Advances in hybridoma preparation using electrofusion technology

Jiaqian Kou[1], Jianzhong Shen [1], zhanhui wang[1], and Wenbo Yu[1]

[1]China Agricultural University

August 22, 2022

Abstract

As a rapidly developing cell engineering technique, cell electrofusion has been increasingly applied in the field of hybridoma preparation in recent years. However, electrofusion is a certain degree of difficulty to completely replace the polyethylene glycol-mediated cell fusion. The key elements limiting electrofusion in the field of hybridoma preparation are practical complicated. This review summarizes the state of art of cell electrofusion in hybridoma preparation based on recent published literatures, mainly focusing on electrofusion instruments and their components, process control, cell treatment, and process characterization. The review provides new information and insightful commentary, critically important to the promotion of further electrofusion development in the field of hybridoma preparation.

Advances in hybridoma preparation using electrofusion technology

Jiaqian Kou, Jianzhong Shen, Zhanhui Wang*, Wenbo Yu*

College of Veterinary Medicine, China Agricultural University, Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, and Beijing Laboratory for Food Quality and Safety, Beijing 100193, People's Republic of China

*Author to whom correspondence should be addressed:

Wenbo Yu: yuwenbo@cau.edu.cn

Zhanhui Wang: wangzhanhui@cau.edu.cn

Abstract

As a rapidly developing cell engineering technique, cell electrofusion has been increasingly applied in the field of hybridoma preparation in recent years. However, electrofusion is a certain degree of difficulty to completely replace the polyethylene glycol-mediated cell fusion. The key elements limiting electrofusion in the field of hybridoma preparation are practical complicated. This review summarizes the state of art of cell electrofusion in hybridoma preparation based on recent published literatures, mainly focusing on electrofusion instruments and their components, process control, cell treatment, and process characterization. The review provides new information and insightful commentary, critically important to the promotion of further electrofusion development in the field of hybridoma preparation.

Keywords: Electrofusion, Hybridoma, Cell Engineering, Monoclonal Antibody

Contents

Introduction

In 1975, a British scientist Milstein and a German scientist Köhler successfully fused myeloma cells and antibody-producing lymphocytes into a hybridoma[1]. Both of the cell lines have the property of producing monoclonal antibodies against only a specific antigenic determinant, in addition to the property of unlimited proliferation in myeloma cells. This hybridoma technology, for which the two scientists were awarded the Nobel Prize in medicine in 1984, ushered in a new era of monoclonal antibody preparation and use. To date, hybridoma technology remains one of the most important methods for preparing monoclonal antibodies. Based on the induction characteristics, cell fusion methods can be classified into biological[2] (Sendai virus and Japanese hemagglutination virus), chemical[3] (polyethylene glycol (PEG)-mediated fusion method), and physical[4] (centrifugation, vibration, and electrofusion) methods. Among them, PEG-mediated fusion method plays a dominant role in the hybridoma preparation process. However, over time, it was found that the PEG-mediated fusion method still had limitations, such as low fusion efficiency, high cytotoxicity, and high requirements for professional skills. As a new cell engineering technology developed in the 1980s[5], cell electrofusion has become an important technique in modern biomedical research due to its advantages of high fusion yield, low cytotoxicity, and is easily standardized among different laboratories[6]. It has been applied in many fields, such as biological breeding[7,8], cell cloning[9], drug screening[10], cancer therapy[11,12], and hybridoma preparation[13]. However, the influential parameters and factors affecting the electrofusion yield, especially in hybridoma preparation, have not been systematically reviewed by analyzing the relevant literature. Hence, our work providing a timely response to cover recent advances and new trends in cell electrofusion technology for hybridoma preparation.

In this review, we first collected information on available commercial electrofusion instruments for hybridoma preparation and analyzed the parameters of each instrument, followed by an overview of published research literatures on the hybridoma preparation using electrofusion technology. In this review, the following four important aspects involved in the hybridoma preparation using cell electrofusion technology were focused: (I) electrofusion instruments and their components; (II) process control; (III) cell treatment; and (IV) process

characterization. The intrinsic features of these four aspects were discussed, and important case studies were reviewed in depth to Figureure out the challenges of hybridoma preparation using electrofusion technology. Finally, since the hybridoma preparation using electrofusion technology exhibits significant advantages in monoclonal antibody preparation, the prospect of application of this technology was considered.

1 Electrofusion instruments and their components

1.1 Current commercial electrofusion instruments

Cell electrofusion is an important research element in the field of biology and it cannot be developed without corresponding instruments and technologies. With the increasing application of cell electrofusion in recent years, some commercial instruments have been available on the market, such as ECM 2001 LITE/PLUS from BTX (Massachusetts, USA), the Multiporator 4308 from Eppendorf (Sachsen, Germany), the CFB16-HB from BEX (Tokyo, Japan), the CF-150B from BSL (Budapest, Hungary), and CRY-3/3B electrofusion instrument from Scientz (Zhejiang, China).

The current commercial electrofusion instruments all comprise an "electrofusion instrument + fusion chamber" and are connected by electrode wires during operation. The electrofusion instrument is equipped with operating software that controls various electronic parameters during the fusion process, while the fusion chamber plays the role of a cell carrier. The fusion chamber volume varies from microliter- to milliliter-scale. The fusion chambers can be divided into two types according to the number of cells to be fused. Type 1 is a fusion cuvette **(Figure. 1A)** with a volume of 1 mL or more, which is mainly used for electrofusion of intact mouse spleen cells. Type 2 is a fusion microslide **(Figure. 1B)** with a volume of less than 1 mL, which is suitable for precise electrofusion of rare cells[14]. Currently, fusion microslides have been heavily utilized in embryonic stem cell fusion, electroporation of mammalian cells, somatic cell nuclear transfer, and plant protoplast fusion. They have been less often utilized in hybridoma preparation.

< **Figure. 1** >

1.2 Microfluidic electrofusion chip

Although the above-mentioned conventional electrofusion systems can complete a large number of cell electrofusion at one time, they have limited capabilities in cell control at a single-cell level[15,16], leading to precise electrofusion of specific cells. In recent years, the electrofusion systems are gradually developing single-cell methods and improving precision. Microfluidic technology with precisely controlling fluids in nano/microchannels, offers fundamentally new capabilities in the control of cells in space and time and has been already combined with cell electrofusion in some studies due to its advantages with the aim of achieving precise electrofusion at a single-cell level.

1.2.1 Microarray electrofusion chip

Microarray electrofusion chip is designed with a structure in a specific micro-orifice shape to form a non-uniform electric field distribution inside the chip electrodes. Achieving cell arrangement control by utilizing the microfluidic chip at a single-cell level facilitates efficient and precise electrofusion of cells in specific regions of the microarray chip.

The idea of using a micro-orifice for cell fusion was first proposed by the Washizu group in 1989 and then optimized in subsequent studies[17]. The chip consists of a fusion chamber and a pair of parallel electrodes. The chamber is separated by an insulating barrier between two parallel microfluidic channels. A small fusion micro-orifice is set in a specific location[18]. As shown in **Figure. 2A** , the structure enables the electric field to be concentrated at the micro-orifice, where the cells attract and contact each other through dielectrophoresis and are then fused at the micro-orifice. This microarray fusion chip can avoid homologous cell fusion due to the advantages of dividing the microchannel into two separate regions by the microarray. Allowing efficient and precise 1:1 cell electrofusion control. The chip resolved the issues of homologous cell fusion and had a fusion yield of more than 90%. However, due to the limit on the number of micro-orifices in the chip, it is difficult to achieve high-throughput electrofusion. In order to improve its throughput, a

new microarray chip based on previous studies was developed by the same group in 2010[19]. As shown in **Figure. 2B (1)** , this chip has an electrode gap of 400 μm and a series of micro-orifices between the electrodes. Cell fusion throughput can be increased significantly by increasing the number of micro-orifices compared to the previous microarray chip. The cell pairing rate of the chip increases up to 95%. At the end of the electrofusion, the chip is transferred directly from the electrode substrate into the culture medium, so that fused cells stay at the micro-orifice, while the unfused cells are flushed during the transfer process. As shown in**Figure. 2B (2)** , the fused and unfused cells are clearly separated, and the fused cells can also be imaged in real time. The main strength of the study lies in enabling high-throughput cell electrofusion. Moreover, the chip solves the problem of separating fused cells from unfused cells and realizes the visual monitoring of the cell electrofusion process. In 2016, the same group improved the separating step of fused cells from unfused cells by developing a new "air-lock patterning technique", which creates an "adhesion zone" on the micro-orifice bottom[20]. The advantage of this technique is that the fused cells will be located in the "adhesion zone" **(Figure. 2C)** . The separation of the fused and unfused cells can be achieved without extra transfer of the fusion chip, which greatly simplifies the separating procedure.

< Figure. 2 >

Besides the micro-orifice electrofusion chip designed by the Washizu group, channel-type structure-based chips are also used for cell electrofusion. The Hu group at Chongqing University in China has conducted relevant research on this topic. In 2011, they designed a serpentine microfluidic channel **(Figure. 3A)** with a length of 5900 μm. An array of 1015 gold electrodes was arranged in the channel[21]. By introducing this design, a non-uniform electric field distribution was formed inside the electrofusion chip, with the highest electric field intensity near the convex tooth electrode. When flowing along the microfluidic channel, the cells will be attached to the surface of the convex tooth electrode and arranged in the form of a short pearl-chain. The results suggested that the chip structure enabled 99% of the cells to be efficiently arranged in a pearl-chain and 70.7% of the cells formed a 1:1 pairing. Additionally, the special tooth electrode structure of the chip effectively lowered the fusion voltage, that is, the lower fusion voltage only enabled the fusion of the two cells close to the microelectrode. Pearl-chains, which have more than two cells, could not be fused due to insufficient electric field intensity. Hence, this chip can reduce the fusion and avoid multi-cell fusion. However, in this study, the membrane voltage at the end of the pearl-chain was always higher than that at the contact point of the cell chain, which caused cell membrane rupture. To address this problem, the research team improved the electrofusion chip in 2013 **(Figure. 3B)** . In this improved chip, the serpentine microfluidic channel was still used but an additional micro-cavity array was designed on the sidewalls of the microfluidic channel. The purpose of this design is to trap cells and improve the 1:1 cell pairing rate[22]. Moreover, the electric field in the micro-cavity region was relatively concentrated, so that the cell fusion occurred only at the contact point of the cell chain, avoiding the risk of cell rupture. With this improved chip, the 1:1 cell pairing ratio was further improved to 73.9 %. During the electrofusion process in this study, no multi-cell fusion and no cell membrane rupture were observed.

< Figure. 3 >

1.2.2 Microdroplet electrofusion chip

Unlike the microarray electrofusion chip, the microdroplet electrofusion chip utilizes two immiscible fluids, such as oil and water: one as a continuous phase and the other as a dispersed phase. By controlling the flow of the two phases using microfluidic technology, microdroplet generation, transport, fusion, and sorting can be achieved[23,24]. With the properties of monodispersion and picoliter-sized volume, the microdroplet provides a powerful platform for precise cell fusion.

In 2014, the Schoeman group designed a functionally integrated droplet microfluidic chip to finish the droplet encapsulation, droplet pairing, droplet fusion, and droplet shrinkage, followed by cell electrofusion in the droplet[25] **(Figure. 4A)** . The microfluidic chip was designed in the form of a yin-yang-shaped alternating curved rectangular microchannel. It consisted of an elongated funnel that continues into one full loop, followed by three semi loops. The dimensions of the microchannel loops were 27.1 μm high by

50 μm wide, which were spaced 100 μm apart from each other. The microchannel loop structure ensured a single-cell suspension when reaching the T-junction, which was followed by a double T-junction that was responsible for the single-cell encapsulation and droplet pairing. Subsequently, the droplets passed three pitchfork structures to shrink the droplet, so that the two cells remain in close contact in the microdroplets, thereby enabling efficient and precise 1:1 cell electrofusion when they pass through the electrode. The advantage of this microdroplet chip is integration of cell encapsulation, pairing, fusion, droplet shrinkage, and electrofusion, which significantly reduces manual operation and lays the foundation for high-throughput hybridoma preparation in the future. In 2016, the same group designed another cell microfluidic chip similar to that introduced in 2014 **(Figure. 4B)** . The micro channels were also yin-yang-shaped and produced droplets with a volume of 50 pL[26]. The obvious difference from the previous study is that the number of electrodes increased from one to six. All electrodes were able to adequately generate electric field intensity to ensure that cells in droplets can complete the electrofusion as they pass through the electrode region. On one hand, this microfluidic chip can integrate the whole process on one chip. On the other hand, it achieves 1:1 cell pairing and fusion at a single-cell level. Thus, the microdroplet chip has the potential to be a promising platform for precise electrofusion control.

< Figure. 4 >

2 Process control of electrofusion

The electrofusion process usually involves two key stages of cell alignment and cell fusion. First, the cells are arranged in a pearl-chain under an alternating current (AC) electric field, and then the cell membranes are electroporated, followed by electrofusion of adjacent cells. Currently, the commercial electrofusion instruments can set the key electrical parameters of electrofusion process, which include the voltage, pulse duration, frequency, number of pulses, and inter-pulse interval. The information on commonly used commercial electrofusion instruments and their related parameters is shown in **Table 1** . Electrical parameters involved in each stage of the electrofusion process need to be optimized for practical electrofusion research work. Some studies focused on hybridoma preparation have employed the above instruments. Investigators usually need to optimize the parameters in the electrofusion process to be suitable for different types of cells.

< Table 1 >

2.1 Cell alignment

As the primary key step of cell fusion, cell alignment is defined as suspended cells arranged in a pearl-chain by dielectrophoresis force in a non-uniform electric field[27,28]. The principle is that when a voltage is applied to the electrodes, a non-uniform electric field will be formed between the electrodes and the electroneutral cells will be polarized by the electric field to form dipoles. Because the electric field intensity is unequal on two sides of the cell, the polarized dipoles (cells) will migrate to the region of high field intensity. The adjacent dipoles (cells) will make contact with each other via electric field to form a pearl-chain. Three key electrical parameters should be optimized during cell alignment—namely, AC voltage, pulse duration, and frequency—in order to form a pearl-chain with appropriate length, regular arrangement, and minimal damage.

It should be noted that high AC voltage can damage cell membranes and inhibit cell activity, while low AC voltage cannot force cells to contact each other. Therefore, it is necessary to optimize AC voltages in cell alignment. For the preparation of hybridoma for human vasorin protein, Yin et al[29]. used an electrofusion instrument from BEX (Japan) with a 1.5-mL electrofusion chamber (electrode gap of 3 mm, model LF498-3) and observed cell alignment under a microscope (**Figure. 5A**) at four AC voltages of 30 V, 40 V, 50 V, and 60 V (field intensities of 99, 132, 165, and 198 V/cm, respectively). It was found that cells arranged irregularly when AC voltages were too low (30 V and 40 V). The cells were also arranged irregularly at a high AC voltage (60 V) due to the cell membrane damage. Hence, it was concluded that AC voltages that are too high or too low affect the cell alignment. In this study, 50 V (165 V/cm) was chosen as the optimal AC voltage for cell alignment.

The duration of AC voltage application is also an important parameter in cell alignment. Generally, the lower the AC voltages, the longer the duration of AC voltage application required for cell alignment. The recommended AC voltages and time for the ECM2001 LITE electrofusion instrument (BTX, USA) for hybridoma preparation are 70 V and 30 s, respectively. If the duration of AC voltage application is too short, most cells can only form a short pearl-chain rather than a long one. Using a long AC voltage duration, the continuous AC voltages will lead to a temperature change in the fusion chamber, indirectly affecting the cell activity.

As early as the 1980s, German scientists in the Zimmermann group investigated the frequency of electrofusion at the cell alignment stage[30,31]. In the 1990s, the Wang group at Nankai University in China continued to intensively study the frequency at the cell alignment stage[32]. It was found that frequency that is too high or too low has an impact on the cells. The cells were prone to electrolysis when the frequency was too low. Moreover, low frequency may cause a large number of bubbles in the electrofusion chamber. If the frequency was too high, the high frequency will increase the difficulty of design and the manufacturing cost of the circuit. Previous results demonstrated that the optimal frequency in the cell alignment stage for hybridoma preparation was in the range of 1–2 MHz, which was adopted by the most studies[29].

<Figure. 5>

2.2 Cell fusion

Cell fusion is defined as the process in which pearl-chain-arranged cells are activated and generate a membrane potential under a high-intensity electric field. When the external electric field intensity exceeds the threshold of the membrane potential, the cell membrane is transiently reversibly electroporated to form multiple small pores. Cells in contact with each other exchange intracellular substances, and cell membrane fusion occurs simultaneously. By fusion of the myeloma cells with the lymphocytes, hybridoma will acquire the characteristics of unlimited proliferation as well as the ability of secreting specific antibodies. During cell fusion, the electric field intensity, number of pulses, and pulse duration are the key elements of successful electrofusion.

Electric field intensity is one of the most important parameters in cell fusion. As early as 1987, Ohnishi et al[33]. explored the optimal electric field intensity for generation of murine-derived hybridoma. In the study, 10 different electric field intensities (0, 500, 1000, 1400, 2000, 2200, 2700, 3100, 3400, and 3700 V/cm) were used to perform electrofusion of mouse spleen cells with myeloma cells. The optimal field intensity was determined by comparing the number of formed hybridoma clones. The results suggested that the maximum number of clones was observed at 2000 V/cm. It was concluded that the cell membrane structure would not be disrupted so that cell fusion could not be conducted when the electric field intensity was too low. However, the cells would be permanently damaged if the electric field intensity was too high and no effective cell fusion was possible. In 2009, Ušaj et al[34]. investigated the effect of different electric field intensities on fusion yield using two different cell lines: mouse melanoma cells (B16-F1) and Chinese hamster ovary cells (CHO). In the study, the fusion yield of the two cell types was studied using eight different electric field intensities (0, 200, 400, 600, 800, 1000, 1200, and 1400 V/cm) (**Figure. 6A)** . According to the results, the highest electrofusion yield was at 1200 V/cm. Subsequently, other research groups performed similar electrofusion studies using mouse melanoma cells (B16-F1) and mouse embryonic cells. The results showed that the optimal electric field intensity values were 1200 V/cm[35] and 800 V/cm[36], respectively. According to the previous studies, there is a slight difference in optimal electric field intensity in different cells. In general, an optimized electric field intensity in the range of 800–1200 V/cm is suggested as a guidance for highly efficient cell fusion.

The number of pulses and thus the level of electroporation will affect the electroporation efficiency[37]. As previously reported, most investigators directly applied a single pulse (one-time electroporation) or multiple pulses (multiple-time electroporation) for cell electrofusion without optimizing the exact number of pulses. In 2019, Ke et al[38]. systematically compared the effects of single and double pulses on the fusion of B lymphocytes with myeloma cells. In this study, B lymphocytes were mixed with myeloma cells and then

divided equally into two groups, one for single-pulse electrofusion and the other for double-pulse electrofusion. After electrofusion, the cells were transferred to a semi-solid medium, and the number of hybridoma clones in each group was counted after 20 days of culture **(Figure. 6B)** . The results revealed that the electrofusion yield of the double-pulse group was about three-fold higher than that of the single-pulse group, indicating that the double-pulse electrofusion can improve the yield of hybridoma preparation. However, the effect of the number of pulses on electrofusion has not been studied extensively. Investigators can explore this relationship in-depth in the future.

The pulse duration is crucial for cell electrofusion. The Zimmermann group suggested that the pulse duration should not exceed 100 μs, otherwise it would cause irreversible electroporation, resulting in cell lysis. In recent years, microsecond (μs)-level pulses have been widely applied in cell electrofusion experiments[35,36]. Nevertheless, the microsecond-level pulse duration is still relatively long and will lead to a large electroporation radius, causing serious irreversible damage to the cell membrane and ultimately resulting in a significant decrease in cell fusion yield. In contrast, nanosecond (ns)-level pulses have attracted increasing attention in the cell engineering field due to their unique "intracellular electro-manipulation" effect[39]. Moreover, nanosecond-level pulses can produce a large number of nanometer-sized pores on cells, and they are less damaging to the cells compared to the microsecond-level pulses, which are more conducive for cell recovery after electrofusion. However, the pore size on the cell membrane after a nanosecond-level pulse is so small that biomolecules such as DNA cannot easily pass through[40]. Moreover, a short duration is likely to result in rapid recovery from perforation before intracellular substance exchange. Due to certain defects in using only microsecond- or nanosecond-pulses, the application of a "micro/nanosecond composite pulse" that combines the advantages of both will be of great importance for cell electrofusion. In 2019, Wu et al[41]. used mouse myeloma cells to systematically study the cell survival rate and electrofusion yield with different microsecond-level pulses and micro/nanosecond composite pulses. In this study, three groups of single microsecond-level pulses (2 kV/cm, 20 μs, one time; 2.5 kV/cm, 20 μs, one time, and 3 kV/cm, 20 μs, one time) and three groups of micro/nanosecond composite pulses (2 kV/cm, 20 μs, one time + 10 kV/cm, 200 ns, eight times; 2.5 kV/cm, 20 μs, one time + 10 kV/cm, 200 ns, eight times; and 3 kV/cm, 20 μs, one time + 10 kV/cm, 200 ns, eight times) were applied in stained myeloma cell electrofusion and the effects observed under a fluorescent microscope (**Figure. 6C**). The results demonstrated that the survival rate and electrofusion yield with micro/nanosecond composite pulses were increased by 6.4% and 23.3%, respectively, compared to those with single microsecond-level pulses. Wu et al. explained this mechanism of superior method for cell electrofusion based on the micro/nanosecond composite pulses. First, a microsecond-level pulse is used to perforate the junctional zone of cells, and then a nanosecond-level pulse is applied to form small pores. Through the synergistic effect of micro/nanosecond composite pulses, irreversible damage during cell perforation caused by a single use of microsecond-level pulses will be reduced, which is beneficial for improving fusion yield.

After the cell alignment and fusion stage, myeloma cells and lymphocytes are successfully fused into a hybridoma. An additional cell repair process is recommended for some commercial electrofusion instruments, such as application of a lower-frequency and weaker AC voltage to the cell. This can stabilize the state of the heterogeneous cells and promote the repair of the damaged cells to some extent, thus improving cell viability and survival rate. However, relevant research on cell repair after electrofusion has not been detailedly reported.

< Figure. 6 >

3 Cell treatment for electrofusion

The above section summarized the studies on electrical parameters involved in cell alignment and fusion in electrofusion. The optimization of electrical parameters is an important external condition to ensure a smooth electrofusion performance. In addition, a number of studies have been carried out from the perspective of cell treatment to further improve electrofusion yield. Generally, the purpose of cell treatment is as follows: (I) to increase the chance of contact between cells: dispersed cells make contact with each other in the electric field and complete cell membrane fusion and intracellular substance exchange; (II) to enhance the pairing rate

of heterologous cells: only the fusion that occurred between myeloma cells and lymphocytes (spleen cells or B cells) is of interest; and (III) to promote 1:1 cell fusion, if multiple cells are fused at the same time, they will eventually form ineffective giant multinucleated cells, resulting in a lower cell fusion yield. In order to achieve the above goals, the current studies treated cells before electrofusion from the following four aspects: change cell size, improve specific affinity between cells, promote cell confinement, and control cell number and proportion.

3.1 Change cell size

The cells used to produce hybridomas by fusion are usually myeloma cells and lymphocytes (spleen cells). There are diameter differences between the two cell lines: diameters of myeloma cells and spleen cells are about 12 μm and 7 μm, respectively[42]. This complicates the choice of the optimal electric field for fusion of these cells since different electric field intensities are required to perforate membranes of different cell lines[43,44]. In order to address this problem, Kato et al[42]. reduced the difference in size between myeloma and spleen cells before electrofusion. Spleen cells were incubated with a cell stimulator CpG oligodeoxynucleotide to increase the diameter of spleen cells from 7 μm to 9.2 μm, which allowed the field intensity required for spleen cells to be similar to that of myeloma cells with a relatively larger diameter. In this way, the cell fusion yield was successfully raised from 0.00014% of conventional electrofusion to about 0.17%.

3.2 Improve specific affinity between cells

During electrofusion, myeloma cells and lymphocytes are randomly combined and arranged in a pearl-chain under the influence of an AC electric field, which easily causes pairing between homologous cells. The highly specific affinity between biotin and streptavidin was used to enhance the pairing rate between heterologous cells. In 2014, Liu et al[45]. studied the biotin-streptavidin system for enhancing heterologous cell fusion. In this comparison study, three different groups of cells were used to demonstrate the superiority of the biotin-streptavidin method. The electrofusion yield between treated and untreated cells by biotin-streptavidin system was compared. The results indicated that using the biotin-streptavidin treatment increased the fusion yield from 2.18–5.98% to 16.64–31.44%. Therefore, the fusion yield of cells treated with biotin-streptavidin improved five- to ten-fold. In 2016, Wu et al[46]. also applied the biotin-streptavidin system to functionalize the spleen cells and SP2/0 cells before electrofusion. A total of 20 clones were obtained from a hybridoma prepared with the biotin-streptavidin-treated cells, of which 12 clones were able to express specific antibodies. In contrast, only six clones were obtained with untreated cells, of which only two expressed specific antibodies. The results demonstrated that the biotin-streptavidin significantly improved the electrofusion yield in both the total number of hybridoma clones and the number of positive hybridoma clones (secreting an antigen-specific antibody). It is noteworthy that biotin-streptavidin has a certain level of cytotoxicity, and a high concentration or long incubation duration are likely to cause deterioration of the cell state, resulting in a reduced fusion yield.

3.3 Promote cell confinement

The number of myeloma cells and lymphocytes is usually maintained at a level of 10^6–10^7 cells during electrofusion, so that a large number of cell pearl-chains are likely to undergo multi-cell fusion, resulting in the formation of giant multinucleated cells that cannot survive[47]. In addition, homologous cell fusion cannot be avoided even if commercial fusion chambers were used. In order to minimize multi-cell and homologous cell fusion, Schoeman et al[26]. trapped two heterologous cells in a single droplet using a microfluidic chip to ensure that the two cells (HL60) are electrofused at a single-cell level. The results revealed that electrofusion of the two cell types by means of trapping in the microdroplets with a picoliter volume could increase the chance of contact between the two cells, resulting in a 1:1 cell pairing rate of up to 77% and cell fusion yield of as high as 95%. Similar to the study by Schoeman et al., Gel et al[19]. trapped myeloma cells and spleen cells on each side of the micro-orifice using microporous array chips to ensure 1:1 cell pairing **(Figure. 2B)** . In this study, the pairing rate of heterologous cells was up to 95–100%, and the fusion yield was up to 95%, which greatly improved the electrofusion yield. It can be noted that electrofusion with cell confinement can

largely solve the problem of multi-cell and homologous cell fusion events, which enhances the heterologous and 1:1 pairing rate.

3.4 Control cell number and proportion

It is very important to control the number and proportion of cells for electrofusion. In hybridoma preparation using the PEG-mediated fusion method, it is believed that the number of cells should be maintained at a level of 10^4–10^8 cells, and the proportion of myeloma cells to lymphocytes should be maintained at 1:(1–10) [48]. At present, no unified standard for the proportion of myeloma cells to lymphocytes has been reported in the electrofusion literature. In this section, the studies on the number and proportion of cells used for hybridoma preparation by electrofusion are summarized (**Table 2**).

Some researchers have explored the optimal number and proportion of myeloma cells and lymphocytes in electrofusion experiments. In the preparation of hybridoma for human vasorin protein using electrofusion, Yin et al[29]. explored the optimal proportion of myeloma cells to spleen cells. Myeloma cells were mixed with spleen cells at the ratios of 1:1 and 1:2, respectively. The total number of cells was maintained at 1.8×10^8–2.5×10^8, and electrofusion was carried out in a 1.5-mL electrofusion chamber. The results suggested that when myeloma cells and lymphocytes (spleen cells) were electrofused at a ratio of 1:2, the fusion yield was about two-fold higher than that of the 1:1 fusion group and about four-fold higher than that of the PEG-mediated fusion group. The optimal fusion yield was 0.31% when 2.5×10^7 cells were used at a proportion of 1:2. However, only two cell proportion values were employed in this study. Therefore, on the basis of this research study, Yu et al[4]. systematically optimized the proportion of human myeloma cells (HMMA2.5) to lymphocytes (B cells) in preparation of virus-specific human hybridoma by electrofusion. The number of HMMA2.5 cells was maintained at a level of 6×10^6 cells. They were then mixed with lymphocytes (B cells) at the ratios of 2:1, 1:1, 1:2, and 1:3, and electrofusion was carried out in a 0.5-mL fusion chamber. The results demonstrated that the fusion yield of the four groups was 0.43%, 0.23%, 0.17%, and 0.014%, respectively. Accordingly, the optimal fusion proportion of myeloma cells to lymphocytes (B cells) was 2:1. The fusion yield was significantly increased two- to 30-fold compared to that of the other groups. Interestingly, the conclusions drawn in this work were completely opposite to those in the Yin et al. study, which stated that the fusion yield is better when the number of myeloma cells is higher than the number of B cells. Studies on electrofusion report different and sometimes contradictory results about the number and proportion of cells.

In other studies of hybridoma preparation using electrofusion, investigators did not optimize the proportion of myeloma cells to lymphocytes, but directly drew on the PEG-mediated fusion experience. For example, Li et al[38]. mixed myeloma cells with lymphocytes (from the spleen) at a ratio of 1:5 for electrofusion and adjusted the number of cells to 10^6. Electrofusion was then carried out in a 2-mL chamber, and satisfactory electrofusion results were also obtained. Song[44] and Cold Spring Harbor Laboratory[47] also performed electrofusion in a 2-mL electrofusion chamber with the number of cells maintained at 1×10^6. They utilized spleen-derived lymphocytes and myeloma cells at a 1:1 proportion and also achieved satisfactory fusion results. It should be particularly noted that for the electrofusion microfluidic chip, the electrofusion is usually performed at a single-cell level, with the total volume in the nL–pL range, so that myeloma cells and lymphocytes can be fused at a 1:1 proportion[26].

According to the current findings, the optimal number and proportion of cells selected for electrofusion vary widely and differ significantly from the conventional PEG-mediated fusion method. Therefore, we suggest that the number and proportion of cells should be carefully compared and optimized during electrofusion in future studies.

<Table 2>

4 Characterization of electrofusion process

Complete cell fusion occurs via membrane fusion and nuclear fusion. In the stage of membrane fusion, the membranes of the two cells must be in close contact to achieve electroporation in the AC electric field for intracellular substance exchange, but the cells still retain two independent nuclei after membrane fusion.

Cell nuclear fusion is a random process and usually takes place within 1–2 weeks after membrane fusion[49]. Only cells that have undergone nuclear fusion are truly fused successfully. Accurate characterization of membrane fusion and nuclear fusion processes is beneficial for effective assessment of the rationality of the electrofusion scheme. Currently, cell imaging and fusion yield calculations are commonly used to characterize cell electrofusion. Usually, cell imaging is used to observe and evaluate cell morphology and state under an optical or fluorescent microscope. Fusion yield refers to the ratio of the number of colonies formed by hybridoma to all cells. In the current studies, researchers regarded fusion yield as the "gold standard" for determining the success of electrofusion. Therefore, improving the fusion yield is an important goal.

4.1 Cell imaging

4.1.1 Observation and evaluation of cell state

The microscope is the most commonly used imaging device for observing cell changes during electrofusion. In 2021, Yin et al[29]. observed the whole process of cell alignment under an AC electric field in real time with the help of an optical microscope. They optimized the AC voltage based on the cell alignment.

As shown in **Figure. 5A,** when AC voltage is low (**Figure. 5A1 and 5A2**), the interaction between the cells is weak, resulting in cells arranged in a short pearl-chain or failure to arrange. When AC voltage is increased to 50V, the cells are arranged regularly and form a longer pearl-chain (**Figure. 5A3**). However, at a higher AC voltage (**Figure. 5A4)** , cell membranes are damaged and cannot form a regularly arranged pearl-chain. In this study, the authors believed that the regular arrangement of cells in a relatively long pearl-chain (**Figure. 5A3**) was the most suitable cell state for electrofusion. In 2022, Song et al[44]. also observed the state of cell alignment under an optical microscope (**Figure. 5B**). When AC electric field (AC voltage) is high (**Figure. 5B1 and 5B2**), cells arranged in a longer pearl-chain. Unlike Yin et al, Song et al did not choose the electric field intensity that would make cells form a relatively long pearl-chain as the optimal condition. Instead, they chose the field intensity that would make cells form a short pearl-chain (**Figure. 5B3**) as the optimal condition for electrofusion. They concluded that a relatively short cell pearl-chain would be more conducive to obtaining a binucleated hybridoma after electroporation. In 2016, Wu et al[46]. observed the membrane fusion process of SP2/0 cells with spleen cells under an optical microscope. As indicated by arrows in **Figure. 5C** , the two cells changed the membrane state from contact to fusion after electrofusion. Since the organelles of the cells cannot be observed under an optical microscope, it is impossible to further determine whether cell nuclear fusion has occurred.

4.1.2 Fluorescence cell staining

Observing stained cells using a fluorescence microscope is another important means of cell imaging. In some electrofusion studies, the cells are stained with different fluorescence agents prior to electrofusion. In 2018, Schoeman et al[26]. stained HL60 cells with different fluorescence agents in order to accurately assess the process of cell membrane fusion and nuclear fusion in droplets (**Figure. 7A**). Half of the HL60 cells were stained with a green membrane dye (green fluorescent protein) and a blue nuclear dye, and the other half with the same green membrane dye, but with a red nuclear dye (red fluorescent protein). The whole process of electrofusion was then monitored and analyzed using a fluorescence microscope. Schoeman et al. concluded that a blue and a red nuclear signal observed in one cell after electrofusion was an indication of two successfully electrofused cells. In 2019, Ke et al[38]. performed nuclear staining of B lymphocytes using Hoechst 33342 (blue), followed by staining dead cells with PI (red) to exclude interference. SP2/0 cells (not treated with fluorescent staining) larger than B lymphocytes were easily distinguished under a microscope.

Previous studies usually considered that blue-stained B lymphocytes in unstained SP2/0 cells (**Figure. 7A**) meant that electrofusion was successful. However, it was noted that cells may be spatially distributed in different solution layers (overlapping upper and lower positions) without successful fusion. Three types of spatial distribution are shown in **Figure. 7B** . In all three cases, the cells may be misidentified as successful fusion due to a visual illusion. The type 1 and 2 cells (B lymphocytes are located above or below SP2/0) are located in different layers of the solution and will be mistaken for successful fusion. The cell membrane of the blue fluorescent B lymphocytes is still clearly visible after careful observation, so type 1 and 2 cells cannot be

regarded as fused cells. Only type 3 cells can be considered as successfully fused since B lymphocytes (blue) are located inside SP2/0 cells and the cell membrane is barely visible. Therefore, more careful observation of the cell membrane is required to accurately determine whether the electrofusion of the two cells is successful.

< Figure. 7 >

4.2 Calculation of fusion yield

Fusion yield refers to the ratio of the number of hybridoma colonies to the total number of cells, and researchers regard the fusion yield as the "gold standard" for determining the success of electrofusion. After the myeloma cells fuse with the lymphocytes, the cells are resuspended at a concentration of 10^4–10^5 cells/well in 20% hypoxanthine-aminopterin-thymidine (HAT) medium for selective screening[29]. Most researchers consider the number of hybridoma colonies in all wells as the number of successfully fused hybridomas for the calculation of fusion yield. However, there is no well acceptable calculation formula for the fusion yield in reported studies. Foung et al. [50], and Yin et al[29]. used the ratio of the number of successfully fused hybridoma colonies to the number of lymphocytes as the fusion yield. Song et al[44]. and Ke et al[38]. used the ratio of the number of successfully fused hybridomas to the myeloma cells as the fusion yield. In general, there is no difference between the two fusion yield results when myeloma cells are electrofused with lymphocytes at a ratio of 1:1, but the results will be different if the two cells are fused at a different proportion.

5 Summary and prospects

In this paper, the application of cell electrofusion technology in hybridoma preparation was summarized and discussed on the basis of literature research. Compared to the conventional PEG-mediated fusion method, electrofusion technology has unique advantages, such as high fusion yield, low cytotoxicity, easy operation and standardization, and high controllability. Despite the above advantages, surprisingly, cell electrofusion is still not dominant in the field of hybridoma and monoclonal antibody preparation. This is likely because the influential parameters and factors affecting electrofusion yield are inconsistently reported, underestimated, not yet completely known, and not optimized.

In this paper, we provided a detailed overview of electrofusion technology used in hybridoma preparation, electrofusion instrument and its components, process control, cell treatment, and process characterization. The application of electrofusion technology in hybridoma preparation will develop in the following directions in the future: (I) electrofusion technology for hybridoma preparation will be widely applied in different fields, especially in the field of monoclonal antibody preparation, which allows pharmaceutical companies to systematically develop antibody drugs and antibodies for diagnostics; (II) systematic studies on the electrical parameters, cell treatment, and characterization in electrofusion will provide basic and detailed experimental data, as well as theoretical basis, which will be conducive to popularizing electrofusion technology in different application scenarios of hybridoma preparation; and (III) current mature micro-electromechanical systems technology should be fully integrated with electrofusion to provide excellent single-cell tools and platforms, micro-electromechanical systems technology can realize the more precise manipulation of electrofusion at the single-cell level and micro-nano scale. The above review discussed the four important aspects of the electrofusion process that can provide new ideas for hybridoma preparation. It is expected that this technology will be improved continuously through subsequent scientific research and will play a great role in the field of monoclonal antibody preparation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This project has received funding from the National Science Foundation of China under Grant 32172910.

Data availability statement: Data derived from public domain resources

Reference:

1. Kohler, G., Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256 (5517), 495-497.

2. Amy, G., Joseph, M., Yuri, L. (2010). V-fusion: a convenient, nontoxic method for cell fusion. BioTechniques. 49 (4), 747-750.

3. Lentz, B. R. (2007). PEG as a tool to gain insight into membrane fusion. European Biophysics Journal with Biophysics Letters.36 (4), 315-326.

4. Yu, X. C., McGraw P. A., House F. S., Crowe J. E. (2008). An optimized electrofusion-based protocol for generating virus-specific human monoclonal antibodies. Journal of Immunological Methods.336 (2), 142-151.

5. Glassy, M. (1988). Creating hybridomas by electrofusion. Nature. 333 (6173), 579-580.

6. Kanduser, M., Usaj, M. (2014). Cell electrofusion: past and future perspectives for antibody production and cancer cell vaccines. Expert Opinion on Drug Delivery. 11 (12), 1885-1898.

7. Sun, Y. Q., Zhang, X. L., Nie, Y. C., Guo, X. P., Jin, S. X., Liang, S. G. (2004). Production and characterization of somatic hybrids between upland cotton (Gossypium hirsutum) and wild cotton (G-klotzschianum Anderss) via electrofusion. Theoretical and Applied Genetics. 109 (3), 472-479.

8. Gaynor, P., Wells, D. N., Oback, B. (2005). Couplet alignment and improved electrofusion by dielectrophoresis for a zona-free high-throughput cloned embryo production system. Medical & Biological Engineering & Computing., 43 (1), 150-154.

9. Ogura, A., Inoue, K., Takano, K., Wakayama, T., Yanagimachi, R. (2000). Birth of mice after nuclear transfer by electrofusion using tail tip cells. Molecular Reproduction and Development. 57 (1), 55-59.

10. Low, L., Mander, A., McCann, K., Dearnaley, D., Tjelle, T., Mathiesen, I., Stevenson, F., Ottensmeier, C. H. (2009). DNA Vaccination with Electroporation Induces Increased Antibody Responses in Patients with Prostate Cancer. Human Gene Therapy. 20 (11), 1269-1278.

11. Mali, B., Miklavcic, D., Campana, L. G., Cemazar, M., Sersa, G., Snoj, M., Jarm, T. (2013) Tumor size and effectiveness of electrochemotherapy. Radiology and Oncology. 47 (1), 32-41.

12. Adrian, J. E., Kamps, Jaam., Poelstra, K., Scherphof, G. L., Meijer, D. K. F., Kaneda, Y. (2007). Delivery of viral vectors to hepatic stellate cells in fibrotic livers using HVJ envelopes fused with targeted liposomes. Journal of Drug Targeting. 15 (1), 75-82.

13. Elder, P. A., Gaynor, P. T., Lewis, J. G., Bodger, P. S., Bason, L. M. (1997) Generation of monoclonal progesterone antibodies by electrofusion techniques. Bioelectrochemistry and Bioenergetics. 43 (1), 35-40.

14. French, A. J., Adams, C. A., Anderson, L. S., Kitchen, J. R., Hughes, M. R. Wood S. H. (2008). Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. Stem Cells. 26 (2), 485-493.

15. Whitesides, G. M. (2006). The origins and the future of microfluidics. Nature 442 (7101), 368-373.

16. Battat, S., Weitz, D. A., Whitesides, G. M. (2022). An outlook on microfluidics: the promise and the challenge. Lab on a Chip. 22 (3), 530-536.

17. Masuda, S., Washizu, M., Nanba, T. (1989). Novel method of cell-fusion in field constriction area in fluid integrated-circuit. Ieee Transactions on Industry Applications. 25 (4), 732-737.

18. Techaumnat, B., Tsuda, K., Kurosawa, O., Murat, G., Oana, H., Washizu, M.(2008). High-yield electrofusion of biological cells based on field tailoring by microfabricated structures. IET Nanobiotechnol. 2 (4), 93-99.

19. Gel, M., Kimura, Y., Kurosawa, O., Oana, H., Kotera, H., Washizu, M. (2010). Dielectrophoretic cell trapping and parallel one-to-one fusion based on field constriction created by a micro-orifice array. Biomicrofluidics. 4 (2).

20. Sakamoto, S., Okeyo, K. O., Yamazaki, S., Kurosawa, O., Oana, H., Kotera, H., Washizu, M. (2016). Adhesion patterning by a novel air-lock technique enables localization and in-situ real-time imaging of reprogramming events in one-to-one electrofused hybrids. Biomicrofluidics. 10 (5), 054122.

21. Hu, N., Yang, J., Qian, S., Joo, S. W., Zheng, X. (2011). A cell electrofusion microfluidic device integrated with 3D thin-film microelectrode arrays. Biomicrofluidics. 5 (3), 34121-341212.

22. Hu, N., Zhang, X. L., Yang, J., Joo, S. W., Qian, S. Z. (2013). A cell electrofusion microfluidic chip with micro-cavity microelectrode array. Microfluidics and Nanofluidics. 15 (2), 151-160.

23. Mazutis, L., Gilbert, J., Ung, W. L., Weitz, D. A., Griffiths, A. D., Heyman, J. A. (2013). Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc. 8 (5), 870-891.

24. Anggraini, D., Ota, N., Shen, Y. G., Tang, T., Tanaka, Y., Hosokawa, Y., Li, M., Yalikun, Y. (2022). Recent advances in microfluidic devices for single-cell cultivation: methods and applications. Lab on a Chip. 22 (8), 1438-1468.

25. Schoeman, R. M., Kemna, E. W., Wolbers, F., Berg, A. (2014). High-throughput deterministic single-cell encapsulation and droplet pairing, fusion, and shrinkage in a single microfluidic device. Electrophoresis. 35 (2), 385-392.

26. Schoeman, R. M., Beld, W. T. E., Kemna, E. W. M., Wolbers, F., Eijkel, J. C. T., Berg, A. (2018). Electrofusion of single cells in picoliter droplets. Sci Rep., 8 (1), 3714

27. Sen, M., Ino, K., Ramon-Azcon, J., Shiku, H., Matsue, T. (2013). Cell pairing using a dielectrophoresis-based device with interdigitated array electrodes. Lab on a Chip. 13 (18), 3650-3652.

28. Wu, C. H., Chen, R. F., Liu, Y., Yu, Z. M., Jiang, Y. W., Cheng, X. A. (2017) planar dielectrophoresis-based chip for high-throughput cell pairing. Lab on a Chip. 17 (23), 4008-4014.

29. Yin, Moli., Nie, Yuanwang., Liu, Hao., Zhang, Shuang., Liu, Lei., Li, Xiuchun., Wang, Huiyan. (2021). Preparation of mouse monoclonal antibody against human vasorin (VASN) protein by high-efficacy electrofusion-based protocol. Chinese journal of cellular and molecular immunology. 37 (3), 265-270.

30. Zimmermann, U., Vienken, J., Pilwat, G. (1982). Electric-field-induced fusion of cells. Studia Biophysica. 90, 177-184.

31. Mehrle, W., Hampp, R., Zimmermann, U., Schwan, H. P. (1988). Mapping of the field distribution around dielectrophoretically aligned cells by means of small particles as field probes. Biochimica Et Biophysica Acta., 939 (3), 561-568.

32. Guo, M., Pan, Z., Wang, H. (1998). Establishment of hybridoma secreting anti-mycobacteria monoclonal antibody by using electrofusion technique. Acta microbiologica Sinica. 38 (5), 393-395.

33. Ohnishi, K., Chiba, J., Goto, Y., Tokunaga, T. (1987). Improvement in the basic technology of electrofusion for generation of antibody-producing hybridomas. Journal of Immunological Methods. 100 (1), 181-189.

34. Usaj, M., Trontelj, K., Miklavcic, D., Kanduser, M. (2010). Cell-cell electrofusion: optimization of electric field amplitude and hypotonic treatment for mouse melanoma (B16-F1) and Chinese Hamster ovary (CHO) cells. J Membr Biol. 236 (1), 107-116.

35. Suo, L., Wang, F., Zhou, G. B., Shi, J. M., Wang, Y. B., Zeng, S. M., Tian, J. H., Zhu, S. E., Liu, G. S. (2009). Optimal Concentration of Calcium and Electric Field Levels Improve Tetraploid Embryo Production by Electrofusion in Mice. Journal of Reproduction and Development. 55 (4), 383-385.

36. Trontelj, K., Usaj, M. (2010). Miklavcic, D., Cell electrofusion visualized with fluorescence microscopy. Journal of visualized experiments. JoVE.41

37. Li, C. X., Ke, Q., Yao, C., Yao, C. G., Mi, Y., Wu, M., Ge, L. P. (2019). Comparison of Bipolar and Unipolar Pulses in Cell Electrofusion: Simulation and Experimental Research. Ieee Transactions on Biomedical Engineering. 66 (5), 1353-1360.

38. Ke, Q., Li, C., Wu, M., Ge, L., Yao, C., Yao, C., Mi, Y. (2019). Electrofusion by a bipolar pulsed electric field: Increased cell fusion efficiency for monoclonal antibody production. Bioelectrochemistry. 127, 171-179.

39. Buescher, E. S., Schoenbach, K. H. (2003). Effects of submicrosecond, high intensity pulsed electric fields on living cells-intracellular electromanipulation. Ieee Transactions on Dielectrics and Electrical Insulation. 10 (5), 788-94.

40. Li, H., Liu, S., Yang, X., Du, Y., Luo, J., Tan, J., Sun, Y. (2019). Cellular Processes Involved in Jurkat Cells Exposed to Nanosecond Pulsed Electric Field. Int J Mol Sci., 20 (23).

41. Li, C. X., Ke, Q., Yao, C., Mi, Y., Liu, H., Lv, Y., Yao, C. (2018). Cell electrofusion based on nanosecond/microsecond pulsed electric fields. PLoS One. 13 (5), e0197167.

42. Kato, M., Sasamori, E., Chiba, T., Han, Y. (2011). Cell activation by CpG ODN leads to improved electrofusion in hybridoma production. J Immunol Methods. 373 (2), 102-210.

43. Sukhorukov, V. L., Reuss, R., Zimmermann, D., Held, C., Muller, K. J., Kiesel, M., Gessner, P., Steinbach, A., Schenk, W. A., Bamberg, E., Zimmermann, U. (2005). Surviving high-intensity field pulses: strategies for improving robustness and performance of electrotransfection and electrofusion. J Membr Biol. 206 (3), 187-201.

44. Song, G. Y., Huang, L. R., Huang, Y., Liu, W. M., Wang, M. H., Hua, X. D. (2022). Electrofusion preparation of anti-triazophos monoclonal antibodies for development of an indirect competitive enzyme-linked immunosorbent assay. Journal of Immunological Methods. 500, 113184.

45. Li, J., Yu, X., Wagner, T. E., Wei, Y. A. (2014). biotin-streptavidin-biotin bridge dramatically enhances cell fusion. Oncol Lett. 8 (1), 198-202.

46. Wu, M., Liu, Z. H., Ding, Y. C., Ge, L. P. (2016). The Study of modified electrofusion method to prepare hybridoma cell. Sichuan Journal of Animal Science and Veterinary Medicine. 43, 26-28.

47. Greenfield, E. A. (2019). Electro Cell Fusion for Hybridoma Production. Cold Spring Harb Protoc.

48. Chadseesuwan, U., Sangdokmai, A, Pimpitak, U., Puthong, S., Palaga, T., Komolpis, K. (2016). Production of a monoclonal antibody against aflatoxin M1 and its application for detection of aflatoxin M1 in fortified milk. J Food Drug Anal.24 (4), 780-787.

49. Weber, H., Forster, W., Jacob, H. E., Berg, H. (1981). Microbiological implications of electric-field effects .3. stimulation of yeast protoplast fusion by electric-field pulses. Zeitschrift Fur Allgemeine Mikrobiologie. 21(7), 555-562.

50. Foung, S. K. H., Perkins, S. (1989). Electric field-induced cell-fusion and human monoclonal-antibodies. Journal of Immunological Methods. 116(1), 117-122.

Figure Captions:

Figure. 1 The selected types of commercial fusion chambers: (A) Fusion cuvettes with 2-mL (black) and 9-mL (blue) volumes. (B) Fusion microslides with different fusion volumes. From right to left: 20 μL, 40 μL, and 600 μL. The data and pictures were obtained from the BTX website (https://www.btxonline.com)

Figure. 2 Schematic representations of 1:1 cell fusion via micro-orfices employing electric field: (A) High-yield electrofusion of biological cells based on field tailoring using micro-orfices structures. (B) 1 Schematic

of microfluidic chip and cell pairing process. 2 Schematic of fused cell transfer and culture. (C) Schematic of fused cells located on adhesion zones for time-lapse imaging. Part A is reprinted with permission from Techaumnat et al. [18]. Part B is reprinted with permission from Gel et al. [19]. Part C is reprinted with permission from Sakamono et al. [20].

Figure. 3 Schematic of microfluidic device channel structure for cell electrofusion by Hu et al.: (A) Schematics of 3D thin-film microelectrode array. (B) Schematics of microfluidic chip have two chiasm-shaped microelectrode arrays fabricated on Silicon-On-Insulator wafer substrate. Parts A and B are reprinted with permission from Hu et al. [21, 22].

Figure. 4 Schematic of microfluidic platform for cell electrofusion in droplets by Schoeman et al.: (A) Schematic representation of microfluidic design showing yin-yang-shaped alternating curved microchannels. 1 Overview of the double T-junction with single-cell encapsulation in picoliter droplets and automatic droplet pairing. 2 Electrocoalescence of the paired droplets, due to the electric field produced by the electrode pair on either side of the microchannel. 3 Droplet shrinkage caused by one to three pitchforks structures in a row, of which one is shown. 4 Cell fusion due to an electrode pair across the microchannel. (B) Microfluidic platform for cell electrofusion in droplets. 1 A schematic overview is shown with a yin-yang shaped cell solution inlet that functions as an elongated funnel to gradually introduce the cells into the channel to prevent the cells from clogging up the channel, and colored inserts of the diferent functionalities of the chip. 2 In green, cell encapsulation in droplets. 3 In red, droplets containing two cells passing the electrode array, consisting of six electrode pairs, all capable of giving a pulse of certain preset strength. Parts A and B are reprinted with permission from Schoeman et al. [25, 26].

Figure. 5 Schematic representation of cell contact observation and evaluation in electric field: (A) and (B) Effect of alternating electric field on cell contact. (C) Schematic of membrane fusion between SP2/0 and spleen cells. Part A is reprinted with permission from Yin et al. (29). Part B is reprinted with permission from Song et al. [44]. Part C is reprinted with permission from Wu et al. [46].

Figure. 6 Schematic representation of the study on electrical parameters for cell electrofusion: (A) 1 Three-channel microscopic cell image. 2 Schematic of electroporation efficiency under different electric fields. (B) 1 Cell staining experiment in SP2/0 cells and lymphocytes. 2 Hybridoma cell yield based on bipolar pulse and unipolar pulse. (C) 1 Survival and fusion rates of microsecond pulsed electrofusion cells. 2 Survival and fusion rates of micro-/nanosecond pulsed electrofusion cells. Part A is reprinted with permission from Ušaj et al. [26]. Part B is reprinted with permission from Ke et al. [38]. Part C is reprinted with permission from Wu et al. [41].

Figure. 7 Schematic representation of cells with fluorescence staining: (A) Overview of electrofused HL60 cells inside the droplet containing two nuclei with different colors and a rearranged cell membrane. (B) Comparison of cells in different layers vs. cells in the same layer. Part A is reprinted with permission from Schoeman et al. [26]. Part B is reprinted with permission from Ke et al. [38]

Table 1 Summary of key electrical parameters in hybridoma preparation using commercial electrofusion instruments

Company	**Model**	**Parameters**	**Cell alignment process and cell fusion process**
BTX (USA)	ECM2001 PLUS ECM2001 LITE	Voltage	Alignment: 0-75 V; Fusion: 5-3000 V
		Pulse durations	0-99 s
		Frequency	0.2-2 MHz
		Number of pulses	1-99 pcs
		Inter-pulse interval	0.1-10 s

Company	Model	Parameters	Cell alignment process and cell fusion process
Eppendorf (Germany)	Multiporator 4308	Voltage	Alignment: 5-300 V; Fusion: 1-10 V
		Pulse durations	0-95 s
		Frequency	2 MHz
		Number of pulses	1-99 pcs
		Inter-pulse interval	1 min
BEX (Japan)	CFB16-HB	Voltage	Alignment: 0- 100 V; Fusion: 0-1500 V
		Pulse durations	0-120 s
		Frequency	400 KHz
		Number of pulses	0-100 pcs
		Inter-pulse interval	100 ms-10 s
BSL (Hungary)	CF-150C	Voltage	Alignment: 10-150 V; Fusion: 20-150 V
		Pulse durations	10-200 μs
		Frequency	100 Hz-1 MHz
		Number of pulses	1-9 pcs
		Inter-pulse interval	/
Scientz (China)	CRY-3 CRY-3B	Voltage	Alignment: 1-58 V; Fusion: 5-600 V
		Pulse durations	10-5000 μs
		Frequency	30-3000 KHz
		Number of pulses	1-9 pcs
		Inter-pulse interval	/

Table 2 Summary of SP2/0 and lymphocyte ratio in monoclonal antibody preparation by electrofusion

Total number of cells	Myeloma cells:Lymphocytes	Electrofusion Chamber	References
2×10^7	1:1, 1:2*	1.5 mL	29
9×10^6	2:1*, 1:1, 1:2, 1:3	0.5 mL	4
1.2×10^6	1:5	2 mL	46
3×10^6	1:5	2 mL	38
2×10^6	1:1	2 mL	44
2	1:1	50 pL (microfluidic droplet)	26

*optimized condition