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# Microfluidic Library Screening for Mapping Antibody Epitopes

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**The capability to screen molecular libraries using disposable microfluidic devices provides the potential to simplify and automate reagent generation and to develop integrated bioanalytical systems for clinical diagnostics. Here, antibody epitopes were mapped using a disposable microfluidic device to screen a combinatorial peptide library composed of $5 \times 10^8$ members displayed on bacterial cells. On-chip library screening was achieved in a two-stage, continuous-flow microfluidic sorter that separates antibody-binding target cells captured on microspheres through dielectrophoretic funneling. The antibody fingerprints identified were comparable to those obtained using state-of-the-art commercial cell sorting instrumentation.**

Peptide libraries have been used previously to identify autoantibody antigens or mimotopes with diagnostic and therapeutic value.[1] For example, serum autoantibody signatures obtained using mimotope arrays were recently shown to provide improved performance for prostate cancer diagnosis when compared with the widely used marker, prostate-specific antigen.[2] The use of cell-displayed peptide libraries could simplify screening for antibody fingerprinting. Furthermore, whole cells can be used as reagents to purify reactive antibodies from serum.[3] Previously, we and others have demonstrated the facile mapping of antibody epitopes using a library of peptides displayed on the surface of *Escherichia coli* in conjunction with library screening via fluorescence-activated cell sorting (FACS).[3,4] While FACS has proven to be highly effective, because of the high cost and limited accessibility of instrumentation, there exists a need for economical, closed-system sorters. Furthermore, disposable sorters could prevent cross-contamination of patient samples and contain potential biohazards in both research and clinical settings. Toward this goal, we have developed a methodology for mapping antibody epitopes using cell-displayed peptide libraries and a microfluidic sorting device (Figure 1).

## EXPERIMENTAL SECTION

A library of *E. coli* cells, each displaying a random 15-mer peptide fusion within the second extracellular loop of outer membrane protein OmpX, was constructed essentially as described previously[5] using PCR and a low-copy pBAD33-based vector, with the exception that the primer contained 15 degenerate NNS codons. Library diversity was $\sim 10^{10}$ independent transformants. The cell surface expression of peptides was induced by the addition of arabinose (0.02% w/v) to a log-phase culture for 45 min at 37 °C in LB medium with 25 $\mu$g/mL chloramphenicol. Approximately $10^9$ cells were centrifuged (2650*g*, 5 min) and resuspended in 500 $\mu$L of 0.25× PBS, 0.5% BSA (PBSB) containing 20 nM biotinylated mAb (anti-T7·tag, Novagen; anti-FLAG BioM2, Sigma). Following 1-h incubation at 4 °C, the cells were centrifuged as before, resuspended in 500 $\mu$L of PBSB containing 20 nM biotinylated goat anti-mouse IgG-Fc polyclonal antibody (Sigma), and incubated for 1 h at 4 °C. For the T7·tag screening, the secondary antibody labeling was found to be unnecessary. Cells were then washed twice in 500 $\mu$L of PBSB and resuspended in 500 $\mu$L of PBSB with 10 $\mu$L ($\sim 10^6$ beads) of streptavidin-coated, 5.6-$\mu$m Proactive microspheres (Bangs Lab) that had been previously washed four times in BlockAid (Invitrogen/Molecular Probes). Following overnight incubation on an inversion shaker at 4 °C, 10 $\mu$L of 10 mM m-biotin-PEG5000 (Nektar) was added to block remaining free biotin-binding sites and decrease nonspecific interactions during the sort. The cell/bead suspension was washed once with 800 $\mu$L of sorting buffer (0.1× PBS, 1% BSA, 20% glycerol) and passed through a BD Falcon cell strainer 35-$\mu$m mesh filter.

The two-stage device fabrication and the operating conditions for dielectrophoretic sorting were similar to those described previously for the one-stage chip.[6] Briefly, cell suspension and sheath buffer were introduced to the sorting device by syringe pump at 150 $\mu$L/h each, and voltage was applied sinusoidally at 500 kHz and 20 V peak to peak. The height of the fluidic channels in the chip was 20 $\mu$m, and the fluid path from inlet to outlet was ~16 mm. Beads were typically collected at the output for 3 h in

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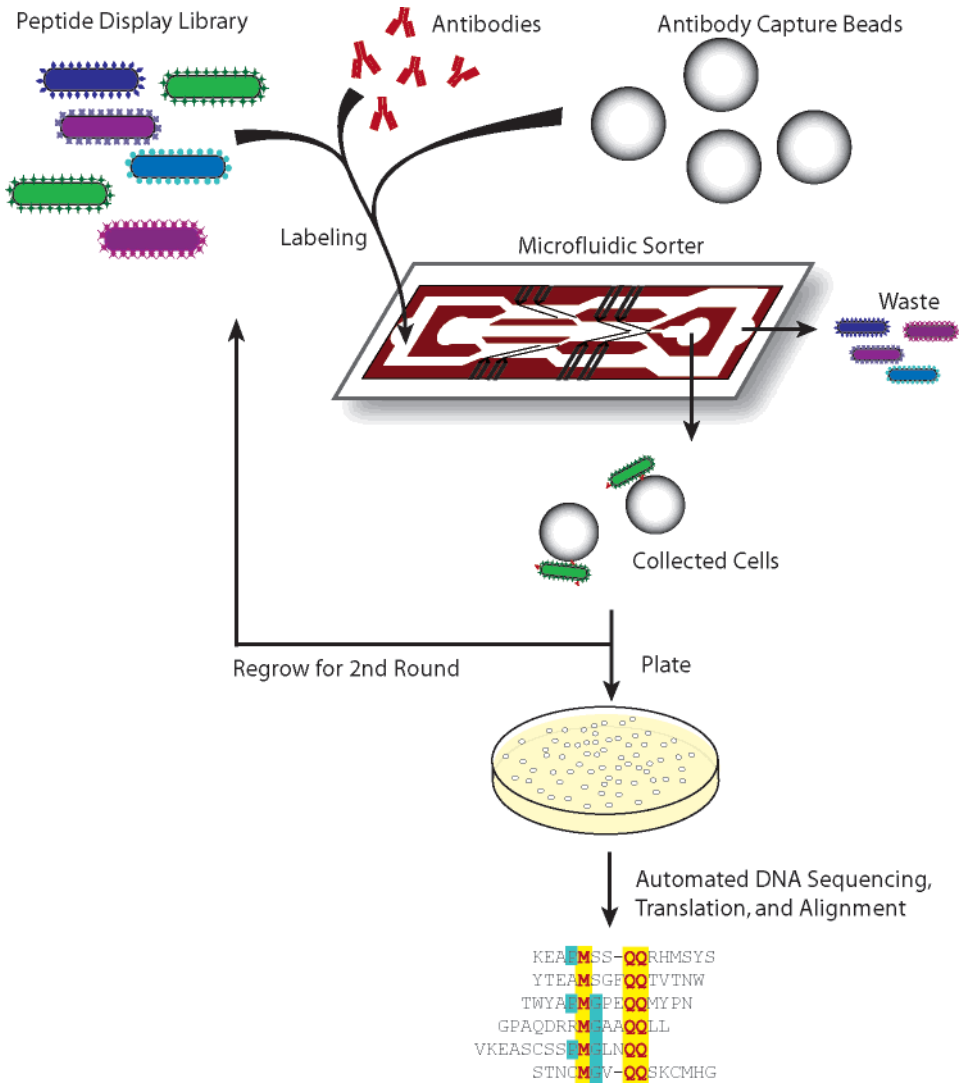


**Figure 1.** Schematic depiction of antibody fingerprinting using a microfluidic sorting device (not to scale). Bacterial cells displaying peptides complementary to the antibody-binding region are captured on polymeric beads, allowing continuous-flow separation by dielectrophoresis. The binding population is then either amplified by growth for a further round of labeling and sorting or plated on solid media to isolate single clones for sequence determination.

the first round, corresponding to a throughput of ~5 × $10^8$ cells. Collected beads with attached cells were added to LB medium with 0.2% glucose for overnight growth. A second round of sorting was performed as for the first, but with 5 nM T7mAb or 20 nM FLAGmAb (antibody concentrations were determined empirically).

Sequential magnetic selection (MACS) and FACS were performed as described previously[4] with the following modifications. Library cells were induced for 20 min at 37 °C. One round of MACS was performed using $10^{11}$ cells, 5 nM biotinylated mAb, and 7 × $10^7$ streptavidin-coated magnetic beads (Invitrogen/Dynal M-270 SA 2.8 $\mu$m Dynabeads). The first round of FACS (BD FACSAria) involved labeling with 1 nM biotinylated mAb and 1 nM anti-biotin mAb-PE (Miltenyi). In the second round of FACS, cells were labeled with 0.1 nM biotinylated mAb and 1 nM anti-biotin mAb-PE. All labeling was carried out in PBS containing 0.5% BSA. Peptide sequences were deduced from DNA sequencing of isolated clones following sorting. Sequence alignment, residue shading, and consensus determination were performed using AlignX software (Invitrogen/Informax).

## RESULTS AND DISCUSSION

For antibody epitope mapping, a bacterial display library of random 15-mer peptides was constructed as insertions within an extracellular loop of the outer membrane protein OmpX. Compared to previous studies using an OmpA-displayed library, the smaller, single-domain OmpX allows for both increased display level[5] and improved binding to capture beads (data not shown). To identify binding peptides, the library was incubated with biotinylated antibodies and subsequently with streptavidin-functionalized 5.6-$\mu$m polystyrene microspheres such that those library members displaying peptides complementary to the antibody-binding pocket were captured on the beads. The bead-captured cells were then separated from the nonbinding cells using a two-stage microfluidic sorter that separates cells using dielectrophoresis (DEP). Dielectrophoresis-activated cell sorting (DACS) relies on the difference in the ability of the polymeric capture beads to be polarized relative to the sorting buffer.[6,7] In a nonuniform

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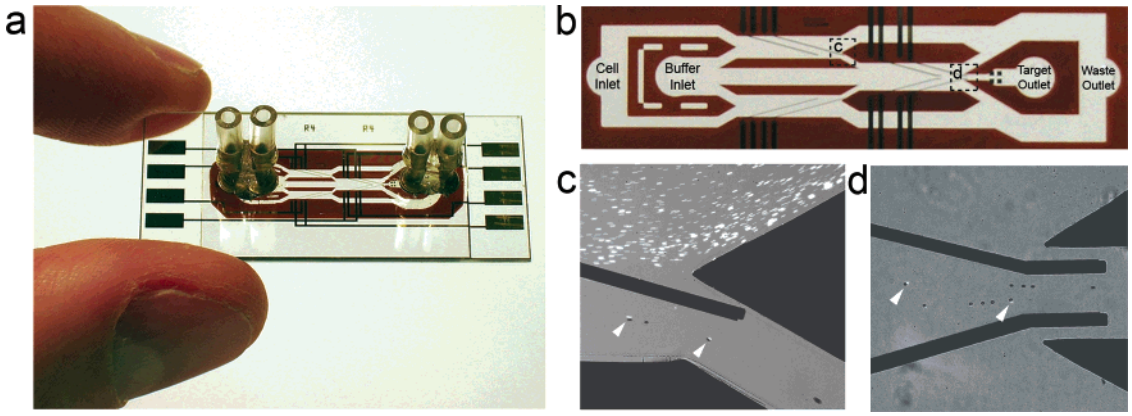


**Figure 2.** Two-stage DACS device. (A) Photograph of a sorting device with the inlet and outlet tubing attached. For operation, the electrodes are connected to a function generator via card edge connectors, and fluid is delivered by a dual-syringe pump through 0.02-in.-i.d. tubing. (B) Closeup photograph of the two-stage DACS device after fabrication, but before attachment of the tubing connectors. Two glass slides sandwich the polyimide spacer (red areas) that defines the fluid channels (white areas). In this top view, each angled dark line is a pair of gold electrodes, deposited on the top and bottom glass surfaces of the channels, that essentially do not impinge the fluid flow. (C) Micrograph of the sorting device in operation showing the first sorting stage. Laminar fluid flow (from left to right) prevents mixing of the buffer and sample streams. Beads, along with any attached cells (shown with arrows), are deflected by DEP into the buffer stream (bottom of photo) toward the second stage, whereas unattached cells pass the electrodes without being deflected. Both transmitted light and epifluorescence were captured in order to visualize the nonfluorescent beads and fluorescently labeled bacterial cells. Both target and nontarget cells were made to express a green fluorescent protein (AlajGFP1),[20] and images were captured at 10× magnification by a CCD camera. (D) Micrograph of the second stage at the collection point. Beads are focused toward the target outlet channel, and fluorescent cells are clearly visible attached to beads (shown with arrows).

electric field, such a difference results in a net dielectrophoretic force on the beads. In our case, the operating conditions were chosen to achieve negative DEP, wherein the beads are repelled from the electrodes. The electrode pairs are aligned on the top and bottom of the microfluidic channel, generating an electric field gradient at an angle such that the horizontal component of the DEP force is greater than the hydrodynamic force. As a result, the bead-labeled cells are deflected from a sample stream into the collection channel through the dielectrophoretic "funnel". Meanwhile, unlabeled cells pass through the force field and into the waste channel. To enhance sort purity, serial sorting stages were designed into a single microfluidic chip (Figure 2). In the first stage, the capture beads are deflected from the sample stream into the buffer stream and toward the center channel, while the vast majority of unlabeled cells follow the streamlines along the outer channels to the waste outlet. Likewise in the second stage, beads and cell/bead conjugates are again funneled into the collection channel by the DEP force field, while stray, unlabeled cells follow the fluid path that reconverges with the waste stream. After sorting, the collected target cells could then be regrown for a further round of sorting or plated to isolate single clones for DNA sequencing analysis (Figure 1).

Two cycles of library sorting in the microfluidic sorter enabled identification of a family of peptide ligands specific for monoclonal antibodies raised against either the T7·tag or the FLAG peptide (Table 1). Following microfluidic sorting for cells binding to the anti-T7·tag mAb, 25 of 27 randomly picked clones exhibited binding to the mAb, as measured using flow cytometry. Clone T7-D5 was the most abundant in the sorted population, representing 14 of 25 sequences. In the case of FLAG, 11 of 16 clones were positive after two rounds of sorting, with clone FLAG-D2 representing 5 out of 10 sequences. In the case of the FLAG monoclonal antibody, a secondary labeling with biotinylated anti-mouse polyclonal antibodies resulted in improved separation relative to that obtained using only a biotinylated FLAG mAb. Interestingly, the DACS protocol did not enrich library clones that bind directly to the streptavidin-functionalized capture beads used here, a problem that can complicate library screening using MACS and FACS. For comparison with DACS, the library was screened using state-of-the-art cell sorting methods consisting of sequential MACS (for pre-enrichment) and FACS (Table 1). For both microfluidic and conventional cell sorting, the identified consensus sequences were in excellent agreement with previous studies,[4,8−10] demonstrating that microfluidic sorting provided an effective means to screen bacterial display libraries for antibody epitope mapping.

The anti-FLAG M2 antibody[11] has been mapped previously using both phage display and DNA display, revealing a consensus sequence of DYKXXD.[8,10] In vitro alanine scanning confirmed the importance of residues in positions 1, 2, 3, and 6 of the FLAG peptide sequence (DYKDDDDK),[9] and NMR analysis of free versus mAb-bound peptides indicated close contact of aspartate residues at the first, fourth, and sixth positions.[12] Here, screening of the OmpX display library by DACS identified a five-residue consensus of DYKDXD, in agreement with other display technologies. In the case of the T7·tag antibody, screening yielded both MGXXQQ- and MGXQQ-containing sequences, with the QQ motif strongly conserved, in agreement with a previous study using a different display scaffold.[4] The ability to identify four or five consensus residues using on-chip, cell display library screen-

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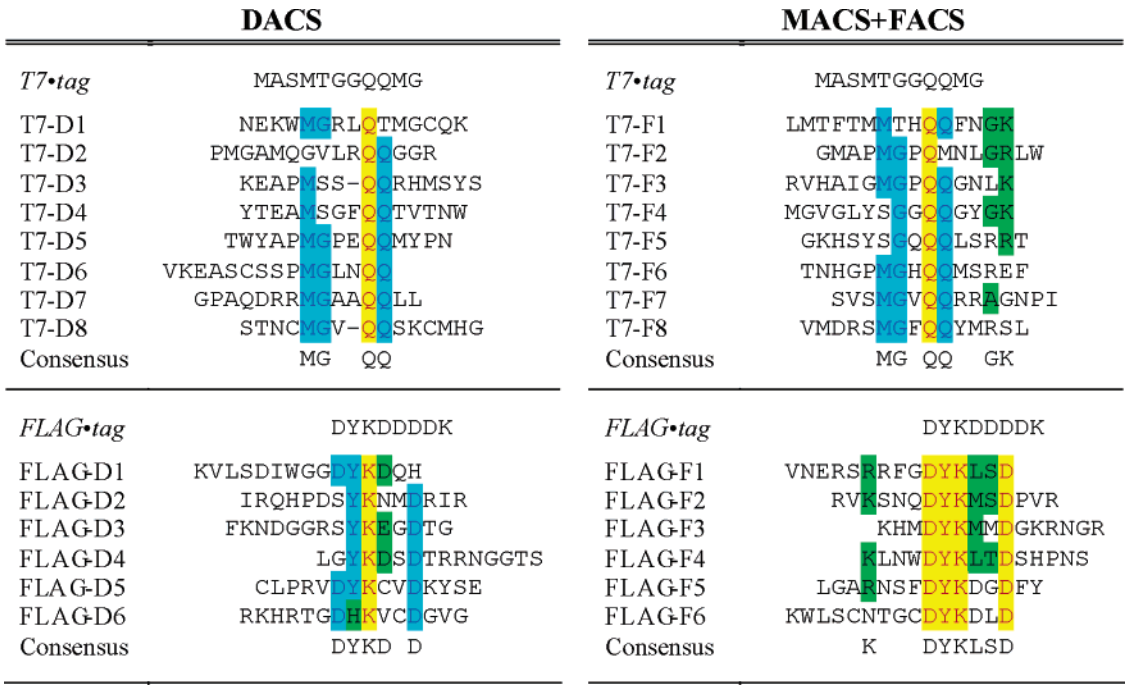
**Table 1. Peptide Sequences of Clones Selected for Binding to Anti-T7·Tag or Anti-FLAG M2 Monoclonal Antibodies, after Two Rounds of DACS, or One Round of MACS Plus Two Rounds of FACS[a]**




[a] The T7·tag and FLAG peptides, against which the antibodies were raised, are shown for comparison.

ing compares favorably with well-established peptide display methods.[13]

The microfluidic sorting chip described here incorporates two sequential stages (Figure 2) as a means to enhance purity. The second stage enabled removal of nontarget cells that were inadvertently captured in the first stage, thus allowing for increased tolerance to flow disturbances and microbubbles, as well as more robust purity performance when using high cell concentrations at the inlet. Even in the two-stage device, enrichment was limited by nonspecific capture of nontarget cells on the capture beads, rather than by the device performance (data not shown). Microspheres prepared with alternative materials and surface coatings specifically designed for DACS could potentially reduce nonspecific binding, as well as provide increased DEP force to enable increased throughput. Even so, the frequency of antibody binding library clones after two cycles of enrichment was comparable to that obtained using sequential MACS and FACS.

Serial staging of sorting devices in cell separations represents an approach to obtain both high purity and throughput, which can be difficult using conventional batch separations. The use of a single-channel, two-stage device enabled processing of $>10^8$ cells/h—sufficient to accommodate libraries of typical size. Throughput could potentially be increased further, without sacrificing purity or recovery, by using parallel channels fabricated on a single chip. Furthermore, multistage DACS is well suited for integration into fully automated microanalytical devices that incorporate operations for upstream reagent delivery and labeling and downstream on-chip manipulations including cell culture, single cell PCR, and DNA sequencing.[14−16] In such a case, having bead-labeled cells could be an advantage since bead-based DNA sequencing can be accomplished in subnanoliter wells.[17,18]

We have demonstrated effective epitope mapping of purified monoclonal antibodies using two rounds of microfluidic sorting and sequencing a small number of clones. Extending this technique to polyclonal signatures of serum antibodies for disease profiling will require a larger number of sequences in order to extract consensus groups, therefore underscoring the motivation for an integrated chip-based approach. Multiple sequence alignments resulting from library screening could then also be combined with computational mimotope analysis to identify conformational epitopes on antigens[19] for identification of possible therapeutic targets. This microfluidic-based library screening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technology is not limited to antibody fingerprinting applications, as one could envision its application to other affinity-based screens using substrate-functionalized beads, as well as for automated reagent generation, wherein ligands for a given protein or cell type could be discovered using self-regenerating libraries. Furthermore, the technology is amenable to sorting other molecular display platforms, such as phage, yeast, and ribosomes. In summary, antibody fingerprinting by microfluidic sorting yielded results matching those obtained using commercial cell-sorting instrumentation, but in a disposable, closed-system format. Sorting of molecular libraries by using a continuous-flow microfluidic chip represents an important step in the miniaturization and integration of laboratory operations in self-contained devices.

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