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Sedimentation field flow fractionation purification of immature neural cells from a human tumor neuroblastoma cell line

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Abstract

The use of stem cells for therapeutic applications is now an important objective for the future. Stem cell preparation is difficult and time-consuming depending on the origin of cells. Sedimentation field flow fractionation (SdFFF) is an effective tool for cell separation, respecting integrity and viability. We used the human neuroblastic SH-SY5Y clone of the SK-N-SH cell line as a source of immature neural cells. Our results demonstrated that by using SdFFF cell sorter under strictly defined conditions, and immunological cell characterization, we are now able to provide, in less than 15 min, a sterile, viable, useable and purified immature neural cell fraction without inducting cell differentiation.

Keywords: Sedimentation field flow fractionation; Cell sorting; Neurons

1. Introduction

In many life science domains, analysis and sorting of a sufficient number of equal cells with a high degree of purity, viability and sterility is required. This is particularly true if cell cultures for functional aspects or biotechnological applications are to be investigated, or if cell transplantation is needed as is the case for stem cells therapeutic applications. Modern cell separation techniques have been essential to many advances for these applications and a wide panel of techniques and methodologies are available, such as centrifugation or elutriation, electrophoresis, flow cytometry (FACS) or magnetic-activated cell sorting (MACS) which take advantage of biophysical criteria (size, density), electrical charge and immunological markers [1–5].

Field flow fractionation (FFF) methodology, introduced in the late 1960s by J.C. Giddings, is described as the one of the most versatile separation techniques [6–9]. This chromatographic-like separation family, in particular sedimentation-FFF (SdFFF), appears to be particularly well suited for isolation and characterization of micron-sized species

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such as cells [1,5,7–9]. By taking advantage of biophysical properties (size, density, shape), SdFFF sorts, in a few minutes, viable cells which can be cultured for further use without specific cell labeling of any kind [1,5,10,11]. Like all other FFF methods, the fundamental principle of SdFFF is based on the differential elution of species in a liquid (mobile phase) flowing through a ribbon-like capillary channel on a laminar mode [6–8]. SdFFF separation depends on specific particle susceptibility to an external field applied perpendicularly to the flat surface of the ribbon, and by consequence perpendicularly to the flow direction [6–8]. If the external field, which is a gravitational one, is lower or equal to earth gravity, the method is described as ''Gravitational-FFF or GFFF''. If the field is greater than one, the method is called ''Centrifugal-Multigravitational- or sedimentation-FFF (SdFFF)'' [1,7]. SdFFF elution mode for cells is described as ''Hyperlayer'' [7,9,12–19]. In such a mechanism, cell size, density, shape and rigidity are involved, as are channel geometry and flow-rate characteristics [1,7]. SdFFF elution mode for cells is described as ''Hyperlayer'' [7,9,12–19]. At constant flow-rate and external field strength, larger, or less dense particles, are focused in faster streamlines and are eluted first [1,7].

Since the pioneering report of Caldwell et al. [9], which defined most of the basic rules and methodologies for cell separation, SdFFF and other FFF techniques have shown a great potential for cell separation and purification with major biomedical applications including hematology, cancer research, microorganism analysis and molecular biology [1,3–5,9,10,20–30]. Recently, we investigated a new and important application for SdFFF cell sorting in neurology [10], where we demonstrated the effectiveness of SdFFF to provide, in less than 10 min, an enriched, sterile, viable and usable neuron fractions [10].

The goal of this work is the use of SdFFF as a safety cell sorter to provide purified immature neural cells which could be used as a tool in further works to investigate cell differentiation processes and the ability of neural cell transplantation to repair brain or spinal lesions in experimental models of traumatic or neurodegenerative diseases. We used the human neuroblastic SH-SY5Y clone of the SK-N-SH cell line as a source of immature neural cells [31]. SH-SY5Y is routinely used as a model in the study of neural cell function, development, differentiation or apoptosis [32]. In culture SH-SY5Y cells display two morphologically distinct cell types. In particular, we observe the presence of small spherical superficial cells that are suspected to be undifferentiated neuroblastic cells or precursors of neural cells.

Our results demonstrated for the first time, the SdFFF cell sorting capacity to obtain a useful, viable and purified immature neural cell fraction in less than 15 min without induction of cell differentiation.

2. Theory

Two models of SdFFF particle elution modes are described for micron-sized species: ''Steric'' and ''Hyperlayer'' [7,9,12–19]. In the ''Hyperlayer'' mode, the flow velocity/channel thickness balance generates a hydrodynamic lift force, which drives the particles away from the accumulation wall. Species are then focused into a thin layer, which corresponds to an equilibrium position in the channel thickness where the external field is exactly balanced by the hydrodynamic lift forces [7,9,12–19]. At equivalent density, large particles generate more lift forces and are focused in faster streamlines to be eluted first. The different average velocities of the different species are compared by means of the observed retention ratio R_{obs}: ratio of the void time versus the retention time [13,14]. Retention ratio (R_{obs}) is flow-rate and external field dependent. At constant field, the increase in flow-rate induces an increase in R, and at constant flow the increase in field decreases R. If the external field can be increased sufficiently, or flow-rate decreased sufficiently to offset lift forces, micron-sized particles are confined into a very thin layer close to the accumulation wall. This elution mode is described as ''Steric'' [7,9,12–19], and appears as a limit case of ''Hyperlayer''. By driving particles close to the accumulation wall, the ''Steric'' elution mode leads to harmful cell/channel wall interactions associated with a decrease in cell viability and recovery.

''Hyperlayer'' mode descriptions predict that sample will not be in close contact with the accumulation wall [7,9,12–19]. By using the following equation

$$R = \frac{6s}{\omega} \quad (1)$$

in which R is the retention ratio, ω is the channel thickness (125 μm), and s the distance from the center of the focused zone to the channel wall [17], we calculated the approximate average cell elevation (s) using the 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 [9,17]. If s is greater than particles radius, cells are eluted under the ''Hyperlayer'' mode [5,9,10,17].

3. Materials and methods

3.1. Cell culture and viability

The human neuroblastic SH-SY5Y sub clone from the SK-N-SH cell line (ATCC, Manassas, VA, USA) was cultured as described before [32] in RPMI culture medium (Gibco-BRL, Cergy Pontoise, France) supplemented with 10% decomplemented fetal calf serum (Seromed, Gibco), 2 m*M* sodium pyruvate (Gibco), 50 U/ml penicillin–50 μg/ml streptomycin (Gibco), 2 m*M* L-glutamine (Gibco) and 0.1% fungizone (Gibco). Cells were grown in flask (10^6 cells plated onto a 25-cm^2 flask) at 37 °C in a humidified 5% CO_2–95% air atmosphere incubator until subconfluence (6 days). Before using, the purity and the neuronal lineage of SH-SY5Y were confirmed by indirect immunofluorescence studies with a mouse monoclonal anti-neurofilament antibody (2F11, Dako, Trappes, France), a specific marker of neurons which stained all the cells, in contrast to a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Dako), a specific marker of astrocytes or to a rabbit anti-fibronectin antisera (Dako), a fibroblastic marker which did not label the cells.

Freshly trypsinized cells were resuspended in the SdFFF mobile phase: sterile phosphate-buffered saline (PBS) solution, pH 7.4, supplemented with 1% penicillin–streptomycin and 1% fungizone. Aliquots of cells were counted ($n=6$) using a Malassez haemocytometer and tested for viability by the trypan-blue dye exclusion method. Trypan blue was added to the cell suspension at a final concentration of 0.1%. Cells that stained blue were considered non-viable and cell density was adjusted to 10^6 cells/ml. These cell suspensions constituted the total SH-SY5Y cell population. A part of this cell suspension was used for SdFFF sorting and the other one constituted the control SH-SY5Y cell population. After SdFFF elution, control and SdFFF fractions were centrifuged and cell pellets were resuspended in culture medium, cell viability measured and culture performed as described above. About 10^4 cells from each collected fraction were seeded on to eight-well plates (Lab-Tek, Nalge Nunc International, Rochester, NY, USA) for immunofluorescence studies. To obtain a sufficient quantity of cells for culture (5×10^5 cells) and immunofluorescence studies, we performed eight successive SdFFF cumulative fraction collections.

3.2. SdFFF device and cell elution conditions

The SdFFF separation device used in this study was derived from those previously described and schematized [10,11,26,32]. The separation channel was made up of two 870×30×2-mm polystyrene plates, separated by a Mylar spacer in which the channel was carved. Channel dimensions were 785×10×0.125 mm with two V-shaped ends of 70 mm. The measured total void volumes (channel volume + connection tubing + injection and detection device) were 960±5 μl ($n=15$). Void volumes were calculated after injection and retention time determination of an unretained compound (0.1 g/l of benzoic acid, UV detection at 254 nm). Inlet and outlet 0.254 mm I.D. Peek tubing (Upchurch Scientific, Oak Harbourg, USA) were directly screwed to the accumulation wall. Then, polystyrene plates and Mylar spacers were sealed onto a centrifuge basket. The channel-rotor axis distance was measured at $r=13.8$ cm. Sedimentation fields are expressed in units of gravity, 1 $g=980$ cm/s^2, and calculated using rotational speed (rpm: rotation per minute) and r as described before [10,11,26]. Two rotating seals were drilled to allow 0.254 mm I.D. Peek tubing to fit in. A Spectroflow 400-ABI Kratos chromatographic pump (ABI-Kratos, Ramsey, NJ, USA) was used to pump the sterile mobile phase. A M71B4 Carpanelli engine associated with a pilot unit Mininvert 370 (Richards Systems, Les Ullis, France), controlled the rotating

speed of the centrifuge basket. Sample injections were done by means of a Rheodyne 7125i chromatographic injection device (Rheodyne, Cotati, CA, USA). Cleaning and decontamination procedures, as well as devices involved in these processes, have been described in previous reports [10,11,26]. The elution signal was recorded at 254 nm by means of a Water 484 tunable absorbance detector (Water, Milford, MA, USA) and a 14-byte M1101 (100 mV input) acquisition device (Keithley Metrabyte, Tauton, MA, USA) operated at 2 Hz and connected to Macintosh computer.

The SH-SY5Y cell suspension elution conditions were set up from 30 to 60 *g* as external field strength and from 0.4 to 1.0 ml/min as mobile phase flow-rate. The optimal elution conditions (''Hyperlayer'' mode) have been experimentally determined and are: flow injection through the accumulation wall of 100 μl SH-SY5Y cells (10^6 cells/ml); flow-rate, 0.6 ml/min; mobile phase, sterile PBS, pH 7.4, supplemented with 1% penicillin–streptomycin and 1% fungizone; external multi-gravitational field strength, 40.00 ± 0.03 *g*; spectrophotometer detection at $\lambda = 254$ nm. Cell fractions were collected and designated as follows: (1) the total peak fraction: TP, from 3 min 40 s to 7 min 15 s; (2) peak fractions 1, 2 and 3 (PF_n): PF_1, 3 min 40 s/4 min 30 s; PF_2, 5 min 00 s/6 min 00 s; PF_3, 6 min 15 s/7 min 15 s. A gap without collection was performed during PF1, 2 and 3 preparation to increase the selectivity of cell fraction collection.

Finally, the average diameter of SdFFF eluted cells was estimated by using a calibrated flow cytometer EPICS Profile II (Coulter, Hialeah, FL, USA), set up as previously described [11,30].

3.3. Immunocytochemical analysis

The control and SdFFF eluted SH-SY5Y cells were fixed with 4% paraformaldehyde (v/v) in PBS, pH 7.4, for 30 min at room temperature and then seeded on glass coverslips for immunofluorescence studies. Briefly, after washing three times with PBS (pH 7.4), cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min on ice. After washing three times with PBS, the non-specific binding sites were blocked by incubation of all coverslips with 10% (v/v) sheep serum in PBS for 30 min at room temperature. After an additional three washes, cells were incubated during 30 min at room temperature, with a monoclonal phycoerythrin-labelled anti-N-CAM antibody (NKH-1, mouse IgG1, Coulter, Fullerton, CA, USA) or with FITC-labeled anti-Fas antibody (B-G 27, mouse IgG2a, Diaclone, Besançon, France), 1/5 diluted in a 10% sheep serum in PBS solution. In some experiments, double-staining was performed with anti-N-CAM and anti-Fas antibodies. Controls were performed in parallel with irrelevant purified mouse monoclonal IgG1 and IgG2a (Immunotech, Marseille, France). Then, coverslips were washed again thrice in PBS–Tween 20 (0.1%) and mounted with glycerol–gelatin medium (Sigma, Saint Quantin-Fallavier, France). Cells were characterized by fluorescence microscopy using a Zeiss microscope (Le Pecq, France).

4. Results and discussion

Since the past decade, SdFFF and related technologies have demonstrated an important potential for cell sorting in major biomedical applications such as hematology, cancer research or molecular biology [1,3–5,9,10,20–30]. Recently, we developed a new application in neurology in which we showed that SdFFF could provide a sterile, viable and enriched (>98%) neurons fractions [10].

Unfortunately, neurons lose their capacity for cell division during their differentiation and maturation, which occurs during nervous system development [33]. Thus, mature neurons cannot be involved in regeneration after injury or degeneration of the nervous system. Because immature neural cells are present at very low levels and in few localized areas in the adult nervous system, they cannot easily participate in its regeneration [33]. Options for neurodegenerative disease therapy could include the activation of endogenous stem cells to provide self-reparation [34], or the transplantation of exogenous stem cells, which then could differentiate, under local environmental stimulation, into specific mature neuron type able to restore nervous system functions [34]. One of the main goals in neurology is to prepare immature neural cells in order to investigate the mechanisms implied in cell differentiation and for clinical purposes. Nevertheless, immature neural

cell preparation is time consuming (many weeks), expensive, difficult to achieve, and hazardous, depending on the origin of cells [34–36].

In this study we investigated, for the first time, the ability of our laboratory designed SdFFF device, used as a cell sorter, to provide purified immature neural cell fractions which were distinguishable based on morphological and immunohistochemical characteristics from a mixed differentiation stage cell culture of the human neuroblastic SH-SY5Y cell line.

4.1. SH-SY5Y cell line characteristics

The human neuroblastic SH-SY5Y subclone from the SK-N-SH cell line is routinely used as a model of neural cell function studies [31,37]. As shown in Fig. 1, before separation, cultured SH-SY5Y cells displayed two morphologically distinct cell types. The first one was composed of flattened adherent cells with neuritic expansions, a feature of neuronal cells during differentiation. The second one corresponded to small spherical cells without neurite outgrowth, which were regrouped to form some neurospheres.

First, the neural origin of the SH-SY5Y cell line was confirmed by immunocytochemistry (see Section 3). Immunohistochemistry studies performed on the total SH-SY5Y population showed that only the large and flattened cells were stained by anti-N-CAM antibody and that small cells were only recognized by anti-Fas antibody (Fig. 2). These findings could correlate with the stage of cell differentiation as suggested by N-CAM expression which is associated with adhesion of neurons after migration. By mediating cell adhesion and signal transduction, N-CAM is associated with neurite outgrowth and fasciculation of several axonal expansions, which are correlated with neuron maturation and target recognition in the developing nervous system [38,39]. In addition, it has been suggested that the role for N-CAM and N-CAM ligands is to inhibit proliferation and to induce differentiation of neural progenitor cells [40]. In contrast, neural precursors did not express this mature form of N-CAM. Interestingly, these small

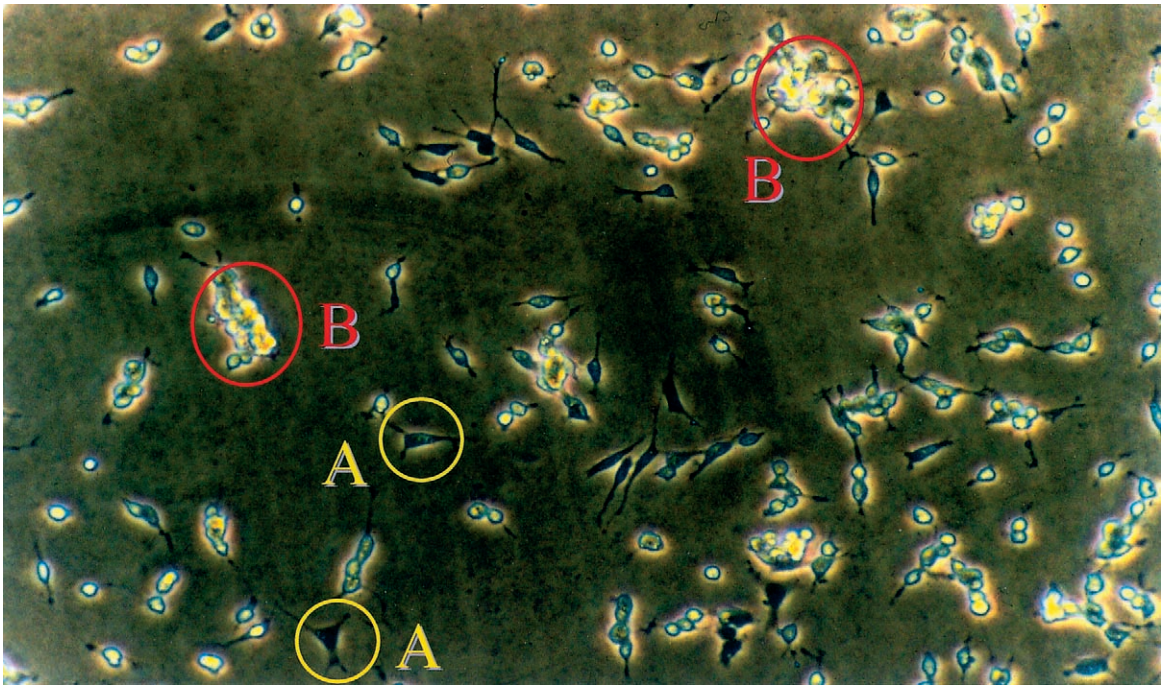

Fig. 1. Microscopic observation of SH-SY5Y cell culture. Cells were cultured for 6 days as described in Section 3. (A) neuroblastic cells: flattened and adherent cells with neuritic expansions, a feature of neuronal cells during differentiation. (B) Small spherical cells without neurite outgrowth, regrouped to form neurospheres. Photographs were taken with a Nikon TMS microscope (magnification, ×160).

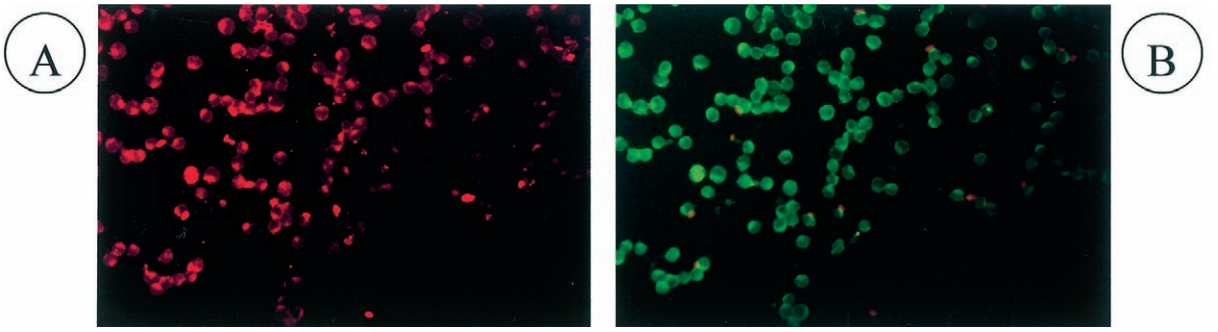

Fig. 2. Immunological characterization of N-CAM and Fas expression in total SH-SY5Y cell population. Cell labeling was done on SH-SY-5Y neuroblastoma cell line using: (A) phycoerythrin-labeled anti-NCAM Ab (clone NKH1); and using (B) fluorescein-labeled anti-FAS Ab (clone BG27). Photographs were taken with a Zeiss microscope (magnification, ×400).

neural cells expressed Fas on their membrane as shown by using the monoclonal B-G27 antibody which is directed against human Fas protein (or CD95, Fig. 2B). Fas is known to induce apoptosis in some cell types when it is activated by its natural ligand (FasL) or by an agonist antibody. The expression of Fas by some tumoral cells such as neuroblastoma cell lines is known [41]. This membrane protein is also expressed by normal neurons, especially motor neurons [32,42], but the pattern of Fas expression during neuronal differentiation is not known. Other functions that apoptosis were related to Fas protein during normal cellular activation and proliferative response, has been reported for T- and B-lymphocytes [43–45].

4.2. SdFFF cell elution

SdFFF cell separation and sorting requires some specific considerations. As well as for the other cells, immature or stem cell separation must take into account and respect cell functional integrity such as cell adhesion properties, surface receptors and antigen expression as well as metabolism specificity. Secondly, high level of short and long term viability are needed. In this case SdFFF should not induce apoptosis which depends both on a drastic limitation of cell-accumulation wall interactions, and on strict observance of cleaning procedures. Finally, the maturation and differentiation stage of eluted cells should not be altered [5]. Nevertheless, as specific labeling is not necessary for cell sorting, SdFFF is particularly interesting for applications in which cell-labeling with antibodies could interfere with further cell using, especially by inducing cellular activation and/or differentiation. Therefore, SdFFF seems to be an adapted method to obtain precursor cells. Thus, because SdFFF cell sorting effectiveness is simply based on the intrinsic biophysical characteristics of the cells, it could provide an advantage over FACS or MACS for stem cell sorting [4,5,11,20,26,30]. Moreover, SdFFF cell separation must also fulfil the following requirements: (1) high repeatability and reproducibility, in particular if routine stem cell preparation is needed; (2) maximal recovery; and (3) sterile collected fractions which are essential to further cell cultures and transplantation.

To achieve these goals, specific SdFFF methodologies have been developed [5,20,26]. SdFFF device setup and elution conditions (see Section 3): injection mode, mobile phase flow-rate and composition, external field strength, composition of channel walls, cleaning and decontamination procedures, were used to obtain better sub-population separation in association with a drastic limitation of cell-accumulation wall interactions by promoting the safety ‘‘Hyperlayer’’ elution mode, because interactions lead to channel poisoning with worst consequences on cell integrity [5,20,26]. Moreover, daily cleaning and decontamination procedures [26] are performed to overcome channel poisoning and microorganism contamination and no cell culture contamination was observed throughout the experiments.

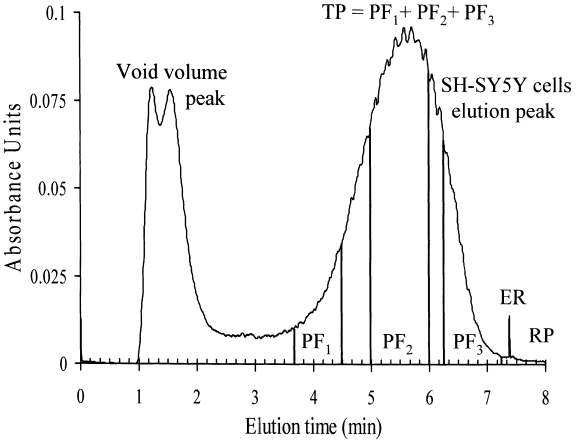

Fig. 3. Representative fractogram of SH-SY5Y cells after SdFFF elution. Elution conditions: flow injection of 100 μl SH-SY5Y cells (10^6 cells/ml); flow-rate, 0.6 ml/min (sterile PBS, pH 7.4, 1% penicillin–streptomycin, 1% fungizone); external multi-gravitational field, 40.00±0.03 *g*; spectrophotometric detection at λ = 254 nm. Fractions were collected as follows: TP, from 3 min 40 s to 7 min 15 s; PF_1, 3 min 40 s/4 min 30 s; PF_2, 5 min 00 s/6 min 00 s; PF_3, 6 min 15 s/7 min 15 s. ER corresponds to the end of channel rotation, in this case the mean externally applied field strength was equal to zero gravity, thus RP, a residual signal, corresponds to the release peak of reversible cell-accumulation wall sticking.

Fig. 3 shows a specific and representative SH-SY5Y cell elution fractogram obtained by using optimal elution condition. Two major peaks were seen. The first corresponded to the elution of unretained species ($R_{obs} \approx 1$; $n = 15$). The second corresponded to the elution of SH-SY5Y cells with $R_{obs} = 0.287 \pm 0.007$ ($n = 15$). At the end of the fractogram when channel rotation was stopped (ER, Fig. 3) and mean gravity was equal to zero (external field applied = 1 *g*), we observed a residual signal (RP, Fig. 3), which represented cell release from the separating channel. This corresponds to a reversible cell adherence linked to a low interaction between cells and the accumulation wall [10]. Then, cells can easily release from the wall under the conjugated effect of mobile phase flowing and channel rotation stop. Irreversible cell sticking is a strong cell-accumulation wall interaction that does not reverse in these conditions and therefore cannot be monitored on the fractogram [10]. Irreversible sticking leads to channel poisoning and can be avoided by systematic cleaning and decontamination [5,10,26].

As described, optimal elution conditions were selected to promote the ''Hyperlayer'' elution mode to reduce cell-accumulation wall interactions. In order to determine if SH-SY5Y cells followed this elution mode, we measured the pattern of R_{obs} for the SH-SY5Y peak under different elution conditions (Table 1). As shown in Table 1, at constant external field strength (40 *g*), the increase in flow-rate is correlated to an increase in R_{obs}.

Under the optimal elution conditions, the approximate average cell elevation decrease from $s = 9.1 \pm 0.2$ μm at the beginning of the SH-SY5Y peak (Fig. 3) to $s = 5.5 \pm 0.2$ μm at the end of the elution peak (Fig. 3). The estimation of the average eluted cell diameter, by using calibrated flow cytometer as described [11,30] (see Section 3), showed that the mean cell diameter varied from 7.7±0.5 μm ($n = 4$) for cells eluted in F_1 (Fig. 3), to 4.7±0.5 μm ($n = 4$) for cells eluted in F_3 (Fig. 3). Under these conditions, the approximate average cell elevation value *s* (9.1±0.2 to $s = 5.5 \pm 0.2$ μm) is greater than the estimate mean cell radius (3.8–2.4 μm).

Thus according to the SdFFF elution mode description for micron-sized species [7,9,12–19], we can assume that our results (flow-rate dependence of R_{obs}, $s >$ cell radius) demonstrated that SH-SY5Y cells were eluted under the ''Hyperlayer'' elution mode. Finally, the effectiveness of this mode to

Table 1
Retention ratio R_{obs} and ''Hyperlayer'' elution mode

	Mobile phase flow-rate (ml/min)			
	0.4	0.6	0.8	1.0
R_{obs}	0.232±0.004	0.287±0.007	0.336±0.005	0.391±0.006

Elution conditions: channel, 780×10×0.125 mm (polystyrene walls); flow injection of 100 μl SH-SY5Y cells (10^6 cells/ml); mobile phase, sterile PBS, pH 7.4, 1% penicillin–streptomycin, 1% fungizone; external multi-gravitational field, 40.00±0.03 *g*; spectrophotometric detection at λ = 254 nm. Results are expressed as mean±SD for $n = 15$.

reduce particle-accumulation wall interactions is shown in part by the absence (Fig. 3) or the very low level of the corresponding cell release peak at the end of the fractogram.

4.3. Characterization of SH-SY5Y eluted cells

The use of these optimal conditions in association with cleaning and decontamination procedures should allowed SH-SY5Y cell elution while respecting functional integrity, viability, sterility, and without induction of cell differentiation. However, we did not know if cell sorting of the different SH-SY5Y subpopulations was achieved, and in particular, if we were able to sort the small suspended cells from this unique elution peak (Fig. 3). As shown in Fig. 3, four cell fractions were collected (1) the total peak fraction (TP) which corresponded to the total SH-SY5Y population eluted; and (2) peak fractions 1, 2 and 3 (PF_n) which are the time-dependent collected fractions of the retained peak profile.

Cell viability, culture and specific antigen expression studies were conducted both on SdFFF eluted fractions and on control SH-SY5Y population in order: (1) to determine if its possible to sort small cells in a purified fraction; and (2) to define whether or not these cells could be defined as neural undifferentiated or immature cells.

First, after SdFFF elution and before cell culture, short-term cell viability was determined and results showed that the mean viability of cells eluted in TP, PF_1, PF_2 and PF_3 fractions was 91.0±0.5% ($n=15$) and 95.0±3.0% for the control SH-SY5Y population ($n=15$). In culture, three cell populations: control SH-SY5Y, TP and PF_2 fractions; behave similarly. As usually observed for SH-SY5Y cells [32,37], we obtained cell adhesion in less than 48 h and cells demonstrated a typical growth pattern that led to a subconfluent culture in 4–6 days. Optical microscopy observation of these cultures showed, as for the SH-SY5Y population (Fig. 1), the presence of two distinct cell types with large neuroblastic adherent cells and small spherical suspended cells that formed neurospheres. After 6 days culture, SdFFF eluted cells (TP and PF_2 fractions) have almost the same mortality 4±1%, as control SH-SY5Y cell 4±2% ($n=15$). These results demonstrated that SdFFF elution did not appear to be involved in a significant increase in cell death, and did not modify cell adhesion or cell growth properties and respected cell functional integrity and viability.

For the first eluted fraction (PF_1), cultured cell behaved differently. We observed the presence of large adherent neuroblastic cells, and a very low percentage of small spherical cells. This cell adhesion was not associated with significant cell growth, even if cells were still alive during the entire the culture period.

For the third eluted fraction (PF_3), after 48 h culture, we only saw the presence of small spherical suspended cells in culture. As for the PF_1 fraction, cells were viable throughout the culture period and cell growth led to the formation of neurospheres without apparition of neuroblastic adherent cells (data not shown).

Thus, SdFFF elution, fraction collection and cell culture showed that: (1) according to the ‘‘Hyperlayer’’ elution mode description [7,9,12–19], large cells are eluted first (at equivalent density); (2) according to microscopic observations, SH-SY5Y cells displayed two morphologically distinct size populations; and finally (3) SdFFF is an effective tool to sort these two subpopulations.

Now, the principal question was to determine if, as expected, large cells are neuroblastic differentiated cells and if small cells are undifferentiated or precursor cells.

Fig. 4 displayed representative immunofluorescent staining corresponding to specific N-CAM and Fas expression. As described for cell culture, three populations: control, TP and PF_2 fractions, had a similar level of N-CAM expression (Figs. 2A, 4A, 4C and 5). The same observation could be done concerning Fas expression (Figs. 2B, 4E, 4G and 5).

In the PF_1 fraction (Figs. 4B and 5), such as in control or TP fractions, N-CAM-expressing cells belonged to the fraction of large cells: 96.8±2.1% (ED, $n=4$), which displayed some features of differentiated cells (neurite outgrowth and adhesion, Fig. 1). In contrast, in the PF_3 fraction, we observed few N-CAM positive cells (Figs. 4D and 5).

As shown in Figs. 4H and 5, the PF_3 fraction showed a specific Fas expression by the smaller cells: 98.0±1.2% (ED, $n=4$), contrasting with low Fas expression in the PF1 fraction (Figs. 4F and 5).

Altogether, these findings suggest that SdFFF

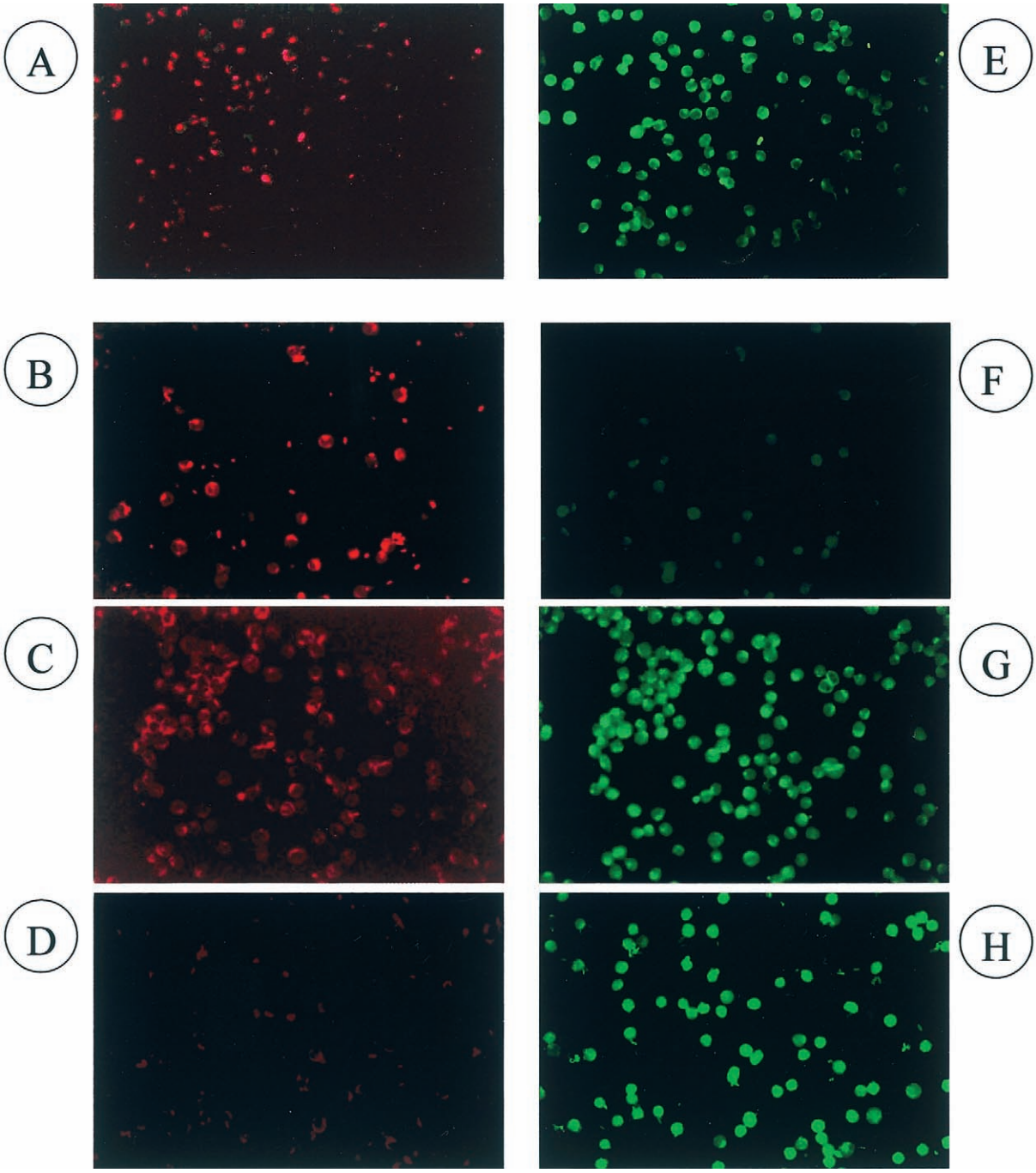

Fig. 4. Immunological characterization of N-CAM and Fas expression in SH-SY5Y cells. Cell labeling was done on SH-SY-5Y neuroblastoma cell line after FFF separation, using phycoerythrine labeled anti-NCAM Ab (clone NKH1) for (A)–(D); and using fluorescein-labeled anti-FAS Ab (clone BG27) for (E)–(H). Photographs were taken with a Zeiss microscope (magnification, ×400). A,E, PT; B,F, PF_1; C,G, PF_2; D,H, PF_3.

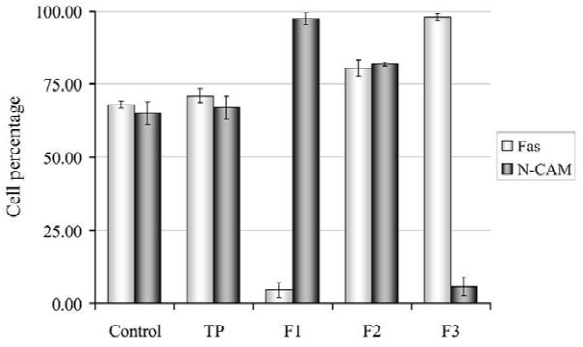

Fig. 5. NCAM and FAS cell percentage in SdFFF collected fractions. After immunological characterization, fluorescent cells were randomly counted and results, for each condition studied (control, TP, PF_1, PF_2, PF_3 fractions), were expressed as mean±ED for $n=4$. Here, n = number of independent SdFFF cell separations and collections performed. Moreover, each cell immunological characterization was performed in triplicate either for control or for SdFFF eluted cells, and for each experiment, 1000 randomly selected cells were counted.

allows us to purify two maturation stages of neural cells with different properties with regards to Fas or N-CAM expression, adhesion and ability to constitute neurospheres. Immature cells were non-adherent cells, forming neurospheres cells as described in the case of neural precursors [46,47]. These results show that the SH-SY5Y cell line is heterogeneous with the presence of distinct morphological cell types, which were correlated, with different steps of cell differentiation. The large neuroblastic cells, eluted in PF_1, appeared as differentiated cells. PF_2 could be defined as a mixture between neuroblastic differentiated N-CAM-expressing cells, small undifferentiated Fas-expressing cells, associated to a predominant cell population expressing both Fas and N-CAM, as revealed by double-staining experiments, which could be defined as neural cells which are an intermediate step of differentiation. Moreover, immunocytochemistry studies demonstrated that cells eluted in the last fraction, the small suspended cells, could be defined as undifferentiated or immature cells.

Finally, results concerning the TP fraction, were very similar to that obtained for a control SH-SY5Y population, which demonstrated that SdFFF elution process did not promoted cell sorting by extra phenomena such as selective cell killing, specific apoptosis induction, or specific cell trapping in the device of one of the subpopulations. Cell sorting of the different SH-SY5Y subpopulation depends only on the difference in cell size, density and shape as predicted by the ‘‘Hyperlayer’’ elution mode [7,9,12–19].

Two other important points have to be noted. The first is that cell culture results suggest that particular cell cooperation exist between adherent differentiated cells and suspended immature cells to obtain cell differentiation. The study of such mechanism, which is now possible by using SdFFF cell sorting, could therefore play an important role in understanding stem cell proliferation and differentiation to their daughter cells. The second point is that immature cells eluted in the last fraction seem to be able to proliferate as observed with the formation of neurospheres. This indicates that SdFFF elution process respects cell functional integrity without adding external factors of differentiation, such as those which are employed with some other method of purification (i.e., antibody used during cell purification by FACS).

5. Conclusion

SdFFF takes advantage of intrinsic biophysical properties of eluted cells and (1) because of no complex mobile phase, no specific cell preparation labeling are needed, (2) because elution and sorting is very fast, and (3) because the device can be easily and rapidly setup (less than 2 h) to obtain optimal elution conditions for each new separation problem, SdFFF could be more useful than many other cell sorters to provide purified stem cell fractions [1–3,5,20].

Taken together, our results demonstrated that by using SdFFF as a cell sorter under strictly defined conditions, in association with major biological tools for cell characterization such as immunohistochemistry, we are now able to provide, in less than 15 min, a sterile, viable, useable and purified immature neural cell fractions without induction of cell differentiation.

This work, with those previously published [3,48], provided the basis for the use of stem cell. Interestingly, these separated-cells can be cultured, and

therefore it is now possible to specifically study different factors involved in stem cell differentiation and the ability to induce different neuronal population such as motor neurons, or dopaminergic neurons. These studies could be performed both in vitro using cell culture and in vivo by using cell grafts in experimental neurodegenerative animal models. In addition to grafts applied to cell therapy research, this procedure could be used in cancer research. By separating cellular fractions at different stages of maturation, it is now possible to study each isolated fraction from a tumor and therefore determine the proliferative and invasive properties of each cellular component of the tumor. Thus the mechanisms allowing the differentiation of immature tumor cells, and the drug-sensitivity of these fractions will be considered.

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References

[1] T. Chianea, N.E. Assidjo, P.J.P. Cardot, Talanta 51 (2000) 835.
[2] J. Bauer, J. Chromatogr. B 722 (1999) 55.
[3] X.B. Wang, J. Yang, Y. Huang, J. Vykoukal, F.F. Becker, P.R. Gascoyne, Anal. Chem. 72 (2000) 832.
[4] P.J.P. Cardot, S. Battu, T. Chianea, S. Rasouli, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), Encyclopedia of Separation Science, Academic Press, London, 2000, p. 2267.
[5] S. Battu, J. Cook-Moreau, P.J.P. Cardot, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 2193.
[6] J.C. Giddings, Sep. Sci. 1 (1966) 123.
[7] J.C. Giddings, in: M.E. Schimpf, K. Caldwell, J.C. Giddings (Eds.), Field-Flow Fractionation Handbook, Wiley, New York, 2000, p. 3.
[8] M.N. Myers, J. Microcol. Sep. 9 (1997) 151.
[9] K.D. Caldwell, Z.Q. Cheng, P. Hradecky, J.C. Giddings, Cell Biophys. 6 (1984) 233.
[10] S. Battu, W. Elyaman, J. Hugon, P.J.P. Cardot, Biochim. Biophys. Acta (Gen. Subj.) 1528 (2001) 89.
[11] P. Cardot, S. Battu, A. Simon, C. Delage, J. Chromatogr. B 768 (2002) 285.
[12] P.S. Williams, S. Lee, J.C. Giddings, Chem. Eng. Commun. 130 (1994) 143.
[13] X. Tong, K.D. Caldwell, J. Chromatogr. B 674 (1995) 39.
[14] X. Tong, K.D. Caldwell, J. Chromatogr. A 831 (1999) 51.
[15] M. Martin, P.S. Williams, NATO ASI Ser. Ser. C 383 (1992) 513.
[16] M.R. Schure, K.D. Caldwell, J.C. Giddings, Anal. Chem. 58 (1986) 1509.
[17] J. Chmelik, J. Chromatogr. A 845 (1999) 285.
[18] J. Plockova, J. Chmelik, J. Chromatogr. A 868 (2000) 217.
[19] K.D. Caldwell, in: M.E. Schimpf, K.D. Caldwell (Eds.), Field-flow fractionation handbook, Wiley, New York, 2000, p. 79.
[20] P.J.P. Cardot, T. Chianea, S. Battu, in: J. Cazes (Ed.), Encyclopedia of Chromatography, Marcel Dekker, New York, 2001, p. 742.
[21] L.E. Schallinger, J.E. Gray, L.W. Wagner, S. Knowlton, J.J. Kirkland, J. Chromatogr. 342 (1985) 67.
[22] S. Hoffstetter-Kuhn, T. Rosler, M. Ehrat, H.M. Widmer, Anal. Biochem. 206 (1992) 300.
[23] V. Yue, R. Kowal, L. Neargarder, L. Bond, A. Muetterties, R. Parsons, Clin. Chem. 40 (1994) 1810.
[24] J.M. Metreau, S. Gallet, P.J. Cardot, V. Le Maire, F. Dumas, A. Hernvann, S. Loric, Anal. Biochem. 251 (1997) 178.
[25] F. Bouamrane, N.E. Assidjo, B. Bouteille, M.F. Dreyfuss, M.L. Darde, P.J.P. Cardot, J. Pharm. Biomed. Anal. 20 (1999) 503.
[26] S. Battu, A. Roux, S. Delebasee, C. Bosgiraud, P.J.P. Cardot, J. Chromatogr. B 751 (2001) 131.
[27] S. Saenton, H. Lee, Y.-S. Gao, J.F. Ranville, S.K.R. Williams, Sep. Sci. Technol. 35 (2000) 1761.
[28] A. Khoshmanesh, R. Sharma, R. Beckett, J. Environ. Eng. ASCE 127 (2001) 19.
[29] M. Hoyos, L.R. Moore, K.E. McCloskey, S. Margel, M. Zuberi, J.J. Chalmers, M. Zborowski, J. Chromatogr. A 903 (2000) 99.
[30] R. Sanz, P. Cardot, S. Battu, M.T. Galceran, Anal. Chem. 74 (2002) 4496.
[31] J.M. Muller, S.J. Lolait, V.C. Yu, W. Sadee, J.A. Waschek, J. Biol. Chem. 264 (1989) 3647.
[32] F.H. Yi, C. Lautrette, C. Vermot-Desroches, D. Bordessoule, P. Couratier, J. Wijdenes, J.L. Preud'homme, M.O. Jauberteau, J. Neuroimmunol. 109 (2000) 211.
[33] A.J. Blaschke, K. Staley, J. Chun, Development 122 (1996) 1165.
[34] F.H. Gage, Science 287 (2000) 1433.
[35] N. Uchida, D.W. Buck, D. He, M.J. Reistsma, M. Masek, F.H. Gage, I.L. Weissman, Proc. Natl. Acad. Sci. USA 19 (2000) 14720.
[36] S.F. Pagano, F. Impagnatiello, M. Girelli, L. Cova, E. Grioni, M. Onofri, M. Cavallaro, S. Etteri, F. Vitello, S. Giombini, C.L. Solero, E.A. Parati, Stem Cells 18 (2000) 295.
[37] M.O. Jauberteau, I. Brindel, N. Baumann, J.L. Preud'homme, J. Neuroimmunol. 48 (1993) 53.
[38] H. Cremer, G. Chazal, C. Goridis, A. Represa, Mol. Cell. Neurosci. 8 (1997) 323.

[39] L.C. Ronn, V. Berezin, E. Bock, Int. J. Dev. Neurosci. 18 (2000) 193.
[40] M.C. Amoureux, B.A. Cunningham, G.M. Edelman, K.L.J. Crossin, Neurosci. 20 (2000) 3631.
[41] E. Goillot, J. Raingeaud, A. Ranger, R.I. Tepper, R.J. Davis, E. Harlow, I. Sanchez, Proc. Natl. Acad. Sci. USA 94 (1997) 3302.
[42] C. Raoul, C.E. Henderson, B. Pettmann, J. Cell Biol. 147 (1999) 1049.
[43] J.C. Rathmell, S.E. Townsend, J.C. Xu, R.A. Flavell, C.C. Goodnow, Cell 87 (1996) 319.
[44] I.M. Catlett, G.A. Bishop, J. Immunol. 163 (1999) 2378.
[45] N.J. Kennedy, T. Kataoka, J. Tschopp, R.C. Budd, J. Exp. Med. 190 (1999) 1891.
[46] F.J. Roisen, K.M. Klueber, C.L. Lu, L.M. Hatcher, A. Dozier, C.B. Shields, S. Maguire, Brain Res. 890 (2001) 11.
[47] M.S. Rao, Anat. Rec. 257 (1999) 137.
[48] E. Urbankova, A. Vacek, J. Chmelik, J. Chromatogr. B 687 (1996) 449.