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Separation of living red blood cells by gravitational field-flow fractionation

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ABSTRACT

The field-flow fractionation technique, using the earth's gravitational field, has been applied to peripheral blood cell populations. A more or less symmetrical, gaussian-like, elution peak is generally observed for the red cell population. The bimodal cell population obtained after a massive transfusion is shown to result in a shoulder on the red blood cell elution profile. In one case where a similar shouldering peak was obtained from a non-transfused donor, the existence of an immunological double population has been demonstrated. This suggests that field-flow fractionation has some potential for complementary biomedical diagnosis.

INTRODUCTION

The technique of field-flow fractionation (FFF) was introduced more than twenty years ago by Giddings [1]. It is a chromatography-like separation method that makes use of very thin, empty channels with a rectangular cross-section. The components of the sample mixture to be separated are introduced at one end of the separator and they are transported along the separator by the flow of a carrier liquid. There is no stationary phase inside the channel, but an external force field, directed perpendicularly to the channel axis, induces a lateral, non-uniform distribution of the concentration of each sample component. Because of the liquid velocity profile, generally parabolic, existing in the channel in laminar flow conditions, each sample component is transported downstream at a characteristic velocity, which may lead to the separation of the various components at the outlet.

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One can distinguish various FFF techniques according to the nature of the force field. Up to now, experiments have been performed by means of electrical, magnetic, multigravitational (centrifugational) fields, of a thermal gradient or of a transversal flow through semi-permeable membranes [2]. All these techniques cover a wide range of applications, from relatively small molecules with molecular masses of the order of magnitude of a thousand daltons, to sub-micrometre particles. In all these cases, it is observed that the retention increases with increasing molecular mass or particle size.

The Earth's gravitational field appears to be sufficiently intense to allow the fractionation of micrometre particles according to size [3]. However, in this case, it is seen that the larger particles elute before the smaller ones [4]. The retention order is thus reversed compared with what is observed with the other FFF techniques for the analysis of sub-micrometre particles. This phenomenon can be explained as resulting from the steric exclusion of the particles from the vicinity of the channel walls [3], and/or from hydrodynamic effects supposedly associated with the inertia of the liquid [5]. This inertial effect offers the possibility of fractionating particles not only according to their size but also according to their density, which has been demonstrated [6].

This gravitational FFF technique has allowed the separation of various kinds of micrometre-sized particles, such as lamellar (talc) or globular (clay) mineral particles [7], fine coal particles [8], residues from coal liquefaction [9] or polystyrene latex particles [6]. The application of sedimentation FFF to human and animal cells, both fixed and viable, has also been shown [10]. Although, in this last case, the fractograms were mostly obtained with force fields about three to ten times larger than the earth's gravitational field, the flow dependence of the retention factor clearly indicated that the retention mechanism was controlled by the inertial effect, as it is for gravitational FFF. Because obtaining pure cell fractions is a problem of increasing importance for biological, biomedical and biotechnological applications, and owing to the limitations of the classical cell separation techniques, we have investigated the influence of various operational parameters on the gravitational FFF behaviour of living human blood cells. During the course of this study we observed the fractionation effects on red blood cells that are described below.

EXPERIMENTAL

Two FFF channels were used in this study. They are similar in construction to channels previously described [4]. Two mirror-polished glass plates were clamped together over a Mylar sheet in which the channel space was cut. This space was rectangular with tapered ends. The breadth was 2.0 cm and the distance between the apices of the tapered ends was 96 and 110 cm for channels I and II, respectively. Two holes, located at these apices, were drilled through the glass plates to allow the flow of the eluent through the channel, as well as sample introduction at

one end and connection to the detector at the other. The spacer thickness, and thus the channel thickness, was 0.175 and 0.100 mm, respectively, for channels I and II. The pumping system consisted of a peristaltic pump (Model Minipuls, Gilson, Villiers-le-Bel, France) to or a high-performance liquid chromatography (HPLC) alternative piston pump (Model 112, Beckman, Berkeley, CA, USA). The detector was a UV–visible photometer (Model 440, Waters, Milford, MA, USA) operating at either 313 or 515 nm. The glass plates were treated with a biocompatible silicone polymer (Silbione, Rhône-Poulenc, France) to prevent adhesion of the cells to.the walls.

Fresh blood samples collected from about fifteen subjects were analysed. Some were obtained from patients under medical treatment and hospital control. The others were from apparently healthy people who voluntarily donated a fraction of their blood for the purpose of later transfusion or for providing a reference material for systematic FFF studies. In order to avoid confusion, these latter blood samples will, in what follows, be refered to as "donor" blood samples and will be differentiated from the "patient" blood samples. An ACD solution, frequently used in transfusion, based on sodium citrate and glucose, or an EDTA solution, were used as both *in vitro* anti-coagulation and calcium-complexing substrates. The FFF analyses were all performed within 24 h after the taking of blood. The samples were stored at 10°C. Before injection, they were diluted *ca.* 100 to 1000 times in the carrier liquid, which gave a concentration of *ca.* 5000–50 000 cells per mm^3. The diluted blood suspension was injected into the flowing eluent at the channel inlet by means of a micro-syringe through a septum-injector. The injection volume was generally 100 mm^3. An isotonic phosphate-buffer saline (PBS) solution at pH 7.2 (8 g/l NaCl, 1.12 g/l anhydrous Na_2HPO_4, 0.2 g/l anhydrous KH_2PO_4) was used as the eluent. The eluent flow was not stopped after the sample injection.

A Coulter counter (Coulter Model TA II, Coultronics, Margency, France) with a 100 μm I.D. orifice and a microscope (Nikon, Model Alphaphot, Prosciences, Paris, France) were used for the analysis of some blood samples or collected fractions.

RESULTS AND DISCUSSION

The fractogram of a blood sample, which is the signal of the detector *versus* time, has typically the profile shown in Fig. 1. The two first peaks are usually present in all fractograms of particulate samples, although their relative heights may vary. For that reason they can be labelled as "system peaks". They generally correspond to species too small to be significantly affected by the applied external force field, at least during the course of their migration along the channel. The first peak starts to elute when a volume of eluent equal to two thirds of the channel volume has flowed through the channel. The elution volume of the second peak is equal to the channel volume. When a diluted sample of whole blood is

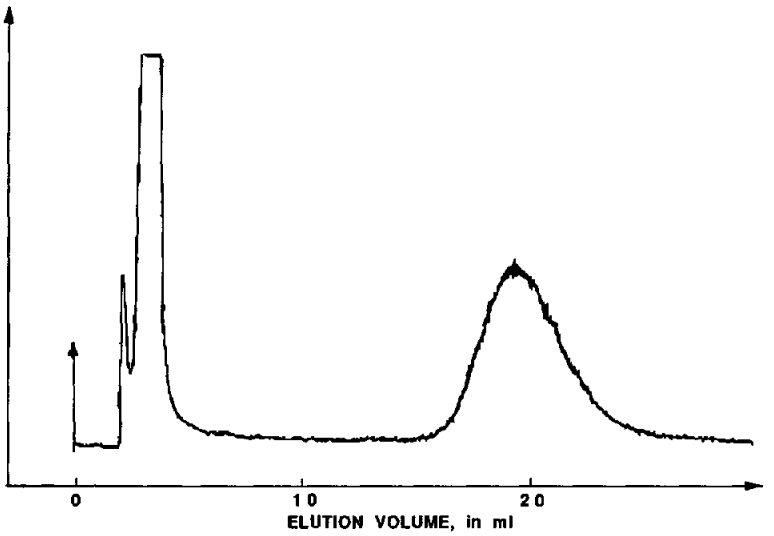

Fig. 1. Fractogram of whole peripheral blood from a healthy donor. Channel I; flow-rate, 0.1 ml/min; eluent, PBS solution at pH 7.2.

injected, this second peak is associated with the elution of the plasma proteins. The third peak observed on the fractogram appears somewhat later. It corresponds to the elution of the erythrocytes, as was subsequently demonstrated by the microscopic examination of the effluent fraction collected during the elution of this peak. Not all the erythrocytes injected into the channel have exactly the same physico-chemical characteristics, and each individual erythrocyte species has its own retention volume. Therefore, the overall profile of all individual erythrocyte species in the fractogram can be characterized by an average retention volume, which for simplicity can be defined as the elution volume corresponding to the maximum of the overall profile. This retention volume depends on the flow-rate, which indicates that the migration of the red blood cells along the channel is influenced by hydrodynamic forces, as has been previously demonstrated for rigid, spherical, micrometre-sized particles [5,6,11,12]. Generally, the erythrocyte peak is approximately symmetrical with a gaussian-like shape. The platelets are too small to be retained by the gravitational field and elute within the two first peaks of the fractogram, and the concentration of the white blood cells in the samples is too low to give a detectable peak. A microscopic examination of the fractions collected and treated with the coloured May Grünwald–Giemsa reactant [13] has shown a total absence of nucleate corpuscles during the elution of the third peak in Fig. 1, which indicates that leucocytes do not co-elute with erythrocytes. Furthermore, it was subsequently established that the white cells elute well before the red cells [14].

A systematic study of the repeatability of the retention characteristics of the erythrocyte peak has shown that the relative standard deviation of the retention factor is *ca.* 2–3% [14].

Exogenous double red cell population

During the systematic studies of the influence of various operational parameters on the retention of the erythrocytes in gravitational FFF, elution profiles of erythrocytes differing markedly from the "normal" profile shown on Fig. 1 have sometimes been observed. Especially while evaluating the influence of the average cellular volume on retention of red cells from samples from different donors or patients under the same experimental conditions, the type of fractogram shown in Fig. 2 was obtained. A relatively important shoulder is observed on the fronting edge of the red cell peak. Examination of the hospital file later revealed that the patient had been previously transfused. A massive transfusion of blood to a patient can lead to the presence in the plasma of a double red cell population because the physico-chemical characteristics of these cells (*e.g.* cell size and density) are known to vary from one individual to another and because the *in vitro* storage of blood leads to a modification of these characteristics [15]. Since gravitational FFF separates particles according to some discriminating parameter that combines the size, shape and density of the particles [12], one may expect the fractogram of the blood sample from a previously transfused patient to present some indication of a more or less pronounced double red cell population. It is, therefore, reasonable to attribute the shoulder observed on the red cell peak of Fig. 2 to the presence of a non-homogeneous red cell population resulting from the previous transfusion. The Coulter counter analysis of this blood sample has not, however, been able to show a significant difference between the red cells based on the size or, more correctly, volume criterion alone, which indicates that cell shape and/or density variations play an important role in the discrimination ability of gravitational FFF for red cells.

During the course of this study, we have also analysed a blood sample from

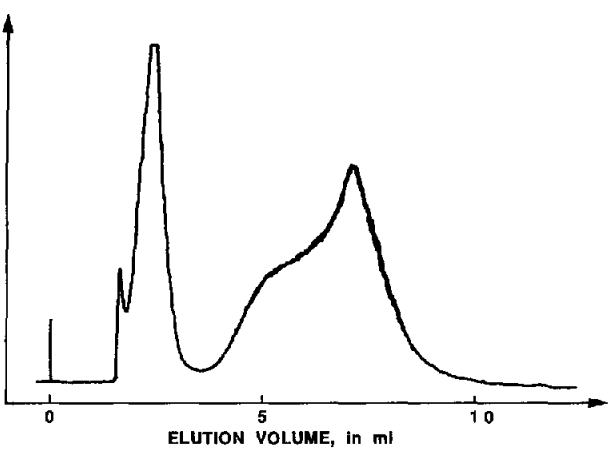

Fig. 2. Fractogram of a human blood sample from a transfused patient. Channel II; flow-rate, 0.2 ml/min; eluent, PBS solution at pH 7.2.

another patient known to have received a massive transfusion in the days preceding blood sampling and FFF analysis. The corresponding fractogram is presented in Fig. 3. Again, it displays a clear and relatively important shoulder on the fronting edge of the erythrocyte peak, which also can be interpreted as being due to the presence of a double cell population in the injected sample, due to the transfusion. The retention volume of the erythrocyte peak in Fig. 2 is considerably smaller than the corresponding one in Fig. 3, because of the larger channel thickness and the lower flow-rate used in the later case. In spite of the reduction of the volume gap between the peak of unretained species and the major erythrocyte peak in that case, the selectivity of the separation is still sufficiently large for detection of the shoulder on the erythrocyte peak profile due to the double red cell population.

In order to demonstrate the ability of gravitational FFF to discriminate human red cells from different origins, two blood samples of the same group (A group) were mixed *in vitro*, one from a donor in our laboratory and the other from a transfusion-stock population collected five days before in a citrate phosphate adenine dextrose (CPAD) nutritive buffer solution and stored at 10°C. The concentration of this population was about twice that of the laboratory donor. The 1:1 (v/v) mixture was then diluted 1000 times in the carrier liquid and injected. In that case, apart from the two initial "system" peaks, the fractogram, which is shown in Fig. 4, contains two partially separated peaks corresponding to the elution of the erythrocytes. Since, as seen in Fig. 1, the injection of a blood sample from a single donor leads to only one red cell peak, the bimodal distribution of the retention times of the erythrocytes in Fig. 4 undoubtedly illustrates the separation of the erythrocytes of the two donors.

Furthermore, it has been found that the signal of the UV photometric detector is roughly proportional to the concentration of the erythrocytes [14]. This is due

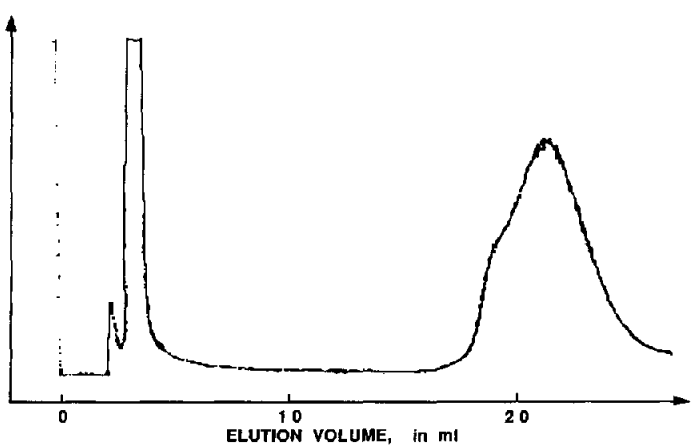

Fig. 3. Fractogram of a human blood sample from another transfused patient. Channel 1; flow-rate, 0.1 ml/min; eluent, PBS solution at pH 7.2.

to a combination of two factors. Firstly, the red cell size is significantly larger than the wavelength of the UV light, so that the scattering regime of detection is such that the signal is approximately proportional to the projected surface area of the cells in the beam direction. Secondly, there is essentially no cell obscured by another because the red cell suspension in the effluent is highly diluted. Therefore, the detector signal is approximately proportional to the projected area of all particles contained at a given time in the detector cell and, hence, to the concentration of the red cells in the effluent. Although the detector response as well as the dilution of the sample components associated with the migration through the FFF channel may slightly differ from one red cell population to another, the large difference between the two erythrocyte peak heights in Fig. 4 is most likely due to the difference in the concentrations of the two red cell populations in the injected sample. The higher first peak corresponds therefore to the blood sample taken earlier and the second lower peak to the laboratory donor's blood. This is confirmed by the observation that the erythrocyte peak of the laboratory donor's blood has a very similar retention volume to that of the second erythrocyte peak

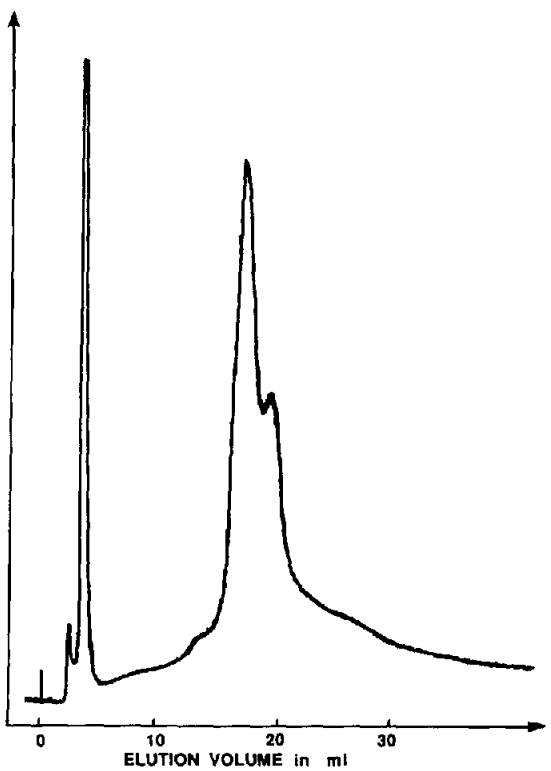

Fig. 4. Fractogram of a mixture of two human blood samples from different donors of the A group. One of the samples was collected five days before injection into the FFF channel and stored at 10°C in a CPAD solution. Channel I; flow-rate, 0.1 ml/min; eluent, PBS solution at pH 7.2.

in Fig. 4 when analysed in the same channel, with the same mobile phase and flow-rate conditions.

It is known that, during the *in vitro* storage of blood, the red cells become more spherical and rigid [16]. The earlier elution of the erythrocytes stored in the CPAD solution, seen in Fig. 4, is consistent with the recent observation that erythrocytes with a spherical shape elute before red cells with a biconcave discoidal shape [14]. This also indicates that the red cells eluting under the shoulders observed on the fronting edges of the erythrocyte profiles in Figs. 2 and 3 correspond to those arising from the transfusion received by the patients. Their proportion at the time of blood withdrawal from patients was probably too low for the fractogram to exhibit two maxima in the overall erythrocyte profiles.

Endogenous double red cell population

While the shouders observed in Figs. 2 and 3 correspond to double populations of exogenous origin due to a previous blood transfusion, another similar anomaly was observed on the erythrocyte elution profile (Fig. 5) for a blood sample from a non-transfused donor of A group, which again is strongly indicative of a double red cell population. In this case, also the Coulter counter analysis has not demonstrated a significant bimodal size distribution. However, the microscopic examination of the red cells of this donor in the presence of A-specific antibodies, according to the Beth–Vincent immunological technique frequently used for blood group determination [16], revealed the presence of both small red cell aggregates and numerous individual erythrocytes, leading to the diagnosis of a double red cell population according to the definition of a double population used in haematology.

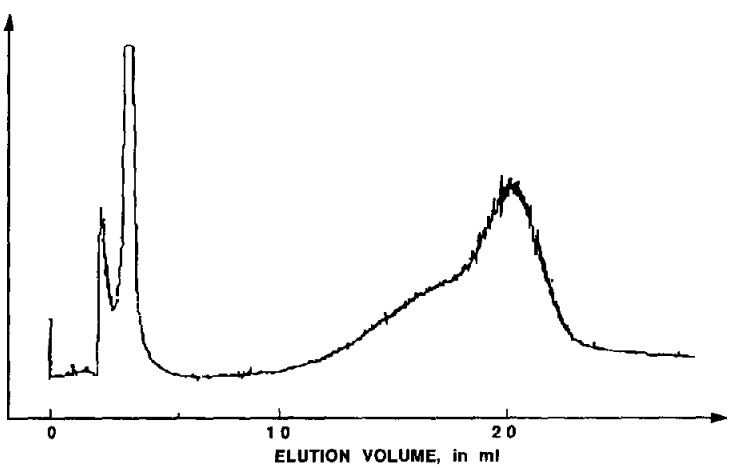

Fig. 5. Fractogram of a human blood sample from a healthy donor, for which a diagnosis of a double red cell population has been subsequently established. Channel I; flow-rate, 0.1 ml/min; eluent, PBS solution at pH 7.2.

The origin of this double population has not been established. However, among the various causes of such a diagnosis, one can most likely eliminate the possibility that the red cells of the donor have a weak A phenotype, which would be too weak to provoke the formation of large aggregates in the presence of anti-A antibodies. Indeed, this case does not correspond to a double red cell population on a physico-chemical basis and cannot explain the significant shoulder observed on the red cell peak profile in Fig. 5. Similary, because the donor was healthy, one can discount some unlikely causes associated with a serious pathological behaviour (leukemia, intestinal cancer, anemia), which lead to a reduction of the agglutination process induced by anti-A antibodies, or with a recent blood transfusion or bone marrow transplantation.

It has not been possible to make further studies of the blood of this donor. Various possibilities remain to account for the diagnosis of a double red cell population. There might be a true double population of genetic origin (endogenous chimerism), with one major group of erythrocytes bearing the A antigen on the cell membrane and a small group of red cells having neither A nor B antigens. A modification of the antigen structure at the surface of a fraction of the red cell population, leading to the diagnosis of a double population in presence of anti-A antibodies, may also be induced by either *in vivo* or *in vitro* bacterial contamination. Finally, an accidental laboratory contamination cannot be absolutely ruled out.

Whatever the exact origin of the double red cell population immunologically diagnosed, it is linked to a double population of the chemical structure and/or configuration of the antigens present in the erythrocyte membranes. Such cell surface modifications can induce only very tiny changes in the overall physico-chemical properties of the red cells, such as size and density, responsible for the primary control of retention in gravitational FFF. These changes alone are too small to explain the significant shoulder observed on the erythrocyte profile in Fig. 5. However, the membrane modification may lead to some changes in the shape of the cell and in their rheological properties at the microscopic level (membrane cell viscosity and stiffness). As the retention in gravitational FFF is heavily dependent on the magnitude of the inertial lift force pushing the particles away from the walls, one expects that a change in the rheological properties of particles, such as red cells, which are deformable and have a surface viscous membrane separating the outer eluent from the inner fluid hemoglobin, sufficiently modifies the hydrodynamics of the fluid–particle system in the FFF channel to induce a significant change in retention. Such a high sensitivity to a relatively small surface modification would not be observed for a solid particle, or even for fixed red cells, but should be a unique property of living cells due to the cell membrane circulation and inner liquid closed flow.

Clearly, further studies are required to confirm this hypothesis and to elucidate the relationship between the red cell double population and the resulting erythrocyte peak profile in gravitational FFF. Nevertheless, the partial separation shown

in Fig. 5 indicates that this technique may constitute a useful clinical diagnosis tool for erythrocyte anomalies, complementing existing immunological and classical rheological methods.

CONCLUSION

The experiments described above demonstrate that gravitational FFF, which is the simplest to implement of the FFF techniques, making use of the Earth's gravitational field, enables the fractionation of human peripheral red blood cells according to some of their specific characteristics, such as the degree of sphericity. Some further studies have shown that the biological activity of the red blood cells is maintained after their migration through the FFF channel [14].

The separation selectivity is attributed to modifications of the physical characteristics of the red cells (some combination of the size, density and shape) of the transfused blood. A previous blood transfusion received by a patient was detected from an anomaly (presence of a shoulder) of the erythrocyte peak profile in the fractogram of a sample of blood from this patient. In the fractograms of Figs. 2–4, corresponding, respectively, to two samples of previously transfused patients and to one artificial mixture of fresh blood from one donor and blood from another donor's transfusion sample, the first eluted erythrocyte population is attributed to the erythrocytes that were for some time a part of a transfusion stock, because red cells become more rigid and spherical when stored. These separations illustrate the potential of the FFF technique for the detection of a previous transfusion, and this suggests that the technique could also be used to determine the fraction of the transfused blood present at a given time in the blood of a patient and to study the kinetics of its disappearance.

Although the *a posteriori* demonstration that an anomalous shoulder in the erythrocyte FFF peak profile of a blood sample was associated with an immunological double population has to be confirmed by more systematic studies on this kind of anomaly, the fractionation effect observed on Fig. 3 indicates that gravitational FFF may find some use among the variety of techniques of blood diagnosis. It is a relatively fast and inexpensive technique, which may serve as a preliminary tool indicating when a further, more lengthy diagnosis must be developed, and which may give some guidance on the immunological method to be employed. Furthermore, since some small molecular modifications on the cell membrane seem to produce significant macroscopic changes of the cells behaviour in the FFF channel due to the extreme sensitivity of the system of the hydrodynamic and rheological properties at the microscopic level, it may become possible to use gravitational FFF to study the thermodynamics and kinetics of interactions of living cells with various molecular, colloidal or cellular substrates.

It should be noted that, whereas the present study deals with fractionation of human erythrocytes of the peripheral blood, the biological application of gravitational FFF is not restricted to this kind of cells but can undoubtedly be extend-

ed to various types of human, animal or plant living cell. FFF, and especially gravitational FFF, has some specific advantages over some other analytical techniques for cell characterization. It is a gentle technique, which can be operated in such a way that low shear stresses are exerted on the cells. In contrast to some other techniques, such as density-gradient sedimentation, the sample environment is kept unchanged, since the carrier liquid has a constant composition during the residence time of the sample in the FFF channel. This is especially important with samples, such as living cells, that may exchange material with the surrounding liquid through their semi-permeable membrane and are sensitive to osmotic effects. The particles are indeed much better characterized if their physico-chemical properties are unchanged during the analysis. In addition, FFF offers the possibility of collecting fractions of purified living cells in viable conditions, as has been shown for the erythrocytes [14], for further *in vitro* or *in vivo* use. Thus the gravitational FFF method, as well as the companion multigravitational (or sedimentation) FFF technique, may well become a useful tool in cell biology.

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