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REVIEW

The emerging role of biosensor-based epitope binning and mapping in antibody-based drug discovery

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ABSTRACT

Introduction: Surface plasmon resonance (SPR) for affinity/kinetics measurements of drug candidates has been a mainstay application for characterizing drug candidates for many years. Recently, with the growth of monoclonal antibodies (mAbs) as a drug class and the availability of higher-throughput biosensors, the role of label-free biosensors has evolved to include epitope characterization in the early drug discovery process through epitope binning and mapping of mAbs.

Areas covered: This manuscript outlines the importance of using epitope characterization early in the drug discovery process and describes a strategy for success in discovering drug leads. Updated practices for integrating epitope characterization with other biochemical/biophysical data, cell-based functional data, and computational prediction tools are also discussed.

Expert opinion: The authors propose using epitope characterization during early drug discovery by: (1) using epitope binning of mAbs following the pre-screening of clones to assure selection of mAb candidates with epitope diversity, (2) binning the maximum number of mAbs in order to fully define epitope engagement profiles, and (3) integrating epitope binning/mapping data with binding affinity, kinetics, cell-based functional assays, etc. to better describe functional epitope. This approach, together with structural and binding prediction data, will improve the quality of leads and improve the selection speed for clinical candidates.

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1. Conventional drug discovery process for therapeutic monoclonal antibodies

With a market value of over $50 billion in 2014 and with seven of the top 10 selling drugs, therapeutic monoclonal antibodies (mAbs) have begun to challenge the dominance of small molecules in sales [1,2]. Therapeutic mAbs now treat most major disease classes including cancer, infectious, autoimmune, cardiovascular, pulmonary, and inflammatory disease [3]. Hundreds of mAb-based drugs are under clinical evaluation, where in some disease classes like cancer, they account for over half of the new drugs in trials [3]. Independent of the sustained growth of full-length mAb sales, therapeutic space for antibody fragments and scaffold-based biologics is poised to expand further [4]. Complicating the development of a commercially viable therapeutic is the highly competitive landscape for newly uncovered targets. Therefore, therapeutics to the same target antigen require sophisticated approaches to identify and develop mAbs that are superior to competitor molecules [3,5]. Here, we describe the conventional therapeutic mAb discovery process and lay out new techniques for epitope characterization that provide innovative opportunities to improve the discovery workflow for next generation therapeutic mAbs.

1.1. mAb generation platforms

The growth of the therapeutic mAb market has been followed with a corresponding growth in the antibody generation platforms such as transgenic rodent models, B-cell clonal expansion followed by sequencing by Ig-seq, phage/yeast display, unique avian and camelid hosts [6–8]. Innovative antigen presentation systems also have been developed [9,10]. During early mAb discovery, 5,000-20,000 mAb clones are screened resulting in thousands of potential mAb candidates. These numbers are projected to increase as mAb generation platforms are developed and refined. The growth of these novel mAb discovery platforms will continue to require improvement in screening strategies to sustain this growth.

1.2. Conventional screening strategy

In a conventional drug-discovery process, see Figure 1(a), mAbs are generated and screened using endpoint assays such as ELISA® or Luminex®. ELISA and Luminex assays are capable of quickly performing a primary screening of thousands of mAbs expressed in crude conditioned media (CM) with minimal human intervention [11–14]. While ELISA and Luminex assays are rapid and robust, they are endpoint assays, and thus are limited both informationally and in accuracy where they

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Article highlights

- Surface plasmon resonance (SPR) for affinity/kinetics measurements of drug candidates has been a mainstay application for characterizing drug candidates for many years.
- Screening therapeutic monoclonal antibodies by epitope has become the best screening criteria during the early mAb drug discovery process.
- Epitope characterization through binning improves intellectual property (IP) positions and increases R&D productivity, and guides engineering allowing for best in class therapeutic development.
- Epitope binning is not a stand-alone tool and requires additional supporting evidence for informed screening decisions including binding kinetics/affinity, cross-reactivity, cell-based functional assay, and other biophysical/structural data.
- Binning and mapping will also change how antidrug antibodies are detected and characterized.

This box summarizes key points contained in the article

commonly possess higher degree of false negative/positive results due to the labeling of reagents and/or non-specific coating of the antigen to the ELISA plates [15,16]. Secondary screens of mAb drug candidates are commonly performed on up to a 1,000 candidates using functional assays and binding affinity/kinetics. Additional technologies that are making inroads for screening are high-throughput flow cytometry and next-generation sequencing (NGS) of B-cell clones [2,11,12,17–21]. In the lead selection and candidate selection stages of discovery, a detailed characterization of mAbs are performed through biophysical characterization, *in vitro* cell-based assays and then *in vivo* disease models, (see Figure 1(a)) [22]. Epitope characterization of mAbs is traditionally performed during the later stages of drug discovery owing to the historically low-throughput nature of these techniques [22].

1.3. Challenges involved with conventional screening

Several factors have been shown to govern the clinical success of therapeutic mAbs such as binding affinity/kinetics, mode of action, immunogenicity, and pharmacokinetics/pharmacodynamics of the candidate mAb [4]. A successful mAb candidate binds with both high specificity and appropriate affinity to a functional epitope [4]; however, conventional screening does not characterize the epitope of mAb drug candidates until late in the drug discovery process. Recent manuscripts support the idea of means for target epitope engagement as crucial for clinical success of mAb drug candidates [23–25]. Conventional affinity-based mAb screening strategies can lead to the exclusion of mAbs binding to less immunogenic and rare epitopes, which may be the more relevant functional epitope, Figure 1(b). Only a limited number of epitopes on an antigen are functional and correlate with *in vivo* therapeutic effects. The epitope targeted by a mAb is an innate property and is not currently able to be engineered 'by design' d*e novo*. Simply put, while other factors governing the clinical success of a mAb do present opportunities for improvement through common molecular biology techniques, the paratope on a mAb presently cannot be readily engineered to engage a specific epitope.

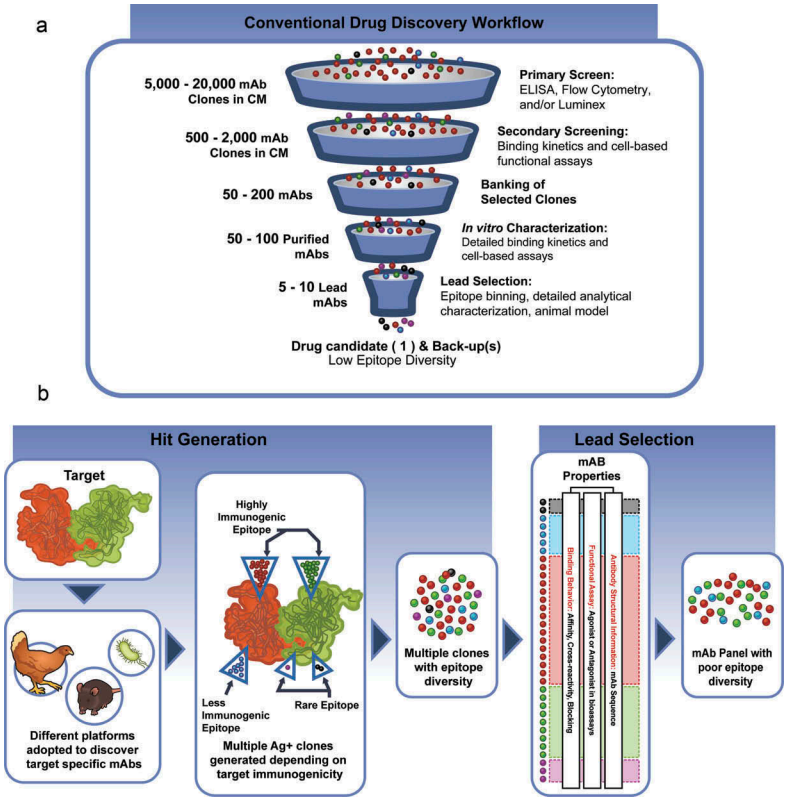

Figure 1. (a) Conventional mAb drug discovery workflow. (b) Graphic illustration of conventional mAb screening: In the absence of epitope information, it is challenging to shortlist mAbs from a large panel of primary clones and identify lead clinical candidate. Identification of functional epitope is the key.

2. The value of epitope characterization

Here, we posit that epitope-based selection is more valuable than affinity-based selection when narrowing down large numbers of antibodies to a few, high-quality leads [24,26]. Using an epitope-based screen where numerous epitopes are identified allows the researcher the ability to move antibodies that bind to functional epitopes into the next phases of drug discovery and development. Thus, epitope-based screening can be used to identify leads while maintaining epitope diversity and reducing epitope bias [23]. In previous manuscripts, we detailed the opportunity that high-throughput, epitope characterization of antibodies provides by increasing the probability of success in Phase II and III [24,27]. This increased probability of success reduces overall costs and increases R&D productivity [27].

Additional benefits in R&D productivity can be realized when considering that therapeutic leads are not developed in a vacuum but rather require many additional reagents such as back-up candidates, surrogate molecules for studies in model species, and general reagents to progress preclinical and clinical assays. Data from a single epitope characterization study have the potential to define reagents for all of these requirements in contrast to the conventional approach that often would require separate follow-up efforts to generate necessary reagents. Epitope characterization is also a highly effective way of assessing candidate diversity early in the discovery process for either a single platform or multi-platform mAb generation approach [28].

Moving beyond discovery into the commercialization space, epitope diversity and understanding provides a basis for intellectual property (IP) protection and differentiation [27]. With efforts underway at multiple biopharmaceuticals find therapeutics against the same target, selecting from a pool of functional candidates with limited knowledge of epitope is fraught with both IP and commercialization obstacles [29,30].

2.1. High-throughput label-free biosensors

The traditional role of label-free biosensors for measuring biomolecular interactions is expanding as newer high-throughput biosensor platforms are entering the market. Array-based Surface plasmon resonance imaging (SPRi) and biolayer interferometry (BLI)-based platforms are routinely being used to perform screening and characterization assays for large mAb libraries [23,28,31]. Each biosensor platform has different working principles to measure protein–protein interactions and exhibit unique capabilities that can easily streamline assays used in drug discovery.

There are generally two types of real-time, label-free biosensor platforms available that are suited for epitope characterization. SPRi systems such as the IBIS MX96 and the Horiba XelPlex are flow-based biosensors that can simultaneously monitor up to 384 binding interactions. Although these platforms monitor the same SPR phenomenon as traditional Biacore biosensors, they do so using a much greater number of distinct ligand locations per surface area. Such platforms provide a unique opportunity to immobilize an array of mAbs onto the sensor surface and examine 96 or more competition profiles from a single injection. Moreover, apart from exhibiting unmatched throughput, these platforms require minimal amounts of reagents and maintain low consumable costs [23].

BLI-based systems are also favored for high-throughput experiments, particularly after the launch of ForteBio HTX instrument. Rather than flow, these instruments utilize sensor tips immersed in an orbitally mixed well plate of samples. Experiments can be performed in up to 96-tip detection mode on the HTX. To handle assay requirements of multiple well plates, robotic arms can be integrated. Fresh sensors can be used after each cycle, thus avoiding the challenges associated with the optimization of regeneration conditions, with the caveat of increased biosensor tip consumption costs [23].

2.2. Epitope binning using real-time label-free biosensors

Epitope binning is a competitive, pairwise immunoassay where a panel of mAbs are assessed for blocking against all other antibodies in the same panel to determine which antibodies block the same or similar epitopes on a target antigen [32]. A blocking profile for each antibody against all other antibodies in the panel is generated with antibodies possessing shared blocking profiles being placed in the same 'bin' [23]. Binning is commonly performed on SPR or BLI biosensors, as the assay can be conducted using only unlabeled antibody and antigen, which are routinely available at even the earliest stages of drug discovery. Other techniques such as ELISA or Luminex-style assays are also available to generate binning data, though data is not real-time in nature and requires labeling or other secondary detection schemes to monitor binding [13,32,33]. Additionally, the lack of real-time data monitoring precludes some understanding of apparent binding kinetics between antibody and antigen as well limits the ability study weaker-affinity mAbs, which may not remain bound at the conclusion of washing steps. For example, dissociation rates of antibody from antigen can be gleaned from biosensor based epitope binning studies with relative ease providing an additional layer of candidate data without the need for a separate assay. The emergence of several high-throughput, real-time biosensor systems has further pushed the status of these platforms as the preferred means of epitope binning.

Competitive epitope binning studies are performed using three different techniques: premix, classical sandwich, and tandem blocking [31], described in Figure 2. In a premix binning assay, mAb is immobilized on the biosensor surface followed by flowing a premixed solution of antigen with an excess amount of a second antibody over the first mAb surface. In a classical sandwich assay, an antibody is captured or coupled onto the biosensor surface, then an antigen is flowed over the surface, and finally, a second antibody is passed over the surface which comprises the immobilized antigen/mAb complex. In the case of the tandem assay, the target antigen is immobilized on the surface and the two mAbs are injected sequentially. Premix and tandem binning formats both require saturation of antigen binding sites to accurately assess competition, but are amenable to assay formats where the antigen is multivalent. The sandwich format does not require saturation of binding sites, but is complicated if the antigen is not monovalent. SPR platforms utilizing arrays of surface immobilized antibodies are well suited for the

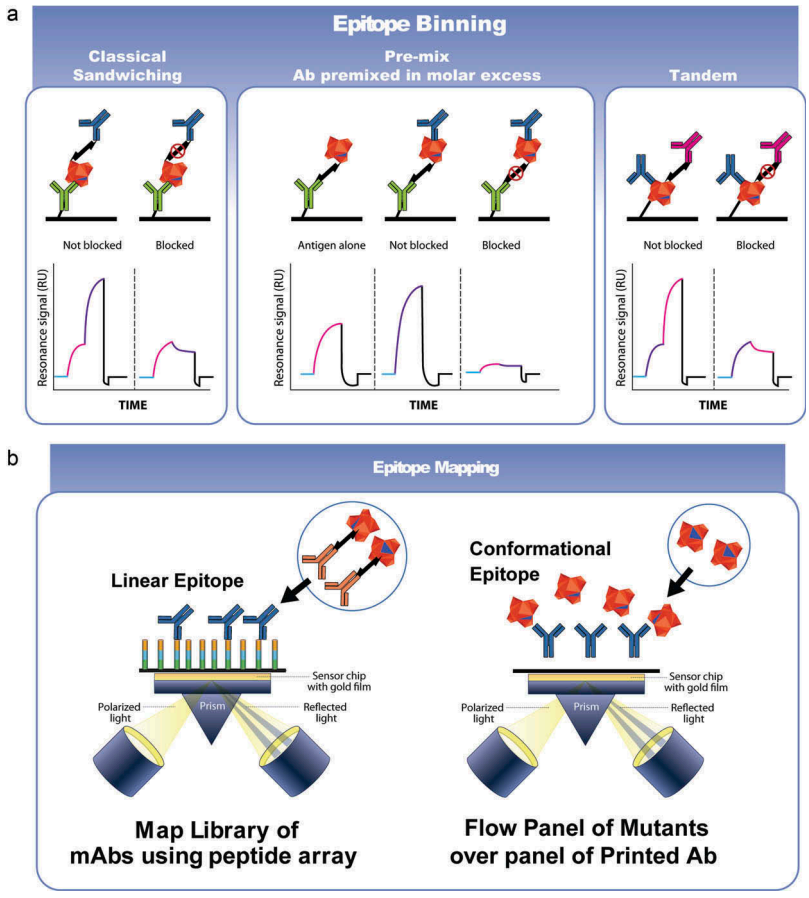

Figure 2. (a) Graphic illustrating the types of epitope binning assays and their corresponding sensorgrams. (b) Graphic illustrating the types of epitope mapping.

premix and sandwich assay formats by taking advantage of the one-on-many injection routine, whereas BLI-based Octet platforms are more efficiently run using the tandem format [23]. Every assay format has its strengths and weakness and it is important to adopt the appropriate assay format and platform to generate epitope binning data [23,22].

The inclusion of real-time biosensors for epitope characterization at the initial stages of mAb screening provides opportunities to monitor for much more diverse binding phenomenon than traditional workflows currently facilitate. A distinguishing characteristic of using SPR or BLI platforms for epitope binning is that data is acquired in real-time, unlike endpoint assays such as ELISA. Sensorgram (for SPR) or interferogram (for BLI) binding profiles offer detailed insight into antigen:mAb engagement. In terms of kinetic rate constants, the rate of dissociation (kd) can be gleaned from a binning study without any significant changes in assay design. Depending on the assay format, it is also possible incorporate an ascending titration of antigen to measure rate of association (ka) in addition to kd, and ultimately calculate affinity (KD). Therefore, from a single experiment mAbs can be viewed in both the context of their epitope relative to other mAbs, as well as ranked for their affinity toward these epitopes.

In addition to direct monitoring of regeneration efficiency, non-specific binding (NSB), and kinetics, such phenomenon as affinity disparity, where a high-affinity mAb can outcompete a lower-affinity mAb for antigen and cause a drop in signal not necessarily related to epitope competition, can be observed using real-time screening approaches. This aspect of epitope binning is particularly valuable for understanding competitive relationships that are dependent on order of binding and is relatively straightforward to identify using existing software tools. In a traditional endpoint assay, non-reciprocal competition may simply arise from fast off-rates, but in real-time data collection, these behaviors can be tracked more closely and assessed for validity [15]. A real world use of this type of behavior is in identification of allosteric modulating antibodies, which remains challenging in current workflows at early stages with hundreds of candidates [34]. In the cross-competitive format that epitope binning affords, additional pathway reagents can be readily introduced into the assay. Once epitopic groupings are identified (see Section 2.2.2), those that modulate antigen/pathway reagent binding, yet group amongst mAbs that may otherwise show no apparent effect on the pathway in question, make excellent candidates for allosteric follow-up studies, as the data would suggest they are not necessarily steric in mechanism of action (MoA).

2.2.1. Important considerations in epitope binning experimental design and implementation

Selection of the label-free biosensor platform (SPRi or BLI), appropriate binning technique (classical sandwich, premix, or tandem), and optimization of experimental conditions is key to developing robust epitope binning assay that can be

routinely employed as a frontline screening tool in drug discovery. In order to confidently screen large numbers of mAbs, the binning format and immobilization strategies must be thoroughly vetted in light of the strength of biosensor platform used in order to maximize throughput. Regardless of the binning format, immobilization of mAb, antigen and/or their capture/couple reagents onto the sensor surface can appreciably impact outcome of the experiment (see references for additional information) [23] [22]. Pre-assay routines such as regeneration scouting and NSB determination can greatly reduce complexities in large data sets and highlight antibodies with properties that are potentially challenging from a development perspective [22].

Identification of an optimal regeneration condition that can be used over multiple cycles is one of the most critical requirements during the design of binning experiment with immobilized proteins [22,36]. Regeneration is the process of disassociating analyte bound to the immobilized ligand and ideally results in complete removal of analyte while retaining the full binding capacity of the ligand. Regeneration scouting can be performed by exposing ligand surface to various regeneration solutions and can be fairly straightforward for mAbs since they typically demonstrate a similar tolerance for low pH. In addition to acidic reagents, presence of salts can also aid in removal of bound analytes [28]. Recent manuscripts have described approaches to regeneration scouting, though they more focused on methodologies than identification of one ideal solution as appropriate regeneration properties vary significantly for different proteins [36,37]. Therefore, regeneration scouting remains very much an empirical process, though as mentioned previously the tolerance of mAbs for low pH means that a much more focused set of reagents can be used to identify a satisfactory condition, particularly for epitope binning [23]. If the tandem assay format is being utilized with immobilized antigen surface, regeneration scouting can be more challenging as antigen may have significant sensitivity to regeneration conditions. In such cases, capturing antigen using specific tag is the preferred choice of experiment design or in the case of BLI, discarding of biosensor tips between cycles when feasible. In some scenarios, BLI sensor tips can be regenerated using separate solutions, but does require scouting be performed for each candidate [23]. For additional information beyond the scope of this review, van der Merwe has detailed a thorough list of regeneration solutions suitable for biosensor applications [38].

Practically speaking, it is very challenging to find one regeneration condition that is uniformly effective for a panel of diverse mAbs, but there are opportunities to anticipate issues in the assay set-up. Monitoring surface activity over the course of the experiment is an excellent approach toward tracking data quality parameters. This is achieved, for example, in the classical assay format, by periodically binding antigen alone to the sensor surfaces to monitor for changes resulting from either incomplete regeneration or ligand inactivation. Small percentage losses of mAb surface activity due to incomplete regeneration of antigen or inactivation of mAb, however, do not significantly impact epitope binning data and data analysis tools can accommodate for slight changes in ligand binding capacity. Additionally, the classical and premix binning formats typically represent each mAb as both a ligand and analyte in the assay, so that analyte competitive profiles are still obtained for each mAb even if ligand activity is absent.

In addition to regeneration, identification of NSB is essential for increased confidence in the quality of binning data and ensuring assay robustness. One approach for assessing NSB is to pre-screen antibodies in a given panel against themselves without the presence of antigen, though this does require an additional step before epitope binning. As mAbs are fully competed in binning experiments, evaluation of the competition of a mAb against itself can also provide clues regarding the degree of NSB present. Injection of antigen across the sensor surface prior to the start of a full binning study can also highlight issues with unwanted surface interactions. NSB signals, detected as binding to reference locations lacking immobilized ligand, can be subtracted from signals in the full binning assay to correct for modest background binding [39]. If NSB appears to be ubiquitous across the entire panel, optimization of buffer conditions or supplementing the buffer with additives may mitigate NSB. Schaasfort and Tudos [40] provides a more detailed description on the selection of buffer additives to overcome NSB without disrupting the interaction of analyte and ligand, with increased salts, detergents, and/or blocking proteins such as bovine serum albumin (BSA) being some of the more common additives to employ. As a last resort, if the level of NSB is too great, mAbs exhibiting high NSB can be eliminated from analysis in the binning study. Candidates demonstrating extensive NSB toward biosensor surfaces may highlight issues that could exclude these antibodies from further development where they will likely continue to bind in non-specific fashion for other biochemical and biophysical assays.

Another challenge with screening early in the discovery process is that antibodies may be present in crude supernatants, unless investments have been made in small scale purification processes [41]. Epitope binning from CM can be performed using SPRi or BLI, but can be challenging owing to lower mAb titers in CM. To some extent media components may complicate assays as well if they compete, for example, with ligand sites. Additionally, if the antigen is multivalent, difficulties can arise in ascertaining saturation of binding sites for premix and tandem formats, particularly when the fraction of active mAb is lower that than that of total titer. For workflows where mAb titers are beyond the biosensor limit of detection, single step purification is one potential solution that simultaneously enriches mAb concentration while removing potentially interfering media components, but does add an additional stage in the process. However, since both SPRi and BLI are real-time platforms and do not require any labeled reagents, they allow faster time-to-data due to ease of assay set-up and reduced development time necessary for binding assays requiring labeled secondary detection reagents and thus may offset this additional sample processing step. Improvements in sensitivity for high-throughput real-time biosensors should address the challenges with low titers found in CM. In addition to enhanced sensitivity, simplified assay reagent needs and throughput enhancements will continue to push real-time biosensors as screening tools into the pre-purification stages of discovery.

2.2.2. Presentation of epitope binning data

The magnitude of data generated during an epitope binning experiment necessitates different approaches to analyzing and representing data. A fully competed panel of 96 mAbs will yield over 9,200 sensorgrams. Fortunately, software tools are adapting to these requirements and offering increased options for data display. Figure 2 provides examples of sensorgram data for the three types of epitope binning studies. Based on user defined thresholds, these responses can be classified as sandwiching, intermediate, or blocking. As it can be challenging to control fractional activity of ligand mAbs in the assay, a good practice for automating the categorization of responses is to normalize antigen binding responses within each ligand. Once the response scale is normalized, numerical thresholds can be consistently applied across all ligands to assess the degree of competition. This also affords the opportunity to easily exclude poorly behaved data such as mAbs that cannot compete with themselves and subsequently may have NSB or other issues. Collectively these responses are first structured as heat map that can represent overall competitive profiles in a single view.

If mAbs are fully competed, meaning they are represented as both ligands and analytes (or in the case of the tandem format as both the primary and secondary analytes), then the heat map matrix is bidirectional in the sense that each mAb is listed both row- and column-wise. While epitope binning can be run in a simple unidirectional fashion, alternating the order of binding events both provides a higher level of confidence in the data set and also has the potential to uncover nuanced behaviors such as non-reciprocal competition and allosteric effects.

In Figure 3, a typical cross-competition heat map for a panel of 96 mAbs is shown. This also provides a good representation of the complexity of epitope binning data, particularly when unsorted, and the need for software tools capable of applying sorting routines to structure similar antibodies within the panel. Sorting of heat maps has traditionally been done by hand and can add unnecessary subjectivity to the analysis. Normalized sensorgram responses were processed by grouping mAbs based on their discrete competitive binding profiles. Green color indicates no competition (sandwiching) between the mAb pair, while red highlights competition and yellow shows values intermediate to those of sandwiching and blocking. In Figure 3(a), the heat map is unsorted, meaning it largely follows the experimental ligand order and analyte order. When a sorting routine is applied to the matrix (Figure 3(b)), mAbs are both clustered based on their competitive similarities and well as aligned so that the self–self comparisons are positioned on the diagonal, denoted in darkened boxes. Many sorting routines are available to carry out this function and in this instance, a binary sorting routine based on either sandwiching or competing outcomes was applied [42,43].

Hierarchical clustering routines are next applied to the sorted heat map to in order to build a convergent structure of competitive behaviors, resulting in dendrograms as shown in Figure 3(b) [23,26]. While hierarchical clustering tools have enjoyed applications in other fields, their use in understanding the epitopic relationships of mAbs is relatively new [43]. At the lowest points on the y-scale of the dendrogram, referred to as leaves, all competitive differences amongst mAbs are represented on the dendrogram structure. Moving up the y-scale of the dendrogram, mAbs showing similar, but not necessarily identical behaviors converge and differences are ignored. In this approach, all mAbs are initially treated as unique and through an agglomerative process, those with comparable competition profiles are progressively grouped together.

As the mAb panel increases in size, so does the opportunity to identify more uniquely competitive mAbs via increased resolution of epitope diversity [23]. However, larger cross-competition heat map matrices can significantly increase the complexity involved with data interpretation. Such large data sets are inherently challenging to interpret and require additional tools to simplify representation of binning data to a broader audience. Representation of heat map/dendrogram data as network plots can provide graphical cues that more effectively communicate competitive relationships behaviors.

The epitope binning network plot shown in Figure 4(a) provides a simplified, user-friendly representation of the cross-competition matrix relationships from the heat map. These networks are established using detection algorithms designed to identify relatedness amongst large sets of data [44]. The discrete nature of the binning network forces the assignment of mAbs into separate bins on a binary basis of

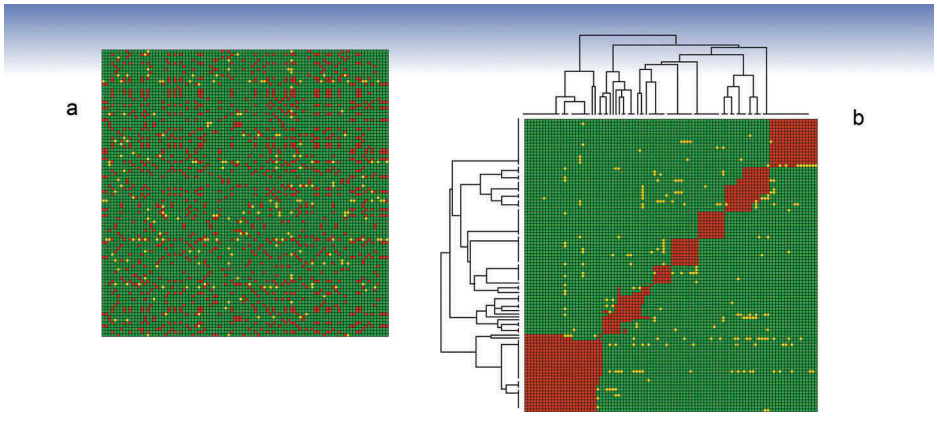

Figure 3. (a) Unsorted heat map of 96 mAb from epitope binning. (b) Sorted heat map from (a) with clustered dendrogram.

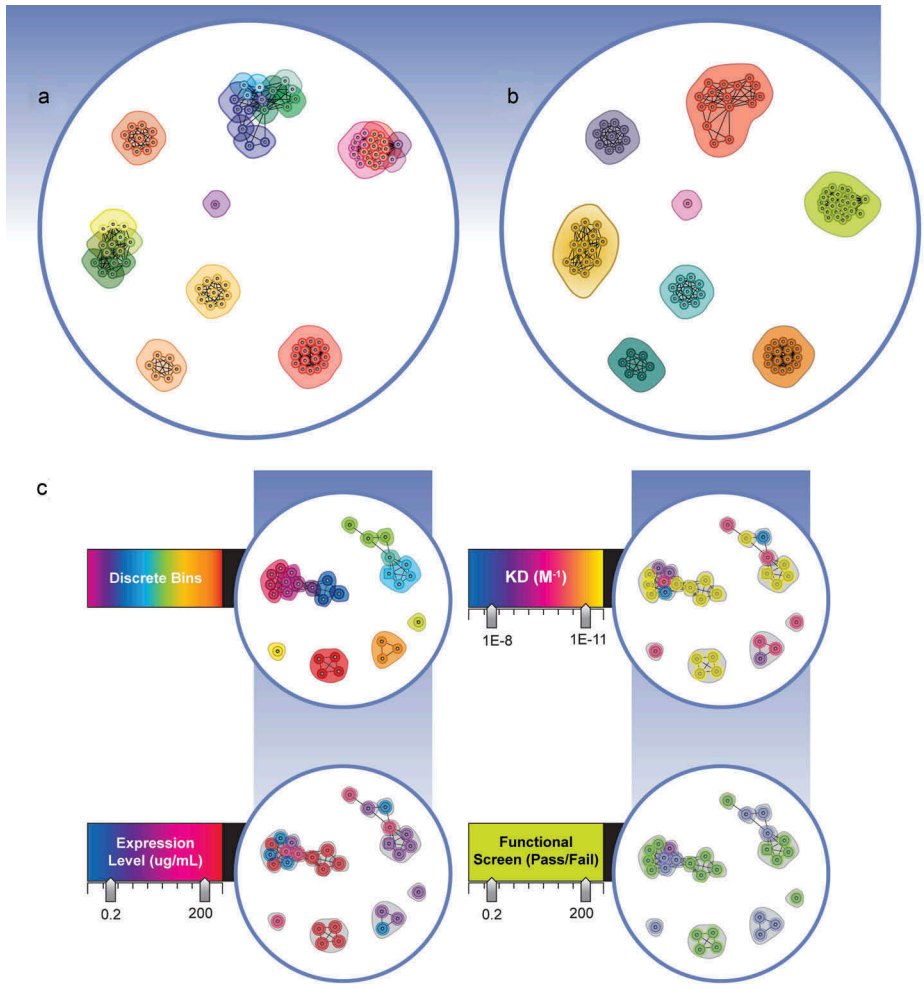

Figure 4. (a) Binning network plot representing discrete competitive relationships observed in a 96 mAb generated from heat map from Figure 3. Solid lines represent blocking between mAbs. (b) Epitope binning relationships plotted as community networks. Graphical cues are the same used in (a); however, hierarchical clustering creates networks based on degrees of similarity. Highlevel trends in shared competitive/non-competitive profiles dictates groups of mAbs. (b) Overlays of functional assay data on the pre-defined binning network relationships.

either sandwiching or blocking. Put another way, mAbs belonging to the same bin share identical competition profiles in the assay. Nodes represent mAbs and individuals in the same bin are highlighted using a shared color scheme. Solid connectors signify symmetrical competition and dashed connectors indicate asymmetric competition. Referring back to the dendrogram in Figure 3(b), the lower leaves on the y-scale reflect the bin level of network representation. Horizontal lines identify mAbs which share identical competition profiles in the assay, with even one difference in blocking causing mAbs to separate into discrete bins. While this is the most objective way to represent the data, it can be complicated by intermediate responses that are not clearly assignable as sandwiching or blocking. Multiple factors such as antigen size, binding affinity, partially overlapping epitopes, steric/allosteric effects, and antigen surface density can contribute to these intermediate responses. To overcome this, looking at higher branch points on the dendrogram can identify mAbs with very similar, but not necessarily identical competition profiles.

Community network plots (Figure 4(b)) borrow many of the visual and spatial cues from binning network plots, but are dynamic in that they allow for small differences in competition profiles to be ignored, thus permitting identification of broad relationships among mAbs. The height chosen along the y-scale continuum of the dendrogram largely dictates whether a network plot represents binning level data or else community level data, the user can evaluate the network trends in both circumstances. Representation of mAb competition profiles using community plots overcomes the challenges in applying absolute and discrete criteria is defining relationships among mAbs, which can be particularly complicated by the aforementioned intermediate responses. Both objective (binning network) interpretations of mAb competition as well as subjective (network) plots are readily available. This is a highly effective way to identify major epitopic trends at the outset of a mAb discovery campaign and use informed selection criteria to categorize and reduce candidate numbers.

While epitope bins and communities are good starting points for describing similar/dissimilar trends in antigen engagement for a panel of mAbs, they are most effective when combined with data from of other assays. Additional data can be represented as 'lenses' on the network plot as shown in Figure 5. Network plot lensing is emerging as a new tool to represent the intersection of

epitope with various biophysical, biochemical, and cell-based data. As an example, binding affinity, expression levels, and functional assay data can be easily represented in the lensed plots, thus allowing quick selection of lead mAb(s) with desired traits. It also offers researchers insight into the granularity of individual bin/communities and emphasizes that these groupings are not necessarily uniform, but can reflect a diversity of steric and energetic mechanisms underlying each mAbs means of engaging the antigen. For example, functional activity may not be uniformly present or absent within each bin suggesting that competing mAbs in that bin may reside on the functional periphery for an epitope or else their binding is functionally disruptive through processes other than direct competition (Figure 5). Lensed network plots such as this also highlight mAbs that may make for excellent tool regents in development as they bind near the site of action, but do not have functional effects.

2.3. Epitope mapping

While powerful and instructive toward identifying groups of mAbs with similar epitopes, epitope binning does not delineate the specific residues or regions of amino acids involved in an antibody–antigen interaction. Epitope mapping, a process that characterizes the mAb binding site on an antigen, allows for localization of the amino acid residues at the binding interface between mAbs and antigen [34]. The two main routes for epitope mapping in biosensors are either (1) epitope mapping wherein mAb is assessed for binding against an N-mer peptide library with overlapping coverage along the entire sequence of the target or (2) antigen mutagenesis epitope mapping, in which binding of mAb is tested against different mutants of target created using site directed mutagenesis [45]. Here, we will briefly discuss the advantages/disadvantages of each method (Figure 2).

Peptide epitope mapping most commonly consists of coupling an overlapping biotinylated 12- to 18-mer antigen peptide library to a streptavidin-coated sensor surface and flowing over antibodies to quickly identify both linear and conformationally sensitive binders, and in the case of the linear binders demonstrate which regions of sequence are involved. While this type of experiment can be performed with ELISA, the real-time nature of label-free biosensors can provide important binding properties such as kinetics constants and/or affinity depending on assay design. Although specific residues involved in the binding interaction cannot be implicated from this assay alone, the magnitude of responses, particularly for those of adjacent peptides, indicate preference for certain regions of sequence and can suggest differential means of epitope engagement for a panel of otherwise similar mAbs. The major limitation is the lack of direct binding site localization for conformational binders, but in conjunction with epitope binning studies, conformational mAbs can be confidently excluded from certain regions based on how they compete with linear epitope mAbs [46].

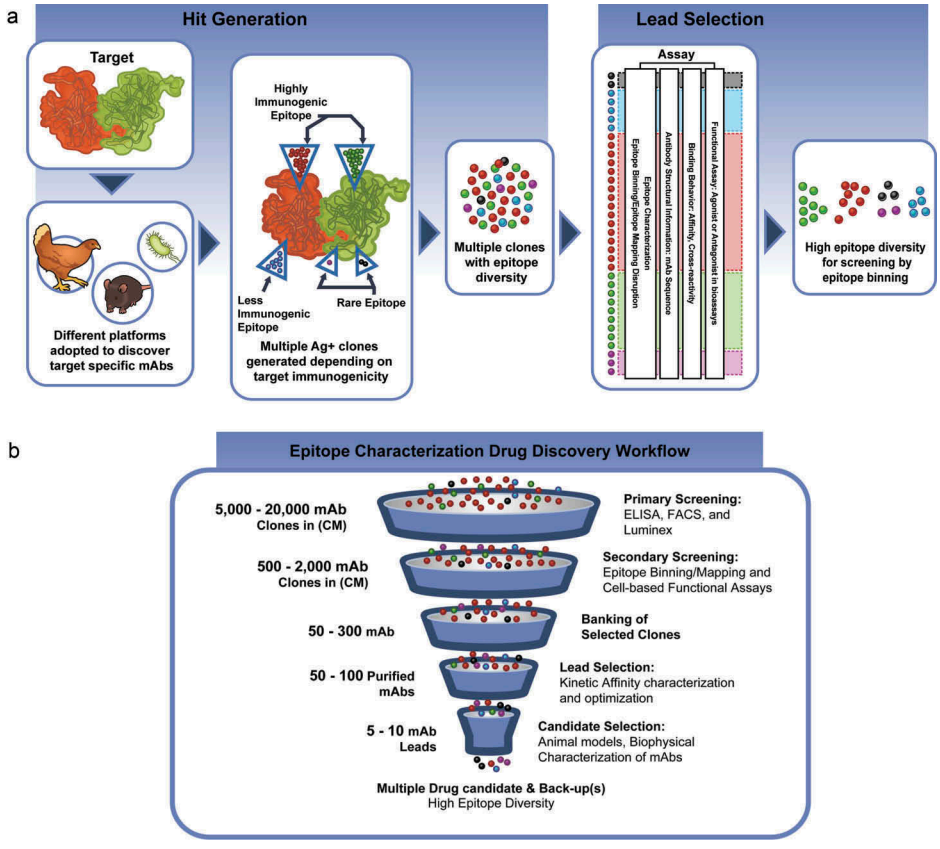

Figure 5. (a) Graphic illustration of epitope characterization focused mAb screening. With epitope information, epitope diversity is maintained throughout the drug discovery process. (b) Epitope characterization focused mAb drug discovery workflow.

In mutagenic epitope mapping, insertions or deletions are made at key residues in predicted epitopes that ideally should both protect the general structure of the antigen while modifying residues involved with mAb binding. Structure and binding analysis through biophysical prediction software can facilitate the mutagenic design and layout. A common approach is alanine scanning where side chains are changed to alanine and the binding properties are assessed [47–49]. Although mutagenic epitope mapping provides important complimentary information by discerning the regions of interaction on a target, it does require resources for mutant design, expression, and purification, which historically has limited its implementation during the early stages of drug discovery. Expression, stability, and misfolding issues can all contribute to challenges in representing the full breadth of mutations being sought, but platforms are available for more rapid screening and generation of these reagents [45]. Despite the challenges, mapping via mutagenesis can yield insight into conformationally sensitive epitopes as well as elucidating behaviors that require structural integrity that are not discernable using the peptide epitope mapping approach. Characterization of epitopes using mapping provides an additional level of understanding at the site of antigen engagement and informs epitope localization for bins and communities.

An additional approach to epitope mapping approach that mimics the presentation of altered antigen like mutagenic mapping, and has been adapted to real-time, label-free biosensors is the differential antigen disruption (DAD) assay [26]. In DAD, target antigen is treated with specific chemicals and/or enzymes thereby modifying the structural and chemical composition of antigen. Presence or absence of binding to modified antigen can allow for identification of epitopically similar mAbs. One challenge of the DAD format is that disruption is required in order identify mAbs sharing epitope and therefore epitopes not affected by treatments may remain ambiguous using this approach. Additionally, the assay is not capable of detecting unique changes in structure such as allostery. However, depending on the assay configuration, DAD can achieve very high levels of throughput.

2.4. Complimentary epitope characterization assays (non SPR/BLI methods)

At the earliest stages of drug discovery, epitope characterization is most efficiently achieved using rapid screening approaches such as SPR and BLI to inform candidate selection. At the later stages of drug discovery, when candidate numbers are reduced and yields are scaled up, alternate techniques become available for characterizing epitope. Nuclear magnetic resonance (NMR) imaging [50,51], hydrogen–deuterium exchange (also called H/D exchange) mass spectrometry [52], X-ray crystallography, and cryo-electron microscopy are additional techniques available to assess binding site engagement [53–56]. Of these techniques, X-ray crystallography provides some of the most detailed visualization of mAb–antigen binding and identifies both strong and weak contributions to the interaction. However, X-ray crystallography remains resource and time intensive and is typically performed on only a few lead mAbs. Although all of these techniques can provide exquisite resolution, they become feasible at a point where detailed epitope characterization is necessary, but does not significantly factor into epitopically diverse lead selection.

3. An evolving drug discovery paradigm to streamline mAb discovery and accelerate mAbs from bench to clinic

Here, we advocate a modernized screening paradigm that incorporates epitope characterization in the earliest stages of the drug discovery, as opposed to later stages which is more akin to the conventional drug discovery process. Figure 6(a,b) provide a more detailed schematic of this updated therapeutic mAb discovery workflow. No major alterations in pre-screening processes such as target validation and screen designs are warranted and this new strategy can be easily adapted to various mAb generation platforms. Once mAbs are generated against a specific target, the newly proposed primary screening workflow will be:

(1) Antigen specificity and species cross-reactivity screening of mAbs using ELISA or Luminex,
(2) Epitope binning and off-rate ranking/kinetic screening in CM or after single step purification,
(3) *Optional*: Epitope mapping using overlapping peptide libraries and/or mutant screening,
(4) Binding kinetics of mAbs (if not determined from binning studies) and high-throughput cell-based assays on antigen positive clones, and
(5) Epitope network lensing using all available criteria and selection of candidate mAbs from each functional epitope bin; use of epitope data to also identify supporting tool reagents.

Following this screening paradigm, we recommend selection of 50–100 epitopically diverse mAbs for further detailed characterization as purified reagents. Following purification, 5–10 lead mAbs can be chosen based on downstream selection criteria such as expression yield, stability, immunogenicity, etc. With currently available platforms, this workflow will easily accommodate epitope screening of 500 clones. Brute force through use of multiple platforms in parallel can push these numbers into the thousands. Technological gains in throughput will eventually make epitope binning in the thousands a routine process that will increase the odds of success at the clinical stage.

3.1. Incorporate epitope binning during primary screening to drive selection of epitopically diverse mAbs

Binning therapeutic mAbs based on target binding epitope is an ideal early stage screening tool that provides unique advantages. Binning experiments do not require a priori knowledge of antibody's function/epitope or secondary detection reagents and can be performed on several commercially available systems using multiple formats (tandem, classical sandwich, or premix) [23,24]. In addition to the primary goal of identifying and ranking functional leads, epitope characterization also results in a wealth of knowledge regarding tool mAbs to support development, avoiding allocation of precious

resources for follow up screening. We have detailed how binning increases the work in process queue thereby reducing 'dead-end' candidates, and increases likelihood of success in Phase II and III [57,58]. Epitope binning of 500 or more mAbs using crude CM is no longer an insurmountable obstacle.

Although we advocate epitope binning as the early screening tool, measurement of mAb binding kinetics continues to be critical. The new mAb discovery paradigm will promote affinity ranking of mAbs; but within the bin/community, so that we can identify a subset of mAbs for additional screening assays. Kinetic/affinity data can be drawn from the binning studies when the experimental design accommodates or else run in separate assays. This approach is of particular importance when therapeutic endpoints or MoA is not well defined and selection of diverse collection of mAbs is preferred – both in binding affinity and epitope. Moreover, weak-affinity mAbs binding to functional epitopes can be affinity matured and progressed further in the pipeline. Following the integration of all the data generated from this modern drug discovery paradigm, better decisions can be made much earlier in the drug discovery process compared with the conventional screening approach. Characterizing mAb binding epitopes at an earlier stage will facilitate a better understanding of function and MoA which in turn will assist in a more guided discovery of successful clinical candidates.

3.2. Maximize the capacity of epitope binning

The historical position of epitope binning in drug discovery has been primarily for characterization of competitive attributes amongst a small subset of functional leads prior to final candidate selection. The chance of all leads at this stage having discrete epitopes is not very likely given the bias in traditional screening platforms. The rationale for large-scale binning studies in early phase screening operates under a much different pretense of having to both confidently group competitively similar mAbs while at the same time identifying diverse candidates from a pool of hundreds of hits. In order to maximize evidence of diversity, or lack thereof, between two mAbs, it is essential to have as many data points as possible. In doing so competitive network plots become highly informed by monitoring the epitopic footprint of each mAb against all others in the panel, amplified hundreds of times. This can be seen in a heat map as 'ragged edges' for groups of mAbs sharing similar competitive profiles (Figure 3(a)). Even more intuitively, these can be seen in network plots as mAbs that fall under the same community as others having similar competition profiles, but spatially they are distant from the core of the community, reflecting the unique underlying properties they possess. Thus, the resolving power of epitope binning data typically increases with the inclusion of greater numbers of mAbs that can begin to uncover the subtle differences in competition that are indicative of distinct steric and energetic traits. Abdiche et al have demonstrated the enhanced resolution of epitopes when additional antibodies are included in a competitive epitope binning format [23]. This supports the concept that 'more is more' in competitive epitope binning. While we have previously reported the challenges involved in binning large numbers of mAbs [26], recent advances in high-throughput, label-free biosensors provide affordable options to perform epitope binning of large numbers of mAbs in a reasonable period of time [28], making epitope characterization of large panels of mAbs much more approachable. Although it is unclear if there exists a point in which the size of a mAb candidate panel yields diminishing returns in terms of epitope diversity, it is anticipated that the increasing adoption of competitive epitope binning toward diverse targets will provide insight in the near future.

Increasing the numbers of mAbs epitopically characterized also provides major opportunities to explore engagement of functionally relevant hypervariable proteins such as viral surface antigens. Variability of disease mediating antigens had been a key challenge in fields such as virology and oncology [59–61]. Larger antibody panels that provide full coverage of all major antigen epitopes enable monitoring of both dynamic changes in structure across antigen isoforms as well as binding kinetics. Collectively, this data can be used to evaluate similarities and differences between antigen isoforms and also identify functional mAbs that remain active across these species. Assessing the reactivity of mAbs against multiple forms of antigen using real-time label-free biosensors can be achieved by simply including these reagents during initial epitope characterization studies.

3.3. Inform epitope networks with orthogonal data

Epitope binning, although data rich, is most valuable in when integrated with other data sets to drive highly informed selections. Lensed representation of data such as binding kinetics, cross-reactivity, expression yields, and cell-based function (as shown in Figure 5) on epitope networks advances the thoughtful prioritization of candidates and establishes target epitopes as the central paradigm around which as discovery program can visualize and select lead candidates., This also highlights the power of merging functional data with epitope binning, to fully understand how functional activity of mAbs relates to epitope and in conjunction with mapping may uncover differential residue engagement for mAbs in the same bin/community. Lastly, the cross-competitive nature of epitope binning studies has the ability to uncover mAbs exhibiting allosteric properties via asymmetric competition, which would manifest as being functionally active, but in bins or communities not otherwise identified as functional. Without being placed in the context of epitope, these mAbs could very well appear similar to all other functional hits, but their MoA is quite unique and could provide novel agonistic/antagonistic opportunities to be uncovered using this approach.

3.4. Model epitope data computationally

For novel targets, crystal structures are often not available at the outset of a drug discovery campaign. In lieu of crystal structure, homology models provide an excellent opportunity to visualize MoA and potentially drive engineering of optimized leads [62]. Often the implementation of informatics support for therapeutic program comes after lead selection activities have begun to conclude, allowing for little recourse if modeling were to uncover additional opportunities. While epitope characterization does not require structure in order to be implemented as a screening tool, the relationships

derived from binning and mapping studies provide valuable data for modeling antigen engagement by mAbs. Epitope binning data can further be bolstered by inclusion of native ligands or receptors in the competition assays, particularly if there is information available to suggest where these proteins may bind on the target. Similar to this epitope mapping data can highlight regions of antigen that are being engaged by mAbs and provide insight into conformational versus linear epitopes. If target structure is available, then modeling capabilities can be highly refined and possibly compared with competitor molecules for among other things, differentiation strategies. As epitope characterization becomes more widely adopted, numerous inroads will be made in modeling of this data to inform drug discovery and development.

3.5. Utilize mAb epitope to protect commercialization strategies

IP concerns play an important role in the drug discovery process and companies routinely evaluate the freedom to operate (FTO) on a particular target before ramping up development. One of the pivotal factors that mandate target FTO is the pre-existing claim on the binding epitope of the competitors' drug molecule. The strategy used to discover therapeutic mAb is often not considered a patentable invention in many jurisdictions [63]. But mAb sequence, especially the complementarity-determining region (CDR) along with its binding epitope provides the foundation for IP claims and protection [24]. Patent applications are typically drafted with a broad scope to prevent competitors from marketing a functionally equivalent mAb [63], thereby dictating the necessary FTO and limiting competitors' ability to design mAbs with the same epitope and function. Therefore, it is crucial to identify functional epitope by correlating epitope binning data with cell-based functional data. Over the last two decades, significant number of companies have established numerous IP claims on their 'first-in-class' (FIC) clinical molecules and the biopharmaceutical industry is currently striving on the development of 'best-in-class' (BIC) therapeutic mAbs by showcasing its differentiation based on binding epitope, PK/PD profile, efficacy, and immunogenicity [63]. Companies are currently investing significant amount of resources for extensive mAb engineering in an effort to demonstrate such differentiation. Though protein engineering tools can assist in modulating various mAb properties, it is currently not possible to engineer mAb binding to a specific epitope, thus making identification of unique functional epitope a critical factor for developing BIC molecules.

4. Expert opinion

Strategies for discovery and development of therapeutic mAbs need to evolve to meet the demands of increasingly challenging targets as well as growing commercial competition. The implementation of screening assays based on ease of use or historical precedence does not justify missed opportunities in selecting diverse mAb candidates for progression to the development phase. Detailed epitope characterization using real-time label-free biosensors is beginning to change the way that mAb screening is conducted. The key enabler to this approach is the emergence of biosensor platforms capable of handling the screening requirements in early drug discovery. Using the updated screening paradigm we have laid out here, a much more detailed understanding of the process of antigen epitope engagement can be established for large panels of candidate mAbs. Data supporting candidate selections then become epitope-centric, with other biophysical, biochemical, and functional attributes being integrated around this key trait. While this review has focused on mAbs, it is worthy of mention that therapeutic moieties will continue to evolve including the use of scaffold-based formats and fragments. Although structurally dissimilar to mAbs, these emerging formats will still require detailed understanding of where and how target epitope is engaged, making this workflow adaptable to future therapeutic campaigns.

Binning and mapping data overlapped with functional data generates a critical overlay of epitopic and functional information. We advocate screening higher numbers of antibodies – not just in the dozens or even hundreds, but into the thousands. Up until now, binning dozens of mAbs was challenging, but in the near term, up to 384 will be realistic as biosensor throughput increases with screening of 1,536 in the medium term. Upcoming publications from our group and others will show the discriminating power of binning to resolve fine differences in function and epitope with larger panels of antibodies especially in regards to allosteric targets. In practice, merging epitope characterization data with functional data from all of the antibodies in a panel as the primary screening mechanism in a project provides research the best opportunity to make informed decisions. The future integration of improved, predictive protein–protein binding algorithms with binning/mapping data will further enhance the decision-making process and is driving current research efforts in epitope characterization in our group. Moreover, binning and mapping will also change how antidrug antibodies are detected and characterized. These changes, especially increased throughput in binning, will improve mAb screening workflows with the ultimate goal of decreasing development time and cost for life-saving therapeutics.

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Declaration of interest

N Ditto and B Brooks are employed by Wasatch Microfluidics and have a financial interest in the company. Wasatch sells instruments that are used for epitope binning and epitope mapping. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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