

# Fluorescent *Eimeria bovis* sporozoites and meront stages in vitro: a helpful tool to study parasite–host cell interactions

Carlos Hermosilla · Ivonne Stamm · Anja Taubert · Kathleen Lutz · Horst Zahner · Christian Menge

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**Abstract** A fluorescence-based technique was established to trace intracellular sporozoites of *Eimeria bovis* for tests on gliding motility, invasion, replication and quantification of infection rates in cultured bovine umbilical vein endothelial cells (BUVEC) by laser scanning confocal microscopy and flow cytometry (FCM) analyses. Employing the fluorescent dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), we determined its effects on sporozoites at various concentrations and duration of staining. More than 98% of sporozoites were labelled with the dye at a concentration of 2.5  $\mu\text{M}$ . Staining was predominantly found in refractile bodies and presumptive micronemes. Upon infection of BUVEC, CFSE-labelled sporozoites developed into fluorescent immature macromeronts, which were traceable inside the cells until 22 days postinfection (p. i.). Consistent with a peripheral localisation of the fluorescence signal in macromeronts merozoites released from these lacked detectable fluorescence. As example of use, a multicolour FCM approach for the simultaneous determination of *E. bovis* infection and host cell surface molecule expression was established. The approach proved suitable to quantify major histocompatibility complex (MHC-I) and MHC-II expression, thereby clearly distinguishing between infected and uninfected BUVEC up to day 14 p. i. In conclusion, CFSE labelling of *E. bovis* sporozoites facilitates monitoring of intracellular

stages in vitro and will be a highly useful tool for studying host cell responses towards parasite invasion.

## Introduction

Reliable methods for tracing intracellular parasites in vivo and in vitro are of great interest in experimental biology concerning *Eimeria* spp. Attempts for stable transfections with fluorescent marker proteins used for other Apicomplexa, such as *Plasmodium* spp. (VanWye and Haldar 1997; Natarajan et al. 2001), *Toxoplasma gondii* (Bradley et al. 2004; Gubbels et al. 2004) or *Sarcocystis neurona* (Gaji et al. 2006), have failed in *Eimeria*. As an alternative, staining techniques have not been developed to a standard level so far. Fuller and McDougald (2003) employed cell membrane labelling using a fluorescent dye (PKH-67 GL) for tracking *Eimeria tenella* sporozoites in vitro. Stained parasites indeed invaded host cells. However, the authors were unable to unveil whether the staining persisted in dividing parasites. As another option, membrane-permeant reactive tracers, such as 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) seemed promising. CFSE is intracellularly converted into a green-fluorescent molecule by esterases and binds covalently to amino groups (McCabe et al. 2002; Spadaro et al. 2007). This staining was recently shown to be useful for tracing *Cryptosporidium* spp. sporozoites in vitro (Feng et al. 2006).

In the current work, we tested CFSE staining of *Eimeria bovis* sporozoites with respect to the parasites viability, invasive and developmental capabilities in primary cultured bovine umbilical vein endothelial cells (BUVEC). Additionally, we tested CFSE-labelled sporozoites for gliding motility, a prerequisite for the cell invasion process of apicomplexan parasites (Soldati et al. 2004; Wetzel et al.

C. Hermosilla (✉) · A. Taubert · K. Lutz · H. Zahner  
Institute of Parasitology, Justus Liebig University Giessen,  
Rudolf-Buchheim-Str. 2,  
35392 Giessen, Germany  
e-mail: Carlos.R.Hermosilla@vetmed.uni-giessen.de

I. Stamm · C. Menge  
Institute of Hygiene and Infectious Diseases of Animals,  
Justus Liebig University Giessen,  
Giessen, Germany

2005). CFSE-labelled sporozoites infect BUVEC and develop into fluorescent macromeronts (22 days postinfection (p. i.)). The technique allows the quantitation of infection rates by flow cytometry (FCM) analyses and permits many other options for studying parasite–host cell interactions. To demonstrate the usefulness of this technique, we established a multicolour FCM assay for the separate evaluation of major histocompatibility complex (MHC) expression on infected and uninfected cells in heterogeneous cultures.

## Materials and methods

### Parasites

*E. bovis* strain H was maintained by passages in Holstein–Friesian calves (Fiege et al. 1992). Oocysts were isolated from faeces and sporulated according to the method of Jackson (1964). Sporozoites were excysted from sporulated oocysts and purified as previously described (Hermosilla et al. 2002). After excystation, sporozoites were washed three times in phosphate-buffered saline (PBS), resuspended in endothelial cell growth medium (ECGM; PromoCell) and counted in a Neubauer haemocytometre.

### Host cells

Bovine umbilical vein endothelial cells (BUVEC) were isolated according to Taubert et al. (2006) and cultured in modified ECGM [0.4% endothelial growth supplement–heparin, 0.1 ng epidermal growth factor per millilitre, 1 ng basic fibroblast growth factor per millilitre, 1 µg hydrocortisone per millilitre (all PromoCell), 50 µg gentamicin–amphotericin B solution per millilitre (Sigma), 2% fetal calf serum (FCS; Gibco)]. Cells were seeded into 25 cm<sup>2</sup> tissue culture flasks (Nunc) and incubated at 37°C in 5% CO<sub>2</sub> atmosphere. Cells were released from the tissue culture flasks by trypsinisation (37°C, 5 min) for further analyses. In the case of laser scanning confocal microscopy (LSCM), analyses cells were seeded on glass coverslips located in six-well culture plates (Nunc) and cultured (37°C, 5% CO<sub>2</sub> atmosphere) until confluent.

### Labelling of *E. bovis* sporozoites

Freshly excysted sporozoites were suspended in ECGM without FCS at a concentration of 1×10<sup>6</sup> sporozoites per millilitre and pulsed with various concentrations of CFSE (0.075 µM, 0.75 µM, 2.5 µM, 7.5 µM, Invitrogen) prediluted in dimethyl sulfoxide (Merck). Sporozoites were suspended in the dye by gently shaking and incubated for 5 min, 1, 2 and 3 h (37°C, 5% CO<sub>2</sub> atmosphere). In order to stop the labelling process, an equal volume of ECGM with

10% FCS was added and CFSE-labelled sporozoites (sporozoites<sup>CFSE</sup>) were washed four times (400×g, 10 min) in ECGM, resuspended in ECGM plus FCS and protected from light before use.

### Demonstration of gliding motility of *E. bovis* sporozoites<sup>CFSE</sup>

Sporozoites<sup>CFSE</sup> were resuspended in ECGM plus FCS, dropped (20 µl) on glass slides and allowed to move on the glass slides for 1 h (37°C, 5% CO<sub>2</sub>). After fixation (4% [w/v] paraformaldehyde [Merck], 10 min, dark), slides were washed three times in PBS, mounted in Mowiol (Sigma) and stored in the dark at 4°C until use. The gliding trails of sporozoites<sup>CFSE</sup> were visualised using LSCM (TCS SP2 Leica) equipped with an argon laser. Laser excitation wave lengths were 496 nm.

### Host cell infection

For the FCM analyses, confluent BUVEC monolayers grown in 25 cm<sup>2</sup> tissue culture flasks (Nunc) were infected with 2.5×10<sup>5</sup>, 7.5×10<sup>5</sup> and 1.5×10<sup>6</sup> sporozoites<sup>CFSE</sup> or unstained sporozoites. For LSCM analyses, 1×10<sup>4</sup> sporozoites<sup>CFSE</sup> were used to infect BUVEC monolayers grown on glass coverslips in six-well culture plates (Nunc). Culture medium was changed 24 h after infection and thereafter every third day.

To determine whether CFSE staining influenced the infectivity of sporozoites, confluent BUVEC isolates (25 cm<sup>2</sup> culture flasks) were infected with 2.5×10<sup>5</sup> labelled or unlabelled sporozoites. To determine infection rates, intracellular sporozoites were counted microscopically in five randomly selected vision fields at 3 h p. i.

### *E. bovis* sporozoites<sup>CFSE</sup> development in vitro

To control the long-term persistence of CFSE labelling, sporozoites<sup>CFSE</sup>-infected BUVEC were analysed on days 1, 3, 8, 14 and 22 p. i. For this purpose, infected BUVEC grown on coverslips were fixed (4% [w/v] paraformaldehyde [Merck], 10 min, dark), washed three times in PBS, mounted in Mowiol (Sigma) and stored in the dark at 4°C until use. For fluorescence detection by LSCM, a microscope (TCS SP2, Leica) and a phase-contrast videomicroscope (DMIRB, Leica) equipped with argon lasers were used. The laser excitation wave lengths were 488 nm.

### MHC expression on BUVEC infected with *E. bovis* sporozoites<sup>CFSE</sup>

The MHC-I and MHC-II expression on sporozoites<sup>CFSE</sup>-infected and uninfected control BUVEC was determined on days 1, 3, 8 and 14 p. i. by a multicolour FCM approach.

*E. bovis*-infected and noninfected BUVEC monolayers were trypsinised and washed in PBS (400×g, 10 min, 4°C). For immunolabelling, anti-bovine MHC-I (mouse immunoglobulin G2a (IgG2a), clone IL-A88) and anti-bovine MHC-II (mouse IgG1; clone J-11; both hybridoma cell lines kindly provided by J. Naessens, International Livestock Research Institute, Nairobi, Kenya) monoclonal antibodies, biotin-conjugated anti-mouse goat IgG1 and anti-mouse goat IgG2a antibodies (BD Biosciences) and allophycocyanin (APC)-conjugated streptavidin (BD Biosciences) were employed. Immunolabelling was performed according to Menge et al. (2001). Briefly, cell pellets were resuspended in 50-µl PBS as negative control or with 50-µl supernatant of the hybridoma cell lines (MHC-I and MHC-II), incubated for 30 min on ice, subsequently washed once with PBS and incubated (30 min on ice) with biotin-conjugated anti-mouse goat IgG1 (1:800) or with biotin-conjugated anti-mouse goat IgG2a (1:400) as appropriate and with APC-conjugated streptavidin (1:40, 30 min on ice). Thereafter, cells were washed twice with PBS and submitted to FCM analyses.

#### FCM analyses

A FACSCalibur (BD Biosciences) equipped with an argon laser and a standard filter setting was used. Laser excitation wave lengths were 488 nm for the green fluorescence (CFSE) and 635 nm for the red fluorescence (APC). In each sample, 10,000 events were analysed for fluorescent signals utilising the Cell Quest Pro software (BD Biosciences). Subsequent data processing was performed with the FCS express V2 software (DeNovo Software).

## Results

#### CFSE labelling of *E. bovis* sporozoites

Incubation of sporozoites with 7.5-µM CFSE for 5 min resulted in a bright fluorescence of >99% of the parasites. Increasing the incubation time led to increased fluorescence intensity as demonstrated by FCM analyses (data not shown). By using 0.75 µM, a prolonged incubation period was necessary: >95% of the sporozoites were stained only after 1 h. Staining of sporozoites with 0.075-µM CFSE for up to 3 h resulted in 7% stained sporozoites. In general, CFSE staining led to a strong homogenous fluorescence of the sporozoites. By fluorescence microscopy (Figs. 1a, 2b and 3a,b), the refractile bodies showed the strongest fluorescence and a more weakly stained cytoplasm interspaced with more intensely stained granule-like structures in the apical region of the parasite (Fig. 1a), probably reflecting the micronemes. The *E. bovis* sporozoites were

labelled for 5 min with 2.5-µM CFSE in the subsequent experiments.

*E. bovis* sporozoites<sup>CFSE</sup> show gliding motility and release fluorescent material

Sporozoites<sup>CFSE</sup> showed typical movements on glass slides and released fluorescent gliding trails (Fig. 1e). Occasionally, sporozoites<sup>CFSE</sup> shedding fluorescent material from the apical tip were found (Fig. 1f). Thus, proteins involved in the gliding process of sporozoites, such as microneme proteins were obviously stained by CFSE as well.

When determined by light microscopy, at 3 h p. i., 9.6±1.2% (mean±SD of *n*=3 experiments) of exposed BUVEC were infected with sporozoites<sup>CFSE</sup>. Control infections with untreated sporozoites resulted in slightly enhanced infection rates of 12.8±0.8% (mean±SD of *n*=3 experiments) but differences were not significant (Fig. 3c).

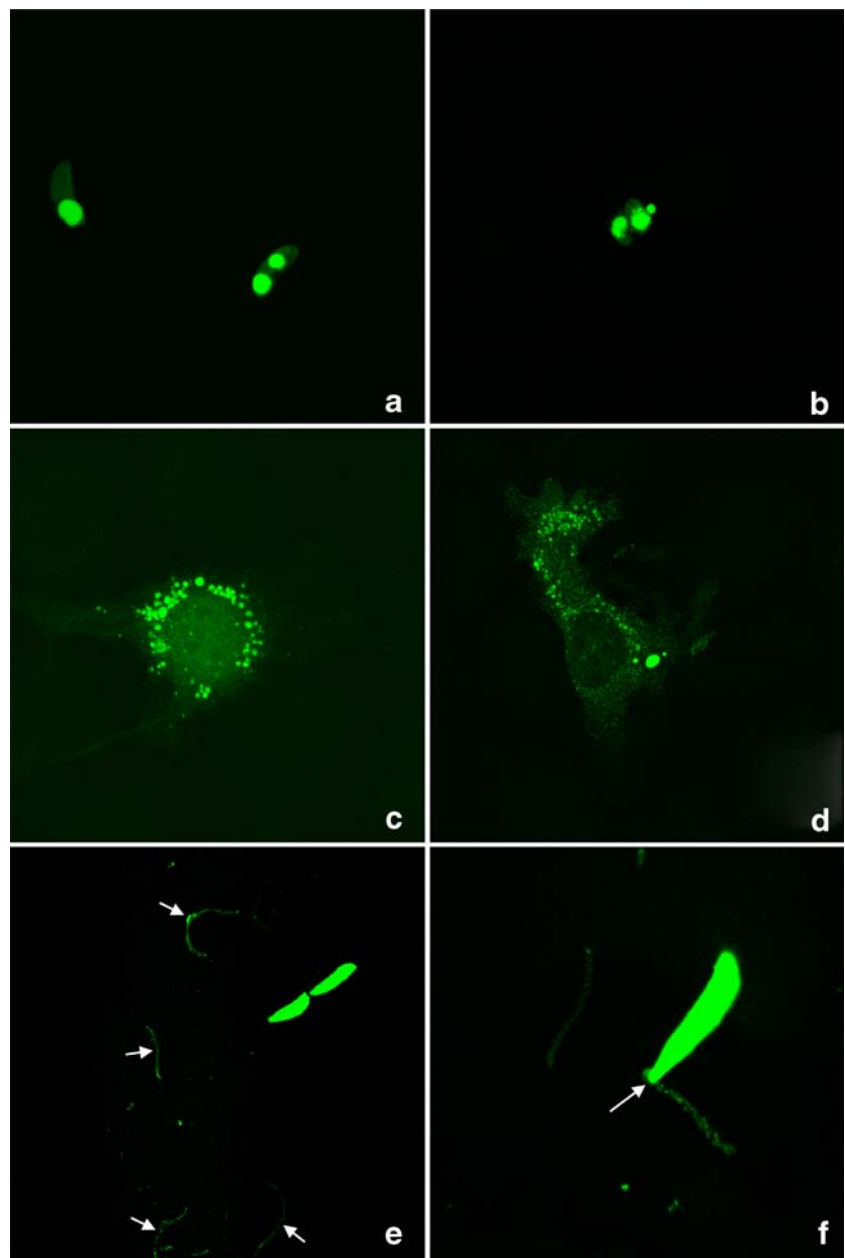
*E. bovis* sporozoites<sup>CFSE</sup> develop into fluorescent trophozoite and meront stages

Intracellular sporozoites<sup>CFSE</sup> displayed a strong green fluorescence of the refractile bodies and a weak staining of the cytoplasm as illustrated in Fig. 1a. In general, the anterior refractile body had disappeared and only the posterior had remained. On day 3 p. i., intracellular sporozoites<sup>CFSE</sup> were shortened and had rounded up into trophozoite stages (Fig. 1b). Most of the remaining refractile bodies still showed a bright fluorescence. In addition, fluorescing material was found in the cytoplasm of the parasite (Fig. 1b). As also holds true for non-labelled sporozoites, the majority of sporozoites<sup>CFSE</sup> remained as intracellular sporozoite or trophozoite stages, but some sporozoites<sup>CFSE</sup> initiated asexual replication resulting in bright fluorescent immature *E. bovis* meronts on day 8 p. i. (Fig. 1c). These immature meronts showed a marked fluorescence, visible as green dots, probably due to the dispersion of the refractile bodies. Fluorescent *E. bovis* macromeronts were detectable on day 14 p. i. (Fig. 1d) and showed fluorescing granules mainly concentrated at the periphery. First-generation *E. bovis* merozoites were released from mature macromeronts 22 days p. i. and onwards (Fig. 2a). Free merozoites, however, lacked LSCM-detectable fluorescence although the staining persisted in the macromeronts (see Fig. 2b).

Analysis of *E. bovis* sporozoites<sup>CFSE</sup>, infected and noninfected BUVEC cells by FCM

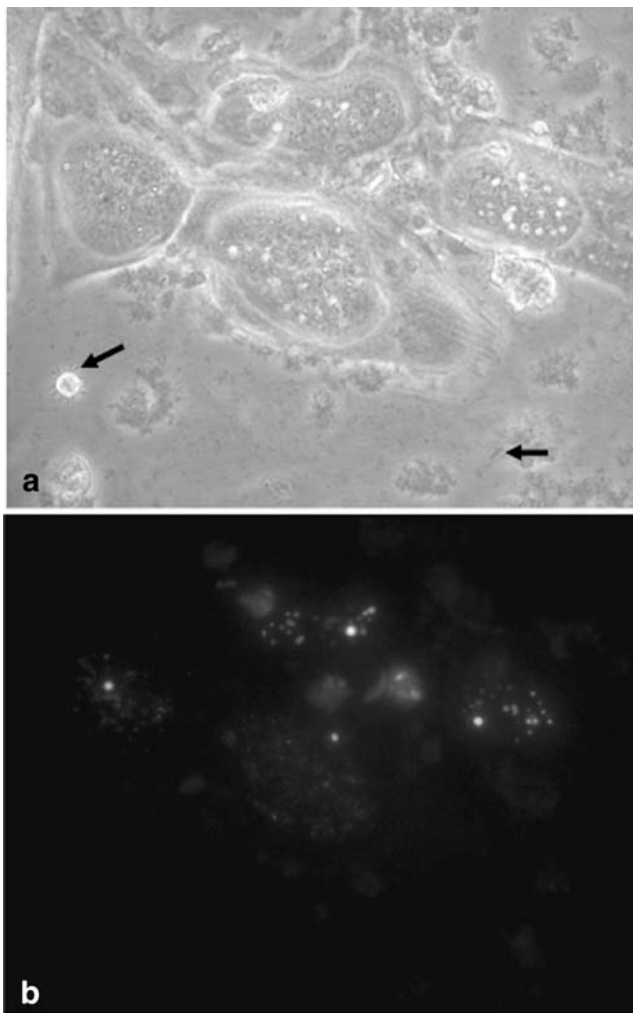
In order to assess whether sporozoite<sup>CFSE</sup>-infected BUVEC can be distinguished from noninfected cells within the same culture by FCM, BUVEC monolayers were infected with *E.*

**Fig. 1** CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* stages (sporozoites, trophozoites, meronts) and fluorescent gliding trails detected by confocal laser scanning microscopy. BUVEC were grown to confluence and infected with CFSE-labelled sporozoites ( $2.5 \times 10^5/\text{flask}$ ). Infected BUVEC were investigated on 1, 3, 8 and 14 days p. i. CFSE-labelled sporozoites were dropped on glass coverslips and analysed after 1-h incubation ( $37^\circ\text{C}$ ;  $5\% \text{CO}_2$ ). **a** intracellular stained sporozoites with fluorescent refractile bodies and cytosol at 1 day p. i., **b** intracellular stained trophozoites with fluorescent refractile bodies and cytoplasm at 3 days p.i., **c** fluorescent immature meront at 8 days p. i., **d** fluorescent macromeront at 14 days p. i. (**a to d**  $\times 600$  magnification), **e** extracellular stained sporozoites and fluorescent gliding trails (*arrows*;  $\times 400$  magnification), **f** stained extracellular sporozoite shedding fluorescent material from the apical part and fluorescent trails (*arrow*;  $\times 640$  magnification)



*bovis* sporozoites<sup>CFSE</sup> overnight and subsequently detached by trypsinisation. The expression of MHC-I was used as a marker for bovine cells. Immunodetection of MHC-I molecules was performed and the resulting suspension was submitted to FCM analyses (“BUVEC sporozoites<sup>CFSE</sup>” in Fig. 4). For controls, suspensions of sporozoites<sup>CFSE</sup> cultured in the absence of BUVEC and of BUVEC without sporozoites<sup>CFSE</sup> (see “sporozoites<sup>CFSE</sup>” and “BUVEC”, respectively, in Fig. 4) were analysed. Plotting of the forward scatter height signal (corresponding to the size of the particles) versus the side scatter height signal (corresponding to the granularity) allowed the definition of three regions. Region one (R1) was devoid of events when a sporozoite suspension was analysed but was found to

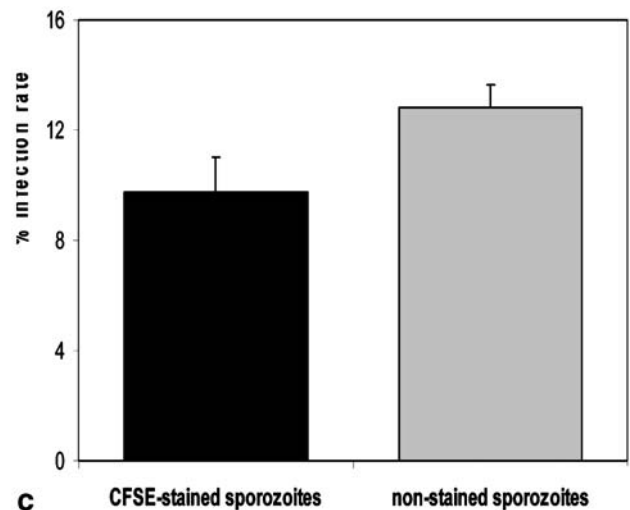
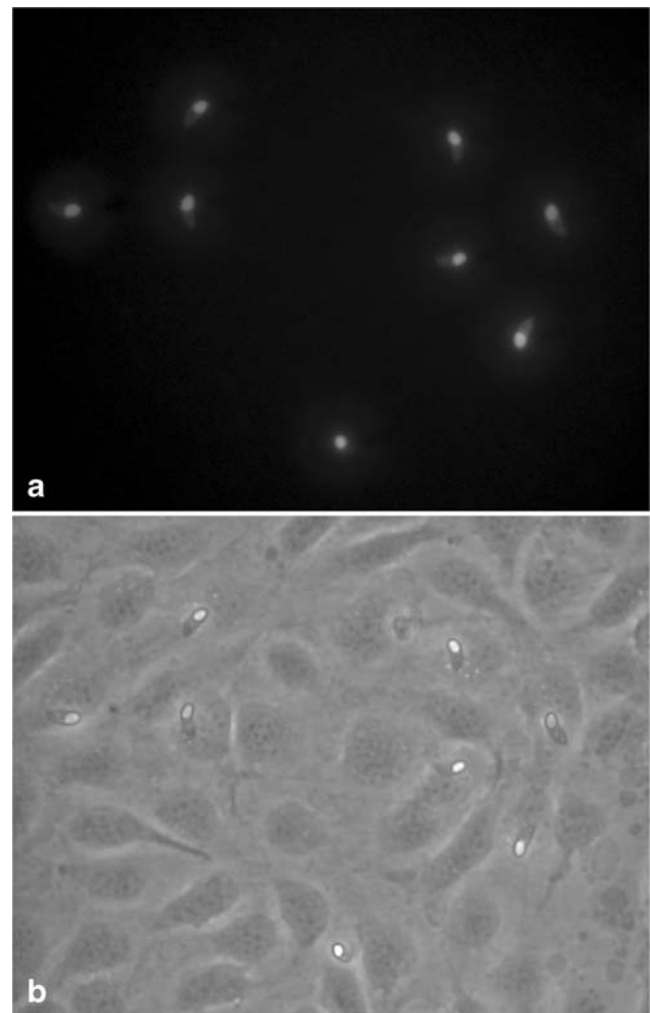
contain events almost all of which being MHC-I-positive when suspensions derived from noninfected or infected BUVEC cultures were analysed. Analysis of a noninfected BUVEC culture resulted in 98.87% MHC-I-positive events in R1 that lacked a specific CFSE (fluorescence 1 height (FL1-H)) signal. In turn, 3.02% of the events in R1 were CFSE (FL1-H)/MHC-I-APC (FL4-H) double-positive when a suspension derived of sporozoite<sup>CFSE</sup>-infected BUVEC cultures were analysed. These staining patterns of the events in R1 are consistent with the interpretation of R1 just comprising viable endothelial cells that were or were not sporozoite-infected. Sporozoite<sup>CFSE</sup>-infected endothelial host cells could be clearly distinguished from uninfected cells by their significantly different CFSE (FL1-H) signal.



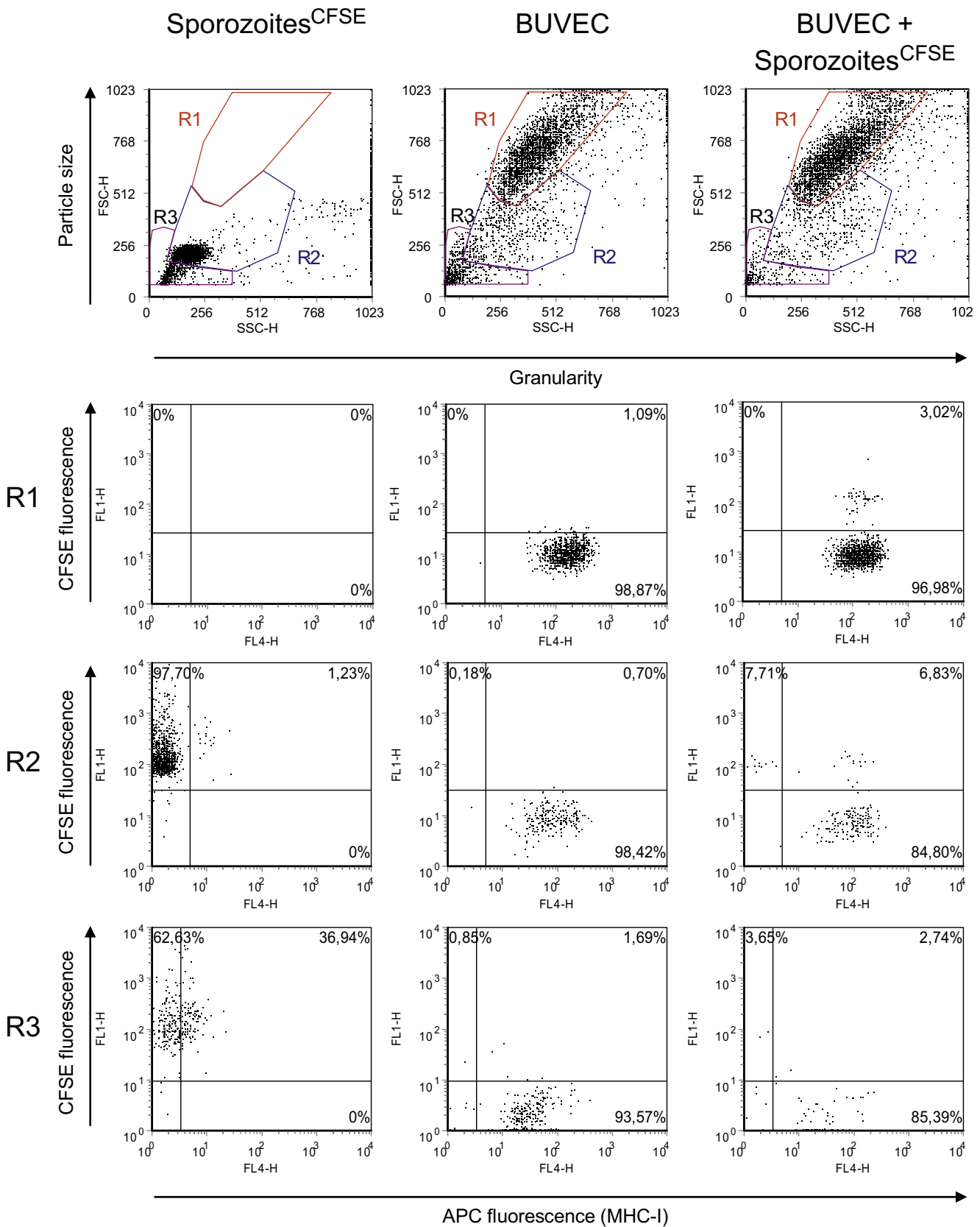
**Fig. 2** CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* macromeronts and free released merozoites. BUVEC were grown to confluence and infected with CFSE-labelled sporozoites ( $2.5 \times 10^5/\text{flask}$ ). Infected BUVEC were investigated at 22 days p. i. **a** four macromeronts and free, released merozoites (arrows) detected by phase-contrast microscopy, **b** the same CFSE-labelled macromeronts detected by fluorescence microscopy ( $\times 400$  magnification)

Sporozoites that were not cell-associated were not included in R1 because CFSE-positive events lacking a MHC-I-APC signal could not be detected in R1 during the analysis of neither suspension.

The vast majority of cell-free sporozoites<sup>CFSE</sup> could be found in region two (R2) when analysing a sporozoite<sup>CFSE</sup> suspension. Accordingly, cell-free sporozoites<sup>CFSE</sup> appeared in R2 together with uninfected and infected endothelial host cells when a suspension of an infected BUVEC culture was submitted to FCM analysis. Compared to R1, however, the endothelial cells in R2 displayed a reduced particle size consistent with morphological alterations occurring during cellular death (Menge et al. 2001). Region three (R3) finally defined very small events that, according



**Fig. 3** Intracellular CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* sporozoites. BUVEC were grown to confluence, infected with CFSE-labelled sporozoites ( $2.5 \times 10^5/\text{flask}$ ) and investigated 3 h p. i.; **a** intracellular fluorescent sporozoites detected by fluorescence microscopy, **b** by phase-contrast microscopy ( $\times 200$  magnification); **c** comparison of microscopically determined infection rates using CFSE-stained and non-stained sporozoites (arithmetic means of infection rates in three different BUVEC isolates and standard deviations)



**Fig. 4** Morphological and fluorescence patterns of CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* sporozoites (left column), uninfected BUVEC (middle column) and BUVEC infected with CFSE-labelled *E. bovis* sporozoites (right column) detected by flow cytometry analyses. BUVEC were detached by trypsinisation and submitted to FCM analysis. Relative sizes (*FSC-H*), granularities (*SSC-H*), *FL1-H* fluorescences and *FL4-H* fluorescences of 10,000 particles (i.e. sporozoites, BUVEC cells and debris) are recorded. Fluorescence intensities are displayed separately for events defined by different regions (*R1*, *R2*, *R3*) set according to the *FSC-H* and *SSC-H* signals. Numbers refer to the percentages of events in the respective region that displayed a specific fluorescence signal (above the threshold indicated by the quadrants) for *FL1-H* only, for *FL4-H* only and for both *FL1-H* and *FL4-H*, respectively

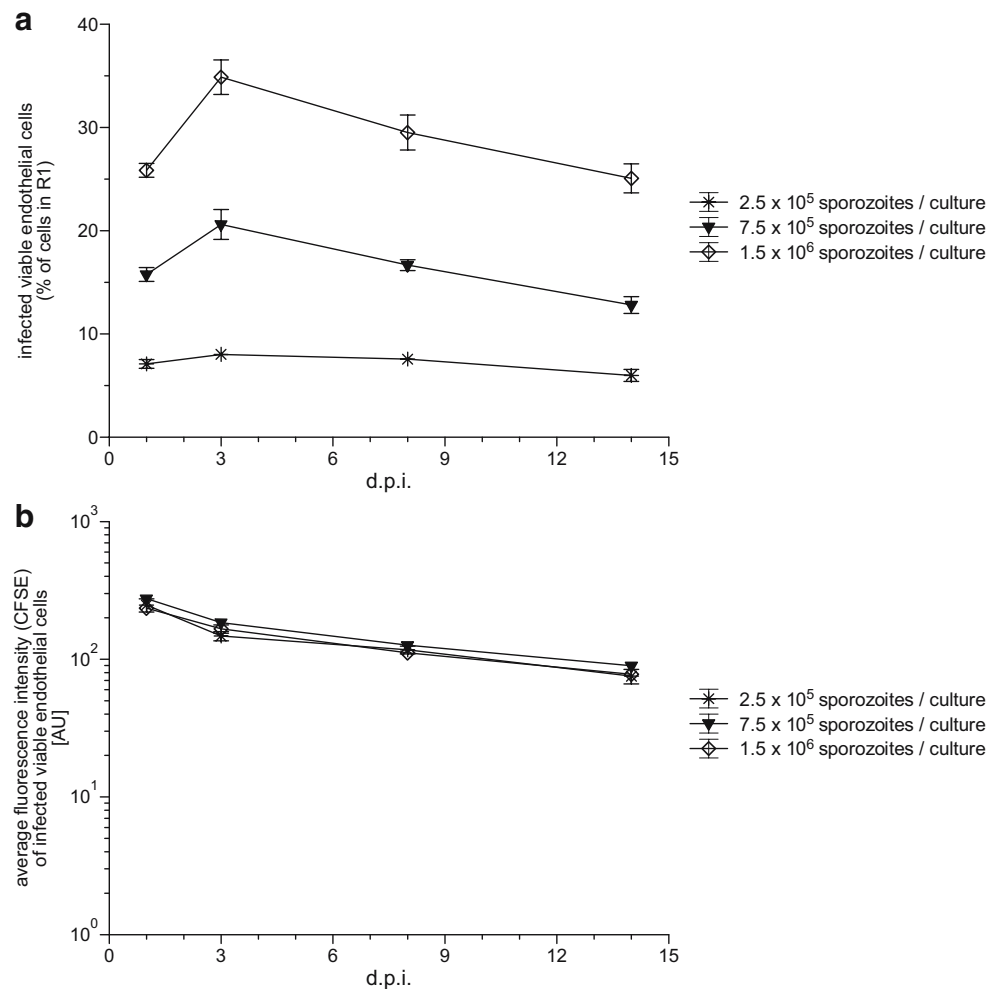
to the staining pattern, represented cellular debris derived from sporozoites and/or endothelial cells.

Application of FCM to qualitatively and quantitatively monitor the infection process of *E. bovis* sporozoites<sup>CFSE</sup> in BUVEC

Analysis of events in *R1* was applied to monitor BUVEC cultures infected with various doses of sporozoites<sup>CFSE</sup> for

up to 14 days of incubation. The percentage of infected viable BUVEC (i. e. percentage of CFSE-positive, MHC-I-APC-positive events of all events located in *R1*) was utilised as a read-out to quantify the *E. bovis* infection rates in BUVEC at different infection doses. A clear distinction between infected and uninfected cells was possible throughout the entire observation period because the CFSE fluorescence intensity of infected cells at day 14 p. i. was found to be reduced by only approximately threefold compared to cells analysed at day 1 p. i. (Fig. 5b). Quantifying by means of FCM analysis the percentage of infected BUVEC, a positive correlation between the sporozoite<sup>CFSE</sup> infection dose and the number of infected host cells was observed (Fig. 5a). Lowest infection rates of approximately 7% were obtained at day 1 p. i. with  $2.5 \times 10^5$  sporozoites per approximately  $1.5\text{--}1.6 \times 10^6$  cells per 25-cm<sup>2</sup> culture flask. Infection with  $7.5 \times 10^5$  sporozoites<sup>CFSE</sup> led to approximately 16% and with  $1.5 \times 10^6$  sporozoites<sup>CFSE</sup> to 26% infected endothelial cells at day 1 p. i.. While at the lowest infection dose, the infection process appears to have been completed within the first 24 h p. i.; the percentage of infected cells raised to 20% and 35% in cultures infected

**Fig. 5** Kinetics of **a** the percentage of viable endothelial cells (as defined by region *R1* in Fig. 4) infected with CFSE-labelled *Eimeria bovis* and **b** the average intensity of the CFSE signal of infected cells. At day 0, BUVEC cultures (containing approximately  $2 \times 10^6$  cells) were infected with different numbers of CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* sporozoites as indicated. Data are depicted as mean $\pm$ SD of sixfold determinations from one out of two independent experiments with similar results. Missing error bars are within symbols



with  $7.5 \times 10^5$  and  $1.5 \times 10^6$  sporozoites<sup>CFSE</sup>, respectively, at day 3 p. i. However, the percentage of infected, viable endothelial cells returned to the levels observed at day 1 p. i. in either case by the end of the observation period. This phenomenon might be explained by numerous sporozoites leaving host cells after inadequate infection trails (using high infection doses we often observe multiple infected host cells that may not permit development of all sporozoites) and being rinsed off by the feeding of the BUVEC cultures.

#### Application of FCM to quantify MHC expression on BUVEC infected with *E. bovis* sporozoites<sup>CFSE</sup>

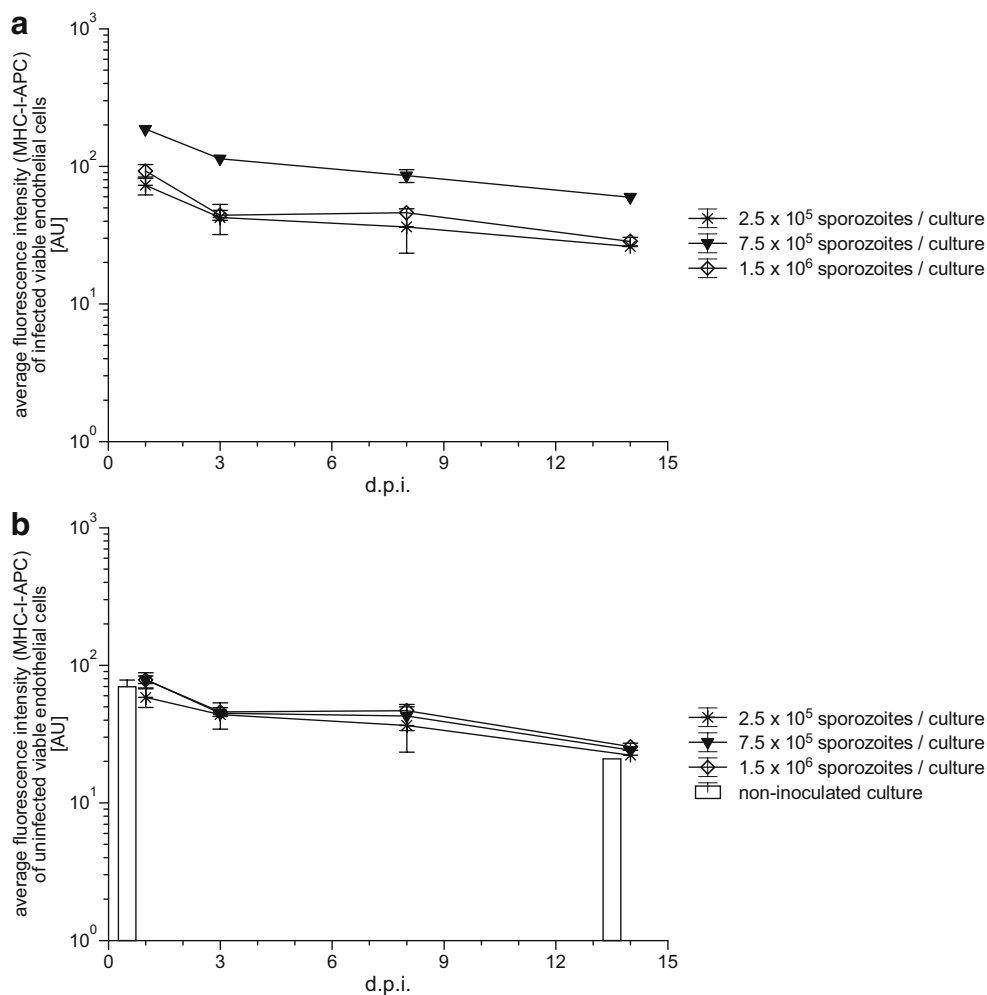
The multicolour FCM approach allowed for the simultaneous quantitation of MHC-I molecule expression on infected and uninfected BUVEC over the entire observation period. Irrespective of the number of sporozoites used to infect the BUVEC cultures, the average number of MHC-I molecules on the surface of uninfected cells, as assessed by the mean APC fluorescence intensity, was similar to the expression by cells from non-inoculated cultures (Fig. 6b). Even though the percentage of endothelial cells expressing MHC-I remained

above 95% throughout, the average expression level per cell somewhat declined over time. Interestingly, the MHC-I expression by infected BUVEC (Fig. 6a) was found to be higher as compared to uninfected cells within cultures that were inoculated with  $7.5 \times 10^5$  sporozoites<sup>CFSE</sup>. The same difference in expression levels also occurred with regard to MHC-II molecules (expressed by approximately 90% of the cells throughout; data not shown). Even though the relative increase in MHC expression by infected BUVEC at intermediate infection doses was less apparent in the repetition of the experiment, this observation indicates the potential of the method to independently quantify the expression of surface molecules on infected and uninfected cells in the same cultures.

#### Discussion

Here, we describe a simple and sensitive fluorescent-based approach to trace and quantitate *E. bovis* infection in cultured BUVEC monolayers. *E. bovis* sporozoites were easily and quickly stained with CFSE and maintained their

**Fig. 6** Kinetics of the average fluorescence intensity for the detection of MHC-I antigens (labeled with APC, detected by FL4-H) on the surface of BUVEC in cultures infected with CFSE-labelled ( $2.5 \mu\text{M}$ ) *Eimeria bovis* sporozoites cultures: **a** *E. bovis*-infected BUVEC, **b** BUVEC that remained uninfected, i.e. cells exhibiting a CFSE signal not exceeding autofluorescence. At day 0, cultures were inoculated with different numbers of CFSE-labelled *E. bovis* sporozoites as indicated. Cultures not inoculated were used as controls (“non-inoculated culture”). Data are depicted as mean $\pm$ SD of triplicate determinations from one out of two independent experiments with similar results. Missing error bars are within symbols





gliding motility, infectivity and developmental capacity. The CFSE fluorescence within *E. bovis* sporozoites was segregated upon first merogony, resulting in bright fluorescent macromeronts until 22 days p. i.. The fluorescence in stained sporozoites and trophozoites was particularly intensive in the refractile bodies and structures in the apical area, possibly micronemes. In maturing and mature macromeronts, the staining was concentrated to small spherical peripheral bodies. Refractile bodies are prominent organelles in sporozoites of Eimeriidae and proteome analysis confirmed that these organelles are reservoirs for proteins probably involved in host cell invasion (de Venevelles et al. 2006). During merogony, refractile bodies of *Eimeria* spp. diminish in size by either releasing small amounts of refractile material or by forming several smaller spherical bodies (Doran 1982; Danforth and Augustine 1989), which could be an explanation for the staining patterns observed in fluorescent *E. bovis* macromeronts.

The predominant staining of refractile body components might also explain the lack of fluorescence in merozoites as these organelles are restricted to sporozoites and trophozoites. Considering the extreme replication rate in macromeronts, the binding of CFSE to other sporozoite molecules is obviously insufficient to be detected in the offspring or has been discharged prior to cell invasion as demonstrated by fluorescent gliding tracks. The latter observation supports the above interpretation that CFSE-stained structures in the apical area of sporozoites represent micronemes as microneme proteins are released onto the host cell surface to promote adhesion of the parasites (Soldati et al. 2004). The reason for a preferential labelling of particular organelles with CFSE is unknown. It has not been observed when *Cryptosporidium* spp. sporozoites were stained (Feng et al. 2006). The dye binds to aminomoiety (McCabe et al. 2002) and therefore should predominantly react with basic proteins, particularly to those with many moieties, e. g. histones, hyaluronates, glycosaminoglycans or amino sugars (Suniara et al. 2000; Spadaro et al. 2007). However, recent proteomic studies on refractile bodies of *E. tenella* (de Venevelles et al. 2006) rather suggest a predominance of acidic proteins than basic ones and to our knowledge other studies on the composition of refractile bodies are not available.

BUVEC infected with sporozoites<sup>CFSE</sup> became brightly fluorescent and could easily be distinguished from uninfected cells by FCM analysis. Corresponding results were reported with CFSE-stained *Cryptosporidium parvum* and *Cryptosporidium hominis* sporozoites (Feng et al. 2006). Also in accordance with our results, the authors demonstrated that CFSE-stained *C. parvum* sporozoites discharge fluorescent material during gliding motility.

Moreover, CFSE has successfully been used to investigate drug effects against stained parasites, e. g. the proliferation of CFSE-stained *Leishmania infantum* promastigotes

after exposure to allopurinol (Kamau et al. 2000) and dinitroaniline compounds by FCM analyses (Kamau et al. 2001), indicating the potential applicability of CFSE in pharmacological screenings.

In a study using the fluorescent dye PKH-67 GL for labelling sporozoites of *E. tenella* (Fuller and McDougald 2003), infectivity of parasites was not affected, but no further development into meronts was observed. PKH-67-GL-labelled sporozoites did not lose fluorescence intensity after prolonged storage at 4°C, but stained parasites were neither exposed to higher temperatures nor longer period of time as it was the case in the current study (37°C, 22 days p. i.). Even the incubation of *E. bovis* sporozoites<sup>CFSE</sup> at 60°C for 30 min did not affect their labelling intensity (data not shown).

Alternative techniques to visualise *Eimeria*, e.g. antibody-based immunoassays, have some limitations (Augustin and Danforth 1984; Danforth and Augustine 1989), such as the requirement of specific antibodies, fixation and permeabilisation techniques to allow access of antibodies to parasitic antigens. Whereas stable transformation of parasites requires laborious experiments and is often unsuccessful due to sexual replication of the parasite, the CFSE procedure offers a simple and useful alternative method. CFSE shows low cytotoxicity and a slow loss of fluorescence. The covalent bonds formed by the dye prevent transfer of dye to host cell cytoplasm. These advantages are of obvious benefit for the study of any other *Eimeria* species in ruminants replicating in long terms (e. g. *Eimeria zuernii*, *Eimeria auburnensis*, *Eimeria bakuensis*, *Eimeria ashata*, *Eimeria arloingi*, *Eimeria ninakohlyakimovae*).

The simultaneous detection of MHC-I or MHC-II expression on BUVEC infected with sporozoites<sup>CFSE</sup> showed the applicability of this staining technique for phenotypical studies of single host cells. In contrast to the active downregulation of MHC molecules, as described for *T.-gondii*-infected host cells (Lüder et al. 1998, 2001, 2003) and for *Leishmania-amazonensis*-infected murine macrophages (Courret et al. 2001), data collected here from *E.-bovis*-infected BUVEC indicated, if at all, a slight increase in MHC-I expression when compared to controls, and this held true for the whole course of first merogony in vitro. However, this effect was exclusively observed in the case of  $7.5 \times 10^5$  sporozoites per flask but not in the highest dosage. At the moment, we do not have a plausible explanation for this phenomenon and further investigation using this tool will be needed to dissect these reactions. Nonetheless, a possible explanation may be a reciprocal effect caused by overdosage or the fact that increasing infection doses do not result in a proportional increase of infection rates owing to multiple infection of host cells.

In principle, *E. bovis* sporozoites might use other evasion strategies than the active downregulation of MHC expression. One possible mechanism might be the diminishment of the recruitment of leukocytes to the site of *E. bovis* infection. In this context, we could show suppressive effects of *E. bovis* sporozoites on tumour-necrosis-factor- $\alpha$ -mediated polymorphonuclear leukocyte adhesion (Hermosilla et al. 2006) and lower activation of infected BUVEC when compared to *T. gondii* or *Neospora caninum* infections (Taubert et al. 2006).

Taken together with the present work, we showed that *E. bovis* sporozoites<sup>CFSE</sup> offer a simple tool to investigate parasite–host cell interactions. CFSE facilitated visualisation and separation of single infected host cells by FCM and LSCM. Infection rates could be easily determined. Therefore, the CFSE labelling technique could be helpful not only for phenotypical studies of *Eimeria*-spp.-infected host cells but also for immunobiological investigations and pharmacological screening studies.

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## References

- Augustin PC, Danforth HD (1984) Use of monoclonal antibodies to locate *Eimeria* sporozoites (Protozoa) in intestinal sections. *Proc Helminthol Soc Wash* 51:361–362
- Bradley PJ, Li N, Boothroyd JC (2004) A GFP-based motif-trap reveals a novel mechanism of targeting for the *Toxoplasma* ROP4 protein. *Mol Biochem Parasitol* 137(1):111–120
- Courret N, Frehel C, Prina E, Lang T, Antoine JC (2001) Kinetics of the intracellular differentiation of *Leishmania amazonensis* and internalization of host MHC molecules by the intermediate parasite stages. *Parasitol* 122(3):263–279
- Danforth HD, Augustine PC (1989) *Eimeria tenella*: use of a monoclonal antibody in determining the intracellular fate of the refractile body organelles and the effect on *in vitro* development. *Exp Parasitol* 68:1–7
- De Venevelles P, Francois Chich J, Faigle W, Lombard B, Loew D, Pery P, Labbe M (2006) Study of proteins associated with the *Eimeria tenella* refractile body by a proteomic approach. *Int J Parasitol* 36:1399–1407
- Doran DJ (1982) Behavior of coccidia *in vitro*. In: Long PL (ed) *The biology of the coccidia*. University Park Press, Baltimore, pp 229–285
- Feng H, Nie W, Bonilla R, Widmer G, Sheoran A, Tzipori S (2006) Quantitative tracking of *Cryptosporidium* infection in cell culture with CFSE. *J Parasitol* 92(6):1350–1354
- Fiege N, Klatte D, Kollmann D, Zahner H, Bürger HJ (1992) *Eimeria bovis* in cattle: colostral transfer of antibodies and immune response to experimental infections. *Parasitol Res* 78:32–38
- Fuller AL, McDougald LR (2003) Cell membrane labelling of *Eimeria tenella* sporozoites with the fluorescent dye PKH-67 GL for tracking parasite–host interactions. *Parasitol Res* 87:521–525
- Gaji RY, Zhang D, Breathnach CC, Vaishnav S, Striepen B, Howe DK (2006) Molecular genetic transfection of the coccidian parasite *Sarcocystis neurona*. *Mol Biochem Parasitol* 150:1–9
- Gubbels MJ, Wieffer M, Striepen B (2004) Fluorescent protein tagging in *Toxoplasma gondii*: identification of a novel inner membrane complex component conserved among Apicomplexa. *Mol Biochem Parasitol* 137(1):99–110
- Hermosilla C, Barbisch B, Heise A, Kowalik S, Zahner H (2002) Development of *Eimeria bovis in vitro*: suitability of several bovine, human and porcine endothelial cells lines, bovine fetal gastrointestinal, Madin–Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. *Parasitol Res* 88:301–307
- Hermosilla C, Zahner H, Taubert A (2006) *Eimeria bovis* modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. *Int J Parasitol* 36:423–431
- Jackson ARB (1964) The isolation of viable coccidial sporozoites. *Parasitology* 54:87–93
- Kamau SW, Hurtado M, Muller-Doblies U, Grimm F, Nunez R (2000) Flow cytometric assessment of allopurinol susceptibility in *Leishmania infantum* promastigotes. *Cytometry* 40:353–360
- Kamau SW, Nunez R, Grimm F (2001) Flow cytometry analysis of the effect of allopurinol and the dinitroaniline compound (Chloralrin) on the viability and proliferation of *Leishmania infantum* promastigotes. *BMC Pharmacol* 1:1
- Lüder CG, Lang T, Beuerle B, Gross U (1998) Down-regulation of MHC class II molecules and inability to upregulate class I molecules in murine macrophages after infection with *Toxoplasma gondii*. *Clin Exp Immunol* 112:308–316
- Lüder CG, Lang C, Giraldo-Velasquez M, Algner M, Gerdes J, Gross U (2003) *Toxoplasma gondii* inhibits MHC class II expression in neural antigen-presenting cells by down-regulating the class II transactivator CIITA. *J Neuroimmunol* 134(1–2):12–24
- Lüder CG, Walter W, Beuerle B, Maeurer MJ, Gross U (2001) *Toxoplasma gondii* down-regulates MCH class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1 alpha. *Eur J Immunol* 31:1475–1484
- McCabe D, Cukierman T, Gabay JE (2002) Basic residues in azurocidin/HBP contribute to both heparin binding and antimicrobial activity. *J Biol Chem* 277:27477–27488
- Menge C, Stamm I, Wuhler M, Geyer R, Wieler LH, Baljer G (2001) Globotriaosylceramide (Gb3/CD77) is synthesized and surface expressed by bovine lymphocytes upon activation *in vitro*. *Vet Immunol Immunopathol* 83:19–36
- Natarajan R, Thathy V, Mota MM, Hafalla JCR, Ménard R, Vernick KD (2001) Fluorescent *Plasmodium berghei* sporozoites and pre-erythrocytic stages: a new tool to study mosquito and mammalian host interactions with malaria parasites. *Cell Microbiol* 3(6):371–379
- Soldati D, Foth BJ, Cowman AF (2004) Molecular and functional aspects of parasite invasion. *Trends Parasitol* 20(12):567–574
- Spadaro M, Curcio C, Varadhachary A, Cavallo F, Engelmayr J, Blezinger P, Pericle F, Forni G (2007) Requirement for IFN- $\gamma$ , CD8, T lymphocytes, and NKT cells in talactoferrin-induced inhibition of neu. tumors. *Cancer Res* 67:6425–6432
- Suniara RK, Jenkinson EJ, Owen JJT (2000) An essential role for thymic mesenchyme in early T cell development. *J Exp Med* 191:1051–1056
- Taubert A, Zahner H, Hermosilla C (2006) Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites. *Vet Parasitol* 142:214–222
- VanWye JD, Hardar K (1997) Expression of green fluorescent protein in *Plasmodium falciparum*. *Mol Biochem Parasitol* 87:225–229
- Wetzel DM, Schmidt J, Kuhlenschmidt MS, Dubey JP, Sibley LD (2005) Gliding motility leads to active cellular invasion by *Cryptosporidium parvum* sporozoites. *Infect Immun* 73(9):5379–5387