Supplementary Information

Experimental section

Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) dissolved in chloroform was purchased from Avanti Polar Lipids, Inc. Anhydrous *n*-decane was purchased from Sigma. Ovalbumin conjugated to AlexaFluor647 (OA647), fluorescein di-β-D-glucuronide (FDGlcU), AlexaFluor 488-conjugated BSA (BSA-A488), and calcein were obtained from Thermo Fisher Scientific. RNasin ribonuclease inhibitor was obtained from Promega. *n*-Hexadecane and cholesterol were purchased from Nacalai Tesque. β-Glucuronidase (GUS) was purified via a his-tag [1]. Plasmids encoding green fluorescence protein (GFP), α-hemolysin (AH), EmrE, and E14C mutant were constructed previously [2-4].

Chamber design and lipid-oil solution preparation

The chambers were fabricated using an acrylic plate using a micromilling machine (MM-100, Modia Systems) with dimensions as indicated in the results section. The lipid-oil solution was prepared by evaporation of chloroform in a lipid/chloroform solution under vacuum (-80 kPa) for 2 h, with the product then dissolved directly in oil. The same lipid-oil solution was used for no longer than one week. Fluorescence stereoscopic microscopy (Leica M205 FA) was used for image recording, and the obtained images were analyzed using ImageJ (NIH).

In vitro transcription and translation

For protein synthesis, 10 nM DNA (PCR product) was added to IVTT supplemented with 0.4 unit/μL RNasin (Promega). The PCR products used for IVTT were amplified using plasmids by KOD-plus (TOYOBO, KOD-201) with the primers T7F (5'-TAATACGACTCACTATAGGG-3') and T7R (5'-GCTAGTTATTGCTCAGCGG-3'). The PCR product was then purified using a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen).

The reconstituted IVTT used for the data shown in Figures 1 and 2 was prepared as previously reported [5, 6]. We used the kit developed in our lab, as it can synthesize up to 0.5 mg/mL of protein in a batch reaction [5, 6], which is higher than commercially available reconstituted IVTTs. Commercially available reconstituted IVTT, *i.e.* PUREfrex1.0 (Gene Frontier Co., Chiba, Japan), was used for the AH and EmrE synthesis shown in Figure 3. We obtained similar results with both IVTTs and no obvious differences were observed, indicating that the developed system can be reproduced easily in other laboratories with

commercially available reconstituted IVTT. When using PURExfrex1.0, 200 mM sucrose was added to both droplets which was essential for DIB stabilization.

Quantification of AH and EmrE at the DIB supplied by IVTT

For calculating the amount of AH at the DIB, two diffusion constants $\boldsymbol{D}$ = 1.44 x 10^{-6} m^2 h^{-1} [7] for FDGlcU and $\boldsymbol{D}$ = 1.49 x 10^{-6} m^2 $h^{-1}$for EtBr [8] were used. It has been reported that diffusion constant may differ between freely diffusing substance and those diffusing through small pores [9]. According to the previous work, the difference in diffusion constant is dependent on the relative size of the pore and the molecule that penetrates through the pore. Based on the relative size of AH pore and either FDglcU or EtBr, the diffusion constant may be overestimated by at most two-fold. This results in a two-fold underestimation of the amount of AH at the DIB.

In order to calculate the amount of EmrE at the DIB, we assumed that each µg of EmrE can transport 7.5 nmol EtBr/min, the same rate reported for methyl viologen [10].. In the previous paper [10], it is not clearly stated whether this value is a V_{max} *(i.e.*, measured under substrate saturation condition) or not. The K_M value for methyl viologen has been reported as 260 µM [11], and thus is likely to reach a V_{max} around 500 µM. The EtBr concentration used in this study was 2.5 µM, 200-fold lower than 500 µM. This lead to the possibility that the transport activity might be overestimated by 200-fold, leading to a significant underestimation of the amount of EmrE molecules at the DIB.

Even after considering the underestimation of the amount of AH at the DIB and the EmrE at the DIB, our conclusions that (1) only a very small fraction (in the order of 10^{-2} to 10^{-6} %) is located at the DIB and (2) the amount of functional AH at the DIB is smaller than that of EmrE are not affected.

Supplementary Figures

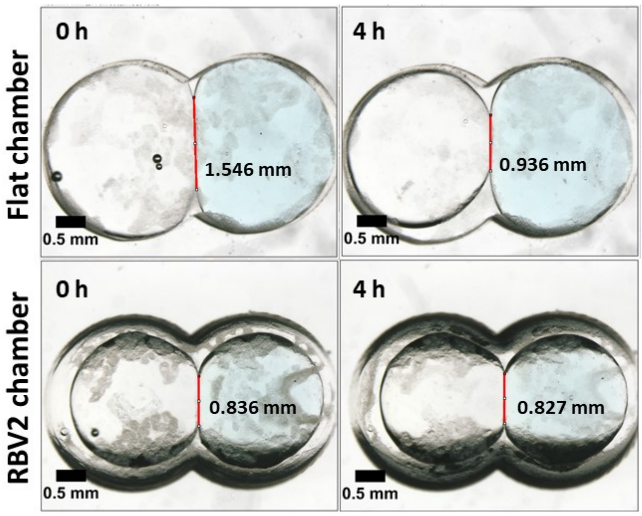

Fig. S1: Contact length measurement. (a) Representative images of the DIB at 0 h and 4 h in the FB (flat chamber) and RB2 (round-bottom V2) chamber, respectively. The contact length between the droplets is indicated by the red line. The images were analyzed by ImageJ.

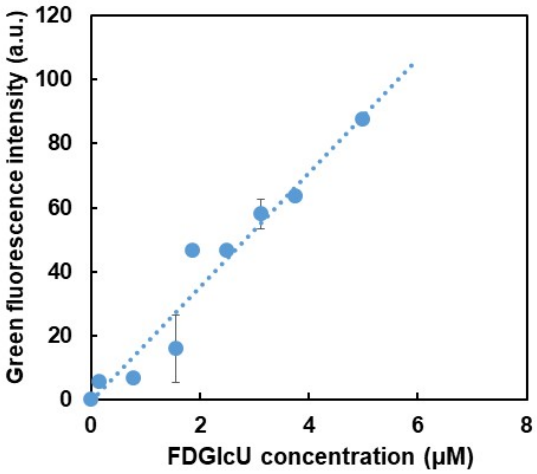

Fig. S2: Correlation between the hydrolyzed FDGlcU concentration and green fluorescence intensity inside the droplet. A 200 μM FDGlcU concentration was hydrolyzed by GUS to completion, and the resulting solution was diluted with an IVTT solution to the concentration indicated on the horizontal axis. The 3.5 μL water-in-oil (W/O) droplets were prepared and observed under a microscope, and the fluorescence intensity was measured.

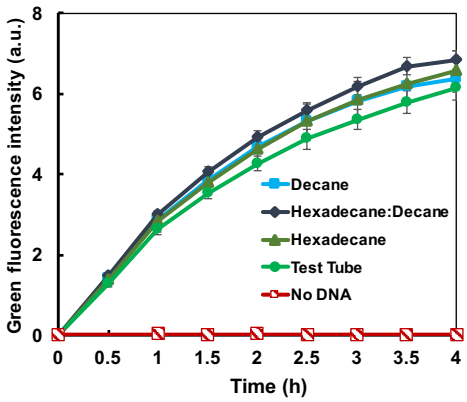

Fig. S3: GFP synthesis in W/O droplets. Time of GFP synthesis by IVTT inside a test tube or a W/O droplet at 37ºC. For the data with the test tube, the synthesis was first performed and the W/O droplet was prepared for measurement. The average and standard error of triplicate experiments are shown. The result shows that the irrespective of the oils, protein synthesized inside droplet using IVTT is indistinguishable from that synthesized in a test tube.

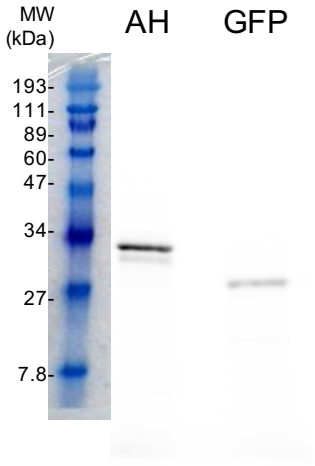

Fig. S4: Quantifying the amount of AH synthesized using IVTT. AH synthesis using IVTT was performed in the presence of [^{35}S]-methionine, with an AH DNA concentration of 10 nM at 37ºC for 2 h. The total amount of AH synthesized was quantified from SDS-PAGE, followed by autoradiography. GFP was synthesized as a control.

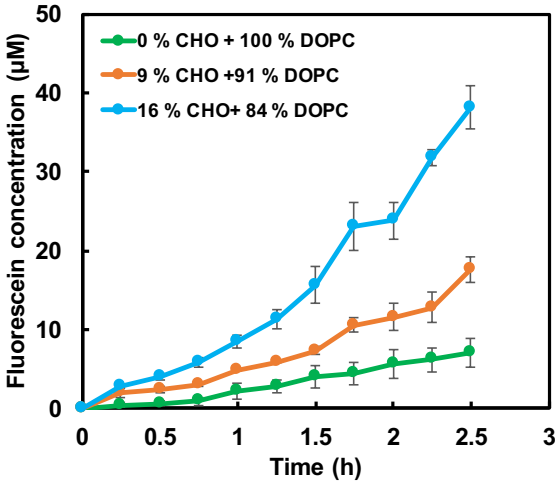

Fig. S5: Cholesterol concentration affected the rate of substrate flux through AH pore. AH synthesis was first performed using IVTT in a test tube, and DIBs with different lipid compositions—100% DOPC, 91% (mol/mol) DOPC+9% cholesterol, or 84% DOPC+16% cholesterol, all dissolved in 10:1 (v/v) hexadecane and decane—were prepared. Measurement was performed in the RB2 chamber and at 25°C. Increasing the concentration of cholesterol increased of the rate of substrate flow.

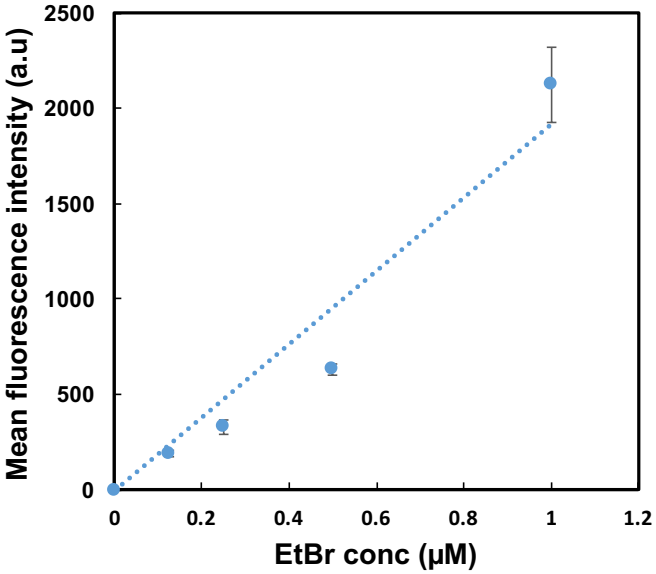

Fig. S6: Correlation between the EtBr concentration and fluorescence intensity inside the droplet. Different concentration of EtBr was added to the right droplet (Fig. 3AB). The DIBs were prepared and observed under a microscope, and the fluorescence intensity was measured.

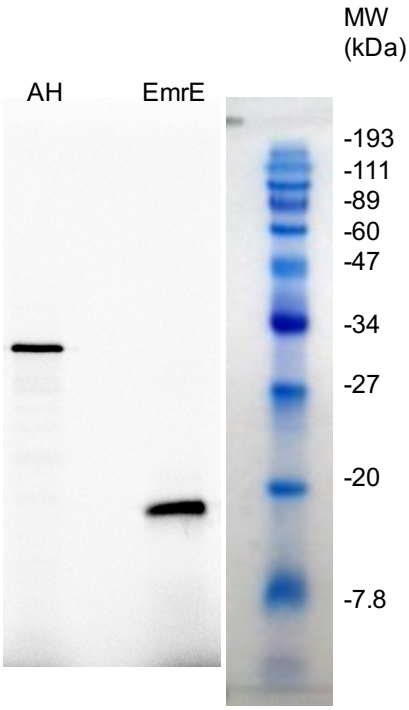

Fig. S7: Quantifying the amount of AH and EmrE synthesized using IVTT (PURExpress1.0). AH and EmrE synthesis using IVTT was performed in the presence of [^{35}S]-methionine, with a DNA concentration of 10 nM at 37ºC for 2 h. The total amount of AH synthesized was quantified from SDS-PAGE, followed by autoradiography.

References

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