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Note

A novel fluorescence imaging technique combining deconvolution microscopy and spectral analysis for quantitative detection of opportunistic pathogens

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Abstract

A novel fluorescence imaging technique based on deconvolution microscopy and spectral analysis is presented here as an alternative to confocal laser scanning microscopy. It allowed rapid, specific and simultaneous identification of five major opportunistic pathogens, relevant for public health, in suspension and provided quantitative results.

Keywords: Deconvolution; Spectral imaging; Confocal microscopy; Pathogens; FISH

Opportunistic pathogens are often ubiquitous. They can be found in drinking water and have a significant role in nosocomial infections resulting in severe disease in newborns, the elderly, AIDS patients and other immunocompromised individuals (Cohn et al., 1999). Specific detection of such organisms in distributed drinking water or food stuffs is therefore crucial for assessing risk for public health. Cultivation on selective media is still the accepted method of choice for isolation and detection of pathogens, while several of them are fastidious, impairing their consistent detection. Due to increased awareness of problems associated with culture-based assays (Vives-Rego et al., 2000), fluorescence *in-situ* hybridization (FISH) using specific fluorescently labeled nucleic acid probes has recently arisen as a rapid, accurate and highly specific technique for the identification of microorganisms without the need for cultivation (Kalmbach et al., 2000; Manz et al., 1993; Amann et al., 1990a,b). Simultaneous detection and identification of pathogens by FISH requires multi-color fluorescence microscopy, commonly utilizing confocal laser scanning microscopy (CLSM). However, CLSM has some limitations. The aperture pinhole, by physically removing out-of-focus light in CLSM images, prevents acquisition of the entirety of the fluorescence emitted by stained specimens, reducing detection sensitivity, especially at low fluorescence levels. Moreover, the required raster scanning of the sample with high light

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Table 1
Sequence and specificity of 16S rRNA oligonucleotide probes with their corresponding fluorophore

Probe	Probe sequence (5′ to 3′)	Fluorophore	Specificity	Reference
MaMB	CCA AAC TCC CGA CGC GCT GCG	Marina Blue	*Mac*†	Collins et al. (1997)
MAPA488	ATG TGG TTG CTG TGT TGG ATG G	Alexa® 488	*Map*†	Naser et al. (2004)
CdA594	AAT ACC GCA TAA AGT TAA G	Alexa® 594	*C. difficile*†	Franke-Whittle et al. (2005)
ECA647	CAT GCC GCG TGT ATG AAG AA	Alexa® 647	*E. coli*†	Huijsdens et al. (2002)
LA660	CGG AGG TTC CGC AAA AGA TG	Alexa® 660	*L. mono.*†	Furrer et al. (1991)

†*Mac*: *Mycobacterium avium complex*; *Map*: *Mycobacterium avium paratuberculosis*; *C. difficile*: *Clostridium difficile*; *E. coli*: *Escherichia coli*; *L. mono: Listeria monocytogenes*.

intensity may bleach the dyes. Furthermore, multi-color imaging with CLSM can be prohibitively expensive (Maierhofer et al., 2003). Deconvolution microscopy (DM, McNally et al., 1999) is a sensitive and affordable alternative to CLSM (Manz et al., 2000; Carrington et al., 1995). In addition, combination of DM and interferometer-based spectral imaging is an efficient approach to simultaneously detect several fluorescent signals in a given sample (Hiraoka et al., 2002) and has been successfully used for human karyotyping (Schröck et al., 1996). Such an approach would provide a novel, sensitive and affordable multi-color fluorescence imaging technique for simultaneous identification of pathogens in suspensions. This study focused on five major pathogens constituting a potent risk for public health: *Listeria monocytogenes* (*L. monocytogenes*; foodborne listeriosis; FSIS, 2002; Adak et al., 2002; Ericsson et al., 1997), *Escherichia coli* (*E. coli*, waterborne gastroenteritis, Cohn et al., 1999; Mitchell, 1972), *Mycobacterium avium paratuberculosis* (*Map*; Crohn's disease; Covert et al., 1999; Collins, 1997; Mishina et al., 1996), *Mycobacterium avium complex* (*Mac*; waterborne pulmonary disease; Von Reyn et al., 1994; Du Moulin and Stottmeier, 1986; Sobsey and Olsen, 1983), and *Clostridium difficile* (*C. difficile*; nosocomial infectious diarrhea; Loo et al., 2005; Barbut et al., 1996). Mycobacterium species (*Mac* and *Map*) were grown in Bactec 12B bottles containing 4 mL 7H12 Middlebrook broth. Mycobactin J was added (1 μg/ mL medium) for cultivation of *Map*. *E. coli* and *C. difficile* were grown on tryptic soy broth and *L. monocytogenes* was grown on cooked meat. Mixed suspensions of these pathogens were fixed in a 1:3 (v/v) solution of 4% paraformaldehyde and phosphate buffer saline (PBS) at

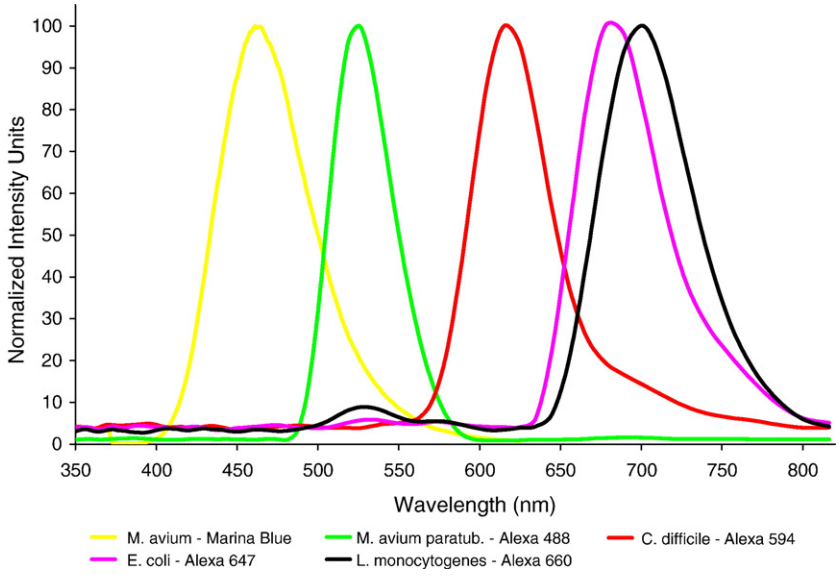

Fig. 1. Normalized fluorescence emission spectra of bacteria FISH-stained with specific 16S rRNA oligonucleotide probes obtained with interferometer-based ASI imaging system. Yellow: *Mycobacterium avium complex* labeled with Marina Blue; Green: *Mycobacterium avium* paratuberculosis labeled with Alexa®488; Red: *Clostridium difficile* labeled with Alexa®594; Purple: *E. Coli* labeled with Alexa®647; Black: *Listeria monocytogenes* labeled with Alexa®660. 600× total magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 °C for 90 min. Based on the protocol of Manz et al. (1993), FISH was performed in microcentrifuge tubes by gently mixing 8 μL of hybridization buffer (containing 0.9 M NaCl, 20 mM Tris–HCl, 30% formamide, 0.01% SDS) with 5 μL of fixed bacterial suspension and by adding 1 μL (or 25 ng) of each probe of interest for 90 min. at 46 °C. Cocktails of specific, fluorescently-labeled 16S rRNA oligonucleotide probes (Sigma-Genosys, The Woodlands, TX) were used to hybridize these bacteria (Table 1). Hybridized bacterial suspensions were then incubated in 200 μL of prewarmed washing buffer (containing 0.1 M NaCl, 20 mM Tris–HCl, 0.01% SDS) for 15 min. at 48 °C, to remove unbound probes. Upon removal of the washing buffer by centrifugation, stained cells were rinsed and resuspended in 10 μL of ice-cold sterile deionized water, which volume was spotted onto a microscope slide, air-dried in a biosafety cabinet for 15 min., mounted in ProLong® Gold antifading agent (Invitrogen, Carlsbad, CA) and finally covered with a coverslip and sealed with nail polish. The fluorescence emission peaks of the fluorophores used to label these probes were: Marina Blue®, Alexa® 488, Alexa® 594, Alexa® 647, Alexa® 660 (Invitrogen Carlsbad, CA), are presented in Fig. 1. An Applied Spectral Imaging (ASI, Migdal Ha'Emek, Israel) platform — comprising an Olympus BX61

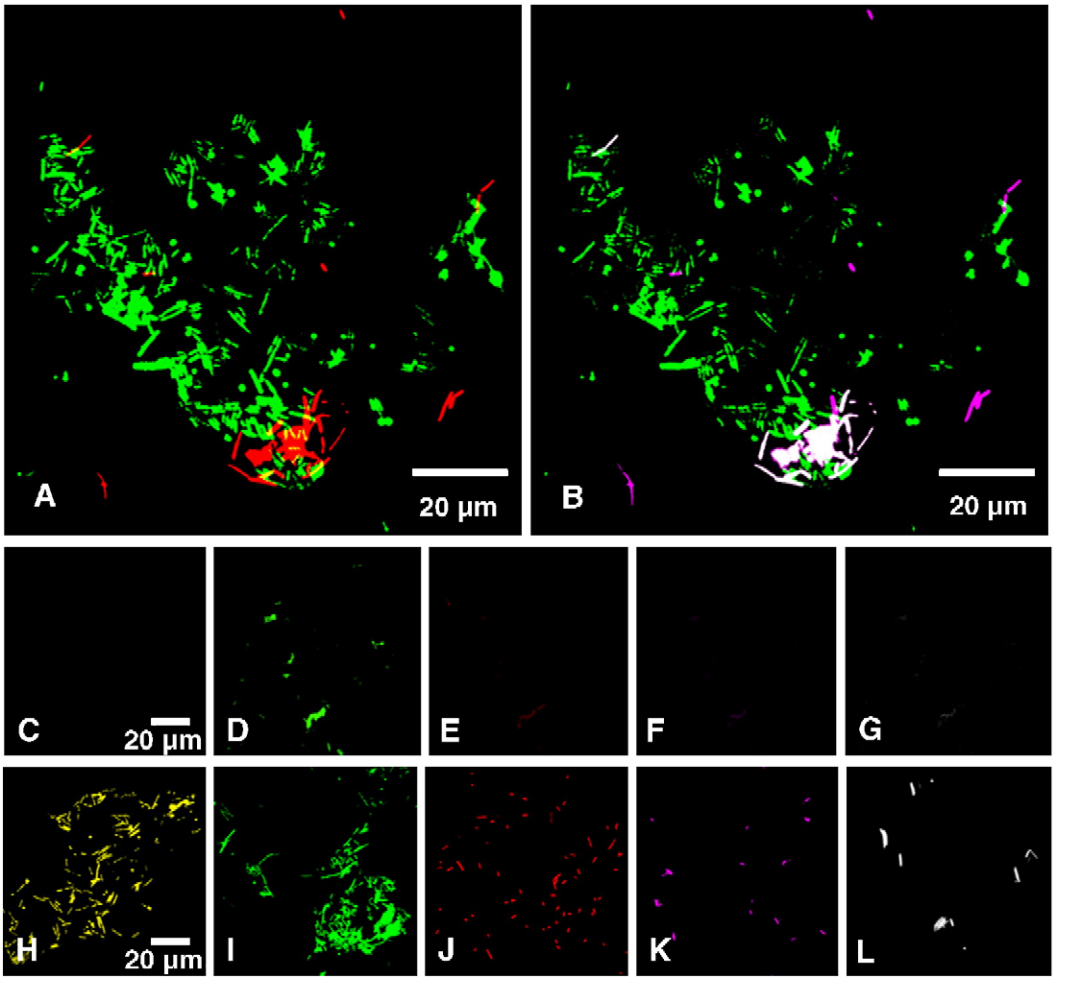

Fig. 2. (A–B) Images in the same field of view of mixed suspension of *Map*, *E. coli* and *L. monocytogenes*, FISH-stained with a cocktail of specific 16S rRNA oligonucleotide probes, labeled with Alexa®488, Alexa®647 and Alexa®660, respectively. A. Image before spectral separation: Alexa®647 and Alexa®660 in red, Alexa®488 in green; B. Image after spectral separation: Alexa®647 in purple, Alexa®660 in white, and Alexa®488 in green; (C–G) Images in the same field of view of suspension of *Map* and of (H–L) mixed suspension of *Mac*, *Map*, *C. difficile*, *E. coli* and *L. monocytogenes*. FISH-stained with cocktail of 16S rRNA oligonucleotide probes C and H. Yellow: Marina Blue channel for probe targeting *Mac*; D and I. Green: Alexa® 488 channel for probe targeting *Map*; E and J. Red: Alexa® 594 channel for probe targeting *C. difficile*; F and K. Purple: Alexa® 647 channel for probe targeting *E. coli*; G and L. White: Alexa® 660 channel for probe targeting *L. monocytogenes*; 600× total magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

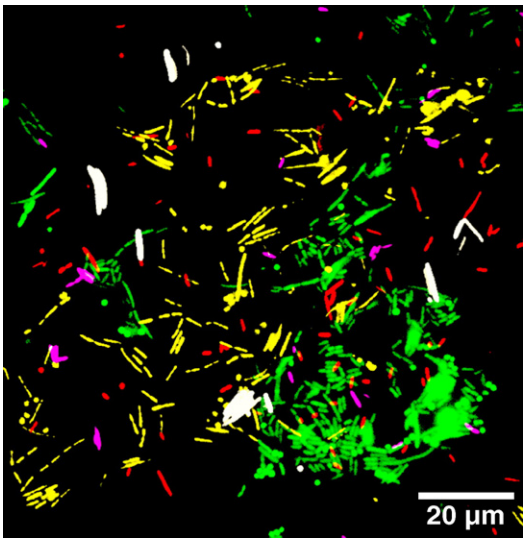

Fig. 3. Composite image in the same field of view of mixed suspension of *Mac*, *Map*, *C. difficile*, *E. coli* and *L. monocytogenes* FISH-stained with cocktail of specific 16S rRNA oligonucleotide probes. Yellow: Marina Blue channel for probe targeting *Mac*; Green: Alexa® 488 channel for probe targeting *Map*; Red: Alexa® 594 channel for probe targeting *C. difficile*; Purple: Alexa® 647 channel for probe targeting *E. coli*; White: Alexa® 660 channel for probe targeting L. monocytogenes; 600× total magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deconvolution microscope, an ASI interferometer-based spectral head and a high definition charge-coupled device camera (VDS Vosskühler GmbH, Osnabrück, Germany) — was used for image acquisition at 600× total magnification (60× objective). A sequential scan of a field of view for a given stained slide was performed using filter cubes that allow acquisition and separation of the proposed fluorophores in different channels. A DAPI filter cube was used to obtain the fluorescence signal from Marina Blue. Two ASI dual-band pass filters were also used. The first filter collected emitted light in the ranges 455–525 nm and 560–665 nm, detecting the fluorescence signal from Alexa® 594. The second filter acquired emitted light in the ranges 500–590 and 660–800 nm, detecting the fluorescence signal from Alexa® 488, Alexa® 647, and Alexa® 660. The imaging platform allowed acquisition of specific spectral signatures for each pixel of the image. As an example, a control experiment was carried out with a suspension of *Map*, *E. coli* and *L. monocytogenes* FISH-stained with a cocktail of specific probes for these bacteria (Table 1). Using the second dual-band pass filter, the spectral signatures from Alexa® 488 (green), as well as Alexa® 647 and Alexa® 660 (red) were obtained (Fig. 2A). Using the spectral information for each image, it was possible to separately target the individual signals of Alexa® 647 and Alexa® 660 and identify them with distinct false colors (Fig. 2B). Specificity experiments were also conducted to guarantee that each probe specifically binds to its target and not to other bacteria. A suspension of *Map* was FISH-stained with a cocktail of all the probes (Table 1). The images were screened for signals from all probes. Fig. 2(C–G) shows that only signals from Alexa® 488 (corresponding to the specific probe for *Map*), were detected, confirming that the other probes did not bind to this organism. Similar results were obtained for all the other probes. Based on these results, a suspension of all the bacteria presented in Table 1 was FISH-stained with a cocktail of all the probes respectively specific for these organisms. The images were screened for the fluorescence signal of each probe. Fig. 2 (H–L) show the individual channels corresponding to each probe. Fig. 3 shows a composite image, in which all the channels were recombined. Based on previous work (Chang et al., 2003; Biggerstaff et al., 1997), a quantitation protocol was derived using AutoDeblur® (AutoQuant Imaging Inc., Watervliet, NY). Fluorescence areas were quantified for each channel to obtain the relative proportions of each bacteria present on the combined image (Fig. 4A). The area occupied by each organism was also calculated and the number of each

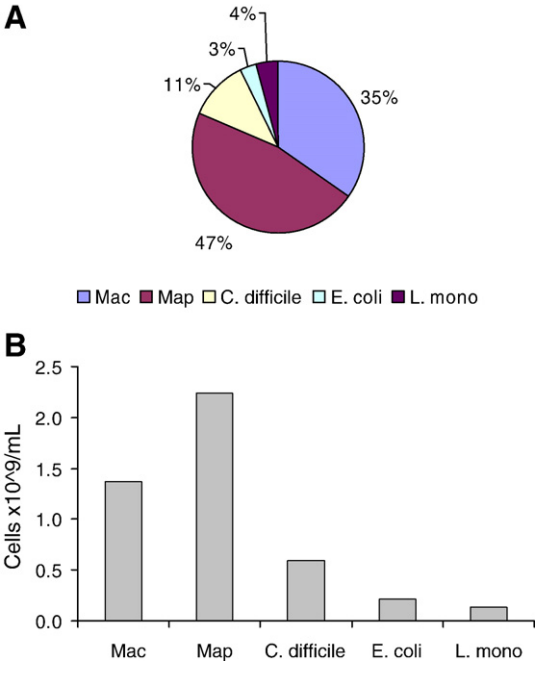

Fig. 4. Mixed suspension of *Mac*, *Map*, *C. difficile*, *E. coli* and *L. monocytogenes* FISH-stained with cocktail of specific 16S rRNA oligonucleotide probes. A: Relative proportions; B: Quantitative bacterial count.

bacterial species was extrapolated, providing a quantitative assessment of cell numbers in the mixed suspension (Fig. 4B). These results demonstrate that this technique allows for quantitative 5-color FISH in mixed bacterial suspensions.

References

Adak, G.K., Long, S.M., O'Brien, S.J., 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. Gut 51, 832–841.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990a. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Amann, R.I., Binder, B.J., Olson, R.J., Chrisholm, S.W., et al., 1990b. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Barbut, F., Corthier, G., Charpak, Y., Cerf, M., Monteil, H., Fosse, T., Trevoux, A., De Barbeyrac, B., Boussougant, Y., Tigaud, S., Tytgat, F., Sedallian, A., Duborgel, S., Collignon, A., Le Guern, M.E., Bernasconi, P., Petit, J.C., 1996. Prevalence and pathogenicity of *Clostridium difficile* in hospitalized patients: a French multicenter study. Arch. Intern. Med. 156, 1449–1454.

Biggerstaff, J., Amirkhosravi, A., Francis, J.L., 1997. Three-dimensional visualization and quantitation of fibrin in solid tumors by confocal laser scanning microscopy. Cytometry 29, 122–127.

Carrington, W.A., Lynch, R.M., Moore, E.D., Isenberg, G., Fogarty, K.E., Fay, F.S., 1995. Superresolution three-dimensional images of fluorescence in cells with minimal light exposure. Science 268, 1483–1487.

Chang, Y.C., Le Puil, M., Biggerstaff, J., Randall, A.A., Shulte, A., Taylor, J.S., 2003. Direct estimation of biofilm density on different pipe material coupons using a specific DNA-probe. Mol. Cell. Probes 17, 237–243.

Cohn, P.D., Cox, M., Berger, P.S., 1999. Health and aesthetic aspects of water quality, Water quality and treatment: a handbook of community water supplies, 2–7 and 2–8, American Water Works Association, 5th ed. McGraw Hill, Inc., New York.

Collins, M.T., 1997. Mycobacterium paratuberculosis: a potential food-borne pathogen? J. Dairy Sci. 80, 3445–3448.

Collins, D.M., Cavaignac, S., de Lisle, G.W., 1997. Use of four DNA insertion sequences to characterize strains of the *Mycobacterium avium* complex isolated from animals. Mol. Cell. Probes. 11, 373–380.

Covert, T.C., Rodgers, M.R., Reyes, A.L., Stelma Jr., G.N., 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. 65, 2492–2496.

Du Moulin, G.C., Stottmeier, K.D., 1986. Waterbrone mycobacteria: an increasing threat to health. ASM News 52, 525–529.

Ericsson, H., Eklöw, A., Danielsson-Tham, M.-L., Loncarevic, S., Persson, I., Unnerstad, H., Tham, W., 1997. An outbreak of listeriosis suspected to have been caused by rainbow trout. J. Clin. Microbiol. 35, 2904–2907.

Food Safety and Inspection Service, U.S. Department of Agriculture., 2002. May 2000. Revised action plan for control of *Listeria monocytogenes* for the prevention of foodborne listeriosis. Available at: http://www.fsis.usda.gov/OA/topics/lm_action.htm. Accessed 20 Mar. 2006.

Franke-Whittle, I.H., Klammer, S.H., Insam, H., 2005. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J. Microbiol. Methods 62, 37–56.

Furrer, B., Candrian, U., Hoefelein, C., Luethy, J., 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by in vitro amplification of haemolysin gene fragments. J. Appl. Bacteriol. 70, 372–379.

Hiraoka, Y., Shimi, T., Haraguchi, T., 2002. Multispectral imaging fluorescence microscopy for living cells. Cell Struct. Funct. 27, 367–374.

Huijsdens, X.W., Linskens, R.K., Mak, M., Meuwissen, S.G.M., Vandenbroucke-Grauls, C.M.J.E., Savelkoul, P.H.M., 2002. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J. Clin. Microbiol. 40, 4423–4427.

Kalmbach, S., Manz, W., Szewzyk, U., 2000. Development of a new method to determine the metabolic potential of bacteria in drinking water biofilms: probe active counts (PAC). In: Flemming, H.-C., Szewzyk, U., Griebe, T. (Eds.), Biofilms: investigative methods and applications. Technomic Publishing, Lancaster, pp. 107–121.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.-M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., René, P., Monczak, Y., Dascal, A., 2005. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. N. Engl. J. Med. 353, 2442–2449.

Maierhofer, C., Gangnus, R., Diebold, J., Speicher, M.R., 2003. Multicolor deconvolution microscopy of thick biological specimens. Am. J. Pathol. 162, 373–379.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K.H., Stenström, T.-A., 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S r RNA-directed fluorescent oligonucleotides probes. Appl. Environ. Microbiol. 59, 2293–2298.

Manz, W., Gernot, A., Schumann-Kindel, G., Szewzyk, U., Reitner, J., 2000. Widefield deconvolution epifluorescence microscopy combined with fluorescence in situ hybridization reveals the spatial arrangement of bacteria in sponge tissue. J. Microbiol. Methods 40, 125–134.

McNally, J.G., Karpova, T., Cooper, J., Conchello, J.A., 1999. Three-dimensional imaging by deconvolution microscopy. Methods 19, 373–385.

Mishina, D., Katsel, P., Brown, S.T., Gilberts, E.C., Greenstein, R.J., 1996. On the etiology of Crohn's disease. Proc. Natl. Acad. Sci. U. S. A. 93, 9816–9820.

Mitchell, R., 1972. Water pollution microbiology. John Wiley & Sons, New York.

Naser, S.A., Ghobrial, G., Romero, C., Valentine, J.F., 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet 364, 1039–1044.

Schröck, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Bar-Am, I., Soenksen, D., Garini, Y., Ried, T., 1996. Multicolor spectral karyotyping of human chromosomes. Science 273, 494–497.

Sobsey, M., Olsen, B., 1983. Microbial agents of waterborne disease. In: assessment of microbiology and turbidity standards for drinking water. In: Berger, P.S., Argamon, Y. (Eds.), EPA570/9-

83-001. U.S. Environmental Protection Agency, Office of Drinking Water, Washington, D.C.

Vives-Rego, J., Lebaron, P., Nebe-von Caron, G., 2000. Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol. Rev. 24, 429–448.

Von Reyn, C.F., Maslow, J.N., Barber, T., Falkinham III, J.O., Arbeit, R.D., 1994. Persistent colonisation of potable water as a source of Mycobacterium infection in AIDS. Lancet 343, 1137–1141.