

Protocols for the Flow Cytometric Analysis of Mycoplasmas

Hazel Davey¹ and Patricia Assunção²

¹Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion, SY23 3DD Wales, UK

²Unidad de Epidemiología y Medicina Preventiva, Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria, ULPGC, Arucas, Spain.

INTRODUCTION

Flow cytometry is a technique whose development has been driven by studies of human, mainly clinically-relevant, cell types. Nonetheless, it has found many applications in the analysis of bacteria (reviewed in Davey and Kell 1996 and Winson and Davey 2000). The small size and correspondingly lower amounts of cellular constituents pushes bacterial cells to the limit of detection of many commercial instruments. However, with on-going developments in the range and brightness of fluorescent stains that are available to the flow cytometrist / microbiologist, experimental possibilities continue to expand.

Amongst the most challenging microbes in the context of microbial flow cytometry are the mycoplasmas. These are particularly difficult organisms to work with as many of them are difficult to grow in culture and those that will grow do so at a much slower rate than our more well known “model (micro)organisms” such as *Escherichia coli* and *Bacillus subtilis*. However, the most challenging aspect from the viewpoint of flow cytometry is the small size of the mycoplasma cell. Whereas “standard bacteria” are 1-2 µm in diameter (or length for non-spherical species), mycoplasmas, in contrast, can be as small as 0.2 µm and are typically ~0.5 µm in diameter. Furthermore, a common target for fluorescent stains is the nucleic acid content of a cell. The genome size of *Mycoplasma* spp ranges from 6×10^5 - 1.35×10^6 base pairs (cf. *E. coli* = 4×10^6 and *H. sapiens* 3×10^9) – see Figure 1. Consequently, the signal from fluorescent DNA probes bound to the DNA of a mycoplasma will be much lower and closer to the lower detection threshold of many commercially-available instruments.

Given these difficulties the obvious question is ‘Why study mycoplasmas?’ Firstly, mycoplasmas cause a range of human diseases, for example *M. pneumoniae* is an important cause of pneumonia and other respiratory disorders and possible roles for a range of mycoplasma species in chronic fatigue, fibromyalgia and Gulf War syndromes have been suggested (Endresen 2003). Secondly mycoplasmas are the cause of considerable economic losses associated with livestock. For example, *M. agalactiae*, *M. mycoides* subsp. *mycoides* large colony-type, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* are the causative agents of contagious agalactia of small ruminants (goats and sheep) which suffer a variety of clinical syndromes including mastitis, arthritis, kerato-conjunctivitis and,

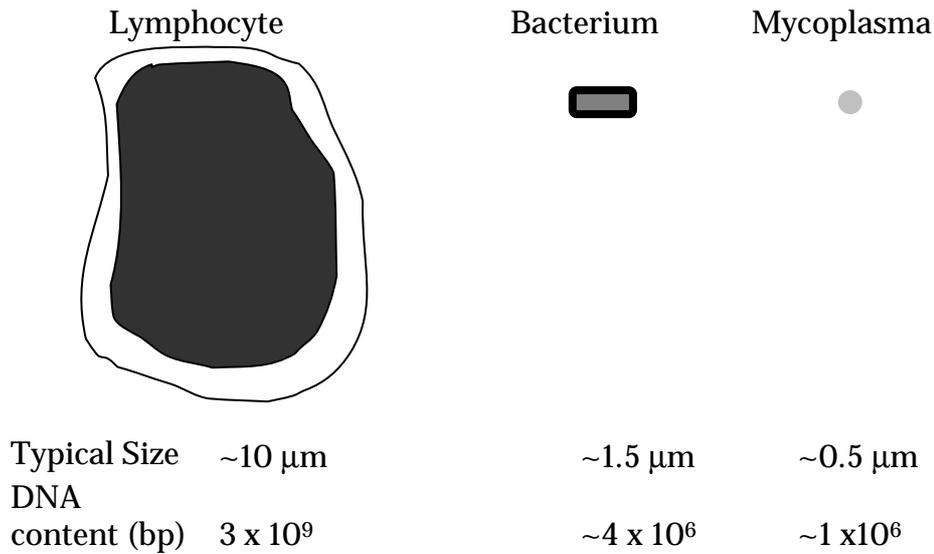


FIGURE 1: Comparison in the size and DNA content of human lymphocyte, bacterial and mycoplasma cell

occasionally, abortion and respiratory disease (Nicholas 1995; Bergonier *et al.* 1997) and *M. hyopneumoniae* is the etiological agent of porcine enzootic pneumonia, a chronic respiratory disease with worldwide distribution (Kobisch and Friis, 1996). Thirdly, and perhaps most relevantly to non-microbial flow cytometrists, mycoplasmas are a common contaminant of cell cultures. The small size of mycoplasmas coupled with their lack of a shape-restricting cell wall, means that they can pass through the filters that are used to sterilise cell culture media.

We have recently developed a number of protocols that permit the flow cytometric analysis of mycoplasmas for the purposes of detection, enumeration and viability determination. These are presented here in the hope that they will be of use to the wider flow cytometry community.

PROTOCOL 1: STAINING OF MYCOPLASMAS FOR DETECTION / ENUMERATION

Although, as described above, the DNA content of mycoplasmas is very small (when compared to more frequent targets of flow cytometric analysis) we have found that the DNA fluorochrome Sybr green-I (SYBR, Amresco, Ohio, USA) gives sufficient fluorescence to distinguish a range of mycoplasma species from background.

Mycoplasma are grown in the laboratory according to their species-specific growth requirements. 10 μl of the mycoplasma culture is then diluted to 1 ml with sterile-filtered saline solution (0.85% NaCl) and stained with SYBR at a final concentration 1:10,000 (vol/vol) of the commercial stock solution. NB - SYBR is obtained from the manufacturer as a stock solution and neither the molecular weight nor the chemical formula are provided.

The samples are then incubated for 15 min at room temperature in the dark to allow the membrane-permeant SYBR to penetrate all of the cells (this occurs in a viability-independent manner).

These samples are amenable to analysis using a Coulter Epics Elite flow cytometer (Beckman-Coulter Ltd, Luton, U.K.) equipped with an air-cooled 488 nm argon-ion laser. We have also successfully performed equivalent analyses using a Coulter Epics XL-MCL but other suitably-equipped instruments with equivalent sensitivity may be appropriate. Fluorescence from SYBR is collected via a 550 nm dichroic filter and a 525 nm band pass filter. Typically, it is also useful to collect side scatter (SSC) and forward angle scatter (FSC) signals. In all cases data should be acquired on a four-decade logarithmic scale using FSC to trigger collection.

Where enumeration of mycoplasmas is required samples can be spiked with beads of a known concentration as reference and the resulting number of cells counted can be converted to cells.ml⁻¹ by reference to the standard.

Results

As shown in Figure 2 below, a 2D dotplot of SYBR fluorescence versus side scatter allows good resolution of the mycoplasma cell population from the background.

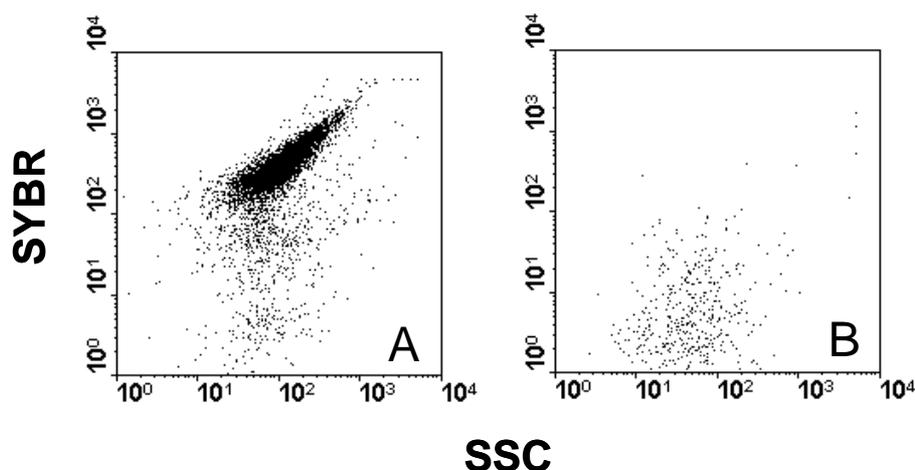


FIGURE 2. Use of flow cytometry to detect *Mycoplasma agalactiae* cells. The cells were stained with SYBR as described above. Dotplots of SYBR vs SSC allow clear resolution of *M. agalactiae* (A) against the background broth medium (B).

PROTOCOL 2: STAINING OF MYCOPLASMAS FOR VIABILITY DETERMINATION

In order to distinguish viable and non-viable cells we have found the most effective method to be combining the use of SYBR with Propidium Iodide (PI). Cells are grown and treated as described above except that PI is added along with the SYBR at a final concentration of 10 µg.ml⁻¹.

The samples are then incubated as described above and whilst the membrane-permeant SYBR penetrates all cells irrespective of their viability, PI is able only to enter cells that have suffered membrane damage – a state that correlates well with cell death in mycoplasmas.

Flow cytometry is performed as described above save for the acquisition of a second fluorescence signal at ~675 nm which corresponds to the emission of PI.

Results

Staining of a live population of mycoplasma cells with a combination of SYBR and PI results in a population with fluorescence values defined by region R1 (Figure 3A). However, heat injury of the cells (60°C for 1 hour) prior to staining shifts the cell population into region R2 (Figure 3B). An artificial mixture of live and heat-treated cells (Figure 3C) shows good separation of the two populations between regions R1 and R2. The broth medium background is shown in (D).

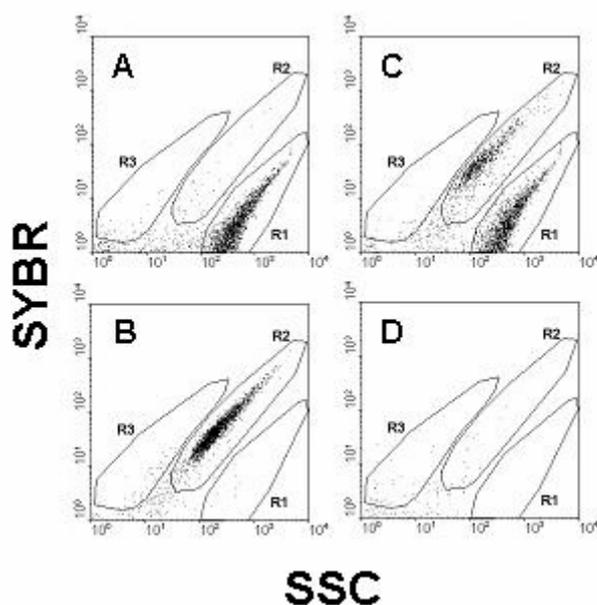


FIGURE 3: Discrimination between live and dead *Mycoplasma mycoides* subsp. *mycoides* large-colony type (LC) using SYBR and PI. An exponentially growing culture was used as the live control (A) and dead mycoplasma cells were prepared from this by heat-injury at 60°C for 1 hour (B). An artificial mixture of live and dead mycoplasma cells is shown in (C) and the broth medium background is shown in (D). R1 corresponds to live cells, R2 corresponds to dead cells. R3 was not used in this analysis.

APPLICATION OF THESE PROTOCOLS

We have recently used these protocols for determining the antibiotic susceptibility of a range of *Mycoplasma* species (Assunção *et al.* 2006a/b/c). The methods presented here are also applicable to detection of mycoplasmas in veterinary samples (Assunção *et al.*, in preparation).

PRACTICAL CONCERNS

Although flow cytometric analysis of mycoplasmas has the potential to be a very useful approach for the diagnosis of infections of both animals (including humans) and cell cultures it is necessary to highlight the potential for cross contamination of cell lines that are subsequently sorted on the flow cytometer. Most of the work that needs to be done for diagnosis / antibiotic susceptibility testing etc could be done on an analysis instrument thus avoiding these problems. However, where mycoplasmas are analysed on a sorter that will be subsequently used to isolate cells for culturing effective cleaning of the machine (and verification of its efficacy) will be required.

REFERENCES

Assunção, P., Antunes, N.T., de la Fe, C., Rosales, R.S., Poveda J.B & Davey, H.M. (2006a). Flow cytometric determination of the effect of antibacterial agents on *M. agalactiae*, *M. putrefaciens*, *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* large colony-type. *Antimicrobial Agents and Chemotherapy* **50**: 2845-2849.

Assunção, P., Antunes, N.T., Rosales, R.S., Poveda, C., Poveda J.B & Davey, H.M. (2006b). A flow cytometric method for the assessment of the minimal inhibitory concentrations of antibacterial agents to *Mycoplasma agalactiae*. *Cytometry* **69A**:1071-6.

Assunção, P., Antunes, N.T., Rosales, R.S., Poveda, C., Poveda J.B & Davey, H.M. (2006c). Application of flow cytometry for the determination of minimal inhibitory concentration of several antibacterial agents on *M. hyopneumoniae*. *Journal of Applied Microbiology* **102**: 1132-1137.

Bergonier, D., Berthelot, X., & Poumarat, F. (1997). Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. *Revue Scientifique et Technique Office International des Epizooties* **16**: 848-873.

Davey, H.M. & Kell, D.B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations-the importance of single-cell analyses. *Microbiological Reviews* **60**: 641-696.

Endresen, G.K.M. (2003). Mycoplasma blood infection in chronic fatigue and fibromyalgia syndromes. *Rheumatology International* **23**: 211-215.

Kobisch, M. & Friis, N.F. (1996). Swine mycoplasmoses. *Revue scientifique et technique (International Office of Epizootics)* **15** : 1569-1605.

Nicholas, R. (1995). Contagious agalactia. *State Veterinary Journal* **5**: 13-15.

Winson, M.K. & Davey, H.M. (2000). Flow cytometric analysis of microorganisms. *Methods: Methods in Enzymology* **21**: 231-240.