1 B-cell epitope discovery: the first protein flexibility-based algorithm –
2 Zika virus conserved epitope demonstration

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20 **Abstract**

21 Antibody-antigen interaction – at antigenic local environments called B-cell epitopes – is a prominent
22 mechanism for neutralization of infection. Effective mimicry, and display, of B-cell epitopes is key to
23 vaccine design. Here, a physical approach is evaluated for the discovery of epitopes which evolve slowly
24 over closely related pathogens (conserved epitopes). The approach is 1) protein flexibility-based and 2)
25 demonstrated with clinically relevant enveloped viruses, simulated via molecular dynamics. The ap-
26 proach is validated against 1) seven structurally characterized enveloped virus epitopes which evolved
27 the least (out of thirty-eight enveloped virus-antibody structures) and 2) eight preexisting epitope and
28 peptide discovery algorithms. Rationale for a new benchmarking scheme is presented. A data-driven
29 epitope clustering algorithm is introduced. The prediction of eleven Zika virus epitopes (for future explo-
30 ration on recombinant vaccine technologies) is demonstrated. For the first time, protein flexibility is shown
31 to outperform solvent accessible surface area as an epitope discovery metric.

32 **Author summary**

33 The human immune response provides a robust defense against a multitude of infection-causing agents.
34 Interaction between an antibody and antigen (a pathogenic protein, for example) is a critical component
35 to this defense. Antibody-antigen interaction occurs at antigenic local environments termed B-cell
36 epitopes. As such, identification of B-cell epitopes could drive the development of world altering medi-
37 cines (e.g. vaccines). Structure-based approaches have emerged as promising (resource efficient) path-
38 ways toward B-cell epitope identification. However, a scarcity of structural data has hampered advance-
39 ment of the field. Here, we develop the first protein flexibility-based B-cell epitope discovery algorithm
40 with clinically relevant enveloped viruses. We show flexible B-cell epitopes are preferentially bound by a
41 subset of clinically relevant antibodies and demonstrate our approach on the Zika virus.

42 **Introduction**

43 B-cell epitopes are localities of antigens targeted by the humoral immune response, via antibodies and
44 B-cell receptors, to protect the extracellular space (e.g. the blood plasma).[1] Molecules which effectively

45 mimic B-cell epitopes are vaccines.[2-4] Consequently, structure-based B-cell epitope discovery has
46 emerged as a promising foundational step in rational vaccine design.[5, 6] Despite promise, 1) ambiguity
47 surrounding the definition of an epitope, 2) limitations of current performance benchmarking approaches,
48 3) a scarcity of benchmark datasets, and 4) an overabundance of solvent accessible surface area-based
49 metrics suggest there is ample room for improvement within the field. For example, while some envel-
50 oped virus[7] epitopes (like those of the Zika virus[8] (ZIKV))[9-11] have been studied, a comprehensive,
51 quantitative, uniform, and structure-based epitope analysis of a clinically relevant enveloped virus (like
52 ZIKV) has yet to be explored. Likewise, 1) “cryptic” epitopes hidden within virus structures,[12] 2) patho-
53 gen morphological diversity,[13] 3) full pathogen structural dynamics,[14] and 4) possible links between
54 protein flexibility and immunogenicity[15, 16] suggest an overreliance on solvent accessible surface area-
55 based epitope discovery metrics[5] may be a major oversight within the field, especially when it comes
56 to highly flexible, clinically relevant antigens (like ZIKV).

57 In addition to the limitations (within the field) which have already been noted, structure-based methods
58 for the discovery of conserved epitopes (epitopes which evolve the least across closely related patho-
59 gens) have yet to be developed. Ineffectiveness of current vaccine technologies in eliciting antibodies
60 able to bind a diverse set of closely related pathogens has been a challenge in vaccine design.[17, 18]
61 Multivalent, live-attenuated vaccines have been introduced to solve the complications mentioned above;
62 however, simultaneous vaccine-based display of related pathogen epitopes has resulted in unbalanced
63 prophylactic protection – suggesting new approaches are needed.[19] One promising, new approach is
64 the presentation (or display) of conserved epitopes on recombinant vaccines.[20]

65 Here, previous studies of *in silico* immunology[16, 21] are extended by evaluating an algorithm for con-
66 served epitope discovery, based on protein flexibility – as measured via root-mean-square fluctuation
67 (RMSF) of isolated protein and virus-like particle (VLP) protein residues. The method is developed with
68 flaviviruses simulated via molecular dynamics (MD). The approach is validated against 1) seven struc-
69 turally characterized flavivirus epitopes (from thirty-eight flavivirus-antibody structures) with the lowest
70 phylogeny-based evolutionary rates (described by Ashkenazy, H., et al. (2016)[22] as the rate at which
71 a structurally aligned residue changes over a phylogenetic tree of proteins with shared ancestry) and 2)

eight preexisting epitope and peptide discovery algorithms.[5] To enhance the epitope dataset (for the prediction of currently uncharacterized ZIKV epitopes), epitopes from seven flaviviruses are structurally aligned. Using 1) a new (data-driven) clustering algorithm, 2) a new epitope organizational model, 3) a new epitope discovery performance benchmarking scheme (which addresses bias in previous methods), and 4) a new epitope discovery benchmark dataset – all presented here for the first time – the prediction of eleven mostly continuous ZIKV epitopes (which provide starting points for future presentation on recombinant vaccine technologies) is demonstrated. Physical insights identified here 1) supply context for understanding (seemingly contradictory) previous reports on protein flexibility’s role in the humoral immune response[15, 16, 21, 23] and 2) shed new light on immunologically relevant distinctions between clinically relevant epitope subsets. Notably, for the first time,[23, 24] protein flexibility is shown to outperform solvent accessible surface area as an epitope discovery metric.

Results/Discussion

Epitope discovery performance benchmarking and rationale for a new method

Epitope discovery performance benchmarking on a compilation of all ZIKV-aligned epitopes and associations between isolated protein convex hull scores vs. other metrics are shown in Table 1. For comparison, benchmarking on individual ZIKV-aligned epitopes is shown in S1 Table.

Table 1. Epitope discovery performance benchmarking on a compilation of epitopes.

Metric	ROCAUC	±	PRAUC	±	Rho	p
hvlp	0.75	0.07	0.57	0.04	0.43	< 0.01
hmon	0.70	0.07	0.57	0.06	---	---
dtope	0.72	0.04	0.54	0.07	0.61	< 0.01
opia	0.70	0.07	0.52	0.09	0.61	< 0.01

epro	0.68	0.08	0.52	0.09	0.88	< 0.01
bpro	0.66	0.02	0.52	0.06	0.73	< 0.01
fmon	0.66	0.03	0.49	0.05	0.51	< 0.01
fvlp	0.63	0.05	0.46	0.05	0.48	< 0.01
tfac	0.57	0.08	0.40	0.02	0.45	< 0.01
ppisp	0.54	0.06	0.34	0.03	0.59	< 0.01
iupred	0.49	0.05	0.32	0.05	0.11	0.03

Metrics are ordered from the top to bottom in terms of highest ROCAUC and PRAUC product. Spearman rho (r) and p-values are shown for associations between isolated protein convex hull scores (hmon) vs. VLP protein convex hull scores (hvp), DiscoTope scores (dtope), Epitopia scores (opia), ElliPro scores (epro), BEpro scores (bpro), isolated protein RMSF (fmon), VLP protein RMSF (fvlp), temperature factors (tfac), cons-PPISP scores (ppisp), and IUPred scores (iupred).

Comparison of new epitope discovery performance indicator, Precision-Recall Area Under the Curve (PRAUC), with preexisting performance indicator, Receiver Operating Characteristic Area Under the Curve (ROCAUC), shows ROCAUC can overestimate epitope discovery performance when epitopes are analyzed individually, as expected. The mean ROCAUC was 0.15 ± 0.07 above baseline and the mean PRAUC was 0.06 ± 0.05 above baseline (over all metrics) when benchmarking was performed on individual epitopes 1) supporting the idea epitopes are much smaller than the entirety of an antigen (comprising only ~2% (the PRAUC baseline) of the number of antigen residues here) and 2) telling us, despite strong capacity to rank non-epitope residues below epitope residues (a high ROCAUC mean), the average metric performed poorly at pinpointing individual epitopes from other known epitopes (a low PRAUC mean) (S1 Table).[25] For comparison, the mean ROCAUC was 0.15 ± 0.08 above baseline and mean PRAUC was 0.16 ± 0.08 above baseline (over all metrics) when benchmarking was performed on a

106 compilation of epitopes telling us 1) the proportion of epitope residues and non-epitope residues had
107 parity when epitopes were compiled and 2) the average metric had a higher capacity to pinpoint a com-
108 pilation of epitopes (a high PRAUC mean) than individual epitopes (Table 1). These results make sense
109 as structural metrics are associated with the entirety of a protein antigen and antigens contain multiple
110 epitopes.[26] These results are also important for interpreting previous epitope discovery benchmarking
111 attempts.[5] From this point forward, all performance benchmarking discussion will refer to results ob-
112 tained using the compilation of epitopes benchmarking scheme.

113 Epitope discovery performance benchmarking comparison shows solvent accessible surface area-based
114 metrics (layered convex hulls, ElliPro,[27] DiscoTope,[28] BEpro,[29] Epitopia,[30] and cons-PPISP[31])
115 performed both the strongest and weakest of all metrics against all thirty-eight ZIKV-aligned flavivirus
116 epitopes (Table 1). More specifically, VLP protein convex hull scores performed with one of the strongest
117 capacities to rank epitope residues above non-epitope residues (the highest ROCAUC mean) and to
118 pinpoint epitope residues (one of the highest PRAUC means) likely because 1) flavivirus structures ma-
119 ture as they are secreted from the cell into the extracellular space,[32] 2) the mature ZIKV structure was
120 used to model the VLP examined here,[8] and 3) the humoral immune response protects the extracellular
121 space from foreign objects via B-cell antibodies[33]. For comparison, a moderate Spearman correlation
122 and overlapping performance were observed between isolated and VLP protein convex hull scores ($r =$
123 0.43 , $p < 0.01$, $n = 402$) perhaps because 1) the transmembrane protein region protrudes from the iso-
124 lated protein, enhancing convex hull scores of residues which are also exposed on the VLP,[8] or 2) the
125 extracellular space sees a diverse ensemble of flavivirus morphologies (outside the mature cryo-EM
126 reconstruction), as supported by recent studies.[12-14] Overall, these results 1) join growing support for
127 the hypothesis accessibility to antibody binding is one prerequisite for an epitope's existence[5, 28, 34]
128 and 2) suggest morphological diversity may challenge our assumptions as to which regions of a pathogen
129 are accessible to antibody binding.

130 Protein flexibility-based metrics (isolated protein RMSF, VLP protein RMSF, and temperature factors),
131 on the other hand, 1) were moderately correlated with and 2) performed below most solvent accessible
132 surface area-based metrics (against all thirty-eight ZIKV-aligned flavivirus epitopes) (Table 1). Despite

133 relatively weak performance (in comparison with solvent accessible surface area-based metrics), flexi-
134 bility-based metrics still retained some capacity to pinpoint ZIKV-aligned flavivirus epitope residues
135 (PRAUC means above the ~0.32 baseline). Comparison of the Spearman correlation between isolated
136 and VLP protein flexibility shows the two metrics were strongly correlated (Spearman $r = 0.76$, $p < 0.01$,
137 $n = 402$). RMSD equilibration plots indicate, despite performance overlap, differences in flexibility-based
138 epitope discovery performance (between the isolated protein vs. the VLP proteins) were likely due to
139 steric hindrance between adjacent VLP proteins, a 0.15 Å RMSD standard deviation (on average) for all
140 VLP proteins (analyzed individually) vs. a 0.37 Å RMSD standard deviation for the isolated protein (over
141 the last 20 ns; Fig 1). These results suggest 1) epitopes can be flexible (as measured via RMSF) and 2)
142 pathogen morphological diversity may complicate our understanding of protein flexibility's role within the
143 humoral immune response.

144

145 **Fig 1. ZIKV structural equilibration at 300 K.** a) RMSD from starting configuration for a) the
 146 ZIKV VLP (black trace), a single VLP protein (gray trace), and b) the isolated ZIKV protein are
 147 shown. Vertical lines represent trajectory bounds used for RMSF calculation.

148 Although protein flexibility-based metrics failed to outperform solvent accessible surface area-based met-
 149 rics (in terms of epitope discovery performance against all ZIKV-aligned epitopes), 1) very strong corre-
 150 lations between isolated protein flexibility of seven structurally related flaviviruses (a mean Spearman r
 151 of 0.80 ± 0.04 and $p < 0.01$ over flavivirus structures; S1 Fig) and 2) seemingly contradictory conclusions
 152 from previous studies (examining flexibility's role in the humoral immune response)[15, 16, 21] suggest

an external factor such as antibody flexibility (as hypothesized here) influences the relationship between protein flexibility and epitopes.[35, 36]

To understand how consideration of protein flexibility differences across antibodies[35, 36] could reveal a stronger association between protein flexibility and epitopes, one must examine the combined influence of antibody flexibility and epitope flexibility on antibody-antigen binding affinity – as epitopes are, by definition, antigenic local environments bound by antibodies. First, one way of obtaining high antibody-antigen binding affinity is through maximization of contact between an antibody and an antigen. Second, to maximize contact between an antibody and an antigen, either the antibody or the antigen (more specifically the partner epitope on the antigen) must have flexibility. For example, when an antibody and an epitope are both rigid, contact is likely limited via steric hinderance, and, when an antibody and an epitope are both flexible, contact is likely limited via a high conformational entropy barrier. Using this logic, the relationship between protein flexibility and epitopes should be enhanced for epitopes bound by antibodies with relatively high rigidity compared with all antibodies. Broadly neutralizing antibodies, for example, have recently been shown to affinity mature through a process which rigidifies the antibody, while, at the same time, enhancing antibody-antigen binding specificity[35, 36] suggesting affinity matured, broad-spectrum antibodies may preferentially target flexible regions of pathogens.

Conserved epitope discovery performance benchmarking

In light of results here and previous suggestions of flexibility's influence on broadly neutralizing antibody affinity maturation,[35, 36] epitope discovery performance benchmarking on a compilation of the top seven most conserved flavivirus epitopes (an epitope subset which 1) evolves slowly over structurally related pathogens and 2) is likely targeted by broad-spectrum antibodies) was examined (Table 2).

Table 2. Conserved epitope discovery performance benchmarking.

Metric	ROCAUC	±	PRAUC	±	Rho	p
fmon-fvlp	0.86	0.07	0.41	0.19	0.54	< 0.01

fmon	0.69	0.06	0.45	0.12	---	---
epro	0.65	0.12	0.34	0.23	0.53	< 0.01
opia	0.61	0.06	0.25	0.08	0.53	< 0.01
hmon	0.61	0.11	0.23	0.12	0.48	< 0.01
ppisp	0.67	0.14	0.15	0.15	0.31	< 0.01
bpro	0.58	0.04	0.11	0.09	0.58	< 0.01
hmon-hvlp	0.64	0.10	0.09	0.04	0.48	< 0.01
fvlp	0.54	0.08	0.08	0.04	0.76	< 0.01
dtope	0.60	0.11	0.07	0.02	0.65	< 0.01
iupred	0.50	0.18	0.05	0.03	0.04	0.44
tfac	0.52	0.15	0.04	0.02	0.37	< 0.01
hvlp	0.39	0.05	0.03	< 0.01	0.12	0.02

175 Metrics are ordered from the top to bottom in terms of highest ROCAUC and PRAUC product
 176 calculated against the top seven most conserved ZIKV-aligned epitopes. Spearman rho (r) and
 177 p-values are shown for associations between isolated protein RMSF (fmon) vs. (fmon-fvlp) the
 178 linear difference of isolated and VLP RMSF, ElliPro scores (epro), EpiToPIA scores (opia), iso-
 179 lated protein convex hull scores (hmon), cons-PPISP scores (ppisp), BEpro scores (bpro),
 180 (hmon-hvlp) the linear difference of isolated and VLP convex hull scores, VLP protein RMSF
 181 (fvlp), DiscoTope scores (dtope), IUPred scores (iupred), temperature factors (tfac), and VLP
 182 protein convex hull scores (hvlp).

183 Epitope discovery performance benchmarking comparison shows isolated protein flexibility performed
184 above all other lone flexibility-based metrics (VLP protein RMSF and temperature factors) and all solvent
185 accessible surface area-based metrics (layered convex hulls, ElliPro, DiscoTope, BEpro, EpiTope, and
186 cons-PPISP) against the top seven most conserved flavivirus epitopes – the first report of a flexibility-
187 based epitope discovery metric outperforming solvent accessible surface area-based metrics (Table
188 2).[5] Examination of epitope discovery performance against the top two most conserved flavivirus
189 epitopes shows isolated protein flexibility also maintained almost perfect capacity to 1) pinpoint residues
190 from the top two conserved epitopes (a PRAUC mean of 0.85 ± 0.30) and 2) rank residues from the top
191 two conserved epitopes above all other residues (a ROCAUC mean of 1.00 ± 0.01). Closer inspection
192 reveals 1) isolated protein flexibility of seven structurally related flaviviruses retained comparable capac-
193 ity to pinpoint residues from the top seven conserved epitopes (a mean PRAUC over flavivirus structures
194 of 0.41 ± 0.04 ; S2 Table), 2) isolated protein flexibility from a ZIKV protein simulation without disulfides
195 performed with a lower capacity to pinpoint residues from the top seven conserved epitopes (a lower
196 PRAUC mean, 0.35 ± 0.22) than is isolated protein flexibility with disulfides, supporting separate studies
197 suggesting disulfides play a structural role in flavivirus immunogenicity,[37-41] 3) IUPred, a sequence-
198 based measure of intrinsically disordered protein regions (which one might associate with protein flexi-
199 bility),[42, 43] performed no better than chance against the top seven conserved epitopes (baseline
200 ROCAUC and PRAUC means), and 4) ElliPro had overlapping performance with isolated protein flexibil-
201 ity in terms of capacity to pinpoint residues from the top seven conserved epitopes (despite a lower
202 PRAUC mean).

203 Strong conserved epitope discovery performance with isolated protein flexibility comes as no surprise,
204 as 1) conserved protein regions are necessary for successful flavivirus infection[44] and 2) protein flexi-
205 bility (linked with major morphological shifts) is also necessary for successful flavivirus infection. For
206 example, conformational dynamics are thought to facilitate flavivirus endosomal escape, and antibodies
207 which block or alter these dynamics have been shown to neutralize infection.[45]

208 Despite performance overlap and compared with isolated protein flexibility alone, VLP protein flexibility-
209 based metrics (VLP protein RMSF and temperature factors) performed weakly at pinpointing conserved

210 epitope residues (PRAUC means around baseline). However, when combined, the linear difference of
211 isolated and VLP protein flexibility performed with stronger capacity to rank conserved epitope residues
212 above all other residues (a distinctly higher ROCAUC distribution) compared with ElliPro ($t(8) = -3.04$, p
213 $= 0.02$) and isolated protein flexibility ($t(8) = -3.56$, $p < 0.01$) (Table 2).

214 Examination of differences between isolated protein flexibility and protein flexibility from a partially iso-
215 lated protein simulation (incorporating the transmembrane region and two M proteins; denoted as par-
216 tially isolated protein flexibility here) reveals, unlike isolated protein flexibility, linear combination of par-
217 tially isolated and VLP protein flexibility resulted in little to no performance enhancement over partially
218 isolated protein flexibility alone (a PRAUC mean of 0.49 ± 0.15 and ROCAUC mean of 0.71 ± 0.08).
219 These results imply rotational and translation freedom of the fully isolated protein, dampened upon as-
220 sembly into the mature virus structure, was one facet of the linearly combined, flexibility model's capacity
221 to rank conserved epitope residues above all other residues. Like previously mentioned, broadly neutral-
222 izing flavivirus antibodies have been shown to mute or disrupt the dynamics of mature flavivirus struc-
223 tures, obstructing conformational motions required for endosomal escape and successful infection.[45]

224 Visualization of the linearly combined, flexibility model on the ZIKV cryo-EM reconstruction[8] highlights
225 a radial spiral in metric amplitude originating at the five-fold rotational icosahedral symmetry axes (Fig
226 2), a local environment thought to hold stress[46] of icosahedral virus curvature. Visual analysis indicates
227 conserved epitopes may be important for 1) stabilizing the mature flavivirus morphology before endoso-
228 mal escape and 2) destabilizing the mature flavivirus morphology for endosomal escape, as part of a
229 morphological transition state. This makes sense as isolated protein flexibility (isolated from other enve-
230 lope proteins, M proteins, and the transmembrane region) is likely, at least somewhat, indicative of the
231 flexibility sampled during flavivirus morphological transitions (e.g. immature to mature or mature to en-
232 dosomal escape morphologies).

Fig 2. Visualizing the linearly combined, flexibility model via the mature ZIKV cryo-EM reconstruction. From left to right: weighted isolated protein flexibility, weighted VLP protein flexibility, and the linearly combined, flexibility model are depicted over the mature ZIKV cryo-EM reconstruction (PDB ID: 5IRE)[8] (scales shown). VLP structures are oriented with the five-fold rotational icosahedral symmetry axis coming out of the figure plane.

Inspection of the weights which optimized performance for the linearly combined, flexibility model shows optimal performance was achieved when weights were chosen as to minimize the root-mean-square difference between individual metrics (Fig 3). For example, weights which minimized the root-mean-square difference between isolated and VLP protein flexibility (0.325 and 0.675, respectively) were almost identical to weights which optimized performance for the linear combination (0.328 and 0.672, respectively). These results suggest the linearly combined, flexibility model was representative of the relative flexibility difference between the isolated protein and VLP proteins after baseline flexibility differences were accounted for.

248 **Fig 3. Visualizing the linearly combined, flexibility model via RMSF profiles.** On the left,
249 plots of the weighted isolated (blue trace) and VLP (red trace) protein flexibility metrics vs. resi-
250 due number are overlaid. On the right, a plot of the linearly combined, flexibility model vs. residue
251 number is shown.

252 For comparison, solvent accessible surface area-based metrics performed with relatively weak capacity
253 to rank conserved epitope residues above all other residues (relatively low ROCAUC means) and to
254 pinpoint conserved epitope residues (relatively low PRAUC means) (Table 2). In fact, although isolated
255 protein convex hull scores had some capacity to rank conserved epitope residues above all other resi-
256 dues (a ROCAUC mean above baseline), VLP protein convex hull scores ranked all other residues above
257 conserved epitope residues (a ROCAUC mean ~ 0.10 below baseline). Additionally, in contrast with pro-
258 tein flexibility, the linear difference of isolated and VLP protein convex hull scores performed with 1)
259 similar capacity to rank conserved epitope residues above all other residues (similar ROCAUC means)
260 and 2) weaker capacity to pinpoint conserved epitope residues (a lower PRAUC mean) compared with
261 isolated protein convex hull scores alone. Cons-PPISP, a metric calibrated to typical protein-protein in-
262 terfaces, performed with a stronger, although relatively low, capacity to pinpoint epitope residues against
263 the top seven most conserved epitopes (a PRAUC mean above the ~ 0.04 baseline) than against all
264 thirty-eight epitopes (a PRAUC mean around the ~ 0.32 baseline). These results signify 1) protein-protein
265 interaction sites play a possibly limited role in the existence of conserved epitopes, 2) differences be-
266 tween isolated and VLP protein convex hull scores were accentuated via performance benchmarking
267 against conserved epitopes, and, relatedly, 3) it is evolutionarily advantageous to partially or transiently
268 conceal conserved epitopes for immune evasion.[44] Interestingly, despite potential partial or transient
269 concealment, engineered presentation of conserved epitopes on recombinant vaccines can still induce
270 broadly reactive humoral immune responses[20] – highlighting the potential significance of 1) conserved
271 epitope discovery methods in vaccine design and 2) engineered presentation of conserved epitopes on
272 vaccine, protein scaffolds.

273 Although the flavivirus epitope dataset provides no quantitative validation for how well the combined
274 isolated and VLP protein flexibility conserved epitope model (identified here) generalizes to structurally

unrelated pathogens, performance reliability shows isolated protein flexibility pinpoints conserved epitope residues well across seven structures (a mean PRAUC of 0.41 ± 0.04 over flavivirus structures; S2 Table). Additionally, 1) a common ancestral link between class II fusion proteins (e.g. the flavivirus envelope protein),[47] 2) previous observations of protein flexibility's role in broadly neutralizing antibody maturation,[36] 3) previous observations of protein flexibility's role in human papillomavirus immunogenicity,[16] and 4) the first principles nature of protein flexibility-based metrics suggest physical insights discovered here may have broader generalizability.

Conserved epitope organization and epitope discovery demonstration

Of the seven highly conserved epitopes, 14% were associated with ZIKV, 0% were associated with dengue virus serotype 1, 14% were associated with dengue virus serotype 2, 14% were associated with dengue virus serotype 3, 29% were associated with dengue virus serotype 4, 29% were associated with West Nile virus, and 0% were associated with Japanese encephalitis virus (Table 3).

Table 3. Flavivirus epitope mean evolutionary rates.

PDB	ER	PDB	ER	PDB	ER	PDB	ER
5JHL	-1.05	5LBS	0.12	5GZO	0.66	5UHY	0.90
3IXX	-0.91	4AL8	0.16	5KVF	0.70	2R69	0.94
4BZ1	-0.87	4UT9	0.19	3UZV	0.71	4L5F	0.95
3I50	-0.71	4UTB	0.43	3UZE	0.72	5KVE	0.97
3IXY	-0.62	5VIG	0.47	3UYP	0.73	5YWO	1.06
4ALA	-0.55	3UAJ	0.48	3IYW	0.73	5KVG	1.14
4AM0	-0.55	5H37	0.57	5Y0A	0.86	5VIC	1.21

4UT6	-0.24	5YWP	0.58	4FFZ	0.89	1ZTX	1.36
5KVD	-0.19	3UZQ	0.63	4FFY	0.89	5GZN	1.48
4UTA	0.02	5AAW	0.65	---	---	---	---

Flavivirus-antibody structures from which conserved epitopes were identified are ordered from the top to bottom and left to right in terms of mean residue evolutionary rate (ER).[22] PDB IDs of the associated structures are shown.

Examination of ZIKV-aligned epitope organization shows conserved flavivirus epitopes fall on the lower end of the epitope distribution, in terms of epitope residue quantity and epitope discontinuity. Conserved flavivirus epitopes had a mean of 3 ± 1 total residues and 2 ± 0 subsequences with 1 ± 1 residues. All thirty-eight ZIKV-aligned flavivirus epitopes had a mean of 7 ± 5 total residues and 4 ± 2 subsequences with 2 ± 2 residues. For comparison, a previous analysis of seventy-six antibody-antigen structures[48] found epitopes had a mean of 16 ± 3 total residues and 5 ± 2 subsequences with 2 ± 2 residues. These results further support the idea partial or transient concealment of conserved epitopes is evolutionarily advantageous,[44] emphasizing the potential value of 1) conserved epitopes and 2) conserved epitope discovery methods in vaccine design.

Prediction of eleven (mostly continuous) ZIKV epitopes with a mean 2 ± 0 total residues was demonstrated. Visual inspection of predicted epitopes on the ZIKV cryo-EM reconstruction shows a spiral pattern focused around the five-fold rotational icosahedral symmetry axes (Fig 4). The isolated and VLP protein flexibility linear difference threshold which minimized the root-mean-square difference between known conserved epitope organization and predicted conserved epitope organization was $0.10 \text{ \AA}$ (Fig 3). The highest performing epitope clustering residue-residue distance cutoff which minimized the root-mean-square difference between known conserved epitope organization and predicted conserved epitope organization was $4 \text{ \AA}$. The conserved epitopes predicted were [residues [104, 105, 106, 107], [347, 348], [149, 150, 151], [73, 74, 75], [330, 331], [111, 112], [311, 312], [100, 102, 103], [384, 385], [195, 196], [316, 317, 318]] (Fig 4).

310

311 **Fig 4. Conserved epitope prediction demonstration.** Predicted conserved epitopes are
 312 shown as enlarged spheres (yellow) on the ZIKV cryo-EM reconstruction backbone (red) (PDB
 313 ID: 5IRE).[8] The cryo-EM reconstruction is shown with the five-fold rotational icosahedral sym-
 314 metry axis coming out of the figure plane.

315 Comparison of the similarity between full, predicted epitopes (clustered from predicted epitope residues)
 316 and structurally characterized epitopes shows several epitope predictions overlap with structurally char-
 317 acterized epitopes which were (not necessarily within the top seven conserved epitopes but still) bound
 318 by cross-reactive, neutralizing antibodies. Predicted epitopes with the highest similarity to structurally
 319 characterized epitopes were: [[[347, 348], '5KVF', 67%], [[316, 317, 318], '3UZE', 38%], [[316, 317, 318],
 320 '3UZQ', 33%], [[104, 105, 106, 107], '5JHL', 33%]], [[316, 317, 318], '3UYP', 29%]] (format: [[predicted
 321 epitope], PDB ID of characterized epitope, Jaccard Similarity index[49]]). Antibody 4E11 (PDB ID: 3UZE,
 322 3UZQ, and 3UYP) cross-neutralizes all four dengue serotypes.[50] Predicted epitope [73, 74, 75] over-
 323 laps with a structurally uncharacterized epitope bound by antibody 1C19 – which also cross-neutralizes
 324 all four dengue serotypes.[51] Antibody 2A10G6 (PDB ID: 5JHL) cross-neutralizes both ZIKV and den-
 325 gue virus.[52]

326 **Methods**

327 **Model preparation and molecular dynamics simulations**

328 ZIKV chains A, B, C, and E were extracted from a 3.80 Å resolution, cryogenic electron microscopy (cryo-
329 EM) structure (Protein Databank (PDB) ID: 5IRE).[8] Protein coordinates were run through Pulchra[53]
330 to reconstruct missing and sterically clashing atoms. Protein coordinates from chain A (residues 1-402)
331 were used to construct a soluble, isolated protein simulation starting structure. An analogous process
332 was applied to construct soluble, isolated protein simulation starting structures for six related flaviviruses
333 (PDB: ID 4CCT (dengue virus serotype 1),[54] 3J27 (dengue 2),[55] 3J6S (dengue 3),[56] 4CBF (dengue
334 4),[57] 2HG0 (West Nile virus),[58] and 5WSN (Japanese encephalitis virus)[59]). Protein coordinates
335 from ZIKV chains A, C, and E (residues 1-402) and an associated symmetry matrix (taken from PDB ID:
336 5IRE) were used to build a soluble ZIKV VLP simulation starting structure. For comparison, protein co-
337 ordinates from ZIKV chain A (residues 1-502) and two chain Bs (touching chain A after application of the
338 associated symmetry matrix) were used to construct a partially isolated ZIKV protein simulation starting
339 structure (with transmembrane region and associated M proteins).

340 Using GROMACS 5.0.4,[60] explicit-solvent (TIP3P)[61] MD simulations were run on the flavivirus pro-
341 teins and ZIKV VLP to a local structural equilibrium on the Indiana University Big Red II and III super-
342 computers (Fig 1). Attainment of local structural equilibrium was assessed as a steady time-average in
343 backbone atom root-mean-square deviation (RMSD) relative to the starting configuration.[21] The
344 CHARMM27[62] forcefield was used with a 1 fs time step and periodic boundary conditions. The
345 LINCS[63] algorithm was used to fix hydrogen bond lengths and the distance from protein to the edge of
346 the cubic box was set to 1.5 nm. Steepest decent energy minimization was employed before simulation
347 runs.

348 Flavivirus proteins were simulated in 142 mM NaCl (physiological) water baths. Simulation temperatures
349 were coupled to 300 K (room temperature) using the Nosé-Hoover[64] thermostat. The coupling time
350 constant was set to 0.5 ps, and ten Nosé-Hoover chains were used. The pressure was coupled to 1 bar
351 using the Parrinello-Rahman[65] barostat. The coupling time constant and compressibility were set to 1

352 ps and 4.5E-5 bar. Short-range electrostatic cutoffs were set to 1.4 nm; long-range electrostatics were
353 managed using the reaction-field[66] method. Epsilon-r (the relative dielectric constant) and epsilon-rf
354 (the dielectric constant beyond a 1.4 nm reaction sphere cutoff) values were set to 1 (protein) and 78
355 (water).

356 Six cysteine residues pairs ([3, 30], [60, 121], [74, 105], [92, 116], [190, 291], and [308, 339]) which had
357 sulfurs positioned 6 Å or less apart (overall mean sulfur separation distance of 3.5 ± 1.2 Å) on the ZIKV
358 starting structures were simulated as disulfide bonds.[8] Disulfides, which aligned with those simulated
359 for ZIKV, were also simulated for dengue virus serotype 1-4, West Nile virus, and Japanese encephalitis
360 virus isolated protein starting structures. For comparison, the isolated ZIKV protein was also simulated
361 without disulfides.

362 For simulations, the protonation state of titratable residues was chosen using a pH value of 8 (the pH
363 associated with PDB ID: 5IRE).[8] To identify biologically relevant pKa values for titratable flavivirus pro-
364 tein residues, a four-step procedure was performed as follows:

365 Step 1. Initial pKa guesses were estimated with PROPKA3.1[67] using the starting model.

366 Step 2. Protonation states were assigned to titratable residues and a simulation was started.

367 Step 3. pKa values were predicted again using simulated protein coordinates averaged over an early
368 portion of the trajectory. The simulation was continued if the pKa predictions matched pKas chosen in
369 Step 1. Steps 2-3 were repeated with the new pKa values if predictions differed from pKas chosen in
370 Step 1.

371 Step 4. pKa values were predicted again using simulated protein coordinates averaged over an equili-
372 brated portion of the trajectory. Step 2-4 were repeated with the new pKa values if predictions differed
373 from pKas found in Step 3.

374 **Protein flexibility**

375 Protein flexibility, measured as root-mean-square fluctuation (RMSF), was considered as an epitope dis-
376 covery metric. Flavivirus backbone atom coordinate RMSF was calculated over structurally equilibrated
377 5 ns MD trajectory segments using GROMACS[60] as follows:

$$378 \quad RMSF = \sqrt{\frac{1}{N} \sum_{n=1}^N \frac{1}{T} \sum_{t=1}^T |\vec{r}_n(t) - \langle \vec{r}_n \rangle|^2} \quad (1)$$

379 where $\vec{r}_n(t)$ is the position of backbone atom n of a residue with N backbone atoms at time t and $\langle \vec{r}_n \rangle$ is
380 the time average position of atom n and T is the total number of discrete time steps. Rotation and trans-
381 lation of the isolated flavivirus proteins, the soluble portion (residues 1-402) of the partially isolated ZIKV
382 protein, and each ZIKV VLP protein were subtracted before RMSF calculation. For the VLP, mean RMSF
383 amplitudes were calculated for each residue over all proteins to enhance statistical significance, as all
384 proteins which comprise the VLP are quasi-equivalent.[68]

385 **Layered convex hull depths**

386 Solvent accessible surface area of the ZIKV isolated protein and VLP proteins were analyzed using a
387 series of layered convex hulls. A convex hull is the smallest convex polygon which encloses all of the
388 points within a dataset.[69] Each protein residue, (1-501) from chain A, C, and E, was reduced to its
389 center of mass to construct three isolated ZIKV protein models for isolated protein convex hull[70] anal-
390 ysis (S2 Fig). Each residue (for the soluble portion (residues 1-402) of chain A, C, and E) was reduced
391 to its center of mass and run through a symmetry matrix taken from PDB ID: 5IRE to construct a soluble
392 ZIKV VLP model for convex hull analysis (S3 Fig).

393 Convex hull scores were calculated using an extension of a method described by Zheng, W., et al.
394 (2015)[71] as follows:

395 Step 1. The SciPy[72] ConvexHull module was run on all model coordinates.

396 Step 2. Model coordinates which comprised the convex hull were given a score and then removed from
397 the remaining model coordinates.

398 Step 3. Steps 1-2 were repeated until no coordinates remained.

399 Step 4. Residues in the first hull were given a score of one. Residues in the last hull were given a score
400 of zero. Residues in the remaining hulls were given an evenly spaced score between zero and one based
401 on the associated iteration of Steps 1-2. The mean of the scores was calculated for each residue.

402 **Preexisting structural epitope discovery metrics**

403 ElliPro,[27] DiscoTope,[28] BEpro,[29] Epitopia,[30] and cons-PPISP[31] structure-based epitope and
404 peptide discovery algorithms were run using default settings on ZIKV chain A, C, and E individually (res-
405 idues 1-501), and the mean of the results was calculated for each residue. ZIKV temperature factors of
406 chain A, C, and E were obtained from PDB ID: 5IRE,[8] the square root was taken for each value, and
407 the mean was calculated for each residue. Sequence-based intrinsically disordered protein region pre-
408 diction algorithm, IUPred2A,[42, 43] was run using default settings. Spearman correlations between met-
409 rics were calculated using the SciPy[72] spearmanr module. T-tests were performed using the SciPy
410 ttest_ind module (with equal_var set to False).

411 **Linear combinations of metric pairs**

412 Linear combinations of select pairs of structural metrics were calculated using an extension of a method
413 described by Kringelum, J. V., et al. (2012)[73] as follows:

414 For a pair of metrics, difference linear combinations (DLC) were calculated as

$$415 \quad \quad \quad DLC(r, \alpha) = \alpha FS(r) - (1 - \alpha) SS(r) \quad \quad \quad (2)$$

416 where FS(r) represents the residue amplitude of the first partner of the pair, SS(r) represents the residue
417 amplitude of the second partner of the pair, and α represents a weight from a grid of weights ranging
418 from zero to one in increments of 0.005.

419 **Structurally characterized, ZIKV-aligned flavivirus epitopes**

420 Structurally characterized flavivirus epitopes were quantified using an extension of a method described
421 by Stave, J. W. and K. Lindpaintner (2013)[6] and aligned to the ZIKV structure as follows:

422 Step 1. All available ZIKV, dengue virus, West Nile virus, and Japanese encephalitis virus envelope
423 protein-antibody structures[12, 45, 50, 52, 74-91] were compiled from the National Institute of Allergy
424 and Infectious Diseases Immune Epitope Database[92] (IEDB) and the PDB.

425 Step 2. The pairwise distance between each envelope protein atom and each antibody atom was calcu-
426 lated for each antibody within each structure.

427 Step 3. Envelope protein residues which had at least one atom within 4 Å from an antibody atom were
428 identified and compiled for each structure, providing one epitope for each structure.

429 Step 4. Epitope residues were renumbered according to a structural sequence alignment between ZIKV
430 and related flaviviruses reported by Kostyuchenko, V. A., et al. (2016).[8]

431 No envelope protein atoms were found within 4 Å of an antibody for PDB ID: 5LCV[85] and 4C2I[80].

432 For conserved epitope quantification: ZIKV residue evolutionary rates were estimated using the cryo-EM
433 chain A with ConSurf[22] (default settings) and the mean amplitude was calculated over each epitope.
434 The sets of two and seven epitopes with the lowest mean residue evolutionary rates[12, 52, 76, 78] were
435 compiled (Table 3).

436 **Epitope discovery performance benchmarking and rationale for a new method**

437 Because of the high structural similarity between ZIKV and other flaviviruses,[8] epitopes of other fla-
438 viviruses were considered as benchmark epitopes for ZIKV.

439 Epitope discovery performance benchmarking was extended from methods described by Ponomarenko,
440 J., et al. (2008)[27] and Kringelum, J. V., et al. (2012)[73]. Two limitations of previous epitope discovery
441 performance benchmarking approaches were addressed here. One limitation is the use of receiver op-
442 erating characteristic curves on imbalanced datasets (a higher proportion of non-epitope residues (neg-
443 atives) to epitope residues (positives)), which can overestimate how well a metric performs at identifying
444 epitope residues (positives).[25] This was overcome by benchmarking performance with precision-recall
445 area under the curve (PRAUC)[93] analysis in conjunction with receiver operating characteristic area

446 under the curve (ROCAUC)[94] analysis. A second limitation of previous studies is 1) measuring perfor-
447 mance using individual epitopes and 2) then calculating the mean of the results. This was overcome by
448 1) benchmarking performance on a compilation of all epitopes using five stratified train-test data splits
449 and then 2) calculating the mean of the results.

450 To understand why using of receiver operating characteristic curves to analyze epitopes individually can
451 overestimate how well a metric performs at identifying epitope residues, one has to look at 1) the size
452 discrepancy between an epitope and a pathogen protein and 2) the meaning of ROCAUC:

453 First, the average ZIKV-aligned flavivirus epitope is only 1.83% of the total number (402) of soluble en-
454 velope protein residues.

455 Second, ROCAUC quantifies, over all thresholds, the combined probability of finding an epitope residue
456 (positive) above a threshold over all epitope residues (positives) (true positive rates) and the probability
457 of finding a non-epitope residue (negative) below the same threshold over all non-epitope residues (neg-
458 atives) (true negative rates) (Fig 5). A 0.50 ROCAUC provides a baseline for performance no better than
459 random chance. Although ROCAUC is a valuable benchmark, when the proportion of non-epitope resi-
460 dues (negatives) is more pronounced than the proportion of epitope residues (positives), the probability
461 of finding a non-epitope residue (negative) below a threshold over all non-epitope residues (negatives)
462 (true negative rate) holds more weight within the combined probability than the probability of finding an
463 epitope residue (positive) above a threshold over all epitope residues (positives) (true positive rate). In
464 this case, ROCAUC provides more information about metric capacity to rank non-epitope residues (neg-
465 atives) below epitope residues (positives) (true negative rates) than metric capacity to rank epitope res-
466 idues (positives) above non-epitope residues (negatives) (true positive rates), which can mask weak true
467 positive rates.[25]

468

469 **Fig 5. ROCAUC and PRAUC benchmarking.** a) Possible prediction outcomes (used for
 470 ROCAUC and PRAUC benchmarking) (e.g. true positives) are depicted with black and white
 471 wedges. The vertical center line separates all positives and all negatives within the dataset. The
 472 center circle contains prediction outcomes for elements above a metric amplitude threshold. b)
 473 Rates (e.g. precision and recall) associated with possible prediction outcomes (used for
 474 ROCAUC and PRAUC benchmarking) are depicted with wedges and division bars.

475 Because flavivirus epitopes are much smaller than the isolated proteins, benchmarking on individual
 476 epitopes (as done in the development of other epitope discovery algorithms)[5, 27] results in datasets

477 with a larger proportion of non-epitope residues (negatives) to epitope residues (positives) – introducing
478 bias.

479 To understand why the use of precision-recall curves can more transparently reveal how well a metric
480 performs at identifying epitope residues (positives) than the use of receiver operating characteristic
481 curves, one must look at the meaning of PRAUC. PRAUC quantifies, over all thresholds, the combined
482 probability of finding an epitope residue (positive) above a threshold over all epitope residues (positives)
483 (recalls or true positive rates) and the probability of finding an epitope residue (positive) above the same
484 threshold over all residues (positives and negatives) also above the same threshold (precisions) (Fig 5).
485 The proportion of epitope residues (positives) in the dataset provides a baseline for performance no
486 better than random chance. PRAUC is less biased than ROCAUC when there is a higher proportion of
487 non-epitope residues (negatives) to epitope residues (positives) because, unlike ROCAUC, the proba-
488 bility of identifying non-epitope residues (negatives) below a threshold over all non-epitope residues
489 (negatives) (true negative rate) is given no weight.[25]

490 Epitope discovery benchmarking was performed as follows:

491 Step 1. In the first case, positives were defined as all structurally characterized, ZIKV-aligned flavivirus
492 epitope residues and negatives were defined as all remaining flavivirus protein residues (from ZIKV en-
493 velope protein residues 1-402) (Table 1). In the second case, positives were defined as residues from
494 the two and seven most conserved ZIKV-aligned flavivirus epitopes (Table 2).

495 Step 2. The epitope dataset was split into test (20%) and training (80%) datasets five times using the
496 scikit-learn[95] StratifiedKFold module (n_splits = 5 and shuffle = True).

497 Step 3. For lone metrics, ROCAUC and PRAUC were calculated on the test datasets using the scikit-
498 learn[95] roc_curve, precision_recall_curve, and auc modules and the means were taken.

499 Step 4. For linear combinations of metric pairs (as described in the *Linear Combination Metric Pairs*
500 section above), ROCAUC and PRAUC were first calculated on the training datasets.

501 Step 5. The mean linear combination weights which produced the highest mean PRAUC and then the
502 highest mean ROCAUC over the training datasets in Step 4 were identified.

503 Step 6. Using the optimized weights obtained in Step 5, ROCAUC and PRAUC were calculated against
504 the associated test datasets and the means were taken.

505 **Epitope organization and epitope discovery demonstration**

506 After epitopic residues have been identified they must be connected to create whole epitopes – in the
507 same way a collection of notes must be connected to form a song. Epitopes can be thought of as collec-
508 tions of subsequences along protein chains e.g. a continuous epitope has one subsequence and a dis-
509 continuous epitope with two parts has two subsequences. From this, each epitope was considered to
510 have three major organizational properties: 1) a number of distinct subsequences, 2) a distribution of the
511 number of residues in each subsequence, and 3) a number of total residues.

512 Experimentally characterized epitope subsequences were defined here through an extension of a defi-
513 nition described by Rubinstein, N. D., et al. (2008) and Sivalingam, G. N. and A. J. Shepherd (2012)[96,
514 97] as one or more positives (including internal stretches of two or less negatives) bordered by stretches
515 of three or more negatives on either side of the protein chain (when all epitopic residues were assumed
516 to reside on an isolated protein). Predicted epitope subsequences were defined here as one or more
517 residues above the prediction threshold (including internal stretches of two or less residues below the
518 prediction threshold) bordered by stretches of three or more residues below the prediction threshold on
519 either side of the protein chain.

520 Epitope discovery was extended from methods described by Ponomarenko, J., et al. (2008)[27] and
521 Kringelum, J. V., et al. (2012)[73] and performed as follows:

522 Step 1. For the top performing metric, a grid of metric amplitude thresholds (determined via the scikit-
523 learn[95] precision_recall_curve module) and residue-residue distance cutoffs (1-10 Å in increments of
524 1 Å) was constructed.

525 Step 2. An amplitude threshold and residue-residue distance cutoff were chosen from the grid.

526 Step 3. Residues above the threshold chosen in Step 2 were clustered using the chosen residue-residue
527 distance cutoff and a clustering method described by Ponomarenko, J., et al. (2008).[27]

528 Step 4. The mean number of distinct subsequences, the mean number of residues in each subsequence,
529 and the mean number of total residues were calculated for residue clusters identified in Step 3.

530 Step 5. The mean number of distinct subsequences, the mean number of residues in each subsequence,
531 and the mean number of total residues were calculated for experimentally characterized epitopes.

532 Step 6. The root-mean-square difference between quantities obtained in Step 4 and 5 was taken.

533 Step 7. Steps 2-6 were repeated until all grid points were exhausted.

534 Step 8. Residue clusters associated with the grid point which minimized the root-mean-square difference
535 calculated in Step 6 (sans those which only consisted of residues 401-402 because the transmembrane
536 region was not included in soluble protein simulations) were predicted as epitopes.

537 To compare the similarity of predicted epitopes with characterized epitopes, the Jaccard Similarity in-
538 dex[49] was calculated using residue numbering along the protein chain.

539 **Author contributions**

540 DWB, JSG, and PJO were involved in conceptualizing and writing an early draft of the manuscript. DWB
541 was involved in all other aspects of the manuscript including the discoveries involving protein flexibility
542 and conserved epitopes.

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Supporting information

801

802 **S1 Fig. Flavivirus isolated protein flexibility correlation plots.** Correlation plots for ZIKV
803 isolated protein RMSF vs. dengue serotype 1 (DENV-1) isolated protein RMSF, dengue sero-
804 type 2 (DENV-2) isolated protein RMSF, dengue serotype 3 (DENV-3) isolated protein RMSF,
805 dengue serotype 4 (DENV-4) isolated protein RMSF, West Nile virus (WNV) isolated protein
806 RMSF, and Japanese encephalitis virus (JEV) isolated protein RMSF are shown. Spearman rho
807 (r) and p-values are also shown.

808

809 **S2 Fig. ZIKV isolated protein convex hull scoring.** The a) lowest scoring (most inner) and b)
 810 median scoring (middle) and c) highest scoring (most outer) isolated protein residue center of
 811 masses obtained from convex hull analysis are shown.

812

813 **S3 Fig. ZIKV VLP protein convex hull scoring.** The a) lowest scoring (most inner) and b)
 814 median scoring (middle) and c) highest scoring (most outer) VLP protein residue center of
 815 masses obtained from convex hull analysis are shown.

Metric	ROCAUC	±	PRAUC	±	Rho	p
hmon	0.74	0.18	0.21	0.16	---	---
opia	0.69	0.13	0.14	0.14	0.61	< 0.01

bpro	0.64	0.12	0.10	0.12	0.51	< 0.01
fmon	0.71	0.18	0.07	0.15	0.48	< 0.01
hvlp	0.66	0.24	0.07	0.10	0.43	< 0.01
epro	0.72	0.20	0.06	0.11	0.88	< 0.01
tfac	0.58	0.22	0.05	0.09	0.59	< 0.01
dtope	0.70	0.16	0.04	0.03	0.61	< 0.01
fvlp	0.62	0.21	0.04	0.04	0.45	< 0.01
iupred	0.54	0.15	0.02	0.02	0.08	0.12
ppisp	0.52	0.17	0.02	0.02	0.11	0.03

S1 Table. Epitope discovery performance benchmarking on individual epitopes. Metrics are ordered from the top to bottom in terms of highest ROCAUC and PRAUC product. Spearman rho (r) and p-values are shown for associations between isolated protein convex hull scores (hmon) vs. Epitopia scores (opia), BEpro scores (bpro), isolated protein RMSF (fmon), VLP protein convex hull scores (hvlp), ElliPro scores (epro), temperature factors (tfac), DiscoTope scores (dtope), VLP protein RMSF (fvlp), IUPred scores (iupred), and cons-PPISP scores (ppisp).

Metric	ROCAUC	±	PRAUC	±	Rho	p
zikv	0.69	0.06	0.45	0.12	---	---
jev	0.61	0.10	0.46	0.13	0.76	< 0.01
denv2	0.57	0.11	0.43	0.22	0.82	< 0.01

wnv	0.58	0.11	0.41	0.14	0.77	< 0.01
denv4	0.68	0.05	0.33	0.12	0.86	< 0.01
denv3	0.54	0.13	0.41	0.19	0.85	< 0.01
denv1	0.57	0.11	0.38	0.26	0.76	< 0.01

S2 Table. Flavivirus isolated protein flexibility: conserved epitope discovery performance

benchmarking. Isolated protein flexibility of seven flavivirus structures is examined for epitope discovery performance against the top seven ZIKV-aligned, conserved flavivirus epitopes. Metrics are ordered from the top to bottom in terms of highest ROCAUC and PRAUC product. Spearman rho (r) and p-values are shown for associations between ZIKV isolated protein RMSF (zikv) vs. Japanese encephalitis virus isolated protein RMSF (jev), dengue serotype 2 isolated protein RMSF (denv2), West Nile virus isolated protein RMSF (wnv), dengue serotype 4 isolated protein RMSF (denv4), dengue serotype 3 isolated protein RMSF (denv3), and dengue serotype 1 isolated protein RMSF (denv1).