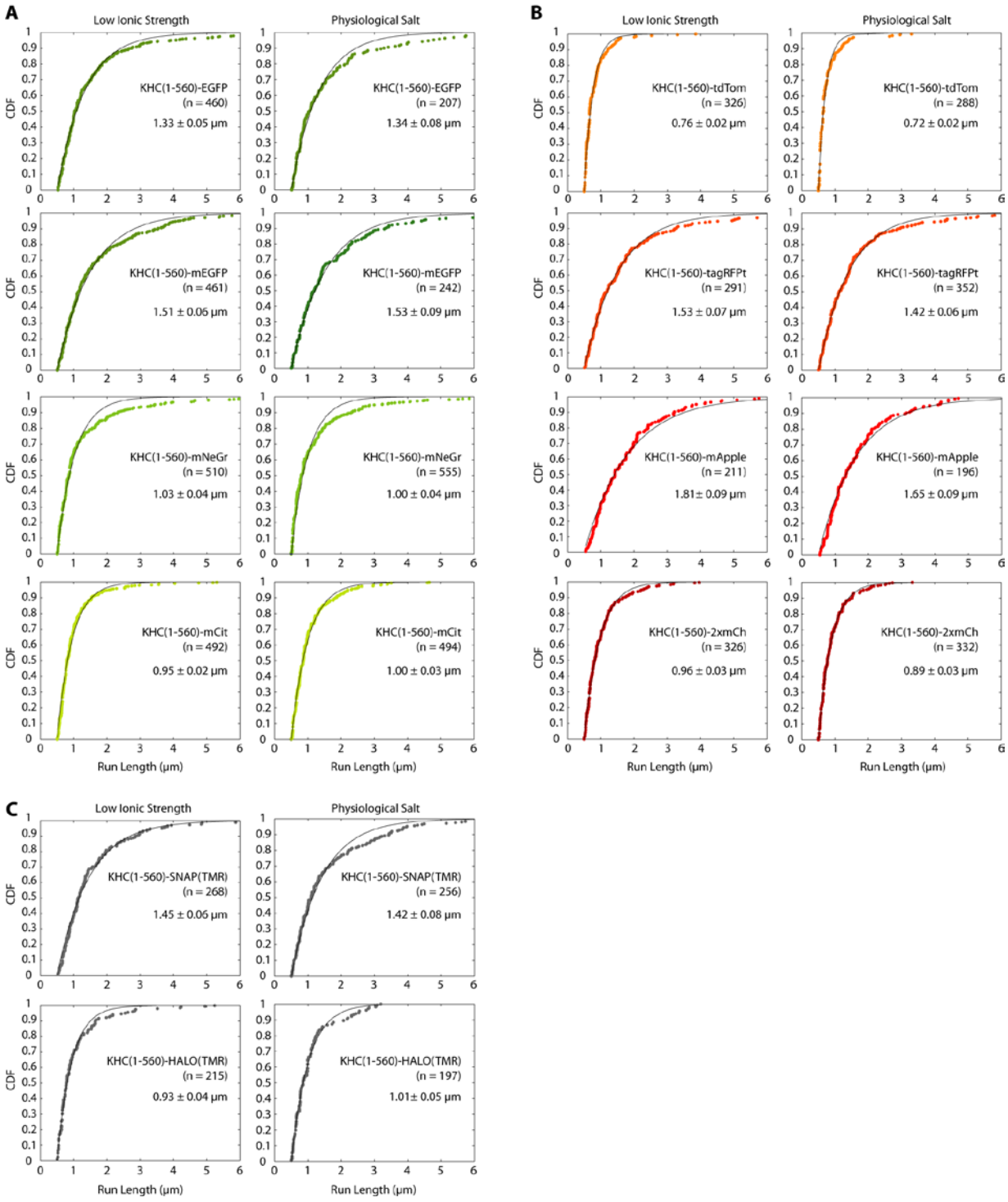
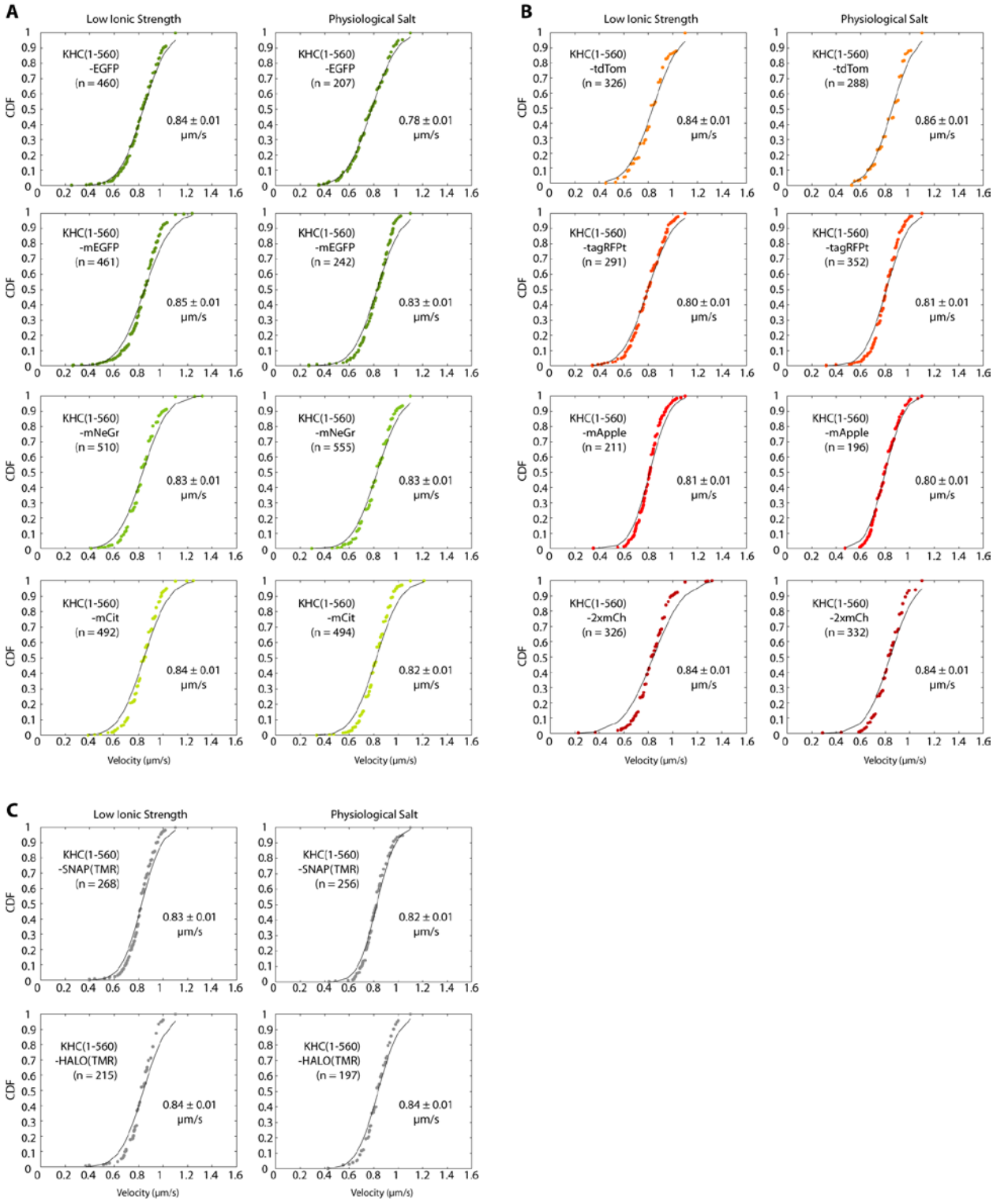
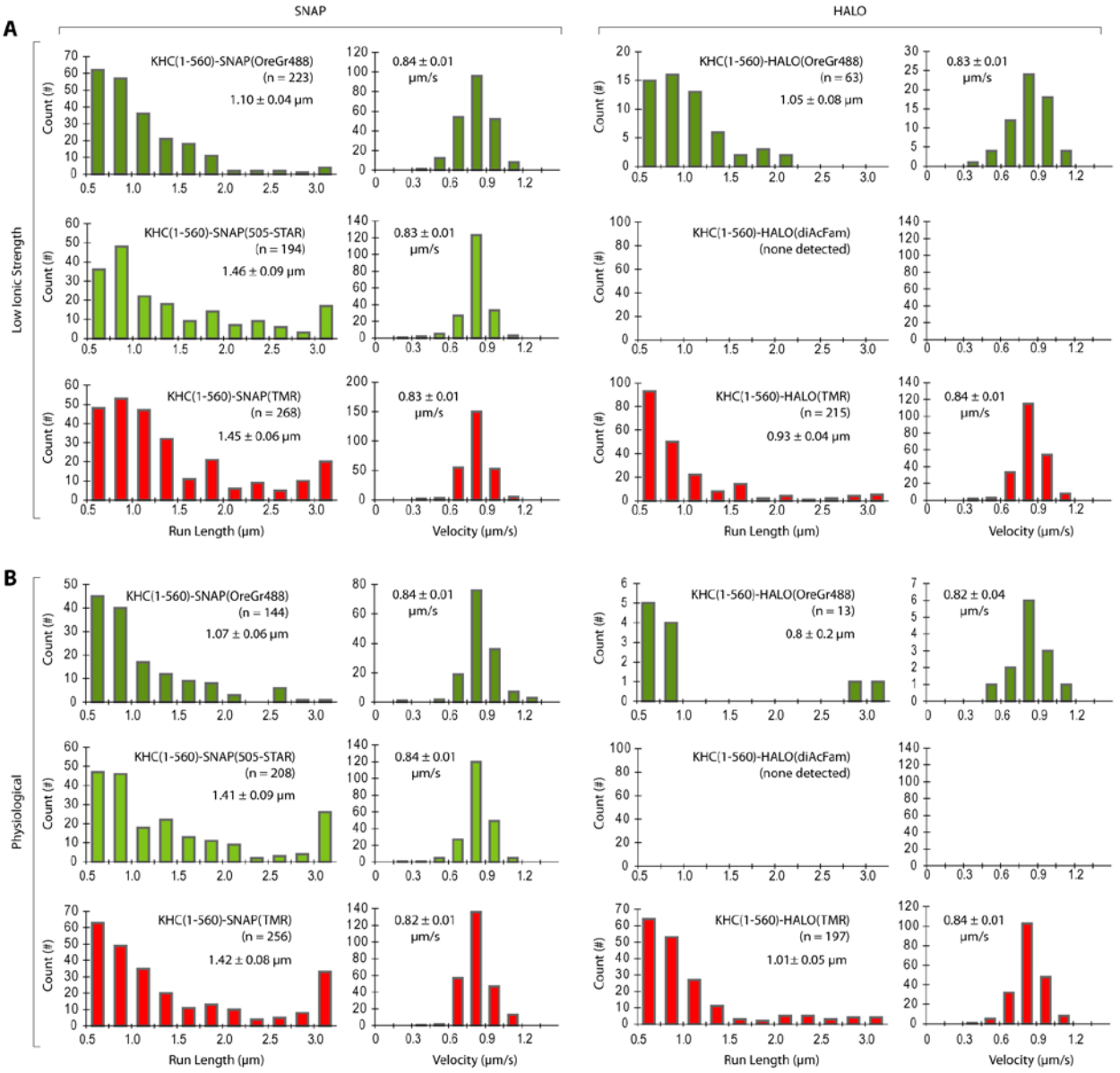


Supplementary Figure S1. Cumulative Distribution Functions (CDFs) for Run Length Distributions. **(A-C)** Run length CDFs for green FP-labeled KHC(1-560) motors **(A)**, red FP-labeled KHC(1-560) motors **(B)**, and SNAP-TMR-labeled KHC(1-560) motors **(C)**. A least-squares fit was performed in MATLAB (overlaid as a black dotted line) to determine mean run lengths (inset). Data are presented as the mean ± the standard deviation from bootstrapping.



Supplementary Figure S2. Cumulative Distribution Functions (CDFs) for Velocity Distributions. **(A-C)** Velocity CDFs for green FP-labeled KHC(1-560) motors **(A)**, red FP-labeled KHC(1-560) motors **(B)**, and SNAP-TMR-labeled KHC(1-560) motors **(C)**. A least-squares fit was performed in MATLAB (overlaid as a black dotted line) to determine mean run lengths (inset). Data are presented as the mean ± the standard deviation from bootstrapping. Gaps in velocity CDFs represent duplicate velocity data points, which are a result of pixel-limited kymograph analysis.



Supplementary Figure S3. Characterization of different SNAP and HALO ligands. **(A-B)** Motility properties for various SNAP (left) and HALO (right) ligands were compared in standard P12 motility buffer **(A)** and under physiological buffer conditions **(B)**. The mean run length and velocity values (insets) were obtained by CDF fit (see Supp. Fig. S1-S2). Error is reported as the standard deviation from bootstrapping. SNAP- and HALO-Oregon Green ligands were barely detectable at 488 nm excitation, likely leading to shorter run lengths than other SNAP-ligands, and HALO-diAcFAM was not detectable at 488 nm. SNAP-505-STAR, SNAP-TMR, and HALO-TMR ligands were readily detectable at 488, 561, and 561 nm excitation, respectively

Supplemental Table S1. Compiled data for fluorescently-tagged KHC(1-560) motors.

FP (dye)	Ionic Strength*	Velocity (µm/s) ± Bootstrap S.E.	RL (µm) ± Bootstrap S.E.	n (events)	Landing rate [events/(µm*s*nM)] ± S.E.M.	Motor oligomeric state from photo-bleaching
EGFP	Low	0.84 ± 0.01	1.33 ± 0.05	460	0.35 ± 0.03	Mostly dimer
EGFP	Phys.	0.78 ± 0.01	1.34 ± 0.08	207	0.26 ± 0.02	
mEGFP	Low	0.85 ± 0.01	1.51 ± 0.06	461	0.43 ± 0.03	Dimer + Tetramer
mEGFP	Phys.	0.83 ± 0.01	1.53 ± 0.09	242	0.30± 0.03	
mNeGr	Low	0.83 ± 0.01	1.03 ± 0.04	510	0.51 ± 0.03	Dimer
mNeGr	Phys.	0.83 ± 0.01	1.00 ± 0.04	555	0.58 ± 0.05	
mCit	Low	0.84 ± 0.01	0.95 ± 0.02	492	0.48 ± 0.03	Dimer
mCit	Phys.	0.82 ± 0.01	1.00 ± 0.03	494	0.44 ± 0.04	
tdTom	Low	0.84 ± 0.01	0.76 ± 0.02	326	0.31 ± 0.03	Dimer
tdTom	Phys.	0.86 ± 0.01	0.72 ± 0.02	288	0.31 ± 0.05	
tagRFPt	Low	0.80 ± 0.01	1.53 ± 0.07	291	0.33 ± 0.03	Dimer + Tetramer
tagRFPt	Phys.	0.81 ± 0.01	1.42 ± 0.06	352	0.30 ± 0.02	
mApple	Low	0.81 ± 0.01	1.81 ± 0.09	211	0.18 ± 0.01	
mApple	Phys.	0.80 ± 0.01	1.65 ± 0.09	196	0.21 ± 0.05	
2xmCh	Low	0.84 ± 0.01	0.96 ± 0.03	326	0.33 ± 0.03	Dimer
2xmCh	Phys.	0.84 ± 0.01	0.89 ± 0.03	332	0.27 ± 0.02	
SNAP (OreGr488)	Low	0.84 ± 0.01	1.10 ± 0.04	223	0.18 ± 0.01	
SNAP (OreGr488)	Phys.	0.84 ± 0.01	1.07 ± 0.06	144	0.12 ± 0.02	
SNAP (505-STAR)	Low	0.83 ± 0.01	1.46 ± 0.09	194	0.14 ± 0.02	
SNAP (505-STAR)	Phys.	0.84 ± 0.01	1.41 ± 0.09	208	0.16 ± 0.03	
SNAP (TMR)	Low	0.83 ± 0.01	1.45 ± 0.06	268	0.33 ± 0.04	
SNAP (TMR)	Phys.	0.82 ± 0.01	1.42 ± 0.08	256	0.32 ± 0.06	
HALO (OreGr488)	Low	0.83 ± 0.01	1.05 ± 0.08	63	0.024 ± 0.005	
HALO (OreGr488)	Phys.	0.82 ± 0.04	0.8 ± 0.2	13	0.005 ± 0.002	
HALO (TMR)	Low	0.84 ± 0.01	0.93 ± 0.04	215	0.25 ± 0.03	
HALO (TMR)	Phys.	0.84 ± 0.01	1.01 ± 0.05	197	0.20 ± 0.02	

*Low ionic strength (I.S.) buffer: 12 mM PIPES/KOH, 1 mM EGTA, and 2 mM $MgCl_2$, pH 6.8, I.S. = 28 mM. Physiological (Phys.) ionic strength buffer: 25 mM HEPES/KOH, 115 mM potassium acetate, 5 mM sodium acetate, 5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4, I.S. = 145 mM.

Supplemental Table S2. Statistical significance of differences in velocity between fluorescently-tagged KHC(1-560) motors in low ionic strength buffer[a].

FP	EGFP	mEGFP	mNG	mCit	tdTom	tagRFPt	mApple	2xmCh	SNAP-OreGr	SNAP-505STAR	SNAP-TMR	HALO-OreGr	HALO-TMR
EGFP													
mEGFP	n.s.												
mNG	n.s.	n.s.											
mCit	n.s.	n.s.	n.s.										
tdTom	n.s.	n.s.	n.s.	n.s.									
tagRFPt	***	***	***	***	***								
mApple	*	***	*	***	*	n.s.							
2xmCh	n.s.	n.s.	n.s.	n.s.	n.s.	***	*						
SNAP-OreGr	n.s.	*	n.s.	*	*	n.s.	n.s.	n.s.					
SNAP-505STAR	n.s.	*	n.s.	*	*	*	n.s.	n.s.	n.s.				
SNAP-TMR	n.s.	*	n.s.	*	n.s.	*	n.s.	n.s.	n.s.	n.s.			
HALO-OreGr	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
HALO-TMR	n.s.	n.s.	n.s.	n.s.	n.s.	***	*	n.s.	n.s.	n.s.	n.s.	n.s.	

[a]Low ionic strength (I.S.) buffer: 12 mM PIPES/KOH, 1 mM EGTA, and 2 mM $MgCl_2$, pH 6.8. Mann-Whitney-Wilcoxon test was used to compare each velocity data set, where each indicated data set on the vertical axis is compared to the indicated data set on the horizontal axis. *** denotes $p < 0.001$, * denotes $p < 0.01$, n.s. denotes not significant ($p > 0.01$).

Supplemental Table S3. Statistical significance of differences in run length between fluorescently-tagged KHC(1-560) motors in low ionic strength buffer[a].

FP	EGFP	mEGFP	mNG	mCit	tdTom	tagRFPt	mApple	2xmCh	SNAP-OreGr	SNAP-505STAR	SNAP-TMR	HALO-OreGr	HALO-TMR
EGFP													
mEGFP	n.s.												
mNG	***	***											
mCit	***	***	*										
tdTom	***	***	***	***									
tagRFPt	n.s.	n.s.	***	***	***								
mApple	***	***	***	***	***	***							
2xmCh	***	***	*	n.s.	***	***	***						
SNAP-OreGr	*	*	***	***	***	*	***	***					
SNAP-505STAR	n.s.	n.s.	***	***	***	n.s.	***	***	***				
SNAP-TMR	*	n.s.	***	***	***	n.s.	***	***	***	n.s.			
HALO-OreGr	*	*	*	n.s.	***	*	***	n.s.	n.s.	***	***		
HALO-TMR	***	***	*	n.s.	***	***	***	n.s.	***	***	***	n.s.	

[a]Low ionic strength (I.S.) buffer: 12 mM PIPES/KOH, 1 mM EGTA, and 2 mM $MgCl_2$, pH 6.8. Mann-Whitney-Wilcoxon test was used to compare each run length data set, where each indicated data set on the vertical axis is compared to the indicated data set on the horizontal axis. *** denotes $p < 0.001$, * denotes $p < 0.01$, n.s. denotes not significant ($p > 0.01$).

Supplemental Table S4. Statistical significance of differences in velocity between fluorescently-tagged KHC(1-560) motors in physiological buffer[a].

FP	EGFP	mEGFP	mNG	mCit	tdTom	tagRFPt	mApple	2xmCh	SNAP-OreGr	SNAP-505STAR	SNAP-TMR	HALO-OreGr	HALO-TMR
EGFP													
mEGFP	*												
mNG	***	n.s.											
mCit	*	n.s.	n.s.										
tdTom	***	*	***	***									
tagRFPt	*	*	*	n.s.	***								
mApple	n.s.	*	*	*	***	n.s.							
2xmCh	***	n.s.	n.s.	*	*	***	***						
SNAP-OreGr	***	n.s.	n.s.	*	n.s.	*	***	n.s.					
SNAP-505STAR	***	n.s.	n.s.	n.s.	*	*	***	n.s.	n.s.				
SNAP-TMR	*	n.s.	n.s.	n.s.	***	n.s.	*	n.s.	n.s.	n.s.			
HALO-OreGr	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
HALO-TMR	***	n.s.	n.s.	n.s.	*	*	***	n.s.	n.s.	n.s.	n.s.	n.s.	

[a]Physiological buffer: 25 mM HEPES/KOH, 115 mM potassium acetate, 5 mM sodium acetate, 5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4, I.S. = 145 mM. Mann-Whitney-Wilcoxon test was used to compare each velocity data set, where each indicated data set on the vertical axis is compared to the indicated data set on the horizontal axis. *** denotes $p < 0.001$, * denotes $p < 0.01$, n.s. denotes not significant ($p > 0.01$).

Supplemental Table S5. Statistical significance of differences in run length between fluorescently-tagged KHC(1-560) motors in physiological buffer[a].

FP	EGFP	mEGFP	mNG	mCit	tdTom	tagRFPt	mApple	2xmCh	SNAP-OreGr	SNAP-505STAR	SNAP-TMR	HALO-OreGr	HALO-TMR
EGFP													
mEGFP	*												
mNG	***	***											
mCit	***	***	*										
tdTom	***	***	***	***									
tagRFPt	n.s.	n.s.	***	***	***								
mApple	***	n.s.	***	***	***	*							
2xmCh	***	***	n.s.	*	***	***	***						
SNAP-OreGr	n.s.	***	***	*	***	*	***	***					
SNAP-505STAR	n.s.	n.s.	***	***	***	n.s.	*	***	*				
SNAP-TMR	n.s.	n.s.	***	***	***	n.s.	*	***	***	n.s.			
HALO-OreGr	n.s.	*	n.s.	n.s.	*	*	*	n.s.	n.s.	*	*		
HALO-TMR	***	***	*	n.s.	***	***	***	*	n.s.	***	***	n.s.	

[a]Physiological buffer: 25 mM HEPES/KOH, 115 mM potassium acetate, 5 mM sodium acetate, 5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4, I.S. = 145 mM. Mann-Whitney-Wilcoxon test was used to compare each run length data set, where each indicated data set on the vertical axis is compared to the indicated data set on the horizontal axis. *** denotes $p < 0.001$, * denotes $p < 0.01$, n.s. denotes not significant ($p > 0.01$).

Movie S1. Representative movies of kinesin-1 motors labeled with green fluorescent proteins. Lysates of COS7 cells expressing KHC(1-560) labeled with green fluorescent proteins in standard P12 motility buffer were imaged by TIRF microscopy at room temperature (Ti-E/B; Nikon). Frames were acquired continuously with 100 ms exposure. Top panel, KHC(1-560) labeled with EGFP; second panel, KHC(1-560) labeled with monomeric EGFP; third panel, KHC(1-560) labeled with mNeGr; bottom panel, KHC(1-560) labeled with mCit. Scale bar = 5 μm.

Movie S2. Representative movies of kinesin-1 motors labeled with red fluorescent proteins. Lysates of COS7 cells expressing KHC(1-560) labeled with red fluorescent proteins in standard P12 motility buffer were imaged by TIRF microscopy at room temperature (Ti-E/B; Nikon). Frames were acquired continuously with 100 ms exposure. Top panel, KHC(1-560) labeled with tdTomato; second panel, KHC(1-560) labeled with tagRFPt; third panel, KHC(1-560) labeled with mApple; bottom panel, KHC(1-560) labeled with 2xmCh. Scale bar = 5 μm.

Movie S3. Representative movies of SNAP-tagged kinesin-1 motors labeled with various SNAP ligands. Lysates of COS7 cells expressing ligand-labeled KHC(1-560)-SNAP in standard P12 motility buffer were imaged by TIRF microscopy at room temperature (Ti-E/B; Nikon). Frames were acquired continuously with 100 ms exposure. Top panel, KHC(1-560)-SNAP labeled with SNAP-OreGr488; middle panel, KHC(1-560)-SNAP labeled with SNAP-505-STAR; bottom panel, KHC(1-560)-SNAP labeled with SNAP-TMR. Scale bar = 5 μm.

Movie S4. Representative movies of HALO-tagged kinesin-1 motors labeled with various HALO ligands. Lysates of COS7 cells expressing ligand-labeled KHC(1-560)-HALO in standard P12 motility buffer were imaged by TIRF microscopy at room temperature (Ti-E/B; Nikon). Frames were acquired continuously with 100 ms exposure. Top panel, KHC(1-560)-HALO labeled with HALO-OreGr488; middle panel, KHC(1-560)-HALO labeled with HALO-diAcFAM; bottom panel, KHC(1-560)-HALO labeled with HALO-TMR. Scale bar = 5 μm.