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Pathogen Detection and Remediation for Safe Eating

Detection of immunomagnetically captured 4',6-diamidino-2-phenyl-indole (DAPI)-labeled *Escherichia coli* O157:H7 by fluorescent microscopic imaging

ABSTRACT

Live cells of *E. coli* O157:H7 were captured by goat anti-*E. coli* O157 serum coated on the surface of polystyrene based immunomagnetic beads (IMB). The captured bacteria were labeled by 4',6-diamidino-2-phenylindole (DAPI), a nucleic acid stain, for observation by epifluorescent microscopy. The beads with captured bacteria were then concentrated by magnetic separators. The efficiency of this magnetic concentration step was less than that of using high speed centrifugation. The antibody-captured and IMB-immobilized bacteria were then applied on HF-treated, bovine serum albumin (BSA)-coated microscope slides mounted on an automated stage, and magnetically aligned before fluorescence distribution was measured by a cooled CCD attached to an inverted microscope. Since the beads were concentrated and linearly aligned along the edge of the magnetic field, image capture along the edge for a few field widths was sufficient to account for most of captured bacteria. We applied this approach to determine the bacterial counts in spiked beef hamburger patties. The results showed that after a 6-hour enrichment, sufficient number of the bacteria could be detected from the samples spiked with 1 CFU of *E. coli* O157:H7 per gram of the hamburger.

Keywords: fluorescent imaging, *Escherichia coli*, food safety, bacterial detection, immunomagnetic beads, DAPI labeling.

1. INTRODUCTION

The presence of pathogenic microorganisms in foods is a major concern in the United States (Federal Register, 60: 6781, 1995). Testing of foods for the absence of pathogenic contaminants along the distribution chain is necessary to reduce the incidence of foodborne illnesses. While significant improvements have been made in developing new analytical/biological techniques for food safety assurance, development of rapid, sensitive and specific bacterial detection processes is still needed.

Current approaches for the detection include immunoassay^{1,2}, immunofluorescence microscopy³, nucleic acid-based tests^{4,5}, optical⁶ and piezoelectric sensors⁷, enzyme-linked immuno-electrochemical methods^{8,9}, etc. These methods are capable to detect low levels of pathogens under appropriate conditions. Often the sensitivity of applied methods may be appreciated only after proper separation and concentration of target pathogens. For example, a process known as antibody-direct epifluorescence filter technique (Ab-DEFT), involves concentrating the bacteria on polycarbonate filters, followed by labeling of trapped bacteria with fluorescent antibody, and then enumerating bacteria count with fluorescence microscopy. This method has been successfully used to detect about 16 CFU of *E. coli* O157:H7 per gram of ground beef¹⁰ and one to ten thousand cells/ml of *E. coli* O157:H7 in milk and apple juice³.

In addition to membrane filtration, immunomagnetic beads (IMB) have been successfully applied to rapidly and conveniently separate target cells and bacteria from complex biological and food matrices^{11,12,13}. More recently, IMB separation of target bacteria from solutions and IMB concentration of captured bacteria at the surface of the detector have been applied in a very sensitive but modified electrochemical detection process for *Salmonella*, 8×10^3 cells/ml¹⁴. The IMB separation and

concentration processes have been incorporated into a commercially available immunomagnetic electro chemiluminescent instrument (ORIGEN, Igen Inc., Gaithersburg, MD.) that exhibited rather impressive sensitivity (10^2 to 10^3 cells/ml) for pathogen detection¹⁵.

In this work, we report a new application of IMB separation and concentration process for pathogenic bacterial detection. *E. coli* O157:H7 cells were first captured by proper IMB and then concentrated with magnets. Captured bacteria were treated with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent nucleic acid stain. The IMB captured labeled-bacteria were further aligned by a magnetic field for fluorescent microscopic imaging using a cooled CCD camera. Since the image capture process was automated, the data collection and analysis could be completed in a few minutes with excellent sensitivity. Applications of this process could detect 1 CFU of *E. coli* O157:H7 per gram of beef hamburger after a 6-hour enrichment at 37 °C.

2. MATERIALS AND METHODS

Culture of *Escherichia coli* O157:H7. *E. coli* O157:H7 (strain B1409, Center for Disease Control and Prevention, Atlanta, GA.) were cultured according to standard procedures in BHI media (DIFCO Laboratories, Detroit, MI.). Aliquots of 25 ml of the BHI solution in 50 ml flasks were inoculated from slants of *E. coli* cultured from selective media (MacConkey Agar, Sigma, St. Louis, MO). The flasks were placed in an incubator and shaken at 160 rpm for 18 hr at 37 °C. The cell density was checked first by light scattering measurement (absorbance at 600 nm) using a Beckman DU-70 spectrophotometer (Beckman Instruments, Palo Alto, CA) and then with plate culture counting method. Typically, *E. coli* O157:H7 cultures after 18 hr at stationary phase had absorbance readings about 0.2 after 30 fold dilution and contained about 2×10^9 CFU/ml as determined by plating on BHI media with agar. The CFU counts were determined by the use of a Model 500a Spiral System bacterial colony counter (EXOTECH, Gaithersburg, MD.). The bacteria were collected by centrifugation and then serially diluted to desired concentrations (cultured samples) with a buffer containing 10 mM Tris and 150 mM NaCl (TBS buffer).

Capture of *E. coli* on beef Hamburger. To 225 ml of modified EC media, 25g of hamburger and 1 ml of diluted *E. coli* O157:H7 with known CFU were added. The media contained 4 mg/ml of sodium novobiocin (Sigma, St. Louis, Mo.) to prevent growth of non-*E. coli* bacteria. The hamburger suspension was shaken at 37 °C at 160 rpm for 6 h. Aliquots were removed at different time intervals and filtered through glass wool to remove large fat globules and meat particles. The filtered solutions were then treated with anti *E. coli* O157 IMB for capturing of the bacteria. To 1 ml bacterial samples in 1.5 ml Eppendorf tubes, 1.5×10^6 of anti-*E. coli* O157 IMB in 15 μ l of water were added (Dynal Inc, Oslo, Norway). These tubes were vortexed for 30 minutes and then placed in a magnetic particle concentrator (Dynal Inc) which was placed on a rocker and shaken for 3 minutes. Supernatant was removed and the bacteria-IMB pellets were suspended in 1 ml of the TBS buffer solution.

Labeling of bacteria. An aliquot of 30 μ l of DAPI solution (1 mg/ml in water) was added to 970 μ l of the cultured bacterial samples or bacteria-IMB conjugates from hamburger experiments in under gentle vortex. After 10 min, DAPI-labeled bacteria were collected by centrifugation and then suspended in 1 ml of TBS. For cultured samples, the labeled bacteria were

Bacterial Capture and Labeling of *E. coli* O157:H7

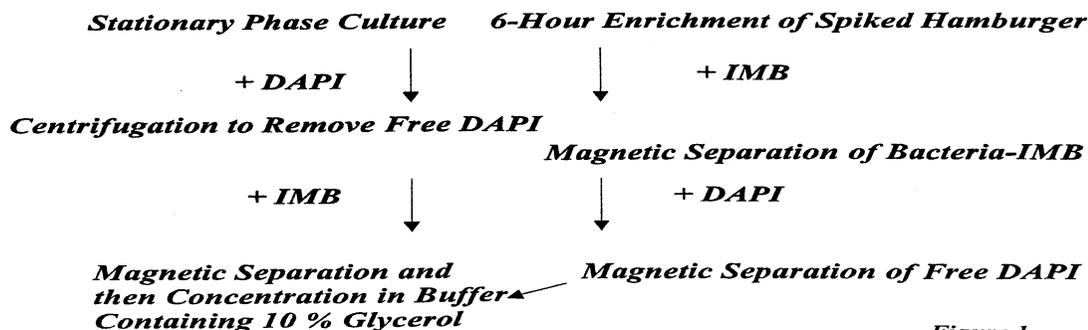


Figure 1

captured with the IMB and concentrated by a magnetic separator as described above. For hamburger samples, IMB along with labeled bacteria were separated from free DAPI by the use of a magnetic concentrator. The magnetically concentrated IMB containing DAPI-labeled *E. coli* O157:H7 were suspended in 20 μ l of TBS buffer supplemented with 10% glycerol. The described procedures for cultured samples and beef hamburger are illustrated in Figure 1.

Counting of Bacteria by Fluorescent Microscopy. Microscope slides coated with teflon grids containing 80 square wells (2 x 2 mm) from Cell-Line Associates (Newfield, NJ) were briefly etched with 5M HF + 1M HCl to increase well volumes. A neodymium boron magnet (Edmund Scientific, Barrington, NJ) was glued to the underside of the slide at the edge of an etched well. The wells were rinsed with 1% bovine serum albumin in the TBS buffer. An aliquot of 1 μ l of DAPI-labeled bacteria-IMB (~75,000 beads) suspensions in the glycerol containing buffer was placed in a well. A coverslip was laid on the slide (edge on well border) and briefly supported (20 seconds) by another coverslip opposite the magnet. This created a wedge of fluid between the well bottom and coverslip in which the beads could migrate. The cover slip was then slid out (See Figure 2 for illustration).

**Microscopic Arrangement for Enumeration of Magnetic Alignment of IMB
Captured, DAPI-Labeled *E. coli* O157:H7**

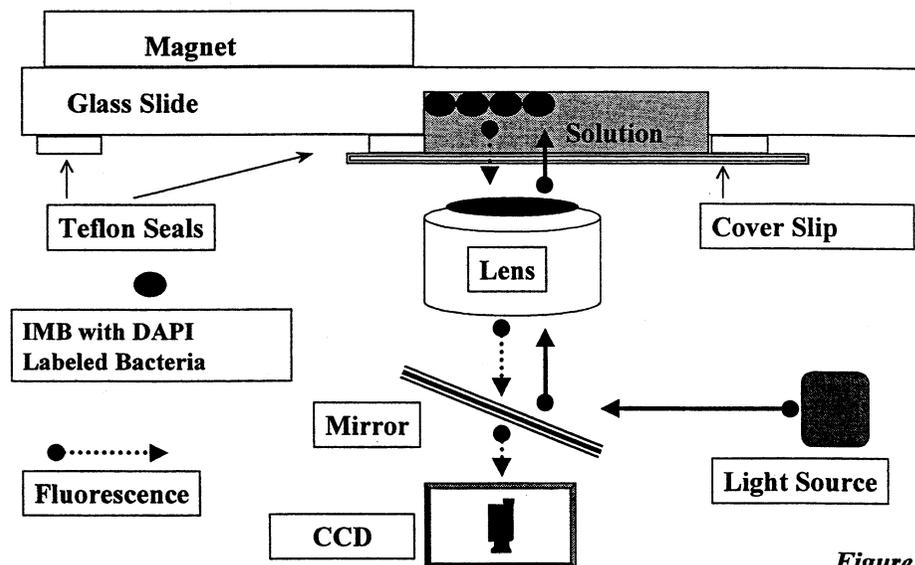


Figure 2

The slide was placed on a Nikon Diaphot inverted microscope (Garden City, NY) with a 20X UV-corrected fluorescent objective lens and the fluorescence of the eleven fields along the edge of a well closest to the magnet (one field covers an area of 272 μ x 187 μ) was measured, field by field, by moving the slide 187 μ each step with an automatic XY stage (Ludl Electronic Products, Hawthorne, NY). A script was written to move the stage in increments and capture field images so that the entire area along the edge of the well was recorded. Iterations of this script were used to capture the entire area of the well.

Samples were illuminated by a 100 watt mercury burner with the light passing through a Nikon DM400 UV1A cube (ex:365 \pm 10 nm, 400 nm dichroic, em:>400 nm). The images were digitally captured by a Pentamax 1317 cooled CCD with 1 MHz data transfer rate using Kodak 1400 chip (Princeton Instruments, Trenton, NJ) then processed using IPlab 3.1 software (Signal Analytics, Vienna, VA). Images were threshold to approximate the perimeter of the fluorescent bacteria. Area with fluorescent intensity higher than the threshold was used to enumerate antibody immobilized and DAPI-labeled bacteria secured by the magnetic beads. For rod-shaped *E. coli* cells with dimensions approximately as 0.5 μ (diameter) x 2.0 μ (length), the possible minimal (circular) and maximum (rectangular) area of the fluorescent image of a cell are ~0.2 μ^2 and 1 μ^2 , respectively. Since the bound bacteria on the surface of the IMB assumed random orientations, the projected fluorescent area for a bound cell

should vary between the maximum and the minimal areas. Thus, an average of $0.6 \mu^2$ was chosen to estimate the number of IMB-captured fluorescent bacteria.

3. RESULTS

IMB capture of the bacteria. The use of Dynal immunomagnetic beads (IMB) to separate heat-killed or radiation-killed *Salmonella typhimurium* for electrochemical detection has been reported ¹⁴. Using the same approach, we applied IMB to separate *E. coli* O157:H7 from aqueous buffers. As detailed in our previous report ¹⁶, under employed experimental conditions, the capture efficiency of IMB (1.5×10^6 beads) to the bacteria (4×10^5 to 4×10^6 CFU/ml) is about 75% efficient as collecting the bacteria by centrifugation at $10,248 \times g$. Our previous study also indicated that the binding to the IMB, did not significantly affect the viability of the bacteria.

DAPI labeling of bacteria. DAPI, a fluorescent dye for staining of nucleic acid ^{17, 18}, was used to label live *E. coli* O157:H7. We found that the labeling inhibited the growth and respiratory of the bacteria ¹⁶. However, the growth is considerably more sensitive to the treatment. For example, treating the bacteria with $30 \mu\text{g/ml}$ of DAPI reduced the activities of respiration and growth by about 65 and near 100%, respectively. The labeled bacteria showed strong fluorescence around 460 nm upon excitation at 359 nm. With a luminescence spectrometer, the fluorescence of 10^5 labeled cells per ml was easily measured suggesting that DAPI labeling would detect the presence of 100 bacteria in a sampling volume of $1 \mu\text{l}$ or less. This size of sampling volume is typical for microscopic experiments.

Magnetic field assisted detection of labeled bacteria. As described in Materials and Methods, the $1 \mu\text{l}$ suspensions used for microscopic study contained about 75,000 IMB. DAPI-labeled *E. coli* O157:H7 captured by IMB could be viewed by a fluorescence microscope (Panel A, Figure 3). The image demonstrates that the IMB have multiple sites for binding of the

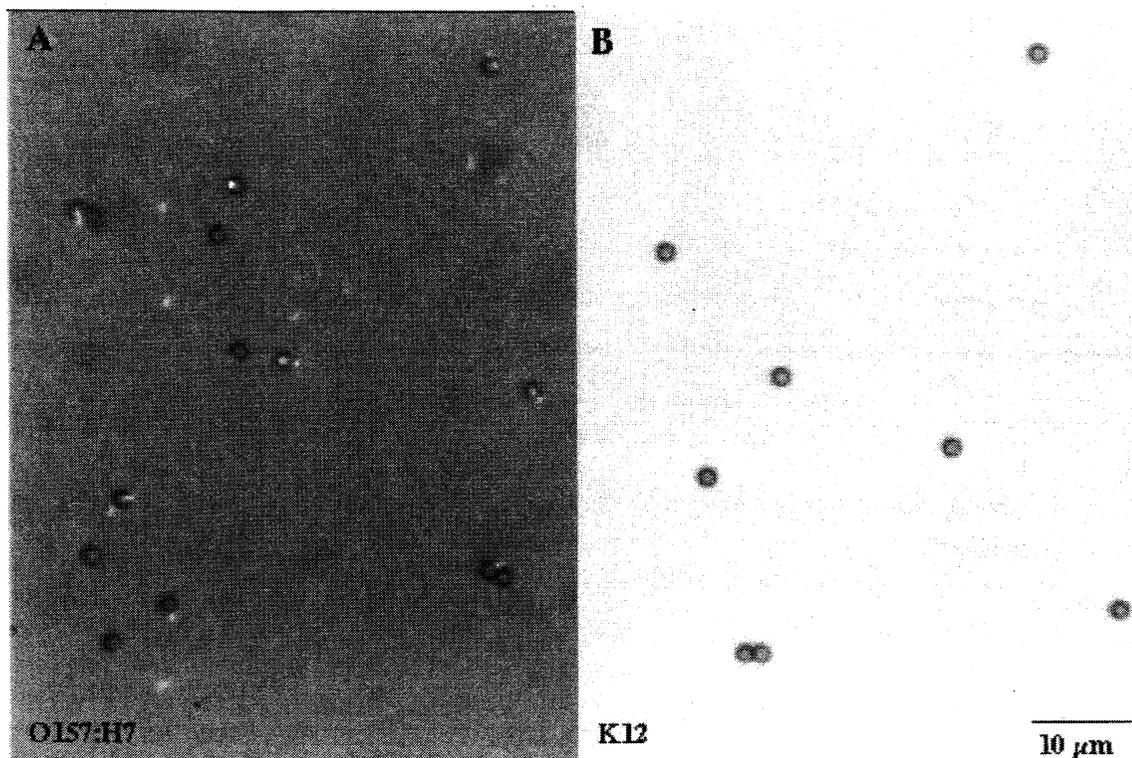


Figure 3. Specification of the IMB. (Left Panel) Capture of *E. coli* O157:H7 and (Right Panel) No binding with K12.

bacteria. It was reported by Tortorello and Gendel³ that the anti-O157 serum (proprietary formulation of Dynal) used to coat the applied IMB, exhibited very little cross reactivity toward other bacteria. With other strains of labeled *E. coli*, e.g., K12, no binding to the IMB was observed (Panel B, Figure 3). The results also indicate that the visualization of a few labeled bacteria was possible by the microscopic method.

Manual fluorescent microscopy is generally considered as a very convenient and cost-effective approach for bacterial enumeration. However, fatigue of operators caused by repetitive counting of targets is a drawback. Methods to simplify the counting process are therefore desirable. For this purpose, the design shown in Figure 2 of applying another magnetic field to concentrate and align IMB under microscope was developed.

The effects of the magnet attachment to the microscope slide are illustrated in Figure 4. Without the magnet, the IMB assumed a random distribution on the slide. However, the magnet attracted the IMB and aligned the beads along the magnetic

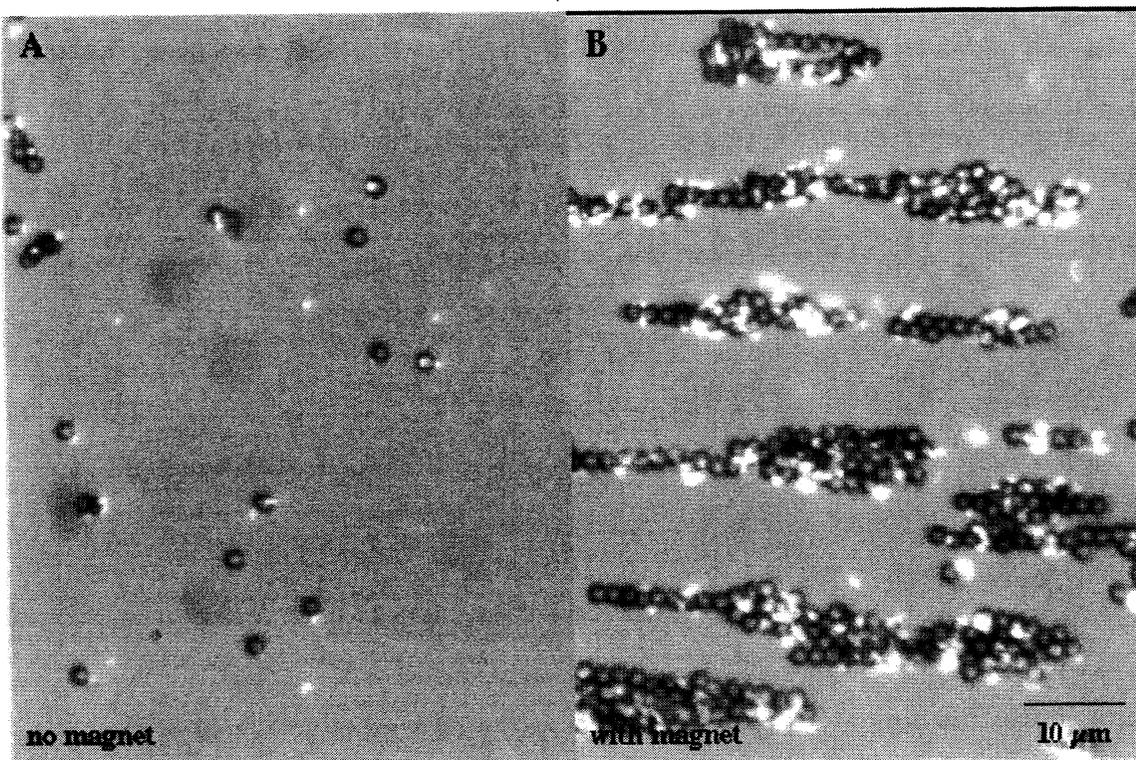


Figure 4. Effects of the Magnet Attached to The Microscope Slide on the Alignment of IMB Captured and DAPI-labeled Bacteria, (Left Panel) no magnet and (Right Panel) with magnet.

field and concentrated along the well edge close to the magnet. Using the described magnetic assembly, the bright-field images of IMB (diameter = 2.8 μm) and the fluorescent images of captured *E. coli* were obtained (Figure 5). The neodymium-iron-boron magnet attached to the microscope slide had a field strength of ~ 750 gauss at the well edge (Catalog # 38684, Edmund Scientific, Barrington, NJ). As shown, the magnetic field concentrated and also aligned the fluorescent targets for easier viewing. This arrangement of having labeled bacteria concentrated in near linear fashion, allows the automatic microscopic stage to be programmed to take consecutive images of fluorescent objects in the well in a shorter period of time and less computer memory for image storage since not the whole area of the well need be examined by the CCD. However, it should be mentioned that the attachment of bacteria to IMB decreased the migration rate of the beads toward the magnet¹⁶. Thus, with increased ratio of

labeled bacteria to IMB, more well area need be counted.

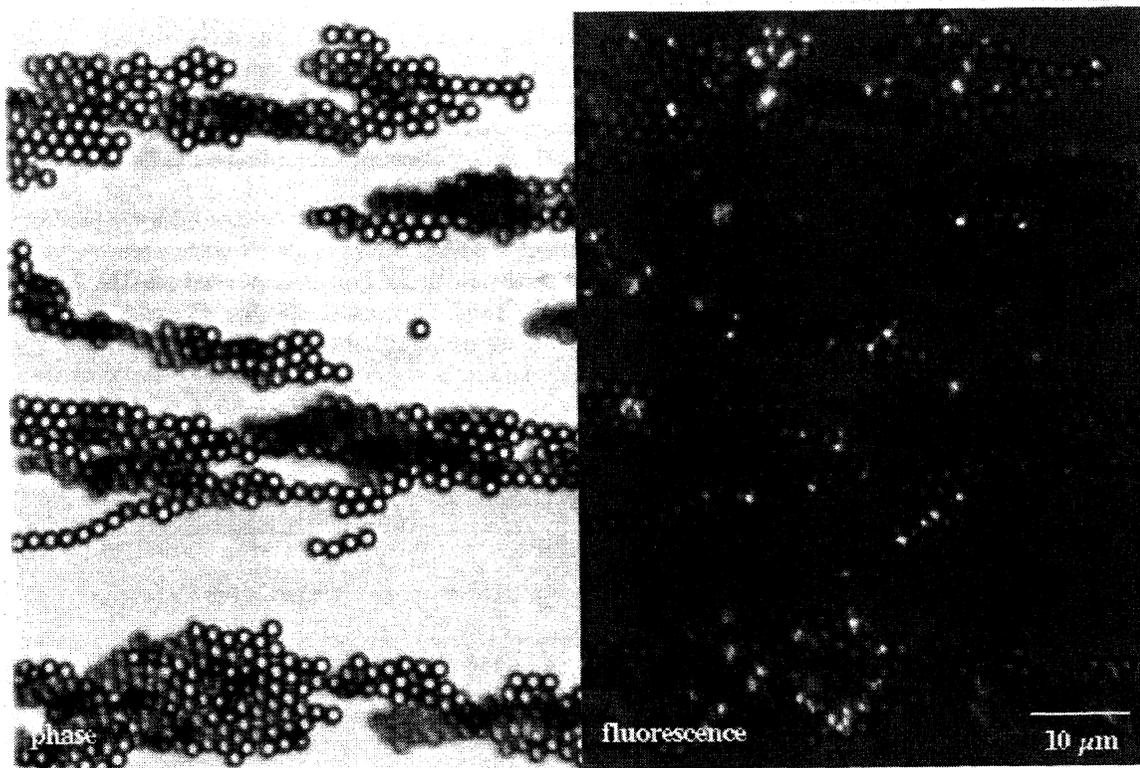


Figure 5. Magnetic Alignment of IMB with Captured, DAPI-labeled *E. coli* O157:H7. (Left Panel) The bright field image of aligned IMB. (Right Panel) The fluorescence image of the labeled bacteria captured by the IMB.

Enumeration of captured *E. coli* O157:H7. With a 20X objective lens, the observation field of utilized CCD camera is 800 (width) x 550 (length) pixels, where a pixel is a square of $0.34 \mu \times 0.34 \mu$. The dimension of the sample well is 2 mm x

Imaging of DAPI-labeled *E. coli*

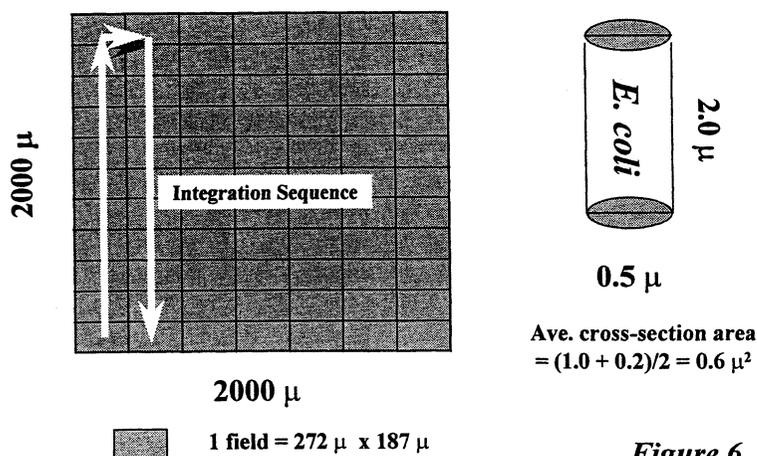


Figure 6

2 mm or $2,000 \mu \times 2,000 \mu$. Thus, to cover the whole area, 77 observations (7 columns, with 11 observation fields in a column) are necessary (Figure 6).

However, using the magnetic arrangement shown in Figure 2, more than 50% of IMB-captured, DAPI-labeled bacteria were found in the first column. In contrast, without the magnet, the beads appeared to uniformly distribute among all 77 observation fields. The fluorescent area associated with first 3 columns was then integrated. The total number of labeled bacteria in the well was estimated from integrated area and the average cross-section area of the *E. coli* cells.

Applications to spiked beef hamburger. To test the applicability of developed method for bacterial detection in food systems, we have spiked beef hamburger patties with 0 to 4 CFU of *E. coli* O157:H7 per gram of the meat. After a six-hour enrichment at 37 °C in EC media that contained 4 mg/ml of sodium novobiocin (Sigma, St. Louis Mo) to prevent growth of non-*E. coli* bacteria, aliquots were removed at different time intervals and filtered through glass wool to remove large fat globules and meat particles. The filtered suspension contained numerous small particles of various shapes that could be labeled by DAPI of which only a small fraction was bound to the IMB (Left Panel, Figure 7). Presumably, only the targeted *E. coli* O157:H7 cells were captured by the IMB. After magnetic concentration of the IMB, most of DAPI labeled contaminants could be removed from the suspensions (Right Panel, Figure 7)

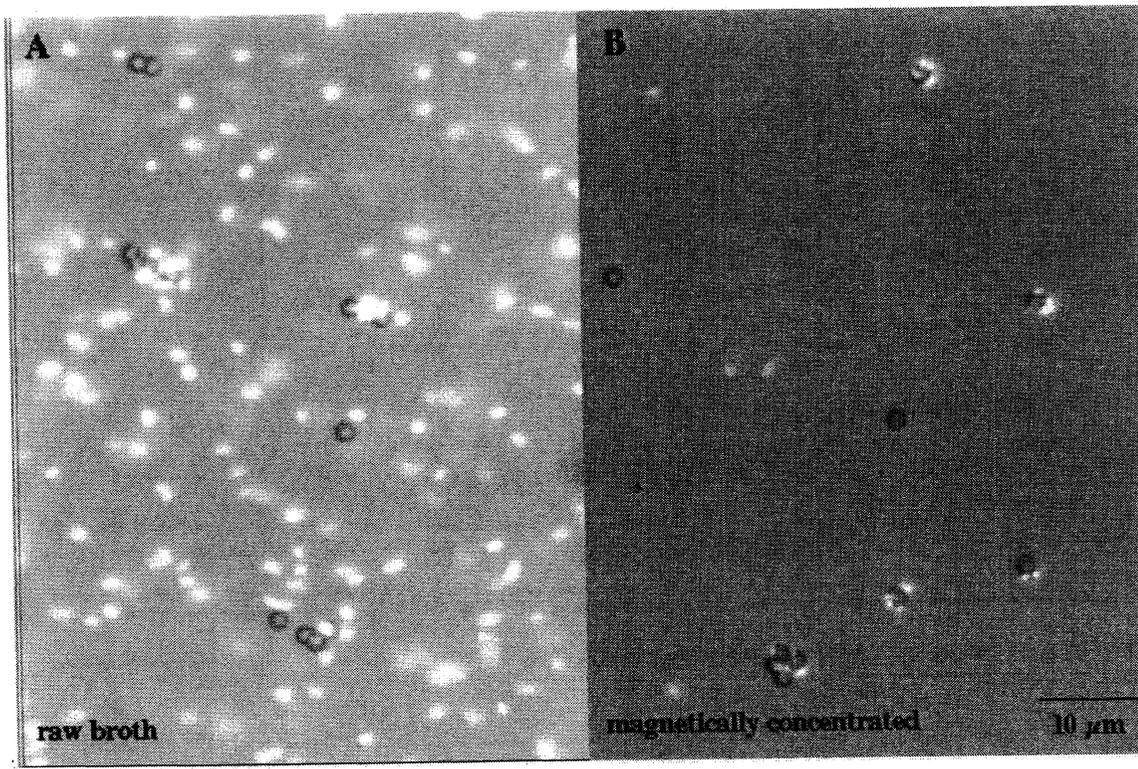


Figure 7. Non-interference of DAPI-labeled Contaminants in Enrichment Media. (Left Panel) Total DAPI labels in the enrichment media including IMB-captured *E. coli* O157:H7. (Right Panel) IMB-captured bacteria after concentrated by magnetic separator and then suspended in TBS buffer.

Indeed, after a 6-hour enrichment, the control samples (with 0 CFU of the *E. coli* added) generated negligible binding between DAPI labeled materials and the IMB. Using the fluorescent area measurements described in prior section, we obtained the enumeration results as depicted in Table 1.

Table 1. Fluorescence Imaging of IMB-captured DAPI-labeled *E. coli* O157:H7 from Artificially Spiked Beef Hamburger¹

Inoculation Dosage	Detected Fluorescent Area (μ^2)	Estimated Counts of the bacteria	Expected Counts from growth properties (Doubling time = 20 min)
0 CFU/g	no binding to IMB observed	0	0
1 CFU/g	7,419	12,360	6,556
25 CFU/g	25,656	42,760	163,840

- ¹. Beef hamburger spiked with pure culture of *E. coli* O157:H7 with indicated dosages. After a 6-hour enrichment at 37 °C, the bacteria were capture by IMB and labeled with DAPI. The enumeration of the bacteria was performed as described in text.

We have determined that the doubling time of the *E. coli* is about 20 min under enrichment conditions. Thus, the numerical results shown in Table 1 are within the prediction from the growth properties of *E. coli* O157:H7.

4. DISCUSSION

The use of IMB to increase the sensitivity of bacterial detection by electrochemical methods has gained considerable interest (Gehring et al. 1996, Yu and Bruno 1996). The basis of the enhancement is to utilize a magnetic field to concentrate IMB captured bacteria on the surface of an electrochemical detection mechanism. Thus, the concentration of the signaling compounds could rapidly reach a level sufficient for the detection mechanism. In the current work, we applied the same general principle to concentrate targeted bacteria in solutions as well as on microscope slides for fluorescent digital imaging. This approach relies on the fact that bacterial cells contain appreciable amounts of DAPI reactive cell components, e.g., AT-rich regions in nucleic acids, acidic domains of proteins and negatively charged phospholipids. Furthermore, the application of a magnetic field to linearly align IMB on the slides may considerably decrease the time needed for enumeration. As shown in Figures 2 and 4, the fluorescence imaging approach may be used to detect the presence of low numbers of intact cells of *E. coli* O157:H7 in beef hamburger after a 6-hour enrichment at 37 °C. It is expected that with higher incubation temperature, e.g., 42 °C, the enrichment time can be further reduced. After the enrichment, the process of microscopic imaging analysis requires no more than 30 min.

The practicality of developed fluorescent imaging method may be significantly improved, when a few technical issues are resolved. First, the capture efficiency of the bacteria by the IMB may be affected by the content of the culture media which changes continuously during the enrichment. The contents may also change the size and shape of the bacteria and thus affect the accuracy of cell enumeration from the described fluorescent area measurements. Nevertheless, the findings with spiked beef hamburger experiments demonstrated that the described approach could be used as a convenient method for qualitatively determination of the presence of *E. coli* O157:H7 in a 8-hour shift, a standard industrial operation.

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