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# Fluorescent in situ hybridization for the localization of viruses, bacteria and other microorganisms in insect and plant tissues

Adi Kliot, Murad Ghanim *

*Department of Entomology, The Volcani Center, Bet Dagan 50250, Israel*

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ABSTRACT

Methods for the localization of cellular components such as nucleic acids, proteins, cellular vesicles and more, and the localization of microorganisms including viruses, bacteria and fungi have become an important part of any research program in biological sciences that enable the visualization of these components in fixed and live tissues without the need for complex processing steps. The rapid development of microscopy tools and technologies as well as related fluorescent markers and fluorophores for many cellular components, and the ability to design DNA and RNA sequence-based molecular probes and antibodies which can be visualized fluorescently, have rapidly advanced this field. This review will focus on some of the localizations methods which have been used in plants and insect pests in agriculture, and other microorganisms, which are rapidly advancing the research in agriculture-related fields.

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## 1. Introduction

Non-florescent in situ hybridization (ISH) methods were simultaneously developed in two research group independently in the late 1960s [1,2]. The development of non-radioactive fluorescent labels that could be used with ISH 20 years later made the methods of visualizing genes, their transcripts and proteins, in dissected tissues and tissue sections, very popular and powerful tools, and over the years these methods became sophisticated with the development of many fluorescent molecules that could be attached to almost every cellular components such nucleic acids, proteins, antibodies and more [3]. Long, robust probes were in use during the early days of FISH, later their length was reduced to 200 nucleotides or less in length. There were many drawbacks of using long probes such as that they often contained repetitive sequences that would induce background signal; generate secondary structures and self-hybridize, plus false positive results due to unspecific binding (this was somewhat mitigated by pre-treatment with non-labeled nucleic acids that bound to non-specific binding sites) [4]. Advances in gene sequencing technologies enabled identifying unique discriminating sites and resulted in development of shorter, but more specific probes for detection of single-copy genes and their transcripts, and detection of closely-related species in mixed samples. Databases were developed to assist in better design of species- and strain-specific probes such as the ARB database for Ribosomal RNA sequences [4,5]. Further advances in these methods came in the form of microscopy hardware and computational analysis developments complemented with chemical improvement and increase in the number of chemical fluorophores, enabling the simultaneous detection of several different targets in the same sample, with quantitative accuracy [4]. In addition, hardware advances in the microscopy field now allows whole mount sample preparation and visualization, instead of the use thick or thin microscopic sections.

FISH probing has several advantages which make it highly popular in certain research fields such as cytogenetics, microbiology and genetic diagnostics. It offers a non-invasive cultivation-independent method for identification, quantification, localization and spatial distribution of several cellular targets including nucleic acids, proteins, cell membranes, vesicles and more. In its early days, it was vastly used in cytogenetic to probe chromosomes, their aberrations, diversity and study them in various organisms and in different life stages of cellular development [6–8]. In microbiology, FISH methods have been in use to study marine microbial ecology, sludge microbial diversity but also for the study of microbial communities in the human oral cavity, gut flora and in the study of lung infections (reviewed in [3]. In diagnostics, these methods are used to identify possible genetic mutations and aberrations; either for the diagnosis of possible genetic defects in embryos and children or as known markers for possible canceration [6,9–11].

* Corresponding author.
*E-mail address:* ghanim@agri.gov.il (M. Ghanim).

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## 2. Practical considerations

Before starting a FISH experiments there are a few important factors that need special attention and design for successful labeling and identification.

### 2.1. Probe design

Most probes used nowadays for detection of microorganisms and mRNAs are 15–30 nucleotides long and are labeled with a fluorophore at their 3′ or 5′ end. The choice of target site and probe sequence must be done very carefully to aim for species/gene specific sites. In the case of probing mRNA, the probe site does not get edited or spliced when translated into mRNA. When probing for microorganisms, the probe used should be the one that is most inclusive for the phylum, but still exclusive and not probing bacteria from other phyla. The probe for example, EUB338 is designed as an all-inclusive eubacteria probe often used as control, however, some microorganisms such as planctomycetales are not always bound to it, and so new probes to replace EUB338 have recently been designed [3]. One such case of careful probe design can be found in the work of Raquin et al. [12]; where several probes were designed to detect as many Dengue virus isolates as possible but not closely related Flaviviruses such as Yellow fever and West Nile Fever [12].

### 2.2. Consideration of fluorophore selection in plants

Over the years, many fluorophores of different excitation and emission wavelengths, chemical characteristics and binding affinities have been developed. Some are directly bound to the nucleic acids while other bind via a reporter molecule, such as digoxygenin (DIG) that is then detected by a fluorescently labeled anti-digoxygenin antibody. A table summarizing the different fluorophores can be found in [3]. The sensitivity of the probe signal can be increased in various ways. One method is to conjugate the probe with several fluorophores (for instance, one at the 3′ end and several at the 5′ end with sufficient space between them). Another way is to combine direct labeling and helper labeling. Finally, the tyramide signal amplification (TSA) protocol is used for signal enhancement, although it demands permeabilization of tissues with proteinase K prior to probing, which could pose problems in some cases. In the TSA protocol, probes are labeled with DIG and antibodies against DIG are infused with Horseradish Peroxidase (HRP). HRP catalyzes fluorophores in its vicinity. By adding tyramide labeled with a fluorophore the catalyzation process is boosted and fluorescent signal is enhanced. For additional enhancement, biotin-labeled tyramide can be added and labeled using fluorophore-bound streptavidin. This protocol is often termed Catalized Reporter Deposition (CARD-FISH) [3,13,14]. Several attempts were made for improving the efficiency of hybridization by designing probes that are not nucleotides. One such attempt was the use of peptide nucleic acids (PNAs). These uncharged nucleotide chain analogues bind to nucleic acids much stronger than DNA probes, however their high production price and the slight improvement in the results they provide hampered their popularity and development [5]. Another such example is the use of Locked nucleic Acids (LNA) while constructing FISH probes. LNA probes showed better binding affinity and detection compared to DNA probes when tested on the whitefly, *Bemisia tabaci* [15]. Indirect approaches to enhance signal were also developed, such as the ways to increase ribosomal activity prior to fixation and probing [5].

In all FISH experiments, several obstacles are expected and these include: possible lack of specificity of the probe designed, insufficient penetration of the probe into the tissue resulting in false negative results, photobleaching of the fluorophores, possible probe hybridizations problems due to 3D DNA and RNA structures (such as loop and hairpin formations) and background to noise ratios caused by unbound fluorophores or by auto-fluorescence of the tissue being studied. However, the most common and difficult problem is auto-fluorescence. Auto-fluorescence is caused when the labeled tissue itself projects a fluorescent signal, causing background noise interference and in some cases this may mask the fluorophore signal. Auto-fluorescence is a widely known problem in plants (chloroplasts) and insect (eyes, cuticle), tissues that have similar emission wavelengths as many of the chemical fluorophores used, resulting in false and masking signals. In plants therefore, initial thorough bleaching of the tissue is recommended (as described in [16]) in addition to smart probe design; avoiding fluorophores such as GFP and cy5 whose emission wavelengths are close to that of chlorophyll. In insects, bleaching of the cuticle can greatly reduce the unspecific binding (protocols described in [17,18]) although in the case of cuticles the process can take even months. Nonetheless, hardware and computational tools are also being development to help overcome this problem [4].

## 3. Localization of viruses in insects and plants

The study of various Arthropod borne viruses (Arboviruses), causing diseases in both animals and plants, has been tackled using FISH for studying the routes and infection sites these viruses reach within the insect vectors, plants and animal tissues. Some viruses are transmitted in a circulative mode through their vector; actively enter gut cells after being acquired with the food. Those viruses enter the blood stream (hemolymph in insects), circulate in the blood/hemolymph and finally reach the salivary glands, from which they will exit onto the next host. This type of transmission is therefore persistent, meaning that once the insect vector acquired the virus and the virus completed this route, transmission will continue for the rest of the insect life. On the other hand, some viruses only attach to the mouth parts of their vectors, thus causing a temporary, transient attachment to the insect tissues. The discovery of these different transmission modes and their routes was aided with FISH microscopy methods. A good example is the detection of Dengue fever in *Aedes albopictus* tissues and cell line. Three DNA probes were designed to fit as many Dengue isolates as possible but not to the closely related Flaviviruses. All three probes showed positive, specific results- probing virus in lateral lobes of the salivary glands of infected mosquitoes but not healthy ones or ones infected with Flaviviruses [12]. FISH probing of Dengue virus was also successfully applied on human samples of various diseased tissues [19].

Phloem and xylem insect feeders, such as aphids, whiteflies, leafhoppers and mealybugs and other sap sucking insects such thrips, are the most important insect group that transmit plant viruses important in agriculture. The transmission of many begomoviruses, exclusively transmitted by whiteflies, was studied using both DNA probes and antibodies. Localization of *Tomato yellow leaf curl virus* (TYLCV) in the midgut of its whitefly vector, *Bemisia tabaci*, using antibodies to its coat protein was used to demonstrate co-localization and possible linkage with proteins encoded by the insect such as the Heat Shock Protein 70 kDa (HSP70) [20]. DNA probes were designed and used to localize various gene transcripts of the virus, both on the virus strand and the complementary strand for demonstrating that virus can replicates within its vector [21], a subject that have been under debate for many years. The results showed that the virus transcripts localize and accumulate in the insect midgut [21]. FISH on TYLCV was also used in a quantified manner to show difference in acquisition and

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