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Antibody arrays for high-throughput screening of antibody–antigen interactions

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We have developed a novel technique for high-throughput screening of recombinant antibodies, based on the creation of antibody arrays. Our method uses robotic picking and high-density gridding of bacteria containing antibody genes followed by filter-based enzyme-linked immunosorbent assay (ELISA) screening to identify clones that express binding antibody fragments. By eliminating the need for liquid handling, we can thereby screen up to 18,342 different antibody clones at a time and, because the clones are arrayed from master stocks, the same antibodies can be double spotted and screened simultaneously against 15 different antigens. We have used our technique in several different applications, including isolating antibodies against impure proteins and complex antigens, where several rounds of phage display often fail. Our results indicate that antibody arrays can be used to identify differentially expressed proteins.

Keywords: antibody, array screening, high throughput, single-chain variable fragment (scFv), phage display

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The availability of large phage–antibody libraries[1] has provided a source of binders to almost any antigen, including many that were previously considered difficult targets, such as self-antigens[2–4] or cell surface proteins[5–7]. Phage selection involves repeated rounds of growth, panning, and infection, which selects both for binding and for antibody fragments that are well expressed on phage. In addition, when selecting against highly complex targets (e.g., whole cells, cell lysates, or membrane preparations[8–10]), there is often a strong bias for antibodies directed against immunodominant epitopes and abundant proteins. To isolate the maximum diversity of binders it is therefore advantageous to analyze clones as early as possible in the selection procedure. Depending on the library and the number of input phage, the first round of phage selection generally yields 10^3–10^7 clones. Thus, conventional enzyme-linked immunosorbent assay (ELISA) screening using 96-well plates would only allow a small percentage of the selected clones to be screened. One way to increase throughput would be to use larger filter-based assays that allow up to 10^6 clones to be simultaneously screened on a single filter.

Mass screening was first described using expressed proteins in lytic λ-phage plaques[11] and later applied to screening libraries of recombinant antibodies[12,13]. Antibodies are absorbed (or captured on a "generic" ligand that binds all antibodies regardless of target ligand specificity[14]) on a filter that is left in contact with a lawn of lytic phage plaques. The filter is then probed with labeled antigen. Skerra and colleagues[15] showed that the addition of a second filter reduced the background caused by bacterial debris. Here, bacterial clones are grown on a filter and the antibodies they secrete are captured on a second filter that is coated with a generic ligand[15] or the target antigen[16] and placed directly beneath the first filter. This second filter can then be removed and probed with labeled antigen (where the filter is coated with a generic ligand) or with labeled generic ligand (where the filter is coated with target antigen).

Although such screening techniques enable large numbers of clones to be screened against a single antigen, because the plaques/bacteria are spread at high densities, it is difficult to identify genuine positives and isolate them from neighboring negatives. In practical terms, groups of clones (that correspond to the regions where a potential positive signal was observed) have to be deconvoluted by screening further lifts to identify a single binding antibody. Furthermore, it is often difficult to rescue viable clones from bacterial colonies that have been induced for protein expression[16]. Finally, such techniques are not suitable for screening against several antigens, because duplicate filters are difficult to produce and signals produced by the same antibodies against different antigens are hard to compare. This can lead to the isolation of a large number of false positives, because "sticky" or cross-reactive clones cannot be excluded during the initial screen.

To create a more robust screen for antibody–antigen interactions, whereby many antibodies are screened in parallel against many antigens, we wondered whether filter-screening techniques could be applied to ordered arrays of antibodies generated by robotic picking and gridding.

Results

Creation of high-density antibody arrays. We compared the signals derived by direct capture of scFvs on antigen-coated filters followed by probing with a labeled generic ligand with those derived by capture on a generic ligand-coated filter followed by probing with labeled antigen. We found that direct capture on antigen gave a consistently higher signal-to-noise ratio (data not shown). Furthermore, coating the antigen on the filter removes the need to label every antigen, which is time consuming and can be difficult for certain antigens.

Although, in principle, any antibody library could be used as a source of binders, we used a scFv library in which all members are well expressed and bind the generic ligands protein A and protein L (see Experimental Protocol). Because all scFvs in this library have binding sites for proteins A and L, we can determine both the extent of antigen binding (by capture on antigen and detection with protein A or L conjugates) and scFv expression (by capture on protein L and detection with a protein A conjugate, or vice versa).

One round of phage selection was performed with this library using purified bovine serum albumin (BSA) as the target antigen. A

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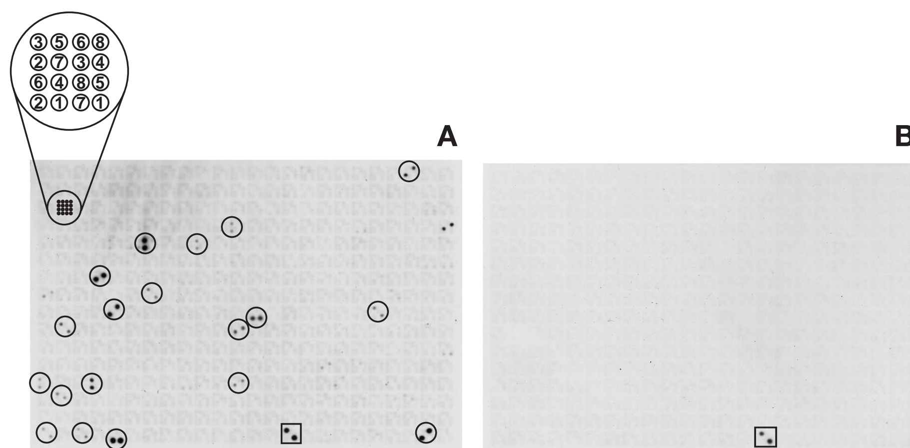

Figure 1. Antibody array screening after one round of phage selection using BSA. (A) Screening for BSA-binding scFvs. (B) Screening for HSA-binding scFvs. The panel depicted here consists of 2,304 clones each double spotted plates (384 x 6 x 2, in this example plates 7 and 8 were not used). Bacterial clones were arrayed in a 4 × 4 pattern (see expanded panel), BSA-specific antibodies are circled. The box indicates a BSA/HSA dual reactive clone. The fraction of bacterial clones expressing functional antibodies was also determined by array screening, by capturing the expressed scFvs on a Protein L-coated filter followed by detection with Protein A–HRP. This showed that about 50% of the clones expressed functional antibodies (data not shown).

total of 1.9×10^4 phage were eluted from the immunotube and infected into fresh bacteria. Clones (8,448, a number which represents almost half of the phage recovered from the first round of selection) were robotically picked and gridded. To identify BSA-specific binders as well as any cross-reactive clones, scFvs were screened on filters coated with either BSA (Fig. 1A), human serum albumin (HSA, Fig. 1B), or a mixture of irrelevant proteins (in this case HeLa cell proteins, data not shown), and then detected with protein L–horseradish peroxidase (HRP). We found that 49 of the 8,448 arrayed clones recognize only BSA (0.6%), and 2 out of 8,448 clones (0.02%) recognize both BSA and HSA but not the mixture of irrelevant proteins. Because BSA and HSA are 76% homologous on the amino acid level, it is not surprising that some antibodies recognize both proteins. To confirm whether these antibodies bind their respective antigens in conventional ELISA, 30 were taken from the master 384-well plates, grown, and induced in liquid culture. Nineteen of the 30 clones gave a clear positive signal on BSA or BSA/HSA and were negative on the mixture of irrelevant proteins, indicating that the high-density filter screen was indeed able to identify specific antibodies that bind as soluble fragments. Subsequent analysis of these antibodies indicated that most bind to native but not to denatured BSA (data not shown).

Array screening versus conventional selection and ELISA screening. To compare the utility of a mass array screen following a single round of phage selection to a conventional ELISA screen following one or two rounds of phage selection, purified recombinant ubiquitin (antigen T, see Experimental Protocol) was used as an antigen for library selection, either undiluted or diluted at different levels in a bacterial lysate. After two rounds of phage selection, ubiquitin binders were identified by conventional ELISA screening of clones selected using either 100% or 20% ubiquitin (Table 1). However, where only 2% or 0.2% was used, no anti-ubiquitin scFvs were identified by conventional screening even though significant phage–antibody enrichment was observed after the second round of selection (these antibodies are presumably directed against components of the bacterial lysate). In contrast, mass array screening after a single round of selection yielded specific ubiquitin binders for all dilutions, including 0.2% (Table 1). Forty-eight clones identified as being ubiquitin specific by array screening were picked, induced in liquid culture, and tested in conventional ELISA. Of these, 34 clones were confirmed as strong binders to ubiquitin (but were negative using an irrelevant bacterial lysate as the antigen; see Table 1). Thirteen of these clones were sequenced, and all were found to be different (Table 2), demonstrating that a wide diversity of binders was present. Several of these were tested on western blot analysis of recombinant ubiquitin and many gave a strong signal (data not shown).

Antibody arrays against complex antigens. Next, we tested whether array screening could be performed on clones selected using mixtures of three unpurified bacterial lysates containing the recom-

Table 1. Comparison of array screening with multiple rounds of phage display

Single antigen, recombinant ubiquitin (T)

	Round 1			Round 2	
Percentage protein[a]	Phage titer[b]	Array screening[c]	ELISA screening[d]	Phage titer	ELISA screening[d]
100	5×10^5	98/3,072 (3.2%; 17/18)	2/24 (8.3%)	6×10^8	24/24 (100%)
20	4×10^5	19/3,072 (0.6%; 9/18)	0/24 (0%)	4×10^8	9/24 (37.5%)
2	5×10^5	9/3,072 (0.3%; 6/8)	0/24 (0%)	6×10^7	0/24 (0%)
0.2	2×10^5	4/3,072 (0.1%; 2/4)	0/24 (0%)	3×10^8	0/24 (0%)

Mixture of antigens D, M, and T

	Round 1		Array screening	
Percentage protein[a]	Phage titer[b]	D positives[c]	M positives[c]	T positives[c]
0.2	4×10^4	7/3,072 (0.2%; 6/7)	1/3,072 (0.03%; 1/1)	30/3,072 (1%; 4/5)
0.05	4×10^4	7/3,072 (0.2%; 5/7)	7/3,072 (0.2%; 7/7)	71/3,072 (2.3%; 7/10)
0.005	4×10^4	4/3,072 (0.1%; 4/4)	4/3,072 (0.1%; 4/4)	10/3,072 (0.3%; 6/7)
0.0005	4×10^4	7/3,072 (0.2%; 4/7)	1/3,072 (0.03%; 0/1)	3/3,072 (0.1%; 1/2)

[a]The approximate percentage of target protein present in the bacterial lysate during phage selection.
[b]The approximate number of phage recovered after each round of selection. Input phage titers were routinely 10^{12}.
[c]The number of clones identified by array screening (as a percentage of the total in parentheses) that give a signal on the target antigen but not on any of the other antigens tested (including irrelevant antigens). In each case, several of these clones were picked and induced for soluble expression. The number that were confirmed as binding strongly to their target antigen in conventional ELISA (and were negative for the other antigens) is also given in parentheses along with the number tested.
[d]As an alternative to array screening, clones were randomly picked from the various selections and directly tested by conventional soluble ELISA. The number of positives is given (as a percentage in parentheses).

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binant proteins, D, M, or T, at concentrations of ~0.2%, 0.05%, 0.005%, or 0.0005% of total protein (see Experimental Protocol). In total, 12,288 clones (thirty-two 384-well plates) were picked and gridded, and identical copies of this array were screened separately against each of the three lysates using a coating concentration on the filters of ~100 μg/ml of total protein (containing antigens D, M, or T at approximate concentrations of 5, 5, and 10%, respectively; if screening was performed using 0.5, 0.5, and 1% D, M, and T, respectively, only about a tenth of the binding scFvs gave a detectable signal).

Specific binders to D, M, and T were identified (Fig. 2 and Table 1), and a limited number of positive clones, selected using different concentrations of recombinant D, M, and T, were picked and tested in conventional ELISA for binding to their respective antigens. Specific binding of soluble scFvs was confirmed for D (19/25 tested clones), M (12/13), and T (18/24) (see Table 1), including some that were selected using only 0.005% (M11, M12, T23, T24, T25, T27, and T28) or 0.0005% of recombinant protein in the lysate (D7, D9, and T29). Thirty-five of the confirmed positives were sequenced and all were different (Table 2), indicating that a wide diversity of binders was present. The 12 confirmed anti-M scFvs were tested for binding on BIAcore (see Experimental Protocol), and one (M12) was found to bind strongly to purified antigen M (with a solution affinity of 21 nM).

Our success in selecting antibodies against dilute components in complex cellular mixtures suggested that the same procedure could be used to select antibodies against targets present in natural cell extracts. Furthermore, using parallel screens it should be possible to identify those antibodies that recognize proteins that are differentially expressed between cell types. The scFv libraries were therefore selected on a lysate of HeLa cells. Clones (9,216) were gridded and tested for binding to filters coated with the HeLa cell lysate, a yeast cell lysate, or a bacterial lysate. From the screen, antibodies were identified that bound the HeLa lysate alone (27), HeLa and yeast lysates (4), or HeLa, yeast and bacterial lysates (75), the last probably corresponding to cross-reactive or "sticky" binders. The 27 Hela cell-specific clones were isolated and further tested by western blot analysis of HeLa and

Table 2. Antibody sequences of clones identified using array screening

Clone	Library[a]	Antigen	CDRH2	CDRH3	CDRL2	CDRL3
Single antigen T (recombinant ubiquitin)						
U1	DVT	T	TISTSGGYTYYADSVKG	GGSSFDY	TASALQS	QQTYSYPST
U2	DVT	T	NIGYDGSSTDYADSVKG	GASAFDY	AASTLQS	QQTYNSPST
U3	DVT	T	TISYSGAYTNYADSVKG	GGGSFDY	NASTLQS	QQTYSNPYT
U4	DVT	T	TISDAGGATYYADSVKG	NASAFDY	SASSLQS	QQSANSPYT
U5	DVT	T	SIGGTGSSTYYADSVKG	GSGCFDY	AASSLQS	QQSYSTPNT
U6	DVT	T	TIAASGYDTYYADSVKG	GASAFDY	AASDLQS	QQSSSDPYT
U7	DVT	T	NISYSGYNTNYADSVKG	NASAFDY	SASSLQS	QQSYSTPTT
U8	DVT	T	TIYGNGYATAYADSVKG	GASYFDY	SASSLQS	QQSYATPAT
U12	DVT	T	TIAATGNYTAYADSVKG	GGGSFDY	AASSLQS	QQSYSTPNT
U13	DVT	T	AIGSYGGNTYYADSVKG	TNTSFDY	NASTLQS	QQCSGSPCT
U16	DVT	T	TISNDGTTTYYADSVKG	GGSSFDY	SASALQS	QQAYASPST
U17	DVT	T	SIAGTGATTDYADSVKG	SASAFDY	TASTLQS	QQTTDTPAT
U19	DVT	T	TISDSGNNTTYADSVKG	AASSFDY	SASSLQS	QQSADTPYT
Mixture of antigens D, M and T						
D1	NNK	D	AIDSPGARTTYADSVKG	NSRQFDY	RASRLQS	QQWRVFPMT
D2	NNK	D	SIS*RGLNTLYADSVKG	VTPGFDY	GASALQS	QQLAWRPRT
D4	NNK	D	SIATTGRGTKYADSVKG	PRLIFDY	SASTLQS	QQKHAPPST
D5	NNK	D	GIDAGGSYTSYADSVKG	SGDSFDY	SASSLQS	QQCSYSPDT
D6	NNK	D	SIS*TGRKTLYADSVKG	QRGVFDY	LASELQS	QQLSPPPGT
D7	NNK	D	SISKTGTVTIYADSVKG	QGSSFDY	RASRLQS	QQGPGDPAT
D9[b]	DVT	D	n.a.	n.a.	SASALQS	QQSYTCPCT
D10	NNK	D	TITRSGM*TKYADSVKG	QSIFFDY	KASALQS	QQTRHAPRT
D11	NNK	D	LISARGN*TRYADSVKG	YIKRFDY	AASSLQS	QQSYSTPNT
D3n	NNK	D	NISNRGYKTAYADSVKG	RQPMFDY	SASVLQS	QQNAKRPST
M4	DVT	M	NITYNGSSTYYADSVKG	SSYSFDY	SASTLQS	QQDGNSPST
M5	DVT	M	NITYSGNYTSYADSVKG	SNSSFDY	DASTLQS	QQNSSSPST
M6	DVT	M	NISYSGNYTTYADSVKG	STTSFDY	SASSLQS	QQNSNSPGT
M7	DVT	M	NISYTGSYTAYADSVKG	ASYSFDY	DASTLQS	QQATSSPGT
M8	NNK	M	*ISYTGNYTAYADSVKG	RALRFDY	KASVLQS	QQSINQPST
M10	DVT	M	NISYSGNYTTYADSVKG	STTSFDY	SASSLQS	QQNSNSPGT
M11	DVT	M	GITYNGYTTTYADSVKG	ASTSFDY	SASSLQS	QQNYSSPGT
M12	DVT	M	GISYNGSSTTYADSVKG	ATYNFDY	YASWLQS	QQAYSNPNT
T12	NNK	T	AIAHSGD*TTYADSVKG	SMRTFDY	RASHLQS	QQIQRHPLT
T13	DVT	T	TIADSGTATYYADSVKG	GAAAFDY	AASSLQS	QQTYAYPAT
T14	DVT	T	TIGYDGNNTSYADSVKG	SASAFDY	AASTLQS	QQSSYTPYT
T15	DVT	T	TISYDGGGTYYADSVKG	AASAFDY	SASSLQS	QQSSSSPTT
T16	DVT	T	AIGYDGANTGYADSVKG	GSGTFDY	SASTLQS	QQGYSAPAT
T17	DVT	T	TISSGGDTTYYADSVKG	GANAFDY	TASSLQS	QQTNTAPTT
T19	DVT	T	TISSDGSNTYYADSVKG	SAGAFDY	AASNLQS	QQSNSSPTT
T20	DVT	T	TIGNSGDNTYYADSVKG	SASAFDY	GASTLQS	QQNNSYPST
T21	DVT	T	NIGSGGTTTAYADSVKG	GAGAFDY	AASTLQS	QQAYSNPTT
T22	DVT	T	TIGSSGDNTYYADSVKG	GASNFDY	NASYLQS	QQSAGTPYT
T23	DVT	T	TIAYDGTNTYYADSVKG	NAGAFDY	AASALQS	QQAYSTPNT
T24	DVT	T	TISYAGDYTYYADSVKG	GGAAFDY	AASTLQS	QQNYNSPTT
T25	DVT	T	SISTDGYYTYYADSVKG	GGGTFDY	SASSLQS	QQNSSSPST
T27	DVT	T	TIAASGYDTYYADSVKG	GASAFDY	AASSLQS	QQTSTYPST
T28	NNK	T	TISMNGSLTTYADSVKG	GASMFDY	RASDLQS	QQHMSMPTT
T29	DVT	T	SIGTSGDYTYYADSVKG	GGGNFDY	TASGLQS	QQATNDPYT
T14n	DVT	T	TISSDGATTYYADSVKG	NASAFDY	AASNLQS	QQSYSDPYT
HeLa lysate						
2M15	NNK	HeLa protein	TITDNGASTAYADSVKG	NKYYFDY	NASSLQS	QQAYSAPAT
2G11	DVT	HeLa protein	YIASAGDGTSYADSVKG	GAGTFDY	GASSLQS	QQSYSSPTT
8F20[c]	DVT	HeLa protein	SIAASGYYTDYADSVKG	NYYYFDY	TASSLQS	QQAAANPST
14H10	DVT	HeLa protein	SIYDSGDSTGYADSVKG	GAYYFDY	DASTLQS	QQANTSPTT
20M8	DVT	HeLa protein	NINYNGNYTNYADSVKG	NYYYFDY	DASYLQS	QQGYNTPAT
16P1[c]	DVT	HeLa protein	SINGAGTYTSYADSVKG	NYYSFDY	NASGLQS	QQYNTYPTT
17L13[c]	DVT	HeLa protein	NISTNGYGTYYADSVKG	AYDSFDY	AASSLQS	QQYGSNPNT
21L23[c]	DVT	HeLa protein	SISAYGSSTNYADSVKG	NYYYFDY	n.d.	n.d.

[a]All clones were selected from libraries based on a single human framework comprising the heavy-chain germline genes V3-23/DP-47 and J_H4b and the κ-light chain genes O12/O2/DPK9 and J_κ1 with side chain diversity (either NNK or DVT encoded) incorporated at 18 different positions in the antigen binding site (underlined). Depending on the antigen, the scFv clones are derived mainly from the NNK (antigen D) or the DVT (antigens M and T) encoded libraries. *Indicates a TAG stop codon that is read as glutamine in the TG1 suppressor strain. n.d., Sequence not determined. n.a., not applicable.

[b]Clone D9 is a light chain single domain.

[c]These clones have a different sequence but a similar pattern on a western blot of HeLa cells (see Fig. 3).

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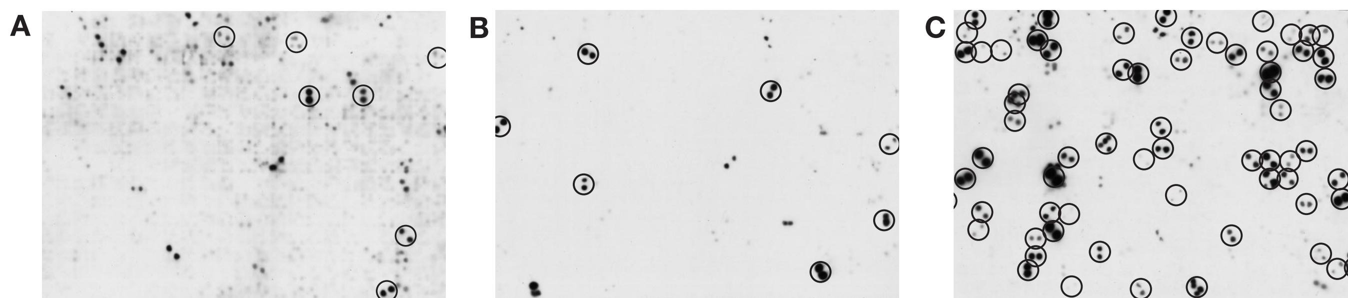

Figure 2. Antibody array screening after one round of phage selection using a mixture of three unpurified recombinant bacterial lysates. The panels depicted consist of 3,072 double-spotted clones (selected with 0.05% each target antigen concentration) screened for binding to lysates that contain antigens (A) D (B) M, or (C) T, respectively. D, M, or T-specific antibodies are circled. Several clones seem to be specific but have not been circled. On longer exposures (data not shown), these were found to bind all three recombinant lysates and an irrelevant bacterial lysate and therefore probably bind components of the bacterial lysate or correspond to cross-reactive, or "sticky" binders. The difference in the signals produced by these clones is almost certainly due to experimental variation caused by uneven coating of the antigen, poor contact between the two filters, or air gaps between the filters and the agar plate used for induction.

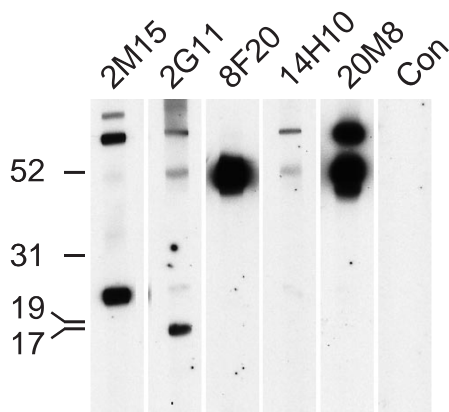

Figure 3. Western blot analysis of HeLa cells probed with scFv clones 2M15, 2G11, 8F20, 14H10, and 20M8 and Con (a non-binding scFv control). Molecular weight markers (in kilodaltons) are indicated. Three other clones 16P1, 17L13, and 21L23 (see Table 2) have different sequences but gave the same banding pattern as 8F20 on the immunoblot (data not shown). The different bands recognized by each antibody could represent degradation products of a single protein, alternative splice products, or similar epitopes present on different proteins.

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yeast lysates, thus identifying eight different clones (Table 2) that gave five distinct banding patterns on HeLa but not on yeast (Fig. 3). The reason that so few HeLa-specific binders were confirmed is almost certainly due to limitations of protein-based screens, where the affinities compared with nucleotide-based screens are much lower (see Discussion) and vary depending on the interactions being studied. Thus, although very low concentrations of protein can be used to select binding antibodies (down to 0.0005%), their *efficient* detection requires a much higher concentration (perhaps as high as 5% for some antibody–antigen interactions). As a consequence, it is likely that antibodies against many targets in the HeLa lysate were in fact selected (and are therefore present in the array) but that only those that bind abundant targets were able to be detected.

Discussion

Using robotic picking and gridding together with filter screening of bacterially expressed scFvs on antigen-coated filters, we have generated a range of high-density antibody arrays for parallel screening against many different antigens, including native or denatured proteins, impure antigens, and whole-cell extracts. These arrays have the advantage that because each clone has a unique position, 18,342 individual colonies can be double spotted on one 22 × 22 cm filter and, using our current robotic system, up to 15 identical filters can be generated simultaneously. The ability to create double spots and duplicate arrays not only means that false positives and cross-reactive clones can be eliminated during the initial screen but also enables antibodies against differentially expressed targets to be easily identified. The screen could be applied to any source of recombinant antibodies, for example, antibody genes cloned from immunized mice[17], from patient libraries[18–20], or from "mini" libraries created by random mutagenesis[21], chain shuffling[22], CDR (complementarity determining region) diversification[23], or rounds of phage selection. By varying the antigen concentration on different filters it should also be possible to identify binders that have higher affinities and/or improved expression characteristics.

We found that unlike conventional selection procedures, that typically require three to four rounds and often yield a limited range of binders to the target antigen, our approach, using a single round of selection followed by mass array screening, enabled the isolation of a wide diversity of binders to BSA, HSA, and several recombinant human proteins. Most of these bind as soluble fragments in conventional ELISA, and many also give strong signals on western blot analysis of the cognate protein, demonstrating their utility as immunochemical reagents. Even when the dilutions of the target antigens were very low (0.0005%, or 1 in 200,000), we were still able to select specific binders after a single round of selection, whereas with several rounds, these clones tended to be outcompeted with binders to other (more abundant) components of the mixture. In this regard, it is interesting to note that the degree of enrichment for binders did not decrease linearly with decreasing target antigen concentration. We wondered whether this might be due to the presence of a high proportion of specific binders in the unselected library. However, on screening a similar number from the unselected library no specific antibodies were identified (data not shown), confirming that some form of selective enrichment must have taken place. Indeed, the high affinity of the M12 clone (which was selected using 0.005% antigen M in the bacterial lysate) confirms that genuine selection of a rare species must have occurred.

In addition to high-throughput screening of antibody–antigen interactions, we envisage that recombinant antibody arrays will have a wide range of applications in proteomics, ranging from detection of protein modifications to profiling protein expression, a field that, to date, has been dominated by two-dimensional (2-D) electrophoresis (see Blackstock and Weir[24]). Unlike 2-D gels, antibody arrays can be used to analyze the expression of one or more proteins in a mixture such as a cell extract, without the need to physically separate the individual components, analyze the differences in the patterns and then determine the identity of the corresponding proteins.

DNA array technology is already used extensively as an indirect assay for protein expression by profiling mRNA expression, for

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example, in *Saccharomyces cerevisae* and to compare healthy versus diseased human tissues (see Brown and Botstein[25]). Because the affinity of the nucleotide–nucleotide interaction is determined by the extent of base pairing between the two substrates, low concentrations of mRNA can be detected and relative amounts of different mRNAs can be easily quantified. Nevertheless, there are several limitations with DNA chip technologies, which could be addressed using antibody arrays. First, mRNA concentrations do not always correspond to protein concentrations. Different proteins are translated from their mRNA precursors at different rates and may have quite different degradation characteristics. Furthermore, proteins are subject to a range of posttranslational modifications, such as phosphorylation and glycosylation, which cannot be identified by nucleotide-based screens. Antibody arrays have the potential to profile both protein expression concentrations and posttranslational modifications. In addition, antibodies can also be used to detect smaller molecules, such as peptide hormones[6,26] or carbohydrates[27,28].

Here, by doping recombinant proteins into bacterial lysates we have shown that phage–antibody libraries can be used to select binders to very rare components in a complex antigen but that their detection requires much higher concentrations of target antigen. Thus, although we were able to perform a differential screen and isolate antibodies against a handful of proteins present in human but not in yeast cells, for the array to be a truly useful tool for fingerprinting the tens of thousands of different proteins that comprise a cellular extract, the sensitivity of detection will need to be improved at least 100-fold, and perhaps a 1,000-fold. Several strategies are now being explored to achieve this goal including scFv multimerization[29,30]and signal amplification, for example, using biotin tyramine[31,32]. Alternatively, the screen could be miniaturized enabling higher concentration cellular extracts to be used.

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Experimental protocol

]\s and cell lysates. Purified BSA and HSA were obtained from Sigma (St. Louis, MO; A-7638 and A-3782, respectively). Three bacterial cDNA clones: a human chloride ion current inducer (D, RZPD clone name MPMGp800E04369Q3), one with an unknown function (M, RZPD clone name MPMGp800B12492Q3), and ubiquitin (T, RZPD clone name MPMGp800D17184Q3) were taken from a human brain cDNA library hEX1 (ref. 33) and expressed as described[34]. Crude cell lysates were collected and used for selection. Expression levels of each clone were tested by SDS–PAGE followed by western blot analysis and detection of the recombinant protein using an anti-RGS-His antibody (Qiagen, Valencia, CA; 1/2000 in 2% skimmed milk powder in phosphate-buffered saline, MPBS) and an anti-mouse antibody conjugated to HRP (Dako, Carpinteria, CA; 1/2000 in 2% MPBS). Blots were developed with a chemiluminescent detection reagent (ECL; AP Biotech, Piscataway, NJ). Recombinant D, M, and T were expressed at concentrations corresponding to ~5%, 5%, and 10% of total bacterial protein, respectively. Recombinant ubiquitin (from clone T) was purified through the hexahistidine tag using immobilized metal chelate affinity chromatography (IMAC) (ref. 35) as described[34]. HeLa whole-cell extract was prepared as described[36]. Yeast cell lysate was prepared by enzymatic lysis as described[37].

Phage–antibody selections. The phage–antibody library is based on a single human framework comprising the germline heavy-chain genes V3-23/DP-47 and J_H4b and the κ-light chain genes O12/O2/DPK9 and $J_κ$1 with side chain diversity (either DVT or NNK encoded) incorporated at positions in the antigen-binding site that make contacts to antigen in known cocrystal structures and are highly diverse in the mature repertoire (18 different amino acid positions in total). This fold is highly expressed in vivo[38] and binds the generic ligands, protein L and protein A, which facilitate the capture and/or detection of the antibody fragments without interfering with antigen binding. The libraries have been preselected in phagemid/scFv format for binding to protein A and protein L so that most clones are functional. After preselection, the respective library sizes are 1.47×10^8 (DVT encoded–Library I) and 1.37×10^8 (NNK encoded–Library J) (I.M. Tomlinson and G. Winter, in preparation). The two libraries were mixed to form a single library and selected as described[4], except that KM13 helper phage (which contains a pIII protein with a protease cleavage site between the D2 and D3 domains) was used and phage were eluted with 1 mg/ml trypsin in PBS. The addition of trypsin cleaves the pIII proteins derived from the helper phage (but not those from the phagemid) and elutes bound scFv–phage fusions by cleavage in the c-myc tag, thereby providing a further enrichment for phages expressing functional scFvs and a corresponding reduction in background[39].

Selections were performed using immunotubes (Maxisorp; Nunc, Roskilde, Denmark) coated with either BSA (100 μg/ml PBS), purified recombinant ubiquitin (100 μg/ml), or three dilutions thereof in a bacterial lysate (~20 μg/ml, 2 μg/ml, or 0.2 μg/ml of ubiquitin in 100 μg/ml of lysate, that is, 20%, 2%, or 0.2% target antigen:total protein); an approximately equimolar mixture of the three recombinant lysates D, M, and T (~2.5 μg/ml of each recombinant protein in 1.3 mg/ml total protein, that is, 0.2% each target antigen:total protein), or three dilutions thereof in an irrelevant bacterial lysate (~250, 25, and 2.5 ng/ml of each recombinant protein in 500 μg/ml total protein, that is, 0.05%, 0.005%, or 0.0005% each target antigen:total protein); or a lysate from HeLa cells (200 μg/ml, equivalent to ~10^7 cells).

Picking and gridding. Selected clones were plated onto a large square plate (230 × 230 mm, Nunc plates containing TYE , 100 μg/ml ampicillin, 1% glucose) at a density of about 10^4 colonies/plate, and grown overnight at 30°C. Colonies were picked (BioRobotics colony picker, Cambridge, UK) into 384-well plates (Genetix, Christchurch, UK, containing TYE, 100 μg/ml ampicillin, 1% glucose, 8% glycerol, 75 μl/well) and then grown without shaking overnight at 37°C. These were then gridded (Genetix, Q-bot) in a 4 × 4 pattern (such that each clone is gridded twice in each 4 × 4 grouping, see Fig. 1A) onto a large square plate (Genetix Q-tray, containing TYE, 100 μg/ml ampicillin, 1% glucose) covered with a nitrocellulose filter (Protran BA85 - Schleicher & Schuell, Keene, NH) that had been blocked in 2% MPBS for 30 min at room temperature (RT), briefly washed in PBS, and soaked in 2× TY . This procedure prevents binding of recombinant antibodies to this filter. The gridded plates were then grown overnight at 37°C. In the meantime, a second filter was coated overnight at 4°C in 100 ml of PBS with either 0.5 μg/ml of the generic ligand protein L (Affitech, Oslo, Norway), 100 μg/ml of BSA, HSA, purified recombinant ubiquitin, or recombinant bacterial lysates D, M, or T (total protein concentration 100 μg/ml with ~5, 5, and 10% recombinant D, M, and T, respectively) or HeLa, yeast, or bacterial cell lysates. This second filter was then blocked in 2% MPBS for 1 h at RT, washed 3× in PBS, soaked in 2× TY, and then transferred onto a large square plate (230 × 230 mm, Nunc plates containing TYE, 100 μg/ml ampicillin, 1 mM isopropyl β-D-thiogalactoside (IPTG)). The first filter containing the grown colonies was then transferred onto the plate covered with the second filter making sure no air was trapped between the two filters. These plates were incubated for 3 h at 30°C to induce expression of scFvs and to facilitate their binding to the second filter.

Probing of filters. The top filter was discarded and the bottom filter washed two times with PBS/0.05% Tween (PBST) and then blocked with 2% MPBS for 30 min at RT, followed by two washes with PBST. To detect bound scFvs, the filter was incubated with protein L-HRP (Affitech, 1/4000) in 2% MPBS for 1 h at RT, washed three times with PBST, and then developed with ECL reagent. To determine the levels of scFv expression, a filter coated with protein L was used to capture scFvs and was then probed with protein A–HRP conjugate (Amersham), 1/4,000 in 2% MPBS for 1 h. The filters were then washed three times with PBST and developed with ECL reagent. All incubations were performed in 50 ml of buffer on a gently agitating shaker.

ELISA, immunoblotting, and sequencing. Conventional ELISAs were performed as described[3], except that 96-well ELISA plates were coated overnight at 4°C using 10 μg/ml purified antigens or 100 μg/ml (total protein concentration) unpurified recombinant bacterial lysates. Two percent Tween/PBS was used as a blocking buffer and bound scFvs were detected with protein L–HRP. Western blot analyses of HeLa or yeast cell extracts were performed as described[3], except that scFvs were detected with protein L–HRP, and blots were developed using ECL reagent. PCR amplification and sequencing of scFv clones was performed as described[38] using the primers LMB3 (CAG GAA ACA GCT ATG AC) and pHEN seq (CTA TGC GGC CCC ATT CA).

Purification of scFvs and determination of affinity constants. scFv fragments were purified from the supernatant of 50ml inductions using protein A–Sepharose (Pharmacia, Uppsala, Sweden) columns as described[37]. Affinities were determined by solution phase competition[40] on a BIAcore 2000 biosensor according to Nieba and colleagues[41].

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