**A Generic Residue-Numbering system for Microbial Rhodopsins  
– Unifying Structural Frameworks and Functional Mapping**

**A Generic Residue-Numbering System to Unify Structure-Function Analysis across Microbial Rhodopsins**

**A Generic Residue-Numbering system for Microbial Rhodopsins**

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# Abstract

Microbial rhodopsins are photoreceptors with a seven-transmembrane architecture that use the chromophore retinal—a vitamin A derivative—to convert light absorption into ion transport, signaling, and enzymatic activity. Since their discovery half a century ago, genome sequencing has uncovered thousands of these proteins across archaea, bacteria, eukaryotes, and viruses, revealing a diverse functional repertoire that includes ion pumps and channels, transducer activators, and enzyme/channel regulators. Although the structure of the seven-transmembrane bundle and the retinal-binding pocket is highly conserved, the sequence identity across major classes is low. This low sequence identity, combined with the rapidly expanding number of microbial rhodopsins identified by metagenomics, makes direct comparisons across the superfamily increasingly difficult. Here, we introduce a generic residue-numbering system for microbial rhodopsins (GRN-MR): a structure-based framework anchored to the retinal-binding pocket that enables consistent residue comparison across even the most divergent family members. Analyzing 129 structures and approximately 40,000 sequences, we demonstrate its utility by analyzing sequence–structure–function relationships across the superfamily, including previously uncharacterized members. GRN-MR provides a common language for organizing the growing repertoire of microbial rhodopsins, integrating structural, spectroscopic, and electrophysiological data, and engineering next-generation optogenetic tools.

# Introduction

The microbial rhodopsin superfamily comprises light-sensitive proteins that use the chromophore retinal—a vitamin A derivative—to convert photon absorption into concerted cellular events. Since the discovery of the proton pump bacteriorhodopsin in *Halobacterium salinarum* (*Hs*BR) over fifty years ago1, microbial rhodopsins have revealed a striking diversity of functions. These proteins are found across all domains of life—Archaea, Bacteria, and Eukaryota— and viruses2, 3, 4functioning as proton, chloride, or sodium pumps, cation and anion channels, activators of downstream signal transducers, and even as enzyme/channel/transporter regulators (when fused to catalytic domains). Their photochemical properties are equally varied: while in most microbial rhodopsins light isomerizes all-*trans* retinal to the 13-*cis* form, some produce 9-*cis* or 11-*cis* isomers as photoproducts2, 5, 6, 7.

Despite this functional and photochemical diversity, all microbial rhodopsins share a core architecture: a tight bundle of seven transmembrane helices (TMs) surrounding the retinal-binding pocket, with retinal bound via a Schiff base to a conserved lysine on TM7. Some families form oligomers ranging from dimers to hexamers8, 9, while others have an additional TM or an inverted membrane topology6, 10, 11. Remarkably, this structural conservation persists even though sequence identity between major families can be as low as 10–12%12, with a nearly identical orientation of retinal in the binding pocket. Such conservation despite extreme sequence divergence13 suggests powerful evolutionary constraints on the architecture required for retinal binding and photoisomerization.

Microbial rhodopsins are often classified, annotated, and compared by tracking characteristic sequence motifs that are coupled to their function. However, the high sequence variability within the family complicates the analysis of large datasets obtained in metagenomics studies, particularly the comparison of distant subfamilies. Researchers in the field often resort to developing *ad hoc* sequence numbering systems using a reference rhodopsin, such as *Hs*BR, channelrhodopsin-2 from *Chlamydomonas reinhardtii* (*Cr*ChR2, the most common optogenetics tool)14, or C1C2 also from *Chlamydomonas reinhardtii* (the first channelrhodopsin structure, a chimera between channelrhodopsin-1 and 2)15. This makes it challenging to recognize equivalent positions and conserved motifs in different rhodopsins throughout the scientific literature. Other fields have overcome this hurdle by adopting generic residue-numbering (GRN) systems. A prime example is the field of G protein-coupled receptors (GPCRs), in which the Ballesteros–Weinstein GRN system16—or its descendant GPCRdb system17—assign a unique index to equivalent positions in different proteins. Similar systems developed for microbial rhodopsins are limited to a few specific families18 and cannot be applied across the entire superfamily.

In this work, we develop a GRN system for microbial rhodopsins (GRN-MR) based on the structure of the retinal-binding site, rather than on sequence conservation. This numbering system provides a consistent framework for comparing residues across even the most divergent rhodopsins. Using a dataset of 129 structures and approximately 40,000 sequences, we demonstrate its utility by mapping functionally important residues across ion pumps, channels, and sensory rhodopsins, and by identifying conserved sequence signatures in both characterized and previously uncharacterized rhodopsin families.

# Results

## Assembly of a dataset of microbial rhodopsin structures

Comparative structural analysis of microbial rhodopsins has been somewhat hindered by limited structural coverage of certain functional classes and taxonomic groups. To address this limitation, we assembled a comprehensive dataset of 129 structures, combining 69 experimentally determined structures and 60 computationally predicted models **(Fig. 1)**. The dataset includes rhodopsins from all domains of life—archaea (27, 21%), bacteria (42, 33%), and eukaryota (50, 39%)—and also includes synthetic variants (5, 4%) and viral rhodopsins (5, 4%). Functionally, the dataset spans proton pumps (42, 33%), cation channels (28, 22%), regulatory rhodopsins with fused domains (14, 11%), anion channels (12, 9%), chloride pumps (12, 9%), transducer activators (8, 6%), sodium pumps (6, 5%), and rhodopsins of undefined function (7, 5%). Due to biases in available structural databases, proton pumps are overrepresented in the experimentally determined structures (31/69, 45%). To balance functional coverage, we enriched underrepresented families through predictive structural modeling: enzyme/channel/transporter regulators (12 predictions), cation channels (11), proton pumps (11), anion channels (8), chloride pumps (6), transducer activators (6), sodium pumps (3), and rhodopsins of undefined function (3).

The dataset reflects known taxonomic distributions of rhodopsin functions. Channelrhodopsins—both cation-conducting (23/28) and anion-conducting (10/12)—are concentrated in eukaryotes, consistent with their restriction to algae and related protists19. Similarly, enzyme/channel/transporter regulatory rhodopsins are predominantly eukaryotic (12/14), consistent with enzyme rhodopsins having been identified mostly in fungi, green algae, and choanoflagellates10. Sodium pumps appear exclusively in bacteria (6/6), consistent with their known distribution in marine flavobacteria and related lineages20. Chloride pumps are found in both archaea and bacteria, reflecting independent evolutionary origins in these lineages21. Proton pumps show the broadest distribution across Archaea, Bacteria, and Eukaryota, consistent with ancestral sequence reconstruction studies suggesting that the last common ancestor of microbial rhodopsins functioned as a light-driven proton pump22.

## Validation of the structural predictions

As nearly half of our dataset consists of predicted models, we first assessed their accuracy using two complementary validation sets of experimental structures: benchmark set A (n = 42), composed of structures deposited before September 2021 (which may have been used in the training of the structure prediction methods), and blind set B (n = 27), composed exclusively of structures deposited after the model training cutoff. We assessed the accuracy of the models by comparing the protein backbone, the retinal-binding pocket, and retinal **(Supplementary Fig. 1)**. The predicted models have high accuracy. Models in benchmark set A had a mean backbone Cα RMSD of 0.61 ± 0.23 Å, binding-pocket iRMSD of 0.51 ± 0.28 Å, and retinal L-RMSD of 0.41 ± 0.24 Å. Models in blind set B had a mean backbone Cα RMSD of 0.80 ± 0.24 Å, binding-pocket iRMSD of 0.75 ± 0.28 Å, and retinal L-RMSD of 0.61 ± 0.27 Å. The errors in blind set B are consistently higher, but the mean differences between the predicted models and the reference structures remain below 1 Å.

To illustrate the accuracy of the predicted models, we show experimental and predicted structures for six representative rhodopsins spanning different functional classes **(Fig. 2)**.In all cases, the overall fold was accurately reproduced, with loops largely correctly positioned **(Fig. 2a).** The retinal-binding pocket was also consistently modeled at sub-angstrom precision **(Fig. 2b)**, including accurate handling of unusual 6-s-*cis* β-ionone ring conformations (as observed in the cation channelrhodopsin *Kn*ChR), consistent with the non-G rule23, 24, 25. These results show that current structure prediction methods reliably capture both the global rhodopsin fold and the details of the retinal-binding pocket, supporting the inclusion of predicted structures to increase the diversity of our dataset.

## Overall structural conservation of the microbial rhodopsin fold

To analyze structural conservation across the dataset, we calculated pairwise RMSDs between Cα atoms of the transmembrane helices for all structure pairs. The resulting RMSD matrix **(Fig. 3)** reveals substantial structural conservation across the superfamily. However, sequence identity is low, averaging 21 ± 9% in the TM bundle (min = 7%) and 19 ± 9% in the full sequence (min = 6%). Hierarchical clustering of the RMSD matrix identified a single main structural cluster, confirming that microbial rhodopsin fold is highly conserved across the superfamily. A generic residue-numbering system for microbial rhodopsins should therefore be based on structural features rather than sequence.

## Development of the generic residue-numbering system

The GRN-MR system was inspired by the Ballesteros-Weinstein system for G protein-coupled receptors but adapted to the unique architecture of microbial rhodopsins. GRN-MR codes consist of two numbers: the first denotes the helix (1-7) and the second the position within the helix relative to the residue closest to retinal, defined as position 50. For example, position 7.46 denotes a residue in TM7, four residues before the Schiff base-forming lysine (defined as 7.50).

The first task in developing the GRN-MR system was identifying the closest residues to retinal in each helix **(Fig. 4a),** which serve as the ‘anchors’ of the numbering system . The distance profiles along each helix **(Fig. 4b)** exhibit the expected systematic variations, reaching their minima at central positions and increasing toward the N- and C-termini. The binding pocket (<3.5 Å around retinal) is formed by residues from TM3-7. Only two residues in TM2 are part of the extended pocket (<6 Å), while TM1 is at a peripheral location within the 7-TM bundle and further from retinal. The closest residue to retinal in each helix is designated as an anchor **(Fig. 4b, vertical lines)**. The sequence variability around the anchor positions **(Supplementary Fig. 2b)** shows that they lie within or adjacent to regions of elevated sequence conservation despite the overall sequence divergence of the superfamily. These data support the choice of a ligand-centric approach to capture structurally and functionally critical positions across diverse microbial rhodopsins **(Supplementary Fig. 3)**.

## Application of the generic residue-numbering system

To demonstrate the application of the GRN-MR system, we first focus on residues that form the retinal-binding pocket, using *Hs*BR as a reference **(Fig. 5a)**. Retinal is covalently bound to K2167.50 (GRNs are shown as superscripts) via a Schiff base linkage and is surrounded by 18 additional residues from TM3-7 (5 from TM3, 3 from TM4, 4 from TM5, 4 from TM6, and 2 from TM7). Two highly conserved carboxylates, D3.45 and D7.46, located near K7.50 (D853.45 and D2127.46 in *Hs*BR), are historically referred to as the “Schiff base counterions” and stabilize the positive charge of the protonated Schiff base. Residues at positions 3.53, 4.51, 6.54, and 7.49—known as the L/Q, N/LI, G/P, and A/TS switches, respectively—contribute to spectral tuning in microbial rhodopsins26, 27, 28 (Wang et al., JBC, 2003; Shimono et al., JBC 2003). In addition, position 4.54 (G1224.54 in *Hs*BR) plays an important role in determining retinal planarity: when a non-glycine residue occupies this position, steric clash with the retinal β-ionone ring can induce rotation and favor a 6-s-*cis* retinal conformation (the “non-G rule”)23, 24, 25. Two other notable positions near the β-ionone ring are 5.44 and 5.47. Although retinal is enclosed by TM3-7, many microbial rhodopsins have a small lateral opening between TM5 and TM6 that connects the retinal pocket to the lipid bilayer15, 29, 30, 31, 32. The shape of this opening varies among microbial rhodopsins: in some bacterial ion-pumping rhodopsins, residue 5.47 contributes to its formation, whereas in heliorhodopsins (HeR) both 5.44 and 5.47 contribute. Functionally, 5.44 has been implicated in retinal uptake from the membrane—the G1715.44W mutation in *Ts*HeR inhibits this process31— while 5.47 is involved in binding the carotenoid antenna pigment, as evidenced by the G1785.47W mutation in *Gloeobacter* rhodopsin, which inhibits carotenoid binding33, 34.

The GRN-MR system also facilitates comparative analyses across subfamilies. As an example, in ion-pumping rhodopsins, the identity of the transported substrate and the direction of transport are primarily influenced by three residues at positions 3.45, 3.49, and 3.56, which are located on one face of TM3 **(Fig. 5b)**2, 4, 21. Ion-pumping rhodopsins containing D3.45, T3.49, and D3.56—known as the DTD motif—predominantly function as outward proton pumps. Upon photoactivation, the proton is released from the Schiff base to the extracellular bulk solvent via T3.49, D3.45, and the proton release group composed of EECL3 and E7.38, accompanied by a conformational change of the key arginine residue R3.42. A new proton is subsequently supplied from the intracellular bulk solvent to the Schiff base via D3.56, thereby accomplishing net transport of a single proton from the intracellular to the extracellular side. In contrast, inward proton-pumping rhodopsins, which comprise schizorhodopsins (SzRs) and xenorhodopsins (XeRs), lack one of the two counterions near the Schiff base. Specifically, D853.45 in *Hs*BR is replaced by F3.45 in SzRs, whereas D2127.46 in *Hs*BR is replaced by P7.46 in XeRs. The D3.45–T3.49–D3.56 motif is replaced by FSE in SzRs and by DTA, DTL, or DTS in XeRs, respectively, and the proton release group conserved in outward proton pumps is absent in both SzRs and XeRs35, 36. Inward chloride pumps have T3.45-S3.49-A/D3.56 or N3.45-T3.49-Q3.56 motifs, and the loss of negative charge caused by replacement of D3.45 with T3.45 or N3.45 is compensated by binding of the chloride substrate37, 38, 39. Outward sodium pumps share the N3.45-D3.49-Q3.56 motif, in which transient proton transfer from the Schiff base to D3.49 enables the sodium ion to pass through the Schiff base region20, 30, 40, 41.

The GRN-MR system is equally applicable to rhodopsins with non-canonical architectures. Heliorhodopsins (HeRs), for instance, have an inverted topology, with an intracellular N-terminus and an extracellular C-terminus (**Fig. 5c**). Despite this inversion, our structural alignment and annotation approach remains effective. At the sequence level, HeRs retain a TM3 counterion at position 3.45, whereas the TM7 counterion at 7.46 is replaced by serine. While the molecular functions of most HeRs remain unclear, a recent study suggested that V2HeR3 functions as a proton transporter42, and the GRN-MR system may facilitate future comparative analyses as more functional data become available.

Channelrhodopsins, widely used in optogenetics, present a challenge for systematic comparison: mutagenesis and protein engineering have generated numerous functional variants, while metagenomics continues to uncover natural diversity. The GRN-MR system enables mapping of this diversity onto a common structural framework, as illustrated for C1C2 structure **(Fig. 5d)**. Unlike ion-pumping rhodopsins, the TM3 residues at positions 3.45, 3.49, and 3.56 are not essential for channel function in canonical dimerics channelrhodopsins; substitutions at these and nearby positions, such as E3.45T or H3.56R, primarily affect channel kinetics43, 44. Instead, ion selectivity and kinetics are largely determined by five glutamates (E1–E5, corresponding to E2.46, E2.47, E2.54, E2.61, and E2.65) aligned along TM245, 46, 47, 48. The DC gate, formed by D4.47 and C3.50, controls channel closure kinetics and light sensitivity, enabling bistable optical control of neuronal activity49, 50, 51. **Fig. 5d** summarizes additional positions implicated in spectral tuning, conductance, and kinetics23, 52, 53, 54, 55, 56, 57, 58, 59, 60 (Kamiya et al., Chem. Phys. Lett., 2013; Scholz et al., 2017; Kato et al., Nature 2018; Lahore et al., Nat. Commun., 2022), and **Supplementary Fig. 4** summarizes positions that affect properties in trimeric channelrhodopsins24, 61, 62, 63 (Tucker et al., Nat. Commun., 2022; Duan et al., Adv. Sci., 2025).

Transducer-activating rhodopsins (sensory rhodopsins) relay light signals to downstream effectors. Structural, spectroscopic, and mutational studies have clarified the activation mechanism of *Np*SRII64, 65, 66 (**Fig. 5e**). *Np*SRII forms a 2:2 complex with its transducer HtrII through an interface formed by TM6 and TM7 of *Np*SRII and TM1 and TM2 of HtrII. In *Np*SRII, retinal photoisomerization induces structural changes in residues Y1746.53, L2007.45, D2017.46, and T2047.49, which are transmitted to HtrII via the interaction between Y1997.44 and N74 of HtrII66, 67. Notably, the key residue T2047.49 in *Np*SRII is not conserved in *H. salinarum* SRI (*Hs*SRI), suggesting that this signaling pathway may be specific to *Np*SRII rather than a general feature of transducer-activating rhodopsins68.

Enzyme-fused rhodopsins, such as rhodopsin-guanylyl cyclases and rhodopsin-phosphodiesterases, couple light detection to enzymatic activity. A characteristic feature of these proteins—shared with some channel-fused rhodopsins—is an additional TM0 helix at the N-terminus of the seven-transmembrane core6, 69. The GRN-MR system annotates the seven helices forming the retinal-binding domain, excluding TM0. For NeoR, the longest-wavelength rhodopsin reported to date, functional studies have identified three acidic residues essential for activity: E1363.45, D1403.49, and E2627.46 70 **(Fig. 5f)**. As structural and spectroscopic data on enzyme-fused rhodopsins accumulate, the GRN-MR system will facilitate comparative analysis of their activation mechanisms.

The GRN-MR system also accommodates microbial rhodopsins that exhibit gaps or insertions arising from local structural distortions **(Supplementary. Fig. 5)**. For example, ChRmine, a trimeric cation-conducting channelrhodopsin71, contains a constricted 310 helical turn within TM6 that results in a gap in the sequence alignment61 **(Supplementary. Fig. 5, salmon)**. In this case, the ‘missing’ position (6.48) is simply skipped. Conversely, TaraRRB-R1, a channel-regulating rhodopsin6, contains a bulged π-helical turn in TM5 that introduces an additional residue between 5.45 and 5.46 **(Supplementary. Fig. 5, green)**, which designated 5.451.

Together, these applications demonstrate that the GRN-MR system provides a unified framework for comparing rhodopsins across functional classes, taxonomic origins, and structural variations.

## Application of the GRN-MR system to a large dataset of microbial rhodopsins

To evaluate the GRN-MR system at scale, we applied it to a large sequence dataset that includes both characterized and uncharacterized rhodopsins. We combined the 129 functionally characterized rhodopsins in our curated dataset with a large collection of uncharacterized rhodopsin-like sequences, yielding an initial set of over 300,000 sequences from archaea, bacteria, eukaryotes, and viruses (Nishimura et al., in preparation). After curation, we obtained approximately 40,000 non-redundant sequences. From pairwise sequence similarities, we constructed a protein similarity network in which nodes represent individual sequences and edges connect pairs exceeding a defined similarity threshold **(Fig. 6a)**. The network resolved 14 clusters containing functionally characterized rhodopsins and 17 clusters containing only uncharacterized rhodopsins (**Supplementary Fig. 6**). The 14 characterized clusters comprise six major groups—archaeal/eukaryotic, bacterial, viral (VirR), canonical channelrhodopsins (ChRs), canonical histidine kinase-fused rhodopsins (HKRs), and canonical heliorhodopsins (HeRs)—and eight minor groups: schizorhodopsins (SzRs), cryptophyte anion-conducting channelrhodopsins (ACRs), alveolate ChRs, pump-like channelrhodopsins (PLCRs)72, guanylate cyclase-/phosphodiesterase-fused rhodopsins (RhoGC/RhoPDEs), bestrophin-fused rhodopsins (BestRs), noncanonical HKRs, and eukaryotic/viral HeRs (**Supplementary Fig. 6**).

To validate the clustering and explore uncharacterized rhodopsins, we predicted structures for 11 sequences from the periphery of 5 of the 14 functionally characterized clusters, along with one representative member from each of the 17 uncharacterized clusters (**Supplementary Fig. 7a**). In total, we generated structural models for 28 rhodopsin-like proteins (**Supplementary Fig. 7b**). All 11 characterized and 13 of the 17 uncharacterized sequences (uncharacterized clusters 1–13) adopted a rhodopsin fold and were retained for further analysis. Of the 13 uncharacterized rhodopsins, two lacked the conserved Lys7.50 and one exhibited an incorrectly predicted retinal-binding pose. Several uncharacterized clusters exhibited distinctive structural features: for example, the rhodopsin in cluster 2 has an unusually long TM1, whereas the rhodopsin in cluster 10 contains β-strands in ECL2 and ECL3 that form an extracellular β-sheet (**Supplementary Fig. 7b**).

To examine sequence conservation, we generated sequence logos in selected clusters **(Fig. 6b,c; Supplementary Fig. 8 and 9**). Conserved residues grouped near the X.50 anchor positions at approximately four-residue intervals, consistent with enrichment on the retinal-facing side of each helix; lipid-exposed positions showed lower conservation. Across all clusters, residues in TM3, TM6, and TM7 were more conserved than those in other helices. The arginine at position 3.42 is highly conserved across all functionally characterized clusters—with the exception of the PLCR cluster—consistent with its known key functional roles73, 74, 75, 76 (Kubo et al., Photochem. Photobiol., 2008). Residues forming the retinal-binding pocket (positions 3.46, 6.50, 6.53, and 6.54) and the Schiff base counterions (positions 3.45 and 7.46) are well conserved within characterized clusters; however, position 7.46 is more conserved than 3.45. Most characterized clusters have a highly conserved Asp at 7.46, but the alveolata ChR, bacterial/archaeal HeR, and eukaryotic/viral HeR clusters show highly conserved Asn, Ser, and Ser, respectively. Positions 3.50 and 3.51 vary markedly between characterized clusters—while remaining conserved within each—suggesting that these positions are linked to cluster-specific functions and serve as characteristic sequence signatures for these rhodopsin families. Similar conservation patterns were observed in uncharacterized clusters, although position 3.50 in cluster 1 and position 7.46 in cluster 4 showed reduced conservation.

# Discussion

The repertoire of microbial rhodopsins continues to expand. With estimates of global microbial diversity ranging from 106 77 to 1012 species78, and over two million bacterial and archaeal genomes now sequenced79, the number of identified rhodopsin genes—already exceeding 10,00080—will undoubtedly grow further. This expanding diversity, combined with ongoing optogenetics engineering efforts, has made a unified residue-numbering system increasingly necessary.

The limitations of existing numbering approaches become apparent when comparing across functional classes. Ion-pumping rhodopsins have historically been described using BR-based residue numbering, whereas channelrhodopsins are typically numbered according to ChR2- or C1C2-based systems. The three-residue TM3 motif introduced by Yoshizawa et al. provides a useful signature for classifying ion-pump functions21; however, in channelrhodopsins, functional properties are determined primarily by other residues, including five glutamates in TM2, rather than the TM3 motif. These differences underscore the need for a broadly applicable numbering system, which motivated the development of the GRN-MR system.

The sequence similarity network provides insight into the evolutionary relationships among microbial rhodopsins **(Fig. 6a, Supplementary Fig. 6)**. Rhodopsins from archaea and eukaryotes form a single major cluster (the archaeal/eukaryotic rhodopsin cluster), whereas bacterial rhodopsins occupy a distinct cluster (the bacterial rhodopsin cluster). This pattern indicates that eukaryotic microbial rhodopsins are more closely related to archaeal than to bacterial rhodopsins, consistent with previous phylogenetic and structural analyses81 (Brown, 2004; Sharma et al., 2006; Zabelskii et al., 2021).

Rhodopsins within the same cluster but with different functions likely diverged from a common ancestor, whereas functionally similar rhodopsins in different clusters likely arose through convergent evolution. For example, both the archaeal/eukaryotic and bacterial clusters include chloride-pumping rhodopsins, yet their TM3 motifs differ (T3.45-S3.49-A3.56 versus N3.45-T3.49-Q3.56, respectively), suggesting that chloride-transport function evolved independently in these lineages. Within clusters, oligomeric assembly and sequence motifs are conserved despite functional diversification. This conservation likely explains why researchers have been able to interconvert functions through relatively few mutations, even among rhodopsins sharing only ~30% sequence identity47, 48, 67 (Sasaki et al., 1995; Hasemi et al., 2016; Inoue et al., 2016; Takeno et al., bioRxiv, 2025).

The conservation of oligomeric assembly within clusters raises a question: what determines oligomeric state? All characterized microbial rhodopsins form specific oligomers, ranging from dimers to hexamers, and rhodopsins within the same clade consistently adopt the same oligomeric state8, 31, 61 (Shinohara et al., 2018; Ikuta et al., 2018; Inoue et al., 2020; Kishi et al., 2022; Mannen et al., 2023). Yet residues on the outer, lipid-exposed surface are generally poorly conserved, even in helices that participate in oligomeric interfaces. This suggests that oligomeric state is not dictated by a fixed set of conserved interface residues. In some rhodopsins, such as *Np*HR, outer-facing residues do contribute to interprotomer contacts and stabilize oligomerization82, but these residues are not conserved across clusters, implying that they fine-tune oligomer stability within a family rather than specifying oligomeric state.

Instead, we propose that the type of oligomer formed is largely determined by helix-packing geometry, specifically how the transmembrane helices are tilted and arranged relative to one another. Helix tilt can be influenced by glycine and proline residues within helices, as well as loop architecture. In particular, the length and orientation of the extracellular loop 1 (ECL1) have been proposed to distinguish trimeric from pentameric or hexameric assemblies81. However, pentameric rhodopsins such as GR and TR form trimers in DDM micelles, suggesting that the ECL1 may bias helix tilt toward particular arrangements without rigidly fixing protomer number. This observation implies that the membrane or detergent environment also contributes to stabilizing a given assembly.

Beyond evolutionary and oligomeric insights, our study also assessed the utility of structure prediction for rhodopsin analysis. Structure prediction methods performed well for rhodopsins. Predictions captured the overall fold and the detailed architecture of the retinal-binding pocket, often correctly reproducing the retinal configuration and orientation, including whether the chromophore adopts an all-*trans* geometry or a 6-*s-cis* conformation consistent with the non-G rule24, 25. Our backbone RMSDs (0.61 Å for Set A and 0.80 Å for Set B) are comparable to those reported for Boltz-1 in the Runs N' Poses benchmark83 (0.56 Å) and lower than AlphaFold210.1038/s41586-021-03819-2 predictions of GPCRs84 (1.12–1.55 Å). The accuracy of retinal placement is particularly notable: our mean retinal RMSDs of 0.41 Å (Set A) and 0.62 Å (Set B) are substantially lower than the 2.30 Å mean ligand RMSD that Boltz-1 achieved on diverse protein-ligand complexes in the Runs N' Poses benchmark. This likely reflects the strong structural constraints imposed by the retinal-binding pocket, including the covalent Schiff base linkage to the conserved lysine.

However, inaccuracies were frequently observed in fine structural details, including the placement of loop regions and side-chain rotamers. For example, in the predicted structure of *Kn*ChR, the retinal chromophore is not covalently linked to the Schiff base-forming lysine (K7.50) **(Fig. 2b and Supplementary Fig. 10a**). In *Gt*ACR1, the β-ionone ring of the retinal adopts an incorrectly twisted conformation85 **(Supplementary Fig. 10b**). In Tara-RRB R2, the cytoplasmic ends of TM0, TM1, TM2, and TM7 show marked deviations from the experimentally determined structure **(Fig. 2a and Supplementary Fig. 10c**). In *Hc*KCR2, the architecture of the potassium selectivity filter is incorrectly predicted24 **(Supplementary Fig. 10d**). These issues may become problematic when predicted structures are used e.g. as inputs for molecular dynamics simulations or for interpreting spectroscopic measurements, where accurate loop conformations and side-chain rotamers are critical. Thus, while current structure prediction methods are sufficiently accurate for comparative analyses and for capturing the overall architecture and retinal environment, they do not replace experimental structure determination when precise atomic detail or characterization of conformational dynamics is required.

The GRN-MR system, validated here on 129 structures and approximately 40,000 sequences, provides the microbial rhodopsin field with a common structural language. As the repertoire of rhodopsins continues to expand through metagenomics and structural prediction, we anticipate that it will become a standard tool for comparing residues, interpreting functional data, and guiding optogenetic engineering.

# Methods

## Structural Dataset

We assembled a comprehensive structural dataset of 129 microbial rhodopsins spanning all major functional classes—proton pumps, chloride pumps, sodium pumps, cation channels, anion channels, sensory rhodopsins, and enzyme rhodopsins—with representatives from archaea, bacteria, and eukaryotes. Experimentally determined structures were obtained from the Protein Data Bank, supplemented by eight recently solved channelrhodopsin structures (n = 69). The experimental structures were partitioned into two validation cohorts based on PDB deposition date relative to the Boltz-110.1101/2024.11.19.624167 training data cutoff (September 2021): a benchmark set (Set A, n = 42) potentially overlapping with training data, and a blind test set (Set B, n = 27) comprising structures deposited after the cutoff. For each experimentally characterized protein, we generated Boltz-1 predictions to enable systematic assessment of prediction accuracy. We additionally predicted structures for 60 functionally characterized rhodopsins lacking experimental structures. All analyses were performed on chain A, with retinal chromophores identified by residue name and validated by proximity (≤6.0 Å to protein atoms).

## Structure Prediction and Validation

For Boltz-1 predictions, input sequences were trimmed to transmembrane domain boundaries when exceeding 400 residues. Retinal was specified as all-trans-retinal without the aldehyde oxygen (SMILES code: CC=C(C)C=CC=C(C)C=CC1=C(CCCC1(C)C)C), allowing Boltz-1 to predict the covalent Schiff base linkage to the conserved lysine. Prediction accuracy was assessed by comparing each model to its experimental counterpart using the combinatorial extension alignment algorithm (Cealign)10.1093/protein/11.9.739 (window size = 8, maximum gap = 30) with a two-pass refinement procedure: an initial global alignment using all Cα atoms, followed by refinement restricted to residue pairs with inter-Cα distance ≤3.0 Å after the initial superposition. This excluded flexible regions with large structural divergence but did not excluderesidues outside intrinsically disordered loop and tail regions. Accuracy was quantified using three metrics: backbone Cα RMSD (overall fold accuracy), binding pocket RMSD (Cα atoms within 6.0 Å of retinal, the local accuracy in the chromophore environment), and ligand RMSD (mean closest-atom distance between experimental and predicted retinal coordinates).

## Structure Conservation Analysis

We aligned the shared TM bundle of all members of our dataset. Pairwise structural similarities -calculated using CEalign- were computed as Cα RMSD across TM 1–7 to generate a symmetric distance matrix. Our structure analysis leverages the conserved microbial rhodopsin fold by using the closest residue in each helix (TM1-TM6) and the Schiff-base lysine in TM7 as anchors. We then performed hierarchical clustering. For each GRN position, two distance profiles were calculated: (i) side-chain distances, from heavy atoms to the nearest retinal atom, and (ii) Cα distances, from backbone Cα to the nearest retinal atom. Equivalent residue pairs for all pairwise alignments were stored to create the blueprint of the GRN-MR system.

## Generic Residue-Numbering System

We developed the GRN-MR system by assigning numbers based on structural equivalence relative to a reference structure, 7BMH. This structure was selected based on its small mean RMSD to all other structures, while also not having any high error alignments amongst all structure comparisons. In the GRN-MR system, residues are numbered relative to the helix-specific anchors using the format <Helix>.<Position> (e.g., 3.49, 3.50, 3.51). After annotating the reference structure, we used the stored residue pair equivalences to assign GRNs to all other structures. The resulting preliminary GRN system was curated to correct alignment artifacts, including helix truncations and register shifts arising from the repetitive nature of α-helical structures. Then, the less conserved outer parts of the helices were annotated up until the end of the helices by determining helical residues based on phi-psi angles of the backbone. The non-helical loop regions and tails received systematic identifiers**.**

## Microbial Rhodopsin Sequence Search

To construct Hidden Markov Models (HMMs) for rhodopsin sequences, we searched for archaeal-, eukaryotic-, and viral-derived rhodopsin sequences in NCBI, the UniProt Archive86, and the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP)87. This search yielded 3,173 sequences, designated subAEV. We also identified bacterial-derived rhodopsin sequences (subB; 2,275 sequences) and heliorhodopsin sequences (subH; 720 sequences).

Profile HMMs for subAEV, subB, and subH were constructed by aligning sequences using MAFFT (v7.453) 88 with the "--genafpair" and "--maxiterate 1000" options. Using these rhodopsin HMMs, we searched the UniParc database (as of November 2020) and 10,032 metagenomic samples for rhodopsin genes. Sequences were identified using hmmsearch (HMMER v3.3)89 with an e-value threshold of <1e-5 and a length threshold of ≥174 amino acids corresponding to the HMM length. This yielded 309,570 sequences, which were reduced to 41,136 non-redundant sequences using CD-HIT90 (90% identity threshold).

Structure-based sequence alignment was performed using 10 channelrhodopsin structures determined by cryo-electron microscopy (C1C2, bReaChES, *Co*ChR, ChroME2s, *Cn*ChR2, *Kn*ChR, *Ts*ChR, *Gt*ACR1, *A1*ACR1, and *R2*ACR [manuscript in preparation]) and 60 additional microbial rhodopsins with published structures, totaling 70 structures (Set-1). These were aligned using US-align91 to generate a multiple sequence alignment (MSA).

## Network Analysis Visualization

For protein network analysis, we collected reference rhodopsins (n=147) that were functionally and/or structurally characterized, along with nonredundant sequences from Set-2 rhodopsins. Because enzymatic rhodopsins contain additional domains, the rhodopsin regions of both Set-1 and Set-2 sequences were extracted using hmmsearch (HMMER v3.3)89 with the three HMMs (subAEV, subB, and subH). To remove redundancy within Set-2, protein clustering was performed using MMseqs2 (ver. 15-6f452)92 at 60% identity and 50% coverage, with options “--min-seq-id 0.6 -c 0.5 --cluster-mode 2 --cov-mode 0”. Cluster representatives (n=5,168) were combined with the reference rhodopsins (total n=5,315).

Normalized pairwise similarity was computed using CPAP (https://github.com/yosuken/cpap), which performed all-against-all BLASTp with options “-evalue 1e-2 -dbsize 100000000” and quantified normalized similarity scores between all possible protein pairs based on high-scoring segment pairs (HSPs) with ≥20% identity and ≥20 amino acids in length, as follows:

Let be the normalized similarity score between proteins and .

Let be the length of protein .

Let be the length-normalized bit score (i.e., bit score per position) of the HSP covering position i of protein , where BLASTp is performed using as the query and as the subject. If multiple HSPs cover position i, the maximum value is used.

()

The resulting network was visualized using Gephi (ver. 0.10.1). Edges were drawn between protein pairs with normalized similarity scores ≥ 0.2.

## Sequence Logo Generation

Sequence logos were generated using WebLogo393. Sequences were aligned according to their GRN positions, and logos were computed for each transmembrane helix. For cluster-specific analyses, sequences were grouped based on their assignment in the sequence similarity network, and separate logos were generated for functionally relevant regions (e.g., TM3 and TM7). Amino acid frequencies are displayed as information content in bits.

## Statistical Analysis and Data Availability

Correlations between structural and sequence metrics were computed using Pearson's correlation with two-tailed significance testing. All analyses (structural alignment, superposition) were performed in Python 3.10 using Biopython. Dataset management and CIF parsing were performed using ProtOS (Hidber et al., manuscript in preparation, https://github.com/flurinh/protos). Structure coordinates, alignments, RMSD matrices, GRN tables and all analysis code are provided at <https://github.com/flurinh/mogrn>. The structure analysis data is available at https://doi.org/10.5281/zenodo.18147121.

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# Author Contributions

F.S.H., S.T., K.E.K., X.D., and H.E.K. discussed and defined the GRN-MR numbering system. Under the supervision of X.D., F.S.H. performed the structure comparison analysis and built the GRN-MR framework. Under the supervision of H.E.K., S.T. analyzed sequence alignments and structures. Y.N. and S.Y. mined in-house metagenome and transcriptome databases to identify rhodopsin-like genes. Under the supervision of S.Y. and H.E.K., S.T. and Y.N. constructed and analyzed the sequence similarity network. F.S.H., S.T., X.D., and H.E.K. wrote the manuscript and prepared the figures with input from all authors. X.D. and H.E.K. supervised the project.

# Competing Interests

The authors declare no competing interests.

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