Single-vesicle estimation of ATP-binding cassette transporters in microfluidic channels

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Supplementary Figure S1. The process flow for the immobilization of P-glycoprotein vesicles in parylene-coated PDMS channels. (Step 1) Fabrication of PDMS using standard soft lithography procedures. (Step 2) Coating the parylene onto the inner surface of the PDMS channels. (Steps 3 and 4) Attaching glass coated with single-stranded DNA to provide DNA tethers within the PDMS channels. (Step 5) Immobilizing the P-glycoprotein vesicles within the channels through the formation of DNA duplexes.
Supplementary Figure S2. The time-course of the Rh123 influx obtained by the fluorescence-based batch assays. The time-courses of the influx in the presence of ATP and AMP are shown by the red and blue lines, respectively. The error bars represent the s.d. for 5 measurements collected after various incubation times.
**Supplementary Figure S3.** The standard Rh123 fluorescence curve. The image capturing conditions (exposure time and receiver gain) were the same as those used to collect the data for Fig. 3e. Rh123 was added to 50 mM MOPS-Tris buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂ at pH 7.0) to make 1, 2, 3 and 5 μM solutions. The fluorescence increased in proportion to the Rh123 concentration. The hillslope of the best-fit line was determined to be 9.5 FU/μM.
Supplemental Methods

Device fabrication – Straight microchannels were used in this study. The cross-sectional width and height of the channels were 500 μm and 50 μm, respectively, and the channel length was 12 mm. The microfluidic devices were fabricated using standard soft lithography procedures (supplemental Fig. S1, step 1). Briefly, we cast a poly(dimethylsiloxane) (PDMS) substrate (Sylgard 184, Dow Corning) on an SU-8 (Nippon Kayaku) master mold fabricated on a 50.8-mm silicon wafer, degassed it for 20 min and baked it at 75°C for 90 min. We then punched holes (1.5 mm diameter) in the PDMS microchannels and placed the PDMS chip on a 3-( trimethoxysilyl)propyl methacrylate (A174) coated glass slide, which leads to an enhanced adhesion of a poly-p-xylylene derivative (parylene N), without O₂ plasma treatment (supplemental Fig. S1, step 2). The PDMS chip was then coated with parylene N to prevent the absorption of rhodamine 123 (Rh123) onto the inner surface of the PDMS channels (supplemental Fig. S1, step 2). The parylene deposition in the PDMS microchannels was performed using a commercially available parylene coater (PDS2010, Parylene Japan) under 4–9 Pa of pressure. The temperatures used for vaporization, pyrolysis and deposition of the parylene N were 165–175°C, 650–690°C and room temperature, respectively. The thickness of
the parylene layer, measured on an external standard, was 1.2 μm. Following the parylene deposition, the PDMS chip was detached from the glass slide and attached to an aldehyde-coated glass with single-stranded DNA (ssDNA) tethers (supplemental Fig. S1, steps 3 and 4). The parylene burrs of PDMS chip after the glass detachment was suppressed by using A174-coated glass slides because of the enhanced adhesion of parylene N to the slides.

**Preparation of the A174-coated glass** – Glass slides (76 mm × 26 mm, thickness 0.8–1.0 mm; Matsunami) were heated at 140°C for 15 min to dehydrate their surface and then immersed in 3% (v/v) A174 (Tokyo Chemical Industry) in toluene for 1 day. After rinsing the glass slides with toluene, 2-propanol and water, they were dehydrated at 120°C for 3 min.

**Preparation of the aldehyde-coated glass with ssDNA tethers** – First, glass slides (30 mm × 40 mm, 0.12–0.17 mm thick; Matsunami) were silanized by treatment with 4% (v/v) 3-aminopropyl-trimethoxysilane in acetone for 5 min at room temperature. The silanized glass was then functionalized with an aldehyde by immersion in 5% (v/v) glutaraldehyde in 0.1 M sodium bicarbonate *aq.* for 15 min at room temperature. The aldehyde moiety at the glass
surface was used to form a Schiff base with the primary amine moiety of the ssDNA now immobilized on the glass. To immobilize the ssDNA, whose sequence was NH$_2$-5’-CAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGT-3’ (BEX), a solution of 20 μM ssDNA dissolved in a PBS solution (8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl and 2.7 mM KCl at pH 7.4) was spotted (10 μl) onto the aldehyde-coated glass, which was then heated to 120°C for 15 min. After washing the glass with 0.2% (w/v) SDS _aq_, the residual aldehydes on the glass surface were deactivated by treatment with 0.25% (w/v) sodium borohydride in PBS:ethanol (3:1, v/v).

_Immobilization of the P-gp vesicles in the microfluidic channels_ – P-gp vesicles, obtained from GenoMembrane, Inc. (http://www.genomembrane.com/english_Home.html), were tethered to a microfluidic channel via DNA duplexes using a modification of an immobilization procedure reported previously.$^2$ Our immobilization procedure is as follows: First, the microfluidic channels were filled with 10% (v/v) fetal bovine serum (FBS) in 10 mM PBS for 30 min to prevent the nonspecific adsorption of the P-gp vesicles onto the inner surface of the channels. After rinsing the channels with 100 μl of a 50 mM MOPS-Tris buffer (50 mM
MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂ at pH 7.0), a cholesterol-modified ssDNA (8 µl of a 7.3 µM solution) in a 50 mM MOPS-Tris buffer was applied to the channels. The sequence of the cholesterol-modified ssDNA was 5’-ACTGACTGACTGACTGACTGACTGACTGACTGACTGACTG-3’ (BEX), with a 3’ triethylene-glycol (TEG) cholesterol modification. This modified ssDNA formed duplexes with the complementary ssDNA tethered to the glass surface, exposing the cholesterol moiety in the channels. After a 30 min incubation (at room temperature) to allow the duplex to form, the channels were rinsed with 100 µl of 50 mM MOPS-Tris buffer and filled with P-gp vesicles (8 µl, with a total protein concentration of 1 mg/ml; GenoMembrane, Inc.). For the methyl-β-cyclodextrin (MβCD) (Sigma) treatment, the P-gp vesicles were incubated with 1 mM MβCD at room temperature for 60 min prior to the vesicle injection. After a 30 min incubation at room temperature, the P-gp vesicles were immobilized in the channels via the cholesterol, which acted as a membrane anchor (supplemental Fig. S1, step 5).

*Rh123 transport assay* – The samples for the microscopic Rh123-transport analyses were prepared by adding the 10 µl of an Rh123 stock solution (5 µM in DMSO) to a 990 µl of 50 mM
MOPS-Tris buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl$_2$ at pH 7.0) in the presence of either AMP (10 mM) or ATP (10 mM) (plus 1 mM MβCD). The final Rh123 concentration used for the experiments was 50 nM. For the inhibition analyses, the required amounts of digoxin (5 mM), verapamil (1 mM), cyclosporine A (1 mM) and quinidine (1 mM) stock solutions in DMSO were mixed with the Rh123 solution. The concentrations of the inhibitors used in the inhibition experiments were as follows: (digoxin) 0.3 μM, 1 μM, 2 μM, 3 μM, 4 μM and 10 μM; (verapamil) 0.5 μM, 1 μM, 2 μM, 3 μM, 6 μM and 10 μM; (cyclosporine A) 0.1 μM, 0.3 μM, 0.6 μM, 1 μM and 3 μM; and (quinidine) 0.3 μM, 0.5 μM, 0.7 μM, 1 μM, 5 μM and 7 μM. The Rh123 solutions (8 μl) were injected into the channels and incubated at 37°C on a temperature-controlled plate (ThermoPlate MATS-52NLR, Tokai Hit). To minimize the contribution from the Rh123 transport kinetic factors, the inhibition analyses used the fluorescence of the P-gp vesicles after the Rh123 uptake was saturated. The incubation time was 60 min because the time-course data for the Rh123 transport revealed that the uptake was saturated in ca. 20 min (Fig. 2). After the incubation, the channels were rinsed with 40 mM MOPS-Tris buffer (40 mM MOPS-Tris, 70 mM KCl at pH 7.0, 100 μl).

The fluorescence-based batch assays (supplemental Fig. S2) were performed by adding the
25.5 µl of reaction mixture (9.8 µM Rh123, 19.6 mM ATP (or AMP) in 50 mM MOPS-Tris buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂ at pH 7.0)) to P-gp vesicles in 50 mM MOPS-Tris buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂ at pH 7.0, 24.5 µl, with a total protein concentration of 2 mg/ml). The assay mixtures were incubated at 37°C for the appropriate time (0 min, 15 min, 60 min and 180 min). After the incubation, 200 µl of a chilled 40 mM MOPS-Tris buffer (40 mM MOPS-Tris, 70 mM KCl at pH 7.0) was added to stop the reaction, and then the assay mixtures were suction filtered by a 96-well glass fiber filter plate (Whatman). The residual vesicles on the filter were washed 5 times by the 200 µl of chilled 40 mM MOPS-Tris buffer, and dissolved with a 50 µl of 10% (w/v) SDS solution. The dissolved vesicles were collected to a fluorometer plate (Nunc) placed under the filter plate by centrifuging the plates at 2,000 r.p.m. for 1 min, and the fluorescence intensity of each well was measured on a Corona Electric MTP-800Lab fluorescence microplate reader using an excitation/emission wavelength filter of 490/530 nm.

Microscopy and data analyses – The confocal imaging was performed on a Leica TCS SP5 confocal microscope using a 100× (NA 1.40–0.70) oil objective. For the influx time-course,
drug inhibition and single-vesicle analyses, an inverted microscope (IX-71, Olympus) mounted with a 512 × 512 pixel iXonEM+897 EMCCD camera (Andor Technology) with a 40× (NA 0.60) objective was used. The fluorescence signals from the P-gp vesicles were identified by comparing the fluorescence (16-bit) and differential interference contrast (DIC) images. The polygonal definition of the fluorescence areas of the vesicles and the collation of the P-gp fluorescence, which was defined as the integrated intensity of the vesicles above the background fluorescence outside of the channel, were obtained using Andor iQ 1.9.1 imaging software (Andor Technology). The mean intensities of the fluorescence distributions were used to determine the time at which half of the ATP-dependent transport had been completed (T1/2) in the time-course experiments (Fig. 2b) and the half-maximal inhibitory concentration (IC50) in the drug-inhibition experiments (Figs. 3a–3d). Gaussian fittings were performed to determine the mean intensities of the distributions using the Prism 4 commercial software package (GraphPad Software). The IC50 values were determined by fitting the mean fluorescence (I) to the following equation:
where $I_{\text{background}}$ and $I_0$ are the mean fluorescence values when the Rh123-influx suffers complete and no inhibition, respectively, \([\text{drug}]\) is the drug concentration and hillslope is the slope of the fitting curve.

**Single-vesicle substrate transport estimation** – We assumed that the Rh123 concentration and the fluorescence per volume ($\alpha$) within the MβCD-treated vesicles were constant in the equilibrium state. The $\alpha$ value was determined by fitting the vesicle intensity per area ($I_{\text{vesicle}}/A$) to the following equation:

\[
\frac{I_{\text{vesicle}}}{A} = \frac{2}{3}\alpha d + \frac{4I_{\text{background}}}{\pi d^2}
\]

(2)

where $d$ is the diameter calculated from the area of vesicles by assuming a spherical shape and $I_{\text{background}}$ is the vesicle fluorescence when no Rh123 is transported. The best-fit values for the
plot (Fig. 3e) are $\alpha = 5.599 \pm 0.1484$ fluorescence-units (FU)/$\mu$m$^3$ and $I_{\text{background}} = 95.04 \pm 21.31$ FU (mean ± s.d.; $R = 0.69$). Combined with the Rh123 standard curve (supplemental Fig. S3), which shows that Rh123 fluoresces at 9.5 FU/$\mu$M, the Rh123 concentration within the 1 $\mu$m$^3$ vesicles was 0.59 $\mu$M in the equilibrium state. Rauch and Pluen have mathematically predicted that the average surface density of P-gp in multidrug resistant cancer cells$^3$ is ca. 16 molecules/$\mu$m$^2$. This means that the P-gp density in our P-gp vesicles, which are the Sf9 insect cell membranes containing P-gp to 2–3% of total membrane protein$^4$, is at least 10–20-fold higher than that observed in mammalian cancer cells. Hence, the net number of Rh123 molecules transported into the 1 $\mu$m$^3$ vesicles by a single P-gp could be estimated to be 0.25–0.5 molecules/$\mu$m$^3$.

References

