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High-throughput strategies for monoclonal antibody screening: advances and challenges

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Abstract

Antibodies characterized by high affinity and specificity, developed through high-throughput screening and rapid preparation, are crucial to contemporary biomedical industry. Traditional antibody preparation via the hybridoma strategy faces challenges like low efficiency, long manufacturing cycles, batch variability and labor intensity. Advances in molecular biology and gene editing technologies offer revolutionary improvements in antibody production. New high-throughput technologies like antibody library display, single B cell antibody technologies, and single-cell sequencing have significantly cut costs and boosted the efficiency of antibody development. These innovations accelerate commercial applications of antibodies, meeting the biopharmaceutical industry's evolving demands. This review explores recent advancements in high-throughput development of antibody, highlighting their potential advantages over traditional methods and their promising future.

Keywords High-throughput, Antibody preparation, Display system for antibody libraries, Single B cell antibody, Single-cell sequencing

Introduction

Initially, antibodies were produced by stimulating animal immune systems with target antigens, which suffered from limitations such as inconsistent specificity and significant batch-to-batch variability [1]. Hybridoma technology addresses these challenges by fusing host spleen cells with myeloma cells to create hybridoma cells. which can continuously proliferate and produce large amounts of specific, high-affinity monoclonal antibodies in vitro. Monoclonal antibodies (mAb) are highly stable, reproducible, and specific, making them valuable for research, drug development, and diagnostics [2]. However, this technology is limited by low-throughput mAb production due to the complex and labor-intensive hybridoma cell fusion process, along with time-consuming screening and dilution steps [3, 4].

High-throughput mAb production technologies utilize advanced biotechnologies, including high-throughput screening, single-cell sequencing, and antibody library display, among others, to quickly obtain coding sequences for target antibody variable regions [5, 6]. Furthermore, these technologies enable the precise optimization, modification, and expression of antibodies through genetic engineering techniques. Through these methods, high-affinity and highly specific antibodies can be rapidly and massively produced [7]. With

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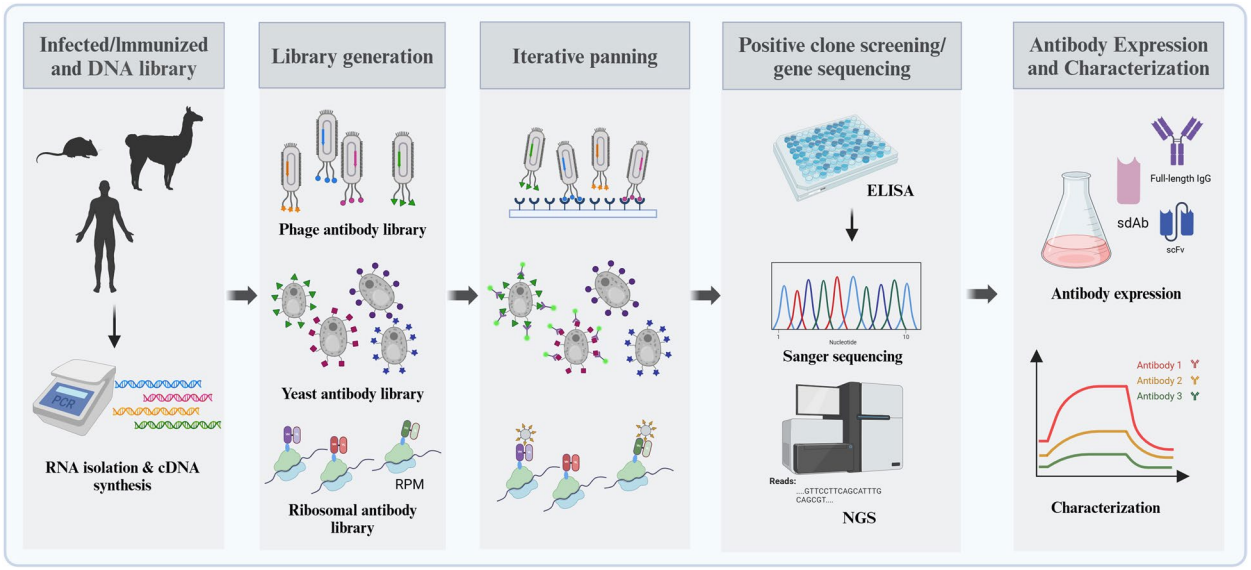

Fig. 1 The high-throughput antibody production scheme of the antibody library display technology. Antibody libraries from immunized animals and humans are displayed on a variety of vectors (e.g. phage, yeast, ribosomes, etc.) to construct diverse antibody libraries, and high-affinity binding vectors are isolated through multiple rounds of iterative screening. The gene sequences of the variable regions of the antibodies were determined by ELISA identification and sequencing analysis. The gene sequences of the variable regions are integrated into the designated expression vectors for antibody production, followed by downstream characterization and analysis

the deepening of biomedical research and clinical applications, high-throughput mAb production technologies are expected to become the mainstream method for antibody development, bringing further breakthroughs and progress in the fields of disease diagnosis, treatment, and drug discovery [8, 9]. This review summarizes recent advancements in high-throughput monoclonal antibody screening, highlighting their advantages over traditional methods and discussing future applications in antibody production.

Antibody library display technology

Due to somatic DNA recombination, the mammalian B-cell antibody repertoire can generate a diversity of 10^{12} to 10^{18} unique antibodies to recognize and combat a wide range of microbial pathogens [10]. The antibody library display technology presents antibody fragments from all variable regions of the antibody repertoire on the surfaces of vectors (such as phages, cells, or ribosomes, etc.), enabling the generation of an antibody library. Through multiple rounds of specific selection and screening parameter optimization, it facilitates the rapid and efficient production of antibodies targeting specific antigen conformations or epitopes [11, 12]. The high-throughput screening process for antibody library display technology is shown in Fig. 1

Phage display antibody library technology

Phage display antibody library technology is one of the most widely used methods for antibody development. Clackson et al. first demonstrated that the variable regions of antibodies could be displayed on the phage surface [13]. Additionally, Barbas III and Lerner enabled the construction of large combinatorial antibody libraries by fusing antibody gene fragments to phage coat proteins, allowing antibodies to be displayed on the phage surface [14]. By incubating the phage library with antigens and performing panning, phages with specific binding affinities are selected. Finally, high-affinity antibody genes are obtained through positive phage clone identification and DNA sequencing, providing a basis for further genetic engineering and antibody expression [15, 16].

Initially, antigen presentation and selection were carried out using solid-phase carriers such as ELISA plates and magnetic beads. With the development of technology, the introduction of automated microplate-based screening, magnetic bead processors, and robotic workstations has optimized the workflow and enhanced throughput [17–19]. Additionally, array-based formats further improve screening efficiency [20]. For instance, Pérez-Gamarra et al. reported an in-well array assay for multiparametric screening of phage display antibody libraries [21]. Fluorescence-activated cell sorting (FACS) and microfluidics integrated with phage display antibody libraries have further enhanced automation

and high-throughput screening [22, 23]. Hemadou et al. employed multiplex flow cytometry for high-throughput screening of scFvs with precise binding specificity, reducing the number of screening rounds and simultaneously obtaining a broader diversity of clones [24]. Philpott et al. designed the μCellect platform, which utilizes microfluidic selection to identify picomolar-affinity antibodies within just two rounds of screening [25]. Additionally, studies have reported that combining phage antibody display with FACS-based yeast surface display not only enables the generation and screening of large libraries but also allows for flexible quantification and efficient enrichment of antibodies with specific binding properties [26, 27]. To reduce multiple rounds of screening and improve efficiency. Keyser et al. employed biosensors to present antigens to nanobody-displaying phages within wells, leveraging an automated Octet bio-layer interferometry sensor for high-throughput selection with precise control over each step [28]. Hornsby described an optimized automated phage display pipeline that generated approximately 3,000 sequenced antigen-binding domains with high affinity, significantly increasing the throughput of antibody discovery [29].

To enhance parallel analysis capacity and increase screening diversity and precision, next-generation sequencing (NGS) has been integrated into phage display selection. Lee et al. constructed a single-domain antibody (sdAb) phage display library and employed NGS for high-throughput analysis, validating library diversity and identifying multiple high-affinity sdAbs [30]. Sasso et al. co-incubated phages with activated lymphocytes to select clones binding to CD27, BTLA, and TIGIT, followed by NGS-based VH region sequencing, enabling large-scale parallel screening [31]. Nakada-Masuta et al. integrated selection pressure with NGS analysis to effectively reduce expression bias and maintain antibody library diversity [32]. Furthermore, Barreto et al. developed an NGS-assisted antibody discovery platform, demonstrating that rare clones identified by NGS exhibited superior affinity and specificity compared to high-abundance clones detected by Sanger sequencing [33]. These advancements have significantly enhanced the efficiency of phage display selection, driving its widespread application in antibody development for cancer, viral infections, and autoimmune diseases [34–38].

Cell display antibody library technology

Cell display antibody library technology utilizes engineered cell surface display systems to present antibody fragments, providing an alternative to phage display. The major cell display platforms include yeast display, bacterial cell display, and mammalian cell display, each offering distinct advantages in terms of expression efficiency and screening capabilities [39].

Yeast display antibody library technology

Yeast display antibody library technology is a well-established method for displaying antibody fragments on the yeast cell surface and constructing antibody libraries. It typically employs high-throughput screening methods, such as FACS or microfluidics, to isolate high-affinity antibody clones for further analysis or subsequent applications [40, 41]. After multiple rounds of screening and amplification, high-affinity antibody clones can be obtained. The automation of high-affinity antibody characterization, sequencing, and expression, along with engineering advancements, has significantly accelerated the antibody production process [42]. Yeast's eukaryotic nature provides an ideal environment for antibody display, facilitating proper folding and post-translational modifications like glycosylation, which enhances the solubility and expression of disulfide-bonded and natively glycosylated antibodies or antibody fragments [43, 44]. Bowley et al. directly compared the same HIV-1 immune scFv cDNA library expressed in both phage and yeast display vectors, finding that yeast display yielded three times more specific scFv clones than phage display, and included all the clones recovered via phage [45]. This result demonstrates that yeast display can capture a broader and more functional diversity of antibodies. In addition, this technology based on cell surface antibody library display (including the mammalian cell surface display antibody library discussed below) integrates high-throughput screening and analysis methods, allowing for the high-throughput screening and characterization of tens of thousands of candidates [46]. This capability offers significant benefits in affinity, discovery of rare antibody clones, and improved cost-effectiveness and efficiency. Holliger et al. recently utilized the Illumina HiSeq platform to sequence yeast antibody libraries, which greatly accelerated the identification of high-affinity candidate sequences, screening for 108 antibody-antigen interactions within 3 days [47]. It is important to note, however, that while the identification process using NGS data can be accomplished quickly, the subsequent steps (e.g., gene synthesis, cloning, expression, and functional validation) are still time- and resource-intensive. The entire process of physically synthesizing and validating high-affinity antibodies typically takes several weeks, and cloning for synthesis and expression is costly [48]. This is largely due to the lack of cost-effective, high-throughput gene synthesis and expression solutions, which limits the scalability of the antibody discovery pipeline.

Mammalian cell display antibody library technology

Mammalian cell display antibody library technology involves inserting the target antibody gene into an expression vector and introducing it into mammalian cells (like CHO or HEK293) via transfection or viral infection. This allows the surface expression of the exogenous proteins, creating display libraries [49]. The high-throughput antibody screening of mammalian cell display following library construction are similar to yeast display. The mammalian cell display technology, owing to its endogenous eukaryotic secretion mechanism, partially mitigates the issues of low effective activity and misfolding that arise from the absence of post-translational modifications inherent in phage display. Theoretically, complex and highly stable antibodies (including Fab, scFv, and full-length IgG antibodies) can be displayed directly on the cell surface using mammalian cell surface display systems [50]. Robertson et al. developed an antibody discovery platform based on mammalian cell display technology for the natural conformation of membrane proteins, thereby increasing the opportunities to obtain high-affinity antibodies [51]. Furthermore, mammalian cell antibody display libraries can be designed in a secreted antibody format to enhance the efficiency of antibody screening. The secreted antibody repertoire has been integrated with various high-throughput auxiliary screening techniques, including gel microdroplet-fluorescence-activated cell sorting, droplet microfluidics, nanopores, and microarrays, among others [52]. Recently, Doerner et al. created a display-secretion switch system for pre-enriching highly manufacturable antibodies and conducting functional screening [53]. In the aforementioned context, antibody therapeutics utilizing mammalian cell display technology have undergone significant development in recent years [54–56]. However, it should be noted that conventional mammalian cell display antibody libraries struggle to meet all expectations of an ideal library. Researchers may need to employ targeted strategies to enhance library quality, thereby increasing the likelihood of identifying ideal antibodies during the screening process. For instance, enhancing the library's capacity by utilizing immunized animals, and achieving stable integration and transcriptional normalization of library mutations through programmable nucleases such as CRISPR/Cas9 and TALENs [57, 58]. Furthermore, this technology involves cell transfection and transmembrane secretion during library construction, which still requires further optimization to enhance the overall efficiency of antibody development.

Bacterial cell display antibody library technology

Bacterial cell display antibody library technology provides a simple and rapid alternative for the efficient transformation and functional screening of large peptide libraries. However, in bacterial display systems, proteins must traverse two membranes or contend with a thick peptidoglycan cell wall, which poses significant challenges for the display of large proteins or antibodies [59]. Although Francisco and Lee et al. demonstrated that scFv fragments can be functionally displayed on the surface of *E. coli* and sorted using flow cytometry, the outer membrane remains a barrier [60, 61]. As a result, only a few bacterial display systems can effectively present antibody fragments, limiting yield and complicating large-scale production. Mazor and Lombana et al. innovatively described a bacterial cell antibody library method in which IgG antibodies were immobilized on the periplasm-facing inner membrane. Using FACS, they successfully selected specific antigen binders, obtaining a diverse set of high-affinity IgG clones within 3–4 weeks [62, 63]. The dominant screening method for bacterial cell display antibody libraries is FACS, as it enables the assessment of expression levels, antigen binding, and estimation of antibody affinity [64]. While FACS excels in throughput and high-affinity binder isolation, it presents technical challenges when screening highly diverse libraries. To address this, researchers have integrated phage display as a preselection step to reduce library size, allowing subsequent rounds of FACS screening in *E. coli*. This approach facilitates the selection of a highly diverse repertoire of binders while enabling real-time monitoring and optimization of the screening process [65, 66]. In addition to the high-throughput FACS screening method, MACS is also widely used for bacterial cell display antibody libraries due to its faster library processing speed and the absence of expensive laboratory equipment [67, 68]. Furthermore, by incubating the bacterial antibody library with cells expressing the target antigen, bacteria can adhere to the target cells, enabling specific clone selection based on target cell binding [69]. This live-cell screening is particularly suitable for the development of nanobodies targeting tumor surfaces [70]. Overall, bacterial cell display antibody libraries are emerging as an efficient, cost-effective, and scalable antibody screening platform, overcoming technical bottlenecks and expanding into broader applications.

Ribosome display antibody library technology

Ribosome display technology enables the screening and identification of functional proteins by forming"protein-ribosome-mRNA"(PRM) ternary complexes in vitro, which links newly synthesized proteins to their corresponding mRNA molecules [71]. Ribosome display Antibody libraries are typically screened using antigen-functionalized magnetic beads. After the screening process, the eluted mRNA undergoes RT-PCR to synthesize

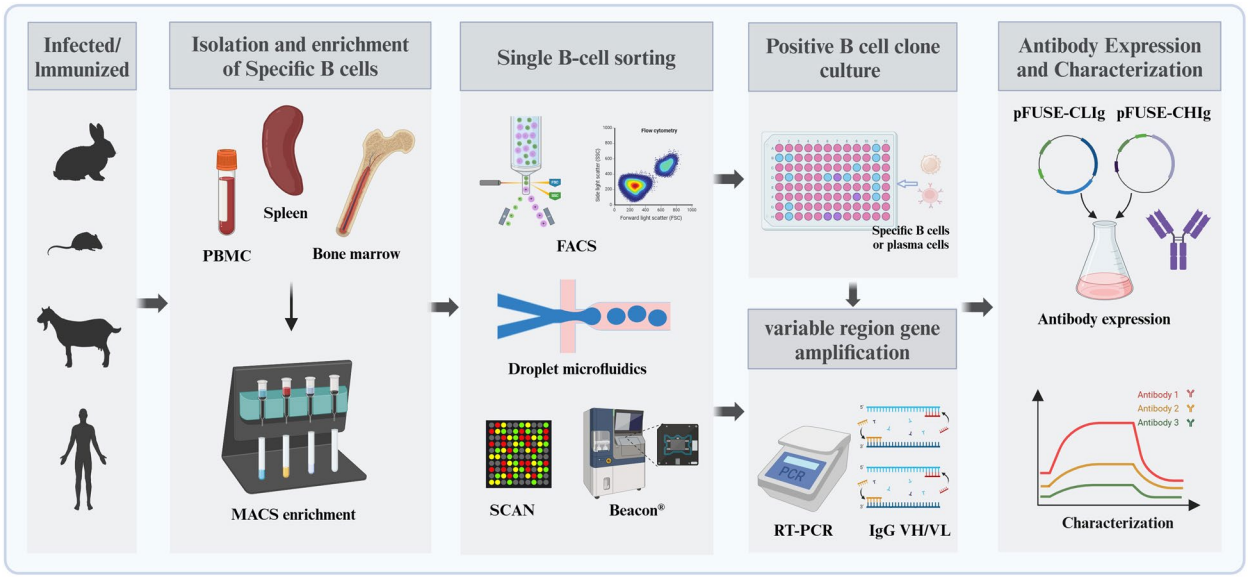

Fig. 2 The high-throughput antibody production scheme of the single B cell antibody technology. In the context of isolating PBMCs or splenocytes from immunized animals, as well as PBMCs from convalescent patients, various techniques such as MACS can be employed to enhance the concentration of target B cells. Subsequently, antigen-specific single B cells be isolated using methods such as FACS and microfluidics. The antibody variable region genes can be cloned through RT-PCR to facilitate antibody expression and subsequent analysis

cDNA, which could serve as the template for subsequent antibody expression [72].

Library display technology, with its advantages in high-throughput screening and the rapid generation of high-affinity antibodies, has greatly advanced the development of therapeutic antibodies. However, display techniques that present antibody fragments on the surface of living cells or viruses face the challenge of low efficiency in transfecting large DNA libraries [73]. Ribosome display libraries overcome these limitations by constructing the library in a cell-free system and introducing methods such as error-prone PCR and DNA recombination. This approach significantly enhances molecular genetic diversity and facilitates the affinity maturation and evolution of antibodies in vitro [74]. Despite its advantages, the effective function of ribosome display technology is heavily reliant on the integrity of the PRM complex. One significant challenge is the susceptibility of mRNA within the PRM complex to degradation in vitro, which can lead to decreased stability of the complexes, thereby affecting antibody selection efficiency, since incomplete or unstable complexes may not be effectively selected [75]. To address this issue, Ohashi et al. invented the PURE system for protein synthesis in vitro. This optimized system, which contains fewer nucleases and proteases, improves mRNA recovery and produces more stable ternary complexes [76]. Additionally, Wagner et al. developed the PolyMap high-throughput platform, which combines bulk binding of ribosome display libraries with scRNA-seq for pairwise mapping of protein–protein interactions, characterizing unique antibody clones without the need for mRNA recovery, thereby improving the efficiency of antibody development [77]. However, ribosome display technology is considered less optimal compared to cell- or phage-based antibody library display technologies due to reduced fidelity in cell-free reactions. For instance, cell-based display technologies leverage the natural mechanisms of living cells, offering higher expression efficiency and more reliable screening outcomes. In contrast, ribosome display often faces challenges related to stability and fidelity in vitro, which can negatively impact antibody selection and optimization processes [78, 79].

Single B cell antibody technology

Mammalian B-lymphocyte are extremely diverse and can produce as many as 10^{12} antibody clonotypes [80]. Unlike traditional antibody display technologies (e.g. phage display), which rely on iterative screening within a vast antibody library, single B-cell antibody technology allows for the direct isolation of individual antigen-specific B cells from animal tissues or peripheral blood [81]. The specific process of this technology(outlined in Fig. 2) includes antigen-specific B cell isolation, antibody gene amplification, and the expression and purification of recombinant antibodies. Compared with traditional antibody production techniques, single B cell antibody production technology offers the advantage of rapidly obtaining naturally paired light and heavy chain variable regions

through high-throughput screening of antigen-specific B cells from animals or humans [82]. With the progression of technology, downstream expression methodologies in single B-cell antibody technology, including the utilization of expression plasmids such as the pFUSE series, as well as various engineered cell lines like CHO-K1 and HEK293, have become well-established [83–85]. In addition, matured nested PCR enables high-fidelity amplification of antibody variable region genes, thereby serving as a crucial tool for the generation of single-cell antibodies [86, 87]. However, the cell sorting method plays a crucial role in isolating individual B cells with high antigen affinity and specificity from heterogeneous cell populations, which significantly influences the specificity and affinity of the antibodies produced. In this section, we provide a comprehensive review of various single B-cell sorting techniques and their applications in antibody production.

Single B-cell antibody preparation based on fluorescence activated cell sorting

FACS is a widely recognized and highly efficient technique that employs a flow cytometer for the multiparametric detection and classification of cells. This method enables selective separation of target B cells through the establishment of screening"gates."The sorting principle is as follows. Initially, fluorescently labeled antigens and antibodies targeting various cell surface markers are utilized to immunolabel the antigen-specific B cells in the single-cell suspension. Then, the labeled cellular samples are introduced into a flow chamber operating under high-pressure conditions, wherein the sheath fluid arranges the cells into a single row. The cells then traverse a fluorescence detection channel, where they are exposed to ultrahigh-frequency vibrations to generate single-cell droplets. These droplets are subsequently sorted into individual cells utilizing an electrostatic deflection system [88]. The individual cells are then isolated into distinct wells of cell culture plates. Following cell lysis for RNA extraction, cDNA synthesis and amplification of antibody variable region genes can be performed. In fact, FACS technology is capable of screening individual cells at rates of up to 10,000 cells per second and can precisely isolate specific B cells [89]. The principal advantage of FACS technology in single B-cell antibody preparation is its ability to swiftly and accurately identify individual antigen-specific B cells through multicolor fluorescence. This capability has significantly facilitated the development of a wide array of antibodies in research. Carbonetti et al. rapidly and efficiently isolated, cloned, and produced monoclonal antibodies from immunized mice, which was achieved through the utilization of multichannel screening techniques employing markers such as B220-PacBlue, CD38-APC, and IgM-FITC, among others [90]. Additionally, Fan et al. employed multicolor FACS to generate humanized monoclonal antibodies targeting seven distinct subtypes of BoNT/F, resulting in antibodies that could neutralize multiple antigenic variants [91]. This approach exemplifies how FACS technology can streamline the production of highly specific and versatile monoclonal antibodies.

Nonetheless, the low sensitivity and high detection noise of FACS often lead to challenges associated with false positives (FP) and false negatives (FN), which in turn increase the workload of subsequent antibody characterization and validation processes [92]. To improve the specificity of sorting individual B cell, the integration of MACS with FACS is increasingly employed. Zhou et al. utilized magnetic beads to selectively enrich specific B cell populations before dual fluorescent dye sorting, which markedly diminished the interference from other cell types within the sample [93]. This antibody development strategy not only improves sorting specificity but also reduces the subsequent workload associated with antibody expression and characterization. Moreover, to further reduce the impact of FN and FP, it is crucial to enhance the identification of positive clones through single B cell culture and the analysis of culture supernatants [94]. Moreover, antibody-secreting cells (ASCs) not only exhibit higher antibody affinity compared to memory B cells but are also present in significantly larger quantities in vivo [95]. However, their low expression of surface markers poses substantial challenges for isolation and identification using FACS, as this method heavily relies on marker expression for cell sorting. These technical difficulties have greatly hindered the widespread application of FACS in studying ASCs [96]. Although the hetero-functional particles developed by Ramirez et al. integrated with the FACS platform enable high-throughput isolation of specific ASCs and significantly enhance the enrichment efficiency of antibody-associated cells, this method is relatively complex to design and requires skilled operation to fully optimize the platform's capabilities [97]. Moreover, the application of FACS screening may be limited by unclear or absent cell surface markers, which can lead to the loss of ideal antibody clones. For instance, rabbit-derived lymphocytes are currently constrained by the lack of useful surface markers on their cell membranes for effective screening. While Starkie et al. introduced a two-color antigen staining method for the recognition of antigen-specific rabbit memory B cells. the intricate design of the screening process led to challenges in ensuring the specificity of the identified positive cells, necessitating extensive downstream validation efforts, including a substantial amount of antibody cloning and validation work in subsequent stages [98]. In addition, FACS often requires a large starting number of cells for

the screening of antigen-specific B cells, and manipulations in the pre-treatment may affect cell viability, resulting in difficulties in the identification of downstream antibody affinity and antibody activity.

Microfluidic sorting-based single B-cell antibody preparation technology

To address the limitations of traditional FACS methods in sorting single B cells for antibody development, microfluidics-based sorting technologies offer significant advancements in both efficiency and precision. Microfluidics technology is categorized into microdroplet and microwell systems according to their operational principles [99]. These microfluidic platforms enable the detection and isolation of specific B cells at the single-cell level and support the cultivation of positive clones within a controlled nutrient environment [100]. Compared to traditional antibody production methods, microfluidics-based single-cell antibody production technologies offer superior cell sorting precision, throughput, and integration capabilities. These advantages make microfluidics a key tool for large-scale antibody screening and optimization, underscoring its potential to advance the fields.

Based on droplet-based microfluidics

Microdroplet microfluidics utilizes an aqueous phase containing cells and analytical reagents, in conjunction with an oil phase that isolates individual cell droplets. Within the flow channel, these droplets are swiftly analyzed and identified using a high-frequency laser, while an electric field classifies them based on their unique characteristics [101]. A notable advantage of this technique for antibody screening is that each droplet not only functions as an independent microreactor but also has an extremely small volume (in the picoliter range), effectively preventing cross-contamination between droplets and ensuring that the cells and reagents within each droplet can undergo efficient and precise reactions in a confined environment [102]. Additionally, precise manipulation of droplets through merging, splitting, and sorting enables high-throughput separation and recovery of cells. This technique allows for the generation of droplets in the order of 10^7 per experiment, processing thousands per second. Consequently, it significantly reduces the screening cycle from months to a single day, greatly enhancing assay efficiency [103].

Microfluidic platforms, celebrated for their efficient sorting capabilities, have significantly propelled the advancement of antibody development. In the realm of scientific research, Eyer et al. have elucidated a droplet-based microfluidic technique known as DropMap. This technique facilitates massively parallel kinetic analysis of individual ASCs by integrating immobilized picoliter-sized droplets with highly sensitive assays. DropMap enables precise assessment of antibody secretion rates, specificity, and affinity for antigens, thereby enhancing the capabilities of antibody evaluation and characterization [104]. For commercial applications, the Cyto-Mine® platform developed by Sphere Fluidics Co., Ltd. offers a significant advantage in antibody screening. This system utilizes fluorophore-labeled donor and acceptor probes to enable fluorescence resonance energy transfer (FRET) for the detection of cellular secretions. Antibodies secreted by cells within droplets are captured by these probes; one fluorophore is excited by a laser, transferring energy to the second fluorophore. This technique provides precise quantification of target antibodies and facilitates the efficient production of recombinant homologous paired antibody candidates for secondary screening [105]. According to Doerner et al., the Cyto-Mine® platform provides several key advantages: 1) It enables the rapid identification of antigen-specific positive cells through FRET technology, with direct applicability to full IgG formats. 2) It can efficiently analyze up to one million single antibody-secreting cells, achieving a screening rate of 250 drops per second. 3) The microdroplet technology minimizes mechanical damage to cells, thereby maintaining cell viability for subsequent antibody performance evaluations. 4) The entire process, from cell preparation to recombinant antibody confirmation, is completed within a four-week timeframe [106]. Similarly, Brenan et al. introduced the Celli GO technique, which combines fluorescence-based single-cell bioanalysis with single-cell barcoding in droplets to visualize specific ASCs. This technique allows sorting of droplets at velocities up to 600 per second, resulting in the generation of 77 recombinant antibodies from identified sequences [107]. The MTX-COVAB antibody drug developed by Hillenbrand et al. DROPZYLLA® platform has demonstrated significant efficacy in vivo, further highlighting the impact of microdroplet microfluidic technologies in antibody development [108]. Additionally, Adler et al. introduced an emulsion droplet microfluidic approach that enables the isolation of millions of single B cells while preserving the natural pairing of heavy and light chains, facilitating the affinity screening of rare antibodies from murine libraries [109]. Furthermore, DeKosky et al. developed a single-cell, emulsion-based microfluidic technology capable of generating VH-VL amplicons for NGS [110]. These advancements further expand the applications of droplet microfluidics in antibody discovery and lay the foundation for single-cell immune repertoire sequencing.

Recent advancements in microdroplet microfluidic platforms have significantly enhanced the capabilities for antibody development, yet several critical

challenges remain. Key issues include the need for greater simplification of technology and the integration of automated systems. Addressing these challenges is crucial for the continued progress of droplet microfluidic antibody discovery platforms. In this context, Wippold et al. have introduced a rapid assessment platform that leverages integrated microfluidic technology to enable high-throughput, single-cell resolution identification of neutralizing antibody libraries in response to viral infections [111]. Wang et al. have developed a sophisticated signal capture system that addresses cell viability issues arising from iterative enrichment processes and overcomes some limitations associated with existing commercial plasma cell enrichment reagents [112]. In a related development, Wang et al. have successfully combined an autocrine-based lentiviral transduction system with microfluidic droplet technology, enhancing both efficiency and capacity. This innovation facilitates the screening of low-frequency functional antibodies and the characterization of bispecific antibodies [113]. Mazutis et al. have employed FRET-based droplet microfluidics to screen individual antibody-secreting cells, achieving sorting efficiencies of up to 97.5% and an 800-fold enrichment of target cells in a single sorting round, thus significantly accelerating the antibody discovery process [114]. Additionally, Ahmadi et al. have pioneered a novel method for on-demand co-encapsulation of cells, providing new insights into the automation of droplet microfluidic antibody discovery techniques [115]. These advancements highlight the rapid progress in droplet microfluidic platforms for antibody discovery, showcasing their transformative potential in high throughput antibody research. High-throughput microdroplet technologies can now generate and screen approximately ten million picoliter-scale microdroplet reaction units in a single experimental iteration. This unprecedented capacity not only accelerates throughput but also shortens development timelines and reduces operational costs, making these platforms highly attractive for large-scale antibody screening. However, significant challenges persist that must be addressed to unlock the full potential of this technology. A major limitation is the occurrence of false-negative results, often attributed to the inefficient encapsulation of ASCs within the defined microdroplet volumes. Furthermore, the intrinsic constraints of picoliter-sized droplets, such as limited reagent availability and dilution effects, can compromise detection sensitivity and accuracy. Overcoming these barriers will require innovations in droplet design, cell encapsulation efficiency, and signal amplification strategies. Future advancements in these areas are essential for enhancing the reliability and scalability of droplet microfluidics, paving the way for its broader application in antibody discovery and beyond.

Based on microwell-based microfluidics

Antibody discovery and development have made significant strides with the advent of microfluidic technologies. Single-Cell Antibody Nanopores (SCANs) represent a synthesis of microwell chips devices and high-throughput screening techniques, offering a novel approach to isolating and analyzing individual B cells. The original microengraving and immunospot array assay on a chip (ISAAC) is represents a sophisticated technique for SCAN applications. Among these, microengraving technology enables the precise separation and isolation of individual cells in microfluidic chip chambers by exploiting variations in cell size, shape, or surface markers, ensuring that a single cell is present in each chamber. These miniature chambers are meticulously designed to offer an optimal cell culture environment, encompassing essential nutrients, growth factors, and extracellular matrix components, thereby facilitating the growth and maturation of individual B cells. Antibodies secreted by individual B cells are transferred to a protein microarray for antigen-specific screening and are mapped to the corresponding target cells. Subsequently, the target cells are isolated from the wells through micromanipulation techniques, facilitating high-throughput cell sorting [116]. ISAAC technique utilizes anti-IgG or specific antigens immobilized on the surface of a microarray. Antibodies secreted by the cells bind to the immobilized anti-IgG or specific antigens, resulting in the formation of distinct circular spots that are readily distinguishable from non-specific signals [117]. Using this technique, ASCs are identified within 3–4 days and subsequently analyzed through clonal expansion or RT-PCR [118].

SCANs can screen up to 100,000 polyclonal B cells simultaneously, thereby providing remarkable high-throughput capacity, sensitivity, and specificity [119]. The use of fluorescently labeled antigens for screening and identification reduces extensive validation of antibody clones [120, 121]. Additionally, the multi-nanopore design of SCANs technology enables the simultaneous study of specific antibodies targeting a diverse array of distinct antigens on a single chip. Esfandiary et al. employed SCAN screening to identify high-frequency cell clones producing specific IgG isotypes targeting anti-SSA/Ro60 and anti-SSB/La antigens, illustrating the capability of SCAN to isolate multiple antigen-specific B cells on a single chip [122]. Currently, researchers have enhanced the microwell system to improving the efficiency of antibody development. For instance, Li et al. introduced CSMN, a nanopore-based, semi-antigen-specific rabbit ASC selection method, enabling the

acquisition of target antibody gene sequences within a 5-day screening period [123]. Abali et al. employed a nanopore platform for screening, enabling the generation of high-titer antibodies. This process, from the introduction of single cell and the measurement of antibody yield to the production of fully amplified clones, was completed within a timeframe of 4 to 6 weeks [124]. However, this technique necessitates intricate and precise experimental procedures, including the extraction of cells from microwells and the application of antibody coatings on the chip surface. Furthermore, the design and fabrication of these chips involve substantial costs. As a result, the technology platform has limited applicability. Another important consideration is the high cell density within the miniature device, which may result in cross-contamination between cells and subsequently impact the accuracy of the experimental outcomes.

In contrast to SCANs, the commercial Beacon® single B-cell screening platform exhibits superior automation capabilities. Introduced in 2016 by Berkeley Lights, Co., Ltd., the Beacon® platform offers fully automated and high-throughput functionality, establishing itself as a pivotal tool for high-throughput antibody screening [125]. This platform integrates advanced opto-electropositioning (OEP) technology, nanoscale microreactor systems, and innovative microfluidic design. This combination enables precise single B-cell sorting, nanoscale single B-cell culture, and validation of positive clonal antibody secretion—all within a compact, centimeter-scale chip [126]. The experimental procedure for this technology platform consists of four sequential stages. In the first stage, 10,000 to 20,000 B cells are introduced onto the Beacon® chip via microfluidics. Next, using OEP technology, individual B cells are isolated and transferred to NanoPen™ culture chambers for cultivation. Each NanoPen™ chamber can only accommodate one cell, ensuring a monoclonality rate of 99% or higher while maintaining high cell viability. In the third stage, the platform performs real-time detection of antibody binding strength, specificity, and affinity within the NanoPen™ chambers to identify effective clones. Finally, specific B cells are selected based on culture and real-time analysis results, extracted using OEP technology, and recovered into multi-well plates for further expansion [127].

The Beacon® single B cell OEP platform enables full automation of the entire single-cell experimental workflow. Winters et al. employed this platform to generate antibodies targeting unique reagents [128]. Remarkably, the entire workflow, encompassing chip-based cell import, antibody screening, and data export for discovery, was completed in less than 5 h. Furthermore, the complete discovery process, encompassing immunization to recombinant antibody expression, can be accomplished in as few as 40 days. Douet et al. utilized the Beacon platform to generate efficient CHO clones in a reduced timeframe, thereby enhancing both the throughput of the Beacon® system and the identification of high-producing clones [129]. Compared to traditional single-cell antibody preparation platforms, the Beacon® single B-cell screening platform enables automated cell sorting, cultivation, and positive clone identification, significantly reducing manual intervention associated with traditional methods. This advancement not only dramatically accelerates antibody development but also improves stability and reproducibility. Additionally, the Beacon® single B-cell screening platform can simultaneously process tens of thousands of cells, providing exceptional scalability and significantly enhancing antibody development efficiency. Given these advantages, the platform has become a pivotal tool in the development and industrialization of therapeutic and diagnostic antibodies [130, 131].

Notably, while both microwell-based microfluidics and the commercial Beacon® System facilitate the cultivation and characterization of individual specific ASCs within microwell or NanoPen™ chamber, amplification and sequencing of variable region genes is still performed off-chip, this separation increases the risk of ASC loss and cross-contamination. To address these issues, Zhang et al. introduced an innovative modular design for the SSMA chip, which allows for the simultaneous acquisition of paired variable region genes from a single ASC within a single SSMA chip, thereby reducing the risks of cell loss and cross-contamination [132]. Nevertheless, microwell-based microfluidics and commercial beacon® systems remain prohibitively expensive and complex for antibody development. Future advancements in user-friendliness, cost efficiency, and increased throughput may offer substantial opportunities for the field of single cell antibody development.

Antibody production technology for high-throughput single-cell sequencing

The complex processes of V(D)J gene rearrangement, somatic hypermutation (SHM), and class switching generate a vast repertoire of variable region sequences in antibody heavy and light chains, contributing to the huge capacity of the B cell immune repertoire [133]. Generally, the antibody preparation technique based on high-throughput single-cell sequencing includes the following processes: 1) Isolation and enrichment of specific B cells from peripheral blood mononuclear cells (PBMCs) or splenocytes obtained from animal models. 2) In water-in-oil microdroplets, gel beads or magnetic beads with tagged sequences are used to capture single B cells, followed by the extraction of RNA from each cell, which is then reverse-transcribed into cDNA

for sequencing library construction. 3) Sequencing of B cells by single-cell NGS technology to obtain large-scale single-cell resolution data. 4) Bioinformatics tools are used to analyze the sequencing data from individual B cells to obtain the paired IgG heavy and light chain variable region genes [134]. Figure 3 depicts the technical workflow of high-throughput antibodies preparation based on high-throughput single-cell sequencing. This technology exemplifies the synergy between state-of-the-art sequencing techniques and advanced bioinformatic methodologies. Such integration underscores the sophistication and depth of modern antibody repertoire research. By offering a comprehensive framework for analyzing the B cell antibody repertoire, this approach markedly advances the development of high-throughput antibody solutions.

The introduction of Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) in 2017 marked a significant breakthrough in the field of single-cell sequencing [135]. Building on the success of CITE-seq, Setliff et al. proposed linking B-cell receptors to antigen specificity through sequencing (LIBRA-seq). The core of LIBRA-seq is the addition of fluorescent modifications and oligonucleotide labeling (including PCR processing, barcode sequences, and capture sequences) onto antigens. By incubating B cells with different antigens and using FACS to sort antigen-specific B cells. Subsequently, microfluidic technology is employed to encapsulate individual B cells in droplets containing magnetic beads, followed by single cell RNA sequencing (scRNA-seq). The magnetic beads are coated with universal cell barcodes, which facilitate the linking of antigen-specific barcodes and B cell receptor (BCR) transcripts, enabling the identification of both the BCR sequences and their corresponding antigen specificity [136]. This method offers several advantages, including rapid, high-throughput antibody screening, the establishment of direct correlations with antigen specificity, and the simultaneous identification of broad-spectrum neutralizing antibodies. These broad-spectrum antibodies are capable of recognizing and neutralizing a number of different antigens or antigenic variants, which is particularly valuable against different strains of pathogens or against multiple epitopes of a single antigen. Kramer et al. used LIBRA-seq technology to isolate a monoclonal antibody, 54,042–4, from COVID-19 convalescents that was effective in neutralizing SARS-CoV-2 viruses including multiple SARS-CoV-2 variants [137]. Additionally, Pilewski et al. employed LIBRA-seq to study the antibody repertoire of individuals co-infected with chronic HIV-1 and HCV, identifying five antibodies with broad neutralization activity and potent functional effects against both HIV-1 and HCV [138]. Recently, substantial progress has been made in LIBRA-seq. Shiakolas et al. incorporated barcode-labeled antigenic ligands into the LIBRA-seq platform, thereby establishing a comprehensive three-dimensional

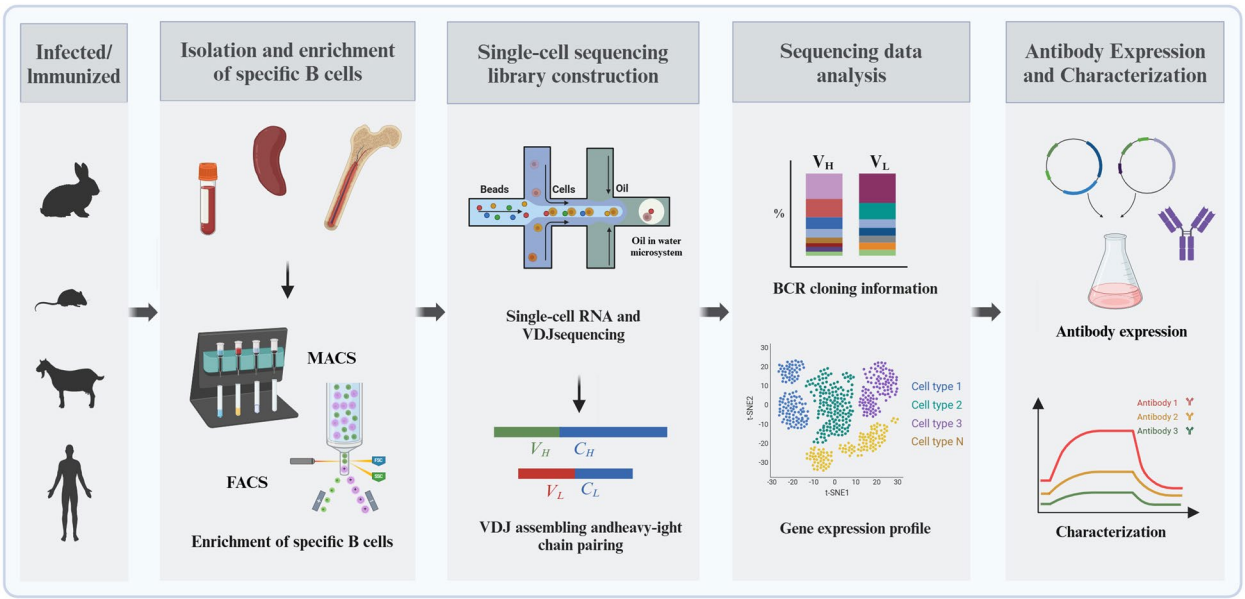

Fig. 3 The high-throughput antibody production scheme of the single-cell sequencing. In the context of isolating PBMCs or splenocytes from immunized animals, as well as PBMCs from convalescent patients. Target B cells were enriched using MACS or FACS. Single-cell sequencing was conducted on the enriched cells to obtain 5'-end transcriptome data and immune repertoire data. Bioinformatics analysis was then employed to identify high-affinity antibody gene sequences from a vast amount of single-cell sequencing data. Specific vectors were subsequently incorporated for antibody expression and downstream characterization

correlation among antibody sequences, antigens, and neutralization effects. This advancement has significantly improved the efficiency of neutralizing antibody screenings [139]. Additionally, Walker et al. employed a panel of antigens with point mutations to map the relationship between antigenic epitopes and antibody sequences. This approach has refined the antigenic epitopes of thousands of B cell receptors simultaneously, thereby elevating the functionality and utility of LIBRA-seq [140]. LIBRA-seq has now achieved remarkable success in the development of antibodies against a diverse array of viruses [141–143]. Overall, LIBRA-seq represents a powerful and innovative method for antibody development. However, its application may be constrained in certain scenarios due to the complexity of recombinant antigen design and the discontinuous nature of its experimental workflow.

In contrast, the 10 ×Genomics single-cell sequencing platform has shown great potential in simplifying experimental workflows and providing broader and more comprehensive antibody discovery. As a premier platform for single-cell antibody library sequencing, it utilizes sophisticated microfluidic technology and efficient molecular identification methods to support large-scale antibody development and optimization, thereby offering a dependable solution for high-throughput antibody screening [144]. The platform leverages microfluidic technology to encapsulate single cell, gel beads, and reaction reagents within a"water-in-oil"droplet microenvironment, each tagged with molecular identifiers (UMIs) and cellular barcodes. Upon cell lysis within the droplets, mRNA containing Poly(dA) sequences are released, subsequently binding and being reverse-transcribed into cDNA that carries both the cellular barcode and UMI. After oil dissolution, a portion of the resulting cDNA is used for the construction of a 5'transcriptome library, while the remainder undergoes PCR amplification for V(D)J gene analysis, facilitating the construction of an immune repertoire library [145, 146]. The 10 ×Genomics platform facilitates high-throughput single-cell V(D)J sequencing, providing the capability to accurately capture and pair the complete variable region sequences of both light and heavy chains with high precision. This technology not only facilitates detailed single cell analysis but also provides comprehensive expression profiling information. With its significant advantages, including high resolution and efficiency, the platform is widely used by researchers to develop antibodies with high affinity and specificity, significantly advancing the field of antibody discovery and development [147, 148].

Compared to other antibody development techniques, high-throughput single-cell sequencing offers a faster and more efficient solution by bypassing intermediate steps and directly obtaining high-quality antibody data [149]. However, the vast volumes of sequencing data generated by high-throughput single-cell sequencing technologies can be overwhelming. A critical challenge in identifying and isolating high-efficiency antibodies lies in enhancing the proportion of target-specific or high-value information within these datasets. Developing efficient strategies to prioritize and refine these datasets is essential for advancing antibody discovery. To address this challenge, researchers often focus on enriching target B cells specific to the antigen before sequencing. This approach helps reduce interference from low-affinity B cells and other cellular components, thereby enhancing the quality and pertinence of the data. For instance, Cao et al. improved the success rates for high-efficiency neutralizing antibody screening by using magnetic bead-specific enrichment of patient sera followed by 10 ×Genomics single-cell sequencing, identifying 14 high-efficiency neutralizing antibodies within two weeks [150]. Similarly, Wang et al. employed FACS to isolate memory B cells bound to recombinant Staphylococcus aureus antigen, followed by 10 ×Genomics single-cell sequencing to identify 10 distinct IgG antibodies [151]. These studies highlight the effectiveness of pre-sequencing enrichment strategies in reducing non-informative noise from low-affinity B cells and other contaminants. By selectively enriching target cells prior to sequencing, this approach significantly enhances the likelihood of identifying high-performance antibodies, thus advancing the development of potent therapeutic antibodies.

To fully leverage the advantages of high-throughput single-cell sequencing technology, implementing pre-sequencing enrichment strategies is crucial for improving data quality and relevance. However, these strategies alone are inadequate. Equally important is the bioinformatics analysis of the downstream data produced by single-cell sequencing. Generally, data from single-cell immune repertoire sequencing must be processed to infer B-cell population structures and quantify detailed features [152]. Various tools have been developed for BCR analysis, including IgBLAST and IMGT/HighV-QUEST for V(D)J gene annotation, as well as AbNum and AbRSA for antibody numbering [153–155]. Research indicates that various tools exhibit differing levels of performance. For example, a study by Smakaj et al. identified MiXCR as the most efficient tool for sequence processing when compared to alternatives such as IMGT/HighV-QUEST and IgBLAST [156]. The large volume of sequences poses a significant processing challenge. Zong et al. recently introduced Abalign (http://cao.labshare.cn/abalign/), based on AbRSA, which significantly reduces the time required for high-throughput BCR data analysis from weeks to hours [157]. Additionally, machine learning and deep learning have extensive applications for

Table 1 Characteristics of different antibody high-throughput preparation techniques

Platform		Throughput	Automation	Chain Pair	Affinity	Screening period	Potential improvements	Representative
Antibody Library Display		Relatively low	Low	N	Low	1–2 month	Affinity of antibodies; Library quality	Phage Display [15]
Single B-cell sorting platform	FACS	Medium (10, 000cell/s)	Low	Y	Relatively Lower	16–18 weeks	Low screening specificity; Difficult to screen for ASCs	BD FACSAria™ [89]
	Microdroplet	High (millions cell/s)	Medium	Y	High	4–6 weeks	False negatives; Limited droplet volume	Cyto-Mine [105]
	Microwell	Medium (1–200,000/ chip)	Low	Y	Medium		The technology is complex; contamination between cells	ISAAC [117]
	Micro-Chamber	Medium (1–20, 000/ chip)	Hight	Y	High		Expensive; contamination between cells	Beacon® [125]
Single-cell sequencing		160,000cell/run	Hight	Y	High	1–2 weeks	An efficient method for analyzing sequencing data	10 × Genomic [144]

antibody data analysis. Researchers are developing artificial intelligence models that integrate these techniques with traditional bioinformatics methods to extract valuable information from immunome libraries [158]. Bai et al. has reviewed major machine learning techniques and their applications, including computational predictions of antibody structures, antigen interfaces, and interactions [159]. These advanced methods enhance data processing efficiency and support high-throughput antibody production and screening. By leveraging these tools, researchers can rapidly identify and optimize therapeutically valuable antibodies, thus accelerating the antibody development process and advancing the discovery of novel antibody drugs. The application of these technologies is ushering in a new era in high-throughput antibody screening, making the transition from discovery to product more efficient and precise.

Summary and outlook

High-throughput antibody screening has gained widespread attention for its rapid, efficient, and scalable capabilities in antibody preparation. With continuous advancements in bioinformatics, structural biology, antibody engineering, artificial intelligence, and automation, these technologies are increasingly focusing on integration, automation, and intelligence. In this systematic review, we summarize current high-throughput antibody screening technologies, including antibody library display-based screening strategies, single-cell technologies, and single-cell sequencing. While several recent reviews have delved into various strategies for monoclonal antibody discovery, such as antibody display technologies, microfluidics, and single B cell technologies, they typically focus on individual strategies without offering a comprehensive comparison within a high-throughput framework [160–162]. In contrast, our review not only summarizes these strategies but also emphasizes how they integrate and work synergistically within modern high-throughput antibody discovery pipelines.

Table 1 provides a summary of the characteristics of antibody library technology, single B cell antibody production, and single-cell sequencing in the context of antibody development. Overall, compared to the initial complex procedures that required multiple rounds of antibody library screening, the integration of automation and higher-throughput methods (such as microfluidics and NGS) has transformed antibody library display technology into a powerful platform for innovative drug discovery. However, it is important to note that the quality of the antibody display library has a significant impact on screening outcomes. In contrast, single-cell antibody preparation technology offers high-throughput screening capabilities, enabling the generation of naturally paired antibodies with both light and heavy chain variable regions. This technology is known for its speed and high affinity, making it one of the primary methods for industrial-scale antibody production. Despite these advantages, there are still challenges, such as ensuring

the accuracy of positive clone screening, as well as the complexity and cost of the required instruments. Among these techniques, high-throughput single-cell sequencing stands out as the most efficient method for antibody preparation. It can provide gene expression profiles and immune repertoire data from a large number of single cells in a single run. However, the complexity and volume of immune repertoire sequencing data present significant challenges. While existing bioinformatics tools, along with the integration of machine learning and deep learning techniques, offer promising solutions for data analysis and mining, there are still limitations. Further improvements and enhancements to algorithms, scoring functions, databases, and benchmarking tools are necessary to better address these challenges.

Abbreviations

mAb	Monoclonal antibodies
NGS	next-generation sequencing
HAMA	human anti-mouse antibody
scFv	single chain antibody fragment
SdAb	single-domain antibodies
FACS	fluorescence activated cell sorting
MCS	multiple cloning site
PCR	polymerase chain reaction
PRM	protein-ribosome-mRNA
cDNA	complementary DNA
FP	false positives
FN	false negatives
MACS	Magnetic Activated Cell Sorting
ASCs	antibody-secreting cells
FRET	fluorescence resonance energy transfer
SCANs	Single-Cell Antibody Nanopores
ISAAC	immunospot array assay on a chip
OEP	opto-electro-positioning
SHM	somatic hypermutation
LIBRA-seq	linking B-cell receptors to antigen specificity through sequencing
CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by sequencing
scRNA-seq	single cell RNA sequencing

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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N/A.

Consent for publication
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