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Hyphenation of sedimentation field flow fractionation with flow cytometry ☆

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Abstract

Interest in the development of field flow fractionation (FFF) systems for cell sorting recently increased with the possibility of collecting and characterizing viable cellular materials. There are various tools for the analysis of cell characteristics, but the reference is small- and large-angle light scattering often coupled with fluorimetric measurements. The well-known flow cytometry (FC) cell analysis techniques can be associated with FFF leading to the possibility of collecting information provided by a remarkable separation technique for micron-sized particles (cells) operating in the steric-hyperlayer elution mode with multiparametric detection provided by flow cytometry. Moreover FFF derived cell characteristics can be correlated with FC characteristics to describe in a unique way the nature of the eluted materials. Experimental demonstrations are described herein using nucleated cells (HL-60 cell lineage) and human red blood cells (HRBC).

Keywords: Sedimentation field flow fractionation; Flow cytometry; Hyphenation

1. Introduction

1.1. Cell sorting

The possibility of developing elution-based cell sorting methodologies is based on the theoretical and instrumental development of a unique separation concept invented by Giddings [1], i.e. field flow fractionation (FFF), which integrates three basic notions. The first was established by Caldwell and coworkers [2] in the early 1980s, by demonstrating the possibility of separating cells of different characteristics by multigravitational sedimentation field flow fractionation (SdFFF). The second was initiated by a group lead by Chmelik [3] who isolated mouse hematopoietic cells and retransplanted them after irradiation, while the third was developed by Cardot and coworkers to set up specific biocompatible

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SdFFF instrumentation [4] for cell separation as well as to define sterilizable separators and appropriate methodological procedures to collect viable and reusable purified cells [5]. Various reports demonstrated the possible use of FFF separation techniques for monitoring specific pathologies in hematology [6,7] or human parasitology [8,9]. The possible hyphenation of FFF with flow cytometry (FC) for cell sorting was first described and experimentally demonstrated by Cardot [10,11] in the late 1980s and by Gascoyne [12].

1.2. Field flow fractionation

Conceptualised in the late 1960s by Giddings [1], the root principle of field flow fractionation lies in the differential migration of cells in the parabolic flow profile established in flow height dimension of a ribbon-like channel as shown in Fig. 1A. An external field is applied perpendicularly to the larger surfaces of the channel. Under its effect, species of different characteristics, introduced at the channel inlet, are distributed in different streamlines in the channel. The interesting aspects of this wide family of separation techniques reside in the diversity of external fields, e.g. those created by simple gravity or by centrifugal forces [1]. The differential elution of micron-sized species is described as ''steric-hyperlayer'' separation [1,13,14]. This model originates in the demonstration of complex flow dependent forces due to lubrication or inertia (called ''lift forces'' in the FFF literature), that are opposed to the external field direction and focus the species into hyperlayers located in different flow streams. The combination of the focusing with the parabolic differential flow profile in the direction of the channel drives the separations. Micron-sized species therefore reach an equilibrium position in the channel when the magnitude of the lifting force is exactly balanced by the force created by the external field, as shown in Fig. 1B. This ''steric-hyperlayer'' migration depends mainly on the size, shape, density and mass of the particles leading to extraordinary separation and selectivity. Literature data [1,13,14] show that so far, balanced effects of size, density and shape are not completely mastered in terms of separation development for micron-sized species such as cells. The

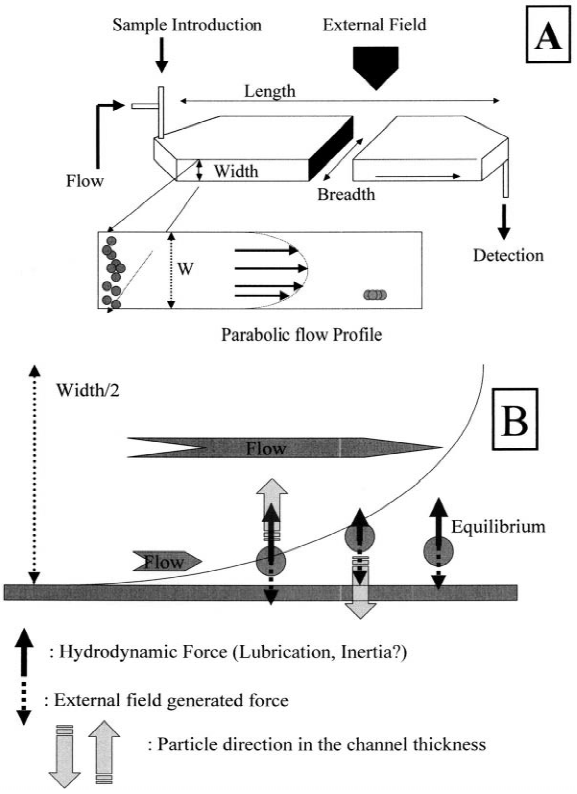

Fig. 1. Principle of micron-sized species separation by field flow fractionation. (A) Separation channel general design. Classically the inlet tubing is screwed to the depletion (upper) wall as shown in the figure. The inlet tubing can also be connected to the opposite wall (not shown). Length varies in sedimentation FFF from 50 to 100 cm; breadth from 1 to 2 cm and thickness as low as 80 μm can be used. FFF connection tubing must be less than 10% of the channel void volume. (B) Steric-hyperlayer elution mode. Half of the channel thickness is schematised from the accumulation wall, with the parabolic flow profile. From left to right: (A) particle positioned below to its equilibrium position and submitted to the resulting balance of the force generated by the external field and the hydrodynamic force. The particle is submitted to a vertical acceleration away from the accumulation wall. (B) Particle positioned above its equilibrium position, with a vertical acceleration towards the accumulation wall. (C) Particle at equilibrium.

main interest of the elution mode for cell sorting is that cells are focused according to their biophysical characteristics in different flow stream lines. Model calculations show, at given average flow rate and external field intensity, that the closer the cells are to the accumulation wall, the more intense are the lift forces. As a consequence, the possible cell–wall

interactions are considerably reduced. These interactions may lead to a reversible or irreversible trapping or to some cellular activation or differentiation process.

The separation procedure is analogous to chromatographic procedures. A mixture of species, e.g. cells, is introduced at the channel inlet; the cells are eluted under the combined effects of the external field and flow. Species are detected at the outlet of the channel by means of an appropriate detector, and sterile fractions can be collected for further characterisation or use. Compared to classical SdFFF design commercialised or described in the literature, the separator instrumental modification of the inlet zone, described in this report, led to simplified elution procedures.

1.3. Flow cytometry

Light scattering techniques for the analyses of biological species such as cells in a flowing system, whether or not they are associated with other detection techniques such as fluorescence, are now considered as standard instrumentation in biology [15]. The refinement of such techniques has led to the commercialisation of sophisticated instrumentation [16]. Signals obtained from the forward angle (FS) (low angle, 1.5–19°) as well as the side scattering techniques (SS) (90°) are now used routinely to provide particle information for cell characterisation [17]. The light scattering principle involves collecting the light diffused by an illuminated particle and generating a signal which is a function of the particle size, surface characteristics, shape and particle composition. With more than 25 years of experiments, FS is considered to provide a signal function of the particle diffracted light which is proportional to the size and can be theoretically described by the Mie Law [18]. Size calibration of the FS signal by means of particles of known average size and polydispersity is therefore possible. The 90° side scattering (SS) consists of refracted and reflected light and provides information on the particle composition, surface and shape [19]. However, so far, calibration of SS signal intensities appears very complicated because of the complexity of the response function. Instrumentation combines SS and FS signals to generate histograms, whose *x*-axis describes the diffused light intensity, while the *y*-axis describes the particle number.

1.4. FFF/FC hyphenation

Three different approaches can be used. The first considers only the possible correlations between information provided by flow cytometry and the information of the fractogram. The second is an off-line approach of FFF/FC hyphenation, in which FFF eluted fractions are systematically analysed by means of flow cytometry. The third considers an on-line coupling of FFF with FC, leading to the concept of a multidimensional hyphenated fractogram.

It is the objective of this report to demonstrate that hyphenation of the remarkable separation power of FFF with the unmatchable multiparametric particle characterisation offered by FC may lead to new particle analyses domains.

2. Experimental

2.1. Samples

2.1.1. HL-60 cell culture

The human promyelocytic leukemia cell line HL-60 was derived from cells obtained from a patient with acute promyelocytic leukemia [20] and was purchased from the American Type Culture Collection (Biovalley, France). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 m*M*) (all from Gibco, Cergy Pontoise, France). HL-60 cells were routinely cultured in 75-cm^3 culture flasks and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell cultures were passed twice weekly, and exhibited characteristic doubling times of ~24 h. Cell viability was assessed by trypan blue exclusion and exponentially growing HL-60 cells were used for experiments.

2.1.2. Human red blood cells (HRBC)

A peripheral blood sample was drawn from a healthy and informed donor with K_3EDTA as anticoagulant, and stored at 4 °C. Samples used for

separations were made of a simple dilution of that total blood suspension in an isotonic medium prior to utilisation. Standard latex beads (Duke Scientific, Palo Alto, CA, USA, lot no. 22388) of 7.00-μm nominal average diameter (6.992±0.050 μm) and size standard deviation of 0.07 μm with 0.1% C.V., and claimed average density of 1.05 g/cm^3 were diluted in the FFF carrier phase prior to injection. Benzoic acid, at a final concentration of 0.05 g/l, was added to the working suspension as void volume probe.

2.2. Field flow fractionation and flow cytometry

2.2.1. Field flow fractionation system

The SdFFF separation device used in this study was derived from those previously described and schematized [4,5]. The separation channel used for cell separations was made of two 870×30×2-mm polystyrene plates, separated by a Mylar® spacer in which the channel was carved. Channel dimensions were 780×10×0.125 mm with two V-shaped ends of 70 mm. The measured total void volumes (channel volume+connection tubing+injection and detection device) were 935.5±10.2 μl (n=15). Void volumes were calculated after injection and elution time determination of a non-retained compound (0.1 g/l of benzoic acid, UV detection at 254 nm). Inlet and outlet 0.254-mm I.D. Peek® tubings (Upchurch Scientific, Oak Harbourg, USA) were mounted directly out to the accumulation wall. Such inlet and outlet tubing connection positions are specific of the separators designed in Limoges. The polystyrene plates and Mylar spacer where then sealed into a centrifuge basket. The channel–rotor axis distance was measured as r=13.8 cm. Sedimentation fields are expressed in units of gravity (1 g=980 cm/s^2), and calculated from the rotational speed (rpm, rotations per minute) and r as:

$$G = \left(\frac{\text{rpm} \times 2\pi}{60}\right)^2 \times r \quad (1)$$

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 deliver the sterile mobile phase. An M71B4 Carpanelli motor associated with a pilot unit Mininvert 370 (Richards Systems, Les Ullis, France) controlled the rotation speed of the centrifuge basket. Sample injections were made using a Rheodyne® 7125i chromatographic injection device (Rheodyne, Cotati, CA, USA). Cleaning and decontamination procedures, as well as the devices involved in these processes, have been described elsewhere [5]. The elution signal was recorded at 254 nm by means of a Spectra 200 spectrophotometer (Spectra-Physics) and a 14-byte M1101 (100-mV input) acquisition device (Keithley Metrabyte, Tauton, MA, USA) operated at 2 Hz and connected to a Macintosh computer.

2.2.2. Flow cytometry

An EPICS Profile II (Beckman Coulter France, Villepinte, France) with 256 channels and nine different gains was used. The forward scattering (FS) and small angle scattering (SS) signal histograms were systematically recorded. Minimum 50-μl samples or fraction suspensions were analysed. A sample introduction speed of 25 μl/min with a sheath pressure of 7.5 p.s.i. was used.

3. Results and discussion

To define the characteristics of a micron-sized particle population is a tricky procedure by analogy with what is known in polymer sciences; these populations can be characterised by a multidimensional matrix of parameters describing the multi-polydispersity of the analysed population [10].

3.1. Flow cytometry and fractogram information: the latex bead example

Latex spherical particles of reduced size dispersity are systematically used as reference for establishing FFF separation or to demonstrate the separation power of FFF for micron-sized particles. However, only partial information is available for 7.00-μm latex beads as described in the Experimental section. The size distribution profile must be established, and some other characteristics are not provided including smoothness of the surface and density distribution. Such partial information can be given independently by FC and FFF. Fig. 2 shows the SS and FS signals

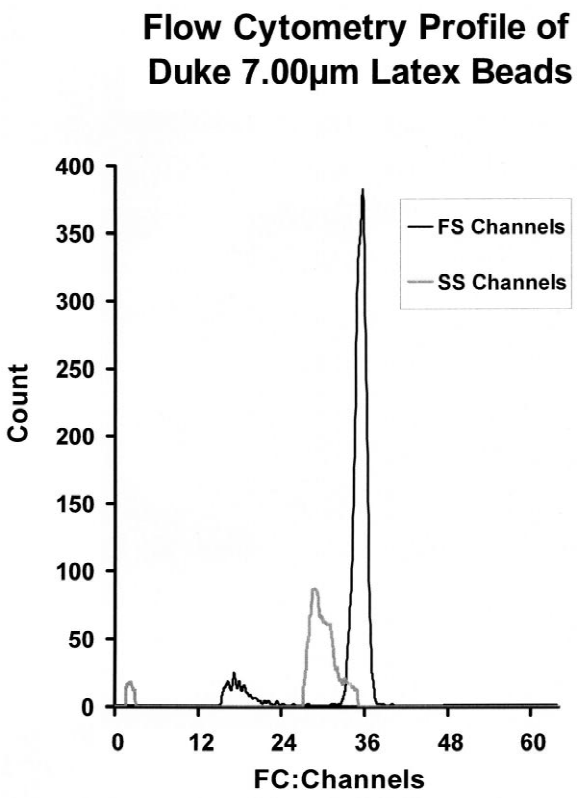

Fig. 2. SS and FS flow cytometry pattern of Duke® latex beads of 7.00-μm nominal diameter. SS and FS gain were chosen to obtain maximum intensity at median channels; *x*-axis: 64 channels, SS and FS signal intensity resolution; *y*-axis: particle count. FS gain = 5, SS gain = 3.

obtained from flow cytometry. It must be noted here that the FS signal amplification gain is chosen to describe with precision the FS distribution. Amplification gain is chosen first to center the signal maximum at the histogram medium, and second to obtain the maximum number of channels with a significant signal. It is observed that the FS main signal is very sharp in intensity distribution. It describes the reduced dispersity of this standard population. At first glance, Duke latex bead suspensions, considered as standard, generated a bimodal signal. As observed in Fig. 2, signals of channels 15–24 would correspond to particles of reduced size compared to those of channels 30. The low channel signals (15–24) must be considered as FC-FS ''noise'' and are generated by the FC/sample medium composition differences. This can be filtered by deconvolution analysing of a blank sample free of particles. Duke latex particles appear therefore monomodal and centered on channel 36. The calibration of FS channels at any gain in terms of particle volume/size is possible using series of calibrated beads. An analogous procedure is applied to generate the SS signal. In contrast, its signal intensity distribution appears with a different dispersity pattern indicating variances of characteristics not evidenced by other techniques such as microscopy. So far, the information provided by SS signals is only qualitative. Therefore, although the latex bead population appears relatively monodisperse in size, it is also associated with ''different'' dispersities whose resultant is expressed in term of SS signal intensity distribution. One can easily imagine the beginnings of a multipolydispersity matrix in using the information provided by the FC signal in Fig. 2 [10]. Moreover FS and SS signals can be correlated to describe in a tridimensional map (bidimensional projection) the latex bead pattern as shown in Fig. 3. It must be noted here that FS-SS orthogonality pattern in Fig. 3 depends not only on the particle

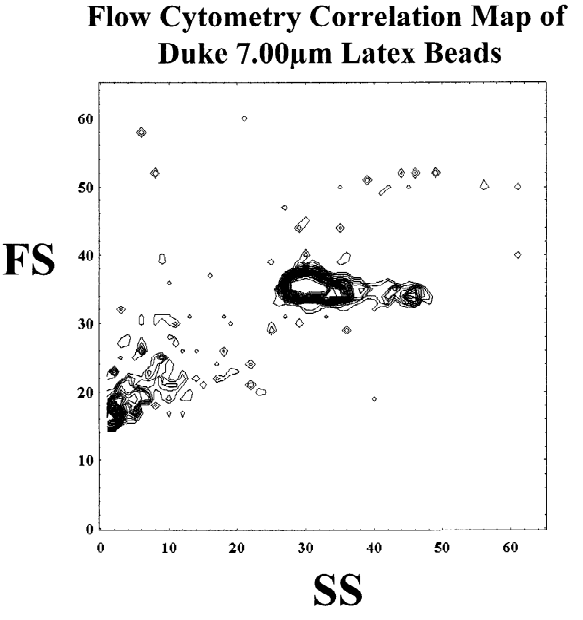

Fig. 3. SS and FS correlation map of Duke latex beads of 7.00-μm nominal diameter. Bidimensional projection of a tridimensional FS/SS/count profile (*x*-axis: SS; *y*-axis: FS; and *z*-axis: count (20 000 particles counted). FS and SS gains are given in Fig. 2.

population characteristics but also on the gain matrix (FS, 5; SS, 3 in Figs. 2 and 3).

Signals located in the FS-SS matrix range $\begin{matrix}10 & 0\\24 & 10\end{matrix}$ shown in Fig. 3, are associated with ''noise''. Duke latex particles appear monomodal on both the FS and SS axes.

This correlation map describes very clearly the size and some other patterns of the latex bead population. Such particle description can be empirically ''correlated'' to the FFF elution profile of that population. Fig. 4 shows the elution characteristics of these latex beads in SdFFF optimised conditions (channel thickness of 80 μm). Flow injections are performed while average flow rate and external field are established. It can be observed that, in the experimental conditions described in the legend, Duke latex beads are retained from the void volume probe. These micron-sized particles are assumed (Duke sample technical data) and controlled (FC-FS) to be relatively monodisperse in size. Their elution signal produced a sharp peak, even sharper than that of the void volume probe, i.e. benzoic acid. Such a profile can be interpreted as being due to two phenomena. The first is linked to the considerably lower diffusion coefficient of micron-sized latex beads compared to benzoic acid in the carrier phase. The second is supported by the ''steric-hyperlayer'' elution mode which postulates an effective localisation of the particles in the channel thickness [1]. However to be objectively rigorous, and to be able to apply retention characteristics to particle properties, average density and density distribution descriptions should be added. These complementary latex characterisation processes are beyond the scope of this report. Nevertheless, it appears that ''standard species'' have to be more and more precisely described; flow cytometry provides only part of the necessary information.

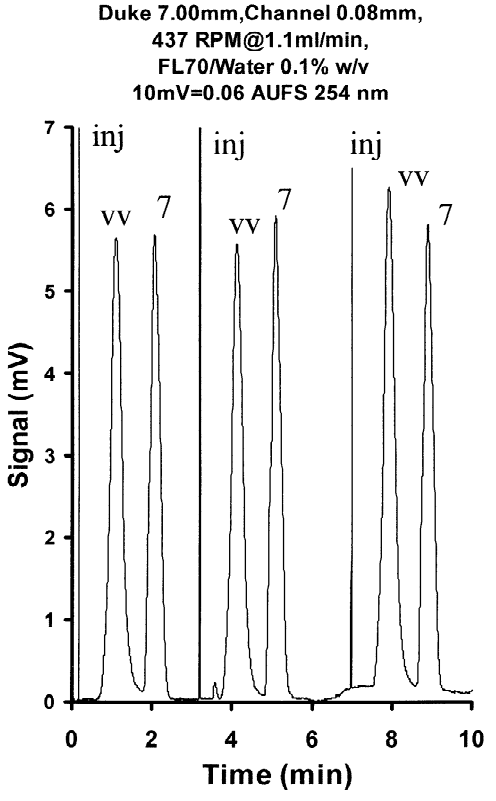

Fig. 4. Reproducibility of Duke latex beads of 7.00-μm nominal diameter fractograms. Channel void volume: 1.08 ml, flow injection (25 μl of 0.02% suspension in carrier phase supplemented with benzoic acid at 0.05 g/l). Channel to rotation axis distance ~14 cm. Polystyrene channel walls. VV, void volume peak; 7, latex elution peak.

3.2. HL-60/HRBC SdFFF separation development

HL-60 nucleated cells can be detected using a classical chromatographic photometer without any specific preparation. At constant external field, retention ratio increases with flow-rate increases, as shown in the fractograms in Figs. 5 and 6, indicate a ''steric-hyperlayer'' elution mode for HRBC and HL-60. It is postulated in the ''steric-hyperlayer'' elution mode, that the particle average position in the channel thickness depends on the balance of the external and lifting forces [13]. Retention ratio modifications indicate therefore modifications in the average position of the particles in the channel thickness. For a given particle population eluted at constant external field, retention ratio modifications with flow-rate modifications signal modifications in lifting force intensities [14]. The fractograms in Figs. 5 and 6 suggest strongly that the less intense the flow rate, the less intense the lifting forces, causing reduced retention ratio.

Moreover, the signal intensity observed after elution after the field is stopped shows a low release of cellular material as seen in Figs. 5 and 6. More than 80% of the injected HL-60 are recovered after

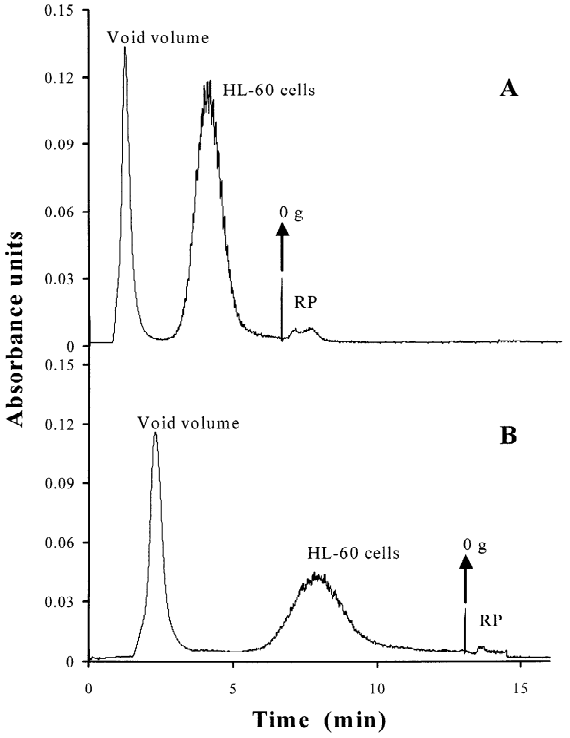

Fig. 5. Representative fractograms of HL-60 cell suspensions. Elution conditions: channel: 780×10×0.125 mm; flow injection of 100 μl HL-60 cell (10^7 cells/ml); flow rate: A: 0.80 ml/min (sterile PBS pH 7.4, 0.1% w/v BSA), B: 0.40 ml/min (sterile PBS pH 7.4, 0.1% w/v BSA); external multi-gravitational field: 60.00±0.01 *g*; spectrophotometric detection at λ=254 nm. The "0 *g*" label corresponds to the end of channel rotation; in this case external field was equal to earth gravity, thus RP corresponds to the release peak of reversible cell–accumulation wall sticking.

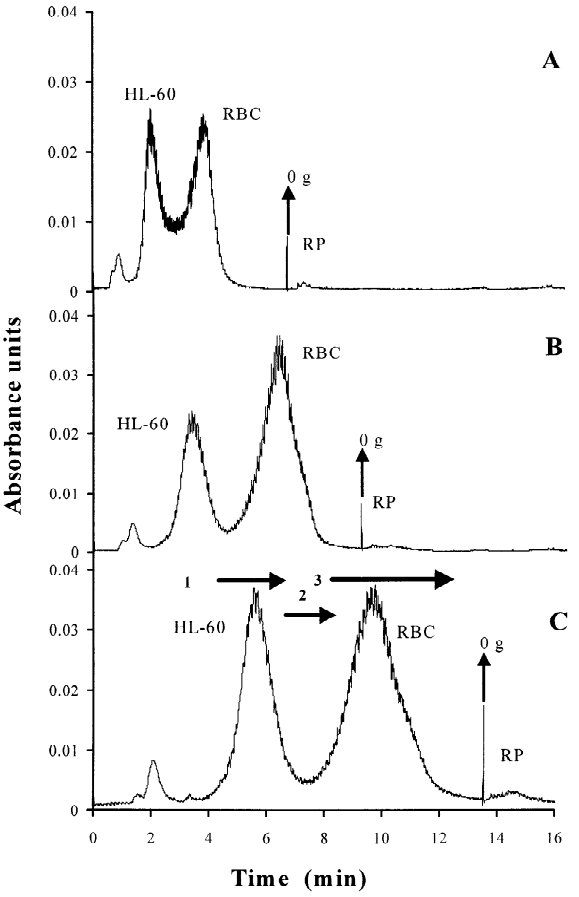

Fig. 6. Representative fractogram of HL-60 cell suspensions/RBC separation by SdFFF elution. Elution conditions: channel: 780×10×0.125 mm; flow injection of 100 μl of HL-60/RBC mixture (90% of HL-60 suspension at 10^7 cells/ml and 10% of 1/1000 dilution of total blood); flow rate: A: 1.00 ml/min (mobile phase: sterile PBS pH 7.4, 0.1% w/v BSA), B: 0.70 ml/min; and C: 0.50 ml/min; external multi-gravitational field: 25.00±0.01 *g*; spectrophotometric detection at λ=254 nm. The "0 *g*" label corresponds to the end of channel rotation; in this case external field was equal to earth gravity, thus RP corresponds to the release peak of reversible cell–accumulation wall sticking. Arrows show fraction collecting time.

elution indicating that an accumulation wall made of polystyrene helps to enhance proper biocompatibility of the system if combined with cleaning and sterilisation procedures already described [5]. The low loss of cellular material evidenced when the field is stopped does not appear flow-rate dependent, suggesting limited cell–accumulation wall interactions favoring the recovery of cells for further characterisation or use.

Such recovery appears to be due to the injection procedure described above. This is in contrast with the methodology classically utilised in most of the literature [1,2,13,14]. One must bear in mind that in the early days of SdFFF samples were directly introduced into the channel by means of a syringe, which obliged the experimenter to operate with field and flow stopped [1,2]. The syringe injection device was located on the channel upper wall (opposite the accumulation wall). SdFFF injection procedure development saw the removal of the injection device on the basket rotor and allowed introduction of the sample into the channel with field established via the upper wall. Once the sample was introduced into the

channel, flow was stopped to allow particles to sediment across the channel thickness. Elution is consequently, in both cases, started by resuming flow rate. The process of introducing the sample into the channel and submitting it to the field action for a given time in the absence of flow rate is called the ''primary relaxation step''. Such procedures required highly trained experimenters. In terms of cell sorting, these classical procedures led to some difficulties. First cells are forced to the surface of the accumulation wall generating ''transduction ''signals able to modify the physiology of the cells. Second, cell suspension can be over concentrated under the field effect generating cell–cell interactions of unmastered effects.

However reproducibility can be obtained only if an additional parameter is considered. If a low reversible trapping of mechanical or adsorption origin is easily observed at field stopped, what conclusions can be drawn from irreversible trapping? Previous work has demonstrated that cells could be irreversibly trapped in the SdFFF system [21]. The long-term impact of such a problem can be solved by methodological strategies, which include rigorous channel cleaning or regenerating procedures as described recently by Battu et al. [5]. As the retention characteristics of HRBC have been extensively described in previous reports [6,7,10,22]; it is possible to set up artificial mixtures of HRBC and HL-60 and to determine empirically the optimal separation conditions as shown in the sequence in Fig. 6. With appropriate elution conditions, determined semi-empirically using the ''steric-hyperlayer'' elution rules whose guidelines were given by Caldwell et al. [2], an almost base line resolution of HL-60 versus HRBC can be obtained. Such a fractogram (Fig. 6C) should be compared with that pioneered almost 20 years ago using fixed RBC and HeLa cells [2]. To complete the determination of the elution mode of HL-60, systematic retention ratio versus average flow-rate measurements were performed as shown in Fig. 7. These retention curves of HRBC and HL-60 are of major importance in determining the optimal separation conditions. One can observe at high external field (60 *g*) that HL-60 retention ratio increases with flow rate indicating that the elution mode is still ''hyperlayer''. This linear increase with average flow rate, a characteristic experimental effect which defines the ''hyperlayer'' mode, is an argument which supports the instrumental interest of introducing species through the accumulation wall authorising established flow injections. However this

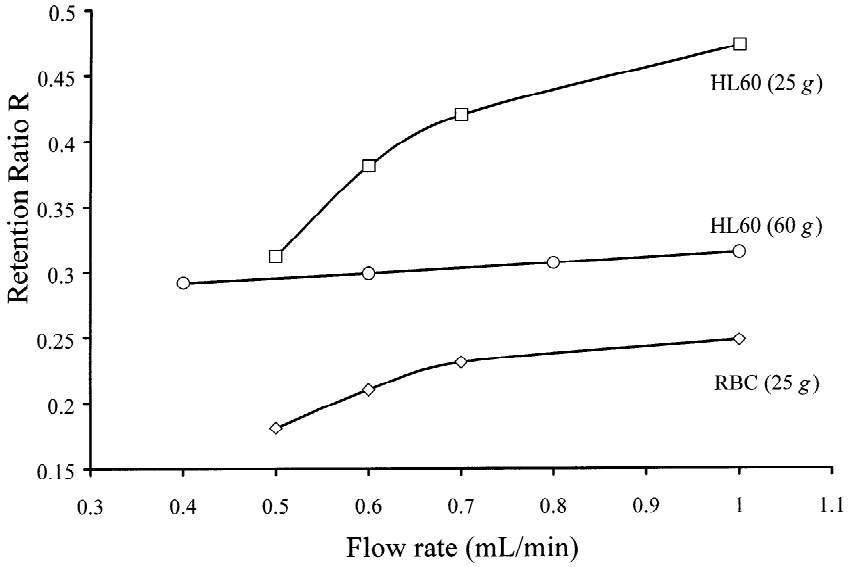

Fig. 7. Retention ratio *R* and steric-hyperlayer elution mode. Elution conditions: channel: 780×10×0.125 mm; flow injection of red blood cells (RBC; 5×10^5 RBC, 100 μl of 1/1000 dilution of total blood in PBS pH 7.4/0.1% BSA) or HL-60 cell line (1.10^6 cells, 100 μl of a 1.10^7 cell suspension in PBS pH 7.4/0.1% BSA); external field: multigravitational field set to 25 or 60 *g* (1 *g* = 9.81 cm/s^2); mobile phase: sterile PBS pH 7.4/0.1% BSA; photometric detection at $\lambda = 254$ nm.

is only a clear advantage if intense external fields are used. The non-linear curves obtained at low external field (25 *g*) for HRBC and HL-60 are in contrast with those of Caldwell et al. [2] and are explained by the two different methodological procedures explored in this report. The first one is the flow injection, which may lead to a relatively complex trajectory of the cells across the channel during elution, while the second is linked to the use of a 125-μm thick channel.

3.3. Hyphenated fractogram: off-line flow cytometry characterisation of cellular populations

The HL-60/HRBC mixture can be characterised by means of a flow cytometry FS and SS correlation map as shown in Fig. 8. In the experimental measurement conditions the flow cytometry correlation map allows one to clearly discriminate two different populations. That observed at low SS signal intensity with a large FS intensity distribution is associated with HRBC, while that at mid FS and SS intensities is associated with HL-60.

Once HL-60/HRBC elution conditions are determined, as shown in Fig. 6C, it is possible to collect elution-time dependent fractions. Three different fractions were collected whose elution time characteristics are indicated on Fig. 6C by arrows. Fraction 1 runs from 4 to 6.5 min, fraction 2 from 6.5 to 8 min and fraction 3 from 8 to 12 min; the flow cytometry correlation maps of these three fractions are given in Fig. 9 (1,2,3). Fraction 2 corresponds to the intermediate elution period between the two major peaks shown in Fig. 6C. Classically and by analogy with chromatography, one can expect a flow cytometry pattern analogous to that observed for the mixture. A careful observation of the HL-60 pattern shows clearly that the HL-60 population of fraction 2 does not have the same FS and SS characteristics as the original mixture.

If the fraction 1 correlation map is analysed, its main characteristics (FS and SS) determine that HL-60 cells were eluted in the first peak, and that no HRBC interfered. If the HL-60 populations of fraction 1 and fraction 2 are compared, it appears that these two HL-60 subpopulations have different characteristics. Using FS properties, it is possible to state that the HL-60 cells of fraction 2 are significantly smaller than those of fraction 1. Fraction 3 contains HRBC with no HL-60 pollution. When the

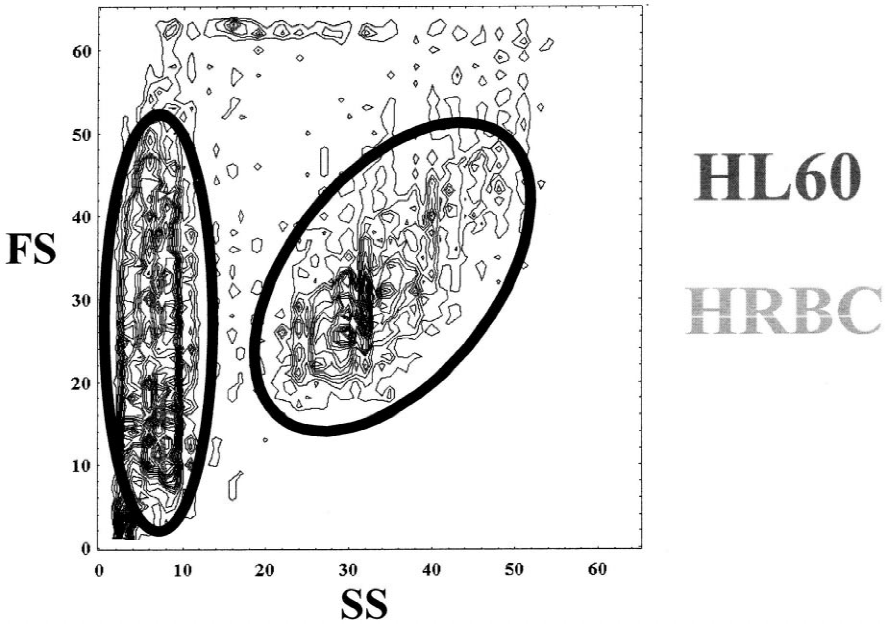

Fig. 8. SS and FS correlation map of the 50% HL-60 HRBC mixture. FC measurement conditions are given in Figs. 2 and 3. Ovoids indicate the patterns of HRBC and HL-60. Signals not included in these regions of interest are considered as noise. Light grey: HRBC (SS<10); dark grey: HL-60 (20<SS<50).

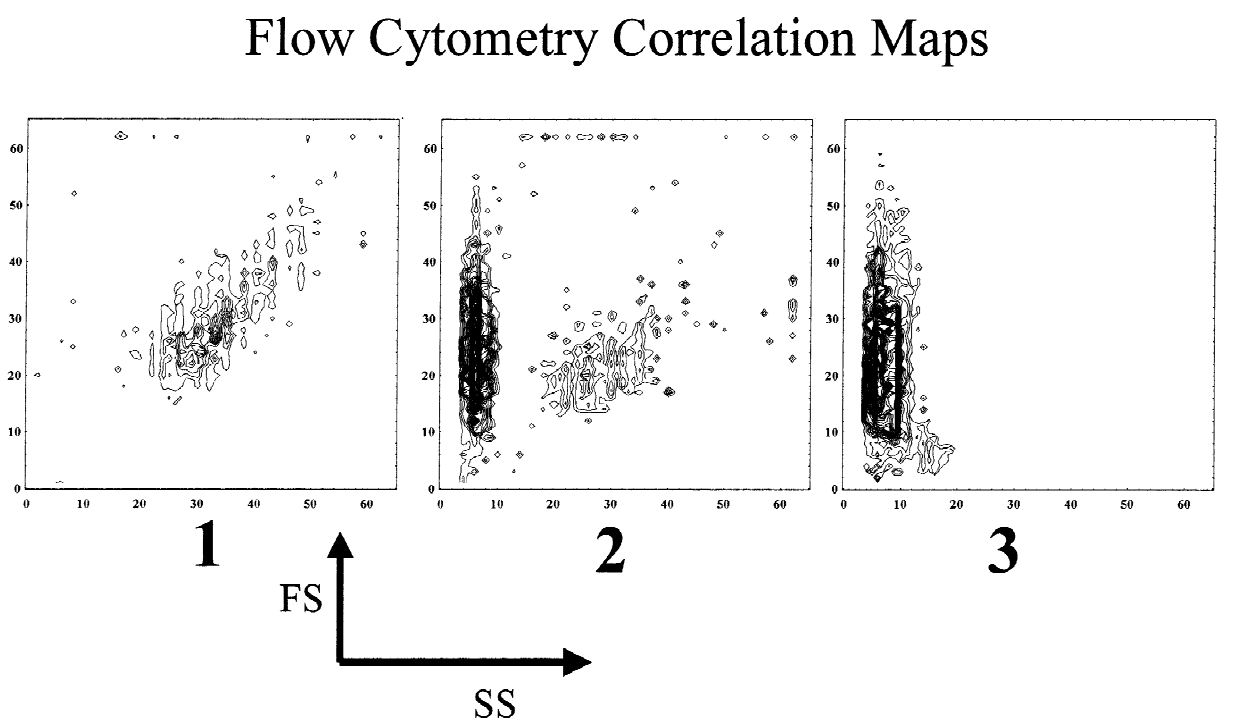

Fig. 9. HL-90/HRBC hyphenated fractogram sequence. FC measurement conditions are given in Figs. 2 and 3. Correlation map 1: HL-60 pattern of subpopulation 1; correlation map 2: HL-60 pattern of HL-60 subpopulation 2 and HRBC subpopulation 1; correlation map 3: HRBC subpopulation 2.

HRBC patterns of fractions 2 and 3 are observed, one finds that RBCs of fraction 2 have a different signature to those of fraction 3. Fraction 2 cannot be considered as an ''inter peak'' mixture but demonstrates particular cell characteristics. In fact there is evidence that two HL-60 populations were revealed, one in fraction 1 and the second in fraction 2. By analogy one can state that two HRBC fractions were evidenced between fractions 2 and 3. Cardot in gravitational field flow fractionation already demonstrated such a result of a continuous separation of subpopulations where HRBC from peripheral blood of different ages have been sorted into fractions of different ages [23].

It is possible, by means of continuous fraction collections of reduced volumes, and analysis by means of flow cytometry to define a fractogram described by a succession of correlation maps associated to an elution time. Such representation can be described as an FFF/FC hyphenated fractogram. This approach allows one to imagine an on-line coupling of SdFFF separation techniques with FC, whose data are not limited to FS and SS signals as defined earlier [10,11].

4. Conclusions

Sedimentation field flow fractionation, with the inlet tubing connected to the accumulation wall, avoids stop-flow relaxation time. The simplicity of the separation process, which becomes totally equivalent to a chromatographic separation step consisting of injection, elution, detection and fraction collection steps, makes such a technique methodologically available to any one trained in bio separations. The factors essential for success in cell separations or purification are sterilisation and cleaning/regeneration procedures. Off-line coupling of FFF with flow cytometry will lead to enhanced cell subpopulation characterisation. It indicates the usefulness of combining SdFFF and FC on-line. Such a technical combination must methodologically accomplish some basic steps, the most evident being calibration

of the FC detector. If we only consider the FS signal, which is correlated to the particle size, it is possible to calibrate this FS signal with latex beads of different average size. However other parameters such as SS or fluorescence, are more complex to define. Any calibration attempts for the purpose of FFF/FC hyphenation must consider the overall description of the sample using the concept of multipolydispersity. In terms of cell purification other ''detectors'' can be imagined principally in the off-line mode, some being adapted to describe cell functional properties such as apoptosis induction, stem or precursor functional characteristics. One can therefore imagine functional detectors based on histochemical or immunochemical properties. Some of these functional detectors already exist in flow cytometry (labeling). As some FC systems are equipped with sorting devices, one can also imagine a double hyphenation system where cells are separated according to their biophysical properties, then detected by means of, for example, some functional properties (cell labeling) and resorted by FC.

The information provided by combined FFF and FC, associated with functional characterisation, opens up new opportunities in cell sorting particularly if elution procedures can be simplified to ''chromatographic procedures'' by means of specific instrumentation development.

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