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Array-in-well binding assay for multiparameter screening of phage displayed antibodies

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ABSTRACT

Phage display is a well-established and powerful tool for the development of recombinant antibodies. In a standard phage display selection process using a high quality antibody phage library, a large number of unique antibody clones can be generated in short time. However, the pace of the antibody discovery project eventually depends on the methodologies used in the next phase to identify the clones with the most promising binding characteristics e.g., in terms of specificity, affinity and epitope. Here, we report an array-in-well binding assay, a miniaturized and multiplexed immunoassay that integrates the epitope mapping to the evaluation of the binding activity of phage displayed antibody fragments in a single well. The array-in-well assay design used here incorporates a set of partially overlapping 15-mer peptides covering the complete primary sequence of the target antigen, the intact antigen itself and and appropriate controls printed as an array with 10 × 10 layout at the bottom of a well of a 96-well microtiter plate. The streptavidin-coated surface of the well facilitates the immobilization of the biotinylated analytes as well-confined spots. Phage displayed antibody fragments bound to the analyte spots are traced using anti-phage antibody labelled with horse radish peroxidase for tyramide signal amplification based highly sensitive detection. In this study, we generated scFv antibodies against HIV-1 p24 protein using a synthetic antibody phage library, evaluated the binders with array-in-well binding assay and further classified them into epitopic families based on their capacity to recognize linear epitopes. The array-in-well assay enables the integration of epitope mapping to the screening assay for early classification of antibodies with simplicity and speed of a standard ELISA procedure to advance the antibody development projects.

1. Introduction

Recombinant antibodies based drugs have become an elementary part of the pharmaceutical toolbox to combat various diseases including cancers and autoimmune disorders. Also, the use of recombinant antibodies, in diagnostic assays and as research tool, is rapidly increasing. Recombinant antibodies can be obtained by cloning the rearranged variable domain genes from hybridoma cell line or B-cells for expression in a recombinant host cell [1]. Another widely used approach utilizes an *in vitro* platform involving large man-made repertoires of antibody molecules and use of a display technique for enrichment of the clones with desired properties [2,3]. Phage display is the longest-standing and still the most widely used display technique. Subjecting a suitable high quality phage display antibody library to a few iterative rounds of selection, generally results in a strong enrichment of binders against the selection target. In this technique, the target can be freely chosen including self-antigens and toxic molecules [3,4]. A single phage display selection experiment against the target of interest typically yields a strongly enriched antibody phage pool, in many cases, containing a large number of different target specific antibodies.

After the rapid phage display based enrichment phase, the individual target specific binders are identified in a screening process which typically involves picking the individual colonies, expressing antibody, and revealing the positive clones by binding assays such as conventional ELISA [5]. The number of colonies taken to the screening often ranges from hundreds to thousands, and for practical reasons, the screening is typically performed in two stages: potentially interesting clones are first identified by a simple and coarser primary screening assay and the binding activity of the shortlisted clones is then verified in a more diligent secondary screening assay. The unique antibody clones can then be identified by sequencing and the promising ones directed to more detailed analysis pertaining to e.g. the strength and specificity of the target recognition. After the screening, there can be tens or even hundreds of active binders deserving further characterization, and compared to the fairly straightforward and rapid phage display selection phase, the screening and especially the following closer characterization is often considerably slower and labor-intensive process, constituting a major bottleneck in the antibody development process.

Information on the epitope, antibody's binding site in the target protein, is useful for many applications. Such information has several important uses, such as advancing the understanding of the possible mechanism of action, identifying functional antibody pairs for immunoassay development and securing intellectual property rights [6].

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Moreover, understanding the nature of the epitope, whether linear or conformational, is valuable for many purposes. The most accurate approach to map an epitope is the crystallographic analysis of the antigen-antibody complex. However, this method is time-consuming and technically challenging [7]. Other methodologies for epitope mapping include hydrogen-deuterium exchange coupled to mass spectrometry [8], site directed mutagenesis [9] and mimotope analysis with phage display peptide library [10]. Each of these having their own technical limitations, making them too laborious and slow for routine analysis of a large number of antibody clones. The most widely used and simple epitope mapping approach use consecutive, overlapping synthetic peptides that cover the complete primary sequence of the protein antigen. Each peptide can be immobilized on ELISA wells [11,12] or the peptides can be printed or directly synthesized as an array on cellulose membranes [13,14], slides [15], glass chips[16], polyethylene pins [17] or single-walled carbon nanotube field-effect transistors [18]. The peptide-based approach is primarily suited for identification of linear epitopes, although the identification of conformational epitopes can be promoted by constrained, cyclized, peptides [19]. The advantages of overlapping synthetic peptides based assays include that the interpretation of results is straightforward and performing the assay itself is typically technically less demanding compared to other epitope mapping techniques. Owing to the advances in peptide synthesis process, the reagents for such assays are today reasonably affordable.

Here, we present an array-in-well concept [20] based method for simultaneous assessment of binding activity and epitope specificity of phage displayed antibodies. This method allows reliable analysis of an antibody clone against a number of targets such as various proteins and a series of antigen-derived peptides in a single microtiter plate well. The assay can help to develop more efficient and economic screening processes with high information content.

2. Material and methods

2.1. Phage antibody production

Phage displayed- single-chain variable fragment (scFv) antibodies were isolated from the synthetic antibody phage library scFvP [21] by selections against HIV-1 p24 protein antigen (NEXT BIOMED Technologies, Finland). The first round of phage display selection was performed with 250 μg of streptavidin magnetic beads (Dynabeads ®MyOne™streptavidin T1, Invitrogen, Norway) saturated with the biotinylated p24 antigen and 5.0×10^{12} colony forming units (cfu) of phage from the library. The phage pool was first incubated with the beads without the antigen for 1 h to eliminate undesired antibodies against streptavidin, and the unbound phage were then let to react with the antigen containing beads for 30 min on rotatory mixer at room temperature. After washing, the bound phage were eluted from the beads by adding 50 μL of 50 μg/ml trypsin (Sigma Aldrich, USA) and incubating 10 min at 37 °C with shaking. Trypsin activity was blocked by adding 12.5 μg Trypsin inhibitor (Sigma Aldrich) and incubation for 15 min. The scFv-genes were rescued from the eluted phage by PCR based amplifications. The PCR mixture included 0.5 μM primers JLe01_s (5′CGGCAGCCGCTGGATTGTTATTAC) and JLe01_as (5′-ACCAGAACCGCCACGACCTTC), 200 μM dNTP′s, 25 U/mL Pfu DNA polymerase, 1× Pfu buffer with $MgSO_4$ and eluted phage (heat-treated at 95 °C for 5 min prior the PCR amplification) in the total reaction volume of 360 μL. The PCR consisted of 2 min initial denaturation at 95 °C, followed 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 2 min at 72 °C, the final extension10 min at 72 °C. The PCR products were purified with geneJET™ purification kit (Fermentas, Lithuania) according to manufacturer instruction. The products were digested with 20 U SfiI enzyme in 40 μL. The reactions were incubated overnight at 50 °C. The SfiI digestion product of 768 bp was isolated from 0.8% agarose gel, and ligated to 100 ng of PEB32x vector (molar ratio 1:3) overnight at 16 °C. After inactivation of ligase at 65 °C for 10 min, 2 μl of ligation mixture was used to transform to electrocompetent cells XL1-blue *E. coli* [*rec*A1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F' *pro*AB *lacI*^{q}ZΔM15 Tn10 (Tet^r)]] (Stratagene, USA). The electroporation was conducted with Bio-Rad Genepulsed (Biorad, USA) with settings 200 Ω, 1.25 kV, 25 μF. After recovery in 1 mL SOC at 37 °C at 100 rpm for 1 h, samples were plated on LA (25 μg/mL cm, 10 μg/ml tet and 1% glc) and incubated overnight at 37 °C. The cells were used as inoculum for 20 mL phage production as described by Brockmann et al. [2]. The second round of phage display selection was performed with 50 μg of magnetic beads saturated with the biotinylated p24 antigen and 3×10^{11} cfu of phage from the first round. After the two selection rounds, 960 individual bacterial colonies were randomly selected from a culture plate to produce individual phage antibody cultures in a 96-plate format as described by Brockmann et al. [2]. Monovalent phage antibodies were obtained using VCS M13 helper phage (Agilent Technologies, USA) and multivalent phage antibodies with a genetically pIII-deficient helper phage, known as hyperphage M13 K07ΔpIII (Progen Biotechnik, Germany) [22].

2.2. Time resolved fluorescence (TRF) based immunoassay

The binding activity of the enriched phage pools and individual phage antibody clones towards the antigen was measured by TRF immunoassay. Streptavidin-coated microtiter plates, assay buffer and wash solution used in TRF immunoassay were procured from Kaivogen Oy (Finland). The total volume of the immunoassay was 100 μL and all incubations were performed at room temperature (RT) in shaking, 900 rpm. First, 30 ng of biotinylated p24 antigen or assay buffer for blank were added to the streptavidin wells and incubated for 1 h. The wells were washed four times, followed by the addition of 100 μL of diluted phage pools (1×10^9 cfu) for the evaluation of the phage pools or 100 μL of 1:10 dilution of culture supernatant for the evaluation of individual clones. After 1 h incubation, the wells were washed four times and 25 ng of Eu-N1-labelled anti-M13 monoclonal antibody 9E11 (Turku University) was added to trace the bound phage. After 1 h incubation, the wells were again washed four times and 200 μL of DELFIA enhancement solution (Perkin Elmer, Finland) was added for signal development. Time-resolved fluorescence signal was measured after 15 min with Victor 1420 multilabel counter (Perkin Elmer), using Europium program (λ excitation = 615 nm, λ emission = 340 nm, delay time 400 μs and window time 400 μs)

2.3. Sequence analysis

For DNA analysis, the phage-displayed antibody clones were grown in a 96-culture plate in Super Broth media (1% glucose, 10 μg/ml tetracycline, 25 μg/ml chloramphenicol), incubated at 30 °C with 70% humidity for 16 h at 700 rpm shaking in Multitron shaker incubator (INFORS, Germany). The scFv antibody containing DNA segments were amplified by PCR from 2 μL cell culture supernatant containing phage particles using sense primer (WO375: 5′-TCACACAGGAAACAGCTATGAC-3′) and antisense primer (pak400rev: 5′-CGCCATTTTTCACTTCACAG-3′). The PCR mixture consisted of 0,5 μM of primers, 200 μM dNTPs, 1 U Taq DNA

polymerase (Thermoscientific, Lithuania), 1× Taq buffer $(NH_4)SO_4$, 2 mM $MgCL_2$, and the reaction volume was 50 µL. The amplification reaction consisted of 3 min initial denaturation at 95 °C, followed 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 2 min at 72 °C, and a 10 min final extension at 72 °C. The PCR products were treated with 20 U Exonuclease I and 2 U Fast thermosensitive alkaline phosphatase to remove leftover primers and dNTPs. The reaction was incubated at 37 °C for 15 min and inactivation was performed at 85 °C for 15 min. For DNA sequencing, 5 µL of treated (Exo I and FastAP) PCR product was mixed with 5 µL of 5 µM sequencing primer (pAKfor: 5′-TGAAATACCTATTGCCTACG-3′). The DNA was sequenced by Macrogen (The Netherlands).

2.4. Array-in-well immunoassay

An overlapping peptide library to cover the whole primary sequence of HIV-1 p24 antigen and its variants was custom synthesized by JPT Peptide Technologies (Germany). The peptide length was 15 amino acids (aa) and there was 12 residues overlap between consecutive the peptides. All the peptides were N-terminally biotinylated with the help of Ttds-Linker (N-(3-{2-[2-(3-Amino-propoxy)-ethoxy]-ethoxy} -propyl)-succinamic acid) and a glycine amide was added at the C-terminus. In addition to the peptides representing the consensus sequence of the HIV-1 p24, peptides containing some frequently observed mutations (E71D, V83 M/L) were included in the library. Also, the peptides containing the residue C198, were represented as two versions having either the original cysteine or a serine instead. The sequence and related information of 96 synthetic peptides is provided as supplementary information (Supplementary file 1: Table S1). The biotinylated peptides (50 µM peptide in a buffer containing 10% glycerol, 250 mM NaCl, and 50 mM Tris-HCl, pH 8) and antigens were printed in an array (Fig. 1) on to the bottom of streptavidin-coated microtiter plate wells (Thermo Scientific; USA) using QArray 2 microarray printer (Genetix Limited, UK). The biotinylated anti-M13 monoclonal antibody and an irrelevant antibody were printed as controls.

Array wells were blocked with blocking solution (5% BSA, 100 µM biotin, TBS (50 mM Tris, 150 mM NaCl, pH 7.7) at RT for 1 h. anti-M13 antibody was conjugated with horseradish peroxidase (HRP, Thermo Scientific) according to the manufacturer′s instructions. The antibody-phages were diluted 1:100 and the HRP-anti M13 1:5000 with assay buffer (5% BSA, 100 µM biotin, 0.05% Tween 20 in TBS). The wells were washed four times with TBST (TBS, 0.05% Tween 20) using plate washer (Biohit, Finland). After washing, 25 µl of diluted antibody-phage and 25 µl of HRP-anti-M13 were added into each array well. After 1 h incubation at RT with shaking (600 rpm), the wells were washed four times. Tyramide signal amplification (TSA) plus Cyanine 3 tyramide (Perkin Elmer) was diluted 1:200 in Amplification diluent (Perkin Elmer) and 25 µl of that dilution were added per well and incubated for 10 min at RT. The wells were washed four times and the plate was dried by centrifugation at 1500 rpm for 1 min, upside down. The plate was scanned with LS400 microarray scanner (Tecan, UK) and the images were analysed with Array-Pro Analyzer software (Media Cybernetics Inc., USA).

2.5. Evaluation of soluble antibodies

The scFv sequences from phage display plasmid were cloned into the expression vector pLK06H [23]for production of soluble scFv antibodies in fusion with alkaline phosphatase. The antibodies were produced and purified using Ni-NTA column as described earlier [24] except using 300 mM of imidazole in the elution buffer. Purified antibodies (2 µg/mL) were evaluated for binding to a series of concentrations (0.032, 0.16, 0.8, 4, 20, 100 nM) of different biotinylated HIV-p24 immobilized on a streptavidin-coated microtiter wells, using Europium-labelled anti-alkaline phosphatase pAb (0.5 µg/mL) as tracer. All the steps of the immunoassay were performed at RT with shaking 900 rpm for 1 h, followed by 4 × wash. Finally, TRF-signals were measured as in Section 2.2. EC_{50} values for the soluble antibodies were determination as described by Sebaugh [25].

3. Results

3.1. Isolation of antibodies against p24 of HIV-1

The synthetic human antibody repertoire, scFv_P [21], was interrogated by phage display to identify specific binders against the p24

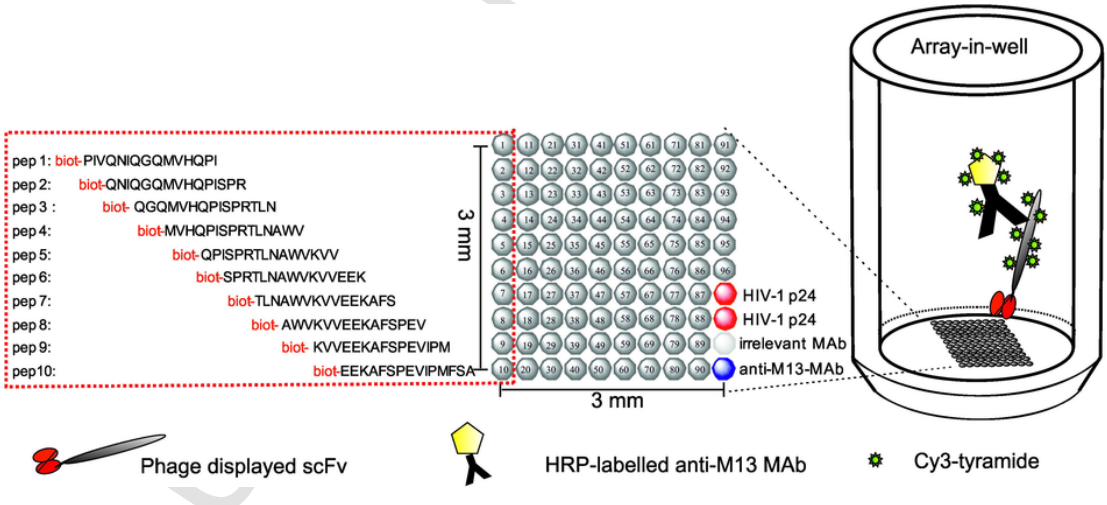

Fig. 1. Scheme of the array-in-well binding assay. The array consists of 100 spatially separated spots generated by immobilizing the biotinylated targets on the streptavidin-coated bottom of a 96-format microtiter well. The targets include a library of overlapping peptides covering the whole primary sequence of HIV-1 p24 protein, intact p24 protein (in two concentrations) and controls: irrelevant antibody and anti- M13 phage antibody. The antibodies are tested for binding to the array as displayed on the filamentous phage M13, and the bound phage are traced with HRP-labelled anti-phage antibody. The HRP-catalyzed tyramide signal amplification reaction results in the incorporation of Cy3 dye which is detected by a fluorescence scanner.

protein of HIV-1. The biotinylated p24 antigen was coupled to magnetic streptavidin beads, and used for two rounds of phage display selection. Enrichment of specific binders was evident as indicated by strong immunoreactivity of polyclonal scFv-phage against the target antigen; testing the output phage pool from the 2nd selection in a phage binding assay resulted in signal-to-background ratio of almost 500 (Fig. 2a). Altogether, 960 individual colonies were picked and used to produce single clone scFv-phage cultures on the 96-well culture plates. Testing of the scFv-phage clones for binding to p24 protein by immunoassay resulted in 848 positive hits (Fig. 2b). Next, 95 candidates were randomly selected for sequence analysis, and among the 94 successfully sequenced clones 91 unique scFvs with differences in the complementarity determining regions (CDRs) were found, three clones occurring twice (Supplementary file 2: Table S2). As deduced from the presence of identical heavy chain CDR3 sequences in some clones, a part of the clones was apparently formed by the shuffling of the CDR segments among the antibodies during the PCR-based rescue of the scFv-genes at the first panning round. The same phenomenon was also observed in the recent study by Lövgren et al. [26].

3.2. Array-in-well binding assay design

Array-in-well (AinW) based assay was established for the analysis of the binding characteristics of antibodies displayed on phage. As shown in Fig. 1, the array consisted of 100 spatially-separated addressable spots of targets immobilized onto a 96-format microtiter plate well. Biotinylated antigens were printed in nanoliter volumes on the streptavidin-coated bottom of the well on which the antigens were rapidly bound generating spots strictly confined around the site of printing. Most of the spots (96) in the array were reserved for a set of partially overlapping 15-mer peptides covering the entire primary sequence of HIV-1 p24 protein. Due to three residues N-terminal offset, there was a 12 residues overlap between the consecutive peptides in the array. The remaining four spots in the array contained proteins: two of these spots were occupied by the intact p24 protein in two different concentrations, one spot was for a non-specific control antibody to monitor the level of unspecific binding of the phage and the last spot contained an anti-M13 antibody to verify the presence of phage particles in the sample. The detection of the antibody displaying phage bound to the arrayspots was based on the use of an HRP-labelled anti-M13 antibody in combination with tyramide signal amplification (TSA) method [27]. The Cy3 dye incorporated by the TSA reaction was read with a fluorescence scanner.

3.3. Effect of phage display valency on array-in-well assay performance

We were interested in whether the valency of the phage, i.e. the number of scFvs displayed per phage particle, plays a role in the ability of phage to recognize its target on the array. Therefore, the aforementioned 94 (91 unique + a duplicate for 3 clones) p24 binding clones were produced both as monovalent and multivalent phage versions. The monovalent scFv-displaying phage were produced using the conventional VCSM13 helper phage to elicit phage production by the cells harbouring the scFv-gene in a phagemid vector. This approach is known to yield phage population dominated by phage displaying a single antibody molecule and those not displaying antibody at all. The multivalent antibody-displaying phage were generated with the hyperphage technology described to give a forced display of 3–5 scFvs per phage particle [22].

The ability of the scFv-phage clones to bind to the targets in the AinW assay was studied using 1:100 diluted supernatants from the microtiter plate based the phage cultures. All the evaluated antibody phage candidates, both mono- and multivalent, gave signal from the two spots containing the intact p24 protein (Fig. 3). The signals from the spot with the higher antigen concentration were practically saturated for the most of the clones, irrespective of the valency of the display. In the case of the spot with lower antigen concentration, the signals obtained with the monovalent display were considerably lower for most of the clones. However, with the multivalent display the signals were in the similar high level as in the spot with the higher antigen concentration reflecting the enhanced binding of the multivalent antibody-displaying phage to the antigen on the array. With the monovalent display, there also was a clear positive correlation between the signals from the p24 spot (low antigen concentration)and the anti-M13 spot, indicating that the antigen-specific signal was dependent on the phage titer.

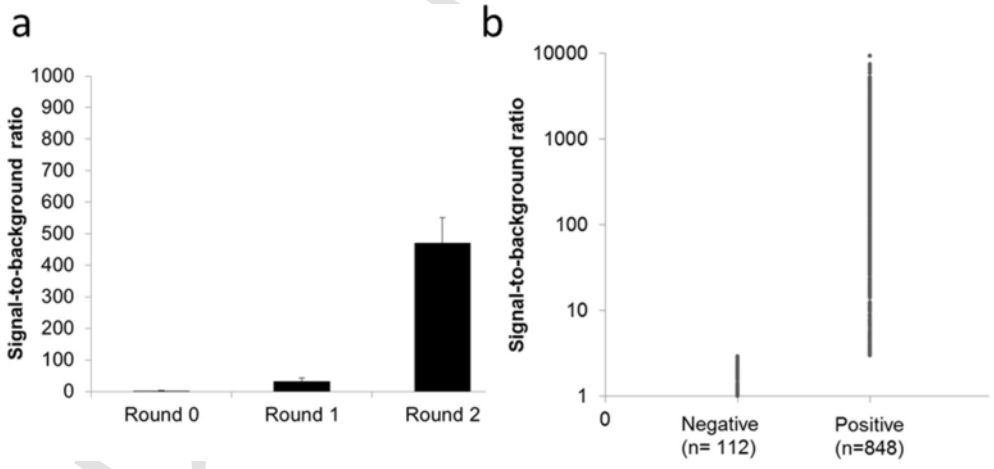

Fig. 2. Immunoreactivity of synthetic antibody library derived antibody-phage against the target antigen, HIV-1 p24 protein. a) Immunoreactivity of polyclonal phage pool after the first and second round of phage display based selection against p24. (Round 0 refers to the original library not challenged with the antigen.) b) Result of the initial activity screening where individual antibody-phage clones produced from 960 randomly picked bacterial colonies (after the 2nd selection round) were tested for binding to p24. Signal-to-background ratio of three was arbitrarily chosen as cut-off value to select positive binders. The assays were performed in the wells of a streptavidin coated 96-format microtiter plate on which the biotinylated p24 was immobilized. The bound phage were traced with europium-labelled anti-phage antibody for time-resolved fluorescence based detection. The phage were obtained using helper phage VCSM 13 for rescue.

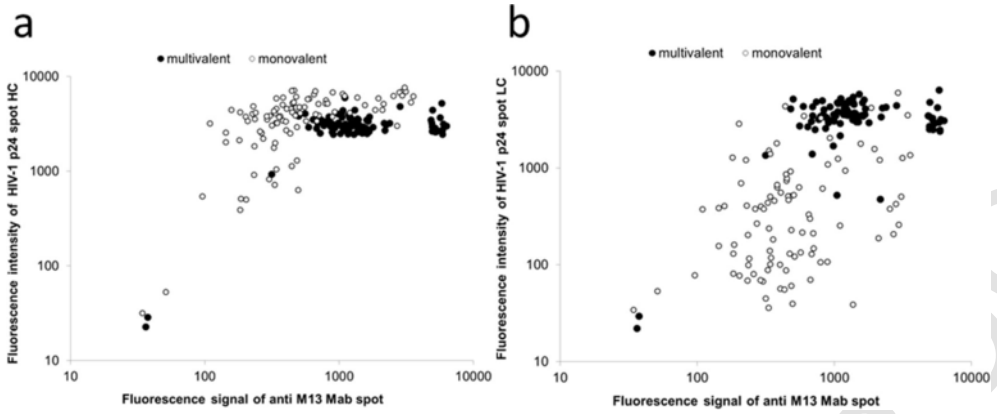

Fig. 3. Comparison of the fluorescence signal intensities between AinW spots coated with p24 protein and anti-M13 antibody. a) p24 spot containing higher concentration (HC) and b) lower concentration of the antigen (LC).

3.4. Epitope analysis

Binders were found against peptides representing four different regions in the sequence of p24. Peptides 4, 5 and 6 were recognized by several antibody phage clones, both in mono and multivalent phage format. However, significantly stronger signals without notable increase of background were observed with multivalent phage (an example shown in Fig. 4). For all the other peptide epitopes recognized by any binder, a detectable signal was obtained only with multivalent display format.

On the basis of binding to the array, the antibody clones were classified into four families with different specificity profiles. The first family was formed by the binders recognizing the overlapping peptides 4, 5 and 6 sharing the minimum epitope SPRTLNAWV. This family comprised a total of seven different antibody clones 1E2, 1F3, 1C8, 2F9, 2D11, 2F8 and 2H2 (Fig. 5a). At primary structure level these binders showed by markedly different sequences in the complementarity determining region 3 of the heavy chain (CDR-H3), and amino acid differences were also found in the other CDRs in the light and heavy chain (i.e., CDR-L1, CDR-L3, CDR-H1 and CDR-H2). The second family was constituted by the clones 2E9 and 2C1 which exhibited binding to the peptides 40–47 (including peptides representing frequent mutations, with substitutions in the position 83: V→L and V→M) and 56–58, and their minimal epitopes were AGPIAP and NPPIPVGI (Fig. 5b). The clones 2A6 and 2B7 recognizing the peptide 70–73 delineating the minimal epitope DRFYKT formed the third epitope-specificity family (Fig. 5c). Finally, the fourth category is constituted by the remaining 80 binders

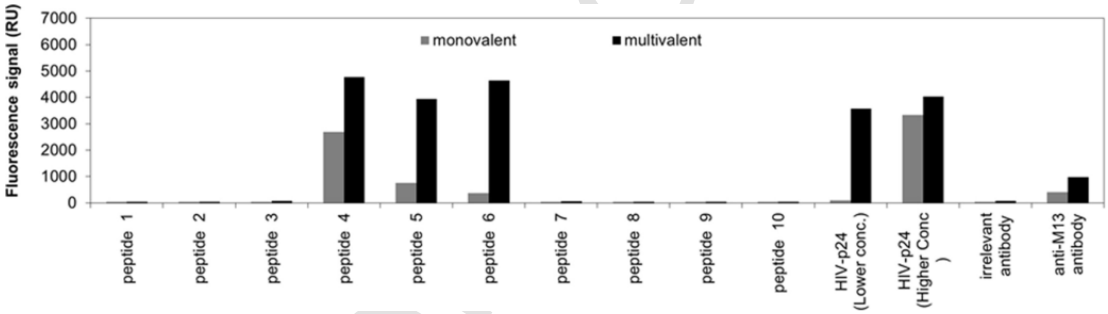

Fig. 4. Influence of the valency of the phage on the signal levels in AinW assay. As an example, the figure shows the observed signal levels for the scFv clone 2F9 displayed either in monovalent (rescued with VCSM13 helper phage) or multivalent (rescued with hyper phage) format.

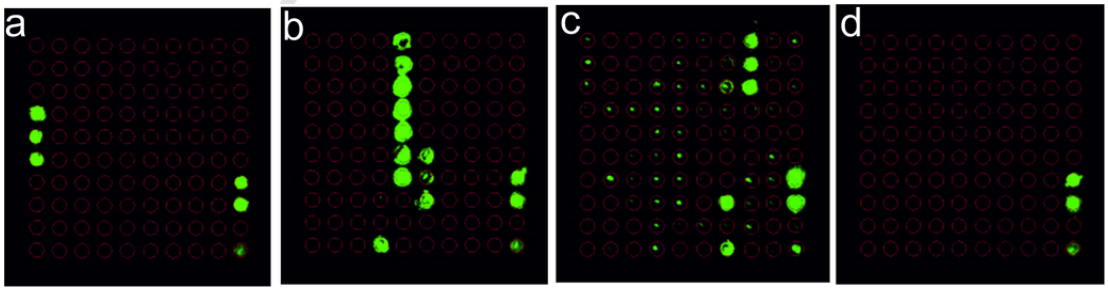

Fig. 5. Typical AinW assay readouts. Results are shown for four clones with different epitope specificities. a) Binder recognizing peptides 4, 5 and 6. b) Binder recognizing peptides 40–47 and 56–58, c) Binder recognizing peptides 68, 70–73 and d) Binder recognizing only intact HIV-1 p24 protein.

that did not show binding to the linear peptides but recognized the intact HIV-1 p24 protein (Fig. 5d).

3.5. Analysis of binders in soluble format

The scFv genes of six phage displayed antibodies, including representatives from all the three identified families against linear epitopes, were cloned into the expression vector pLK06H to produce these antibodies as a soluble protein in fusion with bacterial alkaline phosphatase (scFv-AP). Testing the purified scFv-AP antibodies against a series of immobilized antigen concentrations showed that all the six clones maintained their antigen binding capacity also in the soluble format (Fig. 6).

4. Discussion

In this study, we describe an array-in-well binding assay for phage displayed antibodies to perform simultaneous secondary screening and epitope mapping in a single well format. Various characteristics of recombinant antibodies such as binding affinity, specificity profile and stability can be optimized by further engineering of a lead compound [2,28]; however, the epitope cannot be altered. Obtaining information on the epitope specificity at early phase can significantly facilitate the antibody development process by helping to narrow down the number of promising binder candidates taken further in the pipeline. In many phage display selection campaigns, as also observed in this study, the proportion of active, antigen specific, binders within the final enriched phage pool is very high, justifying the use of the AinW assay already as a primary screening tool. Due to the high information contents from a single AinW assay, secondary screening could then, in many cases, be completely omitted.

The AinW assay enables very sensitive detection of the bound phage particles, largely conferred by the use of two types of signal amplification strategies in an additive manner. Firstly, each phage is recognized by a number of HRP-labelled detection antibody molecules as the used antibody targets the major coat protein gVIIIp, present in up to 2800 copies per a (wild type) filamentous phage particle [29]. Secondly, the highly efficient HRP-catalyzed tyramine radical generation based signal amplification technology is employed. Upon enzymatic activation, several activated fluorophore-conjugated tyramides with short half-lifetime become covalently anchored to surrounding proteins in a spatially confined manner, increasing the specific signal and avoiding diffusion artifacts [30]. Thus, the binding of several fluorophore-conjugated tyramides increases the original signals, and, for example, Garrigues et al. found a TSA-based method to be 500-fold more sensitive than conventional fluorescent methods [31]. Owing to the high sensitivity, the AinW assay can be performed using a small sample from a miniscale, microtiter well-based, phage culture. Moreover, the possibility to use the same microtiter plate format throughout the screening process, including both the binder expression and the analysis steps, reduces the need of liquid handling and facilitating the automation of the process.

The avidity of phage display was found to be influential on the determination of the epitopes for binders. From the analysis, five different clones were found to bind consecutive peptides in both the monovalent and multivalent display formats. Additionally, six more binders against linear peptides were identified exclusively with multivalent display. Thus, avidity enables a more efficient capture of phage antibodies, increasing the signal levels for the discovery of binders that might have lower affinity to the peptide [22]. While multivalent phageseem to provide benefits in the recognition of linear peptides in the array, testing also in monovalent phage format might help in identification of higher affinity binders. In our AinW assays, the binding of monovalent phage was found to be more dependent on the amount of the intact p24 antigen in the spot, or the titer of phage particles in the sample) than the binding of the multivalent phage. Conceivably the antigen-specific signal in proportion to the anti-M13 signal could provide an estimate for the relative affinity of the clone for its antigen. However, even the monovalent phage pool obtained using VCSM13 helper typically contains a small fraction of bivalent and maybe trivalent phage particles, and the proportion of such higher valency phage might vary by the expression levels of the displayed antibody clone. Therefore, it remains to be sorted out to which extent the signals of the phage produced with VCSM13 helper phage reflect the actual binding affinity of the displayed antibody and what is the role of the antibody expression properties.

AinWs were prepared on the wells of 96-format microtiter plates, a very common tool used in routine laboratory work. Compared to the conventional peptide arrays synthesized *in situ* or printed into different solid surfaces, such as glass slides [15,32] and cellulose mem-

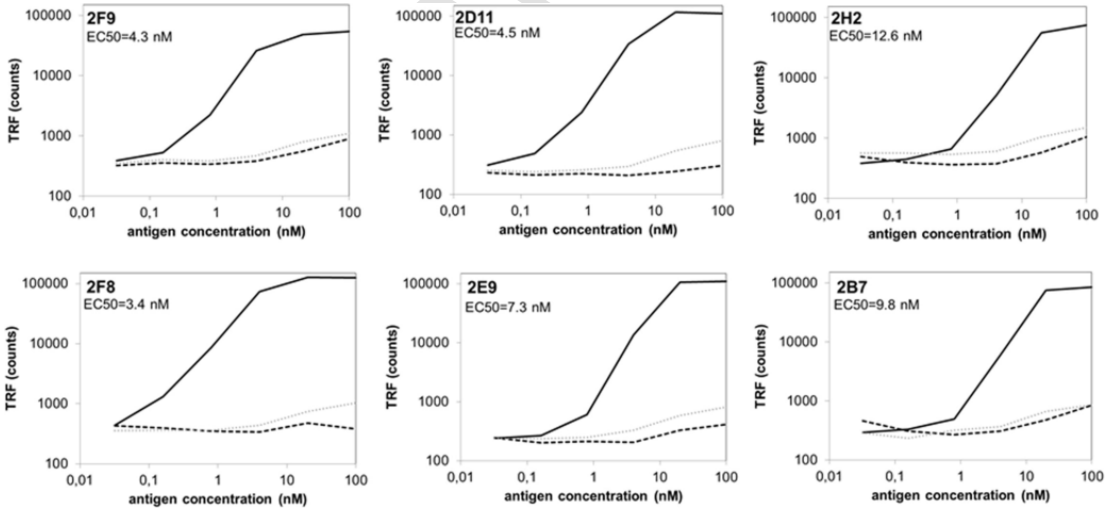

Fig. 6. Binding of soluble scFv-AP antibodies to HIV-1 p24 protein. The scFv-AP bound to the immobilized antigens p24 (solid line), HIV-1 envelope protein (dashed line) and GST (dotted line) was traced with europium labelled anti-AP antibody for time-resolved fluorescence based detection.

brane [14], AinW provides by far more practical platform for accessing multiple parameters. It allows large-scale parallel testing of multiple samples with ease and convenience of typical ELISA assays but in a more convenient and economical fashion utilizing considerably less sample and reagents than conventional immunoassay formats [33]. A microtiter well has capacity to accommodate an array composed of a series of overlapping peptides needed for epitope mapping studies, and streptavidin-biotin based chemistry used here allows convenient immobilization of peptides in well-exposed fashion. Importantly, due the low reagent antigen consumption, biotinylated peptides may be obtained through a miniscale synthesis with affordable price. An array with higher spot density than used in this study can be considered in the case of larger proteins. These features make the assay concept an attractive tool for large scale screening campaigns. Moreover, this methodology could facilitate the early identification and classification of antibodies for their epitopes. In this study, the array data allowed classification of the binders in four categories and this information could be used e.g., in the development of a sandwich immunoassay for p24. Altogether, 12% of the synthetic scFv isolates bound to linear epitopes which is similar with earlier findings that 5–10% of monoclonal antibodies raised against native antigens bind to linear epitopes [11]. Although only a small fraction of binders (11 out of 91 unique clones) recognized linear peptides, it could be possible to increase epitope coverage by sampling more clones. Furthermore, the information that an antibody does not bind a linear epitope is also useful, and such antibodies should, for example, not be considered for western blot type application.

The array in this study used only linear peptides and thus lacked the ability to identify conformational or discontinuous epitopes. However, there are publicly available online tools (see e.g. The Immune Epitope Database and Analysis Resource, http://www.iedb.org/ and Epitopia, http://epitopia.tau.ac.il) to predict such complex epitopes. Cyclization of peptides through disulfide bridges has long been recognized as a simple method to constrain their conformational freedom to better mimic protruding loop structures on protein. [34] Commercial peptide synthesis services also use proprietary epitope prediction tools to offer their customers improved synthetic mimics of native protein epitopes. Also discontinuous epitope mimics can be constructed by attaching two or more separate peptides from the original protein sequence onto organic scaffolds with the hope that the configuration emulates the native discontinuous epitope (see e.g. CLIPS – Chemical LInkage of Peptides onto Scaffolds, http://www.pepscan.com/custom-peptide-synthesis/clips-constrained-peptides). Thus, by complementing the linear peptide set with selected higher-order synthetic peptide constructs, one could potentially use the array also in identifying conformational and discontinuous epitopes.

This methodology can be further customized for specific purposes. For example, it can include multiple replicates of the same target, the same protein with post-translational modifications, closely related or non-related proteins to monitor off-target binding and assess specificity, cross-reactivity, or even to test constrained peptides. Furthermore, by performing the assay using a series of antigen concentrations, it might be possible to obtain information on the relative affinities of the antibody clones. A potential risk associated with the screening in phage format concerns the possible loss of antibody's binding activity upon conversion to soluble format. However, we have not observed such a problem with the clones from the single framework antibody library used in this study, and indeed all six clones converted to soluble scFv-AP format here were functional.

5. Conclusions

The study describes an efficient method for the simultaneous evaluation of binding activity and assessment of the epitope of phage displayed antibodies. This automation-amenable approach can facilitate the characterization of antibody candidates from phage display libraries accelerating the identification of lead antibodies for further development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2016.12.004.

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