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Highly Avid Magnetic Bead Capture: An Efficient Selection Method for de novo Protein Engineering Utilizing Yeast Surface Display

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Abstract

Protein engineering relies on the selective capture of members of a protein library with desired properties. Yeast surface display technology routinely enables as much as million-fold improvements in binding affinity by alternating rounds of diversification and flow cytometry-based selection. However, flow cytometry is not well suited for isolating de novo binding clones from naïve libraries due to limitations in the size of the population that can be analyzed, the minimum binding affinity of clones that can be reliably captured, the amount of target antigen required, and the likelihood of capturing artifactual binders to the reagents. Here, we demonstrate a method for capturing rare clones that maintains the advantages of yeast as the expression host, while avoiding the disadvantages of FACS in isolating de novo binders from naïve libraries. The multivalency of yeast surface display is intentionally coupled with multivalent target presentation on magnetic beads—allowing isolation of extremely weak binders from billions of non-binding clones, and requiring far less target antigen for each selection, while minimizing the likelihood of isolating undesirable alternative solutions to the selective pressure. Multivalent surface selection allows 30,000-fold enrichment and almost quantitative capture of micromolar binders in a single pass using less than one microgram of target antigen. We further validate the robust nature of this selection method by isolation of de novo binders against lysozyme as well as its utility in negative selections by isolating binders to streptavidin-biotin that do not cross-react to streptavidin alone.

Keywords

yeast surface display; combinatorial library screening

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Introduction

The growing applications of yeast surface display (YSD) reflect several advantages over alternative technologies.¹ YSD mimics the protein processing machinery of higher eukaryotes, thereby minimizing expression biases against the complex protein structures that require foldases and chaperones for efficient assembly, and allowing presentation of sequence variants and structural architectures less accessible to procaryotic hosts.² In a recent study, in which the same cDNA library was transformed into both phage and yeast display systems, YSD identified three times as many binding clones, and when cloned back into phage, these additional sequence variants could not be expressed as functional protein.³ Additionally, YSD is amenable to engineering increased expression, stability, pH sensitivity, and enzymatic properties such as enantioselectivity.^{4–6} Thus, yeast serve as an excellent host organism for presentation of proteins for diverse engineering goals.

Flow cytometry (FACS) is the method of choice for quantitative selection. FACS permits facile comparison between display level and antigen binding, allowing for selection without expression level bias, and discrimination between clones with very fine differences in affinity.⁷ FACS also allows the stringency of the selection to be set in live time based on actual sample data rather than being rigidly predefined. Unfortunately, while well suited for affinity maturation, FACS is problematic for the isolation of de novo binding interactions. Flow cytometry simply cannot directly query a library for the ability to recognize a target antigen, but instead relies on the acquisition of fluorescence as a proxy for antigen binding. Most commonly this is attempted by tagging the antigen and using a fluorescent secondary reagent to identify the clones that have bound the target antigen. However, the naïve libraries used in de novo selections are not biased toward any particular epitope or target molecule, and any binding interaction resulting in acquired fluorescence will be selected. Accordingly, direct interactions between the yeast-displayed construct and fluorescent reagents satisfy the selection criteria. In fact, such an interaction regrettably represents the fittest solution to the selective pressure. Additionally, an interaction with the reagent may also be the most accessible result as the fluorophore itself may present the most electronically distinct epitope, and may be present at multiple sites, granting it an avidity advantage relative to the desired interaction with the target molecule. These factors promote binding to the secondary reagent above and beyond any target quality issues such as the heterogeneity, glycosylation, questionable purity, or improper folding that may trouble antigens produced for the purpose of such selections.

To avoid such undesirable outcomes, steps are generally taken to minimize the chances of isolating secondary binders and to bias the selection to favor an interaction with the desired target. For instance, incubation with secondaries can be performed for a minimum amount of time and at a minimum concentration, the target itself can be directly conjugated with the fluorescent tag using a site-specific modification,⁸ and positive selections against target and secondary can be alternated with negative selections against the secondary alone. However, even these modifications may not prove sufficient to steer the system toward isolating the desired interaction.

The efficiency of FACS selections compounds these difficulties and poses a serious impediment to the isolation of de novo interactions. FACS machines analyze and sort on the order of 10^8 cells per hour. To ensure that almost all clones are analyzed at least once, a 10-fold excess of the library diversity should be analyzed, requiring a day of sort time for a library of 10^8 cells, and setting this size as a reasonable upper limit for the diversity that can be easily analyzed with this degree of diligence.

Unfortunately, by one estimate, a library of 10^{10} cells may be necessary to obtain binders with affinities in the nanomolar range.⁹ As a recent example, a library of 2.3×10^7 clones was FACS selected to identify binders to lysozyme, and the initial hit from this search had micromolar affinity—so low that it could not accurately be measured using FACS.¹⁰ The likely absence of nanomolar binders against any given *de novo* target is a considerable obstacle for two reasons. First, weaker binding interactions require that more antigen be present in the incubation steps to be captured, as by definition only half of the antigen binding sites will be occupied when a micromolar binder is incubated in a micromolar concentration of antigen. For a weak interaction, this can translate into a significant quantity of antigen. For example, FACS selections of naïve libraries typical require on the order of 10 nmole (hundreds of micrograms) of target antigen.

Additionally, weak interactions have very fast dissociation rates. The half-life of a micromolar interaction is generally on the order of 5 s, and nanomolar binders only a few minutes. Such a rapid dissociation rate is extremely unfavorable for a detection scheme requiring a separate incubation in fluorescent secondary followed by a lengthy selection on a flow cytometer. While preincubating the secondary with the antigen before labeling the yeast or using a directly conjugated antigen can help to offset such a rapid dissociation rate, it increases the probability of isolating binders to either the secondary or the conjugated fluorophore as described previously.

A new selection methodology surpassing these barriers to isolating *de novo* binders from naïve libraries is needed. Given the confounding factors described here, the ideal yeast selection platform would first use no tags, secondary reagents, or proxy read-outs of antigen binding—preventing alternative solutions to the selective pressure. Secondly, it would allow rapid screening of upwards of 10^{10} cells, so that the size of the library that can be thoroughly searched is limited by library construction rather than by the selection method. Third, it would have the ability to capture even the very weak binders likely to be present in a naïve library. Finally, it would do so in a rapid, inexpensive, and straight-forward manner without requiring the production of large quantities of the target antigen.

Accordingly, we have considered alternatives to FACS with the goal of defining a technique for isolation of even very weak binders from large populations using a minimum amount of antigen and secondary reagents. To capture weak interactions, we have coupled the multivalency of YSD with antigen multivalency—allowing avidity effects to drive the selection. Simple mathematic and geometric treatments of bivalent binding interactions demonstrate the dramatic effects of avidity.¹¹ In a simplified system involving two binding sites separated by a flexible linker, bivalency boosted the effective affinity of the interactions by four orders of magnitude.¹² As yeast typically display approximately 50,000 copies of the molecule to be engineered, there is likely the opportunity to reach interactions that are highly multivalent, and thus have overall dissociation rates of days as opposed to their monovalent dissociation rates of seconds.

A concomitant benefit of multivalent antigen display is that it greatly increases the effective concentration of the antigen. In fact, the high effective concentration drives the apparent increase in affinity. Once a yeast cell associates with surface bound antigen, it encounters a very high local concentration of antigen, favoring the formation of additional interactions. This fortuitously allows a much smaller amount of antigen to be used in the selection. As long as the multivalent antigen and yeast are well mixed—affording each yeast cell the opportunity to contact and associate with the antigen during the selection, high local concentrations can substitute for the high systemic concentrations used in a FACS selection, dramatically decreasing the amount of antigen required.

As a means to present multivalent antigen and capture weak interactions we have investigated the use of solid surfaces. Successful in phage selections for years, *E. coli* selections more recently,¹³ and having precedence in yeast selection in the form of mammalian cell-based selections employing density gradient centrifugation^{14,15} or monolayer panning,¹⁶ the use of synthetic multivalent surfaces represents a logical extension of past methodology. Here we test a variety of surfaces and demonstrate that extremely low affinity binders can be captured with high efficiency from large populations using multivalency and we validate multivalent antigen presentation as a selection method by successfully isolating confirmed de novo binders from naïve libraries.

Materials and Methods

Yeast strains and plasmids

All plasmids were transformed into EBY100 yeast using the EZ Yeast Transformation II kit (Zymo Research D2004) according to the manufacturer's instructions. Surface displayed constructs were all expressed as *aga2p* fusions on the galactose-inducible, tryptophan selectable plasmids pCT-CON or pCTCON2 and grown and induced as described previously.¹⁷ Table 1 lists the plasmids used in this study.

Surfaces

Nickel and streptavidin-coated wells (Qiagen), immuno-tubes (Nunc), tissue culture plastic (BD Biosciences), glass slides and nitrocellulose membrane (VWR International), agarose talon resin (Clontech), and magnetic beads (Invitrogen 101-01D, 110-47, and 162-03) were used as multivalent surfaces.

Flow cytometry

Cells were labeled for FACS analysis as necessary.¹⁷ Lysozyme-binding clones were identified via labeling with biotinylated hen egg lysozyme (Sigma) followed by streptavidin-PE (Invitrogen), or via their c-myc tag by means of a chicken anti-c-myc antibody (Invitrogen) followed by goat anti-chicken 488 secondary (Invitrogen). His tagged clones were labeled with biotinylated mouse anti-his6 antibody (Qiagen) followed by streptavidin-PE. Samples were analyzed on a Coulter Epics XL, or sorted on FACS Aria cytometers after labeling.

Pilot selections

Briefly, populations of binding and nonbinding yeast, usually at a ratio of 1:100 or 1:1000, were mixed and incubated with the materials, allowed to bind, and then washed. Growth media was added to the material and bound yeast were allowed to divide off for a period of time before enrichment and yield were measured by either FACS or plating, as described previously.²²

Agarose Talon Resin—Binding and nonbinding yeast were mixed and incubated with 2 mL of washed and equilibrated resin in a 15 mL conical for 1 h. The yeast slurry mixture was then poured into a column and washed and eluted per the manufacturer's instructions. Fractions were diluted into growth media, induced, and FACS was performed to determine the prevalence of binders after the selection.

Plastic, Glass, Nitrocellulose, and Immuntubes—These materials were incubated in a concentrated solution of lysozyme (>2 mg/mL) in PBS for at least 2 h at room temperature, or 4°C overnight on a rocker platform, allowing coating to occur by passive adherence. A western blot was performed on the nitrocellulose sample in to confirm the lysozyme coating. The surfaces were washed with PBS-BSA (0.1%) three times for 5 min to remove unbound lysozyme. Binding and nonbinding yeast were incubated with the materials for at least 1 h at

room temperature on a rocker platform. Washes were performed to remove unbound yeast, and growth media was added allowing bound yeast to divide off the surfaces before an aliquot was removed for subsequent analysis by either FACS or plating.

Nickel and Streptavidin Coated Wells—These functionalized plastics were handled similarly except nickel wells were left bare, and streptavidin wells were incubated with biotinylated lysozyme for the selection.

Biotin Binder Magnetic Beads—Except where otherwise noted, 2.5×10^6 streptavidin coated beads (Invitrogen 110-47) were washed twice in PBS-BSA (0.1%) and then incubated in 100 μ L of 350 nM biotinylated lysozyme overnight at 4°C in a microfuge tube on a rotator. Beads were washed twice and then mixtures of binding and nonbinding yeast were added to the beads and allowed to bind for 1–3 h at 4°C on a rotator. The beads were then washed and diluted and plated to quantitatively determine enrichment and yield.

M-270 Amine Magnetic Beads— 6×10^8 amine functionalized beads (Invitrogen 162-03) in DMSO were incubated with a 100-fold excess of solid, dry fluorescein-PEG-NHS (Nektar), or p-SCN-Bn-DOTA (Macrocyclics) and a 1,000-fold excess of TEA and incubated at room temperature overnight on a rotator, then washed twice with DMSO. Bound DOTA was loaded with either gallium or yttrium by adding a 100-fold excess of metal and incubating in a 37°C shaker for 5 h in 0.4 M ammonium acetate. Conjugated beads were then washed with PBS-BSA (0.1%) and used in selections as described above.

Talon Magnetic Beads—Twenty-five microliter of talon beads (Invitrogen 101-01D) were washed twice in PBS-BSA (0.1%) and then mixed with populations of yeast and handled as described above.

De Novo library selection

Two fibronectin libraries, with variation in both loop length and sequence were used to isolate de novo lysozyme binders. $1\text{--}2.5 \times 10^9$ yeast from each library were first incubated with 1×10^7 bare biotin binder beads for 1 h as a negative selection. Unbound yeast were then removed and incubated with 4×10^7 lysozyme-coated biotin binder beads for at least 1 h as a positive selection for lysozyme binders. Unbound yeast were removed and discarded. Beads and bound yeast were then resuspended in 5–50 mL of growth media and yeast allowed to grow off the beads overnight. For successive rounds of selection, the procedure was repeated as described with the exception of decreasing the number of lysozyme-coated biotin binder beads to 1×10^7 . In addition, the number of cells used in each round was modified to reduce the number of cells processed yet maintain at least tenfold sampling of library diversity. After two selections, a flow cytometric sort for c-myc positive cells was performed to prevent the accumulation of truncation clones. The library was then diversified as described.¹⁰ Two further bead selections and a c-myc flow cytometric selection were subsequently performed. Plasmids were isolated from the yeast cells, transformed into bacteria, individual clones were sequenced and their binding properties analyzed and confirmed by FACS.

Similarly, selections against biotin-streptavidin were performed essentially as described above, except that multiple negative selections against streptavidin were performed each round.

Results

The multivalency of yeast display was coupled with multivalent target presentation utilizing a variety of solid surfaces, as diagrammed in Figure 1A, to identify a selection method capable of isolating rare, weak interactions among large nonbinding populations. Each surface was

coated with a target molecule and then incubated with a mixture of yeast, a small fraction of which expressed a protein capable of binding to the target. Unbound yeast were removed by washing the surface and the captured yeast were analyzed in order to determine the ability of the surface to enrich binding clones from the initial mixture.

Profile of materials tested

Figure 1B presents the ability of each surface tested to enrich binding yeast. Nonfunctionalized surfaces were coated by passive adherence of the target protein, lysozyme, to the surface, and the lysozyme-binding fibronectin clone L3.3.1 (nanomolar affinity) was used as the binding population. Cobalt, or Talon[®] conjugated surfaces were simply washed and then used to select yeast with a surface displayed his6 tag. Streptavidin coated surfaces were incubated with biotinylated lysozyme and used to enrich L3.3.1-expressing yeast. The surfaces tested included nitrocellulose, several plastics, glass, agarose, and magnetic beads. Binding yeast were mixed with a 10 to 1,000-fold excess of nonbinding yeast, such as EBY100 yeast lacking any display construct plasmid, or yeast expressing a nonbinding construct. Surfaces were incubated with the mixed yeast population, washed to remove unbound yeast, and the selected yeast populations were then either plated or analyzed by FACS to determine the final prevalence of binding clones. The ability of each surface to select binding yeast was determined by evaluating the enrichment of the binding clone population (final prevalence/initial prevalence), which could then be compared to the theoretical maximum enrichment to purity. For all materials, a number of wash and incubation conditions were tested, and several materials, including each of the functionalized surfaces, had some ability to enrich binding yeast.

Interestingly, none of the passively coated materials were able to enrich the lysozyme binding clone L3.3.1. This inability may be due to our use of a relatively small protein target and a binder with a conformationally sensitive epitope. Passive adherence may have partially or completely denatured the lysozyme and destroyed the epitope recognized by L3.3.1. Although larger proteins may avoid complete denaturation when passively attached, partial unfolding is still likely to both abolish native and generate novel epitopes—a highly undesirable presentation of the target antigen in a de novo selection method. So, although a tag-free means to perform selections is desirable, the use of tag is preferable to altering the conformation of the target antigen—especially as a tag may be necessary for purification of the antigen even if unnecessary for the selection. Additionally, if the tag is present at only a single site and is used to immobilize the target on the selection material, much of its surface may be buried or occluded.

The two surfaces with the best selective profiles identified from these initial tests were agarose and magnetic beads, and these materials were characterized more extensively. First, a mixture of yeast expressing either a his6 tagged scFv or a c-myc tagged fibronectin domain at a 1:4 ratio were incubated with Talon agarose in order to test enrichment of the his6-expressing population. The yeast:bead slurry was poured into a column and washed and eluted. Fractions were then labeled for FACS analysis to determine the relative prevalence of each population in the various fractions. Figure 1C presents the percentage of each yeast population present in each column fraction and clearly shows the ability of agarose beads in a column format to be used in selections.

As a direct means of comparison, his6-tagged yeast were mixed with nonbinding yeast at a ratio of 1 in 1,000 and then applied to both agarose and magnetic Talon-functionalized beads. Figure 2 presents the profile of the initial population as well as that of the yeast eluted from each type of bead. Despite being present below the threshold of detection in the initial population (red trace), binding yeast have been enriched to approximately 1 in 4 yeast by agarose beads (blue trace), and to near purity by magnetic beads (green trace), indicating the excellent ability of these materials to isolate binders.

However, since one of the criteria for the selection methodology is use of a minimum amount of antigen, agarose beads, which by design have a high binding capacity due to extensive porosity, while ideal for protein purification, are nonideal for target antigen presentation as many of these binding sites are likely to be inaccessible to the yeast. A less porous surface with fewer contours, such as magnetic beads is better suited for efficient presentation of the target antigen in this regard.

Having demonstrated the ability of several multivalent surfaces to isolate binding yeast, we next determined whether magnetic beads, the most promising of these surfaces, met the other criteria for naïve library selections.

Testing criteria for successful de novo selections

First, we desired a method capable of isolating extremely weak binders, so magnetic beads were coated with lysozyme and used to select a series of lysozyme-binding fibronectin domains with affinities spanning a million-fold range from μM to pM . The weakest clone, L0.7.1, was identified by FACS selections of soluble multivalent lysozyme,¹⁰ and has such a low affinity that it could not accurately be measured by FACS, while the tightest binder, L7.5.1 interacts with 3 pM affinity. Figure 3A presents the enrichment of lysozyme binding clones from nonbinding clones at a starting ratio of 1:1,000 (gray bars). The million-fold decrease in affinity has no effect on the ability of the beads to select the binding clone.

In contrast, when yeast are first incubated with soluble lysozyme and then quickly washed and allowed to bind to beads as was done previously in the protocol of Yeung and Wittrup,²² enrichment is highly dependent on affinity (black bars). In fact, this seemingly minor protocol alteration drastically reduces the ability to enrich the clone with the weakest binding, which is likely most representative of the binders present in naïve libraries. In each case, selection depends on the ability to generate and maintain an interaction between yeast and bead, and the ability of each yeast to interact multivalently with a bead is dependent on the surface density of the interacting molecules. In utilizing a soluble target incubation, multivalency is limited by affinity as only some subset of surface displayed molecules will be bound to the target antigen, and capable of interacting with the beads. Alternatively, the degree of multivalency that can be achieved using target-coated beads is set by either the display level of the yeast, or the coating density of the target, and is therefore an inherent property of system, and these parameters can be set to maximize possible valency.

As a means to estimate the lower affinity limit for capture with a target-coated surface, a rough calculation of the effective concentration of the target can be made by quantifying the amount of antigen on each bead and dividing that value by the bead volume, giving an approximate local concentration. The magnetic beads used in this study present the target antigen at an effective local concentration in the millimolar range, and therefore may be able to isolate extremely weak interactions.

Having established the ability to isolate very weak interactions, we investigated the effect of rarity on enrichment. Binding and nonbinding yeast were mixed at ratios varying from 1:10 to 1:100,000 and were bead selected, resulting in the enrichment values presented in Figure 3B. For both interactions between a his6 tag and chelated cobalt, and the L0.7.1 fibronectin clone and lysozyme, enrichment scaled well with the theoretical maximal enrichment for each initial prevalence (lines), with a maximal increase in prevalence from 1 in 100,000 cells to 1 in 3 (30,000-fold enrichment). Thus, magnetic bead selection easily captures rare clones.

One of the limits of FACS selections is that it restricts the size of a population that can be sorted; thus, the number of yeast that can be screened effectively by magnetic beads was investigated. Lysozyme-binding (L0.7.1) and nonbinding yeast were mixed at a ratio of 1:1000

at total population sizes varying from 5×10^7 to 4×10^9 , and enrichment values after 1 and 3 washes were determined for each population (Figure 3C). Consistent enrichment values were found across all population sizes, and demonstrate that four billion yeast can be straightforwardly surveyed in a single micro-centrifuge tube in 1 h. Whereas scaling up FACS selections requires either multiple sorters or significantly more time, magnetic bead selections can be scaled up by simply using a larger vessel or running several microcentrifuge tube-sized selections in parallel—allowing even the largest yeast libraries to be screened to high coverage multiplicity with ease.

To evaluate the flexibility of magnetic bead selections, the ability to enrich clones displaying several different scaffolds capable of interacting with various targets was investigated. Figure 3D shows the enrichment values of clones expressing either fibronectin or scFv domains that recognize both small molecule and protein targets. Proteins were immobilized on streptavidin-coated beads via a biotin tag, while small molecules were chemically conjugated to amine-functionalized beads. Binding yeast were efficiently isolated from a larger population of nonbinding yeast, independently of their displayed scaffold, size of the target antigen, and whether the targets were chemically conjugated or immobilized via a tag.

Finally, we tested the yield of bead selections in order to determine the probability that each binding yeast cell would be captured. Yield is a critical selection parameter in two respects. The chances of capturing each binding yeast will first influence the coverage multiplicity used, and secondly determine the ability of the method to act as an effective negative selection, wherein yeast with undesirable binding properties are removed from the library population. Figure 4A shows the percent of binders present that were recovered from selections of binding yeast (L0.7.1) present at a ratio of 1 in 1,000 nonbinders. In most cases, more than three-quarters of the binders present were successfully recovered, and this fraction declined significantly only when the number of binders present was comparable to the binding capacity of the beads (several million binders) decreasing the likelihood that binding yeast would encounter available binding sites.

Similarly, Figure 4B shows the number of yeast isolated from repeated rounds of selection against streptavidin. After each selection, yeast in the supernatant were reapplied to fresh streptavidin beads. The number of binders isolated from each round is approximately equal to 80% of the total number present. Thus, after 4 rounds of selection, ~99.8% of streptavidin-binding clones have been captured, leaving only 0.16% in the population. The ability to thoroughly deplete the population of streptavidin binders via such negative selection serves as a highly efficient means to constrain the undesirable selection of reagent binders and steer the selective pressure of each positive selection toward the intended target. This restraint of alternative solutions to the selective pressure represents a significant advantage over negative selections by FACS, particularly as extremely weak interactions can be captured and because the yeast do not require subsequent regrowth and induction before positive selection. Therefore, negative selections using magnetic beads coated with reagents would be useful when paired with any method of positive selection in order to improve the chances of isolating the desired binding interaction.

Thus far, we have demonstrated that magnetic beads can be used to isolate highly rare and very weak binders to both protein and small molecule targets with high quantitative yield from very large populations. To further profile the factors affecting these selective abilities, we carried out a series of experiments in which parameters such as the time of incubation and surface density of interacting molecules were varied. Figure 5A presents the enrichment of lysozyme binding clones (L0.7.1) from nonbinding yeast after 1 wash when incubated at a starting ratio of 1:1,000 for varying periods of time. The magnetic beads rapidly isolate the binding population, showing near maximal enrichment after 1 h.

Additionally, since the binding affinities that can be captured using this method are so low, and because in most tests the nonbinding population consisted of yeast that did not express a surface-displayed construct, we undertook extensive testing of negative controls in order to ensure that only specific interactions were selected. Beads coated with a given target were used in tests against yeast expressing constructs capable of interactions with other targets. Four such mismatched pairs were tested, and all prevalences after selection were comparable to the initial prevalence of 1 in 1,000 (Figure 5B), demonstrating that the enrichment of binding yeast represents a specific interaction and is not an artifact of a biased comparison, nor the effect of a promiscuous system.

Lastly, as described previously, this method depends on the number of molecular interactions or degree of multivalency that can be achieved, and is therefore predicted to be impacted by both the density of the target ligand coating the beads, and the density of the construct displayed on the surface of the yeast. Accordingly, Figures 5C,D show the effect of construct display level and target presentation level on enrichment. As expected, the higher the surface density of interacting molecules—whether limited by the bead or the yeast, the more likely a multivalent interaction can be achieved resulting in higher enrichment values. Because the yeast expression level is a significant factor in determining enrichment, care should be taken to ensure use of cultures that have been induced to express as highly as possible.

Demonstrations of method utility in de novo isolation

To fully test the method, we also sought to select de novo binders from two naïve libraries, including the same fibro-nectin library utilized to generate the clones presented in Hackel et al., using lysozyme-coated beads as opposed to the FACS methodology applied previously, allowing direct comparison of the two methods. In the bead selections carried out here, each library was first depleted of streptavidin or bead binders by an initial incubation with beads as a means to avoid enriching reagent-binding clones. Nonbinding yeast were removed from the supernatant and applied to beads coated with lysozyme. These beads and the yeast bound to them were then directly transferred into growth media and subsequently induced for the next round of the process. After two such selections, a FACS sort to isolate c-myc positive clones was performed to remove truncated clones from the population. The clones were diversified as described,¹⁰ and the resulting population was bead selected twice followed by a c-myc positive FACS sort. Several clones were isolated, sequenced, and characterized.

Interestingly, L0.7.1, the initial binder isolated by FACS selections was also identified in these bead sorts. However, this clone was not the dominant member (present once in eight sequenced clones) of the selected population, and therefore it appears as though the bead selections were able to identify additional binders that were not isolated by FACS. As we cannot determine accurate affinity values by FACS for such weak binders, it is unclear whether these additional clones represent stronger binders that happened to be missed by FACS, or weaker ones that FACS could not isolate. In either case, the inclusion of additional diversity in subsequent rounds of evolution is likely to be beneficial, especially given the use of a loop shuffling protocol.

A second fibronectin library was bead sorted in parallel, and clones with nM binding affinities were isolated following the same protocol—allowing isolation of validated lysozyme-binding clones from two different libraries in 1 week using only about 100 pmol (10 µg) of target antigen. The supplemental information contains additional instructions for performing selection from naïve libraries.

Additionally, as a means to demonstrate the excellent ability of the method to function in negative selections, bead selections were used to isolate clones capable of discriminating between streptavidin and biotin-streptavidin. Multiple negative selections against streptavidin

with its biotin-binding site unoccupied were performed prior to each positive selection against biotin-streptavidin. After six selections and two rounds of mutagenesis, clones with nanomolar binding affinity to biotin-streptavidin, but undetectable affinity to streptavidin were isolated (data not shown). This result shows the robust nature of beads in both positive and negative selections, and the ability to isolate interactions of interest with a high degree of specificity.

The excellent capabilities of this multivalent selection method establish it as the method of choice for de novo selections. Bead-based selections followed by mutagenesis can be iterated, and the success of each round can be assessed by comparing binding to reagent-coated beads to antigen-coated beads. Once a population displays increased binding to the target antigen, it can be mutagenized and selection stringency can be increased in the following rounds. Stringency may be tuned by altering the duration and frequency of wash steps, decreasing expression of the surface-displayed construct or decreasing antigen density on the beads. Alternatively, as long as thorough negative selections are performed, a soluble antigen incubation as in the method described by Yeung and Wittrup can be utilized to increase stringency. Once FACS-detectable (nM) binders have been isolated via rounds of bead selection with increasing stringency, affinity maturation can proceed via FACS. Figure 6 presents a schematic diagram of the process for generating high affinity binders with high efficiency from naïve libraries.

Overall, by allowing multivalent presentation of the target antigen locally on a particle surface, the bead selection method described here is able to sort large populations and quantitatively isolate even weak binders in a highly rapid and efficient manner.

Discussion

Because of their robust protein processing machinery, yeast are an excellent host organism for protein engineering efforts. FACS, while an optimal tool for making fine discrimination among variants,⁷ is less well suited for de novo selections. Accordingly, we sought an alternative method for early selection rounds that would avoid the limitations posed by FACS. These limitations include the size of the population that can be analyzed, the affinity of binding clone that can be reliably captured, the amount of target antigen needed, and the likelihood of capturing alternative solutions to the selective pressure. Here we have demonstrated a method for capturing rare clones that maintains the advantages of YSD, while avoiding the disadvantages of FACS in isolating de novo binders from naïve libraries. Coupling the multivalency of YSD with multivalent target presentation allows screening of billions of yeast in a microcentrifuge tube in an hour and is able to capture even interactions of micromolar affinity.

While previous studies have utilized magnetic beads (MACS) rather than FACS for the first round of library selection, they have done so to select from larger sized populations, and not to capture weak interactions, and have thus incubated yeast with soluble antigen rather than precoating the magnetic beads. While such a method still possesses some advantages over FACS, such as less time for target dissociation during the selection and the ability to screen a larger population, it remains less suitable for naïve selections than utilizing antigen-coated beads because it requires considerably more antigen, only captures clones of affinities similar to the concentration of antigen achieved in the soluble incubation, and has a high risk of selecting reagent binders.

While the ability to capture weak binders is generally only necessary when no suitably strong binders are present in a population, the additional inclusion of weak clones is likely to provide benefits due to the uneven topology of the sequence-fitness landscape, in which the fittest final

solution does not necessarily arise from the fittest initial solution as some sequence variants have a higher inherent capacity for improvement.

Moreover, there are some circumstances in which it may be necessary or beneficial to work with low affinity interactions. For example, when T cell receptors are engineered to interact with specific peptide-MHC complexes, variants with high affinity to the complex often interact with moderate affinity to MHC independent of the presence of the cognate peptide leading to nonspecific stimulation, or stimulation when other peptides are present.²³ To maintain a requirement for peptide, weak interactions may be necessary. Because such weak interactions are not reliably detectable by FACS, use of the multivalent bead method described here may be advantageous.

Beyond the ability to isolate weak interactions, the use of multivalent beads permits a high local concentration of the target antigen to substitute for a high systemic concentration, decreasing the amount of antigen required for initial selections by almost 2 orders of magnitude. FACS selections often proceed at a 5 μ M systemic concentration, while beads require far less antigen yet present the target at a mM local concentration—potentially allowing isolation of binders with extremely weak affinities.

While the method described here does require the use of tags and other reagents, isolation of reagent-binding clones can be avoided by the use of bead-based negative selections. This means to scrub populations of reagent binders dramatically increases the probability of isolating clones capable of interacting with the desired target. In fact, the ability to remove some 99.8% of even extremely weak reagent binders in 4 h makes the method useful as a negative selection regardless of the method used for positive selections. Such an effective negative screen is in stark contrast with attempts to use FACS as a method for negative selections. Negative selections by FACS are generally ineffective not only because of errors made in droplet sorting, but because the regrowth and subsequent induction required allows reagent-binding clones with low expression during the negative selection a second chance during subsequent reinduction to fall into the sort window. For example, even if negative selections by FACS were able to reliably decrease the prevalence of reagent binders 100-fold, subsequent regrowth and the practice of analyzing a 10-fold excess of library diversity would diminish the effect of the negative selection to a 10-fold reduction in the total number of reagent binders. Given sort windows typically drawn to capture the top 0.1 to 1% of the population, once present, reagent-binders are generally impossible to remove from the library by FACS. Conversely, negative selection by the multivalent bead method described here is able to reduce reagent binders almost 1,000-fold in 4 h, after which a positive selection can be carried out without regrowth—greatly diminishing the chances of enriching these undesirable solutions to the selective pressure.

An improved means to isolate binders from naïve libraries also increases the utility of both alternative scaffold and human scFv libraries, and decreases reliance on immunization of mice or other animals followed by subsequent humanization for initial binding clones. This advance ought to allow isolation of novel binders with greater ease, speed, and higher probability of success than previous techniques.

Conclusions

Multivalent antigen presentation on magnetic beads allows microgram quantities of antigen to identify and select even extremely weak binders with high efficiency from populations of billions of yeast. Magnetic bead selection combines the advantages of yeast as a protein engineering host and complements the subsequent use of FACS to isolate variants with improved properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Literature Cited

1. Gai SA, Wittrup KD. Yeast surface display for protein engineering and characterization. *Curr Opin Struct Biol* 2007;17:467–473. [PubMed: 17870469]
2. Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotechnol* 1997;15:553–557. [PubMed: 9181578]
3. Bowley DR, Labrijn AF, Zwick MB, Burton DR. Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. *Protein Eng Des Sel* 2007;20:81–90. [PubMed: 17242026]
4. Lipovsek D, Lippow SM, Hackel BJ, Gregson MW, Cheng P, Kapila A, Wittrup KD. Selection of horseradish peroxidase variants with enhanced enantioselectivity by yeast surface display. *Chem Biol* 2007;14:1176–1185. [PubMed: 17961829]
5. Piatasi A, Howland SW, Rakestraw JA, Renner C, Robson N, Cebon J, Maraskovsky E, Ritter G, Old L, Wittrup KD. Directed evolution for improved secretion of cancer-testis antigen NY-ESO-1 from yeast. *Protein Expr Purif* 2006;48:232–242. [PubMed: 16563796]
6. Wittrup KD. Protein engineering by cell-surface display. *Curr Opin Biotechnol* 2001;12:395–399. [PubMed: 11551469]
7. VanAntwerp JJ, Wittrup KD. Fine affinity discrimination by yeast surface display and flow cytometry. *Biotechnol Prog* 2000;16:31–37. [PubMed: 10662486]
8. Tanaka T, Yamamoto T, Tsukiji S, Nagamune T. Site-specific protein modification on living cells catalyzed by Sortase. *Chem-biochem* 2008;9:802–807.
9. Griffiths AD, Duncan AR. Strategies for selection of antibodies by phage display. *Curr Opin Biotechnol* 1998;9:102–108. [PubMed: 9503596]
10. Hackel BJ, Kapila A, Wittrup KD. Picomolar affinity fibronectin domains engineered utilizing loop length diversity, recursive mutagenesis, and loop shuffling. *J Mol Biol* 2008;381:1238–1252. [PubMed: 18602401]
11. Crothers DM, Metzger H. The influence of polyvalency on the binding properties of antibodies. *Immunochemistry* 1972;9:341–357. [PubMed: 4113719]
12. Klemm JD, Pabo CO. Oct-1 POU domain-DNA interactions: cooperative binding of isolated subdomains and effects of covalent linkage. *Genes Dev* 1996;10:27–36. [PubMed: 8557192]
13. Jung ST, Jeong KJ, Iverson BL, Georgiou G. Binding and enrichment of Escherichia coli spheroplasts expressing inner membrane tethered scFv antibodies on surface immobilized antigens. *Biotechnol Bioeng* 2007;98:39–47. [PubMed: 17657772]
14. Baccarini M, Bistoni F, Lohmann-Matthes ML. In vitro natural cell-mediated cytotoxicity against *Candida albicans*: macrophage precursors as effector cells. *J Immunol* 1985;134:2658–2665. [PubMed: 3882833]
15. Richman SA, Healan SJ, Weber KS, Donermeyer DL, Dossett ML, Greenberg PD, Allen PM, Kranz DM. Development of a novel strategy for engineering high-affinity proteins by yeast display. *Protein Eng Des Sel* 2006;19:255–264. [PubMed: 16549400]
16. Wang XX, Shusta EV. The use of scFv-displaying yeast in mammalian cell surface selections. *J Immunol Methods* 2005;304:30–42. [PubMed: 16099466]
17. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD. Isolating and engineering human antibodies using yeast surface display. *Nat Protoc* 2006;1:755–768. [PubMed: 17406305]
18. Corneillie TM, Fisher AJ, Meares CF. Crystal structures of two complexes of the rare-earth-DOTA-binding antibody 2D12.5: ligand generality from a chiral system. *J Am Chem Soc* 2003;125:15039–15048. [PubMed: 14653738]
19. Lubic SP, Goodwin DA, Meares CF, Song C, Osen M, Hays M. Biodistribution and dosimetry of pretargeted monoclonal antibody 2D12.5 and Y-Janus-DOTA in BALB/c mice with KHJJ mouse adenocarcinoma. *J Nucl Med* 2001;42:670–678. [PubMed: 11337558]

20. Boder ET, Midelfort KS, Wittrup KD. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci USA* 2000;97:10701–10705. [PubMed: 10984501]
21. Nieba L, Nieba-Axmann SE, Persson A, Hämäläinen M, Ede-bratt F, Hansson A, Lidholm J, Magnusson K, Karlsson AF, Plückthun A. BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal Biochem* 1997;252:217–228. [PubMed: 9344407]
22. Yeung YA, Wittrup KD. Quantitative screening of yeast surface-displayed polypeptide libraries by magnetic bead capture. *Biotechnol Prog* 2002;18:212–220. [PubMed: 11934287]
23. Colf LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, Garcia KC. How a single T cell receptor recognizes both self and foreign MHC. *Cell* 2007;129:135–146. [PubMed: 17418792]

Figure 1. Profile of multivalent selection methods

(A) Diagram of multivalent interaction between yeast and surface. (B) Yeast can be selected by multivalent surfaces: The ability of each multivalent surface to select and capture binding yeast from a larger population of nonbinding yeast was determined. In each case, the enrichment ratio was determined and is presented as a fraction of the maximum theoretical enrichment (enrichment from 1 in 100 or 1 in 1,000 to purity). Non-functionalized materials had no ability to enrich binders, perhaps due to denaturation of the target protein on the surface. Functionalized surfaces, including agarose and magnetic beads showed a strong ability to select binders and were characterized further. (C) Selection on agarose resin in column format: Talon agarose resin was used to select yeast expressing a His6 tag from a larger population of c-myc positive yeast. The percent of each clonal population recovered from wash (fractions 1–8) and elution (fractions 9–11) is given and demonstrates the ability to isolate binders specifically.

Figure 2. FACS profile of selected populations

FACS data presenting the prevalence of His6 yeast before (red) and after selection on Talon agarose resin (blue) and magnetic beads (green) demonstrating the excellent enrichment profiles of these multivalent surfaces.

Figure 3. Efficiency of magnetic bead sorting

(A) Affinity: Magnetic bead enrichment is not dependent on affinity. Lysozyme binding clones with affinities spanning 6 orders of magnitude were mixed with nonbinding EBY100 at a ratio of 1 in 1,000 and incubated with either soluble lysozyme for 1 h followed by a wash and 2 min incubation with streptavidin coated beads (black bars), or were incubated with lysozyme-coated beads for 1 h (gray bars). The ability of each clone to be selected via the soluble incubation method is highly dependent on affinity, while lysozyme-coated beads enrich all clones at a very high efficiency regardless of affinity. (B) Rarity: Magnetic bead sorting enriches binding yeast with an efficiency close to the theoretical maximum. Yeast expressing a low affinity (micromolar) lysozyme-binding fibronectin domain were mixed with nonbinding

EBY100 at varying initial prevalences ranging from 1 in 100 to 1 in 100,000, and bead selected with resulting enrichment ratios that scaled well with the theoretical maximum (line), yielding up to 30,000-fold enrichment in a single selection. *Data not available. (C) Population size: Magnetic bead sorting easily and quickly processes large populations of yeast. Varying numbers of yeast at a ratio of 1 low affinity lysozyme binder per 1,000 nonbinders were bead selected. Enrichment ratios at each population size are presented after 1 and 3 washes, demonstrating that 4×10^9 yeasts can be screened with no loss in enrichment. *Data not available. (D) Target: Yeast expressing scFv or fibronectin recognizing a range of protein and small molecule targets were mixed with nonbinding EBY100 yeast at a ratio of 1:1,000 and bead selected. Magnetic beads were able to enrich for all scaffolds, targets and binding interactions tested.

Figure 4. Yield of magnetic bead sorting

(A) Yield: Magnetic bead sorting retrieves binding yeast in high quantitative yield. Low affinity lysozyme-binding yeast were mixed with nonbinding EBY100 at a ratio of 1:1,000, and the size of the total population sorted was varied between 1.2×10^7 and 4×10^9 . Bead selections were performed and the total number of binding yeast retrieved after each selection was compared to the number initially present. Under all conditions, bead selection captured the majority of binding yeast present, and was not highly dependent on the total number of yeast present in the selection. (B) Yield of negative selections: Magnetic bead sorting retrieves binding yeast in high quantitative yield. A fibronectin library was repeatedly incubated with streptavidin-coated beads as a means to deplete clones capable of binding to the beads independent of the target ligand. Each negative selection captured 75–80% of the streptavidin-binding population present. After the 4th negative selection, only about 0.16% of bead binders remain and yeast can be incubated with target-coated beads with a dramatically reduced chance of isolating reagent-binders.

Figure 5. Dependencies of magnetic bead sorting

(A) Time: Magnetic bead sorting selects binding yeast rapidly. A population of 10^7 yeast at a ratio of 1 low affinity lysozyme binder in 1,000 nonbinding EBY100 were bead selected for various times and washed once, yielding a half-life of association under 30 min. (B) Specificity of Interaction: Despite the very low affinity requirements, selection of yeast is highly specific. Mismatched pairings of yeast expressing scFvs and target-coated beads were selected in order to determine the specificity of interaction necessary for enrichment. The line represents the presort prevalence. (C) Yeast Valency: Enrichment is dependent on yeast valency. Low affinity lysozyme binding yeast were induced for varying periods of time resulting in different densities of surface expression and then bead selected. A clear dependence on the yeast expression level can be observed. (D) Bead Valency: Enrichment is dependent on target valency. Beads were coated with varying densities of lysozyme and used to select a low affinity lysozyme-binding clone. A clear dependence on the density of ligand on the beads is observed regardless of yeast expression level.

Figure 6. Selection profile for successful molecular evolution

A flow diagram for successful molecular evolution showing the complementary roles of bead-based and FACS-based selections in the isolation of proteins with desired binding properties.

Table 1**Plasmids Used in this Study**

Clone	Scaffold	Target	Target type	Citation	Affinity
L0.7.1	Fibronectin	Lysozyme	Protein	10	> 1 μ M
L1.5.1	Fibronectin	Lysozyme	Protein	10	~1 μ M
L3.3.1	Fibronectin	Lysozyme	Protein	10	8 nM
L7.5.1	Fibronectin	Lysozyme	Protein	10	3 pM
2D12.5	scFv	DOTA	Small molecule	18,19	10 nM
4m5.3	scFv	Fluorescein	Small molecule	20	48 fM
his6 tag	scFv	Chelated cobalt	Small molecule	21	~1 μ M
		Anti-his antibody	Protein	n.a.	n.a.