

1 **Relevance of host cell surface glycan structure for cell specificity of influenza A virus**

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3 Markus Kastner^{1,*}, Andreas Karner^{1,2,*}, Rong Zhu^{1,*}, Qiang Huang³, Dandan Zhang⁴, Jianping
4 Liu³, Andreas Geissner^{5,6}, Anne Sadewasser⁷, Markus Lesch⁸, Xenia Wörmann⁸, Alexander
5 Karlas⁸, Peter Seeberger^{5,6}, Thorsten Wolff⁷, Peter Hinterdorfer^{1,2,§}, Andreas Herrmann^{9,§} and
6 Christian Sieben^{9,10,§}

7

8 ¹ *Institute for Biophysics, Johannes Kepler University Linz, 4020 Linz, Austria*

9 ² *Center for Advanced Bioanalysis GmbH, A-4020 Linz, Austria*

10 ³ *State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai
11 200433, China*

12 ⁴ *Shanghai Supercomputer Center, Shanghai 201203, China*

13 ⁵ *Department for Biomolecular Systems, Max Planck Institute for Colloids and Interfaces, Potsdam,
14 Germany*

15 ⁶ *Institute of Chemistry and Biochemistry, Free University, Berlin, Germany*

16 ⁷ *Div. of Influenza and other Respiratory Viruses, Robert Koch-Institute, 13353 Berlin, Germany*

17 ⁸ *Molecular Biology Department, Max Planck Institute for Infection Biology, 10117 Berlin, Germany*

18 ⁹ *Department of Biology, Molecular Biophysics, Humboldt Universität zu Berlin, 10115 Berlin,
19 Germany*

20 ¹⁰ *Current address: Laboratory for Experimental Biophysics, School of Basic Sciences, École
21 Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland*

22

23

24 * These authors contributed equally to this work

25 § Corresponding authors:

26 christian.sieben@epfl.ch (C.S.)

27 andreas.herrmann@rz.hu-berlin.de (A.H.)

28 peter.hinterdorfer@jku.at (P. H.)

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35 **Abstract**

36 Influenza A viruses (IAV) initiate infection *via* binding of the viral hemagglutinin (HA) to
37 sialylated glycan receptors on host cells. HAs receptor specificity towards sialic acid (SA) is
38 well studied and clearly critical for virus infection, but the contribution of the highly complex
39 cellular plasma membrane to the cellular specificity remains elusive. In addition, some
40 studies indicated that other host cell factors such as the epidermal growth factor receptor
41 might contribute to the initial virus-cell contact and further downstream signaling¹.

42 Here we use two complementary methods, glycan arrays and single-virus force spectroscopy
43 (SVFS) to compare influenza virus receptor specificity with actual host cell binding.
44 Unexpectedly, our study reveals that HAs receptor binding preference does not necessarily
45 reflect virus-cell specificity. We propose SVFS as a tool to elucidate the cell binding
46 preference of IAV thereby including the complex environment of sialylated receptors within
47 the plasma membrane of living cells.

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59 **1. Introduction**

60 Influenza A viruses circulate in aquatic birds, their large natural host reservoir, but have also
61 established stable lineages in various mammalian species such as pigs. Although animal
62 influenza viruses are usually confined to their natural host species, they can cause zoonotic
63 infections in humans on rare occasions². Such trans-species transmissions can result in
64 clinically severe or even fatal respiratory disease in humans as illustrated by the outbreaks of
65 avian-origin H7N9 subtype viruses in China occurring since 2013³. Zoonotic transmission
66 events can, in fact, largely influence the epidemiology of human influenza directly if the virus
67 succeeds to spread among humans as was observed in 2009 for the pandemic swine-origin
68 H1N1 strain (pdmH1N1)⁴. Although the genetic requirements for crossing the species barrier
69 are still incompletely understood, it is accepted that interspecies transmission of influenza A
70 viruses partially depends on the capability of viral hemagglutinin (HA) to recognize specific
71 sialylated-glycan receptors on the host cell surface. In general, HA of avian viruses
72 preferentially binds to α -2,3-linked sialic acid (SA) (avian-type receptor)⁵ whereas HA of
73 human-adapted strains strongly bind to terminal α -2,6-linked SA (human-type receptor)⁵.
74 Several studies have determined that alterations in HA receptor binding specificity are a
75 crucial step in host adaptation and interspecies transmission for several IAV subtypes^{6, 7, 8}.
76 However, it is not well established if those adaptive mutations (1) provide an actual
77 advantage in virus-cell binding during entry, or (2) whether they are necessary to confer
78 transmission (i.e. by evading decoy receptors lining the human airway mucus) or (3) to avoid
79 triggering of innate immune signaling. Regarding the first point, several studies suggest a
80 much higher complexity of virus-cell interaction beyond the level of HA-SA binding (for a

81 review see⁹). Consequently, it was hypothesized that human influenza viruses bind to a more
82 structurally diverse set of SA linked carbohydrates than avian viruses which goes beyond the
83 general preference of α -2,3 or α -2,6 linkage.

84 Currently, glycan arrays with libraries of synthesized glycan structures are widely utilized for
85 the characterization of IAV glycan specificity. In particular, due to direct exposure of
86 receptors on the array, sialic acid specificity can be studied with high precision on structural
87 glycan properties. However, the cellular glycome has been recently studied for human and
88 swine respiratory tract tissue, showing that its complexity might not be well represented by
89 current glycan arrays^{13, 14}. Indeed, influenza virus infection in the absence of sialic acid
90 suggests other possible attachment factors involved in virus binding. Candidates molecules
91 are C-type lectins (L-SIGN and DC-SIGN), which were found to participate in influenza
92 virus attachment independent of SA specificity¹². Hence, complementary approaches to
93 directly assess viral receptor specificity within the complex environment of the cell surface
94 are necessary to reach a more comprehensive understanding of the initial stage of virus
95 infection. As we have recently shown, atomic force microscopy (AFM)-based single-virus
96 force spectroscopy (SVFS) allows to measure the binding of individual IAV to living host
97 cells at the molecular level^{15, 16, 17}. In this type of analysis, intact influenza viruses are
98 covalently attached to AFM cantilevers, which are then lowered on single living cells (Fig. 1).

99 Cycles between cantilever-cell approach, cell binding and cantilever retraction, allow direct
100 characterization of virus cell binding, while revealing kinetic and thermodynamic properties
101 of the interactions^{15, 16}. Thus, SVFS allows to investigate virus-cell binding in an
102 experimental system that closely mimics the natural situation.

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104 Here, by using a set of five different influenza A virus strains, we systematically address
105 whether virus-cell specific binding patterns as determined by SVFS are reflected in their
106 receptor specificity observed by glycan array analysis. Our data indicate that results obtained
107 from *in vitro* glycan arrays may not be directly transferred to virus-cell binding. We suggest
108 that host cell specificity does not solely depend on the sialic acid configuration of the cell
109 surface, but is more complex and depends on the specific environment of the receptor and
110 possibly involves additional attachment factors or co-receptors with yet unknown functional
111 roles.

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113 **2. Results**

114 **Receptor specificity of influenza A virus strains studied by glycan arrays.** To
115 investigate and compare the SA receptor specificity of different virus strains, we performed
116 an *in vitro* glycan array study utilizing a library of 15 glycans (Fig. 2). Regarding specific
117 IAV receptors, our library included three α -2,3-linked (avian-type) SA conjugates as well as
118 three α -2,6-linked (human-type) SA conjugates. The glycan number nine, sialyl-Lewis^X
119 (SLe^X) has due to its fucosylation a different topology and was, although α -2,3-linked to SA,
120 separated from the two groups shown on the left side in Fig. 2.

121 We found that the zoonotic AH1 strain and the pandemic H1N1 virus recognized all six SA
122 conjugates with a preference for α -2,6-linked (human-type) receptors. Such a dual-binding
123 behavior was already observed before for pdmH1N1 (A/California/04/2009 and
124 A/Hamburg/5/2009). The receptor binding preference of the human H7N9 isolate

125 A/Anhui/1/2013 (AH1) is still under debate. It was shown that AH1 exhibits increased human
126 receptor binding while still preferring avian receptors ²⁰, others reported on the specificity for
127 human-type receptors ²¹. Interestingly, when we looked at the cumulative difference between
128 the tested virus strains (Fig. S1), receptor specificity of AH1 and pdmH1N1 was most similar
129 (i.e. the lowest difference).

130 FPV recognized all three avian-type conjugates, but bound only one human-type conjugate,
131 which is in line with previous findings using glycan arrays ²².

132 We further tested H3N2/X31 as well as H1N1/WSN. H3N2/X31 carries the HA of the human
133 pathogenic strain A/Aichi/68, which was also previously shown to prefer α -2,6-linked
134 (human-type) receptors ²³. In line with that, we found that H3N2/X31 only recognized
135 human-type SA conjugates on the glycan array. The lab-adapted H1N1/WSN was previously
136 shown to prefer α -2,6-linked (human-type) receptors over α -2,3-linked SA on re-sialylated
137 erythrocytes ²⁴. In our hands H1N1/WSN bound all six receptors with no obvious preference.
138 Its worth mentioning that among all glycans, we observed the strongest binding for
139 pdmH1N1, H1N1/WSN and AH1 to the kinked, fucosylated glycan SLe^X (Fig. 1). SLe^X is
140 well-known as an integrin receptor on leucocytes ²⁵, but was also shown to be recognized by
141 different IAV subtypes ²⁶. However, FPV was shown to bind only the sulfated form of SLe^X,
142 which is in line with our findings ²².

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144 **Cell specificity of influenza A virus strains studied by SVFS.** Next, we used SVFS to
145 characterize virus binding to two different cell types: First, we studied living A549 cells, a
146 model cell line derived from the lower human respiratory tract, expressing both major SA

147 receptor types on the cell surface as shown by lectin binding^{15, 18}. Secondly, CHO cells lack
148 an α -2,6-specific sialyltransferase and only express α -2,3-linked SA. Hence, we chose them
149 as a comparative model for studying viral binding to cells displaying only avian-type
150 receptors¹⁵. For SVFS, intact viruses were covalently attached to AFM cantilevers as
151 previously reported (Fig. 1)¹⁷. Binding to cells was measured in a dynamic range of
152 increasing loading rates, i.e. pulling velocities to determine the dissociation rate at zero force
153 k_{off} . Unbinding events were recorded and analyzed to obtain the rupture force F as well as the
154 effective spring constant k_{eff} , defined as the slope of the force-distance curve at rupture (Fig.
155 3a). From k_{eff} , the loading rate r (force per time) was calculated by multiplication with the
156 retraction velocity v . Notably, we used an adapted data analysis procedure, which takes the
157 variable local conditions of a living cell surface into account²⁷. Briefly, although the loading
158 rate r should be constant for a given pulling speed v , recent studies have shown that the
159 heterogeneity of a living cell surface leads to a broad distribution of observed loading rates²⁷.
160 Hence, to account for this effect, our approach does not rely on binning of loading rates, but
161 takes each individual force-distance curve into account (Fig. 3c). By fitting the force spectra
162 to a single energy barrier model (Fig. 3c, d, see *Materials and Methods*), we obtained the
163 thermodynamic properties of the interaction such as the dissociation rate k_{off} , and the
164 separation of the receptor-bound state to the energy barrier x_u (summarized in table 1). The
165 dissociation rate k_{off} and its reciprocal, the bond lifetime τ_{off} , provide information about the
166 stability of the underlying virus-cell interaction. The results for all virus-cell interaction pairs
167 are illustrated in Fig. 2d. For details, please see *Materials and Methods*.
168 Fig. 3b shows a typical rupture force histogram and the accompanying probability density

169 function (pdf) of the interaction between influenza AH1 and A549 cells with a binding
170 probability of 29.7 % ($v = 500$ nm/sec). The pdf shows a single peak at ~ 23 pN indicating
171 specific interaction (red curve in Fig. 3b). After cell surface SA deprivation by neuraminidase
172 (NA) treatment, the binding probability was reduced to 5-13 % while the pdf peak position
173 was unchanged (red curves and inset in Fig. 3b). This verifies the specificity of our
174 measurement for receptor interaction. The binned histograms are shown for comparison along
175 with the fitted pdf.

176 For AH1, we observed pronounced binding to both tested cell lines, with rupture forces,
177 between 10 and 100 pN depending on the applied loading rate (Fig. 3c, d). However, we
178 found an about 40 % reduced dissociation rate for A549 compared to CHO cells, indicating
179 preferential binding of human-type cell surfaces. We confirm this binding preference of AH1
180 by measuring binding to living MDCK cells, which express, similar to A549 cells, both
181 human-and avian-type receptors. We observed preferential binding to MDCK cells compared
182 to CHO cells (Fig. S2). For FPV, we observed about three times lower dissociation rates
183 compared to AH1, with preferential binding to A549 (see table 1). pdmH1N1 virus showed
184 similar dissociation rates as FPV, but without pronounced cell type preference. H3N2/X31 as
185 well as H1N1/WSN were already studied by SVFS in our previous study¹⁵ but reanalyzed
186 using the improved fitting procedure described above. The fitting values are reported in table
187 1. H3N2/X31 showed stronger attachment to CHO cells, while binding of H1N1/WSN to
188 A549 and CHO cells was almost identical¹⁵.

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190 **3. Discussion**

191 Among other methods, solid-phase binding assays or glycan arrays represent a widely used
192 state-of-the-art way to analyze HA receptor specificity ²⁸. The desired ligand is coupled to a
193 flat surface and can either be probed with intact viruses ^{7,8} or purified HA ¹⁹, which is then
194 detected using antibody binding. This makes them a powerful tool to screen large glycan
195 subsets. Choosing the right library is critical and since recent glycomics studies indicate a
196 large heterogeneity of host cell-specific glycans, this choice is not easily made ^{14, Byrd-Leotis, 2014}
197 ^{#5}. Also, the presentation (i.e. orientation and density) of the glycan is critical ²⁹, a factor that
198 can be estimated by performing avidity studies along with the glycan array ³⁰. In our study,
199 we have performed glycan array binding to evaluate the receptor specificity of various virus
200 strains along with testing their cell specificity using SVFS. While our glycan array results are
201 largely in line with previous findings, SVFS results, as summarized in table 1 and Fig. 4a,
202 suggest that HA's preference for human or avian-type receptors does not necessarily correlate
203 with expected binding patterns to cell lines modelling the surfaces of human or avian cells
204 (see below). We compared the specificity of virus binding measured in a glycan array (i.e.
205 receptor specificity) with that measured by SVFS (i.e. cell specificity). For pdmH1N1 and
206 H1N1/WSN, we found that the SVFS data (for H1N1/WSN see ¹⁵) are in good agreement
207 with results obtained from glycan array binding as neither strain displayed strong preference
208 for human or avian-type receptors or a particular cell model.

209 However, we observed contradicting preferences for H3N2/X31, AH1 and also for FPV.
210 H3N2/X31 was found to preferentially bind avian-type cell surfaces, while only recognizing

211 α -2,6-linked (human-type) receptors on the glycan array. AH1, similarly to pdmH1N1,
212 recognized both receptor types on the glycan array and showed good binding to all six
213 presented specific glycans on the array, while SVFS indicated a preference for human-type
214 cell surfaces. FPV recognized all three avian, but only one human-type receptor on the glycan
215 array, while showing preferential binding to human-type cell surfaces in SFVS. However,
216 binding to the recognized human-type receptors (receptor 3 in Fig. 1) was about 2-3 fold
217 stronger compared to the avian-type receptors (receptor 5 in Fig. 1), which might explain the
218 stronger binding to A549 cells. In conclusion, our results suggest that the HAs receptor
219 preference as tested in glycan array binding may not be a good predictor for preferred binding
220 to human-type over avian-type cell surfaces.

221 The findings described above raised the possibility that non-sialic acid receptors contribute to
222 a larger than expected extent to virus cell binding. However, SVFS analyses of cells after
223 pre-treatment with neuraminidase to remove sialic acid structures showed that the binding
224 probability was strongly reduced, leaving the unbinding force unchanged (Fig. 2B). This
225 indicates that the viruses indeed mainly bind to sialic acid of the cell surface, but that the
226 local environment of the receptor or other cell surface molecules alters the macroscopic cell
227 specificity leading to the observed differences. The stronger binding of AH1, pdmH1N1 and
228 H1N1/WSN to fucosylated glycan SLe^X with α -2,3-linked to SA in comparison to the other
229 α -2,3-linked (avian-type) SA of our glycan array is indicative for the relevance of the local
230 environment. On the viral side, cumulating evidence suggests a role of the viral
231 neuraminidase (NA) in contributing to cell binding via sialic acid ³¹. In our SVFS
232 measurements, NA was kept active and, hence, it cannot be excluded as a binding mediator, a

233 feature that could be tested in future experiments.

234 We also took a closer look at the thermodynamic properties of the virus cell interaction (table
235 1). Comparison of the transition state distance x_u revealed values around 2-10 Å for viruses
236 binding to A549 cells (mean 5.8 Å). In contrast, on CHO cells, we found higher transition
237 state distances between 2-13 Å (mean 8.1 Å). This interesting feature was also previously
238 observed for IAV H3N2/X-31¹⁵ and suggests a differently shaped energy barrier. Correlation
239 of x_u and k_{off} revealed no apparent clustering of H1 or H7 viruses (Fig. 4A) indicating a
240 dynamic interaction potentially involving multiple different receptor sites. However, some
241 correlation can be observed suggesting that binding to human-type cell surfaces tends to
242 result in lower bond energy and shorter unbinding distance. Indeed, x_u could be a possible
243 parameter to explore structural differences underlying virus-cell specificity. The observed x_u
244 values fall within the distance regime between receptor and its binding pocket. As an
245 example, Fig. 4C shows snapshots of a force-distance molecular dynamics simulation
246 between HA from influenza A H3N2/X31 and its human-type receptor (taken from ¹⁵). The
247 distance between the terminal SA and Asn137 (magenta), part of the critical loop 130, is
248 shown and scales between 4-20 Å. The corresponding value pair for x_u and k_{off} is shown in
249 Fig. 3B (green).

250 **4. Conclusion**

251 Recent glycomics approaches and the use of *ex vivo* tissue culture revealed new insights into
252 the complexity of the living cell surface^{13, 14}. Since sialic acid was first identified as an
253 influenza virus attachment factor³², many studies have focused on HA-SA binding. Although

254 this interaction is clearly important, not only infection of desialylated cells ¹¹, but also the
255 recent characterization of non-SA binding hemagglutinin encoded by a bat-derived H17N10
256 virus³³, and the discovery that 1918 pandemic virus unaffectedly binds to primary human
257 airway cells even when its HA is engineered to bind exclusively to avian-type SA receptors ³⁴
258 suggested that other molecular determinants within the plasma membrane are also critical in
259 initiating influenza A virus infection. For characterizing virus specificity, we suggest a dual
260 complementary approach: (1) *in vitro* binding assays with synthetic glycans to precisely
261 identify the preference of HA (or NA) for a specific sialic acid structure and (2) SVFS as
262 demonstrated here to unravel the cell specificity, modulated by the local environment of the
263 living host cell. We have recently demonstrated this complementary approach for an adapted
264 mutant of pdmH1N1 ¹⁷. While glycan array analysis could not identify a switch in receptor
265 preference, SVFS revealed that the adaptive mutation in HA strongly reduced the binding
266 strength without changing the cell specificity. These binding properties are not accessible and
267 might be hidden when only using *in vitro* specificity assays. The use of new methods such as
268 SVFS ¹⁶ and *ex vivo* tissue culture in combination with global glycomics and proteomics
269 approaches could help to identify essential components of the plasma membrane facilitating
270 influenza virus cell interaction.

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276 **Materials and Methods**

277 **Cell and virus propagation.** Chinese hamster ovary (CHO) cells and human alveolar A549
278 cells were grown in DMEM (PAA) supplemented with 1% penicillin/streptomycin and 10%
279 FCS (PAA) in plastic petri dishes. For sialic acid digestion, we used neuraminidase (NA)
280 from *Clostridium perfringens* (Sigma) solved in PBS buffer. The cells were treated for 10
281 min at 37° C with 1 U/mL NA. Influenza A viruses were grown on 10-day old chicken eggs
282 and purified from allantoic fluid by gradient centrifugation through a 20-60 % (w/v) sucrose
283 gradient. The A/Anhui/1/2013 strain was inactivated by UV irradiation before gradient
284 centrifugation.

285 **Glycan array.** Glycan array preparation was performed as described previously ^{35, Wermann, 2016}
286 ^{#192}. Briefly, glycans containing a primary amino linker were dissolved at a concentration of
287 0.1 mM in printing buffer (50 mM sodium phosphate, pH 8.5) and printed on
288 N-hydroxysuccinimide activated glass slides (CodeLink slides, Surmodics, Edina, MN, USA)
289 using an S3 robotic microarray spotter (Scienion, Berlin, Germany). Slides were incubated
290 overnight in a humidity saturated chamber and remaining reactive groups were quenched by
291 incubating with 100 mM ethanolamine, 50 mM sodium phosphate at pH 9.0 for 1 h at room
292 temperature. Slides were washed with water, dried by centrifugation and stored at 4 °C until
293 use. Before loading, the array was washed with DPBS. Virus was diluted as indicated into
294 sterile binding buffer containing 1% BSA, 0.05% Tween 20 (MERCK), CaCl₂ (492 μM) and
295 MgCl₂ (901 μM) at pH 7.0. 30 μl of diluted virus were pipetted in each well and the array
296 was incubated in a moist chamber for 24 h at 4 °C. Each well was then washed three times
297 with washing buffer containing DPBS and 0.1% Tween 20 (DPBS-T). Subsequently, wells

298 were blocked with DPBS containing 1% BSA for 2 h at 4 °C and permeabilized using
299 DPBS-T containing 0.3% Triton-X100. To stain the bound virus the array was incubated with
300 a primary monoclonal antibody against the viral NP protein (1:1000, clone AA5H, AbD
301 Serotec, Oxford, UK) at 4 °C overnight. Primary antibody was removed and wells were
302 washed three times with DPBS-T. Secondary Cy3-coupled goat anti-mouse IgG (1:100,
303 product-code: 115-165-146, Jackson ImmunoResearch Laboratories, West Grove, PA, USA)
304 was added and incubated at RT for 1 h. The array was washed three times with DPBS-T and
305 dipped into distilled water before scanning. Glycan array fluorescence images were obtained
306 using a GenePix 4300A microarray scanner (Molecular Devices, Sunnyvale, CA, USA).
307 Fluorescence intensities of spots were evaluated with GenePix Pro 7.2 (Molecular Devices).

308 **AFM tip chemistry.** Commercially available AFM cantilevers (MSCT, Bruker) were amine
309 functionalized by using the room-temperature method for reaction with APTES³⁶. A
310 heterobifunctional PEG linker, acetal-PEG₈₀₀-NHS (N-hydroxysuccinimide) (Fig. 1B), was
311 attached by incubating the tip for 1.5-2 h in 0.5 mL of chloroform containing 2 mg/mL
312 acetal-PEG-NHS and 8 μL triethylamine, resulting in acylation of surface-linked APTES by
313 the NHS group. The terminal acetal group was converted into an amine-reactive aldehyde by
314 incubation in 1% citric acid as described previously³⁶. After rinsing with water for 3 times,
315 once with ethanol and drying under a stream of nitrogen, the tips were incubated in a mixture
316 of 19-25 μL of approximately 0.6-1.6 mg/mL influenza A virus in PBS (without Ca⁺⁺) and
317 1-2 μL of 1 M NaCNBH₃ (freshly prepared by dissolving 32 mg of solid NaCNBH₃ in 500
318 μL of 10 mM NaOH) for 60 min. The tips were then washed in 3mL PBS for 3 times and
319 stored in PBS at 4 °C. All other chemicals and reagents were purchased from different

320 commercial sources in the highest purity grade available.

321 **SVFS measurement.** As illustrated in Fig.1, AFM-based force spectroscopy was performed
322 with an Agilent 5500 AFM. The Petri dish with cells was mounted with the AFM, which was
323 put on the optical microscope through a specially designed XY stage. Before force
324 measurements, the cantilever with a nominal spring constant of 10 pN/m functionalized with
325 influenza A virus was incubated in 5 mg/mL BSA for 30 min in order to minimize the
326 nonspecific interaction between the cantilever tip and the cell surface. Measurements were
327 performed in PBS buffer at room temperature. After the cantilever tip approached to the cell
328 surface, force distance curves were repeatedly measured with Z-scanning range of 2 μm ,
329 cycle duration of 0.5-8 s, 500 data points per curve, and typical force limit of about 40-70 pN.
330 The spring constants of the cantilevers were determined by using the thermal noise method³⁷.

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332 **Fitting of SVFS data.** Similar to single molecule force spectroscopy (SMFS), also in SVFS
333 studies, several hundred force distance cycles are recorded in a dynamic range of increasing
334 loading rates under identical conditions. For each of these force curves showing unbinding
335 events, the unbinding force F_i and the effective spring constant k_{eff} (slope at rupture)
336 were determined. The loading rates r were determined by multiplying the pulling velocity v
337 with the effective spring constant k_{eff} (i.e. $r = v * k_{eff}$). Additionally, a rupture force
338 probability density function (pdf) (Fig. 1d) was calculated and a Gaussian distribution was
339 fitted to the main peak of the pdf. Subsequently, all unbinding events within $\mu \pm \sigma$ of the fit
340 have been selected to create a loading rate dependence scatter plot (Fig. 1c-f) for further
341 calculations of k_{off} and x_u .

342 Generally, the loading rate r is constant for a fixed pulling speed, which implies, that the
343 effective spring constant k_{eff} does not vary significantly. However, for force spectroscopy
344 measurements on live cells it is known, that k_{eff} could show a broadened distribution
345 caused by local variations of the spring constant of the cell surface, leading to a convolution
346 of the rupture force distribution and further influences the calculations for the dissociation
347 rate constant, k_{off} , and the separation of the receptor-bound state to the energy barrier, x_u .
348 To circumvent this influence, we applied a maximum likelihood routine to fit the SVFS data
349 to the Evans-model²⁷, in order to obtain k_{off} and x_u (Table 1).

350 Accordingly to the single energy barrier binding model, the probability p that the complex
351 breaks at a certain force, F , is given as³⁸:

$$352 p(F) = \frac{k_{off}}{r} \exp \left[\frac{F x_u}{k_B T} - \frac{k_{off} k_B T}{r x_u} \left(\exp \frac{F x_u}{k_B T} - 1 \right) \right] \quad (3)$$

353 The parameters x_u and k_{off} were determined by applying a maximum likelihood approach,
354 in which the negative log likelihood nll was minimized by modifying k_{off} and x_u , with p
355 based on Equation (3) defined in the single barrier model³⁸:

$$356 nll = - \sum_t \log p(k_{off}, x_u, F_t, r_t) \quad (4)$$

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366 **Additional information**

367 Supplementary information is available. Correspondence and requests for materials should be

368 addressed to C.S., A.H. or P.H.

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370 **Competing financial interests**

371 The authors declare no competing financial interests.

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Table 1 | Dissociation rate k_{off} , separation from the energy barrier x_u , and average bond lifetime τ_{off} obtained by fitting the SVFS data to a single energy barrier binding model as described in Methods (see also Fig. 2D).

Cell (receptor type)	x_u (Å)	k_{off} (s ⁻¹)	τ_{off} (s)
Virus AH1 (H7N9)			
CHO (<i>avian-like</i>)	13.2 ± 0.016	1.17 ± 0.001	0.85
A549 (<i>human-like</i>)	9.24 ± 0.006	0.69 ± 0.0008	1.43
Virus FPV (H7N1)			
CHO (<i>avian-like</i>)	2.40 ± 0.004	0.33 ± 0.001	3.03
A549 (<i>human-like</i>)	5.74 ± 0.016	0.24 ± 0.001	4.15
Virus pdmH1N1			
CHO (<i>avian-like</i>)	12.5 ± 0.004	0.19 ± 0.001	5.20
A549 (<i>human-like</i>)	5.2 ± 0.016	0.2 ± 0.001	5.00
Virus X31 (H3N2)			
CHO (<i>avian-like</i>)	9.54 ± 0.18	0.66 ± 0.05	1.51
A549 (<i>human-like</i>)	6.42 ± 0.09	1.27 ± 0.07	0.78
Virus WSN (H1N1)			
CHO (<i>avian-like</i>)	2.77 ± 0.04	0.62 ± 0.04	1.61
A549 (<i>human-like</i>)	2.67 ± 0.04	0.85 ± 0.05	1.18

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412 **Figure legends:**

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414 **Figure 1 | Schematic diagram of the SVFS experimental setup using atomic force**
415 **microscopy (AFM).** (A) General principle of AFM-based SVFS. Cells grow in a plastic
416 culture dish that is attached to three step motors that allow movement with high accuracy.
417 The cantilever acts as a Hookean spring and hence bending can be translated into applied
418 force. The force-induced deflection of the cantilever is measured by pointing a laser on the
419 back of the cantilever while detecting the reflection on a quadrant photo diode (QPD). (B)
420 For SVFS, influenza A virions are covalently attached to the cantilever using an
421 acetal-PEG₈₀₀-NHS crosslinker ³⁹. (C) The cantilever is lowered on a single cell until
422 touching the cell surface. The combination with light microscopy, allows identification of the
423 cantilever with its pyramidal cantilever tip (C, inset shows a graphical illustration) and
424 thereby precise positioning. Subsequently, the cantilever is retracted at a defined velocity v .
425 In case of an interaction, the cantilever will bend towards the sample until the underlying
426 bond fails and the cantilever returns into the zero-force position (see also Fig. 2a).

427

428 **Figure 2 | Binding characteristics of the indicated viruses to sialic acid-conjugated**
429 **receptors quantified by glycan arrays.** Equal amounts of the indicated viruses were bound to
430 glycan arrays, spotted with 15 different sialic acids and printing buffer as negative control.
431 Staining of bound viruses was achieved using a NP-specific primary antibody and a
432 Cy3-coupled secondary antibody. The results represent the mean + SD for two independent

433 experiments.

434

435 **Figure 3 | SVFS measurements of H7 and pdmH1N1 viruses interacting with receptors**

436 **on living cells.** (A) Force trace of H7N9 AH1 virus-cell interactions measured by AFM-based

437 SVFS showing a characteristic single unbinding event. After treating the cells with

438 neuraminidase (NA), the binding probability was strongly decreased (see also inset in B),

439 causing a high number of force traces showing no interaction (inset in a). (B) Force histogram

440 (left Y axis) and overlaid force probability density function (pdf, right Y axis) of AH1

441 virus-A549 cell interaction before and after NA treatment. The observed force values were

442 found to be very similar, but the binding probability was strongly decreased (inset). (C)

443 Scatter plots showing unbinding force F plotted against the loading rate r of every individual

444 force curve from AH1 virus-A549 cell interaction. The red dotted line shows the fitting to the

445 single energy barrier model (see Methods). (D) Overview of the fittings used to determine

446 values for k_{off} and x_u (see table 1 and Methods) for all virus-cell combinations.

447

448 **Figure 4 | Comparing cell with receptor specificity and summary of the thermodynamic**

449 **parameters obtained from SVFS.** (A) To allow comparison between cell and receptor

450 specificity, we show results from SVFS (blue bars) as Δk_{off} (avian/human) as well as results

451 obtained from glycan arrays (red bars). The red bars indicating receptor specificity were

452 placed according to the number of recognized glycans as well as the strength of binding, as

453 discussed in section 2. (B) Correlation of k_{off} and x_u shows no apparent clustering for H7 (red)

454 or H1 (black) viruses indicating a very dynamic interaction (as shown in ¹⁵) supposedly
455 including various receptors. The distance to the transition state x_u provides a parameter to
456 characterize the interacting cellular receptor as the distance between receptor and HA falls
457 within this range. As an example, (C) shows snapshots of a force-probe molecular dynamics
458 simulation between HA from influenza A/X31 (green) and a human-type receptor (red) (taken
459 from ¹⁵). The distance between the terminal SA and Asn137 (magenta) is shown and scales
460 between 4-20 Å. The measured values for x_u and k_{off} for the corresponding virus-cell
461 combination are shown in **B** (green).

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473 **Supplementary Figure Legends**

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475 **Supplementary Figure S1. Cumulative receptor binding difference between H7N9/AH1**

476 **and the other on glycan arrays tested virus strains.** For each glycan, the binding intensity

477 was compared to the value of H7N9/AH1. The summed difference is shown for each virus

478 strain revealing that H7N9/AH1 shares most similarities with pdmH1N1.

479 **Supplementary Figure S2. SVFS dynamic force spectra of H7N9 AH1 interacting with**

480 **single receptors on living MDCK cells.** Scatter plot showing unbinding force F plotted

481 against the loading rate r of every individual force curve. From those data, the values for k_{off}

482 and x_u were determined to be $0.256 +/- 0.00169 \text{ s}^{-1}$ and $6.160 +/- 0.0146 \text{ \AA}$, respectively.

483 **Supplementary Table S3. Comparing SVFS results obtained using two different fitting**

484 **approaches.** Loading rates LR can either be obtained by using the mean effective spring

485 constant $\langle k_{eff} \rangle$ for each pulling velocity v ($LR = v * k_{eff}$) or, using a more adapted approach

486 reported previously and now used in this study, calculated for each individual force-distance

487 curve. Each fitting approach results in slightly different fitting parameters. Dissociation rate

488 k_{off} , separation from the energy barrier x_u , and average bond lifetime τ_{off} obtained by fitting

489 the SVFS data to a single energy barrier binding model.

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Figure 1

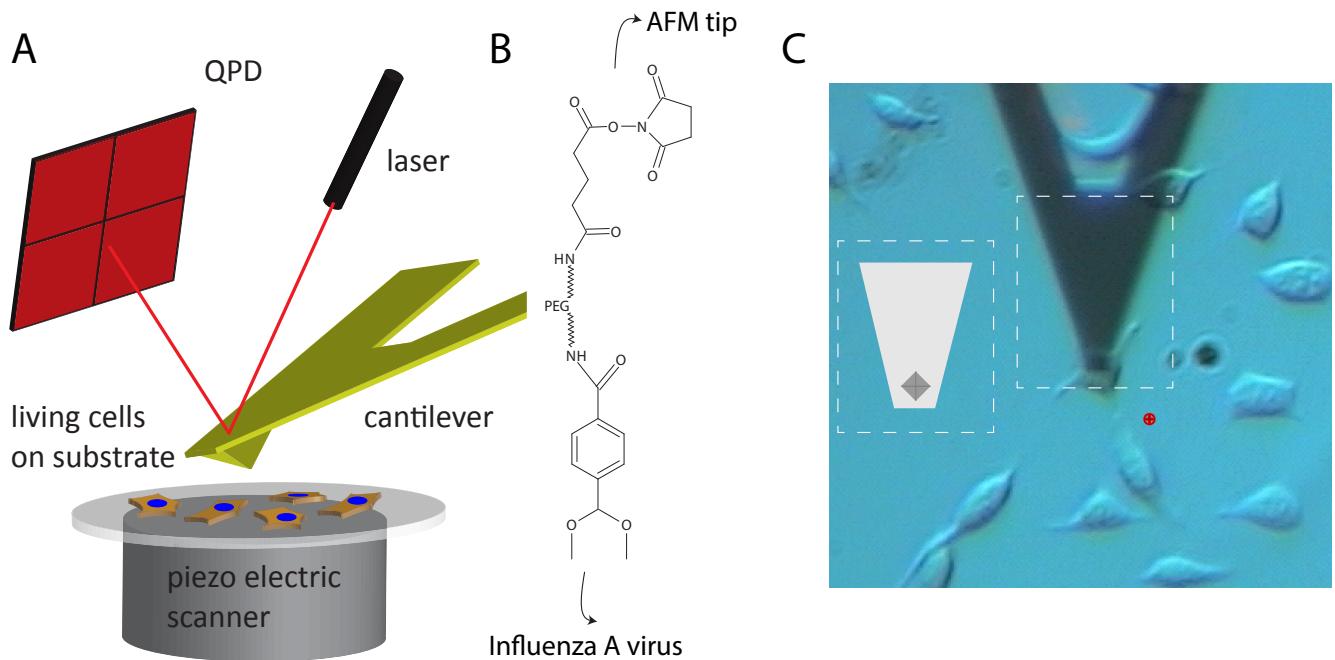


Figure 2

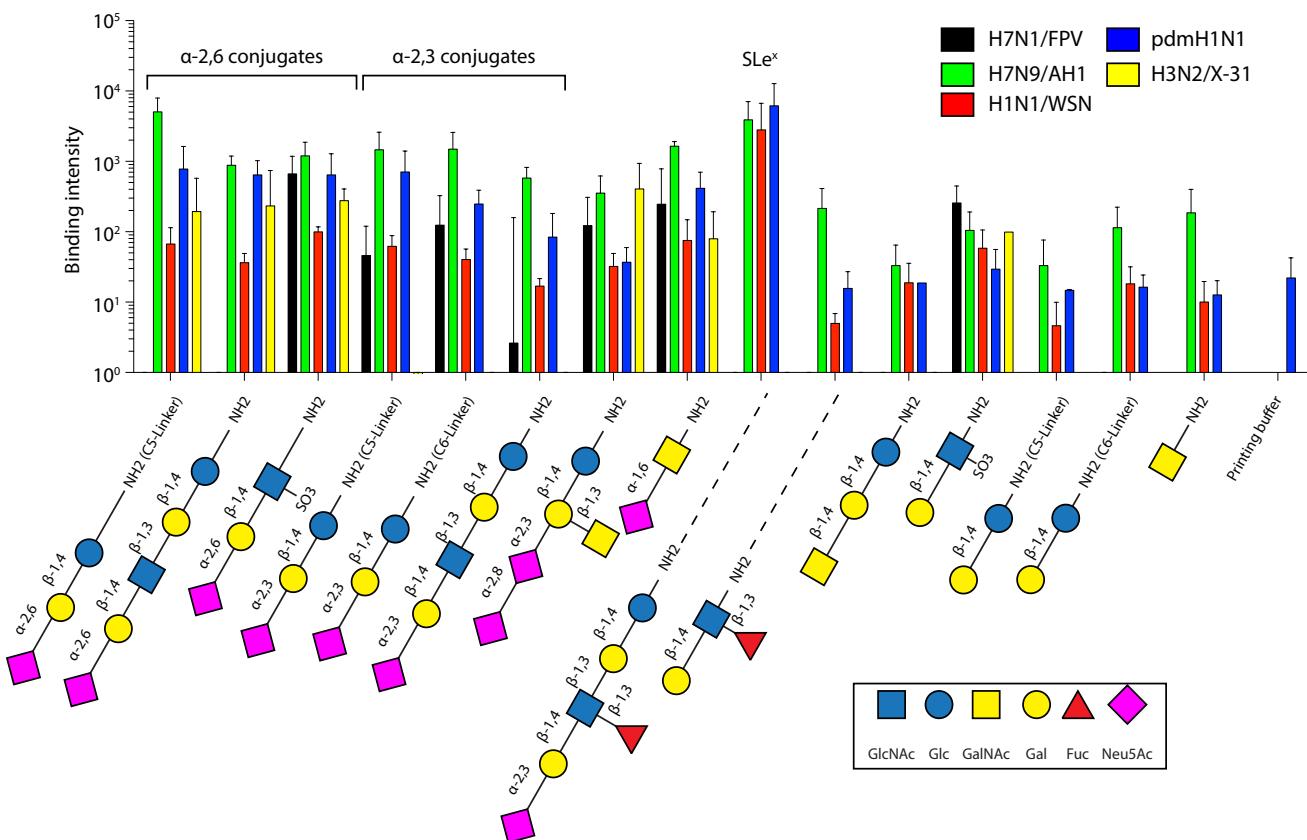


Figure 3

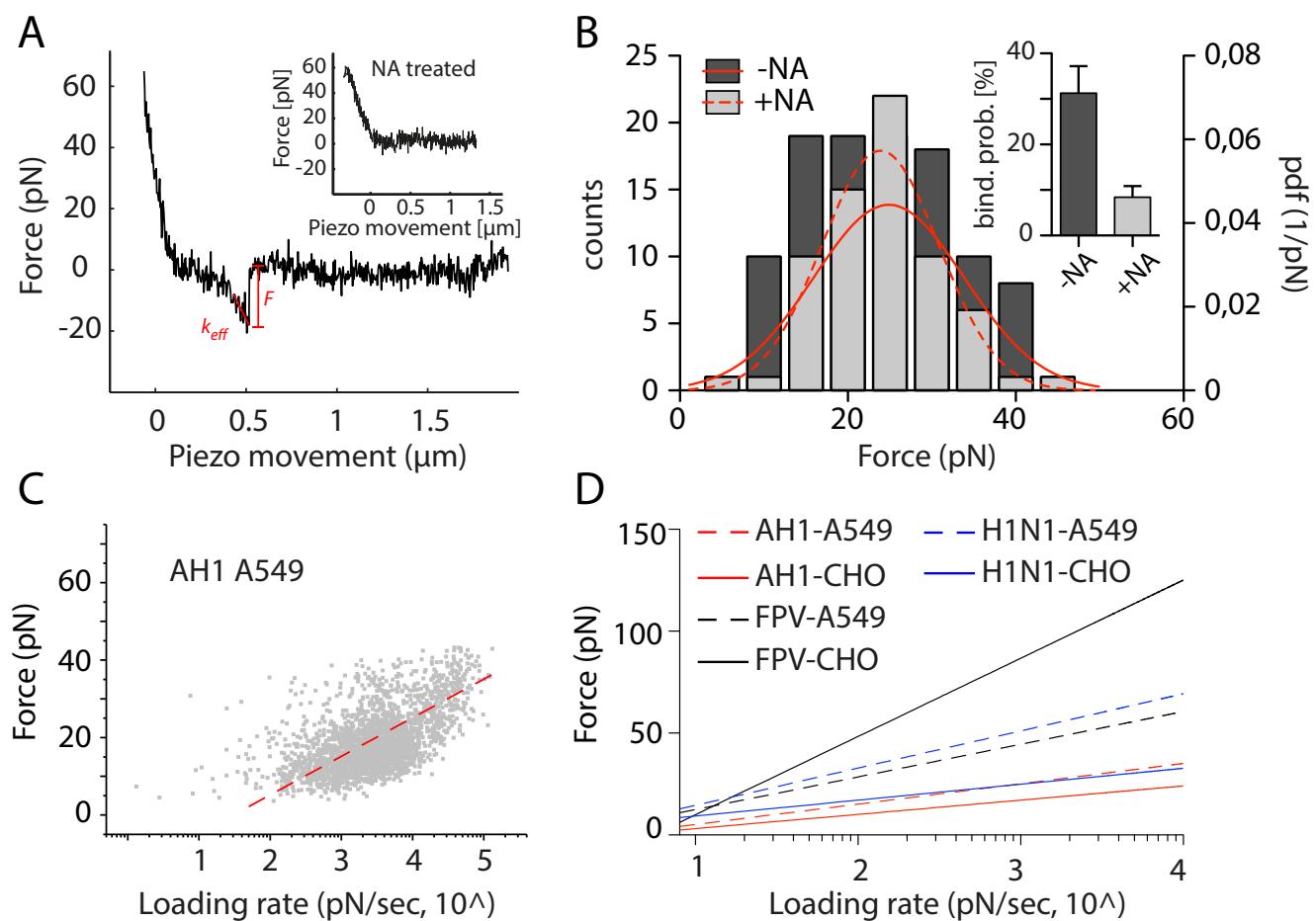
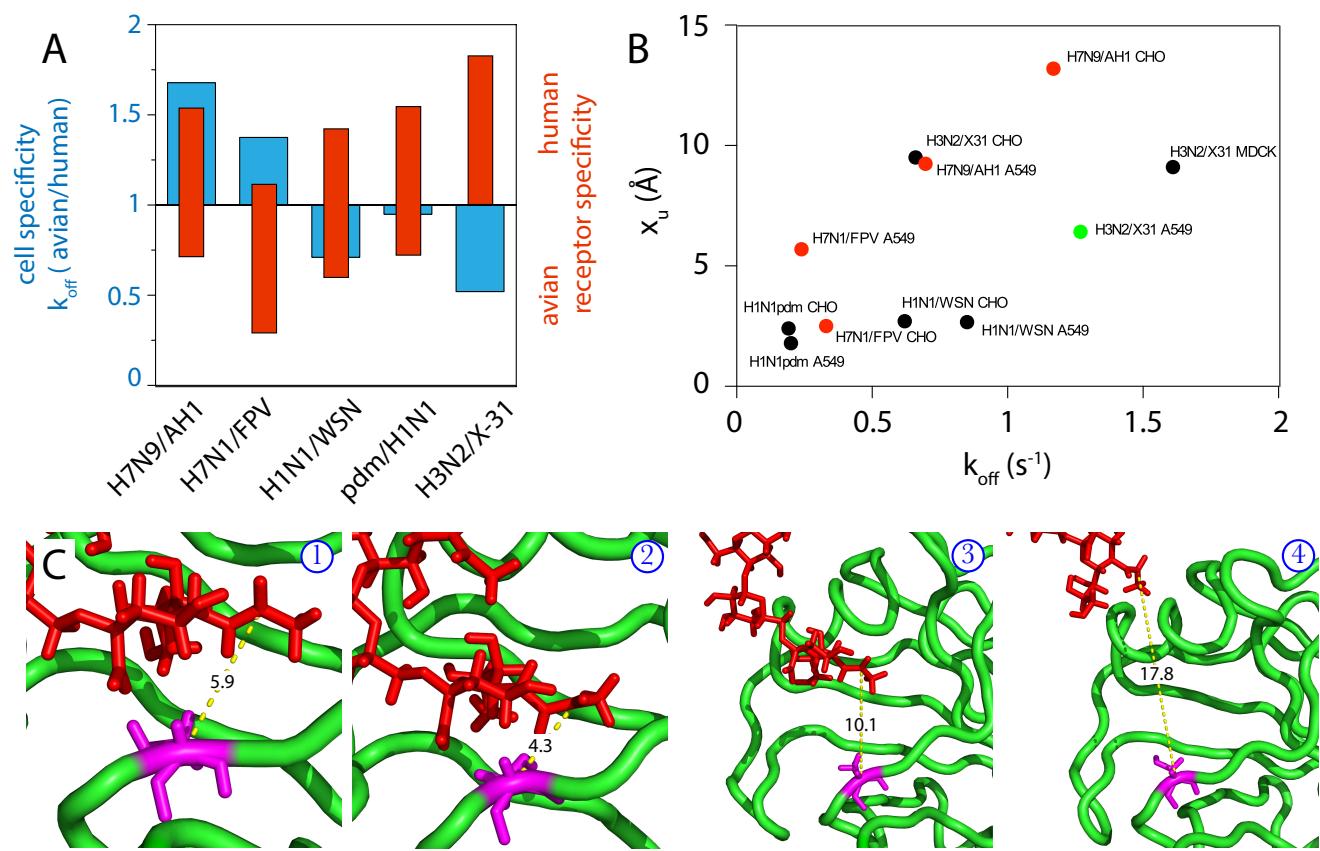


Figure 4



Supporting Information

Relevance of host cell surface glycan structure for cell specificity of influenza A virus

Markus Kastner^{1,*}, Andreas Karner^{1,2,*}, Rong Zhu^{1,*}, Qiang Huang³, Dandan Zhang⁴, Jianping Liu³, Andreas Geissner^{5,6}, Anne Sadewasser⁷, Markus Lesch⁸, Xenia Wörmann⁸, Alexander Karlas⁸, Thorsten Wolff⁷, Peter Hinterdorfer^{1,2,§}, Andreas Herrmann^{9,§} and Christian Sieben^{9,10,§}

¹ *Institute for Biophysics, Johannes Kepler University Linz, 4020 Linz, Austria*

² *Center for Advanced Bioanalysis GmbH, A-4020 Linz, Austria*

³ *State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China*

⁴ *Shanghai Supercomputer Center, Shanghai 201203, China*

⁵ *Department for Biomolecular Systems, Max Planck Institute for Colloids and Interfaces, Potsdam, Germany*

⁶ *Institute of Chemistry and Biochemistry, Free University, Berlin, Germany*

⁷ *Div. of Influenza and other Respiratory Viruses, Robert Koch-Institute, 13353 Berlin, Germany*

⁸ *Molecular Biology Department, Max Planck Institute for Infection Biology, 10117 Berlin, Germany*

⁹ *Department of Biology, Molecular Biophysics, Humboldt University Berlin, 10115 Berlin, Germany*

¹⁰ *Current address: Laboratory for Experimental Biophysics, School of Basic Sciences, École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland*

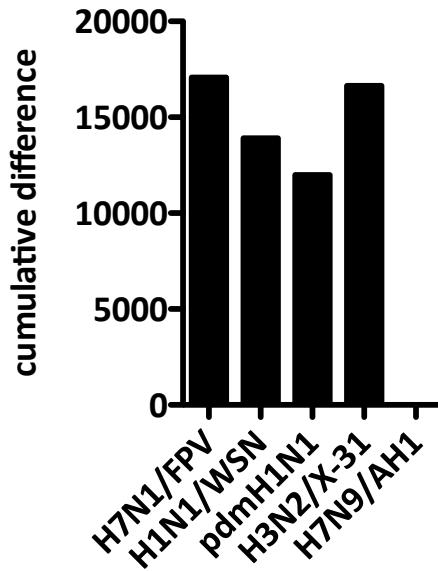
* These authors contributed equally to this work

§ Corresponding authors:

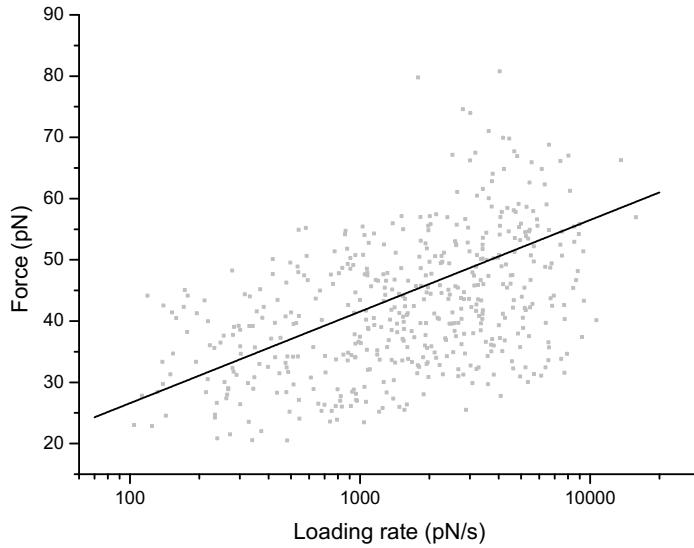
christian.sieben@epfl.ch (C.S.)

andreas.herrmann@rz.hu-berlin.de (A.H.)

peter.hinterdorfer@jku.at (P. H.)



Supplementary Figure S1. Cumulative receptor binding difference between H7N9/AH1 and the other on glycan arrays tested virus strains. For each glycan, the binding intensity was compared to the value of H7N9/AH1. The summed difference is shown for each virus strain revealing that H7N9/AH1 shares most similarities with pdmH1N1.



Supplementary Figure S2. SVFS dynamic force spectra of H7N9 AH1 interacting with single receptors on living MDCK cells. Scatter plot showing unbinding force F plotted against the loading rate r of every individual force curve. From those data, the values for k_{off} and x_u were determined to be $0.256 +/- 0.00169 \text{ s}^{-1}$ and $6.160 +/- 0.0146 \text{ \AA}$, respectively.

New Analysis, MLE from F vs. LR scatter plots, 2017

Virus	Cell	k_off (1/s)	x_beta (A)	tau (s)		
H1N1_WSN	A549	0.851	0.0525	2.67	0.0445	1.1751
H1N1_WSN	CHO	0.62	0.0355	2.77	0.0397	1.6129
H3N2_X31	A549	1.27	0.0704	6.42	0.0897	0.7874
H3N2_X31	CHO	0.66	0.0472	9.54	0.183	1.5152
H3N2_X31	MDCK	1.61	0.141	9.11	0.198	0.6211
						0.0544

Old Analysis, fit with grouped loading rates, 2012

Virus	Cell	k_off (1/s)	x_beta (A)	tau (s)		
H1N1_WSN	A549	1.22	0.32	1.8	0.3	0.8197
H1N1_WSN	CHO	1.16	0.13	2.4	0.3	0.8621
H3N2_X31	A549	0.64	0.52	4.2	4.1	1.5625
H3N2_X31	CHO	0.18	0.17	26.1	31.1	5.5556
H3N2_X31	MDCK	0.45	0.35	8.4	7.9	2.2222
						1.7284

Supplementary Table S1. Comparing SVFS results obtained using two different fitting

approaches. Loading rates LR can either be obtained by using the mean effective spring constant $\langle k_{eff} \rangle$ for each pulling velocity v ($LR = v * k_{eff}$) or, using a more adapted approach reported previously and now used in this study, calculated for each individual force-distance curve. Each fitting approach results in slightly different fitting parameters. Dissociation rate k_{off} , separation from the energy barrier x_u , and average bond lifetime τ_{off} obtained by fitting the SVFS data to a single energy barrier binding model.