Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supporting Information

Endogenous Exosome Labelling with an Amphiphilic NIR-Fluorescent Probe

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Methods

Synthesis

NIR-AZA probe **1** was synthesized following previously reported procedure.[1]

Cell culture

KellyCis83cells were grown in complete RPMI 1640 medium (supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin or 1% Gentamicin and 1% Glutamine, all purchased from Gibco), at 37 °C, 5% CO_2.

Endogenous exosome labelling with NIR-AZA 1.

KellyCis83 cells were grown in complete RPMI 1640 medium until 70-80% confluence was reached, and washed thrice with sterile PBS. Cells were incubated for 2 h with 10 ml of staining medium (RPMI complete medium added with NIR-AZA **1**, 5 μM) and then rinsed three times with sterile PBS. Finally, 10 ml of serum free medium was added to each flask and exosomes were purified following 24 h incubation at 37 °C, 5% CO_2.

Unstained exosome harvest

KellyCis83 cells were grown in complete RPMI 1640 medium until 70-80% confluence was reached. Complete medium was removed and cells were washed thrice with sterile PBS. Cells were then added with 10 ml of Serum Free RPMI 1640 (supplemented with 1% penicillin/streptomycin or 1% gentamycin and 1% glutamine, all purchased from Gibco) and exosome were purified after 24 h of incubation.

Exosome isolation

Both NIR-exosomes and unlabelled exosomes were purified from cell-conditioned serum free medium using several differential centrifugation steps: 800 *g* x 30 minutes (to pellet larger EVs such as apoptotic bodies and cell debris) and 16,000 *g* x 45 minutes to pellet large EVs (microvesicles). The remaining supernatant containing smaller EVs (exosomes) were then concentrated using centrifugal filters (Amicon Ultra-15 with a MWCO of 100 kDa), following manufacturer's instructions. Exosomes were pelleted by ultracentrifugation at 100,000 *g* x 2 h.

Exosome Nanoparticle Tracking Analysis

NTA was performed using a Malvern Nanosight NS300 equipped with a blue laser and a quartz chamber for sample injection (O-Ring top plate model). Each exosome sample was diluted in sterile, ultrapure grade water and measured for 60 sec. Measurement parameters were set using 100 nm polystyrene-latex beads as standards and kept constant between samples; dilution factor was tuned in order to keep a particle number per frame ~ 30, according to NS300 standard operational procedures, and varied between 1:100 and 1:500.

Exosome purity and titration through colloidal gold nanoplasmonics

Exosome purity and concentration were assessed using a test based on colloidal gold nanoplasmonics (CONAN assay) (Fig S3A, B) as previously reported.[2] Exosomes and NIR-exosomes were resuspended in sterile PBS, diluted 1:100 with MilliQwater and analyzed using a test based on colloidal gold, CONAN assay (Fig S3A, B). The assay exploits three aspects of gold nanoparticles (AuNPs) - nanoplasmonics, nanoparticles/lipid membrane interaction and protein corona, to assess purity and concentration of exosome samples. In CONAN assay, the exosome purity and concentration are linked with the aggregation state of AuNPs in solution, which is expressed through a numerical value called Aggregation Index (AI).

Exosome biochemical analysis

For biochemical analysis, NIR-exosomes and unlabelled exosomes were resuspended in 50 µl of 100 mM Tris, 150 mM NaCl, 1 mM EDTA supplemented with 1:1000 protease inhibitor cocktail (P.I.). 10 µl of loading buffer 6x were added and samples were boiled 5 min at 95°C. Twenty µl of samples were electrophoresed (120V x 90 min) in sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred on a PVDF membrane (100V x 60 min), which was incubated 45 min at 37°C in PBS + tween 0.05% + fat-free milk 5%. The membrane was then analyzed by Western Blot (WB), using the following antibodies: mouse rabbit α GM130 1:1000 (Origene), mouse α TSG101 1:500 (Santa Cruz Biotechnology), mouse α Annexin-V 1:500 (Santa Cruz Biotechnology), mouse α CD81 1:500 (Santa Cruz Biotechnology). PVDF membrane was incubated under mild agitation with primary antibodies for 90 min, washed three times with PBS and then incubated for 60

min with HRP-conjugated secondary antibodies (provided by Bethyl Laboratories), diluted 1:10000 prior to use. Both primary and secondary antibodies were diluted into PBS + tween 0.05% + fat-free milk 1%.

Exosome fluorimeter analysis

To check fluorescence after NIR-AZA **1** loading, similar amounts (between 1.5 and 2.0 x 10^{10} particles/ml, according to NTA) NIR-exosomes and unlabelled exosomes were re-suspended in 200 µl of sterile PBS and analyzed with a Jasco UV-Vis-NIR fluorometer. Samples were measured in quartz microcuvettes (Perkin-Elmer, optical path length: 10 mm, chamber width: 1 mm); fluorophore was excited at λ =680 nm and fluorescence was collected between λ=690 nm and λ=900 nm.

Exosome atomic force microscopy

Exosomes and NIR-exosomes samples re-suspended in sterile PBS were diluted 1:10 in milliQ water and 7-10 µl were spotted on freshly cleaved mica substrates and let dry at room temperature in a Petri dish. Mica sheets were then analyzed with a NaioAFM (Nanosurf, Liestal, Switzerland) atomic force microscope, equipped with MultiGD-G probes (BudgetSensors, Sofia, Bulgaria) and run in dynamic mode. Scanning parameters were tuned according to instrument and probes' manufacturers. Images were processed using WSxM 5.0[3] software.

Exosome flow cytometry analysis

NIR-Exosomes and unlabelled exosomes were diluted into sterile-filtered PBS for analysis with a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lake, New Jersey, U.S). For stability studies NIR-Exosomes in PBS with 10% FBS added were incubated at 37 °C and analyzed at time points of 1h, 6h and 24h. Forward scatter threshold was set to its minimum value. EV flow rate was set on slow; illumination was provided by a standard 635 nm red laser and fluorescence was collected through a APC-Cy7-A filter. Data were processed with FACSDiva software. Downstream of acquisition, data was analysed in Summit 5.2 software. Overlays and boxplots were generated in R using pre-quantified data exported from Summit 5.2.

Live cell fluorescence microscopy

KellyCis83 cells were cultured in 8-well plates (μ-slide 8-well plates, Ibidi, Martinsried, Germany) suitable for live imaging, until 60% confluence was reached. NIR-AZA **1** was then added to each well (final concentration 5 μM) and its uptake was followed for 30 minutes on an Olympus IX73 epi-fluorescent wide field microscope fitted with an Andor iXon Ultra 888 EMCCD, using a 100x/ 1.40 oil PlanApo objective (Olympus Corporation, Shinjuku, Tokyo, Japan) controlled by MetaMorph (v7.8). Fluorescence illumination was provided by a Lumencor Spectra X light engine containing a solid state light source, and a 640 nm excitation filter. NIR fluorescence emission was collected using a 705 nm emission filter. Images in the NIR channel were then acquired using 75 ms exposure, 1000 x gain, and 60% laser power.

Microvesicle labelling with NIR-AZA 1.

KellyCis83 cells were grown in complete RPMI 1640 medium until 70-80% confluence was reached, and washed three times with sterile PBS. Cells were incubated for 2 h with 10 ml of staining medium (RPMI complete medium added with NIR-AZA **1**, 5 μM) and then rinsed three times with sterile PBS. Finally, 10 ml of serum free medium was added to each flask and microvesicles were purified following 24 h incubation at 37 °C, 5% CO_2.

Unlabelled microvesicle isolation

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Microvesicle analysis by flow cytometry

NIR-microvesicles and unlabeled microvesicles were diluted into sterile-filtered PBS or with 10% FBS for up to 24 h at 37 °C and analyzed with a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lake, New Jersey, U.S). Forward scatter threshold was set to its minimum value. EV flow rate was set on slow; illumination was provided by a standard 635 nm red laser and fluorescence was collected through a APC-Cy7-A filter. Data were processed with FACSDiva software. Downstream of acquisition, data was analysed in Summit 5.2 software. Overlays and boxplots were generated in R using pre-quantified data exported from Summit 5.2.

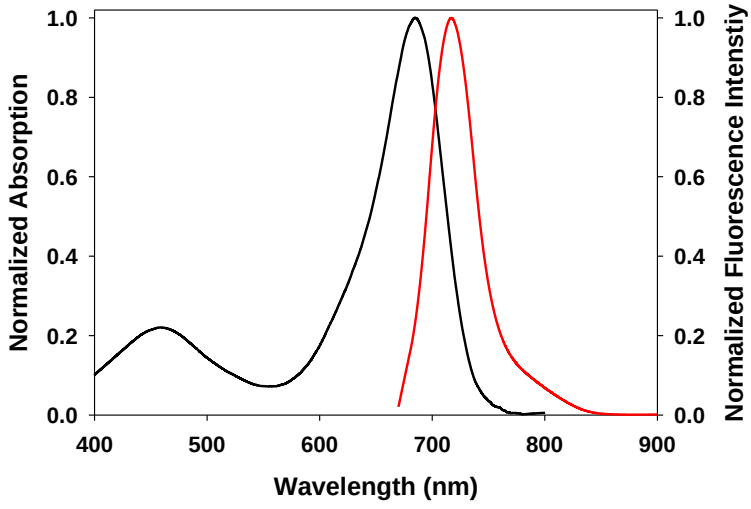

Fig. S1. Absorption and emission spectra of NIR-AZA **1** (5 µM) in MeOH.

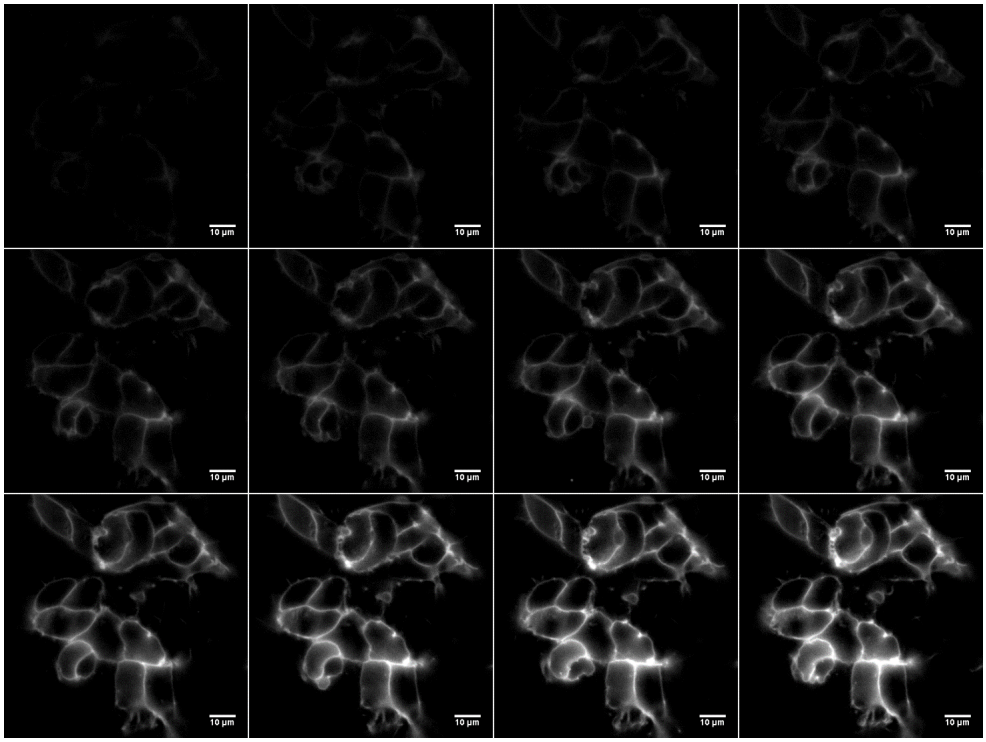

Fig. S2 Z-Stack images of Fig. 2B (fluorescence shown in white for clarity).

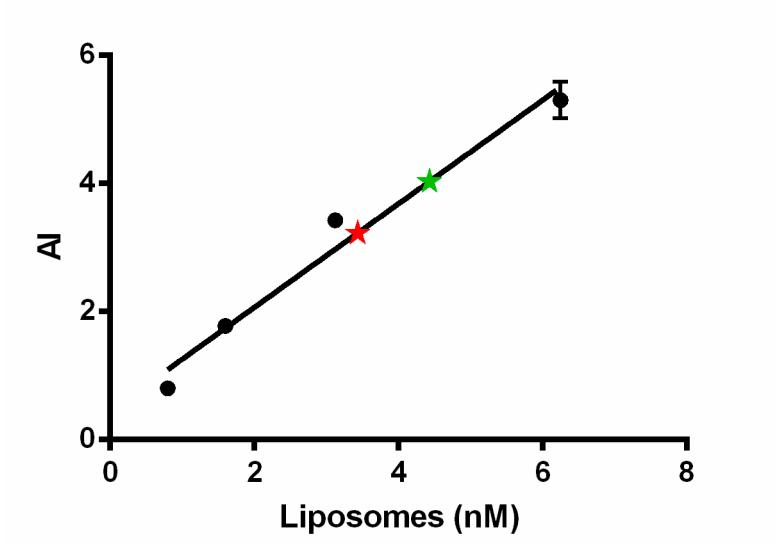

Fig. S3A Calibration line obtained by plotting the NP Aggregation Index calculated from the standards of liposomes at known concentration (black dots) and linear regression fit (black line, R^2 = 0.974). Errors bars indicate standard error of three different replicates. The star points highlight the intercept of the AI value of the exosomes (red star) and NIR-exosomes (green star) samples with the regression line. The point projection on the Y-axis allows for the extrapolation of the unknown concentrations, which resulted consistent (within the experimental uncertainty of CONAN, about 20%), being equal to 3.4 nM for exosomes and 4.4 nM for NIR-exosomes, respectively.

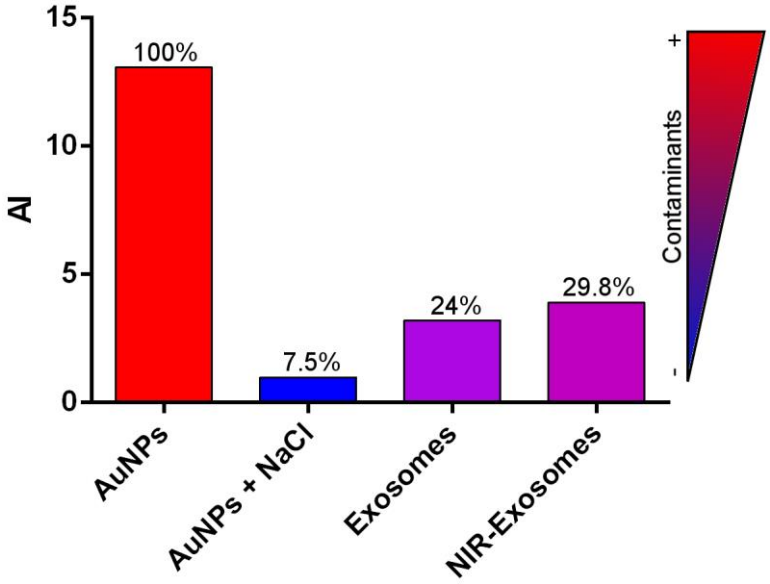

Fig. S3B Purity analysis of exosome samples performed through CONAN assay. The Aggregation Index (AI) of exosome (purple column) and NIR-exosome (violet column) samples are compared with the AI of pure, monodisperse AuNPs (red column) and reported as percentages of it.

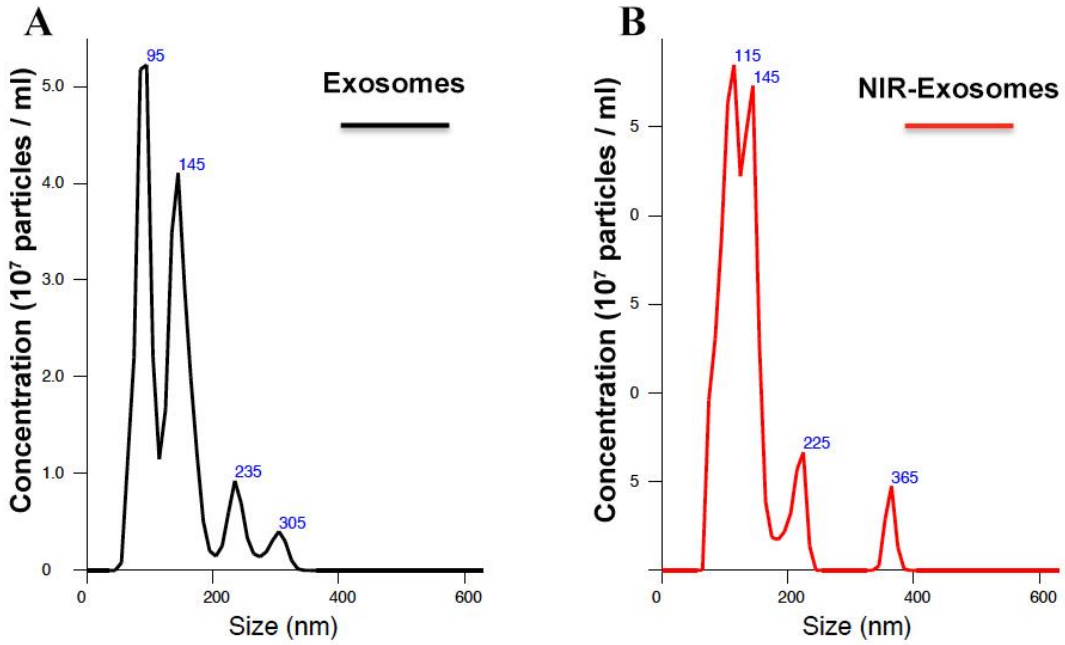

Fig. S4. Additional independent experimental NTA data of (A) Exosomes and (B) NIR-exosomes.

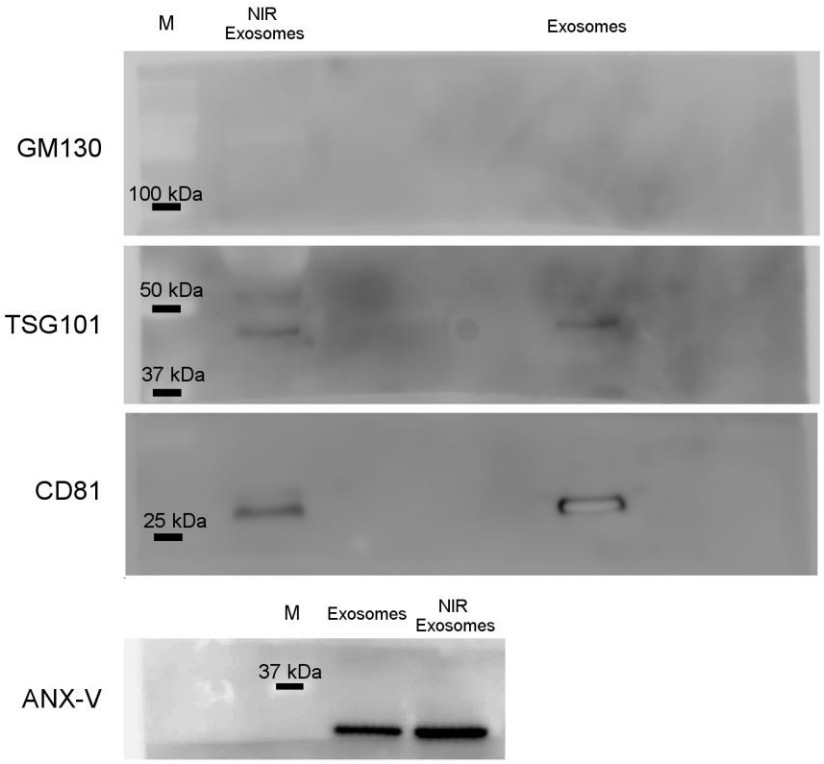

Fig. S5 Western blot membranes for exosomes and NIR-exosomes.

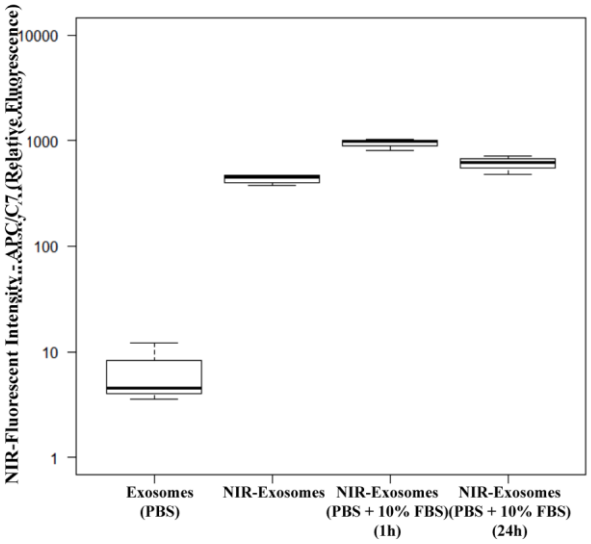

Fig. S6. Exosome and NIR-Exosome characterisation by flow cytometry A) Median florescent intensity analysis of the exosomes with flow cytometry Unlabeled exosomes were used to calibrate background autofluorescence and have a low median fluorescent intensity. The NIR-exosome fluorescent intensity was dramatically increased. Notably the fluorescent intensity remained constant after the exposure to 10% FBS for up to 24 h of exposure at 37°C. Error bars represent standard deviation from the mean value estimated from at least three individual measurements.

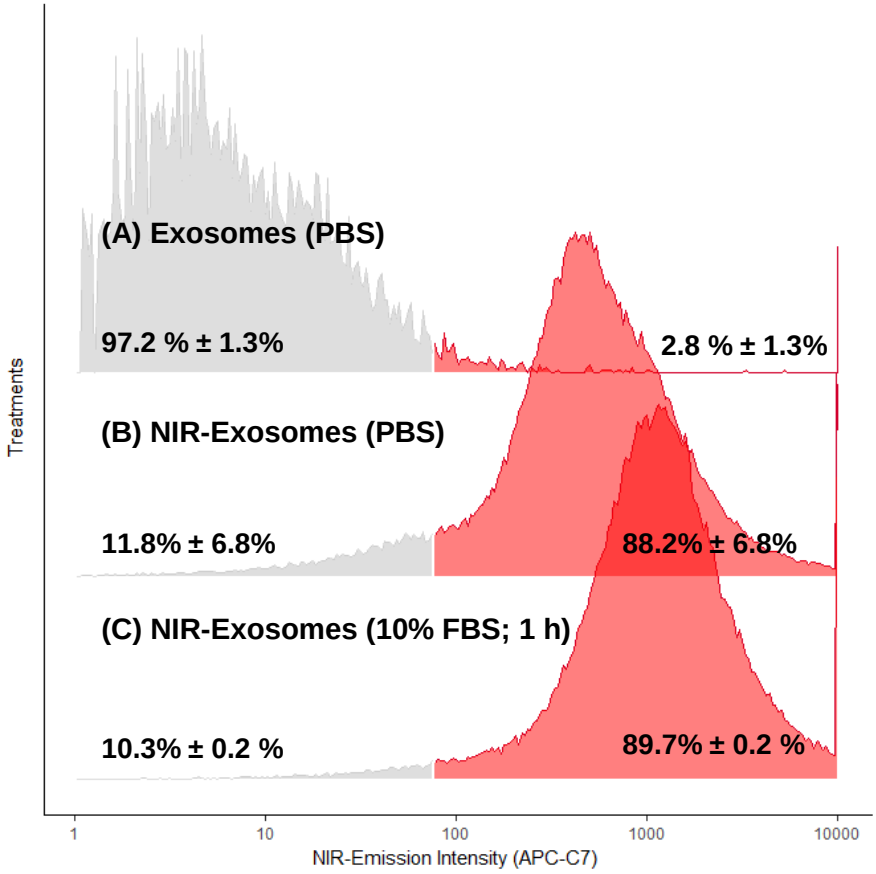

Fig. S7. Quantification of NIR-labelled exosomes determined from flow cytometry data for a constant number of exosomes and NIR-exosomes. A post-analysis gate region (horizontal line) was set in A-C as threshold for fluorescence labelling.
(A) Unlabelled exosomes.
(B) A comparison between the exosomes and NIR fluorescence intensity showing that the NIR-exosomes have a greater fluorescent intensity than the unlabelled exosomes. A population of 11.8 % +/- 6.8% (n=3) of the labelled exosomes have fluorescent intensity indistinguishable from unlabelled exosomes, indicating that 88.2 +/- 6.8% of all exosomes are labelled.
(C) Analysis showed 89.7 +/-0.2% labelled post incubation for 1 h at 37 °C in PBS/10% FBS.
Data shown is a representative individual run and values are an average of a triplicate of experiments. (The same post analysis gate for fluorescence was applied Fig. S8 panels D-I below).

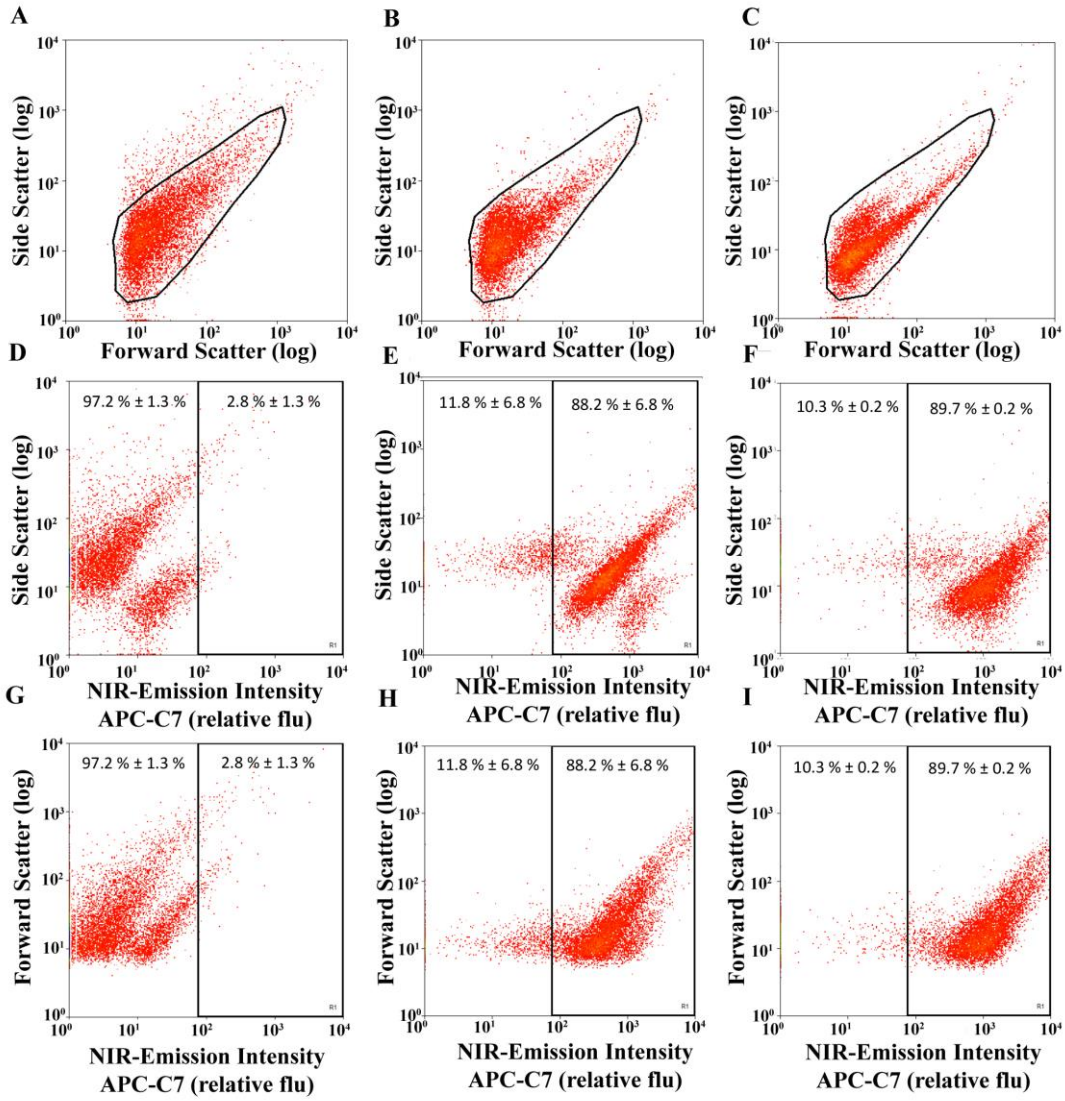

Fig. S8. Flow cytometry characterisation of exosome labelling.

(A) FSC/SSC unlabelled exosomes, (B) FSC/SSC NIR-exosomes, (C) FSC/SSC NIR-exosomes incubated for 1 h at 37 °C in PBS/10% FBS. Post-analysis gated region (black outline in A-C) shown for comparison.

(D) Side Scatter (SSC)/Fluorescent Intensity (FI) of unlabelled exosomes, (E) SSC/FI of NIR-exosomes, (F) SSC/FI of NIR-exosomes incubated for 1 h at 37 °C in PBS/10% FBS. A post-analysis gate region (horizontal line) was set in D-I as threshold for fluorescence labelling. (The same post analysis gate for fluorescence was applied throughout the manuscript). Data shown is a representative individual run and values are an average of a triplicate of experiments.

(G) Forward Scatter (FSC)/FI unlabelled exosomes, (H) FSC/FI NIR-exosomes, (I) FSC/FI NIR-exosomes incubated for 1 h at 37 °C in PBS/10% FBS. A post-analysis gate region (horizontal line) was set in G-I as threshold for fluorescence labelling. The same post analysis gate for fluorescence was applied throughout the manuscript. Data shown is a representative individual run and values are an average of a triplicate of experiments.

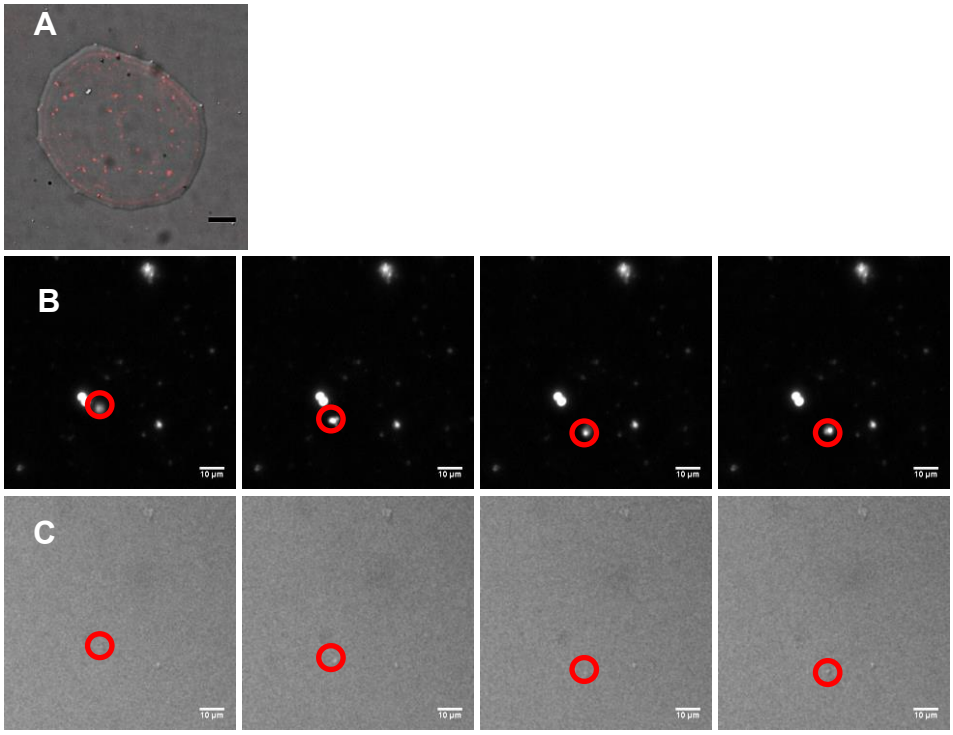

Fig. S9. (A) Overlay of differential interference contrast (DIC) and fluorescence (red colour) images of PBS sample containing NIR-exosomes as shown in Fig 6B. (B) Time course of images of an expanded region of the sample showing (B) fluorescent exosome and corresponding DIC images allowing the visualization of some exosomes and their positional correlation with the images in (B). Scale bars 10 µM.

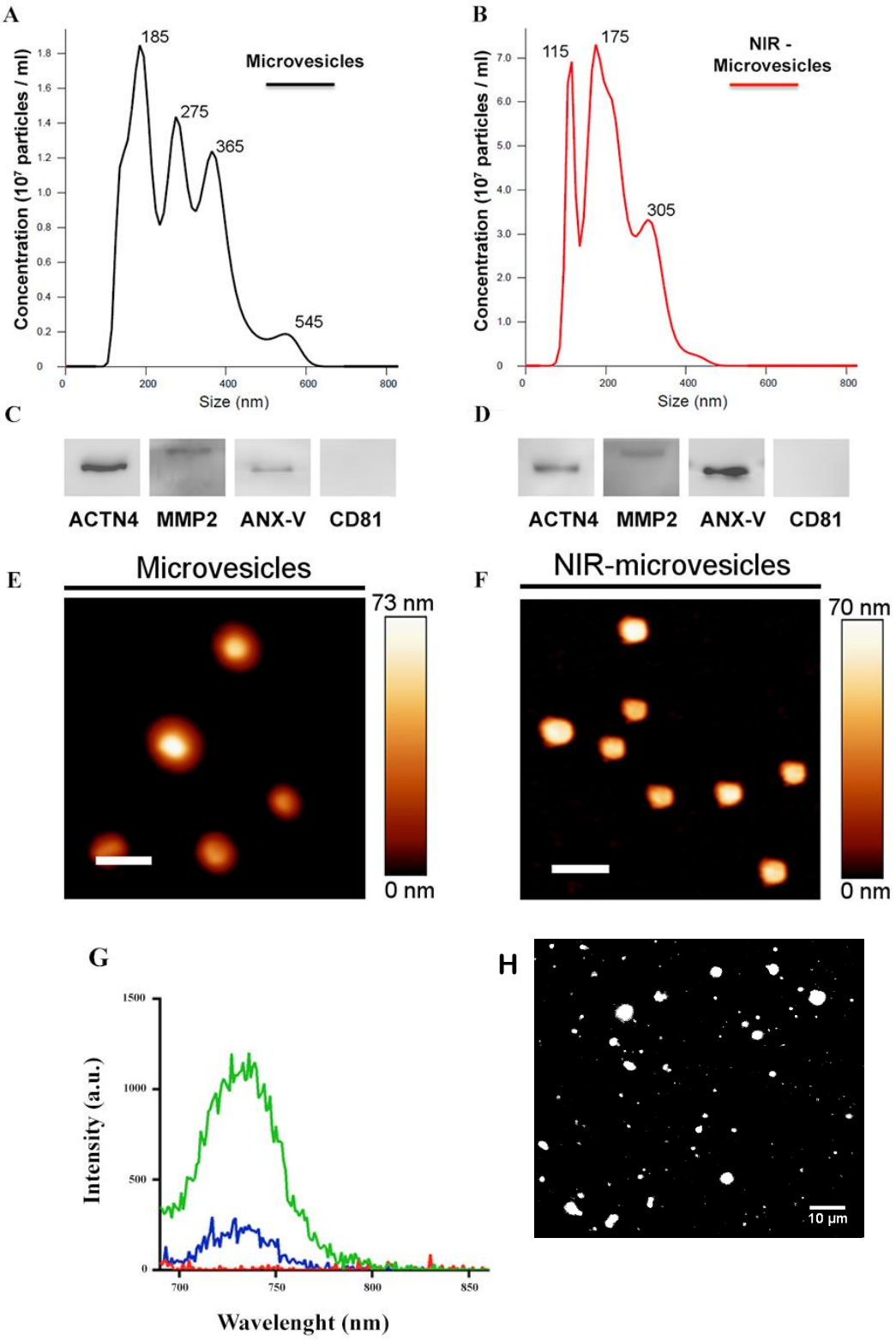

Fig. S10. Physiochemical and biochemical characteristics of microvesicles and NIR-microvesicles. (A,B) NTA data for exosomes (grey trace) and NIR-microvesicles (red trace). (C, D) Western blots of microvesicles and NIR- microvesicles. (E, F) AFM image of microvesicles and NIR- microvesicles. (G) Emission spectra of NIR-NIR-microvesicles (green trace), microvesicles + 5mM of NIR AZA **1** (blue trace), unlabelled microvesicles (red). (H) Widefield microscopy imaging of NIR-microvesicles.

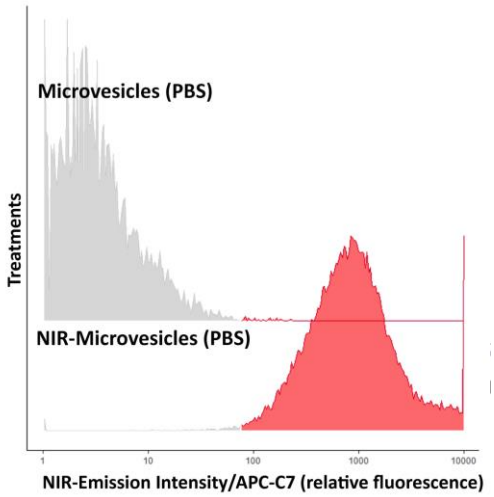

Fig. S11. Flow Cytometry data for a constant number of unlabelled microvesicles and NIR-microvesicles in PBS.

ESI Movie Legends

Movie S1. Live cell imaging of KellyCis83 cells shown in Fig. 2B.

Movie S2. Continual imaging of exosomes as shown in Fig. 6B for 1 min.

References

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