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Research Article

Combined capillary electrophoresis and DNA-fluorescence *in situ* hybridization for rapid molecular identification of *Salmonella* Typhimurium in mixed culture

CE, long a staple in analytical chemistry for molecular separations, has recently been adapted for separating heterogeneous mixtures of microbial cells based on intrinsic differences in cell morphology and surface charge. In this application, CE enables effective separations of both relatively broad categories of cells, as well as of more similar cell types. As a phenotypic approach, CE may be less applicable to certain populations, including those comprised of pleiomorphic cells or chain-forming cells, where differences in cell size, shape, or chain length may lead to broad, "unfocusable" distributions in cell surface charge. At the other end of the spectrum, closely related species having similar surface charge profiles may not be separable *via* CE alone. Successful combination of microbial CE with a compatible method for generating cell-specific signals could address these limitations, increasing the diagnostic power of this approach. Fluorescence *in situ* hybridization (FISH) is a rapid molecular technique for fluorescence-based labeling of whole target cells. In this work, we combined a simple CE-based presence/absence test with FISH to develop a bacterial detection assay having an additional "layer" of molecular specificity. Using this approach, we were able to differentiate *Salmonella* Typhimurium from *Escherichia coli* in mixed populations *via* CE. Both hybridizations and CE run times were short (10–15 min), bacterial populations were highly focused (~2–3 s peak width) and there was no need for a posthybridization wash step. As few as three injected cells of *S*. Typhimurium were detected against a background of ~300 injected *E. coli* cells, suggesting the possibility for single-cell detection of pathogens using this technique. This proof of concept study highlights the potential of CE-FISH as a promising new tool for molecular detection of specific bacterial cells within mixtures of closely related, physiologically inseparable populations.

Keywords:
In situ hybridization / Rapid detection / *Salmonella*

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1 Introduction

Improved techniques for the detection of pathogenic microorganisms are needed to help ensure the safety of the global food supply. The efficiencies of today's food harvesting, processing, and distribution networks emphasize the need for rapid and accurate detection of foodborne pathogens [1]. Access to suitable methods may help reduce the incidence of food product recalls and outbreak-related disease. On the clinical side, use of rapid detection methods can lead to timelier diagnosis of disease, which can translate directly into cost savings (shorter hospital stays, less waste due to inappropriate antimicrobial therapies) and improved patient outcome [2]. Existing rapid methods include immunological tests, which often lack both sensitivity and specificity, and nucleic acid amplification techniques such as PCR, which may be inhibited by factors within the sample matrix [3]. A method combining the specificity of nucleic acid-based tests with the rapidity, simplicity, and matrix-independent robustness of an antibody-binding assay would be valuable to both the food and healthcare sectors. Fluorescence *in situ* hybridization (FISH) is a rapid whole cell method for the detection

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Abbreviations: CB, Columbia broth; **FAM,** 6-carboxyfluorescein; **FISH,** fluorescence *in situ* hybridization; **NBF,** neutral buffered formalin; **rRNA,** ribosomal RNA; **SB3-10,** caprylyl sulfobetaine; **TSB,** trypticase soy broth

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of specific pathogens. The FISH approach utilizes fluorescently labeled oligomer probes targeted to complementary RNA sequences located on ribosomes within whole, permeabilized cells [1]. Because microbial cells contain thousands of ribosomes, recognition of ribosomal RNA (rRNA) by a FISH probe results in bright and specific fluorescent labeling of whole target cells, while nontarget cells remain dark. FISH-based labeling of target cells is rapid and is no more complex than existing "shake and bake" methods used for antibody-based labeling. Therefore, this technique has multiple benefits, including rapidity, simplicity, and nucleic acid-based specificity.

CE has long been a workhorse technology in analytical chemistry labs. CE provides rapid and efficient separations of biologically relevant molecules or compounds, can be used to analyze small sample volumes and typically requires minimal sample preparation [4]. Recently, CE-based analyses have been used for genetic detection and/or characterization of pathogenic bacteria or other microorganisms. These approaches include detection of "signature" single strand conformational polymorphisms (SSCPs) generated *via* PCR from bacterial 16S or 16S–23S spacer region rRNA [5], use of a combined PCR-ligase detection reaction-CE approach targeting bacterial 16S rRNA genes [6], in-solution hybridization to mRNA with fluorescently labeled riboprobes, followed by detection of probe–target hybrids *via* CE-LIF [7] and multiplex mRNA transcript analysis of microbial cultures using CE-detectable oligonucleotide probe pools [8].

These approaches target extracted nucleic acids in solution, but CE may also be applied to separations of whole microbial cells [4, 9]. This application takes advantage of the fact that microbial cells are essentially amphoteric colloidal particles, and intrinsic differences in cell size, shape, and surface charge among different microbial species can be exploited for separation of different cell types in an electrical field [4]. The ability of CE to separate different microbial cell types from each other and from complex sample matrices may facilitate the detection of specific bacteria, including pathogens, within foods, or clinical samples [10–12]. Although CE represents a promising approach for such microbial separations, it is essentially a phenotypic approach and therefore may suffer from certain limitations as a diagnostic method. For example, pleiomorphic or chain-forming cells may not be resolved adequately *via* CE, due to the wide range of cell surface charge values occurring within these populations [4, 13]. Conversely, it may be difficult to separate closely related cell types, such as *Salmonella* Typhimurium and *Escherichia coli* due to their physiological similarities.

In this work, we sought to combine an existing CE-based method for determining the presence or absence of microorganisms in a sample with FISH-based labeling using group-specific DNA probes. Specifically, we used probes targeting *Salmonella* spp. to facilitate detection of *S*. Typhimurium in mixed cultures also containing *E. coli*. In order to provide an approach that could be extrapolated to other microorganisms for which FISH probes exist or may be designed, we based our methods on the CE protocols described by Lantz *et al.* [3] and Rodriguez *et al.* [14]. In this protocol, injected cells are coated with a dilute solution of a cationic detergent (CTAB), which induces cathodic migration of the originally negatively charged cells. After crossing the sample and spacer zones, these cells contact an oppositely migrating plug of a zwitterionic blocking agent, caprylyl sulfobetaine (SB3-10). Once in contact with the blocking agent, their electrophoretic mobility is lost and the cells are compacted into a tightly focused aggregate that is subsequently passed in front of the detector. Because all cell types are aggregated, this method obviates problems associated with analyzing cells of different size, shape, or surface charge. It is anticipated that any potential effects on cell migration stemming from use of alternate, differently charged dyes for DNA probe labeling would also be minimized by this aggregation step. Our combined approach for CE-FISH capitalizes on the demonstrated benefits of CE as a rapid analytical tool, while providing additional, nucleic acid-based specificity lacking in other published reports of CE-based detection of pathogenic microorganisms.

2 Materials and methods

2.1 Buffers and stock solutions

Tris, sodium hydroxide, hydrochloric acid, and CTAB were all purchased from Aldrich (Milwaukee, WI). Citric acid was obtained from Fisher Scientific (Itasca, IL). SB3-10, absolute ethanol, and 10% neutral buffered formalin (10% NBF) were purchased from Sigma (St. Louis, MO). Histochoice MB formalin-free tissue fixative was from Amresco (Solon, OH). Uncoated fused-silica capillaries with an id of 100 μm and an od of 365 μm were obtained from Polymicro Technologies (Phoenix, AZ). Trypticase soy broth (TSB) and Columbia broth (CB) were from Difco Laboratories (Detroit, MI).

2.2 Bacteria

E. coli ATCC 25922 and *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 13311 (type strain) were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

2.3 Cell growth and fixation

Bacterial cultures were grown overnight (~20–22 h) at 30°C in either TSB or CB to a density of $\sim 1 \times 10^9$ cells/mL (verified by plating). Fixed cells were prepared from both individual or mixed cultures of *E. coli* and Typhimurium. For cell mixtures, the concentration of *E. coli* was held steady at 10^6 CFU/mL, and the *S*. Typhimurium culture was serially diluted in this suspension of nontarget cells. This yielded a dilution series with *S*. Typhimurium concentrations ranging from 10^7 to 10^1 CFU/mL, against a background of

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10^6 CFU/mL *E. coli*. Individual cultures of *S*. Typhimurium and *E. coli* were at 10^8 CFU/mL. One milliliter portions of individual or mixed cultures were dispensed into 1.5 mL polypropylene microcentrifuge tubes and cells were pelleted for 7 min at 7000 rpm (~4000 × *g*) using a Spectrafuge 16 M microcentrifuge (Labnet International, Woodbridge, NJ). All but the last few microliters of supernatant (spent growth medium) was poured off. The cell pellet was resuspended in this remaining liquid with thorough vortexing and 1 mL of fixative (Histochoice MB, or a 60:40 mixture of absolute ethanol and PBS) was added, and the sample vortexed again. Whenever changing liquids (*e.g.*, when introducing a fixative or different buffer), it was essential to thoroughly resuspend (*via* vortexing) the cell pellet in a minimal portion of the "outgoing" liquid system. This was key to ensuring even suspensions of indiviual cells when preparing fixed cells for analysis. Cells were incubated in fixative at room temperature for as little as 5 min or up to 1 h, pelleted again, and all but the last few microliters of fixative removed. Cells were again resuspended in the remaining liquid (fixative) and further reconstituted to 1 mL in a cell storage medium consisting of a 50:50 mixture of absolute ethanol and PBS. These preparations were stored at −20°C until needed.

2.4 DNA probes and hybridization

Two DNA probes targeting the rRNA of *Salmonella* spp. were used: Sal3: 5′-AAT CAC TTC ACC TAC GTG-3′ [15], and Salm63: 5′-TCG ACT GAC TTC AGC TCC-3′ [16]. Probes were synthesized, labeled at the 5′-end with 6-carboxyfluorescein (FAM) and HPLC-purified by Integrated DNA Technologies (Coralville, IA). After receipt, probes were resuspended to a working stock concentration of 500 ng/μL in nuclease-free "low-TE" buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA). Small aliquots of these probe suspensions were dispensed into polypropylene microcentrifuge tubes and stored at −20°C until needed. For hybridization, a suspension of previously prepared fixed cells was removed from storage at −20°C, vortexed thoroughly to resuspend loosely associated cell flocs, and 100 μL portions were dispensed to new microcentrifuge tubes and pelleted as described above. After pelleting, all but the last few microliters of the supernatant (cell storage medium) was discarded and cell pellets were resuspended in this remaining liquid. One hundred microliters of hybridization buffer (0.7 M NaCl, 0.1 M Tris (pH 8.0), 0.1% SDS, 10 mM EDTA) containing probe were added and the mixture vortexed. For single-probe hybridization, Sal3-FAM was added at 5 ng/μL. Dual probe hybridizations (Sal3-FAM and Salm63-FAM) were carried out with each probe ranging from 1.25 to 2.5 ng/μL. Cell/probe suspensions were hybridized at 55°C for 15 min on a heat block (Eppendorf Thermomixer R). At 15 min, 500 μL "wash" buffer (hybridization buffer without probe, preheated to 55°C) was added and the samples were vortexed thoroughly, then incubated for up to five additional minutes. Cells were pelleted as previously, and all but the last few microliters of supernatant (wash buffer) was removed. Cell pellets were resuspended in this remaining liquid with thorough vortexing and the samples were transported to the CE analysis facility. Samples were either analyzed immediately, or stored at 4°C, protected from light, for up to 2 days without any apparent impact on the results. Prior to CE analysis, samples were pelleted *via* centrifugation (1380 × *g*, 5 min) and the supernatant was removed. Cells were then washed with CE run buffer, pelleted, and resuspended in 0.5 mL run buffer for analysis.

2.5 CE

CE was carried out as described by Lantz *et al.* (Fig. 1) [3]. Briefly, CE separations were performed on a Beckman Coulter P/ACE MDQ CE system equipped with a 488 nm laser and a photo-DAD. The capillaries used in these experiments were 30 cm in length (20 cm to the detector) with an id of 100 μm. When a capillary was first used, it was rinsed with water for 30 s, 1 N NaOH for 5 min, and the running buffer for 2 min. Stock solutions of 10 mM Tris/3.3 mM citric acid were prepared and diluted 10 × for a working buffer concentration of 1 mM Tris/0.33 mM citric acid as needed. Surfactants were added to these working concentration buffers in the appropriate concentrations as they were needed daily. Sodium hydroxide and hydrochloric acid were used to adjust the pH when necessary. The standard running buffer concentration was 1 mM Tris/0.33 mM citric acid with 1 mg/mL CTAB and was evaluated at both pH 7.0 and 9.0. The blocker plug contained 1 mM Tris/0.33 mM citric acid with 10 mg/mL SB3-10. For each run, the capillary was filled with the running buffer containing CTAB, and the following injections were made in order: bacterial sample (158 nL) for 5 s at 0.5 psi, spacer plug of running buffer for 4 s at 0.5 psi, and blocker plug of SB3-10 for 2 s at 0.1 psi. Run voltage was −2 kV in reverse polarity (current – 1.4 μA) due to reversal of the EOF by CTAB [3]. This method has been shown previously to coalesce cells of a wide variety of bacteria and fungi into a single focused zone [3]. Detection was either by absorbance and Mie scattering at 449 nm or by fluorescence at 516 nm.

3 Results and discussion

3.1 Impact of growth medium

Preliminary experiments using cells grown in either TSB or CB yielded slightly better S/Ns for FISH-labeled *S*. Typhimurium cells grown in CB. It was not clear whether this was simply due to better growth of *S*. Typhimurium in this richer medium, or due to other factors. These data suggest that the choice of growth medium is not critical, provided that the growth medium supports production of sufficient rRNA within target cells.

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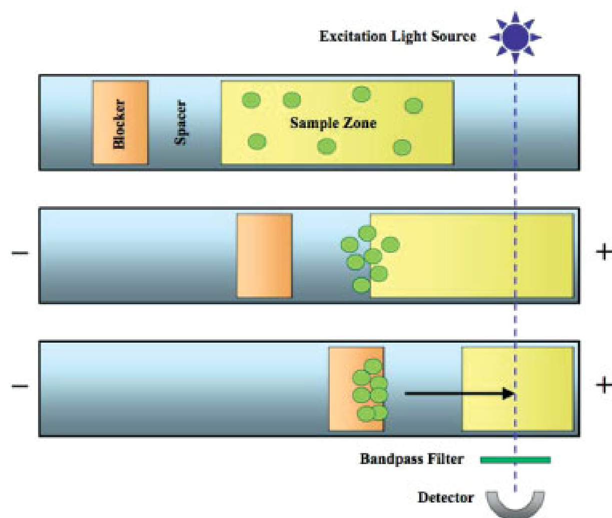

Figure 1. Scheme of sample loading and bacterial peak formation within capillaries. Prior to application of voltage, three injections are made: a sample plug containing FISH-labeled bacterial cells in running buffer (158 nL), a spacer plug of running buffer, and a short plug of blocking agent (SB3-10). Upon application of voltage, CTAB-coated bacterial cells migrate toward the cathode, traversing both the sample and spacer zones. Once cells contact the blocker plug, their electrophoretic mobility is lost, and they aggregate into a tightly focused peak that subsequently passes in front of the detector. Method and figure adapted from Lantz *et al.* [3].

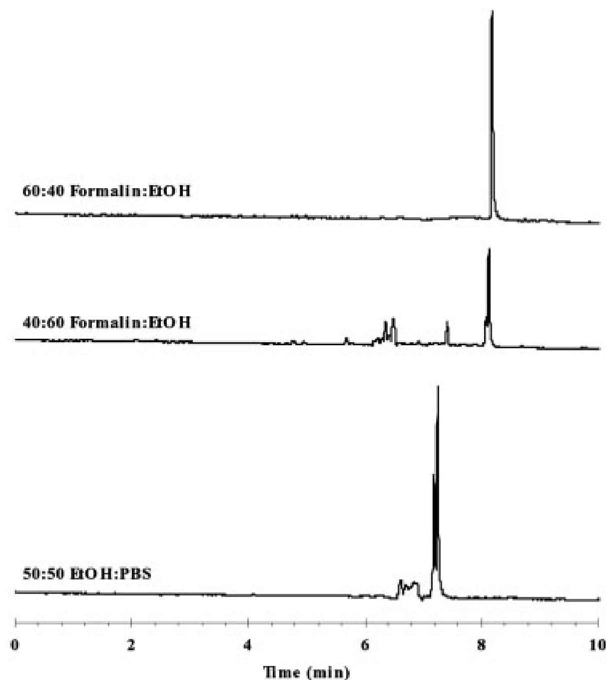

Figure 2. Effect of fixation method on cell sweeping and stacking of *S.* Typhimurium *via* CE. A 60:40 mixture v/v of 10% NBF and absolute ethanol was found to be optimal for tight peak formation. This fixation protocol also enabled effective FISH probe uptake. Detection by absorbance and Mie scattering at 449 nm.

3.2 Cell fixation

Initial experiments suggested that fixation in 10% NBF, a process we have used successfully for other FISH-based analyses (*e.g.*, fluorescence microscopy, flow cytometry), interfered with effective cell sweeping, and formation of discrete peaks. As a cross-linking aldehyde fixative, formalin may act to alter cell surface properties, including charge distribution. Therefore, a noncross-linking tissue fixative (Histochoice MB) was also evaluated, but found to be unsatisfactory for this application (data not shown). In an effort to find a suitable fixative, a series of ethanol/NBF mixtures was evaluated, with ethanol concentrations ranging from 0 to 100% v/v, in 10% increments. Cell storage buffer (a 50:50 mix of ethanol and PBS) was also evaluated as a fixative. In the ethanol/NBF mixtures, ethanol concentrations above 60% led to problems with cell precipitation and clumping during fixation and were not evaluated further. Subsequent analysis of the rest of this series *via* CE demonstrated that a mixture of 60% (of the 10% NBF solution) and 40% ethanol yielded excellent cell sweeping and focusing (Fig. 2). Results were evaluated in run buffers at both pH 7.0 and 9.0. At pH 9.0, small broad peaks appeared both before and after the main cell peak (data not shown). Therefore, all subsequent samples were run at pH 7.0. In addition to being compatible with CE analysis, fixation in 60:40 NBF/ethanol was also found to be suitable for permeabilization of cells to DNA probes. For most experiments, cells were fixed for 1 h prior to hybridization. However, in an effort to streamline the assay, shorter fixation times were also examined, and cell preparations fixed for only 5 min yielded comparable results (data not shown). These data suggest that sample fixation time, and therefore overall assay time, may be shortened considerably.

3.3 Sonication

Early in the development of this assay, the use of ultrasonication as a means for disrupting any potential cell aggregates was investigated [17]. Briefly, fixed cell suspensions were sonicated (100 W, 42 kHz) for various times prior to analysis. Sonication of fixed *S.* Typhimurium suspensions produced multiple and broadened peaks as a function of exposure time, suggesting cellular damage and lysis (data not shown). These observations are in agreement with the study of Dai *et al.* [18], who found similar results for sonication of *E. coli*. It was subsequently determined that suspensions of individual cells could be formed reliably with vortexing alone, and this method was used throughout the remainder of the study.

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3.4 Hybridization

We have previously determined *via* flow cytometry that FISH-labeling of *Salmonella* occurs very rapidly, with cells reaching near-maximal fluorescence values within only 15 min (Bisha and Brehm-Stecher, unpublished results). In typical applications of FISH, a wash step is also included to remove any nonspecifically bound probe that may interfere with the assay [19]. In our work, we found there was no need for a formal wash, but that simple dilution of the sample with 0.5 mL of wash buffer (hybridization buffer without probe) was sufficient to remove interference from non-specifically bound probe. In an effort to boost *Salmonella*-specific signal, and increase our LOD for *S*. Typhimurium, we used two probes targeting the rRNA of *Salmonella* (Sal3-FAM and Salm63-FAM). In initial experiments, when Sal3 and Salm63 were used together at 2.5 ng/μL each, we observed an approximate doubling of *Salmonella*-specific signal intensity. However, because the signal from nontarget (*E. coli*) cells was also doubled under these conditions, this approach resulted in no net improvement in LOD for *S*. Typhimurium and was not elaborated on. Toward the end of this study, preliminary experiments demonstrated that the dual probe approach did lead to improved peak area ratios (*S*. Typhimurium/*E. coli*) when each probe was used at only 1.25 ng/μL (83.5 for Sal3/Salm63 *vs*. 42.3 for Sal3 alone). These data suggest that the dual probe approach may be used to maximize the sensitivity of our assay in future work, provided that probe levels are properly titrated.

3.5 Detection of *S.* Typhimurium in pure culture

We first examined the specificity of our CE-FISH approach using pure cultures of both *E. coli* and *S*. Typhimurium. Probe-reacted, pelleted cells of *S*. Typhimurium or *E. coli* (10^7 CFU/pellet) were suspended in 0.5 mL of running buffer and injected for analysis as described. Hybridization with Sal3-FAM for 15 min led to very bright peaks for *S*. Typhimurium (RFU values in 10^5–10^6 range) and relatively dim peaks for *E. coli* (RFU values 10–100-fold lower). Figure 3 illustrates typical results for these experiments.

3.6 Detection of *S.* Typhimurium in mixed culture

Once we had successfully combined CE and FISH for labeling of *S*. Typhimurium alone, we sought to evaluate the ability of CE-FISH to detect *S*. Typhimurium in the presence of nontarget cells. Figure 4 shows the results obtained from our CE-FISH analysis of the *S*. Typhimurium/*E. coli* dilution series described in Section 2. We were able to detect as few as $\sim$3–3×10^1 injected *S*. Typhimurium cells against a constant background of $\sim3\times10^2$ injected cells of *E. coli*.

Our results suggest the capacity of CE-FISH to detect injected *S*. Typhimurium at near-single cell levels in the presence of nontarget bacteria. It is important to note, however, that the actual concentration of *S*. Typhimurium in the original sample (overnight broth cultures and dilutions thereof) was much higher (10^5–10^8 CFU/mL *S*. Typhimurium; 10^6 CFU/mL *E. coli*). Sample dilution was necessary prior to analysis, as the CE instrument we used required a minimal sample volume of 0.5 mL for proper injection. Therefore, the commercial instrumentation used placed an inherent physical limitation on the sensitivity of our system. As with other microscale approaches, additional limitations stem from the small volumes sampled (158 nL injected sample). Despite these limitations of dilution and small sample volume, substantial cell concentration *within* the capillaries is possible with our approach. Rodriguez *et al.* [14] determined that sample injection size was limited by the ability of all injected cells to effectively reach the blocking plug during the analysis time. Using a 30 cm long capillary (100 μm id), the limit of sample injection was shown to be 9 s at 0.5 psi (284 nL). Assuming the cells in such samples stack within the volume of the blocking plug (2 s at 0.1 psi, 12.65 nL), this represents

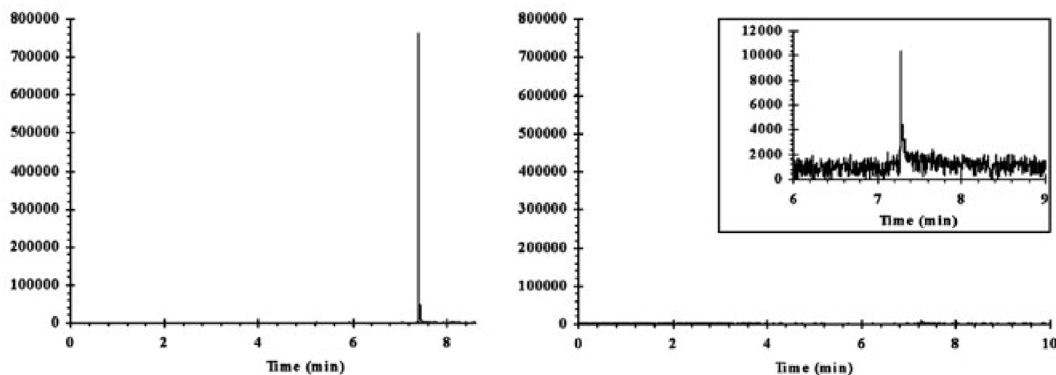

Figure 3. Electropherograms of $\sim3\times10^4$ injected cells of *S*. Typhimurium (left) and *E. coli* (right) hybridized for 15 min with the Sal3-FAM DNA probe as described in the text. The inset on the right shows the *E. coli* peak, rescaled for comparison. Fluorescence detection performed at 516 nm.

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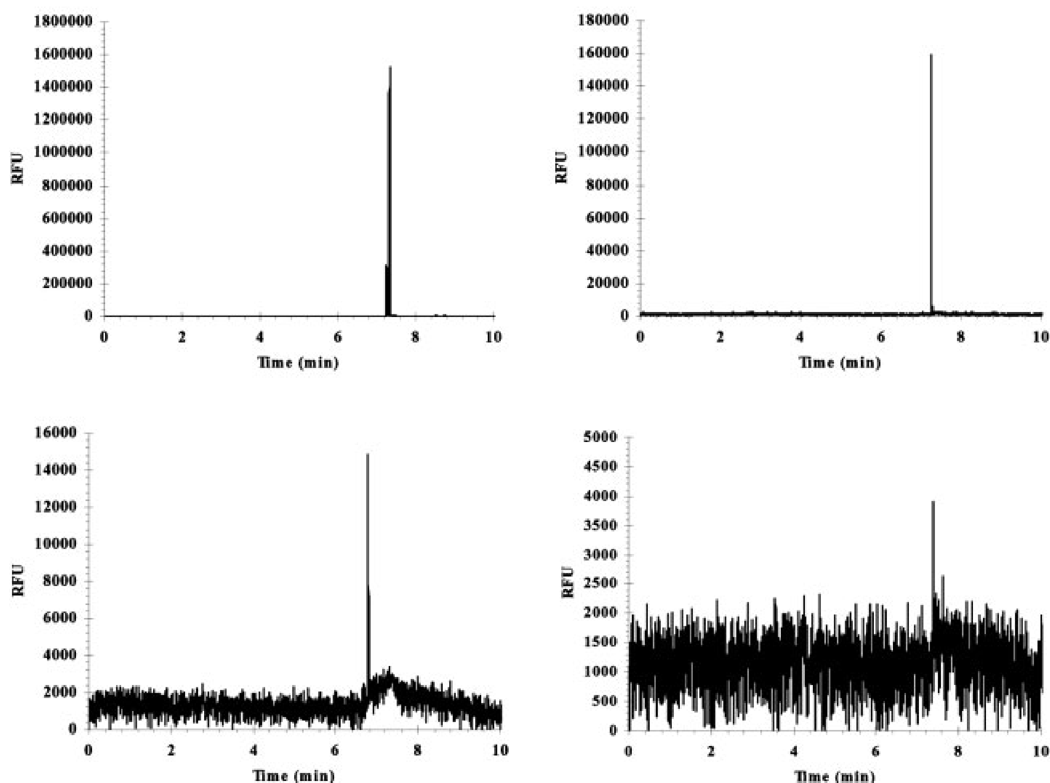

Figure 4. Dilution series of *S.* Typhimurium (ST) containing a constant 10^6 cells/0.5 mL *E. coli* background ($\sim 3\times10^2$ injected cells). Upper left: 10^7 cells/0.5 mL *S.* Typhimurium ($\sim 3\times10^3$ injected cells), upper right: 10^6 cells/0.5 mL *S.* Typhimurium ($\sim 3\times10^2$ injected cells), lower left: 10^5 cells/0.5 mL *S.* Typhimurium ($\sim 3\times10^1$ injected cells), lower right: 10^4 cells/0.5 mL *S.* Typhimurium ($\sim 3\times10^0$ injected cells). Samples were hybridized with Sal3-FAM DNA probe and detected *via* LIF at 516 nm. Slight differences in peak times are the result of the minor variations in the EOF commonly seen with CE.

a $\sim 22.5\times$ concentration of the sample. However, because the cells stack primarily at the front of the blocking plug, this concentration factor is in reality much higher. The sample injections on average have peak widths of $\sim$2.5 min, while stacked cell peaks are quite sharp, with widths as low as 0.015 min. This corresponds to a concentration factor of $167\times$.

In terms of LOD, our results (approximately three injected cells) were similar to those obtained by Shintani *et al.* [10] using the bright, nonspecific nucleic acid dye SYTO 9. These authors examined both *S.* Typhimurium and *S.* Enteritidis *via* CE-LIF and found that they migrated several minutes apart, suggesting substantial differences in cell surface charge between these closely related species. These authors also used a fluorescently labeled polyclonal antibody raised against the *Salmonella* common structural antigen to label *S.* Enteritidis and detect it *via* CE-LIF. Although they were able to demonstrate specific labeling of *S.* Enteritidis using this approach, unbound antibody in the system led to formation of a large, broad peak coinciding with the migration position of *S.* Typhimurium, making it unclear whether or not this approach would enable simultaneous detection of *S.* Typhimurium. Although they demonstrated the specificity of the antibody for *Salmonella* spp. using nontarget cultures, these authors did not present data showing detection of *Salmonella* spp. within mixtures of target and nontarget cells [10]. Kourkine *et al.* [11] combined fluorescent antibody staining with CE-LIF for the detection of *E. coli* O157:H7 in enrichment cultures from meats. Unfortunately, the observed cell peaks were very broad (5 min or greater), preventing the direct detection of labeled target cells. The source of the extremely broad peaks was attributed to the affinity of the labeled antibody for not only whole cells, but cell fragments, as well [11].

Beyond issues with sensitivity, specificity (*e.g.*, cross-reactivity) or interference from cellular fragments, expression of the cell surface antigens recognized by antibodies may also vary widely according to growth conditions or exposure to environmental stresses [1]. In contrast, Fang *et al.* [20] found FISH to be remarkably robust to several different types of stresses typical of food environments, including high salt concentrations, freezing, and low pH (pH 3.3). One drawback of PCR is that it can be inhibited by components of the food matrix (proteins, fats, divalent cations, polyphenolic compounds) or components of selective media used to enrich for various pathogens (bile salts, esculin, acriflavin, ferric ammonium citrate) [1]. FISH, on the other hand, is

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largely independent of matrix-specific effects. Fang *et al.* [20] found no matrix-related impact on FISH-based detection of *Salmonella* in 18 different kinds of foods as diverse as eggs, meats, cheese, milk, butter, ice cream, fish, and vegetables [20]. The genus *Salmonella* is comprised of over 2300 serovars, many of which are known or assumed to be capable of causing human infection [15]. Therefore, we sought to use broadly reactive FISH probes to facilitate detection of most (Sal3) or all (Salm63) serovars belonging to *Salmonella*'s seven DNA subgroups [15, 16]. Although the specificities of rRNA probes may range from the broadest grouping (universal), to domain, genus, species, or even subspecies levels [21], the ultimate level of specificity possible varies among target organisms, which can be a limit of the rRNA approach. FISH-based approaches directed to alternate nucleic acid targets (*e.g.*, mRNA, tmRNA, plasmids, *etc.*) using newly developed, highly sensitive FISH protocols may help address these limitations [22].

Given the advantages of CE in terms of speed, sensitivity, efficiency, and automation [4], our CE-FISH assay may yield a valuable new tool for rapid and specific detection of pathogenic microbes from foods or clinical specimens. Remarkably, the basic concepts behind electrokinetic manipulation of bacteria date back to as early as 1909, with Russ's investigations into "The electrical reactions of certain bacteria, and an application in the detection of tubercle bacilli in urine by means of an electric current" [23]. These experiments were carried out in both a "glass cell" comprised of sandwiched cover slips containing a bacterial suspension and fitted with electrodes, and in larger U-shaped tubes ("U-tubes"). In the glass cell apparatus, electrically induced bacterial migration could be monitored microscopically, whereas the U-tubes enabled macroscopic visualization of bacterial migration as a function of turbidity at the attractive electrode [23]. Russ examined the migration of a variety of bacteria, including "*Bacillus coli*" (*E. coli*) in the presence of various electrolytes and postulated that "... If bacteria possess electric charges, they might be attracted to the electrode of the opposite sign, and migration of the bacilli would ensue ...." Almost 100 years later, we are again taking advantage of the basic principles first described by Russ, only now, the key components needed for this work are readily available from commercial sources, including a more diverse array of available chemicals, fused-silica capillaries with defined diameters and laser-equipped CE instruments with digital data output. Additionally, such separations are now *much* faster ($\sim$10 min *vs.* the $\sim$21 h reported by Russ) and have the potential to be carried out in high-density formats [24].

Microbial applications for CE are diverse, and include separations of bacteria, yeasts, and viruses [4, 9, 25, 26]. Although FISH-based detection of individual viruses is not currently practical, screened and validated FISH probes have been published for many different bacteria and fungi, opening the possibility for ready use of the approach described here in targeting other organisms [27]. It is likely that DNA-FISH may be used in combination with existing CE separations, providing them with an additional dimension of specificity, with minimal need for further assay development beyond optimization. Potential applications may include diagnostics for food and environmental testing, as well as for clinical use. Examples in this latter category might include detection of *Candida albicans* in blood, pathogenic bacteria present in urinary tract infections, or *H. pylori* in gastric fluid samples [4, 28].

4 Concluding remarks

In this proof-of-concept work, we have described the use of FISH as a means to "overlay" an additional dimension of molecular specificity onto an existing CE-based assay for bacterial detection [3]. FISH was compatible with the whole cell format of the CE assay, and hybridization could be carried out rapidly (within 15 min). We found that both pre- and posthybridization steps (cell fixation and washing, respectively) could also be carried out within 5 min each, further abbreviating the assay. Strong *Salmonella*-specific signals could be detected in cellular aggregates comprised of either *S.* Typhimurium alone or mixtures of *S.* Typhimurium *E. coli*. The LOD for injected *S.* Typhimurium approached the single cell level, even in the presence of a 100-fold excess of nontarget cells.

Our CE-FISH technique answers the need for specificity in current CE-based microbial detection assays and avoids the pitfalls of previously published assays incorporating fluorescently labeled antibodies, such as broad or interfering peaks from unbound antibody [10, 11]. As noted, FISH-labeling is more robust than antibody-based staining, where target expression may vary widely according to cell growth conditions and physiological stress. Although the current assay uses a low-throughput, single-color format, existing off-the-shelf CE-based DNA sequencing technology (four colors plus one internal standard) could potentially be adapted to enable high-throughput, multispecies bacterial detection *via* CE-FISH. Ultimately, it may be possible to carry out all sample preparation steps, including microbial enrichment, fixation, and hybridization within 96-well microtiter plates, followed by automated, high-throughput analysis using a CE-based DNA sequencer as the readout instrument.

Alternatively, our CE-FISH approach could also be adapted for use at the microscale, in miniaturized, "lab on a chip" applications [4, 29]. Such an approach may be especially attractive for single cell detection of microbial pathogens [3, 30]. Because macroscale sample preparation steps such as centrifugation, pipetting, and resuspension are not appropriate for the micro- or nanoscale analyses needed for such a single cell approach, sequential steps such as cell fixation and hybridization would likely need to be carried out within the capillary itself. The increasing availability of compact, inexpensive, near-monochromatic and long-lived LED-based detectors may also play a role in the further development of such miniaturized, single cell analysis systems [31].

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Our CE-FISH approach represents a promising advance in CE-based microbial detection assays. However, an *ideal* detection method would be label-free, or "reagentless" [32]. Looking further into the future, it may be possible to couple CE with detection of specific microorganisms *via* in-line Raman microspectroscopy [32]. This could be a powerful means for detecting a large variety of target cells, would not require the use of extrinsic labels, and could potentially be carried out on either separately migrating peaks (multiple or single cells) or mixed-species microbial aggregates.

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