SUPPORTING INFORMATION

Tetranuclear polypyridylruthenium(II) complex as a selective stain for extracellular vesicle penetration through brain microvascular endothelium

Kartika Wardhani,[a] Aviva Levina,[a] Biyun Sun,[b] Georges E. R. Grau,[c-f] F. Richard Keene,[g,h] J. Grant Collins,[i] Peter A. Lay*[a,c-e,j]

[a]School of Chemistry, The University of Sydney, Sydney, NSW, 2006, Australia.

[b]Dementia Research Centre, Department of Biomedical Science, Faculty of Medicine and Health Sciences, Macquarie University, Sydney, NSW, 2109, Australia.

[c]Sydney Nano, [d]Sydney Cancer Network, [e]Marie Bashir Institute, The University of Sydney, Sydney, NSW, 2006, Australia.

[f]Vascular Immunology Unit, Department of Pathology, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, 2006, Australia.

[g]Department of Chemistry, School of Physical Sciences, The University of Adelaide, Adelaide, SA, 5000, Australia.

[h]Australian Institute of Tropical Health and Medicine/Centre for Molecular Therapeutics, James Cook University, Townsville, QLD, 4811, Australia.

[i]School of Science, The University of New South Wales, Australian Defence Force Academy, Canberra, ACT, 2612, Australia.

[j]Sydney Analytical, The University of Sydney, Sydney, NSW, 2006, Australia.

EXPERIMENTAL SECTION

Materials and Stock Solutions. Tetranuclear polypyridylruthenium(II) complex-based stain, **Rubb7-TNL** (Figure 1a, main text) was synthesized and characterized as previously described.[16-18] Stock solutions of Ru complex (10 mM Ru) in high-purity (>99.9%) dimethyl sulfoxide (DMSO, Merck Cat. No. 5439001000) was stored at 295 K, protected from light and moisture; its stability over time (at least six months) was verified by electronic absorption spectroscopy.[16-18] Other Analytical grade (>99% purity) reagents and HPLC grade solvents from Sigma-Aldrich or Merck were used without further purification, and water was purified by the Milli-Q technique.

Cell culture. The human cancer cell line: THP-1 (human acute monocytic leukemia, ATCC TIB-202) was purchased from the American Type Culture Collection (ATCC). Immortalized endothelial cells of human brain microvessels (hCMEC/D3 cell line) were previously developed as an *in vitro* model of blood-brain barrier (BBB),[23] and supplied by Prof. Georges Grau (Faculty of Medicine and Health, University of Sydney). Pre-sterilized solutions and plasticware for mammalian cell culture were purchased from Thermo Fisher Scientific (TFS), unless specified otherwise. The human cells were cultured under sterile environment, using standard techniques.[15,S1] Cancer THP-1 cells were cultured in Advanced RPMI 1640 (TFS 12633012), supplemented with GlutaMax (TFS 35050061; equivalent to 2.0 mM L-glutamine), antibiotic-antimycotic mixture (TFS 15240062; 1.0 U mL^{-1} penicillin, 1.0 mg mL^{-1} streptomycin and 2.5 μg mL^{-1} amphotericin B) and fetal calf serum (FCS; Gibco 10500064; 2.0% vol.), which is referred to as the growth medium. Immortalized non-cancer cells (hCMEC/D3) were cultured in EBM-2 medium (Lonza CC3121), supplemented with FCS (5.0% vol.), hydrocortisone (Sigma H0888; 1.4 μM), ascorbic acid (Aldrich 255564, 5.0 μg mL^{-1}), chemically defined lipid concentrate (LTA 11905031; 1.0% vol.), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, (HEPES; Astral Scientific BIOHB0264; 10 mM), and bFGF growth factor (recombinant human protein; TFS 13256029; 1.0 ng mL^{-1}), which is referred to as EBM-2 endothelial cell medium. Plasticware used for the culture of hCMEC/D3 cells was pre-coated with collagen type 1 (TFS A1048301; 0.15 mg mL^{-1} solution in phosphate-buffered saline) overnight at 310 K. The hCMEC/D3 cells were subcultured at 60-70% confluence, and the passage numbers 28-40 were used for experiments. Cell viability was assessed by the trypan blue assay together with the Invitrogen Countess Haemocytometer.

Isolation of monocytic large extracellular vesicles (EVs) from LPS-stimulated immune system THP-1 cells. In a typical procedure, THP-1 monocytes were grown to the density of 2×10^6 cells mL^{-1} in growth medium (20 mL) within 175 cm^2 cell culture flasks. The cells were pelleted by centrifugation (2 min at 600 *g*) and re-suspended in 20 mL of serum-free medium (same as the growth medium, but FCS was entirely replaced with 1.0 mg mL^{-1} of AlbuMax, TFS 11020021). Serum-free medium was used to avoid the interference of EVs that are present in serum.[26] The resultant suspension was divided between two 9-cm cell culture dishes (10 mL per dish), and bacterial lipopolysaccharide (LPS; Sigma L6529; concentration in the medium 100 ng mL^{-1}) was added into each of the dishes. The dose of LPS 100 ng mL^{-1} was selected as the optimal dose for monocytic EV release without significantly compromising THP-1 parental cell viability.[11,15,22] After overnight incubation, stimulated cell suspensions were collected into two separate centrifuge tubes, and cells were removed by centrifugation (2 min at 600 *g*). The supernatants were then sequentially centrifuged for 5 min at 1200 *g* (295 K) to remove the cell debris, and for 60 min at 20,000 g (277 K) to pellet the LPS-stimulated monocytic EVs, as previously described.[11,15,22] The LPS-stimulated monocytic EV pellets were re-suspended in 5.0 mL of phosphate buffered saline (PBS) and re-centrifuged (60 min at

20,000 *g* and 277 K). The resultant LPS-stimulated monocytic EV pellets were re-suspended in 0.20 mL PBS and kept frozen at 253 K until they were prepared for staining and further characterization.

Dynamic light scattering (DLS) measurements. The particle size distribution of the LPS-stimulated monocytic EV pellets suspended in 0.20 mL PBS was measured by DLS, using Malvern ZetaSizer NanoS instrument (173° scattering angle, 298 K) and Malvern ZEN0040 disposable cuvettes. The measured parameters were the averages of 12-15 scans (scan time, 3 s). DLS results were 611±100 nm and 699±167 nm (p = 0.2) for unstained and **Rubb$_7$-TNL**-stained LPS-stimulated monocytic EVs, respectively.[15]

Staining of isolated LPS-stimulated monocytic large EVs with polypyridylruthenium(II) phosphophore. Typically, 1.0 μL of **Rubb$_7$-TNL** solutions in DMSO (2.0 mM Ru) were added to 0.20 mL of LPS-stimulated monocytic EV suspension in PBS (final Ru concentration, 10 μM), and the suspensions were kept in the dark for 1 h at 298 K. After that, stained LPS-stimulated monocytic EVs were pelleted by centrifugation (60 min at 20,000 *g* and 277 K) and washed twice with 1.0 mL PBS by re-suspending and re-centrifugation under the same conditions. The purified stained LPS-stimulated monocytic EV pellets were re-suspended in 0.20 mL PBS and stored at 253 K until they were used for *in vitro* BBB study (Figure 1b, main text). Ru uptake by EV was measured in three random samples of EVs released from LPS-stimulated THP-1 cells. Each LPS-stimulated monocytic EV pellet was lysed in 0.10 mL of 0.10 M NaOH (overnight at 277 K), and an aliquot of the lysate (5.0 μL) was used for protein content determination using Bradford assay with bovine serum albumin (BSA) as a standard. The remaining lysate was mixed with 0.10 M HCl (0.90 mL), and Ru content in the resultant solution was determined by ICPMS (Perkin-Elmer Nexion 350X spectrometer), using standard Ru solution (Aldrich 207446) and ^{193}Ir peak as an internal standard. The measured values of Ru uptake by LPS-stimulated monocytic EV during staining with **Rubb$_7$-TNL** were 12 ± 4 nmol Ru per mg protein (average and standard deviation for three separate LPS-stimulated monocytic EV samples).[15]

Application of flow cytometry for the detection of stained LPS-stimulated monocytic EVs penetration through endothelial cell layers. Monolayers of hCMEC/D3 cells were grown in 1.0 μm pore size cell culture inserts (Merck Millipore MCRP24H48), as previously described.[23-25] The pore size was chosen to pass most of the EV, based on size distribution (DLS) measurements.[15] The inserts were pre-coated with collagen, ~10^4 cells were seeded in each insert in 0.20 mL of EBM-2 endothelial medium (with 0.90 mL of the same medium in the lower chamber) and grown for twelve days (with three changes of medium) to form a tight monolayer that resembles BBB.[23-25] Integrity of the monolayer was tested using fluorescein-labelled dextran (FITC-dextran, 70 kDa size, Sigma FD70) according to a standard procedure.[23-25] Collagen-coated inserts that did not contain cells were used as controls. A solution of FITC-dextran (40 μL of 5.0 mg mL^{-1} in PBS) was added to 0.20 mL of medium in each insert, and 10 μL aliquots of media from the lower chambers were taken at 15, 30, 45 and 60 min after the addition of FITC-dextran (the cells were kept at 310 K between sampling). Each aliquot was placed in a well of a 96-well plate, mixed with 90 μL of 10 mM aqueous $NaHCO_3$, and the fluorescence was measured using ClarioStar plate reader (BMG Labtech) with standard FITC settings (490 nm excitation, 520 nm emission). Permeability of cell monolayers was ~1% of that of the control inserts (Figure 3, main text, and Figure S1, SI). To test the permeability of LPS-stimulated monocytic EV through endothelial cell monolayers, **Rubb$_7$-TNL**-stained EV pellets isolated from LPS-stimulated THP-1 cells were suspended in 0.20 mL of serum-free EBM-2 endothelial medium (supplemented with 1.0 mg mL^{-1}

Albumax) and placed into the inserts that contained hCMEC/D3 cell monolayers (0.90 mL of the same medium was placed into the lower chambers). After 24 h of incubation, media from upper and lower chambers was collected into separate tubes and directly used for flow cytometry (Figure 1b and Figure 2, main text).

Analysis of stained monocytic EVs penetration of the endothelial BBB by flow cytometry. Analyses were performed using a Guava EasyCyte 6-2L benchtop flow cytometer (Merck Millipore). Typical flow cytometer settings were as follows: 488 nm laser on; green, yellow and red emission channels on (high detection level, gain 10); forward and side scattering gain, 10; forward scattering threshold, 0; flow rate, 0.12 $\mu L\, s^{-1}$; counting 5000 gated events. Suspensions of serum-free EBM-2 endothelial medium containing stained LPS-stimulated monocytic EVs from upper and lower chambers were used for flow cytometry (Figure 1b and Figure 2, main text). The fluorescence threshold was set at 10^2 units based on preliminary flow cytometry data for stained and unstained EVs, as previously reported.[15] Blank serum-free EBM-2 endothelial medium was used to gate out the electronic noise, and 0.46 μM and 1.1 μM polystyrene beads (Sigma LB5 and LB11) diluted 50,000-fold with PBS were used for calibration, as previously described.[15,S2] All the plots were gated to exclude non-specific background "electronic noise" (measured using serum-free EBM-2 endothelial medium as the background, Figure S2e, SI).

For all the experiments described in this paper, consistent results were reproduced in multiple independent experiments. The same procedures and instrument settings were applied to avoid interexperimental variations.

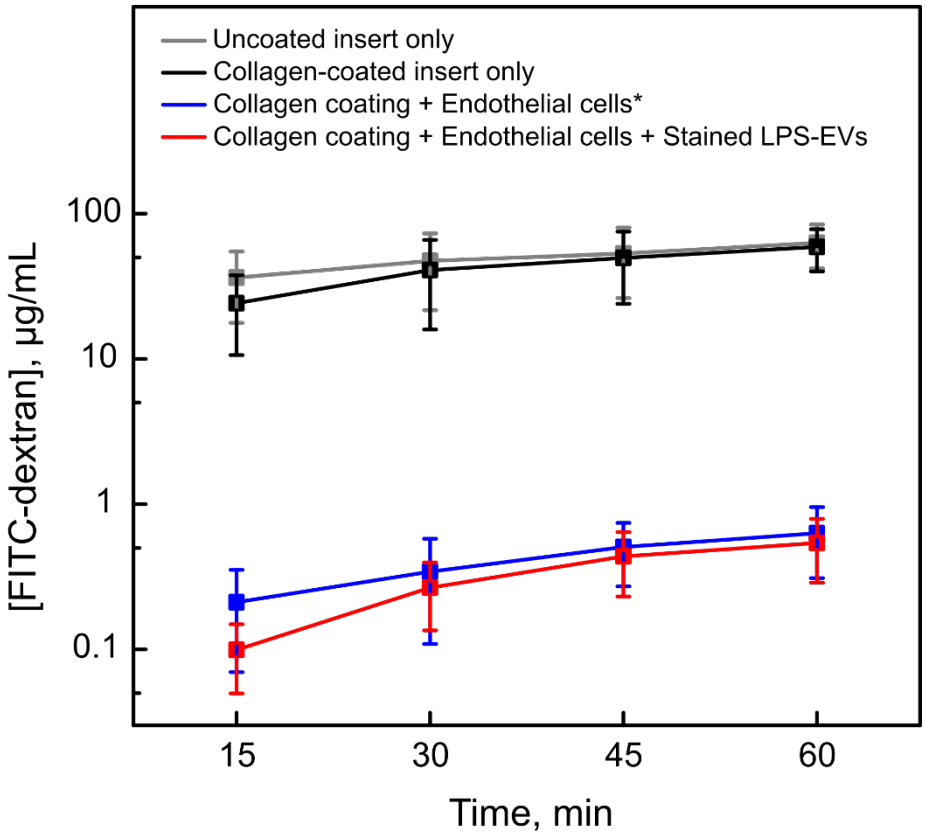

Figure S1. Time-dependent permeability of cell culture inserts (1.0 µm pore size) with and without monolayers of hCMEC/D3 cells. The star sign (*) shown in the legend of the figure corresponds to the 70 kDa FITC-dextran permeability in the hCMEC/D3 cell monolayers before co-incubation for 24 h with **Rubb$_7$-TNL**-stained LPS-stimulated monocytic EVs. The results are expressed as average values and standard deviations of three independent experiments for each treatment group. EV samples were isolated from three different batches (n = 3) of LPS-stimulated THP-1 cells.

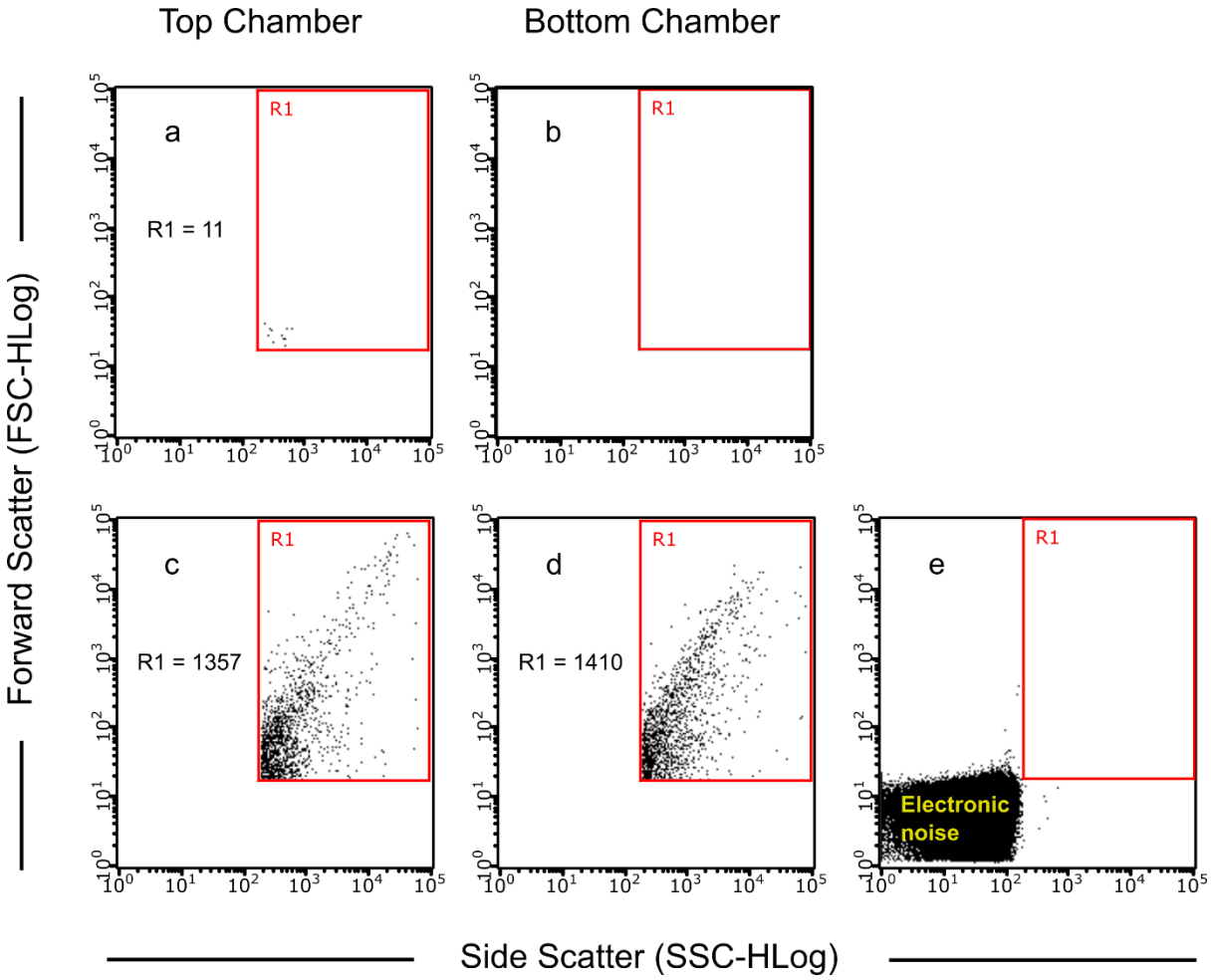

Figure S2. Flow cytometry forward vs. side scatter intensity plots of (a-b) the collagen-coated inserts only and (c-d) with the presence of the endothelial cell monolayer and with no added photoluminescent LPS-stimulated monocytic EVs. (e) The area of interest events was gated based on FSC and SSC (R1, red outlined region, right panels) using serum-free EBM-2 endothelial cell medium to exclude non-specific background "electronic noise".

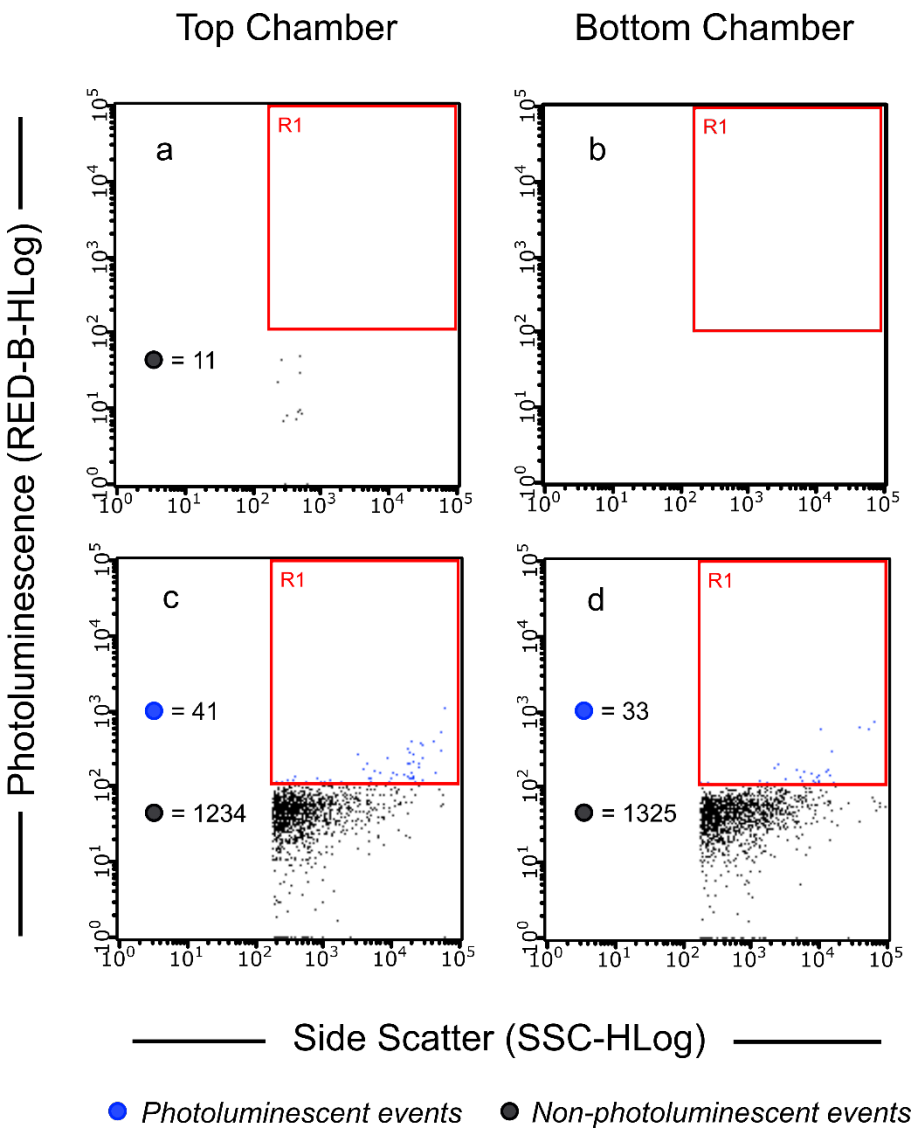

Figure S3. Comparison of photoluminescence vs. side scatter intensity plots for representative samples of: (a-b) the collagen-coated inserts only and (c-d) with the presence of the endothelial cell monolayer and with no added photoluminescent LPS-stimulated monocytic EVs. Populations were gated to exclude non-specific background "electronic noise" (Figure S2e, SI).

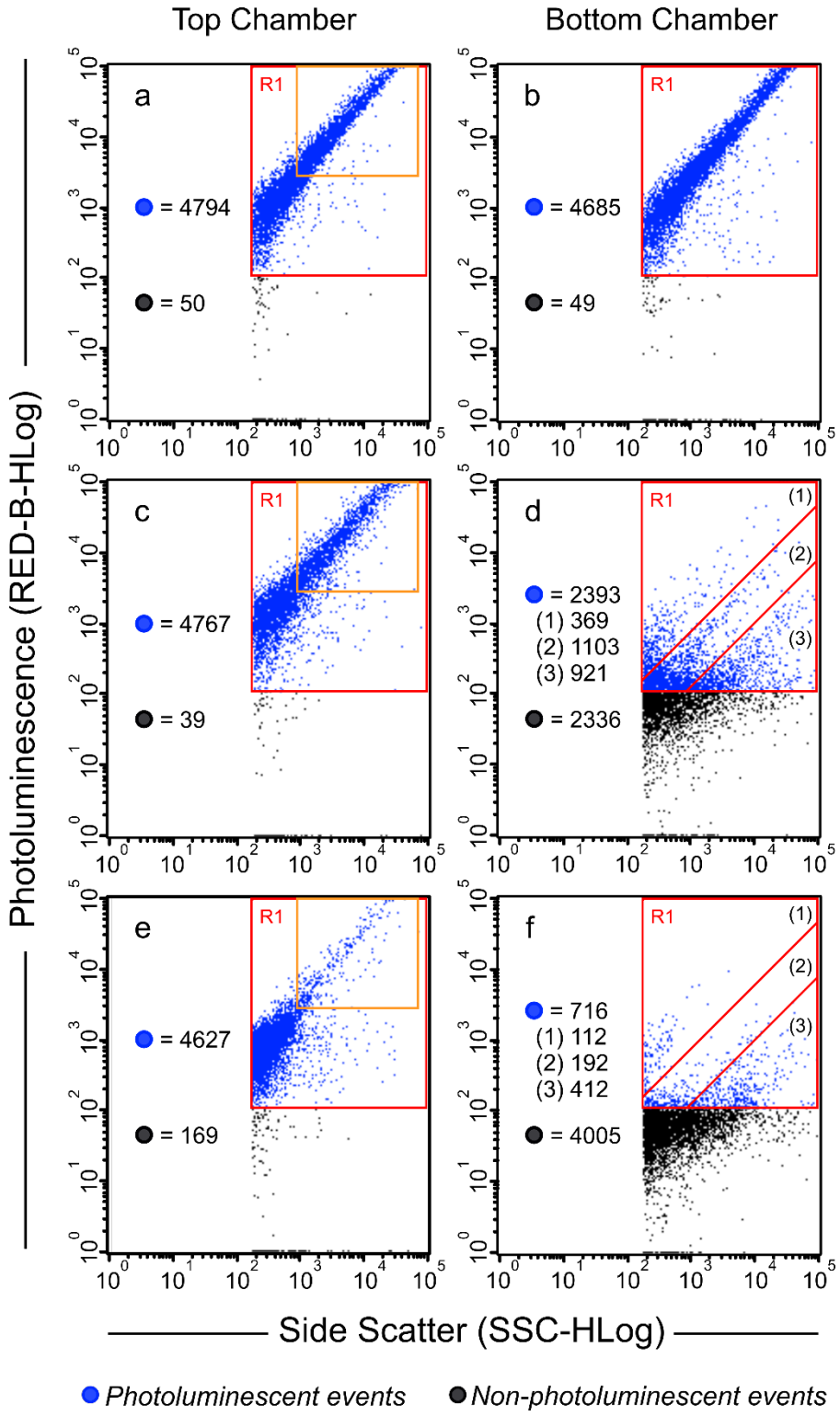

Figure S4. Typical flow cytometry results for the endothelial cell permeability assays. (a-b) Uncoated and (c-d) coated-collagen inserts without endothelial cell layer and with added **Rubb7-TNL**-stained LPS-stimulated monocytic EVs; (e-f) collagen-coated inserts with the layer of endothelial cells and with the addition of **Rubb7-TNL**-stained LPS-stimulated monocytic EVs. The numbers are calculated photoluminescent LPS-stimulated monocytic EVs in each gate R1 and non-photoluminescent events. The orange rectangle corresponds to photoluminescent events with high photoluminescence and SSC characteristics. All the plots were gated to exclude electronic noise.

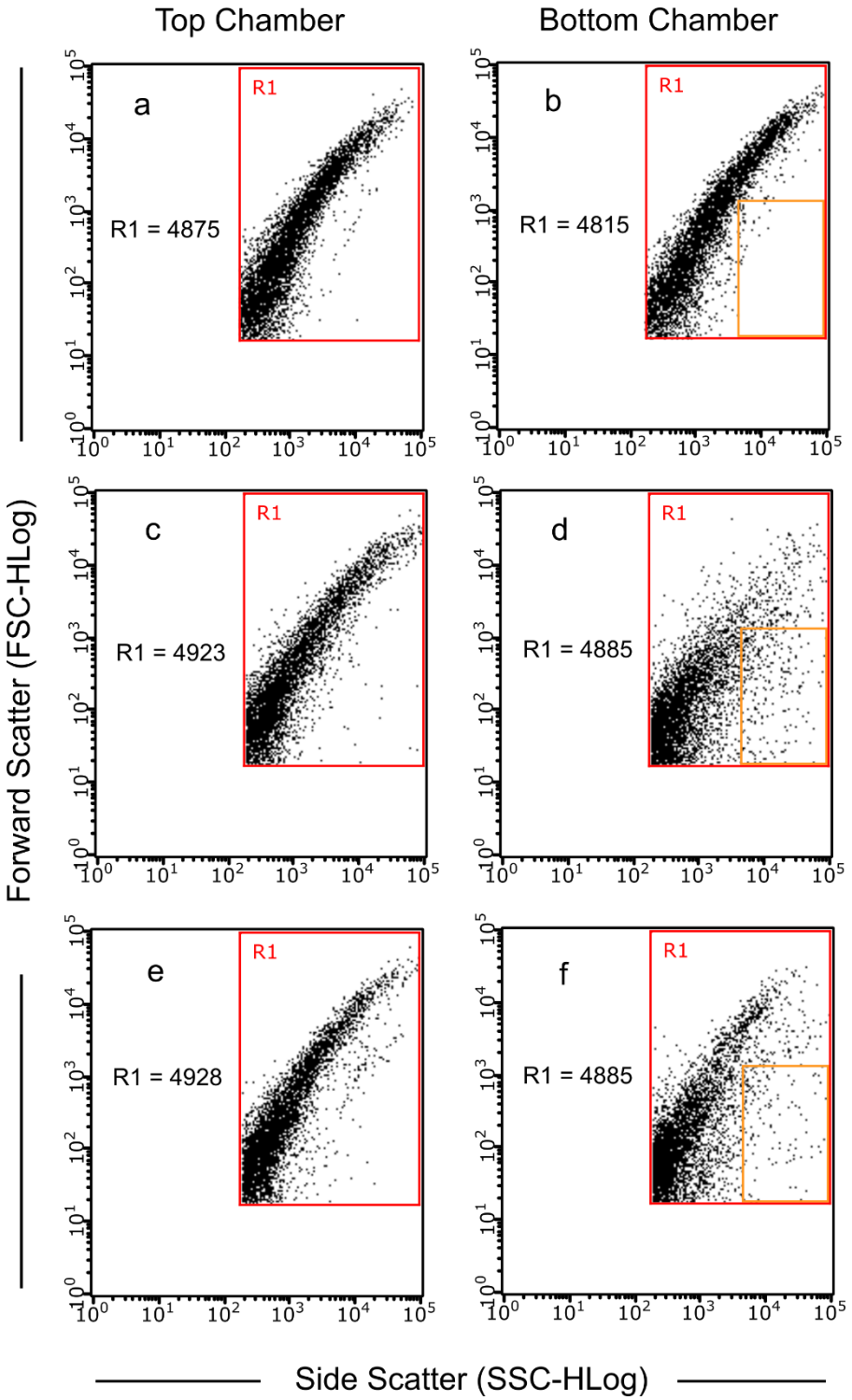

Figure S5. Flow cytometry forward vs. side scatter intensity plots for the uptake and permeability of photoluminescent LPS-stimulated monocytic EVs using the *in vitro* human BBB model. (a-b) Uncoated and (c-d) coated-collagen inserts without endothelial cell layer and with added **Rubb7-TNL**-stained LPS-stimulated monocytic EVs; (e-f) collagen-coated inserts with the layer of endothelial cells and with the addition of **Rubb7-TNL**-stained LPS-stimulated monocytic EVs. Populations were gated based on FSC (size) and SSC (granularity) using a rectangle scale (R1, red outlined region, right panels) to gate out background "electronic noise" (Figure S2e, SI). The orange rectangle corresponds to the events with high SSC but low FSC characteristics.

References

[S1] A. Levina, H. H. Harris and P. A. Lay, *J. Am. Chem. Soc.* 2007, **129**, 1065-1075.

[S2] H. C. van der Heyde, I. Gramaglia, V. Combes, T. C. George and G. E. Grau, *Methods Mol. Biol.* 2011, **699**, 337-354.