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Selection of bead-displayed, PNA-encoded chemicals

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Abstract

The lack of efficient identification and isolation methods for specific molecular binders has fundamentally limited drug discovery. Here, we have developed a method to select peptide nucleic acid (PNA) encoded molecules with specific functional properties from combinatorially generated libraries. This method consists of three essential stages: (1) creation of a Lab-on-Bead™ library, a one-bead, one-sequence library that, in turn, displays a library of candidate molecules, (2) fluorescence microscopy-aided identification of single target-bound beads and the extraction – wet or dry – of these beads and their attached candidate molecules by a micropipette manipulator, and (3) identification of the target-binding candidate molecules via amplification and sequencing. This novel integration of techniques harnesses the sensitivity of DNA detection methods and the multiplexed and miniaturized nature of molecule screening to efficiently select and identify target-binding molecules from large nucleic acid encoded chemical libraries. Beyond its potential to accelerate assays currently used for the discovery of new drug candidates, its simple bead-based design allows for easy screening over a variety of prepared surfaces that can extend this technique's application to the discovery of diagnostic reagents and disease markers.

Keywords

PNA-encoded chemicals; DNA-encoded chemicals; drug discovery; fluorescence; beads; small molecule screening; microscopy; micropipette

INTRODUCTION

Combinatorial techniques can produce large numbers of small molecule drug candidates, increasing the need for techniques to identify which small molecules bind specifically to important targets. High throughput screening techniques close the gap between library synthesis and identification of lead compounds. However, these methods consume large amounts of material and require expensive robotic systems (Sundberg, 2000; Melkko *et al.*, 2007a). Given these considerations, there is a continuing need to multiplex and miniaturize

screening techniques to reduce material consumption and increase screening efficiency (Kapur *et al.*, 1999; Haupts *et al.*, 2003; Melkko *et al.*, 2007a).

Recent advances using DNA- or PNA- (peptide nucleic acid) encoded chemical libraries offer new opportunities for screening libraries of unprecedented size at low concentrations. These chemical libraries can be synthesized with DNA or PNA tags covalently conjugated to each small organic molecule and these tags serve as identification bar codes for the unique conjugated chemical (Brenner and Lerner, 1992; Gartner *et al.*, 2002, 2004; Halpin and Harbury, 2004a,^b; Halpin *et al.*, 2004; Harris *et al.*, 2004; Harris and Winssinger, 2005; Urbina *et al.*, 2006; Debaene *et al.*, 2007; Melkko *et al.*, 2007a,^b; Wrenn and Harbury, 2007; Buller *et al.*, 2008; Kleiner *et al.*, 2008; Pianowski and Winssinger, 2008). While diverse libraries of such nucleic acid-encoded molecules can now be created (Gartner *et al.*, 2002, 2004; Harris and Winssinger, 2005; Urbina *et al.*, 2006; Debaene *et al.*, 2007; Kleiner *et al.*, 2008; Pianowski and Winssinger, 2008), subsequent isolation of the fittest library members remains challenging, mainly due to two key reasons. First, though the available libraries display larger and larger diversity, the libraries contain only a few members of each species. Thus, screening methods that use little material are required. Second, most nucleotide-based or peptide-based screening methods, such as SELEX-based approaches (Conrad *et al.*, 1995; Fitzwater and Polisky, 1996; Forst, 1998; Stoltenburg *et al.*, 2007; Kim *et al.*, 2008), or phage display (Smith, 1985; Clackson *et al.*, 1991; Boder and Wittrup, 1997; Yin *et al.*, 2004; Silacci *et al.*, 2005), utilize iterative steps of selection, amplification, and re-selection. These iterative steps require large amounts of material to be continually produced, which can involve time-consuming cloning or nucleotide purification steps. Also, conventional resin-based affinity selection of binders may introduce significant bias into the selection step by reducing the number of high affinity binders identified (Mendonsa and Bowser, 2004), though capillary electrophoresis SELEX addresses this shortcoming. Overall, a method that identifies binders in a single cycle, without intermediate amplification and cloning, is desirable.

A single cycle selection method has been previously described using ligands immobilized onto an agarose microarray surface (Childs-Disney *et al.*, 2007; Disney *et al.*, 2008). This system effectively eliminates iterative rounds of selection, amplification, and re-selection; however, it also has the drawback of requiring identified binders to be gel extracted from the agarose surface. This extraction technique requires the surface to be rehydrated after read out and limits the quantities of RNA that can be recovered. Moreover, this technique limits the number of chemicals that can be screened by requiring that the combinatorial chemical space be confined to only two dimensions.

In this work, we describe an improved single cycle molecule-screening method, NanoSelection, which harnesses the robust nature and extreme sensitivity of DNA detection methods, exploits the nucleic acid bar codes, allows chemical space to be displayed in three dimensions, and employs a novel combination of a fluorescent microscope and micropipette manipulator to effectively and efficiently select, identify, and extract target-binding molecules from large nucleic acid encoded chemical libraries. Beyond its potential to multiplex, miniaturize, and accelerate assays currently used for the discovery of new drug candidates, its simple bead-based design allows for easy screening over a variety of prepared surfaces, including agarose-based microarrays, thus extending this technique's application to the discovery of diagnostic reagents and disease markers.

MATERIALS AND METHODS

PNA and DNA sequences

Biotin and fluorescein isothiocyanate (FITC) conjugated PNAs were a kind gift from Nicholas Winssinger (Pianowski and Winssinger, 2008) from his PNA encoded chemical library. PNA 1: biotin-TGATGACGAACGG (N- to C-terminal) and PNA 2: FITC-CAAATGAGCAGCC (N- to C-terminal). Two 107 nucleotide (nt) single strand DNA sequences containing a complementary binding region to one of these PNAs, and an additional 92 nt DNA sequence containing no PNA binding region were synthesized (Table 1, Bioneer). Each DNA was composed of three critical regions: (i) a 5'-NH₂ bead linker region, (ii) two 29 nt polymerase chain reaction (PCR) handles (Table 1, italics), and (iii) a PNA binding (capture) region (Table 1, underlined). Additionally, for these experiments, a unique restriction site was added to each sequence for more rapid identification of the sequences identity, *Bam*HI for dPNA1 and *Xho*I for dPNA2.

Formation of bead libraries

Both yellow green and yellow orange Fluoresbrite® Carboxylate Microspheres (1 µm, Polysciences) were conjugated with the single strand DNA sequences by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) coupling similar to Iannone *et al.* (2000). Briefly, 2 µL of the bead slurry was washed in 100 µL of 0.1 M 2-morpholino-ethanesulfonic acids (MES) pH 4.5, centrifuged for 3 min at 10 000×g, then resuspended again in 50 µL of 0.1 M MES. Then, 1 µL of 100 µM DNA solution was added to the tube and vortexed briefly. Finally, 10 µL of 50 mg/mL EDC solution was added, vortexed briefly, and incubated at room temperature in the dark for at least 2 h. After incubation, the DNA-bead solution was centrifuged and the supernatant was carefully removed, and the beads were washed three times with 1 mL of PBS (10 mM NaH₂PO₄, 150 mM NaCl), 0.02% Tween® 20, pH 7.2. After the final wash, the beads were resuspended in 50 µL of PBS and stored at 4°C. Final bead suspension was counted with a counting chamber and stored for no longer than a month.

Flow cells and surfaces

Sample chambers were prepared and cleaned as described in Joo and Ha (2008). Two 0.75 mm diameter holes were drilled into a glass slide to form an inlet and outlet port. Glass slides were sonicated in 10% alconox, acetone, then 1 M KOH, while coverslips were sonicated in 1 M KOH. Both were washed with copious amount of water and dried with N₂. Removable double-sided tape or parafilm was used to seal the chamber. Vacuum was applied to the outlet port to remove solution from the chamber.

For the selection of biotin-PNA, ~20 µL of 1 mg/mL of biotinylated BSA in 10 mM Tris-HCl pH 8.0 and 50 mM NaCl (T50) was added into the inject port and incubated for 5 min. The chamber was then washed with 100 µL of T50 buffer, then ~20 µL of 0.4 mg/mL neutravidin in T50 was added to the chamber and incubated for 2 min. The neutravidin solution was washed from the chamber with 100 µL of T50.

For the selection of FITC-PNA, ~20 µL of 1 mg/mL of anti-FITC antibody (Pierce) in PBS was added into the inject port and incubated for 5 min. The chamber was then washed with 100 µL of PBS buffer.

PNA hybridization

On the day of the experiment, a 10 µL aliquot of bead suspension, containing ~2×10⁶ beads, was centrifuged for 3 min at 10 000×g, and the supernatant was carefully removed. Beads were resuspended in 20 µL of 10 mM NaH₂PO₄, pH 7.2. To this suspension, 1 µL of 100

μM PNA in 10 mM NaH_2PO_4 , pH 7.2 was added and vortexed briefly. The PNA-bead suspension was incubated at 42°C for 30 min then centrifuged for 3 min at $10\,000\times g$ and the supernatant was carefully removed. This bead pellet was then washed three times in the same PBS solution used above, and then resuspended in 10 μL of PBS. In addition to the specific PNA–DNA reaction, beads coated with nonbinding DNA strand, dNull, were also incubated with either the PNA1 or PNA2 sequence, as above. All bead solutions were sonicated prior to selection to reduce aggregation.

Selection of PNA/DNA beads

After PNA hybridization and sonication, three types of selection solutions are prepared: (1) with 1 μL of positive binding beads, PNA1-hybridized dPNA1 beads, and 10 μL of nonbinding, PNA1-hybridized dNull beads, for selection off neutravidin surfaces, (2) with 1 μL of positive binding beads, PNA2-hybridized dPNA2 beads, and 10 μL of nonbinding PNA2-hybridized dNull beads, for selection off anti-FITC antibody surfaces, and (3) with 1 μL of positive binding beads, PNA2-hybridized dPNA2 beads, and 10 μL of nonbinding, PNA1-hybridized dPNA1 beads, for selection off anti-FITC antibody surfaces.

The selection solution was added to the washed sample chambers and incubated for 1–2 min. The beads were then removed from the sample chamber and washed with 400 μL of T50 for the neutravidin surfaces or 800 μL of PBS for the anti-FITC antibody surfaces. Application of the wash buffer to the inlet port was carefully maintained, such that the solution meniscus was not pulled through the chamber with the vacuum.

During the incubation and washing, the presence of beads and surface binders was verified by fluorescence microscopy with an inverted Nikon Ti-U microscope. Yellow green beads were excited with a xenon lamp with a standard FITC filter set, and the yellow orange beads were also excited by the xenon lamp with a standard Cy3 filter set. Images were acquired with a Hamamatsu digital camera.

Micropipette micromanipulator pick up of surface-bound beads

The micropipette tip size must be matched to the diameter of the beads that the user was attempting to pick up. Pipette tip sizes were modified by the pipette pulling procedure, by the type of heating filament in the pipette puller, and by the size of the capillary tubes used as the base material for the micropipettes. Here, a Sutter P-87 micropipette puller with a 1.5 mm $\times$ 2.0 mm box filament, with borosilicate glass capillaries (Sutter) with an outer diameter of 1 mm and an inner diameter of 0.5 mm was used. The following pipette puller settings were used: Heat=ramp (461), pull=0, time=200, pressure=300, velocity=40 for the pull and 74 for the second.

Tip diameters can be confirmed by imaging the tips in a scanning electron microscope, or by performing a bubble test. Bubble tests are carried out by inserting a pipette into methanol in a glass container, and then applying pressure from a nitrogen gas cylinder, and increasing the back pressure on the pipette until a bubble forms at the tip. Calibrated curves for the tip diameter as a function of the bubble pressure are provided by Sutter Instruments. Here, a pipette tip size of 0.80 μm was used.

For pick up, the surface of the microscope slide was brought into focus to identify a bead, then the microscope objective was raised so that the microscope objective was focused at a plane above the target bead. The micropipette tip was then roughly centered over the imaging area by eye, and the tip was lowered toward the target with the manipulator. The pipette was angled at about 20° with respect to the horizontal plane, so the tip was the first part of the pipette to come into contact with the surface. Once the tip was within one or two bead diameters of the bead, a vacuum valve was opened that provides suction at the tip. The

bead was then sucked on to the tip by the negative pressure. Once a bead was secured, it can be expelled into a nearby well by the application of positive pressure to the micropipette tip. Alternatively, the pipette tip can be inserted into a microtube and snapped off, insuring the bead has been deposited in the tube.

PCR amplification of extracted beads

For identification of the bead species selected from the surface, PCR amplification of the DNA sequence conjugated to the bead was required. Given the low number of single-stranded DNA molecules conjugated to the surface, two rounds of PCR, with nested primers, were utilized to provide highly specific amplification of the DNA conjugated to the selected bead. Primer mix 1 (Table 2) adds an additional 19 bps (base pairs) to the overall length of the DNA (126 bps total, or 128 bps with *Taq* amplification). The second round of PCR utilizes primer mix 2 (Table 2), which is 10 bps internal to the previous set and results in a final PCR product of (107 bps, or 109 bps with *Taq* amplification).

Each single extracted bead was PCR amplified with AmpliTaq Gold (Applied Biosystems). To each PCR tube containing a single bead was added 1 AmpliTaq Gold reaction buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM primer mix 1, 0.25 mL of AmpliTaq Gold. After 25 PCR cycles, with an annealing temperature of 53°C, 1 µL of the PCR product was removed and added to a new PCR tube containing 1× AmpliTaq Gold reaction buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM primer mix 2, 0.25 µL of AmpliTaq Gold. It was again subjected to 25 PCR cycles with an annealing temperature of 53°C.

Identification of surface binders

The PCR products from the second round of PCR were run on a 17% native acrylamide gel at 150 V for 1 h and 30 min. The native gel was stained with Sybr Green I nucleic acid stain, and the bands were visualized with a UV transilluminator. If necessary, restriction digest was used to identify similar sized bands. Ten microliters of the second PCR product was digested with *Xho*I at 37°C for 16 h. PCR products used for sequencing were gel extracted with the QiaEX II kit (Qiagen) with the second round PCR primers (Table 2) used as sequencing primers.

RESULTS

Our novel NanoSelection technique (Peng *et al.*, 2007), overviewed in Figure 1, can be divided into three essential stages: (1) creation of a Lab-on-Bead library: a one-bead, one-sequence library that, in turn, displays a library of candidate molecules, (2) surface selection and extraction – wet or dry – of the target-bound beads and their attached candidate molecules, and (3) identification of the target-binding candidate molecules via amplification and sequencing.

Overall, the multiplexed, miniaturized nature of NanoSelection stems from its use of Lab-on-Bead libraries. The micrometer- to nanometer-sized plastic or glass beads (Chen *et al.*, 2000; Nifli *et al.*, 2006) in these Lab-on-Bead libraries serve as display handles for the molecules being screened and can contain fluorescent dyes for facile visualization during the screening process.

A variety of standard methods can be used to generate Lab-on-Bead libraries. For this study, Lab-on-Bead libraries of PNA-encoded chemicals were created by EDC coupling individual aminated DNA sequences to 1 µm carboxylated latex beads, such that each bead displayed multiple copies of the same single stranded DNA sequence. The individual Lab-on-Bead members were then pooled into a library and reacted with their complementary PNA-encoded chemicals. This type of coupling scheme can easily be adapted into a 96-well

reaction format to match the increasing size of PNA-encoded chemical library pools, or a variant of the BEAM (beads, emulsion, amplification, magnetics) method could be employed to create Lab-on-Bead libraries in a single reaction well.

Once a suitable Lab-on-Bead library has been created, it can be screened with target molecules that are bound to a surface. Specifically target-binding beads can be distinguished from nonspecific binders through washing, since bound-beads remain immobilized longer in the presence of flowing buffer, than nonspecific binders. Once bound-beads are located by fluorescence monitoring, they can be extracted by a micropipette manipulator.

Here, a micropipette manipulator (Sutter Instruments, MP-285 Motorized Micromanipulator with Rotary Optical Device) was integrated with a fluorescence microscope (Supplemental Figure 1). The manipulator allows the user to control the position of a micropipette in discrete 40-nm steps in the *x*, *y*, and *z* directions, with a dynamic range large enough to allow a selected bead to be moved several centimeters. Beads were extracted from the surface with negative pressure applied through the micropipette tip, which has approximately the same diameter as the bead to be extracted. For the 1 μ m diameter range beads used in this study, borosilicate glass capillaries (OD 1 mm, ID 0.5 mm) were pulled with a Sutter P-87 micropipette puller to a tip size of 0.8 μ m, confirmed by bubble testing (for details see Supplemental Materials). Figure 2 shows the selection, extraction, handling, and expulsion of a 1 μ m fluorescent, DNA-conjugated microsphere with the micropipette manipulator/fluorescence microscope combination. Subsequently, each extracted bead was subjected to PCR to amplify the identification bar codes contained in the oligonucleotide to a detectable amount, and this PCR product was sequenced to precisely identify the target-binding molecule.

With this NanoSelection platform, we can effectively and efficiently select target binding molecules from surfaces. To demonstrate the capabilities of this improved platform for selecting nucleic acid-encoded chemical libraries, we have created Lab-on-Bead pools for two PNA-encoded chemicals, biotin and FITC, and selected for binding to surfaces coated with neutravidin or anti-FITC antibody.

The PNA-encoded chemicals were bound to the beads as follows. First, Lab-on-Bead capture libraries were created by conjugating single-strand DNA sequences (107 bases) to Yellow Green or Yellow Orange Fluoresbrite® Carboxylate Microspheres (1 μ m, Polysciences) by EDC coupling (Iannone *et al.*, 2000). The sequences contain three segments: (i) a 5'-NH₂ bead linker region, (ii) two 29 nt PCR handles (Table 1, italics), and (iii) a PNA binding (capture) region (Table 1, underlined). Three different bead types were created. One that captures PNA 1, biotin-TGATGACGAACGG (N- to C-terminal); another one that captures PNA 2, FITC-CAAATGAGCACGG (N- to C-terminal); and a third, null bead library, with a 92 bp DNA sequence containing no PNA capture region (Table 1).

We performed three types of experiments to demonstrate the viability of the NanoSelection technique to select these PNA-encoded chemicals from surfaces. First, we examined high affinity interactions by selecting the biotin–PNA conjugate (PNA1) from 10:1 mixture of null beads to PNA1 beads off neutravidin substrates. Substrates were prepared as described in the Materials and Methods Section and images of these experiments are shown in Supplemental Figure 3. Second, we examined a lower affinity interaction by selecting the FITC–PNA conjugate (PNA2) from a 10:1 mixture of null beads to PNA2 beads off anti-FITC antibody surfaces (Figure 3), prepared as described in the Materials and Methods Section. Finally, we again selected the FITC–PNA conjugate (PNA2) from a 10:1 mixture of PNA1 to PNA2 beads off anti-FITC antibody surfaces (Figure 4).

Flow cells for bead selection were prepared and cleaned as described in Joo and Ha (2008) and the Materials and Methods Section. Briefly, two 0.75 mm-diameter holes are drilled into a glass slide to form an inlet and outlet port, and removable double-sided tape or parafilm is used to seal the chamber. Chambers are coated with biotinylated BSA then neutravidin for the selection of biotin-PNA (PNA1) or by nonspecifically adsorbing anti-FITC antibody to the surface for the selection of FITC-PNA (PNA2). The concentrations used to coat the surfaces, 0.4 µg/mL neutravidin or 1 mg/mL anti-FITC antibody, were optimized to produce surfaces with a large number of well-spaced, single surface binders. These amounts can easily be increased or decreased to produce more or fewer surface binders. Vacuum is applied to the outlet port to remove solution from the chamber; however, buffer is continuously applied to the inlet port such that the solution meniscus is not pulled through the chamber by the vacuum.

On the day of the experiment, a 10 µL aliquot of bead suspension, containing $\sim 2 \times 10^6$ beads, was hybridized with 1 µL of 100 µM PNA at 42°C for 30 min in 10mM NaH₂PO₄, then any excess PNA was washed from the beads with 10mM NaH₂PO₄, 150mM NaCl, 0.02% Tween® 20, pH 7.2. In addition to the specific PNA-DNA hybridization, beads coated with nonbinding DNA strand were also incubated with either the PNA1 or PNA2 sequence as above, these hybridization conditions resulted in very little nonspecific binding of PNA1 or PNA2 to the null sequence or the DNA sequence not containing their specific complementary region. We also tested the hybridization specificity of the PNA by incubating in a mixture of both null sequence beads and PNA-specific beads (1:1 and 10:1) and no appreciable difference in nonspecific binding on the surface was observed (data not shown).

The bead solutions were then combined to create the correct ratio of analytes and added to the washed sample chambers and incubated for 2 min. The unbound beads were then removed from the sample chamber, which was subsequently washed with 400µL of 10mM Tris-HCl pH 8.0 and 50mM NaCl for the neutravidin surfaces or 800µL of PBS for the anti-FITC antibody surfaces. During the incubation and washing, the presence of beads and surface binders was verified by fluorescence microscopy (Figure 5 and Supplemental Figure 2).

Initially, different colored beads were utilized to determine the number of specific and nonspecific surface binders during incubation and washing steps. As shown in Figures 3 and 4 (and also, Supplemental Figures 3 and 4), the PNA/DNA hybridized beads clearly bind to the surface; however, there is some nonspecific binding observed on both surfaces (Figure 5). For these studies, the incubation period (Supplemental Figure 2) and washing volumes (Figure 5) were optimized to stringently select for positive binders, while providing a large number of well-spaced, single bead binders for pick up with the micropipette. As shown in Figure 5, these conditions can be relaxed significantly to allow for more binding events. However, as incubation periods increase there is also an increase in the number of bead clumps observed on the surface (Supplemental Figure 2), which will slow pick up by micropipette.

Overall, we found more nonspecific binding events at lower wash volumes for the anti-FITC antibody surfaces, than for the neutravidin surfaces, which may be attributed to the nonspecific adsorption of the antibody. However, both surfaces provide a significant number of surface binders for analysis with the short incubation times and stringent washing used here (Figure 5).

After washing the majority of the nonspecific beads out of the chamber, the flow cells were carefully disassembled and the micropipette manipulator was used to extract the binders

from the surface. All of the beads picked up off the surface and PCR amplified were taken from experiments that utilized a *single* color bead for both specific and nonspecific binders, so no sampling bias was introduced. An inverted fluorescence microscope (Zeiss Axiovert 200, Hamamatsu EMCCD camera) was used to excite the fluorescent beads and identify beads at the surface of the microscope slide for pick up. The micropipette tip was then roughly centered over the imaging area by eye, and the tip was lowered toward the target with the manipulator. The pipette was angled at about 20° with respect to the horizontal plane, so the tip was the first part of the pipette to come into contact with the surface. Once the tip was within one or two bead diameters of the bead, a vacuum valve was opened that provides suction at the tip, and the bead was then sucked on to the tip. Once a bead was secured to the tip, it was drawn up through the meniscus and was either expelled by positive pressure onto a glass shard and placed in a microtube, or deposited into a microtube by inserting the pipette tip and snapping it off. Both techniques were utilized for the bead pickups shown in Figures 3 and 4 (and Supplemental Figures 3 and 4), with no differences observed. For the experiments shown in Figures 3 and 4, 15 and 16 beads, respectively, were extracted from the surfaces and subjected to two rounds of PCR with nested PCR primers (described in Supplemental Materials). In all three of the experiments described, we did not select any nonspecific binders off the surfaces with the extensive washing conditions used. This is confirmed for the PNA and null bead experiments by the PCR product size (Figure 3, Supplemental Figure 3), and for the third FITC-PNA versus biotin-PNA experiment by restriction digest of the PCR product and sequencing (Figure 4, Supplemental Figure 4).

DISCUSSION

As demonstrated by these experiments, the NanoSelection platform is an effective and efficient tool for low (subpico- to femtomolar) concentration high-throughput screening of nucleic acid encoded chemicals. While the reaction conditions presented here represent very stringent reaction and binding conditions for selecting high affinity interactants, the NanoSelection platform can easily be adapted for a range of binding affinities or even multiplexed to allow selection from surfaces with multiple targets.

The number of surface binders can easily be improved by increasing the number of target molecules on the surface, increasing the incubation time, or decreasing the wash volumes. Flow cells can be constructed with mylar spacers (12 μm , 3M) to decrease the height of the chamber and increase the likelihood of the beads being exposed to the surface. Alternatively, beads with a higher density, e.g., magnetic beads, could be used to decrease buoyancy in the chamber and therefore increase the exposure of the beads to the surface. These changes could potentially increase the number of nonspecific surface binders; however, they can be easily integrated into the NanoSelection platform described, and optimized for the desired number of surface binders. Furthermore, with new materials and surfaces continuing to emerge for DNA and protein microarray applications, protein targets could be printed onto surfaces and multiple protein targets could be screened in a single experiment. The modular nature of the NanoSelection technique allows any coverslip-based surface to be utilized.

This novel method has several potential advantages as a selection tool over current screening techniques. The relatively simple design allows rapid and simple sample preparation, while requiring orders of magnitude less material than other high throughput screening techniques. The bead-based design allows for easy screening over a variety of prepared surfaces, and by employing strategies that conjugate multiple copies of a unique sequence on a bead, a higher avidity multivalent interaction with the surface results, which is a significant advantage over current strategies. Additionally, since the creation of the Lab-on-Bead libraries relies on robust and well-described conjugation chemistries, such as biotin-streptavidin interactions or EDC coupling, each bead can be easily functionalized with a different clonal set of

molecules. Further, as nucleic acid libraries continue to grow in size, the synthesis of bead libraries could be greatly increased by using techniques such as BEAMing PCR (Diehl *et al.*, 2006; Li *et al.*, 2006). The instrumentation can be integrated into any inverted fluorescence microscope. It is inexpensive, as compared to AFM (Peng *et al.*, 2007) and other screening techniques, and its components are readily available from several commercial vendors. The integration of the fluorescence microscope with the micropipette manipulator allows for individual surface binders to be extracted and unambiguously identified quickly. The tip sizes of the micropipettes can be adjusted to accommodate a wide range of different bead sizes. The micropipette can extract beads from dry surfaces, and from within solution and can draw the bead through the surface tension at the meniscus without losing the bead (Figure 2). The resolution of the method is extremely fine (40 nm) yet its range is very large (2–3 cm) making it very flexible; this allows for selection of small beads from very large libraries. The micropipette can use suction or positive back pressure to pick up or expel a bead from the tip, allowing for multiple bead pickups without changing the pipette. With this method, it is possible to extract 10–20 beads per hour – even higher by the incorporation of microfluidics.

Our technique may also be combined with microarrays, for example, to select drug candidates from multiple targets spotted on a microarray. Though microarrays are also used for drug discovery, our technique is, in a sense, the inverse of microarrays, which may be an advantage or disadvantage, depending on the application. In our technique, the target is attached to the surface and the library of candidates is flowed over the target area; then single, bound candidates are selected and identified. In a microarray, ultimately, the candidates (or decoders) are arrayed on a surface, and the targets are flowed over the array. Target-bound candidates are then identified in bulk according to their binding spot on the array.

The key distinction of our technique is that we can pick up and identify single, target-bound, nanoscopic beads each with a single sequence on it. While our technique still requires amplification after selection, iterative rounds of selection, amplification, and re-selection are not required. Moreover, as sequencing technologies continue to improve, our unique clonal bead libraries could provide enough copies of a single selected sequence to allow direct sequencing of the binder from the extracted bead, streamlining the selection, and identification process even further.

CONCLUSIONS

As shown here, NanoSelection with Lab-on-Bead has the potential to dramatically miniaturize and accelerate assays currently used for the discovery of new drug candidates, diagnostic reagents, and disease markers. At this stage, we can screen small libraries of aptamers (Peng *et al.*, 2007) and as described in this work, nucleic acid encoded libraries. In principle, the low cost and simple design of the NanoSelection platform could allow its application to be extended beyond nucleic acids based systems to protein libraries as well (Shlyakhtenko *et al.*, 2007). Additionally, the compartmentalized design of the NanoSelection platform could be readily adapted to microfluidics, since it can operate with wet samples, or integrated with incubation systems for screening of binders to targets on cell surfaces. Taken together, NanoSelection with Lab-on-Bead provides researchers with an adaptable and flexible tool for screening molecular binders quickly, efficiently, and at low concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Overview of the NanoSelection method.

Figure 2.

Bead pick-up with micropipette manipulator. Beads are 1 μm in diameter. The top panels depict pick-up of a targeted bead from the first bead-containing well. The focal plane is at the surface of the glass slide, and out-of-focus beads can be seen in the surrounding solution. Vacuum applied through the pipette is seen drawing the bead to the tip. The middle panels show the pipette and bead pulling out of the first solution. The bottom panels show the pipette and bead exiting the solution and entering a buffer-containing well. The objective is changed (from 40 $\times$ to 20 $\times$) to follow the pipette as it moves to the empty well.

Figure 3.

(A) Fluorescence microscope images of the 10:1 null beads to FITC-PNA2 beads incubation taken with 20 $\times$ objective. The FITC-PNA2 beads are shown in green, imaged with a 535/40 emission filter, and the null beads in the same area are shown in red, imaged with a 620/60 emission filter. (B) After washing with 800 μ L of PBS, a few specific FITC-PNA beads remain bound to the surface while the null beads are washed away. The inset images are taken with a 60 $\times$ objective and distinguish between single and clumped surface bound beads. (C) A 17% native acrylamide gel of the PCR products from 10 representative extracted beads and two controls of the dPNA2 sequence (109 bps) and the null sequence (94 bps).

Figure 4.

(A) Fluorescence microscope images of the 10:1 biotin-PNA1 beads to FITC-PNA2 beads incubation taken with 20 $\times$ objective. The biotin-PNA1 beads are shown in green, imaged with a 535/40 emission filter, and the FITC-PNA2 beads are shown in red, imaged with a 620/60 emission filter. (B) After washing with 800 μ L of PBS, a few specific FITC-PNA2 beads remain bound to the surface while the biotin-PNA1 beads are washed away. (C) A 17% native acrylamide gel of the *Xho*I digested PCR products from 10 representative extracted beads and two controls of the dPNA2 and dPNA1 sequence (109 bps). Digest of the dPNA2 yields a 71 bp fragment and a 38 bp fragment. (D) Sequencing results for beads 2 and 6 are displayed. Both clearly show the complementary binding sequence for PNA2 (red letters).

Figure 5. Number of surface binders observed after subsequent washing steps for (A) biotin-PNA and (B) FITC-PNA.

Table 1

Lab-on-Bead DNA sequences

Name	Sequence
dPNA1	5'-NH ₂ -TCCCGCGGAAATTAAATACGACCATGTTATGATAGTCAACAGTTTAAACCGCTTCGTCATCAATGTTGGTTACGGATCCACA GTTAAATTCA GATAGCTGCA GAGCTCCAGC
dPNA2	5'-NH ₂ -TCCCGCGGAAATTAAATACGACCATGTTATGATAGTCAACAGTTTAAACCGCTGCTCATTTGATGTTGGTTACCTCGAGACAGTTAAATTCA GATAGCTGCA GAGCTCCAGC
dNull	5'-NH ₂ -TCCCGCGGAAATTAAATACGACCATGTTATGATAGTCAACAGTTTAAAGTCTGACTTTTACGATACAGTTAAATTCA GATAGCTGCA GAGCTCCAGC

Italic - capture PCR handles; underlined - PNA capture region.

Table 2

Lab-on-Bead primer sequences

Name	Sequence
Primer 1 F	5'-GCTATGCATGTCCCGCGAAA
Primer 1 R	5'-CTCGTCACAGCTGGAGCTC
Primer 2 F	5'-TCCCGCGAAATTAATACGACCATGTTATG
Primer 2 R	5'-GCTGGAGCTCTGCAGCTATCTGAATTAAC