

Sequencing the functional antibody repertoire —diagnostic and therapeutic discovery

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Abstract | The development of high-throughput DNA sequencing technologies has enabled large-scale characterization of functional antibody repertoires, a new method of understanding protective and pathogenic immune responses. Important parameters to consider when sequencing antibody repertoires include the methodology, the B-cell population and clinical characteristics of the individuals analysed, and the bioinformatic analysis. Although focused sequencing of immunoglobulin heavy chains or complement determining regions can be utilized to monitor particular immune responses and B-cell malignancies, high-fidelity analysis of the full-length paired heavy and light chains expressed by individual B cells is critical for characterizing functional antibody repertoires. Bioinformatic identification of clonal antibody families and recombinant expression of representative members produces recombinant antibodies that can be used to identify the antigen targets of functional immune responses and to investigate the mechanisms of their protective or pathogenic functions. Integrated analysis of coexpressed functional genes provides the potential to further pinpoint the most important antibodies and clonal families generated during an immune response. Sequencing antibody repertoires is transforming our understanding of immune responses to autoimmunity, vaccination, infection and cancer. We anticipate that antibody repertoire sequencing will provide next-generation biomarkers, diagnostic tools and therapeutic antibodies for a spectrum of diseases, including rheumatic diseases.

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Introduction

Antibodies are a major component of the adaptive immune system and have critical roles in protective and pathogenic immune responses. In response to microbial infection, vaccination, autoimmune disease or cancer, the immune system generates distinct antibody repertoires. Analysis of these antibody repertoires, particularly those contributing to functional immune responses, can provide important information on protective and pathogenic immunity. In autoimmune diseases, including autoimmune rheumatic diseases, antibody characterization has enabled the identification of autoantigens and has provided insights into the underlying mechanisms of disease; furthermore, detection of autoantibodies has become a cornerstone of modern diagnostics.^{1–3} In infectious diseases, in which antibody responses are usually protective, there is growing interest in isolating antibodies that could be developed as novel therapeutic agents^{4,5} and in using microbial antigens and epitopes targeted by antibody responses to develop vaccines.^{4,6,7} A challenge in understanding, as well as in diagnostically and therapeutically harnessing, antibody responses is the identification of antibodies that underlie functional immune responses, that is, antibodies that directly contribute to an immune outcome, such as neutralizing a

microbial pathogen or mediating autoimmune tissue injury. This Review provides an overview of technologies for large-scale sequencing of antibody repertoires, and discusses how these technologies can be applied to characterize immune responses and identify antibodies of therapeutic, diagnostic or mechanistic relevance to autoimmune diseases, including rheumatic diseases.

Antibody responses

Antibodies are comprised of an immunoglobulin heavy chain (IgH) and light chain (IgL), each containing an antigen-binding domain that is generated by the recombination, junctional diversification and somatic hypermutation of variable (V), joining (J) and/or diversity (D) gene segments during B-cell development.^{8,9} The complementarity-determining regions (CDRs), CDR1, CDR2 and CDR3, as well as the surrounding framework regions, together form the antigen-binding site of the antibody.¹⁰ In the primary B-cell repertoire, substantial diversity in antibody specificity comes from the IgH CDR3 owing to its generation both from combinatorial gene segments and from N-region diversity.¹⁰ In an ongoing immune response to an antigen, B cells that produce antibodies specific for that antigen further diversify by undergoing clonal expansion and by somatic hypermutation of their antibody variable regions. In this process, termed affinity maturation, B cells that express antibodies with an increased affinity for the

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Competing interests

The author declares that he is a member of the Board of Directors, consultant for, and owner of equity in Atreca, Inc.

Key points

- The development of high-throughput DNA sequencing has enabled large-scale analysis of antibody repertoires and responses
- Critical parameters for characterization of functional antibody repertoires include the methodology, the B-cell population characterized, clinical characteristics of the individuals analysed and the bioinformatic analysis
- High-fidelity analysis of full-length paired heavy and light chains expressed by individual B cells is critical for the characterization of functional antibody repertoires
- Analysis of coexpressed functional genes can identify antibodies that contribute to functional immune responses
- Sequencing antibody repertoires is transforming our understanding of pathogenic and protective immune responses in autoimmunity, infection, vaccination and cancer
- Antibody repertoire sequencing should lead to the generation of biomarkers, diagnostic tools and therapeutic antibodies for various diseases, including rheumatic diseases

activating antigen as a result of somatic hypermutation are selected for further expansion (Figure 1).^{11–13} These antigen-activated, affinity-matured B cells are then released from germinal centers into the blood as short-lived or long-lived plasmablasts, which migrate to other secondary lymphoid organs and sites of tissue injury, and can differentiate into long-lived memory B cells and plasma cells.^{14,15}

Identifying antigens

For most diseases, knowledge of the antigens and epitopes targeted by antibody responses is limited. For example, autoimmune diseases affect 3% of the global population, yet in most of these diseases the pathogenic autoantigens have not been identified.¹⁶ In infectious diseases, most of the critical microbial antigens and epitopes targeted by protective antibody responses are also unidentified,¹⁷ with the exception of HIV antigens, influenza and some other microbes.¹⁸ With regards to cancer, T-cell-mediated responses are considered the primary form of immunity. Although immunotherapies are now revolutionizing cancer therapy,^{19,20} the specific targets of the immune response to these therapies are unclear, as is the contribution of antibody responses to effective anticancer immunity.

A variety of ‘array and display’ approaches have been developed for investigating antibody responses, including protein and peptide arrays,²¹ aptamer and peptoid arrays,²² random peptide arrays,²³ large-scale peptide arrays,²⁴ human protein arrays,²⁵ and human proteomic display arrays.²⁶ One major challenge of these approaches is the use of serum or plasma samples as the source of antibodies analysed. Serum (and plasma) samples contain a pool of all the antibodies produced by an individual, including antibodies that are heterogeneous, bind to many different antigens, and have differing affinities and avidities, thereby increasing background reactivity and diluting potential antibodies of interest. Furthermore, most serum antibodies are produced by plasma cells generated in prior immune responses, and are not produced by the plasmablasts or plasma cells responding to the immunogenic antigen of interest.

Another major challenge is that most approaches involve screening the binding of blood antibodies against panels of preselected antigens. For example, important autoantigens in rheumatoid arthritis (RA) are post-translationally citrullinated,^{27,28} and in systemic lupus erythematosus (SLE) are post-translationally cleaved or phosphorylated;²⁹ therefore, preselecting antigens might result in the failure to detect pathogenic autoantibody clonal families. Antigen-agnostic approaches (those that are antigen-independent) are therefore needed for identifying important functional antibodies and their targets. An ideal solution is to isolate, recombinantly express, and characterize antibodies produced by the specific subset of B cells participating in functional immune responses to vaccination, infection, cancer or other diseases, in humans or other animals. In this Review, I describe several DNA sequencing approaches that are currently used to achieve this end.

B-cell populations

Selection of B-cell populations is an important consideration for antibody sequencing. The best B cells for characterizing the functional antibody repertoire include plasmablasts, plasma cells, memory B cells and tissue-infiltrating B cells (Figure 1). Of these cells, plasmablasts and memory B cells are the two populations that are present in peripheral blood and are therefore most easily isolated from humans.

Plasmablasts and plasma cells

Plasmablasts and plasma cells are the antibody-producing cells of the immune system. During an immune response, plasmablasts differentiate from antigen-activated B cells and are released from germinal centers to transiently circulate in the bloodstream before migrating to secondary lymphoid organs or to inflamed tissues, where they can differentiate into longer-lived tissue-resident plasma cells.^{30–32} Plasmablasts in peripheral blood form not only from newly generated naive B cells, but also from reactivated memory B cells. Together with the relative ease of isolating plasmablasts, their origin from either naive or memory B cells make them an ideal B-cell population to analyse when searching for antibodies associated with any given immune response. For example, studies sequencing the antibody response to influenza vaccination have detected a transient increase in peripheral blood plasmablast numbers, >75% of which produced anti-influenza antibodies.^{33,34}

Although autoimmune diseases are chronic, evidence exists that underlying autoantibody responses involve continuous activation and reactivation of new B-cell clones,³⁵ such that peripheral blood plasmablasts could produce new pathogenic antibodies at any stage of the disease. Indeed, in patients with RA, the proportion of plasmablasts in the blood correlates positively with disease activity,^{27,36} and these cells produce anti-citrullinated protein antibodies (ACPAs)^{27,28} that are known to contribute to the pathology of RA.^{37,38} Tissue-resident plasma cells and plasmablasts might also produce ACPAs and might even be generated *in situ* in

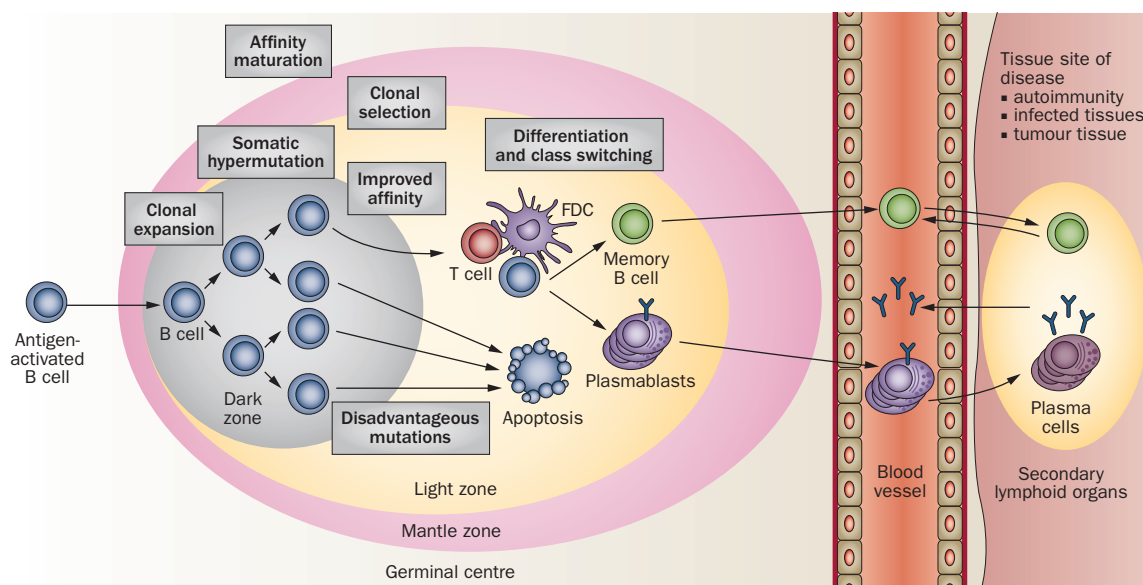


Figure 1 | The B-cell response. B cells undergo clonal expansion and affinity maturation after encountering antigen and T-cell help or co-stimulatory signals, a process that generally occurs in germinal centres within secondary lymphoid organs and results in the generation of clonal families of B cells.^{12,13} Affinity maturation involves two processes, somatic hypermutation and clonal selection. Somatic hypermutation is a cytidine-deaminase-mediated process in which antibody CDRs are mutated ~1–2 times per cell division. Clonal selection involves competition of B cells for antigen and growth factors in germinal centres, resulting in B cells that express the highest-affinity antibodies being selected to expand and survive.^{12,13} After multiple rounds of somatic hypermutation and clonal selection, antibody-expressing B cells produce antibodies with increased affinity for their target antigen.¹⁰ B cells that express high-affinity antibodies respond further to growth factors and other signals that induce differentiation into plasmablasts and memory B cells.^{11,14,15} Plasmablasts transiently circulate in the blood and migrate to secondary lymphoid organs and tissues involved in the disease process, including tissues under autoimmune, infectious or malignant attack. Memory B cells circulate in the blood, and enable rapid recall responses upon re-exposure to their target antigen, whereas plasma cells localize primarily in the bone marrow and lamina propria, where they secrete antibodies. Abbreviations: CDRs, complementarity determining regions; FDC, follicular dendritic cell.

the synovial tissue of patients with RA; however, these tissue-resident cells are not easily accessible.^{39–41}

Memory B cells

Memory B cells form during germinal centre reactions and provide an accelerated antibody response upon re-exposure to a target antigen.⁴² Whereas >75% of peripheral blood plasmablasts are specific for the antigens targeted by the ongoing immune response,^{33,34,43} antigen-specific memory B cells are present at much lower frequencies; approximately 1 of 2,500–100,000 memory B cells in blood are specific for the inciting antigen.^{42,44,45} These memory B cells can be isolated by antigen-sorting (by fluorescence-activated cell sorting [FACS], with fluorescently-labelled antigen baits to sort the cells by the specificity of their membrane immunoglobulins) of memory B cells from peripheral blood; however, because of the low frequency of antigen-specific memory B cells, such sorting requires large numbers of peripheral blood mononuclear cells. The substantial differences in the antigen load, prior exposure history, route of exposure, and associated immunostimulatory molecules in different vaccines, microbial infections, and autoimmune diseases influence the magnitude and properties of antibody responses; modifications of the specific B-cell populations analysed and the timing of their isolation might be needed to effectively characterize antibody responses in these conditions.

Tissue-infiltrating B cells

Analysis of tissue-infiltrating B cells could also help to characterize functional antibody responses. Despite being more difficult to access than peripheral blood B cells, characterization of tissue-infiltrating B cells in both autoimmune disease^{46,47} and in cancer⁴⁸ has identified disease-associated antibodies. Integrated analysis of the B-cell antibody repertoires in the synovium and blood of patients with RA, and the brain and blood of patients with multiple sclerosis,^{46,47} is providing insights into the sites of B-cell activation and affinity maturation in these diseases.

Small-scale sequencing

An approach used in the 1990s and 2000s for the analysis of antibody repertoires involves reverse transcriptase polymerase chain reaction (RT-PCR) and Sanger sequencing of antibodies expressed by individual B cells. Because the experimental steps in this technique require manual handling, in practice this approach can only characterize tens to hundreds of B cells per experiment. Despite low throughput, the technique has been used to identify antibodies that neutralize clinically important pathogens such as HIV-1 and influenzae viruses,^{5,33,49–52} or autoantibodies that contribute to autoimmune disease.^{46,53}

A variety of approaches have been used for single-cell RNA sequencing,^{54–57} several of which enable sequencing

of coexpressed genes and use next-generation technology. However, these assay platforms and short-read sequencing technologies, for example, HiSeq™ platforms (Illumina, USA), read considerably fewer base pairs than are necessary (>500) for sequencing the entire IgL and IgH variable regions.⁵⁸

Large-scale sequencing

A variety of high-throughput next-generation sequencing technologies have now been developed^{59,60} and are transforming the analysis of B cells and their antibody repertoires.^{4,58,61–63} These technologies are applied in a variety of ways, and important parameters for characterization of functional antibody responses include the methodological approach, the B-cell populations characterized, the clinical characteristics of the individuals analysed, the approach to bioinformatic analysis and the objective of the experiment.

Monitoring specific B cells

A number of research groups have developed approaches for deep-sequencing IgH (or in some cases the IgL), or solely the CDR3, in genomic DNA or cDNA generated from bulk RNA isolated from individuals with autoimmune disease,^{47,64} infections⁶⁵ or cancer,^{66,67} or from vaccine recipients.⁶⁸ In some instances, molecular ‘barcodes’ are used to enhance single-chain or CDR3 sequencing, for example, use of random hexamers as primers^{10,69,70} or adapters with unique molecular identifiers^{54,55} to tag each cDNA. Molecular barcoding enables the correction of base-calling errors inherent to the sequencing platform or of PCR bias, although not the correction of RT-PCR errors or accurate identification of the clonal proportions of the B cell pool.

Sequencing immunoglobulin genes in genomic DNA is challenging because as many as 50% of B cells have multiple rearranged immunoglobulin gene loci, a result of nonproductive rearrangements and receptor editing.^{71,72} Furthermore, receptor editing, an important feature in B-cell maturation that helps to prevent autoimmunity, can lead to allelic inclusion at the immunoglobulin IgL loci and the development of B cells that coexpress two different immunoglobulin IgLs, thereby confounding the sequencing of IgH and IgL cDNA from individual B cells.^{71,72} Moreover, although rapid, these methods usually involve the sequencing of only one immunoglobulin chain and, therefore, provide limited information about the antibodies generated during an immune response. Although both IgHs and IgLs can be sequenced simultaneously in bulk mRNA, pairing of the IgH and IgL sequences obtained in this way can only be attempted according to similarities in sequence frequency;⁷³ this pairing is not always accurate. Without the accurate pairing of the cognate IgHs and IgLs, the information generated about an antibody response is incomplete.

Although not optimal for analysis of antibody repertoires, single-chain sequencing from bulk RNA can be used to track malignant or clonal populations of B cells by detecting their unique IgH, IgL or CDR3

sequences.^{74,75} This method is proving to be powerful for detecting and monitoring the recurrence of B-cell malignancies and has better sensitivity for detecting residual disease or disease recurrence than flow cytometry or other conventional approaches.^{66,67}

IgH CDR3 sequencing is also used in the search for biomarkers of autoimmune diseases and in characterizing B-cell responses to infection and vaccination, and to immunomodulation in patients with cancer. Results from bulk IgH sequencing of unsorted B cells suggest that direct analysis of isolated B-cell subsets might be necessary for obtaining the most informative data regarding diseases of immune function.⁷⁶ Additional data are needed to assess whether sequencing of a single chain or a single-chain CDR3 will provide biomarkers with clinical utility.⁷⁷

The functional antibody repertoire

Many challenges must be overcome to understand and identify functional antibody repertoires (Table 1). In addition to high throughput analysis, a comprehensive survey of the B-cell repertoire requires sequences that enable accurate recreation of endogenous, bioinformatically selected antibodies, so that their antigen specificity, binding and functional properties can be characterized. To accurately recreate recombinant versions of endogenous antibodies, one must sequence and correctly pair the cognate IgHs and IgLs expressed by individual B cells, ensure that the sequencing covers the entire variable regions encoding the CDR1, CDR2, CDR3 and framework regions of both the IgHs and IgLs, and ensure that the sequences are error-free.^{10,61,78,79} High-process fidelity and quality are major challenges for the robust analysis of antibody repertoires, and are further discussed in this article. Furthermore, to enable bioinformatic identification of important functional antibodies, antibody repertoire sequencing must be applied to the appropriate B-cell subsets derived from individuals with an immunological phenotype of interest (Table 1).

Several new single-cell sequencing methods can preserve the endogenous pairing of IgHs and IgLs (Figure 2). These methods include linkage-PCR based, barcode-based, bead-based, microwell-based and droplet-based methods, with most approaches integrating more than one of these methods.

Linkage-PCR

A method for physically linking and thereby sequencing IgH and IgL pairs from individual B cells is overlap-extension, also known as linkage PCR.^{80,81} Several groups have adapted this technique for high-throughput sequencing of IgH and IgL pairs.^{82,83} One such method involves depositing single B cells in high-density microwell plates, capturing their mRNA with oligo-dT beads, and then using emulsion-based linkage PCR to physically link and amplify IgH and IgL pairs before sequencing their CDR3 regions (Figure 2a).⁸² Integrating long-read sequencing technologies, cell barcodes and universal 5' priming into linkage-PCR-based approaches creates the potential to yield robust antibody repertoire datasets.

Table 1 | Challenges and solutions to sequencing the functional antibody repertoire

Challenge	Details of challenge	Solution
Analyse the functional repertoire	Identify clinical phenotype	Focus analysis on individuals with immune phenotypes of interest
	Analyse B cells participating in an immune response of interest	Focus analysis on functional B cell populations, such as plasmablasts, antigen-specific memory B cells, plasma cells or tissue-infiltrating B cells
	Perform integrated analysis of coexpressed functional genes	Cell barcode to enable analysis of coexpressed functional genes in B cell subsets
Overcome the scale necessary for comprehensive repertoire analysis	Analyse thousands of B cells per experiment	Use high-throughput approaches
Demonstrate function	Accurately pair IgH + IgL expressed by individual B cells	Use cell barcoding and linkage PCR methods
	Sequence full-length variable regions	Sequence entire variable regions
	Have error-free sequences (correction of both RT-PCR and sequencing errors)	Error correct using cell barcodes to enable correction for RT-PCR and sequencing errors (molecule barcodes enable correction of sequencing, but not RT-PCR errors)
Overcome poor fidelity and quality	V-gene primers fail to sequence some antibodies	Use template switching or other non-V-gene primer approach to add 5' barcode
	PCR bias distorts clonal proportions	Use cell barcodes
	RT-PCR and sequencing errors yield artefact clonal families	Use cell barcodes to enable correction of both types of error
	PCR contamination produces artefacts	Use cell or molecule barcodes
Abbreviations: IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction.		

Cell barcoding

In my laboratory, we developed a cell-barcoding method for antibody repertoire sequencing.^{27,34,43,84} In this approach, all cDNA generated from each individual B cell is labelled with a unique cell barcode by using a universal 5' adapter (Figure 2b, Figure 3a), which is added via the template-switching activity of reverse transcriptase, and enables full-length and unbiased sequencing of the antibody variable regions. Because each B cell has multiple copies of mRNA encoding its IgH and IgL genes, cell barcoding creates consensus IgH and IgL sequences for each cell and thereby enables correction not only of errors inherent to the sequencing platform, but also of those arising from RT-PCR (Figure 3b). Furthermore, cell-barcoding enables accurate calculation of B-cell clonal proportions (Figure 3c), providing error-corrected sequences of the full-length V regions of natively paired IgHs and IgLs and enabling direct gene synthesis of the variable regions of the antibodies for recombinant expression. My laboratory has used this approach to understand human plasmablast antibody responses to RA,²⁷ *Staphylococcus aureus* infection,⁴³ and influenza vaccination.³⁴ Bioinformatic analysis of sequence datasets can be used to generate phylogenetic trees to guide selection and recombinant expression of plasmablast antibodies, including ACPAs implicated in the pathogenesis of RA,²⁷ or antibodies that mediate opsonophagocytosis of *Staphylococcus aureus*⁴³ or that neutralize influenza.³⁴ With this method, rapid and comprehensive characterization of antibody responses is possible, as is the generation of affinity-matured, monoclonal human antibodies with high efficiency relative to

hybridoma and other classical approaches for generating monoclonal antibodies. Wardemann and colleagues developed a 2D barcode primer matrix PCR that utilizes distinct sets of barcoded V-gene forward primers and barcoded constant-region reverse PCR primers for multiplex cell-based barcoding of single-cell-sorted B cells (Figure 2c).^{61,85}

Droplet PCR

Several groups are working to develop droplet-based approaches that utilize beads, linkage-PCR or barcodes to enable the sequencing of IgH and IgL pairs expressed by individual B cells (Figure 2d).^{61,83} These techniques offer the potential to increase throughput analysis and reduce costs. Furthermore, droplet PCR, also termed digital PCR, isolates and amplifies individual DNA molecules in emulsion droplets and thereby reduces PCR bias.⁸⁶

Bioinformatic analysis of clonal families

Sampling depth

An important requirement of next-generation single-cell sequencing technologies is that they generate datasets with IgH and IgL pairs from sufficient numbers of B cells to enable bioinformatic generation of phylogenetic trees and the identification of clonal antibody families (Figure 4). The sampling depth necessary to characterize a functional antibody response depends on the population of B cells analysed. For example, antigen-specific memory B cells are present in blood at frequencies of 1:2,500–100,000 cells;⁴² therefore, a relatively large number of cells must be obtained from the blood (or in some cases by leukapheresis) to isolate memory

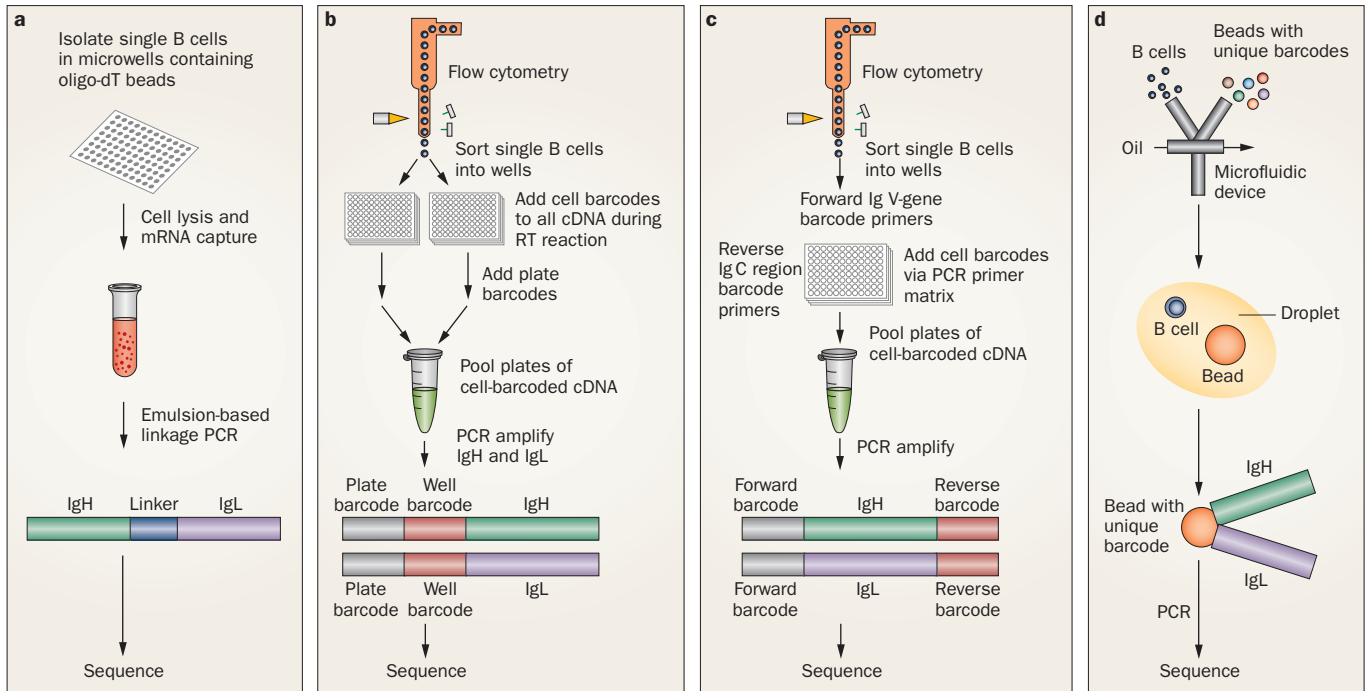


Figure 2 | Approaches for high-throughput sequencing of functional antibody repertoires. Comprehensive characterization of the functional antibody repertoire necessitates high-throughput, full-length and error-free sequencing of IgH and IgL pairs expressed by individual B cells. **a** | Cell barcoding via linkage PCR. Single B cells are isolated and lysed, then their RNA is captured by poly-T beads. cDNAs of the IgH and IgL expressed by individual B cells are then linked by emulsion to RT-PCR, and then pooled and sequenced.⁸² **b** | Cell barcoding via template switching. Single B cells are sorted, then the template switching activity of RT adds a unique cell-specific barcode to all cDNAs generated from an individual B cell. Plate-specific barcodes are then added, resulting in cDNAs that have compound cell barcodes. Finally, the compound cell-barcoded IgH and IgL genes are amplified by PCR, pooled and sequenced.^{27,34,43} **c** | Cell barcoding by forward and reverse primer matrix. Single B cells are sorted, then V-gene forward primers and C-region reverse primers are used to add cell-specific barcodes to, and amplify by PCR, IgH and IgL cDNA generated from an individual B cell. Single-barcoded immunoglobulin genes are then pooled and sequenced.⁸⁵ **d** | Microfluidic combination of beads with unique barcodes and single B cells into individual droplets. Using microfluidics, single B cells and beads with unique barcodes are combined in individual droplets, followed by lysis of the B cell, PCR and sequencing.^{61,83} Abbreviations: C, constant; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; PCR, polymerase chain reaction; RT, reverse transcriptase; V, variable.

B cells with a specific antigen specificity, or to identify clonal families of memory B cells. By contrast, plasmablasts produced during an active antibody response have extensive redundancy in clonal families, and a number of groups have reported that >75% of peripheral blood plasmablasts express antibodies specific to the antigenic target of an ongoing immune response.^{33,34,43,87} As a result, when sequencing the plasmablast antibody repertoire of an individual with an ongoing functional immune response, analysis of hundreds to thousands of plasmablasts (obtained from as little as 5–15 ml blood, or just 1–3 ml from infants) is sufficient for robustly identifying and characterizing the clonal families.^{27,34,43}

Bioinformatic methods

A number of bioinformatics methods have been developed for V(D)J gene assignment and antibody repertoire analysis,^{88,89} and clonal families can be defined as B cells that share IgH VJ and IgL VJ usage. In stricter definitions of clonal families, the IgH VJs and IgL VJs must have identical CDR3 lengths or the amino acid sequences must have a certain degree of homology, suggesting that

the B cells that express these antibodies arose from the same B cell progenitor.⁹⁰ Clonal families can be generated by expansion of a common progenitor B cell, by independent *de novo* recombination events, or by convergent evolution of B-cell responses, that is, accumulation of mutations in B cells that express IgH V(D)J and IgL VJ of different genomic origins, resulting in convergence of encoded antibody sequences.^{91,92} Regardless of their particular ontology, B cells that express high-affinity antibodies are able to ‘out-compete’ other B cells in the germinal centre for antigen and growth factors during affinity maturation, thereby generating clonal families.^{14,15} Cross-patient analysis of antibody repertoires of individuals with a common immune phenotype has proven to be a powerful method of identifying shared IgH V(D)J and IgL VJ that encode antibodies with functional activity at an increased frequency, as compared with identification of clonal families alone.^{51,65,93}

Analysis of coexpressed B-cell functional genes

An important direction for antibody repertoire sequencing is the integration of analysis of coexpressed genes, in

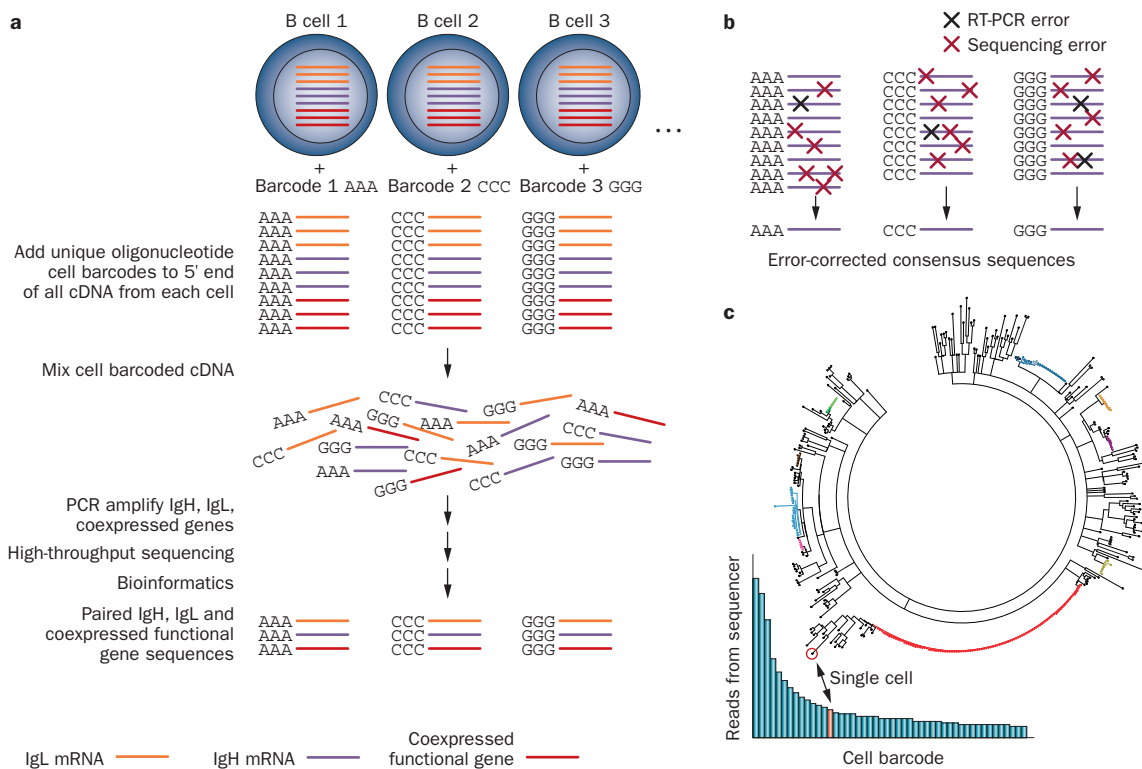


Figure 3 | Cell barcodes enable robust antibody repertoire sequencing. Cell barcodes (represented by AAA, CCC and GGG) added through the template switching activity of RT^{27,34,43} have many advantages, including preservation of the IgH and IgL pairs expressed by individual B cells, and enabling accurate determination of the error-corrected sequence and of clonal proportions. **a** | In the approach presented, all cDNA generated from each cell receives the same unique barcode; therefore, coexpressed functional genes indicative of functional subsets of B cells (or T cells) can also be characterized.^{27,34,43} Both cell and molecular barcodes enable quantitative analysis of mRNA in individual cells. **b** | Multiple copies of each mRNA are expressed by each cell, and for each cell all mRNA receives the same cell barcode; therefore, cell barcoding enables correction of both PCR and high-throughput sequencing errors. **c** | Cell barcodes enable accurate determination of clonal proportions. Cell barcodes enable binning of the IgH and IgL chain reads from each B cell, thereby providing accurate determination of clonal proportions. By contrast, molecular barcodes do not enable RT-PCR error correction, IgH and IgL pairing, analysis of coexpressed genes or accurate determination of clonal proportions. Abbreviations: IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; RT-PCR, reverse transcriptase polymerase chain reaction.

an approach analogous to the simultaneous sequencing of T-cell receptors and functional genes that demarcate T-cell subsets.^{94,95} Investigators in my laboratory are now simultaneously sequencing the IgH and IgL genes along with a set of functional B-cell subset genes (Figure 3), a strategy that will integrate information about functional B-cell subsets into our antibody repertoire data and thereby enhance our ability to identify functional antibodies. For B cells, coexpressed functional genes include transcription factors and effector molecules indicative of differentiation into plasma cells, memory B cells, long-lived plasmablasts or other matured B-cell subsets. For quantitative analysis of coexpressed functional genes that are indicative of distinct functional B-cell (or T-cell⁹⁴) subsets, both cellular and molecular barcoding are needed^{54,55} to quantitatively measure the expression of the coding mRNAs in each cell.

Need for high-fidelity high-quality datasets

Obtaining large-scale, high-fidelity and high-quality sequencing data is a critical challenge in sequencing antibody repertoires, and is essential for biomarker analysis

and for the generation of diagnostic and therapeutic antibodies. To achieve these ends, accurate determination of B-cell clonal proportions is needed, as is error correction for provision of high-fidelity sequences, comprehensive analysis of all variable regions expressed in the chosen B-cell subset for capturing the entire repertoire, and the ability to detect PCR contamination (Table 1).

Analysis of clonal proportions

Accurately measuring the size of clonal families is important for estimating the degree to which the immune system has selected and expanded a B cell expressing a particular antibody. Selection by the immune system is indicative of a high affinity of B-cell membrane immunoglobulins for cognate antigens, and thereby out-competing other B cells for antigen and growth factors in the germinal centre (Figure 1). In studying the antibody response to influenza vaccination with a cell-barcoding approach (Figure 2b), investigators in my laboratory found that large clonal families encode antibodies that bind to influenza haemagglutinin with higher affinities, and in certain cases are more effective at neutralizing

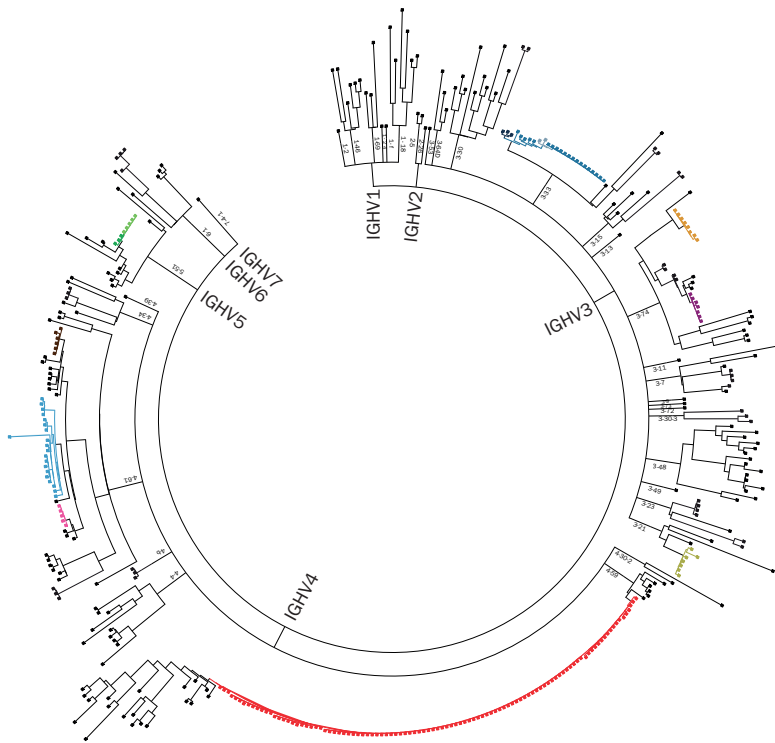


Figure 4 | Phylogenetic tree of the antibody repertoire. Example of a phylogenetic tree of a human plasmablast response generated using the cell barcoding method developed in my laboratory.^{27,34,43} Phylogenetic trees reveal clonal families and guide the expression and screening of the identified antibodies. Each major branch represents a single IgH V(D)J, outer branching is determined by the IgL VJ sequence, and each terminal leaf represents the error-corrected, full-length, paired IgH and IgL sequences expressed by an individual B cell. Clonal families (colour coded) contain antibodies that share IgH VJ and IgL VJ sequences. Immunodominant (red), intermediate and rare (other colours) families are identified. Single nucleotide substitutions can substantially alter antibody binding specificity and affinity^{11,7}, and in order to evaluate the spectrum of antibodies encoded within each clonal family recombinant antibodies representing the range of variants represented within each family should be expressed and characterized. Abbreviations: D, diversity; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; J, joining; V, variable.

influenza virus than antibodies that are not part of large clonal families.³⁴ PCR bias, in which certain templates amplify more efficiently than other templates, can distort clonal proportions.⁹⁶ In addition, activated B cells, such as plasmablasts, express as much as 100-fold more RNA than memory B cells and other resting B cells,⁹⁷ which, in the absence of cell-based barcoding²⁷ or amplification of each cell's cDNA within an individual emulsion droplet,⁹⁸ might suggest artificial differences in clonal proportions. Analysis of antibodies expressed by individual cells, with cell barcodes^{27,34,43,54,55} (Figure 3c) and emulsion droplets,⁹⁸ can correct or minimize PCR bias to enable accurate determination of clonal proportions.

Biological variation versus error

Robust analysis of datasets is highly dependent on obtaining high-fidelity, error-free sequences. Sequence errors arise from both RT-PCR and the sequencing platform.⁷⁸ Several groups have demonstrated, by sequencing DNA plasmids that encode single antibodies (or single T-cell

receptors), that the error from next-generation sequencing alone results in artefactual identification of clonal families.^{10,79,99} A variety of approaches have been developed for error correction, two of which use molecule^{10,54,55,70,100} or cell barcodes.^{27,34,43,54,55} Cell barcodes provide multiple advantages over molecular barcodes, including error correction of both RT-PCR and base-calling sequencing errors as well as accurate assessment of clonal proportions (Table 1, Figure 3).

Analysis of antibody variable regions

Most approaches for sequencing antibody repertoires use 5' V-gene primers, which fail to amplify variable regions containing V genes encoded by germline sequences with insufficient homology or that have acquired somatic hypermutations in the residues to which such primers hybridize. Indeed, in my laboratory's antibody repertoire analysis of plasmablasts from influenza vaccine recipients,³⁴ we found that ~10% of all antibodies analysed had an IgH or IgL variable region that would not have been amplified by state-of-the-art V-gene-specific primers. Capturing such highly mutated antibodies is important because they might have acquired variable region mutations that confer binding and functional activity.

Ability to detect PCR contamination

Most next-generation sequencing approaches for antibody repertoire analysis utilize PCR to amplify DNA isolated, or cDNA generated, from B cells. PCR exponentially amplifies sequences, and is highly prone to contamination by amplicons from prior experiments.¹⁰¹ As the sequences isolated by antibody repertoire analysis have a high degree of nucleotide sequence homology, detecting PCR contamination can be difficult.²⁷ Both cell and molecule barcodes provide a means to bioinformatically monitor and detect PCR contamination.

Applications

Generation of recombinant antibodies

Monoclonal antibodies are a mainstay of diagnostic tests, therapeutics and research tools. High-throughput sequencing of antibody repertoires is a powerful new approach to generate recombinant monoclonal antibodies directly from humans or other animals during a functional immune response. Therapeutic monoclonal antibodies have revolutionized care for patients with autoimmune diseases (anti-TNF, anti-IL-6, anti-IL-1 and anti-IL-12p40 monoclonal antibodies), infection (anti-respiratory syncytial virus monoclonal antibody) and cancer (anti-CD20, anti-CTLA4, and anti-programmed-death 1 [PD-1] monoclonal antibodies). Investigators in my laboratory used barcode-enabled antibody repertoire sequencing (Figure 2b) of plasmablasts isolated from *Staphylococcus aureus*-infected humans to generate recombinant antibodies that bind and mediate killing of the bacteria.⁴³ Others have isolated individual B cells in microwells, then performed linkage RT-PCR and sequencing (Figure 2a) to generate tetanus toxoid (TT) reactive recombinant antibodies from TT-sorted peripheral blood plasmablasts isolated from a TT-vaccinated human.⁸²

Monitoring immune responses

More-effective approaches are needed to monitor immune responses in health, autoimmunity, microbial infection, biothreat detection, vaccination and cancer. Monitoring immune responses provides opportunities for early diagnosis, predicting flare, monitoring remission and assessing responses to immunomodulatory therapies and vaccines.

Autoimmunity

Investigators in my laboratory used barcode-enabled antibody repertoire sequencing (Figure 2b) of peripheral blood plasmablasts derived from patients with RA to identify sequences encoding autoantibodies that target citrullinated fibrinogen and citrullinated enolase.²⁷ Others have used IgH (single-chain) sequencing of bulk RNA isolated from synovium of patients with RA to identify dominant clones utilizing the IGHV4–34 gene segment and having CDR3s longer than those of antibodies expressed by naive B cells.¹⁰² Sequences encoding pathogenic autoantibodies have the potential to be used as predictive biomarkers to identify individuals likely to develop disease, experience an autoimmune disease flare or respond to immunomodulatory therapy.⁷⁷ A leading hypothesis is that mucosal sites, including the lung and oral cavity, might be critical in the initiation of ACPA responses that lead to the development of RA.¹⁰³ Some antibody repertoire sequencing technologies^{27,34,43} generate sequencing reads that extend sufficiently into the antibody constant region to identify the antibody isotype and subclasses, thereby enabling IgA ACPA-expressing B cells to be pinpointed to gain insight into the potential environmental exposures and microbial infections that initiate their production in RA. Finally, these sequences also have the potential to be used as pharmacodynamic biomarkers to monitor the response to an immunomodulatory drug, or to expedite clinical development by demonstrating the activity of these drugs in proof-of-concept studies.⁷⁷

Vaccine development

A great need exists for the development of vaccines for a variety of pathogens. Antibody repertoire sequencing is used to identify the microbial antigens and epitopes targeted by effective antimicrobial antibody responses that naturally control infection in humans and other animals,^{4,6,43,104,105} thereby enabling the development of vaccines based on these antigens. In addition, antibody repertoire sequencing is used in clinical proof-of-concept trials to demonstrate that a candidate vaccine, adjuvant or vaccination regimen can induce protective immune responses.¹⁰⁶

Immunomodulatory drug development

Immunomodulatory ‘checkpoint inhibitors’ are revolutionizing the care of patients with autoimmune diseases and cancer. Abatacept (CTLA4-Ig) blocks CD28-mediated activation of T cells and is efficacious in the treatment of RA.¹⁰⁷ In patients with metastatic melanoma and other cancers, ipilimumab (anti-CTLA4

antibody) and pembrolizumab (anti-PD-1 antibody) are therapeutically effective by blocking inhibition of T cells.^{19,20,108} Antibody repertoire analysis and sequencing provides an approach to monitor and characterize the immune responses induced by candidate immunomodulatory drugs¹⁰⁹ or vaccines.¹¹⁰ Checkpoint inhibitor therapy in cancer results in the induction of autoimmunity in a subset of patients, and antibody repertoire sequencing has the potential to be used to identify individuals at risk for developing autoimmune disease and to characterize the autoimmune response in individuals that do.^{19,20,108}

B-cell repertoire development

By analysing the sequences of antibody repertoires in an individual and comparing them to the sequences of the corresponding germline antibody genes, the lineage development of B cells and evolution of antibody responses can be tracked.^{42,58,61,62} Using this analysis, one can model and investigate the evolution of functional antibody responses in health and disease, reveal mechanisms underlying the development of native B-cell repertoires^{111,112} and the activation, development and trafficking of B cells in immune responses of interest.^{47,113,114} My laboratory, and several others, are in the process of utilizing antibody repertoire sequencing to investigate the development of anti-citrullinated-protein-reactive B cells in RA and anti-nuclear-antigen-reactive B cells in SLE. Such studies might identify autoantigens and provide insight into disease initiation and progression in these and other diseases.

Antigen and epitope discovery

Identification of the critical antigens and epitopes targeted by functional antibody responses is needed. Most approaches require prior knowledge of the target antigen, which is used for screening vast numbers of hybridomas, B cells or panels of candidate antibodies.¹¹⁵ Antibody repertoire sequencing, by contrast, does not require such knowledge. When applied to plasmablasts derived from a human or other animal with an immune phenotype of interest, this method enables the bioinformatic identification of clonal families of antibodies within a functional B-cell response without having to express or screen the antibodies. Such clonal antibody families define important antigens, as determined by the immune response. Recombinant antibodies generated from plasmablast clonal families can be used to identify the antigenic specificity of these cells. Such antigen discovery can uncover not only the targets of antibodies but also those of T cells, because B cells and T cells coordinate responses against the same macromolecular antigen complexes.¹¹⁶ Comprehensive datasets generated by large-scale sequencing of antibody repertoires, in combination with identification of the corresponding antigen targets of important antibodies, is anticipated to enable bioinformatic modelling of antibody–antigen structure-binding relationships that will ultimately lead to antibody variable region sequences being used to predict antibody specificity.

Conclusions

Effective characterization of the antibody repertoire requires high-fidelity analysis of the full-length IgH and IgL pairs expressed by individual B cells, to enable robust bioinformatic analysis of the repertoires and accurate recombinant generation of selected antibodies that can be used to identify target antigens and to investigate the mechanisms of their protective and pathogenic functions. Technologies that utilize cell-barcodes and analyse

full-length paired IgH and IgL are anticipated to provide the greatest utility in terms of identifying biomarkers and gaining new insights into the pathogenesis of RA, SLE, other autoimmune rheumatic diseases, and other diseases. Antibody repertoire analysis will transform our understanding of immune responses to autoimmunity, vaccination, infection and cancer, providing new biomarkers and diagnostic tools, and enabling efficient generation of therapeutic antibodies.

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