An introduction to fluorescence *in situ* hybridization in microorganisms

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Abstract

Fluorescence *in situ* hybridization (FISH) is a molecular biology technique that enables the localization, quantification and identification of microorganisms in a sample. This technique has found applications in several areas, most notably the environmental, for quantification and diversity assessment of microorganisms, and the clinical, for the rapid diagnostic of infectious agents. The FISH method is based on the hybridization of a fluorescently-labelled nucleic acid probe with a complementary sequence that is present

inside the microbial cell, typically in the form of ribosomal RNA (rRNA). In fact, a hybridized cell is typically only detectable because a large number of multiple fluorescent particles (as many as the number of target sequences available) are present inside the cell. In here, we will review the major steps involved in a standard FISH protocol, namely fixation/permeabilization, hybridization, washing and visualization/detection. For each step the major variables/parameters are identified and, subsequently, their impact on the overall hybridization performance is assessed in detail. .

1. Introduction

During the 1940's, Chargaff observed that the concentration of the four DNA bases inside the cell was similar for the adenine-thymine and cytosine-guanine pairs, a work that laid the foundation for the selective base-pairing rules of nucleic acids [1]. This base recognition specificity is the basis of the principle of hybridization, a process where two complementary (or near complementary) sequences of nucleic acids are able to interact and stay together via the action of non-covalent forces. When the hybridization process occurs inside the cells, it is said to occur *in situ*. Whereas researchers have quickly seized the immense possibilities of *in situ* hybridization for biotechnological processes, namely for detecting and tracking specific nucleic acid sequences, a robust method to report a successful hybridization was still needed. The most widely-used method to detect hybridization at the present involves coupling a fluorescent molecule, also known as fluorochrome or fluorophore, to the nucleic acid sequence of interest. Fluorescence *in situ* hybridization (FISH) is now routinely used to assess microbial diversity in environmental samples [2], rapidly detect specific pathogens in clinical diagnostics [3], or identify spoilage microorganisms in industrially-relevant bioprocesses [4], among other applications.

The origins of the detection of microorganisms by FISH can be traced back to the late 1980's, when DeLong *et al*. used oligonucleotides (i.e. short nucleic acid sequences containing typically between 15-35 bases) as probes to target the 16S ribosomal RNA (rRNA) of bacteria [5]. Previously, the same group had used radiolabeled probes to identify microorganisms [6], but concluded that with FISH a higher resolution and faster analysis were obtained. In both studies, the FISH method involved 4 major steps: fixation/permeabilization, hybridization, washing and visualization/detection. The fixation/permeabilization step objective is to render the cell wall permeable to the nucleic acid probe entry, while at the same time guarantee that cell lysis and extensive nucleic acid degradation will not occur. During hybridization, the probe is placed in contact with the target cells, and if complementary (or near-complementary) sequences are present, it hybridizes. The specificity of this binding is guaranteed by the stringency conditions set for the hybridization step and, later, at the washing step, where all loosely-bound probes are washed away. Finally, visualization/detection by either fluorescence microscopy or flow cytometry allows the researcher to observe if a successful hybridization has occurred.

In spite of being one of the most widely-used molecular biology techniques at the present, those working with FISH are aware that not everything is simple in protocol development. A large part of the problem has to do with the high number of experimental variables that are needed to adjust in order to obtain a successful hybridization. Most of these variables are interconnected, which means that alterations in one of them might implicate unexpected changes in others.

2. Parameters involved in a FISH method

Each of the four steps involved in a FISH method and described in the previous section contains multiple variables (Table 1). Most of them can be user-defined (e.g. hybridization temperature and duration), but others are characteristics of the system that is being studied or are restricted to the conditions available in the laboratory (e.g. target microorganisms and type of equipment available).

2.1 Fixation/Permeabilization

There are several agents that can be employed during fixation/permeabilization. The choice for the agents to be used in this step is mostly dependent on three factors: the type of microorganism (mainly the cell envelope), the type of nucleic acid probe used, and the target cells spatial location (i.e. whereas cells are in slides, filter membranes or in suspension). This multifactor dependency has caused all efforts to create a universal fixation method for microorganisms to fail [2, 7].

By far, the two most common fixation agents are ethanol and formaldehyde [e.g. 8, 9]. The first is a precipitating fixative and as such acts by reducing the solubility of macromolecules, which in turn inactivates enzymes and stabilizes nucleic acid structures. It also helps on dissolving the lipidic layer, so ethanol can be regarded as a permeabilization agent too. The second, formaldehyde, induces the formation of covalent bonds between molecules, which will also inhibit the action of enzymes, but might decrease membrane permeability and consequently target accessibility. Typical concentrations for ethanol vary between 50% and 100 % (vol/vol) with contact times of less than 30 minutes, whereas for formaldehyde the concentration is usually very close to 4% (wt/vol) and exposure can take from as little as 10 minutes to last overnight, depending on the type of sample and the FISH technique [10, 11]. For the case of ethanol, it is not uncommon to subject the samples to solutions with increasing ethanol concentrations in order to obtain a complete dehydration without the destruction of cell structure [12]. In studies employing DNA probes, fixation is at times performed at low temperatures (~4 ºC). Other less common fixatives are methanol and glutaraldehyde, but their effect in the cell is expected to be quite similar to the one of ethanol and paraformaldehyde, respectively.

As for permeabilization agents, the method of choice usually involves enzymes, even though mild acid hydrolysis has been used as well [12, 13]. Lysozyme is the most widely enzyme used, and acts by degrading the peptidoglycan present in bacterial cell walls. Standard protocols tend to use this enzyme mainly on Gram+ bacteria at a concentration of 10 mg/ml, since these bacteria have a

thicker peptidoglycan layer that might be hard to permeate. Lipase, proteinase K, mutanolysin, lysoesthaphin or streptolysin have also been tested, particularly for microorganisms associated with permeabilization difficulties, such as those possessing mycolic acids in their cell walls, spore-forming bacteria or mycobacteria [12, 14]. Other studies make use of a combination of enzymes that will act over different components of the cell envelope. For instance, FISH methods for *Staphylococcus* apply very often a mixture of lysozyme and lysostaphin [15]. Mixtures of mutanolysin/lysozyme or lipase/proteinase K have been applied to mycolic-acid-containing bacteria (e.g. actinomycetes) [12]). These microorganisms are particularly difficult to permeabilise [12] because mycolic acids are long-chain fatty acids that form a capsule-like, high hydrophobic layer. The role of capsule and capsule-like external structures of the cell envelope in the permeabilization/fixation procedures is still poorly understood, especially because these layers are poorly characterized for many bacterial species. Other less common permeabilization protocols might include detergents. Triton X-100, SDS, EDTA and other detergents, are frequently used to permeabilize the membranes by extracting the lipids membrane or destabilizing the lipopolysaccharides by removing divalent cations by chelation [16].

For combinations of particular microorganisms and nucleic acid probes, the use of permeabilization was found to be unnecessary [17, 18]. For instance, a protocol that uses peptide nucleic acid (PNA) probes to detect *Staphylococcus aureus* was able to dispense with this step altogether. In the case of PNA, this might be attributed to the neutral charge of the molecule and the small size of the probes (15 bp) that makes cells penetration easier [18]. Nonetheless, similar results have been found for charged probes. LNA/2'-Omethyl RNA probes have been introduced into unfixed *Helicobacter pylori* cells [17], whereas DNA probes were applied to both Gram-positive (*Bacillus* sp.) and Gram-negative bacteria (*Ruegeria* sp. and *Pseudovibrio* sp.) [19]. Apart from *H. pylori*, which seems to have a more permeable envelope (as no additional permeation steps were applied); for the other species, DNA probes were introduced into live cells by means of chemical transformation. This

showed that fixation and permeabilization steps might not be mandatory if innovative strategies are used to deliver probes inside cells. However, when cell fixation is not a concern (i.e. when viable cells are not necessary for further tests), fixation/permeabilization protocols are likely to deliver a more robust FISH outcome.

2.2 Hybridization and washing

Hybridization is probably the most complex step of FISH due to the number of variables that can affect the binding of the probes to the sequence target - the duplex formation. This reaction is thought to proceed in two steps. First, a nucleation event leads to the formation of a few correct base pairs between the two complementary sequences and, then, a rapid zipping occurs, during which the rest of the base pairing proceeds [20]. Since the zipping step is very quick, it is thought that nucleation is the rate-limiting step, so it is important to understand how FISH variables will affect this reaction. As FISH protocols usually use probe concentration in excess relatively to the number of target sequences [21], the concentration of the target molecule (typically the rRNA) will have a tremendous impact on the FISH outcome.

The target RNA

The physiological state of the microorganism as well as the species, will define the content of target rRNA molecules in the cells. In growing bacterial cells, as many as 10^4 to 10^5 ribosomes per cell can be found [22]. For fast growing bacteria, such as *Escherichia coli* with a doubling time of 24 min, the number of rRNA copies per cell has been estimated at about 72,000 ribosomes/cell; nonetheless, the same *E. coli* species growing more slowly (doubling time, 100 min) contained about 6,800 ribosomes/cell [23]. The slow-growing bacteria *Mycoplasma pneumoniae*, *Spiroplasma melliferum* and *Rickettsia prowazekii* have shown values of 300, 1000 and 1500 ribosomes per cell, respectively [24-26]. These values show the huge variation one can face when applying FISH to different species and at different metabolic states. In environmental samples, rRNA content can be

even more challenging as metabolic processes of cells can be reduced to minimal levels, and because some matrices can present a strong background that requires brighter signals for a proper detection. As an example, the detection limit of conventional FISH with Cy3-labeled Eubacteria probe was found to be 370 ± 45 16S rRNA molecules per cell for *Escherichia coli* hybridized on standard glass microscope slides; but increased to 1,400 ± 170 16S rRNA copies per *E. coli* cell in activated sludge [27]. Also, if an mRNA target is intended, the concentration is by default a problem when comparing with the rRNA content, and is also highly depend on the induction of a particular gene. In these cases, the number of hybridized probes will be obviously lower and cells might not be detected by a standard FISH procedure. Thus, researchers might need to resort to other FISH techniques intended to detect low copy numbers of the target.

In addition to the concentration, the access to the target RNA is another parameter that will have a major impact on duplex formation. The rRNA naturally forms a complex secondary structure that might hinder the probe access to the target sequence. This structure has many loops and helices and embedded ribosomal proteins leaving some stretches of the rRNA more accessible to probes than others. Fuchs *et al.* has proposed an "accessibility map" of the *E. coli* 16S rRNA, describing regions with strong or weak accessibility that allows researchers to anticipate limitations on probe access to the target [28, 29]. Nonetheless, regions described as inaccessible can be made more accessible by using helper-oligonucleotides (unlabelled oligonucleotides which bind in close vicinity to the target site of the labelled probe, opening the rRNA secondary structure) [28]. Accessibility to rRNA is also strongly affected by the salt concentration in solution (ionic strength), the denaturant, the temperature and the type of nucleic acid probes being used. While the temperature and denaturant effects are quite obvious, as high values will destabilize the rRNA secondary structures making the probe access easier; the other two are more complex. Salts are essential for stabilising both the rRNA secondary structure and the probe-target duplex. So, while the reduction in salts will help with accessibility, it might simultaneously affect the duplex (target-probe) formation. This is

particularly true for natural nucleic acid probes, such as DNA probes, for which repulsive forces between the phosphate groups in the strand backbones need to be blocked/reduced by salts for a successful pairing. With the introduction of some apolar synthetic nucleic acids, this task has become easier for several reasons, as detailed in the next sections. While accessibility to rRNA can be improved resorting to probes made of modified nucleic acids, the composition of the hybridization solution needs to be significantly adapted. Hybridization solutions are substantially different from technique to technique, which sometimes simply reflects the cumulative knowledge/experience of each research group, but in many cases is related with the FISH variant applied. The composition of the hybridization solution, as well as the effect of salt, denaturants and type of nucleic acid, will be discussed below in more detail.

The probes

Probe design is the parameter that has a stronger effect on the method performance, namely on the specificity and sensitivity of the method. In diagnostic applications, specificity refers to the probe ability to correctly discriminate the target from the non-target sequences/species, whereas sensitivity refers to the ability to correctly detect all strains/sequences within the same species/taxonomic group. In this regards, properties such as the length of the probe, GC percentage, melting temperature (Tm), specificity, sensitivity, self-complementarity, number of mismatches with close sequences, should be assessed in the design stage to increase the odds of success of the method.

The length of the probe and GC percentage have a direct impact on the probe Tm. It is well-known that duplex thermal stability is highly dependent on base composition as three hydrogen bonds occur between guanine/and cytosine bases, in opposition to two hydrogen bonds between adenine/thymine bases. The importance of G/C content is typically more pronounced for shorter probes and in general is kept between 40 and 60%.

Theoretically, the shorter the probe, the more rapidly diffusion across the cellular envelope occurs and the more discriminating the probe becomes. For instance, short oligonucleotides can discriminate between closely related target sequences that differ by as little as one base, simply because a single base will have a stronger effect on the overall affinity and Tm of a shorter molecule than in a longer one. However, the discriminating power might not translate into increased specificity for very short molecules, as very small sequences will present higher odds of being found in other organisms. As such, a balance between the discriminating power and specificity of the sequence should be achieved. Probes with as low as 10-bp have been described and proved specific [17]; but these low values would definitely need extra care in terms of specificity assessment. Most works use 12 to 20-bp long probes depending on the nature of the nucleic acid being used. For unnatural nucleic acids (nucleic acid mimics), the probe size is usually shorter (12 to 15 bp) because their thermal stability and affinity per base pair is usually higher.

Regarding target specificity, a probe is typically designed to have an exact-match to the target. As such, one of the main parameters affecting probes performance are mismatches found in sequences of non-target organisms. A mismatch is a position within the probe where the base is unable to bind with the base in the corresponding position in the target molecule. Mismatching delays the hybridization rate and may even inhibit hybridization altogether for shorter probes or if a significant number of mismatched positions are involved. Assuming a correct design, mismatches could be a result of a point mutations in the target organism, or simply a natural polymorphism of the sequence. They are very important for probe design as they can affect sensitivity. In this case we want to avoid mismatches. However, they can (and should) be used to discriminate our target sequence/taxonomic group from closely related ones. As such, mismatched positions need to be well evaluated during probe design so we can both avoid them within the target sequences, and look for them to choose regions with high discriminating power for closely related non-target sequences. When mismatches are present, the probes performance will also be greatly dependent on two other

features: 1) the stringency of the hybridization and 2) the type of probe being used. If the stringency is relaxed, mismatched probes will be able to hybridize with non fully-complementary sequences. The stringency of the system can be relaxed or increased by simply changing the hybridization temperature. The more the hybridization temperature is reduced below the probe Tm, the more mismatches will be tolerated [30]. However, if stringency is too high, probe binding is compromised even for perfect matches. In addition to temperature, stringency is affected by different factors that are mainly dependent on the composition of the hybridization and washing solutions to be used in FISH. The composition of those solutions, and its effects on strigency will be explored in the next sections.

Regarding the type of probe, as mentioned earlier, traditional FISH applications resort to probes made of natural nucleic acids, in particular DNA. Advances on nucleic acid technology has resulted in the appearance of different generations of nucleic acid mimics, which are synthetic molecules that hybridize with natural nucleic acid obeying to the same base-paring rules [31]. They provide several advantages over the traditional molecules, from which the most relevant for FISH application include: 1) higher thermal stability, so higher hybridization temperature which will help with accessibility; 2) higher affinity towards RNA than RNA or DNA, so they might be able to displace secondary structures in the target region; 3) neutral backbone for some particular synthetic nucleic acids (in this particular case, the PNA) that allow hybridization to occur with low or no salts at all (something that will also help with accessibility to the target rRNA); and 4) resistance to nucleases degradation due to their unnatural nature.

Among the synthetic molecules most used in FISH application, PNA, locked nucleic acids (LNA) and 2’-O-methyl-RNA (2'OMe-RNA) have a prominent role [31, 32]. Modifications in these synthetic molecules are very diverse, including: backbone substitutions, conformational lock, ring substitution or the entire backbone replacement. The most singular advantage of PNA is related with its neutral backbone as mentioned above. It makes the probes-target duplex less dependent on

the salts stabilization effect because there are no negatively-charged phosphate groups on the probe. This effect is translated in the use of hybridization solutions with low salt, usually around the 10 mM NaCl, or with no NaCl at all. This will cause the rRNA secondary structure to open, improving drastically the accessibility.

Regarding LNA, several studies have shown that these monomers can increase affinity toward DNA and RNA molecules, improving the overall signal-to-noise ratio, sensitivity and specificity [33, 34]. Also, DNA duplexes containing LNA monomers present higher thermal stability and increase the melting temperature in about 2 °C to 10 °C (against RNA), per single LNA nucleotide incorporation [33, 35]. Because of the significant increase of the Tm per single LNA nucleotides incorporation, LNA probes are typically not completely made of LNA, as this would cause melting and hybridization temperatures to rise to values that could destroy the structure of the cells. Most often, LNA monomers are combined with DNA or with another RNA mimics, such as the 2'OMe- 2'OMe- has also a great affinity for RNA/DNA targets, but increases on Tm are less prominent comparing with LNA [36, 37]. Consequently, probe design with LNA or 2'OMe- has great flexibility, as mixed synthesis resorting to the intercalation of different monomers allows the fine-tuning of the probe thermodynamic parameters. This means that minor adjustments in the thermodynamic parameters can be achieved by changing the type of nucleotide in a particular position. In fact, such combinations are not limited to LNA and 2'OMe-, as there is an array of new nucleic acid mimics with potential for combination/mixed synthesis that have been applied to FISH [31, 38] or even to to antisense approaches (i.e. the use of complementary nucleic acids to bind/block a specific messenger RNA) [39, 40].

The hybridization solution

Hybridization solutions have some common features among the different FISH procedures. In addition to the probe, their basic composition should always include a denaturant, a buffer and salt.

As described above on the “target RNA” section, salts play an important role on the stability of the duplex probe-target and on the rRNA secondary structures. The buffer role is to keep the pH of the hybridization step fairly constant, usually between 7 and 9. While the pH effect has not been fully disclosed, it is known to affect the ionization of the nucleotides [41]. From pH 5 to 9 all bases are uncharged so hybridization should occur without interference. For lower values, such as pH 4, the hybridization has proved possible for instance for LNA probes [17]. However, it is known that some common fluorochromes are affected by pH and such values might compromise their performance [42]. For values higher than 10, hybridization/pairing might be disfavoured as most bases will be deprotonated. Nonetheless, a pH around 10 has shown to be beneficial for the hybridization of PNA probes in some Gram-negative bacteria. This effect was probably linked with the viscosity of the hybridization solution that decreased for higher pH (a variable that will be further discussed below).

Temperature and denaturant concentration, are typically the variables that are first changed when trying to reach the desired performance. Formamide at concentrations ranging from 30 to 50% (vol/vol), is by far the most commonly denaturant applied in FISH procedures. Nonetheless, some studies have attenpted to optimize this parameter and have shown that the concentration range can be broader (5 to 70% vol/vol),depending on the type of probe and cell envelope of the microorganisms [43]. Formamide destabilizes the double-stranded molecules by interfering with hydrogen bond formation. Thus, it reduces the Tm of the probe and, consequently, the hybridization temperature (Th) in a linear manner (Th is expected to decresase 0.75–1.0 ℃ for each 1% (vol/vol) of formamide added [44]. It is therefore common to use Th near the 50 and 60ºC, using probes that have theorethical Tm above 70ºC. However, one should be aware that formamide is toxic and volatile at the temperatures commonly used in hybridization procedures. In the last decade other chemicals have been introduced to either use non-toxic compounds, or to try to accelerate the nucleation reaction. In the first case, urea has been the elected denaturant of choice for replacing the

toxic formamide, specially when in *vivo* applications are intended [45]. Usually its concentration ranges from 0.5 to 6 M. Regarding the acceleration of the hybridization kinetics, ethylene carbonate has been tested and proved efficient for targeting specific sequences in human tissue samples [46]. However, until now its superiority over ureia or formamide for application in microorganisms is not apparent [47, 48].

Denaturants, salts and buffers were listed above as the basic components of the hybridization soluation, but these solution might include several other compounds that can interact with each other. These might include detergents/surfactants, chelators or polymers. For instance, the anionic polymer dextran sulfate is a common ingredient in many hybridization solutions. It appears to increase the rate of hybridization by forcing the probe into the cell, as it will fill the physical space creating an osmotic pressure that will drive probes toward the target [48]. However, dextran sulfate, in particular the ones with high molecular weight, will render the hybridization solution highly viscous and that might also interfere with macromolecular diffusion [49]. Dextran sulphate with molecular weights raging from 0.5 to 500 kDalton and concentrations from 2 to 10% (wt/v) have been applied. Both the molecular weight and concentration might be adjusted according to the target cell properties [48]. A similar effect can be obtained with the inclusion of Denhardt's solution, which is part of the recipe of several hybridization solutions. This solution includes a mixture of high-molecular weight polymers that artificially increase the concentration of available probe; but it also also helps reducing background signal. It acts as a blocking agent, since polymers are capable of saturating non-specific binding sites reducing non-specific binding. While these reagents are more common on Northern and Southern Blot techniques (to block membrane unspecific binding), they persist in some FISH recipes, but, apart from techniques requiring enzymatic conjugates for signal amplification or the techniques performed in filter membranes, their role is probably not crucial for most FISH variants.

Washing

Washing steps ensure that excess probe molecules are washed away and, when properly optimized, prevent unspecific binding by removing also loosely-bound probes. Parameters such as temperature, salt and washing time are manipulated to ensure a stringent wash. Depending on the nature of the probe and the stability of the duplex, these 3 parameters might need adjustments, but their values are in general very similar to the ones used in the hybridization step for each experiment. Other components of the washing solution are buffers and detergents, such as SDS, Tween-20 or Nonidet P-40. Denaturants can also be part of the washing buffers solutions, with formamide being the most commonly used compound. Adjustments in this step usually depends on the FISH outcome. For instance, if cross-hybridization is noticed, temperature can be increased or denaturant can be added. If a foggy image is obtained (or if impurities are noticed), the number and time of the washes can be increased. Protocols resorting to 3 short washings or one longer wash are both quite common for different FISH variants.

2.3 Visualization/detection

The importance of the visualization/detection step in the FISH procedure is often underestimated, but the selection of appropriate settings can be crucial for a successful detection of the cells. FISH samples are typically evaluated by fluorescence microscopy, resorting to filter units containing excitation and emission filters that allow the discrimination of different colour channels (usually blue, green and red channels). The properties of the filters should be evaluated even before ordering the probes, as ordering a probe with a red fluorochrome does not mean it will work efficiently with any red filter set. This is even more relevant now because of the wide range of fluorochromes available. If the fluorochromes do not fit perfectly the filters setting, the excitation efficiency can be reduced drastically, which will compromise the emission signal. Equally, if the emission signal is not collected at the peak of the fluorochrome emission spectrum, fluorescence intensity might be

very low. Presently, many fluorescence systems make use of lasers for excitation, so instead of an excitation range, they have a fixed excitation wavelength. The relevance of the equipment setting described here for microscopy is also true for other fluorescence detection systems such as flow cytometry, has they rely on the same excitation/emission strategies.

The choice of the equipment to be used for visualization/detection will mainly depend on laboratory resources/access to equipment and also on the main goal of the study. If a quantification is intended, flow cytometry will provide a more straightforward approach; while for observing complex population three-dimensional structures, microscopy is the obvious choice. Both equipments have observed a huge evolution in the last decade. Flow cytometry systems offer the possibility of sorting cells with a specific fluorescence signal (a functionality usually called as fluorescence-activated cell sorting [19, 50]); while advanced microscopy systems allow the 3D observation of microbial communities and present highly multiplexed (i.e. simultaneous detection of multiple fluorochomes) capabilities.

The visualization equipment, in particular microscopy, can also be combined with other techniques so that, in addition to genetic information, data on the cells metabolic activity are collected. These techniques usually resort to labelled substrates [51]. They provide functional analysis at a single-cell level in complex microbial communities. One of the first methods being introduced was MAR-FISH. This well-established approach has been followed by Raman- or SIMS-FISH. Differences between the methods rely on the techniques used to detect the labelled substrates, that resort to either Secondary Ion Mass Spectrometry measurements for SIMS-FISH, bright- or phase-contrast microscopy for MAR-FISH or Raman spectroscopy for Raman-FISH. Within the technology advances on fluorescence imaging, works focusing on super-resolution microscopy have also opened up a new window into bacterial cells, allowing the study of intracellular processes at an unprecedented level of spatial resolution. Single-molecule fluorescence tracking is now possible and is teaching us about dynamic cellular processes of microorganism. An overview on single-

molecule fluorescence imaging and subsequent application to the study of bacteria biology, can be found in Gahlmann, et al. (2014) [52].

CONCLUSIONS

In this chapter basic concepts of nucleic acid hybridization, as well as parameters that influence FISH performance/outcome, were reviewed. Overall, it is clear that probe design, type of fixation/permabilization and composition of hybridization solution, are the steps with major influence on FISH. Nonetheless, information that was taken for granted a few years ago, is now being put into question. The need for a fixation/permeation step, the role of denaturants; or, even, the optimal salt concentrations for a successful hybridization, are all examples of parameters that had very well-defined ranges, which are now much more flexible. For decades de development of FISH techniques have resorted to an empirical experimental design that was mainly based on previous observations. However, further developments on 1) the nucleic acids properties, 2) hybridization modelling/prediction, 3) fluorescence imaging/detection systems and 4) on the databases of genomic data; have triggered a new systematic approach on FISH development. New design strategies have emerged for nucleic acid mimics and modelling its hybridization kinetics became a necessity for exploring the properties of this new arrays of molecules and its behaviour on the different FISH reagents. At the same time, the resolution of fluorescence detection and imaging systems have faced a huge evolution boosting the design of new FISH strategies. In that field, the emergence of fluorochromes with brighter, stable and narrow emission spectra, has also played a major role. The detection and separation of single cells with a particular FISH profile became a reality and, subsequently, the quantification of RNA content and the tracking of single molecules was also possible. Last, but not the least, the databases of genomic data, as well as the bioinformatics tools, have perfected probe design, allowing the expansion to other genomic regions

with higher specificity. All these advances have paved the way for the diversity of FISH procedures available today and are still having a major role on FISH progress.

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Tables

Table 1 Variables typically involved in a standard FISH method development, classified according to the steps to which they are relevant. A more thorough description on the specific impact of these and other variables can be found in the main text.

Step	Variables	Examples
Fixation Permeabilization	Fixation or permeabilization agent	Ethanol, formaldehyde, lysozime,
	Concentration of the agent	50 % (vol/vol) ethanol, 4% (wt/vol) paraformaldehyde
	Number and sequence of fixation/permeabilization agents	Typically 1-3 agents are used
	Contact time	5-60 minutes depending on the agent and sample
	Cell wall	Gram-, Gram +,
	Cell spatial location	Adhered (membrane, slide), suspension
Hybridization	Type of probe	DNA, PNA, LNA
	Probe sequence	Self-complementarity, GC content, Tm
	Probe length	15 to 35 bases
	Hybridization Temperature	37-70 ºC
	Salt concentration	0-1M
	Formamide concentration	0-50% (vol/vol)
	Probe self-complementarity	Hairpin structures, number of self-

		complementary bases
	Target molecule	mRNA, rRNA, DNA
	Cell physiological state	Latent, stationary, exponential
	rRNA target	Secondary structure
	Contact time	30-180 min
	Cell spatial location	Adhered (membrane, slide), suspension
	Probe concentration	200-400 nM
	pH	7-9
	Viscosity	Presence of polymers in solution (eg. dextran sulphate of different molecular weights)
Washing	Contact time	15-90 min
	Temperature	40-70 ºC
	pH	7-9
Visualization/ detection	Sample autofluorescence	Type of sample, mounting media,
	Equipment	Fluorescent filters, light source
	Fluorescence detection limit	Equipment settings; fluorochrome properties
	Filters wavelength	Band pass/long pass, fit to the fluorochromes excitation and emission spectra, cross-talk
	Type of Fluorochrome	Cy3, FITC, Alexa Fluor, cross-talk
	Fluorochrome quenching	User expertise, type of fluorochrome, light source