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Rapid Multitarget Immunomagnetic Separation through Programmable DNA Linker Displacement

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Supporting Information

ABSTRACT: Immunomagnetic separation has become an essential tool for high-throughput and low-cost isolation of biomolecules and cells from heterogeneous samples. However, as magnetic selection is essentially a "black-and-white" assay, its application has been largely restricted to single-target and single-parameter studies. To address this issue, we have developed an immunomagnetic separation technology that can quickly sort multiple targets in high yield and purity using selectively displaceable DNA linkers. We envision that this technology will be readily adopted for experiments requiring high-throughput selection of multiple targets or further adapted for selection of a single target based on multiple surface epitopes.

Magnetic separation technologies have played a critical role in a variety of biomedical applications ranging from molecular diagnostics to cell-based therapy.[1] In contrast to other separation technologies, such as spatial separation via microarrays[2] and optical separation using fluorescence-activated cell sorting,[3] magnetic separation offers major advantages in terms of throughput and cost.[4,5] However, as selection is based on a single parameter (magnetization), only one target can be isolated at a time. Thus, intricate protocols are necessary to separate multiple targets from a sample (multitarget) or to isolate a single target based on multiple surface epitopes (multiparameter).[6] Since emerging research demands interrogation of increasingly complex and heterogeneous systems, in particular within the fields of immunology and oncology, there is a clear need for innovative magnetic separation technologies that enable multiplexed target sorting with high throughput, purity, and yield.

Several strategies have been proposed to incorporate multiplexing potential into magnetic separation. One promising approach is to use the size-tunable properties of magnetic nanoparticles for simultaneous isolation of several targets.[7] For example, Adams et al.[8] described a multitarget magnetic-activated cell sorting (MACS) technique that applied microfluidics and high-gradient magnetic fields to separate two bacterial targets using two distinct magnetic tags in >90% purity with >500-fold enrichment. However, multitarget sorting through "physical" encoding of magnetic particles requires sophisticated instrumentation and remains highly limited by the number of discrete magnetic tags that can be reliably separated. In a more straightforward approach, multiplexed separation can be achieved through multiple sequential rounds of single-target magnetic selection (Figure 1a). As an example, Semple et al.[9] used this method to sort CD4+ and CD19+ lymphocytes in a 4 h procedure.

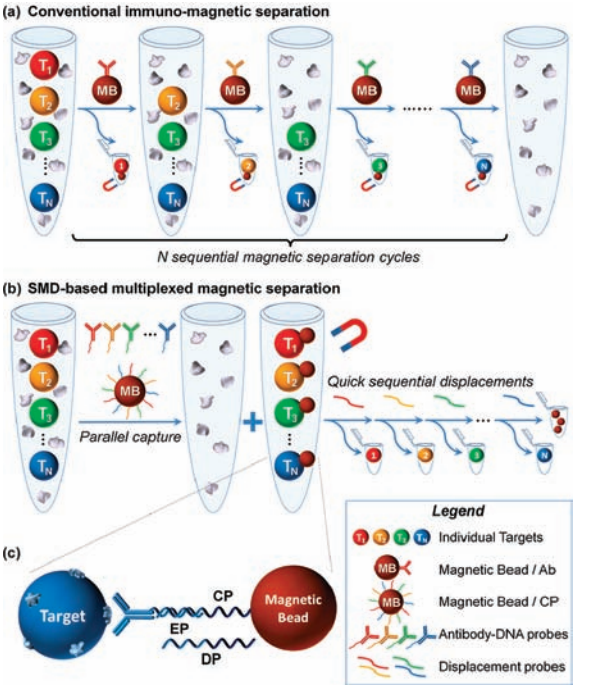

Figure 1. Schematic of multitarget immunomagnetic sorting. (a) Conventional sorting of multiple targets involves lengthy sequential magnetic isolation steps. (b) In contrast, SMD-based sorting technology captures all targets of interest simultaneously and then rapidly sorts them through release of the magnetic bead (MB)−target link. (c) The target is captured through immunorecognition by a DNA-encoded antibody and partial hybridization with the capture probe (CP) on the MB. Selective target release is achieved through sequence-specific encoding probe (EP) displacement due to a more favorable hybridization between the CP and the displacement probe (DP).

Nevertheless, despite its simplicity, sequential sorting is time-consuming, and lengthy separation protocols often result in an alteration of the biological state of the target (e.g., gene expression and/or viability of cells),[10] rendering such an approach unsuitable for many applications.

Complementary to the challenge of spatial or temporal segregation of target-carrying magnetic particles is the issue of incorporating multiplexing capability within the target capture method itself. Magnetic selection can be applied in one of two formats: (1) direct selection, where the affinity ligand is directly

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coupled to the magnetic nanoparticle, and (2) indirect selection, where targets are first incubated with an excess of primary affinity ligand and then captured by magnetic particles via a secondary affinity ligand. As the indirect method allows for optimal affinity ligand orientation on target, a "signal amplification" effect is observed, improving the yield and purity.[5] Furthermore, the indirect method enables the utilization of a wide range of commercial affinity ligands without the need for further modification. At the same time, this approach is particularly challenging to multiplex, given the limitations in selectivity of primary–secondary affinity ligands (e.g., biotin–streptavidin and primary–secondary antibody links). In this regard, DNA–antibody conjugates represent a powerful tool for multiplexed indirect selection, as first demonstrated by Heath and co-workers[2] on a DNA microarray platform and recently applied for characterization of secreted proteins from single cells, opening exciting opportunities in study of human immune cell responses.[11] However, the small surface area of microarray chips hampers large-scale sorting applications. In this context, incorporation of molecular encoding *into* the conventionally single-parameter magnetic selection platform holds the key to achieving truly multiplexed, high-throughput target sorting.

Here we report a rapid multitarget immunomagnetic separation technology that combines the extensive multiplexing capacity of DNA–antibody conjugates and the high selectivity, throughput, and simplicity of magnetic isolation by employing a unique approach involving strand-mediated displacement (SMD) of DNA linkers. Our key insight is that the combination of spatial and temporal segregation could offer simultaneous selection of multiple target populations from a heterogeneous sample followed by quick isolation of individual targets through SMD, inspired by the fast kinetics and selectivity of SMD in DNA motors and walkers.[12] The major steps of SMD for multitarget sorting are illustrated in Figure 1b. In the first step, antibodies encoded with distinct DNA sequences [encoding probes (EPs)] bind their identifying antigens on target populations. Next, magnetic beads (MBs) coated with capture probes (CPs), which partially hybridize with their corresponding EPs, enable simultaneous magnetic selection of all targets of interest. In the second step, the magnetically enriched targets are released one population at a time through SMD upon addition of a displacement probe (DP). Probe assembly is schematically illustrated in Figure 1c. The DP binds selectively to an exposed "toehold" region on the CP and then "unzips" the EP from its original binding site as a result of the longer complementarity between the CP and DP, thus breaking the MB–target link. In this way, multiple targets can be quickly isolated through serial addition of DPs. In direct contrast to the conventional multicycle magnetic separation (Figure 1a), SMD technology employs only a single round of slow immunorecognition, and the following SMDs are remarkably rapid (with displacement half-times of only ~0.2 s[13]). Therefore, with sorting of N targets ($N > 1$), our technology should offer a significantly shortened assay time (1 h + rapid SMDs vs N h), which would be highly desired for preserving the native states and biofunctionalities of the isolated targets.

Although SMD-based magnetic sorting represents a platform technology applicable to a wide range of analytes, the proof-of-concept work reported here most closely resembles the conditions necessary for sorting of live cells, which is a significant application of this technology. In our setup, four-color fluorescent beads of size similar to mammalian cells were used as a model system for the development of the SMD technology.

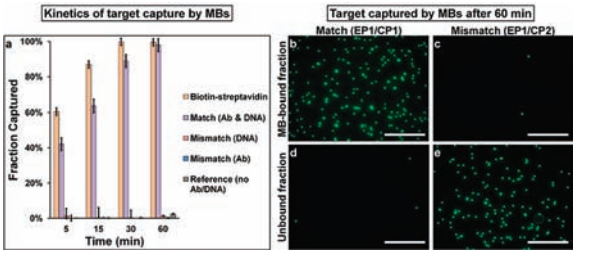

Figure 2. Kinetics and specificity of target capture. (a) Quantitative analysis of target capture through DNA hybridization. Averages of three separate experiments are shown. Error bars indicate one standard deviation. (b–e) Qualitative evaluation of target capture using fluorescence microscopy. Targets (green) were retained in the MB-bound fraction for the complementary EP1/CP1 link (b, d) but not for the noncomplementary EP1/CP2 link (c, e). Scale bar = 250 μm.

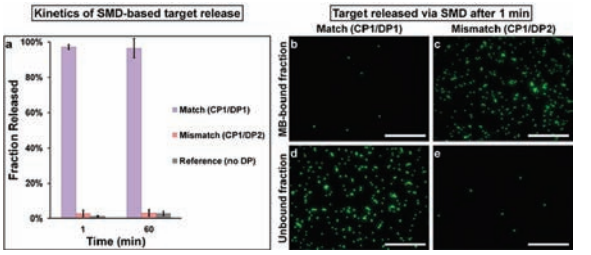

Figure 3. Kinetics and specificity of target release. (a) Quantitative analysis of SMD-based target release kinetics. Averages of three separate experiments are shown. Error bars indicate one standard deviation. (b–e) Fluorescence microscopy evaluation of target release yield and specificity. Nearly complete release of targets (green beads) into the supernatant was obtained with complementary (b, d) but not noncomplementary (c, e) DPs. Scale bar = 250 μm.

Fluorescent beads are easy to identify and count and thus are ideal for technology characterization and validation. Three of the colored beads were surface-modified with a species-specific immunoglobulin G (IgG), which served as an identifying antigen; the fourth bead was unmodified and served as an impurity population to be removed (Figure S1 in the Supporting Information). To demonstrate multitarget enrichment and isolation, three sets of DNA sequences were designed with 16 base pair (bp) overlap between the CP and EP and a 6 bp toehold region, allowing for a total of 22 bp DP binding (Table S1).

Considering that the length of target separation methods can dramatically impact the biological characteristics of the target, we first investigated the kinetics and specificity of the magnetic capture and SMD-based release using single-color (green) microspheres. As shown in Figure 2a, target capture through hybridization between EPs and CPs slightly lagged behind that mediated by the gold-standard streptavidin–biotin interaction, but both reached nearly complete target capture (>98%) at the 1 h time point. Representative fluorescence micrographs taken for the unbound (supernatant) and MB-bound fractions for matched and mismatched DNA sequences after a 60 min incubation period are shown in Figure 2 b–e. Clearly, the matched EP1/CP1 pairs led to nearly complete capture of target beads in the MB-bound fraction, whereas the mismatched sequences (CP2) produced only negligible nonspecific binding. High specificity of target capture through DNA hybridization was

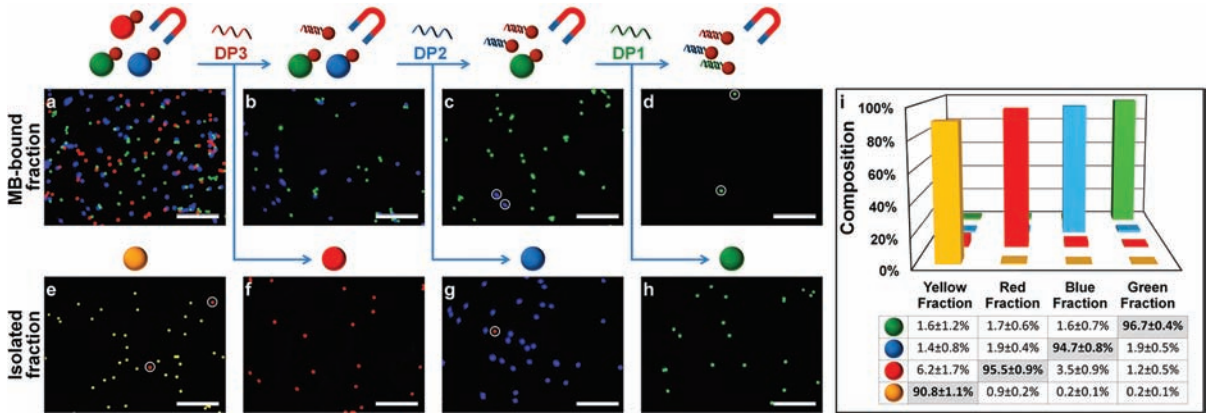

Figure 4. Rapid multitarget SMD-based sorting. (a−h) Fluorescence microscopy of (a−d) MB-bound and (e−h) isolated fluorescent bead fractions at different stages of SMD-based sorting. Immunomagnetic isolation of three targets (red, green, and blue beads) retained the targets within the MB-bound fraction (a), leaving the "impurity" (yellow beads) in the supernatant (e). Sequential introduction of target-specific DPs led to selective release of a target into the supernatant (f−h), leaving nondisplaced targets in the MB-bound fraction (b−d). Impurities are indicated by white circles. Images were processed according to procedure outlined in Figure S4. Scale bar = 100 μm. (i) Quantitative analysis of the purities of the isolated fractions using flow cytometry. Averages and standard deviations from three separate experiments are shown.

observed with a set of systematically designed negative controls (Figure 2a, Table S2, and Figure S2).

Following the investigation of magnetic capture via DNA linkers, we probed the specificity and kinetics of target release via SMD (Table S3). Target release was measured after incubation periods of 1 or 60 min (Figure 3a). Remarkably, even after 1 min of SMD, release of the target beads into the supernatant was nearly complete (97%) for the complementary DP, while only minimal nonspecific release (<3%) was observed for the mismatched DP and the reference, demonstrating the high selectivity and speed of SMD despite potential issues such as steric hindrance. Fluorescence microscopy (Figure 3b−e) and further control studies (Figure S3) corroborated this conclusion. The outstanding kinetics and selectivity of SMD results from three fundamental features of this technology. First, as mentioned earlier, DNA displacement with longer complementary strands is an extremely selective and fast process. Second, diffusion of small DNA strands to microbeads is much faster than diffusion between two microparticles. Third, a profound impact originates from the differential concentrations in the separation reaction. In the case of conventional immunomagnetic separation, the two "reactants" are MBs and targets, which are often used in the femto- to picomolar concentration range.[10,14] In our SMD approach, on the other hand, the two "reactants" are MB−target complexes and single-stranded DNA with concentrations typically in the micromolar range, which is 6−9 orders of magnitude higher than that of the MBs, thus promoting the reaction rate. It is noteworthy that there was virtually no nonspecific target release for incubation periods of 1 and 60 min, indicating that the 16 bp EP−CP overlap offers sufficient long-term stability (under gentle rotating agitation). However, vigorous washing resulted in more noticeable nonspecific target release with DNA links relative to the biotin−streptavidin bond (data not shown). Such behavior is not surprising, as the DNA−DNA binding strength (20−50 pN for 10−30 bp)[15] is considerably lower than that of the biotin−streptavidin bond (300 pN).[16] Poor bond stability can be partially addressed by using longer DNA probes while the maintaining binding specificity through careful sequence design. Furthermore, this effect may be negligible for separation of smaller analytes (proteins, bacteria), as shear forces decrease along with the particle size.

Next, we proceeded to demonstrate the utility of SMD technology for quick sorting of multiple targets from a mixed sample, transforming conventional single-parameter magnetic separation into a multiplexed format. To begin, four populations of fluorescent beads were pooled into a single sample at even proportions. Beads of the three primary colors were tagged with distinct antigens on the surface (green with rabbit IgG, blue with mouse IgG, and red with human IgG). Unmodified yellow beads served as an impurity to be removed. Three antibodies specifically recognizing those surface antigens were tagged with unique encoding oligonucleotides (EP1, EP2, and EP3) and incubated with the mixture sample. In parallel, MBs were modified with CP1, CP2, and CP3 complementary to the respective EPs. According to the procedure schematically illustrated in Figure 1b, the red, blue, and green beads were simultaneously enriched magnetically and subsequently isolated one at a time by sequential addition of the cognate DPs. Qualitative evaluation using fluorescence microscopy (Figure 4a−h) and quantitative analysis using flow cytometry (Figure 4i) revealed excellent purity (red, 95.5%; blue, 94.7%, green, 96.7%) and reproducibility (standard deviation < 1%) for each of the isolated fractions. We also calculated the overall yield of each target (red, 68.2 ± 13.8%; blue, 74.5 ± 8.5%l green, 61.4 ± 8.2%) by dividing the number of beads collected by the number in the reference sample, which did not undergo the magnetic separation procedure. The sources of the loss may include dead volume in pipet tips, retention of beads on centrifuge tube walls/caps, incomplete magnetic capture, nonspecific release, and incomplete release of targets. These parameters deserve further optimization, especially for separation and characterization of rare targets such as stem cells or circulating tumor cells. Nevertheless, the yield and throughput reported herein mark a considerable improvement over previous multitarget magnetic selection methods[8] while requiring no sophisticated instrumentation.

To highlight further the benefits of SMD technology, we contrasted our approach with conventional sequential immunomagnetic sorting (Figure S5). Here, using biotinylated antibodies and streptavidin-coated MBs, we again isolated three targets from an initial mixture of four bead populations. Interestingly, not only was this protocol time-consuming (5 h vs 1.5 h for SMD-based sorting), using a single secondary affinity label (streptavidin) resulted in a marked decrease in target purity, as any antibody-biotin-labeled target not captured during its corresponding magnetic selection step

becomes an impurity for subsequent targets. In contrast, the SMD sorting protocol demonstrated higher purities because complete target release via SMD is more favorable than complete target capture by MBs as a result of improved reactant kinetics, and the unique DPs prohibit cross-reactivity between sequential isolation steps. At the same time, the two procedures share major sources of target loss, thus offering similar overall yields for each target.

Finally, we tested the dynamic range for multitarget cell sorting, as large variations in target concentration can be encountered in certain practical applications. Here we found that changing the ratio of two targets from 1:1 to 1:100 resulted in a considerable decrease in purity of the rarer target during both the capture (98 to 43%) and release (98 to 20%) steps (Figure S6). Inferring from the control studies (Figures S2 and S3) and the similar performance of biotin−streptavidin-mediated capture (Figure S6), we believe that the major causes for this effect are antibody cross-reactivity, incomplete washing, and rupture of DNA links. Therefore, further protocol optimization is required for applications where targets are present in a high dynamic range.

In summary, we have developed a simple yet robust multitarget immunomagnetic separation technology based on the clever concept of DNA strand-mediated displacement. Magnetic separation serves as a high-throughput platform for sorting a wide array of targets, while DNA−antibody conjugates enable highly multiplexed indirect selection, which confers important benefits of high target yield and purity. Finally, rapid target sorting is enabled by SMD technology via fast DNA binding and displacement, fast diffusion of relatively small DPs, and high concentrations of DNA reactants. Overall, the combination of these critical components provides a unique solution to a long-standing problem in magnetic separation: multitarget sorting with high yield, purity, and throughput.

We believe that the versatility of this SMD-based separation platform will enable a number of powerful applications, such as live-cell sorting, as both target capture through DNA hybridization and SMD can be carried out in a range of cell- and biocompatible buffers and under ambient or chilled conditions.[2] Furthermore, SMD technology might streamline the implementation of conventional immunomagnetic selection, where MBs must be removed to avoid interference with further analysis or adverse effects on the target biological state[17] as well as to allow further isolation via a different surface epitope of the same target, thus enabling multiparameter selection. Finally, the availability of DNA−antibody conjugates on the target surface following MB release should enable the isolation of rare targets by applying several selective rounds of magnetic capture and SMD release.

■ ASSOCIATED CONTENT

Supporting Information. Abbreviations; experimental materials and methods; validation of antigen coverage on target beads; study of target capture and release specificity; multitarget sorting via standard sequential magnetic capture; and evaluation of SMD technology for separation of targets at varying abundance. This material is available free of charge via the Internet at http://pubs.acs.org.

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