

Preparation protocol for epifluorescence microscopy when working close to the detection limit

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Abstract

Working with low density, low biomass material can be challenging, especially when working near the detection limit. Although background contamination is a universal consideration in microbiological research, its impact is increased when the cells under assessment approach the same concentration as the background contamination. The aim of this work was to identify and remove laboratory sources of background contamination in the cell mounting process for epifluorescence microscopy to improve the reliability of cell counting for low biomass samples. Microscope slides and coverslips were assessed before and after autoclaving, washing with detergent and rinsing with ethanol solution. The solutions used in sample mounting; 4',6-diamidino-2-phenylindole, phosphate buffered saline, and immersion oil, were tested before and after autoclaving as well as both single and triple filtering with a 0.2 µm membrane filter. Using a combination of detergent and ethanol rinses of glassware and triple filtering of all solutions, we were able to reduce the background contamination by almost two orders of magnitude, down from $1 \times 10^4 (\pm 4.3 \times 10^3)$ cells to 302 (± 312) cells per filter paper. This method was then validated with low biomass glacial sediment samples from Renardbreen, Svalbard, with cell concentrations of $1.8 \times 10^5 (\pm 2.9 \times 10^4)$ cells g⁻¹, close to the reported detection limit of epifluorescence microscopy.

Impact Statement

This study addresses one of the core challenges of cell enumeration routinely encountered in low volume, low biomass samples using epifluorescence microscopy. It outlines a straight-forward and cost-effective preparation that reduces background contamination of non-target cells by almost two orders of magnitude. This enables the reproducible enumeration of cells from environmental samples where cell counts are close to the detection limit.

Keywords: enumeration; method development; microbial detection methods; microbial contamination; environmental microbiology

Introduction

Enumeration of cells using DNA staining fluorophores such as 4',6-diamidino-2-phenylindole (DAPI) is a standard method in environmental microbiology (e.g. Porter and Feig 1980, Kirchman et al. 1982, Muthukrishnan et al. 2017). However, such methods generally assume cleanliness and the use of sterile materials to limit background contamination. In practice, however, and often in shared laboratories with multiple occupants, this can sometimes be challenging. There are several well-known primary sources of background contamination that can affect sample preparation for microbiological studies in laboratory settings.

- (i) The sampling environment—Contamination arising in the sampling environment during collection from improper PPE and aseptic technique is a particular concern when working with low biomass samples (e.g. Willerslev et al. 2004, Makinson et al. 2016).
- (ii) The laboratory environment—Contamination can arise easily inside busy and/or drafty laboratory environments including those with active air condition-

ing systems, where settled contaminants can be resuspended in air (known as bioaerosols) and deposited on samples or other laboratory equipment (e.g. Nikfarjam and Farzaneh 2012, Sanders 2012, Ghosh et al. 2015).

- (iii) Equipment contamination—Ineffective cleaning practices, improper storage, as well as the presence of bioaerosols can contribute to contamination of laboratory equipment (e.g. Eisenhofer et al. 2019, Cando-Dumancela et al. 2021).
- (iv) Reagent contamination—Although typically purchased as sterile, common laboratory reagents have been known to contain contaminants contributing background cells and DNA (Salter et al. 2014, Eisenhofer et al. 2019). It is therefore best practice to filter any reagents, even those listed as sterile, to remove any potential contaminating cells (Kallmeyer 2011).
- (v) Cross-contamination between samples—Ineffective cleaning practices of equipment between samples and splashing of reagents when samples are in close

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contact can result in cross-contamination (e.g. Abatenh *et al.* 2018, Cando-Dumancela *et al.* 2021).

- (vi) Researcher-contamination—Poor technique, improper PPE, and human error can result in contamination of the sample with a researcher's own microbiome (e.g. Eisenhofer *et al.* 2019, Hornung *et al.* 2019).

Each of the above sources of contamination can vary in the same laboratory environment over time, thus frequent and comprehensive background controls during sample preparation are critical for reliable results (Eisenhofer *et al.* 2019, Weyrich *et al.* 2019). Due to the number of potential sources of contamination, when compounding components for cell mounting and enumeration, it can also be difficult to identify at which specific step contamination may be occurring. For example, regardless of the sterility of the fluorescing compound, washing solution, and immersion oil, a contaminated glass coverslip will result in anomalously high cell counts. In addition, a completely sterile glass coverslip will be of little benefit if all the solutions at the preparation stage contain some degree of contamination.

The detection limits of cell counting by fluorescence microscopy have been cited at $\sim 10^3$ – 10^6 cells ml⁻¹ evenly distributed on a filter membrane (Broadaway *et al.* 2003a, 2003b, Lisle *et al.* 2004, Madrid and Felice 2005, Barton *et al.* 2006, Chae *et al.* 2008, Pascaud *et al.* 2009, Singh *et al.* 2016, Bedrossian *et al.* 2017a, 2017b, Muthukrishnan *et al.* 2017, Hoyles *et al.* 2018, Tyagi *et al.* 2022). High biomass environmental samples such as soils, have estimated prokaryote cell counts of $> 10^9$ cells g⁻¹ which is often enough for the enumeration of cells even with relatively high detection limits (Torsvik *et al.* 1990, Dunbar *et al.* 2002, Smith *et al.* 2006, Liebner *et al.* 2008, Tecon and Or 2017). However, in low biomass environments (see Table 1), time consuming and complex cell separation techniques are required, and often large volumes of sample are needed to exceed the detection limit. This is not always possible for samples taken from remote and extreme environments where sample volume is limited. Thus, lower limits of detection must be improved and background contamination reduced to achieve reliable and reproducible results.

In published works, the degree of background contamination present in an epifluorescence microscopy protocol, and indeed the detection limit of a particular study are often not reported. This leaves a significant knowledge gap making it difficult to achieve reproducible results using published protocols. To address this issue, we focussed on reducing the amount of background contamination present in the laboratory and materials used for cell mounting. The overall aim being to develop a simple and cost-effective protocol which reduced background contaminant cells to a value below the detection limit of the instrument/protocol that can be employed by others in a reproducible way.

Materials and methods

The following protocol applied sequential additions of each cell mounting component using different cleaning and sterilization methods to determine the most effective protocol for reducing background contamination. Aseptic techniques, the use of laboratory gloves when handling consumables and samples and the frequent changing of gloves to reduce cross-contamination and sterile single-use plastic materials were

used throughout. To reduce the impact of background contamination from the laboratory environment, it is also recommended that filtering and sample preparation is performed in a class II microbiological safety cabinet where possible.

For the purposes of this investigation, a standard microscopy slide preparation consisted of; a (i) glass microscope slide, (ii) glass coverslip, (iii) Whatman Cyclopore track-etched membrane (0.2 µm pore size), and (iv) immersion oil (Sigma-Aldrich). An initial analysis of all preparatory materials for microscopy was conducted prior to any cleaning procedures. A vacuum manifold with a diameter of 18.5 mm was used to stain, rinse, and dry filter papers using a gentle vacuum (≤ 0.20 millibar pressure). All components of the vacuum manifold were cleaned with detergent (Detergent 8, Alconox), rinsed well with sterile water, and allowed to dry between uses to prevent cross-contamination between samples. This provided a baseline of background contamination present in the current laboratory setting.

Cleaning protocol

Prior to the cleaning protocol, a 10-fold dilution series using a stock solution of *Escherichia coli* (*E. coli*) culture was performed to determine detection limits. The solution was prepared in 5 ml Luria-Bertani broth and incubated for 24 h. A 10-fold dilution series was prepared for microscopy analysis using the slide and enumeration procedure below, counting cells per fields of view (FOV) until a stable mean had been established. Sample 1 being the most concentrated stock solution and each step thereafter prepared with 100 µl of the previous sample and 900 µl of sterile phosphate buffered saline (PBS). This protocol was also repeated after the cleaning protocol to verify the background levels of contamination.

The first step in the cleaning process was to remove any contaminating cells or auto-fluorescing dust and particles from glassware (Fig. 1). Both microscope slides and coverslips were washed with detergent then thoroughly rinsed in a stream of 95% ethanol while held with sterile tweezers and allowed to dry.

The solutions used in the preparation of the samples; (i) Gibco pH 7.4 PBS, (ii) immersion oil (Sigma-Aldrich), and (iii) 5 mg ml⁻¹ solution of DAPI (Thermo Fisher Scientific) could also be sterilized and the following sterilization techniques were tested (Fig. 1):

- (i) Single filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 µm).
- (ii) Triple filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 µm).
- (iii) Autoclaving immersion oil and PBS followed by triple filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 µm).

Preparation of slides and enumeration of cells

To stain the samples, a 5 mg ml⁻¹ solution of DAPI was prepared using PBS and stored at -20°C for up to 1 month. A volume of 10 µl of DAPI solution was added to a Whatman Cyclopore track-etched membrane (0.2 µm pore size) and incubated in the dark for 5 min. A glass coverslip without immersion oil was then placed on top of the filter membrane and the slide was analysed. To test the sterility of PBS, 1 ml of sterile certified on purchase PBS was added to the filter membrane in a vacuum manifold and a gentle vacuum (≤ 0.20

Table 1. Example cell counts, reported detection limits, and reported FOV counts from the literature of low biomass ecosystems.

| Sample type | Cell count | Method for checking background contamination | Reported number of FOV counts | Reference |
|--------------------|--|---|-------------------------------|-----------------------------|
| Air | 1×10^4 cells m^{-3} | Not reported | 400 cells or 86 FOV | Bauer et al. (2002) |
| | 3.4×10^4 cells m^{-3} | Control samples reported as one order of magnitude lower than cell counts | 10 FOV | Xia et al. (2013) |
| Sea ice | $6.5\text{--}9 \times 10^5$ cells m^{-3} | Not reported | 10 FOV | Dong et al. (2016) |
| | $1.1\text{--}3.2 \times 10^5$ cells m^{-3} | Negative controls | 20 randomly selected | Hu et al. (2020) |
| | 1.4×10^{11} cells m^{-3} | Negative controls | 200 cells and 10 FOV | Sullivan et al. (1984) |
| | 1.0×10^5 cells ml^{-1} | Not reported | Not reported | Piontek et al. (2021) |
| Soil and sediments | $1.15 \times 10^8\text{--}2.13 \times 10^9$ cells g^{-1} | Not reported | Not reported | Garcia-Pichel et al. (2003) |
| | $1.2\text{--}23 \times 10^8$ cells g^{-1} | Not reported | 1000 cells and 10 FOV | Kobabe et al. (2005) |
| | $2\text{--}6 \times 10^8$ cells g^{-1} | Not reported | 10 FOV | Buckeridge et al. (2013) |
| | $10^{6\text{--}8}$ cells g^{-1} | Not reported | Not reported | Thomas et al. (2021) |
| | $2\text{--}7 \times 10^6$ cells g^{-1} | Subtracting the average reagent control from 20 FOV of each blank control | 10-20 FOV | Foght et al. (2004) |
| | 1.6×10^7 cells mg^{-1} of organic matter | Not reported | Not reported | Kaštovská et al. (2007) |
| | 10^7 cells g^{-1} | Not reported | Not reported | Lanoil et al. (2009) |
| | $1.22\text{--}4.42 \times 10^7$ cells g^{-1} | Negative controls | 2500 cells and 50 FOV | Pearce et al. (2013) |
| Glacier and snow | $1.3 \times 10^5\text{--}2.6 \times 10^6$ | Negative controls | 25-60 FOV | Davis et al. (2023) |
| | $10^1\text{--}10^5$ cells ml^{-1} | Not reported | Not reported | Harding et al. (2011) |
| | 39-363 cells ml^{-1} | Not reported | 300 cells | Dayal et al. (2023) |
| | $10^2\text{--}10^4$ cells g^{-1} | Negative controls | 60 FOV | Montross et al. (2014) |
| | $2 \times 10^3\text{--}2 \times 10^6$ cells ml^{-1} | Blanks with no cells counted in parallel | 300 cells | Stibal et al. (2015) |
| | $6.6 \times 10^4\text{--}3.7 \times 10^5$ cells ml^{-1} | Field blank of sterile Nanopure water in parallel | Not reported | Skidmore et al. (2005) |

millibar pressure) was applied. Potential contaminating cells present in the PBS captured on the filter membrane were then stained with 1 μ l of 5 mg ml^{-1} DAPI and incubated for 5 min. The filter membrane was then rinsed twice with 1 ml of PBS to remove excess DAPI with a gentle vacuum applied between rinses. A glass coverslip without immersion oil was then placed on the filter membrane glass slide and analysed immediately. The same preparation procedure was repeated to test the sterility of the immersion oil but before adding the glass coverslip a drop of immersion oil was placed in the centre of the filter membrane using a sterile pipette tip and a glass coverslip placed on top. The sample was then analysed immediately. Samples were analysed at 40 \times magnification across a full vertical and horizontal transect of the filter paper accounting for potential cell clumping at the edges of the filter paper where the vacuum effect is strongest. Cell counts were obtained using a running mean to achieve a stable mean value. This equated to an average of 35 FOV on a fluorescence microscope (Olympus BX51) using UV light. Each method was also analysed using three independent replicates by three different researchers to remove any potential counting bias related to human error. An estimate of total cells on the membrane filters was calculated using the following equation (Equation 1):

$$\begin{aligned} & \text{Cells per filter paper} \\ &= \text{Average cells per FOV} * \frac{\text{Area } (\pi R^2) A}{\text{Area } (\pi R^2) B}, \quad (1) \end{aligned}$$

where Area A is the area of the filter membrane exposed to solution (268.8 mm²) and Area B is the area of the FOV

(0.22 mm²) providing a conversion factor (CF) to extrapolate the total cells on the membrane (CF=1221).

Environmental sample

To validate the protocol, the triple filtered consumables, and detergent cleaned glassware was used to enumerate cells from a glacial low volume, low biomass environment. Sediment samples were collected from the surface of Renardbreen, Svalbard, originating from a thrust structure within the ice. Samples were collected using sterile gloves into Fischerbrand 20 ml sterile syringes and stored in sterile Whirlpak bags at -20°C .

Cells were extracted from the sediment in three independent replicates using 0.51-0.55 g of sediment vortexed for 2 min in 2 ml Eppendorf tubes with PBS. A negative control was also implemented at this stage consisting of 2 ml of the PBS only. Samples and control were then centrifuged at 6764 g for 1 min to separate the suspended sediment from the solution containing the cells. The resultant supernatant was then pipetted onto a Whatman Cyclopore track-etched membrane. Excess liquid was removed via gentle vacuum before being stained with 10 μ l of DAPI solution and incubated in the dark for 5 min. Samples were then washed twice with 0.5 ml of PBS and excess liquid removed via vacuum before mounting on a microscope slide with 1 drop of immersion oil and a cover slip. The three replicate samples were analysed by counting cells in 35 FOV on each sample at 40 \times magnification on an epifluorescence microscope using UV light. The cell concentration in the sample could then be compared with the

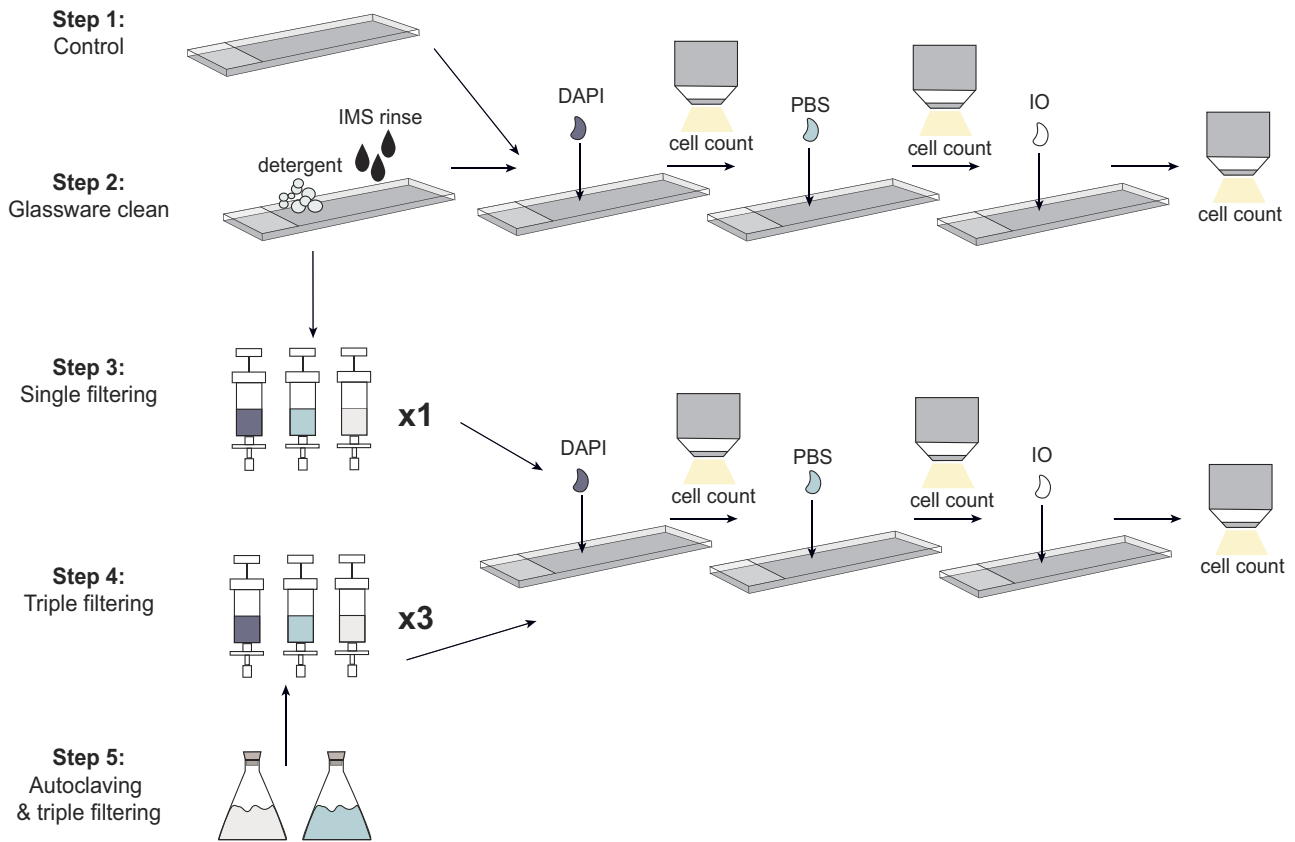


Figure 1. Schematic workflow of the methodological approach of this study detailing the sequential steps and analyses employed to test the sterility at different points of the cell mounting process. DAPI, 4',6-diamidino-2-phenylindole; PBS, Gibco pH 7.4 phosphate buffered saline; IO, immersion oil; and IMS, industrial methylated spirit 95% ethanol.

negative control which would reflect the level of background contamination.

Results and discussion

Glassware preparation

Cleaning of glassware with detergent before rinsing with ethanol and drying on a hot plate in a sterile petri dish removed any autofluorescence from small fibres and reduced the number of apparent contaminant cells (Table 2). This alone reduced the apparent background contamination from 8.89 cells per FOV or $1 \times 10^4 (\pm 4.3 \times 10^3)$ cells per filter paper to 3.30 cells per FOV or $4.04 \times 10^3 (\pm 2.97 \times 10^3)$ cells per filter paper. The continued presence of contaminant cells after cleaning of the glassware was likely introduced through the solutions required for the cell mounting process i.e. air, DAPI, PBS, and immersion oil. This effect was suggested by the gradual increase in number of background cells observed after the cleaning process with the addition of each new component.

Solution sterilization

Single filtering of DAPI, PBS, and immersion oil produced a reduction in background contamination in all steps of the process, with a statistically significant difference seen in cell counts per FOV and per filter paper between filtered and unfiltered consumables ($P = < 0.01$, $t = 1.98$, $n = 105$) (Table 2). The largest improvement in background contamination was seen when all solutions underwent a triple filter

technique with a statistically significant difference obtained between the single filtering and triple filtering ($P = < 0.01$, $t = 1.98$, $n = 105$) (Fig. 3 and Table 2). The triple filtering technique combined with the clean glassware reduced background contamination by almost two orders of magnitude with the total number of cells present on the filter membrane and cover slip down from 8.89 cells per FOV or $1 \times 10^4 (\pm 5.2 \times 10^3)$ cells per filter paper to 0.25 cells per FOV or 302 (± 312) cells per filter paper using all solutions and cell mounting components.

Autoclaving of PBS and immersion oil before triple filtering was also tested to see if further improvements in the reduction of background contamination could be achieved. However, triple filtering after autoclaving showed similar results to triple filtering alone. In fact, the lowest cell concentration was observed using the triple filtered method with no autoclaving (Table 2). This demonstrated that the addition of autoclaving the solutions did not sterilize the materials further and therefore the added labour and cost of autoclaving was not beneficial in this instance.

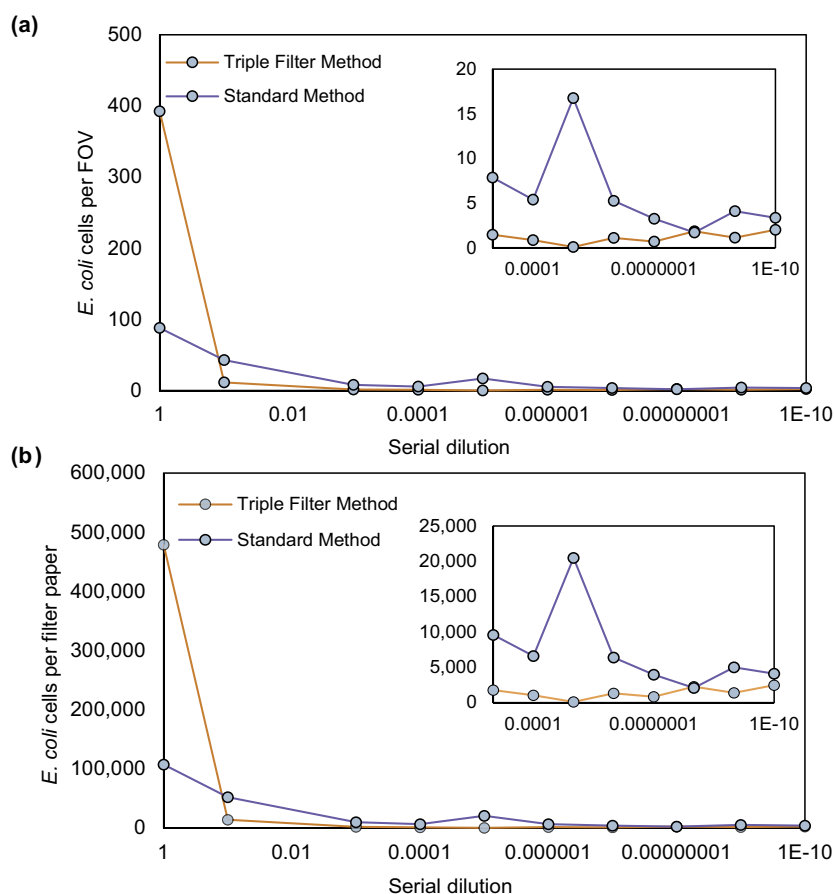
Background contamination

Background contamination before employing the cleaning protocol was measured using an *E. coli* dilution series (Fig. 2) and then again with no sample but all components of the cell mounting process for microscopy (Table 2, Fig. 3). This method showed increasing levels of background contamination highlighting the importance of cleaning/sterilizing all materials used in the cell mounting process to ensure

Table 2. Average cells per FOV in each step of the cleaning protocol identifying the most effective way to reduce background contamination in microbial microscopy preparation.

| Cleaning protocol | Components | Average cells per FOV (\pm SD) | Average cells per filter paper |
|--|----------------------------|-----------------------------------|---|
| Control (no cleaning or sterilization) | DAPI + PBS + Immersion oil | 8.89(\pm 3.52) | 1.08×10^4 ($\pm 4.30 \times 10^3$) |
| Cleaning of glassware with detergent and ethanol | DAPI + PBS + Immersion oil | 3.30(\pm 2.4) | 4.04×10^3 ($\pm 2.97 \times 10^3$) |
| Single filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 μ m) | DAPI | 1.89(\pm 1.09) | 2.30×10^3 ($\pm 1.33 \times 10^3$) |
| | DAPI + PBS | 3.03(\pm 2.22) | 3.70×10^3 ($\pm 2.71 \times 10^3$) |
| | DAPI + PBS + Immersion oil | 1.61(\pm 1.27) | 1.97×10^3 ($\pm 1.55 \times 10^3$) |
| Triple filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 μ m) | DAPI | 0.2(\pm 0.2) | 2.1×10^2 ($\pm 2.3 \times 10^3$) |
| | DAPI + PBS | 0.61(\pm 0.36) | 744(\pm 436) |
| | DAPI + PBS + Immersion oil | 0.25(\pm 0.26) | 302(\pm 312) |
| Autoclaving immersion oil and PBS followed by triple filtering with a sterile syringe fitted with a Filtropur S attachment (0.2 μ m) | DAPI | 0.02(\pm 0.01) | 23.60(\pm 16.69) |
| | DAPI + PBS | 0.20(\pm 0.11) | 246(\pm 130) |
| | DAPI + PBS + Immersion oil | 0.36(\pm 0.29) | 438(\pm 352) |
| | | | |

Cells per filter paper were calculated using Equation 1.

**Figure 2.** Calibration graphs of serial dilutions of *E. coli* to test background contamination levels. (a) Average cells per FOV in a 10-fold dilution series using the standard method and the triple filter method. (b) Average cells per filter paper in a 10-fold dilution series using the standard method and triple filter method.

low background contamination and a reduction in detection limits.

Prior to any cleaning procedure, the background contamination value fell within the previously stated detection limit of $\sim 10^3$ – 10^6 cells ml^{-1} for fluorescence microscopy (e.g. Broadway et al. 2003a, Chae et al. 2008, Pascaud et al. 2009, Bedrossian et al. 2017a, Bedrossian et al. 2017b, Muthukrishnan et al. 2017, Hoyles et al. 2018). Thus, when working close to this lower limit of detection, reducing the background

contamination below this level is paramount to achieving accurate and reproducible counts. This was further confirmed using an *E. coli* dilution series which showed cell numbers of 0.08–2 cells per FOV or 3.9×10^2 – 9.8×10^3 per filter paper at 3–10-fold dilution after employing a slide washing and triple filtering cleaning protocol compared to the previous 1.7–16.8 cells per FOV or 8.3×10^3 – 8.2×10^4 per filter paper at 3–10-fold dilution prior to any cleaning protocol (Fig. 2).

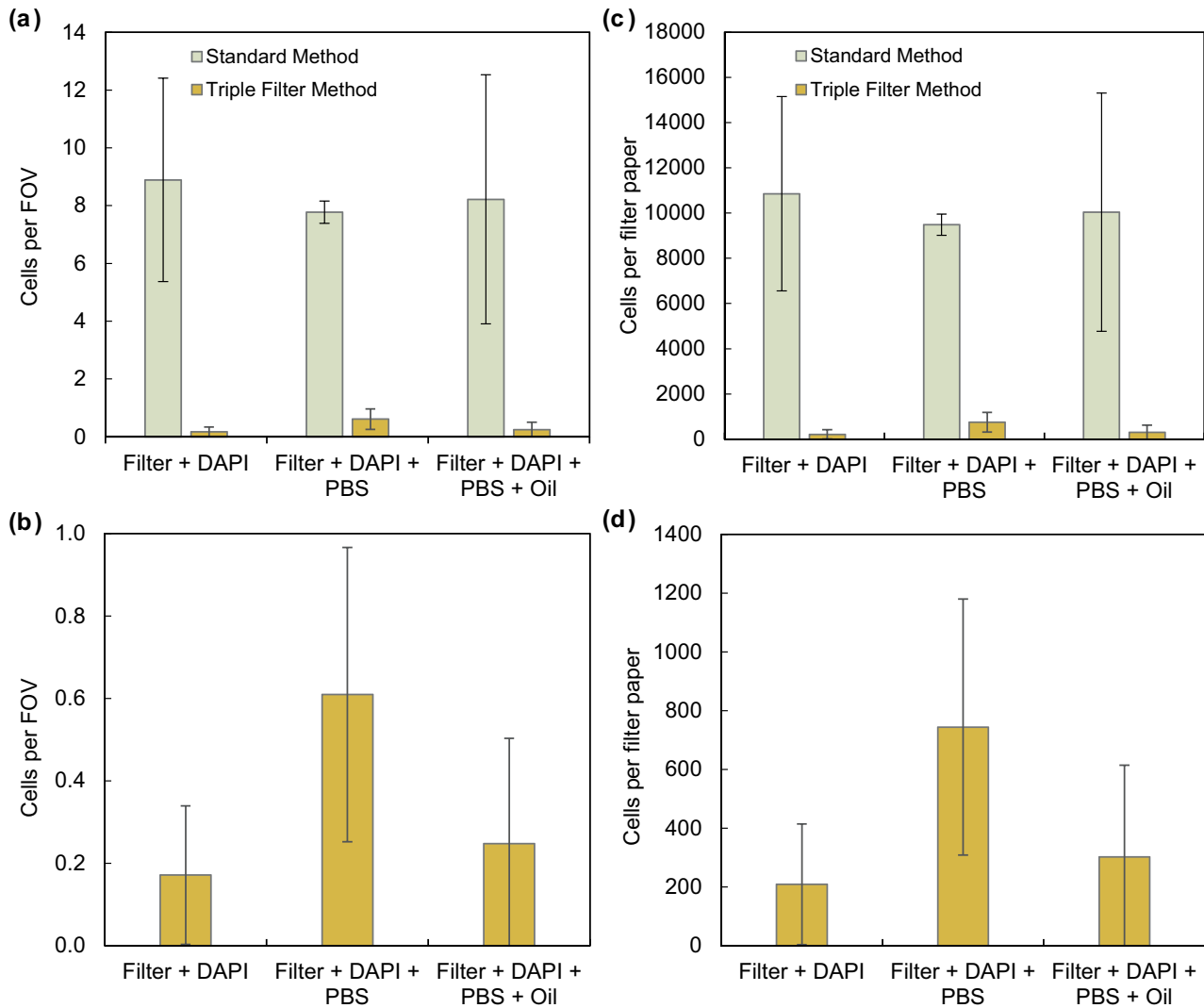


Figure 3. (a) Average cells per FOV of cell mounting components at each stage of the cell mounting process. Error bars represent the standard deviation. (b) Cells per FOV of the triple filtered consumables only. (c) Average cells per filter paper of cell mounting components at each stage of the cell mounting process. Error bars represent the standard deviation. (d) Cells per filter paper of the triple filtered consumables only.

In previous works background contamination during cell enumeration has been accounted for by subtracting cell numbers of negative controls from sample counts (e.g. Foght et al. 2004, Xia et al. 2013, Davis et al. 2023). However, when working with low biomass and thus low cell number samples, there is potential for background contamination to be equal to or surpass those of the actual sample.

Applications for environmental samples

In remote polar environments ice, snow, and glacial sediment samples have a generally low biomass with reported cell abundances of 10^1 – 10^6 cells ml^{-1} of water or g^{-1} of sediment (see Table 1 and references therein). Similarly, ultra-low biomass environmental aerosol samples have a reported cell abundance of 10^1 – 10^5 cells m^3 (see Table 1 and references therein).

Therefore, to validate this protocol, environmental low biomass samples from a glacial environment were also analysed using the triple filtered and detergent washed consumable protocol. Negative controls were used alongside the samples which comprised all the cell mounting compo-

nents with no sample. The average cell concentration across the three replicates in these sediment samples was $1.8 \times 10^5 (\pm 2.9 \times 10^4)$ cells g^{-1} . The standard deviation of this value fell within the same level as the background contamination before any cleaning or sterilization protocol. Whereas the negative control using the most optimal protocol outlined here remained low with an average cell concentration of $44 (\pm 1.9 \times 10^2)$ cells per filter paper. This sample falls close to the detection limit of the epifluorescence microscopy method of $\sim 10^3$ cells ml^{-1} . By reducing the background contamination, with a negative control slide two–four orders of magnitude lower than the sample, we were able to confidently enumerate cells in this low biomass environmental sample.

Further considerations for protocol optimisation

Further considerations for optimizing cell enumeration using fluorescence microscopy include the use of other cell stains. Fluorescence *in-situ* hybridization allows for fluorescent probes to bind to RNA of the small ribosomal sub-unit

(SSU) of cells (Rivera et al. 2022). The probe EUB338 is one such universal probe that binds to bacterial SSU (Amman et al. 1995, Fuchs et al. 1998, Rivera et al. 2022). Such probes and analyses aid in the differentiation of true bacterial cells and fluorescing artifacts. Differentiation of live and dead bacteria can also be achieved using 5-cyano-2,3-ditolyl tetrazolium chloride which is absorbed by active cells and reduced to the fluorescent formazan (CTF) product detectable using fluorescence microscopy (Rodriguez et al. 1992, Schaul et al. 1993). Both compounds can be coupled with DAPI as outlined here to investigate cell viability.

Background contamination may also be reduced by employing and reporting on aseptic laboratory techniques used to; reduce the impact of researcher contamination through the proper use of PPE (e.g. Willerslev et al. 2004, Makinson et al. 2016) and cross-contamination between samples as well as other contaminated surfaces or equipment (e.g. Abatenh et al. 2018, Eisenhofer et al. 2019, Hornung et al. 2019, Cando-Dumancela et al. 2021). Further description of such techniques in future publications as well as routine reporting of background contamination levels and detection limits will better facilitate reproducible cell enumerations.

Conclusions

The protocol outlined here reduced background contamination from $1 \times 10^4 (\pm 4.3 \times 10^3)$ cells per filter paper to $302 (\pm 312)$ cells per filter paper. When this protocol was applied to an *E. coli* dilution series the cells per FOV were reduced from 1.7–16.8 cells to 0.04–1.7 cells per FOV. This is a reduction by almost two orders of magnitude. This makes low biomass samples such as the glacial sediment samples presented here, fall within a detectable range above the background contamination. This protocol is particularly applicable to studies whereby background contamination is accounted for by subtracting background cell values in controls from those measured in the sample.

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Authors contribution

Rebecca McCerery (Conceptualization [Supporting], Data curation, [Lead], Formal analysis [Lead], Investigation [Equal], Methodology [Lead], Project administration [Supporting], Supervision [Supporting], Validation [Lead], Visualization [Lead], Writing - original draft [Lead], Writing - review & editing [Lead]), Lewis Hall (Data curation [Supporting], Investigation [Supporting], Methodology [Supporting], Validation [Supporting], Writing - review & editing [Supporting]), Jithin Omanakuttan (Data curation [Supporting], Investigation [Supporting], Methodology [Supporting], Validation [Supporting], Writing - review & editing [Supporting]), Hui Zhi Chai (Data curation [Supporting], Investigation [Supporting], Methodology [Supporting], Validation [Supporting], Writing - review & editing [Supporting]), James Lawrence (Validation [Supporting], Writing - original draft [Supporting], Writing - review & editing [Supporting]), John Woodward (Funding acquisition [Equal], Supervision [Equal],

Writing - original draft [Supporting], Writing - review & editing [Supporting]), and David A. Pearce (Conceptualization [Lead], Data curation [Supporting], Funding acquisition [Equal], Methodology [Supporting], Project administration [Lead], Resources [Lead], Supervision [Lead], Validation [Equal], Writing - original draft [Supporting], Writing - review & editing [Supporting]).

Conflicts of interest: None declared.

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Data availability

All data presented in this paper is available in an open access repository, Open Science Framework, at: 10.17605/OSF.IO/952ZA.

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