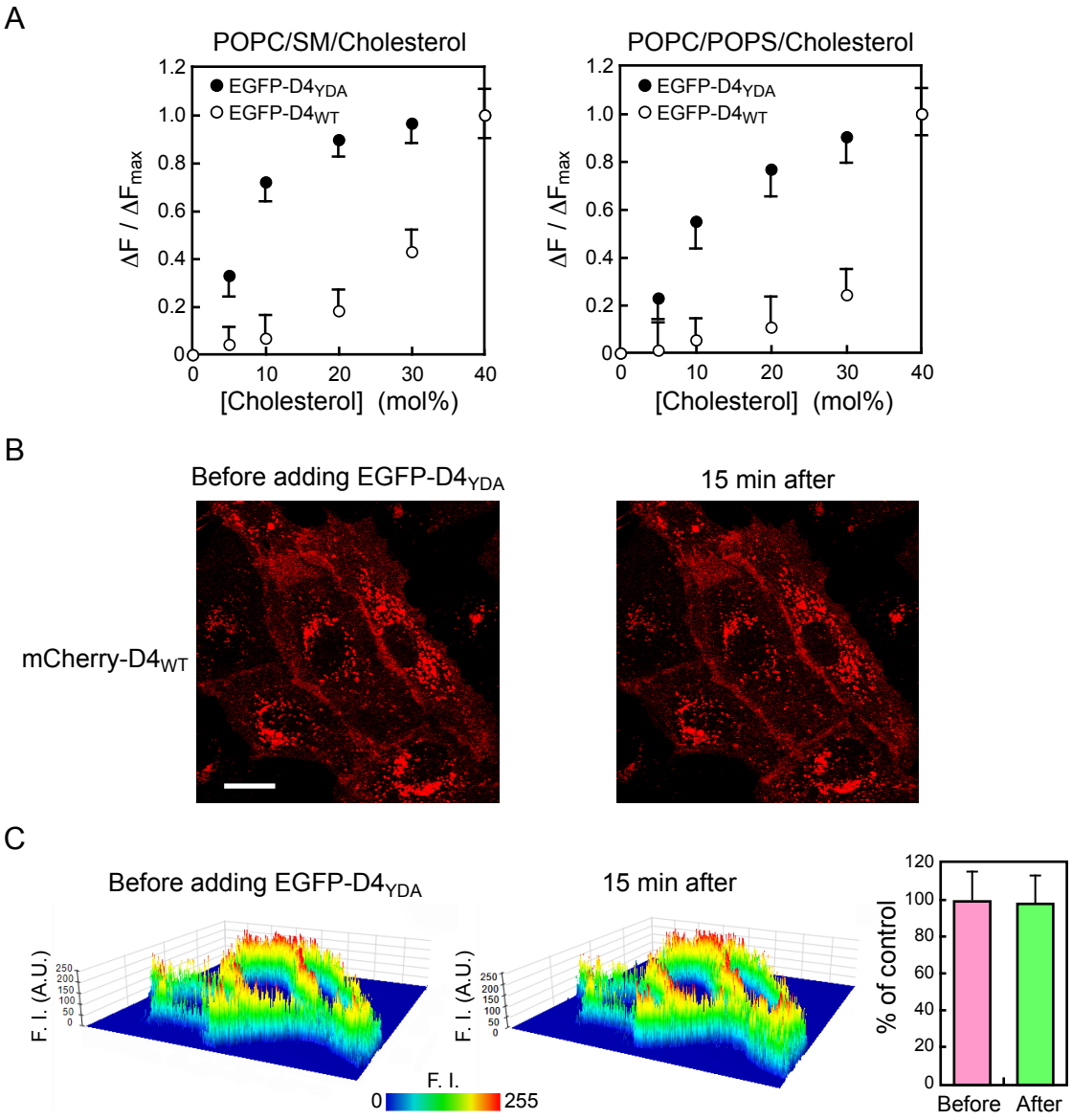

Fig. S1. Cellular properties of D4-based cholesterol probes. (A) Cholesterol affinity of the wild-type (WT) D4 and mutated D4 (YDA). EGFP-D4$_{WT}$ and EGFP-D4$_{YDA}$ bounded to both POPC/SM/ cholesterol (70-x/30/x: x = 0-40 mol%) and POPC/POPS/cholesterol (80-x/20/x: x = 0-40 mol%) giant unilamellar vesicles (GUVs) in a cholesterol concentration-dependent manner, and EGFP-D4$_{YDA}$ had a significantly higher cholesterol affinity than EGFP-D4$_{WT}$. Vesicle binding of the probes was monitored in terms of the increase in fluorescence intensity (ΔF) normalized against the maximal ΔF (ΔFmax). GUVs were prepared as previously described (Yamamoto et al. J Cell Sci, 2013) and the cholesterol probes were added to them at a concentration of 50 μg of proteins/mL. Values are the mean ± S.D. of 10 samples. (B) Microscopic images of HPAECs expressing mCherry-D4$_{WT}$ before and 15 min after the extracellular addition of EGFP-D4$_{YDA}$ protein (50 μg/mL). Scale bar represents 20 μm. (C) Quantitative analyses of mCherry-D4$_{WT}$ signal. Using Image Pro 10 with reference to the methods described by Liu et al. (Nat Chem Biol, 2016), we set up a region of interest over the plasma membrane and measured fluorescence intensity of mCherry-D4$_{WT}$. Spatially resolved fluorescence intensity (F. I.) on the cross-section of a representative cell showed that the extracellular addition of EGFP-D4$_{YDA}$ did not affect the signal from mCherry-D4$_{WT}$ bound to the inner layer of the plasma membrane in HPAECs. A pseudo-coloring scheme with red representing the highest and blue the lowest signal is used to better illustrate the spatial signal heterogeneity. Spatially averaged fluorescence intensity of mCherry-D4$_{WT}$ are displayed in the right panel. Values are the mean ± S.D. of 30 samples. These findings indicate that EGFP-D4$_{YDA}$ does not cross the plasma membrane in HPAECs under the present conditions.

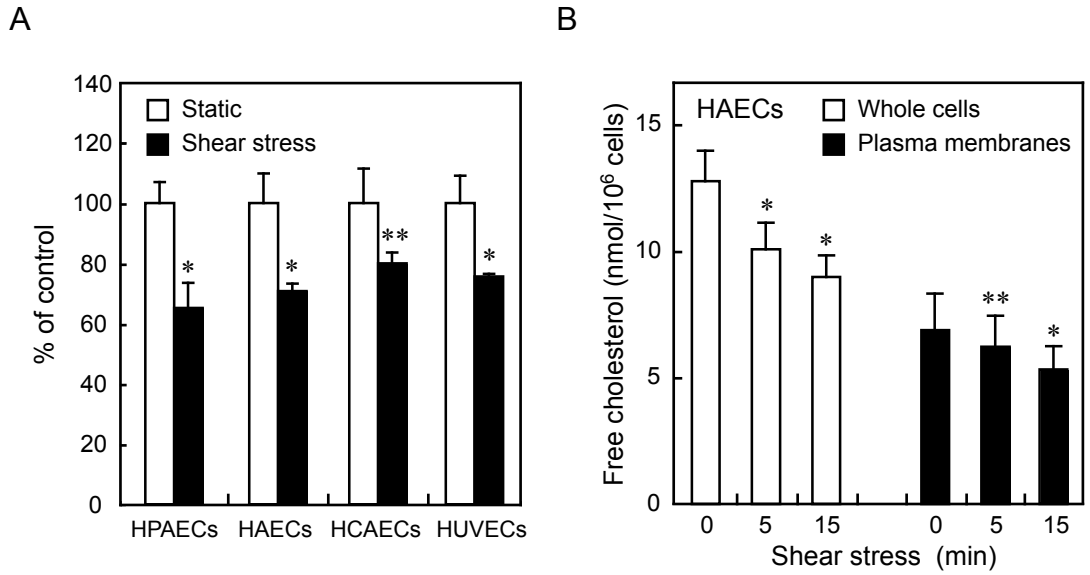

Fig. S2. (A) Shear-stress-induced changes in cholesterol in the outer bilayer as quantified using flow cytometric analyses of cells labeled with EGFP-D4$_{YDA}$. The application of shear stress (15 dynes/cm^2) for 15 min significantly decreased the cholesterol levels in human aortic ECs (HAECs), human coronary artery ECs (HCAECs), and human umbilical vein ECs (HUVECs) as well as in HPAECs. The values are the mean ± S.D. of 6 samples obtained in two separate experiments; *$P < 0.01$, **$P < 0.05$, compared with a static control (zero shear stress). (B) Shear-stress-induced changes in the free cholesterol contents of whole cells and isolated plasma membranes. Biochemical assays showed that shear stress (15 dynes/cm^2) significantly decreased the cholesterol contents of both the whole cells and the plasma membranes in HAECs. Values are the mean ± S.D. of 6 samples; *$P < 0.01$, **$P < 0.05$, compared with a static control (zero shear stress).

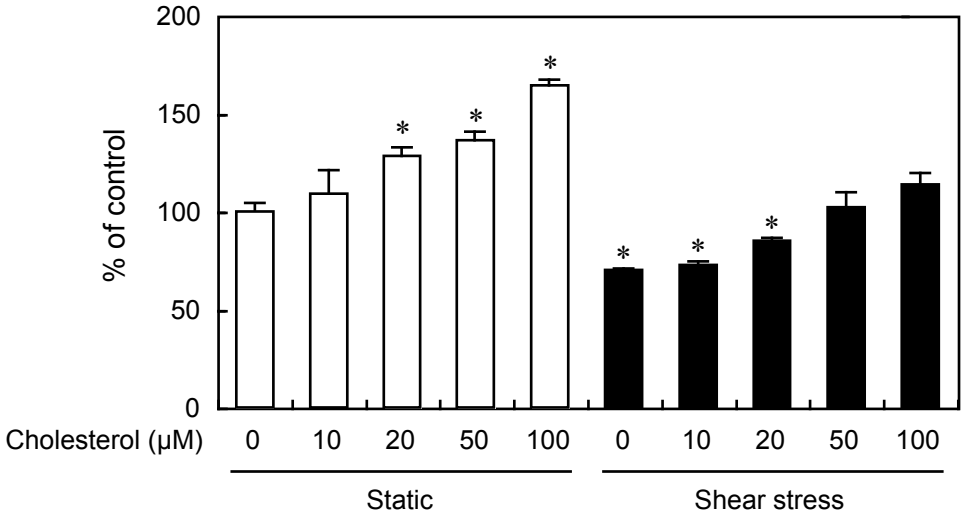

Fig. S3. Effects of cholesterol addition on the cholesterol content of the plasma membrane. HPAECs were treated with cholesterol (Sigma-Aldrich C4951) at different concentrations for 60 min, then labelled with EGFP-$D4_{YDA}$. Changes in the cholesterol content of the outer bilayer were examined using flow cytometry. The addition of cholesterol increased the cholesterol levels in the static cells and inhibited the decreasing effects of shear stress on the cholesterol levels in a dose-dependent manner. The addition of 50 μM of cholesterol offset the cholesterol-reducing effect of the shear stress, equalizing the cholesterol level with that observed in the control. The results are presented as the mean ± S.D. of 9 samples obtained in three separate experiments. $*P < 0.01$ compared with the control (zero cholesterol) in static cells.

Supplementary materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), porcine brain sphingomyelin (SM), and cholesterol were purchased from Avanti Polar Lipids. Human aortic ECs (HAECs), human coronary artery ECs (HCAECs), and human umbilical vein ECs (HUVECs) were purchased from Lonza Walkersville Inc. (Product code: cc-2535, cc-2585, and cc-2517, respectively).