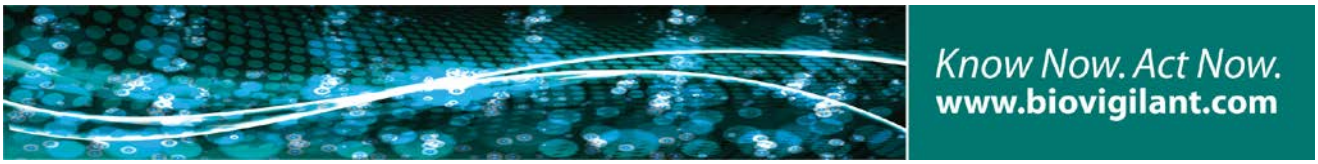

BioVigilant®
Instantaneous Microbial Detection

Understanding *Fluorescence Sensitivity*: A Key Parameter in IMD-W™ Detection Performance

Executive Summary

The IMD-W™ system has been designed to provide continuous, online monitoring of ultrapure water systems for bioburden. The Online Water Bioburden Analyzer (OWBA) work group of Pharmaceutical stakeholders released a User Requirements Specification in March 2013 that outlines the application, value, and technical requirements for such an instrument. [A]

The target sensitivity suggested in the OWBA specification is based upon a unit of microbe equivalent (µeq) which, depending upon the technological approach to detection, is tantamount to a planktonic (single, free-floating) microbe. Detection of planktonic organisms is vital to the core principle of rapid detection, in that these organisms are the earliest indicators of potential biofilm formation, and also the earliest indicators of potential changes within a water system. As such, the mere detection of a clump of organisms (e.g. from a biofilm detachment/dispersal event) is insufficient to understand microbial changes within a Purified Water (PW) or Water for Injection (WFI) system.

For this reason, the IMD-W technology is based upon the principle of using Mie scatter and autofluorescence to detect microbes on a particle-by-particle basis (as opposed to bulk detection used by certain chemical methods). This approach does not imply that 100% detection of all microbes is inherently achieved, as the challenge to detect a microbe varies with its size, autofluorescent properties, metabolic state, and other properties. In accordance, the OWBA Requirement Specification document targets a Limit of Detection of 1 µeq/mL.

To test and ensure the IMD-W system to be fit for its intended application, BioVigilant uses several microparticle suspensions from third-party vendors for calibration and suitability checks of the instrument. Among these particles, the commercially-available Spherotech Yellow Low Intensity 0.8µm particle is used as a benchmark for *minimum* instrument fluorescence performance. [B]

These suspensions, analyzed on a particle-by-particle basis during calibration and system suitability checks, ensure the system's capability for detection of single, waterborne microbes at a rate suitable for the intended application.

IMD-W System and Bead Selection

The IMD-W system evolved from the core technological principles of its predecessor, the IMD-A® system from BioVigilant. Both instruments use 405nm laser-induced scatter and autofluorescence to detect and discriminate microbes on a particle-by-particle basis.

Due to the increased complexity of an optical system for handling water compared to air, signals from particles and microbes are significantly lower and harder to detect in water. This is a key reason why the IMD-W system was developed subsequent to the IMD-A system. To improve specificity and reduce the possibility of detecting interferent particles, the IMD-W system uses two PMT detectors to discriminate particle signals based upon spectral content.

Flow cytometers are a similar class of instruments used to detect scatter and fluorescence of waterborne particles, and there are many vendors of reference particles for these applications. However, flow cytometers almost exclusively detect *extrinsic* fluorescence that is applied to a sample using stains, reagents, highly-fluorescent conjugate particles, etc. They are not designed to detect the ultra-low levels of autofluorescence from microbes. As such, the fluorescent particles designed for flow cytometer applications are largely inappropriate for an instrument like the IMD-W system, as fluorescent levels from these particles can be ten times, or even 10^5 times and beyond, the autofluorescence from a microbe. The tradeoffs for this enhanced signal strength are the requirement for sample prep and inability for a system to operate continuously in-situ. Even if such particles do not saturate the fluorescence detectors on an instrument like the IMD-W system, they do little to confirm performance in the intended application. One exception is the 0.8 µm Spherotech Yellow Low Intensity (STY) bead. Not only is the bead size similar to that of microbes, the fluorescence spectrum and intensity when excited with 405nm is on the same order of magnitude as microbes. These features make it particularly attractive as a general reference for size and fluorescence, and underscore its use as a standard calibration bead on the IMD-A system.

[A] OWBA URS Document v1.3

[B] Part Number FL-0852-2 from Spherotech, Inc. (Lake Forest, IL)

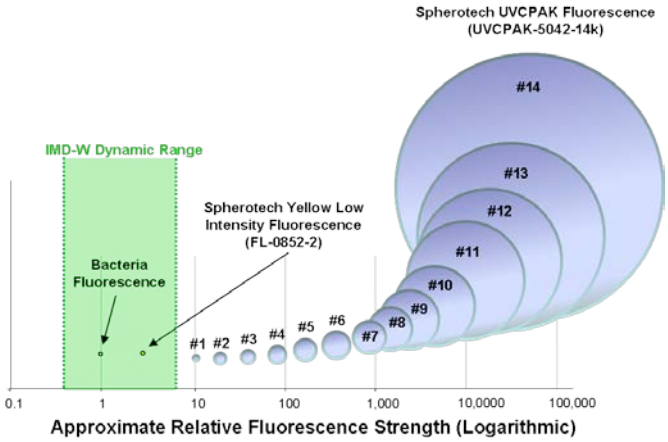

Figure 1: Comparison of relative fluorescence levels for bacteria and various Spherotech beads.

To put the fluorescence of the STY bead in context with beads relevant to common flow cytometer applications, **Figure 1** illustrates the approximate relative levels of fluorescence from a microbe, the STY bead, and a 14-peak series of beads from Spherotech for flow cytometers with violet excitation.[C]

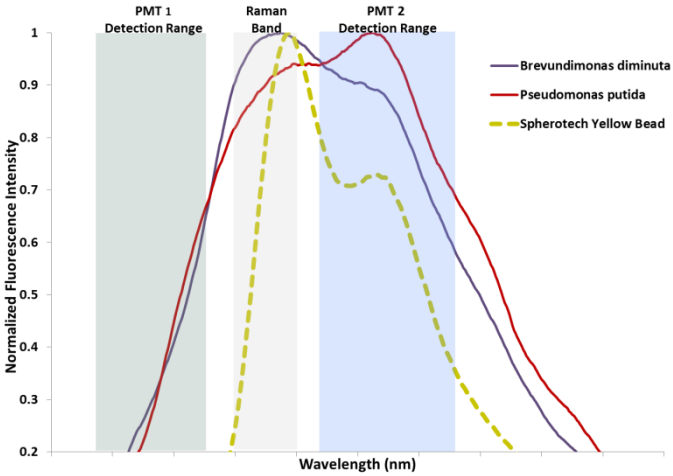

Figure 2: Comparison of microbial and STY fluorescence within the IMD-W detection range.

Figure 2 illustrates the normalized intensity for the STY bead and two waterborne microbes as a function of fluorescence emission wavelength (for 405nm excitation). Superimposed on the graph are bands that represent spectral regions for the IMD-W's two fluorescence detectors. The center band represents the region where Raman (vibrational) fluorescence is inherently generated from the background water and must be excluded. As can be seen, the integrated fluorescence on the PMT-2 channel for the microbes is much greater than that for the PMT-1 channel. This is generally true for microbes as well as the STY bead, so the PMT-2 channel is the primary channel to use for comparing microbial and bead fluorescence. When any such fluorescent particle is measured, each PMT produces a signal whose magnitude is linked to the total fluorescence which falls within the respective spectral band.

IMD-W System and Microbial Response

During development and testing of the IMD-W system, many microbes have been tested, including seven compendial microbes specified in the OWBA Testing Protocol.[D] To interpret the relative fluorescence intensity from these microbes and the STY bead, **Figure 3** shows overlaid PMT-2 signal histograms empirically measured on the IMD-W system.

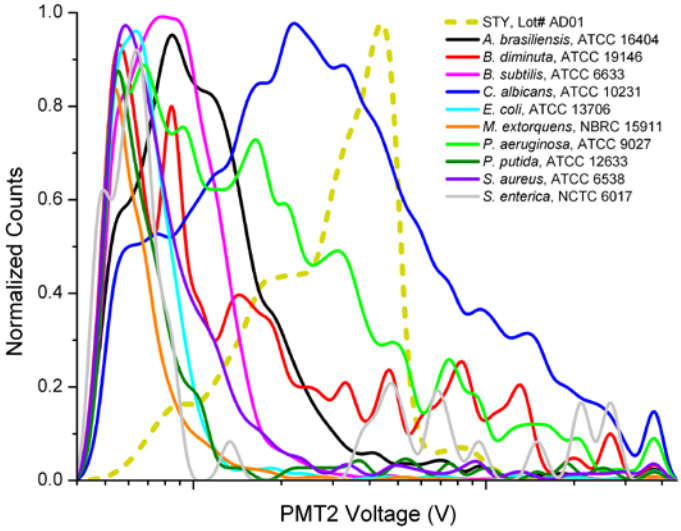

Figure 3: IMD-W fluorescence histograms for OWBA microbes and STY bead.

The vertical axis of the graph is linear and represents the normalized counts for each sample, while the horizontal axis shows the signal intensity on a logarithmic scale. The logarithmic scaling enhances visual distinction of each curve, and also aids comparison to Figure 1 which gave a pictorial context for relative fluorescence. The graph clearly shows that the ten microbes have measured fluorescence on the same order as the STY bead.

An important point of the graph is that the STY histogram peak is slightly higher than the microbes' peak locations in the experimental setup.

These microbes were evaluated as homogeneously dispersed, planktonic suspensions; this is the most challenging scenario from a detection standpoint. In a real-world environment, microbes may also be found in clumps and/or with an extracellular matrix (i.e. from

[C] Part Number UVCPAK-5042-14k from Spherotech, Inc. (Lake Forest, IL)

[D] OWBA Testing Protocol Document v1.5

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a biofilm) which would yield more fluorescence and thus be easier to detect.

Also, the STY bead is only one of several beads which are employed to fully calibrate the IMD-W instrument. Among these, a different, proprietary bead has been developed by BioVigilant to also closely resemble microbial size and fluorescence. This special bead is used during both IMD-W calibration as well as the automated suitability check routine which can be performed on-demand by the end user to ensure that the instrument is performing nominally.

Conclusion

- The IMD-W system is the result of years of research and development to produce an instrument capable of detecting the ultra-low levels of autofluorescence produced by microbes in an aqueous environment.
- While many types of microparticles are commercially available for diagnostic applications, few are appropriate for a system like the IMD-W.
- The Spherotech Yellow Low Intensity bead is one exception, whose characteristics make it appropriate as a *minimum* benchmark surrogate for microbial auto-fluorescence.
- This bead, among others, helps confirm that the IMD-W system is fit for its intended purpose of providing the user instantaneous data about the quality of their ultrapure water streams, and is available to the end user during evaluation and testing of the IMD-W system.

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