Fluorescence In Situ Hybridization

EMD Team Fact Sheet—November 2011

This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.

Why is fluorescence in situ hybridization relevant?

Fluorescence in situ hybridization (FISH) is a molecular biology technique that can be used to detect microorganisms known to biodegrade contaminants. When combined with traditional measuring of changes in contaminant concentration over time, FISH provides project managers valuable information for site management, including site conceptual model development, remedy selection, and optimization and determination of contaminant attenuation rates.

What does FISH do?

To implement FISH, environmental samples (soil, water, sediment) are taken to a laboratory and in a series of steps a fluorescent dye is attached to a particular gene of interest in microorganisms or families of microorganisms. These targeted microorganisms can then be observed and their abundance and spatial distribution determined under a microscope by detecting the fluorescent light emitted. For the purposes of environmental investigation, the targeted microorganisms are typically ones capable of degrading specific contaminants.

Originally developed in the 1990s, FISH is routinely used in medical fields such as genetic counselling, disease identification, and microorganism species identification. More recently, it has been applied to the study of environmental processes, mostly in the wastewater treatment field, but also for contaminant biodegradation at sites with coal tar, herbicides, or chlorinated solvents, as well as at wetlands used to treat acid mine drainage. The FISH technique also has potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

How are the data used?

FISH can provide valuable insights for environmental remediation alone and in combination with other EMDs, like polymerase chain reaction (PCR). For example, this method can reveal whether key organism(s) needed for biodegradation are present in the sample material and allow estimation of their abundance, similar to other EMDs. However, this method can also allow investigators to explore their structure, form, and spatial distribution and association with other microorganisms. FISH signals can provide some information about activity of the target organisms although no precise rate information can be obtained.

Example Environmental Remediation Questions FISH Can Help Answer

- **Site Characterization**
    - Are the right microorganisms and/or genes present that are capable of degrading the contaminant? If so, how many and where?
    - What other microorganisms are present in the environment, and what impact do they have on the microorganisms or processes of interest?
- **Monitoring**
    - Does the microbial community change in response to an amendment?

How does it work?

Short sequences of single-stranded nucleic acids (such as DNA), called “gene probes,” are designed to match a portion of a gene or metabolic product of the organism or population of interest. A fluorescent dye is attached to the probe so that when the probe binds to target sequences within a cell, it emits fluorescent light that can be observed through a microscope (i.e., using an epifluorescent microscope) or sorted with flow cytometry. Cells emitting a fluorescent light are called “hybridized cells.” In flow cytometry, labeled cells are diluted or concentrated (depending on the initial cell concentration in the sample) so that individual cells pass through a laser beam that detects and counts fluorescently labeled cells. Flow cytometry can be significantly more efficient than counting cells using a microscope. Cells in environmental samples are handled in such a way that the cell structure remains intact while still allowing the comparatively large gene probe to enter the cell and bind to the target gene within the microorganism of interest (Figure 1). Under ideal conditions, only cells that contain the target gene are recognized by the probe and become fluorescently labeled. Various cell staining procedures are sometimes combined with FISH probes to allow quantification of various parameters such as the total number of microorganisms or the presence of specific compounds. Table 1 presents selected FISH probes and cellular stains.

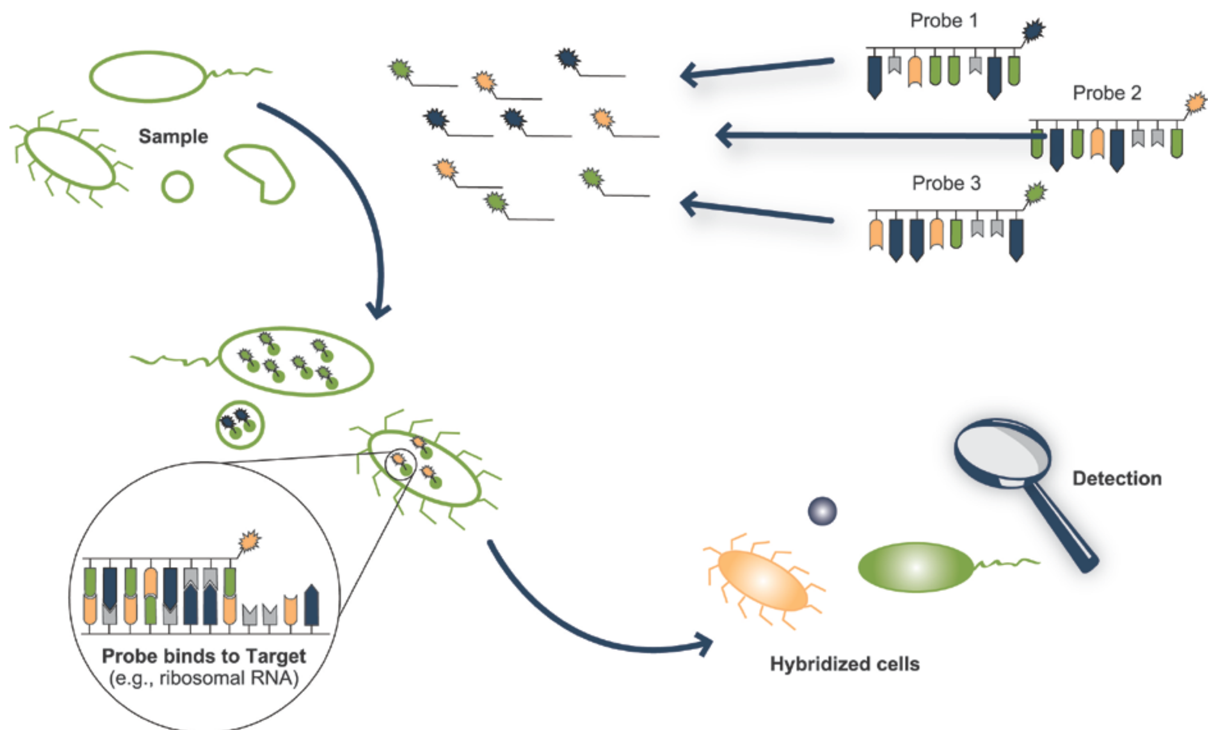

Figure 1. Fluorescence in situ hybridization method.

How are the data reported?

Depending on the method, FISH results can be presented in two different ways:

- If FISH is evaluated using a microscope and manual counting of labeled cells, the results are presented as cells per unit (liter of liquid or gram of solid) analyzed.
- If FISH is evaluated with advanced microscopy techniques and digital image processing, the results are usually presented on a relative volume or area basis, which can be converted by the laboratory to cells per unit of liquid or solid.

Table 1. Selected FISH probes or cellular stains

FISH probes or cellular stains	Contaminants	Target microorganism(s)	Reference
DAPI	NA	DNA of all microorganisms (live and dead)	This is a very common laboratory cellular stain. Not unique to environmental contaminants.
Acridine orange	NA	DNA of all microorganisms (live and dead)	This is a very common laboratory cellular stain. Not unique to environmental contaminants.
Dhe1259t	Chlorinated solvents	Some *Dehalococcoides* spp. 16S rRNA	Yang and Zeyer 2003
Dhe1259c	Chlorinated solvents	Some *Dehalococcoides* spp. 16S rRNA	
KT1phe	Trichloroethene	*Ralstonia eutropha* KT1 16S rRNA	Tani et al. 2002
Ac627BR	Naphthalene	Naphthalene dioxygenase (*nahAc*) mRNA	Bakermans and Madsen 2002
RhLu	s-Triazine herbicides	*Rhodococcus wratislaviensis* 16S rRNA	Grenni et al. 2009

Advantages

- FISH does not require cultivation of the organisms or any technology-based gene amplification (see PCR Fact Sheet), which can lead to false negatives and positives.
- In contrast to some other EMDs, FISH allows visualization of whole cells that are important to environmental remediation activities. FISH can thus provide complementary information to other EMDs, such as morphology of the cells or association of groups of microorganisms with relationship to one another.
- FISH can target several different genes simultaneously, for example, genes associated with specific degrading species of interest (e.g., *Dehalococcoides*) and broader microbial groups, such as methane-producing organisms.
- Depending on the species, and in combination with other appropriately validated activity-targeted approaches, FISH can provide general information about the activity of the organisms or populations of interest.
- FISH enables single-cell microbial studies and allows for subsequent studies, such as gene sequencing (see the Microbial Fingerprinting Methods Fact Sheet).

Limitations

- The detection limit of FISH is high (~10^6 cells/mL). However, in some cases high detection limits can be corrected by sample concentration or cell extraction methods which lower the detection limits to a few hundred cells per concentrated sample.
- Validated probes and FISH procedures are not available for a wide range of organisms within the bioremediation field. Additionally, standard protocols for sample collection and storage prior to FISH analysis have not yet been developed.
- FISH can also be used to target not only ribosomal genes (which indicate the type of organism) but also functional genes (via mRNA) relevant in bioremediation. These other genes indicate what the microorganisms can do with regards to contaminant biodegradation, for example, naphthalene dioxygenase or reductive dehalogenase. However, laboratory protocols are often time-consuming and complicated and not yet validated for field applications.
- The FISH method is not widely commercially available. Currently, mainly specialized research laboratories are performing these analyses to explore and optimize the potential of FISH for validated and cost-effective applied studies.
- The FISH method is currently expensive because of the expertise and labor needed for development of validated FISH protocols and direct microscopic counting. Once validated protocols have been

developed, FISH can be automated to some extent by using flow cytometry to count target cells more efficiently, reducing the analytical costs. However, when using flow cytometry for cell counting, all information regarding spatial relationships (among and between the cells) is lost.

Sampling Protocols

Sample matrices that can be analyzed by FISH include most kinds of environmental samples, such as wastewater, groundwater, filters, soil, and sediments. However, depending on the sample type, different types of sample preparations and FISH protocols may have to be employed. Basic sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at an appropriate temperature (e.g., 4°C during handling and transport to the laboratory). Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance/quality control information available (such as results for positive and negative controls). Sample collection, preservation, and laboratory protocols for FISH have been standardized for only certain types of organisms and ecosystems.

Additional Information

Darby, I. A., and T. D. Hewitson, eds. 2006. *In Situ Hybridization Protocols*, 3rd ed. Totowa, N.J.: Humana Press.

Lee, N., and F. Löffler. 2011. “Fluorescence In Situ Hybridization,” in *Encyclopaedia of Geobiology*, 1st ed., J. Reitner and V. Thiel, eds. New York: Springer-Verlag. www.springer.com/earth+sciences+and+geography/book/978-1-4020-9212-1.

Liehr, T., ed. 2009. *Fluorescence In Situ Hybridization (FISH): Application Guide*. Berlin: Springer.

Lebrón, C. A., C. Acheson, C. Yeager, D. Major, E. Petrovskis, N. Barros, P. Dennis, X. Druar, J. Wilkinson, E. Ney, F. E. Löffler, K. Ritalahti, J. Hatt, E. Edwards, M. Duhamel, and W. Chan. 2008. *An Overview of Current Approaches and Methodologies to Improve Accuracy, Data Quality and Standardization of Environmental Microbial Quantitative PCR Methods*. SERDP ER-1561. www.serdp-estcp.org.

References

Bakermans, C., and E. L. Madsen. 2002. “Detection in Coal Tar Waste-Contaminated Groundwater of mRNA Transcripts Related to Naphthalene Dioxygenase by Fluorescent In Situ Hybridization (FISH) with Tyramide Signal Amplification (TSA),” *Journal of Microbiological Methods* **50**: 75–84. PMID 11943360.

EPA (U.S. Environmental Protection Agency). 2004. *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples.* EPA/815/B-04/001. Office of Water. www.epa.gov/nerlcwww/qa_qc_pcr10_04.pdf.

EPA. 2011. "Glossary of Technical Terms." www.epa.gov/oust/cat/tumgloss.htm#a.

Grenni, P., A. Gibello, A. B. Caracciolo, C. Fajardo, M. Nande, R. Vargas, M. L. Saccà, M. J. Martinez-Iñigo, R. Ciccoli, and M. Martín. 2009. "A New Fluorescent Oligonucleotide Probe for In Situ Detection of *s*-Triazine-Degrading *Rhodococcus wratislaviensis* in Contaminated Groundwater and Soil Samples," *Water Research* **43**(12): 2999–3008. PMID 19476963.

Madigan, M. T., J. M. Martinko, D. Stahl, and D. P. Clark. 2010. *Brock Biology of Microorganisms*, 13th ed. San Francisco: Pearson Benjamin-Cummings.

Tani, K., M. Muneta, K. Nakamura, K. Shibuya, and M. Nasu. 2002. "Monitoring of *Ralstonia eutropha* KT1 in Groundwater in an Experimental Bioaugmentation Field by In Situ PCR," *Applied and Environmental Microbiology* **68**(1): 412–16. PMID 11772654.

Yang, Y., and J. Zeyer. 2003. "Specific Detection of *Dehalococcoides* Species by Fluorescence In Situ Hybridization with 16S rRNA-Targeted Oligonucleotide Probes," *Applied and Environmental Microbiology* **69**(5): 2879–83. PMID 12732561.

Glossary

biodegradation—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

***Dehalococcoides*—**A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of PCE and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls.

DNA (deoxyribonucleic acid)—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

epifluorescent microscope—A type of microscope that uses a high-energy light source (e.g., ultraviolet light) and specialized filters to visualize fluorescently stained specimens. Epifluorescent microscopy procedures can be used to determine both the total number of cells and total number of viable or active cells in a sample.

FISH probes—Short sequences of single-stranded DNA carrying a fluorescent label. When the probe binds to the target DNA/RNA sequence of the microorganism(s) of interest in an environmental sample, the target cell fluoresces and can be visualized and counted using a specialized microscope or a flow cytometer.

flow cytometry—A method whereby cells or particles move in a liquid stream past a laser or light beam and a sensor detects the relative light scattering and fluorescence of the particles.

functional gene—A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene *tceA* encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of TCE. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

gene—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

labeled cell—A microorganism in which a gene probe has bound to a matching sequencing within the microorganism and released a fluorescent dye, resulting in a cell that is emitting fluorescent light.

metabolic product—Products generated by a microorganism whose structure and function are defined by DNA sequences also called genes. Example metabolic products include RNA and proteins or enzymes.

probes—(1) short DNA strands (see *microarray probes*, Microarray Fact Sheet; *FISH probes*; *qPCR probes*, qPCR Fact Sheet); (2) surrogate compounds (see *enzyme activity probes*, EAP Fact Sheet).

RNA (ribonucleic acid)—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

whole cell—The entirety of a microbial cell, without extraction of DNA, RNA, etc. A whole-cell preparation does not modify the cell but evaluates it as unit.

EMD Team Contact

Robert Mueller, Team Leader

New Jersey Department of Environmental Protection

bob.mueller@dep.state.nj.us, (609) 984-3910

ITRC is affiliated with the Environmental Council of the States

ACKNOWLEDGEMENTS

The members of the Interstate Technology & Regulatory Council (ITRC) Environmental Molecular Diagnostics (EMD) Team wish to acknowledge the individuals, organizations, and agencies that contributed to this set of fact sheets.

As part of the broader ITRC effort, the EMD Team effort is funded by the U.S. Department of Energy, U.S. Department of Defense, and the U.S. Environmental Protection Agency and through ITRC's Industry Affiliates Program.

The EMD Team wishes to thank the ITRC external reviewers and the peer reviewers who contributed comments and suggestions that were of great help to the team in finalizing the fact sheets. The EMD Team also wishes to recognize and thank Bonnie Pierce, formerly of the Wyoming Department of Environmental Quality, who was our team co-leader during 2010 and whose leadership helped guide the development of these fact sheets.

The EMD Team worked hard to develop, review, and revise this set of fact sheets. The team recognizes the great value of teamwork and thanks everyone who participated—named and unnamed, ITRC staff, ITRC Point of Contact, or team member.

The EMD Team recognizes the efforts and important contributions of the following state environmental personnel: James Fish, Alaska Department of Environmental Conservation; Christine Brown, Vivek Mathrani, Sara Michael, and Claudio Sorrentino, California Department of Toxic Substance Control; Cleet Carlton, California Regional Water Quality Control Board; Leslie Smith, Florida Department of Environmental Protection; Amanda Howell and Undine Johnson, Georgia Environmental Protection Division; Robert Mueller, New Jersey Department of Environmental Protection, EMD Team Leader; and Ramesh Belani, Pennsylvania Department of Environmental Protection.

The EMD Team recognizes the efforts and valuable contributions of the following stakeholder and academic representatives: Peter Strauss, PM Strauss & Associates; Michael Hyman, North Carolina State University; Frank Löffler, University of Tennessee; Paul Philp, University of Oklahoma; Kerry Sublette, University of Tulsa; and Jennifer Weidhaas, West Virginia University.

The EMD Team recognizes the efforts and valuable contributions of the following federal personnel: Adria Bodour and John Gillette, AFCEE; Ann Miracle, DOE, Pacific Northwest National Laboratory; Hans Stroo, SERDP/ESTCP; Cheryl A. Hawkins and Ann Keeley, U.S. EPA; and Carmen Lebrón, U.S. Navy.

The EMD Team recognizes the efforts and valuable contributions of the following consultants and industry representatives: Stephen Koenigsberg, Adventus Americas, Inc.; Rebecca Mora, Chad Roper, Matthew Mesarch, and Jing Zhou, AECOM Environment; Jessica Goin, Anchor QEA; Caitlin Bell, Rula Deeb, and Denice Nelson, ARCADIS; Ramona Darlington, Battelle Memorial Institute; Stephanie Fiorenza, BP; M. Hope Lee, Tamzen Macbeth, and Ryan Wymore, CDM; David Duncklee, Duncklee and Dunham; William Berti, DuPont; Eric Raes, Engineering and Land Planning Associates, Inc.; Devon Rowe, ENVIRON;

David Major and Erik Petrovskis, Geosyntec Consultants; Ioana Petrisor, Haley & Aldrich, Inc.; Sophia Drugan, Kleinfelder, Inc.; Brett Baldwin, Dora Ogles, and Greg Davis, Microbial Insights, Inc.; Pat McLoughlin Microseeps, Inc.; Lesley Hay Wilson, Sage Risk Solutions, LLC; and Paul Hatzinger, Shaw Environmental.

ABOUT ITRC

The Interstate Technology & Regulatory Council (ITRC) is a public-private coalition working to reduce barriers to the use of innovative environmental technologies and approaches so that compliance costs are reduced and cleanup efficacy is maximized. ITRC produces documents and training that broaden and deepen technical knowledge and expedite quality regulatory decision making while protecting human health and the environment. With private- and public-sector members from all 50 states and the District of Columbia, ITRC truly provides a national perspective. More information on ITRC is available at www.itrcweb.org.

ITRC is a program of the Environmental Research Institute of the States (ERIS), a 501(c)(3) organization incorporated in the District of Columbia and managed by the Environmental Council of the States (ECOS). ECOS is the national, nonprofit, nonpartisan association representing the state and territorial environmental commissioners. Its mission is to serve as a champion for states; to provide a clearinghouse of information for state environmental commissioners; to promote coordination in environmental management; and to articulate state positions on environmental issues to Congress, federal agencies, and the public.

DISCLAIMER

This material was prepared as an account of work sponsored by an agency of the U.S. Government. Neither the U.S. Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the U.S. Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the U.S. Government or any agency thereof, and no official endorsement should be inferred.

The information provided in documents, training curricula, and other print or electronic materials created by the Interstate Technology & Council ("ITRC Products") is intended as a general reference to help regulators and others develop a consistent approach to their evaluation, regulatory approval, and deployment of environmental technologies. The information in ITRC Products is formulated to be reliable and accurate. However, the information is provided "as is," and use of this information is at the users' own risk.

ITRC Products do not necessarily address all applicable health and safety risks and precautions with respect to particular materials, conditions, or procedures in specific applications of any technology. Consequently, ITRC recommends consulting applicable standards, laws, regulations, suppliers of materials, and material safety data sheets for information concerning safety and health risks and precautions and compliance with then-applicable laws and regulations. ITRC, ERIS, and ECOS shall not be liable in the event of any conflict between information in ITRC Products and such laws, regulations, and/or other ordinances. ITRC Product content may be revised or withdrawn at any time without prior notice.

ITRC, ERIS, and ECOS make no representations or warranties, express or implied, with respect to information in ITRC Products and specifically disclaim all warranties to the fullest extent permitted by law (including, but not limited to, merchantability or fitness for a particular purpose). ITRC, ERIS, and ECOS will not accept liability for damages of any kind that result from acting upon or using this information.

ITRC, ERIS, and ECOS do not endorse or recommend the use of specific technologies or technology providers through ITRC Products. Reference to technologies, products, or services offered by other parties does not constitute a guarantee by ITRC, ERIS, and ECOS of the quality or value of those technologies, products, or services. Information in ITRC Products is for general reference only; it should not be construed as definitive guidance for any specific site and is not a substitute for consultation with qualified professional advisors.

Regulatory Acceptance for New Solutions

Documents, free Internet-based training, contact information

www.itrcweb.org