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Research paper

# Antibody discovery via multiplexed single cell characterization

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ABSTRACT

The secreted immunoglobulin footprint of single hybridoma cells, containing ~10 fg of antibody purified *in situ*, has been probed for 9 properties concurrently by use of detection labels comprising 280 nm combinatorially colored fluorescent latex beads functionalized with proteins. Specificity of each individual hybridoma cell's product has thereby been assessed in a primary screen. Varying the density of antigen on beads to modulate the avidity of the interaction between bead and secreted antibody footprint allowed rank ordering by affinity in the same primary screen. As more criteria were added to the selection process, the frequency of positive cells went down; in some cases, the favorable cell was present at <1/50,000. Recovery of the cell of interest was accomplished by plating the cells in a viscous medium on top of a membrane. After collecting the antibody footprint on a capture surface beneath the membrane, the immobilized cells were transferred to an incubator while the footprints were analyzed to locate the hybridoma cells of interest. The desired cells were then cloned by picking them from the corresponding locations on the membrane.



## 1. Introduction

Hybridoma screening technology today is very similar to the technique first described in 1980 (de StGroth and Scheidegger, 1980). After fusion of immunized mouse spleen cells to an immortal myeloma cell line, and limited growth under conditions selecting for hybrids, the hybridoma library is plated out at a dilution yielding a small number of clones per well, trading off noise from multiple clones in a single well against cost of screening additional wells at true limiting dilution (a process described by Poisson statistics wherein about two thirds of the wells have no cells). Following growth of the clones, and accumulation of secreted immunoglobulin, supernatant is sampled for antigen binding in an ELISA format. Although a fusion may yield up to 10,000 independent clones, economic constraints often prohibit screening the entire library, particularly at sufficient over-sampling to assure identification of rare favorable cells. Furthermore, supernatant ELISA based methods require clones to produce sufficient antibody to be detected, a fact that skews primary screening away from low producing, slow growing clones which in fact may have desirable binding characteristics.

For strong immunogens, standard practice is often sufficient, as recently stimulated B cells are preferentially preserved in the hybridoma formation process (Schmidt et al., 2001). This can result in a high enough frequency for isolation of useful antibodies. Many immunogens do not generate a robust response, however, including conserved antigens, integral membrane proteins with small extracellular exposure, and peptides. Furthermore, quality criteria typically include affinity as well as specificity within a protein family or among different epitopes on the target protein. Even for immunogenic antigens, raising the antibody quality threshold to encompass all these parameters naturally leads to lowering the frequency of favorable clones.

One approach to overcoming the problem of low frequency antibodies is to increase the number of cells screened by shrinking the well size from the standard 96

*Abbreviations:* BSA, bovine serum albumin;PBS, phosphate buffered saline;ELISA, enzyme linked immunosorbent assay;ECD, extracellular domain;HA, hemagglutinin.

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well microplate format. For example, wells have been prepared via microlithography that are only large enough to accommodate one cell (Love et al., 2006). Fluid handling difficulties rise as the well size decreases, however, including differential evaporation between wells at different locations, and difficulty in achieving uniform washing. Assaying supernatant precisely is thus difficult in such formats. Without replicates, both false positive and false negative rates rise, reducing the value of the technique. Noise level issues also limit flow cytometry as a technique to extract favorable clones, particularly as the frequency of those clones drops (Gross et al., 1993). Although flow cytometry is in principle capable of considerable multiplexing, in practice, the absolute signal and the dynamic range (which impacts signal to noise) decline as multiplexing increases, further limiting utility.

A different approach to increasing the fraction of the immune repertoire surveyed is direct examination of primary B cells. In an illustrative example of this approach, antigen coated erythrocytes are lysed by locally high concentration of antibody around a lymphocyte secreting antibody specific for an antigen conjugated to the erythrocytes (a hemolytic plaque assay) (Babcook et al., 1996). This technique is poorly quantitative, and is limited by the inability to measure multiple parameters in order to define antibody quality.

Finally, it is possible to bypass animal immunization altogether and generate very large recombinant antibody libraries, often comprising 10 million or more independent antibody sequences. In general, the initial hope (Lerner et al., 1992) that this diversity source would eliminate the need for *in vivo* immunization has not been fulfilled. Such libraries are typically screened by phage display methods in a single parameter assay, relegating specificity screening to more laborious secondary screening (Hoogenboom, 2005). Natural immunization remains a particularly effective method for generating high quality antibodies, due in large part to the highly parallelized screening against all human antigens along with interative selection, that takes place in vivo (Or-Guil et al., 2007). Screening recombinant libraries in a similarly parallelized, iterative fashion is well beyond our current capability to reproduce in the laboratory.

In spite of these limitations, monoclonal antibodies have become commonplace as research reagents over the past 25 years, and therapeutic antibodies have become the fastest growing segment of the pharmaceutical industry over the past 10 years (Reichert and Valge-Archer, 2007). The quality threshold for therapeutic agents is higher than for research reagents, requiring more exacting methods to meet therapeutic requirements. Thus, there is a need for reliable and efficient generation of antibodies meeting strict quality criteria, especially for poorly immunogenic antigens. Since specificity is one major aspect of quality, screening approaches based on a primary assay against a single antigen are not as likely to succeed as is a multiplexed primary screen.

The technology described here assays single cells using digital microscopy to read multiplexed probes bound to the secreted antibody footprint formed around individual cells. In effect, a microscopic virtual well is created *in situ* around each cell. The technique is readily useable for rapidly screening 100,000 to 1 million clones at a very high specificity threshold, enabling rapid examination of multiple libraries prepared by alternative immunization strategies. Modification of the probes to vary the avidity effect inherent in the bead based assay format enables rank ordering of clones by affinity in the primary screening step. Further customization of the probes allows screening for reactivity to solubilized integral membrane antigens. The high signal to noise of the assay format is particularly useful for this difficult class of antigens.

## 2. Materials and methods

### 2.1. Immunization protocols

Anti-peptide libraries were prepared by subcutaneous injection into Balb/c mice of cocktails of 4 or 5 peptides from the extracellular domain of cMet, each conjugated to Keyhole Limpet *Hemocyanin* (KLH) (Calbiochem). Four mice were used for each peptide pool, with 200 μg of the cocktail injected every other week for 10 weeks. Complete Freund's adjuvant was used for all injections except the final boost. Spleens were harvested 3 days after the final boost and lymphocytes fused to the myeloma line Ag8.653.P3 by standard methods. For immunizations with recombinant protein (cMet, RON, Protein Kinase C, Nerve Growth Factor), mice were injected in the footpad weekly with 5 μg protein in adjuvant for 5 weeks, with recovery of popliteal lymph nodes 3 days after the final boost. Immunizations with cMet-expressing A549 cells were done with $5\times10^6$ washed cells that were pelleted, resuspended in a minimal volume, and injected subcutaneously into mice every other week for 10 weeks. All animal work was conducted by Antibody Solutions, Inc (Mountain View, CA), in compliance with animal welfare regulations.

### 2.2. Preparation of bead probes

Multihued particles of 6 distinguishable types were used in these experiments. Functionalization was performed by incubating aldehyde derivitized particles with a 3-fold excess of purified protein (determined by total surface area calculation). After overnight incubation, a reductive amination reaction was performed to stabilize the covalent bond between the bead and protein amino groups. Beads were washed to remove unbound protein and then placed into buffered 2% BSA to promote colloidal stability. For peptide-bead probes, the peptides were first conjugated to BSA at a 1/1 mass ratio prior to bead conjugation. Functionalized beads are stored at ~0.1% solids by volume.

### 2.3. CellSpot assay

CellSpot 96 well assay plates were prepared by coating with goat anti-mouse IgG (Jackson ImmunoResearch), 2 μg/ml, and blocking with BSA. Cells were washed and counted prior to being dispensed into the assay plate. For cells that had been growing in bulk culture, any number between 100 and 10,000 cells were placed into each well of a 96-well plate; the optimal number was determined by the frequency of CellSpot-generating cells (i.e. frequency of clones recognized by any of the bead-antigen probes). More than 200 CellSpots per well proved to be difficult to image and analyze due to overlapping footprints. When the frequency of positives was unknown, cells were plated at several dilutions, and the