Laser scanning detection of FISH-labelled *Escherichia coli* from water samples

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**Abstract**

The development of rapid and accurate methods for the detection and quantification of bacteria without cultivation is of increasing importance for water monitoring. The aim of this study was to develop a solid phase cytometry detection method for DVC-FISH labelled *Escherichia coli* cells. In order to allow automatic detection with ChemScan RDI, the fluorescein-tyramide was combined with an oligonucleotide probe directly labelled with horseradish peroxidase to increase the fluorescence intensity. The method developed was tested for the enumeration of pure cultures, for GAC-filtered and drinking water samples. The method, which appeared to be equivalent to the culture method, was less sensitive than the DVC-FISH method followed by microscopic analysis. Research is underway to further optimise the labelling conditions.

**Keywords** DVC-FISH; enumeration; *Escherichia coli*; solid phase laser cytometer

**Introduction**

Water monitoring is largely based on the detection of indicators of faecal contamination with *Escherichia coli* being the most widely used bacterial indicator. The enumeration of coliforms and *E. coli* are, therefore, the most commonly performed assays for water monitoring. Conventional microbiological methods are culture-based and require 2–5 days incubation before results are obtained. The detection of total coliforms (TC) and *E. coli* relies on the fermentation of lactose included in selective agar media after incubation at 37°C (TC) and 44°C (*E. coli*) with the latter requiring confirmation to preclude false positives.

The development of rapid and accurate methods for the detection and quantification of specific bacteria without cultivation is of increasing importance in water microbiology. As an alternative to the conventional methods, fluorescent indicators of cell viability have the advantage of allowing a rapid and direct enumeration. They may be based on membrane integrity, enzyme activity, membrane potential, respiration or pH gradient. Such a method for detecting total coliforms and *E. coli* developed by Chemunex (France), based on the detection of specific enzyme activity and the use of the laser-scanning apparatus (ChemScan RDI), aims to yield same-day results (Van Poucke and Nelis, 2000 a, b; Nelis and Van Poucke, 2000).

Recently, oligonucleotide probes targeting rRNA with various specificity and combined with fluorescent dyes have been used. There are many advantages to the use of rRNA as a target for species identification as it is present in all organisms and there is a large amount of sequence data available. Furthermore, it enables some organisms to be identified in their natural habitat without the need for enrichment techniques. The rRNA molecule contains regions which are conserved or semi-conserved and others which are highly variable, thus allowing probes of varying specificity to be designed. The fluorescent *in situ* hybridisation (FISH) method is based on the hybridisation of a fluorescently labelled probe on a specific genetic sequence of the targeted microorganism (Amman et al., 1995). Usually, the specific sequences of the target are located within the 16S rRNA. By means of microscopic observation, the presence of the target microorganism can be visualised and quantified. The FISH method is very sensitive, specific and rapid but does not allow a direct determination
of the viability of the detected microorganism. A step in which water samples are incubated with nutrients (yeast extract) in the presence of an antibiotic (nalidixic acid) has been proposed (Kogure et al., 1979). The culturable bacteria sensitive to nalidixic acid elongate during incubation because other cellular components (RNA and proteins) are not affected. A FISH-DVC method has been developed for the specific detection of E. coli (Regnault et al., 2000) and results can be obtained within the day of sample reception.

Although the FISH method produces results within a working day, the microscopic detection has the following limitations: (i) only a fraction of the membrane surface can be analysed thereby reaching detection limits that may not be appropriate for the analysis of drinking water samples that contain small numbers of bacteria and (ii) microscopic observation is time-consuming and leads to operator eye fatigue. The new possible way to detect FISH-labelled bacteria is based on solid-phase cytometry by using a ChemScan RDI (Chemunex) (Baudart et al., 2001). In this system, the laser beam scans the entire surface of the filtration membrane to detect fluorescent signals. It allows detection of as few as one labelled cell on a membrane in 3 min. Consequently, the use of solid-phase cytometry rather than direct microscopic detection should result in improved sensitivity and rapidity.

The aim of this study was to develop a solid phase cytometry detection method for FISH-labelled E. coli cells. The classical FISH microscopic detection method is based on the use of FITC-labelled nucleotide probes. As FITC fluorescence intensity is too low to allow the automatic detection with the ChemScan RDI, the first part of this work aimed at (i) increasing the fluorescence intensity of labelled bacteria using pure cultures and (ii) finding optimal operational parameters of the ChemScan RDI (in order to differentiate the labelled bacteria from the autofluorescent particles based on their optical and electronic characteristics). The second part of this work focused on testing the developed method with different types of water samples.

Materials and methods

Enumeration of bacteria
Analyses were performed according to the AFNOR 90-414 standard method (EN ISO 9308-1). The sample (2 × 100 mL) was filtered (cellulose membrane, 0.45 µm) and the membranes incubated on selective TTC-tergitol lactose agar medium (48 h at 37ºC for total coliforms, TC, and 44ºC for faecal coliforms, FC). After the incubation period, the typical lactose fermenting colonies were confirmed using the oxidase test (TC) and indole test for E. coli identification.

Probes
The E. coli probe sequence (COLINSITU, GAGACTCAAGATTGCCAGTATCAG) has been described (Régnault et al., 2000). The fluorochrome and double biotin-labelled oligonucleotides (COLINSITU-FITC and COLINSITU-2B) were purchased from MWG-Biotech (AG, France) and the HRP-labelled oligonucleotide (COLINSITU-HRP) was purchased from Interactiva (Ulm, Germany).

DVC procedure
Samples were filtered through a polycarbonate HTTP membrane (0.4 µm) and incubated (4 h) on a pad saturated with trypto-casein-soy (30 g/L) medium supplemented with yeast extract (6 g/L), nalidixic acid (10 µg/mL) and ciprofloxacine (1 µg/mL) antibiotics.

DVC-FISH coupled with epifluorescent microscopy
After the DVC step, the membranes were used for FISH labelling as previously described (Regnault et al., 2000). Elongated E. coli cells were enumerated using an epifluorescence
microscope (Leitz DMRB, Leica, France) connected to a numeric camera (Olympus DP50). Image analysis was performed using software (Studio Lite Version 1.0, Olympus France) set at a 10 s exposure time.

**DVC-FISH coupled with laser scanning system**

After the DVC step, the cells were fixed overnight (4°C, PBS with 3% paraformaldehyde) followed by *in situ* hybridisation. Cells were first dehydrated by successively placing the membranes on pads saturated with 50%, 80% and 95% ethanol (4°C, 4 min each). The membranes were then covered with 1mL of lysozyme solution (0.1 mg/mL lysozyme in Tris-EDTA, pH 8 warmed to 37°C) for 20 min at room temperature before stopping the reaction by rinsing 3 × with sterile water.

In the case of HRP-labelled probes hybridisation, the membranes were placed on pads saturated with the hybridisation buffer warmed to 42°C, covered by the probe (40pmol COLINSITU-HRP/filter) and, after 2 h at 42°C, the membranes were submitted to a stringent washing step with buffer (20 mM Tris-HCl, pH 7.2, 0.01% w/v SDS, 0.1 M NaCl, 5 mM EDTA) for 20 min at 48°C. Membranes were equilibrated by incubating 3 × with TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 5 min after which 300 µL freshly prepared fluorescein-tyramide solution (1:50 v/v) in the amplification diluent (DuPont, NEN Research Products, Boston, Mass) was added. After 10 min at room temperature in the dark, the membranes were rinsed 3 × with 1 mL TNT buffer and analysed using the ChemScan RDI.

In the case of biotin-labelled probes hybridisation, the membranes were placed on pads saturated with the hybridisation buffer (42°C) and covered by the probe (10 pmol COLINSITU-2B/filter). After 2 h at 42°C, the membranes were submitted to a stringent washing step with buffer (20 mM Tris-HCl, pH 7.2, 0.01% w/v SDS, 0.1 M NaCl, 5 mM EDTA), incubated (20 min, 51°C) and then saturated with TNB buffer (0.1M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking buffer) for 30 min at room temperature. Streptavidine-HRP solution diluted 1:250 v/v in TNB buffer (DuPont, NEN Research Products, Boston, Mass) was added and after 30 min at room temperature, membranes were equilibrated 3 × with TNT buffer for 5 min. Freshly prepared fluorescein-tyramide solution (300 µL 1:20 v/v in amplification buffer, DuPont, NEN Research Products, Boston, Mass) was added. After 10 min at room temperature in the dark, the membranes were rinsed 3 × with 1 mL TNT buffer and analysed using the ChemScan RDI.

**Labelled-cells detection by the ChemScan RDI**

The solid-phase laser cytometer (ChemScan RDI) was used for direct detection of labelled cells of *E. coli*. Membranes were scanned by an argon laser (488 nm emission wavelength) with fluorescent events emitting at 505 nm being detected. The application developed by Chemunex for coliform detection (Nelis and Van Poucke, 2000; Van Poucke and Nelis, 2000b) was used to discriminate bacteria from the background.

**Results and discussion**

The development of the protocol was performed on strain *E. coli* A58 isolated from the river Marne in the suburbs of Paris during a previous study. As FITC-labelling gave weak fluorescence intensity, it did not permit the use of the solid phase laser cytometer for the detection of fluorescence-labelled cells. Different ways to increase the fluorescence intensity of labelled bacteria were first evaluated on pure cultures using microscopic quantification of the fluorescence intensities.
Permeabilisation
In order to facilitate the penetration of the probe and the SA-HRP into the cell, dehydration and permeabilisation steps were added after the fixation. Dehydration was performed by placing the membranes on pads saturated with 50%, 80% and 95% ethanol (4°C, 4 min). For the permeabilisation step, different concentrations of lysozyme were tested (10, 5, 1 and 0.1 mg/mL in Tris-EDTA (100 mM Tris-HCl, 50 mM EDTA, pH 8). The results showed that 0.1 mg/mL was the optimal lysozyme concentration for permeabilisation (data not shown).

Biotinylated oligonucleotide probe and tyramide signal amplification
The amplification technique based on tyramide signal amplification (TSA) was compared with the FITC labelling technique. An indirect labelling was performed by linking biotin to the 5’ and 3’ ends of the oligonucleotide probE. coliNSITU via a 6-carbon spacer arm. The TSA kit was used to label cells. The suppliers of the TSA advocated use of SA-HRP (between 1:100 and 1:500 v/v) and fluorescein-tyramide solution at 1:50 v/v with an incubation time from 3–10 min. These three parameters needed to be optimised to obtain optimum fluorescence intensity. Different dilutions of the SA-HRP solution were tested (1:100, 1:250 and 1:500 v/v) with the fluorescence intensity best with the 1:250 dilution (results not shown). The fluorescein-tyramide solution was tested at different concentrations at (a) 1:50 v/v dilution with 6 min incubation (manufacturer’s recommendations) and (b) also using dilutions 1:30 and 1:20 v/v with 10 min incubation. The maximum fluorescence intensity of the labelled cells was obtained with the following conditions: 10 pmol COLINSITU 2B/membrane, 1:250 v/v SA-HRP solution in TNB buffer and 1:20 v/v fluorescein tyramide solution in amplification diluent with an incubation time of 10 min (data not shown).

Membranes containing E. coli cells labelled using the biotinylated probe and the TSA-kit were tested for automated detection with ChemScan RDI. No bacteria could be detected indicating that the fluorescence intensity was too weak. Furthermore, an important background of fluorescence was observed, due to the non-specific deposition of tyramine.

Combination of the horseradish peroxidase (HRP)-labelled oligonucleotides and tyramide signal amplification
In order to obtain a fluorescence intensity detectable by the ChemScan RDI, the oligonucleotide probE. coliNSITU was directly labelled with HRP. The fluorescein-tyramide was used as a substrate for the probe labelled with HRP. This method had been reported to allow a 10-20-fold increase in signal intensity over FITC-monolabelled probes (Schönhuber et al., 1997). In order to obtain the optimal conditions that give fluorescence intensity detectable by ChemScan RDI, different concentrations of HRP labelled probe (10, 20, 30 and 40 pmol/filter) and different dilutions of the fluorescein tyramide (1:40, 1:50 and 1:60 v/v) were tested. The best conditions were found to be 40 pmol of HRP-labelled probe/filter and 1:50 (v/v) fluorescein tyramide (Figure 1).

Comparison of the efficiency of FITC- and HRP-labelling
Identical E. coli cultures were labelled using the probes COLINSITU-FITC as a control and COLINSITU-HRP. The results showed that labelling using COLINSITU-HRP combined with tyramide signal amplification (TSA) gave the same enumeration as FITC labelling. All bacteria labelled with the FITC probe were also labelled with the HRP probe (Figure 2).

Detection and enumeration of labelled-cells using ChemScan RDI
Two types of membranes were compared: polycarbonate HTTP white membranes and black Chemfilter CB04 membranes (Chemunex). The HTTP membranes appeared to be the
most appropriate for the detection by solid phase laser cytometry because a higher number of bacteria were detected.

Pure culture cells of *E. coli* were labelled using COLINSITU-HRP with TSA in the conditions developed in this study (40 pmol COLINSITU-HRP/filter, 1:50 v/v fluorescein tyramide). The results from this assay (performed in triplicate) showed that 89% of the fluorescent events detected were validated as targeted fluorescent bacterial signal while the remainder were autofluorescent particles. The peak intensity of the bacteria was around 1,000 Fluorescent Units with a very weak background observed.

Identical *E. coli* cultures labelled with COLINSITU-HRP with TSA were enumerated using ChemScan RDI and epifluorescent microscopy. The culture dilutions filtered onto the membrane were calculated in order to obtain 500–1,000 cells/membrane. Microscopic counts were higher than ChemScan counts (Table 1). This could be explained by heterogeneous labelling as previously mentioned (Lebaron *et al*., 1997; Schönhuber *et al*., 1997). This phenomenon was conducive in the non-detection of a part of the bacterial population but may not be noticeable with eye detection. A possible way to avoid heterogeneous labelling would be to increase the salinity of the buffers used after hybridisation in order to maintain osmolarity and keep integer cells in order to increase the labelling rate.

![Figure 1](image1.png) Epifluorescence micrographs of *E. coli* cells hybridised with three differently labelled probes (A = COLINSITU-FITC probe; B = specific biotinylated probe *E. coli*NSITU-2B and the TSA system; C = specific HRP probe *E. coli*NSITU-HRP; each used at optimal levels; magnification ×1,000)

![Figure 2](image2.png) Comparison of pure culture enumeration using COLINSITU-FITC labelling (black) or COLINSITU-HRP labelling (grey)

**Table 1** Comparison of pure culture enumeration using ChemScan and microscopic detection

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>E. coli</em>/100 mL</th>
<th>Ratio of ChemScan:Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChemScan</td>
<td>Microscopy</td>
</tr>
<tr>
<td>1</td>
<td>2.0 × 10⁹</td>
<td>1.2 × 10¹⁰</td>
</tr>
<tr>
<td>2</td>
<td>6.3 × 10⁸</td>
<td>7.5 × 10⁹</td>
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<tr>
<td>3</td>
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<td>7.5 × 10⁹</td>
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<tr>
<td>4</td>
<td>4.2 × 10⁹</td>
<td>1.1 × 10¹⁰</td>
</tr>
<tr>
<td>5</td>
<td>6.6 × 10⁹</td>
<td>1.8 × 10¹⁰</td>
</tr>
</tbody>
</table>
Detection and enumeration of *E. coli* from environmental samples

A complete drinking water treatment file includes coagulation, flocculation, settlement, rapid sand filtration, ozonation, granulated activated carbon (GAC) filtration and final chlorination. Samples taken after GAC and after final chlorination were analysed for *E. coli* levels by the standard culture method and by DVC-FISH followed by microscopic or ChemScan enumeration. In the case of GAC water samples, the results after analysis by the standard culture method or by DVC-FISH followed by ChemScan varied from 0–10 *E. coli*/100 mL. Of ten samples analysed, seven were positive after culture and nine with FISH-microscopy; four samples were positive with both methods. In contrast, the results obtained by DVC-FISH followed by microscopic analysis were significantly higher and all samples, except one, were positive (Figure 3).

For most drinking water samples, no *E. coli* cells could be detected with the three methods. However, with six of these samples, DVC-FISH followed by microscopic analysis led to counts of 10–40 *E. coli*/100 mL. Among these, results were negative for four samples with the two other methods (Figure 4). Delabre *et al.* (2001) have previously shown the higher sensitivity of DVC-FISH followed by microscopy compared to culture enumeration for drinking water.

The DVC-FISH method followed by ChemScan analysis developed in this study gave results similar to culture counts but appeared to be less sensitive than DVC-FISH followed by microscopic detection. The method has to be improved in order to obtain similar results by microscopic and ChemScan analysis. Further experiments will consist of testing two

![Figure 3](image-url)  
**Figure 3** Detection of *E. coli* in GAC-filtered water samples by DVC-FISH followed by microscopic (back) and ChemScan (middle) enumeration and culture method (front)

![Figure 4](image-url)  
**Figure 4** Detection of *E. coli* in drinking water samples by DVC-FISH followed by microscopic (back) and ChemScan (middle) enumeration and culture method (front)
possible ways to improve the method: (i) to increase the salinity of buffers used after hybridisation in order to avoid labelling leakage and (ii) to decrease the minimum peak intensity parameter for ChemScan analysis in order to detect bacteria for which fluorescence is weak. While doing this, the balance of the two components will have to be maintained in order to avoid the detection of a high number of autofluorescent particles.

Conclusion
The DVC-FISH method developed in this study allowed (a) the intensity of the fluorescent signal to be increased and (b) detection of E. coli using a solid phase cytometer (ChemScan RDI). The combination of the DVC-FISH method and ChemScan RDI cytometer analysis resulted in an interesting gain of rapidity compared to direct microscopic detection. However, the method, equivalent to culture enumeration, requires further improvement in order to obtain the same sensitivity as obtained with direct microscopy.

Acknowledgements
The authors thank F. Laurent, J.-L. Drocourt and P. Cornet of Chemunex (Ivry-sur-Seine, France) for their helpful discussions and assistance in technical aspects of the ChemScan RDI.

References