Application of Microfluidic Technology in Field of Antibody Preparation

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Abstract

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Abstract: Microfluidic technology is a science and technology that can accurately manipulate fluids in micro-sized channels. In recent years, microfluidic devices have attracted wide attention due to its easy manipulation, miniaturized size, high throughput and precise control, which provide a potential platform for antibody screening. This review paper provides an overview of recent advances in microfluidic methods application in the field of antibody preparation. While hybridoma technology and four antibody engineering techniques including phage display, single B cell antibody screening, antibody expression and cell-free protein synthesis are mainly introduced, important advances of experimental models and results are also discussed. Furthermore, the authors expound on the limitations of current microfluidic screening system and present future directions of antibody screening platform based on microfluidics. Antibody preparation on microfluidics combined with other technologies has huge application potential in the field of biomedicine, and it is anticipated to be further developed.

Keywords: Microfluidics, Antibody screening, Hybridoma technology, Antibody engineering

1.Introduction

Over the past few decades, the importance of antibodies in the fields of biochemistry, molecular biology, passive immunotherapy has continued to increase, there has been a dramatic increase in demand for antibodies. With the advancement of science and technology, antibody products that have undergone review or approval are growing rapidly (Kaplon, Muralidharan, Schneider, & Reichert, 2020; Kaplon & Reichert, 2019). Since the emergence of hybridoma technology (Köhler & Milstein, 1975), antibody preparation has been an object of research. Antibody engineering techniques that have emerged in recent years including phage display antibody libraries (Kretzschmar & Rüden, 2002), ribosome antibody libraries (Kanamori, Fujino, & Ueda, 2014), yeast surface display (Miller, Pefaur, & Baird, 2008), and transgenic mice (Jakobovits, Amado, Yang, Roskos, & Schwab, 2007) have been successfully applied to the manufacture of monoclonal antibodies (Saeed, Wang, Ling, & Wang, 2017). However, far too little attention has been paid to achieving high efficiency and automating the preparation method of antibodies.

Microfluidic chip, also known as lab-on-a-chip, is a micro-electromechanical technology-based, multi-dimensional network microfluidic channel as structural features (Beebe, Mensing, & Walker, 2002). The goal is to transfer and integrate the basic operating units involved in the fields of traditional chemistry or biology onto a small chip, and ultimately achieve miniaturization and integration of conventional chemistry or biological laboratories (Dittrich & Manz, 2006). It is also widely used in the field of cells, the micro-scale two-dimensional or three-dimensional channels in the microfluidic chip provide a size-matched structure for the manipulation of a single cell (Hummer, Kurth, Naredi-Rainer, & Dittrich, 2016; Lagus & Edd, 2013; N. Shembekar, Chaipan, Utharala, & Merten, 2016). According to different needs, the operation unit required for single cell research, such as micropump, microdroplet, microvalve technology related to cell manipulation, single cell capture technology, and detection and analysis unit, can be designed and integrated into a chip platform (Guo, Rotem, Heyman, & Weitz, 2012; Joensson & Andersson Svahn, 2012; Lagus & Edd, 2013; Seah, Hu, & Merten, 2018). The microfluidic chip has the advantages of high separation efficiency, fast analysis speed, few required samples, wide application range, and high degree of automation. It fully reflects the development trend of miniaturization, integration, automation and portability of equipment. The successful development of microfluidic technology ability to facilitate the process of monoclonal antibodies discovery and development (Fitzgerald & Leonard, 2017; Khalid, Arif, Kobayashi, & Nakajima, 2019; O'Kennedy et al., 2018).

This review aims at summarizing the latest advances in the application of different microfluidic systems in the preparation and screening of monoclonal antibodies (Figure 1), such as the traditional hybridoma technology, phage display, single B cell technology, antibody expression technology and cell-free protein synthesis technology. Finally, the prospects of microfluidic application in antibody preparation techniques are discussed.

2.Hybridoma technique in the microfluidic device

2.1 Principle of hybridoma technique

The hybridoma technology has established from its conception in 1975 (Kohler & Milstein, 1975) into the most widely used and successful method for producing monoclonal antibodies. As shown in Figure 2, B lymphocytes and myeloma cells are fused into hybridoma cells. Then the resulting hybridoma cells have the characteristics of amphiphilic cells, and monoclonal antibodies can be obtained by further screening and isolation hybridoma cells. High-purity and defined specificity monoclonal antibodies can be prepared by using

hybridoma technology (Taggart, 1987). Nevertheless, long screening period, large sample consumption, low screening efficiency, cumbersome operation steps, and the antibodies produced are strictly murine proteins, which extremely limits the use of this technology. The next generation of improved hybridoma technology is already in development (Tomita & Tsumoto, 2011), but the challenge of lacking high-throughput monoclonal antibody production platform remains (Layton, Laverty, & Nice, 2013).

2.2 Cell Electrofusion within microfluidic devices

The production of hybridoma cell line appears to be the key factor affecting the production of monoclonal antibodies, accordingly, cell fusion method plays an important role in hybridoma technology. Methods such as virus-induced cell fusion (Nagata, Yamamoto, Ueno, Kurata, & Chiba, 1991), polyethylene glycol (PEG) -induced cell fusion (Lane, 1985), and electrical pulse-induced cell fusion (Karsten et al., 1985) have been developed for cell-to-cell fusion. To control the fusion process of cells, electrofusion has been widely used. However, during electrofusion, the cells are randomly aligned into pearl-chains, resulting in the efficiency of the electrofusion method for producing hybridomas is still low. Microfluidic system enables precisely to control individual cells to achieve high-throughput cell pairing and cell electroporation in the process of cell fusion (D. T. Chiu, 2001; N. Hu, Yang, Joob, Banerjeeb, & Qian, 2013). The 3D thin film microelectrode array was wrapped on the wall of the serpentine microchannel of the chip, which induced electroporation and electrofusion at a lower voltage (˜9 V) to reduce the damage of cells and avoid multi-cells fusion (N. Hu, Yang, Qian, Joo, & Zheng, 2011), resulting in improvements in fusion efficiency compared with the PEG method.

Skelley (Skelley, Kirak, Suh, Jaenisch, & Voldman, 2009) proposed a microfluidic device based on hydrodynamic trapping suitable for different types of cell fusion, which contains a dense array of weir-type passive hydrodynamic cell trap. The device achieved a pairing efficiency of up to 70% and a fusion yield of 90%, and cells after fusion were continuously survived in culture, which is necessary for subsequent antibody secretion. Moreover, these devices can also dynamically monitor the process of cell fusion to understand the interaction between cells (Dura, Liu, & Voldman, 2014). To verify the functionality of the device, a chip with 783 traps was used for human $CD19^{+}$ B cells and mouse myeloma cells (Figure 3A) (Kemna, Wolbers, Vermes, & van den Berg, 2011). After optimizing the size of the traps and electrofusion parameters, the highest fusion efficiency was 50.6 +- 11.1% (103/198) when applying six DC pulses of 100 μs (2.5 kV/cm) with AC, the final yield of hybridoma in long-term conditioned culture was 1.2% (2/166) (Figure 3B-C).

Lu (Lu1, Pendharkar, Lu, Chang, & Liu, 2015) combined hydrodynamic trapping with positive dielectrophoretic force to develop a chip consisting of 960 pairs of trapping channels. The results indicated that the pairing efficiency of 68% was achieved under the electric field of 1 kV/cm, and the electric fusion efficiency was 64% under the electric field of 100 μs (2 kV/cm). In particular, it is easier to operate for cell loading and unloading (Figure3D). As shown in Figure 3E-G, another study introduced the unconnected gold plating pads as high density electric traps to complete the hybridoma fusion under high density cells (Hamdi, Francais, Subra, Dufour-Gergam, & Le Pioufle, 2013).

He (He et al., 2019) presented a complete microfluidic system that integrated cell fusion modality and cell culture modality to reduce possible contamination during cell transfer and provided an environment for the long-term cultivation of hybridoma cells (Figure 4A). As an alternative method, a droplet-based microfluidic system can also be created for a high-throughput hybridoma production platform, including cell encapsulation, droplet pairing, droplet fusion and droplet shrinkage, then the cells form intimate contact for subsequent electrical fusion (R. M. Schoeman, Kemna, Wolbers, & van den Berg, 2014), as shown in Figure 4B. The results confirmed that the fusion rate of around 5%, droplet electrofusion was a promising cell fusion platform (Rogier M. Schoeman et al., 2018).

2.3 Hybridoma cells screening within microfluidic device

After electrofusion of cells in microfluidics at the single-cell level, it is also necessary to consider separation of fused cells from the mixture of unfused cells. Therefore, complete hybridoma technique of monoclonal antibody screening platform in all other respects, including analysis of hybridoma cells, detection of secreted antibody and sorting of hybridoma cells, are the most important in separating of hybrid cells from the mixture. Microfluidic technologies have emerged as a great solution for hybridoma cell screening. In the following sections, we will specify the development status and analysis of hybridoma cells screening technology based microfluidic chips.

2.3.1 Detection and analysis of hybridoma cells

Appropriate and accurate specific monoclonal antibody detection and characterization method are critical for downstream hybridoma cell screening in a short period of time. Conventional enzyme-linked immunosorbent assay (ELISA) methods require microliter volumes of reagents with the interaction of the labeled secondary antibody and the substrate to detect antibodies by enzymatic reaction in microplate(Cervino, Weber, Knopp, & Niessner, 2008; Kazuhiro Sasaki, Thomas R. Glass, & Ohmura, 2005). Moreover, surface plasmon resonance (SPR) and bio-layer interferometry (BLI) are the most widely used technique for the characterization or antibody screening, which extensively facilitate the discovery and development of monoclonal antibodies (Karlsson, Pol, & Frostell, 2016; Lad et al., 2015). It can be directly applied for analyzing the kinetic process between antigens and antibodies or screen high affinity antibodies based on binding kinetics.

Some advanced high-throughput microarray-based methods have been developed to identify monoclonal antibodies in hybridoma cell supernatants (Love, Ronan, Grotenbreg, Veen, & Ploegh, 2006; Rieger, Cervino, Sauceda, Niessner, & Knopp, 2009; Staudt, Muller-Sienerth, & Wright, 2014). However, these methods are involved in manual mixing and washing steps, which are cumbersome and complicated, and the entire experiments is a time-consuming process. Here, Lai (Lai et al., 2004) reported a compact disk (CD) microfluidic device by controlling the rotation speed of the CD to automatically perform ELISA assay for rat immunoglobulin G (IgG) identification. The flow sequence of different solutions in the process was controlled by the centrifugal and capillary forces. The result showed that IgG from hybridoma cell culture was effectively detected in this platform, and the detection limit was 5 mg/L (31 nM). The most striking result was less reagent consumption and shorter assay time compared to conventional protocol. In addition, Espulgar (Espulgar, Tadokoro, Tamiya, & Saito, 2019) reduced the reaction time and operation time of the centrifugal microfluidic ELISA by using the rotational speed to control the fluid flow. The results showed that the detection limit of IgA was 6.16 ng/mL.

Cohen (Cohen, Sabhachandani, Golberg, & Konry, 2015) introduced the microsphere-based assay into a microfluidic device for detecting anti-TNF-α antibody concentration dynamic changes without washing steps required by standard immunoassays. Furthermore, microfluidic was more effective than traditional SDS-PAGE in determining the purity and size of IgG in hybridoma cell culture (Ohashi, Otero, Chwistek, & Hamel, 2002).

Droplet-based microfluidic devices have great advantages in detecting antibodies secreted by hybridomas, the small volumes of droplets enable the concentrations of secreted molecules to rapidly attain detectable levels in several hours (Koster et al., 2008). As shown in Figure 5A, Joensson (Joensson, Zhang, Uhlen, & Andersson-Svahn, 2012) used a fluorescently labeled peptide (Fc-III) as a tracer to measure molecular rotation for fluorescent polarization (FP) analysis in droplets to detect antibodies, as the rotation of fluorescein slows down while FP increases. Results indicated that containing 5 μM antibodies from droplets were detected by the droplet-based FP assay with an accuracy of 95% for distinguishing a droplet containing rabbit IgG from a droplet without antibodies. Besides, FP measurement was also used to antigen-antibody interaction analysis and dissociation constant determination (Figure 5B) (Choi, Kang, Park, deMello, & Chang, 2012).

In addition to droplet-based microfluidics, Romanuik (Romanuik et al., 2010) developed a hydrodynamic microfluidic to trap antibody-secreting cells. The device was designed concave traps inset into serpentine

bends and spirals with 5-20 mouse B cell hybridoma cells/well effectively trapped in 10 minutes. Moreover, cells remained viable for 24 h by using a Cr-free substrate, which was essential for the surface plasmon-resonant (SPR) to real-time identification of those secreting high-affinity antibodies against the target antigen. Subsequently, extraordinary optical transmission (EOT) was used to detect antibodies secreted by trapped hybridoma cells (Romanuik et al., 2011). This system is similar to SPR and has great potential for label-free, real-time, and rapid detection. However, this system required high-density hybridoma cells to generate sufficient antibodies for detection by sensors and did not distinguish between individual antibody secreted cells. As shown in Figure 5C, Anupam (Anupam Singhal, Charles A. Haynes, & Hansen, 2010) described a microfluidic fluorescence bead assay for antibody-antigen binding kinetics measurement in single cells. A single mouse hybridoma cell was trapped by the microfluidic, then protein A beads coated anti-mouse antibodies were introduced into the adjacency of a hybridoma cell for 1 h of co-incubation, and the antibody-of-interest was captured by the beads. Subsequently, antibody-antigen binding kinetics assays were performed by washing with fluorescently labeled antigens for direct assays and indirect assays by replacing the saturated free-labeled antigens with fluorescently labeled antigen. Fluorescent intensity analysis data showed that rate constants were consistent with SPR spectroscopy. Surprisingly, detection limits and sample consumption were reduced by four orders of magnitude compared to SPR, which was exceedingly beneficial in directly high-throughput characterization low-abundance antibodies secreted by single hybridoma cells.

2.3.2 Sorting of hybridoma cells

In addition to the hybridoma cell analysis and detection system, a cell sorting system should be included in the hybridoma screening platform, which can accurately and high-throughput sorting antibody-secreting cells that bind to the target based on upstream detection results. A flow cytometer or fluorescence-activated cell sorter (FACS) is a widely used tool for cell sorting (Herzenberg et al., 2002), but the limitation is that requires fluorescent labeling on the cell surface or inside to be used for hybridoma screening (Dippong et al., 2017). It can be drastically improved by combining ultrahigh-throughput droplet-based microfluidics with the screening capacity of flow cytometry (Lim & Abate, 2013). Usually, signal amplifying agents are added into the droplet to increase the detection sensitivity to detect the target signal in a short time. It follows that droplet-based microfluidics provides an alternative method for hybridoma cells screening, which includes a microfluidic chip encapsulating cell for droplet generation (Clausell-Tormos et al., 2008; Collins, Neild, deMello, Qun, & Ai, 2010) and a microfluidic chip for cell sorting (Baret et al., 2009; Frenzel & Merten, 2017) (Figure 6A and B respectively).

Drops were generated on a microfluidic chip using a 2-phase system, in which cell was compartmentalized in an aqueous microdroplet (1 pL to 10 nL) surrounded by an immiscible oil with biocompatibility surfactants. Meanwhile, the recovery procedures of encapsulated cells was established and the entire microfluidic-based process remained cell viability and survival rates over a period of several days (Clausell-Tormos et al., 2008; Eric Brouzes, 2009). As shown in Figure 6C and D, The screening and sorting of hybridomas expressing inhibitory antibodies were performed on this platform based on the difference in fluorescence intensity (El Debs, Utharala, Balyasnikova, Griffiths, & Merten, 2012). 4E3-hybridoma cells, producing monoclonal antibody inhibit angiotensin converting enzyme 1 (ACE-1), were encapsulated with ACE-1 in the same droplet. After incubation, the droplets were fused with a second droplet containing fluorogenic ACE-1 substrate in another microfluidic device. Subsequently, low fluorescence intensity droplets (indicating antibody inhibit ACE-1 activity) were sorted by fluorescence-activated droplet sorting (FADS) system and cells were recovered from droplets for cell culture supernatants ACE-1-inhibitory activity analysis. The approach enabled the sorting rate to reach at 5×10^4cells per hour and a 9,400-fold enrichment of hybridoma cells and the desired properties antibodies strong inhibitory effect were observed in cell supernatants.

In contrast to inhibition assay, Mazutis (Mazutis et al., 2013) described a protocol for a binding sandwich assay without washing steps to sort antibody production from single mouse hybridoma cells. Single 9E10 hybridoma cell and detection antibodies were co-compartmentalized in 50-pl droplets with streptavidin bead, which was covered with capture antibodies against the Fc fragment of mouse IgG antibodies. After only incubation for 15 min, the secreted antibodies reached the detectable levels. The capture of secreted antibodies

on the bead and bound of the fluorescence detection antibodies in a sandwich assay resulting in a clearly distinguishable high intensity fluorescence peak (Figure 6E). Then fluorescent signals on the bead via based on the electric force of dielectrophoresis for fluorescence activated droplet sorting. More importantly, this protocol required only 2-6 hours to efficient screening and enrichment one million cells and the sorting rate reached above 1 kHZ.

Droplet-based microfluidic high-throughput screening is usually performed at a single cell level, where dual-color sorting in one droplet with two different cells was reported. According to Poisson statistics, theoretically only 13.5% possible single droplet contains two cells, however the efficiency is as high as 87% after dual-color sorting (H. Hu, Eustace, & Merten, 2015). Here shown in Figure 7A, Shembekar (Nachiket Shembekar, Hu, Eustace, & Merten, 2018) developed dual-color normalized fluorescence readout system in droplet-based microfluidics for screening antibodies against cell-surface receptors, in which OKT9 hybridoma cell and CTV-labeled leukemic K562 target cell were co-encapsulated in droplets (100 μm aqueous droplets in oil). Fluorescently labeled goat anti-mouse antibody in the droplets were utilized to generate sufficient binding signal for laser spectroscopy. In case the specific antibodies secreted by the hybridoma cells bound to the cell-surface receptors, then fluorophores of the secondary antibodies got localized on the target cell. Droplets were sorted by another dielectrophoresis mechanism FADS microfluidic device based on the narrow high-intensity fluorescence peak detected by laser spectrometer. The real-time PCR analysis result showed that after sorting target droplets could be enriched up to 220-fold and antibody concentrations as little as 50 ng/mL could be sorted in high-throughput using this device. Furthermore, by reducing the size of the droplets to reach the concentration detection limit, the time required for detection can be further reduced to ensure cell viability in the droplets. An alternative method can further improve two cells pairing and co-encapsulation efficiency, first generating droplets of two different types of cells respectively and eliminated empty droplets via preliminary sorting. Then, the two different types of droplets were paired on a one-to-one basis and merged to generate droplets that contain exactly two different cells (Chung, Núñez, Cai, & Kurabayashi, 2017). Subsequently, an integrated microfluidic system that performs merging of the two droplet populations was developed for the screening of neutralizing antibodies. In this platform, a single hybridoma cell was encapsulated in the droplet produces neutralizing antibodies against murine hepatitis virus. The merged droplet of the hybridoma cell and the virus was then merged with the host cell droplet and the hybridoma cells were screened based on the prototype fluorescence imaging of the infection of the host by the virus (Wippold et al., 2020) (Figure 7B). These microfluidic systems are promising tools to generate cell pairs for studying cell-cell interactions or screening hybridoma cells against surface receptors.

Screening throughput and automation have been significantly improved in droplet microfluidic systems. However, the limitation of this platform is that detection reagents and hybridoma cells are usually co-encapsulated in the same droplet, consequently unbound fluorescent labels are difficult removed from the droplet, resulting in a high background signal. Akbari (Akbari & Pirbodaghi, 2014) encapsulated single hybridoma cells in alginate microparticles and three capture antibodies were added (Figure 7C). The antibody secreted by the cell formed a macromolecules complex with the fluorescent antigen and the capture antibody. After breaking the emulsion, the porous nanostructure of alginate enabled the cell and the antigen-antibody molecule complex remained inside the particle, and unbound fluorescent antigen diffused out of the particle, extremely improved detection efficiency. The results indicated that the anti-TNFα antibody secreting hybridoma cells were screened from 9E10 hybridoma cell mixture. (Figure 7D).

Zhang (Zhang et al., 2020) developed a novel hydrodynamic force based microfluidic method that could perform multiple rounds of screening processes, including trapping, proliferation, transfer of hybridomas and fluorescent identification of antigen-antibody binding (see Figure 8). As a verification of the method, high-producing anti-CD45 hybridoma cells were successfully screened after two rounds of screening. More importantly, the entire device reduced the time cost from 14 days required for a plate-based limited dilution to 5 days and with higher antibody yield.

The hybridoma technique, as the most representative monoclonal antibodies manufacture method, is demonstrated in the microfluidic system. Table 1 summarizes the advantages and drawbacks of the microfluidic

applied in hybridoma technique. It is expected to be a useful platform for rapid determination and screening of hybridoma cells. A short overview of some recent advances is outlined in Table 2. Currently, nearly all devices are model screening and verifying by incorporating irrelevant hybridoma cells, and it is still in the laboratory stage. Despite this, microfluidic showing great potential in high-throughput screening. In order to broaden its applications, the development of hybridoma technology on a chip in the future should construct an effortless method and increase throughput, and even to eliminate the false positive.

3.Antibody engineering technique in the microfluidic device

Compared with hybridoma technology, recombinantly engineering antibody techniques have contributed enormously to the field of monoclonal antibody production in rapidly obtaining humanized transformations of candidate antibodies and reducing the required cost and development cycle. The researchers have utilized the high integration and the ultra-high throughput of microfluidic chips to develop recombinant antibody screening based on microfluidic chips. Here we specify the progress of four antibody engineering techniques using the microfluidic platform: phage display, single B cell antibody screening, antibody expression and cell-free protein synthesis.

3.1 Phage display technology

In the past few decades, phage display technology has been developed into a mature and widely used method for antibody discovery (Nixon, Sexton, & Ladner, 2014; Rahbarnia et al., 2017). Principle of antibody phage display: (a) The whole set of antibody genes of the antibody-secreting cells are amplified by PCR, (b) the foreign DNA fragment is inserted into an appropriate phage vector, and express and amplify the target gene library by phage transfecting bacteria, (c) Enriching phage expressing specific antibodies by several rounds of panning, (d) the target phage is sequenced to obtain the antigen-specific human antibody gene sequence, and finally the specific fully human therapeutic antibody is generated by using genetic engineering technology (Hammers & Stanley, 2014). In order to obtain antibodies with the desired specificity, the selection process or panning step of phage antibody libraries is a key step in the phage display technology (Figure 9A). Microfluidic is a powerful device allowing screening of phage, which integrated incubation, washing, cell lysis, and lysate collection steps into one chip for eliminating contamination and improving screening efficiency (J. Wang et al., 2011).

A study by Persson showed antigen-coated beads based microfluidic chip for the efficient enrichment of phages displaying specific binders from the single chain antibody fragment (scFv) phage display libraries (Persson, Augustsson, Laurell, & Ohlin, 2008) (Figure 9B). It achieved 1000-fold enrichment efficiency in a single chip comprising two serially coupled separation channels in a single round of selection. This automated high throughput of the chip-based microfluidic wash system allowed fast and efficient separation of bound and unbound phages. Similarly, microfluidic panning has been optimized to identify high-affinity phage display peptides of multiple targets in one round without requiring any bacterial culture (Cung et al., 2012). The entire screening took only four hours to complete, which resulted in less time and labor consumption. Additionally, Figure 9C shows an example of panning conditions can also be precisely controlled during phage selection (Liu et al., 2009). The elution kinetics of M13 phage was evaluated on a microfluidic platform to optimize parameters for elutriation, highly specific binding phages could be obtained at critical washing drag force and washing time of 5.9×10^{-8} dyne and 1.1 min respectively (Park, Lee, & Nam, 2016).

Automation of microfluidic screening will speed up the phage antibody library screening process and improve efficiency, but the washing process results in the loss or inability to be enriched of clones. As shown in Figure 9D, Hsiao (Hsiao et al., 2016) proposed a continuous microfluidic driven by two alternating orthogonal electric field drives for the screening and sorting of scFv displayed on phages without washing, elution or magnetic beads. The phage was electrophoresed on an agarose gel immobilized with the target antigen, and phage clones were enriched at different outlets according to the difference in electric field force and

affinity. In 40 minutes, 10^5 CFU specific phages against VEGF-R2 were isolated from a phage library with a capacity of 3×10^9, which provide an automated high-throughput platform for the future development of phage antibody libraries. In contrast, Zheng (Zheng et al., 2018) described a method of separating phage display antibodies based on the activity or function of the antibody, without requirement for the binding affinity of the antibody to the antigen. This method used microfluidic to generate a micro-ecosystem by encapsulating 10 pL droplets of target cells and phage-producing bacteria, combined with FACS to select functional antibodies from diverse antibody repertoire.

3.2 Single B cell antibody screening technology

With the development of genomic technology and protein technology, the emergence of single B-cell antibodies has evolved as an newly arisen screening tool, especially for the monoclonal antibody (Tiller, 2011). Compared with phage display, single B cell antibody screening technology improve the effectiveness of the antibody as well as yield. Single B cell antibody preparation technology is to clone and express the genes of antibodies encoded by a single antigen-specific B cell in vitro (Carbonetti et al., 2017; Huang et al., 2013; Tiller, Busse, & Wardemann, 2009). This method retains the natural pairing of the light and heavy chain (B. Wang et al., 2018), and has significant advantages of genetic diversity (Starkie, Compson, Rapecki, & Lightwood, 2016), high efficiency (Boehmer et al., 2016), completely humanized (Smith et al., 2009). Therefore, monoclonal antibodies can be obtained in a way that does not require immortalization of B cells or fusion with myeloma cells, simultaneously increasing the likelihood of finding valuable rare antibodies. However, the processing of a large number of B cells is laborious and time-consuming. Microfluidics provides an option that isolation of single B cells and the high-throughput processing of single B cells without required any cell proliferation or immortalization (El Debs et al., 2012; Nachiket Shembekar et al., 2018). A spiral microfluidic chip was used to isolate human peripheral blood B lymphocytes, which facilitated the high-throughput (1.3×10^5 cells/min) separation with cell viability of up to 95% (P. L. Chiu, Chang, Lin, Tsou, & Li, 2019).

Adler (Adler, Mizrahi, Spindler, Adams, Asensio, Edgar, Leong, Leong, & Johnson, 2017) used an emulsion droplet microfluidics combined with antibody engineering method to screen antibody repertoire from millions of single B cells. B cells were isolated from immunogen immunized lymph node of mice or peripheral B cells of human donors, and then encapsulated into droplets by co-flow emulsion droplet microfluidic chip for mRNA capturing, PCR amplification of antibody gene sequence. Subsequently, the native pairing of heavy and light chain amplicons was expressed using yeast for single-chain variable fragment (scFv) display and identified high affinity scFv by several rounds of FACS. Finally, deep sequencing of the scFv libraries was carried out to identify all clones and functional verification of Full-length antibodies expressed in Chinese hamster ovary cells was performed (Figure 10A).

Rare, high-affinity mouse anti-PD-1 antibodies were rapidly screened from the B cells of immunized Balb/c and SJL mice (Adler, Mizrahi, Spindler, Adams, Asensio, Edgar, Leong, Leong, & Johnson, 2017). After construction of the scFv library, 269 strong scFv binders against PD-1 were discovered and antibody sequences were further characterized, 17 scFvs were finally selected to express as full-length monoclonal antibodies. The binding specificity and affinity of all antibodies against PD-1 with K_D was less than 500 nM. Surprisingly, one antibody was $K_D < 1$ pM. This method is promising to enrich more candidates for immune checkpoint therapy. Additionally, natively paired human antibody repertoires have been successfully identified from peripheral B cells of human donors using this platform. Anti-influenza A and anti-pneumococcus full-length antibodies were produced, while the selected monoclonal antibodies inhibition of respective pathogens/serotypes has been demonstrated by functional validation (Adler, Mizrahi, Spindler, Adams, Asensio, Edgar, Leong, Leong, Roalfe, et al., 2017). Notably, the pneumococcus vaccinated library showed higher affinity maturation frequency than the non-vaccinated library in human antibody libraries, which was not limited by the low affinity antibody produced by the natural B cell. Similarly, the natively paired versus randomly paired of heavy and light chain of antibody was further extensive studied (Adler et al., 2018). The results demonstrated that the natively paired antibodies had more advantages in binding specificity and affinity than the randomly paired antibodies. On the other hand, both two paired methods generated

more distinct antibodies, which ensured the diversity of antibodies. Recently, it was also shown that using different immunization protocols in parallel yielded greater immunoglobulin diversity of candidate antibodies (Asensio et al., 2019). In summary, by using this method, the construction of the entire scFv library required only three weeks, and the full-length antibody libraries could be used for high-throughput parallel function screening. Thus, the droplet-based microfluidic B-cell screening platform offers a huge tool for a broad antibody repertoires analysis and monoclonal antibody development. However, there was no specific identified of B cells before amplification and clonal antibody genes, and the affinity sorting was identified until the entire scFv library was constructed, which still resulted in a lot of reagent consumption and redundant labor in this process.

Due to the rate and affinity of antibody secretion by B cells, it is difficult to perform early functional experimental verification of large-scale B cells at the single cell level. The presence of a droplet-based microfluidic technology allows massively parallel kinetic analyses of secreted antibody, with simultaneous measurement of antibody secretion rate over time in single B cells from mice immunized with tetanus toxoid (Eyer et al., 2017). After encapsulating B cells with paramagnetic nanoparticles and fluorescently labeled detection reagents in droplets, a Fluorescence-based sandwich immunoassay in-droplet was performed for quantification IgG and calculated the secretion rate and the strength of the interaction (Figure 10B). It could detect the secretion rate and affinity of the antibody secreted by a single B cell in just 30 minutes (Bounab et al., 2020). The protocol enabled analyze of more than 300,000 droplets in one two-dimensional droplet array and could be scalable up to millions of droplets. Subsequently, a droplet microfluidic, CelliGO, capable of single cell screening and droplet sequencing based on the measurement of secreted IgG at the single B cell level was described (Gerard et al., 2020). Finally, 77 kinds of recombinant antibodies were generated from the sequences determined by the system.

Winters (Winters et al., 2019) described a complete, commercially available nanofluidic optoelectronic single B lymphocyte antibody screening technique. B cells were imported into individual nanopens chip, then a bead-based two-color fluorescent binding assay was used to identify IgG specificity and B cell screening via gravity or light-induced dielectrophoresis. It only took approximately five hours to complete this process. The variable region gene sequence of antibody was subsequently amplified and monoclonal antibodies were expressed recombinantly. However, while this technology offers a novel, integrated platform, due to the fixed number of nanopens on the chip, typically no more than 3513 cells can be processed. Better performance can be expected by further microfluidic chip design and optimization.

The latest research shows that neutralizing antibodies against SARS-CoV-2 were isolated from B cells in combination with microfluidic high-throughput screening methods (Jones et al., 2020). Taken together, Single B cell antibody technology based on microfluidics has become an attractive method, a highly diverse antibody library is capable of generating high affinity matured and specific antibodies. With the optimization and development of related technologies, we believe that single B cell antibody preparation technology will be further developed and matured in the field of producing fully human antibodies.

3.3 Antibody expression technology

Recombinant antibodies have occupied an increasingly important position in the field of antibody production. Immunoglobulin molecules are appropriately processed and transformed at the genetic level, and reassembly of antibody molecules are expressed after introduction into recipient cells. The strategy of combining cells expressing target proteins with sorting equipment is used to quickly and accurately obtain recombinant protein expression. In order to select high-yield and stable transgene cell lines from a heterogeneous cell population, Droz (Droz et al., 2017) developed a method that combines a microfluidic cartridge with ClonePix to efficient enrichment of cell populations secreting very high levels of a recombinant protein. In this approach, CHO cells, transfection to stably expressing the Trastuzumab IgG, were sorted based on magnetic markers of target proteins (Figure 11A). Another strategy used Gel microdroplet (GMD) – fluorescence activated cell sorting (Yanakieva et al., 2020). P pastoris expressing full-length monoclonal antibodies and mammalian target cells were co-encapsulated in GMDs, then IgG antibodies bound to the epidermal growth factor

receptor (EGFR) target cells were detected by fluorescently labeled antibodies for FACS screening (Fang, Chu, Ackerman, & Griswold, 2017) (Figure 11B). As a proof of concept, anti-EGFR antibody clones were efficiently enriched 4000-fold in three days.

Admittedly, expression yield is a complex trait, which is affected by many factors. For example, cell growth metabolism directly determines the yield and quality of antibody expression in cell culture. Scale production of antibodies requires strict control of cell culture microenvironment that affects productivity. The high degree of control of cell culture conditions by microfluidic systems has been widely reported in literatures (Mehling & Tay, 2014; W, Huangb, & Lee, 2010). The subsequent development of microfluidic-based microbioreactors offer numerous advantages, including easy operation, automated, and low cost (Ladner et al., 2017). The first considerations when culturing cells on microfluidics are adherent culture conditions for adherent cells. Barbulovic-Nad (Barbulovic-Nad, Au, & Wheeler, 2010) developed a complete CHO-K1 cell culture digital microfluidic system including cell seeding, growth, media exchange, cell subculture, and transfection. Indeed, fibronectin modification of the device facilitated better adherent growth of cells. Subsequently, the fibronectin modification was also used to culture CHO cells on a continuous flow bioreactor to produce Infliximab (against α-TNF cell receptor) and each microdevice produced 14.4 μg monoclonal antibody in 24 h, at a flow rate of 0.005 mL/min (Garza-Garcı´a et al., 2013). Productivity has been significantly improved 100 times higher than the full-scale systems. After optimized the geometry and flow rate of the microdevice, the production rate of this antibody increased by 3 orders of magnitude (Garza-García et al., 2014).

Another approach, Poly-D-lysine as a cell adhesion molecule in microfluidics has been proved (Peñaherrera et al., 2016). Bourguignon (Bourguignon et al., 2018) presented a microfluidic structure with cisterns microchannels for the production of monoclonal antibodies. CHO-K1 cells were able to adherently cultured on the surface of microchannels coated with poly-D-lysine for up to two months, which was perfectly suitable for the period of recombinant antibody expression. The CHO cell line recombinantly expressing chimeric antibody anti-hIFN-α2b was further evaluated using this system. Results showed antibody expression levels increased over time, reaching a maximum value (166.4 μg/mL) at 24 days and the yield was significantly higher (5.89-fold) than cell cultures in T-flasks without affecting the quality attributes of the antibody. Similarly, the microfluidic device was also suitable for HEK cells culturing and antibody expressing (Peñaherrera et al., 2016). Interestingly, the expression level of monoclonal antibody by CHO was significantly higher than that of HEK cell lines under the same conditions (Bourguignon et al., 2018).

Alternatively, microfluidic devices provide a suitable environment for growing suspended microbial cells for production of monoclonal antibodies. For example, Pichia pastoris were cultured over 11 days for continuous and stable expression of heterologous proteins (Mozdzierz et al., 2015). An advantage of the microfluidic bioreactor compared to the macroscale bioreactors was that the former has higher productivity (Brás, Chu, Aires-Barros, Conde, & Fernandes, 2017). Finally, automated purification and separation of recombinantly expressed proteins were realized with the integrated chromatography module on the microfluidic chip (Millet, Lucheon, Standaert, Retterer, & Doktycz, 2015). In conclusion, the novel antibody expression platform has the potential to increase the efficiency of biopharmaceutical processes and promote the improvement of antibody drug production level and product quality.

3.4 cell-free protein synthesis system

The cell-free protein synthesis system (CFPS) has become a powerful technology platform, which can rapidly and accurately synthesize desired therapeutic proteins without being affected by host cells and transfection efficiency. This technique uses exogenous DNA or mRNA as a template, supplements substrates and energy substances, and synthesizes antibody under the action of various enzymes provided by cell extracts (Stech & Kubick, 2015).

Microfluidic was reported in the cell-free protein synthesis system that achieves high-throughput protein expression while reducing reagent consumption, thereby reducing protein synthesis costs (Figure 12A) (Damiati, Kompella, Damiati, & Kodzius, 2018; Damiati, Mhanna, Kodzius, & Ehmoser, 2018). Running for 10

hours on the serpentine channel continuous microfluidic reactor, the protein yield increased by 40% compared with conventional batch reactions (Timm, Shankles, Foster, Doktycz, & Retterer, 2015). Further optimization of microfluidics, integrated dialysis membrane in the array system, passive exchange of reaction solution and reaction by-products through the dialysis membrane to improve protein yield (Jackson, Jin, & Fan, 2015). As shown in Figure 12B, Xiao (Xiao et al., 2018) developed an on-chip protein synthesis device that integrates protein purification functions. As a result, one chip directly harvested 10.1 μg of pure protein. In summary, the system has proven its potential to be used for antibody production by simply replacing template DNA.

The antibody protein candidates synthesize in vitro also need to be further evaluated to obtain high-affinity molecules. Tabata (Tabata et al., 2009) performed a Biacore microfluidic chip for selection of high affinity and specificity anti-p53 and anti-MDM2 scFv from 10^{12} molecules. This approach enabled 10^6 to 10^8-fold enrichment per round. However, the process of coating the complex antigen on the chip is still cumbersome. Seefeld (Seefeld, Halpern, & Corn, 2012) constructed on-chip protein-coated biosensors through cell-free protein synthesis and could be used for antibody measurement (Figure 12C). The entire process took just a few hours to complete, and integrating it into an antibody synthesis chip will have the potential to perform real-time, rapid measurement of antibodies.

Traditional antibody screening methods have been proven successful, but there are obvious limitations of these methods. The novel antibody screening platforms based on the combination of technology in a flexible way have emerged. A comparison of antibody engineering technology between traditional methods and microfluidic methods is given in table 3. In summary, the use of microfluidic system-based antibody engineering screening is considered to be a more effective method.

4.Conclusion and perspectives

After decades of development, a variety of monoclonal antibody production technology (including hybridoma technology, phage display, yeast display, cell-free display, and single B-cell antibody technology) is becoming more and more mature. These methods are designed to accelerate the screening process of antibody development and obtain better quality antibodies. Microfluidics technology is recently a highly interesting platform in the field of antibody preparation, which can solve the high dependence of these technologies on equipment. This article summarizes the research progress of microfluidic-based devices in antibody preparation and screening and compares traditional methods with various antibody technologies based on microfluidic.

The microfluidic chip has wide applications by virtue of properties such as low sample and reagent consumption, high throughput, fast operation speed and low application cost. More importantly, this integrated microchip-type automatic analysis platform can effectively avoid errors caused by human operations, and the data obtained is more reliable. In the application of hybridoma technology, microfluidics based single-cell manipulation performs universally in cell electrofusion and hybridoma cell screening. It has played an important role in improving the efficiency of cell fusion to increase in number of hybridoma cells. After exerting a high-throughput hybridoma cell screening chip, it is expected to obtain more hybridoma cell lines capable of secreting specific antibodies. However, there are some limitations. In order to achieve the desired function, an appropriate microfluidic structure and control system needs to be designed. The current microfluidic platforms are modular with different functions, and a complete set of integrated equipment for cell fusion, cell sorting, and antibody detection has not be created. On the other hand, combining traditional antibody engineering technology with microfluidic platform can also be executed on microfluidic platform, enriching antibody preparation methods. The cost and complexity of engineering antibody production are reduced with a microfluidic system. Integrating microfluidic technology with other mature antibody detection and characterization technologies, this powerful technology can be broadened for the development of various types of antibodies. However, the technology is still in the laboratory stage, and it needs further polishing from the actual application. The focus in the future is to integrate microfluidic platform with different functions and develop complete and highly automated device. Therefore, the whole process of antibody preparation will

probably be realized on a chip. It is reasonable to believe that antibody preparation based on microfluidic devices would be a very meaningful development for the biopharmaceutical field.

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Author contributions

Conceptualization: H.S. and J.W.; literature research: H.S. and N.H.; graphing: H.S.; writing (review and editing): H.S., N.H., and J.W.; supervision: J.W.

Conflicts of Interest

The authors declare no conflict of interest.

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