The ganglioside GM1a functions as a coreceptor/attachment factor for dengue virus during infection

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Dengue virus (DENV) is a flavivirus causing an estimated 390 million infections per year around the world. Despite the immense global health and economic impact of this virus, its true receptor(s) for internalization into live cells has not yet been identified, and no successful antivirals or treatments have been isolated to this date. This study aims to improve our understanding of virus entry routes by exploring the sialic acid–based cell surface molecule GM1a and its role in DENV infection. We studied the interaction of the virus with GM1a using fluorescence correlation spectroscopy, fluorescence crosscorrelation spectroscopy, imaging fluorescence correlation spectroscopy, amide hydrogen/deuterium exchange mass spectrometry, and isothermal titration calorimetry. Additionally, we explored the effect of this interaction on infectivity and movement of the virus during infection was explored using plaque assay and fluorescence-based imaging and single particle tracking. GM1a was deemed to interact with DENV at domain I (DI) and domain II (DII) of the E protein of the protein coat at quaternary contacts of a fully assembled virus, leading to a 10-fold and 7-fold increase in infectivity for DENV1 and DENV2 in mammalian cell systems, respectively. We determined that the interaction of the virus with GM1a triggers a speeding up of virus movement on live cell surfaces, possibly resulting from a reduction in rigidity of cellular rafts during infection. Collectively, our results suggest that GM1a functions as a coreceptor/attachment factor for DENV during infection in mammalian systems.

Dengue virus (DENV) is a mosquito-borne enveloped virus, from the Flaviviridae family. It is transmitted from human to human by the bite of an infected mosquito (Aedes aegypti, and occasionally Aedes albopictus) with symptoms ranging from fever, muscle and joint pain (dengue fever), to a more life-threatening hemorrhagic fever or shock syndrome, in both adults and children alike (1–3). It has been estimated that there are as many as 390 million dengue infections per year, spread throughout 128 countries, of which 96 million manifest clinically with symptoms (4–6), posing a major global health impact.

DENV has four identified serotypes (DENV1, DENV2, DENV3, and DENV4). It has a diameter close to 500 Å with an outermost protein shell that is embedded in the host-derived lipid bilayer, which in turn encapsulates the ~11 kb single stranded positive sense RNA genome. The genomic RNA encodes ten viral proteins, out of which three are structural (capsid (C), the premembrane or membrane (prM or M, respectively) and the envelope (E) proteins) and seven are nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) in function (1, 4, 7–9). The outermost protein shell of mature DENV is made up of 180 copies of E and 180 copies of M proteins arranged in an icosahedral manner (4). The E protein is the major antigenic structure on the surface of the virus and is involved in receptor and attachment factor binding (4, 8, 10–16). Infectious DENV particles interact with attachment factors on the cell membrane, followed by movement along the cell surface in its search for its receptors and coreceptors, which internalize it into its host cell (13–15, 17, 18).

Current research work and literature highlight a number of receptors/coreceptors/attachment factors involved in DENV internalization, with no definitive answer on what factors are truly the most critical for virus internalization. The adhesion molecule of dendritic cells DC-SIGN, endoplasmic reticulum chaperonin GRP-78, the 37/67 kDa high-affinity laminin receptor, heat shock proteins 70 and 90, glycosaminoglycans such as heparan sulfate, heparan sulfate proteoglycans, and lectins, TIM and TAM proteins, mannose receptor of macrophages, lipopolysaccharide receptor CD14, glycosphingolipids, claudin-1 are a few among these explored receptors and attachment factors for DENV in mammalian cell systems (1, 12, 14, 19–22, 23–27). Having a broad range of receptors and entry routes, DENV appears to possess the ability to infect many different types of cells, using many different mechanisms, and may use entry routes more ubiquitous in nature and reflective of the diversity in cell surface composition.

In this work, we focus on the ganglioside GM1a and describe its involvement in DENV infection in mammalian cell

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systems. GM1a is a glycosphingolipid, with a glycan part and lipid portion, which contributes to the glycoalyx and lipidome of cells, respectively. It possesses a terminal sialic acid moiety, which interacts with cargo, such as Cholera Toxin B (CTxB), viruses, and bacteria, to internalize them into cells (28–33). Sialic acids of the ganglioside family are ubiquitously found in most mammalian cells and are important for cell signaling, cell adhesion, and many other cellular functions (34–40). Mammalian cells have a dense glycoalyx, and more often than not, the first point of contact for any pathogen will be the glycan interaction points on cell surfaces. Interestingly, more than half of all known mammalian viruses are reported to interact with glycans during internalization (41–47).

One such virus is the Influenza A virus (Orthomyxoviridae), which shows interaction with sialic acid moieties on host cell surfaces during infection (43, 45, 47–50). The virus surface is decorated with two surface glycoproteins, hemagglutinin (H/HA) and neuraminidase (N/NA). The hemagglutinin is responsible for interacting with the sialic acid moiety (Neu5Ac) on cell surfaces and initiates viral internalization, possibly with the help of another more proteinaceous receptor (51–54). Paramyxovirus (Paramyxoviridae) binds sialic acid residues in a similar fashion to Influenza A virus, where the virus surface hemagglutinin-neuraminidase (HN) glycoprotein binds to sialic acid on host cells, mediating virus internalization (55). Newcastle disease (NDV), Sendai, mumps, and parainfluenza viruses are reported to recognize glycans with the terminal NeuAca2-3Gal linkage (45, 56). Simian virus 40 (SV40) is reported to be highly specific for GM1 binding, where the capsid protein, VP1, forms a complex with the carbohydrate portion of GM1 (45, 50, 57–59). Coronavirus (Coronaviridae) surfaces hold two glycoproteins, a spike protein and hemagglutinin-esterase, which can both interact with glycans as primary/coreceptors or as attachment factors. Feline coronavirus, transmissible gastroenteritis virus, porcine epidemic diarrhea, have all been reported to bind sialic acid host at cell receptors (NeuAc/NeuGc) via the spike protein as a secondary receptor (60). The spike on Human Respiratory Coronavirus (OC43) and Human Coronavirus (HKU1) is reported to interact with NeuAc as a primary receptor, while Middle East respiratory syndrome binds sialic acid with a preference for an α2,3-link (45, 50, 61–63). Picornaviruses (Picornaviridae) Coxsackie A24, human enterovirus 68, and Murine encephalomyocarditis virus, all bind sialic acid receptors at the NeuAca2-6Gal and/or NeuAca2-3Gal sites on N-glycans (45, 64–66). It has been recently shown that sialic acid–containing glycolipids including GM1 mediate binding and viral entry of SARS-CoV-2 (67). Various viruses in the families of Polyomavirus (Polyomaviridae), Parovirus (Paroviridae), Rotavirus and Orthoreovirus (Reoviridae), Caliciviruses (Caliciviridae), and Mammarella viruses in the family of Arenaviruses (Arenaviridae) interact with sialic acid containing glycans during cellular infection (43, 45, 68–73), making sialic acid containing glycan receptors a widely utilized entry route.

In this work, the interaction of DENV1 (PV159) and DENV2 (NGC strain) with the sialic acid ganglioside GM1a was explored and confirmed by fluorescence cross correlation spectroscopy (FCCS), fluorescence colocalization studies, fluorescence single particle tracking (SPT), and imaging fluorescence correlation spectroscopy (FCS) (ImFCS). The interaction site of GM1a with DENV was mapped by amide hydrogen/deuterium exchange mass spectrometry (HDMS) and corroborated by isothermal titration calorimetry (ITC). The impact on infectivity of this interaction of DENVV1 and DENV2 with GM1a was tested by plaque assays, and it is shown that both DENVV1 and DENV2 show increased infectivity in the presence of GM1a. Taken together, our results show that both DENV1 and DENV2 interact with GM1a on mammalian cells, resulting in their increased infectivity. In addition, GM1a functions as an attachment factor/receptor/coreceptor during virus internalization.

Results

Interaction of dengue virus with GM1a on live mammalian cells

The involvement of GM1a during DENV infection was probed using colocalization studies and quasi pulsed interleaved FCCS (qPIE-FCCS) (74) in live Vero cells. Colocalization studies and time-lapse imaging carried out on a confocal microscope showed that DENV1 and DENV2 particles each colocalized and moved together with GM1a labeled with Bodipy FL (GM1a-Bodipy) on live cell surfaces (Fig. S1 and Table S1). For a more direct measurement of interactions, qPIE-FCCS experiments on the confocal microscope were conducted between GM1a-Bodipy and DENV-labeled with Alexa Fluor 555 NHS (Figs. 1 and S2). The degree of interaction in qPIE-FCCS was semiquantitatively evaluated using the so-called q-value, which is proportional to the interaction detected (see Experimental procedures section). As negative control, we used DiIC18(3) and GM1a-Bodipy, two lipids not expected to interact as they partition into liquid disordered or liquid ordered regions, respectively. As positive control, we used PMT-mEGFP-mApple, a plasma membrane targeting sequence where both red and green fluorophores are linked to each other and thus should provide a maximum of cross correlation achievable with the setup (75). While qPIE-FCCS negative and positive controls showed q-values of 0.03 ± 0.01 (Avg ± SEM; cells = 6) and 0.45 ± 0.01 (Avg ± SEM; cells = 10), respectively, DENV1 and DENV2 showed intermediate q-values of 0.15 ± 0.02 (Avg ± SEM; cells = 19) and 0.21 ± 0.02 (Avg ± SEM; cells = 58), indicating their interaction with GM1a. As an additional control, we measured the interaction between GM1a and CTxB, a known GM1a-binding protein (28, 29), which yielded a q-value of 0.25 ± 0.04 (Avg ± SEM; cells = 9). The fluorescent protein tandem dye mEFP-mApple is a standard calibration system we use for live cell measurements. But, it does not fully reflect the maximum amount of cross correlation that could in principle be achieved by the organic dyes Bodipy and Alexa Fluor 555, due to the general problem of fluorescent protein maturation and bleaching (75). However, the q-values calculated give a relative comparison of how well the two DENV strains interact with GM1a under these experimental conditions.
Figure 1. Representative FCCS curves obtained by quasi-PIE FCCS. A, FCCS positive control of PMT-mEGFP-mApple with a q-value of 0.54. B, FCCS negative control of noninteracting partners GM1a-Bodipy and DiIC18 with a q-value of 0. C, GM1a interaction with DENV1 with a q-value of 0.15. D, GM1a interaction with DENV2 with a q-value of 0.40. E, interaction of GM1a with CTXB with a q-value of 0.51. F, q-value distribution of DENV1 and DENV2 interaction with GM1a compared with the negative and positive controls. Overall q-values of 0.15 ± 0.02 (cells = 19), 0.21 ± 0.02 (cells = 58), 0.45 ± 0.01 (cells = 10), 0.03 ± 0.01 (cells = 6) for DENV1, DENV2, positive control of PMT-mEGFP-mApple and negative control with DiIC18 (Avg ± SEM). Error bars represent the SD. All cells were pretreated with D-PDMP to inhibit endogenous GM1a production. DENV, dengue virus; D-PDMP, (+)-D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (hydrochloride); FCCS, fluorescence cross correlation spectroscopy; quasi-PIE, quasi pulsed interleaved.
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*Mapping the binding hotspots of GM1a on DENV E-protein*

We performed HDXMS on free DENV2 and DENV2 in the presence of the GM1a sugar moiety to identify the binding hotspot of GM1a on the DENV viral surface. Thirty-six pepsin proteolyzed peptides were obtained with high signal to noise ratios and covering 63% of the E protein sequence (Fig. S3). We mapped the interactions of E-protein with saturating concentrations of GM1a at (125:1) stoichiometry of GM1a to E-dimer. Comparative deuterium exchange analysis was performed between free and GM1a-bound DENV2 using a deuterium exchange difference plot. A deuterium exchange difference plot (Fig. 2A) shows the difference in deuterons exchanged in DENV2:GM1a compared to free DENV2 at 1 min of deuterium labeling time for each pepsin fragment peptide. In presence of GM1a, peptides 40 to 57, 129 to 136, and 322 to 335 showed lower deuterium exchange (Fig. 2A). Peptides 40 to 57 and 129 to 136 span the domain I (D1) and domain II (DII) of the E protein. Peptide 322 to 335 is localized at the 5-fold symmetry axis of the virion (Fig. 2D). Previous reported crystal structure of the E protein complexed with carbohydrate molecule β-octyl glucopyranoside (βOG pocket) (Protein Data Bank: 1OKE) showed a binding site for sugar molecules spanning DI and DII (76) consistent with HDXMS. We also performed control HDXMS experiments to map interactions of GM1a with recombinant DENV2 E protein/soluble E protein (sE protein). We observed no changes in deuterium exchange in the presence of GM1a (Fig. S4). This indicated that GM1a binding occurs only at quaternary contacts of E-protein in a whole icosahedral assembled virus to bind to the surface. Furthermore, DENV1 and DENV2 both show a ~67% sequence similarity of the E protein (Fig. S5), therefore it can be expected that DENV1 may also bind GM1a in a similar fashion to that of DENV2. It is important to note that the ~37% E protein regions lacking coverage in our experiments that span domain II (peptide 59–92) and domain I/domain III (peptide 280–317) are surface exposed and mediate quaternary contacts in the virion. Due to the lack of pepsin fragment peptide coverage in these regions, we are unable to ascribe any role of these regions in binding to GM1a.

We further used ITC to investigate binding between the recombinant DENV2 E protein and GM1a. As shown in Fig. 6, the isotherms resulting from titrating GM1a into dengue E protein exhibit a biphasic structure. The initial integrated heats of injection show a trend toward increasingly negative enthalpy, while later data show a trend of decreasing enthalpy until saturation is reached. This biphasic appearance is consistent with the system indicating two-site binding (77). The applied asymmetric and sequential two binding site model revealed enthalpies of binding in the range of 10 mM to 1 μM. Since GM1a solution precipitated when prepared at >300 μM concentration, we could not reach the enthalpy saturation in binding isotherms. Therefore, it is difficult to quantify binding enthalpies of both GM1a interacting sites on the E protein. Nevertheless, the obtained biphasic isotherms corroborate the binding between dengue E protein and GM1a.

*GM1a assists and increases infection in DENV1 and DENV2*

The biological significance of the proximity of GM1a and DENV observed by qPIE-FCCS and colocalization studies was further investigated on live baby hamster kidney strain 21 (BHK21) mammalian cells by conducting plaque assays. The infectivity levels were compared for untreated cells, GM1a-depleted cells, and GM1a-enriched cells (Figs. 3 and S7). GM1a depletion was achieved by (+)-D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (hydrochloride) [D-PDMP] treatment, while GM1a enrichment was by bovine serum albumin (BSA) loading (see Experimental procedures). DENV1 and DENV2 both show a similar trend in infectivity with relation to GM1a on cell surfaces, with a significant increase in infectivity seen for the GM1a-enriched cells as compared to the GM1a-depleted cells. DENV1 shows an increase in infectivity in GM1a-enriched cells with an average value of 1.3 × 10^8 PFU/ml, as compared to 5.7 × 10^5 PFU/ml for D-PDMP treated (GM1a depleted) cells (Fig. 3A). While DENV2 shows an increase in infectivity in cells enriched with GM1a, with an average value of 1.3 × 10^5 PFU/ml versus a value of 2.1 × 10^6 PFU/ml in GM1a-depleted plates (Fig. 3B). Both DENV1 and DENV2 show a similar trend of increased infectivity in GM1a enriched cells, as compared to the GM1a depleted and untreated cells. This indicates that the enrichment of GM1a in BHK21 mammalian cells significantly increases infectivity of DENV but is not the only route of entry for the virus, where in the absence of GM1a, the infection is not completely abolished and the virus internalizes by other routes.

*Interaction of GM1a with DENV1 and 2 triggers increased diffusion of virus on the cell surface*

The interaction of DENV with GM1a was further studied using fluorescence based SPT, where the movement of DENV1 and DENV2 was observed in GM1a-depleted and GM1a-enriched (cells were treated with D-PDMP and subsequently enriched with GM1a) live Vero cells. Diffusion coefficients of both DENV1 and DENV2 show an increase in GM1a-enriched cells as opposed to GM1a-depleted cells (Figs. 4, 5 and S8). The diffusion coefficients of DENV1 and DENV2 on cell membranes of GM1a-depleted cells show similarity to each other at 0.005 ± 0.001 μm²/s (Avg ± SEM; tracks = 260) and 0.005 ± 0.001 μm²/s (Avg ± SEM; tracks = 487), respectively. In GM1a-enriched cells, both DENV1 and DENV2 (colocalized with GM1a) showed increased diffusion coefficient of 0.010 ± 0.002 μm²/s (Avg ± SEM; tracks = 86) and 0.015 ± 0.002 μm²/s (Avg ± SEM; tracks = 417), respectively, which is an increase in D from its movement in GM1a-depleted cells (p = 0.0676 and p < 0.0001, respectively, where the latter shows a difference that is statistically significant at 95% confidence interval) (Fig. 4).

The changes in diffusion of DENV on cell membranes might also indicate a change in the mode of diffusion. GM1a is located in lipid rafts of mammalian cell membranes, and any changes in organization may influence the diffusion of cargo associated with GM1a on cell membranes (77, 78). For this
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Figure 2. HDXMS reveal GM1a sugar moiety binding hotspot on DENV2 NGC surface on E protein. A, differences in average number of deuterons exchanged (y-axis) in DENV2:GM1a state and free DENV2 state are plotted in a deuterium uptake difference plot for t = 1 min labeling time. Pepsin digested peptides are listed from N to C terminus (x-axis). Peptide regions showing high protection in the presence of GM1a are highlighted. SDs are shaded gray and plots were generated using DynamX 3.0 software. The data is uncorrected for maximum deuterium content of 90% under experimental conditions and unadjusted to accommodate loss in deuterons exchanged due to back-exchange (average back-exchange ~15% under our HDXMS conditions). B, representative mass spectra of peptides protected in the presence of GM1a, that is, peptide 44 to 58 and 322 to 335 are shown and dotted lines are shown at the centroid of mass spectra. C, differences in deuterium exchange between DENV2-GM1a bound and free DENV2 are mapped onto a E protein dimer (PDB: 3J27) with differences highlighted as per key. D, peptides protected in the presence of GM1a sugar are mapped in blue onto the full virus (PDB: 3J27). Red spheres represent glycans on viral surface. DENV, dengue virus; HDXMS, hydrogen/deuterium exchange mass spectrometry; PDB, Protein Data Bank.
purpose, we performed ImFCS measurements on live Vero cells transfected with the raft marker GFP-GPI and pre-enriched with GM1a, to compare changes that occur before and after overlay of DENV. ImFCS is capable of determining the diffusion mode of cell membrane components by plotting the average time a particle needs to transit an observed area as a function of the area size (79). The intercept of this plot, denoted as $\tau_0$, is 0 for free diffusion but positive for hindered diffusion by trapping in domains. Therefore, GFP-GPI as a raft marker exhibits a positive $\tau_0$ under normal conditions and functions as sensor of changes to the membrane organization. In our case, the GFP-GPI $\tau_0$ values reduced from 0.53 ± 0.30 s before (cells = 6, with three sets of 21 × 21 pixel areas per cell for ImFCS) to 0.34 ± 0.15 s after DENV1 addition ($p = 0.0131$, difference is statistically significant at 95% confidence interval). While in the case of DENV2, a similar but smaller trend was observed where $\tau_0$ showed a reduction from 0.68 ± 0.31 s to 0.51 ± 0.30 s (cells = 5, with three sets of 21 × 21 pixel areas per cell for ImFCS) in the absence and presence of DENV2, respectively ($p = 0.1382$, difference not statistically significant.) (Fig. 6). This reduction in $\tau_0$ for GFP-GPI raft marker indicates a change in the probe diffusion mode from transient domain confined to a free diffusion, which in turn indicates a change of the raft organization tending toward a slightly less rigid organization allowing more freedom of movement for lipids and embedded proteins. This reduction in rigidity could be attributed to the faster movement of both DENV1 and DENV2 when colocalized with GM1a, as observed in 2D SPT trajectory data. These results were compared with ImFCS studies before and after addition of CTxB (labeled with AlexaFlour555) under the same conditions. In the case of CTxB, however, there was an inversion in the GFP-GPI $\tau_0$ value, where there was an increase in $\tau_0$ from 0.58 ± 0.50 s to 1.44 ± 0.75 s (cells = 4, with three sets of 21 × 21 pixel areas per cell for ImFCS) before addition of CTxB and after the addition of CTxB ($p = 0.0032$, difference is statistically significant.) (Fig. 6). This indicates a possible increase in the rigidity of the lipid raft after CTxB binding. CTxB is known to bind multiple GM1a receptors (up to five) and is reported to stabilize raft domains via a lipid crosslinking mechanism (80, 81), leading to an increased rigidity to the lipid raft they reside on, which could lead to the GFP-GPI probe to experience a more rigid raft environment, leading to the increase in $\tau_0$. Interestingly, however, in the case of DENV1 and DENV2, the lipid raft region tends toward decreasing rigidity, indicating a difference in the way the virus binds to GM1a as compared to that of CTxB. The integrity of the plasma membrane after D-PDMP treatment and GM1a enrichment was checked by conducting FCS studies on GFP-
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Discussion

DENV is known to enter mammalian cells using many different entry mechanisms, while utilizing many different types of receptors/coreceptors/attachment factors. The range of receptors and cells DENV is reported to infect, hints toward a more ubiquitous form of entry which is available on many cells. Any virus when approaching the cell surface must find its way to these entry points for successful access into the host system. The mammalian cell membrane consists of a mosaic of lipids and proteins, which are known to form liquid ordered (rafts) and liquid disordered regions, and encompass various receptors both proteinaceous and others. The cell surface is decorated with sugar antennas, which form the glycosylyx, extending outward toward the extracellular environment, to regions beyond the reach of cellular receptors, which forms the first barrier that any virus will encounter as it approaches the cell surface. Thus, it is important to note that any virus that internalizes into the host cell must travel past this first barrier, and therefore, it is biologically important to identify how sugar-based molecules are involved in the virus infection process.

In this work, we focus on GM1a, which is a glycosphingolipid with a sialic acid–based sugar found ubiquitously on mammalian cell surfaces, with its sugar moiety located within the glycosylyx. Sialic acid–based sugars have been widely reported to be involved in internalizing many different types of viruses, including influenza A and SARS-CoV-2. We explore the involvement of GM1a and its sialic acid moiety in DENV internalization by using real-time fluorescence microscopy techniques. GM1a labeled with Bodipy FL colocalizes with both DENV1 and DENV2 on live Vero cell systems, indicating binding of the viral cargo onto the GM1a moiety. This binding of DENV with GM1a is further confirmed by our FCCS results, where a positive $q$ value of 0.15 ± 0.02 and 0.21 ± 0.02 for DENV1 and DENV2 was observed. The biological significance of this binding of DENV with GM1a and its effect on infectivity of DENV1 and DENV2 was then explored by plaque assay, where it was evident that the presence of GM1a on mammalian cell surfaces significantly increases the infectivity of both DENV1 and DENV2 as compared to the GM1a-depleted cells. The association of DENV with GM1a shows a significant effect on the infection process of DENV, and being an ubiquitously available molecule on mammalian cell surfaces, it acts as a more universal interacting partner during virus internalization.

The sialic acid receptor GM1a is reported to bind CTxB protein cargo via the two terminal sugars (galactose and sialic acid) on the receptor, in the form of a two fingered grip, involving ionic interactions between the positively charged protein cargo and the negatively charged sialic acids, along with solvent-mediated hydrogen bonding (28). Hemagglutinin on influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)) with Avian influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)) with Avian influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)) with Avian influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)) with Avian influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)) with Avian influenza viruses binds sialic acid receptors (commonly N-acetyl neuraminic acid (Neu5Ac)).

In this work, the binding of DENV2 virus to the sugar moiety of GM1a occurs in the regions spanning across domain I (DI) and domain II (DII) of the E protein. Recombinant DENV2 E protein alone does not significantly bind GM1a under our experimental HDXMS conditions. This indicates...
that GM1a requires quaternary contacts of a fully assembled virus to bind to the surface. The binding of GM1a sialic acid moiety may follow ionic interactions between the negatively charged sugar and the more positively charged virus surface E protein, along with possible solvent mediated H-bonding to further stabilize the interaction. This proposition was further strengthened by the interaction between GM1a and recombinant DENV2 E protein at high concentrations of both GM1a and E protein in ITC experiments. The E proteins of both DENV1 and DENV2 show sequence similarity of ~67%, indicating preservation of similarity of E protein arrangement of the two variants. Thus, DENV1 interaction with GM1a sialic acid may show a similar binding pattern to that of DENV2.

DENV1 and DENV2, once colocalized with GM1a on live cell surfaces, show movement along the cell membrane. This might be a viable strategy to find other receptors or internalization sites. In this work, both DENV1 and DENV2 show an increase in movement once they are colocalized with GM1a, as opposed to the GM1a-depleted states, indicating that once attached to GM1a, the sialic acid receptor promotes faster movement of the virus on live cell surfaces. Thus, we hypothesize that GM1a acts as an attachment factor or

Figure 5. 2D-SPT trajectories of DENV movement on live Vero cell surface. A, DENV1 in the absence of GM1a (i) trajectory on live cells, (ii) MSD curve. B, DENV1 in the presence of GM1a (i) trajectory on live cells, (ii) MSD curve. C, DENV2 in the absence of GM1a (i) trajectory on live cells, (ii) MSD curve. D, DENV2 in the presence of GM1a (i) trajectory on live cells, (ii) MSD curve. (Scale bar = 2.5 μm). DENV, dengue virus; MSD, mean square displacement; SPT, single particle tracking.
coreceptor for both DENV1 and DENV2 in assisting its internalization and infection process into live mammalian cells.

This increase in movement of DENV1 and DENV2 goes hand in hand with changes in cell membrane organization. Once DENV associates with GM1a, there is an increase in fluidity of the membrane raft regions, indicated by the decrease in GFP-GPI raft marker \( r_0 \) value obtained by ImFCS. This indicates a change in the probe diffusion mode from transient domain confined to a freer diffusion, which in turn indicates a change of the raft organization tending toward a slightly less rigid organization allowing more freedom of movement for lipids and embedded proteins. This change in the lipid rafts possibly allow the DENV–GM1a complex to travel faster on the cell membrane surface. The interaction of DENV with GM1a is thus different to that of CTxB binding to GM1a, as CTxB binding triggers increased crosslinking between GM1a molecules inside the raft region, leading to increased rigidity. Thus, the binding of DENV with GM1a does not appear to require crosslinking of GM1a.

GM1a may act as an important attachment factor/coreceptor for DENV entry into mammalian cells, which support DENV movement to assist finding a receptor or internalization site. Depletion of GM1a reduces, but does not abolish infection, and thus, it is not clear whether GM1a only assists during or is sufficient for infection. The dynamics of DENV binding to GM1a needs to be explored further, as the effect on the cell membrane during binding of virus to GM1a is an interesting phenomenon that is quite different to the normal way GM1a is known to participate in internalizing cargo such as CTxB. Further, the final receptor for DENV1 and DENV2 is not known as of now, and future work may enable identifying “true” receptor(s) involved in GM1a-assisted entry of DENV into mammalian cells.

**Experimental procedures**

**Key resources table**

| Chemical or resource | Source | Identifier |
|----------------------|--------|------------|
| DENV2 New Guinea C strain (NGC) | The laboratory of Michael Rossmann | |
| DENV1 PVP159 strain | The laboratory of Michael Rossmann | |
| Dengue Virus Serotype 2 Envelope Protein | The Native Antigen Company | |
| Hepses (Hyclone) | GE Healthcare life sciences | |
| NaCl | Sigma–Aldrich | |
| AlexaFlour555 NHS ester | Molecular Probes, Life Technologies | A37271 |
| DiI(DIC18(5)-DS) | Molecular Probes, Life Technologies | D12730 |
| 1,10-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiIC18(3)) | Invitrogen | D282 |
| EDTA | Sigma–Aldrich | 60-00-4 |
| Bovine serum albumin (BSA) | Sigma–Aldrich | 9048-46-8 |
| D2O | Cambridge Isotope Laboratory Inc | 7789-20-0 |
| NaOH | Sigma–Aldrich | 1310-73-2 |
| GnHCl | Sigma–Aldrich | 50-01-1 |
| Tris(2-carboxyethyl) phosphate-hydrochloride (TCEP-HCl) | Sigma–Aldrich | 51805-45-9 |
| Titanium dioxide (TiO2) | Sigma–Aldrich | 13463-67-7 |
| RPMI | Sigma–Aldrich | SH30255.FS |
| C6/36 Aedes albopictus mosquito cells | American Type Culture Collection (ATCC) | CRL-1660 |
| Baby hamster kidney fibroblast cells (BHK21) | American Type Culture Collection (ATCC) | CCL-10 |
| Vero (African green monkey kidney cells) | American Type Culture Collection (ATCC) | Vero-81 |
| DYNAXM Ver. 2.0 software | Waters | | |
| D-PDMP[(+)-D-threo-1-Phenyl-2-decanoylamino-3-morpholin-1-propanol (hydrochloride)] | Invitrogen | |
| BODIPY FL C5-Ganglioside GM1 | GE Healthcare | |
| MicroSpin 5-200 HR columns | HyClone, GE Healthcare | |
| Fetal bovine serum (FBS) | Gibco Life Technologies | |
| Penicillin-streptomycin | Thermo Scientific Nunc Lab-Tek Chambered coverglass | |
| 8-well chambered cover glass | HyClone Hank’s 1X Balanced Salt Solutions | |
| HBSS | Gibco | |
| Phenol red free RPMI (with 25 μM Hepses) | Gibco | |
| Ganglioside GM1 (Ovine-Sodium salt) | Avanti Polar Lipids, Inc, Alabama | CAS: 37758-47-7 SKU: 860065P-5mg |
| GFP-GPI | kind gift of Dr John Dangerfield | |
| Electrophoration buffer R and E | Invitrogen, Life Technologies, Singapore | |
| Neon transfection system | Life Technologies | |
| Aquacide-II | Merck | |
| Crystal violet | Sigma–Aldrich | |
| Formaldehyde | Sigma–Aldrich | |
| Defatted BSA | Biowest | |
| TRYPsin-EDTA 10X | Thermo Fisher Scientific | |
| CTXB (Cholera Toxin Subunit B, Alexa Fluor 555 Conjugate) | | |

http://www.waters.com/waters/en_SG/ProteinLynx-Global-SERVER

http://www.waters.com/waters/library.htm?cid=511436&lid=134832928&locale=en_SG

http://www.waters.com/waters/en_SG/ProteinLynx-Global-SERVER

GM1a functions as a coreceptor/attachment factor for dengue
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**Figure 6. Diffusion coefficients and diffusion law intercepts of GFP-GPI raft probe on live Vero cell membrane measured by ImFCS.** A, comparison of diffusion coefficient of the raft-marker GFP-GPI, on cells treated with D-PDMP+GM1a, to observe the effect of DENV1, DENV2, and CTXB interaction with GM1a. B, ImFCS diffusion law intercept \( \tau \), changes of the raft-marker GFP-GPI, on cells treated with D-PDMP+GM1a, to observe the effect of DENV1, DENV2, and CTXB interaction with GM1a. Error bars in both graphs represent the SD. DENV, dengue virus; D-PDMP, (+)-D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (hydrochloride); ImFCS, imaging fluorescence correlation spectroscopy.

**Method details**

**Cell lines**

Vero-81 (RRID:CVCL_0059), C6/36 mosquito cells, and BHK21 fibroblast cells were from the American Type Culture Collection (ATCC). C6/36 were cells derived from larvae of female *Aedes albopictus* mosquito and were adapted to Eagle’s minimum essential medium. Cells were cloned and recloned by seeding single cell suspensions into petri dishes. Further information about the cell lines can be obtained through [Key resources table](#).

**Cell culture**

African green monkey kidney cells (Vero) and BHK21 were cultured in 10% fetal bovine serum (FBS) in RPMI1640 medium with 25 mM Hepes and L-glutamine, with 1% penicillin-streptomycin in 5% CO\(_2\) at 37 °C. Vero and BHK21 cells were a kind gift from Prof Dr Shee-Mei Lok, Duke-NUS Medical School, Singapore. Cells were passaged once they reached 90% confluence, by trypsinization (2 ml 0.25% trypsin-0.03% EDTA solution) for 2 min, and cells were reseeded in new T75 flasks at a 1:5 or 1:10 split ratio with RPMI media (with 10% FBS, 1% PS, 25 mM Hepes and L-Glutamine).

**Virus preparation**

DENV2 NGC and DENV1 PVP159 were produced and purified as previously described (4, 83). C6/36 mosquito cells were infected with DENV2 NGC and DENV1 PVP159 at a multiplicity of infection (MOI) of 0.1 and grown under 5% CO\(_2\). The inoculant was replaced after 2 h with RPMI medium supplemented with 2% FBS and allowed to incubate for 4 days, after which the virus-containing supernatant was clarified from cell debris by centrifugation. The virus particles were collected by precipitation with 8% PEG 8000 in NTE buffer (10 mM Tris–HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA) and purified by centrifugation using a 30% sucrose cushion followed by a 10% to 30% potassium tartrate gradient. The virus band was collected and concentrated with a buffer exchange to NTE, using an Amicon Ultra-4 centrifugal concentrator (Millipore) with a 100 kDa molecular-mass cut-off filter to obtain glycosylated native state virus particles. The virus preparation contained low levels of contamination by immature virus as determined by test for prM with a Coomassie blue–stained SDS-PAGE. The envelope protein concentration was estimated by comparing the corresponding band with that of BSA at different concentrations (4, 82, 83).

**Viral labeling for fluorescence experiments**

Purified DENV samples were labeled using either Alexa Fluor 555 NHS ester or DiI(DiIC18(5)-DS) with ~2.5 x 10^8 PFU of the purified virus in 10 mM Hepes and 150 mM NaCl at pH 7.4 (HN buffer) solution. The labeling was performed at a final concentration of 750 nM and 100 nM Alexa Fluor 555 NHS ester and DiI’ (DiIC18(5)-DS), respectively (molar extinction coefficients of 71,000 M\(^{-1}\) cm\(^{-1}\) and 144,000 M\(^{-1}\) cm\(^{-1}\), respectively), and left at 4 °C (to reduce loss of infectivity due to exposure to room temperature [RT]) for 2 h. The Alexa Fluor 555 NHS ester labels the E protein on the virus, while the DiI’ (DiIC18(5)-DS) labels the viral bilayer (83). The free dye molecules were filtered out by size-exclusion chromatography (MicroSpin S-200 HR columns). The infectivity of labeled virus was confirmed to be unchanged from the unlabeled virus by conducting plaque assays (Fig. S11).

**Preparation of GM1a for cell enrichment**

The GM1a-Bodipy 25 μg powder was first diluted in 50 μl chloroform:ethanol (19:1) solution, transferred to a clean 25 ml round bottom flask, dried under nitrogen gas to evaporate the solvent, and further dried under vacuum for 1 h, after which it was dissolved using 200 μl of absolute ethanol. This ethanol mixture of GM1a-Bodipy/GM1a was then mixed by vortexing into 0.34 mg/ml solution of defatted BSA in Hanks’ buffered salt solution + 10 mM Hepes, pH 7.4 (HBSS/Hepes) to make a 3 μM solution of GM1a in BSA, which was aliquoted as 1 ml batches in Eppendorf tubes and stored at ~20 °C, as recommended by the product information sheet. Similarly,
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Ganglioside GM1a solid was dissolved in a chloroform:ethanol (19:1) solution, to make a 10 mg/ml stock solution. From this, a volume of 40 ml was taken and following the same protocol as aforementioned, a final stock of 5 μM GM1 in BSA was prepared and stored at ~20 °C until experiment date.

Treatment of cells with D-PDMP and enrichment of GM1a

The cells were trypsinized and spun down in a 15 ml falcon tube at 1000 rpm for 3 min to form a pellet. The cells were resuspended in 5 ml RPMI (with 10% FBS, 1% PS, 25 mM Hepes, and L-glutamine) and seeded at ~2000 cells per well in an 8-well chambered coverglass (Thermo Scientific Nunc Lab-Tek Chambered coverglass). A solution of D-PDMP was diluted in 100% ethanol to form a 10 mg/ml solution and diluted in 1xPBS to a final concentration of 0.5 mg/ml stock solution. This stock was diluted in cell culture media to prepare a solution of 10 μM. The cells were left to adhere to the coverglass, and after 2 h, the media was replaced with media containing 10 μM D-PDMP and left for 2 days to inhibit endogenous GM1 synthesis in cells. The cells were rinsed three times with HBSS and were overlaid with 200 μl volume at a concentration of 300 nM or 100 nM GM1a-Bodipy for imaging experiments and FCS/FCCS experiments, respectively, in Hanks’ buffered salt solution + 10 mM Hepes, pH 7.4 (HBSS/Hepes). The GM1a-Bodipy was left on the cells for 30 min at 4 °C and washed three times with phenol red–free RPMI (with 25 μM Hepes). Labeled DENV was then overlaid at an MOI of 50 in phenol red–free RPMI (with 25 μM Hepes) at 4 °C, then was washed and replaced with fresh media for imaging (84).

Transfection protocol

For transfection with GFP-GPI, cells were trypsinized and the volume of one million cells (counted by Biorad tc10 automated cell counter, Bio-Rad Laboratories, Inc) was spun down in a 15 ml falcon tube at 1000 rpm for 3 min to form a pellet. The cells were resuspended in 9 μl electroporation buffer R (Neon transfection buffers), gently mixed in with 500 ng of GFP-GPI plasmid, and transfected using the Neon transfection system with a 10 μl transfection tip using 1200 pulse voltage (v), with 2 pulses. The cells were resuspended in 3 ml RPMI (with 10% FBS, 25 mM Hepes, and L-glutamine) and were seeded ~2000 cells per well in 8-well chambered coverglass (Thermo Scientific Nunc Lab-Tek Chambered Coverglass) and kept at 37 °C in 5% CO2.

DiIC18(3) staining of live cell membrane

DiIC18(3) (1,1′-Diocadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate) was dissolved in dimethyl sulfoxide and diluted to a final DiIC18(3) concentration of 100 nM in 1× HBSS with vortexing. Vero cells were rinsed three times with HBSS and overlaid with 100 nM solution and left at 37 °C in 5% CO2 incubator for 15 min. The cells were then washed three times with phenol red–free media and left in the same media for imaging and FCS/FCCS experiments.

Plaque assay

BHK21 cells were grown till 90% confluency in a T75 flask and trypsinized. The trypsinized cell suspension in RPMI was centrifuged to remove debris and resuspended in 50 ml of RPMI (with 10% FBS, 1% PS, 25 mM Hepes, and L-glutamine). A volume of 1 ml of this cell suspension was seeded per well into two 24-well cell culture plates and left for 1 day to allow cells to form a continuous monolayer. The virus solution was serially diluted in 10-fold dilutions, using RPMI (with 4% FBS, 25 mM Hepes, and L-glutamine). The media was aspirated off of the cell monolayer, and a volume of 100 μl of each dilution was added in triplicate to the wells. This was left to incubate at 37 °C with 5% CO2 for 2 h with plate tilting every 15 min to stop drying of cells. The virus overlay was then aspirated out, and a viscous overlay made up of RPMI with 1% Aquacide-II and 2% FBS was placed over the cell monolayer and left to incubate for 7 days. The plates were tipped to remove the viscous overlay, stained using few drops of crystal violet (0.5% (wt/vol) crystal violet–25% formaldehyde), and was left for 1 h, before washing under a running tap to remove unstained plaque areas. The plaques were counted by visual inspection and the PFU/ml was given by the (average number of plaques/dilution factor × volume of virus added (ml)).

Confocal microscope setup

An FV1200 confocal microscope (Olympus) equipped with a time-resolved FCS upgrade kit (PicoQuant) was utilized in this work. The confocal was equipped with a pulsed 485 nm laser (LDH-D-C-488, PicoQuant) operated at 20 MHz repetition rate, a 543 nm continuous wave (cw) laser (GLG 7000, Showa Optronics), 488 nm cw laser, and 635 nm cw laser. All lasers were operated at 5 μW power before the objective, on live cell membranes, and passed through a 60×, NA 1.2 water immersion objective (UPlanSApo, Olympus), while the fluorescence emission is routed through a 405/488/543/635 dichroic mirror (Chroma Technology), confocal pinhole of one airy unit, and band-pass emission filters 513/17 (Brightline; Semrock, IDEX Health & Science, LLC) and a 615/45 (XF3025 32833, Omega Optical) for green and red emissions in FCS and FCCS experiments. Band pass filter of BA505-525 and BA655-755, for green and red, respectively, was utilized for imaging and SPT experiments. The emission signal was detected by an avalanche photodiode (SPCM-AQR14; PerkinElmer). The photon counts from the detector were registered by a Time-Harp 260 time-correlated single photon counting board (PicoQuant) and processed by the SymPhoTime 64 software (PicoQuant) (85, 86).

FCS measurements on confocal microscope

FCS was performed on Vero cells at 37 °C in phenol red–free media with 25 μM Hepes on a FV1200 confocal microscope (Olympus). FCS measurements in the green channel for GM1a-bodiphy and GFP-GPI were performed by illuminating the sample with a pulsed 485 nm laser. FCS in the red channel for DiIC18(3)–labeled cell membranes were performed by illumination with a 543 nm cw laser. The signal was collected at
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detector without a beam splitter using a single detection channel and correlation functions were calculated using SymPhoTime 64 software (PicoQuant). Determination of dimensions of the effective detection volumes were performed by calibration of FCS measurements in solutions of reference dyes atto488 and atto565 (Atto-Tec) of green and red channels, respectively. The diffusion coefficient of Atto dyes was taken as 400 μm²/s at RT based on previously reported values (85, 86).

Fit models for confocal FCS measurements

For FCS in the confocal, in the simplest case, fluorescent molecules moving in 3D diffusion in the confocal volume is fitted using a single particle (1p) 3D diffusion fit model.

\[
G_{3D,1p}(t) = \frac{1}{N} \left(1 + \frac{t}{\tau_D}\right)^{-1} \left(1 + \frac{t}{K^2 \tau_D}\right)^{-\frac{1}{2}} + G_\infty
\]

(1)

where \( K \) is the structure factor, which defines the shape of the observation volume and \( \tau_D \) is the diffusion time.

In the case of 2D diffusion on cell membranes, the fitting model can be simplified as

\[
G_{2D,1p}(t) = \frac{1}{N} \left(1 + \frac{t}{\tau_D}\right)^{-1} + G_\infty
\]

(4)

If there are multiple diffusion components in the system, the linear sum of all these individual components weighted with their respective mole fractions, gives the \( G_i \), and can be expressed as

\[
G_{2D/3D, multicomponent}(t) = \frac{1}{N} \sum_{i=1}^{n} a_i^2 F_i g_i(t) + G_\infty
\]

(5)

\[
g_{1D}(t) = \left(1 + \frac{t}{\tau_D}\right)^{-1} \left(1 + \frac{t}{K^2 \tau_D}\right)^{-\frac{1}{2}}
\]

(6)

\[
g_{2D}(t) = \left(1 + \frac{t}{\tau_D}\right)^{-1}
\]

(7)

Where \( a_i \) is the ratio of the brightness of the \( i \)th species to that of species 1. \( \tau_D \) is the diffusion time and \( F_i \) is the mole fraction of the \( i \)th species.

The overall autocorrelation function (ACF) is given as the product of all the individual dynamic processes that are present in the system, including the triplet state relaxation of the fluorophore, and can be written as

\[
G_{i}(\tau) = 1 + \left(\frac{F_{\text{trip}}}{1-F_{\text{trip}}} \right) e^{t_{\text{trip}}}
\]

(8)

where \( F_{\text{trip}} \) is the fraction of the triplet state and \( t_{\text{trip}} \) is the relaxation time of the triplet state.

The fitting models for 2D and 3D diffusion with triplet contribution is as follows:

\[
G_{2D,1p\text{t}}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \left(\frac{F_{\text{trip}}}{1-F_{\text{trip}}} \right) e^{t_{\text{trip}}}\right) + G_\infty
\]

(9)

\[
G_{3D,2p\text{t}}(\tau) = \frac{1}{N} \left[ (1-F_2) \left(1 + \frac{\tau}{\tau_{D1}}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_{D1}}\right)^{-\frac{1}{2}} + F_2 \left(1 + \frac{\tau}{\tau_{D2}}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_{D2}}\right)^{-\frac{1}{2}} \right] \left(1 + \left(\frac{F_{\text{trip}}}{1-F_{\text{trip}}} \right) e^{t_{\text{trip}}}\right) + G_\infty
\]

(10)

\[
G_{2D,2p\text{t}}(\tau) = \frac{1}{N} \left(1-F_2 \right) \left(1 + \frac{\tau}{\tau_{D1}}\right)^{-1} + F_2 \left(1 + \frac{\tau}{\tau_{D2}}\right)^{-1} \left(1 + \left(\frac{F_{\text{trip}}}{1-F_{\text{trip}}} \right) e^{t_{\text{trip}}}\right) + G_\infty
\]

(11)

FCCS on confocal microscope

FCCS was performed on Vero cells at 37 °C, in phenol red−free media with 25 μM Hepes on a FV1200 confocal microscope (Olympus). Quasi-PIE FCCS was performed by using pulsed 485 nm and the 543 cw laser to illuminate sample simultaneously. The emission was split by a 560DCXR (Chroma Technology) emission dichroic filter cube to allow green and red emission to travel to the two separate detectors. The after pulsing and spectral crosstalk was corrected by statistical filtering, using the FLCS script for spectral crosstalk removal via FLCCS on SymPhoTime 64 software (PicoQuant) (85, 86).

Confocal FCCS q value evaluation

The degree of crosscorrelation, \( q \) (the ratio of the concentration of the double-labeled species to that of all the particles carrying the less abundant label), was calculated as in equation (12), where the increase in \( q \) indicates a higher level of interaction between the two biomolecules.

\[
q = \frac{\max(N_{E},N_{R})}{N_{E}}
\]

(12)
The derivation of $q$ factor is as follows:

$$G_G(0) = \frac{1}{N_G} = \frac{N_g + N_{gr}}{(N_g + N_{gr})^2} \quad (13)$$

$$G_R(0) = \frac{1}{N_R} = \frac{N_r + N_{gr}}{(N_r + N_{gr})^2} \quad (14)$$

$$G_G(0) = \frac{1}{N_G} = \frac{N_{gr}}{(N_g + N_{gr})(N_r + N_{gr})} \quad (15)$$

$$q = \frac{N_{gr}}{\min\{N_g + N_{gr}; N_r + N_{gr}\}} \quad (16)$$

$$= \max\left[\frac{N_{gr}}{N_g + N_{gr}}; \frac{N_{gr}}{N_r + N_{gr}}\right] \quad (17)$$

$$= \max\left[\frac{G_x(0)}{G_0(0)}; \frac{G_r(0)}{G_0(0)}\right] = \frac{\max\{N_G; N_r\}}{N_x} \quad (18)$$

$N_g$, $N_r$, and $N_x$ are the background corrected particle numbers extracted from the ACFs in the green and red channel and from the cross correlation function, respectively. Ten cell measurements with three 100 s acquisitions each were measured and the SDs of the $q$ mean were calculated for all measurements. The number of individual cells and measurement statistics are provided in the results section.

**2D SPT of DENV on live cell membrane and imaging on confocal**

Vero cells were transfected with GFP-GPI and seeded in 8-well chambered cover glasses. DENV labeled with Dil' (DiIC18(5)-DS) and overlaid on cells at 4 °C for 10 min. 2D SPT was performed by acquiring time lapse image series of labeled DENV on Vero cells at 37 °C. Sample signal was acquired by collecting the fluorescence emission (after passing through the ZT 405/488/561/640pc dichroic mirror by filtering through a laser quad band ZET405/488/561/647m (Chroma Technology). The signal was recorded on an EMCCD Andor iXon3 X-9388 EMCCD camera (128 × 128 pixels, 24 μm pixel size), for 50,000 frames at 0.002 s exposure time per frame. The microscope was fitted with a CO₂/air gas chamber (Live Cell Instrument, FC-5, Chamlide) and sample stage was maintained at 37 °C, with 5% CO₂ for all cell measurements.

**ImFCS setup on total internal reflection fluorescence microscope**

ImFCS was performed on an Olympus Inverted epifluorescence microscope IX83 equipped with a motorized total internal reflection fluorescence (TIRF) illumination combiner (cell-TIRF/IX3-MITICO, Olympus), which allows simultaneous illumination with four laser lines. The system uses a UApOn 100×/1.49 Olympus oil-immersion objective; the excitation laser was passed onto a ZT 405/488/561/640pc (Chroma Technology) dichroic mirror to reflect the laser light on the back focal plane of the microscope objective. The laser light incident angle was adjusted to TIRF mode by help of the Olympus Xcellence software. The GFP-GPI in cell sample was excited with the 488 nm excitation laser (Olympus Cell laser) at 100 μW before the objective, and sample signal was acquired by collecting the fluorescence emission (after passing through the ZT 405/488/561/640pc dichroic mirror) by filtering through a laser quad band ZET405/488/561/647m (Chroma Technology). The signal was recorded on an EMCCD Andor iXon3 X-9388 EMCCD camera (128 × 128 pixels, 24 μm pixel size), for 50,000 frames at 0.002 s exposure time per frame. The microscope was fitted with a CO₂/air gas chamber (Live Cell Instrument, FC-5, Chamlide) and sample stage was maintained at 37 °C, with 5% CO₂ for all cell measurements.

**ImFCS on live cell membranes**

Vero cells where transfected with GFP-GPI and treated with D-PDMP for 2 days to deplete GM1. The cells were then enriched with GM1a. ImFCS measurements were carried out and diffusion law intercept was determined on the same cell for before and after addition of DENV1, DENV2, and CTXB. DENV1, DENV2, and CTXB were labeled with Alexa fluor 555, and the cells measured after addition of DENV1, DENV2, or CTXB were first checked with 561 nm laser illumination (Olympus Cell laser) to confirm the presence of DENV1, DENV2, or CTXB on the cell of interest. A region of interest of 21 × 21 pixels (5 × 5 μm²) was selected from acquired image stacks away from cell edges for ImFCS and diffusion law analysis. The ACFs for all pixels in the image were calculated using a multi-tau correlation scheme (79), and the signal was corrected before fitting, with an exponential of polynomial bleach correction (89). The ACFs were fitted using the model in equation (19) on the home written ImFCS plugin, which runs on ImageJ, to generate diffusion coefficient (D) maps and diffusion law plots.
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**ImFCS diffusion law**

FCS diffusion law (90, 91) exploits the space dependent property of membrane diffusion to explore and identify the mode of membrane organization in living cells. It is capable of differentiating between free diffusion, diffusion in domain partitioning, and meshwork environments. ImFCS (79, 92, 93) is the adaptation of FCS diffusion law, where the dependence of diffusion time of the fluorophore (tD) on observation area (Aeff) is described by tD(Aeff) = τ0 + Aeff/D. Aeff is given by the convolution of the detection area (or pixel area) with the point spread function and different Aeff values were obtained by binning the pixels postacquisition (n x n with n = 1–5). The magnitude and sign of intercept τ0 indicates the mode of membrane diffusion and state of membrane packing. In a diffusion law plot, free diffusion produces a straight line that passes through the origin with τ0 values of 0.0 ± 0.1 s (94).

Heterogeneous systems with domains or meshwork producing different diffusion law plots with characteristic positive and negative τ0 values for domain partitioning and meshwork diffusion, respectively (92, 95).

**Fitting of FCS curves in ImFCS**

FCS curves in ImFCS were fitted using the following equation:

\[ G(τ) = \frac{1}{N} \left( \frac{\text{erf}(p(τ)) + (e^{-p(τ)^2 - 1})}{\sqrt{Np(τ)}} \right)^2 + \left[ 1 + \left( \frac{Ft}{1-Ft} \right) e^{−πτ/4} \right] G_∞ \]

(19)

\[ p(τ) = \frac{a}{\sqrt{4Dτ+ω_0^2}} \]

(20)

where \( G(τ) \) is the temporal autocorrelation function, \( N \) is the number of particles, \( a \) is the pixel size, and \( ω_0 \) is the 1/e2 radius of Gaussian approximation of the microscope point spread function. \( F_t \) is the fraction of particles in triplet state, \( t_F \) is the average time molecules spend in the triplet state, and \( G_∞ \) is the convergence of \( G(τ) \) at long lag times. The fitting parameters were \( N, D, F_t, t_F \) and \( G_∞ \).

**DENV overlay on live Vero cells for imaging and 2D SPT**

Vero cells were plated on 8-well chambered coverglass (Thermo Scientific Nunc Lab-Tek Chambered coverglass) after necessary dilution in media and treated for different experimental conditions (D-PDMP/transfection/Dil staining/GM1a-Bodipy enrichment) as required. DENV was labeled, filtered, and diluted in phenol red–free media with 25 μM Hepes to obtain an MOI of 50 and overlaid on cells, which were washed three times using phenol red–free media. This was left at 4 °C for 10 min to help virus reach the live cell membrane and to halt cellular endocytosis (96). The 8-well chambered cover slides were then transferred to the microscope stage for imaging.

**Deuterium labeling and quench conditions for amide HDXMS**

Purified dengue virus solubilized in NTE buffer at pH 8.0 was incubated at 37 °C in PBS buffer reconstituted in D2O (99.90%) resulting in a final D2O concentration of 90%. For generating DENV2–GM1a complex, viral particles were pre-incubated at a molar ratio of 1:125 (E protein monomer: GM1a sugar) at 37 °C for 30 min prior to each deuterium exchange reaction. Deuterium labeling was performed for 1 min for both free and GM1a–bound state, followed by quenching the exchange reaction by adding prechilled quench buffer. Quench buffer contained 1.5 M GlnHCl and 0.25 M Tris(2-carboxyethyl) phosphine-hydrochloride, and after adding quench buffer, the solution was incubated at 4 °C on ice for 30 s, followed by addition of titanium dioxide to precipitate envelope lipids. Precipitated envelope lipids were removed before injecting the sample for pepsin digestion using 0.22 μm centrifugal filters at 10,000 rpm for 1 min. Deuterium exchange was also performed on free soluble E protein (1 mg/ml, C-terminal (His)6 tag) and sE protein: GM1a (1:50) for labeling times of 1, 10, and 100 min. sE protein was reported to exist dominantly in a monomeric and dimeric state at 37 °C and 22 °C, respectively (97, 98). HDXMS was carried out at 22 °C to map GM1a interactions to the dimeric state of E protein. Same quench conditions were used for both sE protein states with the exception that E protein from intact dengue was treated with TiO2. This was not required for recombinant sE protein as it lacks stem helices and lipid membrane.

FCCS experiments have shown colocalization of GM1a glycosphingolipid with DENV2 and DENV1 showing the stable binding of GM1a to the viral surface. In the absence of equilibrium dissociation constant (Kd), we used high molar ratio of GM1a sugar moiety to E protein (indicated previously) in our deuterium exchange reactions. We observed no significant changes in sE protein (monomer) in the presence of GM1a at 1:50 M ratio either due to weak binding (high Kd) or lack of quaternary contacts akin to a virion. Also, no bimodal mass spectra were observed in sE protein:GM1a state to suggest partial or unsaturated binding of GM1a at virus E protein. Hence, in subsequent deuterium exchange reaction with DENV2, we used a higher concentration of DENV2 (E protein monomer) to GM1a molar ratio (1:125). Peptides showing protection in the presence of GM1a (DENV2:GM1a state)
show unimodal distribution of mass spectra, which suggests uniform binding across viral particle.

**Mass spectrometry and peptide identification**

Quenched samples of 35 pmol and 100 pmol from DENV2 and sE protein (free and GM1a complex states) were injected onto nanoUPLC HDX sample manager (Waters), respectively. Injected samples were proteolyzed in online mode using pepsin immobilized Waters Enzymate column (2.1 × 30 mm) in 0.1% formic acid in water at a flow rate of 100 μl/min. The proteolyzed peptides were trapped in a 2.1 × 5 mm C18 trap (ACQUITY BEH C18 VanGuard Precolumn, 1.7 μm, Waters). Elution of pepsin digested peptides was performed using acetonitrile gradient of 8% to 40% in 0.1% formic acid at a flow rate of 40 μl min⁻¹ into reverse phase column (ACQUITY UPLC BEH C18 Column, 1.0 × 100 mm, 1.7 μm, Waters) pumped by nanoACQUITY Binary Solvent Manager (Waters). Peptides were ionized using electrospray ionization mode and sprayed onto SYNAPT G2-Si mass spectrometer (Waters). HDMS² mode acquisition and measurement was employed. About 200 fmol μl⁻¹ of [Glu¹]-fibrinopeptide B ([Glu¹]-Fib) was injected at a flow rate of 5 μl/min into mass spectrometer for calibration and lockspay correction.

Protein Lynx Global Server v3.0 (PLGS v3.0) was used to identify the peptides from undeuterated mass spectra (HDMS²). Search for peptide identification was performed on sequence database of dengue 2 NGC strain with E, M, and C protein. No specific protease and variable N-linked glycosylation modifications were chosen in search parameters to carry out the sequence identification. Peptide identification parameters were intensity of 2500 for product and precursor ions, minimum products per amino acids of 0.2, and a precursor ion mass tolerance of <10 ppm using DynamX v.3.0 (Waters). Peptides present in at least two out of three undeuterated samples were retained for further analysis. Reported deuterium exchange values are uncorrected for back exchange and all the reactions are performed in triplicates. Three technical replicates were carried out for each deuterium exchange reaction for all states and their average values were used to generate the deuterium exchange plots. SD of deuterium exchange within ± 0.5 Da was observed for all the peptides. Therefore, a deuterium exchange difference of ± 0.5 Da was chosen as significance threshold. List of peptides identified in the current HDXMS experiments is shown Supplementary File 1.

**ITC**

ITC experiments were performed using the MicroCal VP-ITC titration calorimeter (MicroCal-Malvern). The E protein and GM1a solutions were prepared in the PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4 at pH 7.4. GM1a concentrations varying from 2 to 60 μM (18 2 μl injections) were titrated into a stirred reaction cell containing 10 μM dengue E protein at 25 °C. The reference power was 10 μcal/s, and the stirring rate was 750 rpm. All titrations were performed in triplicate with identical concentrations and injection schedules. The protein samples were also injected into buffer as a reference to estimate the heat of dilution. All samples were degassed and spun before the experiment. The binding isotherms were analyzed employing a model that considered a two-site sequential binding model (99) implemented in Origin 9.1 (OriginLab).

**Data availability**

All of the experimental data are contained within the article and figures.

**Supporting information**—This article contains supporting information.

**Author contributions**—S. N. T. and T. W. conceptualization; S. N. T. and T. W. methodology; S. N. T., G. S. A., and T. W. validation; S. N. T., P. V. R., and K. K. S. formal analysis; S. N. T., P. V. R., and K. K. S. investigation; J. C. W. B. resources; S. N. T., P. V. R., K. K. S., G. S. A., and T. W. writing—original draft; S. N. T., P. V. R., and K. K. S. visualization; G. S. A. and T. W. supervision; G. S. A. and T. W. project administration; G. S. A. and T. W. funding acquisition.

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**Abbreviations**—The abbreviations used are: BHK21, baby hamster kidney strain 21; BSA, bovine serum albumin;CtxB, Cholera Toxin B; DENV, dengue virus; D-PDMP, (+)-D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (hydrochloride); FBS, fetal bovine serum; FCS, fluorescence correlation spectroscopy; FCCS, fluorescence cross correlation spectroscopy; HDXMS, hydrogen/deuterium exchange mass spectrometry; ImFCS, imaging FCS; ITC, isothermal titration calorimetry; MOI, multiplicity of infection; qPIE-FCCS, quasi pulsed interleaved FCCS; SPT, single particle tracking; TIRF, total internal reflection fluorescence.

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