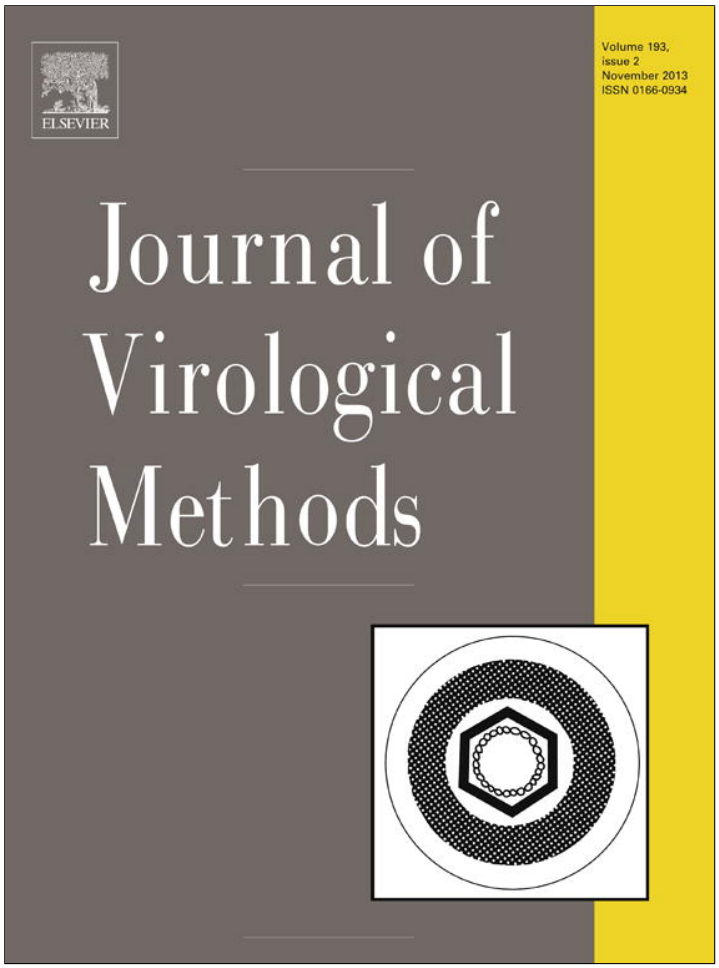

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Optimization of fixed-permeabilized cell monolayers for high throughput micro-neutralizing antibody assays: Application to the zebrafish/viral hemorrhagic septicemia virus (vhsv) model

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A B S T R A C T

A new high throughput centrifugation-free method to estimate viral neutralizing antibody levels in low volumes and large numbers of plasma blood samples is described. Cell monolayers were, (i) plated on poly-D-Lys coated 96-wells, (ii) infected with viruses previously incubated with fish plasma containing antibodies, (iii) fixed with formaldehyde to increase cell recovery and avoid centrifugation steps, (iv) permeabilized with Saponin, (v) immunostained in the presence of Saponin by using a monoclonal antibody (MAb) to viral protein, (vi) digested with trypsin to detach cells from the monolayer, in the absence of Saponin to reduce damage of intracellular MAb-antigen complexes, and (vii) gated by flow cytometry using automatic 96-well batch analysis. The method was applied to the determination of plasma neutralizing antibodies from zebrafish (*Danio rerio*) surviving infections with viral hemorrhagic septicemia virus (VHSV) (an important rhabdovirus of salmonids). This semi-automatic, rapid and practical assay detected anti-VHSV neutralizing antibodies in the plasma (~3 μl per fish) of 95.1% of the zebrafish surviving VHSV infections. The fixed-permeabilized monolayer (FIXPERM) micro-neutralization method might help to analyze sera/plasma from small fish under standarized high throughput conditions.

1. Introduction

There are many methods described to estimate *in vitro* antiviral neutralizing antibody levels by cell cultures. Most of them rely on counting visible plaques of cell lysis (plaque forming units, pfu) after 3–7 days incubation of infected cell monolayers. Overlaid with semisolid media (*i.e.* agarose, methylcellulose, *etc.*) is used to reduce spreading of viral progeny (Hosie et al., 2011; Klasse and Sattentau, 2002; Rainwater-Lovett et al., 2012). Alternatively, viral spreading can be reduced by shorter incubation times, immunostaining and counting focus (focus forming units, ffu) with the aid of an inverted microscope (Chinchilla et al., 2013; Lorenzo et al., 1996). However, those methods counting pfu require larger volumes of sera/plasma than ffu and therefore are not suitable for small fish. Because both pfu and ffu assays require visual counting, theoretically they could be improved by automation of the determination of the numbers of infected cells by flow cytometry.

While a few reports do exist on the use of flow cytometry as an alternative to plaque/focus viral neutralization assays, this method has not been widely used (Kraus et al., 2007; Sashihara et al., 2009). Among other things, the many centrifugation steps required for washing during immunostaining reduce cell recovery and/or increase damage of infected cells. In addition, low sensitivity due to high backgrounds when immunostaining intracellular viral proteins might be a problem depending on the cells, viruses and/or anti-viral antibodies used with each particular assay. On the other hand, to avoid centrifugations some authors have reported methods for cell monolayer fixation and final trypsin digestion to suspend cells before flow cytometry (Grabner et al., 2000). Other authors reported that the best intracellular detection of viral antigens by flow cytometry was obtained by using reversible permeabilization of cellular membranes with Saponin (Gerner et al., 2008). By combining the above two mentioned methodologies (Gerner et al., 2008; Grabner et al., 2000) with fixation to poly-D-Lys coated wells, a high throughput centrifugation-free assay has been developed based on a ffu micro-neutralization method described before (Chinchilla et al., 2013; Lorenzo et al., 1996). The new assay has been applied to zebrafish (*Danio rerio*) and viral hemorrhagic septicemia virus (VHSV, an important rhabdovirus of salmonids) (Encinas et al., 2010; Novoa et al., 2006). The study was

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focused in zebrafish because despite the growing interest in this fish as a disease/immunological model, few studies have addressed their antibody response to viruses. The small amounts of blood that can be harvested from individual zebrafish, the limited access to reagents to detect their antibodies and the scarce number of methods described to estimate viral antibodies in large numbers of samples, increased the difficulties to study zebrafish antibody responses to viruses.

The presence of zebrafish immunoglobulin M (IgM)-dependent neutralizing antibodies in plasma from zebrafish surviving VHSV infections was demonstrated previously by using a micro-neutralization ffu assay (Chinchilla et al., 2013). Micro-neutralization reduced cell culture time and minimized both the use of zebrafish plasma and amounts of reagents. Thus, VHSV-infected ffu of 5–12 cells in 96-wells could be counted under an inverted microscope ~24 h after infection of the cell monolayers (Chinchilla et al., 2013). Although, due to the low amounts of blood this micro-neutralization assay by ffu was more practical to use in zebrafish than any other possible alternative pfu assays (for instance, those using agarose or methylcellulose), the analysis of large numbers of samples still required time-consuming labor-intensive visual counting. Flow cytometry was a possible alternative to speed up the infected cell determinations.

To obtain an maximal differentiation between non-infected and VHSV-infected cells by intracellular immunostaining of VHSV N nucleoprotein, poly-D-Lys plates, fixation and reversible permeabilization of fish monolayers (fixed-permeabilized monolayers, FIXPERM), had to be optimized. By using extracellular trypsin digestion in non-permeabilizing buffers, the immunostained cell monolayers could be then converted to cell suspensions for flow cytometry without altering antigen-antibody complexes. Combination of the FIXPERM method with flow cytometry using 96-well batch analysis allowed for a practical and rapid management of large number of samples (high throughput). Combination of all these methodologies, allowed the determination of anti-VHSV neutralizing antibody levels in low volumes and large numbers of plasma obtained from individual zebrafish surviving VHSV infections. Because zebrafish is susceptible to infections caused by other rhabdoviruses naturally infecting other fish species (LaPatra et al., 2000; Phelan et al., 2005; Sanders et al., 2003), the FIXPERM micro-neutralization assay could be used to follow up any of those rhabdoviral infections. Zebrafish antibodies against other viruses or in any other small fish can be estimated by fine tunning the FIXPERM micro-neutralization method to each case.

2. Material and methods

2.1. Virus and fish cell culture

The viral hemorrhagic septicemia virus (VHSV) strain 07.71 (VHSV-07.71) isolated in France from rainbow trout *Oncorhynchus mykiss* (LeBerre et al., 1977) was replicated in monolayers of EPC cells, from the fathead minnow fish (*Pimephales promelas*), obtained from the American Type cell Culture (ATCC) collection catalog CRL-2872 (Manassas, Virginia, USA). EPC cells were grown at 28 °C with an atmosphere of 5% CO_2 in RPMI Dutch modified cell culture medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum (FCS), 1 mM piruvate, 2 mM glutamine, 50 μg/ml gentamicin and 2.5 μg/ml fungizone (all obtained from Sigma, St. Louis, MO, USA). To prepare VHSV for *in vivo* challenges or to infect EPC cell monolayers, 2% FCS, 10 mM Tris pH 8.0 and no CO_2 atmosphere were employed. To assay for VHSV infectivity, cleared supernatants from VHSV-infected EPC cell monolayers were centrifuged at 60,000 × *g* for 180 min at 4 °C, and pellets resuspended in 50 μl aliquots in cell culture medium and frozen at −70 °C until used (Chinchilla et al., 2013).

2.2. Zebrafish maintenance, immunization and harvest of plasma

Adult "extra-large" (XL) zebrafish weighting 700–900 mg (~4 cm in length) were obtained from a local pet shop (Aquarium Madrid, Madrid, Spain). They were maintained at 24–26 °C in 30 L aquaria provided with biological filters, and fed with a commercial diet.

To obtain zebrafish surviving three consecutive VHSV infections, a previously described protocol was followed (Chinchilla et al., 2013). During VHSV-induced mortalities, fish were monitored 2–4 times daily and those with external hemorrhages killed by an overdosage of methanesulfonate 3-aminobenzoic acid ethyl ester (MS-222, Sigma). Animals were handled in accordance with the National and European guidelines and regulations on laboratory animals care. Animal work was approved by the Ethic Committee of the Instituto Nacional de Investigaciones Agrarias (authorization CEEA 2011/022).

Two months after the last VHSV challenge, the zebrafish were anesthetized prior to handling by immersion in 90 mg of MS-222 per liter. Anesthetized zebrafish were bled by cutting the final end of their tails. Blood was collected in 200 μl of sterilized anticoagulant media (0.64 g sodium citrate, 0.15 g EDTA, 0.9 g sodium chloride per 100 ml of water). The diluted individual blood samples were immediately centrifuged at 1000 × *g* for 3 min to obtain the supernatant plasma. Protein content of plasma was evaluated by nanodrop absorbance at 280 nm. Plasma was de-complemented to avoid individual complement interferences with the neutralization assay by heating to 45 °C for 30 min and kept frozen at −20 °C until used (Chinchilla et al., 2013).

2.3. Optimization of recovery of EPC cells in suspension from fixed-permeabilized monolayers (FIXPERM) by trypsin digestion

EPC cells (50,000 cells per well) were plated in poly-D-Lys 96-well plates (Corning, New York, NY, USA) to form the cell monolayers with increased adherence to the solid-phase and incubated overnight at 28 °C. Monolayers were then fixed and permeabilized during 20 min by using one of these treatments: (i) Methanol at −20 °C; (ii) 50% Methanol in phosphate buffered saline (PBS); (iii) 0.1% Glutaraldehyde in PBS; (iv) 1% Glutaraldehyde in PBS; (v) 0.5% Cytofix in PBS; (vi) Cytofix, 4% formaldehyde (without dilution); (vii) 4% Cytofix and 0.1% Triton X-100; (viii) 4% Triton and 0.1% Tween 20; (ix) 4% Cytofix and 0.05% of Saponin; (x) 10% Formaldehyde and 0.1% Triton X-100 in PBS; (xi) 10% Formaldehyde and 0.1% Tween 20 in PBS and (xii) 10% Formaldehyde and 0.05% of Saponin in PBS. Methanol, Glutaraldehyde, Formaldehyde, Triton X-100 and Tween 20 were obtained from Sigma (St. Louis, MO, USA). Cytofix was obtained from BD-Biosciences Pharmingen (San Diego, CA, USA). Saponin (Quil-A) was obtained from Superfos (Vedbaek, Denmark). Cells were then detached from the monolayers by 5 min digestion with 0.25% trypsin, 0.05 M EDTA in RPMI medium by pipetting up and down until no cells remained in the solid-phase as shown by observation with an inverted microscope. Cells suspensions were then counted and cell recovery calculated by the formula, 100 × number of cells in suspension/50,000.

2.4. Preparation of possible contaminants of zebrafish plasma

Because zebrafish plasma could be contaminated during tail bleeding by mucus and/or hemoglobin, their possible interference with the neutralization assay was assayed. To prepare mucus, 5 zebrafish were consecutively agitated and allowed to move for 1 min into 1 ml of anticoagulant media. The mucus was collected

in the supernatant after centrifugation. To prepare hemoglobin, zebrafish blood was obtained by bleeding 5 fish from the tail into anticoagulant media. The blood was centrifuged at 10,000 × *g* 5 min and the pellet containing the red blood cells was lysed in distilled water with the aid of 2–3 sonication bursts. The hemoglobin containing supernatant was then obtained by centrifugation. The protein content of each of the above mentioned extracts was estimated by nanodrop measurements at 280 nm and adjusted to 2 mg of protein per ml. The extracts were kept at −20 °C until used.

2.5. Estimation of VHSV neutralizing antibodies by using FIXPERM

The procedure was modified from our previous microneutralization assay that used focus forming units (ffu) detected after immunostaining of VHSV-infected cells with diaminobenzidine (DAB) (Sigma) (Chinchilla et al., 2013). EPC cells (50,000 cells per well) were plated and grown overnight at 28 °C in 96-well plates of polystyrene coated with poly-D-Lys (Corning, New York, NY, USA) in 100 μl of cell culture medium to form the monolayers. De-complemented zebrafish plasma 20-fold diluted (∼40 μg of plasma protein per well) were agitated during 2 h and incubated overnight (∼16 h) with 300 ffu of purified VHSV per well in cell culture medium containing 2% FCS, 10 mM Tris pH 8.0 and without CO_2 atmosphere at 14 °C. Cell monolayers were then infected by adding 100 μl of the VHSV-zebrafish plasma mixtures and agitating gently during 2 h at 14 °C. After washing the excess of VHSV by flicking the plate and refilling the wells with cell culture medium containing 2% FCS, 10 mM Tris pH 8.0 and without CO_2 atmosphere, VHSV-infected monolayers were incubated overnight at 14 °C before being fixed with 10% formaldehyde in phosphate buffered saline (PBS) during 20 min.

The fixed monolayers were reversibly permeabilized with 0.05% Saponin, 0.01% N_3Na in PBS during 15 min. To detect the most abundant intracellular nucleoprotein N of VHSV, the monoclonal antibody (MAb) 2C9 (Sanz and Coll, 1992) 500-fold diluted in 2% FCS, 0.05% Saponin, 0.01% N_3Na in PBS (permeabilizing buffer) was added to the wells (100 μl/well) and incubated for 1 h. After washing the cell monolayers with the permeabilizing buffer and manually flicking the plate, 100 μl of rabbit FITC-labeled anti-mouse IgG (Nordic, Tilburg, The Netherlands) was added to each well, and the incubation continued for 30 min. After washing 3 times with the permeabilizing buffer, the monolayers were incubated 5 min with 100 μl of 0.25% trypsin 0.02% EDTA (Sigma, St. Louis, MS, USA). Trypsin digestion was stopped by the addition of 50 μl per well of 1% BSA, 50 mM EDTA, 0.01% N_3Na in PBS. To obtain EPC cell suspensions, the cellular contents of the wells were pipetted up and down several times until most cells were suspended as indicated by observation with an inverted microscope.

The suspended cells were analyzed in a BD FACS Canto II apparatus (Beckton Dickinson, San Agustin de Guadalix, Madrid, Spain) provided with a hightroughput sampler (HTS). Forward (FSC) and side (SSC) scatter threshold values corresponding to damaged cells, cellular debris and/or cellular aggregates (<10% of total events) were eliminated from the analysis by defining an individual cell region. Individual cells were gated for the defined FSC/SSC region and fluorescence. The number of fluorescent cells over a threshold containing 95% (mean + 2 standard deviations) of non-infected EPC cells was then determined by gating a non-infected EPC cell control. An VHSV-infected cell control in the absence of added zebrafish plasma was used to confirm that 40–60% of the cells were infected. After the multiwell autosampler gated 10,000 events per well of the 96-well plates (30–60 min), the automatic batch analysis feature of BD FACSDIVA calculated the percentage of fluorescent (N-positive, VHSV-infected) cells by the formula, 100 × number of cells with fluorescences above the threshold/total number of cells gated per well. The software exported all results to an MS Excel sheet for additional calculations. Linear fitting strength was estimated by calculating the Pearson's correlation coefficient by using the Origin program (OriginPro 8.5.1. SR, Northampton, MA, USA). Comparison of values of percentage of fluorescent cells were performed by using the Student's *t*-test at the $p < 0.05$ level.

3. Results

3.1. Optimization of fixation-permeabilization of monolayers of EPC cells

To differentiate non-infected from VHSV-infected cells by using flow cytometry analysis, the cells need to be in suspension rather than in monolayer. However, preliminary analysis showed that intracellular immunostaining of cells in suspension required numerous washing/centrifugation steps that diminished their recovery, were time-consuming and increased handling errors when processing a large number of samples. To substitute centrifugation steps by "in situ" washing of monolayers, cell adhesion to the polystyrene cell culture surfaces need to be increased. To increase their adhesion, cells were plated, grown, and fixed to poly-D-Lys coated plates. Because antibodies need to get through the fixed cellular membranes to stain intracellular VHSV N nucleoprotein, continuous permeabilization was required during the immunostaining procedure. However, to suspend the cells by trypsin before flow cytometry, cells need to be non-permeable to minimize digestion of intracellular antibody-N complexes. Therefore, different methods to balance conditions of fixation/permeabilization/trypsin-digestion of poly-D-Lys-plated EPC cell monolayers were tested first to search for the best recovery of cells in suspension.

Fig. 1 shows that the highest recovery of EPC cells in suspension obtained after trypsin digestion with different fixing and permeabilizing agents was ∼80%. It was obtained with 10% formaldehyde and 0.05% Saponin. Lower but still significant cell recoveries could be obtained by using Cytofix or Formaldehyde fixation with either Tween 20 or Saponin as permeabilizing agents. In contrast, by using Methanol, Glutaraldehyde and Cytofix or Formaldehyde with Triton X-100, cell recoveries were much lower (10–20%). When using Methanol as fixative/permeabilizer, no cells remained in the solid-phase due to their lysis, as confirmed with the inverted microscope. In contrast, when using Glutaraldehyde, no cells could be detached with trypsin since most of the cell monolayer remained intact as confirmed with the inverted microscope. Therefore, Cytofix and Formaldehyde were selected as potential fixing agents while Saponin was selected as the permeabilizing agent for further experiments.

3.2. Immunostaining of the VHSV N nucleoprotein in fixed-permeabilized monolayers (FIXPERM) of EPC cells

In preliminary experiments, 100 ffu of VHSV per 50,000 EPC cells were used for infection since that multiplicity yielded optimal numbers of ffu per well for counting with the microscope (Chinchilla et al., 2013). The high titer anti-N MAb 2C9 was selected for immunostaining since it showed lower backgrounds than other MAbs or polyclonal antibodies raised in rabbits (data not shown). However, under those infections conditions, when cells were fixed-permeabilized with Cytofix and immunostained in the absence of permeabilization, high backgrounds were obtained after flow cytometry, as shown by the small differences between non-infected (white histogram) and VHSV-infected (gray histogram) cells (Fig. 2A). Those preliminary results suggested that some of the antibodies used for the immunostaining procedure remained intracellular despite the washing steps. In contrast, experiments

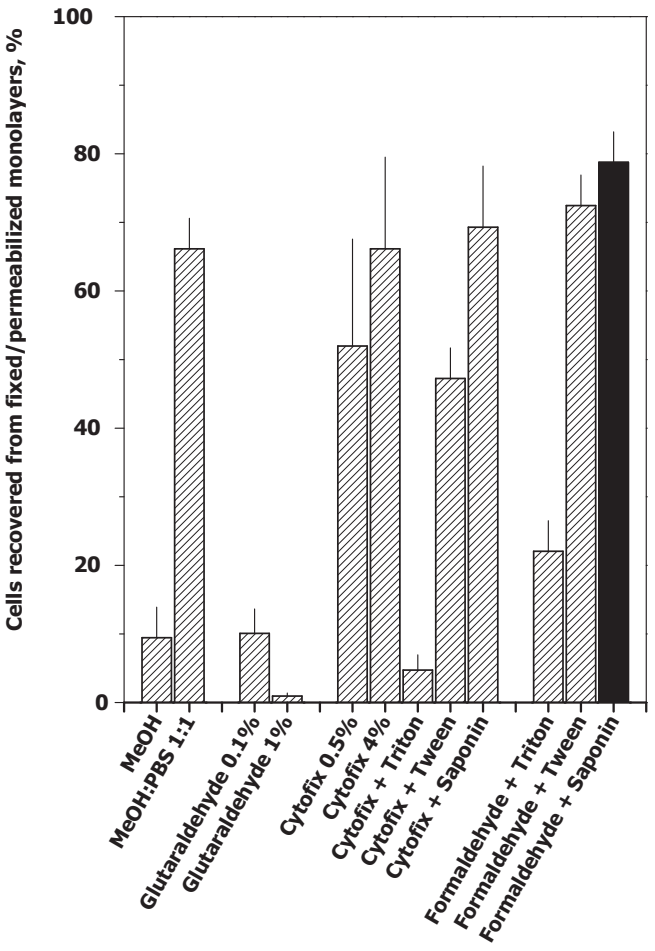

Fig. 1. Recovery of cells in suspension from fixed-permeabilized monolayers by trypsin digestion. EPC cells were plated in poly-D-Lys 96-well solid-phase wells (50,000 cells per well) at 28 °C. After one day, cell monolayers were fixed and permeabilized by different treatments. Cells were then detached by digestion with 0.25% trypsin, 0.02% EDTA and pipetting up and down until no cells remained in the solid-phase. Cells in suspension were then counted and recovery calculated by the formula, 100 × number of cells/50,000. (i) Methanol, −20 °C. (ii) Methanol:PBS 1:1, 50% methanol in phosphate buffered saline (PBS). (iii) Glutaraldehyde, 0.1% in PBS. (iv) Glutaraldehyde, 1% in PBS. (v) Cytofix, 0.5% in PBS. (vi) Cytofix, 4% (without dilution). (vii) Cytofix + Triton, 4% Cytofix and 0.1% Triton X-100. (viii) Cytofix + Tween, 4% Cytofix and 0.1% Tween 20. (ix) Cytofix + Saponin, 4% Cytofix and 0.05% of Saponin. (x) Formaldehyde + Triton, 10% Formaldehyde and 0.1% Triton X-100 in PBS. (xi) Formaldehyde + Tween, 10% Formaldehyde and 0.1% Tween 20 in PBS. (xii) Formaldehyde + Saponin, 10% formaldehyde and 0.05% of Saponin in PBS. Black bar, treatment showing the highest cell recovery.

using cell monolayers fixed with 10% Formaldehyde and then permeabilized with 0.05% of Saponin, and immunostained in the continuous presence of Saponin, increased the differences of mean fluorescences between non-infected and VHSV-infected cells to ~1–2 logs (not shown). When the multiplicity of infection was increased 3-fold, a separation of ~2–3 logs between peak fluorescences of non-infected and VHSV-infected cells were obtained ($n = 17$ experiments) (Fig. 2B). The recoveries of cells in suspension after VHSV infection, immunostaining, trypsin digestion and flow cytometry with respect to the original number of plated cells were of 35.4 ± 6.7% ($n = 95$). Most of the recovered cells remained intact as shown by the corresponding FSC/SSC dot plots (Fig. 2C). Fig. 3 schematizes the final protocol for the FIXPERM micro-neutralization assay.

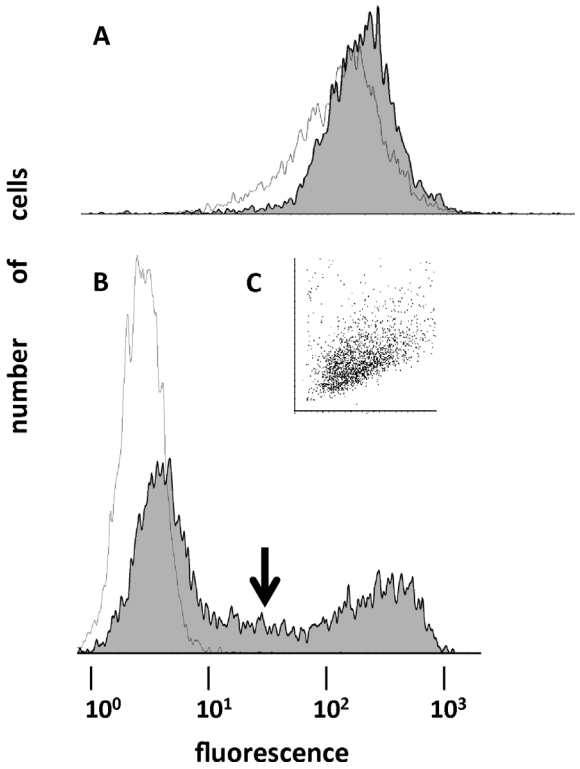

Fig. 2. Flow cytometry of non-infected and VHSV-infected EPC cells fixed-permeabilized and immunostained with anti-VHSV N monoclonal antibody. VHSV was added to EPC cell monolayers and incubated overnight. VHSV infection was stopped by fixation/permeabilization with Cytofix (A) or with 10% formaldehyde and then 0.05% Saponin (B). EPC cells were immunostained with anti-VHSV N nucleoprotein and FITC labeled anti-mouse IgG by using the cells in the absence of permeabilization (A) or in the continuous presence of Saponin (B). Fluorescence profiles of non-infected (white histogram) and VHSV-infected (gray histogram) EPC cells. (C insert) dot plot of forward/side (FSC/SSC) scattering of the EPC cells from assay B. Vertical arrow, threshold between non-infected and VHSV-infected EPC cells calculated by the mean of fluorescences in non-infected cells (white histogram) plus 2 standard deviations to contain 95% of the cells.

3.3. Correlation between focus forming unit (ffu) and fixed-permeabilized monolayer (FIXPERM) assays

A dose-response curve showing linear correlation with a Pearson's coefficient of $r = 0.99$ was obtained between the percentage of fluorescent cells determined by FIXPERM assays and DAB-stained ffu determined by ffu assays at different concentrations of VHSV (Fig. 4A). Thus, by infecting 50,000 cells with 300 ffu of VHSV per well, 40–60% of the cells were fluorescent (fluorescence values above the threshold) in different experiments ($n = 8$). By increasing VHSV to >700 ffu per well, >90% of cells were fluorescent and ffu was outside the range of counting (Fig. 4A). According to these data, 200–500 ffu of VHSV per 50,000 cells was the optimal range required to carried out the FIXPERM micro-neutralization assays.

3.4. Determination of neutralizing antibodies in plasma from zebrafish surviving VHSV infection

To assess for blood neutralizing antibodies, zebrafish surviving three consecutive VHSV infections were individually bleed by cutting their tails into anticoagulant medium. By using this method, a concentration of 2.6 ± 1.2 mg of protein per ml ($n = 64$) of diluted plasma was obtained. Because zebrafish plasma obtained by bleeding from their tails could be contaminated by mucus from the skin and/or hemoglobin from exceptional red blood cell haemolysis, zebrafish mucus and hemoglobin were obtained and assayed for their possible interference with the FIXPERM micro-neutralization

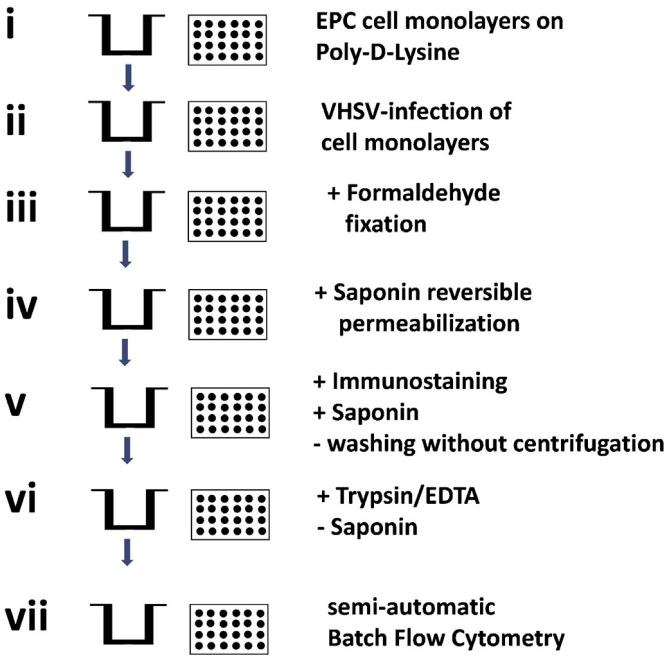

Fig. 3. Scheme of the final design of the FIXPERM method. The steps were: (i) EPC cell monolayers were obtained by plating 50,000 EPC cells per well in 96-well plates coated with poly-D-Lys. (ii) The monolayers were incubated overnight with 300 ffu per well of purified VHSV after previous overnight incubation of VHSV with plasma from VHSV immunized zebrafish. (iii) The monolayers were fixed to the poly-D-Lys solid-phase with formaldehyde. (iv) The monolayers were permeabilized with Saponin. (v) The intracellular immunostaining of the N nucleoprotein of VHSV was performed in the presence of Saponin and washing the monolayers by manually flicking the plates. (vi) The cells were suspended from the fixed-stained cell monolayers by digestion with trypsin-EDTA in the absence of Saponin. (vii) The suspended cells were gated with the hightroughput sampler (HTS) of the BD FACS Canto II and analyzed by the Batch Analysis feature (BD FACSDIVA software).

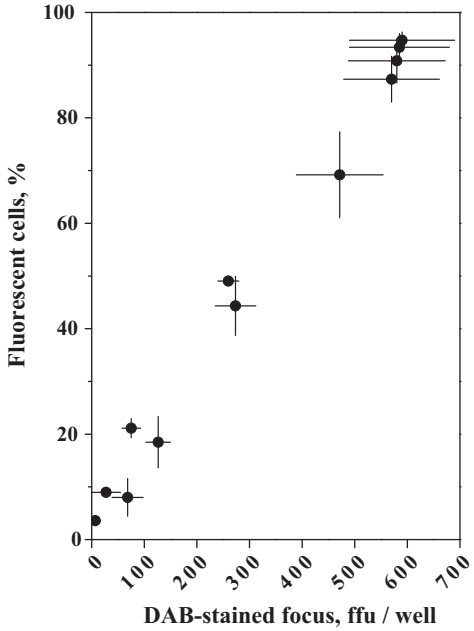

Fig. 4. Relationship between micro-neutralization assays counting ffu (DAB-stained cells) and and FIXPERM (fluorescent cells) at different VHSV dosages. Plasma samples were incubated with VHSV and the mixtures assayed by the focus forming unit (ffu) (Chinchilla et al., 2013; Lorenzo et al., 1996) and by the FIXPERM micro-neutralization assays. The ffu were estimated by DAB-staining and their numbers per well counted with the aid of an inverted microscope ($n = 4$). The percentage of fluorescent (VHSV-infected) EPC cells after flow cytometry were calculated by the formula, number of fluorescent EPC cells in the presence of plasma/number of cells gated. Means and standard deviations ($n = 4$) were represented.

assay. However, nor mucus nor hemoglobin showed any interference with the assay at 40 μg of protein per well, the maximal plasma protein concentration used for the assay (data not shown).

Results obtained from different FIXPERM micro-neutralization assays of plasma samples selected in a wide range of percentage of fluorescent cells, showed a linear correlation of duplicate determinations with a Pearson's correlation coefficient of $r = 0.92$ (Fig. 5A). Fig. 5B shows the distribution profiles of the number of individual zebrafish plasma in function of their percentage of fluorescent cells

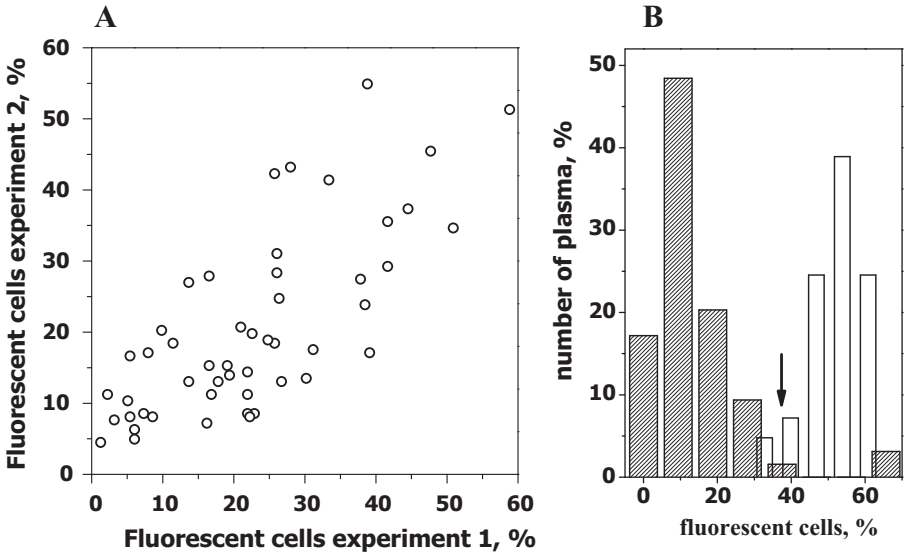

Fig. 5. Distribution of the numbers of zebrafish plasma from non-infected or survivors of VHSV infections assayed by the FIXPERM micro-neutralization assay. (A) Comparison of percentage of fluorescent cells from 57 selected plasma samples from non- infected and survivor zebrafish assayed in two different experiments. Each point corresponds to an individual zebrafish. (B) Comparison of distribution of plasma frequencies of percentage of fluorescent cells from non-infected zebrafish (white bars, $n = 167$) and from zebrafish surviving VHSV infections (hatched bars, $n = 64$). Vertical arrow, threshold of values significantly lower than the mean percentage of fluorescent cells from plasma from non-infected zebrafish at the $p < 0.05$ level (Student's t-test).

from non-infected (hatched bars) and from survivors (white bars) of VHSV infections. The average percentage of infected cells obtained from plasma from non-infected zebrafish were 51.8 ± 7.3% (n = 167) while those from VHSV survivors were 13.0 ± 10.6% (n = 64). Up to 95.1% of the VHSV survivors had significatively higher neutralizing antibodies in their plasma than non-infected zebrafish at the $p < 0.05$ level (Student's t-test) (Fig. 5B).

4. Discussion

A micro-neutralization assay to estimate antibodies to viruses has been developed by using fixed-permeabilized monolayer (FIXPERM) cells for high-throughput flow cytometry analysis. The FIXPERM micro-neutralization assay was used to estimate viral neutralizing antibodies levels in plasma from zebrafish (*Danio rerio*) surviving VHSV infections. Neutralization activity could be detected in the low amounts of blood that could be obtained from individual zebrafish by tail bleeding (corresponding to ~3 μl of blood per zebrafish). The procedure was applied to a large number of individual samples, being capable of reading the results in 30–60 min per 96-well plate, only one day after infection of the cell monolayers.

Due to the absence of any well characterized zebrafish IgM reagents, the detection of VHSV-neutralizing activity is a first approach to study and demonstrate their antibody responses to viruses (Chinchilla et al., 2013). Therefore, the development of a high throughput assay to detect neutralizing antibody levels in zebrafish would increase the use of this fish as an infectious disease model. By using this method, neutralization of VHSV could be demonstrated in 95.1% of plasma from survivors of VHSV infections. In contrast, only 40–50% of the sera from rainbow trout (*O. mykiss*) surviving a VHSV infection showed anti-VHSV neutralizing antibodies by pfu assays (Olesen and Jorgensen, 1986).

For fixing cell monolayers other authors used formaldehyde at concentrations of 0.5% (Grabner et al., 2000) or 3% (Gerner et al., 2008). In the present method, a higher formaldehyde concentration (10%) and poly-D-Lys coated plates were used to increase cell adherence. Increased adherence minimized cell losses during subsequent immunostaining/washing steps allowing the use of a small number of cells in 96-well plates for flow cytometry. To obtain around 1 log difference between non-infected and infected cells, published flow cytometry methods to detect intracellular viruses employed Triton X-100 to permeabilize fixed cells (Kao et al., 2001). However, 2–3 log differences could be obtained in this work by using Saponin to permeabilize fixed cells, confirming that Saponin was the best permeabilizing agent (Gerner et al., 2008). Further to the reports mentioned above, other described methods for instance those using green fluorescent protein recombinant viruses to facilitate neutralization assays, required centrifugation steps for immunostaining before flow cytometry too and could benefit from the FIXPERM method (Earl et al., 2003).

Therefore, among the advantages of the FIXPERM micro-neutralization assay are the possibilities to: (i) carry out all immunostaining steps for multiple samples in 96-well plates minimizing the use of blood plasma and reagents, (ii) wash reagents during immunostaining without centrifugation steps, (iii) perform viral infections overnight, (iv) analyze fluorescent cells semi automatically, and (v) output data automatically to an Excel spread sheet ready for additional calculations. In conclusion, fixed-permeabilized monolayers (Gerner et al., 2008; Grabner et al., 2000) of cells were combined with micro-incubation of viral and host antibodies (Chinchilla et al., 2013; Lorenzo et al., 1996) and centrifugation-free immunostaining, to obtain semi automatic processing of large number of samples to estimate neutralizing antibodies by multiwell flow cytometry.

The FIXPERM micro-neutralization assay may be useful to assay for antibodies to other viruses. However, because no particular fixation/permeabilization protocol is ideal for all types of cell monolayers and/or viruses, some optimization for detection of the main protein antigen for each particular case might be required.

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