Membrane proteins play a ubiquitous role in life sciences, as they are pace makers in a variety of essential cellular processes such as transport, communication, bioenergetics, biosynthesis, motility, and immunity. Being the gatekeepers of life, they are prime drug targets. However, the toolbox of analytical techniques tailored to the need of a parallel analysis of membrane proteins and efficient screening of potential drugs is still in its infancy. Small sample amounts, high sensitivity, and local addressability are best met by surface-based model systems. A variety of these has been developed specifically for membrane proteins, (reviewed in refs 1–3), including, but not limited to, solid-supported lipid bilayers, tethered bilayers, microblack lipid membrane, and native vesicle arrays. Some of them have already become available as commercial setups, but are not yet suitable for multiplexed screening applications of nonelectrogenic transporters. One of the most sensitive methods to characterize membrane channels or transporters is to measure ionic currents by forming a stable electric gradient across the membrane (voltage clamp). But many transporters do not generate significant currents. In addition, parallelization of electric measurements is challenging despite recent advances. To circumvent this problem, the transported species (solute) may instead be monitored by fluorescence techniques (reviewed in ref 18).

During the past decade, nanopores have emerged as an increasingly popular tool to investigate single biomolecules, including the translocation of a nucleic acid molecule through a protein channel and solid-state nanopores. Solid-state nanopores have been prepared by different lithography techniques such as focused ion beam drilling, TEM drilling, and electron beam lithography.

Here, we present a massively parallel system that is suited for fluorescence-based analysis of membrane proteins, in particular nonelectrogenic transmembrane events. In contrast to common previous methods that rely on black lipid membranes (BLM) formed from organic solvents, we create solvent-free (meaning free of organic solvents) suspended lipid bilayers (SLBs) on nanopores by vesicle spreading and fusion. As a major improvement, this enables the compatibility of our approach with the broad spectrum of medically relevant membrane proteins that do not retain their function in the presence of organic solvents. Previous approaches used giant unilamellar vesicles (GUVs, typically 10–100 μm in diameter) for this purpose, as the vesicle diameter has to exceed the pore size to allow the formation of a SLB. However, it is of great advantage to prepare the SLB from large unilamellar vesicles (LUVs, ~400 nm in diameter) instead. In contrast to GUVs, LUVs are easily formed in physiological conditions and can be used for the reconstitution of membrane proteins without the loss of their native structure and function by dehydration. The direct application of proteoliposomes for SLB formation may also abolish the need to deliver membrane proteins to an existing protein-free membrane containing organic solvents. The SLBs formed on the pore arrays by vesicle fusion remain stable over long periods of time (>24 h), which is not only due to the small pore size but also the absence of organic solvents, which reduces membrane prestress.

In this work, we have fabricated chips containing 24 arrays of nanopores (Figure 1a). Each array consists of 2025 (45 × 45) nanopores and femtoliter compartments of homogeneous size arranged in a rectangular grid pattern. The dimensions and distance of the cavities allow for both clear
Addressability and parallel readout in a standard fluorescence microscope setup—all cavities of one array can be resolved in a single image (Figure 1b). The size of the nanopores can be controlled precisely by variation of the electron beam dose during manufacturing.\textsuperscript{30} We exploited this fact to produce nanopores of different size in each array (15–120 nm in diameter) on a single chip (Figure 1c). Each nanopore is connected to a cavity (5 \(\mu\)m edge length) inside the chip, which acts as an individual analytical compartment of 30 fl volume after sealing by a SLB (Figure 1d). The anisotropic etching process leads to a precisely defined geometry of the cavities, since it follows the lattice of the crystalline silicon (100) substrate. This results in a clear-cut cavity shape of an inverted four-sided pyramid. The top layer of the chip (50 nm Si\textsubscript{3}N\textsubscript{4}) is thin enough to allow light transmission through the whole surface area of the nanopore cavities. Notably, the reflective sidewalls of the cavities lead to fluorescence enhancement and therefore greater sensitivity. We prefer silicon nitride over the more commonly used silicon oxide as surface material because of its higher durability. Vesicle fusion on silicon nitride is facilitated by oxidative treatment of the chip, which creates an ultrathin SiO\textsubscript{2}-like surface layer.\textsuperscript{31}

The defined fabrication of nanopores as small as 15 nm in diameter is close to the current technical limit of electron beam lithography, which is evident from the decreasing number of pores in the respective arrays (Figure 1a, top row on the left): at very low e-beam doses, minute deviations in the beam current result in a larger distribution of pore size. Notably, if pores do not fully penetrate the Si\textsubscript{3}N\textsubscript{4} layer, the whole top layer cannot be underetched, which in turn is observed as defects in the grid pattern of the array (Figure 1a).

The principle of our analysis is to detect a flux of molecules across a lipid membrane (further on referred to as translocation). Hence, we initially examined this process in giant unilamellar vesicles (GUVs) as a first proof-of-concept. In these experiments, membrane translocation was facilitated by the pore-forming compound saponin, which associates with cholesterol in lipid membranes\textsuperscript{32} and thereby forms pores of 4–8 nm in diameter. In the absence of pore-forming agents, a membrane-impermeable fluorophore added to the external medium was excluded from the GUV lumen. However, when saponin was added, we observed increasing fluorescence inside the GUVs, corresponding to translocation of the fluorophore across the lipid membrane via the saponin pore. In some cases, the lipid membrane ruptured after addition of saponin (see also Supplementary Figure S1 and Supplementary Movie 1 in the Supporting Information).

Consecutively, we applied this measurement principle to nanopore arrays, where we prepared solvent-free planar SLBs at physiological pH and ion strength (Figure 2a). A solid-supported lipid bilayer forms spontaneously from lipid vesicles if adequate properties of lipids, surface, and the surrounding medium are met.\textsuperscript{33,34} Namely, favorable conditions for supported membrane formation from vesicles include neutral or acidic pH (depending on the lipid), high ionic strength, high vesicle concentration, and a smooth hydrophilic surface. If the aspect ratio between vesicle and pore size is large enough, the resulting planar membrane will not only cover the solid support but also seal the nanopores. Remarkably, these SLBs are stable for more than 24 h, which was confirmed by experiments in which a fluorescent buffer solution was trapped inside the cavities by the sealing lipid bilayer (Figure 2b). After 24 h, almost all pores remain sealed by the SLB, which was concluded from the fluorescence persisting inside the cavities after 1 day. The dye content inside different cavities shows minor variations, which might be related to the recent observations that the efficiency of dye encapsulation in lipid vesicles is de-
dependent on vesicle size, and some vesicles, regardless of size, do not encapsulate any dye at all. Therefore, the fluorophore-containing medium in some cavities could be diluted by “empty” vesicles during SLB formation. Another possibility is that minute surface inhomogeneities, such as roughness, charge, or adsorbed materials, may influence the compartmentalization of dye inside the cavities. However, these small variations have no impact on the efflux kinetics since the monoeXponential decay of fluorescence is independent of the initial dye concentration.

We found that the number of membrane-sealed cavities strongly correlated with the pore diameter \( d \) of the nanopores (Figure 2c). Even the largest pores tested \( (d = 120 \text{ nm}) \) could be sealed by LUV fusion, but the smallest pores were covered most efficiently (\( >94\% \)). We suppose that this is due to a larger fraction of vesicles adsorbing to the surface in such a way that the membrane already covers pores during the prefusion stage (Figure 2a).

In the case of very small pore dimensions \( (d < 30 \text{ nm}) \), the homogeneity of pore size suffers from the technical limitations of e-beam lithography. Deviating pores can be easily discriminated in an optical microscope image depending on their cavity size: For \( d < 30 \text{ nm} \), the removal of silicon through the pore during etching of the cavity becomes limiting, so the cavity size is dependent on \( d \) and therefore considerably reduced for smaller pores. These smaller cavities were omitted when counting the sealed pores. Since only about 66% of the pores corresponding to the first two data points in Figure 2c have the expected cavity size, they appear as “outliers” in the respective data set.

Subsequently, we validated the applicability of nanopore chips for multiplexed membrane transport analyses using two model systems: the aforementioned saponin and the well-characterized channel protein \( \alpha \)-hemolysin. This membrane protein has been widely used as a model membrane protein and biosensor and is known to spontaneously...
insert into lipid membranes in an asymmetric orientation, forming a heptameric pore complex that adopts a mushroom-like shape. The inner channel is composed of a β-barrel structure, with a diameter of 1.4 nm at its narrowest site.

Membrane transport was analyzed on nanopore chips by multiplexed recording of the fluorescent tracer efflux (Figure 3). As shown in Figure 3a, a fluorophore solution was encapsulated in the compartments by sealing the nanopores with SLBs. Subsequently, a pore-forming complex was introduced into the lipid membrane. This triggered the release of fluorophores, which was monitored as decay of fluorescence intensity inside the compartments. Two kinetic regimes of efflux are found - the fast efflux is due to membrane rupture (cf. part b), whereas the slower efflux curves denote efflux through saponin pores inside the lipid membrane. The images correspond to the data points indicated in the graph as bullets (upper row, translocation (black trace); lower row, membrane rupture (red trace)). The data points are connected by lines for clarity. Time resolution 6 s.

Figure 3b shows membrane translocation by α-hemolysin, which forms a heptameric channel in the lipid bilayer. Membrane transport and rupture were observed after addition of the membrane channel. Note that rupture events occur with comparable kinetics to saponin, whereas membrane transport is significantly slower for α-hemolysin because of its smaller inner diameter (1.4 nm). Time resolution 300 ms.

Figure 3c shows examples of representative translocation events through α-hemolysin. In contrast to experiments with saponin (see Supplementary Figure S2b in the Supporting Information), interruptions of dye efflux were observed occasionally for α-hemolysin (arrows), which is likely due to single protein channels leaving the nanopore area by lateral diffusion within the lipid membrane. Time resolution 300 ms.

Figure 3d shows cumulative fluorescence intensity of all cavities of each type of event (pore formation or membrane rupture) in one array. The curves demonstrate that membrane ruptures predominantly occur during the early stages of the experiment, whereas membrane translocation events are distributed more homogeneously on the time axis. Time resolution 300 ms.
that we also have observed in GUVs, (i) transport through the membrane pore or (ii) rupture of the membrane. Since the size of the nanopores is below the diffraction limit, the integrity of the SLBs on the nanopores cannot be directly visualized. Hence, we discriminated between intact and ruptured SLBs by the kinetics of dye efflux from the compartments. Since their monoexponential decay constants are notably different (Supplementary Table 1 in the Supporting Information), the transport traces can be well discriminated from membrane rupture events. As expected, membrane rupture events show comparative kinetics both for saponin and for α-hemolysin, whereas the efflux rates through the two membrane pore complexes differ significantly due to the deviating pore diameters (4–8 nm for saponin vs 1.4 nm for α-hemolysin). Remarkably, some of the fluorescence traces of α-hemolysin mediated efflux show abrupt discontinuities (Figure 3c, denoted by arrows), which we interpret as a single protein channel laterally diffusing in and out of the nanopore region. The α-hemolysin complex can reversibly escape from the nanopores because of its asymmetric structure and directed membrane insertion: the bulky cap domain protrudes out of the membrane for several nanometers, while the opposing rim domain does not interfere with the chip interface. We have not observed this “escape” effect for saponin pores, which can be explained by the larger size (and therefore slower diffusion) of the saponin pore complex and the faster efflux rate (i.e., shorter efflux time of the trapped fluorophore).

Since the insertion of α-hemolysin into the SLB is a spontaneous process, the pore formation occurs at a random point in time. If fluorescence traces are not plotted individually for each cavity, but summed cumulatively for each category (Figure 3d), it becomes obvious that rupture events predominantly occur shortly (during the first 5–10 s) after the addition of hemolysin, whereas translocation events are distributed more homogeneously along the time axis. Analogous results were obtained for saponin.

The diffusion kinetics of a confined solute escaping through a small hole can be fitted monoexponentially (eq 1), if the container volume is much larger than the pore:

\[ c(t) = c_0 e^{-t/\tau} \]  

where \( c_0 \) is the initial solute concentration, \( c(t) \) is the solute concentration at time \( t \), and \( \tau \) is the characteristic efflux time constant. Provided that the particles (fluorophores) are noninteracting, the efflux time corresponds to the single particle escape time:

\[ \tau = \frac{V}{4rD} + \frac{L^2}{2D} \]  

where \( V \) is the container volume, \( r \) and \( L \) are the radius and length of the nanopore (either the solid state nanopore or protein pore), and \( D \) is the particle’s diffusion coefficient. Due to the small dimensions of our system, the second term can be neglected. To evaluate if this model can describe the kinetics of our experiments, we compared it to a 3D random walk simulation within the pyramidal cavity (Figure 4, Supplementary Movies 2 and 3 in the Supporting Information). We found that the analytical solution is able to estimate the results of the random walk model with good accuracy, in particular for small pore sizes. The rate constants determined by the random walk model (membrane rupture, 0.47 s; transport through α-hemolysin, 58 s; see also Supplementary Table 1 in the Supporting Information) are also in reasonable agreement with the experimental values (membrane rupture, 3 s; transport through α-hemolysin, 56 s).

In both cases, the experimental data yield slightly higher values for \( \tau \) than expected from theory, which might be the

FIGURE 4. Simulation of particle escape by a 3D random walk model. (a) Trajectory of an Atto532 molecule inside the pyramidal cavity (5 × 5 µm²) translocated through an α-hemolysin pore (\( r = 0.7 \) nm). The trajectory (yellow line) shows an excerpt of 25 ms from a 25 s simulation. The position of the particle at the beginning of the excerpt (red) and the exit site (protein pore, green) are marked with arrows. Coordinate axes are shown as lines originating from the center of the nanopore. Full simulations are shown as movies in the Supporting Information (Supplementary Movies 2 and 3). (b) Escape rate \( 1/\tau \) determined by the 3D random walk simulation compared to the values predicted from theory. Especially for small pores (below 100 nm), the theoretical predictions agree well with the results of the simulations. The dark area corresponds to the range of pore diameters prevalent on the chip. Simulations were also performed with diffusing channels. However, no significant deviation between a static and dynamic channel is observed for different aperture radii. This is also suggested by the large difference in diffusion coefficients between α-hemolysin and the fluorophore (at least 2 orders of magnitude). For a given time interval of the step length for the protein channel is only about 5% or the step length of the dye molecule.
result of several causes: The simplified model does not take into account the particle’s size and its interaction with the inner pore walls, which can add an energy barrier for its translocation, especially inside the narrow protein channel. Furthermore, the efflux of the fluorophore through the solid-state pore is measured during the rupture process of the membrane. However, the suspended membrane may still be partly intact, so that the hole inside the lipid membrane might be smaller than the solid-state pore in the chip. If we calculate the size of the hole inside the ruptured membrane from the measured escape time, we obtain a radius of only 10 nm, whereas the radius of the solid-state pore is 46 nm. Another possibility is that the membrane ruptures slowly on a time scale that is close to the expected escape time. For translocation through α-hemolysin, the measured escape time corresponds to an estimated pore radius of 0.35 nm (according to the analytical solution), which is in reasonable agreement to the pore dimensions of hemolysin (0.7 nm radius). This provides additional evidence that we indeed observe the translocation through a single, individual membrane channel complex.

The use of fluorescence as readout also facilitates another dimension of multiplexing: several species can be monitored simultaneously by using fluorescent markers of distinct excitation and emission spectra. We exploited this spectral coding to show the selective translocation conveyed by α-hemolysin by trapping fluorescent species of different size inside the cavities (Figure 5a). In contrast to the small fluorophore (Atto532, red channel, ~0.8 kDa), a 10 kDa dextran molecule (Alexa488 conjugate, green channel) cannot permeate the protein channel. Exclusively in the case of membrane rupture, the dextran molecule is able to escape the compartment. By monitoring both fluorescence channels before and after incubation with α-hemolysin, we could distinguish between protein mediated membrane translocation and membrane rupture events (Figure 5b,c): In the absence of α-hemolysin (nanopore NP 1), both markers remained trapped inside the cavity, but the Atto532 fluorescence has been partly bleached during the time between both images. When α-hemolysin was added, the Atto532 fluorescence disappeared completely, e.g., in NP 2 and NP 3, but only in some cases the Alexa488-dextran fluorescence was lost as well (NP 2). Conclusively, this indicates specific efflux through α-hemolysin in NP 3 and membrane rupture in NP 2. This finding is consistent with the Atto532 fluorescence time lapse in the respective regions (Figure 5c): Compartments that lose both markers (trace 2) show much faster efflux rates than those that retain the green marker (trace 3; see also Supplementary Movie 4 in the Supporting Information). The efflux kinetics are consistent with the results of random walk simulations (Figure 4). Intensity histograms of all cavities (Figure 5d) demonstrate that a substantial fraction of the compartments loses the free Atto532 fluorophores (red), but not the larger dextran particles (green), denoting that single translocation events occur in a considerably higher frequency than membrane ruptures. The overall intensity level of red fluorescence is shifted to lower intensities in the second row due to photobleaching (only the red channel is monitored continuously during the experiment).

When all fluorescence traces are plotted cumulatively by category (rupture or translocation), it is evident that rupture events occur most often shortly after addition of the pore-forming compound. Notably, membrane rupture in one nanopore does not affect the analysis of neighboring pores. We believe that membrane ruptures are not a result of osmotic stress, since isoosmolar media are present on both sides of the SLB. Since we observed that ruptures occur predominantly after the addition of membrane pore forming units, we suppose that membrane rupture is induced by high local concentrations at the membrane in this stage of the experiment. After the concentration has equilibrated by diffusion, it is less likely that a high number of the pore-forming species will insert at close proximity in the membrane. Such “concentration spikes” can be avoided by using microfluidics to deliver the substance to the membrane in a more controlled manner. For example, specific vesicle preparations or membrane proteins can be delivered by laminar flow. Spontaneous membrane rupture events were not observed to a significant extent in the absence of membrane-interacting agents, which demonstrates the stability of lipid bilayers suspended on nanopores.

The possibility to monitor several markers simultaneously by fluorescence is especially advantageous for the direct comparison of transported cargo, e.g., to analyze a substrate specificity as well as ligand and inhibitor interactions. Another potential application is to verify whether the transport observed is in fact facilitated by a particular, expected protein of known specificity. The parallel analysis of two or more species also provides the opportunity to correct differences for differences between several experiments, because an additional reference marker may be included. Fluorescence readout not only allows an additional level of parallelism (spectral coding) but can also characterize non-electrogenic membrane transport processes. The experimental setup that is used for optical probing may simultaneously be used for the triggering of photoswitchable membrane transporters, photouncaging of ligands, and manipulation by optical tweezers even inside the compartments. Fluorescence also allows the localization and tracing of single protein molecules inside the membrane, so that transport could be correlated directly with position, number, or interaction state (oligomerization, ligand binding) of membrane proteins.

Since the nanopore chips are fabricated using semiconduc-
tor substrates and techniques, further adaptations to tune the functionality of the system can be envisioned, e.g., additional electrical or mechanical readout. Another intriguing option is patterning of the surface and controlled microfluidic access to individual nanopore arrays, e.g., to deliver different proteins or ligands in a single experiment. The SLBs could be formed on the nanopores from either proteoliposomes or native vesicles directly derived from cells; hence proteins can be studied both in a well-controlled minimalistic model environment (purified...
protein and synthetic lipids) and in their natural surroundings, reflecting their in vivo state.

In conclusion, we demonstrate the massively multiplexed analysis of single membrane pore complexes using a lab-on-chip system that is compatible with direct incorporation of functional transmembrane proteins from proteoliposomes. Its geometry is precisely defined and can be specifically adapted or expanded to extend its functionality. The formation of stable, solvent-free SLBs on nanopore arrays in a single step is a key requirement for accurate and robust

FIGURE 5. Selective transport via single α-hemolysin pores demonstrated by dual-color fluorescence. (a) Size selectivity of α-hemolysin (not drawn to scale). Whereas the free red dye (Atto532, 0.8 kDa) can permeate the protein channel, the green dextran-conjugated dye (Alexa488, 10 kDa) remains trapped inside the femtoliter compartment even in the presence of the channel protein, as long as the sealing lipid membrane stays intact. The dextran particles can escape only after membrane rupture. (b) Simultaneous monitoring of both fluorescence markers by fluorescence microscopy. The upper row depicts the red, green, and merged channels before protein addition; the lower row shows the same chip area after membrane incorporation of α-hemolysin. The three marked compartments are examples for the absence of protein in the nanopore (1); loss of both markers due to membrane rupture (2); and protein-mediated efflux of only the free Atto532 (3). Scale bar 10 µm. (c) Time traces of Atto532 fluorescence intensity corresponding to the three marked nanopore compartments shown in (b). The gallery above the graph shows a zoom-in of the respective compartment before (upper row) and after incubation with α-hemolysin (lower row). The kinetics allows clear discrimination between protein-specific membrane translocation (τ = 56 s), rupture (τ = 3.0 s), and dye bleaching (τ = 513 s). Time resolution 300 ms. (d) Fluorescence intensity distribution of all nanopores in one field of view before (upper row) and after incubation with α-hemolysin (lower row). It is evident that Atto532 fluorescence is in many cases reduced to background level after addition of hemolysin, but loss of Alexa488-dextran fluorescence only occurs in a few cases. The distributions have been fitted with a Gaussian function (black line).
analysis of functional membrane proteins and avoids potential difficulties arising from mechanical or electrical interference, surface modification, or the denaturing of proteins by organic solvents. Hence, the presented nanopore arrays offer the potential for time- and cost-efficient screening of a virtually unlimited variety of therapeutic drug targets.

Acknowledgment. We thank Helge Grossmann for helpful discussions and Drs. Min Chen and Hagan Bayley (Department of Chemistry, University of Oxford, U.K.) for samples of α-hemolysin. We thank Christophe Danelon (Delft University of Technology, Delft, NL) for advice on the preparation of GUVs. This work was supported by the Federal Ministry of Education and Research (BMBF, Nanobiotechnology 0312031/0312034), the German Research Foundation (SFB 807, Transport and Communication across Biological Membranes), the Cluster of Excellence (Macromolecular Complexes) at the Goethe-University Frankfurt, and the European Drug Initiative on Channels and Transporters (EDICT) funded by the EC Seventh Framework Program.

Supporting Information Available. Experimental procedures, graphs of translocation and membrane rupture recordings, images and movie of translocation experiments in GUVs, movies of 3D random walk simulations, and movie of translocation recordings. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES