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ORIGINAL PAPER

An Acridine Orange Spore Germination Fluorescence Microscopy Versus Spectral Paradox

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Abstract The metachromatic fluorophore acridine orange (AO) has demonstrated green fluorescent staining of dormant *Bacillus* spores and orange to red staining of transcriptionally active vegetative cells when used in the mid-micoMolar range. Despite the microscopic observation of numerous bright orange to red fluorescent vegetative cells following germination induction, no clear spectral emission peaks > 590 nm have ever been reported for spectrofluorometric analysis involving AO in conjunction with spore germination. This microscopy versus spectrofluorometry paradox is documented in the present report and hypotheses are put forth to explain the very weak spectral changes in the red region which do not appear to correlate with the abundant orange-red fluorescence of nascent vegetative cells seen through the fluorescence microscope.

Keywords Acridine orange · *Bacillus* · Fluorescence microscopy · Germination · Metachromatic · Quench · Spectrofluorometry · Spore

Introduction

Interest in detecting and tracking bacterial endospore germination stems from purely academic curiosity to a desire by military and civilian organizations to rapidly assess *B. anthracis* spore viability after decontamination procedures. Effective decontamination of building interiors and fomites can prevent inhalation anthrax, but ineffective decontamination could be life-threatening for exposed individuals. Nowhere was this need more dramatically illustrated than in the very expensive and time-consuming decontamination of Senator Tom Daschle's Capitol Hill offices after the 2001 anthrax-laced mailings. This point was further illustrated by the more recent CDC failure to decontaminate *B. anthracis* spores in highly regulated laboratories. Therefore, a simple and portable fluorescence method to assess spore viability by detection of induced germination could be of value in such scenarios.

In the 1990's two groups [1, 2] published the use of the differential metachromatic fluorescent dye acridine orange (AO) to assess spore viability and germination by fluorescence microscopy. AO is primarily known as a differential fluorophore which intercalates into double-stranded (ds) DNA to produce green fluorescence emissions centered around 530 nm. Simultaneously, AO can stain single-stranded (ss) DNA or messenger (m)RNA by complexing with the ss nucleic acids and stacking in metabolically active cells to produce a red emission peak at ~640 nm in addition to any 530 nm green fluorescence [1, 3, 4]. It is not entirely clear what structures stain green in or on *Bacillus* spores, but for uninduced dormant spores, the characteristic green fluorescent ring or "oval" staining pattern around the spore's perimeter strongly suggests surface or subsurface staining of the spore coat or cortex [5]. Once germination is induced by specific nutrients, however, the endospore's ds DNA-filled core begins to become permeable as it sheds dipicolinic acid and absorbs water [6]. Thus, ds DNA in the core of newly germinated spores could also be stained green by AO and contribute the to 530 nm emission peak. The germinated spores tend to take on a more uniformly stained green fluorescent appearance throughout the entire spore cross section during early germination [1, 6].

In Bruno and Mayo's studies [1], a simple 2 % dextrose solution was used to induce *Bacillus* spore germination [7, 8] which resulted in observation of primarily nascent orange-red vegetative cells and green fluorescent dormant or ungerminated spores. Over time, the red signal of an RGB microscope

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camera rose, but not dramatically [1]. Subsequent unpublished spectrofluorometric analyses of *Bacillus* spore germination with AO in a dextrose solution by Bruno and Mayo failed to produce any increase in the red fluorescence signal despite the presence of copious red vegetative cells seen under the fluorescence microscope. Bruno and Mayo did not spend much time on the spectrofluorometric assessment and attributed this spectral anomaly to sub-optimal germination conditions such as insufficient nutrients in the 2 % dextrose solution as well as a lack of oxygenation in the cuvette and room temperature incubation during spectrofluorometry [1].

The present report revisits Bruno and Mayo's original work [1] and further explores the paradox of concentrated bright red vegetative cells seen under a fluorescence microscope which appear to have little or no impact on fluorescence spectra beyond 550 nm when the same samples are evaluated with a spectrofluorometer. Mason and Lloyd [4] have shown that orange-red AO fluorescence beyond 550 nm is correlated with metabolic activity and bacterial viability. In the present work, the author reinvestigates this phenomenon, documents its existence, and attempts to explain the fluorescence microscopy versus spectrofluorometry paradox in terms of spectral emission overlap and the metachromatic nature of AO.

Materials and Methods

Stock 2 % acridine orange (AO) in deionized water was purchased from Sigma-Aldrich Corp. (St. Louis, MO) and diluted 1:1,000 in various sterile culture media to give a 66.3 μM final concentration. *Bacillus thuringiensis* (BT, kurstaki strain) spores were provided by the U.S. Air Force Research Laboratory at Brooks City-Base, TX and kept dried and frozen at −20 °C. Sterile 200 ml Erlenmeyer flasks were loaded with 100 ml of sterile PBS, 2 % dextrose in PBS, or tryptic soy broth (TSB) and 5 mg of BT spores were added to each culture flask. Flasks were stuffed with sterile cotton and placed on an incubated shaker at 37 °C with orbital mixing at 100 rpm.

At various times after inoculation as indicated in the text, 4 ml samples were drawn from each of the three flasks to evaluate fluorescence spectra after addition of 4 μl of the 2 % stock AO solution for 15 min at room temperature with gentle mixing in capped clear polysytrene cuvettes (Thermo Fisher Scientific No. 14-955-129). Fluorescence spectra were obtained by use of a Cary Varian Eclipse spectrofluorometer with excitation at 460 nm (5 nm slits), emission scans from 470 to 700 nm, and a photomultiplier tube (PMT) setting of 500 V. The 460 nm excitation was selected to simultaneously monitor changes in the anticipated green (530 nm; ds DNA) and red (640 nm; ss mRNA) emission peaks.

In some experiments, BT spores and vegetative cells were exposed to 66.3 μM AO for 15 min at room temperature and then washed once or twice in PBS for 5 min and pelleted by centrifugation at 14,500×*g* for 5 min followed by resuspension of the cellular pellet to the original 4 ml in sterile PBS and analyzed by spectrofluorometry as described above. Unstained 1 ml samples were also drawn from each flask at the indicated times post-inoculation and used to evaluate overall growth of each culture by absorbance at 600 nm using a Spectronic GENESYS 10 UV/Visible spectrophotometer which had been blanked against each type of medium (i.e., PBS, 2 % dextrose in PBS, and TSB) as appropriate.

Immediately following spectrofluorometric analyses, a 50 μl sample of well-mixed culture from each of the treatment groups was placed on a glass slide and overlaid with a coverslip for fluorescence microscopic observation at 1,000×. Color digital images were acquired with an AmScope FM 320 fluorescence microscope (United Scope, LLC, Irvine, CA) in either phase-contrast or fluorescence mode using a 490 nm excitation filter and 530 nm long pass emission filter. Images were captured using a Moticam 2000 (2.0 Mega pixel) digital camera (Motic Instruments, Inc., Richmond, British Columbia, Canada) and Motic image analysis software.

The only exception to these experimental descriptions applies to Fig. 1 in which the author's own leukocytes were stained with AO and analyzed by spectrofluorometry and fluorescence microscopy as described in Bruno et al. [9]. The leukocyte staining technique involved the use of 0.25 μM AO in whole blood for 20 min.

Results

Figure 1 illustrates a clear trimodal fluorescence emission spectrum for AO-stained human leukocytes with a clear green emission peak for lymphocytes centered at 520–530 nm and orange-red peaks for granulocytes centered at 550–560 nm and 600 nm. As noted in the Materials and Methods section, leukocytes were stained with less AO (0.25 μM) [9] than the 20 μM AO which Bruno and Mayo originally used on germinating *Bacillus* spores [1] or the 66.3 μM AO final concentration used in the present studies. The author explored AO concentrations as low as 13.25 μM, but noted only green vegetative *Bacillus* cells at the 13.25 μM concentration (data not shown) perhaps because AO could not penetrate the vegetative cells to reach mRNA at this lower concentration without the aid of a detergent to permeabilize the cells [4].

Figure 2 illustrates the repeated observation of increased fluorescence in the orange-red region (>590 nm) from day 0 to days 1 and 2 post-germination induction with 2 % dextrose or TSB. However, unlike the trimodal spectrum in Fig. 1, the curves in Fig. 2 are smooth from 590 to 700 nm and show no distinct spectral peaks in the orange-red region. Curiously too, the 2 % dextrose curves on days 1 and 2 were much higher than the corresponding day 1 and 2 spectra for spores

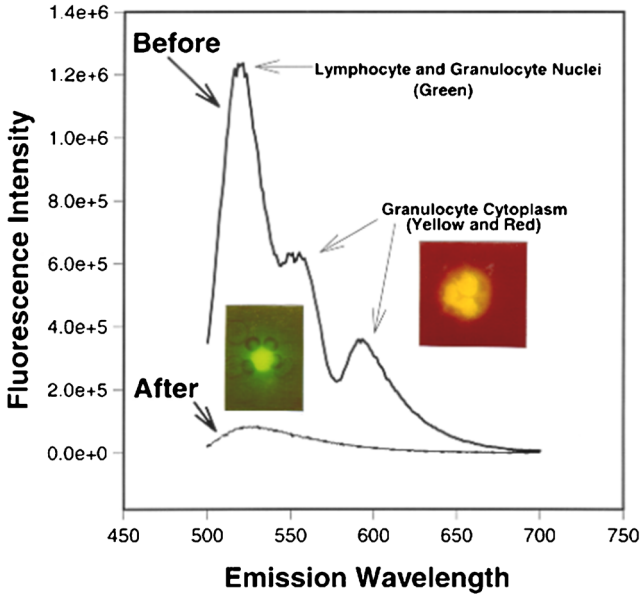

Fig. 1 Fluorescence spectra of AO-stained whole human blood ("before") showing the green fluorescing lymphocyte population at ~530 nm and orange to red fluorescing granulocytes at 550–560 nm and ~600 nm. The "after" spectrum reflects fluorescence after immunomagnetic separation of T lymphocytes as described in reference 9

germinated in TSB which prompted the author to observe the cells under 1,000× phase-contrast microscopy and to compare their overall growth (absorbance values at 600 nm).

The TSB culture appeared much more turbid grossly than the 2 % dextrose culture and this difference in growth was corroborated by the absorbance curves and phase-contrast microscopic observations shown in Fig. 3. Although dextrose can trigger *Bacillus* spore germination [1, 7, 8], dextrose alone is not sufficient to sustain growth for very long unless some residual broth nutrients were present in the BT spore stock. The nutrient deficiency of the 2 % dextrose culture therefore accounts for the paucity of sickly appearing bacilli in the 2 % dextrose photomicrographs in the upper left corner of Fig. 3 and the large difference in growth versus TSB documented on the right side of Fig. 3. Thus, the difference in the peak intensity for day 1 and 2 dextrose-treated cells versus TSB treated cells in Fig. 2 is probably attributable to the dark TSB medium blocking or quenching some of the AO fluorescence emissions. The 2 % dextrose is transparent in the visible range and surely transmits AO fluorescence much better than the dark TSB medium.

Based on the poor growth of 2 % dextrose cultures, the author eliminated the 2 % dextrose treatment group from further experiments. Figure 4. presents results which validate the green staining of ungerminated AO-stained *Bacillus* spores and abundant orange to red vegetative cells in the one day TSB-cultured group and further validate Bruno and Mayo's original published observations [1]. An unstained BT spore population was also examined under the fluorescence microscope (image not shown) to determine the level of green background autofluorescence. However, no spore autofluorescence could be detected visually at the same digital camera exposure and gain settings used for Figs. 4a and b.

In a final experiment, the author attempted to wash one day-germinated vegetative cells from the TSB-induced group in PBS as shown in Fig. 5. One wash appeared to produce a slight shoulder in the red region of the emission spectrum in Fig. 5, but this shoulder was lost after a second wash (Fig. 5). The 2× washed AO-stained TSB-cultured sample yielded much stronger fluorescence than the 1× washed sample in Fig. 5 suggesting some self-quenching of the AO dye in or on the germinated vegetative cells (i.e., fluorescence intensity increases as some of the AO washes out of cells and ceases to self-quench).

Discussion

Fluorescence microscopy of AO-stained germinating *Bacillus* spore populations ([1, 2] and Fig. 4) might very well lead one to believe that the emergence of orange-red vegetative cells should easily be observed as a strong red emission peak at ~ 640 nm by spectrofluorometric analysis which could be useful for evaluating the germination state of the population as a whole. However, a strong orange-red peak >590 nm does not occur as illustrated herein (Figs. 2 and 5). The data presented in this report are representative of the author's many other failed attempts to observe a red spectral fluorescence peak at ~ 640 nm for germinating spores. Indeed, other than a modest increase in the emission curve's shoulder in the red region as a function of time, the author has never observed a distinct peak in the red emission region for germinated AO-stained vegetative cells. The lack of a bimodal emission spectrum, except in the case of AO-stained mixed leukocyte populations (Fig. 1), is somewhat baffling, especially since the orange-red emissions under the fluorescence microscope appear quite strong and distinct to the eye versus the green ungerminated spores (Fig. 4a). However, even in transparent dextrose solution or with washed bacteria (Fig. 5) a clear red peak in the >600 nm range is lacking by spectrofluorometry.

The author had originally wanted to use AO staining of germinating *Bacillus* spores to serve as a sort of fluorescence spectral alarm for military personnel that *B. anthracis* spores in the environment were present and viable [1]. Unfortunately, the author has never been able to distinguish clear evidence for the red emission correlating with mRNA production and transcription except by fluorescence microscopy which is much less convenient and less field deployable than a portable fluorometer. The author originally attributed this paradox to the nutrient poor environment of 2 % dextrose medium and lower oxygen levels in a non-aerated cuvette [1]. However, the present studies were performed in part with bacteria grown in nutrient-rich TSB in well-aerated shaker cultures which

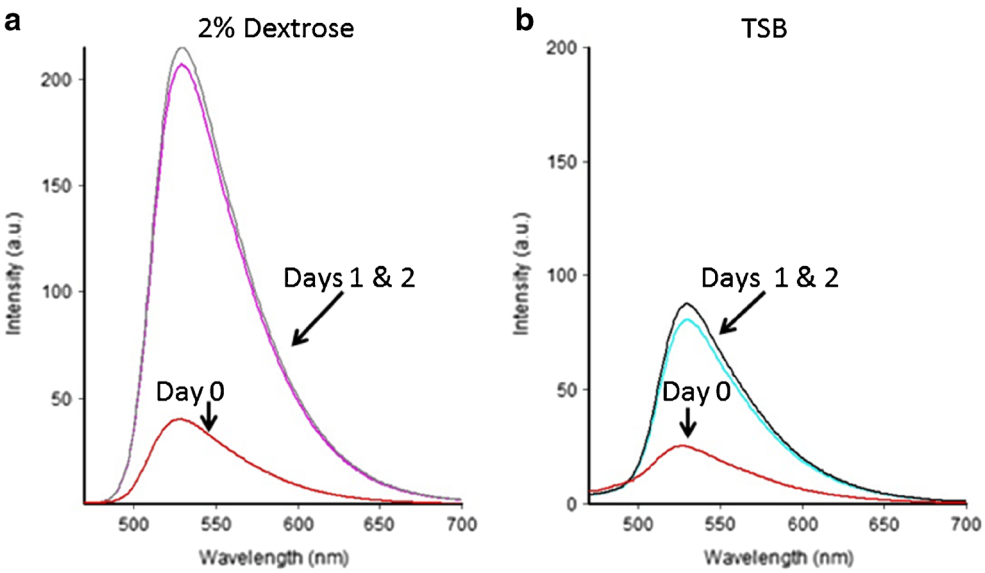

Fig. 2 Fluorescence spectra of BT spores germinated in (**a**) 2 % dextrose in PBS or (**b**) TSB shaker cultures over the course of 2 days. Excitation was at 460 nm and the PMT was set at 500 V throughout

produced many more healthy vegetative cells than their 2 % dextrose-fed counterparts (Fig. 3), but still no red peak was observed by spectrofluorometry even after washing cells from the AO-stained TSB shaker cultures (Fig. 5). This paradox cannot be resolved in terms of physical or chemical quenching of red AO fluorescence by interfering species in the medium [10, 11] either, because one can clearly see orange to red fluorescent vegetative cells under a fluorescence microscope (Fig. 4) in TSB with or without washing in clear PBS buffer and yet the spectrofluorometer detects only a mild fluorescence increase (not a peak) in the red region of the spectrum after one day's growth (Fig. 2) without or with washing (Fig. 5).

The only reasonable explanation in whole or in part appears to be that the expected red peak is buried in the fluorescence emission spectrum's shoulder beyond 590 nm (i.e., overlaps significantly with AO's green-centered emission spectrum tail). Some authors have discussed the buried or obscured red peak for AO-stained chromosome or platelet analyses and even deconvoluted the observed emission spectrum to extract the putative red peak attributable to AO's interaction with ss nucleic acids [3, 12–14].

Still, the question of why a bimodal emission curve like the one seen in Fig. 1 can sometimes be obtained for AO-stained materials or populations and other times it cannot be obtained remains enigmatic. While the smooth unimodal spectra from 590 to 700 nm documented in this report for germinated spores (red vegetative cells) exposed to AO speak to a continuum of metachromatic fluorescence emissions, it still seems quite odd that a noteworthy red peak does not emerge from a population of washed red cells. The author has

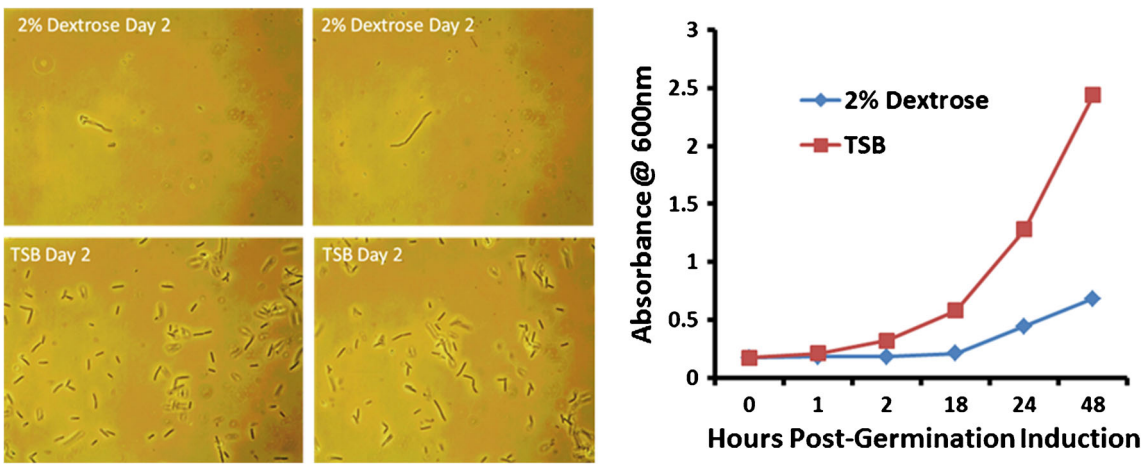

Fig. 3 Left –phase-contrast images at 1,000× comparing populations of germinated BT vegetative cells on day 2 of growth in 2 % dextrose or TSB, Right – growth curves for the 2 % dextrose vs. TSB populations over 2 days in shaker cultures expressed as absorbance at 600 nm of suspended cells. A_{600} readings were blanked against 2 % dextrose in PBS or TSB as appropriate. Spores incubated in PBS did not grow or exhibit an increase in A_{600} values and were therefore not graphed

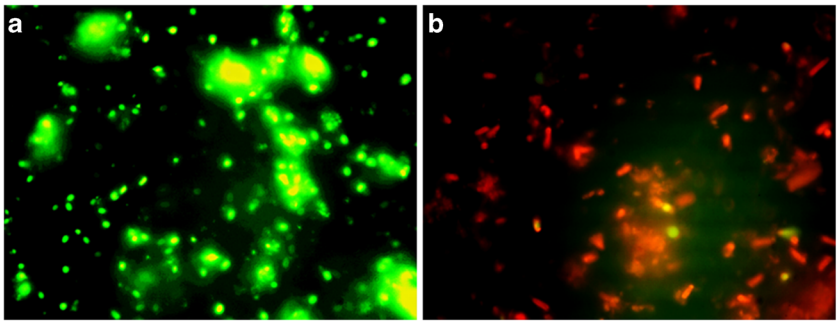

Fig. 4 Fluorescence images of (**a**) predominately green AO-stained ungerminated BT spores and (**b**) a predominately red one day TSB shaker culture of BT vegetative cells at 1,000×. An unstained spore population was also examined (image not shown) to determine the level of green background autofluorescence, but no spore fluorescence could be seen at the same digital camera exposure and gain settings used for panels **a** and **b**

published other spore studies in which populations of red fluorescing *Bacillus* spores have produced distinct peaks in the red region beyond 600 nm [15]. One might argue that the spectrofluorometer was simply not sensitive enough in the red region of the spectrum, but the same spectrofluormeter has been used to detect TYE 665, Amplex Ultra Red and red quantum dots with very high sensitivity in the past [15–18].

Regardless of the reason for the lack of bimodality in the fluorescence spectrum of AO-stained germinated *Bacillus* bacteria, it is clear that AO is not a good choice for spectral detection of germination unless microspectroflorometry of microscopic images is employed [1, 3, 12, 13]. A far better alternative appears to be the use of SYTO 9, 10, 13 or 16 dyes to rapidly detect changes in *Bacillus* spore cortex permeability and the staining of spore DNA upon germination induction by fluorescence microscopy, fluorometry or flow cytometry as reported by other investigators [6, 19–21].

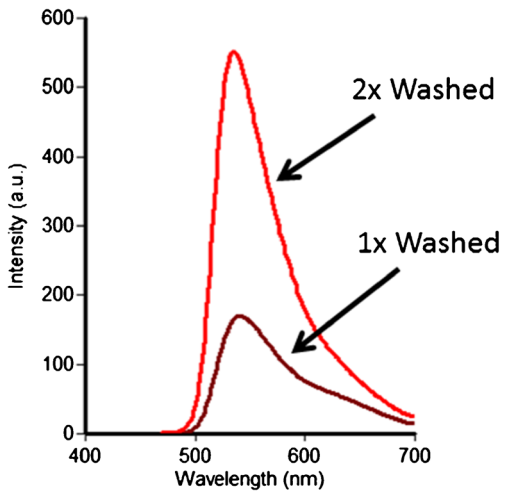

Fig. 5 Spectrofluorometric comparison of once (1×) and twice (2×) PBS-washed AO-stained BT vegetative cells following one day of shaker culturing in TSB. Excitation was at 460 nm and the PMT was set at 500 V

References

1. Bruno JG, Mayo MW (1995) A simple color image analysis method for assessment of germination based on differential fluorescence staining of bacterial spores and vegetative cells by acridine orange. Biotechnic Histochem 70:175–184
2. Sharma DK, Prasad DN (1992) Rapid identification of viable bacterial spores using a fluorescence method. Biotechnic Histochem 67: 27–29
3. MacInnes JW, Uretz RB (1966) Spectral analysis of the binding of acridine orange to polytene chromosomes. Proc Natl Acad Sci U S A 55:1109–1113
4. Mason DJ, Lloyd D (1997) Acridine orange as an indicator of bacterial susceptibility to gentamicin. FEMS Microbiol Lett 153: 199–204
5. Schichnes D, Nemson JA, Ruzin SE (2006) Fluorescent staining method for bacterial endospores. Microscope 54:91–93
6. Kong L, Zhang P, Yu J, Setlow P, Li Y (2010) Monitoring the kinetics of uptake of a nucleic acid dye during the germination of single spores of *Bacillus* species. Anal Chem 82:8717–8724
7. Racine FM, Dills SS, Vary JC (1979) Glucose-triggered germination of *Bacillus megaterium* spores. J Bacteriol 138:442–445
8. Shay LK, Vary JC (1978) Biochemical studies on glucose initiated germination in *Bacillus megaterium*. Biochim Biophys Acta 538: 284–292
9. Bruno JG, Yu H, Kilian JP, Moore AA (1995) Development of an immunomagnetic assay system for rapid detection of bacteria and leukocytes in body fluids. J Molec Recogn 9:474–479
10. Komiyama T, Miwa T, Koizumi M (1966) Studies of the quenching of the fluorescence of acridine orange from the standpoint of charge-transfer. Bull Chem Soc Japan 39:2597–2602
11. Liu SP, Sa C, Hu XL, Kong L (2006) Fluorescence quenching method for the determination of sodium carboxymethyl cellulose with acridine yellow and acridine orange. Spectrochim Acta Part A 64:817–822
12. Golden JF, West SS, Echols CK, Shingleton HM (1976) Quantitative fluorescence spectrophotometry of acridine orange-stained unfixed cells: potential for automated detection of human uterine cancer. J Histochem Cytochem 24:315–321
13. Mahieu-Williame L, Falgayrettes P, Nativel L, Gall-Borrut P, Costa L, Salehzada T, Bisbal C (2010) Near-field microscopy and fluorescence spectroscopy: application to chromosomes labelled with different fluorophores. J Microscop 238:36–43
14. Popov EG, Gabbasov ZA, Gavrilov IY, Pozin EY, Mejlumian AG (1987) Accumulation of acridine derivatives by intact platelets. Thromb Res 47:639–645

15. Bruno JG (2012) Carrillo MP (2012) Development of aptamer beacons for rapid presumptive detection of *Bacillus* spores. J Fluorescence 22:915–924
16. Bruno JG, Carrillo MP, Phillips T, Hanson D, Bohmann JA (2011) DNA aptamer beacon assay for C-telopeptide and handheld fluorometer to monitor bone resorption. J Fluorescence 21:2021–2033
17. Bruno JG, Richarte AM, Phillips T, Savage AA, Sivils JC, Greis A, Mayo M (2014) Development of a fluorescent enzyme-linked DNA aptamer-magnetic bead sandwich assay and portable fluorometer for sensitive and rapid *Leishmania* detection in sandflies. J Fluorescence 24: 267–277
18. Bruno JG, Phillips T, Carrillo MP, Crowell R (2009) Plastic-adherent DNA aptamer-magnetic bead and quantum dot sandwich assay for *Campylobacter* detection. J Fluorescence 19:427–435
19. Cronin UP, Wilkinson MG (2007) The use of flow cytometry to study the germination of *Bacillus cereus* endospores. Cytometry Part A 71A:143–153
20. Welkos SL, Cote CK, Rea KM, Gibbs PH (2004) A microtiter fluorometric assay to detect the germination of *Bacillus anthracis* spores and the germination inhibitory effects of antibodies. J Microbiol Methods 56:253–265
21. Laflamme C, Lavigne S, Ho J, Duchaine C (2004) Assessment of bacterial endospore viability with fluorescent dyes. J Appl Microbiol 96:684–692