Single-vesicle estimation of ATP-binding cassette transporters in microfluidic channels
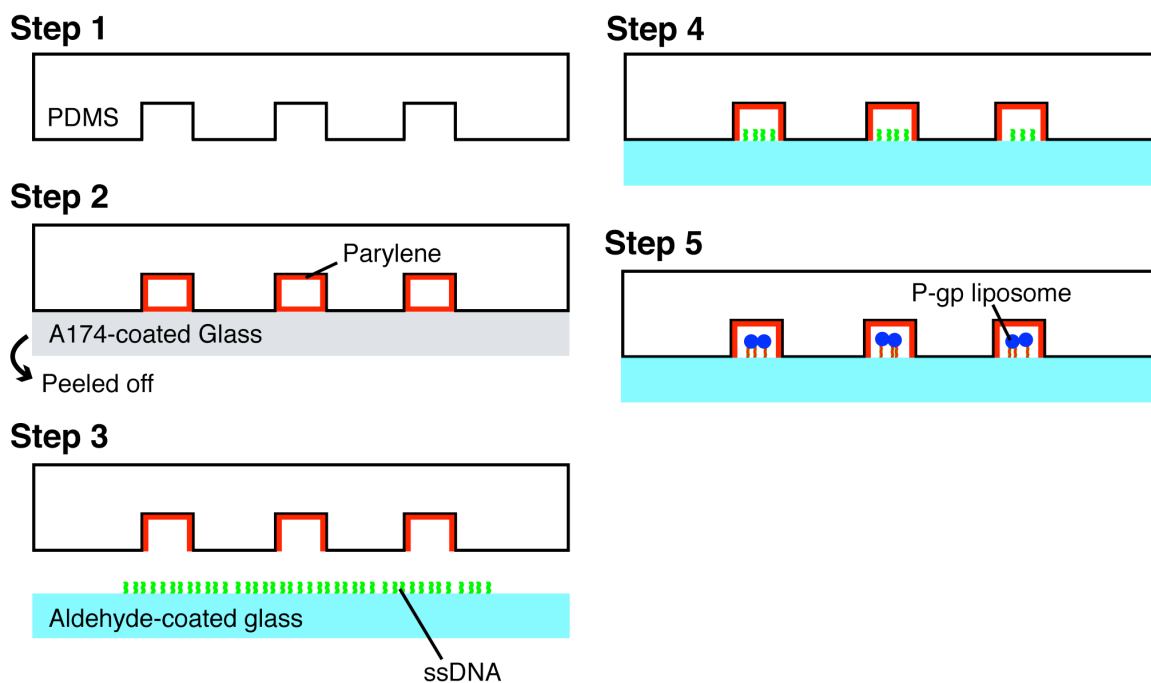
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Supplementary Figure S1	Process flow for the immobilization of P-glycoprotein vesicles in parylene-coated PDMS channels.
Supplementary Figure S2	The time-course of the Rh123 influx obtained by the fluorescence-based batch assays.
Supplementary Figure S3	The standard Rh123 fluorescence curve under the image capturing conditions used in this study.

Step 1

PDMS

Step 2

Parylene

A174-coated Glass

Peeled off

Step 3

Aldehyde-coated glass

ssDNA

Step 4

Step 5

P-gp liposome

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.
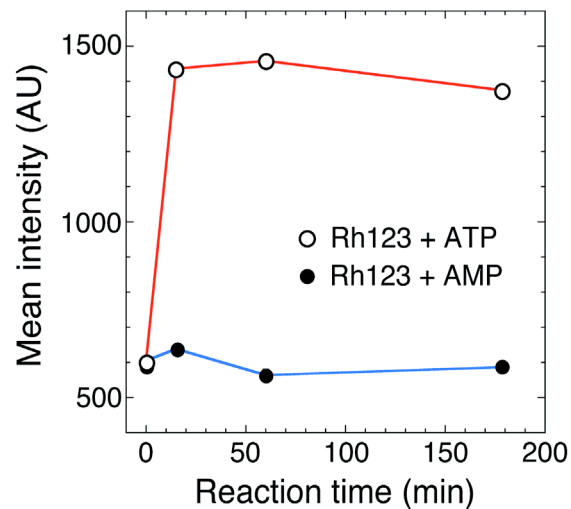
Supplementary Figure S2. The time-course of the Rh123 influx obtained by the fluorescence-based batch assays. The time-courses of the influx in the presence of ATP and AMP are shown by the red and blue lines, respectively. The error bars represent the s.d. for 5 measurements collected after various incubation times.
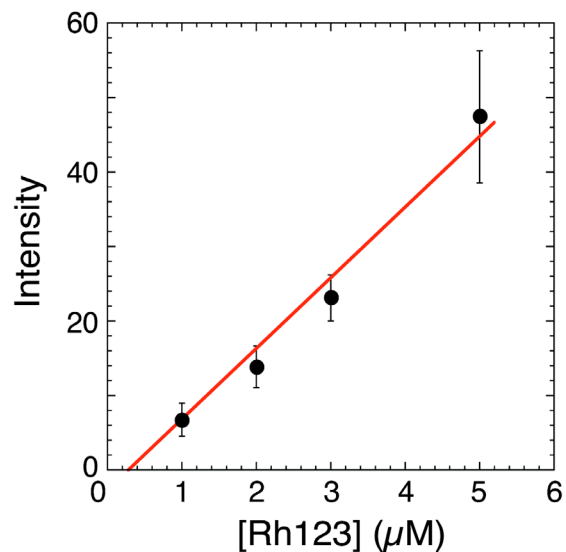
Supplementary Figure S3. The standard Rh123 fluorescence curve. The image

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 $MgCl_2$ 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 O_2 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[1] (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_2HPO_4, 1.5 mM KH_2PO_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 $MgCl_2$ 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'-ACTGACTGACTGACTGACTGACTGACTGACTGACTG-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 $MgCl_2$ at pH 7.0)) to P-gp vesicles in 50 mM

MOPS-Tris buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM $MgCl_2$ 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 ($T_{1/2}$) in

the time-course experiments (Fig. 2b) and the half-maximal inhibitory concentration (IC_{50}) 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 IC_{50} values were determined by fitting the mean fluorescence (I) to the

following equation:

$$I = I_{background} + \frac{I_0 - I_{background}}{1 + 10^{\,(logIC_{50} - log[drug]) \,*\, hillslope}}$$

(1)

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where $I_{background}$ and I_0 are the mean fluorescence values when the Rh123-influx suffers complete

and no inhibition, respectively, [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 (α) within the MβCD-treated vesicles were constant in the

equilibrium state. The α value was determined by fitting the vesicle intensity per area

($I_{vesicle}$/A) to the following equation:

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

(2)

where d is the diameter calculated from the area of vesicles by assuming a spherical shape and

$I_{background}$ 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)/μm^3 and $I_{background} = 95.04 \pm 21.31$

FU (mean $\pm$ s.d.; $R = 0.69$).   Combined with the Rh123 standard curve (supplemental Fig. S3),

which shows that Rh123 fluoresces at 9.5 FU/μM, the Rh123 concentration within the 1 μm^3

vesicles was 0.59 μM 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/μm^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 μm^3 vesicles by a single P-gp could be estimated to be 0.25–0.5

molecules/μm^3.

References

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