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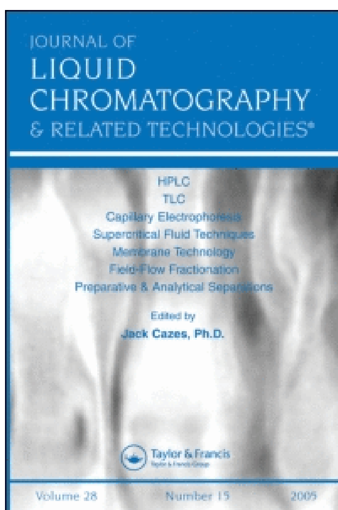

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SEDIMENTATION FIELD-FLOW FRACTIONATION: METHODOLOGICAL BASIS AND APPLICATIONS FOR CELL SORTING

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SEDIMENTATION FIELD-FLOW FRACTIONATION: METHODOLOGICAL BASIS AND APPLICATIONS FOR CELL SORTING

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ABSTRACT

As a cell sorter, sedimentation field-flow fractionation (SdFFF) can be defined as an efficient tool for cell separation and purification which respects cell functional integrity, viability, as well as provides enhanced recovery and purified sterile fraction collection. SdFFF elution should be performed under strictly defined conditions concerning apparatus construction (channel wall materials) and set up (bio-compatible "Hyperlayer" mode) to obtain rapid cell elution, high recovery (negligible cell trapping), short- and long-term viability, and sterile conditions (cleaning and decontamination procedures). As shown recently in various reports, specific characterization of time-dependent

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collected cells have demonstrated the effectiveness of SdFFF to provide, in a few minutes, purified, viable, sterile cells which can be used for many investigations such as transplantation.

Key Words: SdFFF; Hyperlayer; Cell Sorting; Cell population

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INTRODUCTION

The last decades have shown the considerable development of many cell separation techniques which have greatly contributed to many advances in life sciences.[1–6] The principal goal in cell sorting is the preparation of a sufficient number of identical cells with a high degree of purity, viability, and sterility. These requirements are of the utmost importance if cells are needed for fundamental investigations (metabolic activities, cell cycle analysis, apoptosis, or induction of differentiation, . . .), biotechnological applications (production of recombinant proteins), or cell transplantation (stem cell therapy, transgenic mice). Actually, a wide panel of techniques and methodologies are available for cell separation and characterization, such as centrifugation, elutriation, electrophoresis, flow cytometry (fluorescent-activated cell sorting or FACS), or magnetic-activated cell sorting (MACS), which takes advantages of biophysical criteria (size, density, shape, . . .), electrical charge, or specific antigen expression.[1–6]

Among these techniques, field-flow fractionation (FFF) methodologies, introduced in the late 1960s by J. C. Giddings, are described as the one of the most versatile separation techniques.[1,7–9] This chromatographic-like separation family, such as the Sedimentation-FFF (SdFFF) sub-family, appears to be particularly well suited for separation and characterization of micron-sized species such as cells.[1,5,6,8,10–13] Like all other FFF methods, separation using SdFFF is achieved by the combined action of a parabolic flow profile, generated by flowing a mobile phase through a ribbon-like capillary channel, and of an external field applied perpendicularly to the flow direction.[7–9,13–17] While gravitational-FFF (GFFF) uses the Earth's gravity, the SdFFF, also called Centrifugal- or Multi-gravitational-FFF, uses a multigravitational external field generated by the rotation of the separation channel in a more complex device.[7–9,13–17]

The SdFFF elution mode for cells is described as "Hyperlayer". In such a mechanism, cell size, density, shape, or rigidity are involved, as are channel geometry and flow rate characteristics. At constant flow rate and external field strength, larger or less dense particles are eluted first.[8–10,12,14–24]

Since the pioneering report of Caldwell et al.[10] which defined most of the basic rules and methodologies for cell separation, FFF, SdFFF, and related technologies[4,25–29] have shown a great potential for cell separation and purification with major biomedical applications, including hematology,[10,21,29–42] cancer

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research,[25,43] microorganism analysis,[11,44–62] and molecular biology.[11,63–77] More recently, we opened the field of neuroscience with the purification of neurons from a complex cell matrix (rat fetuses cerebral cortices),[78] or purification of neural undifferentiated cells from human cell lines.[79] The main goal of our group is now to explore many cellular functional aspects, such as cell differentiation, development, cell cycle analysis, apoptosis induction, stem cell therapy, or transgenic mice preparation, to demonstrate the effectiveness of the SdFFF as a safe cell sorter.

As for other preparative cell separation techniques, SdFFF must respect cellular functional integrity and viability, as well as provide enhanced recovery and purified fraction collection under sterile conditions. These features are easily achieved when SdFFF elution is performed under specific conditions. Moreover, by taking advantage of biophysical properties of cells, SdFFF appears to be a rapid and effective cell sorting technique in comparison to labeling-dependent methods for specific cell sorting applications, such as stem cell preparation, culture, and transplantation.[79]

In this study, we describe the theoretical, methodological, and practical aspects of SdFFF cell sorting.

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PRINCIPLES OF SdFFF CELL SORTING

Specific Requirements

Like all other separation techniques, SdFFF cell separation and sorting requires some specific methodological and technological features. These requirements are needed to achieve the main goal of cell separation which is the preparation of a homogenous cellular population whose original characteristics are respected. Therefore, SdFFF cell purification must take into account and respect:

(1) Cell functional integrity, such as metabolism specificity, genomic and proteomic capacity, cell adhesion properties, cell differentiation possibilities, . . . ;
(2) High level of cell viability is needed both at short (just after SdFFF elution and collection), and at long term for which SdFFF should not induce cell death by apoptosis or by necrosis in cell culture or transplantation. Limitation of cell death, apoptosis in particular, depends both on a drastic curtailment of cell-accumulation wall interactions, and on strict application of the cleaning procedure;
(3) Along with the first point, the maturation and differentiation stages of eluted cells should not be altered. If the capacity for cell differentiation can be preserved (first point), SdFFF elution process should not induce an uncontrolled differentiation of immature or stem cells. Our previous

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results, obtained with immature neural cells,[79] have demonstrated that SdFFF elution, performed under defined conditions, is very gentle and respects the maturation stage for both immature and differentiated cells. This leads to a possible comparison with other cell separation techniques based on the use of specific cell pre-labeling (FACS or MACS). As specific labeling is not necessary, SdFFF is particularly interesting for applications in which labels could interfere with further cell use (culture, transplantation), when labels do not exist or when labels could induce cell differentiation. Thus, because SdFFF cell sorting effectiveness is simply based on the intrinsic biophysical properties (size, density, shape, . . .),[8–10,12,14–24] it could provide an advantage over FACS or MACS for stem cell sorting. Nevertheless, the off-line hyphenation of SdFFF separation power with specific biophysical characterization by flow cytometry could be a very effective tool.[42]

The SdFFF cell sorting must also fulfill the following requirements:

(1) High repeatability and reproducibility, particularly if routine cell preparations are needed for culture, transplantation, or diagnosis;
(2) Maximal recovery. This is of a great importance in the case of tissue preparation such as chicken embryo olfactive epithelium for which, in contrast to cultured cells, sample preparation is expensive and time consuming, to finally obtain a very small amount of usable cells.[78] Decreased recovery should have two main origins. The first is the use of a flow injection procedure to avoid cell sticking and channel poisoning. This leads to a partial sample loss because some cells do not reach their equilibrium position and are eluted in the void volume. The second reason is linked to the small amount of cell sticking which led, as in its irreversible form, to channel wall surface modification, causing channel aging and poisoning. Thus, in the absence of cleaning procedures, this leads to a decrease in recovery, in signal repeatability, and reproducibility. Channel poisoning also results in peak shape modification and to a possible decrease in cell viability, as in the increase in apoptosis;[5,6,59]
(3) Finally, collected fractions must be sterile, which is essential if cell culture and transplantation are needed. To achieve this goal, an effective decontamination procedure has to be performed, such as using sterile samples and mobile phases.[5,6,59,78,79]

Specific Methodologies, Instrument Setup, and Procedures

To achieve cell separation in agreement with the previously cited requirements, specific SdFFF methodologies have been developed with the

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goal to preserve cell characteristics. These methodologies are performed to obtain better sub-population separation in association with a drastic limitation of cell-accumulation wall interactions which lead to channel poisoning with worst consequences on cell integrity.[5,6,41,59,78,79] Thus, the SdFFF device and elution conditions such as:

- —channel wall materials,
- —inlet tubing position and injection mode,
- —mobile phase composition and flow rate,
- —external field strength, and
- —cleaning and decontamination procedures

must be selected and performed to reduce cell-wall interactions.

Our SdFFF separation apparatus has been extensively described and schematized (Fig. 1).[5,6,59,78,80] Many characteristics have been developed and improved for its bio-adaptation and to facilitate maintenance.

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Channel Wall Material and Mobile Phase Composition

In many published reports,[5,6,41,57,59,80] it has been shown that cell separation is more effective when polycarbonate plates channel wall material, and the mobile phase supplemented with 5–10% bovine serum albumin (BSA) are used. However, some specific cell separations were not achieved under these conditions, such as for rat embryo cortical cells,[78] for which an irreversible and complete cell trapping was observed (data not published). For this reason, polycarbonate plates were replaced with 2 mm thick glass wall polystyrene plates. This very hydrophobic material led to effective cell separation with high recovery for all cell types studied until now, such as nucleated cells, human red blood cells, bacteria, or algae.[42,78,79] Moreover, if polystyrene plates were used, the addition of BSA to the carrier phase was not necessary, and no modification of retention behavior or separation effectiveness was observed (data not published). This is very interesting, because BSA increased channel poisoning and the risk of microorganism contamination and, therefore, the importance of cleaning and decontamination procedures. The use of polystyrene plates simplified the mobile phase preparation to iso-osmotic phosphate buffered saline solution at pH 7.4 (PBS 7.4), which could be added with a classical mixture of antibiotics and anti-fungals used in cell culture.

Injection Procedure and Inlet Tubing

The injection procedure usually used for cell separation is described as a "flow injection procedure", in which the sample is introduced and sorted without

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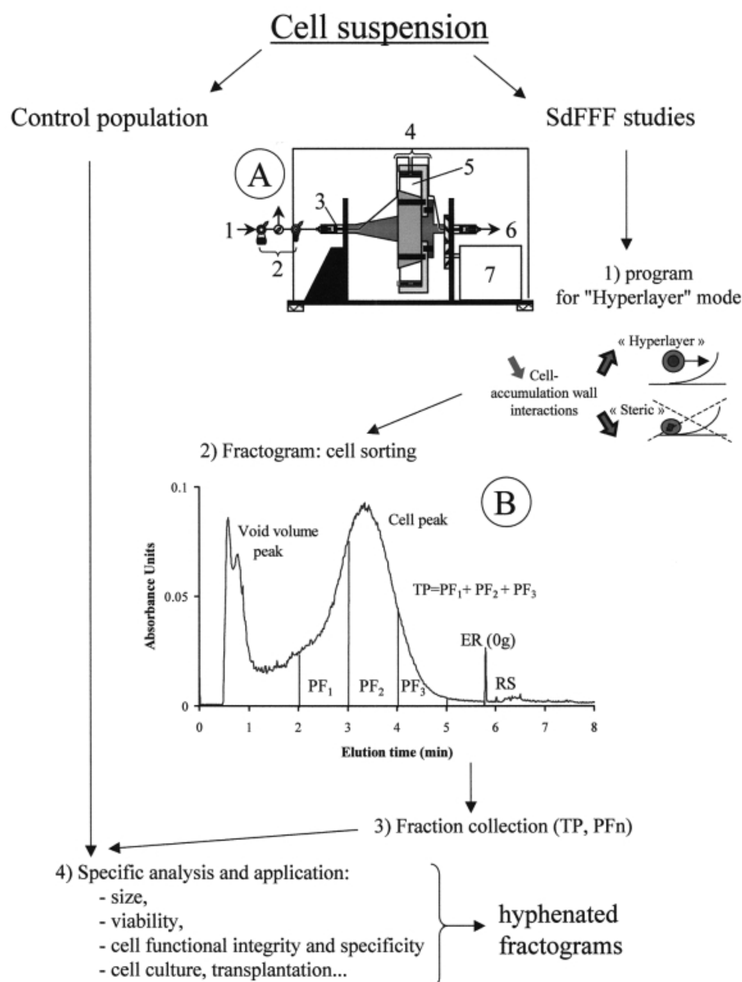

Figure 1. Schematic description of SdFFF cell sorting. A: Schematic representation of our SdFFF device with (1) mobile phase; (2) sample and decontamination injection device; (3 and 3′) Inlet and outlet rotating seals; (4) inlet and outlet tubing; (5) centrifuge basket with separation channel; (6) outlet tubing to detector and fraction collector; (7) motor and speed control command. B: Example of SdFFF cell elution fractogram. Elution conditions: flow injection of 100 μL cortical cell (10^7 cells/mL), flow rate: 1.24 mL/min (sterile PBS pH 7.4, 0.1% w/v BSA, penicillin/streptomycin); external multi-gravitational field: 60 ± 0.01 g, spectrophotometric detection at $\lambda = 254$ nm. TP and PF_n represented the collected fractions. ER correspond to the end of channel rotation, in this case the mean externally applied field strength was equal to zero gravity, thus RS, a residual signal, corresponds to the release peak of reversible cell-accumulation wall adherence.

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interruption of mobile phase flow and without a relaxation step.[41,78,79] The relaxation step procedure, also called "Stop-flow procedure"[1,9,41] consists in driving the sample to the channel inlet and then stopping the flow while the external field is applied. This procedure allows nano-sized particles to reach their equilibrium positions, which is essential to obtain a maximal selectivity for their separation. In the case of micron-sized species (cells), the absence of a flowing stream offsets hydrodynamic forces; particles are then submitted only to the influence of multigravitational forces which drive close to the accumulation wall, and, thus, increasing cell-wall interactions and channel poisoning. Thus, the "flow injection procedure" appears to be the more convenient mode, but to obtain similar selectivity, sample injection in our SdFFF device was done through an inlet tubing directly screwed to the accumulation wall, in contrast to other classical devices. This particular design simulated an elutriation phenomenon which preserved high selectivity with reduced retention time, and without increasing the channel poisoning, cell activation, or destruction.[5,6,35,40–42,78,79] But, as explained, this injection procedure led to decreased recovery due to partial elution of the sample in the void volume peak.

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Elution Mode: Flow Rate and External Field Strength Impact

The elution mode of cells depends on the flow rate/external field balance which generates hydrodynamic lift forces which, in turn, drive particles away from the accumulation wall. Species are then focused into a thin layer at the equilibrium position in the channel thickness, where the risk of cell-wall interaction is negligible. This elution mode is called "Hyperlayer".[8–10,12,14–24] Flow rate and external field strength should be selected to promote the bio-compatible "Hyperlayer" mode against the "Steric" one which can be defined as a limit case of the "Hyperlayer". The "Steric" mode occurs when the external field can be sufficiently increased or when the flow rate is sufficiently decreased to make lift forces negligible, instead of the external field strength. Then, cells are confined to a very thin layer close to the accumulation wall, which leads to harmful cell-channel interactions. Thus, each new cell separation study should begin with a systematic investigation of retention behavior at various flow rates and external field strengths (Fig. 1). Then, the retention ratio R_{obs} is systematically determined. R_{obs} is defined as the ratio of the void time vs. the retention time.[21,22] To determine if cells follow the "Hyperlayer" mode, we measured the pattern of R_{obs} for the specific cell elution peak under these various elution conditions. According to the SdFFF elution mode description of micron-sized species, R_{obs} is flow rate and external field dependent. At a constant field, the increase in flow rate induced an increase in R_{obs}, and an increase of field at a constant flow rate decreased R_{obs}.

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Moreover, "Hyperlayer" mode descriptions predict that sample will not be in close contact with the accumulation wall.[8–10,12,14–24]

By using the following equation,[14]

$$R = \frac{6s}{\omega} \tag{1}$$

in which R is the retention ratio, ω is the channel thickness, and s the distance from the center of the focused zone to the channel wall;[15] we calculated the approximate average cell elevation (s) using R_{obs} values. At a minimum, s is equal to the cell radius and particles are eluted under the "Steric" mode in close vicinity to the accumulation wall.[10,14] If s is greater than the particle radius, cells are eluted under the "Hyperlayer" mode.[10,14,78] But, to use this equation, it is necessary to determine, or to accurately estimate, the mean diameter of eluted particles and their size distribution in the different parts of the peak. Finally, other criteria such as Sd (size distribution selectivity) determination can be used to confirm the elution mode of the cells.[8,9,17,81] Nevertheless, the systematic study of cell retention properties can be performed easily with abundant cellular material, such as cultured cells, but it is more difficult if only small amounts of cells are available, as is the case with primary cell cultures or with cells prepared form tissues such as chicken embryo olfactive epithelium. In the latter situation, the "Hyperlayer" hypothesis was estimated by determination of R_{obs} under two distinct conditions. The first corresponded to the usual flow/strength conditions, and the second was conducted under more drastic conditions: lower flow rate and higher field strength, which led to a significant decrease in R_{obs} and, thus, demonstrating the flow/field dependence of the retention ratio. Moreover, if the cell radius was accurately known, it was possible to demonstrate that the average position of cell(s) in the channel thickness was greater than the cell radius.

Finally, even if the principal aim of SdFFF cell sorting was not the determination of the cell elution mode, it seemed to be important to demonstrate that cells followed the biocompatible and safety "Hyperlayer" mode.

Cleaning and Decontamination Procedures

The effectiveness of instrument design and set up to reduce cell-accumulation wall interactions was demonstrated first by the recovery of cells in the corresponding elution peak (>80%). Secondly, it was shown by conservation of cell viability which was, after SdFFF elution, similar to that of the control population. Finally, reduction of interactions was partially demonstrated by a very low cell release peak which was observed at the end of the fractogram when channel rotation was stopped and the mean gravity was equal to zero (external field applied = 1 g, Fig. 1). This residual signal corresponded to

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reversible cell sticking due to low interactions between cells and the accumulation wall.[5,6,17,41,78,79] Cells could easily be released from the accumulation wall under the effect of the mobile phase flowing, in the absence of channel rotation. On the other hand, irreversible cell trapping, which is due to strong cell-channel interaction, could not be reversed under these conditions. This phenomenon cannot be observed on the fractogram and leads to channel poisoning.

This can be overcome by systematically performed cleaning and decontamination procedures.[5,6,41,59,78,79] Some experiments (not published) have shown that the absence of effective and systematic channel cleaning led to many problems, in particular, an increase in apoptosis in separated cells, even though elution conditions were set up to enhance the "Hyperlayer" mode, because the small portion of definitively trapped cell die, releasing apoptotic signal into the separator which could activate apoptosis in freshly separated cells.

The different steps and instrument setup used for cleaning and decontamination have been extensively described.[5,6,41,59,80] The cleaning procedure is based on the use of osmotic shock and injection of deproteinating agent. The use of polystyrene plates and a BSA-free mobile phase has simplified the previous steps[59] which are now performed as an end-of-day cleaning-decontamination procedure. First, the PBS pH 7.4 was replaced by flushing the entire system with sterile distilled water at high flow rate. Five void volume equivalents of a 0.22 μm filtered protein cleaning agent (used for flow cytometry) were injected. Then, the entire SdFFF device was flushed at 0.7 mL/min for 30 min with a 3–4° sodium hypochlorite solution. The system was rinsed with sterile distilled water for 2 h at 2 mL/min. The system is then ready to use by replacing sterile water with sterile PBS. By implementing this cleaning–decontamination procedure, the same channel can be used for analysis of various cell populations without sample cross contamination and microorganism proliferation.

In conclusion, specific instrument design (polystyrene plate, flow injection through the accumulation wall, ...), optimal elution conditions ("Hyperlayer" mode), and cleaning-decontamination procedures should allow cell separation while respecting functional integrity, viability, recovery, sterility, and without modification of cell differentiation.

CELL SORTING AND CHARACTERIZATION

SdFFF as a Measurement Technique

Giddings[8,9] clearly defined FFF as a measurement technique. As retention is governed by field induced forces, retention measurements can be converted into numerical values of forces by using specific equations.[8,9,23,24] The quantification of these forces can be used to analyze particle properties, such as mass, size,

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density, electrical charge, diffusivity, ... and for populations with a continuum of properties, also described as polydispersed populations.[5,6,9] The properties can be measured, yielding size or density distribution by using specific calibration. This is the case for cell populations for which each measurable characteristic (size, density, ...) can be associated with polydispersity. Thus, by determining the average and variance of each parameter, it is possible to describe cell populations using a multi-polydispersity matrix.[6]

Cell Sorting Effectiveness

Cell sorting effectiveness could be measured and demonstrated only if we were able to separate and purify the different subpopulations of biological interest and, as a first step, the measurement of forces did not indicate, for example, if stem cells were sorted from the whole cell population of a specific epithelium. To achieve this goal, we first determined the best elution conditions based on cellular biophysical properties, and then analyzed the biological properties of the sorted population. The elution conditions had to be designed to obtain a larger retention time distribution corresponding to the broader peak in order to separate the different constituents of the original polydispersed population. Nevertheless, optimal elution conditions are a compromise between a sufficiently long elution time to collect sorted cell populations, and between the smaller cell sticking (Fig. 1). We performed an apparently effective cell separation supported by their differences in biophysical properties as predicted by the "Hyperlayer" mode in which size and density appeared as first order parameters.[8–10,12,14–24] However, obtaining a broader isolated peak did not indicate whether or not cell sorting based on the differences in biological properties was performed. Thus, time dependent fractions were collected (three or more, Fig. 1), analyzed, and cultured in order to characterize cell populations and to determine if previously described requirements were respected.

Cell Population Characterization

Many off-line techniques are then available to achieve this goal; we operated in three different and complementary directions (Fig. 1).

The first corresponded to the control of cell functional integrity including cell adhesion and culture capacities, determination of usual metabolic activities, and properties of cell differentiation. The second was the characterization of cell type in order to evaluate the cell sorting efficiency. These studies depend on the explored biological properties which were usually: specific metabolic activity, particular expression of antigen (surface receptors, differentiation markers), the

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capacity for cell differentiation or, on the contrary, the absence of differentiation for stem cells, specific size distribution, specific apoptosis behavior, the cell cycle, ...[36,39,42,47,57,59,78,79,82] Cell short- and long-term viability were assessed to determine if the elution process induced cell death and, in particular, a specific apoptosis.[78,79]

These studies were performed on each collected fraction (Fig. 1) and results were compared with control population. Two types of fractions were analyzed (Fig. 1):

(1) the total peak fraction (TP) which corresponded to collection of the entire cell elution peak (without void volume and release peaks). It represented the whole cell population after SdFFF elution; and
(2) specific peak fractions (PF_n, Fig. 1) which represented a part of the cell elution peak.

One important point was to verify if the TP fraction behaved similarly to controls. This could indicate that differences observed between control and PF_n fractions were a direct consequence of SdFFF elution efficiency to promote cell sorting by only taking advantage of biophysical properties.[8–10,12,14–24] If control and TP fractions behaved similarly, this demonstrated that cell sorting was not effective due to non specific phenomena, such as a selective sub-population killing, apoptosis induction, or irreversible cell trapping in the separator. If control and TP fractions properties were similar, it showed that cell sorting depended only on the difference in cell size, density, volume, or shape as predicted by the "Hyperlayer" elution mode.[8–10,12,14–24]

Finally, the association between retention parameters (biophysical properties) and specific biological markers led to the concept of a multi-dimensional hyphenated fractogram.[42]

CONCLUSION

SdFFF now appears as a mature technique for cell sorting in many biological applications. In principle, it can be defined as simple to use, to set up, and less expensive than many other cell sorting systems. Unfortunately, significant development and diffusion have not yet been achieved. This could be explained, first, by the small number of published studies and, secondly, it could be due, in part, to the lack of a specific commercial device which led each research group to develop or to adapt their own. Nevertheless, (1) because SdFFF takes advantage of intrinsic biophysical properties of cells and combines the possibilities of flow-driven separation techniques (elutriation, chromatography) and of field induced and focusing techniques (electrophoresis, centrifugation); (2) elution and sorting is very fast (less than 15 min); and (3) because the device

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can be easily and rapidly set up (less than two hours) to obtain optimal elution conditions for each new separation problem.

SdFFF could be more useful than many other cell sorters to provide purified, viable, and usable cell fractions.

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