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Review

Advances in cell separation: recent developments in counterflow centrifugal elutriation and continuous flow cell separation

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Abstract

Cell separation by counterflow centrifugal elutriation (CCE) or free flow electrophoresis (FFE) is performed at lower frequency than cell cloning and antibody-dependent, magnetic or fluorescence-activated cell sorting. Nevertheless, numerous recent publications confirmed that these physical cell separation methods that do not include cell labeling or cell transformation steps, may be most useful for some applications. CCE and FFE have proved to be valuable tools, if homogeneous populations of normal healthy untransformed cells are required for answering scientific questions or for clinical transplantation and cells cannot be labeled by antibodies, because suitable antibodies are not available or because antibody binding to a cell surface would induce the cell reaction which should be investigated on purified cells or because antibodies bound to the surface hamper the use of the isolated cells. In addition, the methods are helpful for studying the biological reasons for, or effects of, changes in cell size and cellular negative surface charge density. Although the value of the methods was confirmed in recent years by a considerable number of important scientific results, activities to further develop and improve the instruments have, unfortunately, declined.

Keywords: Reviews; Cell separation

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1. Introduction

Organisms such as plants or animals consist of a great number of different kinds of cells. Each cell type has characteristic features and fulfills distinct functions within the 'concert' of life of the whole organism. Our understanding of the life of multicellular organisms very much depends on knowledge about each single type of plant, animal and human cells. Thus, it is a permanent task of science to gather knowledge about each kind of cells separately.

Many important studies on defined cell types were and are performed, while the cells of interest remain within their natural environments. Modern microscopical [1–8] or flow cytometrical techniques [9–16] are powerful tools for investigation of features of single cells that are still embedded in their original tissue or surrounded by other cells of their origin body compartments (e.g. blood or liver). With the help of these methods, numerous kinds of cells have already been successfully characterized regarding their expression of genes and gene products or their intracellular content of ions and DNA.

For studies on many cell biological questions, however, a sufficient number of equal cells with a high degree of purity and vitality are required. In particular, if functional aspects are to be investigated and if cells are needed for clinical transplantation or for biotechnological production purposes, interesting populations have to be purified from their natural environment prior to investigation, transplantation or engineering.

2. Cell purification by clonal selection

A high number of equal cells can be obtained by culturing cell lines or cell clones for several days, weeks or even months. A high degree of homogeneity is achieved by selecting distinct cell types under culture conditions that allow only the wanted cells to grow or to grow faster than unwanted ones. Cloned cells are often quite suitable for the investigation of biochemical components and processes and for the fermentation of biological products. A great number of cell lines and cell clones have already been cultured [17–33]. The most famous examples are B-cell hybridomas, which secrete monoclonal antibodies [34]. However, other cell lines derived from different origin cells also have the capability to produce large quantities of various biological substances. Additional cell lines and clones were developed with the aim of providing cell populations that react homogeneously upon induction of gene expression, differentiation or cellular signaling. Studies on cell lines or cell clones contributed a huge amount of knowledge to science. However, cells of cell lines or cell clones are as a rule transformed, unnaturally fast-growing cells. Many of them do not even have a normal set of chromosomes. Thus, results of studies on cell lines or cell clones usually do not provide much information about the in vivo situation of the corresponding normal origin cells. Furthermore, injecting cloned cells into patients for transplantation purposes appears dangerous as they resemble cancer cells very much.

So, there remains a demand to purify cells that are equal but not transformed and represent their kind of cells as they occur in vivo. They have to be isolated within a short time, should not be damaged and, in many cases, should not even receive signals of activation and differentiation during the isolation procedure. Short-term cell isolation procedures need separation criteria that do not depend on growing capabilities but comprise cell type-specific biological capabilities or physical characteristics. Typical biological capabilities that can be used for cell separation are as follows: adhering to surfaces, binding distinct fluorescence dyes and the expression of defined patterns of surface antigens, while specific

cell density, cell size or negative surface charge density are considered to be physical cell parameters.

3. Cell purification after tagging the surface

Many kinds of cells are identifiable by substances that bind specifically to their surfaces. In particular, monoclonal antibodies proved most suitable for the identification of a lot of cell types that express specific patterns of antigens on their surfaces. Cells binding a known substance can be isolated preparatively from single cell mixtures, if this substance is either labeled by fluorescent dyes or by magnetic beads or is coupled to a solid matrix.

3.1. Panning

The selective attachment of cells to solid surfaces is a useful tool in the isolation of specific cells. The method is based on the different rates at which different cell types adhere either directly to plastic and glass surfaces [35] or to peptides or antibodies that are coupled covalently to solid surfaces [36–40]. It is simple and rather inexpensive and, therefore, is commonly used. However, resolution is often unsatisfactory.

3.2. Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) devices and their operating principles have already been described [41]. Instruments are commercially available from Becton and Dickinson (San Jose, CA, USA) or Coulter (Miami, FL, USA). Current state of the art devices are capable of identifying cell types by three to five parameters, e.g. by three to five different fluorescent dyes bound to the cells either directly or via antibodies. On a preparative scale, between 500 and 3000 cells can be divided per second into two or three fractions. So, if suitable antibodies and fluorescent dyes are available, the machines can purify up to 10 million cells per hour, achieving enrichments of 95% or higher. Thus, FACS devices are widely used despite their high cost. Examples of applications are described in [42–53].

3.3. Magnetic-activated cell sorting

An alternative method of preparatively purifying cells that can be distinguished using antibodies is immunomagnetic sorting. Antibodies are coupled to magnetic beads and added to a cell suspension. After the antibodies have bound to their corresponding cell surface antigens, the beads (and, simultaneously, the cells connected with the beads) are separated from the cell suspension with the help of magnetic forces. This method is already in use, but technical improvements are still being sought. Since the magnetic beads bound to the cells often interfere with the experiments that are intended to be performed on the purified cells, positive and negative selection procedures are applied. This means that separation experiments are performed either after labelling the wanted cells, so that they interact with the magnetic field and can be collected, or after labelling the unwanted cells so that only the wanted cells pass the magnetic field. Both procedures have inherent drawbacks. In the first case, the antibodies have to be removed from the purified cells, whereas in the second case, lots of different antibodies are required. Research is also on-going to find the optimal magnetic beads and to construct the optimal separation devices. Frequently used beads are the dynabeads, which are commercially available from Dynal (Oslo, Norway). Usually they are rather large and have diameters of 4.5 μm. Smaller beads, with diameters of 1.5 μm, may be obtained from Advanced Magnetics (Cambridge, MA, USA) and beads with diameters below 1 μm have been developed by Microcaps (Rostock, Germany) [54].

Many immunomagnetic sorter (MACS) devices consist of columns filled with magnetizable material such as steel wool. These columns are fixed within a magnetic field. Cell suspensions treated with antibodies bound to magnetic beads are applied while the magnetic field is active. Under these conditions, unlabeled cells pass through the column, while the magnetic beads are retained by the magnetized column matrix. Then the column is washed, to prevent unspecific cell adhesion. Afterwards, the magnetic field is removed and the beads, together with the cells bound to the beads, are eluted [55–60].

In addition to the column devices, continuously working flow through systems were developed [61].

One of them was constructed according to the continuous flow principle originally realized in free flow electrophoresis [62]. Separation chambers are composed of two plates placed parallel to each other at distances of between 0.2 and 3 mm (Fig. 1). Carrier fluid is pumped through the gap to flow laminarly. Suspensions of cells treated with antibodies bound to magnetic beads are continuously introduced at one side of the separation chamber and are carried by the laminarly flowing fluid through the chamber. Arriving at the other end the fluid film is fractionated continuously. On their way through the chamber, the cells and magnetic beads are exposed to a magnetic field, which acts perpendicularly to the fluid flow so that it induces the magnetic beads to deviate from the laminar flow and to approach the magnet. Hence, unlabeled cells are carried through the chamber following the laminar flow, while magnetic beads, together with cells bound to them, deviate from the stream lines approaching the magnet. Thus, both unlabeled cells and cells bound to beads arrive at different sites at the distant chamber side and are separated.

In an older device, of a continuous immunomagnetic sorter (CIMS), cells and magnetic beads flow upwards through the separation chamber under laminar flow conditions [63]. Magnets are mounted along the midline of the front and the rear plate of the chamber (Fig. 1). They establish a magnetic field with forces oriented perpendicularly to the direction of the fluid flow. Mixed cell suspensions are injected continuously as fine bands into the chamber near both edges. On the way through the chamber, the magnetic beads and the cells coupled to them are deviated according to the direction of the magnetic forces and move from the edge into the center of the separation chamber. Thus, when cells and beads arrive at the top of the chamber, the unlabeled cells arrive near the edges opposite to their site of injection, while labeled cells arrive at a central position. So, fractionation of the various cell types is possible.

Another principle of continuous magnetic-activated cell sorting was recently developed by the Dr. Weber GmbH (Kirchheim, Germany). In this system, a separation chamber is formed by two glass plates, as shown in Fig. 1. However, the magnet is mounted as a magnetic plate on the rear glass plate (Fig. 2) of

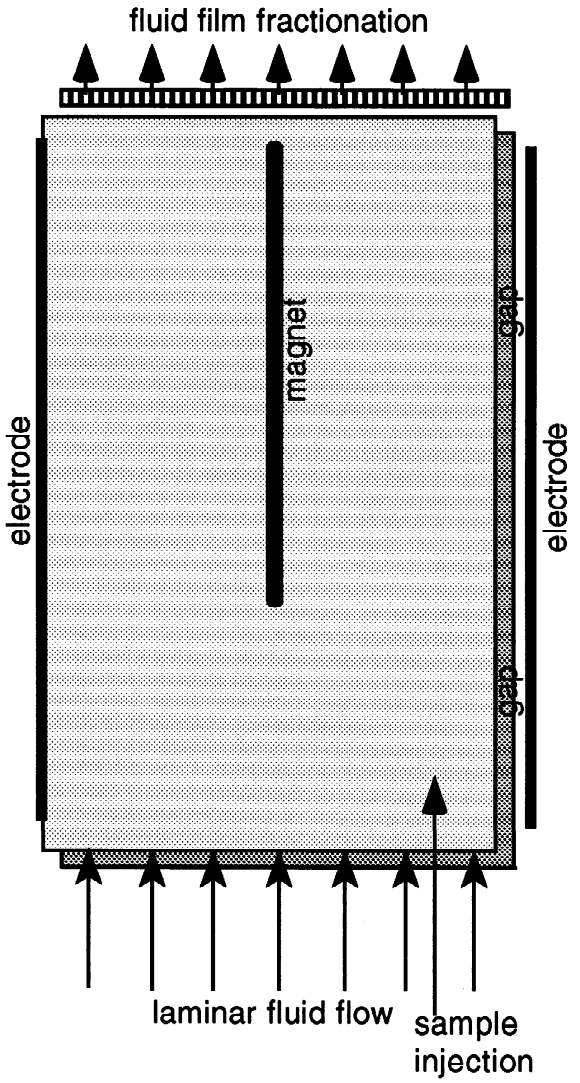

Fig. 1. Principle behind a continuous flow separation chamber. The separation chamber consists of a front (dotted area) and a rear glass plate (gray area), with a gap between them. Gaps may have widths ranging from 0.2 to 3 mm. Separation medium flows laminarly through the gap, as indicated by the arrows. If magnets are mounted along the midlines of the front and rear plates, the chamber can be used for magnetic-activated cell sorting (see Section 3.3). Alternatively, if electrodes are mounted at both edges, free flow electrophoresis may be performed (see Section 4.2).

the free flow chamber. The thickness of the buffer film is divided into two parts. Covering the total breadth, plain separation medium flows through the chamber at the rear plate and, adjacently, cell

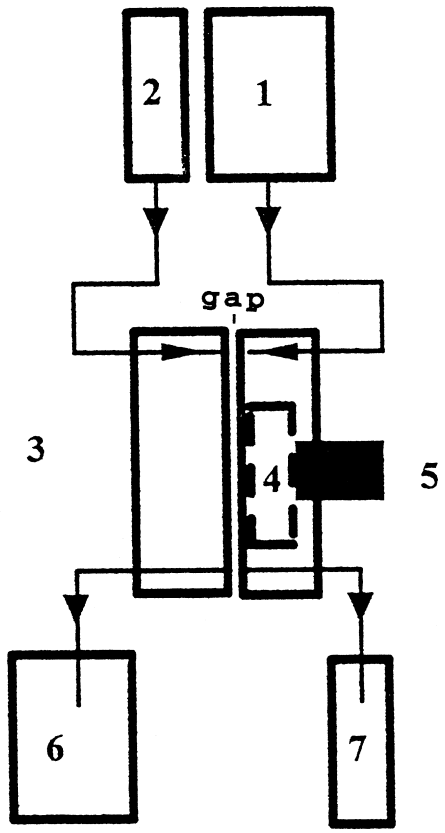

Fig. 2. Scheme of a newly developed continuously operating magnetic-activated cell sorting device with a separation medium container (1), a cell suspension vial (2), a separation chamber formed by two glass plates (3), a magnetic amplifier (4), a magnet (5) and two additional vials for collecting the fraction, which is free of magnetic beads (6) and the fraction that contains the magnetic beads and the cells connected with them (7). (The picture was generously provided by the Dr. Weber GmbH).

suspension flows at the front plate. On their way through the gap, beads, together with coupled cells, are attracted by magnetic forces and leave the rest of the cell suspension, approaching the rear plate, while the unlabeled cells remain near the front plate. At the other end of the gap, the chamber medium is split and labeled cells are separated from unlabeled ones. The new instrument provides a tremendous throughput of at least 10^9 cells/h. In addition, it has the major advantage that cells and the magnet are close together (<1 mm) so that the distances that the beads, or beads and cells, have to be moved are short. Hence, the magnetic forces required for attracting the beads are small and small beads are sufficient for labelling the antibodies.

Basically, MACS devices can be used for separating all kinds of cells that are identifiable by an antibody [55–60]. However, in contrast to FACS devices, only one type of antibody can be used for identifying a cell. Thus, resolution is poorer. Still, due to their lower prices, MACS instruments are used for many cell separation experiments. A very interesting field of application is purification of CD34-positive cells for clinical transplantation purposes [64–69]. In this case, large quantities of cells are required, so that the high throughput of MACS devices provides a real advantage. A major disadvantage, however, of purification of CD34-positive cells with the help of antibodies and magnetic beads is that the labels have to be removed from purified cells before they are injected into patients and many cells are severely damaged during this process.

4. Cell purification without surface tags

4.1. Counterflow centrifugal elutriation (CCE)

CCE separates cells on the basis of different cell size and, to a lesser extent, density. Because separation may take place in culture medium within a short time period, it is gentle and the characteristics of resultant populations reflect the status of the cells applied to fractionation quite closely.

4.1.1. Method

In an elutriation system, the cells loaded into the elutriation chamber are subjected to the centrifugation force generated by the rotation of a rotor in an outward direction and to the fluid force pumped into the separation chamber in an inward direction. At a given centrifugation speed, centrifugation forces are higher at peripheral points of a rotor, but lower at central points. The geometry of the separation chamber is designed so that the fluid forces are highest at the periphery, decrease along about 80% of the chamber length, but increase again near the central end. So, in an elutriation chamber, net sedimentation forces are low at the peripheral bottom where the cells enter the chamber through a narrow inlet. Within the following peripheral part, they rise

maximally and decrease again towards the center of rotation. During the loading process, at constant rotation and pump speed, the cells are sedimented to a position in the separation chamber according to their sedimentation velocity, which depends on the cell size and cell density, as well as on the loading pump and rotation speed. As long as sedimentation forces and the opposite fluid forces remain constant, the cells remain in this position. If the pump speed is increased and/or the rotation speed is decreased, homogeneous populations of cells with a specific size can be eluted. Usually, increasing the pump speed leads to much higher separation quality than decreasing the rotation speed. In any case, a smooth function of the pump driving the counter fluid flow is very important.

To my knowledge, only Beckman Instruments (Palo Alto, CA, USA) sells elutriation equipment. Two classes of rotors are available. The JB-6 rotor has a 4-ml separation chamber and can accommodate about 2×10^8 cells. The JE-5.0 rotor, with a 40-ml separation chamber, can accommodate 2×10^9 cells. Both rotors are mounted on suitable centrifuges, also constructed and sold by Beckman Instruments. In addition, a laboratory-made device has been described in the literature [70], which works quite well when only a rather small number of cells is available. It consists of a benchtop centrifuge, to which a small rotor that has a separation chamber with a volume of 0.5 ml is mounted (Fig. 3). The rotor works with a resolution similar to that of the commercial instruments, when 2×10^5 tissue cells or 2×10^7 mononuclear leukocytes, which may be obtained from 20 ml of peripheral blood, are loaded.

Most experiments described in the literature were performed using a Beckman JB-6 rotor with a 4-ml separation chamber. Usual rotor speeds ranged from 1800 to 3000 rpm. Depending on the actual rotor speed, the counterflow was started at rates of between 10 and 20 ml/min and was increased stepwise for fractional elution up to 80 ml/min. Another series of experiments were performed using a JE-5.0

Fig. 3. Photograph of the separation chamber, with a 0.5-ml volume and a rotor with a diameter of 18 cm, of the laboratory-made elutriator described in Ref. [70]. The rotor may be mounted on a benchtop centrifuge.

rotor with a 40-ml separation chamber. The rotor speed usually was between 1500 and 2300 rpm, while the counterflow rates started at 60 ml/min.

4.1.2. Applications

The method of CCE was described for the first time in 1968. It took about ten years until scientists started to be interested in this method. Meanwhile, 870 manuscripts recorded in MEDLINE mention the method within the title or the abstract. Although this number may only represent a small part of the studies on CCE purified cells or the CCE purification technique, it suggests that the method is not used too often. For comparison, the term 'cell line(s)' was found 116 770 times in the same database, the term 'cell clone(s)' 7850 times and the term 'FACS' 2268 times. However, studies published during the last three or four years confirmed that there are niches where the method of CCE is very important and helps in the gathering of knowledge about the biology of different kinds of cells or about purifying stem cells for transplantation. Articles including the term centrifugal elutriation describe the separation of different cell types from mixed populations as well as the separation of a wide variety of proliferating tumor cell lines into progressive stages of the cell cycle. CCE was used preferentially when antibodies for labeling of the surface of living cells of interest were not available or could not be used, because they appeared to hamper the subsequent use of the purified cells.

4.1.2.1. *Cell cycle analysis*. Measuring the DNA content of cells by flow cytometrical or microscopic methods is widely performed. However, these methods cannot be used for the preparative purification of living cells, since cells have to be killed to make their DNA accessible for intercalated fluorescence molecules. Therefore, an increase in cell size during passage through the cell cycle is still the most dominant difference between living cells that are at different stages of the cell cycle. Obviously it is a biological rule that initiation of DNA replication does not occur in eukaryotic cells until cells reach a minimum size [71]. Before initiation of DNA synthesis, the overall accumulation of cellular proteins and the adjustment of cell size in late G1-phase seems to be required.

For the separation of cells that are at different stages of the cell cycle, mainly cells of homogeneous cell clones are subjected to centrifugal elutriation. Thereby, the small cells that are in G1 phase can be separated from S-phase cells, which have intermediary size, and from the G2/M phase cells, which have the largest size of the cell population. The DNA content of the separated cells is usually verified by flow cytometric analyses. In good experiments, the cells of a cell line are separated in fractions that have 100% G1 phase cells, 80% S-phase cells and 80% G2/M phase cells, respectively [72].

The fractions of cells at different stages of the cell cycle could successfully be used to gather information about the regulation of the progression of a cell cycle. During the last three years, many investigators used elutriated cells to study the role of cyclin and cyclin-associated proteins in the regulation of cell division [73–80], to study the cell cycle-dependent sensitivity of cells to radiation and drugs [81–90], the cell cycle-dependent activity of enzymes that are important for cell division [91–93], the cell cycle-dependent expression of various types of proteins involved in cell cycle regulation (e.g. kinases) or in the de novo synthesis of other proteins (e.g. chaperones) [94–98], and the cell cycle-dependent responsiveness to signals transmitted by cytokines [99,100].

The advantages of synchronizing cells by CCE are obvious. The separation conditions are ideal for isolating populations of cells in specific phases of the cell cycle with minimal metabolic perturbations. No drugs are required to stop the progress of cell division at a distinct point of the cell cycle. Therefore, no drugs are present that could interfere with the genes or enzymes to be investigated. Elutriation takes about 1 h. In this time, cells do not progress very far through the cell cycle. The cells are kept in suspension and signals triggering any change in the progress are avoided. Thus, if a cell population is so homogeneous that all G1 phase cells have similar volume, centrifugal elutriation is a method that seems very suitable for synchronizing cells for further studies of biological and biochemical processes that are important for cell division.

4.1.2.2. *Purification of contact-sensitive cells*. A number of different kinds of cells, such as mononuclear phagocytes, recognize, very unspecifically, foreign molecules and particles entering an organism. If these cells come into contact with any material that does not belong to the host organism, they react very sensitively. Even upon adhesion or upon antibody binding, signals may be delivered to their cell's interior, which trigger a variety of cell activities [62]. So, despite the many alternative methods, such as antibody-dependent sorting or panning or absorption techniques, CCE, which does not include cell adhesion to matrices or to antibodies, is often preferred, to separate monocytes from peripheral blood or bone marrow and to purify macrophages from alveolar tissues or Kupffer cells from liver and to enrich mast cells. The purified cells have been used to investigate their antigen-processing behaviour [101] and their production of reactive oxygen molecules [102–108], their responses to polysaccharides or glycosides [106,108–110] as well as to histamine, nicotine, complement factors [103,107,111–113] or to cytokines [104,114–116]. In addition, monocytes, macrophages, Kupffer cells, basophils or mast cells purified by CCE proved useful in investigations of their actual phenotypic appearance [107,115,117–121], their actual cytokine [110,122,123] or histamine secretion capability [119,121,124], their ability to influence other cells [107,114,125,126] or to kill cancer cells [105,115,127] and their infection by viruses [128–130].

4.1.2.3. *Purification of tissue and cancer cells*. Characterizing the biological behaviour of healthy and malignant tissue cells is still a problem. Most of the different types of tissue cells can barely be purified while keeping their biological function unchanged. Even preparing single cell suspensions often requires very rigorous methods. In order to remove the intercellular matrix, digestion by proteases and collagenases is essential. These enzymes not only digest the extracellular proteins but also attack the membrane proteins, i.e. the cell surface antigens. In addition to enzyme treatment, mechanical disruption usually follows, which, in most cases, is not very gentle on the cells. Sometimes it may be helpful to culture single cells obtained after tissue disruption for a passage in vitro, in order to give them some time to recover before they have to suffer the additional stress of subfractionation. We have shown that a short in vitro incubation between tissue disruption and CCE separation does not change the cellular composition [131].

Tissue cells in general are not as well characterized as, for example, cells of the peripheral blood, of the bone marrow or of the various lymphoid organs. This lack of knowledge is in the areas of biological behaviour and surface antigens. While a battery of monoclonal antibodies against a number of lymphoid cell surface antigens enables one to determine the differentiation status of lymphoid cells exactly, antibodies against tissue cells are still not very abundant. So, fractionation of single cell populations, obtained from healthy or malignant tissues, by CCE, is a competitive way of providing homogeneous cell populations of the tissue for biological, toxicological and pharmacological studies.

Most of the tissue cell purification experiments using CCE were done on liver cells, as single cells can be more easily obtained from the liver. The liver consists of hepatocytes, which represent 60–70% of the liver cell population and are extremely large (20 μm of diameter), bile duct epithelial cells, which make up 2–3% of the liver cell populations and other cell types. Of the total hepatic cell population, 25–40% consist of other cell types, such as fibroblasts, endothelial cells, dendritic cells and sometimes infiltrating erythrocytes and/or leukocytes [132]. Various types of liver cells that were enriched or purified by CCE proved suitable for addressing toxicological questions [133–137] or biological questions regarding membrane potential variations [138] and were also used to investigate various surface receptors [139], the production of biological substances [140] and the progress of differentiation [141]. In addition, the enrichment of distinct liver cell populations was a useful prerequisite for cloning the respective cell type [142].

A further cell type prepared for biological studies by CCE was gastric mucosa cells [143]. Alterations in these cells under the influence of *Helicobacter pylori* were studied. Also, keratinocytes were subdivided into three subtypes in order to see their different abilities to produce interleukin-1 [144]. *Corpus luteus* cell populations were purified to study

their cytokine dependence [145]. Alveolar and lung epithelial cell populations were fractionated for the determination of adenosine 3′,5′-cyclic monophosphate (cAMP) and the enzyme content of various cells, respectively [146,147]. Canine endocrine cells were enriched for the determination of their peptide release capability [148]. Growing characteristics and collagen synthesis capabilities of chondrocytes were also studied after enrichment of these cell types [149–151].

Identifying the causal events and temporal aspects of tumour development requires the ability to separate malignant and normal cells from a single piece of tumour for comparative analyses. Centrifugal elutriation was recently used as a method to further enrich prostate [131] and ovarian cancer cells [152] from suspensions obtained by conventional means of tumor dissociation. Viable mouse type II and Clara cells were purified with the aim of being able to identify cell-specific changes in gene expression or in enzymatic pathways following in vivo or in vitro exposure to environmental carcinogens [153]. Ovarian cancer cells were separated from infiltrating immune cells. Both types of cells were studied with respect to their IL-6 production capability [154]. Human JAr cells and cytotrophoblasts, cocultured for 72 h, were fractionated according to their size by centrifugal elutriation, so that the mutual influence of both interacting cell types could be investigated [155].

4.1.2.4. *Purification of sperm cells*. A considerable number of studies were published recently describing the purification of male germ cells at different stages of maturation. Using CCE, the different cell types of the testis of rats and also of humans were separated from each other in order to optimize the method [156] and to subject purified cells to various studies. In addition to toxicological aspects [157], the expression of histone genes [158–160], major histocompatibility antigen genes [161], genes encoding for neuroactive factors or peptides [162,163] and for insulin-like growth factor [164] were of interest. Furthermore, different growth behaviours of different testis cell populations were found when the *c-mos* gene was overexpressed [165], and age-related testosterone production was investigated [166]. The dependence of various cell populations on hormones such as luteinizing hormone and follicle-stimulating hormone was studied by withdrawing the hormone using antibodies [167,168]. In another series of experiments, the protein pattern of purified spermatids from rat testis was analysed by two dimensional electrophoresis [169,170].

4.1.3. Impact of CCE on cell research

Taken together, the studies reviewed above reveal that CCE is still a competitive method of cell purification, in areas where it is important to enrich cell populations without changing their activation or differentiation status. This is the case if the various steps of the cell cycle or the transient expression of distinct genes are targets of interest and if cells, such as monocytes/macrophages, which are sensitive to contact with foreign material, are to be investigated. Furthermore, the effects of delivering activation or differentiation signals to cells can more easily and reliably be recognized, if these cells are not exposed to excitatory signals during the purification procedure. In addition, CCE has proved helpful in the preliminary purification of tissue cells that are not identifiable by antibodies.

4.1.4. Purification of CD34-positive cells for transplantation purposes

Recently, an interesting new field of application arose, when transplantation specialists found that CD34-positive hematopoietic stem cells were useful in restoring hematopoiesis in patients who had to undergo whole body radiation or strong chemotherapy. In many experiments, anti-CD34 antibodies were used to label the cells of interest, which are present in the bone marrow, and, after mobilization, also within the peripheral blood at a reasonable concentration. The CD34-labelled cells were separated either by panning, immunomagnetic sorting or fluorescence-activated cell sorting [64–69]. All of these techniques, however, had two major drawbacks: labelling the cells specifically was time-consuming and it was difficult to remove the antibody from the cell surface of isolated cells. So, CCE was considered to be an alternative method for CD34-positive stem cell purification.

Various studies have already been performed in order to investigate the feasibility of applying CCE for stem cell purification [171]. In one series of

experiments, the investigators tried to find out which source of cells would be most suitable for further purification, i.e. bone marrow cells or the peripheral blood after mobilization of the stem cells [172–174]. The separation studies revealed that the CD34 cells varied in their volumes. The more immature ones had small volumes, like lymphocytes, and the more mature ones had larger volumes, similar to monocytes. Since the T-cell fractions usually contain killer cells that are often the cause of severe graft versus host reactions, it was of interest to remove the T-cells from the CD34-positive cells. Thus, several investigators tried to take only the fractions containing the large cells for transplantation. However, T-cell depletion through CCE, while causing the loss of only minor proportions of CD34(+) cells and granulocyte-macrophage colony-forming units (CFU-GM), carried the risk of losing the majority of the more immature progenitor cells [175,176]. Therefore CCE of CD34(+) cells was further investigated, with the aim of obtaining the optimal cell population for transplantation [177–180].

The attempts at purification of CD34-positive cells showed that CCE purification of hematopoietic stem cells is desirable, because it is fast and no antibodies bearing labels have to be removed from the cell surface prior to injection into a patient. However, CCE resolution is still not optimal. Further efforts are required to improve to resolution of CCE instruments and/or to include CCE in a multistep procedure such as described in Ref. [62].

4.2. Free flow cell electrophoresis (FFE)

Another method of purifying cell populations according to their natural features is carrier-free electrophoresis. Similar to CCE, this method can be applied without pretreatment of the cells. Two types of carrier-free electrophoresis are suitable for cell purification: free flow [181,182] and column [183] electrophoresis. Within the last ten years, most studies published described the use of FFE.

4.2.1. Method

The basic principle of this method has already been described [181,182]. Thus, in this manuscript, only a short summary of the technique is given. A laminar buffer stream flows between two narrowly spaced parallel glass plates (Fig. 1). An electric current is applied perpendicularly to the carrier flow. A sample solution is injected as a narrow band into the carrier fluid flow near one end of the chamber. Cells exposed to the electric field migrate laterally towards the positively charged electrode in the horizontally flowing buffer. The migration velocity depends on the negative surface charge density. Thus, cells with different negative surface charge densities migrate at different speeds, arrive at different points along the opposite edge and can be collected for preparative isolation.

During the past few decades, several FFE machines have been developed and sold by several companies from all over the world [184]. In addition to the machines destined for normal laboratory use, special instruments have been developed for use in space flights under microgravity [185–187]. At the moment, according to my knowledge, only the OCTOPUS manufactured by the Dr. Weber, GmbH, is commercially available. This machine has a chamber that is 500 mm long, 100 mm wide and 0.2–0.4 mm thick. Compared to earlier devices, it has a few important features which support the performance. Cell harvesting is facilitated by a counterflow implement adjusted to the cell harvest implement. The carrier fluid may be divided into several segments, since the introduction of the medium into the chamber via several inlets is allowed. Therefore, it is possible to add an elevated amount of ions (NaCl) to the separation medium [182,188,189].

4.2.2. Applications

The first attempts to purify cells from heterogenous cell mixtures, such as those found in ascites fluid, were reported more than 34 years ago [190]. Since then, FFE has been used for purifying many kinds of cells [181]. Prior to the production of monoclonal antibodies, FFE was used frequently to purify various cell populations of the immune system. The isolated cells were then studied to determine their various cell types and interactions. However, since it is possible to characterize cells using antibodies and to purify labelled cells from unlabelled ones by FACS, MACS, or panning techniques [35–69], the use of FFE for the separation of whole nucleated cells has dropped dramatically.

Only two aims were pursued using preparative FFE in recent years. One aim of preparative cell electrophoresis was to attempt to separate X and Y sperms [191–196]. However, an isolation procedure for reliable separation has not been found to date. The second reason for using FFE as a tool of cell purification was to study the biological impact of the negative surface charge density. For this purpose, variations in the negative surface charge density within defined cell populations were investigated. Often, analytical cell electrophoresis devices were used as reasonably pure cell populations were available. With the help of analytical cell electrophoresis devices, changes of the negative surface charge density of red blood cells [197–200], cloned tumor cells [201,202] and endothelial cells [203,204], under conditions of sickness, cancer or drugs, were investigated.

Some cell populations were preparatively separated by FFE devices in order to provide different fractions containing cells of one cell type with different electrophoretic mobilities. The cells of these fractions were comparatively characterized. Human erythrocytes were subfractionated and the red blood cells with different electrophoretic mobilities (EPMs) were investigated with respect to their enzyme content [205]. Neutrophils isolated from the peripheral blood were fractionated by FFE and neutrophils with differing EPMs were tested to determine their levels of various activities, such as oxidative burst [206–208]. Other scientists were interested in pituitary gland cellular interaction and activities [209,210]. They separated the cells of the gland either immediately after tissue dissection or after incubation in ground-based laboratories and space shuttles by preparative FFE and characterized the cells with different EPMs comparatively. We looked at the changes of negative surface charge densities that occur when B-cells and macrophages differentiate along their lineage [211–213].

4.2.3. Impact of FFE on cell research

At the moment, FFE has almost no impact on cell research. There are several reasons why preparative cell electrophoresis is so rarely used. The most dominant reason is that cells have to be suspended in low ionic strength media, such as triethanolamine medium, for the period of electrophoresis [214]. Low ionic strength media have disadvantageous effects on the cells [215]. The new device constructed to allow the presence of 50 mM sodium chloride in the separation medium [182,188,189] may provide new possibilities to prepare cells with different EPMs for biological tests. A second problem is that virtually all cells have very similar electrophoretic mobilities [216]. A collection of EPM values for more than 300 cells, which seems to include most of the electrophoretic mobilities measured so far, revealed that most cells have an EPM that is between 50% below and 40% above the EPM of human erythrocytes. This observation may be explained in part by considering that cell electrophoresis is a quite insensitive method. Using sophisticated formulae [204,217,218], it has been determined that major changes in the outer cell surface layer have to occur before the EPM changes. Thus, considerable improvement of the resolution of the FFE method is desirable in order to make it a more helpful tool for investigating the negative surface charge density of cells. Further progress in understanding biochemical components and biophysical laws that build up the cellular negative surface charge density will surely have considerable impact on future cell research and medicine.

5. Conclusion

Recent advances in cell separation include two interesting developments: (i) The principle of a continuous flow separation chamber originally developed for carrier-free electrophoresis could successfully be adapted to magnetic-activated cell sorting with low unspecific absorption and tremendous throughput. (ii) A great number of publications confirmed that cell separation without surface tags by counterflow centrifugal elutriation and/or free flow electrophoresis is most useful, if suitable tags (e.g. antibodies) for identification of wanted cells are not available and if tags bound to cell surfaces would interfere with the use of the isolated cells. However, limited resolution capabilities hamper the use of CCE and FFE in some cell separation problems, such as the enrichment of CD34-positive stem cells or the subfractionation of defined cell populations with varying electrophoretic mobilities. Thus, it appears to

be desirable that instrumental improvements continue to be made.

6. List of abbreviations

FACS	Fluorescence-activated cell sorter
MACS	Magnetic-activated cell sorter
CCE	Counterflow centrifugal elutriation
FFE	Free flow electrophoresis
CIMS	Continuous immuno-magnetic sorter
EPM	Electrophoretic mobility

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