## Supporting Information

## Nanoscale Flow Cytometry for Immunophenotyping and Quantitating Extracellular

## Vesicles in Blood Plasma.

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**Figure S1:** To ensure the absence of autofluorescence, noise, non-specific antibody signal and bleedthrough into other channels the following controls were carried out on the nanoscale flow cytometer: PBS alone, plasma alone and single antibody tests were ran independently of one another. The signal in all detectors of interest were assessed. A compensation value of 3 was applied to the PE channel to counteract bleedthrough into the FITC channel. Controls were carried out on three independent plasma samples. Representative images are shown.



**Figure S2:** Further controls were carried out: (A) antibodies only, (B) isotype controls, (C) and all the antibodies together. (D) To ensure the correct concentration of CD9, CD41 and mammaglobin-a antibodies were being used to acheive saturated and specific signal, the antibody concentration was titrated. (E) The antibody concentration at which the signal begun to decrease was used to label decreasing volumes of plasma to ensure the antibody signal was specific; detected signal decreased by approximatley half upon plasma titration.



**Figure S3:** Graphs in column 1 show the reproducibility between two different experiments using 219 breast cancer plasma samples (run 1 and run 2), and the specificity (isotype control) of nanaoscale flow cytometry for high throughput analysis of breast cancer plasma sample analysis of mammaglobin-a (A), CD9 (B) and CD41 (C) levels. CD9 and mammaglobin-a dual positive EV population reproducibility and specificity is also demonstrated (D & E). Graphs in column 2 show the linear regression analysis for correlation of the values obtained during run1 and run2, the r<sup>2</sup> value is shown as a readout of correlation where a 1.0 would represent a perfect and strong correlation.



**Figure S4:** Two plasma samples were subjected to size exclusion chromatography and the EV fraction was collected followed by fractions (F) 2, 3 and 4. 20 ug of each fraction, and 1.2  $\mu$ g of recombinant mammaglobin-a protein was loaded onto bis/tris gels and Western blotted for EV marker CD63, EV contaminant protein albumin, platelet derived EV marker CD41 and breast cancer marker mammaglobin-a.



cytometry. n=2.



Violet SSC-H Violet SSC-H **Figure S6:** Platelet and mammaglobin-a levels pre- and post-platelet removal. Representative flow plots of plasma before 2,800 x g spin, after 2,800 x g spin, and the resuspended pellet from the 2,800 x g spin.



**Figure S7:** (A) Breast cancer patient plasma samples were labeled for CD9, and the number of platelets in each sample was quantified and associated with the blood draw volume information from the biobank. (B) Decreasing volumes of whole blood, starting from 10 ml, were spun at 2500 x g for 15 minutes. Resultant plamsa was labelled for EVs using CD9 and the number of platelets in the sample analyzed. (C) 10 ml of plasma was centrifuged at different speeds and times. The number of platelets in the resultant plasma was analysed by labelling the plasma EVs with CD9. Outlined in red are the most common plasma isolation protocols used by our biobanks. 1 whole blood bag was used for this study (B & C) and two technical repeats were done.



**Figure S8:** Flow cytometry analysis of mammaglobin-a and CD9 positive platelets in 50 healthy, 56 benign and 219 breast cancer plasma samples. The average of two independent runs was taken for analysis. Representative images of the mammaglobin-a and CD9 positive platelet populations detected by nanoscale flow cytometry.



**Figure S9:** Mammaglobin-a positive platelets in whole plasma samples and platelet isolated samples. (A) Nanoscale flow cytometry based quantification of mammaglobin-a positive platelets in plasma and in resuspended pellets from post 2800xg plasma centrifugation. (B) 20  $\mu$ g of platelet pellet was Western blotted for CD41 and mammaglobin-a alongside 1.2  $\mu$ g recombinant mammaglobin-a protein as a positive control.

| Antibody         | Catalogue number | Company                     | Concentration       |
|------------------|------------------|-----------------------------|---------------------|
| CD9 CF405M       | ab123624         | Abcam                       | Flow 4 µg/sample    |
| CD41 PE          | ab134372         | Abcam                       | Flow 1 µg/sample    |
| CD63 FITC        | Ab18235          | Abcam                       | Flow 1.2 µg/sample  |
| CD42a FITC       | 11-0428-42       | Thermo Fisher<br>Scientific | Flow 0.89 µg/sample |
| Mammaglobin-a    | DCABH-           | Creative diagnostics        | Flow 20 ng/sample   |
| 647              | L4031AF647       |                             |                     |
| Isotype CD9:     | ab126036         | Abcam                       | Flow 4 µg/sample    |
| mouse IgG2a      |                  |                             |                     |
| CF405M           |                  |                             |                     |
| Isotype          | 34528            | Cell Signalling             | 20 ng/sample        |
| mammaglobin-a:   |                  |                             |                     |
| Rabbit IgG 647   |                  |                             |                     |
| Isotype CD41:    | ab91357          | Abcam                       | Flow 1 µg/sample    |
| mouse IgG1 PE    |                  |                             |                     |
| Isotype CD42a:   |                  | Thermo Fisher               | 0.89 µg/sample      |
| mouse IgG1 FITC  | 11-4714-42       | Scientific                  |                     |
| CD63             | ab59479          | Abcam                       | Western 1:1000      |
| Mammaglobin-a    | TA313104         | Origene                     | Western 1:500       |
| CD41             | ab83961          | Abcam                       | Western 1:1000      |
| Human albumin    | ab10241          | Abcam                       | Western 1:2000      |
| Secondary anti   | 926-68070        | LI-COR                      | Western: 1:10000    |
| mouse IgG IRdye® |                  |                             |                     |
| 680RD/800CW      |                  |                             |                     |

## Table S1: Antibody product information and concentrations used in the study.

All antibody concentrations were established using nanodrop quantification.