Supporting Information

Rapid Multitarget Immunomagnetic Separation through Programmable DNA Linker Displacement

Christine E. Probst, Pavel Zrazhevskiy, and Xiaohu Gao*

Department of Bioengineering, University of Washington, Seattle, Washington 98195, United States

** e-mail: xgao@u.washington.edu*

Abbreviations

MACS, magnetic-activated cell sorting; DNA, deoxyribonucleic acid; Ab, antibody; IgG, immunoglobulin G; SMD, strand-mediated displacement; CP, capture probe; EP, encoding probe; DP, displacement probe; MB, magnetic bead; bp, base pair.

Materials

Fluorescent beads (Bangs Laboratories) 5-6 μm in diameter doped with 4 different organic dyes (Glacial Blue 360/450nm excitation/emission maxima, Dragon Green 480/520nm, Suncoast Yellow 540/600nm, and Flash Red 660/690nm) were used as a model system for development and characterization of the SMD technology. Each bead features surface carboxylic acid functional groups (with parking area between 37 and 174 $Å^2$/surface group) suitable for covalent conjugation with biomolecules. Magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen) are 1 μm in diameter and feature streptavidin coating for easy assembly with biotinylated DNA probes. Purified IgG from human, mouse, and rabbit serum as well as whole goat anti-human, anti-mouse, and anti-rabbit IgG were purchased from Sigma-Aldrich. All antibodies were obtained in 1x PBS without carrier proteins or stabilizing reagents. Biotinylated goat anti-human, anti-mouse, and anti-rabbit IgG were either purchased from Sigma-Aldrich or prepared in house using EZ-Link NHS-PEG4-Biotin (Thermo Scientific). DNA probes were purchased from Integrated DNA Technologies. Sequences were optimized to minimize secondary structures and homology with mismatched DNA sequences at room temperature. Encoding probes (EPs) were synthesized with primary amine functional group at the 5' end for covalent conjugation with antibodies. Capture probes (CPs) were synthesized with a biotin tag at the 5' end for assembly with streptavidin-coated MBs. Both CPs and EPs included a 5’ 10A spacer to allow for flexibility at the bead interface. All DNA probes were purified with HPLC, reconstituted in DNase-free water (Thermo Scientific) at 100 μM, and stored at -20^{0}C. Sequences of DNA probes are summarized in Table S1.

Table S1. DNA sequences used for multi-target enrichment and isolation*.

Set	CP, EP, and DP sequences
CP1	5' AAAAAAAAAA**TGTGAA**TAGACTTGCCATACGT 3'
EP1	5' AAAAAAAAAAACGTATGGCAAGTCTA 3'
DP1	5' ACGTATGGCAAGTCTA**TTCACA** 3'
CP2	5' AAAAAAAAAA**TACCGT**AATTCTTGAGACCAGG 3'
EP2	5' AAAAAAAAAACCTGGTCTCAAGAATT 3'
DP2	5' CCTGGTCTCAAGAATT**ACGGTA** 3'
CP3	5' AAAAAAAAAA**GCATTG**TTCCCTAGCGTCATCT 3'
EP3	5' AAAAAAAAAAAGATGACGCTAGGGA 3'
DP3	5' AGATGACGCTAGGGAA**CAATGC** 3'

Methods

Preparation of DNA-antibody conjugates

Functionalization of antibodies with encoding DNA sequences was achieved by covalent conjugation between primary amine groups present on antibody and the 5'-end primary amine group on DNA. First, IgG was activated with S-HyNic (succinimidyl-6-hydrazino-nicotinamide, Solulink) heterobifunctional cross-linker, which introduces aromatic hydrazine group: 100 µL 1 mg/mL IgG in 100 mM PBS was mixed with 2 µL 10 mM S-HyNic (in DMF) and incubated for 2 hours. At the same time, EP was activated with S-4FB (N-succinimidyl-4-formylbenzamide, Solulink) heterobifunctional cross-linker, which converts primary amine into aromatic aldehyde group: 100 µL 50 µM EP in 100 mM PBS was mixed with 2.5 µL 100 mM S-4FB (in DMF) and incubated for 2 hours. Excess cross-linkers was then removed by passing both activated IgG and EP through Zeba spin desalting columns (Thermo Scientific), and buffer was exchanged to 100 mM MES, pH5. Finally, activated IgG and EP were conjugated through formation of bis-arylhydrazone bond between aromatic hydrazine and aromatic aldehyde groups: IgG and EP were mixed together at ~20x molar excess of EP, reacted for 4 hours, and buffer-exchanged into 10 mM PBS with Zeba spin desalting columns. All reactions were performed at room temperature. DNA-antibody conjugates were stored at 4^0C and used within 2 months after preparation.

Preparation of antigen-coated fluorescent beads

IgGs purified from human, rabbit, and mouse serum were covalently linked to the surface of red, green, and blue fluorescent beads, respectively. Covalent conjugation was achieved via 2-step carbodiimide-mediated cross-linking between primary amines on IgG and carboxylic acid groups on bead surface. First, fluorescent beads were washed and suspended in MES buffer (pH 4.7) with 0.01% Tween-20 at 0.1 w/v% (~10^7 beads/mL) and activated for 15 minutes upon addition of 10 mg/mL 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich) and 10 mg/mL *N*-hydroxysulfosuccinimide (sulfo-NHS, Thermo Scientific). Activated beads were washed by centrifugation (at 3000 g for 2 minutes)

* The toehold region is bolded. 10A spacer at the 5' end of EP and CP is in black.

twice using 50mM Borate buffer (pH 8.4) with 0.01% Tween-20 to remove excess reactants and then incubated with IgG at 2.5 mg/mL in Borate buffer with 0.01% Tween-20 for 4-8 hours for covalent cross-linking. IgG-conjugated microspheres were washed 4 times to remove excess IgG, resuspended in 10mM PBS with 0.5% Bovine Serum Albumin (BSA, Sigma-Aldrich), and stored at 4^0C.

Validation of antigen coating on the surface of fluorescent beads

Presence of target-specific surface antigen (mouse, rabbit, or human IgG) on the surface of each fluorescent bead population was tested via labeling with biotinylated goat anti-mouse, anti-rabbit, or anti-human IgG followed by staining with quantum dot probes functionalized with streptavidin (Qdot 655 streptavidin conjugate, Invitrogen). PBS with 0.5% BSA was used as incubation and washing buffer throughout the experiment. All incubation steps were carried out at room temperature under gentle rotation. All washing steps were done through centrifugation at 3000 g for 2 minutes. Each bead type was resuspended in 400µL buffer at a final concentration of $1x10^6$ beads/mL and split into 4 test volumes 100µL each. To 3 of the 4 samples, biotinylated IgGs were added at 0.2 µg/mL, incubated for 30 minutes, washed 3 times, and resuspended with 100µL buffer. The forth sample (control) was incubated with only 0.5% BSA/PBS and washed in the same fashion. Then QDot655-Streptavidin probes were added to each sample at 1nM final concentration, incubated for 30 minutes, washed with buffer 4 times, and finally washed with water once. Pellets were resuspended in 10µL water, spotted onto glass coverslips, allowed to dry, and imaged at high magnification. Wide UV filter cube was used for imaging of all fluorescent beads and quantum dots (as UV light provides sufficient excitation energy for all fluorophores). Hyper-spectral imaging and further image analysis with Nuance software enabled unmixing of fluorescence signal components and direct quantitative analysis of QDot staining intensity on the surface of fluorescent beads. False-color composite images were obtained by merging individual channels.

Study of target capture and release kinetics

Green fluorescent beads conjugated with Rabbit IgG were used for the study of target capture and release kinetics. PBS with 0.5% BSA was used throughout all steps of this study. All incubation steps were performed under gentle rotation at room temperature unless noted otherwise. The conditions for capture experiments are outlined in Table S2. In step 1a, target beads were incubated with capture antibodies (with biotin or EP) for immuno-recognition: 10^6 fluorescent beads/mL were mixed with capture antibodies at final antibody concentration of 2.5 µg/mL in 100 µL buffer and incubated for 30 minutes. Meanwhile, in step 1b, biotinylated CPs were immobilized onto streptavidin-coated magnetic beads: 10^7 MBs were mixed with CPs at final DNA concentration of 1 µM and incubated for 30 minutes. Fluorescent beads were washed through centrifugation (at 3000 g for 2 minutes) 4 times to remove excess antibody, while MBs were washed 4 times with a magnet to remove excess CPs. Fluorescent beads were then mixed with the magnetic fraction at a ratio of 50 MBs per fluorescent bead. For study of capture kinetics, the mixture was washed 3 times on the magnet following 5, 15, 30, and 60 minute incubation periods; supernatant fractions were pooled, and the MB-bound fraction was resuspended in equal volume. For study of target release kinetics, MB-bound fluorescent beads were prepared using the protocol described above (Condition 2 in Table S2). The conditions for SMD experiments are outlined in

Table S3. Displacement probe (DP) was added to the mixture at 5 μM final concentration, and the solution was gently mixed for ~30 seconds with pipette. MB-bound fraction was separated with a magnet after 1 minute and 60 minute incubation periods. Three independent trials for each experiment were conducted to evaluate reproducibility. For fluorescence measurement samples were placed into a 96-well black flat-bottom plate (Corning). Fluorescence in MB-bound and supernatant fractions was measured with fluorescence plate reader (Infinite M200, Tecan) at 480/541 nm excitation/emission wavelengths and constant gain. Measurements were averaged over 4 quadrants of each well to correct for inhomogeneous sedimentation of fluorescent beads. Equal volume of PBS with 0.5% BSA was used as a baseline, which was subtracted from all fluorescence readings. Fraction captured was calculated as the baseline-corrected fluorescence in MB-bound fraction divided by the sum of the baseline-corrected fluorescence in MB-bound fraction and supernatant.

Table S2. Conditions for the study of target capture kinetics

	Capture antibody type	**MB type**
Condition 1: Biotin-streptavidin mediated capture	Goat anti-Rabbit/Biotin	Streptavidin
Condition 2: Capture via complementary DNA hybridization	Goat anti-Rabbit/EP1	CP1
Condition 3: Non-complementary DNA hybridization	Goat anti-Rabbit/EP1	CP2
Condition 4: Mismatched antibody binding	Goat anti-Mouse/EP1	CP1
Condition 5: Nonspecific binding	None	Streptavidin

Table S3. Conditions for the study of target release kinetics

	DP
Condition 1: Release through complementary DP	DP1
Condition 2: Release through non-complementary DP	DP2
Condition 3: Release with no SMD	None

Study of target capture specificity (from a 4-bead mix)

Three sets of "target" fluorescent beads were prepared: green beads were coated with rabbit IgG, blue beads with mouse IgG, and red beads with human IgG. Uncoated yellow beads were used as an "impurity" fraction. PBS with 0.5% BSA was used as incubation and washing buffer throughout this study. All incubation steps were performed under gentle rotation at room temperature. Purity of isolated fractions was quantitatively measured with flow cytometry (method described separately) and qualitatively evaluated with fluorescence microscopy (method described separately). Three independent trials for each experiment were conducted to evaluate reproducibility.

Part A (Specificity of antibody-antigen recognition for biotinylated IgGs): Four bead populations were pooled in even proportions into a single centrifuge tube to a total final concentration of $4x10^6$ beads/mL

and split into 4 test volumes 100µL each. To 3 of the 4 samples, biotinylated Abs against rabbit, mouse, or human IgG were added at 0.2 µg/mL, incubated for 15 minutes, washed through centrifugation (at 3000 g for 2 minutes) 4 times to remove excess Abs, and resuspended with 100µL buffer. The forth sample (control) was incubated with buffer only and washed in the same fashion. Next, fluorescent beads were mixed with streptavidin-coated MBs at a ratio of 50 MBs per target fluorescent bead and incubated for 30 minutes. Each sample was then separated on a magnet for enrichment and isolation of green, blue, or red beads from the initial 4-color mixture. The magnetic fraction was washed 3 times, the supernatant fractions were pooled together, volumes of magnetic and supernatant fractions were equalized, and the absolute number of fluorescent beads in each fraction was counted with flow cytometry. All conditions tested are summarized in Table S4.

Table S4. Conditions for the study of antibody-antigen binding specificity for IgG-biotin

	Capture antibody type	**MB type**
Condition 1: Blue bead (Mouse IgG coating) capture	Goat anti-Mouse/Biotin	Streptavidin
Condition 2: Green bead (Rabbit IgG coating) capture	Goat anti-Rabbit/Biotin	Streptavidin
Condition 3: Red bead (Human IgG coating) capture	Goat anti-Human/Biotin	Streptavidin
Condition 4: Control (non-specific MB binding)	None	Streptavidin

Part B (Specificity of antibody-antigen recognition for DNA-antibody conjugates): Four bead populations were pooled in even proportions into a single centrifuge tube to a total final concentration of $4x10^6$ beads/mL and split into 4 test volumes 100µL each. To 3 of the 4 samples, DNA-antibody conjugates against rabbit, mouse, or human IgG were added at 2 µg/mL, incubated for 15 minutes, washed through centrifugation (at 3000 g for 2 minutes) 4 times, and resuspended with 100µL buffer. The forth sample (control) was incubated with buffer only and washed in the same fashion. Meanwhile, MBs with CPs (CP1, CP2, or CP3) were prepared by mixing 10^7 MBs with CPs at final DNA concentration of 1 µM, incubating for 15 minutes, and washing 4 times with a magnet. The fluorescent beads were then mixed with CP-MBs complementary to the capture DNA-antibody conjugate at a ratio of 50 MBs per target fluorescent bead and incubated for 30 minutes. Each sample was then separated on a magnet for enrichment and isolation of green, blue, or red beads from the initial 4-color mixture. The magnetic fraction was washed 3 times, the supernatant fractions were pooled together, volumes of magnetic and supernatant fractions were equalized, and the absolute number of fluorescent beads in each fraction was counted with flow cytometry. All conditions tested are summarized in Table S5.

Table S5. Conditions for the study of antibody-antigen binding specificity for IgG-DNA

	Capture antibody type	**MB type**
Condition 1: Blue bead (Mouse IgG coating) capture	Goat anti-Mouse/EP2	CP2
Condition 2: Green bead (Rabbit IgG coating) capture	Goat anti-Rabbit/EP1	CP1
Condition 3: Red bead (Human IgG coating) capture	Goat anti-Human/EP3	CP3
Condition 4: Control (non-specific MB binding)	None	Streptavidin

Part C (Specificity of complementary DNA hybridization for EP-CP oligonucleotide pairs): Each "target" fluorescent bead type was **separately** labeled with its corresponding DNA-antibody conjugate. Fluorescent beads at a final concentration of $1x10^6$ beads/mL in a 100µL test volume were mixed with 2 µg/mL IgG-DNA, incubated for 15 minutes, and washed through centrifugation (at 3000 g for 2 minutes) 4 times. Then the three "target" bead populations and "impurity" yellow beads were mixed together at even proportions to a total final concentration of $4x10^6$ beads/mL. This way, potential nonspecific antigen-antibody binding was circumvented, and specificity of oligonucleotide hybridization could be independently assessed. Meanwhile, MBs with CPs (CP1, CP2, or CP3) were prepared by mixing 10^7 MBs with CPs at final DNA concentration of 1 µM and incubating for 15 minutes, and washing 4 times with a magnet. Magnetic beads with no CP were used as a control. The fluorescent beads were then mixed with CP-MBs corresponding to capture of a single target by DNA hybridization at a ratio of 50 MBs per target fluorescent bead and incubated for 30 minutes. Each sample was then separated on a magnet for enrichment and isolation of green, blue, or red beads from the initial 4-color mixture. The magnetic fraction was washed 3 times, the supernatant fractions were pooled together, volumes of magnetic and supernatant fractions were equalized, and the absolute number of fluorescent beads in each fraction was counted with flow cytometry. All conditions tested are summarized in Table S6.

Table S6. Conditions for the study of EP-CP hybridization specificity for IgG-DNA

	Capture antibody type	**MB type**
Condition 1: Blue bead (Mouse IgG coating) capture	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP2
Condition 2: Green bead (Rabbit IgG coating) capture	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP1
Condition 3: Red bead (Human IgG coating) capture	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP3
Condition 4: Control (non-specific MB binding)	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	Streptavidin

Study of strand-mediated displacement specificity for target release (from a 4-bead mix)

Three sets of "target" fluorescent beads were prepared for this experiment: green beads were coated with rabbit IgG, blue beads with mouse IgG, and red beads with human IgG. Uncoated yellow beads were used as an "impurity" fraction. PBS with 0.5% BSA was used as incubation and washing buffer throughout this study. All incubation steps were performed under gentle rotation at room temperature. **Separately**, each "target" bead type was labeled with its corresponding DNA-antibody conjugate. Fluorescent beads at a final concentration of $1x10^6$ beads/mL in a 100µL test volume were mixed with 2 µg/mL DNA-antibody conjugate, incubated for 15 minutes, and washed through centrifugation (at 3000

g for 2 minutes) 4 times. Then the three "target" bead populations and "impurity" yellow beads were mixed together at even proportions to a total final concentration of $4x10^6$ beads/mL. This way, weak nonspecific antigen-antibody binding/unbinding was circumvented, and specificity of target release via SMD could be independently assessed. Meanwhile, MBs with CPs (CP1, CP2, and CP3) were prepared by mixing $3x10^7$ MBs with CPs at total final DNA concentration of 3 µM, incubating for 15 minutes, and washing on a magnet 4 times. For capture of all 3 targets, fluorescent beads were mixed with CP-MBs at a ratio of 50 MBs per target fluorescent bead and incubated for 30 minutes. Each sample was then separated on a magnet for enrichment and isolation of green, blue, or red beads from the initial 4-color mixture. The magnetic fraction was washed 3 times. Following magnetic capture, displacement probe corresponding to release of a single target (DP1: green, DP2: blue, DP3: red) was added to the sample at 5 µM final concentration, mixed gently with pipette for 1 minute, and then placed immediately on the magnet for collection of released beads in the supernatant. The sample was washed 3 times, and the supernatant fractions were pooled together. Purity of isolated fractions was quantitatively measured with flow cytometry (method described separately) and qualitatively evaluated with fluorescence microscopy (method described separately). Three independent trials were conducted to evaluate reproducibility. All conditions tested are summarized in Table S7.

Table S7. Conditions for the study of SMD specificity

	Capture antibody type	MB type	DP
Condition 1: Blue bead (Mouse IgG coating) release	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP1/CP2/CP3	DP2
Condition 2: Green bead (Rabbit IgG coating) release	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP1/CP2/CP3	DP1
Condition 3: Red bead (Human IgG coating) release	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP1/CP2/CP3	DP3
Condition 4: Control (non-specific target release)	Goat anti-Rabbit/EP1 Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP1/CP2/CP3	None

4-color bead sorting with SMD technology

Three sets of "target" fluorescent beads were prepared for this experiment: green beads were coated with rabbit IgG, blue beads with mouse IgG, and red beads with human IgG. Uncoated yellow beads were used as an "impurity" fraction. The four populations were pooled in even proportions into a single centrifuge tube to a total final concentration of $4x10^6$ beads/mL. DNA-antibody conjugates (Ab-EP) against rabbit, mouse, and human IgG were added to the mixture at a final concentration of 2.5 µg/mL each and incubated for 30 minutes. Meanwhile, 3 different CPs (CP1, CP2, and CP3) were mixed at a 1:2:2 ratio (5 µM total) and incubated with $3x10^7$ MBs for 30 minutes. Fluorescent beads were washed

through centrifugation (at 3000 g for 2 minutes) 4 times to remove excess antibody, and MBs were washed 4 times with a magnet to remove excess CPs. The fluorescent beads were then mixed with MB-CP at a ratio of 50 MBs per target fluorescent bead and incubated for 30 minutes. The sample was then separated on a magnet for enrichment and isolation of green, blue, and red beads from yellow bead "impurity". The sample was washed 3 times, and the supernatant fractions were pooled together. Following magnetic capture, DP3 (corresponding to red beads) was added to the sample at 5 μM final concentration, mixed gently with pipette for 1 minute, and then placed immediately on the magnet for collection of released beads in the supernatant. The sample was washed 3 times, and the supernatant fractions were pooled together. In the same way, displacement probes corresponding to blue and green beads (DP2 and DP1 respectively) were added sequentially, mixtures were washed 3 times, and supernatants were pooled. The remaining MB-bound fraction was also resuspended in equal volume of buffer to evaluate the amount of beads that were not released. PBS with 0.5% BSA was used throughout all steps of this study. All incubation steps were performed under gentle rotation at room temperature unless noted otherwise. Purity of isolated fractions was quantitatively measured with flow cytometry (method described separately) and qualitatively evaluated with fluorescence microscopy (method described separately). Three independent trials were conducted to evaluate reproducibility.

4-color bead sorting with sequential streptavidin-mediated target capture

Three sets of "target" fluorescent beads were prepared for this experiment: green beads were coated with rabbit IgG, blue beads with mouse IgG, and red beads with human IgG. Uncoated yellow beads were used as an "impurity" fraction. The four populations were pooled in even proportions into a single centrifuge tube to a total final concentration of $4x10^6$ beads/mL. First, for red bead capture, biotinylated goat anti-human antibody was added to the sample at 0.2 μg/mL, incubated for 15 minutes, and washed 4 times with centrifugation (at 3000 g for 2 minutes). The sample was then incubated with streptavidin-coated MBs (at 50 MBs per target bead ratio) for 30 minutes. The magnetic fraction was collected with the magnet to isolate captured "target" beads and washed 3 times. The supernatants with unbound beads were pooled together, pelleted through centrifugation (at 3000 g for 2 minutes), and resuspended back to the original sample volume of 100 μL for subsequent magnetic capture steps. In the same way, capture of blue and green "target" beads was performed through serial incubation with biotinylated secondary antibody (goat anti-mouse and goat anti-rabbit respectively), washing, incubation with MB-streptavidin, and magnetic collection. PBS with 0.5% BSA was used throughout all steps of this study. All incubation steps were performed under gentle rotation at room temperature. Purity of isolated fractions was quantitatively measured with flow cytometry (method described separately) and qualitatively evaluated with fluorescence microscopy (method described separately). Three independent trials were conducted to evaluate reproducibility.

Study of SMD-based sorting selectivity at varying target abundance

Two sets of "target" fluorescent beads were prepared for this experiment: red beads were coated with human IgG, blue beads with mouse IgG. Samples were prepared at a blue:red bead ratio at 1:1, 1:5, 1:20, and 1:100 by maintaining the concentration of red bead at $1x10^6$ beads/mL and reducing amount of blue beads accordingly. To evaluate the performance of SMD technology during target capture at

decreasing target concentrations, sample purity achieved with capture via biotin-streptavidin bond formation was compared to sample purity achieved with capture via oligonucleotide hybridization. For each blue:red bead ratio, the fluorescent beads were incubated with either biotinylated antibodies (goat anti-mouse, 0.2 μg/mL) or DNA-antibody conjugates (goat anti-mouse conjugated to EP2, 2.0 μg/mL) for 15 minutes and washed 4 times with centrifugation (at 3000 g for 2 minutes). Meanwhile, CP2-coated MBs were prepared by mixing $1x10^7$ MBs with CP2 at final DNA concentration of 1 μM, incubating for 15 minutes, and washing 4 times with magnet to remove excess CPs. Next, MB-Streptavidin (for samples previously incubated with biotinylated antibodies) or MB-CP2 (for samples previously incubated with DNA-antibody conjugates) were added to the sample at a constant concentration of 50 MBs per red fluorescent bead, incubated for 30 minutes, and isolated with using the magnet. Each magnetic fraction was washed 3 times, and the purity of the blue beads (rare target) in magnetic fraction was quantitatively measured with flow cytometry (method described separately).

To evaluate the performance of SMD during target release, both red and blue target beads were initially captured by MBs and then rare blue target beads were released via SMD. For each blue:red bead ratio, DNA-antibody conjugates against red (goat anti-human Ab/EP3) and blue (goat anti-mouse Ab/EP2) targets were added to the mixture at a final concentration of 2 μg/mL each, incubated for 15 minutes, and washed through centrifugation (at 3000 g for 2 minutes) 4 times. Meanwhile, complementary CPs (CP2 and CP3) were mixed at even proportion (1 μM each) with $2x10^7$ MBs, incubated for 15 minutes, and washed 4 times with a magnet. The fluorescent beads were then mixed with MB-CP at a constant ratio of 50 MBs per red fluorescent bead, incubated for 30 minutes, isolated with a magnet (thus enriching both red and blue targets), and washed 3 times. Following magnetic capture, DP2 (corresponding to release of blue beads) was added to the sample at 5 μM final concentration, mixed gently with pipette for 1 minute, and placed immediately on the magnet for collection of released beads in the supernatant. The sample was washed 3 times, and the supernatant fractions were pooled together. The purity of blue beads (rare target) in the supernatant fraction was quantitatively measured with flow cytometry (method described separately). Three independent trials for each experiment were conducted to evaluate reproducibility. All conditions tested are summarized in Table S8.

Table S8. Conditions for the study of SMD selectivity at varying target abundance

	Capture antibody type	**MB type**	**DP**
Condition 1: Blue bead (Mouse IgG coating) capture via biotin-streptavidin	Goat anti-Mouse/Biotin	Streptavidin	N/A
Condition 2: Blue bead (Mouse IgG coating) capture via DNA-antibody conjugate	Goat anti-Mouse/EP2	CP2	N/A
Condition 3: Blue bead (Mouse IgG coating) release via SMD	Goat anti-Mouse/EP2 Goat anti-Human/EP3	CP2/CP3	DP2

Flow cytometry

Flow cytometry on LSR-II (BD Biosciences) machine was used to count the number of fluorescent beads for calculations of purity and yield of isolated fractions. In order to compare relative bead counts, all samples were reconstituted in the same 100 µL volume, and a 96-well plate setup was used to consistently analyze an equal volume of each sample. A total of 5 channels were used for bead identification and enumeration. Forward scatter (FSC-A) was used to discriminate particles based on size, such that small particulates were not included in further analysis. Four different excitation lasers and 4 band-pass filters were used to uniquely identify a single bead color, as listed in Table S9. With this setup, beads were easily distinguished by their respective channel, and no compensation was necessary. For each specimen, at least 3000 beads were counted (lower counts were obtained for some cases in Figure S6, where low target concentration was used). Flow cytometry data was analyzed in FlowJo 9.3.3 (TreeStar). The total number of fluorescent beads was calculated by summing the counts from each of the four excitation/emission channels. Purities are reported as the number of beads of one color, divided by the total number of fluorescent beads within the sample. The overall yield is reported as the number of beads isolated into their respective fraction, divided by the number of beads of the same color counted within the reference sample that did not undergo magnetic sorting.

Table S9. Flow-cytometry parameters used for bead counting

Microsphere	Laser	Emission filter
Blue	350 nm	450/40 nm
Green	488 nm	530/30 nm
Yellow	561 nm	610/20 nm
Red	641 nm	710/50 nm

Fluorescence imaging

IX-71 inverted fluorescence microscope (Olympus) equipped with true-color camera (QColor5, Olympus) and spectral imaging camera (Nuance, CRI, covering 420-720 nm spectral range) was used for imaging of fluorescent beads. Low-magnification images were obtained with 20x dry objective (NA 0.75, Olympus) and high-magnification with either 40x oil-immersion objective (NA 1.30, Olympus) or 100x oil-immersion objective (NA 1.40, Olympus). Wide UV filter cube (330-385 nm band-pass excitation, 420 nm long-pass emission, Olympus) was used for imaging of blue beads, FITC LP cube (460-500 nm band-pass excitation, 510 nm long-pass emission, Chroma) for green beads, Rhodamine LP cube (530-560 nm band-pass excitation, 572 nm long-pass emission, Chroma) for yellow beads, and Cy5 LP cube (590-650 nm band-pass excitation, 665 nm long-pass emission, Chroma) for red beads. All images were acquired for beads deposited on the surface of a glass coverslip. For 4-color bead imaging, images obtained with individual filter cubes were false-colored and merged into a composite image in Photoshop (Adobe Systems). Background was removed, and brightness and contrast were adjusted for best visual representation and clarity. Representative example of image processing is shown in Figure S4.

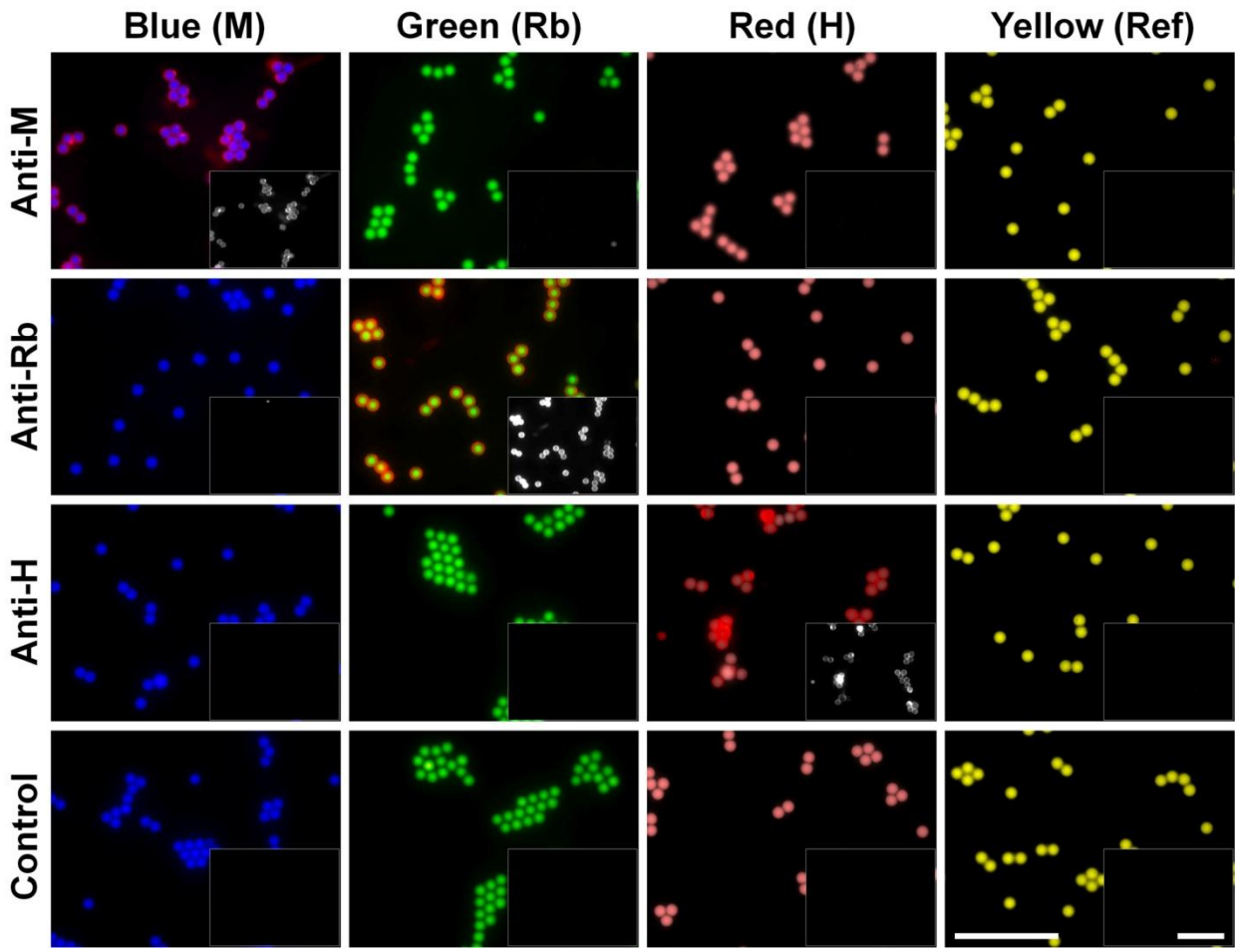

Figure S1. Validation of antigen coating on the surface of fluorescent beads. Presence and density of a specific antigen (IgG from mouse, rabbit, or human serum in this case) on the surface of fluorescent beads was confirmed with 2-step staining using biotinylated secondary antibodies for antigen detection and red-emitting quantum dots for fluorescent labeling. Red quantum dots (emitting at 655 nm) exhibit very large Stokes shift and high brightness, enabling easy unmixing of QDot signal from the fluorescence of target beads and quantitative analysis of staining intensity, while using single UV source for excitation of all samples. Positive staining appears as red ring around target beads on false-color images (merged QDot and fluorescent bead channels) and as high-intensity signal on black-and-white inserts (QDot channel only). All insert images (QDot channel) were normalized by imaging parameters, thus enabling direct comparison of staining intensity between different bead types. Blue, green, and red beads develop bright positive staining only for antigens that were conjugated to their surface (i.e. blue for mouse IgG, green for rabbit IgG, and red for human IgG), while showing no cross-reactivity with other antibodies or QDots alone (control). Unconjugated yellow beads, which were used as an "impurity" population throughout all studies, failed to produce staining with any antibody, as expected. Scale bar 50μm.

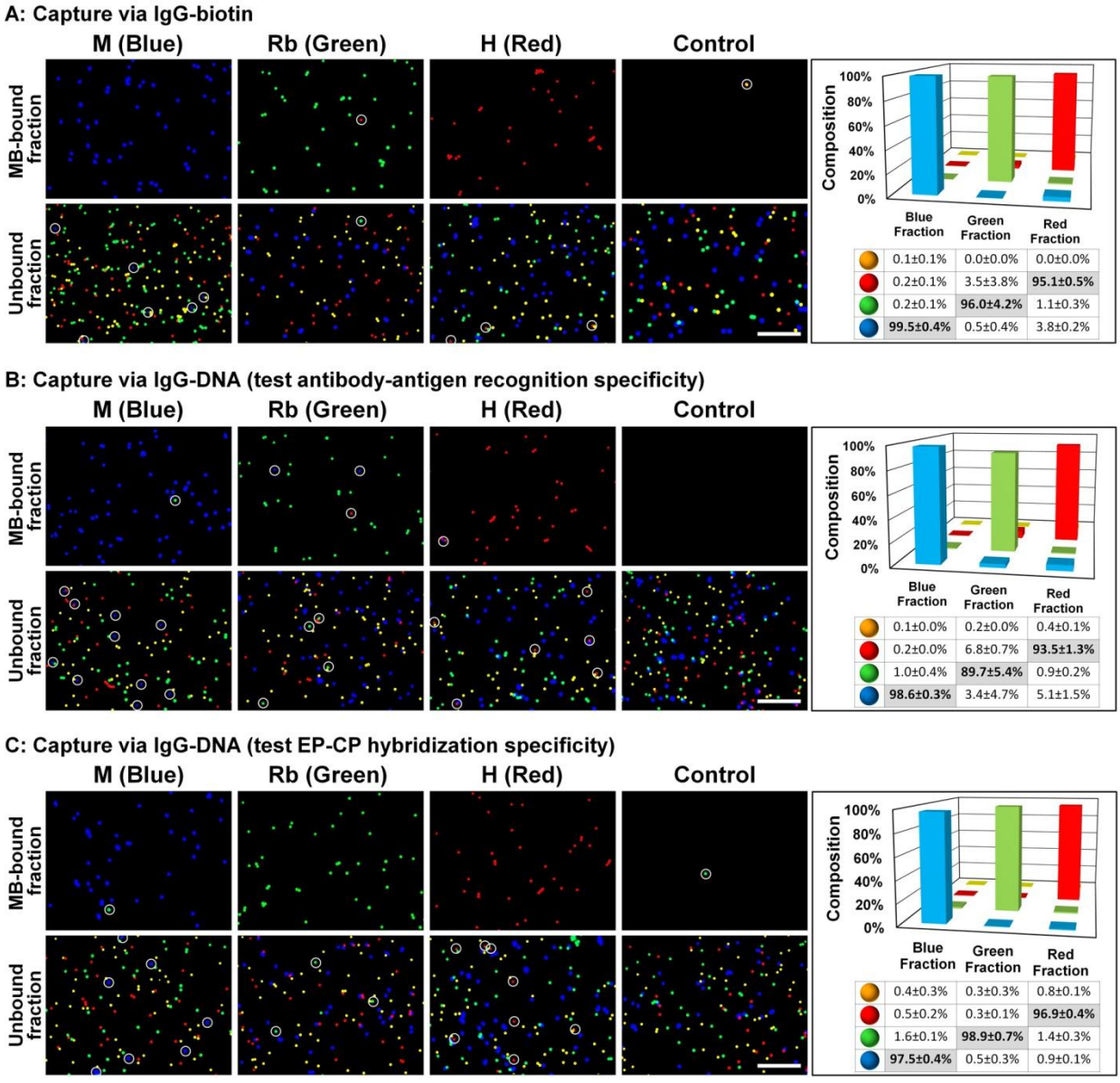

Figure S2. Specificity of antibody-antigen recognition and DNA hybridization during magnetic capture. Specificity of capture of each "target" bead (blue, green, or red) from a 4-bead mix was tested for standard magnetic isolation (via streptavidin-biotin bond formation) as well as for SMD technology. Purity of magnetic fraction captured was used as a measure of the degree of binding selectivity. For standard capture method, specificity of antibody-antigen recognition was tested (A), while for SMD technology, both specificity of antibody-antigen recognition by DNA-antibody conjugates (B) and specificity of DNA hybridization between encoding and capture probes (C) were evaluated. For each study representative fluorescence images are shown. Impurities are indicated by white circles. Scale bar 100µm. Quantitative analysis of purities of isolated fractions is presented in respective histograms and tables (right panels). Average value and standard deviation of 3 independent experiments is reported. As evident from (C), very high selectivity of target capture via EP-CP hybridization is obtained (with purities ranging from 96.9% to 98.9%). At the same time, antibodies exhibit some cross-reactivity,

producing red bead impurity in green bead captured fraction (due to binding of anti-rabbit antibody to human IgG on red beads) and blue bead impurity in red bead captured fraction (due to binding of anti-human antibody to mouse IgG on blue beads) above background values in both streptavidin-biotin bond-mediated capture (A) and SMD-based capture (B). In the control, only a minor non-specific binding is observed between MBs and "target" (blue, green, and red) as well as "impurity" (yellow) beads.

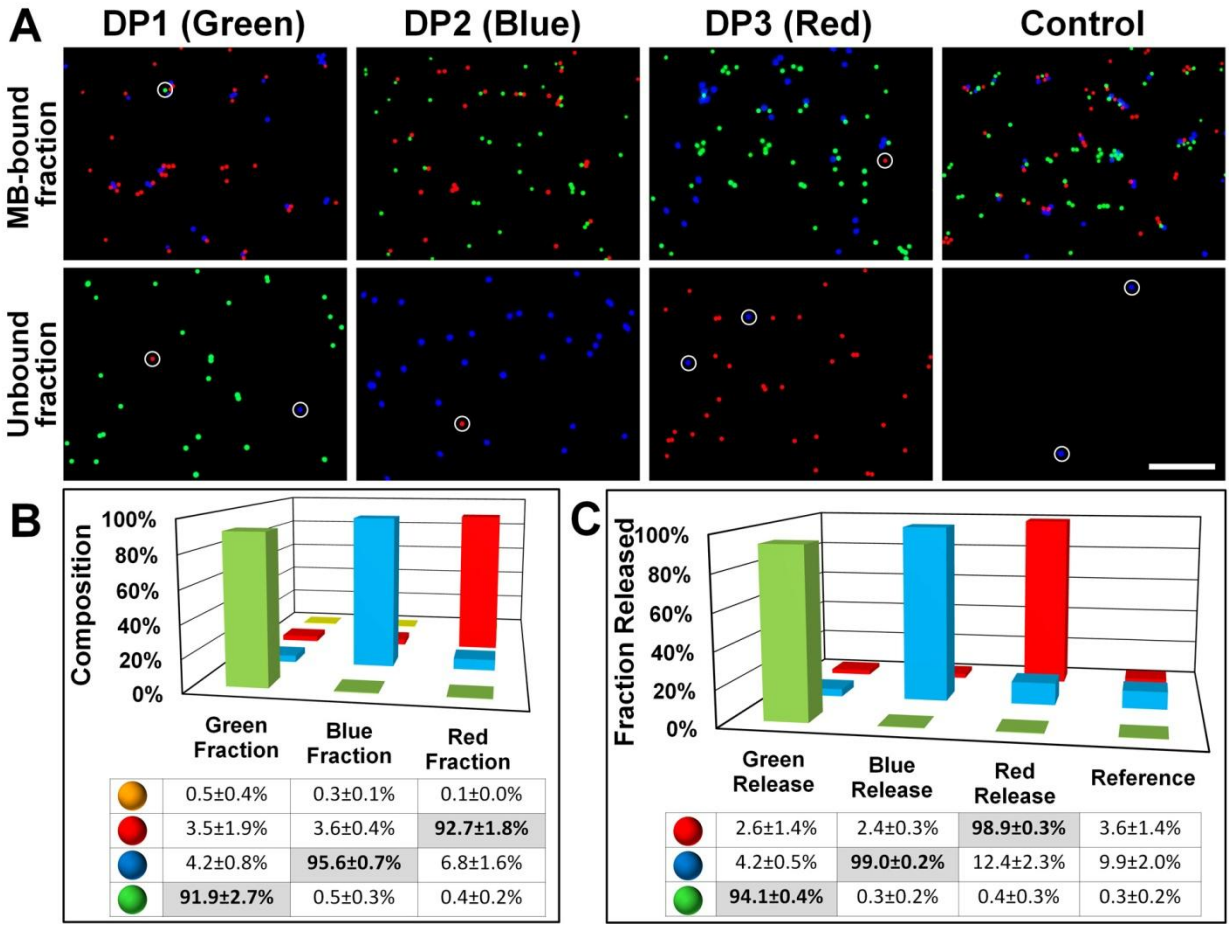

Figure S3. Specificity of strand-mediated displacement during target release. Specificity of SMD-based release of each "target" bead (blue, green, or red) from a MB-captured 3-bead mix was tested. Purity of isolated (unbound) fraction was used as a measure of the degree of DNA link displacement selectivity. Both qualitative evaluation with fluorescence microscopy (A) and quantitative analysis of flow cytometry data (B) indicate good selectivity of SMD, as evidenced by the moderate purities of isolated target fractions above ~92%. Trace amounts of yellow beads are present in all fractions due to initial non-specific capture from a 4-bead mix. Some non-specific release of red and blue targets observed here might, in part, be explained by the lower antigen coating on these beads compared to green beads, as indicated by lower QDot staining intensity in Figure S1. Effect of differential antigen surface coverage can be negated to some extent by adjusting the number of corresponding capture probes on MBs (i.e. increasing CP coverage on MB should improve bonding with targets exhibiting lower density of surface antigen). Following this logic, SMD-based 4-bead sorting study presented in Figure 4 of the main article was performed using a 2:2:1 ratio of CPs on MBs for red, blue, and green targets respectively, yielding an increase in purity above ~95%. To further confirm that non-specific target release results from the rupture of DNA link due to mechanical forces rather than due to SMD by non-complementary DPs, we compared the fraction of beads released in cases with and without DP added (C). As evident from the quantitative analysis of flow cytometry data, non-specific target release does not depend on the presence of DPs, showing no statistically significant difference between levels of impurities in all cases

($p>0.05$ based on two-tailed t-test), except for the blue bead impurity in the green bead fraction, which is actually lower than in control case. Average value and standard deviation of 3 independent experiments is reported in quantitative analysis (B and C). Impurities are indicated by white circles on fluorescence images (A). Scale bar 100µm.

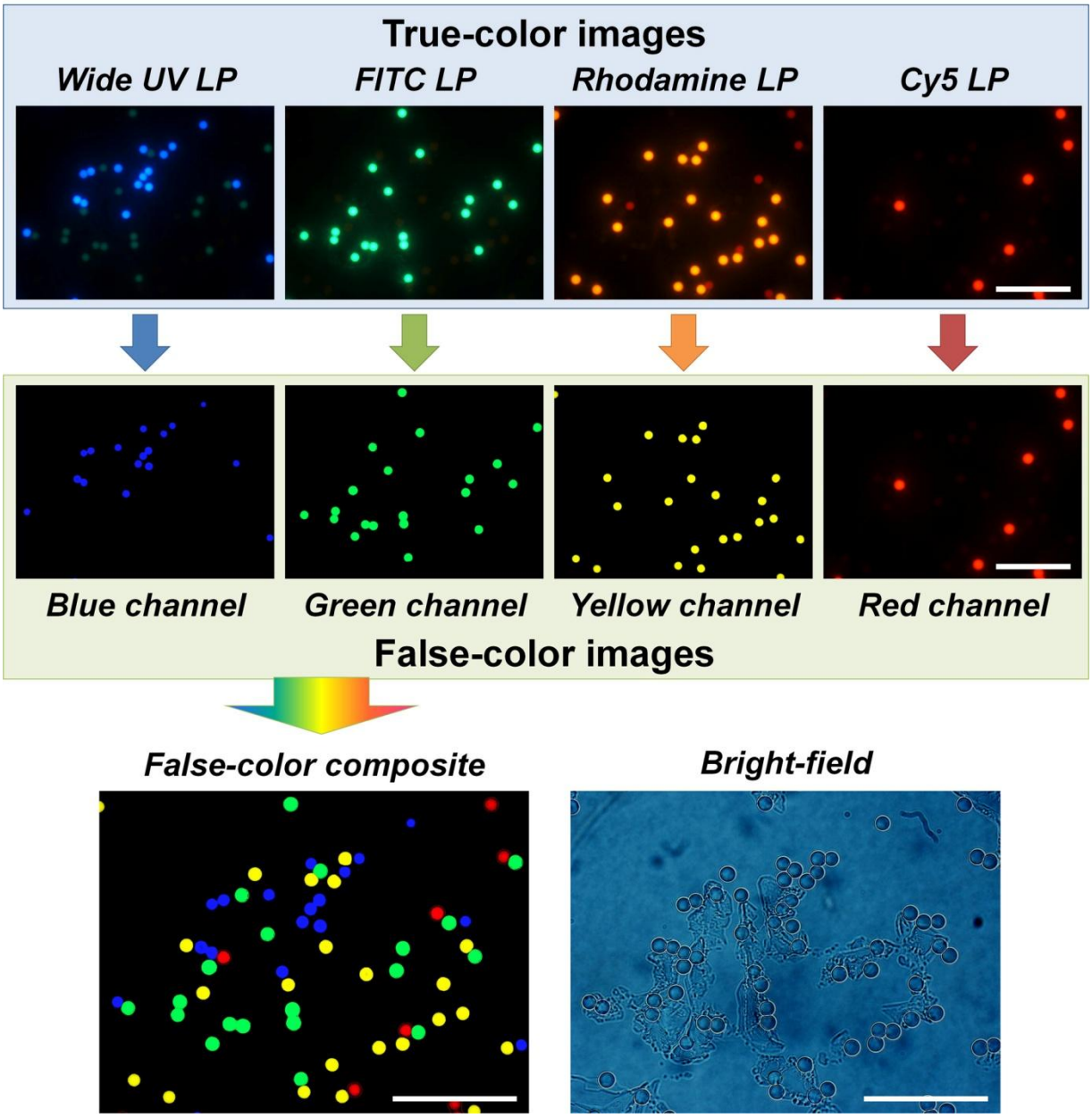

Figure S4. Schematic of fluorescence image processing. Each bead type is imaged with a filter cube best matching excitation/emission profile of a corresponding organic dye. With a set of 4 filter cubes indicated, individual true-color images contain mostly the signal from one fluorescent bead type, with minor spectral leak from adjacent colors. Application of a proper threshold eliminates such leaked signal along with the overall background, leaving only dominating signal from a single bead type. This signal is then false-colored for better clarity of presentation. Merging of four false-color channels produces composite 4-color image, where each fluorescent bead is accounted for (compared to bright-field image) and represented by its corresponding color, thus enabling quick qualitative evaluation of the sample composition. Scale bar 50µm.

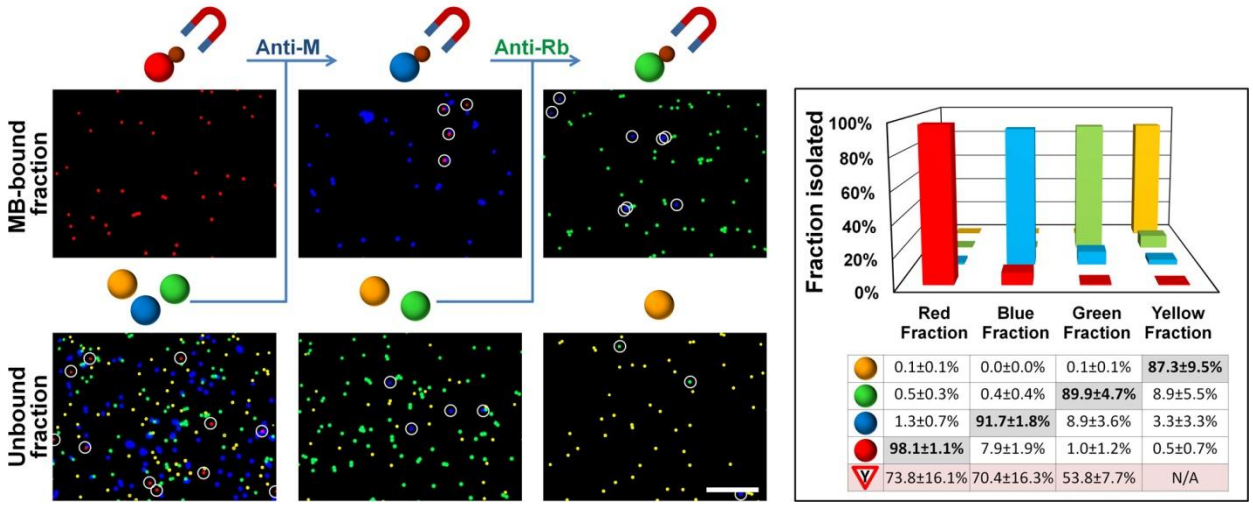

Figure S5. Multi-target sorting using sequential streptavidin-biotin bond-mediated target capture. In order to compare performance of SMD technology to multi-target sorting via sequential magnetic target capture, 4-color bead sorting was done using antigen recognition by biotinylated antibodies followed by magnetic isolation of individual targets via streptavidin-biotin bond formation with streptavidin-coated MBs. From a 4-bead mixture (consisting of 3 "target" fluorescent beads and yellow "impurity" at equal proportions) targets were isolated in the following order: red beads -> blue beads -> green beads, leaving "impurity" yellow beads in unbound fraction. Both qualitative evaluation with fluorescence microscopy (left panels) and quantitative analysis of flow cytometry data (histogram and table on the right) indicate high purity of the first isolated target (red, >98%) and markedly decreasing purity of subsequent isolation cycles (~92% purity for blue and ~90% for green beads), resulting mainly from contamination by the preceding target. Thus, not only is this process time consuming in comparison to SMD (requiring over 1.5 hours for each target isolated, for a total of ~5 hours), but also incomplete magnetic capture of any given target creates impurities for targets isolated in downstream steps. We attributed this effect to incomplete magnetic capture of a given target, where biotinylated antibody remained on the target surface, creating a biotin-labeled impurity for capture via streptavidin during the next isolation cycle. Although this issue can potentially be circumvented by using unique secondary-affinity ligands, availability of such ligands is limited, falling far behind the multiplexing potential offered by DNA. At the same time, potential incomplete target release with SMD does not result in additional impurities in downstream fractions, as unique DPs are used for release of each target. Also, it is worth noting that yields achieved with sequential sorting (red: 73.8 ± 16.1% , blue: 70.4 ± 16.3%, green: 53.8 ± 7.7%) are comparable to SMD approach (red: 68.2 ± 13.8%, blue: 74.5 ± 8.5%, green: 61.4 ± 8.2%, as reported in main article), with losses mainly attributed to bead loss during sample handling (washing/pipetting). Average value and standard deviation of 3 independent experiments is reported in quantitative analysis. Impurities are indicated by white circles on fluorescence images. Scale bar 100µm.

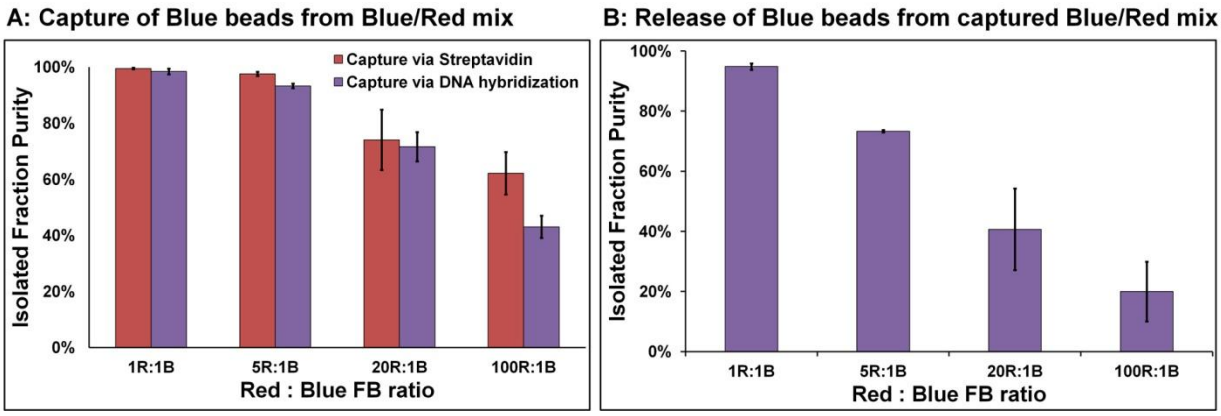

Figure S6. SMD-based sorting selectivity at varying target abundance. Purity of isolated fractions for targets at varying abundance was evaluated using two antigen-coated fluorescent beads: test target blue and reference red beads. Samples were prepared with test-to-reference bead ratio of 1:1, 1:5, 1:20, and 1:100 (holding concentration of reference and MBs constant). Purity of isolated test target fraction was determined by flow cytometry, with an average value and standard deviation of 3 independent experiments reported. Expectedly, purity of target captured from a mix of beads in solution (A) as well as purity of target released from a MB-bound fraction containing multiple bead types (B) suffers from decreasing test target abundance (and increasing proportion of reference beads). With this, target capture demonstrates higher selectivity compared to target release (e.g. purity of capture via DNA hybridization drops from 98.4 ± 1.0% to 43.0 ± 4.0% with target abundance decreasing from 50% to ~1%, whereas purity of release via SMD drops from 94.8 ± 1.0% to 20.0 ± 9.9%). However, it should be noted that an issue of isolation of rare targets at high purity is not unique to SMD technology, as similar drop in target purity at degreasing target abundance is observed with more conventional magnetic isolation via streptavidin-biotin bond formation (showing a drop of captured target purity from 99.5 ± 0.2% at equal bead proportion to 62.1 ± 7.5% at 1:100 test-to-reference bead ratio). Also, it is worth mentioning that the increase in error bar magnitude observed with decreasing test target abundance mainly results from a shot noise introduced by the low absolute number of test beads within the sample, with actual test bead counts from flow cytometry dropping from ~3000 to only ~30 when target abundance is decreased from 1:1 to 1:100.