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Immunoassay/Vital Stain System (IVSS) Proof-of-Concept Final Report

Authors

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December 1999

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Environmental Protection Department
Environmental Restoration Division

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1. Introduction

Certain species of bacteria and viruses are a human health concern due to their potential to cause infection and disease. Diseases caused by microorganisms are well known; *Salmonella* and *E. coli* organisms are two well known examples. It is this infectivity and potential to cause disease that make bacteria and viruses useful as potential weapons of mass destruction. Concern over the use of bacteria and viruses as biological warfare agents currently has a high degree of public and congressional concern. Frequent news reports concerning suspected caches of biological agents in the hands of private citizens for possible terrorist activities, or the possible use of biological agents on troops abroad has further increased public anxiety.

Many government agencies have responded to this threat by putting in place programs to develop technologies for the rapid detection and identification of biological warfare (BW) agents. Most of these technologies rely on genomic or antibody characterization (Olsen & Rice 1996; Johns *et al*. 1994). DNA/RNA-based identification techniques are typically variants of polymerase chain reaction (PCR) methods. In PCR, a small amount of organism's DNA or RNA is amplified, providing a large enough quantity of genetic material to determine base-pair sequences. These base-pair sequences are then compared to a library of such sequences, and the resulting match identifies the organism. Immunological assays are based on creating antibodies to the particular organism of interest and conjugating that antibody to a fluorescent or colored probe. Reaction of the antibody/stain conjugate with the target organism results in a characteristic fluorescence or visible color, indicating the presence of the organism. However, these detection technologies do not determine viability of the organism. For these organisms to be infective and cause disease, they must be viable, that is, alive. This leaves open the possibility of a positive detection of the presence of a biological agent, but should the organism be non-viable, its presence does not provide a human health threat. Therefore, we conducted work to show proof-of-concept for a detection technology that could provide simultaneous agent identification and viability status.

1.1. Microorganisms Of Interest

Microorganisms are ubiquitous inhabitants of all environments. They take many different forms, with their common thread being that they are too small to be seen with the naked eye. There are three different general classes of microbes: (1) bacteria, (2) spores (the dormant form of bacteria), and (3) viruses. The focus of our proof-of-concept work was on bacteria, using species with and without the ability to form spores.

Bacteria are fairly simple organisms that have the ability to reproduce (RNA, DNA) and the machinery to convert food sources into energy (ATP). They lead uncomplicated lives of eating and replicating. Spores are dormant forms of bacteria that are formed when environmental conditions become unfavorable. Not all bacteria form spores, but those that do have a competitive advantage over their neighbors. Spores are made of several thick protective layers, somewhat akin to nutshells, with the actual vegetative material buried deep within; they are metabolically inactive and have tremendous heat, chemical and radiation resistance. When

conditions become favorable (i.e., when there is available food), spores germinate and become vegetative cells once again.

1.2. What Is Viability?

In recent years, techniques in molecular biology have greatly enhanced our ability to identify the presence of specific bacteria. Both immunological assays and DNA/RNA-based techniques allow for the rapid identification of an organism when only minute amounts of genetic material are present (Olsen & Rice 1996; Johns *et al*. 1994). However, the mere presence of an organism says nothing about its ability to cause disease. The potential to cause disease is directly related to the organism's ability to grow and/or reproduce, which results in either the production of toxins (as in the case of bacteria) or host cell death due to viral DNA/RNA replication. In other words, the organism must be "viable" to cause disease. Thus, viability is an important characteristic of microorganisms, and a general operational definition of viability is useful to guide the development of techniques to rapidly determine viability.

Non-spore forming bacterial cells (as well as the vegetative cells of spore-forming bacteria) are probably the most straight forward to consider when developing an operational definition of viability. These organisms are easily thought of as "alive" in the most common use of the term. Each cell is a unique, independent organism which has an active metabolism to utilize food sources to gain the energy required for growth and cell division. While it is possible for such bacteria to go into a dormant, resting, or cryptobiotic state in response to specific environmental stresses (Russell *et al*. 1997), growth and reproduction will recommence once the organisms again find themselves in a favorable environment. For these bacteria, we propose an operational definition of viability to be the ability of the bacteria to undergo cell division. In other words, a viable bacteria cell is one that can undergo cell division given the appropriate environment. The advantage of such a definition is that it incorporates the ability of the organism to grow, as a minimum size is required for cell division.

The concept of viability when applied to spores is more difficult. Because spores are in a resting state, or a period of quiescence (analogous to that found in the seeds of plants), it is difficult to speak in terms of whether the spore is alive or dead. However, once spores are placed in favorable environmental conditions, they desporulate (germinate) and produce vegetative cells. Therefore, the most useful operational definition of a viable spore is one that will germinate into a vegetative cell under favorable environmental conditions.

1.3. Need For Viability Detection Systems

Viability is an important characteristic of the biological threat agent that must be determined in order to adequately respond to a biological threat agent attack. The rapid determination of viability would significantly enhance our ability to detect and respond to a potential threat (Carlsen & Vanderberg, 1998). Col. James Mobley pointed out in his 1995 article "Biological Warfare in the Twentieth Century: Lessons from the Past, Challenges for the Future," that "Reliable verification procedures... need to be developed," (Mobley, 1995). Indeed, methods for verifying decontamination following a terrorist biothreat agent attack are lacking well behind our development of new and rapid biological sensors that do not determine the viability of the agents they detect. Potential users of a viability sensor include first responders to a suspected terrorist

incident, inspectors investigating high risk areas, and decontamination teams attempting to restore an area after a biological warfare event. These potential users may be either civilian or military.

Major General John Doesburg, Commanding General, US Army Soldier Biological and Chemical Command, SBCCOM (formerly CBDCOM) and former Joint Program Manager for the Joint Program Office for Biological Defense, strongly believes in the necessity for developing rapid viability detection technologies. In a recent e-mail to Dr. Vanderberg (Vanderberg, 1999 a) MG Doesburg wrote, "Today our ability to detect biological warfare (BW) agents has several limitations, but two stand out as critical. First we must rely on time consuming, redundant processes to ensure we have correctly identified a BW agent, and, second, after identified, we cannot tell whether the agent (bacteria, virus, toxin, etc.) is alive or dead (viability). Viability detectors are particularly critical from a Domestic Preparedness/Homeland Defense perspective. Our first responders must be able to make quick decisions – what has happened and how to treat this potentially catastrophic event. Without being able to clearly determine the viability of a BW agent, incorrect decisions can and will be made that may further compound a disastrous situation. The same vignette applies to the battlefield, where not only treatment decisions, but decisions on individual protection (Mission Oriented Protective Posture, MOPP), collective protection, decontamination, and contamination avoidance must be made. The consequences on Operational Tempo (OPTEMPO) can and will be dramatic."

1.4. Impact To Potential Users

First responders. Information on viability collected at the time of discovery can assist first responders or intelligence organizations in determining how potentially dangerous the suspected agent is. For example, a terrorist group could send a vial of unknown liquid to a government organization claiming it to be a lethal biological agent and threaten to release the substance unless some demands are immediately met. However, the vial could simply contain dead agent. A similar scenario could be imagined from a group claiming to release a biological agent in a public area with intent on creating infrastructure chaos without the loss of human life. Under both scenarios, antibody and DNA/RNA techniques (such as polymerase chain reaction) would confirm the presence of the biological agent, but could not determine if the agent was viable. In this case, the important questions are "is the agent alive?" (any of it), or "is it dead" (all of it). The expense of mobilizing a decontamination team, the infrastructure damage and chaos resulting from treating a large public area (such as a subway), and the impact to the psyche of the public of such an activity are all substantial, and totally unnecessary if the organisms were non-viable. This is the key question first responders must answer: do they need to do anything?

Additionally, it has been noted that some terrorist groups have enjoyed the threat of a "false alarm" incident. Taking this one step further by actually dispersing "dead" agent (easily done in the case of vegetative bacterial agents) would give them the ability to shut down major transportation systems (i.e., Washington Metro, New York subway, etc.) as well as impact other key diplomatic activities (i.e., Congress/Capitol Building, United Nations, etc.). This could be accomplished with minimal long-term negative publicity since no lives would be lost. However, the incident would scare the public, cost money, and cause time delays while we all wait at least 48 hours for the initial results from laboratory culturing of samples.

Inspectors of high risk areas. During inspection of high risk areas or suspected biological weapons manufacturing facilities, viability would be an important piece of information for surveillance or inspection organizations. It would provide an indication as to the potential threat should the agent be released. In addition, it could provide intelligence concerning the competency of organizations attempting to create biological weapons, and how advanced their biological weapon capabilities are. In this case, the percentage of the viable threat agent is very useful information. A viability detector could also be applied to characterizing other cellular properties important for developing clinical treatments for those exposed to the threat agent. Antibiotic susceptibility, for example, could be rapidly determined by measurements of loss of viability after short-term exposures of the agent to different antibiotics (Carriere et al., 1997; Pearson et al., 1996).

Decontamination teams. Decontamination efficacy must be confirmed following cleanup of a biologically contaminated site. Following an initial decontamination pass, samples would be collected, returned to the laboratory, and cultured. After several days, any indication of viable organisms would necessitate remobilizing the decontamination team, retreating the area, and again sampling and laboratory culturing. This cycle may need to be repeated several times, stretching the decontamination response out from several days to weeks. Obviously, during this time, the area would need to remain secure. Therefore, the expense of multiple mobilizations of the decontamination team, retaining the team on-site, and keeping the site secure for an extended length of time would be substantial. With the ability to rapidly determine viability, several such decon/testing/decon cycles could be conducted in a single day, with laboratory culturing validation upon completion of the final cycle. This would also eliminate the need for chain-of-custody, preservation packaging, and removal/transportation to an off-site location for laboratory testing (culturing) of potentially large numbers of samples.

1.5. Current State Of The Art

Current technologies to determine biological agent viability are either very slow or non-specific. Laboratory culturing represents the baseline technology, although other technologies do exist. Despite the variety of techniques available for enumeration of viable microbes, none of the accepted clinical tests fulfill a requirement for truly rapid detection (Ulitzur & Kuhn, 1987). Laboratory culturing involves collecting a sample from the suspected affected area (such as a swipe, swab, washing, etc.), introducing the sample into a growth medium specific to the suspected agent, incubating the sample in the laboratory, and enumerating cells or colonies over time. Depending on the organism, this could take several days or longer. However, even on selective growth media, growth of mixed populations may result in false positives from the presence of the indigenous organisms, depending on the existing environmental conditions. Table 1.5 illustrates the numbers of culturable organisms present on a variety of surfaces in an office and a laboratory environment after 24 h and 5 days incubation (Vanderberg 1999b).

Table 1.5. Background levels of culturable organisms in an office and laboratory environment.

Sampling location	Viable microbes (CFU/mL)*	
	24 h	5 days
Computer keyboard	320	650
Copy machine	30	290
Top of bookshelf	0	10
Wall in office	0	0
Sink countertop in lab	200	300
Lab bench	270	290
Lid on –70° freezer in lab	30	40
Lid on toolbox in instrument room	0	40
Control	0	0

*5 x 5 cm areas were swabbed with a sterile cotton swab moistened with phosphate buffer, transferred into 1 mL phosphate buffer and plated on Nutrient Agar. CFU = colony forming unit.

1.6. Non-Specific Viability Detection

ATP production. One of the more recent developments in detection of viable microbes has been in the detection of adenosine triphosphate (ATP) (Carlsen and Vanderberg 1998). ATP is the principal energy carrier in all cells, and only living organisms can produce this molecule. As shown in Figure 1.6a. above, when a cell dies, its ATP is rapidly degraded. For this reason, the determination of ATP can be used to selectively quantify viable cells.

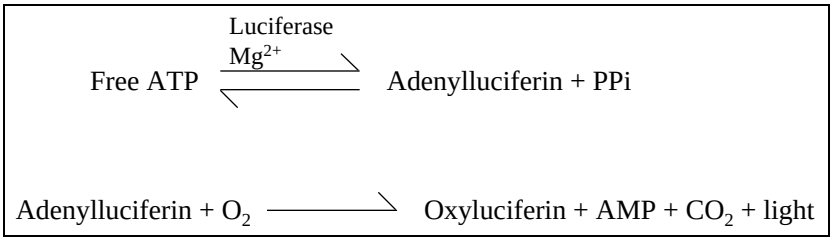

Figure 1.6a. ATP degradation and light production.

ATP detection, as an indicator of viability, has been useful for assessing disinfection and sanitation levels in the health care, pharmaceutical and food industries (Karl, 1980). Monitoring of meat slicer cleanliness, detection of contamination on beef carcasses, and prediction of pasteurized milk shelf life are among the recent applications of the ATP bioluminescence assay (Bautista et al., 1992; Siragusa et al., 1995; Seeger & Griffiths, 1994; Murphy et al., 1998). In clinical settings, this assay has been effectively employed to detect bacterial contamination in blood and urine samples (Karl, 1980). Recently, it has been used to characterize viable *S. cerevisiae* from aerosols (Stewart et al., 1997). ATP bioluminescence assay units are commercially available from companies such as New Horizons Diagnostics. In fact, the ATP bioluminescence assay is accepted as an ASTM standard method for determining bacterial viability in waters, wastewaters and surface waters (ASTM method D4012-81). Its applicability to hot spot localization and restoration operations is clear.

The ATP assay described above is designed to work in solution in a test tube or cuvette. Sampling is the standard method for all viability detection assays that have been developed. Los Alamos National Laboratory (LANL) has been developing an *in situ* ATP assay for use on contaminated surfaces. The ATP assay is being modified in order to produce a sprayable solution by optimizing permeabilization of biological materials, germination of spores (as needed), stabilizing the luciferase enzyme system using water soluble polymers, using the water soluble polymer to provide adhesion of viability reagents to both horizontal and vertical surfaces and employing known methods to detect light emission from viable bacteria in real time.

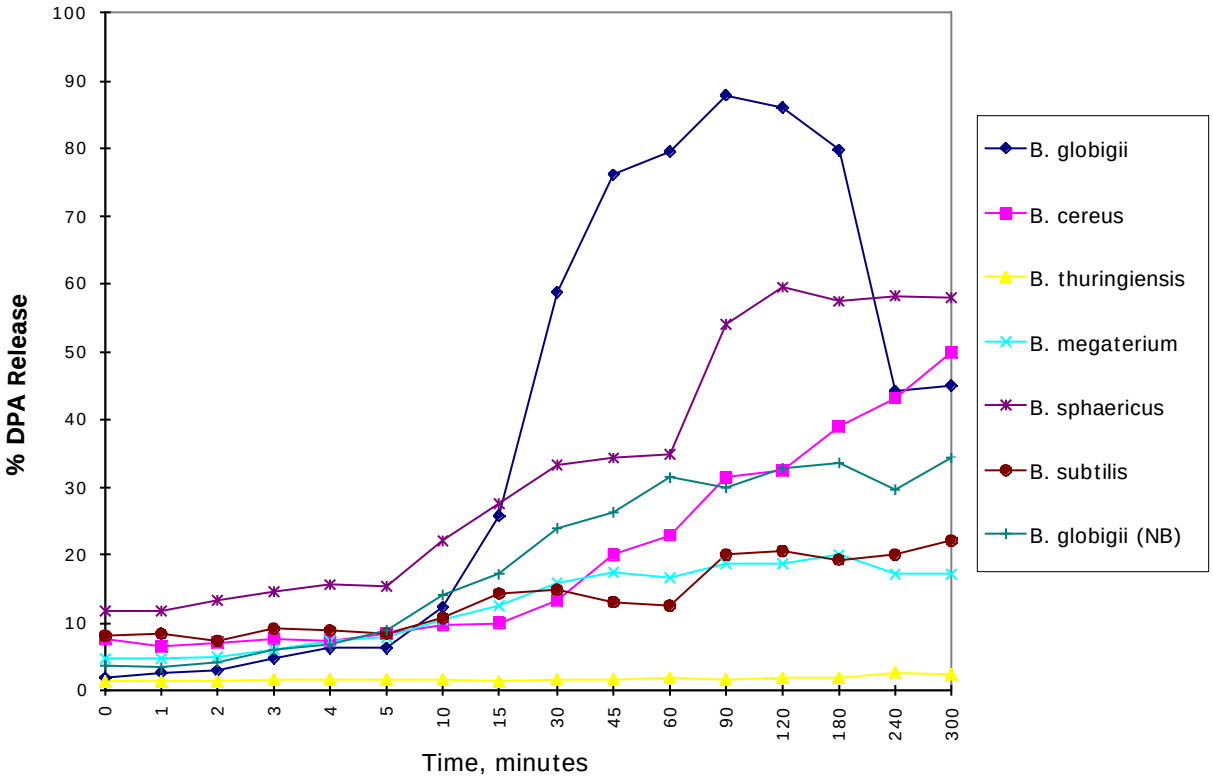

Figure 1.6b. Detection of dipicolinic release acid upon spore germination.

Dipicolinic Acid Detection. Dipicolinic acid (DPA) is a biomolecule unique to bacterial spores, not present in vegetative cells, nor present in fungal cells or spores (Carlsen and Vanderberg 1998). As shown in Figure 1.6b. above, this biomolecule is rapidly released during germination of viable bacterial endospores (Setlow 1981). LANL has developed a simple and rapid assay to detect viable bacterial spores called the LUNG because it mimics the environment of the human lung for the most rapid germination and employs an optimized spore germination solution which is incubated with bacterial spores for 10 minutes. The sample is mixed with a solution of terbium nitrate and analyzed by fluorescence spectroscopy. The DPA-terbium complex has a 10,000-fold increase in fluorescence over the non-chelated materials. Free DPA is detected and concentrations are converted to numbers of viable spores using a standard curve. Using the LUNG assay, we can detect as few as 5.8 x 10^5 viable bacterial spores in less than 15 minutes, start to finish. The LUNG system is currently at the prototype development stage using *Bacillus globigii* as a model spore-forming bacterium. This unique approach can be employed as

a stand alone detection system or added on to a variety of front end collection systems and used in conjunction with identification-type detection systems.

Staining with Flow Cytometric Analysis. There are many "vital stains" that have been used as indicators of viability (Jacobsen et al. 1997, Olsen & Rice 1996, Belosevic et al. 1997, Taghi-Kilani et al. 1996, Lloyd & Hayes 1995). Membrane potential sensitive dyes can be used to indicate the presence of an electrical potential across the cellular membrane which is maintained by active metabolism (Lloyd & Hayes 1995). Fluorogenic esters indicate active metabolism as a consequence of the chromofluor being released and accumulated within the cell, which can be subsequently detected (Schupp & Erlandsen 1987, Smith & Smith 1989).

Another useful indication of viability is the integrity of the cell membrane (Jacobsen et al. 1997, Olsen & Rice 1996, Belosevic et al. 1997, Taghi-Kilani et al. 1996). The LIVE/DEAD *Bac*Light Bacterial Viability assay is a commercial fluorescent dual staining system that may be useful within a viability indicator. This assay, made by Molecular Probes, Inc, utilizes mixtures of green (SYTO9) and red (propidium iodide) fluorescent nucleic acid stains. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO9 stain labels bacteria with both intact and damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, competing with the SYTO9 stain for nucleic acid binding sites when both dyes are present. When mixed in recommended proportions, the SYTO9 stain and propidium iodide produce green fluorescent staining of bacteria with intact cell membranes and red fluorescent staining of bacteria with damaged membranes. Prepared samples can be either viewed using fluorescent microscopy, or analyzed using a fluorescent spectrophotometer or flow cytometer. The ratio of green to red fluorescence intensities (or number of green vs. red cells) provides a quantitative index of bacterial viability.

While this staining system shows great promise, it depends on the assumption that a damaged plasma membrane equates to a dead cell. A more robust test of viability is looking for evidence of cellular metabolism. Fluorescent esters such as carboxy fluorescein diacetate (CFDA) have been used as indicators of metabolic activity. This molecule passes through the cell membrane, where esterases attack it, releasing free fluorescein (Schupp & Erlandsen 1987, Smith & Smith 1989). This fluorescent molecule builds up within the cell, and is readily observable through microscopy or flow cytometeric analysis. However, CFDA has been primarily used with eukaryotic cells, and thus its performance in prokaryotic cells is less well defined.

Flow cytometry is often used to analyze the emission spectra of vital stains (Diaper et al. 1992, Shapiro 1990). Flow cytometric analysis can detect multiple characteristics of each cell, allowing the identification of specific sub-populations of interest in samples containing mixed populations of cells or other particles (Van Dilla et al. 1983). Internal standards can be used to accurately determine the absolute concentration of the target cells in the sample. Due to the small sample volumes and high flow rate, the instrument has the potential to analyze up to or exceeding 50 samples per hour. Both LLNL and LANL have developed fieldable flow cytometer systems. LLNL has built a portable mini-flow cytometer based on a patented in-stream technique for light collection. LANL has designed and fabricated a self-contained, portable mini-flow cytometer for the DoD. In addition, work is being conducted to develop miniature sample preparation modules for use with the flow cytometer.

1.7. Organism-Specific Viability Detection

All of the methods for determining viability discussed above are non-organism specific; that is, they can determine if viable microorganisms are present, but cannot determine the identity of those organisms. In some applications, particularly defense and certain industrial applications, the identity of the viable organism is important. In order to make these viability techniques organism specific, they will need to be combined or followed with either immunological or DNA/RNA based identification techniques. Both immunological assays and DNA/RNA-based identification are well established and have excellent accuracy and precision.

Bacteriophages. Some bacteriophages, viruses that infect bacteria, are host specific (Carlsen and Vanderberg 1998). Phage infection can be used as a means of inserting known genes in the bacterial cell. This process, known as transduction, is routinely used in genetic cloning and is very well understood. The process of phage infection is very rapid. Viral DNA delivery occurs within minutes of phage introduction. Transcription of the viral DNA follows immediately and the translated protein is within minutes following initial infection.

By using bacteriophages as vectors, genes that encode specific detector proteins are moved into bacteria. Green fluorescent protein (GFP) is an ideal reporter protein. It is an autofluorescent, non-catalytic protein that doesn't require substrates or cofactors and seems to have no toxic effect in a wide range of cells (Inoue *et al*., 1995). As the name implies, it fluoresces with a green color. Extremely sensitive and rapid detection can be accomplished using this reporter protein. As shown in Figure 1.7 below, phage infection can be used to introduce the GFP gene into bacteria. Within a short time, the bacteria will produce GFP which can be easily detected. In addition to GFP, several other genes encoding different colored autofluorescing proteins (FP) are commercially available; these include blue FP, red-shift FP, yellow FP, and many other FPs that are in development (personal communication, Quantum Biotechnologies, Inc.; Chisso, Corp.; Clontech). Luciferases are also useful reporter molecules that can be employed in a similar fashion (Sala-Newby et al. 1996, Wood 1995).

Figure 1.7. Detection of viable *E. coli* using the phage cocktail concept.

The specificity of the detection system comes from using bacteriophages that are specific to each biothreat agent, whether naturally occurring or altered for species specificity. For example, naturally occurring phages specific for *Bacillus anthracis, Yersinia pestis* and *Vibrio cholerae* have been identified (Nunes & Saussuna, 1978; Nagy & Ivanovics, 1982; Albert et al., 1996). In addition, several phages that infect more than one species within a given genus exist. Examples that can be altered for improved host specificity include vibriophage KVP40 and bacillus phages CP51 and CP53 (Matsuzaki et al., 1992; Thorne, 1993).

LANL is developing a phage cocktail that can detect and identify viable biothreat agents. A viable mixture of these bacteriophages will be maintained in a cocktail solution that also promotes the rapid growth of introduced potential bacterial hosts. By using bacteriophages as vectors, the genes that encode specific detector proteins can be moved into bacteria.

1.8. The Immunoassay/Vital Staining System (IVSS) Concept

The primary drawback to the use of vital stains with flow-cytometry is that it is not organism-specific. A potential solution to this problem is combining the vital stain with an immunological assay. Immunological assays are based on creating antibodies to the particular organism of interest and conjugating that antibody to a fluorescent or colored probe. Reaction of the antibody/probe conjugate with the target organism results in a characteristic fluorescence or visible color, indicating the presence of the organism. These methods are even more powerful when combined with a flow cytometer, which allows the rapid analysis of large numbers of samples.

The biggest challenge in combining vital stains with immunoassay techniques is identifying fluorescent-labels for the antibody that have different emission characteristics from the viability stains. Other fluorescent chromophores have been successfully used as antibody labels. To be most efficiently used in the viability sensor, all fluorescent chromophores should be excited by a single wavelength, for ease of use with flow cytometers. The 488 nm wavelength line of an argon laser is most commonly used with flow cytometers. At least three dyes that are excited at 488 nm and are easily conjugated to antibodies are available. Phycoerythrin (PE) emits at 570 nm (used in our proof-of-concept experiments), Cy5-Phycoerythrin (Cy5PE) emits at 680 nm, and Cy7-Phycoerythrin (Cy7PE) emits at almost 800 nm. Both Cy5PE and Cy7PE are tandem resonance energy transfer dyes in which phycoerythrin is covalently linked to either Cy5 or Cy7 (Roederer et al. 1996). In the tandem dye, PE is the component that is excited by the argon laser. Emission from the PE then excites the tandem dye, which subsequently emits at a longer wavelength than the PE. The ratio of the two dyes must be carefully selected to maximize the efficiency of the energy transfer from the PE without subsequent quenching of the tandem. Any residual emission from the PE must be compensated for with the PE channel in the FCM. Figure 1.8 below from Roederer (1997) shows excitation and emission spectra for 8 different dyes used in immuno-fluorescence experiments. Spectra are uncorrected for detector sensitivity and are scaled for presentation purposes.

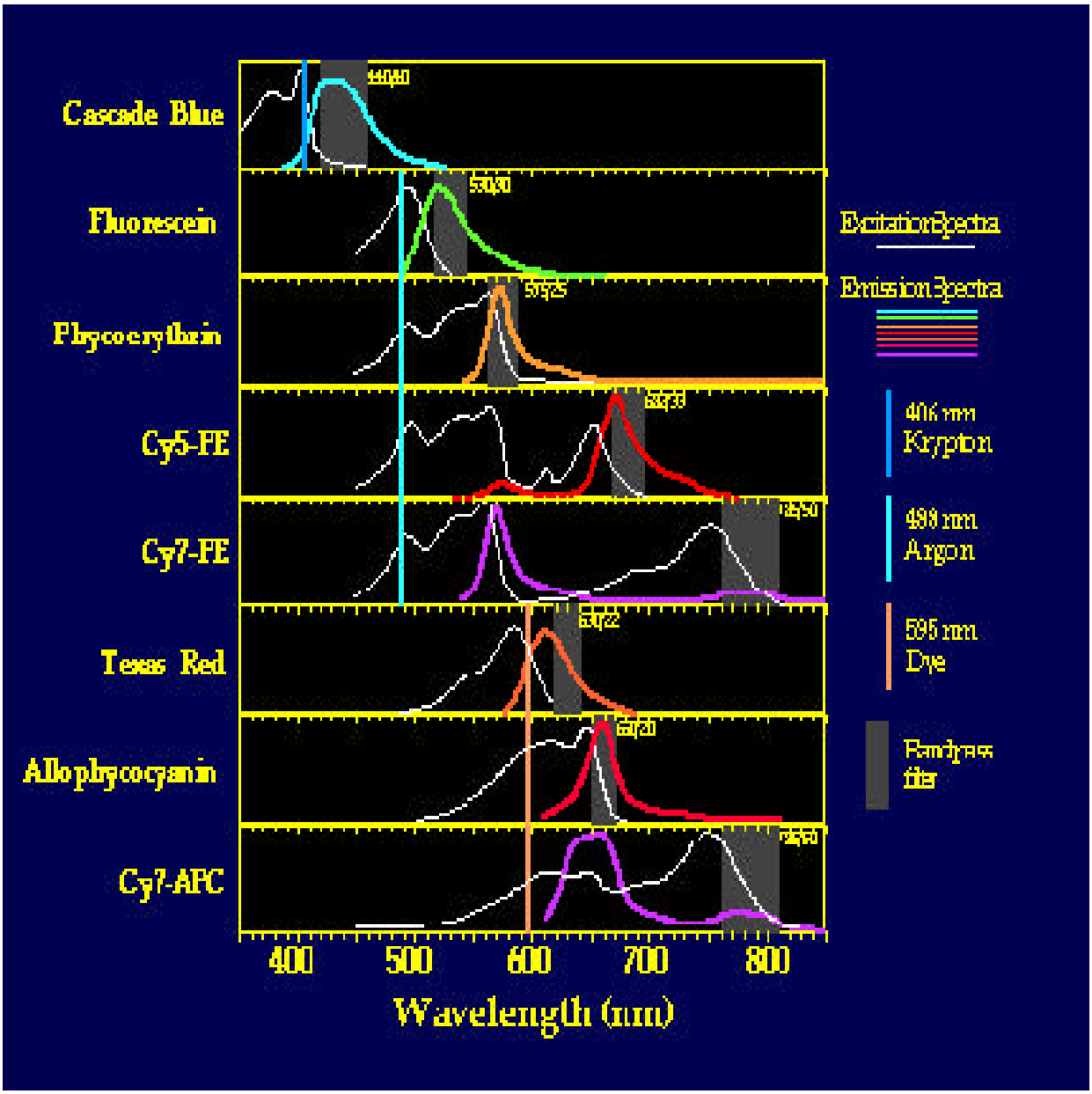

Figure 1.8. Excitation and emission spectra for dyes used in immuno-fluorescence experiments.

2. IVSS Proof-Of-Concept

2.1. Selection Of Surrogates And Culturing

For the IVSS proof-of-concept, it was necessary to prepare both live and dead cultures of the bacterial agents of interest. We concentrated on detecting the viability of vegetative bacteria. We selected common, non-pathogenic bacteria species that had similar characteristics to known biological warfare agents to act as surrogates for biological warfare agents. We worked with two surrogates, *Bacillus globigii* and *Pantoea agglomerans*. *B. globigii* is a surrogate for *B. anthracis*, the causative agent for anthrax. *P. agglomerans* (formerly known as *Erwinia*

herbicola) is a surrogate for *Yersinia pestis*, the causative agent for the plague. Both surrogates were obtained from investigators at the U.S. Army's Dugway Proving Grounds. Table 1 describes the characteristics of the two surrogates, as well as the basic culturing conditions used in the IVSS experiments.

Live cultures: For the preparation of live cultures, the basic technique involved inoculating a 50 mL disposable pre-sterilized centrifuge tube containing 20 to 25 mL of liquid media with the surrogate using standard sterile technique (Benson 1982). The culture was then placed into a temperature-controlled shaker and incubated for 17 to 30 hrs at 30º C while agitating at 150 rpm. Optical density measurements at a wavelength of 600 nanometers (OD_{600}) were obtained on the cultures using a Shimadzu UV160 U spectrophotometer to estimate cellular growth. Cell counts in the liquid cultures were determined using standard plate count techniques (Benson 1982) onto solid media. Cell counts were determined as colony forming units (CFUs). All dilutions were done in 50 mM phosphate buffer solution (PBS) to reduce osmotic shock to cells. Live cultures were also examined using the LIVE/DEAD *Bac*Light Bacterial Viability assay to observe the proportion of live to dead cells and amount of extracellular debris in the cultures. Slides were prepared using the *Bac*Light protocol and observed microscopically (see Plasma membrane integrity indicators below for full description of *Bac*Light and microscopic protocol).

The majority of the live cultures used in the IVSS proof-of-concept experiments were grown in commercially purchased Difco beef extract nutrient broth. Table 2 shows the typical OD_{600} measurements obtained in the nutrient broth. Generally, nutrient broth was adequate to provide sufficient cell numbers for staining and flow cytometry analysis. Although the cellular numbers were relatively low for both surrogates in nutrient broth, they were more than adequate for observation under the microscope without the need for culture dilution. In addition, these cultures had a very high percentage of green staining live cells, very few red staining dead cells, and a low amount of extracellular debris. However, death of the live cells could be observed under the fluorescent lamp, with the internal organelles first beginning to pick up the red propidium iodide stain, then the entire cell becoming red. In addition, in the fresh live cultures, the extracellular debris tended to stain green using the *Bac*light assay.

Although the nutrient broth culturing was generally adequate for the IVSS work, cell growth was quite variable, and at times could be quite low. Therefore, we investigated the use of commercially purchased Difco brain heart infusion media for use with *B. globigii* and laboratory prepared SOC media for *P. agglomerans* (see Table 1 for SOC recipe). The use of these media resulted in OD_{600} readings consistently near 1.0. However, the large number of cells observed under the microscope required dilution for efficient observation and photography. A large amount of extracellular debris was present, as was a fair number of dead cells. However, the live cells remained green fluorescent longer under the fluorescent lamp compared to the cells grown in nutrient broth. Figures 1 and 2 shows the relationship between OD_{600} readings and CFU for *B. globiggi* and *P. agglomerans*, respectively.

To efficiently run multiple IVSS experiments over several days, it would be preferable to be able to use the same live culture over multiple days. Therefore we investigated the longevity of the live cultures. Figure 3 shows the longevity of live cultures grown in nutrient broth and placed into a refrigerator at 4º C after the initial incubation period. All cultures were agitated prior to each OD_{600} reading. As can be seen. *P. agglomerans* cultures lasted up to 25 days in the refrigerator with little change in OD_{600} measurements. The cells remained well suspended in the

culture. *Bac*light observations verified the cultures were alive. Additional debris was noticed in these cultures, and the live cells died very quickly under the fluorescent lamp. *B. globigii*, on the other hand, did not fare well under refrigeration. Cell mass was observed settled at the bottom of the culture tube, and agitation failed to resuspend the cells. *Bac*light observations showed many of the cells to still be alive, but there was a large amount of red and green fluorescing extracellular debris present. The longevity of *B. globigii* and *P. agglomerans* cultures was better when the cultures remained in the agitated incubator (Figure 4). OD_{600} readings remained stable over 12 days. It was not until being placed into a 4° C refrigerator that OD_{600} measurements began to drop for *B. globigii*. *Bac*light observations revealed an increasing percentage of dead cells and extracellular debris over time in the live cultures stored in the incubator. Thus, fresh cultures were used in all the flow cytometry and antibody work on IVSS.

Dead (fixed) cultures: In order to determine the effectiveness of the various vital stains in determining viability, it was necessary to prepare bacterial cultures known to be dead (i.e. fixed cells). This was done using either 95% ethyl alcohol or 70% isopropyl alcohol. Table 3 describes the cell fixing procedures used. All three procedures resulted in fixed cultures that were reasonably stable over time (Figure 5). When the cells were stored in the alcohol, there was an initial reduction in the OD_{600} readings, probably a result of some cell loss due to lysing. However, the remaining cells were quite stable. When cells were fixed in isopropyl alcohol, washed and stored in water, there was no initial cell loss, but the fixed cells were slightly less stable over time. All fixed cultures contained intact cells when observed under the microscope. Cells generally stained yellowish/orange to red using the *Bac*light assay. Extracellular debris tended to stain green.

2.2. Selection Of Vital Stains

The primary challenge to the development of an immunoassay/vital stain system for viability detection is the identification of fluorescent antibody labels and fluorescent vital stains that contain chromofluors with similar excitation characteristics but different emission characteristics. Emissions from the chromofluors must be readily separable using standard three-channel flow cytometers. The flow cytometer used in the IVSS proof-of-concept work was a Becton/Dickenson FACScan three-channel flow cytometer equipped with an argon laser for a 485 nm excitation wavelength. The three channels were configured as follows:

Channel	Emission wavelength (nm)	Color	Typical chromofluor
1	530 + 15	Green	Fluoroscein
2	585 + 21	Yellow/orange	Phycoerythrin
3	>650	red	Propidium iodide

With these three channels, it is theoretically possible to use one channel for antibody detection, one for live cell detection, and one for dead cell detection. However, as we are most interested in answering the question “is anything alive” vs the question “how many are dead”, a system in which only live cells fluoresce and are labeled with an antibody for identification would require only two channels.

We undertook a literature review to identify potential chromofluors for use both as a vital stain and an antibody label. Table 4 summarizes the chromofluors identified in this review. Over eighty chromofluors were identified. Twelve chromofluors have application as potential antibody labels. The remaining sixty-nine dyes had been used in various viability determination applications. These sixty-nine chromofluors included seven membrane potential indicators, forty-two nucleic acid stains, eleven metabolic activity probes and three fluors that are excluded by the cell membrane. Also listed in Table 4 are five non-fluorescent vital stains.

In general, all of the chromofluors listed in Table 4 used as viability indicators can be placed into two classes, those that indicate the integrity of the plasma membrane, and those that indicate metabolic activity. For IVSS proof-of-concept, we focused on two staining systems which indicated the integrity of the plasma membrane and one staining system indicating metabolic activity.

Plasma membrane integrity indicators: The first plasma membrane integrity indicator we investigated was the commercially available LIVE/DEAD *Bac*Light Bacterial Viability assay available from Molecular Probes, Inc. This assay utilizes mixtures of the fluorescent nucleic acid stains SYTO9 and propidium iodide. SYTO9 fluoresces at 500 to 520 nm when excited at 485 nm and is membrane permeable, thus it labels bacteria with both intact and damaged membranes. Propidium iodide fluoresces at 635 nm and penetrates only bacteria with damaged membranes, competing with SYTO9 for nucleic acid binding sites. Thus, these combined stains result in green fluorescent bacteria with intact cell membranes and red fluorescent bacteria with damaged membranes.

Live and dead cultures were prepared as follows for *Bac*Light staining and subsequent microscope or flow cytometery analysis. Culture preparation is also summarized in Table 5. The LIVE/DEAD *Bac*Light ™ bacterial viability kit L-7012 from Molecular Probes, Inc. was used. Component A consists of 300 µl of 3.34 mM SYTO9 in anhydrous DMSO, and Component B consists of 300 µl of 20 mM propidium iodide in anhydrous DMSO. Both components are stored in the freezer at –4º C. Component stains are allowed to reach room temperature and are centrifuged at 10,000 g for 2 mins. Equal volumes of each component are transferred to a microfuge tube, sufficient to provide 3 µL of stain mixture per 1 mL of culture to be stained. This stain mixture is centrifuged for 2 mins. at 10,000 g. 1 mL of live culture is centrifuged for 5 mins. at 6,000 g at 4º C. 1 mL of fixed culture is centrifuged at 10,000 g at 4º C for 5 to 10 mins., or until a cohesive pellet is formed. Liquid is decanted and pellet resuspended in 1 mL sterile water. 3 µL of stain mixture is added to each mL of live or fixed culture, mixed using a vortexer, and allowed to incubate at room temperature in the dark for 15 mins.

For microscopic analysis, 1.5 to 3 µL of stained culture was trapped between a cleaned microscope slide and cover slip. Stained cultures were observed using a Zeiss Axioplan MC100 microscope equipped with 10 X ocular and 1.25 X ocular ring, a Zeiss 50w mercury lamp and a FT 510 filter (excitation at 450-480 nm, emission at 515-585). Slides were observed using the 100 X lens under oil immersion.

Figure 6 shows the *Bac*Light staining for *B. globigii* and *P. agglomerans*, respectively. The *B. globigii* shown in Fig. 6a were from a two-day old culture prepared in BHI media. Note the high percentage of dead cells. The *P. agglomerans* shown in Fig. 6b, on the other hand, is a 7 d old culture, and shows relatively few dead cells. Microscopic analysis was also conducted on

fixed cultures and mixtures of live and dead cultures. In general, live cells could be visually distinguished from dead cells. However, often the dead cells fluoresced more yellowish/orange instead of red. Live cells consistently fluoresced a bright green.

With respect to use in determining viability of biological warfare agents, it is the live cells that are of concern. In addition, the possibility of dead cells fluorescing green and providing a false indication of viability is more acceptable than live cells fluorescing red, incorrectly identifying the sample as non-viable. Therefore, we investigated the use of a non-fluorescent stain to replace the propidium iodide in the *Bac*Light assay. A stain that would only penetrate cells with a damaged membrane and "quench" the fluorescence of the SYTO9 was desired. In this manner, live cells would still fluoresce green, with dead cells becoming non-fluorescent. We investigated three stains as possible quenchers with STYO9. These were malachite green, safranin, and trypan blue. Malachite green and safranin stains are traditional inorganic stains used in gram-staining (Benson, 1982). Trypan blue is a non-fluorescent protein dye (i.e. nucleic acids) that cannot pass through intact cell membranes and is used for viability determination (Sigma-Allied Chemicals, 1999).

To test the gram-staining dyes, a 10% aqueous malachite green solution and a 20% aqueous safranin were used. Live cultures of both surrogates were grown in nutrient broth to an OD_{600} of 0.351 to 0.495. Dead cultures were fixed in a 50mM phosphate buffer solution with 33% ethyl alcohol. Live and dead cultures of both surrogate species were prepared for staining as described for the *Bac*Light assay except that 1.5 µL of STYO9 only was used per mL of culture. The propidium iodide was replaced with 1.5 µL of the malachite green or safranin solutions. For comparison, 1 mL of both live and dead cultures were also incubated with 1.5 µL of SYTO9 only, as well as with the full *Bac*Light assay. Incubation times were as indicated below:

Assay	Incubation time (mins.)
BacLight	15 - 25
SYTO9 only	30 – 35
SYTO9/malachite green	40 – 45
SYTO9/safranin green	60 - 75

The live and dead cultures produced good *Bac*Light results, with the live cells fluorescing bright green and the dead cells all fluorescing red. Using the STYO9 only, both the live and dead cultures fluoresced a bright green. Figure 7 shows the dead *P. agglomerans* culture when stained using the full *Bac*Light assay, and when using SYTO9 only. When the STYO9 was used with the malachite green, the live and dead cells appeared dark green using brightfield microscopy (Figure 8). Under fluorescent microscopy, no fluorescence was observed in either the live or dead cultures, indicating the dye was strongly quenching the SYTO9 in both live and dead cells. This was also observed when the STYO9 was incubated with the safranin dye. In this case, under brightfield microscopy, the live and dead cells were a dark pink, and no fluorescence was observed for either live or dead cultures. Thus, neither malachite green nor safranin were further investigated for use as a quenching stain with SYTO9.

To test trypan blue for use as a STYO9 quenching dye, a 0.4% trypan blue in a 0.85% saline solution was used (purchased from Sigma-Aldrich Chemicals). An initial experiment was

conducted in which 0.5, 1.0 and 1.5 µL of the trypan blue solution was added with the 1.5 µL of SYTO9 to 1 mL of bacterial culture and incubated for 10 minutes. When observed under the microscope, all live cultures were still clearly fluorescent. The dead cultures were also still fluorescent, however, the cultures incubated with the highest trypan blue concentration, the fluorescence was noticiably duller and faded rapidly. This suggested that trypan blue had potential for use as a SYTO9 quencher.

Further experimentation revealed that 100 µL of trypan blue added to 0.9 mL of culture containing 1.5 µL of SYTO9 resulted in substantial quenching of dead cell fluorescence with minimal quenching of live cell fluorescence. Table 5 summarizes the preparation protocol for the STYO9/trypan blue system. For *P. agglomerans*, the dead cells are slightly fluorescent, but noticeably dimmer than the live cells (Fig. 9). For *B. globigii*, very few dead cells stained with the STYO9/trypan blue system are fluorescent and they are barely visible (Fig. 10). However, in some cases, the debris from the lysed cells had bright fluorescence and appeared visually similar to the fluorescence of the live cells.

Metabolic activity indicators: Although both the *Bac*Light and STYO9/trypan blue staining systems show promise for use in an IVSS, these stains suffer from the shortcoming that they indirectly provide evidence of viability through determination of plasma membrane integrity. A more robust approach would be to interrogate the cells for direct evidence of metabolic activity. Thus, we evaluated the fluorescent ester carboxy fluorescein diacetate (CFDA). The CFDA molecule passes through the cell membrane, where esterases attack it, releasing free fluorescein (Schupp & Erlandsen 1987, Smith & Smith 1989). This fluorescent molecule builds up within the cell, and is readily observable through microscopy or flow cytometric analysis. However, CFDA has been primarily used with eukaryotic cells, and thus its performance in prokaryotic cells is less well defined.

Bacterial cultures were stained with CFDA based protocol of Jacobsen *et al.* (1997) and summarized in Table 5. 5(6)-carboxyfluorescein diacetate was purchased from Sigma-Allied Chemicals and a 10 mM solution was prepared in acetone. Ten µL of the CFDA solution was added to 1 mL of bacterial culture which had been resuspended in PBS or tris buffer solution and incubated for 30 minutes in the dark at room temperature. Live and dead cultures of both surrogate species were incubated with the CFDA.

Figure 11 shows live and dead cultures of *B. globigii* that had been incubated with CFDA. As can be observed, live cells were readily apparent under fluorescent microscopy, where as dead cells were not. Bright field microscopy reveals the presence of dead cells which had not accumulated the fluorescein. CFDA staining was less effective on *P. agglomerans*. Although some cells did accumulate the fluorescein, bright field microscopy revealed the presence of a much greater number of cells (Fig. 12). Like *B. globigii*, dead cells of *P. agglomerans* did not accumulate fluorescein. *P. agglomerans* is a gram-negative bacteria, and fluorescently-labeled esterases have been reported to be less effective on this class of bacteria (Fry et al. 1990).

2.3. Selection Of Antibodies

Initial experiments utilized polyclonal antibodies for *B. globigii* spores and vegetative *P. agglomerans* that were obtained from Dr. Bruce Harper at Dugway Proving Grounds, Utah. These antibodies consisted of the immunoglobulin-G fractions from goat for *B. globigii,* and

from rabbit for *P. agglomerans*. These antibodies were conjugated with fluorescein using a standard FITC conjugation kit (Sigma Chemical Company). Fluorescein is a fluorescent dye that is excited by the argon laser within a flow cytometer at 488 nm, with emission data collected at 530 nm. Preliminary experiments using the *B. globigii* spore antibody indicated this antibody was not cross reactive with vegetative cells. Therefore, two additional polyclonal antibodies specifically selected for cross-reactivity with vegetative *B. globigii* were obtained from Jennifer Aldritch at the Naval Medical Research Institute (NAMRI). Both antibodies were supplied as IgG fractions from goat sera.

Flow cytometric characterization of these antibodies was performed by separately labeling samples of each bacterial form. The live vegetative cultures were grown in nutrient broth to an OD_{600} of 0.208 for *P. agglomerans* and 0.696 for *B. globiggi*. The dead cultures were prepared using the Etoh/PBS protocol in Table 3 to an OD_{600} of 0.488 for P. *agglomerans* and 0.225 for *B.globiggi*. Two sources of *B. globigii* spores were tested. Dugway spores were obtained as a dry powder from Dugway Proving Grounds. This material is a crude spore preparation that has been used for many years for aerosol releases. The "clean" spores were prepared at LLNL from fresh cultures. These spores were extensively washed to remove cell debris and residual vegetative cells. The following procedure was used to adjust all preparations to have the same cell concentration for each flow experiment. Flow cytometric light scatter signals from unlabeled cells were used to determine the numbers of cells per second analyzed using each sample. Dilutions with buffer were then used to adjust all preparations to a standard concentration of 10^6 cells per mL.

For the initial experiments, cells were diluted into PBS buffer and directly labeled with the fluoresceinated Dugway antibodies at a concentration of 1 µg/mL. The flow distributions obtained from these experiments showed two potential problems. First, the *B. globiggi* antibody labeled *B. globiggi* spores brightly, while very weakly labeling live or dead vegetative *B. globiggi* cells. This observation suggested that this antibody was specific for spores. Second, dead (but not live) vegetative cells from both species showed moderate cross reactivity with the antibody directed to the other species. This suggests a non-specific uptake of antibodies by Etoh fixed cells. Non-specific labeling of dead cells was eliminated by replacing the PBS buffer with a previously described staining buffer that contains bovine serum albumin and the detergent NP-40 (Langlois et al., 1990). All subsequent flow cytometric experiments utilized cells suspended in this staining buffer.

The results of flow cytometric analyses using the Dugway antibodies to label cells in staining buffer are shown in Figure 13. The two Dugway antibodies showed highly species specific labeling. For *P. agglomerans*, comparable labeling of live and dead vegetative cells showed that the ethyl alcohol treatment did not interfere with antibody binding. It is also clear from Figure 13 that the Dugway antibody for *B. globigii* spores is not cross reactive with *B. globigii* vegetative cells. While this specificity towards spores may be useful in discriminating between spores and vegetative cells, different antibodies are clearly required to label vegetative cells from *B. globigii*.

The two NAMRI antibodies selected for reactivity with *B. globigii* vegetative cells were then evaluated. Indirect fluorescence labeling was used for the NAMRI antibodies. Cells were incubated with 1 µg/mL of primary antibody, washed by centrifugation, and then labeled with 1 µg/mL of fluoresceinated mouse anti-goat secondary antibody. The results of these experiments

are shown in Figure 14. Compared to the Dugway antibodies, the fluorescence of the NAMRI antibodies was less intense. These antibodies labeled both *B. globigii* spores and vegetative cells with minimal cross-reactivity with *P. agglomerans*. One antibody, vb7/6, showed the strongest preference for vegetative cells. The elevated binding of this antibody to Dugway spores vs. clean spores suggests that there is considerable vegetative material in the Dugway powder. Again, there appeared to be no significant effect of the ethyl alcohol on the antibody binding.

2.4. Flow Cytometry Analysis Of Vital Stains

Flow cytometric analysis was conducted on cultures using dual-color nucleic acid labeling (i.e. *Bac*Light), single-color nucleic acid labeling (i.e. STYO9 and trypan blue) and metabolic activity labeling (i.e. CFDA) to indicate cell viability. The live vegetative cultures were grown in nutrient broth to an OD_{600} of 0.496 for *P. agglomerans* and 0.212 for *B. globiggi*. The dead cultures were prepared using the Etoh/PBS protocol in Table 3 to an OD_{600} of 0.488 for P. *agglomerans* and 0.225 for *B.globiggi*.

Figure 15 shows the results of the dual-color nucleic acid labeling using *Bac*Light on *B. globigii*. Results on *P. agglomerans* were similar. Only the green fluorescence was analyzed, as this represents live cells. As can be seen, the live culture shows a large population of live cells (window 3), with a smaller population of dead cells (window 2). The dead culture shows only a single dead population. Using the windows identified in the live and dead cultures, live cells added to the fixed culture down to 1% can be detected in window 3. Thus, the propidium iodide, although not specifically analyzed for in this analysis, effectively quenched the green fluorescence of SYTO9 in dead cells. The green fluorescing live cells are easily detected. As we wished to use the red/orange fluorescence space on antibodies, it was necessary to find a non-fluorescent replacement for propidium iodide if STYO9 was to be used to indicate live cells.

Figure 16 shows the results of the single-color nucleic acid labeling using SYTO9 to indicate live *B. globigii* cells, and the non-fluorescent trypan blue to quench the fluorescence in dead cells. As shown in Figure 16, a two population distribution similar to that observed in the *Bac*Light analysis was observed, although the populations were somewhat less distinct. Trypan blue did quench the fluorescence of the fixed culture, although it was slightly less effective than the propidium iodide, as can be seen by the small amount of fluorescence still observed in window 3.

Figure 17 shows the results of the metabolic activity labeling of *B. globigii* cells using CFDA. Again, the live culture shows two populations, the smaller being the normal amount of dead cells found in a typical culture. Fixed cells clearly show no live populations, and cluster very tightly. As shown in Figure 18, as low as 0.1% of live culture added to the fixed culture can be detected using CFDA. While clearly CFDA labeling appears superior for *B. globigii*, unfortunately it was less effective for *P. agglomerans* (Figure 19). Although live cells can be detected in the live culture, the observed size of the population is much less than the observed "dead" population, which is not expected for a live culture. As shown by microscopic analysis (Figure 12), not all of the live cells are accumulating the fluorescein in this species.

2.5. Flow Cytometry Analysis Of Combined Vital Stains/Antibodies

Because of the superior performance of the CFDA metabolic indicator with vegetative *B. globigii*, it was selected for use in the final experiment which combined the analysis of viability with that of species identification. For this experiment, *B. globigii* was designated the target organism and was grown in brain heart infusion broth to an OD_{600} of 1.141. The dead culture was prepared using the ISO/H2O procedure in Table 3 to an OD_{600} of 1.034. Dead *P. agglomerans* was used as the "background" or non-target organism. It was grown in SOC media and fixed using the ISO/H20 procedure listed in Table 3 to an OD_{600} of 0.597. The Dugway spore preparation was also analyzed for comparison purposes. Indirect immunofluorescent labeling was used for these experiments. *B. globigii* cells were first incubated with the NAMRI antibody vb7/6, washed, and then labeled with a phycoerythrin conjugated rabbit anti-goat antibody. Antibody labeled cells were subsequently incubated with CFDA before flow analysis. In the future, a direct conjugate of phycoerythrin with the primary antibody would allow single step labeling of cells with both antibody and CFDA. Phycoerythrin is excited by the argon laser, and its fluorescence at 570 nm can be clearly separated from the fluorescein emission from the CFDA.

Figure 20 shows the dual-color flow cytometry analysis of the individual cultures when incubated with the *B. globigii* antibody and CFDA. Both dead vegetative cultures show low green fluorescence, whereas the live vegetative *B. globigii* culture shows strong green fluorescence. The two vegetative *B. globigii* cultures shows clear orange fluorescence, whereas the *P. agglomerans* culture does not. The *B. globigii* spore culture shows a smear between all three windows, with the vast majority of cells not labeling as either live or dead *B. globigii*.

Figure 21 shows the results of the dual-color flow cytometry analysis of an artificial mixture of live and dead *B. globigii* in a matrix of dead *P. agglomerans*. Window size and placement were those determined from the results of the pure cultures. The artificial mixture was designed to have a large majority of non-target dead organisms (*P. agglomerans*), and for a small fraction of the target organisms (*B. globigi*) remaining alive. As shown in Figure 21, the observed percentage of the cell types agreed quite well with the calculated percentage. Thus, very small proportions of live target cells can be detected in complex mixtures. This analysis shows the potential for the combined use of vital stains and immunoassay techniques in the rapid, species-specific determination of viability.

3. Future Work For Full IVSS Development

The work done to date clearly shows that the concept of combining vital staining with antibody probes for the simultaneous determination of viability and identity has merit and is worth pursuing. However, our work has also pointed out some challenges that must be overcome before the IVSS concept can be fully developed.

In order to use the single color nucleic acid stain such as SYTO9 to determine viability, additional work needs to be conducted to further enhance the quenching of STYO9 in dead cells

to eliminate false indications of viability. This could be done by attempting to optimize combined STYO9 and trypan blue concentrations, or investigating additional dyes for use as non-fluorescent quenchers with SYTO9.

The use of metabolic activity indicators clearly has advantages over membrane integrity indicators (such as nucleic acid stains). Metabolic activity is a direct indication of viability, whereas membrane integrity is an indirect indication of viability. However, as our work here has shown, additional effort needs to be conducted to identify a metabolic activity indicator that will work with gram negative bacteria.

As our work has also shown, the systems investigated to date for IVSS development do not appear effective in identifying viable spores. However, we have clearly shown the ability to detect viable vegetative bacteria. As the definition of a viable spore is one able to germinate, a rapid germination procedure could be developed and used at the front end of the IVSS procedure, thus allowing the determination of viable vegetative bacteria. Another avenue of investigation is to incubate the spores in a solvent solution to sufficiently loosen the spore coat to allow staining without rendering the viable spore nonviable.

Besides optimizing the systems investigated thus far and adapting them for use on spore cultures, there are additional challenges to the development of a fully-functioning IVSS system. The laboratory-based IVSS would need to be extended to the analysis of actual field samples. Field collected samples are messy systems which will likely contain a variety of “contaminants”. This environmental debris will likely contain particulates comprised of a wide range of substances, from inert mineral particulates, to organic material which may contain proteins or nucleic acids. Thus, the extent to which environmental debris is labeled, the amount of interference, and methods for reducing debris load will need investigation.

In addition, characteristics of post-decontamination samples that may prove a challenge for an IVSS assay. For example, it is necessary to determine how quickly the BW agents die once in contact with the decontaminant so that the latency period between decontamination and decon verification can be established. In addition, it will be necessary to determine if candidate decontamination agents being developed at LLNL and other facilities (such as peroxydisulfate catalyzed by copper, hydrogen peroxide, sodium hypochlorite, n-alkyl dimethyl benzyl ammonium chlorides and n-alkyl dimethyl ethylbenzyl ammonium chlorides) result in a dead signature appropriate for use with the IVSS (such as lethality associated with the disruption of the cell membrane). It will also be necessary to determine if these highly oxidizing decontamination agents interfere with dye and stain expression of the IVSS.

Another important characteristic of post-decontamination samples is the extremely low concentrations of BW agents expected in the samples. These low concentrations may potentially impact three important components of post-decontamination sampling, that is (1) the size of the sample, (2) pre-IVSS analysis handling of the sample, and (3) number of samples collected. The interplay of these three components will determine the ability to detect the single live cell that escaped decontamination efforts. The size of the sample as well as any required pre-IVSS sample handling will in large part be driven by the detection limit of the flow cytometer-IVSS. Therefore, it may be necessary to develop a short-term culturing step prior to IVSS analysis, on the order of less than one hour. Simply allowing for a few generations worth of cell division may greatly increase the accuracy of the analysis.

However, some of the sample size and detection limit shortcomings may be overcome by the number of samples collected. Thus, it will be necessary to develop an effective sampling strategy for use with the IVSS technology using information developed on sample size and detection limits. The sampling strategy should be based upon confidence limits and sampling statistics, and should define protocols based upon regulatory criteria for interpreting the results. One possible solution is to consider sampling to detect the presence of BW threat agents as a "hot spot" detection problem (Gilbert 1987, Singer 1972, Singer 1975).

Development of a fieldable system would need to include sample fluidics capable of handling the sample, viability stains, antibody cocktails and any necessary culturing media. This would feed into a portable field flow cytometer, capable of quickly analyzing the introduced sample. Such a system could be deployed at sites undergoing decontamination to allow real time verification of decontamination activities. Such a system could also be used to verify suspected incident threats.

The rapid determination of species-specific viability clearly would add to our ability to respond to the threat of biological weapons. The IVSS technology represents one solution to this need. While we have shown proof of concept for the IVSS technology, additional work is required before this system is ready for field deployment.

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Tables

Table 1. Surrogates for biological warfare agents.

Agent	Surrogate	Surrogate source	Surrogate Description[a]	Culturing Conditions	Culture media
Bacillus anthracis	*Bacillus globigii*	Dugway Proving Grounds	Bergey's Group 18, rod-shaped and straight, 0.5-2.5 x 1.2- 10 µm, often arranged in pairs or chains with rounded or squared ends. Motile by peritrichous flagella. Endospores are oval, only one spore per cell, very resistant to adverse conditions, sporulation is not repressed by exposure to air. Aerobic or facultatively anaerobic, Gram-positive.	20 – 25ml broth in 50 ml disposable centrifuge tube, incubation for 18 to 24 hrs at 30º C agitating at 150 rpm.	Liquid: Difco Bacto beef extract nutrient broth or brain heart infusion broth containing polymyxin, lysozyme, EDTA, thallous acetate. Solid: Difco Bacto beef extract nutrient agar.
Yersinia pestis	*Pantoea agglomerans* *(Erwinia herbicola)*	Dugway Proving Grounds	Bergey's Group 5, rod-shaped and straight, 0.5-1.0 x 1-3 µm. Motile by peritrichous flagella. Most strains produce a yellow pigment, isolated from plant surfaces, seeds, soil and water. A phytopathogen. Facultatively anaerobic, Gram-negative.	20 – 25ml broth in 50 ml disposable centrifuge tube, incubation for 18 to 24 hrs at 30º C agitating at 150 rpm.	Liquid: Difco Bacto beef extract nutrient broth or SOC broth. Solid: Difco Bacto beef extract nutrient agar.

[a] Holt et. al, 1994.

Difco Bacto Beef Extract Nutrient and Difco Bacto Brain Heat Infusion media purchased from VWR Scientific.

SOC Media

2% Bacto-tryptone	10 mM $MgCl_2$
0.5% Bacto-yeast extract	10 mM $MgSO_4$
10 mM NaCl	20 mM glucose
2.5 mM KCl	

Combine tryptone, yeast extract, NaCl and KCl in pure water and autoclave 30-40 minutes. Make a 2M stock of Mg^{2+}, comprised of 1M $MgCl_2$ and 1M $MgSO_4$. Sterilize by filtration through a 22µ membrane. Prepare a 2M stock of glucose similarly and store at –20º C. Just prior to use combine the media with Mg and glucose and sterilize by filtration through a 22µ membrane. For solid media, add 7.5 g Bacto-agar to 500 mL SOC media. Recipe taken from DNA Cloning, volume 1, edited by D. M. Glover. P127. IRL Press.

Table 2. Live culturing of surrogates

Surrogate	Culture Media[a]	Volume[b] (mLs)	Incubation Time (hrs)	OD_{600}	Cell Count[c]	Observations
B. globigii	Nutrient broth	20 - 25 (n = 26)	17-30	0.173 – 0.944	OD_{600}: 0.891 = 1 x 10^8 CFU OD_{600}: 0.212 = 3 x 10^7 CFU	Healthy live rods clearly visible under scope. Using 100X lens, lower OD_{600} readings result in good number of cells for microscopic analysis. Very few dead cells. More extracellular debris than observed for *P. agglomerans*, in fresher cultures staining green using *Bac*light, in older cultures the debris stains red. Live cells begin to dye and take up propidium iodide if left under mercury lamp for greater than a minute.
	Brain Heart Infusion	25 (n=8)	17-24	0.941 – 1.418	OD_{600}: 1.091 = 3 x 10^8 CFU	Healthy live rods stain bright green using *Bac*Light assay. Higher OD_{600} readings require culture to be diluted prior to microscopic analysis. A fair number of red staining dead cells in fresh cultures, although in older cultures a higher percentage of live cells compared to nutrient broth. Live cells stay green longer under fluorescent lamp compared to cultures grown in nutrient broth. More extracellular debris present, especially at higher OD_{600} readings.
P. agglomerans	Nutrient broth	20 - 25 (n = 25)	17-30	0.345 – 0.862	OD_{600}: 0.804 = 4 x 10^8 CFU OD_{600}: 0.345 = 2 x 10^7 CFU	Healthy live rods clearly visible under scope. Smaller and more numerous than *B. globigii* for same OD_{600}. Using 100X lens, lower OD_{600} readings result in good number of cells for microscopic analysis. Very few dead cells. Less extracellular debris than observed for *B. globigii*, in fresher cultures staining green using *Bac*light, in older cultures the debris stains red. Live cells begin to dye and take up propidium iodide if left under mercury lamp for greater than a minute.
	SOC	25 (n=6)	17-24	0.703 - 1.168	OD_{600}: 1.004 = 8 x 10^8 CFU	Healthy live rods stain bright green using *Bac*Light assay. Higher OD_{600} readings require culture to be diluted prior to microscopic analysis. A fair number of red staining dead cells. Live cells die faster under fluorescent lamp than compared to cultures grown in nutrient broth. Higher proportion of live cells in older cultures compared to nutrient broth. More extracellular debris present, especially at higher OD_{600} readings.

[a] Media:
NB: Difco Laboratories Bacto beef extract nutrient broth
BHI: Difco Laboratories Bacto Brain heart infusion liquid media
SOC: SOC liquid media

[b] All culturing was performed in 50 mL disposable pre-sterilized centrifuge tubes at 30º C agitated at 150 rpm.

[c] All cell counts determined by standard plate counts on nutrient agar plates; CFU = colony forming units.

Table 3. Description of cell fixing procedures

Procedure Description	Observations
Etoh/PBS • Grow 10 mL of fresh live culture in liquid media to an OD_{600} of 0.3 to 1.0. • Centrifuge at 10,000 g for 10 mins. Decant liquid. • Resuspend pellet in 10 mL of 50mM potassium phosphate buffer solution. • Slowly add 5mL of 95% ethyl alcohol 1 mL at a time, vortexing after each addition. • Store at room temperature. • Results in a 33% dilution from original OD_{600} measurement.	No biomass clumping during alcohol addition. At lower OD_{600} readings (<0.5), majority of fixed cells remain suspended in solution. Cells easily resuspend. Intact cells observed under microscope. Using *Bac*Light staining, stains yellow which changes to orange/red within 30 to 120 s under fluorescent light. Some green staining debris visible immediately after fixing, but is not observed over time.
Iso/H2O • Grow 20 mL of fresh live culture in liquid media to an OD_{600} of 0.3 to 1.0. • Centrifuge at 10,000 g for 10 mins. Decant liquid. • Resuspend pellet in 2 mL of sterile millipore water. • Bring to 20 mL volume with 70% isopropyl alcohol. • Incubate for 1 hr, mixing every 15 mins. • Centrifuge at 10,000 g for 10 mins. Decant liquid. • Resuspend pellet in 20 mL sterile millipore water. • Centrifuge at 10,000 g for 10 mins. Decant liquid. • Resuspend pellet in 20 mL sterile millipore water. • Store at room temperature. • Results in no dilution from original OD_{600} reading.	No biomass clumping during alcohol addition. At OD_{600} readings near 1.0, some settling of cells, but easily resuspends. Intact cells observed under microscope. Using *Bac*Light staining, stains yellow which changes quickly (<15 s) to orange/red under fluorescent light. Some green staining debris visible immediately after fixing, but is not observed over time.
Iso/Iso • Grow 20 mL of fresh live culture in liquid media to an OD_{600} of 0.3 to 1.0. • Centrifuge at 10,000 g for 10 mins. Decant liquid. • Resuspend pellet in 2 mL of sterile millipore water. • Bring to 20 mL volume with 70% isopropyl alcohol. • Store at room temperature. • Results in no dilution from original OD_{600} reading.	No biomass clumping during alcohol addition. At OD_{600} readings near 1.0, some settling of cells, but easily resuspends. Intact cells observed under microscope. Using *Bac*Light staining, stains yellow which changes quickly (<15 s) to orange/red under fluorescent light. Some green staining debris visible immediately after fixing, but is not observed over time.

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
Antibody labels							
fluorescein (FITC)	small organic molecule, antibody conjugation	485 +/-11	>=530	According to ref 7, fluorescein and rhodamine emissions are not well separated, making simulataneous FCM difficult	MP Omega O-5717, 5852, 5854, 5855, 5856	Conjugated with antibody in ref 6 and used with SYTO 9 for simultaneous id (green ring) of C. parvum and viability determination (red throughout)	1, 2, 6,7
Cy7			far red (780)	narrow excitation and emission bands		indotricarbocyanine (Cy7), very high extinction coefficient, high quantum efficiency	9
Cy5		630	red	narrow excitation and emission bands	MP Omega O-5856	indodicarbocyanine (Cy5), very high extinction coefficient, high quantum efficiency	1,9
Cy5PE	tandem resonance energy transfer, antibody conjugation	488 and 633	682 +/- 33			excite at the phycobiliprotein wl, emit at cy wl (r9)	2,9
Cy7PE	tandem resonance energy transfer, antibody conjugation	488	785 +/- 50	low autofluorescence in this part of spectrum, brightness comparable to fluorescein, no significant nonspecific binding characteristics (r9)		excite at the phycobiliprotein wl, emit at cy wl (r9)	2,9
Cy7APC	tandem resonance energy transfer, antibody conjugation	600-647	780	low autofluorescence in this part of spectrum, brightness comparable to fluorescein, no significant nonspecific binding characteristics (r9)		excite at the phycobiliprotein wl, emit at cy wl (r9)	9
Cascade Blue	antibody conjugation	351/ 361, 405	440 +/140				2
Phycoerythrin (PE)	photosynthetic accessory pigment, large phycobiliprotein (240 kDa) w/25 fluors. antibody conjugation	488 and 533 (r2), 490-560 (r7)	575 +/- 25 (r2), 570 (r7)			"bilins" are the fluorescent group (r7), broad excitation range (r7)	2,7
Phycocyanins (PC)	photosynthetic accessory pigment, 240 kDa phycobiliprotein, antibody conjugation	580-620	640			broad excitation range	7
Allophycocyanin (APC)	photosynthetic accessory pigment, 80 kDa (r2) - 110 kDa (r3) phycobiliprotein, antibody conjugation	595-605, 633 (r2), 600-650 (r7), 600-647 (r9)	660 +/- 20 (r2), 680 (r7)	emits in region with low cellular autofluorescence		huge absorption coefficient and almost perfect quantum efficiency (r2), broad excitation range (r7)	2,7,9
Texas Red	small organic molecule, antibody conjugation	595-605, 567 (r2)	630 +/- 22 (r2)		MP Omega O-5854, 5855, 5856 (r1)		1,2,9
YOYO-1	antibody conjugation	485 +/-11	>=530		MP Omega O-5717		1
Membrane potential indicators							

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
tetramethyl-rhodamine (r1)/ Rhodamine 123 (r7, 8)	lipophilic cationic membrane potential sensitive dye, viability dependent uptake	514.5 subopt (r7), 488 (r5)	red (r7), 520 (r5)		MP Omega O-5852 (r1)	Unacceptably low fluorescence on L. monocyto-genes (r5), may be toxic or unpredictable (r8)	1,5,7,8
Cyanine	lipophilic cationic membrane potential sensitive dye, viability dependent uptake						8
Oxonol VI	membrane potential sensitive dye, anionic oxonol, excluded by undamaged membrane		red (r8)	emits away from autofluorescence		not as toxic or unpredictable (in terms of uptake or active flux) as cationic fluorophores	8
JC-1	membrane potential sensitive dye, anionic oxonol, excluded, green but dimerized to red form		red			at high concentrations gives semi-quantitative measure of local differences in transmembrane potential in mitochrondria	8
Dihexyloxacarb-ocyanine	transmembrane electrochemical potential						8
Di-8-ANEPPS	transmembrane electrochemical potential						8
DiBaC4(3)	transmembrane electrochemical potential						8
Nucleic acid dyes							
acridine orange	nucleic acid binding	470 +/- 18	>=515		MP Omega O-5712		1,8
BRG-1	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 98 and 100% of heat-killed oocysts	6
BRG-2	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 94 and 100% of heat-killed oocysts	6
CY 365	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 92 and 100% of heat-killed oocysts	6
CY 369	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 86 and 94% of heat-killed oocysts	6
FUN-1	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
Hexidium	nucleic acid dye, did not pass through C. parvum oocyst (6) or G. muris cycst (10) membrane	490	520 (but stains orange-red? r10)	Ozone, chlorine and chlorine dioxide treatments did not interfere with staining (r6)	Nikon epi-fluores. scope, B-1A filter combination	detected between 90 and 100% of heat-killed oocysts, indicated stained red, incubation 60 min or >	6,10
MPR 71058	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 88 and 96% of heat-killed oocysts	6
MPR 71044	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
MPR 71045	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
MPR 71016	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
MPR 71047	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
MPR 71048	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
MPR 71049	nucleic acid dye, did not pass througy G. muris cyst (r10)	490	520		Nikon epi-fluores. scope, B-1A filter combination		10
propidium iodide (PI)	phenanthridinic fluorochrome, nucleic acid dye, does not pass through membrane	490	635	stained after heat and BTA treatment, but not after chlorine or monochloramine treatment (r6)	MP Omega O-5745, 5852, 5854, 5855, 5856 (5712, 5717)		1,6,8
SYTO 9	nucleic acid dye, will pass through membrane in BacLight	480	500		MP Omega O-5745, 5852, 5854, 5855, 5856 (5712, 5717)		1
SYTO 9	nucleic acid dye, did not pass through C. parvum oocyst (6) or G. muris (10) membrane	490 (r6,10)	520 (r6,10) stains yellow (r10)	Ozone, chlorine and chlorine dioxide treatments did not interfere with staining (r6)	Nikon epi-fluores. scope, B-1A filter combination	detected between 98 and 100% of heat-killed oocysts, nonviable oocysts stained light green-yellow, incubation 60 min or > (r6)	6, 10
SYTO 11	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	508 (r3) 490 (r6,10)	527 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 80 and 94% of heat-killed oocysts	3, 6,10

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
SYTO 12	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	499 (r3) 490 (r6,10)	522 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 70 and 80% of heat-killed oocysts (r6), exhibited toxic behavoir (r10)	3, 6,10
SYTO 13	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	488 (r3) 490 (r6,10)	509 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 78 and 90% of heat-killed oocysts	3, 6,10
SYTO 14	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	517 (r3) 490 (r6,10)	549 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 64 and 85% of heat-killed oocysts	3, 6,10
SYTO 15	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	516 (r3) 490 (r6,10)	546 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 76 and 82% of heat-killed oocysts	3, 6,10
SYTO 16	nucleic acid dye, did not pass through C. parvum oocyst (r6) or G. muris cyst membrane (r10)	488 (r3) 490 (r6,10)	518 (r3) 520 (r6,10)		Nikon epi-fluores. scope, B-1A filter combination	detected between 76 and 92% of heat-killed oocysts (r6), exhibited toxic behavoir (r10)	3, 6,10
S-7572	nucleic acid dye, did not pass througy G. muris cyst (r10)	490 (r10)	520 (r10)		Nikon epi-fluores. scope, B-1A filter combination		10
SYTO 18	nucleic acid dye	490	507				3
SYTO 20	nucleic acid dye	512	530				3
SYTO 21	nucleic acid dye	494	517				3
SYTO 22	nucleic acid dye	515	535				3
SYTO 23	nucleic acid dye	499	520				3
SYTO 24	nucleic acid dye	490	515				3
SYTO 25	nucleic acid dye	521	556				3
SY 756	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 80 and 94% of heat-killed oocysts	6
SY 1169	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 88 and 94% of heat-killed oocysts	6
SYBR-1	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490	520		Nikon epi-fluores. scope, B-1A filter combination	detected between 80 and 90% of heat-killed oocysts	6
SYTO 59	nucleic acid dye	630	645				4

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
SYTO 59	nucleic acid dye, in this ref did not pass through C. parvum oocyst membrane	490 (?)	520 (?)	Ozone, chlorine and chlorine dioxide treatments did not interfere with staining (r6)	Nikon epi-fluores. scope, B-1A filter combination	detected between 99 and 100% of heat-killed oocysts, indicated stained red, incubation 60 min or >	6
SYTO 60	nucleic acid dye	652	678				4
SYTO 61	nucleic acid dye	628	645				4
SYTO 62	nucleic acid dye	652	676				4
SYTO 63	nucleic acid dye	657	673				4
SYTO 64	nucleic acid dye	599	619				4
SYTOX Green	nucleic acid dye	485 +/- 11	>=530		MP Omega O-5717		1
Activity probes							
Fluorescein diacetate (FDA)	fluorogenic ester, requiring 6-24 hr incubation time to accumulate fluorescein	485 +/- 11 (r1)	>=530 (r1)			original molecule non-fluorescent, enters cell by diffision, metabolized (esterase) to release free fluorescein, which accumulates in cell (r6)	5,1,6,8
carboxyfluorescein diacetate (CFDA)	fluorogenic ester, requiring 6-24 hr incubation time to accumulate fluorescein	488	520			Good correlation between fluorescence and viability of L. monocytogenes, increased with exponential growth	5
CTC	activity probe (activity induced uptake)		green				8
Chemchrome B	fluorogenic ester, requiring 6-24 hr incubation time to accumulate fluorescein, bacteria	488	520			Good correlation between fluorescence and viability of L. monocytogenes, increased with exponential growth	5,8
Chemchrome Y	activity probe, fungi		green				8
BCECF-AM	fluorogenic ester, additional alkyl groups facilitating uptake	488	520			Unacceptably low fluorescence on L. monocytogenes	5
FDA-PI	see data on FDA and PI			the use of chemical disinfectants resulted in unsatisfactory results		Combined use of FDA (resulting in accumulation of fluorescein in live cells) an propidium iodide which only stains dead cells	6, 10
Calcein acetoxymethyl ester	esterase activity and membrane integrity						8
Fluorescein di-B-D-glactopyranoside	B-glactosidase activity and membrane integrity					FCM sorting can discriminate between viable and non-viable bacteria	8

IVSS Table 4

Table 4. Summary of literature review on potential stains and dyes for use with IVSS.							
Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
Hydroethidine	oxidation of ethidium bromide and subseqent DNA binding						8
Tetrazolium	fluorescent type used as a respiratory chain activity probe		green			dye reduction	8
Misc fluorescent dyes							
BODIPY FL ceramide		485 +/- 11	530 +/- 15, >=590		MP Omega O-5745		1
dye exclusion vital stains							
DAPI	dye exclusion (r8)				MP Omega O-5855, 5856	used in combintion with PI to detect C. parvum oocysts using FCM (r6)	1,6,8
ethidium homodimer	dye exclusion						8
eosin	exclusion, acidic dye, anionic chromophore, will not stain membrane (r11)						6,10, 11
Non-fluorescent vital stains							
Calcafluor white	undefined						8
Malachite green	used in spore staining (r11) requiring heat, inorganic						
Methylene blue	basic dye, cationic chromophore, will stain cell membrane (r11)					useful way of rapidly assessing dead organisms, esp. when used with Safranin O	8
Safranin O	counterstain for gram staining, probably a cationic chromophore (r11)						8
Trypan blue	non-fluorescent nucleic acid stain (r12)						
				Flow cytometer lasers		References	
				Dye Laser: 595-605 (2) Krypton Laser: 567, 405 (2)		1. Molecular Probes Product Information Sheet MP 7007 10/04/96. LIVE/DEAD BacLight Bacterial Viability Kits	
				Argon laser: 488, 351/361 (2)		2. M. Roederer, Conjugation of monclonal antibodies. November 13, 1997. http://cmgm.standford.edu/~roederer/abcon/	

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Dye	**Type**	**Exit wl nm**	**Emit wl nm**	**Background interference**	**Filters**	**Comments**	**Refs**
				HeNe laser: 633 (2)		3. Molecular Probes Product Information Sheet MP 07572 11/24/97. SYTO Green Fluorescent Nucleic Acid Stains	
						4. Molecular Probes Product Information Sheet MP 11340 11/24/97. SYTO Red Fluorescent Nucleic Acid Stains	
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						10. Taghi-Kilani, R., L. L. Gyurek, P. J. Millard, G. R. Finch and M. Belosevic. 1996. Nucleic acid stains as indicators of Giardia muris viability following cyst inactivation. Inter. J. Parasitology 26(6):637-646.	
						11. Benson, H.J. 1982. Microbiological applications complete version: a laboratory manual in general microbiology. Wm. C. Brown Company, Dubuque, Iowa.	
						12. Out of Sigma catalog	

Table 5. Summary of staining methods

*Bac*light assay	SYTO9/trypan blue	CFDA
• Obtain *Bac*Light assay kit L-7007 or L-7012 from Molecular Probes, Inc. Store at –4º C. • Bring *Bac*Light assay stains to room temperature - Component A: 300 µL of 3.34 mM SYTO9 in anhydrous DMSO - Compenent B: 300 µL of 20 mM propidium iodide in anhydrous DMSO • Centrifuge both components for 2 mins. at 10,000 g. • Add 1.5 µL of each component to a microfuge tube for each 1 mL of culture to be stained. Centrifuge for 2 mins. at 10,000 g. • Centrifuge 1 mL fresh live culture (OD_{600} 0.300 – 0.800) for 5 mins. at 6,000 g at 4º C. Decant liquid and resuspend in 1 mL sterile MiliQ (deionized) water. • Centrifuge 1 mL fixed culture (OD_{600} 0.300 – 0.800) at 10,000 g at 4º C for up to 20 mins. or until cohesive pellet is formed. Decant liquid and resuspend in 1 mL sterile MiliQ (deionized) water. • Add 3 µL stain mixture to 1 mL of culture. Mix (vortex). • Incubate in darkness at room temperature for 15 mins. • Trap 1.5 – 3 µL of stained culture between cleaned microscope slide and cover slip (use lower amount if streaming is a problem). • Observe immediately under a fluorescent microscope equipped with a fluorescein or other appropriate filter or analyze using flow cytometry.	• Bring Component A of the *Bac*Light assay (SYTO9) to room temperature. • Centrifuge for 2 m at 10,000 g. • Obtain a 0.4% solution (w/v) of trypan blue in a 0.85% saline solution (available commercially). • Centrifuge 1 mL fresh live culture (OD_{600} 0.300 – 0.800) for 5 mins. at 6,000 g at 4º C. Decant liquid and resuspend in 0.9 mL sterile MiliQ (deionized) water. • Centrifuge 1 mL fixed culture (OD_{600} 0.300 – 0.800) at 10,000 g at 4º C for up to 20 mins. or until cohesive pellet is formed. Decant liquid and resuspend in 0.9 mL sterile MiliQ (deionized) water. • Add 1.5 µL SYTO9 (Component A) and 100 µL trypan blue solution to each culture. Mix (vortex). • Incubate in darkness at room temperature for 10 mins. • Trap 1.5 – 3 µL of stained culture between cleaned microscope slide and cover slip (use lower amount if streaming is a problem). • Observe immediately under a fluorescent microscope equipped with a fluorescein or other appropriate filter, or analyze using flow cytometry.	• Prepare a 10 mM solution of CFDA in reagent grade acetone. • Centrifuge 1 mL fresh live culture (OD_{600} 0.300 – 0.800) for 5 mins. at 6,000 g at 4º C. Decant liquid and resuspend in 1 mL of 50 mM PBS or 0.5 M Tris-HCL (pH 7.4) buffer solution. • Centrifuge 1 mL fixed culture (OD_{600} 0.300 – 0.800) at 10,000 g at 4º C for up to 20 mins. or until cohesive pellet is formed. Decant liquid and resuspend in 1 mL of 50 mM PBS or 0.5 M Tris-HCL (pH 7.4) buffer solution. • Add 10 µL of CFDA solution to each mL of culture. Mix (vortex). • Incubate in darkness at room temperature for 30 mins. • Trap 1.5 – 3 µL of stained culture between cleaned microscope slide and cover slip (use lower amount if streaming is a problem). • Observe immediately under a fluorescent microscope equipped with a fluorescein or other appropriate filter or analyze using flow cytometry. • If samples cannot be immediately analyzed after incubation, centrifuge at 8,500 g for 10 mins., decant liquid, and resuspend in 1 mL of buffer solution. Store on ice in the dark for no longer than 45 minutes before analysis.

Figures

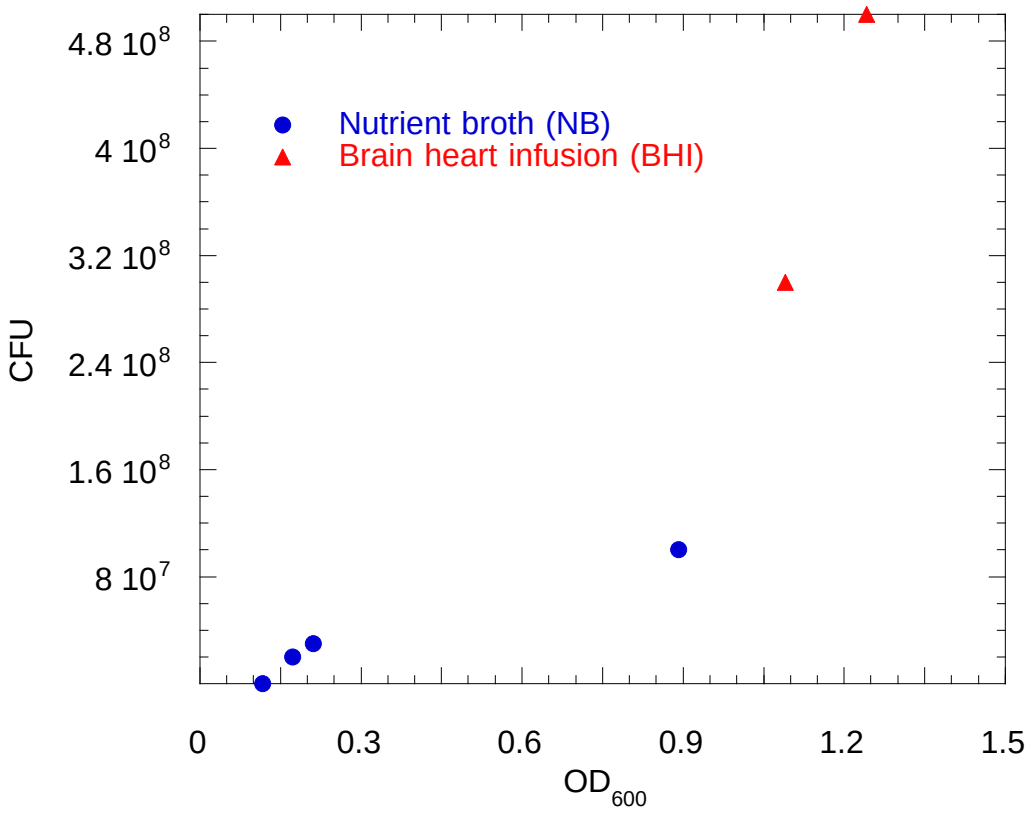


Figure 1. Relationship between OD_{600} and CFU for *B. globigii* cultured in 20-25 ml of media for 17-23 hr @ 30° C, agitated at 150 rpm.

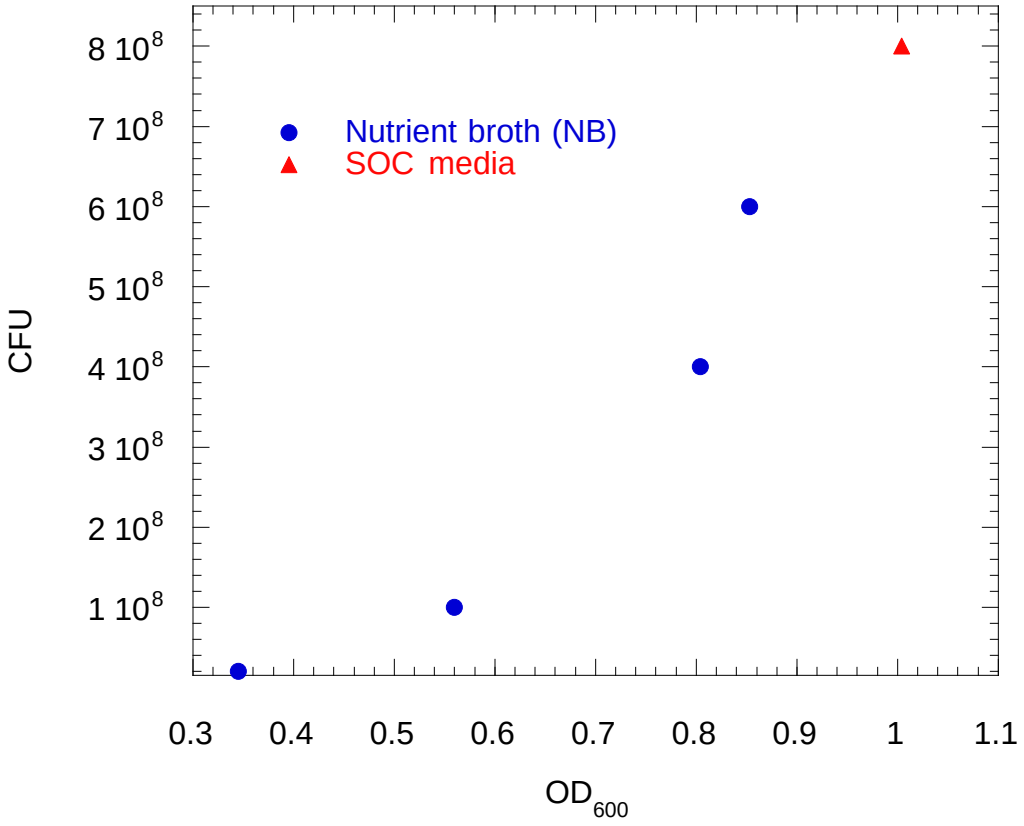


Figure 2. Relationship between OD_{600} and CFU for *P. agglomerans* cultured in 20-25 ml of media for 17-23 hr @ 30° C, agitated at 150 rpm.

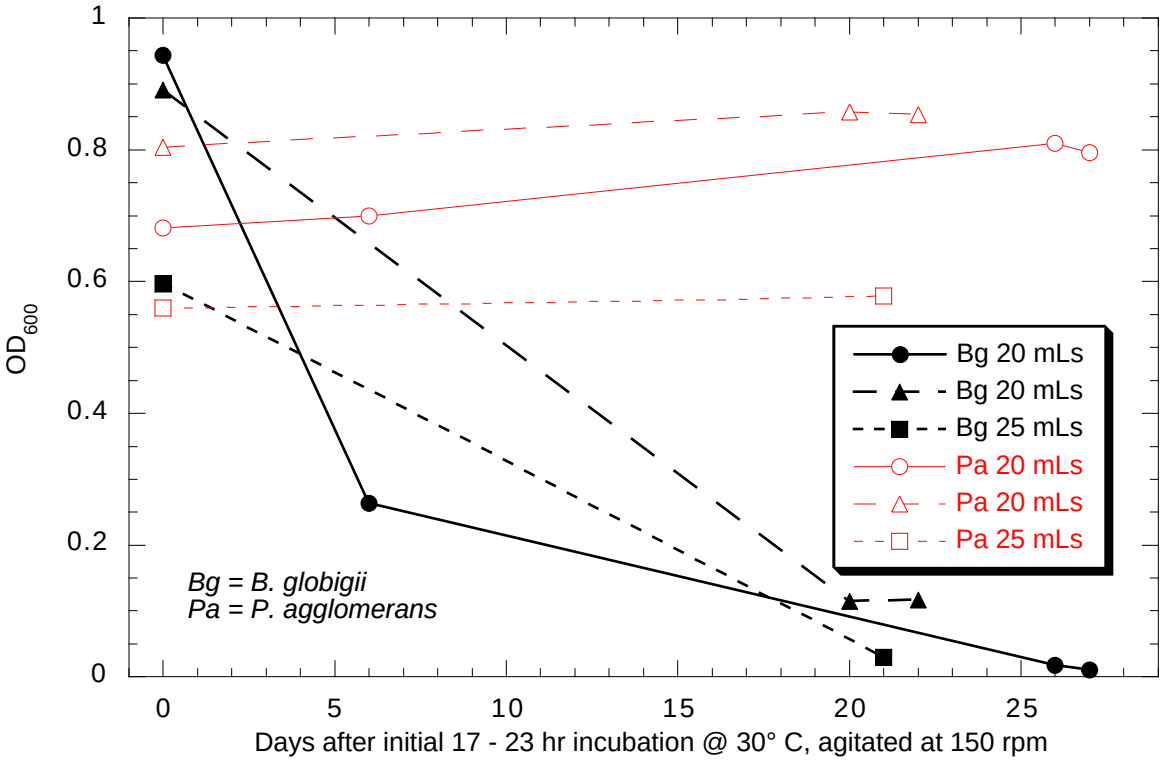


Figure 3. Longevity of live cultures grown in 20 - 25 mLs of nutrient broth and stored at 4° C (without agitation) after initial incubation.

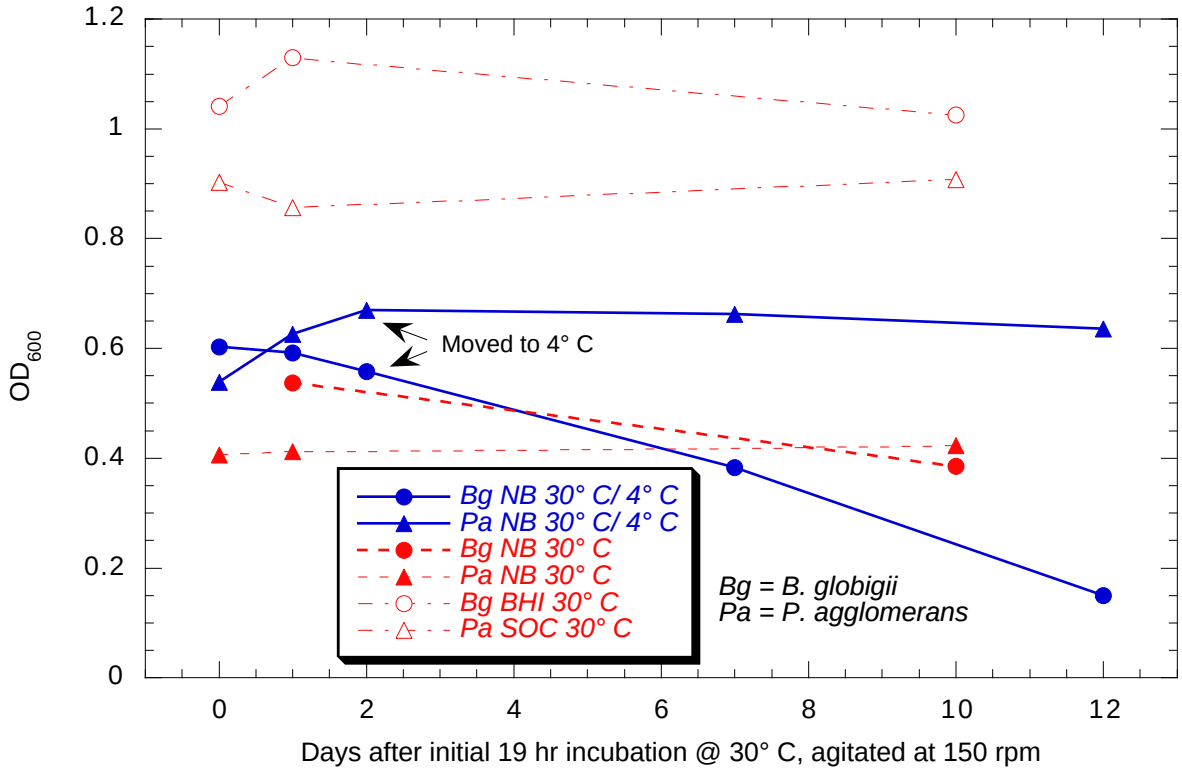


Figure 4. Longevity of live cultures grown in 25 mLs of media (nutrient broth [NB], brain heart infusion [BHI] or SOC) and stored at 30° C (agitated at 150 rpm) or 4° C (without agitation) after initial incubation.

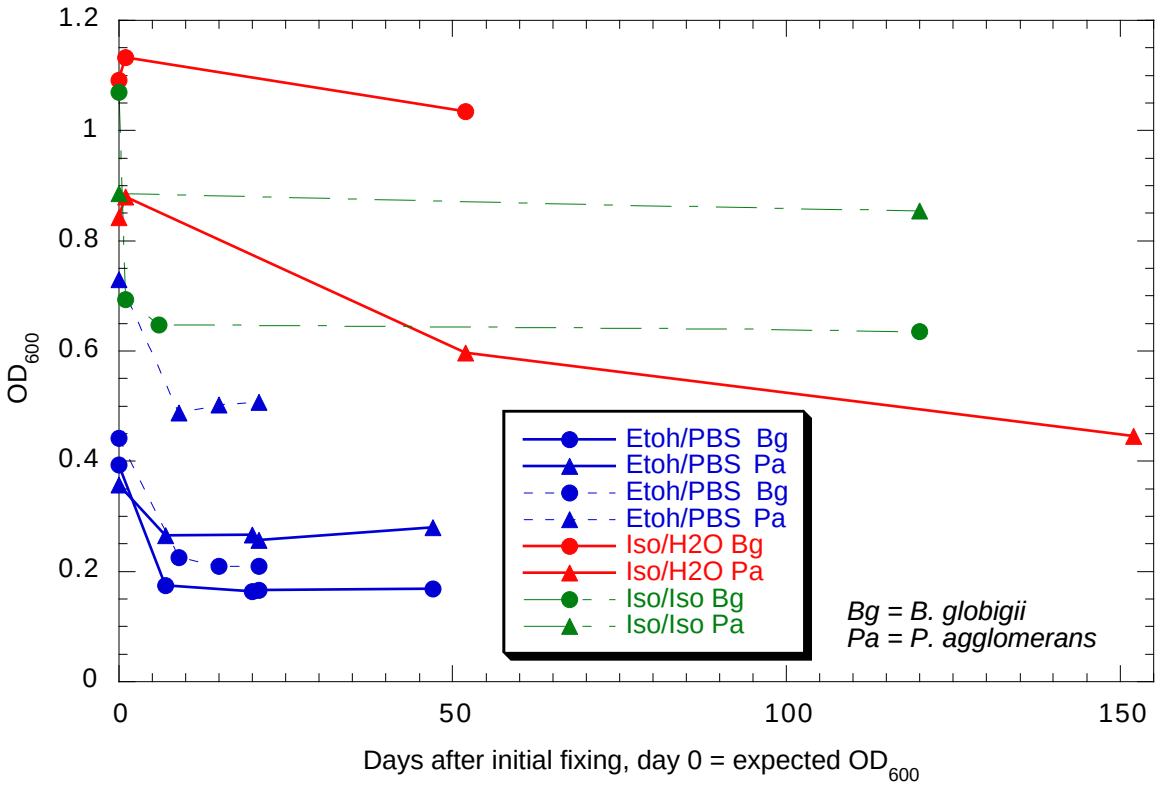


Figure 5. Longevity of fixed cultures. See Table 3 for description of fixing procedures.

A. B.

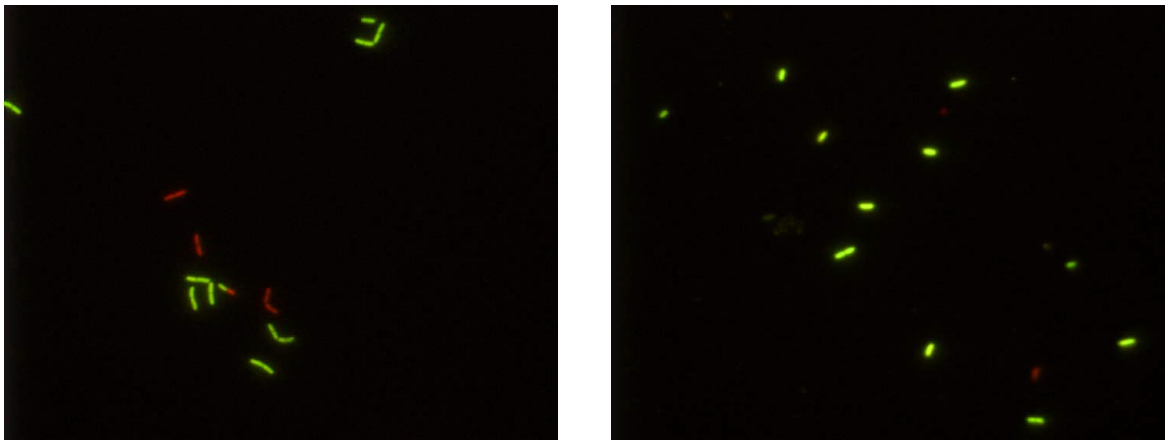


Figure 6. Fluorescent photomicrographs of *Bac*Light staining of *B. globiggi* (A) and *P. agglomerans* (B).

A. B.

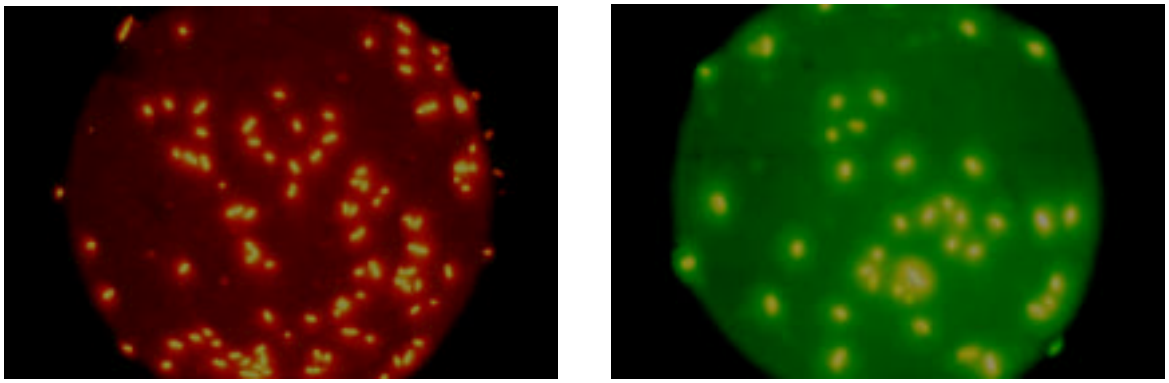


Figure 7. Fluorescent photomicrographs of dead (fixed) vegetative *P. agglomerans* culture stained with the full *Bac*Light assay (A) and with SYT09 only (B). Photomicrographs have been electronically compensated for overexposure.

A. B.

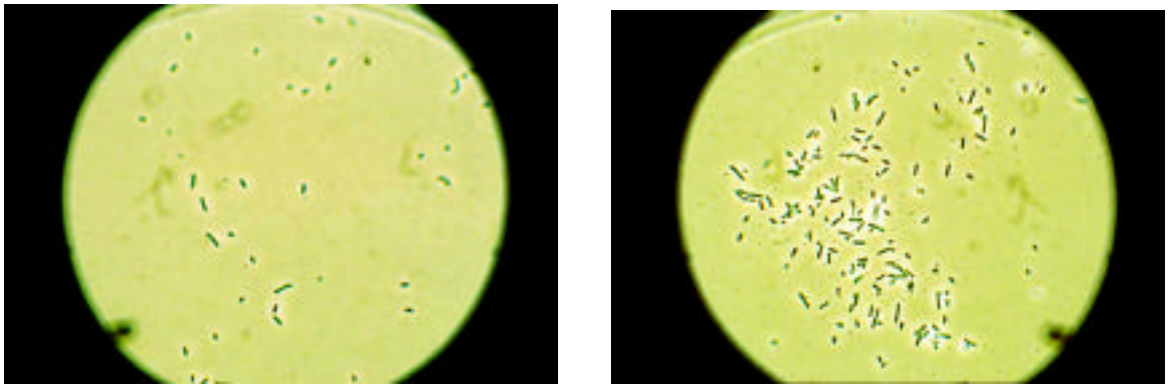


Figure 8. Brightfield photomicrographs of live (A) and dead (B) cultures of vegetative *P. agglomerans* stained with SYT09 and malachite green.

A. B.

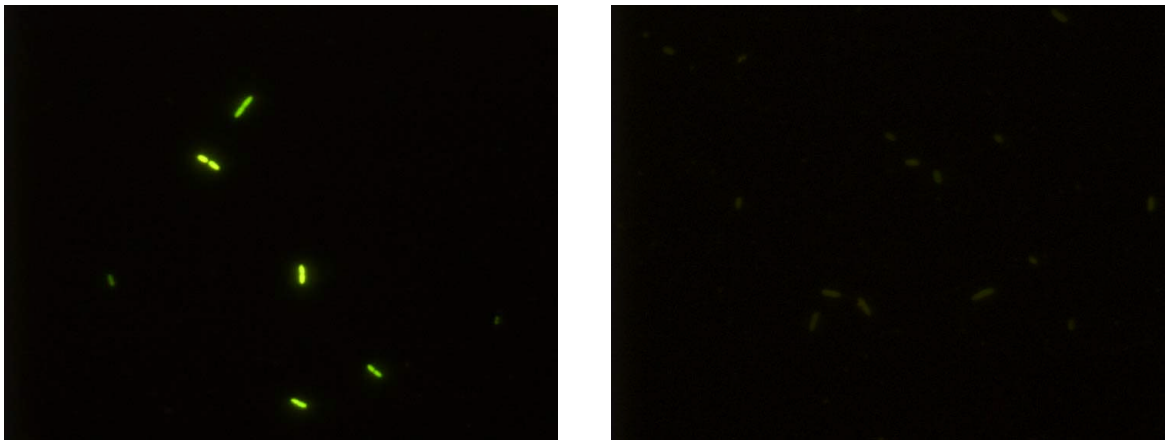


Figure 9. Fluorescent photomicrographs of live (A) and dead (B) *P. agglomerans* stained with SYTO9/trypan blue.

A. B.

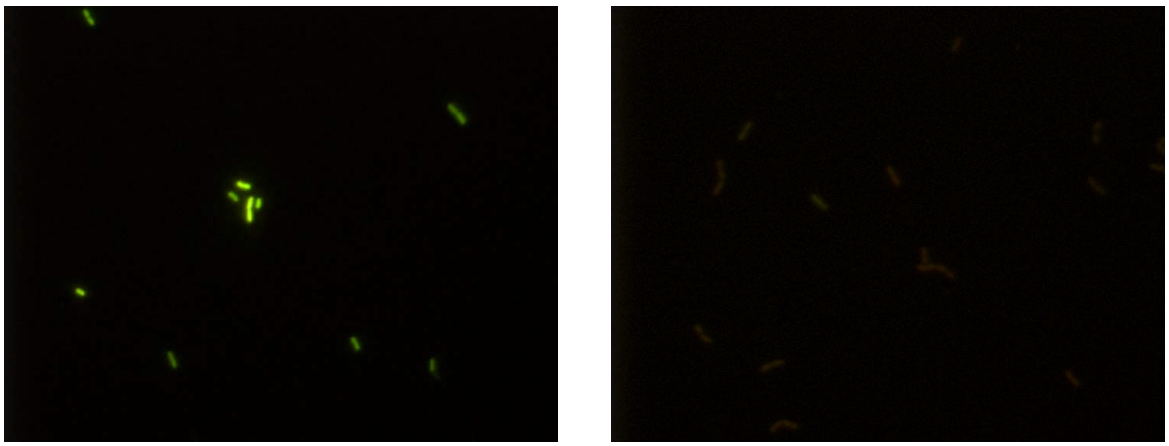
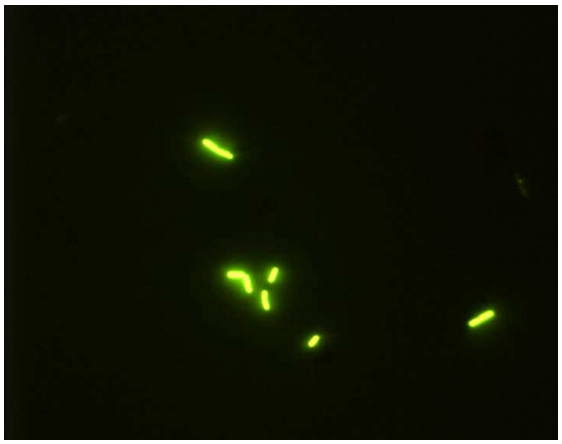


Figure 10. Fluorescent photomicrographs of live (A) and dead (B) *B. globigii* stained with SYTO9/trypan blue.

A.



B.

C.

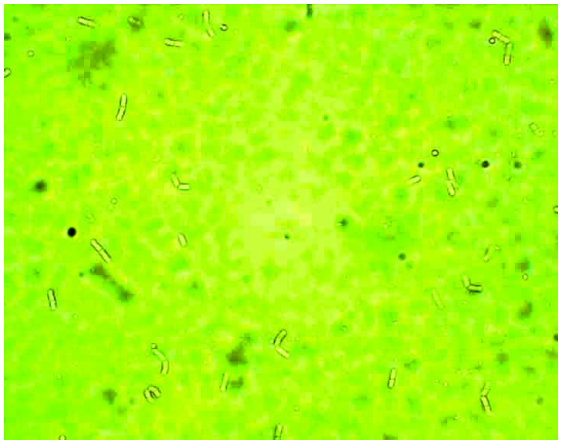
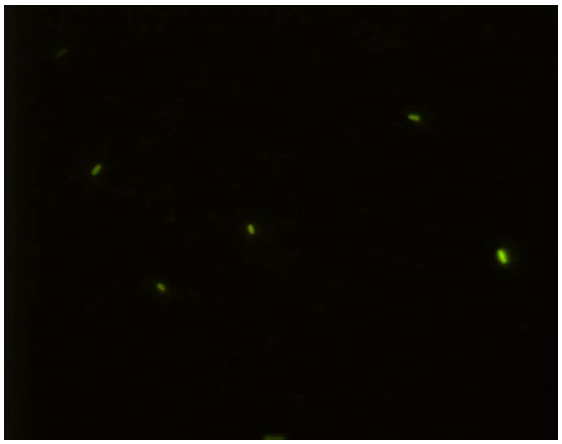
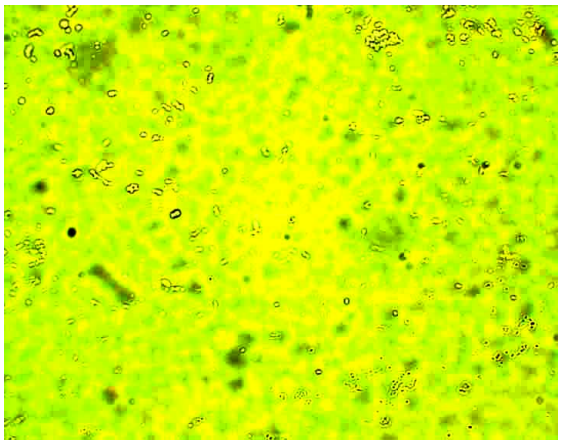


Figure 11. Fluorescent (A and B) and bright field (C) photomicrographs of live (A) and dead (B and C) *B. globigii* cultures incubated with CFDA. Cells in figure C are the lighter colored rods.

A.



B.



C.

D.

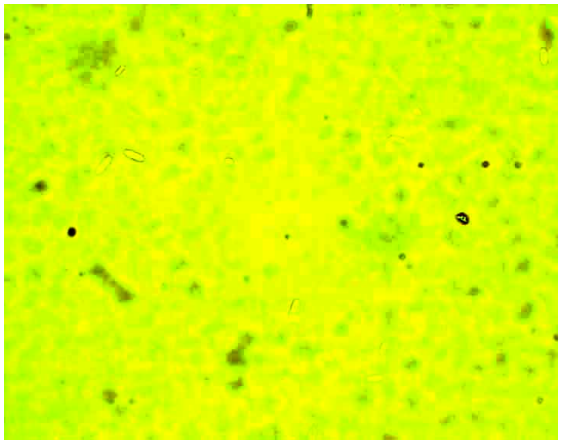


Figure 12. Fluorescent (A and C) and bright field (B and D) photomicrographs of live (A and B) and dead (C and D) cultures of *P. agglomerans* incubated with CFDA. The cells in the bright field photomicrographs are the lighter colored rods.

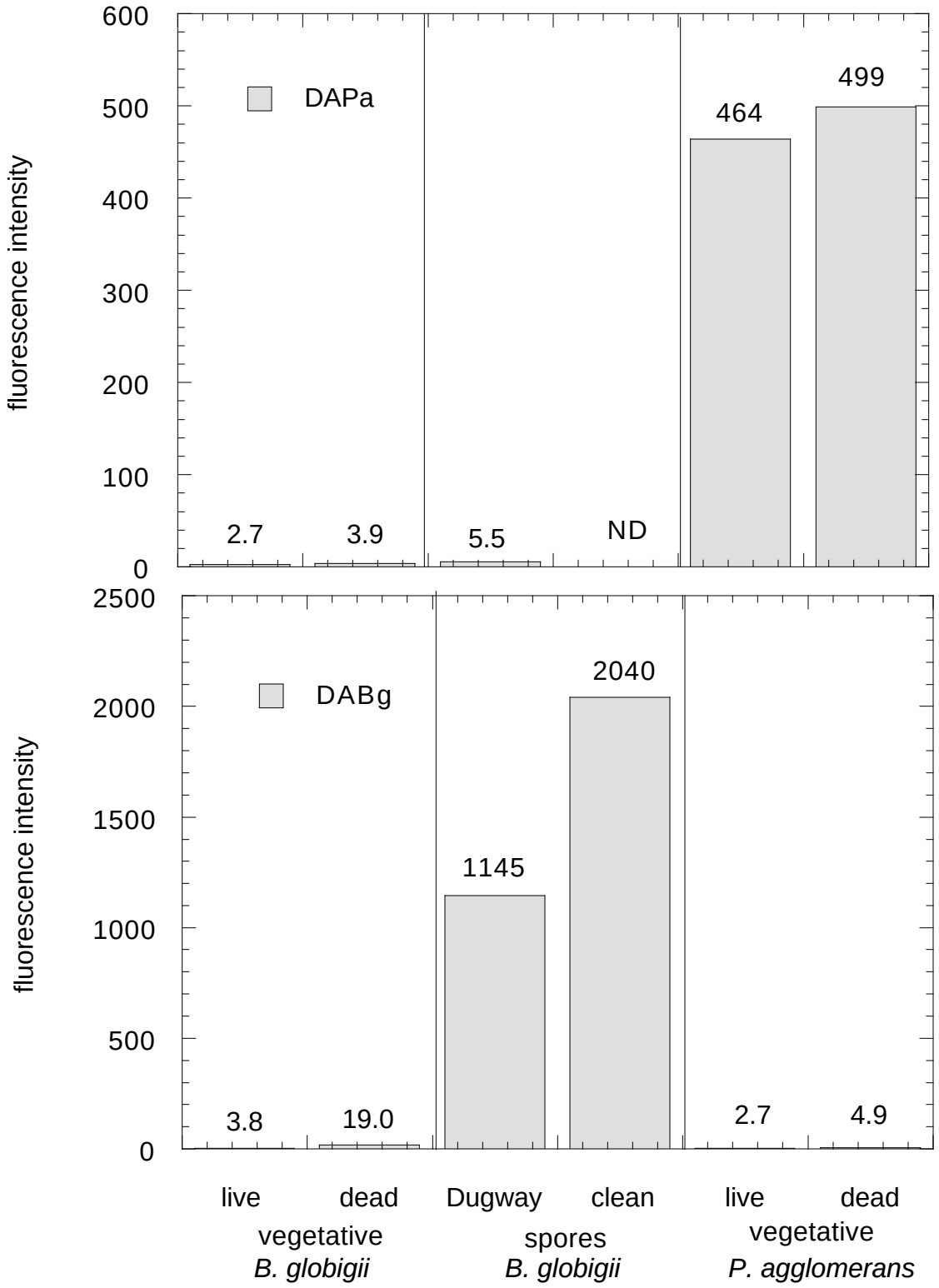


Figure 13. Tests of Dugway antibodies labeled with fluorescein on various cell cultures.
DAPa = Dugway antibody for *P. agglomerans*
DABg = Dugway antibody for *B. globigii*

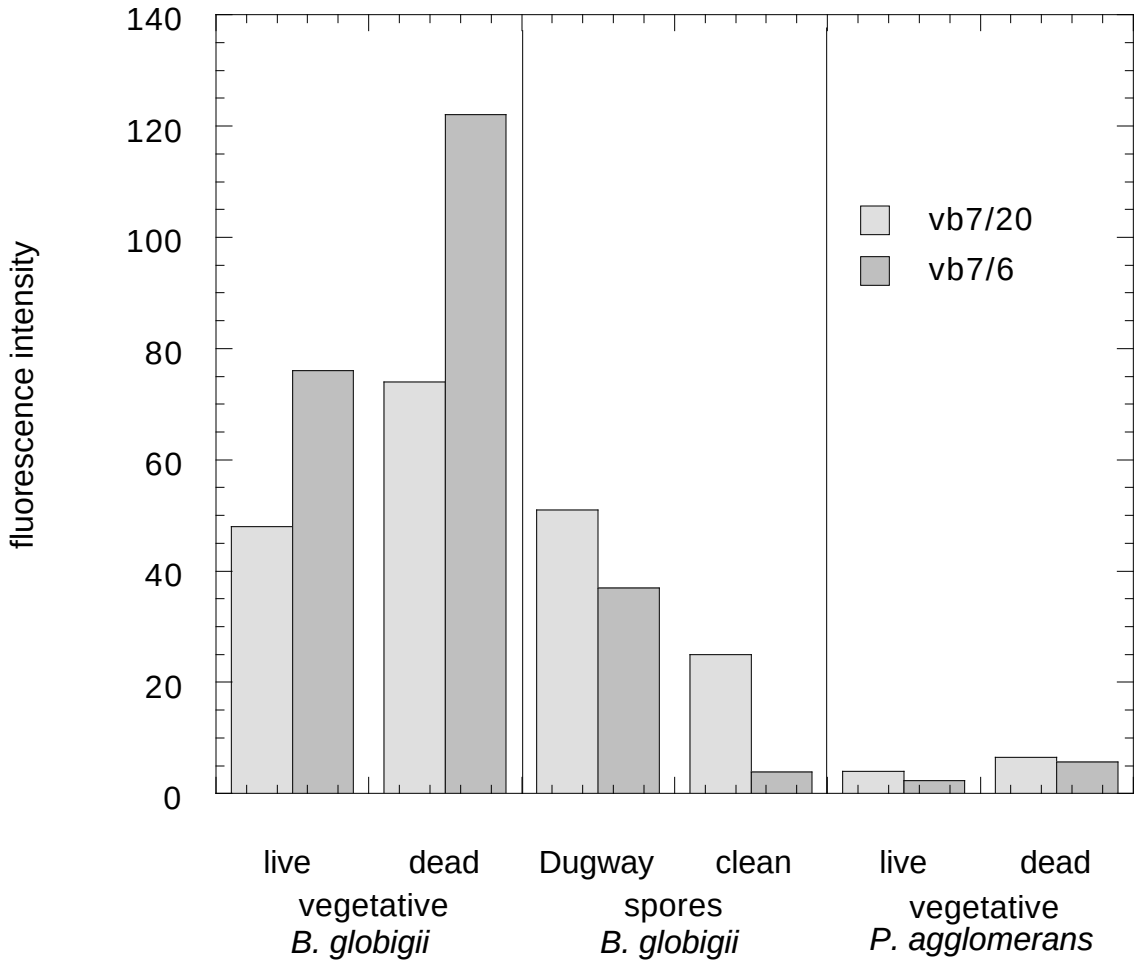
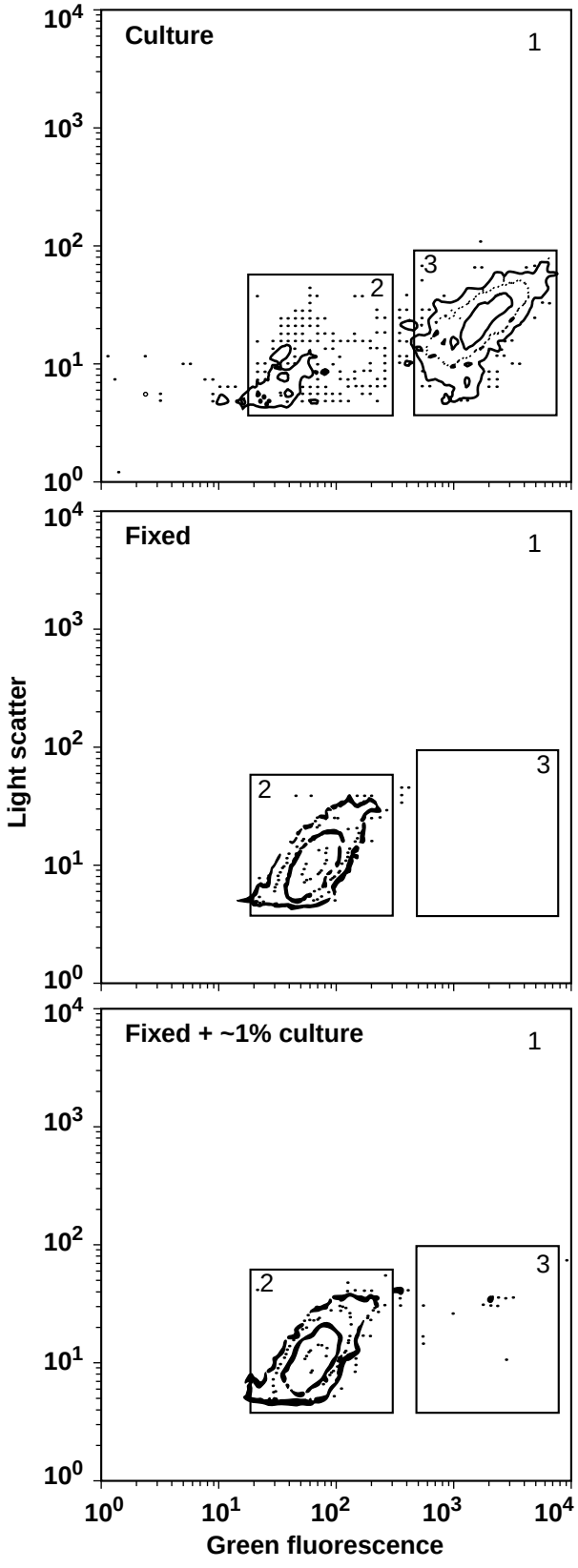


Figure 14. Tests of two NAMRI vegetative *B. globigii* antibodies (vb7/20 and vb7/6) labeled with fluorescein to various cell cultures.



ERD-S3R-99-0142

Figure 15. Dual-color nucleic acid labeling of vegetative *B. globigii* using the *Bac* Light assay (SYTO 9 and propidium iodide).

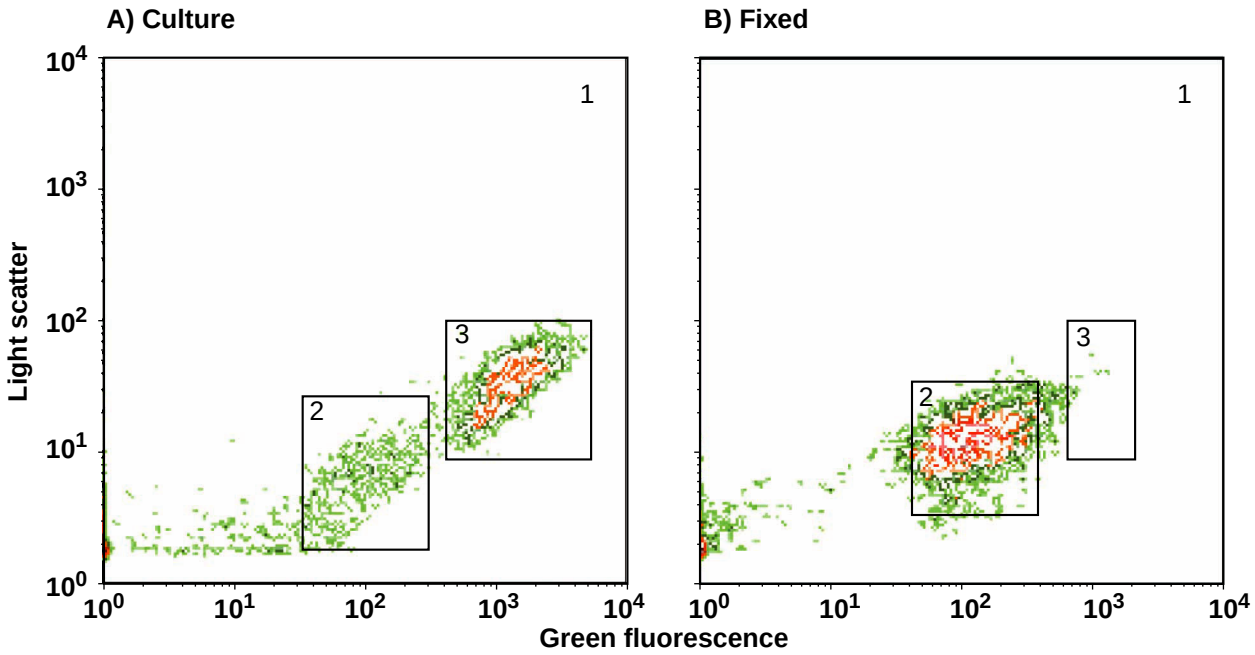
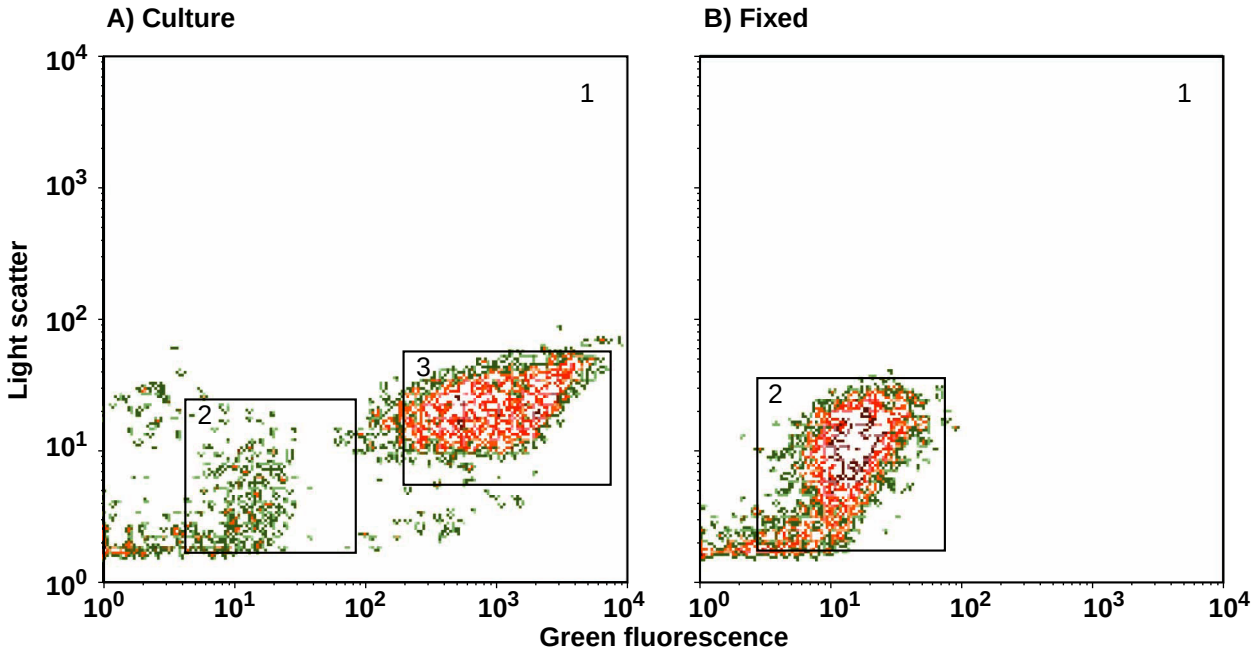


Figure 16. Single-color nucleic acid labeling of vegetative *B. globigii* using SYTO 9 and trypan blue.



ERD-S3R-99-0118

Figure 17. Metabolic activity labeling of vegetative *B. globigii* using CFDA.

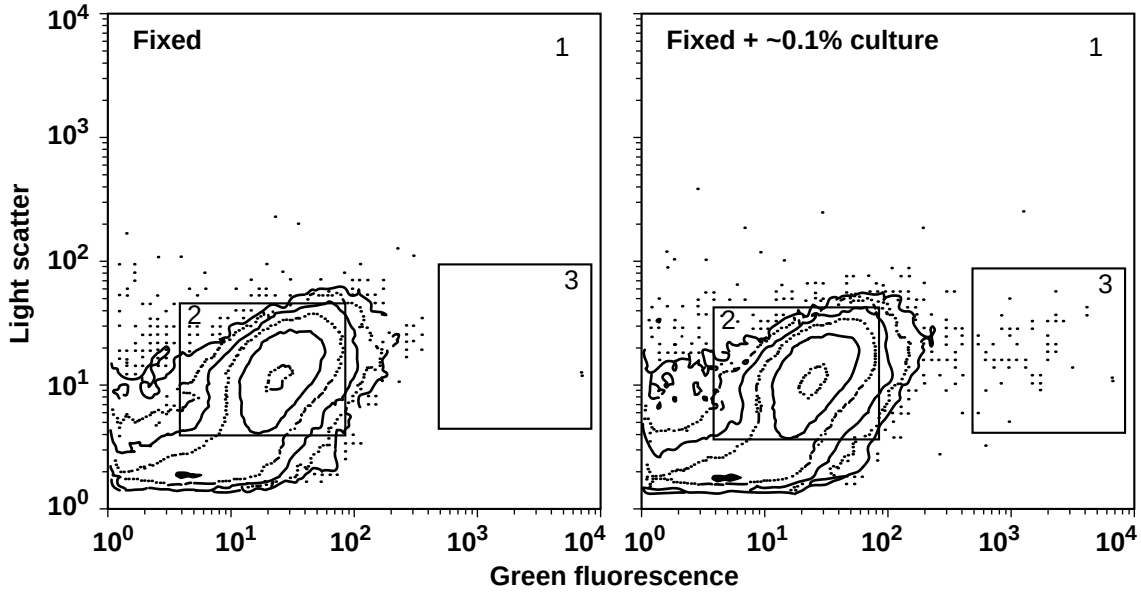
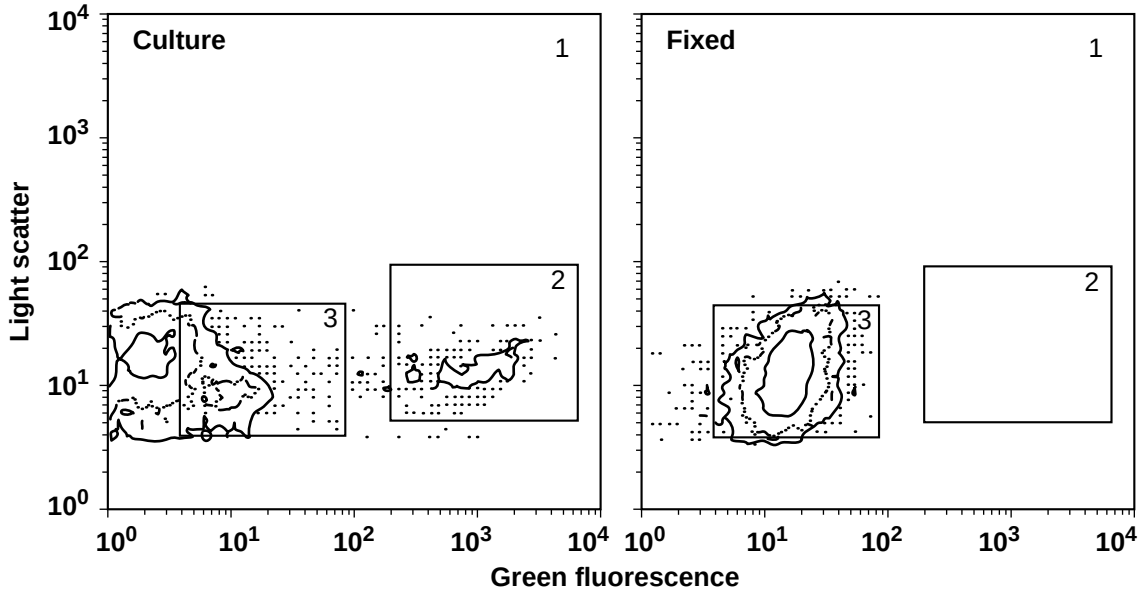
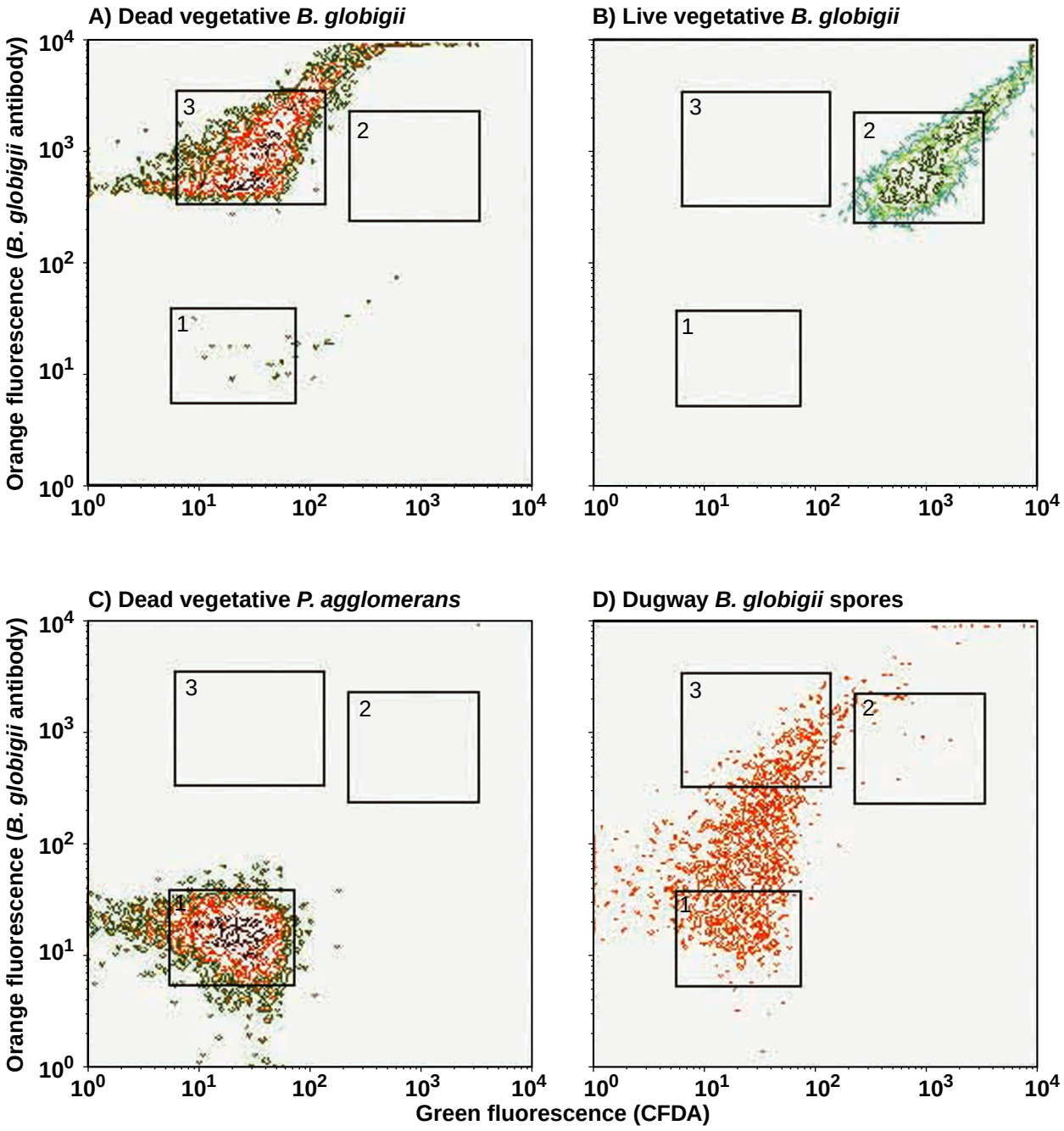


Figure 18. Metabolic activity labeling of fixed and fixed with ~0.1% live cultures of vegetative *B. globigii* using CFDA.



ERD-S3R-99-0144

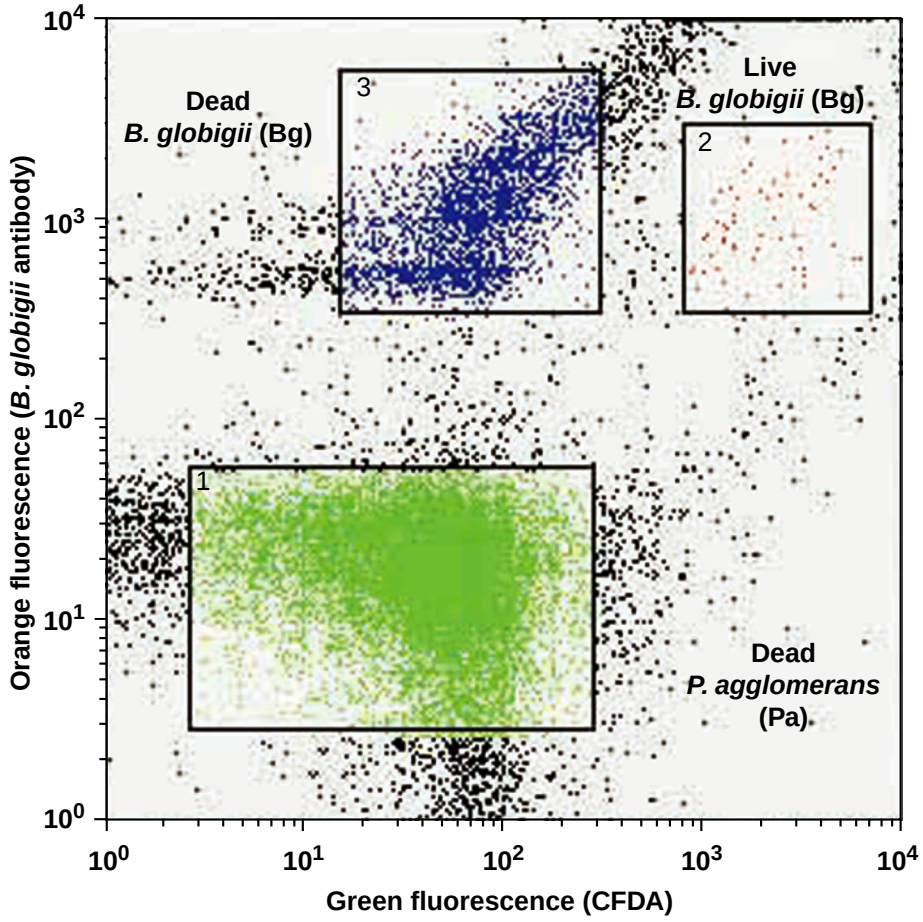
Figure 19. Metabolic activity labeling of vegetative *P. agglomerans* using CFDA.



ERD-S3R-99-0119

Figure 20. Flow cytometry analysis of various cultures incubated with metabolic activity label CFDA and phycoerythrin-labeled vegetative *B. globigii* antibody.

Cell type	Calculated percentage	Observed percentage
Dead Pa	86.5	84.6
Dead Bg	13.0	15.0
Live Bg	0.5	0.4



ERD-S3R-99-0120

Figure 21. Flow cytometry analysis of artificial mixtures of vegetative cultures incubated with the metabolic activity indicator CFDA and antibody for vegetative *B. globigii*.