

Scintillation Proximity Assay: Instrumentation Requirements and Performance

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ABSTRACT

Recently, a new immunoassay and receptor binding analysis technology has been introduced. This methodology, scintillation proximity assay (SPA), belongs to a family of ligand binding techniques known as "sandwich assays." SPA technology employs a scintillation microsphere as the solid support, but requires no separation step. In addition, no conventional liquid cocktail is needed, minimizing safety hazards and disposal costs.

The unusual demands this technology places on currently available liquid scintillation instrumentation will be discussed. We will concentrate on the ability to accurately quantitate low levels of a low energy isotope, such as tritium, with adequate sensitivity. Since the recommended total sample volume is no more than 0.4 mL, acceptable sensitivity requires excellent light collection efficiency. Additionally, this technology is intended for high volume applications. Throughput, to a significant degree, is influenced by counting efficiency, and hence, instrument performance. Finally, the unique scintillation properties of the fluor material may affect instrument counting efficiencies, leading to poor quality data. Because assay sensitivity may be affected by the instrument employed, it is important that the technology is evaluated on several types of liquid scintillation counters. The results of such a study are presented. Alternative instrumental methods for counting SPA samples, providing increased sample throughput, are discussed.

INTRODUCTION

Classical immunodiagnostic and ligand-binding methodology using beta- and/or gamma-emitting radionuclides, is widely recognized in the industry for its sensitivity and specificity for the analyte of interest.^{1,2} Several general techniques, such as direct and competitive assays, exist for a variety of applications. A third category is typically referred to as a sandwich assay.³ Here, the analyte (usually an antigen) is bound to two different antibodies. One antibody is labeled with a radioactive tracer (typically ^3H or ^{125}I), while the other is permanently bound to a solid support structure such as a polymeric microsphere. When incubated together, these three elements form an Ab-Ag-Ab complex bound to the microsphere. The fraction of radiotracer bound to the

ligand can be separated from the tracer in free solution via a number of techniques including centrifugation, filtration, charcoal adsorption, or magnetic separation. One significant disadvantage in these RIA methods is the time and effort required for this separation step.⁴ A key challenge over the past 20 years has been to simplify the sample preparation steps, especially separation, required prior to assaying the tubes or vials.

A new technique, scintillation proximity assay (SPA), has recently been introduced for immunodiagnostic and ligand-binding assays. This method exploits the very low radioactive energy of the common beta emitting radionuclide tritium (³H) or the Auger electrons emitted by ¹²⁵I. Because tritium decays energy on the average of 6 keV, beta electrons formed during the decay process travel only a short distance (4 μm in water). Therefore, in order to create a scintillation event, a tritium label must be in intimate contact with the scintillating medium.⁵

SPA technology employs a scintillating microsphere as the solid support structure. Formation of the Ab-Ag-Ab complex, which requires the presence of Ag in free solution, brings the radiolabeled antibody close to the scintillating particle, excites it, and causes photon emission. If there is little or no Ag in free solution, the complex will not be formed to any large degree. In this situation, radiolabeled Ab will not be bound close to the scintillating microsphere, and therefore, will not cause appreciable excitation of the fluor,^{6,7} thus, the number of detected scintillation events is directly related to the amount of Ag in the sample. This effectively separates the bound fraction from the free fraction, without any need for manual separation steps.⁸ Samples can be assayed directly in a conventional liquid scintillation counting system, following an appropriate incubation period. As a result, assay precision is ultimately improved due to fewer sample processing steps.

While SPA technology has obvious advantages in the areas of sample preparation, incubation, and separation, it also places unusual demands on commercially available liquid scintillation counting instrumentation. Most important is accurate quantitation of low levels of tritium with adequate sensitivity for routine immunoassay and receptor binding applications. Acceptable sensitivity requires both excellent tritium counting efficiency and low background count rate.

A primary factor influencing these requirements is the fact that typical SPA samples have a total volume of 0.4 mL and are prepared in 7 mL LS vials. A compounding factor is that the bound (scintillating) fraction consists of only a few milligrams of material which rapidly fall to the bottom of the vial. Sample geometry, as presented to the liquid scintillation counter, is therefore quite poor. This will tend to limit the photon collection efficiency, and thus the effective radionuclide counting efficiency. A low overall counting efficiency reduces the statistical accuracy with which one can measure a sample at a given count time. In order to compensate, the investigator or clinician must increase the sample count time or the sample volume to achieve better statistics. This either decreases sample throughput or increases assay cost. Furthermore, over-

all counting efficiency is critical in the determination of the signal to noise (S/N) ratio for the instrument employed in the assay. Poor efficiencies will limit the S/N ratio. As a result, assay sensitivity might be adversely affected.

A third factor influencing the net performance of this technology is the ability of a liquid scintillation counter to directly assay samples contained in 7 mL vials. Most currently available instrumentation is capable of loading and counting these vials directly. However, older counters may only be capable of counting samples in 20 mL vials. This necessitates placing the small SPA vial within an adaptor and assaying it as a large vial. This significantly reduces sample throughput.

Another factor influencing SPA performance is the fluor employed as the solid support medium. Experimental evidence indicates that the fluorescent emission of the scintillating microsphere occurs over a relatively long period of time compared to the emission from conventional LS samples. In addition, pulse height distributions obtained from typical SPA samples show that the fluorescent emissions average a slightly higher energy level than those usually encountered from conventional scintillation fluors. Currently available liquid scintillation systems incorporate various pulse discrimination schemes, based on pulse height and width, in order to optimize instrument performance. These systems have been optimized for use with existing scintillation chemicals.

The novel pulse decay characteristics of the scintillator used in SPA technology has broad implications for the signal processing techniques that discriminate true beta decay events from PMT thermal noise. Conventional liquid scintillation counters accomplish this via coincidence circuitry in conjunction with narrow pulse width scintillators. The design of conventional LSCs has been based on, and limited by, these requirements. By using a scintillator with a wider decay pulse, as shown below, patented time-resolved pulse discrimination techniques, using only a single PMT per detector, can discriminate against noise events. Radiochromatography counting systems based on this technology are well known.

The experiments detailed in the following section describe recent work done to better characterize SPA technology performance on existing liquid scintillation counting equipment. In addition, the evaluation has been carried out on experimental time-resolved counting equipment, using single PMT detectors. The results of these experiments are also presented.

EXPERIMENTAL

All experiments described herein were performed using either the Thromboxane B2 (code # TRK.951) or 6-Keto-prostaglandin F1a (Code # TRK.952) SPA kits available from Amersham Corporation. These kits contain all of the reagents necessary to prepare SPA samples. The components are listed in Table 1.

Table 1. Contents of SPA Reagent Kit

Item	Component	Volume (μL)
1	Assay buffer (PBS + gelatin + thimerosal)	100
2	Tracer (H-3 labeled antibody)	100
3	Antiserum	100
4	SPA protein a reagent (coupled to scintillating microspheres)	100
5	Standards (a through E, with varying amounts of antibody)	100 each

The instructions included with the reagents describe two sample preparation protocols—one day and one overnight. A preliminary study was done in which these two protocols were directly compared, to find the optimum preparation protocol. Triplicate sets of standard, NSB, and Bo tubes were prepared per the instructions provided for each protocol. All samples were prepared in 7 mL polyethylene LS vials (Packard, #6000192), and incubated with mixing for the appropriate period using a commercially available multi tube vortexer. All tubes were then assayed in a Packard Tri-Carb 2500TR liquid scintillation analyzer using a region setting of 0 to 200 keV. The results of this experiment, illustrated in Figure 1, indicated that few significant differences in sample count rate exist between the two protocols. For this reason, and for reasons of convenience, the overnight procedure was chosen as the preparation protocol to be used in all further experiments.

Because most current-generation liquid scintillation counting systems are

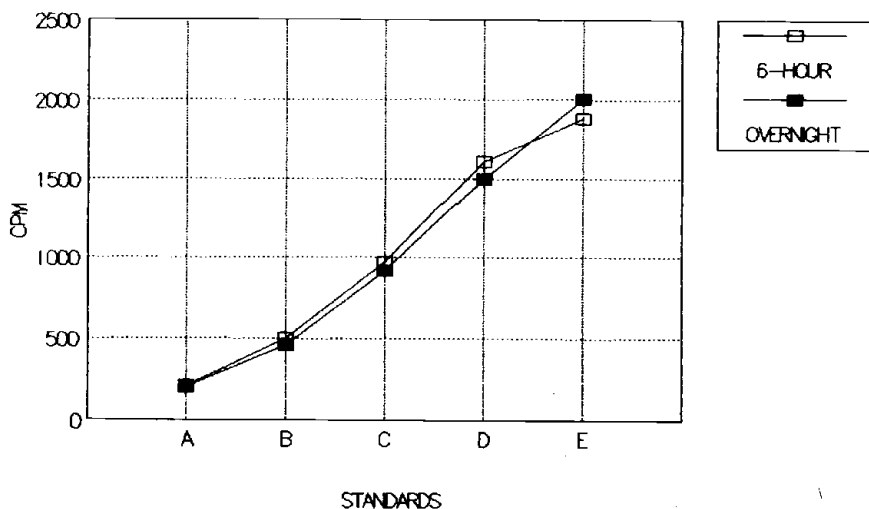


Figure 1. Comparison of 6 hr and overnight SPA incubation protocols.

equipped with MCA technology, counting regions can be optimized for the particular radionuclide/fluor combination being assayed. SPA technology employs a unique combination of radiolabeled tracer and solid scintillator. This can be illustrated by comparing an oscilloscope trace for a typical SPA sample to a conventional LS sample, as illustrated in Figure 2. Therefore, an initial experiment was performed to evaluate the spectral characteristics of a typical SPA sample. A Bo tube was prepared, incubated, and assayed in the Tri-Carb 2500TR LSA. A representative sample spectrum was collected and plotted (Figure 3). This plot indicates that preset ^3H counting regions will not capture the entire SPA sample spectrum. It is necessary to manually set the counting window to a region encompassing 0 to 30 keV.

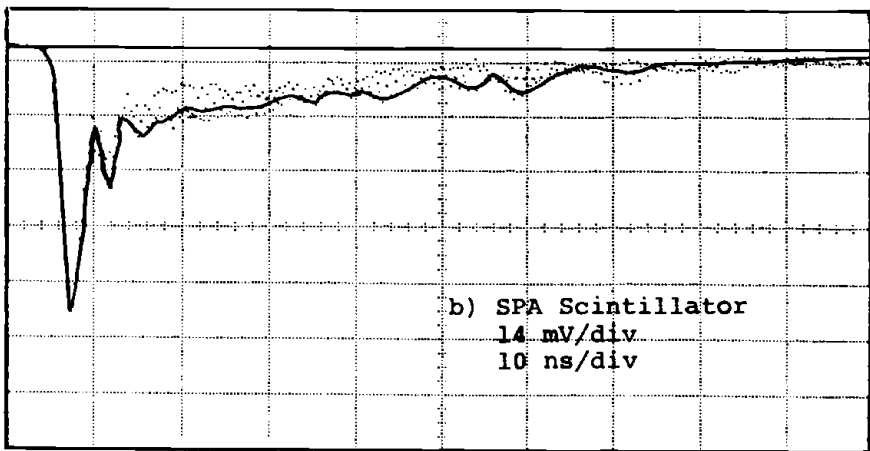
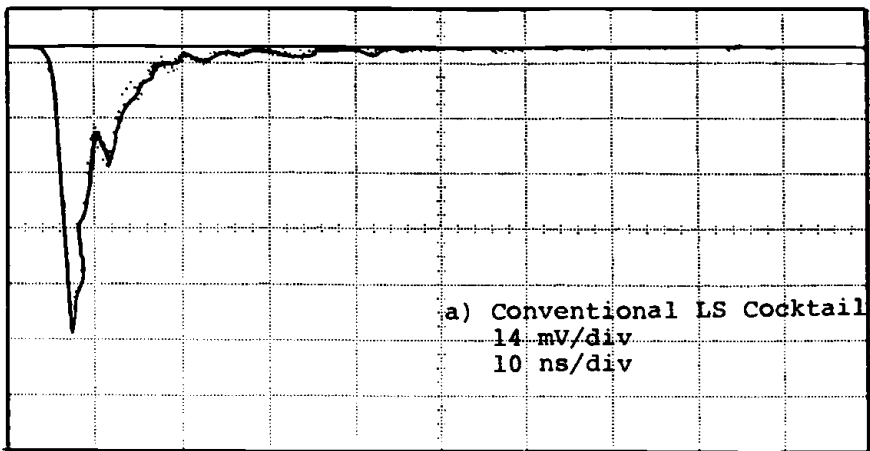


Figure 2. Pulse shape characteristics: a) conventional LS cocktail, b) typical SPA sample.

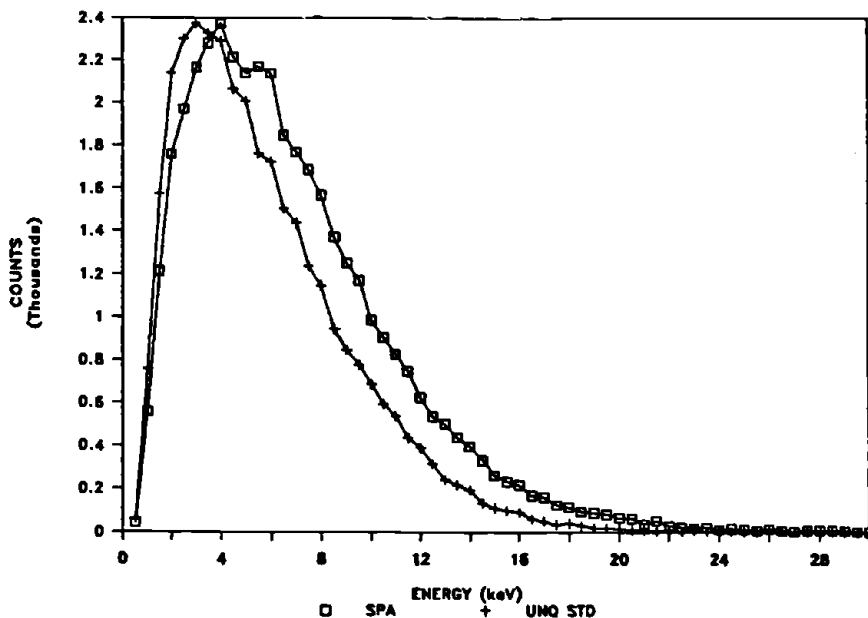


Figure 3. Normalized ^3H spectra.

The first set of experiments was designed to determine the long-term stability of typical SPA samples after preparation. Triplicate sets of standard, NSB, and blank tubes were prepared per the previously qualified overnight protocol. Following incubation, all tubes were repeatedly assayed in the aforementioned liquid scintillation analyzer for a period of 60 hr. The data obtained from these assays indicated that the count rates of the samples, as a group, decreased by a significant amount over the 60 hr period. Figure 4 illustrates this for standards A-E. Furthermore, it was noted that the rate and function of this decrease varied with the initial count rate of the sample.

Because of the time dependency of the sample count rates, it becomes necessary to normalize the results of any one assay to an appropriate point in time relative to the incubation period. Therefore, a mathematical function was developed for each sample type (Stds A-O, and NSB) that relates the sample CPM to the time after incubation, when the sample was assayed. These functions were found to be fourth-order polynomials. These functions were used to calculate normalization factors based on sample type and time of assay.

A counting system using the aforementioned single-PMT discrimination scheme has been developed and constructed.⁹ Experiments performed during development have demonstrated that efficiencies and backgrounds approaching those of conventional LSC can be achieved using a fluor with a relatively long decay constant. SPA technology employs such a scintillator. Therefore, a series of experiments was performed to evaluate SPA technology using this type of counter. Triplicate sets of Thromboxane B2 standards were prepared

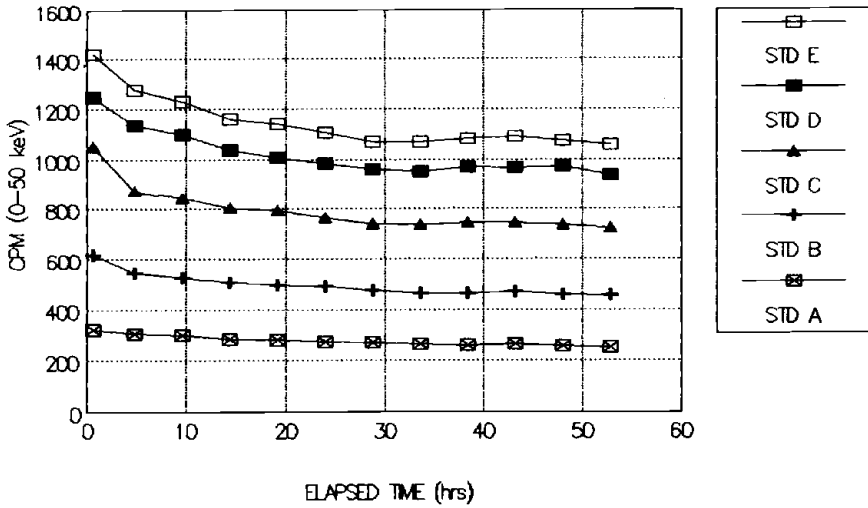


Figure 4. SPA sample stability after overnight incubation.

per the overnight protocol and assayed in the Tri-Carb 2500TR LSA. All samples were then assayed in the experimental counting system. Raw count results were normalized to time zero using the relationships described above. The normalized results for each test instrument were then compared to the reference instrument (the Tri-Carb 2500TR) by dividing each result by the count rate obtained in the reference system. The resulting CPM ratios are illustrated in Figure 5.

The data obtained from the above trials were also used to calculate figures

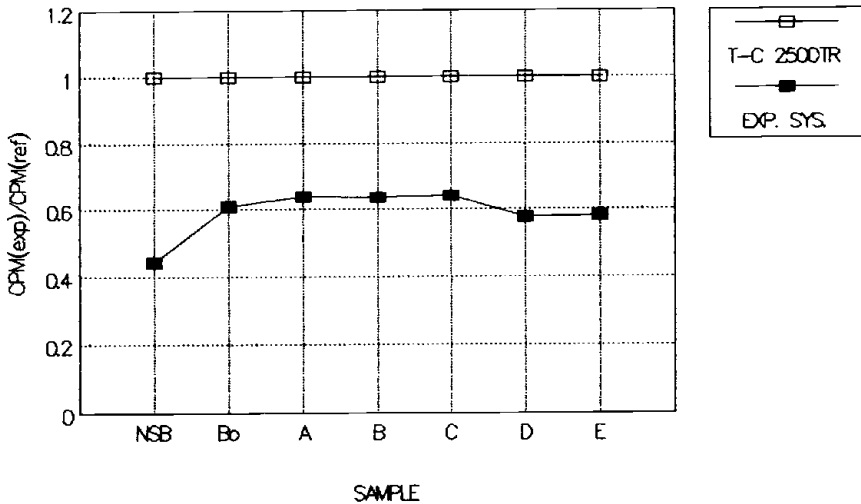


Figure 5. Normalized CPM ratios for reference and experiemetal counters.

Table 2. Figures of Merit for SPA Counting Systems

Parameter	TRI-CARB 2500TR	Experimental System
CPM, Bo tube	1911.3	1160.0
CPM, blank tube	20.1	3.1
FOM $\left(\frac{\text{Bo-BLK}}{\text{BLK}}\right)$	94.1	373.2

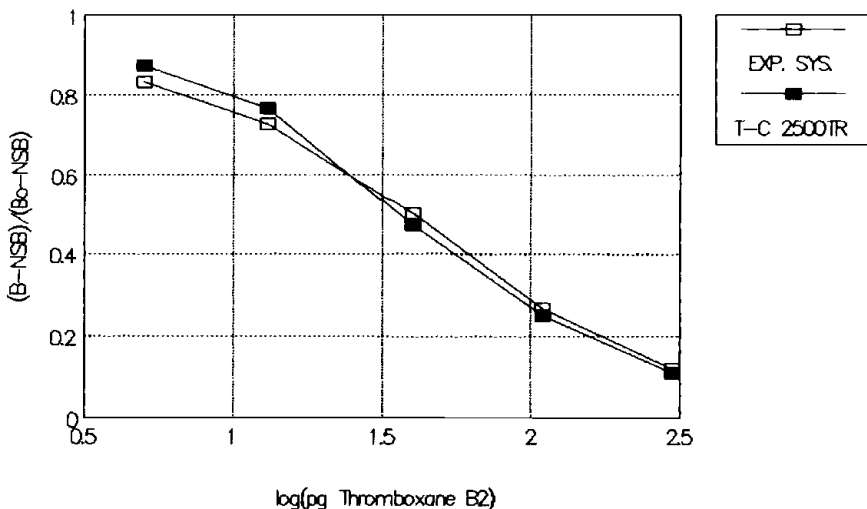
of merit for each instrument. To evaluate the sensitivity of the counter, the following equation was employed:

$$\text{FOM} = \frac{\text{CPM}(\text{Bo}) - \text{CPM}(\text{blank})}{\text{CPM}(\text{blank})}$$

The value of this parameter is directly proportional to the dynamic range of the instrument for this application; that is, a maximum value is indicative of excellent performance. The values for each of the instruments employed in this study are displayed in Table 2.

Finally, standard curves were plotted for each of the trials, according to the format recommended in the SPA instruction booklet (Figure 6). Note that while count rates for the experimental system were generally about 40% lower, the resulting standard curves are almost identical.

The count rates obtained for each of the runs were then plotted against each other in order to directly compare instrument performance. Figure 7 illustrates these results. Here, we observe a high degree of correlation between the experimental system and the liquid scintillation counter. This suggests that a commercial system of the type described above would be ideally suited for SPA applications.

**Figure 6.** Thromboxane B2 standard curves.

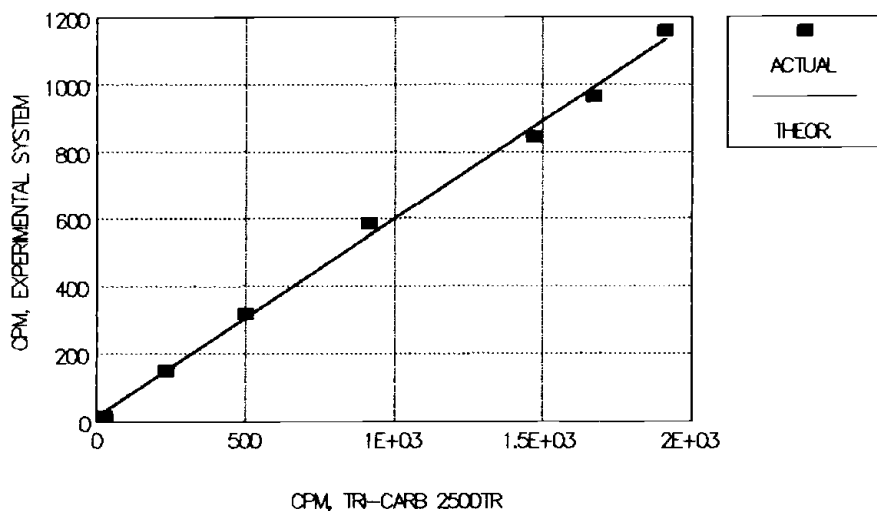


Figure 7. SPA count rate correlation.

RESULTS AND CONCLUSION

SPA technology represents a significant development in the field of radio-metric assay. Because of the lack of a separation step in the sample preparation, labor costs for laboratories running routine ligand binding assays can be markedly reduced. Although most current assay kits are based on gamma-emitting radionuclides such as ^{125}I , the use of a beta-emitter such as ^3H is attractive due to its longer shelf life and lesser radiological hazard. Previous ^3H assays required separation steps and conventional liquid scintillation cocktails. These too represent both a cost and a hazard to many laboratories.

Scintillation proximity assay addresses these issues by eliminating both of them. In doing so, however, the technology forces a critical evaluation of the instrumentation required to perform these assays. The nature of the samples, and particularly the scintillator employed in them, is unique in comparison to classical LS samples and cocktails. While most commercially available liquid scintillation counting systems are capable of counting samples of this type, it is important to realize that SPA technology creates somewhat unusual counting requirements.

The data presented above clearly indicate that a typical current-generation liquid scintillation counter can effectively analyze SPA samples with adequate efficiency and sensitivity. Commercially available immunoassay data reduction packages such as RiaSmart (Packard Instrument Company) can readily be joined to the LSC to provide an integrated immunoassay environment. One critical drawback, however, is the net sample throughput. Scintillation proximity assay is intended for high volume applications such as receptor binding and drug screening. In addition, the homogeneous nature of SPA technology theo-

retically allows the user to perform kinetic measurements rather than endpoint determinations, effectively reducing incubation times. Conventional LSCs, possessing only a single detector, limit the number of samples that can be analyzed in a given period and make it impossible to perform kinetic measurements.

It has also been demonstrated that unique instrument technologies can count SPA samples. While classical coincidence counting, using two PMTs, is quite effective, new techniques, based on the unique scintillation properties of the fluor, are equally if not more effective for this type of sample. Single-PMT noise discrimination schemes can offer comparable counting efficiencies with reasonable background levels. One such scheme, used in the above experiments, resulted in an approximate quadrupling of the figure of merit. This ultimately results in greater assay sensitivity.

The effectiveness of time-resolved single PMT designs has major implications for the instrument technologies suited for scintillation proximity assay. Because the samples are of limited volume, large detectors are not required. In addition, the noise discrimination circuitry employed in the above experiments does not require massive lead shielding. These factors can lead to the development of extremely compact dedicated instrumentation. In addition, multiple detector instrumentation, which uses this technology, can address the problem of sample throughput and enhance assay performance through kinetic measurements.

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