Supplementary Information

Highly parallel transport recordings on a membrane-on-nanopore chip at single molecule resolution

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Experimental Procedures

Design of nanopores and femtoliter cavities. 5-inch silicon-on-insulator (SOI) wafers were prepared with photolithography for the processing of approximately 1,150 individual chips (Fig. 1*A*,*B*). The SOI chips were subsequently structured by reactive ion etching (RIE) resulting in arrays of 250,000 uniform cylindrical cavities in the silicon (diameter: 0.95 µm; depth: 10 µm; volume: 6.5 fL; Fig. 1*C*). The cavities are arranged in a rectangular pattern on each chip with a center-to-center distance of 4 µm (Fig. 1*D*). In a third step, a silicon dioxide top layer is formed by chemical vapor deposition, narrowing the cavity opening to a pore in the nanometer range (Fig. 1*E*). Importantly, the chip features an optically intransparent top layer and a transparent glass support. Detailed information about the chip fabrication can be found in the patent WO 2012006995 A1 and chips are commercially available (nanoFAST E100, Nanospot GmbH, Münster). After wafer dicing, the cleaned chips were integrated into an 8-well sample holder (ibidi GmbH, Planegg/Martinsried, Germany) for use on inverted fluorescence microscopes. Nanopore chips were analyzed by atomic force microscopy (AFM) and scanning electron microscopy (SEM). For AFM studies, a Nanowizard II (JPK Instruments AG, Berlin, Germany) was used in contact mode. The surface roughness of the silicon dioxide top layer of the chips was determined as the root-mean-squared roughness (R_q) from multiple AFM scans (n = 40). Sample areas of 2 x 2 μm^2 were analyzed.

Liposome preparation. Large unilamellar vesicles (LUVs) were prepared as described.[1,2] In brief, lipids (SoyPC20, *E. coli* polar lipids, DOPC; Avanti Polar Lipids Inc.) dissolved in chloroform were dried by a rotary evaporator for 2 h under vacuum at room temperature. The lipid film was rehydrated in Tris-buffer (20 mM Tris, 150 mM NaCl, pH 8.0) for 45 min at 50°C to a final concentration of 20 mg/mL, followed by sonication for 4 min in a water bath ultrasonicator at room temperature. Subsequently, seven freeze-thaw cycles were performed with rapid freezing in liquid nitrogen and thawing in a water bath at 50°C, respectively. Aliquots of 1 mL were stored at -80°C.[3]

Protein reconstitution into liposomes. $MscL^{G22C}$ was purified and reconstituted as described.[2] In short, after thawing on ice, liposomes were extruded 17-times through a 400 nm polycarbonate filter. Liposomes were destabilized by addition of a final concentration of 0.25% Triton X-100 (Affymetrix) for 5 min at 50°C. $MscL^{G22C}$ and detergent-saturated liposomes were mixed at 1:20 (w/w) ratio, respectively, and incubated for 30 min at 50°C. Subsequently, a Tris-buffer with or without the self-quenching dye calcein (200 mM) was added to $MscL^{G22C}$-liposome mixture in a 1:1 volume ratio and supplemented with 16 mg (wet weight) Biobeads (SM-2 Absorbents) per µl detergent (Triton X-100). For detergent removal, the sample was incubated overnight (16 h) at 4°C on a rotating plate. Proteoliposomes with an average diameter of 185 nm were formed (Fig. S1) as determined by nanoparticle tracking (NanoSight LM14, Nanosight Ltd.). Calcein-encapsulated proteoliposomes were used in fluorescence dequenching assay to confirm the activity of the $MscL^{G22C}$ channel after reconstitution.[2]

Pore-spanning solvent-free suspended lipid bilayers. Nanopore biochips were treated with air plasma for 5 min. The cavities were pre-wetted with ethanol for 20 min. Ethanol was gradually exchanged again calcium containing Tris buffer (20 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ pH 8.0). Liposomes or proteoliposomes were added to a final concentration of 0.5 mg/mL and incubated for 1 h at room temperature. Excess of non-spread liposomes and fluorescent dye were removed by washing with calcium-free buffer.

Fluorescence recovery after photobleaching. FRAP was performed on a Leica DMRE upright microscope (Leica Microsystems) equipped with a Leica HCX APO L 63×/0.90 water-immersion objective, a Leica TCS SL confocal scanner and an argon ion laser (488 nm, 25 mW). Images with an edge length of 60 µm and a resolution of 0.116 µm/px were

acquired at 10% laser power every 346 ms. After recording ten pre-bleach images, a central rectangular region of 7.4×7.4 μm^2 was bleached for 346 ms (1 frame) at 100% laser power, and 180 post-bleach frames at intervals of 346 ms were acquired. Data analysis of rectangular bleach area was performed as previously described using GNU Octave 3.6.2.[4]

Channel efflux experiments. Cavities were prefilled with buffer containing 10 µM of Oy647 (Luminartis) as transport solute. 5 µM of OregonGreen-labeled Dextran 70 kDa (Life Technologies) was used as non-permeable control solute. Proteoliposomes were supplemented with 0.1 mol% ATTO390-labeled DOPE (ATTO-Tec) during liposome preparation. Efflux of the fluorescent substrate was initialized by the addition of cysteine-specific, positively charged compound MTSET (3 mM). Reaction of cysteine 22 of $MscL^{G22C}$ with MTSET introduces five positively charged choline groups within the pore and forces this channel into an open state.[5] Images were recorded every 10 s over a period of 90 min. Data processing of the images was performed using ImageJ. Transport kinetics were analyzed according to an efflux process with first order rate constant k_{flux} using the Nanocalc software (Nanospot GmbH) for curve fitting.[6] Gaussian fits of the histogram were calculated using Origin 8.6 Pro (OrginLab). All efflux experiments were performed on an automated NyONE microscope (SynenTec GmbH) equipped with a 20x Olympus air objective (Olympus UPlan SApo 20x/0.75). Additional experiments were carried out with a DMI6000 TIRF microscope (Leica Microsystems) equipped with a 40x Leica air objective (Leica HCX PL FLUOTAR 40x/0.60 CORR).

References

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Supplementary Figures

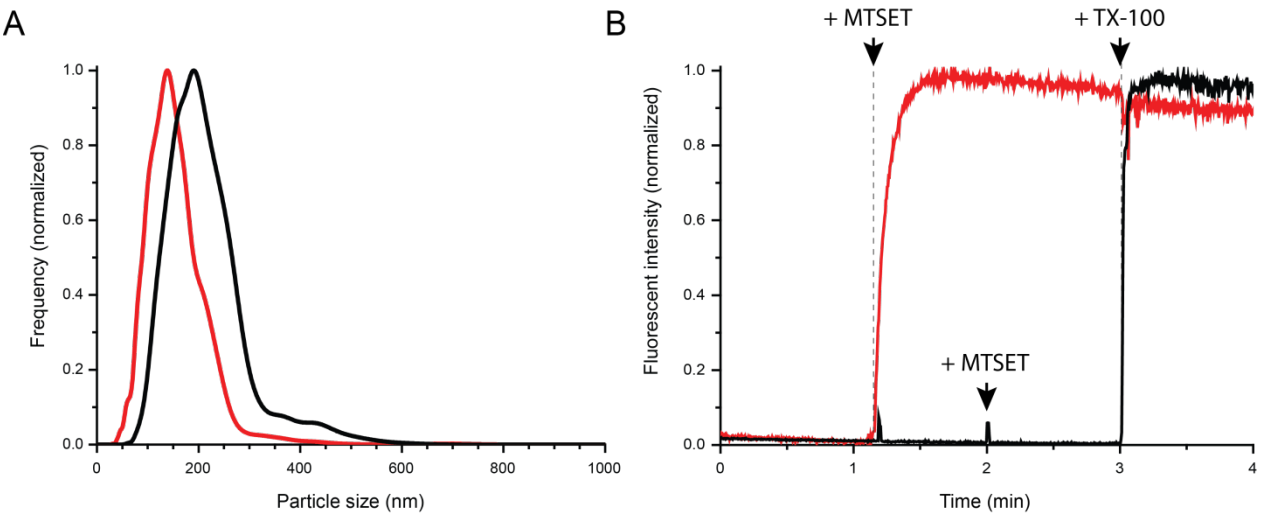

Figure S1. Size distribution of proteoliposomes and MscL function. A) Vesicles were analyzed by nano-particle tracking analysis. LUV samples were examined before (black trace) and after reconstitution of MscL (red trace). After LUV preparation, a mean particle size of 214 ± 70 nm is achieved. The size is decreased during reconstitution, resulting in proteoliposomes of 158 ± 60 nm. B) MscL activity was validated after reconstitution by an efflux assay.[2] Self-quenched calcein is released from proteoliposomes through MTSET-mediated opening of the MscL channel, leading to dequenching of the fluorophore (red) and a rapid increase of the fluorescence intensity. Liposomes without protein show no such efflux, even after repeated MTSET addition (black). Subsequent detergent-introduced solubilization (TX-100) of the liposomes releases all previously encapsulated dye.

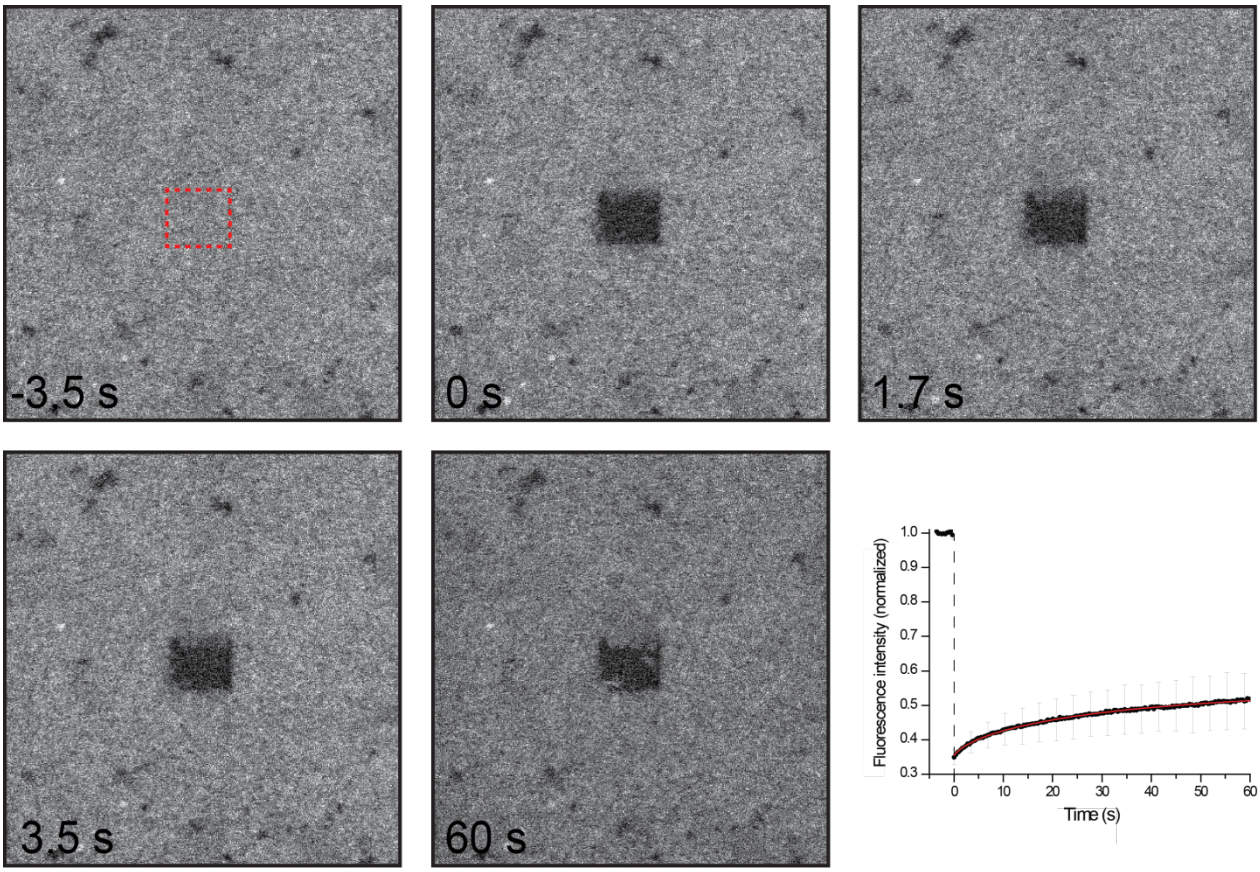

Figure S2. If 0.05 mg/mL lipid (ten times less than in Fig. 2) is used for bilayer formation, only small bilayer patches intermixed with regions of adsorbed, non-fused LUVs are observed. These isolated LUVs cannot recover by lateral diffusion after bleaching (D = 0.2 ± 0.3 µm^2 s^{-1}) and account for an immobile fraction of up to 90%. Small bilayer patches showed a diffusion coefficient of 1.7 ± 0.4 µm^2 s^{-1} and an immobile fraction of 5 ± 5% in good agreement to the continuous bilayers described in Fig. 2.

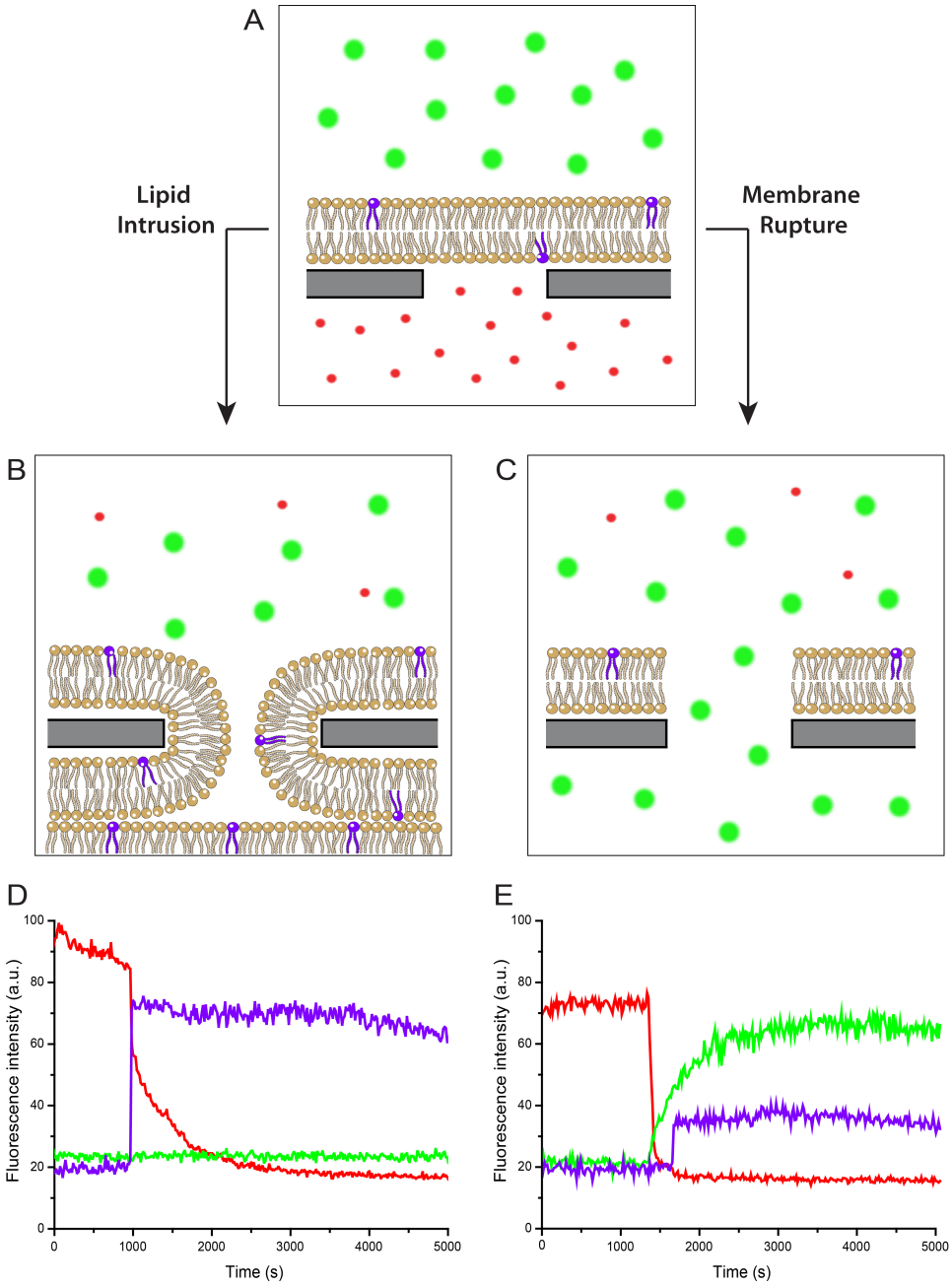

Figure S3. Different events distinguished by three-color detection. Translocated solute (red), control solute (green) and a lipid dye (violet) can be spectrally discriminated allowing for unbiased data selection. A) Sealed cavities harboring no membrane protein show no flux events under the experimental conditions described for the MscL channel recording (see Fig. 2A). The fluorescent readout is stable for all channels. B) Ill-defined suspended lipid bilayers are able to intrude the cavity. C) Membrane rupture results in discontinuous lipid bilayer spanning the nanopore fissures. Thereby, the cavity space is no longer separated from the buffer compartment. Entrapped dyes (red) evade the cavity down the concentration gradient. The control solute (green), previously unable to pass across the membrane, enters the cavity. D) Example for a lipid intrusion event. While the fluorescent signal for the lipid dye (violet) increases due to the membrane invading the cavity space, the translocation solute is pushed out of the cavity (red), leading to a decrease in fluorescent signal. The nanopore is blocked by the lipid bilayer, preventing the passage of the control substrate (green). E) During membrane rupture, the enclosed transport solute rapidly evades from the cavity (red), while the control solute diffuses in (green).

Supplementary Movie

Movie M1. Long-term stability of nanopore suspended lipid bilayers. A small fluorophore (ATTO488) was entrapped inside the cavities by SLB formation. LUVs (1.0 mg/ml POPC/POPG/ cholesterol 8:1:1 w/w) were spread on the chip surface, non-enclosed dye diluted out by consequent washing steps with calcium free buffer (20 mM HEPES, 150 mM NaCl, pH 7.6). Images were taken every 15 min for 48 h. Shown is a time-lapse video with 20 frames per second. After 48 h of originally 1755 sealed cavities 73.1 % of pore spanning membranes remain intact, still retaining the encompassed dye.