HHS Public Access

Author manuscript

Curr Opin Immunol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Curr Opin Immunol. 2015 August ; 35: 89–97. doi:10.1016/j.coi.2015.06.009.

Serology in the 21st Century: The Molecular-Level Analysis of the Serum Antibody Repertoire

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Abstract

The ensemble of antibodies found in serum and secretions represents the key adaptive component of B-cell mediated humoral immunity. The antibody repertoire is shaped by the historical record of exposure to exogenous factors such as pathogens and vaccines, as well as by endogenous host-intrinsic factors such as genetics, self-antigens, and age. Thanks to very recent technology advancements it is now becoming possible to identify and quantify the individual antibodies comprising the serological repertoire. In parallel, the advent of high throughput methods for antigen and immunosignature discovery opens up unprecedented opportunities to transform our understanding of numerous key questions in adaptive humoral immunity, including the nature and dynamics of serological memory, the role of polyspecific antibodies in health and disease and how protective responses to infections or vaccine challenge arise. Additionally, these technologies also hold great promise for therapeutic antibody and biomarker discovery in a variety of settings

Antibody Serology: Past Achievements

Serology is classically defined as the study of proteins, predominantly antibodies, found in blood and secretions such as saliva. The genesis of serology dates to the end of the 19th century and the pioneering “serum therapy” of Emil von Behring and Paul Ehrlich, followed for decades by elegant studies on the specificity of serological reactions by Karl Landsteiner. But it would take Landsteiner until the twilight of his career to formally demonstrate that an anti-serum does not comprise merely a single antibody but rather a mixture of different antibody populations of unknown complexity[1]. It was another decade before the plasma cell, which is responsible for the secretion of antibodies, was discovered,

and then it was only 50 years ago, in 1965 that it was convincingly shown that antibodies are produced by B lymphocytes [2]. This latest discovery coincided with the development of new technologies in protein chemistry and the advent of molecular biology that, together, catalyzed a remarkable pace of progress in the understanding of B cell development and antibody formation. We now know that long-lived plasma cells constitute the (most likely) irreversible end-point of B cell development, show little or no evidence of proliferation and produce copious amounts of antibodies for years, and quite possibly for decades, in humans [3]. Long-lived plasma cells reside predominantly but not exclusively in the bone marrow, surviving within specialized anatomical niches with the help of anti-apoptotic signals provided by stromal cells [4]. Of note, a fraction of bone marrow plasma cells have been recently reported to lack CD19 expression and to be protected from mobilization and replacement by newly formed antibody producing cells following infection[5•], underscoring the heterogeneity of the long-lived compartment of plasma cells and, by extension, the pool of serum immunoglobulins.

The compendium of antibodies produced first by long-lived plasma cells and second by transient waves of short-lived plasma cells or plasmablasts (elicited in response to pathogen, vaccine or autoantigen stimulation) constitutes the two main components of antibody serological immunity. A third component is contributed by natural antibodies which recognize common pathogen antigens such as galactose-α-1,3-galactose (Anti-Gal) and have an innate-like protective function [6•,7]. The relative contribution of antibodies from long-lived plasma cells, transient plasmablasts and by “natural antibody”-producing cells (rodent B1 and marginal zone B cells, and perhaps the human analogues of B1 cells, although these have yet to definitively identified) to the serological immunity is poorly understood. The antibody concentration in blood is homeostatically controlled. For example, the Immunoglopbulin G (IgG) concentration in healthy adults is maintained at roughly between 7–17 mg/ml. The level of IgG produced by long-lived plasma cells changes very slowly over time [8]. Plasmablast bursts following vaccine or infection are accompanied by a dramatic increase in the antigen-specific antibody titer but generally seem to have little impact on the total concentration of immunoglobulin in blood. It is noteworthy that the fate of the large number of plasmablasts present in circulation at the peak of the plasmablast wave has never been quantitatively followed although it is well established that the majority apoptose and only a relatively small fraction mature to long lived plasma cells and are able to take residence in niches so that they can contribute to serological memory[9]. Finally, the fraction of natural antibodies in blood or secretions is not known, nor is it known how the total level of natural antibody (comprising mostly IgM but also some IgG and IgA) vary as a function of time in health or in inflammation.

Antibody Serology: Future Directions

The essence of serological immunity is predicated on the existence of a diverse repertoire of antibodies, elicited over the life of the host and representing the integrated response to numerous antigenic stimuli. Due to the complexity and temporally dynamic nature of the antibody repertoire, the identification of its component immunoglobulins represents a formidable challenge. All serological studies to date have relied on the detection of an *ensemble* of antibodies that either could be resolved by a certain analytical technique or

bound to a specified antigen (Fig. 1). Among the most useful metrics for assessing humoral immunity, the presence of neutralizing antibodies in the serum following vaccination or infection represents the best correlate for vaccine efficacy and for protection during invasive infections [10,11,12]. The limitations imposed by the inability to resolve complex serum antibody mixtures into their constituent clonal representatives and the need to have pre-established the identity of antigens of potential interest have obscured central questions of profound basic and clinical significance, some of which are outlined below:

- First and foremost, there is nearly no information on the number of sequences (clonal diversity), functions and relative concentrations of the individual antibodies in serum. Upon reflection it is surely striking that after over a century of immunology research that the clonality of serum antibodies (number of distinct antibody clonotypes having same IGHV and IGHJ segments and highly homologous CDR-H3 sequences) in blood and in secretions has not been established experimentally even to a first approximation, although a remarkably prescient estimate of antibody diversity was first proposed by Talmage based on theoretical arguments, over half a century ago [13]. Nor do we know anything about how the clonality of serum antibodies changes as a function at extreme ages and disease status, or about the concentration distribution of individual antibodies in biological fluids. Without a detailed description of the identities and relative amounts of the antibodies that comprise the serological repertoire it is not possible to establish precisely how the B cell developmental program shapes protective immunity.

  Information on the serological antibody repertoire is particularly pertinent to vaccine design/evaluation and to antibody discovery from patients. In an effort to better delineate the serological response to influenza by taking into account antigenic variation among strains circulating in humans, Fonville et al. sought to establish correlations between serum mediated neutralization against panels of different hemagglutinins and their phylogenetic distance [14]. The authors further developed a visualization tool for describing complex serological data by plotting antibody-mediated immunity as a function of the antigenic relationships among viruses (landscape plot).

  For rapidly evolving viral pathogens such as HIV-1 or influenza, immortalization techniques and single cell cloning coupled with high throughput micro-neutralization assays of peripheral plasmablasts or memory B cells have led to the discovery of numerous broadly neutralizing antibodies (bNAbs) displaying neutralization breadth towards many or even nearly all known clinical isolates of a particular virus [15•,16•,17,18•,19]. To provide a first-order approximation of component antibody neutralization specificities in sera, Georgiev et al.[20•] proposed matching bNAb and serological neutralization potency fingerprints towards HIV-1 isolates.

  Identification of bNAbs coupled with the reconstruction of the evolutionary route that led to the development of phylogenetically-related antibodies from an un-mutated common ancestor (UCA) is essential for the design of vaccine

immunogens with the potential of eliciting broad protection. However, the fact that a DNA encoding a bNAb has been isolated from peripheral B cells does not mean that the respective antibody is present in serum or that it is produced at physiologically relevant amounts (i.e. at a concentration near the equilibrium dissociation constant such that the antibody can bind antigen). This divergence was described initially by Burnet stating that there is no requirement in the clonal selection theory that every cellular receptor has a corresponding secreted globulin [21]. As a matter of fact, considering that the BCR repertoire diversity in the memory and plasmablast compartments is orders of magnitude greater than that of the serological repertoire [22••,23] it follows that the overwhelming majority of peripheral B cell-encoded antibodies are unlikely to be present in detectable amounts as soluble proteins in blood or secretions and thus could not have contributed to humoral immunity.

- Second, while it is well established that a significant fraction of antibodies display polyreactivity and that these antibodies have important physiological functions in processes such as the clearance of cell debris and in pathogen recognition[24,25], there is a paucity of methods for quantifying and characterizing the polyreactive fraction of the serological response. It is noteworthy that, in addition to natural antibodies, which are typically polyreactive, IgG antibodies encoded by human memory B cells [26] and even bone marrow plasma cells and also bNAbs elicited by anti-viral immune responses in response to HIV-1 infection [27] often recognize multiple antigens. There is a clear need to understand the mechanisms that drive polyreactivity and its implications in health and disease. One possible explanation is that polyreactivity originates from B cells that were not removed from the repertoire during B-cell development. For some pathogens, notably HIV, polyreactivity may confer a selective advantage to pathogen–specific antibodies [26,28].
- Third, in many instances the antigens that are recognized by serum antibodies are not known *a priori*. The significance of identifying antigens, antigen surrogates (i.e. antigen-mimics that can be chemically distinct from the antigens that elicited an antibody response) and immunosignatures [29] for disease diagnosis is being increasingly recognized. Additionally, mapping the serum antigen reactivity profile in a comprehensive manner is key to understanding which environmental exposures play a more dominant role in shaping humoral immunity.

Antigen and Immunosignatures Discovery

Earlier methods for antigen discovery include the use of peptide libraries either immobilized in array format or displayed on M13 phage and serological analysis of recombinant cDNA expression libraries (SEREX), which employs cDNA libraries from tumor tissues for cancer antigen discovery and finally, antigen arrays [29,30•,31,32]. More recently advances in library screening methodologies and separately, in high throughput gene and peptide synthesis technologies have led to an explosion of high-resolution methods for the discovery of autoantigens and antigen surrogates. Daugherty and coworkers developed a technology that capitalized on *E.coli* display [33] of very large random peptide libraries for the

identification of peptide motifs that bound to antibodies from patient sera. This technique led to the discovery of a diagnostically-relevant epitope for celiac disease that exhibited a high degree of amino acid identity to deamidated gliadin, a key antigen in celiac disease. In a separate study the same group reported a 7 peptide panel of highly statistical diagnostic significance for preeclampsia [34,35]. Using massively parallel DNA synthesis Larman et al. [36] constructed libraries of >410,000 overlapping 26-mer peptides spanning all ORFs in the human genome on T7 phage. Phage was immunopreciptated with patient sera and diagnostic immunosignatures were determined by NextGen sequencing. In an alternative approach very high density printed arrays of synthetic random peptide arrays were used for immunosignature discovery and were reported to exhibit higher accuracy than tiled peptide epitope arrays [37,38]. Since peptides may not be able to capture antibodies that bind to post-translational modifications and conformational epitopes Kodadek and coworkers pioneered the use of libraries of unnatural molecules and specifically peptoids (N-substituted oligoglycine polymers) for the discovery of surrogate antigen biomarkers from patient sera [29,39]. Other powerful methods that are currently used for antigen discovery include antigen microarrays [40,41], nucleic acid programmable protein arrays (NAPPAs) produced using *in-vitro* transcription/translation[42], peptide arrays [43] or finally arrays based on a different targets such as DNA, peptides and recombinant/native proteins [44].

Finally, for very complex conformational antigens on proteins that cannot be immobilized on arrays (e.g. integral membrane proteins) or contain complex post-translational modifications or for which a peptide or synthetic surrogate is not available, novel solution phase assays are being developed for diagnostic purposes. For example radio-binding assays (RBA) [45] using *in vitro* transcription/translation RBAs have been used for detection of autoantibodies. In one notable example Joseph and co-workers, studied the association of scleroderma with an humoral immune response to cancer. They found an association of genetic alterations the RNA polymerase III subunit (RPC1) encoding gene (POLR3A) in patients with autoantibodies to RPC1 suggesting that POLR3A mutations triggered cellular immunity and cross-reactive humoral immune responses [46]. Alternatively, light-emitting recombinant antigens (LIPS) [47] have been developed and used for the profiling of 16 autoantigens in SLE patients [48]. Solution phase assays are useful for diagnostic purposes but are difficult to implement for antigen discovery since thousands of soluble proteins having a native conformation would need to first be produced and validated

The Serological Antibody Repertoire

Until recently determining the sequence and relative concentration of the antibodies in the serum repertoire was considered a nearly impossible task: biological fluids contain many thousands of different antibodies all of which are chemically very similar, having an overall high degree of sequence identity, and whose concentrations can vary by several orders of magnitude in a dynamic fashion. Starting in 2008 LC-MS/MS instrumentation began to be used to detect immunoglobulin tryptic fragments in serum samples [49,50]. However, the Ig-derived peptides detected in these earlier studies, were overwhelmingly derived from the framework regions and did not provide sufficient information to piece together complete antibody sequences. By restricting the diversity of the antigen-specific antibody pool from the serum of immunized rabbits using antigen-affinity chromatography under stringent

elution conditions, Polakiewicz and coworkers succeeded in using MS to identify overlapping antibody peptides that could help assemble a complete V gene. Combinatorial pairing of separate VH and VL sequences deduced from LC-MS/MS was then used then used to produce several antibodies displaying high affinity for antigen, first from rabbits and subsequently from humans [51,52]. In an alternate approach, dating to 2011, our lab invented a technology for determining the serological repertoire to a specified antigen [53] by combining: (i) NextGen V gene sequencing from peripheral memory B cells and plasmablasts to first create an archive of the antibodies encoded by an individual; (ii) enrichment of the pool of antigen-specific antibodies by affinity chromatography; (iii) determining the CDR-H3 peptides and other informative peptides as needed (e.g. CDR-H2) by LC-MS/MS bottom up proteomics; and (iv) the use of stringent informatics filters to assign the informative mass spectra to the entire VH gene with the help of the sequenced antibody gene archive from (i) above [54•,55]. In parallel we developed methods for the massive sequencing of the natively paired VH:VL repertoire, first in 10s of thousands and later on, in millions of peripheral B cells[56,57••] In this manner, the mass spectrometric identification of a unique peptide in a serum antibody could be mapped into the paired VH:VL repertoire archive to reveal the complete sequence of that antibody which then could be produced and studied *in vitro*. Thanks to the exquisite sensitivity of modern MS instrumentation, individual serum antibodies can be detected semi-quantitatively at levels as low as 0.4 ng/ml[22••]. Given that for an antibody to bind to antigen it has to be present at a concentration at or above its equilibrium dissociation constant, which is estimated to have a ceiling of around 0.1 nM or [58] the approach outlined above has more than adequate resolution for the detection of the repertoire of physiologically relevant antibodies in a sample.

As with any analytical technique, the experimental pipeline described above has certain limitations. First, a fraction of antibodies in the repertoire may evade discovery due to constraints inherent in LC-MS/MS proteomics. Such fault may arise when proteolysis fails to produce unique peptides for some sequences, physicochemical properties such as exceptional length limit peptide observation, or MS/MS spectral identification is ambiguous or incorrect. Additionally, unanticipated post-translational modifications or an incomplete sequence database may prevent assignment of some spectra. We estimate that the fraction of CDR-H3 peptide spectra that cannot be assigned is <20%, based on semiautomated inspection of the MS/MS data. Antibodies that evade detection using the proteomic approach outlined above may potentially be identified through use of alternative proteases and instrument fragmentation methods[59]Second, bottom up proteomic approaches reveal the antibody clonotypes (i.e. the family of antibodies having near identical CDR-H3s and V and J segments and likely derived from the same precursor B cell) in the repertoire. Somatic variants of antibodies having the same CDR-H3 are determined based on the NextGen sequencing data but cannot be unambiguously quantified proteomically. Third, the analysis of the antibody repertoire is both technically demanding and expensive, requiring access to VH:VL paired repertoire sequencing, high-end (Thermo-Electron Obritrap or equivalent mass accuracy) LC-MS/MS instrumentation and finally the requisite informatic and data storage infrastructure. Nonetheless, the above technical limitations notwithstanding, we now

have the capability to delineate the serum antibody repertoire with medium-high resolution and with excellent sensitivity.

What's Next? Addressing Fundamental Questions in Humoral Immunity

What kind of information can we glean by utilizing these new tools? We are at the dawn of a new era in the molecular-level understanding of the serological response and numerous questions wait to be answered. Examples of some major outstanding issues we expect to be addressed in the near future are listed below:

- The size of the serological antibody repertoire: We have argued based on the amount of total IgG in healthy humans and the fact that physiologically relevant antibodies must exist at concentrations at or above K_D, that a possible upper bound for the number of distinct clonotypes in human blood can be estimated to be $\geq 10^4$ [23]. Preliminary MS analyses by our lab place the size of the IgG repertoire for a middle age adult at <20,000 distinct clonotypes consistent with previous numbers presented by Talmage during the late 50's [13]. To put this number in perspective, the serological memory repertoire to vaccines we have analyzed so far such as tetanus[22••] TIV seasonal influenza, inactivated polio and pneumonococcal polysaccharide typically comprises between 50–400 antibody clonotypes each. In turn these results suggest that as much as 10–15% of the blood IgG pool corresponds or is "taken up" by vaccine-induced antibodies. How clonotypic diversity of the IgG pool changes as a function of age especially in the elderly and during early development and the relationship between overall antibody diversity and susceptibility to infection will need to be determined. Also it will be interesting to determine whether vaccination with a new vaccine impacts the established serological memory antibody repertoire to an earlier vaccine.
- Concentration distribution of circulating antibodies: In biological fluids individual antibodies are present at widely different concentrations. For example we have found that out of the 80–120 antibody clonotypes that typically constitute the serum titer to the tetanus toxoid vaccine a single clonotype can account for as much as 15–30% of antigen-specific response [22••]. We have observed a similar polarization of the serological repertoire, manifest by the presence of one or a few highly abundant antibodies with a number of vaccine antigens. Obviously, such antibodies must be the product of large plasma cell expansions. The mechanisms that dictate the extent of expansion of certain B cell clones following antigen stimulation are not clear. Since highly abundant antibodies display comparable binding affinity as much less prevalent clones, differences in the degree of plasma cell expansions observed in peripheral blood at day 7 (the typical peak of plasmablast response in serum) clearly cannot be solely due to antigen affinity. Of note, extremely highly abundant antibody clones (clinically defined as globulin level >30 mg/ml) are observed in about 3% of asymptomatic adults over 50 yr old and in multiple myeloma patients[60]. There is a documented reciprocal change with age in the antibody titer to extrinsic and intrinsic antigens [61], but whether this is related to the presence of age-associated highly expanded serum antibodies remains to be determined.

- The relationship and contribution of the different isotype repertoires to humoral immunity: In addition to IgG, human blood contains an appreciable level of IgM and IgA. IgD is present at a level 1/100 of that of IgA and IgE is found at an even lower concentration [62]. The relationships among the isotypic repertoires in serum need to be elucidated. For example to what degree is the antigen-specific IgG repertoire clonally related to the respective IgA repertoire? Additionally, there might be isotypic "layers" to human B cell memory, as recently described in the mouse, wherein particular memory B-cell subsets are predisposed to differentiate into plasma cells while others re-enter germinal center reactions [63]. Are features such as degree of somatic hypermutation, V gene usage, CDR-H3 length etc comparable among the class switched repertoires? While some of these issues are beginning to be explored for peripheral B cells through the use of NextGen sequencing approaches [64,65] there is presently no information for the repertoire at the protein level. A related immunological puzzle which could be clarified using Ig-seq proteomics is the extent to which peripheral blood $IgM^+IgD^+CD27^+$ circulating B cells are germinal center-derived memory B cells or are instead related to marginal zone B cells found in human spleen, and whether, analogous to the mouse, they constitute a distinct B-cell lineage harboring a pre-diversified repertoire that is responsible for predominating anti-polysaccharide responses against encapsulated bacteria such as *S. pneumonia*. Finally an equally intriguing question is the degree to which the antigen-specific repertoires in blood and in secretions are clonally related and whether they are directed to the same epitopes.
- Significance of antibody post-translational modifications: Post-translational modifications impact all aspects of antibody function including antigen-binding, stability to aggregation and degradation, interaction with Fc binding proteins and effector functions and half-life[66•]. Antibody post-translational modifications have been studied in the context of quality control of therapeutic antibodies, a key issue for the biopharmaceutical industry. How post-translational modifications influence the selection and function of circulating antibodies is not known. Certainly, oxidation must be quite prevalent in circulating antibodies since they originate from oxidatively stressed plasma cells and then are exposed to the high redox potential of the serum for many days. It is quite plausible that surface Ig on B cells is also subject to oxidation and perhaps oxidized residues may contribute to antigen affinity and B cell selection during the germinal center reaction. Fc glycosylation is well established to impact the effect of antibodies on the innate immune system via the modulation of Fc receptor interactions[67]. While glycosylation heterogeneity in monoclonal antibodies has been studied extensively [66•] recent advances now enable deep analyses of human glycosylation in complex mixtures. For example, Pučić et al. used hydrophilic interaction chromatography and mass spectrometry to investigate correlation between gender, age and heredity on the relative abundance of serum IgG N-glycan structures for a cohort of 2298 individuals[68]. Though this work focused solely on the characterization of the pooled N-glycans from each sample, MS based methods

may enable the future study of serum Ig glycosylation at the individual antibody level[69].

- The polyspecific antibody repertoire: As mentioned above, the immunological significance of antibody polyreactivity or polyspecificity is being increasingly appreciated [24,70]. However, polyspecificity as a feature of the serum antibody response has not been investigated in a systematic way. Part of the problem is inherent to the diversity of the antibody repertoire of normal serum. Perhaps this is an area where lessons and tools from the biotherapeutics industry which has developed a number of widely used metrics and probes to evaluate polyspecificity in the context of therapeutic antibody development can be applied to molecular immunology[71]., Cell-based studies have shown that up to 50% of B cells found in cord blood of human newborns, and approximately 20% of peripheral blood B cells circulating in human adults encode polyreactive antibodies[72,73]. Furthermore, although significantly reduced by putative checkpoint mechanisms, the terminal repertoire of bone marrow plasma cells still encompasses ≥10% polyreactive antibodies [74] which suggests that a similar observation awaits discovery at the level of the serum repertoire (Fig. 1).

Conclusions

We are on the verge of a new and exciting research era in serology. For the first time, newly developed technologies provide us with the means to interrogate the adaptive immune response at unprecedented resolution to determine both the spectrum of antigens towards which the serological response is directed and the antibodies that comprise the respective polyclonal antibody pools towards these antigens. Together with new tools for the analysis of B cell subsets and BCR repertoires we can now begin to envision the possibility of a fairly complete description of the nature of antibody responses in health and disease. Needless to say numerous challenges remain: First off, the molecular level dissection of humoral immunity is technically demanding and requires a combination of tools that are currently available only in a very small set of laboratories. It will be interesting to see how and whether these techniques may be “industrialized” to become available for clinical use and for use by the wider research community. Second, these studies result in very large and complex data files and a major effort will be required for data storage, annotation, storage and meta-analysis. Third, numerous experimental challenges still need to be addressed: how to develop appropriate probes for the quantification and for characterizing the composition of the polyspecific antibody repertoire, how to best characterize the antigenic determinants in complex pathogens (e.g. *S. aureus* and other bacterial pathogens, parasites etc) how to efficiently identify the neutralizing antibodies present in serum (as contrasted to relying on B cell cloning which as was argued above may not necessarily yield the biologically relevant antibodies in a patient) and many others. Finally and perhaps most importantly the deluge of data that can be generated by the tools described above and by other systems immunology approaches should by no means overshadow the need for first formulating testable hypotheses and well posed questions that in turn can provide definitive insights into the working of the immune system. Critical thinking, appreciation of the biological context

and, for disease biology a clear understanding of the clinical need must be the driving force behind any large scale science including in this case modern serology.

Acknowledgments

Work on immune profiling in our lab was supported by grants HDTRA1-12-C-0105 from DTRA, RO1CA106006 and 5U19AI057234-09 from NIH and by the Clayton Foundation.

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polyclonal serum could be viewed as the combined effect of the neutralization fingerprints of the constituent monoclonal antibodies. They demonstrated the neutralization fingerprints for 30 neutralizing antibodies on a panel of 34 diverse HIV-1 strains and showed that similarity in neutralization fingerprint correlated with similarity in epitope. Furthermore, they used these fingerprints to delineate specificities of polyclonal sera from 24 HIV-1-infected donors. Collectively, they suggested that epitope delineation based on neutralization fingerprints may provide a transformative strategy for screening sera or characterizing antibody specificities induced upon infection or vaccination against HIV-1 as well as other viruses. [PubMed: 23661761]

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Highlights

- New technologies have revolutionized serological antibody analysis
- NextGen sequencing and shotgun proteomics help identify and quantify serum mAb's
- Clonality and dynamics of serum mAb repertoire in various states can be addressed
- New tools available for identifying novel antigens that elicit serological response

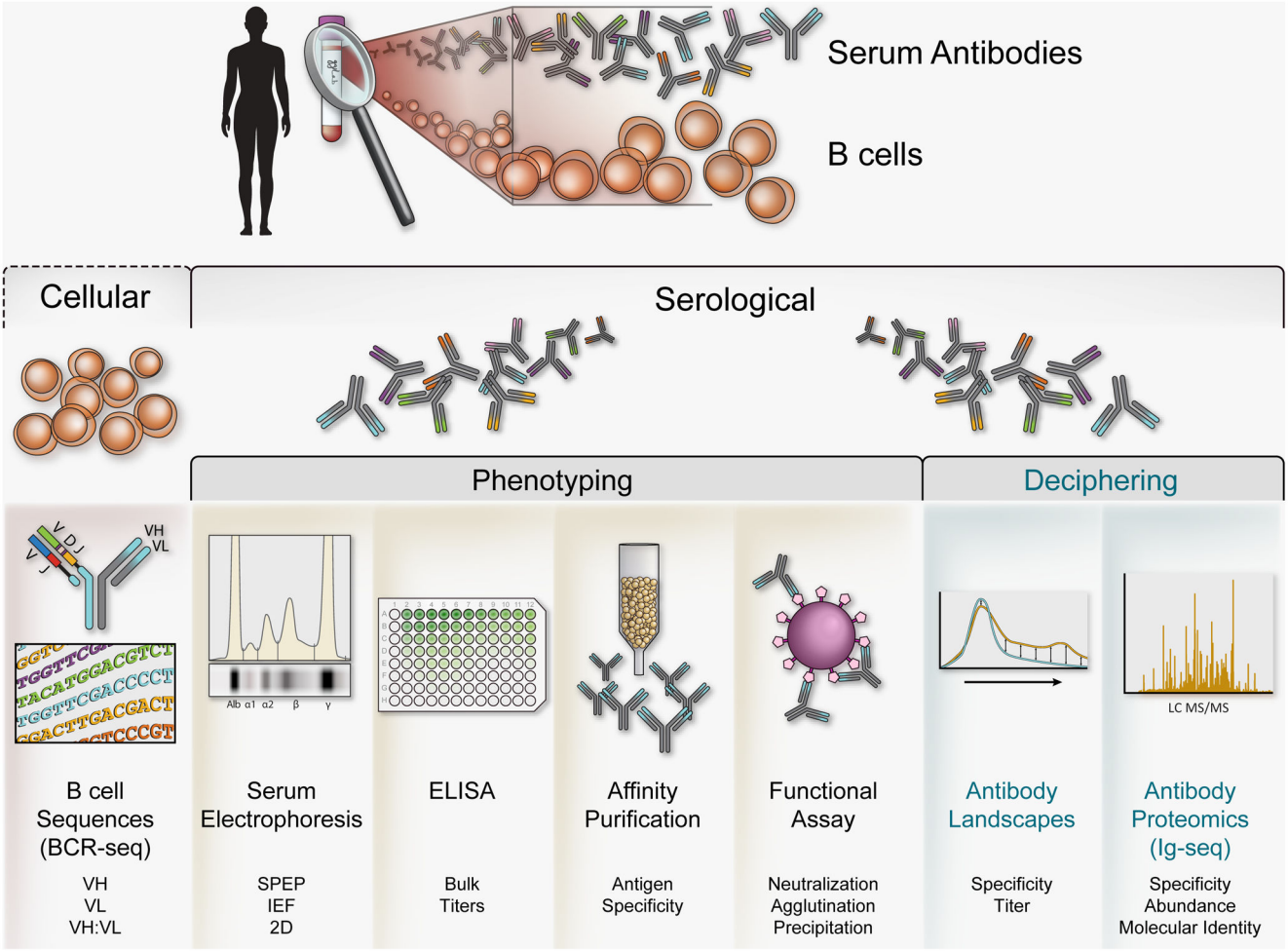

Figure 1. Approaches for the analysis of human antibody repertoires

Isolated B cells are sorted into several subsets based on expressed cell markers that correspond to the developmental stage of the B cell. These populations can be further processed for high-throughput sequencing to generate the antibody repertoire encoded by B cells (cellular repertoire, left side of the figure). The corresponding serum immunoglobulins are isolated from the samples and can be analyzed by various methods including well established technologies such as 2D gels or by recently established methodologies such as high resolution shotgun proteomics (serological repertoire, left side of the figure). The methodologies for serological immunoglobulin analysis can be broadly based upon the phenotype of an antibody subpopulation (e.g., ELISA titer of antigen-specific fraction) or upon decipherment of the molecular identity and sequence determination of an antibody subpopulation (e.g., LC-MS/MS immunoglobulin sequencing, Ig-seq).