Supplementary Figure S1. Testing Fab monobiotinylation. Fab0.35 with the tag for biotinylation only on the light (Fab0.35-LAPb) or only on the heavy chain (Fab0.35-HAPb) was analyzed by SDS-PAGE and Coomassie staining, with or without reduction and gel-shift by core streptavidin (SA).

Supplementary Figure S2. Relationship between Fab0.11 antibody concentration and number of antibodies bound per cell. Flow cytometry from BT474 stained with the indicated concentration of monobiotinylated Fab0.11, before detection with monovalent streptavidin (mSA-488). This staining allowed quantitation of the number of Fab bound per cell by calibration with beads bearing known numbers of AlexaFluor488 per particle.

Supplementary Figure S3. Cell recovery sensitivity was enhanced by twin biotin tags on the antibody. A, Testing Fab monobiotinylation. Fab0.11 with the tag for biotinylation only on the heavy chain (Fab0.11-HAPb) was analyzed by SDS-PAGE and Coomassie staining, with or without reduction and gel-shift by core streptavidin (SA). B, Dependence of cell recovery on the number of biotinylation sites. BT474 were incubated with the indicated concentration of Fab0.11 bearing 1 or 2 biotinylated tags, before magnetic isolation with streptavidin-beads and quantification of recovered cells (mean of triplicate ± 1 SD).

Supplementary Figure S4. Chemically biotinylated antibody also gave efficient cell isolation. A, Testing Fab chemical biotinylation. Fab0.35 bearing multiple biotins from incubation with an amine-reactive biotin (Fab0.35-bio_n) was analyzed by SDS-PAGE and Coomassie staining with or without reduction and gel-shift by core streptavidin (SA). B, Comparison of cell isolation using enzymatically or chemically biotinylated antibody. BT474 were incubated with the indicated concentration of Fab0.35 with 2 biotinylated AP tags or Fab0.35 chemically biotinylated, before

magnetic isolation with streptavidin-beads and quantification of recovered cells (mean of triplicate ± 1 SD).

Supplementary Figure S5. Effect of cholesterol on surface staining for HER2. BT474 were untreated, loaded with cholesterol, or depleted of cholesterol by methyl-β-cyclodextrin (MβCD) for 1 hr, before staining for surface HER2 expression with Fab0.11 and streptavidin-PE and analyzing by flow cytometry. Fab0.11 was omitted in the "No Fab" sample, where the signal represents principally the cell autofluorescence. B, Quantification of results in A (mean of triplicate ± 1 SD).

Supplementary Figure S6. Cholesterol loading and direct bead linkage enhanced the recovery even with lower affinity antibody. BT474 were incubated with 0.1 µg/ml Fab1.4 with secondary antibody linkage without cholesterol loading, with direct linkage without cholesterol linkage, or with direct linkage combined with cholesterol loading, before quantification of recovered cells (mean of duplicate ± 1 SD). Controls are shown for each condition where Fab1.4 was omitted (No Fab).

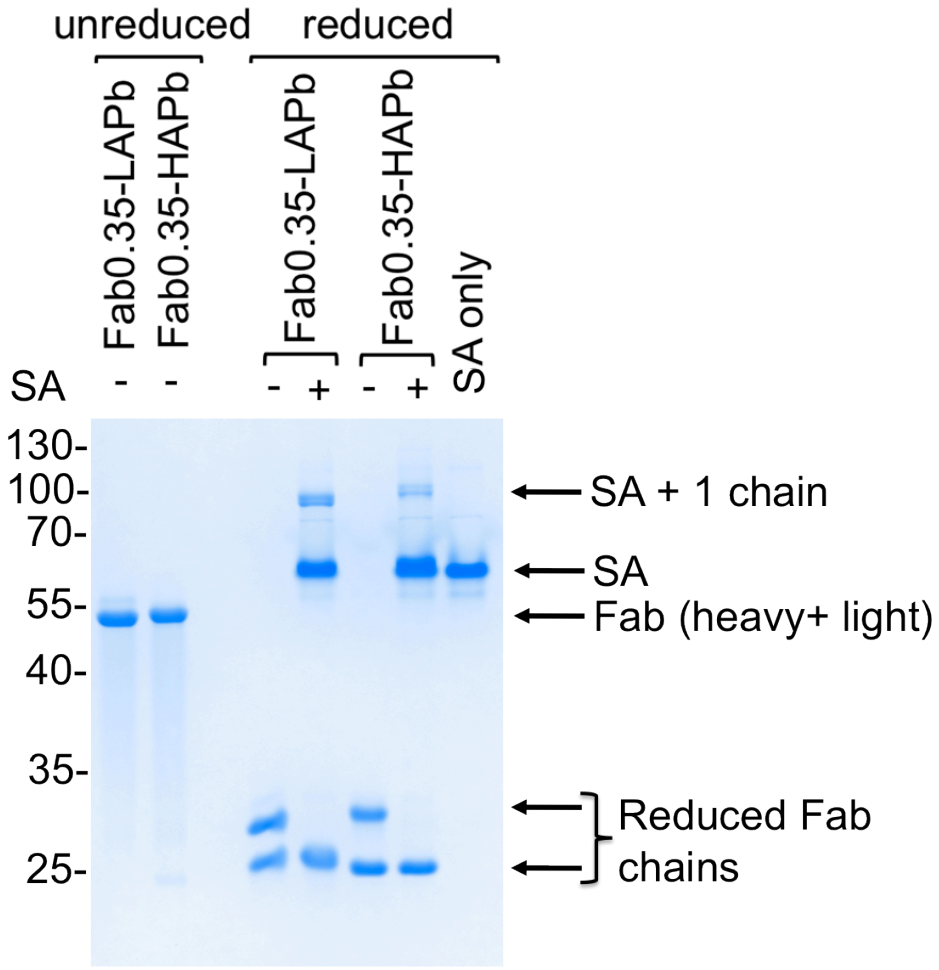
Supplementary Figure S7. Effect of individual changes on recovery of cells varying in HER2 expression. A, Cells were untreated or loaded with cholesterol, before incubating with 1 µg/ml biotinylated Fab0.11 and magnetic isolation using streptavidin-magnetic particles (mean from triplicate measurements ± 1 SD). B, Cells were incubated with 1 µg/ml biotinylated Fab0.35 or Fab0.11 and isolated using streptavidin-magnetic particles (mean from duplicate measurements ± 1 SD). C, For the "Secondary antibody linkage" condition, cells were incubated with 1 µg/ml non-biotinylated Fab0.11, followed by streptavidin-magnetic particles coated with biotinylated secondary antibody. For the "Direct streptavidin-biotin linkage" condition, cells were incubated with 1 µg/ml biotinylated Fab0.11, followed by direct

linkage to the magnetic bead via streptavidin. Recovery is shown as mean from duplicate measurements ± 1 SD.

Supplementary Figure S8. Cholesterol loading and direct bead linkage enhanced isolation of cells expressing low levels of EpCAM. A, Flow cytometry of cell-lines showed variable EpCAM expression. Cells were stained with anti-EpCAM antibody (+mAb) or primary antibody was omitted (No mAb), before labeling with a PE-conjugated secondary antibody. B, Cells were incubated with or without cholesterol for 1 hr and isolated using direct linkage (from biotinylated anti-EpCAM to streptavidin-beads) or secondary linkage (with non-biotinylated anti-EpCAM to streptavidin-beads coated with biotinylated anti-mouse IgG). Recovered cells were quantified (mean of duplicate ± 1 SD). Each condition was also compared with primary antibody omitted.

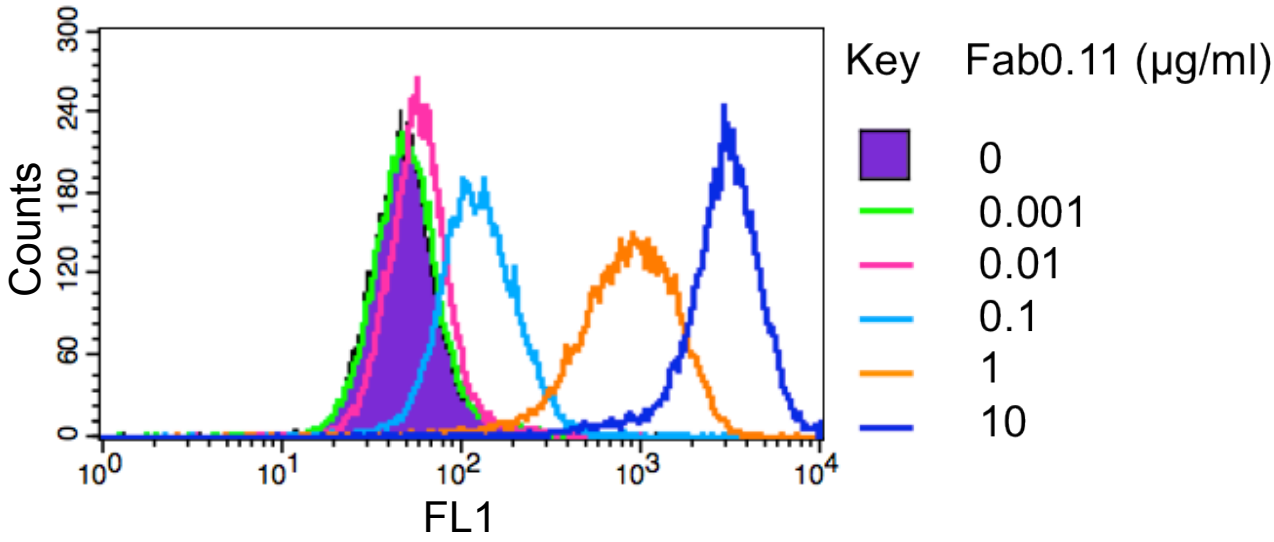
Supplementary Figure S9. Specificity of recovery of cancer cell-lines from blood. Cancer cell-lines of varying HER2 expression levels were spiked into rabbit blood and then isolated using enhanced conditions with or without Fab0.11 (mean from triplicate measurements ± 1 SD). In the absence of Fab, signals were too low to be seen.

Supplementary Figure S1



Supplementary Figure S2

A

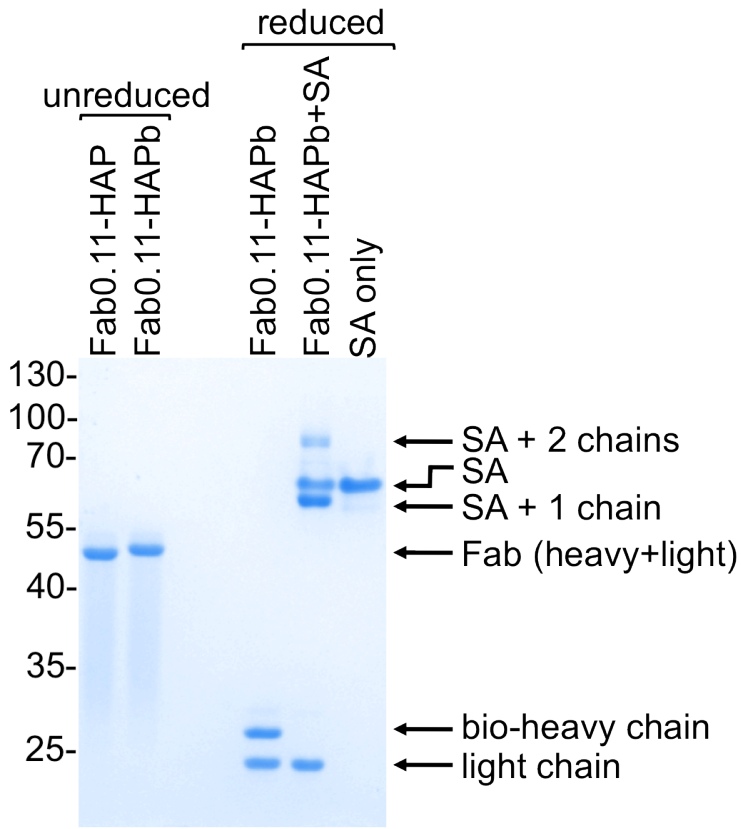


B

[Fab0.11] (µg/ml)	10	1	0.1	0.01	0.001	0
Fab bound per cell (mean ± 1 s.d., *n* = 3)	762,000 ± 14,000	218,000 ± 2,100	21,700 ± 530	2,460 ± 106	79 ± 170	0

Supplementary Figure S3

A



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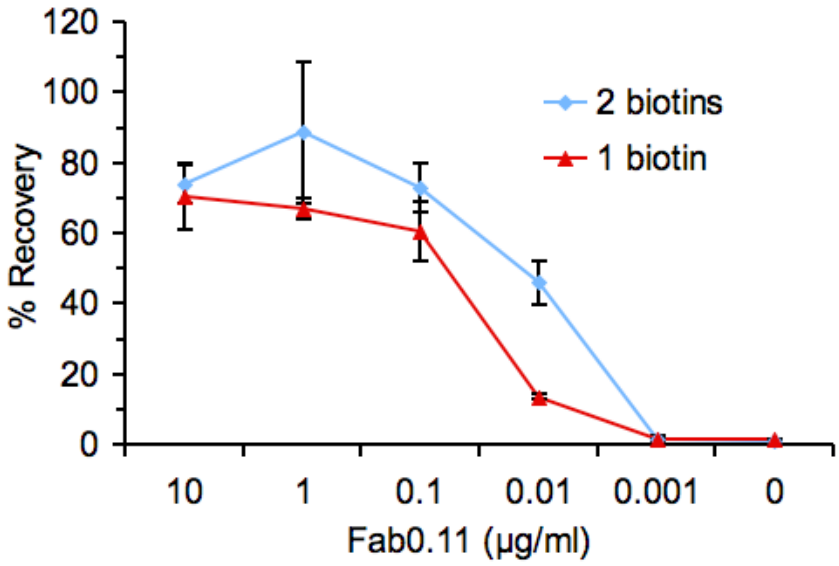
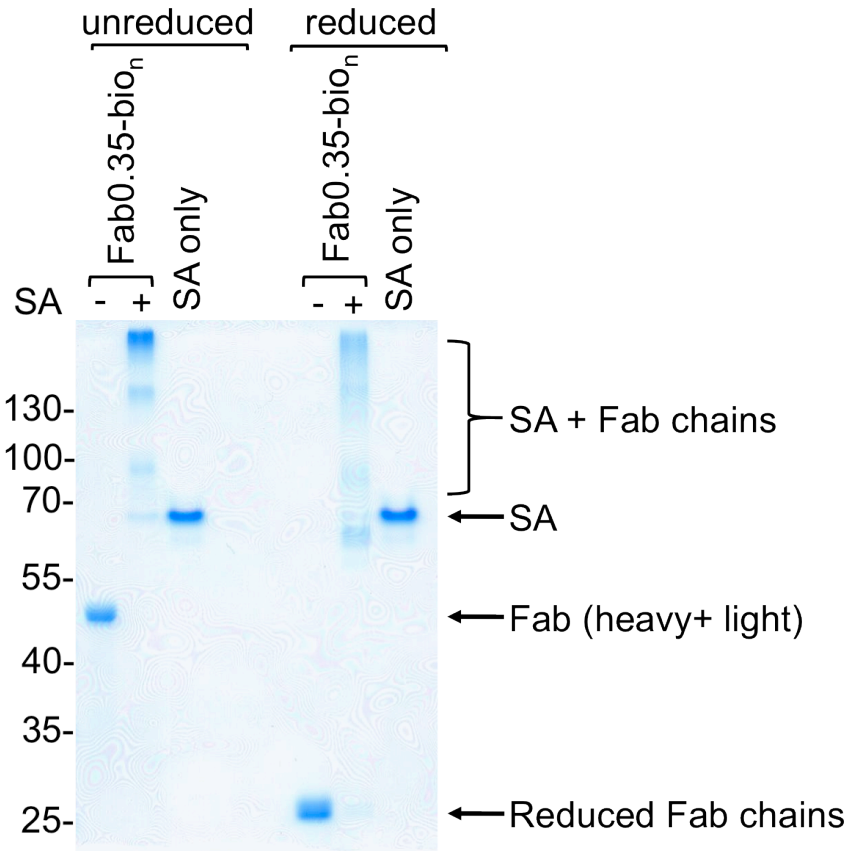


Supplementary Figure S4

A



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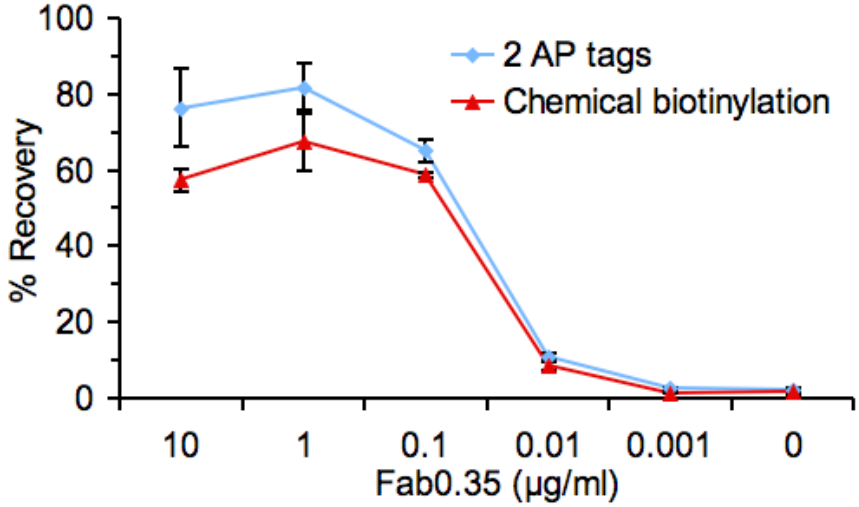

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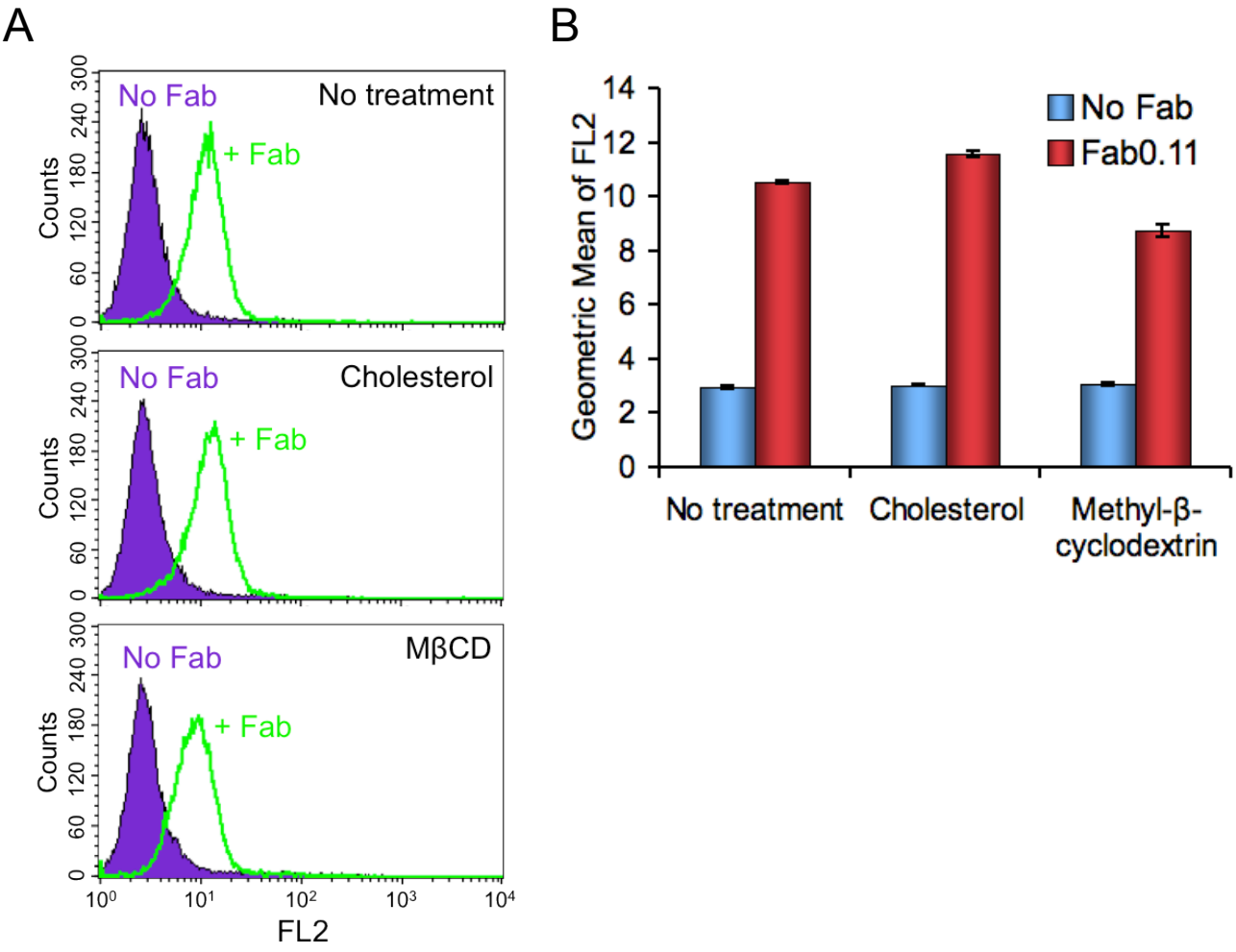
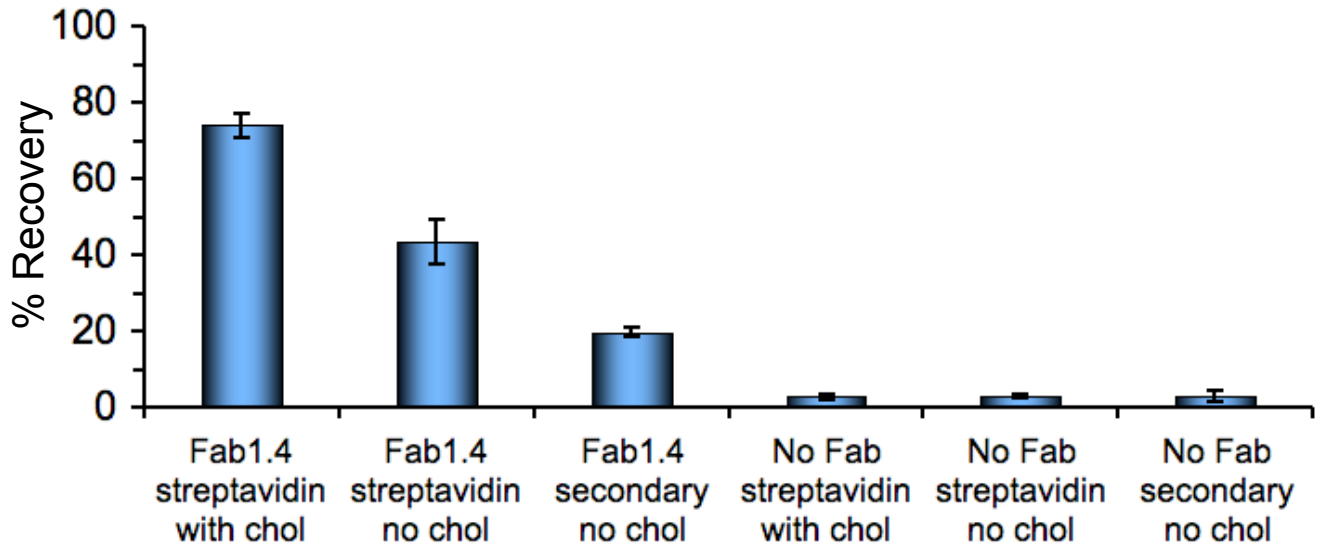
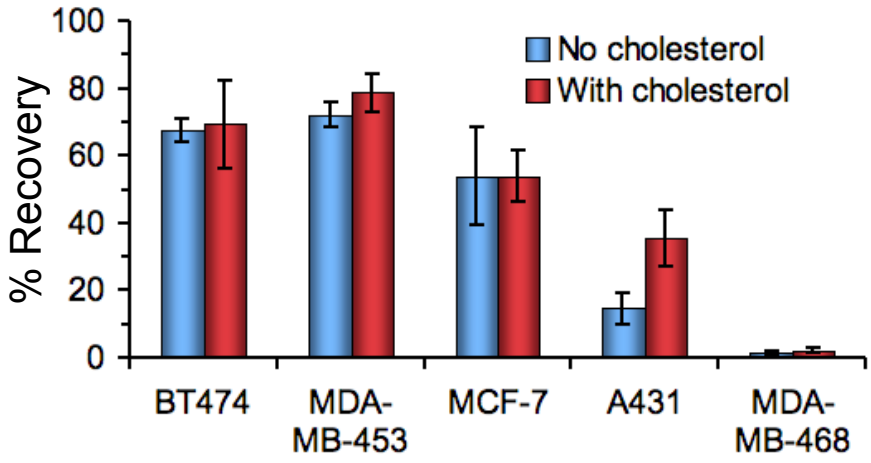


Supplementary Figure S6



Supplementary Figure S7

A



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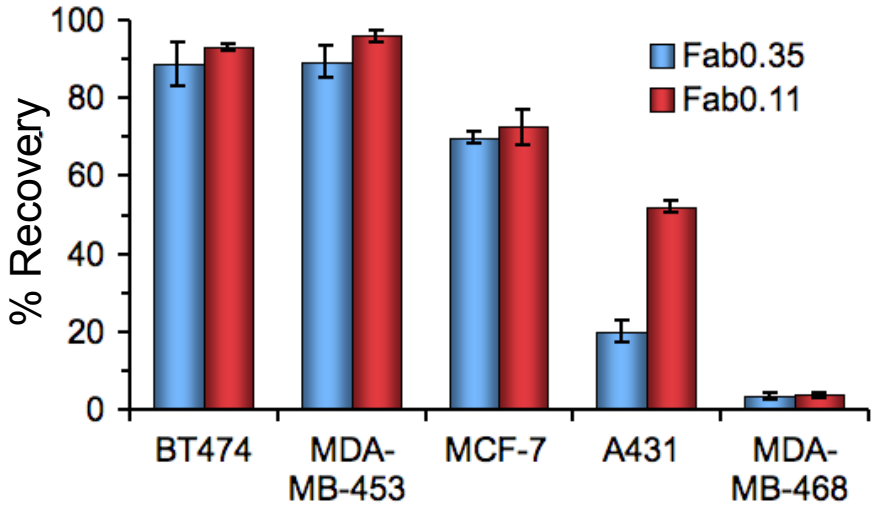

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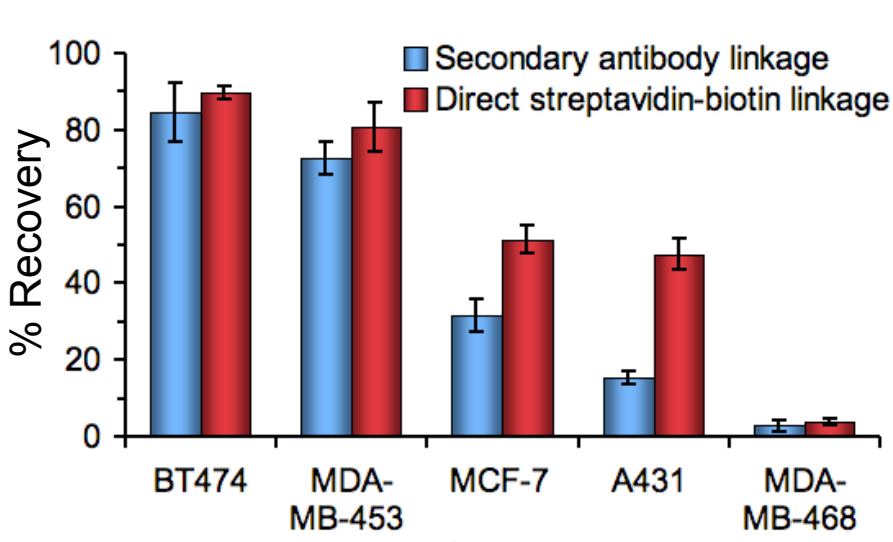
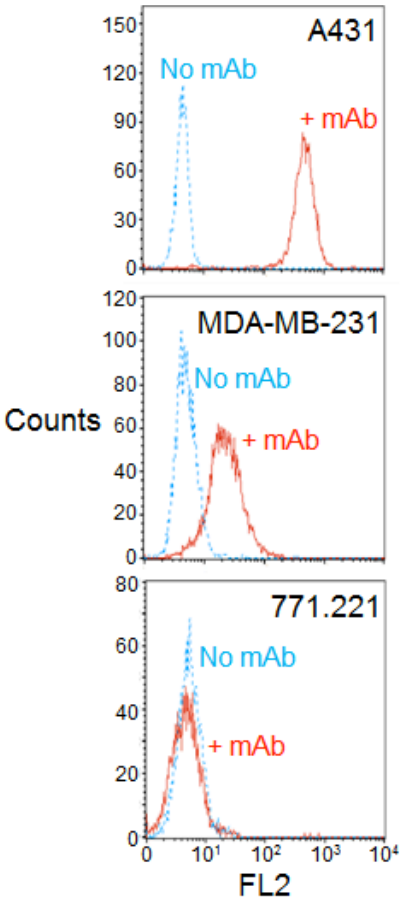


Supplementary Figure S8

A



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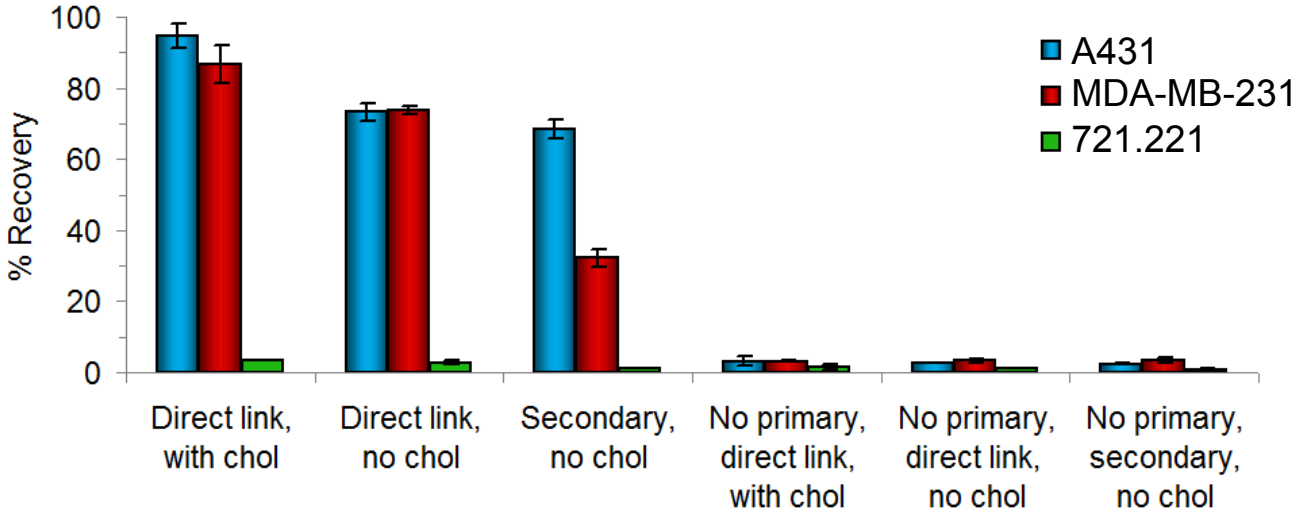
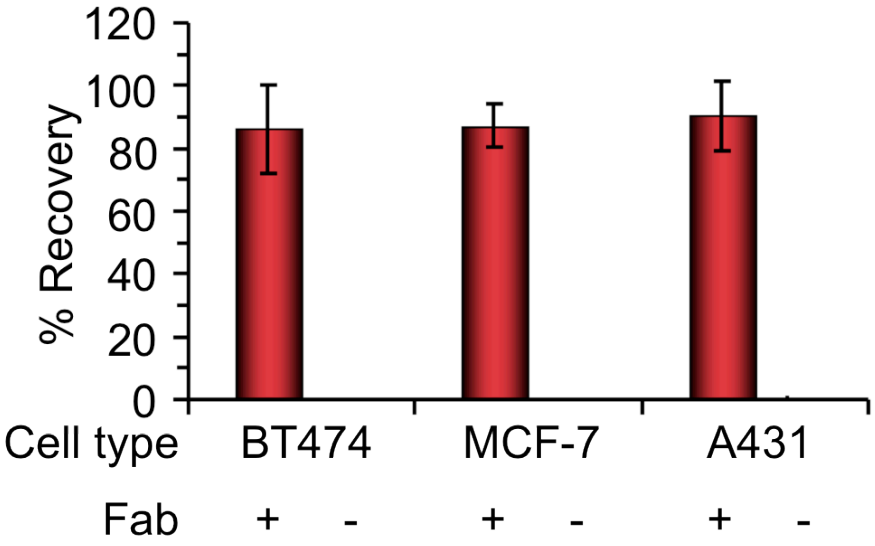


Supplementary Figure S9



Supplementary Methods

Jain, Veggiani & Howarth

Cholesterol Loading and Ultrastable Protein Interactions Determine the Level of Tumor Marker Required For Optimal Isolation of Cancer Cells

Cloning

PCRs were performed with KOD Hot Start DNA polymerase (Merck). All constructs and mutations were verified by sequencing.

Antibody construct cloning

The required hu4D5 construct was assembled by DNAWorks gene synthesis (51). The PCR products were inserted into pOPINVH and pOPINVL, kind gifts of Ray Owens (University of Oxford) (52), via restriction sites *KpnI* and *DraIII* (for V_H) and *KpnI* and *PmeI* (for V_L). Primers 5'-GATCGGTACCGGAGAGGTGCAGCTGGTAGAGTCCGGAGGCGGCCTCGTGC-3'and 5'-GATCCACTTAGTGTTATTAATGATGATGGTGGTGATGCTCATGC-3' were used for the heavy chain insertion and 5'-GATCGGTACCGGA GACATCCAGATGACCCAGAGTCCCTCTT-3'and 5'-GATCGTTTAAAC TTATTACTCGTGCCATTCGATCTTCTGCG-3' for light chain insertion. Going from the N-terminus, the heavy chain construct contained a signal sequence (cleaved in the endoplasmic reticulum), V_H domain, C_H1 domain, AP tag (53) and then a His_6 tag. The light chain constructs had a signal sequence, V_L domain, Cκ domain, and an AP tag.

To generate point mutants of Fab0.35, overlap extension PCR was carried out using primers 5'-CCGATGGGGCGGATGGGGCTTCTATGCCATG-3' and 5'-

CATGGCATAGAAGCCCCATCCGCCCCATCGG-3'for D102W to make Fab0.11, 5'-GGAGACGGCTTCTTTGCCATGGACTACTGGG-3' and 5'-CCCAGTAGTCCATGGCAAAGAAGCCGTCTCC-3' for Y105F to make Fab1.4, 5'-CTGCCAACAGCACGCCACCACCCCCCCTAC-3' and 5'-GTAGGGGGGGTGGTGGCGTGCTGTTGGCAG-3' for Y92A to make Fab2.7, and 5'-CTACTGCCAACAGGCCTACACCACCCCCCC-3' and 5'-GGGGGGGTGGTGTAGGCCTGTTGGCAGTAG-3' for the H91A mutation to make Fab55 (54;55). The same forward and reverse primers as for Fab0.35 were used for insertion into pOPINVH and pOPINVL.

Unless indicated, all Fabs were bis-biotinylated. Non-biotinylated Fab (Fig. 3 and 5) had the same sequence as bis-biotinylated Fab but was not incubated with BirA. To produce monobiotinylated Fab0.35 (Fab0.35-HAPb) for quantitative flow cytometry (Fig. 1D, Fig. 5A, Table 1, Table 2), 5'-GATCGTTTAAAC CTATTA GCACTCGCCCCTGTTGAAGCTC- 3' was used as the reverse primer to produce the light chain without the AP tag. We also made Fab0.35-LAPb with the heavy chain lacking the AP tag using primer 5'-GATCCACTTAGTGCTATTAGTGGTGGTGATGATGGTGGCAAGACTTGGGC TCTACCTTC-3' (Supplementary Fig. S1). To produce monobiotinylated Fab0.11 for quantitative flow cytometry and immunomagnetic cell isolation (Supplementary Fig. S2, Supplementary Fig. S3, Supplementary Fig. S5), the light chain without the AP tag was transfected along with the D102W mutation in the heavy chain plasmid. Structures of the antibody-receptor complex were rendered in PyMOL (DeLano Scientific).

Expression of antibody

HEK 293T cells were grown in roller bottles (Greiner) in 250 ml D10 (DMEM with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin). HEK 293T were transfected in DMEM with 50 U/ml penicillin and 50 μg/ml streptomycin using 1.15 mg polyethyleneimine (25 kDa, Sigma) with 160 μg heavy chain plasmid and 160 μg light chain plasmid, each endotoxin-free prepared using the Fisher Maxiprep kit. 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 3.8 mM valproic acid (Sigma) and 4 mM glutamine (Life Technologies) were added at the time of transfection (56). Four days after transfection, the supernatant was harvested by centrifugation at 2,000 rpm for 15 min. 25 ml of 10× PBS and $MgCl_2$ to a final concentration of 100 μM were added to the supernatant and then His_6-tagged Fab was purified on Ni-NTA superflow resin (Qiagen) before dialyzing three times into PBS. Protein concentrations were measured from A_{280} with extinction coefficients from ExPASy ProtParam.

Expression of BirA and Streptavidin

The plasmid for glutathione-S-transferase linked to biotin ligase (GST-BirA) was a kind gift from Christopher O'Callaghan (University of Oxford) and was expressed in *E. coli* and purified as described (57), before dialysis into PBS. Core streptavidin (tetravalent) and monovalent streptavidin (mSA) (58) were expressed in *E. coli* and purified as described (59), before dialysis into PBS.

Antibody enzymatic biotinylation

Purified Fab was biotinylated in PBS with 5 mM $MgCl_2$, 1 mM ATP, 50 μM biotin and 1 μM GST-BirA for 2 hr at 25°C. GST-BirA was removed with

glutathione-agarose beads (Qiagen). Excess biotin was removed by dialyzing three times into PBS.

SDS-PAGE

SDS-PAGE was performed on 12% polyacrylamide gels, using an XCell SureLock (Life Technologies) at 200 V. 1 µg Fab in PBS was mixed with 6× SDS-PAGE loading buffer and heated at 95°C for 3 min with or without 120 mM 2-mercaptoethanol in a Bio-Rad C1000^{TM} Thermal Cycler, before loading onto the gel. For gel-shift assays, Fab that had been boiled in SDS loading buffer was incubated with 5.4 µM core streptavidin for 2 min at 25°C, before loading on the gel without any further heating. Gels were stained with InstantBlue Coomassie (Triple Red) and imaged using Gel DocXR+ Imaging System (Bio-Rad) and Image Lab 3.0 software (Bio-Rad).

Dye labeling

To prepare core streptavidin-488, 200 µl of 14 µM core streptavidin was mixed with 20 µl 1 M $NaHCO_3$ pH 8.3 and 7.5 µl AlexaFluor488 succinimidyl ester (10 mg/ml in DMSO, Life Technologies) and incubated for 4 hr at 25°C. To prepare mSA-488, 300 µl of 35 µM mSA was mixed with 30 µl 1 M $NaHCO_3$ pH 8.3 and 6 µl AlexaFluor488 succinimidyl ester (10 mg/ml in DMSO, Life Technologies) and incubated for 4 hr at 25°C. Free dye was removed by gel filtration with Sephadex G-25 (Sigma) and dialyzing three times into PBS.

Quantitative flow cytometry

For quantification of bound Fab, Quantum AlexaFluor488 MESF (Molecules of Equivalent Soluble Fluorochrome) beads (Bangs Laboratories) with five different levels of MESF (0, 3507, 32348, 196948, 941222) were used. The beads were analyzed on FACScalibur, the background from blank beads subtracted, and a best-fit line of the geometric mean of the bead's FL1 fluorescence was plotted against their respective MESF values, using Microsoft Excel and passing through (0,0). After subtracting the geometric mean fluorescence of blank cells (0 µg/ml Fab) from the other samples (10 – 0.001 µg/ml Fab), MESF for cells was calculated using the 488-bead standard curve. The obtained MESF value was then divided by the number of dye molecules conjugated per mSA molecule, to give the final number of mSA molecules bound to cells at various Fab concentrations. The number of AlexaFluor488 conjugated per mSA molecule was calculated from the absorption of mSA-488 at 280 nm and 495 nm following the dye manufacturer's instructions (Life Technologies). All samples were processed in triplicate. The gain was set so that the lowest fluorescence bead was between 10^0 and 10^1 on the FL1 scale, which meant that the cell autofluorescence rendered the background signal from cells in the middle of the FL1 scale.

For quantification of bound Fab on the different cell-types, a standard curve using the Quantum AlexaFluor488 MESF beads was plotted as described above. Keeping the FL1 setting consistent, and subtracting the geometric mean fluorescence of blank cells (0 µg/ml Fab) from the labeled cells (10 µg/ml Fab), MESF for cells was calculated using the 488-bead standard curve as above.

Microscopy

The DeltaVision Core fluorescent microscope used a 40× oil-immersion lens for Fig. 1B and Fig. 2D, but a 4× lens for Fig. 5E. CFSE/AlexaFluor488 (490DF20 excitation, 528DF38 emission, Chroma 84100bs polychroic), TRITC/PE (555DF28 excitation, 617DF73 emission, Chroma 84100bs polychroic) and brightfield images were collected and analyzed using softWoRx 3.6.2 software (Applied Precision). The typical exposure time was 0.25 - 1 s and fluorescence images were background-corrected. Different samples from the same experiment were prepared, imaged and analyzed under identical conditions.

Chemical biotinylation of Fab0.35

To chemically biotinylate Fab0.35 on reactive surface amines, 400 µl of 21.4 µM unbiotinylated Fab0.35 was mixed with 40 µl 1 M $NaHCO_3$ pH 8.3 and 4 µl biotinamidohexanoic acid N-hydroxysuccinimide ester (10 mg/ml in DMSO, Sigma). The reaction was incubated for 4 hr at 25°C. The biotinylated Fab was separated from small-molecule forms of biotin by gel filtration with Sephadex G-25 (Sigma) and by dialyzing three times into PBS.

BT474 were incubated with indicated concentrations of chemically biotinylated Fab0.35 (Fab0.35-bio_n) or Fab0.35 bearing biotins on both AP tags and then isolated with streptavidin-beads, before quantification as previously (Supplementary Fig. S4B).

Similarly BT474 were incubated with indicated concentrations of Fab0.11 biotinylated on both AP tags or with biotin only on the heavy chain and then isolated with streptavidin-beads, before quantification as previously (Supplementary Fig. S3B).

Testing of inhibitors on cell isolation

For the inhibitor experiments, we used 50 μM cytochalasin D (Sigma), 16.5 μM nocodazole (Sigma), 10 mM methyl-β-cyclodextrin (Sigma), 10 μM AG825 (Merck), 1 mM sodium azide (Merck), or 250 μg/ml cholesterol (Sigma). The cholesterol was water-soluble as it contained a 1:7 molar ratio of cholesterol to methyl-β-cyclodextrin (60). 250,000 BT474 cells were incubated in D1 in triplicate with the respective inhibitor for 1 hr at 25˚C. 100 μl Fab0.11 was added at 0.01 μg/ml, incubated for 10 min at 25˚C and the beading was carried out as described above, but with the inhibitor present in D1 throughout the experiment.

Beading of cell-lines with variable HER2 expression

For cell panel testing, cell-lines were harvested as described above. Under the enhanced conditions, samples were treated with 250 μg/ml cholesterol for 1 hr at 25˚C, before incubation with 1 μg/ml of Fab0.11 and then streptavidin-beads were used for cell isolation. Under the standard conditions, samples were incubated with 1 μg/ml unbiotinylated Fab0.35 and cells were isolated using biotinylated goat anti-human kappa chain antibody coated on streptavidin-beads as above.

Effect of cholesterol on HER2 staining by flow cytometry

To check whether cholesterol treatment changes Fab binding to cells, BT474 cells were harvested by trypsinizing and washed once in D1 before resuspending in D1 at 2.5×10^6 cells/ml. Cells in 50 μl D1 (125,000 cells/sample) were treated with either 10 mM methyl-β-cyclodextrin, 250 μg/ml cholesterol or water control, in triplicate, for 1 hr at 25^o C. 0 or 0.01 μg/ml of monobiotinylated Fab0.11 was then added to cells and incubated for 10 min at 25^oC. Cells were centrifuged to remove

excess Fab and resuspended in 50 µl of 0.41 µM Streptavidin-R-Phycoerythrin (Invitrogen) in FACS-A and incubated on ice for 15 min. After washing twice with 100 µl FACS-A, cells were resuspended in 0.4 ml FACS-A on ice and analyzed on a FACScalibur flow cytometer with CellQuest Pro version 5.2.1. The signal from 20,000 cells was recorded on the FL2 channel (488 nm excitation with 585±15 nm emission).

Flow cytometry on EpCAM expression level

To determine the expression levels of EpCAM on different cell lines, cells were washed once in D1 before resuspending in D1 at 2.5 x 10^6 cells/ml. 125,000 cells/sample were incubated with 10 µg/ml mouse anti-human EpCAM (eBioscience, clone 1B7) in FACS-A buffer for 30 min at 25°C. Cells were washed thrice with 100 µl of FACS-A buffer. Labeling was performed by adding 100 µl 1:100 goat anti-mouse IgG-phycoerythrin (Invitrogen) in FACS-A and incubating cells for 20 min on ice. After washing thrice with 100 µl of FACS-A buffer, cells were resuspended in 0.3 ml FACS-A on ice and analyzed on a FACScalibur flow cytometer with CellQuest Pro version 5.2.1 (Becton Dickinson).

Immunomagnetic isolation using anti-EpCAM

A431, MDA-MB-231 and 771.221 cells were resuspended at 2.5 x 10^6 cells/ml in D1. 100 µl of cells were used for each condition. To compare the effect of cholesterol on cell recovery and the importance of the antibody linkage to the beads, samples were incubated with or without 250 µg/ml cholesterol for 1 h at 25°C, before incubation with 1 µg/ml of mouse anti-human EpCAM.

Samples were treated with or without cholesterol loading and with both the biotinylated or non-biotinylated version of anti-EpCAM (eBioscience, clone 1B7). Cells treated with 1 µg/ml biotinylated anti-EpCAM antibody were isolated by direct linkage to streptavidin-beads. Samples incubated with non-biotinylated anti-EpCAM mAb were captured by pre-coating streptavidin-beads for 30 min at 25°C with a 1:4 solution of a biotinylated goat anti-mouse IgG antibody (Sigma). After incubation in a 1.5 ml microcentrifuge tube at 25°C for 30 min with end-over-end rotation (Stuart Equipment), 100 µl of the cell-bead mixture was removed for counting. The tube with the remaining cells was placed on a magnet (MagRack 6, GE Healthcare) and the unbound cells after 2 min were pipetted out, for counting of flowthrough cells. The cells bound to the magnetic beads were washed once with 400 µl D1 and resuspended in 100 µl D1. Cells in the bead-cell mixture, flowthrough and recovered cells were counted on a Coulter Counter as above.

Statistical analysis

Statistical tests were unpaired t-tests and were carried out using GraphPad Software QuickCalcs.

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