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Separation of *Leishmania*-infected macrophages by step-SPLITT fractionation

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ABSTRACT

After a primary infection protocol of macrophages with *Leishmania amazonensis*, the percentage of infection drops as infection progresses and the uninfected population of macrophages mask the effects of infection for electrophysiological studies. In order to increase or maintain the infection percentage, we introduce an enrichment process after primary infection, which increases the possibility of following the infection longer times than any known process. A membraneless separation technique, step-SPLITT fractionation, implying flow and transverse gravity field in a ribbon-like channel, was used for enriching samples of macrophages infected with particles and with *L. amazonensis*. We demonstrate the capability of the s-SPLITT of generating, from a mixture resulting from a primary infection, an enriched and a depleted fraction with infected cells, without using any selective labeling pre-processing. It is also shown that a continuous sorting is possible without damaging cells and the losses of matter into the separation chamber is minimal.

1. Introduction

Parasites of the *Leishmania* genus are responsible for severe morbidity and mortality in developing countries, affecting approximately 12 millions people around the world [1]. *Leishmania* species are intracellular obligatory parasites of macrophages (innate immune cells). After entry into its mammalian host, the parasite is taken (phagocytosed) by macrophages and confined to a compartment, known as a phagolysosome (PG) [2]. We have shown that infection with *Leishmania amazonensis* of macrophage-like cells (J774A.1) alters macrophage ion permeability [3,4] which contributes against macrophage ability to activate. However, one of the problems we have encountered in our experimental model is that during the 4 days after infection, the percentage of infected cells drops from 75% to barely 30% and the uninfected population of macrophages partially, or could even totally, mask the effects of infection, especially when measuring biochemical parameters such as nitrite detection and/or cytokine secretion where the signal from uninfected macrophages cannot be discriminated from that of infected cells. In order to maintain the percentage of infection, near to 70% or superior, we added a filtration process as a second stage after the primary infection protocol. The primary protocol is standard and similar to that used by other laboratories. However the 70% infection level is only achieved at 24 h post-infection, longer periods are associated with infection level drop and measurements such a nitric oxide production required minimum 48 h post-infection. For this purpose we used the technique called gravitational step-split flow lateral transport thin fractionation (s-SPLITT) [5,6] in order to deplete the population of uninfected cells, from mixtures of uninfected and infected macrophages obtained by primary infection process. The s-SPLITT device is a new development of the classical SPLITT fractionation channel proposed by Giddings [7], which belongs to a family of separation techniques called by Giddings F+ [8]. The motivation of using this device for macrophage sorting is based on the fact that infected cells are bigger than non-infected ones [3]. In principle, species with different buoyancy factor $\phi_B \sim \Delta\rho d^2$ where $\Delta\rho$ is the difference between the particle and the liquid densities and d the particle size, will settle at different velocities. In this study two sets of experiments were conducted. The first one demonstrates that macrophages are amenable for separation experiments in s-SPLITT channels by testing their viability. It also shows separation of macrophages after phagocytosis of latex and silica particles from control macrophages, a situation that mimics some of the effects generated by *Leishmania* infection, on these cells. The second set of experiments demonstrates the capability of this

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hydrodynamic technique to enrich fractions of *Leishmania*-infected macrophages.

2. Materials and methods

2.1. Cell culture

The murine macrophage-like cell line J774A.1 was obtained from the European Cell line and Hybridoma Bank Collection (EECACC No. 91051511) and the ATTC (TIB-67) and maintained as a monolayer in 25–75 cm^2 flasks at 37 °C, 5% CO_2 for up to 4 weeks. Cells were kept in RPMI 1640 culture medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS, Invitrogen). Cell viability was checked by Trypan blue exclusion. Cultures with viabilities above 98% were used for experiments.

2.2. Latex and silica beads phagocytosis

In order to make a first approach to macrophage (Mϕ) separation we conducted experiments using macrophages which have phagocytosed polystyrene latex 5 ± 0.02 μm and 1.05 g/cm^3 density, Duke Scientific, and silica beads, 5 ± 0.5 μm, 2.42 g/cm^3 density, Duke Scientific (PMϕ). Eighty percent confluent cell cultures were exposed to latex and silica beads in a ratio 1:10:10 macrophage:latex:silica and incubated at 35 °C, 5% CO_2 for 4 h. Excess beads were washed out using free serum RPMI. The percentage of phagocytosis was measured under light inversed phase contrast microscopy, with magnification 20×, and was calculated as the number of cells that did phagocytosis per 100 cells. Once the percentage of phagocytosis was calculated, we mixed this population with control Mϕ. Two mixtures with fractions 57–43% and 70–30% PMϕ–Mϕ were used. Size distributions of control and cells after phagocytosis were determined by a Coulter counter, Z2 Beckman Coulter, FL. Given that the cell size range was between 5 and 25 μm, the Coulter aspiraton tube used had a hole of 50 μm. The number of cells counted at each run was minimum 2000 in 0.1 mL which was the volume of suspension analysed by the Coulter.

2.3. Parasite culture and infection

A *L. amazonensis* isolate, kindly donated by Dr. Nancy Gore Saravia (FLA/BR/67/PH8, CIDEIM, Cali, Colombia), was used. Promastigotes, at an initial concentration of 1×10^6, were cultured at 24 °C in 25 cm^2 flasks in TC-100 or Schneider's medium (Invitrogen) supplemented with 10% FBS [9]. Promastigotes were allowed to reach their stationary stage (stationary growth, rosette formation and good infectivity) and either diluted to maintain the culture or harvested for infection. Eighty percent confluent macrophage cultures were exposed to stationary phase promastigotes at a ratio of 1:10 and incubated at 34 °C, 5% CO_2 for 4 h. Excess parasites were washed out with serum free medium and the cultures kept for 48 h post-infection (48 hpi) as a monolayer in 25–75 cm^2 flasks at 35 °C, 5% CO_2. Infected cells (IMϕ) were visualised by Giemsa staining and percentage of infection was measured under light microscopy and calculated as the number of infected cells per 100 cells. Two mixtures with fractions 53–47% and 70–30% IMϕ–Mϕ were used.

2.4. Macrophage and parasite viability

Infected macrophages onto glass cover slips were loaded with 20 μg/mL FITC and 10 μg/mL propidium iodine in RPMI for 30 min at 35 °C and washed. Cells were visualized with a fluorescent inverted microscope Zeiss IM-35 with the filters sets GFP (excitation 472 ± 11 nm; emission 520 ± 15 nm). Viable parasites fluoresce green.

2.5. Separation technique and experimental device

The two main methods F+ are Field Flow Fractionation, FFF [10] for analytical separation, and SPLITT fractionation for continuous sorting [7,11,12]. The F+ techniques are membraneless therefore well adapted to micron-sized particulate materials. These techniques combine an axial flow through a ribbon-like channel generating a parabolic velocity profile, and a transverse field force applied all along the channel. The fact that F+ techniques use low shear rates up to 100 s^{-1} in classic working condition, makes them particularly suitable for separating fragile materials like living cells. It is worth noting that the averages physiological shear rate is 600 s^{-1}; at this rate, platelets can be activated and cells deformed. By using F+ techniques, cells can be preserved from physiological and mechanical modifications.

Regarding FFF, many subtechniques are reported depending on the field force applied: gravitational, electric and magnetic [10]. This technique is used only for analytical purposes while our interests are focused on continuous fractionation. Concerning the possibilities of fractionating macrophages in function of the infection, FFF could be used in hyperlayer mode where buoyancy is in competition with lift-forces, or eventually in steric mode of operation [10]. The former is related to the very poorly known problem of lift-forces of deformable and non-spherical objects, more over, the buoyancy parameter $\Delta \rho d^2$ is not really known in our samples, the macrophage density changes when they are infected. The steric mode of operation in FFF, is related only to the fractionation in size and, as we shall see later, infected and non-infected cells may have the same size. In any case, our mayor argument of using SPLITT-like fractionation technique is the capability of preparative separations.

The fluidic separation chamber called step-SPLITT channel or s-SPLITT [5,6], is a Hele-Shaw-like cell, *i.e.* high aspect ratio $B/w > 10$, with B the breadth and w the thickness, provided with two inlets and two outlets as schematized in Fig. 1. It was fabricated at the PMMH laboratory by superimposing Plexiglas plates and Mylar spacers intercalated at two levels. The channel is composed of an inlet part at the bottom consisting of a pre-chamber which allows the inlet carrier to spread out the total breadth and to merge with the sample injected at the top part under the fluid thin lamina. The outlet part is analogous to the inlet one; the flow splits out into two thin laminas at the step vicinity. The part of the channel where the separation takes place is $L = 10$ cm length, $B = 1$ cm and $w = 356$ μm; the channel volume is thus $V_{ch} = 0.356$ mL. The plastic plates are bolted between two metallic plates in order to keep the parallelism and neoprene glue is used between plates in order to prevent leakages. The two inlets are configured out of face and are separated by an inlet step. This configuration allows separate injection of the sample and the carrier which merge at the step vicinity forming a virtual stream plane called Inlet Splitting Plane, ISP. A symmetrical configuration is used at the channel outlet for dividing the flow into two fractions and the stream surface dividing both

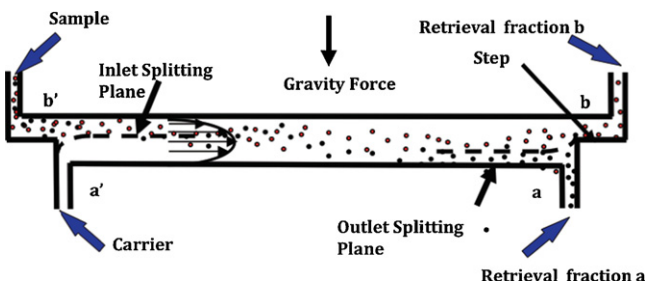

Fig. 1. Schematic view of the step-SPLITT channel and the separation method. Species having mobility high enough for crossing the distance between the two splitting planes, will elute at the bottom outlet.

fractions is called Outlet Splitting Plane, OSP. Let us note that the *step* in this channel replaces the splitter used in classical SPLITT fractionation [11]. Several contiguous steps can be implemented in a s-SPLITT chamber in order to generate multiple outlets for multiple compound fractionation. Let's note that classical SPLITT does only binary separations. The dificulty to implement a chamber where multiple metallic sheets are superimposed for generating multiple outlet is related to the mechanical deformations of metal sheets which generates 3D flow disturbances making very difficult predicting the separation capabilities of the device. It is a simpler way to use steps as detailed in [5]. Work using multi-outlet s-SPLITT fractionation is going on.

The sample is injected through the inlet labelled a' with a flow rate $Q_{a'}$, while the inlet b' is feed by the carrier with a flow rate $Q_{b'}$ (Fig. 1). At the point where the step is placed, both laminar fluids merge. In the configuration used in our experiments, the flow rate $Q_{b'} > Q_{a'}$ and particles are compelled to accumulate at the vicinity of the A wall, just up the ISP. The control of the outlet flow rates Q_a and Q_b, allows determining the OSP position. The ISP and OSP position may be calculated by the following relationship:

$$\frac{Q_{a'}}{Q} = 3\left(\frac{w_{a'}}{w}\right)^2 - \left(\frac{w_{a'}}{w}\right)^3 \quad (1)$$

where the total flow rate $Q = Q_{a'} + Q_{b'} = Q_a + Q_b$. $w_{a'}$ is the ISP position, the origin being the wall adjacent to the inlet a, and w is the channel thickness. The OSP position w_a can be easily computed by Eq. (1). The distance $\delta w = w_a - w_{a'}$ is called transport lamina thickness. Species mobile enough to cross this distance, will elute at the outlet b, whereas other species will be collected at the outlet a as indicated in Fig. 1. The experimental ISP and OSP we used have been deduced from the control parameters: $Q_{a'}/Q = 0.1$ and $Q_a/Q = 0.4$ corresponding to $w_{a'}/w = 0.19$ and $w_a/w = 0.43$, leading to a value of the transport lamina thickness $\delta w = 0.34w$. Considering that the channel is 356 μm thick, $\delta w \sim 120$ μm. In s-SPLITT fractionation, the time taken by the species crossing the transport lamina thickness fixes the order of magnitude of the residence time, *i.e.* the time the particle lasts under the influence of the transverse field force. The transport lamina thickness being of in our experimental configuration very thin, around 120 μm, and the sedimentation velocity of macrophages, around 5 μm/s, the residence time is smaller than 30 s, which places the SPLITT as a fast continuous separation technique. The transport lamina plays the role of a liquid membrane, which is permanently renewing. Its thickness could be considered as analogue to membrane porosity. Selectivity in s-SPLITT fractionation is generated because each species composing a mixture has different transversal mobility, which characterizes the interaction between species and the field force. When the transverse field is the gravity, the settling velocity becomes the selective process. In this case, species do not require any special pre-treatment before being injected what is always desirable in the separation process of biological materials. Under the influence of the gravitational field, species composing a dilute suspension axially migrating across the channel will settle at different velocities following the Stokes law for the sedimentation of an isolated sphere:

$$v_s = \frac{\phi_B g}{18\,\eta} \quad (2)$$

where v_s is the sedimentation velocity g is the acceleration of gravity, η is the dynamic viscosity of the carrier and the buoyancy factor:

$$\phi_B = (\rho_m - \rho_f)d^2 \quad (3)$$

where ρ_m and ρ_f are the macrophage and the suspending fluid densities, respectively, and d the cell diameter. Therefore, by controlling the ISP and OSP, when all species have the same density, we can fix for instance, a cut off diameter d_c, which drive species bigger that d_c toward the outlet b. The diameter d_c is thereby defined by [13]:

$$d_c^2 = \frac{18\eta\,(Q_a - Q_{a'})}{LBg\,(\rho_m - \rho_f)} \quad (4)$$

The model used so far for determining the operating parameters in s-SPLITT neglects some hydrodynamic effects like inertial lift-forces [14,15], shear induced hydrodynamic diffusion [16,17,12] instabilities [18], hydrodynamic drafting [19] and channel imperfections [20], all more or less detrimental for separations. These effects favour a non-specific cross over, *i.e.* cross-stream migration of particles independently of any external field [6]. Indeed, a few works deal with these important topics and because this study is the first one devoted specifically to macrophages infected with *Leishmania*, it is not possible to consider a theoretical approach for predicting separations; the complexity of that task deserves a thorough study that is beyond this work. Nevertheless, all those effects are minimized if diluted samples and low flow rates are used. We took care of those aspects by using volume fractions under 1% and flow rates smaller than 5 mL/min, which are the limit values found in the literature related to SPLITT fractionation. The SPLITT technique has been previously used for cell separation [21,22], with low impact in cell viability and high output recovery [22,23].

In this study we are not dealing with high throughput separations, we are only concerned with the separation capability and the availability of cells after the separation.

2.6. Cell size and fraction analysis

The experimental set up is composed of a s-SPLITT channel and three syringe pumps KDS 100 (KD scientific), two at both inlets and one at one of the outlets, the second outlet is left free for pressure compensation. s-SPLITT fractionation is normally run in a continuous mode, *i.e.* species are continuously injected at the inlet a' and clear fluid injected at the inlet b'. For practical purposes, given that our interest is not so far high throughput fractionations, we used the pulse injection chromatographic mode. By using a third syringe pump and switching valve, we could easily control the sample-injected volume V_{sample} which was in each run 200 μL. Note that the channel volume (365 μL) is just a little higher than the volume of the injected sample and we cannot consider the separation as analytical but either in continuous mode of operation. This is not really relevant in our experiments, only may partially justify the use of the ideal theoretical model of SPLITT fractionation for fixing the flow parameters. Control parameters including flow rates used during the experiments are presented in Table 1.

For data analysis we used a Coulter counter Z2. Size distributions of control macrophages before the infection, after the infection and the mixtures were determined. Based on size distributions, we fixed the flow parameters and the cut off diameter in order to obtain the best enrichment. Infection as well as phagocytosis percentage cannot be determined using only size distribution, and therefore this parameter was evaluated by light microscopy, as the number of cells that have phagocytosed parasites or particles per 100 cells. In the case of infection, cells were dyed previously with Giemsa. In

Table 1
Employed samples and parameters for the carried out experiments. In all cases $Q_{a'}$ was set as 0.2 mL/min.

Type of sample	Q (mL/min)	Q_a/Q
Mφ	1,1.5,2,2.5	0
Mφ	1,1.5,2,2.5	0.2,0.4,0.5,0.8
PMφ:Mφ (57%:43%)	2	0.4
PMφ:Mφ (63%:47%)	2	0.4
IMφ:Mφ (53%:47%)	2	0.4
PMφ:Mφ (70%:30%)	2	0.4

all cases images of at least 400 cells were acquired, the infection and phagocytosis percentage were determined before cell injection, as well as at channel outputs.

3. Results and discussion

3.1. Leishmania infection profile in J774A.1 macrophage-like cells

The *Leishmania* infection profile in our macrophage model shows relatively high percentages of infection after the first 24 h post-infection (pi) of approximately 83 ± 3%. As infection progressed we observed a decrease of percentage of infection (Fig. 2a). We cannot attribute this drop to alterations in parasite number (Fig. 2b) or viability (Fig. 2c) since the parasite number increase during the first 72 h post-infection and the viability is over 80% and remains constant during the observation period. Therefore our interpretation is that the drop in percentage of infection can be attributed to a wash out of the infection due to the continuous replication of uninfected macrophages. These data does not agree with previous reports using a related macrophage cell line, where the authors maintain continuous infection over weeks [24]. However, these authors suggest that given some conditions uninfected macrophages can replicate fast enough to get the wash out effect described in this study.

We have observed that infected macrophages have larger diameters compared to uninfected ones [3]. Based on the principle that species with different ϕ_B will settle at different velocities, and that the sedimentation velocity depends on the square of the particle diameter, separation might occur in s-SPLITT channel because of the hydrodynamic diameter of an infected macrophage might exceed 50% that of an uninfected one. It is nevertheless, nontrivial to use this physico-chemical parameter because is its necessary to take into consideration that infected macrophages modify their actual density, which probably decreases with respect to uninfected cells, partially compensating the size increase. Moreover, macrophages are not monodisperse in size, generating situations where the biggest uninfected macrophages could have comparable sizes to smaller infected ones leading to a poor differentiation for hydrodynamic separation needs. It is worth noting that size of infected cells, may be due to phagolysosome volume, parasite/cell number, parasite size, among other reasons. In this study, cell size distribution was determined by a Coulter counter and phase contrast microscopy in order to evaluate the relevant separation parameters.

3.2. Cell viability and recovery

In order to determine cell viability in our model, we injected uninfected macrophages using different flow rates in the s-SPLITT channel (see Table 1). At the channel output we measured the percentage of recovery (cells/mL), determined by the Coulter counter, as well as cell viability, by using the classic Trypan blue assay. In all cases we have recovered percentages equal or higher than 80% of the injected cells (Fig. 3). The cell viability does not suffer any change with respect to control cells, which have not been injected into the s-SPLITT channel and was equal or superior to 90% (Fig. 3). Both facts confirm the suitability of s-SPLITT for macrophage separation.

3.3. Cells mixtures and size distributions

In order to do a first approach to infected macrophage separation we used a mixture of two populations, the first one was composed for macrophages that have phagocytosed silica and latex beads (PMϕ), as was described previously and the second one was composed only of control macrophages (Mϕ). Mixtures show

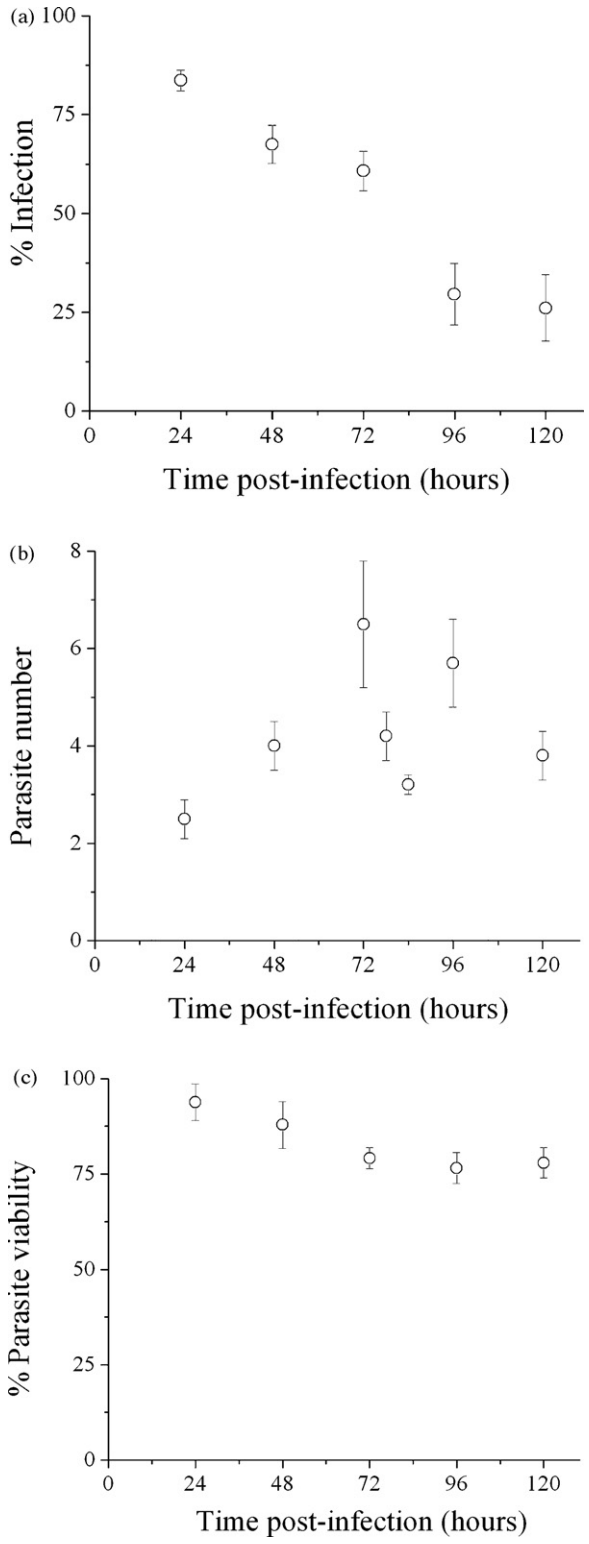

Fig. 2. *Leishmania* infection behavior in J774A.1. (a) Percentage infection. Data represent mean ± SE, $n = 12$. (b) Parasite number per macrophage. Data represent mean ± SE, $n = 12$. (c) Parasite viability. Data represent mean ± SE, $n = 3$.

wider size distributions compared to these ones observed in the control macrophage population as well as, a shift of the diameter mean value; for control macrophages the mean diameter is approximately 12 ± 0.5 μm while the mean value for the mixture is 14 ± 1 μm. Similar differences in diameter were observed between infected cells and uninfected cell populations. The mean diameter

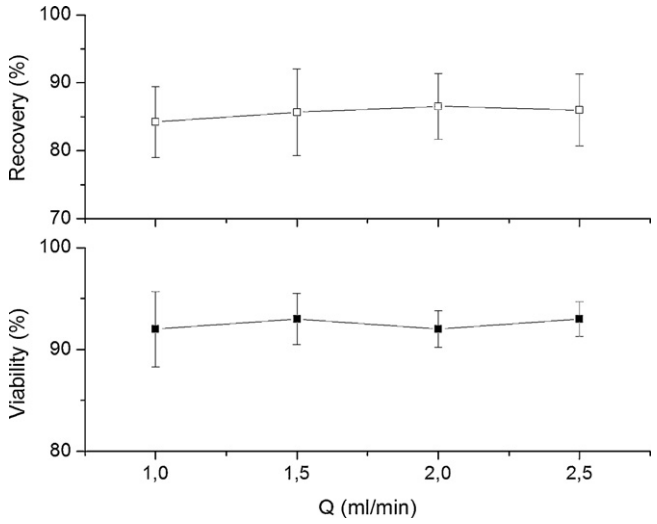

Fig. 3. Percentage of recovery and cell viability of a control macrophage population injected into the step-SPLITT channel at different flow rates.

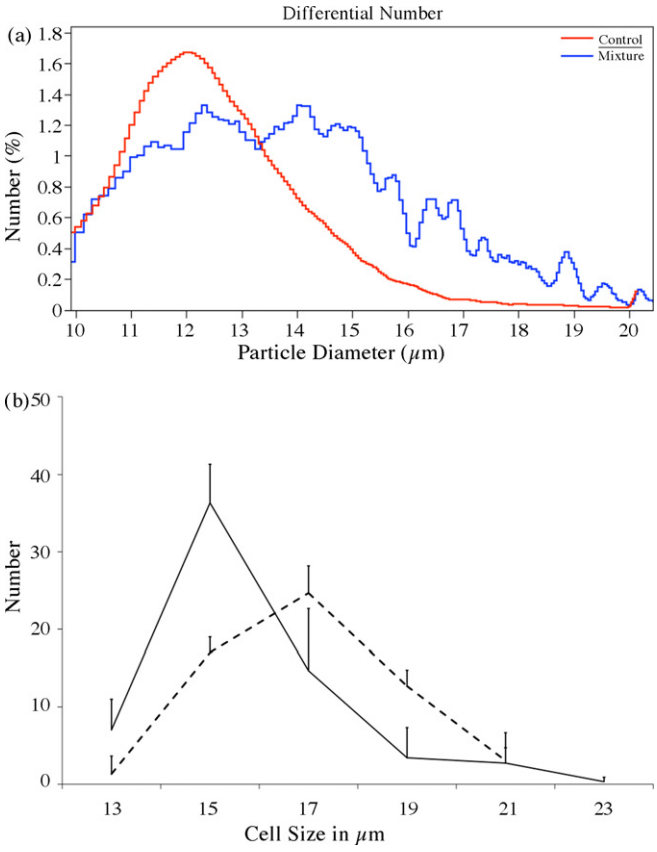

Fig. 4. (a) A cell size distribution of Mϕ (narrow distribution) and cells that have phagocytosed latex and PMϕ (broaden distribution). (b) Cell size distribution of Mϕ (plain line) and *L. amzonensis* infected macrophages (IMϕ, dashed).

for infected cells is $14 \pm 2\,\mu m$ at 48 h post-infection (Fig. 4b). This fact confirms the suitability of phagogytosis of beads to mimic at least in size, the infected cells (Fig. 4a). It is important to notice that there is an overlap between control cell size distribution and cells that have phagocytosed latex and silica beads, and the same occur between uninfected and *L. amazonensis* infected cells 48 hpi. Because of this, it is necessary to employ a methodology altering the Coulter counter when the efficacy of separation is measured.

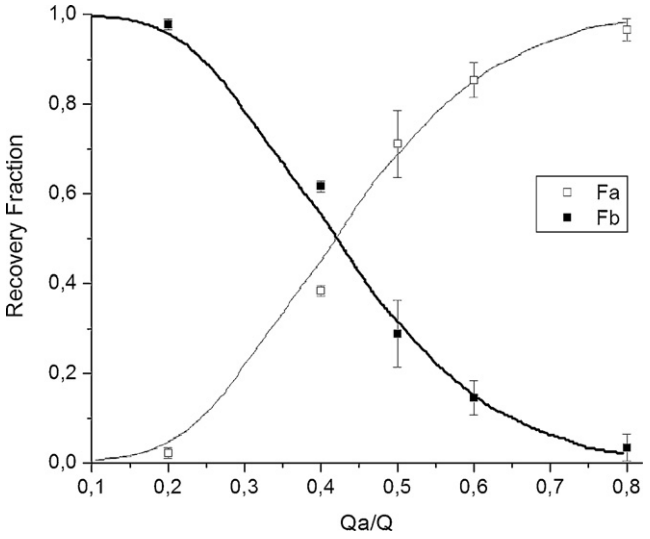

Fig. 5. Recovery fractions obtained theorically (solid lines) and experimentally (*Fa* open squares and *Fb* solid squares).

In this case we have chosen light microscopy observation, image capture and image analysis.

3.4. Control cell behavior inside the s-SPLITT channel

In order to determine the behavior of control marcrophages alone inside the s-SPLITT channel we made a series of experiments using different flow rates and changing the Q_a/Q ratio, as was previously described in Materials and Methods. The experimental results were compared with the theoretical ones obtained through the proceeding described in [15]. We study the fraction recovery by evaluating the trajectories of a number of particles equivalent to the estimated number of J774A.1 cells introduced in each injection ($\sim 10^5$). In order to mimic the cell size distribution, the diameter of each particle was chosen randomly according to a Gaussian distribution fitted to the percentual distribution shown in Fig. 4. This fit indicates a mean value $\langle d_{cel} \rangle = 12.4\,\mu m$ and a standard deviation $\sigma = 2.6\,\mu m$. The result of that comparison is depicted in Fig. 5. It is important to mention that theoretical curves were obtained by using the cell density as an adjustable parameter; in Fig. 5 we settled $\rho_{cel} = 1.07\,g/cm^3$, which is a reasonable value compared to those established by using Percoll gradients. As can be noticed the experimental behavior of control cells inside the s-SPLITT channel is similar to that expected theoretically, this fact supports the hypothesis that macrophages are in fact affected by the gravity force. In the case of infected cells and cells that have phagocytosed latex and silica beads it was not possible to observe separately from control cells their behavior into the channel because using infection and phagocytosis existing protocols, it is not possible to obtain pure populations, this is as mentioned before the goal of this work.

3.5. Separation of macrophages that have phagocyted latex and silica beads

An enrichment of 30 ±12% (Fig. 6) in the population of cells that have phagocytosed particles was observed at *b* output, when the following parameters were used: $Q = 2\,mL/min$, $Q_{a'}/Q = 0.1$, $Q_{b'}/Q = 0.9$ and $Q_a/Q = 0.4$. In order to confirm that the enrichment is due to gravity through the channel and not to other unspecific crossover phenomena we did experiments using two different phagocytosis percentages in the feeding sample. In the first case a proportion 57–43% PMϕ:Mϕ was used (Fig. 6a) and in the second one we used a proportion 70–30% (Fig. 6b). In

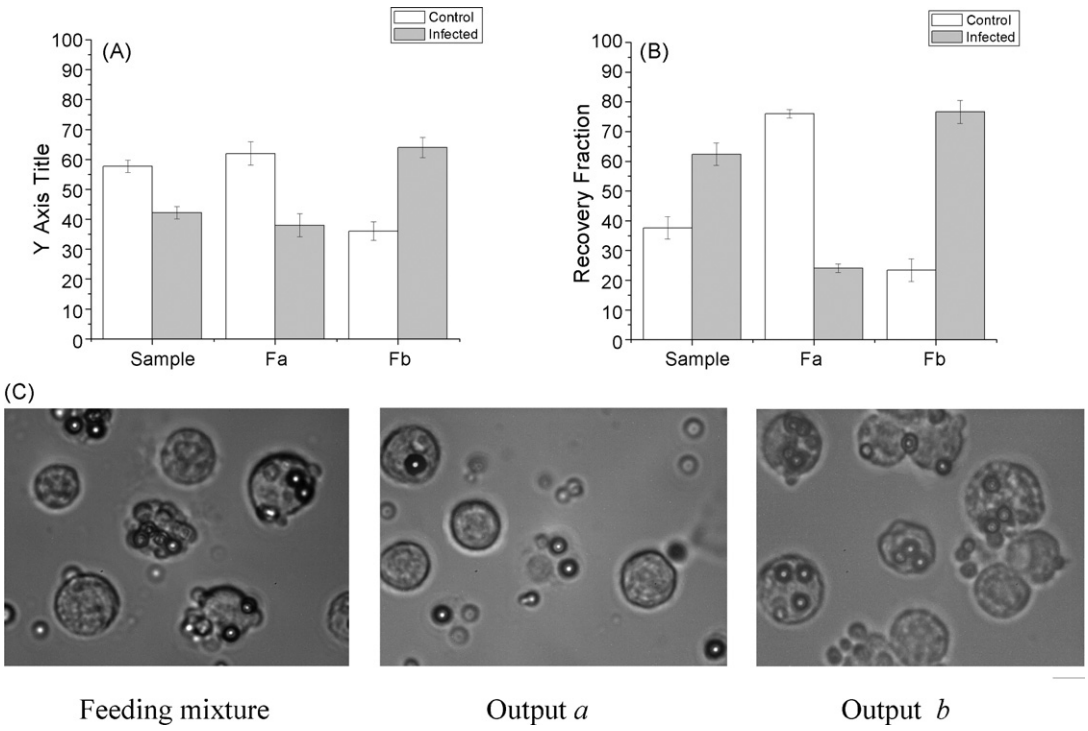

Fig. 6. Recovery percentage of control cells (open bars), and cells that have phagocytosed latex and silica particles (grey bars), obtained by Coulter counting, at the outputs of s-SPLITT channel. (a) In this particular case a proportion 57–43% between control and phagocytocis cells was employed as feeding sample. (b) In this experiment a mixture 37–63% control and phagocytosis was used as feeding sample. (c) Images obtained from feeding mixture and enriched fractions obtained at *a* and *b* output the bar represents 5 μm.

both cases we obtain a roughly similar enrichment in phagocytosis percentage; this fact suggests that the obtained enrichment is due to the action of the gravity force through the cell and not to an unspecific phenomena. Fig. 6c shows example pictures, obtained by phase contrast microscopy, of the feeding mixture and both collected fractions. In all pictures we clearly observe control macrophages and cells with particles. We can see that macrophages after phagocytosis appear larger than controls. We

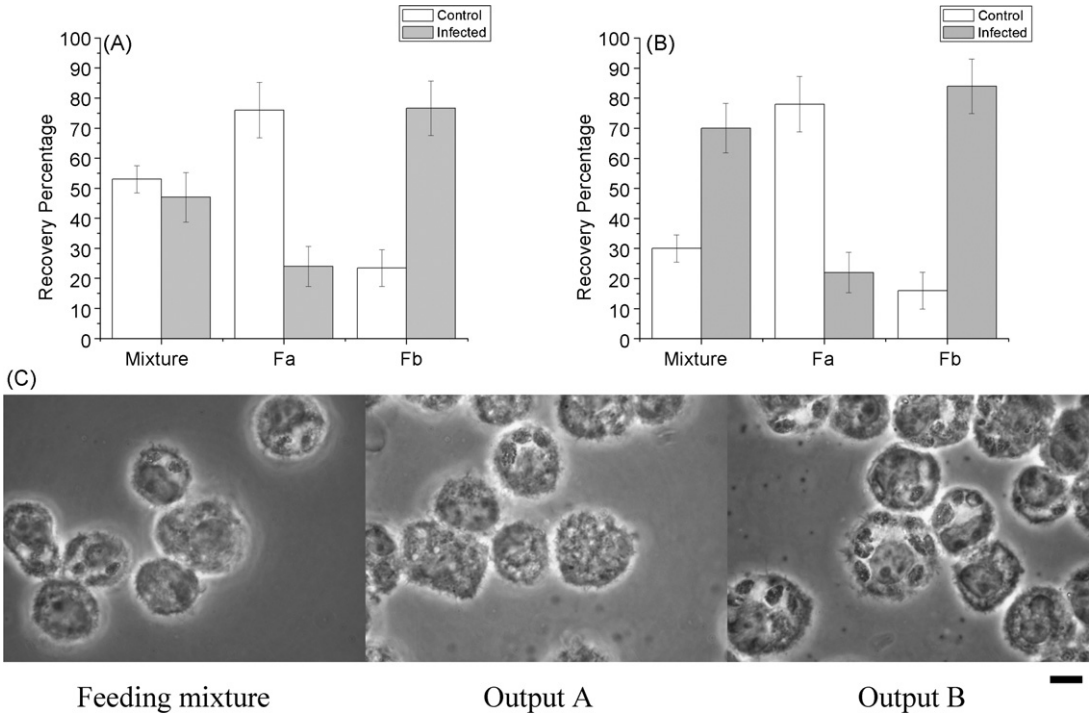

Fig. 7. (a) Recovery percentage of control cells (open bars) and *L. amazonensis* infected cells (grey bars) at the outputs of the s-SPLITT channel. In this particular case a proportion 53–47% between control and infected cells was employed as feeding sample. (b) In this experiment a mixture 70–30% control and infected cells was used as feeding sample. (c) Images obtained by phase contrast microscopy, from feeding mixture and fractions obtained at *A* and *B* output. The bar (at the lower right part of the output *B* picture) represents 5 μm.

observed that fraction *b* is enriched with infected cells, respect to the feed mixture. Fraction *b* seems to contain mostly cells with more than two phagocyted particles. In addition there are isolated particles which have not been phagocyted by cells. The set of pictures shown is only one example of more than one hundred analysed.

3.6. Separation of infected macrophages

After optimizing the parameters to achieve the enrichment in phagocytosis percentage, the technique performance was evaluated for *L. amazonensis* infected macrophages. Using the initial mixture of 53% control and 47% with phagocytosis (Fig. 7a), an enrichment of 21 ± 10% in the infection percentage was observed at the *B* output (Fig. 7b), in the same way the infection percentage enrichment does not depend of the infection percentage in the feeding mixture, the same experiment was performed for two different mixtures, the first one with approximately 45% (Fig. 7a) and the second with 70% of infected cells (Fig. 7b) and after the SPLITT a similar percentage of enrichment was obtained. Fig. 7c shows example pictures of the feeding mixture and both collected fractions. In all pictures we observe control macrophages and infected ones; *L. amazonensis* appear as dark points that have to be resolved from the cell granularity. Fraction *b* is enriched with infected cells with respect to the feed mixture. There are also isolated parasites which have not been phagocyted by cells. The set of pictures shown is only one example of more than one hundred analysed.

4. Conclusion

In this work, we were focused in showing the possibility of using a secondary enrichment process of macrophages infected with *L. amazonensis*. In doing so, we used for the first time the membraneless step-SPLITT fractionation. While primary infections rarely reach more than 70% using the primary infection protocol which is no fully controlled, the secondary enrichment by s-SPLITT fractionation gave concentrations higher than 80% from samples initially composed around 50–50% infected-uninfected macrophages. Results presented here of macrophages after the phagocytosis of silica and latex particles gave us important information about the possibility of using the technique for *L. amazonensis* infected cells, and allowed us to set up the operational parameters for succeeding enrichments. Viability of cells was close to 100% and material lost into the channel was less than 5%. The s-SPLITT technique may become a new quick, cheap and easy way of increasing infection percentages in preparative mode for biotechnological applications.

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