

Electronic Supplementary Information (ESI)

Sensing protein antigen and microvesicle analytes using high-capacity biopolymer nano-carriers

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Supplementary Methods

Materials

His-tagged human fascin (cf. ²⁸) was purchased from Novus Biologicals, Novus Europe, UK. Anti-human CD45 monoclonal antibody (Clone: T29/33, α -CD45) was purchased from Universal Biologicals LTD, Cambridge, UK. Anti-rabbit IgG (H&L, Goat, α -rIgG) was purchased from Rockland Immunochemicals, Gilbertsville, USA. Tetramethylrhodamine-isothiocyanate (TRITC) labeled goat anti-mouse IgG (2–8 fluorophores/IgG), was purchased from Invitrogen, Stockholm, Sweden. Rabbit IgG (rIgG) and all other chemicals were of analytical grade and, unless otherwise stated, purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden. Rabbit IgG was labeled with Rhodamine (Rh) as described previously.¹⁴ Actin monomers (G-actin) were dissolved in G-actin buffer (2 mM Tris base, pH 8.5, 0.2 mM Na₂ATP, 0.5 mM DTT, 0.2 mM CaCl₂, 3 mM NaN₃). A stock solution was prepared of conjugation catalyst buffer (100 mM aniline in conjugation buffer (100 mM (2-(*N*-morpholino)ethanesulfonic acid (MES), 150 mM KCl, 5 mM MgCl₂, 1 mM Na₂ATP, pH 6.0)).

Protein preparations

Myosin II was purified from rabbit fast skeletal muscle and heavy meromyosin (HMM) was prepared by digestion of myosin using α -chymotrypsin.^{14,61,62} Actin was prepared from rabbit skeletal muscle⁶² and monomeric actin was polymerized into actin filaments (F-actin) as described previously. F-actin was fluorescently labeled using Alexa Fluor[®]-488 phalloidin (APh) or Tetramethylrhodaminisothiocyanate-phalloidin (RhPh) (molar ratio: 1:1.5; actin:APh or actin:RhPh). The phalloidin derivatives were obtained from Molecular Probes Invitrogen (Eugene, OR). All experiments using animal material were performed in accordance with national and EU-legislation and were approved by the Regional Ethical Committee for Animal experiments (reference # 96-11), Linköping, Sweden.

Preparation of flow cells

Flow cells were constructed from trimethylchlorosilane (TMCS) derivatized coverslips (motor binding substrate) and non-derivatized glass cover slips as described previously.¹⁴ In brief, glass cover slips (No. 0, 24 x 24 mm, Menzel-Glaser, Braunschweig, Germany) were cleaned with

piranha solution (70% H₂SO₄ and 30% H₂O₂; *Caution! Piranha solution is a highly corrosive acidic solution that can react violently with organic materials. Do not store in a closed container, and take appropriate safety precautions*) at 80 °C for 5 min and washed sequentially with deionized water (three times), methanol, dry acetone and dry chloroform (2 min each). The slide was then dried (in N₂ gas stream) and dipped in a freshly prepared TMCS solution (5% TMCS in dry chloroform) for 2 min and washed twice with dry chloroform. *Caution: TMCS is highly flammable and reacts violently with water!* TMCS derivatized substrate were kept in deionized water for more than 30 min before they were dried and assembled into flow cells by using double-sided adhesive tape as spacers.¹⁴ Successful TMCS derivatization was verified on basis of advancing and receding contact angles for water droplets.¹⁴

Antibodies conjugated to F-actin

F-actin, conjugated with anti-human CD45 monoclonal antibody (a-CD45) or a-rIgG was prepared using heterobifunctional cross-linkers.¹⁴ Briefly, the C6-succinimidyl 6-hydrazinonicotinate acetone hydrazone (C6-SANH) modified F-actin was incubated with C6-succinimidyl 4-formylbenzoate (C6-SFB) modified antibodies for 6 h at room temperature (21-23 °C) using 2:1, G-actin:a-rIgG or a-CD45 molar ratio. Moreover, 10 mM catalyst buffer was added from stock solution¹⁴ and later, after stipulated time, removed by dialysis at 4 °C against G-actin buffer. Samples were flash frozen in liquid nitrogen and stored at -80 °C.

The conjugated G-actin monomers (actin-a-CD45 or actin-a-rIgG) were co-polymerized with non-conjugated monomers to form filaments with 1:3 molar ratio of conjugated to non-conjugated actin. The co-polymerization reaction was performed at 4 °C (3 h) by addition of KCl, MgCl₂ and ATP to final concentrations of 100 mM, 2 mM and 3.3 mM, respectively. The co-polymer was fluorescence-labeled with APh or RhPh (molar ratio: 1:1.5; actin:APh or RhPh) at 4 °C, overnight (see above).

In vitro motility assays and characterization of antibody-labeled fascin-actin bundles

In vitro motility assays were performed according to standard principles.^{14,29,61} All solutions were based on buffer A: 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 50 mM KCl, 1 mM MgCl₂ and 0.1 mM K₂-ethylene glycol tetraacetic acid (EGTA), pH 7.4. The flow cells were sequentially incubated with (i) HMM (120 µg mL⁻¹) for 5 min, (ii) 1 mg mL⁻¹ BSA for 5 min,

(iii) wash with buffer B (10 mM MOPS, 45 mM KCl, 1 mM MgCl₂, 0.1 mM K₂EGTA, pH 7.4), (iv) F-actin (10-15 nM) or fascin-arIgG-(or-aCD45)-actin bundles (60 nM) labeled with RhPh or APh in buffer B (10 mM MOPS, 45 mM KCl, 1 mM MgCl₂, pH 7.0) supplemented with an anti-bleach system (10 mM dithiothreitol (DTT), 3 mg mL⁻¹ glucose, 20 units mL⁻¹ glucose oxidase and 460 units mL⁻¹ catalase). Then, we added antigen, either with Rh-rIgG for 1 min, or with DND41 MVs or MG63 MVs for 5 min. The latter had been labeled with CFSE for 20 min. Flow cells were then washed with buffer B with anti-bleach mixture, and (vi) incubated with assay solution (1 mM Mg-ATP in buffer B with anti-bleach system). The sliding of F-actin or fascin-actin bundles with or without a-rIgG or aCD45 was imaged using an epi-fluorescence microscope (Eclipse TE300, Nikon, Tokyo, Japan) equipped with a temperature regulated (100x, 1.4 NA) oil immersion objective and TRITC (Ex. 540/25, DM 565, BA 605/25) as well as FITC (Ex. 465–495, DM 505, and BA 515–555) filter sets. Image sequences were recorded using an EMCCD camera (C9100; Hamamatsu Photonics, Hamamatsu, Japan). In some experiments we instead used an Axio Observer DI inverted microscope, with 1.4 N.A. 63× planapochromat objective (Zeiss, Jena, Germany), similar filter sets as for the Nikon microscope but a cooled CCD camera (C4742-95, Orca-ER, Hamamatsu Photonics).

The filament sliding velocity was measured as described previously.⁶³ The coefficient of variation (CV) of the frame-to-frame velocity was similar at all Rh-rIgG incubation concentrations except 10 nM. The CV values (mean ± SEM) were: 0.18 ± 0.10 (N=27); 0.22 ± 0.12 (N=31), 0.23 ± 0.12 (N=30) at 0, 0.1 and 1 nM, respectively and 0.30 ± 0.09 (N=10) at 10 nM Rh-rIgG.

For fluorescence microscopy based characterization of antibody content of fascin-actin bundles, the flow cells were pre-incubated as for in vitro motility assay: (i) HMM (120 μg mL⁻¹) for 5 min, (ii) 1 mg mL⁻¹ BSA for 5 min, (iii) wash with buffer B (10 mM MOPS, 50 mM KCl, 1 mM MgCl₂, 0.1 mM K₂EGTA at pH 7.0). Next, however, the the following procedure followed: (iv) fascin-a-rIgG-actin bundles (60 nM) labeled with APh in buffer C (10 mM MOPS, 45 mM KCl, 1 mM MgCl₂ at pH 7.0 with an anti-bleach system), (v) wash with buffer C, (vi) 0, 0.1, 1 and 10 nM rhodamine labeled rabbit IgG in buffer D for 1 min, and (vii) wash with buffer C. The image sequences were recorded using an EMCCD camera as described above. The number of

fluorescent antigens per bundle and the antibody/actin subunit ratio was calculated from the corrected (Fig. S9) ratio of the background subtracted Rh-rIgG and APh fluorescence intensities.

CD45 expression: screening of cell lines and MVs isolation

Screening of CD45 expression in B and T leukemic cell lines was performed by flow cytometry analyses (Fig. S5). DND41 cells were selected as positive control with 100% of CD45 (all cells were positive for CD45) expression. The MG63 osteosarcoma cell line was chosen as negative control; lack of CD45 expression was confirmed by FACS analysis (Fig. S6).

MVs isolation and preservation in MOPS buffer

MVs were isolated by centrifugation from both DND41 and MG63 cell medium, stained with CFSE and examined by FACS to assure vesicles membrane integrity (only vesicles with an intact membrane will retain CFSE). Isolated MVs were then labeled with CD45 antibody and CD45 expression was detected by cytometry, confirming high CD45 expression for MVs released from DND41 cells and lack of CD45 expression for MVs of MG63 cells, as expected (Fig. S7-S8, CD45 signal reported in gate P6). For MVs FACS analyses, 3 μ m beads were used as size reference (Fig. S7-S8, gate P3). CFSE+ events were detected (P5) after gating on MVs morphological parameters (P2).

MVs were re-suspended in a MOPS based buffer (buffer A) and vesicle membrane integrity was (re)checked by CFSE staining and flow cytometry analysis. PBS buffer is not ideal in a device because high concentration of inorganic phosphate appreciably increases ionic strength and affects ATP turnover of biomolecular motors. Therefore, MVs were re-suspended in MOPS buffer, suitable in motility assays, and MVs preservation in MOPS buffer has been confirmed after 2 hours (Fig. S10).

Optimization of CFSE staining

For MVs detection in the fluorescence microscope CFSE staining was optimized. To detect MVs by flow cytometry, a final concentration of 1 μ M CFSE was used. This concentration did not allow a clear detection in fluorescence microscopy. The CFSE concentration was therefore

increased to 10 μ M to better discriminate MVs from the background; moreover, another washing/centrifugation step was added to decrease the CFSE background signal (Fig. S11).

MVs maintenance in PBS buffer

In order to use CFSE+ MVs to perform experiments some days after their isolation, we checked MVs conservation during time. CFSE+ DND41 MVs were stored in PBS 1X at 4 °C. Their number and integrity were checked at several points by FACS analyses and a good maintenance was detected as reported in Fig. S12.

Supplementary Figures

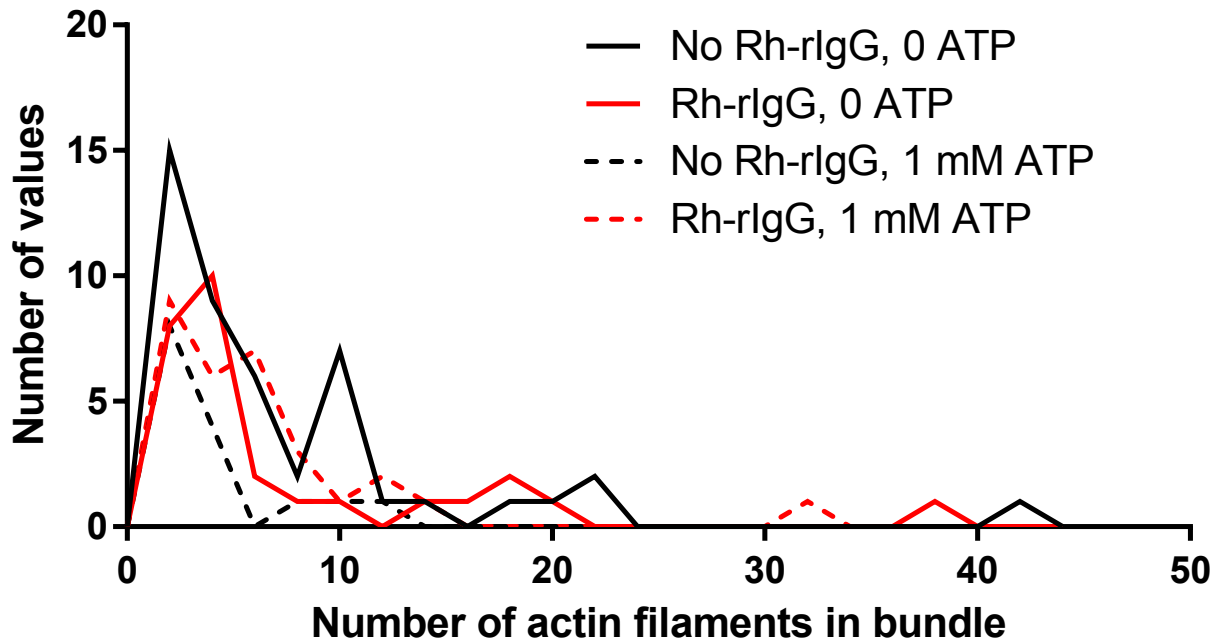


Fig. S1 Size distributions for fascin-actin bundles formed from antibody-conjugated actin filaments. Data shown both before ATP addition (full lines) and 5 min after ATP-addition (dashed lines).

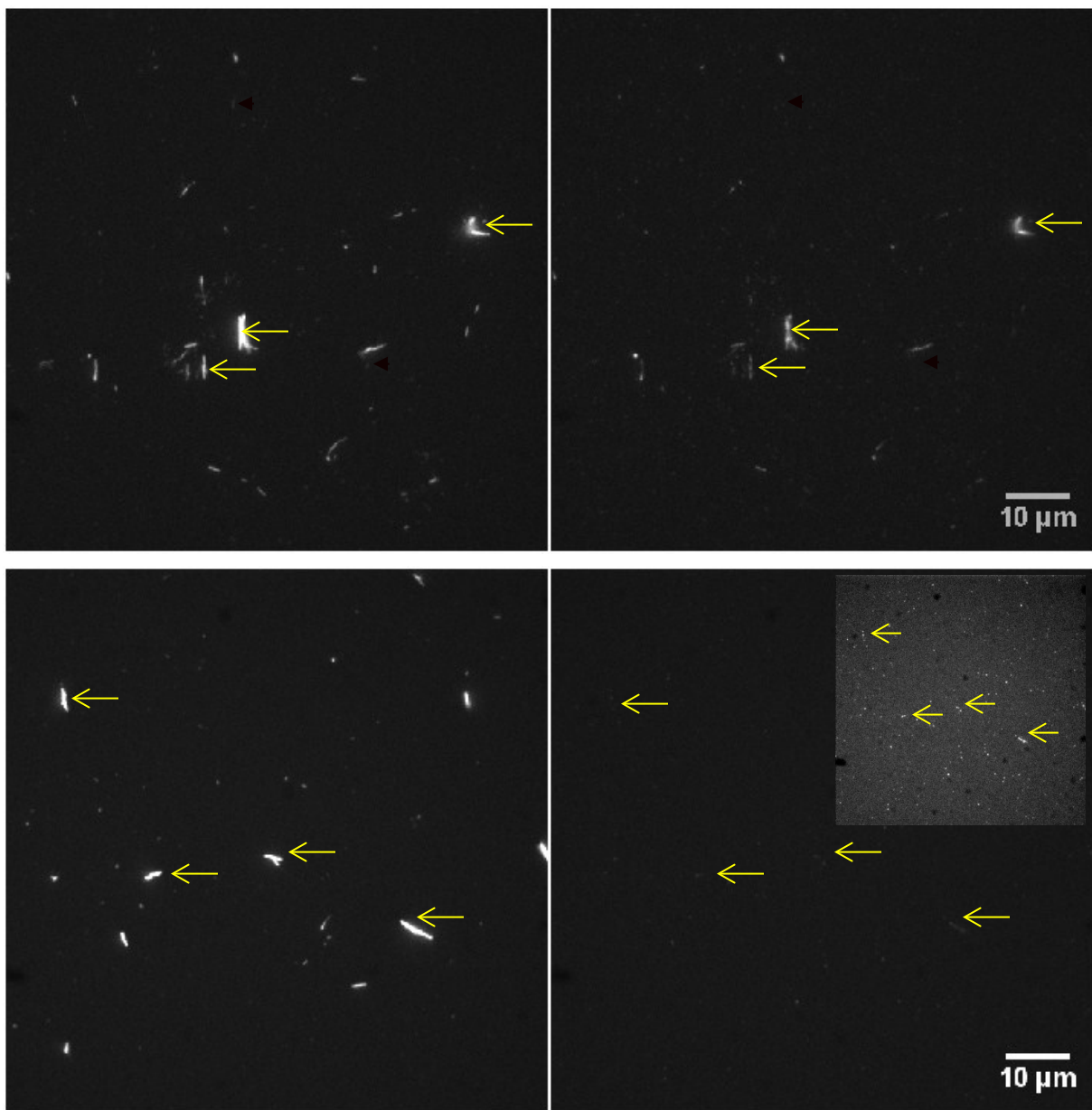


Fig. S2 Image frames from which main Fig. 2a-d was derived. For further information, see legend of main Fig. 2.

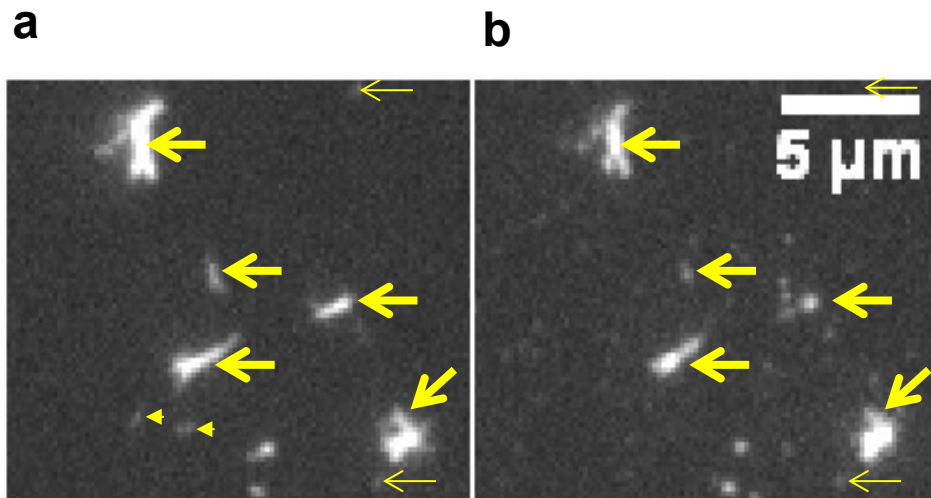


Fig. S3 Fascin actin-APh-labeled bundles and single APh-labeled actin filaments after incubation with 10 nM Rh-rIgG and 1 mM MgATP. (a) APh labeling observed using a FITC filter set in epi-fluorescence microscope. (b) Same region as in (a) a few seconds later after switch to TRITC filter set for observation of Rh-rIgG antigens captured by a-rIgG antibodies. Full length bold and thin arrows indicate non-motile bundles and single filaments, respectively. Arrow-heads indicate motile single actin filaments. Similar exposure time and gain in (a) and (b) (exposure time 0.1 s). Both images histogram stretched to the same extent to optimize brightness and contrast. The filaments and bundles are bound to a TMCS-surface coated with HMM in the presence of 1 mM MgATP. Note that all (stationary) bundles are branched to different degrees, indicating non-polar actin cross-linking presumably via a-rIgG-Rh-rIgG- a-rIgG links.

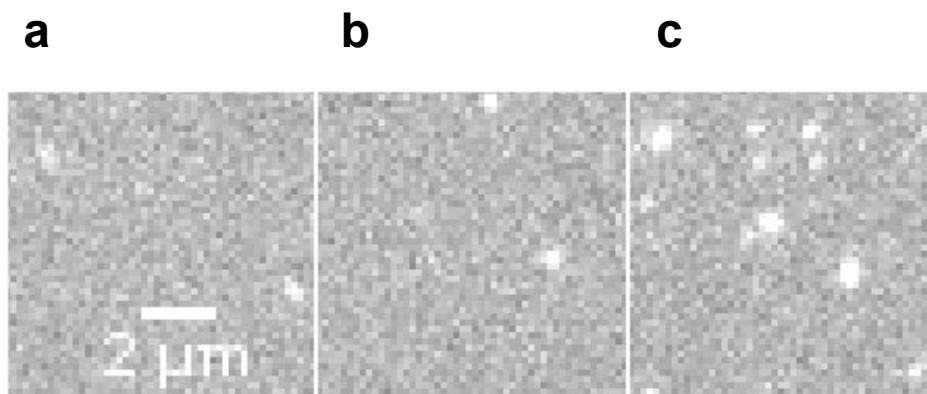


Fig. S4 Fluorescence micrographs obtained using TRITC filter sets of representative areas of a HMM coated surface without actin filaments or fascin-actin bundles in the field of view. (a) Before incubation with Rh-rIgG and MgATP. (b) After incubation for 1 min with Rh-rIgG at 1 nM and after subsequent addition of 1 mM MgATP (assay solution). (c) After incubation for 1 min with Rh-rIgG at 10 nM and after subsequent addition of 1 mM MgATP (assay solution). Fluorescent spots, observed before Rh-rIgG incubation, are of unknown origin but are observed also on pure glass cover slips. Note increased non-specific Rh-rIgG adsorption with increased Rh-rIgG incubation concentration. All images histogram-stretched to the same degree to optimize brightness and contrast.

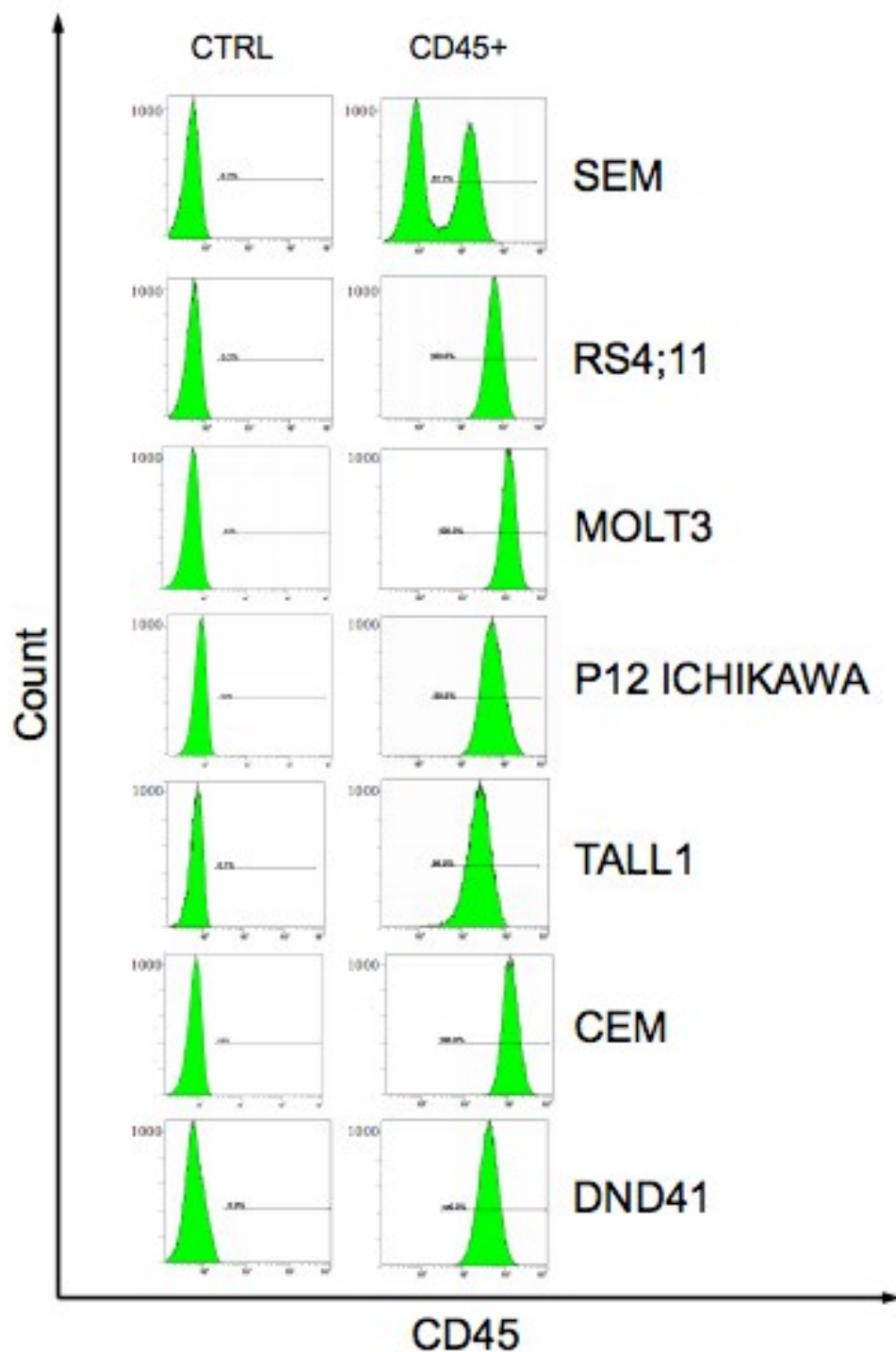


Fig. S5 Screening of CD45 expression in B and T leukemic cell lines (labels to the right). For each cell line, the CD45 signal (horizontal axis; Texas-Red fluorescence intensity proportional to number of anti-CD45 antibodies) was detected by flow cytometry analysis, for negative controls and CD45+ stained cells.

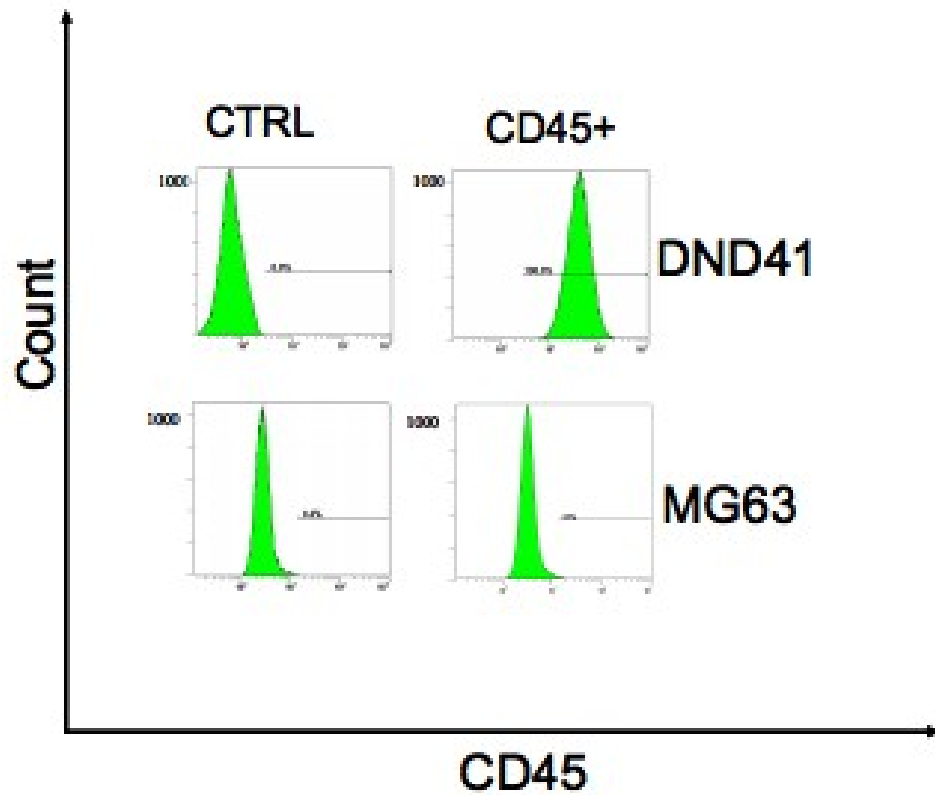
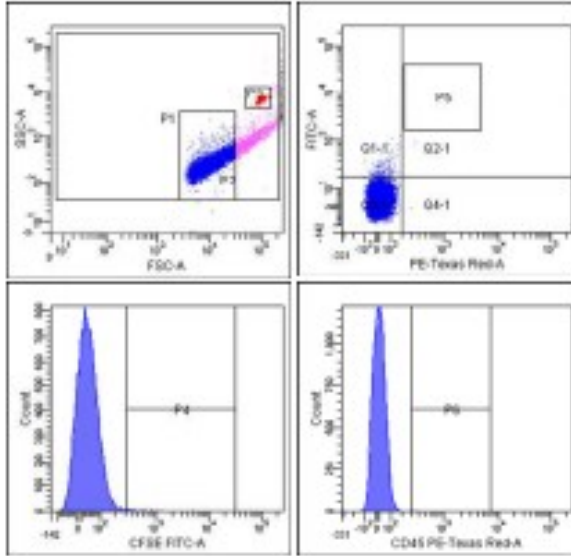


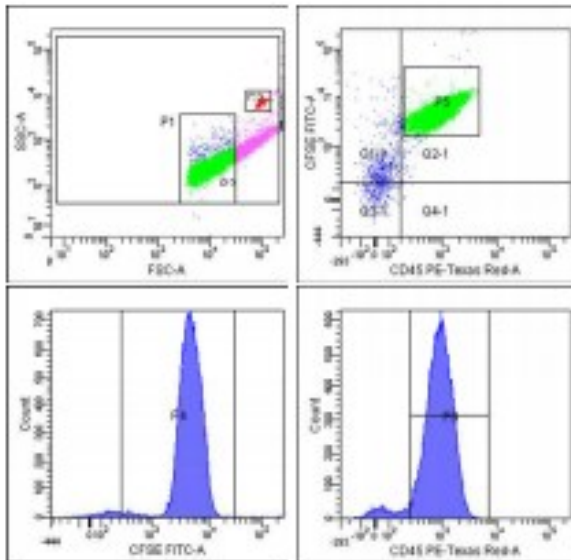
Fig. S6 Flow cytometry CD45+ signal detection in selected *in vitro* models. DND41 cell line has been selected as positive control (100% of CD45 expression) and MG63 cell line has been chosen as negative control (0% of CD45 expression). The plots show the number of microvesicles vs the fluorescence intensity attributed to anti-CD45 antibodies.

a



DND41 MVs control		
Population	Events N°	Total %
All events	36669	100.0
P1	36189	98.7
P2	20900	57.0
P3	10000	27.3
P4	33	0.1
P5	0	0.0
P6	2	0.0
Q1-1	935	2.5
Q2-1	44	0.1
Q3-1	35206	96.0
Q4-1	4	0.0

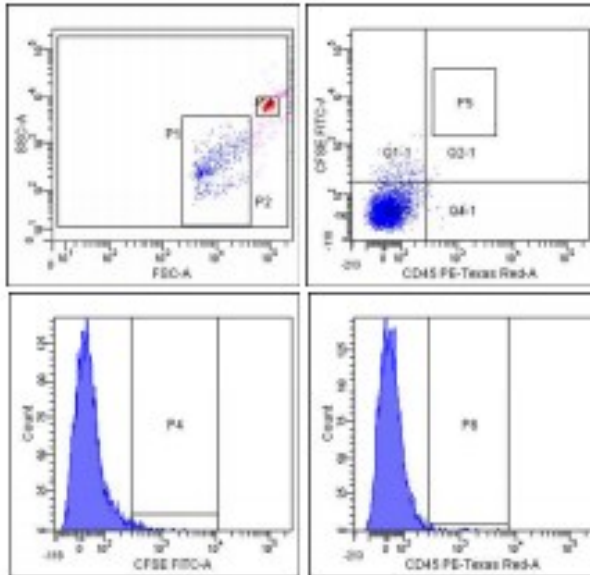
b



CFSE+ CD45+ DND41 MVs		
Population	Events N°	Total %
All events	38869	100.0
P1	38063	97.9
P2	21311	54.8
P3	10000	25.7
P4	27109	69.7
P5	20313	52.3
P6	20419	52.5
Q1-1	2892	7.4
Q2-1	27111	69.7
Q3-1	7979	20.5
Q4-1	81	0.2

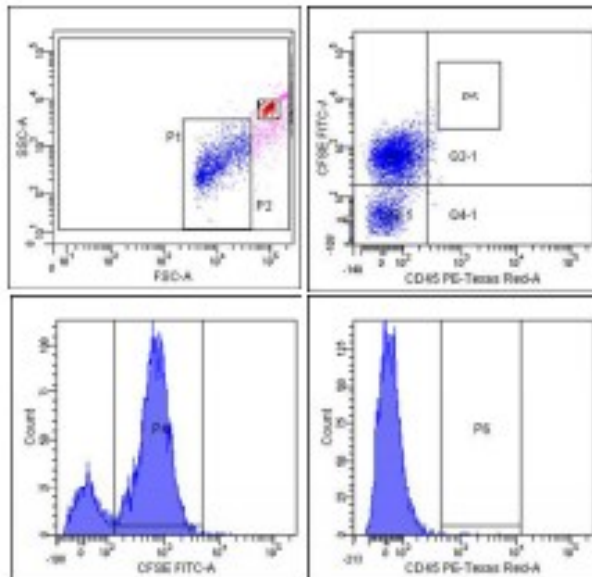
Fig. S7 DND41 MVs unstained control (a) and DND41 MVs stained with CFSE and CD45 antibody (b). P2: MVs gate; P3: 3 μ m beads gate; P4: CFSE+ signal; P5: CFSE+ MVs; P6: CD45+ signal; Q2-1: CFSE+/CD45+ MVs.

a



MG63 MVs control		
Population	Events N°	Total %
All events	10721	100.0
P1	10655	99.4
P2	463	4.3
P3	10000	93.1
P4	445	3.1
P5	0	0.0
P6	9	0.1
Q1-1	142	1.3
Q2-1	0.0	0.0
Q3-1	10513	98.1
Q4-1	0	0

b



CFSE+ CD45+ MG63 MVs		
Population	Events N°	Total %
All events	12083	100.0
P1	11894	98.4
P2	1370	11.3
P3	10000	82.8
P4	5987	49.6
P5	5	0.0
P6	129	1.1
Q1-1	9.796	81.8
Q2-1	29	0.2
Q3-1	2.069	17.1
Q4-1	0	0.0

Fig. S8 MVs isolated from MG63 cell line. No CD45+ MVs have been detected both in unstained control (a) and in the CFSE+ CD45+ stained MVs (b). P2: MVs gate; P3: 3 μ m beads gate; P4: CFSE+ signal; P5: CFSE+ MVs; P6: CD45+ signal; Q2-1: CFSE+/CD45+ MVs.

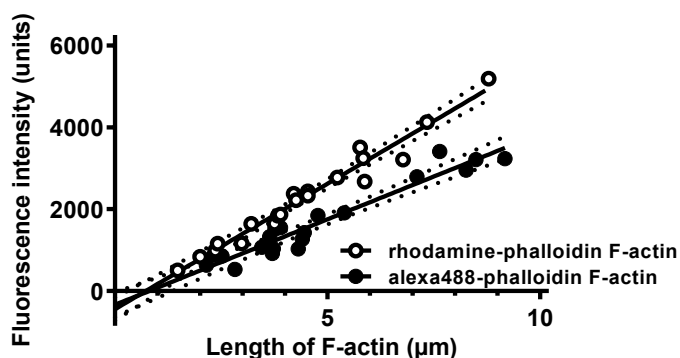
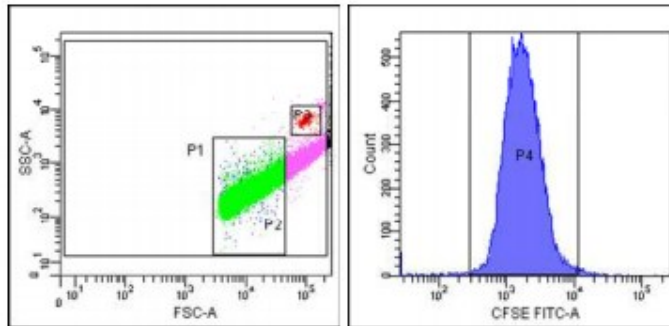


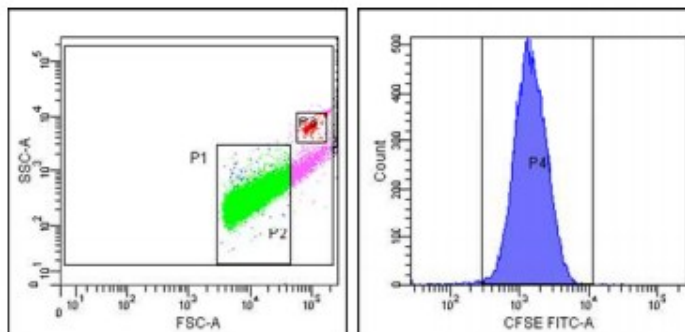
Fig. S9 Fluorescence intensity vs. filament length for several RhPh and APh labeled actin filaments in given fields of view. The ratio between the slopes gives a conversion factor to transform the fluorescence intensity per RhPh-molecule to that per APh-molecule. This factor takes into account all bases of this difference, including different extinction coefficient, different quantum yield and higher intensity of TRITC caused by higher excitation intensity (due to a spectral line in the Hg-lamp). Fluorescence microscopy data for actin filaments dually labeled by APh and Rh-rIgG then allowed estimate of the rIgG labeling density by comparing the total fluorescence intensity due to APh and Rh-rIgG on a given filament or bundle. This calculation assumed 362 APh molecules per 1 μm of an actin filament, 2 TRITC molecules per Rh-rIgG and the conversion factor between single molecule APh and Rh-rIgG fluorescence derived here.

a



DND41 MVs control in PBS1X buffer		
Population	Events N°	Total %
All events	37132	100.0
P1	36309	97.8
P2	21914	59.0
P3	10000	26.9
P4	25557	68.8

b



DND41 MVs control in MOPS buffer		
Population	Event N°	Total %
All events	29584	100.0
P1	29376	99.3
P2	17868	60.4
P3	10000	33.8
P4	19287	65.2

Fig. S10 CFSE+ DND41 MVs stability in PBS (a) and in MOPS buffer (buffer A) (b). MVs stained with 1 mM of CFSE.

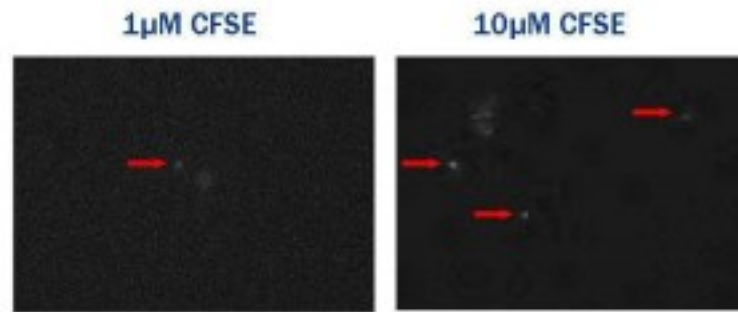


Fig. S11 Optimization of CFSE+ MVs staining for confocal microscopy detection. On the left, an example of MVs stained with 1 μ M of CFSE. On the right, the optimized concentration of 10 μ M.

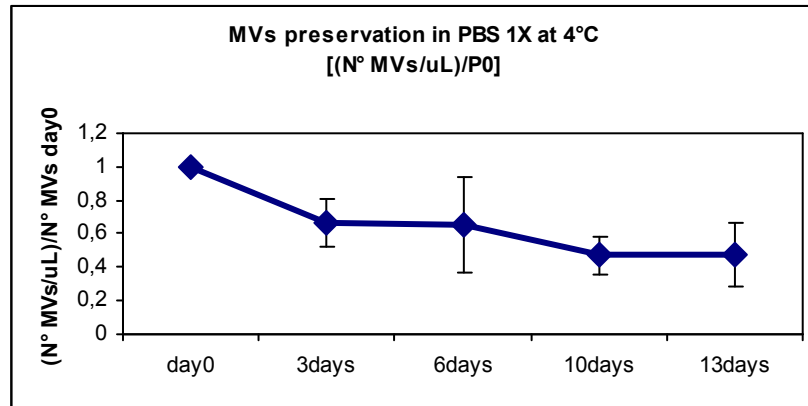


Fig. S12 CFSE+ DND41 MVs were storage in PBS 1X at 4 °C. Their number and integrity were checked at several points by FACS analyses and sufficient solidity of MVs was detected as reported below (number of MVs/ μ L divided by number of MVs at day 0). Three independent experiments have been performed.

Supplementary Movie legends

All movies shown in real time with size of movie frame, 66 x 66 μ m² in Movies S1-S4 and 53 x 53 μ m² in Movies S5-S6. The temperature was in the range 20-23 °C.

Movie S1 Motion of anti-rIgG-actin-fascin-bundles (green) and several anti-rIgG conjugated single filaments in the absence of rhodamine-rIgG on the heavy meromyosin-coated surface in the in vitro motility assay. Towards end of movie, switching from observation using FITC filter set (to visualize APh labeled filaments and bundles) to TRITC filter set.

Movie S2 Motion of anti-rIgG-actin-fascin-bundles (green) after incubation with 0.1 nM rhodamine-rIgG (red) for 1 min on the heavy meromyosin-adsorbed surface in the in vitro motility assay. Towards end of movie, switching from observation using FITC filter set (to visualize APh labeled filaments and bundles) to TRITC filter set (to visualize Rh-rIgG). Note, no unambiguously verified Rh-rIgG binding to any filament or bundle after 0.1 nM Rh-rIgG in this case.

Movie S3 Motion of anti-rIgG-actin-fascin-bundles (green) after incubation with 1 nM rhodamine-rIgG (red) on the heavy meromyosin-adsorbed surface in the in vitro motility assay. Towards end of movie, switching from observation using FITC filter set (to visualize APh labeled filaments and bundles) to TRITC filter set (to visualize Rh-rIgG).

Movie S4 Motion of anti-rIgG-actin-fascin-bundles (green) after incubation with 10 nM rhodamine-rIgG (red) on the heavy meromyosin-adsorbed surface in the in vitro motility assay. Towards end of movie, switching from observation using FITC filter set (to visualize APh labeled filaments and bundles) to TRITC filter set (to visualize Rh-rIgG).

Movie S5 Unidirectional movement of anti-CD45-actin-fascin-bundles (red) in the presence of microvesicles (green) on the heavy meromyosin-adsorbed surface in the in vitro motility assay. Towards end of movie, switching from observation using TRITC filter set (to visualize RhPh labeled filaments and bundles) to FITC filter set (to visualize CFSE labeled microvesicles).

Movie S6 Small and large aggregates of stationary anti-CD45-actin-fascin-bundles (red) in the presence of microvesicles (green) on the heavy meromyosin-adsorbed surface in the in vitro motility assay. Towards end of movie, switching from observation using TRITC filter set (to visualize RhPh labeled filaments and bundles) to FITC filter set (to visualize CFSE labeled microvesicles).