Electronic Supplementary Information

Large, square-shaped, DNA origami nanopore with sealing function on giant vesicle membrane

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Materials and methods

Preparation and purification of DNA origami structures

All staple strands and TAMRA-modified DNA and Blocker-DNA were purchased from Eurofins Genomics Tokyo (Tokyo, Japan). Single-stranded M13mp18 viral DNA was purchased from Nippon Gene (Tokyo, Japan). Cholesterol-modified DNA (Anchor-DNA) was purchased from Integrated DNA Technologies Japan (Tokyo, Japan).

DNA origami structures were designed using the caDNAno software. The formation of the origami structures was accomplished by mixing 20 nM of scaffold DNA (M13mp18 ssDNA) with ~150 nM of staple strands, TAMRA-modified DNA, and Blocker-DNA in the folding buffer containing 5 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 12.5 mM $MgCl_2$. The mixture was annealed as follows:

1) Heated at 90 °C for 20 s.
2) Cooled from 70 °C to 45 °C at a rate of −0.8 °C/h.
3) Cooled from 45 °C to 24 °C at a rate of −2.0 °C/h.
4) Cooled and stored at 24 °C.

After annealing, the structure was stored at 4 °C away from light sources. The assembled structure was purified with a 100 kDa MWCO centrifuge filter (Amicon Ultra, Merck Millipore, Billerica). Using an Mg^{2+} concentration-reduced buffer (12.5 mM → 1.5 mM), the centrifugation

set of 4500 x g for 5 min and 1000 x g for 2 min with the filter inverted was repeated 3 times. After purification, the Mg^{2+} concentration was immediately recovered.

Electrophoresis

In the agarose gel electrophoresis, the samples were electrophoresed in 1.0% agarose gel containing 5 mM $MgCl_2$ in 0.5x Trisborate-EDTA buffer solution (pH 8.0) at 50 V at 4 °C. In the polyacrylamide gel electrophoresis, the gel was composed of 12% bis-acrylamide, 0.1% tetramethylethylenediamine (TEMED), and 1% ammonium persulfate (APS) in a tri-acetate-EDTA (TAE) buffer. Each DNA sample was mixed in TAE buffer containing 12.5 mM $MgCl_2$. Electrophoresis was performed at 100 V for 100 min at room temperature (≈25 °C). The gels were then imaged with ChemiDOC MP (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, MA) as the staining dye.

nsTEM observation

A 0.2% phosphotungstic acid solution was used as the staining solution. Phosphotungstic acid (NISSHIN EM, Tokyo, Japan) was dissolved in ultrapure water to give a 0.2% solution, and the pH was adjusted to 7.3–7.4 using sodium hydroxide. A copper grid (Collodion COL-C15, Okenshoji, Tokyo, Japan) was used as the observation grid. The grid was hydrophilized by irradiating with a plasma irradiator (SEDE-GE/B, Meiwafosis, Tokyo, Japan) at a voltage of 5 mV for 1 min. The diluted sample (~5 nM) was dropped onto the grid and left to stand for 1 min. The excess solution was then removed, and the grid was washed thrice with the folding buffer. Subsequently, 0.2% phosphotungstic acid solution was dropped onto the grid, left to stand for several seconds, and the excess solution was removed; this was performed twice to complete the dyeing process. The grid was kept in a dry place until the start of the observation. JEM-2100F (JEOL Ltd., Tokyo, Japan) was used for nsTEM observations at an acceleration voltage of 200 kV. The observed images were analysed using Fiji (ImageJ) and EMAN2.

LUV preparation for TEM observation

A lipid film was prepared using dioleoylphosphatidylcholine (DOPC) (NOF Corporation, Tokyo, Japan) and cholesterol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The lipid was dissolved in chloroform at a ratio of 9:1 to generate a 30 µL solution. The mixture was

evaporated in an argon gas atmosphere and then left to stand for at least one night. Afterwards, 300 µL of buffer solution for LUV (1x TAE, 12.5 mM $MgCl_2$, and 300 mM glucose) was added and vortexed for 1 min. By using an extruder (Syringe (1000 µL), Avanti Polar Lipids, Alabama, USA) and a 10 mm / 200 nm filter (10 mm: manufactured by Avanti Polar Lipids; 200 nm: manufactured by Sigma Aldrich Japan, Tokyo, Japan), LUVs with a size distribution of approximately 200 nm were prepared.

GUV preparation for the confocal assay

An aliquot of liquid paraffin (300 µL) was mixed with the lipid film mentioned above, and the lipid was dissolved in liquid paraffin by sonication for 60 min at 50 ℃. Then, 20 µL of the GUV internal buffer (1x TAE, 12.5 mM $MgCl_2$, 150 mM glucose, and 150 mM sucrose) was added to the mixture and vortexed for 1 min to prepare a W/O emulsion. The emulsion was added to 300 µL of the GUV external buffer (1x TAE, 12.5 mM $MgCl_2$, 300 mM glucose, and ~10 µM of the fluorescent dye molecule) kept in a tube and centrifuged at 8,000 x g for 15 min. The precipitated GUVs were collected by puncturing the bottom of the tube. Calcein was purchased from Dojindo Laboratories (Kumamoto, Japan). Dextran 40k and dextran 500k were purchased from Thermo Fisher Scientific (Waltham, MA) and Sigma-Aldrich Japan (Tokyo, Japan), respectively.

Binding nanopores to vesicles

Cholesterol-modified DNA (Anchor-DNA) was incubated at 55 ℃ for 15 min before the start of the experiment to avoid hydrophobic aggregation. Osmotically adjusted Anchor-DNA and GUV or LUV were mixed at a volume ratio of 2:1 and incubated for 30 min to achieve insertion of the Anchor-DNA into the vesicle membrane surface. The incubated solution was mixed with a pre-purified nanopore solution, with osmotic pressure adjusted to a volume ratio of 1:1.5. In the case of the LUV solution for TEM observation, a total of 10 µL of the mixture was incubated for approximately 3 h before grid formation. The binding of the nanopores to the TAMRA-modified DNA was observed using a fluorescent microscope IX-83 (Olympus, Tokyo, Japan).

The GUV dye influx assay

A total of 30 µL of the solution containing GUVs, Anchor-DNAs, and nanopores prepared by the method described above was injected into a moulded plastic chamber (ibidi µ-slide 0.4(VI), ibidi GmbH, Gräfelfing, Germany) immediately after the addition of the nanopore solution. The

osmotic pressure of the reaction system was carefully adjusted to prevent the rupture of the GUVs. Then, 30 µL of the GUV external buffer, containing the fluorescent molecule, was injected into both sides of the chamber, and 7.5 µL of mineral oil was dropped onto each side to prevent evaporation of the sample (details are shown in Fig. S6). Confocal microscope observation was performed using FV1200 (Olympus, Tokyo, Japan).

The time of mixing of the nanopores into the GUV was set to T = 0 min, and the GUV sedimentation and confocal time-lapse setting were performed for 30 min. The confocal microscope recording was then performed for 12 h (T = 30–750 min). Each sample was photographed at multiple coordinates (around 20 places) and heights (2–3 slices) in 10 min intervals using an automatic XY-stage (H117, Prior Scientific Instruments, Cambridges hire, USA). The number of influxes of GUVs was counted visually, and Fiji was used for numerical quantification. Among the inflowing GUVs, those with significant membrane deformation or rupture were excluded from the influx count (Fig. S7).

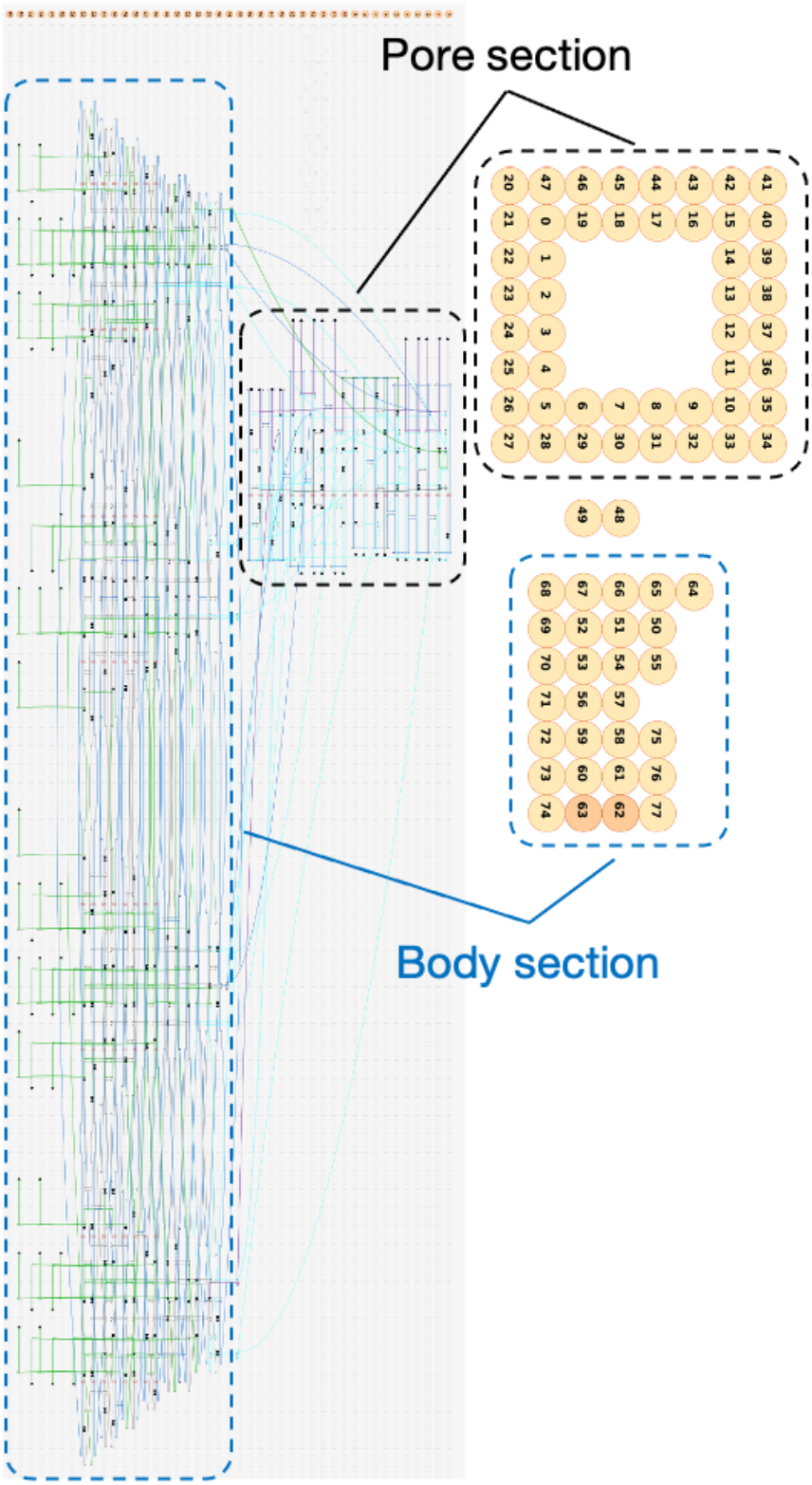

Fig. S1. The caDNAno artificial nanopore design. The nanopore consists of a pore section and a body section.

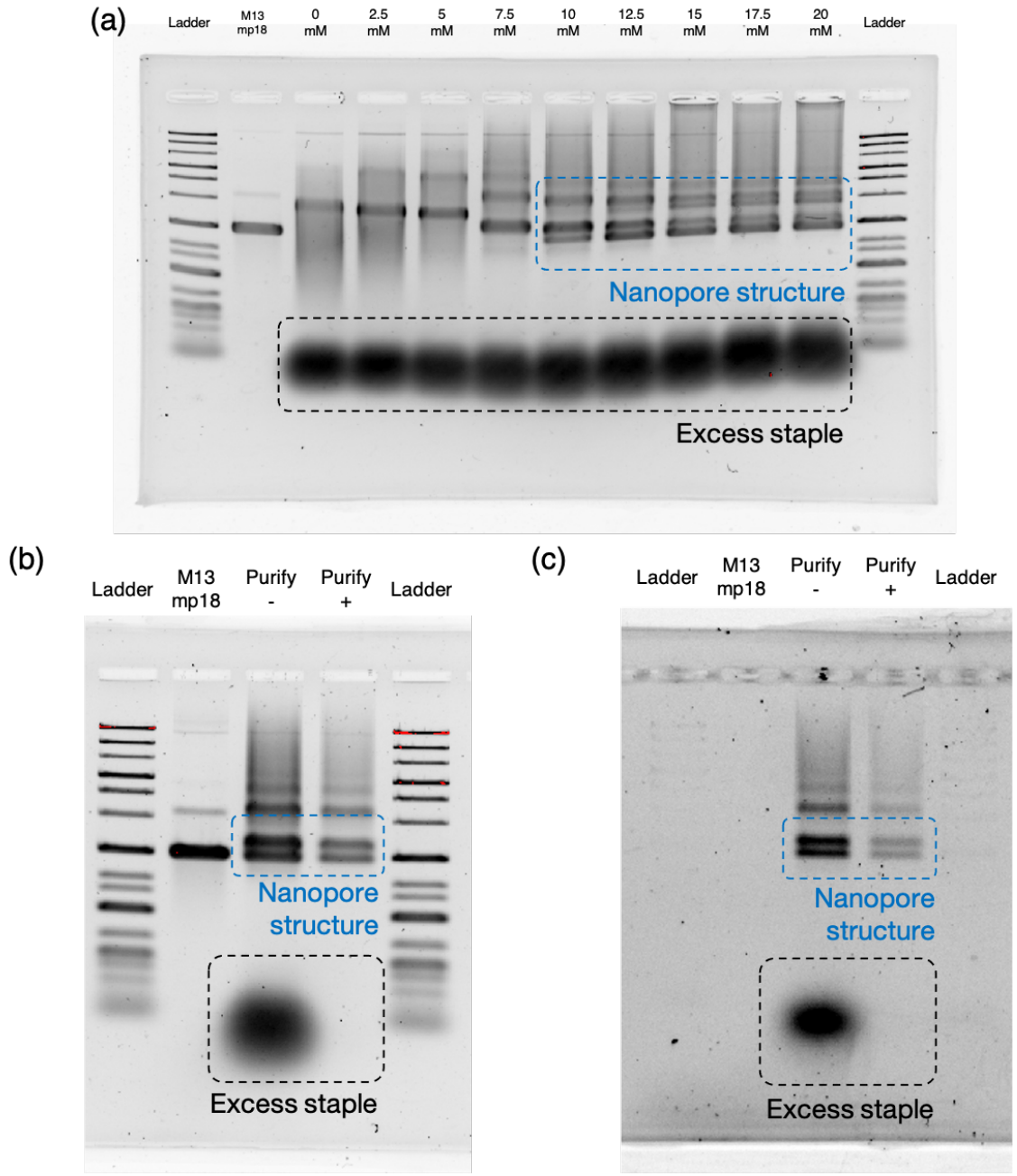

Fig. S2. The agarose gel electrophoresis analysis of the artificial nanopore. (a) The analysis of the structures in the folding buffer with different Mg^{2+} concentrations. (b) The analysis of the structures before and after purification. (c) The confirmation of the TAMRA fluorescent label (taken before staining with SYBR Gold).

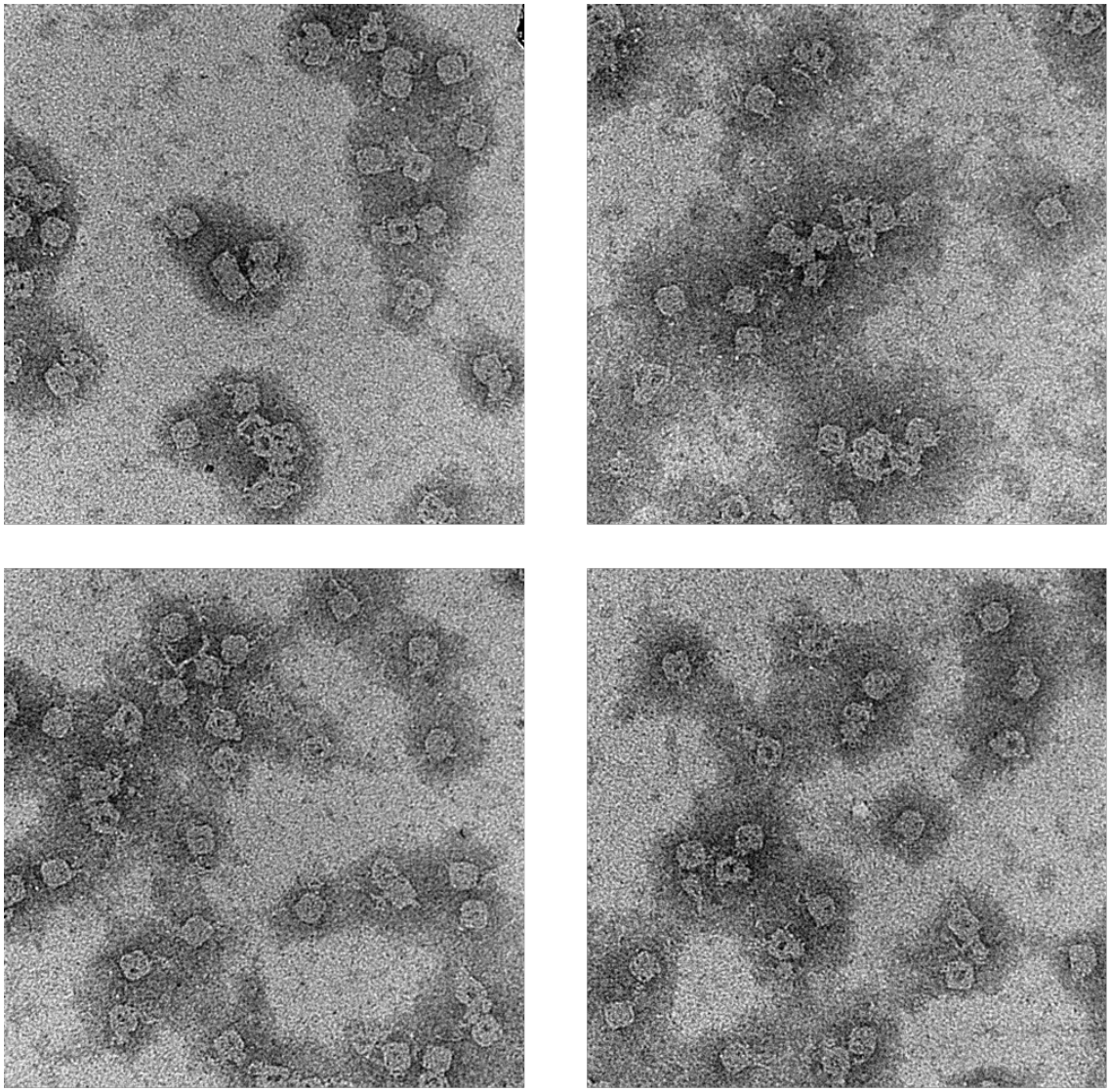

Fig. S3. The raw images of the nanopores observed by nsTEM (stained with 0.2% phosphotungstic acid, at an acceleration voltage of 200 kV). Scale bar: 100 nm

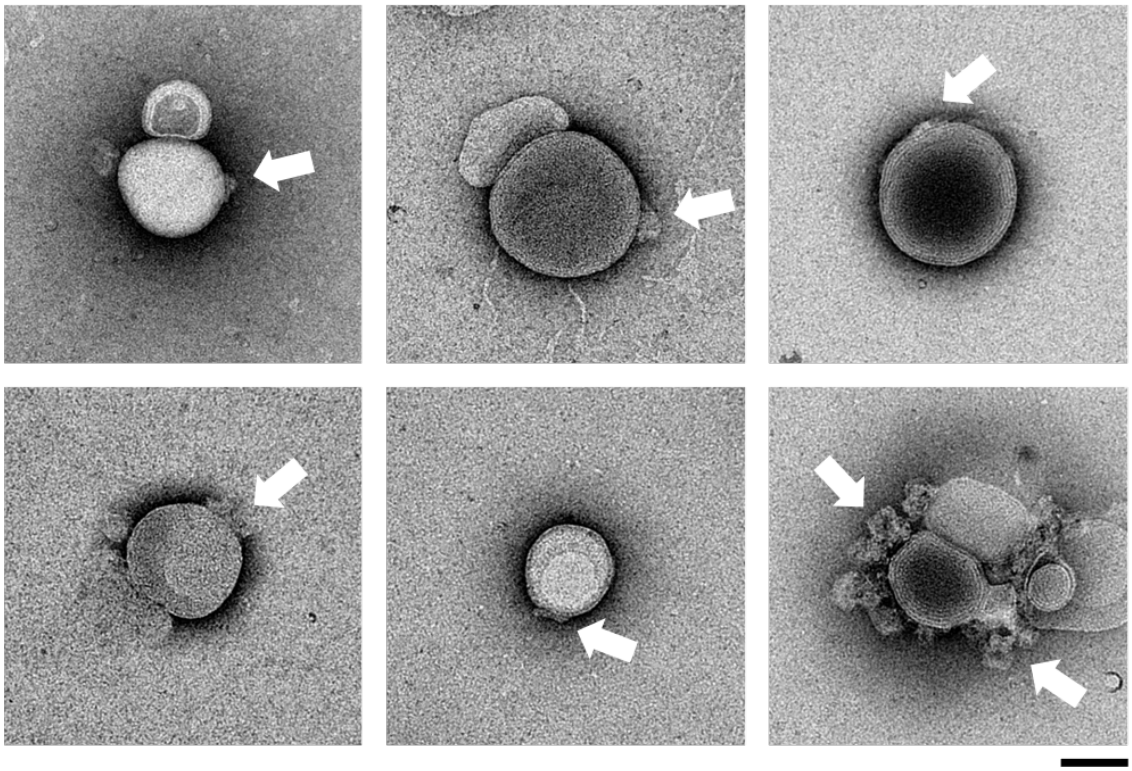

Fig. S4. The negative-staining TEM images of the vesicles with the DNA origami nanopores. Scale bar: 100 nm

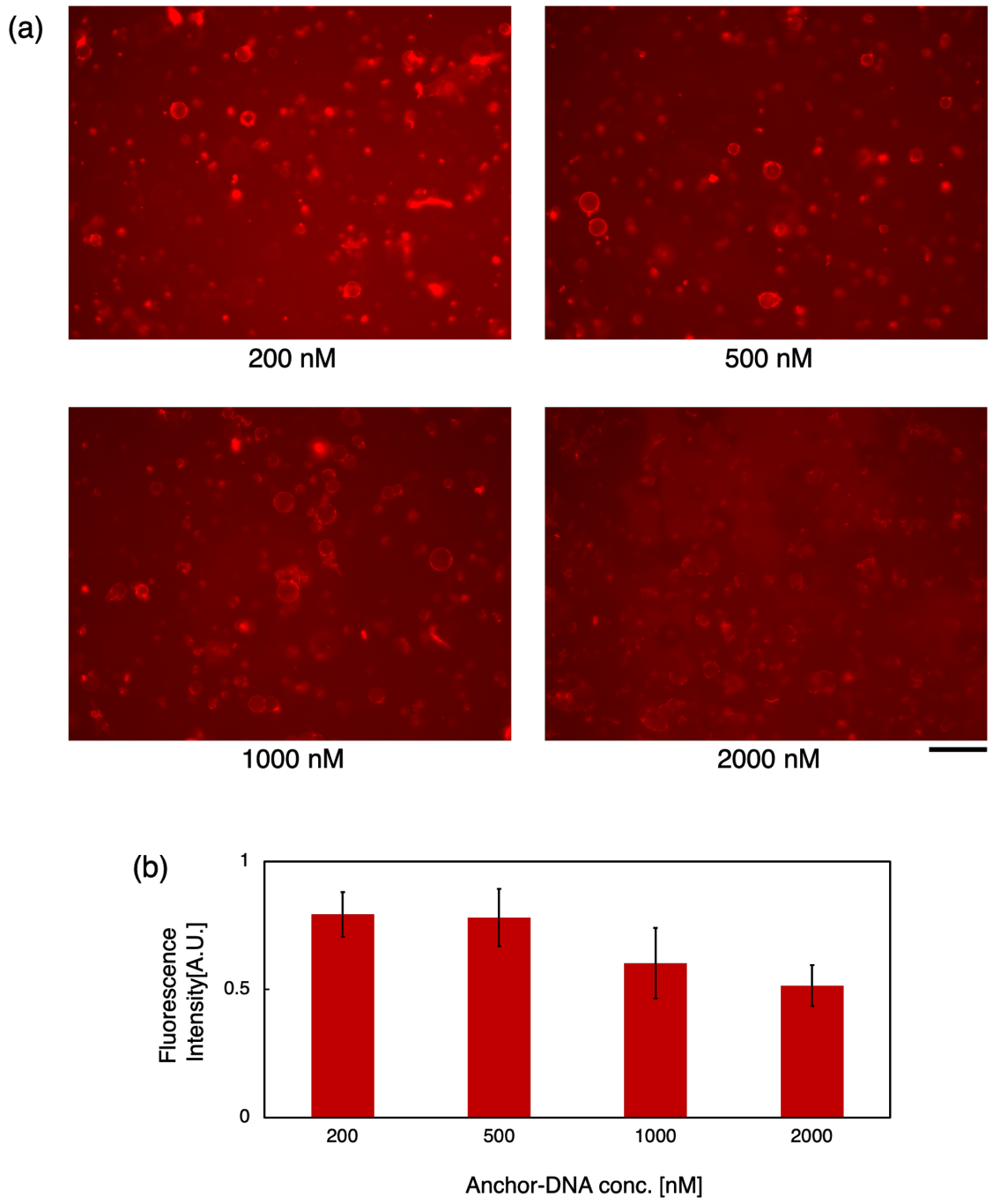

Fig. S5. Binding of the nanopore at multiple concentrations of Anchor-DNA

(a) Representative fluorescence confocal microscopic images of the artificial nanopores and GUVs at different concentrations of the Anchor-DNA. Scale bar: 50 µm. (b) Average fluorescence intensity of GUV membranes at different Anchor-DNA concentrations (N = 10).

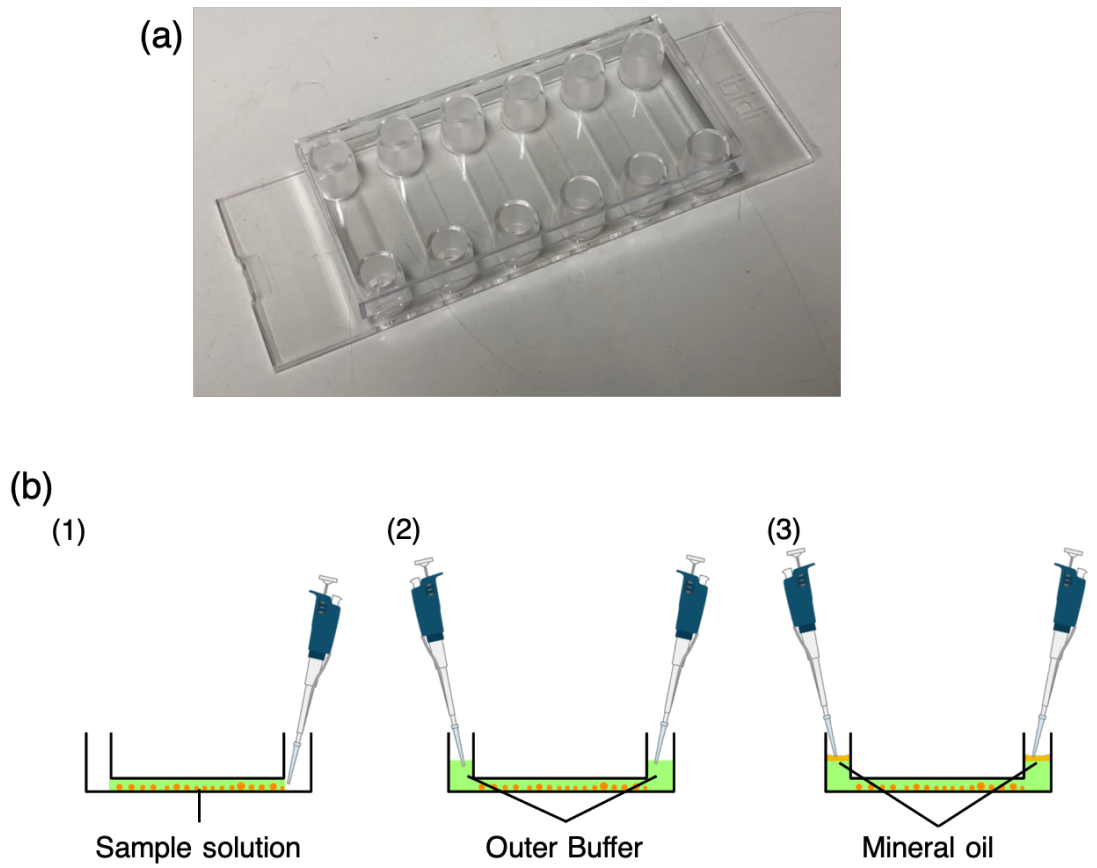

Fig. S6. The microchamber setup for the dye influx assay. (a) The moulded plastic chamber (ibidi µ-slide 0.4(VI)) used in the assay. (b) A schematic of the chamber preparation. (1) 30 µL of the mixture solution was added to the lane. (2) 30 µL of the GUV external buffer was injected into both sides of the chamber. (3) 7.5 µL of mineral oil was dropped onto both sides of the chamber to avoid evaporation.

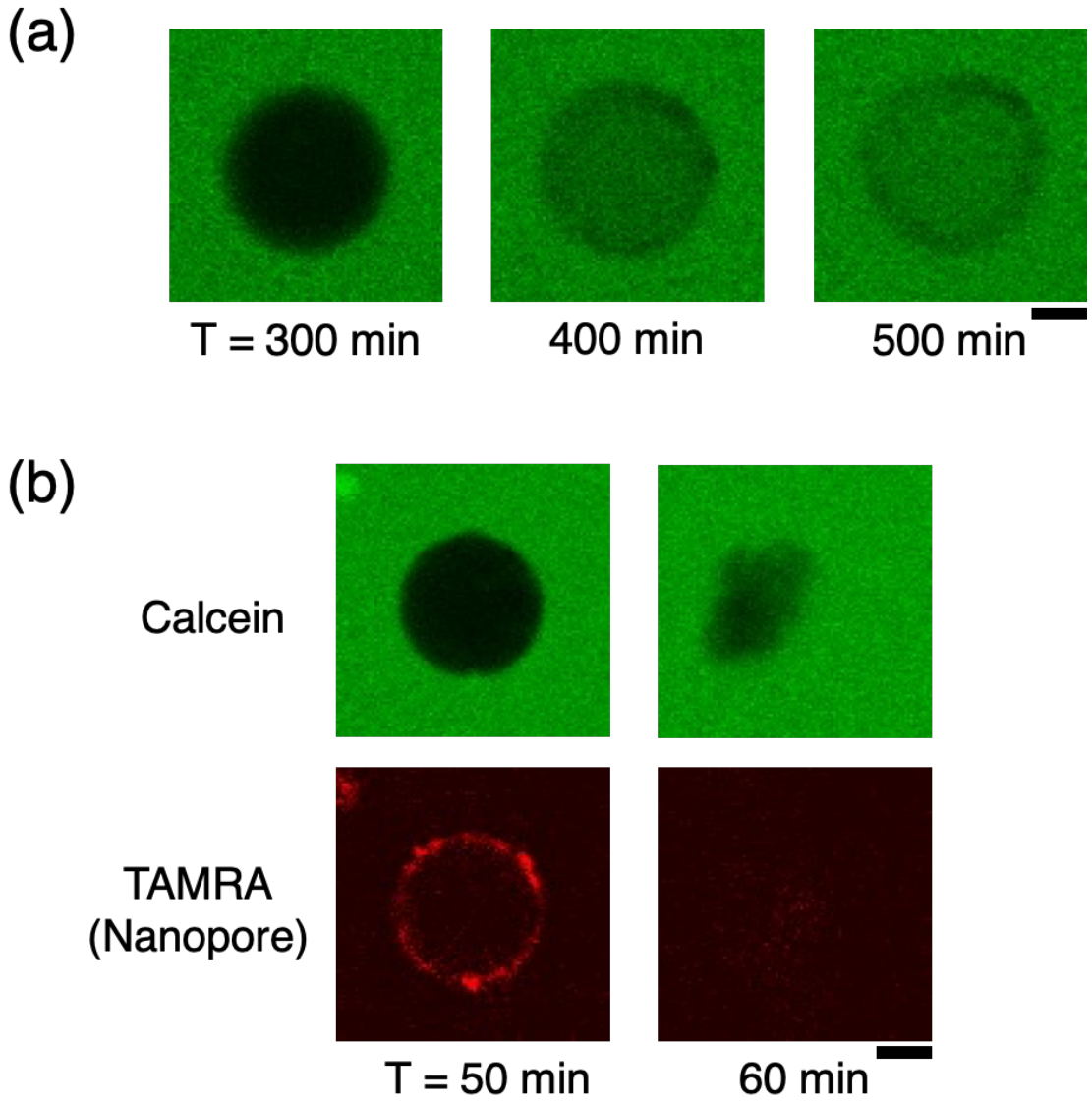

Fig. S7. An excluded example of GUV with dye influx. Representative confocal microscope images of GUVs with an influx of fluorescent molecules, which were not counted because of their false positive behaviour. (a) Confocal microscope image of the GUV with a large deformation of the membrane. (b) A confocal microscope image of the GUV that burst. Such GUVs were considered to possess punctured membrane and therefore, were not included in the analysis. Scale bars: 10 µm

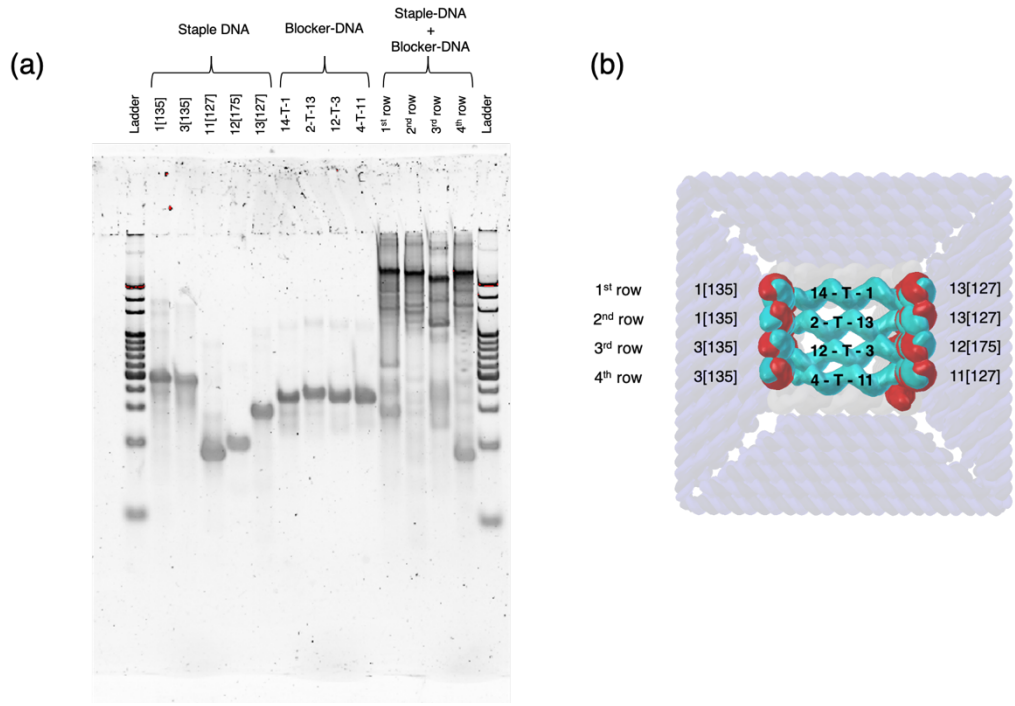

Fig. S8. Confirmation of the lid by binding between staple DNA and Blocker-DNA. (a) The result of the PAGE experiment for staple DNA, Blocker-DNA, and a mixture of both units. (b) Correspondence of each DNA with the position of pore shielding.

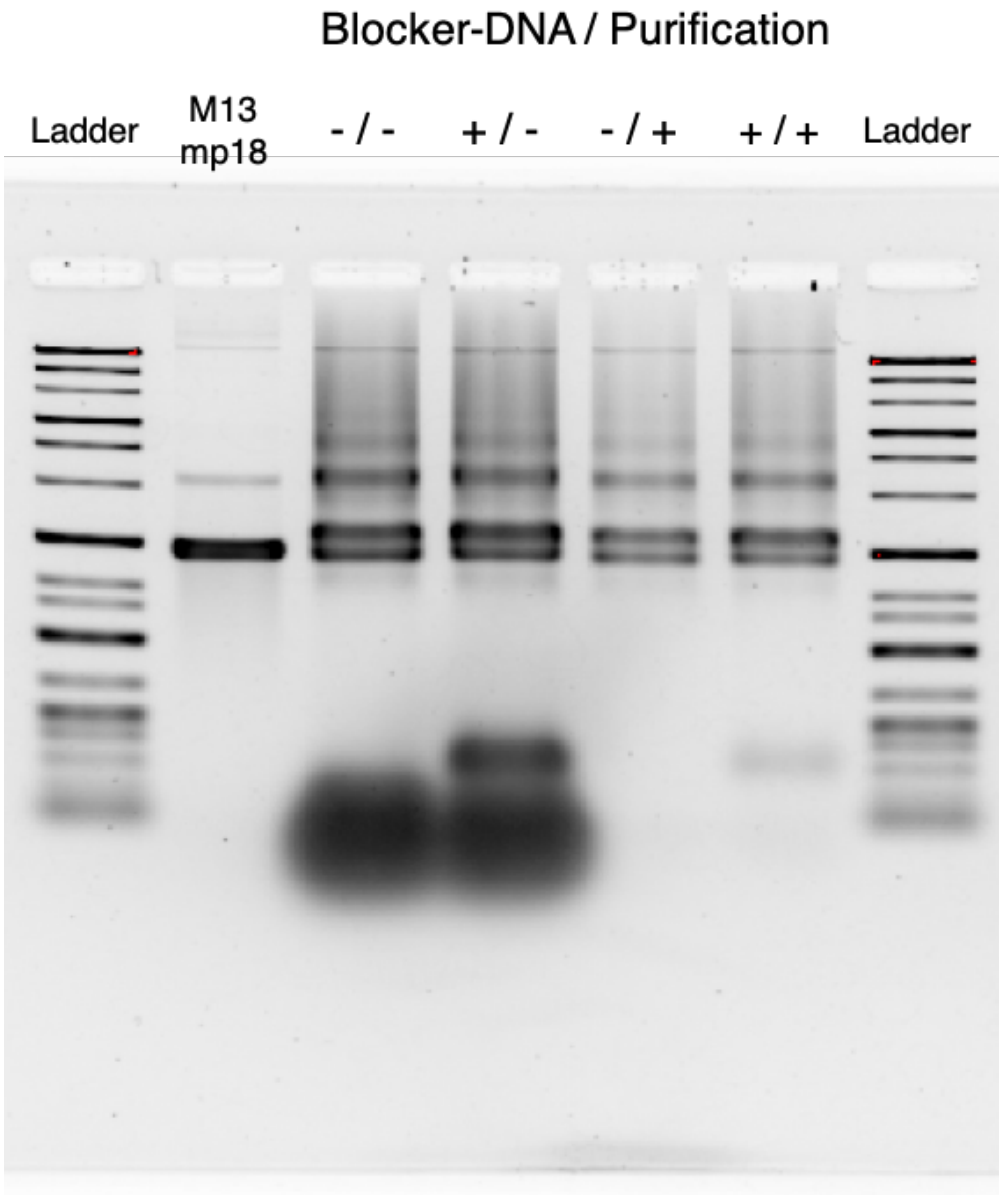

Fig. S9. Electrophoresis of the nanopores with and without Blocker-DNA. Agarose gel electrophoresis results of the nanopores with and without Blocker-DNA before and after purification.

Supporting Note 1: Estimation of the binding limit of cholesterol-modified DNA

To investigate the decrease in the fluorescence intensity of the nanopores on the vesicle surface with an increase in the concentration of Anchor-DNA, shown in Fig. 2d, we calculated and estimated the binding limit of cholesterol-modified DNA to vesicle phospholipids from the values of previous studies using supported lipid bilayer (SLB)[1, 2] and small unilamellar vesicle (SUV)[3].

1. Estimating the binding limit from SLB

Pfeiffer et al. reported the binding behaviours of SLB and cholesterol-modified DNA using QCM-D equipment[1, 3].

The density of lipids forming SLB was calculated to be 468 ng/cm^2 from the frequency variation of QCM-D. Since the lipid molecule (Egg PC) was 768 Da, the density in terms of molecular weight was 0.609 nmol / cm^2.

The area of 1-inch (25.4 mm) QCM, where the SLB is formed, was

$$(1.27\ cm)^2 \times \pi \approx 5.06\ cm^2$$

Therefore, the total lipid molecular weight of the SLB was calculated to be approximately 3 nmol. Assuming that the solution volume of the measurement portion was in the range of 250 to 500 µL, the lipid concentration of the measurement portion was estimated to be 6–12 µM. Under these conditions, the binding limit of the cholesterol-modified DNA was 100 nM, as measured by the frequency fluctuation of QCM-D, so the binding limit for 100 µM of vesicle lipid was 825 to 1650 nM.

2. Estimating the binding limit from SUV

Arnott et al. reported the binding limit value by mixing cholesterol-modified DNA with SUVs of different concentrations and observing the sample by electrophoresis[3].

In their experiments, the binding limit was 2.5 µM cholesterol-modified DNA at 100–200 µM of vesicle lipids.

Thus, the binding limit for 100 µM of vesicle lipids was estimated to be 1250–2500 nM.

3. Comparison of the experiment data

We measured the lipid content of the GUVs using a phospholipid test kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and a spectrophotometer (V-630 BIO, JASCO Corporation, Tokyo, Japan), and the concentration was found to be ~500 μM.

The GUV solution was finally diluted to 20% by mixing the Anchor-DNA with the nanopore, so the concentration of the lipid vesicle in the experimental system was ~100 μM.

The critical concentration of Anchor-DNA ranged from 500 nM to 1000 nM, as shown in Fig. S5b, and this value agrees with the numerical value of the previous study.

The above estimation supports the hypothesis that the binding strength of the nanopore to the GUV, i.e., the fluorescence intensity of the GUV membrane surface, is reduced because the excess Anchor-DNA that cannot bind to the GUV membrane surface binds to the nanopore.

Supporting Note 2: Estimation of the number of pores formed on the GUV membrane

The number of nanopores penetrating the GUV membrane was estimated based on the assumption that the internal/external solutions of GUVs in an actual experimental system were regarded as independent containers and that the containers were bound by nanopores (Fig. S10).

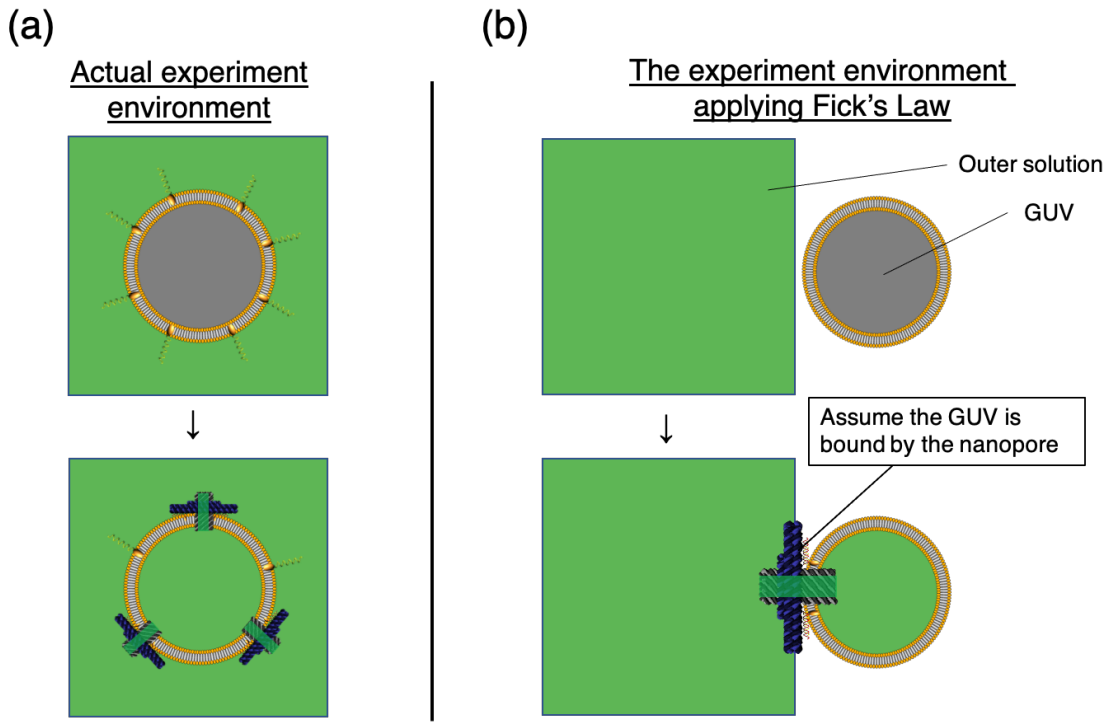

Fig. S10. Influx assumptions using Fick's Law. (a) The actual experiment environment. (b) The experiment environment applying Fick's Law. It is assumed that the GUV and outer solution each had an individual container, and the nanopore linked the containers.

The quantification of the number of nanopores was based on the formula by Krishnan et al[4]. The flux *j* through a single nanopore is given by the formula:

$$j = D\frac{n_o - n_i}{L} \qquad (1)$$

where D is the diffusion coefficient of the dye, L is the length of the nanopore, and n_o and n_i are the concentrations of the fluorescent molecules outside and inside the GUV, respectively (in units of $1/m^3$).

The increase in the concentration of fluorescent molecules per unit time is given by the following equations:

$$\Delta n_i = \frac{jA}{V} = \frac{DA}{LV}(n_o - n_i) =: k_o(n_o - n_i) \quad (2)$$

where A is the cross-sectional area of the nanopores on the lipid membrane, and V is the volume of the GUV.

Here, we defined DA / LV as the influx velocity–time constant, k_o, for a single nanopore.

The number of fluorescent molecules in the outer solution was sufficiently larger than that in the inner solution. Thus, we could assume that the concentration of the fluorescent molecules in the outer solution remained constant, and the concentration of the fluorescent molecules in the inner solution $n_i(t)$ was as follows:

$$n_i(t) = n_o - (n_o - n_i)\, e^{-k_0 t} \quad (3)$$

Since the initial concentration of the inner solution was zero, the preceding equation can be simplified as

$$n_i(t) = n_o\left(1 - e^{-k_0 t}\right) \quad (4)$$

This equation represents the ideal influx by a single nanopore.

Here, the experimental influx velocity–time constant k is given by

$$k = \frac{DA_m}{LV} \quad (5)$$

where A_m is the actual cross-sectional area of the nanopore formed on the GUV membrane.

Since D, L, and V are constants, the value k / k_0 becomes

$$\frac{k}{k_0} = \frac{A_m}{A} \quad (6)$$

From this value, the number and cross-sectional area of nanopores penetrating the GUV membrane can be estimated.

Table S1 shows the parameters used for the estimation of the number of nanopores.

Table S1. Parameters used for the estimation

Parameters	Values
Diffusion coefficient D	3.3×10^{-10} m^2/s
Pore length L	24 nm
The cross-sectional area of the nanopore A	81 nm^2
The cross-sectional area of the nanopore with 2 Blocker-DNAs A'	56.25 nm^2
GUV volume V	(Calculate from the radius of GUV)

The diffusion coefficient D was adopted from previously reported data[5].

The pore length L was obtained from the design value of the nanopore.

The cross-sectional area of nanopore A was determined by considering the Debye length of the DNA. Since the fluorescent molecule calcein is negatively charged, an electrostatic repulsion occurs between the fluorescent molecule and the DNA that constitutes the nanopore.

Woo et al. used DNA origami to estimate the Debye length from a double-stranded DNA and found it to be about 0.5 nm in the presence of 12.5 mM $MgCl_2$ [6].

Taking this into account, the effective cross-sectional area was estimated to be

$$(10 - 0.5 \times 2) \times (10 - 0.5 \times 2) = 81[nm^2]$$

With 2 Blocker-DNAs, the cross-section area of nanopore A' was determined by assuming that each Blocker-DNA blocked 75 % of one row (2.5 nm).

Considering the Debye length to be described above, the cross-sectional area was estimated to be

$$(10 - 2.5 \times 0.75 \times 2) \times (10 - 0.5 \times 2) = 56.25[nm^2]$$

The GUV volume, V, was calculated from the radius of the GUVs analysed using Fiji (ImageJ).

Fig. S11 shows the graph presented in Fig. 3g, with k / k_0 as the vertical axis.

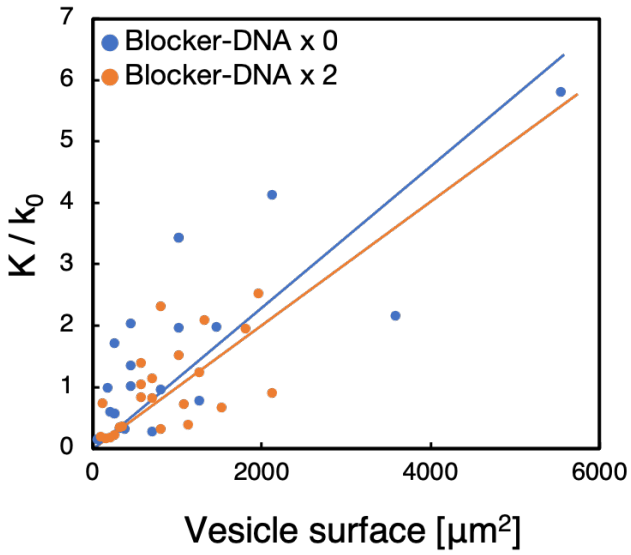

Fig. S11. The plot of the surface area of the GUV versus k / k_0 (the estimated number of nanopores) obtained from the data of calcein with different units of Blocker-DNA

(N = 21 in 0 Blocker-DNA and N = 23 in 2 Blocker-DNAs; the experimental data are same as presented in Fig. 3g).

Supporting Note 3: Dissociation of Blocker-DNA by strand displacement reaction

To extend the controllability of molecular transport in nanopores, we attempted to dissociate Blocker-DNA using a strand displacement reaction (Fig. S12). For performing the strand displacement reaction, the Blocker-DNA was further extended by ten bases from both ends, compared to Fig. 3a, which shows that the staple DNA was extended. The extended strand becomes a toehold for the reaction between Blocker-DNA and its complementary single-stranded DNA (Releaser-DNA), allowing the Blocker-DNA to be dissociated from the nanopore. The reaction between the staple DNA, Blocker-DNA, and Releaser-DNA was confirmed by polyacrylamide gel electrophoresis (PAGE) (Fig. S13).

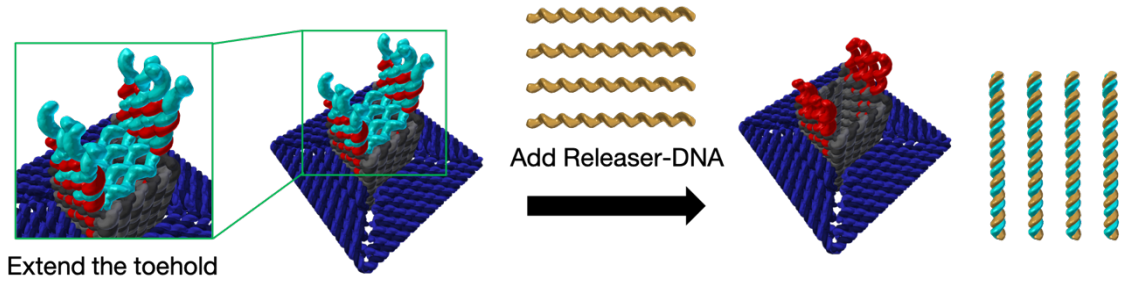

Fig. S12 Schematic illustration of dissociation of Blocker-DNA from the nanopore. The extended part of Blocker-DNA enables the strand displacement reaction between Blocker-DNA and its complementary DNA (Releaser-DNA). The reaction releases the Blocker-DNA from the nanopore, opening the pore.

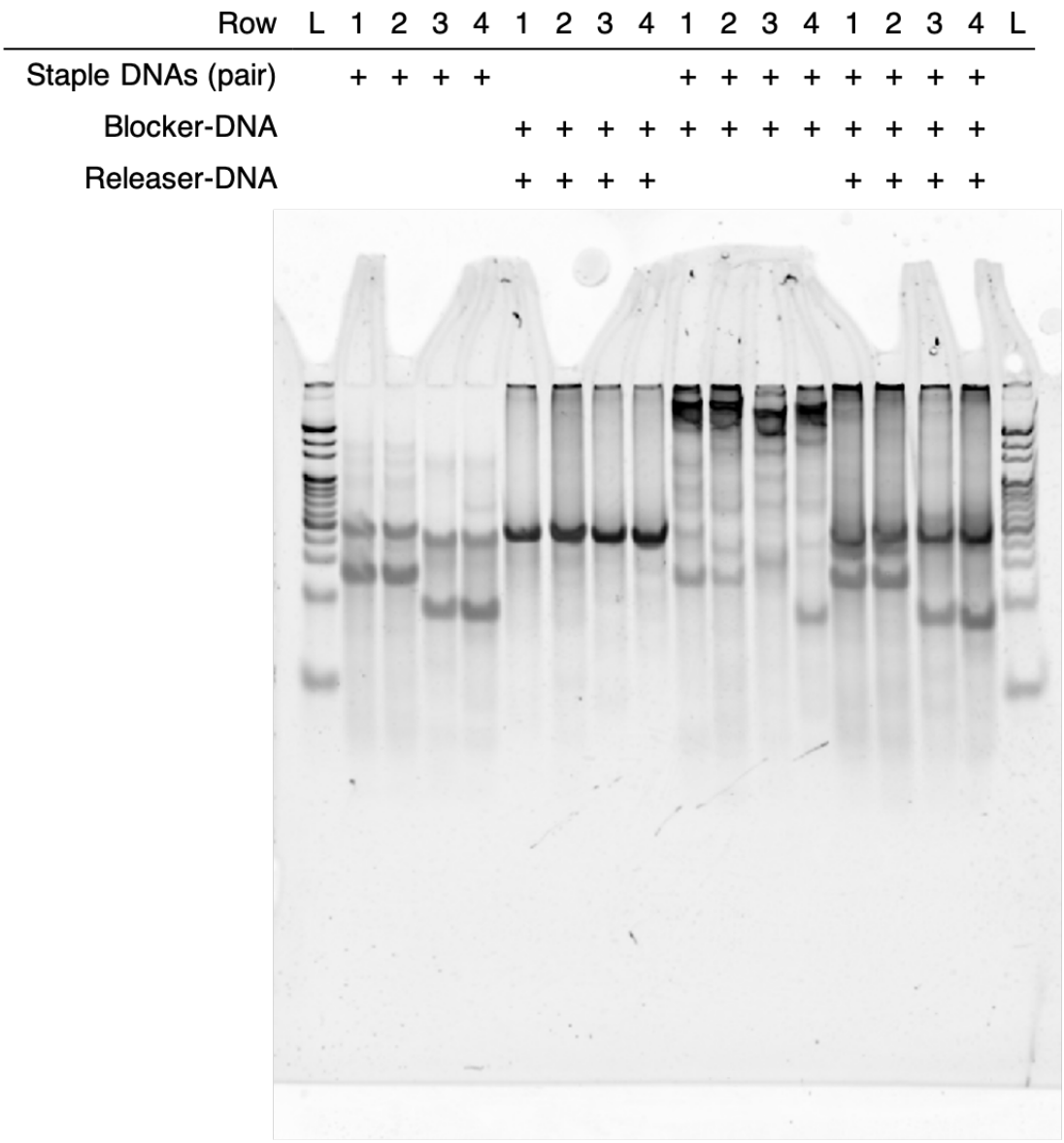

Fig. S13 Confirmation of the strand displacement reaction by Releaser-DNA.

The result of the PAGE experiment for staple DNAs (pair), Blocker-DNA, Releaser-DNA, and a mixture of the units. A sample of staple DNAs, Blocker-DNA with Releaser-DNA, and staple DNAs with Blocker-DNA was prepared by annealing at temperatures increasing from 95 °C to 25 °C at a rate of -1.0 °C/min. The sample consisting of staple DNAs, Blocker-DNA, and Releaser-DNA was prepared by mixing Releaser-DNA with the annealed sample of staple DNAs and Blocker-DNA and then incubating for 3 hours. The correspondence of the four lanes is consistent with the results shown in Fig. S8b. ”L” indicates the 20 bp Ladder.

Using these DNA samples, the same dye influx assay as in Fig. 2 and 3 was performed.

In the influx assay with four Blocker-DNA bound nanopores, the Releaser-DNA corresponding to each lane was mixed with twice the concentration of nanopores (nanopore concentration ≈ 3 nM, each Releaser-DNA concentration = 6 nM) or without mixing (Fig S14). The results of the experiment showed that the ratio of GUVs with influx of calcein increased by mixing Releaser-DNA. On the contrary, no influx of 40k Dextran was observed in both conditions, with and without Releaser-DNA (Fig. S14c).

The increase in the ratio of GUVs with influx of calcein suggests that the dissociation of Blocker-DNA from the nanopore by Releaser-DNA is functioning as designed. All four Blocker-DNAs molecules are designed to be dissociated completely, and the influx of 40k Dextran was expected. However, it was not observed. The result is similar to that of 2 Blocker-DNAs (Fig. 3f, Middle). This suggests that the dissociation of the Blocker-DNA does not take place in all four strands.

Further improvement in the nanopore mechanism is required for the realization of complete and/or repeated opening and closing of nanopores, and improvement of yield.

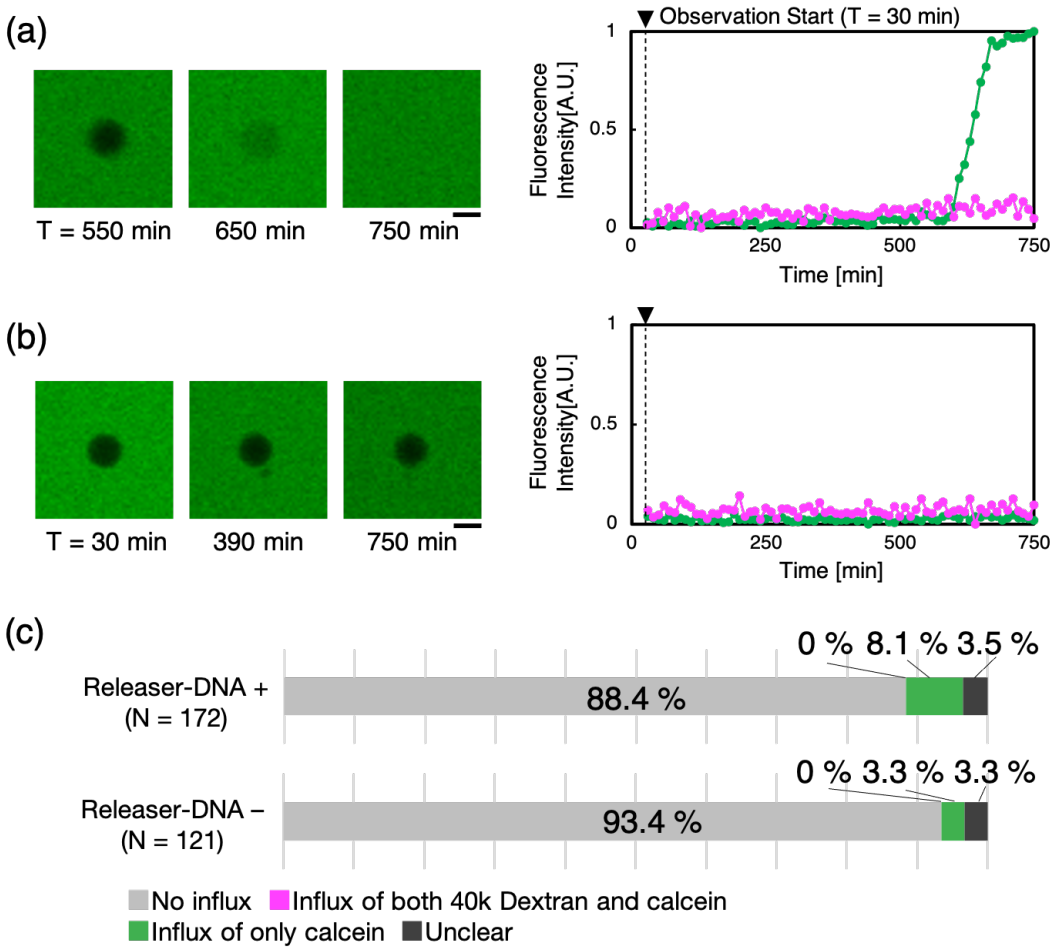

Fig. S14 Evaluation of the activity of Releaser-DNA by the dye influx assay.

(a) and (b) show the representative confocal microscopy time-lapse images and fluorescence intensity of GUVs with Releaser-DNA and without Releaser-DNA, respectively. Scale bars: 10 µm.

(c) The ratio of the vesicle with or without influx of each dye molecule, in the presence or absence of Releaser-DNA.

Table S2. Staple sequences for the artificial nanopore

Name	Sequence (5' → 3')	Length (bases)
Side*0[220]	GAAAGGAACAACTAAAGGAACCACATTCAACTAATCATAGTAA	43
Side*12[183]	AATATCGCATTCAGTGAATAAGGCTTGCCCAAAAATCAGAAGAGGA	46
Side*2[231]	TTTTTGCTAAACAACTTTCTTAAACAGCTTGATACCGATGTGTACTG	47
Side*6[229]	TTTTAGCGTAACGATCTAAAGAGAAGGATAGCATTC	36
Pore*10[159]	TTAAATATCCGCCACCCTCAGAACACCGCCACCCTCAGAAGAGGGTTG	48
Pore*7[160]	ATATAAGTGGATAAGTGCCGTCGATTTATTTCGGAACCTA	40
Anchor*1[184]	TTATCAGCAATAATTTTACTCTTTTTTGACCCCCAGCGTTATCTCTACCTGAAGCGAC	58
Anchor*50[423]	AACGTAACCAGAACCGTTTCATAAAATAATTCCGTAAAACATGTGCTGGTTATCTCTACCTGAAGCGAC	69
Anchor*51[416]	GTGTACAGTAAATTGTGATTCAAAGTTATCTCTACCTGAAGCGAC	45
Anchor*51[96]	AGGGTAGCAGAGGCAAGGAAATTAGTTATCTCTACCTGAAGCGAC	45
Anchor*52[262]	AACGAGGTAAAAATTGTTATCTCTACCTGAAGCGAC	36
Anchor*52[551]	AAACAAAGATTCAACCGTTATCTCTACCTGAAGCGAC	37
Anchor*53[127]	GTCACTTTTTTGAGCCATTTGGGAATTAGGTTATCTCTACCTGAAGCGAC	50
Anchor*53[264]	TTTAGAACCCTCTTTTATTTTAAATGCAGTTATCTCTACCTGAAGCGAC	49
Anchor*54[103]	ACCAATGAAAAGTAAGACGGAATAAGGCGTTTGTTATCTCTACCTGAAGCGAC	53
Anchor*54[263]	AAACATTATAATATTTAGCAAATAAAAGCGCCGTTATCTCTACCTGAAGCGAC	53
Anchor*54[551]	GCGTTTTCAGATAACCTCTACAAAGCATGCCTGTTATCTCTACCTGAAGCGAC	53
Anchor*54[74]	AATCAGTTTTTACAGAATCAAGTAATAAGAGAAGACAAAGGGTACCGGTTATCTCTACCTGAAGCGAC	68
Anchor*55[80]	TTCACAAACAAATAAATCGCCCACTCGGAACG	32
Anchor*56[215]	GAAAATACATATTTTAAGGTGGCAACGGTTTTGGTTATCTCTACCTGAAGCGAC	54
Anchor*56[535]	GGTCATTGCCTGAGAGTCTTTTTCAAACAAGAGAGTTATCTCTACCTGAAGCGAC	55
Anchor*57[279]	TTAAATTTTTGTTAAATCAGCTCATTTTGGTTGATATCTTCGCTGTTATCTCTACCTGAAGCGAC	65
Anchor*57[408]	CCATCAAATCAAAATCTAGGTAAAGTCGAAATGTTATCTCTACCTGAAGCGAC	53
Anchor*57[88]	TTTTAAGAAACCATCGATATTGACAAGAATACGTTATCTCTACCTGAAGCGAC	53
Anchor*58[103]	ATCCAAATACATGTAAACGGGTATTAAACCAAGTTATCTCTACCTGAAGCGAC	53

Anchor*58[135]	TACAAAAAGTAATATAATCGGCTGTCTTTGTTATCTCTACCTGAAGCGAC	50
Anchor*58[263]	GGAAGATCTGCCCTTCCCAGTGAGACTCGTATGTTATCTCTACCTGAAGCGAC	53
Anchor*58[391]	GGGCGCATTGTTTGATCTGCATTAGCGGAATTGTTATCTCTACCTGAAGCGAC	53
Anchor*58[423]	ACCGTAATCCTTATAACCGCTTTCTGATGGCAGTTATCTCTACCTGAAGCGAC	53
Anchor*58[514]	CGAGTAATTTTCCGTCGGATTCTGGTTGAGTTAACTCACTTTGGATTGTTATCTCTACCTGAAGCGAC	68
Anchor*58[551]	TGAACACCCGGAATCACACAATTCGCGTAGATGTTATCTCTACCTGAAGCGAC	53
Anchor*58[575]	AGCGCATTGGATCCCCGAGAGGGTGTTATCTCTACCTGAAGCGAC	45
Anchor*58[583]	AACAGGGAGTATCATATAGCTGTTATACAGTAGTTATCTCTACCTGAAGCGAC	53
Anchor*58[71]	CAAAAATTAATTGAGAACAAGCAAGCCGTGTTATCTCTACCTGAAGCGAC	50
Anchor*59[448]	CCAGGGTTTTCCTTTTCACGACGTTGTAGTTATCTCTACCTGAAGCGAC	49
Anchor*59[584]	AGCTCGAATTCGCCCAATATTTTGCAAATCAGATATAGAAGGGTTATCTCTACCTGAAGCGAC	63
Anchor*60[375]	GGCCAACGCGCGGGGAGAGGTTTTTTGCGTATTGGGTAATTTTAGTTATCTCTACCTGAAGCGAC	65
Anchor*61[120]	TTCGAGCCTAAACAGCTTGCGGGAATATAAAAGTTATCTCTACCTGAAGCGAC	53
Anchor*61[216]	CAATAAACTACCAACGCAGCTACACAAACGTAGTTATCTCTACCTGAAGCGAC	53
Anchor*61[408]	GCAAAATCGGGATAGGAAAGGGGGTAGCATGTGTTATCTCTACCTGAAGCGAC	53
Anchor*61[505]	AGTCCACGATAAATAATAAAGTGTATTTGCAGTTATCTCTACCTGAAGCGAC	52
Anchor*61[536]	ATAAACACCTGAACAACCAAGCTTGGCTATCAGTTATCTCTACCTGAAGCGAC	53
Anchor*61[88]	CAACGCCAAAGAAACGAGAACGCGAGTTTATTGTTATCTCTACCTGAAGCGAC	53
Anchor*62[103]	TCATAGGTCTGAGAGAATTTCAATTACCTGAGGTTATCTCTACCTGAAGCGAC	53
Anchor*62[132]	AGAAGAGTCAATAACAAACATCAAGAAGTTATCTCTACCTGAAGCGAC	48
Anchor*62[159]	ATAGCGATTTTAACAATTTCTTTTGAATTACCTTTTTTAATGGAGTTATCTCTACCTGAAGCGAC	65
Anchor*62[239]	TAGAAGTATAACCTTGCTTCTGTAAATATATGGTCCTGAAGTTATCTCTACCTGAAGCGAC	61
Anchor*62[383]	GGCAAATAGAAGGAATGAATCGTTATCTCTACCTGAAGCGAC	42
Anchor*62[423]	AACCTCAAATATCAAATTATCAGACAGTCGGGGTTATCTCTACCTGAAGCGAC	53
Anchor*62[464]	AAAACCGTCTATCAGAAAAATCTACTGATTGATTAATTGTTATCTCTACCTGAAGCGAC	59
Anchor*62[47]	TATATGTAAATGTTTTTGCAAATCCAATCGCAAGACTCGGGAGA	44
Anchor*62[551]	TTCTGACCTAAATTTAAAGAAATTCACACAACGTTATCTCTACCTGAAGCGAC	53
Anchor*62[583]	ACTTTTTCAAATATATAGATGAATTCCTGTGTGTTATCTCTACCTGAAGCGAC	53

Anchor*62[63]	GGTTGGGTAGTTACAAAATCGCGGTTATCTCTACCTGAAGCGAC	44
Anchor*63[296]	AAAGTTTGAGTATTTTTATCATTTTGCGGTTATCTCTACCTGAAGCGAC	49
Pore*1[200]	GAGGTGAATTTCAACAGTTTCAGCGGAGTTT	31
Pore*10[183]	TATAATGCGAACCAGATTGCTCCATGTTATTTTGCCGG	38
Pore*10[191]	TTGCTGAAACCACCCTCATTTTCACAGTACAAACTACCGTACTCAGGA	48
Pore*10[215]	TTTGCGGAAGGATTAGAGAGTACCTTGCATCAAAAAGATT	40
Pore*10[237]	TTTCTTTTGATAAGAGGTCATTATAGGAACCCATTT	36
Pore*12[199]	AGCCCGAAAAACTCCAACAGGTCTGGCTTAGAGCTTAA	38
Pore*12[237]	TTTAGCAAAGCGGATTTAATTGCTCTTT	28
Pore*13[208]	GTCTTTACCCTGACTATTCAGAAAACGAGAATTAAATATT	40
Pore*14[183]	CCAGAACGCCTTATGCTTCGCCTGAACCAGGCGCATATTTTGGCTGACCTTCATAGGACGTT	62
Pore*14[207]	GACCATAAATCTGACGAGAAACA	23
Pore*14[237]	TTTGCTTTAAACAGTTATAGTCAGATTT	28
Pore*15[168]	GTGAATTAAGTAGTAAATTGGGCTATTCCCAAAGCTGCTC	40
Pore*15[216]	CATTGAATCCCCCTCAATAGTAAAATGTTTAGTTTGCCAG	40
Pore*16[204]	TCCAATACTCTGGCTCATTACAAAATAGCGAGAGGCACTATCAT	44
Pore*17[224]	AGGGGGTAAAAAGGAATTACGAGGGCAGATACATAACGCCTTTTGAGAATA	51
Pore*18[191]	AACCCTCGTTGAGATTTAGGAATATTGCGAATTTGCTTTC	40
Pore*18[207]	GAGCAACTTTTGCAAAAGAAGTACTGGATAGCG	33
Pore*3[184]	ATACAGGAAGTTGCGCCGACAATGGTATCGGT	32
Pore*3[200]	GTAATAAGTTTTTTTTCTGTATGGGATTTTT	31
Pore*4[231]	TTTGACGTTAGTAAATGAAAACGGGGTCAGTGCCTTGAGAGAGGCTG	47
Pore*5[168]	TTATTCTGTGACTTTTTCGTAATGCCACTTTTTAGGCACCAACCTTCCCGTATATTTTGATG	62
Pore*5[184]	AAGTATTATAACAGTGTAAAACGAAAACGGCTAGAAACGTCAAAGCCAGGGTGGCA	56
Pore*5[200]	AGACTCCTCAAGTTTTGTCGTCTTTCCATTT	31
Pore*50[574]	GCTGAGGTCGTCACCTTGAAAATCTAGAAAGA	32
Pore*52[407]	CCGCGACCTCCGGAAGCAGACTTCA	25
Pore*52[543]	TACAACGGAGATTTTTATCATGATTTTAAGAAGCGGAATCGTCA	44
Pore*52[87]	ACTAAAACTTTTCACGTTCTCATTTTCGAAA	31
Pore*55[244]	AAGGCAAAGAATAATGGTCAGTCAATCATTCAGAGCC	37

Pore*56[119]	GAAACGCAAGGTGAATCAAGGCCGCAGAGGCTTTGATTTTTAAATAAACATGATTGCTCAG	61
Pore*6[175]	TACCAGGCATAGCCCGGAATAGGTGTATCACAACGCCTGTTAGGATTAGCGGGGTT	56
Pore*7[208]	CACAGACAACTGAGTTTCGTCACGGGATAGCAAGCCCA	38
Pore*8[175]	GGTTTAGTCGCCACCCTTAAGGGAACCGATTTTACCAACTTTGAA	45
Pore*8[229]	TTTTGTACCGTAACGCCCTCATAGTTTT	28
Surround*50[228]	TTTCATTTGTTTTGCGAGCTGAAAAAATGGAAAGCGCAGTCTCCTCATT	49
Surround*50[399]	TTAGTTTGACCATTTTATACATTTCGCATAGCAAAA	36
Surround*50[559]	GAGTTAAAAATCTTGAACCAGAGC	24
Surround*51[81]	GACAGCAGCATAACCGATATTTTCGGTC	28
Surround*52[239]	GTAAAATACATGAGGATTGCGGGAAATCATACAGGC	36
Surround*52[567]	GATTATACCAAGCGCGGGCCGCTTACTGTAGCCGCCGCCA	40
Surround*53[104]	TTCATTAAAAGACACCCAGATAGCTTATCCCA	32
Surround*53[232]	GAAGCCTTTTACGCAGAACTGGCA	24
Surround*53[392]	ATGCCTGATGTACCCCTTAACCAAGTGTAGAT	32
Surround*53[424]	AGGGTGAGACGGTAATGCGTCTGGGCGGATTG	32
Surround*53[552]	GTTCTAGCTTGAGAGACACAAGAAGAATTAAC	32
Surround*53[73]	AGGGAGGATCAATAGATCTTACCTTTAACGT	31
Surround*54[216]	CAGTATTTTCATTACCATTAGTATCACC	28
Surround*54[247]	TAATACTTAGTTTCCAATAACCTGTTTAGCTATAT	35
Surround*54[255]	TGACCCTGCGCAAGGACGCAGACGTTAAACGG	32
Surround*54[270]	GTACCAATTAAGCAATAAATTTTCAGAGCAGGAACCGC	38
Surround*54[399]	CAGAGCCACCACCTAAAGCTTTTTCGGTT	29
Surround*54[528]	CATAGTTTTCTTATTAGCGTTTGCCATCTCCACCCTC	37
Surround*55[119]	TCAATTCTACTAATTTTAGTAGCAT	25
Surround*55[220]	TAACATCCAATAAGCCAGCACCCAAAAGTATGTTAG	36
Surround*55[395]	CTCCCTCAGAGCCGCCACCCTCTGCGAACGATGAACG	37
Surround*55[432]	AGAGCCACCACCTTTTGAGCCGCCACCA	28
Surround*55[536]	GAACCACCCAAGAACCGGATTTTTATTACCCAAATC	36
Surround*55[560]	GCATTGACAGGAGGTTGAGGCAGGTCAGTTTTTTGGCCTTGATA	44
Surround*56[276]	GAAGATTGTATATGTTAAAACGGCCTCA	28
Surround*56[407]	CAATCATAGTAATGTGACCGGAACAGAGGACAGAGTAGAT	40
Surround*56[439]	ATCGATGAAAAGGCCGGAGATTTTCAAATCACCATCTTTCGGT	43

Surround*56[567]	AGCTATTTTGATAAATAGCGTCAGTTGCGGGACTTGCAGG	40
Surround*56[591]	AGCGCCACAAGAAACAATTTTTTAGCA	27
Surround*56[87]	TTGTCACAGAAGGTAAATAGCAGCACCGT	29
Surround*57[130]	GGAAACCGATTTTACGCAATAATAACGGAATAAAATCAC	39
Surround*57[442]	GCCAGCTTTTTTTAACATTAATTGAGCG	28
Surround*57[532]	CTAATATCAGAGATCGGCATAATATGAT	28
Surround*57[568]	AGCCCAATTTGCCTTTTAATGCCGAGGGCGACATTCTTTTGATTG	45
Surround*57[65]	ATAGCTAAAATTCATATTTTTTACC	25
Surround*58[239]	TCCGGCACCTGAATCTAACATGTTATAGATAATGAGTGAATTAGACTT	48
Surround*58[368]	TGCCATTTTGAGGGGACGACGTGCGCAA	28
Surround*59[104]	TAGCGAACTTCCAAGATTTAGGCATATCAAAA	32
Surround*59[136]	AAGCCTTAAATCTTTTTTAGT	21
Surround*59[201]	TGCTATTAATAATATCTGTCCAGGCTATTAATTAAT	36
Surround*59[224]	ATTTTATCCGCTTCTGTATCAACACAGCTAAT	32
Surround*59[240]	GTGCCGGAGCCAGCTTTGATTAAGGTAAACGT	32
Surround*59[248]	AACCAGGCTTTAAATTACTCCTTATATTTCAA	32
Surround*59[264]	ATTCGCCATCTTTTCAACCGCCTGAGAGCCGTCAAT	36
Surround*59[287]	CTGTTTTTTAGGGCGATCGGTGTGCATC	28
Surround*59[392]	ATTACGCCCGTGCCAGGGTGGTTCCAATATCTGGTCAGTT	40
Surround*59[424]	CAAGGCGACTCACTGCATCAAAAGCCTTGCTG	32
Surround*59[520]	AAACGACCGGAAGCAGGCGTTAAATACC	28
Surround*59[552]	GCAGGTCGTATCCGCTTAATTACTATTTCATC	32
Surround*59[72]	CTTATCCGCTCATCGAGAATCGCCTTAACCTCCGGCTTA	39
Surround*60[119]	CCTTATCACTCCCGACCATATTATCGAACAAAGTTACCAGAA	42
Surround*60[147]	GAAACCAATCAAAGAGAATATTAAGACGCTG	31
Surround*60[215]	CAAGAAATTGCACCCTAACGAGCGTCTTTCCAGTTTTTAATTTGCCAGT	49
Surround*60[287]	CGCCAGGGTGGTTTTTTCAGGCACAGTATTTCGCA	35
Surround*60[407]	AAACCTGTAGCTGGCGTCACGTTGTAGGAACG	32
Surround*60[439]	GCGTTGCGTTAAGTTGAACAAACGCCTTCCTGTA	34
Surround*60[504]	TGGGGTTTTTAATGAGTGAGCGTTGTTCCAGTTTTTTACAAG	42
Surround*60[535]	ATACGAGCGGCCAGTGAGTCAGAGGGTAAATGTGAG	36
Surround*60[55]	TTTTATTTTCATTTTTGGAATCA	23
Surround*60[567]	GAAATTGTACTCTAGAAGACGGGATTGAGTTA	32
Surround*60[604]	TTACCGCGTAATCATGGTCATGCGTTACGAGAAA	34

Surround*60[87]	GTACCGCAGTATTCTAATTTTTTGGAAGCCCT	32
Surround*61[151]	CCGACTTTTGGTAAAGTAATTCCCATCCTAATTTTTTAGCATGTA	45
Surround*61[280]	GAGAGTTGCAGCAAGCGGTCTTTTCTGGT	29
Surround*61[361]	TTGCCCCGAAAGGAATTGAGGAAGGTTTTTTAAAATATC	39
Surround*61[376]	AAAATCCCGTAACCGCGGGCCATCAGAAAAGCTTTTAAAACAG	43
Surround*61[440]	GAGATAGCCGTGGGGGTAACG	21
Surround*61[56]	GTAGGGCTGAAAATAGCAGCTTTTACAGAGAGAATAACATAAA	43
Surround*61[600]	CTTACCAGTATATTTTCAACGCTCAACA	28
Surround*62[194]	TTTCCCTTAGAATCCTTTTAAAC	23
Surround*62[259]	AGATAATACATTATTCGACAACGGGCAACGCCTGTT	36
Surround*62[299]	TTTAGGAGCACTAACAACTAAACGTTAT	28
Surround*62[519]	GACCGTGTTATTAAAGATCAAAATTAAAGCC	31
Surround*63[104]	CAAAAGAAGATGATGAAGTGAATTGAGGCATT	32
Surround*63[136]	AACAAAATTAATTACAAGCTTAGATAAAGTA	31
Surround*63[200]	AACAGTACATAAATCAATCGTCACGACGA	29
Surround*63[240]	TACAAACATGAGGATTGCAGAACGCAGCTGATGCACTCCA	40
Surround*63[264]	TAAATCCTTTGCCCGATAGATTGCCCTGA	29
Surround*63[360]	GAACAAAGAAACCACCCAACAGTTAGCAGGCG	32
Surround*63[392]	ATCATCATATTCCTGACCCTCAATCGAAATCG	32
Surround*63[424]	ATTCATCAATATAATCAAGCATCAAATAGCCC	32
Surround*63[456]	ATACTTCTGAATAATGGAAGTTTTAGAACCTACCATAACGTGGACTCCTTTTTCAAAGGGCGA	63
Surround*63[520]	CGTAAAACAGAAATAATGGTTTGAAATAAGA	31
Surround*63[552]	TTTCAGGTTTAACGTCTTTAGTTAAGAAAAAGCCTGTTTA	40
Surround*63[584]	ACAGTACCTTTTACAAAAGAACGTACAAATT	31
Surround*63[608]	AACAATAACGGATTTTCCTGATTGCTTTGAATACCATATATAAC	44
Surround*63[72]	CAGAGGCGAATTATTCCTACCTTTATATTTAA	32
NegaConnect* 1[135]	TTTCTCCAAAAGGAGCCTTTAATTACAACAACTGAATTTACCGTTTTT	48
NegaConnect* 11[127]	TTTACTAAAGTACGGTGTCAGCTTCAAAGCTGTAGCTCAACATGTT	46
NegaConnect* 12[175]	GTTTTAATTCGTGGAAGTTTCATTCCATTT	30
NegaConnect*	TTTTATAACAGTTGTGAGATGGTTTTTT	28

13[127]		
NegaConnect* 15[127]	TTTAATTTCAACTTTAATCATTGGGAAGAAAAATTT	36
NegaConnect* 17[136]	TTTTCTACGTTAATAAAACACGATAAAAACTACCAGTCTTCAAGAGT	47
NegaConnect* 19[136]	TTTATTATTACAGGTCCAAAAAAAAGGTTT	30
NegaConnect* 19[176]	TTCATCAGTTTACCAGACGGAACTAACGGAACAACTTT	38
NegaConnect* 3[135]	TTTCCAGTAAGCGTCATACATGGCAACAGTTAATGCCCCCTGCCTTTT	48
Connect*1[135]	AGCTAGCTGCTCGAACTCGACCTCCAAAAGGAGCCTTTAATTACAACAACTGAATT TACCGTTCGGCTCAAGCTCGAGATTTTG	84
Connect*15[127]	TGTCATGATTATGGGGCCTAAAATTTCAACTTTAATCATTGGGAAGAAAAATGTCCA TGTTGTGCCACGTCG	72
Connect*3[135]	CCTAGAGAGATGTGCTAGAGACCAGTAAGCGTCATACATGGCAACAGTTAATGCC CCCTGCCTATGGACTGACCTAGCCATTGG	84
Connect*12[175]	GTTTTAATTCGTGGAAGTTTCATTCCATTTCGAGAGTCAATAGGCCTC	48
Connect*13[127]	GGCTAATAAGGTGCTGATCGTTATAACAGTTGTGAGATGGTTTATGCGCGTAGGTC ATGTACCT	64
Connect*17[136]	GGGCTAAACCTGGCTCCAACATCTACGTTAATAAAACACGATAAAAACTACCAGTC TTCAAGAGT	65
Connect*19[136]	ACTGGTTAGCTAGTCGATGCAATTATTACAGGTCCAAAAAAAAGGTAGTACGTACG ACTGCTATCG	66
Connect*19[176]	TTCATCAGTTTACCAGACGGAACTAACGGAACAACCAAGCTTTGTTGTAGCTGACT	56
Connect*11[127]	AGCTAGTCAGGTTTGTAGCACACTAAAGTACGGTGTCAGCTTCAAAGCTGTAGCT CAACATGTT	64
Connect*[14-T-1]	AGGTACATGACCTACGCGCATTTTTTTTTTTTTTTTTTTTTTTTTTGTCGAGTTCGAG CAGCTAGCT	67
Connect*[2-T-13]	CAAAATCTCGAGCTTGAGCCGTTTTTTTTTTTTTTTTTTTTTTTTTACGATCAGCACCT TATTAGCC	67
Connect*[12-T-3]	GAGGCCTATTGACTCTCGAAATTTTTTTTTTTTTTTTTTTTTTTTTCTCTAGCACATC TCTCTAGG	67

Connect*[4-T-11]	CCAATGGCTAGGTCAGTCCATTTTTTTTTTTTTTTTTTTTTTTTTGTGCTACAAACCT GACTAGCT	67
TAMRA-16[255]	TTGACGGGGAAAGCCCGCGAACGT [TAMRA]	24
TAMRA-16[279]	TAAAGGGAGCCCCCGATTTAGAGC [TAMRA]	24
TAMRA-16[303]	CCGTAAAGCACTAAATCGGAACCC [TAMRA]	24
TAMRA-16[327]	ATCAAGTTTTTTGGGGTCGAGGTG [TAMRA]	24
TAMRA-16[351]	CCCACTACGTGAACCATCACCCAA [TAMRA]	24
Anchor-DNA	GTCGCTTCAGGTAGAGATAAC [3CholTEG]	21

Table S3. DNA sequences for the Blocker-DNA dissociation experiment (*Supporting Note 3*)

Name	Sequence (5' → 3')	Length (bases)
14-1st-1_extend	AATACTCTGGAGGTACATGACCTACGCGCATTGTAGTGGTTACTCAGTACACTC TTGTCGAGTTCGAGCAGCTAGCTCGTCACATAC	87
2-2nd-13_extend	CCGCACTGTACAAAATCTCGAGCTTGAGCCGAAATACGCTGAAGTGATCTGTTT GGACGATCAGCACCTTATTAGCCAGGTACTGCT	87
12-3rd-3_extend	TTTCGGGAATGAGGCCTATTGACTCTCGAAAGATTGTTCCGAGCAGTGGAAAGA TGTCTCTAGCACATCTCTCTAGGTGTTTGCAAG	87
4-4th-11_extend	AAATATCACACCAATGGCTAGGTCAGTCCATCTTAACCGAGTAGACTTCCTTTA GCGTGCTACAAACCTGACTAGCTAAGACTTGAG	87
(14-1st-1_extend)'	GTATGTGACGAGCTAGCTGCTCGAACTCGACAAGAGTGTACTGAGTAACCACTA CAATGCGCGTAGGTCATGTACCTCCAGAGTATT	87
(2-2nd-13_extend)'	AGCAGTACCTGGCTAATAAGGTGCTGATCGTCCAAACAGATCACTTCAGCGTAT TTCGGCTCAAGCTCGAGATTTTGTACAGTGCGG	87
(12-3rd-3_extend)'	CTTGCAAACACCTAGAGAGATGTGCTAGAGACATCTTTCCACTGCTCGGAACAA TCTTTCGAGAGTCAATAGGCCTCATTCCCGAAA	87
(4-4th-11_extend)'	CTCAAGTCTTAGCTAGTCAGGTTTGTAGCACGCTAAAGGAAGTCTACTCGGTTA AGATGGACTGACCTAGCCATTGGTGTGATATTT	87

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