
Preliminary experiment for cell count using flow cytometry¹

Yuki Morono,² Jens Kallmeyer,² Fumio Inagaki,² and the Expedition 329 Scientists²

Chapter contents

Abstract	1
Introduction	1
Principles of fluorescent wavelength-based cell discrimination	2
Standardization of flow cytometry cell count protocol for South Pacific Gyre sediment	2
Results and discussion	3
Conclusion and future prospects	6
Acknowledgments	6
References	6
Figures	7

Abstract

One of the major challenges in microbial ecology is to evaluate the accurate number of living cells in a natural environment. Cell count using flow cytometry is a powerful, high-throughput technique that has widely been used for aquatic habitats but not for sedimentary environments because mineral grains interfere with cell detection. During Integrated Ocean Drilling Program Expedition 329, we tested several sample preparation methods for on-board cell counting using flow cytometry with various pelagic sediments from the South Pacific Gyre. The cell numbers acquired from shallow sediments are almost consistent with microscopic direct counts (i.e., $\sim 10^5$ cells/cm³). Yet, the method needs improvement and standardization to reduce the background signal and to lower the minimum detection limit for deep sedimentary habitats with very low cell densities (i.e., < 1000 cells/cm³).

Introduction

Detection and enumeration of microbial life in marine subsurface environments provides primary information for deciphering the extent and habitability of Earth's biosphere. Hence, accurate and precise knowledge of microbial cell abundance at high spatial resolution is a major challenge for the exploration of subsurface life. Cell-counting efforts during drilling expeditions typically depend on direct eye count of fluorescent dye-stained cells using epifluorescence microscopy. The improved cell extraction and filter preparation techniques of Expedition 329 provide reliable cell counts to levels as low as 1000 cells/cm³ of sediment. However, ship-board microscopic observation requires a great deal of time and effort in observing hundreds of microscopic fields of view per sample to produce statistically meaningful data. Processing the large number of samples necessary for such observation causes considerable difficulty given the limited time and manpower on-board. During Expedition 329, experiments with an alternative cell detection and enumeration method using flow cytometry (FCM) equipped with a 96-well autosampler system were conducted. Expedition 329 provided an unprecedented opportunity to test a variety of open-ocean sediment types in the South Pacific Gyre at depths ranging from the seafloor to basaltic basement.

¹Morono, Y., Kallmeyer, J., Inagaki, F., and the Expedition 329 Scientists, 2011. Preliminary experiment for cell count using flow cytometry. In D'Hondt, S., Inagaki, F., Alvarez Zarikian, C.A., and the Expedition 329 Scientists, *Proc. IODP*, 329: Tokyo (Integrated Ocean Drilling Program Management International, Inc.).
doi:10.2204/iodp.proc.329.110.2011

²Expedition 329 Scientists' addresses.



Principles of fluorescent wavelength-based cell discrimination

One of the serious issues for fluorescence-based cell enumeration in geological samples (e.g., sediment and rock) is to distinguish few microbial cells in the forest of nonspecific background fluorescence signals from minerals. Morono et al. (2009) reported that using SYBR Green I fluorescent dye, SYBR Green I-stained cells (hereafter, SYBR-stained cells) are distinguishable from nonbiological background signals from SYBR-stainable particulate matter (SYBR-SPAM) based on the difference in fluorescent wavelength pattern (i.e., most fluorescent spectra from SYBR-SPAM shift to longer wavelengths than those from SYBR-stained cells). Based on the fluorescence characteristic of SYBR Green I stain, it is possible to discriminate cell-derived fluorescence signals from the background by processing green- and red-filtered fluorescent intensity of microscopic images (Morono et al., 2009). Although this method is independent of human variation in cell recognition, it is still necessary to aseptically prepare the membrane filter for image acquisition and processing. Therefore, even using the automated-robotic slide loader system (Morono and Inagaki, 2010), onboard difficulties with potential contamination and unbiased high-throughput cell counting are still present, such as

- Filter preparation requires very careful and aseptic handling, which is in most cases very difficult on ships and take considerable human effort and time for sample processing;
- Deployment of the highly sensitive robotic microscopic system on every microbiological expedition is difficult; and
- Maintaining stable and constant operation of automatic z-focus adjustment on a continuously moving ship is hard.

Although the image-based automated cell count method has been useful and robust for shore-based microbial cell counting, an innovative onboard high-throughput technique for filtration- and eye-independent cell count for geologic samples is required.

Flow cytometry is a powerful tool for identifying and enumerating fluorescence-labeled cells, based on cell size, fluorescent intensity, and wavelength. It is commonly used for medical sciences and aquatic microbial ecology. The application of FCM to seafloor life has great potential for high-throughput and statistically reliable cell counts onboard because discrimination of cell-derived SYBR fluorescence from SYBR-SPAM is essentially the same as with the image-based technique and thus should be possible using

FCM. However, it is necessary to separate cells effectively from most nonbiological particles (e.g., minerals) and prepare SYBR-stained cell suspensions. During Expedition 329, the cell detachment protocols of Kallmeyer et al. (2008) and Morono et al. (2009) were modified and a standardized FCM cell counting method was developed for various types of open ocean sediment using cored samples from the South Pacific Gyre.

Standardization of flow cytometry cell count protocol for South Pacific Gyre sediment

Subsamples were taken from the innermost part of whole-round core sediment samples by a tip-cut sterilized syringe on the catwalk or in the core refrigerator of the R/V *JOIDES Resolution* as soon as possible after core recovery (see “Microbiology” in the “Methods” chapter [Expedition 329 Scientists, 2011a]). Two cubic centimeters of sediment was transferred to a sterile-filtered 15 mL centrifuge tube containing 8 mL of 0.2 μm filtered, 2.5% (w/v) NaCl solution with 2% (v/v) formalin as a fixative and thoroughly shaken to form a homogeneous suspension. The slurry sample was tested with the cell detachment and cleaning steps for FCM cell count according to the following experimental protocols.

Protocol FCM-A (basic protocol prior to modifications)

1. Depending on the expected cell abundance, 50–500 μL of the (1:5) slurry is used for the extraction. When sample volumes are $<500 \mu\text{L}$, 2.5% NaCl solution is added to adjust the sample volume to 500 μL . Then, 50 μL each of detergent mix (100 mM disodium EDTA dihydrate, 100 mM sodium pyrophosphate decahydrate, 1% [v/v] Tween 80) and methanol is added, followed by vigorous vortex mixing for 30–60 min. In cases of extremely low cell abundances, the volume of slurry and all reagents can be increased proportionately.
2. The sample is underlain with a cushion of Nycodenz (50%) and centrifuged (10 min at $3000 \times g$) to separate microbial cells and sediment particles by density.
3. The supernatant is carefully removed with a syringe and stored in separate vial. The pellet is suspended in 400 μL of 2.5% NaCl solution, followed by addition of 50 μL each of detergent mix and methanol.
4. The slurry is subjected to an ultrasonic treatment at 20 W for 30 s.

5. The sample is placed on a vortex mixer for 10–20 min followed by density separation as before (Step 2).
6. The second supernatant is recovered as described before and pooled with the first one.
7. To the pooled supernatants, 100 μL of 1% HF is added and the solution incubated for 20 min at room temperature.
8. To remove HF from the suspension, 120 μL of a 0.5 M each CaCl_2/Na acetate solution is added. Precipitation of CaF_2 occurs immediately.
9. To remove CaF_2 precipitates, the solution is underlain by a cushion of Nycodenz (50%) and centrifuged (10 min at $3000 \times g$). The clear supernatant is removed as described before.
10. The recovered supernatant is diluted (1:5) with 2.5% NaCl solution to decrease density and centrifuged again (10 min at $3000 \times g$).
11. Most of the supernatant is discarded and only $\sim 100 \mu\text{L}$ of the solution at the bottom, containing the cell pellet is kept (in most cases, the pellet is invisible).
12. SYBR Green I solution (2.5 μL ; Lonza Rockland Inc., Maine [USA]), is added and let to sit for 10 min in darkness at room temperature.
13. Tris-EDTA (TE) buffer (pH 8.0; 400 μL) is added. The cell suspension is ready for FCM cell count analysis.

Protocol FCM-B (protocol without CaF_2 precipitation)

For the Steps 1–7, follow Protocol FCM-A.

8. Tris-base solution (100 μL ; 1.5 M) is added to neutralize the solution pH without CaF_2 precipitation.

Continue with Step 10 in Protocol FCM-A.

Protocol FCM-C (protocol without HF treatment)

Follow Steps 1–6 in Protocol FCM-A. Skip Steps 7–9 and continue with Steps 10–13.

Protocol FCM-D (final improved protocol with HF treatment)

Follow Steps 1–11 in Protocol FCM-A.

12. Add 400 μL of 1:40 diluted SYBR Green I in TE buffer and let sit for 10 min at room temperature.
13. Centrifuge for 20 min at $3000 \times g$ and discard supernatant.
14. Wash the precipitates with 1 mL TE buffer.
15. Centrifuge for 20 min at $3000 \times g$ and discard supernatant.

16. Repeat the washing step (Steps 14 and 15) two more times.

17. Add 500 μL TE buffer to the solution.

During Expedition 329, a C6 flow cytometer (Accuri Cytometers, Inc.) equipped with a 96-well autosampler for FCM cell count experiments was used. All cell extraction experiments and FCM operations were performed in the Paleontology Preparation Laboratory on the *JOIDES Resolution*. On the flow cytometer, the stained cells were excited with a 488 nm laser and the forward-scatter, side-scatter, and fluorescent signals at 530 and 610 nm were detected and counted.

Quality assurance and control and flow cytometry cell count data processing

For quality assurance and quality control, all reagents were filtered through 0.2 μm filters. Twice-filtered (0.2 μm) 18.2 M Ω water was used for the blank and FCM-sheath solution. For negative control samples, “cell/DNA-free” kill-control samples were prepared by (1) treating some core sediment with an equal volume of bleach-based commercial cleaning product and (2) treating other sediment by heating at 450°C for 5 h. Both kill-control samples were washed and neutralized twice with TE buffer and subjected to FCM cell counting and direct microscopic cell counting (see “Microbiology” in the “Methods” chapter [Expedition 329 Scientists, 2011a]). The flow cytometer was calibrated prior to data collection for accurate volume measurement using fluorescent microsphere standards at known concentrations (Accuri Volume Validation Beads, Accuri, QA120). Because ship heave prevented automatic volume calibration procedure during FCM, volume-validation bead counts were used to calibrate the flow volume.

Results and discussion

Prior to Expedition 329, the FCM cell counting technique was tested with several organic-rich continental margin sediments (e.g., Nankai Trough and Aarhus Bay) onshore. Using Protocol FCM-A as the basic method, the successful separation of microbial cells from SYBR-SPAM signals on the same flow cytometer was confirmed; these FCM cell count data were stable and highly consistent with direct microscopic cell counts (data not shown). However, at the beginning of Expedition 329, significant differences between shore-based FCM counts and shipboard results on the *JOIDES Resolution* were observed. For example, unexpectedly high background signals with the same or similar fluorescence wavelength of SYBR-stained cells were observed in early shipboard experi-

ments using the South Pacific Gyre sediment. Several factors may contribute to this difference in performance:

1. Cell concentrations in South Pacific Gyre sediment are significantly lower than those that were observed onshore in organic-rich continental margin sediment;
2. Sedimentological characteristics are very different between smectite-rich young continental margin sediment and geologically old zeolite-rich metalliferous sediment of the South Pacific Gyre; and
3. Experimental conditions such as cleanliness, reagent qualities, and machine stability may differ significantly between shore-based and shipboard laboratories.

Nevertheless, Expedition 329 provided an excellent opportunity to establish a more robust fieldwork protocol for FCM cell counting, using various types of cored sediment from the extensive area of the South Pacific Gyre. Here a site-by-site effort to develop the best protocol for FCM cell counting with low background and high sensitivity (i.e., low detection limit) is reported.

Flow cytometry quality control

To verify absolute mechanical background signals of FCM under onboard laboratory conditions, null-control (water) during protocol development and sample measurements were run repeatedly. A “hat-trick cleaning step” for FCM was conducted according to the manufacturers’ recommendation when relatively unstable and/or high background signals were observed during the experiment. Overall, these null-control experiments showed negligible numbers (10 signals per milliliter of water) within the range of fluorescent wavelength of SYBR-stained cells. Surface seawater was used as a positive control sample for FCM cell counting; these surface water counts consistently matched direct microscopic cell count ($\sim 10^5$ cells/mL; data not shown). Based on these seawater sample tests and the standard bead counts on FCM, the effect of ship movement (i.e., pitch and roll) was negligible for onboard FCM cell counting during Expedition 329.

Site U1365

At Site U1365, FCM cell counting Protocol FCM-A was first tested for sediment samples from just below the seafloor to just above the sediment/basement contact. Unexpectedly high background signals were observed, which resulted in a minimum detection limit (MDL) of $\sim 10^4$ cells/cm³. Using Protocol FCM-A, estimates of cell concentrations in sediment <10

meters below seafloor (mbsf) were in good agreement with direct counts using epifluorescence microscopy (Fig. F1; see also Fig. F61 in the “Site U1365” chapter [Expedition 329 Scientists, 2011b]). However, at greater depths no reliable data could be obtained using Protocol FCM-A because all FCM counts were below MDL. These results indicate that (1) cell abundance in organic-poor sediment of the South Pacific Gyre is overall significantly lower than cell abundances in previously studied organic-rich continental margin sediments (D’Hondt et al., 2009), and (2) Protocol FCM-A needs to be improved by lowering the MDL in order to assess more precisely the microbial biomass in this extreme subseafloor habitat.

The reason for high background signals was unclear at this point. One possible factor is interference by CaF₂ precipitates that remained in the final suspension after density centrifugation. The fluorescent particles were examined with epifluorescence microscopy, but neither visible particles nor SYBR-stained cells were observed, suggesting that the fluorescence interference might instead be caused by unknown inorganic precipitates that could only be detected with the relatively wider signal detection capacity of FCM with a very short excitation time. To solve this problem, it was necessary to improve Protocol FCM-A by addressing possible causes of the interference one-by-one during Expedition 329.

Site U1366

Because FCM measurements from Site U1365 had high background signals caused by potential interference of CaF₂ precipitates, Protocol FCM-A was modified by eliminating the addition of 0.5 M each of calcium chloride and sodium acetate and replacing them with 1.5 M Tris-buffer (see Protocol FCM-B). However, the results from Site U1366 contained remarkably high background signals that interfered with cell-derived SYBR fluorescence. Consequently, no or very little improvement of the results was achieved with Protocol FCM-B. These high background signals cannot result from CaF₂ in Protocol FCM-B. Instead, they may be due to fine chemical particles (e.g., silicates and mineral salts) that are sensitive to pH change during the Tris-buffer neutralization step.

Given the high background signals, FCM counts were ~ 1.5 –2 orders of magnitude higher than microscopic direct counts. Because these abiotic background signals concealed cell-derived SYBR-fluorescent signals, no reliable onboard cell counts using FCM for Site U1366 sediment samples were obtained. Clean-up steps on the flow cytometer were repeatedly performed according to the manufac-

turer's instruction, and the null controls (i.e., 18.2 MΩ water and TE buffer) showed negligible signals of SYBR-specific fluorescence wavelength.

Site U1367

At Site U1367, the sample preparation protocol was refined for FCM-based cell counts. Given the results from previous sites, cell-extract solutions without HF treatment steps were prepared (see "[Protocol FCM-C \(protocol without HF treatment\)](#)"). This acid-wash treatment is standardized for better recovery of cells from smectite-rich sediment on continental margins and significantly reduces fluorescent backgrounds caused by amorphous silica when using an epifluorescent microscope (Morono et al., 2009). For FCM counts in South Pacific Gyre sediment, the protocol without HF treatment was found to reduce background signals, and consequently the MDL was lowered to 2.2×10^3 cells/cm³. Using Protocol FCM-C for Site U1367 sediment samples, a decreasing trend in FCM counts with depth were observed. However, these counts were still ~1.5–2 orders of magnitude higher than direct microscopic counts, suggesting that FCM-cell count numbers using Protocol FCM-C may still overestimate the "true" cell numbers with faint nonbiological (i.e., mineral) fluorescence signals from cell extracts. Despite the low MDL, SYBR-SPAM interfered significantly with identification of SYBR-stained cells for unknown reasons. Nevertheless, the preliminary FCM count indicated that cell abundance in South Pacific Gyre sediment (Sites U1365 and U1366) is overall significantly lower than any other previously studied seafloor sediment.

Site U1368

At Site U1368, the cell-staining steps with the SYBR Green I fluorescent were examined. To examine the extent of background fluorescent signals caused by the SYBR Green I dye, a dilution series of SYBR Green I dye solution (1:200, 1:400, 1:1000, 1:10000, and 1:100000) in a phosphate-buffered saline (PBS) buffer, TE buffer, and 2.5% NaCl solution containing 2% formalin was prepared. These solutions were analyzed by FCM without any addition of cells. The analyses indicated that background signals were unexpectedly high in ~1:1000 dilution in PBS and TE buffers. The pattern of background signals using FCM varied in each solution and appeared to overlap with the area of SYBR-stained cell fluorescence (Figs. [F2A](#), [F2B](#), [F2C](#)). The formation of precipitates in PBS buffer was especially critical because PBS buffer is commonly used for many fluorescent dye-staining experiments in medical and molecular ecological studies. This has not been observed in previous experiments using SYBR Green I (Invitrogen) on shore.

The result clearly indicates that the quality of SYBR Green I dye solution is most likely different between the dye from Lonza Rockland, Inc. (used during Expedition 329), and the dye from Invitrogen (used in previous experiments). Although the background signals in 1:1000-diluted solution with 0.5% NaCl plus 2% formalin were less obvious than those in other buffers (Fig. [F2D](#)), the slightly acidic pH (~5.0) weakened the fluorescence of, or even degraded, SYBR Green I. During Expedition 329, only the SYBR Green I fluorescence dye from Lonza Rockland, Inc., was available.

SYBR Green I-derived precipitates were observed on a 0.22 μm filter under epifluorescence microscopy. The precipitates were obviously not cells; instead they appeared as thin squared or dendritic crystals that produce weak green fluorescence. Because FCM is highly sensitive to even weak fluorescence in extremely short exposure time, these chemical precipitates are automatically detected by FCM as "fluorescent particles," like SYBR-stained cells. However, these backgrounds are clearly distinguishable from cell-derived SYBR fluorescence by morphology and fluorescent intensity; hence, the effect of these background signals on FCM are not critical for microscopic direct counts.

Given these results, the nonbiological SYBR precipitates were washed three times after staining with TE buffer (i.e., over 1:100000 dilution) to remove the chemical precipitates as completely as possible. Using modified Protocol FCM-D, samples from Site U1368 were processed for FCM cell counting. The results are in relatively good agreement with the trend of microscopic direct counts (Fig. [F1](#)). However, FCM background signals were still inconstant, hampering statistically reliable data production under these experimental conditions.

Site U1369

At Site U1369, the background signals produced by SYBR Green I were examined using surface seawater. It was found that SYBR Green I produced by Lonza Rockland, Inc., was highly sensitive to solvent salinity. Visible precipitates of SYBR Green I in both PBS and 3% NaCl were detected in the solutions. In addition, the color and DNA-staining capacity of SYBR Green I disappeared in TE buffer (1:10000) when the solution was stored at 4°C for a few days. This indicates that even though no visible precipitates in TE were observed, SYBR Green I might form crystal precipitates in the solution and interfere with analysis on FCM.

Another finding is that it is extremely difficult to eliminate the SYBR-derived precipitates from the suspension. Although attempts to remove these precipi-

tates by quadruplicate washing with TE buffer were performed, only a slight decrease in the background signals was observed (data not shown). Even worse, often a significant decrease in cell-derived signals (e.g., SYBR-stained *Escheria coli* or seawater cells) during the washing step was observed, likely because of cell destruction caused by osmotic and centrifugation stresses.

Subsequently, Protocol FCM-D with no modification for samples from Site U1369 and kill-control samples from Sites U1365–U1369 was applied. Although reasonable FCM-based cell counts in near-surface sediment (~2 mbsf) were obtained, because of the relatively high number of cells ($>10^4$ cells/cm³), background signals were still high enough to conceal the low abundance of cells in deeper South Pacific Gyre subseafloor sediment.

Sites U1370 and U1371

Given the data described above, a new SYBR Green I solution for further methodological improvements of FCM-based cell counting was required, which is impossible to achieve in the middle of the South Pacific Gyre, the place furthest away from continents (and, consequently, the place furthest away from replacement supplies). Because of this, the onboard FCM experiment was terminated. Further analysis will be carried out at a shore-based laboratory.

Conclusion and future prospects

Using FCM together with microscopic observations for cell counting has confirmed that subsurface sediment in the South Pacific Gyre harbors significantly lower microbial concentrations than previously reported for any other subseafloor sediment. The use of FCM for onboard cell counts was tested for the first time in the history of scientific ocean drilling. This technique has a great potential for high-throughput onboard cell enumeration. In addition to providing more robust estimates of cell abundance, cell size data were also collected. This is significant in that cell size can provide more accurate estimates of biomass than cell abundance. The method appears to work well for relatively high numbers of cells ($>10^4$ cells/cm³) in sediment. However, the method still needs methodological improvement and standardization, including detection sensitivity and quality of chemical reagents, for accurate counts of extremely low cell numbers in subsurface habitats, like South Pacific Gyre subsurface

sediment. In a shore-based study, cell numbers of all sediment samples prepared during Expedition 329 will be recounted with a new method using FCM and verified with other established methods, such as image-based microscopic cell count and microscopic direct count.

Acknowledgments

This research used samples and data provided by the Integrated Ocean Drilling Program (IODP) and is supported in part by the JSPS Strategic Fund for Strengthening Leading-Edge Research and Development (to JAMSTEC).

References

- D'Hondt, S., Spivack, A.J., Pockalny, R., Ferdelman, T.G., Fischer, J.P., Kallmeyer, J., Abrams, L.J., Smith, D.C., Graham, D., Hasiuk, F., Schrum, H., and Stancin, A.M., 2009. Subseafloor sedimentary life in the South Pacific Gyre. *Proc. Nat. Acad. Sci., U. S. A.*, 106(28):11651–11656. doi:10.1073/pnas.0811793106
- Expedition 329 Scientists, 2011a. Methods. In D'Hondt, S., Inagaki, F., Alvarez Zarikian, C.A., and the Expedition 329 Scientists, *Proc. IODP*, 329: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.329.102.2011
- Expedition 329 Scientists, 2011b. Site U1365. In D'Hondt, S., Inagaki, F., Alvarez Zarikian, C.A., and the Expedition 329 Scientists, *Proc. IODP*, 329: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.329.103.2011
- Expedition 329 Scientists, 2011c. Site U1368. In D'Hondt, S., Inagaki, F., Alvarez Zarikian, C.A., and the Expedition 329 Scientists, *Proc. IODP*, 329: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.329.106.2011
- Kallmeyer, J., Smith, D.C., Spivack, A.J., and D'Hondt, S., 2008. New cell extraction procedure applied to deep subsurface sediments. *Limnol. Oceanogr.: Methods*, 6:236–245. <http://www.aslo.org/lomethods/free/2008/0236.pdf>
- Morono, Y., Terada, T., Masui, N., and Inagaki, F., 2009. Discriminative detection and enumeration of microbial life in marine subsurface sediments. *ISME J.*, 3(5):503–511. doi:10.1038/ismej.2009.1
- Morono, Y., and Inagaki, F., 2010. Automatic slide-loader fluorescent microscope for discriminative enumeration of subseafloor life. *Sci. Drill.*, 9:32–36. doi:10.2204/iodp.sd.9.06.2010

Publication: 13 December 2011
MS 329-110

Figure F1. Plots of direct microscopic cell counts in South Pacific Gyre sediment using epifluorescence microscopy and flow cytometry (FCM). Red dotted line indicates minimum detection limit (MDL; see “**Microbiology**” in the “Site U1365” chapter and “**Microbiology**” in the “Site U1368” chapter [Expedition 329 Scientists, 2011b, 2011c]). FCM-based cell count used Protocols FCM-A and FCM-D for Sites U1365 and U1368, respectively. Note that FCM-based counts have no statistic support because nonbiological background fluorescent signals inconstantly appeared in the same fluorescent range of SYBR-stained cells (see text). **A.** Site U1365. **B.** Site U1368.

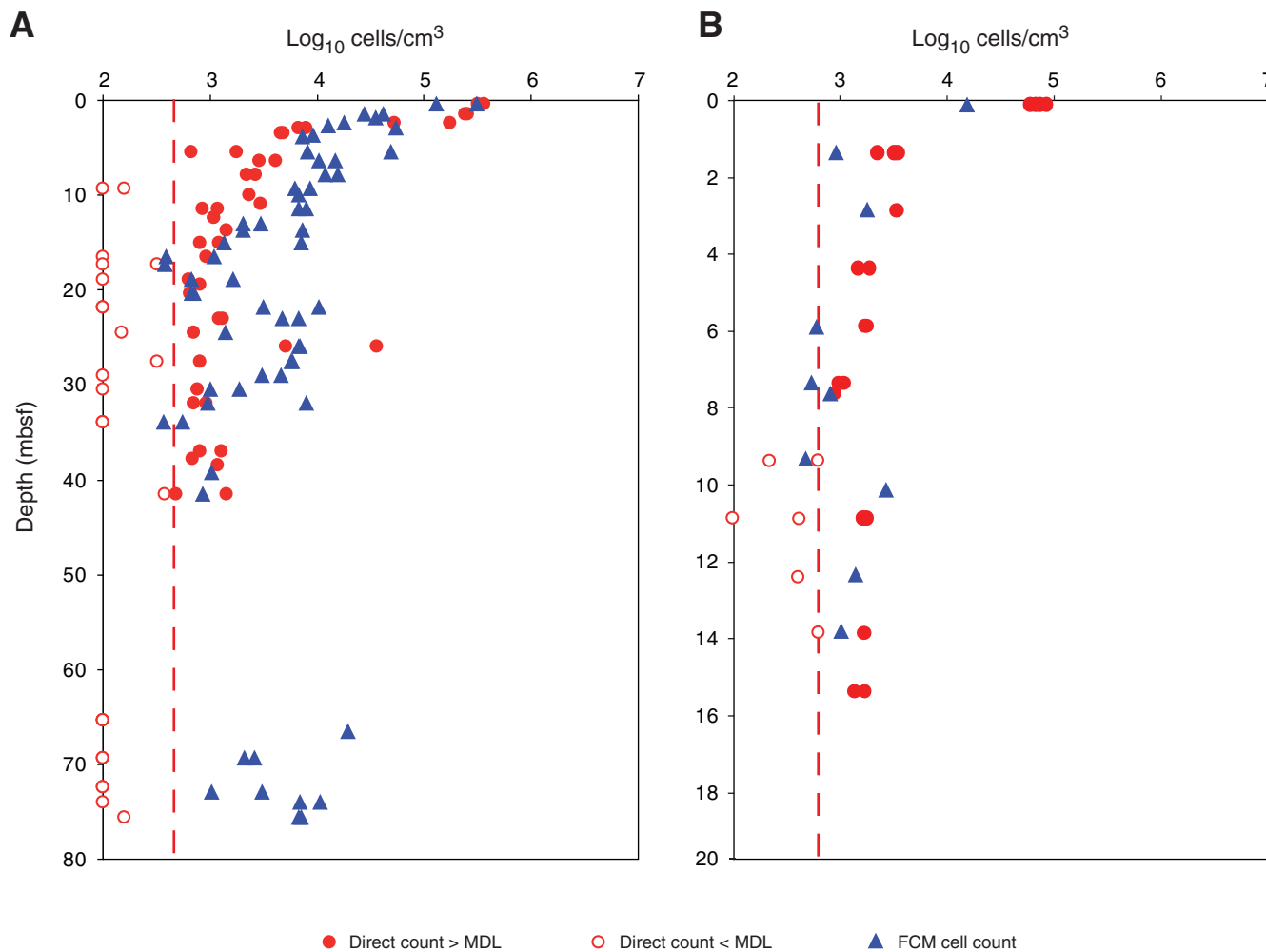


Figure F2. Positive and negative control experiments on flow cytometry using SYBR Green I fluorescent dye. **A.** Surface seawater sample collected from Site U1368. Microbial cells in the seawater sample were stained with SYBR Green I. The high cell density ($\sim 10^5$ cells/mL) does not show significant interference from background fluorescent signals. **B, C.** Cell-free SYBR Green I solution diluted with (B) Tris-EDTA (TE) buffer (1:400) and (C) phosphate-buffered saline (PBS) buffer (1:400). Both negative control samples showed unstable fluorescent background signals derived from unknown chemical reaction of SYBR Green I. These background signals interfere with accurate cell enumeration, especially for low-biomass samples, such as South Pacific Gyre subsurface sediment. **D.** Image of SYBR Green I-derived precipitates in TE buffer, PBS buffer, and 2.5% NaCl + 2% formalin solution. Even at 1:1000 dilution, red to orange precipitates are visually observed. These precipitates are barely removable by washing steps and hence significantly hamper detection of cell-derived fluorescence using flow cytometry.

