A large size-selective DNA nanopore with sensing applications

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Transmembrane nanostructures like ion channels and transporters perform key biological functions by controlling flow of molecules across lipid bilayers. Much work has gone into engineering artificial nanopores and applications in selective gating of molecules, label-free detection/sensing of biomolecules and DNA sequencing have shown promise. Here, we use DNA origami to create a synthetic 9 nm wide DNA nanopore, controlled by programmable, lipidated flaps and equipped with a size-selective gating system for the translocation of macromolecules. Successful assembly and insertion of the nanopore into lipid bilayers are validated by transmission electron microscopy (TEM), while selective translocation of cargo and the pore mechanosensitivity are studied using optical methods, including single-molecule, total internal reflection fluorescence (TIRF) microscopy. Size-specific cargo translocation and oligonucleotide-triggered opening of the pore are demonstrated showing that the DNA nanopore can function as a real-time detection system for external signals, offering potential for a variety of highly parallelized sensing applications.
lipid bilayers are key biological structures, serving as vital barriers for cells and subcellular organelles. To allow translocation of macromolecules and ions across this otherwise impermeable barrier, structural pores serve as transmembrane gatekeepers by creating hollow water-filled channels between the separated compartments. A large variety of protein pores exist in nature with various types of quaternary structure1,2, and several successful examples of modified protein nanopores have driven the technological advancement within label-free biosensing and DNA-sequencing fields3,4. However, the lack of generic design rules for de novo protein design has limited its widespread application so far5,2. This is particularly evident for de novo-designed transmembrane proteins, where only recently computationally designed transmembrane proteins were successfully reported6. As an alternative, DNA has been established as a highly predictable building material for bottom-up de novo nanopore structure creation7, including DNA nanopores8–10. In particular, DNA origami11 and single-stranded tile10,11 techniques have excelled due to their extensive design space, while computer-aided design has further streamlined the rational design of complex three-dimensional DNA nanostructures12. With DNA origami, a single-stranded kilobase DNA scaffold is assembled into the designed structure by using hundreds of shorter staple strands that easily can be modified individually13. Thus, advancements of DNA modifications13–15 and dynamic DNA structures16 have enabled the construction of programmable and functional DNA-based nanomachines17–20. Of particular interest for this study are the biomimetic nucleic acid-based systems developed to manipulate lipid bilayers, including DNA nanopores20–22, DNA-programmed SNARE mimics23–25, and membrane-shaping structures26,27.

Among the intrinsic problems of inserting a DNA nanopore into a lipid bilayer is its negatively charged phosphate backbone, creating an energy barrier known as the Born energy24. To enhance membrane association, DNA structures are usually decorated with hydrophobic moieties25 by including lipid-modified DNA oligonucleotides (LiNA)s as staples or by capture handles.

Existing DNA nanopore structures have primarily been dominated by six-helix bundle (6hb) stem designs20–22,25,29 and mainly focused on ionic current measurements and voltage-gating functionalities. Only recently, smaller24 and larger26,27 DNA nanopores have expanded the structural repertoire, demonstrating cytotoxicity25, translocation of larger molecules26, and charged-substrate or voltage gating28,29 that have expanded the technological repertoire.

In this study, we create a rationally designed DNA nanopore with the largest channel lumen to date, which we have combined with a programmable trigger that significantly expands the functionalities of nanopores. With direct observation and in-depth analysis of the insertion and translocation kinetics of individual nanopores on liposomes, we analyze the functionality of the DNA nanopore and demonstrate a size-selective, modular and responsive gating of molecules between compartments separated by lipid bilayers.

The development of environment-responsive features, enabling autonomous structural actuation and cargo capture/release upon signal sensing, as demonstrated by other DNA devices18,36,37, has the potential to transform the artificial nanopore field by enabling programmable insertion and biosensing of macromolecules.

Results

A dynamic and rigid DNA origami nanopore. For the design of our synthetic DNA nanopore, we defined three important advances, illustrated in Fig. 1. (I) The pore is created from a double-layered pseudosymmetric hexagonal DNA structure with a 9-nm-wide lumen and an outer diameter of 22 nm, allowing translocation of large macromolecules including globular native proteins of more than 150 kDa in size (Fig. 1a). (II) The design contains numerous sites for functionalization in the channel interior, and (III) programmable DNA flaps that can be opened to expose lipid moieties. In the closed state, the flaps are locked by staple strands at the base to shield the hydrophobic moieties from the aqueous environment until activation (Fig. 1b), limiting hydrophobicity-driven aggregation37. When fully complementary key strands are presented by the liposome or added in solution to drive toehold-mediated strand displacement mechanism, the flaps are opened, and the exposure of lipids drives membrane insertion (Fig. 1d).

The three flaps were attached about 12 nm from the base of the 32-nm core channel by single-stranded DNA hinges and locked at the base by two staple strands. Importantly, when introducing these features, care was taken not to compromise the structural stability and introduce obvious kinetic folding traps (Supplementary Note 1 and Supplementary Fig. 1). Based on the theoretical considerations of energetic penalty for inserting a pore of this channel size (Supplementary Note 2), a total of 46 lipidation sites were introduced on the channel and flap surfaces (Fig. 1d and Supplementary Fig. 3). The DNA pore was decorated with both cholesterol (18/46) and palmitoyl (28/46) (Supplementary Figs. 3 and 4) by including the synthesized LiNA staples into the self-assembly mixture. As expected, an increased association to liposomes is observed by increasing the number of hydrophobic moieties on the pore; thus we used the full lipidation scheme (Supplementary Fig. 5).

Using negative-stain transmission electron microscopy (nsTEM), raw images along with subsequent single-particle 2D analysis confirmed the correct folding of the DNA nanopore and function of the programmable flaps (Fig. 2). While measurement of the nanopore length from the 2D-class average estimated the length to be 35 nm, quantification from a limited set of cryoEM images provided a length of 31.6 nm, which is very close to the theoretically expected 32 nm (Supplementary Fig. 12). The lumen is clearly visible, both from the side and top views, and measured 9.6 nm at the widest dimension. From the top view the three flaps are visible as densities attached to the three larger hexagonal sites. In addition, a thermal denaturation assay confirmed high structural stability with a melting temperature of 56°C (Supplementary Fig. 13). To enable hierarchical assembly of an elongated two-way pore, we designed two versions (A and B), each with 27 complemented sticky ends (Fig. 1c). Mixing A and B nanopores in solution resulted in a dimeric pore of the expected size, by aligning the lumens into a continuous elongated channel (Fig. 2 and Supplementary Figs. 6 and 7). Using this approach, dimer structures increased from 15% for noncomplementary A or B pores to a plateau of 70% for end-complementary A + B pores (Supplementary Fig. 14).

To confirm the dynamic properties of the flap structures, both nsTEM and FRET assays were used. The nsTEM analysis clearly showed extended protruding flap structures from the channel connected at the intended hinge region upon incubation with the key strand (Fig. 2c). Positioning of a Cy5–Cy3 FRET pair in a flap compartment further verified efficient and specific flap opening (Supplementary Fig. 15). The addition of the correct key sequence resulted in a 45% decline in the relative FRET signal within the first 5 min, whereas scrambled sequence did not have any effect. We conclude that the designed key strand can efficiently open the locks within minutes, leaving the flaps protruding from the core structure.

Insertion of the DNA nanopore into lipid bilayers. Next, we studied how the pore engages with bilipid membranes. Assembled
nanopores were mixed with small unilamellar vesicles (SUVs) and visualized by nsTEM. Although not a quantitative assay per se, we observed many examples of flap-mediated insertion and interaction by using pre-opened nanopore structures, providing initial evidence of a functional DNA nanopore design (Fig. 2d and Supplementary Fig. 10). Interestingly, by dimerizing A and B nanopores, we were able to connect adjacent SUVs, suggesting a possibility to create channeling gates between vesicles/organelles, which would allow for gated translocation between compartments (Fig. 2d, lower).

To directly image nanopore docking on individual SUVs, pore formation, and to characterize dye translocation kinetically and

Fig. 1 Design of the DNA nanopore. a The pseudosymmetric nanopore is based on a hexagonal origami lattice and has a 9-nm inner pore diameter, a 22-nm outer diameter, and a length of 32 nm. b The three flaps are each locked with two dsDNA hybrids that possess an 8-nt toehold for strand displacement opening. c At the top, 27 staple strands are extended with unique 8-nt sequences to allow specific sticky end-mediated dimerization upon mixing A and B nanopores. d Lipidated nucleic acid staples (orange protrusions) are displayed at the surface of the channel and the flaps when opened. e Schematic illustration of the nanopore inserted into a liposome.

Fig. 2 TEM characterization of the DNA nanopore. a Raw nsTEM image of non-lipidated closed DNA nanopores. b Class-average images of the DNA nanopores in the closed- and c open-flap conformations. Grayed box shows closed and open nanopores assembled with 46 LiNA staples in the flap-channel system. d Raw TEM image cutouts of lipidated DNA nanopores incubated with liposomes. In the lower image, dimerized DNA nanopores have been formed prior to incubation with SUVs. Scale bars in (a) and (d) are 50 nm, while the scale bars in (b) and (c) are 15 nm. Additional raw images are provided in Supplementary Figs. 6–10. Sizes of SUVs have been analyzed by DLS (Supplementary Fig. 11).
thermodynamically, we used total internal reflection fluorescence (TIRF) microscopy\(^\text{38,39}\). In this system, SUVs loaded with the dye ATTO 655 were surface-immobilized on poly(ethylene glycol) (PLL-PEG)-passivated surfaces by using a neutravidin–biotin capture, which maintains the vesicles’ morphology\(^\text{38}\) and essentially leakage-free membranes\(^\text{40}\). Freeze–thaw cycles (see the Methods section) ensure unilamellar SUVs as we showed recently\(^\text{38}\). Here, the immobilized SUVs serve as targets for either pre-opened lipidated DNA nanorods labeled with ten ATTO 488 fluorophore strands or \(\alpha\)-hemolysin as a positive control (Fig. 3a, b). Parallel imaging of two emission channels permitted synchronous imaging of SUVs and DNA nanorods and allowed the direct real-time observation of individual nanopore docking (blue trace) on a SUV followed by dye leakage (red trace). The single-particle setup allows real-time observations of the docking time \(\tau_1\), reorientation time for pore insertion \(\tau_2\), as well as the ATTO 655 flow rate \(k_f\). Histogram of flow rates from 86 recorded ATTO 655 efflux events fitted with a single Gaussian fit to extract the average flow rate. e, f All dye flow rates from SUVs observed separated by the SUV diameter into two populations \(d_{\text{min}}>70\text{ nm}>d_{\text{max}}\) for either \(\alpha\)-hemolysin (e) or DNA nanorope (f). While the \(\alpha\)-hemolysin flow rate shows dependency of SUV size, the DNA nanorope flow rate does not. The \(\alpha\)-hemolysin distribution consists of a total of 182 fully formed and leaking pores. g The probability for the DNA nanorope to form a pore upon docking into liposomes is plotted as a function of SUV size. Detailed data are found in Supplementary Fig. 21b. h Percentage of SUVs docked with non-lipidated or lipidated DNA nanoropes (NL or L). The SUVs are either key- or no-key-decorated (+ or −) and the experiment is performed in a continuous nanopore flow setup (Supplementary Fig. 21a). i Percentage of the DNA nanorope-docked SUVs that successfully result in pore formation and dye flux performed in a continuous nanopore flow setup with the same legend as (h). Asterisks (***) in (h, i) indicate a p-value of \(<10^{-7}\) and reflect a significant difference between the compared populations based on two-sample t test, while error bars show the standard error of the mean. The sample size for (h, i) is \(N=709\) composed from four different experiments. Scale bar in (b) is 20 \(\mu\)m.

### Fig. 3 TIRF studies of DNA nanoropes interaction with SUVs

a) TIRF setup, to monitor pre-opened lipidated DNA nanoropes labeled with ATTO 488 used in (c), (d), (f), and (g) and interacting with ATTO 655-loaded SUVs tethered on PLL-PEG-passivated surfaces by a biotin–neutravidin linkage. Insertion of a DNA nanorope allows ATTO 655 efflux. b) A single field of view images hundreds of surface-tethered ATTO 655-filled SUVs. c) Representative traces of an individual nanorope docking (blue trace) on a SUV followed by dye leakage (red trace). The single-particle setup allows real-time observations of the docking time \(\tau_1\), reorientation time for pore insertion \(\tau_2\), as well as the ATTO 655 flow rate \(k_f\). d) Histogram of flow rates from 86 recorded ATTO 655 efflux events fitted with a single Gaussian fit to extract the average flow rate. e, f All dye flow rates from SUVs observed separated by the SUV diameter into two populations \(d_{\text{min}}>70\text{ nm}>d_{\text{max}}\) for either \(\alpha\)-hemolysin (e) or DNA nanorope (f). While the \(\alpha\)-hemolysin flow rate shows dependency of SUV size, the DNA nanorope flux rate does not. The \(\alpha\)-hemolysin distribution consists of a total of 182 fully formed and leaking pores. g) The probability for the DNA nanorope to form a pore upon docking into liposomes is plotted as a function of SUV size. Detailed data are found in Supplementary Fig. 21b. h) Percentage of SUVs docked with non-lipidated or lipidated DNA nanoropes (NL or L). The SUVs are either key- or no-key-decorated (+ or −) and the experiment is performed in a continuous nanopore flow setup (Supplementary Fig. 21a). i) Percentage of the DNA nanorope-docked SUVs that successfully result in pore formation and dye flux performed in a continuous nanopore flow setup with the same legend as (h). Asterisks (***) in (h, i) indicate a p-value of \(<10^{-7}\) and reflect a significant difference between the compared populations based on two-sample t test, while error bars show the standard error of the mean. The sample size for (h, i) is \(N=709\) composed from four different experiments. Scale bar in (b) is 20 \(\mu\)m.
section): the docking lag ($\tau_1$—time from incubation to SUV docking), the reorientation time ($\tau_2$—time from docking to pore formation), and dye flow rates ($k_f$) from perforated SUVs (Fig. 3c). Imaging optimization ensured minimal bleaching of ATTO 655 within the experimental time frame (Supplementary Fig. 20) and extraction of accurate efflux rates. The nanopore docking lag ($\tau_1$) follows a monoexponential decay process with a decay rate of 1450 s$^{-1}$, suggesting a one-step process of nanopore–SUV interaction (Supplementary Fig. 21a). The reorientation phase on the other hand showed a complex insertion process (Supplementary Fig. 22). The prolonged time from nanopore docking to efflux, as well as the diverse efflux rates, support that the nanopore does not cause destruction of the vesicles upon docking. The flow rates were fitted by using a Gaussian distribution to extract the time derivative of the intensity, which revealed a rate of 0.098 Is$^{-1}$ through the DNA nanopore with a 95% confidence interval of (0.81,0.118) Is$^{-1}$ (Fig. 3d). In comparison, α-hemolysin control traces, a sevenfold lower average flow rate of 0.014 (0.013,0.016) Is$^{-1}$ was determined as expected due to the narrower pore. Looking at the complex system by using a simplified theoretical approach, the flow rate and pore size relationship are found to be approximately following the Hagen–Poiseuille relation (see Supplementary Note 3 for theory and approximation). Splitting the observed flow rates into two populations, SUVs with a diameter ($d_{m}$) above and below 70 nm, enabled us to address membrane tension$^{41,42}$ on the pores and evaluate the relative mechano sensitiveness of α-hemolysin versus the DNA pore (Fig. 3e, f and Supplementary Table 1). In agreement with previous reports$^{43}$, the flow rate of α-hemolysin appears mechanosensitive, exhibiting a flow rate of 0.014 (0.012,0.015) Is$^{-1}$ in small SUVs ($d_{m} < 70$ nm) versus 0.053 (0.046,0.060) Is$^{-1}$ in large SUVs ($d_{m} > 70$ nm) (Fig. 3e). Independent two-sample Kolmogorov–Smirnov test between the two distributions confirmed distinct populations (KS = 1, $P = 1.34 \times 10^{-5}$). In contrast, performing the same analysis with the DNA nanopore did not yield any difference in flow rate between the populations indicating a mechano-insensitive pore (Fig. 3f). Our DNA nanopore is capable of withstanding bilayer-mediated stress as opposed to previous measurements and simulations on single-walled 6hb structures$^{29,30}$, indicating the double-layered DNA pore structure to provide extra rigidity. Additional future experiments are required to verify if the observed mechanoinsensitivity further can suppress voltage gating, as observed with the single-walled 6hb structures. We conclude that our DNA nanopore has a rigid channel, which allows a monodisperse flow rate independent of membrane curvature.

Interestingly, while the liposome population is polydisperse in size and follows a log-normal distribution (Supplementary Figs. 20 and 28), the probability of successful DNA nanopore insertion upon docking has a slight preference for larger liposomes (Fig. 3g, Supplementary Fig. 21b and Supplementary Table 1). This occurs despite the fact that DNA nanopore and α-hemolysin were exposed to identical SUVs. These findings agree with the qualitatively observed SUV insertion/interaction events from the nSTEM experiments (Supplementary Fig. 10), where insertion in smaller liposomes is rarely seen and indicates that the insertion process of the DNA nanopore is mechanoinsensitive. The reorientation phase on the other hand displays shorter lag times for smaller liposomes with higher curvatures (Supplementary Fig. 22) in agreement with earlier studies by us and others, demonstrating curvature-selective penetration of peptides and proteins$^{38,44}$. Looking at the nanopore efficiency of insertion, about 20% of tracked liposomes were docked with DNA nanoropes, from which about 11% was perforated during the experiment (Supplementary Fig. 21b). Although docking of α-hemolysin was not tracked, about 40% of liposomes were perforated by it. Considering that α-hemolysin was used in a 15× higher concentration an approximately equal perforation per pore was obtained.

To test the programmability of DNA nanopore insertion into specific SUV populations decorated with flap-activating LiNA key strands, we conducted the following test: non-lipidated or lipidated DNA nanoropes (NL or L) were subjected to a continuous flow over SUVs preincubated with or without (+ or −) LiNA key strands (Supplementary Fig. 23). As expected, non-lipidated nanoropes showed practically no detectable bilayer docking on plain SUVs (NL−). Meanwhile, 17% of the observed SUVs were docked by nanoropes upon decoration with LiNA key strands (NL+), demonstrating targeted docking only when the flaps were targeted (Fig. 3h). Using lipidated nanoropes (L+), the docking lag was considerably reduced (Supplementary Fig. 23b), an effect that most likely arises from the increased lipidation. Interestingly, no significant difference in docking of lipidated nanoropes on plain SUVs (L−) or key-decorated SUVs (L+) was observed, suggesting a tethering mechanism independent of flap activation for lipidated structures. Importantly, looking at the number of insertion events, defined by the beginning of dye efflux from the liposomes with an associated docked nanopore, we observed a 2.2-fold increase in events by using lipidated DNA nanoropes on key-decorated SUVs (L+) compared with plain SUVs (L−). This indicates that exposure of the lipid groups enhances the fraction of docked pores that successfully insert into the membrane and result in dye efflux (Fig. 3i). Thus, effective DNA nanopore penetration can be controlled by surface-presented key signals, enabling individual addressability of SUVs.

**Nanopore channel size and size-selective gating of molecules.** To further evaluate membrane insertion and molecular transport through the channel, we conducted a dye-efflux assay of surface-immobilized giant unilamellar vesicles (GUVs), as previously described in other nanopore studies$^{26}$ (Fig. 4 and Supplementary Fig. 25). Unlike SUVs, GUVs are experienced as flat lipid bilayers by nanostructures, thus providing a better mimic for physiological cell membranes. In order to track the insertion and channel accessibility, a combination of three differently sized fluorescent molecules were added to the external buffer solution prior to addition of Cy5-labeled DNA nanoropes: a small (558 Da) sulfonforhodamine B (Rh = 0.5 nm; SRB) combined with either a 40- (Rh = 4.8 nm$^{45}$, dFITC-40k) or a 500-kDa (Rh = 15.9 nm$^{46}$, dFITC-500k) dextran–FITC dye (Supplementary Fig. 25). Confocal laser scanning microscopy (CLSM) was used to visualize dye influx into the GUVs over 8 h. In line with the TIRF studies described above, only lipidated nanoropes were inserted into the lipid membranes and this happened within the first 30 min. The SRB can readily pass through the DNA nanopore and is used as the positive readout of influx, and a total of 36 rapid (single frame = 10 min) SRB-filling events with intact membranes were registered and analyzed to gain a qualitative study from simple True/False answers. Slow multiframe filling events were interpreted as leaky bilayer influx and omitted (Supplementary Fig. 26).

As expected from the 9-nm lumen of the nanopore channel, the smaller dFITC-40k dye molecule was also able to translocate into the GUVs, albeit with a slower flux than SRB (Fig. 4a–d). As expected, the large dFITC-500k was unable to translocate across the membrane and only SRB influx was observed (Fig. 4e–h). This result demonstrates size-selective translocation of individual molecules with the expected cutoff value.

Next, we investigated the potential to control channel flux by introducing an addressable molecular plug. Up to ten 20-kDa
PEG polymers (Rh = 4.9 nm\textsuperscript{35}) were immobilized within the DNA pore by using available staple strand overhangs (Supplementary Figs. 16 and 17). As expected, this still allowed translocation of the small SRB dye into the GUVs. In contrast, dFITC-40k was now unable to pass through the PEG-plugged DNA nanopore (Fig. 4a–d), exhibiting a restricted influx similar to that seen for the large dFITC-50k (Fig. 4i–l). This demonstrates that plugging the channel can serve as a size-selective gate, distinguishing between cargo sizes of 15 and 5 nm.

To study the potential for the DNA nanopore to act as a real-time sensing device, we adapted the TIRF setup (see Methods section), performing a synchronous three-color imaging of ATTO 488-labeled nanopore insertion into ATTO 655- and 40-kDa dextran–tetramethylrhodamine (dTMR-40k)-loaded SUVs (Fig. 5a). Pore formation by a non-plugged DNA nanopore allowed full emptying of the SUVs as expected, supporting the existence of a large and rigid channel also observed from the CLSM influx assay (Supplementary Fig. 28). As a control, when using the narrow α-hemolysin pore, only ATTO 655 was able to translocate out of the SUVs whereas dTMR-40k remained encapsulated (Supplementary Fig. 29).

To introduce a controllable unplugging mechanism, we extended the PEG-tethering oligo with a toehold sequence of eight nucleotides, enabling PEG removal by a toehold-mediated strand displacement mechanism using a fully complementary DNA oligonucleotide unplugging strand (Supplementary Figs. 16 and 17). To examine the unplugging of the DNA nanopore, real-time, continuous imaging of DNA nanoparticles, ATTO 655 and dTMR-40k was performed for 9 h. DNA nanopore docking and pore formation into liposomes loaded with ATTO 655 and dTMR-40k resulted in selective initial efflux of the smaller ATTO 655. This in turn resulted in an increase of the TMR signal due to FRET dequenching (Supplementary Figs. 24 and 26). Unplugging strand was added after 4.5 h (dashed line in Fig. 5b) resulting in channel opening and the subsequent flow of dTMR-40k, demonstrating successful sensing capabilities of our nanopore (Supplementary Table 5). Interestingly, using the Stokes–Einstein diffusive model for the diffusive translocation of molecules indicates some hindrance for both dye molecules, probably due to interactions with the pore or hydrophobic interactions with the inner bilayer (Supplementary Note 4 for calculations and approximation). As nanopore docking on liposomes is a stochastic process that may take up to multiple hours, the experimental setup allowed us to sample events where insertion happens after addition of the unplugging strand, in which case the efflux of both dyes is observed simultaneously (Supplementary Fig. 27c). The differential flow rate of ATTO 655 and dTMR-40k and the observed dequenching of dTMR-40k upon ATTO 655 efflux, in addition, verify the integrity of the perforated SUVs.

In conclusion, our experiments demonstrate that controlled unplugging of the DNA nanopore combined with size-selective gating can be used as a sequence-specific sensing mechanism for external oligonucleotides.

**Discussion**

We have successfully designed and prepared a DNA nanopore with a 9.6-nm pore inner diameter, which is significantly larger than existing DNA-based pores and demonstrated actuation-based insertion of the pore into GUVs and SUVs in response to specific keys. In addition, we have designed a plug system that
allowed size-selective gating of translocation through the pore. Finally, we turned the DNA nanopore into a biosensor by demonstrating sequence-specific unplugging and dye efflux. We envision applications where other macromolecules are sensed by aptamer-based switches coupled to fluorescent detection. One of the advantages of the DNA pore is the possibility of spatially controlling virtually any modification in the lumen. For instance, one can imagine adding multiple plugs to the lumen to sense more complex, multicomponent signals or building enzymatic assembly lines.

Another application of the DNA nanopore to be explored further is its ability to create dimeric channels between adjacent lipid bilayers as previously also demonstrated by using engineered α-hemolysin pores\(^6\). In comparison, bifaced DNA pores can be constructed in a programmable fashion, thereby enabling controlled content mixing between liposomes.

Furthermore, our demonstration that the DNA nanocores can be targeted to prespecified SUVs, which can be addressed individually in a 2D TIRF setup, allows us to register thousands of molecular-sensing events simultaneously. This sets the stage for highly parallelized, label-free molecular sensing in the future.

**Methods**

**Materials.** Unless otherwise stated, all DNA oligodeoxynucleotides, including the cholesterol-modified versions, were acquired from IDT (Coralville, Iowa, USA). The dtd(\text{hexanyl})TP was synthesized in-house. Ligation was done with recombinant Terminal Transferase acquired from Roche Applied Bioscience (Indianapolis, Indiana, USA). The azido-palmitoyl building block (\((S)-1\)-azido-3-(palmitoyloxy)propan-2-yl) sulfate 4) was synthesized in 3 steps (Supplementary Note 5). In brief, commercially available (S)-Glycidyl is acetylated with palmitoyl chloride to give compound 2 in 95% yield which is subsequently turned into the corresponding azide by microwave assisted nucleophilic opening of epoxide 2 to provide azide 3 in 88% yield. Microwave assisted nucleophilic attack of the hydroxyl group on Sulphur trioxide followed by ion exchange with tetra-butylammonium hydroxide turns azide 3 into water soluble sulfate 4 in 87% yield. All fatty acids and liposome extruder parts were purchased from Avanti Polar lipids (Alabaster, Alabama, USA). Premade TEM grids were bought from TED-Pella (Redding, California, USA). Uranyl formate was acquired from Polysciences Inc. (Warrington, Pennsylvania, USA) and kept in the dark. M13 was acquired from Bayou Biolabs (Metairie, Louisiana, USA) at 0.50 µg/µl concentration. Amicon Ultra-0.5 ML (100 MWCO) Centrifugal Filters for purification were bought from Merck Milipore (Darmstadt, Hessen, Germany). SYBR Gold was purchased from Invitrogen (Thermo Fisher Scientific (Waltham, Massachusetts, USA)) and SYBR safe from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

All water was type 1 grade produced by MilliQ water purification system (or equivalent) unless stated otherwise and all solvents were p.a. grade. Premade 10xTAE was acquired from Giboco (Thermo Fisher Scientific (Waltham, Massachusetts, USA)).

**DNA origami design and assembly.** The origami structure was designed as described by using CaDNAno, and the crossover pattern optimized through analysis of each staple with in-house written software. To create 3D models and renderings of the structure, Autodesk Maya (http://autodesk.com) with a CaDNAno plugin was used. Further, the 8-nt toeholds have been optimized against internal and unwanted structures with the NUPACK software. Modules of staples for the DNA origami were pooled from the plates acquired from IDT by our Eppendorf pipetting robot by using a custom-written software. Assembly of the structure was done by mixing a final concentration of 10 nM of scaffold with 5–10x excess of each staple strand (219 in total), 10 mM MgCl\(_2\) (or 500 mM KCl if stated), and a 1xTAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3), and diluted with MQ water to the final volume in a PCR tube. A heat-annealing program of 17 h was used. Initially a quick ramp from 90 to 65 °C was used followed by a slow ramp from 65 to 50 °C (−0.1 °C/20 min), and finally a last quick ramp to storage at 10 °C was programmed. Assembly of the origami with functionalities as Cy dyes and hydrophobic moieties was done similarly, by just replacing the non-functionalized oligos with the modified counterparts. DNA nanopore origami CaDNano blueprint, information about the design and staple sequences can be found in Supplementary Fig. 31, Supplementary Note 6, and Supplementary Table 6.

Purification and concentration of the structures was done by 100 kDa MWCO Amicon ultra spin dialysis, prerinsed, and calibrated three times with a TAE buffer (40 mM Tris-acetate, 1 mM EDTA, 5 mM MgCl\(_2\), and 5 mM NaCl) for 5 min with 6 ul of DNA-alkylene (1.5 nmol) in 150 mM HEPES, pH 7.5, 100% lipidatedaza (20 mM in DMSO), and 6 µl of SYBR Safe dissolved in 1xTBE buffer unless stated otherwise. Identical concentrations of TBE and MgCl\(_2\) were used as running buffer.

**Functionalized oligonucleotides.** The diodeo(hexanyl)lthymine triphosphate (dtd(\text{hexa})TP) synthesized in-house was enzymatically ligated with Terminal transferase to the 28 of the 46 lipid oligos (18 bought at IDT with cholesterol). Ligation reactions consisted of a cadocadylate (0.2 M) and Tris-HCl buffer (0.125 M, pH 6.6), CoCl\(_2\) (5 mM) and BSA (0.25 mg/ml), Terminal Transferase (20 U/µl), DNA up to 80 µmol (1.5 mmol), and 15% DIT(hex)[TP in a total volume of 20 µl, incubated for a minimum of 6h depending on DNA amounts at 37 °C and gently vortexed every 1 h for the first 3 h. The reactions were terminated by 20 µm final EDTA (pH 8.0) and ETOH precipitated by adding NaAc (3 M, pH 5.6) and 2.5x volumes of cold EtOH. This was incubated on dry ice for at least 15 min before being pelleted at 17,000 g at 4 °C. The supernatant was removed, and the pellet was resuspended in HEPES buffer (150 mM, pH 7.5) and prepared for subsequent allyne reaction with click chemistry. The standard Cu(II)-catalyzed click reaction was done by mixing 10 µl of click buffer (1.33 mM CuSO\(_4\) (H\(_2\)O), 2.64 mM TBT (DMSO), 50 mM acetic acid (H\(_2\)O), and DMSO to 20 µl—in total 30% DMSO) with 55.8 nM of H\(_2\)O (1.5 mmol in 150 mM HEPES, pH 7.5), 100% lipidated azide (20 mM in DMSO), and 14 µl of DMSO to a final volume of 32 µl and 65% DMSO reacted for 8 h at 50 °C. ETOH was purified again and resuspended in 195 µl.
of 0.1 M TEAA buffer. The reacted product was RP-HPLC purified with a Phe-
nonexem Kinetex XB-C18 column (150 × 4.6 mm) by using a TEAA/MeCN gra-
dient buffer system (Buffer A: 50 mM TEAA, pH 7.0 and 5% MeCN, Buffer B: 
MeCN). Program: 5–100% in 20 min, 0.6 mL/min. Fractions containing the pro-
duct were pooled, lyophilized, and dissolved in 1×TAE to a final concentration of 
100 μM confirmed by UV absorption at 260 nm and ready for use. Similarly, biotin 
was attached by using either NHS-biotin with an amine oligo or using terminal 
transferase and a biotin-11-dUTP (Fenabioscience, Jena, Germany). For PEG 
functionalization of plug, NHS-PEG20k was reacted with the amine oligo and 
purified by using RP-HPLC.

Flow cytometry. Fluorescently labeled DNA nanopores were incubated with 
GUVs prepared by the inverted emulsion assay in the given time. Flow cytometry 
was done by using a Gallios Flow Cytometer (Beckman Coulter, IN, USA) where 
10,000 events were recorded for the statistics. Following this gating was done by 
using relevant software.

Transmission electron microscopy (TEM). A 2% solution of uranyl formate 
was made in 20 mM KOH. Loading and staining of the DNA origamis onto TEM grids 
was done by first putting the grids into a glow discharger for 45 s followed by 
sample incubation for 1–1.5 min (depending on desired coverage) and dried by 
careful blotting. This was quickly followed by staining with freshly thawed ali-
med with the Scipion software package47. For cryoEM, DNA origamis were 
to 150 μl of mineral oil and left for 30 min on ice. Twenty microliters of inner 
and diluted to 5 mg/ml. The chloroform was evaporated in a vacuum rotator for 1 h 
that was evaporated by blowing N2, depositing a lipid 
emulsion from LN2 to a water bath at RT. Extrusion was done with up to 1 ml of 
for 30 min. Slides for confocal assay were prepared by 

TIRF experiments. Single-particle assays. SUVs from DOPC lipids with 1% DOPS charges and 
0.5% biotinylated lipids were subjected to ten cycles of flash-freezing and thawing 
to ensure a unilamellar structure. Afterwards, the SUVs were extruded at 200 nm as 
described before in 400 mM sucrose and 200 mM KCl. The DNA nanoparticles 
demonstrated to be stable for >24 h when diluted in the TIRF solution (Supple-
mentary Fig. 24). ATTO 655 was encapsulated following recently published 
methodology48,49. Glass slides with attached sticky-Slide VI 0.4 from Ibidi were 
functionalized with PLL-g-PEG and PLL-g-PEG–biotin in a 100:1 ratio and con-
sequently covered by a neutravidin layer50. Biotinylated SUVs were flown into the 
system by using a peristaltic pump and left to immobilize for 10 min. Tuning of 
various parameters and equilibrating processes were allowed to achieve 1 × 300 
vessels per field of view. Flowing of buffer removed freely diffusing vesicles.

Long-term DNA nanopore measurements were initiated by flowing into the 
microscope slide ATTO 488-labeled 1.4 pmol opened DNA nanopores that were 
flushed out into the field of view. For other experiments, lipidic key oligos were 
first flown into the system, and settled for about 30 min before rinsing as we 
have done lipidized key oligos recently for lipidated proteins46 and followed by 
slow incubation of DNA nanopores at a flow rate of 10 μl/min with a total flow of 
500 μl in 50 min. To increase throughput, we designed experiments where four 
fields of view were automatically imaged sequentially. In a typical experiment, 
the first field of view is recorded in all relevant imaging channels with exposure time of 
100 ms, followed by the second, third, and fourth fields of view. When the cycle 
was completed, the automated software would return to the first position and initiate 
the cycle again. The temporal resolution for each image was 100 ms followed by 
500-ms change time, resulting in a final temporal resolution of 2.8 s for each cycle. 
A typical experiment lasted for 8 h.

Tracking and localization software of the TIRF experiments. Due to the 
exceptionally long experimental time frame (>9 h), an inevitable stage drift of 
liposomes was observed. To circumvent this problem, we deployed single-particle 
tracking using custom-made plugin TrackPy15,21. First, the TIRF image was 
converted into the TIRF stack and follow the drift of liposomes on the surface. 
This was done by localizing each individual liposome, in each frame for a given experiment, by using a Lagrangian 
Gaussian approximation. Hereafter the localized dots are connected through 
frames, by using Linear Assignment Problem Tracker (LAP Tracker)50, thus 
creating time-resolved trajectories circumventing state drift. By applying the same 
methodology to the nanopore-imaging channel and colocalizing with liposomes, 
we were able to extract docking times and time-resolved intensity traces.

For analysis of three-color experiments (using encapsulated ATTO 655, dTMR- 
40k, and ATTO 488-labeled nanopores), it was necessary to extend and develop the 
methodology. Due to increased noise from adding a third 
fluorophore to 

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inserting each individual frame (512-by-512 pixels) of a given experiment into a bigger, empty frame (562-by-562 pixels) and then moving it according to the average drift (19 for detailed explanation). After drift correction, the signal for each channel would be extracted by using an in-house-developed routine that localizes spots on a surface using TrackPy and subsequently collects the signal from each spot and corrects for background noise.

**Data availability**

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary information files. Additional and relevant data are available from the corresponding authors on reasonable request.

**Code availability**

Codes used for tracking and colocalization in the TIRF setup are available from the Hatzakis group homepage, http://www.hatzakislab.com.

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Author contributions
R.P.T., R.S.S., and J.K. conceived the project and planned the research. R.P.T. and R.S.S. designed the DNA nanopore and R.P.T. did all DNA functionalization and performed the TEM characterizations. R.P.T. and A.O. did the flow cytometry experiments and FRET studies. R.P.T., A.O., and S.K. set up and executed the CLSM GUV studies under supervision from F.C.S. TIRF measurements were planned by M.G.M. and R.P.T. under the guidance of N.S.H. and done primarily by M.G.M. with help of R.P.T. The TIRF data treatment was done by M.G.M., S.S-R.B., and N.S.H. O.R and S.V. provided lipid building blocks for the DNA nanopore functionalization. The paper was written by R.P.T. with inputs from all authors.

Competing interests
The authors declare no competing interests.

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