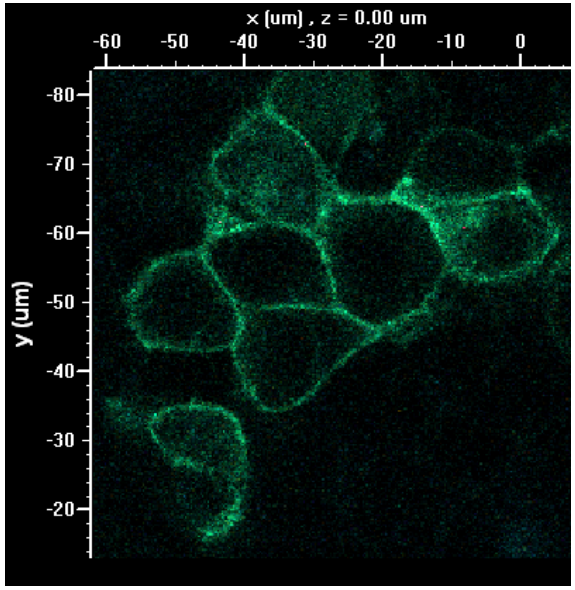
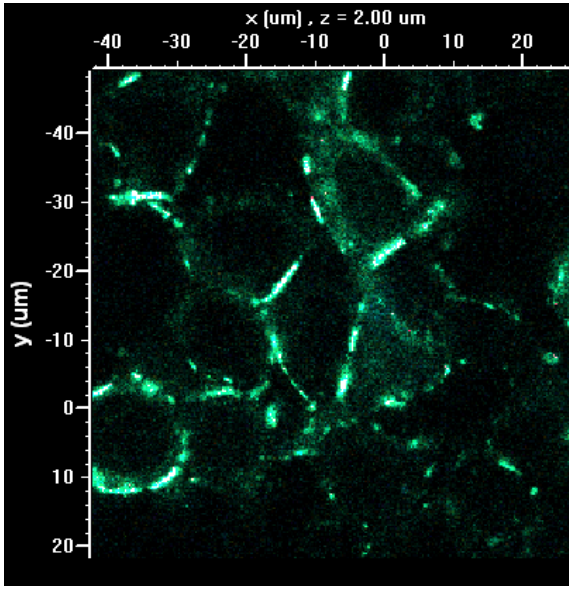
A

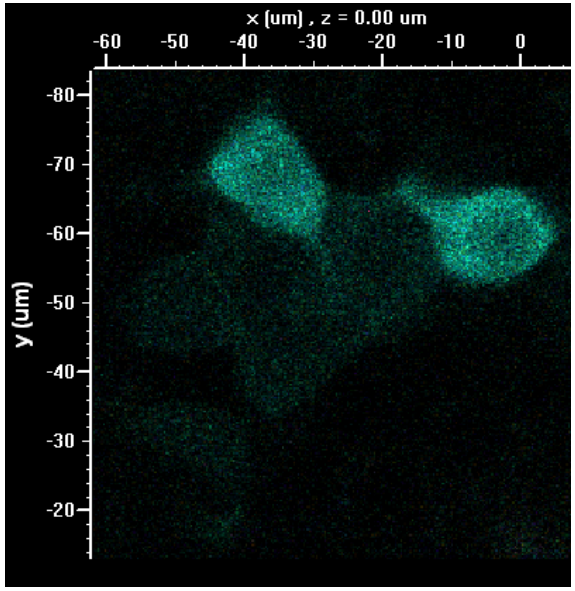
HN GFP - In the presence of zanamivir



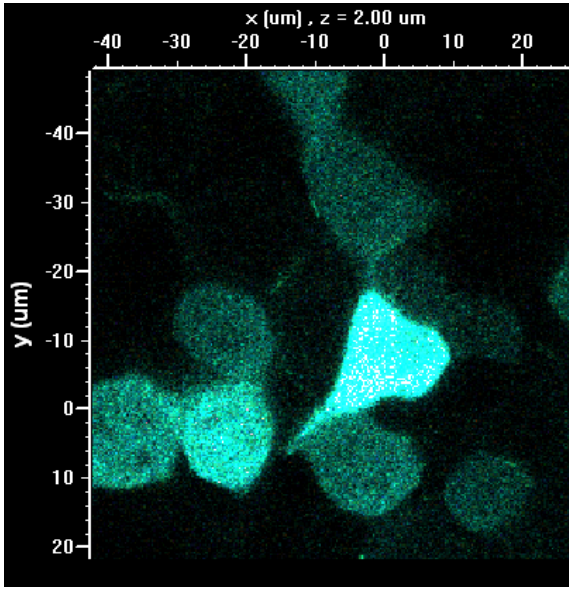
HN GFP - In the absence of zanamivir



B

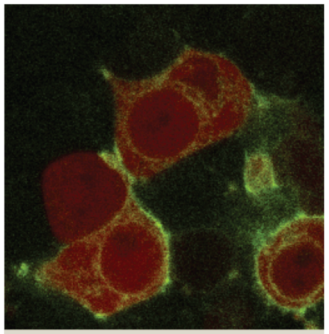


F CFP - In the presence of zanamivir

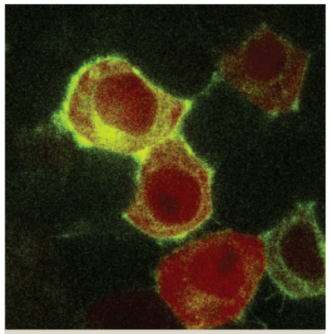


F CFP - In the absence of zanamivir

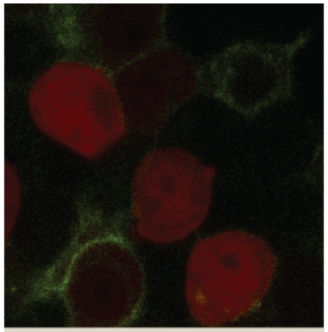
A



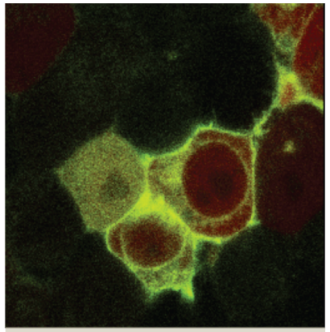
BiFC: WT HN-F



BiFC: H552Q HN -F

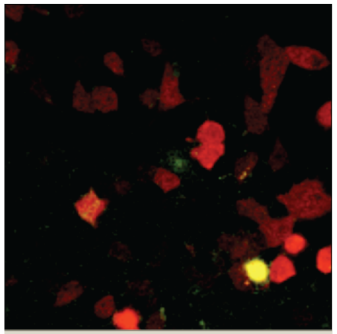


BiFC: WT HN-F
Cold competition: H552Q HN

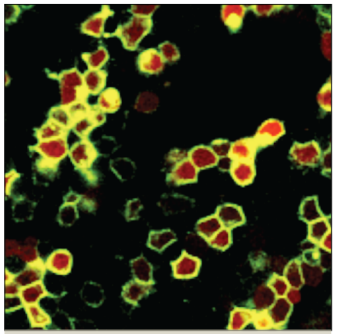


BiFC: H552Q HN -F
Cold competition: WT HN

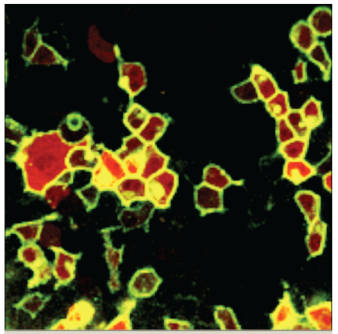
B



BiFC: HA - F



BiFC: HA - HA



BiFC: HA - HA
Cold competition: H552Q HN

Supplemental Figure Legends

Figure S1, related to Figure 3. **HN, alone, forms clusters upon receptor interaction.** (A) HN EGFP was transiently expressed in 293T cells, and localization was visualized using a confocal laser scanning microscope equipped with a spectral detector. The images show fluorescence (green) before receptor engagement (in the presence of 10mM zanamivir) and after receptor engagement (without zanamivir). Images were acquired using EZ-C1 acquisition and analysis software.
(B) F CFP was transiently expressed in 293T cells, and localization was visualized using a confocal laser scanning microscope equipped with a spectral detector. There is diffuse, evenly distributed fluorescence across the cell surfaces both with and without zanamivir.

Figure S2, related to Figure 4. **HN H552Q shows enhanced interaction with HPIV3 F but does not interfere with influenza HA oligomerization.**
(A) Magnified representative plots of the results shown in Figure 4D.
(B) 293T cells co-transfected with (i) HA N-Venus, F C-CFP and pCAGGS; (ii) HA N-Venus, F C-CFP and pCAGGS; (iii) HA wt N-Venus, HA C-Venus and HN H552Q pCAGGS were treated overnight with 10mM Zanamivir, and one hour prior to analysis fresh 10mM Zanamivir and cycloheximide were added. Representative images are provided.

Movie S1 for Figure 3 – HA oligomerizes on the cell surface prior to receptor engagement.
Influenza HA N-Venus and HA C-Venus constructs were transiently expressed in 293T cells, and visualized using a confocal laser scanning microscope equipped with a spectral detector. The Z series projection shows the localization of oligomerized HA on the cell surface (green) and the RFP internal reference (red) in the presence of cycloheximide and 10mM zanamivir to block receptor engagement. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Movie S2 for Figure 3 – Influenza HA and HPIV3 F do not interact. Influenza HA N-Venus and HPIV3 F C-CFP constructs were transiently expressed in 293T cells, and visualized using a confocal laser scanning microscope equipped with a spectral detector. The Z series projection shows an absence of fluorescence on the cell surface (green) and the RFP internal reference (red) in the presence of cycloheximide and 10mM zanamivir to block receptor engagement. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Movie S3 for Figure 3 – Influenza HA and HPIV3 HN do not interact. HPIV3 HN N-Venus and Influenza HA C-Venus constructs were transiently expressed in 293T cells, and visualized using a confocal laser scanning microscope equipped with a spectral detector. The Z series projection shows an absence of fluorescence on the cell surface (green) and the RFP internal reference (red) in the presence of cycloheximide and 10mM zanamivir to block receptor engagement. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Movie S4 for Figure 4 – HPIV3 HN and HPIV3 F interact prior to receptor engagement. HPIV3 HN N-Venus and HPIV3 F C-CFP constructs were transiently expressed in 293T cells, and visualized using a confocal laser scanning microscope equipped with a spectral detector. The Z series projection shows fluorescence on the cell surface (green) with RFP internal reference (red) in the presence of cycloheximide and 10mM zanamivir to block receptor engagement. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Movie S5 for Figure 4 – HPIV3 HN and HPIV3 F cluster after receptor engagement. HPIV3 HN N-Venus and HPIV3 F C-CFP constructs were transiently expressed in 293T cells, and visualized using a confocal laser scanning microscope equipped with a spectral detector. The Z series projection shows fluorescence on the cell surface (green) with RFP internal reference (red) in the presence of cycloheximide and 10uM HRC peptide, which allows receptor engagement but blocks the F protein's progression towards fusion. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Movie S6 for Figure 4 – HPIV3 HN and HPIV3 F are together prior to and during fusion, but seem to separate after fusion is accomplished. HPIV3 HN N-Venus and HPIV3 F C-CFP constructs were transiently expressed in 293T cells, and their localization was visualized using a confocal laser scanning microscope equipped with a spectral detector. The time lapse movie follows fluorescence for 90 minutes after receptor engagement is allowed (by the removal of zanamivir). The movie shows fluorescence on the cell surface (green) which clusters after receptor engagement, then once the fusion event has occurred – visualized by the RFP internal reference (red) - the green fluorescence decreases. Images were acquired as a Z stack (0.1 μm slices) and assembled as a projection using EZ-C1 acquisition and analysis software.

Table S1 for Figure 4 - Values calculated on entire field as shown in movie S6.

Time in min.	Mean fluorimetric ratio
0	1.79
10	1.84
20	2.08
30	2.11
40	2.09
50	1.84
60	1.76
70	1.69
80	1.55
90	1.45