Development of a Novel Rapid Immunoassay for *Chlamydia Trachomatis*

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Background

There are ~3 million cases of *Chlamydia trachomatis* (CT) in the US each year, costing in excess of $2.8 billion for diagnosis and treatment. Current, FDA-approved rapid immunoassays for CT utilize a colorimetric sandwich capture immunoassay on a nitrocellulose (NC) substrate, but such mechanisms are insufficiently sensitive for female urine samples, thus limiting their use for point-of-care testing (POCT). The requirement of cervical swab samples for FDA-approved rapid assays (RA), therefore necessitating a pelvic exam, is a barrier to routine rapid screening of females in the ED and non-clinical venues. A POC-RA utilizing urine as a specimen would make screening much more feasible in areas outside primary care, and such samples are currently used for nucleic acid amplification assays (NAAT). However, results are not available at the time of clinic or emergency department visit and NAAT assays require expensive equipment and trained personnel. A rapid assay utilizing a fluorescent compound and fluorometer for detection should offer much greater sensitivity.

Objective

To develop a POCT substrate-based rapid immunoassay sensitive enough for urine analysis by utilizing an antibody to CT lipopolysaccharide (LPS), labeled with fluorescent compounds and a small, portable fluorometer.

Methods

We chose NC, polyvinylidene fluoride (PVDF) and FL-PVDF (PVDF modified for fluorescent probing) as the substrates to test due to cost effectiveness and availability. The fluorometer, GloMax®-Multi Jr. (Promega) was chosen due to its relative inexpensiveness, portability and ease of use.

. Step 1: Each substrate was illuminated by the four fluorescent optical filter modules: UV (Ex:365, Em: 410-450), Blue (Ex:460, Em: 515-570), Green:(Ex: 525, Em: 580-640), Red (Ex:625, Em: 660-725), to determine the substrate-wavelength combination with the lowest autofluorescence.

. Step 2: A commercially obtained fluorescent dye (DyLight-fluorochrome (fc)-488) corresponding to the lowest substrate emittance was then covalently bound to a mouse monoclonal LPS antibody, which recognizes 15 serovars of *C. trachomatis* using a micro-scale labeling kit (Pierce).

. Step 3: Anti-LPS-fc conjugates were then incubated with chlamydial elementary bodies (EBs), and binding efficiency determined by confocal microscopy and flow cytometry. The anti-LPS-fc-EB complexes were serially diluted onto the substrate to determine detection limits of the GloMax® instrument.
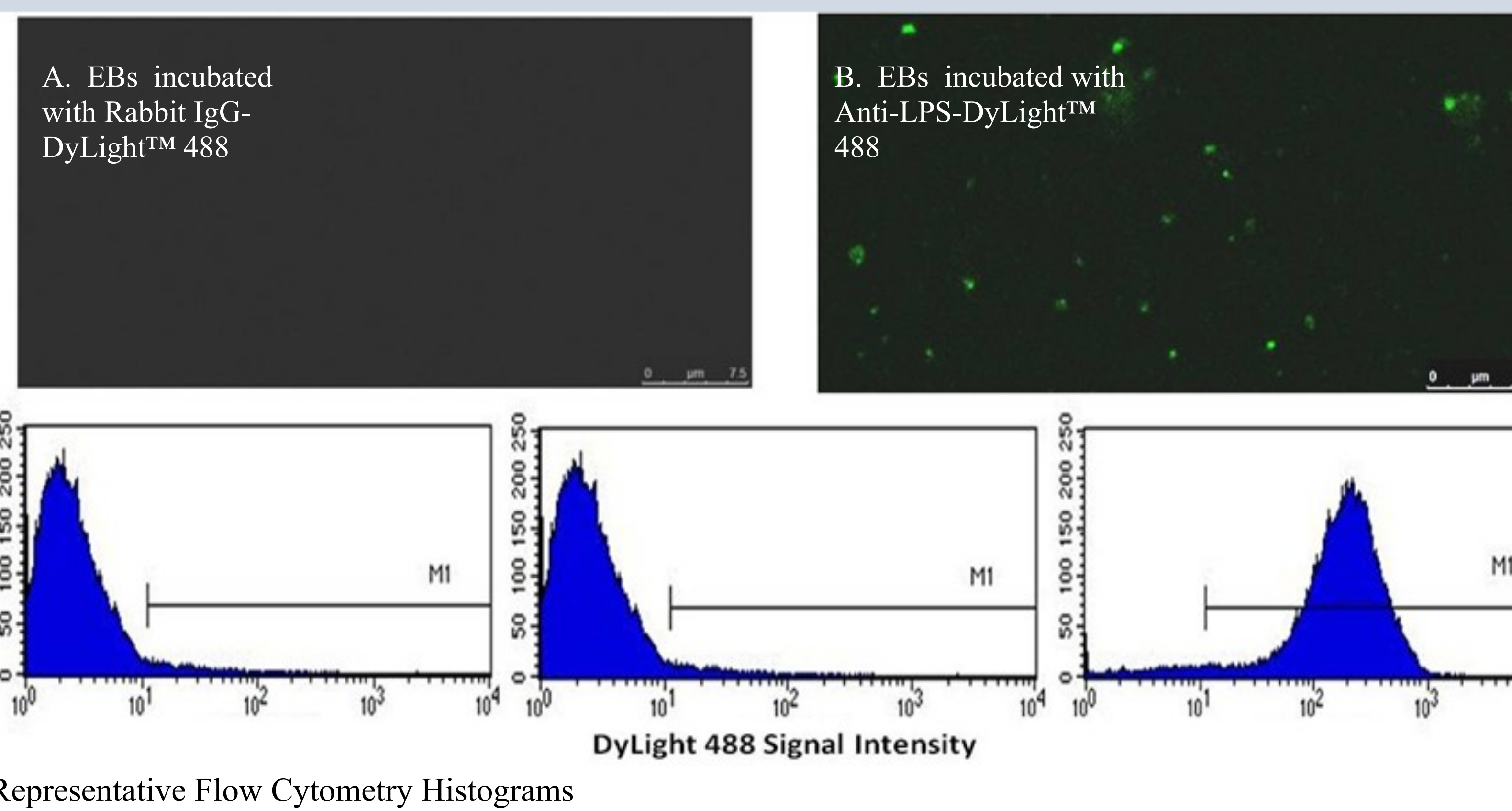
Results– Step 1

Emission capture within the blue spectrum displayed the lowest background fluorescence (measured by fluorescent standard units, FSUs; Figure 1). Though FL-PVDF had the lowest autofluorescence, the need for methanol pre-wetting precluded its utility and NC was chosen.

Figure 1. Background Fluorescence of Membrane Substrates. FSUs of nitrocellulose, low-fluorescence PVDF (FL-PVDF) and PVDF membranes were determined at 4 different excitation/emission wavelengths using the GloMax® Jr. instrument. A minimum of 20 serial measurements were taken for each membrane type using each filter module. Results are plotted as Mean ± STDEV.
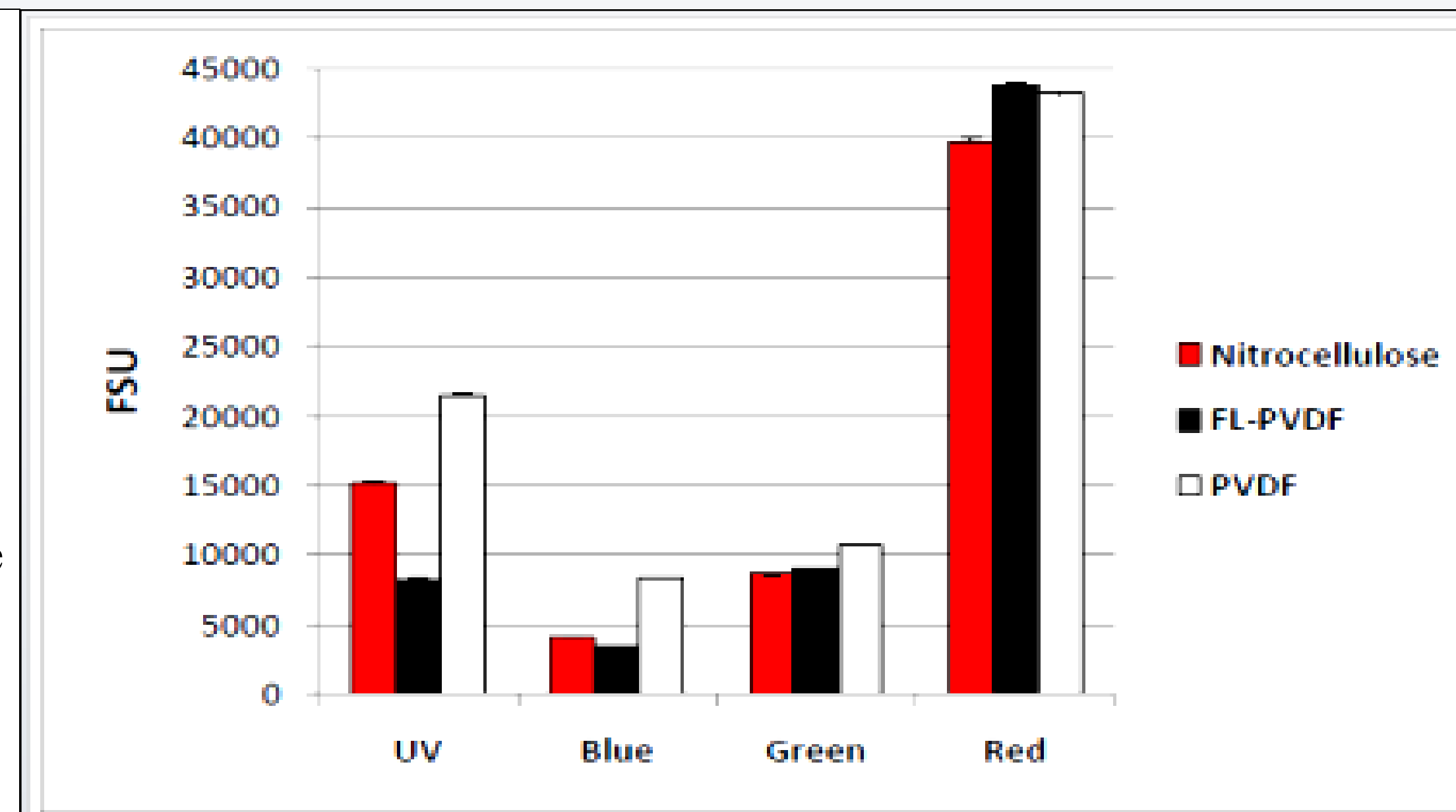
Results—Steps 2-3

The fluorescent dye, DyLight™-488, most closely matched the minimal absorbance/emission characteristics of the blue filter module with NC. This fc was conjugated to the LPS antibody with a binding efficiency of ~1 fluor per molecule. Similar binding efficiency was evident for the isotype control antibody, Rabbit IgG. C. trachomatis elementary bodies (EBs) were incubated with either the fc-labeled control antibody or fc-labeled anti-LPS. Confocal microscopy confirmed positive reactivity,


A. EBs incubated with Rabbit IgG-DyLight™ 488
B. EBs incubated with Anti-LPS-DyLight™ 488
C. Representative Flow Cytometry Histograms

Figure 2. Binding efficiency of anti-LPS-DyLight™ 488 to EBs. Equal volumes (1 X10^{7}) of EBs were incubated with anti-LPS-DyLight™ 488 (Panel A) or Rabbit IgG (Panel B). Images were captured with Leica TCS SP5 spectral laser scanning confocal microscope using a 100X oil immersion objective. (C) Representative histograms from flow cytometry analysis of EBs either un-reacted, (left panel), reacted with isotype control Rabbit IgG-DyLight™ 488 (middle) or EBs + LPS-DyLight™ 488 (right).

Results-Steps 2-3 (cont.)

specifically to EBs and substantially above background fluorescence (Figure 2A & B). Figure 2C illustrates the difference of fluorescent intensity between un-reacted EBs (left panel) and EBs reacted with Rabbit IgG DyLight™ 488. Compared to the control antibody, 97% of the EBs were positively reactive for LPS-DyLight™ 488. This represented ~90% of the population analyzed. To determine if the presence of a carrier protein was necessary or inhibitory for antibody detection procedures, EBs were incubated with fc-labeled anti-LPS with and without 0.1% bovine serum albumin (BSA). BSA was found to have negligible effects on binding efficiency; representative mean fluorescent intensities (MFI) are shown below (Figure 3). Overall, MFI increased by ~5.7 fold above background (Figure 3). Anti-LPS-488 labeling of EBs was >97% efficient as measured by flow cytometry. LPS-labeled-EBs were consistently detectable with the fluorometer of choice; however, serial dilutions produced inconsistent readings.

Figure 3. Comparisons of Mean Fluorescent Intensities (MFI) of EBs labeled with Rabbit IgG-DyLight 488™ vs. anti-LPS-DyLight 488™. The black histogram indicate EBs incubated with non-immune Rabbit IgG-DyLight™488 (MFI = 40.1) which served as an isotype control for antibody staining. The pink histogram designates EBs bound to anti-LPS-DyLight 488™reacted in the presence of 0.1% BSA (MFI = 222.4); blue histogram indicate EBs bound to anti-LPS-DyLight 488™ with no carrier protein (MFI = 236.4).
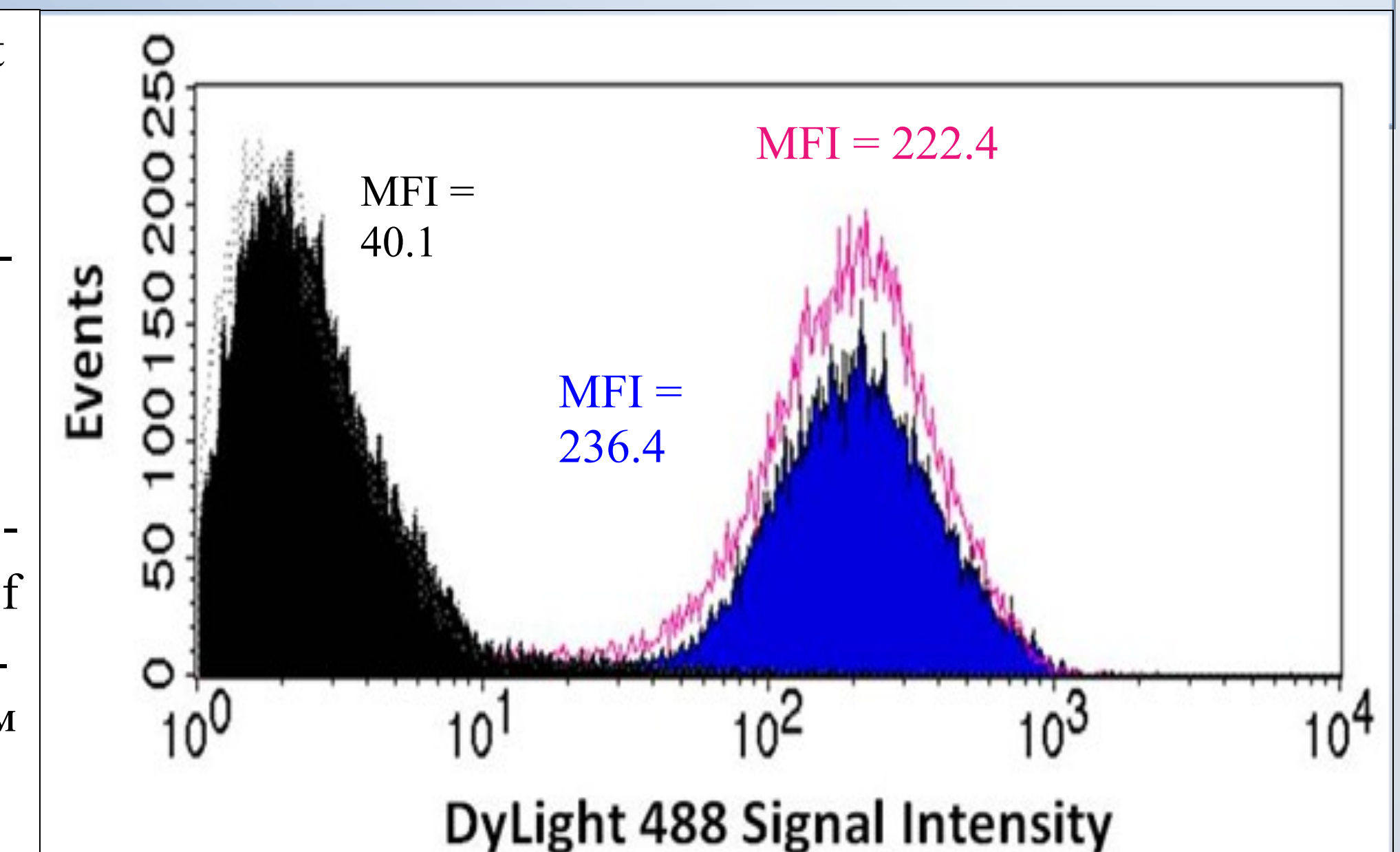
Conclusions

We identified two substrates with low background autofluorescence (FL-PVDF, NC). FL-PVDF was precluded due to its requirement for methanol pre-wetting—a likely barrier to moderate complexity assay requirements. We were also able to successfully label anti-LPS monoclonal antibodies for use as the detection agent, and bind them to previously harvested CT elementary bodies. However, the attempt to determine a detection limit (number of labeled EBs required for 2 ± STD DEV above baseline fluorescence) was stymied by inconsistent results. These were not resolved before project funding was expended. Further research needs to be performed in the successful binding of fc-labeled antibodies to EBs and their subsequent handling and dilution. Furthermore, the development of a test assay mechanism, and refinement of the detection method (fluorometer type and specification) need to be addressed.