

SUPPLEMENTAL INFORMATION

Reversible lipid mediated pH-gating of connexin-46/50 by cryo-EM

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SUPPLEMENTAL (TABLES, FIGURES AND LEGENDS)

Supplemental Table 1

Data Collection and Processing Information

Collection:	1	1	1	5	6	6	6	2
Condition:	pH 5.8	pH 5.8	pH 5.8	pH 5.8	pH 7.4	pH 7.4	pH 7.4	pH 5.8
State:	Gated	Destabilized -Open	Stable -Open	Asymmetrically Gated	Gated	Destabilized -Open	Stable -Open	Stable -Open
Magnification	29,000	29,000	29,000	105,000	105,000	105,000	105,000	105,000
Voltage (kV)	300	300	300	300	300	300	300	300
Electron Exposure (e-/Å ²)	50	50	50	50	50	50	50	50
Defocus Range (µm)	-0.8 to -2.2	-0.8 to -2.2	-0.8 to -2.2	-0.5 to -1.8	-0.5 to -1.8	-0.5 to -1.8	-0.5 to -1.8	-0.8 to -2.2
Pixel size (Å)	0.8015	0.8015	0.8015	0.8220	0.8220	0.8220	0.8220	0.665
Symmetry imposed	D6	D6	D6	C6	D6	D6	D6	D6
Initial particle images (no.)	2,503,619	2,503,619	2,503,619	6,838,630	4,002,015	4,002,015	4,002,015	502,309
Final particle images (no.)	106,741	218,088	42,656	162,334	15,342	75,136	311,313	127,102
Map resolution (Å)	2.3	2.2	2.5	2.4	2.7	2.2	2.0	2.7
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.1 – 5.4	1.9 – 4.5	2.1 – 5.5	1.8 – 5.5	2.5 – 6.7	1.8 – 5.5	1.8 – 4.0	1.4 – 9.3

Connexin-46: Refinement and validation statistics

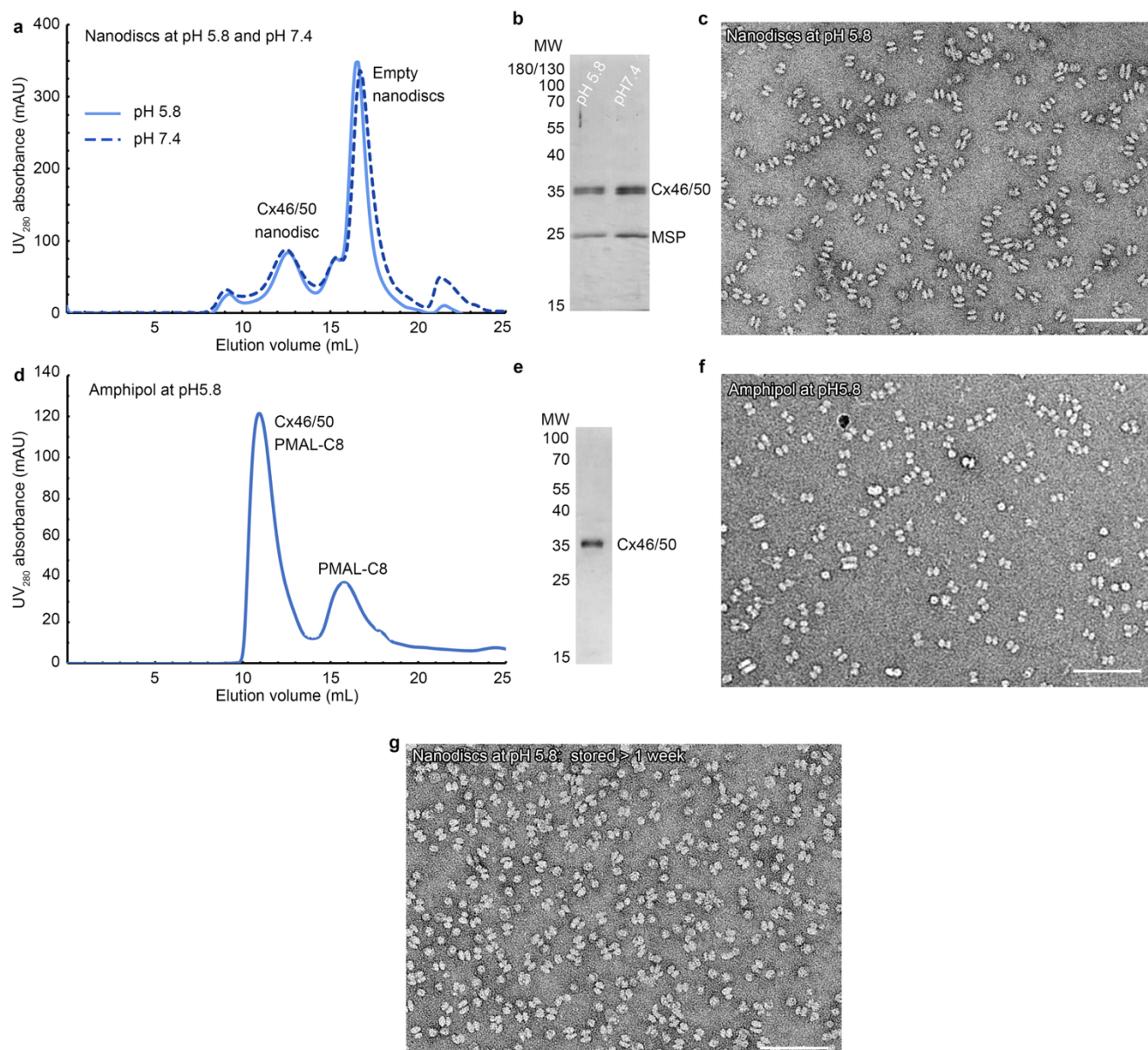
Collection:	1	1	1	5	6	6	6	2
Condition:	pH 5.8	pH 5.8	pH 5.8	pH 5.8	pH 7.4	pH 7.4	pH 7.4	pH 5.8
State:	Gated	Destabilized -Open	Stable -Open	Asymmetrically Gated	Gated	Destabilized -Open	Stable -Open	Stable -Open
Initial model used (PDB code)	7JJC	7JJC	7JJC	7JJC	7JJC	7JJC	7JJC	7JJC
Model resolution (Å)	2.4	2.3	2.5	2.5	2.7	2.2	2.0	3.0
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Model resolution range (Å)	2.2 - 2.4	2.2 - 2.4	2.3 - 2.5	2.2 - 2.5	2.4 - 2.7	1.8 - 2.2	1.8 - 2.0	2.5 - 3.0
Map sharpening B-factor (Å ²)	51.99	93.24	101.26	70.79	43.40	86.94	73.02	129.36
Model composition								
Non-hydrogen atoms	47,508	45,288	42,252	44,016	43,752	45,504	48,432	35,592
Protein residues	2220	2244	2196	2208	2208	2244	2280	2184
Ligands	396	288	180	252	192	276	312	0
B factors (Å ²)								
Protein	51.99	93.24	101.26	70.79	43.43	86.94	73.02	129.36
Ligand	83.90	111.45	114.72	107.74	47.26	104.67	103.67	-
R.M.S. deviations								
Bond lengths (Å)	0.005	0.004	0.005	0.004	0.006	0.004	0.004	0.007
Bond angles (°)	0.960	1.319	1.284	1.336	0.958	1.121	1.434	1.178
Validation								
MolProbity score	0.93	0.84	1.14	1.03	1.05	0.87	1.06	1.30
Clashscore	1.74	1.21	3.47	2.47	2.61	1.36	2.72	5.61
Poor rotamers (%)	0.67	0.60	0.62	0.64	0.67	0.60	0.59	0.62
Ramachandran plot								
Favored (%)	99.45	98.36	98.32	98.70	98.33	98.91	98.39	98.31
Allowed (%)	0.55	1.64	1.68	1.30	1.67	1.09	1.61	1.69
Disallowed (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Connexin-50: Refinement and validation statistics

Collection:	1	1	1	5	6	6	6	2
Condition:	pH 5.8	pH 5.8	pH 5.8	pH 5.8	pH 7.4	pH 7.4	pH 7.4	pH 5.8
State:	Gated	Destabilized -Open	Stable -Open	Asymmetrically Gated	Gated	Destabilized -Open	Stable -Open	Stable -Open
Initial model used (PDB code)	7JJC	7JJC	7JJP	7JJC	7JJC	7JJC	7JJP	7JJP
Model resolution (Å)	2.4	2.3	2.5	2.5	2.7	2.2	2.0	2.9
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Model resolution range (Å)	2.2 - 2.4	2.2 - 2.4	2.3 - 2.5	2.2 - 2.5	2.4 - 2.7	1.8 - 2.2	1.8 - 2.0	2.5 - 2.9
Map sharpening B-factor (Å ²)	54.08	92.73	101.81	64.84	44.84	88.34	71.70	131.24
Model composition								
Non-hydrogen atoms	47,304	45,108	42,612	43,524	40,404	45,264	48,132	35,412
Protein residues	2220	2244	2220	2202	2208	2244	2280	2184
Ligands	396	288	180	252	2192	276	312	0
B factors (Å ²)								
Protein	54.04	92.73	101.81	64.84	44.84	88.34	71.70	131.24
Ligand	88.99	111.45	112.75	105.35	47.26	103.02	101.48	-
R.M.S. deviations								
Bond lengths (Å)	0.014	0.004	0.004	0.005	0.005	0.009	0.006	0.003
Bond angles (°)	1.149	1.342	1.255	1.358	0.596	1.378	1.425	0.558
Validation								
MolProbity score	1.16	0.90	0.99	0.99	1.03	1.12	0.95	1.20
Clashscore	3.69	1.59	2.18	2.19	2.46	3.24	1.88	4.14
Poor rotamers (%)	0.00	0.00	0.60	0.63	0.66	0.59	0.57	0.60
Ramachandran plot								
Favored (%)	100.0	92.73	99.45	98.89	100.0	98.36	98.92	99.44
Allowed (%)	0.00	1.09	0.55	1.11	0.00	1.64	1.08	0.56
Disallowed (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplemental Table 1. Cryo-EM data collection, refinement, and validation statistics.

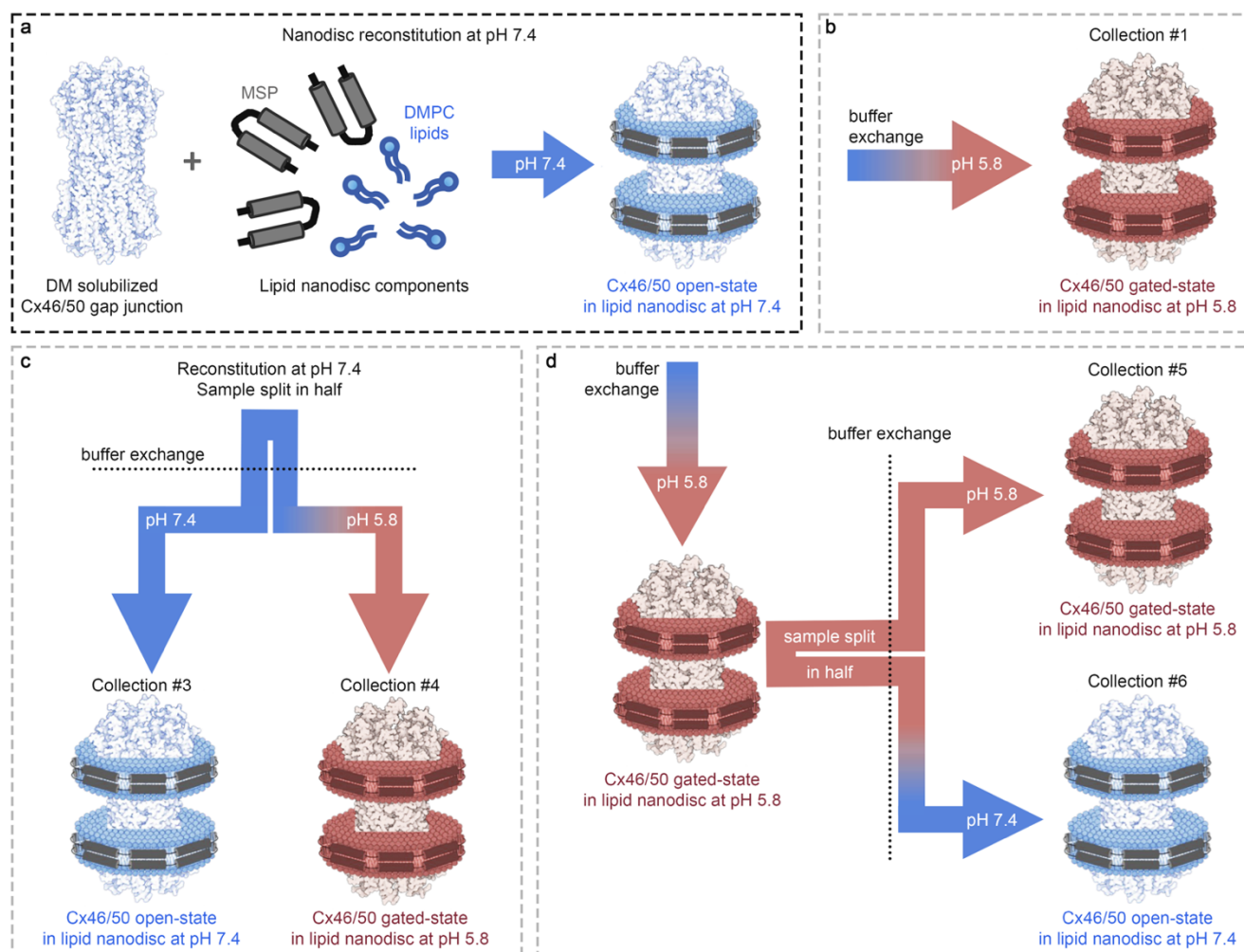
Supplemental Figure 1



Supplemental Figure 1. Purification and reconstitution of connexin-46/50 in nanodiscs and amphipols. **a** Gel filtration chromatography (Superose S6) traces monitored by UV absorbance at 280 nm. Peaks containing native Cx46/50 gap junctions embedded in DMPC lipid nanodiscs (MSP1E1) and empty nanodiscs are labeled. Gel filtration traces at pH 5.8 (light blue) and pH 7.4 (dashed, dark blue) are nearly identical. **b** Silver stained SDS-PAGE of the gel filtration peak containing Cx46/50 embedded in nanodiscs, with molecular markers indicated. MSP1E1 migrates at ~24 kDa (predicted MW ~27.5 kDa), and native Cx46/50 gap junctions from the lens core migrate together as a band at ~38 kDa. **c** Electron micrograph of negatively stained particles from the pH 5.8 gel filtration fraction, containing Cx46/50 in nanodiscs (scale bar = 100 nm). Structures characteristic of Cx46/50 in nanodiscs are observed⁴¹. **d** Gel filtration chromatography (Bio-Rad ENrich SEC 650) trace monitored by UV absorbance at 280 nm. Peaks corresponding to native Cx46/50 reconstituted into amphipol (PMAL-C8) and PMAL-C8 micelles are labeled. **e** Silver stained SDS-PAGE of the gel filtration peak containing Cx46/50 in PMAL-C8 (band at ~38 kDa), with molecular weight markers indicated. **f** Electron micrograph of negatively stained particles from the pH 5.8 gel filtration fraction, showing Cx46/50 in amphipol (scale bar = 100 nm). Particles appear identical to those previously reported for Cx46/50 in amphipol at pH 7.4⁴⁰. **g** Electron micrograph

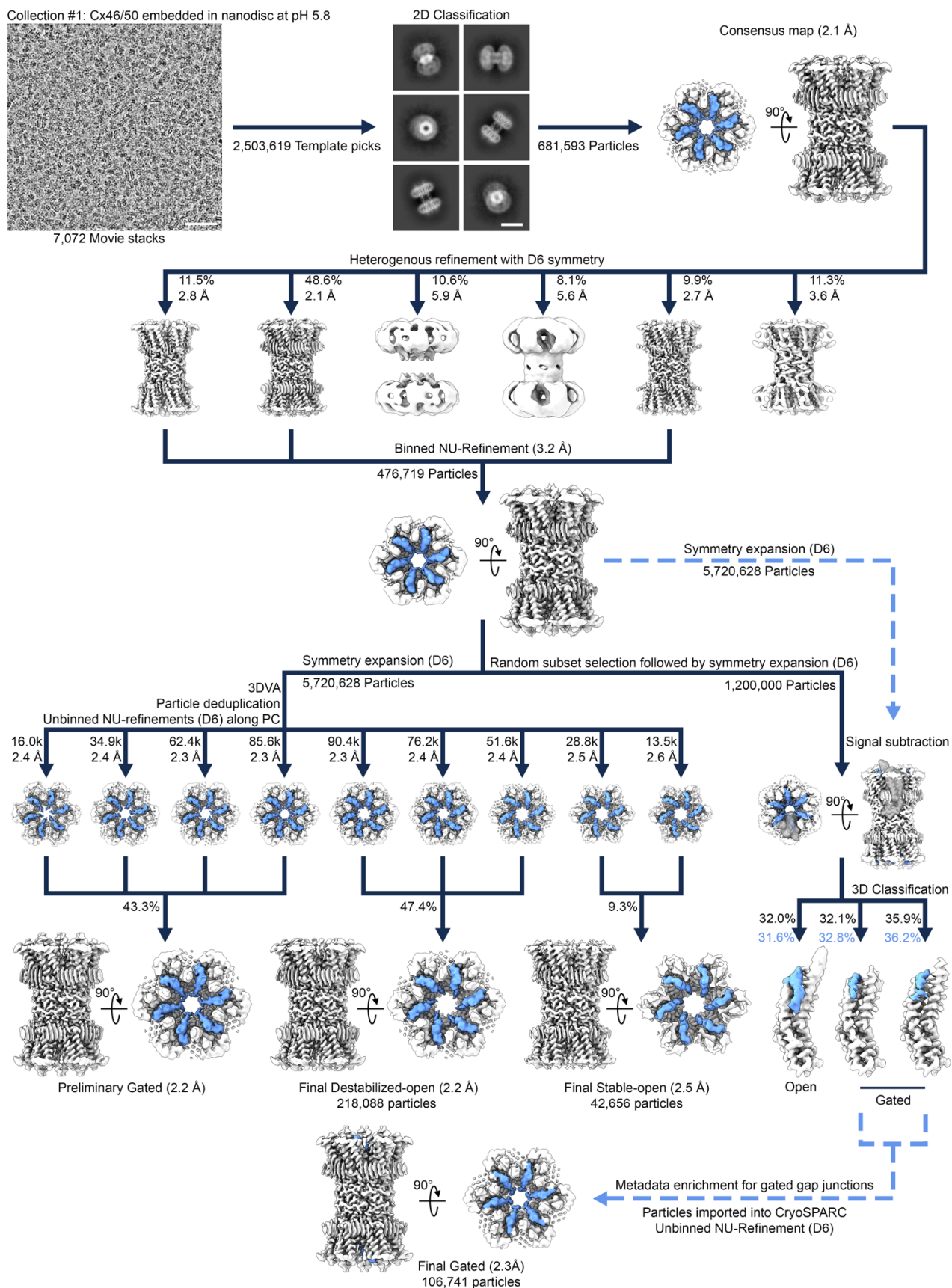
of negatively stained particles from the pH 5.8 gel filtration fraction, containing Cx46/50 in nanodiscs stored at 4°C for over one week prior to imaging (scale bar = 100 nm). Particle morphology under these conditions appears consistent with freshly purified samples (see panel c), indicating that the protein–nanodisc complex remains stable at pH 5.8.

Supplemental Figure 2

**Supplemental Figure 2. Schematic of experimental design for pH- and lipid-dependent structural studies.**

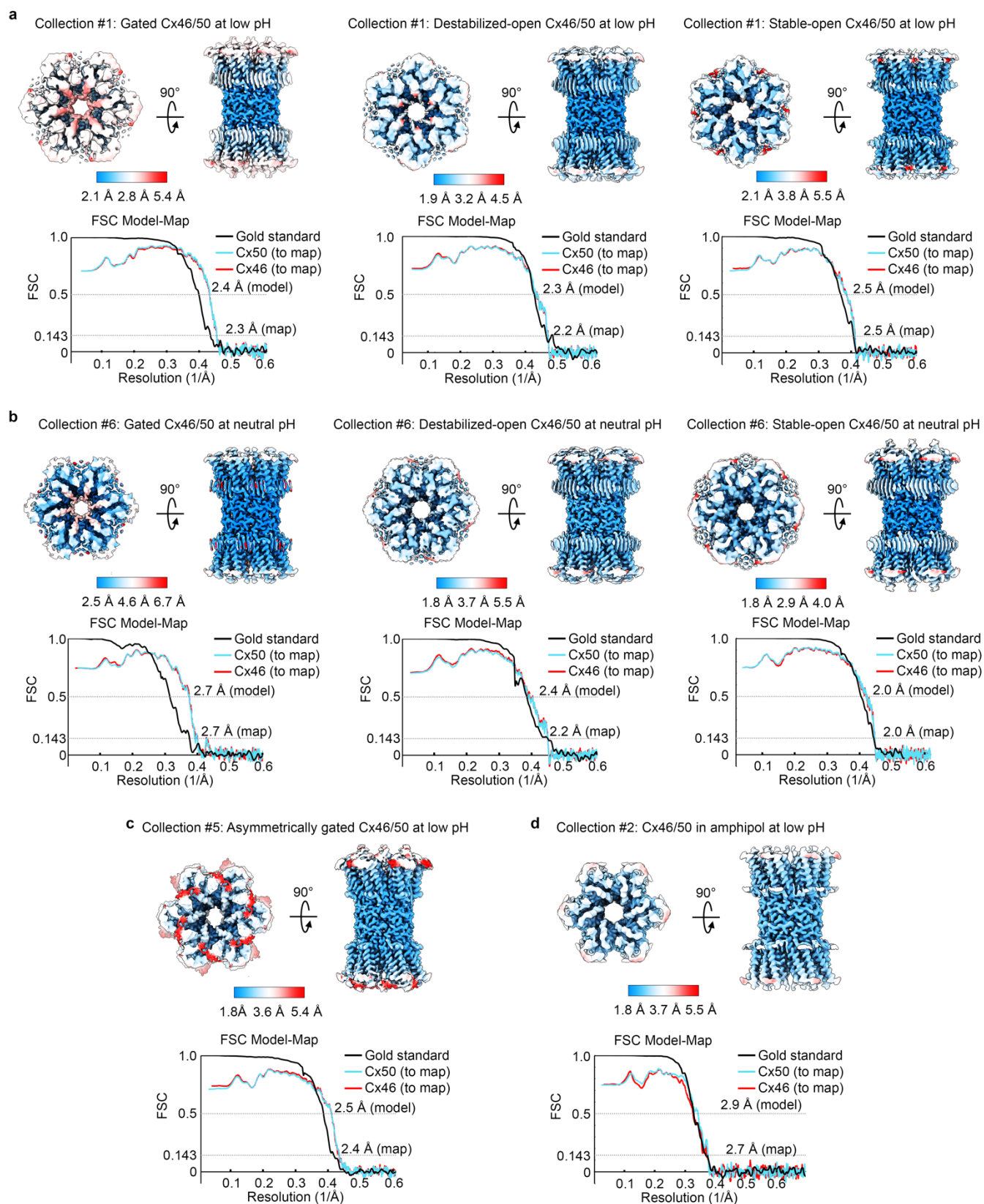
a Native Cx46/50 gap junction channels were reconstituted into MSP1E1 nanodiscs containing DMPC lipids at pH 7.4. **b** For Collection #1, nanodisc-embedded Cx46/50 channels at pH 7.4 (panel a) were buffer exchanged by gel filtration to pH 5.8 and imaged by cryo-EM. **c** After reconstitution into nanodiscs at pH 7.4 (panel a), the sample was split in half and buffer exchanged by gel filtration to pH 7.4 (control, Collection #3) or pH 5.8 (Collection #4), followed by cryo-EM imaging. **d** Nanodisc-embedded Cx46/50 channels reconstituted at pH 7.4 were exchanged to pH 5.8 by gel filtration to induce gating. The sample was then split: one half was dialyzed into the same pH 5.8 buffer (control, Collection #5), while the other half was returned to pH 7.4 by dialysis (Collection #6). Both samples were then imaged by cryo-EM. Not shown: Cx46/50 gap junctions were reconstituted into amphipols at pH 7.4 and subsequently buffer exchanged to pH 5.8 by gel filtration (Collection #2).

Supplemental Figure 3



Supplemental Figure 3. Cryo-EM image processing workflow for the low pH nanodisc dataset (Collection #1). Workflow for the low pH in nanodiscs containing DMPC lipids dataset (Collection #1). Representative cryo-EM micrograph (physical pixel size 0.802 Å²; total dose ~50 e⁻/Å²) and 2D classes are shown with 50 nm and 10 nm scale bars, respectively. The dataset was processed using CryoSPARC, with representative maps shown at various stages of the pipeline. After blob picking and template generation (not shown), particles were selected using template picking followed by iterative 2D classification and particle deduplication. Particles underwent successive non-uniform (NU) refinements (D6 symmetry) and unbinning to yield a high-resolution consensus map. Heterogeneous refinement (D6 symmetry) was used to isolate high-resolution (< 3.0 Å) particles, which were subsequently binned (1.5 Å pixel size) and subjected to NU-refinement (D6 symmetry). To assess particle heterogeneity at the subunit-level, signal subtraction (mask of ROI shown in grey) was performed on a random subset of symmetry expanded (D6) gap junction particles, yielding 1.2M monomer particles for 3D classification. 3D classification revealed primarily gated-state monomers, with a minor population of open-state monomers. To assess particle heterogeneity at the gap junction-level, particles were symmetry expanded (D6) and subjected to 3D variability analysis (3DVA). A mask generated to exclude the nanodisc was used. The primary principal component was divided into nine non-overlapping frames, each locally refined using D6 symmetry. Particles were then deduplicated and subjected to NU-refinement (D6 symmetry). Particles yielding similar 3D reconstructions (classified as gated, destabilized open, and stabilized open-states based on conformation of the NT domain – blue) were pooled and subjected to a final round of NU-refinement with applied D6 symmetry. To further enrich for a more homogeneous gated state gap junction particle set all high-resolution particles were symmetry expanded, signal subtracted and 3D classified at the subunit-level (light blue dotted line). The particle metadata was exported, analyzed and filtered for gap junction particles with ≥10 gated subunits. The enriched gated particle metadata was imported, particles were unbinned and NU-refined using D6 symmetry to achieve the final gated-state map (2.3 Å resolution).

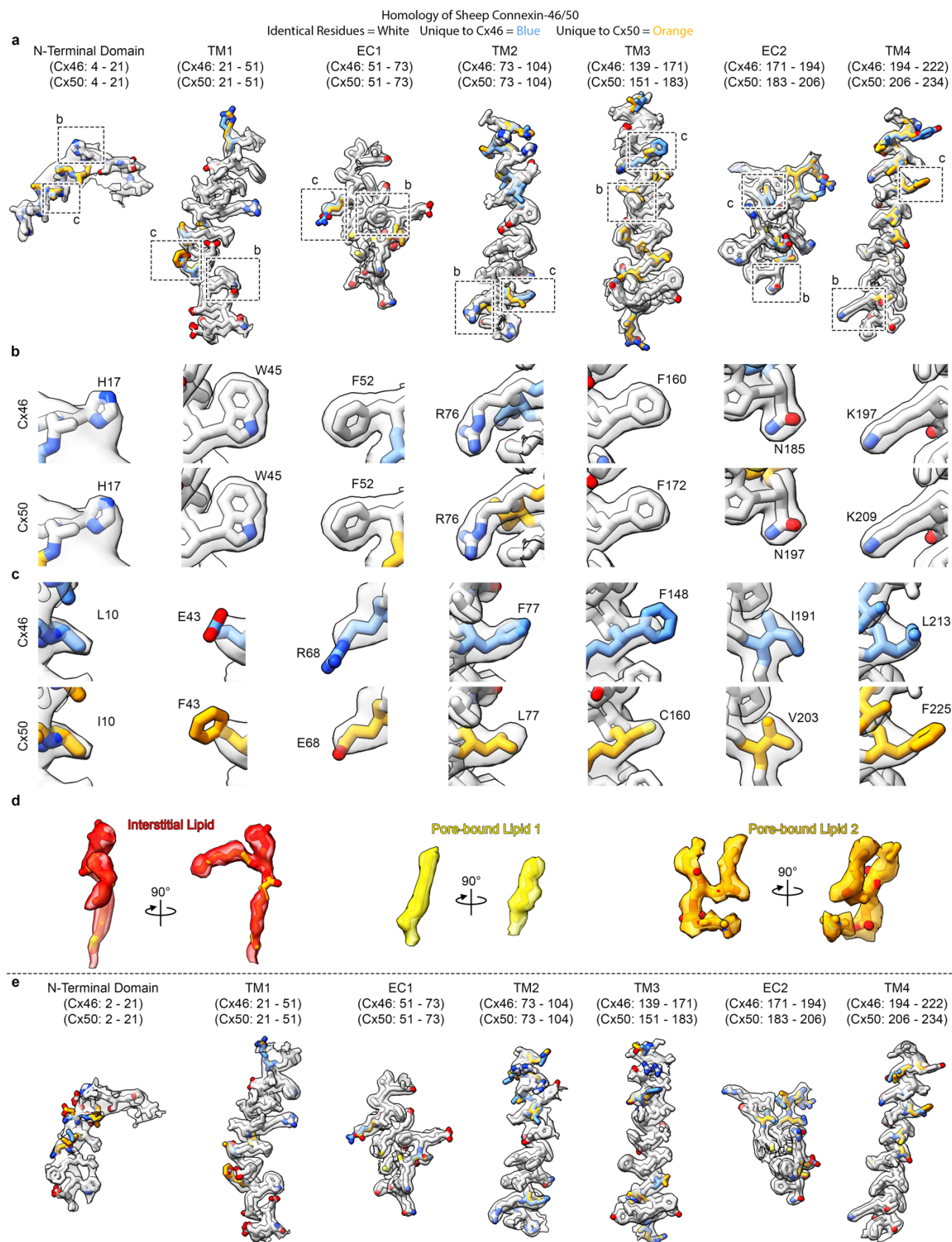
Supplemental Figure 4



Supplemental Figure 4. Resolution assessment of cryo-EM 3D reconstructions. Local resolution analysis of final cryo-EM 3D reconstructions using CryoSPARC, displayed by colored surface. Fourier Shell Correlation (FSC) analysis obtained from final 3D reconstructions. Gold-Standard FSC (black) indicating global resolutions

(0.143 cut-off). FSC curves comparing atomic models for Cx46 (red) and Cx50 (blue) fit to the respective final cryo-EM 3D reconstruction (0.5 cut-off). Resolution assessment is shown for: **a** (Collection #1) *Left*, gated-state; *middle*, destabilized open-state; *right*, stabilized open-state obtained at low pH. **b** (Collection #6) *Left*, gated-state; *middle*, destabilized open-state; *right*, stabilized open-state obtained at neutral pH. **c** (Collection #5) Representative asymmetrically gated-state obtained at low pH. **d** (Collection #2) Stabilized open-state obtained at low pH in amphipol.

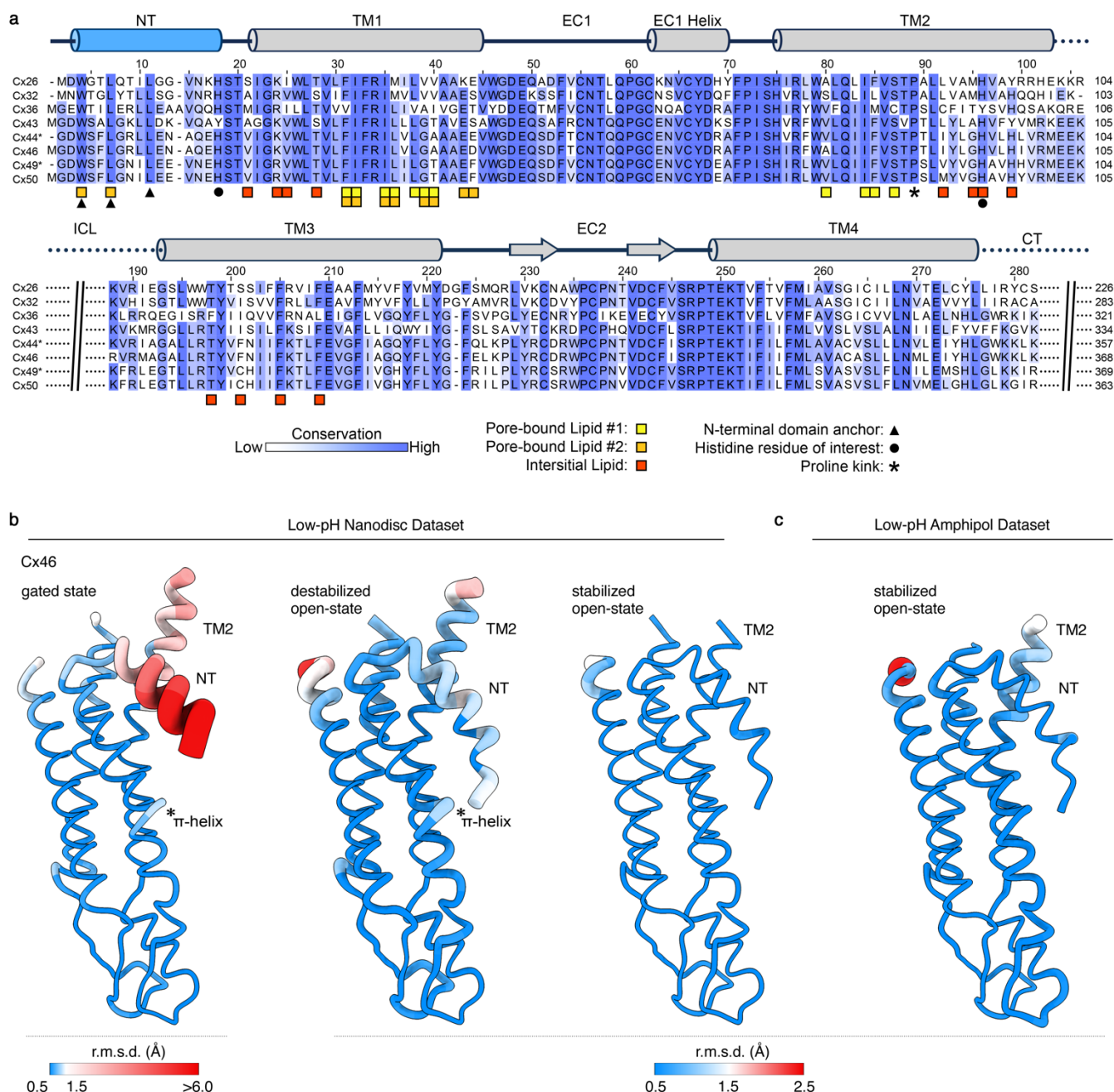
Supplemental Figure 5



Supplemental Figure 5. Fit of atomic models to the cryo-EM density map of the low pH gated-state. a Segmented cryo-EM map for the low pH gated-state with atomic models for sheep connexin-46 (Cx46) and

connexin-50 (Cx50) fit to the experimental densities derived from Collection #1 (2.3 Å, D6-symmetry), including regions for the N-terminal domain, transmembrane domains 1-4 (TM1-4) and extracellular domains 1-2 (EC1-2). Cx46 and Cx50 models are colored according to their pair-wise sequence homology, as being identical (white), or unique (blue for Cx46; orange for Cx50). For the gated-state models of both Cx46 and Cx50, residues 2–3 were not modeled, and the sidechains of residues 4-16 in the NT domain were truncated to the C β position, reflecting limited local resolution in this region. **b,c** Windows show zoom-views corresponding to boxed regions of the segmented maps, highlighting representative sidechain densities and fit to the atomic models. Regions of identical amino acids (panel b) are fit equally well by both models, whereas regions where the sequence of Cx46 and Cx50 differ (panel c), sidechain density is typically weaker or show features due to imposed averaging of two different sidechains in these areas (or relative flexibility of solvent exposed sidechains). **d** Segmented cryo-EM map for the interstitial lipid (red) and pore-bound lipids – PL1 (yellow) and PL2 (orange). **e** Segmented cryo-EM map for the low pH destabilized open-state with atomic models for sheep Cx46 and Cx50 fit to the experimental densities derived from Collection #1 (2.2 Å, D6-symmetry), including regions for the N-terminal domain, TM1-4, and EC1-2, colored as in panel a.

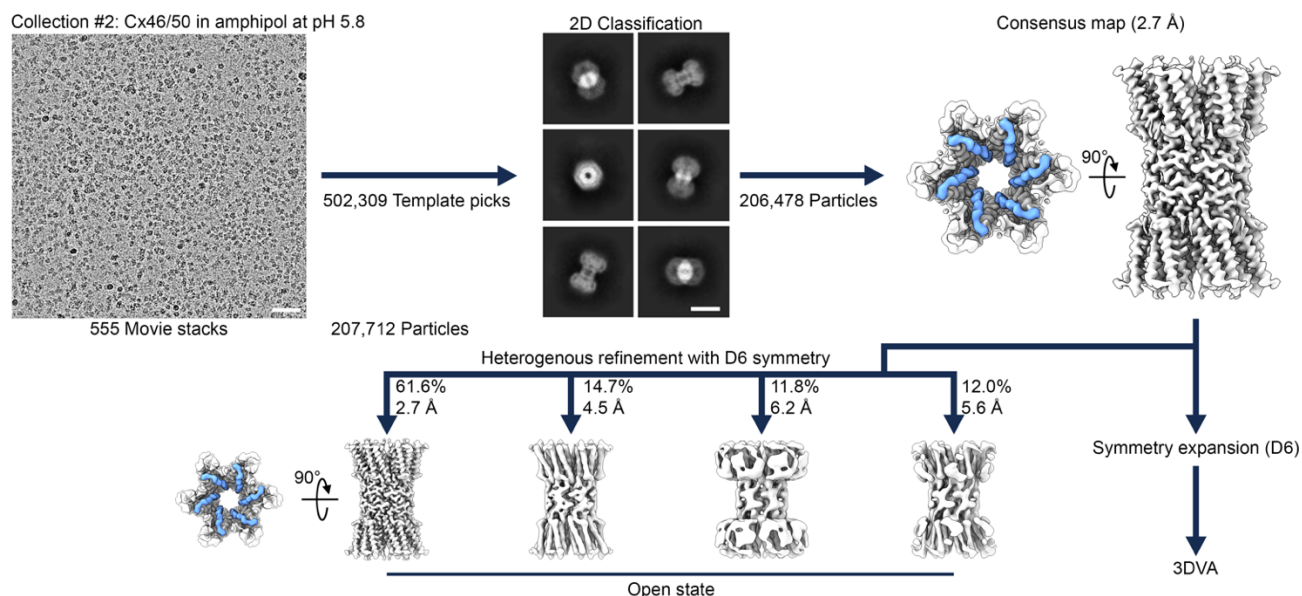
Supplemental Figure 6



Supplemental Figure 6. Annotated sequence and structural alignments. **a** Sequence alignment and secondary structure topology of representative human and ovine (Cx44 and Cx49) connexin isoforms, colored by conservation. Conserved hydrophobic residues involved in N-terminal domain anchoring of the stabilized open-state of Cx46/50 are marked with triangles. Conserved histidine residues highlighted in this study (H17 and H95, Cx46/50 numbering) are indicated with circles. The conserved proline kink on TM2 is marked with an asterisk. Residues interacting with pore-bound lipids (PL1 and PL2) are annotated with yellow and orange squares, respectively. Residues interacting with the interstitial lipid (IL) are marked with red squares. **b** Structural alignment and C α root mean square deviation (r.m.s.d.) analysis of Cx46 in a lipid nanodisc environment obtained in the low pH gated (left) destabilized open (center) and stabilized open (right) states versus the stabilized open state obtained at neutral pH (7JKC). **c** Structural alignment and C α r.m.s.d. analysis of Cx46 in an amphipol environment obtained at low pH versus the stabilized open state obtained at neutral pH (7JKC). For panels b and c, r.m.s.d. values are visualized by color gradient (blue to red, indicating low to high values) and

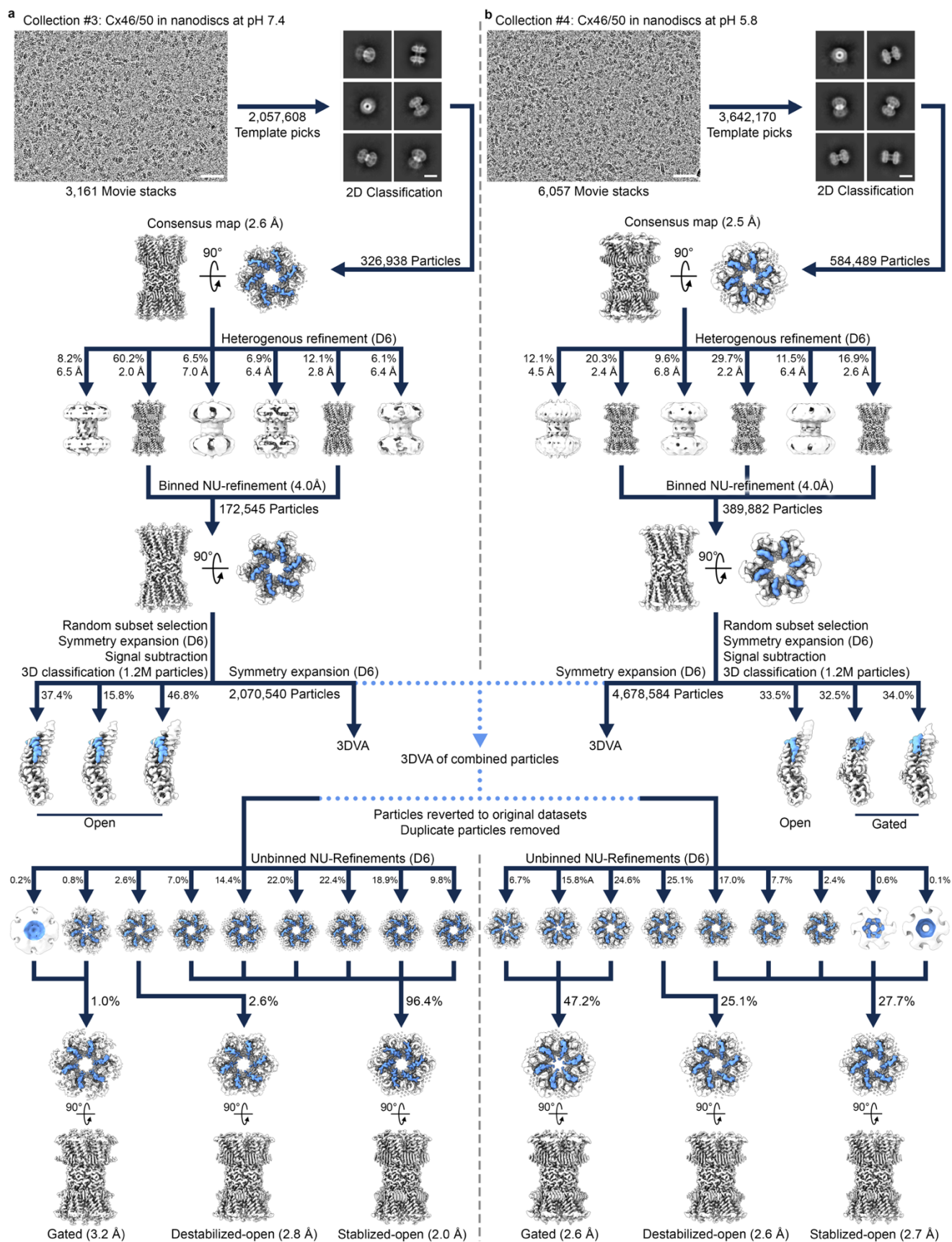
by the thickness of the backbone in 'worm' representation produced in ChimeraX. NT and TM2 domains are labeled. A key for r.m.s.d. values for each set of comparisons is displayed. Asterisk indicates region of minor variability around a π -helix that forms a kink in TM1 (residues ~39-41) observed in the gated and destabilized open-states.

Supplemental Figure 7



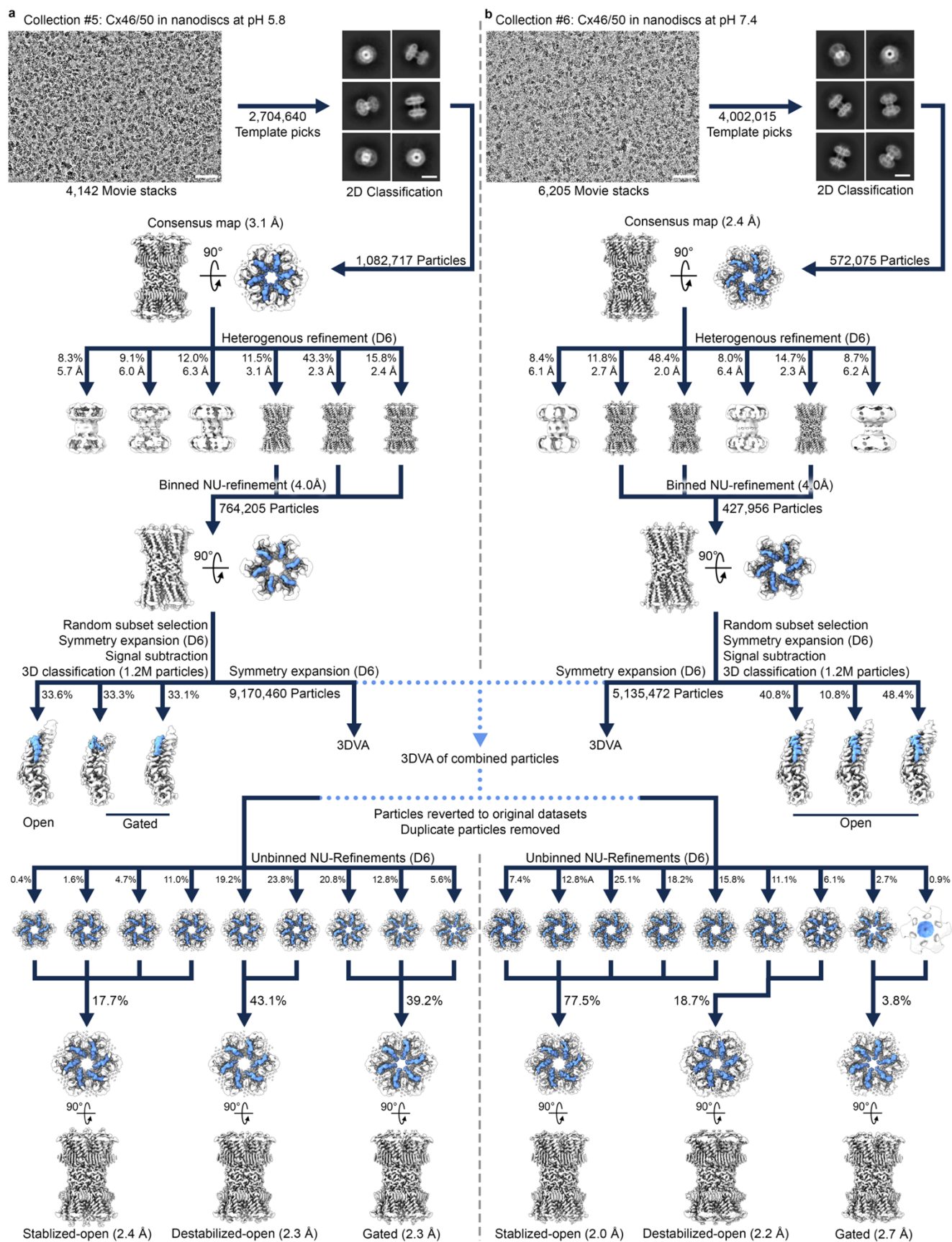
Supplemental Figure 7. Cryo-EM image processing workflow for the low pH amphipol dataset (Collection #2). Workflow for the low pH in amphipol dataset (Collection #2). Representative cryo-EM micrograph (physical pixel size $0.665 \text{ \AA}^2$; total dose $\sim 80 \text{ e}^-/\text{\AA}^2$) and 2D classes are shown with 50 nm and 10 nm scale bars, respectively. The dataset was processed using CryoSPARC, with representative maps shown at various stages of the pipeline. After blob picking and template generation (not shown), particles were selected using template picking followed by iterative 2D classification and particle deduplication. Particles underwent successive non-uniform (NU) refinements (D6 symmetry) and unbinning to yield a high-resolution consensus map. Heterogenous refinement (D6 symmetry) was used to isolate high-resolution ($< 3.0 \text{ \AA}$) particles. This resulted in one class representing the stable-open state. To assess particle heterogeneity at the gap junction-level, particles from the consensus 3D reconstruction were symmetry expanded (D6) and subjected to 3D variability analysis (3DVA). A mask generated to exclude the detergent micelle was applied.

Supplemental Figure 8



Supplemental Figure 8. Cryo-EM image processing workflows for the split neutral (Collection #3) and low pH (Collection #4) datasets. **a** Workflow for the neutral pH dataset (Collection #3). Representative cryo-EM micrograph (physical pixel size 0.788 Å²; total dose ~50 e⁻/Å²) and 2D classes are shown with 50 nm and 10 nm scale bars, respectively. **b** Workflow for the low pH dataset (Collection #4), following the same general procedure as the neutral pH dataset. Representative cryo-EM micrographs and 2D classes are shown at equivalent scale. Both datasets were processed using CryoSPARC, with representative maps shown at various stages of the pipeline. After blob picking, and template generation (not shown), particles were selected using template picking followed by iterative 2D classification and particle deduplication. Particles underwent successive non-uniform (NU) refinements (D6 symmetry) and unbinning to yield a high-resolution consensus map. Heterogeneous refinement (D6 symmetry) was used to isolate high-resolution (< 3.0 Å) particles, which were subsequently binned (2.0 Å pixel size) and subjected to NU-refinement (D6 symmetry). To assess particle heterogeneity at the subunit-level, signal subtraction was performed on a random subset of symmetry expanded (D6) particles, yielding 1.2M monomer particles for signal subtraction and 3D classification. For the neutral pH dataset (Collection #3), 3D classification only revealed open-state monomers. For the low pH dataset (Collection #4), 3D classification revealed primarily gated-state monomers, with a minor population of open-state monomers. To assess particle heterogeneity at the gap junction-level, particles were symmetry expanded (D6) and subjected to 3D variability analysis (3DVA). A mask generated to exclude the nanodisc was used for both datasets independently and on the combined datasets. The primary principal component for the combined datasets was divided into nine non-overlapping frames, each locally refined using D6 symmetry. Particles were then reverted to their original datasets, deduplicated, and subjected to NU-refinement (D6 symmetry). Particles yielding similar 3D reconstructions (classified as gated, destabilized open, and stabilized open-states based on conformation of the NT domain – blue) were pooled and subjected to a final round of NU-refinement with applied D6 symmetry.

Supplemental Figure 9



Supplemental Figure 9. Cryo-EM image processing workflow for the reversibility of lipid-mediated pH gating low pH (Collection #5) and neutral pH (Collection #6) datasets. **a** Workflow for the low pH dataset (Collection #5). Representative cryo-EM micrograph (physical pixel size 0.822 Å²; total dose ~50 e⁻/Å²) and 2D classes are shown with 50 nm and 10 nm scale bars, respectively. **b** Workflow for the neutral pH dataset (Collection #6), following the same general workflow as the low pH dataset. Both datasets were processed using CryoSPARC, with representative maps shown at various stages of the pipeline. After blob picking and template generation (not shown), particles were selected using template picking followed by iterative 2D classification and particle deduplication. Particles underwent successive non-uniform (NU) refinements (D6 symmetry) and unbinning to yield a high-resolution consensus map. Heterogeneous refinement (D6 symmetry) was used to isolate high-resolution (< 3.0 Å) particles, which were subsequently binned (2.0 Å pixel size) and subjected to NU-refinement (D6 symmetry). To assess particle heterogeneity at the subunit-level, signal subtraction was performed on a random subset of symmetry expanded (D6) particles, yielding 1.2M monomer particles for signal subtraction and 3D classification. For the neutral pH dataset (Collection #6), 3D classification only revealed open-state monomers. For the low pH dataset (Collection #5), 3D classification revealed primarily gated-state monomers, with a minor population of open-state monomers. To assess particle heterogeneity at the gap junction-level, particles were symmetry expanded (D6) and subjected to 3D variability analysis (3DVA). A mask generated to exclude the nanodisc was used for both datasets independently and on the combined datasets. The primary principal component for the combined datasets was divided into nine non-overlapping frames, each locally refined using D6 symmetry. Particles were then reverted to their original datasets, deduplicated, and subjected to NU-refinement (D6 symmetry). Particles yielding similar 3D reconstructions (classified as gated, destabilized open, and stabilized open-states based on conformation of the NT domain – blue) were pooled and subjected to a final round of NU-refinement with applied D6 symmetry.