance (λmax) from sequence data is challenging due to the complex interactions within the retinal binding site. We introduce LAMBDA (Light Absorption Modeling via Binding Domain Analysis), a computational framework that uses Message Passing Neural Networks (MPNNs) to accurately predict λmax values for animal and microbial opsin for dark and activated states. LAMBDA integrates residue embeddings from large language models with generic residue numbering (GRN) systems to build detailed graphs of the retinal binding site. By mapping GRNs, our model infers structural positions from sequence data, enabling comparisons across species and protein folds. We have used LAMBDA to release a dataset comprising over 40,000 opsin sequences with predicted λmax values. This resource provides new tools to study the evolution and functional diversity of opsins.

Authors

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Poster 49 >> Francisco Miguel Picarra Leisico (Paul Scherrer Institute, Switzerland)

Production of the bistable GPCR Melanopsin for structural studies

Melanopsin is a photoreceptor located at the membrane of the intrinsically photosensitive retinal ganglion cells. Its phototransduction regulates important biological processes such as photoentrainment and visual perception. The light-sensitivity of Melanopsin is ensured through the binding of a retinal molecule. The photocycle of Melanopsin is bistable, with retinal remaining bound through its isomerization reaction to the active and inactive states of Melanopsin. Activation of Melanopsin by light can relay signaling through different G protein families. The particularities of bistablility and G protein selectivity renders Melanopsin a great potential as an optogenetic tool to control with spatiotemporal precision the activity of G protein-coupled receptors, one of the most important targets in drug development. However, little is known about the molecular mechanisms driving Melanopsin phototransduction and G protein bias. The main limitation has been the recombinant production of Melanopsin, which prevents functional and structural studies. In this work, I will show the outcome of my efforts to express and purify mammalian Melanopsin combining different expression systems, fusion strategies, partners co-expression and purification methodologies. These efforts are essential to obtain a pure and homogenous sample of Melanopsin and to understand its function at the molecular and atomic level.

Co-authors

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Poster 50 >> Probal Nag (Hebrew University of Jerusalem, Israel)

Understanding light-gating mechanism of potassium-selective channelrhodopsin

Channelrhodopsins (ChRs) are light-activated ion channels that allow ions to pass through the cell membrane upon photoillumination. ChRs contain seven transmembrane (TM) helices with a retinal moiety bound to the TM7 via a Schiff base linkage with a lysine residue. Upon photoactivation, the retinal undergoes isomerization, leading to conformational changes that create a pore across the protein, thereby enabling conductance of ions. The ion flow may not be selective and can conduct multiple ion types simultaneously. Control over the conductance process makes ChRs an ideal protein for applications in the field of optogenetics.

Potassium channels constitute an important class that selectively allow K+ ions to flow through the cell membrane. A change in the ion gradient leads to membrane hyperpolarization of neurons. Hence, it is interesting to control the potassium flow through

the membrane, thereby controlling neuronal activity. Reports on potassium channels show that the gated activity of the channel is largely ligand- and voltage-dependent. Recently, Govorunova et al., reported a natural light-gated potassium channel, known as Kalium channelrhodopsins (KCRs), from the family of *Hypochytrium catenoides* (*Hc*KCR). The interesting aspect is that it is specific to K+ ions and the conduction mechanism follows a similar pathway as that of the channelrhodopsins instead of being voltage-gated.

The channel opens on the sub-millisecond time scale and closes within a few milliseconds. This rapid closing of the channel made it hard to gain structural insights of the open structure using the Cryo-EM method. Recently, it was found that replacing the highly conserved cysteine residue in TM3 with alanine resulted in >1000-fold decrease in the rate of channel closing, thereby enabling freeze-trapping of the illuminated structure.

Using atomistic molecular dynamics simulations, we demonstrate ion conductance occurring in the mutant structure of HcKCR with the help of the computational electrophysiology (CompEL) protocol. Our simulations show that the dark state does not conduct whereas the illuminated structure shows ion conduction towards both intracellular as well as extracellular directions, depending on the transmembrane voltage. We are also able to identify the pore opening channel and the binding sites of the cation that aids in migration through the protein.

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Poster 51/Talk J1 >> Andrey Rozenberg (Technion - Israel Institute of Technology, Israel)

Carotenoid antennas in proton-pumping rhodopsins from bacteria and archaea

The phenomenon of light-harvesting antennas in microbial rhodopsins, although known for two decades now, has remained an idiosyncrasy of two related proteins binding ketolated carotenoids from the xanthorhodopsin family: Salinibacter ruber xanthorhodopsin and Gloeobacter rhodopsin. By exposing diverse rhodopsin proton pumps to environmental samples of carotenoids, we discovered that a range of rhodopsins from freshwater and marine environments bind common hydroxylated carotenoids and that such antenna-rhodopsin complexes are much more widespread in aquatic environments. Fluorescence measurements show that the energy is transferred between the antenna carotenoids bound non-covalently to the surface of the protein and the retinal moiety, itself a carotenoid derivative, inside the protein. A total of three different rhodopsin families: xanthorhodopsins, proteorhodopsins and a distinct proteorhodopsin-like family from

Asgard archaea (HeimdallRs) are found to be able to recruit hydroxylated carotenoid antennas. Different proteins demonstrate varying specificities with respect to the type of carotenoids with HeimdallRs appearing to bind the broadest range of antennas from lutein to the epoxycarotenoid fucoxanthin. We demonstrate that antennas increase pumping activity in these rhodopsins and given a ubiquitous distribution of rhodosin pumps with the potential to bind such antennas, we hypothesize that this phenomenon has a global impact on the amount of sunlight utilized by photoheterotrophs in aquatic microbial communities.

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Poster 52 >> Giorgia Fiorini (Paul Scherrer Institute, Switzerland)

Mechanistic studies on Rhodopsin using Electron Diffraction.

Vision is initiated by light-sensitive membrane proteins belonging to the rhodopsin G protein-coupled receptors (GPCRs) family. To date, the molecular mechanism behind the first step of vision is not fully understood and recently, time-resolved X-ray crystallography revealed some ultrafast events. However, due to limitations of X-ray diffraction, some atomic-level molecular details are still missing (e.g., hydrogen localisation).

Recent studies have shown that microcrystal electron diffraction (MicroED) can be used for the determination of protein structures. As opposed to X-ray crystallography, MicroED has the potential of enabling hydrogen localisation, and there is growing interest in further developing this technique as a complementary method to X-ray crystallography. To obtain high-quality MicroED data and minimise scattering events, thin homogenous nanocrystals are required6.

Rhodopsin crystals, as many other membrane proteins, are usually grown in a lipidic phase that mimics the cellular membrane. The lipidic phase, or any other carbonated additives, may increase the diffraction background thus decreasing the quality of the data collected with MicroED. Studies have been reported where FIB-SEM milling has been used to obtain thin nanocrystals in order to optimise the signal- (near to single scattering electron event) to-noise (strict reduction of hydro-carbonated molecules to the crystal entity) ratio.

The aim of our project is to validate the possibility to use FIB-SEM milling and MicroED for rhodopsin structure determination and mechanistic investigation and, to contribute to the development of MicroED methods that could be used for other proteins of interest.

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Poster 53 >> Andreea Pantiru (University of Manchester, UK)

The optogenetic potential of photocyclic rhodopsin

Vertebrate rhodopsins are photosensitive proteins which have been widely used in the development of optogenetic tools. One of the main limitations of opsins is the requirement of 11-cis retinal replenishment. Sakai and co-workers identified a single amino acid mutation from glycine to cysteine at position 188 (G188C) in bovine rhodopsin overcomes this limitation. G188C mutation leads to photoconversion to the active state, which then thermally relaxes back to the dark state, revealing photocyclic behaviour in bovine rhodopsin and bypassing the need for retinal replenishment (Sakai et al., 2022). To explore its potential as an optogenetic tool, we introduced the G188C mutation into human

rhodopsin. Multiple G188C variants of human rhodopsin exhibited robust photocyclic activity, maintaining stable activation across repeated light stimulations in HEK-293 and Neuro2A cell lines. Furthermore, human rhodopsin G188C based chimeras were developed, which retained the photocyclic behaviour of G188C mutation and the G-protein activation profile of the endogenous receptor. These findings highlight the G188C mutant of human rhodopsin as a promising optogenetic tool for answering physiologically relevant questions due to its advantages for in vivo application.

Poster 54/Talk J3 >> María del Carmen Marín Pérez (Technion-Israel Institute of Technology, Israel)

Light-harvesting by antenna-containing xanthorhodopsin from an Antarctic cyanobacterium

Microbial rhodopsins are a versatile family of light-sensitive proteins crucial to various phototrophic and sensory processes in microorganisms. Xanthorhodopsins, notable for their dual chromophore system involving retinal and carotenoids, have been predominantly studied in halophilic bacteria where they facilitate light-driven outward proton pumping and enhanced light-harvesting. However, there is a significant gap in understanding their presence and function in cyanobacteria. Here, we report the discovery and characterization of a novel xanthorhodopsin in Antarctic cyanobacteria that uniquely binds a hydroxylated carotenoid. Utilizing bioinformatic analysis, spectroscopic techniques, and functional assays, we elucidate the properties and ecological role of this xanthorhodopsin. Our findings reveal that this xanthophyll-binding xanthorhodopsin represents an evolutionary adaptation, enhancing the ability of cyanobacteria to thrive in diverse and fluctuating light environments, potentially contributing to their ecological success. Differently to the previously characterized xanthorhodopsin from the cyanobacterium *Gloeobacter violaceus*, which binds a ketolated carotenoid (echinenone), the newly identified xanthorhodopsin binds lutein, a hydroxylated carotenoid. Our discovery highlights the potential ecological significance of xanthorhodopsins in supporting the success of these organisms in their natural habitats.

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Poster 55 >> Michal Koblizek (Institute of Microbiology Czech Acad Sci, Czech)

Cold-loving bacterium from a mountain lake harvests light using both proton-pumping xanthorhodopsin as well as bacteriochlorophyll-containing photosystems

Bacterium *Sphingomonas glacialis* AAP5 isolated from the alpine lake Gossenköllesee contains genes for anoxygenic phototrophy as well as proton-pumping xanthorhodopsin. We found that BChl is expressed between 4°C and 22°C in the dark, whereas xanthorhodopsin is expressed only at temperatures below 16°C and in the presence of light. Thus, cells grown at low temperatures under a natural light–dark cycle contain both BChl-based photosystems as well as xanthorhodopsins. The purified xanthorhodopsin contains auxiliary carotenoid nostoxanthin, which absorbs the blue part of the solar spectrum. Time-resolved fluorescence excitation analysis documented that nostoxanthin transfers excitation to retinal with approx. 50% efficiency. Its photosystems consist of circular light harvesting complex 1 composed from 16 homodimeric subunits surrounding the type-2 bacterial reaction center.

Flash photolysis measurements proved that both systems are photochemically active.The captured light energy is used for ATP synthesis and stimulates growth. Thus, *S. glacialis* AAP5 represents a chlorophototrophic and a retinalophototrophic organism. Our analyses suggest that simple xanthorhodopsin may be preferred by the cells under higher light and low temperatures, whereas larger BChl-based photosystems may perform better at lower light intensities. This indicates that the use of two systems for light harvesting may represent an evolutionary adaptation to the specific environmental conditions found in alpine lakes characterized by low nutrients and large changes of irradiance and temperature.

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Poster 56 >> Deborah Walter (Paul Scherrer Institute, Switzerland)

Engineering an OptoGPCR based on a bistable rhodopsin for optogenetic applications

Jumping spider rhodopsin (JSR1) is a light-sensitive G protein-coupled receptor (GPCR). Due to its bistable properties, it is an interesting target for optogenetic applications, which allows rapidly switching the GPCR signal on and off. Here, we use JSR1 as a model to derive strategies for engineering bistable rhodopsin with different primary G protein selectivity. JSR1 couples to the jumping spider Gq protein and can couple to human Gi and Gq proteins. Since Gs signaling is most distinct from Gq signaling, we exchanged parts of the intracellular and G protein-interacting sequences with the sequence of known Gs-coupled receptors. These engineered chimeras are validated with expression tests and time-resolved cellular signaling assays. The first results show strong cAMP activation that can be switched on and off with different wavelengths. In the next step, chimeric proteins will be purified to obtain biophysical characteristics and assess the binding to purified G proteins. With structural and biophysical analysis of those bistable chimeric receptors, we hope to improve the overall understanding of GPCR signaling selectivity and the design of engineered light-controlled bistable GPCRs that mimic specific GPCR signaling profiles.

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Poster 57/Talk J4 >> Shin-Gyu Cho (Sogang University, Seoul Korea)

Heliorhodopsin-mediated light-modulation of ABC transporter

Rhodopsins are light-driven seven-transmembrane proteins with a retinal chromophore that binds to a specific Lys residue, and are found in various organisms. Six years ago, heliorhodopsins (HeRs) had been discovered and formed a distinct clade, separate from the rhodopsins. Especially, HeRs have been hypothesized to have widespread functions. Recently, the functions for few HeRs have been revealed; however, the hypothetical

functions remain largely unknown. Here, we investigate light-modulation of heterodimeric multidrug resistance ATP-binding cassette transporters (OmrDE) mediated by *Omithinimicrobium cerasi* HeR (OcHeR). In this study, we classified genes flanking the HeR-encoding genes in the same operon into 10 groups and identified highly conservative residues for protein–protein interactions. Next, we investigated relationship between OcHeR and OmrDE in one of the 10 groups. Our results revealed a binding between OcHeR and OmrDE, as determined by isothermal titration calorimetry analysis to derive thermodynamic parameters. Specifically, positively charged residues within an intracellular loop of OcHeR played a crucial role in the binding. Based on the thermodynamic parameters, we propose a positive cooperatively sequential binding mechanism. Moreover, a conformational change induced by light in OcHeR enhanced OmrDE drug transportation, as measured by changes in enzyme kinetic parameter, drug transport capacity in membrane vesicle, cell viability assay, and half-life of conformational changes. These results suggest that the conformational change derives dynamic energy and further leads protein structural change in OmrDE. Hence, the binding may be crucial to drug resistance in *O. cerasi* as it survives in a drug-containing habitat. Overall, we revealed a function of HeR as regulatory rhodopsin for multidrug resistance. Our findings suggest potential applications in anticancer therapy and optogenetic technology.

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Poster 58 >> Jonas Mühle (Paul Scherrer Institute, Switzerland)

A high-throughput screening platform for the biophysical characterization of visual pigments

Understanding the determinants of bistability and color tuning in visual pigments is essential for designing optogenetic tools. This understanding often requires extensive mutagenesis studies to investigate the functions of individual residues within the chromophore binding pocket. We present a robust, baculovirus-based high-throughput screening platform designed to evaluate expression levels and protein stability. This platform employs analytical fluorescence-detection size exclusion chromatography (FSEC) using 500 µl of cell suspension. Additionally, we perform a small-scale purification from 25 ml of cell suspension to determine the biophysical properties of visual pigments, including the UV/Vis spectra of both the ground and excited states.

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Poster 59 >> Yoshitaka Kato (The University of Tokyo, Japan)

Xenorhodopsin with a new sequence motif lacking the characteristic proline

Xenorhodopsin (XeR) is a light-driven inward proton pump characterized by a proline at the position of Asp212 in TM7 in bacteriorhodopsin (BR) [1, 2]. While the aspartic acid at this position is highly conserved in almost all microbial rhodopsins, which function as ion pumps, ion channels, phototaxis sensors, and enzymes, only XeR and heliorhodopsin (HeR) have proline and serine at this position, respectively [3]. In the case of light-driven outward proton pumps such as BR, two carboxylic residues (Asp85 and Asp212) are located near the protonated retinal Schiff base (PRSB), which increases the tendency of proton movement toward the extracellular side. In contrast, in XeR, where Asp212 is replaced by proline, the proton is translocated to the cytoplasmic side. Therefore, it is believed that the proline plays an essential role in the inward proton transport of XeR.

Here, we report novel rhodopsin discovered from a salt pond near Eilat in Israel (EltXeR). Although EltXeR is classified in the XeR group based on the phylogenetic sequence analysis, the proline characteristic for XeR is not conserved and is replaced with alanine. Despite the Pro to Ala replacement, EltXeR exhibits inward proton transport activity and a slow photoreaction with transient accumulation of M intermediate with deprotonated retinal Schiff base, similar to the previously reported XeR [2]. These results indicate that the inward proton transport function is tolerant to the replacement of the proline. Furthermore, the finding that a microbial rhodopsin with alanine at the position of Asp212 in BR exhibits the molecular function provides new insights into the diversity of microbial rhodopsin. Additionally, the absorption maxima of EltXeR differs from previously reported XeR. The absorption maximum of EltXeR is 525 nm, which is 25–52 nm shorter than the reported values for other XeR (550–577 nm). The absorption maxima of inward proton pumps were thought to be less diverse than those of outward proton pumps [4]. Our finding could provide new insights for the spectral-tuning mechanism of inward proton pumping rhodopsins.

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Poster 60/Talk K2 >> Matthias Mulder (Paul Scherrer Institute, Switzerland)

Structural insights into the opening mechanism of Channelrhodopsin C1C2

Channelrhodopsins are light-gated cation channels that enable mobile algae cells to locate suitable conditions for photosynthesis. Beyond their natural role, scientists have shown interest in channelrhodopsins as tools in optogenetics. The first crystal structure of a channelrhodopsin was that of C1C2, a chimera of channelrhodopsin 1 and channelrhodopsin 2 from Chlamydomonas reinhardtii. The channel opening of C1C2 is controlled by two gates, the intracellular and central gates, which connect to an extracellular vestibule to form a continuous channel upon activation. Although C1C2 remains one of the most extensively studied channelrhodopsins, the molecular details of its channel opening are still largely unknown.

In this study, we have used serial crystallography to resolve the structural changes upon light-induced retinal isomerization in C1C2. We observe large conformational movements in the retinal binding pocket and the adjacent central gate. Rearrangements in the counterion network suggest that the structure represents an early deprotonated intermediate, where the channel has partially opened, but significant bottlenecks remain at the extracellular gate. Our study provides direct structural insights into the initial rearrangements upon photoactivation, bringing us closer to an atomistic understanding of cation conduction in channelrhodopsins.

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Poster 61 >> Shino Inukai (Nagoya Institute of Technology, Japan)

Early photoreaction mechanism in jellyfish rhodopsin with the counterion at an atypical position

Animal rhodopsins commonly have 11-cis-retinal as a chromophore, which forms a protonated Schiff base stabilized by a negatively charged amino acid counterion. In vertebrate rhodopsins, the counterion is located at transmembrane helix-III (TM3), while in invertebrate rhodopsins, it is located at the extracellular 2nd loop. Vertebrate rhodopsins activate Gt(i/o) proteins, whereas invertebrate rhodopsins interact with Gq proteins. Box jellyfish rhodopsin (JelRh), an invertebrate rhodopsin, has gained attention due to its unique counterion position at TM2, which evolved independently from other animal rhodopsins. Additionally, it is the only animal rhodopsin known to activate the Gs protein, making it a novel optogenetics tool. Recent studies using low-temperature light-induced FTIR spectroscopy have shown that the structural features of the initial photo-intermediate formed upon retinal isomerization in JelRh are similar to those in vertebrate rhodopsin.

To elucidate the relationship between different counterion positions and G protein activities, it is necessary to investigate the effects on the intermediates formed during the photoreaction process. In this study, we successfully detected the structural changes during the transition to the Lumi intermediate following the initial photo-intermediate. For vertebrate rhodopsin, this transition involves the relaxation of the distorted retinal structure and changes in the peptide backbone, including TM3. In contrast, for JelRh, the structural change of the Lumi intermediate was comparatively smaller, suggesting that the structural changes in JelRh differ from those in vertebrate rhodopsin, unlike the initial photoreaction. Additionally, FTIR spectra comparison between vertebrate and invertebrate rhodopsins revealed JelRh-specific C=O stretching vibration bands originating from the carbonyl group.

To assign the C=O stretching vibration bands of the carbonyl group, we performed systematic mutant measurements. Interestingly, when we mutated the two glutamines at positions 86 and 122 (which correspond to methionine and glutamic acid, respectively, in vertebrate rhodopsin and are important for its activation) to alanine, we observed differences in the peptide backbone changes during Lumi intermediate formation. Notably, in the Q122A mutant, there was an unexpectedly larger α-helical structural change than in the wild type. This suggests that Q122 acts to suppress structural changes during Lumi intermediate formation. In our presentation, we will discuss the differences in structural changes specific to the early photoreaction process of JelRh, as well as the molecular mechanism by which only JelRh can activate the Gs protein, in conjunction with results from other asparagine and glutamine mutants.

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Poster 62 >> Masae Konno (The University of Tokyo, Japan)

High-throughput engineering of microbial rhodopsin mutants using an automated liquid-handling system

Microbial rhodopsin is a membrane protein that has seven transmembrane helices and an all-*trans*-retinal as chromophore. The molecular functions of microbial rhodopsins are very diverse including ion transport, light sensing, and enzyme. Mutant engineering for controlling properties such as spectral tuning and ion transport activity plays a crucial role in understanding molecular mechanisms of microbial rhodopsin and advancing optogenetic tool development. Recently, AI-driven protein design methods have been used to predict amino acids critical for spectral tuning in microbial rhodopsins [1, 2]. To achieve more efficient protein design, it is important to repeat cycles of examining many mutants proposed by the AI and refining the prediction model by feeding back the results. However, manually producing all required mutants is impractical due to the large number of involved amino acids.

To address this challenge, our study aims to streamline mutant production by developing an automated system using a liquid handling platform. As a model experiment, we engineered spectral-tuning *Gloeobacter* rhodopsin (GR) mutants. Site-directed mutations were introduced into GR using PCR-based mutagenesis, followed by transformation into *E. coli* cells. Transformants were screened using the limiting dilution method, and plasmids were extracted. DNA sequencing confirmed that nearly all clones successfully introduced the desired mutations.

The high-throughput potential of this automated system will be demonstrated, highlighting its efficiency and scalability for rhodopsin mutant production.

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Poster 63 >> Yuma Ito (Nagoya institute of technology, Japan)

The initial intermediate of an inward proton pumping rhodopsin NsXeR exhibits relaxed 13-cis retinal configuration

Xenorhodopsin (XeR) is the first light-driven inward proton pump family found in nature. The function is clearly opposite to that of bacteriorhodopsin (bR), which is a typical microbial rhodopsin and performs a light-driven outward proton pump. The most characteristic difference in the molecular structure between XeRs and bR is a proline residue at the second counterion position (Asp212 in BR). *Ns*XeR is a member of the XeR family found from a *Nanosalina*. Although *Ns*XeR has similar residues to other XeRs around the retinal chromophore, it exhibits much higher inward proton pump transport activity than other XeRs. However, the molecular mechanism underlying the efficient pumping activity of *Ns*XeR remains unclear. In this study, we applied light-induced difference FTIR spectroscopy at 77K on *Ns*XeR and compared the results with those of bR, other XeRs and *Ns*XeR mutants. The FTIR at 77K can reveal tiny structural differences between the initial intermediate and the dark state, which allows us to discuss structural changes in the initial processes upon the retinal isomerization.

The FTIR experiments revealed two interesting features after photoisomerization in NsXeR. The first one was hydrogen-out-of-plane (HOOP) band which represents the twisting of retinal after photoisomerization. The previous experiments on bR and other XeRs showed strong HOOP bands in the initial intermediate state, but *Ns*XeR barely showed HOOP band. It means that the retinal conformation in NsXeR was not twisted after photoisomerization. The second one is strength of hydrogen bonds around the protonated retinal Schiff base. In the X-D stretching vibration region, N-D stretching vibration of the protonated Schiff base and O-D stretching vibrations of water molecules are observable and can be assigned by use of Lys-15N and D218O, respectively. Interestingly, *Ns*XeR has weaker hydrogen bonds around the protonated retinal Schiff base than those in BR and other XeRs. These are also unique features which have not been reported for other proton pump rhodopsins. To deepen understanding of the molecular mechanism, the effects of mutation of proline at the second counterion position (P209A and P209E) were examined. These *Ns*XeR mutants exhibited lower inward proton pump activity than WT. Concomitantly, it was revealed that these mutants showed distinctive HOOP bands in the initial intermediate and have strong hydrogen bond around the protonated retinal Schiff base in the dark state. These results suggest that the less distorted photoisomerization in *Ns*XeR is a key feature for the high inward proton transport activity.

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Poster 64/Talk K3 >> Han Sun (Leibniz-Forschungsinstitut für Molekulare Pharmakologie, Germany)

Channel opening and ion conduction mechanism in channelrhodopsin C1C2, ChR2, and iChloC

Channelrhodopsins are photoreceptors that act as ion channels upon illumination. Over the last two decades, channelrhodopsins have become an essential tool in optogenetics, enabling precise manipulation of neurons, neural circuits, and animal behavior through the use of light. Although structural biology studies have provided important structural insights into channelrhodopsins, a detailed study of their ion permeation mechanism has been challenging due to the lack of the experimentally resolved open-state structures.

In this study, we employed a multi-scale simulation approach comprising molecular dynamics (MD) based computational electrophysiology, quantum-mechanics/molecular mechanics (QM/MM), and constant pH simulations to obtain the fully open state of three channelrhodopsins. This approach was facilitated by leveraging the experimentally resolved protonation state of the open-state in the simulations. Starting from the open-state simulations, a significant number of spontaneous ion permeation events were observed in C1C2, ChR2 and iChloC, where the ion conduction mechanism differs from that of most prokaryotic and eukaryotic cation channels, which function as multi-ion, single-file pores. Analyses of the ion binding sites in these open-state channelrhodopsins provided key insights into their ion selectivity. Our study presents a robust computational approach to establish the fully open-state structures of channelrhodopsins, addressing a formidable challenge in structural biology.

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Poster 65 >> Zibo Li (University of Liège, Belgium)

Spectral tuning evolution and adaptation of crustacean long-wavelength r-opsins

Revealing the functional basis of sensory receptor evolution allows us to ask how genetic and molecular changes can explain invertebrate ecological adaptations. In particular, the ability to perceive complex light spectra from short to very long wavelengths depends on

the number of G-protein coupled opsin receptors expressed in an animal's eye and their molecular structure and specific interactions with a retinal chromophore, which altogether define spectral absorbance properties towards short- (350-450 nm), middle- (450-510 nm), or long wavelength light (LW, >510 nm).

The development of optimized expression systems to characterize Gq opsins, including from insects and the stomatopod crustacean Neogonodactylus oerstedii, expands our ability to tease apart genotype–phenotype relationships underlying functional variation and spectral tuning of visual invertebrate opsins [1,2].

Stomatopods possess a complex visual system forming up to sixteen photoreceptor types depending on lineages [3,4] and express a large number of duplicate opsin genes thought to detect short ultraviolet to very long wavelengths (LW) of light. This opsin diversity may be related to adaptations to diverse aquatic light environments, from shallow clear-water coral reefs to deep or murky waters, and may contribute to their complex visual behaviors, such as territoriality and predation.

Here we focus on stomatopod species from superfamilies Gonodactyloidea, Lysiosquilloidea and Squilloidea, with distinct behaviours, body colorations and ecologies including aquatic habitat depths ranging 0 to 200 m. We leverage recently assembled eye transcriptomes, heterologous expression, homology modeling, and mutagenesis to comparatively investigate the functional diversity, molecular tuning mechanisms and mode of evolution of selected LW opsin clades (L13-L14-L15). These results will contribute to link genetics to evolutionary adaptations and sensory ecology by providing insights into how light environments shape species interactions, evolutionary processes, and the use of visual systems by marine animals to adapt to specific ecological niches, and may also reveal new types of LW opsins with potential for transversal applications.

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Poster 66 >> Seiya Tajima (The University of Tokyo, Japan)

Structural Analysis of Ion Selectivity and Property Enhancement in Potassium Channelrhodopsins HcKCRs

The recent discovery of potassium-selective channelrhodopsins (KCRs) has been a long-awaited breakthrough in optogenetics, providing neuroscientists with new opportunities to enable efficient neural inhibition. Despite the potential, the underlying mechanism of ion selectivity in KCR has remained elusive due to the lack of a well-conserved motif typically responsible for forming a symmetric selectivity filter among potassium channels. In this study, we resolved the cryo-EM structures of the first-discovered potassium-selective channelrhodopsins, HcKCR1 and HcKCR2 to elucidate the structural foundation of ion selectivity. In combination with electrophysiological, spectroscopic, and computational analyses, we reveal the unique selectivity mechanism of KCR, which involves three key components; asymmetric selectivity filter on the extracellular side, an ion gradient under physiological conditions, and a dehydration system on the intracellular side. Additionally, we succeeded in enhancing the KCR properties by the introduction of specific mutations. To investigate the mechanism behind these mutational enhancements, we performed the structural and computational studies. We will discuss the updated understanding of ion selectivity and the structural mechanisms underlying the improvement of KCR properties.

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Poster 67 >> Xuchun Yang (University of Siena, Italy)

Theory of Photochemical Efficiency in Vertebrate and Invertebrate Visual Photoreceptors

The light-driven isomerization of bovine rhodopsin (Rh) is driven by a vibrationally coherent process resulting in a quantum yield (Φcis-trans) of almost 70%. However, the extent to which the precise relationship between coherent motion and photoproduct formation is unique to Rh or extends to other vertebrate and invertebrate visual rhodopsins remains unknown. We lack a comprehensive understanding of the mechanisms underlying the light sensitivity of visual rhodopsins. In this study, we selected two vertebrate rhodopsins and two invertebrate rhodopsins, employing hundreds of quantum-classical trajectories for each. Our findings reveal that in vertebrate visual rhodopsin, a quantum effect induces the splitting of the coherent population into subpopulations propagating with different velocities and Φcis-trans contributions. In contrast, this quantum effect is diminished in invertebrate rhodopsin due to the absence of the counter-ion E113. This study provides fundamental insights into the correlation between amino acid sequence and light sensitivity.

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Poster 68/Talk K4 >> Shunki Takaramoto (The Institute for Solid State Physics, The University of Tokyo, Japan)

ApuRhs, a new family of anion channelrhodopsin from apusomonads

Current understanding of light-sensing systems and rhodopsin diversity in heterotrophic flagellates (HFs) is limited, with notable examples in marine stramenopile species, non-photosynthetic algae, and choanoflagellates. One under-researched group is apusomonads, bacterivorous biflagellate protists found in freshwater and marine sediments worldwide. Apusomonads are closely related to opisthokonta, which includes animals, fungi, and their unicellular relatives, retaining ancestral traits valuable for studying early opisthokont evolution.

Recently, two extensive culturing efforts isolated a significant diversity of apusomonads, introducing four new genera and seven new species. These studies generated transcriptomic data to resolve phylogenetic relationships and examine key traits through comparative genomics. This study has aimed to uncover hidden microbial rhodopsin diversity in HFs by screening all available apusomonad omics data and environmental data. We discovered 14 microbial rhodopsins forming a new family, ApuRhs, unique to apusomonads. Phylogenetic analysis revealed that ApuRhs constitute a new, independent family within type-1 microbial rhodopsins. They possess conserved DAQ and (A/I/M/C)TQ motifs in the transmembrane helix 3.

Here, we tested their photoactivity by electrophysiological experiments, which demonstrated that ApuRhs have absorption peaks between blue and UV ranges (385–489 nm). Patch clamp experiments showed that ApuRhs function as ion channels transporting anions showing particularly high ion permeability for monovalent anions such as Cl^- and NO_3^-. An interesting point is that ApuRhs with the DAQ motif exhibit absorption maxima in the UV region, suggesting that the retinal Schiff base is deprotonated. Despite this, the excitation in the UV region induces ion channel activity, indicating that the photoreaction starts from the deprotonated retinal Schiff base and structural changes during this process lead to channel gating. Furthermore, transient absorption measurements on ApuRh with the DAQ motif revealed that the photocycle is initiated upon excitation in the UV region. These findings suggest that ApuRhs represent a new, deep-branching family of microbial rhodopsin channels, independently evolved from the well-known channelrhodopsins family. This research highlights that significant microbial rhodopsin diversity remains undiscovered within heterotrophic flagellates.

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Poster 69 >> Anika Spreen (Humboldt University of Berlin, Germany)

Rhodopsin-based optogenetic silencing

Rhodopsin guanylyl cyclases (RhGCs) are membrane-bound enzyme rhodopsins that convert GTP into the second messenger cGMP upon light activation. In optogenetic applications, RhGCs are valuable as light-sensitive modules in silencing systems, where their co-expression with cGMP-gated K+ - channels enables light induced cellular hyperpolarization. We developed the two-component optogenetic silencer RoCK, which combines CaRhGC (from Catenaria anguillulae), producing cGMP in response to green light, with SthK2.1 (from Spirochaeta thermophila), a highly selective K+ - channel modified to preferentially open upon cGMP binding. In ND7/23 cells, a 10 ms pulse of 525 nm triggered outward currents up to 6 nA with an operational light sensitivity of 1.8 mW/mm^2. RoCK has been tested in rabbit cardiomyocytes and mouse hippocampal neurons, successfully modulating cellular contractions and action potentials. Additionally, RoCK inhibited coiling activity in zebrafish embryos by targeting spinal motor neurons. To enable activation at longer wavelengths and lower light exposure, we modified RoCK's light-sensitive domain. First, we replaced CaRhGC with the recently identified GhRGC2 (from Gorgonomyces haynaldii), which has a red-shifted maximal activation wavelength of 571 nm. A single mutation near the Schiff base (T138A) further shifted this peak up to 595 nm. Co-expression of SthK2.1 with GhRGC2 or GhRGC2(T138A) in ND7/23 cells generated robust currents induced by a 10 ms pulse at 580 nm and 635 nm, respectively. However, both variants exhibited reduced light sensitivities and current amplitudes compared to CaRhGC. Since light sensitivity in RhGCs is influenced by the duration of the active state, we next targeted the aspartate-cysteine pair (DC gate), a functional motif near the retinal C13=C14 bond, which affects photocycle kinetics. Substitution of D163S in GhRGC2 increased current amplitudes up to 14 nA—more than fivefold compared to WT GhRGC2—while light sensitivity was significantly increased. The corresponding CaRhGC mutation (D283S) has previously been shown to extend the active state, which might also cause the enhanced efficacy of GhRGC2(D163S). Notably, the double mutation GhRGC2(T138A/D163S) enabled activation at 635 nm and even 660 nm, with high operational light sensitivities of 0.6 mW/mm^2 and 1.7 mW/mm^2, respectively. Additionally, mean current densities were over four times higher for GhRGC2(T138A/D163S) compared to CaRhGC. In conclusion, we highlight the advantages of optogenetic silencing systems using RhGCs for their extensive tunability. Given the successful application of CaRhGC-based RoCK in several systems, we recommend further testing of GhRGC2(T138A/D163S)

as a promising light-sensing module, particularly in scenarios requiring deep brain targeting with low light intensities.

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Poster 70 >> Seiwa Nakamura (The University of Tokyo, Japan)

Understanding light-sensitive mechanism of non-visual opsins

Opsins are photoreceptive proteins that are essential for both visual and non-visual responses to light in animals, translating light signals into various biological processes. Among the opsins, visual opsins, such as rhodopsin, are primarily expressed in photoreceptor cells and play a critical role in visual function. The structure of rhodopsin revealed in previous studies has visualized the molecular features of how rhodopsin converts light information into G-protein signals and elucidated the molecular functions of visual opsins through both spectroscopic analysis and cell-based assays, marking significant advances in opsin research. Compared to visual opsins, non-visual opsins are expressed in different tissues, including brain, liver and muscle, and their functions are diverse, including circadian rhythms, thermoregulation, melanogenesis and pulmonary vasorelaxation. These diverse biological phenomena of non-visual opsins are caused not only by differences in the location of expression, but also by the characteristics of each opsin molecule, such as absorption wavelength and G-protein selectivity. However, the molecular understanding of the recently identified non-visual opsins is currently limited due to their low expression levels and poor thermal stability. In this study, we attempted structural and functional analyses of human non-visual opsins to elucidate their light-sensitive mechanisms. We also discuss strategies to improve the structural determination of these proteins.

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Poster 71 >> Linda Tillert (Charité - Universitätsmedizin Berlin, Germany)

Gating mechanisms and ion selectivity of bestrhodopsins

A recent discovery from metagenomic studies revealed a completely novel kind of microbial rhodopsin: rhodopsin-bestrophin fusion proteins, called bestrhodopsins (Rozenberg et al. 2022). The cryo-EM structure of a bestrhodopsin revealed that they form pentameric megacomplexes with the bestrophin channel being surrounded by rhodopsin pseudodimers. Interestingly, they possess the rare ability among microbial rhodopsins to absorb far-red light, which is highly desired for deep tissue penetration in the field of optogenetics. So far only one member of the bestrhodopsin family, Karlodinium veneficum-rhodopsin-rhodopsin-bestrophin (KvRRB), shows light-induced anion currents, but the mechanism of activation is still unknown. We explore this new family of microbial rhodopsins in detail regarding their gating mechanism, conductivity and ion selectivity to gain insight into their natural function and pave their way for possible applications in optogenetics.

To understand the unknown gating mechanisms of bestrhodopsins, we investigated both the role of channel modulation by the rhodopsins and the contribution of pore-region residues. Using single-turnover electrophysiological measurements, we examined channel opening and closing kinetics and show that transient charge displacement currents in the rhodopsin precede the channel opening and closing, indicating a transmission of conformational changes from the rhodopsin to the bestrophin. Additionally, we identified key residues in the proposed pore region of the bestrophin channel that are essential for the induction of photocurrents. We further examined the anion selectivity of KvRRB and found that, like other eukaryotic bestrophin channels, it conducts HCO_3^-, suggesting a potential involvement in the CO_2-concentrating mechanism that enhances photosynthesis.

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Poster 72/Talk L4 >> Johannes Vierock (Charité Universitätsmedizin Berlin, Germany)

pHRoG: pH Regulating optoGenes for all-optical control of subcellular pH

Life is organized in cells and even smaller compartments that create functional environments for signal processing and enzymatic reactions. In most subcellular organelles, such as mitochondria and lysosomes, proton concentrations are tightly regulated, and

prolonged changes in lysosomal pH are associated with neurodegenerative diseases such as Alzheimer's or Parkinson's, cellular aging, and the adaptation of different types of cancer to their increased metabolic activity. Despite the importance of subcellular pH for cell homeostasis and its role in various diseases, molecular tools for organelle-specific, time-resolved, and quantitative manipulation of subcellular pH remain limited. Chemical drugs like bafilomycin A1 or hydroxychloroquine, used to manipulate endolysosomal pH, affect all organelles along the pathway simultaneously and are slowly taken up and cleared by the cell.

By comparing the targeting and performance of different ion-transporting opsins in subcellular organelles, we developed a suite of pH-Regulating optoGenes, called pHRoG, that, in combination with spectrally complementary sensors for voltage and pH, allow spatially and temporally precise manipulation of organelle-specific physiology. In lysosomes, we show how these tools can be used to manipulate subcellular enzyme activity and study the pH and buffering capacity of individual lysosomes under physiological conditions. We demonstrate bidirectional control of local pH levels in different cell lines and neurons, and quantify both the potential and limitations of subcellular optogenetic pH manipulation using state-of-the-art opsins.

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Poster 73 >> Mako Aoyama (Nagoya Institute of Technology, Japan)

Structural analysis of viral rhodopsin OLPVRⅡ using low-temperature FT-IR spectroscopy

Nucleocytoplasmic large DNA viruses that regulate phytoplankton dynamics containing rhodopsin genes categorized into two distinct groups: viral rhodopsin 1 (VR1) and 2 (VR2). OLPVRI and OLPVRⅡ belong to the VR1 and VR2 groups, respectively. Recently determined crystal structures reveal that both possess a pentagon-shaped hydrogen bonding network around the retinal Schiff base, similar to that of bacterorhodopsin (BR)[1,2]. OLPVRI has been found to function as a cation channel. This year, we conducted low-temperature FTIR spectroscopy on OLPVRI and identified differences in the hydrogen bonding network around the Schiff base compared to BR, despite their similar pentagonal cluster structures[3]. We interpret that these differences may be what distinguish channel function from pump function.

For OLPVRII, the formation of the M intermediate has been observed using transient absorption measurements, although its function remains unknown. In this study, we

performed low-temperature FTIR spectroscopy on OLPVRII to explore difference in the hydrogen bonding network around the Schiff base. The FTIR spectra of OLPVRⅡ revealed that the isomerization mechanism of the retinal chromophore and its structure is similar to those of other microbial rhodopsins. However, the hydrogen bonding environment of the C=O groups in protonated carboxylates, asparagine, and glutamine differs between OLPVR Ⅱ and other rhodopsins.

The presentation will discuss the details of the hydrogen bonding network around the Schiff base specific to OLPVRII by analyzing protein-bound water and conducting measurements on amino acid mutations.

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Poster 74 >> Camille Brouillon (Humboldt University of Berlin, Germany)

Functional characterisation of Neogonodactylus Oerstedii rhodopsins

Rhodopsins that absorb at the far end of the visible spectrum are important for the use in the field of optogenetics for controlling neuronal activity by expressing light-activated proteins in otherwise blind neurons. Infra-red absorbing rhodopsins are useful since the red wavelengths penetrate the tissue and scatter more in the neuronal tissue. Far-red absorbing rhodopsins are scarce in nature, and one of their bearers are stomatopod crustaceans (a famous member is the peacock mantis shrimp), which can detect wavelengths ranging from UV (310 nm wavelength) to the infrared (beyond 700 nm) by using different rhodopsins. This fact makes the mantis shrimp an ideal candidate for isolating specific proteins which detect light outside of the human visual spectrum.

In this project, for the first time we aim to achieve heterological expression of stomatopod rhodopsins and their biochemical characterization for use as optogenetic tools. To this end, 35 genes were selected for expression in human cells, including all the long-wave sensitive rhodopsin from the newly sequenced Neogonodactylus Oerstedii (N.oesterdii). Out of these genes, 20 are expressing well in human HEK-T cells amongst which 17 belong to N.oerstedii (including two middle wave sensitive) showing maximum absorption between 446 nm and 611 nm. Using second messenger bioluminescent assays, we were able to confirm that 9 of them use calcium as a second messenger for cellular signalling and that the middle wave opsins couple also very efficiently to the cAMP inhibitory Gi protein. Using heterologous action spectroscopy based on their calcium response, we found a

maximal absorption of 592nm for the most red shifted opsin, and were able to confirm that it can be toggled off maximally with 487nm, confirming that it is bistable and is yet the most red-shifted animal opsin known to date. Interestingly, we also found that the middle-wave opsins showing maximal activation at 485nm and 479nm were also bistable and were deactivated most efficiently with a 595nm light stimulation.

Our results suggest that the N.oerstedii did not evolve to express rhodopsins capable of maximum absorption above 700 nm, rather it uses the residual absorption from 600nm absorbing rhodopsins.

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Poster 75 >> Alexey Alekseev (University Medical Center Goettingen, Germany)

Automated Screenings of Natural Channelrhodopsin Variants using Planar Patch-Clamp and Spinning Disc Confocal Microscopy

Aiming for advanced channelrhodopsins (ChRs) with properties tailored for specific research and medical applications, we conducted extensive screenings of natural ChR variants. Patch-clamp electrophysiology is the gold standard for characterizing ion channels in biological membranes, while confocal microscopy is widely used for studying expression and subcellular localization. However, the limited throughput of these techniques constrains the scope of ChR screenings. To ovecome this limitation, we have incorporated automated spinning disc confocal fluorescence imaging (Yokogawa CQ1), and an automated planar patch-clamp system equipped with suitable illumination devices (opto-Syncropatch 384) into our research methodology. Optogenetic applications are often associated with phototoxicity and proteostatic stress in the manipulated cells. Minimizing these adverse effects is crucial, particularly in the context of clinical translation. This requires ChRs that allow excitable cells to be reliably activated by low light doses and at moderate expression levels with appropriate plasma membrane targeting. A fundamental ion channel property limiting the efficiency of optogenetic stimulation is the small unitary conductance of ChRs (~30 fS for ChR2 [1, 2] vs. ~7 pS for NaV1.1 [3]). By employing automated workflows, we performed stationary and non-stationary noise analyses with sample sizes sufficient for robust statistical comparisons. Our findings revealed that the bacteriorhodopsin-like ChR (BCCR), ChRmine [3], and its gain-of-function mutant ChReef [4], exhibit a significantly higher unitary conductance (~80-100 fS) compared to the state-of-the-art ChR, CatCh (~30 fS) [2]. Motivated by that finding, we aimed to identify ChRs

with an even higher unitary conductance than ChRmine. To achieve this, we tested 62 previously uncharacterized BCCRs for their biophysical properties and plasma membrane expression. This screening uncovered ChRs with advantageous properties and revealed correlations between these properties and specific features in their amino acid sequences. Using optimized protocols for noise analysis, we aim to determine unitary conductance values of high photocurrent ChRs. Upon finding high unitary conductance ChRs we aim to further optimize them for basic research and future medical applications through combinatorial mutagenesis.

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Poster 76/Talk M1 >> Kirill Kovalev (EMBL Hamburg, Germany)

4D structural studies of the light-driven sodium pump ErNaR

Light-driven sodium-pumping rhodopsins are unique natural active ion transporters also being a great model for the understanding of the molecular mechanism of transmembrane sodium translocation against strong electrochemical gradients. The main type of sodium-pumping rhodopsins are proteins with the NDQ motif, such as KR2 from bacteria

Krokinobacter eikastus[1]. However, even in the case of KR2, the mechanism of sodium translocation remain under debate[2], [3]. Recently, we reported a new subgroup of NDQ rhodopsins comprising an additional glutamic acid residue in close proximity of the retinal Schiff base (RSB), and presented the in-depth characterization of one of its members, ErNaR from Erythrobacter sp. HL-111[4]. The additional glutamic acid (E64 in ErNaR) allowed to make the spectroscopic and functional properties of ErNaR almost pH-insensitive in the wide range of proton concentration. The structural basis for this affect is suggested to be the low-barrier hydrogen bond (LBHB) between E64 and the main counterion of the RSB, D105 in ErNaR, which likely results in a very low pKa value of D105. However, the mechanism of light-driven sodium pumping in the new subgroup of NDQ rhodopsins involving transient protonation the RSB counterion remains unknown. We present the current progress of the structural investigations of ErNaR using cryo-electron microscopy and X-ray crystallography, including time-resolved serial femtosecond and millisecond crystallography methods. The ensemble of the high-resolution ErNaR structures under various conditions and at fs-ms time delays to the optical excitation of the protein allowed us to propose the molecular mechanism of the ErNaR functioning and to show the conformational changes associated with the increase of the pKa value of D105 allowing for proton translocation from the RSB to the counterion. With the example of ErNaR, we will also demonstrate current progress on use of 4D crystallography on microbial rhodopsins at the PETRAIII synchrotron beamlines.

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Poster 77 >> Lea Jänisch (Paul Scherrer Institute, Switzerland)

Structural characterization of UV-absorbing bistable optoGPCRs with X-ray crystallography

Bistable opsins are G protein-coupled receptor photopigments that can switch between active (ON) and inactive (OFF) states using specific wavelengths of light. These properties make them promising tools for optogenetic applications to manipulate cellular signaling events. Bistable opsins are 7-transmembrane proteins using retinal as a natural ligand for light sensing, with distinct absorption spectra at each state due to the differing chemical environments in the retinal binding pocket. Accurately predicting the absorption λmax from the protein sequence alone is challenging.

This project aims to characterize the spectral properties of bistable opsins through time-resolved UV-VIS spectroscopy. Additionally, we aim to resolve the atomic structures of bistable opsins in both active and inactive states by solving crystal structures of bistable opsins to atomic resolution. Solved structures provide key information about the Schiff base link between retinal and opsin proteins. Since absorption λmax is closely tied to the charge distribution around the Schiff base and nearby residues, this structural insight will be of use to tailor the spectral properties of bistable opsins for optimized optogenetic applications.

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Poster 78 >> Aditya Lakshminarasimhan (Arizona State University, USA)

Comprehensive Database of Rhodopsin Conformations and Analysis of their Retinal Binding Pockets

Rhodopsin is a retinal protein and G-Protein Coupled Receptor critical for vertebrate vision. Extensive crystallization efforts of rhodopsin since the publication of its first structure in 2000 has generated 66 structures of different conformational states of rhodopsin with and without its binding partners arrestin, rhodopsin kinase and the G-protein, transducin. This provides us with an opportunity to quantify the interactions of retinal with rhodopsin in different structures on a large scale. We created a database of all bovine rhodopsin structures and classified them into 16 dark-state rhodopsin, 26 opsin, 19 Metarhodopsin (Meta)-II, and 5 other photo-intermediate structures. Across these groups, we also identified 21 entries contain the N2C/D282C substitution mutation that stabilizes the structure. In addition, there are 8 Meta II structures with G-Protein, 4 with arrestin (3

Meta-II and 1 opsin with arrestin), 4 Meta-II with kinase, 9 opsin with transducin. Then, we quantified binding of retinal to these structures through prediction of binding affinities. Using DiffDock and GNINA Minimized Affinity, which are protein-ligand pose prediction and binding affinity calculation tools, we docked each of the 66 rhodopsin proteins against 11-cis-retinal and all-trans-retinal. As expected, Meta-II structures bind better to all-trans-retinal while dark structures bind better to 11-cis retinal conformations. However, we also find that the flexibility of the binding pocket during photo-intermediate states – lumi-rhodopsin and batho-rhodopsin – enable these conformations to dock with higher affinity to both retinal chromophores. Furthermore, opsin and active forms with proteins bound, the binding affinity profile of retinal weakens on average despite adopting conformations similar to canonical inactive and active states. These results provide a novel, quantitative perspective into the rhodopsin activation process.

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Poster 79 >> Jingjing Zang (University of Zurich, Switzerland)

Recoverin in Visual Transduction and Beyond: New Insights in Cone Vision

Recoverin (Rcv) is a neuronal calcium sensor with a low molecular weight, predominantly found in the photoreceptors of the vertebrate retina. In darkness, Ca2+-bound Rcv is suggested to inhibit G-protein-coupled receptor kinase (GRK). Upon exposure to light, Ca2+-free Rcv releases GRK, initiating the phosphorylation of visual pigment, and consequently quenching the visual transduction cascade. While mammals and sauropsids have in general only one recoverin gene (albeit an evolutionary distinct one), amphibians have retained both the sauropsid and the mammalian ortholog, and fish possess up to four recoverin genes. Therefore, it seems likely that efficient phototransduction can be achieved with a single recoverin gene and the increased number of recoverins in teleost species hints towards additional functions for recoverins such as an intracellular Ca2+ buffer or as Ca2+ dependent regulators of other kinases in and outside the visual system. To address this, we utilized a cone-dominant zebrafish retina to explore the function of a cone-specific Rcv through the generation of a CRISPR-Cas9 Knockout (KO) line. Electroretinogram Recording (ERG) results revealed that Rcv did not modulate cone photosensitivity. Interestingly, Rcv KO fish exhibited accelerated response kinetics under medium to low light conditions, with no effects observed under extremely bright light. Additionally, Rcv KO fish demonstrated robust ERG off response across a broad range of light intensities. Collectively, our findings show that Rcv play a role in regulating the visual transduction cascade. Intriguingly, our data suggest an unexpected role beyond the visual transduction cascade.

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Poster 80/Talk M4 >> Kwang-Hwan Jung (Sogang University, Seoul Korea)

Dual roles of proton pumping rhodopsin in Gloeobacter: Energy production and gene regulation

Light is a significant factor for living organisms with photosystems, like microbial rhodopsin - a retinal protein that functions as ion pumps, channels, and sensory transducers. *Gloeobacter violaceus* PCC7421, has a proton-pumping rhodopsin called the *Gloeobacter* rhodopsin (GR). It was involved in energy production by green light with photosynthetic machinery. The helix-turn-helix family of transcriptional regulators has various motifs, and they regulate gene expression in the presence of various metal ions. We show that active proton outward pumping rhodopsin interacted with the helix-turn-helix transcription regulator and regulated gene expression. This interaction is confirmed using ITC analysis (KD of 8 μM) and determined the charged residues required. Using *in vitro* expression experiments with fluorescent and luciferase reporter systems, we confirmed that ATP-binding cassette (ABC) transporters and the self-regulation of *G. violaceus* transcriptional regulator (GvTcR) are regulated by light, and gene regulation is observed in *G. violaceus* using the real-time PCR. These results could expand our understanding of microbial rhodopsin function's natural potential and limitations.

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Poster 81 >> Caspar Jonas Schattenberg (Leibniz-Forschunginstitut für Molekulare Pharmakologie, Germany)

Hybrid Quantum-Mechanics/Molecular-Mechanics Investigation of a Channelrhodopsin Open State Structure

Channelrhodopsins are light-gated ion channels, that enable the control of neuronal activity at the single cell level and have thus become important tools in optogenetic research. Photoexitation induces conforma- tional changes accompanied by proton transfers in the

protein, which ultimately allows an opening of the ion channel and an influx (or efflux) of ions through the cell membrane. However, a detailed understanding of the mechanism of channel opening and the opsin-induced spectral shifts that accompany the process were ham- pered so far by a lack of experimentally and (unbiased) computationally resolved structures of the open-states. However, the combined approach of computational electrophysiology (Comp-EL) – molecular dynamics simu- lations based on experimental evidence of the local structure of the retinal chromophore (13-cis, 15-anti) and the protonation states of the opsin environment has now been used to generate open-state structures of various Channelrhodopsins such as C1C2, Channelrhodopsin-2 and the anion conducting iChloC. These simulations allow for a more in depth analysis of the open-state structures.

Here, we consider the hybrid quantum-mechanics/molecular-mechanics (QMMM) simulations for structural refinement and the subsequent quantum-mechanical calculations of channelrhodopsins including also the open state of C1C2 in more detail. We investigate the structural - and methodological influence on the calculated UV- Vis spectra. Particular attention is also paid to the presence of ions in the channel near the retinal chromophore. We investigate potential factors influencing the opsin-induced shift of these additional charged entities via direct and indirect structural effects.

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Poster 82 >> Sarah Luise Schmidt (Paul Scherrer Institute, Switzerland)

Unravelling ultrafast kinetics behind human colour perception

Human vision is mediated by retinal-binding proteins in the photoreceptor cells of the retina. Rod cells contain rhodopsin, which enables vision under dim light conditions, while cone cells express cone opsins, responsible for high-acuity central vision and color discrimination across varying light conditions. Human cone opsins are classified into three types based on their spectral sensitivity: short (S), medium (M) and long (L) wavelength-sensitive, corresponding to blue, green and red light sensitivity, respectively. Dysfunction of these opsins causes related disorders such as cone-rod dystrophy, achromatopsia, and various forms of color blindness, which can lead to partial or complete vision loss.

Rhodopsin and cone opsins are G protein-coupled receptors (GPCR) that share the same ligand, 11-cis-retinal. Despite this molecular similarity, cone opsins exhibit not only distinct spectral sensitivities but also ultrafast activation and regeneration kinetics, which are

approximately 100-fold faster than rhodopsin. The reasons for this difference remain largely unknown, as cone opsins have been significantly understudied due to their low abundance in the retina and inherent challenges in isolating these proteins for detailed structural and functional analysis.

To address this question, we aim to use a combination of time-resolved structural biology and biophysical methods to capture real-time activation with near-atomic resolution on femtosecond to millisecond timescales. One of the key challenges in this project is isolating highly pure human cone opsins. We have successfully expressed and purified functional human cone opsins in quantities sufficient for spectroscopic and structural analysis.

Poster 83 >> Matthew Joseph Rodrigues (Paul Scherrer Institute, Switzerland)

Activating JSR1 with the all-trans 6.11 retinal analog for structural studies

Jumping Spider Rhodopsin-1 (JSR1) is a green-light sensitive photoreceptor that can be reversibly activated by photoisomerisation of a covalently bound retinal moiety. The receptor is inactive with 11-cis retinal bound and activated when retinal isomerises to the all-trans form. The structure of the inactive protein has previously been solved using X-ray crystallography, however, structural characterisation of the active state has proven elusive. Here we show that a non-natural retinal analog, all-trans retinal 6.11 (ATR6.11), can be reconstituted with JSR and acts as an agonist. ATR6.11 enabled complex formation between JSR1 and a chimeric G protein heterotrimer, enabling the complex structure to be solved by cryo-EM. The structures elucidate similarities and differences in the active state structures of monostable and bistable opsins and may facilitate engineering of bistable opsins for optogenetic applications. We envisage that ATR6.11 may prove to be a useful tool for studies of other bistable opsins.

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Poster 84/Talk N4 >> Wayne Busse (Humboldt University of Berlin, Germany)

Localization of the Fluorescent Rhodopsin NeoR in Fungal Zoospores with Insights into Its Enzymatic Functionality

Rhodopsin guanylyl cyclases (RGCs) control phototactic behavior in motile zoospores of unicellular aquatic fungi. Based on their dimerization, RGCs can be divided into two groups: homo- and heterodimeric. In heterodimeric RGCs, a conventional green-absorbing rhodopsin (RGC1) pairs with the far-red absorbing rhodopsin NeoR to form a functional enzyme. NeoR stands out with its remarkable absorption around the 700 nm mark. In addition, the red-absorbing state is intensely fluorescent, which is reversibly switched upon illumination into a deprotonated UV-absorbing state. Given that the enzyme is activated by green light, the role of NeoR within the heterodimeric assembly remains unclear.

To elucidate the enzymatic mechanism, we performed functional studies with recombinantly expressed proteins combined with introduced point mutations in the pseudo-symmetric enzymatic active centers. By manipulating each binding site individually, we demonstrate that RGC1 is primarily responsible for functionality, while NeoR affects the overall catalytic turnover without contributing to cGMP synthesis. Notably, replacing the metal ion-binding aspartate in the degenerate catalytic center of NeoR led to a six-fold increase in enzyme activity. Additionally, we spectroscopically characterized the photocycle dynamics of RGC1 and two other rhodopsins, the most blue- and red-absorbing NeoR partners known to date. Neither enters a deprotonated M-state, which is expected to be the main signaling state due to its long lifetime. However, the design of various chimeras shows that the heterodimeric RGCs can be converted into functional homodimers by exchanging their catalytic domains without altering the photochemistry, thus giving the rhodopsins a secondary role in the enzymatic functionality. Furthermore, we aimed to study the protein under native conditions. Therefore, we used the original fungus Rhizoclosmatium globosum and observed an intrinsic fluorescence inside the cell that we could experimentally assign to NeoR. We localized NeoR adjacent to the lipid-rich droplet, in a sickle-shaped organelle of the chytrid fungus known as the rumposome, which together form the fungal eye. The photophysical properties further allowed us to study local structure of the rumposome using single-molecule localization microscopy. The ongoing study aims to reveal the number of fluorophores per zoospore and provide further subcellular structural information.

Taken together, we provide an overview of the functionality of each particular binding site in the enzyme, with a detailed spectroscopic characterization of various green-absorbing RGCs, and present the first sensory rhodopsin that can be observed inside its native organism based on its intrinsic fluorescence properties.

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Poster 85 >> Hartmut Oschkinat (Leibniz-Forschungsinstitut für Molekulare Pharmakologie, Germany)

Protons flipping around in the center of bacteriorhodopsin by MAS NMR at 1.2 GHz

Proton translocation across membranes is vital to all kingdoms of life. Mechanistically, it relies on characteristic proton flows and modifications of hydrogen bonding patterns, termed protonation dynamics, which can be directly observed by fast magic angle spinning (MAS) NMR. As a model study, we demonstrate that fast proton displacement takes place in the active site of bacteriorhodopsin already in its equilibrated dark-state, providing new information on the underlying hydrogen exchange processes. In particular, MAS NMR reveals proton exchange at D85 and the retinal Schiff base, suggesting a tautomeric equilibrium and thus partial ionization of D85. We provide evidence for a proton cage and detect a preformed proton path between D85 and the proton shuttle R82. Exchange with water is remarkably uneven between the all-trans and the 13-cis, 15-syn forms. We propose that retinal isomerization makes the observed proton exchange processes irreversible and delivers a proton towards the extracellular release site.

Poster 86 >> Masahiko Taguchi (Tohoku University, Japan)

Insights into light-driven chloride ion pump mechanism of NM-R3 by molecular dynamics simulation

Microbial rhodopsins acting as pumps and channels are retinal-binding membrane proteins with seven transmembrane helices. Recently time-resolved serial femtosecond crystallography (TR-SFX) experiments were performed for the microbial rhodopsins [1-3]. *Nonlabens marinus* rhodopsin-3 (NM-R3) is a microbial rhodopsin which acts as a chloride pump. NM-R3 has a characteristic NTQ (Asn98, Thr102, Gln109) motif. The reaction is initiated by photoisomerization of the retinal from the all-*trans* conformation to the 13-*cis*,15-*anti* conformation. After photoisomerization, a chloride ion is transported from the extracellular side to the intercellular side via various intermediate states with different maximum absorption wavelengths. In this study, we investigated protein responses of NM-R3 upon light-absorption. We built simulation models for dark and light states with all-*trans* and 13-*cis*,15-*anti* retinal, respectively. The initial protein structure of NM-R3 was taken from the SFX structure (PDB ID: 7O8F [3]). The atomic charges of the retinal for all-*trans*

and 13-*cis*,15-*anti* conformations were determined by hybrid quantum mechanical / molecular mechanical (QM/MM) calculations to take into account the electrostatic interaction between the retinal and surrounding environment including a chloride ion. For 13-*cis*,15-*anti* retinal, the two dihedral angles were constrained using the geometry obtained by the QM/MM calculation as reference. For each simulation model, we performed molecular dynamics simulation. Ten independent runs were carried out for 500 ns (a total of 5 μs). We analyzed simulation trajectories to clarify structural changes, hydrogen bonding networks around the retinal, and ion movement upon light-absorption. As discussed about importance of Asn98 and Thr102 at initial phase of chloride pump process in the TR-SFX experiment [3], our simulation showed that hydrogen bonding network between the sidechains of Asn98 and Thr102, and the chloride ion is more stable in the light state than in the dark state. In the light state simulation, the chloride ion frequently moved toward intercellular side reflecting the direction of pronated Schiff base of 13-*cis*,15-*anti* retinal. Concomitant with the movement of these residues, some water molecules entered into the cavity, and then the hydrogen bonding network around the retinal also reorganized. Consequently, these consecutive structural changes around the retinal also induced conformational changes of helices.

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Poster 87 >> Tobias Weinert (Paul Scherrer Institute, Switzerland)

Time-Resolved Crystallography (TR-X): Techniques and Applications for Beginners

Time-Resolved Crystallography (TR-X) is a powerful technique for studying dynamic biological processes at the molecular level, allowing to capture transient states of proteins and other biomolecules in action. This poster introduces key concepts and methodologies in TR-X, starting with single crystal techniques that are fundamental to the field. I cover steady-state measurements, freeze trapping, and the challenges posed by non-isomorphism when using cryo-crystallography, which can complicate data interpretation.

I describe serial crystallography approaches in detail, discussing the use of fixed targets and high-viscosity injectors, outlining their advantages and limitations in capturing time-resolved data. The poster explains how to carry out simple steady-state experiments in both fixed targets and injectors and elucidates how serial TR-X can produce either discrete or continuous timepoint data and explain their advantages and disadvantages.

Additionally, I address the pitfalls associated with analyzing isomorphous difference maps, introduce the refinement of activated states, which is crucial for accurate modeling of dynamic structures. Specific examples, including bacteriorhodopsin, KR2, NM-R3, and visual rhodopsin, demonstrate the potential of these techniques to elucidate the functional mechanisms of retinal proteins, showcasing the impact of TR-X in structural biology.

This overview provides a foundational understanding for beginners to navigate the complexities of TR-X, highlighting both theoretical aspects and practical applications in the field.

Poster 88 >> Tobias Weinert (Paul Scherrer Institute, Switzerland)

Direct observation of coherent azobenzene photochemistry

Molecular photoswitches are versatile natural or synthetic molecules that undergo reversible conformational changes in response to light. In chemistry azobenzenes act as ubiquitous synthetic photoswitches[1] with applications ranging from opto-electronics[2], over molecular machines[3] to photopharmacology[4]. Their isomerization mechanism defines their molecular properties and yet is controversially debated, as the underlying ultrafast photochemistry is challenging to resolve in time and space. In this study, we have used an X-ray Free Electron Laser to observe coherent structural transitions in the prototypical photoswitch azo-combretastatin A4[5] bound to its protein target tubulin. A molecular movie assembled from a crystallographic femtosecond scan and snapshots of kinetic intermediates in the femto-to-nanosecond range show how the cis isomer overcomes energy barriers in the excited state, traverses the conical intersection into a twisted ground state conformation, cools, stretches and finally relaxes into a planarized trans conformer. Our crystallographic data at near-atomic resolution contrasted with femtosecond transient absorption spectroscopic data and quantum chemical trajectories provides an experimental and theoretical description of the ultrafast azobenzene photoreaction. These fundamental insights reveal surprising parallels between natural and synthetic photoswitches opening a route for the design of artificial photoswitching proteins.

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Poster 89 >> Minglei Ma (University of Liège, Belgium)

The adaptational and evolutionary history of spectral sensitivity in ecdysozoan r-opsins

Visual r-opsins originated more than 600Ma in the common ancestor of all protostomes, and underwent multiple duplications within the evolutionary history of Ecdysozoa, leading to a remarkable number of visual pigments in extant insect and crustacean species. Combining phylogenetic, computational and molecular approaches we explore the evolution of visual pigment functions across major duplications throughout Arthropoda (insects, crustaceans, chelicerates, myriapods). We capture key evolutionary transitions, including the terrestrialisation of insects, tardigrades and chelicerates, giving a functional view of opsin adaptation to new visual environments.

All 4 opsin families shared across Arthropoda (LWS-MWS-Rh7 and UV/SWS) originated prior to terrestrialisation of arthropods but also of vascular plants. For shallow water and coastal organisms, the water would likely have been red-shifted, owing to sediment influx without roots to bind the soil. The LWS/MWS to LWS+MWS duplication (second opsin duplication) might have resulted in a bathochromatic LWS opsin adapted to Cambrian coastal environments. Similarly, UV-light has poor depth penetration in saltwater, yet the modern UV/SWS/Rh7 clade originated in the first opsin duplication. As the ancestral ecdysozoan opsin is likely blue-green sensitive, the evolution of LWS and UV/SWS pigments should tell us about the early ecologies of Cambrian shallow marine environments.

A comprehensive phylogeny was built combining large-scale opsin datasets of modern UV-SW-MWS-LWS-Rh7 paralog lineages across arthropods and non-arthropod ecdysozoans.

Ancestral states were robustly calculated using Lazarus with additional Gap Correction, alongside a novel comparative approach to better approximate flanking regions, providing 8 ancestral sequences (around 370-aa long) at key internal nodes representing duplication events preceeding modern arthropod opsin lineages.

Our first ancestral sequence is placed at the origin of all modern opsin lineages, incorporating arthropod-specific families Rh7, UV, SWS, LWS and MWS and non-arthropod ecdysozoans (ancestral 1). Our second (ancestral 2) represents the common ancestor of all arthropod-specific visual r-opsin families, which upon duplication around 599Ma, gave rise to LWS/MWS opsins (ancestral 3) in Chelicerata-Pancrustacea-Myriapoda, and Rh7/UV/SWS opsins in Chelicerata-Pancrustacea (ancestral 4). The LWS/MWS (ancestral 3) lineage evolved into MWS+LWS opsins 590Ma before the split between Chelicerata-Pancrustacea-Myriapoda, with an internal division between Chelicerate and Mandibulate LWS opsins (ancestral 5). Duplications of the RH7/UV/SWS (ancestral 4) lineage resulted in Rh7 and a combined UV/SWS (ancestral 6) 570Ma, followed by evolution of the Chelicerate UV family (ancestral 7), then the UV/SWS split into modern UV+SWS opsins (ancestral 8).

We explore functionally these resurrected opsins by heterologous expression to assess the spectral range of early light-sensitive receptors.

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Poster 90 >> Minglei Ma (State Key Laboratory of Marine Environmental Science, Xiamen University, China)

Proton pump rhodopsin of the coral symbiont Breviolum minutum and its potential role in coping with phosphorus deficiency in future warmer ocean

Global warming can inhibit chlorophyll-based solar energy capturing of phytoplankton by decreasing nutrient supply through upwelling. However, species with proton pump rhodopsin (PPR) can independently convert solar energy to cope with nutrient limitation. Besides prokaryotes, PPR has been documented in dinoflagellates and some species of other algal lineages, and its potential role in compensating for the deficiency of phosphorus has been demonstrated in dinoflagellates. However, PPR has not been studied in the coral reef endosymbiotic Symbiodiniaceae. Here, we report a PPR in Breviolum minutum (BmR). Both phylogenetic analysis and structure prediction results indicate that BmR resembles eukaryotic proton pump rhodopsins, phylogenetically affiliated with the subgroup Xanthorhodopsins. BmR contains the critical residues for proton pumping, retinal binding,

and spectrum tuning for green absorption. To explore BmR's potential roles in responding to phosphorus limitation, we cultured B. minutum under different phosphorus conditions, and monitored physiological and BmR's transcriptional responses. Phosphorus limitation caused decreases in B. minutum population growth, photosynthesis efficiency. Meanwhile, our quantitative PCR showed that BmR expression was strongly upregulated under phosphorus limitation, showing a strong positive correlation with alkaline phosphatase activity and a negative correlation with photosynthetic efficiency. Our findings demonstrate that proton pump rhodopsin occurs in Symbiodiniaceae and BmR has the potential to provide supplementary energy to support cell basal metabolisms when photosynthesis of B. minutum is impaired by phosphorus limitation, thereby enabling corals to better weather climate change.

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Poster 91/Talk K1 >> Quentin Clément Bertrand (Paul Scherrer Institute, Switzerland)

Structural effects of high laser power densities on an early bacteriorhodopsin photocycle intermediate

Time-resolved serial crystallography at X-ray Free Electron Lasers offers the unique opportunity to observe ultrafast photochemical reactions at the atomic level. The new technique has yielded exciting molecular insights into various biological processes including light sensing and photochemical energy conversion. However, to achieve sufficient levels of activation within an optically dense crystal, high laser power densities are often used, which has led to an ongoing debate to which extent photodamage may compromise interpretation of the results. Here we compare time-resolved serial crystallographic data of

the bacteriorhodopsin K-intermediate collected at laser power densities ranging from 0.04 to 2493 GW/cm2 and follow energy dissipation of the absorbed photons logarithmically from picoseconds to milliseconds. Although the effects of high laser power densities on the overall structure are small, in the upper excitation range we observe significant changes in retinal conformation and increased heating of the functionally critical counterion cluster. We compare light-activation within crystals to that in solution and discuss the impact of the observed changes on bacteriorhodopsin biology.

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