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Capture antibody targeted fluorescence in situ hybridization (CAT-FISH): Dual labeling allows for increased specificity in complex samples

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ABSTRACT

Pathogen detection using biosensors is commonly limited due to the need for sensitivity and specificity in detecting targets within mixed populations. These issues were addressed through development of a dual labeling method that allows for both liquid-phase fluorescence in situ hybridization (FISH) and capture antibody targeted detection (CAT-FISH). CAT-FISH was developed using *Escherichia coli* O157:H7 and *Staphylococcus aureus* as representative bacteria, and processing techniques were evaluated with regard to FISH intensities and antibody recognition. The alternative fixative solution, methacarn, proved to be superior to standard solid-phase paraformaldehyde fixation procedures, allowing both FISH labeling and antibody recognition. CAT-FISH treated cells were successfully labeled with FISH probes, captured by immunomagnetic separation using fluorescent cytometric array beads, and detected using a cytometric array biosensor. CAT-FISH treated cells were detectable with LODs comparable to the standard antibody-based technique, (~10^3 cells/ml in PBS), and the technique was also successfully applied to two complex matrices. Although immunomagnetic capture and detection using cytometric arrays were demonstrated, CAT-FISH is readily applicable to any antibody-based fluorescence detection platform, and further optimization for sensitivity is possible via inclusion of fluorescently tagged antibodies. Since the confidence level needed for positive identification of a detected target is often paramount, CAT-FISH was developed to allow two separate levels of specificity, namely nucleic acid and protein signatures. With proper selection of FISH probes and capture antibodies, CAT-FISH may be used to provide rapid detection of target pathogens from within complex matrices with high levels of confidence.

1. Introduction

Pathogen detection using biosensors is commonly limited due to the need for both sensitivity and specificity in detecting targets within the mixed populations present in complex samples (Lim et al., 2005). The relatively high detection limits inherent in most systems are influenced by many factors, including low target concentrations, poor capture efficiencies, non-target detection (false positives/negatives) and interference by organic/inorganic constituents, all of which precludes adequate detection (Simpson-Stroot et al., 2008). Additionally, for those systems using immunochemistry for target capture or reporting (e.g. antibody sandwich assays), non-target cross-reactivity issues can be problematic, and adequately specific antibodies are often not available.

Some of these limitations may be overcome by combining two different molecular signature techniques, which would bestow added confidence for identifying the presence of targeted pathogens — in particular, the use of specifically-targeted fluorescently-labeled 16S rRNA gene oligonucleotide probes in conjunction with specifically-targeted antibodies. The dual level specificity (nucleic acid and protein) allows two levels of accuracy for detection and/or confirmation, as well as addressing cross-reactivity. For example, if the antibody available for a given target cross-reacts with other bacteria (related or otherwise), it could still be used for antibody capture-dependent biosensors, as long as a labeled nucleic acid specific probe is used to generate the signal (as opposed to a labeled detector antibody). This probe would only provide fluorescent signal to the appropriate target. Thus, the binding of unlabeled non-target cells to the antibodies becomes a null issue as no signal is generated.

The use of fluorescence in situ hybridization (FISH) to phylogenetically identify microorganisms without cultivation based on either 16S or 23S rRNA has become a mainstay of microbial ecology since its introduction (DeLong et al., 1989; Amann et al., 1990a,b; Amann,

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1995; Amann et al., 2001; Wagner et al., 2003; Daims et al., 2005). FISH has also been reported as a rapid method for pathogen identification in clinical and food settings (Kempf et al., 2000; Hartmann et al., 2005; Kempf et al., 2005; Peters et al., 2006; Wellinghausen et al., 2006; Bisha and Brehm-Stecher, 2009a,b). Although the predominant FISH application has been to study microbial community structure and spatial arrangements on solid supports, some applications have explored its usefulness for flow cytometric analyses (Amann et al., 1990a,b; Wallner et al., 1993, 1997; Fuchs et al., 1998; Hartmann et al., 2005; Kempf et al., 2005). This adaptation to a liquid phase processing for flow cytometry lends itself to facilitating biosensor applications, provided that conditions allowing for both probe and antibody recognition are met.

Traditionally, samples to be processed by FISH are fixed with paraformaldehyde (PFA) to stabilize and preserve them (Daims et al., 2005). PFA acts as a strengthening agent on the membranes of Gram-negative bacteria by cross-linking proteins to prevent lysis during hybridization, but can make Gram-positive bacteria highly resistant to probe uptake (Leong, 1994; Daims et al., 2005). Additionally, this cross-linking activity, while giving stability and excellent conditions for FISH, can severely inhibit any subsequent immunochemistry. To circumvent these problems, combined bacterial applications of FISH and immunostaining have typically involved extensive antibody incubation times or involved processing steps to overcome the fixative effects (Aβmus et al., 1997; Li et al., 1997; Ramage et al., 1998; Oerther et al., 1999), limiting their utility for rapid and high-throughput testing situations.

As formaldehyde and its derivatives are well known in the histopathology community to inhibit molecular analyses (e.g. immunostaining, immunohistochemistry or nucleic acid analysis), alternative tissue fixatives have been explored that are more conducive to downstream processing (Baumgärtner et al., 1988; Leong, 1994; Shibutani et al., 2000; Srinivasan et al., 2002; Cox et al., 2006). Methacarn solution has been found to be a non-cross-linking protein-precipitating fixative that does not appear to affect polynucleotide or protein analysis of fixed tissues and usually will give superior immunohistochemical results (Shibutani and Uneyama, 2002). This success with tissues suggests that methacarn solution may also be successful with fixation of bacterial cells and lend itself to facilitating the use of FISH in combination with immunolabeling for biosensor detection.

In the work described herein, we demonstrate a modified liquid FISH processing method used in conjunction with capture antibody targeted detection (CAT-FISH) to increase the specificity for biosensor assays. Detection of pathogens in pure cultures and seeded matrices was demonstrated on a cytometric bead array biosensor, using bead-bound capture antibodies with FISH labeled cells. Since the applications of both FISH and immunochemistry have been well established for use with complex sample matrices, this method should be easily adapted to other bacteria and biosensor platforms. The use of FISH in conjunction with antibody based biosensor assays for pathogen detection has not been previously reported.

2. Materials and methods

2.1. Microorganisms

Escherichia coli O157:H7 (ATCC 35150) and *Staphylococcus aureus* (ATCC 25923) were cultured at 37 °C with shaking in brain heart infusion broth (BHI) (Difco, Becton Dickinson, Franklin Lakes, NJ). Cells were cultured for ~18 h prior to experiments and then diluted 1:100 in fresh BHI and incubated as described. Cells were harvested at 0.8 O.D. (600 nm) and washed once with 1× phosphate buffered saline (PBS −11.9 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4 [Fisher BioReagent, Suwanee, GA]). Cell pellets were then fixed immediately (PBS samples) or diluted into complex matrix prior to fixation.

2.2. Fixation

Harvested bacteria were fixed by one of two methods: Control cells were fixed with 4% ice-cold paraformaldehyde (PFA) for 2 h at 4 °C, collected by centrifugation (9000×*g*) for 90 s, decanted and resuspended in 50% (v/v) ethanol in 1× PBS according to standard FISH protocols (Daims et al., 2005). Test cells were fixed with methacarn solution (6:3:1 parts absolute methanol, chloroform, and glacial acetic acid) made fresh daily (Leong, 1994). All manipulations involving the methacarn solution were performed in a chemical hood. Cell pellets were resuspended in methacarn, incubated at room temperature (22 °C) for 10 min, collected by centrifugation (9000×*g*) for 90 s, decanted, and washed once in 80% (v/v) ethanol and once in 100% ethanol. Cell pellets were dried in the dark via rotation for 20 min at 46 °C to drive off remaining ethanol (Enviro-Genie, Scientific Industries, Inc., Bohemia, NY). Samples containing spinach rinse (spiked with *E. coli* O157:H7 or un-amended) were processed in the same manner. For *Staphylococcus* samples, prior to alcohol dehydration steps, cells were treated with lytic agents as follows: after centrifugation, cells were washed once in 10 mM Tris HCl pH 8.0, centrifuged and resuspended in lysozyme (1 mg/ml in 10 mM Tris HCl pH. 8.0) and incubated 15 min at 30 °C; then cells were collected as described and resuspended in either lysostaphin (10 μg/ml in 10 mM Tris HCl pH 8.0) and incubated 5 min at 30 °C or nisin (0.1 g of 2.5% powder in 10 ml 0.02 N HCl pH 2.0) diluted into PBS for final concentration of 25 μg/ml).

2.3. Liquid fluorescence in situ hybridization (LQ-FISH)

Fluorescently-labeled oligonucleotide hybridization probes targeting the 16S rRNA for the domain *Eubacteria* (EUB338; 5′ gctgcctcccgtaggagt 3′) and species *S. aureus* (SAU349; 5′ gaagcaagcttctcgtccg 3′) were obtained from Molecular Probes (Invitrogen, Carlsbad, CA) and labeled with either Alexa Fluor 532 or Cy3 at the 5′ end (Loy et al., 2007). Alexa Fluor 532 labeled probe was used for cytometric bead array assays, and Cy3 labeled probe was used for optimization assays, image capture and image analysis. The probes were diluted to 50 ng/μl with dH_2O, and stored in 100-μl aliquots at −20 °C in the dark. Fixed and dried sample pellets (pure cultures or spiked matrix) were resuspended in 200 μl of hybridization buffer containing the labeled probe at a final concentration of 5 ng/μl. The hybridization buffer also contained 20% (v/v) formamide, 0.9 M NaCl, 0.1% SDS and 100 mM Tris HCl [pH 7.0] (Daims et al., 2005). Hybridization was conducted in the dark for 1 h in a 46 °C water bath. Subsequently, samples were centrifuged (9000×*g*) for 90 s, the supernatant was removed, and cells were resuspended in 500 μl of pre-warmed washing buffer followed by 10 min incubation in a 48 °C water bath in the dark. The washing buffer contained 215 mM NaCl, 20 mM Tris HCl [pH 7.0] and 5 mM EDTA (Daims et al., 2005). After washing, samples were centrifuged and the supernatant removed. Treated pellets were resuspended in PBS for downstream processing (microscopy, antibody labeling, or immunomagnetic capture and cytometric analysis).

2.4. Standard fluorescence in situ hybridization

Variations in oligonucleotide conferred fluorescence intensity based upon fixative affect were measured. Cells fixed by either PFA or methacarn were placed on 10-well heavy Teflon coated microscope slides (Cel-Line Associates, New Field, NJ) and processed by standard FISH protocols (Amann et al., 1990a,b; Oerther et al., 2000) using the Cy3-labeled EUB338 probe. Lytic agents were applied to the *Staphylococcus* samples as described in Section 2.2. The hybridization step was 1 h and the washing step was 30 min. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/ml for 1 min, rinsed with dH_2O, air dried, and

mounted with Cargille immersion oil (Type FF, Cedar Grove, NJ) and a cover slip.

2.5. Antibody binding assays

Fixed cells were used in adsorption enzyme linked immunosorbent assays (ELISA) to compare antibody recognition after the different fixation techniques. Bacteria were fixed with either PFA or methacarn as described above and split, with half used as controls and half processed alike through the entire liquid FISH procedure. Additional controls consisted of untreated cells that were harvested and stored on ice in PBS from the same initial test cultures. After treatments, cells were enumerated by direct microscopic count, normalized to 1×10^8 cells/ml and serially diluted to 10^3 cells/ml in PBS. Preliminary experiments indicated that cell concentrations below 10^2 cells/ml did not produce measurable signal and were omitted from further tests (data not shown). Cells were then applied in triplicate to an ELISA plate, incubated at 4 °C ~18 h and processed using a QuantaBlu Fluorogenic Peroxidase kit (PIERCE, Rockford, IL) according to manufacturer's instructions with the following exceptions: volumes for each step were normalized to 100 µl and incubation times were reduced to 30 min each. Reporter antibodies included affinity purified peroxidase labeled polyclonal goat anti-*E. coli* O157:H7 [1 µg/ml] (KPL, Gaithersburg, MD), purified rabbit anti-*S. aureus* [10 µg/ml] (AbD Serotec, Raleigh, NC) and affinity purified peroxidase labeled goat anti-rabbit [1 µg/ml] (KPL). ELISA plates were washed using an EL_x50 auto-strip washer (Bio-Tek Instruments Inc., Winooski, VT) and end product detection performed on a SpectraMax GeminiXS Fluorometer (Molecular Devices, Silicon Valley, CA). Results are reported as signal to noise (S/N) ratios for test samples versus PBS, where an S/N ratio above 3 indicates a positive detection result.

2.6. Simultaneous FISH and reporter antibody labeling

E. coli O157:H7 and *S. aureus* samples were individually fixed in methacarn and processed according to the liquid FISH method described in Section 2.3 either with or without Cy3-labeled EUB338 probe. Treated cells were resuspended in 500 µl PBS containing either 4 µg/ml goat anti-*E. coli* O157:H7 (KPL- for *E. coli* O157:H7 samples) or 4 µg/ml biotin conjugated rabbit anti-*S. aureus* (AbD Serotec — for *S. aureus* samples), and incubated at room temperature for 30 min in the dark with end over end rotation (24 rpm). The cells were recovered by centrifugation (9000×*g* for 90 s) and washed once in 500 µl PBS with 5 min end over end rotation. *E. coli* and *S. aureus* samples were then resuspended in 500 µl PBS containing 2 µg/ml Cy2-labeled donkey anti-goat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or 2 µg/ml streptavidin conjugated fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch), respectively. Cells were incubated at room temperature for 30 min in the dark with end over end rotation, washed, resuspended in 100 µl PBS, and visualized using an epifluorescent microscope.

2.7. Cytometric assays

A method was developed to incorporate immunomagnetic separation (IMS) with cytometric bead arrays for direct detection of FISH labeled cells (IMS-CAT-FISH) using a specialized flow cytometer. This method is a modification to standard cytometric bead array (CBA) assays, which rely on a labeled detector antibody for target detection. MagPlexC magnetic microbeads (6.5 ± 0.2 µm, fluorescence region 33, Luminex, Austin, TX) were used for all assays, and for all manipulations beads were separated from liquid solutions using the 3-in-1 Magnetic Particle Separator (PureBiotech, LLC, Middlesex, NJ). Magnetic microspheres were coupled to goat anti-*E. coli* O157:H7 antibody (KPL) or rabbit anti-*S. aureus* (AbD Serotec) using the amine coupling kit (BioRad, Hercules, CA), as per the manufacturer's instructions and stored at 4 °C until used.

Coupled magnetic microspheres were used to capture target cells from sample suspensions following the LQ-FISH procedure. Antibody-coupled microbeads (1000 per sample) were added to 1 ml of sample and briefly mixed by vortex action on low setting. Mixed samples were centrifuged (6000×*g*, 60 s) and target allowed to bind during a 5 min stationary incubation at 22 °C. Samples were briefly mixed after incubation and beads were separated from the supernatant. Recovered beads were washed twice with 500 µl PBS containing 0.05% Tween 20 (PBST) in the dark using 5 min rotations. After washing steps, beads were separated from the sample supernatant and resuspended in 100 µl PBST. Beads were placed into 96-well round-bottom plates, and analyzed using the Bio-Plex 200 reader (BioRad), as per the manufacturer's instructions using the high PMT setting. Fluorescence signals were expressed as the mean fluorescence intensity of 100 beads per well.

Positive signals were determined using signal above limit of detection (SALOD) values. SALODs were calculated by taking the average sample signal and subtracting the limit of detection (LOD) value. The LOD value was calculated by averaging sample blanks and adding three times the standard deviation of the sample blanks. All experiments were performed in duplicate or triplicate.

2.8. Simulation of complex testing situations

2.8.1. Spinach rinse matrix preparation

Spinach rinse was generated to simulate a field wash sample as *E. coli* O157:H7 contamination is a concern in commercial produce products. Fresh bundled spinach was purchased from a local wholesale market (Tampa, FL) and processed within one day. Spinach rinse was generated and stored at −20 °C until used. Rinses were tested for background *E. coli* O157:H7 contamination according to established FDA-BAM procedures (Feng et al., 1998), and determined to be devoid of endogenous *E. coli* O157:H7 prior to initiation of experiments. Serially diluted samples were plated on BHI agar using the spread plate method to determine microbial background loads.

Prior to IMS-CAT-FISH, *E. coli* O157:H7 was diluted into spinach rinse, mixed 1:1 with 2× modified buffered peptone water with pyruvate (mBPWp), (Feng et al., 1998), and enriched overnight at 42 °C, statically. After enrichment, spiked and un-spiked samples were plated onto CT-SMAC agar to verify growth in spiked samples and absence of target in controls. Before sampling, enriched samples were mixed briefly and allowed to stand for 1 min to allow for sand and large particulates to settle out. Samples for analysis were collected from the resultant supernatant, and processed for IMS-CAT-FISH, as described. Assay samples included bead only controls, un-spiked PBS blanks, spiked PBS samples, and un-spiked spinach rinse blanks to evaluate possible background interference from non-specific binding.

2.8.2. Blood cultures

Citrated sheep blood (Fisher Scientific) was used as a surrogate for human blood. Blood samples were spiked with *S. aureus* at concentrations of ~2 CFU/ml (verified by plate counts on BHI agar), and diluted 1:5 into BACTEC-derived blood culture media, which was prepared in-house and contained 3% soybean-casein digest, 0.3% yeast extract, 0.01% beef extract, 0.1% sucrose, 0.0005% hemin, 0.00005% menadione, 0.001% Pyridoxal HCL, 0.04% sodium bicarbonate, and 0.035% sodium polyanetholsulfonate (all w/v). Samples were enriched overnight at 37 °C, with aeration, and plated on BHI agar to verify bacterial growth in spiked samples, and absence of bacteria in non-spiked samples. 500 µl of the blood culture sample was used for each assay. The sample was mixed with 500 µl water and incubated for 5 min at room temperature to permit lysis of

remaining erythroid cells. Samples were then centrifuged to pellet bacteria, re-suspended in 1 ml PBS, and processed according to the IMS-CAT-FISH procedure. Prior to IMS capture, samples were diluted 1:100 in order to dilute particulates resulting from remaining blood components.

2.9. Microscopic imaging and data analysis

Samples for image analysis were placed on 10-well microscope slides (Cel-Line) and either observed under wet mount or air-dried. Air dried cells were mounted with Cargille immersion oil (Type FF) and a cover slip. Oligonucleotide and/or antibody conferred fluorescence were visualized with upright epifluorescence microscopes, either a Leitz DiaPlan (Heerbrugg, Switzerland) or an Olympus BX60F (Center Valley, PA). Digital images were captured using Spot-FLEX CCD cameras and image modifications (for publication purposes only) were performed using Spot Software 4.6 (Diagnostic Instruments, Inc., Sterling Heights, MI). Image modifications consisted of cropping, scale bar calibrations and pixel multiplicative brightness adjustments. Brightness adjustments were scaled up a factor of 2 or 3 dependent upon image series, however within each series the multiplicative factor was the same, so that relative intensities were comparable. All data analysis was performed on raw images without modifications. Fluorescent images were evaluated with the *daime* 1.3.1 software package (Daims et al., 2006). Measure objects analysis calculated the number of cells detected, as well as the mean fluorescent intensity and standard deviations for each cell. SigmaPlot® 10 (Systat Software, Inc., San Jose, CA) was used for graphic analysis. Student's *t* test was used to determine if significant differences occurred between fixative types.

3. Results

3.1. Optimization of processing conditions

Preliminary evaluations of multiple fixatives and conditions, including standard FISH fixation with PFA, did not facilitate sufficient antigen–antibody binding as required for target capture and detection on biosensors (data not shown). Experiments attempting to ascertain antigen–antibody binding capabilities for stored PFA fixed *E. coli* cells were unsuccessful in producing any antibody binding (data not shown). Thus all subsequent experiments used "fresh" fixed cells for evaluation purposes, in that cells were processed immediately after fixation steps without holding or storage. Methacarn fixation was the most promising candidate for both fluorescence and antibody binding and was subsequently optimized for use in the LQ FISH protocol.

3.2. Influence of fixative upon fluorescent signal

E. coli O157:H7 and *S. aureus* were fixed in either methacarn or PFA to determine if methacarn fixation produced cells capable of binding probe and emitting fluorescence signals comparable to standard protocols. Cells from both fixative treatments (methacarn and PFA) were mounted on slides and subjected to standard FISH. In addition, cells were processed by the LQ-FISH method using methacarn fixation, and spotted onto separate slides (Fig. 1). Relative fluorescence intensity means for *E. coli* O157:H7 cells (200 per treatment) were: 61 ± 12 for PFA, 68 ± 11 for methacarn and 58 ± 10 for LQ-FISH. Analyses indicated that there were no significant differences between the PFA and LQ-FISH treatments, but the methacarn treated cells were significantly brighter ($p \leq 0.0004$) than both PFA and LQ-FISH cells. Relative fluorescence intensity means for *S. aureus* cells (150 per treatment) were: 48 ± 14 for PFA, 56 ± 18 for methacarn and 100 ± 28 for LQ-FISH. Analyses indicated that the PFA treated cells were significantly dimmer ($p \leq 0.003$) than both methacarn and LQ-FISH cells, and LQ-FISH treated cells were significantly brighter than the slide based methacarn treated cells ($p \leq 0.003$).

3.3. Influence of fixative upon antibody binding

Adsorbent ELISAs were used to evaluate PFA and methacarn fixation effects. In addition, fixed cells were subjected to further hybridization conditions (LQ FISH) to determine the effect of hybridization treatments on antibody binding for *E. coli* O157:H7 and *S. aureus* (Fig. 2). Antibody binding was significantly reduced for PFA fixed *E. coli* as compared to control (non-fixed) and methacarn fixed cells for all concentrations below 10^8 cells/ml (Fig. 2A). Positive S/N ratios were observed for methacarn, control, and PFA at 10^4, 10^5, and 10^6 cells/ml, respectively. Methacarn treatment achieved better binding than controls at concentrations below 10^5 cells/ml and showed no difference at higher concentrations. After LQ-FISH hybridization treatments, methacarn treated cells produced higher S/N ratios than PFA treated cells; however, the minimum concentration producing a positive signal was one log higher as compared to non-hybridized cells (Fig. 2B). No change in minimum concentration was observed for the PFA treated cells after hybridization. Antibody binding for *S. aureus* produced no significant differences between controls and methacarn or PFA fixed cells, and positive S/N ratios were achieved at 10^6 cells/ml for all conditions (Fig. 2C). After hybridization, the methacarn fixed cells produced slightly higher S/N ratios, but were not significantly different from PFA samples (Fig. 2D).

3.4. Simultaneous visualization of oligonucleotide and antibody fluorescence

Both labeling methods were used in conjunction to examine labeling coverage after methacarn fixation with LQ-FISH hybridization. *E. coli* O157:H7 and *S. aureus* were simultaneously labeled with rRNA probes and antibodies and generated signals that were sufficient for microscopic visualization (Fig. 3). Appropriate un-labeled controls were included ($\pm$probe and/or antibody) and verified (data not shown). Visual examination of *E. coli* cells indicated that they were uniformly labeled with both CY3 FISH probe and CY2 antibodies, providing ample coverage for dual level specificity detection. *S. aureus* cells also exhibited dual labeling, but the signal intensities appeared to be more variable for both fluorescent markers.

3.5. Immunomagnetic cytometric bead array detection

A cytometric bead array biosensor was selected as an example platform to demonstrate the utility of the dual specificity target detection. Cells were labeled using LQ-FISH and captured by IMS using antibody-coupled magnetic cytometric array microspheres (IMS-CAT-FISH). Bead-associated fluorescence signal was then determined using the cytometric array reader. Initially, limit of detection assays for *E. coli* O157:H7 and *S. aureus* were performed in PBS, and microscopic evaluation of *E. coli* O157:H7 was used to visually verify labeling and capture. Utility of the method was subsequently verified using a more complicated testing matrix appropriate for each organism. In addition, *S. aureus* limit of detection assays were performed using either species specific lytic treatments (lysostaphin) or general gram-positive lytic treatment (nisin), incorporated into the LQ-FISH procedure.

3.5.1. E. coli O157:H7 detection assays

We previously reported that standard cytometric bead array (IMS-CBA) assays using labeled detector antibodies are useful for rapid detection of *E. coli* O157:H7 in both PBS and a more complex testing scenario (raw spinach testing via enrichment of rinse samples) (Leach et al., 2010). However, in spinach rinse enrichment samples, some non-

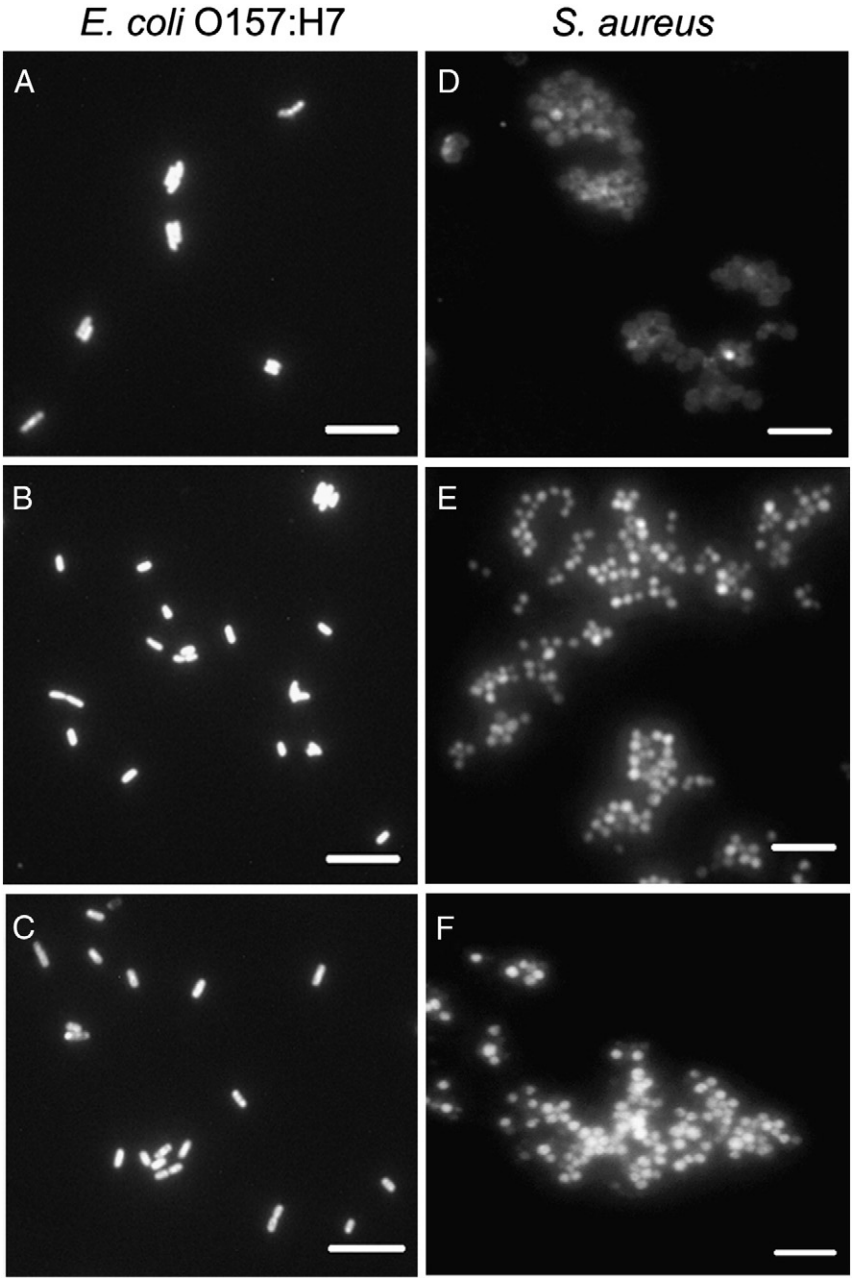

Fig. 1. Comparison of relative fluorescence intensities for *E. coli* O157 and *S. aureus* treated with different fixatives. All cells were labeled with EUB338 Cy3 rRNA FISH probe. Cells fixed with either PFA or methacarn were fixed prior to mounting on slides for FISH processing. Cells treated via LQ-FISH were mounted on slides after the process was complete. A and D) PFA treatment; B and E) methacarn treatment; C and F) LQ-FISH treatment. Scale bar is 5 μm.

specific background signals were encountered, suggesting the need for a more specific detection procedure (Leach et al., 2010). Toward this end, CAT-FISH was applied.

Microscopic evaluation was first conducted to evaluate target capture and labeling for both the standard IMS-CBA assay (antibody label) and the IMS-CAT-FISH method (FISH label). All samples (bead only controls, un-spiked PBS blanks, spiked PBS samples, and un-spiked spinach rinse blanks) were spotted onto slides and examined (Fig. 4). Beads incubated without target or reporter produced no detectable signal in either assay (not shown). Detector-associated signal for PBS blanks was absent from CAT-FISH treatments (i.e., no labeled cells present, not shown), but a dim signal was visible after processing according to standard array assay method (Fig. 4A). Beads incubated with control cells produced highly visible signal for both treatments. *E. coli* detected with the standard assay produced images with visible halos surrounding the surface of the captured cells, corresponding to the binding of labeled detector antibody to bead-captured cells. Labeled cells were easily distinguishable from the bead surfaces (Fig. 4C). *E. coli* cells detected with CAT-FISH produced images of solidly labeled cells bound to the surface of the beads (Fig. 4D). Non-spiked spinach blanks treated with CAT-FISH produced no detectable signal (not shown). Interestingly, there was observable signal on beads after the standard assay was performed on the un-spiked rinse. Fluorescence images displayed relatively large patches of labeled non-target particulate material attached to the cytometric array beads (Fig. 4B), thus demonstrating non-specific binding of spinach debris to the beads and the reporter antibody.

As microscopic evaluation indicated successful labeling and capture, a cytometric array biosensor (BioPlex 200) was used to evaluate target signals obtained using the IMS-CAT-FISH assay. Detection was sporadically observed at 10^3 cells/ml and routinely observed at

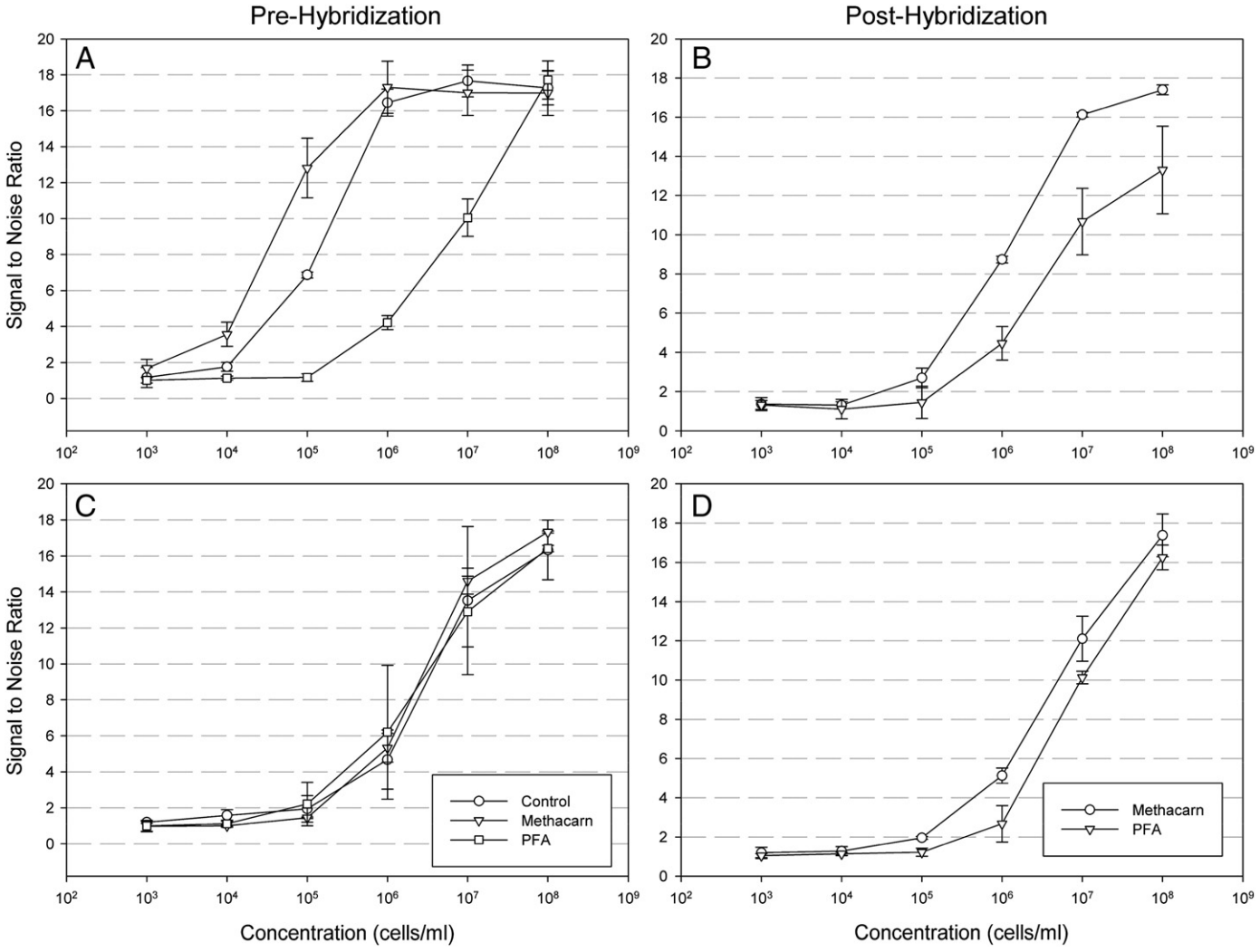

Fig. 2. ELISA comparison of fixative effect on antibody binding for liquid FISH hybridized bacteria. A) Pre-hybridized *E. coli* O157:H7 fixed with methacarn or PFA and unfixed control; B) post-hybridized fixed *E. coli* O157:H7 after liquid FISH treatment steps; C) pre-hybridized *S. aureus* fixed with methacarn or PFA and unfixed control; D) post-hybridized fixed *S. aureus* after liquid FISH treatment steps. (n = 9, error bars represent standard deviations).

10^4 cells/ml (Table 1). A one log increase in signal strength was observed for each log increase in cell concentration until saturation was reached at 10^6 cells/ml, after which no significant increases were observed. These detection limits are similar to those previously reported for the standard cytometric array assay (Kim et al., 2009, 2010; Leach et al., 2010) using a reporter antibody.

Next, the ability of the CAT-FISH dual labeling technique to mitigate matrix and antibody cross-reactivity problems was tested using enriched spinach rinse cultures. Two independent batches of spinach rinse were prepared according to the standard FDA-BAM protocol (Feng et al., 1998), and either left un-spiked or spiked with 0.9 CFU/ml *E. coli* O157:H7. After overnight enrichment, all samples were processed according to the CAT-FISH method, and FISH-labeled target cells were captured using anti-*E. coli* O157:H7 coupled magnetic cytometric array beads. Average relative fluorescent signals reported were 21.4 ± -2.3 for PBS controls (n = 3), 26.1 ± -6.2 for un-spiked samples (n = 12), and 583.1 ± -200.6 (n = 12) for spiked samples.

3.5.2. S. aureus detection assay

S. aureus samples were treated with either a species specific or generalized lytic agent to improve probe accessibility. Both were demonstrated as potential modifications of the assay, dependent upon whether the presence of *Staphylococcus* species is suspected (e.g. in blood samples gram + cocci growing in clusters are assumed to be *Staphylococcus* species). PBS was spiked with targeted concentrations of 5×10^7 cells/ml *S. aureus* (direct count) and serially diluted to 10^1 cells/ml with PBS. Final spike concentrations were confirmed by BHI plate count at $4.9 \pm 1.3 \times 10^7$ CFU/ml.

IMS-CAT-FISH assays produced consistent positive signal for all cell concentrations greater than 10^3 cells/ml for both lysostaphin and nisin (Table 1). Control cells (absent probe) did not produce any positive signals. A one log increase in signal strength was observed for each log increase in cell concentration until saturation was reached at 10^6 cells/ml, after which signal decreases were observed. A two-fold higher signal per concentration was generated using the *Staphylococcus* specific lysostaphin than was observed with the nisin.

As a further test for process utility, *S. aureus* was spiked into sheep blood at approximately 2 CFU/ml (verified by viable counts) to simulate a low-level bloodstream infection. Samples were cultured overnight according to standard blood culture practices and further processed, as described. In preliminary experiments, antibody cross-reactivity for non-*S. aureus* species was observed (not shown), so the dual-level specificity allowed by CAT-FISH was demonstrated through the use of an *S. aureus*-specific probe. Two independent blood culture samples were each processed in triplicate according to the IMS-CAT-FISH method. The average relative fluorescent signals

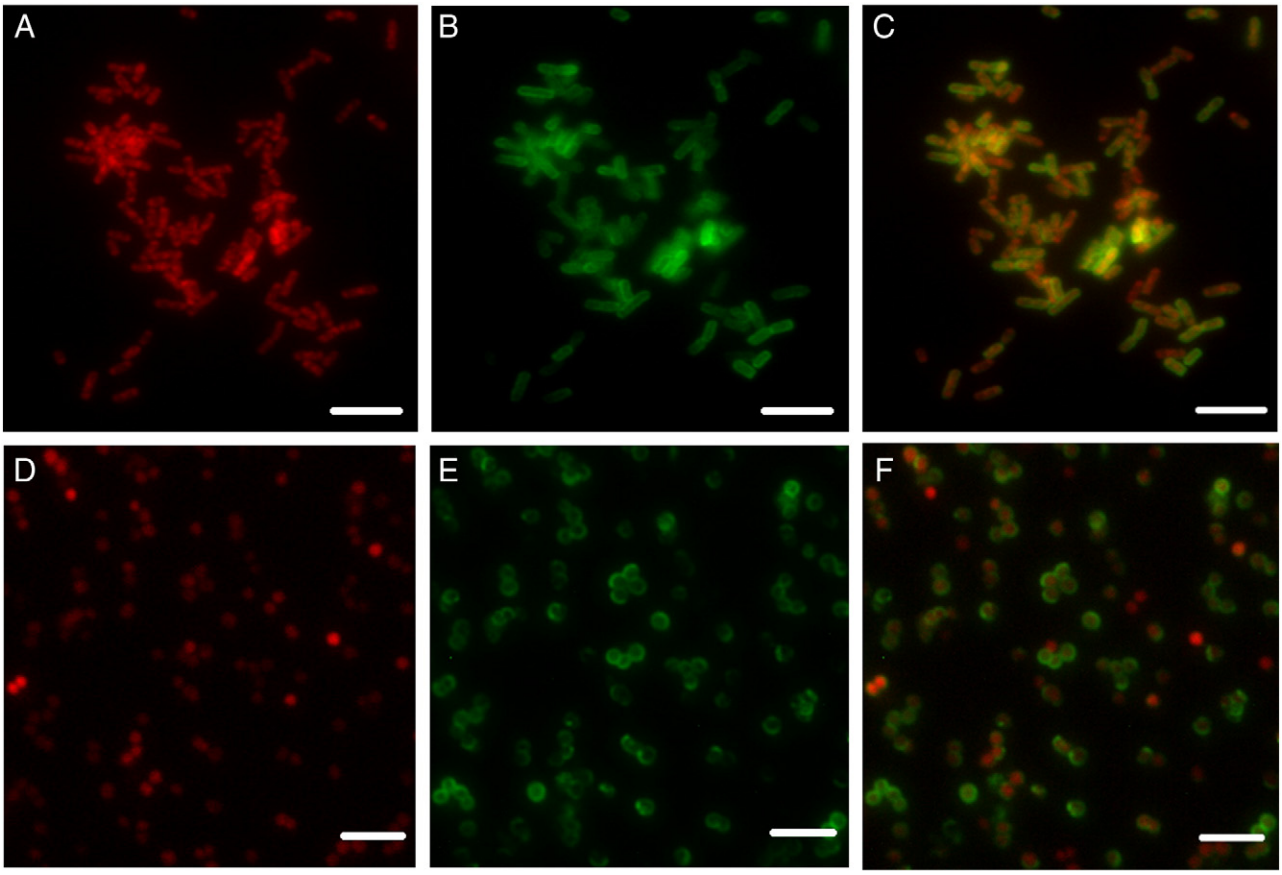

Fig. 3. CAT-FISH dual labeled *E. coli* O157:H7 and *S. aureus*. A) *E. coli* labeled internally with EUB338 CY3 rRNA FISH probe; B) *E. coli* labeled externally with goat anti-*E. coli* O157:H7 primary antibody and rabbit anti-goat CY2 labeled secondary antibody; C) merged A and B images; D) *S. aureus* labeled internally with EUB338 CY3 rRNA FISH probe; E) *S. aureus* labeled externally with biotin conjugated rabbit anti-*S. aureus* primary antibody and streptavidin conjugated fluorescein isothiocyanate (FITC) secondary antibody; F) merged D and E images; Scale bar is 5 μm.

generated after capture were 35.2 ± − 2.1 for buffer controls (n = 3), 32.4 ± − 4.3 for un-spiked blood controls (n = 6), and 562.2 ± − 106.4 for *S. aureus* samples (n = 6).

4. Discussion

A new method for simultaneous detection and identification of pathogens is presented using a modified fluorescence in situ hybridization technique, which internally labels targeted nucleic acids, while still permitting antibody binding to surface antigens. This dual level specificity permits two levels of accuracy in determining presence or absence of pathogens within a complex matrix. Comparison of LQ-FISH processing to standard PFA slide based FISH indicated that comparable fluorescent signal was obtained for both *E. coli* O157:H7 and *S. aureus* cells. This denotes that the fixation and hybridization processing steps of CAT-FISH work with both Gram positive and Gram negative bacteria. While the fluorescent intensity of the *Staphylococcus* cells tended to be more variable than those of *E. coli*, this is not unexpected as *Staphylococci* are known for hybridization difficulties due to membrane permeability issues (Amann et al., 1990a,b; Roller et al., 1994). Permeability issues are typically overcome by use of lytic enzyme treatments prior to hybridization (Roller et al., 1994; Hogardt et al., 2000; Peters et al., 2006), and the use of lytic enzymes was observed to improve signal intensity for *S. aureus* in our assays (not shown). While Kempf et al. demonstrated the utility of lytic treatments, they also indicated that permeabilization can lead to cell destruction during centrifugation (Kempf et al., 2000, 2005). However, with careful modulation, this can be overcome, as demonstrated here, and improved signal can be generated for gram-positive targets. In this study, both specific and general lytic agents were used to demonstrate improved probe accessibility options dependent upon whether or not the specific target is known to the investigator. As a further potential modification, Hartmann et al. have demonstrated the utility of peptide nucleic acid probes (PNA FISH) for overcoming staphylococcal permeability problems. Although PNA FISH probes were incompatible with our detection system, they may have utility when other biosensors are used in conjunction with the CAT-FISH method (Hartmann et al., 2005).

Methacarn treatment of *E. coli* allowed better antibody binding than that observed for untreated cells and two log better binding than that of cells treated with PFA prior to hybridization. After hybridization treatments, the methacarn treated cells still out performed PFA treated cells, but a one log reduction in sensitivity was observed (as compared to non-hybridized methacarn treated cells). This is likely due to the inclusion of formamide in the hybridization solution. The similarity in sensitivity for PFA treated cells (pre versus post hybridization) suggests that the surface antigens were already damaged sufficiently, such that no further reduction in antibody binding was observed after formamide addition. Although possible to reformulate the hybridization conditions to maintain stringency with the exclusion of formamide, this would deviate from standard practices, and was out of the scope of this study (Stahl and Amann, 1991). The lack of significant differences between treatments for the *S. aureus* cells is likely due to the cell membrane and permeability issues as discussed previously. The two log decrease in sensitivity for *S. aureus* as compared to *E. coli* is most likely due to poorer quality of the antibody, since a 10-fold more concentrated stock was necessary to achieve detection, even for the untreated control.

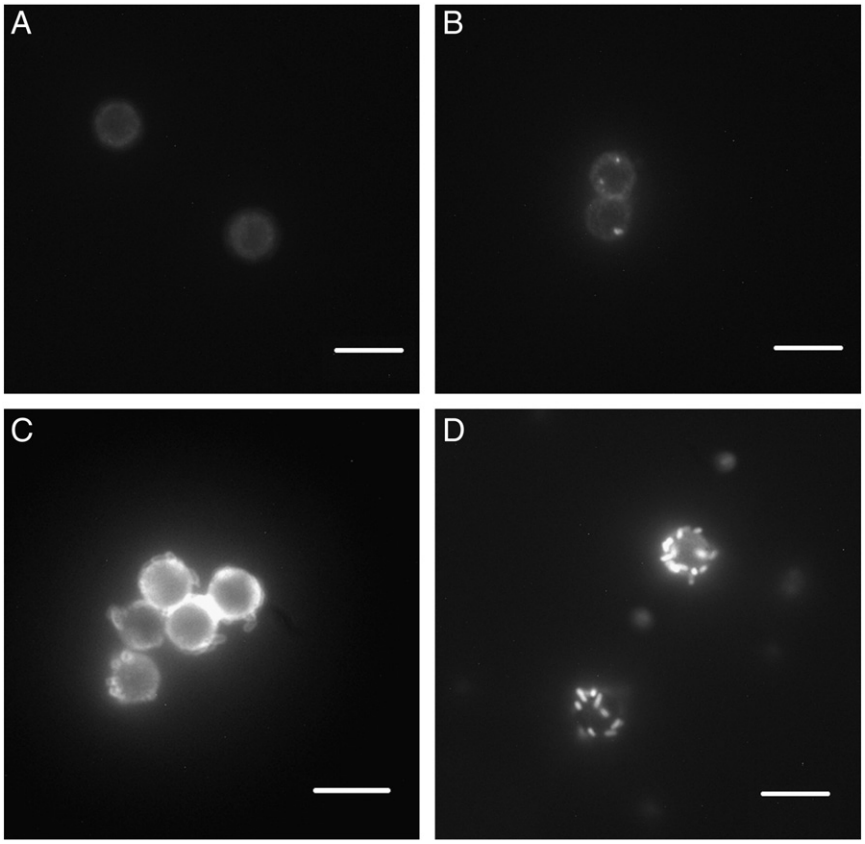

Fig. 4. Cytometric bead fluorescence images with or without *E. coli* O157:H7. A) Standard assay with non-spiked PBS; B) standard assay with non-spiked spinach rinse; C) standard assay with *E. coli* O157:H7; D) CAT-FISH assay with *E. coli* O157:H7. Negative controls (no target and/or no reporter) are not shown due to lack of fluorescence. Scale bars are 10 μm.

LQ-FISH and antibody binding conditions were evaluated separately, with antibody binding assessed using a standard ELISA kit that was not based on FISH signal. Once both conditions were optimized and detection confirmed individually, both detection types were combined (CAT-FISH) and samples examined for labeling coverage. Both *E. coli* and *S. aureus* were sufficiently labeled by probe and antibody for easy visual detection. Whereas *E. coli* cells were fairly uniform in coverage, there seemed to be an alternating pattern for *S. aureus*. *S. aureus* cells that had high 16S rRNA signal had low antibody signal and vice versa. This may be due in part to growth phase of the cells in regard to ribosomal content and protein expression or a limitation to the current protocol.

The potential for CAT-FISH utility was further demonstrated on a commercially available cytometric bead array biosensor. CAT-FISH treated cells were successfully captured by magnetic array beads (IMS-CAT-FISH), and detected via signals emitted from the fluorescent probe. The BioPlex 200 was selected due to its popularity and the versatility of cytometric bead arrays, overall, but it should be noted that this platform is not optimized for detection of the CAT-FISH signal. Specifically, the BioPlex 200 is pre-set and optimized for the detection of cells externally labeled using a phycoerythrin-tagged antibody, not internally labeled with Alexa Fluor 532. The fluorophore used was one that matched detector specifics as closely as possible but it was not possible to exactly match the optimum

Table 1
CAT-FISH detection of *E. coli* O157:H7 or *S. aureus* in PBS using a cytometric bead array biosensor.

	E. coli O157:H7		*S. aureus*			
			Lysostaphin		Nisin	
	Signal	SALOD[a]	Signal	SALOD	Signal	SALOD
PBS buffer	13.9 ± 1.2	17.3[b]	32.7 ± 2.5	40.3[b]	32.7 ± 2.5	40.3[b]
10^1/ml	–	–	31.9 ± 3.5	−8.3	31.2 ± 4.8	−9.1
10^2/ml	–	–	38.2 ± 10.6	−2.0[c]	37.7 ± 14.6	−2.6[c]
10^3/ml	19.3 ± 6.2	2.0[c]	125.7 ± 80.5	**85.4**	67.4 ± 33.1	**27.1**
10^4/ml	48.4 ± 18.0	**31.0**[d]	646.7 ± 196.5	**606.4**	364.0 ± 180.7	**323.7**
10^5/ml	365.9 ± 179.0	**348.6**	3370.2 ± 591.6	**3329.9**	1650.7 ± 670.4	**1610.4**
10^6/ml	2277.8 ± 658.7	**2260.5**	5464.8 ± 1086.3	**5424.5**	2923.2 ± 1178.9	**2882.9**
10^7/ml	2163.4 ± 642.0	**2146.0**	3318.3 ± 1509.2	**3278.0**	2498.6 ± 1338.3	**2458.3**

n = 9, LOD = blank average plus 3 times the standard deviation.
[a] SALOD — signal above limit of detection.
[b] LOD — baseline limit of detection value used to calculate sample SALOD values.
[c] Sporadically positive.
[d] Bold numbers indicate positive SALOD values.

detection conditions, since the excitation and emission wavelengths of the reader are pre-set and could not be altered.

The aforementioned compatibility discrepancies notwithstanding, similar detection limits were achieved with IMS-CAT-FISH as compared to the standard assay for *E. coli* O157:H7 in PBS buffer, and similar detection limits for *S. aureus* were also observed. This suggests that methacarn fixation permits sufficient target capture from a fluid matrix using antibody-coupled beads, and LQ-FISH generated fluorescent signals were ample enough to result in minimal to no loss in sensitivity. Notably, preliminary experiments to compare bead binding to PFA treated cells were unsuccessful in producing any signal and were not pursued.

Inasmuch as there may be problems between how well a detection method works in a laboratory buffer sample versus a real-world sample, additional sample matrices were tested. We have previously shown that detection of *E. coli* O157:H7 in enriched spinach rinse samples is possible using the cytometric array system, albeit with some difficulties due to background signals likely associated with the antibody used (Leach et al., 2010). We found that CAT-FISH is successful in detecting *E. coli* O157:H7 when used in conjugation with established spinach testing methods (rinsing and enrichment). It is interesting to note that microscopic evaluation of samples indicated that non-specific signals in standard antibody assays are due to binding of debris to antibody-coupled beads, as opposed to non-target cells (Fig. 4B). Thus, in this instance it was possible to use a general probe for the CAT-FISH procedure (since it labels only cells) to successfully eliminate the background problem.

We also evaluated the utility of CAT-FISH for *S. aureus* detection in an appropriate complex matrix. As bloodstream infections are commonly caused by *S. aureus*, we mimicked established blood testing conditions by spiking sheep blood with *S. aureus* and culturing in standard blood-culture media. We have observed cross-reactivity of our antibody with other *Staphylococcus* species (data not shown), so we chose to demonstrate the dual-label utility of CAT-FISH via incorporation of both a specific antibody and a specific probe. Our results indicated that specific antibodies can be used in association with specific probes to detect *S. aureus* in blood cultures via CAT-FISH.

Modification to further improve detection includes mainly optimizations specific to individual instruments, such as alignment of probe/detector excitation/emission pairs (e.g. BioPlex detection presented herein). Additionally, alteration of types and/or number of oligonucleotide probes used during hybridization may also be applied [e.g. dual labeled FISH probe (DOPE-FISH) (Stoecker et al., 2010); peptide nucleic acid probes (Hartmann et al., 2005; Almeida et al., 2010) or multiple probes targeting different rRNA regions (Amann et al., 1990a,b)]. CAT-FISH also has the potential for transition to kit and/or multi-well plate formats, which would significantly reduce the amount of time required for processing per sample. Furthermore, should additional confirmation be necessary, treatment of bacterial cells with methacarn does not preclude assessment by PCR methods, and auxiliary (or alternate) analysis is possible prior to hybridization steps (data not shown).

5. Conclusion

In summary, CAT-FISH should be applicable to any antibody-based fluorescence detection biosensor platform with optimal performance (i.e., high specificity) with the use of compatible rRNA probe fluorophore and fluorescence detector. The confidence level needed in a positive identification of a detected target is often paramount, and thus CAT-FISH is based on two separate levels of specificity, namely nucleic acid and protein signatures. With proper selection of FISH probes and capture antibodies, CAT-FISH may be used to provide rapid detection of target pathogens from within complex matrices with high levels of confidence.

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